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## Multicomponent Bioluminescence Imaging with Naphthylamino Luciferins

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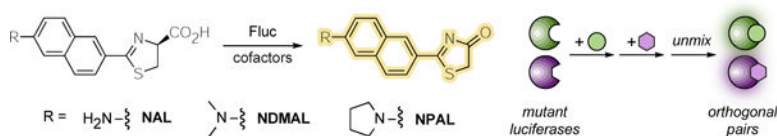
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### Abstract

Bioluminescent tools have been used for decades to image processes in complex tissues and preclinical models. However, few distinct probes are available to probe multicellular interactions. We and others are addressing this limitation by engineering new luciferases that can selectively process synthetic luciferin analogs. In this work, we explored naphthylamino luciferins as orthogonal bioluminescent probes. Three analogs were prepared using an optimized synthetic route. The luciferins were found to be robust emitters with native luciferase *in vitro* and *in cellulo*. We further screened the analogs against libraries of luciferase mutants to identify unique enzyme-substrate pairs. The new probes can be used in conjunction with existing bioluminescent tools for multi-component imaging.

### Graphical Abstract



Naphthylamino luciferins were used for rapid, multi-component bioluminescent imaging. The analogs were synthesized using a modular route, and activities were measured *in vitro* and *in cellulo*. Mutant luciferases were identified that could differentiate the substrates, and the unique enzyme-substrate pairs were compatible with existing bioluminescent probes. These substrates add to the growing set of bioluminescent tools for multiplexed imaging.

### Keywords

bioluminescence; imaging; luciferase; luciferin; orthogonal

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Supporting information for this article is given via a link at the end of the document.

## Introduction

Bioluminescence imaging (BLI) is a versatile method for monitoring cellular processes.<sup>[1–4]</sup> Among the most popular BLI probes are derivatives of firefly luciferase (Fluc), an enzyme that catalyzes the activation and oxidation of a small molecule substrate, D-luciferin (D-luc, Figure 1A).<sup>[5,6]</sup> Fluc/D-luc and related pairs are routinely used for monitoring biological events, including stem cell proliferation<sup>[7]</sup> and cancer metastasis.<sup>[8]</sup> While powerful, many of these probes cannot be deployed simultaneously owing to overlapping emission spectra or substrate cross-reactivities.<sup>[9]</sup>

We and others are addressing the need for more distinguishable bioluminescent tools by developing substrate-selective (i.e., “orthogonal”) luciferases.<sup>[10–20]</sup> In this strategy, enzymes are engineered to process chemically distinct luciferin analogs. We previously targeted luciferins comprising steric appendages at C4' and C7' (Figure 1C).<sup>[10]</sup> Iterative screening of the scaffolds with mutant luciferase libraries revealed unique combinations of enzymes and substrates for multi-component imaging.<sup>[12]</sup> Other luciferase-luciferin pairs have been similarly identified and are now used in a variety of applications.<sup>[17–19]</sup>

Selectivity between orthogonal luciferase-luciferin pairs has been historically achieved via mutual exclusion: “unmatched” luciferins and luciferases do not interact. We have recently found, though, that perfect selectivity among the probes is not required for multi-component imaging.<sup>[11,12]</sup> Rather, unique *patterns* of light emission are sufficient. If the dimmer substrate is imaged first, the change in brightness when the second substrate is added allows for two unique luciferases to be resolved (Figure 1B).<sup>[11]</sup> Multi-component imaging via this approach still requires diverse substrates, though, as distinct emission profiles are required. Unique patterns of photon output are most readily achieved using molecules that “look” different.

To broaden the scope of multi-component imaging, we set out to identify additional luciferins that could be differentially processed by mutant luciferases. Herein we report that naphthylamino luciferins are useful scaffolds for orthogonal imaging. These classic motifs are robust emitters and structurally distinct from other popular luciferins, making them attractive probes for multi-component studies.<sup>[21,22]</sup> Three naphthylamino luciferins were synthesized and evaluated *in vitro* and *in cellulo*. The luciferins were also screened against a library of mutant luciferases to identify complementary enzymes. The luciferase hits were capable of discriminating subtle changes in luciferin structure. The naphthylamino luciferins were also readily applied in tandem with other established analogs. Overall, this work expands the number of distinct and bright luciferins to facilitate multi-component imaging applications.

## Results and Discussion

### Design and synthesis of luciferin analogs

We were drawn to naphthylamino luciferins for a variety of reasons. The naphthalene core is small and rigid, and the light emitter can adopt a planar structure in the enzyme active

site, ensuring efficient photon output. Previous studies have also shown that naphthalene can substitute for the benzothiazole unit in D-luc. For example, Branchini and co-workers synthesized a naphthyluciferin variant (Scheme 1, R = OH) and demonstrated its ability to produce light with Fluc.<sup>[21]</sup> Additional modifications to the naphthalene core were also pursued. Nagano and colleagues synthesized naphthyluciferins bearing amine substituents in place of the C6' hydroxy group (**NAL**, **NDMAL**).<sup>[22]</sup> **NAL** and **NDMAL** were robust emitters with Fluc, making them well suited for biological application. The probes also exhibited red-shifted emission compared to the parent naphthyluciferin. Similar changes in spectral output and binding have been observed upon amination of other Fluc substrates. [13, 23, 24]

Given the robustness of the naphthylamino analogs and their unique aromatic cores, we reasoned that they would be useful for orthogonal imaging. To our knowledge, though, **NAL** and **NDMAL** have yet to be applied in cellular contexts. We thus aimed to prepare these luciferins and examine their utility for multi-component cellular imaging. We also decided to synthesize an analog comprising a pyrrolidine (**NPAL**) substituent. Such cyclic amino motifs have been previously shown to enhance luciferin binding to complementary luciferases.<sup>[13,14,25]</sup> To access the panel of probes, we devised a short and modular route. Previous attempts to prepare N-substituted naphthylamino luciferins relied on double reductive amination of **1a**,<sup>[22]</sup> yielding complex mixtures of both mono- and di-functionalized products. To circumvent this issue, we envisioned using a Bucherer reaction to install diverse groups at C6' (Scheme 1).<sup>[26]</sup> Simply changing the amine base could thus provide the desired structures. Using this approach, dimethylamine and pyrrolidine were successfully leveraged to prepare analogs **1b** and **1c**, respectively. The amino products were then subjected to copper-mediated cyanation to install the requisite nitriles. Condensation with D-cysteine ultimately afforded the panel of desired probes.

### Naphthylamino luciferins are competent light emitters *in vitro* and *in cellulo*

Once synthesized, the naphthylamino luciferins were tested with purified Fluc to verify activity. All three probes provided robust photon outputs (Figure 2), corroborating previous observations. **NDMAL** and **NPAL** emitted the most light with the native enzyme. The light output from **NPAL** was detected even at picomolar concentrations (Figure S1), suggesting that the probe is suitable for sensitive imaging applications. Collectively, these data indicated that the naphthylamino luciferins were robust emitters and suitable candidates for developing unique bioluminescent pairs.

We next investigated whether the naphthylamino luciferins would be sufficiently cell permeable for biological use. The scaffolds were incubated with multiple cell types stably expressing Fluc, and bioluminescent outputs were recorded (Figure 3, S2-S3). Light emission was observed with all three substrates. **NPAL** and **NDMAL** exhibited light on par with D-luc at saturating concentrations. At low concentrations, photon outputs for **NPAL** and **NDMAL** were larger than those of D-luc (Figure S2-S3). These results are significant as several luciferin analogs are orders of magnitude dimmer than the native substrate and thus not suitable for the most sensitive imaging applications. High concentrations of **NDMAL** resulted in decreased photon outputs likely due to product inhibition. **NAL** exhibited the

least amount of light among the naphthylamino analogs, likely due to reduced permeability. Cellular light emission of the analogs was also monitored over time (Figure 3B) and in a tissue mimic (Figure S4). In all cases, sustained photon outputs were observed.

### Structurally diverse analogs enable orthogonal imaging

The robust activity of naphthylamino luciferins *in cellulo* suggested that they were good candidates for multiplexed imaging, provided that unique (i.e., orthogonal) enzymes to process the luciferins could be identified. Toward this end, the luciferins were screened against a 220-member library of engineered luciferases (Figure S6).<sup>[12]</sup> Each mutant was tested and light emission was recorded (Figure S7). Several enzymes capable of discriminating the naphthylamino luciferins were identified using a cross-compare algorithm (Figure S7).<sup>[12]</sup> One set of orthogonal enzymes (mutants 20 and 83) was further analyzed in a multi-component format. Each of the luciferases was treated with the distinct luciferins (Figure 4). **NAL** (preferentially processed by mutant 20) was administered first, and signal was recorded. **NDMAL** (preferentially processed by mutant 83) was then immediately added, and a second image was acquired. The change in light emission upon **NDMAL** addition enabled the pairs to be readily resolved using a published algorithm.<sup>[27]</sup> Interestingly, the same set of mutants could not be distinguished using 6'-aminoluciferin and 6'-dimethylamino luciferin (the benzothiazole equivalents of **NAL** and **NDMAL**, Figure S8). These results suggest a key role for the naphthalene ring in selective enzyme processing. The naphthylamino probes are also structurally distinct from (and thus compatible with) previously synthesized analogs, enabling expanded multiplexed imaging. As shown in Figure 5, **NDMAL** and one of its complementary luciferases (mutant 12) could be used in conjunction with two existing luciferins (**PhOH-Luc**<sup>[20]</sup> and **7'Pyr-Luc**<sup>[16]</sup>) and their associated luciferases (mutants 207 and 213, respectively). Sequential addition of the unique substrates to the mutant samples enabled triple-component imaging in under 30 minutes.

### Conclusion

We demonstrated that naphthylamino luciferins are viable substrates for multi-component imaging with orthogonal luciferases. These probes comprise unique structures and exhibit robust light emission profiles, making them well suited for cellular application. In this work, three analogs were synthesized and characterized using both *in vitro* and cell-based assays. Screening studies revealed luciferase mutants capable of selectively processing the analogs. Pairs of the mutants could be discriminated based on their unique patterns of light emission with the analogs. The naphthylamino luciferins were also used in conjunction with existing bioluminescent probes for rapid triple-component imaging. Collectively, the naphthylamino probes add to the growing collection of bioluminescent probes for multiplexed detection.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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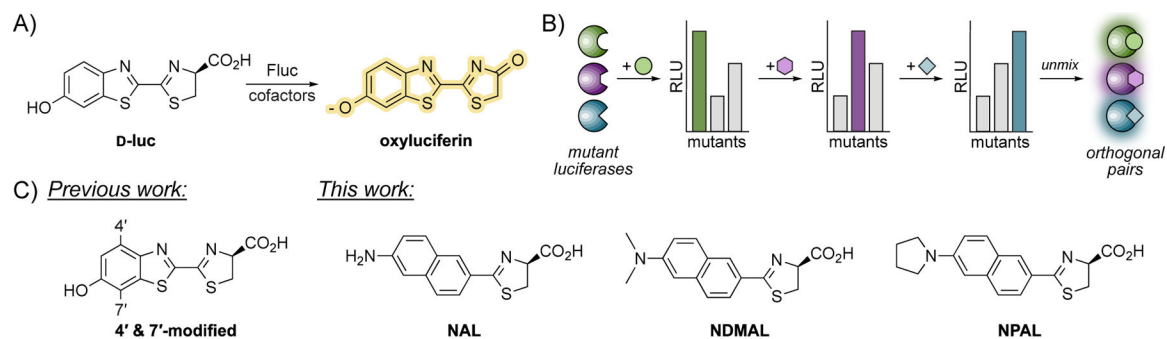
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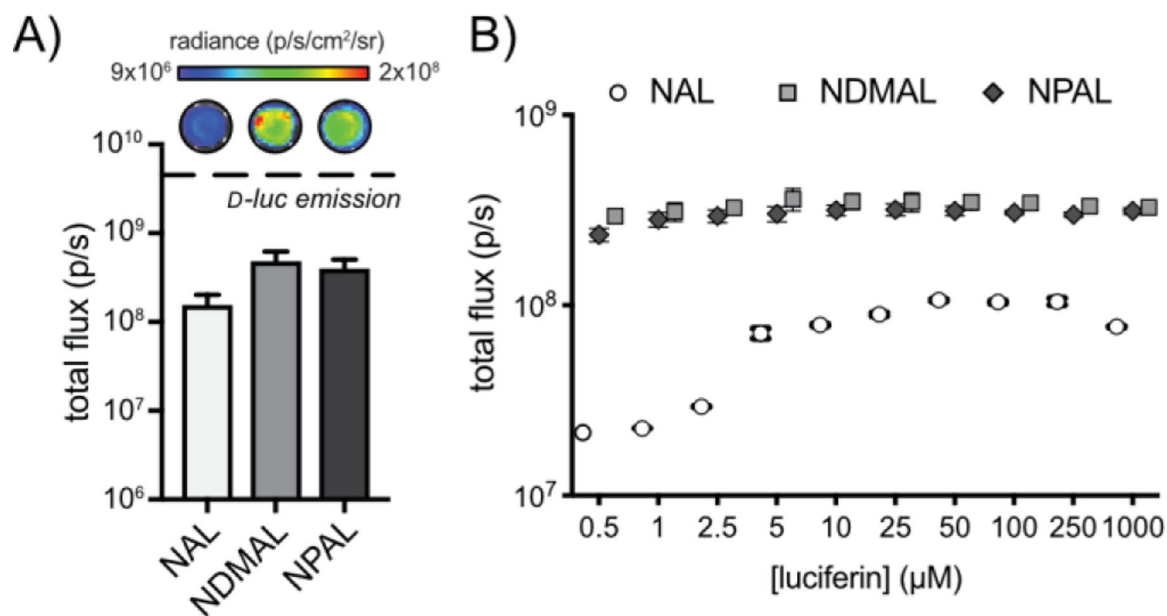
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**Figure 1. Bioluminescence imaging with unique luciferases and luciferins.**

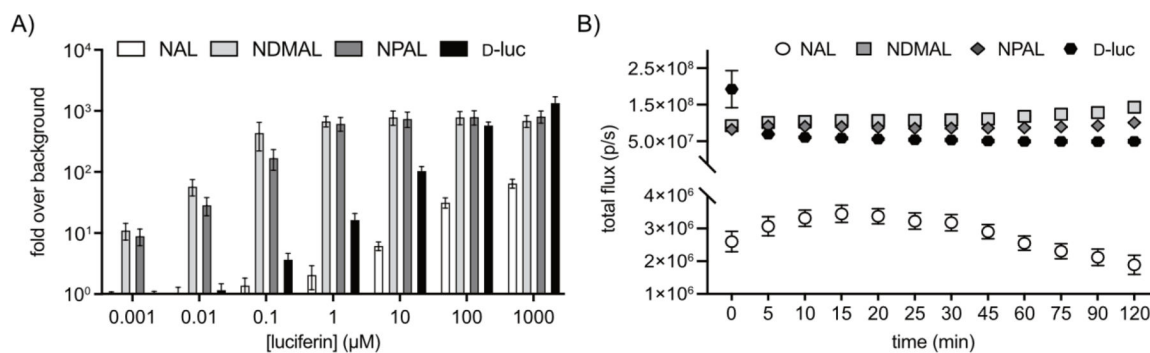
(A) Firefly luciferase (Fluc) catalyzes the transformation of D-luciferin (D-luc) to oxyluciferin, resulting in light emission. (B) Distinct luciferases can be differentiated upon sequential luciferin administration. Changes in bioluminescent intensity can be used to resolve the identities of unique luciferin-luciferase pairs. (C) Previous work evaluated sterically modified luciferins. This work showcases naphthylamino luciferins for multi-component imaging.





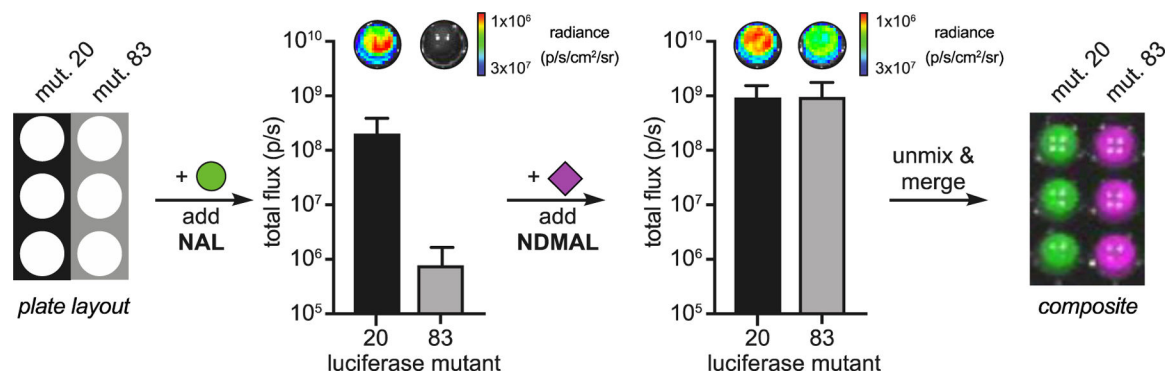
**Figure 2. Naphthylamino luciferins are competent light emitters.**

(A) Bioluminescent light emission from naphthylamino luciferins and D-luc (100  $\mu\text{M}$ ) incubated with Fluc (1.2  $\mu\text{g}$ ). Error bars represent the standard deviation of the mean for  $n=3$  measurements. (B) Naphthylamino luciferins emit light over a broad range of concentrations. NAL, NDMAL, and NPAL (0–1 mM) were incubated with recombinant Fluc (1.2  $\mu\text{g}$ ) and light emission was recorded. Error values represent the standard deviation of the mean for  $n=3$  measurements.



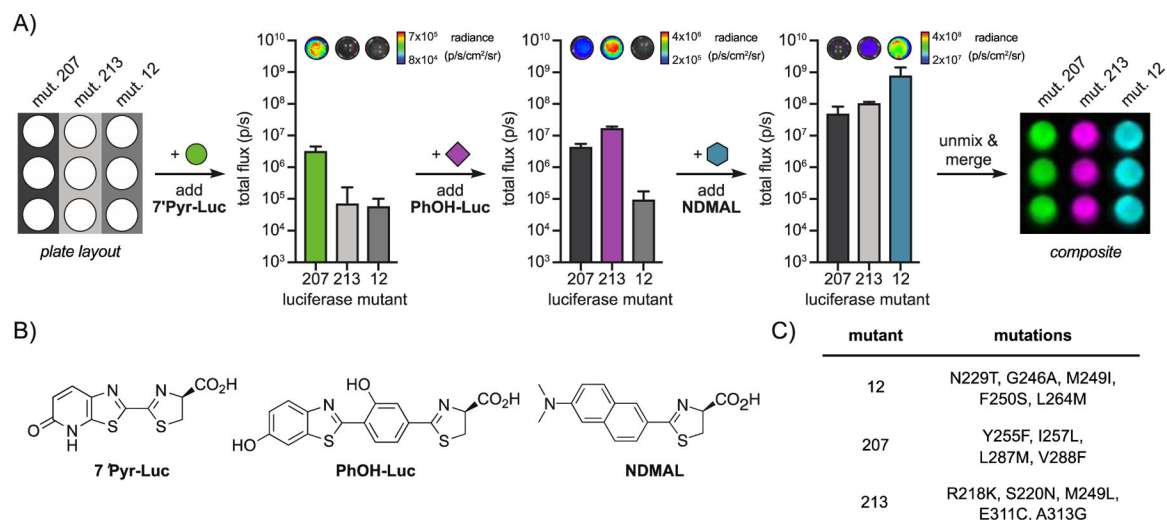
**Figure 3. Naphthylamino luciferins emit light *in cellulo*.**

(A) NAL, NDMAL, NPAL, and D-luc were incubated with Fluc-expressing HEK cells at multiple concentrations. Data represent light emission measured 20 minutes post-luciferin administration. Error bars represent the standard deviation of the mean for n=3 measurements. (B) Light emission was sustained for >2 h at all concentrations examined. Data shown are from cells treated with 100 μM luciferin. Error values represent the standard deviation of the mean for n=3 measurements.



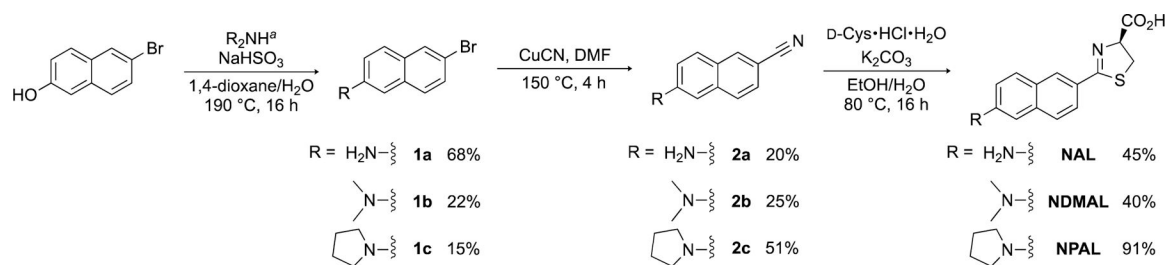
**Figure 4. Mutant luciferases can discriminate naphthylamino luciferins.**

(A) *E. coli* cells expressing mutant 20 or 83 were lysed, treated with luciferin analogs, and imaged. The mutants were resolved upon sequential addition of the corresponding substrates. **NAL** was administered to both mutants first, followed by **NDMAL**. The images were unmixed and false colored to create a composite image. Error bars represent the standard deviation of the mean for  $n=3$  measurements.



**Figure 5: Triplet set of orthogonal pairs.**

(A) *E. coli* cells expressing mutant 207, 213, or 12 were lysed and imaged. The mutants were resolved upon sequential addition of the corresponding substrates. **7'Pyr-Luc** was administered to all mutants first, followed by **PhOH-Luc** and **NDMAL**. These images were unmixed and false colored to create a composite image. Error bars represent the standard deviation of the mean for  $n=3$  measurements. (B) Structures of luciferin analogs used in this experiment. (C) Mutants used for tri-component imaging.



<sup>a</sup>NH<sub>4</sub>OH for **1a**, Me<sub>2</sub>NH (40% aq) for **1b**, pyrrolidine for **1c**.

**Scheme 1.**  
Synthesis of naphthylamino luciferins.