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Advances in bioluminescence imaging: New probes from old recipes

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Abstract

Bioluminescent probes are powerful tools for visualizing biology in live tissues and whole animals. Recent years have seen a surge in the number of new luciferases, luciferins, and related tools available for bioluminescence imaging. Many were crafted using classic methods of optical probe design and engineering. Here we highlight recent advances in bioluminescent tool discovery and development, along with applications of the probes in cells, tissues, and organisms. Collectively, these tools are improving *in vivo* imaging capabilities and bolstering new research directions.

Introduction

Bioluminescent enzymes (luciferases) are among the most sensitive probes for imaging in thick tissues and whole organisms.[1] Luciferases catalyze light emission via the oxidation of small molecule substrates (luciferins). Since no external light is required, the background emission is virtually zero, enabling sensitive imaging *in vivo*. Bioluminescence has long been used to track cells, gene expression, and other biological features in tissues and whole organisms.[2] The emitted light is inherently weak, though, compared to conventional fluorescent tools. For this reason, luciferases are typically used in conjunction with fluorescent reporters. The bioluminescent enzymes survey processes on the macro scale and in heterogeneous environments. The fluorescent probes capture events at the micro scale or *ex vivo* – environments where excitation light is more efficiently delivered.

Historically, the most popular bioluminescent reporter for imaging *in vivo* has been firefly luciferase (Fluc). This enzyme emits the largest percentage of tissue-penetrant light with its cognate luciferin (D-luciferin, Figure 1A).[3] Other luciferases, including *Renilla* luciferase (Rluc) and *Gaussia* luciferase (Gluc) have also found broad utility in biological research.[4] These enzymes oxidize coelenterazine and emit blue light in the process. Rluc and Gluc

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require no additional cofactors (other than oxygen), making them well suited for extracellular work. Compared to their fluorescent protein counterparts, though, luciferases have been less frequently employed in bioimaging studies. Fewer bioluminescent probes have been developed and even fewer have been optimized for application *in vivo*. There is a constant demand for more bioluminescent colors, improved enzymes, and more biocompatible substrates.

Advances in protein engineering and chemical syntheses are addressing voids in the bioluminescent toolbox. The past few years, in particular, have seen an uptick in the number of sensitive and substrate-selective luciferases available for use. Much of the progress mirrors trends in fluorescent protein development, including identifying mechanistically distinct probes in nature and subsequently evolving for new function.[5] Systematic efforts to engineer fluorescent probes for altered colors of emission, photo-switching capabilities, and other features ultimately enabled new studies in biology. This iterative cycle of tool development and biological discovery is similarly driving the field of bioluminescence. Below we highlight recent efforts to discover and evolve new bioluminescent tools, and showcase their application to biological sensing.

Discovering new luciferases and luciferins

Thousands of luminescent species exist in the natural world, but only a fraction of the associated luciferases and luciferins have been characterized in detail.[4,6] Even fewer have been coopted for use in heterologous systems.[1] Continued efforts to mine new luciferase and luciferin architectures from natural sources are expanding the number of available tools. For example, the luciferase gene from *Photinus scintillans* was recently cloned.[7] *P. scintillans* emits predominantly orange light, in contrast to the well-known North American firefly (which emits predominantly yellow-green light). The unique spectrum was traced to a single amino acid change (Y255F) in the luciferase structure. In 2016, Sharpe and colleagues reported the isolation of bioluminescent, crystalline protein assemblies in the Japanese firefly squid.[8] The crystals comprise three different—but homologous—proteins that catalyze light emission with coelenterazine-disulfate and ATP (Figure 1B).

New luciferin scaffolds and light-emitting mechanisms have also been elucidated in recent years (Figure 1B). One example includes the peptide-like luciferin from *Fridericia heliota*. Yampolsky and colleagues speculated that this molecule undergoes oxidative decarboxylation in the light-emitting reaction (similar to D-luciferin), despite its highly divergent structure.[9] This same group also discovered a new bioluminescent mechanism operative within glowing fungi. Some species convert 3-hydroxyhispidin to a putative endoperoxide en route to light emission[10*]. This scaffold is distinct from dioxetanones and other intermediates observed in classic bioluminescent reactions. Such unique luciferins and light-emitting mechanisms are potentially useful for multi-component imaging.[11]

The majority of luciferin biosyntheses remain unknown, but advances in genome sequencing are beginning to shed light on these historical mysteries. Unraveling the biosynthetic pathways would be a huge boon to *in vivo* imaging efforts: both enzyme production *and* substrate generation could potentially be genetically encoded in mammalian cells. Cells

would thus be autoluminescent without the need for exogenous substrate (similar to the *lux* operon for bacterial imaging).[12] Weng and coworkers have taken key steps toward this goal by harvesting the light-emitting organs from fireflies and other insects. Transcriptome profiling revealed multiple conserved genes that are likely involved in the de novo synthesis of D-luciferin.[13] Related efforts to elucidate coelenterazine biosynthesis have also been reported.[14]

Generating a palette of bioluminescent probes

The discovery and characterization of native bioluminescent systems, while important, have often not kept pace with the demand for user-friendly imaging tools. Thus, efforts to engineer bioluminescent probes with desirable properties have been critical to fill voids in the imaging toolbox. Many of the approaches have mirrored those in fluorescent protein development: mutagenesis and screening for desired properties such as thermostability, turnover, and color. Some of the most impactful luciferase engineering work in recent years has centered on NanoLuciferase (Nluc).[15] Nluc is a small (16 kDa) luciferase derived from the luminous sea shrimp *Oplophorus gracilirostris.* Nluc was evolved to process a stabilized coelenterazine analog (furimazine) in the light-emitting reaction. The Nluc-furimazine pair has been widely adopted for imaging studies in diverse fields, due to its brightness and stability. Split versions of Nluc have also been reported.[16] Like other split reporters, these tools have been useful for analyzing protein-protein interactions in cells[16] and screening inhibitors.[17]

Nluc has also proven to be a versatile platform for broadening the palette of bioluminescent probes. Much like fluorescent proteins, distinct bioluminescent reporters are desirable for applications in multicellular imaging. An enhanced set of colors can be achieved via bioluminescence resonance energy transfer (BRET, Figure 2). BRET involves luminescent reactions that excite acceptor fluorophores, resulting in altered emission spectra. The process is analogous to Förster resonance energy transfer (FRET), where energy transfer processes between two fluorophores can tune emission spectra. Nagai and colleagues recently generated a set of Nluc-fluorescent protein conjugates for BRET imaging. These chimeras were inspired by earlier Rluc-fluorescent protein conjugates (i.e., the “Nano-lanterns”).[18,19] The cyan- and green-emitting Nluc lanterns exhibited quantum yields on par with (or exceeding) Nluc itself and enabled real-time colorimetric imaging.[20*] Fluorescent dyes are also suitable BRET acceptors. Johnsson and coworkers pioneered a strategy to append different fluorophores to Nluc using SNAPtag and HaloTag technologies (Figure 2B). The suite of resulting probes provided a bioluminescent portrait reminiscent of the famous fluorescent protein collection.[21] The Nluc chimeras were also shown to be well suited for multi-component imaging in cells.[22**]

Pushing the frontiers in noninvasive imaging, the Lin lab reported an Nluc BRET construct (Antares) suitable for *in vivo* work.[23**] Antares comprises Nluc flanked by 2 copies of an orange fluorescent protein (CYOFP1). This construct produces ~20-fold more tissue-penetrant photons (>600 nm) compared to Nluc, enabling sensitive imaging in rodents. Further engineering and analog optimization yielded a second-generation reporter: Nluc (teLuc) that uses a modified furimazine analog (DTZ, Figure 2C). teLuc and DTZ provide

improved spectral overlap with CYOFP1. The optimized BRET construct (termed Antares2) exhibited enhanced red-shifted light emission and more robust bioluminescence in deep tissues.[24] Related BRET probes with Rluc have similarly provided bioluminescent probes that emit in the near-infrared regime.[25]

Efforts to produce multi-spectral tools have historically focused on the luciferase enzyme, although modifying the luciferin architecture offers another viable route. Changes to the luciferin chromophore can directly impact the color of light released. For example, extending the conjugation of the luciferin pi system or altering heteroatom substituents can alter emission wavelengths.[26–28] Both blue- and red-shifted analogs have been synthesized in recent years, although most remain weak emitters with native luciferases.[26,27,29] Engineering enzymes to better process the modified analogs—and thus recover light intensity—has been successful in some cases.[27,29,30]

Engineering orthogonal luciferase-luciferin pairs

Discriminating among wavelengths *in vivo* is challenging, as the perceived color changes with depth. Multi-component bioluminescence imaging has thus been most often achieved using *substrate*-resolved luciferases versus spectrally resolved pairs. For example, Fluc and Rluc oxidize completely different luciferins and can therefore be readily distinguished in two-component assays.[31] The Fluc/Rluc combination has further inspired the expansion of orthogonal bioluminescent tools. Unique patterns of substrate use, rather than color, can serve as diagnostic fingerprints for collections of cells or other features (Figure 3A). In our own lab, we synthesized dozens of chemically distinct luciferins and screened them against a panel of Fluc mutants.[32,33] A computer algorithm was used to identify orthogonal enzyme-substrate pairs. Substrate selectivity was maintained in both mammalian cells and in mouse models, enabling multi-cellular imaging *in vivo* (Figure 3B). Additional screening analysis further revealed triplet sets and higher-order orthogonal combinations.[33**] Simultaneous engineering of enzymes and substrates has also been applied to luciferases that use coelenterazine.[34]

Luciferase-like enzymes are further expanding the number of orthogonal probes. Luciferases belong to the ANL (Acyl-CoA synthetases, NRPS adenylation domains, and Luciferase enzymes) superfamily; these enzymes use a common mechanism to activate carboxylates as adenylates. These intermediates can be displaced with biological thiols (e.g. CoA) or, in the case of firefly luciferase, react with molecular oxygen.[35] Most ANL enzymes do not catalyze light-emitting reactions with their cognate substrates. Excitingly, though, Miller showed that some fatty acyl-CoA synthetases exhibit “latent” luciferase activity when supplied with a luminogenic substrate.[36] For example, AbLL (a synthetase from the non-luminous beetle *Agrypnus binodulus*) catalyzed light emission with a panel of synthetic luciferin analogs (Figure 3C).[36**] Many of the latent luciferases exhibit unique patterns of substrate use, expanding the number of new and orthogonal tools.

The functional and sequence similarity between luciferase and other ANL enzymes is further enabling luciferase-engineering efforts. ANL enzymes are known to be promiscuous,[35] and can potentially serve as starting points to identify new luciferase-luciferin pairs.

The Leconte lab used the homologous enzymes in combination with a previously developed bioinformatic method, statistical coupling analysis (SCA)[37,38] to guide the design of mutant luciferase libraries. SCA was used to analyze amino acid positions that were mutable and functionally important, along with networks of potentially synergistic interactions.[39*] In a single round of selection, mutants with desirable emission spectra and improved thermostability were identified. Mutants with >50-fold changes in specificity for modified luciferins were also found.

Identifying new enzyme-substrate pairs requires rapid access to diverse collections of luciferins. Such molecules have historically been difficult to synthesize from common routes. Recent advances in luciferin chemistry, though, are beginning to address this issue. Modular coupling reactions to outfit D-luciferin with diverse steric modifications have been reported.[32,40*] Ring-closing metathesis and carbene insertions have also been used to produce a series of conformationally restricted and pi-extended coelenterazines.[41–43] Many these probes exhibited red-shifted emission or other desirable photophysical properties.

Monitoring new facets of biology

Advances in luciferase engineering have ushered in a flurry of new sensors for metabolites and enzyme activities.[44–47] Many of the probes have parallels to classic fluorescent sensors, but are more tailored for *in vivo* work. A notable example is CalfluxVTN, a BRET-based calcium sensor comprising Nluc and Venus fluorescent protein (Figure 4A). In the absence of Ca^{2+}, Nluc emission is observed. Upon Ca^{2+} binding, the sensor undergoes a conformational change and BRET is observed. CalfluxVTN enabled sensitive imaging of calcium flux in response to stimulation of a rhodopsin photoreceptor.[48] Such measurements were refractory to FRET, as external light interfered with receptor activation. Johnsson and coworkers further developed a universal BRET sensor platform for analyte detection. They fused various antibody fragments to an Nluc-fluorophore pair; upon binding of a complementary analyte, a conformational change was induced, accompanied by a change in emission color.[49] The modularity of this system could enable point-of-care diagnosis for a variety of antigens.

Advances in luciferin synthesis have also enabled access to new probes of cellular function, including “caged” luciferins. These molecules typically contain a bulky group (i.e. “cage”) that renders the molecule non-emissive with luciferase. Upon removal of the cage (typically from an enzymatic reaction), an active luciferin is revealed and available for light emission. “Caged” luciferins have recently been used to detect biologically relevant metal ions and other species.[50–53**] (Figure 4B). Some have also been used to profile cell-cell interactions[54] and improve delivery.[55] The caged luminophore concept has recently been expanded to craft novel chemiluminescent sensors.[56] Some of these probes comprise embedded dioxetanes that are cleavable—and thus emit light—in response to a variety of triggers.[57*,58] Unlike canonical caged luciferins, these reporters do not require a luciferase to produce light. Such probes further diversify the portfolio of tools for biological imaging.

Conclusions and Future Directions

Many advances in bioluminescent probe technology have mirrored trends in fluorescent probe development. Dozens of luciferases have been evolved for new functions via iterative mutagenesis and screening. Collections of robust and structurally distinct luciferins have also synthesized. A variety of unique bioluminescent mechanisms have further been uncovered in the natural world, providing platforms from which to craft new tools. The continued discovery and development of bioluminescence probes, like other optical imaging agents, promises to expand what researches can “see” in cells and tissues.

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Highlights

- Advances in bioluminescent tool production that mirror trends in fluorescent probe development.
- New bioluminescent platforms based on naturally occurring luciferases and luciferins.
- Engineered probes that enable deep tissue and multi-component bioluminescence imaging.
- Applications of bioluminescent tools to cellular biosensing

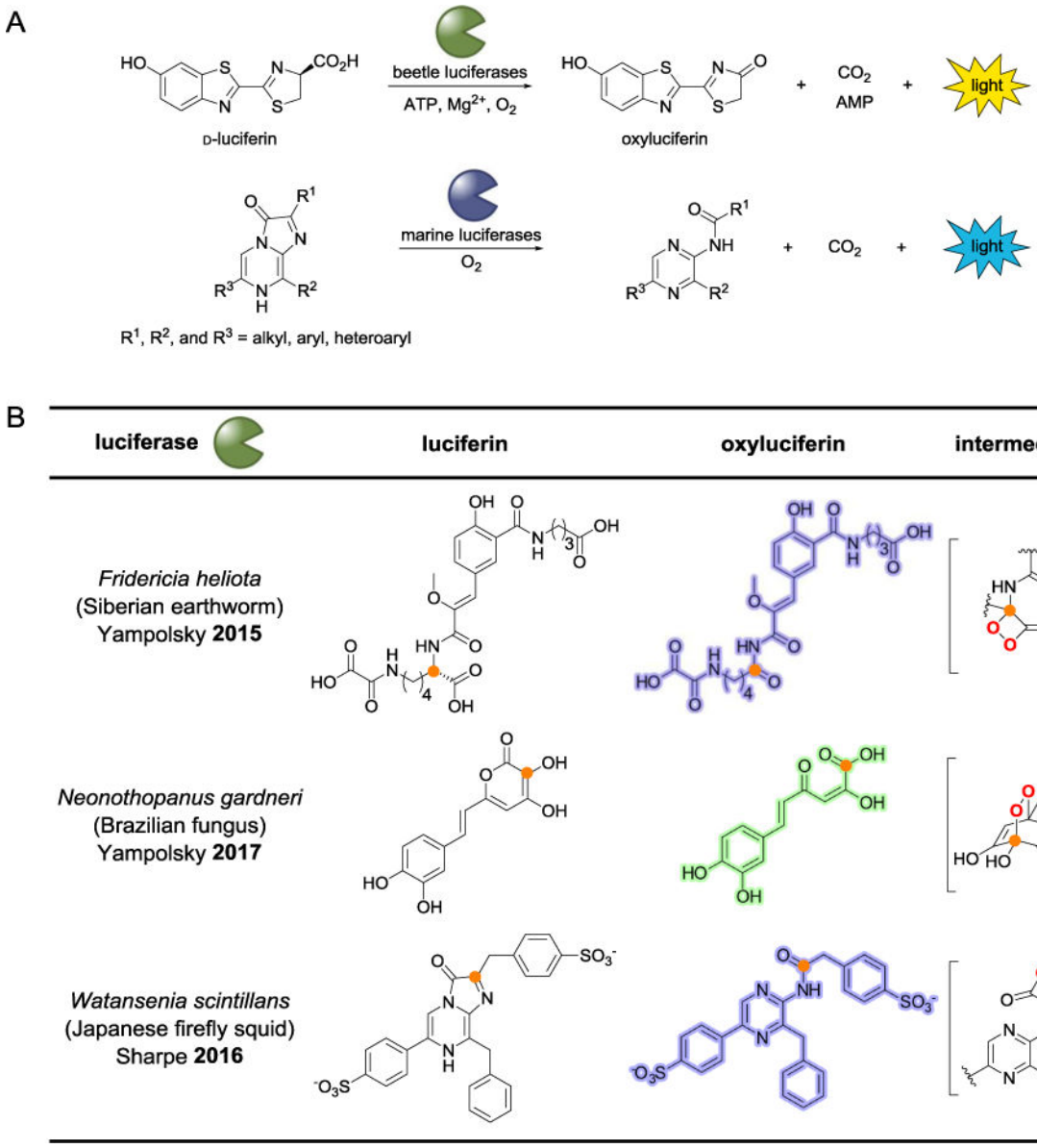

Figure 1. Luciferase-luciferin pairs in nature

(A) Beetle luciferases oxidize D-luciferin using ATP and O_2, generating primarily yellow-green light. Marine luciferases release blue photons via the oxidation of imidazopyrazinone analogs. **(B)** Recently characterized luciferases exploit unique molecules and mechanisms to produce light. The relevant luciferins and oxyluciferin products are shown. Orange dots mark the sites of oxidation.

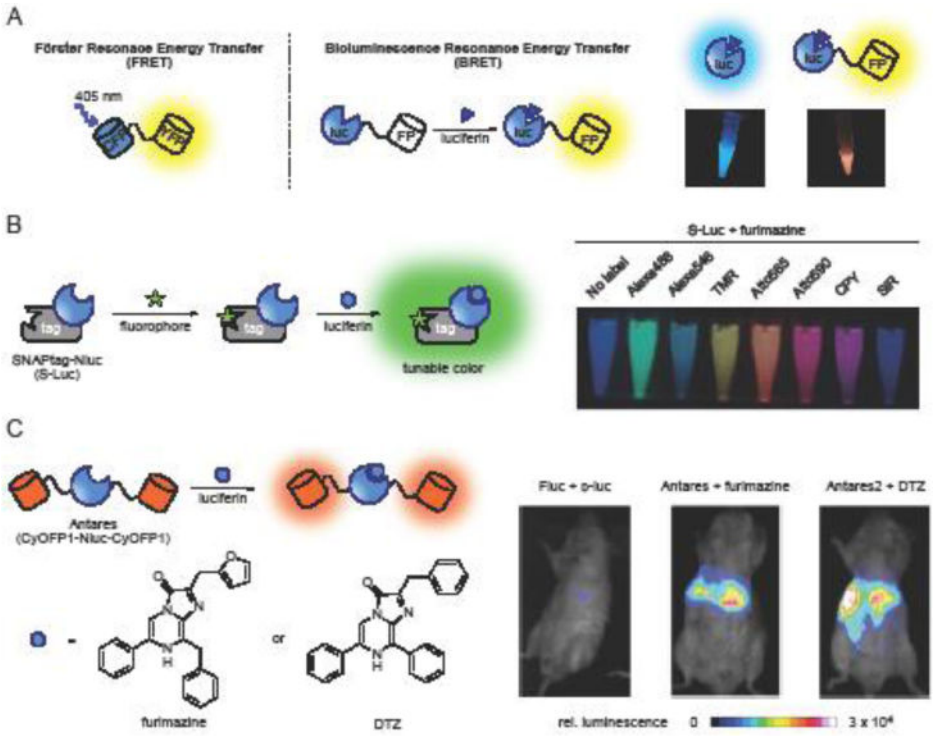

Figure 2. Expanded palette of bioluminescent probes

(A) Resonance energy transfer can tune optical emission spectra. In FRET, donor fluorophores (e.g., CFP) are excited with external light. Emission from the acceptor fluorophore (e.g., YFP) is observed. In BRET, luminescent reactions can excite acceptor fluorophores, resulting in altered emission spectra. A sample BRET construct (ReNL, pairing Nluc with tdTomato) is shown. **(B)** Nluc-fluorophore chimeras expand the palette of bioluminescent probes. Fluorescent molecules were appended to Nluc via SNAPtag ligation (left), generating a colorful array of S-Luc tags (right). S-Luc images were reproduced with permission from ref. 22. **(C)** Far red-emitting BRET constructs enable sensitive imaging *in vivo*. Antares comprises Nluc and two copies of a fluorescent protein (CyOFP1). Red-shifted light is produced upon luciferin administration. Antares and a related construct (Antares2) were expressed in mice following hydrodynamic transfection. Light emission was observed upon furimazine or DTZ administration (3.3 μmol i.p.). Mouse images were reproduced with permission from ref 24.

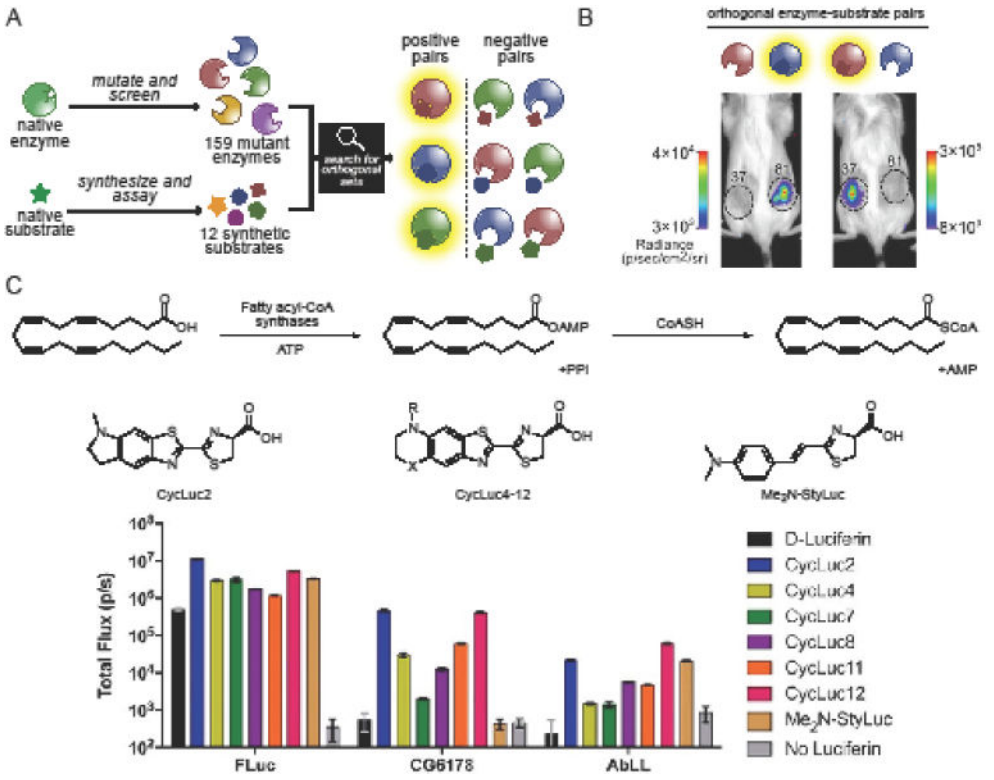

Figure 3. Substrate-selective luciferases for multi-component imaging

(A) Orthogonal luciferases were identified via parallel screening of luciferase mutants and luciferin analogs. **(B)** Dual imaging with engineered luciferase-luciferin pairs. DB7 cells expressing orthogonal mutants (37 and 81) were inoculated in opposing flanks. The populations were readily distinguished upon administration of the complementary luciferins. Bioluminescence images were reproduced with permission from ref. 33. **(C)** Fatty acyl-CoA synthetases from non-luminous organisms (e.g., CG6178 from *D. melanogaster* and AbLL from *A. binodulus*) exhibit luciferase-like behavior with synthetic luciferin analogs. Bar graph was reproduced with permission from ref. 36.

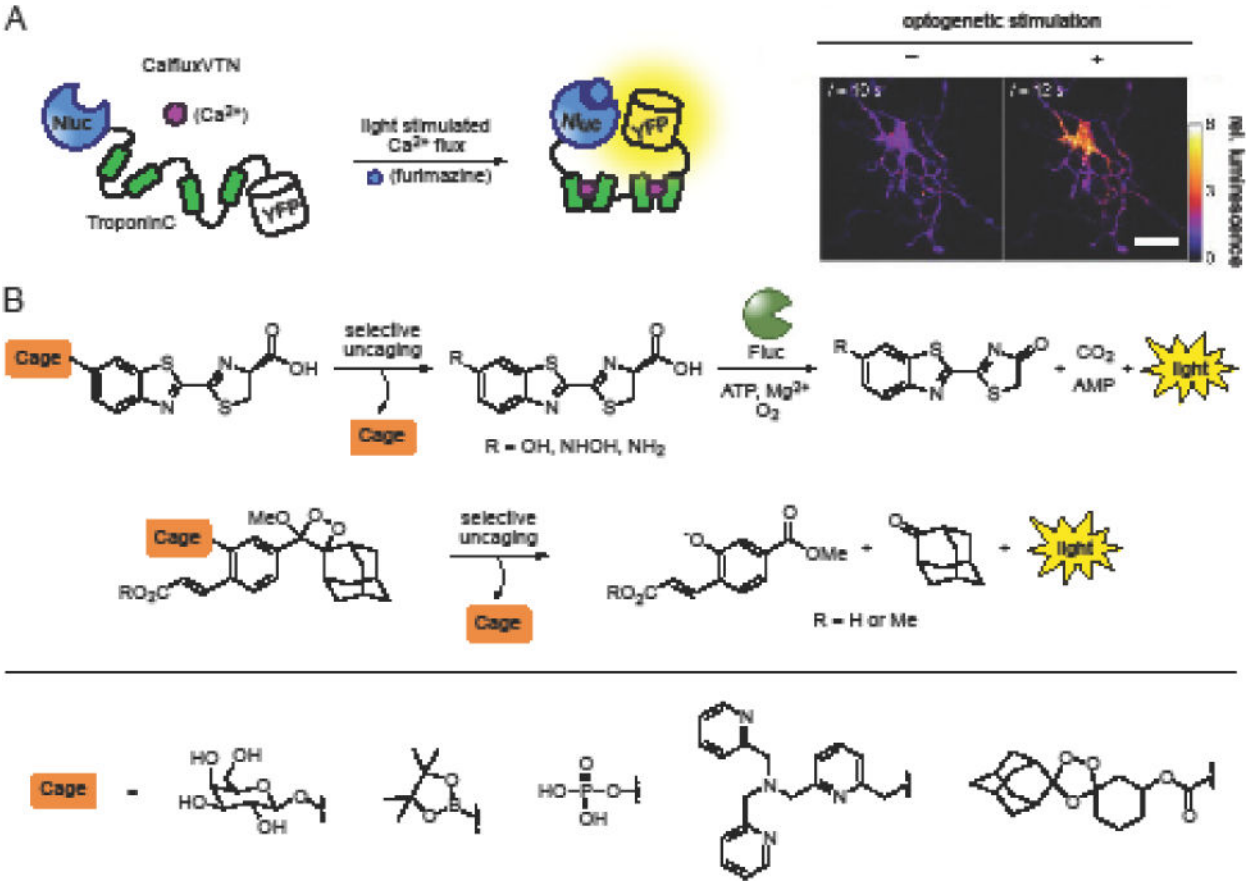

Figure 4. Visualizing cellular species with bioluminescent sensors

(A) CalfluxVTN comprises a calcium-binding protein (TroponinC) flanked by Nluc and YFP (left). Ca^{2+} binding induces a conformational change in the sensor, resulting in BRET. ClafluxVTN was expressed in neurons and used to monitor Ca^{2+} flux following photoreceptor firing (right, scale bar = 20 μm). Cellular images were reproduced with permission from ref. 48. **(B)** Caged luciferins and dioxetanes can report on cellular activities. Selective removal of the caging groups provides an active luminophore. Light emission via luciferase oxidation (top) or direct chemiluminescence (bottom) thus provides a readout on the uncaging enzyme or analyte of interest.