Video Article

In Situ Measurement and Correlation of Cell Density and Light Emission of Bioluminescent Bacteria

Eveline Brodl[1], Johannes Niederhauser[1], Peter Macheroux[1]

[1]Institute of Biochemistry, Graz University of Technology

Correspondence to: Peter Macheroux at peter.macheroux@tugraz.at

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Abstract

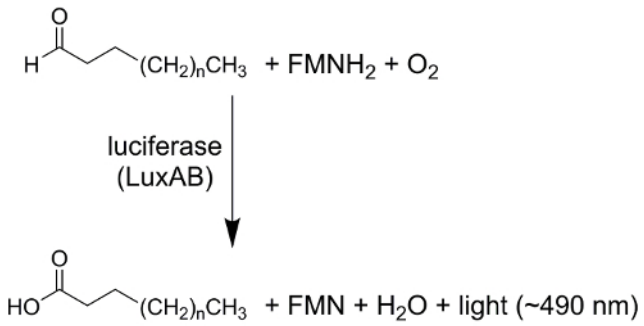
There is a considerable number of bacterial species capable of emitting light. All of them share the same gene cluster, namely the *lux* operon. Despite this similarity, these bacteria show extreme variations in characteristics like growth behavior, intensity of light emission or regulation of bioluminescence. The method presented here is a newly developed assay that combines recording of cell growth and bioluminescent light emission intensity over time utilizing a plate reader. The resulting growth and light emission characteristics can be linked to important features of the respective bacterial strain, such as quorum sensing regulation. The cultivation of a range of bioluminescent bacteria requires a specific medium (*e.g.,* artificial sea water medium) and defined temperatures. The easy to handle, non-bioluminescent standard-research bacterium *Escherichia coli* (*E. coli*), on the other hand, can be cultivated inexpensively in high quantities in laboratory scale. Exploiting *E. coli* by introducing a plasmid containing the whole *lux* operon can simplify experimental conditions and additionally opens up many possibilities for future applications. The expression of all *lux* genes utilizing an *E. coli* expression strain was achieved by construction of an expression plasmid via Gibson cloning and insertion of four fragments containing seven *lux* genes and three *rib* genes of the *lux-rib* operon into a pET28a vector. *E. coli* based *lux* gene expression can be induced and controlled via Isopropyl-β-D-thiogalactopyranosid (IPTG) addition resulting in bioluminescent *E. coli* cells. The advantages of this system are to avoid quorum sensing regulation restrictions and complex medium compositions along with non-standard growth conditions, such as defined temperatures. This system enables analysis of *lux* genes and their interplay, by the exclusion of the respective gene from the *lux* operon, or even addition of novel genes, exchanging the *luxAB* genes from one bacterial strain by another, or analyzing protein complexes, such as *luxCDE*.

Video Link

The video component of this article can be found at https://www.jove.com/video/57881/

Introduction

The emission of light by living organisms (bioluminescence) is a fascinating process found in bacteria, fungi, insects, nematodes, fish, and squids[1]. Bioluminescent light emission emerges from a chemiluminescent reaction in which chemical energy is (partially) transformed into light energy ("cold light"). In bioluminescent bacteria, the heterodimeric enzyme luciferase catalyzes the monooxygenation of long chain, aliphatic aldehydes, such as tetradecanal, to the corresponding acids accompanied by light emission with a maximum at 490 nm[2,3].



n = 4, 6, 8, 10

Figure 1: General reaction scheme of bacterial luciferase. The bacterial luciferase (LuxAB) catalyzes the monooxygenation of long chain aldehydes ($CH_3(CH_2)_nCHO$) by utilizing reduced flavin mononucleotide ($FMNH_2$) and molecular oxygen (O_2), yielding the products long chain acids ($CH_3(CH_2)_nCOOH$), flavin mononucleotide (FMN), water (H_2O), and the emission of light centered at 490 nm. Please click here to view a larger version of this figure.

The energy released during this oxidation causes an excited state FMN-4a-hydroxide, which serves as the light emitting luciferin[4]. The proteins involved in bacterial bioluminescence, namely LuxCDABEG, are encoded by the *lux* operon and are highly conserved over various bacterial strains[2,5]. The genes *luxA* and *luxB* encode for the heterodimeric luciferase; *luxC's*, *luxD's*, and *luxE's* gene products are components of a fatty acid reductase complex; and *luxG* encodes for a flavin reductase[6]. A number of bioluminescent *Photobacteria* (*e.g.,Photobacterium mandapamensis* 27561) carry the additional *luxF* gene. It was reported that LuxF is a homodimeric protein that binds the unusual flavin derivative 6-(3'-(*R*)-myristyl)-FMN (myrFMN)[7,8,9,10,11,12]. Additional genes have been identified that are responsible for riboflavin synthesis (*e.g., ribEBH*) and furthermore regulatory genes have been reported, that play a role in quorum sensing regulation of bioluminescence, especially for *Vibrio fischeri* and *Vibrio harveyi*[6,13]. Despite the highly conserved gene order, bioluminescent bacteria show high variations in characteristics like growth behavior, intensity of light emission, or regulation of bioluminescence[2,5,14].

Several modified strains or plasmids containing parts or whole *lux* operons are known, exploiting bioluminescence as reporter systems. Various applications such as determining promoter activity, monitoring of bacterial contaminations in environment or food samples, Bioluminescence Resonance Energy Transfer (BRET), *in vivo* imaging of infections in eukaryotic organisms, pyrosequencing, and so forth were established[15,16,17]. Interestingly, the three most frequently used bioluminescent reporter systems are derived from the North American firefly (*Photinus pyralis*), the enteric pathogen of nematodes (*Photorhabdus luminescens*), and the sea pansy (*Renilla reniformis*). None of those systems has a bacterial origin, but the use of *lux* genes and operons from bacterial origins is gaining more interest for applied research[16]. The less abundant application of bioluminescence proteins from bacterial sources is mainly due to lower stability and longevity of bacteria derived luminescent proteins which can be related to their marine habitats. Bioluminescent bacteria of marine habitats are not cultivable under standard lab conditions. These bacteria require specific growth media and conditions, such as artificial sea water medium and lower growth/incubation temperatures (*e.g.,* 28 °C).

To simplify comparison of *lux* operon characteristics or single *lux* genes of a range of different bioluminescent bacterial strains, a method to standardize *lux* operon expression and analysis is a prerequisite. Thus, the idea of integrating the whole *lux-rib* operon into the standard-research bacterium *Escherichia coli* (*E. coli*) emerged. For this purpose, Gibson assembly proved to be a useful tool to integrate multiple linear, overlapping fragments into one expression vector without the need for specific restriction sites. This method is also suitable when DNA inserts are too large (*e.g.,P. mandapamensis* 27561 *luxCDABFEG-ribEBH*; ~ 9 kb operon size) to be amplified via PCR. A *lux* operon can be separated into multiple overlapping fragments, then be assembled into one expression plasmid and finally the sequence verified assembly product can be directly transformed into an appropriate *E. coli* system for high yield protein expression[18,19,20]. In addition to the easy to handle *E. coli* based *lux* gene expression, a simple method combining recording of cell growth and bioluminescent light emission remained to be established. The method described here allows the *in situ* measurement and correlation of cell density and light emission of bioluminescent bacteria.

The analysis of *lux* genes and *lux* operon order and regulation of various bioluminescent bacteria with, on the one hand, an artificial bioluminescent *E. coli* system containing the whole *lux-rib* operon of *P. mandapamensis* 27561 and, on the other hand, a newly developed plate reader assay combining the *in situ* recording of cell density and light emission, helps to gain more information on the various bacterial *lux* systems. This fundamental characterization and comparison of luciferases and related enzymes may lead to alternatives to the already established reporter systems with enhanced stability and activity.

Protocol

1. Design, Preparation, and Expression of the *lux* Operon in *Escherichia coli*

Note: See **Table of Materials** for information on commercial kits used in this section.

1. For transferring the *lux* operon into *E. coli* choose a standard pET vector with appropriate restriction sites and antibiotic resistance gene of interest (*e.g.,* pET28a; NcoI, XhoI, kanamycin).
2. Design the fragments and overlapping primers for Gibson assembly based on the DNA sequence of *Photobacterium mandapamensis* 27561 (GenBank: DQ988878.2).

3. Set up a standard PCR reaction with the designed primers and the isolated genomic DNA of *Photobacterium mandapamensis* 27561 as template (see **Supplemental Material** for primers and conditions).
   NOTE: Isolation of genomic DNA of the respective bacterial strain enhances PCR efficacy.
4. Purify the PCR product via spin-column purification.
5. Perform a restriction digestion of the isolated pET28a vector with NcoI and XhoI at 37 °C for 45 min.
6. Purify the linearized vector and the PCR fragments via agarose gel electrophoresis and subsequent spin-column purification.
7. Determine the DNA concentration of each fragment and the linearized vector and calculate the optimal quantities for the assembly according to the protocol[20,21].
   NOTE: Efficacy of assembly depends on fragment size and number and has to be adjusted according to manufacturer's protocol[20,21].
8. After combining all fragments and the buffer in a PCR tube, incubate the assembly mixture in a PCR machine at 50 °C for 1 h.
9. Transform the assembled vector product according to standard transformation protocols for *E. coli* bacterial plasmid transformation into an appropriate *E. coli* system for high yield plasmid replication (*e.g., E. coli* TOP10 or XL-1).
10. Pick colonies from the transformation plate and streak on new plates for DNA isolation.
11. Isolate plasmid DNA according to standard protocols.
12. To verify the correct assembly of the plasmid including all fragments, first perform a colony PCR according to standard protocols using primers specific for every assembled fragment.
13. Additionally to the colony PCR and subsequent agarose gel electrophoresis, prepare all isolated assembly vectors for DNA sequencing to verify the correct assembly and the correct DNA sequences.
14. Transform the verified plasmid according to standard transformation protocols for *E. coli* bacterial plasmid transformation into an appropriate *E. coli* system for high yield protein production (*e.g.,E. coli* BL21).
    NOTE: Continue directly with expression protocol below. For longer storage, the preparation of a glycerol stock is recommended.

2. Expression of Modified *E. coli* Strains

1. Prepare an overnight culture (ONC) for expression by inoculation of an appropriate volume of LB medium (*e.g.,* 100 mL) with the previously prepared glycerol stock of the *E. coli* BL21 cells transformed with the assembled plasmid or directly from a transformation plate. Add 100 µL of kanamycin (50 mg/mL; antibiotic resistance gene of pET28a) and incubate the ONC at 37 °C and 120 rpm in an incubator shaker overnight.
2. Inoculate the main expression culture (*e.g.,* 800 mL of LB medium) with 8 mL of the ONC and add 800 µL of kanamycin (50 mg/mL).
3. Incubate the main culture at 37 °C and 120 rpm in an incubator shaker until the cell density reaches an OD_{600} of 0.6 - 0.8 (approximately 2.5 h).
4. Reduce the incubation temperature to 28 °C.
5. Induce protein expression by adding IPTG to a final concentration of 0.1 mM.
   NOTE: Empirical tests showed that the reduction of the temperature to 28 °C gave the highest light intensity.
6. Observe cells until they start shining (approximately 1 h).
   NOTE: Depending on the purpose of the expression, the cells are grown until the next day and are then harvested, or the cells can be kept shaking as long as they are shining (maximum 48 h). Harvesting the cells and purification of any proteins can be done according to standard procedures.

3. Expression of Bacterial Bioluminescent Strains

NOTE: Bacterial bioluminescent strains require specific growth medium/artificial sea water medium for growth and light production.

1. Prepare artificial sea water medium, composed of two separately prepared medium components.
   NOTE: The preparation of the artificial sea water medium was adapted from the original protocol[22]. The following amounts are for 1 L liquid medium or 1 L agar medium.
   1. For artificial sea water medium, weigh in the following salts: 28.13 g NaCl, 0.77 g KCl, 1.60 g $CaCl_2 \cdot 2H_2O$, 4.80 g $MgCl_2 \cdot 6H_2O$, 0.11 g $NaHCO_3$, and 3.50 g $MgSO_4 \cdot 7H_2O$.
   2. Add 1 L of distilled water and dissolve all components.
   3. For LB medium, weigh in the following ingredients: 10 g yeast extract, 10 g peptone, and for agar plates, an additional 20 g agar.
   4. Add 250 mL of tap water and dissolve components.
   5. Autoclave both prepared media separately at 121 °C for 20 min.
   6. For agar plates, combine 250 mL of LB medium with 750 mL of artificial sea water medium directly after autoclaving and prepare plates.
   7. For liquid medium, combine 250 mL of LB medium with 750 mL of artificial sea water medium either directly after autoclaving or when cooled down.
      NOTE: The artificial sea water medium may get turbid through salt precipitation.
2. Streak the bacterial bioluminescent strains on artificial sea water medium agar plates and incubate overnight at 24 - 30 °C.
   NOTE: Long time storage of bacterial strains is normally achieved through freezing glycerol stocks of the bacterial culture. Strains should always be streaked on agar plates first to assure uniform starting conditions for all strains, prior to usage for liquid cultures, due to a lag phase in growth after thawing.
3. Prepare an ONC by inoculating 100 mL artificial sea water medium with a single colony from the plate. Incubate the ONC at 24 - 30 °C and 120 rpm in an incubator shaker overnight.
4. Inoculate 800 mL of artificial sea water medium with 8 mL of ONC.
5. Incubate the bacterial cells at 24 - 30 °C and 120 rpm in an incubator shaker.
   NOTE: The light intensity profile of bioluminescent bacteria strongly varies with temperature. Depending on the regulatory mechanisms of the light production of the respective bacterial strain, light emission may start after approximately 1 - 6 h.
6. Observe bacterial cell culture until they start shining (approximately 1 - 6 h).

NOTE: Depending on the purpose of the expression, the cells are grown until the next day and are then harvested, or the cells can be kept shaking as long as they are shining. Harvesting the cells and purification of any proteins can be done according to standard procedures.

4. *In Vivo* Activity Assay for Bacterial Bioluminescent Strains and Modified *E. coli* Strains

NOTE: Long time storage of strains is normally acieved through freezing glycerol stocks of the bacterial culture. Strains should always be streaked on agar plates first to assure uniform starting conditions for all strains, prior to usage for liquid cultures, due to a lag phase in growth after thawing.

1. Streak the desired bioluminescent bacterial strain or modified *E. coli* strain on an agar plate and incubate at 28 °C overnight.
   NOTE: The incubation temperature can vary from strain to strain and has to be evaluated empirically. To be able to compare bioluminescent bacterial strains and modified *E. coli* strains, growth conditions have to be identical.
2. Inoculate 3 mL of medium with the respective strain with a single colony from an agar plate and incubate the cells at 28 °C and 180 rpm in an incubator shaker for approximately 1 - 2 h.
3. Measure the cell density of a 1:10 dilution of the liquid culture at 650 nm. Calculate the ratio and volume for 1 mL culture with an OD_{650} of 0.05.
   NOTE: The subsequent plate reader assay will determine the cell density at 650 nm to avoid interference by the light emission of the strains.
4. Pipette the calculated volume of culture and medium into a 24-well black-walled plate with glass bottom. For the modified *E. coli* strain, add 1 µL of kanamycin (antibiotic resistance of pET28a vector) and 1 µL of IPTG (induction of gene expression) to the samples. Place a lid on the plate to avoid evaporation during the measurements.
   NOTE: To assure that the pET28a vector containing the whole *lux* operon does not get lost by the *E. coli* culture, kanamycin must be added to each *E. coli* sample in the plate wells and to assure that light production of the *E. coli* cells can be measured, the gene expression must be induced by IPTG. To avoid crosstalk and measurement interference, black-walled well plates with glass bottom and transparent lid showed best results. Nevertheless, crosstalk can be observed and well positions have to be chosen carefully.
5. Start the measurement in a plate reader.
   NOTE: The plate reader protocol is based on a script specially developed for this assay (see **Supplemental Material**) that combines two measurements, absorbance and bioluminescence. Data points are collected every 10 min with permanent shaking between the measurements and a constant temperature of 28 °C.

Representative Results

The gene order of the *lux* operon - *luxCDABFEG* - is highly conserved over various strains[2,5,14]. For the design of the plasmid, the sequence information was taken from the bioluminescent bacterial strain *Photobacterium mandapamensis* 27561 and its gene order was kept the same, and, also, noncoding sequences between single genes were considered. A schematic overview of the applied Gibson cloning strategy is depicted in **Figure 2**. Four fragments in total, *luxCDAB*, *luxF*, *luxEG,* and *ribEBH*, with 20 - 40 base pair overlapping sequences were generated. After following all steps of the Gibson assembly[20], DNA sequencing confirmed the correct assembly of the plasmid, including all fragments. The vector map of the final assembly product pET28a containing the *lux-rib* operon is depicted in **Figure 3**. A significant advantage of this modified pET28a vector is the utilization of standardized *E. coli* growth conditions and controlled induction with IPTG.

To measure light emission of bioluminescent bacteria and the respective cell density, a plate reader based method was developed. The method for the plate reader was generated combining single measurement scripts for light intensity and cell density. This novel script enabled the measurement of OD_{650} and light intensity every 10 min for a user defined time frame, which has to be adjusted to the generation time of the bacteria used for the respective analysis (*e.g.,* 10 h). The measurement of the optical density was performed at 650 nm to avoid interference with the light emission. As a proof of concept and to assure the health and the correct growth behavior of the *E. coli* cells, reference measurements were performed. In **Figure 4** the comparison of *E. coli* BL21 cells, *E. coli* BL21 cells containing an empty pET28a vector, and *E. coli* BL21 cells containing the pET28a vector with the *lux-rib* operon insert are presented. For the latter strain, no IPTG was added to analyze the light emission due to the leakiness of the T7 promoter. All three reference measurements show a sigmoidal growth curve with three growth phases (lag, exponential, and stationary phase). Only the *E. coli* BL21 cells containing the pET28a vector with the *lux-rib* operon insert start to emit light, but in contrast to the measurements where expression is induced by addition of IPTG and light is emitted after 30 min, the non-induced cells only start to shine after approximately 5 h and show a much lower light emission (ca. 4-fold) compared to the induced system.

Figure 5 gives a comparison of growth curves and light intensities of the *lux* operon expressed in *E. coli* and the bioluminescent bacterial strain *P. mandapamensis* 27561, either in LB medium or in artificial sea water medium, using the novel established *in situ* method. To compare these bacteria, the measurements were performed at an incubation temperature of 28 °C. This temperature decreases the growth rate of the modified *E. coli* strain in LB medium as well as artificial sea water medium, but for bioluminescent bacterial strains lower temperatures are crucial. This temperature dependence is visible in **Figure 5A**, as *P. mandapamensis* 27561 shows a much higher cell density than *E. coli*. Furthermore, while for *E. coli* strains, LB medium allows generation of higher cell densities, for natural bioluminescent bacterial strains, artificial sea water medium is preferred and essential for bioluminescence. The recorded cell densities correlate with the respective light intensities, as shown in **Figure 5B**. Noteworthy, the bioluminescent *E. coli* cells reach similar light emission maxima in both LB medium as well as artificial sea water medium, although the highest intensities were recorded at different time points. In contrast to this observation, *P. mandapamensis* 27561 is viable with highly reduced growth rates in LB medium, but the bacterial cells did not emit light at all (**Figure 5B**). In artificial sea water medium, *P. mandapamensis* 27561 shows a maximum of light emission at around 1×10^4 counts per second, which is nearly a factor of 200 lower than *E. coli*. **Figure 5C** represents relative light units where the bioluminescence is normalized by the OD. These results confirm that not only was the insertion of a plasmid containing the *lux* operon into *E. coli* successful and functional, but also that this modified *E. coli* strain is a valid alternative with even higher light emission yields and without the limitation of bacterial bioluminescence of marina bacteria, such as a complex seawater medium and lower temperatures.

Additionally, a long-term measurement of the *E. coli* based *lux-rib* gene expression over 24 h was performed to analyze the longevity of the light emission (**Figure 6**). Light emission lasted for 19.5 h, much longer than the bacterial strains (*e.g., P. mandapamensis* 27561) where a gradual decrease was observed resulting in very low light emission after 10 h.

To illustrate the limitations of the developed assay, **Figure 7** shows the measurement results of three bioluminescent bacterial strains, namely *Photobacterium mandapamensis* S1, *Photobacterium mandapamensis* TH1 and *Vibrio harveyi* 14126. For the first strain (S1), the method works very well and shows a maximal bioluminescence intensity of nearly 2 x 10^6. For the other two strains (TH1 and 14126), the maximal light intensity cannot be determined because the light intensity generated by both exceeded the detection limit of the used instrument settings. The gain value defined for the developed method (script) for these two strains was set too high. Nevertheless, the onset of bioluminescence activity can be compared with each other. *P. mandapamensis* TH1 and *P. mandapamensis* S1 start shining after approximately 1 h and an OD_{650} value of 0.1 - 0.2, respectively. In contrast, *V. harveyi* 14126 starts to emit light after approximately 5.5 h at an OD_{650} value of 1.0. The observed onset of light emission is accompanied by an exponential increase in OD as well as bioluminescence. It is known that bioluminescence of *V. harveyi* 14126 underlies quorum sensing regulation and therefore a specific cell density allowing the activation of the *lux* operon, which can be clearly observed in **Figure 7**[13]. This result demonstrates that with this novel *in situ* plate reader assay it is possible to easily compare bioluminescent bacteria and also roughly define a regulatory mechanism of these strains by determining whether a quorum sensing regulation can be observed or not.

Figure 8 shows an example of an expression culture of *E. coli* BL21 cells harboring the assembled pET28a plasmid containing the *lux* operon after induction with IPTG. After approximately one h after induction, the *E. coli* cells start shining with a blue-green color. **Figure 8A** shows the *E. coli* expression culture photographed in the light and **Figure 8B** gives the same culture in the dark. **Figure 8C** depicts an agar plate of artificial sea water medium with *P. mandapamensis* S1 glowing in the dark in the same blue-green color characteristic for bacterial bioluminescence.

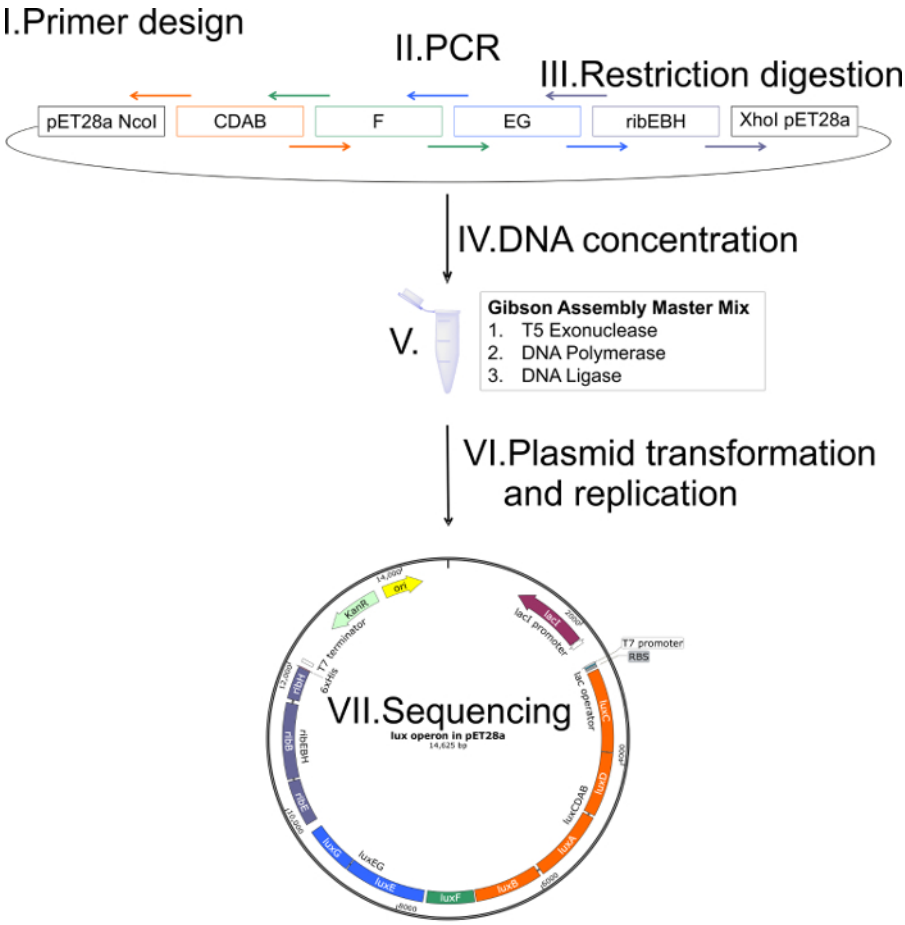


Figure 2: Schematic representation of the applied Gibson cloning strategy. Step (I): Overlapping primers (colored arrows; ca. 20 - 40 base pair overlap) are designed. Overlapping primers contain annealing sequences consisting of the respective 5' and 3' region of one fragment and the respective 3' and 5' region of the adjacent segment. **Step (II):** The designed fragments for assembly are generated via standard PCR reactions. **Step (III):** The target vector is linearized by restriction digestion (*e.g.,* NcoI, XhoI). **Step (IV):** The DNA concentrations of all fragments and the linearized vector have to be determined to adjust concentration appropriate for Gibson assembly (according to the manufacturer's protocol). **Step (V):** All fragments and the linearized vector with optimized DNA concentrations are combined with the Gibson assembly master mix (T5 exonuclease, DNA polymerase, and DNA ligase) and are incubated at 50 °C for 1 h. **Step (VI):** The assembly product is transformed according to standard protocols into an appropriate *E. coli* strain for high yield plasmid replication (*e.g., E. coli* TOP10 or XL-1). **Step (VII):** To verify the correct assembly of the plasmid, DNA sequencing of the assembled plasmid has to be performed. Please click here to view a larger version of this figure.

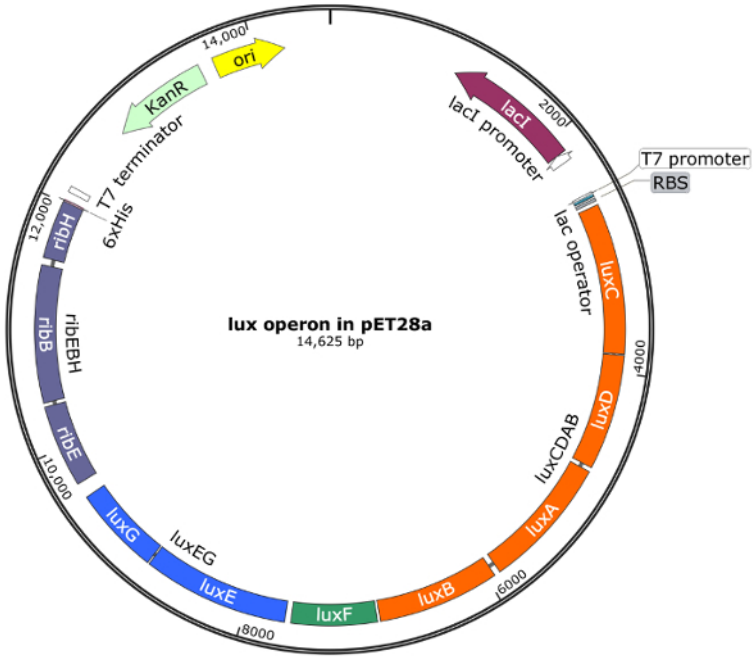


Figure 3: Vector map of pET28a containing the *lux-rib* operon. The *lux-rib* operon of *P. mandapamensis* 27561 is inserted in the multiple cloning site of pET28a in the original gene order (*luxCDABFEG-ribEBH*). Restriction sites used for cloning are NcoI and XhoI. Fragments used for Gibson assembly of the operon are *luxCDAB* in orange, *luxF* in green, *luxEG* in blue and *ribEBH* in lavender; genes within a fragment are shown as a separate box. Noncoding sequences between each gene of the operon are included in the applied cloning strategy. The final plasmid size of pET28a containing the whole *lux-rib* operon is 14,625 base pairs. Please click here to view a larger version of this figure.

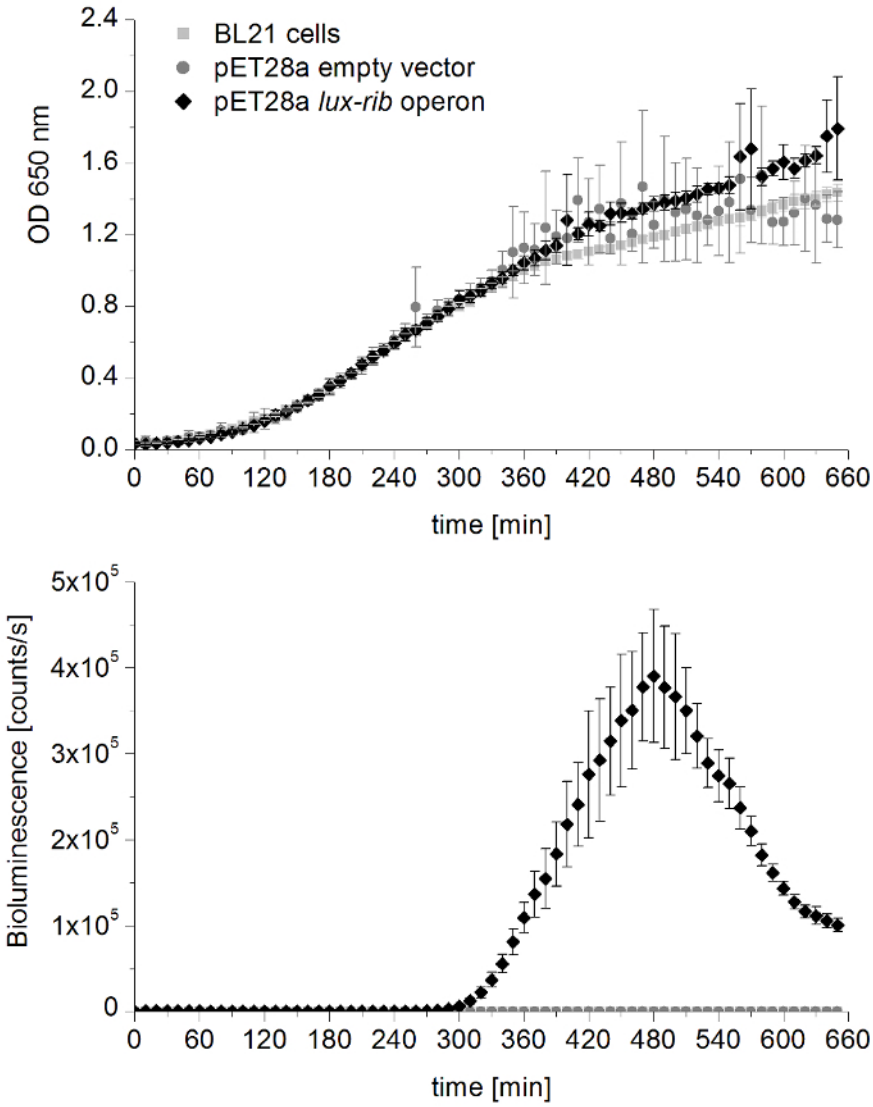


Figure 4: Comparison of growth curves and light intensities of reference strains. The OD at 650 nm and the bioluminescence intensity in counts per second were measured every 10 min over 10 h at 28 °C. All measurements are mean values of three biological replicates with four technical replicates each. Error bars represent standard deviations. *E. coli* BL21 cells (grey squares), *E. coli* BL21 cells containing an empty pET28a vector (grey circles), and *E. coli* BL21 cells containing the pET28a vector with the *lux-rib* operon insert (black diamond) were analyzed to assure correct growth behavior of our *E. coli* cells. Please click here to view a larger version of this figure.

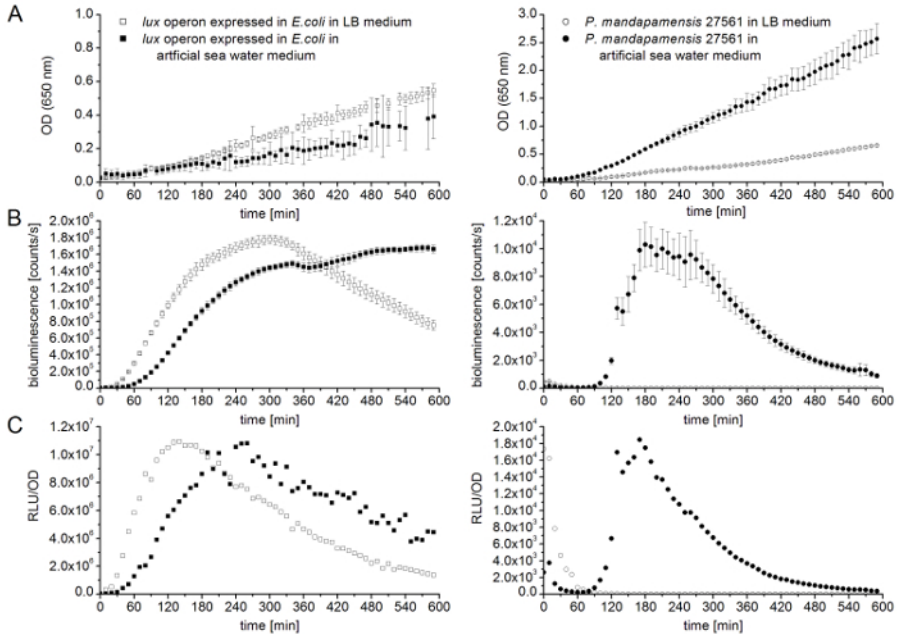


Figure 5: Comparison of growth curves and light intensities of the *lux* operon expressed in *E. coli* (squares) and *P. mandapamensis* 27561 (circles) in LB medium (open symbols) or artificial sea water medium (filled symbols). All measurements are mean values of three biological replicates with four technical replicates each. Error bars represent standard deviations. All experiments were performed at an incubation temperature of 28 °C. **(A)** Optical density (OD) measurements at 650 nm were performed every 10 min for 10 h. *E. coli lux* operon expression (left panel) is compared to *P. mandapamensis* 27561 (right panel) in LB medium and artificial sea water medium. Cell densities are determined at 650 nm to avoid bioluminescence-interference. **(B)** Measurement of light intensity (bioluminescence [counts/s]) was performed every 10 min for 10 h. *E. coli lux* operon expression (left panel) is compared to *P. mandapamensis* 27561 (right panel) in LB medium and artificial sea water medium. **(C)** Relative light intensities (RLU/OD) of the *lux* operon expressed in *E. coli* (left panel) and *P. mandapamensis* 27561 (right panel) are determined by normalizing bioluminescence to cell density. Please click here to view a larger version of this figure.

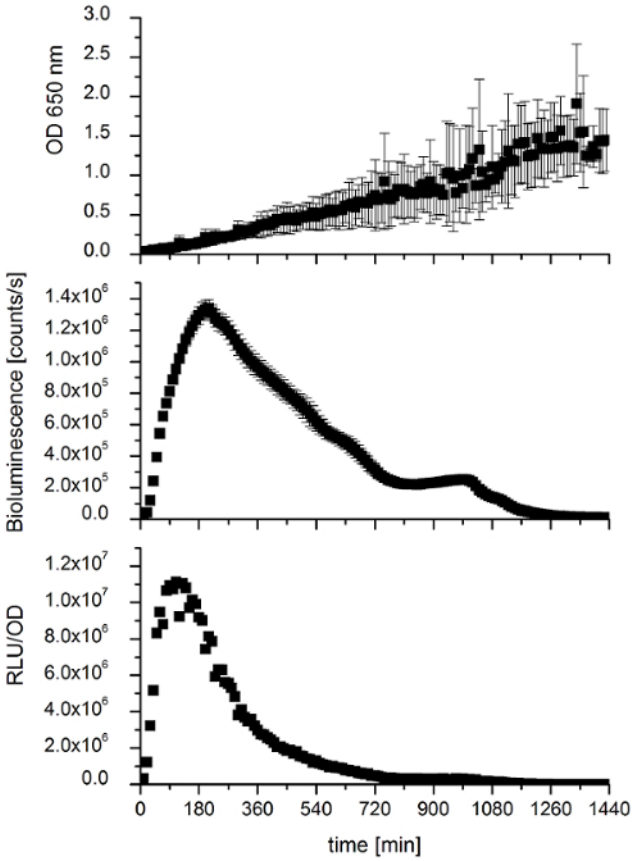


Figure 6: Comparison of growth curves and light intensities of *E. coli* based *lux* gene expression for 24 h. The OD at 650 nm and the bioluminescence intensity in counts per second were measured every 10 min over 24 h at 28 °C. All measurements are mean values of three biological replicates with four technical replicates each. Error bars represent standard deviations. Additionally, the relative light intensities (RLU/OD) where bioluminescence is normalized by cell density are represented. Please click here to view a larger version of this figure.

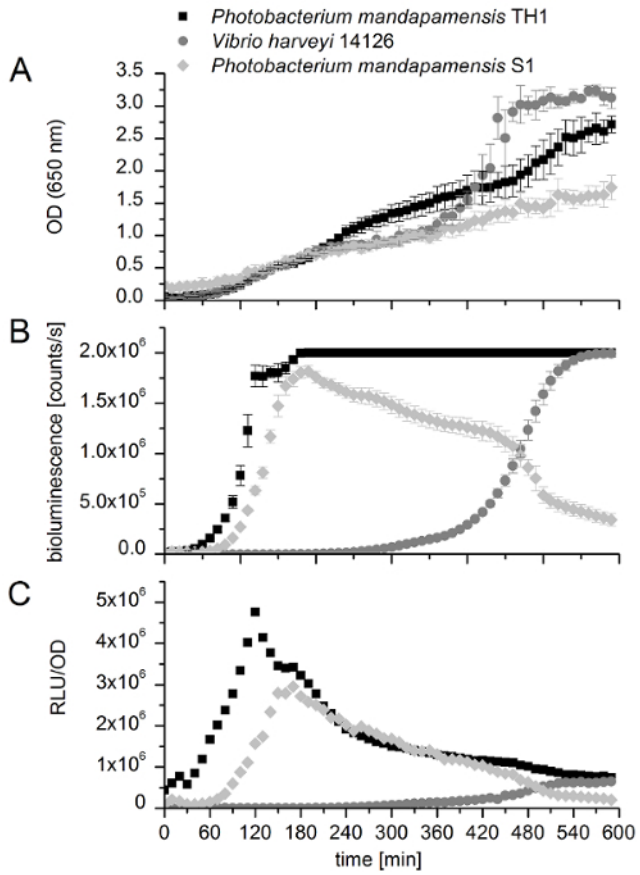


Figure 7: Comparison of bioluminescent bacteria to evaluate potential quorum sensing regulation. Light emission and cell density are measured every 10 min for 10 h and represent mean values of three biological replicates with four technical replicates each. Error bars represent standard deviations.Measurements of *Photobacterium mandapamensis* TH1 (black squares), *Vibrio harveyi* 14126 (grey circles) and *Photobacterium mandapamensis* S1 (grey diamonds) were compared to each other; **(A)** depicts the optical density (OD) at 650 nm, **(B)** the light intensity (bioluminescence [counts/s]), and **(C)** relative light intensities (RLU/OD). Please click here to view a larger version of this figure.

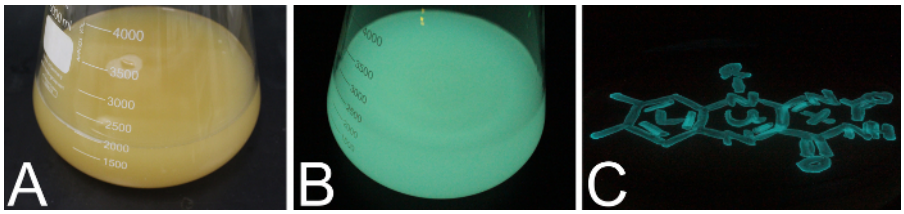


Figure 8: Bioluminescence in liquid media and on agar plates. (A) 5 L flask with 2 L LB medium inoculated with *E. coli* BL21 cells expressing the pET28a *lux* operon plasmid photographed in light. **(B)** The same culture as in (A) photographed in the dark. Pictures A and B were taken approximately 2 h after induction of expression. **(C)** Artificial sea water medium agar plate with streaked culture of *P. mandapamensis* S1 photographed in the dark. Please click here to view a larger version of this figure.

Discussion

The construction of an expression plasmid containing four fragments composing the whole *lux-rib* operon of *P. mandapamensis* 27561 was achieved via Gibson cloning. This *E. coli* based *lux* gene expression enables *E. coli* cells to emit light. Avoiding quorum sensing regulation and non-standard growth conditions are substantial advantages of this system.

The advantages of using Gibson cloning strategy are the easy assembly of multiple linear DNA fragments, high flexibility and no need for specific restriction sites[18,19,20]. This method allows to easily change a *lux* operon, excluding single genes or gene clusters or introducing new genes or exchanging genes from one strain with another. A prerequisite for the application of this method is the availability of the respective *lux* operon gene sequence. Only for a small number of bioluminescent bacteria is the complete DNA sequence of the respective *lux* operon known and/or available. Many of these sequences are fragmented because they were generated using shotgun sequencing. In the case of the investigated *P. mandapamensis* 27561 the complete DNA sequence of the *lux* operon is known and available from the NCBI gene database (GenBank: DQ988878.2).

One critical step in the protocol of the Gibson assembly is the calculation of the DNA concentration of the single fragments. In the assembly protocol of the manufacturer it was recommended to use 0.2 - 0.5 pmols and a total volume of 20 µL for 4 - 6 fragments[20,21]. In our case, where *luxCDABFEG-ribEBH* is comprised of 4 fragments, the concentrations and total volumes were 0.1 pmol and 45 µL, respectively, depending on the yield of PCR products. On the one hand, the concentration had to be reduced and on the other hand, the volume had to be increased. Nevertheless, the assembly worked very well and DNA sequencing confirmed the correct insertion at the first attempt. This finding confirmed Gibson assembly as a robust and appropriate method for our investigations, which can easily be modified[18,19,20].

The newly established plate reader assay is an easy to handle method. It enables a simple primary analysis of new or (un-)known bioluminescent bacterial strains and gives already a first hint on the regulatory mechanism of light production (*e.g.,* lag in luminescence at low cell densities). Additionally, growth conditions in combination with light production can easily be evaluated by simply changing growth medium or temperature.

The measurements with the plate reader were performed at 28 °C. The reason for this unusual setting is the temperature sensitivity of bioluminescent bacterial strains, where temperatures above 30 °C lead to less or even no growth and/or light emission. Note, that the plate reader is capable of keeping a defined temperature over time with the limitation of a missing active cooling system. Therefore, the ambient temperature has to be below the temperature of the measurement.

As a proof of concept, the comparison of the *E. coli* construct with the bioluminescent strain *P. mandapamensis* (**Figure 5**) on the one hand and the reference measurements (**Figure 4**) on the other hand were performed and confirm the reliability of this newly established system. Additionally, the long-term measurement assured the longevity of the light emission (**Figure 6**). But one has to consider that OD values are not reliable above a certain optical density, depending on the characteristics of the used measurement device (approximately 15 h in the present case) where an appropriate dilution would be needed for a measurement in the linear range of the detector. These high variances in OD values make these long time measurements not reliable. Therefore, shorter measurements such as 10 h are recommended using the experimental setup reported here.

Limitations of the established plate reader assay can be seen in **Figure 7**. The difficulty is to find an appropriate setting of the measurement. The 'gain value' adjusts the sensitivity of the photo multiplier tube (PMT). The gain is the amplification of the signal in the PMT, meaning that a higher gain factor will increase the signal. If the gain is set too low, the signal to noise ratio becomes bigger and light intensities of "low" shining bioluminescent bacteria cannot be measured any more (signals close to zero, data not shown). The challenge in a bioluminescent assay is to set the gain so the measurement results for all bacterial strains stay within the range of the instrument. Additionally, the measurement range for luminescence depends on the measurement interval time (*e.g.,* maximum of 2,000,000 for 1 s).

The gain was set to 2800, which was empirically tested and chosen for the specific plate reader used for the establishment of this method. The used gain setting allows the recording of maximum emitted light by the bioluminescent *E. coli* system, *P. mandapamensis* 27561, and *P. mandapamensis* S1 without an overflow, but for the strains *P. mandapamensis* TH1 and *V. harveyi* 14126 the gain was too high. Therefore, these latter strains exceed the detection limit and the real maximal light intensity cannot be measured. This technical limitation might prevent the comparison of bioluminescent bacteria, which show high variations in maximum light emission, although growth conditions and cell densities might be comparable.

Positioning of the analyzed bacterial strains within the used well plates has to be evaluated empirically. Although black well plates with glass bottoms were used, crosstalk between the samples was observed. The light intensity of specific strains is so high, that all neighboring wells will show false positive light emissions (*e.g.,* blank). Therefore, it is important to measure two different strains either separately or with a certain spatial separation to each other.

There are already many modified *E. coli* strains known that contain parts of the *lux* operon and are mainly application-oriented[15,16,17]. The methods described here, aim at fundamental research, for example the possibility of analyzing each *lux* gene separately. Although research of bioluminescence has a long history, there are still many open questions. By excluding or introducing genes from the *lux* operon, exchanging the *luxAB* genes with genes from another strain, or analyzing protein complexes, embedded in the easy to handle *E. coli* system and further application of the plate reader assay, it might be possible to gain more information on regulatory processes and the functions of *lux* genes.

Disclosures

The authors have nothing to disclose.

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