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FIRST REACTIONS

Diversifying the Glowing Bioluminescent Toolbox

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Diversification strategies for bioluminescence imaging can provide bio-orthogonal tools for illuminating multicomponent in vivo processes.

Advances in chemical design, genetic engineering, and biomedical technologies have contributed to impressive progress in bioluminescence imaging (BLI) of complex biological systems from cells to living mammals. While the technique is prevalent in small animal *in vivo* molecular imaging applications, most tools have been limited to only a few sets of bioluminescent systems. Now, the Prescher lab at the University of California, Irvine, has introduced an elegant interdisciplinary approach that paves a new path for the diversification of the bioluminescence toolbox.[1]

Bioluminescence involves the production of light via the enzymatic oxidation of small-molecule substrates by luciferase enzymes. The process has been exploited for noninvasive and highly sensitive optical imaging of small animal models. Selective integration of luciferase into mammalian systems, either by implanted cells or through linked expression to promoter genes, has allowed for a wealth of applications including tumor cell tracking, monitoring tissue-specific gene expression, and reporting on circadian rhythms.[2] Moreover, chemical modification of the light-emitting substrates have further expanded the range of BLI applications to analyzing small molecules, assessing drug delivery, and studying enzyme activity.[3,4] Compared with other *in vivo* imaging modalities, BLI is inexpensive, requires no excitation light source, and is easy to use. These factors coupled with the versatility of its potential applications has resulted in its widespread implementation across biological sciences. However, only a small subset of bioluminescent substrate−enzyme pairs is amenable to *in vivo* BLI, limiting its use for multicomponent applications.[5]

Expanding the bioluminescent toolbox requires optimization of the biochemistry (substrate−enzyme interactions), photophysics, and both the biodistribution and bioavailability of the substrate. Research in recent years has generated modified substrates with improved *in vivo* emission properties

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(e.g., red-shifted light which better penetrates through mammalian tissue or brighter emission to improve the signal-to-noise ratio) or biodistribution. Yet, the majority of these developments have focused on improved substrates for the widely used firefly luciferase enzyme.

Achieving multicomponent imaging poses an added challenge of requiring bioluminescent pairs that are orthogonal—meaning they do not cross-react with one another. To this end, emerging work has reported on new or mutant luciferase enzymes that can act more favorably with unnatural substrates. Efforts to optimize substrate−enzyme interactions have led to discoveries such as the furimazine−Nanoluc pair, a promising system which addresses challenges in stability and brightness that has plagued imidazopyridine−luciferase systems (e.g., coelenterazine−*Renilla* luciferase).[6] In other pioneering work, Miller and co-workers identified mutant luciferases that more readily interact with substrates based on a cyclized scaffold termed CycLuc over the parent D-luciferin.[7] Interestingly, these mutant luciferases can process CycLuc derivatives more readily than firefly luciferase. These examples highlight the promise of parallel modifications of both enzymes and substrates to achieve multicomponent BLI.

The Prescher lab recently introduced a direct approach for finding bio-orthogonal bioluminescent pairs.[8] They generated a panel of derivatives of D-luciferin and screened them against a library of mutant luciferases, identifying functional substrate−enzyme systems. From there, the degree of orthogonality of pairs of enzymes with cognate substrates was assessed with a

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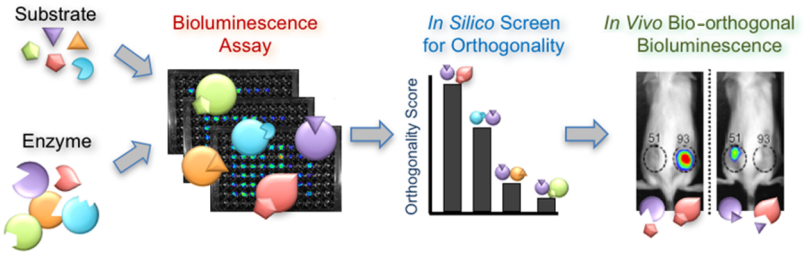

Figure 1. A parallel screening strategy utilizing *in vitro* data points to identify bio-orthogonal bioluminescent probes expands the potential for bioluminescence imaging of multiple features *in vivo*.

computer algorithm, revealing a new bio-orthogonal bioluminescent pair. The discovery of this bio-orthogonal pair set the stage for their latest work published in this issue of *ACS Central Science*, where the authors have now streamlined the discovery of bio-orthogonal bioluminescent systems (Figure 1).[1] Substrate selectivity was utilized as the primary screening parameter to generate hits focused on orthogonality. The authors developed an *in vitro* medium-throughput plate-based imaging strategy to test 12 luciferin substrates against 159 luciferases, generating 1908 data points. These data points were then input into a powerful algorithm to sort pairs of substrate−enzyme systems based orthogonality scores. The algorithm generated a ranked list of 829 026 orthogonal sets, and at least 1000 of these sets exhibited at least a 10-fold difference in bioluminescence *in vitro*. The *in silico*-based orthogonality ranking was impressively validated from the top to lower-selectivity hits, demonstrating the predictive power of the approach for both selecting and excluding potential candidates. Among the top-ranked pairs, three pairs were further tested and positively validated in cell and animal models. Taking it a step further, the authors applied this innovative strategy to the identification of orthogonal triplets with the top hit being pulled out on >144 million possible combinations and validated in cell culture.

With more information, the authors could start drawing design principles: for instance, orthogonality seems influenced by reduced photon output of negative pairings more so than increased photon output of positive ones. As the library of bioluminescent substrates and enzymes grows, this powerful new approach transforms an otherwise laborious task of cross-evaluating each system with one another to a rapid in silico approach based on in vitro data to confidently select for functional bio-orthogonal BLI probes. The strategy has added impact on data mining of substrate−enzyme interactions beyond BLI provided that high-throughput *in vitro* readouts are available.

While Prescher's work utilized a substrate panel with similar emission profiles, one can envision applying the parallel screening approach to substrates with varied emission wavelengths, a growing area in luciferin substrate design. By expanding the *in vitro* screen to assess filtered emission, the algorithm could potentially uncover multicolored orthogonal pairs to expand the color palette of BLI probes. Such an advance could revolutionize BLI as a versatile imaging modality as multiple features could be simultaneously imaged in the same animal over time.

> Diversification strategies for bioluminescence imaging can provide bio-orthogonal tools for illuminating multi-component *in vivo* processes.

Despite the historical lack in tool diversity, bioluminescence has impacted our ability to understand and follow biology and disease pathology. Prescher's work draws attention not only to the need for expanding the scope of bioluminescence but also demonstrates that new solutions are at hand. As the BLI toolbox grows, an existing challenge lies in the need for genetic modification of animals for expression of mutant luciferase enzymes lest the discoveries be limited to implanted cell lines. However, progress in BLI can be further bolstered with the recent advent of genome-editing technologies. BLI involves combined expertise in biochemistry, chemistry, genetic engineering, imaging technologies, and now, computational data mining, and collaborations and open communication by investigators in these different areas will foster exciting breakthroughs. BLI already plays a crucial role in basic research in small animal models; the opportunity for multicomponent BLI can expect to transform approaches for understanding dynamic interactions of living systems.

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