Current advances in the use of bioluminescence assays for drug discovery: an update of the last ten years

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Abstract

Introduction: Bioluminescence is a well-established optical detection technique widely used in several bioanalytical applications, including high-throughput and high-content screenings. Thanks to advances in synthetic biology techniques and deep learning, a wide portfolio of luciferases is now available with tuned emission wavelengths, kinetics and high stability. These luciferases can be implemented in the drug discovery and development pipeline, allowing high sensitivity and multiplexing capability.

Areas covered: This review summarizes the latest advancements of bioluminescent systems as toolset in drug discovery programmes for *in vitro* applications. Particular attention is paid to the most advanced bioluminescence-based technologies for drug screening in the latest 10 years (from 2013 to 2023) such as cell-free assays, cell-based assays based on genetically modified cells, bioluminescence resonance energy transfer (BRET) and protein complementation assays (PCA) in 2D and 3D cell models.

Expert opinion: The availability of tuned bioluminescent proteins with improved emission and stability properties is vital for the development of bioluminescence assays for drug discovery, spanning from reporter gene technology to protein-protein techniques. Further studies, combining

machine learning with synthetic biology will be necessary to obtain new tools for sustainable and highly predictive bioluminescent drug discovery platforms.

Keywords: Bioluminescence, BRET assays, cell-free assays, drug screening, multiplexing, protein-complementation assays, reporter gene assay, 3D cell models

1. Introduction

High-throughput screening (HTS) and high-content screening (HCS) are routinely employed in early-stage drug discovery and represent valuable tools for the drug industry. The identification and validation of scientifically sound drug screening models for de-risking new drugs is of utmost importance, also considering the necessity to reduce the number of animals. In accordance with recent legislation, such as the US FDA Modernization Act 2.0, which for the first time refuted the Federal Food, Drug, and Cosmetics Act (1938), allowing the use of alternative models, the development of predictive cell-based assays and *in silico* models is a high priority to avoid or, more realistically, reduce animal-based laboratory tests [1].

Several drug screening *in vitro* and *in vivo* platforms have been developed relying on optical detection techniques, such as fluorescence (FL), chemiluminescence (CL) and bioluminescence (BL) [2], that have proved to be suitable for HTS in terms of rapidity and detectability using small volumes of samples and reagents. A plethora of technologies relying on genetically modulated cells, resonance energy transfer (RET), protein complementation assays (PCA) and *in vivo* imaging have been applied to drug screening. BL represents an optical readout platform widely employed in biochemical assays and routinely exploited in drug discovery programmes. BL is a fascinating natural phenomenon occurring in terrestrial and marine organisms, spanning from fireflies, fungi, bacteria to several abyss species. BL is highly attractive thanks to its intrinsic high-signal-to-noise ratio, high dynamic range, equipment simplicity, suitability to multiplexing. Differently from FL, BL emission does not require an external excitation light source, and it does not suffer of FL signal background present in biological samples.

The light emission is due to a chemical reaction catalyzed by an enzyme, the luciferase, and a substrate, the luciferin. Different luciferin-luciferase systems have been studied or de novo designed and are now available for implementation into drug screening platforms.

Luciferase from the North American firefly *Photinus pyralis* (PpyLuc) is by far the most investigated luciferase, enabling the development of highly sensitive methods for ATP. Thanks to the progress of

synthetic biology techniques [3] and deep learning [4], a wide portfolio of luciferases is now available with tuned emission wavelengths, kinetics and high stability [5–12]. An updated list of luciferin/luciferase systems and their emission properties has been recently provided elsewhere [2,13].

Advancements in development of BL assays for drug discovery in the last 10 years (from January 2013 to April 2023) are provided in this review, with a critical overview of the different BL approaches available up to now with potential applicability in drug screening, including new or improved cell-based assays for HTS based on genetically modified cells, cell-free assays, protein-proteins interaction assays exploiting the Bioluminescence resonance energy transfer (BRET) and the Protein complementation assay (PCA) (Figure 1). New luciferases suitable for multiplexing and predictive BL 3D models are also discussed as new alternative tools to revolutionize preclinical research [14].

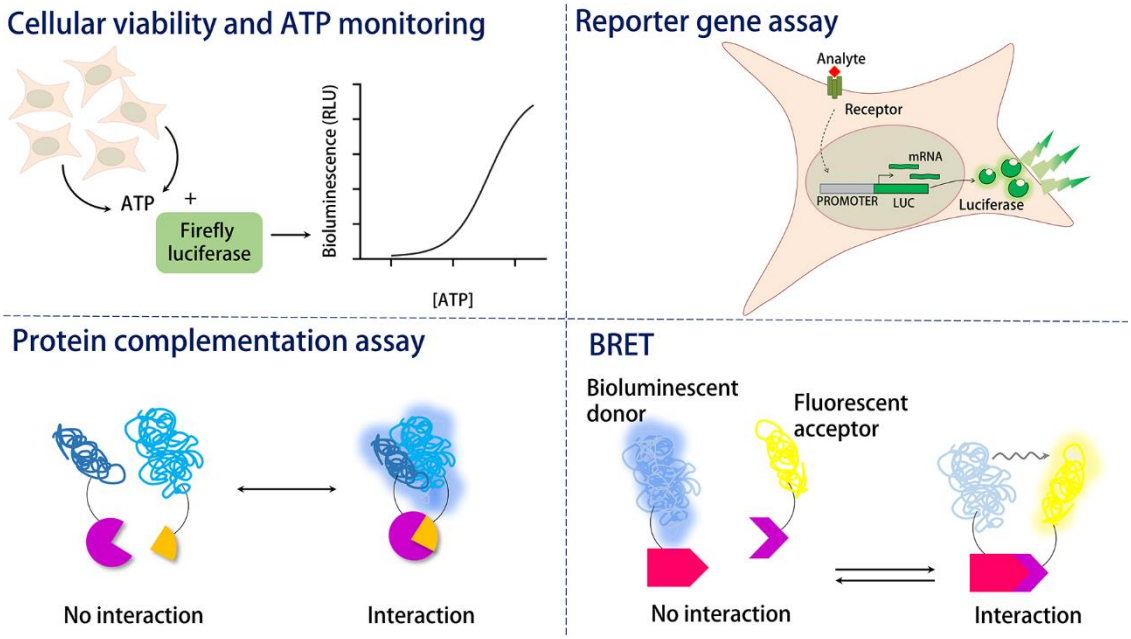

Figure 1: Schematic view of bioluminescent assays used in the early phases of drug discovery.

2. Cell-free assays

PpyLuc has been widely used for adenosine triphosphate (ATP) detection, especially for hygiene monitoring and cytotoxicity assays [15–17]. Since intracellular ATP concentration rapidly drops upon

cell death, a subsequent loss of BL can be observed with luciferase/D-Luciferin systems (Figure 2). ATP is thus considered an indicator of cell viability and BL assays for ATP detection have been widely used for cytotoxicity to screen compound libraries (Figure 1) [18]. Thanks to the availability of improved Luc enzymes in terms of brightness and stability, the sensitivity and robustness of HTS assays has been significantly improved.

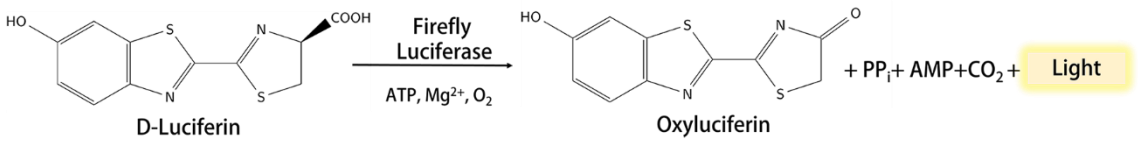

Figure 2: Schematic representation of luciferase/D-Luciferin system reaction.

In addition, BL ATP assays can be exploited for quantifying enzyme activities through the consumption or generation of ATP. For example, BL assays are powerful tools for screening kinase inhibitors since most kinases consume ATP for the phosphorylation reactions[19–21]. However, these methods do not selectively differentiate the source of ATP consumption, potential artifacts can be generated due to the presence of ATPases in the sample. This is an issue that is encountered when using BL proteins in both cell-free and cell-based assays; several molecules in fact can directly interfere with the catalytic activity or affect the stability of the luciferase, thus producing artefactual results. To avoid interferences due to the presence of potential compound with an inhibitory activity on *P. pyralis* luciferase [22], a Published Kinase Inhibitor Set (PKIS) was released by GSK helping the interpretation of assay results derived from the use of this library comprising of 367 molecules ATP-competitive kinase inhibitors [23,24]. A LUMinescent AntiBody Sensor (LUMABS) platform based on bioluminescence resonance energy transfer (BRET) [25] for small molecules and antibodies detection was developed by Arts et al. The protein sensors were designed for detecting four therapeutic antibodies, trastuzumab, rituximab, obinutuzumab and cetuximab, and are composed by the NanoLuc luciferase connected to the fluorescent acceptor mNeonGreen via a semiflexible linker (Figure 3A). The proposed sensor is designed to have donor and acceptor in close proximity thanks

to the interaction established between the proline rich peptide and the Src Homology 3 domain. In the presence of the target antibody, this interaction is disrupted, the distance between NanoLuc and its acceptor increases, thus reducing the energy transfer [26].

A similar approach has been developed based on an homogeneous immunoassay platform, called RAPPID (Ratiometric Plug-and-Play Immunodiagnostics), relying on complementation of split NanoLuc fragments induced by the analyte (Figure 3B). In this system NanoLuc fragments were conjugated to an antibody sandwich pair that to Protein G adapters. The inclusion of a control luciferase provided improved robustness, with the applicability to different target analytes, including therapeutic antibodies, anti-drug antibodies, SARS-CoV-2 spike protein, and anti-SARS-CoV-2 antibodies at the low picomolar level [27].

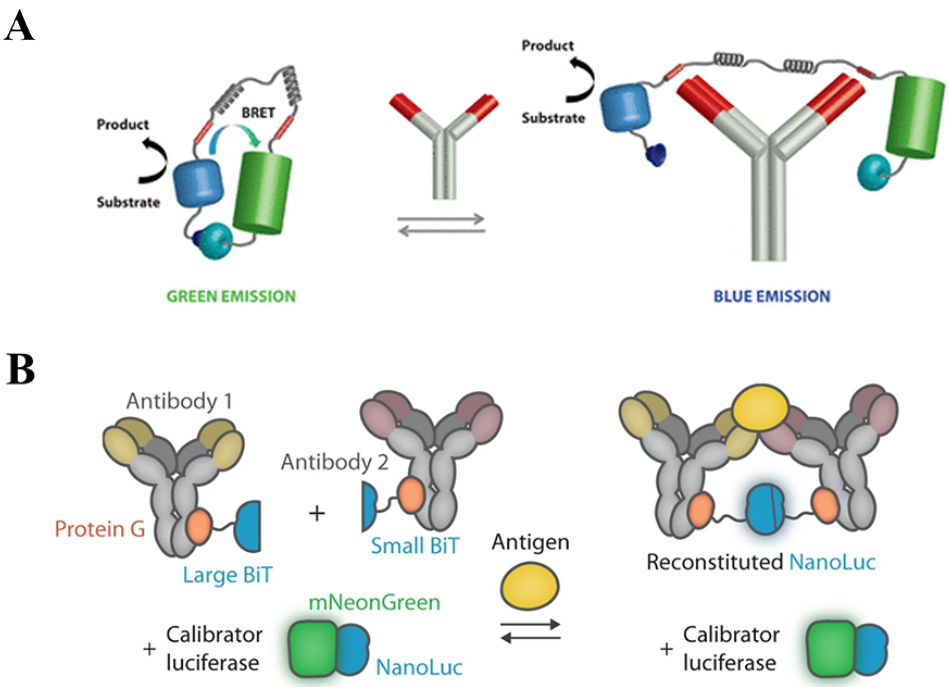

Figure 3: A) Schematic illustration of the LUMABS platform based on the NanoLuc luciferase as BL donor and the mNeonGreen as FL acceptor. Reprinted with permission from [22] Copyright (2016) American Chemical Society; B) Schematic illustration of the RAPPID immunoassay based on antibodies functionalized with the split version of the NanoLuc luciferase. Reprinted with permission from [24].

3. Cell-based assays

An invaluable tool for the early stages of the drug discovery process is represented by cell-based assays, enabling to screen potential inhibitors or inducers of biological processes and to identify bioactive molecules interacting with molecular targets. Highly suitable for HTS and HCS formats, cell-based assays are used to understand gene, protein or cellular functions, their regulatory mechanisms and to localise an effect or event within the cell physiological environment. Moreover, cell death mechanisms, including apoptosis, autophagy or oxidative stress can be easily monitored in cell cultures. Exploiting BL as detection principle various cellular events can be monitored, providing a complete picture of how and why cells die.

3.1 Bioluminescent reporter gene assays

Reporter gene assays are widely employed for monitoring at cellular level relevant events for drug discovery associated with gene expression. Thanks to reporter gene technology a luciferase reporter protein, can be expressed under the regulation of a specific promoter sequence or enhancer elements, thus enabling correlation of reporter protein expression, measured as light signal, and transcriptional regulation [28], providing quantitative information about target bioavailability and specific bioactivity, even in multiplexed format [29–31].

About 33% of currently marketed drugs target G protein coupled receptors (GPCRs) [32] and can be monitored using a cAMP response element (CRE) positioned upstream of a luciferase reporter gene. When activated, the GPCR causes an increase in intracellular cAMP, in turn activating protein kinase A to phosphorylate CRE binding protein, leading to an increase in the transcription and transduction rate of the BL reporter protein, with a proportional BL signal. As concerns cell-based assays targeting nuclear receptors, a BL reporter protein can be placed downstream a promoter containing a hormone response element and the luciferase gene expression is activated by the binding of the nuclear receptor in the proximity of the promoter. Bioassays relying on yeast or mammalian cells genetically engineered to express reporter protein in the presence of compounds with androgenic and estrogenic activities have been used since more than 30 years, however the predictivity of using yeast cells remains questionable [9,33–35]. The chemically activated luciferase gene expression

(CALUX®) bioassay represents the gold standard for the analysis of dioxin-like activity and endocrine disrupting chemicals. Recently, by using CALUX® assays, researchers explored receptors involved in major toxicity pathways targeted by carcinogenic heterocyclic aromatic amines [36], and the combination effects of binary and ternary mixtures of anti-androgenic fungicides [37].

NanoLuc luciferase was exploited to design a BL estrogen screen biosensor based on *Saccharomyces cerevisiae* cells genetically modified to express a yeast codon-optimized variant of NanoLuc under the regulation of human estrogen receptor α activation. The proposed system allowed to obtain in 1 h quantitative evaluation of the 17β-estradiol in small samples reaching a limit of detection (LOD) of 0.08 nM [34].

Thanks to the internal signal amplification due to intracellular signal transduction and gene transcription, BL reporter assays can be designed for highly sensitive HTS providing simple and efficient measurements of cellular physiology without sacrificing data quality. To minimize the false-positive hits during antagonist screening, a second luciferase gene can be added as internal control [29]. The implementation of such controls is crucial to avoid biases in the interpretation of results. In particular Auld et al. analysed a library 700000 compounds identifying that luciferase inhibitors represent about the 3% of all screened compounds. This finding suggests the need for implementing orthogonal assays in drug discovery [38].

An affordable BL assay for drug discovery was developed by Benitez et al. who genetically engineered a bloodstream *Trypanosoma brucei brucei* parasite to constitutively express a thermostable red emitting firefly luciferase[6]. The simplicity and robustness of the BL 96-well-plate HTS phenotypic assay, validated using a small compound library to identify several hits with EC50 ≤ 10 μM, allowed to estimate the number of metabolically active parasites as a function of BL signals, with the potential to be scaled up to a 384-well-plate format [39].

ATP-independent luciferase NanoLuc was molecularly engineered to develop a class of genetically encoded kinase-modulated bioluminescent indicators (KiMBIs) for noninvasive longitudinal imaging of drug activity by kinase inhibitors in the brains of living mice. This represents an important step

due to the high interest in developing new specific inhibitors for kinase pathways in the brain and to discriminate brain-penetrant and nonpenetrant MEK (mitogen-activated protein kinase kinase) inhibitors [40].

The first BL cellular reporter for monitoring of human Sonic Hedgehog protein (SHh) autoprocessing was developed by Ciulla et al. [41]. The assay combines intracellular SHh precursor's C-terminal enzymatic domain autoprocessing using endogenous cholesterol with extracellular secretion of the NanoLuc luciferase. The authors reported the design of an additional mutant reporter line lacking a conserved general base critical for the activation of the cholesterol substrate, useful for screening of small molecules activators. The detection of the BL signals in the cell culture media of 1536-well plate was performed thanks to a new synthetized anionic phosphonylated coelenterazine substrate.

A substrate-free, self-bioluminescent assay based on human embryonic kidney cells (HEK293ARE/Gal4-Lux) was developed to identify compounds with androgenic activity by self-initiating a BL signal [42]. Natural and synthetic androgenic compounds, including the 5α-dihydrotestosterone, the testosterone, the 17α-methyltestosterone, the 4-androstenedion and the mifepristone, were tested with the proposed reporter system obtaining for the natural androgen 5α-dihydrotestosterone an EC_{50} value of 7.6×10^{-10} M with the same order of magnitude as the median EC_{50} of 1.5×10^{-10} M obtained with other mammalian cell reporter gene assays reported by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) meta-analysis[43]. As concerns sustainability and total assay cost, this represents an interesting approach to reduce additional cost due to the addition of D-luciferin (D-LH_2) substrate.

4 Bioluminescence for Protein-Protein interactions

All physiological and pathological events, including the regulation of different cellular processes, such as the transcription, the translation, signal transduction and the oncogenic transformation, are led by protein-protein interactions (PPI). Two hybrid systems, split reporter protein complementation,

or Protein-fragment complementation assay (PCA), and reconstitution or resonance energy transfer (RET)-based assays, represent viable approaches for monitoring PPIs.

4.1 Protein-fragment complementation assay

PCA is a well-established technique for *in vitro* screening of compounds causing protein-protein interactions, with great potential also for *in vivo* applications. In PCA assays a FL or BL reporter is generally splitted into two halves and the cDNA encoding for these fragments are genetically fused to the two proteins which interaction is under investigation. After target protein interaction, the reporter fragments reassemble, and the functionality of the reporter protein is restored. PCA assays based on BL reporters are highly useful for screening molecules targeting PPI and have been widely applied for GPCR [44]. The use of split-luciferase complementary assay is advantageous for discovering inhibitors in a large-scale HTS format [45]. The assay is characterized by high sensitivity because of the light signal is emitted only when the protein-protein interaction occurs. Split luciferase complementation has been exploited to monitor early-stage of apoptosis [46], apoptosome complex formation [47] and structural characterization during inflammation [48]. Despite split luciferases have been exploited to obtain quantitative data, artefactual results can be generated. To solve issues related to aspecific reconstitution of the splitted luciferase, leading to false positive results, a new technology was developed, called NanoLuc® Binary Technology (NanoBiT). Taking advantage of NanoLuc luciferase Dixon et al. designed the binary reporter system NanoBiT, in which the two subunits, the SmBit 1.3 kDa peptide, and the LgBit 18 kDa polypeptide, associate very weakly and, being so small, do not lead to steric hindrance, thus providing high detection sensitivity [49].

Up to now NanoBiT has been widely used to design BL-cell-based assays for studying PPIs with the aim to obtain accurate results about protein interactions under physiological conditions [9].

A cell-based assay useful to interrogate the binding of the cellular LDL receptor (LDLR) with its extracellular protein ligand, the proprotein convertase subtilisin/kexin type 9 (PCSK9), was developed (Figure 4A). BL assay based on cells stably expressing LDLR with an extracellular complementary tag for the PCSK9 that, after binding to the receptor, generate a bright luminescence

signal. The interaction is directly detected at the cell membrane with a simply add-and-read procedure without the use of additional washing steps. This assay technology represents a useful tool to study other molecular targets-with their extracellular protein ligands, opening a new way to develop new therapies [50]. To discover new inhibitors compounds for influenza A and B viruses acting on polymerase PA-PB1 interactions, an *in vitro* PCA based on firefly luciferase was developed in a 96-well plate format for HTS. The optimized assay was characterized by a simple mix-and-measure procedure, used to screen 10.000 compounds against a panel of influenza A and B viruses [51]. Recently, several studies reported the use of BL to study receptor internalization[52]. HER2 is a well-known RTK receptor, considered a therapeutic target in breast and ovarian cancer, and its internalization process can be monitored through the NanoBiT [53]. The authors described an interesting approach to quantify in real-time the recycling and the membrane protein internalization in living systems, providing a useful method also to study the antibody-mediated internalization. The catalytic activity of NanoLuc luciferase could be modulated exploiting interactions between phosphorylated substrates (pSub) and phosphopeptide-binding domains (PBDs) [40]. In presence of an active phosphorylating kinase, the PBD-pSub interaction prevents SmBiT reassembly with LgBiT. In presence of a kinase inhibitor, the equilibrium of conformational states was shifted towards the LgBiT-SmBiT reconstitution, resulting in light production (Figure 4B).

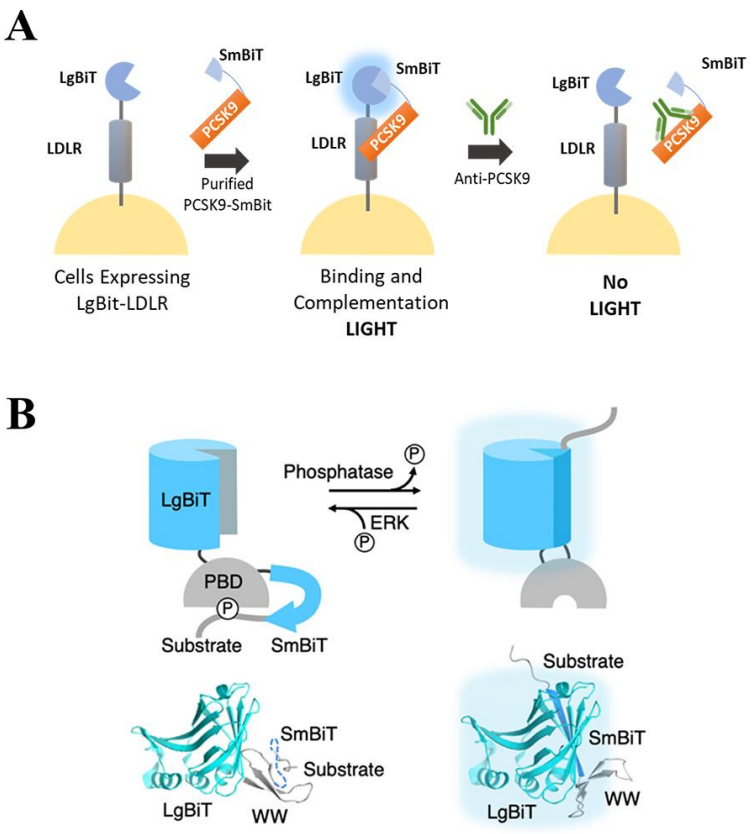

Figure 4: A) Schematic representation of the PCSK9-LDLR binding assay [50]. B) Domain arrangement and model of an ERK KiMBI. Reprinted with permission from [40] (Copyright © 2023 The Authors. Published by American Chemical Society).

4.2 Bioluminescence Resonance Energy Transfer (BRET)

BRET technique involves a transfer of energy from a light-emitting protein, acting as a donor, to a FL acceptor. This energy transfer occurs when the distance from the donor to the acceptor is between 1-10 nm with an efficiency inversely proportional to the sixth power of the distance between donor and acceptor [54]. Other important issues for an efficient transfer are the overlap of the emission spectrum of the donor with the excitation spectrum of the acceptor and the relative dipole-dipole orientations of BRET pairs. For this reason, in the last years this proximity-based assay has gained popularity and it is considered one of the most versatile techniques for monitoring PPIs and conformational rearrangements in living cells. BRET has been exploited for studying molecular

interactions of soluble or transmembrane proteins occurring in living cells at "physiological" expression levels, and to follow dynamic interactions in real-time [54,55].

Luciferases from *Gaussia princeps* (GLuc)*, Renilla reniformis* (RLuc) and NanoLuc have been widely used as BL energy donors in combination with Green Fluorescent Protein (GFP) and its variants as FL energy acceptors. One of the major advantages of this technique is the non-invasive nature of the analysis, allowing to monitor PPI in the physiologically relevant environment. BRET has been used to study many transmembrane receptor classes of pharmacological importance including GPCRs dimeric/oligomeric forms [6,56,57] and to elucidate the conformational states of β-arrestins associated with GPCR activation in living cells [58,59].

BRET technology does not require an external light source, which is a non negligible advantage, but one of the major limitations in BRET imaging is related to the obtainment of high-resolution images. This is due to the energy transfer efficiency of the selected sensor pair and the kinetics of the phenomenon. To widen the field of BRET imaging applications and obtain higher resolution BRET imaging, recent improvements in the design of BRET probes were achieved by optimizing the imaging protocols [54].

BRET assay was exploited to evaluate Ribosomal P proteins and proteins involved in mRNA processing, in perspective to discover new molecular targets for anti-trypanosomal treatments and to replace the outdated therapies to fight infection by *Trypanosoma cruzi* [60].

BRET assays are considered more sensitive than luciferase PCA assays where only 20-50% of the functional recovery of the split luciferases occurs. Furthermore, misfolding of the protein obtained after complementation or non-specific interactions between the split fragments may cause both false-positive and false-negative signals [61].

Traditional BRET platforms are based on an ATP–independent luciferase (e.g., RLuc8) as energy donor and a FL acceptor as the green fluorescent protein variant (e.g., Venus), using coelenterazine h as substrate. One limitation of this system is the relatively large size of the luciferase that potentially could cause steric hindrance, thus preventing the interaction [62] or interfering with the trafficking of

G protein–coupled receptors (GPCRs) to the plasma membrane [63]. The NanoBRET platform overcomes these issues providing a new strategy to design assays for a broad range of molecular interactions. This system relies on the use of NanoLuc Luciferase as the donor and an HaloTag® NanoBRET fluorescent Ligand as the acceptor.

NanoLuc produces exceptionally bright luminescence light of about ~150-fold greater that RLuc luciferase, with a slightly blue-shifted of about 20 nm and narrower emission spectrum (~20%), resulting in high spectral resolution [64].

Several interactions between protein partners in different cellular compartments have been studied with the NanoBRET technology, including membrane proteins [65,66], signaling of protein kinases [67], and transcription regulators [68]

The red-shifted HaloTag fusion protein [69], an alternative to GFP-based acceptors in BRET experiments, has been used as acceptor in the pairing with the NanoLuc luciferase, showing minimal spectral overlap, high signal to noise ratio, and dynamic range, confirming its applicability for PPIs [70].

5. Bioluminescence for 3D cell models

Cell-based assays in 2D formats represent invaluable tools widely employed in drug discovery programmes. Despite they are still considered the “gold” standard in drug screening, 2D cell models do not replicate the cellular architecture, comprising the extracellular matrix (ECM) microenvironment and cell–cell interactions, and the *in vivo* complexity, thus providing misleading data and reducing predictivity of the results. Many of the limitations of traditional 2D cell culture systems are overcome by 3D cell models with the capability to reproduce intra- and inter-cellular signalling networks, and diffusion and transport conditions, which are extremely important for various cellular function such as differentiation and proliferation; thus, they can be considered the most suitable model to mimic *in vivo* physiology to understand the impact of a potential drug candidate before it enters animal and clinical trials [14]. In line with the “three Rs principle” of Russell and Burch, recently not only the European Union but also the USA and many other countries strongly

encourage the implementation of *in vitro* assays and other approaches to replace animal testing [71,72].

In the last years, highly predictive assays based on 3D cellular models have been shown suitable for identifying molecules with potential bioactivity interacting with molecular targets, representing a very promising and reliable tool to decode intra- and inter-cellular signalling and for effect-based analysis [9,29,73,74].

Different methods can be employed to obtain 3D cell models, relying on the use of biocompatible supports [75] and the standardization of 3D cell models is a vital factor in obtaining robust and reproducible results especially in terms of size and shape. Biocompatible poly-l-lactic acid supports were exploited for monitoring BL implanted neural stem cells in corticectomized rat models [76].

An easy monitoring system to evaluate cytotoxicity in 3D hepatocyte models by a continuous nondestructive BL measurement have been developed by Yasanuga et al. [77]. To evaluate therapeutic agents against diabetes, BL imaging in 3D models was performed to visualize quantitatively the insulin secretion from living cells using as reporter protein GLuc luciferase from *Gaussia Princeps* [78].

BL 3D models have been developed to study the Nuclear Factor-kappa B (NF-kB) signal transduction pathways that has a pivotal role in the regulation of cell-cycle/growth, inflammation, apoptosis, and immunity[79] acting as crucial player in many steps of cancer initiation and progression.

An enhanced chimeric firefly luciferase-inspired enzyme (PGL2)[80] and a new powerful luciferase mutant BgLuc developed by Michelini's group [17] have been exploited as BL reporter of NF-KB pathway activation in 3D models for longitudinal studies [31] and for monitoring inflammation [29,74] in micropatterned 96-well plate format. With the aim to upgrade conventional 2D cell-based assay and to obtain multiple pieces of information via a single assay, a multiplexed assay relying on BL 3D spherical microtissues was developed to evaluate four different activities of a single sample, i.e. inflammatory, antioxidant, toxic activities and the presence of heavy metals [74].

Inflammation induced by tumor necrosis factor α (TNF-α) was studied in jejunum-derived NF-κB reporter organoids, by recreating the cellular composition of the organ using different intestine regions [81].

PCA based on a split NanoLuc reporter was exploited to develop an intracellular nanosensor for studying the activation state of the androgen receptors in 3D cell models and for fast and sensitive detection of new androgenic-like compounds or endocrine disrupting molecules [9].

6. New bioluminescent tools for multiplexing

The need to simultaneously monitor multiple targets and mechanisms of actions requires the development of new reliable and cost-effective drug screening assays. Multicolor luciferase reporter assays represent a powerful tool to monitor expression of several genes [82–87].

The discovery of new BL systems[88,89], the availability of new synthetic luciferases with altered spectral properties [17,90–92], and the design of luciferin substrates assisted by computational models [93] have expanded the BL toolbox for more challenging applications in drug screening.

To achieve multiplexing, two or more luciferases can be introduced in the assay, and their emission separated either by spectra, spatial, or temporal resolution [29,74,94]. Spectral unmixing by using suitable emission filters is certainly the most explored solution [95], however partial overlapping of the emissions or reduced light output of the red emitters can reduce the sensitivity of the assays. The use of luciferases requiring different substrates is also a viable option [96]. BL multiplexed assays, including viability controls able to correct the analytical signal according to cell viability, or to monitor two or more analytes, have been developed [29,82,94].

An elegant work by Tarnow et al. reported the development of a new luciferase-based reporter system suitable for the simultaneous and time-resolved measurement of Aryl Hydrocarbon Receptor (AHR) and the Estrogen Receptor (ER) activations in living cells. This simple and cost-effective multiplexed assay exploited two luciferases emitting at different wavelengths, the emerald luciferase (ELuc), a green emitting luciferase from the Brazilian click beetle *Pyrearinus termitilluminans,* and the “stable

luciferase red" (SLR) luciferase, a red-emitting luciferase from the railroad worm *Phrixothrix hirtus*. Thanks to the use of the same D-LH_2 substrate, which is cell permeable, nontoxic, and highly stable, time-resolved quantitative measurements in living cells were obtained. The system allowed to screen single substances and mixtures able to activate AHR and ER and to perform continuous readings over extended periods of time as well as to provide a powerful tool to investigate AHR/ER signaling crosstalk [97].

The combination of multicolor BL with the availability of optimized luminogenic substrate was exploited to develop a rapid and cost-effective antimalarial drug screening assay. A BL gametocyte assay to simultaneously assess the viability of parasites was developed by Cevenini et al. (Figure 5A). This dual-luciferase assay is based on immature and mature *Plasmodium falciparum* gametocyte stages expressing green- and red-emitting luciferases from *Pyrophorus plagiophthalamus*, and represents an interesting bioanalytical method for the drug stage-specific evaluation in a HTS format [94,98].

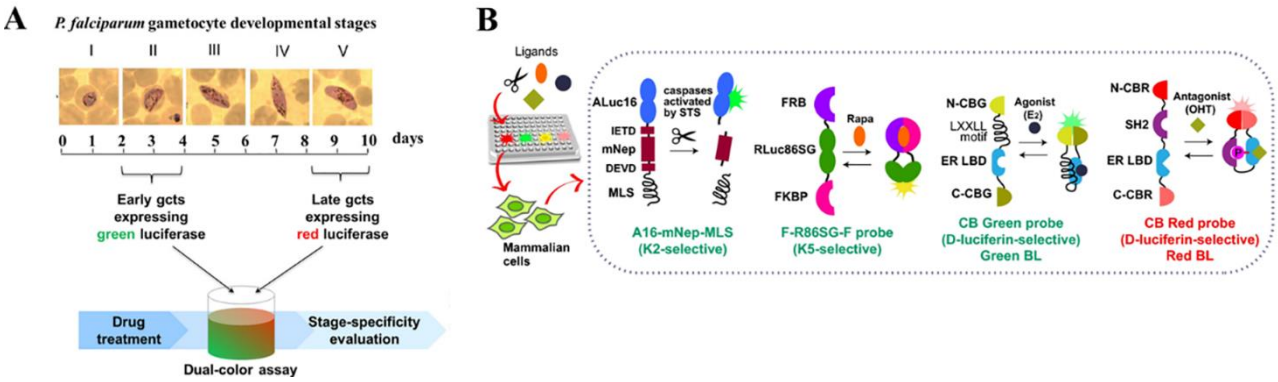

Figure 5: A) Schematic representation of the multicolor gametocyte assay based on immature and mature *P. falciparum* gametocyte stages. Reprinted with permission from [94] (Copyright © 2022 American Chemical Society). B) The working mechanism of the multiplex quadruple bioluminescent assay system developed by Kamiya et al. Reproduced with permission from [99].

Two highly stable mutant luciferases, the PpyGR-TS and the PpyRE-TS luciferases, were exploited by the same group to develop a smartphone based multicolour BL 3D cell biosensor platform [92].

In the proposed biosensor the green emitting PpyGR-TS luciferase and the red emitting PpyRE-TS luciferase were used as viability control and inflammation reporter, respectively. Thanks to advantageous properties of these two luciferases, in terms of high emission intensities, glow-type kinetics, and high pH stability, as well as the well separated emission spectra using the same D-LH_2 substrate, enabled the development of an assay highly cost effective enabling the analysis of complex biological samples [100]. The obtainment of ratiometric measurements allowed to obtain more robust analyses since it normalizes signal variability due to different number of cells and cell viability, providing a LOD for tumor necrosis factor α of 0.15 ng/mL and an EC_{50} of 1.0 ng/mL.

A pioneristic work for examining multiple cellular pathways was reported by Sarrion-Perdigones et el. who developed an hextuple reporter assay based on six luciferases which activities can be uniquely determined thanks to the combination of orthogonal substrates, exploiting selective quenching of the BL and spectral deconvolution [101].

Multiplexing can be also achieved by using newly synthetized substrate analogues. Disubstituted luciferins have been designed for orthogonal detection [24]. Kim's group synthetized coelenterazine substrate analogues for a multiplex quadruple assay system containing four single-chain BL probes. In this configuration each BL probe is able to emit a BL signal only in the presence of the target ligand and the specific coelenterazine analogue [99]. This strategy allowed to minimize optical signal cross-leakages, enabling screening of specific ligands in the mixture (Figure 5B).

7. Expert Opinion

BL reporters have been used for more than 20 years to identify chemical modulators acting on cellular pathways in HTS or HCS formats. New luciferin and luciferases have enabled uncharted applications in drug screening, leading to highly efficient and robust assays.

The great advancements in protein engineering, and light detector technology led to new BL systems and new assay formats. More recently the implementation of 3D cell models coupled with red emitting luciferases provided a new level of predictivity. However, it is not a mystery that 80% of *in vitro* drug screening fails, mostly due to assay artifacts caused by aspecific effects on fluorescence

and bioluminescence reporters or assay reagents. About ten years ago coincidence bioreporter circuits provided a new tool to unravel potential interactions with the reporter an avoid false positives and false negatives [102]. Nevertheless, the potential for multiplexing is still lagging behind that offered by the GFP variants. Besides the cost related to the use of one or more substrates surely represents an issue, both in terms of cost and sustainability. In scientific literature the greenness of the assays is seldom reported or quantitatively evaluated, while it should be a key factor to be considered in the choice of the assay format. In this regard de novo protein design could create luciferases on demand having the suitable features for the selected applications. This approach proved feasible with a synthetic luciferin, diphenylterazine (DTZ), and in the future more efforts should be put in this direction to guide the development of new luciferases with artificial intelligence. The recent cloning of the BL autobioluminescent systems from fungi also provides a new promising alternative to the well-known commercial tools [103]. The integration of BL proteins, 3D cell models, and miniaturized platforms represents a promising approach which could provide sustainable and cost-effective organ-on a chip platforms not only for predictive drug screening but also for drug development and precision medicine applications [104,105].

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