Characterization of a Fluorescent Probe for Imaging Nitric Oxide

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Key Words

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Abstract

Background: Nitric oxide (NO), a potent vasodilator and anti-atherogenic molecule, is synthesized in various cell types, including vascular endothelial cells (ECs). The biological importance of NO enforces the need to develop and characterize specific and sensitive probes. To date, several fluorophores, chromophores and colorimetric techniques have been developed to detect NO or its metabolites (NO₂ and NO₃) in biological fluids, viable cells or cell lysates. **Methods:** Recently, a novel probe (NO₅₅₀) has been developed and reported to detect NO in solutions and in primary astrocytes and neuronal cells with a fluorescence signal arising from a nonfluorescent background. **Results:** Here, we report further characterization of this probe by optimizing conditions for the detection and imaging of NO products in primary vascular ECs, fibroblasts, and embryonic stem cell- and induced pluripotent stem cell-derived ECs in the absence and presence of pharmacological agents that modulate NO levels. In addition, we studied the stability of this probe in cells over

time and evaluated its compartmentalization in reference to organelle-labeling dyes. Finally, we synthesized an inherently fluorescent diazo ring compound (AZO₅₅₀) that is expected to form when the nonfluorescent NO₅₅₀ reacts with cellular NO, and compared its cellular distribution with that of NO₅₅₀. **Conclusion:** NO₅₅₀ is a promising agent for imaging NO at baseline and in response to pharmacological agents that modulate its levels.

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Introduction

Endothelium-derived nitric oxide (NO) is a potent signaling molecule that is critically involved in maintaining metabolic and cardiovascular homeostasis [1–3]. In addition to its role as a potent endogenous vasodilator, we and others have shown that NO plays a key role in regulating vascular smooth muscle cell growth, as well as the interaction of the vessel wall with circulating blood elements. Because NO suppresses the expression of endothelial adhesion molecules and chemokines, it reduces endothelial adhesiveness for monocytes [4–6]. Furthermore, NO suppresses platelet reactivity [7, 8] and vascular smooth muscle cell proliferation [9, 10]. Because NO suppresses

key processes in vascular lesion formation, enhancement of NO synthesis is associated with resistance to atherogenesis and myointimal hyperplasia [11–13]. By contrast, pharmacological or genetic suppression of NO synthesis is associated with reduced vascular compliance [14] and an acceleration of vascular disease in preclinical models [15, 16]. Notably, enhancement of NO synthesis is associated with insulin sensitivity [17] whereas genetic knock-down of endothelial NO synthesis increases insulin resistance [18]. These preclinical studies suggest that endothelium-derived NO is critical for vascular health. Indeed, accumulating evidence from epidemiological studies indicate that humans with impaired endothelial NO synthase (eNOS) activity are at increased risk for major adverse cardiovascular events and mortality [19–21].

It is, therefore, due to this essential role of NO in biological systems and cardiovascular health that substantial amount of research has focused on developing methods to differentially detect and quantify its concentration in biological samples. Some of these methods include the Griess colorimetric assay, which measures total NO (as NO₃ and NO₂) in various biological fluids and cell culture media; electrochemical measurement using a current-based electrode system [22]; electron paramagnetic resonance spectrometry by complexing NO with chemicals such as iron and hemoglobin [23]; an NO-sensitive porphyrin-based electrode [24]; a chemiluminescent technique which quantifies NO following its reaction with luminol [25]; dual-photon microscopy [26], and an HPLC-based system [27]. However, the colorimetric technique detects NO indirectly, lacks real-time measurement in viable cells and only detects micromolar concentrations of the molecule, and many of the other techniques generally require instrumentation and expertise that are not readily available in many laboratories. Meanwhile, small molecule-based fluorescent techniques that use cell-permeable probes have been developed to quantify NO in viable cells and in vivo. Some of these probes include diaminobenzene-based fluorophores such as diaminofluoresceins (DAFs) [28], diaminonaphthalene (DAN) [29, 30], diaminorhodamine (DAR-4M) [30], diaminoanthraquinone [31]; chemical element-based probes such as the boron-based chromophore BODIPY [32, 33], copper-based fluorophore (CuFL) [34] and a lanthanide-based time-resolved luminescence probe [35]; synthetic dye-based fluorophores such as cyanine-based probes (DACs) [36], and others such as the nanocrystal-based quantum dots (QDs) [37] and carbon nanotube-based sensors (SWNTs) [38] (table 1). Their sensitivity to NO at nanomolar concentrations, ease of use, real-time measure-

ment, and their application in living cells have made these fluorescent techniques a premium choice for the detection and imaging of NO in mammalian cells. However, high pH dependence, high background signal due to side reactions with other nitrogen/oxygen species, and fluorescence quenching confound the measurements obtained by many of these fluorophores. In this study, we evaluated the use of a new probe (NO₅₅₀) [39] to detect signals associated with NO levels produced by vascular cells (fig. 1). Chemically, the nonfluorescent NO₅₅₀ reacts with oxidized NO to form a fluorescent product (AZO₅₅₀). The preliminary study by Yang et al. [39] provided important insights about the NO₅₅₀ probe. However, the study did not address the possibility of imaging NO in nonneuronal cells, e.g. vascular ECs and fibroblasts. In addition, their study was limited in examining the ability of the probe to sense changes in NO levels as a result of pharmacological manipulation of pathways that are major sources of NO, including the NOS and xanthine oxidoreductase (XOR) pathways. Moreover, the study by Yang et al. [39] did not address the biochemical stability of the NO₅₅₀ probe in cells. Accordingly, we designed a study to test the following hypotheses: (1) the NO₅₅₀ probe will be able to detect NO produced by a variety of cell types in response to pharmacological agents that modulate NO; (2) NO₅₅₀ will be able to distribute in cellular compartments in line with NO localization and comparable to commercially available NO detection dyes such as DAF-FM diacetate, and (3) NO₅₅₀ is a chemically stable probe when loaded to cells and may be useful to study NO kinetics over time.

Materials and Methods

Primary human microvascular endothelial cells (HMVECs; p3), EC maintenance media (EBM2), and growth supplements were purchased from Lonza (Walkersville, Md., USA). Dulbecco's modified Eagle's medium (catalog No. 11995) was purchased from Invitrogen (Grand Island, N.Y., USA). Human foreskin (BJ) fibroblasts were purchased from ATCC Bioresource Center (Manassas, Va., USA) and human dermal fibroblasts (HUF) were obtained from the laboratory of Dr. Renee Reijo Pera (Stanford University). NO probes (NO₅₅₀ and AZO₅₅₀) were from the laboratory of Dr. Eric Anslyn (University of Texas at Austin) and were dissolved in stock DMSO (Sigma D2650) to obtain a stock concentration of 10 mM prior to diluting in media to final concentrations of 20 (AZO₅₅₀) and 50–100 μM (NO₅₅₀). The final DMSO concentration was 0.2% for AZO₅₅₀ and 0.5–1% for NO₅₅₀. DAF-FM diacetate was purchased from Molecular Probes (Eugene, Oreg., USA; catalog No. D-23844) and was dissolved in stock DMSO to a 5-mM concentration as recommended. The organelle-labeling dyes LysoTracker and ER-Tracker were purchased from Invitrogen (cata-

log Nos. L7528 and E34250, respectively) and were diluted in 1 mM stock solution to the desired final concentration in fresh media. N^G-nitro-L-arginine methyl ester (L-NAME), allopurinol, TNF- α , 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), and a toxicity assay kit were purchased from Sigma (St. Louis, Mo., USA); a Griess assay kit for NO quantification was from Assay Designs (Ann Arbor, Mich., USA); RT-PCR primers were from Applied Biosystems (Foster City, Calif., USA), and the anti-eNOS antibody was purchased from BD Transduction Laboratories (San Jose, Calif., USA; catalog No. 610296).

Statistical tests between two groups were performed using unpaired t test of the mean values and expressed as means $\pm$ SEM. Multiple group comparison was performed using one-way ANOVA followed by the Bonferroni post hoc test. Data were considered statistically significant at $p < 0.05$.

NO Imaging Assay

HMVECs were seeded in 75-cm² tissue culture flasks containing growth factor-fortified (including 5% fetal calf serum) EBM2 media and incubated at 37°C/5% CO₂ to the desired degree of confluency. Once ready, the conditioned media were aspirated and the cells were briefly washed with PBS. Subsequently, the cells were exposed to the NO₅₅₀ probe (premixed with fresh media) to a final concentration of 50, 60, 75, or 100 μ M for various time points (1, 2, 3, 4, and 24 h). For kinetics studies, cells were imaged under a fluorescent microscope by removing the plates from the incubator at predetermined time points. For an endpoint study, the cells were exposed to a defined concentration of NO₅₅₀ (60–75 μ M), and Hoechst 33342 (Sigma B2261; 5 μ g/ml final concentration) was used to stain nuclei. The same procedure was followed to expose cells to chemically synthesized AZO₅₅₀ (20 μ M) or the NO imaging agent DAF-FM diacetate (1 μ M), and cells were imaged under the same excitation/emission spectra as NO₅₅₀. Background fluorescence was studied by incubating NO₅₅₀ with the same media in the absence of cells and was followed over time.

Image Acquisition and Analysis

Images were captured at the indicated time points under the same image acquisition settings (with the exception of the 24-hour image set in fig. 2g, where the exposure time for all the samples at that time point was reset to minimize image saturation) at excitation/emission spectra of 488/519 nm for NO₅₅₀/AZO₅₅₀/DAF-FM diacetate and at 358/461 nm for the nuclear Hoechst staining using a standard inverted fluorescence microscope (Nikon Eclipse TE 2000-U) equipped with SPOT (2.2.1/RTPS-IN; Diagnostic Instruments, Sterling Heights, Mich., USA) and QImaging (RETIGA-2000R FAST) cameras. The exposure time for the color images was set using SPOT Advanced (Diagnostic Instruments;) and the black/white acquisition parameters were set using the QCapture Pro software (QImaging, Surrey, B.C., Canada). Finally, regions of interest were placed over each of the individual cells in the field of view, and the average fluorescence value was analyzed using ImageJ software (National Institutes of Health, NIH) [40] under the same settings. In each case, the average background at zero time point was used to subtract from the average fluorescence value for the whole time series due to minimal changes in background fluorescence over time as described in the Results section below.

All images were taken at $\times 20$ objective magnification (0.45 numerical aperture) unless stated otherwise. All the scale bars are 50 or 100 μ m unless indicated otherwise.

Enhancement or Inhibition of NO Levels

ECs were exposed to agents that negatively (L-NAME/allopurinol: final concentration from 100 μ M to 1 mM each; NO scavenger: PTIO at a final concentration of 10–100 μ M) or positively [acetylcholine (ACh; 10 μ M), the NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP; 10 μ M) or TNF- α (10 ng/ml)] influence intracellular NO levels. The chemicals were dissolved in stock DMSO to 100 mM and then further diluted in PBS as needed while recombinant human TNF- α was dissolved in PBS containing 1% BSA to 1 μ g/ml stock concentration and was further diluted in PBS. The diluted reagents or diluted DMSO control solution were then added to fresh media and prewashed adherent cells cultured in 12-well plates were exposed to these agents either alone or in combination (such as allopurinol + L-NAME + NO scavenger) at the stated final concentrations. Subsequently, NO₅₅₀ was added to image the changes in NO levels in response to these agents. Images were captured at various time points during the treatment course. Image analyses were performed as described above. Meanwhile, the standard Griess assay was used to quantify total NO in cells similarly treated with vehicle, L-NAME/allopurinol, PTIO or the L-NAME/allopurinol/PTIO combination.

Compartmentalization of NO₅₅₀

In order to assess the intracellular compartmentalization of NO₅₅₀, we used cell-permeant organelle-selective fluorescent probes to label the lysosomes (using the LysoTracker Red DND-99 dye) and the endoplasmic reticulum (ER; using the ER-Tracker Red dye). ECs were loaded with NO₅₅₀ (60 μ M final concentration) for 30 min and then LysoTracker or ER-Tracker dyes were added to the cells at the preoptimized final concentrations (0.5 and 1 μ M, respectively) for these cells. The loaded cells were incubated at 37°C/5% CO₂ for additional 2–3 h. Finally, the cells were incubated with Hoechst for 5 min to stain the nuclei. Images were acquired at excitation/emission wavelengths of 358/461, 488/519, and 595/615 nm for DAPI, NO₅₅₀ and Lyso-/ER-Tracker dyes, respectively, as described above and were merged for colocalization studies.

Toxicity Assay

To assess the degree of cellular toxicity of the NO₅₅₀ probe, ECs were seeded on 12-well plates overnight and then exposed to various concentrations of the probe (60–100 μ M) or vehicle for 4 h. The cells were washed and dissociated by Accutase solution (Sigma, catalog No. A6964) treatment, pelleted by centrifugation at 500 g for 3 min and then the viable cells were counted in replicates using an automated cell counter (TC10; Bio-Rad, Hercules, Calif., USA) after staining with trypan blue (0.4%) to exclude dead cells. In addition, cytotoxicity was also assessed using an ELISA-based lactate dehydrogenase (LDH) leakage assay as recommended by the supplier. In brief, ECs were seeded at a density of 3×10^4 cells/well in 12-well plates overnight and then washed and exposed to NO₅₅₀ (60–100 μ M) or vehicle control for 4 h prior to harvesting the conditioned media. The supernatant was clarified by centrifugation at 250 g for 4 min and 25 μ l of each sample, including blank media, were transferred into a 96-well plate in duplicate for cytotoxicity assays. Absorbance, proportional to the amount of LDH released, was measured using a plate reader (M1000; Tecan, Männedorf, Switzerland) at 490 nm. The background absorbance (measured at 690 nm) was subtracted from the respective absorbencies at 490 nm and the data (indicating cell membrane integrity) were analyzed using GraphPad Prism (La Jolla, Calif., USA).

Fig. 1. Schematic diagram illustrating the regulation of NO production and reactivity to the NO₅₅₀ probe to generate a fluorescent AZO₅₅₀ product.

Comparison of NO₅₅₀ with DAF-FM Diacetate

NO₅₅₀ was compared with the commercially available DAF-FM diacetate fluorophore for the detection and imaging of intracellular NO in primary ECs. In brief, HMVECs were cultured and the detection of NO using the NO₅₅₀ probe was performed as described above. In parallel experiments, HMVECs were incubated with 1 μ M of freshly diluted (from DMSO stock) DAF-FM diacetate at 37°C/5% CO₂ for 20 min as recommended by the supplier. Excess probe was removed by rinsing and the cells were switched to fresh media or PBS prior to imaging.

RT-PCR and Western Blot Analyses of eNOS

Baseline gene and protein expression profiling of eNOS in ECs, human induced pluripotent stem cells (hiPSCs) and hiPSC-derived ECs were performed by RT-PCR and Western blot, respectively. The gene expression study was performed as described previously [41, 42]. For the Western blot study, 6 μ g of total protein were transferred into Eppendorf tubes and 1 $\times$ LDS buffer and reducing agent were added to the samples prior to boiling the mixtures. The samples were resolved on a 4–12% precast gel at 125 mA for 2 h. The samples were then transferred to nitrocellulose membrane using a semidry iBlot (Invitrogen) system and the blot was blocked with 5% nonfat milk (in 0.1% TBST) for 1 h at room temperature (RT). Subsequently, the blot was incubated with 1:2,500-diluted mouse anti-eNOS primary antibody at 4°C overnight. The next day, the blot was washed with 0.1% TBST (4 $\times$ 5 min each wash) and incubated with HRP-conjugated goat anti-mouse secondary antibody (1:5,000 diluted; GE Healthcare, Little Chalfont, UK, catalog No. NA931V) for 1 h at RT. Finally, the blot was washed, ECL Plus detection reagent was added for 5 min at RT, and the film was developed using an automated film processor. eNOS protein (140 kDa) expression was normalized to human β -actin housekeeping protein (42 kDa) by stripping the blot and reprobing it with rabbit anti-actin antibody (1:1,500 diluted; Sigma, catalog No. A2066).

Differentiation of ESCs and iPSCs into ECs

Mouse ESCs (mESCs) and human iPSCs were differentiated into ECs and purified by fluorescence-activated cell sorting using the EC markers CD144 (for mESC-derived ECs) [41] and CD31 (for hiPSC-derived ECs) [42] as we have previously described. The hiPSC-derived ECs were maintained using a similar protocol and reagents used for the maintenance of primary ECs. The mESC-derived ECs were maintained in growth medium containing alpha minimum Eagle's medium, fetal bovine serum, penicillin/streptomycin, and β -mercaptoethanol [41].

Results

NO₅₅₀: Mechanism of Detecting NO

Oxidation reaction of the nonfluorescent NO₅₅₀ probe with NO generated a fluorescent azo compound (AZO₅₅₀; fig. 1) presumably through the stepwise generation of nitrosamine- and hydroxyhydrazine-derived compounds as previously described [39].

Detection of NO: Dependency on NO₅₅₀ Probe Concentration and Incubation Time

The ability of NO₅₅₀ to detect oxidized NO in ECs was studied over a range of probe concentrations. We found that at a final probe concentration of 50 μ M, a modest intracellular fluorescence signal was observed as early as 60 min after addition of the probe and was progressively enhanced when the probe concentration or incubation time was increased (fig. 2). Importantly, despite the increase in intracellular fluorescence intensity upon incubation of the probe with the cells over time, there was negligible change in background fluorescence when the probe was incubated with media only in the absence of cells (online suppl. fig. S1; for all online supplementary material, see www.karger.com/doi/10.1159/000356445). When ECs were incubated with the NO₅₅₀ probe at concentrations >100 μ M, cytotoxicity and/or enhanced background fluorescence was observed (data not shown).

In addition, the probe was also successfully used to image oxidation products of NO in primary fibroblasts, and ESC- as well as iPSC-derived ECs (online suppl. fig. S2). Interestingly, hiPSCs showed NO production preferentially at the border of colonies (online suppl. fig. S3); these border cells are known to be susceptible to spontaneous differentiation compared with cells in the center of colonies. Such cells may have begun to express functional NOS [43]. Our RT-PCR and Western blot data also show that only the differentiated cells (ECs and iPSC-derived ECs) but not the iPSCs express eNOS (online suppl. fig. S4).

Fig. 2. a–d Fluorescence microscopy demonstrating the imaging of NO produced by HMVECs over a range of probe concentrations 2 h after loading the cells with 50 (**a**), 60 (**b**), 75 (**c**) and 100 μM (**d**) of NO_{550} probe. **e–h** Fluorescence microscopy showing the

detection of intracellular NO generated by HMVECs over time after loading the cells with 75 μM of NO_{550} probe for 30 min (**e**), and 3 (**f**), 5 (**g**) and 24 h (**h**).

Fig. 3. Stimulation of HMVECs with DMSO vehicle (**a**) or pharmacological agents: ACh (10 μ M; **b**) or the NO donor SNAP (10 μ M; **c**). NO₅₅₀ (60 μ M) was added at the time of treatment with these agents. **d** Fluorescence intensity quantification is expressed as arbitrary units (AU; means $\pm$ SEM; * $p < 0.05$ vs. vehicle).

Stimulation or Inhibition of NO Production

In addition to imaging physiological levels of oxidized NO produced intracellularly, cells were also treated with various agents that promote or inhibit NO levels. Treatment of ECs with ACh (which activates NOS) and the NO donor SNAP (which releases NO) increased fluorescence intensity (fig. 3). Treatment of the cells with inhibitors of NO production such as L-NAME and allopurinol blunted the fluorescent signal compared to the vehicle control (fig. 4) consistent with the hypothesis that the probe was detecting NOS- and XOR-dependent production of NO. However, the fluorescent signal was only completely antagonized when the NO radical scavenger PTIO [44, 45] was added (fig. 4c), suggesting the existence of alternative NO sources that are insensitive to NOS/XOR inhibition. This finding was corroborated by an established Griess assay quantifying NO levels under these conditions (fig. 5). Similarly, we found that cytokine-mediated induction of NO signaling could be effec-

tively abolished by cotreatment with the NO scavenger PTIO (online suppl. fig. S5).

Compartmentalization of NO₅₅₀

We studied the localization of NO₅₅₀ in intracellular compartments using organelle-labeling fluorescent dyes. Simultaneous labeling of the nuclei and acidic organelles such as the lysosomes in NO₅₅₀-stained cells revealed that despite its vesicle-like staining, NO₅₅₀ only partly appears to emanate from this compartment (fig. 6). Labeling of the ER with a fluorescent dye that binds to sulfonylurea receptors (subunits of the ATP-sensitive K⁺ channels that are abundantly expressed in the ER) indicated that the uptake and/or trafficking of NO₅₅₀ was impaired in the majority of cells stained with the ER-Tracker dye (fig. 6).

Cytotoxicity of NO₅₅₀

We estimated the degree of in vitro cytotoxicity of the NO₅₅₀ probe by counting viable cells and biochemically

Fig. 4. Treatment of HMVECs with NOS/XOR inhibitors and NO scavenger. **a** Vehicle. **b** L-NAME + allopurinol (100 μ M each for 24 h). **c** L-NAME + allopurinol + PTIO. **d** PTIO (10 μ M for 1 h). The final concentration of NO₅₅₀ loaded was 75 μ M.

by an ELISA-based LDH release assay. These studies revealed that NO₅₅₀ could be used to image NO produced by primary vascular ECs when cells are incubated at relatively high probe concentration for at least 4 h (online suppl. fig. S6). Similarly, ATP production studies following the loading of astrocytes and a neuronal cell line with NO₅₅₀ also found no differences in the metabolic activity of NO₅₅₀-loaded cells compared to controls [39].

Imaging of NO with NO₅₅₀ Compared to DAF-FM

Our direct comparison of NO₅₅₀ with a commercially available and relatively optimized fluorophore for NO imaging, DAF-FM diacetate, indicated that while NO₅₅₀ has minimal extracellular fluorescence, DAF-FM diacetate produced very bright fluorescence intensity even when used at 75-fold lower concentration (fig. 7). Meanwhile, our attempt to reduce the background signal and nuclear entry associated with DAF-FM diacetate by varying the incubation temperature (from 37 to 4°C), incubation

time (from 60 to 10 min), and probe concentration was not successful. These results suggested that some inherent nonspecific reaction(s) and/or spontaneous removal of the acetyl groups in the starting reagent might have yielded the relatively more fluorescent intermediate product, DAF-FM [46].

On the other hand, despite its weaker fluorescence intensity, the signal produced by the NO₅₅₀ probe was punctate and cytoplasmic in its distribution, consistent with the extranuclear localization and particulate association of NOS (fig. 7; online suppl. fig. S7).

Discussion

The NO pathway is centrally involved in endothelial homeostasis as well as many other cellular processes. As a messenger involved in several of these physiological and pathological conditions, NO is a profoundly important

Fig. 5. The effect of pharmacological agents on NO production. HMVECs were treated with vehicle, L-NAME/allopurinol (100 µM each; 24 h), PTIO (10 µM; 1 h) or the L-NAME/allopurinol/PTIO combination. Total nitrite (NOx) was measured using the Griess reaction. Means ± SEM from duplicate experiments. * $p < 0.05$ vs. vehicle control.

signaling molecule. Unfortunately, due to the exquisitely short half-life of NO [47], lack of selective reagents and robust techniques to directly measure its concentration, and the requirement of specialized instrumentation by some of the developed techniques, the degree and distribution of intracellular NO under basal and stimulated conditions has been a challenge. In addition, since many of the reported fluorophores permeate into the nuclear membrane of cells, they likely interfere with DNA replication and cell survival, limiting longer kinetic studies when the cells are loaded with these imaging agents. Recently, Yang et al. [39] developed and characterized a novel imaging probe (NO₅₅₀) by applying a technique that yields a fluorescent product (AZO₅₅₀) from the oxidative reaction of a nonfluorescent probe (NO₅₅₀) with NO. The probe was able to react with bubbled NO gas in solution and with oxidation products of endogenously produced NO in neuronal cells and astrocytes while showing no pH dependency over a biologically relevant range (pH 4.5–9). In the present study, NO₅₅₀ is further characterized by demonstrating its application in the imaging of NO in vascular ECs. Functionally, we described the utility of this probe to perform kinetic studies by demonstrating the real-time imaging of NO derivatives in cells incubated with various

concentrations of the probe over a period of time as well as in response to various pharmacological agents that enhance or inhibit NO production. However, there are some discrepancies between our findings and those of Yang et al. [39] in that the localization and distribution pattern of the fluorescent signal appears to be distinct between the studies. It is possible that such differences might have arisen as a result of the species from which the cells were derived (mouse vs. human cells), developmental stages of the cells used (neonatal vs. adult), the cell types studied (astrocytes vs. vascular ECs), and the dominant NOS isoforms expressed by these cells (neuronal NOS vs. eNOS) as well as the mechanism by which NO production was stimulated (cytokines and nerve growth factor vs. vasoactive pharmacological agents). Further studies are warranted to investigate these technical and/or biological differences with respect to the use of NO₅₅₀.

In our study, we found that the probe has a unique extranuclear distribution but generates very weak signal intensity requiring the need for longer incubation and/or high probe concentrations compared to established fluorophores such as DAF-FM diacetate. In addition, we found that the reacted fluorescent product (AZO₅₅₀) is biochemically stable in primary vascular ECs, which indicates its potential as an imaging agent in longer kinetic studies of agents that may regulate vascular function. Interestingly, our comparative study of chemically synthesized AZO₅₅₀-loaded cells with NO₅₅₀-loaded cells indicated that NO₅₅₀ has a unique and limited distribution pattern compared with its fluorescent product AZO₅₅₀ (online suppl. fig. S8) suggesting that the nonfluorescent NO₅₅₀ may not be able to effectively diffuse into some NO-producing intracellular compartments.

Furthermore, our follow-up of NO₅₅₀-loaded cells over time demonstrates that a progressively increasing fluorescence signal is observed (e.g. between 1 and 24 h) in live cells, suggesting that unreacted probes in the culture may still be able to react with newly generated NO.

Treatment of cells with the NOS activator ACh increased fluorescence intensity whereas treatment with the inhibitors of NOS (L-NAME) or XOR (allopurinol) diminished the fluorescence signal, consistent with expected changes in NO synthesis [48–50]. The lack of complete inhibition of the fluorescence signal by L-NAME and allopurinol may reflect the presence of NO stores, such as nitrosothiol and nitrosoheme moieties [51–53]. Such stores have been shown to exist in ECs and would not be sensitive to transient inhibition by allopurinol or L-NAME as in this study. Indeed, we demonstrated that the NOS-/XOR-insensitive fluorescent signal can be effec-

Fig. 6. Compartmentalization of NO_{550} . HMVECs were costained with NO_{550} (green), Hoechst (blue) and LysoTracker (red; **a**) or ER-Tracker (red; **b**). Colors are shown online.

Fig. 7. Comparison of NO_{550} with DAF-FM diacetate. HMVECs were loaded with NO_{550} (75 μM ; **a**) or DAF-FM diacetate (1 μM ; **b**). Images were captured 1 h after NO_{550} loading and 20 min after loading with DAF-FM diacetate. Excess DAF-FM diacetate probe was washed off in PBS (pH = 7.4). Colors are shown online.

tively eliminated when an NO scavenger is present. For example, the small molecule PTIO scavenges NO by forming a stoichiometric radical-radical interaction [44]. Cultured ECs generate less NO with ACh stimulation than with agents that activate the inducible isoform of NOS [54]. Indeed, our data show that induction of the inducible isoform of NOS using the cytokine TNF- α robustly stimulated NO production and enhanced the fluorescent signal.

Our comparison of NO_{550} to the commercially available and widely used fluorescent NO probe, DAF-FM diacetate, indicated that NO_{550} has some significant advantages. This probe provided minimal background outside

cells and showed distinct cytoplasmic and perinuclear punctuate fractions without crossing the nuclear membrane. In contrast, DAF-FM diacetate displayed significant extracellular background and permeation into the nuclei, as demonstrated in a previous report using DAF-2 diacetate [39]. Since there is no concrete evidence that NO is physiologically present in the nuclei of human ECs, the presence of a signal with DAF-FM diacetate dye in the cell nuclei is likely a nonspecific reactivity of the dye. On the other hand, several reports have identified the existence of active eNOS in multiple extranuclear compartments in ECs, including in plasmalemmal caveolae, Golgi bodies [55], and the cytoskeleton [56, and the refer-

Table 1. Comparison of fluorophore-based imaging agents for NO

Detection agent	Limitation	Turn ON signal (fold)	Ex/Em, nm	Detection limit, nM	pH dependency	Ref.
DAF-2	Permeates into nuclei; high background; phenol red, Ca ²⁺ /Mg ²⁺ interferences; photostability	184 ^a	495/515	5	Less efficient below pH 7 and above pH 7.5	28, 39, 61, 62
DAN reagent	Not highly reactive to NO; inefficient at alkaline pH; hemoglobin, phenol red and BSA interfere with signal	ND	375/415	10–30	Effective at neutral pH	63, 64
DAN-1 EE	Potential permeation out of cells	300 ^a	365/445	N/D	Demonstrated in neutral pH only	29
DAR-4M	DHA and Ca ²⁺ interfere with signal; high background	840	560/575	7	No dependency above pH 4	30, 61
DAF-FM	Photobleaching; permeates into nuclei; autofluorescence	160	495/515	3	Effective above pH 6	46, 65, this study
BODIPY	Not demonstrated to detect cellular NO	370 ^a	495/515	0.8 ^b	Independent at pH 3–13	32, 33
CuFL	Permeates into nuclei; potentially reactive to S-nitrosothiols	290 ^a	503/530	5	Demonstrated at pH 7 only	34
DACs	Weak signal	14	750/850	N/A	Slightly sensitive to pH above 6	36
SWNTs	Extended probe incubation (12 h) prior to imaging; oxidative species interfere with signal	ND	658/850	70	Only demonstrated at neutral pH	38
QDs	Not demonstrated in cells; weak signal; excitation/emission overlap; low sensitivity	10	595/595	3,000	Not reported	37
NO ₅₅₀	Significant serum-binding; rapid elimination after washing loaded cells; low hydrophilicity	1,500	470/550	30	No pH dependency between 4.5 and 9	39, this study

DHA = Dehydroascorbic acid; Ex/Em = excitation/emission; N/D = not determined.
^a Calculated based on the provided quantum yield of fluorescence in the respective studies.
^b Calculated based on the relative yield comparison with DAF-2.

ences therein]. Although NO₅₅₀ has an advantage over other probes in that it provides a signal that is localized to compartments that are expected to generate NO, it does have some flaws. Its poor water solubility, low sensitivity (by comparison to DAF-FM i.e. 30 vs. 3 nM; table 1), and potential reactivity with oxidation products of NO such as nitrates (NO₃), nitrites (NO₂), peroxynitrite (OONO⁻), nitrosoheme and/or nitrosothiols (R-SNO) in cells are weaknesses that may need to be addressed with second-generation analogues.

Interestingly, we also found that physiological (PBS; pH 7.4) rinsing of cells loaded with the NO₅₅₀ probe resulted in immediate disappearance of the fluorescent signal from subcellular compartments. This rapid elimination of the signal by a simple wash step suggests that the probe might largely be localized in the caveolae. Caveolins are ubiquitously expressed membrane proteins known to form the caveola membrane of mammalian cells in order

to allow internalization and trafficking of exogenous molecules independent of surface receptors. Caveolin-1 is primarily expressed in vascular smooth muscle cells, fibroblasts and ECs, and is well known to interact with eNOS and influence NO production [56–58].

This observation warrants further mechanistic studies to delineate the site(s) of interaction and precise subcellular compartmentalization of the NO₅₅₀ probe. In the meantime, our colocalization study using cell-permeant organelle-specific dyes to label the lysosomes indicates that NO₅₅₀ does not appear to be emitted from these acidic compartments. To our knowledge, there is no scientific evidence demonstrating the presence of NO in these vesicles in ECs under baseline conditions. In addition, our compartmentalization study of NO₅₅₀ in conjunction with an ER-tracking dye caused progressively increasing cell death and reduced NO₅₅₀-associated fluorescence intensity suggesting that either the active drug (gliben-

clamide) conjugated to the ER-Tracker dye has impaired the ability of the cells to actively uptake NO₅₅₀ probe [59] or ER stress, which is known to impair NO production, has been induced [60].

Finally, this probe might be useful for conducting large-scale kinetic studies to profile the effect of drugs on NO release and vascular function as well as for diagnostic use of NO-related diseases and for performing high-throughput drug screening in search of novel small molecules that regulate NO provided that the selectivity, hydrophobicity, serum binding, and brightness issues could be optimized by a series of organic chemical studies.

In conclusion, we have evaluated the utility of the NO₅₅₀ probe in imaging NO produced by primary vascular ECs and ECs derived from PSCs. In addition, we demonstrated that NO₅₅₀ is reasonably stable in cells and is able to discriminate changes in NO in response to treatment of cells with pharmacological agents that increase or reduce NO levels. Finally, we found that the NO₅₅₀ probe has a unique distribution pattern in subcellular compartments that is limited to perinuclear areas compared to the commercially available DAF-FM diacetate probe.

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Disclosure Statement

J.P.C. and Y.T.G. are inventors of a patent owned by Stanford University that protects the use of agents that modulate the NOS pathway for therapeutic use.

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