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Article

Characterizing quorum sensing-induced bioluminescence in variable volumes with *Vibrio fischeri* using computer processing methods

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SUMMARY

Bacteria use the mechanism of quorum sensing to gather information about the density of other cells in their surroundings. Quorum sensing, in which bacteria sense chemical signals that increase in concentration as a function of surrounding cell density, leads to downstream changes in bacterial gene expression. These changes may include the activation of virulence or bioluminescence. In this study, we used *Vibrio fischeri* as a model to study the activation of bioluminescence by quorum sensing. We inoculated the same number of bacterial cells in 3 different culture volumes (1mL or small volume, 3mL or medium volume, and 5mL or large volume) and measured luminescence of the cultures over time. Using digital image processing to determine changes in luminescence over time, we found that cultures in smaller volumes reached peak illumination densities in less time than those in larger volumes. These results suggest that *V. fischeri* luminesce in response to cell density information in the environment and that we can use computer processing methods to study quorum sensing-induced bioluminescence. Studying the mechanisms by which bacteria use quorum sensing to activate gene expression may facilitate the development of therapies to inhibit bacterial virulence in the context of infectious disease.

INTRODUCTION

Cell-to-cell communication that is used to share information about bacterial cell density in the surrounding environment is called quorum sensing (1). Bacterial cells release autoinducers, which are chemical signaling molecules that other bacterial cells sense and use to collect information about cell density in the environment. Based on the acquired cell density information, bacteria behave collectively in a synchronous manner to regulate gene expression, which results in the activation of specific traits such as virulence or bioluminescence. Physiological activities that are regulated by quorum sensing include symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation (2).

Vibrio fischeri is a Gram-negative marine organism that was initially used to investigate quorum sensing (3). When population density reaches a certain threshold *V. fischeri* activate bioluminescence through a concerted response process (4). In the quorum sensing cycle of *V. fischeri* (**Figure 1**) the main responsible genes are luxR and luxI (5). The luxI gene codes for an autoinducer synthase that synthesizes the acyl-HSL autoinducer (6). The luxR gene encodes for a LuxR protein that recognizes autoinducers released into the environment by other bacterial cells (5, 7). The lux operon is an inducible operon that is comprised of genes that are responsible for bioluminescence. Once the autoinducer is bound to the LuxR protein, the complex activates the lux operon, luxCDABE (6, 8). luxA and luxB encode for subunits of luciferase, which catalyzes the bioluminescence reaction (5). luxCDE encode for a fatty acid reductase, which synthesizes the aldehyde substrate. The luciferase enzyme is used to catalyze the reaction of the aldehyde substrate, diatomic oxygen, and flavin mononucleotide to trigger bioluminescence (9). The binding process of the autoinducers and luxR results in a positive-feedback mechanism that further increases the concentration by inducing the transcription of the cognate luxI signal synthase gene (8).

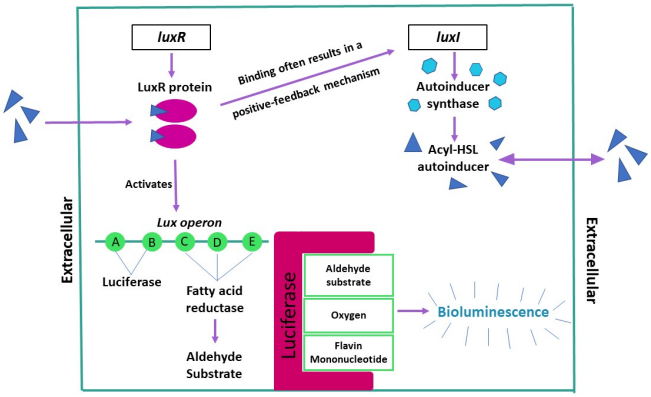

Figure 1. A schematic of the quorum sensing cycle in *V. fischeri*.

The activation of the bioluminescence in *V. fischeri* is especially important to the bobtail squid, a nocturnal creature that uses the light emitted from the bacteria to hide from predators. Studies have also shown that quorum sensing can be triggered in other species of bacteria even for a single cell confined in a micro droplet as a result of the accumulation of signaling molecules produced by the confined cell (10).

Quorum sensing depends on cell density, defined as the total number of cells per milliliter. We hypothesized that quorum sensing is triggered much faster when bacteria is cultured in smaller volumes than when cultured in larger volumes of media. In this study we validated this hypothesis by first showing that quorum sensing can be activated at

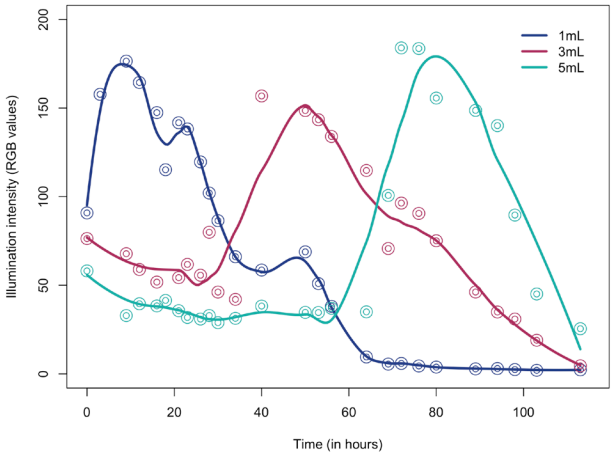

Figure 2. Progression of the 1mL, 3mL, and 5mL cultures toward maximum illumination. After peaking at maximum RGB intensity, bioluminescence gradually declined toward an RGB value of 0. Each point represents the mean illumination intensity of multiple images taken at the same time. The curve was developed by the 'loess' function in R which locally fitted illumination data against time in hours.

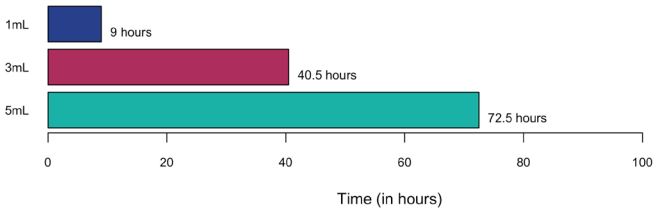

Figure 3. Bar plot of the time that each volume took to reach maximum illumination. Greater volumes took significantly more time to reach their peak intensity than smaller volumes.

quorum sensing using computer processing of high-quality digital images. Finally, we characterized the variation in the quorum sensing lifecycle of *V. fischeri* in small, medium, and large volumes. We showed that, with the same starting number of cultured cells, higher densities of cells were attained more quickly in smaller culture volumes than in larger culture volumes. This study proved the dependency of quorum sensing on cell density by using computer processing techniques on captured digital images.

RESULTS

To investigate the effect of quorum sensing in *V. fischeri*, cultured in variable volumes, we inoculated the same number of bacterial cells in 3 different culture volumes (1mL or small volume, 3mL or medium volume, and 5mL or large volume) and measured luminescence of the cultures over time. RGB color values were utilized to characterize illumination levels of digital images taken for *V. fischeri* cultures. Values were averaged for all pixels of an image and were shown to precisely evaluate illumination levels across images.

Volume (mL)	Average illumination (RGB intensities)		Time to peak
	At starting time	At peak time	illumination (hours)
1	90.8	176.5	9.0
3	76.4	156.8	40.5
5	58.1	183.9	72.5

Table 1. Average illumination at starting and peak time for all three volumes expressed as RGB pixel intensities. Time intervals in hours from starting to peak time are shown.

Volume (mL)	Avg. final illumination (RGB intensities)	Time from peak to loss of illumination (hours)
1	4.5	67.5
3	4.5	73.0
5	25.5	41.0

Table 2. Average illumination at final time for all three volumes expressed as RGB pixel intensities. Time intervals in hours from peak to loss of illumination are shown.

Time (hrs.)	Trend	Slope	Std Error	*P* Value
1mL				
0 - 9	Upward	8.603	1.371	< 0.001
9 - 69	Downward	-2.609	0.091	< 0.001
3mL				
30 - 40	Upward	11.702	1.393	< 0.001
> 40	Downward	-2.335	0.103	< 0.001
5mL				
64 - 72	Upward	18.306	3.913	< 0.001
> 72	Downward	-4.418	0.341	< 0.001

Table 3. Slope estimates of illumination intensity on time in hours. Slopes were estimated for volumes 1, 3 and 5 mL from the time of quorum sensing activation to peak illumination and from the time of peak illumination to loss of illumination.

Full quorum sensing cycles of cell culture volumes of 1, 3, and 5 mL are shown in **Figure 2**. Because higher cell densities were attained earlier in smaller volumes, the starting and peaking times for quorum sensing were different for the three volumes studied. In smaller volumes, quorum sensing was activated and peaked at a faster rate than in larger volumes. The average times to reach peak illumination were 9, 40.5, and 72.5 hours for volumes 1, 3, and 5 mL, respectively (**Figure 3**). The RGB values of the volumes were low at starting time and reached their highest at peak time (**Table 1**). To characterize illumination after its peak for each of the three volumes, the average time between peak and loss of illumination and average RGB values at final illumination were calculated. Final illumination of the 5 mL volume averaged higher than other volumes because of its shorter time span between peak and final illumination (**Table 2**).

We studied the rate of illumination progress toward the peak versus the rate of illumination decline after the peak. The rate of the progress toward the peak was higher than the rate of decline after the peak in all volumes (**Figure 2**). We estimated slopes of illumination intensity over time for volumes 1, 3 and 5 mL during the intervals from the activation of quorum sensing to peak illumination (upward trend) and from the time of peak illumination to total loss of illumination (downward trend). These slope estimates showed that the

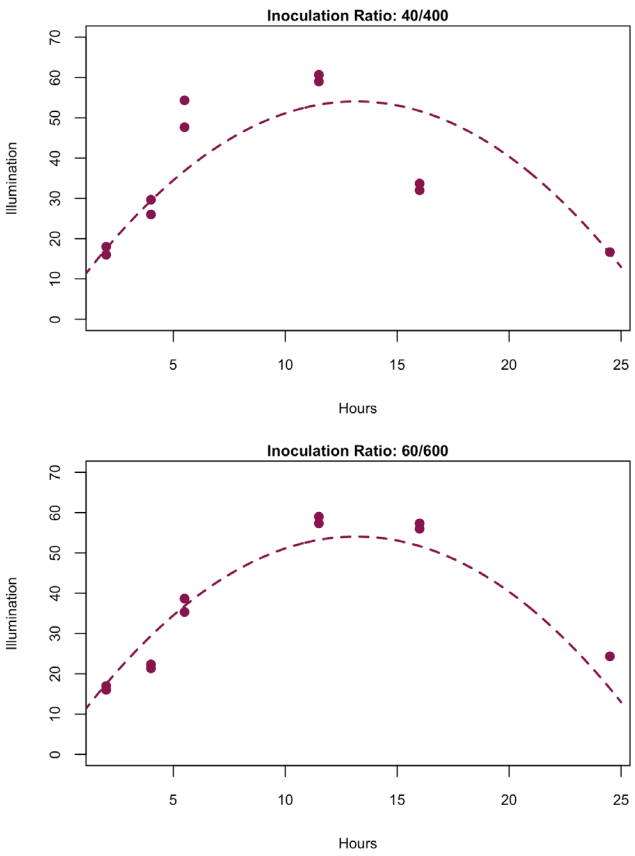

Figure 4. Plot of pixel intensities of each of 2 images taken at each time for the 2 sets of vials with proportional inoculation. Curves shown modeled the quadratic trend of quorum sensing using a 2nd degree equation estimated from data. Maximum illumination occurred at the same time (11.5 hours) in the two volumes because of their equal starting cell densities.

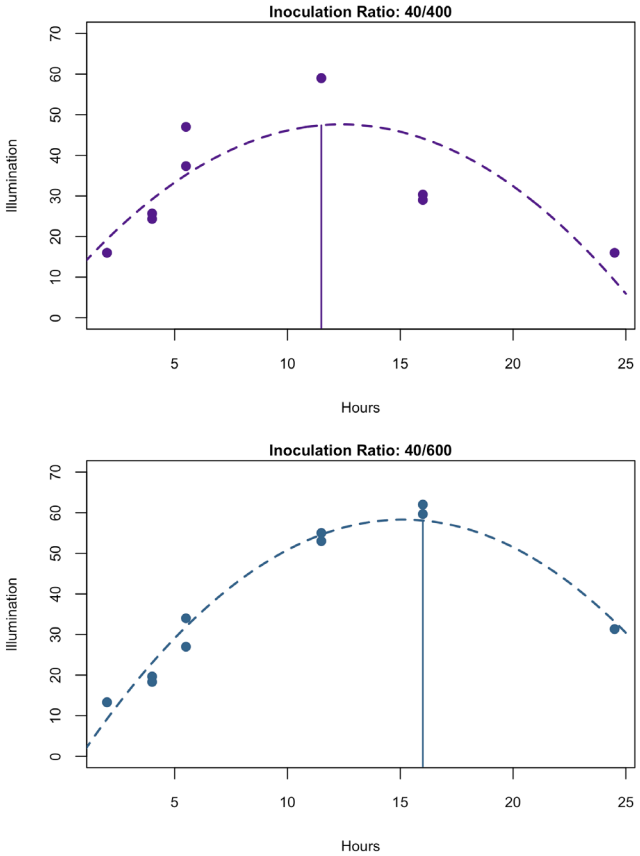

Figure 5. Plot of pixel intensities of each of 2 images taken at each time for the 2 sets of vials with fixed inoculation amount in small and large volumes of media. Curves shown modeled the quadratic trend of quorum sensing using a 2nd degree equation estimated from data. Maximum illumination occurred 4.5 hours earlier in the small than in the larger volume of media.

rate in the upward trend was 3 to 5 times greater than the absolute rate in the downward trend. *P* values were less than 0.001, indicating that slope estimates were significantly different from 0 (**Table 3**).

As explained earlier (**Figure 1**), the binding process of the acyl-HSL autoinducers and LuxR results in a positive-feedback mechanism that further increases the concentration of autoinducers. Results in **Table 3** of the slopes agreed with the positive feed-back mechanism which causes quorum sensing to accelerate at a higher rate toward peak illumination.

As expected, when the starting cell densities in small and large volumes of media were the same due to proportional inoculation, quorum sensing was triggered simultaneously. However, when the starting cell densities were different due to fixed inoculation in variable volumes, quorum sensing was triggered earlier in the media with greater starting cell density (**Figure 4**). Maximum illumination occurred after 11.5 hours in both volumes because of the equal starting cell densities (**Figure 4**). On the other hand, an earlier illumination could be found for the smaller volume after 11.5 hours versus 16 hours for the greater volume (**Figure 5**). The smaller volume peaked earlier as a result of its higher starting cell densities.

Based on these results, we chose to keep the starting amount of culture constant, while varying volumes. Throughout the study smaller volumes implied higher starting cell densities, with the hypothesis of reaching the required threshold for quorum sensing earlier than larger volumes.

DISCUSSION

By varying volumes of growth media while keeping the amount of the starting culture fixed, we demonstrated that quorum sensing was triggered earlier in smaller volumes when compared to larger volumes. *V. fischeri* cultures in smaller volumes reached their maximum illumination intensity in shorter periods of time than in larger volumes, as a result of the higher starting cell density and the faster progress toward the density threshold required to trigger quorum sensing. Moreover, in all volumes, upward trends toward peak illumination were found to display an accelerated rate when compared against downward trends, which was a common feature in quorum sensing cycles of all volumes studied. This could have been the result of the positive-feedback mechanism, however, there could be other explanations for these results, for example, the bioluminescence state might be very stable once it is triggered, and it requires more time before it turns off.

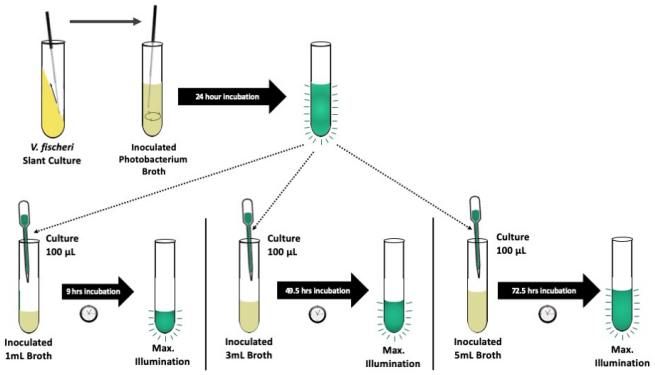

Figure 6. Experimental design. First, *V. fischeri* slant culture was used to prepare a stock of bacteria. Second, 3 different volumes of photobacterium broth were inoculated from the stock and incubated for 80 hours.

Figure 7. An example cropped image displayed to show how its pixel intensities were calculated and analyzed in R.

In this study, we developed a method to characterize quorum sensing in *V. fischeri* using RGB illumination intensity values of pixels in digital images. The RGB approach was shown to be precise in quantifying illumination based only on a high-resolution camera and image processing software. While it is common to use a luminometer to measure bacterial bioluminescence (e.g., 11), we introduce a simpler and more informative approach of gathering illumination data from digital images.

Studying the mechanisms by which bacteria use quorum sensing to activate gene expression may facilitate the development of therapies to inhibit virulence in bacterial strains in the setting of infectious disease.

Although *V. fischeri* is not disease-causing bacteria, it serves as an experimental model for studying and controlling quorum sensing. Methods to deactivate quorum sensing in *V. fischeri* can be tested while illumination levels are more efficiently tracked using image processing approaches introduced here. This makes it easier to then transfer quorum sensing inhibitors studied to disease-causing strains of bacteria at a later research phase (12, 13).

MATERIALS AND METHODS

Broth Preparation

Gram-negative *V. fischeri* and dehydrated photobacterium broth were purchased from Carolina Biological Supply Company. To prepare the photobacterium broth, 15.2 mL of distilled water were added to every gram of dehydrated photobacterium broth. The broth was mixed thoroughly to ensure that all of the solute was dissolved to achieve a hydrated photobacterium broth with a concentration of 65.8 mg/mL. The prepared broth was then sterilized by placing it into a pressure autoclave for 50 minutes.

Culture Preparation

Living *V. fischeri* bacteria cultured on photobacterium agar at 25°C in a tube were obtained from Carolina Biological Supply Company. Sterilized broth was then pipetted in test tubes which were inoculated with the living *V. fischeri* bacteria using a sterilized inoculating loop. The test tubes were incubated at 25°C for 24 hours on an orbital shaker with 200 rotations per minute. The purpose was to prepare liquid bacterial cultures to use in subsequent experimental steps, which required precise concentrations of bacterial cultures to inoculate broth in tubes with variable volumes.

Experimental design

One objective of the current study was to use variable volumes to validate the dependency of quorum sensing on cell density. In a smaller volume, less time is needed for autoinducers to reach the threshold required for bioluminescence, while in a larger volume, more time is needed for autoinducers to reach the same threshold. Volumes 1, 3, and 5 mL were chosen throughout the experiment to represent small, medium, and large culture volumes. Smaller culture volumes were expected to reach the autoinducer density threshold of bioluminescence faster than larger volumes, as volume and density are indirectly related, where cell density = total number of cells/volume.

To measure time to bioluminescence per volume, 2 test tubes per volume were prepared for a total of 6 tubes of sterile photobacterium broth. Each of the six test tubes was inoculated with 100 µL of the liquid bacterial culture prepared earlier (**Figure 6**). All test tubes were incubated in a dark chamber with no external light source at 25°C on an orbital shaker set to 200 rpm. At the end of each experiment, live cultures were disposed of properly after bleaching all test tubes.

Acquisition of digital images

High resolution images were taken using a Canon EOS Rebel T6 camera with a 30-second exposure at ISO 3200. All images were taken with no zoom using the same settings and placement for both the camera and test tubes. High resolution still digital images were taken for the bacteria in triplicate in a completely dark chamber every 1-2 hours to monitor the progression of bioluminescence levels for a total of 80 hours. The 80-hours monitoring period was the time necessary for all three volumes to go through the full bioluminescence cycle

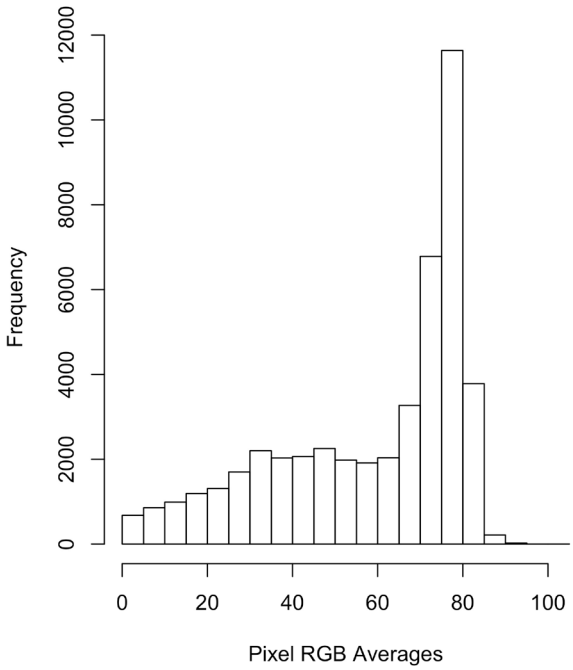

Figure 8. Bar plot of pixel intensities of an example image. The bar plot shows a high frequency for pixels with intensities ranging from 65 to 85 in their average RGB values.

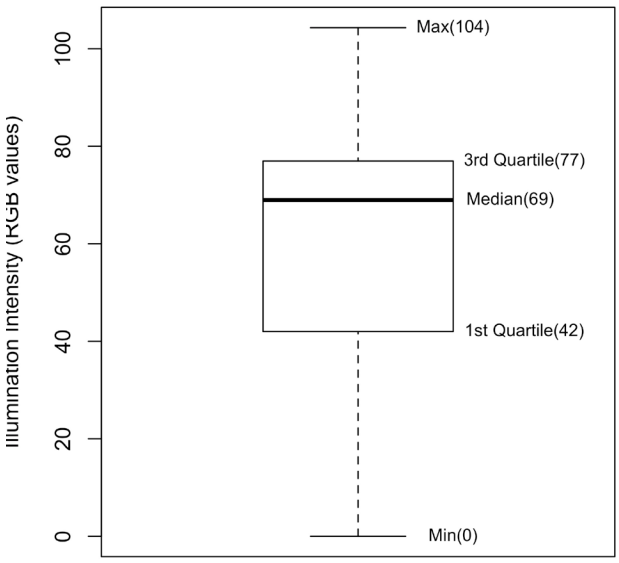

Figure 9. Box plot of pixel intensities of an example image. The box plot shows RGB range of values from 0 (completely dark) to a maximum of 104 (fairly luminescent). Summary statistics are also labeled on the box plot.

of reaching peak illumination and gradually dimming down to zero light emission.

Detection and measurement of illumination intensity in digital images

Images were converted into quantities amenable for statistical analysis by averaging the R, G, and B values of each pixel (**Figure 7**). The R, G, and B values of each pixel in the image were averaged to quantify pixel intensities. To

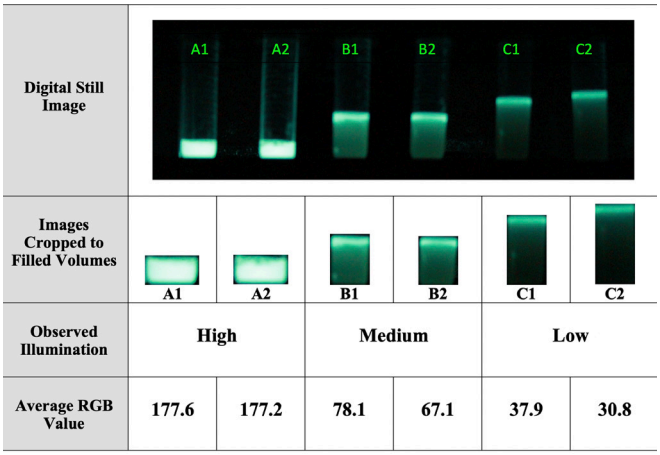

Figure 10. Test tubes with *V. fischeri* illuminating at different intensities. Corresponding average pixel RGB values of the filled volumes are shown.

study image illumination further, a histogram of pixel intensities was created (**Figure 8**). The histogram shows pixel intensities, ranging from 65 to 85, to be of the highest frequency in the image. Finally, a boxplot of pixel intensities was developed and labeled to show the summary statistics of pixel intensities in the image (**Figure 9**). The boxplot shows the minimum and maximum values as well as the mean and the median; it also displays the 1st and 3rd quartiles of pixel intensities in the image. The R code used to read images and transform them into RGB data is available online (14).

Digital still images were taken for test tubes with *V. fischeri* grown in photobacterium medium while illuminating. Images of 6 test tubes at specific illumination levels were taken. The objective was to interpret colors in images taken into meaningful values to differentiate between high and low illumination intensities. To achieve this objective, the RGB color values of each pixel in the images were measured as shown above. The RGB color values quantify the combination of Red, Green, and Blue colors, where their average associates directly with the intensity of light emitting from each pixel in the image. The average was preferred to the median because of its sensitivity towards extremely low- or high-illumination pixels.

The average RGB values of tubes A1 and A2 were near 177, of tubes B1 and B2 were 67 to 78, and of tubes C1 and C2 were 31 to 38 (**Figure 10**). Average pixel RGB values of the filled volumes in the tubes were shown to correctly identify the level of illumination intensity observed. Therefore, average pixel RGB values were used throughout this experiment as a measure for light emission.

Proportional versus fixed starting culture

To decide between using fixed or proportional inoculum, a short study was performed to validate the hypothesis that smaller volumes reach higher cell densities faster than larger volumes when the amount of the starting culture is the same. The impact of proportional versus fixed starting

culture volumes was compared in two pilot trials. In the first trial, 40 and 60 µL of starting culture were used to inoculate 400 and 600 µL of sterile media in vials, respectively. This proportional inoculation was performed to have the same starting cell densities in small and large volumes of media. In the second, a fixed amount of 40 µL of starting culture was used to inoculate another set of 400 and 600 µL of sterile media. The fixed-volume inoculum in two different volumes was performed to have variable starting cell densities. Two vials of each combination of inoculum and media volumes were used with images taken over a period of 24 hours.

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