

UNIVERSITY OF CALIFORNIA,
IRVINE

Parallel screening for rapid identification of orthogonal bioluminescent tools

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Organic Chemistry

by

Colin Michael Rathbun

Dissertation Committee:
Associate Professor Jennifer Prescher, Chair
Professor Scott Rychnovsky
Professor Chris Vanderwal

2012

© 2012 Colin Michael Rathbun

DEDICATION

(Optional dedication page) xxx To ...

TABLE OF CONTENTS

	Page
LIST OF FIGURES	iv
LIST OF TABLES	v
ACKNOWLEDGMENTS	vi
CURRICULUM VITAE	vii
ABSTRACT OF THE DISSERTATION	ix
1 Introduction	1
1.1 Bioluminescence Basics	2
1.2 Building better bioluminescent tools	4
1.3 Luciferase-luciferin pairs for multicellular imaging	9
1.4 Bioluminescent reporters for cell-cell interactions	11
1.5 Bioluminescent reporters for analyte detection <i>in vivo</i>	12
1.6 Conclusions and future directions	13
Bibliography	14
A Appendix Title	17
A.1 Lorem Ipsum	17

LIST OF FIGURES

	Page
1.1 Popular luciferase-luciferin pairs for cellular imaging	3
1.2 Recently synthesized luciferin heterocycles	5
1.3 Palette of luciferin analogues	6
1.4 BRET for deep-tissue imaging	8
1.5 Orthogonal luciferin-luciferase pairs for multicomponent imaging	10

LIST OF TABLES

Page

ACKNOWLEDGMENTS

I would like to thank the National Science Foundation for funding through the Graduate Research Fellowship Program (grant No. DGE-1321846, and Allergan for funding through the Allergan Graduate Fellowship. I would also like to thank the members of the Prescher lab, Bernard Choi, and Bruce Tromberg, for very helpful discussions. xxx

You also need to acknowledge any publishers of your previous work who have given you permission to incorporate that work into your dissertation. See Section 3.2 of the UCI Thesis and Dissertation Manual.)

CURRICULUM VITAE

Colin Michael Rathbun

EDUCATION

Doctor of Philosophy in Computer Science	2012
University name	<i>City, State</i>
Bachelor of Science in Computational Sciences	2007
Another university name	<i>City, State</i>

RESEARCH EXPERIENCE

Graduate Research Assistant	2007–2012
University of California, Irvine	<i>Irvine, California</i>

TEACHING EXPERIENCE

Teaching Assistant	2009–2010
University name	<i>City, State</i>

REFEREED JOURNAL PUBLICATIONS

Ground-breaking article 2012
Journal name

REFEREED CONFERENCE PUBLICATIONS

Awesome paper Jun 2011
Conference name

Another awesome paper Aug 2012
Conference name

SOFTWARE

Magical tool <http://your.url.here/>
C++ algorithm that solves TSP in polynomial time.

ABSTRACT OF THE DISSERTATION

Parallel screening for rapid identification of orthogonal bioluminescent tools

By

Colin Michael Rathbun

Doctor of Philosophy in Organic Chemistry

University of California, Irvine, 2012

Associate Professor Jennifer Prescher, Chair

The abstract of your contribution goes here.

Chapter 1

Introduction

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.[23] 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 report on biological processes.[22] The 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. Because 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.[22] 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.

1.1 Bioluminescence Basics

Millions of luciferases exist in the natural world, but phylogenetically related enzymes use the same luciferin.[16] The identities of such luciferins remain exceedingly difficult to characterize, and of those reported to date, only two have found routine application in mammalian cell imaging.[22] The first, Dluciferin (Figure 1.1A), is used by the firefly and a number of other terrestrial organisms to produce light. The second, coelenterazine (Figure 1.1B), is a molecule found in bioluminescent sea creatures, including the sea pansy *Renilla reniformis* and deep-sea copepods *Gaussia princeps* and *Oplophorus gracilirostris*.

Despite their distinct chemical structures, D-luciferin and coelenterazine use a similar mechanism for light production: 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 1.1). The color of light released is primarily dictated by the small molecule luciferin. In the case of D-luciferin, the aromatic core is sufficiently extended to provide

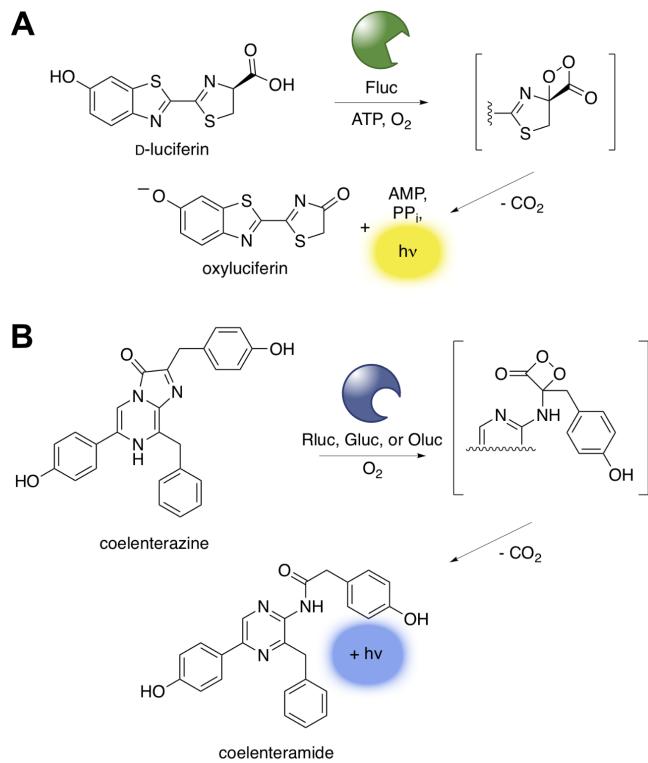


Figure 1.1: Popular luciferase-luciferin pairs for cellular imaging. (A) DLuciferin is oxidized by firefly luciferase (Fluc) to produce oxyluciferin and a photon of light. (B) Coelenterazine is oxidized by a variety of marine luciferases, including Renilla luciferase (Rluc), Gaussia luciferase (Gluc), and Oplophorus luciferase (Oluc). These luciferases, unlike Fluc, require only oxygen as a cofactor in the light-emitting reaction.

yellow-green (≈ 560 nm) light. For coelenterazine, the π 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.[29] Discriminating among related luciferases *in vivo*, though, remains difficult because of their broad emission spectra and the strong absorption of >650 nm light.[31]

1.2 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.[22] 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 multicellular or other multicomponent 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 and 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 historic challenge. The chemical complexity of Dluciferin and coelenterazine has also frustrated efforts to rapidly diversify these scaffolds to produce new analogues. In recent years, improved methods for producing luciferins have been reported. In the case of D-luciferin, we developed a streamlined method for accessing the requisite benzothiazole core using Appel's salt and modern C-H activation (Figure 1.2).[17]

This approach improved the yield and generalizability of the synthesis, eliminating the need for multiple functional group conversions used in previous routes. The strategy has enabled multigram syntheses of D-luciferin and has proven to be quite modular. In fact, we and others have used this chemistry to produce more than 30 distinct analogues.[30] Many of these scaffolds also comprise key functional handles for late stage diversification (Figure 1.2). Efforts to expediently synthesize amino luciferins[10] and related analogues[2] are similarly facilitating

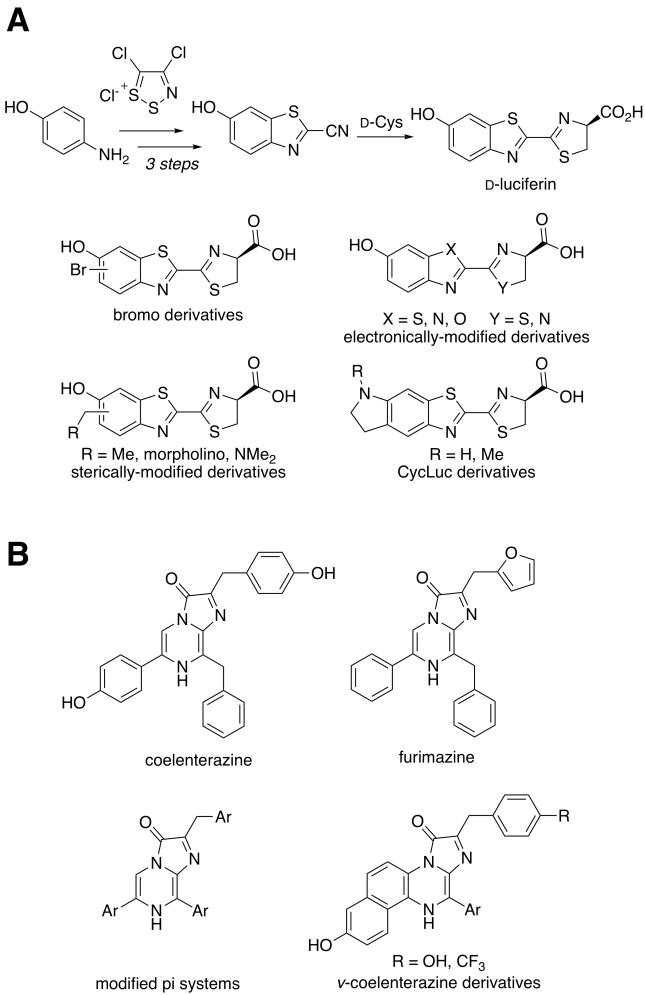


Figure 1.2: Recently synthesized luciferin heterocycles. (A) D-Luciferin and related analogues can be accessed using Appel's salt and C-H activation chemistry. (B) A diverse array of coelenterazine analogues have been synthesized in recent years.

more widespread use of bioluminescent tools. Improved methods for accessing coelenterazine analogues 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 analogues. Kirkland and colleagues identified milder conditions to access the coelenterazine core via Horner-Wadsworth-Emmons olefination.[28] The method proved to be both scalable and generalizable, providing a range of useful analogues. Recent advances in cross-coupling methodology are similarly enabling access to diverse coelenterazine architectures.[12]

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.[22] Recent years, though, have seen a shift in focus to modulating the small molecule (Figure 1.3).

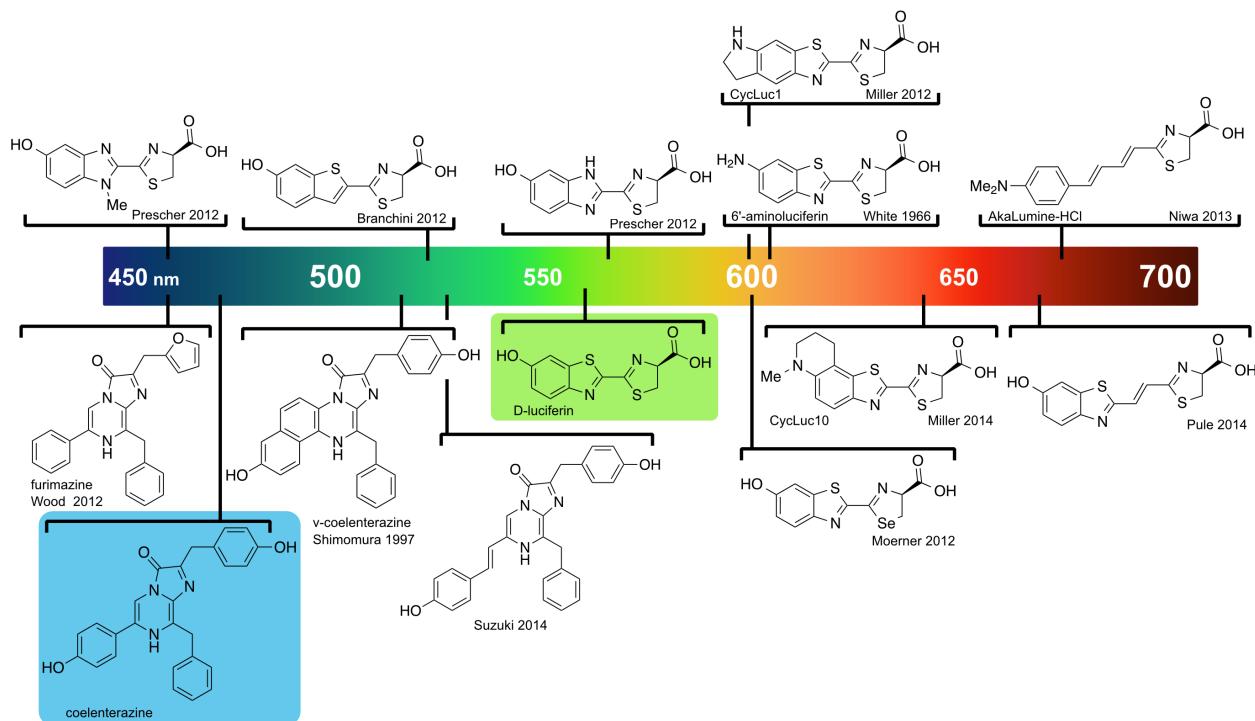


Figure 1.3: Palette of luciferin analogues. Native luciferins are highlighted with colored boxes. Brackets denote the wavelength (nanometers) of maximal bioluminescence emission observed upon incubation of the compound with luciferase. While many analogues can provide unique colors of light, most are not efficiently processed by native luciferases.

Because the structure of the luciferin emitter influences bioluminescent output, methods for modulating 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 Dluciferin, replacing the rotationally flexible 6' electron-donating group with a confor-

mationally locked amine.[25] This configuration enabled enhanced donation of electrons to the luciferin core and more robust, red-shifted emission compared to that of related probes.[3] Moreover, many of the cyclic luciferin (CycLuc) scaffolds were viable substrates for Fluc, highlighting the promiscuity of the enzyme. Fluc can also process luciferin analogues with extended aromatic cores and modified heteroatoms, providing additional avenues for wavelength alteration.[17, 30, 4] π -Extended chromophores typically emit red-shifted light emission.[15] In the case of the vinyl-extended luciferins (Figure 1.3), the shift was >100 nm. It should be noted, though, that these and other extended analogues 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. Redshifted 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.[31] Inouye and colleagues recently developed a streamlined synthesis of a conformationally locked coelenterazine for improved imaging.[12] Others have explored luminophores with extended π -conjugation to achieve more tissue-penetrant wavelengths of light.[21, 6] 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 CyOFP1 [dubbed Antares (Figure 1.4A)] resulted in an emission shift of 114 nm (Figure 1.4B).[3]

Antares is one of the most red-shifted BRET pairs reported to date, enabling enhanced deep-tissue imaging *in vivo* (Figure 1.4C). In related work, Nagai and colleagues linked a mutant Renilla luciferase to Venus, a version of yellow fluorescent protein.[27] Unexpectedly, this construct (dubbed Nanolatern) produced 6-fold more photons than the luciferase alone and shifted emission into the yellow-green region (530 nm). Additional Nanolatern colors have been described.[29] Collectively, these and other BRET probes are enabling sensitive imaging of many biological processes, including membrane voltage[13] and gene expression.[11]

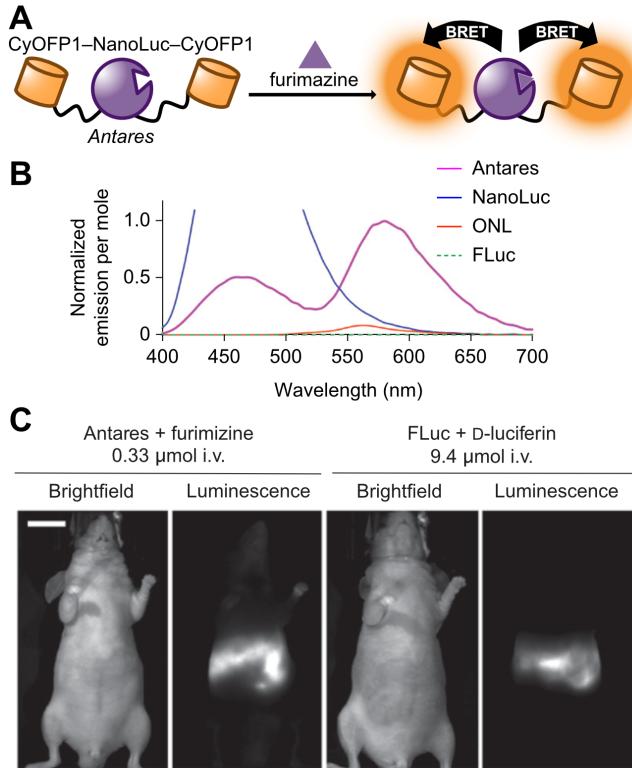


Figure 1.4: BRET for deep-tissue imaging. (A) Antares comprises a fusion of NanoLuc with two cyan-excitable orange fluorescent proteins (CyOFP1). (B) The BRET fusion exhibits red-shifted emission relative to NanoLuc. ONL denotes Orange Nanolatern. (C) Antares enabled sensitive imaging with low luciferin levels in mice. Antares and Fluc genes were delivered to the liver via hydrodynamic injection. Luciferins were supplied intravenously. Adapted with permission from ref [3].

In Vivo Improvements

Luciferins can access most tissues, but their distribution and cell permeability are suboptimal and not uniform.[1] 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*.[5] 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).[18] Once the probe is in the brain, the amide bond can be cleaved by

endogenous fatty acid amide hydrolase (strongly expressed in brain tissue), releasing a functional luciferin.

1.3 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,[24] 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 analogues 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.[9] The authors later identified a mutant that preferred a luciferin analogue over Dluciferin, setting the stage for developing substrate-responsive enzymes.[19] Further work by the Miller group revealed latent luciferase activity in a fatty acyl-CoA synthetase from the fruitfly.[20] Interestingly, this enzyme did not emit light with Dluciferin—it was able to use only a CycLuc substrate—opening the door to pairing unnatural luciferin analogues 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).[8] Early work in this area was motivated by the need for improved coelenterazines (molecules prone to autoxidation 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 1.3)] was more stable in media and lysate and less susceptible to nonspecific oxidation. Directed evolution was used to select an enzyme that could readily catalyze light emission from 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 point-of-care diagnostics with simple cell phone cameras.[7] 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,[31] and bioluminescence spectra are broad,[26] necessitating an alternative approach. Substrate resolution is one such strategy (Figure 1.5A).

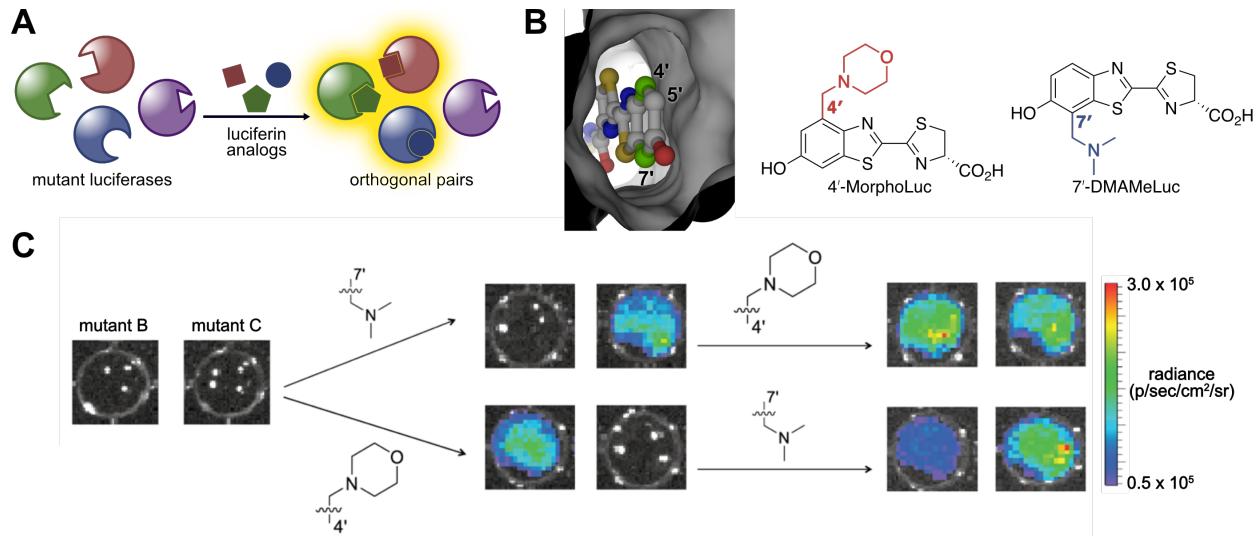


Figure 1.5: Orthogonal luciferin-luciferase pairs for multicomponent imaging. (A) A strategy for multicomponent bioluminescence imaging with luciferin analogues and mutant luciferases. (B) The 4? and 7? sites of D-luciferin were targeted to preclude binding to native Fluc. (C) Substrate resolution was achieved in mouse DB7 cells using 4?- and 7?-modified luciferins and mutant luciferases identified from screening. Adapted with permission from ref [14].

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 marine luciferases, along with their requisite substrates), and many have been adapted for dual-imaging studies. To expand and expedite the search for orthogonal pairs,³⁰ we turned to producing non-natural analogues and enzymes. We synthesized a panel of luciferins, with additional steric bulk at the 4? and 7? positions (Figure 6B). We screened these analogues 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 to be 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 bioluminescence imaging with coelenterazine analogues.³²

1.4 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 are necessary. The development of tools that report on cell?cell contacts has been inspired by classic methods for reporting on biomolecule activities and interactions. For example, caged probes have been used widely to report on small molecule analytes in cells. We and others³³ have shown that such cages can be repurposed 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.³⁴ 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 half in another. Light emission in this case tracked with distance between the cell populations (Figure 7C).

1.5 Bioluminescent reporters for analyte detection *in vivo*

Bioluminescence has long been exploited for detecting enzyme activities and low-abundance 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*.^{36,37} 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 Dluciferin. 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.³⁶

1.6 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.

Bibliography

- [1] S. T. Adams and S. C. Miller. Beyond D-luciferin: expanding the scope of bioluminescence imaging in vivo. *Curr. Opin. Chem. Biol.*, 2014.
- [2] J. C. Anderson, H. Grounds, A. P. Jathoul, J. A. H. Murray, S. J. Pacman, and L. Tisi. Convergent synthesis and optical properties of near-infrared emitting bioluminescent infra-luciferins. *RSC Adv.*, 7(7):3975–3982, 2017.
- [3] J. Chu, Y. Oh, A. Sens, N. Ataie, H. Dana, J. J. Macklin, T. Laviv, E. S. Welf, K. M. Dean, F. Zhang, B. B. Kim, C. T. Tang, M. Hu, M. A. Baird, M. W. Davidson, M. A. Kay, R. Fiolka, R. Yasuda, D. S. Kim, H.-L. Ng, and M. Z. Lin. A bright cyan-excitable orange fluorescent protein facilitates dual-emission microscopy and enhances bioluminescence imaging in vivo. *Nat. Biotechnol.*, 34(7):760–767, July 2016.
- [4] N. R. Conley, A. Dragulescu Andrasi, J. Rao, and W. E. Moerner. A Selenium Analogue of Firefly D-Luciferin with Red-Shifted Bioluminescence Emission. *Angewandte Chemie International Edition*, 51(14):3350–3353, 2012.
- [5] M. S. Evans, J. P. Chaurette, S. T. Adams, G. R. Reddy, M. A. Paley, N. Aronin, J. A. Prescher, and S. C. Miller. A synthetic luciferin improves bioluminescence imaging in live mice. *Nat. Methods*, 11(4):393–395, Apr. 2014.
- [6] K. M. Grinstead, L. Rowe, C. M. Ensor, S. Joel, P. Daftarian, E. Dikici, J.-M. Zingg, and S. Daunert. Red-Shifted Aequorin Variants Incorporating Non-Canonical Amino Acids: Applications in In Vivo Imaging. *PLOS ONE*, 11(7):e0158579, July 2016.
- [7] R. Griss, A. Schena, L. Reymond, L. Patiny, D. Werner, C. E. Tinberg, D. Baker, and K. Johnsson. Bioluminescent sensor proteins for point-of-care therapeutic drug monitoring. *Nat. Chem. Biol.*, 10(7):598–603, June 2014.
- [8] M. P. Hall, J. Unch, B. F. Binkowski, M. P. Valley, B. L. Butler, M. G. Wood, P. Otto, K. Zimmerman, G. Vidugiris, T. Machleidt, M. B. Robers, H. A. Benink, C. T. Eggers, M. R. Slater, P. L. Meisenheimer, D. H. Klaubert, F. Fan, L. P. Encell, and K. V. Wood. Engineered Luciferase Reporter from a Deep Sea Shrimp Utilizing a Novel Imidazopyrazinone Substrate. *ACS Chem. Biol.*, 7(11):1848–1857, Nov. 2012.
- [9] K. R. Harwood, D. M. Mofford, G. R. Reddy, and S. C. Miller. Identification of Mutant Firefly Luciferases that Efficiently Utilize Aminoluciferins. *Chemistry & Biology*, 18(12):1649–1657, 2011.

- [10] J. R. Hauser, H. A. Beard, M. E. Bayana, K. E. Jolley, S. L. Warriner, and R. S. Bon. Economical and scalable synthesis of 6-amino-2-cyanobenzothiazole. *Beilstein J. Org. Chem.*, 12:2019–2025, 2016.
- [11] T. Horikiri, H. Ohi, M. Shibata, M. Ikeya, M. Ueno, C. Sotozono, S. Kinoshita, and T. Sato. SOX10-Nano-Lantern Reporter Human iPS Cells; A Versatile Tool for Neural Crest Research. *PLOS ONE*, 12(1):e0170342, Jan. 2017.
- [12] T. Hosoya, R. Iimori, S. Yoshida, Y. Sumida, Y. Sahara-Miura, J.-i. Sato, and S. Inouye. Concise Synthesis of v-Coelenterazines. *Org. Lett.*, 17(15):3888–3891, July 2015.
- [13] S. Inagaki, H. Tsutsui, K. Suzuki, M. Agetsuma, Y. Arai, Y. Jinno, G. Bai, M. J. Daniels, Y. Okamura, T. Matsuda, and T. Nagai. Genetically encoded bioluminescent voltage indicator for multi-purpose use in wide range of bioimaging. *Sci. Rep.*, 7:42398, Feb. 2017.
- [14] K. A. Jones, W. B. Porterfield, C. M. Rathbun, D. C. McCutcheon, M. A. Paley, and J. A. Prescher. Orthogonal Luciferase-Luciferin Pairs for Bioluminescence Imaging. *J. Am. Chem. Soc.*, 139(6):2351–2358, Feb. 2017.
- [15] T. Kuchimaru, S. Iwano, M. Kiyama, S. Mitsumata, T. Kadonosono, H. Niwa, S. Maki, and S. Kizaka-Kondoh. A luciferin analogue generating near-infrared bioluminescence achieves highly sensitive deep-tissue imaging. *Nat. Commun.*, 7:11856, 2016.
- [16] S. Martini and S. H. D. Haddock. Quantification of bioluminescence from the surface to the deep sea demonstrates its predominance as an ecological trait. *Sci. Rep.*, 7:45750, Apr. 2017.
- [17] D. C. McCutcheon, M. A. Paley, R. C. Steinhardt, and J. A. Prescher. Expedient synthesis of electronically modified luciferins for bioluminescence imaging. *J. Am. Chem. Soc.*, 134(18):7604–7607, May 2012.
- [18] D. M. Mofford, S. T. Adams, G. S. K. K. Reddy, G. R. Reddy, and S. C. Miller. Luciferin Amides Enable in Vivo Bioluminescence Detection of Endogenous Fatty Acid Amide Hydrolase Activity. *J. Am. Chem. Soc.*, 137(27):8684–8687, July 2015.
- [19] D. M. Mofford, G. R. Reddy, and S. C. Miller. Aminoluciferins extend firefly luciferase bioluminescence into the near-infrared and can be preferred substrates over D-luciferin. *J. Am. Chem. Soc.*, 136(38):13277–13282, Sept. 2014.
- [20] D. M. Mofford, G. R. Reddy, and S. C. Miller. Latent luciferase activity in the fruit fly revealed by a synthetic luciferin. *Proc. Natl. Acad. Sci. U.S.A.*, 111(12):4443–4448, Mar. 2014.
- [21] R. Nishihara, H. Suzuki, E. Hoshino, S. Suganuma, M. Sato, T. Saitoh, S. Nishiyama, N. Iwasawa, D. Citterio, and K. Suzuki. Bioluminescent coelenterazine derivatives with imidazopyrazinone C-6 extended substitution. *Chem. Commun.*, 51(2):391–394, Dec. 2014.

- [22] M. A. Paley and J. A. Prescher. Bioluminescence: a versatile technique for imaging cellular and molecular features. *Med. Chem. Comm.*, 5(3):255–267, Feb. 2014.
- [23] W. B. Porterfield and J. A. Prescher. Tools for visualizing cell-cell 'interactomes'. *Curr. Opin. Chem. Biol.*, 24:121–130, Feb. 2015.
- [24] K. V. Purtov, V. N. Petushkov, M. S. Baranov, K. S. Mineev, N. S. Rodionova, Z. M. Kaskova, A. S. Tsarkova, A. I. Petunin, V. S. Bondar, E. K. Rodicheva, S. E. Medvedeva, Y. Oba, Y. Oba, A. S. Arseniev, S. Lukyanov, J. I. Gitelson, and I. V. Yampolsky. The Chemical Basis of Fungal Bioluminescence. *Angew. Chem., Int. Ed.*, 54(28):8124–8128, July 2015.
- [25] G. R. Reddy, W. C. Thompson, and S. C. Miller. Robust light emission from cyclic alkylaminoluciferin substrates for firefly luciferase. *J. Am. Chem. Soc.*, 132(39):13586–13587, Oct. 2010.
- [26] K. A. Rumyantsev, K. K. Turoverov, and V. V. Verkhusha. Near-infrared bioluminescent proteins for two-color multimodal imaging. *Sci. Rep.*, 6:36588, Nov. 2016.
- [27] K. Saito, Y.-F. Chang, K. Horikawa, N. Hatsugai, Y. Higuchi, M. Hashida, Y. Yoshida, T. Matsuda, Y. Arai, and T. Nagai. Luminescent proteins for high-speed single-cell and whole-body imaging. *Nat. Commun.*, 3:1262, Dec. 2012.
- [28] A. Shakhmin, M. P. Hall, J. R. Walker, T. Machleidt, B. F. Binkowski, K. V. Wood, and T. A. Kirkland. Three Efficient Methods for Preparation of Coelenterazine Analogues. *Chem.—Eur. J.*, 22(30):10369–10375, June 2016.
- [29] K. Suzuki, T. Kimura, H. Shinoda, G. Bai, M. J. Daniels, Y. Arai, M. Nakano, and T. Nagai. Five colour variants of bright luminescent protein for real-time multicolour bioimaging. *Nat. Commun.*, 7:13718, Dec. 2016.
- [30] C. C. Woodroffe, P. L. Meisenheimer, D. H. Klaubert, Y. Kovic, J. C. Rosenberg, C. E. Behney, T. L. Southworth, and B. R. Branchini. Novel Heterocyclic Analogues of Firefly Luciferin. *Biochemistry*, 51(49):9807–9813, Dec. 2012.
- [31] H. Zhao, T. C. Doyle, O. Coquoz, F. Kalish, B. W. Rice, and C. H. Contag. Emission spectra of bioluminescent reporters and interaction with mammalian tissue determine the sensitivity of detection *in vivo*. *J. Biomed. Opt.*, 10(4):041210, 2005.

Appendix A

Appendix Title

Supplementary material goes here. See for instance Figure A.1.

A.1 Lorem Ipsum

dolor sit amet, consectetur adipisicing elit, sed do eiusmod tempor incididunt ut labore et dolore magna aliqua. Ut enim ad minim veniam, quis nostrud exercitation ullamco laboris nisi ut aliquip ex ea commodo consequat. Duis aute irure dolor in reprehenderit in voluptate velit esse cillum dolore eu fugiat nulla pariatur. Excepteur sint occaecat cupidatat non proident, sunt in culpa qui officia deserunt mollit anim id est laborum.

“I am glad I was up so late,
for that’s the reason I was up so early.”
William Shakespeare (1564-1616), British dramatist, poet.
Cloten, in Cymbeline, act 2, sc. 3, l. 33-4.

Figure A.1: A deep quote.