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Development of a Ruthenium(II) Complex Based Luminescent Probe for Imaging Nitric Oxide Production in Living Cells

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Abstract: A unique ruthenium(II) complex, bis(2,2′-bipyridine)(4-(3,4-diaminophenoxy)-2,2′-bipyridine)ruthenium(II) hexafluorophosphate ([(Ru$(bpy)_2$(dabpy)][PF_6]$_2$), has been designed and synthesized as a highly sensitive and selective luminescence probe for the imaging of nitric oxide (NO) production in living cells. The complex can specifically react with NO in aqueous buffers under aerobic conditions to yield its triazole derivative with a high reaction rate constant at the 10^{10} $M^{-1}s^{-1}$ level; this reaction is accompanied by a remarkable increase of the luminescence quantum yield from 0.13 to 2.2%. Compared with organic probes, the new Ru^{II} complex probe shows the advantages of a large Stokes shift (>150 nm), water solubility, and a wide pH-availability range (pH independent at pH>5). In addition, it was found that the new probe could be easily transferred into both living animal cells and plant cells by the coincubation method, whereas the triazole derivative was cell-membrane impermeable. The probe was successfully used for luminescence-imaging detection of the exogenous NO in mouse macrophage cells and endogenous NO in gardenia cells. The results demonstrated the efficacy and advantages of the new probe for NO detection in living cells.

Keywords: analytical reagents · imaging agents · luminescence · nitric oxide · ruthenium

Introduction

Nitric oxide (NO), a highly reactive, ubiquitous, and paramagnetic free-radical molecule with an unpaired electron in the π* orbital, is biosynthesized in organisms and exerts diverse physiological and pathological effects by reacting rapidly with free radicals and metal-containing proteins in animals, plants, fungi, and bacteria. In animal systems, NO molecules are endogenously generated from L-arginine by nitric oxide synthase (NOS). It is known that NO at low concentrations plays an important role as a intra- and intercellular signaling molecule in the cardiovascular, immune, and nervous systems,[1] whereas at a high level it can react with various reactive oxygen species (ROS) to form reactive nitrogen species (RNS),[1c,f,2] which cause damage to DNA, lipids, and proteins. In plant systems, there are two types of process that are known to produce NO, enzyme (nitrate reductase and NOS) and nonenzyme ones.[3] It has been reported that NO is implicated in many key physiological processes in plants, including growth regulation, cell differentiation, stomatal closure, phytoalexin accumulation, and plant responses against a variety of abiotic stress factors, such as wounding, salinity, drought, and hypoxia.[3b–e,4]

To understand the complicated functions of NO in living systems, a number of methods have been developed to monitor endogenous NO, including electron paramagnetic resonance spectroscopy[5] and colorimetric,[6] fluorometric,[5d,7] electrochemical,[8] and chemiluminescence techniques.[9] Of these methods, the fluorometric assay with a specific fluorescence probe is considered to be one of most promising methods for the detection of NO production at cellular level, due to its high sensitivity and selectivity. Most NO-specific fluorescence probes are designed by linking organic fluorophores to an electron-rich *o*-diaminophenyl group that can effectively quench the fluorescence of the fluorophores by a process known as photoelectron transfer (PET).[7] When the *o*-diaminophenyl group of the probe reacts with NO-derived by-products such as N_2O_3 formed by the autooxidation of NO, a strongly fluorescent benzotriazole derivative can be formed. Based on this strategy, a number of fluorescence probes for NO have been synthesized and applied to NO detection.[7] Among them, 4,5-diaminofluorescein diacetate (DAF-2-DA)[7a] and 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-DA)[7b] are the

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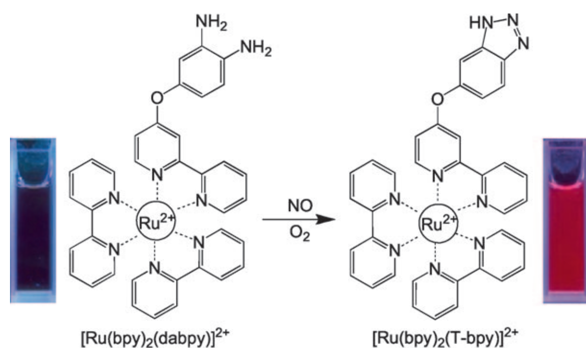
most successful probes for NO detection in living systems. These probes are cell-membrane permeable and can be deacetylated by intracellular esterases to form 4,5-diaminofluorescein (DAF-2) and 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) in the cells. However, some characteristics of the organic probes are undesirable, such as lower photostability and pH-dependent fluorescence. The small Stokes shift of an organic fluorescence probe is also unfavorable because it can cause self-quenching and measurement errors by excitation and scattered lights.[10]

An increasingly important class of luminescent sensing materials is the transition-metal complexes, especially those of Ru^{II}, Re^{I}, and Ir^{III}.[11] As luminescent bioprobes, Ru^{II} complexes have several very desirable features, including intense visible excitation and emission, a large Stokes shift, high photo-, thermal, and chemical stabilities, and very low cytotoxicity.[12] Owing to these outstanding photochemical and photophysical properties, a variety of sensitive and specific luminescence probes based on Ru^{II} complexes, especially the Ru^{II}–bipyridine complex, have been developed in recent years for the detection of anions,[13] metal cations,[14] and molecular oxygen.[15] However, Ru^{II} complexes that can be applied to monitor the behavior of reactive small molecules in living species have rarely been reported.[16] In this work, a novel Ru^{II} complex based luminescence probe that is specific for NO, $[Ru(bpy)_2(dabpy)][PF_6]_2$ (bpy: 2,2′-bipyridine; dabpy: 4-(3,4-diaminophenoxy)-2,2′-bipyridine), was designed and synthesized. In the complex, the 3,4-diaminophenyl group was used as a specific reactive moiety for NO, and the Ru^{II}–bipyridine complex acts as a fluorophore. This complex is almost nonluminescent due to the strong luminescence quenching effect of the 3,4-diaminophenyl moiety in the ligand, and it can specifically react with NO under aerobic conditions to form the highly luminescent triazole derivative $[Ru(bpy)_2(T\text{-}bpy)]^{2+}$ (T-bpy: 4-(triazolephenoxy)-2,2′-bipyridine), with a remarkable luminescence enhancement (Scheme 1). To the best of our knowledge, this is the first Ru^{II} complex based luminescence probe for ROS.

The luminescence intensities of $[Ru(bpy)_2(dabpy)]^{2+}$ and $[Ru(bpy)_2(T\text{-}bpy)]^{2+}$ are pH independent at pH > 5, which shows the wide pH-availability range of the probe. A strong luminescence response was only found upon reaction of $[Ru(bpy)_2(dabpy)]^{2+}$ with NO, but not with other ROS and RNS, such as hydrogen peroxide, hydroxyl radicals, hypochlorite anions, or singlet oxygen, nitrite, nitrate, peroxynitrite, and superoxide species; this indicates an extremely high specificity for NO. Furthermore, it was found that $[Ru(bpy)_2(dabpy)]^{2+}$ could be easily transferred into cultured animal and plant cells by the coincubation method, whereas $[Ru(bpy)_2(T\text{-}bpy)]^{2+}$ is cell-membrane impermeable. To demonstrate the utility of the new luminescent probe for the imaging of NO production in living systems, $[Ru(bpy)_2(dabpy)]^{2+}$-deposited mouse macrophage cells and gardenia cells were prepared and used for luminescence imaging of exogenous and endogenous NO in the cells. The results demonstrated the efficacy of the new probe for sensitive luminescence imaging of NO production in living cells and highlighted the advantages of the Ru^{II} complex based luminescence probe.



Scheme 1. Reaction of $[Ru(bpy)_2(dabpy)]^{2+}$ with NO under aerobic conditions. The photos show the luminescence colors of solutions of the two complexes under a 365 nm lamp.

Results and Discussion

Design, synthesis, and characterization of the Ru^{II} complex probe: The Ru^{II} complex luminescence probe specific for NO was designed by incorporating an NO-specific reactive moiety, *o*-diaminophenyl, into a luminescent tris(2,2′-bipyridine)ruthenium(II) ($[Ru(bpy)_3]^{2+}$) complex. Unlike the reported organic-dye-based probes, the Ru^{II}–bipyridine complex is employed as a luminescence moiety because it has some unique chemical, photochemical, and photophysical properties, such as high chemical and photochemical stabilities, intense luminescence, a large Stokes shift, visible-light excitation, red emission wavelengths, good water solubility, and low cytotoxicity. These properties make it potentially invaluable as a luminescent probe for cellular imaging. The *o*-diaminophenyl moiety, a well-known response scaffold that is specific for NO, is electron rich and can effectively quench the luminescence from the excited fluorophore ($*[Ru(bpy)_3]^{2+}$). In addition, the two moieties are bridged by an electron-donating oxygen atom, which facilitates the PET process. Thus, the probe is almost nonluminescent, but it can become highly luminescent after it reacts with NO under aerobic conditions because the triazole ring in the product is electron poor so PET can not occur.

The probe can be easily synthesized by a three-step procedure (Scheme 2). Briefly, complex **2** was the sole product of the reaction between complex **1** and 4-amino-3-nitrophenol at room temperature. After reduction with Pd/C and hydrazine hydrate, $[Ru(bpy)_2(dabpy)][PF_6]_2$ was obtained with a high yield. The corresponding triazole derivative, $[Ru(bpy)_2(T\text{-}bpy)][PF_6]_2$, was also synthesized by treating $[Ru(bpy)_2(dabpy)][PF_6]_2$ with $NaNO_2$ in hydrochloric acid. All complexes were confirmed by NMR spectroscopy, ESI-MS, and elemental analysis. The probe is stable, both in the solid state and in buffers, for at least several months.

The luminescence properties of $[Ru(bpy)_2(dabpy)]^{2+}$ and $[Ru(bpy)_2(T\text{-}bpy)]^{2+}$ are listed in Table 1. The two com-

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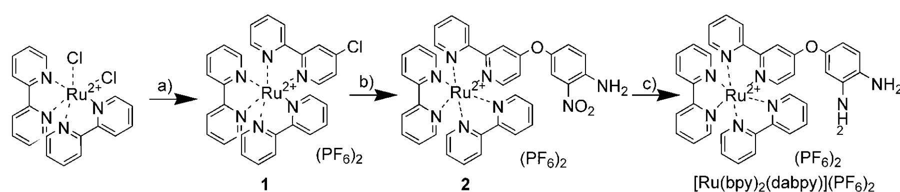

Scheme 2. Synthesis procedure for $[Ru(bpy)_2(dabpy)][PF_6]_2$: a) Cl-bpy, methanol, reflux, 6 h, 71 %; b) 4-amino-3-nitrophenol, NaH, acetonitrile, RT, overnight, 90 %; c) 10 % Pd/C, hydrazine hydrate, ethanol, reflux, 4 h, 95 %.

electron density of the 3,4-diaminophenyl group is decreased due to protonation of the amino group, which leads to luminescence enhancement of the $[Ru(bpy)_2(dabpy)]^{2+}$ complex. Unlike the organic probes, DAF-2[7a] and DAF-FM,[7b] the luminescence intensity of $[Ru(bpy)_2(T\text{-}bpy)]^{2+}$ is pH independent in the range of pH 1–13; this shows that $[Ru(bpy)_2(dabpy)]^{2+}$ can work well as an NO luminescence probe in weakly acidic, neutral, and basic buffers.

Table 1. Luminescence properties[a] of $[Ru(bpy)_2(dabpy)]^{2+}$ and $[Ru(bpy)_2(T\text{-}bpy)]^{2+}$.

Complex	$\lambda_{ex,max}$ [nm]	$\varepsilon_{455\,nm}$ [$cm^{-1}M^{-1}$]	$\lambda_{em,max}$ [nm]	ϕ[b]
$[Ru(bpy)_2(dabpy)]^{2+}$	455	13000	610	0.0013
$[Ru(bpy)_2(T\text{-}bpy)]^{2+}$	455	9540	616	0.022

[a] All data were obtained in 0.1 M borate buffer at pH 7.4 and room temperature. [b] The luminescence quantum yield was measured by using $[Ru(bpy)_3]Cl_2$ ($\phi = 0.028$)[17] as a standard.

plexes have a strong visible absorption peak at 455 nm (Figure 1) and emission peaks at 610 and 616 nm, respectively; these values are characteristic of the metal-to-ligand charge transfer (MLCT) based luminescence that is typically observed in the spectra of Ru^{II}–diimine complexes. As expected, $[Ru(bpy)_2(dabpy)]^{2+}$ itself is almost nonluminescent, with a very low luminescence quantum yield (0.13 %). Upon reaction with NO in air-saturated buffer to form $[Ru(bpy)_2(T\text{-}bpy)]^{2+}$, the complex becomes highly luminescent with a 16.9-fold increase in the luminescence quantum yield. This indicates that the 3,4-diaminophenyl moiety of the probe can effectively act as a chemically irreversible 'Off–On' switch for NO.

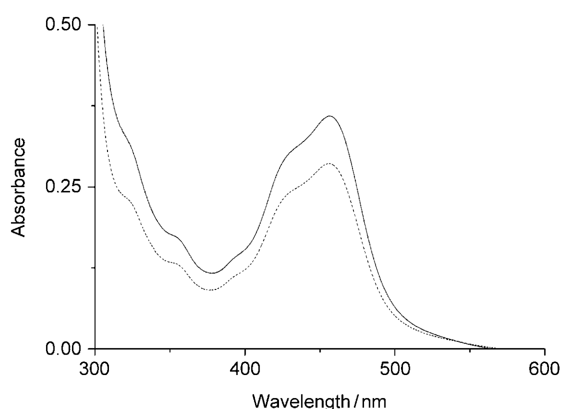

Figure 1. Absorption spectra of $[Ru(bpy)_2(dabpy)]^{2+}$ (solid line, 30 μM) and $[Ru(bpy)_2(T\text{-}bpy)]^{2+}$ (dashed line, 30 μM) in 0.1 M borate buffer at pH 7.4.

Figure 2 shows the effect of pH value on the luminescence intensities of $[Ru(bpy)_2(dabpy)]^{2+}$ and $[Ru(bpy)_2(T\text{-}bpy)]^{2+}$. The luminescence intensity of $[Ru(bpy)_2(dabpy)]^{2+}$ is weak and stable at pH > 5. When the pH value is less than 5, the

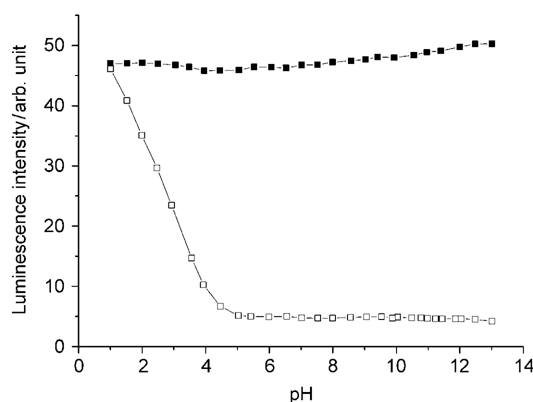

Figure 2. Effect of the pH value on the luminescence intensities of $[Ru(bpy)_2(T\text{-}bpy)]^{2+}$ (10 μM, ■) and $[Ru(bpy)_2(dabpy)]^{2+}$ (10 μM, □) in 0.1 M phosphate buffers with different pH values.

To evaluate the reaction specificity of $[Ru(bpy)_2(dabpy)]^{2+}$ with NO under aerobic conditions, the reactions of $[Ru(bpy)_2(dabpy)]^{2+}$ with different ROS and RNS were examined by using a solution of 10 μM $[Ru(bpy)_2(dabpy)]^{2+}$ in 0.1 M borate buffer at pH 7.4. As shown in Figure 3, $[Ru(bpy)_2(dabpy)]^{2+}$ did not give any observable response upon the addition of H_2O_2, $^{\bullet}OH$, OCl^-, $^1O_2$, NO_2^-, NO_3^-, $ONOO^-$, O_2^-, whereas the luminescence intensity was significantly increased after the reaction of $[Ru(bpy)_2(dabpy)]^{2+}$ with NO. This result demonstrates that the luminescence response of the $[Ru(bpy)_2(dabpy)]^{2+}$ complex is highly specific for NO.

The reaction rate constant of $[Ru(bpy)_2(dabpy)]^{2+}$ with NO was also measured in 0.1 M borate buffer at pH 7.4 under aerobic conditions. As shown in Figure 4, after addition of NO, the luminescence intensity of the complex rapidly increased and reached the maximum value within approximately 2 min. A good linearity between the NO concentration and the corresponding initial reaction rate is obtained, with a slope of $(2.7 \pm 0.1) \times 10^{-1}$ (Figure 5). The total reaction rate constant (k_{tot}) of the $[Ru(bpy)_2(dabpy)]^{2+}$/NO reaction in air-saturated buffer is calculated to be $(2.7 \pm 0.1) \times 10^{10}\,M^{-1}s^{-1}$, which indicates that the reaction of $[Ru(bpy)_2(dabpy)]^{2+}$ with NO in air-saturated buffer is remarkably

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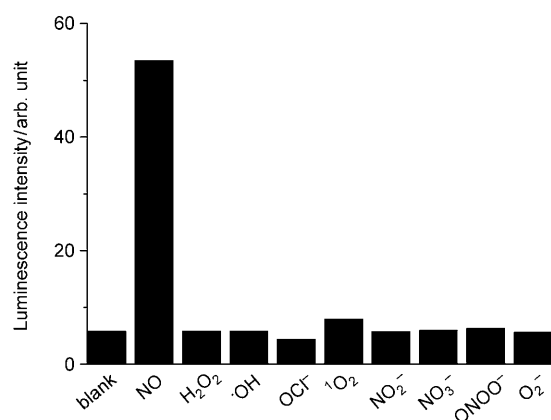

Figure 3. Luminescence intensities of the products of $[Ru(bpy)_2(dabpy)]^{2+}$ (10 μM) treated with various ROS and RNS in 0.1 M borate buffer at pH 7.4. NO: 40 μM; H_2O_2: 100 μM; ·OH: 100 μM H_2O_2 + 100 μM ferrous ammonium sulfate; OCl^-: 100 μM NaOCl; $^1O_2$: 100 μM H_2O_2 + 100 μM NaOCl; NO_2^-: 100 μM $NaNO_2$; NO_3^-: 100 μM $NaNO_3$; $ONOO^-$: 100 μM NaONOO; O_2^-: 100 μM KO_2.

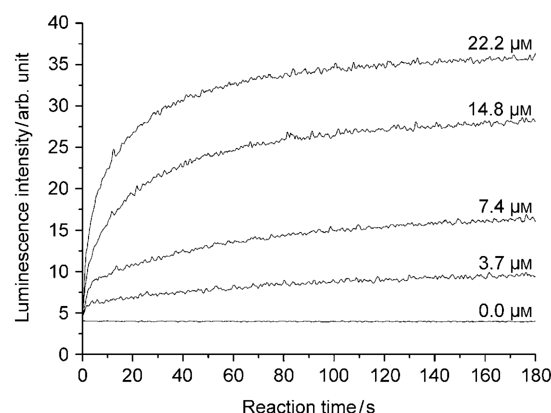

Figure 4. Reaction kinetic curves of $[Ru(bpy)_2(dabpy)]^{2+}$ (10 μM) with different concentrations of NO. The measurements were carried out on a Perkin–Elmer LS 50B luminescence spectrometer with an excitation wavelength of 455 nm, an emission wavelength of 616 nm, an excitation slit of 10 nm, and an emission slit of 5 nm.

rapid, so detection errors derived from the reactions of NO and other reactive molecules can be minimized.

Detection of NO in aqueous media: To investigate the performance of $[Ru(bpy)_2(dabpy)]^{2+}$ for the detection of NO in aqueous media, a luminescence titration experiment was conducted with an NO-saturated borate buffer and a $[Ru(bpy)_2(dabpy)]^{2+}$ solution (10 μM) in 0.1 M borate buffer at pH 7.4. Figure 6 shows the excitation and emission spectra

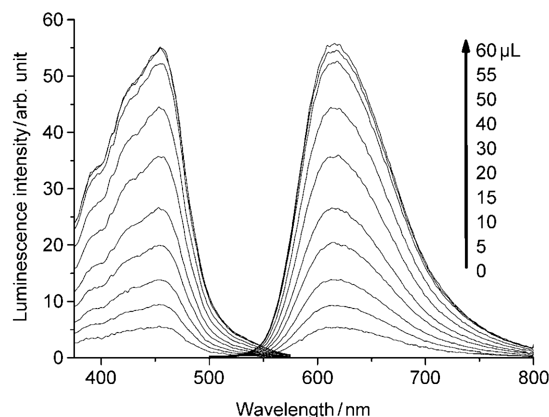

Figure 6. Excitation and emission spectra of $[Ru(bpy)_2(dabpy)]^{2+}$ treated with various amounts of saturated NO solution at room temperature in 0.1 M borate buffer at pH 7.4. The spectra were measured after the addition of the NO aqueous solution (2.2 mM; 0–60 μL) to a solution of $[Ru(bpy)_2(dabpy)]^{2+}$ (10 μM, 3.0 mL) for 30 min.

of $[Ru(bpy)_2(dabpy)]^{2+}$ treated with various amounts of NO in the air-saturated buffer. Upon addition of the NO solution, the luminescence intensity of $[Ru(bpy)_2(dabpy)]^{2+}$ is significantly enhanced, and the dose-dependent luminescence enhancement shows a good linearity (Figure 7). The detection limit for NO, calculated as the concentration corresponding to three standard deviations of the background signal, is 2.7×10^{-7} M. Although the detection limit is higher than that of DAF-2 probe (the detection limit of the DAF-2

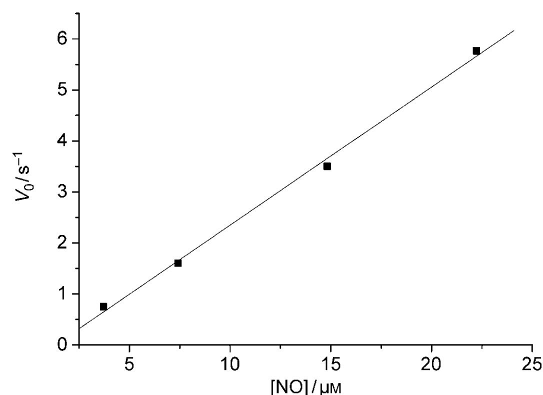

Figure 5. The plot of initial rates (V_0) of the $[Ru(bpy)_2(dabpy)]^{2+}$/NO reaction versus different concentrations of NO.

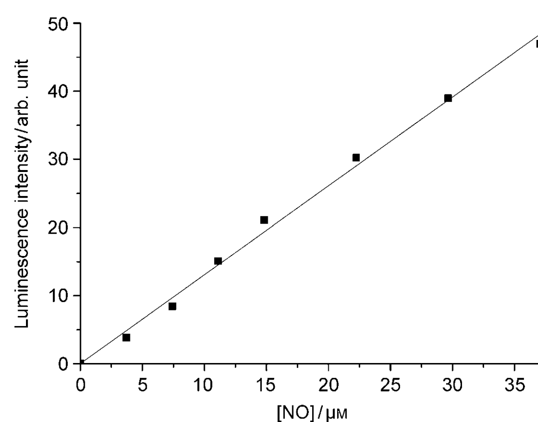

Figure 7. Calibration curve for NO detection.

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method is 5 nM),[7a] the larger Stokes shift (>160 nm; that of DAF-2 is less than 25 nm) and red emission wavelength (616 nm; that of DAF-2 is 513 nm) of the new Ru^{II} complex probe enable it to be more favorable for cell-imaging applications.

Luminescence imaging of NO production in living cells: The luminescence imaging technique is a very useful tool for in vivo and in situ detection of NO. Although several methods for NO detection have been reported in recent years, probes that can be used to monitor NO production in living cells are rarely reported. Owing to their cell-membrane permeability, low cytotoxicity, and luminescence stability in the intracellular environment, Ru^{II} complexes have increasingly been applied for luminescence cell imaging since the cellular-uptake method was explored by Puckett and Barton.[18] Unfortunately, these complexes have a disadvantage in that they can be washed out of the cells. To evaluate the availability of our probe for cell imaging, its performance for the imaging of NO production in animal and plant cells was investigated.

First, the probe was applied to the luminescence imaging of NO in cultured mouse macrophage cells. After the cells were incubated with the complex for 4 h, the complex-deposited cells were washed and further incubated with a solution containing NOC 13 (1-hydroxy-2-oxo-3-(3-aminopropyl)-3-methyl-1-triazene, an NO donor with a half-life of 13.7 min)[19] for another 0.5 h. The cells were then subjected to luminescence detection. Figure 8 shows the bright-field and luminescence images of the cells in the absence and presence of NOC 13. In the presence of NOC 13, clear red intracellular luminescence is observed, and the luminescence intensity in the cytosol is almost the same as that in the nucleus. This indicates that the new Ru^{II} complex probe can permeate through the cell membrane into the cells for in vivo luminescence imaging of NO.

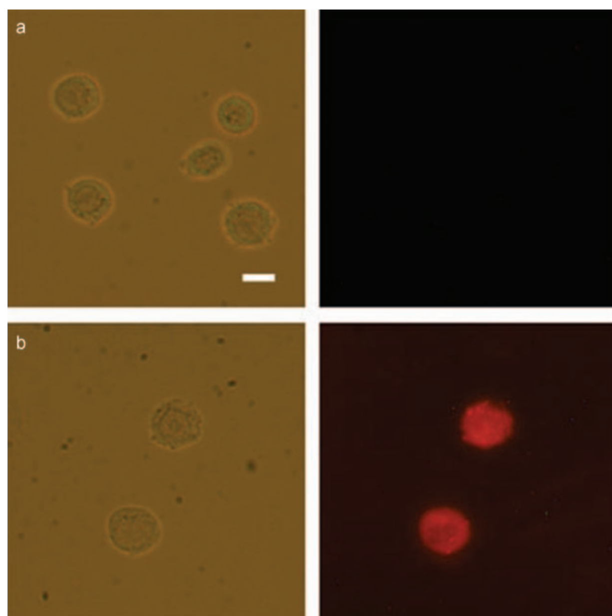

Figure 8. Bright-field (left) and luminescence (right) images of the $[Ru(bpy)_2(dabpy)]^{2+}$-deposited mouse macrophage cells in the absence (a) and presence (b) of NOC 13. The scale bar represents 10 μm.

It has been reported that the plant cells can produce substantial amounts of NO naturally when they are subjected to slightly biotic or abiotic stressor.[3c] In this work, the kinetic process of NO generation in gardenia cells with shaking was real-time monitored by using $[Ru(bpy)_2(dabpy)]^{2+}$ as a probe. As shown in Figure 9, red intracellular luminescence could be observed after the cells were incubated with $[Ru(bpy)_2(dabpy)]^{2+}$ for 1 h, and the luminescence intensity gradually increased with the increase in incubation time. This result suggests that the NO generation in the cells should be a continuous process: the longer the cells are incubated, the more NO molecules are generated. It can be also seen that the luminescence intensity in the nucleus is remarkably higher than that in the cytosol. This observation implies that NO generation mainly occurs in the nucleus region of the plant cells, which is in agreement with the reported results from the use of DAF-2 DA as the NO probe.[3a–c]

The HPLC analysis of the product extracted from the gardenia cells incubated with $[Ru(bpy)_2(dabpy)]^{2+}$ demonstrates that the luminescence surge is attributed to $[Ru(bpy)_2(T\text{-}bpy)]^{2+}$ rather than autofluorescence of the cell components or degradation products of the probe (Figure 10). In addition, no luminescence could be observed when gardenia cells were directly incubated with $[Ru(bpy)_2(T\text{-}bpy)]^{2+}$ (Figure 11), which demonstrates that the product of $[Ru(bpy)_2(dabpy)]^{2+}$ reacted with NO, $[Ru(bpy)_2(T\text{-}bpy)]^{2+}$, is cell-membrane impermeable and that the intracellular luminescence only derived from the reaction of $[Ru(bpy)_2(dabpy)]^{2+}$ with NO in the cells.

Conclusion

We have successfully developed the first Ru^{II} complex based luminescence probe specific for NO, $[Ru(bpy)_2(dabpy)]^{2+}$, by incorporating an *o*-diaminophenyl group into a luminescent Ru^{II}–bipyridine complex. The new probe can specifically and rapidly react with NO to form its triazole derivative, $[Ru(bpy)_2(T\text{-}bpy)]^{2+}$, which results in remarkable luminescence enhancement. The results of luminescence imaging to monitor the time-dependent NO production in living plant cells demonstrated the utility of the probe for the in vivo detection of NO. Compared with previously reported organic-dye-based NO probes, the advantages of this probe's high selectivity, water solubility, reaction rate constant, wide pH-availability range, and large Stokes shift suggest that it should be widely useful for the luminescence detection of NO in various chemical and biological systems.

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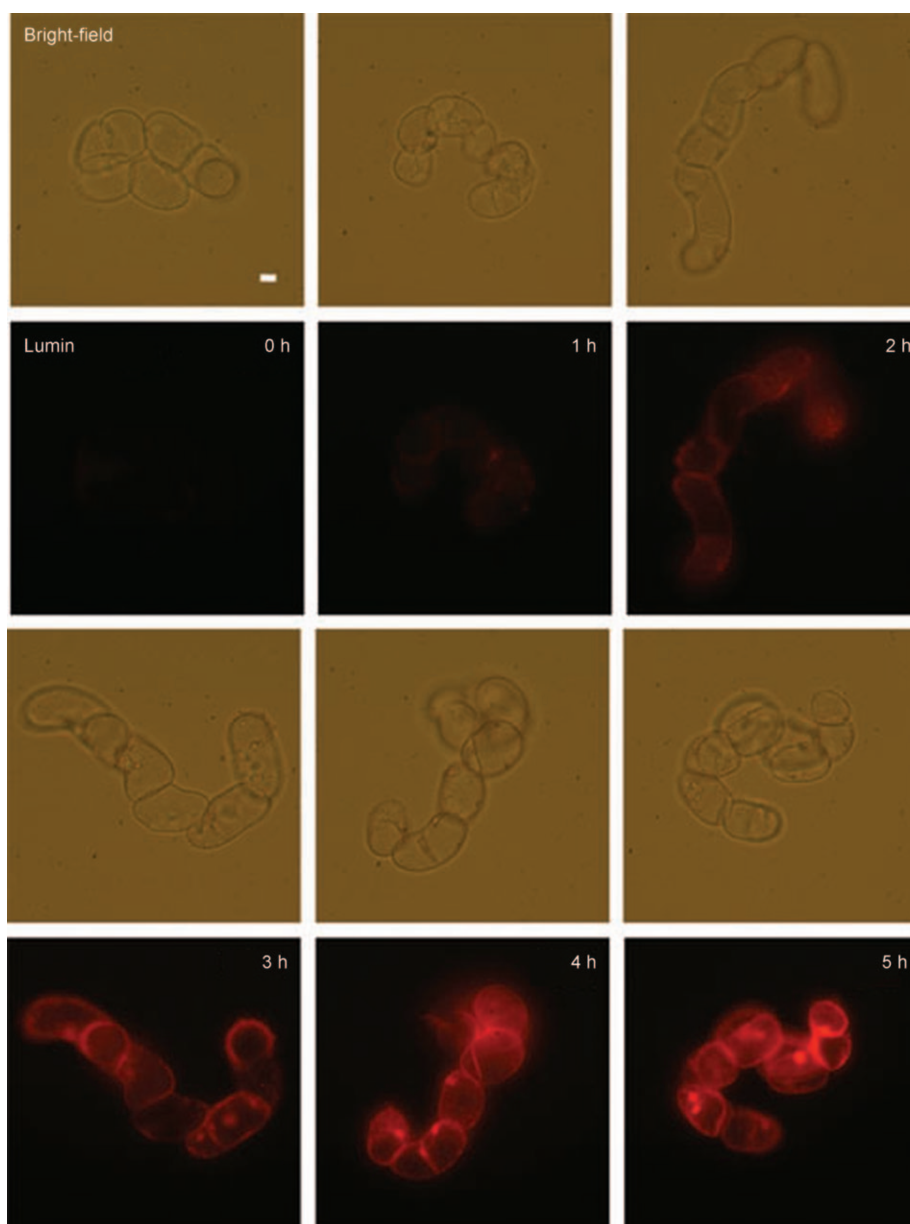

Figure 9. Bright-field and luminescence images of gardenia cells incubated with $[Ru(bpy)_2(dabpy)]^{2+}$ for different incubation times. The scale bar represents 10 μm.

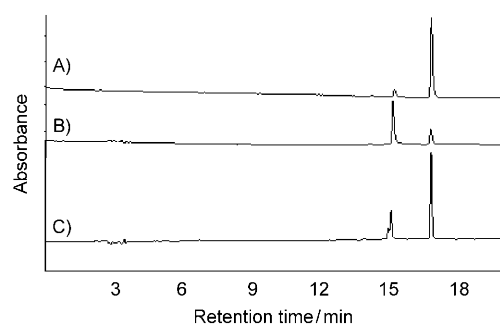

Figure 10. HPLC analysis of the product extracted from the gardenia cells incubated with $[Ru(bpy)_2(dabpy)]^{2+}$. Line A: Pure $[Ru(bpy)_2(T\text{-}bpy)][PF_6]_2$ solution (3.73×10^{-4} M); line B: the extracted solution from the $[Ru(bpy)_2(dabpy)]^{2+}$-incubated gardenia cells; line C: a mixed solution of A (15 μL) and B (85 μL).

Experimental Section

Materials and physical measurements: 4-Chloro-2,2′-bipyridine (Cl-bpy)[20] and *cis*-$Ru^{II}(bpy)_2Cl_2\cdot 2H_2O$[21] were synthesized by using the literature methods. The NO donor, NOC 13, was synthesized by using a literature method.[19] The NO aqueous solution was prepared by passing NO gas through a 0.1 M argon-deoxidized borate buffer at pH 7.4 for 3 h. The NO concentration was measured by using the Griess method.[22] The mouse macrophage cells (RAW 264.7) and gardenia cells were obtained from the Department of Biotechnology, Dalian Institute of Chemical Physics, Chinese Academy of Sciences. Unless otherwise stated, all chemical materials were purchased from commercial sources and used without further purification.

NMR spectra were recorded on a Bruker Avance spectrometer (400 MHz). ESI-MS spectra were measured on a HP1100 LC/MSD MS spectrometer. Absorption spectra were measured on a Perkin–Elmer Lambda 35 UV/Vis spectrometer. Elemental analysis was carried out on a Vario-EL analyser. Luminescence spectra were measured on a Perkin–Elmer LS 50B luminescence spectrometer with an excitation slit of 10 nm and an emission slit of 5 nm. HPLC analysis was carried out on a Sino-Chrom ODS-BP 5 μm (4.6×250 mm) column by using an HPLC system composed of two pumps (P230) and a detector (UV 230+). All bright-field imaging and luminescence imaging was carried out on a laboratory-use luminescence microscope.[23] The microscope, equipped with a 100 W mercury lamp, B-2A filters (excitation filter: 450–490 nm; dichroic mirror: 505 nm; emission filter: >520 nm), and a color CCD camera system, was used for the luminescence-imaging measurements with an exposure time of 25 s (animal cells) or 10 s (plant cells).

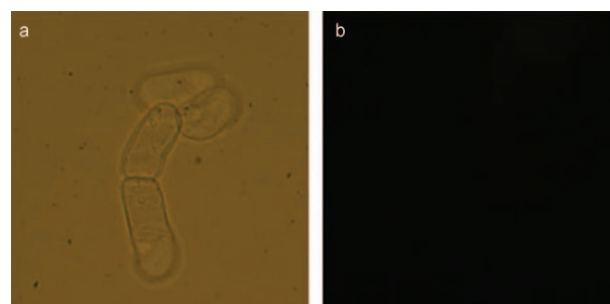

Figure 11. Bright-field (a) and luminescence (b) images of gardenia cells incubated with $[Ru(bpy)_2(T\text{-}bpy)][PF_6]_2$ for 5 h.

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Synthesis of bis(2,2′-bipyridine)(4-chloro-2,2′-bipyridine)Ru^{II} hexafluorophosphate (1): A mixture of Cl-bpy (38.1 mg, 0.2 mmol) and *cis*-Ru^{II}-(bpy)$_2$Cl$_2$·2H$_2$O (104.1 mg, 0.2 mmol) in methanol (20 mL) was heated to reflux temperature for 6 h. After the solvent was evaporated, the residue was purified by silica gel column chromatography with MeCN/H$_2$O/KNO$_3$(sat.) (100:7:0.5 v/v/v) as the eluent. The fractions containing the target product were collected, and the solvent was evaporated. The resulting solid was dissolved in a small amount of CH$_3$CN/H$_2$O (1:1), and a saturated solution of NH$_4$PF$_6$ was then added to give a red precipitate. The product was filtered and washed with small amount of water. Compound **1** was obtained (126.9 mg, 71% yield); ^{1}H NMR (400 MHz, CD$_3$CN): δ = 7.37–7.44 (m, 6H), 7.63 (d, *J*(H,H) = 6.0 Hz, 1H), 7.69–7.78 (m, 5H), 8.0 (m, 5H), 8.49 (d, *J*(H,H) = 8.0 Hz, 5H), 8.57 ppm (d, *J*(H,H) = 2.0 Hz, 1H); ESI-MS (*m/z*): 749.1 [$M-$PF$_6$]$^+$, 302.0 [$M-2$PF$_6$]$^{2+}$; elemental analysis: calcd (%) for C$_{30}$H$_{23}$ClF$_{12}$N$_6$OP$_2$Ru·H$_2$O: C 39.51, H 2.76, N 9.21; found: C 39.32, H 2.77, N, 8.77.

Synthesis of bis(2,2′-bipyridine)(4-(4-amino-3-nitrophenoxy)-2,2′-bipyridine)Ru^{II} hexafluorophosphate (2): After a mixture of 4-amino-3-nitrophenol (38.5 mg, 0.25 mmol), NaH (10 mg, 60% in purity, 0.25 mmol), and anhydrous acetonitrile (30 mL) had been stirred at room temperature for 45 min under a nitrogen atmosphere, a solution of **1** (89.4 mg, 0.1 mmol) in anhydrous acetonitrile (5 mL) was added. The mixture was stirred overnight. After the solvent had been evaporated, the residue was purified by silica gel column chromatography with MeCN/H$_2$O/KNO$_3$(sat.) (100:7:0.5 v/v/v) as the eluent. The fractions containing the target product were collected, and the solvent was evaporated. The resulting solid was dissolved in a small amount of CH$_3$CN/H$_2$O (1:1), and a saturated solution of NH$_4$PF$_6$ was then added to give a red precipitate. The product was filtered and washed with small amount of water. Compound **2** was obtained (91 mg, 90% yield); ^{1}H NMR (400 MHz, CD$_3$CN): δ = 6.66 (s, 2H, NH$_2$), 6.9 (m, 1H), 7.10 (d, *J*(H,H) = 9.2 Hz, 1H), 7.27 (m, 1H), 7.34–7.46 (m, 6H), 7.73 (m, 4H), 7.81 (d, *J*(H,H) = 5.2 Hz, 1H), 7.89 (d, *J*(H,H) = 2.8 Hz, 1H), 7.97–8.08 (m, 6H), 8.40 (d, *J*(H,H) = 8.0 Hz, 1H), 8.47–8.52 ppm (m, 4H); ESI-MS (*m/z*): 867.2 [$M-$PF$_6$]$^+$, 361.1 [$M-2$PF$_6$]$^{2+}$; elemental analysis: calcd (%) for C$_{36}$H$_{28}$F$_{12}$N$_8$O$_3$P$_2$Ru·H$_2$O: C 41.99, H 2.94, N 10.88; found: C 42.23, H 3.10, N, 10.62.

Synthesis of [Ru(bpy)$_2$(dabpy)][PF$_6$]$_2$: After a mixture of **2** (101.2 mg, 0.1 mmol), 10% Pd/C catalyst (50 mg), and ethanol (50 mL) had been stirred at room temperature for 30 min, hydrazine hydrate (15 µL) was added. The mixture was heated to reflux temperature for 4 h, and the catalyst was removed by filtration. The solvent was evaporated. The resulting solid was dissolved in a small amount of CH$_3$CN/H$_2$O (1:1), and a saturated solution of NH$_4$PF$_6$ was then added to give a red precipitate. The product was filtered and washed with small amount of water. [Ru(bpy)$_2$(dabpy)][PF$_6$]$_2$ was obtained (93.3 mg, 95% yield); ^{1}H NMR (400 MHz, CD$_3$CN): δ = 6.33 (m, 1H), 6.42 (d, *J*(H,H) = 2.4 Hz, 1H), 6.69 (d, *J*(H,H) = 8.4 Hz, 1H), 6.81 (m, 1H), 7.34–7.45 (m, 6H), 7.69–7.76 (m, 4H), 7.81 (d, *J*(H,H) = 5.6 Hz, 1H), 7.99–8.08 (m, 6H), 8.35 (d, *J*(H,H) = 8.0 Hz, 1H), 8.47 ppm (m, 4H); ^{13}C NMR (100 MHz, CD$_3$CN): δ = 108.18, 110.76, 113.41, 115.75, 117.16, 125.22, 128.56, 134.28, 138.25, 138.67, 146.47, 152.72, 153.49, 158.06, 159.12, 168.37 ppm; ESI-MS (*m/z*): 837.1 [$M-$PF$_6$]$^+$, 346.1 [$M-2$PF$_6$]$^{2+}$; elemental analysis: calcd (%) for C$_{36}$H$_{30}$F$_{12}$N$_8$OP$_2$Ru: C 44.05, H 3.08, N 11.41; found: C 43.81, H 3.42, N, 11.27.

Synthesis of [Ru(bpy)$_2$(T-bpy)][PF$_6$]$_2$: A solution of NaNO$_2$ (3.2 mg) in H$_2$O (0.5 mL) was added to a mixture of [Ru(bpy)$_2$(dabpy)][PF$_6$]$_2$ (23 mg, 0.024 mmol) in 2 M HCl (20 mL) at 0 °C. The mixture was stirred for 2 h and neutralized by 4 M NaOH. After the solvent had been evaporated, the residue was purified by silica gel column chromatography with MeCN/H$_2$O/KNO$_3$(sat.) (100:10:1 v/v/v) as the eluent. The fractions containing the target product were collected, and the solvent was evaporated. The resulting solid was dissolved in a small amount of CH$_3$CN/H$_2$O (1:1), and a saturated solution of NH$_4$PF$_6$ was then added to give a red precipitate. The product was filtered and washed with small amount of water. [Ru(bpy)$_2$(T-bpy)][PF$_6$]$_2$ was obtained (11.2 mg, 47% yield); ^{1}H NMR (400 MHz, CD$_3$CN): δ = 6.92 (m, 1H), 7.27 (d, *J*(H,H) = 8.4 Hz, 1H), 7.27–7.48 (m, 5H), 7.51 (d, *J*(H,H) = 6.4 Hz, 1H), 7.72 (m, 5H), 7.85 (d, *J*(H,H) = 5.2 Hz, 1H), 7.95–8.09 (m, 7H), 8.35 (d, *J*(H,H) = 8.0 Hz, 1H), 8.47–8.51 ppm (m, 4H); ^{13}C NMR (100 MHz, CD$_3$CN): δ = 113.72, 116.58, 125.24, 125.50, 128.55, 128.80, 129.75, 132.29, 138.71, 152.76, 153.82, 157.70, 158.09, 159.62 ppm; ESI-MS (*m/z*): 848.1 [$M-$PF$_6$]$^+$, 351.4 [$M-2$PF$_6$]$^{2+}$; elemental analysis: calcd (%) for C$_{36}$H$_{27}$F$_{12}$N$_9$OP$_2$Ru: C 43.56, H 2.74, N 12.70; found: C 43.06, H 3.02, N, 12.23.

Reactions of [Ru(bpy)$_2$(dabpy)]$^{2+}$ with different ROS and RNS: All reactions were carried out in 0.1 M borate buffer at pH 7.4 with the same [Ru(bpy)$_2$(dabpy)]$^{2+}$ concentration (10 µM) for 0.5 h at room temperature. Hydrogen peroxide (H$_2$O$_2$) was diluted immediately from a stabilized 30% solution and was assayed by using its molar absorption coefficient of 43.6 M^{-1}cm^{-1} at 240 nm.[24] Hydroxyl radicals (·OH) were generated in the Fenton system from ferrous ammonium sulfate and hydrogen peroxide.[25] Peroxynitrite was synthesized from sodium nitrite (0.6 M) and H$_2$O$_2$ (0.65 M) in a quenched-flow reactor (excess H$_2$O$_2$ was used to minimize nitrite contamination). After the reaction, the solution was treated with MnO$_2$ to eliminate the excess H$_2$O$_2$. The concentration of the ONOO$^-$ stock solution was determined by measuring the absorbance at 302 nm with a molar extinction coefficient of 1670 M^{-1}cm^{-1}.[26] Singlet oxygen was chemically generated from the $^-$OCl/H$_2$O$_2$ system in buffer.[27] Freshly prepared aqueous solutions of NaOCl, NaNO$_2$, and NaNO$_3$ were used as hypochlorite anion ($^-$OCl), nitrite (NO$_2^-$), and nitrate (NO$_3^-$) sources, respectively.

Detection of NO in aqueous media: The reaction of [Ru(bpy)$_2$(dabpy)]$^{2+}$ with NO was performed in 0.1 M borate buffer at pH 7.4 at room temperature. Various amounts of NO solution (2.2 mM; 0–60 µL) were added to buffer solution (3.0 mL) containing 10 µM [Ru(bpy)$_2$(dabpy)]$^{2+}$. The excitation and emission spectra were measured after the addition of the NO solution for 30 min.

Luminescence imaging of NO in cultured cells

Animal cells: Mouse macrophage cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, Inc.), supplemented with 10% fetal bovine serum (Corning Inc.), 1% penicillin, and 1% streptomycin (Gibco) at 37 °C in a 5% CO$_2$/95% air incubator. After the freshly prepared [Ru(bpy)$_2$(dabpy)]$^{2+}$ complex solution in dimethylsulfoxide (DMSO) had been diluted with the culture medium to a concentration of 1.0 mM (final DMSO concentration: 0.1% v/v), the cultured cells, in a 25 cm^2 glass culture bottle, were washed with the culture medium and then incubated with the above Ru^{II} complex solution (5 mL). The bottle was incubated for 4 h at 37 °C in a 5% CO$_2$/95% air incubator. The cells were washed 4 times with an isotonic saline solution consisting of 140 mM NaCl, 10 mM glucose, and 3.5 mM KCl. The cells were then further incubated with isotonic saline solution containing 1.0 mM NOC 13 for 0.5 h before being subjected to luminescence microscopy imaging.

Plant cells: Fresh gardenia cells cultured in Murashige and Skoog (MX) medium[28] were harvested by filtration of the cell suspension through a 200-mesh nickel screen and rinsed twice with distilled water. The cells were suspended in an isotonic saline solution consisting of 500 µM [Ru(bpy)$_2$(dabpy)]$^{2+}$ or [Ru(bpy)$_2$(T-bpy)]$^{2+}$, 140 mM NaCl, 10 mM glucose, and 3.5 mM KCl with a density of 3.5×10^5 cells per mL. The cell suspension was incubated at room temperature with slow shaking. At different incubation times, the cells were centrifuged for 5 min at 400 rpm at 4 °C and washed 4 times, before being spotted on a glass slide for luminescence microscopy imaging.

HPLC analysis of the product of [Ru(bpy)$_2$(dabpy)]$^{2+}$ after reaction with endogenous NO in gardenia cells: Gardenia cells (1 mL; 1.5×10^6 cells per mL) in 0.1 M phosphate buffer at pH 7.0 were incubated with [Ru(bpy)$_2$(dabpy)]$^{2+}$ (500 µM) for 5 h at room temperature. The cells were then centrifuged for 5 min at 10000 rpm and 4 °C. The precipitate was ground with liquid nitrogen and quartz sands for 3 min, and the mixture was stirred in 0.1 M phosphate buffer (1.0 mL; pH 7.0) containing 3.5 mM KCl, 140 mM NaCl, and 10 mM glucose for 30 min. After centrifugation for 5 min at 10000 rpm and 4 °C, the supernatant was subjected to HPLC analysis with aqueous CH$_3$OH (10%)/HOAc (1%) as the eluent for 5 min followed by aqueous CH$_3$OH (30%)/HOAc (1%) for 40 min. The UV/Vis detector wavelength was 460 nm.

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