Comparison of Firefly and Click Beetle Luciferase to Maximize Wavelength of Bioluminescence Imaging

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Abstract:

The accuracy of bioluminescence imaging (BLI) is currently compromised by many of the photons emitted being absorbed by the surrounding tissue in the subject. However, increasing the wavelength of the light emitted has been proven to reduce tissue absorption and improve accuracy. One of the factors affecting the wavelength of the light produced is luciferase, which is the catalyst used in the reaction to create light. This paper studied the relationship between the wavelength and the luciferase to determine whether Luc2 or CBR created a longer average wavelength. The wavelengths from previously conducted experiments on BLI were collected from a range of peer-reviewed academic papers and the mean wavelength of each luciferase was calculated along with a statistical test to test correlation. The results concluded that CBR produced an average wavelength of 642.67 nanometers (nm) which was higher that Luc2, which produced an average wavelength of 603.10 nm, meaning CBR, in terms of wavelength, would likely reduce photon absorption in the tissue more than Luc2 and therefore be more accurate at tracking cancer in comparison.

Introduction:

Currently, breast cancer is the most common type of cancer in the world, affecting one in eight women in the United States alone. This type of cancer can often spread to other parts of the body through a process known as metastasis, which can be difficult to track, and if gone unnoticed, metastasis of cancer is deadly (Zhao et al., 2013). The five-year survival rate of a cancer is the percentage of people who survive at least five years after first discovering the cancer within their body. In 2018, the five-year survival rate of breast cancer was 99% if it

stayed localized within the breast, but if it spread to other parts of the body, the five-year survival rate dropped to 27%, meaning roughly only one in every four people survived for at least five years after the breast cancer was first discovered. Most commonly, breast cancer can metastasize to the bone, lungs, or liver, which can cause complications in the treatment of the cancer since it is no longer localized to a single area of the body (Siegel, et al., 2019). Therefore it is important to track if the breast cancer has metastasized.

However, breast cancer tracking is currently an expensive, difficult, and invasive process. Various methods to visualize and track tumor growth and metastasis are currently in use, but they can be expensive, invasive, slow, or unreliable. PET scans are expensive, costing around $5000, and they use radioactive material, which can be dangerous and harmful to the patient's body (Sadikot, et al., 2005). MRI scans are expensive as well, costing up to $3500, and they take a long time to complete. CT-scans use x-rays which can be harmful to the body if used repeatedly and ultrasounds are not very accurate and have a low visual resolution making them unreliable (Dikmen et al., 2004). To combat these issues, two new types of noninvasive and relatively cheap imaging systems based off of natural reactions have emerged, fluorescence imaging and bioluminescence imaging.

Fluorescence Imaging:

Fluorescence imaging (FLI) is an optical imaging system that uses the fluorescence probes and highly sensitive cameras known as a charge-coupled device (CCD) to visualize cells within the body. Fluorescence imaging uses light created by the excited state of the fluorescence to produce an image. In addition, it has a wide array of different fluorescence probes, giving

scientists more options to research and test in different environments (Aswendt et al., 2014). However, its high background and easily excitable state lead to low contrast and resolution. When using a green fluorescent protein from a type of jellyfish in a study conducted at the University of Texas, researchers discovered FLI produced a high noise-to-signal ratio, making accuracy of FLI poor because the background of the the fluorescence imaging was too high to observe where the area being imaged was. In addition, the signals released by the proteins had issues penetrating to the tissue and were often absorbed before they could be detected by the CCD (Chudakov, et al., 2010). Despite FLI generating images of poor accuracy, over the past decade, a different cheap, noninvasive, and highly sensitive method to tracking cell growth has emerged known as bioluminescence imaging (BLI).

Bioluminescence Imaging:

BLI is a tracking method that can be used in vivo (within the body) and in vitro (outside the body) using natural light emission properties of animals and insects. This process is able to visualize, track, and quantify tumor growth and other cell or bacterial growth. The process of light emission involves an enzyme and a substrate, the most common of which being firefly luciferase and D-luciferin, combined within the subject to emit photons that are detected by highly sensitive CCDs. Since BLI is based on natural reactions, this method of tracking different processes is also noninvasive and easily attainable. In addition, it is relatively cheap

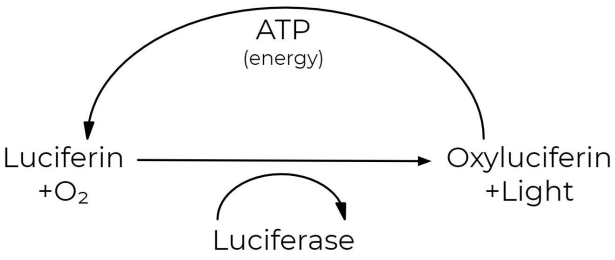


Fig 1: Chemical reaction of bioluminescence that leads to the production of light

when compared to the other types of imaging systems. Establishing an entire functional BLI system for experimental use has only costed around $100 to 200k which is relatively cheap when compared to the PET and MRI systems which can cost up to $1 million (Wu & Gambhir, 2010). Currently, the common enzyme used in BLI is firefly luciferase and the common substrate used is D-luciferin. Firefly luciferase is used due to its accessibility and naturally high wavelength. Unaltered, these substances together create a wavelength of about 560 nm in vivo and roughly 600 nm in vitro. However, these substances, when chemically altered, can produce different intensities and wavelengths. In addition, other enzymes such as beetle luciferase and substrates such as infra-luciferin can be used in BLI.

The luciferase is a protein, or an enzyme, contained within the bioluminescent organisms that acts as a catalyst to produce light during the bioluminescent reaction. Enzymes are the proteins within an animal that act as catalysts to bring about a specific chemical reaction. In this case, the enzyme is used to assist in the production and release of photons. This enzyme is used as a marker for gene expression and acts as a reporter gene binding to the cells that the luciferase has been chosen to track. The luciferin is the substrate and the substance that is used up in the reaction while the luciferase, being a catalyst, is not consumed in the reaction (Anderson et al., 2017). Substrates react with the enzyme which break down to produce the luminescence in the form of photons. As seen in Fig. 1, in a normal reaction that takes place in a bioluminescent organism, such as a firefly, the luciferin reacts with an excess of oxygen gas within the cells to produce an oxidized luciferin known as oxyluciferin along with light in the form of a photon. Then, the oxyluciferin is converted back into luciferin. The luciferase is not consumed so it can be reused to recreate the reaction any number of times with the presence of oxygen gas and

luciferin. When used in BLI, the cells of interest are located and attached to the luciferase using plasmid vectors to obtain a selectable marker (Doraz, et al., 2017). Since a plasmid vector was used to attach the luciferase to the cell, everytime the cell reproduces itself, the luciferase is also reproduced. This way, since the luciferase is never consumed when producing light, BLI can be used to image the cells to track cell growth such as tumor growth multiple times after injecting the luciferase into the subject once (Cosette, et al., 2016). Because of this, cancer caught in the early stages can use BLI to track the growth of the tumor and identify if the cancer spreads throughout the body since the cells that reproduce and spread to other parts of the body will carry the luciferase and light up when imaged. This technology is inserted into the subject by separately injecting both luciferin and luciferase into the body, to emit photons, that are then detected by the highly sensitive CCD. BLI works when both injected substances combine

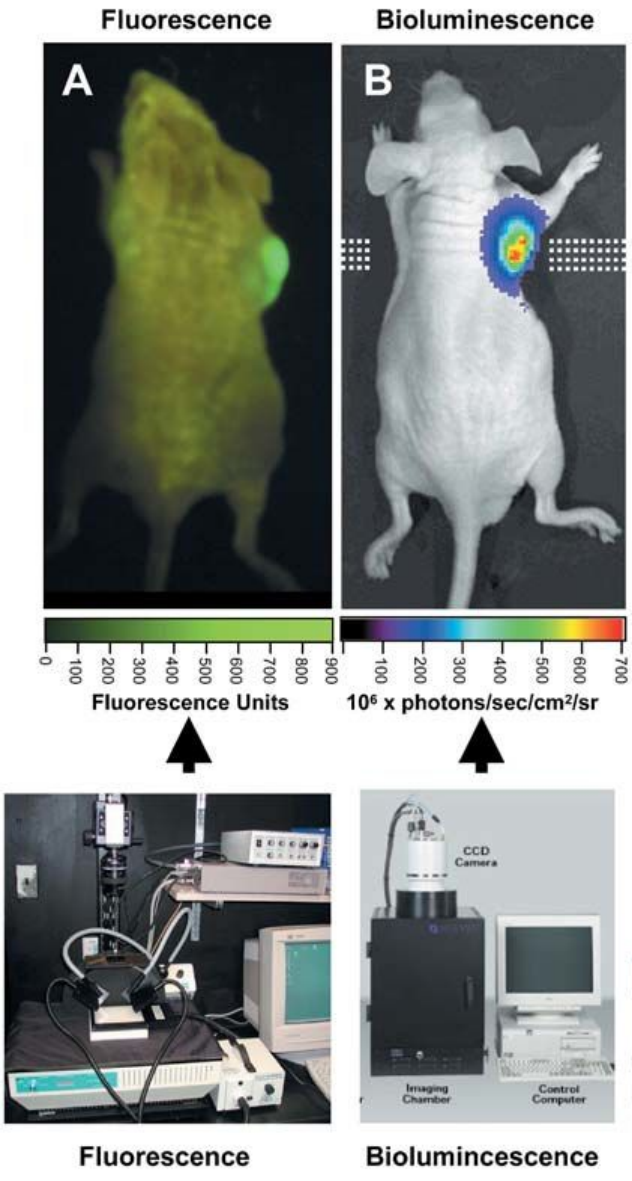


Fig. 2: side-by-side comparison of fluorescence imaging, pictured on the left, to bioluminescence imaging, pictured on the right (Ponomarev, et al., 2004)

within the body, catalyzing the reaction and releasing a photon to produce light within the body to be detected (Sadikot & Blackwell, 2005).

As previously mentioned, a similar method, FLI, exists which has a broader array of known fluorescent probes than the amount of luciferases BLI currently has available for use. However, bioluminescence is more sensitive in vivo than fluorescence imaging which makes it more accurate and produces a lower background than fluorescence imaging, increasing accuracy and contrast between the subject matter and surrounding tissue (Mofford et al., 2014). As seen in Fig. 2, fluorescence imaging on the left has a higher background than bioluminescence imaging. Although both methods visualize the same growth, the image pictured on the right which utilizes BLI, shows a much clearer and higher contrast between the growth and the rest of the subject's body. In the picture on the left which uses FLI, the contrast between the specific cells that are being imaged and the ones that are not is not clear making quantitative and qualitative accuracy unreliable. On the contrary, in the picture using BLI, the differentiation between what is being imaged and what is not has much more contrast making quantitative and qualitative values easier to identify and evaluate. The higher the contrast and accuracy of the imaging, the more accurate researchers can estimate the quantitative values and the physical properties of the growth. The more accurate the information doctors have on cancer tumors, the better they can treat them. Although BLI is more accurate than FLI, there are still issues in the accuracy of this method.

Current Issues:

Currently, BLI is only used in small animals because larger amounts of surrounding tissue make it harder for the photons to penetrate through and be detected by the cameras

(Cosette et al., 2016). Many of the photons are absorbed by the surrounding tissue instead, creating low visual resolution and quantitative inaccuracy (Zhao et al., 2013). Photons are absorbed when they are reflected or when they repel off the tissue before making their way out of the body.

One of the factors to high photon absorption is a low photon yield. The fewer the number of photons that are produced, the fewer the amount of photons that are not absorbed by the surrounding tissue. However, if the bioluminescence produces more photons, even if some photons are absorbed, there will be a higher number of photons that are not absorbed and therefore make a more accurate picture (Mofford, et al., 2014). Generally, the more tissue between the area of interest and the CCD, the higher the absorption and the lower the photon yield. In addition, the type of tissue, not just the amount of tissue affects the amount of photon absorption. Denser tissue such a stomach lining and dense bone makes photons much more susceptible to being absorbed since the photon has a higher chance of being reflected by the denser packed cells (Dikmen, et al., 2004).

Hemoglobin and melanin within the body also tend to absorb and scatter the light throughout the body making visual accuracy difficult. In specific, hemoglobin tends to absorb blue to green light but the absorption of wavelengths longer than 600 nm decreases significantly when compared to the blue to green light which has wavelengths averaging around 400 to 500 nm (Zhao, et al., 2005). Although wavelengths of blue and violet while they can travel farther through water, they are easily stopped by solid masses including tissue due to their high energy and frequency which makes the photon more susceptible to collision with other particles. Wavelengths of the photons range from blue to red with blue being shorter, averaging around

400 nm and red being the longest wavelengths, averaging around 630 to 700 nm. Red wavelengths are the longest wavelengths within the visible spectrum. Longer wavelengths can penetrate through skin and tissue easier and farther than shorter wavelengths because they are lower energy and not as reflective as well as being safer to the body due to their lower energy. Therefore, wavelengths of the light closer to infrared would have longer wavelengths and would be more visible to the detection systems (Jathoul et al., 2014). In relation, the wavelength would reduce the number of photons absorbed and would make them increasingly visible and easier to be picked up by the camera (Sadikot & Blackwell, 2005). As seen in Fig. 3, the solid black line, representing the longest wavelength of 650 nm, has the highest photon emission as represented by the y value of the graph. The solid line consistently shows higher emissions of photons even

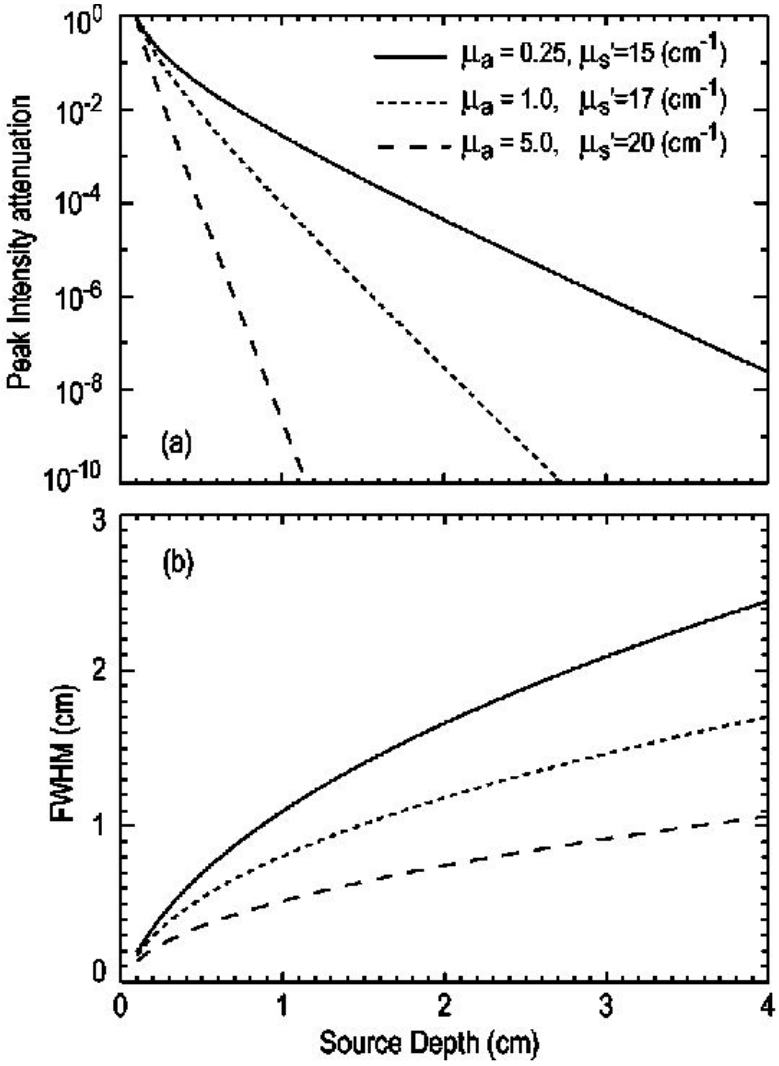


Fig. 3: depicts three different wavelengths tested at the same depth in vivo and their corresponding photon output of the photon's not absorbed by the surrounding tissue and detected by the highly sensitive cameras. The solid black line is a wavelength of 650 nm, the short dashed line represents a wavelength of 590 nm, and the long dashed line represents a wavelength of 550 nm.

as the depth increased than the lines representing wavelengths of 590 nm and 550 nm. The x-axis represents depth that the photon emission took place while the y-axis represents the number of photons emitted in vivo that were not absorbed by the surrounding tissue and were detected by the camera. Fig. 3 supports that longer wavelengths yield higher photon emission and therefore less absorption than lower wavelengths do. Therefore, increasing the wavelength of the bioluminescence would make it easier for the photons to pass through the surrounding tissue. However, due to the short wavelengths and low photon yield currently being produced by BLI, this imaging technique is still limited to small animals since the photons cannot travel far enough without being absorbed for BLI in larger animals to be accurate or effective.

Possible Solutions:

Increasing the wavelength of the bioluminescence would make it easier for the photons to pass through the surrounding tissue. Longer wavelengths can penetrate through skin and tissue easier and farther than shorter wavelengths because they are not as reflective and are also safer. Currently, naturally, firefly luciferase and D-luciferin produce an average wavelength of around 500 nm. However, modified firefly luciferase known as “red-shifted” has peak wavelengths of over 600 nm (Anderson et al., 2017). In addition, chemical alterations to the D-luciferin with the red-shifted firefly luciferase may increase the wavelength. By increasing the wavelength of the bioluminescence using variations of luciferases, using BLI in larger animals or deeper tissue would become more likely.

Firefly luciferase and D-luciferin naturally produce a broad band of wavelengths from 530–640 nm and with the peak wavelength at 562 nm. However, modified firefly luciferase

known as “red-shifted” has peak wavelengths of over 100 nm more, around 700 nm or more (Sadikot & Blackwell, 2005). In addition, chemical alterations to the D-luciferin with the red-shifted firefly luciferase have been shown to increase the wavelength as well. Identifying a luciferase or luciferin that will produce a higher photon yield or increasing the wavelength of the bioluminescence would possibly make using BLI in larger animals or deeper tissue possible in the future.

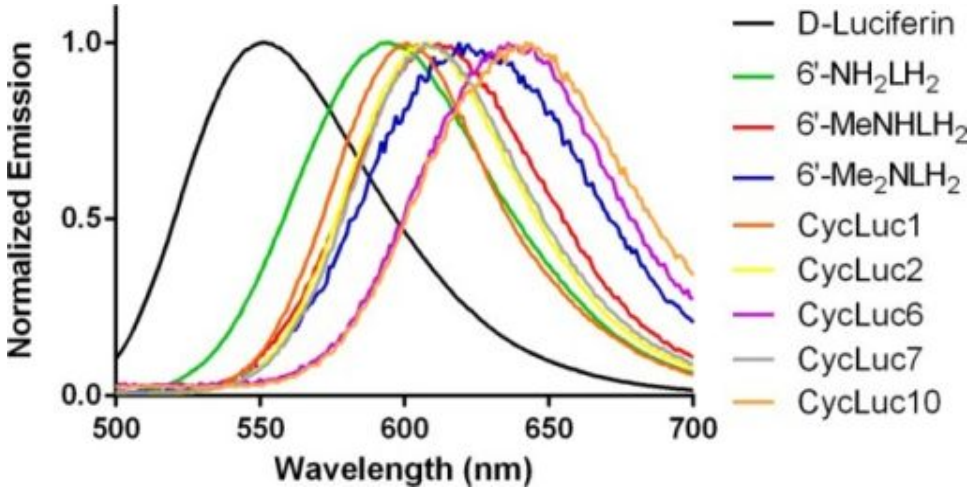


Fig. 4: As shown in the emission spectrum, D-Luciferin is used as a control to test and compare altered versions of the luciferin to produce the longest wavelength (Mofford, etal., 2014)

Fig. 4 shows the emissions spectrum of nine different luciferins and firefly luciferase. In this graph, D-luciferin, the only naturally occurring substrate in fireflies, is used as a control to compare to the eight altered luciferins. The luciferins used besides the D-luciferin were either chemically or genetically mutated to produce higher wavelengths. Fig. 4 presents an example of how changing one of the reactants, the luciferin, can increase the wavelength produced in each experiment.

While many studies exist with the purpose to produce a wavelength near infrared, the purpose of this paper was to compile and compare these studies on the different wavelengths obtained from the different luciferases used in each paper. Unlike other preexisting papers, it compares specifically firefly luciferase and click beetle luciferase to determine which will yield longer wavelengths along with the altered luciferins. By identifying the highest wavelengths produced, this paper will hypothesize theoretical luciferase and luciferin based upon previous papers, therefore adding to the preexisting knowledge of BLI. This paper will investigate the questions: Is there a correlation between luciferase and wavelength and if so, which luciferase produces the average wavelength closest to infrared, Firefly luciferase (Luc2) or Click-beetle luciferase (CBR)?

By answering this question, it may help in the future to improve the accuracy and success of BLI imaging in vivo in larger subjects. As previously mentioned in this paper, longer wavelengths are less harmful to live cells and can penetrate deeper through tissue than shorter wavelengths can. Identifying and isolating which luciferase will produce the longest wavelength can help with future research by reducing tissue absorption and making visualizing cells more successful through improving the quantitative and qualitative accuracy of BLI. This can help to produce a cheap, reliable, accessible, and noninvasive way to quickly and accurately report activity on the cellular level, possibly to track fatal diseases such as cancer tumors from early stages.

Purpose:

The purpose of this study was to identify the optimal luciferase that would yield the longest wavelength by comparing a type of firefly luciferase (Luc2 or Fluc) and a type of red shifted click beetle luciferase (CBR) since longer wavelengths reduce photon absorption during in vivo use and are safer to the body than smaller, higher energy wavelengths. Although there are many pre-existing papers individually studying click beetle luciferase or firefly luciferase, not many existing papers compare the two luciferases together. In addition, although many papers acknowledge that longer wavelength lowers the amount of tissue absorption of photons, many papers focus on altering luciferins to increase wavelength without focusing on which luciferase is used. The purpose of the meta-data analysis in this paper was able to identify the connection between luciferase and wavelength to contribute further to the pre-existing knowledge on firefly and click beetle luciferase and determine which luciferse should be used in further research in vivo.

Alternative Hypothesis:

There is a correlation between wavelength and luciferase and click beetle luciferase produces an averagely longer wavelength when compared to firefly luciferase, reducing harm to surrounding tissue and tissue absorption to improve accuracy on BLI use in vivo.

Null Hypothesis:

Neither luciferase produces a longer average wavelength than the other or there is no significant correlation between which luciferase used in the experiment and the wavelength that is produced.

Methods:

This study was completed using numerical data from various academic peer-reviewed papers that have been published within the last decade, from 2009 to 2019, in order to ensure the information used was relevant to current research on the topic. Utilizing systematic literature review and meta data analysis, all sources of data were obtained from the electronic databases of ScienceDirect, NCBI, EbscoHost, Google Scholar, ResearchGate, Nature, and PubMed. The search terms and keywords used were bioluminescence imaging, firefly luciferase, FLuc, Luc2, click beetle luciferase, click beetle red, CBR, in vivo, wavelength, and near infrared to find academic peer-reviewed articles on different bioluminescence imaging combinations that had data on the wavelengths.

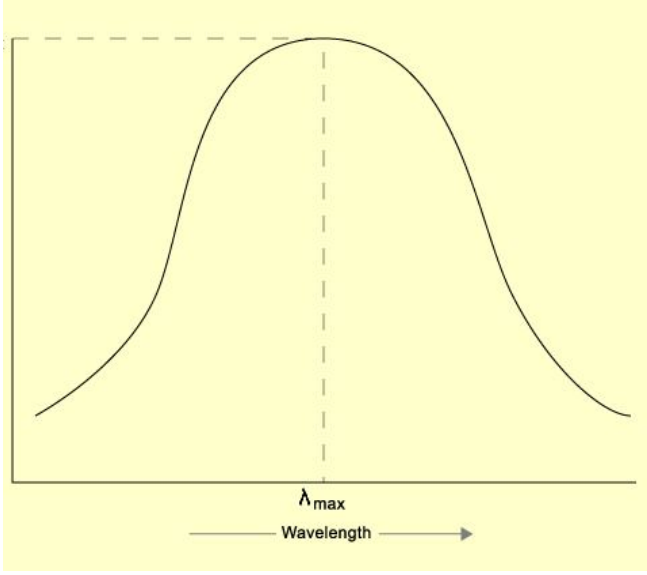


Fig. 5: the value of λmax is pictured as the x-value of the peak of the normal curve

The only luciferases used in this study were a type of commonly firefly luciferase and a type of click beetle luciferase due to the naturally high wavelength yielding nature of the two luciferases and to also limit the

variables of this research, despite other additional luciferases being used in other current research on BLI. The type of firefly luciferase used in this research was a type that is commonly used for BLI known as Luc2 and Fluc. The click beetle luciferase used in this research was a type of red-light-yielding click beetle luciferase that has become increasingly popular for BLI known as click beetle red or CBR. All wavelengths collected were quantitative values measured by nanometers containing three significant figures and were compared using the wavelength emitted at peak photon emission also known as lambda maximum (λmax). Since a single trial produces a range of wavelengths visualized in a normal curve, as seen in Fig. 5, λmax is the wavelength that is produced at the point of highest photon emission, or peak emission. This research only took into consideration the λmax values produced in each of the experiments of the papers for each luciferase. In addition, all wavelengths were from in vivo tests and were tested in mice to reduce the variables. Luc2 and CBR luciferase tested in vitro often produced longer wavelengths than in vivo testing. Since using BLI to tracking cancer cells would be in vivo and the need to produce longer wavelengths is to reduce tissue absorption is in current vivo tests, the wavelengths studied in this paper were from experiments in vivo.

The conclusion in this research identified which luciferase, Luc2 or CBR, produced the highest average wavelength to be used for in vivo research. The conclusion made in this paper was based upon the data from previous papers and therefore was theoretical. To find the average wavelength of each luciferase, the sum of the wavelength values found were divided by the total number of wavelengths, producing two mean wavelength values, one for each luciferase.

After finding the average wavelengths of the luciferases, a two-tailed, two variable t-test was conducted using Excel on the wavelengths in order to test how strong the correlation

between the wavelength produced and the luciferase used. All wavelength values were input into a spreadsheet with separate columns representing each luciferase. This test determined that the difference in average wavelength of each luciferase was significant and not due to random chance and proved there was a correlation between wavelength and luciferase used. This confirmed that the average wavelengths found in this paper could be used as legitimate values to test whether one luciferase over the other produced a longer average wavelength.

Results:

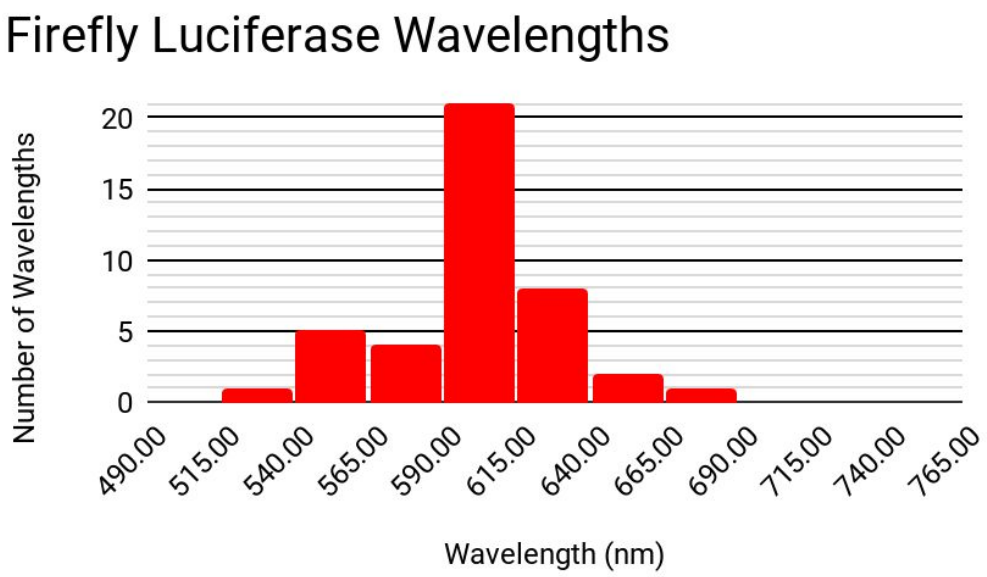
Table 1: wavelengths of Luc2 and CBR collected from various academic peer-reviewed papers measured in nanometers

Firefly Luciferase, Luc2, FLuc Wavelength (nm)	Click Beetle Luciferase, CBR Wavelength (nm)
578	618
612	618
560	614
591	614
603	617
603	664
607	730
551	728
594	758
610	743
619	618
601	618
609	620
598	615
607	618
635	615
636	620

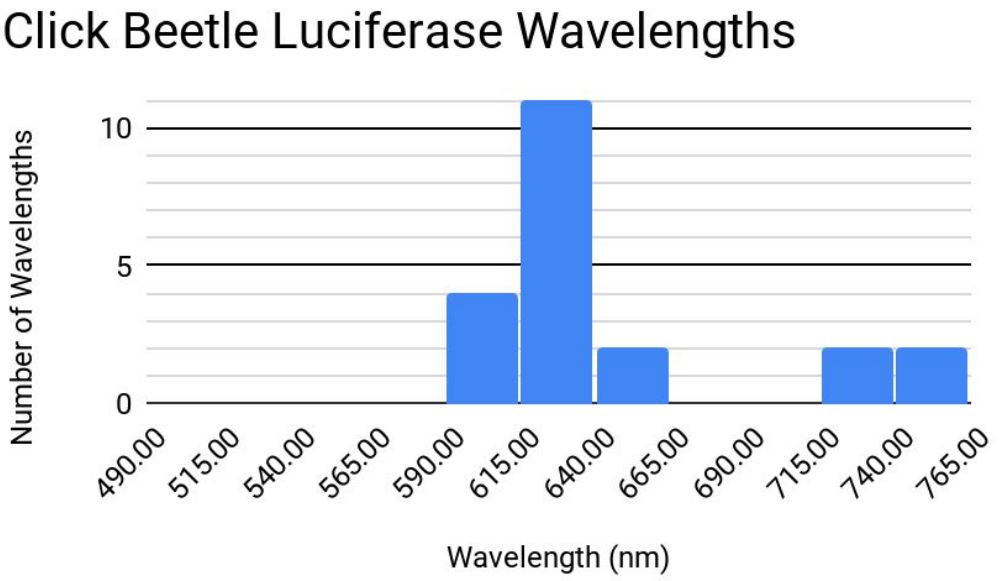
Luc2 Wavelength (nm) con.	CBR Wavelength (nm) con.
607	640
610	615
627	613
642	600
631	
625	
538	
600	
650	
559	
608	
678	
610	
580	
600	
612	
610	
617	
620	
612	
578	
560	
580	
600	
562	
$\bar{x}$=603.0952381	$\bar{x}$=642.6666667
Standard Deviation=27.68887205	Standard Deviation=50.05430384

Fig. 5: histograms of wavelengths found of Fluc (A) and CBR (B)

A.



B.



The average value calculated as a mean of all wavelengths collected for the firefly luciferase was 603.10 nm compared to the click beetle luciferase at 642.67 nm. The $\bar{x}$ Luc2 subtracted by the $\bar{x}$ CBR produces a difference of -39.6 nm.

The histograms in Fig. 5 of both luciferases were graphed on the same x-axis to show the comparison between the averages of each luciferase. The x-axis depicts the wavelength measured in nanometers and it increases from left to right. It depicts the wavelengths between the measurement of 490.00 nm and 765.00 nm. The histogram is split into sections of 25 nm and the number of wavelengths within the span of the 25 nm were counted on the y-axis. The n for Histogram A is 42. The n for Histogram B is 21.

Statistical Analysis:

The standard deviation for the firefly luciferase was 27.69 and the standard deviation of the click beetle luciferase was 50.05, as seen at the bottom of Table 1. The p-value calculated by the two-tailed two variable t-test was 0.000147.

Discussion:

The average wavelength of the click beetle luciferase is longer than the average wavelength of Luc2 by a difference of 39.6 nm. In addition, the range for the p-value that this data was compared to was a value of 0.01. The p-value obtained of 0.000147 was below 0.01 giving an over 99 percent confidence that a strong correlation between wavelength and the type of luciferase used exists (Table 1). Therefore, the null hypothesis that there is no correlation between luciferase and wavelengths can be rejected. The alternative hypothesis that there is a

correlation between the two can then be accepted. The data supports the hypothesis that CBR has an average longer wavelength than Luc2 and should therefore be used for in vivo imaging.

The majority of the wavelengths used in this paper when using Luc2 were in the range of 590 nm to 615 nm (Fig. 5, A). The n of the range equalled twenty-one, meaning twenty-one experiments out of forty-two experiments that used Luc2 produced a wavelength at λmax that was within 590 nm to 615 nm. This observation agrees with the average wavelength found when using Luc2 which was 603.10 nm, since it lies within the range of 590 nm to 615 nm (Table 1). In comparison, the histogram of wavelengths produced using CBR had the highest number of wavelengths in the range of 615 nm to 640 nm, with the n in the range equalling eleven out of a total n of twenty-one (Fig. 5, B). Out of twenty-one experiments using CBR luciferase, eleven produced wavelengths at λmax within the range of 615 nm to 640 nm. Unlike Luc2, however, the mean value of CBR produced wavelengths was not located within the range of 615 nm to 640 nm and had a value of 642.67 nm, higher than the peak range on the histogram (Table 1). This may have been due to the distribution of wavelengths in the histogram for the Luc2 was a relatively normal distribution, while the distribution for the CBR luciferase was skewed to the left. The distribution of CBR had a more data in a smaller range on the left of the x-axis range than on the right so the actual mean wavelength was expected to be higher than the average value estimated by the histogram.

Limitations:

According to the conclusions of this paper, CBR would be a more accurate luciferase to use instead of Luc2. However, as mentioned before, this conclusion is only theoretical and based

upon wavelength. In addition, there are many factors not directly studied in this report besides luciferase and wavelength that affect the accuracy of BLI when used in vivo. Luciferin, photon yield, temperature, depth of tissue, and density of tissue are a few of the variables that affect the accuracy of BLI when used in vivo. However, since this report only focused on luciferase and wavelength, these variables were not taken into account. The conclusion that CBR is a more accurate luciferase, as a result, is only developed by the two variables in this paper and may not be true when other variables are taken into account.

Due to the large amount of research existing using FLuc or Luc2 and a lower amount of research on CBR, the results of this meta-data analysis contained about twice as much data on Luc2 then on CBR which also may have affected the results. While a two variable two-tailed t-test was performed to ensure the values could be applied to create a conclusion, the lesser amount current research on CBR still may have skewed the results due to a small n value, as seen in the histogram of the CBR wavelengths where the distribution is skewed right.

Conclusion and Further Work:

Overall, this study concludes that click beetle luciferase in comparison to firefly luciferase yields longer wavelengths. This conclusion leads to the belief that future research should focus more on click beetle luciferase for in vivo use rather than firefly luciferase in order to decrease the photon absorption of surrounding tissues and reduce damage to the cells surrounding the area of interest. Much of the current research is conducted using firefly luciferase because it naturally has a high wavelength and was easily attainable. However, this paper has determined that CBR should be used instead because it has a longer average

wavelength than firefly luciferase. Conducting experiments using longer wavelength in vivo would be able to increase quantitative and qualitative accuracy due to less photon absorption by the surrounding tissues making the research more accurate on all fronts and therefore more reliable. In addition, it may make using BLI in larger subjects a possibility. Due to the decrease in photon absorption, the wavelength would be able to travel through deeper tissue without getting absorbed as easily. If it were to be used in humans for example, diseases such as breast cancer would be able to be tracked easier, safer, faster, and for a smaller cost than the methods currently used. My research has concluded that using CBR as opposed to the commonly used Luc2 would accomplish this and help future research. CBR in terms of wavelength presents a promising resource in the effort to improve BLI to be able to be used in humans. However, since there is much less current research using CBR, more focus on this luciferase than the existing research would be required. In addition, luciferase and wavelength only play a small role in the success and accuracy of BLI. Therefore, moving forward, if more research is completed on examining the relationship between factors such as temperature, luciferin, and photon yield, BLI has the potential become an extremely useful tool in helping to save lives.

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