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Spectral-Resolved Gene Technology for Multiplexed Bioluminescence and High-Content Screening

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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 wavelengths 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 *Luciola italica* has been recently cloned, and thermostable red- and green-emitting mutants were 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 *Photinus 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 nonsecreted 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.

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 messenger 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 multistep 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]

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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 *Luciola 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 is the spectral unmixing that, when using more than two luciferases requiring the same substrate, does not allow complete separation of 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 that combines spectral unmixing of green- and red-emitting luciferases with a secreted luciferase requiring a different substrate, thus allowing us 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 *L. 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 the model analyte. Once evaluated that both 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 *Escherichia 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.

EXPERIMENTAL SECTION

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 *G. princeps* luciferase was a kind gift of Dr. Bruce Bryan (NanoLight Technologies, Pinetop, AZ). Ampicillin, IPTG, CDCA, phenylmethanesulfonyl fluoride (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

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as a starting point to clone the Ppy WT and Ppy GR-TS into the pQE30 backbone (Qiagen).

The plasmid pGL3CYP7A1-Luc containing the portion of the human CYP7A1 promoter −1887/+24 and *P. pyralis* luciferase wild-type as reporter gene was kindly provided by Prof. John Y.L. Chiang (Northeastern Ohio Universities College of Medicine). The plasmid pSOH4.3 containing a 4.3-kb portion of 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 TAT*GGTACC*CCAGGGATCAGATGACTGG (*Kpn*I) and the reverse primer RevCyp27 TCT*AAGCTT*ACCTCAGCCTCGCGCAG (*Hind*III); restriction sites are shown in italic type. The optimized conditions for the PCR were as follows: 1× Pfx buffer, 2× 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 s, 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′-GC*AAGCTT*ATGGAAACGGAAAGGGAGGA-3′ (forward primer containing a *Hind*III site, in italic type) and primer *Xba*ILitRETSrev, 5′-A*TCTAGA*TTACCCCCCGGCTTGTGGTTTCT-3′(reverse primer containing a *Xba*I site, in italic type). 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 the 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 Expression 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 of 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 $OD_{600\ nm}$ 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 (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1 mM PMSF, pH 7), and sonicated using 10 10-s bursts with a 15-s cooling period on ice between each burst. The lysate was then centrifuged at 5000 rpm for 1 h at 4 °C to pellet cellular debris, and the supernatant was saved to proceed with protocol for purification under native conditions. The cleared lysate was mixed with 1 mL of the 50% Ni-NTA slurry, loaded into a polypropylene column (Qiagen), and washed twice with 4 mL of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 1 mM PMSF, pH 7). The 500-μL aliquots were eluted in elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 7). Protein concentration was determined by a Bio-Rad microassay procedure using bovine serum albumin as the standard. The activity of the purified proteins was evaluated using a luminometer (Luminoskan Ascent, Labsystem) using 4 μL of eluted protein, 100 μL of 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 was added. After 5-min incubation at room temperature, bioluminescent emission spectra were collected as previously described. The pH

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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 1.0×10^{-6}−1.0×10^{-1} mM). Bacteria were incubated at 30 °C for 2 h. Luminescence measurements were taken with 5-s 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 of LB medium to midlog phase (A_{600} nm 0.6). Then cultures were induced with 0.1 mM IPTG for 2 h. The following luciferase pairs were used: Ppy WT and Lit RE-TS, PPy GR-TS and Lit RE-TS, and 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 sodium citrate buffer solution at pH 5.0, was 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 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 minimum essential medium with Earle's salts (MEM) supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 0.1 mM nonessential 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 feceptor (FXR), in order to obtain clones that stably overexpressed the receptor. Transfection was performed with the calcium phosphate method, and after 72 h, 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 1.5×10^5 HepG2 cells per well was 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 administered 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 5 μM in PBS and 5-s acquisition) and viable cell count. All transfections were performed in triplicate.

(31) Chroma-Glo Luciferase Assay System, Technical Manual No. TM062, Promega Corp., 2003; pp 7−9.

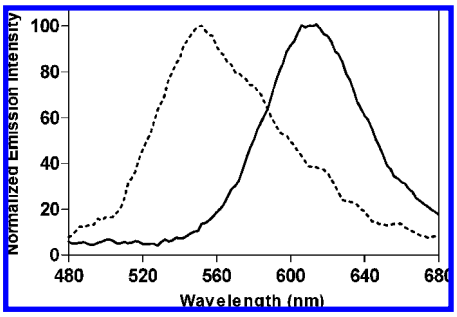

Figure 1. Normalized BL emission spectra of Lit GR-TS (dotted line) and Lit RE-TS (straight line) obtained at pH 7.8. Conditions for recording the emission spectra are described in the Experimental Section.

Dual and Triple Luciferase Mammalian Assays. Approximately 1.5×10^5 HepG2-FXR cells were seeded per well in 24-well cell culture plates 1 day before transient transfection. Cells were washed with PBS and then transiently transfected or cotransfected 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 cotransfected 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 cotransfection and treatment (usually 20 h) of the cells with the analyte, 10 μL of the medium was transferred in triplicate to a 96-well microtiter plate for GLuc activity measurements, and 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 was transferred to the 96-well plate. Each lysate was analyzed sequentially for the presence of the green-emitting luciferase (Ppy WT) and red-emitting luciferase (Lit RE-TS) by the addition of 100 μL of 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, 5 μM in PBS, and 5-s 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 Experimental 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

Table 1. Bioluminescent Emissions of the Purified Luciferases (in Vitro) and of *E. Coli* Cells Expressing the Luciferases (in Vivo)

enzyme	in vitro (pH 7.8) max (nm)	bandwidth 50%	bandwidth 20%	in vitro (pH 6.3) max (nm)	bandwidth 50%	bandwidth 20%
Ppy WT	557[a]	68[a]	113[a]	560	66	108
Ppy GR-TS	546[a]	66[a]	110[a]	551	68	106
Lit GR-TS	550	79	130	565	92	127
Lit RE-TS	613	66	114	613	52	88

[a] See Branchini et al.[19]

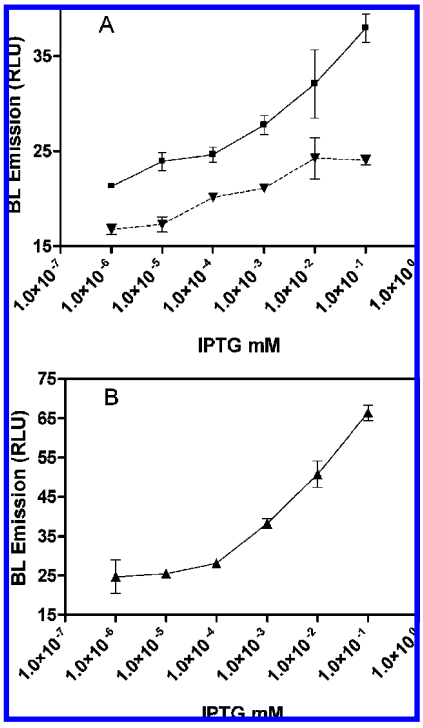

Figure 2. Dose−response curves for IPTG obtained using JM109 cells harboring plasmid with Lac promoter driving the expression of Lit RE-TS (A, ■), Ppy WT (A, ▼), or Lit GR-TS (B, ▲).

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 1.0×10^{-6}–1.0×10^{-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 mutants, the light emission was proportional to the amount of added IPTG over 4−5 orders of magnitude. The limits of detection for IPTG (blank plus three times the standard deviation) were $(1.0 \pm 0.2) \times 10^{-6}$ mM with Lit RE-TS, $(5.0 \pm 0.3) \times 10^{-6}$ mM with Ppy WT, and $(1.0 \pm 0.2) \times 10^{-5}$ 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 emissions obtained with purified proteins and emissions

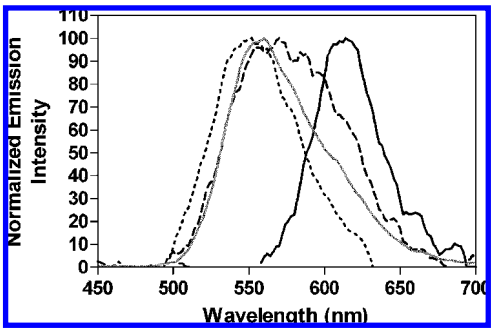

Figure 3. Normalized BL emission spectra obtained after addition of 100 μL of 1 mM D-luciferin in 0.1 M sodium citrate buffer at pH 5 to 100 μL of JM109 *E. coli* cells expressing Ppy WT (gray line), Ppy GR-TS (dotted line), Lit GR-TS (dashed line), and Lit RE-TS (straight line) in pQE30 vector.

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 whether an eventual red shifting or spectrum broadening caused by pH lowering could interfere with the signal separation. At the measured pH, emission maximums 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 and 62 nm when expressed in *E. coli* cells, respectively.

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

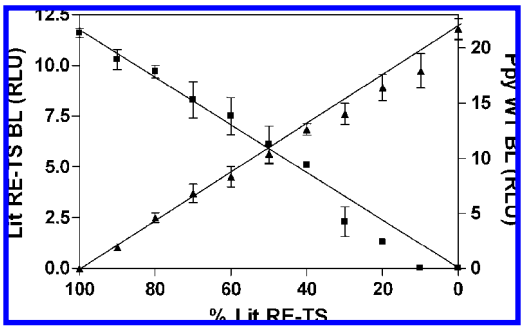

Figure 4. BL emissions of Lit RE-TS (■) and Ppy WT (▲) expressed in *E. coli* JM109 cells grown at 37 °C. Mean values are plotted, with standard deviations indicated by error bars.

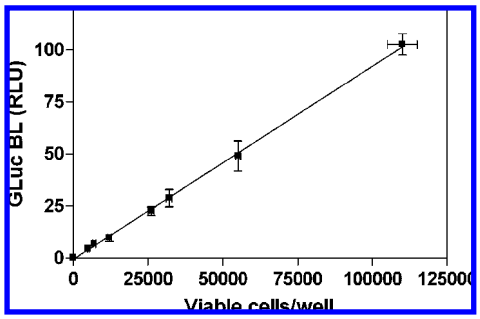

Figure 5. Correlation of BL emission in cell medium with viable cell number in the presence of increasing concentrations of G418. HepG2 cells were transfected with pcDNAhGluc and treated with G418 according to the Experimental Section. Cell viability was determined by trypan blue exclusion. Linear regression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

well-separated emission maximums. 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 percentage 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.

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.

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 harboring 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 a pGL3 backbone. The

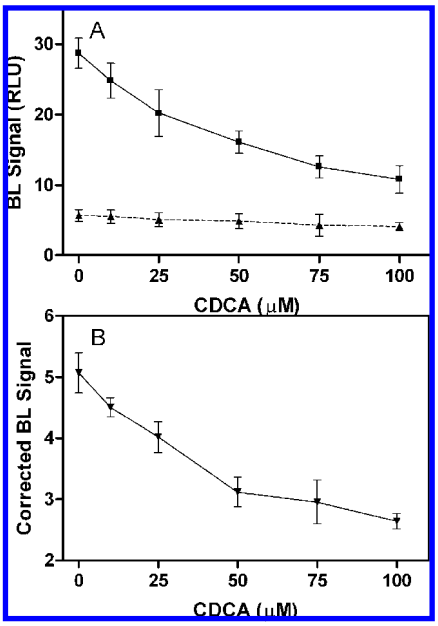

Figure 6. Effects of CDCA on CYP7A1 transcription. (A) Bioluminescent emission of Ppy WT (■) under the regulation of CYP7A1 promoter and emission of the constitutively expressed Lit RE-TS (▲) in the presence of increasing concentrations of chenodeoxycholic acid. Values are the mean ± standard deviations of triplicate samples measured with the green and red filters in place. (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.

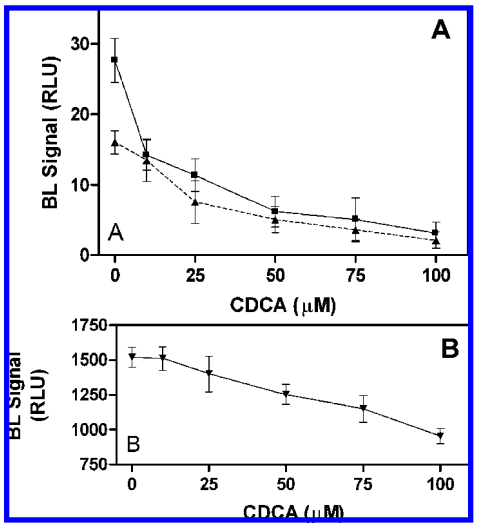

Figure 7. Effects of CDCA on CYP7A1 and CYP27A1 transcriptions. (A) BL emission of Ppy WT (■) under the regulation of CYP7A1 promoter and BL emission of Lit RE-TS (▲) under the regulation of CYP27A1 promoter in the presence of increasing concentrations of chenodeoxycholic acid. Values are the mean ±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

plasmid pGL3-Lit RE-TS was transiently cotransfected with the plasmid pGL3CYP7A1-Luc in HepG2-FXR cells. At 48 h after cotransfection, cells were treated with CDCA, an 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 us to correct the response of the Ppy WT using the ratio of Ppy WT emission over Lit RE-TS emission (Figure 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 ~10 μM. Similar results were obtained cotransfecting HepG2 cells; IC_{50} of 30 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 interassay variability of 15.4% ($n = 6$) were obtained.

A triple assay was then developed using three BL reporters: Ppy WT, Lit RE-TS and *G. 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 7 shows the noncorrected BL emissions of the two reporters (Figure 7A) and the BL emission of the vitality control GLuc (Figure 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 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).

(32) Palmeira, C. M.; Rolo, A. P. *Toxicology* **2004**, *203*, 1−15.

(33) Chiang, J. Y.; Kimmel, R.; Weinberger, C.; Stroup, D. *J. Biol. Chem.* **2000**, *275*, 10918−24.

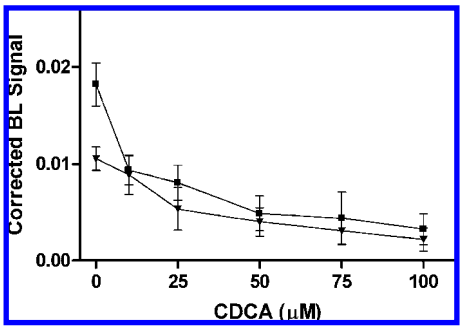

Figure 8. Corrected dose−response curves for CDCA on CYP7A1 (■) and CYP27A1 (▼) transcriptions (ratio of Ppy WT/Lit RE-TS emission over GLuc emission against [CDCA]).

The response was reproducible at fixed concentrations of CDCA (10 μM), with an intra-assay variability of 15.0 and 18.3% and an interassay 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 been pointed out by Wu et al.,[24] who 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), the absence of interference with measurements of intracellular reporter proteins, and 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 multicolor reporter gene technology and the ease of use of secreted proteins.

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 the deconvolution process due to overlapping emissions. Therefore, this triple-reporter assay, the first reported in the 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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