Abstract
Bioluminescence (BL) relies on the enzymatic reaction between luciferase, a substrate conventionally named luciferin, and various cofactors. BL imaging has become a widely used technique to interrogate gene expression and cell fate, both in small and large animal models of research. Recent developments include the generation of improved luciferase–luciferin systems for deeper and more sensitive imaging as well as new caged luciferins to report on enzymatic activity and other intracellular functions. Here, we critically evaluate the emerging tools for BL imaging aiming to provide the reader with an updated compendium of the latest developments (2018–2020) and their notable applications.

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Novel luciferase and luciferins
Imaging cellular processes or molecular reactions within the deep tissue of animals is optimal when the BL enzymatic reaction has a high quantum yield and when emitted BL falls under the so-called optical window for in vivo imaging (wavelengths higher than 650 nm) [1–3]. Conversely, photons associated with wavelengths lower than 650 nm are readily scattered or absorbed by components of the tissue. This is why the latest research in BL concentrates on the development of brighter and red-shifted BL systems with the aim of generating BL systems that can be combined for multicolor applications, as is the case for fluorescent proteins and dyes [4–7] (Figure 1).

Terrestrial luciferases
Among the terrestrial luciferases, mutated red-emitting click beetles (CBRs) offer the additional benefit of achieving greater brightness for imaging in deep tissue. In particular, CBR2 luciferase has improved the sensitivity in deep tissue, giving a bright, near-infrared, signal ($\lambda_{\text{max}} = 730$ nm) with the NH$_2$-NpLH$_2$ luciferin substrate [8,9].

The last few years have also witnessed the emergence of synthetic luciferase enzymes, one example being Akaluc luciferase [10]. Differing from firefly luciferin (Fluc) in 28 amino acids, Akaluc enables more sensitive imaging than Fluc in preclinical models when paired with AkaLumine-HCl. Akaluc/AkaLumine-HCl pairing has been named the AkaBLI system ($\lambda_{\text{max}} = 650$ nm). Interestingly, this system was used to transduce the...
right striatum of a marmoset brain and to visualize BLI for over a year (Figure 1).

### Marine luciferases

Further improvements were also reported with optimized marine luciferases, yielding brighter BL. Starting from NanoLuc luciferase [11], Yeh et al. [12] re-engineered NanoLuc to react with a coelenterazine (CTZ) analog named diphenylterazine (DTZ) ($\lambda_{\text{max}} = 502$ nm). The novel teLuc luciferase with DTZ achieved greater brightness and sensitivity subcutaneously than Fluc/ D-luciferin (D-Luc) and NanoLuc/ furimazine (FRZ) [12]. In an analogy to the Antares luciferase, teLuc was fused in between the two domains of the fluorescent protein cyOFP to achieve intramolecular BL resonance energy transfer (BRET). This fusion created Antares 2 luciferase, which emits more photons at wavelengths higher than 600 nm compared with Antares and leads to improved performances compared with simple teLuc/DTZ for liver imaging. More recently, 8pyDTZ, a pyridine analog of DTZ ($\lambda_{\text{max}} \sim 490$ nm) like marine luciferases, and the operon can be entirely inserted in the genome of the heterologous organism. Although the photon yield of such a system is low, the independence from the exogenous administration of luciferin makes its use particularly interesting. A recent advancement in bacterial luciferases was the design of the ilux operon by Gregor et al. [15,16], enabling single-cell imaging.

### Fungal luciferases

A breakthrough in the BL field was the discovery of the entire biochemical pathway involved in generating BL in fungi. In particular, the wild-type Neonothopanus nambi luciferase (nnLuc) uses a $\alpha$-pyrone 3-hydroxypispidin substrate and emits green light ($\sim 520$ nm) in the

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| Luciferase     | Luciferin                                      | Emission wavelength (nm) | Application                                      |
|---------------|-----------------------------------------------|--------------------------|-------------------------------------------------|
| Beetle luciferase | Click beetle mutants NH$_2$-NpLH2            | 659/740                  | Dual color BL in depth with one substrate       |
|                | Fluc and mutants Infraluciferin               | 690/706                  | Dual color BL in depth with one substrate       |
|                | Akaluc Akalumine-HCl                          | 660                      | Imaging large animal                            |
| Bacterial luciferase | ilux operon (Photobahdus luminescens)          | FMNH$_2$ + long-chain fatty aldehyde | 590 | Effect of antibiotics on cell viability |
| Marine luciferase | Nanoluc-CyOFP (Antares) Furimazine            | -600                     | Antares-calcium sensitive probe                 |
|                | Nanoluc-CyOFP (Antares) Fluorofurimazine      | -580                     | Two population imaging combined to Akaluc/BLI  |
|                | Nanoluc-CyOFP (Antares) Hydrofurimazine       | -600                     | Highly sensitive BL                             |
| Fungal luciferase | NnLuc $\alpha$-pyrone 3-hydroxypispidin      | -520                     | Autonomous glowing plants                       |

Schematic illustration of emerging tools in bioluminescence imaging. BLI, bioluminescence imaging.
presence of O₂ [17]. Interestingly, this system was engineered into plants to produce glowing plants without exogenous administration of a substrate [18,19] (Figure 1).

**D-Luc analogs**

Recent years have also seen remarkable advancements in the generation of luciferin analogs to improve the brightness of the BL system, cell permeability, and blood–brain barrier penetration, as well as to modulate color emission. Several modifications have been introduced on D-Luc to achieve these goals (e.g. π-extended luciferins) [20]. In that direction, Wu et al. [21] developed a series of Fluc substrates, namely, N-cycloalkylaminoluciferins (cybLucs). To enhance cellular uptake, the lipophilic N-cycloalkyl group was introduced in aminoluciferin. The intravenous injection of cybLuc in luciferase-expressing transgenic mice revealed a fourfold extended circulatory half-life and brighter bioluminescent signal intensity relative to D-Luc. In terms of signal stability within the brain hippocampus, where the BL emission from D-Luc was found to fade within 5 min, cybLuc’ generated emission persisted for more than 30 min, making it a preferred substrate for brain imaging (Figure 2).

**Pyridone luciferins** have been generated with the aim of developing novel orthologous bioluminescent systems for multicolor application. Zhang et al. [22] developed luciferin analogs that had their original D-Luc scaffold modified at pyridone cores. For many of them, Km values were >100-fold higher than those of D-Luc when using firefly luciferase (Fluc). To overcome that, Fluc mutants, namely, 24 and 166, were generated. Nevertheless, in _in vitro_ studies, pyridine analogs (oxy4) with mutant luciferase 24, and especially 166, attained the near to equivalent binding affinity seen in native Fluc, the reason being F295l mutation. Second, in live cells, authors found the emission spectra of oxy4 with Fluc ($\lambda_{max} = 530$ nm) were close to those of Fluc/D-Luc.

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**Chemical structure of novel bioluminescent substrates.** (1) Mitochondria-activatable luciferin (MAL) [32]. (2) Bioluminescent glucose-uptake probe (BiGluc) [31]. (3) γ-Glutamyl transpeptidase (GGT) probe [28]. (4) N-cyclobutylaminoluciferin (cybLuc), a cycloaminoluciferin analog [21]. (5a and 5b) Pyridone luciferin analog 3 and 4 [22]. (6) Selenocysteine probe (BF-1) [27]. (7) Hikarazine, a coelenterazine analog [24]. (8a and 8b) Hydrofurimazine and fluorofurimazine, furimazine analogs [23]. (9) 8-Pyridyl diphenylterazine (8pyDTZ), a diphenylterazine analog [13].
CTZ derivatives

Other interesting developments are the FRZ analogs described in the work of Su et al. by targeting polar substitutions on phenyl rings [23]. Hydrofurimazine (3'-hydroxy substitution), under its saturation dose, gave a fourfold higher BL than that generated from FRZ. Furthermore, with fluorine substitutions on analogs' phenyl rings, the authors designed fluorofurimazine. These analogs were formulated in Poloxamer-407 (solubilizing formulation), which enabled intraperitoneal injection of higher doses of the substrate and ensured good biodistribution in animals. Antares/Fluorofurimazine and AkablI systems were used to achieve dual-color population imaging in vivo by using Antares-expressing tumor cells and Cat-2T cells expressing Akaluc [25]. Other interesting novel CTZ analogs include hikarazines, which are O-acetylated luciferins that are highly stable at room temperature for up to two years. Hikarazines can react with NanoLuc luciferins. However, their performance for in vivo imaging still needs to be further evaluated [24].

Caged D-Luc substrates

Caged luciferin substrates are used to monitor enzymatic activities and other molecular events on the release of D-Luc. Making use of this phenomenon is a sensor for detecting in vivo fibroblast activation protein-α activity, which was designed by Lin et al. [25]. Here, N-carbobenzyloxy-Gly-Pro-OH (probe-1) was used to mask the substrate recognition site on aminoluciferin, making it inaccessible to Fluc. The peptidase activity of membrane-bound fibroblast activation protein-α allows it to cleave the amide bond after proline within probe-1, making aminoluciferin available. In a tumor xenograft mice model involving U87MG-Fluc cells, injecting 5 mg/kg of SP-13786 inhibitor led to fourfold decrease in BL from the test group relative to the control group with the vehicle (saline + probe-1).

Other similar recent developments are caged luciferins to monitor peptide uptake [26], to detect biothiols [27], for real-time in vivo detection of selenocysteine [28] and γ-glutamyl transpeptidase [29] and also photoactivable substrates [30] (Figure 2).

A bioluminescent glucose uptake reporter (BiGluc) [31] and mitochondrial membrane potential (ΔΨm) sensor (mitochondrial-activated luciferin [MAL]) [32] were designed by harnessing the reaction efficiency of Staudinger ligation to report molecules’ bioavailability through BL. Both the reporters used caged luciferintriphenylphosphine (TPP) and an azide- or azido-linked molecule of interest. TPP, being a lipophilic cation caging luciferin, could be passively taken up through the lipid bilayer. In the MAL sensor, both its components, luciferin and organic azide, were linked to TPP for permitting mitochondrial matrix accumulation. In proximity, the reaction of chemical moieties results in the uncaging of luciferin and BL emission when reacting with cytosolic luciferase. For example, in FVB-luc+ mice (ubiquitous luciferase expression under the β-actin promoter), testing of MAL3 reporter (superior variant) in the presence/absence of butylated hydroxytoluene and nigericin aided successful visualization of mitochondrial membrane potential depolarization and hyperpolarization [32] (Figure 3).

Emerging BL technologies

Bioluminescent nanoparticles

The formulation of bioluminescent nanoparticles has emerged as an attractive application to combine bioimaging and nanomedicine (Figure 4a). Usually, BL quantum dots, relying on BRET, are applied for imaging purposes [33,34]. Recently, D-Luc has been linked via a disulfide bond to the surface of ferritin-conjugated nanoparticles, with the aim of monitoring the delivery of drugs, with high sensitivity, into the tumor site [35]. In fact, the presence of a high concentration of glutathione inside the cells leads to the release of D-Luc, making the BL remission dependent on nanoparticle uptake. Another interesting application is the development of biocompatible near-infrared-II bioluminescent probes (Figure 4b). These probes integrate a BRET process with a two-step fluorescence resonance energy transfer (FRET) process, to image blood vessels and lymphatics with a high signal:noise ratio at near-infrared-II wavelengths (1000–1700 nm). The nanoparticle-based probe consists of NanoLuc luciferase and three fluorescent acceptors for sequential energy transfer (BRET-FRET-FRET) (Figure 4c) [36].

Automated image processing and analysis

BL images are usually analyzed by manually selecting and drawing region of interests on bioluminescent areas. Therefore, the robustness of the measurements might be compromised by multiple factors such as the operator, the animal positioning relative to the camera, the spatial location of the reporter, and the imaging view. In an interesting work, Klose and Paragas [37] successfully developed an automated image processing tool: a body-fitting animal shuttle and a statistical mouse atlas conjugated by a multispectral BL tomography technique, which aided the quantification of an in vivo distributed bioluminescent reporter. This tool will contribute to collect more throughput data and being consistent with measurements in vivo.

Multiplexing in deep tissue

Combining luciferases with different emission properties and visualizing multiple molecular events...
simultaneously is one of the most interesting applications for BLI [38,39]. This can be carried out by combining orthogonal luciferase−luciferin pairs. The selectivity of the substrate for each luciferase guarantees specificity and distinct bioluminescent signals [40]. The most common combination uses D-Luc substrate analogs paired with terrestrial luciferases such as Fluc and CTZ analogs paired with marine-derived luciferases [41]. However, multiple substrate injections and longer imaging sessions can be stressful for animals. In addition, errors in data analysis can also be introduced because of differences in solubility, biodistribution, and clearance of the substrates used [42]. These issues can be resolved using a single substrate that activates two luciferases and results in spectrally separated emissions. Recently, dual-color BLI using a single substrate could be performed using D-Luc analogs for near-infrared emission and mutant luciferases. The far-red−/near-infrared−shifted color of luciferase mutants using infraluciferin or naphthyl-luciferin in vivo measurements allowed for dual-color imaging in deep tissues [43,44].

**CRISPR/Cas knock-in**

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-associated protein Cas9 based gene editing has revolutionized biotechnology. The knock-in of genes up to 2 Kb can now be performed with high specificity and efficiency, thus enabling the monitoring of protein expression at the endogenous level [45–47]. For example, researchers successfully attempted to use the HiBiT peptide as a luminescent reporter tag of endogenous proteins at a cellular level [48]. More recently, in a mouse model affected by Duchenne muscular dystrophy, the Luc gene was inserted in frame

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**Figure 3**

**Molecular mechanism of a mitochondria-activatable luciferin (MAL) probe.** Active components of the MAL reporter are D-Luc and an azido moiety, each of which are linked with triphenylphosphine, triphenylphosphine-caged luciferin (TPP-CL) and triphenylphosphine-linked azido (Azido-TPP). Within the mitochondrial matrix, Staudinger ligation reaction is triggered whenever TPP-CL and Azido-TPP are in proximity, uncaging D-Luc. Free luciferin (D-Luc) is then exported out to the cytoplasm where firefly luciferase is expressed. Finally, reaction between Fluc and D-Luc, in the presence of ATP and Mg²⁺, generate the bioluminescent signal, thus reflecting kinetics of mitochondrial membrane potential. D-Luc, D-luciferin.
with the C-terminus of the dystrophin gene. In the model, disruption of the dystrophin gene extinguishes luciferase expression. After gene therapy, the group could monitor the restoration of the dystrophin gene in deficient mice via BLI [49].

Photouncaging and bioluminolysis
BRET can also be used to turn on functions in living cells. Chang et al [50] and Lindberg et al [51] demonstrated that an excited state of a coumarin compound can trigger hydrolysis to uncage a target molecule (bioluminolysis). The excitation of coumarin is achieved with an efficient BRET from NanoLuc–HaloTag chimera protein to a coumarin substrate. This application of BLI opens up the possibility to image and control the delivery of bioactive small molecules in vivo.

Conclusion and outlook
The emerging luciferase–luciferin pairs have expanded the bioluminescent palette, enabling deep-tissue imaging and detection of fewer number of cells with more reliability in vivo. The discovery of new bioluminescent systems [52,53] and the design and optimization of synthetic ones by computational models [54] will expand the BL toolbox for more challenging applications in vivo. In addition, the production of noninvasive systems to detect protein–protein interaction (i.e. antibody/target in vivo) will be of great interest to further
evaluate therapeutic efficacies [55]. We expect, in the near future, more accurate biosensors for optogenetic studies [56,57], to visualize single cells unequivocally in deep tissues, especially if novel, using more sensitive detection systems. Moreover, the function of small molecules and the control and release of drugs can now be imaged in vivo with a high level of sensitivity.

Such innovations and tools, especially if disseminated widely in the scientific community, will accelerate the advancement of the BLI field.

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G.Z. conceptualization, writing-original draft; C.C. conceptualization, writing-review and editing, supervision.

Declaration of competing interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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