Hindawi Publishing Corporation
Journal of Nanomaterials
Volume 2014, Article ID 523646, 13 pages
http://dx.doi.org/10.1155/2014/523646

Review Article

In Vivo Imaging of Nitric Oxide by Magnetic Resonance Imaging Techniques

Rakesh Sharma,[1] Jeong-Won Seo,[2] and Soonjo Kwon[3]

[1] *Center for Nanomagnetics and Biotechnology, Tallahassee, FL 32310, USA*
[2] *Department of Ophthalmology, Hallym University Dongtan Sacred Heart Hospital, Hwaseong 445-907, Republic of Korea*
[3] *Department of Biological Engineering, Inha University, 100 Inharo, Nam-gu, Incheon 402-751, Republic of Korea*

Correspondence should be addressed to Soonjo Kwon; soonjo.kwon@inha.ac.kr

Received 23 June 2014; Accepted 28 June 2014; Published 17 July 2014

Academic Editor: Ki-Joon Jeon

Nitric oxide (NO) biosensors are novel tools for real-time bioimaging of tissue oxygen changes and physiological monitoring of tissue vasculature. Nitric oxide behavior further enhances its role in mapping signal transduction at the molecular level. Spectrometric electron paramagnetic resonance (EPR) and fluorometric imaging are well known techniques with the potential for *in vivo* bioimaging of NO. In tissues, NO is a specific target of nitrosyl compounds for chemical reaction, which provides a unique opportunity for application of newly identified NO biosensors. However, the accuracy and sensitivity of NO biosensors still need to be improved. Another potential magnetic resonance technique based on short term NO effects on proton relaxation enhancement is magnetic resonance imaging (MRI), and some NO biosensors may be used as potent imaging contrast agents for measurement of tumor size by MRI combined with fluorescent imaging. The present review provides supporting information regarding the possible use of nitrosyl compounds as NO biosensors in MRI and fluorescent bioimaging showing their measurement limitations and quantitative accuracy. These new approaches open a perspective regarding bioimaging of NO and the *in vivo* elucidation of NO effects by magnetic resonance techniques.

1. Introduction

Nitric oxide (NO) as a metabolic nitrogen compound in bound gas form plays an important role in physiological regulation of the cardiovasculature in our body [1, 2]. Since endothelium-derived relaxing factor (EDRF) was first identified in 1980, biological and chemical evidence has suggested that EDRF is nitric oxide (NO), a potent vasodilator [3]. NO is released through the intermittent catalytic action of constitutive NO synthase (cNOS) [4]. In addition, large transient production of NO at sites of inflammation is derived from inducible NO synthase (iNOS) and related to host defense against infection [5]. *In vivo* imaging of NO as a biosensor is an emerging monitoring technique that employs EPR, fluoroscopy, and MRI [6]. The success of this method depends on visualizing free radical distribution of *in vivo* spin-trapped NO. NO imaging techniques primarily utilize magnetic resonance (MR), electron paramagnetic resonance (EPR) spectrometry, and fluorometry. NO is a diatomic free radical that contains one unpaired electron derived from L-arginine via the catalytic action of NOS. The *in situ* visualization of NO using bioimaging techniques provides information pertaining to the production and diffusion processes of NO [7, 8]. Real-time bioimaging techniques using EPR, fluorescent indicators, chemiluminescence, real-time MRI, and functional MRI (fMRI) have recently been introduced [9–11].

Physiological Basis of Bioimaging of NO. NO is synthesized by neuronal NOS, endothelial NOS, which is commonly referred to as cNOS, and other types of iNOS specific to macrophages and microglia through stimulation by cytokines and endotoxins at sites of inflammation. NOS isoforms are generally classified as either cNOS (Ca^{2+}) or iNOS (Ca^{2+} independent) [12]. NO is a highly unstable molecule that is rapidly oxidized into nitrite (NO^{2-}) and nitrate (NO^{3-}) in the presence of oxygen, especially in the liquid phase. Synthesized NO combines with oxygen within the membrane

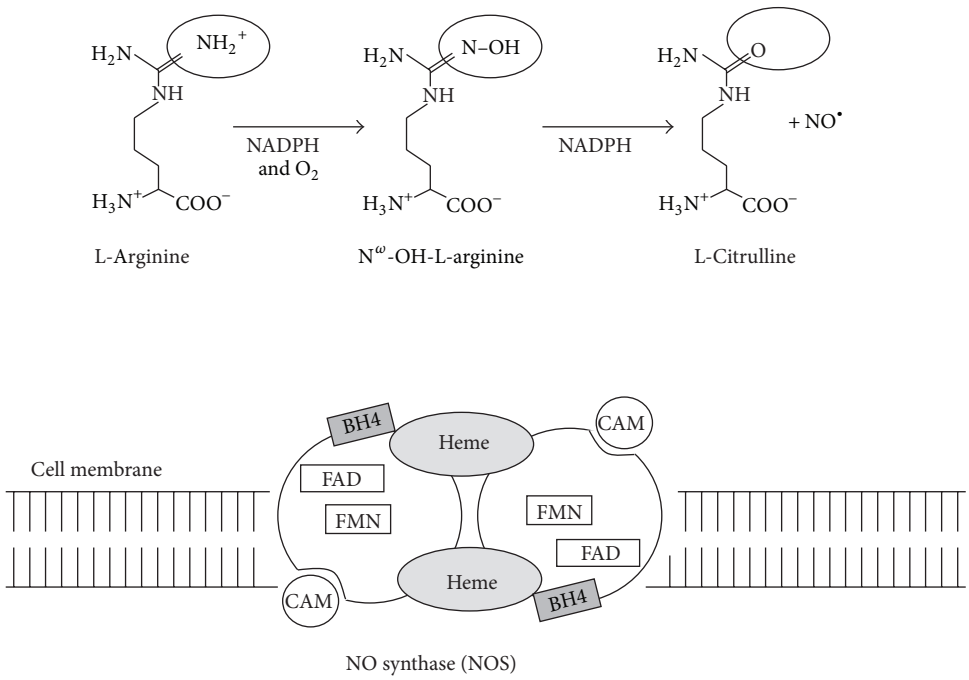

Figure 1: Proposed mechanism of NO production from epithelial cells. $NO^{\bullet}$ is released by several isoforms of the enzyme, NOS, which catalyzes the 5-electron oxidation of the guanidino nitrogen moiety of a nonaromatic amino acid (L-arginine) to citrulline via N^{ω}-hydroxy-L-arginine. The enzyme utilizes O_2 and NADPH as cosubstrates and thiol, tetrahydrobiopterin (BH4), FAD, and FMN as cofactors. NOS is unique among eukaryotic enzymes in being a dimeric, calmodulin-dependent or calmodulin-containing isozyme.

and blood [13]. As shown in Figure 1, NO is released from L-arginine via catalytic action of the membrane-bound enzyme, NO synthase (NOS). NO is a physiologically important mediator in metabolically active organs and tissues, as well as in neurotransmitters in central and peripheral neurons *in vivo* [14].

1.1. Bioimaging of NO in Cardiovascular System

1.1.1. Electrochemical Measurement of NO. NO biosensors were initially developed for high resolution electrochemical measurement methods of NO by several groups [15–17]. These NO biosensors enable evaluation of dynamic changes in NO concentration in solutions and tissues in response to agonists, NO-generating reagents, and physical stimuli [18–20]. However, their electrochemical applications are primarily limited to short term recording of NO sensitive myocardial changes for monitoring the effect of cardiac potassium channel blockers [21]. Use of this technique is also limited due to its poor sensitivity.

1.1.2. NO Biosensing by Electron Paramagnetic Resonance (EPR) Spectrometry. EPR spectroscopy is a specific technique for measurement of *in vivo* NO free radicals by spin-trapping compounds (spin-traps) [22]. A number of derived pyrroline oxide and dithiocarbamate compounds have been shown to be potential EPR spin-trap biosensors (Table 1).

1.1.3. NO Biosensing by Fluorometry. Recently reported fluorescent indicators allow real-time bioimaging of NO with high spatial and temporal resolution. Diaminorhodamines and diaminofluorescein compounds undergo specific reactions with NO in cardiovascular tissues and may serve as potential biosensors in fluoroscopy [23, 24].

1.1.4. Spin-Trapping Technique in NO Biosensing. The presence of NO radicals at greater than the EPR detection limit (0.1–0.01 μM) can be detected by *nitrone traps*: 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO), α-phenyl-*N*-*tert*-butylnitrone (PBN), and α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN), as well as by *nitroso traps*: 2-methyl-2-nitrosopropane (MNP) and 3,5-dibromo-4-nitrosobenzenesulfonic acid (DBNBS) [25–28].

1.1.5. In Vivo EPR Detection by Biosensing Free Radicals. EPR spectrometers operating at S-band (1.6–4 GHz) and L-band (0.4–1.6 GHz) microwave frequency and at radio frequency (0.2–0.4 GHz) are utilized for *in vivo* measurements of the whole body of small animals. The electronic configuration of NO with 11 valence electrons is $(K^2\,K^2)(2s\sigma^b)^2(2s\sigma^*)^2(2p\pi^b)^4(2p\sigma^b)^2(2p\pi^*)^1$. NO is a free radical with one unpaired electron in the antibonding π orbital; therefore, EPR is considered to be the most appropriate tool for its detection. The electronic ground state of NO is expressed by the term symbol $^2\prod_{1/2,3/2}$ [8, 22, 29]. EPR signals from large biological samples cannot be detected with a conventional X-band spectrometer due to its poor sensitivity and detection limit. However,

Table 1: Various complexes as nitric oxide biosensors and NO bioimaging contrast applications and limitations.

Complexes bound with NO	NO bioimaging contrast applications and limitations
(N-Methyl-D-glucamine)2-Fe(II)-NO complex	EPR low contrast and MRI high contrast
5-Diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide	NMR imaging with good possibility
5,5-Dimethyl-1-pyrroline N-oxide (DMPO)	EPR spin-trap with possibility of EPR imaging
5-Diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide	EPR spin-trap with possibility of EPR imaging
α-Phenyl-N-tert-butylnitrone (PBN)	EPR spin-trap with possibility of EPR imaging
α-(4-Pyridyl-1-oxide)-N-tert-butylnitrone	EPR spin-trap with possibility of EPR imaging
nitroso traps: 2-methyl-2-nitrosopropane (MNP)	EPR spin-trap with possibility of EPR imaging
3,5-Dibromo-4-nitrosobenzenesulfonic acid	EPR spin-trap with possibility of EPR imaging
3-Carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-yloxyl	EPR spin-trap with possibility of EPR imaging
Dithiocarbamate derivatives (Fe-DTCs)	EPR spin-trap with possibility of EPR imaging
Pyrrolidine dithiocarbamate (PDTC)	EPR spin-trap with possibility of EPR and MRI
N-Methyl-D-glucamine dithiocarbamate (MGD)	EPR spin-trap with possibility of EPR imaging
N-(Dithiocarboxy)sarcosine (DTCS)	EPR spin-trap with possibility of EPR imaging
N-Methyl-L-serine dithiocarbamate (MSD)	EPR spin-trap with possibility of EPR imaging
L-Proline dithiocarbamate (ProDTC)	EPR bioimaging
Disulfiram (disulfide of DETC)	EPR bioimaging
Diglutathionyl dinitrosyl iron complex, $[DNIC\text{-}(GS)_2]$	EPR bioimaging
$Fe(III)(DTCS)_3$ and $NO\text{-}Fe(II)(MGD)_2$	MRI, EPR, and chemiluminescence bioimaging
Fe-DETC trap	EPR imaging of cultured alveolar cell
$[^{14}N]$ISDN or $[^{15}N]$ISDN	EPR-CT bioimaging
Dinitrosyl dithiolate iron complex	EPR-CT bioimaging
$NO\text{-}Fe(DTC)_2$	EPR-CT bioimaging
(MGD)2-Fe(II)-NO complex	MRI, NMR, and EPR bioimaging
Diaminonaphthalene: DAN	Fluorescent biosensor
Dichlorofluorescein: DCFH	Fluorescent biosensor
Iron(II)-quinoline pendant cyclam	Heme fluorescent reporter biosensor
Co complex: $[Co(NO)_2(^{R}DATI)]$	Fluorescent biosensor
Cheletropic traps: FNOCTs	ESR and fluorophobic bioimaging
Diaminofluoresceins: DAFs	Fluorometry
Diaminorhodamines: DARs	Fluorometry

EPR spectrometers operating at lower frequency are now applied to *in vivo* measurements of EPR signals from the whole body of small animals. In this method, a nitro-compound spin-trap generates a frequency sensitive electron resonance signal due to the hydroxyl ion change in spin-trap energy [30]. By application of a suitable analog to digital simulation (Monte Carlo simulation), all digitized simulations generate a colorful spot image at different locations that results in a whole body *in vivo* animal image [31]. *In vivo* EPR imaging experiments using 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-yloxyl(4-hydroxy-TEMPO) and 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-yloxyl (carbamoyl-PROXYL) have been extensively developed as a promising approach to oximetry for noninvasive measurement of tissue oxygen status. This technique also has the possibility of physiological oxygen imaging application to NO-based fMRI in the near future. For *in vivo* detection of hydroxyl radical using a DEPMPO spin-trap in mice, iron complexes with dithiocarbamate derivatives (Fe-DTCs) were used as spin-traps for NO adduct $[NO\text{-}Fe(DTC)_2]$ [32–34].

1.1.6. Trapping Target Complexes of Nitric Oxide. The complex form of NO (e.g., nitrosothiol) has a relatively longer half-life than free NO. NO is rapidly oxidized into nitrite or nitrate in the presence of oxygen, especially in the liquid or tissue phase, and must be trapped by chemical NO biosensors to monitor its physiological concentration. In previous studies, different levels of NO concentrations were measured in different organs or tissues as tissue specific NO targets (Table 2). Figure 2 shows the different levels of effector molecules or free radical induced cyclic guanosine monophosphate (cGMP), which are proportional to NO concentration. We previously reported guanylate and adenylate cyclase activity and intracellular levels of cGMP proportional to NO concentration in alveolar epithelial cells and hepatocytes [35, 36]. NO concentration is proportional to the intracellular specific tissue responses that reflect the measurable physical properties of tissue metabolic state detectable using routine spin-trap imaging modalities of CT, EPR, MRI, and optical techniques. NO concentration in cardiovascular tissue is in the range of nanomoles and picomoles. These new biosensor approaches have opened a new realm of nanomolar and

Table 2: In tissues, concentrations of NO are shown in different organs or tissues up to the level of nanomol/L and picomol/L.

Specific organs/tissues	Mean concentrations	References
Brain	250 pmol/L	[112, 113]
Postischemic muscle	227 nmol/L	[109]
Breast cancer tissue	36–70 mmol/L	[103]
Serum	22.5 nmol/L	[116]
Liver	600 nmol/L	[104]
Endothelium	120–68 nmol/L	[106]
Alveoli	1500 pbb	[117]
Erythrocytes	4.38 to 14.60 mmol/L	[118]
Articular cartilage	20–140 nmol/mg protein	[105]
Subchondral bone	0.45–2.9 μmol/mg protein	[105]
Trabecular bone	0.5–0.75 μmol/mg protein	[105]

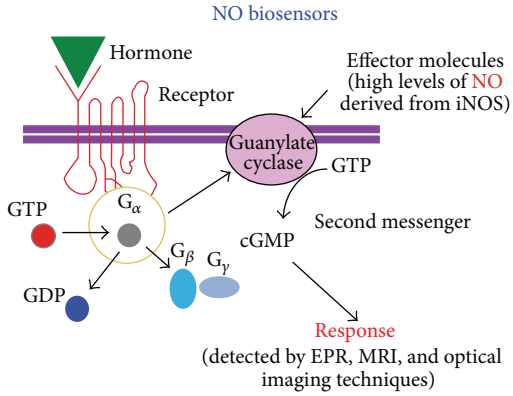

Figure 2: Nitric oxide-induced cascade with a role of guanylate cyclase to generate physiological response detectable by NO bound biosensor compounds (modified from the reference) [115]. GTP: guanosine triphosphate, GDP: guanosine diphosphate, cGMP: cyclic guanosine monophosphate, G_α: G protein alpha, G_β: G protein beta, and G_γ: G protein gamma.

picomolar scale molecular imaging that has yet to be fully developed.

1.2. NO Specific Spin-Trapping and Biosensing Active Groups in Bioimaging. Different types of NO biosensors have active chemical groups that capture nitric oxide at various levels of sensitivity in the body. The ability to capture NO depends on the active chemical structure of nitrone or cheletropic groups and the oxidation state of inorganic iron elements present in the biosensing molecule. The oxidation state of nitric oxide is crucial to its capturing property and its sensitivity as a bioimaging target. Accordingly, various trapping chemicals are routinely used.

1.2.1. NO-Trapping Reagents. The major classes of compounds are nitrone (DMPO) and nitroso (MNP, DBNBS) spin-traps, NO cheletropic traps (NOCTs), *o*-quinodimethane, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-yloxyl-3-oxide (PTIO), ferrous iron complexes such as Hbs and Fe-DTC complexes, Fe(III) hemoproteins, and porphyrin complexes [37–40]. Consider

$$\text{Fe (III) (Heme)} + 2\text{NO} \longrightarrow \text{NO} - \text{Fe (II) (Heme)} + \text{NO}^{+} \quad (1)$$

Cytochromes c' are hemoproteins common in denitrifying and photosynthetic bacteria that have high affinity for NO and can be used as biosensors for NO. Recently, NO-selective photometric or electrochemical biosensors have been developed using cytochrome c' immobilized on an optical fiber or electrode or encapsulated in sol-gel glass [41].

1.2.2. Dithiocarbamates. Pyrrolidine dithiocarbamate (PDTC), *N*-methyl-D-glucamine dithiocarbamate (MGD), *N*- (dithiocarboxy)sarcosine (DTCS), *N*-methyl-L-serine dithiocarbamate (MSD), L-proline dithiocarbamate (ProDTC), disulfiram (disulfide of DETC), *N*,*N*-diethyldithiocarbamate (DETC), and diglutathionyl dinitrosyl iron complex [DNIC-$(GS)_2$] are major nitric oxide biosensor com pounds. Pyrrolidine dithiocarbamate (PDTC) inhibits oxidative activation of nuclear transcription factor κB (NF-κB) to develop immunity, stress responses, inflammation, glial and neuronal function, and the inhibition of apoptosis. Therefore, inhibition of NF-κB activation by PDTC and DETC causes various biological phenomena, including inhibition of iNOS expression, inhibition of apoptosis in thymocytes, leukemic cells, and L929 fibroblasts, and induction of heme oxygenase-1 gene expression [42–49].

1.2.3. Iron-Dithiocarbamate Complexes as NO-Trapping Reagents. NO-Fe(II)$(DTCS)_2$ (>100 mM) and NO-Fe(II) $(MGD)_2$ (<1 mM) showed specific ability for NO-trapping of NO-Fe(II)$(MGD)_2$ and NO-Fe(II)$(DTCS)_2$ complexes [50–52].

1.2.4. Reactions of NO, NO^+, NO^-, and NO_2^- with Fe-DTC Complexes. NO reacts with disulfiram derivative Fe(II)$(DTC)_2$ complex to form NO-Fe(II)$(DTC)_2$ as the primary product and Fe(III)$(DTC)_3$ as a secondary product as shown below. Consider

$$\text{NO} + \text{Fe (II) (DTC)}_2 \longrightarrow \text{NO} - \text{Fe (II) (DCT)}_2 \quad (2)$$

$$\text{Fe (III) (DETC)}_3 + \text{NO} \longrightarrow \text{NO} - \text{Fe (II) (DETC)}_2 + \text{DETC}^{*} \quad (3)$$

Basically, there are three mechanisms responsible for the reductive nitrosylation of Fe(III)$(DETC)_3$ complexes to form Fe(III)$(DETC)_2$ [53]. Consider

$$2\text{DETC}^{*} \longrightarrow \text{bis (DETC)} \quad (4)$$

The reductive nitrosylation of $Fe(III)(MGD)_3$ in the presence of thiols generates NO-$Fe(III)(MGD)_2$, which is further oxidized into NO-$Fe(II)(MGD)_2$ products. Consider

$$2NO + Fe\,(III)\,(MGD)_2 \xrightarrow{H_2O(^*OH)} NO - Fe\,(II)\,(MGD)_2 + NO_2^- \tag{5}$$

$$NO + Fe\,(III)\,(MGD)_2 \xrightarrow{\text{Reducing Equivalent}} NO - Fe\,(II)\,(MGD)_2 \tag{6}$$

The reducing equivalent refers to endogenous reducing agents such as ascorbate, hydroquinone, and thiol. MRI, EPR, optical, and chemiluminescence, LC-electrospray mass spectroscopy showed that bioimaging of NO could be accomplished using $Fe(III)(DTCS)_3$ and NO-$Fe(II)(MGD)_2$ [54]. In another mechanism, unreacted $Fe(III)(DTCS)_3$ donates an electron to the NO-Fe(III) complex to form NO-$Fe(II)(DTCS)_2$ and $Fe(IV)(DTCS)_3$ in the presence of ascorbate and glutathione, suggesting that Fe-DTC complexes are efficient as NO traps *in vivo* and should be suitable for *in vivo* real-time measurements of NO [55]. Consider

$$[Fe\,(III)\,(DTCS)_3]^{3-} + NO \longrightarrow [NO - Fe\,(III)\,(DTCS)_3]^{3-} \tag{7}$$

$$[NO - Fe\,(III)\,(DTCS)_3]^{3-} + [Fe\,(III)\,(DTCS)_3]^{3-} \longrightarrow [NO - Fe\,(II)\,(DTCS)_2]^{2-} + [Fe\,(IV)\,(DTCS)_3]^{2-} + DTCS^{2-} \tag{8}$$

$$[Fe\,(IV)\,(DTCS)_3]^{2-} + DTCS^{2-} \longrightarrow [Fe\,(III)\,(DTCS)_3]^{3-} + \frac{1}{2}[bis\,(DTCS)]^{2-} \tag{9}$$

The overall reaction is as follows:

$$[Fe\,(III)\,(DTCS)_3]^{3-} + NO \longrightarrow [NO - Fe\,(II)\,(DTCS)_2]^{2-} + \frac{1}{2}[bis\,(DTCS)]^{2-} \tag{10}$$

The oxidized NO^- nitroxyl ion donor molecule with Fe-MGD complex and $Fe(II)(MGD)_2$ complex is EPR, which can be used to visibly distinguish NO and reduced to molecular NO_2^- under low pH conditions such as tissue ischemia [56].

1.3. In Vitro and Ex Vivo EPR Detection of NO Using Fe-DTC Traps. Current studies of nitric oxide bioimaging are mainly focused on detection of NO in cultured cells and using EPR and fluoroscopy methods to generate images of small animals. The following sections provide an account of both detection and bioimaging of NO in cultured cells, tissues, and organs.

1.3.1. In Vitro Detection of NO from Cultured Cells Using Chemiluminescence Method. NO is highly unstable in the presence of oxygen and is rapidly converted into NO_2^- and NO_3^- in the liquid phase. To detect NO, both NO_2^- and NO_3^- were converted into NO using a reducing agent (vanadium (III) chloride). In cultured cells, NO was measured based on the chemiluminescence (Model 280 NOA, Sievers, Inc., Boulder, CO). To achieve high conversion efficiency, the reduction was performed at 90°C [4, 5].

1.3.2. Detection of NO in Resected Tissues and Organs. In the last decade, Fe-DETC traps have also been applied to measure NO concentrations in the liver, kidney, intestine, spleen, heart, and lung, as well as in regenerating rat liver, mouse stomach during adaptive relaxation, and rat jejunum and ileum under ischemia reperfusion [57–59]. The Fe-MGD trap detected NO formation from nitrovasodilators including glyceryl trinitrate, isosorbide dinitrate (ISDN), and SNP [60, 61].

1.4. In Vivo EPR Detection and Imaging of NO in Living Small Animals. In vivo EPR bioimaging is used for visualization of iron bound nitrone and dithiocarbamates. NMR imaging provides higher resolution than EPR imaging, enabling observation of spatial distribution of nitrone free radicals due to NMR sensitivity to iron paramagnetic behavior in the body.

1.4.1. Instrumentation and Imaging Techniques for In Vivo EPR Measurements. The three-dimensional EPR image (i.e., EPR-CT) was constructed based on Lauterbur's method [62]. In this method, a pair of magnetic field gradient coils for the x-, y-, and z-axes are attached to the surface of the main magnet to obtain one set of EPR-CT images. The microwave circuit was constructed with a signal source, a VSWR bridge, a phase shifter, a preamplifier, and a double-balanced mixer for homodyne detection. The projection spectra were obtained by changing the angles of the field gradient sequentially under a fixed gradient intensity in one plane. The direction of the field gradient was rotated in 20° steps, and projections were collected. The obtained data for nine spectra of each two-dimensional projection constituted a three-dimensional set of images. Arbitrary slice planes (i.e., CT images) can be cut from the three-dimensional data. Thus, data on 81 projection spectra were needed under the selected field gradients to obtain one set of EPR-CT images [63, 64].

1.4.2. In Vivo EPR Detection of Endogenous NO. In vivo real-time detection of NO in the mouse tail was reported using a Fe-MGD trap and a Fe-DETC trap with an L-band (1.14 GHz) EPR spectrometer. *In vivo* NO detection at the head region models of sepsis and bacterial meningitis by the Fe-DETC trap for NO-$Fe(DETC)_2$ suggested that the NO-$Fe(DETC)_2$ signal is dependent on iNOS induced by IFN-γ [65–71].

1.4.3. In Vivo EPR Imaging of Endogenous NO. In vivo EPR imaging was used by applying the Fe-DTC traps for three-dimensional EPR imaging of NO in ischemia-hypoxia. EPR

images from the frozen resected brain were obtained by employing a Fe-DETC trap and an EPR imaging system with a microwave frequency of 1.2 GHz [72]. An *in vivo* EPR imaging system with 700 MHz microwave unit was designed for bioimaging with a two-gap and loop-gap resonator using NO-Fe$(DTC)_2$ complexes as a spin probe or an imaging agent [73]. Our continued interest in *in vivo* EPR and MRI imaging of endogenously produced NO in the abdominal region in a mouse encouraged us to use a NO-Fe-DTCS trap to image free radicals. Another approach focused on using [^{14}N]ISDN or [^{15}N]ISDN to image the liver and kidney. A more complex multimodal imaging set of EPR-CT images in the z-x plane showed the upper abdomen of a mouse due to ^{15}N substitution in the liver [74].

1.5. EPR Detection and Imaging of Endogenous NO-Relevant Complexes

1.5.1. Nitrosylheme Complexes Produced from Infused Nitrite. An approach employing a combination of nitrite compound and a Fe-MGD trap to detect NO in ischemia using L-band (1.3 GHz) EPR imaging of a heart subjected to cardiopulmonary arrest was reported [8].

1.5.2. Dinitrosyl Dithiolate Iron Complex Administered as a Spin Probe. Dinitrosyl dithiolate iron complex (DNIC) consists of paramagnetic molecules that exhibit a characteristic EPR spectrum in both the solution state at room temperature and the frozen state in tissues at low temperature. Physiologically, DNIC and nitrosothiol (RSNO) compounds stabilize and transport NO in biological systems. *In vivo* real-time detection and three-dimensional EPR-CT imaging of DNIC-$(GS)_2$ in the abdomen in a 700 MHz EPR system showed NO in blood and NO delivery to the abdomen and liver [75].

1.5.3. NO-Fe$(DTC)_2$ Complexes as Spin Probes and Imaging Reagents. Paramagnetic NO-Fe$(DTC)_2$ complexes serve as spin probes or imaging reagents for *in vivo* EPR imaging. Using these compounds, a 700 MHz EPR-CT system generated a two-dimensional image of blood circulation in the coronal section of the rat head (spatial resolution = 6.0 mm) in which the high-intensity area (ventral side) was clearly distinguished from the low-intensity area (dorsal side) [73]. EPR-CT imaging in the mouse abdomen was accomplished using NO-Fe$(DTCS)_2$, NO-Fe$(MGD)_2$, and NO-Fe$(DETC)_2$ complexes as spin probes with the 700 MHz EPR system. EPR-CT images showed the utility of NO-Fe$(DTCS)_2$ and NO-Fe$(DTC)_2$ complexes (spatial resolution, 3.6 mm) [73].

1.6. Approaches to NO Evaluation by Magnetic Resonance Imaging (MRI) Techniques.

Recently, EPR-NMR techniques employing a proton-electron-double-resonance-imaging (PEDRI) hybrid technique showed enhancement of proton NMR signal intensity in the presence of radicals through the Overhauser effect or relaxation of neighboring protons such as the nitrosyl iron complex. This method may be useful as a functional MRI contrast agent specific for NO in living organisms [76–79]. L-Arginine increased the cerebral blood volume in hypertensive rats, while ISDN increased both tumor blood flows on the NO images via magnetic resonance techniques [11]. We propose that another use of NO exposure to hemoglobin may be capturing fMRI BOLD signal hyperintensities on T1-, T2-, and T2*-weighted images due to the addition of aqueous NO, nitrite, or dithionite and nitrite to the hemoglobin in the blood, that is, metHb and NO-Hb. However, additional studies are needed to confirm this.

Multimodal In Vivo NO Spin-Trapping MRI-EPR Experiments. In Vivo MRI imaging of Fe(II)-chelate spin-trapped nitric oxide by N-methyl-D-glucamine dithiocarbamate- (MGD-) NO mapping revealed radical distribution to localize nitric oxide in liver [80, 81]. Synthase (iNOS) is the main source of NO. At the optimal concentration of $(MGD)_2$-Fe(II) [MGD: 100 mM, Fe: 20 mM], MR images on a GE 2-T CSI and IBM PC20 MiniSpect measured millimolar relaxivity of (MGD)2-Fe(II)-NO at parameters of TR 500 msec, TE 10 msec, NEX 2, 4 mm slice thickness, 1 mm slice gap, field of view 12 × 3 × 12 cm, and matrix 256 × 256. A 20 MHz Jeol JES-FG2XG EPR spectrometer (microwave frequency, 9.4 GHz; incident microwave power, 20 mW; 100 kHz modulation amplitude, 2 G; sweep width, 100 G; scan time, 2 min) was used for EPR imaging.

Several assumptions were made regarding multimodal *in vivo* NO spin-trapping MRI-EPR experiments: (1) spin-trapped NO is stable *in vivo*, (2) its contrast enhancement properties in MRI have been assessed, and (3) simultaneous visualization and mapping of free radicals are possible by MRI. The NO complex $(MGD)_2$-Fe(II)-NO is stable in tissues and organs for MRI imaging and subsequent L-band EPR measurements. The liver is the most sensitive to NO complex upon X-band EPR [80, 81]. The $(MGD)_2$-Fe(II)-NO complex shows remarkably strong proton relaxation enhancement because of its paramagnetic properties. The strong magnetic moment of the unpaired electron promotes both spin lattice and spin-spin relaxation of the surrounding water protons, resulting in a decrease in their spin-lattice (T1) and spin-spin (T2) relaxation times. These effects can be exploited to enhance signal intensity in T1 or T2 weighted MR images *in vivo* in areas in which NO is trapped [78, 79].

The NO complex acts as an effective intrinsic contrast agent, enhancing its contrast in the images of several organs. MRI analyses have shown that the NO complex can be a potentially useful NO specificcontrast agent. Mapping the site of NO generation is possible by L-band EPR, combined with MRI spin-trapping, for the direct detection of NO radicals *in vivo*. Here, we propose a multimodal MRI-EPR-fluorometry approach to map NO radicals within tissues and organs at much higher spatial resolution. The spin-trapped adduct, $(MGD)_2$-Fe(II)-NO, a NMR contrast agent, has the potential to provide much higher spatial resolution than with EPR. NO is known to bind to iron compounds to form generally stable complexes such as $(MGD)_2$-Fe(II)-NO. *In vivo*, hemoglobin is normally a natural NO spin-trap. Specifically, NO tends to bind with hemoglobin or to oxidize the hemoglobin, after

which it was converted to nitrosyl-hemoglobin or methemoglobin, both of which are paramagnetic species. When the brain is stimulated to generate NO, it is quite possible that (paramagnetic) nitrosyl-hemoglobin and methemoglobin are formed. Signal intensity enhancement in functional MRI (fMRI) is believed to result from changes in blood flow. However, for blood flow independent effects in MRI, the paramagnetic relaxation from spin-trapped NO might provide a new fMRI contribution using 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO). This multimodal methodology not only is suitable for mapping NO, but also might be valid for other important free radicals *in vivo* when combined with appropriate spin-trapping reagent techniques [11, 82, 83].

1.7. Fluorometric Imaging of NO by Fluorescent Probes for NO

1.7.1. Diaminonaphthalene (DAN). NO is readily oxidized into NO_2^- and NO_3^- as final products in the presence of O_2. The fluorometric assay for the quantification of NO_2^-/NO_3^- up to 10 nM excited at 375 nm and emitted at 415 nm is based on the reaction of NO_2^- with 2,3-diaminonaphthalene (DAN) to form the fluorescent product 1-(*H*)-naphthotriazole (NAT). This method can serve as a tool for defining the role of NOS in both normal and pathophysiological processes. However, the method cannot be adapted for NO bioimaging because it causes serious damage to living cells [84].

1.7.2. Dichlorofluorescein (DCFH). 2,7-Dichlorofluorescein (DCFH) is oxidized by NO to dichlorofluorescein. This compound is a nonfluorescent species that may be used in monitoring of intracellular NO formed in neuronal cells but is not suitable for bioimaging [85]. DCFH has been shown to readily react with all reactive oxygen species. Hence, the overall fluorescence level of DCFH would be specific to all levels of reactive oxygen species and not exclusive to any individual of them [86].

1.7.3. Iron Complexes. The iron(II)-quinoline pendant cyclam, a fluorescent probe for NO, is not convenient for NO detection in biological systems. This probe mimics the activation site of guanylate cyclase if used as a fluorescent biosensor of NO. The iron(II) complex further showed poor fluorescence emission at 460 nm, which was quenched by NO from NO releasing agents. 2,2,6,6-Tetramethylpiperidine-*N*-oxyl (TEMPO) labeled with acridine and $Fe(II)(DTCS)_2$ complex can be used to monitor direct production of NO in biological systems but has not yet been applied in bioimaging [87, 88].

1.7.4. Heme Domain with Fluorescent Reporter Dye. Cytochrome c' labeled with a fluorescent reporter dye containing fluorescent microspheres can serve as a ratiometric sensor of intracellular macrophage NO levels in phagocytosis. NO-selective sensors were reported as a heme domain of guanylate cyclase (sGC) labeled with a fluorescent reporter dye. The fluorescence intensity indicated the sGC heme domain's characteristic binding of NO. The formation of NO from NOS in endothelial cells has a detection limit of 8 μM NO [89, 90].

1.7.5. Cobalt Complex: $[Co(NO)_2(^RDATI)]$. Aminotroponiminates (H^RATIs) with a dansyl fluorophore serve as a fluorescent NO biosensor, and paramagnetic Co^{2+} complexes quench the fluorescence. The $[Co(NO)_2(^RDATI)]$ increases fluorescence intensity, which is ideal for fluorescent NO sensing but not for bioimaging [91].

1.7.6. Fluorescent NO Cheletropic Trap (FNOCTs). FNOCTs react with NO in a formal cheletropic reaction. NO was detected by this method in alveolar macrophages [92].

1.8. Fluorescein Biosensors as NO Bioimaging Probes

1.8.1. Diaminofluoresceins (DAFs). Diaminofluoresceins (DAFs) are used as novel probes for NO. DAFs change to triazole forms (DAF-Ts) with changes in the absorbance maxima of NO fluorescence intensity due to the conversion of DAF-2 to DAF-2 T by NO in the presence of O_2. Major compounds include DAF-4 M1, 4 M2, 5 M1, and 5 M2 fluorinated fluorescein derivatives. These compounds are derived amino acids (DAN) with aromatic groups. However, use of these compounds in NO bioimaging is in its infancy because DAN leaks easily through cell membranes after loading. Nevertheless, the use of esterified DAN has shown promise in studies of NO bioimaging [93, 94]. DAF has emerged as a reliable fluorophore for real-time NO detection in live cells. However, DAF is highly cytotoxic. Thus, DAF-based measurements are only accurate if measured within first 15–20 minutes. Beyond that it significantly affects the viability of the cells. In some of the research studies, to overcome the cytotoxicity of DAF, DAF was coincubated with serum. This approach significantly improved the quality of cells following DAF incubation. However, most of the DAFs had reacted with serum to form fluorescent product, which imposed limitations related to reproducibility of the measurement.

1.8.2. Diaminorhodamines (DARs). Fluorescent rhodamine B fluorophore imaging with DAR-1 AM, DAR-1 EE, DAR-M, DAR-M AM, and DAR-4 M has shown little success [95].

1.8.3. Emission Mechanism. DAF shows the photoinduced electron transfer (PET) process of fluorescence quenching or reduced fluorescence of the fluorophore. The mechanism was reported using 9-[2-(3-carboxy) naphthyl]-6-hydroxy-3*H*-xanthen-3-one (NX) and 9-[2-(3-carboxy) anthryl]-6-hydroxy-3*H*-xanthen-3-one (AX). NX is highly fluorescent, whereas AX is almost nonfluorescent [96].

1.9. Biological Applications of DAFs and DARs

1.9.1. Cardiovascular Tissue. Current studies are focusing on fast real-time nitric oxide biosensing by electrochemical

methods, as recently reported by electron transfer across multiassembly of hemoglobin-montmorillonite with polymer as biosensors with high reproducibility [97]. Low nitric oxide levels are considered potent markers of sickling and major factors responsible for the inability of red blood cells to relax arteries and oxygen deprivation. Nitric oxide is now on the market as a nutrient supplement. Nitric oxide levels were elevated following *in vivo* correction of cardiac ischemia, and NO capture was detected by a nanobiosensor (Nafion, m-phenylenediamine and resorcinol) based amperometric technique [98]. The nitric oxide content in arteries was determined by measuring superoxide anion from superoxide dismutase enzyme at levels of up to 10 nM nitric oxide by using an enzyme biosensor based amperometric method.

Nitric oxide is an organically produced signaling molecule that regulates blood pressure, clots that cause stroke and heart attack, and atherosclerosis. This molecule penetrates across membranes with biological signals and messages, influencing every organ, including the lungs, liver, stomach, genitals, and kidneys. New technological developments such as nanotechnology have led to great advances in nitric oxide biosensing through use of fiber optic chemical sensing, carbon nanotubes, and metalloporphyrin biosensors [99–102].

1.9.2. Breast Cancer Tissue. Recently, nitric oxide was evaluated as an angiogenesis marker in breast cancer patients with the potential for generation of a biomarker of prognosis [103].

1.9.3. Liver. The major application of liver bioimaging was established by nitric oxide and asymmetric dimethylarginine in human alcoholic cirrhosis [104]. The mouse abdomen was imaged in three dimensions by localization of NO-rich regions in the liver [74].

1.9.4. Bone and Cartilage. The use of NO biosensor in bone and cartilage application is still in its infancy and limited. A recent report indicated the possibility of using NO biosensors as a method of detection of NO in bone and for cartilage characterization [105].

1.9.5. Endothelial Cells. DAF-FM is a useful tool for visualizing the temporal and spatial distribution of intracellular NO. Endogenous ATP plays a central role in HTS-induced NOS in BAEC. Endothelial cNOS, a Ca^{2+}/calmodulin-dependent enzyme, is critical to vascular homeostasis and generates a detectable basal level of NO production at low extracellular Ca^{2+}. Actin microfilaments in PAEC regulate L-arginine transport, which can affect NO production by PAEC. DAR-4 M should be useful for bioimaging of samples that have strong autofluorescence [106, 107].

1.9.6. Smooth Muscle Cells. DAF-2 DA and DAF-FM T enhance fluorescence intensity [108]. This sensitive method enables their use for detection of spontaneous and substance P (active coronary artery protein) induced NO release from isolated porcine coronary arteries. This NO release was entirely dependent on the NOS activity in vascular endothelial cells. Furthermore, fluorescence images of cultured smooth muscle cells in the rat urinary bladder were captured after loading with DAF-FM DA [109]. In cells pretreated with cytokines, the fluorescence intensity increased with time after DAF-FM loading.

1.9.7. Brain. DAF-2 DA was used for direct detection of NO in the CA1 region of the hippocampus by imaging techniques. DAF-FM DA was also applied to imaging of NO generated in rat hippocampal slices [110, 111]. Recently, the use of NO bioimaging for assessment of cortical impact injury was evaluated and physiological concentrations of target NO were monitored [112, 113].

1.9.8. Ion Channels. Voltage-gated Na^+ channels and the mechanisms by which they enable signaling across cardiac tissue are not well understood. However, NO is an endogenous regulator of persistent Na^+ current. NMDA-receptor-(NMDAR-) associated ion channel has been reported to be modulated by exogenous and endogenous NO. Endogenous *S*-nitrosylation may regulate ion channel activity [114].

New Emerging Information Regarding Nitric Oxide. Nitric oxide plays a unique role in the body, and its rapid real-time biosensing and measurement may reveal a great deal of new information in time. In the body, nitric oxide is known to

(i) fight bacteria, viruses, and parasites,

(ii) suppress proliferation of some types of cancer cells,

(iii) prevent serious complications in diabetic patients, particularly in association with impaired blood flow,

(iv) play a major role in memory,

(v) act as a neurotransmitter,

(vi) increase sexual functioning,

(vii) act as a powerful antioxidant, deactivating free radicals that contribute to cancer, diabetes, heart disease, and stroke.

NO plays important roles in inflammatory processes. For example, increased expression of iNOS mRNA causes increased NO production at sites of inflammation. *Drosophila* utilizes components of the NO/cGMP signaling pathway, and chemical sensors are specific to endothelial nitric oxide and nitric oxide synthase enzyme systems. A new class of biosensors that are multifunctional and multimodal has the ability to perform as nitric oxide detectors and to monitor tissue response to nitric oxide synthase biochemical mechanisms. Recently, DAF-2 DA has been reported as a useful biosensor of hypoxia. Adenovirus-mediated gene transfer of eNOS in adrenal zona glomerulosa (ZG) cells results in the expression of active endothelial NOS enzyme, decreasing aldosterone synthesis. Moreover, γ-irradiation at doses of 2–50 Gy stimulates the expression of iNOS, which is accompanied by an increase in the fluorescence of DAF-2. NO production by mitochondrial NOS plays a role in respiration, as well as apoptosis in PC 12 and COS-1 cells. DAF-2 can

be used to image real-time intracellular NO production in retina specific synapses. *Kalanchoe daigremontiana* and *Taxus brevifolia* showed NO-induced apoptosis upon application of DAF-2 DA, while L-NMMA suppressed NO production and apoptosis [114].

2. Conclusion

This review highlights the biosensing of NO by multimodal *in vivo* EPR/MRI/fluorometry based on the potential use of NO biosensors. Fluorescent biosensors such as DAFs and DARs visualize the production of intracellular NO and enable observation of the temporal and spatial distribution of intracellular NO as a nitric oxide map. Additionally, the currently available data indicate that more attention should be given to *in vivo* real-time imaging of NO, which could be developed based on a combination of EPR and NMR techniques as NO sensitive fMRI. Amperometric and electrochemical methods using nanotechnology and advanced electronics appear to be a breakthrough in nitric oxide real-time measurement. Currently, DAFs and DARs are good candidates for bioimaging of NO in terms of specificity, sensitivity, and handling. Therefore, the NO detection method depends on reactive oxygen species such as NO_2^-, NO_3^-, ROS, superoxide, hydrogen peroxide, and $ONOO^-$ to yield any fluorescent product. Ratiometric probes are other options for intensity measurements. Overall, further studies on the development of novel ratiometric NO bioimaging probes are warranted.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

Funding was provided by Inha University Research Grant (INHA-47289-01), Republic of Korea.

References

[1] A. Suhardja, "Mechanisms of disease: roles of nitric oxide and endothelin-1 in delayed cerebral vasospasm produced by aneurysmal subarachnoid hemorrhage," *Nature Clinical Practice Cardiovascular Medicine*, vol. 1, no. 2, pp. 110–116, 2004.

[2] K. M. Naseem, "The role of nitric oxide in cardiovascular diseases," *Molecular Aspects of Medicine*, vol. 26, no. 1-2, pp. 33–65, 2005.

[3] R. F. Furchgott, "Introduction to EDRF research," *Journal of Cardiovascular Pharmacology*, vol. 22, supplement 7, pp. S1–S2, 1993.

[4] S. Kwon, R. L. Newcomb, and S. C. George, "Mechanisms of synergistic cytokine-induced nitric oxide production in human alveolar epithelial cells," *Nitric Oxide*, vol. 5, no. 6, pp. 534–546, 2001.

[5] S. Kwon and S. C. George, "Synergistic cytokine-induced nitric oxide production in human alveolar epithelial cells," *Nitric Oxide—Biology and Chemistry*, vol. 3, no. 4, pp. 348–357, 1999.

[6] F. Hyodo, R. Murugesan, K. Matsumoto et al., "Monitoring redox-sensitive paramagnetic contrast agent by EPRI, OMRI and MRI," *Journal of Magnetic Resonance*, vol. 190, no. 1, pp. 105–112, 2008.

[7] H. Fujii and L. J. Berliner, "Nitric oxide: prospects and perspectives of *in vivo* detection by L-band EPR spectroscopy," *Physics in Medicine and Biology*, vol. 43, no. 7, pp. 1949–1956, 1998.

[8] P. Kuppusamy, R. A. Shankar, V. M. Roubaud, and J. L. Zweier, "Whole body detection and imaging of nitric oxide generation in mice following cardiopulmonary arrest: detection of intrinsic nitrosoheme complexes," *Magnetic Resonance in Medicine*, vol. 45, no. 4, pp. 700–707, 2001.

[9] X. Zhang, "Real time and in vivo monitoring of nitric oxide by electrochemical sensors—from dream to reality," *Frontiers in Bioscience*, vol. 9, pp. 3434–3446, 2004.

[10] K. Liu, G. Ning, and X. Zheng, "In vivo detection of nitric oxide in rat hippocampus," in *Proceedings of the 27th Annual International Conference of the Engineering in Medicine and Biology Society (IEEE-EMBS '05)*, pp. 1039–1042, Shanghai, China, September 2005.

[11] F. Di Salle, P. Barone, H. Hacker, F. Smaltino, and M. D'Ischia, "Nitric oxide-haemoglobin interaction: a new biochemical hypothesis for signal changes in fMRI," *NeuroReport*, vol. 8, no. 2, pp. 461–464, 1997.

[12] X. Liu, M. J. S. Miller, M. S. Joshi, H. Sadowska-Krowicka, D. A. Clark, and J. R. Lancaster Jr., "Diffusion-limited reaction of free nitric oxide with erythrocytes," *Journal of Biological Chemistry*, vol. 273, no. 30, pp. 18709–18713, 1998.

[13] J. R. Lancaster Jr. and L. J. Ignarro, *Nitric Oxide Biology and Pathobiology*, Academic Press, San Diego, Calif, USA, 2000.

[14] J. L. Dinerman, C. J. Lowenstein, and S. H. Snyder, "Molecular mechanisms of nitric oxide regulation: potential relevance to cardiovascular disease," *Circulation Research*, vol. 73, no. 2, pp. 217–222, 1993.

[15] T. Malinski and Z. Taha, "Nitric oxide release from a single cell measured *in situ* by a porphyrinic-based microsensor," *Nature*, vol. 358, no. 6388, pp. 676–678, 1992.

[16] K. Shibuki, "An electrochemical microprobe for detecting nitric oxide release in brain tissue," *Neuroscience Research*, vol. 9, no. 1, pp. 69–76, 1990.

[17] Z. Taha, F. Kiechle, and T. Malinski, "Oxidation of nitric oxide by oxygen in biological systems monitored by porphyrinic sensor," *Biochemical and Biophysical Research Communications*, vol. 188, no. 2, pp. 734–739, 1992.

[18] S. Mochizuki, Y. Chiba, Y. Ogasawara et al., "Direct in situ evaluation of nitroglycerin-derived nitric oxide production in the canine and rat vascular walls at high temporal and spatial resolutions," *Cardiovascular Engineering*, vol. 1, no. 2, pp. 85–91, 2001.

[19] S. Mochizuki, M. Goto, Y. Chiba, Y. Ogasawara, and F. Kajiya, "Flow dependence and time constant of the change in nitric oxide concentration measured in the vascular media," *Medical and Biological Engineering and Computing*, vol. 37, no. 4, pp. 497–503, 1999.

[20] P. Vallance, S. Patton, K. Bhagat et al., "Direct measurement of nitric oxide in human beings," *The Lancet*, vol. 346, no. 8968, pp. 153–154, 1995.

[21] D. J. Pinsky, S. Patton, S. Mesaros et al., "Mechanical transduction of nitric oxide synthesis in the beating heart," *Circulation Research*, vol. 81, no. 3, pp. 372–379, 1997.

[22] L. J. Berliner and H. Fujii, "*In vivo* spin trapping of nitric oxide," *Antioxidants & Redox Signaling*, vol. 6, no. 3, pp. 649–656, 2004.

[23] N. Suzuki, H. Kojima, Y. Urano, K. Kikuchi, Y. Hirata, and T. Nagano, "Orthogonality of calcium concentration and ability of 4, 5-diaminofluorescein to detect NO," *The Journal of Biological Chemistry*, vol. 277, pp. 47–49, 2002.

[24] X. Ye, S. S. Rubakhin, and J. V. Sweedler, "Simultaneous nitric oxide and dehydroascorbic acid imaging by combining diaminofluoresceins and diaminorhodamines," *Journal of Neuroscience Methods*, vol. 168, no. 2, pp. 373–382, 2008.

[25] A. L. Kleschyov, P. Wenzel, and T. Munzel, "Electron paramagnetic resonance (EPR) spin trapping of biological nitric oxide," *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 851, no. 1-2, pp. 12–20, 2007.

[26] J. Weaver, S. Porasuphatana, P. Tsai, T. Budzichowski, and G. M. Rosen, "Spin trapping nitric oxide from neuronal nitric oxide synthase: a look at several iron-dithiocarbamate complexes," *Free Radical Research*, vol. 39, no. 10, pp. 1027–1033, 2005.

[27] P. G. Winyard, I. A. Knight, F. L. Shaw et al., "Chapter 8 determination of s-nitrosothiols in biological and clinical samples using electron paramagnetic resonance spectrometry with spin trapping," *Methods in Enzymology*, vol. 441, pp. 151–160, 2008.

[28] M. Ziaja, J. Pyka, A. MacHowska, A. Maslanka, and P. M. Plonka, "Nitric oxide spin-trapping and NADPH-diaphorase activity in mature rat brain after injury," *Journal of Neurotrauma*, vol. 24, no. 12, pp. 1845–1854, 2007.

[29] P. Kuppusamy, P. Wang, A. Samouilov, and J. L. Zweier, "Spatial mapping of nitric oxide generation in the ischemic heart using electron paramagnetic resonance imaging," *Magnetic Resonance in Medicine*, vol. 36, no. 2, pp. 212–218, 1996.

[30] B. Y. Jin, J. L. Sartoretto, V. N. Gladyshev, and T. Michel, "Endothelial nitric oxide synthase negatively regulates hydrogen peroxide-stimulated AMP-activated protein kinase in endothelial cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 41, pp. 17343–17348, 2009.

[31] C. F. Karney, J. E. Ferrara, and S. Brunner, "Method for computing protein binding affinity," *Journal of Computational Chemistry*, vol. 26, no. 3, pp. 243–251, 2005.

[32] M. Hardy, A. Rockenbauer, J. Vásquez-Vivar et al., "Detection, characterization, and decay kinetics of ROS and thiyl adducts of mito-DEPMPO spin trap," *Chemical Research in Toxicology*, vol. 20, no. 7, pp. 1053–1060, 2007.

[33] E. Linares, S. Giorgio, and O. Augusto, "Inhibition of in vivo leishmanicidal mechanisms by tempol: nitric oxide down-regulation and oxidant scavenging," *Free Radical Biology and Medicine*, vol. 44, no. 8, pp. 1668–1676, 2008.

[34] K. J. Liu, M. Miyake, T. Panz, and H. Swartz, "Evaluation of DEPMPO as a spin trapping agent in biological systems," *Free Radical Biology & Medicine*, vol. 26, no. 5-6, pp. 714–721, 1999.

[35] S. Kwon and R. Sharma, "Role of effectors on hypoxia due to nitric oxide production in human alveolar epithelial cells and oxygen depletion in human hepatocytes. Part II. Possible Mechanism of Cancer," *Cancer Research Journal*, vol. 4, pp. 85–101, 2009.

[36] S. Kwon and R. Sharma, "Role of effectors on hypoxia due to nitric oxide production in human alveolar epithelial cells and oxygen depletion in human hepatocytes," *International Journal of Medical and Biological Frontiers*, vol. 15, pp. 425–441, 2009.

[37] Y. R. Chen, C. L. Chen, X. Liu, H. Li, J. L. Zweier, and R. P. Mason, "Involvement of protein radical, protein aggregation, and effects on NO metabolism in the hypochlorite-mediated oxidation of mitochondrial cytochrome c," *Free Radical Biology and Medicine*, vol. 37, no. 10, pp. 1591–1603, 2004.

[38] Y. Chen, C. Chen, W. Chen et al., "Formation of protein tyrosine ortho-semiquinone radical and nitrotyrosine from cytochrome c-derived tyrosyl radical," *The Journal of Biological Chemistry*, vol. 279, no. 17, pp. 18054–18062, 2004.

[39] A. F. Vanin, A. Huisman, E. S. G. Stroes, F. C. de Ruijter-Heijstek, T. J. Rabelink, and E. E. van Faassen, "Antioxidant capacity of mononitrosyl-iron-dithiocarbamate complexes: implications for NO trapping," *Free Radical Biology and Medicine*, vol. 30, no. 8, pp. 813–824, 2001.

[40] R. Cammack, J. K. Shergill, V. A. Inalsingh, and M. N. Hughes, "Applications of electron paramagnetic resonance spectroscopy to study interactions of iron proteins in cells with nitric oxide," *Spectrochimica Acta A: Molecular and Biomolecular Spectroscopy*, vol. 54, no. 14, pp. 2393–2402, 1998.

[41] D. J. Blyth, J. W. Aylott, J. W. B. Moir, D. J. Richardson, and D. A. Russell, "Optical biosensing of nitric oxide using the metalloprotein cytochrome c'," *Analyst*, vol. 124, no. 2, pp. 129–134, 1999.

[42] A. Mulsch, B. Schray-Utz, P. I. Mordvintcev, S. Hauschildt, and R. Busse, "Diethyldithiocarbamate inhibits induction of macrophage NO synthase," *FEBS Letters*, vol. 321, no. 2-3, pp. 215–218, 1993.

[43] W. Eberhardt, D. Kunz, and J. Pfeilschifter, "Pyrrolidine dithiocarbamate differentially affects interleukin 1β- and cAMP-induced nitric oxide synthase expression in rat renal mesangial cells," *Biochemical and Biophysical Research Communications*, vol. 200, no. 1, pp. 163–170, 1994.

[44] F. J. Bedoya, M. Flodström, and D. L. Eizirik, "Pyrrolidine dithiocarbamate prevents IL-1-induced nitric oxide synthase mRNA, but not superoxide dismutase mRNA, in insulin producing cells," *Biochemical and Biophysical Research Communications*, vol. 210, no. 3, pp. 816–822, 1995.

[45] J. T. Wolfe, D. Ross, and G. M. Cohen, "A role for metals and free radicals in the induction of apoptosis in thymocytes," *The FEBS Letters*, vol. 352, no. 1, pp. 58–62, 1994.

[46] R. Bessho, K. Matsubara, M. Kubota et al., "Pyrrolidine dithiocarbamate, a potent inhibitor of nuclear factor κB (NF-κB) activation, prevents apoptosis in human promyelocytic leukemia HL-60 cells and thymocytes," *Biochemical Pharmacology*, vol. 48, no. 10, pp. 1883–1889, 1994.

[47] C. Victor Jongeneel, "Bcl-2 protects from oxidative damage and apoptotic cell death without interfering with activation of NF-κB by TNF," *FEBS Letters*, vol. 351, no. 1, pp. 45–48, 1994.

[48] Y. Hattori, K. Akimoto, Y. Murakami, and K. Kasai, "Pyrrolidine dithiocarbamate inhibits cytokine-induced VCAM-1 gene expression in rat cardiac myocytes," *Molecular and Cellular Biochemistry*, vol. 177, no. 1-2, pp. 177–181, 1997.

[49] B. M. Altura and A. Gebrewold, "Pyrrolidine dithiocarbamate attenuates alcohol-induced leukocyte-endothelial cell interaction and cerebral vascular damage in rats: possible role of activation of transcription factor NF-κB in alcohol brain pathology," *Alcohol*, vol. 16, no. 1, pp. 25–28, 1998.

[50] S. V. Paschenko, V. V. Khramtsov, M. P. Skatchkov, V. F. Plyusnin, and E. Bassenge, "EPR and laser flash photolysis studies of the reaction of nitric oxide with water soluble NO trap Fe(II)-proline-dithiocarbamate complex," *Biochemical and Biophysical Research Communications*, vol. 225, no. 2, pp. 577–584, 1996.

[51] H. Nakagawa, N. Ikota, T. Ozawa, T. Masumizu, and M. Kohno, "Spin trapping for nitric oxide produced in LPS-treated mouse using various new dithiocarbamate iron complexes having substituted proline and serine moiety," *Biochemistry and*

Molecular Biology International, vol. 45, no. 6, pp. 1129–1138, 1998.

[52] H. Fujii, T. Yoshimura, and H. Kamada, "ESR studies of A_{1u} and A_{2u} oxoiron(IV) porphyrin π-cation radical complexes. Spin coupling between ferryl iron and A_{1u}/A_{2u} orbitals," *Inorganic Chemistry*, vol. 35, no. 8, pp. 2373–2377, 1996.

[53] T. Yoshimura and Y. Kotake, "Spin trapping of nitric oxide with the iron-dithiocarbamate complex: chemistry and biology," *Antioxidants and Redox Signaling*, vol. 6, no. 3, pp. 639–647, 2004.

[54] S. Fujii and T. Yoshimura, "Detection and imaging of endogenously produced nitric oxide with electron paramagnetic resonance spectroscopy," *Antioxidants and Redox Signaling*, vol. 2, no. 4, pp. 879–901, 2000.

[55] Q. H. Gibson and F. J. Roughton, "The kinetics of dissociation of the first ligand molecule from fully saturated carboxyhaemoglobin and nitric oxide haemoglobin in sheep blood solutions," *Proceedings of the Royal Society of London B: Biological Sciences*, vol. 147, no. 926, pp. 44–56, 1957.

[56] J. L. Zweier, P. Wang, A. Samouilov, and P. Kuppusamy, "Enzyme-independent formation of nitric oxide in biological tissues," *Nature Medicine*, vol. 1, no. 8, pp. 804–809, 1995.

[57] L. A. Reinke, D. R. Moore, and Y. Kotake, "Hepatic nitric oxide formation: spin trapping detection in biliary efflux," *Analytical Biochemistry*, vol. 243, no. 1, pp. 8–14, 1996.

[58] S. Lecour, V. Maupoil, O. Siri, A. Tabard, and L. Rochette, "Electron spin resonance detection of nitric oxide generation in major organs from LPS-treated rats," *Journal of Cardiovascular Pharmacology*, vol. 33, no. 1, pp. 78–85, 1999.

[59] A. M. Komarov, J. H. Kramer, I. T. Mak, and W. B. Weglicki, "EPR detection of endogenous nitric oxide in postischemic heart using lipid and aqueous-soluble dithiocarbamate-iron complexes," *Molecular and Cellular Biochemistry*, vol. 175, no. 1-2, pp. 91–97, 1997.

[60] A. Mulsch, A. Bara, P. Mordvintcev, A. Vanin, and R. Busse, "Specificity of different organic nitrates to elicit NO formation in rabbit vascular tissues and organs in vivo," *British Journal of Pharmacology*, vol. 116, no. 6, pp. 2743–2749, 1995.

[61] A. Mulsch, P. Mordvintcev, E. Bassenge, F. Jung, B. Clement, and R. Busse, "In vivo spin trapping of glyceryl trinitrate-derived nitric oxide in rabbit blood vessels and organs," *Circulation*, vol. 92, no. 7, pp. 1876–1882, 1995.

[62] P. C. Lauterbur, "Image formation by induced local interactions: examples employing nuclear magnetic resonance," *Nature*, vol. 242, no. 5394, pp. 190–191, 1973.

[63] H. Yokoyama, N. Tsuchihashi, T. Ogata, M. Hiramatsu, and N. Mori, "An analysis of the intracerebral ability to eliminate a nitroxide radical in the rat after administration of idebenone by an in vivo rapid scan electron spin resonance spectrometer," *Magnetic Resonance Materials in Physics, Biology, and Medicine*, vol. 4, no. 3-4, pp. 247–250, 1996.

[64] H. Yokoyama, S. Fujii, T. Yoshimura, H. Ohya-Nishiguchi, and H. Kamada, "In vivo ESR-CT imaging of the liver in mice receiving subcutaneous injection of nitric oxide-bound iron complex," *Magnetic Resonance Imaging*, vol. 15, no. 2, pp. 249–253, 1997.

[65] H.-W. Pfister, U. Koedel, S. Lorenzl, and A. Tomasz, "Antioxidants attenuate microvascular changes in the early phase of experimental pneumococcal meningitis in rats," *Stroke*, vol. 23, no. 12, pp. 1798–1804, 1992.

[66] A. R. Tunkel and W. Michael Scheld, "Pathogenesis and pathophysiology of bacterial meningitis," *Annual Review of Medicine*, vol. 44, pp. 103–120, 1993.

[67] U. Koedel, A. Bernatowicz, R. Paul, K. Frei, A. Fontana, and H. W. Pfister, "Experimental pneumococcal meningitis: cerebrovascular alterations, brain edema, and meningeal inflammation are linked to the production of nitric oxide," *Annals of Neurology*, vol. 37, no. 3, pp. 313–323, 1995.

[68] K. M. K. Boje, "Inhibition of nitric oxide synthase attenuates blood-brain barrier disruption during experimental meningitis," *Brain Research*, vol. 720, no. 1-2, pp. 75–83, 1996.

[69] Y. S. Kim and M. G. Tauber, "Neurotoxicity of glia activated by gram-positive bacterial products depends on nitric oxide production," *Infection and Immunity*, vol. 64, no. 8, pp. 3148–3153, 1996.

[70] R. F. Kornelisse, K. Hoekman, J. J. Visser et al., "The role of nitric oxide in bacterial meningitis in children," *Journal of Infectious Diseases*, vol. 174, no. 1, pp. 120–126, 1996.

[71] S. L. Leib, Y. S. Km, S. M. Black, J. H. Tureen, and M. G. Täuber, "Inducible nitric oxide synthase and the effect of aminoguanidine in experimental neonatal meningitis," *Journal of Infectious Diseases*, vol. 177, no. 3, pp. 692–700, 1998.

[72] A. M. Komarov, "In vivo on-line detection of no distribution in endotoxin-treated mice by l-band ESR.," *Cellular and Molecular Biology*, vol. 46, no. 8, pp. 1329–1336, 2000.

[73] T. Yoshimura, "In vivo EPR detection and imaging of endogenously produced and exogenously supplied nitric oxide," *Analytical Sciences*, vol. 13, pp. 451–454, 1997.

[74] S. Fujii, Y. Suzuki, T. Yoshimura, and H. Kamada, "In vivo three-dimensional EPR imaging of nitric oxide production from isosorbide dinitrate in mice," *The American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 274, no. 5, pp. G857–G862, 1998.

[75] T. Ueno, Y. Suzuki, S. Fujii, A. F. Vanin, and T. Yoshimura, "In vivo distribution and behavior of paramagnetic dinitrosyl dithiolato iron complex in the abdomen of mouse," *Free Radical Research*, vol. 31, no. 6, pp. 525–534, 1999.

[76] M. A. Foster, I. Seimenis, and D. J. Lurie, "The application of PEDRI to the study of free radicals in vivo," *Physics in Medicine and Biology*, vol. 43, no. 7, pp. 1893–1897, 1998.

[77] A. Mülsch, D. J. Lurie, I. Seimenis, B. Fichtlscherer, and M. A. Foster, "Detection of nitrosyl-iron complexes by proton-electron-double-resonance imaging," *Free Radical Biology and Medicine*, vol. 27, no. 5-6, pp. 636–646, 1999.

[78] S. Rast, A. Borel, L. Helm, E. Belorizky, P. H. Fries, and A. E. Merbach, "EPR spectroscopy of MRI-related Gd(III) complexes: simultaneous analysis of multiple frequency and temperature spectra, including static and transient crystal field effects," *Journal of the American Chemical Society*, vol. 123, no. 11, pp. 2637–2644, 2001.

[79] A. E. Merbach and E. Toth, *The Chemistry of Contrast Agents in Medical Magnetic Resonance Imaging*, John Wiley & Sons, Chichester, UK, 2001.

[80] T. Yoshimura, H. Yokoyama, S. Fujii, F. Takayama, K. Oikawa, and H. Kamada, "In vivo EPR detection and imaging of endogenous nitric oxide in lipopolysaccharide-treated mice," *Nature Biotechnology*, vol. 14, no. 8, pp. 992–994, 1996.

[81] A. F. Vanin, P. I. Mordvintcev, S. Hauschildt, and A. Mülsch, "The relationship between L-arginine-dependent nitric oxide synthesis, nitrite release and dinitrosyl-iron complex formation by activated macrophages," *Biochimica et Biophysica Acta*, vol. 1177, no. 1, pp. 37–42, 1993.

[82] Y. Kotake, "Continuous and quantitative monitoring of rate of cellular nitric oxide generation," *Methods in Enzymology*, vol. 268, pp. 222–229, 1996.

[83] A. F. Vanin, "Iron diethyldithiocarbamate as spin trap for nitric oxide detection," *Methods in Enzymology*, vol. 301, pp. 269–279, 1999.

[84] T. P. Misko, R. J. Schilling, D. Salvemini, W. M. Moore, and M. G. Currie, "A fluorometric assay for the measurement of nitrite in biological samples," *Analytical Biochemistry*, vol. 214, no. 1, pp. 11–16, 1993.

[85] P. G. Gunasekar, B. G. Kanthasamy, J. L. Borowitz, and G. E. Isom, "Monitoring intracellular nitric oxide formation by dichlorofluorescin in neuronal cells," *Journal of Neuroscience Methods*, vol. 61, no. 1-2, pp. 15–21, 1995.

[86] O. Myhre, J. M. Andersen, H. Aarnes, and F. Fonnum, "Evaluation of the probes 2′,7′-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation," *Biochemical Pharmacology*, vol. 65, no. 10, pp. 1575–1582, 2003.

[87] N. Soh, Y. Katayama, and M. Maeda, "A fluorescent probe for monitoring nitric oxide production using a novel detection concept," *Analyst*, vol. 126, no. 5, pp. 564–566, 2001.

[88] X. Ye, S. S. Rubakhin, and J. V. Sweedler, "Detection of nitric oxide in single cells," *Analyst*, vol. 133, no. 4, pp. 423–433, 2008.

[89] S. L. R. Barker, H. A. Clark, S. F. Swallen, R. Kopelman, A. W. Tsang, and J. A. Swanson, "Ratiometric and fluorescence-lifetime-based biosensors incorporating cytochrome c′ and the detection of extra- and intracellular macrophage nitric oxide," *Analytical Chemistry*, vol. 71, no. 9, pp. 1767–1772, 1999.

[90] S. L. Barker, Y. Zhao, M. A. Marletta, and R. Kopelman, "Cellular applications of a sensitive and selective fiber-optic nitric oxide biosensor based on a dye-labeled heme domain of soluble guanylate cyclase," *Analytical Chemistry*, vol. 71, no. 11, pp. 2071–2075, 1999.

[91] K. J. Franz, N. Singh, and S. J. Lippard, "Metal-based NO sensing by selective ligand dissociation this work was supported by a grant from the national science foundation and a fellowship to N.S. from the undergraduate research opportunity program (MIT). We thank Prof. Roger Tsien for valuable discussions at the inception of this project," *Angewandte Chemie—International Edition*, vol. 39, pp. 2120–2122, 2000.

[92] P. Meineke, U. Rauen, H. de Groot, H.-G. Korth, and R. Sustmann, "Nitric oxide detection and visualization in biological systems. Applications of the FNOCT method," *Biological Chemistry*, vol. 381, no. 7, pp. 575–582, 2000.

[93] L. J. Ignarro, J. M. Fukuto, J. M. Griscavage, N. E. Rogers, and R. E. Byrns, "Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 17, pp. 8103–8107, 1993.

[94] H. Kojima, N. Nakatsubo, K. Kikuchi et al., "Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins," *Analytical Chemistry*, vol. 70, no. 13, pp. 2446–2453, 1998.

[95] H. Kojima, M. Hirotani, N. Nakatsubo et al., "Bioimaging of nitric oxide with fluorescent indicators based on the rhodamine chromophore," *Analytical Chemistry*, vol. 73, no. 9, pp. 1967–1973, 2001.

[96] R. Y. Tsien, A. Waggoner, and J. B. Pawley, *Handbook of Biological Confocal Microscopy*, Plenum Press, New York, NY, USA, 1995.

[97] J. Pang, C. Fan, X. Liu, T. Chen, and G. Li, "A nitric oxide biosensor based on the multi-assembly of hemoglobin/montmorillonite/polyvinyl alcohol at a pyrolytic graphite electrode," *Biosensors and Bioelectronics*, vol. 19, no. 5, pp. 441–445, 2003.

[98] J. K. Park, P. H. Tran, J. K. T. Chao, R. Ghodadra, R. Rangarajan, and N. V. Thakor, "In vivo nitric oxide sensor using non-conducting polymer-modified carbon fiber," *Biosensors and Bioelectronics*, vol. 13, no. 11, pp. 1187–1195, 1998.

[99] K. Balasubramanian and M. Burghard, "Biosensors based on carbon nanotubes," *Analytical and Bioanalytical Chemistry*, vol. 385, no. 3, pp. 452–468, 2006.

[100] T. Imanishi, A. Kuroi, H. Ikejima et al., "Effects of pioglitazone on nitric oxide bioavailability measured using a catheter-type nitric oxide sensor in angiotensin II-infusion rabbit," *Hypertension Research*, vol. 31, no. 1, pp. 117–125, 2008.

[101] R. Wadsworth, E. Stankevicius, and U. Simonsen, "Physiologically relevant measurements of nitric oxide in cardiovascular research using electrochemical microsensors," *Journal of Vascular Research*, vol. 43, no. 1, pp. 70–85, 2006.

[102] D. H. P. Hedges, D. J. Richardson, and D. A. Russell, "Electrochemical control of protein monolayers at indium tin oxide surfaces for the reagentless optical biosensing of nitric oxide," *Langmuir*, vol. 20, no. 5, pp. 1901–1908, 2004.

[103] D. Konukoglu, M. S. Turhan, V. Celik, and H. Turna, "Relation of serum vascular endothelial growth factor as an angiogenesis biomarker with nitric oxide & urokinase-type plasminogen activator in breast cancer patients," *Indian Journal of Medical Research*, vol. 125, no. 6, pp. 747–751, 2007.

[104] P. Lluch, B. Torondel, P. Medina et al., "Plasma concentrations of nitric oxide and asymmetric dimethylarginine in human alcoholic cirrhosis," *Journal of Hepatology*, vol. 41, no. 1, pp. 55–59, 2004.

[105] M. R. van der Harst, S. Bull, P. A. J. Brama, A. Barneveld, P. R. van Weeren, and C. H. A. van de Lest, "Nitrite and nitrotyrosine concentrations in articular cartilage, subchondral bone, and trabecular bone of normal juvenile, normal adult, and osteoarthritic adult equine metacarpophalangeal joints," *Journal of Rheumatology*, vol. 33, no. 8, pp. 1662–1667, 2006.

[106] P. Kleinbongard, A. Dejam, T. Lauer et al., "Plasma nitrite concentrations reflect the degree of endothelial dysfunction in humans," *Free Radical Biology and Medicine*, vol. 40, no. 2, pp. 295–302, 2006.

[107] H. Kojima, Y. Urano, K. Kikuchi, T. Higuchi, Y. Hirata, and T. Nagano, "Fluorescent indicators for imaging nitric oxide production," *Angewandte Chemie International Edition*, vol. 38, pp. 3209–3212, 1999.

[108] Y. Itoh, F. H. Ma, H. Hoshi et al., "Determination and bioimaging method for nitric oxide in biological specimens by diaminofluorescein fluorometry," *Analytical Biochemistry*, vol. 287, no. 2, pp. 203–209, 2000.

[109] F. Stoffels, F. Lohöfener, M. Beisenhirtz, F. Lisdat, and R. Büttemeyer, "Concentration decrease of nitric oxide in the postischemic muscle is not only caused by the generation of O_2," *Microsurgery*, vol. 27, no. 6, pp. 565–568, 2007.

[110] H. Kojima, N. Nakatsubo, K. Kikuchi et al., "Direct evidence of NO production in rat hippocampus and cortex using a new fluorescent indicator: DAF-2 DA," *NeuroReport*, vol. 9, no. 15, pp. 3345–3348, 1998.

[111] O. Yermolaieva, N. Brot, H. Weissbach, S. H. Heinemann, and T. Hoshi, "Reactive oxygen species and nitric oxide mediate plasticity of neuronal calcium signaling," *Proceedings of the*

National Academy of Sciences of the United States of America, vol. 97, no. 1, pp. 448–453, 2000.

[112] C. N. Hall and D. Attwell, "Assessing the physiological concentration and targets of nitric oxide in brain tissue," *Journal of Physiology*, vol. 586, no. 15, pp. 3597–3615, 2008.

[113] L. Cherian and C. S. Robertson, "L-arginine and free radical scavengers increase cerebral blood flow and brain tissue nitric oxide concentrations after controlled cortical impact injury in rats," *Journal of Neurotrauma*, vol. 20, no. 1, pp. 77–85, 2003.

[114] Y. Choi, L. Tenneti, D. A. Le et al., "Molecular basis of NMDA receptor-coupled ion channel modulation by S-nitrosylation," *Nature Neuroscience*, vol. 3, no. 1, pp. 15–21, 2000.

[115] Y. Wang-Rosenke, H. Neumayer, and H. Peters, "NO signaling through cGMP in renal tissue fibrosis and beyond: key pathway and novel therapeutic target," *Current Medicinal Chemistry*, vol. 15, no. 14, pp. 1396–1406, 2008.

[116] L. Corzo, R. Zas, S. Rodríguez, L. Fernández-Novoa, and R. Cacabelos, "Decreased levels of serum nitric oxide in different forms of dementia," *Neuroscience Letters*, vol. 420, no. 3, pp. 263–267, 2007.

[117] H. Shin, P. Condorelli, and S. C. George, "Examining axial diffusion of nitric oxide in the lungs using heliox and breath hold," *Journal of Applied Physiology*, vol. 100, no. 2, pp. 623–630, 2006.

[118] F. A. Recchia, T. R. Vogel, and T. H. Hintze, "NO metabolites accumulate in erythrocytes in proportion to carbon dioxide and bicarbonate concentration," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 279, no. 2, pp. H852–H856, 2000.