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# Novel application of pH-sensitive firefly luciferases as dual reporter genes for simultaneous ratiometric analysis of intracellular pH and gene expression/location

Gabriele V. M. Gabriel[a,b,c] and Vadim R. Viviani[*a,b,c]

[a]Laboratory of Biochemistry and Biotechnology of Bioluminescence, Department of Physics, Chemistry and Mathematics, Federal University of São Carlos (UFSCar), Sorocaba, SP, Brazil.

[b]Graduate School of Biotechnology and Environmental Monitoring, Federal University of São Carlos (UFSCar), *Campus* of Sorocaba, Sorocaba, SP, Brazil.

[c]Graduate School of Evolutive Genetics and Molecular Biology, Federal University of São Carlos (UFSCar), São Carlos, SP, Brazil.

*V. R. Viviani: Department of Physics, Chemistry and Mathematics, Federal University of São Carlos (UFSCar), *Campus* of Sorocaba, Sorocaba, SP, Brazil. Phone/Fax: +55 15 3229 5983. E-mail address: viviani@ufscar.br

**Abstract**

Firefly luciferases are widely used as bioluminescent reporter genes for bioimaging and biosensors. Aiming at simultaneous analyses of different gene expression and cellular events, luciferases and GFPs that elicit distinct bioluminescence and fluorescence colors, have been coupled with each promoter, making dual and multicolor reporter systems. Despite their wide use, firefly luciferase bioluminescence spectra are pH-sensitive, resulting in a typical large red shift at acidic pH, a side-effect that may affect some bioanalytical purposes. Although several intracellular pH-indicators employ dual color and fluorescent dyes, none has considered benefiting from the characteristic spectral pH-sensitivity of firefly luciferases to monitor intracellular pH-associated stress, an important indicator of cell homeostasis. Here we demonstrate a linear relationship between the ratio of intensities in the green and red regions of the bioluminescence spectra and pH using firefly luciferases cloned in our laboratory *(Macrolampis* sp2 and *Cratomorphus distinctus*), allowing to estimate *E. coli* intracellular pH, thus providing a new analytical method for ratiometric intracellular pH-sensing. This is the first dual reporter system that employs a single luciferase gene to simultaneously monitor intracellular pH using spectral changes, and gene expression and/or ATP concentration using bioluminescence intensity, showing great potential for real time bioanalysis of intracellular processes associated with metabolic changes such as apoptosis, cell death, inflammation and tissue acidification, among other physiological changes.

**Key-words:** *Macrolampis* sp2; pH measurement; spectral changes; bioluminescence

## Introduction

Various luciferins and luciferases obtained from bioluminescent organisms have been widely employed as bioanalytical reagents and their genes, as reporter genes for bioimaging and biosensors by virtue of their sensitivity, selectivity, low cost and promptness.[1,2]

A bioluminescent biosensor relies on luciferases or photoproteins, which are enzymes that require oxygen to oxidize luciferin, producing light in a quantitative manner.[3,4] Bioluminescent biosensors can be grouped in enzymatic, where the enzyme is immobilized and directly or indirectly used to detect a specific analyte; or cellular, in which the luciferase is constitutively expressed or induced by a specific analyte. Whole cell bioluminescent biosensors can be further classified as *light off* and *light on* systems. In the *light off* biosensor, the luminescence is inhibited by specific agents that affect the metabolism leading to decreased intensity of the measured signal. In the case of *light on* biosensors, the luciferase gene is under control of an inducible promoter, being quantitatively expressed in the presence of the inducer (specific analyte).[5,6,7,8,9,10]

The luciferases from bacteria, fireflies and jelly fishes have been extensively used in a variety of metabolic biosensors to monitor the redox metabolism, ATP production and calcium fluxes.[1,11,12,13,14] Bacterial and firefly luciferases, for example, encountered applications as whole cell biosensors for their dependence on metabolic power to produce light: bacterial luciferase consumes $FMNH_2$ whereas firefly luciferase consumes ATP. Bioluminescent bacteria have long been used as biosensors of a plethora of toxicants[15,16] through the well-known Microtox® kit, including metal contaminants in the environment such as mercury, zinc, copper, nickel and cadmium. Similarly, *Escherichia coli* carrying the reporter plasmid reproduces the bioluminescence assay with sensitivity, specificity and accuracy.[17,18,19] Firefly luciferases are the most extensively used enzymes in ATP assays, biomass estimation, enzyme assays, reporter gene assays, bioimaging and biosensors.[2]

Among intracellular biosensors, pH biosensors are very important to assess cell homeostasis.[20] The intracellular pH is generally kept constant in the cytoplasm, whereas some organelles like mitochondria, chloroplasts, peroxisomes and lysosomes may display pH fluctuations according to their metabolic state. In some cases, intracellular pH changes reveal major pathophysiological changes, hence the importance of monitoring the pH of cell microenvironments. For such purposes, specific fluorescent dyes have been developed.[20,21,22,23,24,25] However, low molecular weight fluorescent dyes may have phototoxic effects and affect the physiology of the cell. On the other hand, fluorescent proteins have been recently engineered and used to estimate intracellular pH. A recent report showed the first use

of a GFP which undergoes pH-dependent change of spectra for ratiometric measurement.[26] However, as any fluorescent dye, GFPs must be previously irradiated with blue light in order to emit its fluorescence, which may have drawbacks such as the photodamage of the cell and also the interference of self-absorption and endogenous fluorescence.[23,27] Thus for this purpose, a bioluminescent protein or luciferase which lack these side-effects would offer the advantage to produce its own light, without need of irradiation and competition from endogenous luminescent sources.

The bioluminescence spectra of firefly luciferases are reportedly pH-dependent, prone to undergo a large red-shift at acidic pH, high temperatures and presence of divalent metals as well.[28,29] The pH-sensitivity has been shown to result from pH induced luciferase conformational changes that affect the microenvironment around the oxyluciferin emitter, thereby affecting the bioluminescence colors.[30] Interestingly, the pH-sensitivity has been usually considered an undesired property for some bioanalytical applications. Despite the increasing demand for sensitive probes to estimate intracellular pH, no one has ever considered to take advantage of firefly bioluminescence spectral pH-sensitivity to monitor intracellular pH.

Noteworthy, observations by some researchers showed that when fireflies are dying, they emit an orange-red-shifted glow instead of their usual yellow-green flash. Among Brazilian fireflies, the twilight firefly *Macrolampis* sp2 displays an unusually broad and bimodal bioluminescence spectrum[29] that undergoes a slight red shift during flying activity and at higher temperatures. These findings lead us to clone and characterize its luciferase[31,32] aiming to elucidate the molecular origin of the pH-sensitivity and to prospect its use as indicator of pH-associated intracellular stress.[33] The analytical use of bioluminescence spectral changes together the light intensity widely used to report gene expression or ATP concentration, could append a new dimension in reporter gene technology. Here we investigated the suitability of the expression of the *Macrolampis* sp2 firefly luciferase and other recombinant firefly luciferases cloned in our laboratory as intracellular pH probes in live bacteria, thus providing the first dual luciferase reporter genes for simultaneous ratiometric analysis of intracellular pH, gene expression and/or ATP.

## Material and Methods

### Reagents

1,4-Dithio-D-threitol (DTT) was purchased from Thermo Fisher Scientific (Waltham, MA, USA); ATP from Ambresco (Solon, OH, USA); D-luciferin and Tris base from Promega (Madison,

WI, USA); ethylenediaminetetraacetic acid (EDTA) and $MgSO_4$ from Synth (Diadema, SP, Brazil); glycerin from Ecibra (São Paulo, SP, Brazil); isopropyl β-D-1-thiogalactopyranoside (IPTG) from Fermentas (Burlington, Ontario, Canada); Luria–Bertani (LB) broth and agar Miller from Himedia (Curitiba, PR, Brazil). Monosodium and disodium phosphate from Cromoline (Diadema, SP, Brazil); and Triton X-100 from Riedel-de-Haen (Berlin, Germany). Ultrapure MilliQ water was used in all assays.

### *Escherichia coli* and plasmids

The plasmids pPro-Mac (pPro, Invitrogen, Waltham, MA, USA) harboring the luciferase cDNA from *Macrolampis* sp2 was previously constructed in our laboratory.[31,34] The plasmids harboring the cDNAs from *Cratomorphus distinctus* (pBl-Crt) and *Amydetes vivianii* (pSP-Amy) luciferases were also previously home-cloned,[35,36] and the commercial luciferase from *Photinus pyralis* was purchased from Promega (Madison, WI, USA). For expression of high levels of luciferases, *E. coli* BL21-DE3 cells were used.

### Bacterial culture for *in vivo* assays

Bacteria transformed with the above-mentioned plasmids were grown on LB/agar plates supplemented with ampicillin at 37 °C overnight. In the next day, replicas of the colonies grown were obtained on nitrocellulose membranes and induced over LB/agar plates supplemented with 1 mM IPTG during 6-12 h at 22 °C. Liquid cultures were also grown on LB medium supplemented with ampicillin at 37 °C until absorbance reaching 0.4 at 600 nm, and then induced with 0.4 mM IPTG at 18-20 °C during 6-12 h. The induced bacteria were used for bioluminescence photography and spectrum measurements, respectively.

### Luciferase expression

Bacteria transformed with pPro-Mac, pSP-Amy, and pBl-Crt were grown in LB medium/Ampicillin liquid cultures at 37 °C until $A_{600} \approx 0.40$, and then induced with 1 mM IPTG until $A_{600} \approx 1.5$ at 20 °C.

### Luciferase extraction

Bacteria expressing luciferases were harvested by centrifugation at 2,500 g for 15 min at 4 °C and resuspended in extraction buffer (25 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 2 mM DTT and protease inhibitor cocktail), and then freeze-

thawed, ultrasonicated (Misonix, Nova York, USA) five times and finally centrifuged at 15,000 g for 15 min at 4 °C.

**CCD imaging**

Bioluminescence imaging of bacteria in liquid cultures or in semi-solid medium was done after adding 10 mM D-luciferin in 0.10 M sodium citrate buffer at pH 5.0, using a Light Capture CCD II Camera (ATTO, Tokyo, Japan), at different exposure times and sensitivities depending on the bioluminescence intensity of the sample.

**Luminometric assays of *in vivo* and *in vitro* bioluminescence**

Bioluminescence activities of liquid cultures of *E. coli* expressing firefly luciferases and luciferase *in vitro* assays were done using an AB2200 luminometer (ATTO, Tokyo, Japan) using the intensity values measured in *cps* (counts per second) during 10 s. For measuring *in vivo* bioluminescence of liquid cultures and suspensions, 10 µL of 10 mM D-luciferin in 0.10 M sodium citrate buffer pH 5.0 were mixed to 90 µL of culture in the luminometer tube. For *in vitro* assays, 80 µL of 0.10 M Tris-HCl buffer at pH 8.0 were mixed with 5 µL of 10 mM D-luciferin, 10 µL of luciferase and 5 µL of a solution containing 40 mM ATP and 80 mM $MgSO_4$. The reported values are the result of three independent assays, each one measured in triplicate.

**Bioluminescence spectra**

Bioluminescence spectra were recorded using a Hitachi F4500 spectrofluorometer with the excitation lamp *off* and the emission shutter open at the scanning speed of 2400 nm/min. The *in vitro* bioluminescence spectra were traced by mixing 50 µL of bacterial extracts with 400 µL of assay solution (10 mM luciferin, 40 mM ATP, 80 mM $MgS0_4$, in 0.10 M Tris-HCI, pH 8.0). The reported spectra are the result of three independent experiments (expressions and extractions), each one measured in triplicate. In order to obtain the values of the maximum wavelengths of the green and red emissions for each luciferase, the bioluminescence spectra were decomposed. According, the peaks of bioluminescence spectra in the green and red regions for different luciferases were the following: (*Macrolampis* sp2) $\lambda_{green}$= 563 nm and $\lambda_{red}$= 616 nm; (*Cratomorphus distinctus*) $\lambda_{green}$= 554 nm and $\lambda_{red}$= 614 nm.

**Ratiometric analysis of bioluminescence spectra**

The ratio (R) of intensities ($I_{green}/I_{red}$) of bioluminescence spectra were obtained from the intensities at the maximum wavelength of the red and green bands estimated for each luciferase (*Macrolampis* sp2: $I_{563}/I_{616}$; *Cratomorphus distinctus*: $I_{554}/I_{614}$; *Amydetes vivianii*: $I_{537}/I_{596}$) at different pH. Then the effect of pH was plotted as a logarithmic function of the R values. The dependence log R on the pH was also evaluated in order to estimate the pKa of the spectral transition. The reported values are the average of three independent assays each one measured in triplicate.

**Effect of divalent heavy metals on bioluminescence spectra**

The effect of divalent metals on bioluminescence spectra of firefly luciferases was tested at the final metal concentration of 2 mM, using the following salts: $ZnSO_4$, $CuSO_4$, $NiSO_4$, $AgNO_3$ and $CaCl_2$.

## Results and Discussion

***In vivo* bioluminescence spectra of firefly luciferases**

As previously reported, firefly luciferases display a flash-like kinetics in the presence of saturating luciferin and ATP.[32] The *in vivo* bioluminescence color and spectra of *E. coli*-expressing firefly luciferase is initially reddish gradually shifting towards the yellow region along the time (**Fig. 1D**). This is a general property of firefly luciferases which are pH-sensitive and may result from sudden intracellular pH changes caused by intracellular proton import by luciferin in acidic buffer. In the case of *Macrolampis* sp2 luciferase, however, the *in vivo* bioluminescence spectrum is predominantly reddish with a slight blue-shift (**Fig. 1A**). This might be related to the fact that this luciferase naturally displays a broader and red-shifted spectrum, therefore red emission predominates inside the cell environment. On the other hand, the more blue-shifted luciferase from *C. distinctus* inside bacteria initially emits in the orange region and gradually shifting to the yellow-green region (**Fig. 1C**). Similar trend is observed for the *Macrolampis* sp2 luciferase N354E mutant (results not shown), whose spectral properties resemble those of *P. pyralis* luciferase.[31] Higher temperatures also tend to red-shift the emission spectrum elicited by this luciferase.

**Figure 1**

**Effect of pH on bioluminescence spectrum of *Macrolampis* sp2 and other firefly luciferases**

As expected, the firefly luciferases *in vitro* reaction in 0.10 M phosphate buffer undergoes a red shift at lower pH (**Fig. 2**). These luciferases differ in the degree of pH sensitivity and prevailing red light emission at pH 8.0. Thus, the pH-sensitivity and red light predominance increases when studying the most blue-shifted towards the most red-shifted luciferases in the following order: Amy<Crt<Ppy<Mac (**Fig. 2**).

**Figure 2**

**Ratiometric measurement of pH**

The ratio of bioluminescence intensities in the red and green regions clearly increases at acidic pH (**Fig. 3**). Therefore we analyzed the relationship between pH and the ratio of intensities at the peak of green emission and red emission ($R= I_{green}/I_{red}$) estimated by spectral decomposition for each luciferase, in order to find out whether such relation could be used to estimate intracellular pH in living cells. If one considers that R is related to $[A^-]/[HA]$, where HA is inferred to be a luciferase group acting as a weak acid, then the Henderson-Hasselbalch equation plotted as log R *versus* pH gives the pKa related to the spectral transition for each luciferase. According to such analysis, the pKa for *Macrolampis* sp2 luciferase was estimated as 6.8, shifting to more acidic values for the luciferase with more blue-shifted bioluminescence spectra (**Fig. 3**). It is tempting thus to propose that the most red-shifted luciferases display a higher pKa attributable to a phototropic amino acid residue directly implicated in the pH-sensitivity.

**Figure 3**

**Intracellular estimation of pH in living bacteria**

Next, to check whether the R ($I_{green}/I_{red}$) value could be applied to estimate intracellular pH value, we used the bioluminescence spectrum of *E. coli* expressing *Macrolampis* sp2 luciferase (**Fig. 2B**) and estimated the intracellular pH using the corresponding R value and the linear relationship of log R and pH obtained in phosphate buffer as shown in **Fig. 3**. As shown, the time course of bioluminescence color in living bacteria varies from reddish to yellow-green, which reflects an intracellular pH change, probably due to luciferin intracellular proton import. Thus, we estimated the intracellular pH in living *E. coli* at the onset of light

emission after spraying D-luciferin in citrate buffer pH 5.0, when a fast intracellular acidification is expected due to luciferin-mediated proton import, and after 120 s, when the light emission is more yellow-green, reflecting the recovery of physiological pH (~7.0). The estimated pH value for the *E. coli*-expressing *Macrolampis* sp2 firefly luciferase was ~6.77 at the onset of light emission, and ~7.1 after 120 s, the latter value agreeing reasonably well with the value reported by Lam *et al.*[37] for the intracellular pH of *E. coli* (7.0). By using *C. distinctus* luciferase, the estimated pH values were 6.7 at the onset and 7.05 after 120 s. Thus, the intracellular pH estimation using the ratiometric curves for the above luciferases agreed reasonably well. On the other hand, the green-emitting *Amydetes vivianii* luciferase is very resistant to pH changes and therefore is not suitable to estimate intracellular pH ratiometrically.

Although our calculation in the current format is oversimplified, once other factors such as phosphate concentration, presence of metals and temperature may also interfere with the *in vivo* bioluminescence spectrum, the results presented here clearly show the feasibility of use of *Macrolampis* sp2 and other pH-sensitive luciferase for ratiometric estimation of intracellular pH.

In order to analyze the contribution of other variables on the *in vivo* bioluminescence spectrum, we further analyzed the effect of [phosphate], [$Me^{+2}$] and temperature on light emission.

**Effect of phosphate on bioluminescence spectrum**

Phosphate buffer is known to red-shift the bioluminescence spectrum of firefly luciferases.[28] In the case of *Macrolampis* sp2 firefly luciferase, the spectrum in 0.10 M Tris-HCl buffer pH 8.0 is narrow peaking at 564 nm, whereas in 0.10 M sodium phosphate buffer it is much broader and red-shifted (**Fig. 4**). Because one of the main buffers inside living cells is phosphate, the *in vivo* bioluminescence spectrum is naturally expected to be broader and red-shifted. The intracellular phosphate concentration inside normal living *E. coli* is around 5 mM,[38] therefore very little phosphate effect on the overall bioluminescence spectrum is expected. After cell death, however, the phosphate concentration may considerably increase due to nucleotide phosphates and other polyphosphate hydrolysis, which may also decrease the pH, consequently contributing for red-shifting and broadening of the bioluminescence spectra.

**Figure 4**

**Effect of divalent heavy metals**

Divalent metal cations such as $Zn^{+2}$, $Cu^{+2}$ and $Hg^{+2}$ are also known to red shift the bioluminescence spectra of *P. pyralis* firefly luciferase.[28] This led us to test their effects on *Macrolampis* sp2 firefly luciferase (**Fig. 5**). Only $Zn^{+2}$ and $Ni^{2+}$ ions at the concentration of 2 mM resulted in an appreciable red-shift, whereas the other metals at the same concentration had appreciable effect. Considering that the $Zn^{+2}$ concentration inside *E. coli* is *c.a*. 20 pM, it is unlikely that it may have an appreciable effect on *in vivo* bioluminescence spectra. On the other hand, in mammalian cells such as neurons the concentrations of $Zn^{+2}$ ion are in the upper nanomolar range, thus in these cells this metal it may have a moderate effect on bioluminescence spectra.

**Figure 5**

**Effect of temperature**

Finally, we also analyzed the effect of temperature on the R value. Higher temperatures red-shift the spectrum to lower R values (**Fig. 6**). Noteworthy, a clear linear relationship between R and temperature was obtained.

**Figure 6**

These findings support the use of *Macrolampis* sp2 and other firefly luciferases as temperature sensors in the range 5-40 °C. They also alert for caution when measuring intracellular pH as the assays must be performed in temperature controlled environments to avoid temperature mediated artifacts on the spectra.

**Sensitivity of the spectral bioluminescence assay**

The sensitivity of the bacterial *in vivo* assay using this system *E. coli*/pPro-Mac was previously analyzed by CCD imaging and luminometry, by counting the minimal number of colony forming units (CFU/mL) able to produce a detectable signal.[39] Using luminometry, up to 10 CFU/mL producing a signal of 500 cps could be detected. Using a sensitive spectrophotometer, the minimal amount of bacterial cells estimated to produce a detectable

spectrum was $5.10^4$ CFU, corresponding to a signal of $1.10^5$ cps. Harnessing the sensitivity of this method using *E. coli*-expressing luciferase for intracellular analysis in eukaryotic cells, especially mammalian cells, is promising. Taking into account that (i) eukaryotic cells may have as much as 1000 times the volume of a bacterial cell, (ii) a mitochondria has the approximate volume of a bacteria, and (iii) a typical mammalian cell such a hepatocyte may have as much as 1000-2000 mitochondria, upon cellular targeting of luciferase to mitochondria, one could in principle assess the spectral changes occurring in as little as 10 hepatocytes. By increasing the time and sensitivity of the detection system and luciferase activity, this limit could be easily set close to a single mammalian cell, thereby providing an innovative technology for single cell real time imaging.

The application of bioluminescent proteins (luciferases or photoproteins) for intracellular pH-analysis is recent. Hattori *et al.*[40] constructed a photoinactivable bioluminescent indicator based on combination of a luciferase-fragment complementation with a Phototropin-1 (LOV2) for measurement of intracellular acidification. Light irradiation decreases the bioluminescence activity, whereas in the dark state bioluminescence is gradually recovered. The authors showed that the recovery time was correlated with pH, independently of luciferin and ATP concentrations, providing real-time *in vivo* methodology for recording acidification in apoptotic and autophagic cells. Whereas the methodology is of potential applicability, it requires the participation of two distinct proteins (LOV-2 and luciferase complementation) with different biochemical requirements (ATP and $FMNH_2$), and photoirradiation by blue light with its potential photochemical effects, increasing the complexity of the analysis.[40]

Here we demonstrated for the first time the feasibility of the use of firefly luciferases and their spectral pH-sensitivity for ratiometric analysis of intracellular pH in the range between pH 6 and 9 in live *E. coli*. This methodology provides a simple and direct approach which uses a single pH-sensitive firefly luciferase gene to estimate intracellular pH in the physiological range. The luciferase of the Brazilian fireflies *Macrolampis* $sp_2$ and *Cratomorphus distinctus* turned to be especially suited for such purposes.

Additionally, this methodology provides the first dual reporting bioluminescent system using a single firefly luciferase gene, which can be harnessed to simultaneously give information based on (i) bioluminescence intensity in one dimension (cell location, gene expression analysis and ATP analysis), and (ii) spectral changes in another dimension, providing information about pH variation and intracellular stress. Since the concentrations of ATP and D-luciferin do not affect the bioluminescence spectrum, the ratiometric analysis of

pH is insensitive to the fluctuations of these substrates inside the cell. Thus, considering that the quantum yields for some beetle luciferases are currently known, the amounts of intracellular luciferase or ATP concentrations could be in principle estimated with precision. Finally, firefly luciferin displays low toxicity and has been used for decades for reporter gene assays in cell cultures and live animals. Therefore, firefly reporter genes could be used in different eukaryotic cells, and targeted towards distinct intracellular compartments such as mitochondria, chloroplasts, lysosomes and peroxisomes, and used as biosensors to simultaneously report pH and ATP variations associated with metabolic changes in normal and pathological cellular processes, including muscle lactic fermentation, apoptosis, inflammation, thus providing the first dual reporter system that employs a single luciferase gene.

Whereas the use of pH-sensitive firefly luciferases genes for intracellular pH estimation and dual reporting is promising, there are some potential drawbacks to be overcome, such as their use in pigmented samples which display differential self-absorption properties. Furthermore, the implementation of this technology may require the use of relatively expensive equipments such as filter based luminometers or CCD-cameras, or new imaging systems with coupled spectrometers. With the recent advances of sensitive biophotonic technologies such as BLI and bioluminescence microscopy, we anticipate that this new single luciferase-based reporter gene technology could be increasingly used in cell assays and real time *in vivo* imaging, providing important additional information about intracellular physiology in living and dying cells/tissues and in pathological processes. Work is in progress to analyze the suitability of this dual reporter gene system to examine other kind of cells, including mammalian cells.

**Concluding remarks**

We show here that the pH-sensitive luciferases of *Macrolampis* $sp_2$ and *Cratomorphus distinctus* fireflies are useful for ratiometric indication of intracellular pH in living bacteria. This approach could be associated with the bioluminescence intensity for usual reporting gene expression and/or ATP analysis, providing the first dual reporter system employing a single firefly luciferase gene, for simultaneous analysis of intracellular pH and ATP analysis/or gene expression. The use of a single bioluminescent reporter gene displaying two parameters (ratio of bioluminescence intensities at different wavelengths, and bioluminescence intensity) can replace the time consuming and more expensive use of different reporter genes for analysis of

distinct cellular events in eukaryotic cells, showing promising applications for mammalian cell assays and real time imaging.

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**Figure 1.** *In vivo* bioluminescence spectra of different firefly luciferases in *E. coli*: (**A**) *Macrolampis* sp2; (**B**) *Amydetes vivianii* and (**C**) *Cratomorphus distinctus* at 0 sec ( ), 30 sec ( ), 60 sec ( ), 90 sec ( ) and 120 sec ( ) and (**D**) bioluminescence color of *E. coli* colonies expressing firefly luciferases after addition of D-luciferin: (**1-4**) *Macrolampis* sp2: (**1**) immediately after spraying 1 mM D-luciferin, and after (**2**) 30 s, (**3**) 60 s and (**4**) 90 s; and (**5-8**) *Cratomorphus distinctus*: (**5**) immediately after spraying 1 mM D-luciferin, and after (**6**) 30 s, (**7**) 60 s and (**8**) 90 s.

**Figure 2.** Effect of pH on the bioluminescence spectra of firefly luciferases in 0.10 M phosphate buffer: (**A**) *Macrolampis* sp2 firefly luciferase, (**B**) *Amydetes vivianii*, (**C**) *Photinus pyralis* and (**D**) *Cratomorphus distinctus* firefly luciferases; (black line) 0.10 M phosphate buffer pH 6.0; (dark gray line) pH 7.0 and (light gray line) pH 8.0. In the upper pannel **A** we can see the *in vitro* bioluminescence color at different ps using *Macrolampis* sp2 firefly luciferase.

**Figure 3.** Ratiometric relationship between R ($I_{green}/I_{red}$) and pH in different firefly luciferases: (**A**) *Macrolampis* sp2 luciferase, inset ratiometric relationship between pH 6.0 and 7.0; and (**B**) *Cratomorphus distinctus* luciferase, inset ratiometric relationship between pH 6.0 and 7.0.

**Figure 4.** Effect of 0.10 M phosphate (gray) and Tris-HCl (black) buffers at pH 8.0 on bioluminescence spectra of firefly luciferases: (**A**) *Macrolampis* sp2, (**B**) *Amydetes vivianii*, (**C**) *Photinus pyralis* and (**D**) *Cratomorphus distinctus* luciferases.

**Figure 5.** Effect of divalent metals at the concentration of 2 mM on bioluminescence spectrum of *Macrolampis* sp2 firefly luciferase: (black) no addition; (dark gray) $NiSO_4$ and (light gray) $ZnSO_4$.

**Figure 6.** (**A**) Effect of the temperature on the bioluminescence spectrum of *E. coli* expressing *Macrolampis* sp2 luciferase: (**A**) effect of the temperature on the bioluminescence spectra; 5 °C ( ); 10 °C ( ); 25 °C ( ) and 35 °C ( ); and (**B**) effect of temperature on the ratio (R) of bioluminescence intensities at 563 and 616 nm (correlation coefficient = -0.992).

**Figure 1**

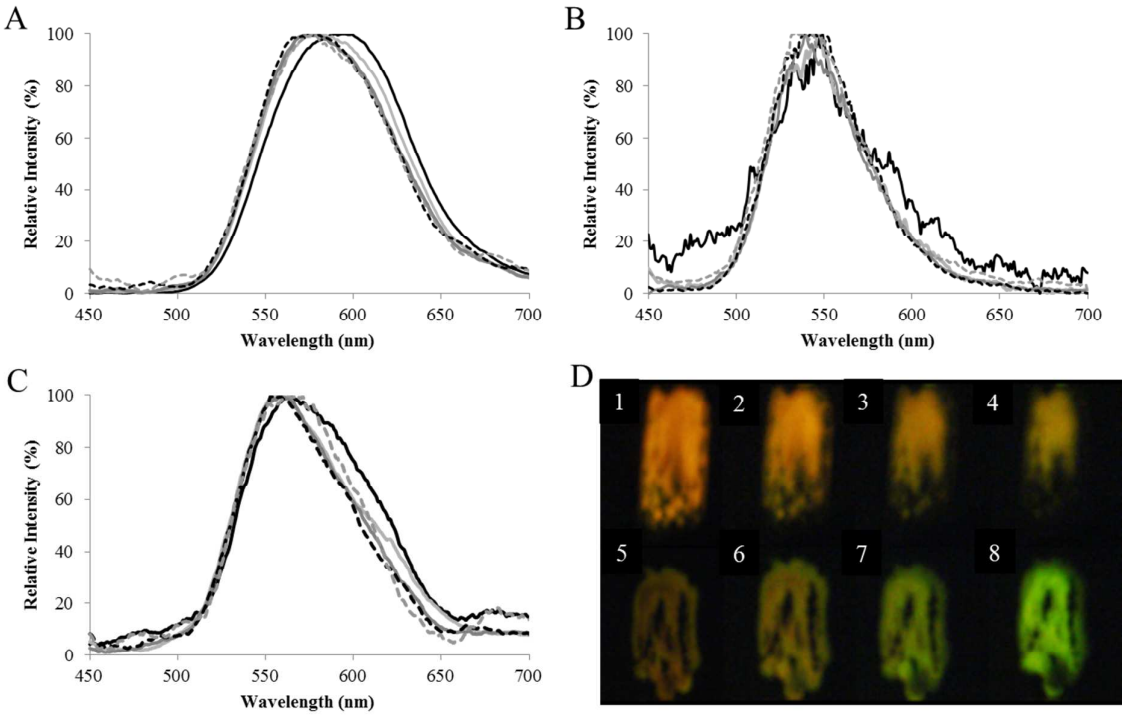


**Figure 2**

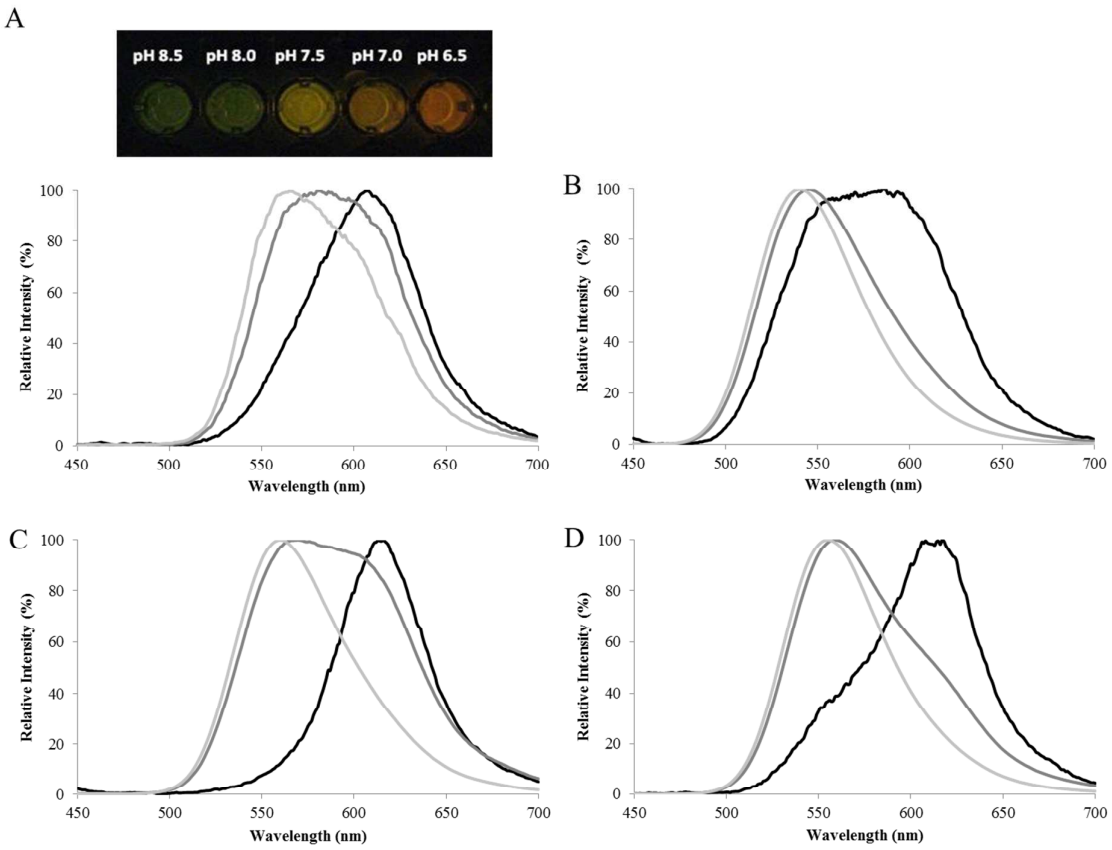


**Figure 3**

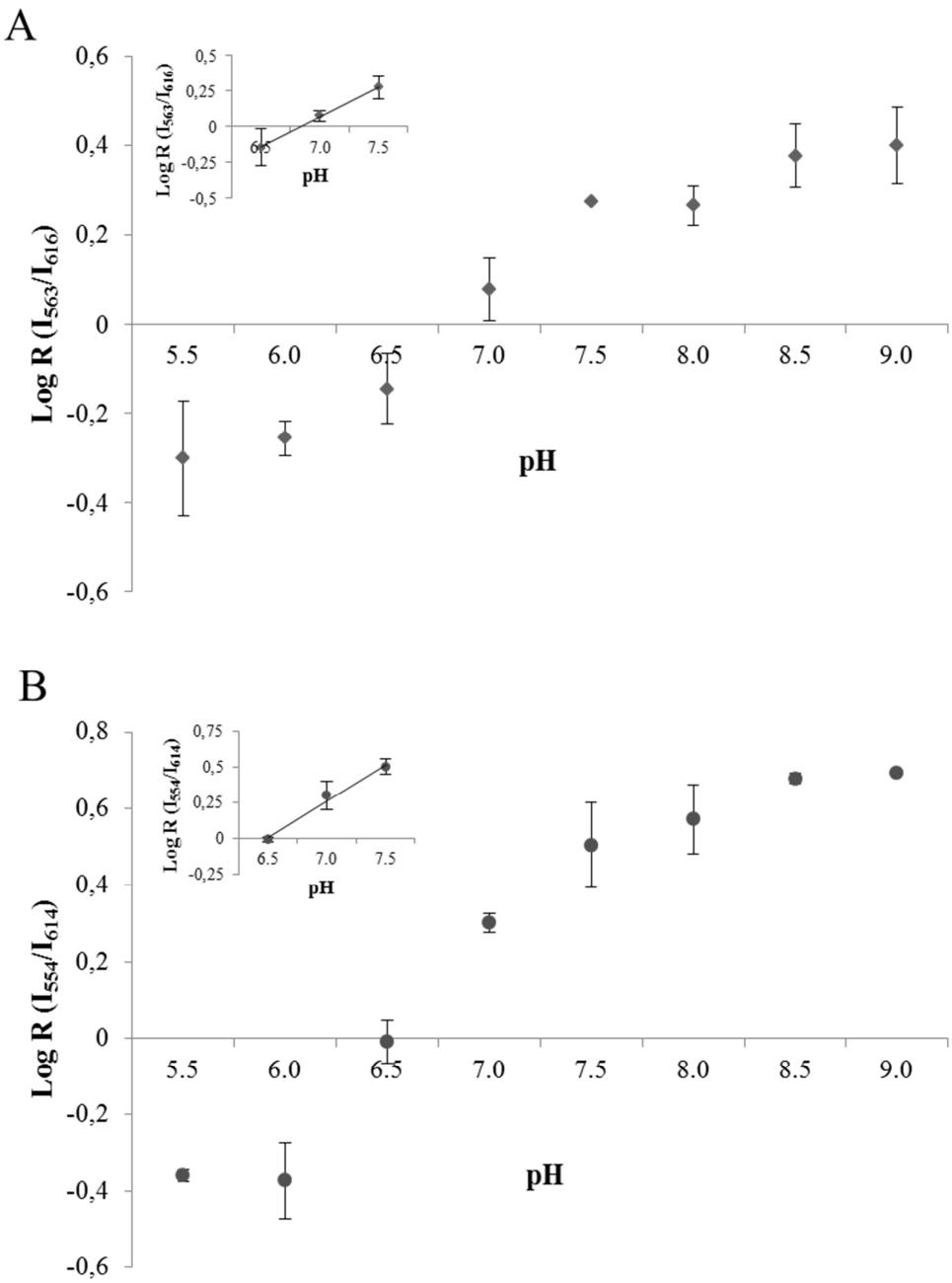


**Figure 4**

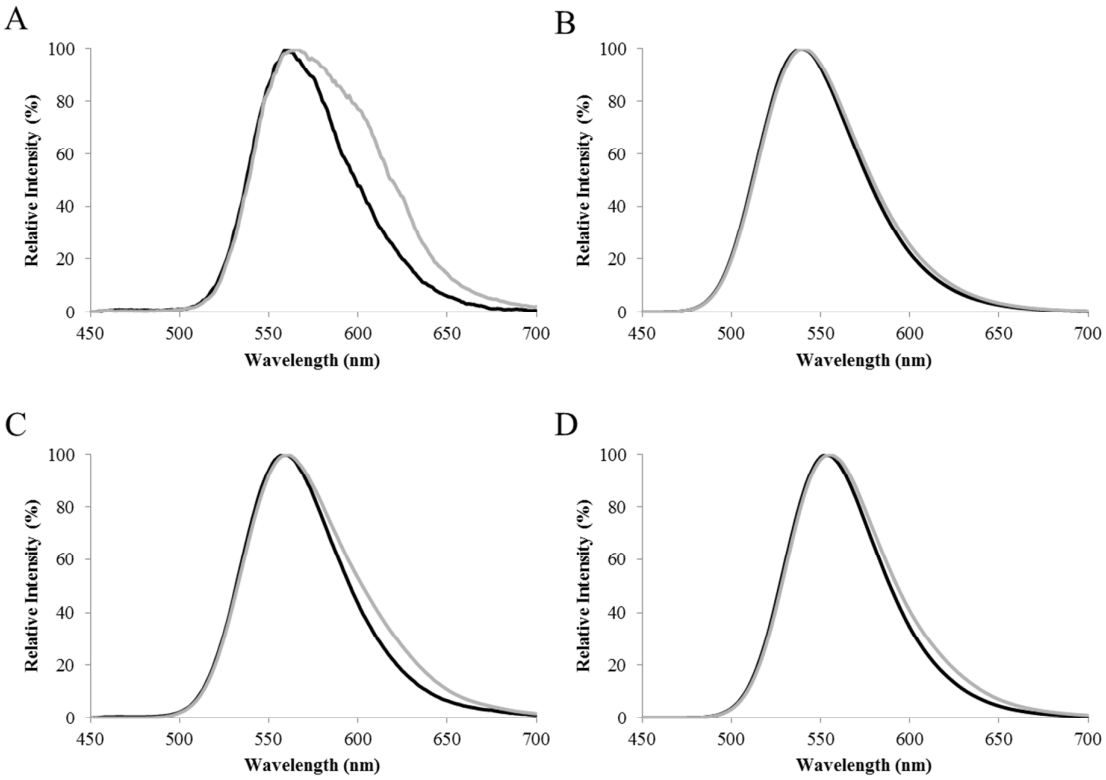


**Figure 5**

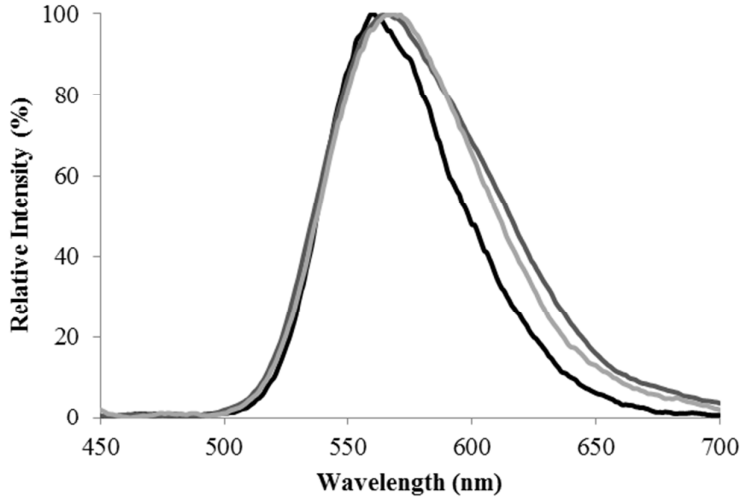
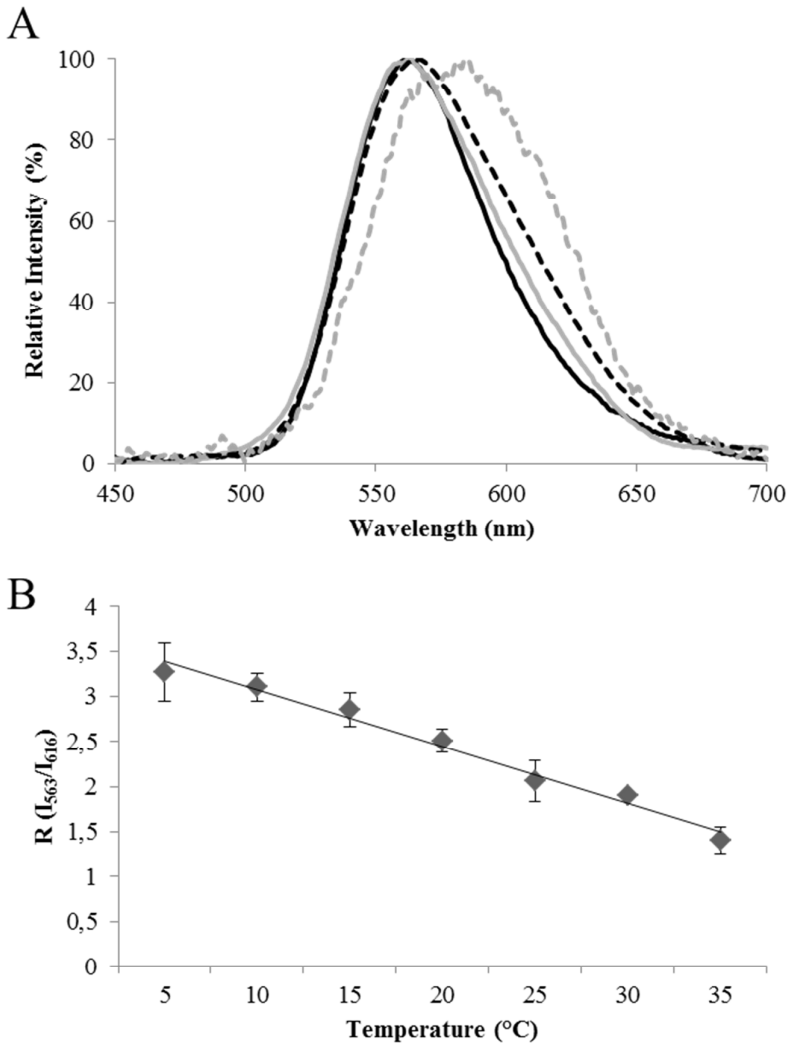
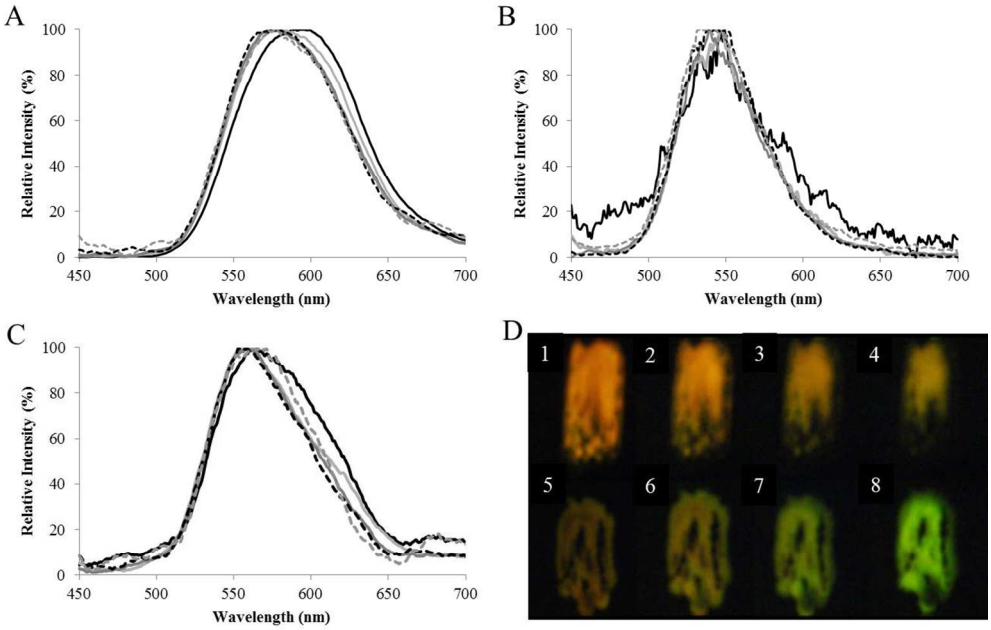


**Figure 6**






*In vivo* bioluminescence spectra of different firefly luciferases in *E. coli*: (A) *Macrolampis* sp2; (B) *Amydetes vivianii* and (C) *Cratomorphus distinctus* at 0 sec ( ), 30 sec ( ), 60 sec ( ), 90 sec ( ) and 120 sec ( ) and (D) bioluminescence color of *E. coli* colonies expressing firefly luciferases after addition of D-luciferin: (1-4) *Macrolampis* sp2: (1) immediately after spraying 1 mM D-luciferin, and after (2) 30 s, (3) 60 s and (4) 90 s; and (5-8) *Cratomorphus distinctus*: (5) immediately after spraying 1 mM D-luciferin, and after (6) 30 s, (7) 60 s and (8) 90 s.
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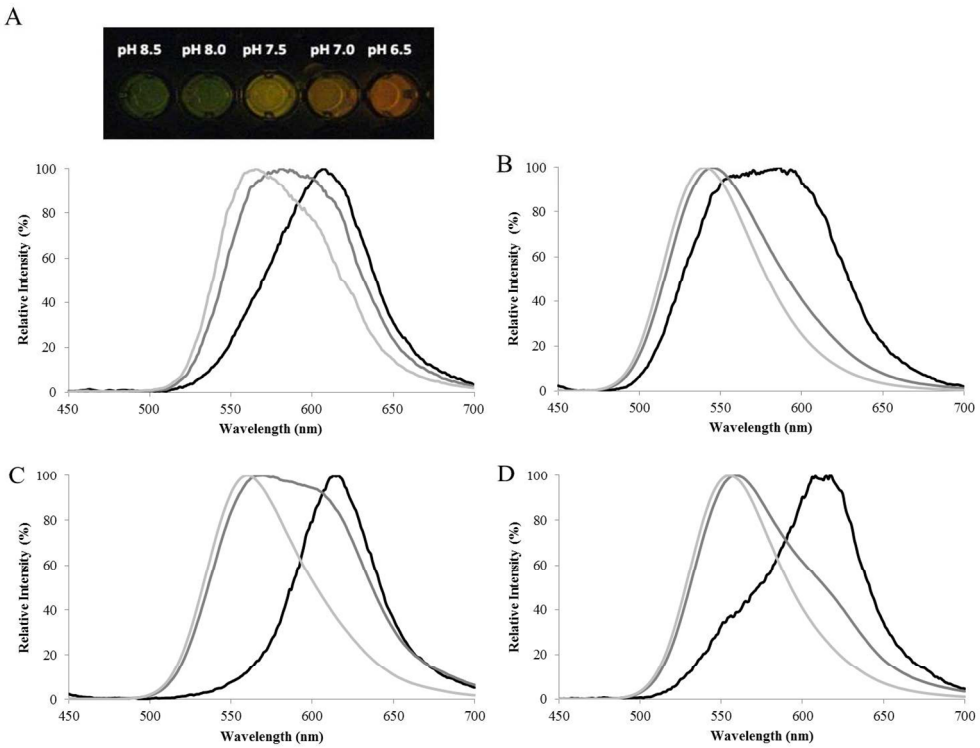
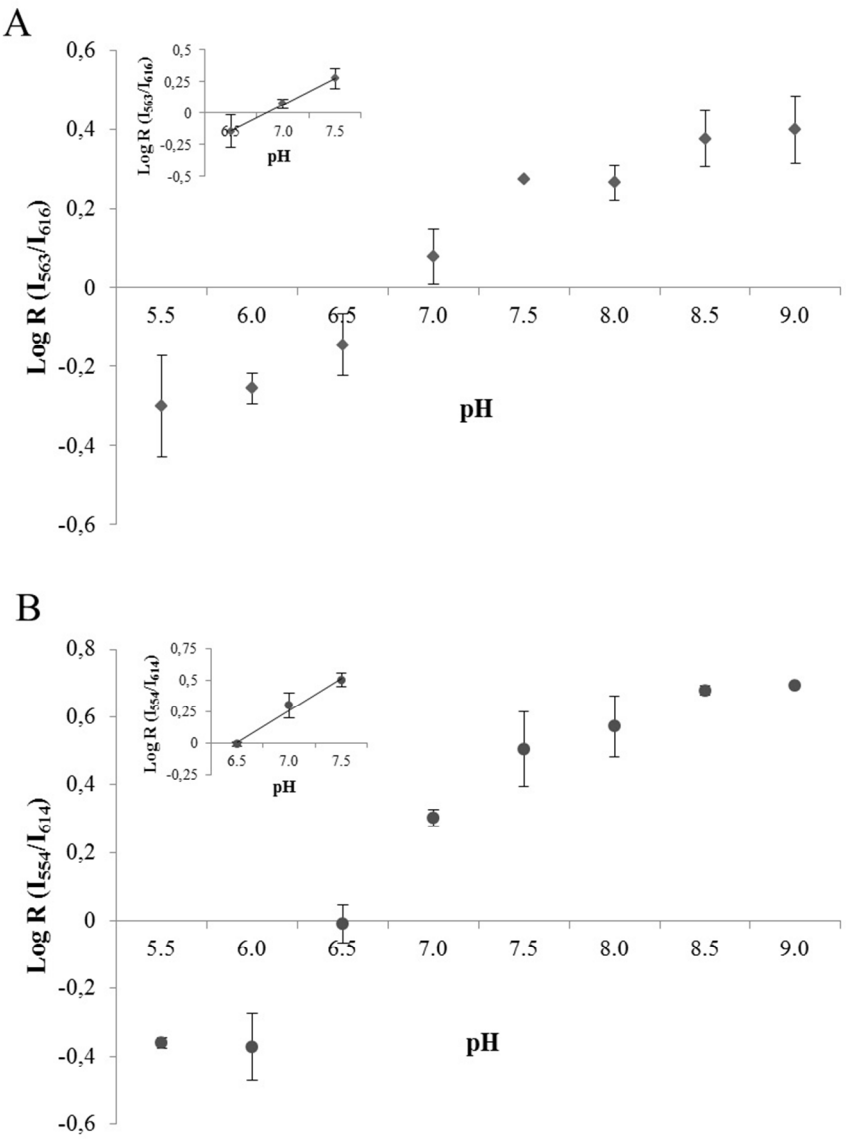



Effect of pH on the bioluminescence spectra of firefly luciferases in 0.10 M phosphate buffer: (A) *Macrolampis* sp2 firefly luciferase, (B) *Amydetes vivianii*, (C) *Photinus pyralis* and (D) *Cratomorphus distinctus* firefly luciferases; (black line) 0.10 M phosphate buffer pH 6.0; (dark gray line) pH 7.0 and (light gray line) pH 8.0. In the upper pannel A we can see the *in vitro* bioluminescence color at different ps using *Macrolampis* sp2 firefly luciferase.
253x194mm (150 x 150 DPI)




Ratiometric relationship between R ($I_{green}/I_{red}$) and pH in different firefly luciferases: (A) *Macrolampis* sp2 luciferase, inset ratiometric relationship between pH 6.0 and 7.0; and (B) *Cratomorphus distinctus* luciferase, inset ratiometric relationship between pH 6.0 and 7.0.
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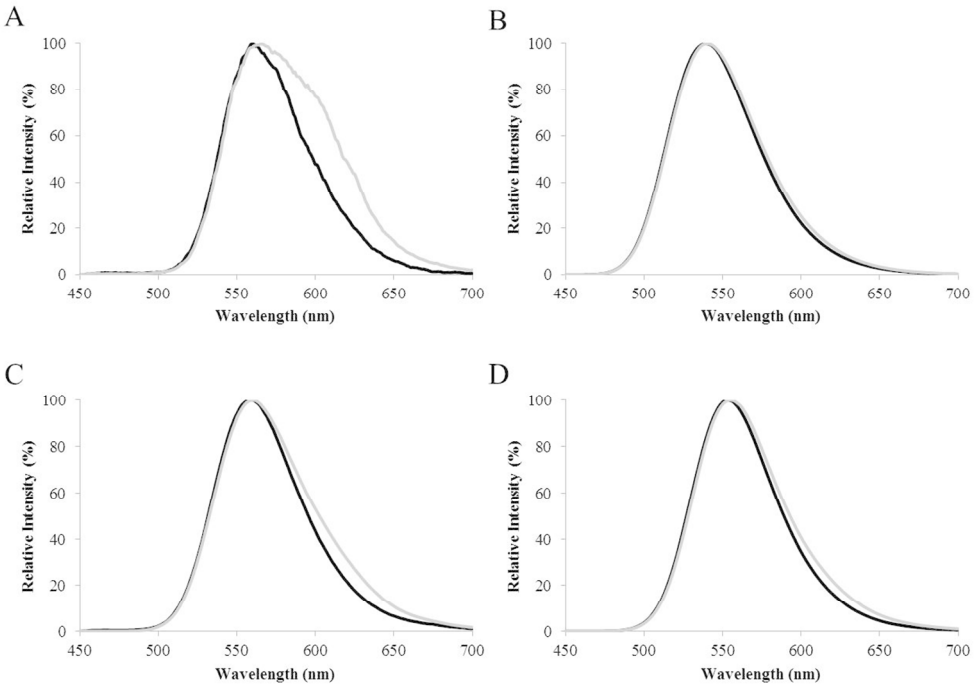
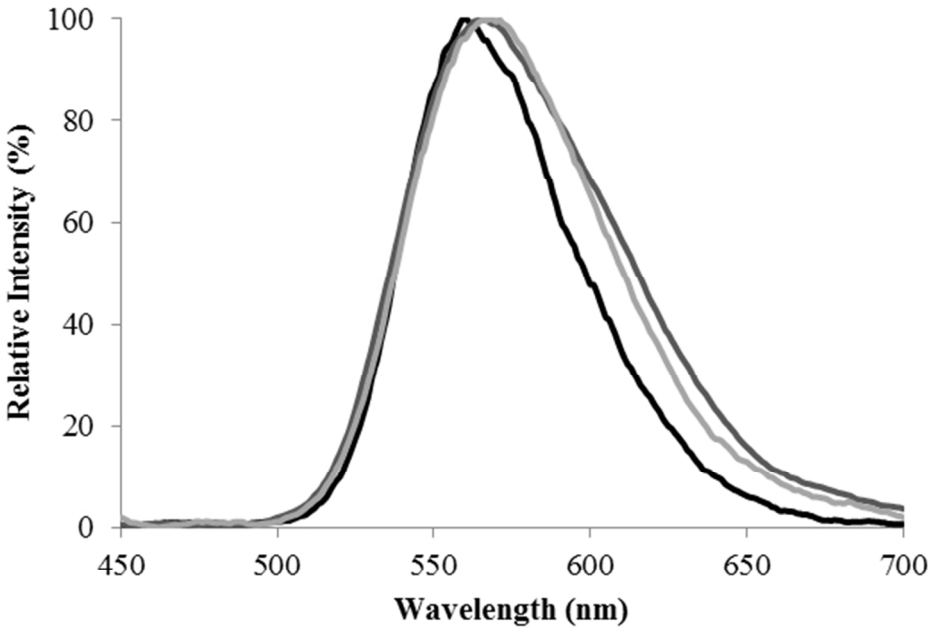
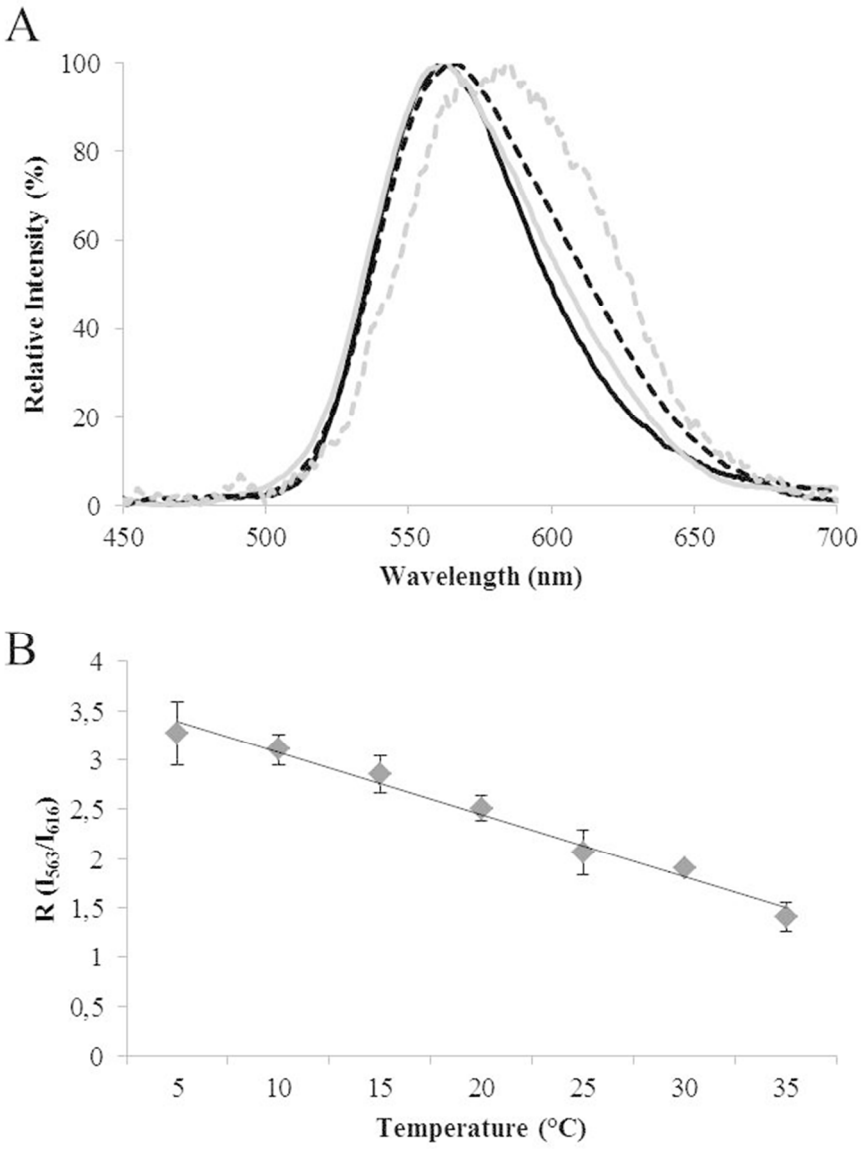



Effect of 0.10 M phosphate (gray) and Tris-HCl (black) buffers at pH 8.0 on bioluminescence spectra of firefly luciferases: (A) *Macrolampis* sp2, (B) *Amydetes vivianii*, (C) *Photinus pyralis* and (D) *Cratomorphus distinctus* luciferases.
218x156mm (150 x 150 DPI)




Effect of divalent metals at the concentration of 2 mM on bioluminescence spectrum of *Macrlampis* sp2 firefly luciferase: (black) no addition; (dark gray) $NiSO_4$ and (light gray) $ZnSO_4$.
111x76mm (150 x 150 DPI)




(A) Effect of the temperature on the bioluminescence spectrum of *E. coli* expressing *Macrolampis* sp2 luciferase: (A) effect of the temperature on the bioluminescence spectra; 5 °C ( ); 10 °C ( ); 25 °C ( ) and 35 °C ( ); and (B) effect of temperature on the ratio (R) of bioluminescence intensities at 563 and 616 nm (correlation coefficient = -0.992).
117x154mm (150 x 150 DPI)