

# Expanding the breadth of 4-amino-1,8-naphthalimide photophysical properties through substitution of the naphthalimide core

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**Abstract** Fluorescent sensors that illuminate specific molecules and chemical events allow the selective and sensitive study of the cellular environment. At the centre of this technology lies the fluorescent reporter molecule, and it is therefore crucial to provide a breadth of fluorophores with varying photophysical and biological behaviour. 4-Amino-1,8-naphthalimides are commonly employed in fluorescent sensors, but the narrow range of structural derivatives limits versatility of application. Here we report the synthesis and investigation of a set of twelve 4-amino-1,8-naphthalimides bearing an additional substituent on the aromatic core. Photophysical characterisation and Time-Dependent Density Functional Theory studies provided insights into the structure-photophysical property relationships of these derivatives, which show an expanded range of emission wavelengths and other photophysical properties. These compounds could all be visualised within cells by confocal microscopy, showing cytoplasmic or lipid droplet localisation. Our studies have demonstrated that simple structural modification of 4-amino-1,8-naphthalimides provides derivatives with considerable breadth of behaviour that lend valuable versatility to the design of fluorescent sensors.

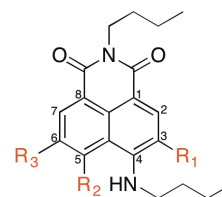
## Introduction

Fluorescent molecules are indispensable tools in chemical biology for imaging cellular structure, environment and function.<sup>1</sup> There are many fluorophore scaffolds that are commonly used as exogenous fluorescent reporters in biological sensors, many of which have been extensively modified and characterised to provide a large range of derivatives. These include rhodamines,<sup>2</sup> fluoresceins,<sup>3</sup> BODIPYs,<sup>4</sup> cyanines,<sup>5</sup> and coumarins,<sup>6</sup> and the diversity in photophysical and biological properties afforded by their variants allows flexibility in the design of a wide range of sensors incorporating these dyes. It is important to continue to identify and expand the range of available fluorescent scaffolds to provide further options for successful fluorescent probe design.

Naphthalimides are an interesting class of fluorophore comprising a naphthalene ring and a dicarboxyl imide moiety. Previous studies into naphthalimides have investigated the impact on the photophysical properties of varying the imide position,<sup>7,8</sup> giving rise to many sub-classes of fluorophore. Of the 1,8-naphthalimides the introduction of electron-donating substituents such as hydroxyl,<sup>9</sup> and amino<sup>10</sup> groups (e.g. Figure 1) has been shown to give rise to enhanced fluorescence through an intramolecular charge transfer mechanism.<sup>11</sup>

Amongst the reported naphthalimides, 4-amino-1,8-naphthalimides have found common use as fluorescent reporters in biological sensors, as thoroughly reviewed by Duke *et al.*<sup>12</sup> Despite several advantageous properties, including the ability to readily attach additional functionality at the imide and amino positions; the intrinsic chemical and photostability; high quantum yields; and a large Stokes shift, 4-amino-1,8-naphthalimides remain a poorly explored fluorophore. Most previous studies into 4-amino-1,8-naphthalimides have focused on the effect of varying the alkyl chain at the C4 group and the imide, which produces only small changes in photophysical properties.<sup>10, 13-15</sup> While the trisubstituted 1,8-naphthalimide Lucifer Yellow is commercially-available, its photophysical properties do not diverge considerably from mono-substituted compounds,<sup>16</sup> and there is a poor understanding of the effects of substitution of the 4-amino-1,8-naphthalimide core.

This work aims to manipulate and expand the fluorescent properties of 4-amino-1,8-naphthalimides by investigating the impact of di-substituting the aromatic core with a variety of electron-donating and electron-withdrawing groups. Here we present the synthesis of 12 derivatives of the core naphthalimide structure, substituted with  $-\text{NH}_2$ ,  $-\text{NO}_2$ ,  $-\text{SO}_3^-\text{K}^+$ , and  $-\text{Br}$  substituents at the C3, C5 and C6 positions (Figure 1, Table 1), whilst maintaining the crucial 4-butylamino substituent. Full photophysical characterisation and theoretical investigation of these derivatives reveals the impact of varying the core aromatic ring structure of this donor-acceptor fluorophore, enabling us to draw conclusions regarding structure-photophysical relationships. This expanded range of 1,8-naphthalimide fluorophores was assessed for suitability in biological imaging experiments.



**Figure 1.** Structure of 4-amino-1,8-naphthalimides with positions of substitution labelled.

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**Table 1.** Library of synthesised naphthalimides with functional group substitution patterns indicated

Compound	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
<b>N1</b>	H	H	H
<b>N2</b>	SO <sub>3</sub> <sup>-</sup> K <sup>+</sup>	H	H
<b>N3</b>	H	SO <sub>3</sub> <sup>-</sup> K <sup>+</sup>	H
<b>N4</b>	H	H	SO <sub>3</sub> <sup>-</sup> K <sup>+</sup>
<b>N5</b>	NO <sub>2</sub>	H	H
<b>N6</b>	H	NO <sub>2</sub>	H
<b>N7</b>	H	H	NO <sub>2</sub>
<b>N8</b>	NH <sub>2</sub>	H	H
<b>N9</b>	H	NH <sub>2</sub>	H
<b>N10</b>	H	H	NH <sub>2</sub>
<b>N11</b>	Br	H	H
<b>N12</b>	H	Br	H
<b>N13</b>	H	H	Br

## Results and Discussion

### Synthesis

Many of the naphthalimides with substituents at the C3 and C5 position have either been previously reported,<sup>17-19</sup> or were readily accessible by adaptation of previous methods<sup>20</sup> (Scheme S1). Substitution at the C6 position, however, is rarely reported in the literature and was less straightforward for many of the substituents. In the case of sulfonation, substitution at the C6 position over C3 was achieved by changing the steric and electronic influence of the directing group at the C4 position, which was varied from an amine to bromine (Scheme 1A).

However, other electrophilic aromatic substitution reactions, such as nitration, do not proceed in the same manner. Preparation of compounds **N7** and **N10** with nitro and amino substituents at the C6 position required nitration of the sulfonated naphthalimide **5**, and subsequent nucleophilic aromatic substitution of the sulfonate group of **6** to give the C6-substituted **N7**, followed by reduction of the nitro group to give the aniline **N10** (Scheme 1B). This nucleophilic aromatic substitution reaction has been reported for C4 nitro groups on naphthalimides,<sup>21</sup> a pathway that we exploited in the synthesis of the C6 bromo-substituted naphthalimide **N13** (Scheme 1C), but to the best of our knowledge it has not previously been observed at sulfonate-substituted carbons on the naphthalimide skeleton. Using this sequence of steps and novel synthetic pathways, we were therefore able to generate the desired set of C6-substituted naphthalimides.

### Photophysical properties

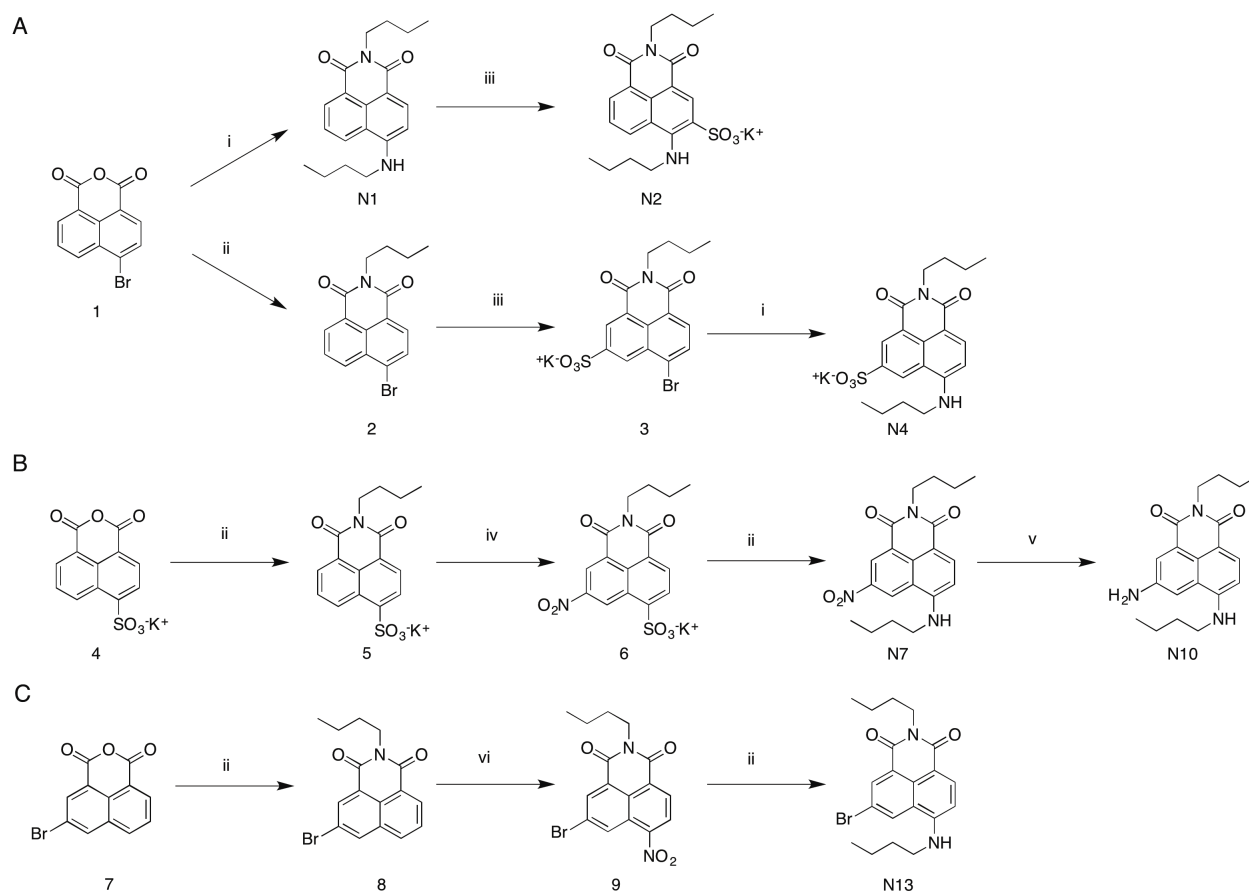
With the 13 naphthalimides **N1-N13** in hand, their photophysical properties were determined and analysed in order to understand the relationships between their structure and fluorescence. The absorbance and fluorescence emission spectra for each naphthalimide were recorded in ethanol (Table 2). The extinction coefficient ( $\epsilon$ ) and the quantum yield ( $\Phi$ ) were also measured, and the product of these measurements ( $\epsilon \times \Phi$ ) gave the overall brightness ( $B$ ) of each dye, a convenient value to quantify the photon yield of a fluorophore.<sup>16</sup> In addition to this, the lifetime decays of the compounds in ethanol ( $\tau$ ) were measured and fit to give the fluorescence lifetimes.

**Table 2.** Absorption and emission maxima of the naphthalimide fluorophores in ethanol, and the corresponding extinction coefficient ( $\epsilon$ ), quantum yield ( $\Phi$ ), brightness ( $B$ ) and lifetime ( $\tau$ ).

	$\lambda_{\text{abs}}$ (nm)	$\lambda_{\text{em}}$ (nm)	$\log_{10}\epsilon$ (M <sup>-1</sup> cm <sup>-1</sup> )	$\Phi$	$B$ (x 10 <sup>3</sup> M <sup>-1</sup> cm <sup>-1</sup> )	$\tau$ (ns)
<b>N1</b>	443	527	4.2	0.63	11.0	9.6
<b>N2</b>	431	517	3.9	0.47	4.0	8.1
<b>N3</b>	471	573	4.0	0.05	0.3	2.1
<b>N4</b>	446	530	3.4	0.59	1.5	10.0
<b>N5</b>	396	448	4.4	<0.01	<sup>b</sup>	4.0
<b>N6</b>	450	464	3.9	0.01	0.01	3.5
<b>N7</b>	477	<sup>a</sup>	4.0	<0.01	<sup>b</sup>	<sup>a</sup>
<b>N8</b>	497	532	3.9	<0.01	<sup>b</sup>	4.9
<b>N9</b>	452	504	4.3	0.42	8.0	6.8
<b>N10</b>	469	546	3.7	0.69	3.7	10.1
<b>N11</b>	422	519	3.9	0.17	1.2	5.0
<b>N12</b>	449	545	4.1	0.03	0.4	1.4
<b>N13</b>	455	549	4.1	0.11	1.5	6.0

<sup>a</sup>Quenched in ethanol <sup>b</sup>Could not be calculated

Di-substituting the parent compound (**N1**) significantly tunes its photophysical properties. In general, the nitro-substituted compounds **N5-N7** show greatly quenched fluorescence, reflected in their low quantum yields. The quenching effect of nitro groups is a commonly-observed phenomenon for organic fluorophores, such as fluoresceins<sup>22</sup> and pyrenes.<sup>23</sup> The bromo-substituted dyes **N11-N13** are also significantly less bright than the parent compound, presumably due to the heavy atom effect.<sup>24</sup> The brightness of the sulfonated naphthalimides **N2-N4** and amino naphthalimides **N8-N10** varies depending on the position of substitution. The lifetimes of the naphthalimides do not appear to follow any particular structural trends in relation to either the



**Scheme 1.** Synthesis of 6-substituted naphthalimides. (A) Changing the directing group at the C4 position from an amine to a bromine changed the directing effect from C3 to C6. (B) Nucleophilic aromatic substitution at the C4-substituted sulfonate led to the synthesis of N7, and subsequent reduction gave N10. (C) Substitution of the C4 nitro group to give N13. (i) *n*-Butylamine, EtOH, CuSO<sub>4</sub>, 80 °C, 16 h, 70% (ii) *n*-Butylamine, EtOH, 80 °C, 3 h, 60-75% (iii) H<sub>2</sub>SO<sub>4</sub>.SO<sub>3</sub>, 90 °C, 3 h, 79% (iv) HNO<sub>3</sub>, H<sub>2</sub>SO<sub>4</sub>, 0 °C, 5 h (v) Pd/C, H<sub>2</sub>, MeOH, rt, 4 h, 89% (vi) NaNO<sub>2</sub>, H<sub>2</sub>SO<sub>4</sub>, rt, 16 h.

nature of the substituent nor the position of substitution. There is a general relationship between the fluorescence lifetimes and the quantum yields of the naphthalimides (Figure S1), with shorter lifetimes correlating with lower quantum yields for the dyes that could be compared. This indicates that these dyes have a similar mechanism of fluorescence and rate of radiative decay, and the differentiating contribution to their lifetime is the rate of non-radiative decay, the same factor that determines quantum yield.

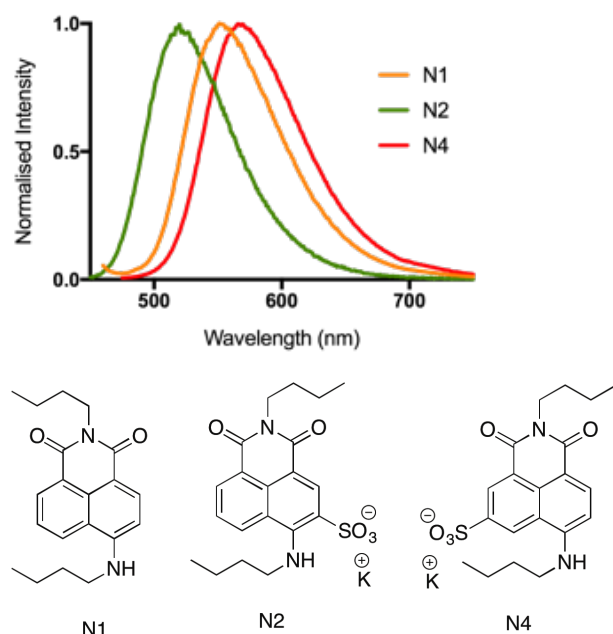
It is evident from the data that both the position of substitution and the electron-withdrawing or -donating ability of the substituent strongly influence the emission wavelength. In the sulfonate, bromo and nitro cases, the C3-substituted naphthalimides are more blue-shifted than the parent compound, and the C6-substituted naphthalimides are more red-shifted (Figure 2, S2, S3). The effect of substitution at position 5 does not follow simple trends, as it involves a more complicated combination of electronic and steric factors. For instance, an excited state model of N9 (Figure S4), shows that in this disubstituted compound, the amino substituent at C5 does not contribute to the ICT in the same way as the C4 butylamino substituent, because the *sp*<sup>2</sup> planar

geometry required for both to undergo electron-donation would cause steric clash between their two protons. This leads to a more blue-shifted emission than the parent compound N1. The C5 sulfonated naphthalimide N3, however, is one of the most red-shifted naphthalimides, potentially due to the stabilising electrostatic interaction between the sulfonate negative charge and the forming positive charge on the butylamino substituent during charge transfer.

In order to rationalise the observed trends, theoretical 0-0 energies of the naphthalimides were modeled in acetone (Table S1), using methods shown to yield trends largely consistent with experimental data.<sup>25-29</sup> These modeled 0-0 points were compared to the absorption and emission cross-over points of the synthesised derivatives in the same solvent. For the dyes for which theory and experiment comparisons can be made (all except N6- see Table S1), a mean signed error of 0.018 eV and a mean absolute error of 0.022 eV was obtained, highlighting the accuracy of the theoretical model. The linear correlation between theoretical and experimental values gives an *R* value of 0.86. All these values are within the expectations of error for the selected

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level of theory.<sup>25</sup> Density difference plots were computed for all compounds (Figure S5), and intramolecular charge transfer parameters, and ground and excited state dipoles calculated for the different dyes (Table S2).

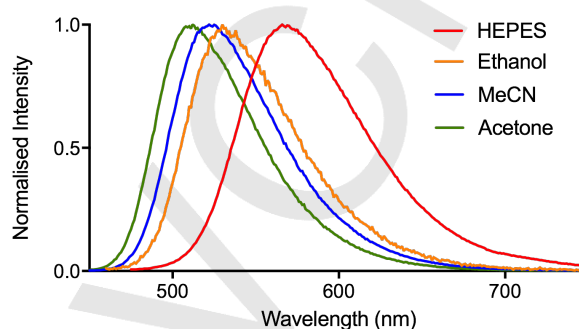


**Figure 2.** Fluorescence emission of **N1**, **N2** and **N4** in HEPES buffer (pH 7.4, 20 mM).

As can be seen for **N1** (Figure S5), there is a strong re-organisation of the electrons upon absorption, corresponding to a charge transfer (CT) distance of 2.1 Å, with the C4 amine acting as a donor group and the keto groups as acceptors. Adding a sulfonate group tends to have a small impact on this charge transfer, except for **N3**, where the C5-sulfonate acts as a secondary acceptor. Nitro group substitution causes much larger changes, as it becomes the main electron-accepting moiety in the molecule. In **N5**, the ICT becomes small (computed CT distance of 1.2 Å) due to the proximity between the donor and acceptor groups, explaining the blue-shifted emission compared to **N1**. In the case of **N6**, TD-DFT optimisation shows a partial excited state intramolecular proton transfer (ESIPT) between the nitro and amino substituents. This process gives a very strong excited state relaxation and therefore negligible emission. The small amount of experimentally-observed fluorescence in aprotic media is most probably due to emission from a non-relaxed excited state. In **N7**, the donor and acceptor groups are sufficiently separated that the CT distance is similar to that of **N1** (2.2 Å). In contrast, the addition of amino groups provides a secondary CT donor. This additional electron donor has a large impact at the C3 and C6 positions (**N8** and **N10** respectively), whereas steric factors inhibit electron donation at C5 (**N9**), as discussed above. The impact of the bromine atom on the electronic re-organisation is relatively small, leading to less variation in properties for **N11** to **N13**.

The absorbance and fluorescence spectra of the naphthalimides were also recorded in HEPES buffer (pH 7.4), in order to assess their potential for applications in biological imaging (Table S3). Notably, emission wavelengths in HEPES buffer range from 450-605 nm, significantly expanding on the previously reported

naphthalimide derivatives, which tend to have emission wavelengths between 520-560 nm. The spectra were also recorded in acetonitrile and acetone, and for a majority of the naphthalimides a strong solvatochromism typical of ICT fluorophores<sup>30</sup> can be observed across the screened solvents (Figure S6-S18). In almost every case, a red-shift in the emission wavelength occurs with increasing solvent polarity and hydrogen-bonding ability, for example as in **N4** (Figure 3).



**Figure 3.** Emission of **N4** in various solvents, showing general solvatochromic trend.

This is consistent with the increase of dipole moments when going from the ground to the excited-state that is predicted by TD-DFT (Table S4). The extent of this solvatochromism ( $\Delta\lambda$ ) was defined by the difference between the maximum emission in HEPES buffer and the maximum emission in acetone (Table S4). The values for these fluorophores ranged from 17 – 55 nm.

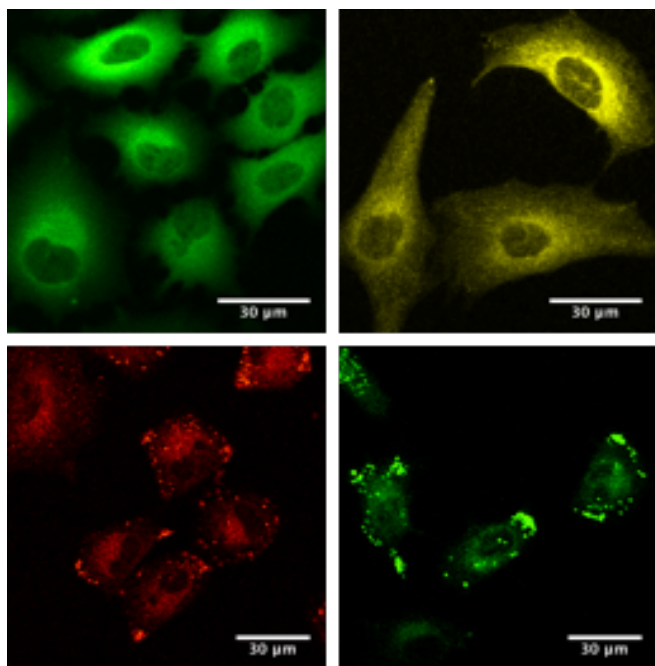
Of the few naphthalimides that did not follow this solvatochromic trend, most were substituted at the C3 position. In the case of **N2** (Figure S7), no change in emission wavelength was observed in the four screened solvents, potentially due to an intramolecular hydrogen bond between the ICT donor amine at C4 and the sulfonate substituent contributing more significantly to the excited state stabilisation than the surrounding environment. Another exception is **N8**, which displays multiple solvent-dependent emission bands (Figure S13), possibly indicative of several mechanisms of fluorescence, with the most red-shifted emission band (at ~ 620 nm) strongly quenched in more polar environments.

### Cellular behaviour

As one of the main aims of this work was to provide new fluorophores appropriate for biological imaging applications, we sought to characterise the cellular behaviour of **N1-N13**, since we had confirmed sufficient emission of most compounds in aqueous solution (Table S3). Firstly, the non-toxicity of the compounds was confirmed by incubating A549 cells with the dyes at 100  $\mu\text{M}$  concentrations for 90 mins, followed by treatment with Alamar Blue cell viability reagent. No dye was found to be toxic under these conditions (Figure S19), which far exceeded those subsequently used in imaging studies. Therefore, A549 cells were incubated with the synthesised naphthalimides (10  $\mu\text{M}$ , 20 min) and cellular fluorescence was visualised by confocal microscopy following excitation with 405 or 488 nm lasers. All dyes could be observed in cells, and despite only small structural changes, there are significant differences in their cellular localisation (Figure S20-S32). A majority of the dyes, such as **N2** (Figure 4A) and **N10**

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(Figure 4B), exhibit cytoplasmic localisation. However, some of the more lipophilic derivatives, such as **N8** (Figure 4C) and **N11** (Figure 4D), show localisation in lipid droplets, which was confirmed by colocalisation studies with Nile red (Figure S33-S37). Interestingly, despite the fact that **N8** emission is completely quenched in HEPES buffer (Table S3), this compound could be clearly seen in lipophilic areas of the cell at wavelengths corresponding to its spectrum in acetonitrile or acetone.



**Figure 4.** Confocal microscopy images of A549 cells incubated with (A) N2 (10  $\mu\text{M}$ ,  $\lambda_{\text{ex}}$  = 405 nm,  $\lambda_{\text{em}}$  = 500-600 nm), (B) N10 (1  $\mu\text{M}$ ,  $\lambda_{\text{ex}}$  = 405 nm,  $\lambda_{\text{em}}$  = 500-600 nm), (C) N8 (10  $\mu\text{M}$ ,  $\lambda_{\text{ex}}$  = 488 nm,  $\lambda_{\text{em}}$  = 550-650 nm) (D) N11 (10  $\mu\text{M}$ ,  $\lambda_{\text{ex}}$  = 405 nm,  $\lambda_{\text{em}}$  = 480-580 nm), for 20 minutes. Colours represent the maximum emission wavelength.

## Conclusions

The development of novel fluorophores with varying photophysical and structural properties is key to enabling versatility in the design of chemical biology tools. We have reported here that simple structural modification of 4-amino-1,8-naphthalimides leads to compounds with a variety of photophysical properties and a greatly expanded range of emission wavelengths. The structure-photophysical property relationships that we have been able to determine will enable the rational design of further improved fluorophores in the future. Importantly, all new naphthalimides showed good intracellular fluorescence and low cellular toxicity, with localisation in the cytoplasm or lipid droplets. These fluorophores are therefore appropriate for incorporation into a wide range of fluorescent sensors. The breadth of photophysical and biological behaviour will enable selection of the most appropriate fluorophore for each specific application. With this expansion in the scope of 4-amino-1,8-naphthalimides, the future looks bright for this class of fluorophore.

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**Keywords:** aromatic substitution • fluorescence • naphthalimide

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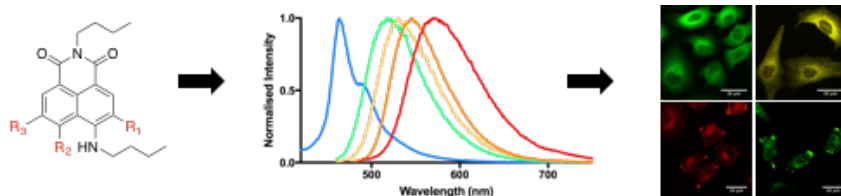
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Substitution of the 4-amino- 1,8-naphthalimide core tunes both the photophysical properties and biological behaviour of this versatile fluorophore