# Bioluminescence of Firefly Luciferase in the Presence of Biologically Relevant Cations

by
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## Abstract

The bioluminescent protein firefly luciferase is a possible biological intracellular pH reporter because it has light emission dependent on the pH of its environment. For firefly luciferase to be reliable as an intracellular pH reporter, its emission should not be significantly disturbed by the presence of biologically relevant cations. To evaluate firefly luciferase's behavior in an environment where $K^+$, $Na^+$, $Mg^{2+}$, and $Ca^{2+}$ are present, luciferase was combined with its substrate and different concentrations of these cations in a microplate and analyzed on a plate reader to determine the emission of 550±10 and 610±10 nm light, which was used to obtain a 550:610 nm ratio. It was discovered that the plate reader's sensitivity to low levels of bioluminescence was inconsistent, meaning that more experimentation with solutions containing higher concentrations of firefly luciferase is needed to confidently conclude that biologically relevant cations do not significantly alter the bioluminescence of firefly luciferase. However, the experiments with monovalent cations produced promising results that fell within the expected range of magnitude for the plate reader and showed small fluctuations in the 550:610 nm ratio. Although the results for divalent cations were inconclusive, firefly luciferase is still relevant for further study of possible intracellular pH reporters.

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# Introduction

The types of reactions that occur in cells contribute to each tissue's unique function. Different cells have specific enzymes and intracellular environments to support their respective reactions (Robinson 2015). A single cell also requires specific local intracellular conditions to support the cell's overall function. For instance, the cytosolic environment for many cells is neutral (pH 7.0-7.4) while organelles, like lysosomes and mitochondria, require a more acidic environment (pH 4.6-6.0) (Dechant and Peter, 2014). Because local cellular environments are highly specific, it is vital to have a reliable method of evaluating environments inside of living cells to better understand biology.

One important aspect that differs in intracellular environments is pH. Because pH is the negative $\log_{10}$ of the concentration of hydrogen ions ($H^+$) in a solution, the ability to measure intracellular pH ($pH_i$) would contribute to a deepened understanding of the function of a cell or cellular component. For example, the $pH_i$ of the mitochondrial matrix compared to the pH of the intermembrane space points to the use of a proton gradient as a driving force (Jonckheere and Smeitink 2011). Furthermore, the $pH_i$ can help predict what types of proteins might be present and in what conformations. For example, the protein lysozyme takes on different conformations at different pHs because proton concentrations affect the binding free energy of lysozyme (Schaefer, et al 1997). Therefore, having an accurate measure of $pH_i$ can allow for conclusions to be drawn about the conformation of proteins present and the function of the cell.

Common tools currently used to measure pH include probes, color test strips, and fluorescent indicator chemicals. Using a pH probe is a simple method to monitor the changes of pH of a solution *in vitro* but requires maintenance and may yield varying results across different probes if they are not standardized appropriately. Color test strips are the most convenient method of measuring pH, but they are subjective and can only measure pH once at a given time. Probes and color strips are also unusable for experiments *in vivo* because they cannot physically be inserted into a cell. In contrast, fluorescent dyes can be used with living cells to monitor $pH_i$ by measuring emitted light (Takahashi, et. al. 2018). However, this method requires physical administration of the dye into the organism being tested and location-specific evaluation of the emitted fluorescence from the target cell/tissue.

An alternative to these approaches is the use of an engineered, integrated reporter, such as a bioluminescent protein, to monitor $pH_i$. This method would involve engineering an organism to produce proteins that have bioluminescent capabilities with the stipulation that the wavelength of emitted light changes with $pH_i$. This approach was first tested using an engineered bioluminescent and fluorescent protein to obtain a ratio of bioluminescence to fluorescence, which then was used to determine $pH_i$ (Zhang, et. al. 2012). The engineered protein, "pHlash", was derived from *Renilla* luciferase, a bioluminescent protein produced by marine organisms (Tzertzinis, et al 2012). While the pHlash experiment demonstrated that it is possible to use emitted light to measure pH, it is unreliable for long term experimentation because its substrate, coelenterazine, is not very soluble in water and breaks down over time (Teranishi and Shimomura, 1997). An alternative approach that would require less protein engineering, use a less expensive, and

more water-soluble substrate, and deliver more consistent results may be to instead use firefly luciferase as a bioluminescent monitor.

Firefly luciferase is a bioluminescent protein produced by fireflies that gives them their signature yellow glow. This enzyme catalyzes the oxidation of luciferin to oxyluciferin, which emits light (Shimomura 2012). Luciferase has a binding site for excited-state and ground-state oxyluciferin, meaning the enzyme can modify the light emitter to emit red or green-yellow light, depending on the organism (Shimomura 2012). Additionally, the pH of the environment in which firefly luciferase acts correlates with a distinct light emission (Figure 1A). The pH dependance of firefly luciferase is precisely why it may have applications for measuring $pH_i$. Using a protein to indicate $pH_i$ is advantageous because the organism being studied would produce the protein itself, as opposed to having to administer an external chemical like a fluorescent dye. It would also allow for extreme specificity in terms of sub-cellular environments.

Additionally, for *in vivo* use of firefly luciferase as a $pH_i$ reporter, it is not necessary to obtain a full emission spectrum of bioluminescence. A correlation can be made between $pH_i$ and bioluminescence by measuring the ratio of light emitted at specific wavelengths. For example, the ratio of emission used for the pHlash protein was obtained by measuring light emitted at 525 and 475 nm (Zhang, et al. 2012). This is also true for firefly luciferase and emission at wavelengths of 550 and 610 nm (Figure 1B).

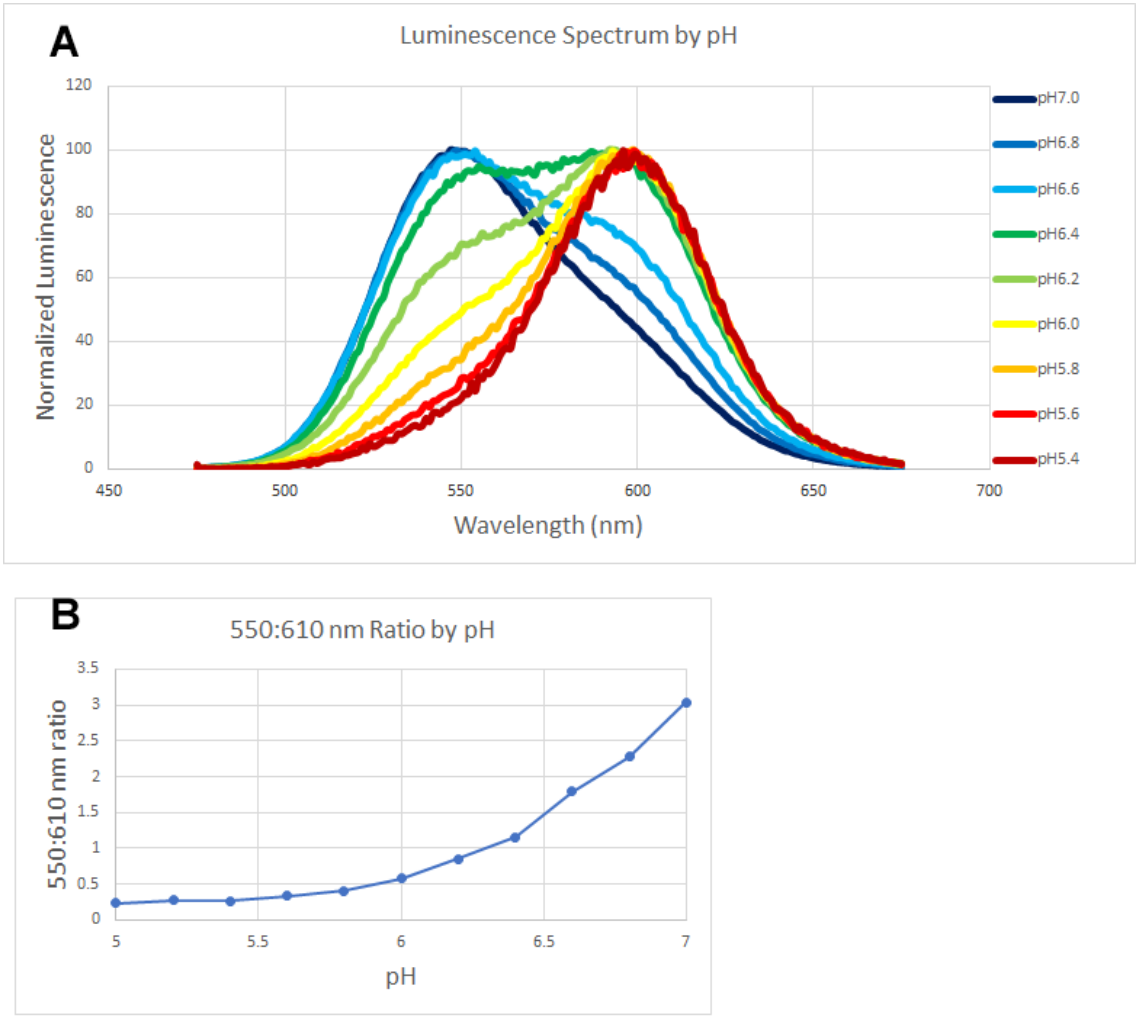


**Figure 1. Luminescence Spectrum of Firefly Luciferase by pH.** The pH at which firefly luciferase metabolizes its substrate has a correlational relationship to a particular pattern of light emission (Samaha and Robertson, unpublished). **A)** The full spectrum of light emission from purified firefly luciferase measured in 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer at different pH. The peak luminescence from each sample was normalized to a relative value of 100. **B)** The 550:610 nm ratio at different pH calculated from data shown in panel A.

Because firefly luciferase's bioluminescence is dependent on pH, it is possible that the presence of cations other than $H^+$ may also alter the character of its bioluminescence (Viviana and Bechara, 1995). For firefly luciferase to be a dependable $pH_i$ reporter, it must have minimal or no interactions with other intracellular cations that may cause a change in the character of bioluminescence it emits. The goal of this project was to assess the relationship between the presence and concentration of intracellular cations ($K^+$, $Na^+$, $Mg^{2+}$, and $Ca^{2+}$) and firefly luciferase's light emission. This was accomplished by quantifying firefly luciferase's bioluminescence as a ratio of emission of 550±10 nm and 610±10 nm light as intracellular cations were included independently. My hypothesis was that the ratios of emitted light measured would not show significant changes when cations were added to a solution of firefly luciferase and its substrate, which would validate its use as an intracellular pH reporter.

## Materials and Methods

*Protein Production*

Prior to this work, Nicholas Samaha in the Robertson lab created a plasmid which contained the coding sequence for firefly luciferase (from pGL4.2 by Promega) under the control of an IPTG-inducible promoter in the kanamycin resistant expression vector pNIC28-Bsa4 from the Gileadi lab at Oxford (Savitsky, et al., 2010). This plasmid was transformed into the BL21-de3 strain of *Escherichia coli* (*E. coli*) which was used as the source of luciferase protein for this work. These cells were grown from a frozen stock overnight at 37 degrees Celsius on an LB (Luria-Bertani) plate (1% Tryptone, 1% NaCl, 0.5% Yeast extract) supplemented with 50 µg/mL kanamycin. A single colony from the plate was added to a test tube containing 5 mL of autoclaved LB media using sterile technique. The test tube was incubated in a shaking incubator at 37°C overnight. The contents of the test tube were added to 150 mL of LB media containing 50 µg/mL kanamycin. A 1 mL sample of LB media was added into a cuvette to serve as a blank for spectrophotometry set to measure absorbance at 600 nm. After the blank, a 1 mL sample of the inoculated *E. coli* culture was measured. The inoculated *E. coli* culture was kept in a 37°C shaking incubator between sampling, and the culture was sampled every 30 minutes. When the culture reached an absorbance in the range of $A_{600}$ = 0.6-0.8, the culture was inoculated with 120 µL of a 500 mM stock solution of Isopropyl β- d-1-thiogalactopyranoside (IPTG) for a final concentration of 0.0004 M and incubated at 16°C shaking incubator overnight.

*Protein Isolation*

The 150 mL *E. coli* culture was separated into three conical tubes containing 50 mL each and centrifuged for 25 minutes at 4°C and 4,500 g. The supernatant was decanted without disturbing the cell pellets, which were washed with Wash Buffer #1 (50 mM sodium phosphate, 300 mM NaCl, pH 7) and combined into one conical tube. This solution was centrifuged for 30 minutes at 4°C and 4,500 g. The supernatant was decanted and 15 mL of lysis buffer (20 mM sodium phosphate, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMFS), pH 7) were added to the cell pellet, which was resuspended into the lysis buffer. This solution was incubated on ice for several minutes before sonification was performed using a Fisherbrand™ Model 705 Sonic Dismembrator with a 6 mm microtip at 50% amplitude for eight seconds of sonification followed by one minute of resting on ice, which was repeated for a total of eight cycles of sonication and ice. The lysed cell solution was then centrifuged for 40 minutes at 4°C and 4,500 g. The cell lysate supernatant was combined with 1 mL bed volume of TALON Metal Affinity Resin (Takara Bio Inc.) suspended in Wash Buffer #1 in a 50 mL conical tube. This solution was rocked horizontally in an ice bath for 20 minutes and then centrifuged for three minutes at 4°C and 1000 g. The supernatant was removed, and the TALON bead pellet was left undisturbed. The bead pellet was then washed with 10 mL of Wash Buffer #1 and allowed to rock on an ice bath for 10 minutes. This solution was added to an empty 10 mL Thermo Scientific™ Pierce™ disposable column and the beads were allowed to settle to the bottom of the column while the column was capped. After the beads settled, the buffer was drained from the column while avoiding letting the

TALON beads become exposed to the air. This series of adding 10 mL of Wash Buffer #1 and draining was repeated twice more. Five mL of Wash Buffer #2 (50 mM sodium phosphate, 300 mM NaCl, 5 mM imidazole, pH 7) were then added to the column and drained similarly to previous steps, then this step was repeated. Four mL of Elution Buffer (50 mM sodium phosphate, 300 mM NaCl, 150 mM imidazole, pH 7) were added to the column and left undisturbed for one minute, then the elution solution containing enriched luciferase was collected. This solution was divided into two Amicon® Ultra-2 mL centrifugal filters to begin buffer exchange with 100 mM MOPS (pH7). The columns were centrifuged for an hour at 4°C and 4,500 g. The flowthrough was removed from the columns and the ~ 1 mL of concentrated protein remaining in the two columns was collected and diluted in 3 mL of 100 mM MOPS (pH7) to bring the volume back up to the original 4 mL. This process was repeated twice more to yield the enriched luciferase protein, which was used in luminescence assays.

*Luminescence Analysis*

Separate solutions containing 100 mM MOPS (pH7) and 1 M NaCl, KCl, $MgCl_2$, or $CaCl_2$ were prepared. Stock 1 M salt solutions were added as appropriate in triplicate to the wells of a 96 well black microplate with well volumes of 200 µL to perform a bioluminescence analysis on luciferase in the presence of 0, 10, 22, 57, 123, and 300 mM of each cation. At the time of experimentation, a luciferase/luciferin solution was made with 1.65 mL of 0.1 M MOPS, 1.65 mL of firefly luciferase (255 µg/mL for monovalent cations and 75.9 µg/mL for divalent cations), 3.3 µL of 100 mM luciferin, and 6.6 µL of ligase buffer (500 mM Tris-HCl, 100 mM MgCl2, 100 mM DTT, 10 mM ATP). 100 µL of this solution were added to each well along with appropriate volumes of each 1 M salt

solution being analyzed and 100 mM MOPS (pH 7) to maintain constant total volume in each well (Table 1).

**Table 1. Composition of solutions added to the wells of a microplate.**

| Desired cation concentration | 0 mM | 10 mM | 22 mM | 57 mM | 123 mM | 300 mM |
|---|---|---|---|---|---|---|
| Volume of luciferase/ luciferin mix | 100 µL | 100 µL | 100 µL | 100 µL | 100 µL | 100 µL |
| Volume of 1 M salt solution added | 0 µL | 1.52 µL | 3.09 µL | 8.11 µL | 17.65 µL | 42.86 µL |
| Volume of 100 mM MOPS added | 42.86 µL | 41.34 µL | 39.11 µL | 34.75 µL | 25.21 µL | 0 µL |
| Total Volume in Well | 143 µL | 143 µL | 143 µL | 143 µL | 143 µL | 143 µL |

The microplate was analyzed on a BM6 Labtech Clariostar Plus plate reader that was paired with MARS software and set to measure luminescence emission at 550±10 nm and 610±10 nm. The shaking specifications were set to 30 seconds at 300 rpm and the integration time was 1 second at a focal height of 11 mm. The emissions were recorded and the 550:610 nm ratio calculated, which was used for interpretation.

Buffer solutions containing 100 mM MES were pH-adjusted using NaOH to produce 11 buffers of pH 5.0-7.0 which increase in increments of 0.2 pH units. Each of these buffers were added in duplicate to wells of a 96 well black microplate so that each well contained 130 µL of the respective buffer. A luciferase/luciferin solution was made with 1.15 mL of 75.9 µg/mL firefly luciferase, 1.15 µL of 100 mM luciferin, and 2.3 µL of ligase buffer (500 mM Tris-HCl, 100 mM MgCl2, 100 mM DTT, 10 mM ATP) and 50 µL of this solution were added to each of the wells. The microplate was analyzed on a BM6 Labtech Clariostar Plus plate reader using the same specifications outlined above.

*Protein Concentration Assay*

Serial dilution of bovine serum albumin (BSA) was performed using 2000 µg/mL stock solution to obtain standard solutions of 1000, 500, 250, 125, 25, and 0 µg/mL (Table 2).

**Table 2. Serial dilution of BSA to create protein standards.**

| BSA concentration | Volume of BSA added | Volume of DI water added |
|---|---|---|
| 1000 µg/mL | 100 µL of 2000 µg/mL | 100 µL |
| 500 µg/mL | 100 µL of 1000 µg/mL | 100 µL |
| 250 µg/mL | 100 µL of 500 µg/mL | 100 µL |
| 125 µg/mL | 100 µL of 250 µg/mL | 100 µL |
| 25 µg/mL | 100 µL of 125 µg/mL | 400 µL |
| 0 µg/mL | 0 µL | 400 µL |

To separate tubes of 1 mL of Coomassie Reagent (Pierce™), a protein sample containing either 20 µL of standard (see Table 2) or 20 µL of firefly luciferase was added. The tubes were capped, inverted, then incubated for 5 minutes. The solutions in the microcentrifuge tubes were transferred to disposable cuvettes and the absorbance at 595 nm was recorded using a spectrophotometer.

## Results

To test the hypothesis that the firefly luciferase emission 550:610 nm ratio would be independent of cation presence and concentration, purified luciferase was combined with different amounts of 1 M salt solutions, 0.1 M MOPS buffer, ATP, and its substrate in a microplate and analyzed for luminescence at 550±10 nm and 610±10 nm. The cations of interest in these experiments were $Na^+$, $K^+$, $Mg^{2+}$, and $Ca^{2+}$ because these are biologically relevant cations that have the potential to interfere with the application of firefly luciferase as an intracellular pH reporter. To begin this study we first needed to quantify the amount of protein in each protein preparation.

To determine the concentration of luciferase present in the protein solutions used, a standard curve was plotted using bovine serum album (Figure 2). The equation of the standard curve was used to determine the concentration of luciferase. Protein sample A had 255 µg/mL and was used in the subsequent monovalent cation experiment, and protein sample B had 75.9 µg/mL and was used in the subsequent divalent cation experiment (Table 3). Having confirmed successful protein enrichment and quantification, we next moved to testing the ability of monovalent and divalent cations to alter the luciferase emission profile.

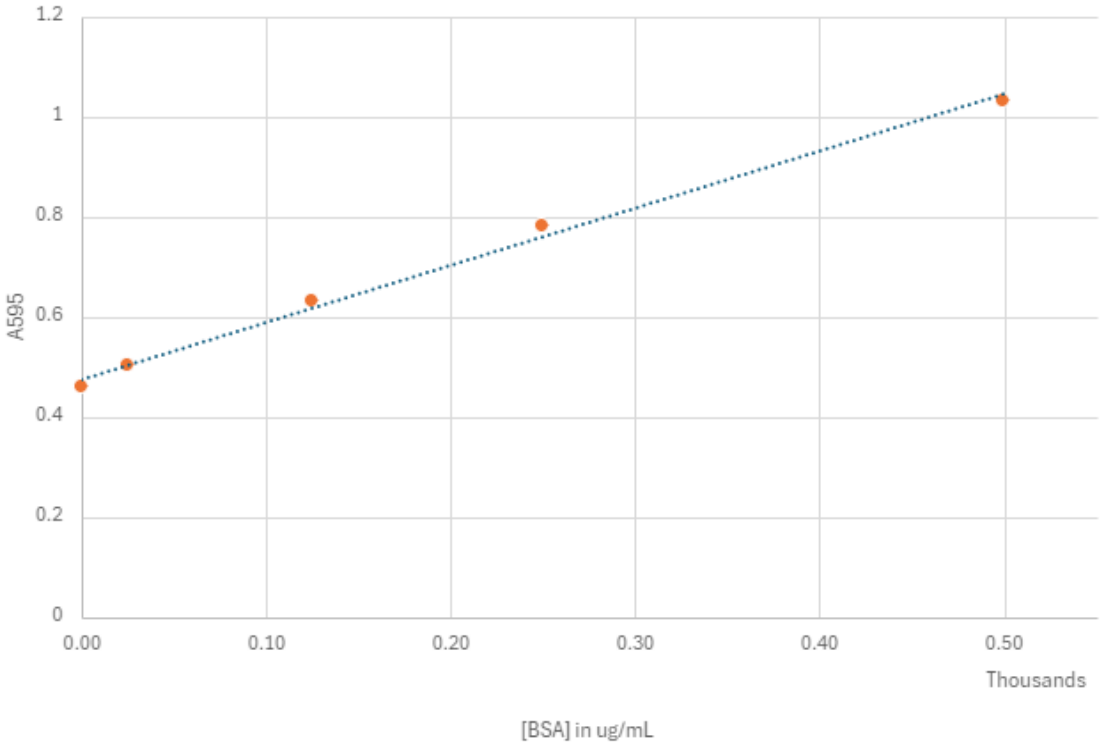


**Figure 2. Standard curve of protein used to determine luciferase concentration.** Serial dilution was performed to obtain standard concentrations of BSA protein, which were added to 1 mL of Coomassie Reagent and measured with a spectrophotometer at 595 nm. This process was repeated with protein samples used in the experiments and their absorbance values were recorded. The concentration of luciferase was determined using the equation of the standard line ($y = 0.0011x + 0.4745$).

**Table 3. Concentration of luciferase in the protein samples.**

| Sample | A595 | Protein Concentration |
|---|---|---|
| A | 0.756 | 255 μg/mL |
| B | 0.558 | 75.9 μg/mL |

Sodium is generally present in animal cells at an extracellular concentration of approximately 140 mM and an intracellular concentration of approximately 15 mM (Inglese, et al. 2013). Potassium is generally present in animal cells at 3.5-5.5 mM extracellularly and 140-150 mM intracellularly (Zacchia, et al. 2016). Because both monovalent cations have biological relevance up to 150 mM, the concentrations of cations tested were 0, 10, 22, 57, 123, and 300 mM. These concentrations were kept for consistency when testing divalent cations, even though magnesium is present in mammalian cells at a lower concentration of 17-20 mM (Romani and Scarfa, 1992) and calcium is generally present in animal cells at concentrations less that 1 mM intracellularly (Bagur and Hajnóczky, 2017).

When sodium concentrations were varied between 0 and 300 mM, we observed a small change in the 550:610 nm ratio at 0, 10, 22, 57, and 300 mM with the largest change at 123 mM (Figure 3, Table 4). When we added different concentrations of potassium, we observed less variability than for sodium with very small changes at all tested concentrations (Figure 3, Table 5). Next, we investigated the effect of adding different concentrations (0-300 mM) of divalent cations on emission. When magnesium was added, we observed large inconsistencies regarding the 550:610 nm ratio, which produced very large values of standard deviation (Figure 4, Table 6). Similarly, calcium addition showed large inconsistencies with slightly less deviation (Figure 4, Table 7). However, the difference in ratio consistency between monovalent and divalent cations may be due to the variation in magnitude of results obtained from the plate reader for emission at 550 nm and 610 nm. This difference in magnitude may be due to the difference in protein concentration between the two samples of enriched luciferase.

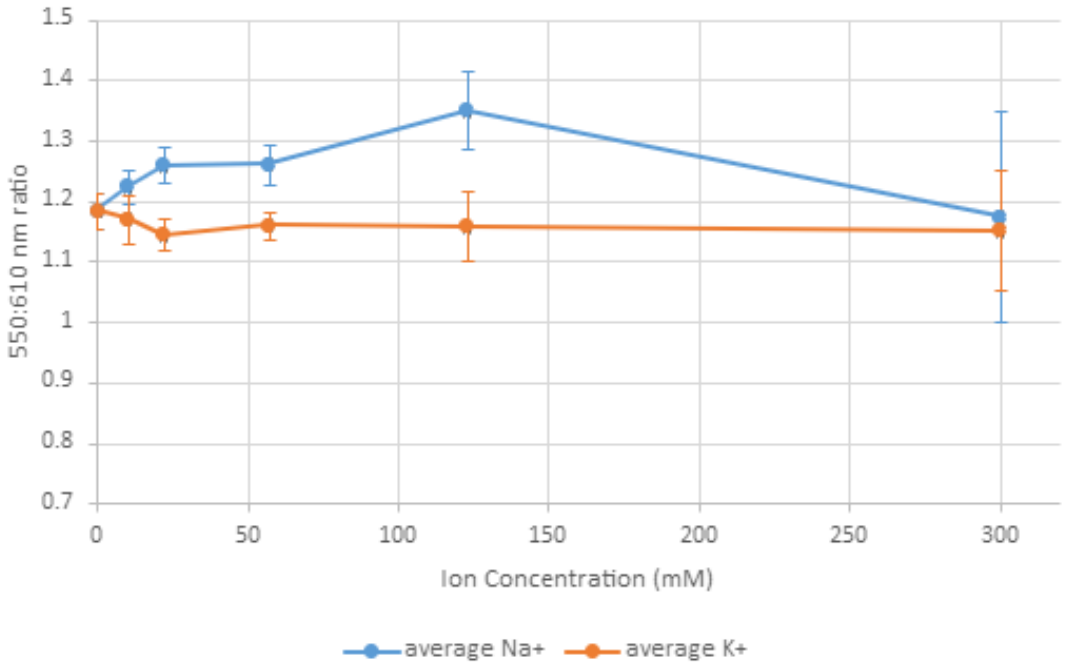


**Figure 3. The 550:610 nm ratio at different concentrations of sodium and potassium cations.** Constant volumes of luciferase, luciferin, and ATP were added to a 96 well plate reader along with varied concentrations of NaCl and KCl to achieve a final volume of 143 µL per well. The variables were measured in triplicate. The parameters were specified to measure the absorbance of 550±10 nm and 610±10 nm wavelengths emitted from the wells with an integration time of 1 second. These emissions were used to determine the 550:610 nm ratios, which were plotted using the average of the triplicate data with standard deviation error bars.

**Table 4. Ratio of 550:610 nm emitted light at different concentrations of NaCl.**

| [$Na^+$] | 0 mM | 10 mM | 22 mM | 57 mM | 123 mM | 300 mM |
|---|---|---|---|---|---|---|
| **Replicate 1** | 1.1737 | 1.2383 | 1.2866 | 1.2957 | 1.3943 | 1.2038 |
| **Replicate 2** | 1.2180 | 1.1928 | 1.2663 | 1.2584 | 1.2764 | 1.3306 |
| **Replicate 3** | 1.1628 | 1.2398 | 1.2260 | 1.2276 | 1.3784 | 0.9879 |
| **Average** | 1.1848 | 1.2237 | 1.2596 | 1.2606 | 1.3497 | 1.1741 |
| **Standard Deviation** | 0.0292 | 0.0267 | 0.0307 | 0.0340 | 0.0639 | 0.1732 |

**Table 5. Ratio of 550:610 nm emitted light at different concentrations of KCl.**

| [$K^+$] | 0 mM | 10 mM | 22 mM | 57 mM | 123 mM | 300 mM |
|---|---|---|---|---|---|---|
| **Replicate 1** | 1.1736 | 1.1448 | 1.1132 | 1.1346 | 1.1386 | 1.0829 |
| **Replicate 2** | 1.2180 | 1.1500 | 1.1621 | 1.1697 | 1.1151 | 1.2659 |
| **Replicate 3** | 1.1628 | 1.2140 | 1.1596 | 1.1759 | 1.2236 | 1.1059 |
| **Average** | 1.1848 | 1.1696 | 1.1450 | 1.1601 | 1.1591 | 1.1516 |
| **Standard Deviation** | 0.0292 | 0.0385 | 0.0275 | 0.0222 | 0.0571 | 0.0996 |

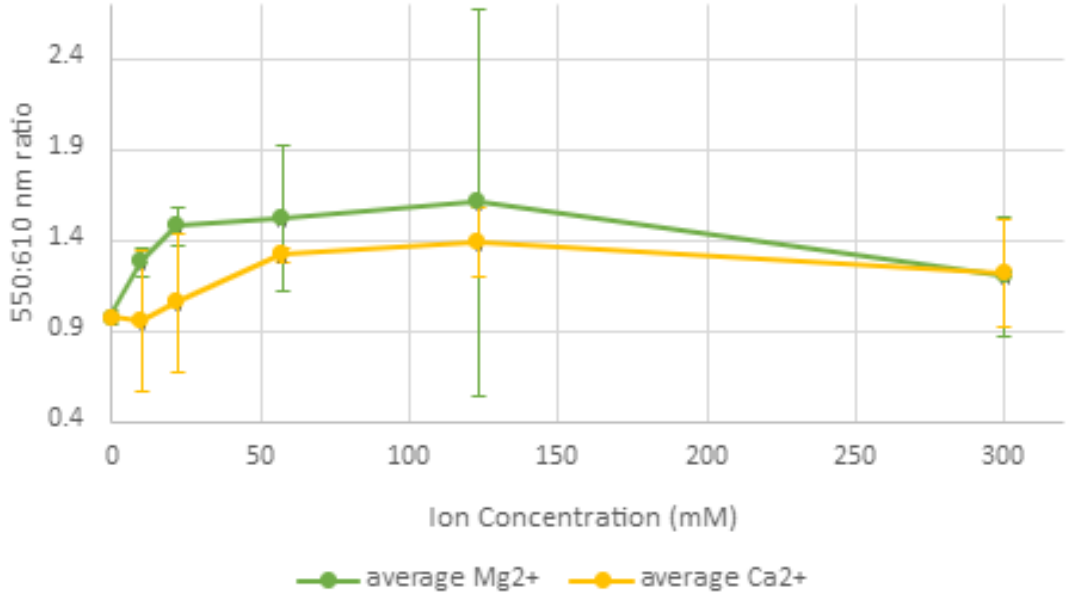


**Figure 4. The 550:610 nm ratio at different concentrations of magnesium and calcium cations.** Constant volumes of luciferase, luciferin, and ATP were added to a 96 well plate reader along with varied concentrations of $MgCl_2$ and $CaCl_2$ to achieve a final volume of 143 µL per well. The variables were measured in triplicate. The parameters were specified to measure the absorbance of 550±10 nm and 610±10 nm wavelengths emitted from the wells with an integration time of 1 second. These emissions were used to determine the 550:610 nm ratios, which were plotted using the average of the triplicate data with standard deviation error bars.

**Table 6. Ratio of 550:610 nm emitted light at different concentrations of $MgCl_2$.**

| $[Mg^{2+}]$ | 0 mM | 10 mM | 22 mM | 57 mM | 123 mM | 300 mM |
|---|---|---|---|---|---|---|
| **Replicate 1** | 0.9424 | 1.2938 | 1.4237 | 1.2555 | 0.9466 | 0.9600 |
| **Replicate 2** | 0.9534 | 1.3532 | 1.6018 | 1.9824 | 2.8333 | 1.0689 |
| **Replicate 3** | 1.0174 | 1.1881 | 1.4160 | 1.3186 | 1.041 | 1.5660 |
| **Average** | 0.9711 | 1.2784 | 1.4805 | 1.5188 | 1.6070 | 1.1983 |
| **Standard Deviation** | 0.0404 | 0.0836 | 0.1051 | 0.4026 | 1.0630 | 0.3230 |

**Table 7. Ratio of 550:610 nm emitted light at different concentrations of $CaCl_2$.**

| $[Ca^{2+}]$ | 0 mM | 10 mM | 22 mM | 57 mM | 123 mM | 300 mM |
|---|---|---|---|---|---|---|
| **Replicate 1** | 0.9424 | 1.3970 | 0.7058 | 1.2926 | 1.4035 | 1.5500 |
| **Replicate 2** | 0.9534 | 0.6739 | 1.0000 | 1.3043 | 1.1860 | 1.1219 |
| **Replicate 3** | 1.0174 | 0.7894 | 1.4545 | 1.3658 | 1.5757 | 0.9736 |
| **Average** | 0.9711 | 0.9534 | 1.0534 | 1.3209 | 1.3884 | 1.2152 |
| **Standard Deviation** | 0.0404 | 0.3884 | 0.3771 | 0.0393 | 0.1952 | 0.2992 |

Because we observed very small variation in the 550:610 nm ratio in the monovalent experiment but very large variation in the divalent experiment, we conducted an experiment in attempt to recreate the pH dependent change in emission ratio that was demonstrated using an instrument engineered to detect very small light emission (Figure 1B). To do this, sample B was added to buffers of various pHs ranging from 5.0-7.0 in duplicate and analyzed, using our plate reader, for emittance of 550±10 nm and 610±10 nm wavelengths. Duplicate samples were measured instead of triplicate samples because the quantity of luciferase protein was limited. The result of this experiment (Figure 5) did not mirror the expected result (Figure 1B) which supports our suspicion that both/either the plate reader is not sensitive enough and/or the protein concentration in sample B is not large enough to provide reliable results. Based on all these data, which are somewhat conflicting, more study is needed to resolve this question.

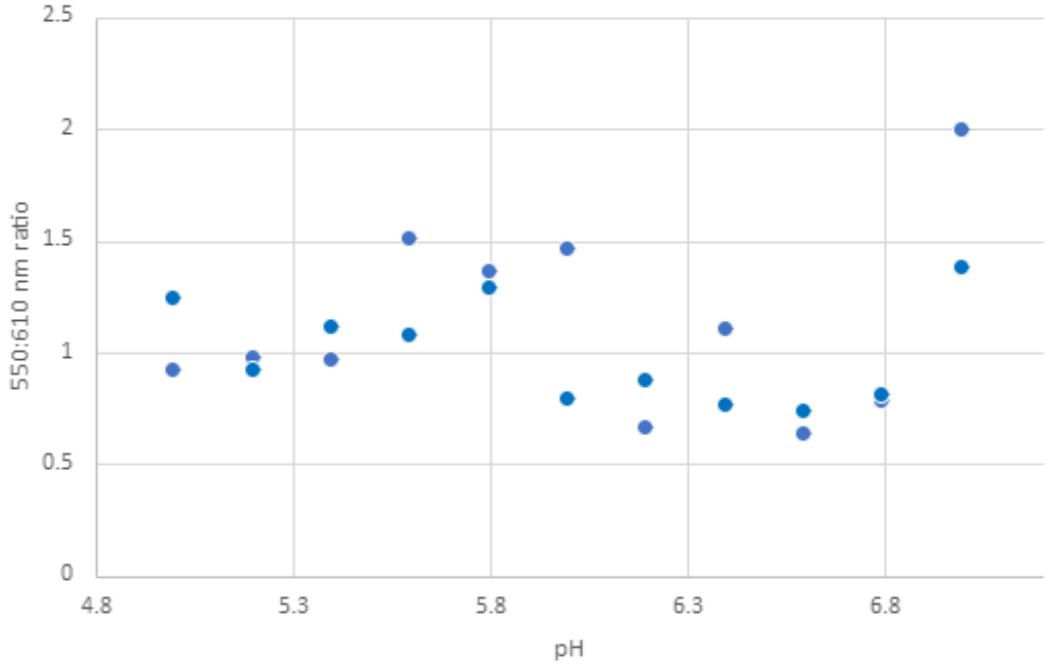


**Figure 5. The 550:610 nm ratio produced from luciferase/luciferin solutions containing buffers of various pHs.** Constant volumes of luciferase, luciferin, and ATP were added along with buffers of various pHs into a 96 well plate reader to a final volume of 180 µL. The experiment was performed in duplicate, and the plate was analyzed in a plate reader. The parameters were specified to measure the absorbance of 550±10 nm and 610±10 nm wavelengths emitted from the wells with an integration time of 1 second. These absorptions were used to determine the 550:610 nm ratios, which were plotted against the pH of the buffer that was added to the respective well.

## Discussion

The goal of these experiments was to determine if the character of bioluminescence produced by firefly luciferase would be disturbed when different monovalent and divalent cations were present in the environment. This is relevant because of the potential of luciferase to be used as an intracellular pH reporter, which would require that it have no interactions with intracellular cations that would skew the portion of bioluminescence emission that is used to measure $pH_i$. To quantify the character of bioluminescence at different cation concentrations, a ratio of 550±10 nm and 610±10 nm light emitted was used. Samaha and Robertson found that these particular wavelengths correlate to the peaks of emission spectra when measured at pHs ranging from 5 to 7, respectively (Figure 1). The benefit of using a ratio of two wavelengths is that 1) the ratio can serve as a surrogate for measuring the entire emission spectrum, and 2) that it self-normalizes for variations in the intensity of light emitted.

The magnitude of measured emission at 550 and 610 nm for the monovalent experiment was consistent with expected magnitudes for other modalities of the plate reader instrument like fluorescence, which typically yields values in the hundreds and thousands (Table A.1). After computation, small fluctuations in the emission ratio were observed, which supports my hypothesis and the potential future use of firefly luciferase as an intracellular pH reporter. However, the results from the divalent experiment were largely inconclusive because the magnitude of measured emission was vastly lower than that seen in the monovalent experiment (Table A.1). This difference in magnitude is

likely the source of the extreme variation in emission ratio, which leads us to believe that the divalent experiment is less reliable than the monovalent experiment.

The potential reasons that the magnitudes of the emission values were starkly different between the monovalent and divalent experiments are either that the plate reader's ability to detect low light emission is inconsistent or that the concentration of luciferase in sample B, which was used for the divalent and pH experiment, was too low to produce sufficient bioluminescence. This question is complicated by the results of the attempt to replicate the pH-dependent emission spectra demonstrated by Samaha and Robertson (Figure 1B) using sample B and our plate reader, because the attempt was not successful. To make this debate less convoluted, further replicate attempts should be conducted on the plate reader using protein samples with higher concentrations than sample B to determine if the pH-dependent emission spectra could be replicated.

While it seems to be the case that monovalent cations do not disrupt the ratio, it is essential to have a reliable plate reader that can detect luminescence at very low levels and protein samples with high concentrations of firefly luciferase to make further assertions. For future work in this area, to maximize the luminescence, it would be beneficial to use freshly enriched protein and avoid storing the enriched protein in a buffer for long periods of time. When luciferase was stored in 100 mM MOPS (pH7) buffer for more than 2 months, the solution became cloudy which indicates precipitation or microbial contamination.

Despite the inconclusiveness of these experiments, it is still the case that an intracellular pH reporter is a technology that would be beneficial to other researchers and further our understanding of biology and biochemistry. The higher magnitude results of

the monovalent cation experiment indicate that any further experimentation should be conducted with freshly enriched firefly luciferase with a concentration above 200 μg/mL. The low level of fluctuation in the 550:610 nm ratio produced from the monovalent experiment supports the claim that biologically relevant cations would not significantly skew firefly luciferase's emission if it is used an intracellular pH reporter. Additionally, firefly luciferase's substrate, luciferin, is stable and preservable. Thus, firefly luciferase is still a promising protein for potential use as an intracellular pH reporter.

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## Appendix

### A.1. 550±10 nm and 610±10 nm light emitted from luciferase solutions with different concentrations of cations.

| $[Na^+]$ | 550±10 nm | | | 610±10 nm | | |
|---|---|---|---|---|---|---|
| 0 mM | 18706 | 19138 | 18603 | 15938 | 15712 | 15998 |
| 10 mM | 16540 | 16880 | 14680 | 13356 | 14151 | 11840 |
| 22 mM | 16540 | 15173 | 15056 | 12856 | 11982 | 12280 |
| 57 mM | 10405 | 9377 | 9797 | 8030 | 7451 | 7980 |
| 123 mM | 4826 | 5370 | 5096 | 3461 | 4207 | 3697 |
| 300 mM | 1577 | 1646 | 902 | 1310 | 1237 | 913 |
| $[K^+]$ | 550±10 nm | | | 610±10 nm | | |
| 0 mM | 18706 | 19138 | 18603 | 15938 | 15712 | 15998 |
| 10 mM | 14316 | 14457 | 14627 | 12505 | 12571 | 12048 |
| 22 mM | 13226 | 13075 | 12420 | 11880 | 11251 | 10710 |
| 57 mM | 7332 | 8371 | 7947 | 6462 | 7156 | 6758 |
| 123 mM | 3811 | 3788 | 3791 | 3347 | 3397 | 3098 |
| 300 mM | 1005 | 971 | 240 | 928 | 767 | 217 |
| $[Mg^{2+}]$ | 550±10 nm | | | 610±10 nm | | |
| 0 mM | 295 | 307 | 175 | 313 | 322 | 172 |
| 10 mM | 273 | 272 | 221 | 211 | 201 | 186 |
| 22 mM | 168 | 17 | 177 | 118 | 108 | 125 |
| 57 mM | 113 | 113 | 120 | 90 | 57 | 91 |
| 123 mM | 71 | 102 | 76 | 75 | 36 | 73 |
| 300 mM | 48 | 62 | 83 | 50 | 58 | 53 |
| $[Ca^{2+}]$ | 550±10 nm | | | 610±10 nm | | |
| 0 mM | 295 | 307 | 175 | 313 | 322 | 172 |
| 10 mM | 95 | 62 | 60 | 68 | 92 | 76 |
| 22 mM | 36 | 48 | 80 | 51 | 48 | 55 |
| 57 mM | 53 | 60 | 56 | 41 | 46 | 41 |
| 123 mM | 80 | 51 | 52 | 57 | 43 | 33 |
| 300 mM | 62 | 46 | 37 | 40 | 41 | 38 |

**A.2. 550±10 nm and 610±10 nm light emitted from luciferase solutions with various pH.**

| pH | 550±10 nm | | 610±10 nm | |
|---|---|---|---|---|
| **5.0** | 32 | 52 | 35 | 42 |
| **5.2** | 60 | 42 | 62 | 46 |
| **5.4** | 63 | 41 | 66 | 37 |
| **5.6** | 72 | 50 | 48 | 47 |
| **5.8** | 57 | 68 | 42 | 53 |
| **6.0** | 70 | 61 | 48 | 78 |
| **6.2** | 48 | 63 | 73 | 73 |
| **6.4** | 77 | 83 | 70 | 110 |
| **6.6** | 70 | 71 | 111 | 97 |
| **6.8** | 72 | 80 | 93 | 100 |
| **7.0** | 123 | 118 | 62 | 86 |