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Publication Date

2019-12-01

DOI

10.1016/j.copbio.2019.05.002

Peer reviewed

Caged ~~Luciferins for Bioluminescent Activity-Based S~~luciferins for bioluminescent activity-based sensing

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Bioluminescence imaging is a powerful modality for *in vivo* imaging owing to its low background and high signal-to-noise ratio. Because bioluminescent emission occurs only upon the catalytic reaction between the luciferase enzyme and its luciferin substrate, caging luciferins with analyte-reactive triggers offers a general approach for activity-based sensing of specific biochemical processes in living systems across cell, tissue, and animal models. In this review, we summarize recent efforts in the development of synthetic caged luciferins for tracking enzyme, small molecule, and metal ion activity and their contributions to physiological and pathological processes.

Current Opinion in Biotechnology 2019, **60**:xx–yy

This review comes from a themed issue on **Chemical ~~B~~biotechnology**

Edited by **Thomas Ward** and **Sven Panke**

https://doi.org/10.1016/j.copbio.2019.05.002

Introduction

Life arises from a complex symphony of concerted and dynamic biochemical activities in space and time. As such, an unmet grand challenge is to monitor and characterize such processes with molecular-level specificity and spatial and temporal resolution [1,2]. In this regard, synthetic optical probes for activity-based sensing (ABS) are promising biotechnologies to meet this need, where the intrinsic chemical activity of a given bioanalyte of interest, rather than lock-and-key molecular recognition, provides the basis for a selective optical readout [3]. Indeed, ABS imaging agents originated from the chemodosimeter work of Czarnik [4], which inspired the earliest examples of utilizing chemical reactivity for promiscuous reactive nitrogen species and selective hydrogen peroxide detection in cell culture [5,6]. However, the use of ABS for imaging in thick tissues or whole animals remains difficult owing to issues of tissue penetration and high background scattering of excitation and emission light. Along these lines, bioluminescence imaging is an attractive alternative imaging modality for *in vivo* ABS because light is generated from the decomposition of chemically excited luciferin molecules; as such, there is essentially no background from excitation light scattering, resulting in a higher signal-to-noise contrast. While the limited tissue penetration of emitted light remains a challenge, substantial efforts are underway to red-shift the bioluminescent emission wavelength to mitigate such issues [7,8].

Most ABS bioluminescent probes operate by caging luciferin, the natural substrate for the firefly luciferase enzyme, with an analyte-reactive trigger group at the 4-~~ or 6~~'position or 6'-position (Figure 1). Aminoluciferin is also employed in some instances. In the caged state, the trigger group disables luciferin-~~–~~luciferase catalysis such that no light emission occurs. Recognition and reactivity of the trigger moiety by a biochemical event (e.g.~~enze~~ enzyme or small molecule flux) releases~~s~~ the free luciferin molecule, which undergoes subsequent adenylation and oxidation to generate the transient dioxetanone intermediate. Subsequent decarboxylation leads to an excited state oxyluciferin molecule that then emits a photon upon relaxation to the ground state. The luminescent signal is ~~a~~proportional to the degree of uncaging and thus provides a functional readout of the activity of a particular enzyme or molecule. Moreover, because luciferase expression can be genetically encoded, caged luciferin-~~–~~luciferase pairs can be employed to sense biomolecular activity under specific conditional gene expression environments [9].

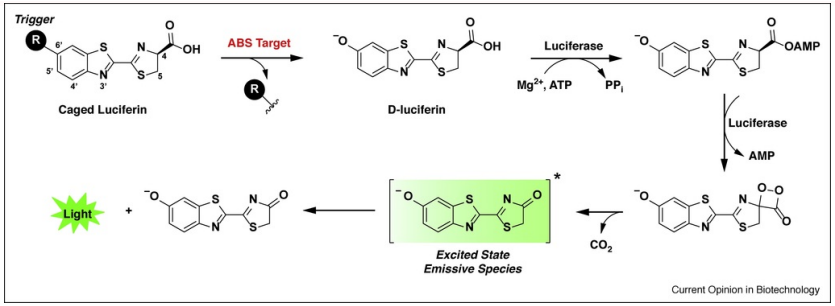

Figure 1 General design strategy for developing caged luciferins for bioluminescent activity-based sensing (ABS). Caged luciferins are typically functionalized with chemoselective and cleavable trigger groups at either the 4- ~~or 6~~position or 6'-position. Interaction with the ABS target (e.g. enzyme, small molecule, or metal ion) results in trigger cleavage to liberate free ~~D~~D-luciferin, which is the bioluminescent substrate for firefly luciferase enzymes and can go on to produce light through a biocatalytic reaction readout. Aminoluciferin can also be employed in place of ~~D~~D-luciferin as a substrate for caging.

alt-text: Figure 1

Here we focus on strategies for developing caged luciferins for activity-based sensing in bioluminescent systems. Many excellent reviews on bioluminescent imaging have been written from the perspective of mutating luciferases or improving the optical properties of the luciferin substrate [10–12], whereas others have provided a broader perspective that includes recent innovations in transgene engineering and signal acquisition [13]. The following sections will survey recent bioluminescent ABS probes for tracking enzyme, reactive small molecule, and metal ion fluxes and summarize how these tools have been employed to characterize their contributions to healthy and disease states in animal models.

Caged luciferins for bioluminescent activity-based sensing of enzyme function

Several caged luciferins have been developed for monitoring enzyme activity *in vitro*, *in cellulo*, and *in vivo* (Figure 2). The general approach for designing bioluminogenic sensors for enzyme activity is to functionalize either the 4- ~~or 6'-position of D~~position or 6'-position of D-luciferin with a chemical substrate for the enzyme of interest, where enzyme activity leads to trigger cleavage and release of free ~~D~~D-luciferin for inducing luciferase-dependent bioluminescence. For *in vitro* studies, enzyme activity is quantified by adding recombinant firefly luciferase to cell culture or lysate, then using luminescent readout as an indirect measure of enzyme-mediated luciferin uncaging. Seminal early work by Miska and Geiger reported synthetic caged luciferins for evaluating enzyme activity *in vitro* by installing phosphate, sulfate, methyl ester, and amino acid groups onto ~~D~~D-luciferin for the detection of alkaline phosphatase, aryl sulfatase, esterase, and carboxypeptidase activity, respectively [14]. The authors later extended their caging platform to sense β-galactosidase [15] and chymotrypsin [16] activity. From this starting point, several other ABS probes have been created for assessing the activity of a wide panel of enzymes *in vitro,* including monoamine oxidase [17], glutathione ~~S~~S-transferase [18], cytochrome P450 [19], caspases [20], and aldehyde dehydrogenase [21].

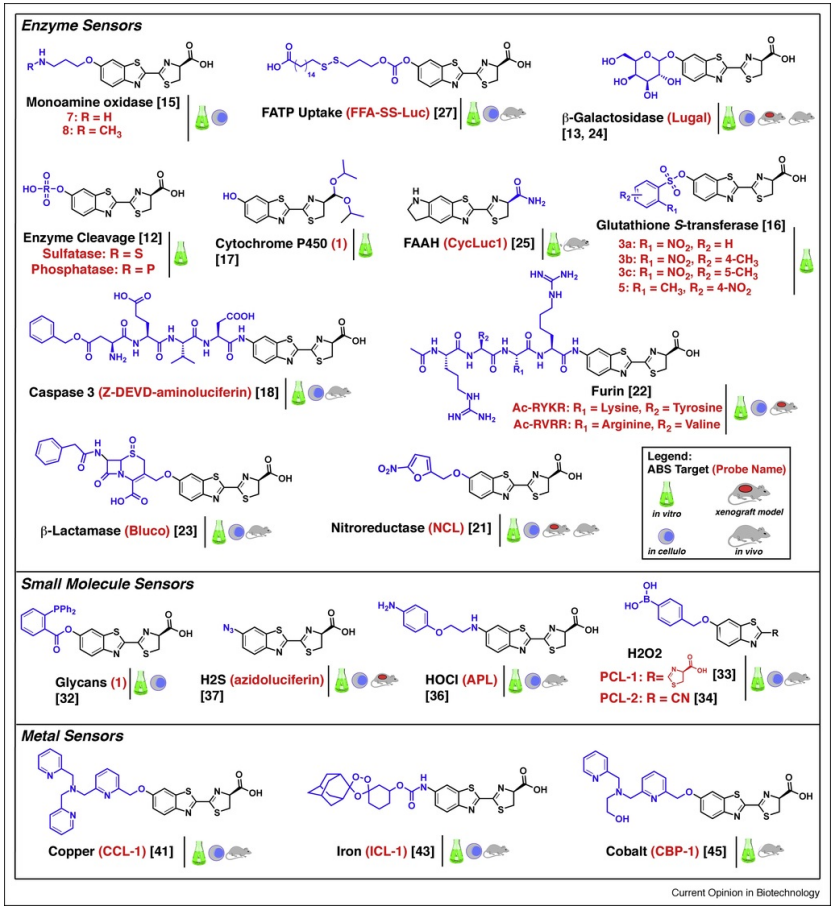

Figure 2 Representative examples of caged luciferins for bioluminescent activity-based sensing (ABS) of enzyme activity, reactive small molecules, and metal ions. Structures for the reactive bioluminogenic triggers are shown in blue. The ABS target for each probe is written in black, with the prob name written in red. The graphic icons next to each probe name denote the biological settings that each probe has been employed in, ranging from solution-phase bioluminescence assays (*in vitro*, green flask), luciferase-expressing cell lines in culture (*in cellulo,* gray/blue cells), luciferase-expressing mice (*in vivo*, gray mouse), to luciferase-expressing tumor xenografts in mice (xenograft model, gray mouse with red circle).

alt-text: Figure 2

As *in vitro* assays can only report on endpoint measurements of enzyme activity, engineering cells and animals to express luciferase in conjunction with the use of synthetic caged bioluminogens can enable monitoring of enzyme activity in real time and space. Such bioluminescent imaging studies in mouse models typically fall into one of two main categories: 1) luciferase-expressing cell xenografts in immunocompromised mice, or 2) transgenic mice that express luciferase via transfection with adeno-associated viral (AAV) luciferase vectors or animal breeding from fertilized embryos injected with the luciferase transgene. A major application for ABS luciferin probes has been to study the contributions of specific enzymes in tumorigenesis and tumor progression. For example, Weissleder ~~and co-workers~~*et al.* applied the caspase-3/7-sensitive DEVD peptide trigger to demonstrate the therapeutic efficacy of a tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) protein in selective tumor apoptosis [22]. Significant turn-on in bioluminescent signal was observed in TRAIL-treated xenografts owing to caspase activation along with a dramatic reduction in glioma burden. Likewise, Dubikovskaya and colleagues developed a caged luciferin to evaluate nitroreductase activity in tumor xenograft models [23], whereas the Rao group created a caged aminoluciferin to study the role of the furin endoprotease in tumor progression [24] and interrogated models of bacterial resistance to beta-lactam antibiotic drugs using β-lactamase-caged luciferins [25].

In addition to evaluating the activity of specific tumor-promoting or tumor-diminishing enzymes, caged luciferins can also illuminate cell–cell interactions in tumor metastasis and immunooncology. In an elegant study, the Prescher laboratory utilized a galactose-caged luciferin (Lugal) to study interactions between xenografted immune cells and tumor cells *in vivo* [26]. Lugal cleavage by β-galactosidase-expressing immune cells releases ~~a~~D-luciferin. Subsequent interception by luciferase-expressing tumor cells results in light emission, where signal intensity scales with the proximity between tumor and immune cells (Figure 3a). This platform was applied to monitor immune responses to tumor metastasis and finding areas of co-localized immune and cancer cells.

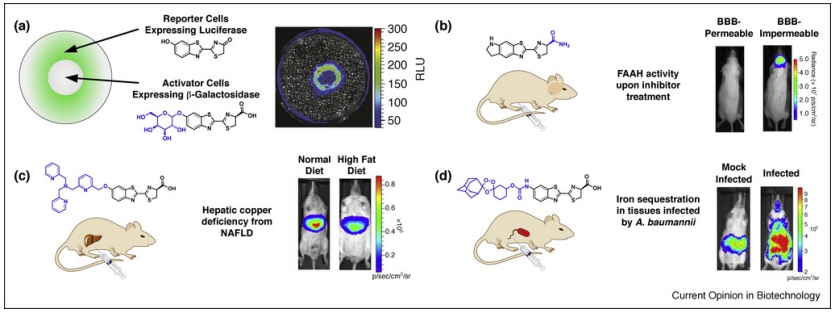

Figure 3 Representative examples of luciferin activity-based sensing (ABS) probes to evaluate physiological and pathological processes in cell and animal models. ~~(a)~~(a) Lugal uncaging by the β-Gal expressing activator cells enables luminscent emission from the luciferase expressing reporter cells serving as a model for cell-to-cell communication. Figure adapted from Ref. [26]. ~~(b)~~(b) FAAH converts CycLuc1 amide to the bioluminogenic CycLuc1 carboxylate. Bioluminogenic signal is mice expressing luciferase in the brain is not observed upon treatment with BBB-permeable FAAH inhibitors. Figured adapted from Ref. [25]. ~~(c)~~(c) CCL-1 identifies hepatic copper deficiency that develops in a diet-induced murine model of non-alcoholic fatty liver disease (NAFLD). Figure adapted from Ref. [43••]. ~~(d)~~(d) ICL-1 shows elevations in tissue levels of labile iron as a host response to *A. baumannii* bacterial infection. Figure adapted from Ref. [45••].

alt-text: Figure 3

Several innovative platforms have exploited caged luciferin probes to characterize enzyme activity in animal model systems beyond cancer. For example, fatty acid amide hydrolase (FAAH) inhibition is a promising avenue for new therapeutics for pain and anxiety relief, and as such, it is highly desirable to establish platforms for sensing FAAH activity in the brain. Miller ~~and co-workers~~*et al.* cleverly functionalized a blood-brain barrier (BBB) permeable CycLuc1 with an amide group at the 4-position to sense FAAH activity, where amide hydrolysis via FAAH generates the luciferase-active CycLuc1 (Figure 3b) [27••]. Various FAAH inhibitors and the CycLuc1 amide were injected into mice that express luciferase in the brain. BBB-permeable FAAH inhibitors resulted in low luminescent brain signal, whereas BBB-impermeable FAAH inhibitors resulted in a high signal response.

Another focus area has been to create bioluminogenic luciferins that report on cell membrane transporter-specific recognition and internalization [28]. Along these lines, the Stahl and Bertozzi laboratories synthesized a fatty acid transport protein (FATP)-targeted luciferin analog FFA-SS-Luc, where recognition of the long-chain fatty acid trigger and subsequent intracellular disulfide reduction releases free ~~D~~D-luciferin [29]. Fatty acid uptake can be mapped from the luminescent signal produced from FFA-SS-Luc administration in mice that express luciferase in all organs. FFA-SS-Luc has been used to elucidate the role of macronutrient uptake in metabolic disorders [~~28~~30]. In a similar vein, Alrefai ~~and co-workers~~*et al.* developed cholic acid-functionalized cleavable luciferins to target bile acid transport proteins and quantify bile acid transporter activity in cell culture [31].

Caged luciferins for small molecule and metal ion activity-based sensing

Transient fluxes of small biomolecules, including reactive oxygen/sulfur/nitrogen/carbon species and metal ions, are dynamic chemical signaling agents that regulate fundamental biological processes [32,33]. Developing tools to monitor changes in their spatiotemporal concentrations and distributions can help decipher their roles in physiology and pathology. As an early example of this approach, Bertozzi ~~and co-workers~~*et al.* synthesized an arylphosphine-functionalized luciferin to quantify metabolic incorporation of azidosaccharides in LNCaP-luc cells [34]. Staudinger ligation between the azidoglycan and phosphine resulted in release of ~~D~~D-luciferin, which could diffuse into cells and interact with intracellular luciferases.

Hydrogen peroxide (H_2O_2) is a major reactive oxygen species (ROS) that plays central roles in redox stress and signaling. To sense the activity of this important ROS, our laboratory in collaboration with Bertozzi developed Peroxy Caged Luciferin-1 (PCL-1), a boronate-based reagent that releases ~~D~~D-luciferin in the presence of H_2O_2 [35]. PCL-1 identified elevated peroxide levels upon steroid-induced stimulation of inflammation in LNCaP-luc tumor xenografts within live mice. We also synthesized PCL-2 as a split luciferin variant, where intracellular reaction with ~~D~~D-cysteine generates the caged luciferin. The split luciferin system offers opportunities for dual-analyte sensing if used in concert with a caged ~~D~~D-cysteine molecule. In a proof-of-concept experiment, we injected PCL-2 and a caged ~~D~~D-cysteine with a caspase substrate to illuminate the concerted roles of caspases and hydrogen peroxide spillover during inflammation and cell death [36]. In addition, PCL-2 was recently used to characterize the role of H_2O_2 in *A.*~~-~~*cinetobacter baumannii* infection models [37]. Urano~~Nagano, and colleagues~~ *et al.* created caged ABS luciferins for sensing hypochlorite, another important reactive oxygen species, and used their probe to image respiratory bursts of neutrophils upon phorbol 12-myristate 13-acetate stimulation [38]. Beyond reactive oxygen sensing, caged luciferin probes for detection of reactive sulfur [39] and nitrogen species [40••] have also been developed.

Finally, an emerging goal of bioluminescent activity-based sensing probes is to target metals, which are essential nutrients for life. ~~At the same time, d~~Dysregulated metal ion homeostasis is implicated in a variety of aberrant disease processes such as cancer, metabolic disorders, genetic disorders, neurodegeneration, and infection [33,41,42]. As such, there is significant interest in developing bioluminescent platforms for longitudinally monitoring the activity of dynamic metal pools over the course of disease progression and treatment. To meet this need, our laboratory has developed caged luciferin activity-based sensors to probe various transition metals and their roles in physiology and pathology. For example, our laboratory and Stahl's laboratory developed Caged Copper Luciferin-1 (CCL-1), where Cu^+ binding to a tris(2-picolyl)amine moiety followed by oxidative cleavage liberates ~~D~~D-luciferin to give a readout for the concentration of loosely-bound Cu^+ stores that represent the labile copper pool [43••]. CCL-1 was used to longitudinally monitor

hepatic copper availability in a diet-induced model of non-alcoholic fatty liver disease (NAFLD, Figure 3c). CCL-1 imaging revealed that a hepatic-specific copper deficiency develops ~~prior to~~before the formal onset of fatty liver symptoms, suggesting a possible copper-dependent role in NAFLD pathogenesis and a path forward to develop copper-targeted diagnostics and therapeutics for NAFLD and related metabolic diseases. Indeed, CCL-1 was subsequently employed to monitor the efficacy of a targeted ionophore metal supplement (TIMS) strategy for selective copper delivery to the liver [44]. We also developed Iron Caged Luciferin-1 (ICL-1), an activity-based sensing probe for monitoring labile iron pools. This probe utilizes a bioinspired endoperoxide trigger that releases ~~D~~D-aminoluciferin upon reaction with Fe^{2+} [45••]. ICL-1 was used to study change in the labile iron pool in a model of systemic bacterial *A. baumannii* infection, establishing an increase in iron accumulation in host tissues upon infection (Figure 3d). These findings correlated with transcriptional changes with elevations in iron acquisition and storage proteins in the infected tissues, suggesting a potential nutrient starvation mechanism by the host to protect against bacterial invasion. ~~Based on~~On the basis of fluorescent activity-based sensing probes [4,46], caged luciferins for sensing Co^{2+} and Hg^{2+} activity *in vivo* have been developed by Li and co-workers [47,48]. Taken together, the growing toolbox of bioluminescent activity-based sensing platforms for longitudinally assessing metal status in living animals provides a powerful technology for studying the contributions of metal metabolism and signaling to healthy and disease states.

Conclusions and ~~Future D~~future directions

The development of synthetic caged luciferins for bioluminescent activity-based sensing offers a versatile and powerful approach to selectively tracking the dynamic activity of enzymes, small molecules, and metal ions in living systems. Such *in vivo* bioluminescent tools can be used to complement existing *ex vivo* approaches to develop a holistic understanding of a diverse array of physiological and pathological processes. Many avenues of research will expand the field. First and foremost, new triggers for many biomolecules are continually being developed and will further diversify the bioluminescent ABS toolkit. Future frontiers beyond trigger development include multiplexed luciferin imaging and purely chemical luminescent sensors. Indeed, developing strategies for multiplexed luciferin imaging will enable simultaneous multi-analyte monitoring in animal models where two or more caged luciferins can be used at once. One potential approach is to red-shift bioluminescence emission by modifying the luciferin scaffold [7,49,50] to enable multi-color ABS imaging. Another route is to establish orthogonal luciferase~~–~~–luciferin pairs for multiplexed ABS [51••,52,53,54••]. Functionalizing these red-shifted or orthogonal luciferins with the appropriate triggers from Figure 2 would enable the activity of many biomolecules to be imaged in concert. Chemiluminescent sensing is another promising area of innovation for *in vivo* ABS [55,56]. These probes do not require luciferase for adenylation and oxidation of the luciferin substrate (Figure 1); instead, the reactive dioxetane bridge is pre-installed in the chemiluminescent scaffold. Trigger cleavage initiates an intramolecular charge transfer process that generates the excited emissive species. Chemiluminescent ABS probes possess untapped clinical potential, as they do not require transgene expression. *In vivo* chemiluminescent probes have been developed for β-galactosidase [57], nitroreductase [58], and cathepsin B [59] as well as small molecules such as hydrogen peroxide [60], formaldehyde [61], and peroxynitrite [62]. Future innovations to improve quantum yield, control probe localization, and increase aqueous stability offer new opportunities for improving chemiluminescent ABS systems. To close, bioluminescent activity-based sensing with caged luciferins offers a powerful and growing toolkit for tracking the activity of enzymes, small molecules, and metal ions *in vivo*, and advances in molecular design and protein engineering will bring new tools for understanding the complex and dynamic reactions between molecules in physiological and pathological contexts.

Uncited ~~References[30•].Acknowledgements~~reference

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Acknowledgements

This work was supported by the National Institutes of Health (GM79465 and ES4705 to C.J.C.). T.A.S. is supported by an NIH Ruth L. Kirschstein NRSA Fellowship (F32 GM122248). K.J.B. is supported by an NSF~~ graduate fellowship~~ graduate fellowship. C.J.C. is an Investigator of the Howard Hughes Medical Institute.

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