Portable light detectors for bioluminescence biosensing applications: A comprehensive review from the analytical chemist's perspective

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Abstract

Bioluminescence, that is the emission of light in living organisms, has been extensively explored and applied for diverse bioanalytical applications, spanning from molecular imaging to biosensing. The unprecedented technological evolution of portable light detectors opened new possibilities to implement bioluminescence detection into miniaturized devices. We are witnessing a number of applications, including DNA sequencing, reporter gene assays, DNA amplification for point-of care and point-of need analyses relying on BL. Several photon detectors are currently available for measuring low light emission, such as photomultiplier tubes (PMT), charge-coupled devices (CCD), complementary metal oxide semiconductors (CMOS), single photon avalanche diodes (SPADs), silicon photomultipliers (SiPMs) and smartphone-integrated CMOS. Each technology has pros and cons and several issues, such as temperature dependence of the instrumental specific noise, the power supply, imaging capability and ease of integration, should be considered in the selection of the most appropriate detector for the selected BL application.

These issues will be critically discussed from the perspective of the analytical chemist together with relevant examples from the literature with the goal of helping the reader in the selection and use of the most suitable detector for the selected application and to introduce non familiar readers into this exciting field.

Keywords: bioluminescence, portable light detector, biosensor, analytical device, smartphone

1. Introduction

The on-site monitoring of analytes of environmental, clinical, and forensic interest still represents a challenge and most of analyses are performed with sophisticated equipment and highly trained personnel, which renders real-time, cost-effective routine monitoring cumbersome, time-consuming and costly. Cutting-edge research of the last decade has provided excellent concepts and new molecular tools to obtain sensitive biosensors for rapid on site-analysis. Biosensors, defined as "devices that use specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals" according to the IUPAC definition provide an unbeatable arsenal of front-line tools for point-of-care and point-of-need analysis [1]. No doubt electrochemical detection, thanks to its peculiar advantages of sensitivity and portability, plays a major role in biosensing, with the glucose sensor being the archetype [2]. Optical detection also proved to be a highly sensitive detection principle, with the possibility of implementation into low-cost and miniaturized analytical devices, with several successful examples [3-6], mostly relying on fluorescence (FL) [7-9] and colorimetric detection [6, 10-11]. Due to size constraints of such devices, also the amounts of sample and reagents are reduced, thus decreasing the analytical signal generated by the biorecognition event. This requires additional efforts to improve the sensitivity.

Bioluminescence (BL), that is emission of light in living organisms, is appearing as a suitable alternative to the more widespread FL. The ability to emit photons without the need of photoexcitation renders BL highly appealing for the implementation into portable and miniaturized devices. BL provided a formidable manifold system relying on different luciferases and luciferin analogues with a wide range of applications spanning from molecular imaging to biosensor development [12-16]. Several portable light detectors were explored for measuring the light emitted by BL reactions including photomultiplier tubes (PMT), charge-coupled devices (CCD), complementary metal oxide

semiconductors (CMOS), single photon avalanche diodes (SPADs), silicon photomultipliers (SiPMs), and smartphone-integrated CMOS.

The selection of a detector to measure photons produced by BL reactions requires some considerations related to important issues, among others the intensity of the emitted light, the kinetics of photon emission, and the emission wavelength, the latter also connected to multiplexing possibility (Table 1). Although in many cases a unique recommendation could be misleading considering the high number of detector models that are now commercially available, guidelines are provided to guide the selection. For instance, the emission wavelength of the BL system should overlap with the detector sensitivity to enable maximum light collection. Ruggedness is also a key factor in on-site applications, and solid state sensors, such as photodiodes and SiPM, are one of the best choices.

In addition, the choice could be also related to the necessity of obtaining spatial information, and in this case imaging systems, such as CCD and CMOS, are required, or only collect as much light as possible, and light-collecting systems are thus employed including highly sensitive PMT and SiPM. As concerns imaging systems, additional constraints are present involving more complex optics to avoid distortions and loss of light collection efficiency.

All these issues will be critically discussed in this review together with relevant examples from the literature covering the last twenty years (2000-2021) with the goal of helping the reader in the choice and use of the most suitable detector for the selected application and to introduce non familiar readers into this promising field which has the potential to radically modify the way on-site analysis is today performed. Since the reader may be interested not only in BL detection but also in chemiluminescence and fluorescence detection, this review will also provide a brief glance on main issues related to the choice of detectors for chemiluminescence and fluorescence.

Device Type	Main features	Applications [Ref].
Photomultiplier (PMT)	• Excellent current amplification • Wide linear dynamic range (10^6) • Low dark current • Requirement of a high voltage stabilized d.c. and power supply • Sensitivity to magnetic fields	Detection of bioluminescent bacteria [49, 112]
Silicon Photomultiplier (SiPM)	• Small size • High quantum efficiency • Low energy requirements • Low bias voltage requirements • Insensitivity to magnetic fields • Rapid response time • Not damaged from light overexposure	Detection of purified luciferases [4, 113]
p-n photodiode	• Fast response time • Broad spectrum wavelength range • Low Price • Small size	Bioluminescence imaging [114-116]
Silicon p-i-n photodiode	• Faster response time • Broad spectrum wavelength range • Small size • Low noise	Bioluminescence detection for parallel diagnostic assays [117-118]
Charge Coupled Device (CCD)	• Very low dark currents • Large areas available for light capture • High pixel uniformity • Blooming effect	Detection of microbial bioluminescence for monitoring of antibiotic residues and water contaminants [91, 96, 119]
Complementary metal-oxide semiconductor (CMOS)	• Cheap manufacturing costs • Great energy efficiency • No blooming effect • Fast data throughput speeds	Reporter gene assays, intracellular ATP monitoring, DNA sequencing [74, 120-121]

Table 1: Features of different light detectors employed for BL measurements in portable devices

2. BL reporters and probes

The components of the optical system (light detector, lenses, filters…) should be designed based on the main features of the BL reporter, for instance emission spectrum, quantum efficiency, and emission kinetics. In the past decades several BL organisms have been object of extensive investigation to elucidate the molecular mechanism of BL reactions (Table 2).

BL reporter protein	Organism	Reporter gene	λmax (nm)	Substrate	Ref.
Bacterial luciferase	*Aliivibrio fischeri*	*Lux*	490	No (decanal)	[41]
Firefly luciferase	*Photinus pyralis*	*Fluc*	556	D-luciferin	[18-19]
Click Beetle luciferase	*Pyrophorus plagiophthalamus*	CBG99	537	D-luciferin	[22]
Click Beetle luciferase	*Pyrophorus plagiophthalamus*	CBR	613	D-luciferin	[22]
Red-emitting Luciola luciferase	*Luciola italica*	*Lit*	610	D-luciferin	[21]
Chimeric Luciferase	*N-terminal domain FLuc and C-terminal domain Lit*	*PLG2*	559	D-luciferin	[21]
Renilla luciferase	*Renilla reniformis*	*Rluc*	480	Coelenterazine	[27]
Metridia luciferase	*Metridia longa*	*MLuc*	480	Coelenterazine	[32]
Railroad-worm luciferase	*Phrixotrix hirtus*	*PxLuc*	542-630	D-luciferin	[23]

Gaussia luciferase	*Gaussia princeps*	*Gluc*	485	Coelenterazine	[28]
NanoLuc	*Oplophorus gracilirostris*	*NLuc*	460	Furimazine	[34-40]
nnLuz	*Neonothopanus nambi*	*nnLuz*	520	Hispidin	[43]

Table 2: Commercially available luciferases whose coding sequence is for reporter gene applications

As of today, there are more than 30 studied BL systems but only 11 luciferin-luciferase systems have been characterized [17] and found practical applications. Luciferase's features differ in terms of enzyme's size, emission wavelength, enzyme thermostability, optimal pH of the reaction, and the need for cofactors, such as ATP. All these factors, summarized in Table 3, should be taken into account in the selection of the luciferase according to the targeted application. Also the choice of the detector will depend, among other factors, on the selected BL reporter. For this choice the emission wavelength of the reporter is one of the first parameters to be taken into account and the spectral sensitivity, i.e. the detector efficiency as a function of the wavelength of the signal, should be either evaluated or checked in the detector's datasheet to guarantee an adequate efficiency at the wavelengths of the BL emission. Of note, the detector sensitivity varies depending on the models, therefore a recommendation for a detector type according to the emission wavelength cannot be provided.

Luciferase	Pros	Cons	Ref.
Bacterial luciferase	Autonomous BL system Improved mutants available also for expression in mammalian cells lines	Heterodimeric Low quantum yield	[41]

Firefly luciferase	Several mutants available with altered emission properties and improved pH and thermostability High quantum yield Can be used for ATP detection	Size of the protein Sensitivity to bivalent metal ions	[18-19]
Click Beetle luciferase	pH-insensitive Several mutants available with altered emission properties Can be used for ATP detection	Tendency for aggregation	[22]
Renilla luciferase	Temperature optimum 18-37°C	Low quantum yield	[27]
Gaussia luciferase	Small size High BL intensity High pH and thermostability	Contain up to five S-S bonds Rapid light decay	[28]
NanoLuc	Small size High BL intensity	Spectral emission profiles non optimal for in vivo imaging	[34-40]
nnLuz	Autonomous BL system Cheap and easy-to-produce luciferin	Membrane protein Low quantum yield	[43]

Table 3: Main features of commonly used luciferases

Firefly luciferase (FLuc) from North American firefly *Photinus pyralis* (*P. pyralis*) is the most studied and widely used luciferase for biological and biotechnological applications. This luciferase has a molecular weight of 61 kDa and catalyzes the oxidation of the substrate D-luciferin (LH_2) in the presence of adenosine-5′-triphosphate (ATP) and molecular oxygen (O_2), yielding emission of yellow-green light that peaks at 560 nm. In the past, several mutants of FLuc were obtained by random and site directed mutagenesis to shift the emission wavelength or improve pH and thermostability [18-19]. Other mutants, ΔFlucs, have been obtained showing higher activities and lower Michaelis-Menten constant (K_m) for D-luciferin and a chimeric luciferase PLG2 was developed by fusing the

N-terminal domain of FLuc and the C-terminal domain of *Luciola italica* luciferase to further improve thermal and pH stability with threefold higher activity than the original FLuc [20-21].

Different BL emission spectra ranging from yellow-green (540 nm) to orange (593 nm) characterize the luciferase reaction from the click beetle *Pyrophorus plagiophthalamus* and luciferases derived from the railroad worm, which emit light that ranges from yellow-green (542 nm) to red (630 nm) [22-23]. The simultaneous use of green- and a red-emitting luciferases with well separated emission spectra using the same D-luciferin substrate allows to monitor multiple molecular targets or signaling events, resulting in increased amount of information from the same cell/well and reduced assay cost/time [24-25]. Coelenterazine-luciferases systems are typical of BL marine organisms where the luciferin molecule is oxidized by O_2 under luciferase catalysis, forming coelenteramide as a product. The majority of these systems contain a natural signal peptide for secretion that allows BL measurements to be performed directly in the medium, without the need for lysing cells or tissues. This approach enables longitudinal analysis with repetitive measurements on the same sample [26]. Several natural coelenterazine–luciferase systems were isolated and Renilla, Gaussia and *Metridia longa* luciferases found broad applications in the past. Renilla luciferase (RLuc) from *Renilla reniformis,* with a molecular weight of 36 kDa and a BL peak at 480 nm, was among the first cloned luciferases. Currently, this luciferase has been widely exploited for bioimaging, it can be expressed virtually in all cell types and several mutants possessing increased stability, brightness and even red-shifted BL spectra have been obtained [27].

Gaussia luciferase (GLuc) from the marine copepod *Gaussia princeps* is a naturally secreted luciferase with a molecular weight of 19.9 kDa and flash-type BL emission at ~473 nm in the presence of coelenterazine substrate. GLuc represents an exceptional tool for non-invasive bioimaging with a BL signal ~100 times brighter than RLuc in mammalian cells under similar conditions and a suitable reporter for bioconjugation and bioanalytical applications [28-30]. However its flash-type emission,

reaching a peak within one second after substrate addition, requires benchtop instrumentation with on-board reagent injection. Red-emitting versions of the GLuc mutant variant have also been obtained exhibiting 10-fold greater intensity relative to the native luciferase and emitting red shifts of up to 33 nm with natural coelenterazine [31].

MLuc luciferase was isolated from the copepod *Metridia longa* and has a molecular weight of approximately 24 kDa [32]. BL catalysed by MLuc is approximately 10 times more stable compared to GLuc luciferase and for this reason it immediately found applications as a secreted reporter protein in *in vitro* and *in vivo* analytical assays.

Another interesting luciferase is from the deep-water shrimp *Oplophorus gracilirostris* (OLuc) with a total molecular weight of 106 kDa, with a structure of two identical subunits (19 kDa and 35 kDa), of which the structure with a mass of 19 kDa actively reacts with coelenterazine with a BL emission spectrum with a peak at 454 nm, showing high luminescence intensity and high quantum yield in comparison with the similar RLuc and photoprotein aequorin and broad substrate specificity [33]. In 2012 Promega developed a set of artificial NanoLuc luciferases characterized by small size, high brightness, high stability, utilizing a synthetic analogue of coelenterazine, furimazine, as substrate widely exploited in several fields [34-40]. Several portable whole-cell biosensors have been developed exploiting NanoLuc luciferase as reporter protein. The absence of post-translational modifications and disulphide bonds enable rapid synthesis and folding of the reporter reducing total assay time [3, 6, 34, 40].

Bacterial BL systems are significantly different from any other BL system, firstly because the bacterial luciferin (myristic aldehyde) is not a true source of light, although it is oxidized during the reaction and secondly, the essential components of the bacterial luciferin–luciferase reaction are nicotinamide adenine dinucleotide (NADH) and flavin mononucleotide (FMN). Bacterial luciferase has a molecular weight of 75 kDa and the photons emission requires a luxCDABE operon that

encodes a heterodimeric luciferase (luxA and luxB) and three biosynthetic enzymes (luxC, lucD and luxE) responsible for the long-chain aldehyde substrate production [41]. The BL system can glow without the addition of exogenous luciferin (blue emission at 490 nm) but is dependent of reduced riboflavin phosphate ($FMNH_2$) and long-chain aldehydes. In addition, the use of Lux as a common BL reporter in heterologous hosts is limited due to the potential toxicity of the long-chain aldehydes. To overcome these problems, improved mutants were obtained for increased BL emission in mammalian cell lines [42].

A completely new biochemical pathway has been recently studied involving nnLuz, responsible for fungal BL. This BL system employs a simple α-pyrone 3-hydroxyhispidin that is oxidised by an insoluble luciferase in a reaction that requires only oxygen. The result is the emission of green light (~520 nm). The nnLuz luciferase resulted to be functional in a variety of heterologous systems and showed similar performance to that of the firefly luciferase [43]. This system was also engineered in plants to obtain glowing plants which do not require the administration of substrates for BL emission [44].

3. BL detection with photomultiplier tubes

Thanks to their high sensitivity to low-light levels and high stability, PMTs are generally the first choice for detecting BL, which radiant flux is generally in the range of 10^4-10^8 photon counts per second [45]. PMTs, composed of a photocathode and an electron multiplier, rely on the external photoelectric effect, i.e., when photons hit a metal or semiconductor in vacuum, electrons are produced from its surface. After the photon hits the photocathode, an electron is emitted and accelerated by electric fields into a dynode, the collision generates new electrons which are in turn accelerated into other dynodes, with an electron gain of about 10^6. The gain is considered the number/

range of electrons emitted per incident photon and it is strictly determined by the number of dynodes, the photocathode sensitivity, power supply voltage, and tube design factors.

The range of optical wavelengths or frequencies in which the detector has a significant responsivity is defined as spectral response of a photodetector. The ultra-fast response and excellent timing performance as well as the high gain and low noise give to the PMT an exceptionally high gain x bandwidth product and makes them suitable for flash-type kinetics.

Since PMTs are characterized by a rapid response to a wide range of wavelengths, filters or monochromators are generally required to improve the sensitivity at specific wavelengths. PMTs are still the optimal choice for laboratory-based applications, however significant drawbacks limit their implementation into portable instruments, including the requirement for high voltages (higher than 1000 Volt) and intrinsic fragility (necessity for sealed vacuum tube) (Table 4). As shown in Table 4, the dark current, which can be expressed in current or in electron/pixel/second, varies significantly depending on the type of detector, the model and manufacturer. This is a critical parameter (the lower the better) for low light level applications in which long integration times are required; in these cases, the implementation of cooling systems can reduce the dark current caused by thermal generation of electrons.

The detector's rise time is defined by the time span required for the output signal to increase from a defined lower percentage to a defined higher percentage, usually from 10%, to 90%, of the final output level when a steady input is instantaneously applied. The rise time is a crucial parameter for photo-physical measurements, but its short timeframe does not affect general BL applications, both relying on flash or glow type emissions.

Device type	Dark current	Gain	Rise time (ns)	Suitable for:	Ref.

Photomultiplier (PMT)	10^{-3}-10^{3} nA	10^{6}	0.1-3	Intensity measurement Flash/glow emissions Weak signals	[49, 112]
Silicon Photomultiplier (SiPM)	90 kcps	10^{5}-10^{6}	10	Intensity measurement Flash/glow emissions Weak signals Rugged devices	[4, 113]
p-n photodiode	0.3 nA	1	$<10^{-3}$	Intensity measurement Flash/glow emissions Rugged devices	[114-116]
Silicon p-i-n photodiode	0.4-1.0 nA	1	10^{3}-10^{6}	Intensity measurement Flash/glow emissions Rugged devices	[117-118]
Charge Coupled Device (CCD)	10^{3} electron/pixel/sec	1	10^{3}-10^{5}	Imaging Glow emissions	[91, 96, 119]
Complementary metal-oxide semiconductor (CMOS)	0.1 electron/pixel/sec	10^{6}	10^{3}-$8x10^{3}$	Imaging Glow emissions	[74, 120-121]

Table 4: Comparison of different light detector parameters employed for BL measurements in portable devices

In a pioneering work, a biomonitoring device was built with recombinant *Escherichia coli* carrying the luciferase gene fused to TOL-Plasmid immobilized at the tip of a fiber optic and connected to a Hamamatsu C2310 PMT [46]. This system was proposed to detect benzene derivatives in drainages of chemical plants. Few years later, Polyak et al. developed a photon counting system implementing a Hamamatsu HC135-01 PMT sensor module optimized for the blue light region with an active area adequate for avoiding additional focusing elements [47]. This module was used, in combination with an optical fiber waveguide to measure BL bacteria, *Escherichia coli* carrying the recA promoter

region fused to the *Photorhabdus luminescens* luxCDABE reporter, used as genotoxicity sensors. By immobilizing BL bacteria within a polymeric matrix and placing them onto an optical fiber, the authors confirmed that light intensity, in response to a specific inducer (mitomycin C), was proportional to the number of bacterial layers. The use of optical fibers provided the additional advantage of enabling remote signal transmission and multiplexing capability.

Multiplexing was extended further with the fabrication of a field-operable photodetector device including a bundle of 22 optical fibers for measuring nine BL bacterial sensors to measure bioavailable metals in standard and real samples [48]. This device, characterized by a high level of integration of all components, still could not be defined as a miniaturized handheld device, since its size was 23 × 25 × 35 cm. A similar optical device, named Lumisens 2, was also reported for on-line detection of pollutants [49]. The Lumisens 2 comprises three parts: i) a fluidic layout, ii) the optoelectronic equipment including a Hamamatsu PMT connected with an optical fiber to iii) a disposable card with immobilized bacterial cells. A dedicated Labview® interface was developed to handle the measurements and it was able to detect the analyte (tributyltin) for 400 min with a continuous flow of sample (after 1 hour incubation with the analyte). The Lumisens 2 had a size (60 x 40 x 35 cm) more similar to a benchtop luminometer than a true biosensor, but with the non-negligible advantage of enabling on-line measurement, thus with future improvements it could be miniaturized to obtain a portable analytical device. At present the implementation of a PMT into a fully integrated miniaturized device integrating BL whole-cell biosensors has not yet reached the market, also due to power consumption and additional electronics required. On the other hand, adenosine triphosphate (ATP) monitoring devices based on luciferin-luciferase reaction are the gold standard for hygiene monitoring of hospital environments and for monitoring organic residues on food contact surfaces. At present, these handheld devices integrate either PMT (e.g., Clean-Trace™ NGi, novaLUM®) or photodiodes (EnSURE™, AccuPoint®, SystemSURE Plus) with similar

performance. A plausible explanation for this discrepancy derives from the difficulty of integrating the biological recognition element, i.e., the whole cell bioreporters, with a decent shelf-life and responsiveness, rather than technical issues related to the required sensitivity of the device.

4. BL detection with solid-state sensors

4.1 Photodiodes

Photodiodes are solid-state detectors composed of silicon p-n junctions that create a depletion region lacking mobile charge carriers. Once a photon is absorbed an electron lone-pair is generated; by applying a reverse bias an electric field is produced across the depletion region causing the charge carriers to move towards the cathode (electrons) or the anode (holes). Thus, in a reverse-biased photodiode the absorption of a photon will generate a flow of current [50]. A similar architecture is present in p-i-n photodiodes which are characterized by improved collection efficiency due to a lightly doped region at the pn-junction. Thanks to their low cost, ruggedness, easy miniaturization and possibility of implementation in arrays, photodiodes are very appealing for bioanalytical applications. In particular, especially thanks to their ruggedness, they represent the first choice for implementation into low-cost robust biosensing devices. Nevertheless, they lack an inherent gain mechanism, and therefore they are not as sensitive as PMTs or SiPMs; this is not an issue for most biological applications, but they are generally a non-viable option for BL typical low light intensities [51]. The use of photodiodes to detect low-light emissions was explored with a-Si:H photodiodes by Caputo and others who reported a remarkable detection of horseradish peroxidase with the luminol/peroxide/enhancer system at the attomole level [52].

4.2 Avalanche Photodiodes (APD) and SiPM

SiPM, thanks to its small size, negligible sensitivity to magnetic fields, low operating voltage (25-70 Volt), fast response time, and good Photon Detection Efficiency (PDE), represents a feasible alternative to conventional PMT [53-54].

After the pioneering work of Daniel et al. who reported the use of a single photon avalanche photodiode (SPAD) working in the Geiger mode to detect BL-based SOS toxin response of *Escherichia coli* bacteria, SiPMs have been proposed as sensitive light detectors for BL [55-57]. SiPMs are arrays of avalanche photodetectors connected in parallel in which each pixel operates in limited Geiger mode, under bias voltage of 10-20% higher than breakdown voltage [58].

Combining the robustness of avalanche photodiodes and unmatched photon number resolution capability with high quantum efficiency and wide sensitive areas, SiPMs replaced PMTs in a high number of applications [59-61]. Thanks to a very high gain (10^6 *vs* 100-200 of avalanche photodiodes) SiPM have a very low electronic noise. The main source of noise is the dark noise which is due to the thermal generation of carriers and to the effects of high electric fields. Like other sensors, a reduction of the SiPM dark rate occurs decreasing the temperature; therefore, in particular for large photosensitive areas (> 1 cm^2), the performance of SiPM is affected when measuring low-light levels [62]. In addition to high gain and sensitivity close to PMTs, SiPMs also have the advantages of low bias voltage, compactness, and mechanical robustness. Differently from APD, which operate in the linear region, SPAD work in the Geiger mode, and even single photons having very low energy (10^{-19} J) can induce both electron and hole impact ionization, whereas for APDs only electrons can produce secondary electron-hole pairs. Differently from SPAD, SiPMs can detect both single photons and several photons by superimposing the response of each SPAD element [56].

One of the first comparisons of PMT *vs* SiPM for BL detection was performed by Li et al. who reported that PMT provided the lowest limit of detection (LOD), while cooled SiPM exhibited a wider linear range using genetically engineered BL *Pseudomonas fluorescens* bioreporters [63]. After a

preliminary investigation of photon-counting performance using a simulated light emitting diode (LED) source, they measured BL emitted by a bacterial strain (*Pseudomonas fluorescens* harboring a plasmid with luxCDABE gene cassette from *Aliivibrio fischeri*) with a PMT with a photosensitive area of 0.5 cm^2 (H7467 series Hamamatsu) and a SiPM consisting of an array of 400 APDs each with cell size of 50×50 μm and photosensitive area of 0.01 cm^2. Interestingly, SiPM provided a uniform gain throughout the entire voltage scan while PMT showed a non-uniform linearity at higher light intensities (>2 x10^6 cps).

Very recently these solid-state sensors were also exploited in a low-cost customized set up for performing aequorin-based intracellular Ca^{2+} measurements [64]. This system showed performance similar to that obtained by a custom-designed PMT tube-based assay, thus supporting the potential use of SiPM devices for low-level BL detection for point-of-need applications. Recently, Jung et al compared the performance of a field-deployable SiPM device (3×3 mm^2 area with thermoelectric cooling of −10°C) with smartphones in detecting pathogens via BL phage reporters. Their result showed that SiPM provided better performance than smartphones in terms of time to detection and signal-to-noise ratio. A stable diode laser source (1 mW) was first used in combination with filters to simulate low-light BL emission and evaluate the photon counting performance of the SipM device, with a reported detectability of the incoming photon flux corresponding to 0.1 pW (3×10^5 photon/s). When applied to spiked food samples, i.e. ground beef samples inoculated with 5 and 50 cells, SiPM provided similar performance compared to a benchtop luminometer, enabling the detection of five and 50 cells in less than 12 and 10 h of incubation at 37°C, respectively. Detection with the smartphone required a 14 and 12 h incubation for samples inoculated with five and 50 cells, respectively [65]. This finding is consistent with a similar comparison reported by us in which the photon counting performance of SiPM device outperformed that obtained with smartphones [4].

Calabretta et al. reported an all-in-one device integrating a SiPM sensor with an active photosensitive area of 1.3x1.3 mm (Hamamatsu MPPC 13360-1325CS) interfaced with an ArduSiPM board and integrated into a 3D printed portable case (Fig. 1A). The performance of this device, called LuminoSiPM was compared with other light detectors comprising a benchtop luminometer, a portable CCD (ATIK 383L+), and a smartphone (Oneplus6). Remarkably, the LuminoSiPM outperformed both the portable CCD and the Oneplus smartphone, reaching a LOD of 1.7×10^{-10} M (8.7×10^{-16} moles) for NanoLuc with a linear range spanning three orders of magnitude [4]. The analytical performance in terms of detectability was also compared with a conventional benchtop luminometer, used as reference instrumentation, providing a LOD of 4×10^{-11} M (3.2×10^{-16} moles). The suitability of LuminoSiPM was also evaluated to detect the activity of AChE inhibitors in liquid samples. Exploiting chemiluminescence detection, an origami sensing paper based on the inhibition of AChE activity by molecules, such as OP pesticides, previously developed [5] was optimized and adapted to fit the LuminoSiPM device. In particular, the proposed origami sensing paper is based on enzyme inhibition exploiting three coupled enzymatic reactions relying on luminol/H_2O_2/HRP system and allows the measurement of AChE inhibitory activity using small volumes of samples and a very straightforward procedure (Fig. 1A). At the end the sample holder containing the origami clamped is then inserted in the LuminoSiPM dark box and closed with the other section of the dark box to acquire the signal.

As reported in this work, the selection of a light detector should be also related to the choice of the BL probe, and *viceversa*, in fact to achieve best results the BL emission should overlap with the maximum PDE of the sensor, which is a parameter that changes depending on the wavelength since the detector response depends on the wavelength of the incident photons. For characterizing the LuminoSiPM sensor two bright luciferases were chosen because their emission was fully covered by the quantum efficiency curve of the sensor, showing a maximum efficiency at 450 nm.

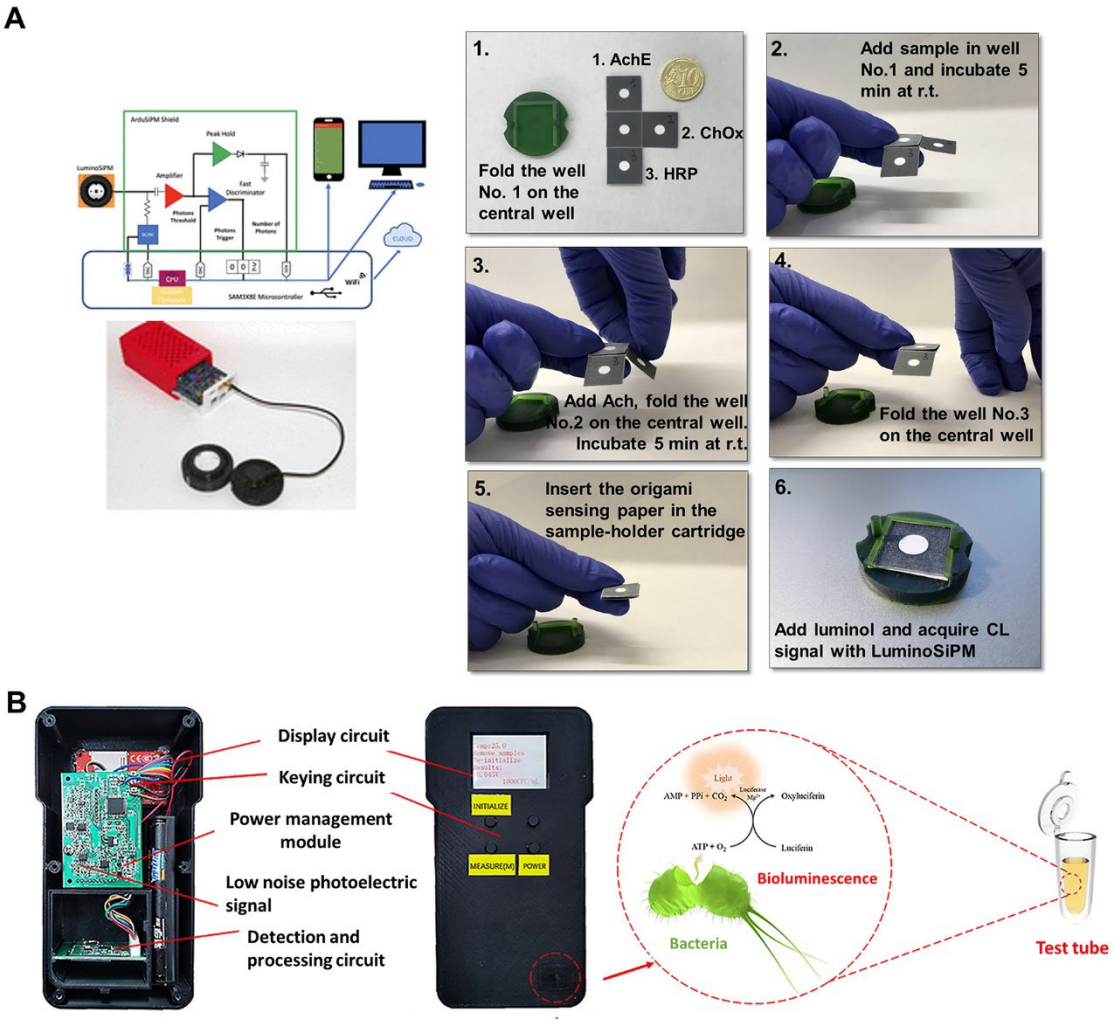

Figure 1: Low-Cost Silicon Photomultiplier Device (LuminoSiPM) for on-field luminescence detection connected to the Arduino DUE board and schematic representation of the assay. Adapted with permission from [4]. Copyright (2021) American Chemical Society; B) Low-cost portable bioluminescence detector based on a SiPM for on-site colony detection: the picture shows the device enclosure, full circuit schematic, circuit board design and the ATP-driven bioluminescence relying on the D-luciferin-luciferase reaction [66]. Copyright (2021) Elsevier B.V.

Very recently a portable BL detector for on-site colony detection based on SiPM was developed by Hu et al. (Fig. 1B). The proposed system adopts an approach relying on balanced chopper modulation and lock-in amplification to improve the signal-to-noise ratio, coupled with a zero-adjustment technique in order to eliminate the dark current of the SiPM and to expand the dynamic range from 1.0×10^3 to 1.0×10^9 CFU/mL [66].

5. BL imaging with CCD and CMOS

The high emission quantum yield of BL reporters combined with the low background noise enabled BL imaging of cells [25,67], tissues, living animals [68] and, more recently, also 3D cell models [69]. The development of more powerful CCD and CMOS systems has also enabled imaging at the single cell and sub cellular level. CCD and CMOS devices are both composed of semiconducting materials in which, when hit by photons, electron-hole pairs are generated with production of an electric charge processed into electronic signals. The main difference is in the reading of the generated charges. While in CCDs the charge produced in each pixel is transported through the chip to an analog-to-digital (A/D) converter, in CMOS sensors the conversion of charge-to-voltage occurs in each pixel, which also integrates transistors. In back-side illuminated CMOS sensors, as those integrated into last generation smartphones, the transistor is placed under the pixel, thus avoiding the noise generated by photons which may accidentally hit the transistor instead of the pixel. Thanks to the parallel output, generally CMOS sensors provide higher image acquisition speeds and reduced energy use. In the past decades, also due to simplified circuit complexity and low power requirements, all smartphones and end-user imaging products adopted CMOS-based sensors. Because of this commercial success and cheaper manufacturing compared to CCD, CMOS sensor performance witnessed a tremendous improvement, reaching those of highly sensitive CCDs in both technical and amatorial applications.

5.1 CMOS-based BL detection

Several field-deployable devices integrating BL whole-cell biosensors with advanced CMOS were reported [70-73]. In particular a CMOS micro luminometer was developed and optimized for detecting low-level BL as part of the BL bioreporter integrated circuit (BBIC), where immobilized bacteria were inserted into an integrated circuit in direct contact with CMOS photodetector. This approach resulted to be advantageous because the entire sensor, including all signal-processing and communication functions, was produced as a single-chip, low-power, rugged, inexpensive device [71]. Exploiting CMOS-based detection, Ma et al carried-out studies relying on immobilized BL bacteria for real-time detection and continuous monitoring of volatile organic compounds. The authors developed an immobilization matrix of calcium alginate that could be incorporated with a CMOS detector, improving the sensitivity and the uniformity of the luminescent bacteria [73].

As with CCDs, lens-free imaging with CMOS can provide significant advantages. A 0.18μm CMOS system-on-chip (SoC) was fabricated as lab-on-chip for luminometry. The peculiar feature of this system was the array pitch matching, i.e., 8x16 pixel array had the same pitch as the assay site array, which ensured reduced loss of sensitivity [74]. This device was validated with pyrosequencing, in which DNA sequencing relies on the detection of inorganic pyrophosphate (PPi) which is released during DNA synthesis. An ATP sulfurylase converts the released PPi into ATP, detected with the luciferin-luciferase system. The synthesized ATP provides the energy for luciferase to generate photons. Then, the enzymatic system is chemically reset through the degradation of unincorporated deoxy-nucleotides and ATP by the enzyme apyrase [74].

5.2 Smartphone-based BL detection

The internet of things (IoT), which integrates everything into network connectivity, is a trend that's been attracting interest from several research fields, with the smartphone being a key player.

The smartphone ubiquitous distribution and connectivity around the world is changing the concept of mobile health with the potential to reshape the biosensor market landscape [75-76]. Smartphones, thanks to their integrated camera, multifunction and geotagging capabilities, and computing power are a sort of evolution of point-of-care devices, providing the possibility to perform tests without any additional equipment, even in remote areas and low resource settings. Several efforts were focused on implementing BL assays into smartphone-based devices including enzymatic assays, whole-cell reporters and protein sensors [45, 77-78]. To use smartphone-integrated CMOS as BL detectors the first requirement is a mini dark box to shield from environmental light and the fabrication of a smartphone accessory to integrate cartridges or sensing papers hosting the bio-specific reactions. In most of the applications, this has been performed exploiting low-cost three-dimensional (3D) printing technology. For example, we recently reported a yeast estrogenic assay based on newly developed yeast biosensors expressing NanoLuc as reporter protein immobilized onto a 3D printed minicartridge and an adaptor to fit the smartphone. This biosensor was used to analyse water samples and complex samples showing limits of detection comparable to those obtained with standard yeast estrogenic bioassays (Fig. 2A) [3]. Arts et al. reported a new smartphone sensing platform, called LUMABS, based on bioluminescence resonance energy transfer (BRET) to detect antibodies in plasma [78]. The LUMABS concept relies on a NanoLuc, used as BRET donor, fused to a green fluorescent protein (mNeonGreen), used as BRET acceptor. Helper domains keep NanoLuc and mNeonGreen close together and the binding of a target antibody disrupts the interaction causing a drastic reduction of BRET signal. This decrease of BRET efficiency is detected with a smartphone via the change of the color of the emitted light, from green blue to blue, with limits of detection in the picomolar range.

The assay configuration and type of application of these two examples are very different, however in both cases NanoLuc was used as BL probe or reporter, this is not a coincidence; NanoLuc, thanks to its high quantum yield and stability, permits to achieve unparalleled sensitivity and this is vital when

using suboptimal portable light detectors [35]. In another work, the NanoBiT splitted variant of nanoLuc luciferase has been exploited in a turn-on system able to detect different circulating miRNA (miR-21. miR-148b, miR-3) via rolling circle amplification (RCA) combined with smartphone detection (Fig. 2B), thus reducing time and cost, with good agreement with standard laboratory methods reverse transcription polymerase chain reaction (qRT-PCR) [79].

In most of the applications in which BL is measured with smartphone either NanoLuc or other highly efficient luciferases, such as *P. pyralis* mutants and chimeric luciferases are used as BL reporters for whole-cell biosensors [24]. The use of two highly stable mutant green- and red-emitting luciferases with advantageous properties, i.e., high emission intensity, glow-type kinetics , and high pH stability enable efficient spectral unmixing. In addition, these optimal spectral features nearly overlap the spectral transmittance of the green and red filters of the Bayer filter mosaic in the smartphone CMOS sensor, allowing an efficient spectral resolution and a sensitive detection of BL signals [80].

In another application NanoLuc was employed to detect isothermal amplification exploiting BRET between NanoLuc (donor) and the green fluorescent protein mNeonGreen (acceptor) for the analysis of tumor-associated circulating microRNAs (miRNAs) in serum samples. Both amplification and detection were implemented into a paper-based format and two papers discs, i.e., the amplification disc and the BRET sensing disc, were produced. The amplification disc contained the lyophilized probes and reagents for the rolling circle amplification and the BRET sensing disc contained reagents for detecting the amplification with specific binding of zinc finger proteins, fused either to donor or acceptor proteins, to their target double-stranded sites. A proof-of-principle system was demonstrated in which the assay was performed by placing the discs in a 96-well microtiter plate and imaged with a smartphone in a 3D-printed darkroom box (Fig. 2C). Besides the excellent performance of the system, the high stability of the paper discs at room temperature provided undoubted advantages for use of the system without strict cold-chain and deployment in remote areas. Despite the great potential

of the approach, yet the obtainment of an all-in-one device, also including a module for sample preparation (i.e., blood separation), was not achieved [81]. The possibilities of the smartphone to image BL reactions occurring in biological targets was pushed forward by Hattori et al who employed a Samsung Galaxy S8+ to monitor Nanoluc chimeric proteins, called Nanolanterns (eNLs) in organelles and whole mouse body (Fig. 2D) [82-83]. An objective lens was also attached to the outer camera of the smartphone to image Ca^{2+} dynamics within cellular organelles. However, this application remains a proof-of-concept until suitable customized apps are developed for elaborating the images to provide quantitative results without the need to transfer images to a computer and analyse them with image processing software packages such as ImageJ.

It must be pointed out that most of the reported smartphone-based biosensors are not fully optimized, especially regarding the design of the optical system and the use of smartphone with small lens apertures [84]. Smartphone-integrated CMOSs are specifically designed for taking photographs and have autofocus mechanisms that do not work perfectly in low-light conditions. Hence, high powerful BL probes together with "tricks" such as the use of chambers and cartridges with reflection materials and plano-convex lenses for efficiently capturing and focusing BL emission are required.

The identification of best conditions permits to improve the LOD of the method and increase the dynamic range. As with other detectors the integration time is crucial and must be carefully evaluated since it may account for significant LOD improvements, as shown in Fig. 3A. Therefore, the most important parameters that should be optimized in smartphone-based sensing platforms are ISO and exposure time, both can be controlled in most recent operating systems. As with all other detectors, a preliminary investigation of the BL system emission kinetics is mandatory before identifying the optimal measurement window. As a general rule, at a fixed exposure time, the increase of ISO permits to decrease the LOD, however aberrant images can be obtained at high concentrations of the target analyte, as exemplified in a recent work based on a paper sensor for ATP exploiting the luciferin-

luciferase system [85]. This paper sensor includes all reagents required for the BL reaction in a lyophilized form except ATP; after 10 min incubation with the sample image is captured with a Oneplus 5 camera using 30 s exposure. As shown in Fig. 3B, the photo taken at ISO 3200 produces aberrant images at high ATP concentrations but permits to detect lower concentrations of ATP than those obtained with ISO 1600. Depending on the expected concentration range of the target analyte a suitable ISO should be selected, in that case the goal is to achieve the lowest detection limit and therefore ISO 3200 has been selected. LOD of 3.8×10^{-14} mol of ATP was achieved with OnePlus 5, one order of magnitude lower than that obtained with a cooled CCD (ATIK 383L) portable camera (LOD of 1.7×10^{-13} mol of ATP) and one higher than that obtained with the benchtop luminometer (10^{-15} mol of ATP). Another issue is the implementation of suitable apps and algorithms to convert the picture taken with the smartphone into quantitative information. Cevenini et al. developed an app running on Android suitable for elaborating the images obtained with a smartphone toxicity sensor relying on genetically modified human embryonic kidney cells expressing a green-emitting luciferase mutant, used as "sentinel cells". This app, which converted the camera photos into a user friendly and quantitative output, was developed in Python using the Kivy Opensource Python library (http://kivy.org/#home). Moreover, the device included not only the cells immobilized into ready-to-use cartridges but also the BL substrate and control sample, which could be easily added with a built-in dispenser [86]. To solve the lack of sensitivity deriving from low exposure times (max 60 s admitted for several smartphone models), a noise-minimization algorithm was developed to accumulate multiple images (max 40) of the same sample, thus mimicking the principle of a long-exposure camera [45]. The algorithm, called noise reduction by ensemble averaging (NREA), also included a compensating step to avoid the increase in the noise signal due to image accumulation. The same authors also provided a very helpful comparison of the performance of different smartphones models, both Android and iOS devices [45]. Using the same camera settings (ISO,

shutter speed and f-number) the iPhone5 and OPO allowed to control the shutter speed over up to 60s while the LG-G2, which provided the best sensitivity for medium levels of light emission, did not allow manual control, thus being non suitable for low-light measurements. The OnePlus provided the best results with a BL bioreporter, with the detection of about ~10^6 CFU/mL with 180 seconds of integration time. More recently others selected the OnePlus series smartphones to develop BL sensors since they provided best results when compared to other models [85,6]. An interesting comparison of the performance of smartphone CMOS (Google Pixel 4a 5G), night vision camera (PVS-12 NL2 night vision monocular), a laboratory grade intensified CCD cameras (IVIS Lumina imaging system) and PMT instrument (CLARIOstar multimode plate) was performed employing continuously bioluminescent human cell lines. The benchtop CCD and PMT detected BL down to a minimum of 200 and 20 cells/well, corresponding to a flux requirement of 4.65×10^5 photons/sec and 1.7×10^5 photons/sec, respectively. The minimum flux requirements of the smartphone and the night vision camera were similar, 6.47×10^6 and 7.37×10^6 photons/sec, respectively, with the same threshold of 2×10^3 cells/well [87].

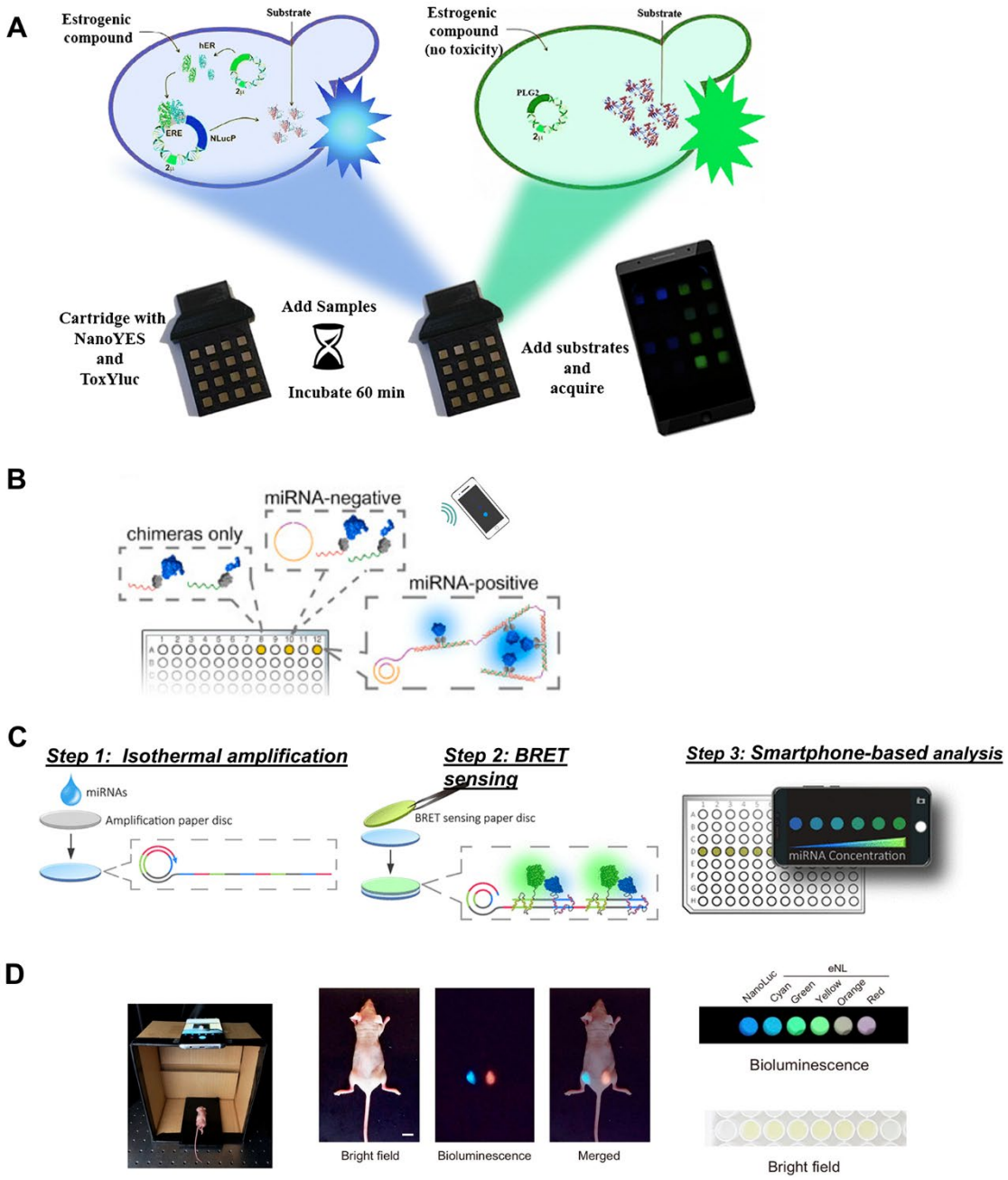

Figure 2: A) Schematic illustration of a low-cost smartphone-based devices principle composed by three strains integrate in a 3D-printed cartridge: nanoYESα, nanoYESβ expressing the reporter protein Nanoluc under the regulation of estrogen receptor α or estrogen receptor β activation, and the viability control strain ToxYLuc constitutively expressing the green-emitting PLG2 luciferase as

reporter protein. Reproduced from [3]; B) Schematic representation of BL smartphone-based assays for detecting miRNA miR-21: a pair of split luciferase-DNA chimeras is constructed and integrated into the miRNA-triggered rolling circle amplification process (RCA). The turn-on bioluminescence response is obtained by the tandem reassembly of split luciferase-DNA chimeras on the RCA products and is available for the smartphone-based detections. Reprinted from [79], Copyright (2021), with permission of Elsevier; C) Schematic representation of the amplified BRET assay for miRNAs on paper detected with a smartphone camera. The paper-based system consists of an amplification paper disc used for miRNA recognition and subsequent isothermal amplification, and a BRET sensing paper disc. By simply rehydrating the amplification paper disc with the miRNA sample, the paper-based RCA process is triggered. By further introducing the BRET sensing paper disc and thanks to the specific recognition of ZFPs to their binding sites, the RCA amplicon can act as a template for assembling a large number of donor–acceptor pairs and the BRET signals are collected with a smartphone. Adapted with permission from [81]. Copyright (2019) American Chemical Society; D) Simultaneous multiple wavelength BL imaging using a smartphone camera of HeLa cell transiently expressing cyan eNL or red eNL and injected subcutaneously into the mouse and wide-field BL image of HeLa cells transiently expressing NanoLuc or each color variant of eNL in a 96-well plate. Reproduced from [83].

5.3 CCD-based BL detection

CCDs are widely employed for different biological applications, having the significant advantage of providing spatial information. Thanks to their sensitivity, CCDs represent the optimal choice for BL imaging in laboratory settings, especially for glow-type emission, for example a cooled charge coupled device camera (VersArray, Roper Scientific Inc) was used to image cell chips and 384-well plates with immobilized recombinant BL bacteria containing different stress promoters fused to *lux*

[88]. These arrays were used as toxicity sensors for measuring chemicals with different mode of action (paraquat, mitomycin C and salicylic acid). Undoubtedly, electron-multiplying CCD (EM-CCD) sensors are the optimal choice for extremely low-light BL imaging in which where photon fluxes may be as low as 0.05 photons per μm^2 of the sensor surface [89]. However, mostly for their cost and size, their use still remains confined to laboratories as benchtop instrumentation. Conversely, several examples of implementation of CCDs in analytical devices have been reported in which lens-less detection via fiber optic tapes was achieved [90-91].

CCDs can be cooled with a Peltier chamber down to -10 °C, allowing a significant decrease of CCD dark current (10^3 electron/pixel/sec) generated by thermal energy and this is vital for low-light measurements. Cooling reduces the detector dark current shot noise, which is the major source of noise approximately in half every 6°C reduction of the chip temperature [92]. Even if cooling improves the detector sensitivity, it consumes power and increases cost and size of the device, thus not suitable for implementation into miniaturized devices. Another issue is related to the optics, generally lower-end CCDs employ simple optics, with optical loss causing the necessity to increase the number of reagents for reaching adequate sensitivity. Conversely, high-end CCD imaging systems do not suffer from this loss but are generally bulkier and more expensive. As an alternative lens-free detection can be achieved by placing the light-emitting species directly onto the imaging device without optics. Several examples of lens-free imaging have been reported by us and others [90, 93-96]. However, CCD optical systems based on lens-free imaging necessitate to operate near room temperature to ensure reactivity of the employed chemistries, thus increasing the dark current.

Nevertheless, room temperature is not a universal definition and, if it can be carefully set in a controlled environment, such as a laboratory (~23–25.5 °C), this is not true in real settings. For all the detectors few degrees of temperature may significantly change the output and thus affect the performance (Fig. 3C) [4]. To balance this effect higher amounts of reagents must be used.

Most of the CCD-based devices were used for imaging BL whole cell bioreporters and applied for environmental monitoring. Recently Kao et al reported the integration of bacterial sensor strains into a CCD-based lens-free optical system, called LumiSense. This device, also integrating microfluidic sample chips and the bacterial incubation chip, was applied to detect ciprofloxacin, used as a model antibiotic, in milk, egg white, egg yolk and chicken essence, providing LODs lower than the allowed values regulated by European Union [91].

Some optical devices relying on PMT, such as the Lumisens 2, described in section "BL detection with photomultiplier tubes", were upgraded by replacing PMT with CCD camera. A CCD camera with a 30-cm focal length lens (1N18, Diagnostic Instruments Inc, INSIGHT™) was used for the Lumisens IV to image a 96-well microplate with transparent bottom [97]. The Lumisens IV was programmed to perform one daily analysis cycle during one week on environmental water samples spiked with metals (mercury, cadmium, arsenic and copper). Thanks to the use of disposable cards with freeze-dried bacteria a detection with 3% of reproducibility was obtained during a 10 day-period Also, the strategy applied by Simpson et al. [71] relying on CMOS has been expanded with the use of CCD camera placed above or below the immobilized cells to perform BL imaging [98]. Exploiting a CCD sensor from a commercial digital camera as a bioluminescence sensor, similar performances were obtained with a biosensor based on luminous marine bacteria, *Photobacterium phosphoreum*, immobilized on an acrylic chip, providing a rapid platform for onsite detection of Biochemical Oxygen Demand (BOD) in environmental samples.

One of the main issues related to CCD sensors, and not present in CMOS sensors (Fig. 3D), is the blooming effect occurring when a portion of the image is overexposed, and leakage of electrical charge occurs between neighboring pixels causing artefacts in the signal [98-100]. Since the blooming effect depends on the integration time, the latter is a crucial parameter for low-light applications and for measuring a wide range of light intensities on the same cartridge, which is the case when real

samples are analyzed without prior knowledge of the expected signals. To achieve the highest sensitivity without having spurious signals, an integration time which represents a compromise between sensitivity and the presence of blooming effects should be selected. The choice of the integration time also depends on the BL system to avoid saturation of the camera. Close et al. performed a comparison between a bacterial luciferase (lux) reporter system engineered for improved expression in mammalian cell lines, and a firefly D-luciferin luciferase-based reporter systems. Yet, this integration time was not sufficient to discriminate low number of cells (~250) from the background. An integration time of 10 s enabled to detect down to 250 cells (Fig. 3A), but simultaneous imaging of low and high BL levels could not be achieved due to camera saturation [42]. The possibility to detect more analytes with a single device is highly appealing. BL, thanks to the high number of different luciferin-luciferase systems available, appears as an optimal choice for multiplexing. Multicolor imaging has been extensively demonstrated and applied in both *in vitro* and *in vivo* settings employing highly sensitive EM-CCD technology [101-102]. Also, when multiple BL systems are employed a crucial factor is the PDE of the detector at the wavelength of the BL systems and, as shown in Fig.3E, for achieving the best sensitivity a significant overlapping is required. Few multiplexing strategies have been reported in BL analytical devices. Besides, spatial multiplexing, relying on the use of separate wells or channels targeting different analytes, the exploitation of different BL systems for multicolor imaging was seldom applied in portable devices. To spectrally resolve the emissions of two BL reporters we previously reported low-cost printed filters obtained with an office printer and transparency films. These filters were used in a lens-free CCD device to separate the emissions of green and red-emitting luciferases expressed in yeast strains for detecting androgenic and estrogenic compounds. As expected, a higher LOD was reported with those printed filters when compared to benchtop instrumentation and hard-coated filters [95]. More recently, multicolor imaging was also achieved with smartphones by using the ImageJ to split images in RGB

channels [24]. Despite these efforts, multicolor devices have not yet reached a maturity level to enable robust and reliable multiplexing.

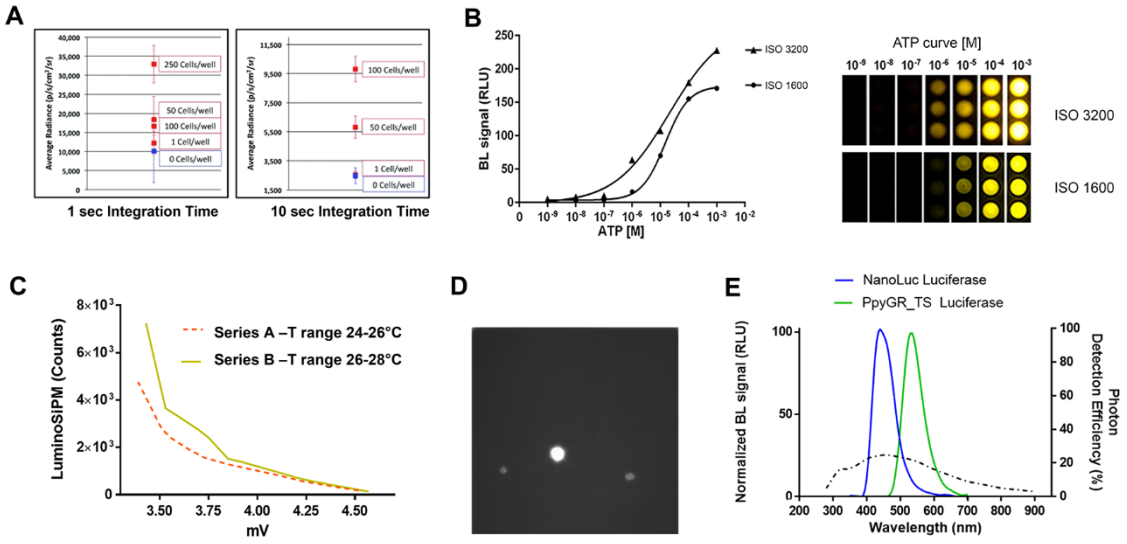

Figure 3: BL detection with portable light detectors: examples of key settings and parameters: A) Short integration times (~1 s) required, if suitable for the intensity of the selected BL reporter, to prevent saturation of the CCD camera. Reproduced from [42]; B) ISO settings in smartphone-integrated CMOS to achieve lowest detection limits. Reprinted from [85] Copyright (2020), with permission of Elsevier; C) SiPM dark noise acquisitions at different temperatures. Adapted with permission from [4]. Copyright (2021) American Chemical Society; D) Natural CMOS anti-blooming effects. Reproduced from [99]; E) Selection of suitable luciferases in according to the PDE of the sensor. Adapted with permission from [4]. Copyright (2021) American Chemical Society.

5.4 BL detection with non-scientific digital cameras

The possibility to use non-scientific grade low-cost digital cameras that provide more functionalities with respect to smartphones-integrated cameras has been also explored as a viable alternative to

smartphones. In addition, the use of these cameras could be advantageous for remote monitoring for environmental applications. For example, Cevenini et al, integrated a cartridge with immobilized yeast-based biosensors, called nanoYES, for endocrine disrupting chemicals detection with an HERO5 wireless action camera that could be remotely controlled with a smartphone App. The proposed biosensor was tested with 17β-estradiol compound providing a LOD of 0.08 ± 0.02 nM in the GoPro-based platform, about one order of magnitude higher compared to those obtained with the nanoYES using conventional benchtop luminometer. This device was intended for rapid monitoring of effluents of wastewater and critical areas, such as agricultural and industrial sites [40].

Xue et al. provided an elegant way to obtain universal reagents composed by genetic fusions of NanoLuc luciferase and antibody fragments using the SNAP-tag for conjugation with a fluorophore labelled antigen acting as competitor for the antibody. In the presence of the suitable antigen, the competitor is displaced from the antibody site and causes disruption of the BRET signal between the BL NanoLuc and the FL acceptor. A paper-based device was developed for analysis of whole-blood samples and, to avoid interference of haemoglobin on BL measurement, they performed a dilution step (1:10 in HEPES buffer) [38]. The use of a digital camera, a Canon PowerShot G1X model, enabled not only the selection of proper setting (exposure time of 10 – 20 s, F value of 2.8 and ISO value of 6400) but also the saving of the image in RAW format for subsequent conversion into PNG format with optimized parameters. This cannot be achieved with pictures obtained with smartphone-integrated cameras.

6. Portable light detectors for other luminescence applications: chemiluminescence and fluorescence

As with BL, light detectors for chemiluminescence and fluorescence-based applications should be compact, sensitive, have low-power requirements and possibly be inexpensive. Among main key

issues to be considered surely the wavelength range, light power, and response time are those requirements to consider in choosing the type of photodetectors for fluorescence. The maturity of fluorescence sensing applications is much higher than that of BL-based applications and several examples can be found in the literature, spanning from food quality monitoring to point-of-care diagnostics [103-105]. Thanks to high signals generated by FL, for most applications the sensitivity of the detector is not a limiting factor and photodiodes, or CCD are generally viable options. Shin et al. published an interesting review with the recent developments in portable fluorescence sensors with an overview of the different applications [106].

Chemiluminescence shares many features with BL and, as a general rule, most of the detectors suitable for BL biosensing also suit chemiluminescence-catalyzed reactions. Also, the device design can be more compact in comparison to a fluorescence-based device requiring the light source dichroic mirrors and objective lenses. In perspective of implementation into portable platforms, great efforts have been devoted to the development of chemiluminescent systems with high intensity and glow type emission kinetics. These chemiluminescence systems have the main advantages of increasing the total light output and provide an extended measurement window or a suitable integration time for signal acquisition. This enhancement effect on both intensity and kinetics was nicely demonstrated by Karabchevsky et al by pumping metallic nanoparticles into a microfluidic device [107]. Most of the detectors that proved suitable for BL detection were also explored for chemiluminescence [4-5, 108-110] .

7. Conclusion and perspectives

The implementation of BL systems into portable analytical devices is leading to lots of unforeseen applications across various sectors. When integrating BL reactions, either recombinant whole-cell biosensors or purified luciferase-based sensors, into miniaturized devices several obstacles related to

the integrity and responsiveness of the biological part of the biosensor, together with technical issues, the majority of which due to extremely low intensities of BL, are faced.

Thanks to their high sensitivity and low manufacturing cost, devices based on both CCD and CMOS technologies have been broadly explored. In some cases, the smartphone-integrated CMOS have totally abolished the requirement for an external light detector, leading to a number of proof-of-principles instrument-free prototypes, especially intended for environmental applications, such as low-cost on-site monitoring of contaminants. However, when smartphones are used as detectors, a dark box must be employed fitting the smartphone model. Therefore the "one size fits all" concept cannot be easily applied in smartphone-based BL biosensing. Considering the high number of models that are on the market and rapid obsolescence of smartphones, wouldn't it better a low cost-device that could fit any smartphone? The answer is not trivial, alternative technologies have been proposed, however the simplicity of using the smartphone as light detector is undeniable. Considering that most of critical issues derive from the intrinsic fragility of the biorecognition element another option to improve the robustness of BL devices is to integrate an orthogonal detection with different sensing principles, for example by coupling BL and colorimetric detection or BL with chromatography [6, 111]. Unfortunately, a detector providing optimal features in terms of detection efficiency over a wide range of wavelengths, low dark counts combined with high portability does not exist, therefore the most suitable detector should be selected case by case. To ensure maximum sensitivity, one of the first issues to be considered is surely the PDE of the detector at the specific emission wavelengths of BL system. In those cases, in which BL reactions yield very few photons and/or the achievements of very low LODs is required, the use of smartphones remains a suboptimal choice. In such situations, the selection of microPMT or SiPM could be a viable option. In addition, the cooling of SiPM with mini Peltier chambers could provide the required sensitivity with a very limited footprint. For multiplexed biosensors exploiting two or more BL reporters emitting at different wavelengths, the

use of filters to spectrally resolve the signals should be included. However, this approach is still underexplored and few prototypes relying on BL detection to distinguish multiple target analytes have been developed. As an alternative, smartphones could be used for multiplexed BL, but customized apps should be developed to obtain quantitative information. Multiplexing is surely one of the aspects that has not been fully explored, with different strategies, including spatial, temporal and spectral resolution, which could be selected or combined together to increase the number of target analytes that can be simultaneously detected. In this context the use of CCDs would enable both spatial resolution and the possibility to integrate signals to achieve high detectability. Flash-type emission, which may generate more than 90% of the light within the first two seconds, still is hard to implement in portable systems, generally solid-state sensors are the best choice for its detection but, to avoid light losses, devices with on-board reagent injection would be required.

In addition, the ideal detector should also have small footprint and low power demand. In fact, such devices, meant for low-cost IoT monitoring to be performed by the general population, are expected to enter the market in the next future and thus their sustainability is mandatory.

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