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BIOANALYTICAL APPLICATIONS OF MULTICOLOUR BIOLUMINESCENCE IMAGING: NEW TOOLS FOR DRUG DISCOVERY AND DEVELOPMENT

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Abstract

The subject of this thesis is multicolor bioluminescence analysis and how it can provide new tools for drug discovery and development. Different applications of bioluminescence imaging using multicolor luciferases are defined in the first part of the thesis while in the second part the development of multicolor cell based assay is shown. Both in vivo and in vitro methods are useful in pharmacological research: cell-based assay are usually employed in high-throughput screening while bioluminescence based mouse models are useful both for target discovery and validation and for preclinical studies of drug efficiency, drug release and biodistribution. The advantage of using luciferases with different emission spectrum, so that the bioluminescence emission peak can extend range between the yellow to the red red region of the UV/visible spectrum, is that multiple analysis can be performed and information can be obtained in one analytical session. Moreover small animal bioluminescence imaging (BLI) is a technique that allows the collection of data with no need for animal sacrifices and permit to perform longitudinal studies on the same animal.

In **Chapter 2** the potential of red-emitting firefly luciferase mutants to enhance bioluminescence imaging experiments is demonstrated. By establishing two mouse cancer models employing the Ppy-RE-TS mutant (λ_{max} =620nm) or WT luciferase (commonly used in bioluminescence imaging studies in vivo), the superior characteristics of the red enzyme for in vivo imaging have been pointed out. That is because the optimal window for in vivo imaging is after 600nm where the tissue absorption (mostly due to haemoglobin) is minimal.

In **Chapter 3** an optimized version of the red luciferase named above, Ppy Re8, has been shown to be useful in imaging gastric emptying in mouse models. Non - pathogenic bacterial expressing the luciferases were administered by gavages to the animals: the bacteria acted as luminescent beads and mixed to the gastric contents. Images of the mice were taken at different time points and used to derive curves for the gastric emptying process. This model demonstrated to be useful for the evaluation of the physiological process and can be employed for the development of new drugs acting of gastric motility and in particular for the study of side effects of drugs. Moreover mice in different pathological conditions can be investigated for physiopathological studies of the gastric emptying process in drug efficacy studies.

In **Chapter 4** Ppy-RE8 enzyme, which is codon optimized for mammalian expression in combination with the green click beetle luciferase, CBG99 for in vitro and in vivo dual color imaging applications has been investigated. The comparison of the spectral characteristics of this dual expression strategy, using a single substrate D-luciferin, in human embryonic kidney cells (HEK293) utilizing different proportions of cells expressing each luciferase, showed the potential of this strategy to follow two distinct events in real time and in vivo.

In **Chapter 5 and 6**. A new luciferase isolated from *L. italica* and thermostable red- and green-emitting mutants obtained by random and site-directed mutagenesis. were tested for their suitability for multicolor assays. A mammalian triple-color reporter model system was then developed using a green-emitting wild-type *P. pyralis* luciferase, a red thermostable mutant of *L. italica* luciferase and a secreted *Gaussia princeps* luciferase (GLuc) to monitor the two main pathways of bile acid biosynthesis. The two firefly luciferases were used to monitor cholesterol 7-α hydroxylase and sterol 27- hydroxylase, while secreted constitutively expressed GLuc was used as an internal vitality control. By treating the cells with chenodeoxycholic acid it was possible to obtain dose-dependent inhibitions of the two specific signals together with a constant production of GLuc, which allowed for a dynamic evaluation of the metabolic activity of the cells. The reported assays were the first triple-color mammalian reporter assay that combines secreted and non-secreted luciferases requiring different substrates, thus avoiding reciprocal interference between different BL signals. This approach is demonstrated to suitable for high content analysis of gene transcription in living cells to shorten the time for screening assays, increasing throughput and cost-effectiveness. Multiple assays can be developed using this strategy fuelling the drug discovery process.

In vivo Bioluminescence imaging has known a rapid progress since its first application no more than 15 years ago. It is becoming an indispensable tool in pharmacological research. Nowadays researchers put a lot of efforts into improvements of instrumentation for light detection and analysis and into the improvements of the bioluminescent probes available. The application of the technology is booming and the author of this thesis is sure that multicolor bioluminescence imaging will boom and improve as well leading to new discoveries in life science, medicine and pharmacological research.

Chapter 1

Introduction

Adapted from : "Luminescent Probes and Visualization of Bioluminescence" in Bioluminescence
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A.Roda Editor, RCS Publishing 2010

"Cell based assay: fuellig drug discovery"
E. Michelini, L. Cevenini, L. Mezzanotte, A.Coppa, A. Roda
Anal Bioanal Chem 2010; 398 (1):227-38 Review

Introduction

Bioluminescence

The term Bioluminescence is generally used for the definition every kind of Chemiluminescence produced by a specific biochemical reaction occuring in a living organism. Bioluminescence reactions involve an enzyme “luciferase” and a substrate “luciferin” and present high quantum yield. The latter is a very important parameter: in theory, every molecule that reacts could produce a photon but only a fraction of them brings to the release of photons. The quantum yield of a bio/chemiluminescence reaction is defined by following the equation:

$\Phi_{bio/chemiluminescence}$ = emitted photons/ n° reacting molecules

For the luciferase/luciferin reaction from Photinus pyralis firefly the value is higher compared to those of chemiluminescence reaction and it is 0.44[1].

One of the unique features of bioluminescence is that, unlike other forms of light, it is “cold light”. In fact, bioluminescent light is produced with very little heat radiation.

The modern study of bioluminescence beagan when Dubois demonstrated the first example of a luciferin/luciferase reaction in 1885 and he concluded that an enzyme and a specific, relatively heat stable substance, which he designated "luciferine" were necessary for the light-emitting reaction. Following the discovery the person who coniated the term “bioluminescence” and made important studies of the phenomenon was E. Newton Harvey (1887-1959) of Princeton University. Harvey traveled widely and studied the bioluminescence of a great variety of luminous organisms, producing over 300 publications. His book Bioluminescence published in 1952 is considered the bible of bioluminescence. In the 20th century many other scientists gave outstanding contribution to the description and the application of bioluminescence in life sciences and medicine: among them Stanley P.E., Kricka L, Mc Capra F. ,Hastings J. W., Mc Elroy W. and Shimomura O. Nowadays the book “Bioluminescence” published in 2008 by the nobel prize O.Shimomura is considered the new bible[2].

Bioluminescence can serve more purposes within a single organism, both offensive such as to lure, confuse or illuminate prey and defensive, such as sturtle, burglar alarm or misdirection of predators as well as mate attraction and recognition. In the

marine environment the predominant bioluminescence colour is blue (around 470nm) because light at this wavelength transmits furthest in water so many organism evolved to be sensitive only to blue light, lacking visual pigments for longer or shorter wavelengths. In the terrestrial environment, green is the predominant bioluminescence colour as a result of an ecological adaptation of bioluminescence to the photic environment [3].

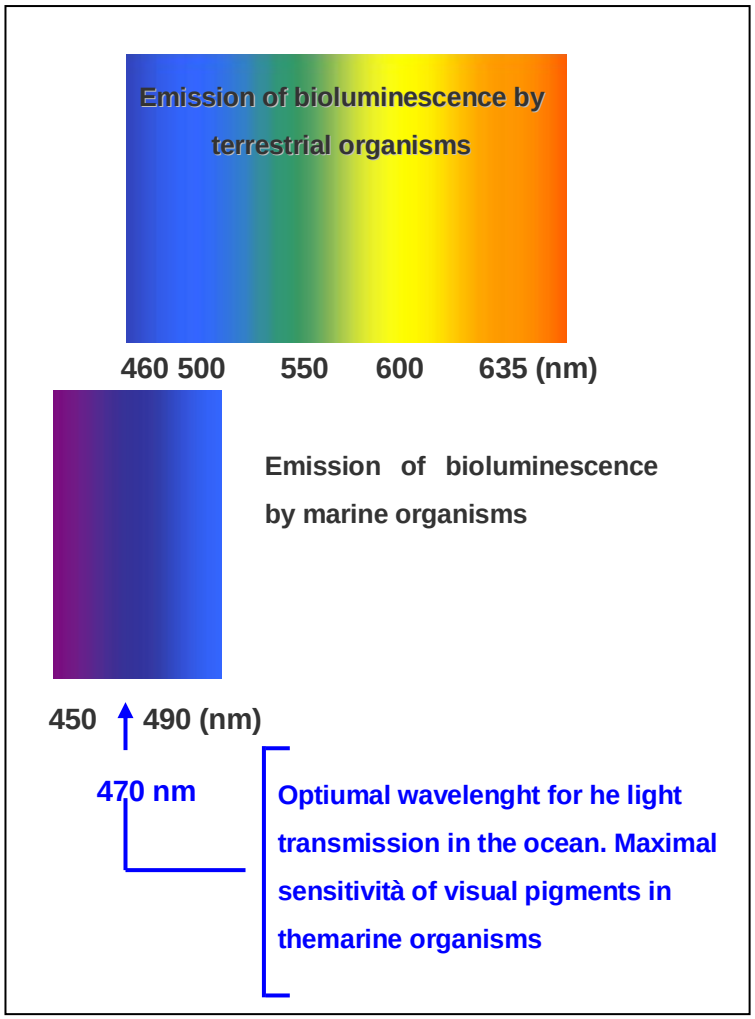

Figure 1 Wavelenghts emission of tereestrial and marine organisms.

Bioluminescent systems

Firefly luciferases and mutants

Luciferases from fireflies family are the most studied and the most commonly used. The luciferin-luciferase reaction of fireflies was first demonstrated by Harvey (1917), although the light observed was weak and shortlasting. Thirty years after Harvey's discovery, McElroy (1947) made a crucial breakthrough in the study of firefly bioluminescence. He found that the light-emitting reaction requires ATP as a cofactor. In 1949, McElroy and Strehler found that the luminescence reaction requires Mg2+ in addition to luciferin, luciferase and ATP. The active luciferin was found to be in the D-form while the L-form is practically inactive. Luciferase from Photinus pyralis belongs to the Lampyridae family that are carachterized by sexual dimorfism. Though the females of some species are similar in appearance to males, larviform females are found in many other firefly species. These females can often be distinguished from the larvae only because they have compound eyes. In the major part of the species male are 1cm long and have wings. The organ for light emission is in the terminal part of the abdomen. In some species this organ is bigger in male individuals but generally both sexes can emit light.

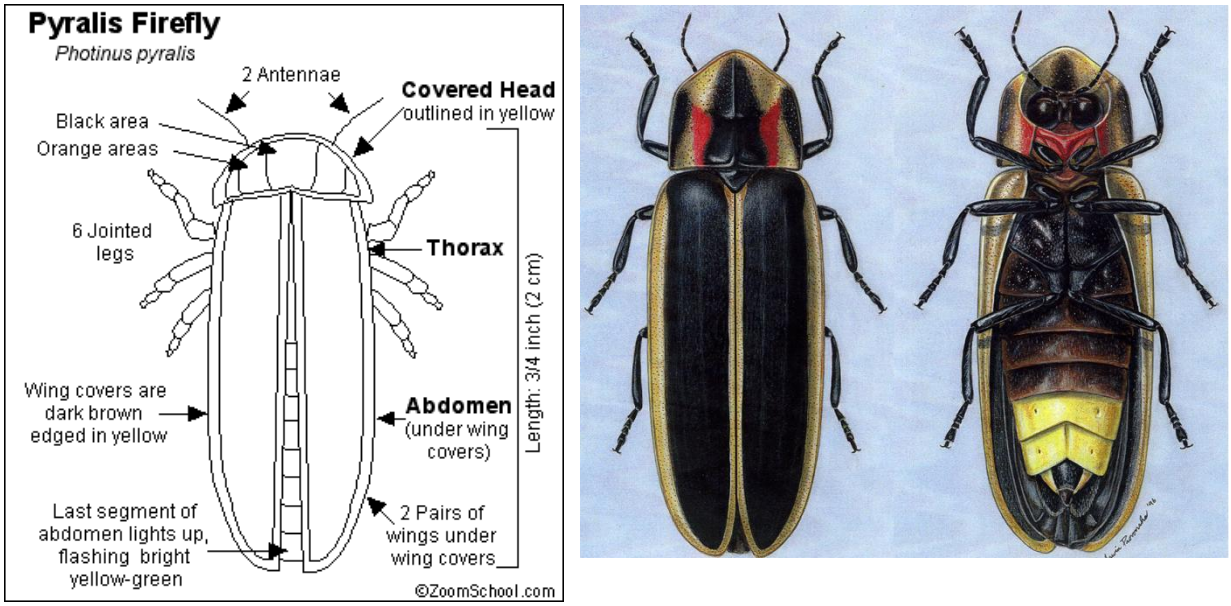

Figure 2: Photinus pyralis

The reaction mechanism is not fully understood yet and it consists in the rapid conversion of the substrate luciferin in luciferyl-adenilate linked to the enzyme in presence of Mg^{2+} and ATP. Pyrophospate is released in this phase then the combination with oxygen lead to the formation of the luciferase-oxyluciferin- adenilate complex and to the elimination of CO_2. The complex is in an “excited state” and it returns to the fundamental state by emitting photons. At the end the complex is dissociated in luciferase, AMP and oxyluciferin (Figure 3)

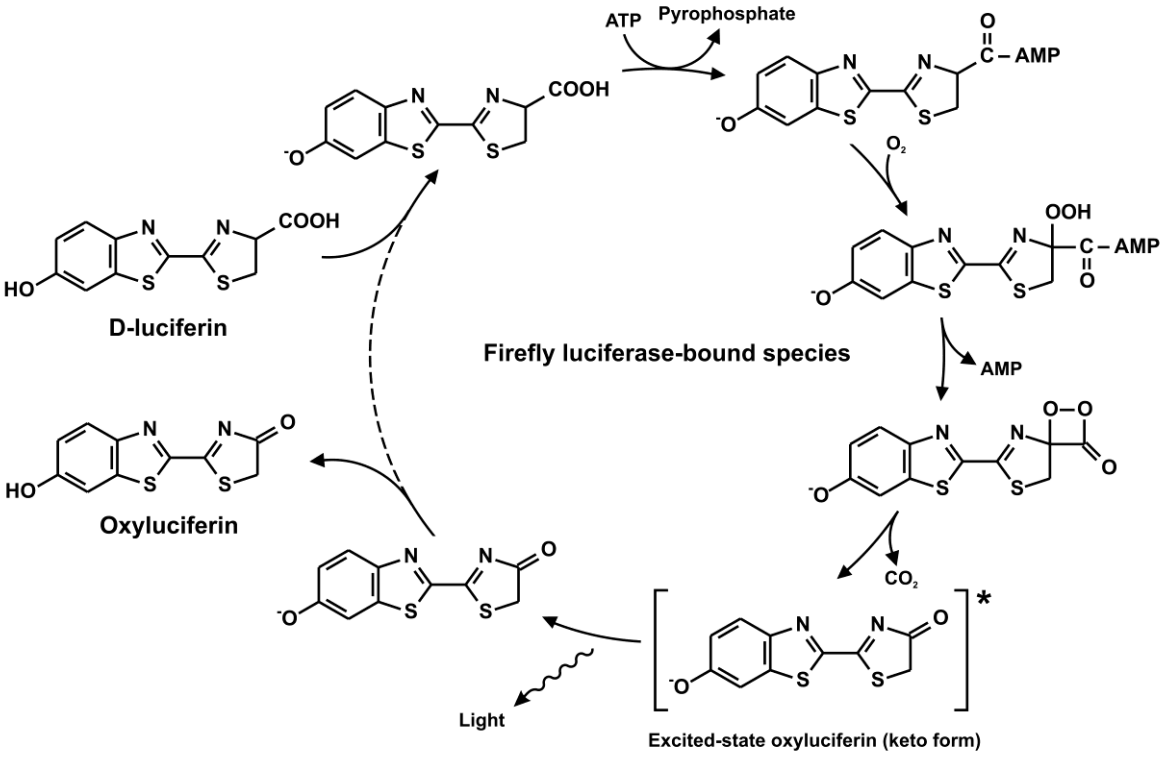

Figure 3: Mechanism of reaction of firefly luciferase and D- luciferin

Since luciferase demonstrated a greater affinity for the oxyluciferin compared to reduced luciferin, high concentration of substrate may inhibit the reaction with a competitive mechanism so that the last step of the reaction result to be the limiting one[4]. Recent studies have demonstrated that in nature Luciferase regenerating enzyme (LRE), peptides of 38 kDa, can convert oxyluciferin in a form that in presence of D-cystein can be converted in luciferin (Figure 4)[5,6] . The reactivation of the enzyme luciferase, instead, is operated by the coenzyme A that removes the adenil-oxyluciferin from the surface of the enzyme[7].

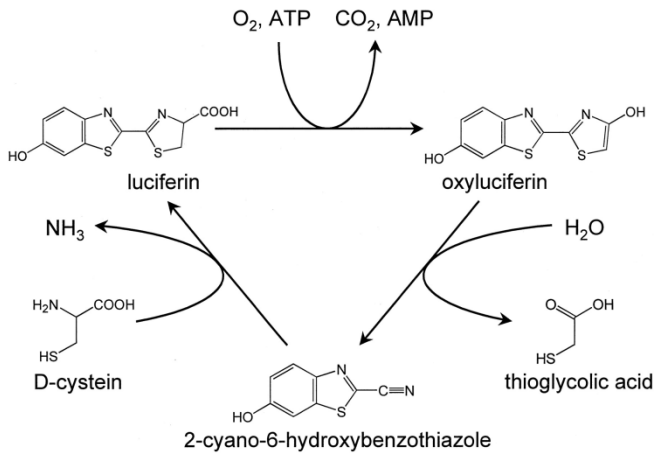

Figure 4. Proposed mechanism of luciferin regeneration

Luciferase from P. pyralis is an enzyme of 62 kDa and with a maximum of emission at 562nm at 25°C and pH 7.8. The enzyme presents two polypeptididic subunit with identical amminoacidic sequences but only one is responsible for bioluminescence activity. The active site create san hydrophobic enviroment with two sulfidric groups essential for the catalytic activity[8]. In the mechanism proposed by Branchini et al. the red emission (λ_{max} 615 nm), observed at pH 6.0 is produced by the chetonic form while the yellow-green emission (λ_{max} 560 nm) at pH 8.0 is produced by the enolate anion form of the excited oxyluciferin[9] (Figure 5,6).

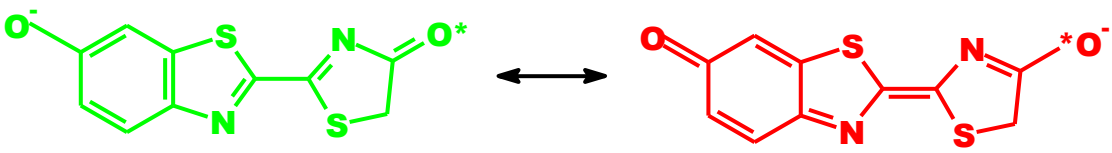

Figure 5. Keto-enolTautomerization of D-luciferin

In nature, beetle luciferases have different emission colors: one of the proposed hypotesis is that the charge delocalization in the excited state cause the different wavelenght emissions. Recently other hypotesis have been proposed and the real cause of the different emission colors is still controversial[1,10]. In order to improve characteristics for analytical application luciferase from Photinus pyralis underwent random and site specific mutagenesis. The group of Prof. B. Branchini generated two variants: Ppy GR-TS and Ppy RE-TS that have a maximum emission wavelength respectively 546nm and 610nm. The former is mutated on the residues Val241Ile,

Gly246Ala and Phe250 Ser, while the latter on the residue Ser284Thr, which is responsible for the red emission, and Thr214Ala, Ala215Leu, Ile232Ala, Phe295Leu and Glu354Lys for increasing stability at 37°C[11]. Moreover additional changes in the amminoacidic sequence have been apported : Arg330Gly e Glu354Lys generated the Ppy RE8 form, with a maximum emission wavelength at 617nm. Such a red shift causes a loss of thermostability and activity of the enzyme so that mutations in the amminoacidic residues Phe465Arg e Ile351Val were necessary. Moreover the CDS sequence of Ppy RE8 enzyme has been codon optimized for mammalian expression, by eliminating repeats, local hairpin and criptic splicing sites. Ppy RE8 has 8 different amminoacid compared to the wild type enzyme and its thermostability rends it useful for cell based assay and in vivo imaging[12].

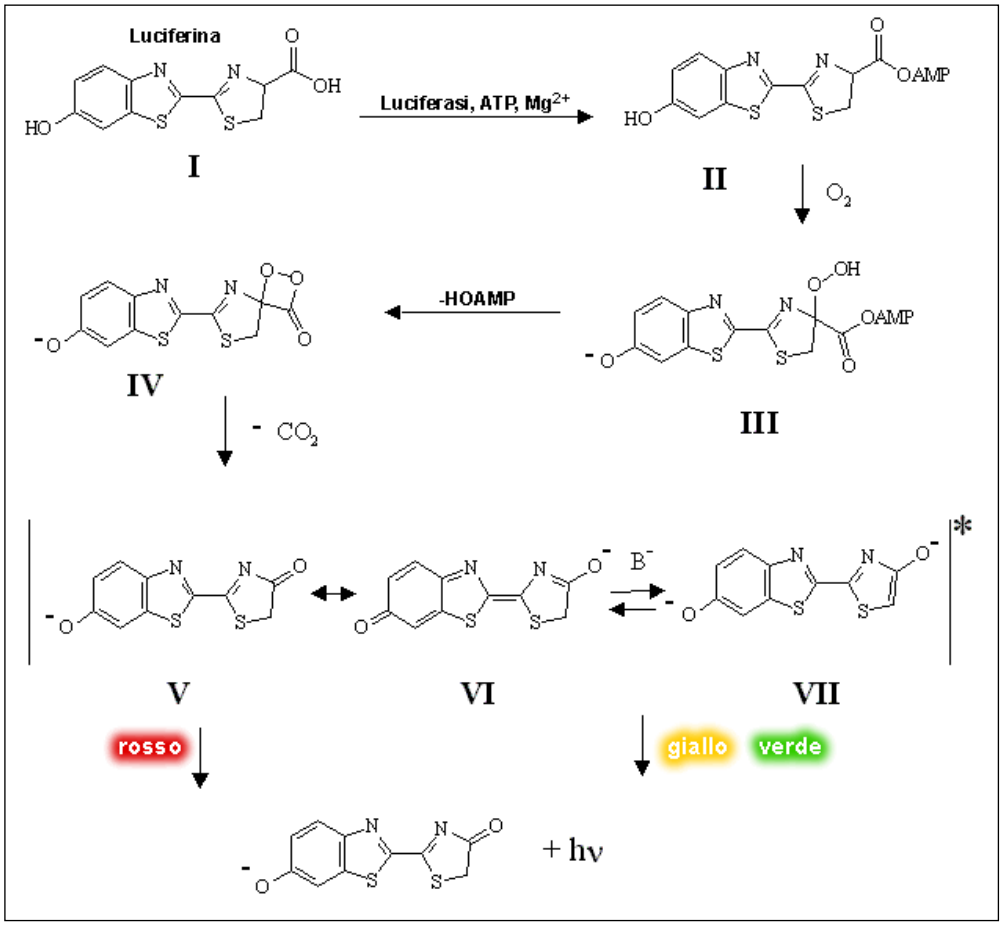

Figure 6 . Mechanism of reaction with different hypotesis on the variation of colour emission.

Click beetle and railroad worm

Click beetle luciferases

The genus *Pyrophorus* belongs to the Eleteridae family and includes 26 bioluminescent species amongst Caribbeans, Central anb south America. The biolumienscence reaction is similar to that of coleoptera and requires the same substrate and cofactors even is the ventral light emission is continuos[13]. They generally eat pollen and little insects like aphids. They lay eggs that are luminescent as larvae that growth up slowly (even years) Pyrophorus plagiophthalamus, the lamaica click beetle present a strong polymorphism in the color of light emission It has two different light organs: a ventral one that emits light in the yellow green (558-562nm) and orange (591-595nm) and two dorsal organs that emit green (544-548nm) and yellow-grren (557-562nm) (figure 7).

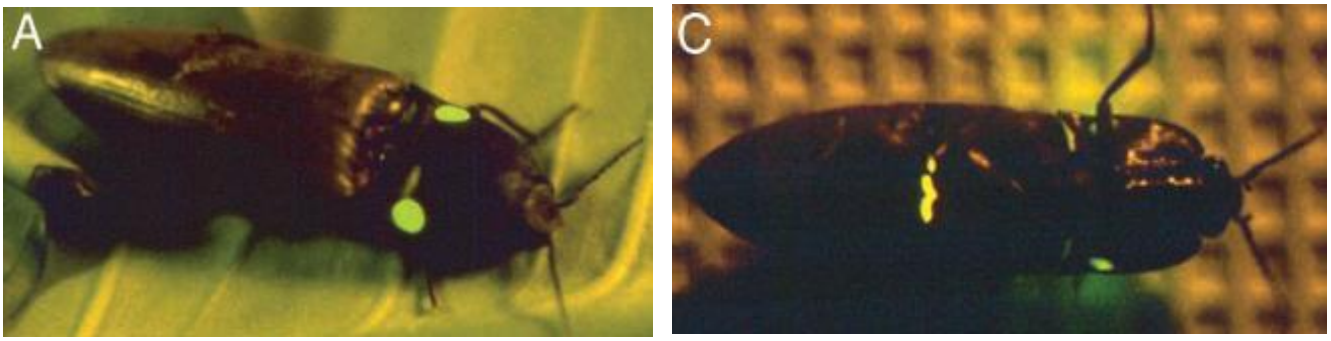

Figura 7: Pyrophorus plagiophthalamus emits green light form the dorsal organ (A) and orange from the ventral ones (B)

Four genes were cloned and mutagenized from this insect that differs one another for emission spectrum and intensity porperties.

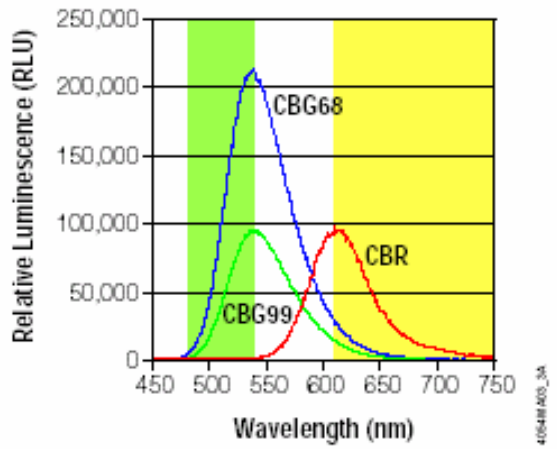

Figure 8: Emission spectra and intensities of Pyrophorus plagiophthalamus mutants(biblio)

Thermostable mutants emits in the red region of the Uv/visible spectrum as CBRLuc or in the green region as CBG68luc and CBG99luc. In every sequence the peroxisome targeting sequence has been removed to obtain a citoplasmatic localization of the espresse enzyme[14,15]. CBG99Luc has a peak of emission at 537 nm while CBRluc at 615 nm at the temperature of 25°C. The most important characteristics is that the emission spectrum of these enzyme is not influenced by variation in the pH like fireflies enzymes(Figure 8).

Railroad worm

The railroad worm *Phrixothrix* is well known for displaying two different colors of luminescence from a single organism. This genus is widely distributed in Central and South America and belongs to the family of *Phengodidae* (Figure 9).The larva of *Phrixothrix* (and also the adult female) emits a greenish yellow light (λ max 535-565 nm) from 11 pairs of luminous organs on the posterior lateral margins of the second to the ninth segment, and a red light (λ max 600-620 nm) from the luminous area on the head. The adult male is a typical beetle and does not show a noticeable luminescence. The bioluminescence systems of these phengodids were essentially the same as that of the fireflies, involving the same luciferin (firefly luciferin), ATP and Mg +. Their emission maxima of luminescence from the lateral and head organs are in the ranges of 535-592nm and 562-638 nm, respectively. The color differences are probably due to the presence of luciferase isoenzymes (Mr about 60,000) according to the authors[16]. Viviani *et al.* (1999) cloned the luciferases from the lateral light organs of *Phrixothrix vivianii* (emission Xmax 542 nm) and the head light organs of *Phrixothrix hirtus* (emission Amax 628 nm). Recently, the latter enzyme has been mutated for improving stability and activity[17].

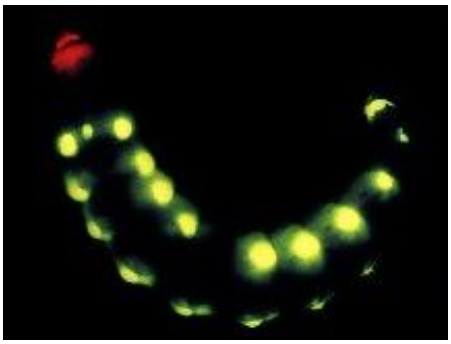

Figure 9. Railroad worm *Phrixothrix hirtus*

Renilla and Gaussia lucifarases

Luciferase from Renilla reniformis

Renilla reniformis, is a marine coral belonging to the family of Renilladae, class of Anthozoa (Figure 10). Renilla luciferase is a monomeric photprotein with a molecular wheight of 36Kd and shares the conserved catalytic triad of residues employed by the dehalogenases, as confirmed by its crystallographic data[18]. The enzymatic reaction does not require ATP and its substrate coelenterazine has a good penetration through cell membrane. The use of Renilla luciferase as reporter gene involves its application in combination with fireflies luciferase for dual reporter assay. Coelenterazine, shown in figure 10, is the common substrate for Renilla luciferase, apoequorin, gaussia and metridia luciferases that have been recently cloned. The luciferase/coelenterazine reaction produces mostly blue light: peak of emission of Renilla luciferase is 480nm. In 2007 A.Loening et al. generated by random and site directed mutagenesis Renilla variants with different emission spectrum and increased activity and stability[19].Rluc8 shows an emission peak at 535nm has an half life of 50h and an activity of 1.4 fold higher than the native enzyme. Consequently Rluc8 improved the sensitivity of the detection in both in vitro and in vivo applications. Moreover they generated a new red shifted mutant Rluc7-535 with an half life comparable to the one of the native enzyme (6.5H) in order to achieve sensitivity in transient gene expression analysis[20]. Renilla luciferase has been widely applied as luminescent donor in bioluminescence resonance Enrgy Transfer method for studing protein-protein interaction[21].

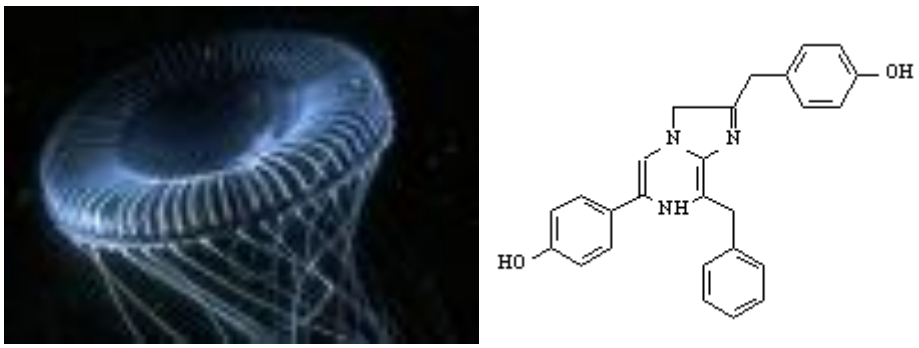

Figure 10. .Renilla reniformis and Coelenterazine structure

Luciferase from Gaussia princeps

Gaussia luciferase (Gluc) is a novel secreted luciferase, cloned from the copepod Gaussia princeps, that catalyzes the oxidation of the small molecule coelenterazine to produce light (Figure 11). Unlike the firefly luciferase systems, these coelenterazine-utilizing luciferases do not require accessory high-energy molecules such as ATP, simplifying their use in a number of reporter applications[22,23]. This luciferase catalyzes the oxidation of the substrate coelenterazine in a reaction that emits light (λem = 470 nm),and has considerable advantages over other reporter genes. Thanks to its pH resistance in a range from 3 to 11 with an optimum at pH 7.8, and its good thermostability (up to 60°C), GLuc is an attractive tool in report gene assays or as an enzyme label for bioanalytical applications, particularly for the development of bioluminescence (BL) cell based assays and for BL imaging animal models. GLuc is the smallest luciferase isolated to date (19.9 KDa) and this small size is a crucial factor for the construction of fusion proteins to avoid steric hindrance. Moreover since Gluc, when expressed into mammalian cells, is secreted into the culture medium the BL measurements are performed by simply addition of coelenterazine in culture medium, without the need for cell lysis.

Figure 11. Gaussia princeps copepod

Bioanalytical application of bioluminescence

The typical bioanalytical applications of bioluminescence (BL) proteins include the investigation of protein–protein interactions, protein conformational changes, protein phosphorylation, second-messengers expression, and, in general, the study of gene expression and gene regulation in vitro and in vivo[24,25]. The expression of a BL protein can be put under the control of tissue-specific regulatory elements allowing non-invasive imaging of physiological and pathological processes like differentiation, apoptosis, tumor progression, and inflammation, even in a 3D fashion by means of BL tomography, which allows 3D BL source reconstruction[26]. Since BL proteins can be detected down to very low levels, they allow ultrasensitive detection of the target analytes and monitoring of the physiological phenomena under investigation. These BL features, associated to instrumental and technical advancements in miniaturization, enable the analysis of small-volume samples, which leads to the development of miniaturized and high-throughput assays.

Bioluminescence imaging in vivo

Bioluminescence in vivo imaging The commercial availability of ultrasensitive imaging systems based on charge-coupled device (CCD) technology together with the high number of BL probes greatly expanded the use of BL in a variety of imaging formats and techniques, spanning from Petri dishes to microtiter plates and to whole-animal imaging. Usually the spatial resolution of the BL signal is in the order of 100–200 μm and may reach 0.4 μm when in combination with optical microscopy, thus similar to that achieved with conventional light imaging.[27] Optical imaging makes it possible to reveal cellular and molecular events in real time, thus tracking biological processes in living animals with a significant reduction in the number of animals needed. Diverse imaging strategies have been developed and successfully employed to study tumour progression and metastasis, infectious pathways of viruses, gene expression patterns, graft-versus-host diseases.[28-31] Thanks to high sensitivity and the availability of new red and BL proteins emitting in the near infra-red (whose emission is scarcely absorbed by animal tissues) in vivo BL molecular imaging is emerging as one of the leading imaging technologies in the areas of cancer biology, cell biology, gene therapy and stem cell research. Moreover, the advancements in molecular biology allowed to obtain organisms in which the expression of a BL protein is under the control of tissue-specific regulatory elements, allowing non-invasive imaging of

selected physiological or pathological processes. Furthermore, BL tomography enables 3D light source reconstruction to obtain, for example, tumor shape in small animals. However, many factors have to be considered when a BL molecular imaging experiment has to be settled up, for instance optical properties (scattering and absorption properties) and thickness of the tissue through which photons are travelling. Haemoglobin is the main chromophore within tissues absorbing light in the visible spectrum (400 to 760 nm) but, if the animal is pigmented, also melanin contributes to light absorption. Haemoglobin light absorption is significantly lower at wavelengths longer than 600 nm. Unfortunately, wild-type BL proteins usually emit in the blue-green spectral region and, therefore, much effort has been recently put to develop of red and near-infrared emitting BL proteins for in vivo imaging applications. Bacterial, firefly and *Renilla* luciferases are the most used BL reporter proteins in whole-body imaging. Bacterial luciferase is the only reporter protein that allows the construction of self-luminescent engineered organisms through the introduction in the cell of the whole lux gene cassette (luxCDABE), which contains the genes encoding both for luciferase and for the enzymes able to synthesize its BL substrate. Genetically engineered BL bacteria (*Escherichia coli, Vibrio cholerae, Salmonella typhimurium*) based on the luxCDABE system have been employed to localize tumors and metastasis in living animals. Such bacteria take advantage of the anaerobic microenvironment and the nutrient-rich growth conditions, being able to replicate in the necrotic central area of tumors.[32] Besides tumor and metastasis localization, self-luminescent bacteria may have others promising applications, for example monitoring of bacteria-mediated gene product delivery systems for therapy of solid tumors. Firefly luciferase is by far the most employed BL protein in several bioanalytical applications. Mutant luciferases with different spectral properties have been developed by random and site-directed mutagenesis of *P. pyralis* wild-type luciferase,[33] and a single amino acidic residue (Ser284) appeared to be the most promising for developing mutants with altered emission properties.[34] Besides emission color, also the thermostability of BL reporters is an important factor for in vivo imaging applications. Indeed, BL measurements performed in cell cultures and other in vitro assays are usually taken at room temperature, on the contrary in vivo imaging has to be performed at body temperature (37°C). A systematic study to investigate the thermostability of commercially available luciferases (FLuc+, CBGr68, CBRed, and hRLuc) was recently performed by Zhao et al, who reported a 34-nm

spectral red shift for the the firefly enzyme.[35] The study showed that for all the investigated luciferases the activity increased with temperature, and the luciferases with the highest increments were CBGr68 (6.4-fold) and CBRed (7.8-fold). Spectral profiles are also affected by temperature and the most evident change was observed for firefly luciferase, whose emission maximum shifted from 578 nm at 25°C to 612 nm at 37°C. A long-term continuous delivery system based on implanted micro-osmotic pumps was also developed to overcome one of the main pitfalls of firefly luciferase, the need for the BL substrate D-luciferin.[36] This system did not require repetitive injections of the BL substrate that, together with substrate pharmacokinetics, put constraints on intervals between image acquisition.

BLI- based mouse models for drug discovery and development

BLI has become a routine modality for use in cancer biology particularly suited for assessing tumor burden and metastatic spread. The most common use of BLI in cancer has been to assess mass and location of xenografted cells constitutively expressing luciferase, providing a robust strategy to monitor effectiveness of anti-tumor drugs in vivo. Whole body BLI using firefly luciferase allows semi-quantitative measurements of tumor load and progression, metastasis and treatment response. Due to the sensitivity of BLI luciferase-expressing tumor cells can be transplanted to at any orthotopic site within a mouse or rat and subsequent tumor development, progression, and possible metastasis can be monitored in a rapid and time-sensitive manner. Also BLI has proven very useful for the early detection of micro-metastases and minimal residual disease states in animal[37.38]. Apart from preclinical studies on cancer BLI-based mouse models are routinely used by pharmaceutic companies since they provide information on where a drug or compound takes part in a specific regulatory pathway related to the disease. A growing number of luciferase expressing animal models are (commercially) available for drug metabolism and toxicology, disease areas like oncology/angiogenesis, metabolic -and neurodegenerative diseases and inflammation[39-45]. That is consequently to the development of the methodology for producing reporter animals by efficiently integrate luciferase gene in the mouse genome under the control of a specific promoter. Luciferase expressing mouse models can highlight the mechanism of many regulatory sequences. BLI can be used to monitor inflammation by driving luciferase with inflammation-specific

regulatory sequences. For instance, Carlsen et al. generated mice expressing luciferase under the control of the regulatory sequences of the NFκB gene, which in its turn is under the direct control of TNFα, a key cytokine produced during inflammation. Using NFκB-luciferase mice, Carlsen et al.[46] monitored osteoarthritic inflammation induced by injection of bacterial lipopolysacharide, and quantified the therapeutic potential of dexamethasone treatment of the arthritic lesion. Moreover luciferase expressing animal models can be employed for studying drug availability and distribution[47].

Cell based assay in drug discovery and development

Cell-based assays include a variety of assays that measure cell proliferation, toxicity, production of markers,motility, activation of specific signalling pathways and changes in morphology.Many of these assays rely on reporter gene technology.

Due to signal amplification of cell-signalling cascades, reporter gene assays are very sensitive, and thus ideal forminiaturization; they have been widely applied in HTS formats[48,49]. Despite these advantages, such assays are based on signal-transduction events that occur downstream of receptor activation and require gene expression. This causes long response times, which span from hours to days for the whole analysis time, and the possibility of interference from other intracellular pathways. For these reasons, alternative approaches relying on the monitoring of the first activation step, e.g., receptor dimerization, for example fluorescence and bioluminescence resonance energy transfer (FRET and BRET) and split complementation assays have also been proposed to satisfy the demands of HT drug discovery[47-50]. Recent improvements in optical imaging instrumentation and the wide choice of bioluminescent and fluorescent probes fuelled the implementation of cell-based assays for high-content screening (HCS). Because a very comprehensive overview has recently been published by Zanella et al. this review will not deal with HCS[51]. Cellular screening still presents a variety of challenges, and key aspects of improving this early phase of the drug-discovery process seem to be predictability, automation, miniaturization, cost-effectiveness, high-speed, and multiplexing.The state of the art, and challenges and future directions,will be discussed in this review, together with an up-to-date overview of recent ameliorations and trends in cell-based assays for drug discovery.

The implementation of reporter gene assays in thedrug-discovery process enables in vitro investigation of ADMET (absorption, distribution, metabolism, elimination, and toxicity) properties well in advance of animal studies. The main ADMET-related genes that have beentargeted are those encoding for drug metabolizing enzymes, for example the cytochrome P450 family(e.g., CYP3A4, CYP2B6, CYP2C9, and CYP2C19) anddrug transporters (e.g., MDR1, MRP2, MRP4, BSEP,BCRP, and NTCP) whose expression is regulated at thetranscriptional level by many of nuclear receptors (e.g., PXR, CAR, GR, and PPARα)[52]. The key advantages of reporter assays are highsensitivity, reliability, convenience, dynamic range, and adaptability to high throughput-screening. The major weakness encountered in their use is the high variability of cell response, mainly caused by sample-aspecificeffects on cell vitality. To improve robustness, an internalor external reference signal must be introduced in order to correct the analytical response and separate the specific signal from nonspecific interferences. This can be easily achieved by introduction of a second reporter gene which is constitutively expressed and whose activity thus parallels cell vitality. Commercial dual reporter assays with bioluminescence detection were introduced commercially to address this issue but, being based on the measurement of firefly and Renilla luciferase in the same sample, they require addition of a reagent to stop one reaction before adding the second substrate. This inevitably increases assay cost and time. More interestingly, use of new BL and fluorescent proteins with altered emission properties enabled simultaneous monitoring of more reporters in the same cell.The use of reporter proteins emitting at different wavelengths facilitates separation of the analytical and the control signals and expands the applicability of these reporters to multiplexed cell-based assays.The major pitfall of reporter gene assays is the possible disengagement between changes in enzyme activity and its corresponding mRNA levels. For example Lim et al. reported that antibiotic rifampicin and the natural furanocoumarin bergamottin (from grapefruit juice) both activate CYP3A4 gene transcription, but enzyme activity is increased by rifampicin and reduced by bergamottin, which is able to covalently inactivate the enzyme[53].This disconnection can be hindered by reporter gene assays, which could thus be combined with other drug screeningassays to increase their predictability.

Multicolor luciferases for multiplexed analysis

HTS reporter gene cell-based assays for lead identification are usually performed in serial or parallel mode and few multiplexed assays have been reported to date. Although the concept of multiplexed screening is well appreciated for image-based technologies, it is emerging as a more feasible option for plate-based, homogeneous assays, because implementation is simple, accessible, and cost effective. An efficient drug-discovery workflow needs the development of reliable information-rich assays. Recently, a cell-based transactivation high-throughput luciferase reporter assay has been developed to identify potential cytochrome P-450 3A4 (drug-metabolizing enzyme) inducers. This 384-well multiplexed homogeneous assay was developed and validated for simultaneous detection of PXR transactivation and HepG2 cell cytotoxicity by combining as fluorescence and bioluminescence readouts. When its analytical performance was compared with that obtained with the conventional singleplex PXR transactivation assay (with separate toxicity assay), using four well-known PXR inducers, the reported EC50 values were not statistically different in either the singleplex or multiplex formats. The authors reported that switching from singleplex to multiplex reduced the overall number of cells by 29% and the consumable costs by 38%. Furthermore, use of cryopreserved cells with multiplexing and automation enabled elimination of a total of 92 processing steps (42% reduction)[54]. Some multiplexed reporter assays have been developed. For example, Nakajima et al. proposed a novel reporter assay system in which three luciferases that emit at different wavelengths (green, orange, and red) in the presence of the same substrate are used as reporter proteins. By using longpass filters and applying a signal processing algorithm, a cell-based monitoring system was developed for simultaneous evaluation of the expression of three different target genes within a cell, achieving a dynamic range of three orders of magnitude[55]. As an alternative, we proposed the combined use of secreted BL reporter proteins, for example Gaussia princeps luciferase, and intracellular proteins (the green-emitting P. pyralis luciferase and a red-emitting thermostable mutant of L. italica luciferase). We developed a multicolour cell-based assay for CYP7A1 and CYP27A1, the two main enzymes responsible for bile acid biosynthesis, and the secreted Gaussia luciferase was used as vitality control under the regulation of a constitutive promoter. The use of a secreted luciferase makes measurement of its activity straightforward, because its expression is measured simply by taking small aliquots of cell culture medium.

Besides, the combination of secreted and intracellular reporters in the same cell-based assay has the advantage of complete absence of interference between the two luminescence signals, which are measured in separate wells of high-throughput microplate formats[56]. This concept could be easily applied in the drug screening workflow to improve consistency of cell-based assay results and reduce cost and time.

Protein-protein interaction studies

In the postgenome era, the analysis of protein expression, protein structure and protein-protein interaction is a much harder task since a global perspective is necessary to understand the complex network of interactions involving proteins, nucleic acids, co-factors and other unknown actors that participate to biochemical and pathological processes. Diverse technologies, ranging from protein affinity chromatography to library-based methods (e.g., phage display, two-hybrid system), clustered together under the term “proteomics”, have been developed to investigate protein expression and function in cells and organisms. Among these, elegant approaches relying on Bioluminescence Resonance Energy Transfer (BRET) and split reporter protein complementation and reconstitution strategies have been employed to investigate protein-protein interactions and explore biological pathways[57]. Bioluminescence Resonance Energy Transfer is a nonradiative resonance energy transfer occurring between a light-emitting luciferase donor and an acceptor fluorescent protein. When donor and acceptor are brought into close proximity (1-10 nm) to one another and are properly oriented each other, the former transfers its energy to the latter, which then emits. Since light emission from the donor takes place at a different wavelength than that from the acceptor, the energy transfer can be easily detected by measuring the ratio of the acceptor to the donor emission intensities. Such ratiometric output allows to compensate for well-to-well aspecific signal variations (e.g., due to different cell numbers in each well or signal decay across the plate). Because BRET strictly depends on the molecular proximity between donor and acceptor, it is suitable for monitoring the activation state of any protein (e.g., receptor or transcription factor) that undergoes association or conformational changes as a consequence of ligand binding. For example, to evaluate receptor dimerization the two subunits of the receptor are genetically tagged

either with the donor (e.g., *Renilla* or firefly luciferase) or the acceptor (e.g., a green fluorescent protein variant). When the activation of the receptor brings the donor and acceptor in a favourable position, BRET will occur. This phenomenon can be either observed *in vitro* using purified proteins or directly within the cells where the fusion proteins were produced[58]. Differently from Fluorescence Resonance Energy Transfer (FRET), BRET does not require a light source, therefore it is not affected by photobleaching, light scattering or autofluorescence, and direct excitation of the acceptor cannot take place. The intrinsic low background of BRET should allow either detection of weak interactions and performing experiments with low concentrated proteins. As of today, several combinations of BRET formats and reagents are available for proteomics applications, including receptor research and mapping of signal transduction pathways[59]. The first application of BRET methodology goes back to 1999 and regarded the study of the dimerization of cyanobacteria circadian clock proteins[60]. Bioluminescence Resonance Energy Transfer has been successfully applied in the study of receptor dimerization, like the insulin receptor[61], and to the evaluation of the homo- and hetero-dimerization of opioid receptors in live cells[62]. Estrogen receptor homodimerization was studied with BRET and BRET^2, a BRET variant in which the energy transfer occurs between *Renilla* luciferase and a green fluorescent protein mutant (GFP^2)[63,64]. Extended BRET (eBRET) is also gaining popularity as a technique that allows the monitoring of protein-protein interactions in real time for many hours[65]. Split reporter-based BL imaging is a newly developed strategy for studying protein-protein and, more generally, intracellular interactions. It relies on the complementation-reconstitution concept, i.e., the spontaneous assembling (in some circumstances) of active/functional proteins from one or more polypeptide fragments. In the split reporter strategy, a BL protein is cleaved into N-terminal and C-terminal fragments and each fragment is linked to one of the target interacting proteins. When protein-protein interaction brings the two fragments close each other, the complete functionality of the BL protein is recovered (Figure 11). This approach works either via protein segment complementation assays through a non-covalent assembly or via intein-mediated reconstitution assays based on covalent binding. A number of reporter proteins have been used for split protein strategies, for example firefly luciferase, *Renilla* luciferase, GFP, β-galactosidase[66-69].

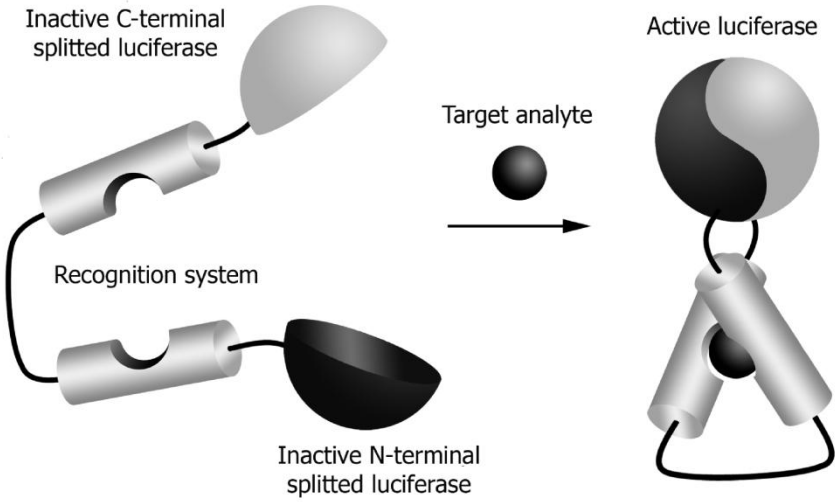

Figure 12: Schematic view of the split reporter-based complementation strategy. The N-terminal and C-terminal halves of a reporter protein (e.g., luciferase) are genetically fused to a recognition element (receptor, ligand binding domain) through a short peptide linker. The interaction with the target analyte causes a conformational change that recovers the reporter protein activity through the protein complementation of split N- and C-terminal fragments.

Recently a rapid screening assay based on a genetically encoded BL biosensor was developed to assess the androgenic effect of ligands by detecting the intramolecular association of the androgen receptor ligand binding domain (AR LBD)[70]. The authors demonstrated for the first time the possibility to use a single molecule-format BL probe to monitor cellular signalling steps. The AR LBD and the N-terminal domain of AR (AR NTD) where sandwiched between the dissected fragments of firefly luciferase. The association of AR LBD and AR NTD in the presence of androgens causes the complementation of the N- and C-terminal fragments of the luciferase, which partially recover its activity. Different chemicals known to possess agonist or antagonistic androgenic activity were assayed with this test, which was able to detect in a short time (20 minutes) as low as 10^{-5}M dihydroxytestosterone (DHT). Recently, Kim and colleagues also validated a single-molecule-format complementation system of split click beetle luciferase (CBLuc) to investigate protein-protein interactions. The ligand binding domain of the androgen receptor was connected to a functional peptide sequence via a flexible linker. This fusion protein was sandwiched between the dissected N- and C-terminal fragments of CBLuc. In the presence of androgens, the association between AR LBD and a functional pepdide causes the complementation of N- and C-terminal fragments of CBLuc. After 20-min stimulation

of the MCF-7 cells carrying the fusion construct with 10^{-5}M DHT luminescence intensities 29 times higher than a control were produced[671] A combinatorial screening approach was used to identify novel firefly luciferase split sites with improved characteristics over the previously published ones[72]. A total of 115 different combinations were screened and the fragments were characterized with five different interacting proteins and an intramolecular folding strategy. A novel firefly luciferase split reporter showing increased sensitivity and complementation was identified, with potential application for the study of protein-protein and other interactions in cells and animal models. An intramolecular luciferase complementation probe for the detection of specific RNAs vas developed by constructing a peptide-inserted firefly luciferase containing short RNA-binding peptide sequences[73]. The same principle was applied by Kanno et al. to investigate the release of proteins from mitochondria toward cytosol[74]. For this purpose, a target mitochondrial protein was fused to a N-terminal fragment of *Renilla* luciferase and a N-terminal fragment of DnaE intein and expressed in the mitochondria of mammalian cells. If, for some reasons, the genetically modified target protein is released from the mitochondria, it interacts with a C-terminal fragment of DnaE interin fused to a C-terminal *Renilla* luciferase present in the cytosol, thus reconstituting an active Rluc. This method allowed high throughput screening of chemicals able to increase or inhibit the release of mitochondrial proteins in living cells and small animals.

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Chapter 2

In vivo bioluminescence imaging of murine xenograft cancer models with a red-shifted thermostable luciferase

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Abstract

Purpose. Conventional in vivo bioluminescence imaging using wild-type green-emitting luciferase is limited by absorption and scattering of the bioluminescent signal through tissues. Imaging methods using a red-shifted thermostable luciferase from *P.pyralis* were optimized to improve the sensitivity and image resolution. In vivo bioluminescence imaging performance of these red- and green-emitting luciferases were compared in two different xenograft mouse models for cancer.

Methods. HepG2 (human hepatoblastoma cell line) and Thp1 (human acute monocytic leukemia cell line) cells were genetically engineered using retroviral vector technology to stably express the red-shifted or the wild-type green luciferase. A xenograft model of liver cancer was established by subcutaneous injection of the HepG2 engineered cells in the flank regions of mice, and a leukemia model was generated by intravenous injection of the engineered Thp1 cells. The cancer progression was monitored with an ultrasensitive charge-coupled device (CCD) camera. The relative intensities of the green- and red-emitting luciferases were measured and the resulting spatial resolutions of the images were compared. Imaging was performed with both intact and scarified live animals to quantify the absorption effects of the skin and deep tissue.

Results. The red-emitting luciferase was found to emit a bioluminescence signal with improved transmission properties compared to the green-emitting luciferase. By imaging the HepG2 models, which contained tumours just beneath the skin, before and after scarification, the percentage of light absorbed by the skin was calculated. The green bioluminescent signal was 75%±8 absorbed by the skin, whereas the red signal was only 20%±6 absorbed. The Thp1 model, which contains cancer cells within the bones, was likewise imaged before and after scarification to calculate the percentage of light absorbed by all tissue under the skin. This tissue was responsible for 90%±5 absorption of the green signal, but only 65%±6 absorption of the red signal.

Conclusion. Two different bioluminescent mouse cancer models demonstrate the utility of a new red-shifted thermostable luciferase, Ppy RE-TS, that improved the in vivo imaging performance when compared with wild-type *P. Pyralis* luciferase. While wild-type luciferase is currently a popular reporter for in vivo imaging methods, this study demonstrates the potential of red-emitting firefly luciferase mutants to enhance bioluminescence imaging experiments.

Introduction

Amongst the recent molecular imaging approaches, including Positron Emission Tomography (PET), Single Photon Emission Computed Tomography (SPECT) and Magnetic Resonance Imaging (MRI), Bioluminescence Imaging (BLI) with luciferase reporters is continuously gaining popularity and is largely applied in preclinical cancer research. BLI is often preferred because it provides a simple, sensitive, robust imaging modality with relatively cost-effective instrumentation. BLI and Fluorescence Imaging (FLI) systems are based on the use of bioluminescent reporter genes such as luciferases or fluorescent proteins expressed in cells (e.g., cancer cells, stem cells, bacteria) that are inoculated into small experimental animals and imaged in vivo with the use of ultrasensitive charge-coupled device (CCD) cameras, able to detect light emission even from deep tissues [1,2,3].Throughout the last decade, BLI has been used in xenograft animal cancer models to investigate the factors involved in malignant transformation, invasion and metastasis, and to examine responses to cancer therapy [4]. Such animal models are quite accessible to researchers, because they are easy to make and simple to use. In vivo BLI with these models allows for direct or indirect physiological imaging of specific cellular and molecular events. To initiate the luciferase reaction, luciferin substrate must be intravenously (i.v.) infused or intraperitoneally (i.p.) administered to the animal in sufficient concentration to saturate the reporter enzyme at the anatomical region of interest. Almost all luciferases require the administration of the substrate with the exception of bacterial luciferase, since the *lux* operon cassette codes not only for the luciferase but also for enzymes to produce the substrate. Unlike fluorescent reporter proteins, which require an excitation source, luciferase reporters generate a highly detectable BL signal with virtually no background noise, resulting in impressive imaging sensitivity. Luciferase from the North American firefly *Photinus Pyralis* provides a particularly strong signal, due to the high quantum yield (~0.41) of the luciferase/luciferin reaction [5]. The choice of luciferase in a BLI system is a crucial means by which to ameliorate and optimize the imaging technique. Though *P. Pyralis* luciferase is the most common choice, luciferase from the sea pansy *Renilla reniformis*, the green- and red-emitting luciferases from the click beetle *Pyrophorus plagiophalamus* and luciferase from the copepod *Gaussia princeps* have also been investigated [6,7]. Most of these enzymes emit in the blue/green region of the UV/vis spectrum, where light is strongly absorbed and scattered by tissues. Consequently, the imaging performance suffers

from poor sensitivity and spatial resolution. While green light is strongly absorbed by haemoglobin, melanin and other pigmented macromolecules, light greater than 600 nm in wavelength is less strongly absorbed [8], and can travel through living tissue over a much greater distance (1/*e* absorption length in tissues of ~ 2cm) [9]. Despite the advanced transmission properties of red-emitting luciferases, the red click beetle luciferase recently proposed for molecular imaging was demonstrated to be non-suitable for in vivo imaging due to its poor thermostability [10]. Recently, a thermostable red-shifted mutant of luciferase from *P. pyralis* was created by random and rational mutagenesis [11].This luciferase, named Ppy RE-TS, has an emission maximum of 612 nm at pH 7.0, a narrow emission bandwidth and excellent thermostability (the half-life at 37°C is 8.8 hrs, versus 0.26 hrs for wild-type luciferase). We report here the in vivo optical and luminescence properties of PpyRE-TS, and we present evidence for its potential to enhance BLI systems if used in place of, or in addition to, *Photinus pyralis* luciferase (WT). These two luciferase reporters were assessed for their applicability in visualizing cancer growth and for 2D localization imaging in two murine cancer models.A liver cancer mouse model, produced for solid tumour monitoring, was developed using HepG2 cells (human hepatoblastoma cell line) stably expressing WT or Ppy RE-TS. The cells were inoculated subcutaneously into the flanks and the upper backs of immunodeficient mice. A leukemia mouse model, produced for monitoring solid metastasizing tumours, was developed using Thp1cells (human acute monocytic leukemia cell line) stably expressing WT or Ppy RE-TS. These cells were intravenously injected into immunodeficient mice.The two luciferase reporters were used to visualize the cancer progression and distribution in both animal models over five weeks, so as to compare green- and red-emitting luciferase performance and to determine the best experimental conditions for in vivo BL imaging.

Material and Methods

Construction and generation of lentiviral particles encoding Ppy RE-TS or WT luciferase from *P. pyralis*

The previously reported pGex plasmid to express Ppy RE-TS (7) was amplified by PCR using the 3' primer-atc*ctcgag*atggaagacgccaaAaacat (*Xho*I) and the 5' primer-gct*agatct*ttactttccgcccTTcTTggc (*Bgl*ii) and inserted into the pMCSVneo plasmid (Clontech, Palo Alto,CA, USA) to create pMCSVPpyred-ts-neo. The *Xho*I and *Bgl*II restriction sites for used for cloning are shown in italics. For the construction of viral particles encoding the WT luciferase gene, the pMMPLuc-neo vector (kindly provided by Prof. A Kung) was used. Retroviral vector particles encoding WT or Ppy RE-TS were produced in the 293T packaging cell line by transient transfection with jetPEI transfection reagent (Polyplus Transfection, Illkirch, France). The viral stock was collected at 48h and 72h post transfection and filtered with a low-protein-binding 0.45 µM filter. The 293T cells were imaged with the low-light imager system LB981 (Berthold technologies, Bad Wildbad, Germany) after addition of D-luciferin (Beetle Luciferin Potassium Salt, Promega, Madison, WI, USA) at 72h to control the efficiency of transfection.

Generation of luciferase-positive hepatoblastoma and acute monocytic leukemia cell lines

Human hepatoblastoma (Hepg2) cells were cultured in DMEM (Sigma, St. Louis, MO,USA) with 10% fetal bovine serum, 2mM L- glutamine, 0.1mM non-essential amino acids, and 1% vitamin solution (Sigma, St. Louis, MO,USA). Human acute monocytic leukemia (Thp1) cells were cultured in RPMI1640 medium with 10% fetal bovine serum. All cultures were incubated at 37°C with 5% CO_2. HepG2 cell lines at 60-70% confluency were transduced by addition of 2 ml viral stock. Thp1 cells were transduced by spinoculation [12]. To facilitate vector penetration of the cells, 8 µg/ml of Polybrene (hexadimethrine bromide, Sigma, St. Louis, MO, USA) was added to virus-containing media. Cells were incubated overnight, then washed twice with PBS (phosphate-buffered saline: NaCl 137mM, KCl 2.7mM, NaH_2PO_4 1.4mM, Na_2HPO_4 4.3mM, pH 7.2). Cultures were grown for another 24h in the original media described above. Cell clones stably expressing luciferase were selected with 1 µg/ml G418 for 14 days. Positive HepG2 and Thp1 cell clones were termed HepG2-Luc or Thp1-Luc

when expressing WT luciferase, and HepG2-Ppyred or Thp1-Ppyred when expressing Ppy RE-TS.

In vitro bioluminescence measurements

Cell clones showing the highest BL emission were selected by the following intact-cell luciferase assay: 100 μl of cell suspension at a density of 10^6/ml was transferred to a white 96-well cell culture microplate and luminescence signals collected by adding 100 μl of the Luciferase Assay System substrate (Promega, Madison, WI, USA) using a spectral scanning multimodal plate reader (Varioskan Flash, Thermo Scientific, Whaltham, MA, USA). The selected luciferase-expressing clones were assayed for bioluminescence emission spectra, as well. A 100 μl sample of cell suspension (10^6/ml) was transferred to a 96 well microplate. After 100 μl of Luciferase Assay System substrate was added, emission spectra were collected from 500-670 nm by measuring the light output at 2 nm intervals for 1 s. Luminescence measurements were performed at room temperature (25°C).To prove that photon emission and viable cell count were linearly related, HepG2-Luc, HepG2-Ppyred, Thp1-Luc, and Thp1-Ppyred cells were each plated in triplicate in a series ranging from $1x10^6$ to $4x10^3$ cells diluted in PBS. After receiving 100 μl of Luciferase Assay System substrate, the cells were incubated for 5 minutes and then imaged for 1 minute with the same region of interest (ROI) used to measure luminescent signals of cells in each well.

Mouse cancer models

Animal experiments were approved by the Bioethics Committee of Bologna University in compliance with international guidelines. The liver cancer model was developed using five 14 week-old NOD/SCID mice purchased from Charles River laboratories (Wilmington, MA, USA). Subcutaneous xenografts were established by injection of $5x10^5$ HepG2-Luc and HepG2-Ppyred cells in the upper backs of the mice. For experiments monitoring both red and green bioluminescence in a single mouse, each mouse was injected also with 10^6 HepG2-Luc cells in the right flank and $2.4x10^6$ HepG2-Ppyred cells in the left flank. The leukemia animal model was developed using ten 6 to 10 week-old NOD/SCID mice. Each mouse was intravenously injected with 5 $x10^6$ Thp1-Luc or Thp1-Ppyred cells and the cancer progression was monitored every 7 days for a total of 5 weeks.

In vivo bioluminescence imaging

Mice anesthetized with 0.3 mg/kg body weight Zoletil 100 (Virbac s.r.l., Carros Cedex, France) were i.p. injected with 150 mg/kg D-luciferin (Synchem OHG, Felsberg, Germany) dissolved in PBS, and were imaged with the low-light imager system LB981. To investigate the kinetics of the in vivo reactions, mice inoculated 14 days prior with HepG2-Luc or Thp1-Luc cells were imaged for 20 minutes with sequential one-minute exposures. Mice were imaged for 20 minutes with sequential one-minute exposures during imaging sessions conducted immediately after i.p. injection of D-luciferin The collected images were used to generate kinetics plots for both luciferases, which were used to determine the optimal temporal windows for subsequent experiments (Fig. 3).Mice inoculated with HepG2-Luc and HepG2-Ppyred cells were placed in prone positions inside the instrument and imaged for three minutes, beginning 8 minutes after the luciferin injection. For each acquisition, the bioluminescent signal from the ROI was depicted as a pseudocolor image superimposed on a greyscale photographic image. Images of mice inoculated with Thp1-Luc and Thp1-Ppyred cells were collected both in prone and supine position for 3 minutes, beginning 8 minutes after the luciferin injection, and the luminescent signal was calculated as the sum of the two acquisitions. Data are reported as the photon flux (ph/s) from a total body ROI of 5000mm^2. At the end of the study, 3 mice of the HepG2 xenograft model, 3 mice of Thp1-Luc and 3 mice of Thp1-PpyRed xenograft model underwent scarification during the imaging sessions. Briefly, animals were anesthetized and injected with D-luciferin as described before, then mice were positioned inside the instrument and imaged for 1 min before and after scarification. The surgery was performed as follows: an incision was made on the back of the mice and the skin layer was removed, the same procedure was used for removing skin layer from the leg of the mice.

Quantitative analysis

The Winlight software version 2.9 of the low-light imager system LB981 was used to analyze and graphically present the BL data. Linear regression analysis was used to determine correlation between bioluminescence signal intensity and number of cells. For the HepG2 xenograft mouse model, bioluminescence intensity and tumour progression are reported as mean ± standard error of the mean (SEM) of the the ROI photon flux (ph/s) after background subtraction. For Thp1 xenograft mouse model,

bioluminescence intensity and tumour progression are reported as the mean ± SEM of the summed prone and supine signals (with subtraction of background for every acquisition) for every mouse. A total of 5 animals were used for HepG2 xenograft mouse model and 10 for Thp1 xenograft mouse model. Statistical analysis was performed using Graph Pad Prism v.5 for Windows, $p<0.05$ was considered significant.

Results

In vitro analysis of luciferases in transfected cell lines

Prior to developing the xenograft models, the Ppy RE-TS and WT luciferases were expressed and characterized in cell cultures for comparitive analysis. Expression of luciferase in Thp1-Luc and HepG2-Luc cell clones transduced with pMMPLuc-neo was monitored in whole cells as described in Materials and methods. Cell viability was monitored with a trypan blue exclusion assay. The bioluminescence emission spectra of intact cell clones are shown in Figure 1. HepG2-Luc cells produced an average signal of 2000 relative light units (RLU), at an emission maximum of 562 nm, whereas HepG2-Ppyred produced an average signal 1000 RLU at an emission maximum of 612 nm. At the same emission maxima, Thp1-Luc cells emitted $2x10^4$ RLU and Thp1-Ppyred cells emitted 600 RLU. The lower BL emission intensity of Ppy RE-TS in both cell lines can be attributed to the lower specific activity, which was approximately 15% that of wild-type luciferase when measured in pure proteins by Branchini et. al. [11]. Nevertheless, this relatively low specific activity is balanced by the extremely high thermostability of the enzyme in both cell lines. This confirms data (37°C half-life=8.8 h) previously reported by Branchini et al., which were obtained using the purified enzyme [11].

Correlation between cell number and photon emission in vitro. Dilution series of selected clones stably expressing WT luciferase or the Ppy RE-TS mutant were imaged in triplicate as described in Materials and methods. The linear relationship between the viable cell count, determined by a trypan blue exclusion assay, and BL signals for each cell clone (HepG2-Luc, Thp1-Luc, HepG2-Ppyred and Thp1-Ppyred) is reported in Figure 2. The correlation coefficient was greater than 0.98 for all cell clones. Using the same quantity of cells, the highest BL intensity was obtained with the Thp1-Luc, followed by HepG2-Luc, HepG2-Ppyred and Thp1-Ppyred.

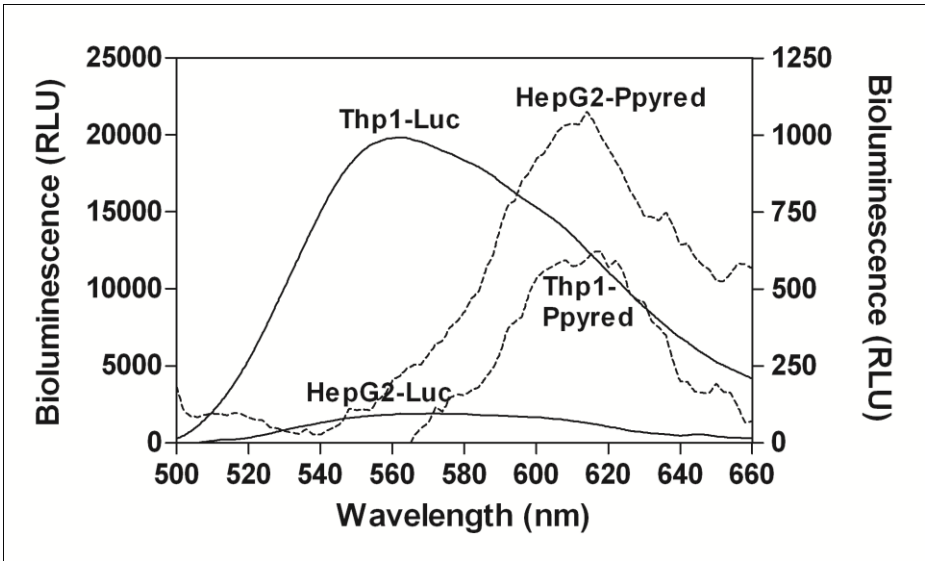

Figure 1. Emission spectra of green-emitting Thp1-Luc and HepG2-Luc clones (straight line, left Y axis) and red-emitting Thp1-Ppyred, and HepG2-Ppyred clones (dot line, right Y axis). Spectra were obtained by addition of Luciferase System Assay substrate to $1x10^5$ intact cells resuspended in PBS in 96-well microtiter plate. Each experiment was performed in triplicate and repeated three times.

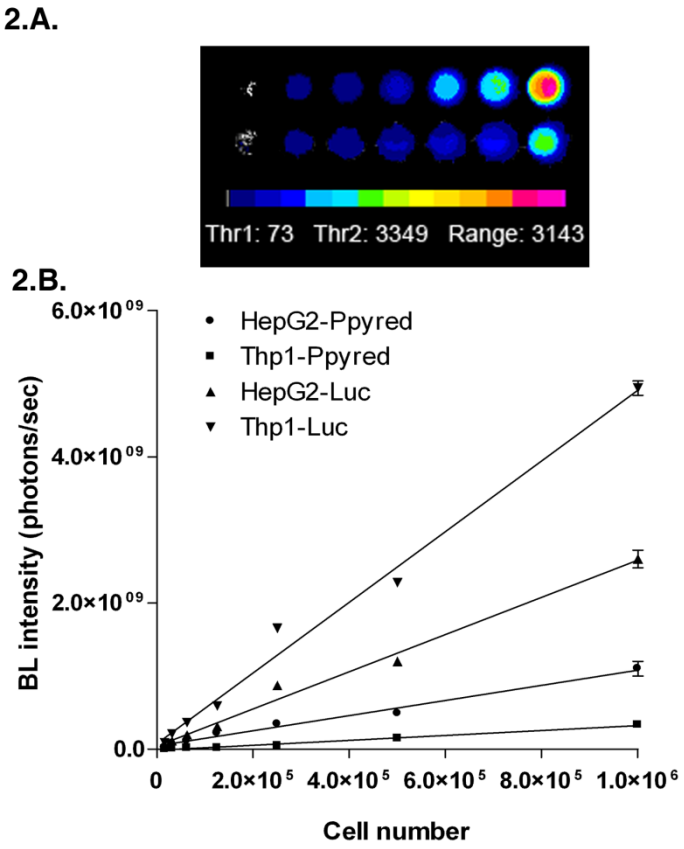

Figure 2.A. Pseudocolor image of flux values in photons per second for serial dilutions of HepG2-Luc and Ppyred ranging from $1.2x10^4$ to$1x10^6$ cells **2.B.** Correlation between the number of cells and

luminescent signals for each cell clone: HepG2-Luc, Thp1-Luc**,** HepG2-Ppyred, Thp1-Ppyred. (R^2> 0.98, P<0.001, n=3)

BLI of the hepatoblastoma (HepG2) xenograft murine model

In vivo comparison between green- and red-emitting luciferases. The difference between red and green light transmission through tissues was evaluated first by inoculating individual mice with two cell cultures, 10^6 of HepG2-Luc cells in the right flank and $2.4x10^6$ of HepG2-Ppyred cells in the left flank. These cell counts were chosen based on the previously described in vitro experiments, which indicated that 10^6 of HepG2-Luc cells and $2.4x10^6$ of HepG2-Ppyred cells bioluminesce with a similar intensity and showed a mean signal of 9,2±0,5 x 10^6 ph/s/mm^2 when detected with the imager. Using one animal with two inoculations made it possible to normalize the initial BL intensity of inoculated cells and thus compare the green and red light transmission through the skin and fur. The whole animal BL emission from the HepG2-Ppyred cells was much higher than that obtained from HepG2-Luc, with a mean recovery of the red photons(2,3±0,3 x10^5ph/s/mm^2) three times higher than that of the green photons (7,8±0,3 x10^4ph/s/mm^2). These results are consistent with previous experiments, wherein mice were injected with nude plasmids encoding WT luciferase and a red-emitting luciferase (S284T)[13].

Serial BLI of tumour burden and substrate-mediated light emission kinetics. The tumour progression and the performance of Ppy RE-TS in comparison with WT luciferase were studied by subcutaneous injections in the flank regions and the upper back of each mouse. Rather than normalizing the cell quantities according to in vitro results, as in the protocol above, HepG2-Luc cells (right flank) and HepG2-Ppyred cells (left flank) were injected in similar quantities ($5x10^5$). The imaging conditions were optimized by adjusting the temporal window of measurement according to the emission kinetics. The kinetic experiments, performed as described in Materials and methods, indicated that the maximum signal intensity is reached 8-12 minutes following the i.p. injection of D-luciferin, followed by a slow decrease of the luminescent signal(Fig. 3). Accordingly, BL images were collected 8-12 min after the i.p injection, which was performed every seven days for five weeks to track cancer progression. By day 7 the BL signal was clearly visible for both the WT and Ppy RE-TS luciferases (Fig. 4), confirming the suitability of the red-emitting enzyme for monitoring early tumour onset and progression. Furthermore, after 20 days the red-

emitting luciferase exhibited an 5-fold greater BL intensity with respect to the WT luciferase.

Scarification studies to compare red and green signal absorption in vivo. To quantify the percentage of light absorbed by the skin, HepG2 mice were imaged before and after scarification. We calculated that 75±8% of the light generated by WT luciferase was absorbed by skin while only 20±6% of the light generated by red-shifted luciferase was absorbed. data reported the mean percentage of six different red and green tumor. This result is in agreement with previous evidence that light emitted from red-shifted luciferase passes through tissues three times more efficiently than does green light [13]. Additionally, the tumour mass evaluation after scarifying the animals revealed differences in tumour dimensions: the red-emitting tumour produced by $1x10^5$ HepG2-Ppyred cells was two times bigger than the green-emitting tumour produced by $1x10^5$ HepG2-Luc cells. The higher replication rate of the selected HepG2-Ppyred clone resulted in superior tumour detectability both at tumour onset and during progression. For example, at day 21 the red BL was $6x10^7$ photon/sec whereas the green BL was $8x10^6$ photon/sec.

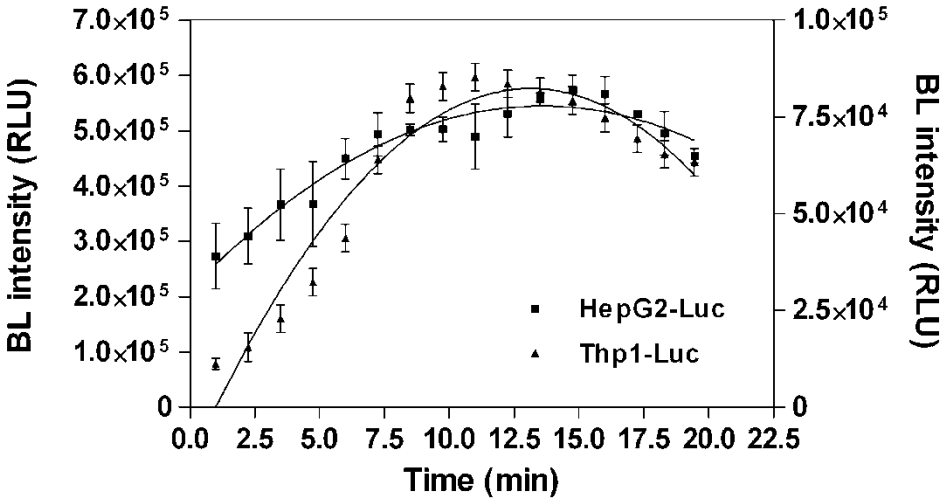

Figure 3. Kinetics measurements of in vivo emissions after intraperitoneal injection of D-luciferin 150 mg/Kg performed by collecting images with 1 minute of exposure for 20 minutes for both xenograft cancer models.

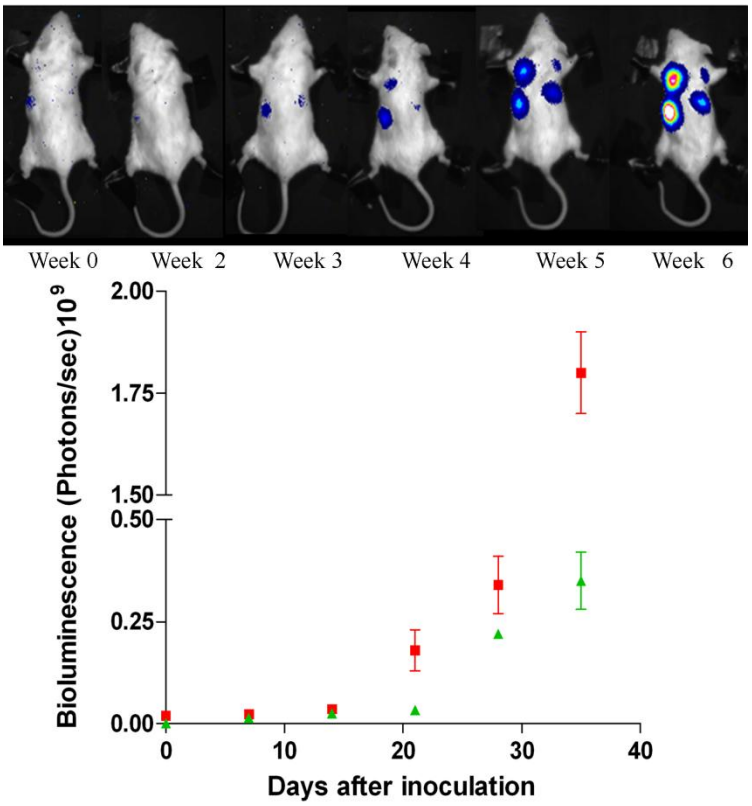

Figure 4. Serial BL imaging of mice inoculated with HepG2-Luc (on the right side) and HepG2-Ppyred cells (on the left side). Subcutaneous tumor on the upper back are generated inoculating different amount of cells but with the same initial emission while tumour on the lower flanks are generated inoculating the same amount of cells. Pseudocolor images have been generated on a common scale.

BLI of the Acute Myeloid Leukemia (Thp1) xenograft murine model

In vivo comparison between green- and red- emitting xenograft models. Unlike HepG2 cells, Thp1 leukemia cells metastasize to unpredictable locations, [14] so it was not possible to study both red- and green-emitting tumours simultaneously in a single mouse. Instead, mice were intravenously injected in the tail vein with $5x10^6$ Thp1-Luc or $5x10^6$ Thp1-Ppyred cells. After 14 days, solid tumours were detected in both groups of mice, and kinetics experiments were performed to optimize the imaging method for subsequent experiments. Following intraperitoneal injection of D-luciferin, the luciferase emission kinetic plot for Thp1-Luc reaches a plateau within 10 min and slowly decreases after the 15 minute point (Fig. 3). Therefore, the selected temporal window was from 8 to 15 min, during which both prone and supine images were acquired (Fig.5). The optimized D- luciferin administered dose was 150 mg/kg

as previously reported [15,16]. Mice were imaged during this 8-15 minute window every seven days for five weeks. Tumour growth was tracked by measuring the increase in bioluminescence signal intensity over time (Figure 6). Primary sites of cell proliferation, determined by immunohistochemistry (data not shown), were bone marrow, femur or backbone while secondary sites were the humerus, spinal cord and, in one individual, the head.

Scarification studies to compare red and green signal absorption in vivo. To evaluate the differences between green and red light emission from bone after skin removal, imaging studies were conducted using three mice inoculated with Thp1-Luc cells and three inoculated with Thp1-Ppyred. Comparing the data from the skinless and intact mice, we calculated that 90%±5 of the green light and 65%±6 of the red light was absorbed by deep tissues when emitted from the bones. Data relative to calculations are reported in Table1.

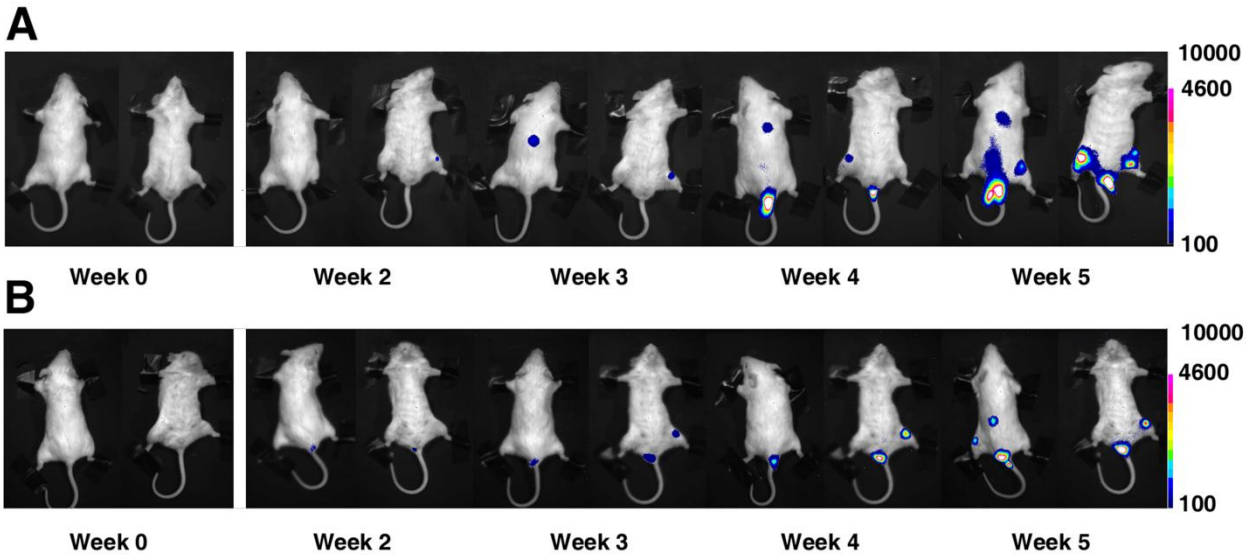

Figure 5. Serial BL imaging of mice inoculated with Thp1-Luc (Upper images) or Thp1 -Ppyred cells (lower images).

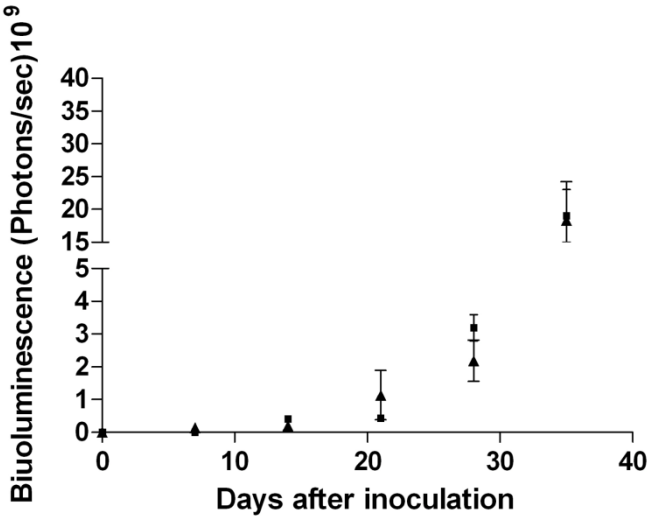

Figure 6. BL intensity signals revealing tumor progression in mice inoculated with Thp1-Luc (triangle)or Thp1 -Ppyred cells (square). Signals were consistent after 14 days, and showed a rapid increase after metastasis formation.

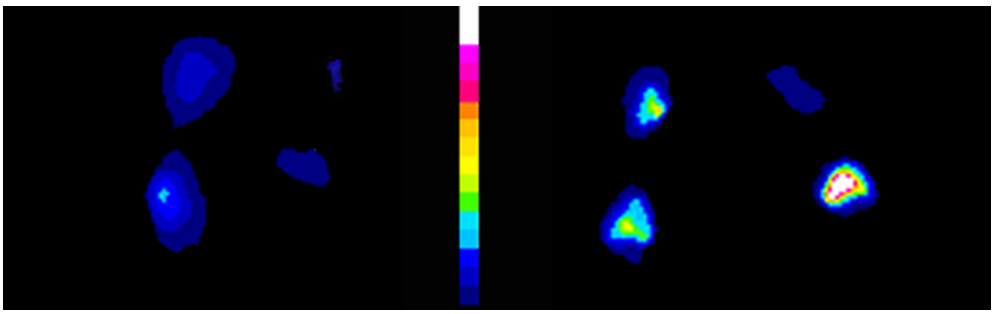

Figure 7. Pseudocolor images on a common scale of HepG2-Ppyred red tumours (on the left) and HepG2-Luc green tumours (on the right) before and after scarification.

Discussion

Seeking an improved probe for BL imaging, we compared the in vivo optical properties of Ppy RE-TS and WT luciferase in two xenograph murine models [17]. Two cell lines, HepG2 and Thp1, were transduced with retroviral particles containing the WT luciferase gene from *Photinus Pyralis* or the red-emitting mutant gene Ppy RE-TS. The long-term stability of luciferase expression in a medium without selective agents was confirmed by monitoring light emission over a 2-week period. For each clone (HepG2-Luc, Thp1-Luc, HepG2-Ppyred and Thp1-Ppyred) luminescence intensity and bioluminescence spectra were measured from intact cell suspensions to confirm the behaviour of WT and red mutant in whole cells as they are inside the

animals. The luminescence intensity of HepG2-Ppyred cells was 2-fold lower than the HepG2-Luc signal, and the Thp1-Ppyred signal was 33-fold lower than the Thp1-Luc signal (Fig. 1). These results in part reflect the relatively low specific activity of Ppy RE-TS (15% of WT) observed in pure protein assays [11].On the other hand different expressing cell clones are due to the use of different plasmid and method used to transduce cells and to G418 selection that bring to a random and possible multiple integration of the constructs inside cell genome. Yet our results also confirmed that cells expressing Ppy RE-TS have a red-shifted bioluminescence spectrum (λ_{max} = 612 nm) compared to cells expressing WT luciferase (λ_{max} = 562 nm) (Fig. 1). Because red light has a lower attenuation rate in living tissue compared to green light, we anticipated that the lower luminescence intensity of HepG2-Ppyred and Thp1-Ppyred in cell assays would be counterbalanced by the red-shifted spectra of these cells in vivo. We also predicted that the superior thermostability of Ppy RE-TS at 37°C could confer some advantage in the in vivo environment, though we did not perform controlled experiments to isolate this variable. In preparation for in vivo experiments, it was necessary to demonstrate a positive correlation between light emission and viable cell count. An impressive linear correlation was obtained for all four clones, validating the use of stable light emission from our models as a quantitative marker of tumour burden (Fig. 2) [18]. Also, we observed a ratio of 2.4 between HepG2-Luc and HepG2-Ppyred emission signals; this value was employed for normalization of light emissions in vivo. In vivo BLI experiments were performed with xenograft murine models for hepatoblastoma (HepG2 cell line) and acute myeloid leukemia (Thp1 cell line). While BLI would be most sensitive in nude mice, we chose white-coated NOD/SCID mice, since they more accurately represent typical xenograft murine models used in other laboratories. We optimized the in vivo imaging technique by performing preliminary kinetics studies (Fig. 3). Because the HepG2-Luc kinetics plot showed an intense and stable signal from 8 min to 15 min, a result consistent with published values [15,16], images were obtained in this temporal window after the injection of D-luciferin. The optical properties of the two luciferases in the hepatoblastoma model were evaluated by inoculation of HepG2-Luc and HepG2-Ppyred cells in the right and left flanks of each mouse, respectively (Fig. 4). By adjusting the injected quantities of red- and green-emitting cells to normalize the two bioluminescence signals, protein specific activity was eliminated as a variable. Immediately following inoculation, the bioluminescence signal produced by the red-

emitting cells was almost 60% higher than the light signal produced by the green-emitting cells, an advantage which can be attributed to the red-shifted spectrum alone. After five weeks the tumour masses were consistent, and 3 mice were imaged before and after scarification to determine the percentage of light absorbed by the skin. We calculated that 75%±8 of the light generated by WT luciferase was absorbed through skin while only 20%±6 of the light generated by red-shifted luciferase was absorbed. This result is in agreement with previous evidence that light emitted from red-shifted luciferase passes through tissues three times more efficiently than green light [13]. As expected, BL signal intensity depended not only on signal transmission, but also on the rate of tumour growth, which varied according to the replication rates of the cell clones selected. The HepG2-Ppyred cells, which showed the highest in vitro replication rate among the four selected clones, produced the brightest BL signal after 20 days. While the higher replication rate of the red-emitting clones was coincidental it illuminates another variable that can be optimized for BL imaging. Xenograft models for acute myeloid leukemia were developed to show the suitability of Ppy RE-TS for probing not only solid tumours, but also metastasizing tumours. NOD/SCID mice were inoculated with Thp1 cells by tail vein injection, and tumour progression was monitored by weekly imaging sessions for five weeks. After 14 days, solid tumours were detected in both groups of mice, with the BL signal localized in the bones (Figure 5). The period until tumour detection depends on the speed of critical cell mass formation and the strength of the in vivo BL signal. Images collected from Thp1-Ppyred model provided the first experimental evidence that the red emitting luciferase can be successfully used to develop xenograft cancer models since the tumor behaviour is analogous to the green one obtained with the conventional wild type luciferase. Moreover tissue absorption of the green and red light emissions was compared by performing BL imaging with and without skin removal, as described in Materials and Methods section. Three Thp1-Luc and Thp1-Ppyred mice were imaged before and after scarification to calculate the signal lost due to tissue attenuation alone. The tissues absorbed 90±5% of the green light generated from the cancerous bones, but only 65±6% of the red light was absorbed. These calculations are further evidence that Ppy RE-TS provides a superior imaging performance to WT luciferase not only when used immediately beneath the skin, but also when used for deep tissue experiments. Thanks to its

reduced scattering the Ppy RE-TS appears to be a viable alternative to WT luciferase for molecular imaging techniques.

Conclusion

After comparing the in vivo imaging performance of wild-type luciferase from *P. Pyralis* with the red-emitting mutant Ppy RE-TS from the same species, we found that Ppy RE-TS produced a more intense and less scattered bioluminescence signal [19]. This enhanced performance was observed in spite of the lower specific activity of Ppy RE-TS relative to the WT luciferase, and therefore attests to the advantage of using a red reporter instead of or in addition to a green reporter for in vivo imaging. An additional advantage that may have contributed to the success of Ppy RE-TS is its high thermostability at 37°C, though the effects of this variable were not isolated in this study. These results strongly suggest that although early xenograft murine models were developed using wild-type *P. Pyralis* luciferase,[20] and most of the published work has used this reporter, red-emitting luciferases could greatly improve BLI systems. The limitations that afflict BL imaging, mainly optical absorption and scattering by tissue, could be overcome by using red-emitting reporters for greater sensitivity. Improved light-collection methods may also advance emerging technologies such as BLI tomography and 3D in vivo imaging, which has not yet been performed with a red-emitting luciferase [21-23].Because the red- and green-emitting luciferases reported here emit light in well-separated spectral regions, it is also possible to use both reporters simultaneously, employing transmission filters to separate the two signals. As demonstrated in these studies, using two luciferases is a convenient method for visualizing two independent physiological events in one animal, since only a single substrate required for injection. To further tailor luciferases for in vivo applications, random and site-directed mutagenesis may be performed to increase enzyme activity or to codon-optimize the genes for greater expression in mammalian cells. Using enhanced luciferase mutants for bioluminescence in vivo imaging will render it an even more powerful tool for studying tumour growth, metastasis and, more generally, for conducting preclinical investigations in animal models.

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Chapter 3

A new gastric-emptying mouse model based on in vivo non-invasive bioluminescence imaging

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Abstract

*Background***:** Different techniques were used to assess gastric emptying (GE) in small animals; most of them require sophisticated equipment, animal sacrifice and are expensive. In the present investigation a simple, non invasive method based on bioluminescence imaging (BLI) is reported to study GE, using light-emitting *E. coli* cells as a marker of the gastric content.

Methods: A new thermostable red-emitting luciferase was chosen as reporter gene to transform *E. coli* cells. Bioluminescent (BL) bacteria were administered to fasting mice, after a solid meal, and in response to different doses of metoclopramide and hyoscine butylbromide. BLI allowed to evaluate the real time 2D spatial and temporal distribution of bacteria along the gastrointestinal tract in animals and to calculate GE rate in basal conditions and following pharmacological stimulation.

Key Results: The administered BL bacteria were easily imaged and localized in the stomach and subsequently followed in the duodenum and upper intestine allowing to accurately calculate GE. GE after the test meal was significantly slower ($T_{1/2}$ 16±3 min) than that obtained in fasting conditions ($T_{1/2}$ 2±1 min); administration of hyoscine butylbromide (1 mg/Kg b.w.) significantly ($p<0.05$) increased $T_{1/2}$ that was delayed up to 25±4 min; metoclopramide (1 mg/Kg b.w.) significantly ($p<0.05$) accelerated $T_{1/2}$, that was achieved within 8±2 min.

Conclusion and Inferences: The reported model is simple, inexpensive, reliable, sensitive and accurate; it can detect both acceleration and slowdown of GE. The model is useful in the investigation of new drug-induced alterations of gastric motility allowing to reduce the number of experimental animals.

Introduction

The mechanisms regulating gastric emptying (GE) are very complex. Abnormal GE often results from impaired myogenic, intrinsic or extrinsic neural, hormonal or central control mechanisms [1-4]. Pathophysiological studies of gastrointestinal motility and the development of drugs acting on the gastric organ motor function require the availability of suitable *in vivo* animal models. The ideal animal model for drug screening should be minimally invasive, reproducible and follow the 3R's roles: replacement, reduction, refinement using as few animals and as responsibly as possible.Mice have been recently re-examined as small animals suitable for gastrointestinal motility studies [5-9] and the more recent availability of transgenic mice largely stimulates their choice [10,11].Different techniques have been used to assess GE in small animals [12-20] and most of them need animal sacrifice to analyze, in the gastric and duodenum content, radioactive beads [18] or marker dyes such as phenol red with spectroscopic techniques [16]. A less invasive technique is based on $^{13}CO_2$ breath analysis in mice after administration of ^{13}C-labelled substrates such as 1-^{13}C-octanoic acid and ^{13}C-acetate as markers of gastric emptying [19,21-23]. This method is minimally invasive but requires a special cage for breath collection over time and isotope ratio mass spectrometry.Imaging techniques are potentially more powerful and scintigraphy and magnetic resonance imaging have been proposed [20, 24-27] in intact animals as well as X-Ray imaging [9]. The application of these technologies for routine screening of drugs in laboratory animals is impractical due to expense and/or slow throughput. An ideal marker for GE evaluation should have peculiar features: firstly it must not interfere with GE rate, it must not adhere to stomach wall or be absorbed by gastrointestinal tract, it must be stable in gastric juice and it should have good imaging properties. We envisaged the possibility to use bioluminescent (BL) cells that, once orally administered, follow the fate of the gastric content and provide a real time image of the kinetics of the gastric content release into the duodenum and upper intestine. We employed for the first time functional in vivo molecular bioluminescence imaging (MBLI) to calculate the GE half-time in control mice and to evaluate the effect of drugs. The light emitted by the cells in part penetrates through tissues and is imaged with an ultrasensitive charge coupled device (CCD) camera. The methodology to prepare bioluminescent cells is well established and instrumentation for MBLI is relatively simple and economic when compared with MRI, PET or scintigraphy. The BLI technology has been recently

successfully applied for the development of xenograft cancer animal models [28,29] providing similar performance to microPET [30]. Here we report the development and validation of a new non invasive mouse model for GE rate evaluation using a bioluminescent non pathogen strain of *E. coli*, expressing a red emitting thermostable mutant of *P. pyralis* luciferase. GE rate was evaluated in physiological conditions and following pharmacological stimulation or inhibition with drugs known to accelerate [31] or to delay [32] GE.

MATERIALS AND METHODS

Bioluminescent bacterial cells

Competent *E. coli* strain JM109 (Invitrogen Corp., Carlsbad, CA, USA) were transformed with the plasmid pGex-6p-2 vector containing the red thermostable luciferase PpyRE8 mutant (GenBank, accession number GQ404465) under the control of Tac inducible promoter [33]. All reagents for bacterial cultures were obtained from Sigma (St. Louis, MO, USA).

In vitro experiments

Bioluminescence characterization of bacterial cultures. JM109 cells harboring pGex-6p-2-PpyRE8 were grown from a single colony in LB broth medium with antibiotic selection (ampicillin 100 μg ml^{-1}) at 37°C overnight and diluted in LB medium to midlog phase (A_{600nm} 0.4). Cultures were induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated at 37°C in an orbital shaker for 1h until an A_{600nm} of 0.6 or grown in the same conditions w/o IPTG. Bioluminescent emission of induced and non induced bacterial cells was measured with a LB 981 NightOWL imaging system (Berthold Technologies, Bad Wildbad, Germany). Briefly, 100 μL of bacterial suspension was imaged for 1 minute immediately after addition of 100μl of D-luciferin (Synchem, Felsberg, Germany) 1 mM in 0.1 M sodium citrate buffer solution at pH 5. Images were collected every minute for 60 minutes to monitor emission kinetics of the bacterial culture. Each experiment was performed in triplicate, and individual experiments were repeated at least 3 times. Spectra of bacterial cells were recorded with a Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, CA, USA) using aliquots of *E. coli* cells expressing the red mutant.

Cells were grown at 37°C to A_{600nm} 0.4, supplemented with 0.1 mM IPTG to induce luciferase expression, and incubated for 2 h at 37°C. Samples were collected, and the A_{600nm} was adjusted to 0.9 using LB. Aliquots (200 µL) were transferred to a 1 mL cuvette, a 400 µL of PBS solution at pH 3.0, 4.0, 5.0 and 7.0 (adjusted with hydrochloric acid when necessary) was added together with 200 µL of 1 mM D-luciferin at pH 5.0 to mimic the in vivo model.

Preparation of bioluminescent cell suspension for oral administration. A 5 ml overnight culture of *E. coli* harbouring pGex-6p-2-PpyRE8 was diluted in 20 ml of fresh LB broth and grown at 37°C to A_{600nm} 0.4, then supplemented with 0.1 mM IPTG to induce luciferase expression and incubated at 37°C to A_{600nm} 0.8. An aliquot (200 µL) of cell culture was mixed 1:1 with D-luciferin solution 1 mM in 0.1 M sodium citrate buffer solution at pH 5.0; this 400µl mixture (containing 1.8x 10^8cells) was called “bioluminescent microbead suspension” and was prepared fresh for every imaging session.

In vivo studies

Animals. All experiments were approved by the Ethical Committee of the University of Bologna. A total of 51 male Balb/c mice (6 weeks old, 20-25 g/body weight) (Charles River, Calco, Milan, Italy) were used for a total of 141 experiments. The animals were housed 2 weeks before the experiments and fed the usual commercial diet and water ad libitum. They were kept at constant light/dark cycling throughout the study in wire-bottom cages and the experiments were started at 9 a.m. Mice were kept in wire bottom cages in the 18 hours previous to the experiments (one each cage) and after the experiments they were moved to solid bottom cages (3 each cage).

Determination of gastric emptying by bioluminescence imaging. The day of the experiment, after i.p. injection of Zoletil (Tiletamine and zolazepam 20 mg Kg^{-1} b.w., Virbac Laboratories, Carros, France) the animals were given 400 µL of the bioluminescent microbead suspension by oral gavage using a stainless bulb tipped gavage needle (20 gauge) (Instetech Solomon Inc, Plymouth, PA, USA). For fed mice, anaesthesia was given immediately after food ingestion and was effective within 5 minutes of the i.p. administration and lasted over 30-40 minutes. The imaging was performed using the LB 981 NightOWL imaging system linked to a PC

running WinLight 32 software (Berthold Technologies). Imaging began 15 min after the addition of D-luciferin to bacterial solution. Signal intensity was quantified as the flux of all the detected photon emissions within the region-of-interest (ROI) of the mouse body. Three animals were used to evaluate loss of signal due to skin and abdominal wall absorption by imaging before and after opening the abdomen. The grey-scale photograph of the mice obtained in the specimen chamber under illumination was overlain with a pseudocolor luminescent image showing spatial distribution of the emission. Data were expressed as photons/s/pixel. Since no significant differences in photon flux were recorded in different selected areas drawn inside the animal or outside of the animal, the background photon flux was defined from a ROI of the same size placed outside the animal. These figures were subtracted from the photon flux in each region to quantify relative luciferase activity as a measure of the amount of bacteria. Reproducibility of the model was investigated using 3 mice. After anesthesia, the animals were given the biomicrobead suspension and imaging started immediately. GE imaging was performed in the same animal at 3 days intervals for a total of 3 determinations for each mouse to evaluate intra-animal variability. Inter-animal variability was determined comparing the $T_{1/2}$ obtained from three different animals.

Evaluation of gastric mucosal integrity after administration of BL bacteria. Increasing concentrations of bacteria (in the range 100-10^9 cells mL^{-1}) were administered by gavage to 18 hours fasted mice to evaluate mucosal damage and/or inflammation. After the microbead suspension emptied from stomach into duodenum, gastric and duodenal mucosa were exposed and the samples were embedded in paraffin for hystological analysis.

In vivo evaluation of gastric emptying of the BL microbeads in fed versus fasting animals A cross-over design was used, each animal was its own control. Six animals were allotted to each group: group A, fed animals (F) and group B, fasting animals (NF); at period 1, A and B animals were used for gastric emptying studies in respectively fed and fasting conditions; in period 2, they were crossed over, respectively group A was used in fasting and group B in fed conditions. A total of 10 days elapsed between the two sets of experiments in order to return to conditions as far as possible similar to the previous ones (see Supporting Material 1). The animals (fed) were taught to eat the solid meal (100 mg Swiss Cheese, 0.36 Kcal, 28% fat,

27% protein, 5.4% carbohydrate) within 5 minutes of the administration. Food was withdrawn 18 hours before the experiment and water was allowed ad libitum. The day of the experiment, group A received the solid test meal and both groups after anaesthesia were given 400 µL of the bioluminescent microbead suspension by oral gavage.

Evaluation of gastric emptying of a solid meal in controls versus metoclopramide and hyoscine butylbromide treatment at different doses. Three groups of animals: C, D, E were used (6 animals each) respectively as controls (CON), metoclopramide (MET), and hyoscine butylbromide (HY) treatment. A cross-over study was adopted (Supporting Material 1 and 2).The wash-out time between each set of experiments was 10 days. Metoclopramide (Sigma) was dissolved in sterile water for injection to provide doses of 0.5-2.5 mg Kg^{-1}. Hyoscine-N-butylbromide (Sigma) was dissolved in sterile water for injection to provide doses of 0.5-2.5 mg Kg^{-1}. For the first set of experiments, 18-hours fasted mice were administered intraperitoneally either placebo (100 µL of saline solution) or metoclopramide (1 mg Kg^{-1}) or hyoscine butylbromide (1 mg Kg^{-1}) twenty minutes before the administration of the test. After eating, animals were anesthetized and 400 µL of "bioluminescent microbead suspension" were administered by oral gavage. Immediately after administration, the animals were imaged. Each animal was its own control: 3 groups of animals were studied (6 animals each group): CON, MET and HYO. Then dose-response studies were performed. In the first group 100 µL of saline was administered according to the protocol above reported; in the MET group, metoclopramide (0.5, 1 and 2.5 mg kg^{-1}) was administered in random order and similarly the HYO group received hyoscine butylbromide at the doses of 0.5, 1 and 2.5 mg Kg^{-1} in random order.

Statistical analysis

For every individual animal, the GE was determined as a function of time from the changes in total bioluminescent emission in the respective ROI, according to the formula

$$GE\%(t) = \left(1 - \frac{I_{stomach}(t)}{I_{total}(t)}\right) \times 100$$

where $I_{stomach}$ is the integrated photon flux within the stomach and I_{total} is the integrated photon flux within the region-of-interest (ROI) at each time point. For each animal, GE kinetics were described using a non linear regression analysis (sigmoidal Boltzman fit) [34] with Prism version 5.02 software (GraphPad Sofwtare, San Diego, CA, USA). The plateau asymptote was constrained to a value of 0. The gastric half-emptying time, $T_{1/2}$ defined as 0.5 GE%, was determined by interpolation. The goodness of fitting was evaluated by running a normality test (D'Agostino-Pearson) on the residuals. Differences between treatment groups were conducted using one-way ANOVA for multiple comparisons. All results are expressed as mean ± SEM, n= number of animals $p<0.05$ was considered statistically significant.

RESULTS

In vitro characterization of the bioluminescent probe The selected BL probe for GE evaluation is based on a luciferase mutant that has two important features that improve the performance of BL in vivo imaging: high thermostability at 37°C and emission in the red region of the visible spectrum. Different culture conditions were evaluated to obtain a standardized cell population with a stable steady-state light emission after substrate addition in the temporal window of GE imaging session. Bacterial cultures were induced with IPTG to increase luciferase expression and the identified optimal temporal window was 20 minutes; BL signal remained stable for at least 1 hour (Figure 1). The effect of pH on PpyRE8 BL intensity and spectrum was also evaluated. As shown in Figure 2.A., at different pH the normalized BL emissions of PpyRE8 showed an unchanged spectrum with λ_{max} 614 nm [33]. On the contrary, the green emitting wild-type luciferase shows a red-shifting and reduced intensity at low pH; this could introduce a bias during imaging of GE since red light is less absorbed and scattered by biological tissues. The kinetic profile remains unchanged with two emission maxima, one after substrate addition and the other after 25±1 min after substrate addition (Figure 2.B).

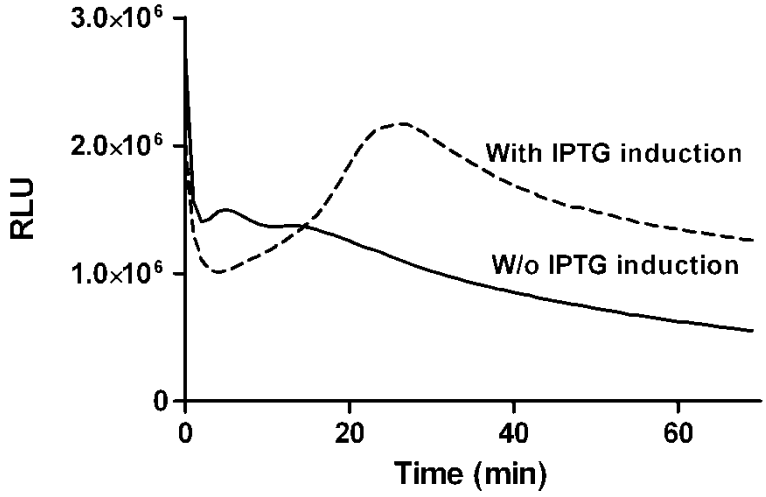


Figure 1: Emission kinetics of JM109 *E. coli* bacterial cultures expressing the PPyRE 8 luciferase under the regulation of a strong IPTG inducible tac promoter after D-luciferin 1mM addition with or without IPTG induction.

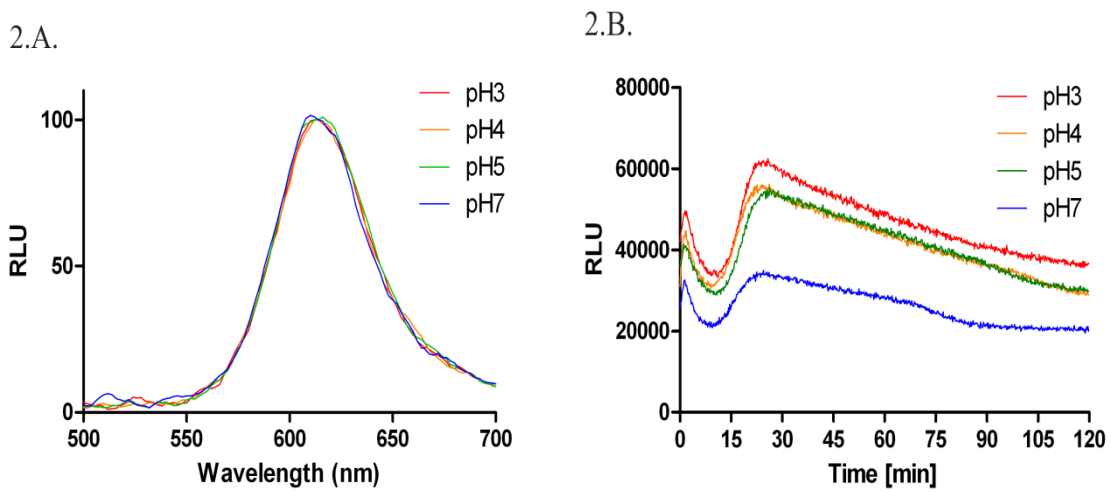


Figure 2: Normalized emission spectra of the "bioluminescent microbead suspension" obtained by mixing bacterial cells expressing PPyRE 8 with D-luciferin at pH 5.0 and a fixed volume of PBS solution at different pH to mime the stomach conditions. Spectra were recorded immediately after D-luciferin addition (**2.A**). Emission kinetics of the "bioluminescent microbead suspension" at different pH (**2.B**).

Determination of GE . The method includes the acquisition of images of the live animal after oral administration of the BL microbeads and BL substrate. Performing repeated bioluminescence image acquisitions on mice was very feasible. The loss of signal due to absorption by skin and abdominal wall was 78%±5, as calculated in Material and Methods section. However, this did not prevent a reliable localization and quantification of the signal. A light anaesthesia with Zoletil was selected to keep the animal still under the CCD imaging device thus avoiding animal handling during the GE monitoring. The high signal-to-noise ratio of bioluminescence allowed to define and draw the ROIs corresponding to the stomach area (Figure 3). GE was clearly assessable on sequential images were the microbeads are released in the duodenum and along the entire intestine (see Supporting Material 3). The basal $T_{1/2}$ evaluated with three separate experiments using the same animal was 17±2 min and the basal $T_{1/2}$ of three animals was 17±4 min, thus demonstrating a good reproducibility.

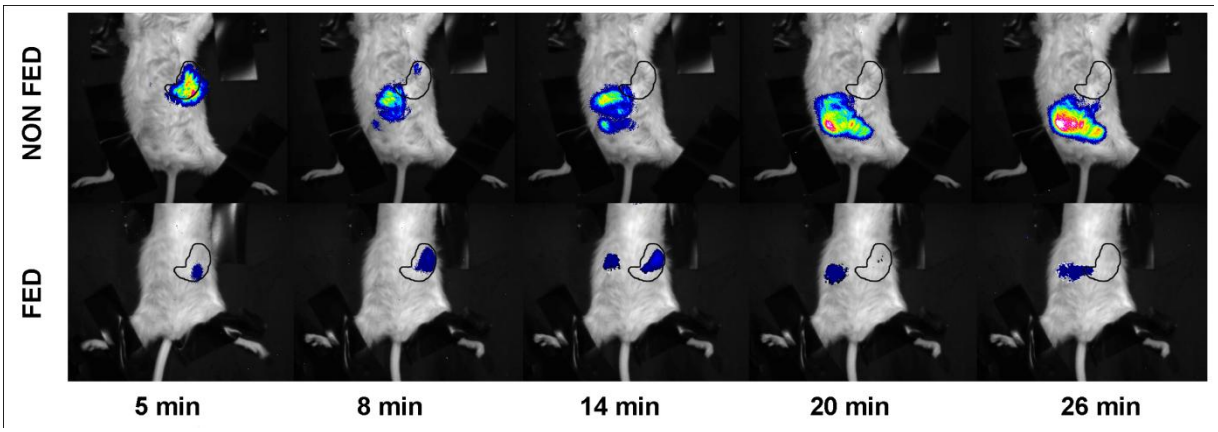


Figure 3: Gastric emptying imaging of fed and non-fed mice using the "bioluminescent microbead suspension". Images were collected every 3 min with exposure time 1 min from 5 to 30 min after food ingestion. A $T_{1/2}$ of 16±3 min has been calculated for the administered solid meal.

Evaluation of mucosal integrity after administration of BL bacteria. The oral administration of bacterial suspensions did not lead to any alteration in the mucosa either in the stomach or in the duodenum. No signs of acute flogosis were apparent. The absence of luminescence from the stomach mucosa and microscopy evidence demonstrated that bacteria did not adhere to the stomach (data not shown).

In vivo evaluation of gastric emptying in fasting versus non-fasting conditions. In fasting conditions, luminescent bacteria appeared simultaneously in the stomach and in the duodenum area, indicating that GE was immediate, consistent with data reported in literature of GE of water [35].In fed animals, after a test meal was given, GE was significantly slower ($T_{1/2}$ 16±3 min) than that obtained in fasting conditions ($T_{1/2}$ 2±1 min). Results were similar before and after the wash out period (10 days), indicating both complete return to the initial conditions and reproducibility of the method (Figure 3).

In vivo evaluation of GE of a solid meal in controls versus metoclopramide and hyoscine butylbromide at different doses.

In control mice, basal $T_{1/2}$ was 17±4 min; administration of hyoscine butylbromide (1 mg Kg^{-1} b.w.) significantly ($p<0.05$) increased $T_{1/2}$ that was delayed up to 25±4 min; on the contrary, metoclopramide (1 mg Kg^{-1} b.w.) significantly ($p<0.05$) accelerated $T_{1/2}$, that was achieved within 8±2 min. BL imaging of mice control-metoclopramide-hyoscine butylbromide 1 mg Kg^{-1} is shown in Figure 4.

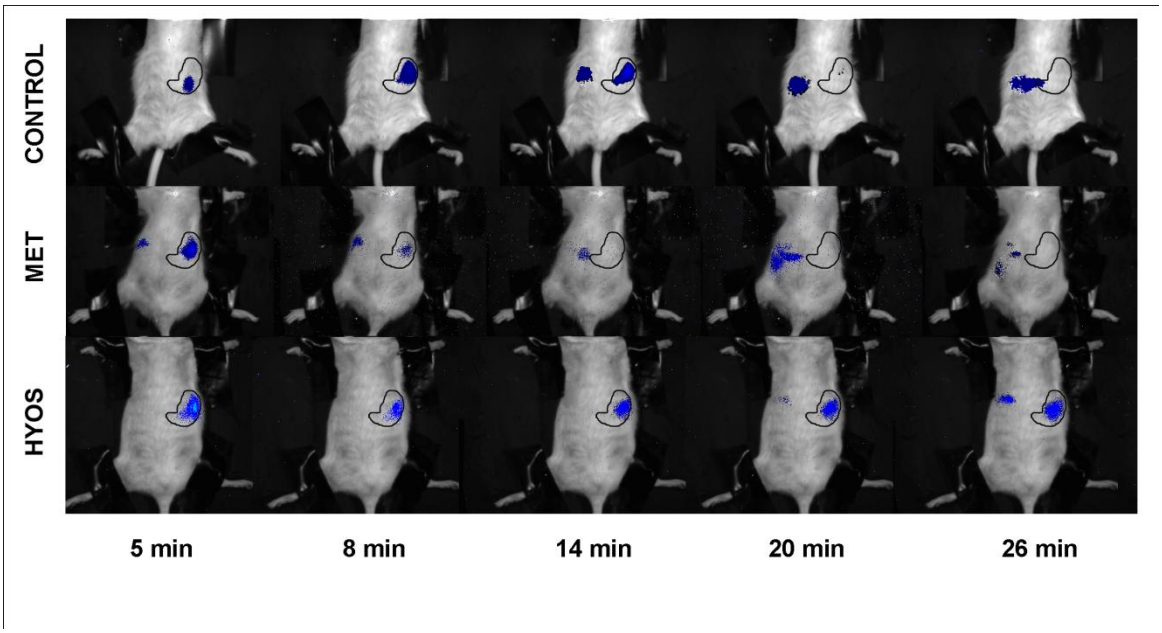


Figure 4: Gastric empting imaging of control mice or treated with metoclopramide (1 mg/kg b.w.) or hyoscine butylbromide (1 mg/kg b.w.) using the "bioluminescent microbead suspension". Images collected every 3 min with exposure time 1 min from 5 to 30 min after food ingestion. In control mice, basal $T_{1/2}$ was 17±4 min; in mice treated with metoclopramide $T_{1/2}$ was 8±2 min while in mice treated with hyoscine butylbromide was 24±5.

Dose-dependent effects of hyoscine butylbromide and metoclopramide on GE are shown in Figure 5. Gastric-emptying curves in fed mice treated with saline solution, metoclopramide 1 mg Kg^{-1} or hyoscine butylbromide 1 mg Kg^{-1} obtained from four to eight mice and the regression lines resulting from modelling the data to a sigmoidal Boltzman function are shown in Figure 6.

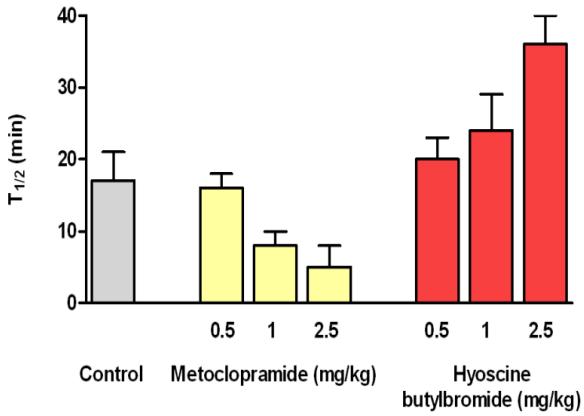


Figure 5: Graph representing $T_{1/2}$ variation in mice treated with different doses of metoclopramide or hyoscine butylbromide resulting from cross-over study. Data represent the mean ± SEM.

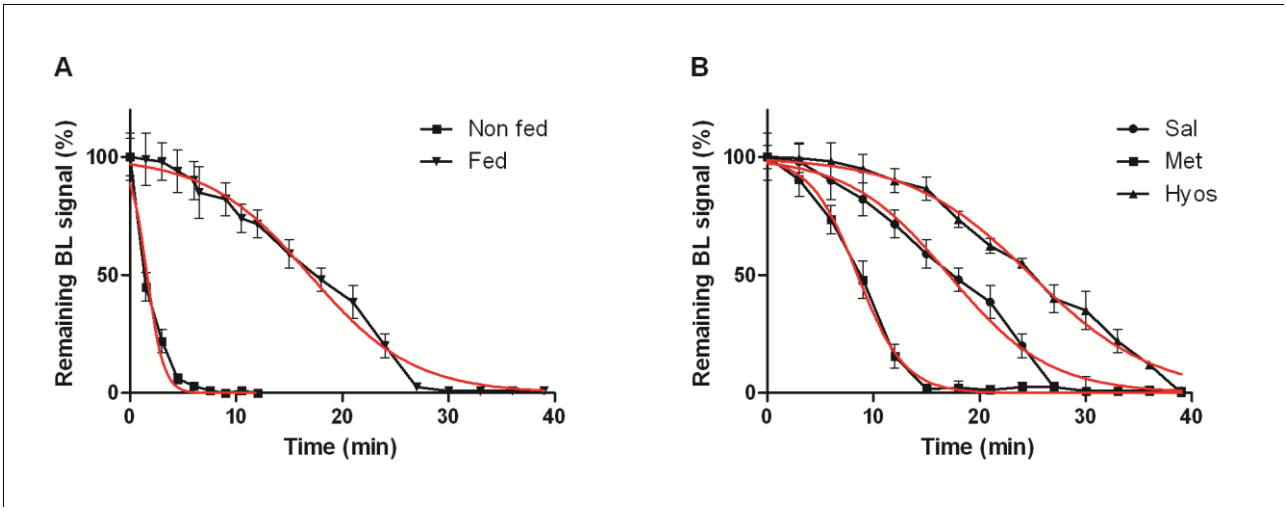


Figure 6. A. Gastric-emptying curves for fed ($T_{1/2}$ 16±3 min) vs fasted mice ($T_{1/2}$ 2±1min). **6. B.** Gastric-emptying curves in fed mice treated with saline solution ($T_{1/2}$ 17±4 min), metoclopramide 1 mg/kg ($T_{1/2}$ 8±2 min) or hyoscine butylbromide 1 mg/kg ($T_{1/2}$ 24±5 min). Drugs were administered intraperitoneally 20 min prior to meal. Each curve represents mean results (± SEM) obtained from four to eight mice. Also shown are the regression lines (red lines) resulting from modelling the data to a sigmoidal Boltzman function.

DISCUSSION

Choice and characterization of bioluminescent bacteria as GE probe We envisaged the possibility to use bioluminescent non-pathogen bacteria as self-luminescent microbeads. Bacteria are a low cost, easy to prepare, dispersed suspension of micrometer size beads that, after mixing with gastric content, can be used as a marker for GE studies. The advantages of bacterial microbeads were combined with reporter gene technology to develop a probe that, by means of in vivo imaging, provides a real time visualization of gastric content release into the duodenum. To characterize and standardize the model, in vitro studies were performed to investigate variations in bioluminescence emission intensity, wavelength and kinetics according to different pH conditions that are present in the gastrointestinal tract. Since green light is more absorbed and scattered by biological tissues, we selected a red-emitting probe (PPyRE8, a mutant of *P. pyralis* luciferase with λ_{max} 615 nm) to improve the performance of in vivo bioluminescence imaging in terms of sensitivity and spatial localization. This allowed an easier definition of ROIs than near Reflectance Imaging models, that in addition suffers from fluorescence emission background [34]. Besides, the selected luciferase mutant has very good thermostability (half-life at 37°C of 3.5 hrs *vs* 0.26 hrs of wild type luciferase), its emission is stable at different pH and its emission kinetics provide a stable and high BL signal during GE monitoring .This stability in terms of emission wavelength and intensity is very important for the reproducibility and sensitivity of the model to avoid misinterpretation of results due to variations in pH along the gastrointestinal tract.

Safety and stability of the BL microbeads. A prerequisite of a marker of GE is that it should not lead to gastric mucosal damage: the stomach which is in continuous contact with host microbial population has evolved an array of strategies for preventing bacterial invasion into deeper tissues. The pH of the stomach of mice is about 3 and 4 respectively in fed and fasting conditions and in the duodenum about 4.9 and 4.7, respectively in fed and fasting conditions. Bacteria survive within a range of pH between 3.0 and 6.0 [36, 37] and similarly the bioluminescent emission produced by the mutant luciferase inside bacteria is stable within this range. It should be pointed out that the introduction of bacterial cells in the stomach, an environment highly different from the laboratory culture conditions make them return to a lag phase. Since they are adapting to a new environment we assumed they do not

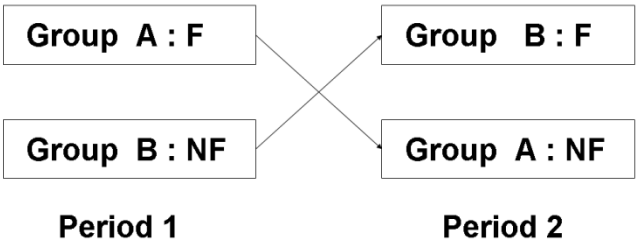
replicate during imaging session. Besides, endoluminal conditions such as oxygen content, number of bacteria and distance from the surface of light-emitting bacteria were taken into consideration for developing the model. These factors mostly affect the bioluminescent imaging in the duodenum, which is deeper and poorer in oxygen than the stomach. Nevertheless after 180 min bacteria present in the colon area still emit light (data not shown) and, with CCD with improved sensitivity, the complete intestinal transit process can be easily visualized. The use of living bioluminescent bacteria that continuously produce light provides a non toxic GE probe that emits light without the need for external light source. Other probes, such as self-illuminating quantum dots, cannot be used due to their in vivo toxicity.

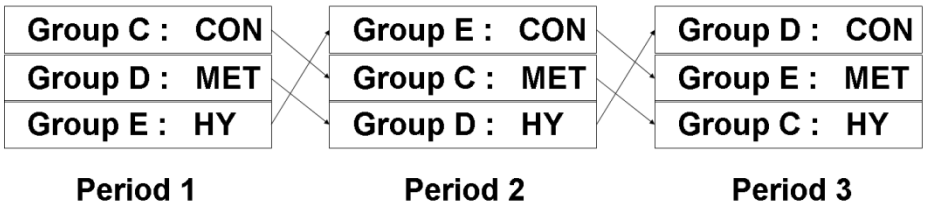
*GE bioluminescence in vivo model***.** The suitability and reliability of this technique to study gastric emptying is demonstrated by the fact that the bioluminescent signal is easily detected, visualized and analyzed. Furthermore the measurements obtained in different experimental conditions were consistent with each other. The microbeads empty from the stomach with a similar pattern in the same animal over time, and different animals show similar emptying rate. Thanks to their micrometer size (far less than 1 mm, the diameter of mouse pylorus), hydrodynamic properties and negligible caloric content (9 calories), bioluminescent bacteria cells mimic non nutrient liquid emptying. This finding confirms the results obtained by Zhao et al., who monitored by optical imaging the progress of *Escherichia coli*-GFP through the mouse gastrointestinal tract [38]. Differently from our model, which is based on oral gavage of 1.6×10^8 cells, they gavaged immunodeficient mice with 10^{11} cells to induce infection; in agreement with us they reported that stomach in non fed mice emptied within 10 min after gavage. In fed animals, the emptying rate is delayed due to mixing of the bioluminescent probe with the gastric content. This indicates that the bioluminescent bacteria can be considered indeed, as a probe to monitor the GE process. The present model has been also demonstrated to be sensitive enough to discriminate among pharmacological modulations of the GE: the prokinetic agent metoclopramide dose-dependently accelerates gastric emptying, while hyoscine butylbromide delays GE, in agreement with a previously published GE model based on optical imaging [34]. In the setting up of the model some variables were carefully considered, among which the meal, handling of the animal, and anaesthesia. A solid meal was preferred over a liquid one because it is more physiological and spontaneously eaten by the animals, allowing the best evaluation of the physiological

mouth to duodenum progression of the food and reducing the stress of the administration. The choice of a solid standard meal was also prompted by the necessity of reducing to a small volume, not exceeding 400 µL, the total volume of the bacterial medium and the liquid formula. The administration of a higher volume can lead to a painful distension of the small stomach of the animal and the reduction to a smaller volume could increase the viscosity of the formula, making difficult its administration through the small cross sectional diameter of the feeding needle (20 gauge). After 18 hours fasting, trained mice ate spontaneously and rapidly within 5 minutes of the administration of food. BL microbeads were not added to the food since mice didn't eat the mixture spontaneously and it took them more than 15 minutes to complete food ingestion. The subsequent immediate administration of the bacteria by oral gavage allowed the standardization of the starting time for imaging. As far as anaesthesia, since high doses of the commonly used isoflurane delay GE in animals and man [39], we have used a combination of zolazepam and tiletamine [40], to obtain a very light sedation of the animal sufficient to make it rest in the dark camera. Handling of the animals has been reported to be a moderate stress inducer thus altering GE therefore, once put in the camera, the animals were not moved throughout the whole GE monitoring. It was possible to take acquisitions every three minutes, resulting in enough time points for tight-fitting algorithms. The developed model thus combines a short monitoring interval, as breath test-based GE models with the possibility of visualizing the whole GE process and obtain quantitative data as with imaging techniques. Furthermore, the model does not require the use of radiopharmaceuticals as scintigraphy or MRI. Compared to a recently reported X-ray GE model that measures stomach volume as parameter to evaluate GE two weeks after the implantation of a gastric fistula, our model is much easier and rapid to perform, moreover there is no need for surgery on the animal and to employ ionizing radiations. Nevertheless, the method can not be used in intestinal inflammation studies because the presence of bacteria could increase the severity of inflammation. A drawback of the model is the need to induce sedation to mice, but this allows to keep the animal still for image acquisition. Alternatively, the model could be applied to non anaesthetised freely moving animals with a recently reported new imaging system [41] . Therefore, the model is a promising imaging tool in drug development and can be proposed in pre-clinical tests in pharmaceutical research and for pathophysiological studies (e.g., diabetics gastroparesis). Besides, we can extend

this model to other rodent species [42] and to transgenic animals (e.g., models of obesity and diabetes). Since an analogous photoprotein has been commercialized as a dietary supplement it is conceivable, in a near future, the use of an encapsulated formulation of purified luciferase-substrate as self-luminescent probe for GE monitoring in humans with non invasive bioluminescence imaging.

Supplementary Material





Supporting Material 1: Design of experimental protocol. A cross-over design was used. A total of 6 animals were allotted to each group: group A: fed animals(F] and group B: fasting animals (NF].

	N° of animals	N° of experiments
Reproducibility studies	3	9
Fed vs Fasting studies	12	24 cross over
Met, Hyos, Control studies	18	54 cross over
Drug response studies	18	54 cross over
Total	51	141

Supporting Material 2: Table of the experiments.

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Chapter 4

Sensitive dual color in vivo bioluminescence imaging using a new red codon optimized firefly luciferase and a green click beetle luciferase

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Abstract

Background: Despite a plethora of bioluminescent (BL) reporter genes being cloned and used for cell assays and molecular imaging purposes, simultaneously monitoring of multiple events in small animals is still challenging. This is due in part to the lack of optimization of the cell expression and spectral resolution of the used color-coupled reporter genes. A new red emitting codon-optimized luciferase reporter gene mutant of *Photinus pyralis*, Ppy RE8 has been developed and used in combination with the green click beetle luciferase, CBG99 for *in vitro* and *in vivo* dual color imaging applications.

Principal findings: Human embryonic kidney cells (HEK293) were transfected with vectors expressing red Ppy RE8 and green CBG99 luciferases. Populations of red and green emitting cells were mixed in different ratios. After addition of the shared single substrate D-luciferin, BL signals were imaged with an ultrasensitive cooled CCD camera using a series of band pass filters (20nm). Spectral unmixing algorithms were applied to the images obtaining a good separation of signals. Then, HEK293 cells expressing the two luciferases were subcutaneously injected in living mice achieving a reliable imaging and quantification of both BL signals in mixed population of cells. These results demonstrated for the first time the *in vivo* molecular imaging of two simultaneous BL signals after the injection of the same substrate (D-luciferin).

Significance: The applicability of Ppy RE8 firefly luciferase in combination with CBG99 click beetle luciferase was demonstrated both *in vitro* and *in vivo*. In respect to previously used dual assays, the present one combines a greater sensitivity thanks to an efficient cell expression with an adequate BL spectral resolution using a suitable algorithm for spectral unmixing. This new D-luciferin-dependent reporter gene couple opens up the possibility to do more accurate quantitative gene expression studies in *vivo* by simultaneously monitoring two events in real time.

Introduction

During the last decade, bioluminescent (BL) imaging has become an indispensable tool for visualizing molecular events at a cellular level both *in vivo* and *in vitro* leading to new advances and discoveries in life sciences[1]. There are many available BL luciferase/luciferin reporter gene systems for *in vivo* imaging: the first used and most popular are the luciferases that require D-luciferin and are ATP dependent (i.e. firefly luciferase, click beetle luciferase). Other luciferases such as Renilla luciferase and Gaussia luciferase that require coelenterazine as a substrate and are ATP independent are nowadays also used[2,3]. In addition, the use of the blue emitting(490nm) bacterial luciferases from *P. luminescens* have been reported[4]. Such luciferases do not require the infusion or administration of the BL substrate but are scarcely expressed in mammalian cells. The codon-optimized version of this luciferase has been recently proposed for *in vivo* imaging but cannot compete yet with firefly luciferase performance[5]. Renilla and Gaussia luciferases emit blue light which in part compromise their *in vivo* performance due to extensive light absorption by the small animal body. Blue light is strongly absorbed by tissue components particularly in highly vascularised tissues where haemoglobin is present[6]. In the case of Renilla, new red shifted and more stable mutants with an emission peak at 535 or 547 nm have been produced[7] by site directed mutagenesis, but dual color imaging still remains difficult to perform due to the relative low quantum efficiency of CCD cameras below 500 nm (30%) where the native enzyme shows the peak of emission. Until now there are no red shifted Gaussia luciferase mutants available but only brighter ones or with a prolonged half-life [4,8,9]. Red-emitting mutants from the railroad worm (*Phrixothrix hirtus*) with higher activity and better stability have been recently proposed for bioluminescence imaging but not fully studied for *in vivo* applications[10]. Regarding click beetle and firefly luciferases, variants with different emission wavelengths have been developed but still lack of optimal characteristics for *in vivo* imaging[11,12]. In particular, red/green couples of reporter proteins for *in vivo* applications must possess intense BL emission with narrow emission spectra resulting in a reasonable separation and with good thermostability at 37°C[12,13,14]. Codon-optimization of the reporter gene is a fundamental prerequisite for improving the BL signal in mammalian cells thus facilitating their detection *in vivo*[15]. Recently it has been reported that dual color BL imaging could be applied *in vitro* using appropriate filters for the separation of BL signals and mathematical corrections for their deconvolution[16,17]. Furthermore, *in vivo* applications using multicolor analysis can be achieved using different substrates or fluorescent proteins[18]. For a simultaneous *in vivo* detection of the fate of two set of cells here we report the use of a novel red codon-optimized luciferase reporter gene mutant of *Photinus pyralis*, Ppy RE8, combined with the green click beetle luciferase, CBG99. Ppy RE8 is characterized by a peak emission at 618 nm and has

an excellent thermostability (half-life of 4,5h at 37°C)[13]. CBG99 is a pH insensitive luciferase with an emission maximum at 537 nm which showed better performance for *in vivo* applications than the widely used *Photinus pyralis* wild type luciferase (PpyWT)[19]. Here , we demonstrate the applicability of the two luciferases *in vitro* and *in vivo* by generating lentiviral vectors for the expression of the genes under the control of the CMV promoter. Multicolor HEK293 cell based assays were developed to evaluate the suitability of simultaneous measurements of the red and green emitting luciferases by spectral unmixing. Both luciferases maintained the same spectrum of emission in cells at 37°C. We also show the applicability of the dual luciferases *in vivo* when HEK293 cells were inoculated subcutaneously in mice and imaged after intraperitoneal injection of D-luciferin. A good separation of the individual signals could be obtained using spectral unmixing algorithms for their deconvolution. Ppy RE8 was demonstrated to be an excellent tool for *in vivo* BL imaging and, in particular, when used in combination with a green luciferase to monitor dual events at the molecular level. The use of a single D-luciferin substrate for the same couple of reporter gene allows time and cost saving in contrast to dual-color luciferase imaging using Firefly and Renilla luciferases in which the addition of a second luminescent substrate, coelenterazine, is warranted.

Results

Emission spectral unmixing of bioluminescence in cell lysates

To evaluate the ability of the two red and green selected luciferase signals to be detected and quantified in a single run analysis using a single substrate, the red codon-optimized luciferase reporter gene Ppy RE8 and the green emitting click beetle CBG99 were expressed transiently under the control of the same promoter in HEK293 cells and lysed after 24 hours. For the same number of cells the light output of red emitting lysate was 2.5 higher than the one of green emitting cells (**Fig 1A**). This is expected since, as previously reported, CBG99 is as bright as CBred but with more stable emission kinetics[11]. Ppy RE8 has been reported to generate more luminescent signals than CBred in a cell-based assay[13]. Moreover, when cell lysates of the red or green expressing cells were plated in different ratios, calculations of the percentages of red and green light in a mixture were possible by applying the spectral unmixing algorithm to the acquired images (images acquired using a series of 20nm band pass filters) as shown in **Figure 1B**. In this set of experiments there were no significant differences between plated and calculated percentage of cell lysates demonstrating the validity of the method. In addition, the algorithm allowed calculating the emission spectra for both luciferases and Ppy WT, which were similar to those obtained when analysed separately **(Fig. 1C)**. A representative image of a spectral unmixing of cell lysates is shown

in **Figure 2**. Lysates from red and green expressing cells were serially diluted in duplicate and mixed in different proportion.

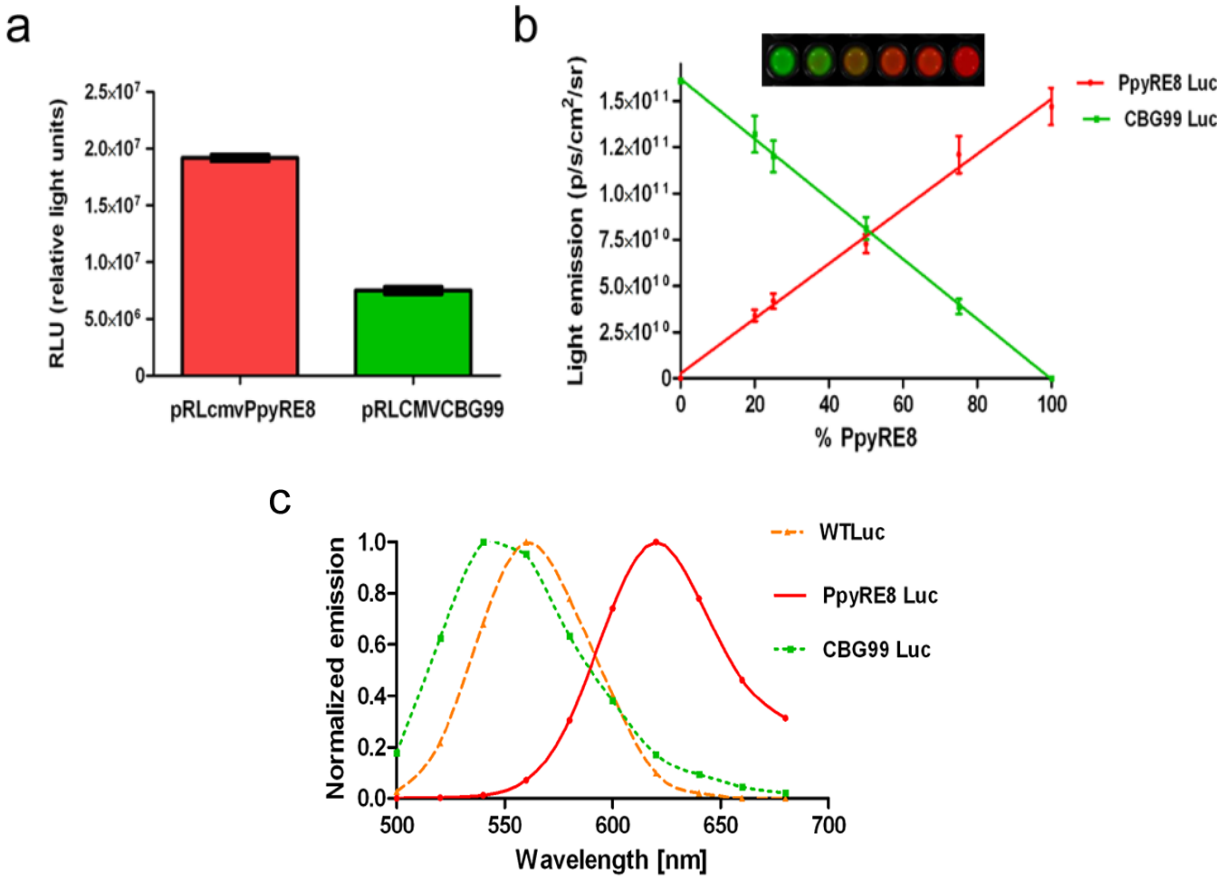


Figure 1 Validation of Ppy RE8 and CBG99 as a bioluminescent couple for multicolor imaging.(a) Level of expression of lentiviral constructs in Hek293 cells. (b) Spectral unmixing of cell lysates mixed in different proportions. Cells were lysed 24 h after transfection with lentiviral constructs. (c) Emission spectra of luciferases calculated with Living Image software in cell lysates at 25°C with the Ppy RE8 peak around 620 nm, CBG99 around 540 nm and WT Luc around 560 nm.

Live cell dual color imaging

This set of experiments was carried out on HepG2 cells to evaluate the performance of this pair of luciferases in living cells. Selected clones of cells stably expressing the luciferase variants can not mirror the expression level of transiently transfected populations, and different promoters vary expression in different cells types. For these reasons a direct comparison between the level of expression of CBG99 and Ppy RE8 luciferase cannot be performed but other relevant parameters such as emission spectra and dynamic range of luminescence signals for *in vivo* application can be evaluated. Ppy RE8 expressing cells showed a 5-fold higher signal than the cells expressing CBG99 at 37°C. A good correlation

between number of cells and light emission was obtained (R^2 =0.98) **(Fig 3).** Moreover the emission spectrum of Ppy RE8 cells did not vary in cells at 37°C.

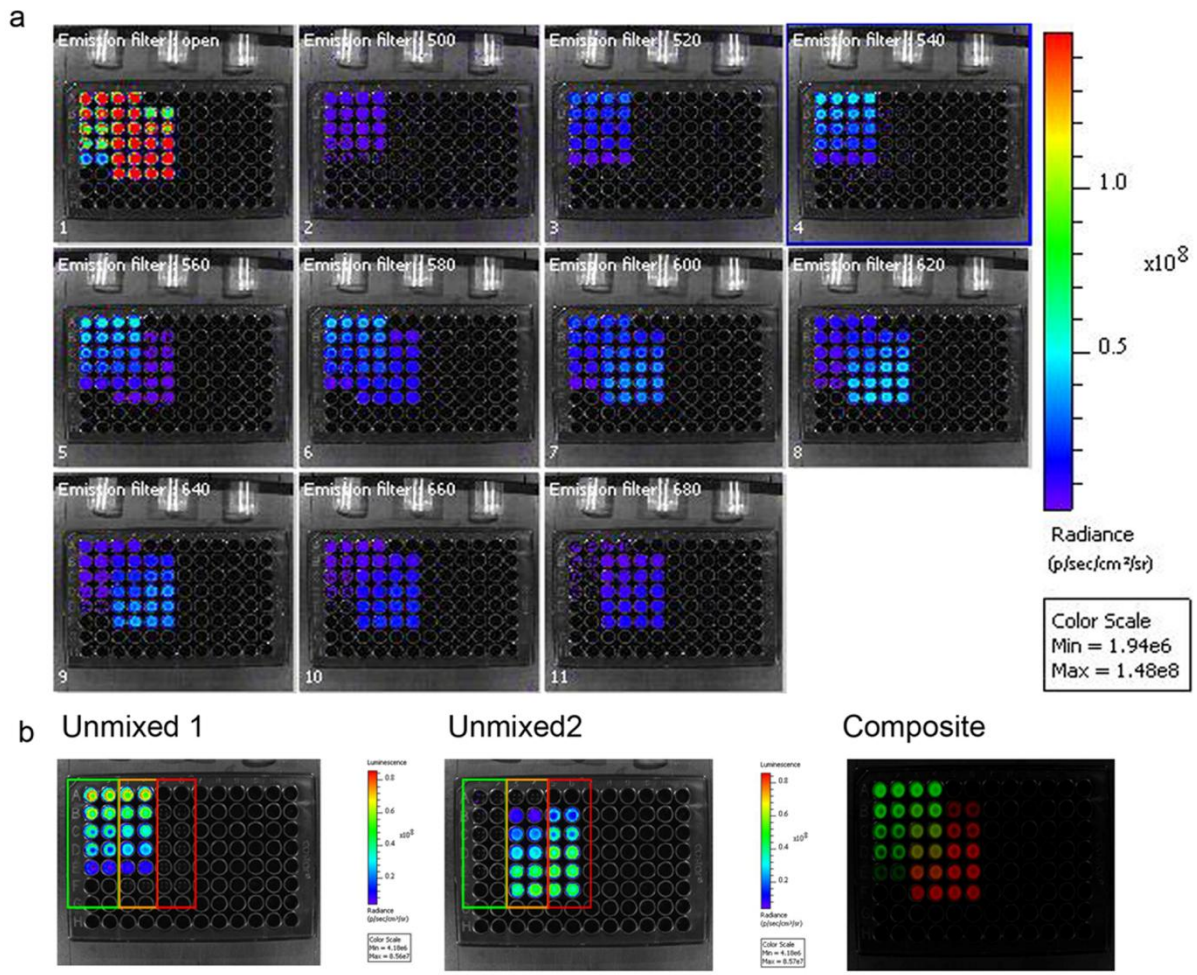


Figure 2 Representative image of emission spectral unmixing of bioluminescence in cell lysates.(a) Multispectral acquisition of red and green emitting cell lysates. In the left part (row 1 and 2)) of the plate dilutions of green emitting lysates were dispensed in duplicate while in the right part (row 5 and 6) dilutions of the red ones. In the middle (row 3 and 4) lysates were mixed in different proportions. The plate was scanned with an open filter and at different wavelengths ranging from 500 nm to 680 nm with a 20 nm interval.

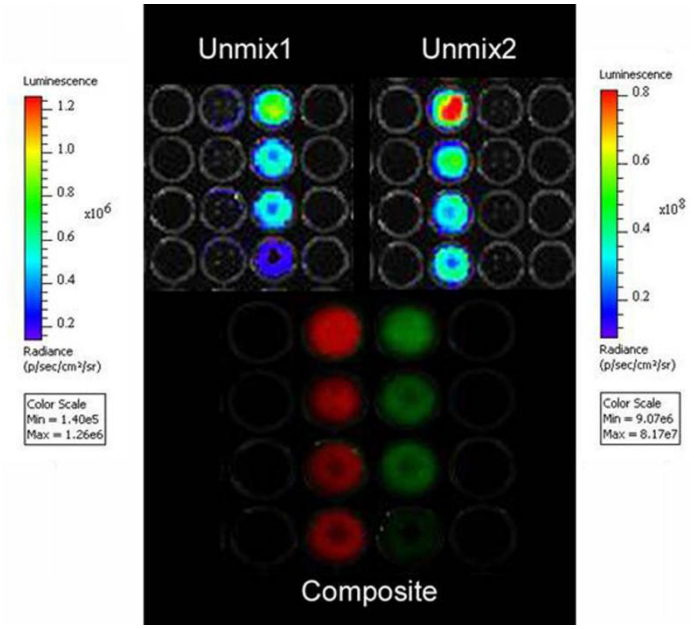
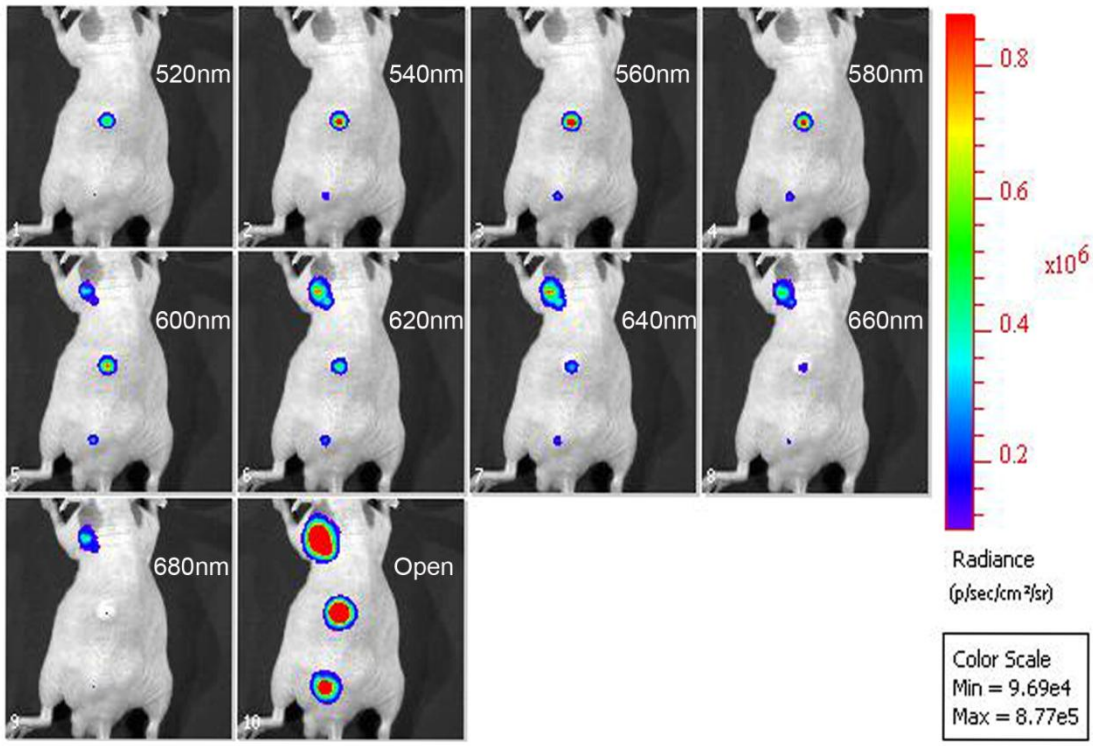


Figure 3 Live cell imaging. Representative spectral unmixing of signals emitted from stable red and green HepG2 cells; 10^5, 7.5 x 10^4, 5 x 10^4 and 2.5 x 10^4 cells were plated for each HepG2 cell line.

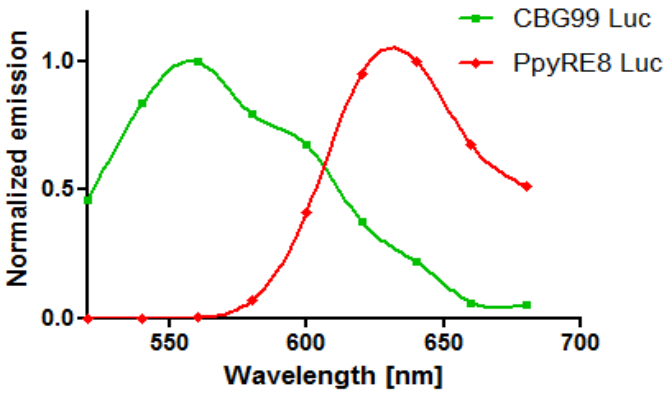
In vivo dual color imaging

In order to test this pair of luciferase for *in vivo* applications, cells expressing each luciferase were injected subcutaneously in mice. Five minutes after substrate injection, a series of images with 30sec aquisitions were obtained. The emission spectra of luciferases calculated from the *in vivo* experiments showed a slight red shift because of absorption and scattering of light generated under the skin **(Fig. 4A).** Intensity of the BL signals allowed the calculation of the red/green cell ratio in a mixed population after applying spectral unmixing algorithms. Average luminescence expressed in photon/sec/sr/cm^2 was determined for the ROI corresponding to the different areas where cells were inoculated. These values were extracted from the unmixed images generated by Living Image software. Experiments carried out in three mice for both independent set of experiments gave reproducible results. The injected cells were 10^5 and 2.5 x 10^4 in the mixture, and the calculated numbers of cells were 2.0 ± 0.4 x 10^4 for CBG99 and 2.4 ± 0.2 x 10^4 for Ppy RE8 **(Fig. 4B).**

a



b



c

Figure 4 Multispectral acquisition of light from live animal. (a) Cells expressing Ppy RE8 and CBG99 luciferases and a mixture was inoculated in the upper part, middle part and lower part of the back, respectively. Emission spectra of luciferases calculated from the same *in vivo* experiments. A slight red shift was noticed for both luciferases. (b) Unmixed and composite images. The injected cells were 10^5 and 2.5 x 10^4 in the mixture. The numbers of cells calculated with Living Image software were 2.0 ± 0.4 x 10^4 for CBG99 and 2.4 ± 0.2 x 10^4 for Ppy RE8.

Discussion

The main advantages of using bioluminescence in bioanalysis are related to the high signal/noise ratios and quantum efficiencies of the luciferin/luciferase system allowing to achieve an extreme high detectability in cell based assays and for in vivo molecular imaging[20,21]. Moreover, the availability of luciferases with different emission wavelengths gives the possibility of performing multicolor and multiplexed assays. Here, we evaluated for the first time a new red-codon optimized luciferase Ppy RE8 in combination with a green click beetle CBG99 luciferase that permitted a simultaneous, sensitive and reliable 2D imaging and quantification of different imaging signals *in vivo* using the same D-luciferin substrate. Issues concerning *in vivo* applications like tracking cells in deep tissues are different from that concerning analysis of gene expression in cell based assays. For this reason we carried out experiments in three different conditions: in cell lysates, in live cells and in whole animals. In order to demonstrate its performance we employed the Ppy RE8 and CBG99 genes for the development of lentiviral expression vectors and used them for transient and stable expression in different cell lines. A major concern was to separate the green emission overlap with the red filter (620 nm) particularly when the two signals have a different intensity. Images were obtained by collecting light using a set of filters (20 nm band pass) from 500 nm to 680 nm and without a filter. This was performed on the IVIS Spectrum (CaliperLS Inc, Hopkinton, MA) and a spectral unmixing algorithm was applied to all the images (Living Image 4 software, CaliperLS, Inc). The spectral unmixing of the images obtained from cell lysates showed the suitability of the use of these red and green luciferases as a BL pair with a single substrate. Images were collected five minutes after substrate addition when signals of both emitting enzymes are stable as indicated by previous studies[11,13]. Then, we performed analysis on stable transfected HepG2 cells to mimic the conditions for *in vivo* imaging. In this case, temperature was set to 37°C and the substrate consisted of 1mM D-luciferin without cofactors normally present in commercial assay buffers for testing cell lysates. Each image generated by a different band pass filter of 20nm was obtained by integrating signals for 30 s since ATP present in living cells represents a limiting factor on bioluminescence intensity. In this set of experiments, acquisition of images requires 5 minutes and detection and quantification of signals were accurate. Moreover, we envisage the possibility to

perform single cell or tissue analysis by using a novel implemented microscope for dual color bioluminescence imaging[22,23].

Finally, we evaluated the pair of luciferases in living animals and reinforce the concept that this pair of luciferases is optimal for *in vivo* imaging. Our results from living mice inoculated with red and green emitting cells demonstrated the possibility to 2D visualization and (semi-)quantification of cells emitting different colors in mixed populations. The light emission of both luciferases underwent a red-shift of 20 nm due to tissue absorption and scattering of light generated under the skin. As previously reported[24], emission spectra of luciferases *in vivo* show dependence from tissue depth and composition due to absorption and scattering of light through tissues. Therefore, the use of the red emitting enzyme is preferential for imaging in deeper tissues. Moreover, future improvement of the analytical performance of spectral unmixing of light signals as in fluorescence applications should lead to a better separation in deeper tissues[25]. Recently, Hida and colleagues applied multicolor luciferases to study protein-protein interaction and proposed *Phrixothrix hirtus* red luciferase (em. Max. 630nm) as an internal control in combination with fusion proteins constructed of different N or C parts of luciferases for a complementation assay. However, no multispectral image acquisition was performed and no unmixing algorithm was applied to images in order to obtain effective quantification of signals *in vivo*[26].In conclusion, Ppy RE8 was demonstrated to be an excellent tool for both *in vitro* and *in vivo* bioluminescence imaging and, in particular, when used in combination with a green luciferase to monitor dual events at the molecular level. Ppy RE8 has a good thermostability at 37°C and is highly expressed in mammalian cells. Differently from the use of click beetle luciferase as a green counterpart, the combined use of Firefly and Renilla luciferase requires the use of different substrates that are luciferin and coelenterazine. Biodistribution and enzyme kinetics with the two substrates are very different making ratio-metric measurements more difficult. Therefore, the described new D-luciferin-dependent red/green couple will allow to perform better (semi-) quantitative gene expression studies *in vivo* and will enable simultaneous tracking of different populations of stem cells, T-cell accumulation in tumor and simultaneous analysis of different molecular pathways. Finally, new dual color transgenic animal models may be generated.

Materials and Methods

Ethics statement

Animal experiments were approved by the Bioethics Committee of Leiden University, the Netherlands in compliance with international guidelines.

Plasmid construction

Self-inactivating lentiviral vectors, pLV.CMV.bc.NEO and pLV.CMV.bc.PURO, were kindly provided by Prof. R. Hoeben. The pLV.CMVPpy RE8.NEO vector was constructed by amplifying the Ppy RE8 gene from pGex Ppy RE8[11], using the following pair of primers: Ppy RE8ForAscI: taggcgcgccgaggacgccaagaacatca and Ppy RE8RevXhoI:aatctcgagtcagatcttgccgcccttctt, and inserted in the MCS of pLV.CMV.bc.NEO. pLV.CMVCBG99.PURO was created by inserting the CBG99 gene, cut with *Nco*I and *Xba*I from the pCBG99basic vector (purchased from Promega, Madison,WI, USA), into the MCS of pLV.CMV.bc.PURO via blunt ligation.

Cell lines

HEK293 and HepG2 cells were cultured in DMEM (Sigma, St. Louis, MO, USA) with 10% fetal bovine serum and 2 mM L-glutamine. The cultures were incubated at 37°C with 5% CO_2. HepG2 cells were transduced by self-inactivating lentiviruses as previously described[27]. Cell clones were selected with 1 mg/ml G418 or 1 µg/ml Puromycin for 14 days.

Imaging

All images were acquired with an IVIS spectrum (Caliper Life Sciences, Hopkinton, MA, USA) with the stage heated to 37°C during live cell imaging. The plates used were black-walled with clear bottoms. Generally, images were acquired at binning 8x8 pixels, f/stop 1, 12.5 cm field of view for the time and with the filter sets indicated. Experiments carried out with a different setup are indicated. Living Image 4 software was employed for generating spectral unmixed images and calculations of signals.

Spectral unmixing of emission wavelengths in cell lysates

Confluent HEK293 cells from a T25-flask were trypsinized and 10^5 cells/well plated in a 6 well plate. The next day, cells were transfected with 1 µg of pLV.CMVPpy RE8.NEO or pLV.CMV.CBG99.PURO using Fugene HD, per the manufacturer's protocol. After 24 h, cells were lysed for 10 min with 0.4 ml of Promega's Passive lysis buffer. Cell debris was pelleted by centrifugation at 13,000 rpm for 10 min. The level of expression of each luciferase was

evaluated in triplicate, and then each luciferase was diluted to a similar level of activity in lysis buffer. Subsequently, 30 µl of each lysate were plated in linear dilutions and in different proportions and imaged after addition of 30 µl of luciferase assay buffer (Promega, Madison, WI,USA) in a 96 black-walled plate with a clear bottom. Images were taken using a set of 20 nm filter steps from 520 nm to 680 nm and without a filter: acquisition time was 2 sec and f/stop 4 at 25°C. Two independent sets of transfections and images were used for calculations. Green and red signals were calculated from unmixed images. Data were plotted using GraphPad Prism 5.

Spectral unmixing of emission wavelengths in living cells

Stably expressing red and green HepG2 cells were trypsinized and resuspended in PBS in the 96 black-walled plates described above. Images were acquired at 37°C 5 min after addition of 1 mM D-luciferin (Synchem OHG, Felsberg, Germany) for 5 sec. Three independent experiments were carried out using the same selected cell lines.

***In vivo* imaging in mice**

HEK293 cells were plated at 2 x 10^5 cells per well in a six well plate. After 24 h, 1 µg of either pLV.CMVPpy RE8.NEO or pLV.CMVCBG99.PURO was transfected as described. After 24 h, the cells were trypsinized, pelleted and resuspended in PBS at 10^5 cells/100 µl. Aliquots were used for testing *in vitro* and *in vivo* imaging in mice. Athymic mice (BALB/c *nu/nu*, 4-6 weeks old) mice were acquired from Charles River (Charles River, L'Arbresle, France), housed in individually ventilated cages while food and water were provided *ad libitum*. Mice were anesthetized by isofluorane, while injected subcutaneously with cells. Images were acquired 10 min after i.p. injection of D-Luciferin (150 mg/kg) using 30 sec exposure with or without filters. Two independent sets of transfections were performed for each of which three mice were tested.

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Chapter 5

Combining intracellular and secreted bioluminescent reporter proteins for multicolor cell-based assays

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Abstract

Bioluminescent (BL) proteins are a promising tool for diverse applications based on reporter gene technology thanks to their high sensitivity and range of linear response. Due to their widespread use in the environmental, medical and agro-food fields, there is a great need for new BL reporter proteins with improved characteristics to provide researches a wide range of suitable reporters. Few efforts have been made in this direction and further improvement of BL reporters features (e.g., thermostability, narrower emission bandwidth, emission at different wavelengths) tailored for specific applications would be a remarkable progress toward the development of ultrasensitive multiplexed assays either *in vitro* or *in vivo*. The suitability of using red- and green-emitting thermostable mutants of *Photinus pyralis* firefly luciferase and two click beetle luciferases in combination with a secreted luciferase from *Gaussia princeps* was evaluated to develop a triple-color mammalian assay. Two triple-reporter model mammalian systems were developed in a human hepatoblastoma cell line to monitor the transcriptional regulation of cholesterol 7-α hydroxylase (cyp7a1), the enzyme that catalyzes the first and rate-limiting step of the main pathway responsible for cholesterol degradation in humans. These model systems allowed us to evaluate the feasibility of using two intracellular BL reporters and a secreted one in the same cell-based assay. The selection of reporter proteins characterized by similar expression levels was identified as a critical point for the development of a multicolor assay.

Introduction

Genetically engineered cells (bacteria, yeasts or mammalian cells) able to produce a bioluminescent (BL) signal in response to the target analyte represent an interesting approach for environmental, medical and food analysis with the great advantages of low cost, rapidity and sensitivity.[1,2] A peculiarity of these analytical devices resides is their ability to measure the bioavailable fraction of the analyte, which is a crucial information difficult to obtain with other analytical techniques. The cells are modified by introducing a reporter gene fused to a regulatory DNA sequence that is activated only in the presence of the analyte (e.g., genotoxic compounds, oxidants, metals, hormones, organic xenobiotics) thus regulating the reporter gene expression. Several whole-cell biosensors and cell-based assays have been developed by employing BL reporter genes such as firefly and bacterial luciferases, *Renilla* luciferase, and aequorin. Different formats were also applied to improve the performance and portability of these analytical devices. Two main typologies may be identified: liquid-phase biosensors featuring bioluminescent cells in suspension in a growth medium[3] or immobilized-phase biosensors in which cells are preserved in a hydrogel matrix.[4,5] Despite the high number of reported applications, few efforts were put on the improvement of the transducer element, i.e. the BL reporter protein. Most of developed whole-cell biosensors rely in fact on the *P. pyralis* luciferase which has a quantum efficiency close to 90%, does not require any post-translational modification for enzyme activity and it is not toxic even at high concentrations, being thus suitable for in vivo applications in prokaryotic and eukaryotic cells.[6,7] Several commercially available luciferase activity assays have been developed permitting single-step reporter activity measurements, also including cell lysis. Alternatively, bacterial luciferase (*lux*) is unique in that it is the only bioreporter system available that generates its own substrate, thus eliminating the need for exogenous substrate addition. However, due to the low expression levels of bacterial luciferase in mammalian cells, to date few *lux*-based mammalian cell-based assays have been developed. New codon-optimized versions of the *luxA* and *luxB* genes from *Photorhabdus luminescens* were also produced to increase the expression levels in mammalian cells.[8] Other BL proteins cloned from different organisms or obtained by random and site-directed mutagenesis are now being explored to expand the applicability and analytical performance of BL assays.[9,10] These mutants are suitable

for improved dual-color reporter assays, biosensor measurements, and in vivo imaging techniques. Besides these mutants, also the cloning of new luciferases from different organisms, like those from *L. italica* and *Cratomorphus distinctus*, obtained will certainly improve current applications and make novel ones possible.[11-15] Few works report the use of different luciferases to develop multiplexed assays, for example Nakajima et al. proposed a novel reporter assay system in which three luciferases that emit at different wavelengths (green, orange, and red) in the presence on the same substrate were used as reporter proteins. By measuring the emission of the three BL proteins with suitable optical filters and applying a signal process algorithm, the authors developed a monitoring system for the simultaneous evaluation of the expression of three different genes within a cell.[16] Quite a few studies compared the performance of different reporter genes. Hakkila et al. investigated the suitability of firefly luciferase, bacterial luciferase (*Photorhabdus luminescens luxCDABE*), green fluorescent protein from *Aequorea victoria*, and red fluorescent protein (*Discosoma sp.* dsRed) in whole-cell bacterial sensors. The lowest detectable concentration of analytes and the fastest responses were achieved with firefly luciferase or *luxCDABE* as reporter genes.[17] The use of secreted BL reporters has very seldom been reported. Recently Wu and coworkers reported a dual-assay with *Gaussia* and *Cypridina* luciferases as secreted reporters.[18] *Gaussia* luciferase (G*luc*) has several advantages over previous luciferases: (i) it possesses a natural secretory signal of 16 aminoacids that drives its secretion into cell medium, thus allowing BL measurements without cell lysis; (ii) it is the smallest luciferase cloned (19.9 kDa); (iii) its codon-humanized version produces a 100-fold higher luminescent signal intensity compared to firefly luciferase.[19] Recently many works have been reported in which secreted reporter proteins are used to monitor protein secretory pathways in living mammalian cells, to detect protein-protein interactions, and to monitor bacterial cells in adverse conditions. [20-22] The BL measure of secreted reporter proteins is in fact easily performed in the cell culture supernatant, allowing repetitive measurements of the same cell sample by simply taking out small medium aliquots (eg., 10 µL). In the present study the suitability of using both intracellular and secreted BL proteins in a mammalian cell-based assay has been investigated. In particular the secreted *Gaussia* luciferase was used in combination with two different couples of red- and green-emitting luciferases (red- and green-emitting thermostable versions of *P. pyralis* luciferases and red- and green-emitting luciferases originally

cloned from the click beetle *Pyrophorus plagiophthalamus*). To evaluate *Gaussia* luciferase as a potential reporter to be used in combination with intracellular luciferases we preliminary transfected the firefly luciferases and *Gaussia* luciferase reporter vectors into mammalian cells and compared the levels of luciferase activity. Two vitality controls (an intracellular firefly luciferase and the secreted Gaussia luciferase) were introduced to first evaluate the suitability of secreted Gaussia luciferase as reporter protein by comparison to the more conventional intracellular luciferases. Since these luciferases need different substrates the signals were measured in separate wells of a 96-well microtiter plate by adding coelenterazine or luciferin. By combining intracellular and secreted luciferases it was in fact possible to simultaneously measure the activities of the two firefly luciferases by splitting their emission with optical filters and measure the *Gaussia* luciferase directly in cell medium aliquots.

Matherials and methods

Materials and plasmids. The Bright-Glo™ luciferase assay system was from Promega. The plasmid pcDNA3-hGluc for expression of humanized *Gaussia princeps* luciferase and pUC19-Gluc were a kind gift of Dr. Bruce Bryan (NanoLight Technologies, Pinetop, AZ, USA). Ampicillin, isopropyl β-D-thiogalactopiranosyde (IPTG) and coelenterazine were obtained from Sigma (St. Louis, MO). Human hepatocarcinoma HepG2 cells were a generous gift from Prof. N. Carulli from the University of Modena, Italy. Materials used for culturing of cells were from Invitrogen. The plasmids pCBG99-Basic and pCBR-Basic were from Promega. The plasmid pCyp7a1-Ppy WT containing the portion of the human cyp7a1 promoter -1887/+24 and *Photinus pyralis* luciferase wild-type (Ppy WT) as reporter gene was a generous gift of Prof. John Y.L. Chiang (Northeastern Ohio Universities College of Medicine, Ohio, USA). The plasmids pGEXPpy GR-TS and pGEXPpy RE-TS expressing red- and green-emitting thermostable mutants of *Photinus pyralis* luciferase have already been described.[23] The red- (CBR*luc*) and the green-emitting luciferase (CBG99*luc*) were inserted into pcDNA3.1 (+) vector backbone (Invitrogen) by mean of a blunt ligation. Obtained plasmids were named pcDNA 3.1-CBR*luc* and pcDNA 3.1-CBG99*luc*. The CBG99*luc* was cloned into pCyp7a1-PpyWT to replace *P. pyralis* wild-type luciferase. The plasmid was named pCyp7a1-CBG99*luc*. All constructs were verified by restriction digestions and DNA sequencing. The bile acid

chenodeoxycholic acid (CDCA), as sodium salt, was purchased from Sigma–Aldrich. All the other reagents were of analytical grade or of the highest purity available.

Bacterial dual reporter systems To investigate the best intracellular luciferase pair for cell-based reporter assays, dual-reporter model systems were first developed. The Ppy WT, PpyGR-TS, and PpyRE-TS were expressed in 5 mL Luria Bertani medium cultures of *E. coli* strain JM109 grown at 37°C overnight and diluted in 20 mL LB medium to midlog phase (A_{600nm} 0.6). Then cultures were induced with 0.1 mM IPTG for 2 hours. The following luciferase pairs were used: Ppy WT and PpyRE-TS, PPy GR-TS and PpyRE-TS. Different proportions of cell cultures expressing the luciferases were transferred in a total volume of 100 µL in a 96-well microtiter plate. All combinations were tested in triplicate. A Luminoskan Ascent equipped with an injector for substrate addition was used for luminescence measurements. An amount of 100 µL of D-luciferin 1 mM in 0.1 M Na citrate buffer solution at pH 5.0 were injected with an automatic dispenser and after a brief shaking luminescence measurements were performed with 5 s integration. Luciferase activities were measured in the absence or presence of two emission filters (537 nm and 612 nm, band pass 20 nm). Light emissions were expressed as relative light units (RLU).

Cell culture and mammalian triple reporter systems. Stable clones of HepG2 cells overexpressing the farnesoid X receptor (FXR)[24] were transiently transfected or co-transfected with the plasmids pCyp7a1-PpyWT, pcDNA3.1-Ppy RE-TS, and pcDNA3-hG*luc* or with pCyp7a1-CBG99*luc*, pcDNA3.1-CBR*luc*, and pcDNA3-hG*luc*. All experiments were run in triplicate and repeated at least four times. HepG2-FXR cells were grown routinely in 5% CO_2 in air in MEM (minimum essential medium with Earle's salts) supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, MEM vitamins and antibiotic/antimycotic solution. Stock solutions of CDCA (10^{-2} M) were prepared in absolute ethanol and diluted in cell culture medium. The final ethanol concentration added to the cells was 0.05% (v/v; the same ethanol concentration, as vehicle, was added to control cells). Briefly, 10^4 cells were seeded in 24-well plates using the cation polymer transfection reagent ExGen 500 (MBI Fermentas, Vilnius, Lituania) following the manufacturer's specifications; in each assay, 0.5 µ/well of each plasmid were cotransfected. All transfections were performed in triplicate. Three days after triple co-transfection and treatment (usually 20 hrs) of the cells with the analyte (CDCA or other bile acids), 10

µL of the medium were transferred in triplicate to a 96 well-microtiter plate for G*luc* activity measurements, cells were washed twice with phosphate-buffered saline and lysed with 200 µL of 1% Triton® X-100 for 5 min at 25°C. After centrifugation, 100 µL of surnatant were transferred to the 96 well-plate. Each lysate was analyzed sequentially for the presence of the green-emitting luciferase (Ppy WT or CBG99*luc*) and red-emitting luciferase (Ppy RE-TS or CBR*luc*) by the addition of 100 µL luciferase assay system (Promega) and reading using the two emission filters described above to quantify the light emitted by each luciferase. The Promega Chroma-Luc "Calculator" was used to determine the contributions of red- and green-emitting luciferases.[25] G*luc* activity was assayed by addition of 20 µL of coelenterazine 5µM in PBS and 5 sec acquisition to 10-µL aliquots of cell medium. Each green signal (Ppy WT or CBG99*luc*) was normalized using the red (Ppy RE-TS or CBR*luc*) or G*luc* signal as an internal control. To analyze the time course of G*luc* synthesis and secretion the cells were transfected with the plasmid pcDNA3-hG*luc* and after 40 hours aliquots of cell medium were analyzed in triplicate for G*luc* activity as already described; after medium replacement BL measurements were taken at different time intervals for an overall period of time of 12 hours.

Results and discussion

Dual-color assays. To develop a simultaneous monitoring system, in which the BL signals of two intracellular luciferases are spectrally resolved and a third BL signal is measured in the cell medium by addition of a different substrate, we first chose the best intracellular luciferase pair that allowed a good signal separation. In theory to reduce spectral overlap the two emitters should have the narrowest bandwidths possible, well-separated emission maxima and similar specific activities. Among available luciferases we selected two thermostable mutants of *P. pyralis* luciferase with red and green emission (Ppy RE-TS and Ppy GR-TS, respectively) and two commercially available red and green luciferases from *P. plagiophalam* (CBG99*luc* and CBR*luc*). The *P. pyralis* thermostable mutants were documented to be well suited for reporter applications, having well-separated emission spectra and similar steady-state kinetic constants.[23] As an alternative, the CBG99*luc* and CBR*luc*, specifically tailored to be used in dual-reporter applications, seem to be the more appropriate candidate for a triple assay.

The suitability of the Ppy mutants was first evaluated in *E. coli*: the red-emitting Ppy RE-TS (maximum emission at pH 7.8 and 25°C 615 nm) was used in combination either with *P. pyralis* luciferase wild-type (Ppy WT, maximum emission at pH 7.8 and 25°C 557 nm) or with the green-emitting Ppy GR-TS (maximum emission at pH 7.8 and 25°C 548 nm). Different amounts of *E. coli* cell cultures expressing the two luciferase pairs (Ppy RE-TS and Ppy GR-TS; Ppy RE-TS and Ppy WT) were mixed in the wells of a 96-well microtiter plate to investigate the spectral resolution using optical filters. A preliminary measurement of the filter correction factors was made by assaying each luciferase separately with no filters, with the green filter and with the red filter. These values provided the calibrations constants for the Promega Chroma-Luc calculator, an Excel spreadsheet designed to calculate corrected luminescence values from samples containing red- and green-emitting proteins.[25] The relative amount of each luciferase was quantitated by simultaneous measurements of the red and green emissions in each well. Figure 1.A shows BL emissions obtained by mixing populations of *E. coli* JM109 cells expressing Ppy RE-TS and Ppy WT grown at 37°C. Simultaneous measurements of green-and red-emitters were performed in intact *E. coli* cells in a high-throughput 96-well microplate format, demonstrating the feasibility of using Ppy WT and Ppy RE-TS as a BL reporter pair. Even though these luciferases have very different relative high specific activities (Ppy RE-TS activity is 15% that of Ppy WT), the lower activity of the red emitter is most likely balanced by its higher thermostability (Ppy RE-TS half-life at 37 °C 8.80 hrs; Ppy WT half-life at 37 °C 0.26 hrs).Next the feasibility of using Ppy RE-TS and Ppy GR-TS was evaluated by measuring the spectral resolution of varying percentages of *E. coli* cells expressing the two luciferases at 37 °C (Figure 1.B). Although these two mutants show similar specific activity and well-separated emission spectra (67-nm separation), their levels of expression differ significantly when the enzymes are expressed in bacterial cells at 37 °C and their BL emissions are measured in intact cell. This is presumably due to the higher level of expression of the mutant Ppy GR-TS if compared to the red one. We concluded that this luciferase pair is not suitable for dual reporter assays and selected as luciferase pairs Ppy RE-TS/Ppy WT and CBG99*luc*/CBR*luc* for developing an cell-based assay with a third secreted reporter protein.

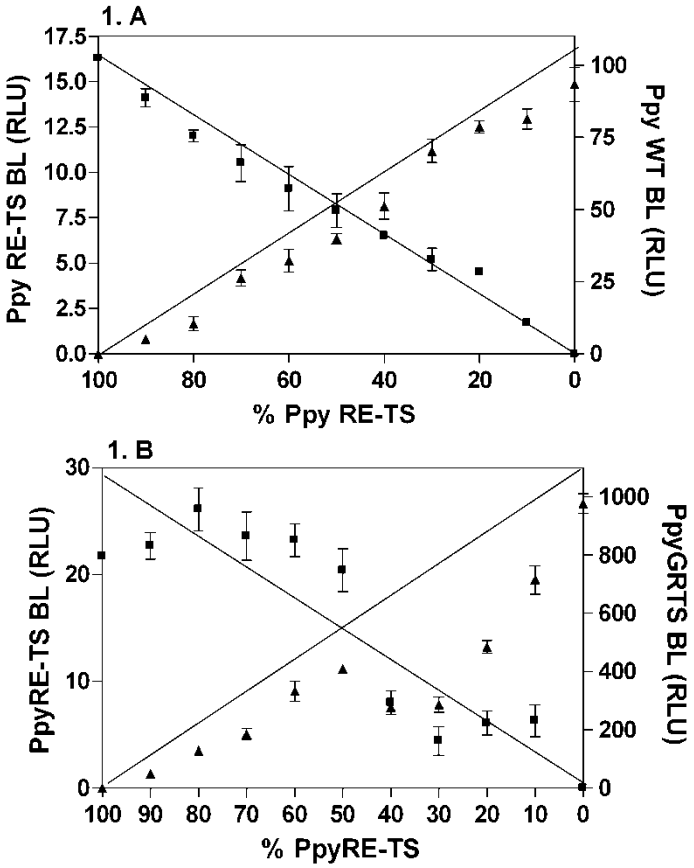

Figure 1. (A) BL emissions of Ppy RE-TS (—■—) and Ppy WT (—▲—) expressed in *E. coli* JM109 cells grown at 37°C. **(B)** BL emissions of Ppy RE-TS (—■—) and Ppy GR-TS (—▲—) expressed in *E. coli* JM109 cells grown at 37°C. Mean values are plotted, with standard deviations indicated by error bars. RLU, relative light units.

Gaussia luciferase measurements in transiently transfected HepG2 cells. Thereafter, we evaluated the expression of these reporter proteins in mammalian cells, with the final goal of using the two red-and green-emitting luciferases together with a secreted luciferase, *Gaussia* luciferase, as third reporter protein. Since this protein is secreted, its BL activity is measured directly into the culture medium after addition of the substrate coelenterazine. In order to study the time required for synthesis and secretion of G*luc*, HepG2 cells were transfected with a plasmid for G*luc* constitutive expression and, after changing the medium, the amount of expressed G*luc* was evaluated by BL measurements in the cell medium over a period of 24 hours. The evaluation of the time required to reach a steady state intensity in the BL signal is important to identify the period of time, after medium replacement, required to obtain a stable BL signal. Figure 2 shows that the time required to get a BL signal

comparable to that obtained after 40 hours post-transfection (without changing growth medium) was approximately 6-8 hours.An analytical approach in order to explore the level of synthesis and secretion of G*luc* was also undertaken by Knappskog et al., who tested the effect of different signal peptides on the expression and secretion of Gluc in transiently and stably transfected CHO cells.[26]Our data confirm results obtained by these authors who reported an increase in BL activity in the medium for an overall period of 40-48 hours, then the signal reaches its maximum intensity.We then proceeded with a proof-of-concept experiment, the green-emitting luciferases were expressed under the regulation of the cholesterol 7-α hydroxylase (cyp7a1) promoter. This promoter is negatively regulated by bile acids. Chenodeoxycholic acid (CDCA), a FXR ligand, is the more active one, being able to inhibit cy7a1 transcriptional basal activity with an IC_{50} of approximately 10 µM (concentration of CDCA required to inhibit 50% of reporter activity).[27]

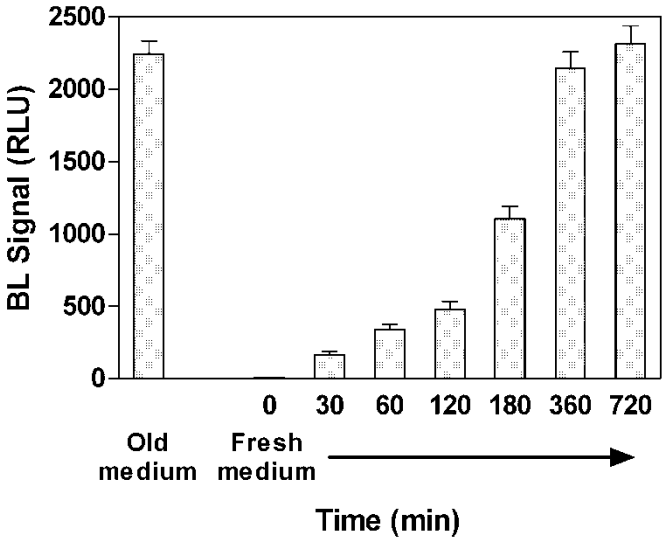

Figure 2. Time course of *Gaussia* luciferase BL signal obtained after cell medium replacement. HepG2 cells were transfected with the plasmid pcDNA3-hGluc. 40-hrs after transfection, the BL emission was evaluated in a 10µL-aliquot of the culture medium. After growth medium replacement, the BL signal was evaluated during a period of 12 hrs.

In the first triple assay the plasmids cyp7a1 Ppy WT, pcDNA3.1-Ppy-RE-TS, and pcDNA3-hG*luc* were co-transfected into HepG2-FXR cells to compare the analytical performance of the Ppy-RE-TS and the G*luc* as internal control reporters. Figures 3.A and 3.B report the concentration-dependent inhibition of cyp7a1 by CDCA in the concentration range 10-100 µM. According to previously published data, treatment of

HepG2-FXR cells with CDCA in concentrations of 100 µM repressed the cyp7a1 Ppy WT reporter activity by more than 50%.

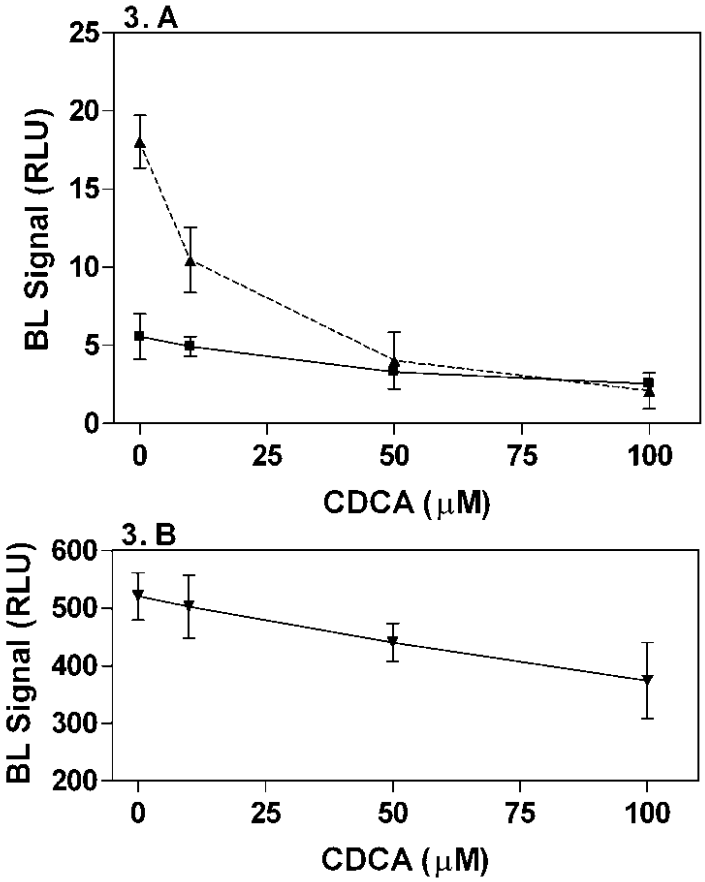

Figure 3. Effects of CDCA on cyp7a1 transcription. **(A)** Bioluminescent emission of Ppy WT (—▲—) under the regulation of cyp7a1 promoter and emission of the constitutively expressed Ppy RE-TS (—■—) in the presence of increasing concentrations of CDCA. **(B)** Emission of the constitutively expressed G*luc* (—▼—) in the presence of increasing concentrations of CDCA. Values are the mean ± standard deviation of triplicate samples measured with the green and red filters. G*luc* BL activity was assayed in 10µL-aliquots of cell medium after addition of coelenterazine.

As shown in Figure 4, corrected signals obtained by calculating the ratio of Ppy WT emission (cyp7a1 transcriptional activity) over Ppy RE-TS emission (internal control) or the ratio of Ppy WT emission over G*luc* emission (internal control) displayed a similar behavior. Both the two luciferases used for internal correction allowed to perform a signal correction according to cell vitality. An IC_{50} of 10 µM for CDCA was obtained with both the reporters, thus confirming the suitability of these luciferases for multiplexed assays. These data confirm that secreted BL proteins may be used in combination with intracellular luciferases to increase the number of information

obtainable from a single cell. Then, we investigated the combination of CBR*luc*, CBG99*luc*, and G*luc* with the same procedure. The green luciferase was used as specific reporter to study cyp7a1 promoter activity, whereas CBR*luc* and G*luc* were both used to monitor cell vitality. Due to its low expression in HepG-FXR cells, BL measurements with the red filter caused a decrease in the CBR*luc* BL signal intensity that dropped almost to zero (0.011 ± 0.004 without filter). This abolished the ability to correct the signal in the presence of 100 µM CDCA (Figure 5.A and 5.B).

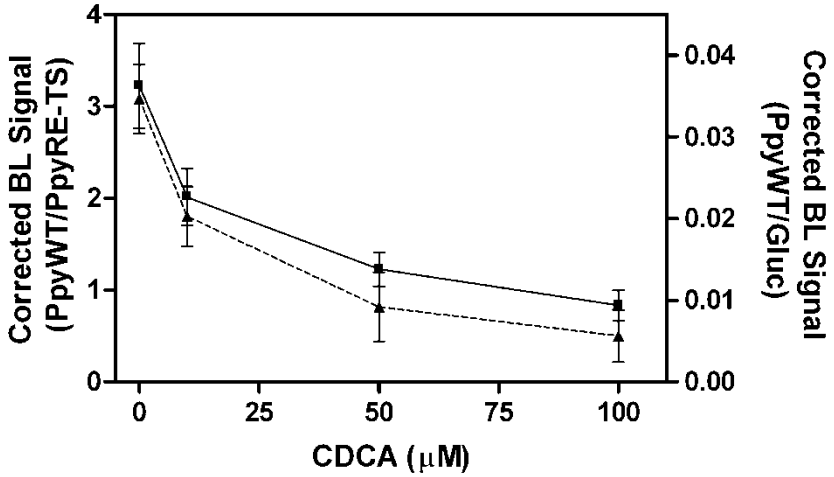

Figure 4. Corrected dose-response curves for CDCA calculated as the ratio of Ppy WT over Ppy RE-TS emissions (—■—, left Y axis) and ratio of Ppy WT over G*luc* emissions (—▲— , right Y axis) against concentration of CDCA. Values are the mean ± standard deviation of triplicate samples measured with the green and red filters.

Also the intensity of CBG99*luc* BL signal under the regulation of cyp7a1 promoter was very low when compared to Ppy WT (basal reporter activity without CDCA 0.254 ± 0.03 vs. 18.03 ± 1.68, respectively). This diversity is certainly due to different levels of expression in mammalian cells and relative specific activities of firefly and click beetle luciferases. A BL signal corresponding to 54 ± 4% of the BL intensity without CDCA was obtained using G*luc* as reporter protein. The high expression of G*luc* in mammalian cells, permits in fact to reach higher sensitivities if compared to CBR*luc* and CBG99*luc*. In a similar approach, a red thermostable mutant of *Luciola italica* has been used together with PpyWT and G*luc* to study the transcriptional regulation of the two pathways of bile acid biosynthesis[28] confirming the feasibility of associating firefly luciferases with G*luc* to develop multiplexed assays

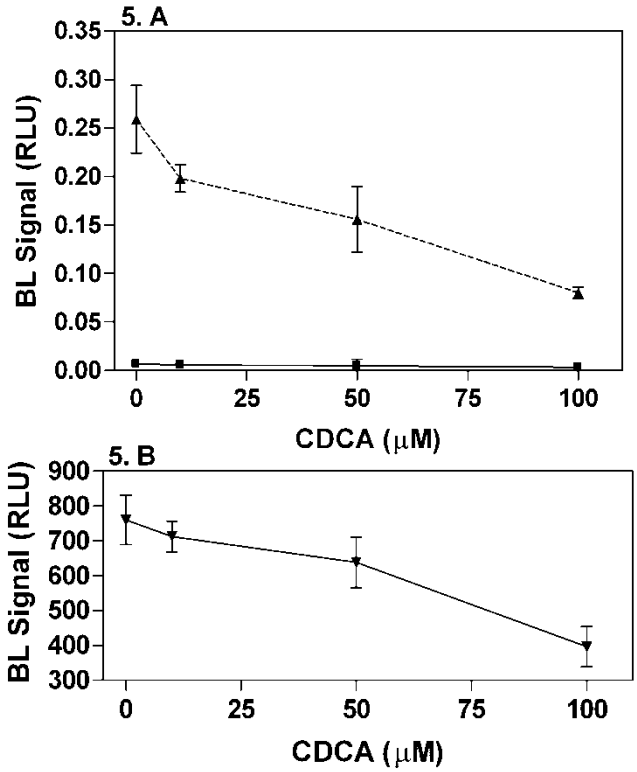

Figure 5. Effects of CDCA on cyp7a1 transcription. **(A)** Bioluminescent emission of CBG99 (—▲—) under the regulation of cyp7a1 promoter and emission of the constitutively expressed CBR (—■—) in the presence of increasing concentrations of CDCA. **(B)** Emission of the constitutively expressed G*luc* (—▼—) in the presence of increasing concentrations of CDCA. Values are the mean ± standard deviation of triplicate samples measured with the green and red filters. G*luc* BL activity was assayed in 10μL-aliquots of cell medium after addition of coelenterazine.

Conclusion

We concluded that the use of *Gaussia* luciferase in combination with *P. pyralis* mutants emitting at different wavelengths allows the development of multiplexed-assays with the peculiar advantage of complete absence of interference between the BL signals. In addition, both the secreted or the intracellular luciferases may be used as internal vitality controls, thus increasing the flexibility of the assay. The use of secreted reporter BL proteins has several advantages if compared to more commonly reported intracellular ones and we demonstrated that mammalian cell-based assays employing both secreted and intracellular luciferases take advantage of the multicolor technology provided by spectral resolution (e.g. when using red- and green-emitting firefly luciferases) and of the straightforward measurement in cell medium allowed by secreted BL proteins. A careful choice of the BL reporters must

be preliminarily taken into consideration since high divergence in the BL reporter expression levels may account for difficulties in performing signal correction. The use of three or more reporter proteins could be extremely useful to develop whole-cell biosensors and cell-based assays with internal correction for multianalyte detection, particularly in environmental and food analysis, for example to simultaneously monitor the presence of different heavy metals or endocrine disruptor compounds.

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Chapter 6

Spectral-resolved gene technology for multiplexed bioluminescence and high-content screening

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Abstract

The availability of new bioluminescent proteins, obtained by cDNA cloning and mutagenesis of wild-type genes, expanded the applicability of these reporters from the perspective of using more proteins emitting at different wavelength in the same cell-based assay. By spectrally resolving the light emitted by different reporter proteins it is in fact possible to simultaneously monitor multiple targets. A new luciferase isolated from *L. italica* has been recently cloned and thermostable red- and green-emitting mutants obtained by random and site-directed mutagenesis. Different combinations of luciferases were used in vitro as purified proteins, and expressed in bacterial and mammalian cells to test their suitability for multicolor assays. A mammalian triple-color reporter model system was then developed using a green-emitting wild-type *P. pyralis* luciferase, a red thermostable mutant of *L. italica* luciferase and a secreted *Gaussia princeps* luciferase (GLuc) to monitor the two main pathways of bile acid biosynthesis. The two firefly luciferases were used to monitor cholesterol 7-α hydroxylase and sterol 27- hydroxylase, while secreted constitutively expressed GLuc was used as an internal vitality control. By treating the cells with chenodeoxycholic acid it was possible to obtain dose-dependent inhibitions of the two specific signals together with a constant production of GLuc, which allowed for a dynamic evaluation of the metabolic activity of the cells. This is the first triple-color mammalian reporter assay that combines secreted and non-secreted luciferases requiring different substrates, thus avoiding reciprocal interference between different BL signals. This approach is suitable for high content analysis of gene transcription in living cells to shorten the time for screening assays, increasing throughput and cost-effectiveness.

Introduction

Reporter gene technology, based upon the splicing of transcriptional control elements to a variety of reporter genes (e.g., green fluorescent protein, β-galactosidase, aequorin, luciferases), has been successfully used to monitor the cellular events associated with signal transduction and gene expression.[1-2] The principal advantages of reporter gene-based assays are their high sensitivity, reliability, convenience, wide dynamic range, and adaptability to high throughput-screening. The choice of reporter gene, however, depends on the cell line used, the nature of the experiment, and the adaptability of the assay to the appropriate detection method (e.g., single cell imaging versus well- or plate-based detection). Together with fluorescent proteins, bioluminescent (BL) proteins are by far the most used reporter proteins for bioanalytical applications, including the investigation of protein-protein interactions, protein conformational changes, protein phosphorylation, second messengers expression and, in general, the study of gene expression and gene regulation.[3-8] Since BL proteins can be detected down to attomole levels,[9] they allow ultrasensitive detection of the target analytes. This also enables the analysis of small-volume samples, which leads to the development of miniaturized and high-throughput assays.

Among BL proteins, luciferase from the North American firefly *Photinus pyralis* is by far the most employed BL reporter gene. The firefly luciferase (Luc) catalyzes the formation of luciferyl-adenylate (LH_2-AMP) from luciferin (LH_2) and ATP. LH_2-AMP is converted through a multi-step oxidative process to excited-state oxyluciferin, the light-emitting product.[10-12] The production of light is very efficient, with a quantum efficiency close to 90%.[13-14]

Luciferase does not require any post-translational modification for enzyme activity and it is not toxic even at high concentrations, being thus suitable for in vivo applications in prokaryotic and eukaryotic cells. Several commercially available luciferase assay formulations have been developed permitting single-step reporter activity measurements, also including cell lysis.

The recent availability of new reporter genes with improved BL properties, together with technical improvements, prompted the development of multiplexed cell-based assays and multicolor in vivo imaging. New BL reporter genes were recently obtained using a random and site-directed mutagenesis approach[15, 16] or by cloning new BL proteins, like luciferase from *L. italica* and *Cratomorphus distinctus*.[17, 18] However, very

few works regarding multicolor reporter assay systems have previously been reported in literature.[19, 20] The bottleneck consists in the spectral unmixing that, when using more than two luciferases requiring the same substrate, does not allow to completely separate the BL signals. In order to perform this kind of calculation, an elegant Java plug-in for ImageJ was also written to deconvolute images composed of signals obtained with different filters.[21]

Self-illuminating quantum dot conjugates have been used as well with potential applications for multiplexing bioluminescence imaging and developing quantum dot-based biosensors.[22, 23]

Alternatively, secreted BL reporter proteins that do not require cell lysis or special equipment (e.g. filtered luminometers) may be used although their expression has a higher variability and expensive substrates are required.[24]

We report here for the first time a triple-color mammalian assay which combines spectral unmixing of green- and red-emitting luciferases with a secreted luciferase requiring a different substrate, thus allowing to measure three separate targets with high sensitivity and rapidity.

Two thermostable red and green-emitting mutants were obtained by site-directed and random mutagenesis of the cDNA encoding the luciferase from the Italian firefly *Luciola italica*. The suitability of these new luciferases as reporter proteins was first assessed in bacterial cells by producing dose-response curves for isopropyl β-D-thiogalactopyranoside (IPTG), used as model analyte. Once evaluated that both the two mutants gave dose-response curves parallel to that obtained with wild-type *P. pyralis* luciferase, spectra obtained with purified luciferases were compared to those produced using bacterial cells expressing the two luciferases. In order to select three luciferases, two intracellular luciferases and a secreted one, to be used for developing a triple mammalian assay, the best couple of intracellular luciferases was first chosen. Different combinations of *P. pyralis* and *L. italica* mutants were mixed in different amounts either using purified proteins or using *E. coli* expressing the BL proteins and spectral resolution was evaluated.

Then, a triple-reporter assay was developed using the green-emitting wild-type *P. pyralis* luciferase, a red-emitting thermostable mutant of *L. italica* luciferase, and a humanized version of *Gaussia princeps* luciferase. This humanized form was specifically produced with humanized codon usage to optimize its expression in

mammalian cells, showing a 200-fold higher signal intensity than the humanized form of Renilla luciferase.[25]

The assay was developed in a 96-well format to in vivo monitor the two pathways of bile acid biosynthesis in a new cell-based perspective for high content screening, thus enabling multiparametric analysis of bioluminescent (and/or fluorescent) indicators to define cellular responses to specific treatments.[26]

Bile acid biosynthesis is in fact a key step of intracellular cholesterol homeostasis and, in turn, affects the rate of cholesterol synthesis in hepatocytes. The "classic" pathway of bile acid formation starts with a 7α-hydroxylation of cholesterol by cholesterol 7α-hydroxylase (CYP7A1) in the liver. The "acidic" pathway starts with a hepatic or extrahepatic 27-hydroxylation by sterol 27-hydroxylase (CYP27A1). In humans, the activity of CYP27A1 is negatively regulated by bile acids, of which chenodeoxycholic (CDCA) is the more active one. An important mechanism of regulation of CYP7A1 activity is believed to take place at the level of gene transcription because changes in enzyme activity were found to parallel those in mRNA levels, although a post-transcriptional regulation seems to be important.[27-29] The transcriptional regulation of these enzymes is usually studied by cell-based reporter gene assays in which a single assay provides information regarding only the regulation of one promoter.

We report here the development of a recombinant HepG2 cell-based luciferase assay with an internal vitality control that enabled us to evaluate the ability of natural and synthetic bile acids and other compounds to activate or inhibit the two bile acid synthesis pathways.

Materials and methods

*Materials***.** Materials used for culturing of cells were from Invitrogen. The Bright-Glo™ luciferase assay system was from Promega. The plasmid pcDNA3-hGLuc for expression of humanized *Gaussia princeps* luciferase was a kind gift of Dr. Bruce Bryan (NanoLight Technologies, Pinetop, AZ, USA). Ampicillin, IPTG, chenodeoxycholic acid (CDCA), phenylmethanesulphonylfluoride (PMSF), coelenterazine and imidazole were obtained from Sigma (St. Louis, MO). Human hepatocarcinoma HepG2 cells were a generous gift from Prof. N. Carulli from the University of Modena, Italy. The plasmids pGEXPpy WT and pGEXPpy GR-TS[19] expressing the *P. pyralis* WT luciferase and its mutant containing the following

mutations Thr214Ala/Ala215Leu/Val241ile/Gly246Ala/Phe250Ser were used as starting point to clone the Ppy WT and Ppy GR-TS into pQE30 backbone (Qiagen).

The plasmid pGL3CYP7A1-Luc containing the portion of the human CYP7A1 promoter -1887/+24 and *Photinus pyralis* luciferase wild-type as reporter gene was kindly provided by Prof. John Y.L. Chiang (Northeastern Ohio Universities College of Medicine, Ohio, USA). The plasmid pSOH4.3 containing a 4.3 kb portion f the human CYP27A1 promoter was a kind gift of Prof. Sebastiano Calandra (University of Modena, Italy).

Construction of Plasmids and Mutagenesis. The following primers were used to amplify a fragment of 531 bp (-500/+31) of the human CYP27A1 promoter using the plasmid pSOH4.3 as template: ForCyp27-531 TATGGTACCCCAGGGATCAGATGACTGG (*Kpn*I) and the reverse primer RevCyp27 TCTAAGCTTACCTCAGCCTCGCGCAG (*Hind*III); restriction sites are shown underlined. The optimized conditions for the PCR were as follows: 1x Pfx buffer, 2x Enhancer solution, 1.5 mM $MgSO_4$, 0.3 mM dNTPs, 0.15 µM Primer RevCyp27, 0.15 µM Primer Forcyp27-431 or ForCyp27-951, 1 µg of DNA template, and 2 units of Platinum Pfx DNA polymerase (GIBCO, Inc., Gaithersburg, MD). The final volume of the PCR mixtures was 50 µL, and they were carried out with a MJ Research PTC 100 thermal cycler (Perkin Elmer). The PCR reactions were carried out with an initial denaturation at 95°C for 5 min followed by 30 cycles of amplification (denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 68°C for 45 sec, with a final extension at 68°C for 5 min). PCR products were purified by gel extraction using the QIAquick Gel extraction kit (Qiagen) and then inserted into pGL3-Control vector (Promega, Madison, WI) which had been previously digested with the restriction enzymes *Kpn*I and *Hind*III to replace SV40 promoter and give the plasmid pGL3-CYP27A1-Luc. The plasmid was sequenced to confirm the introduction of the promoter and verify that no mutation was introduced during PCR amplification. The QuikChange Multi Site-Directed Mutagenesis kit (Stratagene, Heidelberg, Germany) was used to perform site-directed mutagenesis using the *L. italica* luciferase wild-type DNA sequence (Pubmed no. DQ138966) in the pGEX-6P-2 vector as a template. Green- and red-light emitting thermostable (TS) *L. italica* mutants were made and the peroxisome targeting sequences were removed in order to abolish peroxisomal transport and improve their expression in mammalian cells.[30]

The green-emitting mutant (Lit GR-TS) contains the mutations V243I, G248A and F252S; whereas the red-emitting enzyme (Lit RE-TS) has the S286T change. Both luciferases contain the mutations G216A, T217L, S234A, K547G, M548G and E356K. The mutants were inserted into pQE30 (Qiagen) using *Bam*HI and *Pst*I restriction sites.Two paired primers, which included restriction endonuclease sites, were designed to amplify the Lit RE-TS mutant cDNA: Primer *Hind*IIILitRETSFor: 5'-GCAAGCTTATGGAAACGGAAAGGGAGGA- 3' (forward primer containing a *Hind*III site, underlined) and Primer *Xba*ILitRETSrev: 5'-ATCTAGATTACCCCCCGGCTTGTGGTTTCT- 3'(reverse primer containing a *Xba*I site, underlined). The PCR product was then cloned into pGL3-CYP27A1-Luc to replace wild-type luciferase of *P. pyralis* and give the plasmid pGL3-CYP27A1-LitRE-TS. Lit RE-TS and Lit GR-TS mutants were also inserted into pcDNA3.1 (+) vector backbone (Invitrogen) and pGL3 vectors by mean of a blunt ligation. Obtained plasmids were named pcDNA 3.1-LitGR-TS and pcDNA 3.1-LitRE-TS, pGL3-LitGR-TS and pGL3-LitRE-TS. All constructs were verified by DNA sequencing.

*Protein espression in E. coli and purification***.** Lit GR-TS and Lit RE-TS 6his-fusion proteins were first grown in *E. coli* strain BL-21 in 5-mL LB medium with 100 µg/ml ampicillin at 37°C overnight. These cultures were used to inoculate 250 ml cultures at a 1:100 dilution (LB-broth supplemented with 100 µg/ml ampicillin) and grown at 37°C with shaking until an OD_{600nm} of 0.6 was reached. Cultures were transferred to a 22°C incubator, allowed to equilibrate and induced with 0.1 mM IPTG overnight. Qiagen Ni-NTA resins were used for protein purification according to manufacturer's instructions with slight modifications. Briefly the cells were harvested by centrifugation, resuspended in 2 ml of lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10 mM imidazole, 1 mM PMSF, pH 7) and sonicated using ten 10s bursts with a 15s cooling period on ice between each burst. The lysate was then centrifuged at 5000 rpm for 1h at 4°C to pellet cellular debris and the supernatant was saved to proceed with protocol for purification under native condition. The cleared lysate was mixed with 1 mL of the 50% Ni-NTA slurry, loaded into a polypropylene columns (Qiagen) and washed twice with 4 mL wash buffer (50 mM NaH_2PO_4, 300mM NaCl, 20mM imidazole, 1mM PMSF, pH 7). 500-µL aliquots were eluted in Elution Buffer (50mM NaH_2PO_4, 300mM NaCl, 250mM imidazole, pH 7). Protein concentration was determined by Bio-Rad Microassay procedure using bovine serum albumin (BSA) as standard. The activity of the purified proteins was evaluated using a luminometer

(Luminoskan Ascent, Labsystem) using 4 µL of eluted protein, 100 µL phosphate buffered saline (PBS) and 100 µL of Bright-Glo™ Luciferase Assay System (Promega).

Measurement of bioluminescence emission spectra. Emission spectra were obtained using an Eclipse spectrofluorometer (Varian) in 'Bio/Chemiluminescence' mode (excitation source turned off). Reaction mixtures containing purified Lit RE-TS and Lit GR-TS (5-100 µg) in Elution Buffer, 70 µM D-luciferin (Synchem), and 2 mM Mg-ATP were brought to a final volume of 1 mL with 25 mM glycylglycine buffer (pH 7.8). Approximately 1 min after initiation of bioluminescence, spectra were recorded in a 1.0 mL fluorescence cuvette and emission slit of 10 nm. Bandwidths of emission spectra were measured at 50 and 20% of the intensity at the maximum wavelength to investigate luciferase emission behavior and possible spectra broadening due to pH variations or altered cultural conditions. Spectra were also recorded using aliquots of *E. coli* cells expressing the two thermostable luciferases. Briefly, shake flasks (250 ml) containing 20 ml of LB-broth and 100 µg/ml ampicillin were inoculated at a 1:50 dilution using overnight cultures of *E. coli* JM109 harboring pQE30Lit RE-TS or pQE30Lit GR-TS. Cells were grown at 37°C to A_{600} ~0.4, supplemented with 0.1 mM IPTG to induce luciferase expression and incubated for 2 h at 22°C. Samples (1 mL-aliquots) were collected and the A_{600} was adjusted to 0.9 using LB. Aliquots (1 mL) were transferred to a fluorescence cuvette and 200 µL of 0.5 mM D-luciferin were added. After 5-min incubation at room temperature, bioluminescent emission spectra were collected as previously described. The pH of the mixtures was verified after each emission spectrum was measured.

*Bacterial expression and model reporter system***.** To test the feasibility of using Lit RE-TS, and Lit GR-TS as reporter proteins, dose-response curves were produced in *E. coli* for IPTG, used as model analyte, and compared to those obtained with *P. pyralis* wild type luciferase (Ppy WT). Briefly, 90 µL aliquots of freshly grown bacteria in LB broth were transferred to a white 96-well microplate and mixed with IPTG standard solutions (to reach a final concentration of $1.0x10^{-6}$-$1.0x10^{-1}$ mM. Bacteria were incubated at 30°C for 2 h. Luminescence measurements were taken with 5 sec acquisition. Then, dual-reporter model systems were developed to investigate the best luciferase pair for dual reporter assays. The Ppy WT, Ppy GR-TS, Lit RE-TS, and Lit GR-TS were expressed in 5 mL LB medium cultures of *E. coli* strain JM109

grown at 37°C overnight and diluted in 20 mL LB medium to midlog phase (A_{600nm} 0.6). Then cultures were induced with 0.1 mM IPTG for 2 hours. The following luciferase pairs were used: Ppy WT and Lit RE-TS, PPy GR-TS and Lit RE-TS, Lit GR-TS and Lit RE-TS. Different proportions of cell cultures expressing the luciferases were transferred in a total volume of 100 µL in a 96-well microtiter plate. All combinations were tested in triplicate. Luminescence measurements were performed with a Luminoskan Ascent equipped with an injector for substrate addition. An amount of 100 µL of D-luciferin 1 mM in 0.1 M Na citrate buffer solution at pH 5.0 were injected with an automatic dispenser and after a brief shaking luminescence measurements were performed with 5 s integration. Luciferase activities were measured in the absence or presence of two emission filters (537 nm and 612 nm, band pass 20 nm). Light emissions were expressed as relative light units (RLU). The Promega Chroma-Luc "Calculator" was used to determine the contributions of red- and green-emitting luciferases.[31]

Cell culture. HepG2 cells were grown routinely in 5% CO_2 in air in MEM (minimum essential medium with Earle's salts) supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, MEM vitamins and antibiotic/antimycotic solution. Cells were stably transfected with pcDNA3.1-FXR expression vector containing the cDNA encoding human Farnesoid X Receptor (FXR), in order to obtain clones that stably over-expressed the receptor. Transfection was performed with calcium phosphate method and after 72 hrs selection of positive clones was obtained by addition of G418 to transfected cells to isolate clones stably expressing FXR receptor. Cell cultures were split 1:3 when reaching confluency.

Correlation between Gaussia luciferase bioluminescence and cell number Approximately 1.5 x 10^5 HepG2 cells per well were transiently transfected with 0.5 µg of pcDNA3-hGLuc in a 24-well culture plate. Various concentrations of G418 (0, 100, 200, 300, 400, 500, 600, 700 µg/mL) were administrated to cells and incubated for 48 h. Viable cells were then counted by trypan blue exclusion. Linear regression analysis was performed between BL emission measured in 10 µL cell medium aliquots (by addition of 20 µL of coelenterazine 5µM in PBS and 5 sec acquisition) and viable cell count. All transfections were performed in triplicate.

Dual and triple luciferase mammalian assays. Approximately 1.5 x 10^5 HepG2-FXR cells were seeded per well in 24-well cell culture plates one day before transient transfection. Cells were washed with PBS, then transiently transfected or co-transfected with 0.5 µg of pGL3-CYP7A1-Luc and pcDNA 3.1-Lit RE-TS (or pGL3-Lit RE-TS) per well using Exgen500 (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. Alternatively cells were co-transfected with pGL3-CYP7A1-Luc, pGL3-CYP27A1$_{531}$Lit RE-TS and pcDNA3-hGLuc. Several parameters were optimized in order to increase the analytical performance of the bioassay, including cell number, incubation time with the analyte and transfection parameters. Each assay was performed in triplicate, and individual experiments were repeated at least 3 times. Three days after dual or triple co-transfection and treatment (usually 20 hrs) of the cells with the analyte, 10 µL of the medium were transferred in triplicate to a 96 well-microtiter plate for GLuc activity measurements, cells were washed in PBS and lysed with 200 µL of 1% Triton® X-100 for 5 min at 25°C. After centrifugation, 100 µL of surnatant were transferred to the 96 well-plate. Each lysate was analysed sequentially for the presence of the green-emitting luciferase (Ppy WT) and red-emitting luciferase (Lit RE-TS) by the addition of 100 µL luciferase assay system (Promega) and reading using the two emission filters described above to quantify the light emitted by each luciferase. The Promega Chroma-Luc "Calculator" was used to determine the contributions of red- and green-emitting luciferases.[31]GLuc activity was assayed by addition of 20 µL of coelenterazine 5µM in PBS and 5 sec acquisition. Each red (Lit RE-TS) or green (Ppy WT) signal was normalized using the GLuc signal as an internal control.

RESULTS AND DISCUSSION

Overexpression, purification, and bioluminescent emission of luciferase proteins. Red- and green-emitting thermostable mutants of *L. italica* luciferase were obtained by random and site-directed mutagenesis. The two luciferases Lit GR-TS (G216A, T217L, S234A, V243I, G248A, and F252S) and Lit RE-TS (G216A, T217L, S234A, and S286T) were purified to homogeneity in yields of 4-10 mg/L. Normalized bioluminescence spectra, shown in Figure 1, were obtained at pH 7.8 as described in the Materials and Methods section. The relative (to PpyWT) flash height specific

activities of LitRE-TS and Lit GR-TS were 42% and 67%, respectively. Lit GR-TS showed a broader emission spectrum with a maximal emission wavelength at 550 nm, whereas the mutant Lit RE-TS showed a maximum emission at 613 nm with a sharper spectrum (Table 1). Dose-response curves for IPTG were also produced to investigate the feasibility of using the two mutants of *L. italica* luciferase as reporter proteins in bacterial whole-cell biosensors or other bioanalytical applications. Dose-response curves, obtained in the range $1.0x10^{-6}$-$1.0x10^{-1}$ mM, were compared to those produced using the wild-type luciferase of *P. pyralis* as reporter protein. As shown in Figure 2, for both the two mutants the light emission was proportional to the amount of added IPTG over four to five orders of magnitude. The limits of detection for IPTG (blank plus three times the standard deviation) were (1.0 ± 0.2) x 10^{-6} mM with Lit RE-TS, (5.0 ± 0.3) x10^{-6} mM with Ppy WT, and (1.0 ± 0.2) x 10^{-5} mM with Lit GR-TS as reporter protein, demonstrating the feasibility of using these luciferase mutants as BL bioreporters in bacterial cells. Bioluminescence spectra were then collected using *E. coli* cells expressing Ppy WT, Ppy GR-TS, Lit GR-TS, and Lit RE-TS to compare emission obtained with purified proteins and emission obtained within recombinant cells. Figure 3 shows bioluminescent spectra obtained using recombinant bacterial cultures. The previously reported mutant Ppy GR-TS was selected for its excellent thermostability (37°C half-life = 10.5 h) and for its emission properties: an emission maximum of 548 nm at pH 7.8 and 25°C with a bandwidth similar to Ppy WT and a relative (to PpyWT) specific activity of 58%.[19] These desirable characteristics, together with its good specific activity and substrate K_m values similar to Ppy WT, make this mutant the best green-emitting candidate for dual-color reporter systems.The pH of the mixtures, measured after each emission spectrum was collected, ranged from 6.2 to 6.4. Since firefly luciferases are pH sensitive and may change emission wavelength at different pH, the pH was measured to investigate if an eventual red shifting or spectrum broadening caused by pH lowering could interfere with the signal separation. At the measured pH, emission maxima were 560 nm for Ppy WT, 551 nm for Ppy GR-TS, 565 nm for Lit GR-TS, and 613 nm for Lit RE-TS. Actually, the intracellular pH could be higher than the pH of cell mixtures, thus explaining the absence of a marked red-shifting in the emission of Ppy WT. The bioluminescent spectrum recorded for Lit GR-TS expressed in *E. coli* showed a markedly red-shifted emission spectrum which overlapped with the emission of Lit RE-TS . Table 1 shows that Lit GR-TS has also a broader emission

curve when compared to the other three luciferases (e.g., Lit GR-TS has a 50% emission bandwidth of 92 nm vs Ppy WT 50% emission bandwidth of 66 nm). Therefore this mutant was excluded to further investigations for in vivo dual-reporter applications. In contrast, Ppy WT and Ppy GR-TS provided a separation from Lit RE-TS of 53 nm and 62 nm when expressed in *E. coli* cells, respectively.

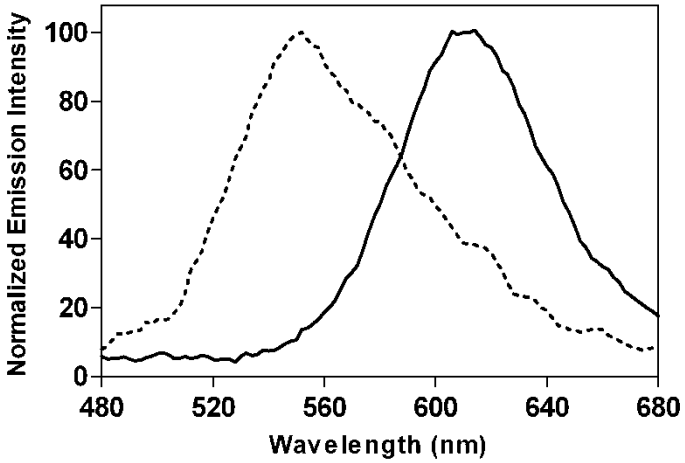

Figure 1: Normalized BL emission spectra of Lit GR-TS (dotted line) and Lit RE-TS (straight line) obtained at pH 7.8. Conditions for recording the emission spectra are described under Materials and Methods section.

Enzyme	In vitro (pH 7.8) Maxima (nm)	Bandwidth		In vivo (pH 6.3) Maxima (nm)	Bandwidth	
		50%			50%	
	20%			20%		
Ppy WT	557*	68*	113*	560	66	108
Ppy GR-TS	546*	66*	110*	551	68	106
Lit GR-TS	550	79	130	565	92	127
Lit RE-TS	613	66	114	613	52	88

* See Branchini et al., 2007.[19]

Table 1 Bioluminescent emissions of the purified luciferases (in vitro) and of *E. coli* cells expressing the luciferases (in vivo).

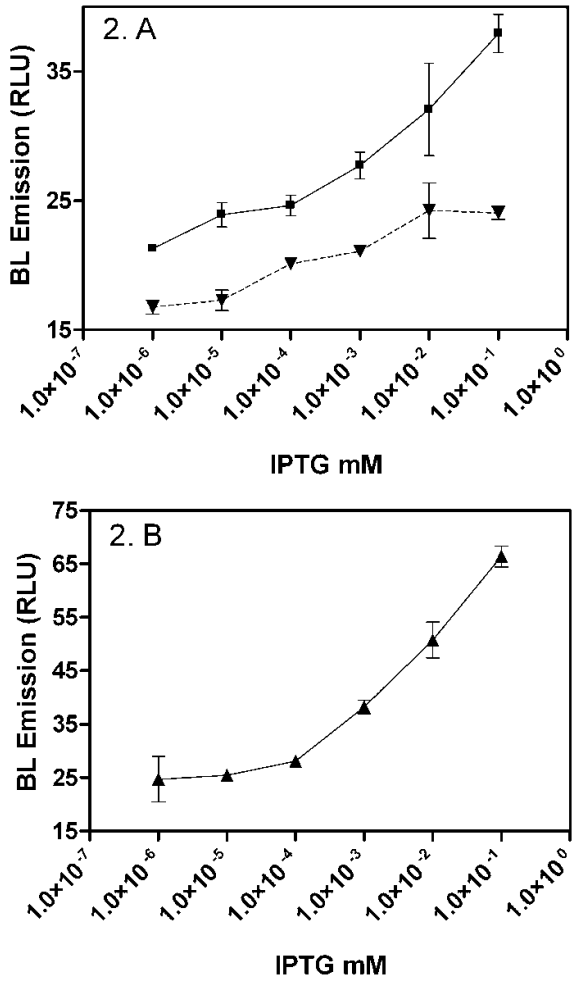

Figure 2: Dose response curves for IPTG obtained using JM109 cells harbouring plasmid with Lac promoter driving the expression of Lit RE-TS (Fig. 2. A —■—), Ppy WT (Fig 2. A - - -▼- - -) or Lit GR-TS (Fig. 2. B. —▲—).

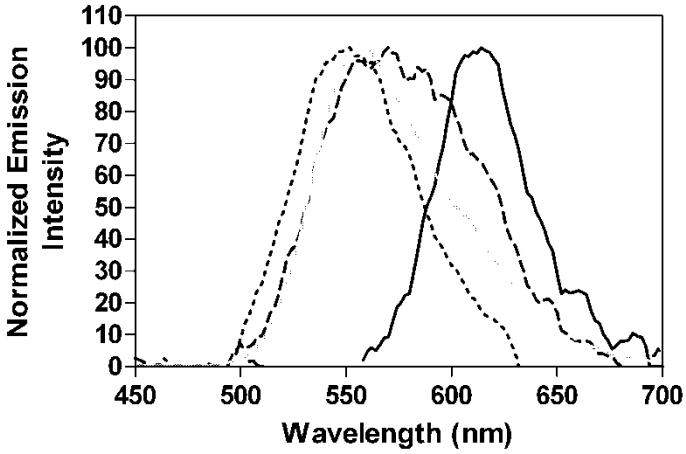

Figure 3: Normalized BL emission spectra obtained after addition of 100 µL of 1 mM D-luciferin in 0.1 M Na-citrate-buffer at pH 5 to 100 µL JM109 *E.coli* cells expressing Ppy WT (grey line), Ppy GR-TS (dotted line), Lit GR-TS (dashed line), and Lit RE-TS (straight line) in pQE30 vector.

Model dual-color assays. After checking that emission spectra obtained using bacterial cells expressing luciferase mutants mirrored those obtained with purified proteins, preliminary experiments were performed to investigate the best luciferase pair to be used in whole-cell dual-reporter applications. Ideally, in a dual-color system the emission spectra of the two reporters would not overlap. Unfortunately two BL proteins requiring the same substrate whose emissions do not overlap at all have not been identified yet. To minimize spectral overlap, the two emitters should have the narrowest bandwidths possible and well separated emission maxima. According to BL emission spectra, Ppy GR-TS and Ppy WT seem to be the more suitable green-emitting candidates for dual-color assays. The red-emitting Lit RE-TS was used in combination with the two green-emitting luciferases, Ppy WT and PPy GR-TS, to quantitate the relative amount of each luciferase by simultaneous measurements of red and green emission. Different amounts of cell cultures expressing the two luciferases were mixed to investigate the spectral resolution using the filter pair already described. A preliminary measurement of the filter correction factors was made by assaying each luciferase separately with no filters, with the green filter and with the red filter. These values provided the calibrations constants for the Promega Chroma-Luc calculator, an Excel spreadsheet designed to calculate corrected luminescence values from samples containing red- and green-emitting proteins. As previously reported, the concept of detection limit in a dual-color assays is not easy to define.[15] In fact, the luminescent signal from one emitter (eg., green) transmitted through the filter used to monitor the other emitter (eg., red), i.e. the interference, must be taken into consideration together with the background noise when calculating the detection limit. This interference is concentration-dependent, meaning that the detection limits and the working range of an emitter are dependent on the concentration of the other. Figure 4 shows BL emissions obtained mixing populations of *E. coli* JM109 cells expressing Lit RE-TS and Ppy WT grown at 37°C. Simultaneous measurements of green-and red-emitters were performed in intact *E. coli* cells in a high-throughput 96-well microplate format, demonstrating the feasibility of using Ppy WT and Lit RE-TS as a BL reporter pair. The deviation at low % of Lit RE-TS is due to the detection limit issues that arise from the overlap of the long wavelength “tail” of the non-Gaussian Ppy green enzyme spectrum. Because the overall activity of the green enzyme is higher than the red, the contribution of the overlapping signal is more significant at low percentages of the red emitter. The

importance of using two luciferases with similar expression levels in the system is well explained using Lit RE-TS and Ppy GR-TS as luciferase pair. Although these luciferases provide the best separation (62 nm), their expression levels in *E. coli* are very different and the very high BL intensity of Ppy GR-TS did not allow an accurate quantitation of low amounts Lit RE-TS (data not shown). In addition we demonstrated that these luciferases could be adapted for use with a single commercial reagent in a standard microplate luminometer protocol without the need to lyse cells, differently form previous works[19] in which the feasibility of using two red- and green Ppy mutants in dual-analyte assays was investigated using *E. coli* lysates from cells grown at 37°C. From these preliminary data, we concluded that Ppy WT and Lit RE-TS are the best luciferase pair for dual-color assay. In fact, these proteins have good spectral separation and similar expression levels, requiring the same synthetic substrate, D-luciferin. In other experiments, either the amount of red- or green-emitting luciferase was held constant while the amount of the other was varied (data not shown) and obtained data confirmed results previously shown.

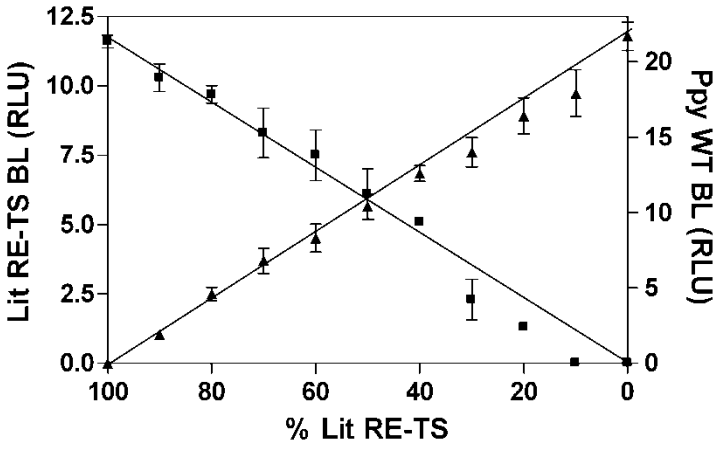

Figure 4. BL emissions of Lit RE-TS (■) and Ppy WT (▲) expressed in *E. coli* JM109 cells grown at 37°C. Mean values are plotted, with standard deviations indicated by error bars. RLU, relative light units.

Correlation between Gaussia luciferase bioluminescence and viable cell number. To preliminary evaluate the possibility to use GLuc as reporter gene in mammalian cell-based assays, the correlation between BL emission and cell viability was investigated.

Figure 5 shows the results of BL measurements in cell medium aliquots and viable cell counts using the trypan blue-exclusion method after treatment with different concentrations of G418 (range 0-700 µg/mL), an antibiotic used to select and maintain stable eukaryotic cell lines. As the numbers of HepG2 cells decreased, the bioluminescence decreased as well (r^2 = 0.9992), demonstrating the feasibility of using GLuc as internal control to monitor cell vitality.

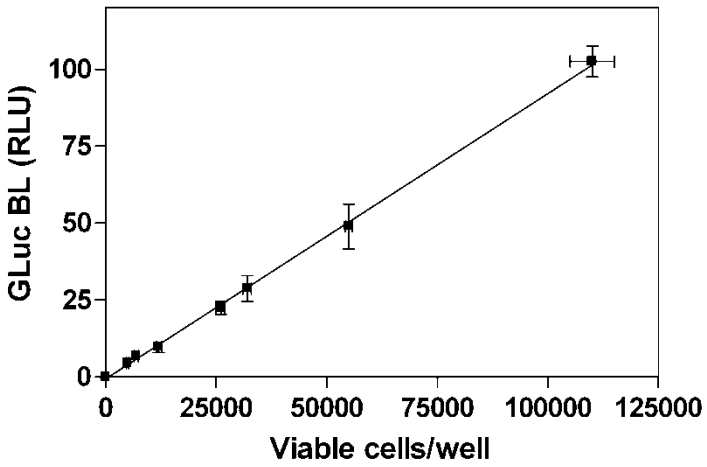

Figure 5: Correlation of BL emission in cell medium with viable cell number in the presence of increasing concentrations of G418. HepG2 cells were transfected with pcDNAhGluc and treated with G418 according to Materials and Methods section. Cell viability was determined by trypan blue exclusion. Linear regression analysis indicated high correlation between cell number and bioluminescence (r^2 = 0.9992).

Dual and triple reporter assays in mammalian cells. Next, we extended the promising results obtained with the bacterial dual-reporter model system based on *E. coli* cells expressing Ppy WT and Lit RE-TS to a mammalian model system. To investigate the feasibility of this system, reporter plasmids harbouring these luciferases were constructed with the final goal of studying the transcriptional regulation of CYP7A1 and CYP27A1, the two main enzymes responsible for bile acid biosynthesis in humans. Since no cell-based assays have been reported to simultaneously monitor the transcriptional regulation of these two enzymes, a reliable and accurate bioassay that allows a rapid and high throughput analysis of compounds able to regulate these two pathways would certainly be of great value. First, the Lit RE-TS was used as internal vitality control under the regulation of SV40 promoter, selected for strong

constitutive expression of the reporter protein, in pGL3 backbone. The plasmid pGL3-Lit RE-TS was transiently co-transfected with the plasmid pGL3CYP7A1-Luc in HepG2-FXR cells. 48-Hours after co-transfection, cells were treated with CDCA, a FXR ligand, in the concentration range 10-100 µM. According to previously reported data, a concentration-dependent inhibition of CYP7A1 by CDCA is shown in Figure 6A. Treatment of HepG2-FXR cells with CDCA in concentrations of 100 µM repressed the pGL3CYP7A1-Luc reporter activity by more than 50%. Due to CDCA toxicity at concentrations higher than 50 µM, an internal correction is mandatory to take into account changes in cell vitality. The introduction of an internal control allowed to correct the response of the Ppy WT using the ratio of Ppy WT emission over Lit RE-TS emission (Fig. 6B). By comparing the BL emissions of Ppy WT and LitRE-TS in the presence of increasing concentrations of CDCA it is evident that the reduced emission of Ppy WT can be partly attributed to the reduction in cell viability (e.g., 36% loss in viability as compared with 81% loss in Ppy WT specific emission for samples containing 100 µM CDCA). The reduction in cell metabolism is a consequence of exposing the cells to sublethal concentrations of the toxicants. In fact, hydrophobic bile acids like CDCA solubilize membrane-bound lipids, leading to damage to cell membranes.[32] The altered overall metabolism may in turn affect protein expression in the cell, including reporter proteins. The concentration of CDCA required to inhibit 50% of reporter activity (IC_{50}) was determined to be approximately 10 µM. Similar result were obtained co-transfecting HepG2 cells, an IC_{50} of 30 µM and 25 µM were determined by transfecting cells with pcDNA 3.1-Lit RE-TS or pGL3-Lit RE-TS, respectively (data not shown). The two plasmids were selected to compare the mammalian expression of Lit RE-TS under the regulation of two different constitutive promoters: the CMV promoter in pCDNA 3.1 backbone and HSV promoter in pGL3 backbone. These values are consistent with previous published results reported by Chiang et al., who studied the effect of CDCA on CYP7A1 transcription in HepG2 cells cotransfected with a CYP7A1/luciferase reporter and an FXR expression plasmid, obtaining an IC_{50} of 25 µM without FXR and 10 µM with FXR.[33]Assay reproducibility was evaluated at a fixed concentration of CDCA (10 µM); an intra-assay variability of 8.9 % and an inter-assay variability of 15.4 % (n=6) were obtained. A triple assay was then developed using three BL reporters: Ppy WT, Lit RE-TS and *Gaussia princeps* luciferase. The introduction of a third reporter protein, GLuc, whose activity can be measured directly in the medium with a different

substrate (coelenterazine), gives the remarkable advantage of a “separate” internal correction. That means that the two luciferases (Ppy WT, Lit RE-TS) can be used for measuring two analytes, and cell vitality is measured by simply taking out an aliquot (10 µL) of the medium and measuring it in the same 96 well-microplate used for the bioassay.

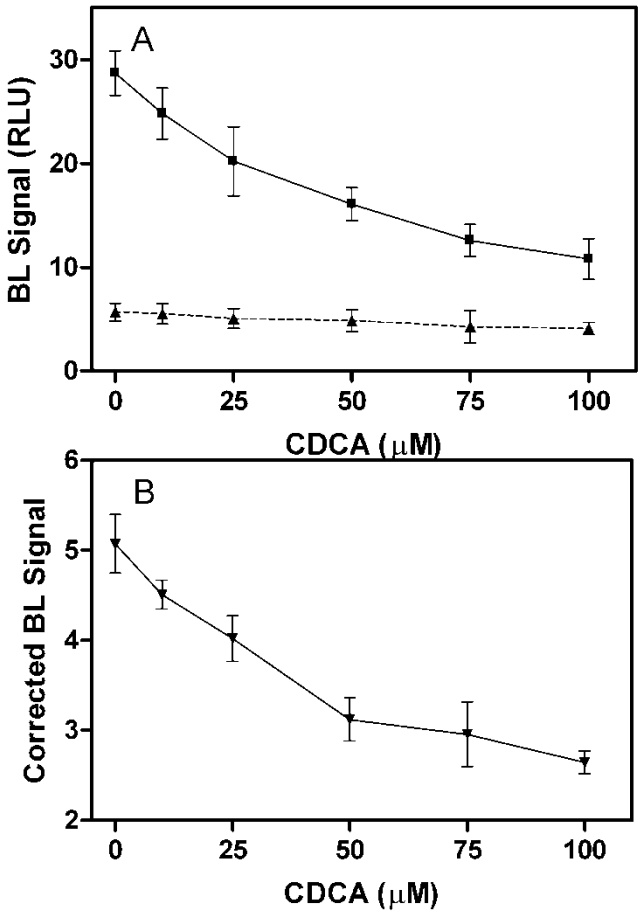

Figure 6. Effects of CDCA on CYP7A1 transcription. **6.A**. Bioluminescent emission of Ppy WT (—■—) under the regulation of CYP7A1 promoter and emission of the constitutively expressed Lit RE-TS (- - -▲- - -) in the presence of increasing concentrations of chenodeoxycholic acid. Values are the mean ± standard deviations of triplicate samples measured with the green and red filters in place. **6.B**. Corrected dose-response curve for CDCA (ratio of Ppy WT emission over Lit RE-TS emission against [CDCA], —▼—). Data are the average ± one standard deviation.

Figure 7 shows the non-corrected BL emissions of the two reporters (Fig.7A) and the BL emission of the vitality control GLuc (Fig 7B) in the presence of increasing concentrations of CDCA. By using the ratio of Ppy WT or Lit RE-TS emission over GLuc emission it was possible to correct both the two specific signals according to cell vitality and cell number in each well. As expected, addition of 50 µM of CDCA inhibited 73% of CYP7A1/Ppy WT and 62% CYP27A1/Lit RE-TS reporter activities, considering a 17 % aspecific loss in cell viability, as determined with GLuc BL activity in the medium (Figure 8). The response was reproducible at fixed concentrations of CDCA (10 µM), with an intra-assay variability of 15.0 % and 18.3 % and an inter-assay variability of 18.1% and 21.8% (n=6) for CYP7A1 and CYP27A1, respectively. Since the signal correction is the major concern of bioassays and whole-cell biosensor applications, a cell vitality control that does not interfere with specific signals, as proposed in the present work, will certainly improve the analytical performance of these assays. Current protocols are mostly based on the dual luciferase reporter assay system (Promega) involving the use of a firefly luciferase for monitoring gene expression and a *Renilla* luciferase as internal control. Unfortunately, this system is laborious and requires expensive reagents. Secreted proteins could be a valid alternative to normalize reporter data in a single, facile step. The applicability of secreted proteins has very recently pointed out by Wu et al.,[24] that reported a dual-reporter assay using *Cypridina* and *Gaussia* luciferases; both these luciferases are secreted into the medium via the endoplasmic reticulum and the Golgi complex. The major advantages of using secreted proteins reside in the rapidity of the assay (no need for cell lysis), absence of interference with measurements of intracellular reporter proteins, and in the possibility to conduct repetitive studies on the same cell population by simply taking out small aliquots (e.g., 10 µL) of cell medium. Furthermore, this has the great advantage of complete absence of interference between intracellular and secreted bioluminescent emission: the two signals are in fact measured in separate wells of a high throughput 96-well microtiter plate. By employing secreted reporters it is therefore possible to implement BL cell-based assays and increase their analytical performance by taking advantage of both multicolour reporter gene technology and the ease of use of secreted proteins.

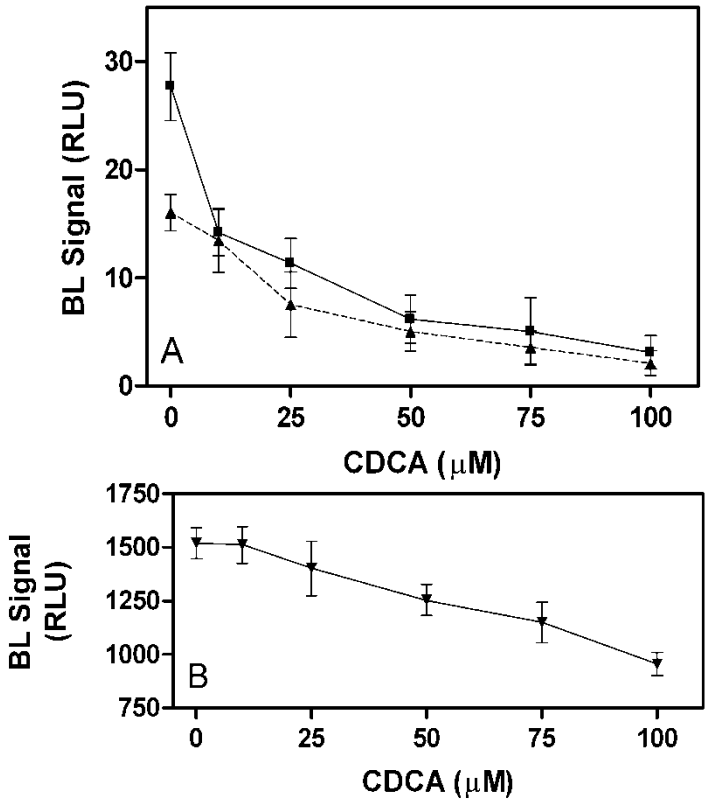

Figure 7: Effects of CDCA on CYP7A1 and CYP27A1 transcriptions. **7.A**. BL emission of Ppy WT (—■—) under the regulation of CYP7A1 promoter and BL emission of Lit RE-TS (- - -▲- - -) under the regulation of CYP27A1 promoter in the presence of increasing concentrations of chenodeoxycholic acid. Values are the mean ± standard deviations of triplicate samples measured with the green and red filters in place. **7. B**. BL emission of constitutively expressed *Gaussia* luciferase (—▼—) used as internal control.

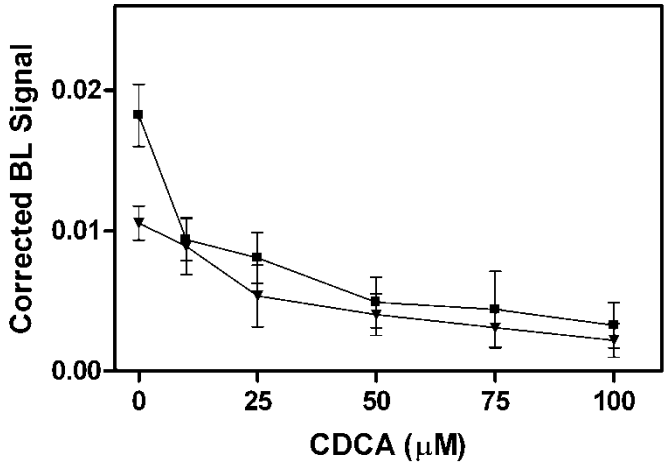

Figure 8: Corrected dose-response curves for CDCA on CYP7A1 (—■—) and CYP27A1 (—▼—) transcriptions (ratio of Ppy WT/Lit RE-TS emission over GLuc emission against [CDCA]).

CONCLUSIONS

A triple-reporter cell-based BL assay was developed in a high throughput 96-well microtiter plate format combining two different intracellular firefly luciferases as reporters and a secreted luciferase as internal control. Green- (*P. pyralis* wild type luciferase) and red-emitting (a thermostable mutant of *L. italica* luciferase) BL proteins were put under the regulation of CYP7A1 and CYP27A1 promoters, respectively, in order to monitor the two main bile acid biosynthesis pathways. In addition the secreted *Gaussia* luciferase, which employed a different BL substrate, was used as vitality control under the regulation of a constitutive promoter. The use of a secreted BL reporter simplified the measure of its activity because it can be separately evaluated on small aliquots of cell-culture medium. The developed assay does not suffer the limitations of previous triple-reporter assays based on green-, orange-, and red-emitting clickbeetle luciferases, such as errors in deconvolution process due to overlapping emissions. Therefore, this triple-reporter assay, the first reported in literature employing both intracellular and secreted luciferases, paves the way for the monitoring of multiple metabolic events for high-content screening.

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Chapter 7

General Discussion

General Discussion

The mechanism of color tuning in bioluminescent reactions is not fully understood yet but it is object of intense research and several hypothesis have been generated. In the past decade key residues of the active site of the enzyme or in the surface surrounding the active site have been identified as responsible of different color emission. Anyway since bioluminescence reaction is stricty dependent from the interaction between the enzyme and its substrate D-luciferin, modification of the substrate can lead to a different emission spectrum too.In the recent years firefly luciferase and other luciferases underwent mutagenesis in order to obtain mutants with different emission characteristics. Thanks to these new discoveries in the bioluminescence field multicolor luciferases can be nowadays employed in bioanalysis for assay developments and imaging purposes. The use of multicolor bioluminescent enzymes expanded the potential of a range of application in vitro and in vivo. Multiple information can be obtained from the same analytical session saving cost and time. This thesis focuses on several application of multicolor bioluminescence for high-throughput screening and in vivo imaging. Multicolor luciferases can be employed as new tools for drug discovery and developments and some examples are provided in the different chapters. New red codon optimized luciferase have been demonstrated to be improved tools for bioluminescence imaging in small animal and the possibility to combine red and green luciferases for BLI has been achieved even if some aspects of the methodology remain challenging and need further improvement. Other potentialities of multicolor bioluminescence in imaging can arise from the application of RET (resonance energy transfer) and Split luciferase technologies involving protein complementation assays (PCA). In the first case luciferase mutants with an emission peak at an opportune wavelengths can be better donor in energy transfer process to acceptor counterparts and lead to emission in the far red or infrared region of the UV/Visible spectrum. An example of this is reported by the use of PpYRE8 as donor for the development of an near-infrared probe relying on the conjugation of luciferase with a fluorescent dye (e.g. AF680) for an efficient intramolecular energy transfer. In the second case luciferase enzyme can be splitted in two inactive polypeptides (split reporter fragments) that will recover activity only when they reconstitute. For instance, Hida and collegues and developed a novel luciferase fragment by random mutagenesis and realized cross complementation between inter- and intra-luciferase fragments with high efficiency

They showed potential applications of the luciferase fragments for real-time and dual imaging in live *Xenopus laevis* embryo. In addition, Villalobos et al. described a set of reversible multicolored heteroprotein complementation fragments based on various firefly and click beetle luciferases that utilize the same substrate, D-luciferin. Luciferase heteroprotein fragment complementation systems enabled dual-color quantification of two discrete pairs of interacting proteins simultaneously or two distinct proteins interacting with a third shared protein in live cells. In analogy, the multicolor optimized luciferases described in this thesis might be used for developing new more sensitive imaging tools for in vivo analisys of protein protein interaction essential for understanding molecular pathways . At the same time the development of more sensitive and implemented microscopes and low-light imager for a better visualization and quantitation of multicolor signals would boost the research and the discoveries in life sciences in general and in drug discovery and devolpment in particular.

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Curriculum vitae

Laura Mezzanotte was born November the 1st 1982 in Rimini. She graduated in Biotechnology in 2004 and obnained her master degree in Pharmaceutical Biotechnology March 2007 at the University of Bologna. She collaborated with the group of Professor Aldo Roda in the analytical and bioanalitycal chemistry laboratory and the department of Gastroenterology of Sant'orsola hospital in Bologna until 2008. In January 2008 she started her PhD research in Pharmaceutical Sciences under the supervision of Prof. Roda. During autumn 2009 and summer 2010 she did part of his research at the department of Endocrinoloy at the Leiden University Medical Center, in Leiden, The Netherlands under the supervison of Prof. C.W.G.M. Lowik , head of the molecular imaging department. She attended at several courses of the molecular imaging field and she won Marlene Deluca prize for your investigator at the 16th Symposium of Bioluminescence and Chemiluminescence 19-23 April, Lyon. She was tutor at student laboratory courses of analytical chemistry for European Bachelor in Chemistry, Biotechnology from 2008 to 2010.

Laura Mezzanotte è nata il 1 Novembre 1982 a Rimini. Ha conseguito l'esame di laurea triennale in biotecnologie in 2004 e ha ottenuto il titolo di dottore magistrale in Biotecnologie farmaceutiche nel Marzo 2007. Ha collaborato con il gruppo del Prof. Aldo Roda nel laboratorio di chmica analitica e bioanalitica e con il dipartimento di Gastroenterologia dell'Ospadale Sant'orsola a Bologna fino al 2008. A gennaio 2008 ha iniziato il dottorato di ricerca in Scienze Farmaceutiche sotto la supervisione del Prof. Aldo Roda. Durente l'autunno 2009 e l'estate 2010 ha eseguito parte della sua ricerca nel dipartimento di Endocrinologia al Leiden University Medical Center a Leiden, Paesi Bassi sotto la supervisone del prof. C.W.G.M. Lowik, direttore del dipartimento di imaging molecolare. Ha frequentato diversi corsi nel campo dell'imaging molecolare e ha vinto il premio Marlene Deluca per giovani ricercatori al 16esimo simposio di bioluminescenza e chemiluminescenza tenutosi a Lione il 19-23 Aprile. Dal 2008 al 2010 ha svolto attività di tutorato ai laboratori didattici per le lauree triennali in Chimica e Biotecnologie nel campo della chimica analitica.