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Article

Fluorescein or Green Fluorescent Protein: Is it possible to create a sensor for dehydration?

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SUMMARY

Dehydration occurs when more fluid leaves the body than enters it. Dehydration can cause symptoms ranging from headaches and dizziness, to more severe symptoms like fever and unconsciousness. Currently there is no early dehydration detection system using temperature and pH as indicators. A sensor could alert the wearer and others of low hydration levels, which would normally be difficult to catch prior to more serious complications resulting from dehydration. The temperature and pH of skin are known to increase and decrease, respectively, with dehydration. These variables are also known to affect the fluorescence of certain fluorophores, which could provide a visible marker of dehydration. In this study, a protein fluorophore, green fluorescent protein (GFP), and a chemical fluorophore, fluorescein, were tested for a change in fluorescence in response to increased temperature or decreased pH. Neither fluorophore was affected by the changes in temperature. However, both lost their fluorescence when the pH of their environment was decreased. We also tested whether the fluorescence returned to its standard brightness when the temperature and pH were normalized. Reversing the pH change did not restore GFP fluorescence, but that of fluorescein was re-established. This finding suggests that fluorescein could be used as a reusable sensor for a dehydration-related pH change.

INTRODUCTION

Dehydration is when more fluid exits the body than enters it. This is a problem because water helps break down nutrients and move waste out of cells, making it essential for health and bodily function. (1, 2). Dehydration can cause symptoms ranging from headaches and lethargy, to fever and unconsciousness (1). Excessive sweating is one of the causes of dehydration, and can be triggered by both exercise and heat (3). For example, football players sweat not only from their many layers of protective equipment, but also as a result of thermoregulation during exercise, a process that maintains the body's core internal temperature. As a consequence, football players sweat 1.5 L/hr, an increase of 2.5% compared to people who do not regularly play sports (4, 5). Increased sweating makes football players more prone to dehydration if they do not replace the water lost through sweat. However, non-athletes can also become dehydrated if they do not consistently drink water throughout the day, resulting in a net loss of fluid. The purpose of this study is to address dehydration in athletes and non-athletes by making a sensor that alerts the dehydrated person to dangerously low fluid levels.

A sensor for dehydration could measure either pH or temperature of the skin to assess whether the person is dehydrated. Normal body temperature is 37 °C and rises to 39.5 °C during exercise (6). The maximum endurable body temperature is 41.1°C (6). An increase in temperature can be used as an indicator for dehydration because as body temperature increases, sweat production increases, which causes dehydration. The pH of skin can also be used as a measure of dehydration. The normal pH of human skin is 6, but sweat causes the pH to become more acidic. During light exercise, skin pH is 5 and further drops to pH 4 during heavy exercise (7). The minimum pH of skin is 3 (7). Acidic skin pH indicates excessive sweating, which can lead to dehydration. In order to monitor hydration levels using temperature and pH, a method must be developed to detect changes in these variables from the physiological ranges mentioned above.

One possible method to detect these changes is by using fluorophores. Fluorophores fluoresce light when their electrons are excited by other light sources. For the fluorescence to be seen, the emitted light must be of a very specific wavelength. One example of a fluorophore is green fluorescent protein (GFP). GFP is a protein that exhibits green fluorescence and comes from the jellyfish Aequorea victoria (8).

Protein fluorophores, like GFP, are known to be highly sensitive to changes in pH and temperature. Proteins rely on electromagnetic bonds between oppositely charged amino acids to help keep their tertiary structure. As the pH of the surrounding environment decreases, the concentration of H+ ions increases (9). Increasing the H+ ions can protonate negatively charged amino acids, thereby disrupting the electromagnetic bonds and causing a conformational change in the protein (9). This conformational change can affect the distribution of electrons, resulting in a loss of fluorescence (9). Temperature changes can also cause a conformational change in GFP. Increased temperature introduces extra energy into the protein. Extra energy can cause the bonds within the protein to vibrate and break, resulting in a change in protein shape and loss of fluorescence (10).

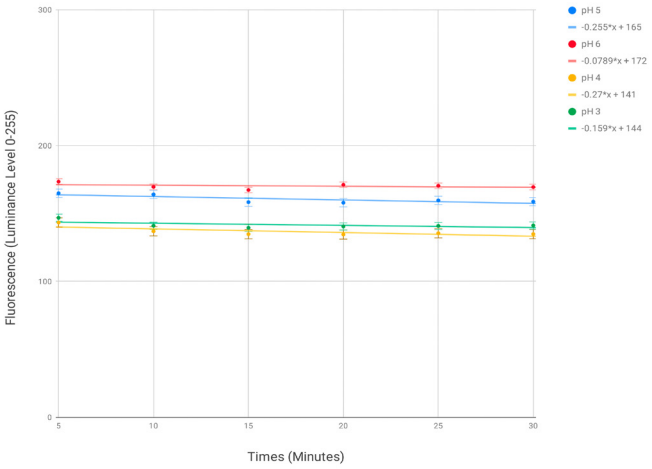

Figure 1: Fluorescence of GFP over time with pH change. This graph shows the change in the fluorescence of GFP when it was held at various pHs measured at intervals of 5 minutes for 30 minutes. GFP fluorescence decreases with decreasing pH, as depicted by the lines with a negative slope for pHs 3 through 5. There were three trials for every pH level, including pH 6, every 5 minutes for 30 minutes, and the averages of the trials were used for the six data points. The error bars represent the standard deviation of the mean of all three trials, showing how much the data varied at each point.

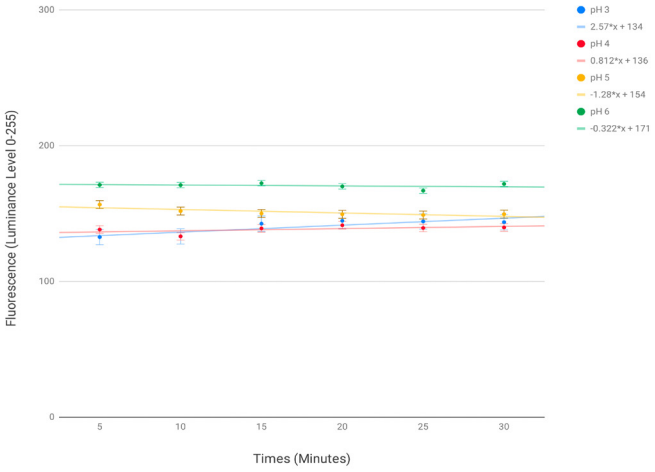

Figure 2: Fluorescence of GFP over time after return to pH 6. GFP fluorescence does not change with increasing pH, as depicted by the horizontal lines for pHs 3 through 5. There were three trials for every pH level, including pH 6, every 5 minutes for 30 minutes, and the averages of the trials were used for the six data points. The error bars represent the standard deviation of the mean of all three trials, showing how much the data varied at each point.

Fluorophores can also be structures other than proteins. Fluorescein is a chemical fluorophore that is used as a synthetic coloring agent and fluoresces yellow-green (11). Fluorescein is less susceptible to a conformational change compared to protein fluorophores, but it still has the potential to be affected by pH and temperature. A decrease in pH may also cause protonation of a chemical similarly to proteins. If fluorescein were to be protonated, the bonds and electron configuration within the molecule would change, thus reducing the fluorescence (12). An increase in temperature could also cause fluorescein to lose its fluorescence. If temperature is increased, the electron configuration of fluorescein could change independently of light input. Changing the electron configuration could affect the chemical's ability to emit light and be interpreted as a loss of fluorescence.

Since both GFP and fluorescein fluorescence are susceptible to changes in pH and temperature within the physiological range, this study aims to measure changes in fluorescence in response to hydration levels. The findings could be used to develop a sensor for dehydration, as change in fluorescent intensity could indicate dehydration. A sensor for dehydration could not only help people live healthier, but could also save lives.

RESULTS

This study used fluorescein and GFP as sensors for dehydration. We tested whether temperature or pH affected the fluorescent intensity of either substance.

After collecting fluorescein and GFP, we put both in tubes and changed their pH and temperature to the experimental groups. The tubes were photographed for 30 minutes. After 30 minutes, the tubes returned to their original temperature and pH and their recovery was photographed for 30 minutes.

The results of this experiment were analyzed using ImageJ to quantify fluorescence.

Fluorescence of GFP Changes with Decrease in pH But Does Not Recover with Return to Original pH

When the pH of GFP was decreased from pH 6 to pH 3 or 4, the fluorescent intensity immediately decreased. When GFP was at pH 5, the brightness decreased, but not as much as at pH 3 or 4. The fluorescent intensity of the control sample at pH 6 did not change. The trendlines for the data for pH between 3 and 5 had a negative slope, showing that the fluorescence decreased over time. The trendline for pH 6 also had a negative slope, but it was closer to 0, so the slight downward trend is likely from the error as shown by the error bars (**Figure 1**).

When the pH was raised to pH 6, the fluorescent intensity of the samples at pH 3 through 5 did not return to the intensity of the control. The fluorescent intensity of the control sample did not change. The trendlines for the data for pH 3 and 4 both had a positive slope, but the final brightness value was smaller than the original fluorescence shown in **Figure 1**. The trendline for pH 5, however, has a negative slope, indicating that the sample decreased in fluorescent intensity rather than returning to its original brightness. Once again, the trendline for pH 6 has a negative slope, but it is a small number and there are a few clear outliers so the negative slope could be due to error (**Figure 2**).

In the ANOVA analysis, the null hypothesis that changing the pH of GFP would not cause the fluorescence to decrease

	GFP	Fluorescein
pH 3	10.014	12.660
pH 4	10.069	13.071
pH 5	2.576	13.879
37 °C	2.004	1.950
39.5 °C	2.000	1.995
41.1 °C	0.960	2.897

Table 1: ANOVA analysis F distribution numbers for the fluorescence of GFP and Fluorescein with changed pHs and temperatures. This table shows the F distribution values for the experimental pHs and temperatures for GFP and fluorescein. If the values are less than 3.11, the null hypothesis is not rejected. If the values are over 3.11, the null hypothesis is rejected.

	GFP	Fluorescein
pH 3	1.039	73.731
pH 4	2.237	36.773
pH 5	1.202	22.739

Table 2: ANOVA analysis F distribution numbers for the fluorescence of GFP and Fluorescein after return to pH 6. This table shows the F distribution values for fluorescein and GFP when the pH was neutralized to pH 6 to determine if they could be reused. If the values are less than 3.11, the null hypothesis is not rejected. If the values are over 3.11, the null hypothesis is rejected.

was rejected, as the F distribution values for pHs 3 through 5 were all above 3.11 (**Table 1**). The results regarding the rejection of the null hypothesis that changing the pH of GFP to 6 would not cause the fluorescence to return to its original brightness were mixed. For pH levels 3 and 4, it was rejected, as the F distribution numbers in these cases were above 3.11. However, for pH level 5, the F distribution number was less than 3.11, indicating that it was not rejected (**Table 2**). This means that the fluorescence of GFP decreased when the pH decreased, but did not return to its original intensity when the pH was neutralized to 6.

Fluorescence of GFP Does Not Change with Temperature Change

The fluorescent intensity of GFP was not affected by any of the experimental temperatures compared to baseline. There were three experimental temperatures (37 °C, 39.5 °C, and 41.1 °C) because they were in the physiological range. GFP was incubated at these three temperatures for 30 minutes. The fluorescent intensity of GFP was expected to drop during these 30 minutes. However, the slopes of all three trendlines from time 0 minutes to time 30 minutes are extremely small and close to zero, signifying that the change in temperature did not affect the fluorescence of GFP (**Figure 3**).

The null hypothesis that changing the temperature of GFP would not cause its fluorescence to decrease was not rejected. For GFP temperature levels of 37, 39 and 41 °C, the F distribution numbers were less than 3.11, clearly indicating the null hypothesis was not rejected and temperature changes did not affect the fluorescence of GFP (**Table 1**).

Fluorescence of Fluorescein Changes with Decrease in pH and Recovers with Return to Original pH

Next we wanted to determine the effect of pH changes on fluorescein. Similar to GFP, when the pH of fluorescein was decreased from pH 6 to pH 3 or 4, the fluorescent intensity immediately decreased. When fluorescein was at pH 5, the brightness decreased, but not as much as at pH 3 or 4. The fluorescent intensity of the control sample at pH 6 did not change. The trendlines for the data for pH between 3 and 5 had a negative slope, showing that the fluorescence decreased over time. The trendline for pH 6 had a positive slope, but there are two outliers for 25 minutes and 30 minutes that are almost 10 units larger than the other four values. We speculated that the lighting in our photo booth may have changed and let in more ambient light, which would have increased the overall light in the picture, appearing as an increase in fluorescence to the image analysis in ImageJ (**Figure 4**).

When the pH was raised to pH 6, the fluorescent intensity of the samples at pH 3 through 5 returned to the intensity of the control. The fluorescent intensity of the control sample did not change. The trendlines for pHs 3-5 in the graph all had a positive slope that resulted in approximately the same fluorescent intensity as baseline at the final timepoint, showing that the fluorescence returned to its original brightness. The trendline for pH 6 is much straighter and, although there is a nonzero slope, it is small enough to assume that any variation

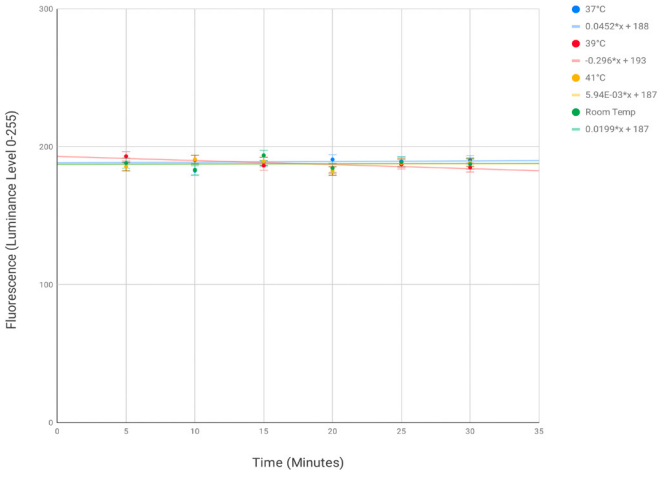

Figure 3: Fluorescence of GFP over time with changed temperatures. GFP fluorescence does not change with increasing temperature, as depicted by the horizontal lines for temperatures 37 °C, 39.5 °C, and 41.1 °C. There were three trials for every temperature, including room temperature (23 °C), every 5 minutes for 30 minutes, and the averages of the trials were used for the six data points. The error bars represent the standard deviation of the mean of all three trials, showing how much the data varied at each point.

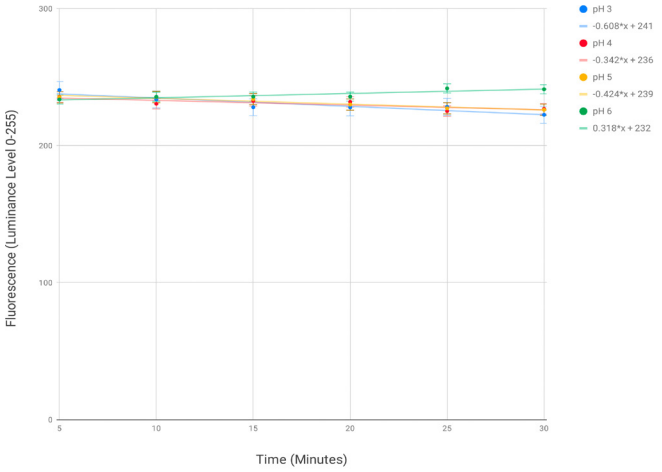

Figure 4: Fluorescence of Fluorescein over time when pH was changed. Fluorescein fluorescence decreases with decreasing pH, as depicted by the lines with a negative slope for pHs 3 through 5. There were three trials for every pH level, including pH 6, every 5 minutes for 30 minutes, and the averages of the trials were used for the six data points. The error bars represent the standard deviation of the mean of all three trials, showing how much the data varied at each point.

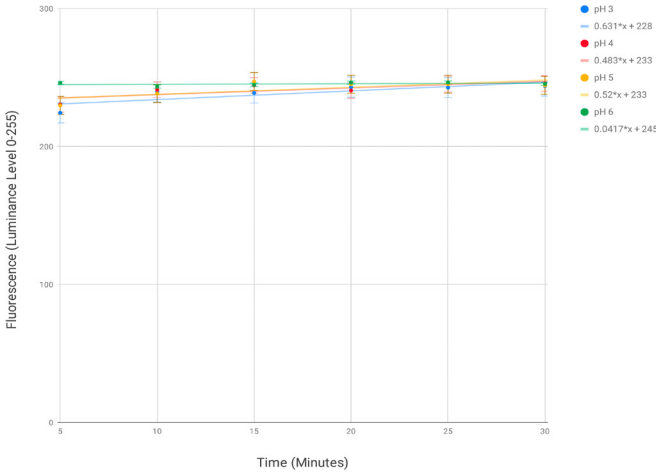

Figure 5: Fluorescence of Fluorescein over time after return to pH 6. Fluorescein fluorescence increases with increasing pH, as depicted by the lines with a positive slope for pHs 3 through 5. There were three trials for every pH level, including pH 6, every 5 minutes for 30 minutes, and the averages of the trials were used for the six data points. The error bars represent the standard deviation of the mean of all three trials, showing how much the data varied at each point.

was a result of error (**Figure 5**).

In the ANOVA analysis, both the null hypothesis that changing the pH of fluorescein would not cause the fluorescence to decrease and the null hypothesis that returning the pH of fluorescein to 6 would not cause the fluorescence to return to its original brightness were rejected. The F distribution values for the fluorescence of fluorescein with the changed pHs in **Table 1** and for the fluorescence of fluorescein after the pH was returned to pH 6 in **Table 2** were above 3.11, rejecting the null hypothesis. This indicates that the fluorescence of fluorescein decreased when the pH decreased and increased when the ph was returned to 6.

Fluorescence of Fluorescein Does Not Change with a Change in Temperature

The fluorescent intensity of fluorescein was not affected by any of the experimental temperatures compared to baseline. Like GFP, there were three experimental temperatures (37 °C, 39.5 °C, and 41.1 °C) because they were in the physiological range. Fluorescein was incubated at these three temperatures for 30 minutes. The fluorescent intensity of fluorescein was expected to drop during these 30 minutes. Instead, the slopes of all the trendlines are extremely small and close to zero, signifying that the change in temperature did not affect the fluorescence of fluorescein (**Figure 6**).

The null hypothesis that changing the temperature of fluorescein would not cause its fluorescence to decrease was not rejected. For fluorescein temperature levels of 37, 39 and 41 °C, the F distribution numbers were lower than 3.11, so the null hypothesis is not rejected, indicating that it could be true and decreasing the temperature does not have an effect on the fluorescence of fluorescein (**Table 1**).

DISCUSSION

In this study, we created a sensor for dehydration that uses changes in fluorescent intensity of a fluorophore to detect changes in pH and temperature. Since dehydration changes temperature and pH of the skin, the fluorescent intensity of the fluorophores GFP and fluorescein were observed at varying temperature and pH. The fluorophores were also tested to see if their fluorescent intensity returned to baseline brightness when their environment returned to the physiologic pH and temperature. This would mean that the sensor could be reusable, making it more cost-effective and therefore a better sensor.

The results for GFP indicated that increasing the temperature of the environment had no effect on its fluorescent intensity. The null hypothesis in the ANOVA analysis was not rejected, indicating that it could be true and decreasing the temperature did not affect the fluorescence of GFP. However, decreasing the pH of the GFP solution caused its fluorescence to decrease. When the pH of the environment was returned to pH 6, the fluorescent intensity of GFP did not return to its baseline brightness. The null hypothesis for when the pH was changed was rejected, meaning that it is not true and decreasing the pH of GFP decreases its fluorescence. However, the null hypothesis for when the pH was neutralized was not rejected, suggesting that it could be true and neutralizing the pH does not affect the fluorescence of GFP. This meant that GFP could be used in a dehydration test, but it would not be reusable.

One plausible explanation for why temperature changes did not affect the fluorescent intensity of GFP was that the range of temperatures tested was small (37 °C to 41.1 °C). However, the range of temperatures must be relevant to the

human body. When the pH of the GFP solution returned to 6, the fluorescent intensity of GFP might not have returned to its baseline brightness because the conformational change caused by low pH was too large.

Similar to the results for GFP, the results for fluorescein indicated that increasing the temperature had no effect on its fluorescent intensity. Likewise, the null hypothesis was not rejected, indicating that decreasing the temperature did not affect the fluorescence of fluorescein. The temperature might not have affected the fluorescent intensity of fluorescein for the same reason as GFP: the range that was tested was not very large because the range of temperatures possible in the human body is not very large. On the other hand, the fluorescence of fluorescein also decreased when the pH decreased. The null hypothesis was, similarly, rejected, meaning that decreasing the pH caused the fluorescence of fluorescein to decrease. Unlike GFP, when the pH of the environment returned to pH 6, the fluorescent intensity of fluorescein returned to its original brightness. The null hypothesis from the ANOVA analysis was rejected, indicating that it is false and the returning the pH to 6 caused the fluorescence to return to its original intensity. Thus, a reusable sensor containing fluorescein may be possible. In dehydrated patients, the pH of their sweat will change, causing the fluorescein brightness inside of the wearable sensor to decrease. When the dehydrated person drinks water after seeing the sensor, their skin pH would return to pH 6 and cause the fluorescent intensity to return to its original brightness, signaling that the person is now rehydrated.

Unlike pH, changing the temperature of both fluorescein and GFP does not change the fluorescence, meaning that they can't be used in a sensor where the factor affecting fluorescence is temperature. However, a sensor based on skin temperature would not be reliable anyway, because body temperature and skin temperature are different, so skin temperature cannot be a reliable indicator of dehydration. Although body temperature while exercising may be 39.5 °C, skin temperature may not be the same. The skin temperature of an athlete is constantly changing depending on activity level and perspiration. Furthermore, one of the symptoms of heat exhaustion is cold and clammy skin, which was not tested. Therefore, skin temperature is an unreliable measure of dehydration. Since our results indicate that skin temperature does not affect the fluorescence of either fluorophore, skin temperature is not an effective indicator of dehydration.

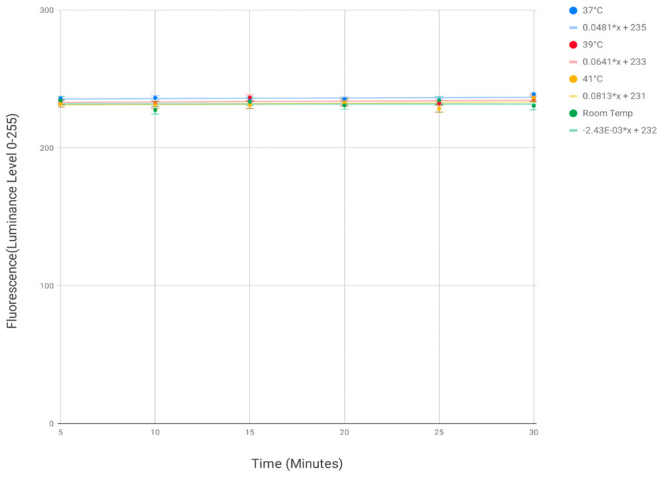

Figure 6: Fluorescence of Fluorescein over time with changed temperatures. Fluorescein fluorescence does not change with increasing temperature, as depicted by the horizontal lines for temperatures 37 °C, 39.5 °C, and 41.1 °C. There were three trials for every temperature, including room temperature (23 °C), every 5 minutes for 30 minutes, and the averages of the trials were used for the six data points. The error bars represent the standard deviation of the mean of all three trials, showing how much the data varied at each point.

Further work on this project would include testing whether changes in pH could have the same effect on fluorescein in a gel. A gel is a mass of liquid where the particles are spread throughout the system evenly. Gels are often used to encase proteins and chemicals. For example, doctors use protein-based hydrogels for tissue engineering and repair because of its structural properties (13). Putting fluorescein in a gel would be the next step in producing a useful sensor, as this would allow the fluorescein to be attached to someone's skin. This experiment would determine whether the required pH changes occur in human subjects. As the environment of the hydrogels is different than water-based buffers, the first step would be to test whether fluorescein can retain its function in this environment. However, one consideration when making this sensor is that some people have a naturally low skin pH, which would make the sensor react even when the person is not dehydrated. In order to solve this problem, before testing a sensor on a person, the pH of their skin must be checked to ensure that the baseline pH is 6.

After testing different temperatures and pHs on both fluorescein and GFP, fluorescein appears to be the most suitable candidate for a dehydration sensor. At the time of writing, the cost of pure fluorescein is almost 150 dollars less than the cost of the materials needed to collect GFP, making it much cheaper. Since GFP can only be used within a short time frame after purification, while fluorescein is more stable, it is even more cost-effective. A sensor containing fluorescein would also be reusable as its fluorescent intensity returns to its original brightness when the pH returns to pH 6, which is not true of GFP. Additionally, the baseline fluorescence of fluorescein is brighter than GFP, making loss of signal during dehydration more obvious. Not only is the baseline fluorescence brighter than GFP, but fluorescein loses its color as well when the pH decreases, making the sensor visible without special equipment. This research suggests a possible cost-effective, reusable solution to prevent severe dehydration, especially in high-risk populations such as the elderly, athletes, and young children.

MATERIALS AND METHODS

Fluorescein Sample Collection

Fluorescein was extracted from a neon yellow highlighter by diluting the ink in 5 mL of water. Fluorescein is the primary ingredient in the ink of these highlighters, and the other ingredient is a soluble binder that prevents the ink from bleeding through pages. The top of the highlighter was removed with pliers in order to get to the tube with the ink in it. This tube was placed into the water until all of the ink had come out and the solution was a mix between fluorescein, the binder, and the water. Neither the water nor the binder, however, is fluorescent, so they would not have an effect on the fluorescence of the mixture. Thus, only the fluorescence of fluorescein was measured in our data.

GFP Sample Collection

A 6 mL liquid culture of HB101 E. Coli with pGLO (a plasmid containing the gene for GFP), ampicillin, and arabinose was grown for 48 hours. After centrifuging the bacteria for 3 min, the pellet was resuspended in 1 mL of Tris-EDTA (TE) Buffer with 0.1 mM EDTA. Then 40 µL of lysozyme was added, and the mixtures were left at room temperature for 30 minutes to lyse the bacteria. To separate the protein from the rest of the bacteria, the lysate was centrifuged for 10 minutes at 16,000 xg. The supernatant was removed and 250 µL of ammonium sulfate (4 M) was added.

The hydrophobic interaction chromatography (HIC) column was equilibrated using 10 column lengths (5 cm) of 2 M ammonium sulfate. The mixture of the supernatant and 4 M ammonium sulfate was added to the HIC column. The column was washed using 1 mL of wash buffer (1.3 M ammonium sulfate) and the GFP was removed from the column by adding 1 mL of elution buffer (TE Buffer).

Temperature Data Collection

Twelve clear tubes, each containing 70 µL of fluorescein, were prepared to determine fluorescence at four temperatures (n=3): room temperature (23 °C), 37 °C, 39.5 °C, and 41.1 °C. One tube was maintained at room temperature as a control.

The tubes were photographed at intervals of 5 minutes for 30 minutes. After 30 minutes, the four temperature groups were moved to room temperature. Their recovery was photographed every 5 minutes for 30 minutes.

The process above was repeated with the GFP samples.

pH Data Collection

Twelve clear tubes, each containing 70 µL of fluorescein, were prepared to determine fluorescence at four different pHs (n=3): 3, 4, 5, and 6. The pH was changed to the listed pHs using HCl, an acid, and NaOH, a base. One tube was maintained at pH 6 as a control. The pH was measured using pH papers.

The tubes were photographed at intervals of 5 minutes for 30 minutes. After 30 minutes, the four pH groups were neutralized to pH 6 using HCl and NaOH. Their recovery was photographed every 5 minutes for 30 minutes.

The process above was repeated with the GFP samples.

Image Analysis

All photos were taken while the tubes were being exposed to UV light. Our photo booth consisted of a black blanket covering the photographer and the samples to minimize the ambient light. The camera used the same exposure settings for all samples.

The change in fluorescence was measured using ImageJ. ImageJ records the brightness of the light from an image. The results were put into a line graph using Google sheets.

The fluorescein samples were too bright to be measured directly by ImageJ, so each image was identically processed to lower the brightness and collect quantifiable data.

Using Photoshop, the image was duplicated as a new layer. The copy was screened, then blurred using a Gaussian Blur filter set to 5.0 pixels. The interaction mode between the copy and the original image was changed from Normal to Multiply. The image was flattened by combining both the original and duplicate layers into one layer. This process reduced the brightness level to within the luminosity range of 0 to 255 for ImageJ analysis.

ANOVA Analysis

An ANOVA analysis was conducted on the fluorescence of both fluorophores at each temperature and pH.

For our data set in the ANOVA analysis, the degrees of freedom in our numerator was 5 and the denominator was 12, giving us a Critical F distribution value of 3.11, $F(5, 12) = 3.11$. If our analysis showed that the F distribution of the data set was less than 3.11 the null hypothesis would not be rejected. If the F distribution were greater than or equal to 3.11, the null hypothesis would be rejected, indicating that our hypothesis was correct.

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REFERENCES

1. M.A., Peter Crosta. “Dehydration: Symptoms, Causes, and Treatments.” Medical News Today, MediLexicon International, 20 Dec. 2017, www.medicalnewstoday.com/articles/153363.php.
2. Ghose, Tia. “Why Is Water So Essential for Life?” LiveScience, Purch, 29 Sept. 2015, www.livescience.com/52332-why-is-water-needed-for-life.html.
3. Baker, Lindsay B. “Sweating Rate and Sweat Sodium Concentration in Athletes: A Review of Methodology and Intra/Interindividual Variability.” Sports Medicine, vol. 47, no. S1, 2017, pp. 111–128., doi:10.1007/s40279-017-0691-5.

4. Shirreffs, S. M. “Hydration: Special Issues for Playing Football in Warm and Hot Environments.” Scandinavian Journal of Medicine & Science in Sports, vol. 20, 2010, pp. 90–94., doi:10.1111/j.1600-0838.2010.01213.x.
5. Godek, S F., et al. “Sweat Rate and Fluid Turnover in American Football Players Compared with Runners in a Hot and Humid Environment * Commentary.” British Journal of Sports Medicine, vol. 39, no. 4, 2005, pp. 205–211., doi:10.1136/bjsm.2004.011767.
6. Trautner, Barbara W, et al. “Prospective Evaluation of the Risk of Serious Bacterial Infection in Children Who Present to the Emergency Department with Hyperpyrexia (Temperature of 106 Degrees F or Higher).” Pediatrics, U.S. National Library of Medicine, July 2006, www.ncbi.nlm.nih.gov/pmc/articles/PMC2077849/.
7. Herrmann, Franz, and Leona Mandol. “Studies of Ph of Sweat Produced by Different Forms of Stimulation.” Journal of Investigative Dermatology, Elsevier, 7 Jan. 2016, www.sciencedirect.com/science/article/pii/S0022202X15486798.
8. Findlay, Kim. “Green Fluorescent Protein(GFP).” What Is Electron Microscopy?, John Innes Center, www.jisc.ac.uk.
9. Campbell TN, Choy FYM, “The Effect of pH on Green Fluorescent Protein: a Brief Review”, Center for Environmental Health, Department of Biology, University of Victoria, Box 3020 STN CSC Victoria, British Columbia V8W 3N5 Canada. Molecular Biology Today (2001) 2(1): 1-4.
10. Zhang C, Liu MS, Xing XH. “Temperature influence on fluorescence intensity and enzyme activity of the fusion protein of GFP and hyperthermophilic xylanase.” Appl Microbiol Biotechnol. 2009 Sep;84(3):511-7.
11. PubChem. “Fluorescein.” National Center for Biotechnology Information. PubChem Compound Database, U.S. National Library of Medicine, 26 Mar. 2005, pubchem.ncbi.nlm.nih.gov.
12. Zhu, H., Derksen, R., Krause, C., Fox, R., Brazee, R., and Ozkan, H., "Fluorescent Intensity of Dye Solutions under Different pH Conditions," Journal of ASTM International, June 2005, Vol. 2, No. 6.
13. Schloss, Ashley C et al. “Protein-Based Hydrogels for Tissue Engineering” Advances in experimental medicine and biology, vol. 940 (2016): 167-177.

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