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## Bioluminescent probes for imaging biology beyond the culture dish

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### Abstract

Bioluminescence with luciferase-luciferin pairs is an attractive method for surveying cells in live tissues and whole organisms. Recent advances in luciferin chemistry and luciferase engineering are further expanding the scope of the technology. It is now possible to spy on cells in a variety of deep tissues and visualize multicellular interactions—feats that are enabling new questions to be asked and new ideas to be explored. This perspective piece highlights recent successes in bioluminescent probe development and their applications to imaging in live cells, tissues, and animals.

### Keywords

Bioluminescence; luciferin; luciferase; imaging; coelenterazine

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Imaging tools enable researchers to “see” inside tissues and cells and monitor biological features in real time. While powerful, most of these probes are confined to monitoring cellular behaviors at the micro scale—in culture dishes and on slides. Visualizing cellular behaviors in more authentic environments requires tools that can function across larger spatial and time scales.<sup>1</sup> Few probes fit the bill, considering the demands placed on biocompatibility and sensitivity in whole tissues and animals. Consequently, basic questions regarding multicellular interactions in immune function, cell migration, and other physiological processes remain unanswered.

Bioluminescent probes can address the need for sensitive imaging on the macro scale. These tools derive from naturally glowing organisms (e.g., fireflies). All bioluminescent species produce light via the luciferase-catalyzed oxidation of a small-molecule luciferin. Luciferase enzymes and luciferin substrates can be imported into diverse cell types and engineered to

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#### Author Contributions

The manuscript was written through contributions of all authors.

report on biological processes.<sup>2</sup> Bioluminescent signal is inherently weak (especially when compared to conventional fluorescence imaging), but there is virtually no background emission. Fluorescence imaging, by contrast, relies on light-based excitation sources that can induce tissue autofluorescence and result in poor signal-to-noise ratios. Since bioluminescence requires no excitation light, it can enable exquisitely sensitive imaging even in heterogeneous tissues. In fact, bioluminescent probes can be preferred to fluorescent tools for long-term cell tracking in rodent and other opaque models. One can serially image luciferase reporters without detriment to organisms and without knowing “when and where” to apply excitation light.

The versatility of bioluminescence has enabled a broad range of biological studies, although limitations persist.<sup>2</sup> This imaging modality has long been plagued by a lack of bright, easily distinguishable probes and poor spatial resolution. However, advances in luciferin chemistry and luciferase engineering have begun to address these issues. This perspective will highlight recent achievements in developing new and improved imaging tools. Collectively, the bioluminescent probes are addressing long-standing voids in imaging capabilities and are being applied to “seeing” biology beyond the culture dish.

## Bioluminescence basics

Millions of luciferases exist in the natural world, but phylogenetically-related enzymes use the same luciferin.<sup>3</sup> The identities of such luciferins remain exceedingly difficult to characterize, and of those reported to date, only two have found routine application in cellular imaging.<sup>2</sup> The first, D-luciferin (Figure 1A), is used by the firefly and a number of other terrestrial organisms to produce light. The second, coelenterazine (Figure 1B), is a molecule found in bioluminescent sea creatures, including the sea pansy *Renilla reformis* and deep-sea copepods *Gaussia princeps* and *Oplophorus gracilirostris*. Despite their distinct chemical structures, D-luciferin and coelenterazine undergo a similar mechanism to produce light: the cognate luciferases oxidize the small molecules to generate excited state oxyluciferins; relaxation of these molecules to the ground state results in photon emission (Figure 1).

The color of light released is primarily dictated by the small molecule luciferin. In the case of oxyluciferin, the aromatic core is sufficiently extended to provide yellow-green (~560 nm) light. For coelenterazine, the pi system of the putative emitter is shorter, resulting in blue wavelengths (~475 nm) of emission. The luciferase environment can further modulate bioluminescent color. Thus, enzymes that use the exact same substrate can emit different wavelengths. In some cases, the emission spectra are sufficiently resolved to enable multicolor imaging.<sup>4</sup> Discriminating among related luciferases in vivo, though, remains exceedingly difficult due to their broad emission spectra and the strong absorption of <650 nm light.<sup>5</sup>

## Building better bioluminescent tools

Luciferases and luciferins have been used for decades to image biomolecules, gene expression, and even whole cells, but the most popular probes are not without limitation.<sup>2</sup>

The poor tissue penetrance of many luciferins has historically precluded sensitive imaging in hard-to-access tissues (e.g., brain). Additionally, only a limited “palette” of bioluminescent probes exists, hindering efforts to visualize multi-cellular or other multi-component processes. Chemical and biological approaches are beginning to address the need for improved and more diverse collections of bioluminescent probes.

### Streamlined and scalable luciferin syntheses

Most bioluminescence imaging studies require an exogenously delivered luciferin. For in vivo work, the doses can range between 1-5 mg per mouse per imaging point. Thus, large—and often prohibitively expensive—quantities of luciferin are required for animal studies. Accessing luciferins in bulk has been a historical challenge. The chemical complexity of D-luciferin and coelenterazine has also frustrated efforts to rapidly diversify these scaffolds to produce new analogs.

In recent years, improved methods to produce luciferins have been reported. In the case of D-luciferin, we developed a streamlined method to access the requisite benzothiazole core using Appel’s salt and modern C–H activation (Figure 2).<sup>6</sup> This approach improved the yield and generalizability of the synthesis, eliminating the need for multiple functional group conversions used in previous routes. The route has enabled multi-gram synthesis of D-luciferin, and has proven to be quite modular. In fact, we and others have used this chemistry to produce over 30 distinct analogs.<sup>7</sup> Many of these scaffolds also comprise key functional handles for late-stage diversification (Figure 2). Efforts to expediently synthesize amino luciferins<sup>8</sup> and related analogs<sup>9</sup> are similarly facilitating more widespread use of bioluminescent tools.

Improved methods to access coelenterazine analogs are also advancing imaging studies. Historically, coelenterazine synthesis relied on condensation of glyoxal derivatives with strong acid and elevated temperatures. These conditions are incompatible with a diverse array of desired analogs. Kirkland and colleagues identified milder conditions to access the coelenterazine core via Horner-Wadsworth-Emmons olefination.<sup>10</sup> The method proved to be both scalable and generalizable, providing a range of useful analogs. Recent advances in cross-coupling methodology are similarly enabling access to diverse coelenterazine architectures.<sup>11</sup>

### Brighter and more colorful probes

Bioluminescence imaging to date has been largely confined to imaging one or two colors at a time. Accessing a full spectrum of emitters has been a long-standing goal, with obvious parallels to fluorescence imaging. Early efforts to modulate color and brightness primarily focused on the luciferase enzyme.<sup>2</sup> Recent years, though, have seen a shift in focus to modulating the small molecule (Figure 3).

Since the structure of the luciferin emitter influences bioluminescent output, methods to modulate color and intensity often parallel those in small molecule fluorophore development. For example, rigidifying the aromatic core can provide improved “push-pull” chromophores and enhanced light emission. Miller and coworkers applied this strategy to D-luciferin, replacing the rotationally-flexible 6’ electron donating group with a

conformationally-locked amine.<sup>12</sup> This configuration enabled enhanced electron donation into the luciferin core and more robust, red-shifted emission compared to related probes. Moreover, many of the cyclic luciferin (CycLuc) scaffolds were viable substrates for Fluc, highlighting the promiscuity of the enzyme.

Fluc can also process luciferin analogs with extended aromatic cores and modified heteroatoms, providing additional avenues for wavelength alteration.<sup>6,7,13</sup> Pi-extended chromophores typically emit red-shifted light emission.<sup>14</sup> In the case of the vinyl extended luciferins (Figure 3), the shift was over 100 nm. It should be noted, though, that these and other extended analogs are often poorly processed by Fluc itself; red-shifted emission comes at the expense of enzyme turnover and thus total photon output.

Parallel developments in coelenterazine synthesis have provided scaffolds that emit different colors of light. Red-shifted probes are particularly desirable for imaging with Rluc and Gluc *in vivo*, as the “normal” blue emission with these enzymes is strongly absorbed by blood.<sup>5</sup> Inouye and colleagues recently developed a streamlined synthesis of a conformationally-locked coelenterazine for improved imaging.<sup>11</sup> Others have explored luminophores with extended pi conjugation to achieve more tissue-penetrant wavelengths of light.<sup>15,16</sup>

Bioluminescent color and photon output can also be drastically modulated via bioluminescent resonance energy transfer (BRET). A recent fusion of NanoLuc (an optimized mutant luciferase, discussed below) to CyOPF1 (dubbed Antares, Figure 4A) resulted in an emission shift of 114 nm (Figure 4B).<sup>17</sup> Antares is one of the most red-shifted BRET pairs reported to date, enabling enhanced deep-tissue imaging *in vivo* (Figure 4C). In related work, Nagai and colleagues linked a mutant *Renilla* luciferase to Venus, a version of yellow fluorescent protein<sup>18</sup> Unexpectedly, this construct (dubbed Nano-lantern) produced six-fold more photons than the luciferase alone and shifted emission into the yellow-green region (530 nm). Additional Nano-lantern colors have been described.<sup>4</sup> Collectively, these and other BRET probes are enabling sensitive imaging of many biological processes, including membrane voltage,<sup>19</sup> and gene expression.<sup>20</sup>

### In vivo improvements

Luciferins can access most tissues, but their distribution and cell permeability is suboptimal and not uniform.<sup>22</sup> Chemical tinkering and med-chem optimization can improve the bioavailabilities of the substrates. For example, many of the CycLuc scaffolds that comprise more lipophilic amino modifications (vs. –OH groups) exhibit improved tissue penetrance *in vivo*.<sup>23</sup> Far less compound is required for a given imaging session compared to native luciferins. Further amidation of a CycLuc probe enhanced its transport across the blood-brain barrier (Figure 5).<sup>21</sup> Once in the brain, the amide bond can be cleaved by endogenous fatty acid amide hydrolase (highly expressed in brain tissue), releasing a functional luciferin.

### Luciferase-luciferin pairs for multicellular imaging

Many improvements to the luciferin small molecules noted above came at the expense of luciferase turnover (and thus light output). Consequently, recent efforts to build improved bioluminescence tools have focused on identifying new substrates and enzymes in parallel.

One source of new luciferase-luciferin pairs is nature itself. Bioluminescent organisms (and potentially new luciferase-luciferin pairs) are continually being described,<sup>24</sup> though the need for optimized probes far outpaces their discovery. Others have turned to engineering existing luciferases to better use chemically modified luciferins. In one example, Miller identified mutant luciferases that can more readily process CycLuc derivatives. These analogs were previously demonstrated to be viable substrates for Fluc, although oxyluciferin products inhibited the reaction. Substrate inhibition was relieved and long-lived light emission restored with mutant enzymes.<sup>25</sup> The authors later identified a mutant that preferred a luciferin analog over D-luciferin, setting the stage for developing substrate-responsive enzymes.<sup>26</sup> Further work by the Miller group revealed “latent” luciferase activity in a fatty acyl-CoA synthetase from the fruitfly.<sup>27</sup> Interestingly, this enzyme did not emit light with D-luciferin—it was only able to use a CycLuc substrate—opening the door to pairing unnatural luciferin analogs with evolutionary relatives of luciferase.

Another engineered bioluminescent enzyme that has seen widespread adoption in recent years is NanoLuc, a derivative of *Oplophorus* luciferase (Oluc).<sup>28</sup> Early work in this area was motivated by the need for improved coelenterazines (molecules prone to autooxidation and exhibiting poor tissue penetrance) and Oluc subunits (enzyme fragments prone to instability). Seeking a brighter and more stable luciferin, Wood and colleagues replaced the electron-rich phenols of coelenterazine with phenyl and furan groups. The resulting molecule (furimazine, Figure 3) exhibited higher stability in media and lysate and was less susceptible to non-specific oxidation. Directed evolution was used to select an enzyme that could readily catalyze light emission the designer luciferin. The “winning” mutant (NanoLuc) contained a total of 16 mutations, an impressive number for a 16-kDa protein. NanoLuc exhibits a high turnover rate with furimazine, providing robust signal output. Such high photon flux values are enabling sensitive imaging in complex tissue samples—even for point-of-care diagnostics with simple cell phone cameras.<sup>29</sup> We anticipate that the popularity of the NanoLuc-furimazine pair will continue to surge in the near term.

Tandem modification of bioluminescent enzymes and substrates is also enabling multicomponent bioluminescence imaging. In contrast to in vitro assays, imaging in vivo often precludes spectroscopic resolution of colored probes. Blood and tissue restrict the passage of wavelengths shorter than red,<sup>5</sup> and bioluminescence spectra are broad,<sup>31</sup> necessitating an alternative approach. Substrate resolution is one such strategy (Figure 6A). This approach requires multiple selective, mutually orthogonal luciferin-luciferase pairs. These pairs produce light together, but will not react with other mutants or enzymes. Orthogonal pairs already exist in nature (e.g. firefly and sea luciferases, along with their requisite substrates), and many have been adapted for dual imaging studies.

To expand and expedite the search for orthogonal pairs,<sup>30</sup> we turned to producing non-natural analogs and enzymes. We synthesized a panel of luciferin analogs. With additional steric bulk at the 4' and 7' positions (Figure 6B). We screened these analogs against libraries of luciferase mutants to produce more than 3000 potential pairings. To find substrate-resolved hits in this milieu, we mined the data with a computer algorithm. This strategy revealed two enzymes that exhibited substrate resolution with two compounds (one 4' and one 7'). This pair proved successful in mammalian cells as well, demonstrating the

robust nature of our screening methodology (Figure 6C). In an analogous approach, Kim and colleagues recently reported substrate-resolved bioluminescent imaging with coelenterazine analogs.<sup>32</sup>

## Bioluminescent reporters for cell-cell interactions

A second challenge being addressed by improved bioluminescent tools is monitoring cellular interactions *in vivo*. Conventional bioluminescence imaging can detect small numbers of cells, but historically has lacked the spatial resolution to precisely pinpoint their locations or interactions in whole organisms. Target recognition and cell-cell contacts are crucial to numerous physiological processes, including neurotransmission, immune function and cell migration, so methods to globally assay such interactions in whole organisms are necessary.

The development of tools that report on cell-cell contacts has been inspired by classic methods to report on biomolecule activities and interactions. For example, caged probes have been used widely to report on small molecule analytes in cells. We and others<sup>33</sup> have shown that such cages can be re-purposed to image interactions between cells (Figure 7A). In a recent example, a “dark” luciferin comprising a 6′ nitro group (Luntr) was used as the cage.<sup>34</sup> This probe could be reduced by one group of cells (expressing nitroreductase) and used by a neighboring group of cells expressing luciferase. Light emission was strongest in areas of cellular contact. Nitroreductase is not expressed endogenously in mammalian cells, making this technology ripe for *in vivo* applications where high spatial sensitivity is required.

A second class of cell contact sensors uses split reporters, originally developed to image protein-protein interactions (Figure 7B). We expanded on this concept to generate split reporters of cell proximity using *Gaussia* luciferase (Gluc)—a secreted protein that functions in the extracellular space. Split fragments of Gluc were fused to leucine zippers Fos and Jun to drive complementation. The N-terminal half was expressed in one cell population, and the C-terminal in another. Light emission in this case tracked with distance between the cell populations (Figure 7C).

## Bioluminescent sensors for analyte detection *in vivo*

Bioluminescence has long been exploited for detecting enzyme activities and low-abundant metabolites. The majority of these studies, though, have been limited to *ex vivo* analyses with cultured cells or excised tissues. Continued advances in bioluminescence technology are enabling new probes to be applied as biosensors *in vivo*.<sup>36,37</sup> In a recent example, Chang and colleagues developed a copper ion sensor for imaging in mice. The probe comprised a caged luciferin, with a bulky chelator group (i.e., the “cage”) attached to the 6′-position of D-luciferin. The sterically encumbered molecule was poorly utilized by luciferase. Upon removal of the cage by copper(I)-dependent oxidative cleavage, a viable luciferin was liberated and available for light emission. Photon production could thus be correlated to copper ion levels. The caged probe was ultimately used for analyte imaging in mouse models of fatty liver disease.<sup>36</sup>

## Conclusions and future directions

Bioluminescence has historically lagged behind fluorescence imaging in terms of the breadth and diversity of available tools. Recent advances in luciferin chemistry and luciferase engineering, though, are beginning to fill this gap. New synthetic methods are providing novel luciferin architectures for improved imaging. Engineered luciferases are enabling the sensitive detection of cells and other analytes *in vivo*. Combinations of designer substrates and mutant enzymes are furthering the range of potential applications. It is now possible to image multicellular features in live animals, visualize cells in difficult-to-access tissues (e.g., brain tissue), and selectively illuminate cell-cell interactions. Moving forward, we anticipate continued advances in red-shifted probes, tandem luciferase-luciferin engineering, and sensors for cellular metabolites. These tools will influence how researchers conduct experiments involving multiple cell types and molecular features beyond the culture dish. Additionally, like other useful imaging agents, the tools will likely facilitate discoveries in a diverse range of fields.

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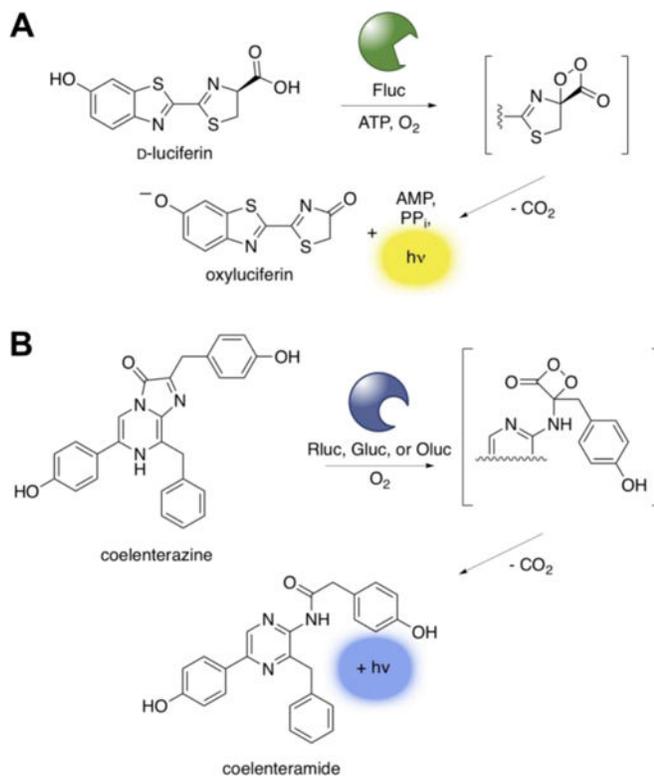
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## References

1. Porterfield WB, Prescher JA. Tools for visualizing cell-cell ‘interactomes’. *Curr Opin Chem Biol.* 2015; 24:121–130. [PubMed: 25461730]
2. Paley MA, Prescher JA. Bioluminescence: A versatile technique for imaging cellular and molecular features. *Med Chem Comm.* 2014; 5:255–267.
3. Martini S, Haddock SHD. Quantification of bioluminescence from the surface to the deep sea demonstrates its predominance as an ecological trait. *Sci Rep.* 2017; 7:45750. [PubMed: 28374789]
4. Suzuki K, Kimura T, Shinoda H, Bai G, Daniels MJ, Arai Y, Nakano M, Nagai T. Five colour variants of bright luminescent protein for real-time multicolour bioimaging. *Nat Commun.* 2016; 7:13718. [PubMed: 27966527]
5. Zhao H, Doyle TC, Coquoz O, Kalish F, Rice BW, Contag CH. Emission spectra of bioluminescent reporters and interaction with mammalian tissue determine the sensitivity of detection *in vivo*. *J Biomed Opt.* 2005; 10:041210.
6. McCutcheon DC, Paley MA, Steinhardt RC, Prescher JA. Expedient synthesis of electronically modified luciferins for bioluminescence imaging. *J Am Chem Soc.* 2012; 134:7604–7607. [PubMed: 22519459]
7. Woodroffe CC, Meisenheimer PL, Klaubert DH, Kovic Y, Rosenberg JC, Behney CE, Southworth TL, Branchini BR. Novel heterocyclic analogues of firefly luciferin. *Biochemistry.* 2012; 51:9807–9813. [PubMed: 23164087]
8. Hauser JR, Beard HA, Bayana ME, Jolley KE, Warriner SL, Bon RS. Economical and scalable synthesis of 6-amino-2-cyanobenzothiazole. *Beilstein J Org Chem.* 2016; 12:2019–2025. [PubMed: 27829906]
9. Anderson JC, Grounds H, Jathoul AP, Murray JAH, Pacman SJ, Tisi L. Convergent synthesis and optical properties of near-infrared emitting bioluminescent infra-luciferins. *RSC Adv.* 2017; 7:3975–3982. [PubMed: 28496975]
10. Shakhmin A, Hall MP, Walker JR, Machleidt T, Binkowski BF, Wood KV, Kirkland TA. Three efficient methods for preparation of coelenterazine analogues. *Chem—Eur J.* 2016; 22:10369–10375. [PubMed: 27305599]

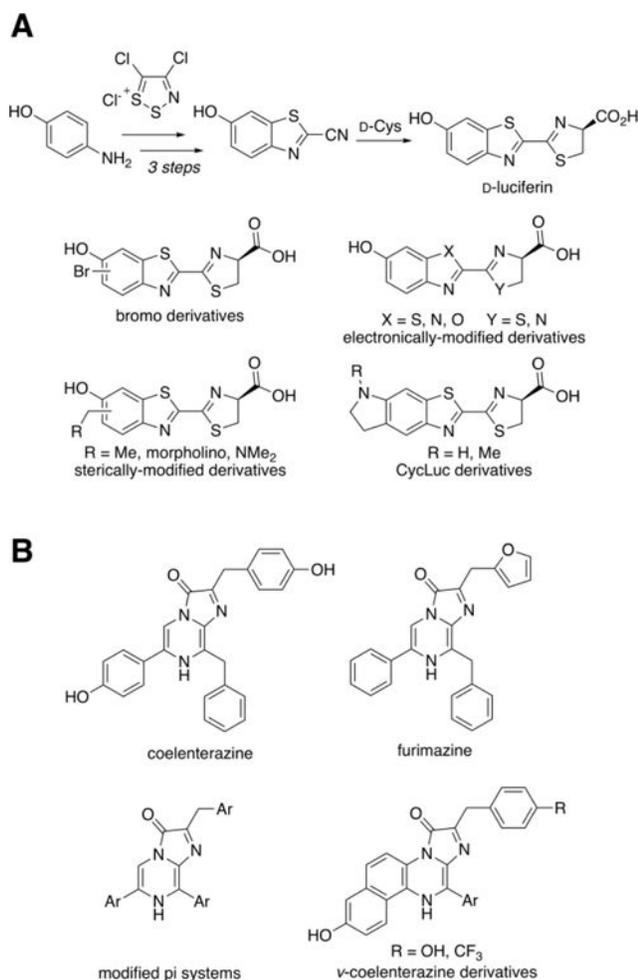
11. Hosoya T, Iimori R, Yoshida S, Sumida Y, Sahara-Miura Y, Sato JI, Inouye S. Concise synthesis of  $\nu$ -coelenterazines. *Org Lett.* 2015; 17:3888–3891. [PubMed: 26194904]
12. Reddy GR, Thompson WC, Miller SC. Robust light emission from cyclic alkylaminoluciferin substrates for firefly luciferase. *J Am Chem Soc.* 2010; 132:13586–13587. [PubMed: 20828122]
13. Conley NR, Dragulescu Andrași A, Rao J, Moerner WE. A selenium analogue of firefly D-luciferin with red-shifted bioluminescence emission. *Angew Chem Int Ed.* 2012; 51:3350–3353.
14. Kuchimaru T, Iwano S, Kiyama M, Mitsumata S, Kadonosono T, Niwa H, Maki S, Kizaka-Kondoh S. A luciferin analogue generating near-infrared bioluminescence achieves highly sensitive deep-tissue imaging. *Nat Commun.* 2016; 7:11856. [PubMed: 27297211]
15. Nishihara R, Suzuki H, Hoshino E, Suganuma S, Sato M, Saitoh T, Nishiyama S, Iwasawa N, Citterio D, Suzuki K. Bioluminescent coelenterazine derivatives with imidazopyrazinone C-6 extended substitution. *Chem Commun.* 2014; 51:391–394.
16. Grinstead, KM., Rowe, L., Ensor, CM., Joel, S., Daftarian, P., Dikici, E., Zingg, JM., Daunert, S. Red-shifted Aequorin variants incorporating non-canonical amino acids: Applications in *in vivo* imaging. In: Jones, DD., editor. *PLoS ONE*. Vol. 11. 2016. p. e0158579
17. Chu J, Oh Y, Sens A, Ataie N, Dana H, Macklin JJ, Laviv T, Welf ES, Dean KM, Zhang F, Kim BB, Tang CT, Hu M, Baird MA, Davidson MW, Kay MA, Fiolka R, Yasuda R, Kim DS, Ng HL, Lin MZ. A bright cyan-excitable orange fluorescent protein facilitates dual-emission microscopy and enhances bioluminescence imaging in vivo. *Nat Biotechnol.* 2016; 34:760–767. [PubMed: 27240196]
18. Saito K, Chang YF, Horikawa K, Hatsugai N, Higuchi Y, Hashida M, Yoshida Y, Matsuda T, Arai Y, Nagai T. Luminescent proteins for high-speed single-cell and whole-body imaging. *Nat Commun.* 2012; 3:1262. [PubMed: 23232392]
19. Inagaki S, Tsutsui H, Suzuki K, Agetsuma M, Arai Y, Jinno Y, Bai G, Daniels MJ, Okamura Y, Matsuda T, Nagai T. Genetically encoded bioluminescent voltage indicator for multi-purpose use in wide range of bioimaging. *Sci Rep.* 2017; 7:42398. [PubMed: 28205521]
20. Horikiri T, Ohi H, Shibata M, Ikeya M, Ueno M, Sotozono C, Kinoshita S, Sato T. SOX10-Nano-Lantern Reporter Human iPS Cells; A Versatile Tool for Neural Crest Research. *PLoS ONE.* 2017; 12:e0170342. [PubMed: 28107504]
21. Mofford DM, Adams ST, Reddy GSKK, Reddy GR, Miller SC. Luciferin amides enable *in vivo* bioluminescence detection of endogenous fatty acid amide hydrolase activity. *J Am Chem Soc.* 2015; 137:8684–8687. [PubMed: 26120870]
22. Adams ST, Miller SC. Beyond D-luciferin: Expanding the scope of bioluminescence imaging in vivo. *Curr Opin Chem Biol.* 2014
23. Evans MS, Chaurette JP, Adams ST, Reddy GR, Paley MA, Aronin N, Prescher JA, Miller SC. A synthetic luciferin improves bioluminescence imaging in live mice. *Nat Methods.* 2014; 11:393–395. [PubMed: 24509630]
24. Purtov KV, Petushkov VN, Baranov MS, Mineev KS, Rodionova NS, Kaskova ZM, Tsarkova AS, Petunin AI, Bondar VS, Rodicheva EK, Medvedeva SE, Oba Y, Oba Y, Arseniev AS, Lukyanov S, Gitelson JI, Yampolsky IV. The chemical basis of fungal bioluminescence. *Angew Chem, Int Ed.* 2015; 54:8124–8128.
25. Harwood KR, Mofford DM, Reddy GR, Miller SC. Identification of mutant firefly luciferases that efficiently utilize aminoluciferins. *Chem Biol.* 2011; 18:1649–1657. [PubMed: 22195567]
26. Mofford DM, Reddy GR, Miller SC. Aminoluciferins extend firefly luciferase bioluminescence into the near-infrared and can be preferred substrates over D-luciferin. *J Am Chem Soc.* 2014; 136:13277–13282. [PubMed: 25208457]
27. Mofford DM, Reddy GR, Miller SC. Latent luciferase activity in the fruit fly revealed by a synthetic luciferin. *Proc Natl Acad Sci USA.* 2014; 111:4443–4448. [PubMed: 24616520]
28. Hall MP, Unch J, Binkowski BF, Valley MP, Butler BL, Wood MG, Otto P, Zimmerman K, Vidugiris G, Machleidt T, Robers MB, Benink HA, Eggers CT, Slater MR, Meisenheimer PL, Klaubert DH, Fan F, Encell LP, Wood KV. Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. *ACS Chem Biol.* 2012; 7:1848–1857. [PubMed: 22894855]

29. Griss R, Schena A, Reymond L, Patiny L, Werner D, Tinberg CE, Baker D, Johnsson K. Bioluminescent sensor proteins for point-of-care therapeutic drug monitoring. *Nat Chem Biol.* 2014; 10:598–603. [PubMed: 24907901]
30. Jones KA, Porterfield WB, Rathbun CM, McCutcheon DC, Paley MA, Prescher JA. Orthogonal luciferase-luciferin pairs for bioluminescence imaging. *J Am Chem Soc.* 2017; 139:2351–2358. [PubMed: 28106389]
31. Romyantsev KA, Turoverov KK, Verkhusha VV. Near-infrared bioluminescent proteins for two-color multimodal imaging. *Sci Rep.* 2016; 6:36588. [PubMed: 27833162]
32. Nishihara R, Abe M, Nishiyama S, Citterio D, Suzuki K, Kim SB. Luciferase-specific coelenterazine analogues for optical contamination-free bioassays. *Sci Rep.* 2017; 7:908. [PubMed: 28424463]
33. Lindberg E, Mizukami S, Ibata K, Miyawaki A, Kikuchi K. Development of luminescent coelenterazine derivatives activatable by  $\beta$ -galactosidase for monitoring dual gene expression. *Chem—Eur J.* 2013; 19:14970–14976. [PubMed: 24105816]
34. Porterfield WB, Jones KA, McCutcheon DC, Prescher JA. A “caged” luciferin for imaging cell-cell contacts. *J Am Chem Soc.* 2015; 137:8656–8659. [PubMed: 26098396]
35. Jones KA, Li DJ, Hui E, Sellmyer MA, Prescher JA. Visualizing cell proximity with genetically encoded bioluminescent reporters. *ACS Chem Biol.* 2015; 10:933–938. [PubMed: 25643167]
36. Heffern MC, Park HM, Au-Yeung HY, Van de Bittner GC, Ackerman CM, Stahl A, Chang CJ. In vivo bioluminescence imaging reveals copper deficiency in a murine model of nonalcoholic fatty liver disease. *Proc Natl Acad Sci USA.* 2016; 113:14219–14224. [PubMed: 27911810]
37. Sekar TV, Foygel K, Massoud TF, Gambhir SS, Paulmurugan R. A transgenic mouse model expressing an ER $\alpha$  folding biosensor reveals the effects of bisphenol A on estrogen receptor signaling. *Sci Rep.* 2016; 6:584.



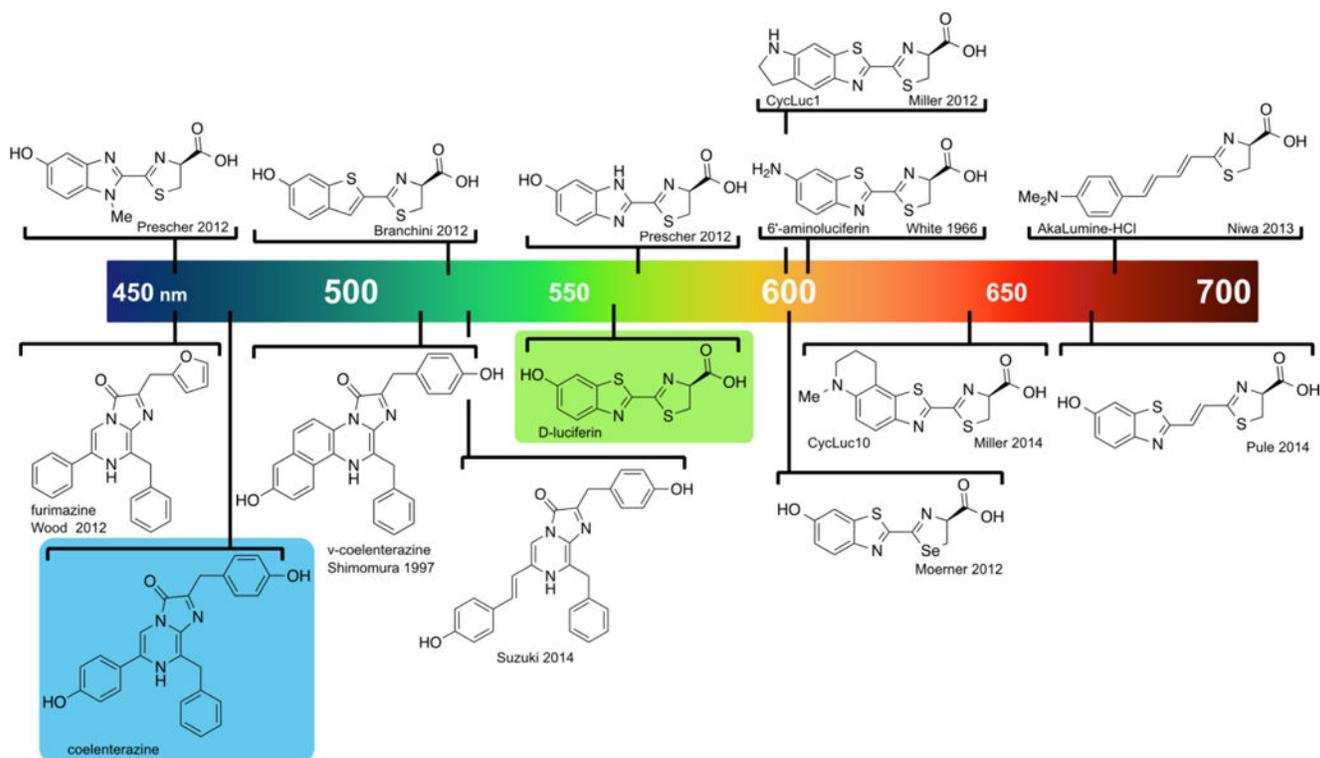
**Figure 1. Popular luciferase-luciferin pairs for cellular imaging**

A) D-Luciferin is oxidized by firefly luciferase (Fluc) to produce oxyluciferin and a photon of light. B) Coelenterazine is oxidized by a variety of marine luciferases, including *Renilla* luciferase (Rluc), *Gaussia* luciferase (Gluc), and *Oplophorus* luciferase (Oluc). These luciferases, unlike Fluc, require only oxygen as a cofactor in the light-emitting reaction.



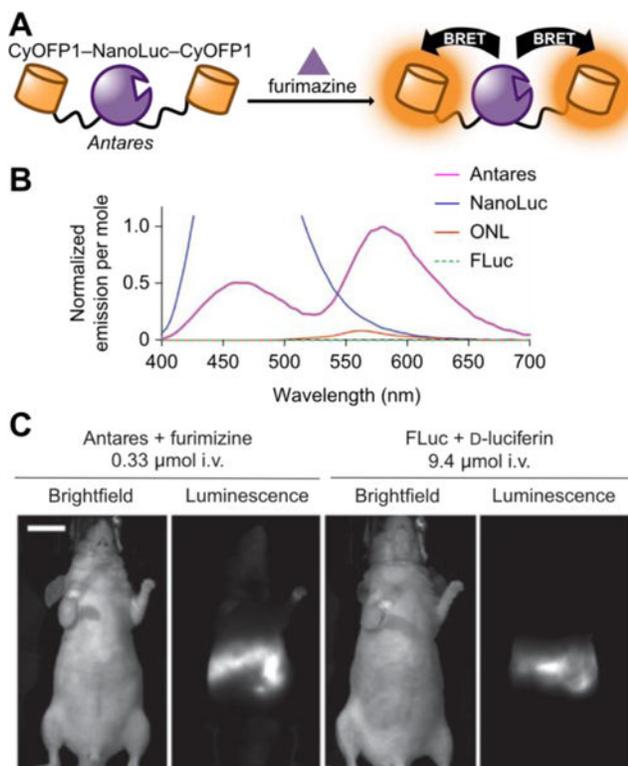
**Figure 2. Recently synthesized luciferin heterocycles**

A) D-Luciferin and related analogs can be accessed using Appel's salt and C-H activation chemistry. B) A diverse array of coelenterazine analogs has been synthesized in recent years.



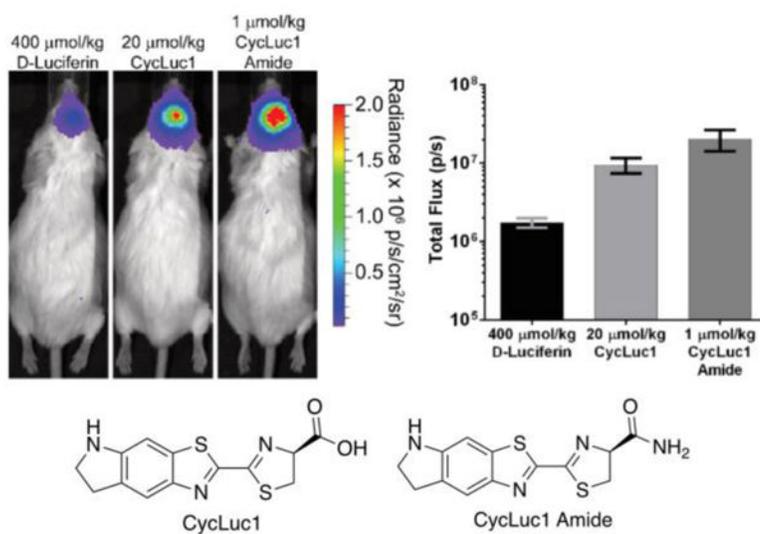
**Figure 3. A “palette” of luciferin analogs**

Native luciferins are highlighted with colored boxes. Brackets denote the wavelength (nm) of maximum bioluminescence emission observed upon incubation of the compound with luciferase. While many analogs can provide unique colors of light, most are not efficiently processed by native luciferases.



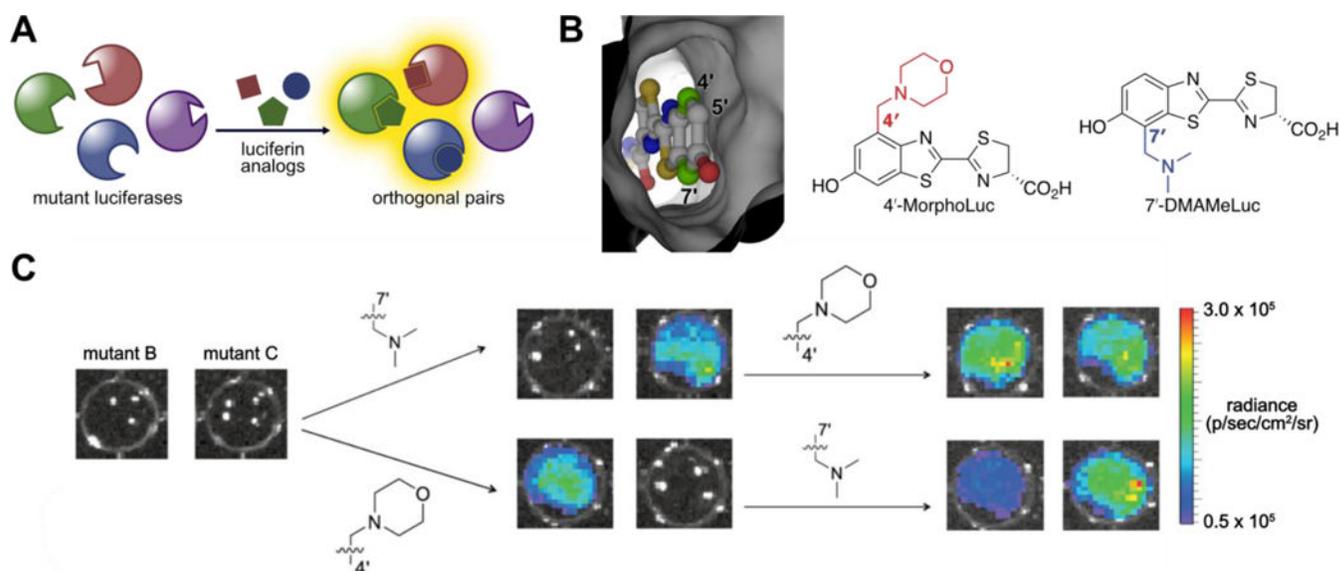
**Figure 4. BRET for deep-tissue imaging**

A) Antares comprises a fusion of NanoLuc with two cyan-excitable orange fluorescent proteins (CyOFP1). B) The BRET fusion exhibits red-shifted emission relative to NanoLuc. ONL: Orange Nano-lantern. C) Antares enabled sensitive imaging with low luciferin levels in mice. Antares and Fluc genes were delivered to the liver via hydrodynamic injection. Luciferins were supplied intravenously. Adapted with permission from ref <sup>17</sup>.



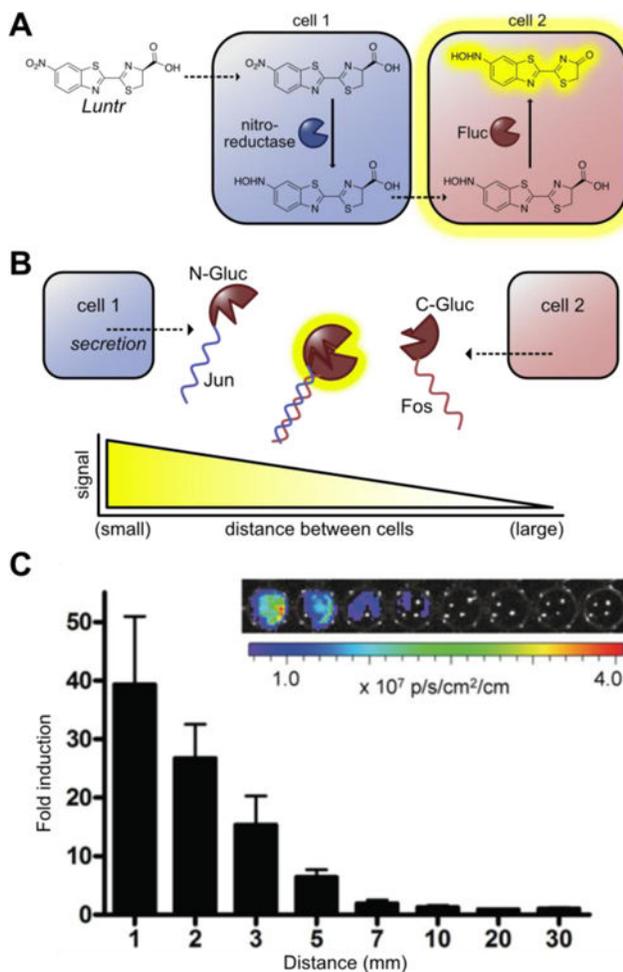
**Figure 5. Tissue-penetrant cyclic aminoluciferins**

(Left) Luciferase expression in brain was achieved via viral transduction. Luciferins were administered via intraperitoneal injection. (Right) Photon flux from mouse brains pictured. Adapted with permission from ref<sup>21</sup>.



**Figure 6. Orthogonal luciferin-luciferase pairs for multicomponent imaging**

A) A strategy for multicomponent bioluminescence imaging with luciferin analogs and mutant luciferases. B) The 4' and 7' sites of D-luciferin were targeted to preclude binding to native Fluc. C) Substrate resolution was achieved in mouse DB7 cells using 4' and 7'-modified luciferins and mutant luciferases identified from screening. Adapted with permission from ref <sup>30</sup>.



**Figure 7. Bioluminescent reporters for cellular interactions**

A) Caged luciferins can report on cell proximity between one cell (expressing an uncaging enzyme) and a second cell (expressing luciferase). In one example, Luntr enabled proximity dependent imaging with nitroreductase- and Fluc-expressing cells. B) Split luciferase constructs can be used to report on cell-cell interactions. The closer the two cells, the more light output is observed. C) Split Gaussia luciferase can report on cell proximity in vitro. As the distance between the cells increased, light emission decreased. Adapted with permission from ref <sup>35</sup>.