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# *Research Progress and Biological Applications of Nitric Oxide Fluorescent Probes*

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***Abstract.*** Nitric oxide (NO) is an important gaseous signaling molecule in the body. It plays dual regulatory roles in a range of physiological and pathological settings, including cardiovascular homeostasis, neural transmission, immune defense, and programmed cell death. Altered NO levels are closely linked to conditions such as atherosclerosis, neurodegenerative diseases, inflammatory disorders, and tumors. Therefore, accurate tracking of NO dynamics holds considerable value for basic research and may also offer clinical translational potential. NO Fluorescent probe technology has become a central tool for visualizing due to its non-invasive nature, high sensitivity, good spatial resolution, and real-time imaging ability. In this review, I summarize recent advances in NO fluorescent probes. I firstly discuss the biological background of NO and its chemical recognition mechanisms. Then, I systematically introduce probe design strategies across various modalities, including fluorescence, phosphorescence, chemiluminescence, and photoacoustic imaging. What's more, I specially introduce organelle-targeting probes that enable imaging at subcellular resolution. Finally, I discuss the NO signaling disturbances in aging-related diseases and offer views on current challenges and future research directions.

***Keywords:*** Nitric oxide, Fluorescent probe, Organelle targeting, Bioimaging, Gaseous signaling molecule

## 1. Introduction

Nitric oxide (NO) is a gaseous signaling molecule with a chemically simple structure but biologically complex functions. In 1992, *Science* magazine named NO the "Molecule of the Year". Six years later, the Nobel Prize in Physiology or Medicine was awarded to three scientists for uncovering NO's signaling role in the cardiovascular system. Since then, research on NO has changed from seeing it mainly as an air pollutant to a topic of great interest in the life sciences [1, 2].

The effects of NO in the body are highly concentration-dependent. At physiological levels, it activates soluble guanylate cyclase (sGC), which helps mediate blood vessel dilation, nerve signaling, and immune regulation. Under pathological conditions, however, NO can build up to much higher levels (micromolar range). There, it reacts with superoxide anion ($O_2^-$) to form peroxynitrite ($ONOO^-$), which leads to protein nitration, mitochondrial dysfunction, and DNA damage, thereby contributing to various diseases [3]. Studies indicate that disturbed NO balance is closely associated with atherosclerosis, Parkinson's disease, Alzheimer's disease, inflammatory

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bowel disease, and cancer [4, 5]. Because of this, being able to precisely monitor the spatial and temporal behavior of NO in complex biological systems is critical for understanding its normal physiological roles and its involvement in disease.

Traditional ways to measure NO include the Griess colorimetric assay, electron paramagnetic resonance (EPR), and electrochemical sensors. Each method has its own limits when it comes to sensitivity, spatial and temporal resolution, or use in living animals [6, 7]. Fluorescent probe technology has become a powerful option for visualizing NO [8]. Relative to other approaches, fluorescent probes are non-invasive, very sensitive, allow real-time imaging, and can even reach subcellular resolution [9, 10]. Over recent years, with the emergence of new imaging modes like near-infrared (NIR) fluorescence, chemiluminescence, and photoacoustic imaging, and as organelle-targeting strategies have improved, research on NO fluorescent probes has changed from simple detection to more accurate measurement.

This review aims to give a systematic overview of recent progress in NO fluorescent probes. I begin with describing the biological basis of NO and its links to disease. Then I explain the chemical design strategies and recognition mechanisms behind the probes. I focus on the status of fluorescent, phosphorescent, chemiluminescent, and photoacoustic probes. I also take a look at organelle-targeting probe designs and their applications. Finally, I discuss the role of abnormal NO signaling in aging-related diseases and provide an outlook on future developments.

## 2. Biological basis of nitric oxide

### 2.1. NO biosynthesis and signal transduction

Endogenous NO is mainly generated during the conversion of L-arginine to L-citrulline, a reaction catalyzed by nitric oxide synthase (NOS). There are three NOS isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) [11, 12]. nNOS and eNOS are calcium/calmodulin-dependent enzymes that continuously produce small amounts of NO (nanomolar range). They respectively participate in neurotransmission and blood vessel regulation. In contrast, iNOS is induced by inflammatory stimuli and produces higher NO concentrations to serve immune defense functions [9].

The traditional view of NO signaling holds that, as a freely diffusible gas, NO crosses cell membranes and activates sGC inside target cells, which then converts GTP to cGMP and triggers the protein kinase G (PKG) cascade [13, 14]. However, recent work has challenged this view. It appears that an NO-ferroheme complex, rather than free NO itself, may be the true signaling species in the blood vessel system. This complex is more stable, can be transferred between proteins, and directly activates sGC—remaining active even in the presence of NO scavengers [15].

### 2.2. Concentration-dependent dual roles of NO

The biological effects of NO depend heavily on its concentration, production site, and duration. This gives NO a typical concentration-dependent dual character [16].

At low levels (<100 nM), NO mainly works through binding to the ferrous iron in heme-containing proteins. In the cardiovascular system, eNOS-derived NO activates the sGC-cGMP pathway, leading to smooth muscle relaxation in vessel walls, inhibition of platelet aggregation and leukocyte adhesion, and maintenance of vascular health [17]. In the nervous system, nNOS-derived NO regulates synaptic plasticity and neurotransmitter release, playing a role in learning and memory [18]. At moderate levels (100–500 nM), NO can modify protein cysteine residues through S-

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nitrosylation to influence various signaling pathways [19]. For example, S-nitrosylation of NF-κB by NO reduces its DNA-binding ability, which has an anti-inflammatory effect [20]; while modification of Ras protein may turn on pro-cancer signals [21]. High NO levels (>500 nM) usually come from iNOS expression and are common in inflammatory environments [22]. At these high concentrations, NO quickly reacts with $O_2^-$ to form peroxynitrite ($ONOO^-$), which causes protein tyrosine nitration, lipid peroxidation, and DNA damage [23, 24]. This nitrosative stress not only helps clear pathogens, but also can also harm tissues, contributing to several diseases [8].

### 2.3. Post-translational modifications involving NO

NO signals through two major types of post-translational modifications. One is S-nitrosylation, where NO covalently attaches to the thiol group of cysteine residues in proteins, forming S-nitrosothiols (RSNO) [25]. This modification can reversibly adjust protein function. Its balance is maintained by denitrosylating enzymes such as S-nitrosoglutathione reductase (GSNOR) [26, 27]. Studies show that GSNOR expression changes with age, leading to an altered pattern of S-nitrosylated proteins, which is linked to age-related neurodegenerative diseases [28, 29]. The other modification is tyrosine nitration, mainly caused by $ONOO^-$, which generates 3-nitrotyrosine (3-NT) as a marker of nitrosative stress [30]. In the brains of patients with Parkinson's disease, tyrosine nitration of α-synuclein promotes its aggregation, speeding up the formation of Lewy bodies [31]; in Alzheimer's disease, nitration of tau protein worsens its tendency to aggregate [32].

### 2.4. Abnormal NO signaling and disease

When NO balance is disturbed, it contributes to many disease processes. In the cardiovascular system, eNOS dysfunction leads to lower NO bioavailability, causing endothelial dysfunction, which is an early key event in atherosclerosis, high blood pressure, and heart failure [33, 34]. In the nervous system, excess NO, through nitrosative stress and abnormal S-nitrosylation, harms mitochondria and synapses to play a role in Parkinson's and Alzheimer's diseases [35, 36]. In the immune system, high NO from iNOS is an important effector for fighting infections, but can also cause autoimmune damage [37, 38]. In cancer, NO has a particularly complex role: low NO levels promote blood vessel growth and tumor growth, while high levels trigger tumor cell death. This concentration-dependent dual behavior provides a basis for NO-based therapies [39, 40].

The complexity of NO's biological functions creates serious challenges for detection methods. It needs high sensitivity covering nanomolar to micromolar ranges, high spatiotemporal resolution to capture fast changes, and biocompatibility for in vivo use all at once [41]. Fluorescent probe technology offers ways to deal with these challenges [15].

## 3. Design strategies for nitric oxide fluorescent probes

### 3.1. Basic structure of probes

Most NO-responsive fluorescent probes contain three core parts: a fluorophore, a recognition moiety, and a linker [42, 43]. The fluorophore determines the optical features, such as excitation/emission wavelengths, fluorescence quantum yield, and photostability. The recognition moiety can make a specific chemical reaction with NO that changes the electronic state or shape of the fluorophore to produce a signal [44, 45]. The linker connects these two parts and can also carry targeting groups for subcellular localization [46, 47].

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### 3.2. Recognition mechanisms

Based on the type of chemical reaction with NO, recognition mechanisms fall into several categories [48]:

(1) **o-Phenylenediamine (OPD) cyclization:** This is the most widely used mechanism [49]. The OPD group quenches the fluorophore's emission through a photoinduced electron transfer (PET) effect. In aerobic conditions, OPD reacts with NO to form a benzotriazole derivative, which is a weaker electron donor. This suppresses PET and restores fluorescence [50, 51]. However, OPD groups can also react with reactive carbonyl species like dehydroascorbic acid and methylglyoxal, so selectivity needs improvement [52].

(2) **N-Nitrosation of aromatic secondary amines:** Aromatic secondary amines react with NO to form N-nitroso derivatives [53]. This mechanism also relies on PET quenching and recovery but is less affected by reactive carbonyl species, giving better selectivity than OPD [54, 55]. In recent years, near-infrared probes based on BODIPY and cyanine platforms that use this mechanism have been reported and successfully used for in vivo imaging [56].

(3) **Deamination of aromatic primary amines:** Aromatic primary amines undergo deamination when reacting with NO, turning into the corresponding phenols or aldehydes. This process can change the intramolecular charge transfer (ICT) effect, enabling ratiometric response. For example, the IPB-OH probe shows a blue shift from 657 nm to 524 nm after reacting with NO, allowing self-calibrated ratiometric detection [57].

(4) **Se–NO bond formation:** Selenium-containing compounds form Se–NO bonds with NO, which changes the conformation of the fluorophore [58].

(5) **Thiosemicarbazide cleavage:** The 4-(4-nitrophenyl)thiosemicarbazide group is cleaved upon NO reaction, releasing a free fluorophore. A probe called DCM-NO based on this mechanism has been used for early diagnosis of NO in a model of idiopathic pulmonary fibrosis [55].

### 3.3. Organelle targeting strategies

To understand NO functions, it is important to monitor its dynamics at the subcellular level. Different organelles have unique physicochemical microenvironments, which can be used to design targeted probes [51].

**Mitochondria targeting:** Mitochondria have a membrane potential of about –180 mV (inside negative). Lipophilic cations can therefore accumulate in the mitochondrial matrix based on the electrochemical gradient. Triphenylphosphonium (TPP) is the most common mitochondrial targeting group [50]. Mitochondria are major sites where NO and $O_2^-$ generate $ONOO^-$, so mitochondria-targeted NO probes are valuable for studying oxidative stress-related diseases [10].

**Lysosome targeting:** Lysosomes have a lumen pH of 4.5–5.5. Weakly basic groups (such as morpholine or tertiary amines) become protonated in this acidic environment and become trapped. Lysosome-targeted NO probes have been used to study autophagy and neuroinflammatory responses [58].

**Endoplasmic reticulum targeting:** The endoplasmic reticulum (ER) can be targeted using p-toluenesulfonamide groups, which have an affinity for ER membrane proteins [51]. The ER is the main site for protein synthesis and folding; NO-induced ER stress is linked to several neurodegenerative diseases [41].

**Golgi apparatus targeting:** Recent work has introduced Golgi-targeting groups such as 4-sulfamoylaniline to design NO probes [58]. The Golgi is rich in NOS, and abnormal NO signaling there contributes to early changes in Alzheimer's disease [37].

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**Lipid droplet targeting:** Amphiphilic molecules can accumulate on the surface of lipid droplets through hydrophobic interactions. Lipid droplets are linked to the degree of neuroinflammation, and combining their detection with NO imaging gives a more accurate assessment of inflammation [34].

### 3.4. Ratiometric probe design

Traditional "turn-on" probes give signals that depend on probe concentration, excitation intensity, and detection efficiency, making accurate quantification difficult. Ratiometric probes overcome these issues by using two wavelength signals for self-calibration [48]. They are often designed based on fluorescence resonance energy transfer (FRET) or ICT modulation. For example, the NOP probe combines coumarin and naphthalimide; when it responds to NO, FRET is turned on, allowing ratiometric two-photon imaging [57]. The IPB-OH probe uses NO-triggered deamination to modulate ICT, causing its emission to shift from red to yellow/green [57].

## 4. Research progress on different modalities of NO probes

### 4.1. Fluorescent probes

Fluorescent probes are the most common type for NO imaging. Based on their emission wavelength, they can be divided into visible-region and near-infrared-region probes [15].

**Visible-region probes:** The classic DAF-2 emits at 515 nm and has been widely used in cell studies, but it suffers from photobleaching, shallow tissue penetration, and background fluorescence interference [54, 55]. Later BODIPY, rhodamine, and naphthalimide probes have shown better photostability and quantum yield; some even offer two-photon imaging capability [56, 58].

**Near-infrared (NIR-I, 650–900 nm) probes:** NIR light scatters less and is absorbed less by biological tissues, allowing deeper imaging [16]. Cyanine dyes (Cy series) and silicon rhodamine (SiR) are common NIR fluorophores. The DAC probe, developed by Nagano's group, uses a tricarbocyanine fluorophore and an OPD recognition group; it was successfully applied to image NO in rat kidneys ex vivo [59]. The SiR-NO probe enabled real-time monitoring of mitochondrial NO in living cells, with emission at 710 nm [58].

**Near-infrared II (NIR-II, 1000–1700 nm) probes:** The NIR-II window further reduces light scattering and tissue absorption, allowing imaging at millimeter depths [16]. A NIR-IIb dual-ratiometric fluorescent probe achieved high-resolution imaging of NO in mouse blood vessels [57].

### 4.2. Phosphorescent probes

Phosphorescent probes are often based on transition metal complexes (e.g., Ir, Ru, Re). They offer long luminescence lifetimes, large Stokes shifts, and high photostability [6]. The long lifetime allows time-gated imaging, which effectively removes interference from short-lived autofluorescence of biological samples [4]. An iridium(III) complex phosphorescent probe was used to monitor mitochondrial NO in living cells in real time [58].

### 4.3. Chemiluminescent and bioluminescent probes

Chemiluminescent probes do not need external light for excitation; they generate excited species through chemical reactions. Because there is no light scattering or autofluorescence from excitation, they provide an extremely high signal-to-noise ratio [16]. An NO-responsive chemiluminescent probe was successfully used for real-time imaging of NO in an inflamed mouse model [57].

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Bioluminescent probes, based on the luciferase-luciferin system, are particularly useful for deep-tissue imaging [16].

### 4.4. Photoacoustic imaging probes

Photoacoustic imaging combines the high contrast of optical imaging with the deep penetration of ultrasound. When a probe responds to NO, its absorption spectrum changes, producing a detectable photoacoustic signal [16].

## 5. Research progress on organelle-targeted NO probes

Organelle-targeted probes enable monitoring of NO dynamics at subcellular resolution, providing powerful tools for dissecting the functions of NO in specific organelles [51, 57]. Representative works are introduced below according to the targeted organelle.

### 5.1. Mitochondria-targeted probes

Mitochondria are the center of cellular energy metabolism and the main site for ROS/RNS production. Using triphenylphosphonium (TPP) as a targeting group, several mitochondria-targeted NO probes have been successfully made. The Mito-NO probe achieved dynamic monitoring of mitochondrial NO in neuronal cells, showing the role of NO in synaptic plasticity [58].

### 5.2. Lysosome-targeted probes

The acidic environment of lysosomes puts special requirements on probe design. The morpholine group is the most commonly used lysosome-targeting moiety [51]. The Lyso-SiRB-NO probe, which uses morpholine as the targeting group and silicon rhodamine as the fluorophore, responds to NO under acidic conditions with a detection limit of 3.1 nM [53].

### 5.3. Endoplasmic reticulum-targeted probes

Endoplasmic reticulum targeting usually uses the p-toluenesulfonamide group [51]. A BODIPY-based probe using methylsulfonamide as the ER-targeting group showed a 65-fold fluorescence increase at 570 nm after reacting with NO, successfully monitoring NO dynamics in the ER [53]. This probe showed an early increase in NO during ER stress.

### 5.4. Golgi apparatus-targeted probes

The Golgi apparatus is rich in NOS, and abnormal NO signaling in the Golgi is closely associated with Alzheimer's disease and Parkinson's disease [37]. The Golgi-NO probe, which use 4-sulfamoylaniline as the Golgi-targeting group and OPD as the recognition moiety, detected significantly increasing NO levels in the Golgi in an Aβ-induced Alzheimer's disease cell model [56]. This finding provides a new view for understanding the role of the Golgi apparatus in neurodegenerative diseases.

### 5.5. Dual-organelle targeted probes

Some probes can dynamically move between different organelles, providing more information [51]. One probe can be protonated at physiological pH and localize to mitochondria. But after it reacts

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with NO, the deprotonated product translocates to the nucleus, achieving NO-triggered imaging of movement from mitochondria to the nucleus [53]. An aggregation-induced emission (AIE) probe can simultaneously localize to mitochondria and lysosomes and dynamically monitor the alternating changes of NO and $H_2S$ [48].

## 6. Conclusion

Over the past twenty years, the field of NO fluorescent probes has come a long way. We have moved from basic visible-light "turn-on" probes to more complex ones operating in the NIR-II region, ratiometric designs, and activatable probes. Detection has gone from qualitative cell-based assays to quantitative imaging inside deep tissues of living animals. And probes have moved from non-specific distribution to precise targeting of organelles [7, 16, 17]. These advances have provided powerful means to clarify NO's complex roles in health and disease.

Still, several challenges remain.

First, probe selectivity and stability need further improvement. Problems include cross-reactivity of OPD groups with reactive carbonyl species, sensitivity of some probes to pH changes within the physiological range, and photobleaching of organic dyes. These have not yet been fully solved [57, 58]. Developing new recognition groups and fluorophores with better chemical selectivity and photostability is an important thing [50].

Second, quantitative accuracy of NO detection must be strengthened. Although ratiometric probes allow self-calibration, the effect of tissue optical heterogeneity on ratiometric measurements in vivo still needs systematic evaluation [57]. Combining multiple imaging method, such as fluorescence-photoacoustic and fluorescence-PET, may provide more accurate quantification [16].

Third, clinical translation is a bottleneck. Most NO probes are still at the basic research stage. Their biosafety, pharmacokinetics, and targeting efficiency need systematic assessment [15]. The potential of activatable probes for early disease diagnosis, surgical navigation, and treatment evaluation still need clinical validation [16].

Looking ahead, NO fluorescent probes could advance in several ways. Developing probes for the NIR-II/III window would allow deeper and higher-resolution imaging [14]. Designing dual- or multi-responsive probes could simultaneously monitor NO and other related species such as $H_2S$, $ONOO^-$, and show their interactions [57]. Creating reversible probes would let us track NO fluctuations in real time. Adding therapeutic functions to theranostic probes would close the loop of diagnosis-treatment-monitoring for NO-related diseases [17]. Promoting clinical translation by screening probes with favorable pharmacokinetic and safety profiles, and exploring their value in inflammatory diseases, cardiovascular diseases, tumors, and other fields, is also important [3, 44].

In summary, research on NO fluorescent probes is at a key stage, moving from "being able to see" to "seeing clearly and understanding deeply." With continued progress in molecular engineering, nanotechnology, and imaging instrumentation, the next generation of NO probes will undoubtedly play an even greater role in uncovering life's mysteries and improving human health.

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