Cell-free NADPH oxidase activation assays: "in vitro veritas"

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Chapter 22

Cell-Free NADPH Oxidase Activation Assays: "In Vitro Veritas"

Edgar Pick

Abstract

The superoxide ($O_2^{\bullet-}$)-generating NADPH oxidase complex of phagocytes comprises a membrane-imbedded heterodimeric flavocytochrome, known as cytochrome b_{558} (consisting of Nox2 and $p22^{phox}$) and four cytosolic regulatory proteins, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and the small GTPase Rac. Under physiological conditions, in the resting phagocyte, $O_2^{\bullet-}$ generation is initiated by engagement of membrane receptors by a variety of stimuli, followed by specific signal transduction sequences leading to the translocation of the cytosolic components to the membrane and their association with the cytochrome. A consequent conformational change in Nox2 initiates the electron "flow" along a redox gradient, from NADPH to oxygen, leading to the one-electron reduction of molecular oxygen to $O_2^{\bullet-}$. Methodological difficulties in the dissection of this complex mechanism led to the design "cell-free" systems (also known as "broken cells" or in vitro systems). In these, membrane receptor stimulation and all or part of the signal transduction sequence are missing, the accent being placed on the actual process of "NADPH oxidase assembly," thus on the formation of the complex between cytochrome b_{558} and the cytosolic components and the resulting $O_2^{\bullet-}$ generation. Cell-free assays consist of a mixture of the individual components of the NADPH oxidase complex, derived from resting phagocytes or in the form of purified recombinant proteins, exposed in vitro to an activating agent (distinct from and unrelated to whole cell stimulants), in the presence of NADPH and oxygen. Activation is commonly quantified by measuring the primary product of the reaction, $O_2^{\bullet-}$, trapped immediately after its generation by an appropriate acceptor in a kinetic assay, permitting the calculation of the linear rate of $O_2^{\bullet-}$ production, but numerous variations exist, based on the assessment of reaction products or the consumption of substrates. Cell-free assays played a paramount role in the identification and characterization of the components of the NADPH oxidase complex, the deciphering of the mechanisms of assembly, the search for inhibitory drugs, and the diagnosis of various forms of chronic granulomatous disease (CGD).

Key words NADPH oxidase, Superoxide, Cell-free assays, Cytochrome b_{558}, Nox2, Noxes, Cytosolic components, $p47^{phox}$, $p67^{phox}$, Rac, Anionic amphiphile, Arachidonic acid, Superoxide dismutase, Isoprenylation, Peptide walking

1 Introduction

> Less is More
>
> Ludwig Mies van der Rohe (German-American architect)

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1.1 Background

Phagocytic cells (principally, neutrophils, monocytes, and macrophages) destroy engulfed bacterial, fungal, and protozoal pathogens by a number of effector mechanisms. Among these, reactive oxygen species (ROS) occupy a prominent position. ROS are derived from the primordial oxygen radical, superoxide ($O_2^{\cdot-}$), which is produced in response to appropriate engagement of membrane receptors by a tightly regulated enzyme complex, known as the $O_2^{\cdot-}$-generating phagocyte NADPH oxidase (briefly, "oxidase") (reviewed in ref. 1). The oxidase catalyzes the formation of $O_2^{\cdot-}$ by the NADPH-driven one-electron reduction of molecular oxygen (O_2). The functionally competent oxidase complex is composed of a membrane-associated flavocytochrome b_{558}, which is a heterodimer of two subunits (Nox2, also known as gp91^{phox}, and p22^{phox}), and four cytosolic components, p47^{phox}, p67^{phox}, p40^{phox}, and the small GTPase Rac1/2 (reviewed in ref. 2). The catalytic component is Nox2, which consists of six transmembrane α-helices linked by three external and two cytosol-facing loops and a cytosolic segment, also known as the dehydrogenase region (DHR). Nox2 is harboring all redox stations carrying the flow of electrons from NADPH to O_2, namely, an NADPH-binding site and non-covalently bound FAD, both present in the DHR, and two hemes, bound to histidine pairs present in the second and fifth membrane helices. In resting phagocytes, the components of the complex exist as distinct entities, oxidase activation being the consequence of the interaction of flavocytochrome b_{559} with cytosolic components, a process requiring translocation of the cytosolic components to the membrane environment of flavocytochrome b_{558}. This process involves a complex set of protein–protein and protein–lipid interactions and is defined as oxidase assembly (reviewed in refs. 3–5) (*see* Fig. 1).

For oxidase assembly, the decisive interaction is that of the DHR of Nox2 with one or more cytosolic components, resulting in a conformational change that initiates electron flow. In one model of oxidase assembly, p67^{phox} is seen as the only cytosolic component responsible for an "activating" interaction with Nox2 [6, 7]. Because p67^{phox} does not possess a membrane attachment signal of its own, it requires the assistance of p47^{phox} and Rac, to be brought in contact with Nox2 [6, 8, 9]. This fact was the reason for naming p47^{phox} and p67^{phox} homologues, associated with some of the non-phagocytic oxidases (Noxes), as Nox organizer 1 (NOXO1), and Nox activator 1 (NOXA1), respectively (reviewed in refs. 3, 5, 10). The roles of p47^{phox} and Rac in helping the association of p67^{phox} with Nox2 are not interchangeable; under certain in vitro conditions, oxidase activation can take place in the absence of p47^{phox} but not that of Rac [11, 12]. These differences in the "assistance" provided to p67^{phox} are of significance in the design and interpretation of cell-free assays and will be discussed in this chapter.

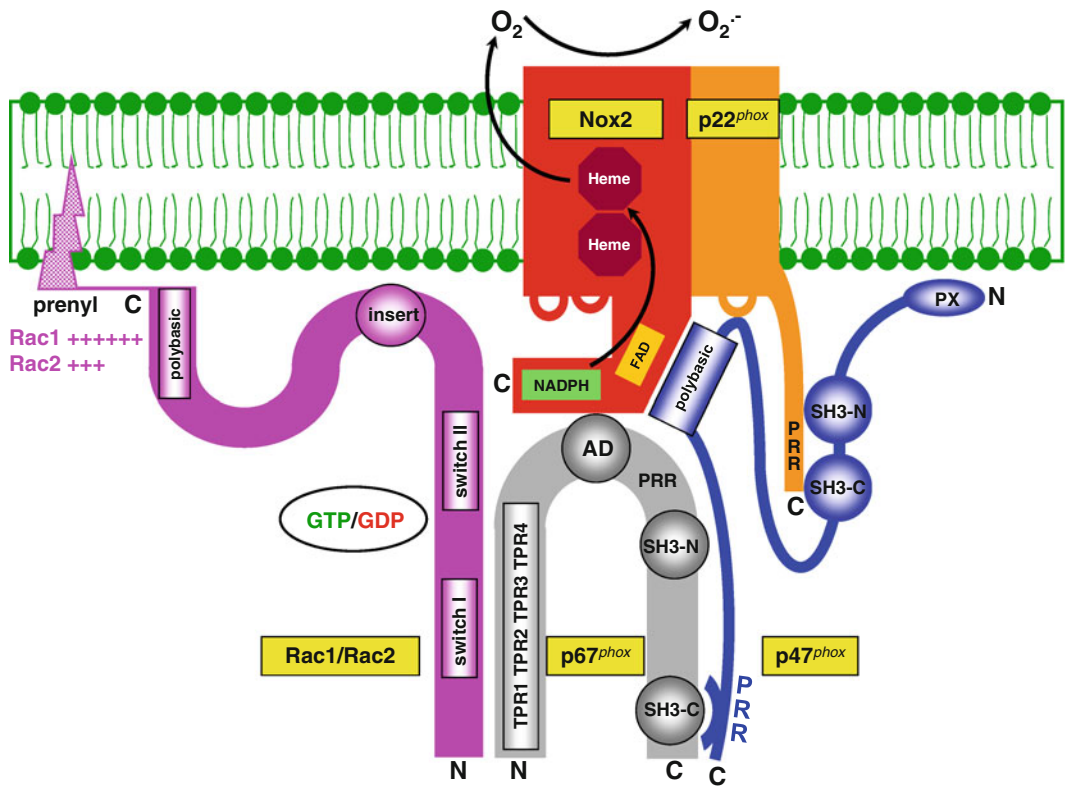

Fig. 1 Schematic representation of the assembled phagocyte NADPH oxidase. TPR = tetratricopeptide repeat; AD = activation domain; SH3 = Src homology 3; PRR = proline rich region; PX = *phox* homology. Reproduced by permission from [124]

The early literature on the assay of NADPH oxidase was rooted in the awareness of the multiplicity of stimulants causing ROS production by phagocytes [13, 14] and on the finding that, independent of the nature of the stimulant, NADPH oxidase activity could be demonstrated in the particulate (membrane) fraction of stimulated but not resting cells [15]. Thus, subjection of phagocytes to a stimulant, followed by cell disruption, separation of the cell homogenate into a particulate (membrane) and a cytosolic fraction, and measurement of NADPH-dependent $O_2^{\bullet-}$ production by the membrane fraction, became the standard procedure for assessing NADPH oxidase activity [16]. In the course of work with the membrane fraction of stimulated phagocytes, two facts became firmly established: first, the physiological substrate of the enzyme was NADPH and not NADH [15], and, second, the enzyme required supplementation with FAD for optimal activity [17]. Working with membranes from stimulated cells was technically difficult because the activity, as expressed in reaction rates, declined rapidly over time, was inactivated at 37 °C, and was sensitive to high salt concentrations and to harsh sonic disruption of the cells [16].

These difficulties prompted a search for an alternative procedure for the assay of NADPH oxidase. An additional stimulus for such a search was the unexplained mechanism responsible for the majority of the autosomal recessive form cases of chronic granulomatous disease (CGD). In the early 1980s, cytochrome b_{558} was the only known component of the oxidase and it was not known that the oxidase was a complex consisting of more than one component (a useful historical perspective of oxidase research "prehistory" appears in ref. 18). Thus, the finding that most patients with autosomal recessive CGD possessed a normal cytochrome b_{558} (found to be missing in the X-linked form of the disease) was puzzling.

This introduction would be incomplete without mentioning the marked increase of interest in non-phagocytic Noxes (reviewed in refs. 3, 5, 10). Nox family members are membrane-associated proteins with an electron carrier function involving the conserved functional modules also present in Nox2: NADPH- and FAD-binding sites, six transmembrane α-helices, and two histidine-bound hemes. The family includes five members, known as Nox1, Nox2, Nox3, Nox4, and Nox5; Nox2 representing the catalytic subunit of the phagocyte oxidase. Noxes 1, 2, and 3 are dependent on or regulated by cytosolic components; Nox4 is constitutively active and cytosol-independent, and Nox5 is Ca^{2+}-activated. Non-phagocytic Noxes generate much smaller amounts of ROS than Nox2, the identity of the activators is much less known, and the availability of purified and recombinant components lags behind that of the phagocyte oxidase. As will become apparent in the following subsections, the design of cell-free assays for non-phagocytic oxidases is a yet only partially fulfilled challenge.

1.2 The Canonical Cell-Free System

As with many discoveries, the design of a system to allow activation of the oxidase in subcellular fractions derived from resting (non-stimulated) phagocytes has a complex parenthood. In the laboratory of the author, the development of a cell-free oxidase activation system was not the result of a focused search for a better methodology, but the consequence of the finding that inhibition of phospholipase A_2 in intact macrophages prevented $O_2^{\cdot-}$ production by many stimulants [19]. These stimulants also caused liberation of arachidonic acid (C20:4) and we proposed that C20:4, generated by phospholipase A_2 action on membrane phospholipids, serves as a second messenger of oxidase activation, leading to the idea that C20:4 might be capable of activating the oxidase in disrupted resting phagocytes. Further encouragement was derived from work on the activation of adenylate cyclase in broken cell preparations by cholera toxin, which exhibited a requirement for both membranes and a cytosolic component [20].

In a paper published in 1984, the NADPH-dependent production of $O_2^{\cdot-}$ by guinea pig macrophage homogenates exposed to a critical concentration of C20:4 and several other long-chain

unsaturated (but not saturated) fatty acids in the ionized (but not in the esterified) forms, was described [21] (*see* **Note 1**). None of the numerous stimulants, eliciting $O_2^{\cdot-}$ production in intact phagocytes were capable of acting as activators in the cell-free system. A finding of paramount importance for future developments in oxidase research was that neither membrane nor cytosolic fractions of the phagocyte homogenate were, by themselves, capable of supporting fatty acid-elicited $O_2^{\cdot-}$ production, but recombining the two fractions resulted in full reconstitution of oxidase activity [21]. The "cell-free concept" was clearly ripe for discovery, as shown by the fact that similar cell-free oxidase activating systems were described within 1 year by three other groups. These were derived from resting horse [22] or human [23, 24] neutrophils, and in all cases, a requirement for cooperation between membrane and cytosol was found. An important finding made by two of these groups was that patients with the X-linked form of CGD possess a defective membrane component but a normal cytosolic component(s) [22, 24]. This observation was followed by the reciprocal finding that most CGD patients with the autosomal recessive mode of inheritance have a normal membrane component but a defective cytosolic component(s) [25]. These clinical correlates were essential for "legitimizing" the cell-free system as reflecting the activity of the $O_2^{\cdot-}$-forming oxidase, known from work in intact phagocytes. It is, perhaps, of interest to mention that the cell-free system was received with skepticism by some experts in the field (see lack of acceptance of the system in ref. 26, in which it was stated that it represents "nonphysiologic changes in the lipophilic environment of the enzyme").

Soon after the introduction of the cell-free system, the mechanism by which fatty acids cause oxidase activation became a subject of intense investigation. One of the first breakthroughs was the discovery that fatty acids act as anionic amphiphiles, as shown by the ability of sodium or lithium dodecyl sulfates (SDS; LiDS) to replace fatty acids as activators in the cell-free system, with very similar dose–response curves [27]. Other anionic detergents, such as sodium cholate, sodium deoxycholate, digitonin, and saponin, containing fused aromatic rings, are inactive. Sodium dodecyl sulfonate, like SDS, consists of an aliphatic hydrophobic moiety and an anionic polar head and thus serves as an activator [27]. Cell-free oxidase activation by fatty acids and some detergents was clearly one and the same and the term anionic amphiphiles became the accepted term for activators belonging to this category. This generalizing thesis was not well received by some reviewers of the manuscript on oxidase activation by SDS, when first submitted for publication (*see* **Note 2**).

The predominant explanation for the oxidase-activating ability of some anionic amphiphiles is that they disrupt an intramolecular bond in $p47^{phox}$ between a C-terminal polybasic region and the Src

homology 3 (SH3) tandem. In the intact phagocyte, this bond is severed by the phosphorylation of critical serines within the polybasic stretch, freeing the SH3 domains of $p47^{phox}$ for intermolecular interaction with a proline-rich region at the C-terminus of $p22^{phox}$, a critical step in the assembly of the oxidase complex [28]. Direct experimental evidence was provided for the induction of a conformational change in $p47^{phox}$ by C20:4 and SDS, a change which is also achieved by phosphorylation of $p47^{phox}$ by protein kinase C in vitro [29].

Some caution is, however, required concerning this "single target" interpretation because of the existence of a massive body of evidence for a direct effect of anionic amphiphiles on cytochrome b_{558}, leading to conformational changes which might also participate in oxidase activation [30–32]. Cell-free oxidase activation can also be induced by the synergistic action of phosphatidic acid (product of phospholipase D) and diacylglycerol (product of phospholipase C), again requiring participation of both the membrane and cytosolic fractions of the phagocytes and involving phosphorylation of $p47^{phox}$ and $p22^{phox}$ [33, 34]. A cell-free oxidase activating system, involving phosphorylation of $p47^{phox}$ (and of an unidentified membrane component) by protein kinase C in vitro was designed with the idea of being as close as possible to the in vivo reality [35, 36]. The latter system led to an activation level inferior to that induced by anionic amphiphiles and did not achieve widespread use.

A methodological but also conceptual revolution was the introduction of the semi-recombinant cell-free system. In this, the membrane is either used in the native form or represented by a purified and relipidated cytochrome b_{558} preparation, but the cytosol is replaced by a mixture of purified recombinant components [37]. This system permits introduction of strict quantification of the components participating in cell-free oxidase activation, the performance of dose–response assays, and control over the ratios among cytosolic components, among these and cytochrome b_{558}, and among the activating amphiphile and the membrane and cytosolic components. The amphiphile-activated cell-free system is the most frequently used and deserves to be coined the "canonical" cell-free assay. A modification of the cell-free system is the "two-step" assay, the purpose of which is to separate the assembly and catalytic phases in oxidase function (*see* **Note 3**).

1.3 Variations on the "Cell-Free" Theme

Recently, variations of the semi-recombinant amphiphile-dependent cell-free system have been introduced in which individual cytosolic components were replaced by chimeric constructs of either [$p47^{phox}$-$p67^{phox}$] [38] or [$p67^{phox}$-Rac1] [7, 39–41], supplemented by the missing third component (Rac1 and $p47^{phox}$, respectively), or by a tripartite chimera [$p47^{phox}$-$p67^{phox}$-Rac1] [42].

These chimeras consist of fusions of either full-length or truncated individual components and will be discussed in more detail further down. A further variation is represented by a cell-free system in which Rac is replaced by a complex of Rac1-GDP and GDP dissociation inhibitor for Rho (RhoGDI) [43].

1.4 A Cell-Free System Based on Cell Cores and Cytosol

The canonical cell-free system is not capable of detecting the participation of $p40^{phox}$ in oxidase activation [44]. To enable this, a system was designed based on permeabilization of neutrophils by streptolysin-O, resulting in the formation of cytosol-free "cores" [45]. These are used as a source of membranes, with the maintenance of membrane morphology and preservation of intracellular granules. Upon supplementation with cytosol, ATP, GTP, and NADPH, a cell-free-like system is generated which responds to stimulants normally acting on intact cells, such as phorbol myristate acetate (PMA), by $O_2^{\bullet-}$ production. Using this system, a role for $p40^{phox}$ in oxidase activation in human neutrophils could be demonstrated [46].

1.5 A Cell-Free System Without Cytosolic Components

A later methodological development was the design of a cell-free system in which macrophage membrane-derived cytochrome b_{558} was relipidated with a mixture of crude (14–23 %) soybean phosphatidylcholine (PC) and pure phosphatidic acid. The relipidated cytochrome b_{558} was found to generate $O_2^{\bullet-}$ in vitro in the presence of FAD, NADPH, and a low amount of anionic amphiphile, in the absence of cytosolic components [47, 48]. The level of $O_2^{\bullet-}$ production was about four times lower than that found in the canonical cytosol-dependent cell-free system, but the discovery of a cytosol-independent oxidase activation process provided definitive functional proof for the presence of all redox stations on cytochrome b_{558}. The cytosol-independent cell-free assay was used successfully for the elucidation of the defect in some cases of the $X91^+$ form of CGD [49].

1.6 Cell-Free Activation in the Absence of an Activator

1.6.1 Relieving Autoinhibition

The elucidation of the mechanism by which anionic amphiphiles induce oxidase activation in the cell-free system (severing the intramolecular bond in $p47^{phox}$ between the $(SH3)_2$ tandem and the polybasic C-terminus) led to the design of a new type of amphiphile-independent cell-free system. In this, truncation of $p47^{phox}$ at residue 286, which removes the polybasic C-terminus [50], or engineered mutations in $p47^{phox}$, which cause unmasking of $(SH3)_2$ [51], make the system amphiphile-independent. The need for amphiphile is circumvented because, in both cases, spontaneous interaction between the $(SH3)_2$ of $p47^{phox}$ and the proline-rich region at the C-terminus of $p22^{phox}$ is made possible. Surprisingly, amphiphile-independent activation involving $p47^{phox}$ truncated at residue 286, required $p67^{phox}$ to be truncated, too, at a residue N-terminal to the N-terminal SH3 domain, suggesting that the amphiphile might also have an effect on $p67^{phox}$ [50].

1.6.2 A Lipid Anchor

We developed a conceptually different amphiphile-independent cell-free system based on the use of prenylated Rac1 [9, 52], which binds to phagocyte membranes with high affinity and serves as a chaperone for $p67^{phox}$, leading to oxidase activation in the absence of amphiphile and without the need for $p47^{phox}$. Later variations of this system are represented by a prenylated [$p67^{phox}$-Rac1-GTP] chimera, which activates the oxidase in the absence of amphiphile and of any other component [7, 41, 53], a tripartite chimera, consisting of the functional domains of $p47^{phox}$, $p67^{phox}$, and full-length prenylated Rac1-GTP [54], and prenylated Rac-GDP, in conjunction with a guanine nucleotide exchange factor (GEF) for Rac and GTP or ATP [55]. The essential difference between amphiphile-dependent and amphiphile-independent, Rac prenylation-dependent cell-free systems is poignantly illustrated by the specific inhibitory effects of a peptide corresponding to the C-terminus of Rac1 (prevents only amphiphile-dependent activation) and of RhoGDI (prevents only amphiphile-independent activation) [7].

1.6.3 "Reversed" Activation: Making the Membrane Anionic

The plasma membrane of mammalian cells contains 15–20 % anionic phospholipids, a fact of considerable importance in leukocyte function (reviewed in ref. 56). Yet another group of cell-free systems was developed, based on the rationale of artificially enriching phagocyte membranes with anionic phospholipids. This is expected to result in an increase in the negative charge at the cytosolic aspect of the membrane and should promote the binding of cationic cytosolic components (or positively charged regions in chimeras resulting from their fusion) to the membrane and, possibly, decrease the electrostatic repulsion of the positively charged cytochrome b_{558}. There are several examples of such cell-free systems. Thus, a combination of a [$p67^{phox}$(1-210)-$p47^{phox}$(1-286)] chimera and Rac1-GTP activates phagocyte membranes enriched in anionic phospholipids, in the absence of amphiphile [38]. The tripartite chimera, [$p47^{phox}$(1-286)-$p67^{phox}$(1-212)-Rac1(192)], is a potent oxidase activator in the absence of anionic amphiphile, provided that the membrane is enriched with one of the anionic phospholipids, phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylserine (PS), or phosphatidylinositol (PI) [42]. Also, enrichment of phagocyte membrane with the anionic phospholipids PG and PA enables oxidase activation in vitro by $p67^{phox}$ combined with [Rac1(GTP)-RhoGDI] complexes [57], and supplementation of membranes with phosphatidylinositol 3,4,5-triphosphate promotes oxidase activation by $p67^{phox}$ and [Rac1(GDP)-RhoGDI] complexes in conjunction with GTP and a GEF [58], both in the absence of an anionic amphiphile and $p47^{phox}$. The fine mechanism behind this form of "spontaneous" activation is not explained by simple electrostatic attraction between the membrane and the cytosolic components and their chimeric variations because the overall charges of [$p67^{phox}$(1-210)-$p47^{phox}$(1-286)] and [$p47^{phox}$(1-286)-$p67^{phox}$(1-212)-Rac1(192)]

chimeras are close to neutral, and thus, it is likely that positively charged domains in the cytosolic proteins and their chimeric constructs are participating in the interaction. An example of the major effect of electrostatics on cell-free activation is illustrated in Table 1.

1.7 Beginning and End

All cell-free systems are reductionist constructions. The systems, in most of their variations, are missing all or part of the initiating transduction mechanism from membrane receptors to the enzyme and also lack the "termination" process occurring in the intact phagocyte. In vivo, NADPH oxidase activity is transient and $O_2^{\bullet-}$ production is regulated by the balance between assembly and disassembly of the complex (reviewed in ref. 59). An in vivo study concluded that the turnover of cytochrome b_{558}-bound p67^{phox} and Rac is very high, indicating a continuous exchange of bound for free cytosolic components [60]. Cell-free systems are not the methodology of choice for the assessment of oxidase complex stability and the apparent decrease or termination of activity, when occurring in short-term assays, is due either to the exhaustion of NADPH or to the consumption of the reagents serving as $O_2^{\bullet-}$ traps. The brief duration of most contemporary cell-free assays also assures that the reaction components are unlikely to be inactivated in the course of the assay. In the past, it was thought that part of the $O_2^{\bullet-}$ generated in the system, which has escaped the intrinsic trap meant to bind the radical, might be dismutated to H_2O_2 and inactivate one or more of the oxidase components. To prevent this, catalase was added to the reaction in order to degrade any H_2O_2 that might have been produced [61], but such supplementation is unnecessary in brief kinetic assays and in the presence of sufficient $O_2^{\bullet-}$-trapping reagent.

In spite of the existing limitations, the stability of the assembled oxidase complex was also studied in cell-free systems, and was found to be significantly increased by chemical cross-linking of membrane and unidentified cytosolic components [62], by chimerization of p47^{phox} with p67^{phox} [38] or with Rac1 [39], and, most pronouncedly, by using a tripartite chimera consisting of functional domains of p47^{phox}, p67^{phox}, and Rac1, as the activator [42]. It, thus, appears that procedures replacing the natural association–dissociation cycles between cytosolic components and between cytosolic and membrane components by covalent bonds, enhances the half-life of the oxidase complex.

1.8 The "Subcellular" in the "Cell-Free"

The "primordial" cell-free assays consisted of mixtures of various membrane preparations and cytosol. Neither of these two components was properly characterized. In parallel with the isolation and characterization of the cytosolic components, the dominant form of cell-free assay became the one coined "semi-recombinant" [37]. This consists of recombinant p47^{phox}, p67^{phox}, and Rac and relipidated cytochrome b_{558}, purified from native or cell-line-derived

Table 1
Amphiphile-independent cell-free oxidase activation by enrichment of membrane with anionic phospholipids

Cytosolic activator(s)	NADPH oxidase activity (mol $O_2^{\cdot -}$/s/mol cytochrome b_{558} heme)			
	Membrane + phosphatidic acid (PA)	Membrane + phosphatidyl-glycerol (PG)	Membrane + phosphatidyl-serine (PS)	Membrane + phosphatidyl-inositol (PI)
No activator (membrane only)	11.70 ± 1.11	2.47 ± 0.13	1.75 ± 0.08	1.87 ± 0.04
$p47^{phox}$ + $p67^{phox}$	32.60 ± 2.60	12.37 ± 1.28	6.81 ± 0.58	2.91 ± 0.19
$p67^{phox}$ + Rac1	95.41 ± 3.64	34.29 ± 5.07	12.80 ± 1.20	5.63 ± 0.40
$p47^{phox}$ + $p67^{phox}$ + Rac1	112.83 ± 12.85	69.95 ± 8.46	48.57 ± 7.73	22.50 ± 3.22
[$p67^{phox}$(1-212)-Rac1(1-192)] chimera[a]	62.46 ± 4.81	12.92 ± 0.55	5.62 ± 0.57	3.11 ± 0.06
[$p67^{phox}$(1-212)-Rac1(1-192)] chimera + $p47^{phox}$	80.03 ± 4.99	44.87 ± 2.43	22.23 ± 0.42	6.12 ± 0.13
[$p47^{phox}$(1-286)-$p67^{phox}$(1-210)] chimera[b]	19.76 ± 0.86	3.21 ± 0.29	1.90 ± 0.35	2.27 ± 0.15
[$p47^{phox}$(1-286)-$p67^{phox}$(1-210)] chimera + Rac1	93.68 ± 2.97	68.94 ± 3.06	48.65 ± 1.46	30.25 ± 3.39
[$p47^{phox}$(1-286)-$p67^{phox}$(1-212)-Rac1(1-192)] chimera[c]	92.05 ± 2.38	50.76 ± 1.01	41.58 ± 1.72	37.80 ± 3.19

Assay mixtures contained solubilized macrophage membrane relipidated with PA, PG, PS, or PI, corresponding to 5 nM heme, and cytosolic activator(s), at 300 nM. $p47^{phox}$, $p67^{phox}$, and Rac1 were full-length proteins. Rac1 and the chimeras were nonprenylated. Rac1 and the chimeras were exchanged to the GTPase-resistant GTP analog, guanylyl imidodiphosphate (GMPPNP). The final concentrations of membrane phospholipid in the assays were 12 μM endogenous membrane phospholipid and 80 μM PA, PG, PS, or PI. Activation was in the absence of an anionic amphiphile. Methodology was as described in ref. 42. The results represent means ± SE derived from three experiments

[a]Reference 40
[b]Reference 38
[c]Reference 42

phagocyte membranes, following the demonstration that the only membrane component participating in cell-free activation is cytochrome b_{558} [63]. Due to the methodological difficulties associated with the preparation and purification of cytochrome b_{558}, membranes or solubilized membrane are routinely used. In the case of human neutrophils, a "whole" membrane fraction contains the plasma membranes, as well as the specific and azurophilic granules. Most of cytochrome b_{558} is found in the specific granules, with a lesser amount present in the plasma membranes [64]. $O_2^{\bullet-}$ production in intact neutrophils occurs both at the level of the plasma membrane and in an intracellular compartment corresponding to granules [65]. An analysis of the subcellular compartmentalization of membranes in the neutrophil, which respond with NADPH-dependent $O_2^{\bullet-}$ generation when exposed to cytosol and an amphiphilic activator, reveals that both plasma membranes and specific granules are involved [66]. To the best of our knowledge, the subcellular nature of macrophage/monocyte membranes participating in cell-free activation has not been ascertained.

One of the great advantages of the cell-free system is that the catalytic component of the enzyme (Nox2) is accessible to all reaction substrates (NADPH, FAD, oxygen) and the reaction product ($O_2^{\bullet-}$) has direct contact with the trapping reagent, maximizing the likelihood that we are measuring the full extent of the oxidase activity. When native membranes are replaced by solubilized membrane liposomes [67] or by purified and relipidated cytochrome b_{558} [63], the accessibility of Nox2 to the substrates and the $O_2^{\bullet-}$ trapping reagent is unknown, but our personal experience is that oxidase activity values are significantly superior to those obtained with native membranes.

1.9 What Are We Measuring in Cell-Free Assays

All cell-free systems are designed to mimic oxidase activation in vivo under in vitro conditions, starting from the equivalent of the state of the enzyme in resting cells. Enzyme activity is expressed as the reaction rate, based on the quantification of a reaction product or on the consumption of a reaction substrate. The most commonly used techniques are as follows:

1. *Reduction of cytochrome c by* $O_2^{\bullet-}$. This method is by far the most reliable, easy to perform, and convertible to a high-throughput assay. It was first described in the landmark paper by Babior et al. [68] on the production of $O_2^{\bullet-}$ by phagocytosing leukocytes. The specificity of cytochrome *c* reduction is checked by its elimination in the presence of superoxide dismutase (SOD). This assay is also used in less common situations, such as in Nox1-based cell-free systems [69], and for assessing the cytosol-independent diaphorase activity of the DHR of Nox4 [70].

2. *Reduction of iodonitrotetrazolium violet (INT)*. This method was introduced with the claim that INT is reduced by electrons originating in $FADH_2$ bound to the DHR of Nox2, by two-electron reduction and, thus, is measuring a step before the reduction of the two hemes and the generation of $O_2^{\bullet-}$ [71]. Most of the INT reduction was described as being SOD-resistant and not being dependent on $p47^{phox}$ (*see* **Note 4**).
3. *Reduction of nitrotetrazolium blue (NBT)*. This method is used almost exclusively for measuring NADPH-dependent diaphorase activities of the DHR of Nox2 and other Noxes, in the presence or absence of cytosolic activators [72–74].
4. *Other artificial electron acceptors*. These include dichloroindophenol, potassium ferricyanide, and cytochrome b_5. Together with INT and NBT, they are used for measuring the constitutive diaphorase activities of the DHR of Nox4 [70, 75].
5. *Measuring the production of* H_2O_2. Quantification of the primordial ROS generated in the cell-free system, $O_2^{\bullet-}$, should, in most cases, be the default choice. On rare occasions, H_2O_2, derived by non-enzymatic dismutation of $O_2^{\bullet-}$, is measured in Nox2 cell-free systems. Unlike Nox2, Nox4 produces mainly H_2O_2 [76] and cell-free systems centered on Nox4 are based on the quantification of H_2O_2 [70], utilizing a fluorescence method, involving the H_2O_2- and horseradish peroxidase-dependent oxidation of Amplex Red [77]. Assay buffers containing horseradish peroxidase should not contain NaN_3, an inhibitor of peroxidases. Oxidase assay buffers intended for quantifying $O_2^{\bullet-}$ production, routinely contain 2 mM NaN_3 (*see* Subheading 2.1.5).
6. *Chemiluminescence assay for measuring* $O_2^{\bullet-}$. On some occasions, an enhanced sensitivity is required for the detection of $O_2^{\bullet-}$ in cell-free assay. For this purpose, lucigenin is used as the chemiluminescent detector and its validity as a specific probe was rigorously demonstrated [78]. Its use in a canonical amphiphile-dependent cell-free system is illustrated in ref. 79.
7. *NADPH consumption*. This is a simple technique, easily applicable to cell-free assays [80]. Its principal use is in situations in which a compound added to the reaction interferes with the detection reagent (*see* ref. 81). As is the case for all substrate consumption assays, it has the disadvantage that the product of the reaction is presumed but not ascertained. However, when applied to the semi-recombinant type of cell-free assay [37], there is an almost absolute certainty that NADPH is used exclusively for $O_2^{\bullet-}$ production.
8. *Oxygen consumption*. This assay is rarely used at present in cell-free systems because of the cumbersome equipment required. It was popular in the early history of the cell-free system in order to establish the stoichiometry between oxygen consumption and $O_2^{\bullet-}$ production [82, 83].

1.10 Uses of Cell-Free Systems

Cell-free assays are extensively used in both basic research and clinical medicine. The tremendous expansion of the field of non-phagocytic NOXes has provided further impetus to their use and to the development of variations of the assay adapted to specific NOXes. At the time of the writing of this review, the original descriptions of the C20:4 and SDS-activated cell-free systems [21, 27] have accumulated a total of 635 citations and many authors cite later applications of the original method. The principal uses of cell-free assays are as follows:

1. The identification, quantification and functional assessment of oxidase components. At present this refers almost exclusively to components produced by recombinant technology and less commonly to those purified from cells. Although cell-free assays, if properly performed, are among the most sensitive techniques for the detection of oxidase components, obtaining quantitative data requires basing these on careful dose–response experiments with highly purified components. Thus, cell-free assays are mainly used to assess the functional competence of recombinant oxidase components.
2. Structure–function studies on recombinant oxidase components, subjected to mutagenesis, truncations, deletions, chimerization, and posttranslational modifications, such as prenylation. This is, at present, one of the most popular applications, due to the very high sensitivity of the system, enabling detection of the effect of minor structural modifications on function.
3. The availability of numerous variations of the cell-free system has opened the way to novel applications that were not possible when only the canonical assay was available. Some examples are: the cell-free system in the absence of cytosolic activator [47, 48], used in the diagnosis of some forms on CGD [49]; the amphiphile- and $p47^{phox}$-independent variations [9, 12, 52], allowing focusing on the interaction of cytochrome b_{558} with $p67^{phox}$, or a system centered on the detection of GEFs [55].
4. Investigating the mechanism of action of oxidase inhibitors. With the increasing interest in the development of Nox inhibitors [84, 85], cell-free assays are key components in the search for such compounds (predominantly in the form of high-throughput screening).
5. Diagnosis of the various forms of CGD and follow up on the success of therapeutic approaches applied to CGD patients. One of the first indicators of the importance of the cell-free system was its application to distinguishing between CGD caused by mutations in cytochrome b_{558} [22, 24] and in the cytosolic components [25].

6. A more recent development is the design of cell-free systems applicable to non-phagocytic Noxes, in either cytosolic components-dependent or -independent variations [69, 70, 75].

Cell-free assays are characterized by simplicity, speed, and repeatability and are ideal for being converted to high-throughput applications.

1.11 Methodological Reductionism Triumphant

Methodological reductionism is the concept that complex biological events should, if possible, be studied at the most elementary level, preferably down to that of interacting molecules [86]. Complex processes, such as activation of an oxidative burst in the intact phagocyte, are deconstructed to its component parts.

The cell-free system is one of the best examples for the successful application of methodological reductionism. Soon after its discovery it was described in the following terms: "What was really needed to achieve an understanding of oxidase activation at the molecular level was a cell-free oxidase activating system that can be taken apart and analyzed component by component using biochemical techniques. In a major advance that has capped years of work on this problem, activation of the oxidase in a cell-free system has at last be realized" [87], and "Through diligent research… whose pace has accelerated greatly since the ground-breaking discovery of a method to activate the respiratory burst oxidase in a cell-free system, the operation of this complex enzyme is now beginning to be understood" [88]. A useful brief review of the role of cell-free methodology in opening the "black box" of oxidase function is provided by ref. 89.

The overwhelming majority of the results obtained employing cell-free methodology fully overlapped those obtained by working with whole cells (transfection) or whole organisms (knock-out or natural disease). Occasionally, results obtained in the cell-free system differ from those obtained in whole cells, providing proof for the statement that "the whole is more than the sum of its parts." Examples for such discrepancies are the findings that truncation of $p67^{phox}$ at residue 246 (which removes both SH3 domains) [90] or deletion of the N-terminal SH3 domain [90, 91] led to the elimination of $O_2^{\bullet-}$ production by stimulated cells transfected with the $p67^{phox}$ mutants whereas both mutants were found to be fully capable of supporting both amphiphile-dependent and -independent $O_2^{\bullet-}$ production in a cell-free system [90, 92].

In this chapter, we describe the basic methodology for performing the different versions of the cell-free assay, commonly used for studying the NADPH oxidase, and shall deal with theoretical considerations to be taken into account, interpretation of results, possible problems and their solutions, available alternatives, and the multiple applications of this approach.

2 Materials (*See* Note 5)

2.1 Chemicals and Reagents

2.1.1 Preparation of Phagocyte Membranes

1. Paraffin oil, weight/mL = 0.85 (highly liquid) or 0.83–0.86 (BDH). This was used for eliciting a sterile peritoneal exudate as a source of macrophages for the preparation of membranes.
2. Earle's balanced-salt solution: 6.8 g NaCl, 0.4 g KCl, 0.125 g $NaH_2PO_4 \cdot H_2O$, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 1 g glucose, 0.2 g $CaCl_2$ (anhydrous), 1.25 g $NaHCO_3$, H_2O up to 1 L.
3. Sonication buffer: 8 mM potassium, sodium phosphate buffer, pH 7.0 (made from 61 parts K_2HPO_4 and 39 parts NaH_2PO_4 stock solutions), 131 mM NaCl, 340 mM sucrose, 2 mM NaN_3, 5 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 1 mM 4-(2-aminoethyl)-benzene-sulfonyl fluoride hydrochloride (AEBSF) (*see* **Note 6**), and 0.021 mM leupeptin hemisulfate. It is best to add DTT (200 mM), AEBSF (100 mM), and leupeptin (2.1 mM) from concentrated stock solutions (concentrations are listed in parentheses) just before using the buffer.
4. 3.5 M KCl solution: made in 20 mM Tris–HCl buffer, pH 7.5, for mixing with sonication buffer to reach a final concentration of 1 M KCl. It is used to wash macrophage membranes for removal of non-integral membrane-attached proteins.
5. Octyl-β-D-glucopyranoside (octyl glucoside).
6. Solubilization buffer: 120 mM sodium phosphate buffer, pH 7.4, 1 mM $MgCl_2$, 1 mM EGTA, 2 mM NaN_3, 10 μM flavin adenine dinucleotide, disodium salt (FAD), and 20 % v/v glycerol. Add 40 mM octyl glucoside (from powder), 1 mM DTT, 1 mM AEBSF, and 0.021 mM leupeptin from concentrated stock solutions just before using the buffer. Unused buffer can be divided in aliquots of 25–50 mL and stored frozen at –20 °C. The same basic buffer, not supplemented with octyl glucoside, FAD, and AEBSF, serves for dialysis of solubilized membranes leading to the formation of membrane liposomes.
7. Sodium dithionite.
8. Phospholipids: 3-sn-phosphatidic acid (sodium salt, from egg yolk, 98 %) and L-α-phosphatidyl-DL-glycerol (ammonium salt, synthetic, 99 %). Dissolve at 5 mM in solubilization buffer containing 40 mM octyl glucoside (but lacking protease inhibitors, DTT, and FAD). Dispense into aliquots of 0.5 mL and store at –75 °C.

2.1.2 Expression of Recombinant Cytosolic Components

For the last 5 years we have switched from glutathione *S*-transferase (GST) fusion proteins to proteins with an N-terminal 6His tag. This applies to $p47^{phox}$, $p67^{phox}$, and Rac1. The basic procedure for

the expression and purification of the recombinant proteins is described elsewhere [54].

1. *E. coli* competent cells (Rosetta 2(DE3)pLysS; Novagen).
2. pET-30a expression vector (Novagen).
3. LB Broth.
4. Isopropyl β-D-1-thiogalactopyranoside (IPTG).
5. Triton X-100 solution: 10 % (v/v) solution in H_2O.
6. Protease inhibitor mixture Complete EDTA-free (Roche).
7. 50 % (w/v) Poly(ethyleneimine) solution (PEI).

2.1.3 Purification of Recombinant Cytosolic Components

1. Imidazole solution: 2 M in H_2O, adjust to pH 7.4 with 3 M HCl.
2. Ni Sepharose 6 Fast Flow (GE Healthcare).
3. *E. coli* lysis buffer and Binding Buffer for metal affinity chromatography on Ni Sepharose: 20 mM sodium phosphate buffer, pH 7.4, 0.5 M NaCl, 20 mM imidazole.
4. Wash buffer: 20 mM sodium phosphate buffer, pH 7.4, 0.5 M NaCl, 40 mM imidazole.
5. Elution buffer: 20 mM sodium phosphate buffer, pH 7.4, 0.5 M NaCl, 300 mM imidazole.
6. FPLC gel filtration columns: HiLoad 10/60 Superdex 75 prep grade (fractionation range: 3–70 kDa) for purification of $p47^{phox}$, $p67^{phox}$ (1-212) and Rac; HiLoad 10/60 Superdex 200 prep grade (fractionation range: 10–600 kDa) for purification of $p67^{phox}$ (1-526).
7. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 Na_2PO_4, 1.4 mM KH_2PO_4, 2 mM NaN_3, pH 7.3.
8. Protein assay concentrate for measuring protein concentration by the Bradford assay [93].
9. Bovine gamma globulin standard: 2 mg/mL for Bradford assay.
10. NuPage 12 % Bis-Tris electrophoresis gels: 1 mm gel thickness (Invitrogen, Life Technologies).
11. NuPage MOPS SDS running buffer (Invitrogen, Life Technologies).
12. NuPage LDS sample buffer (4×), and NuPage reducing agent (10×) (Invitrogen, Life Technologies).
13. Precision Plus SDS-PAGE protein standards, unstained (10–250 kDa) (Bio-Rad).
14. Coomassie Blue protein gel stain.

2.1.4 Reagents Required for Nucleotide Exchange on Rac (See **Note 7***)*

1. Buffer for diluting recombinant Rac1 for nucleotide exchange: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 4 mM $MgCl_2$, 2 mM DTT. PBS is not compatible with high concentrations of $MgCl_2$.
2. Guanylylimidodiphosphate, trisodium salt (GMPPNP): 10 mM stock in H_2O, aliquot, and store –75 °C.
3. Guanosine-5′-*O*-(3-(thio)triphosphate, tetralithium salt (GTPγS): 10 mM stock in H_2O, aliquot, and store –75 °C.
4. (Ethylene-dinitrilo)-tetraacetic acid, disodium salt (EDTA): 0.5 M stock in H_2O. In order to dissolve EDTA, the solution has to be brought to pH 8.0 with 10 M NaOH.
5. $MgCl_2$ solution: 1 M stock in H_2O.
6. Recombinant rat geranylgeranyl transferase I made in *E. coli* (Calbiochem, EMD Millipore, Merck KGaA). One unit transfers 1 nmol geranylgeranyl pyrophosphate to Rho proteins per hour at 37 °C, at pH 8.0.
7. Geranylgeranyl pyrophosphate: 1 mg/mL solution in methanol/10 mM aqueous NH_4OH (7/3).
8. Prenylation buffer: 50 mM Tris–HCl buffer, pH 7.7, 0.1 mM $ZnCl_2$, 5 mM $MgCl_2$, 2 mM DTT.
9. Triton X-114: 10 % (v/v) solution in H_2O.

2.1.5 Cell-Free Assays

1. Cytochrome *c*, from equine heart, 95 %.
2. *p*-iodonitrotetrazolium violet (INT): 10 mM solution in ethanol, keep frozen at –20 °C, in the dark.
3. β-nicotinamide adenine dinucleotide 2′-phosphate reduced, tetrasodium salt, min. 95 % (NADPH): 5 mM stock in H_2O, divide in 1 mL aliquots, and store at –20 °C. Avoid frequent thawing and freezing, and do not store over 2 months.
4. Lithium dodecyl sulfate, >99 % (LiDS): 10 mM stock in H_2O, store at 4 °C for unlimited periods, provided that evaporation is prevented. Unlike SDS, LiDS does not precipitate out of aqueous solutions at low temperature.
5. Superoxide dismutase (SOD), from bovine erythrocytes: 10,000 U/mL stock in H_2O, aliquot in amounts of 100 µL, store at –20 °C.
6. Oxidase assay buffer: 65 mM sodium phosphate buffer, pH 7.0 (made from 61 parts K_2HPO_4 and 39 parts NaH_2PO_4 stock solutions), 1 mM EGTA, 10 µM FAD, 1 mM $MgCl_2$, 2 mM NaN_3, 0.2 mM cytochrome *c* (*see* **Note 8**). The conductivity of this buffer is 7.7 mS/cm. When reduction of INT is measured, cytochrome *c* is replaced by 100 µM INT. When the assay is based on NADPH consumption, the buffer does not contain cytochrome *c* or INT. When the concentration of LiDS to be

used in a large number of assays is known, this can be dissolved in the buffer. Assay buffer with and without LiDS can be divided into batches of 100 mL and stored at –20 °C for unlimited periods of time, in the dark, to prevent damage to FAD. The rationale for the components of the buffer is discussed below.

2.2 Disposable Plasticware

1. 96-well microplates, polystyrene, flat bottom, clear, with a well volume of 382 μL and a maximal height of 10.9 mm. When the wells in these plates are filled with a 0.21 mL reaction volume, the vertical light path is 0.575 cm. Plates intended for use in ELISA assays of medium or high hydrophilic protein-binding capacity are not recommended for use in cell-free assays. For the performance of the NADPH consumption assay, 96-well microplates permitting passage of UV light are recommended (e.g., UV-Star plate, flat bottom).
2. For the preparation of dilutions and the storage of recombinant proteins and membrane liposomes, tubes made of polypropylene are recommended, to reduce binding of the proteins to the tube wall. Glass and polystyrene tubes should not be used.
3. For batch metal affinity purification of 6His-tagged recombinant proteins, we found the disposable centrifuge columns (polypropylene (22 mL capacity) with polyethylene bottom filter (30 μm pore size) very useful.
4. Centrifugal concentrators, 10,000 molecular weight cutoff, 4- and 15-mL, for the concentration of all recombinant cytosolic components and buffer exchange.

2.3 Large and Small Equipment

1. Electronic single channel pipettors (range from 0.5 to 1,000 μL). These have a "dispensing" mode, very useful for adding small equal amounts of reagents to 96-well plates.
2. Multipette Plus (manual) or Multipette Stream (electronic) pipettors and various Combitips (Eppendorf).
3. Finnpipette digital 12-channel pipette (50–300 μL range) (Thermo Scientific).
4. Innova 4230 refrigerated incubator shaker (New Brunswick Scientific).
5. Refrigerated low-speed centrifuge (up to 7,000 × *g*), with a swing-out rotor (e.g., Sorvall RC-3B or RC-3C, and H-6000A rotor).
6. Refrigerated high-speed centrifuge (up to 48,000 × *g*), with fixed angle rotor (e.g., Sorvall RC-5 or RC-5 Plus, and SS-34 rotor).
7. Ultracentrifuge and fixed angle rotor (e.g., Beckman L5-50 ultracentrifuge and 60-Ti rotor).

8. High intensity ultrasonic processor (400- or 500-W) fitted with exchangeable regular and microprobe and cup horn.
9. Akta Basic 10 chromatography system, to be used for FPLC (GE Healthcare).
10. Rotating tube mixer (e.g., Rotamix RM1, ELMI).
11. XCell SureLock Mini-Cell for SDS-PAGE of mini-gels (Invitrogen, Life Technologies).
12. Electrophoresis power supply (e.g., Power Pac 300, Bio-Rad).
13. Microplate spectrophotometer (SPECTRAmax 340 or 190), preferably with PathCheck, fitted with SOFTmax PRO software (Molecular Devices, *see* **Note 9**).
14. Spectrophotometer (double-beam) UV/visible.
15. Thermomixer Comfort, rotary mixer and heater/cooler (Eppendorf).
16. Mini orbital shaker for 96-well plates.
17. Optical microscope (with 10× and 40× magnification objective lenses).

3 Methods

Cell-free assays are used in an almost limitless variety of forms and applications. In the original form of the assay, cytochrome b_{558} was represented by a total macrophage membrane preparation and the cytosolic components, by total cytosol [21]. In the variants developed later, a more sophisticated membrane preparation is utilized or the membrane is altogether replaced by purified relipidated cytochrome b_{558}. In all the assays to be described, we use a modified membrane preparation, originating from guinea pig peritoneal exudate macrophages, and the cytosol is replaced by purified and well-characterized recombinant proteins ($p47^{phox}$, $p67^{phox}$, and Rac). Although a number of anionic amphiphiles can act as activators in cell-free systems (*see* **Note 1** and refs. 21, 27), we shall limit our description to LiDS as the prototype activator. We describe two methods for the detection of ROS, based on the trapping of $O_2^{\cdot-}$ produced in the cell-free system by oxidized cytochrome *c* [68] or INT [71] and one method, based on the consumption of NADPH [80].

3.1 Cytochrome b_{558} (The Membrane Component)

3.1.1 Membrane Preparation

We here describe the preparation of membranes from elicited guinea pig peritoneal macrophages [27, 82]. Considering the paucity of granules in macrophages, differential centrifugation is not required to obtain granule-free pure plasma membrane preparations from these cells. Instead, we prepare a "total" membrane

fraction, defined by its sedimentation at 160,000 × *g*. The use of an uncharacterized membrane preparation is made possible by the fact that, in the cell-free assays to be described, the amounts of membrane added to the assay are based strictly on the cytochrome b_{558} content.

1. Inject guinea pigs (male or female, weighing 300–500 g) intraperitoneally with sterile light paraffin oil (15 mL per animal).
2. After 4 days, sacrifice the animals by CO_2 inhalation, and collect the peritoneal content via a 3-cm-long incision in the abdominal wall and repeated introduction and aspiration of 50-mL volumes of ice-cold Earle's balanced-salt solution.
3. Pass the collected peritoneal lavage through a 180 μm pore size nylon mesh sheet and collect in ice-cooled Erlenmeyer flasks (*see* **Note 10**).
4. Centrifuge fluid for 20 min at 940 × *g* and 4 °C to sediment the cells.
5. Repeat the procedure once more, and suspend the cell sediment in ice-cold distilled H_2O (20 mL to a cellular pellet derived from 400 mL lavage fluid) to lyse red cells.
6. After 3 min, add an equal volume of ice-cold 0.29 M NaCl solution in water (20 mL), resulting in an isotonic NaCl concentration (0.145 M), mix well, and recentrifuge at 940 × *g*, as above. If necessary, the lysis procedure can be repeated once more.
7. Resuspend the cell pellet in Earle's solution (10 mL per animal), and count cells after diluting the suspension 1:10 in 1 % v/v acetic acid in H_2O. The expected cell harvest varies from 1 to 2×10^8 cells per animal.
8. Pellet the cells at 940 × *g* and resuspend in sonication buffer at a concentration of 10^8 cells/mL in 16 × 100 mm polypropylene tubes (4 mL/tube).
9. Sonicate samples (400-W ultrasonic processor), keeping tubes immersed in ice-water, with the microprobe lowered into the cell suspension 2/3 of its entire depth. Submit cells to three cycles of sonic disruption at an amplitude of 10 %, each cycle consisting of sonication for 9 s, followed by a 1-s rest, repeated three times. Check for quality of cell disruption by phase-contrast microscopy at 40× magnification (~90 % cell disruption is expected).
10. Centrifuge the cell homogenate for 10 min at 3,000 × *g* and 4 °C in a swinging-bucket rotor to remove unbroken cells, aggregates, and nuclei. Collect the supernatant (postnuclear supernatant).

11. Centrifuge the supernatant for 2 h at 160,000 × *g* and 4 °C. The supernatant from this step is collected and represents the cytosol. If an ultracentrifuge is not available, centrifuge for 4 h at 48,000 × *g* in a high-speed centrifuge.
12. Resuspend the membrane pellet in sonication buffer supplemented with 1 M KCl (*see* **Note 11**) at a volume identical to the original volume of the homogenate. Resuspend directly in the ultracentrifuge tubes (kept in ice-water) by adding buffer with 1 M KCl in small aliquots (1–2 mL) and very briefly and gently sonicate after each addition, using a microprobe, until all the membrane is suspended (*see* **Note 12**).
13. Centrifuge the mixture for 2 h at 160,000 × *g* and 4 °C.
14. Discard the supernatant and freeze the membrane pellet at –75 °C. The membranes can be kept frozen indefinitely for future use. We found no reason for flash freezing or keeping membranes at a lower temperature.

3.1.2 Preparation of Membrane Liposomes

Although membrane preparations obtained as described above are quite adequate for use, we routinely use solubilized membrane preparations consisting of liposomes of uniform size as our source of cytochrome b_{558} in cell-free assays. Liposomes are obtained by solubilizing membranes with octyl glucoside and then removing the detergent by dialysis [67, 82].

1. Suspend frozen membranes in ice-cold solubilization buffer at a concentration of 5×10^8 cell equivalents/mL using the original ultracentrifuge tubes as containers.
2. Stir suspension with a small magnetic bar by placing the tube in a beaker containing ice-water. Continue solubilization until no intact membrane fragments remain. This might take 3–6 h, and it is important to keep the tubes ice-cooled throughout this period.
3. Centrifuge the solution for 1 h at 48,000 × *g* and 4 °C in a fixed angle rotor.
4. Transfer supernatant containing the solubilized membrane into a fresh tube (appears as a pale-yellow opalescent solution). Discard pellet.
5. Place solution in dialysis tubing with a 25,000 molecular weight cutoff (*see* **Note 13**), and dialyze against a 100-fold excess of detergent-free solubilization buffer for 18–24 h at 4 °C (*see* **Note 14**). The dialyzed detergent-free solubilized membrane consists of liposomes, 250–300 nm in diameter.
6. Measure the concentration of cytochrome b_{558} as described below.
7. Supplement the preparation with 10 μM FAD, divide into aliquots of 1–1.5 mL, and store at –75 °C (*see* **Note 15**). The

preparation is now ready to be used in all forms of cell-free assays (*see* **Note 16**).

3.1.3 Quantification of Cytochrome b_{558} Content

The results of cell-free assays are commonly expressed in turnover values (mol $O_2^{\bullet-}$ produced per time unit [s] per mol cytochrome b_{559} heme). Thus, it is essential that the cytochrome b_{558} content of membranes is known. Cytochrome b_{558} content is expressed by heme concentration.

1. Dilute membrane liposome preparation in solubilization buffer without octyl glucoside and FAD (1:5 or 1:10 dilution). Place diluted samples in spectrophotometer cuvette (*see* **Note 17**).
2. Place cuvette in the sample compartment of a double-beam spectrophotometer, and place a cuvette containing the buffer used for diluting the membrane preparation in the reference compartment.
3. Run an absorption spectrum scan, from 400 to 600 nm, using a band-width of 1 nm, a scanning interval of 1 nm, and a scanning speed of 100 nm/min (*see* **Note 18**). Save this as "oxidized spectrum" in the computer linked to the spectrophotometer.
4. Add a few grains of sodium dithionite, rapidly mix, and run a spectral scan again. Save this as "reduced spectrum."
5. Subtract the "oxidized spectrum" from the "reduced spectrum," and save the resulting "reduced *minus* oxidized spectrum."
6. Detect and record absorbance values of major cytochrome b_{558} peaks, located at 558/559, 529, and 426/427 nm. Detect and record absorbance at the "valley," at 410/411 nm.
7. Calculate heme content, based on the Δ extinction coefficient of the 427 nm peak/411 nm valley pair, using $\Delta\varepsilon_{427-411\ nm} = 200\ mM^{-1}\ cm^{-1}$ [94] (*see* **Note 19**).
8. Normally, we obtain a concentration of cytochrome b_{558} heme of 400 pmol/10^8 cell membrane equivalents. Thus, the membrane liposome suspension of 5×10^8 cell membrane equivalents/mL has a heme concentration of 2 μM (*see* **Note 20**).

3.2 Cytosolic Components

All recombinant cytosolic components are expressed in *E. coli* as N-terminal 6His-tagged proteins. The methodology is described elsewhere [54]. The key steps are briefly summarized below:

3.2.1 Preparation of Recombinant $p47^{phox}$, $p67^{phox}$, and Rac1

1. Transform *E. coli* competent cells (Rosetta 2(DE3)pLysS) with the expression vector pET-30a-6His Kan^R, carrying cDNAs encoding each of the three cytosolic components, following a standard protocol (pET System Manual, 11th edition, Novagen, Merck KGaA).

2. Induce bacteria with 0.4 mM IPTG and grow at 18 °C for 14–16 h in a refrigerated incubator shaker. Induction at 18 °C is important for maximizing the recovery of the recombinant proteins in the soluble fraction after disruption of the bacteria.
3. Sediment bacteria by centrifugation at 3,500 × *g*, resuspend in lysis buffer, supplement with Complete EDTA-free and 1 % v/v Triton X-100 (40 mL for 1 L bacterial culture),
4. Sonicate sample for 5 min in an ice-water-cooled glass beaker, using a 500-W ultrasonic processor with a 12-mm diameter probe, at an amplitude of 20 %, and alternating cycles consisting of sonication for 2 s, followed by a 2-s rest.
5. Centrifuge at 48,000 × *g* for 30 min and decant supernatant containing the soluble protein.
6. Precipitate DNA with PEI at a final concentration of 0.3–0.4 % (w/v) for cationic proteins [$p47^{phox}$, Rac1, and $p67^{phox}$(1-212)] or 0.05 % for anionic proteins ($p67^{phox}$) [95].
7. Sediment DNA–PEI precipitate by centrifugation at 48,000 × *g* at 4 °C for 30 min. Use supernatant for purification on Ni Sepharose.
8. Measure protein concentration by the Bradford assay [93] modified for use with 96-well microplates (*see* Bio-Rad Technical Bulletin 1177 EG and **Note 21**).

3.2.2 Purification of Recombinant Cytosolic Components

Recombinant cytosolic components are purified in two stages. First, by batch affinity metal chromatography on Ni Sepharose, and second, by preparative FPLC gel filtration.

1. Mix 3 mL of washed packed Ni Sepharose beads with soluble fraction derived from sonic disruption of bacteria from 1 L culture. Incubate for 1 h at room temperature with top/bottom rotation using a rotating tube mixer at 10 RPM.
2. Transfer contents into a centrifuge column and allow the fluid to flow by gravity.
3. Wash beads twice with 15 mL volumes of binding buffer and twice with 15 mL volumes of washing buffer.
4. Add 10 mL of column elution buffer, seal bottom and top apertures, and incubate for 30 min at room temperature with top/bottom rotation using a rotating tube mixer at 10 RPM.
5. Allow the eluate to run by gravity into a collecting tube and repeat procedure once or twice.
6. Measure protein concentrations in all eluates and analyze by SDS-PAGE for purity. The purity requirements for the performance of cell-free assays are easily achieved by purification on Ni Sepharose. Purity ≥90 % is adequate, and the only problem

encountered with lower purity preparations is that the actual concentrations of the components present in the assay cannot be accurately determined.

7. When high purity preparations are desired, proceed to purification by gel filtration on HiLoad 16/60 Superdex 200 prep grade for $p67^{phox}$ and tripartite [$p47^{phox}$-$p67^{phox}$-Rac1] chimeras, and HiLoad 16/60 Superdex 75 prep grade for $p47^{phox}$, $p67^{phox}$(1-212), Rac1, and [$p67^{phox}$-Rac1] and [$p47^{phox}$-$p67^{phox}$] bipartite chimeras. The reason for using Superdex 200 for gel filtration of full-length $p67^{phox}$ is the non-globular nature of the protein, which causes its elution at an apparent molecular weight much higher than 67,000 [96].
8. Concentrate eluates from Ni Sepharose to a volume of 2.5–5 mL by centrifugal filters and inject onto the column using PBS buffer and a flow rate of 1 mL/min. Keep the column refrigerated. Record absorbance at 280, and collect 1 mL fractions. Analyze fractions by SDS-PAGE and pool those of highest purity.
9. Supplement purified recombinant proteins with 20 % v/v glycerol, divide in small aliquots in polypropylene tubes with low protein binding quality (Eppendorf, Protein LoBind) and store at –75 °C. Avoid repeated thawing–refreezing. In this state, they are active for an unlimited time period (*see* **Note 22**).

3.2.3 The Preference for Rac1

Rac2 is the predominant form of Rac in neutrophils [97], whereas monocytes and macrophages use Rac1 in oxidase activation [43, 98, 99]. The oxidase can be activated in the cell-free system by both Rac1 and Rac2 in their nonprenylated forms. However, one should be aware that this is an artifact, since nonprenylated Rac does not exist, as such, in phagocytes. Translocation to the membrane of nonprenylated Rac is dependent exclusively on the net positive charge of the polybasic domain at the C-terminus. Because Rac1 contains six contiguous basic residues in this domain whereas Rac2 contains only three that are only partially contiguous, nonprenylated Rac1 is much more active in the cell-free system than Rac2 [8, 54, 100]. Native, recombinant Rac1 contains exclusively GDP [101, 102]. Thus, before use in cell-free assays, subject Rac1 to nucleotide exchange with a non-hydrolyzable GTP analog (GMPPNP or GTPγS; for choosing between the two analogs *see* **Note 23**). Recently, we have started to use predominantly the Rac1 mutant Q61L, which is constitutively in the GTP-bound form [103].

3.2.4 Nucleotide Exchange on Rac

The procedure described here is for exchange to GMPPNP, but the same procedure is used for exchange to GTPγS. It is based on the removal of bound endogenous GDP by chelation of Mg^{2+} and replacement of the bulk of GDP by the GTP analog.

1. Decide on the size of the batch of recombinant Rac that is to be to subject to guanine nucleotide exchange. For use in cell-free assays, we normally perform exchange on aliquots of 10–20 nmol of Rac in Tris–HCl buffer. Place in 1.5- or 2-mL Eppendorf polypropylene tube.
2. Add GMPPNP stock solution in a quantity representing a ten-fold molar excess over the amount of Rac. For example, if you intend to perform exchange on 10 nmol of Rac, add 100 nmol of GMPPNP (10 µL from the 10 mM stock solution).
3. Add EDTA solution to a final concentration of 12.5 mM. Incubate for 30 min at 30 °C in a rotary mixer set at 600 rotations/min.
4. Stabilize the exchanged state of Rac by adding $MgCl_2$ solution to a final concentration of 25 mM.
5. Store exchanged protein frozen at –75 °C (*see* **Notes 24** and **25**).

3.2.5 Prenylation of Rac In Vitro

In the past, 6His-tagged Rac1 was cloned into the baculovirus genome, and this recombinant virus was used to infect cultures of Sf9 cells [9]. In this procedure, prenylated Rac was expressed in the cell membrane and thus had to be purified following membrane solubilization. This procedure was replaced by a much simpler method using in vitro enzymatic prenylation of nonprenylated recombinant Rac1 [53].

1. Add 10 nmol of nonprenylated Rac (*see* **Note 26**) to a 1.5 or 2 mL polypropylene Eppendorf tube.
2. Add 10 µL (20 nmol) of geranylgeranyl pyrophosphate stock solution, 10 µL (10 U) of geranylgeranyl transferase I stock solution, and prenylation buffer containing $ZnCl_2$ (*see* **Note 27**) to a final volume of 0.9 mL.
3. Incubate for 45 min at 37 °C in a rotary mixer set at 600 rotations/min (*see* **Note 28**).
4. Add 60 µL of 70 mM octyl glucoside in H_2O (final octyl glucoside concentration is 4.375 mM), and reincubate for 45 min under the same conditions as above.
5. Sonicate the protein in a 400-W ultrasonic processor fitted with a cup horn filled with ice-water for five cycles of 10 s each at 50 % amplitude.
6. Add 0.24 mL of glycerol to bring the final volume to 1.2 mL. The final concentrations of components are 8.33 µM Rac, 3.5 mM octyl glucoside, and 20 % v/v glycerol (this does not take into account the glycerol carried into the reaction by nonprenylated Rac itself).
7. Prenylated Rac can be stored at –75 °C. After thawing it should be centrifuged for 15 min at 10,000 × *g* to check for the presence of aggregates. If sediment is found, use the supernatant only after measuring its protein content.

3.2.6 Checking the Degree of Rac Prenylation

Prenylation in vitro is a very reliable methodology provided that a trusted source of geranylgeranyl transferase I is available. Nevertheless, it is recommended that until enough experience is acquired, the degree of prenylation should be checked [104].

1. Remove an aliquot from the completed prenylation mixture before adding glycerol. Since the final detection method is based on SDS-PAGE, one has to remove sufficient protein to make detection easy. Normally, one third of a 10 nmol Rac prenylation mixture is used for confirming prenylation (about 3 nmol Rac). The procedure is best performed in 1.5 mL conical microcentrifuge tubes.
2. Add prenylation buffer up to a total volume of 0.9 mL.
3. Add 0.1 mL of 10 % Triton X-114 (1 % final concentration).
4. Place the tube in ice-water for 30 min, vortexing the tube every 5 min.
5. Heat the mixture at 37 °C for 10 min in a heating block or a water bath. Keep the tubes stationary—do not mix. This will cause the solution to become cloudy due to aggregation of Triton X-114 above its cloud point. Amphiphilic proteins, such as prenylated Rac, will associate with the detergent aggregates, whereas nonprenylated Rac will remain in the aqueous phase.
6. Centrifuge the mixture at 10,000–12,000 × *g* in a microfuge at room temperature. This will result in phase separation with the upper (aqueous) phase containing nonprenylated Rac and the lower (detergent-enriched) phase containing prenylated Rac. Transfer upper phase into a fresh tube.
7. Add prenylation buffer to the lower phase to make the total volume equal to that of the upper phase, and mix well.
8. Take equal sample volumes from the two phases and subject to SDS-PAGE.
9. Compare intensity of the Rac bands (21 kDa) in the two phases visually or by densitometry (*see* **Note 29**).

3.3 An Overview of Cell-Free Assay Design

For the proper application of cell-free assays, it is essential to recall a number of theoretical considerations, as outlined below.

1. The $O_2^{\bullet-}$-producing component is cytochrome b_{558} found in the membrane and results are to be related to the amount of cytochrome b_{558} present in the reaction.
2. All cytosolic components must be present at saturating quantities in relation to cytochrome b_{558}. These quantities are determined with dose–response experiments in which the concentration of one or all cytosolic components is varied in the presence of a constant amount of cytochrome b_{558} (membrane).
3. The amphiphile-independent cell-free system is a very useful variant of the canonical system, with specific applications in

situations in which the emphasis is on interaction between Nox2 and $p67^{phox}$. In spite of the fact that it was described more than a decade ago [9], and is technically simple, it has not gained wide acceptance.

4. Normally, amphiphile-dependent cell-free assays are performed with nonprenylated Rac1. However, identical results are obtained with prenylated Rac1, provided that $p47^{phox}$ and $p67^{phox}$ are present in the reaction. In the absence of $p47^{phox}$, amphiphile exerts a paradoxical inhibitory effect in cell-free assays containing prenylated Rac and $p67^{phox}$ [52].
5. All cell-free assays comprise two stages: (a) the stage of oxidase complex assembly, in the course of which cytosolic components translocate to the membrane, leading to the induction of conformational change in Nox2, and (b) the catalytic stage, initiated by the addition of NADPH, resulting in the production of $O_2^{\bullet-}$. In some forms of cell-free assay, the two stages are separated by the interruption of assembly just before the initiation of catalysis. In most assays, the assembly merges with the catalytic stage, although an effort is usually made to bring assembly to completion before the addition of NADPH.
6. Amphiphile-dependent oxidase assembly is time- and temperature-dependent (*see* **Note 30**).
7. Kinetic models of anionic amphiphile-induced oxidase assembly have been proposed both before [105, 106] and after [107] the identification of the components of the oxidase. These contain useful information, which, paradoxically, had relatively little influence on the design and methodological aspects of cell-free assays.
8. Oxidase activation in cell-free systems is reduced by an increase in the ionic strength of the assay buffer (*see* **Note 31**).

3.4 The Canonical Amphiphile-Dependent Cell-Free Assay: "Don't Leave Home Without It"

We describe here the basic methodology for performing cell-free oxidase activation in what is called the "semi-recombinant" system. This is a modification of the original amphiphile-activated (membrane + cytosol) system [21–24, 27]. Since measuring ROS production by phagocytes by microplate spectrophotometers became the standard procedure [108, 109], spectrophotometric kinetic methodology in microtiter plates also became the routine procedure for the performance of cell-free assays. This required adjustment of the assay from the 1–3 mL volumes, used in standard spectrophotometers, to 100–300 μL volumes used with 96-well microtiter plates (96-well plates). We describe a kinetic cell-free oxidase activation assay performed in 96-well plates in which the reaction components comprise solubilized macrophage membrane liposomes, recombinant $p47^{phox}$, $p67^{phox}$, and nonprenylated Rac1.

3.4.1 Cytochrome c Reduction

1. Add 20 μL of solubilized membrane liposomes (50 nM cytochrome b_{558} heme) to the wells of a 96-well plate. This is intended to result in a final concentration of 5 nM cytochrome b_{558} heme in 200 μL (the total volume of the reaction before addition of NADPH) and equals 1 pmol cytochrome b_{558} heme/well.
2. Add 20 μL of a mixture of $p47^{phox}$, $p67^{phox}$, and nonprenylated Rac1-GMPPNP (or Rac1 Q61L mutant), each at a concentration tenfold higher than that desired as the final concentration in 200 μL. As an example, if a final concentration of 100 nM is to be achieved for all three components, add 20 μL of a solution containing 1 μM of each component (*see* **Note 32**). Make all dilutions of membrane and cytosolic components in oxidase assay buffer without LiDS. Dispensing 10 or 20 μL aliquots of membrane or cytosolic components to the wells is best performed with electronic pipettors, in the dispensing mode, or with multichannel pipettors.
3. Add 160 μL/well of assay buffer containing an optimized concentration of LiDS. We typically use a digital 12-channel pipette. For this protocol, the final concentration of LiDS causing maximal activation is 120–130 μM (Fig. 2) (*see* **Note 33**). Because the amphiphile is diluted 1.25-fold by the volumes of

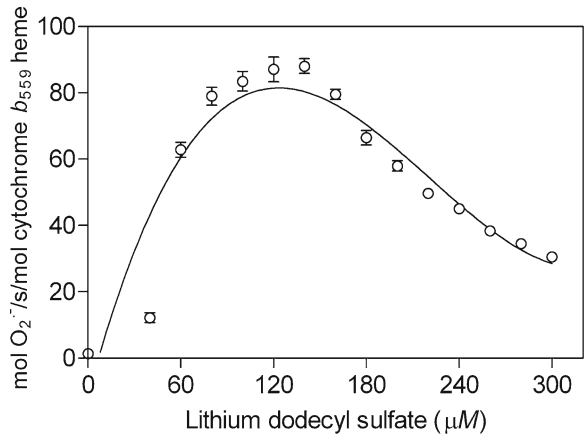

Fig. 2 Dose–response curve of lithium dodecyl sulfate (LiDS) in the amphiphile-dependent cell-free system. Assay mixtures consisting of solubilized macrophage membrane liposomes (5 nM cytochrome b_{558} heme) and recombinant $p47^{phox}$ (100 nM), $p67^{phox}$ (100 nM), and non-prenylated Rac1-GMPPNP (100 nM) were incubated with varied concentrations of LiDS as indicated. $O_2^{\cdot-}$ production was initiated by the addition of NADPH and measured by the kinetic cytochrome *c* reduction assay for 5 min. Results represent means ± SE of three experiments. Reproduced from [142] by permission of Humana Press©2007

membrane and cytosolic components previously added to the wells, the concentration of the amphiphile in the assay buffer has to be adjusted accordingly (as an example, to achieve a final concentration of 130 μM, add 160 μL of a 162.5 μM solution).

4. Place the plate on an orbital shaker and mix contents for 90 s at 500–600 movements/min and room temperature (*see* **Note 34**).
5. Dispense 10 μL of NADPH/well using a multichannel pipettor, as fast as possible. This results in a final concentration of 238 μM NADPH in a total volume of 210 μL per well, which is well above the K_m for NADPH of the oxidase in the cell-free system [21].
6. Transfer the plate quickly to the microplate spectrophotometer and mix contents for 5 s using the mixing option of the reader. The instrument should be set to record increase in absorbance at 550 nm over a time period of 5 min with 28 readings being executed at 0.11 min intervals at room temperature (temperature regulation by the microplate spectrophotometer is set "off"). Include blank wells containing 200 μL assay buffer to which 10 μL of 5 mM NADPH were added simultaneously with its addition to the sample wells.
7. For most instruments, results in the kinetic mode are expressed in $Abs_{550\ nm}$/min units. The software of the instrument calculates these values by dividing the ΔAbs_{550nm} or, preferably, $\Delta mAbs_{550nm}$ over time, by the number of minutes elapsed. Thus, it is essential for the increase in absorbance curve to be linear. The curves turn nonlinear whenever one or more components of the reaction is/are exhausted. Although every effort is made to prevent this from occurring, by choosing the right amounts of enzyme (cytochrome b_{558} in the membrane), cytochrome *c*, and NADPH per well (*see* **Note 35**), it occasionally happens (*see* Fig. 3). In this case, the linear portion of the curve is chosen, and values are recalculated as $\Delta mAbs_{550nm}$/min for the revised time interval (shorter than 5 min). Contemporary microplate readers are fitted with the appropriate software, allowing fast and simple recalculation of the slopes after selecting the linear segment. $\Delta mAbs_{550nm}$/min values are transformed to nnmol cytochrome *c* reduced per min per well content of 210 μL, based on the extinction coefficient $\Delta E_{550} = 21\ mM^{-1}\ cm^{-1}$ for reduced minus oxidized cytochrome *c* (nmol $O_2^{\bullet-}$/min/well) (*see* **Note 36**).
8. Express the final results as "turnover": the amount of $O_2^{\bullet-}$ produced per time unit per mol membrane cytochrome b_{558} heme (mol $O_2^{\bullet-}$/s/mol cytochrome b_{558} heme; *see y*-axis of graphs in most figures in this chapter). This is easily calculated by knowing the nmol $O_2^{\bullet-}$/min/well values and the amount of cytochrome

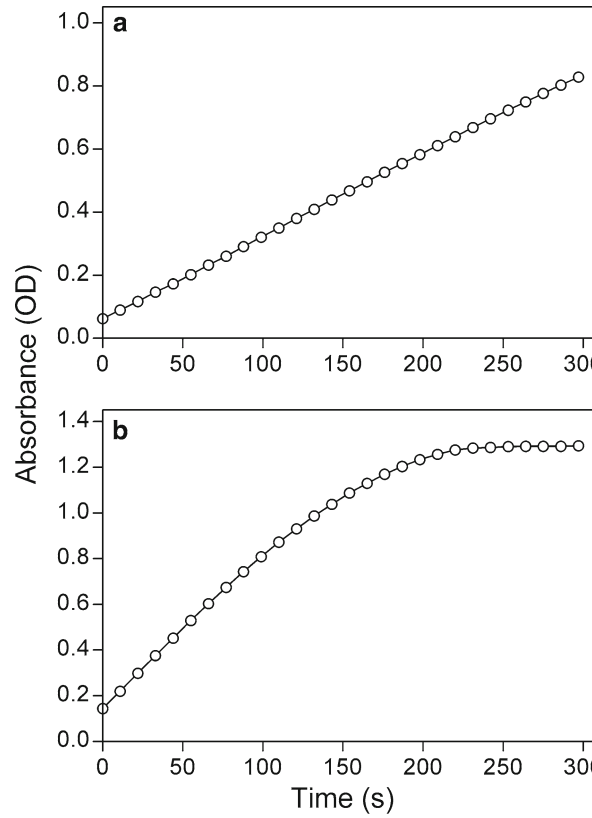

Fig. 3 Actual data displays of the results of two kinetic cell-free assays, showing the increase in absorbance at 550 nm over a 300-s interval. (**a**) Increase is linear throughout the 300 s period. (**b**) Increase is linear up to 120 s, after which time it starts leveling off. In this situation, the ΔAbs_{550nm}/min should be recalculated for the first 120 s. Turnover values were found to be 41 mol $O_2{\cdot}^-$/s/mol cytochrome b_{558}, for panel **a**, and 106 mol $O_2{\cdot}^-$/s/mol cytochrome b_{558}, for panel **b** (recalculated value). Reproduced from [142] by permission of Humana Press©2007

b_{559} heme per well (1 pmol, when 20 μL of membrane, containing 50 nM of cytochrome b_{558} heme, are added per well). For each experimental condition, perform the assay in triplicate wells and make the software calculate mean values and standard deviations (*see* **Note 37**).

9. It is essential to include SOD control wells in cell-free assays to assure that the reduction of cytochrome *c* is indeed due to $O_2{\cdot}^-$. This requires that parallel SOD-containing wells are included for every group of wells in which $O_2{\cdot}^-$ production is detected. Use a large excess of 100 U SOD/mL by adding 10 μL/well of a 2,000 U/mL of SOD solution before the addition of NADPH. Addition of SOD is expected to prevent cytochrome *c* reduction by 95 % or more (*see* **Note 38**).

10. A number of additional control reactions are requirements for the proper execution of cell-free assays, and no assay is complete without the inclusion of reactions wells in which one of the following components is omitted (i.e., anionic amphiphile, NADPH, membrane, and all or each of the individual cytosolic components).

3.4.2 INT Reduction

Cytochrome *c* reduction can be replaced by INT reduction. The method is identical to that described under Subheading 3.4.1, with the exception that the assay buffer contains 100 μM INT instead of cytochrome *c* (*see* **Note 39** for converting increase in absorbance at 490 nm data to $O_2^{\bullet-}$ values).

Three problems are related to the use of the INT technique:

1. The first is the possibility that INT is reduced by electrons originating from reduced Nox2-bound FAD ($FADH_2$) [71]. In our hands, however, when using the canonical semi-recombinant amphiphile-activated cell-free system, at least 80 % of INT reduction is SOD-sensitive and, thus, mediated by $O_2^{\bullet-}$.
2. Second, SDS and LiDS react with INT, forming an unidentified material that absorbs at 490 nm. We have no experience with using C20:4 to replace the anionic detergents.
3. Third, in our hands, INT is about 50 % less effective than cytochrome *c* in detecting $O_2^{\bullet-}$ production in the canonical cell-free assay, under strictly identical conditions. The reason for this is unclear.

INT reduction should, therefore, be used predominantly for measuring the diaphorase activity of the DHR of Nox2 [72–74] and, possibly, other Noxes [70, 75].

3.4.3 NADPH Consumption

The method is similar to that described under Subheading 3.4.1, with the exception that the assay buffer contains no electron acceptor and a negative slope, corresponding to the conversion of NADPH to $NADP^+$, is recorded [80] (*see* **Note 40** for converting *decrease* in absorbance at 340 nm data to $O_2^{\bullet-}$ values). As in the cytochrome *c* and INT assays, the catalytic phase of the reaction is initiated by the addition of NADPH to the wells. A number of issues are to be taken into consideration:

1. The technique is useful when there is evidence for interference by a component of the cell-free reaction with electron acceptors, as illustrated previously for INT [71] and cytochrome *c* [81], or in the presence of a reducing agent.
2. It is ideal for use with amphiphile-dependent semi-recombinant cell-free systems, in which the presence of contaminating NADPH reductases is unlikely. Even in their presence, the absolute dependence on an amphiphile activator makes the assay applicable.

3. The sensitivity of the assay is comparable to that based on cytochrome *c* reduction.
4. A possible limitation is the requirement for microplates allowing passage of UV light.

3.5 Amphiphile-Independent Cell-Free Assays

The ability to activate the oxidase in vitro in the absence of an anionic amphiphile was first reported by Sumimoto et al. [50], based on C-terminal truncation of both $p47^{phox}$ and $p67^{phox}$. Amphiphile-independent systems were also described by Tamura et al. [38], using a chimeric construct consisting of truncated $p67^{phox}$ and $p47^{phox}$, and by Kleinberg et al. [51], who prevented the establishment of intramolecular bonds in $p47^{phox}$, by mutagenesis. The two latter groups and we [42] also observed that acidification of the membrane phospholipid environment made the presence of an anionic amphiphile unnecessary.

A conceptually distinct situation, in which oxidase activation can be achieved in the absence of amphiphile and of $p47^{phox}$, is represented by a cell-free system consisting of membrane liposomes, $p67^{phox}$, and prenylated Rac [9]. We proposed that proper targeting of $p67^{phox}$ to the membrane in conjunction with the induction of a conformational change in $p67^{phox}$ by Rac is sufficient for the initiation of electron flow in Nox2 [7, 9, 53]. Variations of this system include activation by combinations of $p67^{phox}$, prenylated Rac, GTP, and a Rac GEF [55], and the recently described amphiphile-independent oxidase activation by $p67^{phox}$ and prenylated [Rac-RhoGDI] complexes [57, 58].

We describe two methods for amphiphile-independent cell-free oxidase activation. One assay is based on the use of prenylated Rac and does not require the participation of $p47^{phox}$; the other makes use of our ability to modify the charge of phospholipids in phagocyte membranes and works with nonprenylated Rac.

3.5.1 Amphiphile-Independent Cell-Free Oxidase Activation in Mixtures of Membrane, $p67^{phox}$, and Prenylated Rac1

The amphiphile-independent cell-free system is useful for investigating the role of Rac and Rac-$p67^{phox}$ interaction in oxidase assembly. This particular aspect of assembly is more difficult to explore in the presence of $p47^{phox}$, which has not only an assembly-initiating function but also a role in the stabilization of the assembled complex [41]. Other situations in which the amphiphile-independent cell-free system is the assay of choice are when the effects of regulators of Rac are to be explored in vitro. One example is provided by Rac GEF-dependent oxidase activation in a cell-free system consisting of membrane, $p67^{phox}$, prenylated Rac1-GDP, GTP, and a Rac GEF, such as Trio or Tiam1 [55]. Another example is the ability of [prenylated Rac1-RhoGDI] complexes in conjunction with $p67^{phox}$, to activate the oxidase when added to phagocyte membrane liposomes enriched in anionic phospholipids [57] or specific phosphoinositides, in the presence of GTP and a GEF [58], in the absence of amphiphile.

Applications of amphiphile-independent cell-free assays also comprise the study of inhibitors (proteins, peptides, phospholipids, nucleotides, detergents, drugs) on the various stages of oxidase assembly. An example is the study of the effect of the amphiphilic activator LiDS on oxidase activation by $p67^{phox}$ and prenylated Rac1-GMPPNP, in the absence of $p47^{phox}$. We found LiDS to exert a marked dose-dependent inhibitory effect, in the 25–200 μM concentration range, which was relieved by the presence of $p47^{phox}$ [52]. Further examples are the distinct effects of a number of compounds (GTP and GDP, a C-terminal Rac1 peptide, RhoGDI, the p21-binding domain of p21-activated kinase (PBD of PAK), and neomycin sulfate) on amphiphile-dependent and -independent cell-free oxidase assembly, reflecting the existence of different pathways of assembly [52].

1. Subject Rac1 to nucleotide exchange with GMPPNP or use Rac1 Q61L mutant. It is preferable to perform nucleotide exchange before prenylation. This will reduce possible loss of prenylated protein during exchange by binding to surfaces due to hydrophobicity.
2. Prenylate Rac1-GMPPNP, as described above.
3. Add 20 μL/well of solubilized membrane liposomes (50 nM cytochrome b_{558} heme) to the wells of a 96-well plate. This is intended to result in a final concentration of cytochrome b_{558} heme of 5 nM in 200 μL (the total volume of the reaction, before the addition of NADPH) and equals 1 pmol cytochrome b_{558} heme/well.
4. Add 20 μL of a mixture of $p67^{phox}$ and prenylated Rac1-GMPPNP, each at a concentration tenfold higher that that desired as the final concentration in 200 μL. If the requirements of the experiment are to add each component separately, add 10 μL of each component from a 20-fold concentrated stock solution. All dilutions of membrane and cytosolic components are made in assay buffer without LiDS (*see* **Note 41**).
5. Add 160 μL/well of assay buffer without LiDS using a digital 12-channel pipette. Place the plate on an orbital shaker and mix for 90 s at 500–600 movements/min and room temperature (*see* **Note 42**).
6. Dispense 10 μL of NADPH solution/well using an electronic pipettor, in the dispensing mode, or a multichannel pipette. This results in a final concentration of 238 μM NADPH in a total volume of 210 μL per well.
7. Record activity and convert to turnover values as described for the amphiphile-dependent system (*see* Subheading 3.4). An example of such an assay, with the required control mixtures, is illustrated in Fig. 4.

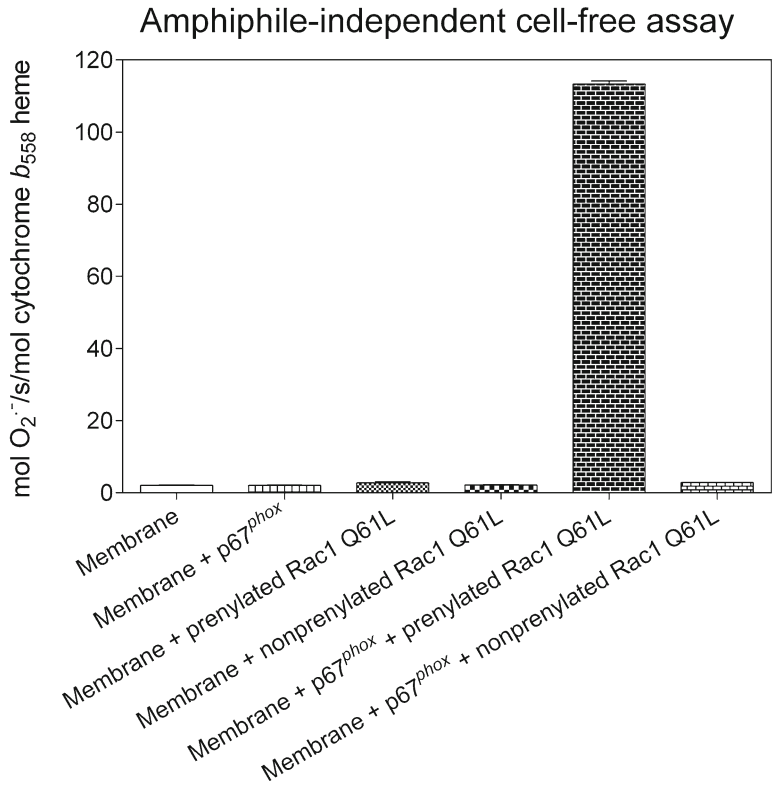

Fig. 4 Typical amphiphile-independent cell-free assay. The complete reaction mixture contained solubilized macrophage membrane liposomes (5 nM cytochrome b_{558} heme), recombinant p67^{phox} (300 nM), and recombinant Rac1 Q61L prenylated in vitro (300 nM). The contents were incubated without amphiphile for 5 min at room temperature. $O_2^{\cdot-}$ production was initiated by the addition of NADPH (238 μM) and measured by the kinetic cytochrome *c* reduction assay for 5 min. The compositions of the control (incomplete) assay mixtures are indicated on the *x*-axis. Results represent means ± SE of three experiments

3.5.2 Amphiphile-Independent Cell-Free Oxidase Activation in Mixtures of Negatively Charged Membrane, p47^{phox}, p67^{phox}, and Nonprenylated Rac1

Preparing Membrane Liposomes Enriched In Anionic Phospholipids

1. Dilute solubilized macrophage membrane in solubilization buffer containing 40 mM octyl glucoside to a concentration of cytochrome b_{558} heme of 1.2 nmol/mL.
2. Add PA or PG, both at a concentration of 5 mM, at a ratio of one part membrane and four parts phospholipids. This results in a final concentration of 240 pmol/ml cytochrome b_{558} heme and 4 mM anionic phospholipids.
3. Dialyze the membrane-phospholipid mixture (*see* **Note 13**) against a 100-fold excess of detergent-free solubilization buffer (also lacking AEBSF and FAD) for 18–24 h at 4 °C (*see* **Note 14**).
4. Measure the concentration of cytochrome b_{558}, and supplement the preparation with 10 μM FAD.
5. Divide into aliquots of 1–1.5 mL and store at –75 °C.

Amphiphile-Independent Cell-Free Oxidase Activation with Anionic Membrane Liposomes

This cell-free assay is a hybrid between the canonical amphiphile-dependent system (from which it borrowed the anionic charge requirement and the fact that Rac is nonprenylated) and the amphiphile-independent assay (based on the use of prenylated Rac).

1. Add 20 μL/well of membrane liposomes enriched in PA or PG (50 nM cytochrome b_{558} heme and about 0.8 mM anionic phospholipid) to the wells of a 96-well plate. This is intended to result in a final concentration of cytochrome b_{558} heme of 5 nM and close to 80 μM anionic phospholipid in 200 μL (the total volume of the reaction, before the addition of NADPH) and equals 1 pmol cytochrome b_{558} heme/well.
2. Add 20 μL of a mixture of $p47^{phox}$, $p67^{phox}$, and nonprenylated Rac1-GMPPNP or Rac1 Q61L mutant, each at a concentration tenfold higher that that desired as the final concentration in 200 μL (*see* **Note 43**). All dilutions of membrane and cytosolic components are made in assay buffer without LiDS. In most situations, concentrations of $p47^{phox}$, $p67^{phox}$, and nonprenylated Rac required for reaching maximal activation in this system are higher than those customary in the canonical amphiphile-dependent assay.
3. Add 160 μL/well of assay buffer without LiDS. Place the plate on an orbital shaker and mix for 90 s at 500–600 rotations/min and room temperature.
4. Dispense 10 μL of NADPH solution/well. This results in a final concentration of 238 μM NADPH in a total volume of 210 μL per well.
5. Record activity and convert to turnover values as described for the amphiphile-dependent system.

3.6 Sense and Sensitivity in Cell-Free Assays

Here, we discuss a number of methodological issues related to the proper way of performing cell-free assays. Emphasis will be placed on untested or unproven assumptions and some "sacred cows" will be questioned.

3.6.1 LiDS, SDS, or Arachidonate?

1. Unless the purpose of performing the cell-free assay is to explore the oxidase activating capabilities of C20:4 itself or of C20:4 isomers or C20:4 oxidation products, there are few occasions justifying the use of C20:4 as an activator.
2. C20:4 acid activates the oxidase only in its ionized salt form, and stock solutions are tedious to prepare and are unstable. We recommend using LiDS or SDS as standard amphiphilic activators.
3. SDS is as good an activator as LiDS, but concentrated solutions of SDS must be kept at room temperature. LiDS and SDS yield more reproducible results than C20:4. We find no basis for the claim that C20:4 is to be preferred because it represents a more "physiologic" form of activation.

3.6.2 To Supplement or Not to Supplement?

1. Calcium: in early experiments, we found that activation was reduced by Ca^{2+} and moderately enhanced by the Ca^{2+} chelator EGTA [21] (*see* **Note 44**). We reinvestigated the necessity of Ca^{2+} chelation in the LiDS-activated and amphiphile-independent systems by examining the effect of EGTA, alone or in association with other supplements. As seen in Fig. 5a, b, EGTA had no enhancing effect on oxidase activation in both the amphiphile-dependent and -independent systems.
2. FAD: a flavin requirement was observed in the oxidase isolated from stimulated phagocytes [17], and, early in the development of the C:20-activated cell-free system, it was found that exogenous FAD enhanced activation [21]. The most likely explanation is that Nox2 lost the noncovalently bound FAD during preparation of membranes, leading to a need to reflavinate cytochrome b_{558}. Here, we compared cell-free oxidase activation in the presence and absence of 10 μM FAD in the assay buffer by using solubilized membrane liposomes, which are routinely supplemented with FAD. As apparent in Fig. 5, FAD enhanced both amphiphile-dependent (Fig. 5a) and amphiphile-independent (Fig. 5b) oxidase activation, the effect being more pronounced on the amphiphile-dependent activation (*see* **Note 45**).
3. Magnesium: a requirement for Mg^{2+} was described early in cell-free studies, and it was suggested that the metal interacted with a saturable oxidase component at a K_m of about 1 mM [105]. The identity of this component was not established at the time, but after the discovery of the involvement of Rac in oxidase assembly, it became common belief that the requirement for millimolar concentrations of Mg^{2+} was related to its role in preventing the dissociation of GTP from Rac [97]. As shown in Fig. 5, supplementation of the assay buffer with 1 mM Mg^{2+} enhanced oxidase activation in both the amphiphile-dependent (Fig. 5a) and -independent (Fig. 5b) systems. Higher concentrations of Mg^{2+} (up to 5 mM) were not more effective than 1 mM (results not shown). Combining supplementation with FAD with that with Mg^{2+} did not result in an additive or synergistic effect; activities were identical to those found with FAD alone. Also, combining supplementation with FAD or Mg^{2+} with EGTA, or adding all three supplements, had no additive or synergistic effect. The almost identical ability of FAD and Mg^{2+} to improve assembly and the lack of an additive or cooperative effect suggest that they act by the same mechanism, most likely related to the stability of the Nox2-FAD bond and not to that of the Rac-GTP bond (*see* **Note 46**).

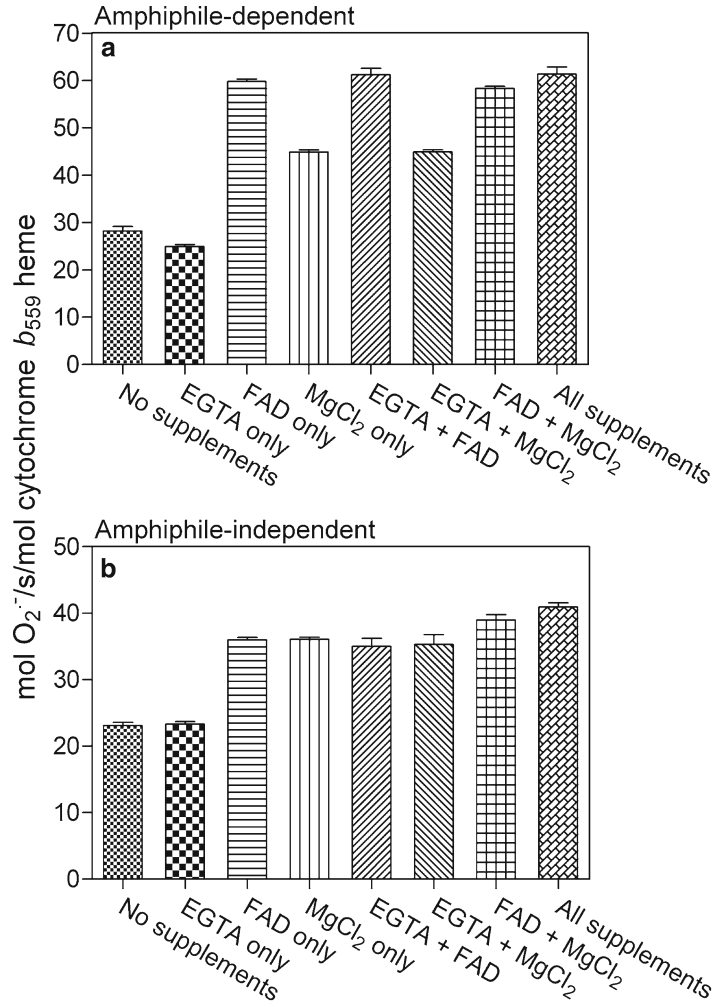

Fig. 5 Which supplements to the cell-free NADPH oxidase assay buffer are essential? Cell-free assays were performed in the canonical amphiphile-dependent system (**a**) and in the amphiphile-independent system, based on the use of prenylated Rac1 (**b**). The basic assay buffer was supplemented with 1 mM EGTA, 10 μM flavin adenine dinucleotide disodium salt, or 1 mM $MgCl_2$, or combinations of two or all three of these, as shown on the *x*-axis of panels **a** and **b**. (**a**) Amphiphile-dependent cell-free systems consisting of solubilized macrophage membrane liposomes (5 nM cytochrome b_{558} heme) and recombinant p47^{phox} (30 nM), p67^{phox} (30 nM), and non-prenylated Rac1-GMPPNP (30 nM) were incubated with 130 μM lithium dodecyl sulfate, as described. (**b**) Amphiphile-independent cell-free systems consisting of solubilized macrophage membrane liposomes (5 nM cytochrome b_{558} heme), recombinant p67^{phox} (300 nM), and recombinant Rac1-GMPPNP prenylated in vitro (300 nM*)* were incubated without amphiphile, as described. In both panels **a** and **b**, $O_2^{\cdot-}$ production was initiated by the addition of NADPH and measured by the kinetic cytochrome *c* reduction assay for 5 min. Results illustrated represent means ± SE of three experiments. Reproduced from [142] by permission of Humana Press©2007

3.6.3 "Measure for Measure": The Intricacies of Dose–Response Studies with Cytosolic Oxidase Components

1. Most cell-free oxidase activation assays follow the principle of a constant amount of membrane and variable amounts of cytosolic components. This leaves open the issue of quantitative relationships among cytosolic components (*see* **Note 47**).
2. A problem we frequently encountered when performing cell-free assays was determining the optimal methodology for relating activity turnover values to the amounts of cytosolic proteins added to a constant amount of membrane. Figure 6 summarizes the two main approaches used in our laboratory. In these experiments, the concentration of the membrane was constant. The concentrations of cytosolic components were either varied all in parallel or individually, in which case the other components were added at the maximal concentration in the range studied. Assays were run either in the amphiphile-dependent system (Fig. 6a) or in the amphiphile-independent system (Fig. 6b). In the amphiphile-dependent system, the concentration of LiDS was kept constant at 130 μM because the optimal activating concentration of LiDS did not vary with the concentration of cytosolic components within the 0–1 μM range when using purified recombinant cytosolic components.
3. It is apparent that when all components are varied in parallel, the dose–response curve has a sigmoidal shape, whereas when a single component is varied in the presence of an excess of the other component(s), the curves are hyperbolic. The highest levels of activation are seen when the concentrations of Rac1 and $p47^{phox}$ (amphiphile-dependent system) and Rac1 (amphiphile-independent system) are varied individually, in the presence of an excess of the other component(s); the lowest activities are found when $p67^{phox}$ is varied individually. The differences are particularly marked at lower concentrations of components.
4. When the purpose of performing cell-free assays is the detection of low concentrations of a cytosolic component, it is best to perform the assay in the presence of a clear excess of the other components, a situation generating hyperbolic curves (*see* **Note 48**).

3.6.4 To Exchange or to Add?

1. In the early period of the use of cell-free assays, it was reported repeatedly that the addition of GTP or non-hydrolyzable GTP analogs (GTPγS or GMPPNP) was an absolute requirement for the expression of oxidase activity. Many of these observations were made in cell-free systems consisting of membrane and total cytosol before the identification of Rac as the small GTPase involved in oxidase activation [110–113].
2. With the advent of the semi-recombinant systems, which involved the use of recombinant Rac1 or Rac2, the "habit" of

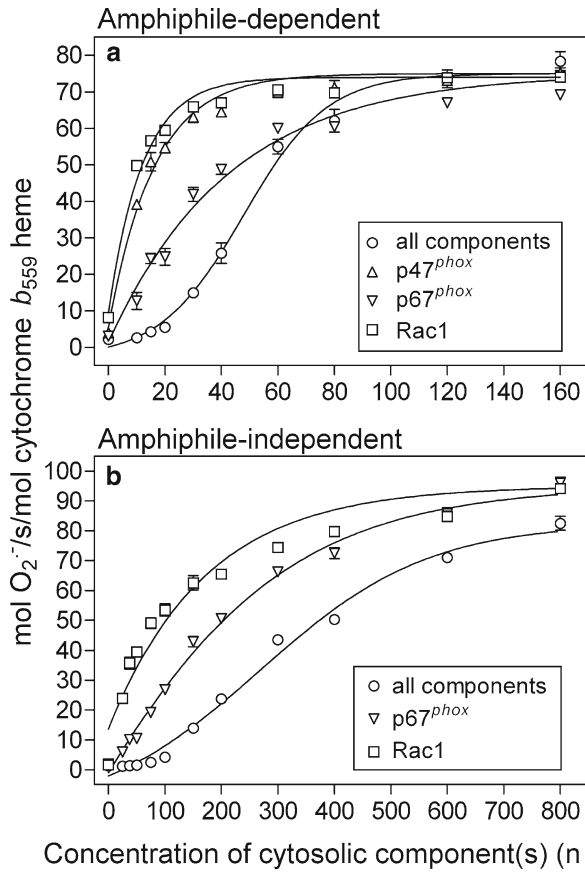

Fig. 6 The effect of concentration ratios among cytosolic components on the nature of the dose–response curves in cell-free assays. (**a**) Four types of amphiphile-dependent cell-free assays, consisting of various combinations of cytosolic components, were performed. All four consisted of solubilized macrophage membrane liposomes (5 nM cytochrome b_{558} heme), recombinant p47^{phox} (varied from 0 to 160 nM), and recombinant p67^{phox} (varied from 0 to 160 nM), and recombinant non-prenylated Rac1-GMPPNP (varied from 0 to 160 nM). The four combinations of components were: (1) All three cytosolic components were present at equal concentrations (varied from 0 to 160 nM); (2) p47^{phox} was varied from 0 to 160 nM, whereas p67^{phox} and Rac1 were both present at a concentration of 160 nM; (3) p67^{phox} was varied from 0 to 160 nM, whereas p47^{phox} and Rac1were both present at a concentration of 160 nM, and (4) Rac1 was varied from 0 to 160 nM, whereas p47^{phox} and p67^{phox} were both present at a concentration of 160 nM. In all cases, the components were incubated with 130 μM lithium dodecyl sulfate, as described. (**b**) Three types of amphiphile-independent cell-free assays, consisting of various combinations of cytosolic components, were performed. All three consisted of solubilized macrophage membrane liposomes (5 nM cytochrome b_{558} heme), recombinant p67^{phox} (from 0 to 800 nM), and recombinant Rac1-GMPPNP prenylated in vitro (from 0 to 800 nM). The three combinations of components were: (1) The two cytosolic components were present at equal concentrations (varied from 0 to 800 nM); (2) p67^{phox} was varied from 0 to 800 nM, whereas Rac1 was present at a concentration of 800 nM, and (3) Rac1 was varied from 0 to 800 nM, whereas p67^{phox} was present at a concentration of 800 nM. The components were incubated in the absence of an anionic amphiphile. In all assays, $O_2{\cdot}^-$ production was initiated by the addition of NADPH and measured by the kinetic cytochrome *c* reduction assay for 5 min. Results illustrated in both panels represent means ± SE of three experiments. Reproduced from [142] by permission of Humana Press©2007

supplementing the assay buffer with GTP analogs persisted when native Rac (Rac-GDP not exchanged to GTP) was present in the reaction. The assumed explanation for this was that added GTP analogs were bound to Rac-GDP in a nucleotide exchange reaction taking place simultaneously with oxidase assembly (*see* **Note 49**).

3. Because the concentration of Mg^{2+} in the assay buffer is prohibitive for spontaneous nucleotide exchange, the ability of prenylated Rac to take up GTP from the medium points to the intervention of a GEF. In a semi-recombinant cell-free system, GEF can originate only in the membrane but its presence, identity, and quantity are unknown parameters in the vast majority of cases and will depend on the animal species and nature of the phagocyte serving as the source for the membrane [114].
4. Another common assumption is that native Rac (Rac-GDP) is inactive in cell-free systems (however, *see* ref. 101). We have shown in the past that this is true only below a certain quantitative threshold and when this is exceeded, significant activity can be achieved. Thus, in the canonical amphiphile-dependent cell-free system, the differences in V_{max} between Rac1-GDP and Rac1-GTPγS were marked at 20 nM Rac but minimal, at 200 nM Rac [115].
5. Figure 7 summarizes studies in which the influence of the following parameters on the ability of Rac1 to support oxidase activation in cell-free systems was examined: (1) GDP vs GMPPNP-bound state; (2) supplementation of the assay buffer with GTPγS; and (3) nonprenylated versus prenylated Rac, corresponding to amphiphile-dependent and -independent assay, respectively.
6. It is apparent that in the amphiphile-dependent system, when the concentration of the cytosolic components is low (30 nM), the difference in activity between native Rac1 (Rac1-GDP) and Rac1 exchanged to GMPPNP (Rac1-GMPPNP) is pronounced (Fig. 7a). When the concentration is raised to 100 nM, the difference in activity between Rac1-GDP and Rac1-GMPPNP is much less pronounced, which is due principally to an increase in activity of Rac1-GDP (Fig. 7b). What is also seen clearly in Fig. 7a, b is that supplementation of the assay buffer with GTPγS (10 μM) has no significant enhancing effect on the activity of Rac1-GDP and does not influence the activity of Rac1-GMPPNP (*see* **Note 50**). In the amphiphile-independent cell-free system, involving the use of prenylated Rac, the difference in the ability to support oxidase activation between Rac1-GDP and Rac1-GMPPNP is marked, with practically no activity being exhibited by prenylated Rac1-GDP (Fig. 7c). The addition of GTPγS

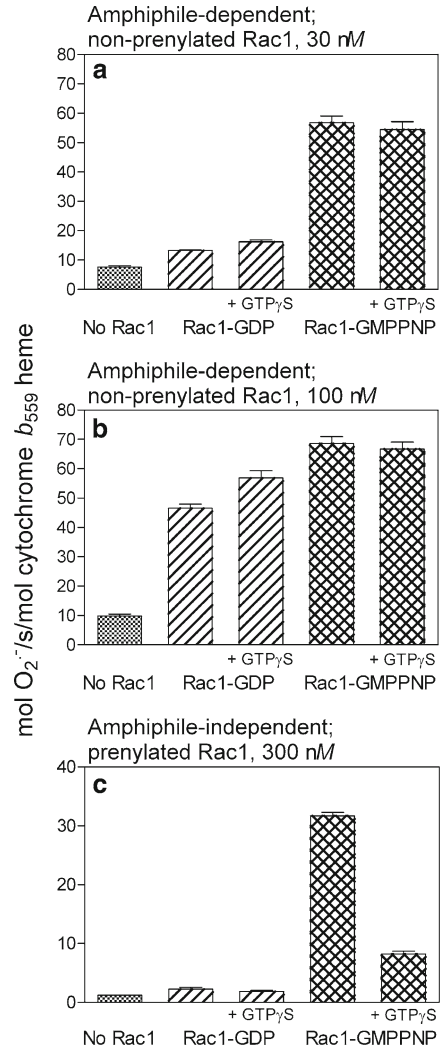

Fig. 7 Does the addition of exogenous non-hydrolyzable GTP analog (GTPγS) affect cell-free NADPH oxidase activation in the amphiphile-dependent and amphiphile-independent cell-free systems? (**a**, **b**) NADPH oxidase activity was measured in a cell-free system (±10 μM GTPγS, as indicated on the *x*-axis) consisting of solubilized macrophage membrane liposomes (5 nM cytochrome b_{558} heme), recombinant $p47^{phox}$ (30 or 100 nM) and $p67^{phox}$ (30 or 100 nM), and recombinant non-prenylated Rac1 (30 or 100 nM), either as native Rac1-GDP or as Rac1-GMPPNP. The components were incubated in the presence of 130 μM LiDS, as described. (**c**) NADPH oxidase activity was measured in a cell-free system (±10 μM GTPγS, as indicated on the *x*-axis) consisting of solubilized macrophage membrane liposomes (5 nM cytochrome b_{558} heme), recombinant $p67^{phox}$ (300 nM), and recombinant Rac1 (300 nM), either as Rac1-GDP or Rac1-GMPPNP prenylated in vitro. The components were incubated in the absence of an amphiphilic activator at room temperature. In all assays, $O_2^{\cdot-}$ production was initiated by the addition of NADPH and measured by the kinetic cytochrome *c* reduction assay for 5 min. Results illustrated in all panels represent means ± SE of three experiments. Reproduced from [142] by permission of Humana Press©2007

(10 μM) did not correct this lack of activity, in contradiction to results obtained with prenylated Rac in the presence of amphiphile [116, 117] (*see* **Note 51**).

7. Overall, we recommend that one should never rely on "in assay" nucleotide exchange, achieved by the addition of GTP analogs to the assay buffer, and always perform quantifiable nucleotide exchange on both nonprenylated and prenylated Rac, before their use in the assays. Following this advice will prevent inconsistent and poorly reproducible results, due both to the lack of conversion of Rac from the GDP- to the GTP-bound form, and to a possible inhibitory effect of free GTP.

3.6.5 Sibling Rivalry: Rac1 and Rac2

1. It is widely accepted today that the isoform of Rac relevant to oxidase function in human neutrophils is Rac2 [97]. Mouse neutrophils have similar amounts of Rac1 and Rac2, but Rac2 has the predominant role in oxidase activation [118, 119]. Human monocytes [99] and guinea pig macrophages [43, 98] use Rac1 in oxidase assembly.
2. Comparative studies of Rac1 versus Rac2 in cell-free assays have been published (*see* **Note 52**).
3. Here, we show a comparative study of the relative abilities of Rac1 and Rac2, in nonprenylated and prenylated forms and in the absence of and following exchange to GMPPNP, to activate the oxidase in the cell-free system. Nonprenylated Rac isoforms were assayed in an amphiphile-activated system whereas prenylated Rac isoforms were used in an amphiphile-independent system. As seen in Fig. 8a, contrary to the dogma, both nonprenylated Rac1-GMPPNP and Rac1-GDP are capable of activation, with V_{max} values of 89 and 83 mol $O_2^{\bullet-}$/s/mol cytochrome b_{558} heme, respectively, and EC_{50} values of 5.74 and 9.70 nM, respectively. Rac2-GMPPNP was clearly less active than Rac1-GMPPNP, as evident in the tenfold higher EC_{50} (53 nM). Rac2-GDP is, for practical purposes, inactive.
4. A similar study made with prenylated Rac1 and Rac2, in the absence of an amphiphilic activator and $p47^{phox}$, revealed that only the GMPPNP-preloaded forms of Rac1 and Rac2 are active. Again, Rac1 was more active than Rac2, as shown by the EC_{50} values of 0.13 and 0.73 μM, respectively (Fig. 8b).
5. Thus, the claim that "Rac-GTP is active; Rac-GDP is inactive" is applicable to prenylated Rac, in the absence of amphiphile and $p47^{phox}$, but is not strictly applicable to the canonical amphiphile- and $p47^{phox}$-dependent system. We believe that what determines the difference between the two situations is the presence or absence of $p47^{phox}$ (*see* **Note 53**). At the practical level, this means that, unless the cell-free assay is specifically meant to be focused on Rac2, the use of Rac1 is recommended, based on the assurance of higher oxidase activities.

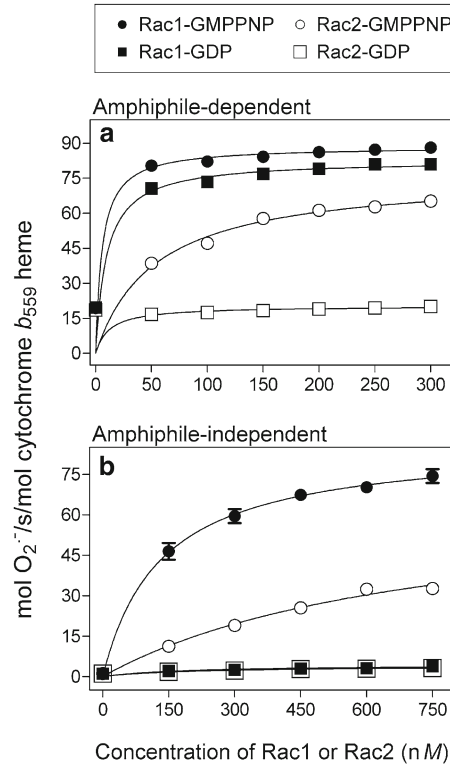

Fig. 8 Differences in the ability of Rac1 and Rac2 to support cell-free oxidase activation in relation to the nature of the bound guanine nucleotide, the absence or presence of prenylation, and the presence or absence of an amphiphilic activator. (**a**) Cell-free assays consisting of solubilized macrophage membrane liposomes (5 nM cytochrome b_{558} heme), recombinant p47^{phox} and p67^{phox} (both at a concentration of 300 nM), and recombinant non-prenylated Rac1 or Rac2, either exchanged to GMPPNP or GDP-bound (varied from 0 to 300 nM) were incubated with 130 μM LiDS, as described. (**b**) Cell-free assays consisting of solubilized macrophage membrane liposomes (5 nM cytochrome b_{558} heme), recombinant p67^{phox} (750 nM), and recombinant Rac1 or Rac2, either exchanged to GMPPNP or GDP-bound and prenylated in vitro (varied from 0 to 750 nM) were incubated in the absence of an anionic amphiphile, as described. In all assays (**a**, **b**), $O_2^{\cdot-}$ production was initiated by the addition of NADPH and measured by the kinetic cytochrome *c* reduction assay for 5 min. Results illustrated in both panels represent means ± SE of three experiments. Reproduced from [142] by permission of Humana Press©2007

3.7 Use of the Cell-Free System for Structure–Function Studies

The cell-free assay system was and continues to be invaluable as a simple and fast method to assess the oxidase activating ability of recombinant cytosolic proteins modified by mutation, truncation, deletion, chimerization, or posttranslational modification. There are uncountable examples of its application. Thus, it was used to test the effect of mutations in domains estimated to participate in functionally important protein–protein or protein–lipid

interactions, in p47^{phox} [42, 54, 120], p67^{phox} [6, 7, 42, 121], and Rac [7, 40, 42, 53, 54, 115, 122]. Testing the modified proteins in careful dose–response experiments, to allow the calculation of V_{max} and EC_{50} values, is essential. The concentration of the native (unmodified) cytosolic components, present in the assay, is also important, as shown by the finding that Rac1 mutations, which caused loss of function, were more easily detectable when the concentrations of p47^{phox} and p67^{phox} were low [115]. Finally, one should keep in mind that no in vitro method fully reproduces the environment of the whole cell. The actual concentrations of the oxidase components in the phagocyte, their compartmentalization within the cell, the presence of auxiliary components and factors missing in vitro, and the physicochemical composition of the intracellular milieu might result in an overall situation quite different from that existing in the cell-free system. This is the likely explanation for the occasions when modified oxidase components function differently in cell-free and whole cells conditions (*see* refs. 90, 91).

3.8 Use of the Cell-Free System for the Discovery of Oxidase Inhibitors

Cell-free systems are ideally suited for identifying potential oxidase inhibitors and for investigating their mechanism of action. The search for oxidase inhibitors received enormous impetus by the accumulating evidence for the involvement of nonphagocytic Noxes in the pathogenesis of a wide variety of diseases (reviewed in refs. 84, 85). So far, cell-free assays appropriate for measuring the activity of non-phagocytic Noxes are few and their use is not widespread. Thus, the cell-free assay is mostly applied to Nox2-based situations, whether in phagocytes or other cells. A central place is taken by synthetic peptide analogs of oxidase components, tested in situations in which they are expected to inhibit oxidase activation in cell-free assays (reviewed in refs. 123–125). Peptide oxidase analogs are used for two purposes: (1) as a mean of locating functional domains in individual oxidase components, and (2) to identify peptides with the potential of being used as therapeutic agents to dampen ROS production in disease situations in which excessive ROS production represents a primary or secondary pathogenic mechanism.

To achieve the first goal, arrays of overlapping peptides "covering" part of or the whole sequence of an oxidase component were tested for an effect on cell-free activation, a methodology that became known as "peptide walking." This was applied to Rac1 [126], p47^{phox} [127], p67^{phox} [92], p22^{phox} [128], and Nox2 [129]. Figure 9 illustrates the results of a typical "peptide walking" experiment, in which overlapping Nox2 DHR peptides were tested for inhibition of cell-free oxidase activation in amphiphile-dependent (Fig. 9a) and -independent (Fig. 9b) systems.

The second goal yielded rather disappointing results, with only one peptide, corresponding to residues 86–94 in the cytosol-exposed loop B of Nox2, found to inhibit oxidase activation in

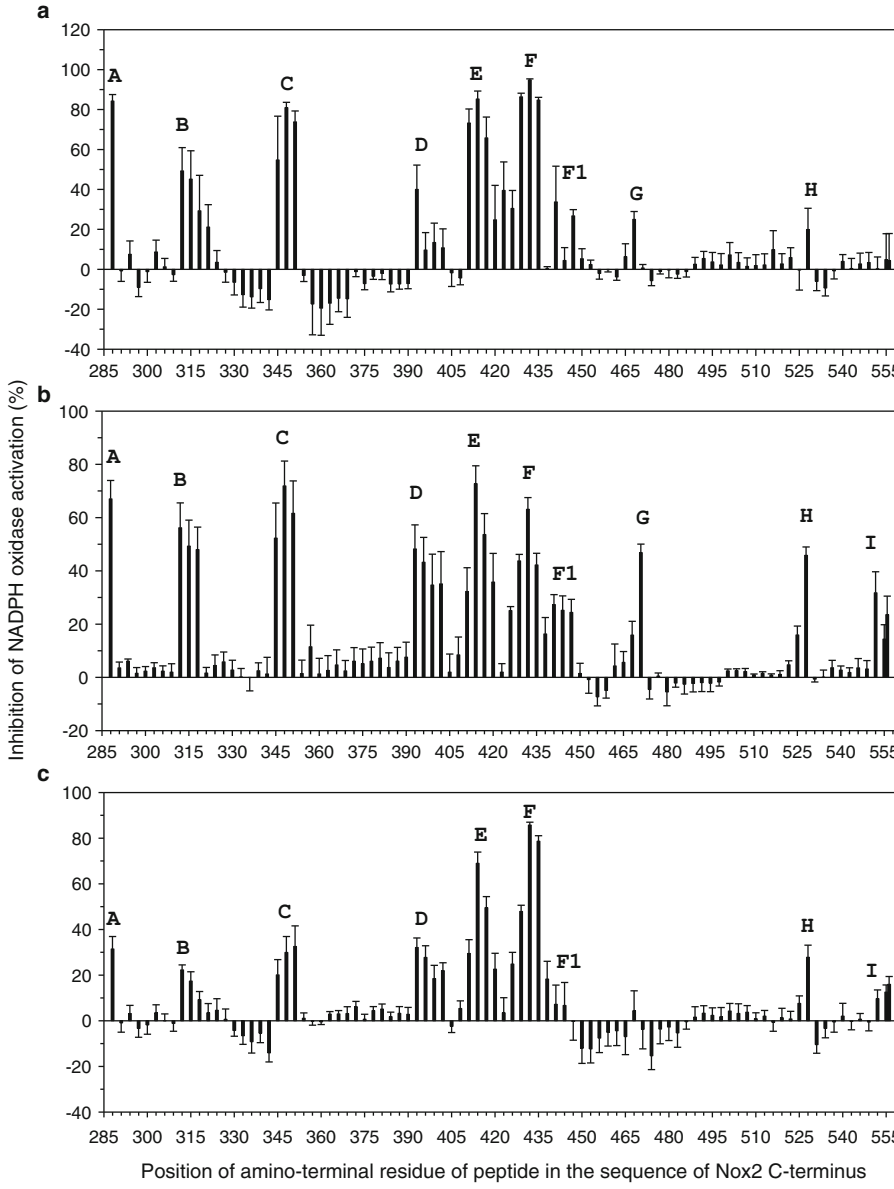

Fig. 9 Inhibition of NADPH oxidase activation by Nox2 dehydrogenase region peptides. (**a**) Inhibition in an amphiphile-dependent cell-free system. Peptides, at a concentration of 10 μM, were tested for the ability to inhibit $O_2{\cdot}^-$ production in a LiDS-activated cell-free system, consisting of solubilized macrophages membrane liposomes (equivalent to 5 nM cytochrome b_{558} heme) and recombinant p47^{phox}, p67^{phox}(1-526), and nonprenylated Rac1 Q61L, all at a concentration of 100 nM. The peptides were preincubated with the cytosolic components for 15 min before the addition of the membrane. Lithium dodecyl sulfate was then added at a concentration of 130 μM, and following incubation for further 1.5 min, $O_2{\cdot}^-$ production was initiated by addition of 238 μM NADPH. (**b**) Inhibition in an amphiphile- and p47^{phox}-independent cell-free system. Peptides were preincubated with p67^{phox}(1-526) and prenylated Rac1 Q61L, each at a concentration of 300 nM, for 15 min before the addition of the membrane. The mixtures were incubated for an additional 5 min before the addition of 240 μM NADPH. (**c**) Effect of C-terminal truncation of p67^{phox} on NADPH oxidase activation inhibition. Inhibition was measured in an amphiphile-and p47^{phox}-independent system, containing p67^{phox}(1-212) instead of p67^{phox}(1-526). All results represent means ± SEM of three experiments. *Uppercase boldface letters A–I* denote the inhibitory peptide clusters. Reproduced by permission from [129]

whole cells and organs and in an animal model, thus exhibiting a therapeutic potential [69].

We shall briefly summarize some of the critical issues to be considered when using the cell-free system for the identification of peptide or other small molecule oxidase inhibitors.

1. When assessing the significance of inhibition results, it is recommended to run peptide dose–response studies in a routine manner. These should be performed within a concentration range to enable the calculation of IC_{50} values. It is also essential that the peptide does not exert a nonspecific inhibitory effect on the actual measurement of $O_2^{\bullet-}$ production. This can be easily tested by adding the peptide to a xanthine/xanthine oxidase $O_2^{\bullet-}$-generating system.
2. Ideally, peptide inhibitors are expected to interfere with oxidase activation in the cell-free system by competing with the intact oxidase component, from which the peptide was derived, for interaction with another component of the oxidase complex. To test such an assumption, kinetic studies are required in order to demonstrate that inhibition is competitive. This was found to be the case with some peptides [100, 127] but, occasionally, what appeared as competition [130] did not withstand kinetic analysis [131].
3. Most Inhibitors active in cell-free systems are expected to interfere with the process of oxidase assembly. Such peptides inhibit only when present before the initiation of assembly and are inactive when added after the completion of assembly. Figure 10 illustrates the marked dose-dependent inhibition of amphiphile-dependent oxidase activation by a Rac1 C-terminal peptide, when added before the initiation but not after the completion of assembly. The preferential inhibition upon peptide addition before assembly is, however, not universal; some peptides and small molecule inhibitors were also found to inhibit when added after assembly, raising the possibility that they may be capable of dissociating assembled complexes [52, 129]. On rarer occasions, peptides interfere with the catalytic (redox) function of Nox2, such as by competing for the binding of cofactors or by another mechanism. To elucidate the mechanism, requires the application to the cell-free system of complex kinetic analysis (*see* ref. 129).
4. It is essential to control the sequence specificity of the inhibitory action of oxidase-analog peptides. This involves testing of scrambled and retro-peptides and unrelated peptides or small molecule compounds of similar size, charge, or hydrophobicity. Charge and hydrophobicity are important parameters in protein–protein and protein–lipid interactions and there are numerous examples of situations in which what was expected

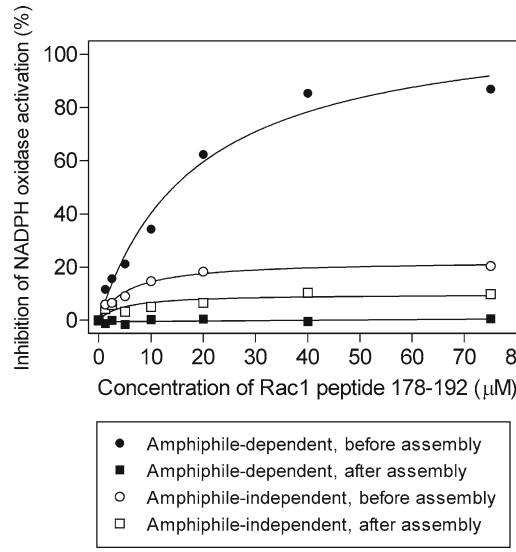

Fig. 10 Inhibition of NADPH oxidase activation in cell-free systems by a synthetic peptide, corresponding to the C-terminus of Rac1 (residues 178–192). The effect of various concentrations of the peptide (from 1.25 to 75 μM) was tested in both amphiphile-dependent and amphiphile-independent systems. The amphiphile-dependent system consisting of solubilized macrophage membrane liposomes (5 nM cytochrome b_{558} heme), recombinant p47^{phox} and p67^{phox}, and recombinant non-prenylated Rac1 Q61L mutant (all at 100 nM) was incubated with 130 μM lithium dodecyl sulfate, as described. The amphiphile-independent cell-free system consisting of solubilized macrophage membrane liposomes (5 nM cytochrome b_{558} heme), recombinant p67^{phox}, and recombinant Rac1 Q61L mutant prenylated in vitro (both at 300 nM) were incubated in the absence of an amphiphilic activator, as described. In all assays, $O_2{\cdot}^-$ production was initiated by the addition of NADPH and measured by the kinetic cytochrome *c* reduction assay for 5 min. The peptide was added either at time 0 (before assembly) or after 90 s of incubation (after assembly) but always preceding the addition of NADPH. The effect of the peptide is expressed as % inhibition of NADPH oxidase activation, with the activity in the absence of peptide being considered as 100 %. The turnover in the amphiphile-dependent system, in the absence of the peptide was 84.42 mol $O_2{\cdot}^-$/s/mol cytochrome b_{559}. In the amphiphile-independent system, the turnover in the absence of peptide was 65.01 mol $O_2{\cdot}^-$/s/mol cytochrome b_{558}. The results represent one characteristic experiment. Reproduced from [142] by permission of Humana Press©2007

to be sequence-specific inhibition by peptides, turned out to be sequence-independent (*see* refs. 52, 128, 129, 132). On the other hand, lack of sequence specificity should not be an automatic disqualifier of the inhibitory peptide for possible practical applications.

5. An important methodological consideration is the type of cell-free assay used for testing inhibitors. Thus, the amphiphile-dependent assay (with nonprenylated Rac1) is to be used when

a charge effect is involved, as illustrated by the inhibitory effect of a positively charged C-terminal Rac1 peptide; the very same peptide is inactive when tested in an amphiphile-independent assay (with prenylated Rac1), in which hydrophobic binding of Rac1 to the membrane is predominant (Fig. 10). In the latter situation, RhoGDI was found to prevent activation but was inactive when tested in the amphiphile-dependent assay (with nonprenylated Rac1) [7, 9].

6. Occasionally, peptides expected to be inhibitory on the basis of the fact they correspond to domains of previously known functional significance, were found to be inactive. Thus, peptides corresponding to the switch I region in Rac1 [126], the proline-rich region in $p22^{phox}$ [128], and the "activation domain" in $p67^{phox}$ [92], were not inhibitory in the cell-free assay.
7. Lack of effect on oxidase activation under cell-free conditions might mean that the hypothetical inhibitor acts upstream of the oxidase, most likely by interfering with phagocyte receptor-ligand interaction or with a transductional step linking the receptor to the oxidase.

3.9 "The End of the Affair"

We have been in the "cell-free business" for three decades. The message we would like to leave with you is as follows: Running cell-free assays is not only useful, simple, reproducible, and economical but, more than anything else, it is really fun. There is nothing like the feeling of taking out four proteins from the freezer and having a $O_2^{\cdot-}$ producing system (at least as good as intact phagocytes, if not better) on your desk in less than an hour. Good luck!

4 Notes

1. In addition to C20:4, oleic (C:18:1), linoleic (C18:2), and linolenic (C18:3) acids were also found to elicit $O_2^{\cdot-}$ production [21]. The cyclooxygenase product prostaglandin E_2 was inactive and the lipoxygenase product 15-hydroperoxy-5,8,11,13-eicostetraenoic acid (15-HPETE) was equal in activating ability to C20:4 [21]. A systematic study of the fatty acid specificity of cell-free oxidase activation revealed that the *trans* forms of C18:1 and C18:2 are active but long chain saturated acids (arachidic (C20:0), stearic (C18:0), and palmitic (C16:0)) are inactive [133]. Shorter chain saturated acids (lauric (C12:0), myristic (C14:0), and, to a lesser degree, palmitic (C16:0)), at high concentrations, were reported to be active [81, 83]. The complexity of the mechanism of oxidase activation is demonstrated by the recent finding that the *trans* form of C20:4 is incapable of cell-free oxidase activation (unlike the *trans* forms of C18:1 and C18:2) and acts as an inhibitor of *cis*-C20:4-induced oxidase activation [134].

2. One of the reviewers of our manuscript describing cell-free activation of the oxidase by SDS recommended rejection of the manuscript and offered the following comments: "the manuscript contains some interesting material…I express some disappointment that the authors do not approach the literature more thoroughly and attempt to relate effects seen here to those observed earlier with numerous detergents on whole cells."
3. In the "two-step" assay, the components of the oxidase are first mixed in a small volume (usually 1/10–20 of the final reaction volume) and exposed to the activating amphiphile at a concentration resulting in maximal activation. This "first-step" mixture does not contain NADPH and the $O_2^{\bullet-}$ detection reagent. After incubation for a determined time interval (leading to the assembly of the oxidase complex), the mixture is supplemented with assay buffer containing the $O_2^{\bullet-}$ detection reagent and NADPH but no amphiphile, which results in the dilution of the amphiphile to a non-activating concentration, and $O_2^{\bullet-}$ generation is measured (this second step represents the catalytic phase). The "two-step" assay is useful for testing the mechanism by which inhibitors affect oxidase activation (such as interference with assembly or competition with substrates) (*see* Subheading 3.8). Examples of its use can be found in refs. 42, 81, 106, 113).
4. The experience of our laboratory with the use of the INT reduction assay in the canonical cell-free assay is that about 80 % of the reduction is SOD-sensitive and, thus, due to reduction by $O_2^{\bullet-}$. A technical problem associated with this assay is the fact that INT precipitates SDS and LiDS, a fact also mentioned in the original description of the method [71]. INT reduction is not the choice method for the quantification of $O_2^{\bullet-}$ but is useful for measuring the spontaneous or cytosolic factors-dependent "activity" of the dehydrogenase region of Nox2 and other Noxes [74, 75].
5. The Subheadings 2 and 3 in this chapter are heavily biased toward semi-recombinant cell-free systems using membranes derived from guinea pig peritoneal exudate macrophages and human recombinant cytosolic components. The rationale behind this rather unusual combination is the ease of preparing large amounts of membranes with high cytochrome b_{558} content, excellent activity in cell-free assays, ease of solubilization, and years-long stability when kept frozen at –75 °C.
6. AEBSF was found to be an inhibitor of oxidase assembly in the cell-free assay [135], with an IC_{50} of 0.87 mM. However, the concentrations of AEBSF carried over into the assays were 250–350 times lower.

7. We have lately replaced the use of Rac1 exchanged to GMPPNP with the Rac1 mutant Q61L, which is constitutively in the GTP-bound form [103].
8. This buffer was first described by us for use in the C20:4-activated rudimentary cell-free system, based on the use of membrane and total cytosol [21] and was modified later [115].
9. A narrow bandwidth improves the sensitivity of the cytochrome *c* reduction assay. SPECTRAmax 340 has a bandwidth of 5 nm; SPECTRAmax 190 has a bandwidth of 2 nm.
10. Analysis of the cellular content of the lavage revealed it consisted of more than 90 % macrophages at 4–5 days following the injection of paraffin oil.
11. The 1 M KCl concentration is achieved by mixing 2.5 volumes of sonication buffer with 1 volume of 3.5 M KCl in 20 mM Tris–HCl, pH 7.5.
12. Washing of membranes with 1 M KCl is intended to remove loosely attached cytosolic components, with special emphasis on the removal of membrane-bound Rac. Indeed, it was reported that omission of the KCl wash step resulted in an apparent decrease in the dependence of cell-free oxidase activation on added Rac, which was due to the presence of membrane-bound Rac [117].
13. A dialysis membrane with a molecular weight cutoff of 25,000 is chosen because octyl glucoside is used for the solubilization of membranes at a concentration of 40 mM, which is well above its critical micellar concentration (CMC) of 25 mM. The micellar molecular weight of octyl glucoside is 8,000 and a large pore size membrane facilitates diffusion of the detergent from the dialysis bag to the surrounding buffer.
14. The dialysis buffer used for removing octyl glucoside and generating the membrane liposomes is identical to the solubilization buffer but contains no octyl glucoside, AEBSF, leupeptin hemisulfate, and FAD. The addition of serine protease inhibitors, especially AEBSF, to membrane preparations is avoided because of the reported likelihood of an interaction with cytochrome b_{558} [135]. FAD is omitted because it is difficult to measure the cytochrome b_{558} content accurately by sodium dithionite reduction in the presence of FAD.
15. Membrane liposomes can be kept frozen at –75 °C for long periods. We found a decrease of 1 % per year storage in the cytochrome b_{558} heme content of frozen membranes. Keeping the material in small aliquots is recommended but thawing and refreezing for up to ten times was not found to cause significant damage. When membrane liposomes are used after long periods of storage or have an uncertain data record, it is

recommended to determine the cytochrome b_{558} content again (*see* Subheading 3.1.3).

16. Routine use of solubilized membranes in the form of liposomes has the built-in advantage of providing a ready-made source of a membrane preparation which elutes as a single well-defined peak by gel filtration on a Superose12 FPLC column (in the exclusion volume). This allows the easy detection, by a variety of means, of translocation of cytosolic oxidase components to the membrane in the course of cell-free activation [9].
17. We use disposable 10 × 4 × 45 mm cuvettetes.
18. We use a Uvikon 943 double-beam spectrophotometer (Kontron Instruments), but any instrument with similar characteristics (scanning ability, narrow band-width, and data analysis (peak detection) capabilities) should be suitable.
19. Quite a number of alternative Δ extinction coefficients for calculating the cytochrome b_{558} heme concentration have been published. Most of these are centered on the 558/559 nm peak. Although this peak is more specific for cytochrome b_{558}, the fact that it is much lower than the 426/427 nm peak increases the chances of error, and we prefer to base our calculations on the 426/427 nm peak. We found no evidence for the presence of significant amounts of *b*-type cytochromes, other than b_{558}, in macrophage membranes [136].
20. The high concentration of cytochrome b_{558} heme (2 μM) might raise the question of whether freeing the solubilized membrane of octyl glucoside by dialysis is a necessary step in preparing membranes intended for the performance of cell-free assays. In these, the concentration of cytochrome b_{558} heme is 5–10 nM, and one might assume that just diluting the membrane 200- to 400-fold is sufficient for reducing the concentration of octyl glucoside well below the CMC, without a need for dialysis. It is our experience, however, that this is not the case. Solubilized membrane preparations, dialyzed free of detergent, are clearly superior in cell-free assays to preparations not subjected to dialysis.
21. It is desirable to use the same protein standard throughout in order to compare protein concentrations over long time periods. No external protein standard reflects the true protein concentration of the recombinant proteins but what is more important is to maintain the same level of "error." We use bovine gamma globulin as the standard.
22. Highly purified cytosolic components have the tendency to self-aggregate, even in the presence of 20 % glycerol. Repeated thawing–refreezing is likely to promote aggregation. Aggregated protein is inactive in cell-free assays and its presence might distort

the results when measuring protein concentration. It is, thus, highly recommended to subject frozen proteins, after thawing, to centrifugation at 12,000 × *g* at 4 °C, for 15 min, in a table top microcentrifuge accommodating 1.5 mL conical tubes. Measure protein concentration in the supernatant and keep the supernatant at 4 °C till used in the cell-free assay.

23. In the Rho GTPase folklore, it is commonly believed that GMPPNP is more resistant to the intrinsic GTPase activity than GTPγS but that the affinity for GTPγS is higher than that for GMPPNP. We are now employing exclusively GMPPNP for generating the GTP-bound form of Rac1 or Rac2.
24. Rac exchanged to GMPPNP was found, in our hands, to be stable for several weeks, and we found no reason to perform the exchange a short time before using the protein in cell-free assays. However, when larger amounts of Rac are subjected to exchange, it is wise to divide it in smaller aliquots, in order to avoid repeated thawing and freezing.
25. It is obvious that, when using this methodology, unbound GMPPNP is not removed and is present in the Rac preparations at a concentration roughly tenfold higher than that of the protein. This means that free GMPPNP is transferred to the cell-free assays, a fact which is to be taken into consideration. When removal of unbound nucleotide is desired to eliminate possible unwanted effects of free GMPPNP, the nucleotide-exchanged Rac is subjected to buffer exchange by centrifugal ultrafiltration, using 4 mL centrifugal filter units fitted with 10,000 molecular weight cutoff membranes. Three volumes of 4 mL each are filtered, using the buffer in which Rac is found, supplemented with 20 % v/v glycerol, and the sample is reconstituted to its original volume. Protein concentration is measured again, to check for some unavoidable loss in the course of ultrafiltration.
26. Prenylation can be performed after and before nucleotide exchange to a non-hydrolyzable GTP analog, but we prefer to prenylate after nucleotide exchange.
27. $ZnCl_2$ is an essential cofactor of geranylgeranyl transferase I.
28. This method can be applied to larger amounts of Rac, provided that non-prenylated Rac is sufficiently concentrated. However, the heated rotary mixer usually accommodates tubes with maximal volumes of 1.5 or 2 mL, which are also convenient for storage.
29. Two additional bands of 48 and 43 kDa are visible in the aqueous phase. These represent the α and β subunits of the enzyme geranylgeranyl transferase I.
30. This was first shown by Ligeti et al. [110] Thus, at 25 °C, full assembly was achieved after 5 min of exposure to the amphiphile

whereas, at close to 0 °C, the process took 30 min. Note, however, that at both temperatures the same level of assembly was ultimately achieved.

31. This observation was first made by Pilloud (Dagher) et al. [106] in a system composed of membrane and whole cytosol, and was thought to be related to the effect of salt on the micellar state of the anionic amphiphile. More recent work in a semi-recombinant system demonstrated that increasing ionic strength prevents binding of nonprenylated Rac1 (via its polybasic tail) to negatively charged phospholipids in the membrane [8]. No effect of ionic strength was seen on Rac2, which possesses a lesser positive charge at its C-terminus. These facts are of practical importance for the design of an optimal assay buffer and are also of relevance for the mechanism of amphiphile-independent cell-free activation of membranes enriched in anionic phospholipids [42, 51, 52].
32. If the requirements of the experiment are to add each component separately or in groupings of two, add 10 μL of each component from a 20-fold concentrated stock solution or 20 μL of a tenfold concentrated mixture of two components and 10 μL of a 20-fold concentrated stock of the third component. This will bring the total volume of the added components to 30 μL and requires the reduction of the volume of added membrane preparation to 10 μL and the proportional increase in the concentration of the membrane stock preparation to 20-fold.
33. The optimization of amphiphile concentration was first discussed in 1989 [106], a paper well worth reading even today. Because anionic amphiphiles act on both cytosolic [29] and membrane [30–32] components, optimization might be a complex issue when setting up radically new conditions and/or when non-purified components are used, some of which might bind the anionic amphiphile. It is a much simpler procedure when using purified recombinant cytosolic components.
34. Optimization of the time required for the amphiphile-dependent assembly of the oxidase complex is discussed in ref. 110. We found 90 s to be sufficient in the overwhelming majority of cases. However, it is important to point out that prolonging the time of assembly for up to 5 min might be advantageous and is, most definitely, not damaging. Thus, when in the preliminary stages of a project involving cell-free activation, exploring longer assembly times is recommended. Once the minimal time assuring full activation is found, this can be used routinely, but adding to it a "safety time supplement" is a wise move.
35. Turnovers in the amphiphile-activated cell-free system rarely exceed 100 mol $O_2^{\cdot-}$/s/mol cytochrome b_{558} heme. This corresponds to

100 pmol $O_2^{\bullet-}$/s/pmol cytochrome b_{558} heme. Thus, in each well containing 1 pmol cytochrome b_{558} heme, 100 pmol $O_2^{\bullet-}$ are produced per s, which means 6 nmol/min, and 30 nmol per 5 min. The total amount of cytochrome *c* present in the well is 40 nmol, which is sufficient for binding all the $O_2^{\bullet-}$ produced in 5 min. A total of 48 nmol NADPH are added to the well, which based on a stoichiometry of 1 mol NADPH supporting the production of 2 mol of $O_2^{\bullet-}$, is more than sufficient for the production of 30 nmol of $O_2^{\bullet-}$. When measuring high activity oxidase preparations, "bending" of the curve nevertheless occurs, in spite of apparently sufficient total amounts of NADPH and cytochrome *c* at the start of the reaction, this being due to the presence of lesser and lesser amounts of NADPH and oxidized cytochrome *c* as we approach the end of the reaction. Our group is using single sources of cytochrome *c* and NADPH as components of the assay buffer (*see* Subheading 2.1.5, **steps 1** and **2**). Because of variations in the degree of purity, the amount of water, and the proportion of oxidized and reduced material, it is wise to verify the actual amount of the two compounds present in the assay buffer. We measure the *total* concentration of cytochrome *c* by performing an absorbance wavelength scan (400–600 nm) on the native and sodium dithionite-reduced solution, determining the difference in absorbance at 550 nm between the reduced and oxidized samples and calculating the concentration by applying the extinction coefficient, $\Delta E_{550} = 21\ mM^{-1}\ cm^{-1}$ for reduced minus oxidized cytochrome *c*. The concentration of *reduced* NADPH is determined in the stock solution in H_2O by assessing the absorbance at 340 nm (the use of quartz cuvettes is required) and using the extinction coefficient, $E_{340} = 6.22\ mM^{-1}\ cm^{-1}$ [137]. In the presence of sufficient cytochrome *c*, no need was found for the addition of catalase (*see* ref. 61) to prevent reoxidation of cytochrome *c* by H_2O_2 originating in the spontaneous dismutation of $O_2^{\bullet-}$ that escaped scavenging by cytochrome *c*. However, if catalase is to be added, the assay buffer should be modified to not contain NaN_3 because of its inhibitory effect on catalase.

36. The extinction coefficient for the absorbance at 550 nm of reduced minus oxidized cytochrome *c*, as applied to a 1-cm path length, must be modified for the vertical path length of the microplate wells. This varies with the dimension and shape of the wells and the volume of the reaction mixture present in the well. Some microplate spectrophotometers (e.g., SPECTRAmax 190, Molecular Devices) have a "PathCheck" sensor, allowing the normalization of absorbance values to a 1-cm path length. This allows the use of the canonical extinction coefficient to calculate the concentration of reduced

cytochrome *c* in the well, without the need for any correction. This has only to be translated into the total amount of reduced cytochrome *c* in 210 μL, which permits the calculation of the turnover. SPECTRAmax 190 also has a wavelength bandwidth of 2 nm, which offers greater accuracy in measuring the "narrow" absorbance peak of reduced cytochrome *c* at 550 nm. With instruments not having the "PathCheck" option, the length of the vertical path length in the well must be determined by other means. Once it is known, the following equation will allow the direct calculation of nmol $O_2^{\bullet-}$ per min per well: nmol $O_2^{\bullet-}$/min/well = $\Delta mAbs_{550nm}$/min × 0.047619 × reaction volume (in mL)/path length (in cm). As an example, when then the reaction volume is 0.21 mL and the path length is found to be 0.575 cm, nmol $O_2^{\bullet-}$/min/well = $\Delta mAbs_{550\ nm}$/min × 0.047619 × 0.21/0.575 = $\Delta mAbs_{550nm}$/min × 0.017391.

37. The proper way to express results of cell-free oxidase activation assays is as turnover values. Unless there is a compelling reason for not doing so, oxidase activities should be related to the heme content of cytochrome b_{558} present in the membrane and not to cell number equivalents, total membrane protein, or the protein concentration of one or the other of the cytosolic components. The, unfortunately, common habit of expressing cell-free assay results as % change relative to a "basal" value can be thoroughly misleading in the absence of the information on the turnover corresponding to that basal value. This is critical when the effect of inhibitors on oxidase activity is expressed. Thus, a 50 % inhibition is meaningless when the basal turnover value is 2 mol $O_2^{\bullet-}$/s/mol cytochrome b_{558} heme but is potentially meaningful when the value is 80 mol $O_2^{\bullet-}$/s/mol cytochrome b_{558} heme.

38. From the advent of semi-recombinant cell-free assays and the increasing rarity of the use of whole cytosol or partially purified cytosolic fractions, the need for the SOD control has been drastically reduced. In our laboratory, in which semi-recombinant cell-free assays are performed routinely, we have not encountered a single occasion of nonspecific cytochrome *c* reduction. When a reducing agent is carried over into the reaction, cytochrome *c* reduction occurs practically at time zero and will be reflected in the absence of the typical kinetics. The presence of a cytochrome *c* reductase in the membrane preparation remains possible but is likely to be independent of the presence of the amphiphile and the cytosolic components. Of course, SOD controls should be used when cell-free assays are utilized as a diagnostic means on unpurified biologic material and in novel experimental situations. Yet another control for the specificity of cytochrome *c* reduction by $O_2^{\bullet-}$, rarely applied today, is the use of acetylated cytochrome *c* as the $O_2^{\bullet-}$ trap [61]. Acetylation

of lysine residues in cytochrome *c* decreases direct electron transfer from reductases, while maintaining the ability of $O_2^{\bullet-}$ to reduce cytochrome *c* [138].

39. The extinction coefficient for the absorbance of reduced INT at 490 nm relevant to a 1 cm path length has to be modified for the vertical path length of the microplate wells. When the microplate spectrophotometer does not have a "PathCheck" sensor, one has to know the total reaction volume per well and the length of the vertical path length. Once these are known, the following equation will allow the direct calculation of nmol INT reduced per min per well: nmol reduced INT/min/well = $\Delta mAbs_{490nm}/min \times 0.095328 \times$reaction volume (in mL)/path length (in cm). If the results are to be expressed in $O_2^{\bullet-}$ equivalents, the values are to be multiplied by 2, to account for the fact that reduction of INT is a two-electron reaction. As an example, when then the reaction volume is 0.21 mL and the path length is found to be 0.575 cm, nmol reduced INT/min/well = $\Delta mAbs_{490nm}/min \times 0.095238 \times 0.21/0.575 = \Delta mAbs_{490nm}/min \times 0.034782$ (0.069564, for $O_2^{\bullet-}$equivalent).
40. For the NADPH consumption test it is recommended to use 96-well plates for work at UV wavelengths (UV-Star plate, flat bottom, Greiner) and a microplate spectrophotometer capable to measure absorbance at 340 nm (we prefer to use the SPECTRAmax 190 (wavelength range 190–750 nm, Molecular Devices) but SPECTRAmax 340 (wavelength range 340–850 nm, Molecular Devices) is also adequate. The minimum absorbance limit of the microplate reader has to be adjusted to a negative value, to allow the recording of negative absorbance kinetics relative to the blank represented by assay buffer with NADPH. The extinction coefficient for the absorbance of reduced NADPH at 340 nm relevant to a 1 cm path length [137] has to be modified for the vertical path length of the microplate wells. When the microplate spectrophotometer does not have a "PathCheck" sensor, one has to know the total reaction volume per well and the length of the vertical path length. Once these are known, the following equation will allow the direct calculation of nmol reduced NADPH consumed per min per well using the following equation: nmol reduced NADPH consumed/min/well = $\Delta mAbs_{340nm}/min \times 0.160771 \times$ reaction volume (in mL)/path length (in cm). If the results are to be expressed in $O_2^{\bullet-}$ equivalents, the values are to be multiplied by 2, to account for the fact that one molecule of NADPH donates two electrons and yields two molecules of $O_2^{\bullet-}$. Note also that the consumption of NADPH will generate negative rate values when related to the reduced NADPH blank value. As an example, when then the reaction volume is 0.21 mL and the

path length is found to be 0.575 cm, nmol reduced NADPH consumed/min/well = $\Delta mAbs_{340nm}/min \times 0.160771 \times 0.21/0.575 = \Delta mAbs_{340nm}/min \times 0.058716$ (0.117432 for $O_2^{\bullet-}$ equivalent).

41. Dispensing 10–20 μL aliquots to the wells is best performed with electronic pipettors in the "dispensing" mode or using a Multipette. In most situations, the concentrations of $p67^{phox}$ and prenylated Rac required for reaching maximal activation, in the absence of amphiphile and $p47^{phox}$, are higher than those necessary for amphiphile-dependent activation in the presence of identical amounts of membrane.
42. In the amphiphile-independent cell-free system, we found on most occasions that prolonging the incubation to up to 5 min resulted in increased oxidase activities. This time interval should, however, not be exceeded.
43. The presence of $p47^{phox}$ is not an absolute requirement for oxidase activity to be detected in this assay, but turnover values are higher in its presence.
44. This effect was thought to be due to an elevation of the Krafft point of the fatty acids in the presence of Ca^{2+} [139].
45. These results support the recent proposal that the stability of the FAD-Nox2 bond is enhanced by anionic amphiphile-induced changes in Nox2 and by the process of assembly with the cytosolic components [140].
46. In the experiments described in Fig. 5, both non-prenylated and prenylated Rac1 were exchanged to GMPPNP and the exchange stabilized by 25 mM $MgCl_2$. Thus, it is unlikely that significant dissociation of GMPPNP from Rac can occur upon dilution of exchanged Rac preparations in assay buffer during the 90-s time interval of oxidase assembly. However, assay buffer is also used for intermediary dilution steps of recombinant cytosolic components, and it is possible that the diluted proteins are, sometimes, kept in assay buffer for longer periods of time. In the particular case of Rac, it is recommended to make such dilutions in the buffer listed, which contains 4 mM $MgCl_2$.
47. There is good evidence for equimolar and simultaneous translocation of the cytosolic components in neutrophils stimulated by two elicitors of a respiratory burst and it was also found that this translocation corresponded temporally with the generation of $O_2^{\bullet-}$ [141].
48. It is of interest that sigmoidal dose–response curves were described in the early period of the use of cell-free assays, when total cytosol was used and its amount was related to activity in the presence of a constant amount of amphiphile [105, 106].

49. This assumption was examined by Heyworth et al. [116] and Fuchs et al. [117], who showed that exchange with GTP added to the assay buffer only takes place with prenylated Rac and that preloading with GTP by Mg^{2+} chelation is required for non-prenylated Rac to work in the amphiphile-dependent cell-free assay.
50. This confirms the contention that no significant nucleotide exchange takes place on non-prenylated Rac upon addition of GTP analogs to the assay buffer [116, 117].
51. Another distinguishing feature of this system is the paradoxical inhibitory effect of added (free) GTPγS (10 μM) on oxidase activation by prenylated Rac exchanged to GMPPNP. The inhibitory effect of free GTP and GTPγS on amphiphile-independent oxidase activation was described by us in the past, but was never satisfactorily explained [52].
52. In one study, it was concluded that non-prenylated Rac1 and Rac2 were poorly active in an amphiphile-dependent cell-free assay in the absence of nucleotide exchange to GTPγS [116]. In another study, it was shown that the non-prenylated form of Rac1 exchanged to GTPγS was much superior to Rac2 exchanged to GTPγS [8]. This difference was clearly attributed to the lesser positive charge at the C-terminus of Rac2, a suggestion also supported by the inhibitory effect of increased ionic strength on the activity of Rac1, but not Rac2, and by the enhancing effect of an increase in the negative charge of the membrane on the effect of Rac1, but not Rac2 [8]. These authors also found that prenylated Rac1 and Rac2, when preloaded with GTPγS, support oxidase activation in an amphiphile-dependent system. In those studies, Rac1 was more efficient at activating the oxidase.
53. We proposed that since both $p47^{phox}$ and Rac function as Nox organizers, thereby contributing to the establishment of a more stable interaction between $p67^{phox}$ and Nox2, the presence of one organizer lessens the dependence on the other. This is expressed in the ability to activate the oxidase in the absence of $p47^{phox}$ [9, 11, 12, 53] and in a more "liberal" interpretation of the requirement for Rac to be in the GTP-bound form, when $p47^{phox}$ is present [41].

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