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# Detecting free radicals post viral infections

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ABSTRACT

Free radical generation plays a key role in viral infections. While free radicals have an antimicrobial effect on bacteria or fungi, their interplay with viruses is complicated and varies greatly for different types of viruses as well as different radical species. In some cases, radical generation contributes to the defense against the viruses and thus reduces the viral load. In other cases, radical generation induces mutations or damages the host tissue and can increase the viral load. This has led to antioxidants being used to treat viral infections. Here we discuss the roles that radicals play in virus pathology. Furthermore, we critically review methods that facilitate the detection of free radicals *in vivo* or *in vitro* in viral infections.

## 1. Introduction

While exhaustive efforts are underway to understand the SARS-CoV-2 pathogenesis researchers have found a connection between SARS-CoV-2 infection and the oxidative stress [1–5]. Such connections are also well-established for several other highly pathogenic and clinically relevant RNA virus, DNA virus and retroviruses where oxidative stress influences pathogenesis, disease progression, severity and anti-viral activity [6]. Infection induced overproduction of highly reactive molecules in a host or the suppression of the host's anti-oxidant defense mechanism lead to oxidative stress upon infection. Association between viral infection and oxidative stress is extremely complex, case specific and it is being extensively studied for the last few decades. There are several older reviews available in the literature which either provide an overview of this vast field [6–8] and articles which focus on a specific aspect within this area such as signaling pathways [9,10] or viral infections of the nervous system [11]. There are two primary reasons for conducting a review on this topic. First, the field of detecting free radicals is evolving quickly and it is crucial to follow how these techniques are used in the field of virology. Second, there is a proven influence of free radicals on viral infections which is very well documented in the literature [12–15]. To this end we will first discuss which chemicals play a role. Then we will elaborate on the specific findings that link viral infections with free radical production. Finally, we will discuss different direct and indirect methods that can be used for detecting free radicals.

Reactive molecules can be categorized in two ways (shown in Fig. 1) either based on the basic element from which they originate (oxygen, nitrogen, carbon) or based on the availability of unpaired free electron in the molecule (paramagnetic free radicals or non-paramagnetic molecules) [16]. Most of the articles which study oxidative stress in viral infection are centered around more or less reactive (the reactivity can vary considerably) molecules that originate from oxygen (ROS) and nitrogen (RNS). These species can be the paramagnetic and non-paramagnetic. In many of these investigations, researchers don't distinguish between the different types of molecules involved in the oxidative stress. They simply probe the amount of all the reactive species present which describes the net redox imbalance caused by the infection. However, this is not sufficient in many cases and specie specific analysis is warranted. For example, the role of nitric oxide (NO*) changes depending on the virus it is interacting with [14]. In particular, antiviral activity of NO* for DNA viruses such as Herpes-simplex virus (HSV), Epstein-Barr virus (EBV) is reported but for several other types of viruses such as Murine Vaccinia Virus or Tickborn Encephalitis Virus (TBE-V), NO* does not demonstrate any appreciable antiviral activity [17–19]. Furthermore, significance of different radical species in a specific viral infection can be different too. For instance, although influenza virus infection elevates the concentration of oxygen radicals [15] the hydroxyl radical concentration remains relatively unaltered [12]. Different species are involved in different signaling pathways in the viral pathogenesis which also renders the species specific analysis very relevant [9].

Within species specific analysis, investigating free radicals is more challenging than studying non-paramagnetic reactive molecules due to their very short lifetime. Phaniendra and co-workers listed the lifetimes

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of cellular ROS and RNS species which clearly highlight that lifetime of free radicals is smaller by several orders of magnitude compared to the mostly stable non-radical species [20]. Modeling of cytosolic ROS showed that superoxide radicals have only 1/6th of the lifetime compared to the hydrogen peroxide which has nearly three times higher diffusion distance [21]. Free radicals are short lived because of their very high chemical reactivity which is why they either get converted into stable reactive molecules or one free radical species gets converted into one another. For example, superoxide radicals get either converted into hydrogen peroxide via enzymatic superoxide dismutase (SOD) catalysis or it reacts with NO* in the fastest known biological reaction ($\sim 5 \times 10^9$ mol/L/s) to give rise to peroxynitrite ($ONOO^-$) [22].

There are multiple standard ROS (free radicals + non-paramagnetic species) detection methods available for intracellular [23–25] and *in vivo* [22,26] ROS detection. However, these methods cannot be directly applied for specifically probing free radicals. The primary limitation being that the signals produced by these standard methods is always dominated by the non-paramagnetic molecules. The reason is the relatively low abundance of short-lived free radicals compared to stable non-paramagnetic counterparts at any instant. Furthermore, free radical specific detection probes can also be oxidized by non-paramagnetic species which leads to a measurement artifact [27,28].

## 2. Brief mechanism of radical generation during virus infection

Cellular free radicals are produced during various metabolic processes, or by cellular redox reactions such as the Haber–Weiss and Fenton reactions. Virus infections related free radicals are mainly generated by the NADPH oxidase family (NOX/DUOX) and nitric oxide synthase (NOS). Herein, we aim to review the detection methods used in virus infection. Thus the radical generation mechanism is briefly illustrated here. A more exhaustive discussion of the mechanism of how the host cells modulate these enzymes was reviewed by Akaike et al. [14], Bedard et al. [29], Vermot et al. [30], Grandvaux et al. [31] and Taylor et al. [32].

*NOX/DUOX:* The NOX family are proteins that transfer electrons across biological membranes. In general, the electron acceptor is $O_2$ and the product of the electron transfer reaction is $*O_2^-$. NOX2, expressed in phagocytic cells, was the first NOX isoform identified. The other six isoforms and homologs, named NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2, in other organisms exhibit a similar structures and functions.

Here, we explain the mechanism based on NOX2 (Fig. 2a), and the difference between the NOX family members is summarized in Fig. 2b. NOX2 consists of a membrane flavocytochrome $b_{558}$ composed of NOX2 and the associated $p22^{phox}$ protein. $p22^{phox}$ and other protein partners stabilize NOX proteins and offer the binding site to regulatory components. Stimulus-dependent (e.g. Respiratory Syncytial Virus [33], Sendai Virus [33], Influenza A [34,35], Covid-19 [36], etc.) activation results in the phosphorylation and subsequent Rac-dependent translocation of cytosolic regulatory subunits, $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$, which associate with the membrane subunits allowing electrons to transfer from NADPH to FAD and ultimately reduce $O_2$ to generate $*O_2^-$. Under physiological conditions, activation of NOX1 and NOX3 requires the presence of the cytosolic factors NOXO1 and NOXA1, respectively, homologous to the $p47^{phox}$ and $p67^{phox}$ NOX2 subunits. NOX5, DUOX1 and DUOX2 are activated by $Ca^{2+}$. Several virus infections, including hepatitis B virus [37] and HIV [38] are involved in modulating $Ca^{2+}$ concentrations. In addition, interferon β (IFNβ) and tumor necrosis factor α (TNFα) synergize to induce DUOX2 and DUOXA2 expression [39]. Unlike other isoforms, NOX4 is constitutively active and mainly produces nonparamagnetic $H_2O_2$.

*iNOS:* NO is produced endogenously in a number of cells and tissues by the enzymatic action of (NOS), which catalyzes the oxidation of L-arginine to NO* and L-citrulline. Inducible NOS (iNOS) which is ubiquitously expressed during host responses to viral replication (Fig. 2c), is the main source of overproduced NO* in virus infection among three cellular NOS types (neuronal NOS, inducible NOS, and endothelial NOS). High amounts of NO* are generated when the iNOS gene is strongly upregulated. Proinflammatory cytokines, such as interferon-γ (IFN-γ), interleukin 12 (IL-12), and tumor necrosis factor-alpha (TNF-α), are considered as mediators that regulate iNOS expression. For instance, Influenza virus and Sendai virus regulate proinflammatory cytokines. In some virus diseases, viral replication or viral components directly upregulate iNOS through a pathway independent of proinflammatory cytokines. For instance, an envelope glycoprotein of HIV and human paramyxovirus respiratory syncytial virus directly trigger the iNOS expression [14,41].

On top of that, mitochondria are essential organelles for the generation of ROS through respiration and function as a crucial signaling platform for various biological responses. However, usually no

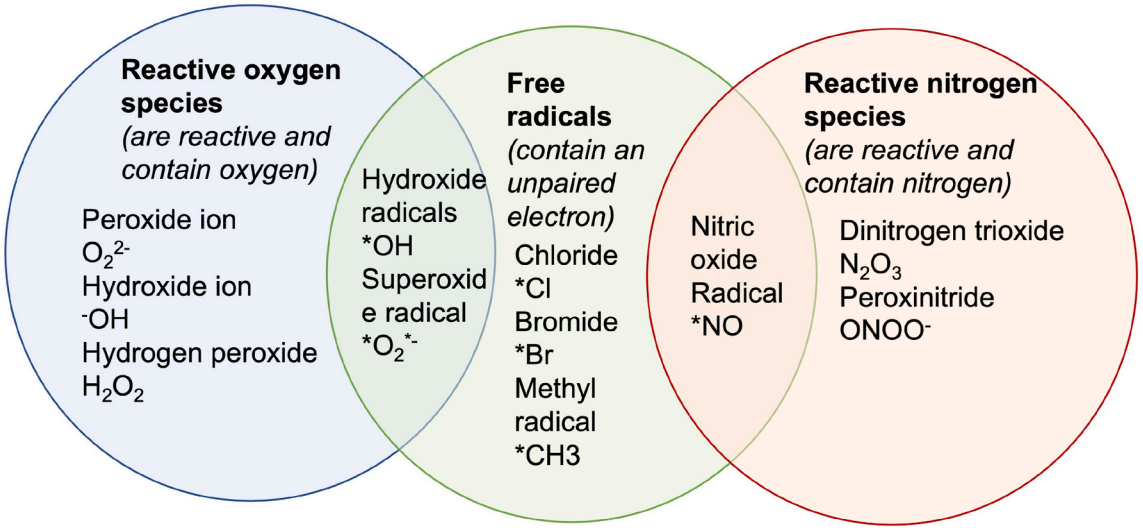


**Fig. 1.** Division of reactive species. Reactive species can be divided based on their chemical structure. Here we define what the respective groups are and give some of the most important examples. Reactive molecules can be divided based on the elements they include. Relevant for viral infections are reactive oxygen and reactive nitrogen species. These groups partially overlap with the group of free radicals which are defined as molecules or atoms with an unpaired electron. Additionally, reactive species can be differentiated based on the presence or absence of one or more unpaired electrons. A reactive molecule with such an unpaired electron is called a radical.

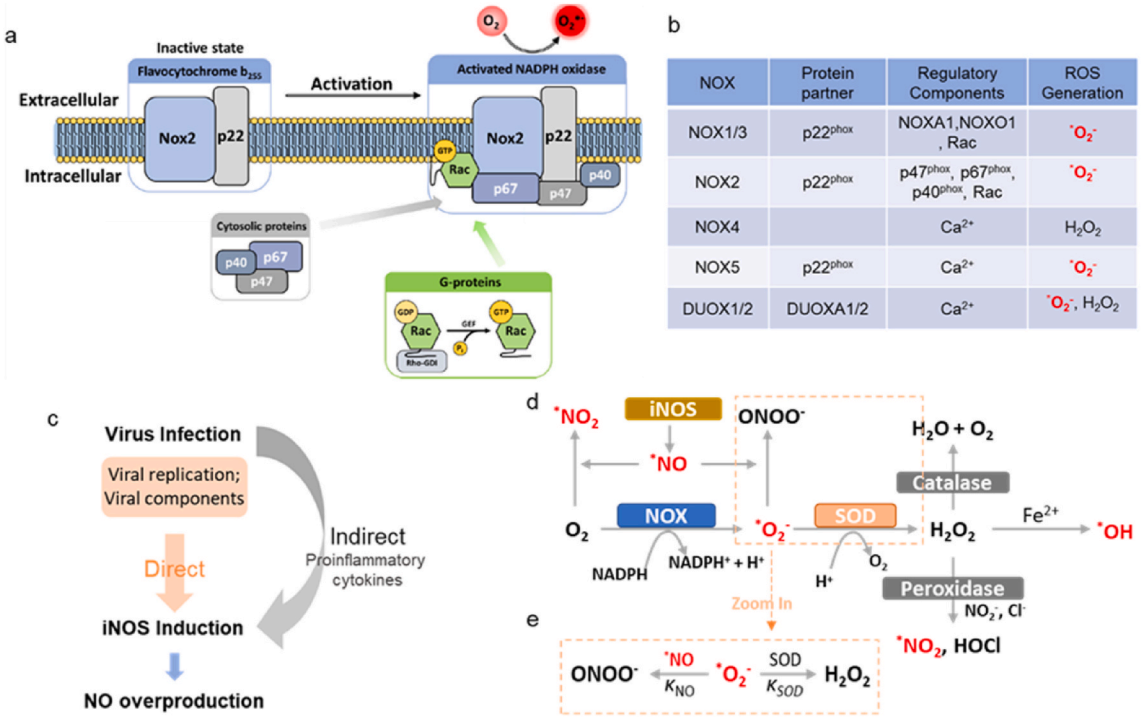


**Fig. 2.** (a) Activation process of NOX2. Detection of a pathogen (e.g. virus) triggers signaling pathways that lead to the phosphorylation of the cytosolic factors (mainly p47phox), inducing their translocation to the membrane-bound components of NOX2 and initiating the catalysis of superoxide production. Reproduced with permission from Vermot et al. [30] (b) Protein partner and regular components of each NOX. NOX 1, 2, 3 and 5 produce mainly $^{\bullet}O_2^-$, NOX4 produces mainly $H_2O_2$, and DUOX1/2 produce both $^{\bullet}O_2^-$ and $H_2O_2$. (c) Mechanisms of iNOS induction in viral diseases. (d) Forming and reaction of ROS and RNS. The free radicals, specifically, the $^{\bullet}O_2^-$, *NO, $^{\bullet}NO_2$, and *OH are marked inred. (e) The formation of $ONOO^-$, which mediates protein oxidation and nitration, lipid peroxidation, mitochondrial dysfunction, and cell death. The competition of forming $ONOO^-$ and $H_2O_2$ depends on the load of cellular *NO and SOD. Upon induction of iNOS, *NO will approach or exceed micromolar levels and will more efficiently compete with SOD, resulting in more significant fluxes of $ONOO^-$ [40].

differentiation between paramagnetic and non-paramagnetic species is made there. Core proteins of some viruses (e.g. hepatitis C virus [42]) cause mitochondrial dysfunction and endoplasmic reticulum (ER) stress resulting in increased ROS production from mitochondria (mtROS) as a result of the $Ca^{2+}$ released from the ER, which further contributes to viral inflammation. A recent study showed that respiratory syncytial virus infection resulted in the impairment of mitochondrial respiration, loss of mitochondrial membrane potential, and increased mtROS. While, mtROS generation can also be suppressed by HIV [43].

Additionally, cellular redox reactions illustrated in Fig. 2d can also

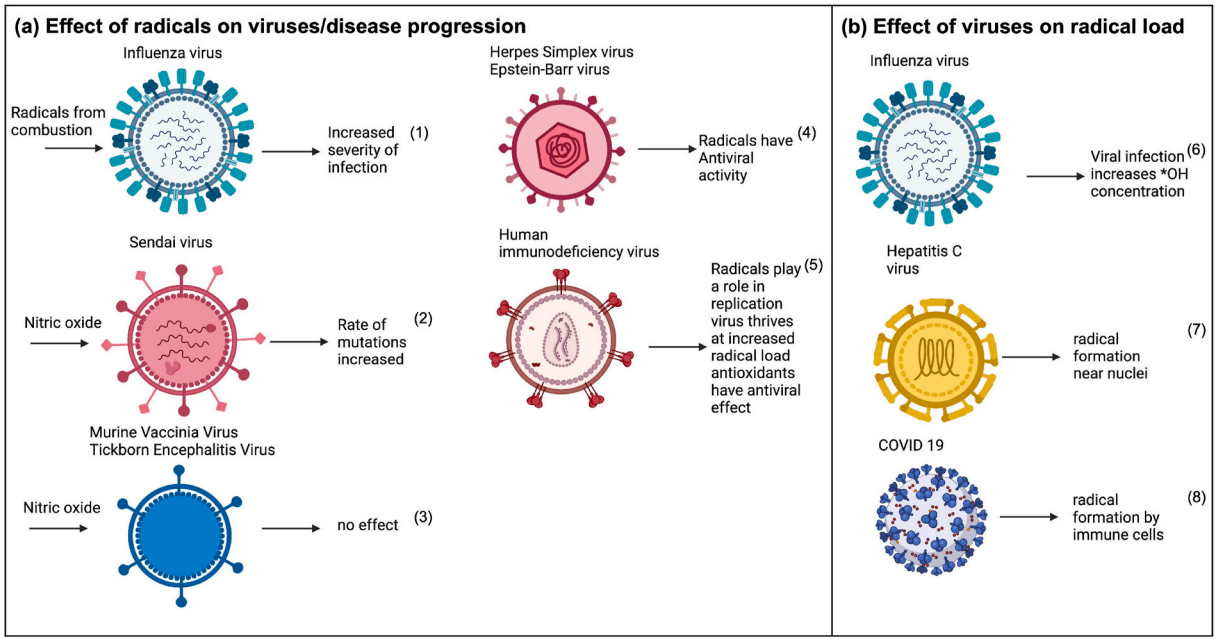


**Fig. 3.** Examples of effects of free radicals on viruses as well as of viruses on radical formation (1) [21], (2) [44], (3) [19], (4) [17], (5) [45], (6) [21], (7) [22], (8) [1].

produce new free radicals form including $^*NO_2$ and *OH, by oxidation of NO* and Fenton reactions, respectively.

## 3. The role of free radicals in viral infection

In this section we will briefly review examples of how free radicals alter the course of viral infection. When it comes to viral infections, free radicals can influence them in multiple different ways as shown in Fig. 3. Most commonly free radicals and other reactive species are generated during viral infections by infiltrating phagocytic cells and xanthine oxidase (XO) expressed in inflamed tissues [13,15,32,41,46]. Especially $^*O_2^-$ and NO* production increases in inflamed tissues. One way is to interact with the immune system. NO* for instance influences immune responses. For example, NO* suppresses type 1 helper T cell-dependent immune responses during infections, leading to type 2 helper T cell-biased immunological host responses [12]. NO*-induced immunosuppression may thus contribute to the pathogenesis of virus infections. Fridovich et al. was able to show the detrimental effect of *OH radicals generated during infection [47].

$^*O_2^-$ is primarily produced by inflammatory neutrophils and macrophages in a biochemical reaction expressing NADPH oxidase and responses involving xanthine oxidase. While elevated radical levels can have an antimicrobial effect [48], these radicals can damage all kinds of biomolecules if present in excess amounts resulting in lipid peroxidation, membrane protein or DNA damage [49]. On the other hand, NO* production primarily occurs by inducible NO* synthase (iNOS), which is expressed by inflammatory phagocytic cells as well as other types of cells [50]. Both antiviral and deleterious effects of free radicals generated by each mechanism are summarized in Table 1. The antiviral function of oxygen and nitrogen radicals on replication inhibition is mainly achieved by regulation of cytokines and protein levels. The deleterious effects are more severe infection symptoms, inflammation, tissue injury, etc.

There are multiple studies which highlight the precise role of oxygen free radicals and nitric oxide on Influenza virus infection and disease pathogenesis studied either *in vitro* or *in vivo* [41,53,67,77–79]. Recently, Lee and co-workers even demonstrated that exposure to combustion generated environmentally persistent free radicals enhances the severity of influenza virus infection [80]. Experimental investigations exploring the free radical – infection interplay are not limited to Influenza viruses alone but many other viruses have a connection with the host's free radical response. For example, the Hepatitis C virus (HCV) increases the production of hydroxyl radicals and peroxynitrite close to the cell nucleus which inflicts DNA damage [81]. Free radical dynamics in neurons in rat model upon Japanese Encephalitis infection [82] and possible involvement of oxygen free radical in cytomegalovirus infection in mice [83] is also confirmed. Additionally, it has been shown that free radicals or their reaction products can increase the mutation rate of viruses. Akaike et al. for instance showed accelerated mutation of the Sendai virus by nitric oxide production during the infection [84].

In light of the important role of free radicals in viral infections and the challenges in species specific quantitative measurements of free radicals, understanding state-of-the-art free radical detection methods in virology is of paramount importance. Below, we review the different techniques that are currently in use for this purpose.

**Table 1**
Antiviral or deleterious effects of free radicals in viral infections.

| Free radical types | Antiviral effects in: | Deleterious effects in: |
|---|---|---|
| NOX-derived $^*O_2^-$ | IAV (H1N1) [51,52], SeV [39], etc. (only by DUOX) | HIV [8,34], IAV [34,35,53,54], RSV [33], SeV [33], Covid-19 [55,56], DENV [34,57], HSV [34,58], RV [34,59], Vaccinia virus [34], etc. |
| iNOS-derived *NO | EVB [14], poxvirus, HSV, IV [60], RSV [61], coxsackievirus [62], Cucumber mosaic virus [63], CCHFV [64], etc. | TBEV [65], IV [66,67], DENV [68], HIV [67,69], etc. |
| Mitochondrial ROS (mtROS) | IAV [51,70], Sindbis Virus [71], Flavivirus [72], HIV-1 [73], etc. | HCV [42], IAV [74], RSV [75], Betanodavirus [76], etc. |

IAV: Influenza A virus; SeV: Sendai Virus; IV: Influenza virus; HIV: human immunodeficiency virus.
DENV: Dengue virus; HSV: Herpes simplex virus; EVB: Epstein-Barr virus; HCV: Hepatitis C virus.
RSV: Respiratory syncytial virus; TBEV: Tick-borne encephalitis virus; RV: Rhinovirus.
CCHFV: Crimean Congo hemorrhagic fever virus.

## 4. Detection techniques

An overview over the most common detection techniques is shown in Fig. 4.

### 4.1. Optical probes

Today, several strategies for designing optical probes, including photoinduced electron transfer (PeT), intramolecular charge transfer (ICT), spirocyclization, and chemiluminescence are well established and have been applied to many probes [85]. An excellent article on different probes and their properties in general can be found here [86]. Most optical probes (including most commercial and newly designed probes) for detecting free radicals are based on the reactions between probes and different types of free radicals due to the high reactivity of free radicals. Even some probes were designed for specific radical detection by utilizing their higher reactivity (for example *OH and $^*O_2^-$.) than the other radicals or by utilizing the unique reaction between probes and radicals (such as NO*). However, the high reactivity is not only exhibited by free radicals but many nonparamagnetic ROS/RNS and cellular enzymes can also oxidize or reduce optical probes and thus show a cross-reactivity [86].

In the next subsections, we review the optical probes used in virology research including their sensing mechanisms and the reactions between probes and free radicals. This way, we discuss clearly whether the probe can specifically detect certain radicals. We also discuss cross reactivity with other nonparamagnetic ROS/RNS and cellular enzymes. In addition, the applications of different probes in virus-related research are summarized in Table 2. It should be noted that, in this review, we focus only on the free radical detecting post viral infections. Hence, probes that can only detect the nonparamagnetic ROS/RNS, such as Amplex Red and Homovanillic acid which detect the cellular $H_2O_2$ in presence of peroxidases, are not included in this section [52,87].

Fluorescence spectrometers [88,89], flow cytometry [78,90–92], and fluorescence microscopy [93] are normally used to read out of the fluorescent signals of optical probes. Fluorescence imaging offers higher spatial resolution and is sensitive and less invasive compared to other approaches. Spectroscopy on the other hand offers possibilities to differentiate probes from other components spectroscopically and is thus more specific. Flow cytometry offers a great advantage by facilitating the single cell analysis thus excellent statistical power. On the other hand, it requires the sample to be in the form of single cells in solution and is thus invasive and offers no spatial and only limited spectral resolution.

#### 4.1.1. Deprotonation of probes

A conjugated system, an arrangement of alternating single and double bonds, is the main structural feature for organic fluorescent molecules. The larger the conjugated system is, the easier it is for the electrons of the delocalized π bond to be excited, and the easier it is to generate fluorescence. Based on the transformation of conjugated systems, several fluorescent probes, including 5-ethyl-6-phenyl-5,6-

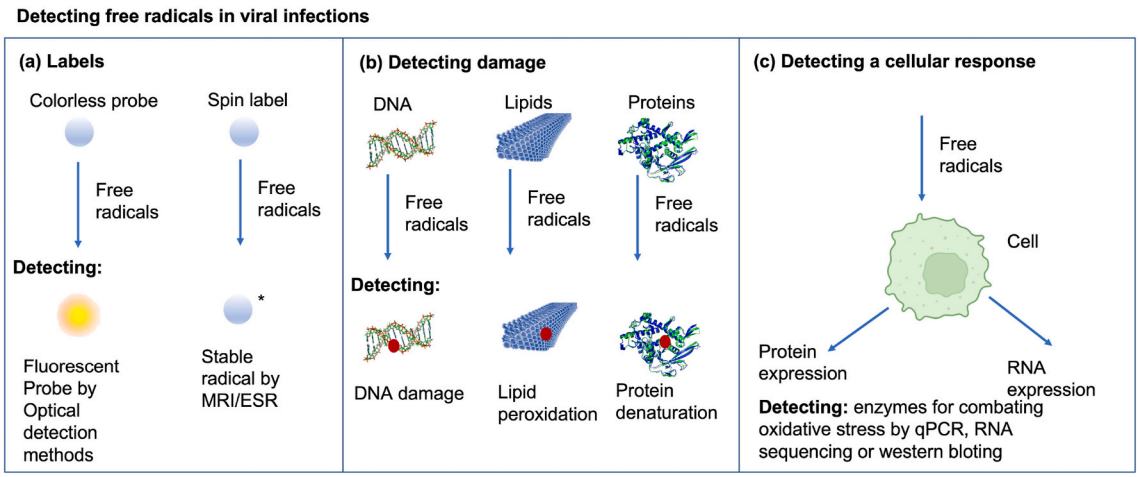


**Fig. 4.** Overview of detection methods for free radicals: There are several options for detecting free radicals in cells that are affected by viruses. (a) shows the possibility of using labels. Generally, there are 2 types of label: labels that react with radicals to form a fluorescent product that can then be detected optically or spin labels which react with free radicals to form a stable radical which can be detected with magnetic resonance techniques. (b) shows different ways of detecting free radicals indirectly via the damage they cause to biomolecules. (c) summarises the possibilities to detect free radicals indirectly by detecting the cells response to the radical generation.

dihydrophenanthridine-3,8-diamine (DHE), 2-(3,6-diacetoxy-2,7-dichloro-9H-xanthen-9-yl)benzoate (DCFH-DA), MitoSOX, methyl 2-(3,6-diamino-9H-xanthen-9-yl)benzoate (DHR), etc. were designed. All these probes have a common feature that an aliphatic ring spaces several aromatic rings, resulting in the suppression of the formation of larger conjugated systems. Free radicals are known for their high reactivity, especially the oxidability, which allows them to snatch the hydrogen atoms (red hydrogen atom in Fig. 5) of these probes. After deprotonation, the extension of the π-conjugation system is formed to yield fluorescence. Besides, the planarity of the conjugated π-electron system of these molecules also affects the transitions strength.

DCFH-DA, DHR, DHE, and MitoSOX are highly used radical fluorescent probes not only in mammalian cell infectious systems but also in plant infection systems. DCFH-DA is the most popular probe used for detecting general intracellular ROS, as it can be oxidized by most ROS (such as *OH, ROO*, and $H_2O_2$), and also by RNS (like *NO and $ONOO^-$) to yield green fluorescence. For instance, Pichili et al. used DCFH-DA to detect the oxidative stress inside astrocytes induced by HIV-1 viral envelope protein gp120 [94]. Besides, DCFDA was also used to detect the intracellular ROS in Neutrophils-Respiratory Syncytial Virus (RSV) [95], *N. benthamiana* epidermal cells-Red Clover Necrotic Mosaic Virus [96], and other infection systems. DCFH-DA, however, is not a suitable probe for radical detection due to its higher reactivity with other non-paramagnetic ROS in presence of cellular peroxidases. The signal is dominated by these non-paramagnetic molecules against paramagnetic free radicals due to the difference in their relative amounts. It is noteworthy that horseradish peroxidase (HRP), xanthine oxidase, and cytochrome *c* can highly increase the formation of DCF. Additionally, the oxidative product DCF can suffer photoreduction in presence of light to generate the semiquinone radical form $DCF^{*-}$ which can react with $O_2$ and originate $^*O_2^-$. In turn, the dismutation of $^*O_2^-$ generates $H_2O_2$, which leads to an amplification of DCF fluorescence [97]. Due to these issues, DCFH-DA has better use as a marker of cellular oxidative stress than as indicator of radicals. Similarly, DHR is also used to evaluate the general oxidative stress inside cells.

In contrast to DCFH-DA and DHR, DHE and MitoSOX have been used as fluorescent probes for detecting $^*O_2^-$ in virology studies, due to their relative specificity. For instance, by using DHE, Lang et al. [98] found that lymphocytic choriomeningitis virus infection led to elevated levels of $^*O_2^-$ producing granulocytes and macrophages in virus-infected liver and spleen tissues. Strengert et al. [52] used DHE on isolated nuclei to reveal that mucosal $^*O_2^-$ are required for anti-Influenza A virus response. However, DHE is unfortunately also not specific for radicals. Tarpel et al. [25] pointed out that (1) cytochrome *c* can oxidize DHE as well, (2) the formation of E+ that exceeds the connection capacity of mitochondrial nucleic acid, which might lead to the increase of substantial fluorescence. Besides DHE, MitoSOX is widely used as well. This probe can be specifically targeted to mitochondria in live cells, and the oxidation product yields red fluorescence. Yanchun et al. [99] found that the hepatitis C virus (HCV) produces a viral core protein that targets mitochondria and increases $Ca^{2+}$-dependent ROS production by using MitoSOX. (More applications of DCFH-DA, DHR, DHE, and MitoSOX in virology research are listed in Table 2.)

Moreover, quinoline-based molecules [100,101] and phenol–quinone interconversion [101] (Fig. 5b and c) were utilized for design of fluorescent probes based on the extension of π-conjugation system. They were already successfully used in intracellular radical detection, and they have good prospects in virus research in the future.

#### 4.1.2. Aromatic diamino fluorescent probes

Aromatic diamino fluorescent probes were developed in attempts to measure NO* generated under physiological conditions. As described in Fig. 6, the relatively nonfluorescent aromatic diamino compound, such as 2,3-Diaminonaphthalene (DAN), reacts rapidly with NO* to yield the highly fluorescent triazole product (DNT). Based on this mechanism, aromatic diamino groups were used to modulate the fluorescence of several widely used fluorophores in biology, such as fluorescein, rhodamine, Boron dipyrromethene (BODIPY), and Cyanine to form diaminofluoresceins (DAFs), Diaminorhodamines (DARs), diaminoBODIPY (DAMBO), and diaminocyanines (DACs). In Fluorescein for example, DAF is converted to the corresponding triazole forms (DAF-Ts) by reaction with NO* causing greatly increased fluorescent intensity. Piccoli et al. [93] reported that expression of HCV proteins causes deregulation of mitochondrial calcium homeostasis. This event occurs upstream of further mitochondrial dysfunction, leading to alterations in the bioenergetic balance and nitro-oxidative stress by using DAF-FM-DA. DAF-2-DA was used by Raymond et al. [102] to evaluate the NO* load of Rift Valley Fever Virus Infected Raw 264.7 cells.

#### 4.1.3. Fluorescent probes based on photoinduced electron transfer

As shown in Fig. 7, 4-amino- or 4-hydroxylphenyl ether moieties are more electron-rich than the xanthene moiety and they were used to quench the fluorescence of rhodamine through a photoinduced electron transfer process (PeT) [130]. 4-minophenylaryl ether and

**Table 2**
Summarized fluorescent and chemiluminescent radical probes used in virology.

| Probes | Ex/Em (nm) | Radicals Detected | Related virus | Nonparamagnetic ROS/RNS Detected |
|---|---|---|---|---|
| DCFH-DA | 500/520 | *OH, NO*, *OOR | HCV [88], HIV [94], RSV [89], Influenza Virus [78], Enterovirus 71 [103], Red Clover Necrotic Mosaic Virus [96], Wheat Yellow Mosaic Virus [104], HSV [90], etc. | $H_2O_2$, HOCl, $ONOO^-$, $^1O_2$ Autooxidation |
| DHR | 505/525 | *OH, NO*, *OOR | HIV [91], Rous Sarcoma Virus [105], HSV [92], Adenoviruses [106], etc. | $H_2O_2$, HOCl, $^1O_2$, $ONOO^-$ |
| DHE | 520/610 | $*O_2^-$ | Lymphocytic choriomeningitis virus [98], Influenza virus A, HBV [52], HCV [107,108], Adenoviruses [109], Rous sarcoma virus [110], etc. | NA |
| MitoSOX | 510/580 | $*O_2^-$ | HCV [99], Influenza virus [70,111], African Swine Fever Virus [112], HSV [113], Encephalomyocarditis Virus [114], etc. | NA |
| DAF | 495/515 | *NO | HCV [93], Rift Valley Fever Virus [102], Tobacco mosaic virus [115,116], white spot syndrome virus [117], Mouse-adapted influenza A virus (H3N2) [118], etc | NA |
| HPF/APF | 490/515 | *OH | HSV [117], HCV [119,120], Sendai virus [115], Influenza A virus [115,120], HIV [121], etc. | $ONOO^-$ and HOCl (only APF) |
| NBT | | $*O_2^-$ | Plum Pox Virus [122], HBV [123], HIV [124], Sunflower Chlorotic Mottle Virus [125], Tobacco Mosaic Virus [126] etc. | NA |

| CL probes | Em (nm) | Radicals Detected | Related virus | Nonparamagnetic ROS/RNS Detected |
|---|---|---|---|---|
| Luminol | 425 | *OH, $*O_2^-$ | HCV [127], Epstein–Barr virus [128], influenza A virus [85] | $H_2O_2$ |
| L-012 | 450 | *OH, $*O_2^-$ | influenza A virus [34,53] | $H_2O_2$ |
| MCLA | 465 | $*O_2^-$ | HIV [129] | $^1O_2$ |

HCV: Hepatitis C Virus, HBV: Hepatitis B Virus, HIV: Human Immunodeficiency Viruses.
HSV: Herpes simplex virus, RSV: Respiratory Syncytial Virus.

4-hydroxylphenyl aryl ether react with highly reactive ROS, namely *OH, HOCl and $ONOO^-$, selectively over other ROS ($H_2O_2$, $^1O_2$, $*O_2^-$, and ROO*) to restore the fluorescence of fluorescein by de-PeT process. Jerry et al. [131] reported that Herpes simplex virus type 1 infection induces oxidative stress of neurons (P19 N cells) using HPF. Yu-Hsiang et al. [132] revealed that Influenza A (WSN) virus infections can induce higher concentrations of ROS in lung cells. Though the use of HPF and APF to detect *OH might be worthwhile, HPF and APF do not reflect the whole radical responses upon virus infection due to their high specificity.

#### 4.1.4. Fluorescent probes based on reducibility of free radicals

The nitroblue tetrazolium (NBT) assay is also widely used to detect the $*O_2^-$ post viral infections. However, the detection mechanism of NBT is based on the reducibility of $*O_2^-$, which is different from all the above fluorescent probes that utilize the oxidability of free radicals. Specifically, the tetrazolium rings of NBT are opened to generate formazan when reduced by $*O_2^-$ (Fig. 7 b). As a result of the insoluble precipitate of formazan, the NBT assay was not used in living cells but in clinical *ex-vivo* tissue samples and plant samples. For instance, Connie et al. [124] reported that neutrophils from asymptomatic HIV-infected patients have an increased NBT reduction, that is an increased production of oxygen radicals. Feng et al. [126] reported significantly enhanced $*O_2^-$ accumulation in tobacco mosaic virus (TMV) infected *Nicotiana Benthamian* plants. However, Francis et al. [133] indicated several limitations of the NBT assay. NBT detects intracellular $*O_2^-$, however, it is less sensitive and specific than DHE. NBT can be reduced by several tissue reductases, and therefore specificity for $*O_2^-$should be confirmed by inhibition of NBT staining by SOD. Besides, the autoxidation of NBT leads to the artifactual generation of $*O_2^-$. For these reasons, detection of $*O_2^-$ in biologic samples should not rely exclusively on NBT reduction.

#### 4.1.5. Chemiluminescence probes

Another method based on optical probes to measure the radical load during viral infection is using chemiluminescence (CL). Unlike fluorescent probes, chemiluminescent probes can adsorb the reaction energy to excite the intermediates, and then emit luminescence, while the products of fluorescent probes still need excitation from light. As described in Fig. 8, Luminol, L-012, Lucigenin and the Cypridina-Luciferin analog (CLA) can emit photons through the excited intermediate of phthalic acid, endoperoxide, oxo-dihydroacridin, and pyrazine acetamide. This approach was used by Fujimori et al. The goal of their study was to assess the antiviral properties of copper(I)iodide nanoparticles against H1N1 influenza virus [134]. In their study they measured hydroxyl radical formation using chemiluminescence and concluded that the antiviral activity was caused by these radicals. To perform the assay, they added horseradish peroxidase and luminol solution to the particles in solution (they did not test this in a biological environment). Under this condition hydroxyl radicals react with luminol forming a chemiluminescent product which was then read out optically. Proskurnina et al. similarly suggest testing antiviral drugs for their antioxidant capacity in solution to better understand their working mechanism [135]. However, Luminol analog probes can react with nonparamagnetic ROS like $H_2O_2$ overestimating the radical concentration.

In another study, CLA were used to evaluate the $*O_2^-$ load post virus infection due to their specificity. Jadwiga et al. [129] reported that the human immunodeficiency virus-Tat (HIV-Tat) can significantly increase Methyl-CLA (MCLA) luminescence. Besides, Lucigenin-enhanced CL (LECL) was also successfully used to detect $*O_2^-$ and *OOH in various cells [136], but it has not been used in a virology study yet. Although MCLA and Lucigenin are more specific to $*O_2^-$ and *OOH, they both are also influenced by $^1O_2$. Besides, CL probes have a common disadvantage: they lack the sensitivity to offer cellular resolution.

### 4.2. Magnetic resonance techniques

Magnetic resonance techniques such as nuclear magnetic resonance (NMR), electron spin resonance (ESR) and magnetic resonance imaging (MRI) are promising for detecting radicals since the free electrons of radicals themselves can be detected via their magnetic resonance. For studying free radicals, magnetic resonance techniques have been used for decades [137]. However, their very short half-life makes such investigations difficult. Hence to circumvent this problem, “spin traps” are used. These are the molecules which form a stable radical after reacting covalently with an unstable radical which in turn can be detected using their magnetic resonance even at room temperature [138]. NMR spin trapping to study biologically relevant free radicals has been reported in the literature [139].

Most of the studies in virology that make use of NMR are focused on studying proteins, structure and physics of viruses [140] such as

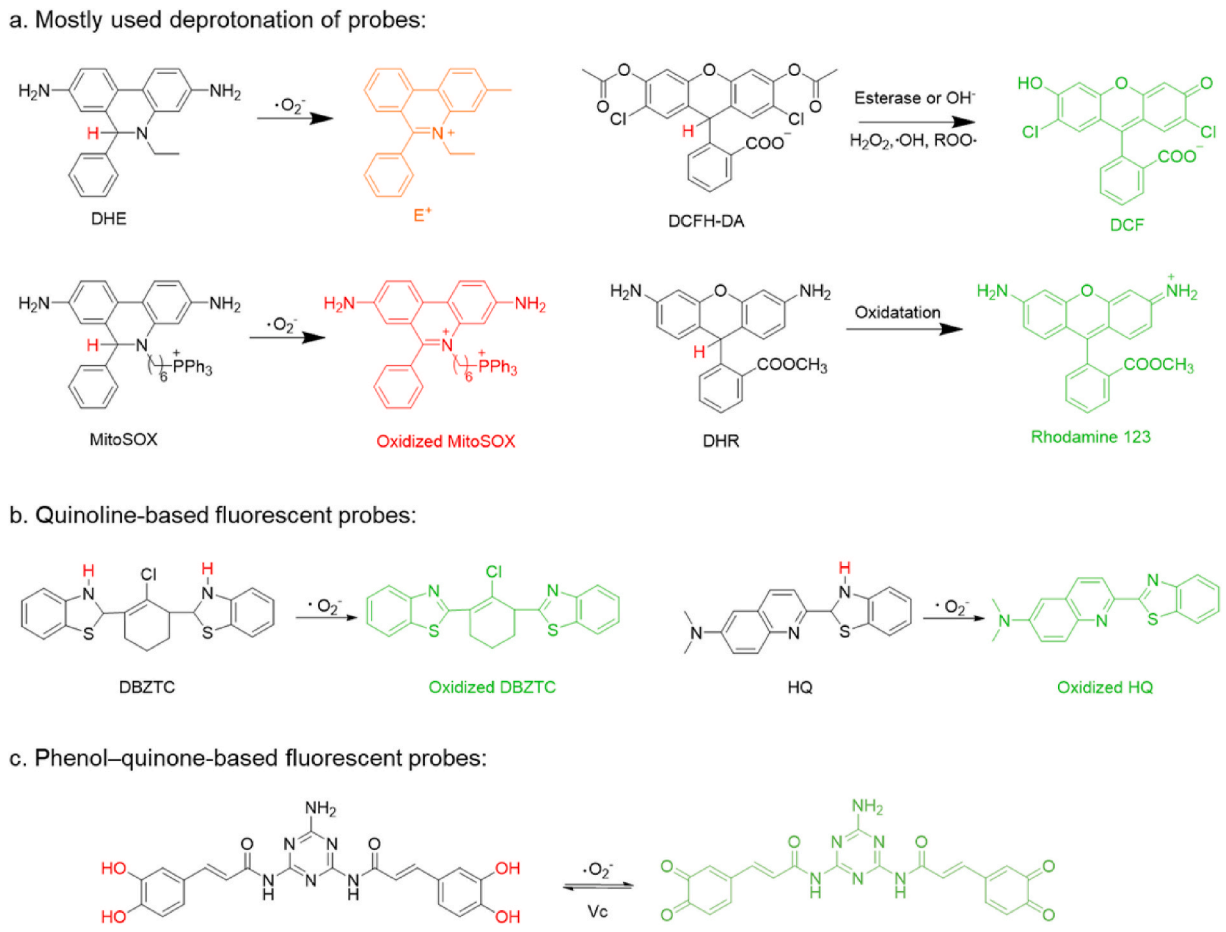


**Fig. 5.** Chemical structure transformations of deprotonation based fluorescent probes when used as free radical probes. (a) most used commerical deprotonation probes including DHE, DCFH-DA, MitoSOX, and DHR. (b) Quinoline-based fluorescent probes, including DBZTC and HQ. (c) An example of phenol-quinone based fluorescent probe. Before deprotonation, all these are nonfluorescent. A double bond is formed after deprotonation which connects nearby aromatic rings and increases the planarity of the molecules, which yields fluorescence. The colors of oxidized products represent the fluorescent colors of these probes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Influenza B [141], Hepatitis B [142,143], SARS-CoV [144], Flavivirus Proteases [145] and Zika [146]. In fact, atomic resolution structural and dynamics of viral systems was revealed using magic angle spinning NMR [147]. Apart from this structural investigations "theme", researchers are also deploying NMR to elucidate details of the infection mechanism via metabolic profiling upon infection. In particular, change in metabolites concentration upon infection is analyzed in turn inferring the infection mechanism or viral pathogenesis from those changes [148,149]. For example, in early 1980s, NMR spectra were found to alter upon introducing the Influenza infection in chicken embryo fibroblasts [150]. More recently, NMR-based metabolic profiling was used for identifying the early stages of Echovirus 11 infection through observing metabolic changes in cells [151]. Furthermore, it was also used to pinpoint the effects of Hepatitis B virus X protein (HBx) on cell metabolism [152]. Not just limited to cellular level events, but metabolic fingerprints of viral infection in bodily fluids are also investigated using NMR. For instance, fingerprints of HCV and HBV infections in human serum [153] or the influence of acute respiratory syncytial virus (RSV) infection on urine composition has been reported in the literature [154]. In the latter case, the hypothesis was that children with RSV infection would have a different metabolomic profile compared to those with bacterial infection or healthy controls. Furthermore, this method was also used to determine the severity of bronchiolitis or to identify a viral from bacterial causes of respiratory distress.

NMR is not only useful for understanding the fundamental viral infection, but also for studying antivirals. Benie et al. reported the application of saturation transfer difference NMR spectroscopy to explore the attachment of antiviral compounds to native human rhinovirus serotype 2 (HRV2) [155]. The role of singlet oxygen in the function of antiviral compounds against Vesicular Stomatitis Virus was also investigated [156]. Further, NMR-based identification of common compound groups or compounds from plants with anti-viral activities against Herpes Simplex Virus (HSV), Cytomegalovirus (CMV) and Human Immunodeficiency Virus (HIV) was carried out [157].

Despite of abundant studies involving the utilization of NMR in virology research, free radical sensing upon viral infection has not been studied using NMR to the best of our knowledge. In cell NMR can be a great technique to specifically study free radicals although the use of the technique has been mostly limited to studying proteins in their native environment [158]. However, one hurdle in the road might be the detection limit of in this method which is in the range of ~100 μM [159]. This detection limit is several orders of magnitude higher than the typical free radical concentrations observed in the cells in the micromolar to nanomolar range [160,161].

Pietraforte et al. used electron spin resonance measurements in combination with spin traps to investigate the effect of recombinant gp120 HIV envelope glycoprotein on the generation of free radicals by monocyte-derived macrophages [162]. They found that these macrophages produced 300% more nitric oxide than cells which were not exposed to the viruses. They did not observe an increase in any other oxygen free radicals that were tested. The authors attributed the increase in radical load to nitic oxide by systematically blocking specific

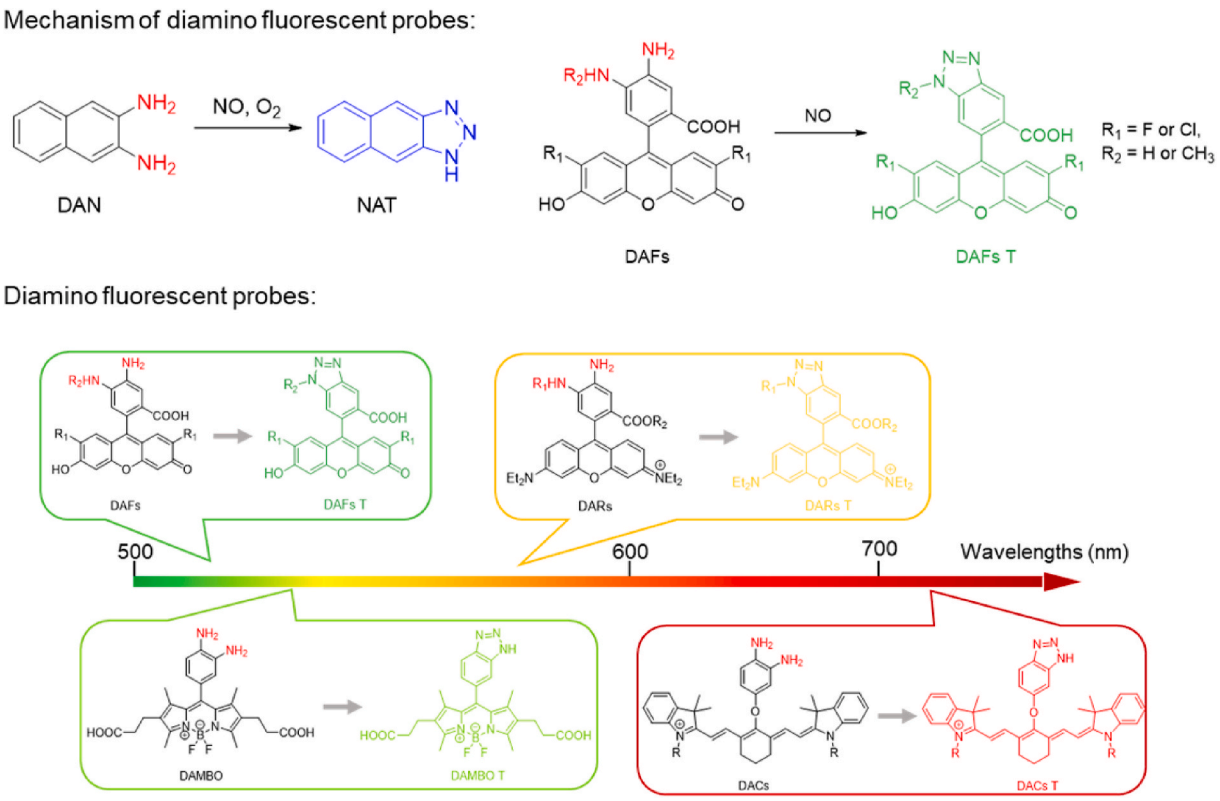


**Fig. 6.** Chemical structure transformations of fluorescent probes. Nonfluorescent aromatic diamino groups (DAN) react rapidly with NO* to yield the highly fluorescent triazole product (NAT). Aromatic diamino groups on different fluorophores originate DAFs, DARs, DAMBO, and DACs which fluoresce at different wavelengths. The reactive aromatic diamino groups are marked as red, and the colors of triazole products represent the fluorescent colors these probes can generate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

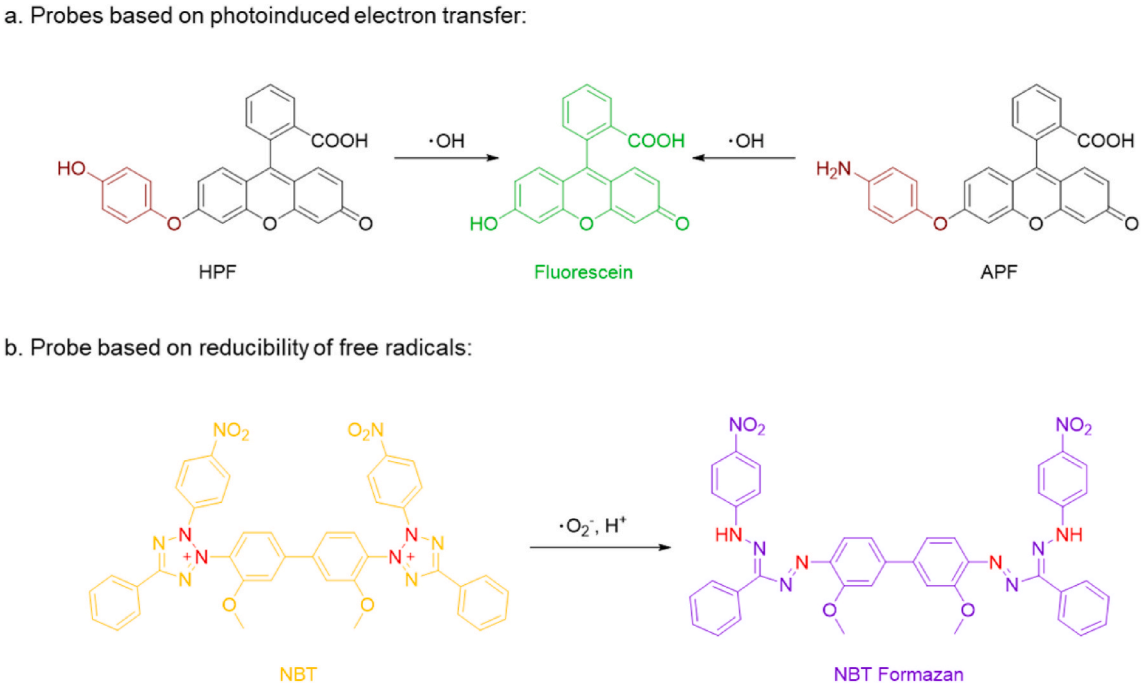


**Fig. 7.** Chemical structure transformations of the fluorescent probes based on the oxidability and reducibility of free radicals. (a) The phenyl aryl ether bond of HPF and APF will be cleaved when it reacts with highly reactive ROS to restore the fluoresce of rhodamine. (b) The tetrazolium rings of NBT are opened to generate formazan when reduced by $*O^{2-}$. Ring opening sites of NBT are marked in red. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

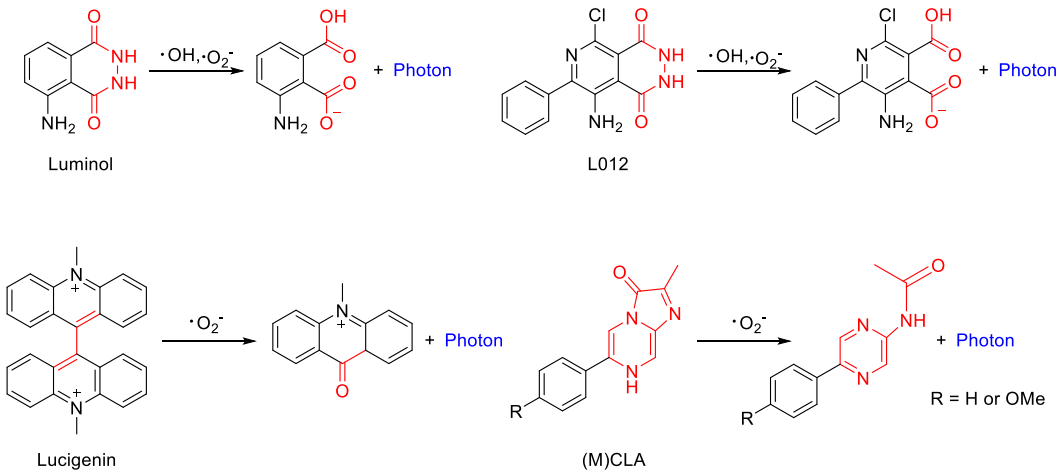


**Fig. 8.** Chemical structure transformations of chemiluminescent probes, namely Luminol, L-012, Lucigenin, and (M)CLA. The reactive sites are marked in red. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

pathways and observing the outcome.

Valgimigli et al. performed an EPR based reactive oxygen species (ROS) measurement in human hepatic tissue. They found that the amount of ROS in healthy human liver was significantly lower than in liver affected by hepatitis B or by hepatitis C [163]. Tomter et al. investigated the properties of the tyrosyl radical in Epstein-Barr virus ribonucleotide reductase small subunit RNR R2 [164]. This is a stable radical that is needed for enzyme turnover. The connection between viral mutation rate in Sendai viruses and the nitric oxide concentration was also studied using EPR. The authors investigated this phenomenon in wild type mice and knockout mice which lacked NO synthase. Both were subjected to the virus but wild type mice showed increased mutation rates. Since most radicals are too short-lived, this is one of very few studies where a biologically relevant radical was studied directly.

In summary, although EPR and MRI are “go-to” techniques in many scientific research streams, they are rarely used to study the cellular free radical/ROS response upon viral infection. This is due to their serious limitations when high spatial resolution is required or only limited amounts of sample are available (inside of a cell for example).

### 4.3. Detecting damage caused by radicals

As mentioned earlier free radicals are short lived and reactive. As a result, they can react with all kinds of biomolecules in their environment and damage them in the process. While this is the reason why an excess of radical production is toxic to cells, this also opens up an opportunity for detecting the radical load indirectly by measuring the damage caused by the radicals. One might also argue, that the damage done by the radicals is the information that is ultimately relevant for assessing the health status of cells or tissues. Unfortunately, also for these methods there is no spatial resolution and the method is destructive.

#### 4.3.1. Detecting DNA damage to assess the radical load

One of the most important techniques is to measure DNA damage. This can be achieved by measuring the 8-hydroxydeoxyguanosine (8-OHdG) content in cells. There are multiple ways to measure the DNA damage such as DNA purification followed by chromatography and analysis [165]. This was for instance achieved by Farinati et al. to assess the radical load during hepatitis virus infections. They measured DNA damage this way in both liver tissues and circulating leukocytes. They found that DNA damage (supposedly from radical damage) was elevated in both hepatitis C virus (HCV) and hepatitis B virus (HBV) patients but greatest for HCV. Alternatively, it is possible to measure DNA damage using enzyme-linked immunosorbent assay (ELISA) kits [166]. This approach has for instance been applied by Muhsin et al. who investigated the effect of sexually transmitted viruses like herpes simplex have on the sperm quality in men [96]. One of the parameters they assessed was the DNA damage which was correlated with poor sperm quality. In another study Jakovljevic et al. used the same approach to reveal that 8-OHdG was elevated in periodontitis lesions infected by Epstein–Barr virus [167]. Kolgiri et al. measured 8-hydroxy-2-deoxyguanosine with 8-hydroxy-2-deoxyguanosine using ELISA kits and found that DNA damage were significantly higher in HIV-positive patients with second-line antiretroviral therapy (ART) and first-line ART than ART-naive patients [168]. Lai et al. found this way that free radical generation is increased in dendritic cells that were infected with dengue viruses (DENV) [169]. Their hypothesis is that DENV infection promotes the release of mitochondrial DNA (mtDNA) into the cytosol and activates TLR9 signaling pathways, leading to production of interferons (IFNs). The DENV-induced mtDNA release involves reactive oxygen species generation and inflammasome activation.

When comparing to other radical detection methods, it is worth mentioning, that this technique is compatible with frozen material and there is no need to work with fresh material. While assessing DNA damage has yielded invaluable insights in free radical biology in viral infections, there are several limitations that need to be mentioned. First, this method only captures radical formation near DNA molecules. While this is a clear advantage for some application, as for instance when studying cancer formation related to hepatitis infections [170], this is not always desired. Second relatively large amounts of material are needed and there is no spatial resolution. Finally, the technique is destructive which renders it unusable in in-vivo studies or when the material is still needed.

#### 4.3.2. Lipid peroxidation

Damage to lipids can also be used to assess the free radical load. To assess this damage which is also called lipid peroxidation, it is common to detect compounds of lipids that react with thiobarbituric acid (TBA) [103]. Due to their reactivity with thiobarbituric acid (TBA) they are also called TBARS (thiobarbituric acid reactive substances). Among molecules that are detected are for instance malondialdehyde (most commonly used), F2-isoprostane, 7-ketocholesterol, and 7β-hydroxycholesterol. These are for instance occurring in elevated levels during influenza virus infection [77,171]. To perform the assay, TBA is added to plasma or tissue lysate samples. The above-mentioned compounds form a precipitate with TBA (as shown in Fig. 9). This precipitate is then extracted with n-butanol and injected into the high-performance liquid chromatography (HPLC) system [107].

This method was used by Klassen et al. [172] to investigate lipid peroxidation in the plasma of dengue virus patients. They found slightly

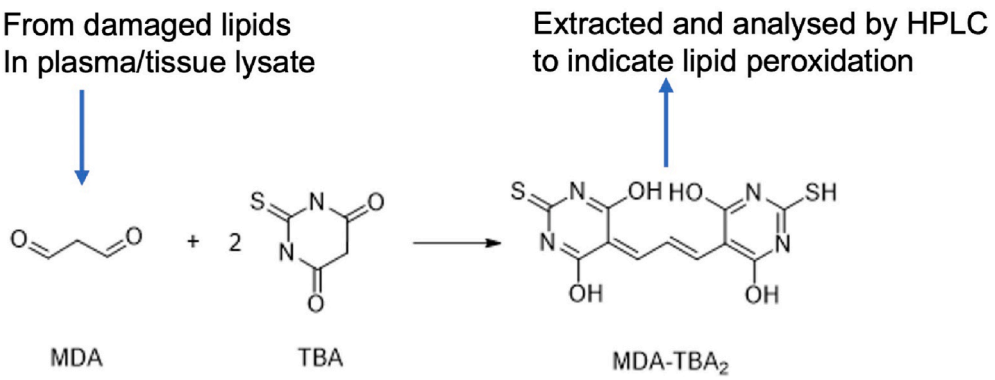


**Fig. 9.** Lipid Peroxidation. Malondialdehyde (MDA) and structurally similar molecules are formed during lipid peroxidation. Then thiobarbituric acid (TBA) is added to tissue lysates or plasma samples. Together they form a precipitate ($MDA\text{-}TBA_2$), which subsequently is extracted and analyzed by high performance liquid chromatography (HPLC).

but non-significantly increased lipid peroxidation when comparing to healthy control samples.

The drawbacks of this method are similar than for detecting DNA damage. The method is destructive and there is no spatial resolution. Furthermore, there is no real time detection since the assay captures all the damage that has been done (the history of the sample) and not just the current status. Further, lipid peroxidation can be caused by a multitude of reactive molecules and is not specific for radicals.

#### 4.3.3. Detecting protein damage

Since proteins are abundant in cells and tissues and readily react with free radicals, measuring protein adducts offers a strategy to detect radicals by the damage they cause [173]. The majority of radical reactions with proteins occur via three major pathways— hydrogen atom abstraction from C–H, S–H, N–H, or O–H bonds, electron abstraction from electron-rich sites, and addition to electron-rich centers (aromatic rings and sulfur species).

Detection methods for the resulting products is usually achieved by chromatography followed by mass spectroscopy or antibody-based detection of adducts. Since radicals can induce a chain reaction that leads to the formation of more radical molecules (see Fig. 10), it is also an option to study protein damage with electron spin resonance. Ohhira et al. for instance used protein adducts to assess liver damage from different sources including viral liver disease [174]. They detected the protein adducts by immunohistochemistry and found that the liver damage in alcoholic liver disease than in viral liver disease. Another example is the work reported by Maki et al. who used protein adducts as biomarkers to assess liver carcinogenesis in hepatitis C virus infected patients [175]. Compared to previously described methods, using protein damage to assess the radical load has several drawbacks. First, there are many proteins and thus many possible adducts can form which renders this method relatively complex. As a result, relatively costly methods are needed for analysis.

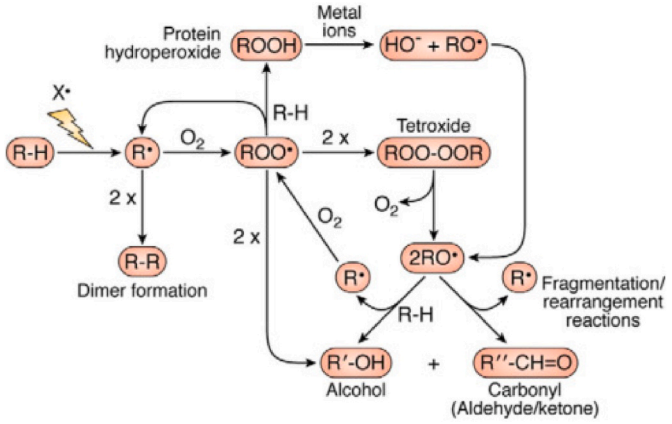


**Fig. 10.** The most important reactions between radicals and proteins and their resulting products. Reproduced with permission from Hawkins et al. [173].

### 4.4. Detecting cellular responses

A powerful alternative for detecting radicals directly is to measure how cells respond to an increased radical load. This can for instance be achieved by following synthesis of antioxidants and enzymes which degrade radicals. This can be done at the RNA or the protein level and has the advantage that enzymes are usually highly specific and thus this is the method with the greatest specificity. Antioxidant defenses include the enzymes superoxide dismutase (SOD, scavenge superoxide radicals), glutathione peroxidases (reduce hydroperoxides arising from lipid oxidation), and reductase. Besides, assessing the activity of $*O_2^-$ generation related enzymes, using NADPH oxidases, is also a promising method to reveal the $*O_2^-$ load.

#### 4.4.1. Detecting glutathione

Glutathione (GSH) is a major compound of the antioxidant defense against intracellular ROS. Normally, a decline in cellular GSH results in an increase in reactive oxygen species (ROS) production. Klassen et al. for instance investigated plasma concentrations of glutathione (important in the defense against toxic injury), which were produced during exposure to dengue viruses [172]. They found that glutathione production was significantly reduced in dengue virus patients compared to healthy controls.

#### 4.4.2. Detecting the activity of superoxide dismutase

SODs, antioxidant enzymes, play vital roles in scavenging $*O_2^-$. Decreased SOD activity results in elevated level of superoxide (Fig. 11). Strycharz-Dudziak et al. investigated free radical formation in patients who were suffering from oropharyngeal cancer associated with EBV infection [176]. In this work, they investigated the amount of

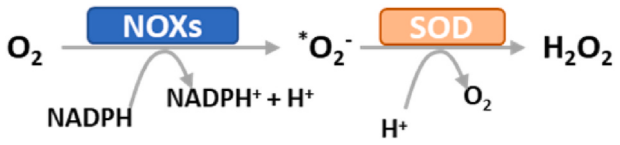


**Fig. 11.** NOXs catalyze $*O_2^-$ generation by a one-electron reduction of $O_2$, using NADPH as electron donor. Increased activity of NOXs leads to an increase of the $*O_2^-$ load. Two molecules of superoxide can react to generate $H_2O_2$ in a reaction known as dismutation, which is accelerated by SOD. Decreased SOD activity results in elevated level of $*O_2^-$.

superoxide dismutase (superoxide radicals), and glutathione peroxidase (not specific for a radical but responsible for metabolizing $H_2O_2$) using commercial kits. The kit called RANSOD for detecting superoxide dismutase (and thus indirectly superoxide radicals) was based on xanthine and xanthine oxidase. These were used to generate superoxide radicals that react with 2-4-iodophenyl-3-4-nitrophenol-5 phenyl tetrazolium chloride (INT) to form a red formazan dye. This dye can then be read out with a UV–vis spectrophotometer.

This approach was used by Dworansky et al. to investigate radical generation in diabetes patients that were also infected with the Epstein-Barr virus [177]. The authors observed that the antioxidant activity was decreased in patients who had suffered from diabetes mellitus type 2 for more than 10 years. Additionally, the antioxidant status of patients with oropharyngeal cancer associated with EBV or HPV infections was investigated [176,178]. Reduced antioxidant levels were also found for instance in cattle which was infected with the food and mouth disease virus [179]. On the other hand in hepatitis C patients increased levels of antioxidants have been observed when compared to healthy controls [180].

Unfortunately, this method also has several limitations. First, one needs to know in advance which radicals are present and how cells respond to the radical load. While this kind of information is available for some radicals and processes, this is not the case for all that might be interesting in the context of viral infections. There is no simple relationship between antioxidant levels and the free radical load. The concentration of antioxidants might be reduced by radicals but they are also produced in response to a high radical load. Further, there is great variability between the concentrations of such molecules in healthy individuals as well which complicates the interpretation of the results. Additionally, the method is destructive and there is no spatial resolution.

#### 4.4.3. Detecting the expression of NADPH oxidases

The NOX family (NOX1-3,5 and DUOX1-2) are known as a major source of $*O_2^-$ since they can catalyze the production of $*O_2^-$ by a one-electron reduction of $O_2$, using NADPH as the electron donor (Fig. 11). Thereby, increased expression of NOXs indicates an increase of the $*O_2^-$ load [181]. The NOX2 complex is the first enzyme complex associated with virus infections. Phosphorylation of $p47^{PHOX}$ is one of the first steps required for the activation of NOX2 in monocytes. Christophe et al. used [32]P labeling, immunoprecipitation electrophoresis and immunoblotting to show that phosphorylation of $p47^{PHOX}$ increased on average 2-fold after stimulation with nonstructural 3 protein (NS3) of HCV as compared to a control [127]. Moreover, Yasuhisa et al. showed that the expression and distribution of NOX2 were significantly up-regulated after EMCV-B infection in microglial cells using reverse transcriptase (RT)-polymerase chain reaction (PCR) and immunohistochemistry [182]. In addition, NOX2 exacerbates virus pathogenicity by overproducing oxygen stress and modulating pro-inflammation [34]. For instance, NOX2 activation is higher in COVID-19 patients compared to controls and higher in severe COVID-19 cases compared to non-severe cases [32,36]. Similarly, the upregulation of NOX1 was detected in HCV and HIV infections using RT-PCR by Mochel et al. [183] and Kovacs et al. [184]. Absolute copy numbers of DUOX2 and DUOXA2 mRNA (measured by by qRT-PCR) of lung cancer cell lines (A549 cells and Calu-3) were higher compared to uninfected cells during Sendai virus infection. However, the expression of DUOX2 depends on the viral strain. In the same work from Flink et al. RSV infection failed to induce detectable levels of the DUOX2 protein [39]. Conversely, DUOX1 and DUOX2 play important roles in innate immune defenses at epithelial barriers. Especially DUOX2 shows antiviral functions on respiratory virus infections [32,39,52]. NOX3 is not known to play a role in immune cells or host defense, and NOX5 is discussed separately in host defense. NOX4 mainly produces $H_2O_2$, which will not be discussed in this section. (More cases of NOX expression detection and their functions are list in Table 3).

In the above mentioned examples, chemiluminescence or fluorescent radical probes were still used to confirm the relative activity of NOX. The reason is that the relations between the radical load are complex and there is not necessarily a clear relationship between the radical load and the enzyme activity.

**Table 3**
Summarized NOX expression detected in virology and their functions.

| NOX | Related-Virus | Functions during viral infections |
|---|---|---|
| NOX1 | HCV [183], HIV [184], etc. | No influence on either viral replication or mechanisms of viral clearance [54]; Increases the severity of infectious disease by modulating pro-inflammation cytokines [183–185] |
| NOX2 | RSV [33], SeV [33], Influenza A Virus [34], Covid-19 [36], EMCV-B [182], etc. | Exacerbates virus pathogenicity by overproduced oxygen stress and modulating pro-inflammation cytokines [32–34,36,53]. |
| DUOX1/2 | Influenza A Virus [52], SeV [39], etc. | Antiviral functions on respiratory virus through cytokine response or through the regulation of the antimicrobial activity in the lumen [31,39,52]. |

HCV: Hepatitis C Virus; HIV: Human Immunodeficiency Virus; Sev: Sendai Virus. RSV: Respiratory Syncytial Virus; EMCV-B: Encephalomyocarditis Virus B.

#### 4.4.4. Detecting the expression of iNOS

Virus infections induce expression of iNOS through proinflammatory cytokines or directly through virus components, which is described in Fig. 2C. Thereby, detecting intracellular iNOS is related to the cellular concentration of NO*. Reverse transcription-PCR (RT-PCR) [186], Western blot analysis [187] and enzyme-linked immunosorbent assay (ELISA) [188] are the widely used techniques for detecting iNOS. Mgbemena et al. uncovered the mechanism of iNOS gene induction by identifying kruppel-like factor 6 (KLF6) during RSV infection and the role of iNOS as a critical host factor regulating apoptosis during RSV infection [186]. Li et al. found that iNOS-derived NO* was increased 2.4-fold in the serum samples of 101 patients infected with influenza A virus in comparison with samples of 105 healthy individuals. In A549 human lung epithelial cells, infection with influenza A virus resulted in increased mRNA and protein levels of iNOS, with subsequent release of NO* [187]. The most recent case where iNOS expression plays a role in viral infection is in COVID-19. Gelzo et al. report that in the first wave, serum iNOS levels increased significantly, in line with the World Health Organization (WHO) severity score. In contrast, in the second wave, iNOS did not change with the severity. The patients of the second wave showed lower levels of iNOS, as compared to the corresponding subgroup of the first wave, suggesting a less severe outcome of COVID-19 in these patients. However, in the severe patients of the second wave, iNOS levels were significantly lower in patients treated with steroids or azithromycin before hospitalization, compared to the untreated patients [188]. This suggests an impairment of the defense mechanism against the virus and potential NO*-based therapies in patients with low iNOS levels.

## 5. Conclusions and outlook

Since there is no simple relation between the radical concentration and viral infection, the presence or absence of radicals does not necessarily indicate presence or absence of viral infection. However, as we have seen, reactive species are very informative for understanding viral infection assessing severity of the infection as well as other processes in the healthy or diseased state of cells or entire organisms. Unfortunately, detecting reactive species remains challenging. Despite the many advances that have been made by using radicals as biomarker this approach still not as widely used as it could be. It is also important to note, that the different assays that are used to assess the radical load or oxidative stress measure very different biomarkers [107,189]. These are for instance present at different parts of the cells/tissues/patients and

sensitive to a different set of molecules. Unsurprisingly, the result is often different depending on the detection method. Thus, it is crucial, to be aware of this and compare between cases within the same method. Apart from techniques that are easier to use with small processing time, the field would greatly profit from techniques which offer single cell resolution. While this is not achieved with most methods several authors have demonstrated for different viruses that there is great variability between individual cells [190–192]. Furthermore, having subcellular resolution which has been achieved recently for studies in cells, would also aid our understanding of viral infection and the role of reactive species [193–195]. Due to their short half-life, especially free radicals are highly diffusion-limited and hence their subcellular site of generation influences the location and nature of their biological action. In fact, it is becoming well understood that cells have evolved ways to compartmentalize ROS production due to the rapid rates of ROS production and removal. It assists in localized subcellular signaling and it avoids the unwanted effects of ROS on chemical reactions occurring in the other parts of a cell [28]. Therefore, a method capable of real-time detection of the species-specific variation in free radical amount with high spatial resolution can significantly help the virology research. A first proof of concept study has recently been published where radical generation during infection has been investigated with subcellular resolution [196].

Another promising area of research is using correlative microscopy and making use of all the features that each technique can reveal. This was for instance done by Bruckman et al. with MRI and fluorescent microscopy for tissues injected with tobacco mosaic virus but they did not investigate radical formation [197]. Finally, most methods do not differentiate between reactive species but are detecting a wide range of reactive species together. So especially radical species which are the most reactive and typically low in abundance remain challenging to measure. When detecting all reactive species together, the signal is dominated by the more stable and more abundant non-paramagnetic reactive molecules.

## Data availability

No data was used for the research described in the article.

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