Title
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Journal
Cell Chemical Biology, 27(8)

ISSN
2451-9456

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Publication Date
2020-08-01

DOI
10.1016/j.chembiol.2020.07.022

Peer reviewed

Published in final edited form as:

Cell Chem Biol. 2020 August 20; 27(8): 904–920. doi:10.1016/j.chembiol.2020.07.022.

Seeing (and using) the light: Recent developments in bioluminescence technology

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Abstract

Bioluminescence has long been used to image biological processes *in vivo*. This technology features luciferase enzymes and luciferin small molecules that produce visible light. Bioluminescent photons can be detected in tissues and live organisms, enabling sensitive and noninvasive readouts on physiological function. Traditional applications have focused on tracking cells and gene expression patterns, but new probes are pushing the frontiers of what can be visualized. The past few years have also seen the merger of bioluminescence with optogenetic platforms. Luciferase-luciferin reactions can drive light-activatable proteins, ultimately triggering signal transduction and other downstream events. This review highlights these and other recent advances in bioluminescence technology, with an emphasis on tool development. We showcase how new luciferins and engineered luciferases are expanding the scope of optical imaging. We also highlight how bioluminescent systems are being leveraged not just for sensing—but also controlling—biological processes.

Graphical Abstract

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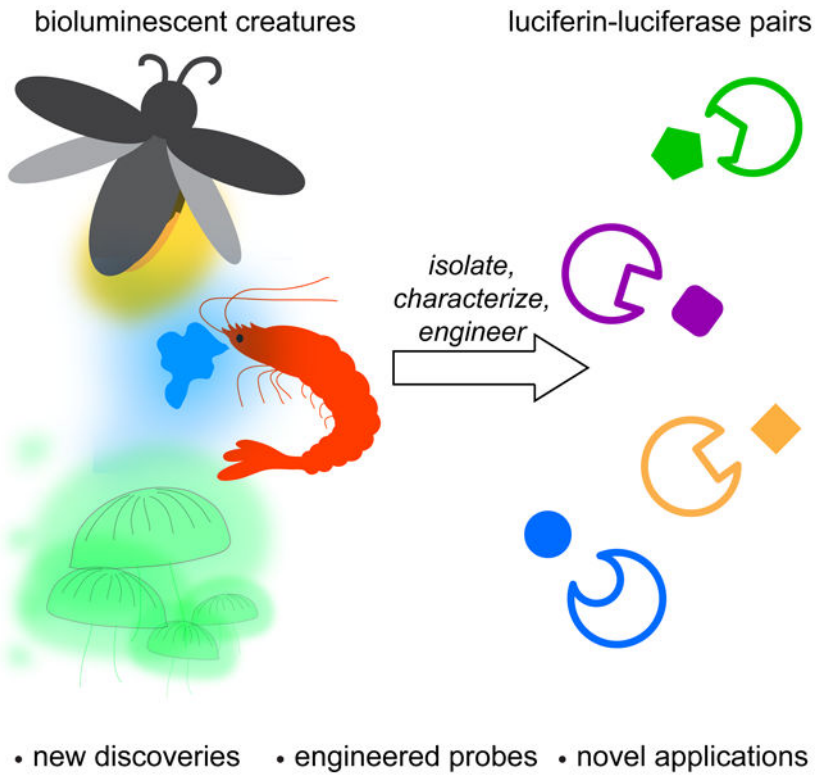

eTOC

Love and Prescher review the discovery and optimization of bioluminescent probes. Such probes have found widespread use in biological imaging, and in recent years, have been used in combination with optogenetic switches.

Introduction:

Bioluminescent probes have been used for decades to report on biological functions (Kaskova et al., 2016; Yao et al., 2018; Yeh and Ai, 2019). These tools emit photons that can illuminate events in cells, tissues, and even live organisms (James and Gambhir, 2012) Bioluminescent light derives from a chemical reaction between luciferase enzymes and luciferin small molecules (Paley and Prescher, 2014). Dozens of luciferase-luciferin pairs from the natural world have been coopted for imaging in heterologous organisms (Oba et al., 2017; Tsarkova et al., 2016; Viviani, 2002; Widder, 2010). The probes employ similar mechanisms to produce photons, but they differ in the wavelengths produced and other optical properties (Kaskova et al., 2016). Bioluminescent light can escape tissue, although it is subject to the same scattering and absorption phenomena as other optical probes (Contag and Bachmann, 2002). However, since bioluminescence does not require excitation light, it can circumvent the high background signals (via autofluorescence) commonly associated with fluorescent proteins in tissues and opaque animals (Contag and Bachmann, 2002). As a consequence, luciferase imaging is often a go-to technique for sensitive imaging in rodents and other preclinical organisms (Contag, 2007; Mezzanotte et al., 2017; Xu et al., 2016). Bioluminescence is particularly well suited for long-term studies, as the probes are largely

nontoxic and not susceptible to photobleaching. Indeed, luciferase-luciferin pairs have been widely used for examining cell trafficking patterns and proliferation *in vivo* (Minn et al., 2005; Prescher and Contag, 2010; Santos et al., 2009). Numerous other applications are also possible, owing to the sensitivity, broad dynamic range, and user-friendly features of bioluminescence.

More biological discoveries are on the horizon, thanks to recent achievements in bioluminescent probe design. Over the past few years, novel luciferases and luciferins have been identified in the natural world and coopted for imaging new targets (Yeh and Ai, 2019). Well-established bioluminescent tools have also been tuned to emit different colors and report on cellular metabolites, among other functions (Rathbun and Prescher, 2017). Additional engineering efforts have provided collections of bioluminescent probes with unique reactivity profiles (Yao et al., 2018). These tools can illuminate multiple targets in cells, tissues, and even whole animals. The expansion of the bioluminescent toolkit mirrors the development of other optical probes. Studies of fluorescent protein structure and function, in particular, paved the way for dozens of new reporters (Rodriguez et al., 2017). Many were engineered to exhibit distinct emission profiles (Heim et al., 1994; Shaner et al., 2004), improved photon outputs (Shaner et al., 2013; Shaner et al., 2008), or light-induced conformational changes (Lukyanov et al., 2005), among other properties (Davidson and Campbell, 2009; Duwé and Dedecker, 2019).

Beyond imaging, bioluminescent light can be harnessed to manipulate biological systems. Luciferases are known to participate in a variety of energy transfer reactions in nature (Titushin et al., 2011). Such processes can potentially be coopted to control both intra- and intercellular functions. In recent years, researchers have integrated luciferases with a variety of light-responsive proteins and molecules, including retinal chromophores and photolabile groups (Berglund et al., 2013; Lindberg et al., 2018). These light-sensitive tools can be employed in a variety of mammalian cells and tissues (Fenno et al., 2011; Pudasaini et al., 2015). When illuminated, the biomolecules undergo conformational changes or cleavage reactions, ultimately triggering gene expression and other downstream functions (Bardhan and Deiters, 2019; Fenno et al., 2011). The necessary excitation light is typically delivered via UV bulbs or LED arrays. Bioluminescent photons can take the place of these external sources, providing a means to activate networks noninvasively, via simple addition of a small molecule. While such bioluminescent optogenetic platforms are only in their infancy, they hold much promise for controlling biological activities in the brain and other deep tissues – locales that are typically refractory to external light and require implantation of fiber optic cables (Doronina-Amitonova et al., 2013).

This review highlights recent advances in bioluminescent probe development that are driving new directions in biomedical research. We begin with a basic overview on bioluminescent platforms, then describe the discovery and characterization of new light-emitting systems. We further highlight synthetic efforts that have afforded novel, bioavailable luciferins with unique photophysical properties. We also discuss parallel advances in enzyme engineering that have provided a broad set of luciferases with improved kinetic and spectral emission profiles. Many of these luciferases are also selective for unique luciferin architectures and can be used for multi-parameter imaging. The last section focuses on bioluminescent probes

used for applications beyond imaging, including optogenetics. These platforms leverage luciferase-luciferin reactions to selectively activate proteins or molecular bonds, enabling precise chemical control over biological processes. Collectively, bioluminescent probes are not only pushing the boundaries of what can be "seen" in living systems, but also what can be controlled.

How nature produces light

Bioluminescence has been observed in organisms across many domains of life (Lloyd, 1983; Widder, 2010). The natural glow is used for a variety of functions (Haddock et al., 2009; Wood et al., 1989a), including mating and defense (Hastings, 1983). Bioluminescence is strikingly prevalent in the deep ocean where sunlight is scarce and luciferase-luciferin pairs can provide a source of photons. In fact, thousands of creatures have evolved to emit blue light, as these wavelengths travel farther in ocean water compared to other colors (Martini and Haddock, 2017). Among the most recognizable bioluminescent sea creatures is *Oplophorus gracilirostris*, a shrimp that spews a bright blue cloud of luminescent liquid to distract predators (Inouye and Sasaki, 2007). Other marine organisms glow due to the presence of symbiotic bioluminescent bacteria (Nealson and Hastings, 1979). The microbes use the host for survival and, in return, provide a shimmer of light to attract prey or camouflage the host (Ruby and Lee, 1998). Still other ocean dwellers produce photons using luciferases (or luciferase-like photoproteins) in combination with fluorescent proteins. A famous example is *Aequorea victoria*, a jellyfish that emits green light via energy transfer between a luciferase-like photoprotein (aequorin) and green fluorescent protein (GFP, Shimomura et al., 1962, 1963). On land, insects such as beetles and fireflies have evolved to emit yellow-green light. The well-known North American firefly, *Photinus pyralis*, uses this light to communicate and attract mates (Lloyd, 1983). Other terrestrial bioluminescent organisms include glowing fungi (Oliveira et al., 2015) and earthworms (Petushkov et al., 2014). The light-emitting mechanisms for these species have only recently been elucidated.

The diversity of bioluminescent organisms contrasts with the surprisingly small number of mechanisms known for light production (Kaskova et al., 2016). All bioluminescent creatures use molecular oxygen and luciferase enzymes to catalyze light emission. The enzyme structures and luciferin substrates vary (Figure 1), although the reactions proceed through similar types of intermediates. The reaction pathways generate excited-state oxyluciferins; relaxation of these molecules to the ground state results in photon emission. Luciferins are "push-pull" chromophores that behave similarly to fluorescent molecules, requiring electron-donating groups and conjugated pi systems for robust light production. The optical properties of bioluminescent reactions are also influenced by the luciferase microenvironment (McElroy et al., 1965). Modulating either the luciferase enzyme or the luciferin small molecule can alter bioluminescent colors and other optical features (Branchini et al., 2010; Hall et al., 2018; Kuchimaru et al., 2016; Rumyantsev et al., 2016).

Despite the prevalence of bioluminescent systems, few have been widely applied *in vivo*. The color of light produced, reporter stabilities, and probe bioavailabilities are often sub-optimal for imaging in tissues (Contag and Bachmann, 2002). Perhaps the most widely used luciferin for imaging *in vivo* is D-luciferin (Yeh and Ai, 2019). This substrate is oxidized by

firefly luciferase (Fluc) and related insect luciferases. The mechanism likely proceeds via single electron transfer (Branchini et al., 2015), producing a high-energy dioxetanone intermediate. Elimination of CO_2 affords the oxidized product and a photon of yellow-green light (in the case of Fluc) (Kaskova et al., 2016). Different colors are produced when D-luciferin is processed by other enzymes. For example, luciferases from the click beetle generate light ranging from green to red (Wood et al., 1989b), underscoring the role of the enzyme microenvironment in modulating emission color. It should also be noted that insect luciferases require ATP to activate the luciferin substrate (Inouye, 2010). This requirement has limited the use of Fluc and related luciferases in extracellular spaces and other areas devoid of large ATP stores.

Similar to insects, most marine luciferases catalyze light emission via dioxetanone intermediates (Markova et al., 2019). Some of the most popular include *Renilla* luciferase (Rluc) and *Gaussia* luciferase (Gluc). The substrate for these (and nearly all) ocean luciferases is the pyrazinone molecule, coelenterazine (CTZ) (Haddock et al., 2009). CTZ, unlike D-luciferin, does not require pre-activation with ATP. Additionally, bioluminescent reactions with CTZ provide primarily blue light (Jiang et al., 2016). The distinct emission spectra and substrate profiles of marine and insect luciferases enables them to be used in tandem for imaging multiple targets (Paley and Prescher, 2014). Multi-component imaging with D-luciferin and CTZ *in vivo*, though, remains challenging. One substrate is typically injected at a time, and signal from the first luciferin must clear prior to administration of the second. The blue light produced by marine luciferases is also poorly tissue penetrant, limiting detection in some cases. Further, CTZ is quite electron rich and prone to non-specific oxidation, resulting in high background emission (Jiang et al., 2016).

Like marine luciferases, bioluminescent bacteria emit blue photons. However, the light-emitting mechanisms are quite distinct. Bacterial bioluminescence is mediated by a suite of enzymes encoded by the *lux* operon (Brodl et al., 2018). Two of the operon genes give rise to a functional luciferase, while the others encode a fatty acid reductase complex and a flavin reductase. The luciferase oxidizes long-chain aldehydes using molecular oxygen and reduced flavin mononucleotides. During the reaction sequence, a flavin conjugate is promoted to the excited state; relaxation to the ground state provides light. Importantly, all the machinery necessary for aldehyde oxidation (and thus bioluminescence) is encoded in *lux* (Brodl et al., 2018). The entire operon can be expressed in heterologous organisms, resulting in a continuous glow (Meighen, 1993) with no exogenous substrate required. The *lux* operon has been used for decades to examine the spread and life cycle of numerous pathogens *in vivo* (Contag et al., 1995; Guijarro and Méndez, 2020; Kadurugamuwa et al., 2003; Na et al., 2019; Sweeney et al., 2019; Wiles et al., 2004). The operon has yet to be routinely applied in mammalian cells, though, owing to aldehyde toxicity and low photon outputs. The Sayler group recently introduced the *lux* genes into a panel of cancer cell lines (Xu et al., 2014). The requisite enzymes were encoded on a single plasmid, separated by self-cleaving elements. The cells exhibited autoluminescence, but light emission was weak. Photon outputs were increased when the expression levels of key enzymes were modulated. Recent engineering efforts have further improved the bacterial bioluminescence platform for imaging in both bacteria (Gregor et al., 2018) and mammalian cells (Gregor et al., 2019).

Contrasting with bacterial bioluminescence, the biosynthetic pathways for D-luciferin and CTZ remain unknown. As a consequence, these substrates must be supplied exogenously in experiments involving common luciferases (Fluc, Rluc, etc.). There remains intense interest in elucidating the origins of D-luciferin and CTZ, to eventually circumvent the need for substrate delivery (Adams Jr and Miller, 2020). Firefly feeding studies with putative precursors suggested that D-luciferin can result from 1,4-hydroquinone condensation with two cysteine residues (Oba et al., 2014). Unfortunately, the exact enzymes involved in the transformation were not determined. In related work, Weng and coworkers undertook large-scale sequencing efforts to probe D-luciferin biosynthesis. The genomes of fireflies and other insects were determined, and transcriptomic analyses revealed candidate biosynthetic genes (Fallon et al., 2018). To date, though, the definitive pathway has not been revealed. Genome mining has also been used to probe CTZ biosynthesis. This luciferin is hypothesized to arise from a tripeptide comprising phenylalanine and two tyrosine residues (i.e., the “FYY” motif) (Francis et al., 2015). A cyclization-dehydration sequence could potentially give rise to a light-emitting substrate. The FYY motif is present in the genomes of many bioluminescent ctenophores, but conspicuously absent from non-luminescent relatives (Francis et al., 2015). Feeding studies with bioluminescent copepods also suggest that CTZ can be produced *de novo* from phenylalanine and tyrosine (Oba et al., 2009; Tessler et al., 2018), although the precise mechanism is unknown.

Despite the inherent challenges, two new luciferin architectures and their associated biosynthetic pathways have recently been solved. The first luciferin derives from the marine polychaetes, *Odontosyllis undecimdonta* (Kotlobay et al., 2019). The Yampolsky and Tsarkova groups elucidated the structure using a combination of mass spectrometry, NMR, and X-ray analyses. The tricyclic luciferin is hypothesized to undergo two separate oxidative pathways—one that results in light emission from canonical luciferase activity. Yampolsky and coworkers also uncovered a second luciferin and novel light-emitting mechanism in the bioluminescent fungus, *Neonothopanus nambi*. The fungus uses 3-hydroxyhispidin as a light-emitting substrate, converting it to caffeylpyruvic acid via a unique endoperoxide intermediate (Kotlobay et al., 2018). Excitingly, the genes involved in 3-hydroxyhispidin biosynthesis were also elucidated. The entire luciferin pathway can thus be expressed in target cells along with the requisite luciferase, enabling light production without the need for exogenous substrate delivery. The fungal bioluminescence system was recently introduced into tobacco and other plants, producing autoluminescent species (Khakhar et al., 2020; Mitiouchkina et al., 2020). Light emission was used to monitor hormone fluxes as well as diurnal flowing patterns (Khakhar et al., 2020). These examples highlight the potential for using fungal bioluminescence in heterologous organisms, and several more applications are anticipated in the near future.

Using nature’s light to “see” biology

Although several bioluminescent systems exist in the wild, only a handful have been widely used for imaging *in vivo*. Firefly luciferase (Fluc) remains one of the most popular, as it emits a larger fraction of red, tissue-penetrant photons compared to other naturally occurring luciferases (Zhao et al., 2005). The Fluc-D-luciferin pair also possesses other desirable features, including low background, excellent bioavailability, and easy handling.

Historically, Fluc has been used to image cell motility and proliferation, along with gene expression, in live animals. The luciferase is simply expressed in target cells of interest. When D-luciferin is administered, the compound can permeate the cells and light is produced. Photon intensities correlate with the amount of luciferase present, enabling biological features to be easily tracked. Seminal studies with Fluc-D-luciferin include tracking metastatic cancers (Jenkins et al., 2003; Scatena et al., 2004), infection (Luker et al., 2002; Schoggins et al., 2012), and other pathologies (Creusot et al., 2008; Swijnenburg et al., 2008) (Figure 2A).

Beyond tracking cells and gene products, bioluminescence can be used to report on enzyme activities and small molecule metabolites *in vivo*. Fluc itself has been engineered to be responsive to certain analytes (Smirnova and Ugarova, 2017). Many analogs of D-luciferin have also been tailored to report on cellular targets (Li et al., 2013; Porterfield and Prescher, 2015). These latter probes typically comprise “cages”, motifs that block Fluc processing and/or light emission. The cages are removed in response to complementary enzymes or metabolites. Once liberated, the luciferin can be used by Fluc to produce photons. Light emission thus correlates with the enzyme or analyte of interest. Promega Corporation has generated a wide variety of caged molecules, including probes for oxidase (Valley et al., 2006; Zhou et al., 2006b) and phosphatase (Zhou et al., 2008) activities, among others (O’Brien et al., 2005; Zhou et al., 2006a). Such molecules have been routinely used to assay enzyme function in lysates, cells, and tissues. Some have further enabled high-throughput screens for inhibitors (Fan and Wood, 2007). Caged probes have also been applied in rodent models. In a recent example, the Miller group designed a caged luciferin to report on fatty acid amide hydrolase (FAAH) (Mofford et al., 2015), a popular therapeutic target. FAAH is expressed in brain tissue and controls the lifetime of key fatty acid metabolites (Blankman and Cravatt, 2013). To detect FAAH activity in live mice, a caged luciferin comprising an amide group (instead of the key carboxylate in D-luciferin) was synthesized. The amide probe is not emissive with Fluc. In the presence of FAAH, though, the amide is hydrolyzed, restoring a functional carboxylate and thus light emission in the presence of Fluc. Miller and coworkers used the probe to visualize FAAH activity and screen drug inhibitors in mice. In related work, the Chang group generated a caged luciferin that was responsive to hydrogen peroxide *in vivo* (Van de Bittner et al., 2010). This probe comprises a 6’ aryl boronic acid moiety, which blocks production of a light-emitting luciferin. The cage is removed in the presence of cellular hydrogen peroxide, restoring the necessary 6’-OH group and thus light emission.

Blue-light emitting luciferases, including *lux*-expressing bacteria and marine luciferase-luciferin pairs, have also been co-opted for imaging *in vivo*. While they emit less tissue-penetrant photons than Fluc, these bioluminescent systems typically exhibit faster turnover kinetics and are thus “brighter” reporters (Bhaumik and Gambhir, 2002; Tannous et al., 2005). Rluc and Gluc also require no additional cofactors, aside from molecular oxygen (Kaskova et al., 2016). Thus, they are well suited for use in the extracellular space where ATP stores are limited. Rluc and Gluc have been used to examine a wide range of biological functions, including T-cell tracking in subcutaneous tumors (Figure 2B) (Jones et al., 2015; Santos et al., 2009). Other recent examples include monitoring stress granule formation in

viruses and the dynamics of biofilm formation *in vitro* (Bonenfant et al., 2019; Charoenviriyakul et al., 2018; Gutierrez Jauregui et al., 2019).

Moving beyond the all-natural

Despite the versatility and ubiquity of bioluminescence in biomedical imaging, limitations remain. As noted earlier, the blue light emission from many luciferases precludes their widespread use in some *in vivo* environments. Blue photons are prone to absorption by hemoglobin and other abundant chromophores in mammals (Raghuram et al., 2020; Yeh et al., 2019; Zhao et al., 2005). CTZ is also prone to autoxidation and can be toxic to cells (Jiang et al., 2016; Shipunova et al., 2018). While D-luciferin is less susceptible to off-target reactions, the turnover rate for insect luciferases is relatively slow, resulting in suboptimal photon outputs. Large amounts of D-luciferin are also required for *in vivo* experiments. Additionally, only a fraction of the photons produced by the Fluc-D-luciferin reaction effectively escape tissue. More sensitive imaging could be achieved with bioluminescent tools capable of producing larger numbers of red photons. There are also only a handful of luciferins that have been optimized for use in live animals, and most cannot be easily used in tandem. As a result, multi-component imaging *in vivo* with bioluminescent reporters remains challenging.

Voids in the bioluminescent toolbox have been addressed with artificial enzymes and substrates. Many recent efforts have focused on expanding the set of viable luminophores. Several groups have generated more stable and/or red-shifted insect luciferins, some of which are pictured in Figure 3A. For example, the Niwa group crafted a collection of red-emitting probes by extending the pi-conjugation of D-luciferin (Iwano et al., 2013). The most extended analog, termed AkaLumine, exhibited a ~100-nm red shift with Fluc compared to the native substrate D-luciferin. The intensity of light emission was low, suggesting that the substrate was poorly processed by Fluc. Some activity was recouped by engineering the luciferase enzyme (Iwano et al., 2018). Similar results were observed with infra-luciferin, a D-luciferin analog comprising an extra vinyl unit between the benzothiazole-thiazole ring juncture (Jathoul et al., 2015). Red-shifted emission has also been achieved with other pi-extended versions of D-luciferin, including naphthyl derivatives (e.g., NH_2-NpLH2 and OH-NpLH2) (Hall et al., 2018). These analogs release 650-750 nm photons with click beetle red luciferase and are excellent candidates for deep tissue imaging.

The Miller group reported a series of cyclic alkylaminoluciferin (CycLuc) analogs that exhibited both red-shifted emission and improved bioavailability (Mofford et al., 2014a; Reddy et al., 2010). CycLuc1, in particular, enabled more sensitive imaging in rodent models when compared to conventional doses of D-luciferin (Evans et al., 2014). CycLuc1 comprises a fused pyrrolidine in place of the hydroxy substituent, a structural perturbation that shifts its emission to ~600 nm (Reddy et al., 2010). The photon output from the Fluc-CycLuc1 reaction was low compared to the native enzyme-substrate pair, but activity was recovered when mutant enzymes were employed (Harwood et al., 2011). Excitingly, synthetic CycLucs were also able to reveal luciferase-like enzymes in non-luminescent organisms (Mofford et al., 2014b). Additional examples of “latent” luciferase activity are likely to be uncovered as new luciferin probes are developed.

Ongoing efforts to produce D-luciferin analogs are benefitting from streamlined and divergent synthetic routes (Kitada et al., 2018; McCutcheon et al., 2015; Sharma et al., 2017). Several of these methods have been used to produce sterically and electronically modified scaffolds (Williams and Prescher, 2019). Importantly, many analogs exhibit preferential activity with certain luciferase enzymes (Jones et al., 2017; Steinhardt et al., 2016; Steinhardt et al., 2017; Zhang et al., 2018). These unique patterns of reactivity can be used to discriminate luciferases in cells and whole organisms, providing an avenue for multi-component imaging that does not rely on color resolution (Rathbun et al., 2017).

A variety of CTZ analogs have also been produced in recent years (Figure 3B). Many efforts have focused on tuning the luciferin architecture to improve brightness, red shift the emission, and mitigate autoxidation. Some of the earliest reported CTZ analogs (e.g., e-CTZ and v-CTZ) included ring fusions at C6. When e-CTZ was paired with Rluc, the luminescence output increased seven-fold compared to CTZ (Inouye and Shimomura, 1997). When v-CTZ was paired with an engineered Rluc variant (Rluc 8.6), the emission spectrum was considerably red-shifted (emission max = 588 nm) (Loening et al., 2007). CTZ has also been modified at other positions to create designer luciferins (Jiang et al., 2016). One example includes CoelPhos, a CTZ analog comprising a phosphorylated appendage at C2 (Lindberg et al., 2013). CoelPhos has limited cell permeability and is thus well suited for visualizing biological events in the extracellular space. Another synthetic analog, ViviRen, is equipped with ester caging groups that temporarily block emission (Yeh and Ai, 2019). These cages can be removed by cellular esterases, releasing a viable luciferin and restoring bioluminescence. ViviRen enables more sustained light emission *in vivo* and is less susceptible to autoxidation than CTZ. These features can be advantageous for imaging in brain and other tissues (Otto-Duessel et al., 2006). Similar to D-luciferin, there have also been efforts to develop more modular syntheses of CTZ analogs (Coutant et al., 2019; Vece and Vuocolo, 2015). Such chemistries are expanding the number of custom luciferins for imaging applications.

Additional improvements to bioluminescent probes have come from modulating the other half of the light-emitting pair, the luciferase. Luciferases with faster kinetics and altered binding pockets have been developed in recent years (Yao et al., 2018; Yeh and Ai, 2019). For example, as mentioned earlier, the Miyawaki group engineered Fluc to process the pi-extended analog AkaLumine. The resulting enzyme, Akaluc, contains 28 mutations and enables more sensitive imaging than Fluc/D-luciferin in thick tissue and preclinical models (Iwano et al., 2018). Akaluc was transduced in the right striatum of a marmoset brain and visualized with AkaLumine. Real-time imaging was possible for over a year – a stunning achievement in bioluminescence technology. Related engineering efforts from Promega Corporation have provided mutant click beetle luciferases that can efficiently process naphthyl-modified luciferins (Hall et al., 2018). The best mutant showed improved activity not only with the naphthyl analogs, but also with D-luciferin. These bright and red-emitting bioluminescent probes will enable ever-more sensitive imaging applications. Mutant luciferases have also been identified that can selectively process infra-luciferin and D-luciferin, enabling two-component imaging in cells and *in vivo* (Stowe et al., 2019).

Several engineering efforts have been directed at marine luciferases to provide more desirable colors of light for imaging in thick tissues. Most efforts have capitalized on bioluminescence resonance energy transfer (BRET, Figure 4A), wherein luciferases are tethered to fluorescent proteins that harbor red-shifted emission spectra (Figure 4B, Yeh and Ai, 2019). In some notable examples, Nagai and coworkers fused Rluc (or an engineered variant) to a panel of fluorescent protein acceptors (Saito et al., 2012; Takai et al., 2015). Several BRET reporters were produced that exhibited a range of colors. One of the brightest conjugates, Rluc tethered to Venus fluorescent protein (yellow Nano-lantern, YNL), produced red-shifted light and ten-fold more photons than Rluc alone (Takai et al., 2015). YNL was further engineered to report on cellular Ca^{2+} levels. The BRET sensor was produced via insertion of the Ca^{2+}-binding domains calmodulin and M13 within the middle of the Rluc sequence. In the presence of calcium, the Rluc fragments can recombine to restore bioluminescence. The yellow emission of YNL enabled the Ca^{2+} sensor to be applied in whole animals (Saito et al., 2012). Additional BRET probes have been produced by pairing marine luciferases with near-infrared emitting fluorescent proteins (Rumyantsev et al., 2016) and quantum dots (Hasegawa et al., 2013) and can be coopted for numerous sensing applications.

Many recent bioluminescent applications have featured NanoLuc (Nluc). Nluc is an engineered variant of the luciferase from the deep-sea shrimp *Oplophorus gracilirostris* (Hall et al., 2012). This enzyme was engineered in combination with the native luciferin, CTZ. The goal was to identify a luciferase that yielded bright luminescence with a substrate that produced less background signal. Toward this end, a variety of CTZ analogs were produced that were more stable and less prone to autoxidation. These molecules were used in screens to identify suitable luciferases. The “winning” pair, NanoLuc and furimazine (FRZ), exhibited robust photon emission with reduced background. This pair also produced “glow”-type kinetics, enabling more sustained imaging in biological environments. Bioluminescent reactions that provide steady light outputs for >30 minutes (Andreu et al., 2010; Fraga, 2008) are popular choices for use in rodent models, where capturing peak light outputs can be challening. Many marine luciferases, by contrast, exhibit “flash” type kinetics. Such reporters produce strong, yet short-lived, (seconds to minutes) signals (Bhaumik and Gambhir, 2002; Tannous et al., 2005). The half-life of luminescent signal from Nluc-FRZ under standard imaging protocols is >2 h, a marked improvement over traditional marine luciferase reporters. The photon output is also ~150-fold higher than Rluc or Fluc under similar conditions, making Nluc well suited for long-term imaging applications. Further, Nluc is also remarkably stable at high temperatures and over a range of pH values, making it a “go-to” reporter in the field. Indeed, Nluc and related variants have been used in numerous applications over the past five years, including pathogen tracking (Azevedo et al., 2014; Sasaki et al., 2018), drug screening (Omachi et al., 2018), and biosensing (Aird et al., 2020; Chang et al., 2020). Nluc is also easily resolved from Fluc and other insect luciferases *in vitro*, based on its unique emission spectrum and substrate profile. Combinations of these reporters have recently been used to monitor multiple signaling pathways at once (Sarrion-Perdigones et al., 2019).

While Nluc has enabled numerous pursuits *in vitro* and *in cellulo*, this luciferase has been somewhat limited *in vivo* (Yeh et al., 2017). The emission maximum of the Nluc-FRZ

reaction (460 nm) is slightly blue-shifted from Rluc and Gluc (Hall et al., 2012). To push the wavelength to more a tissue-penetrant regime, researchers have paired Nluc with red-emitting fluorescent proteins. In one example, the Nagai group fused Nluc to tdTomato to create a red Nano-lantern (termed ReNL) (Suzuki et al., 2016). ReNL has an emission maximum of 590 nm, a 130 nm shift from Nluc itself. ReNL is part of a broader series of "enhanced" Nano-lanterns (eNLs) that comprise Nluc and well-known fluorescent proteins (Figure 4C). The fluorescent proteins were selected to provide high quantum yields and a range of colors. Several of the resulting BRET reporters, including the cyan enhanced Nano-lantern (CeNL) and the green enhanced Nano-lantern (GeNL), were found to emit more photons than the parental luciferase. The palette of eNLs has been co-opted for a variety of applications, including multi-spectral imaging of cellular organelles and calcium sensing (Farhana et al., 2019; Suzuki et al., 2016).

Related work from the Lin group provided Antares, an orange/red-emitting BRET reporter for sensitive imaging *in vivo* (Chu et al., 2016). Antares comprises Nluc flanked by two CyOFP1 fluorescent proteins. CyOFP1 is well suited to be a BRET acceptor with Nluc, as its absorption spectrum overlaps with the Nluc emission profile. CyOFP1 also exhibits a high quantum yield and a large Stokes shift, emitting a large portion of light above 600 nm. Such wavelengths are desirable for imaging in rodents and opaque tissue. Indeed, when a vector encoding Antares was hydrodynamically injected into mice, robust signal was observed in liver tissue. Notably, the signal was 2.6-fold higher than for traditional Fluc/D-luciferin imaging under the same conditions.

Further improvements to Antares were reported by the Ai group, in an effort to identify more robust and red-shifted imaging agents. This group generated libraries of Nluc mutants and screened them with a synthetic CTZ analog (diphenylterazine, DTZ). One of the "winners" (termed teLuc) catalyzed light emission with DTZ, exhibiting a maximum emission of 502 nm, a 60 nm shift from the Nluc-FRZ reaction. DTZ and teLuc enabled more sensitive imaging in thick tissue and mouse models compared to Nluc/FRZ and other CTZ-utilizing luciferases (Yeh et al., 2017). The Ai group also fused CyOFP1 proteins to teLuc to create a new BRET construct termed Antares2. Antares2 emits 3.8-fold more photons above 600 nm when compared to Antares itself, enabling even more sensitive imaging in rodent models.

In addition to classic cell tracking studies, Antares and Antares2 can report on other biological processes *in vivo*. In a seminal example, the Lin group engineered Antares to report on Ca^{2+} levels. Calmodulin and M13 were inserted into the Nluc region of Antares, which reduced photon output from the luciferase (Oh et al., 2019). Ca^{2+} binding to calmodulin triggered a conformational change in the sensor, restoring photon output. The bioluminescent probe was used to study calcium dynamics in different lobes of a mouse liver. The remarkable sensitivity and dynamic range of the sensor bode well for future studies of calcium dynamics *in vivo*, including in difficult-to-image areas like the brain. A related sensor based on Nluc/Antares has also been applied to study GPCR signaling (Oishi et al., 2020), and additional applications for metabolite and biomolecule detection are anticipated.

Similar to fluorescent protein conjugates, Nluc-fluorescent small molecule probes have proven useful sensors. In recent work, the Johnsson group developed a suite of BRET probes combining SNAP-tags and HaloTags with a circularly permuted version of Nluc (Hiblot et al., 2017). The SNAP-/HaloTag labels were used to append a broad range of fluorophores to the Nluc construct. The fusions were engineered such that the small molecules were positioned close to Nluc, optimizing energy transfer between the luciferase and acceptor. The resulting palette of probes was applied to image cellular organelles. Nluc-HaloTag conjugates have also been evaluated with JaneliaFluor dyes (Thirukkumaran et al., 2020). The best BRET combination enabled single-cell microscopic imaging with exposure times of <1 s.

Another set of BRET sensors was developed by the Johnsson group for measuring drug levels in small volumes (Griss et al., 2014). The probes, termed LUCiferase-based Indicators of Drugs (LUCIDs), comprise engineered Nluc-SNAPtag fusions and a receptor for the drug of interest. The SNAPtag facilitates installation of the requisite fluorophore, along with a competing ligand for the receptor. In the absence of drug, the competing ligand binds the receptor, enabling BRET between Nluc and the fluorophore. In the presence of drug, the ligand is released, and BRET is reduced. Such ratiometric readouts enabled sensitive detection of methotrexate and cyclosporin, among other drugs. The modular design of LUCIDs enables them to be applied to many other target analytes. Most recently, this concept was adapted for sensing NADH (Yu et al., 2019) and NADPH (Yu et al., 2018) in patient blood samples. The luminescent readout was on par with the most sensitive conventional methods for metabolite detection, including LC-MS. Importantly, the assays required just a drop of blood and a cell phone camera for detection, showcasing the simplicity and broad applicability of Nluc-based sensors for clinical diagnoses.

Using nature’s light to drive biological functions

Recent advances in bioluminescence technology have harnessed luciferases not just for visualization, but also to activate biological processes. Inspiration has come from the field of optogenetics, where photons from LEDs and other exogenous sources are used to trigger photoresponsive proteins (and often downstream gene expression) (Fenno et al., 2011). Light delivery is quite facile for experiments involving cultured cells or resected tissue. Light delivery *in vivo*, though, can be cumbersome, often requiring surgical implantation of optical fibers (Doronina-Amitonova et al., 2013; Häusser, 2014). The powerful light sources used can also heat surrounding tissue and perturb key measurements (Owen et al., 2019). Luciferases have attracted attention as “cold” light sources to drive optogenetic switches. Activation can be achieved by simple injection of the luciferin, a quick and routine procedure. Bioluminescent probes thus offer the advantage of being “chemogenetic”, in addition to optogenetic (Atasoy and Sternson, 2017). Light can be effectively “dosed” with added amounts of luciferin, enabling a tuned response. Bioluminescence is also functional in deep tissue, where activation of photoresponsive proteins has been historically difficult.

The initial targets for bioluminescence-driven optogenetics have been channelrhodopsins and LOV domains, proteins comprising well-known light-harvesting chromophores (Fenno et al., 2011; van der Horst and Hellingwerf, 2004) (Figure 5). The most common

channelrhodopsin used in optogenetics derives from algae and features a light-sensitive retinal chromophore (Boyden et al., 2005). Retinal absorption of blue light triggers a conformational change, ultimately enabling cation flux into the cell (Sineshchekov et al., 2009). The light-responsive feature of channelrhodopsin has been coopted to control neurons in a variety of settings (Boyden et al., 2005). The gene encoding channelrhodopsin can be expressed in mammalian cells, and upon irradiation with blue light, neurons are triggered to "fire" action potentials.

Flavin-binding proteins have been similarly targeted for bioluminescent optogenetics. Flavins play diverse roles in nature, ranging from catalyzing oxidation-reduction reactions to absorbing light in photocascades (Hemmerich and Nagelschneider, 1970). The most well-known flavin-binding proteins for optogenetics are the LOV domains, the Blue-Light-Using-FAD proteins (BLUFs), and cryptochromes (CRYs) (Losi and Gärtner, 2012). Many organisms use these domains to sense environmental conditions and regulate phototropism (Christie, 2007). Our discussion will focus on LOV domains, as they have been the initial targets of luciferase pairing. LOV domains are found in diverse organisms (Glantz et al., 2016) and play roles in circadian rhythms (Imaizumi et al., 2003) and stress responses (Möglich and Moffat, 2007), among other functions (Huala et al., 1997). These proteins are equipped with a key cysteine residue near the bound flavin (Figure 5). Upon excitation, the cysteine bites into the C4 position of the flavin ring (Christie, 2007; Conrad et al., 2014). Formation of this covalent adduct causes a conformational change in the protein, initiating the phototransduction cascade. Similar to channelrhodopsins, LOV domains have been broadly employed to turn-on gene expression with external light. Both classes of optogenetic probes – and how they have been merged with bioluminescence – are described in more detail below.

Channelrhodopsins.

The first widely used protein for optogenetics, channelrhodopsin (ChR), belongs to a family of ion channels commonly found in light-sensitive algae (Zhang et al., 2011). Like all opsins, ChR binds the chromophore retinal, a vitamin A derivative (Sineshchekov et al., 2009). This polyene is covalently bound to the channel via a Schiff-base linkage. Upon photon absorption, the *all-trans* form of retinal isomerizes to the 13-*cis* configuration. This isomerization changes the conformation of ChR, triggering opening of the pore for cation entrance and downstream activation (Figure 5) (Deisseroth and Hegemann, 2017). Known ChRs absorb blue or green light and are thus good targets for photoactivation by the marine-derived luciferases Gluc, Rluc, and Nluc (Deisseroth and Hegemann, 2017).

The Hochgeschwender group took advantage of the spectral overlap between Gluc and ChR to create a "luminopsin" probe (Figure 6A). Gluc is a bright, blue-emitting luciferase that does not require ATP or other cofactors to produce light, rendering it functional in the extracellular space. The researchers hypothesized that luminopsins could produce action potentials when expressed in neurons (Berglund et al., 2013). The first generation comprised Gluc fused to the N-terminus of channelrhodopsin-2 from *Chlamydomonas* (LMO1, Figure 6A). YFP was fused to the C-terminus to verify channel localization. Upon administration of CTZ, LMO1 action potential firing was observed (Figure 6B). After this initial report, other

luminopsins were developed to potentiate nerve cell firing or inhibition (Berglund et al., 2016; Berglund et al., 2020; Park et al., 2020). Inhibitory opsins block cation passage upon light stimulation, thus blocking action potential firing. Therefore, depending on the desired application, CTZ administration can either activate or prevent neuronal firing.

Luminopsins circumvent the need for external light delivery and are applicable in deep tissue. Thus, they can provide a way to non-invasively modulate brain activity. Luminopsins were evaluated in this context using an established mouse behavior assay (Kilpatrick et al., 1982). This assay involves GABAergic neurons in the substantia nigra. When these neurons are activated in one hemisphere of the brain, mice circle ipsilaterally. By contrast, when these neurons are silenced, mice circle in the opposite direction. Mouse GABAergic neurons were targeted with vectors encoding an activating luminopsin (LMO3) or an inhibitory luminopsin (iLMO) (Berglund et al., 2016). Upon administration of CTZ, mice expressing LMO3 circled primarily ipsilaterally. By contrast, mice expressing iLMO circled contralaterally. This experiment demonstrated the utility of luminopsins for controlling select populations of neurons in living animals, via application of a small molecule. Following this work, the Hochgeschwender group explored new combinations of engineered Gluc enzymes and various rhodopsins (Berglund et al., 2020; Park et al., 2020). Some of the resulting luminopsins comprised GlucM23 fused to more light-sensitive activating and inhibiting channelrhodopsins. Others included Gluc fused to ChR2 harboring step-function mutations. Such probes are expanding the optogenetic toolbox for controlling neuron functions (Petersen et al., 2019; Zenchak et al., 2020).

LOV domains.

Flavins represent another major class of light-harvesting species for bioluminescent optogenetics. These molecules come in a variety of flavors, but all possess a tricyclic isoalloxazine core (Hemmerich and Nagelschneider, 1970). Biologically, flavin exists in three main forms: riboflavin (the free vitamin), flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN). FAD and FMN comprise the majority of flavin-binding proteins, and these molecules are bound via pi-stacking and/or hydrogen bonding interactions (Losi and Gärtner, 2008). Flavin-dependent light-harvesting proteins absorb maximally at 460 nm (Losi and Gärtner, 2008) and have been used as optogenetic platforms (Christie et al., 2012). All absorb light and drive conformational changes in protein structure. We focus exclusively on LOV domains in this section, as they have been targeted with luciferase light sources. As noted earlier, such absorption leads to covalent trapping by neighboring cysteine residues, altering the overall structure of the domain and triggering downstream events. Excited-state flavins can also produce singlet oxygen, and this feature has been similarly exploited in conjunction with bioluminescence.

Some of the earliest examples of harnessing bioluminescence for non-imaging purposes involved LOV domains. For example, Nluc was fused to miniSOG (mini-singlet oxygen generator (Shramova et al., 2016), a LOV domain that was engineered to favor singlet oxygen production over photoadduct formation upon blue light illumination (Shu et al., 2011). miniSOG lacks the key cysteine residue for conventional LOV domain function. Nluc and miniSOG have ideal spectral overlap and, thus, a fusion construct enables cytotoxic

singlet oxygen production “on demand”. When Nluc-miniSOG was expressed in cells, cytotoxicity was observed upon administration of FRZ (Proshkina et al., 2018). Whether or not cells were killed due to singlet oxygen alone, though, remains unresolved (Pimenta et al., 2013; Ruiz-González et al., 2013; Shipunova et al., 2018).

The Ting group capitalized on the conformational switch observed when conventional LOV domains absorb light. Photoadduct formation drives opening of the J helix (Konold et al., 2016). This motif can be engineered to contain proteolytic cut sites or other recognition elements. Thus, upon blue light illumination, the J helix is exposed and available to mediate other biological processes (Kim et al., 2017; Sanchez and Ting, 2020; Wang et al., 2017). The Ting group used an engineered LOV domain in conjunction with Nluc to drive downstream gene expression (Kim et al., 2019). The strategy built off of their previously reported SPARK (Specific Protein Association tool giving transcriptional Readout with fast Kinetics) system to probe protein-protein interactions (Kim et al., 2017). SPARK comprised a transcription factor (Gal4) bound to the membrane via the β-adrenergic receptor (β2AR). This GPCR was tethered to a LOV domain with a TEV protease-cleavage site buried in the J helix (Kim et al., 2017). Upon blue-light activation, the J helix swung open to reveal the cleavage site. SPARK thus required two inputs: (1) external light to activate a LOV domain and (2) protein association (arrestin-β2AR) to drive signal to trigger transcription. SPARK decreased the background of conventional transcription-based detection methods of protein-protein interactions. However, high expression of the TEV-arrestin resulted in unintended cleavage of the protease cut site and artificial signal production.

To tune proteolysis more precisely, SPARK was modified to respond to luciferase-derived light. Nluc was expressed between arrestin and the TEV protease, providing SPARK2. When arrestin binds β2AR, Nluc comes into close proximity to the LOV domain. In the presence of FRZ, bioluminescent light can drive J helix release. Subsequent cleavage by TEV protease liberates the Gal4 transcription factor. Energy transfer between Nluc and LOV domain is highly distance dependent, resulting in reduced background signal and more sensitive readouts on protein-protein interactions. The Ting group was further able to show bioluminescent light activation in a transcellular sense. In this scenario, Nluc was fused to neurexin and ICAM in the extracellular space. These latter proteins drive neuron-neuron interactions. Upon cell contact and FRZ administration, Nluc on one cell activated a photoresponsive version of β2AR (Figure 7A) on a second cell, driving SPARK2. Transcription was modest, likely due to low levels of bioluminescent light. Additional enhancements can likely be achieved using engineered luciferases with higher photon outputs.

One approach to achieve higher power output involves BRET constructs (Parag-Sharma et al., 2020). As noted in the earlier discussion of Nano-lanterns, higher quantum yields from luciferase-fluorescent protein fusions have been observed (Suzuki et al., 2016). The Amelio group capitalized on this feature by developing a Nluc-mCerulean BRET construct known as LumiFluor. LumiFluor produces three-fold higher power outputs than Nluc alone. At physiologically relevant levels, LumiFluor can provide ~mW/m^2 power levels, on par with what is necessary to activate many blue-light sensitive proteins. Indeed, LumiFluor was used in conjunction with flavin-binding heterodimerizers to drive downstream gene expression

(Figure 7B). One of the most exciting results was activation of a light-inducible dCas9 system, suggesting genetic manipulation *in vivo* can be controlled via simple administration of luciferin. Further engineering of the LumiFluor platform will likely expand the number of potential applications.

Bioluminescent light has been used to drive processes beyond conventional optogenetics, including photouncaging reactions. Photolysis has long been used to release drugs and other payloads under spatiotemporal control in cells and tissues (Ellis-Davies, 2007). For example, nitrobenzyl photocages have been extensively employed for targeted activation of gene transcription (Lu et al., 2012), local release of anticancer agents (Dcona et al., 2012), and targeted deposition of imaging probes (Yang et al., 2017). Traditional applications require exogenous UV light, which can damage cells and surrounding tissue. Recent work has focused on developing photocages that are activated with less harmful wavelengths of light, including BODIPY (Peterson et al., 2018) and cyanine scaffolds (Gorka et al., 2018). Bioluminescent activation offers an alternative mechanism for caged molecule release. For example, Nluc can be used to activate ruthenium (Ru) photocatalysts (Lindberg et al., 2018). Such catalysts can cleave pyridinium linkers on caged molecules. Importantly, the photocatalysts are also minimally toxic in living systems, and have good spectral overlap with Nluc. The Winssinger group showed that Nluc undergoes energy transfer with the Ru molecule when in close proximity (Figure 7C). To properly localize the reagents, they used a SNAP-tag conjugate. Incubation with FRZ initiated bioluminescent light production and release of pyridinium-caged materials, including anti-cancer agents. A similar "bioluminolysis" strategy was employed with coumarin-based photocages (Chang et al., 2019), although no catalyst was required. Simple application of a small molecule luciferin could drive drug release. While much remains to be learned about the limits of luciferase-driven reactions, these examples suggest that bioluminescence can be harnessed to manipulate biological systems.

Conclusions and future directions

Bioluminescent tools have been widely applied in recent years – both for imaging and as light sources to activate photocascades. All applications rely on luciferase-luciferin pairs with appropriate emission spectra and robust photon outputs. Synthetic efforts have provided luciferins with altered colors, improved bioavailability, and distinct architectures. Concurrently, dozens of luciferases have been engineered to further modulate photon outputs and selectively process designer substrates. Additional work to uncover novel luciferase-luciferin pairs in nature has further expanded the imaging toolbox and deepened our understanding of how bioluminescence works.

Future imaging experiments will benefit from continued substrate optimization and enzyme engineering. More red-shifted probes will enhance imaging in thicker tissue. Higher photon outputs will also enable fewer cells to be more reliably visualized. With improved tools, it may soon be possible to perform single cell imaging in deep tissues *in vivo*. Additional substrate-specific luciferases will bolster efforts to track multiple cell types and other biological features. This work will continue to benefit from robust synthetic methods to produce collections of luciferins. Molecules deviating from canonical luciferin architectures

are also viable targets. Such chemiluminescent substrates can produce light with alternative oxidases or other cellular enzymes, comprising a distinct class of light-emitting probes (Gross et al., 2009; Hananya and Shabat, 2017; Hananya and Shabat, 2019).

Future optogenetics experiments will benefit from a combination of analytical work and engineering. Much remains to be learned about the mechanisms driving bioluminescent photocascades and the limits of such activation. Signal from bioluminescent-driven systems thus far has been minimal, likely due to the low photon outputs. Such challenges can likely be addressed with additional enzyme engineering and substrate tuning. Synthetic chemistry efforts are likely to yield new luciferins with improved photophysical properties and bioavailability. Most applications to date have relied on CTZ/FRZ, molecules with some liabilities owing to cytotoxicity and optical interference with common reporters. With new probes in hand, we anticipate other photoresponsive proteins and photocages will be pursued in conjunction with luciferases. Such tools will continue to push the boundaries of what is possible with bioluminescent technologies.

Acknowledgements

This work was supported by a grant from the National Institutes of Health (R01 GM107630 to J.A.P.). A.C.L was supported by the BEST IGERT program (National Science Foundation DGE-1144901). We would like to thank members of the Prescher lab for helpful discussions and edits.

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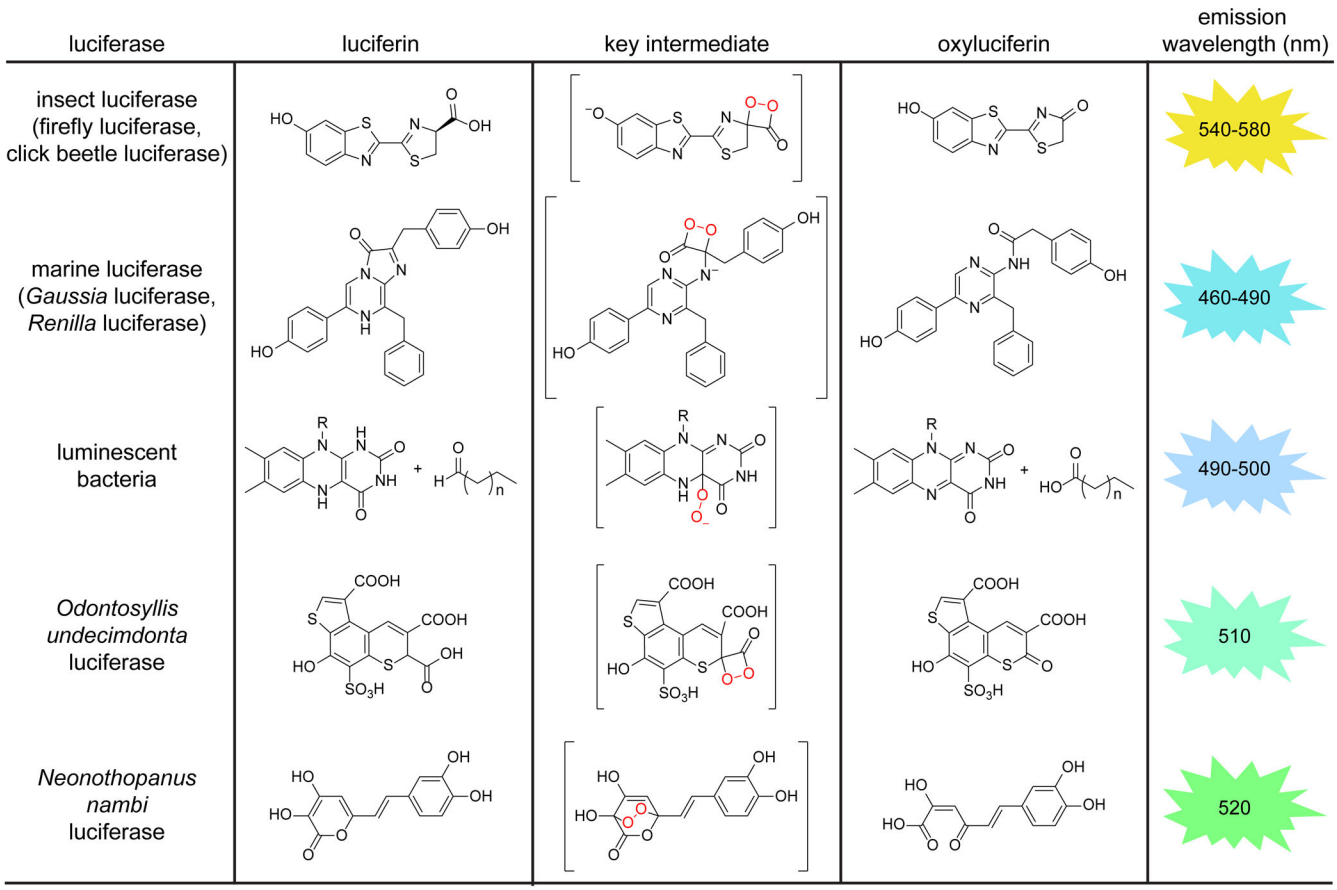


Figure 1. Bioluminescence in the wild.

Examples of naturally occurring luciferase enzymes and luciferin small molecules. All luciferases oxidize their complementary luciferins to yield photons of light, although unique chemistries are employed. Most bioluminescent probes emit light in the blue-green region. (*Emission wavelengths reported at room temperature.)

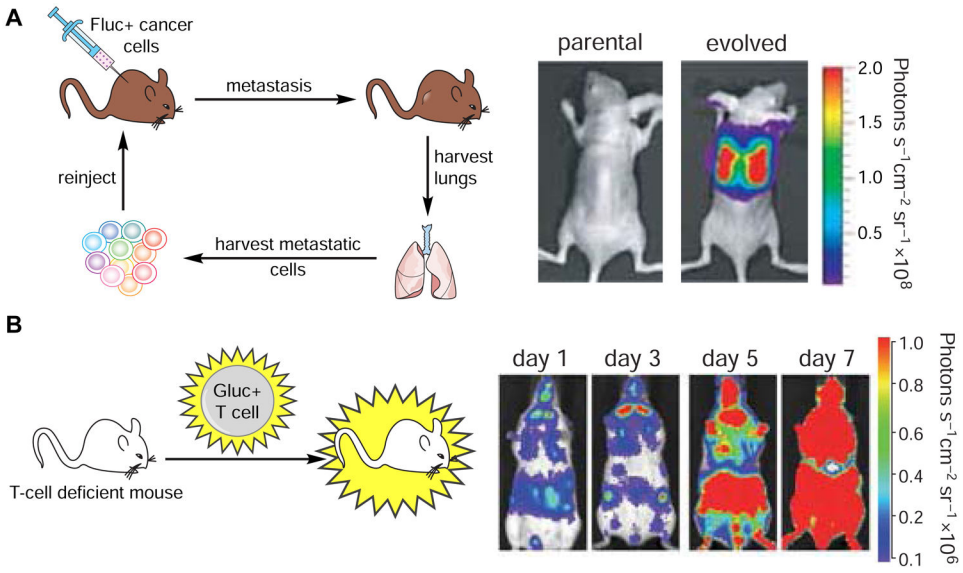


Figure 2. Noninvasive imaging in vivo with bioluminescent probes.

Luciferase-luciferin pairs have been routinely used to track cell migration and proliferation in vivo. (A) Imaging cancer cell proliferation and metastases. Mice were injected with Fluc-expressing MDA-MB-231 cells. Metastatic cells were harvested from lungs and re-injected into recipient mice. These cells exhibited a higher degree of metastatic outgrowth than the parental tumor. Figure reprinted with permission from Minn, et al., 2005 (B) Imaging T cell trafficking. Gluc-expressing T cells were injected into immunodeficient mice. T cell proliferation and homing were visualized over time via bioluminescence. Figure reprinted with permission from Santos et al., 2009.

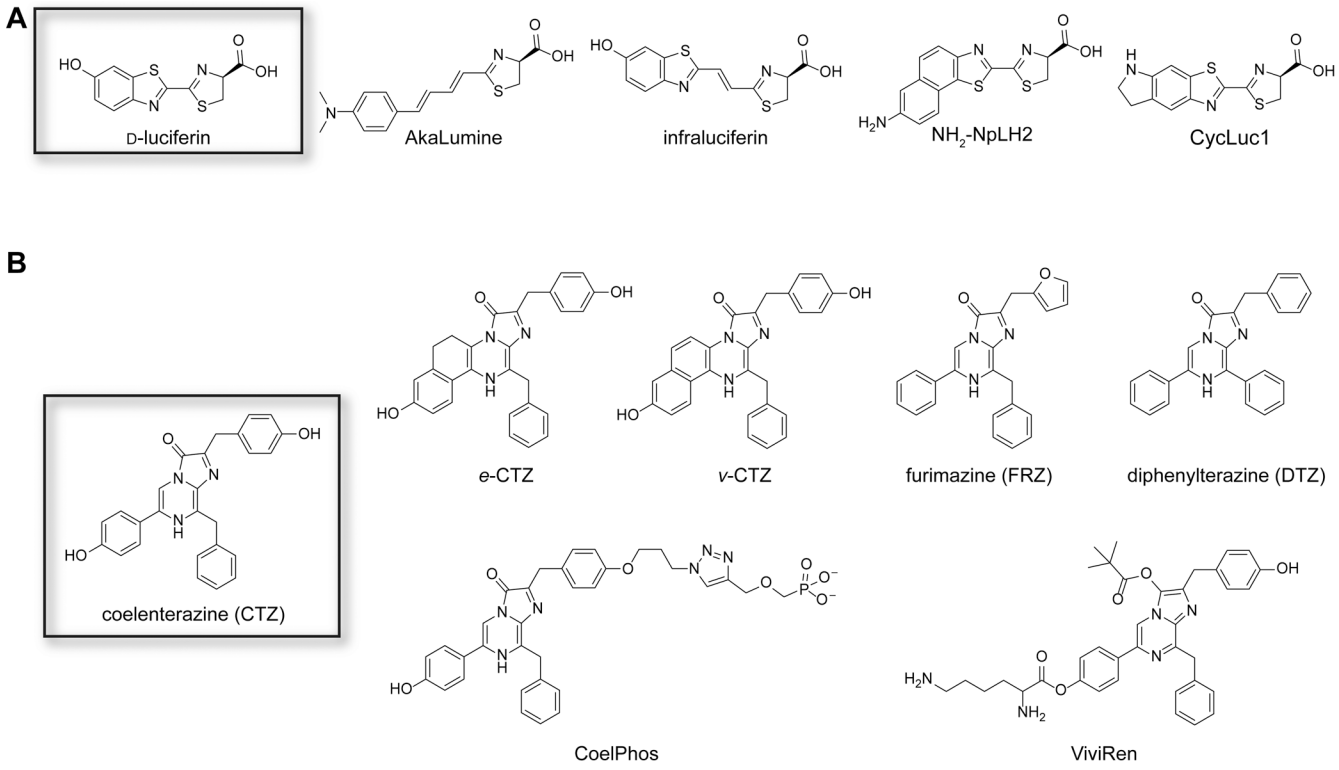
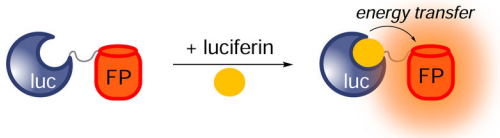


Figure 3. Synthetic luciferins.

(A) Representative analogs of D-luciferin. Some of these molecules exhibit preferential reactivity with mutant luciferases, red-shifted emission, and/or improved bioavailability in organisms. (B) Representative analogs of coelenterazine. Some of these molecules exhibit unique reactivities with mutant luciferases, improved stability and solubility, and/or altered wavelengths of emission.

A



B

Name	BRET pair	Reference
Nano-lanterns	Rluc - various FPs	Takai *et al.* (2015)
enhanced Nano-lanterns	Nluc - various FPs	Suzuki *et al.* (2016)
Antares	Nluc - CyOFP	Chu *et al.* (2016)
Antares2	teLuc - CyOFP	Yeh *et al.* (2017)
LUCIDs	Nluc - Cy3/TMR	Griss *et al.* (2014)

C

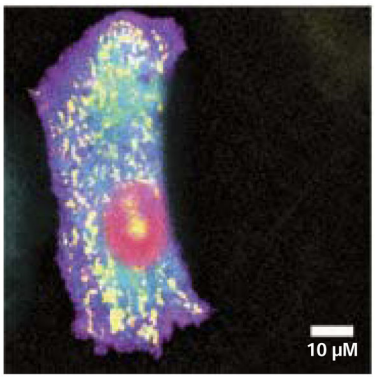


Figure 4. Multicolor imaging with engineered BRET reporters.

(A) A variety of luciferase-fluorescent protein (FP) fusions have been generated. Resonance energy transfer can provide a range of bioluminescent colors. (B) Example BRET reporters. These constructs comprise luciferase donors with either fluorescent protein or small molecule acceptors. (C) A panel of enhanced Nano- lanterns was expressed in HeLa cells, enabling real-time BRET imaging of cellular organelles. Reprinted with permission from Suzuki et al., 2016.

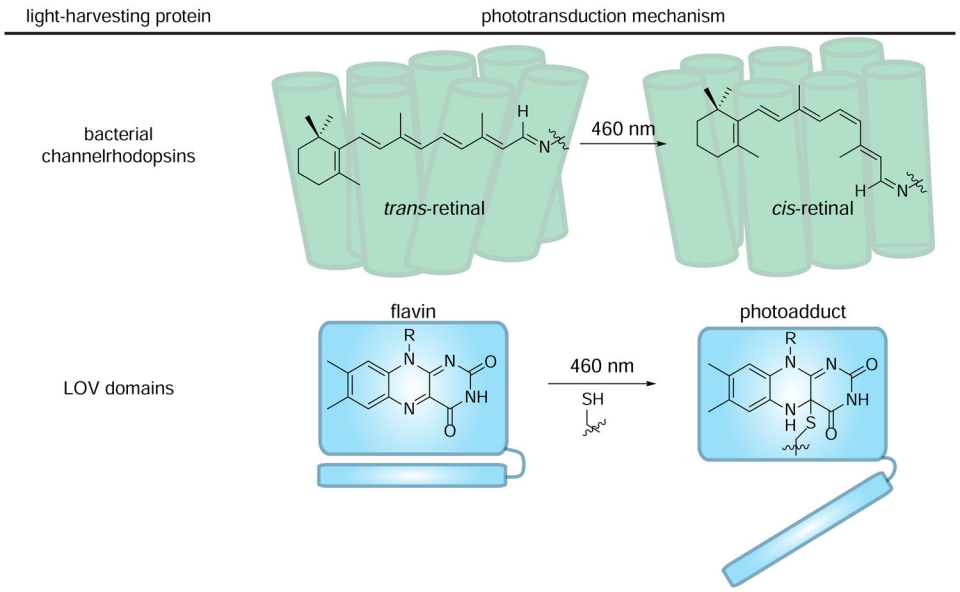


Figure 5. Light-harvesting proteins use unique chromophores to convert light energy to chemical energy.

(top) Channelrhodopsins comprise covalently bound retinal chromophores. Retinal isomerizes when illuminated with blue light, driving the channel pore to open. (bottom) LOV domains comprise noncovalently bound flavins chromophores. When excited with 460 nm light, flavin can be trapped by a nearby cysteine. The resulting conformational change initiates signal transduction.

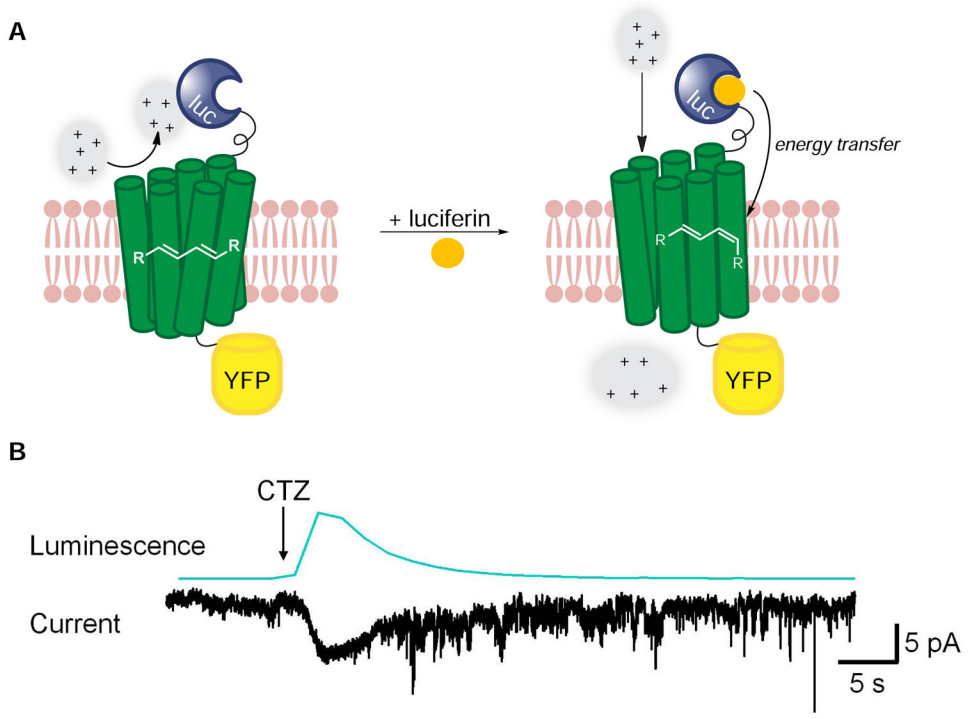


Figure 6. Luciferase-rhodopsin fusions enable spatiotemporal control over ion channel function.
(A) The first-generation luminopsin (LMO1) featured Gluc (blue) tethered to channelrhodopsin2 (ChR2, green). CTZ application potentiates energy transfer, driving retinal isomerization and channel opening. (B) LMO1 was expressed in HEK cells. CTZ application generated photocurrents. Reprinted from Berglund, et al. 2013.

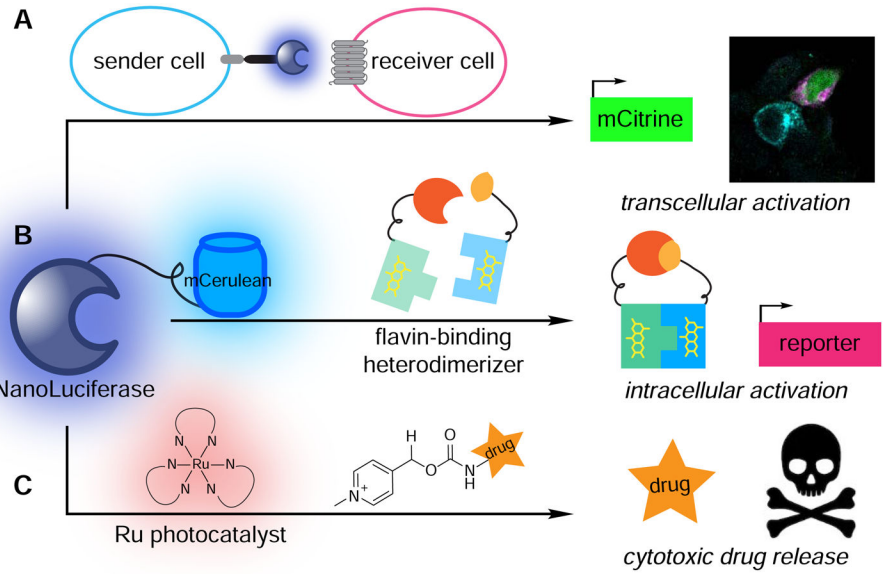


Figure 7. Recent examples of harnessing bioluminescent photons to drive biological processes.

(A) Transcellular activation of gene expression. Nluc expressed in "sender" cells (blue) activated light-sensitive proteins in "receiver" cells (pink), resulting in mCitrine expression (green). (B) Intracellular activation of gene expression. Energy transfer from a Nluc-mCerulean conjugate to a flavin-binding protein facilitated heterodimerization and downstream transcription of reporter genes. (C) Cytotoxic drug release. Energy transfer from Nluc to a ruthenium photocatalyst facilitated bond cleavage reactions and cargo release.