Bioluminescence goes portable: recent advances in whole-cell and cell-free bioluminescence biosensors

Bioluminescence goes portable: recent advances in whole-cell and cell-free bioluminescent biosensors

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Abstract

The recent advancements in synthetic biology, organic chemistry and computational models, allowed the application of bioluminescence in several fields, ranging from well-established methods for detecting microbial contamination to in vivo imaging to track cancer and stem cells, from cell-based assays to optogenetics. Moreover, thanks to recent technological progresses in miniaturized and sensitive light detectors, such as photodiodes and imaging sensors, it is possible to implement laboratory-based assays, such as cell-based and enzymatic assays, into portable analytical devices for point-of-care and on-site applications.

This review highlights some recent advances in the development of whole-cell and cell-free bioluminescent biosensors with a glance on current challenges and different strategies that were used to turn bioassays into biosensors with the required analytical performance. Critical issues and unsolved technical problems are also highlighted, to give the reader a taste of this fascinating and challenging field.

• **Keywords:** Bioluminescence, biosensors, portable analytical device, whole-cell biosensors

Introduction

Bioluminescence (BL), that is the emission of light from living organisms, has been always looked as an amazing natural phenomenon documented in numerous species including bacteria, beetles, molluscs, and coelenterates [1, 2]. Luciferase-catalysed BL is generated through a variety of chemical reactions in which an enzyme (luciferase) oxidises a substrate (luciferin), leading to the emission of photons. All these reactions require molecular oxygen and lead to the production of transient peroxides, for some luciferases these reactions require the presence of specific cofactors (ATP, Mg^{2+}) [3]. Apart from its fascinating aspects BL, thanks to its high sensitivity and independence of external excitation, is a precious bioanalytical tool that, right after the introduction of molecular biology tools, has revolutionized the landscape of basic and applied research. Practical analytical applications of BL span from cell-based high throughput assays for diverse targets such as proteases, kinases, apoptosis, and cytotoxicity, detection of ATP for hygiene monitoring and bacterial contamination to molecular imaging to locate tumors in living organism [4, 5].

Just to mention one elegant example, very recently new autoluminescent plants have been obtained exploiting the newly discovered fungal bioluminescence cell-pathway. A synthetic biology approach has been used to transfer the entire fungal bioluminescent pathway into non-bioluminescent species combining the existing biological components to generate a self-standing bioluminescent process based on caffeic acid conversion into luciferin [6,7].

BL, clearly visible to the naked eye, has naturally evolved in a wide colour palette as a mechanism for mating, defence, and predation and nowadays several luciferase variants cloned by different species are available. Biodiversity evolved in nature combined with random and specific mutagenesis enabled to improve luciferases properties in terms of emission wavelength, intensity and kinetics [8-13]. Integration of luciferases with different BL properties into the same biosensor was successfully proven providing multiplexed analytical tools. Among others, the integration of an internal viability control exploiting luciferases with different emission wavelengths has demonstrated the effective use

of these tools to correct the analytical signal according to cell metabolic state, thus improving biosensor analytical performance [14].

Flash-type kinetics is another problem of bioluminescent biosensors, compared with other optical reporters, in particular for the integration with less fast and precise instrumentation required for on-field applications. Nowadays, thanks to enzyme mutagenesis and substrate modifications this problem is being solved. Indeed, bioluminescent luciferase pairs with stable and glow-type kinetics are commercially available. Reusability over time of bioluminescent whole-cell biosensors is another issue that has been addressed by Cevenini et al., with a long-lasting monitoring of 3D biosensors with non-lysing signal acquisition [15].

Up to now, one of the main unsolved technical problems for bioluminescent biosensors applications is the low reagents' stability in different conditions such as temperature variations and light exposure. Many efforts are being made to overcome this problem, but a solution has not yet been found.

The need for enzymes with improved catalytic activity and high stability prompted studies aimed at the optimization of protein folding and different assay formats were developed, also relying on alternative strategies such as protein-fragment complementation assay (PCA) [16,17]. PCA, originally developed to provide a simple and direct way to monitor protein-protein interactions also in living cells, relies on enzyme refolding from cognate fragments in which reconstitution enzyme activity reflects the occurrence of a specific protein interaction [18].

Among others, NanoLuc Binary Technology (NanoBiT luciferase) seems one of the most promising reporters for protein complementation assays both in cell and cell-free environments [19, 20].

The recent advancements in synthetic biology, organic chemistry and computational models, allowed the application of BL in several fields, ranging from the well-established methods for detecting microbial contamination to *in vivo* imaging to track cancer and stem cells, from cell-based assays to optogenetics. Diverse synthetic biology approaches were pursued, ranging from the use of well-studied inducible systems to more sophisticated designer circuits tuned for specific molecular

recognition, higher sensitivity, and reporter tunability. RNA-based biosensors have been also proposed based on conventional riboregulators or toehold switch models [21].

Inducible gene expression systems have been widely employed to develop new synthetic-biology tools for detecting target analytes and, more recently, to expand the portfolio of detectable targets, genome-wide approaches have been exploited to identify transcription factor-based inducible genes [22]. A tunable modular cascade for intracellular signal amplification has been also designed to boost biosensors output, increasing sensitivity (up to 5,000-fold for whole cell biosensor for mercury detection) and signal intensity of whole-cell biosensors [23]. Moreover, thanks to recent technological progresses in miniaturized and sensitive light detectors, such as photodiodes and imaging sensors, it is possible to implement laboratory-based assays, such as cell-based and enzymatic assays into portable analytical devices for point-of-care and on-site applications.

To this end, new synthetic biology tools and technological solutions have been designed to address limitations of both biological and technological components. Among others, to increase sensitivity, stable bioluminescent proteins having high BL emission have been combined with more sensitive light detectors [24, 25]. Robust immobilization methods have been optimized for different needs to obtain ready-to-use cartridges that can be stored for long period of time (more than 1 year) and can be easily shipped when necessary [26, 27]. Inter-and intra-assay reproducibility has been also increased to cope with the different environmental conditions [14, 28]. More user-friendly and cheap devices for both technological and biological components have been exploited to achieve massive use by consumers [29, 30]. Up to now, low substrate stability and its high cost represent an obstacle for commercialization. To this end, Coutant et al., have recently developed new cheap and stable NanoLuc luciferase substrates which seem promising for real-life applications [31].

This review highlights some recent advances in the development of whole-cell and cell-free BL biosensors with a glance on current challenges and different strategies that were used to turn bioassays into biosensors with the required analytical performance. Critical issues and unsolved technical problems are also highlighted, to give the reader a taste of this fascinating and challenging field.

Bioluminescent whole-cell biosensors

The use of living cells as sensing systems offers the possibility to predict physiological responses to chemicals, drugs and samples [32, 33]. Thanks to the significant advances in detection technology and in the genetic engineering of cells, it is possible to reprogram cells to respond to different analytes or classes of analytes, to provide information about toxicity or specific biological activities. In addition to the well-established reporter gene technology, in which a reporter protein is under the control of a constitutive or inducible promoter, other promising approaches are also emerging, including Bioluminescence Resonance Energy Transfer (BRET) and protein fragment complementation [19, 34].

Genetically modified BL biosensors show excellent analytical performance and flexibility of use and can be turned into real field deployable analytical devices [35, 36].

By taking advantage of the peculiar features of new biocompatible nanomaterials, hybrid devices with immobilized or micropatterned cells, including bacteria, yeast or mammalian cells, have been developed and applied to different fields from drug discovery [37] to environmental monitoring [38] [39,40], food control [41], and anti-doping screening [42].

Bioluminescent bacterial biosensors

Bacteria have several advantages including low-cost, easy of cultivation and genetic manipulation, and fast replication time, allowing the development of several bacterial biosensors that selectively respond to different analytes. Thanks to their easy repurposing to respond to a wide variety of environmental stimuli, engineered bacterial strains have been widely employed as bioreporter entities in many applications [43, 44]. In addition, owing to their small size and robustness, bacterial cell-based sensing systems are amenable to multiplexing, high-throughput, and miniaturization for incorporation into portable devices.

Whole-cell bacterial biosensors rely on bacteria genetically engineered to produce measurable signals (e.g. light) and to respond in a specific manner to particular classes of compounds (e.g. heavy metals

[45], antibiotics [46], aromatic compounds [47, 48], providing a luminescent response to assess toxicity level of a sample and provide information about type of toxicant found therein [49].

Acute (cyto)toxicity evaluation, generally, involves the use of natural bioluminescent bacteria such as *Aliivibrio fischeri*, where the light production is highly dependent on cellular energy levels. In 1967, Serat et al. developed the first biosensors based on immobilized *Photobacterium phosphoreum* to evaluate air pollutants toxicity [50]. Since then, technical advances in genetic engineering allowed the development of new synthetic biology tools and new chassis microbes that, combined with technological progress in photosensitive sensors, greatly contributed to the development of portable biosensors. In 1994, Heitzer et al. designed an inducible biosensor based on immobilized bacteria cells in direct contact with the optical transducer: photomultiplier (PMT) via an optical fibre to detect specific chemicals (i.e., naphthalene and salicylate) in aquatic environments [51].

More recent light detectors, i.e. Charge-Coupled Devices (CCD) and Complementary Metal-Oxide Semiconductor (CMOS), have been widely used to detect the bioluminescence emitted by immobilized bacteria. Simpson et al. reported a BL bioreporter integrated circuit (BBIC), in which immobilized bacteria were inserted into an integrated circuit in direct contact with CMOS photodetector [52]. The main advantage of this approach was that the entire sensor, including all signal-processing and communication functions, was produced as a single-chip, low-power, rugged, inexpensive device. This strategy was further expanded with the use of CCD camera placed above or below the immobilized cells to perform BL imaging. In 2007, Sakaguchi et al. proposed a similar biosensor based on the use of the CCD sensor from a commercial digital camera as a bioluminescence sensor, a mobile PC and luminous marine bacteria, *Photobacterium phosphoreum* immobilized on an acrylic chip. This system, having a similar performance as the imager system, offered a rapid platform for onsite detection of Biochemical Oxygen Demand (BOD) in environmental samples [53].

As an alternative, lensless contact imaging, combined with BL detection, allowed to develop more compact portable devices where cells genetically modified to express BL reporter proteins were immobilized in a polymeric matrix to increase cell shelf-life and to provide ready-to-use disposable

biosensor cartridges [36]. A drawback of this biosensor architecture is that the user has to replace the disposable biosensor cartridges after each analysis, with the need to repeat all bacterial preparation operations, such as growth and immobilization, before performing another experiment. To overcome this technical limitation, several disposable cards containing immobilized BL bacteria were designed to allow continuous analysis [35, 54]. This alternative approach enables to regenerate the cells exposed to the sample with the infusion of the nutrient solution prior to each analysis session.

For example, Horry et al. designed a portable optical biosensors Lumisens 2 used for the online detection of pollutants, that integrates a central unit where the disposable card is inserted for the measurement, and an acquisition unit to control the device. The disposable card with immobilized bioluminescent *E. coli* strain TBT3 harboring the truncated luxAB genes from *Vibrio harveyi*, has been used to specifically detect organotin compounds such as tributyltin or dibutyltin [55]. Despite one limitation of this biosensor is related to the behaviour of tributyltin and its adsorption/desorption rates in agarose interfering with the bioavailability, this strategy opened a new opportunity to detect overall toxicity or specific pollutants. As concerns biosensors in multiplex format, the use of removable multi-well cards represents an interesting approach to improve the workability of biosensors, where portability is combined with the capability to deal with multiple components in a single process. Lumisense 3, an upgraded version of Luminesense 2, was designed to integrate a multi-well card with four bacterial stains *E. coli* DH1 pBzntlux, pBarslux, pBcoplux, and XL1 pBfiluxCDABE immobilized in an agarose matrix, an optical setup for bioluminescence monitoring, a fluidic channel network for media and sample loading, and a computer interface for full automation [35]. This configuration allowed flow of media and samples along the fluidic channels, demonstrating the simultaneous on-line cross detection of several heavy metals (including $CdCl_2$ and As_2O_3) as well as the measurement of the overall toxicity of the sample (Fig. 1).

The use of disposable cartridges is feasible in manually operated systems, but they become unsuitable when automated remotely operated instruments are required. To solve this problem, Elad et al. proposed an integrated system with three parallel microfluidic chips containing 12-wells loaded with

immobilized bacterial bioreporters responding to DNA damage, oxidative stress and heavy metals for continuous water monitoring [56]. This configuration allowed to include sample replicates and controls on the same chip, with the possibility to continuously operate for 10 days with five consecutive sample measurements.

The generation of bacteria having both magnetic and bioluminescent properties provided an alternative to develop cell-based biosensors integrated into microfluidic. A portable toxicity detection system based on a microfluidic analytical device that incorporate genetically engineered bioluminescent magnetotactic bacteria (BL-MTB) was developed by Roda et al. based on *Magnetospirillum gryphiswaldense* strain MSR-1 genetically modified to constitutively express a red emitting click beetle luciferase. Thanks to their intrinsic magnetic properties, these bacteria have been exploited as "natural actuators" and were used for easy on-chip manipulation through magnetic cell concentration, optimizing the chip's analytical performance. The BL-MTB have been integrated in a microfluidic chip, whose bioluminescence detection, directly proportional to bacterial viability, is based on lens-free contact imaging. The chip is placed in contact with a cooled CCD *via* a fiber optic taper to perform quantitative bioluminescence imaging [57].

Another interesting approach has been illustrated by Tsai et al., who developed a portable measurement system, named LumiSense, that use the linear CCD in combination with a miniaturized whole cell sensor array biochip with integrated temperature control components (LumiChip). The Lumichip, composed by a 16-well contained multiple bacteria bioreporter carrying different target specificities, harbours a simple but highly efficient lens-free bioluminescence collection setup, where the samples are infused in an oxygen-permeable microfluidic flow channel to reach the sensor array, where time-lapse changes in the bioluminescence emitted by the array members are measured on a single linear charge-coupled device (CCD). This platform for on-site water pollutant monitoring can detect bioluminescence within 15 to 45 minutes after incubation with the sample. As proof of principle, the authors loaded two *E. coli* bioreporter strains, one for genotoxicity and the other targeting nitroaromatic compounds, demonstrating the possibility to analyse in parallel two or four

samples [58]. In 2019, a successful adaptation of this testing strategy was reported, based on a combination of a microfluidic system for sample acquisition and a mobile phone for low-light bioluminescence imaging [59]. The integral smartphone whole-cell biosensor, named LumiCellSense (LCS), was composed by a miniaturized bioreactor for the induction of bacterial bioreporters, enclosed in a light-impermeable case and a commercial smartphone (iPhone SE). In this work the authors employed bacterial bioreporter based on bioluminescent *E. coli* strain harboring a plasmid-borne fusion of the *E. coli* recA gene promoter to the *Photorhabdus luminescens* luxCDABE gene, which emits light in response to the presence of target analytes, such as fluoroquinolone antibiotic ciprofloxacin (CIP). The phone's camera was used to image the light emitted by the bioreporter cells in response to the presence of target chemicals and a customized phone-embedded application (LCS_Logger) was employed to calculate photon emission intensity. The suitability of the LCS system was confirmed by the detection of residues of CIP in whole milk, showing a remarkable detection threshold of 7.2 ng/mL, a value lower than the allowed maximum limit defined by European Union regulations (Fig. 1).

The combination of recombinant BL bacterial cells with a compact and portable optical detection device has been exploited to design a wireless, low-weight and cheap portable biosensor for 2,4-dinitrotoluene vapors [60]. This is the first optical 2,4 DNT biosensor prototype to detect buried landmines, that employs immobilized recombinant *E. coli* reporter strain genetically engineered. A photodiode, integrated in a sensor chamber the size of a beverage, connected to a transmitter unit located inside an aluminium case and connected to a computer, was used to measure the production of luciferase induced by 2,4-DNT. The detection is possible thanks to recombinant cells immobilized within an agarose hydrogel matrix held by a cage and placed in a case, where the BL light, emitted towards two conic plastic reflectors with a reflection angle of 40°C, directly reflects into the embedded photodiode (Fig. 1).

Bioluminescent reporter bacteria have been used also for direct measurement of air quality. To this end, Eltzov et al. designed a field-operable system based on TV1061 *Escherichia coli* strain

immobilized in a calcium alginate hydrogel set onto optical fibers [61]. Exploiting this concept, the authors proposed a portable biosensor for real-time air quality monitoring in a real indoor environment. This biosensor is composed by a non-disposable part (PMT and liquid light guide) and disposable calcium alginate pads with immobilized BL bacteria elements, sensitive to various toxicants [62]. In particular, the field-operable fiber-optic photodetector device was modified to monitor air toxicities and optimized to acquire BL signal in the blue light region. In addition, a 21 mm diameter active area was used allowing to gather light radiation without the use of optical focusing elements. The receiving and data treatments were operated using a specific driver which allow monitoring of the bioluminescent signal and data handling in real-time (Fig. 1).

The versatility of this system allowed to develop a water toxicity biosensor based on the alternative method to integrate immobilized bioreporter bacteria [63]. The prototype is designed to have a photodetector and a part composed of new calcium alginate pads with immobilized BL bacteria, where their BL activities can be monitored. The induced BL signals, generated after the exposition of BL bacteria to the target sample, coupled into the liquid light guide can be easily carried out to the far end where sits the PMT camera (Fig. 1). The simplicity of the measuring process of the integrated sensitive and portable biosensor system makes it an attractive tool for water toxicity testing and allowing its use also to non-skilled personnel.

Another interesting approach to integrate BL whole cell biosensors into a portable analytical device was reported by Axelrod et al. The proposed biosensor, namely BioPen, was a fully integrated disposable system where BL bacteria trapped within calcium alginate pads and sensitive to metabolic changes, were placed just above a CMOS sensor surface used to monitor cell light activity [64]. The CMOS-alginate-bioreporter based integrated biosensor was validated with a sensitive conventional benchtop commercial luminometer, showing similar performance for all tested chemicals. CMOS based biosensor was able to measure a formaldehyde concentration of 1×10^{-8} mol L^{-1}, comparable to other approaches [65, 66].

Very recently, BL bacterial bioreporters have been encapsulated in poly-dopamine (PD)-coated alginate microbeads to create an optical biosensor module that can be potentially used for monitoring soil contamination from areas suspected of chemical pollution [67]. Two BL reporter strains, harboring an inducible grpE::luxCDABE fusion and a constitutive lac::luxCDABE fusion, were employed to detect increasing toluene concentrations, showing increased and decreased bioluminescence respectively. Despite the improved properties of the PD-coated beads in terms of mechanical strength and stability, the authors observed delayed responses to the inducing toxicant.

An interesting approach to detect Hg^{2+} in water was reported by Sciuto et al. [68] who proposed a new sensor based on BL *E. coli* Hg^{2+} reporter embedded within a single-photon sensitive small-dimension silicon photomultiplier (SiPM) optical detector. The whole-cell based sensing system, packed inside a dark box was composed by a cuvette, containing the *E. coli* reporter cells, placed on the top of a SiPM that collects the bioluminescence signal coming from the induced cells, able to reveal the presence of mercury in water at 0.25 µg/L. The potential of smartphones-based sensor systems has been widely explored as demonstrated by the large body of literature, highlighting the possibility of implementing a variety of biosensors [29, 69,70].

Different strategies were pursued to concentrate the cells or increase the sensitivity of the biosensors, a promising strategy has been very recently proposed combining BL Hg^{2+} sensitive bacteria expressing the bacterial luciferase gene cassette (luxCDABE) under the regulation of transcriptional regulatory protein MerR and blue light-triggered bacterial cell-cell adhesions of bacteria expressing photoswitchable proteins. After Hg^{2+} binding to MerR, bacteria emit blue light that activates the binding of two photoswitchable proteins, nMagHigh and pMagHigh, expressed on the surface, thus inducing bacterial aggregation. This strategy led to up to 10-fold signal enhancement [71].

Photoactivation of the photoswitchable proteins by bioluminescence is surely an intriguing solution that allows to preconcentrate the biosensors upon analyte detection thus increasing the bioluminescence signal and sensitivity. Despite this approach has not yet implemented in portable analytical devices it surely will open new possibilities to improve biosensor sensitivity.

Bioluminescent yeast biosensors

Yeast cells are considered ideal host for whole-cell biosensors, offering many advantages, such as increased robustness, time- and cost effectiveness, easy genetic manipulation and higher-eukaryotic sensing modalities, easier handling, and scalability to high-throughput formats [72]. Moreover, they can tolerate rather harsh environments, especially compared to bacteria, and can be stored for long periods of time in 'active dry' form. All these advantages combined with reporter gene technologies make yeast an excellent choice for the development of cell biosensors able to detect specific compounds and, given their less-demanding growth conditions, making them potentially adaptable to portable devices for in-field testing. Several yeast biosensors have been developed to assess anti-/ estrogenic and anti-/androgenic potencies associated with waste, surface, bottled, and tap waters, as well as with suspended matter and sediments [73-76].

In the last years, portable yeast biosensors based on fluorescent and colorimetric detection have been developed. Some of them have been also integrated into single-use and low-cost microfluidic systems, [77, 78], others have been immobilized in field portable paper devices for point-of-need and point-of-care applications [79, 80].

To simplify the use of biosensors for environmental conditions exploiting BL detection, Roda et al. reported proof-of-principle devices integrating BL yeast cells for endocrine disruptors [36]. The portable device comprises an interchangeable cartridge containing immobilized BL yeast cell biosensors with a new biocompatible matrix that preserved their vitality coupled with a CCD detector via a fiber-optic-based taper. The authors proposed the use of new yeast strain for androgens (green-emitting luciferase) with an internal vitality control (red-emitting luciferase), demonstrating the suitability of the cellular biosensor for a multiplex detection able to measure two resolved BL emission wavelengths using the same substrate. Using a fibre-optic taper, yeast has been placed in direct contact with the cooled CCD sensor, sensor to image and quantify the BL signals (Fig.1).

More recently, a new cell-based mobile platform integrating BL yeast-estrogen screen (nanoYes) coupled with a low-cost compact camera as light detector has been developed by Cevenini et al. [27]. This whole-cell biosensor is composed by Saccharomyces *cerevisiae* cells genetically engineered with a yeast codon-optimized variant of NanoLuc luciferase (yNLucP) under the regulation of human estrogen receptor α activation immobilized in a ready-to-use 3D-printed cartridges. The obtained portable device exploited a compact camera and wireless connectivity enabling a rapid and quantitative evaluation (1-h incubation at room temperature) of total estrogenic activity in small sample volumes (50 μL) reaching a LOD of 0.08 nM for 17β-estradiol. The proposed portable analytical platform found application for the evaluation of water samples and, thanks to its high sensitivity and the possibility to wireless connect the camera with any smartphone model, resulted more versatile than previously reported smartphone-based devices for on-site analysis of endocrine disruptors. Laboratory-based yeast estrogen screening (YES) assays were also implemented into smartphone-based devices [81]. In particular, the authors reported the development of two yeast biosensors expressing human estrogen receptors α and β and employing NanoLuc as the reporter protein and their immobilization in a 3D-printed cartridge for implementation the assay in a portable configuration by exploiting a compact camera as the detector. Moreover, in the same 3D-printed cartridge a viability control expressed for the first time in *Saccharomyces cerevisiae* strain based on a chimeric green-emitting luciferase, PLG2 was introduced. PLG2 is a thermostable luciferase that produces a bright BL signal and is resistant to low pH shifting, making it well suited for cell-based assays [82]. Thanks to the brightness provided by NanoLuc and PLG2 luciferase, the authors implemented the biosensors into low-cost smartphone-based devices reaching excellent sensitivity and a rapid (1h) response and reported on (anti)estrogenic activity via human estrogen receptors α and β as well as general sample toxicity.

Bioluminescent mammalian cell-based biosensors

Mammalian cell lines are characterized by slow growth, lower robustness, and high maintenance cost than microbial cell cultures. Moreover, their viability is highly affected by external factors and for this reason they require controlled environments for survival. Despite these disadvantages, mammalian cells offer highly valuable information about bioavailability [85], cellular metabolism and physiological response relevant to humans [86] or cytotoxic response [87], and they have become essential tools for screening, sensing, and evaluating environment, food, or clinical hazard. Their use is particularly attractive since they represent a better model of human physiology, providing more predictive biological information [88, 89]. The design of mammalian cell-based biosensors that exploits mammalian cell lines as the biorecognition element must take into account that a suitable support is required not just to provide the cells an inert matrix for cell growth but also to confer them protection from external agents and to create a suitable microenvironment. Moreover, optical properties of the selected matrix should not interfere with BL emission.

The implementation of BL mammalian cell biosensors in smartphone-based analytical platforms with 3D-printed cartridge has been explored for the first time in 2016 by Cevenini et al. [29]. The proposed toxicity cell biosensor was based on genetically engineered Hek293 cells used as "sentinel cells", constitutively expressing a green-emitting luciferase and integrated in a 3D-printed device (Fig.2). The portable device has been designed to include all chemical reagents and droppers for the addition of sample and BL substrate and a customized Tox-App application has been developed to provide a stand-alone platform for user-friendly quantitative toxicity testing in just 30 min at room temperature. This report provided the first evidence that smartphone camera can detect BL signals emitted by living cells and represented a very versatile approach thanks to the possibility to create low-cost 3D-printed smartphone adaptors designed for any kind of mobile device**.** Despite this, huge efforts must be required to extend the lifespan of the integrated cells and to improve their responsiveness to reduce the time-to-response signal.

The creation of a more efficient BL system developed by Englad et al. that combine a novel imidazopyrazinone substrate (furimazine) with a new luciferase (NanoLuc), originally cloned from

the deep sea shrimp *Oplophorus gracilirostris,* allowed to develop a smartphone-based BL cell biosensor with improved analytical performances. This luciferase is characterized by a BL signal about 100-fold brighter than that of the firefly luciferase [90]. Moreover, thanks to NanoLuc's small size (19 kDa) and the absence of posttranslational modifications, the synthesis and folding of the reporter enzyme is rapid, reducing total assay time. A smartphone-based BL cell-biosensor for the quantitative assessment of (anti)-inflammatory activity and toxicity was thus developed using Nanoluc as reporter protein [91]. Hek293 T cells, genetically engineered to express NanoLuc in the presence of compounds (i.e., tumour necrosis factor-α) able to activate the NFkB-mediated inflammatory pathway were immobilized into 3D printed cartridge and integrated with the Nokia Lumia 1020 smartphone. Quantitative assessment of (anti)-inflammatory activity and toxicity of white grape pomace liquid extracts was performed with a simple and rapid add-and- measure procedure, confirming the suitability of the smartphone biosensing platform for analysis in just 2 hrs at room temperature of untreated complex biological matrices.

An important issue of these smartphone-based platforms that use mammalian cells as sensing system, is related to their scarce robustness and low stability. To further improve predictivity and robustness of ready-to-use cartridges, 3D-cell culture models were also explored. In the last years, 3D cell culture models have garnered great interest due to their capability to mimic *in vivo* cellular responses to external stimuli. To obtain a portable, predictive, sensitive and robust low-cost 3D cell biosensor, a smartphone-based bioluminescent 3D cell biosensor platform for effect-based analysis was developed [14]. This biosensor exploited the Nuclear Factor-kappa B (NF-kB) signal transduction pathway, which is involved in the regulation of cell-cycle/growth, inflammation, apoptosis, and immunity and is induced by several types of stressors. Immobilized Hek293 spheroid genetically modified to express powerful red- and green-emitting luciferases were exploited as inflammation and viability reporters. The smartphone-based BL 3D cell biosensor provides a LOD for Tumor Necrosis Factor (TNFα) of 0.15 ± 0.05 ng/mL and could represent a useful tool for preliminary on-site environmental samples screening.

Cell-Free bioluminescence biosensors

Cell-free biosensors, generally relying on enzymes or multi-enzyme complexes, have been the first biosensors to be developed. Their use considerably increased selectivity, stability, durability, and efficiency of biochemical transformations, improving cost-effectiveness and significantly extending the range of practical biotechnological applications.

Several examples can be listed since the pioneering works of Leland C. Clark, Jr in 1956, Leland Clark in 1962 and urea biosensors by Guilbault and Montalvo, Jr, in 1969 [92-95].

The easier tunability of enzymes, combined with higher stability in immobilizing systems quickly led to the implementation of these systems into commercially available devices for both laboratory and on-field applications [96-98].

Bioluminescent based cell-free biosensors, compared to others, have many advantages such as lower limit of detection, faster response time, higher catalytic activity, safer procedure and higher signal to noise ratio even in complex matrixes like environmental and biological samples [99].

Bioluminescent proteins can be also used for identification and classification of many analytes. The most exploited ones are activators/inhibitors involved in the mechanism of BL signal productions (e.g. ATP, NADH and Ca^{2+}) [100-101]. Protein engineering has been implemented over the years to increase protein affinity to target compounds or increase catalytic activity or temperature and pH stability [82].

The nicotinamide adenine dinucleotide (NADH) is an important cofactor of many physiological and biochemical processes. Therefore, it can be used as biomarker for the activity of several cell pathways and for metabolite identification. BL detection of NADH has been performed coupling bacterial luciferase (lux) with NADH-dependent dehydrogenases enzyme in different point-of-care biosensors [99]. For example, Roda et al., combined three enzymes (bacterial luciferase, NADH/FMN oxidoreductase and 3a-steroid dehydrogenase) on cellulose support, for identification of bile acids with a smartphone-based device. This biosensor showed a linear response between 0.5−100 μmol/L

of 3α-hydroxy bile acid, a limit of detection of 0.5 μmol/L, within 3 min, demonstrating the suitability for detection of analytes present at medium-high concentration levels [102].

Adenosine triphosphate (ATP) is the energy carrying molecule of all known living systems. ATP concentration is therefore directly correlated to the amount of microorganisms present in a sample and highly important in several fields such as food contaminants detection, environmental monitoring and sterility assurance for air and surfaces [103-105].

The first implementation of this approach was introduced by Moyer et al. and subsequently by Corbitt et al. thanks to the fast response time (10 sec) and a wide/proportional detection range (from 0 to 9999 RLUs), making it a suitable tool for on-site rapid detection [106-107].

Firefly luciferase, luciferin and all necessary cofactor (except ATP) for BL signal production have been immobilized with different techniques to obtain ready-to-use disposable cartridges. For example, Luo et al., developed a microfluidic disposable cartridge with an additional component for sample collection, lysing and extraction. In just 5 minutes all intracellular ATP is released, drops into the microtube and reacts with the enzyme producing a bioluminescence linearly related to bacterial concentration in the sample. Different bacteria were tested (i.e. *E. coli* and *S. aureus*) showing a linear response in the concentration range from 10^3 to 10^8 CFU/ml and CV of 5.92%, using a compact PMT module (Hamamatsu) as portable signal detector and a dark chamber to isolate external light [108].

One of the limiting steps of this technique is the requirement for efficient and user-friendly technique to extract ATP from samples. He et al. developed a fast (5 min) and instrument-free extraction technique using trichloroacetic acid (TCA) as extraction reagent. They were able to increase the dynamic range (10 - 10^7 CFU/ml) and a lower limit of detection, 10 CFU/ml. These increased performances were also obtained thanks to the integration of the flow injection unit with a portable PMT detector [109].

Another important step for bacterial contamination detection in complex matrices is the separation from contaminants and the selective identification of cell types. Unspecific bacterial cell identification is useful for environment monitoring, but useless for health monitoring, since

nonpathogen bacteria cannot be distinguished from pathogen ones, such as *Mycoplasma*, *Chlamydia*, *Group B haemolytic streptococci* (GBS) and *Neisseria gonorrhoeae,* whose detection is a priority, especially in low resource settings. To this end, several portable, user-friendly, low cost and species-specific separation methods have been developed over the years, to isolate bacterial cells from complex matrices. Ngamsom et al. implemented bioluminescent biosensors for ATP detection with a very promising technique for isolation and separation of specific cells, IFAST. IFAST is a microfluidic technique that allows analytes specific isolation and purification thanks to affinity magnetic separation [110-112]. Functionalized magnetic nanoparticles have been used to selectively separate pathogenic bacteria cell from the sample using immiscible liquids spaces (i.e. aqueous sample, olive oil and phosphate buffered saline solution for BL emission). Polydimethylsiloxane (PDMS) and soft lithography were used for cartridge design and dark box, while a photomultiplier was used for signal acquisition (Fig. 3). This method enabled the identification of ATP levels down to 10^{-11} M, corresponding to 10^5 CFU/mL up to 2.3×10^2 CFU/mL of GBS in urine samples, in less than 20 min. A linear response between BL signal and cell concentration was obtained with all cell types [113].

A similar approach was reported used by Chen et al., who combined BL readout with magnetic particles in a double double-enzymes-mediated sensor. They combined alkaline phosphatase (ALP) with luciferase and in the presence of target Ab1−MNPs and Ab2− PS−ALP to form the sandwich which can be magnetically separated into a one step-reaction, decreasing reaction time (less than 1h) and avoiding multiple washing steps of conventional immunoassays. The BL reaction is closely related to the concentration of ATP. The formation of the immunocomplex in the presence of the target analyte causes a decreased BL signal due to ATP dephosphorylation by ALP, that is directly correlated with the biomarker concentration. A limit of detection of 0.045 pg/mL (~3.5 fM) was reached using a portable light detector, compared to 8.4 pg/mL for conventional ELISA [114].

More recently Calabretta et al, integrated an ATP biosensor into a paper-based format for a more economical and environment friendly device. A new lyophilized procedure was optimized to improve

bioluminescent signal emission and long-term stability. A more user-friendly approach was achieved thanks to the integration with smartphone for signal acquisition. The analytical performances of several portable detectors were also compared. In particular two smartphones (OnePlus 5 and Samsung Galaxy S7) and the CCD portable camera ATIK 383L enabled to identify up to 3.8 x 10^{-14} mol, 2.1 x 10^{-12} mol and 1.7 x 10^{-13} mol of ATP, respectively. The best limit of detection was achieved with OnePlus 5 smartphone, being just two orders of magnitude higher than that obtained with a benchtop luminometer (10^{-15} mol of ATP), which an excellent result for a portable, low cost and user-friendly device [26].

The implementation of smartphone camera as signal detector is one of the most important improvement of *point-of-care* application in recent years. Many smartphone cameras are, up to now, able to detect low-light BL emission. Thanks to the implementation of more sophisticate equipment and dedicated smartphone App for long-time exposure, customized algorithms have been also developed, leading to random noise reduction and increase sensitivity [70, 115].

Similar responses were obtained by Santangelo et al., who implemented an ATP biosensor with a SiPM as a detector, a 3D printed microfluidic chip and a computer for data acquisition and elaboration [104].

Protein and cofactor directly involved in bioluminescence processes (i.e. ATP, NADH) are not the only detecable targets with cell-free BL biosensors. Thanks to genetic modification of luciferases and chemical modification of luciferin structure, a wider spectrum of analytes has been identified. Noda et al, implemented a luciferin-modified peptide (benzoyl-Leu-Gly-Arg-aminoluciferin) for selective identification of lipopolysaccharide, an endotoxin component of gram-negative bacteria cell wall. In the presence of the toxin, the modified luciferin with the cleavage point of coagulogen was cut and the BL signal produced. Using a portable luminometer (C-110 from Kikkoman Corporation) they were able to detect toxin concentration from 0.1 EU/ml to 0.0005 EU/ml, with 15 min of incubation time. Using BL detection they were able to increase the sensitivity up to 200-fold compared to the standard endpoint chromogenic method [116].

A different modification was implemented by Arts et al. for the identification of specific antibodies. They modified the luciferase enzyme NanoLuc with a semiflexible linker containing a specific epitope sequence and the fluorescent protein mNeonGreen. In absence of the specific antibody, the linker maintains the two proteins in closer proximity, resulting in an efficient energy transfer (BRET), with a green light emission. In presence of target antibody, the structure and interaction between donor and acceptor is destroyed leading to color change from green to blue. This biosensor called LUMABS, implemented a smartphone as detector, without the necessity of sample pre-treatment. In 30 minutes, it can detect anti-HIV 1p17 antibody from 5 nM to 10 pM. This approach is also easily tunable by changing epitope sequences, bioluminescent proteins and acceptor proteins [117].

The versatility of this approach has been exploited by Griss et al. [118]. The authors implemented an intermolecular BRET system for the identification of drugs, namely LUCID (luciferase-based indicators of drugs). The BRET system is composed by the fusion protein SNAP-Pro30-NanoLuc (NLuc)-cpDHFR linked to a synthetic molecule containing a fluorophore Cy3 and a dihydrofolate reductase (DHFR) inhibitor. In the presence of the target analyte the sensor switches to an open conformation, reducing BRET efficiency. The use of a portable and low-cost digital camera (costing <$100), allowed the detection of methotrexate concentrations from 100 nM to 1.5 mM, covering the therapy range. To demonstrate the versatility of this approach other drugs (tacrolimus, sirolimus, cyclosporin A, topiramate and cardiac glycoside digoxin) were tested, showing good results. This approach also showed a simplified readout and increased sensitivity compared to previously developed methods based on FRET.

The high performance of BRET approaches enabled the integration with other methods for the identification of analytes present at really low concentrations. Li et al, implemented a BRET technique with isothermal amplification to identify and quantify circulating microRNAs (miRNAs) in serum. The authors developed a two-stage paper-based system composed by an amplification paper disc and a BRET sensing paper disc (Fig. 4). The BRET detection was performed with Zinc Finger Protein (ZFP)-fused donors (NanoLuc luciferase) and acceptors (mNeonGreen) with base pairing

specific binding. This paper-based system coupled with a smartphone-based device for signal acquisition was able to detect femtomolar concentrations of all miRNAs tested. In particular, miRNA let-7, miR-500, miR-21, and miR-155 were selected being important biomarkers for different tumors and limits of detection of 1.7 fM, 2.5 fM, 0.8 fM, and 1.3 fM were reported, respectively. Showing results at least 3 orders of magnitude lower than previous methods [119]. A different approach for specific DNA sequencing based on BL detection has been developed by Song et al for on-field detection and monitoring of genetically modified organisms (GMO). In particular, eight genes for modified maize and soya were identified as target for inexpensive and reliable farm test using a small size, portable CCD detector. The pyrosequencing was performed into a small array format (6 W × 2H × 8D mm) and dNTPs dispensation and temperature control (28–30 °C) were performed using the smartphone battery. BL detection provided a remarkable limit of detection of 0.01 % with 35 PCR cycles [120].

As concerns protein biomarker detection in blood, increased performance was obtained by Lui et al, with the development of magnetic BL nanoliposomes (LBM). Liposome doped ATP were coated with antibodies for biomarker detection. Simultaneously, the same target with different monoclonal antibody is bonded with magnet nanoparticle to create a grip for analyte concentration and separation. The ATP is released and BL signal proportional to biomarker concentration is produced. Alphafetoprotein (AFP) was selected as biomarker for hepatocellular carcinoma and using a portable light detector high sensitivity (0.016 ng/mL), wide linear range (0.05-1000 ng/mL) and good specificity were achieved. No interference was reported with other serum proteins and the results obtained were in good agreement with those determined by electrochemiluminescence (relative deviations <10%) [121].

Despite cell-free biosensors are characterized by several advantages, their applications are limited to the identification of compounds without any information about their effects on whole-cell physiology. Thereby, no effect-based information and no data about toxic effects are provided. To overcome this limitation new cell-free systems are being developed, i.e. transcriptional and translational (TX-TL)

cell-free systems. This new approach is based on crude whole-cell extract with all intracellular components (i.e. proteins, enzymes, co-factors and energy sources) in a “cell-free environment” (without genomic DNA and cell membrane). Therefore, it combines the advantages of cell-free systems (practicality, stability and safety) with the ones of whole-cell (predictivity, signal amplification and comprehensive information). Biosensors are recently being developed with this approach proving their potential for both laboratory and on-field applications. Though, as we reported recently, more studies need to be made in this field as their analytical performance cannot compete with the one obtained with whole-cell biosensors. By comparing bioluminescent TX-TL cell-free with whole-cell bioluminescent biosensors for mercury detection in water, the limit of detection was 5 orders of magnitude higher [122]. Recently a new approach was described based on a new chemo-enzymatic cascade that can be used for the de novo synthesis of d-luciferin and, when combined to firefly luciferase, to detect nitro- and halogenated phenols at the ppb level [123]. This method was able to measure target analytes directly in complex biological matrices, without requiring any pre-treatment, thus being very promising for future implementation into portable biosensing platforms.

Conclusions

Thanks to advancements in synthetic biology, organic chemistry and computational models, today bioluminescence is widely exploited in several fields, ranging from the detection of microbial contamination to cell-based assays. Biosensors based on living cells and cell-free systems have been reported and implemented in different portable analytical formats, generally being living cells more advantageous in terms of significance of obtained information. Different levels of complexity can be envisaged according to the required application, starting from single enzymes, enzymes complexes, cell-free TX-TL systems, bacteria, yeast, and human cells lines. Cells undoubtedly provide highly valuable information about the bioavailability of target analyte and, exploiting the intracellular signalling pathways, it is possible to obtain information about the actual biological activity of a

sample. The current challenge is to move from traditional benchtop BL assays to BL biosensors where the cells or the biorecognition elements are integrated into portable biosensing devices.

Thanks to the high maturity level of reporter gene technology and the availability of several bioluminescent proteins with improved features, several approaches were reported, also relying on low-cost technology such as 3D printing and the use of smartphone as light detector. However we are still far from seeing a true exploitation and commercialization of these promising technologies and often these proof-of-principle applications have still limitations, such as those related to the limited shelf-life of cells, or cost for reagents that hamper their commercialization. Indeed the stability of the cells and other biological entities integrated into the portable system represent the limiting factor and we are still far from achieving analytical systems having performance, storability, robustness, and ease-of-use comparable to those of lateral flow immunoassays. In addition, high costs of substrates could hamper commercialization of disposable systems, however self-luminescent systems or the selection of some cheaper substrate alternatives [31] could easily bypass this issue and pave the way towards full exploitation.

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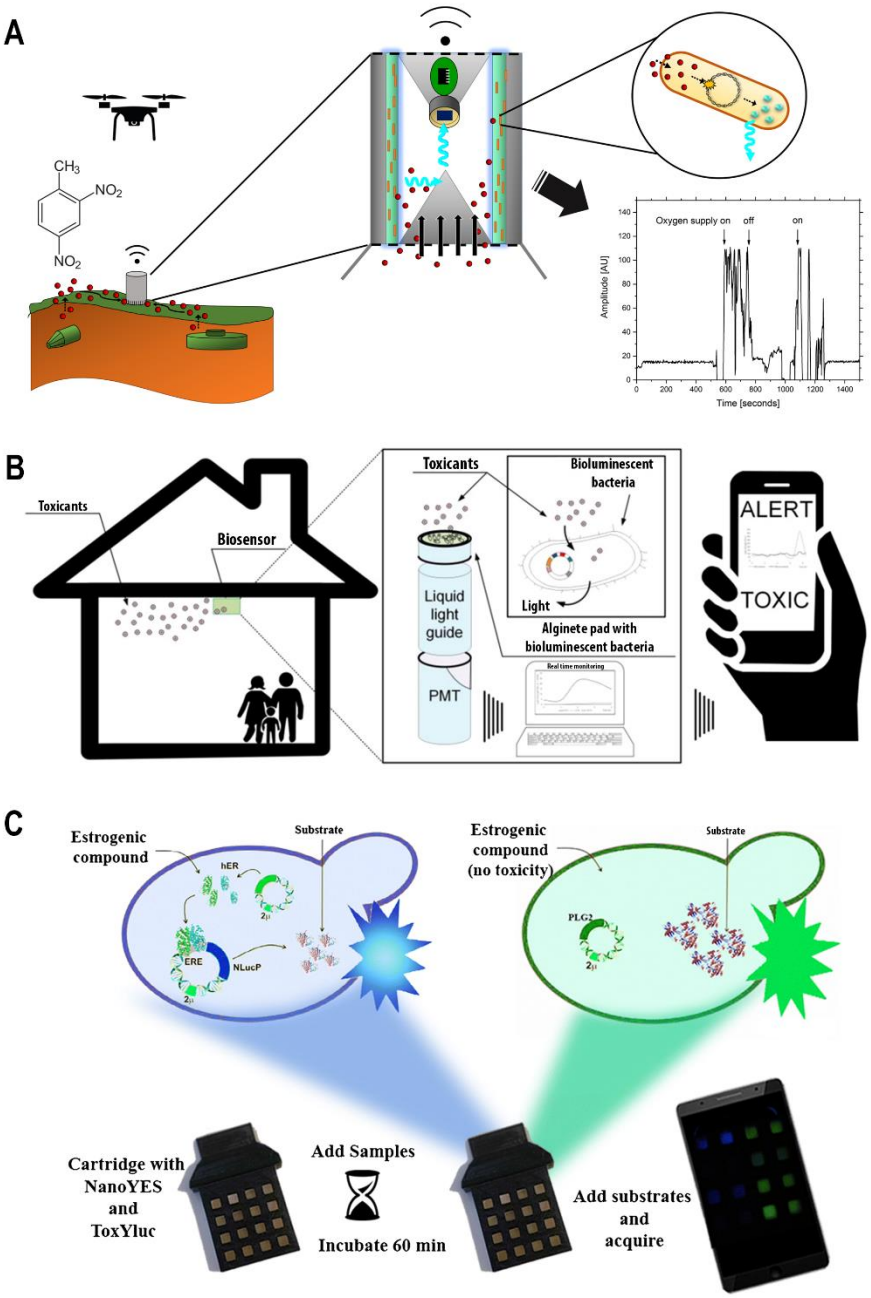


Fig.1 Portable microbial whole-cell bioluminescent biosensor and their applications.

A) Recombinant bioluminescent bacteria cells for sensitive detection of 2,4-dinitrotoluene implemented into a cost effective device for on-site wireless monitoring (adapted with permission from Ref. 60, Creative Common CC BY license, MDPI) **B)** A disposable pad with immobilized bioluminescent bacteria cell exploited for cheap and direct indoor detection of air pollution and toxicity (adapted with permission from Ref. 62, Copyright (2020) American Chemical Society) **C)** On-site user-friendly water monitoring relying on bioluminescent yeast cells integrated into a

disposable cartridge with smartphone interface (adapted with permission from Ref. 81, Springer Nature, license number 4860781493175.)


A
open
Ready to use cartridge
close
Sample addition
Procedure
1) Assemble the smartphone adaptor
2) Add sample to T1-T2 wells
3) Incubate 30 min
4) Add BL substrate
5) Insert cartridge into the adaptor
6) Acquire BL image & Read result
Home
Begin
BL substrate from reservoir
BL substrate addition
Toxicity Test
Sentinel Cells
Smartphone analysis
with dedicated APP

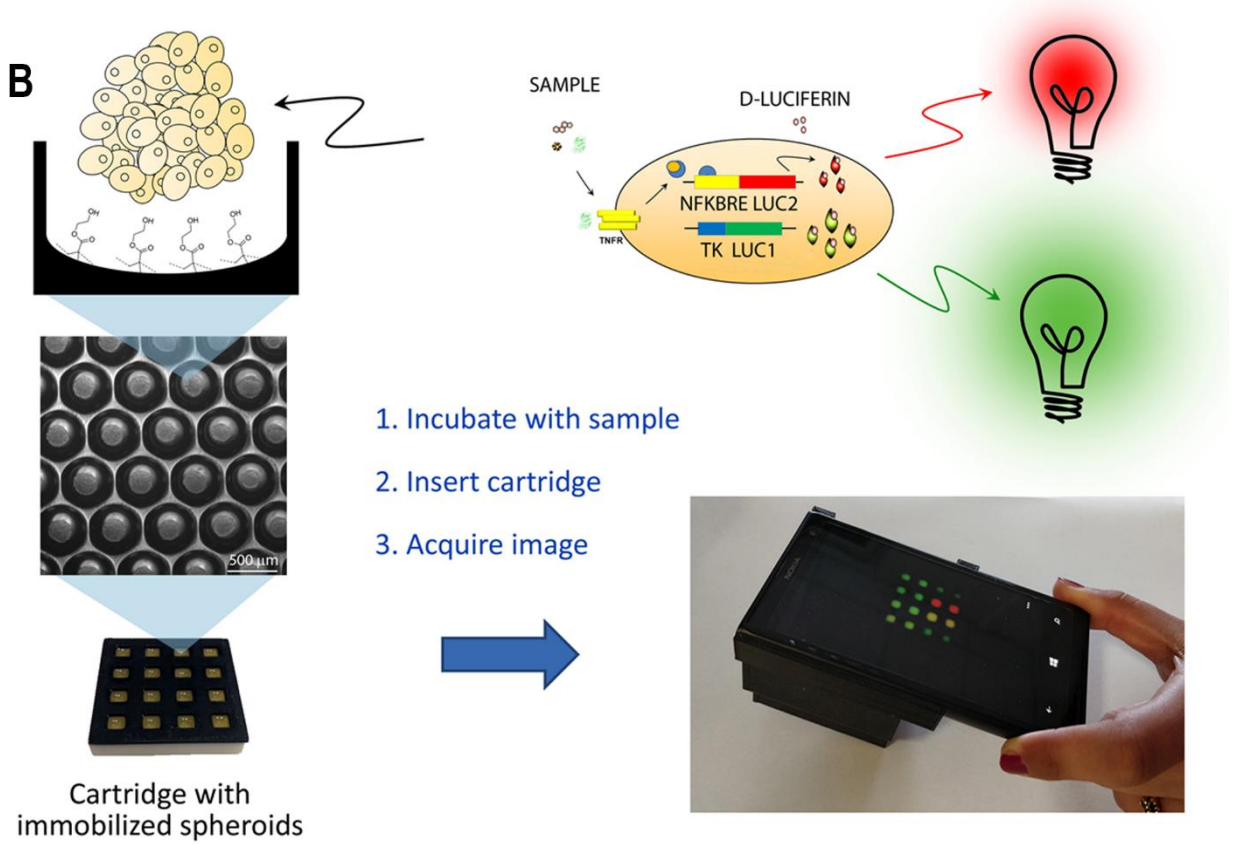
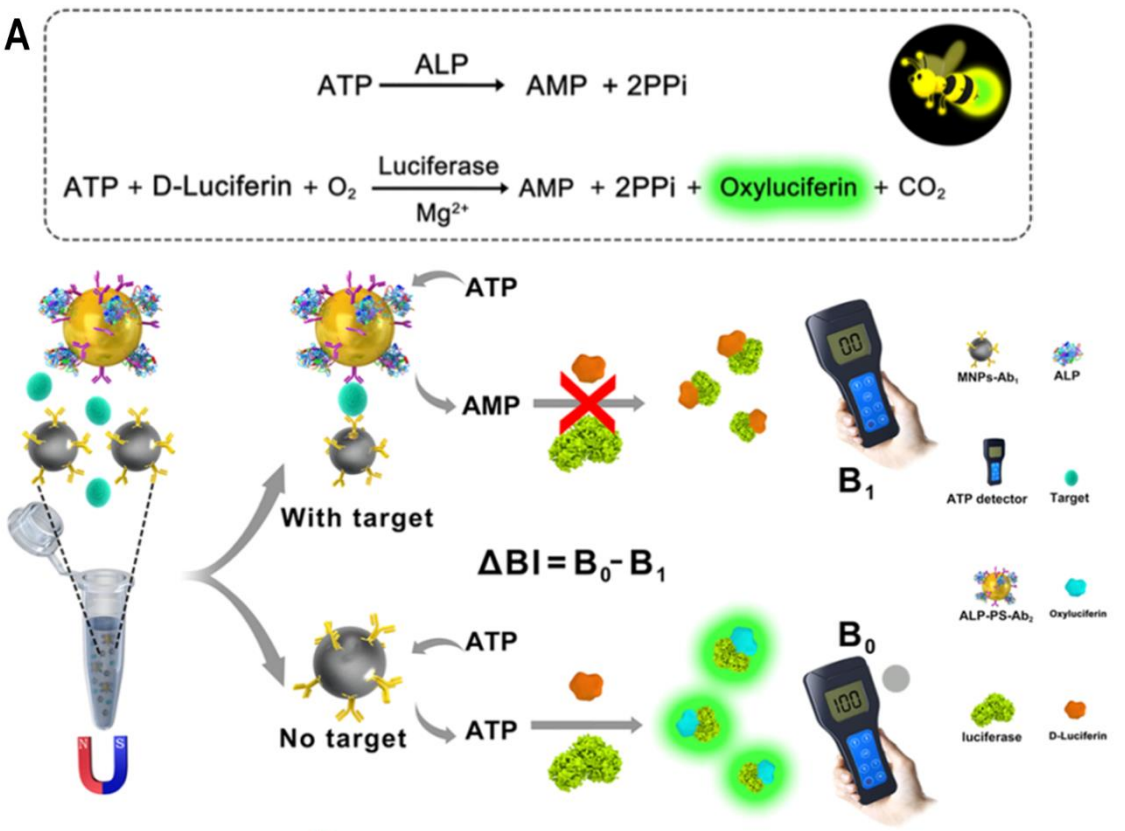
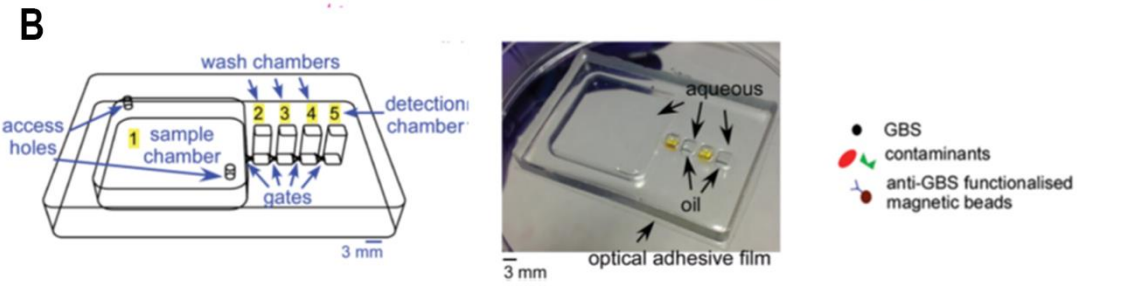
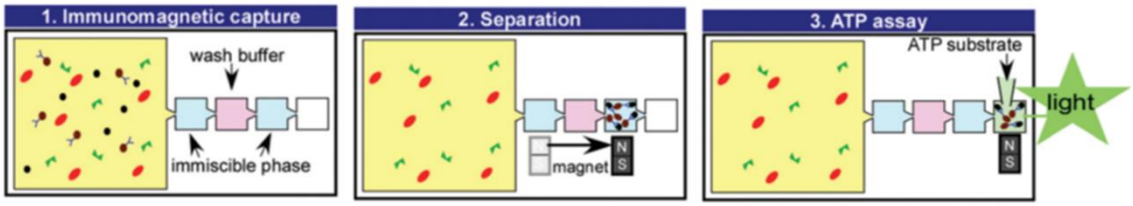

B
SAMPLE
D-LUCIFERIN
NFKBRE LUC2
TK LUC1
TNFR
500 µm
1. Incubate with sample
2. Insert cartridge
3. Acquire image
Cartridge with
immobilized spheroids

Fig.2 Portable bioluminescent biosensors implementing immobilized mammalian cells expressing red- and green emitting luciferases.

A) Schematic representation of steps required for sample toxicity evaluation with mammalian cell biosensors ("sentinel cell") integrated into a portable device with smartphone detection and dedicated App for data analyses (adapted with permission from Ref. 29, Elsevier, license number 4860791030378). **B)** Detection principle of portable biosensors based on 3D cell culture of mammalian cell for inflammatory activity detection (adapted with permission from Ref. 14, Elsevier, license number 4860790371342).


A
ATP
ALP
AMP + 2PPi
ATP + D-Luciferin + O2
Luciferase
Mg2+
AMP + 2PPi + Oxyluciferin + CO2
ATP
AMP
With target
No target
ATP
ATP
ΔBI = B0 - B1
B1
B0
MNPs-Ab1
ALP
ATP detector
Target
ALP-PS-Ab2
Oxyluciferin
luciferase
D-Luciferin


B
wash chambers
access holes
1 sample chamber
2 3 4 5
detection chamber
gates
3 mm
aqueous
oil
optical adhesive film
3 mm
GBS
contaminants
anti-GBS functionalised magnetic beads


1. Immunomagnetic capture
wash buffer
immiscible phase
2. Separation
magnet
3. ATP assay
ATP substrate
light

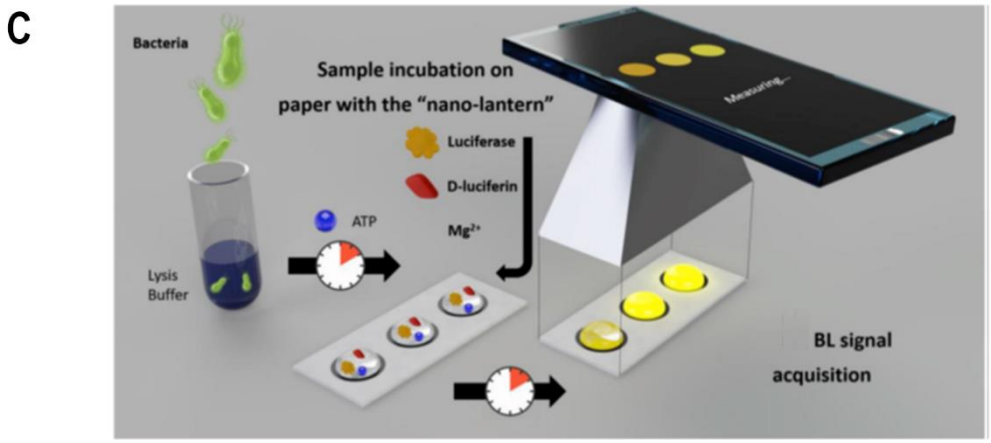
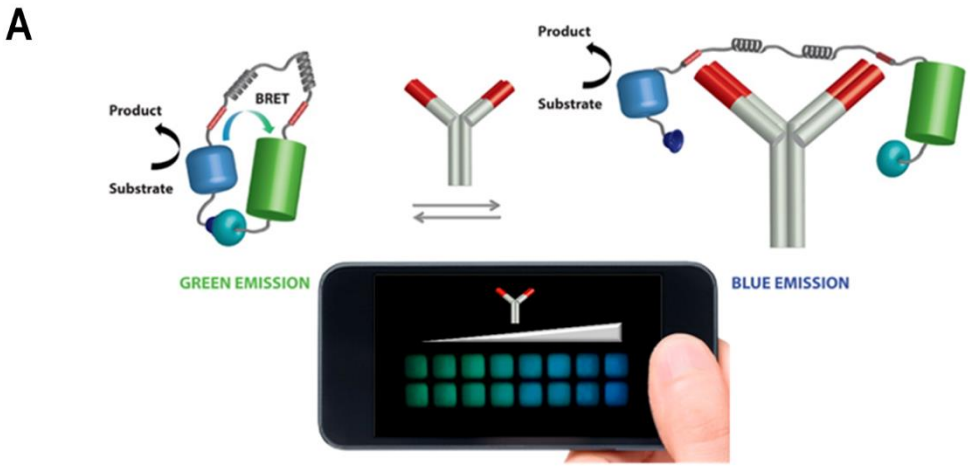
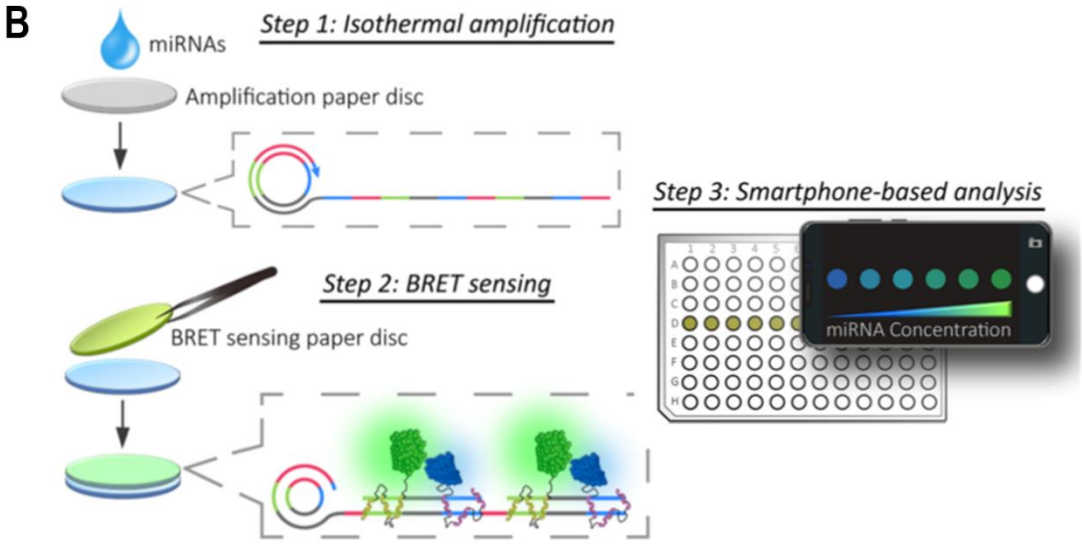

C
Bacteria
Sample incubation on paper with the "nano-lantern"
Luciferase
D-luciferin
Mg2+
ATP
Lysis Buffer
Measuring...
BL signal acquisition

Fig.3 Schematic representations of portable bioluminescent biosensors for ATP detection through firefly luciferase.

A) Scheme of double-enzymes-mediated biosensor exploiting magnetic enrichment of PS−target−MNPs immune-nanocomplex of ALP for ATP degradation to AMP and inhibition of bioluminescent reaction (Adapted with permission from Ref. 114, Copyright 2020. American Chemical Society). **B)** Microfluidic scheme and operating steps for portable bioluminescent detection of GBS by IFAST/ATP assay (Adapted from Ref. 113 with permission from The Royal Society of Chemistry). **C)** Illustration of paper-based bacterial contamination detection in urine sample via smartphone (Adapted with permission from Ref. 26, Elsevier, license number 4837140313120).


A
Product
BRET
Substrate
Product
Substrate
GREEN EMISSION
BLUE EMISSION


B
miRNAs
Step 1: Isothermal amplification
Amplification paper disc
Step 3: Smartphone-based analysis
Step 2: BRET sensing
BRET sensing paper disc
miRNA Concentration

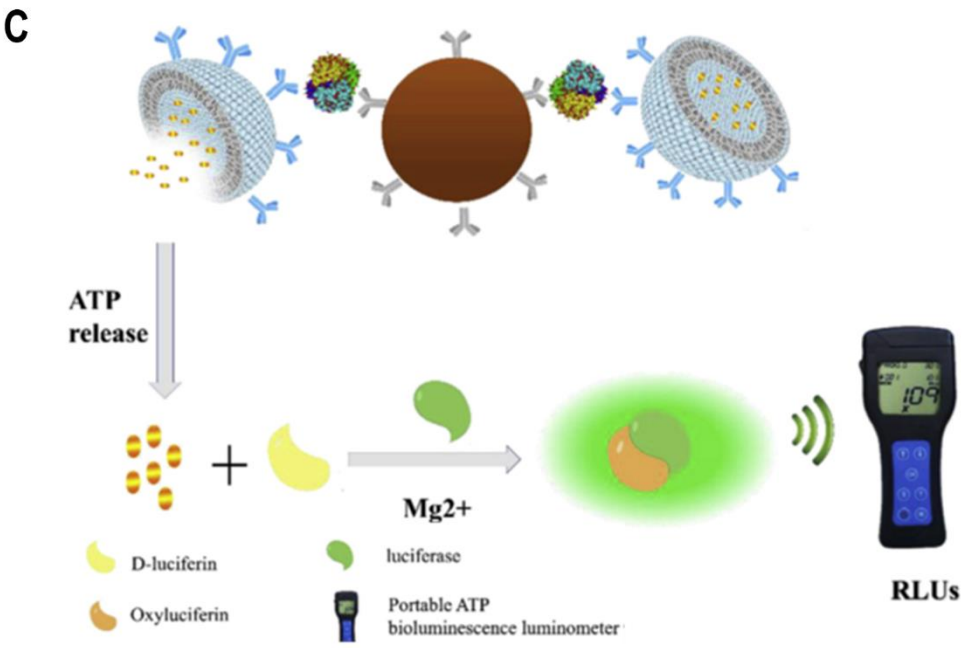

C
ATP
release
Mg2+
D-luciferin
luciferase
Oxyluciferin
Portable ATP
bioluminescence luminometer
RLUs

Fig. 4 Cell-free bioluminescent biosensors for portable biomarker monitoring in blood samples. **A)** Detection principle of smartphone-based BRET cell-free biosensor for antibody detection, using a NanoLuc-mNeonGreen fusion protein (adapted with permission from Ref. 117, Copyright (2020) American Chemical Society). **B)** Illustration of BRET (NanoLuc-mNeonGreen) miRNA detection through on-paper isothermal amplification and specific double-stranded ZFP binding sites (adapted with permission from Ref. 119, Copyright (2020). American Chemical Society) **C)** Schematic representation of magnetic-bioluminescent-nanoliposome complex for ultrasensitive and selective isolation and detection of protein biomarkers (adapted with permission from Ref.121, Elsevier, licence number 4837150418201).

Table 1: Recent examples of microbial bioluminescent biosensors integrated into portable systems relying on different light detectors.

Strain/cell-free system description	LOD and assay time	Reporter protein	Portable light detector	Reference
E. coli for general toxicity	10^{-12} M (toluene)	luxCDABE	Photomultiplier tube (Hamamatsu)	[63]
E. coli for general toxicity	1×10^{-8} M (formaldehyde)	lux*CDABE*	CMOS photodetector-ULS	[64]
Magnetospirillum gryphiswaldense for general toxicity	n.a., 30 min	Click beetle luciferase (CBR)	MZ-2PRO CCD	[57]
Pseudomonas putida for general toxicity	n.a.	luxCDABE	Photon counter Perkin-Elmer	[83]
E. coli for *inorganic mercury (Hg2+)*	0.25 µg/L, 60 min	luxCDABE	Silicon photomultiplier	[68]
E. coli for toluene	0.2%, 16 h	luxCDABE	Luminometer with fiber-optic probe (Turner Design)	[67]
E. coli for *antibiotics*	10 ppb (ciprofloxacin), 90 min	luxCDABE	Portable CCD	[84]
E. coli for gaseous 2,4-dinitrotoluene	50 ppb (180min)	luxCDABE	Photodiode (Luna Optoelectronics)	[60]
E. coli for fluoroquinolone antibiotic	7.2 ng/mL (ciprofloxacin), 120 min	luxCDABE	iPhone SE smartphone	[59]
Photobacterium phosphoreum, for Biochemical Oxygen Demand (BOD)	16 ppm, 20 min	luxCDABE	Fuji film Fine Pix S602 camera	[53]
E. coli sensor array for toxicant detection	15 to 45 minutes	luxCDABE	Portable CCD	[58]
S. cerevisiae for estrogens via hERα	0.08 nM E_2, 60 min	Yeast codon-optimized NanoLuc-PEST luciferase	Portable camera (GoPro HERO 5)	[27]
S. cerevisiae for estrogens via hERβ	0.38 nM E_2, 30 min	Yeast codon-optimized NanoLuc-PEST luciferase	OnePlus 5 smartphone	[81]