

Synthesis and Photophysical Properties of 1,8-Naphthalimide-Based Dual Fluorescence Probe

Susantha K. Ganegamage

sganegamage@lamar.edu

Lamar University

Talysa Ogas

New Mexico Institute of Mining and Technology

Praveen L. Patidar

New Mexico Institute of Mining and Technology

Virginia Chang

Rochester Institute of Technology

Michael D. Heagy

Rochester Institute of Technology

Research Article

Keywords: 1,8-naphthalene diimide, cellular imaging, dual fluorescence, small molecule

Posted Date: May 26th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-9628702/v1>

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Additional Declarations: No competing interests reported.

Abstract

Mono-fluorescent probes are numerous, and their fluorescence mechanisms, photophysical characteristics, and applications are generally well established. In contrast, the design of dual-fluorescent dyes is more challenging, as excited states remain inherently difficult to predict given the milieu of solvent effects, rotational dynamics, and polarity. Consequently, relatively few such systems have been reported, and their mechanisms and applications remain limited. Here, we rationally designed a dual-fluorescent molecule, based on quantum-mechanical calculations, by combining an *N*-aryl-1,8-naphthalimide moiety with a phenyl-1,3-oxazole moiety via a triazole linker via click chemistry. This strategy produced a novel dual-emission probe, **18NIO**, via a multistep convergent synthesis. The photophysical properties, including absorption and emission behavior, as well as key biological properties, were systematically investigated. As predicted, the probe exhibited dual fluorescence emission with a large Stokes shift (> 75 nm), which minimizes self-absorption (inner filter effects), enables cleaner separation between excitation and emission bands, improves the signal-to-noise ratio, and reduces scattered-light interference. Together, these features make the probe well-suited to biological and cellular imaging applications. Furthermore, the probe showed low cytotoxicity at 8 μ M in MDA-MB-231 (triple-negative breast cancer) cells. Thus, it indicates that **18NIO** is internalized via pinocytosis, as evidenced by small vesicular structures along the cell membrane, consistent with uptake pathways typical for large hydrophobic molecules and their subsequent distribution within the cytosol.

Introduction

Monofluorescent dyes such as fluorescein, Alexa Fluor 488, Rhodamine 6G, Cy5, and DAPI are widely used in flow cytometry, confocal microscopy, and nuclear staining applications.^{1–4} Moreover, many probes designed over the past few decades have typically resulted in single-emission systems, reflecting the relative simplicity and reliability of mono-emissive probe design compared with more complex dual-emission platforms.^{5,6} Dual fluorescence is a unique photophysical feature where a single fluorescence molecule emits two color bands (wavelengths) upon single wavelength excitation and resulting in two different excited states.⁷ Compared to intensimetric probes, dual-fluorescence molecules achieve improved accuracy in quantitative detection by eliminating background interference through self-calibration via ratiometric methods, underscoring their importance for biological and environmental detection in complex matrices.^{6,8} Mono fluorescence occurs when a molecule emits from a single relaxed excited state—typically the lowest singlet state—resulting in one emission band.⁹ Dual fluorescence refers to the simultaneous emission from two independent emissive states or species within the same molecular system, typically representing a deviation from Kasha's rule.^{10,11} These emissions may originate from locally excited (LE) and intramolecular charge-transfer (ICT/TICT) states, ESIPT-generated tautomers, distinct conformational states, or aggregated species such as excimers and exciplexes, resulting in two well-resolved emission bands.^{7,12–14} We have studied *N*-aryl-naphthalimide-based dual emissive probes via changing the substitution pattern on either side of the *N*-aryl moiety and naphthalimide moiety, and evaluated their LE vs ICT dual emission behavior.¹⁵ In addition, naphthalimide

and *N*-aryl naphthalimide cores are used in lysosome-targeted probes, cancer detections, enzyme & protein activity probes, neurodegenerative disease probes, chemical and biological sensors (Zn^{2+} , thiol, reactive oxygen species detection).^{16–18} Furthermore, the heterocyclic phenyl-1,3-oxazole moiety possesses a π -conjugated, planar ring system and is commonly found in drugs, fluorescent probes, and other bioactive small molecules. Oxazole-containing scaffolds are frequently utilized in the development of anticancer agents, including tubulin polymerization inhibitors, kinase inhibitor leads, and DNA-interactive oxazole derivatives.^{19–22}

The computational calculations of electronic properties of the **18NIO** showed a prominent absorbance band observed at 320 nm, which can be attributed to $S_0 \rightarrow S_5$ (HOMO-1 $\rightarrow$ LUMO + 2) excitation, and the second weak, longer extended wavelength band at 349 nm is likely due to excitation $S_0 \rightarrow S_3$ (HOMO-2 $\rightarrow$ LUMO). (Fig. 1 and Table 1)

Table 1
Calculated vertical absorption energies (ΔE_{abs}) in eV, wavelengths (λ_{abs}) in nm, and oscillator strengths (f) of selected electronic transitions in L and its protonated forms. Experimental absorption wavelengths ($\lambda_{\text{abs}}^{\text{EXP}}$) in nm. Electronic transitions with low oscillator strengths are omitted for clarity.

Solvent	Excitation and dominant transition		f	ΔE_{abs}	λ_{abs}	$\lambda_{\text{abs}}^{\text{EXP}}$
18NIO	$S_0 \rightarrow S_1$	HOMO $\rightarrow$ LUMO	0.0000	3.18	390	
	$S_0 \rightarrow S_2$	HOMO-1 $\rightarrow$ LUMO	0.0000	3.30	376	
	$S_0 \rightarrow S_3$	HOMO-2 $\rightarrow$ LUMO	0.4577	3.56	349	350
	$S_0 \rightarrow S_4$	HOMO $\rightarrow$ LUMO + 2	0.0174	3.66	339	
	$S_0 \rightarrow S_5$	HOMO-1 $\rightarrow$ LUMO + 2	0.9707	3.87	320	310

Based on our experimental experience and computationally guided design rationale, we developed a novel dual-emission probe, 2-{4-[4-(4-{2,4-dioxo-3-azatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-3-yl}phenyl)-1*H*-1,2,3-triazol-1-yl]phenyl}-*N*-[2-(piperidin-1-yl)ethyl]-1,3-oxazole-4-carboxamide (**18NIO**) by coupling an *N*-aryl naphthalimide unit with a phenyl-1,3-oxazole moiety. The probe framework provides positions for variable amide side-chain modification, offering strong potential for future structural diversification to tune physicochemical properties and biological activity.

The synthesis commenced from commercially available 4-aminobenzamide **1**, which was transformed into oxazole ester **2**, via tert-butyl nitrite-mediated azidation and a modified Blümlein–Lewy oxazole synthesis with ethyl bromopyruvate, followed by base-mediated ester cleavage and amidation with 1-piperidineethanamine shown in Scheme 1.²² The synthesis of alkyne moiety was achieved via condensation dehydration reaction with corresponding 1,8-naphthalic anhydride **5**, with 4-ethynylaniline in pyridine medium according to the reported procedure shown in Scheme 1.²³ The azide **3** and alkyne **5**

moieties were then coupled with copper-catalyzed click coupling reactions (CuAAC) at standard optimum coupling conditions to achieve the probe **18NIO** for initial photophysical property investigation.²² Prior to photophysical characterization, the probe was subjected to extensive purification to obtain HPLC-grade material with > 99% purity (Figure S1).²⁴

As predicted, probe **18NIO** displayed dual emission at 371 nm and 425 nm upon excitation at 310 nm in DMSO medium. The dual-emission property is primarily attributed to the *N*-aryl naphthalimide (alkyne moiety) investigated by our research group, and described by the seesaw photophysical model.^{25–27} As shown in the above Fig. 2, the Stokes shift of the probe is 61 nm and 115 nm with respect to the SW and LW emission, which is higher than the Stokes shift of BRACO-19 (75 nm).²⁸ Having a larger spectral shift and greater photostability are beneficial for biological applications, as they enable capture of the emission wavelength without interference from the excitation source or minimize fluorescence from the complex cellular matrix.²⁹

Table 2
Fluorescence lifetime of **18NIO**. Multi-exponential fitting in the form: ax values are the relative amplitudes (%); $ax = A_x \times t_x$
Lifetimes, t_x , are given in units of picoseconds (ps)

Cpd.	α_1 (%)	τ_1 (ps)	α_2 (%)	τ_2 (ps)	Lifetime (ps)
18NIO	99.19	9.56	0.81	1749.91	23.7
Azide	9.48	88.365	90.52	729.91	669.1
Alkyne	40.05	92.58	59.95	815.65	526.1

As separate chromophores the azide and the alkyne exhibited molecular quantum yields of fluorescence at ($\phi = 0.0383$) and ($\phi = 0.00148$), respectively. Upon combining them together, the **18NIO** probe produced a quantum yield of 0.00119 in DMSO medium. The fluorescence lifetime of probe **18NIO** showed bi-exponential decay with larger, more extensive and smaller lifetime components ($\tau = 1.75$ ns and 9.56 ps) shown in Table 2.

The cytotoxicity of **18NIO** is 8 μ M against MDA-MB-231 triple-negative breast cancer cells shown in Fig. 3. According to the literature, thiazole orange shows a much lower cytotoxic effect, with a reported value of 590 μ M against PC3 human prostate cancer cells, whereas BRACO-19, a DNA G-quadruplex-targeting probe, exhibits substantially higher cytotoxicity, with a reported value of 2.5 μ M against the UXF1138L human uterine carcinoma cell line.³⁰ Lower cytotoxicity is advantageous because the compound is primarily intended for optical detection rather than ligand-based therapeutic activity.^{31, 32}

Figure 3: Confocal microscopy images of fixed MDA-MB-231 cells stained with SYBR Green as nuclear stain (blue) and 10 μ M **18NIO** (green) showing cytoplasmic localization.

Upon treatment of the MDA-MB-231 cells with 10 μ M of compound for 24 h, the imaging compound was done at both $\lambda_{\text{ex}}/\lambda_{\text{em}} = 401/422$ and $\lambda_{\text{ex}}/\lambda_{\text{em}} = 497/520$. Confocal microscopy imaging with the nuclear stain **DAPI** revealed that the compound (**18NIO**) penetrates the cell membrane and selectively localizes in the cytoplasm, specifically avoiding the nucleus and showing minimal to no nuclear accumulation as shown in Fig. 3.

Discussion

Figure 4 displays a zoomed in perspective of the merged images and indicates that **18NIO** undergoes pinocytosis based on the smaller vesicle spots along the cell membrane.^{33, 34} Because pinocytosis vesicles are much smaller than those generated by phagocytosis, such images are consistent with cellular mechanisms involving large hydrophobic molecules and distribution throughout the cytosol via large vacuoles.³⁵ With only a single piperidine unit susceptible to protonation, inclusion of **18NIO** into the nuclear component is less likely. Cytoplasmic localization reduces the risk of direct DNA interaction and associated genotoxic effects, making the probe potentially safer for live-cell imaging and repeated measurements, which further explains the cytotoxicity results. A cytoplasm-targeted probe is highly valuable for monitoring cellular morphology, metabolic activity, organelle distribution, and intracellular transport processes without perturbing nuclear function. In addition, selective exclusion from the nucleus improves spatial contrast between nuclear and cytoplasmic regions, enabling clearer compartmental imaging and more reliable co-staining with established nuclear dyes such as DAPI. Therefore, the compound demonstrates strong potential as a useful and less invasive cytoplasmic imaging probe for biological and mechanistic fluorescence studies.

The novel free probe exhibited dual emission bands at 371 nm and 425 nm, with relatively large Stokes shifts of 61 nm and 115 nm, respectively, in DMSO medium, as shown in Fig. 2. These values are higher than that of the well-known mono-fluorescent G4 probe BRACO-19 reported in the literature, which exhibits a Stokes shift of 75 nm.³⁶ The larger Stoke shift facilitates detection by reducing stray-light interference from the excitation source. The absorbance and emission data provide experimental evidence supporting our hypothesis that the dual fluorescence arises from the alkyne moiety via the seesaw photophysical phenomenon. Preliminary confocal microscopy showed that the probe emits two well-separated emission bands upon cellular infiltration. As predicted, the probe exhibited biexponential decay, attributable to two exciting states.

Materials and Methods

General considerations

All moisture-sensitive reactions were conducted in oven-dried glassware under a dry nitrogen or argon atmosphere. Reaction solvents (CH_2Cl_2 , Et_2O , THF) were dried and degassed by passing through a column of activated alumina in a solvent purification system. N-methyl morpholine was distilled from CaH_2 . Dimethyl formamide (DMF) was dried over activated 4 Å molecular sieves and distilled under a

high vacuum. All other solvents and reagents were purchased from commercial suppliers and used as received unless otherwise specified. Thin layer chromatography (TLC) was performed with glass plates precoated with silica 60 Å F-254 (250 mm) and visualized by UV light, KMnO_4 , phosphomolybdic acid, anisaldehyde or Cerium Ammonium Molybdate stains. ^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR spectra were recorded using a 400 MHz Bruker instrument working at a frequency of 400 MHz for ^1H and 100 MHz for $^{13}\text{C}\{^1\text{H}\}$. Chemical shifts are reported in ppm using residual solvent resonances as internal reference (δ 7.26 and δ 77.0 for ^1H and $^{13}\text{C}\{^1\text{H}\}$ in CDCl_3 , δ 2.50 and δ 39.51 for ^1H and $^{13}\text{C}\{^1\text{H}\}$ in $\text{DMSO-}d_6$). ^1H NMR data are reported as follows: b = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Coupling constants are given in hertz. The purity of all compounds and synthetic intermediates was judged to be 95% or better based on ^1H NMR or RP-HPLC. IR measurements were performed in a Nicolet FTIR on thin films. High-resolution mass spectrometry analyses were conducted at the University of New Mexico Mass Spectrometry facility.

Ethyl 2-(4-azidophenyl)oxazole-4-carboxylate (2) was prepared according to a literature procedure.²² 4-aminobenzamide **1** (5.0 g, 36.72 mmol, 1.0 equiv.) was suspended in THF (300 mL) and cooled to 0°C. Then conc. HCl (18 mL) was added dropwise, followed by *t*-BuONO (12.0 mL, 91.80 mmol, 2.5 equiv.). The reaction was stirred for 2 h, then NaN_3 (14.2 g, 218 mmol) was added in one portion. Then, H_2O (75 mL) was added very carefully, and after ½ h, the reaction was warmed to room temperature. After 2 hrs., a saturated solution of NaHCO_3 (40 mL) was added cautiously, and the reaction was neutralized by adding excess solid NaHCO_3 carefully. The reaction mixture changed its color from yellow, then brown, to pale yellow. The THF was removed in vacuo, and the product was extracted with EtOAc (50 mL x 4). The organic layer was dried (MgSO_4), filtered, and concentrated. The pale-yellow solid was used directly in the next step without further purification. The crude azide (4.12 g, 25.38 mmol) was obtained with the yield of 77%. ^1H NMR (400 MHz, CDCl_3): 7.83 (dt, J = 8.78, 2.51 Hz, 2H), 7.10 (dt, J = 8.78, 2.51 Hz, 2H), 5.73 (bs, 2H). $^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, CDCl_3): 168.2, 143.9, 129.7, 129.2, 119.1. Physical and spectral data were in agreement with the literature.³⁷

The crude azide was then suspended in anhydrous THF (150 mL) and treated with NaHCO_3 (7.47 g, 88.90 mmol, 1.0 equiv.) under an N_2 atmosphere. Then ethyl bromopyruvate (4.70 mL, 37.45 mmol, 1.5 equiv.) was added. The reaction mixture was refluxed overnight. The next day, another portion of ethyl bromopyruvate (4.83 mL, 38.49 mmol, 1.5 equiv.) was added and refluxed for 48 hrs. The reaction mixture was cooled and filtered through Celite using EtOAc as solvent. The filtrate was concentrated and then redissolved in anhydrous THF (150 mL) under an N_2 atmosphere. The mixture was cooled to 0°C, and trifluoroacetic anhydride (15.6 mL, 223 mmol) was added slowly. The reaction was allowed to warm the room temperature and stirred overnight. The next day, a solution of saturated NaHCO_3 (75 mL) was added at 0°C, and another portion of solid NaHCO_3 was added carefully to neutralize the mixture. The reaction mixture was evaporated in vacuo to remove THF. The crude oxazole was extracted with EtOAc (100 mL x 3), which was washed with brine (150 mL), dried (MgSO_4), filtered, and concentrated. The oxazole was recrystallized using EtOAc/petrol solvent system, and the mother liquor was purified by FCC

(Hexane: EtOAc; 9:1 then, 8:2) to obtain a collective pure product **2**, ethyl 2-(4-azidophenyl)oxazole-4-carboxylate, (5.62 g, 21.54 mmol) with the yield of 85%. ^1H NMR (400 MHz, CDCl_3): 8.23 (s, 1H), 8.06 (dt, $J = 9.03, 2.26$ Hz, 2H), 7.08 (dt, $J = 8.78, 2.38$ Hz, 2H), 4.40 (q, $J = 7.29$, 2H), 1.38 (t, $J = 7.03$, 3H). $^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, CDCl_3): 161.6, 161.2, 143.5, 142.8, 134.6, 128.4, 122.9, 119.3, 61.2, 14.2. Physical and spectral data were in agreement with the literature.²²

2-(4-azidophenyl)-N-(2-(piperidine-1-yl)ethyl)oxazole-4-carboxamide (3). The azide **2** (0.7 g, 2.71 mmol, 1.0 equiv.) was dissolved in THF/ H_2O (4.3:1, 15 mL) and cooled to 0°C . LiOH (97 mg, 4.07 mmol, 1.5 equiv.) in H_2O (1.5 mL) was added slowly, then the reaction was stirred for $\frac{1}{2}$ h before being warmed to room temperature. After 3 h, TLC was done (Hexane: EtOAc; 8:2) to evaluate the reaction progress. The reaction mixture was acidified with 10% HCl (pH = 2). The crude acid was extracted with EtOAc repeatedly (50 mL) portions) until all the solids had dissolved. The EtOAc organic layers were combined, dried (MgSO_4), filtered, and concentrated. The crude material was used directly in the next step without further purification or characterization. The crude acid was suspended in anhydrous CH_2Cl_2 /THF (7:1, 16 mL) under an N_2 atmosphere with anhydrous DMF (15 μL) and then cooled to 0°C . Oxalyl chloride (469 μL , 5.47 mmol, 2.02 equiv.) was added slowly dropwise, and after 1 h, the reaction was allowed to come to room temperature and was stirred overnight. The next day, the solvents were removed in vacuo, and the crude brown acid chloride solid was used directly in the next step without further purification. The intermediate crude acid chloride was dissolved in CH_2Cl_2 (15 mL) and then cooled to 0°C . The amine (1-piperidineethanamine) was dissolved in CH_2Cl_2 (8 mL) and slowly added to the reaction mixture at 0°C . The reaction mixture was stirred overnight and then warmed to room temperature (rt) overnight. The reaction was monitored by TLC (CH_2Cl_2 : MeOH; 9:1). A saturated solution of NaHCO_3 ($\sim \frac{1}{2}$ the volume of reaction) was added and extracted with EtOAc (50 mL x 2). The organics were combined, dried (Na_2SO_4), filtered, and concentrated, and the product was purified by flash chromatography CH_2Cl_2 /MeOH (9.5: 5 to 9:1) to afford the pure product (835 mg, 2.45 mmol) with the overall yield of 90%. ^1H NMR (400 MHz, CDCl_3): 8.19 (s, 1H), 8.01 (dt, $J = 9.03, 2.26$ Hz, 2H), 7.48 (bs, 1H), 7.11 (dt, $J = 9.03, 2.26$ Hz, 2H), 3.53 (q, 2H), 2.54 (t, $J = 6.40$, 2H), 2.44 (m, 4H), 1.61 (quint, $J = 5.27$, 4H), 1.48–1.44 (m, 2H). $^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, CDCl_3): 160.6, 160.5, 142.6, 140.5, 137.5, 128.1, 123.3, 119.4, 57.2, 54.3, 35.9, 26.1, 24.4. Physical and spectral data were in agreement with the literature.²²

2-(4-ethynylphenyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (5) was prepared according to a literature procedure.²³ 1,8-naphthalic anhydride (200 mg, 1.01 mmol, 1.0 equiv.), 4-ethynylaniline (177 mg, 1.51 mmol, 1.5 equiv.), and $\text{Zn}(\text{OAc})_2$ (185 mg, 1.01 mmol, 1.0 equiv.) were suspended in dry pyridine in a 10 mL round-bottomed flask. The mixture was heated to reflux for 30 hrs. The reaction progress was monitored by TLC (Hexane: EtOAc; 7:3). Pyridine was removed by Hi-Vac, and the crude was purified by FCC (Hexane: EtOAc; 9:1 then 7:3). The product (**5**) was obtained (228 mg, 0.77 mmol) with a yield of 77%. ^1H NMR (400 MHz, CDCl_3): 8.66 (dd, $J = 7.28, 1.00$ Hz, 2H), 8.30–8.28 (m, 2H), 7.81 (dd, $J = 8.28, 1.00$ Hz, 2H), 7.70–7.66 (m, 2H), 7.32–7.29 (m, 2H), 3.15 (s, 1H). $^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, CDCl_3): 184.2,

135.7, 134.4, 133.1, 131.8, 131.7, 128.8, 128.5, 127.1, 122.7, 122.6, 83.0, 78.0. Physical and spectral data were in agreement with the literature.^{23, 38}

Click chemistry: **2-{4-[4-(4-{2,4-dioxo-3-azatricyclo[7.3.1.0^{5,13}]trideca-1(13),5,7,9,11-pentaen-3-yl}phenyl)-1H-1,2,3-triazol-1-yl]phenyl}-N-[2-(piperidin-1-yl)ethyl]-1,3-oxazole-4-carboxamide (**18NIO**):** The alkyne **5** (40 mg, 0.14 mmol, 1.0 equiv.) and azide **3** (46mg, 0.14 mmol, 1.0 equiv.) were dissolved in THF: H₂O (1:1), 3.0 mL in a dram vial. Then CuSO₄·5H₂O (2 mg, 0.007 mmol, 0.05 eq.) was added, followed by sodium ascorbate (7 mg, 0.034 mmol, 0.25 eq.) dissolved in H₂O (1.0 mL). Then, the reaction mixture was heated at 100°C overnight. The reaction progress was monitored by TLC (DCM: MeOH, 9:1). The reaction was then cooled to room temperature and diluted with H₂O (4 volumes). The suspension was filtered and washed with methanol (5 mL), then EtOAc. The residue was dissolved in water (10 mL) and then washed with DCM (10 mL). The product (**18NIO**) was obtained (79 mg, 0.12 mmol) with a yield of 92%. ¹H NMR (400 MHz, DMSO-*d*₆): 9.54 (b, 1H), 8.77 (b, 1H), 8.53 (d, *J* = 7.78 Hz, 4H), 8.29–8.22 (m, 4H), 8.11 (d, *J* = 8.28 Hz, 2H), 7.93 (t, *J* = 7.48 Hz, 2H), 7.56 (d, *J* = 8.03 Hz, 2H), 3.39 (b, 2H), 3.30 (b, 2H), 2.41 (b, 4H), 1.52 (b, 4H), 1.39 (b, 2H). ¹³C{¹H} NMR (100 MHz, DMSO-*d*₆): 163.6, 159.6, 159.5, 147.0, 142.1, 138.1, 137.4, 135.9, 134.4, 131.4, 130.7, 129.86, 129.7, 127.8, 127.1, 126.1, 127.8, 122.5, 120.4, 119.9, 57.1, 53.7, 35.7, 25.2, 23.7. Elemental Analysis: calculated for C₃₇H₃₁N₇O₄, C 69.69%, H 4.90%, N 15.38%, O 10.04%. HRMS analysis of **18NIO** was not successful using ESI.

2.3 UV–vis and fluorescence spectroscopic studies

Probe **18NIO** was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution at 1.0 mM for subsequent use. The UV–vis and fluorescence spectra were obtained with a Varian Cary 50 scan spectrometer (USA) with a 10 mm quartz cuvette, and Fluorolog®-3(FL-1057) spectrofluorometer (Horiba, Japan) with excitation (*n* = 5) and emission (*n* = 5) slits set for a 5 nm bandwidth at certain wavelength at room temperature relative to the blank (*n* = 5).

Fluorescence Quantum Yield

The fluorescence emission quantum yields were measured with respect to quinine sulfate standard in 0.1 M aqueous sulfuric acid (*φ* = 0.546) at room temperature at a single excitation wavelength at 350 nm and calculated according to the following equation;^{15, 24}

$$\phi_{unk.} = \phi_{std.} \left(\frac{I_{unk.}}{I_{std.}} \right) \left(\frac{A_{unk.}}{A_{std.}} \right) \left(\frac{n_{unk.}}{n_{std.}} \right)^2$$

Where, ϕ_{unk} is the radiative quantum yield of the sample; ϕ_{std} is the radiative quantum yield of the standard; I_{unk} and I_{std} are the integrated emission intensities of the sample and standard, respectively; A_{unk} and A_{std} are the absorptions of the sample and standard at the excitation wavelength, respectively;

and n_{unk} and n_{std} are the indices of refraction of the sample and standard solutions (pure solvents were assumed), respectively.

Fluorescence Lifetime Measurements

Time fluorescence lifetime measurements were obtained using measurements were taken using a Fluorolog®-3(FL-1057) spectrofluorometer (Jobin Yvon, Horiba, Japan) equipped with a 450 W xenon lamp (250 – 700 nm) coupled to an IBH 5000F time-correlated single photon counting (TCSPC) device (Horiba) equipped with a wavelength of 340 nm NanoLED as an excitation source (pulse width < 200 ps, HORIBA). The emission spectra were collected in a right-angle geometry, subsequently scaled for the excitation intensity, and corrected for the spectral sensitivity of the detector.

Computational Studies & Calculations

TD-DFT Calculations for Excitation and Emission

All the excitation and emission calculations were performed with the Gaussian 16 software package. The ground-state geometries were optimized using DFT at Becke's three-parameter hybrid exchange functional with the Lee–Yang–Parr correction functional (B3LYP) method in conjunction with 6-311G + (d, p) basis set.³⁹

Biological Evaluations

Cytotoxicity Studies

MDA-MB-231 cells were plated at a concentration of 4×10^4 cells per well in a 96-well plate and allowed to adhere overnight. RPMI 8226 cells were plated at 4×10^5 cells per well and treated immediately. Cells were treated with a DMSO vehicle control or a serial dilution of **18NIO** for 48 h. MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Alfa Aesar) in PBS (5 mg/ mL) was then added at a final concentration of 0.5 mg/mL to each well and incubated for 2 h at 37°C. For adherent cell lines (MDA-MB-231), the media was removed from each well of the plate, and the resulting formazan crystals were dissolved in 100 µL of DMSO. For RPMI 8226 cells, 200 µL of a DMF/SDS solution (50% DMF, 10% w/v SDS, pH < 4) was used to solubilize the formazan crystals. The resulting optical density (OD) at 490 nm was measured using a ThermoMAX microplate reader. Cells treated with 100 mM phenylarsine oxide (PAO) served as a positive control. Technical replicates of these assays were performed in at least technical triplicate and biological quadruplicate, and the three best values were used for analysis to eliminate outliers. The data presented are half-maximal effective concentration (EC_{50}) values that were calculated using nonlinear regression on the normalized data in GraphPad Prism 6.0 with the corresponding standard deviations.

Confocal Microscopy Studies

MDA-MB-231 cells were seeded on coverslips in 6-well plates (150,000 cells/well) and allowed to adhere overnight. They were treated with 10 μ M 18NIO for 24 h. The cells were then washed with 1X PBS and fixed using ice-cold methanol:acetic acid (3:1, v/v) overnight at -20 °C. Fixed cells were rehydrated in PBS (3x 5 minutes), stained with SYBR Green I diluted (1:25,000 in PBS) for 30 minutes. Cells were washed with 0.05% PBST (3x 5 minutes) followed by a PBS rinse, mounted in 50% glycerol diluted in PBS. Images were acquired using an Olympus FV10i confocal laser scanning microscope with a 60x oil immersion objective using a 2.0 aperture. The 405 nm diode laser was used to excite 18NIO. Images were processed with ImageJ (Fiji).

Declarations

Author Contribution

S.G. wrote the main manuscript text and carried out all of the spectroscopic and synthetic studies..P.P. and T.O. conducted the fluorescence microscopy and MTT studies.V.C. carried out preliminary synthetic studies on the phenylalkyne chemistry.M.H. supported the work and assisted in the revisions

Acknowledgments

The authors acknowledge Dr. Jeff Altig at New Mexico Tech (NMT) for facilitating the molecular modeling lab for computational calculations. MDH thanks Rochester Institute of Technology (RIT) for startup funding and student support. SKG thanks NMT and RIT for the financial support of S.K.G

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Scheme 1

Scheme 1 is available in the Supplementary Files section.

Figures

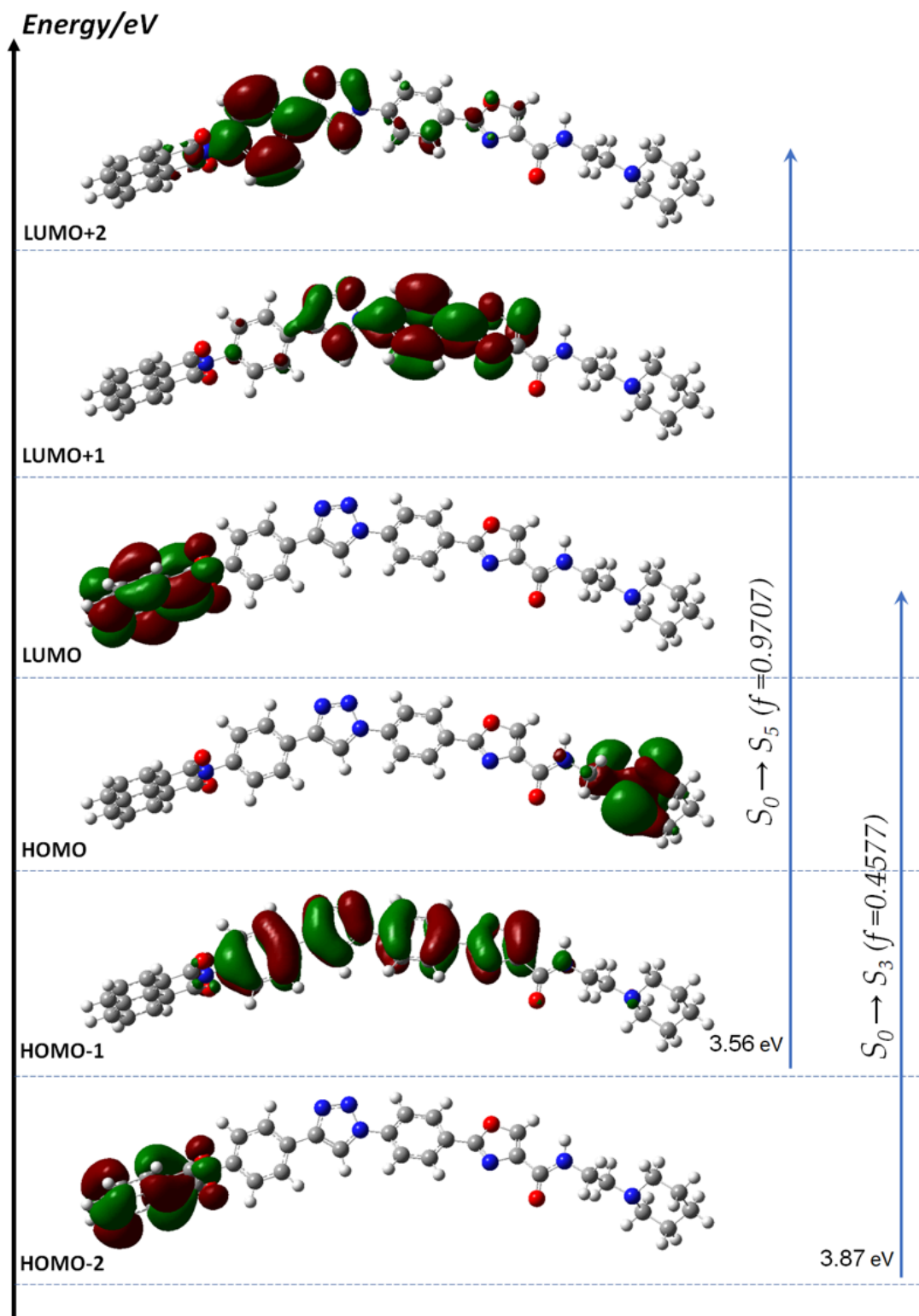


Figure 1

Frontier molecular orbitals of 18NIO at the equilibrium geometry of ground states with the corresponding vertical $S_0 \rightarrow S_3$ and $S_0 \rightarrow S_5$ transitions computed at the 6-311G + (d, p) level of theory in DMSO solvent. H: white, C: gray, N: blue, O: red.

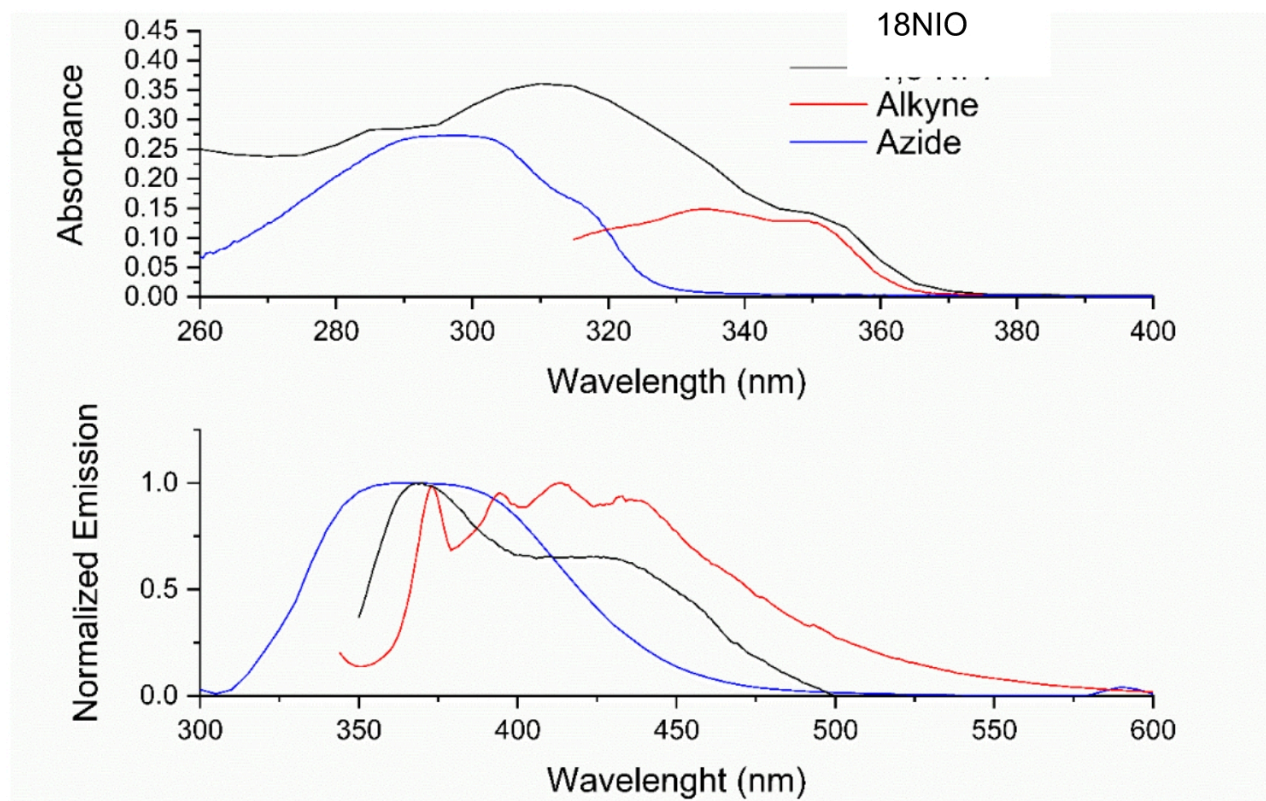


Figure 2

Normalized mean absorbance ($n = 5$) and emission of alkyne ($I_{\text{ex.}} 314 \text{ nm}$), azide ($I_{\text{ex.}} 295 \text{ nm}$) and the final probe, 18NIO probe ($10 \mu\text{m}$) in DMSO ($I_{\text{ex.}} 310 \text{ nm}$)

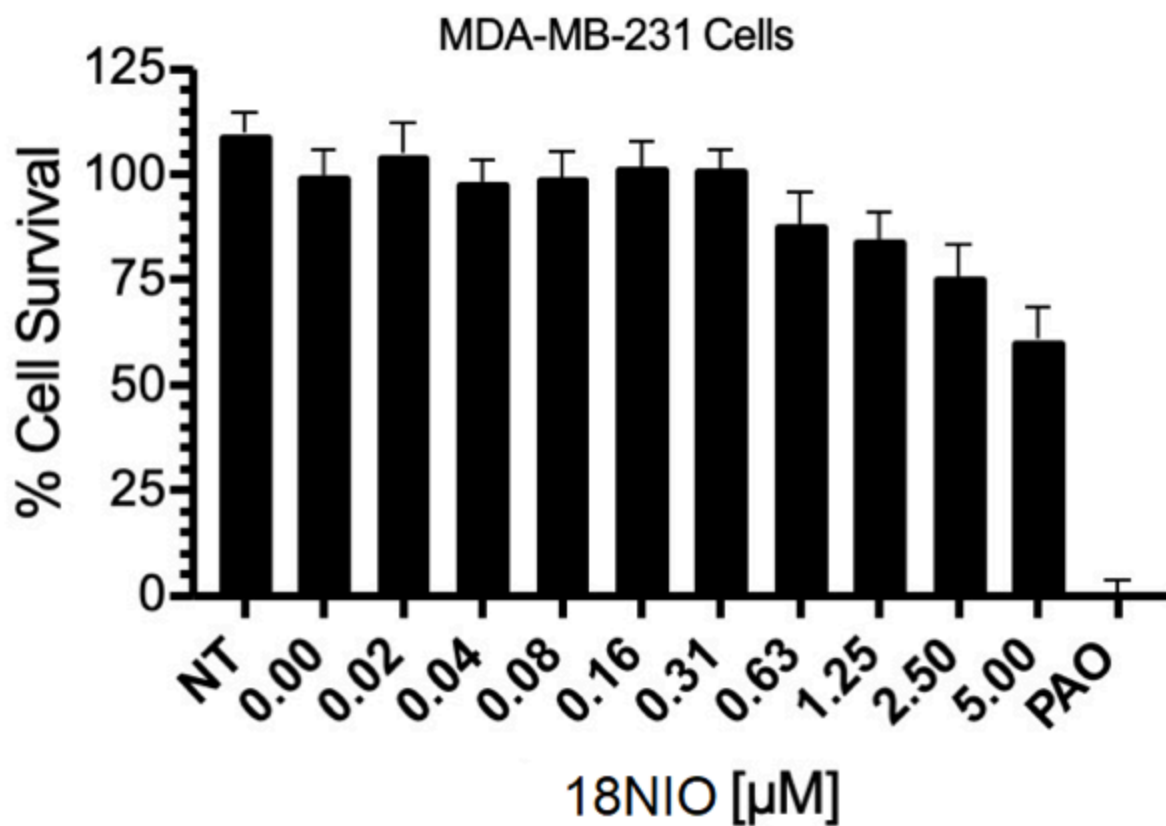


Figure 3

Concentration vs cell survival of 18NIO probe against triple negative breast cancer cells.

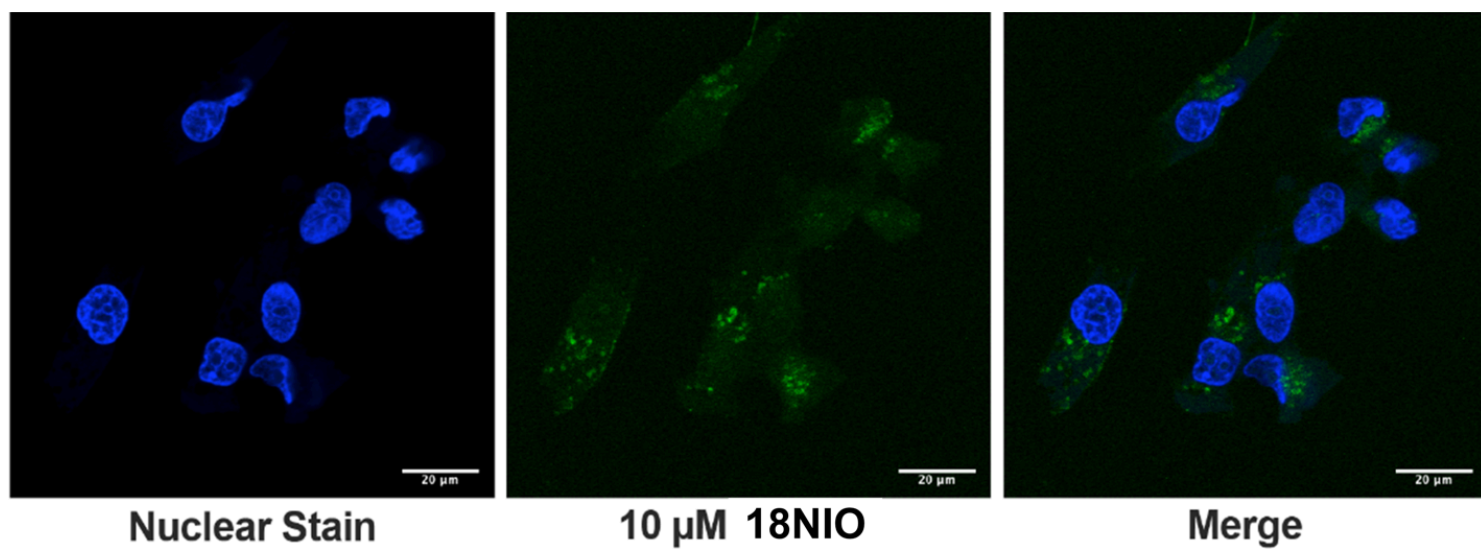


Figure 4

Confocal microscopy images of fixed MDA-MB-231 cells stained with 18NIO (10 μ M) nuclear stain SYBR Green.

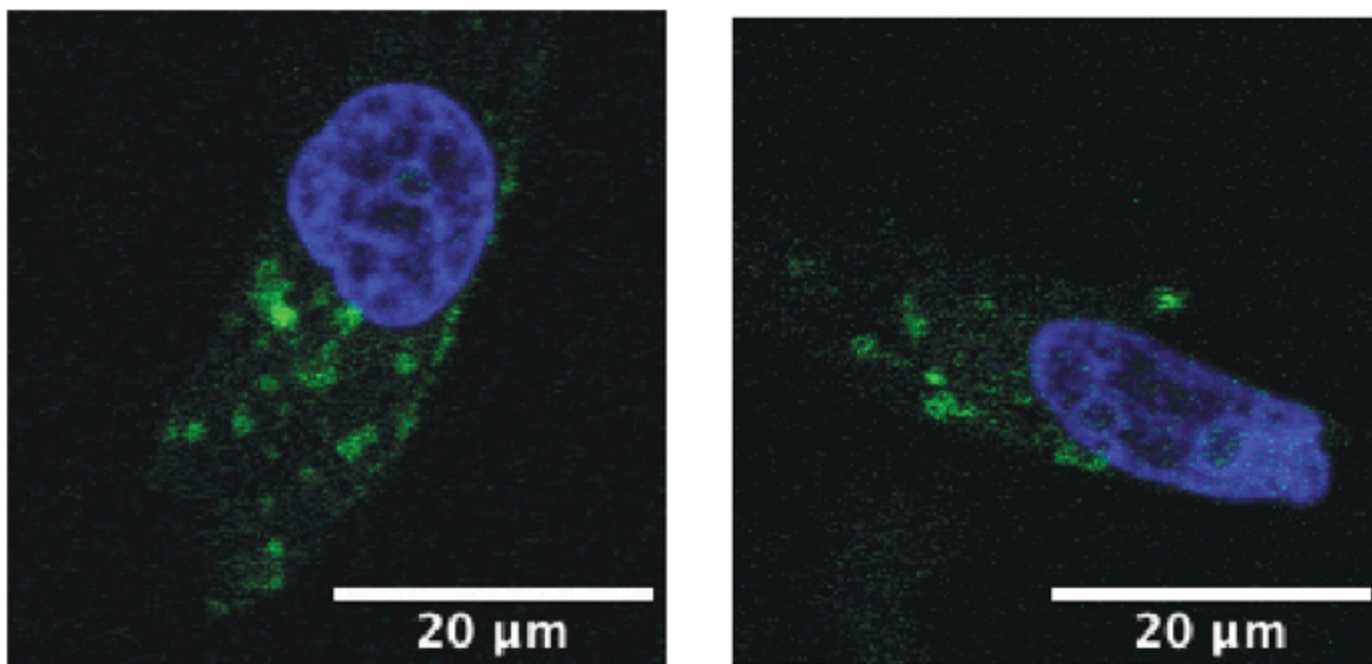


Figure 5

Close up images of **18NIO** showing distribution along cell wall membrane along with larger vacuole inclusion and concentrations that display brighter fluorescence.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SynthesisandPhotophysicalPropertiesofNaphthalimideSupportingInformationV5.docx](#)
- [floatimage2.png](#)