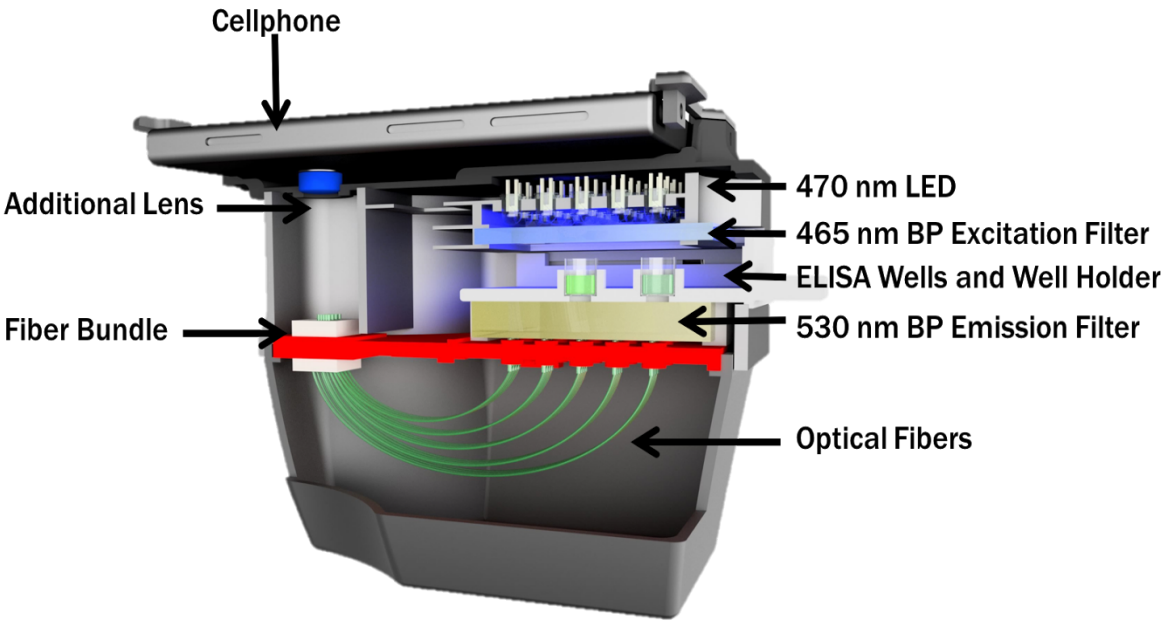
Tear Analysis on a Contact Lens Using a Cell Phone-Based Fluorometric Assay Reader



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I. Abstract

Currently, basal tear extraction serves as the gold-standard method to quantitatively analyze human tear fluid [1]. Lysozyme and lactoferrin are proteins in tear fluid responsible for bacterial defense, and abnormal concentrations can contribute to inflamed eyes, eyelid tumors, dry eye syndrome, among other conditions and diseases [2]. For this reason, lysozyme and lactoferrin measurement can be of considerable diagnostic value. Rather than have a patient undertake an uncomfortable basal tear extraction, in this work we present a simple cellphone-based fluorometric lysozyme and lactoferrin assay reader which can analyze basal tears directly on a contact lens. This reader uses a 3D-printed optomechanical cellphone attachment to extract a signal produced by a commercial fluorescent assay. We tested this mobile platform in comparison to a clinically approved enzyme-linked immunosorbent assay (ELISA) well-plate reader using both artificially incubated contact lenses as well as contact lenses from human participants, and found the mobile platform to have a comparable dynamic range, sensitivity, and specificity for clinically-relevant lysozyme and lactoferrin concentrations [3]. The cost effectiveness, portability, and simple operation allow individuals without medical training to measure their daily lysozyme and lactoferrin concentrations using their disposable contact lenses. Furthermore, this device is a point-of-care platform that could be multiplexed to measure a panel of proteins. By understanding how tear protein levels correspond to changes in an individual's health, this device can advance the field of personalized medicine allowing individuals to make real time measurements, diagnoses, and informed health decisions.

II. Introduction

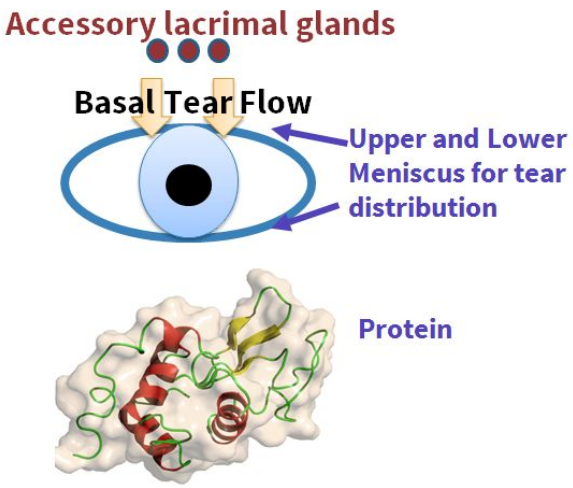


Figure 1: Tear distribution in the eye. The accessory lacrimal glands store and pump tear fluid into the upper and lower meniscus for tear distribution.

Tear fluid proteins and enzymes are vital to ocular health because they serve as antimicrobial molecular complexes, they protect the epithelium from desiccation, and they provide oxygen to the cornea [2]. As shown in Figure 1, tear proteins and enzymes originate in

the lacrimal glands where they are then distributed throughout the hydrophobic ocular surface [4]. These proteins and enzymes can then act as a defense mechanism to protect the eye against a range of microorganisms such as: Staphylococcus (S.) aureus, Herpes Simplex Virus, and Streptococcus pneumoniae [5]. Since the tear film is in contact with the external environment, these tear fluid proteins and enzymes are the first barrier to viruses and pathogens as they try to enter the ocular surface epithelia. Two proteins in particular play an antimicrobial role in tear fluid composition: lysozyme and lactoferrin.

In 1822, Alexander Fleming revealed that lysozyme in human tears killed Gram-positive bacteria [6]. Later, in 1996, Aho et al. found a piece of the rationale behind lysozyme's force and effectiveness in killing Gram-positive bacteria: lysozyme accounts for 20-30% of the total protein in basal and reflex tears [7]. As a result of lysozyme's abundance in human tear fluid it has a higher affinity to break down pathogens in the eye through a series of catabolic reactions. More specifically, in 1999, Lee-Huang et al. were able to explain the series of catabolic reactions between lysozyme and pathogens: lysozyme catalyzes the hydrolysis of the one and four beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine in bacterial cell walls [8]. In other words, lysozyme breaks down bacterial cell walls; thereby, compromising the life of bacterial cells that enter the eye. Likewise, lactoferrin also exhibits very similar properties to lysozyme. In 1983, Janssen and Van Bijsterveld, discovered that lactoferrin also constitutes 20-30% of the total tear protein in basal and reflex tears [9]. While lysozyme breaks down the phospholipid bilayer of cell walls, lactoferrin serves to deprive bacteria of iron (an essential nutrient for growth) [2]. Lactoferrin has the same valence states as iron (a divalent cation) and therefore has a higher affinity to form a complex with iron [10]. When bacteria are deprived of iron, they lack an essential nutrient for life. Therefore, lactoferrin protects the eye through its microbiostatic role (inhibiting the growth of bacteria) [11,33].

Since these two proteins exist in such a large concentration in the eye and play antimicrobial roles, they are of interest for further study to understand how the interactions between lysozyme/lactoferrin and pathogens contribute to ocular health. As both proteins make up 20-30% of total tear proteins, they are easily quantifiable through a series of chemical analyses. Furthermore, according to K. B. Bjerrum, an abnormal concentration of lysozyme or lactoferrin can make people more susceptible to diseases such as: Sjogren's Syndrome, Herpes Simplex Virus, Dry Eye Syndrome, etc. [12, 13,14]. Thus, researchers have been developing techniques to quantify these proteins in human tear fluid. As a means for developing non-invasive methods to quantify these proteins, Daniel Citterio, professor at Keio University in Japan, developed the first paper-based assay to quantify lactoferrin in tear fluid. Essentially, he measured the fluorescent intensity of a lactoferrin-terbium chloride complex when excited by UV light [15, 16]. Despite the novel technique to measure lactoferrin, the paper based assay lacks a modular design to be used for batched analysis and high-throughput diagnostic testing because each paper testing strip has to be recreated for each sample test. As a result, there is a move within the field of biophotonics to replicate the commercial, gold-standard Enzyme-Linked

Immunosorbent Assay (ELISA) Well-Plate Reader because it's function (to measure proteins in solution) can be replicated using optomechanical techniques. Brandon Berg developed a cell-phone based ELISA reader in Professor Aydogan Ozcan's lab at the University of California Los Angeles to quantify herpes, measles, and mumps in solution [3]. Despite Berg's success in being able to parallel his results from his portable mobile-phone based device to the gold-standard ELISA well-plate reader, his device was only calibrated to measure herpes, measles, and mumps. Therefore, there exists a gap in the field: the need for a portable, point-of-care, and high throughput device to measure lysozyme and lactoferrin.

In an effort to fill such a gap, I worked with Professor Aydogan Ozcan at the University of California Los Angeles in the Department of Electrical Engineering, where I built upon both Daniel Citterio's and Brandon Berg's work to create a portable cellphone-based fluorometric assay reader to measure lysozyme and lactoferrin in tear fluid using non-invasive sampling means. Daniel Citterio's work still requires that human participants undergo tear fluid extraction in order to analyze the contents of their tear fluid.This method is not only invasive, but also it only yields a very small sample volume of tear fluid for analysis ~1-2 μL of tear fluid [13]. Thus, I wanted to improve upon the current invasive method of collecting tear fluid by analyzing the tear fluid deposited onto contact lenses. Contact lenses are porous and can easily absorb tear fluid which makes them great candidates as sample collectors [17]. Since the 1990's the interaction between contact lenses and tear fluid has been heavily studied, but never implemented for disease diagnostics [10, 11, 12, 17]. Therefore, to fill the gap within the field I developed the following research question: **Can we improve upon current diagnostic techniques to measure lysozyme and lactoferrin on contact lenses using a cell-phone?**

Contrary to popular belief, I worked on this project very independently such that I was tasked to design my own experimental setup, draw and 3D print spare parts, set up the chemical reactions, etc. In short, I did not work as a lab assistant, but rather I worked as a true researcher while still receiving mentorship from graduate student Zachary Scott Ballard. I conducted this study in two different ways in order to calibrate the chemistry behind the two different fluorescent assays: lysozyme and lactoferrin. To measure lysozyme, I created a 3D printed optomechanical reader that was integrated with a mobile phone (refer to figure 2) and used a commercially available fluorescent assay from EnzCheck [18]. To measure lactoferrin, I created a temporary setup with parts from ThorLabs in order to calibrate the chemistry of the fluorescent assay and take preliminary data (refer to figure 3). For the lactoferrin assay chemistry, I paralleled Daniel Citterio's work exactly except tested his assay in solution rather than using a paper based substrate. For samples, I used known concentrations of lysozyme and lactoferrin in solution, known concentrations of lysozyme and lactoferrin in contact lenses incubated in artificial tear fluid, and human tear fluid on contact lenses (under IRB approval).

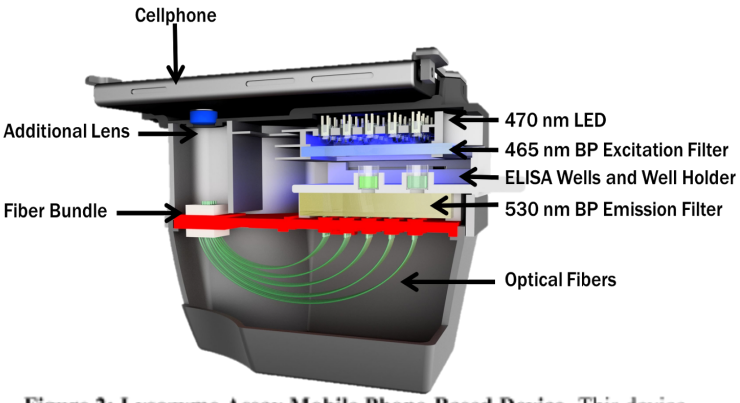


Figure 2: Lysozyme Assay Mobile Phone-Based Device- This device was calibrated specifically to measure lysozyme in solution. It uses a series of optomechanical parts in order to measure the fluorescent assay.

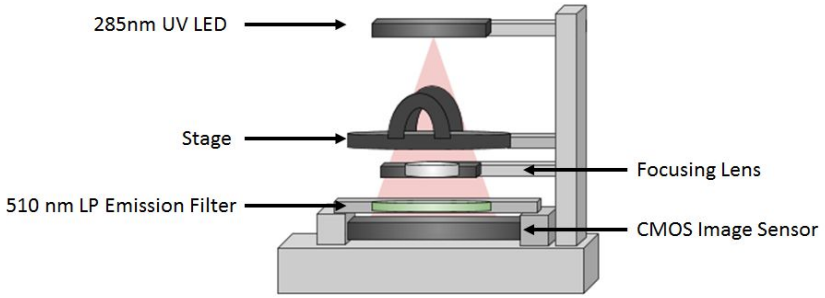


Figure 3: Lactoferrin Set Up- Apparatus for collecting data using optomechanical parts and a UV LED.

III. Literature Review

Before delving into the properties and benefits of mobile-phone readers for disease diagnostics, it is important to understand the fundamental characteristics of lysozyme and lactoferrin. Lysozyme is found in tears, nasal mucus, saliva, blood serum, plasma, and in many other human tissues and secretions [19]. Fleming et al. along with Meyer et al. discovered that lysozyme has a high affinity to impose lytic action on a gram positive coccus, *Micrococcus lysodeikticus*[1] *(micrococcus luteus),* to digest these bacterial substrates (refer to figure 4) [20, 21]. Due to lysozyme is relatively simple biological role, its interactions with bacteria can be easily tracked and replicated in lab settings. Gachon et al. built upon Fleming's foundational study to suggest there exists a correlation between lysozyme concentration and Sjogren's Syndrome[2] [24]. Ohashi et al. identified correlations between lysozyme and lactoferrin levels such that a decrease in lysozyme and lactoferrin levels was attributed to lacrimal gland dysfunction indicating that measuring the concentration of both proteins in the tear fluid is of interest for disease diagnostics [23]. Likewise, Gachon et al. indicate that the correlation between lysozyme and lactoferrin values suggests Sjoren's Syndrome could be more easily identified through a lactoferrin assay (which is more difficult and less precise)[3] [24]. Lactoferrin is an iron-binding glycoprotein that is present in tears [25]. Lactoferrin forms complexes with iron when iron binds

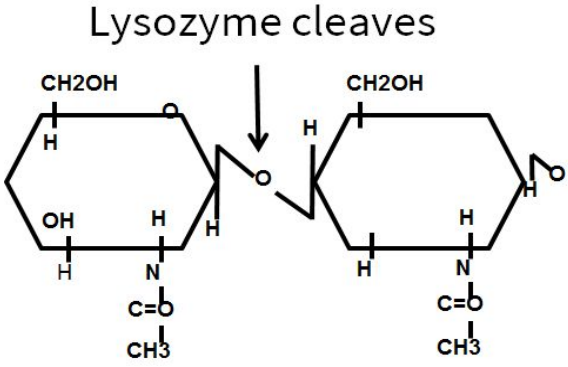


Figure 4: Lysozyme Lytic Action: Lysozyme breaks down the glycosidic linkages within the phospholipid bilayer backbone of cell walls to lysis cell walls.

[1] These cells are known as lysis indicator cells because lysozyme functions to break down their cell walls. I used these same cells in my study to parallel the academic literature.
[2] An immune system disorder characterized by decreased lacrimal gland function, dry eyes, and mouth [22,23].
[3] As part of my research, I worked on improving the sensitivity and dynamic range of the lactoferrin assay.

to lactoferrin's positive binding sites (refer to figure 5). As a result of this iron-binding property, lactoferrin plays a bacteriostatic role in iron uptake by withholding iron from iron-dependent bacteria [25].

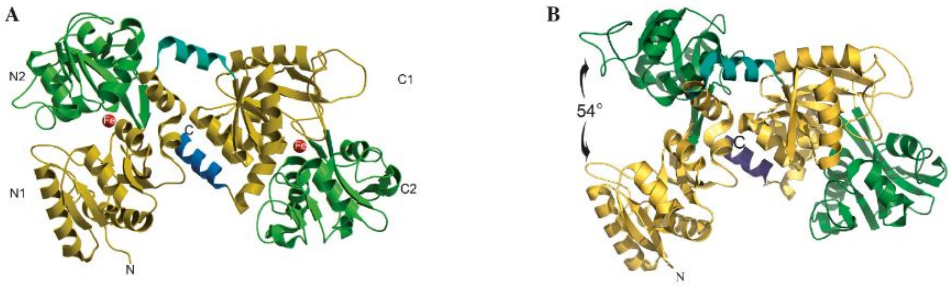


Figure 5:Lactoferrin Binding Properties: Lactoferrin is composed of two lobes: the N lobe and the C lobe each broken down into N1/N2 and C1/C2 respectively. Part A shows the structure of lactoferrin when iron attaches to the binding site between the N1 and N2 lobes. Part B indicates the shape of lactoferrin without the presence of iron. Part B indicates that without iron present, lactoferrin creates space for iron to bind (54 degrees of binding space). Thus when iron binds to lactoferrin, it changes its conformational shape and becomes closed [27].

Realizing the importance of these proteins in ocular health, Daniel Citterio developed the first paper based assay to measure lactoferrin via fluorescence [15]. In order to quantify lactoferrin from a sample of tear fluid, he relied on the chemical interaction of terbium chloride hexahydrate, sodium bicarbonate, and lactoferrin moderated by HEPES buffer because the lactoferrin terbium complex exhibits pH-dependent fluorescence. In short, lactoferrin forms a complex with terbium that begins to fluoresce when excited by 290 nm UV light emitting diode (LED).Therefore, the fluorescent intensity is directly proportional to the lactoferrin-terbium complex concentration. In this way, it is possible to measure the concentration of lactoferrin in tear fluid. In preparing his paper test strips for analysis, Citterio applied 8 printing layers of $TbCl_3$*$6H_2O$ solution, soaked it in poly (vnyl alcohol), allowed the paper to dry, then applied 12 printing layers of 3.75 mM $NaHCO_3$ to the paper, next he added 50mM of HEPES buffer to make the overall pH of the assay 7.4, and lastly he let the finalized paper strip dry (refer to figure 6) [15]. Now that the test

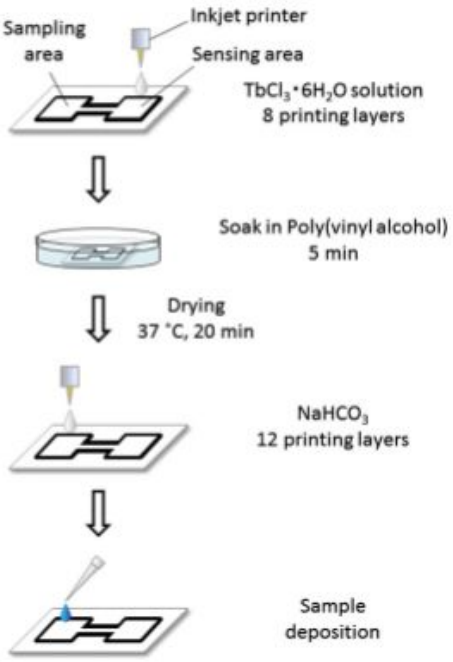


Fig. 2 Schematic illustration of the reagent deposition process during fabrication of final μPADs: $TbCl_3$ and $NaHCO_3$ solutions were printed by using an inkjet printer, while surface treatment of the paper substrate was performed by soaking in poly(vinyl alcohol) solution. All reagents were dissolved in HEPES buffered solution (pH 7.4, 50 mM).

Figure 6: Source: Daniel Citterio et al. 2014.
Description provided by the source.

strips were prepared with substrates for analysis, lactoferrin solutions at various concentrations were placed onto the sampling area where by capillary action would be transferred to the sensing area for analysis. In order to calibrate the device and understand the fluorescent emission spectrum, Citterio tested 6 samples of lactoferrin at various concentrations and generated a fluorescence emission spectra of 100 μM $TbCl_3$ solutions excited at 290 nm. He generated the following fluorescence emission spectra indicating that the peak emission wavelength for the lactoferrin assay resides between 520 and 550 nm for various concentrations of lactoferrin (refer to figure 7). This graph was generated by placing the paper test strips within a gold-standard ELISA Well-Plate reader, therefore it can be assumed that these results are specific and accurate. Furthermore, when blind testing his technique against a gold standard ELISA, Citterio was able to generate minimal percent error between both reader's results such that the largest percent error was -4.8% [15].

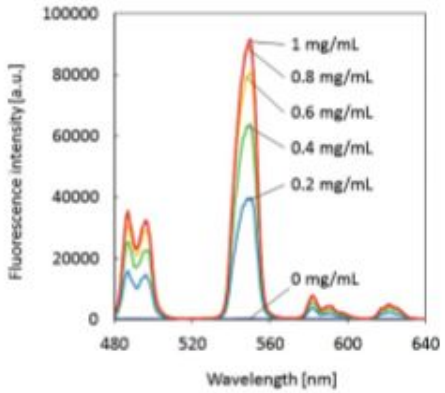


Fig. 3 Fluorescence emission spectra of 100 μM $TbCl_3$ solutions (50 mM HEPES, 3.75 mM $NaHCO_3$, pH 7.4) in the absence and presence of human lactoferrin at various concentrations; $\lambda_{ex} = 290$ nm.

Figure 7: Source: Daniel Citterio et al. 2014. Description provided by the source.

Overall, Citterio found that it is possible to create a user-friendly and low-cost sensing device for analysis of lactoferrin in human tear fluid to yield results within 15 minutes. This very short turnaround time allows for any user to obtain the same results that an ELISA Well-Plate reader can obtain but at a lower opportunity cost, meaning that users can bypass the hours of pipetting, incubation, and washing steps necessary to obtain results [15]. Despite the shortened turnaround time, the paper based assay has inherent limitations such as only being able to be used for a single test rather than for high-batched analysis. Additionally, in order to obtain samples from human participants, they must undergo an invasive tear fluid extraction which must be done in a laboratory setting. If this technology were to be brought to field settings to grant better medical care to those in low-resource areas, there would need to be significant improvement in the modularity of the device. Therefore, in my study, I bridged the gap within this field by using contact lenses as sample collectors for human tear fluid. This method allows for a simple and minimally invasive way to collect human tear fluid without the need for laboratory equipment. In this way, that is the first step I took toward improving Citterio's current work.

To elaborate, with ocular diagnostics, a simple platform for analysis of tear fluid constitutes contact lenses. These hydrogels are porous and easily absorb tear fluid, which makes them viable candidates for tear fluid analysis. Mann et al. reveal biochemical changes brought about by the influence of the contact lens on the tear film can be categorized in two ways. First, the lens can reduce the levels of specific components in the tear film (contributing to a percent error from the literature in my study as some proteins would be denatured) [27]. Second, the lens

can stimulate an increase in tear production (leading to a higher sample volume) [27]. Zhao et al. elaborated on Mann's conclusions in order to conduct experiments with contact lenses analyzing the amount of cholesterol available in tear fluid [28]. Zhao used contact lenses made from different polymers and ensured that each lens was worn on a daily schedule with various lens care solutions. Lipid extracts were separated and cholesterol was quantified using thin layer chromatography, while protein extracts were quantified using a gold-standard technique with a urea substrate [28]. Zhao et al. found that Balafilcon A lenses had the most protein and cholesterol extracted at 4.1-8.2 µg/lens and 5.4-23.2 µg/lens [28]. As a result, Zhao et al. concluded that different contact lens solutions and lenses can affect the quantity of secreted proteins and certain lens care solutions actually decrease protein and lipid production [28,29]. Given the foundational literature, contact lenses make a viable platform for tear fluid sample collectors in order to improve upon Daniel Citterio's invasive tear collection techniques. In my study, I used Acuvue Oasys contact lenses made from Balafilcon A in order to parallel the literature.

To improve the second construct of Citterio's work, repeatability and high-throughput analysis, there is an emerging technique within the field of electrical engineering: using mobile phone cameras for medical diagnostics. According to J.C. Contreras- Naranjo et al., the quality of consumer electronic devices, specifically camera phones, has increased since the early 2000's [30]. According to Moore's Law, from 2000 to 2015 the megapixel count of mobile phone cameras has doubled almost every two years in conjunction with the increase in transistor count of central-processing-units (CPU's) [30]. The evolution of megapixel and transistor count has allowed manufacturers to implement increasingly sophisticated electronic features on mobile devices (phones, computers, etc.) [30]. Now, consumer electronic devices provide users with cost effective and high-performance products that can be used for conducting advanced measurements [30]. As a result of these technological advancements, there has been a growing trend toward the use of cellphone-based devices (CBDs) in bioanalytical sciences [31]. More specifically, immunoassays are increasingly used as tests to measure proteins in solution for healthcare monitoring. In conjunction with mobile phone point-of-care devices, ELISA (microplate reader) technology can be easily adapted to fit a mobile phone [3].

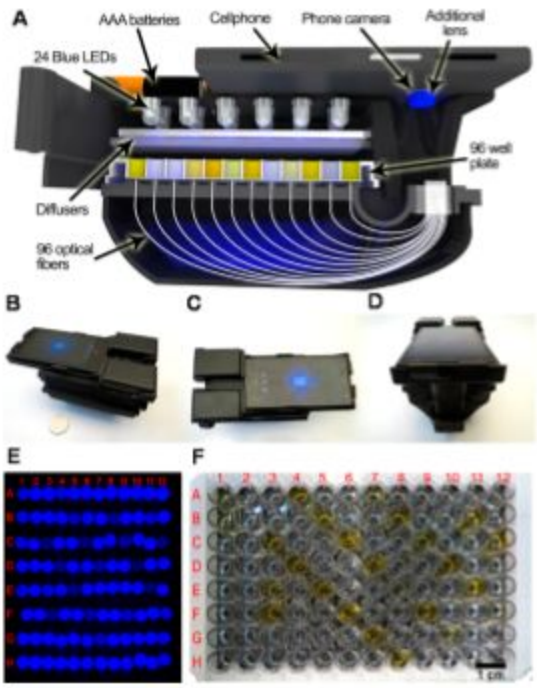


Figure 1. Schematic overview (A) and different perspectives (B–D) of the cellphone based ELISA colorimetric reader. Sample image (E), and sample plate (F). Rows and columns are labeled in (E) to correspond with the plate in (F).

Figure 8: Source: Berg et al. Description provided by the source.

Brandon Berg along with Aydogan Ozcan developed a Cellphone-Based Microplate Reader

for Point-of-Care Testing of ELISA [3]. This device relies on the principles of colorimetry to measure Herpes Simplex Virus 1 and 2, measles, and mumps in solution enabling high-throughput analysis in less than 2 minutes (refer to figure 8) [3]. After performing a Sandwich-ELISA, Berg found that he achieved an accuracy of 99.6%, 98.6%, 99.4%, and 99.4% for mumps, measles, and herpes one and two tests [3]. Therefore he concluded that a cell-phone based colorimetric assay reader could replace a gold-standard ELISA-well-plate reader in order to provide disease diagnostic equipment to low-resource areas.

Building upon the principles of my foundational literature, with a focus on Berg's and Citterio's work, I developed and tested a cell-phone based device to address the current limitations in the field. First, there is no current research discussing a novel technique to measure lysozyme using cell-phone based devices. Therefore, only relying on a commercially available fluorescent assay, my study presents a modified version of Berg's portable device calibrated to measure lysozyme from a contact lens specifically. Second, Citterio's research relies on a paper based assay, my study improves upon his work by implementing a new method to measure lactoferrin in solution.

IV. Methods

The goal of this study is to measure lysozyme and lactoferrin off of tear fluid deposited onto Acuvue Oasys (Balafilcon A) contact lenses. In an effort to parallel the foundational sources, my study followed the same sampling procedures in order to obtain quantitative results. However, because there was no academic literature measuring lysozyme with a paper-based assay or with a cellphone biosensor, I followed the procedure listed in a commercially available lysozyme assay from Enzchek Molecular Biology [18]. The lysozyme assay works on the principle of fluorescence (refer to figure 9) [18]. Whereby I used fluorescent solution, PBS buffer, and varying concentrations of lysozyme for sample tests.

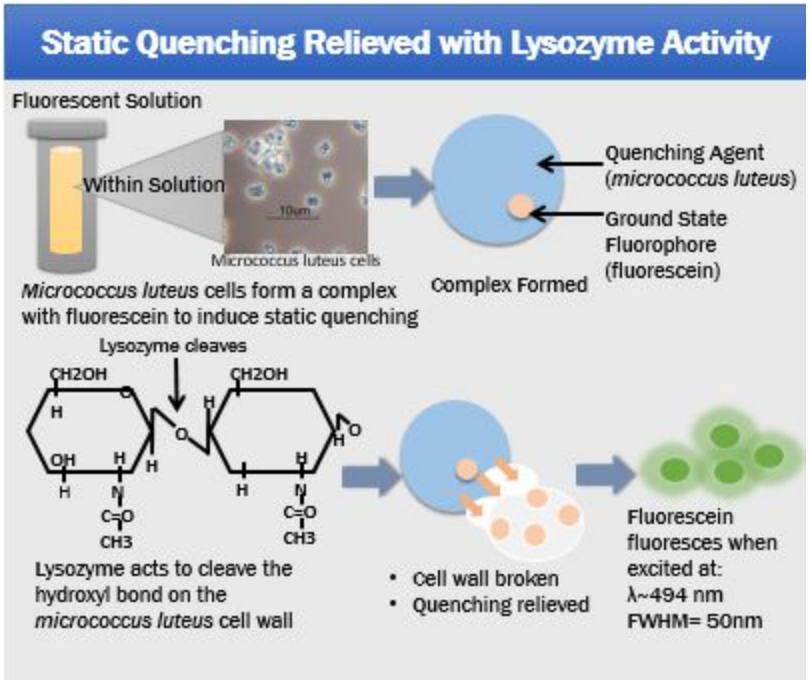


Figure 9:Lysozyme Assay Principle: Micrococcus luteus cells are labeled to quench fluorescence meaning that the cells have been filled with fluorophores (molecular compounds that fluoresce when exposed to a certain wavelength of light). Once lysozyme interacts with the micrococcus luteus cells and breaks down their cell walls, the fluorophores are released and begin to fluoresce when excited by a 494 nm LED. Therefore the fluorescent intensity is proportional to lysozyme activity [33].

Likewise, I modeled Daniel Citterio's procedure to fit my study by using the same reagents, but modifying the assay from a paper-based assay to an assay done in solution. The reagents include, 50 µL of 3.75 mM $NaHCO_3$, 50 µL of 100 µM $TbCl_3$, 50 µL of pH 7.4 HEPES buffer, and 50 µL of varying concentrations of lactoferrin for sample tests[15]. The assay works by capturing the fluorescent light formed by the lactoferrin terbium complex when excited by 290 nm UV light.

Furthermore, after testing the assays in solution only to calibrate the readers, samples were then tested with contact lenses in two ways: incubated lenses and lenses from a human

participant under IRB approval. Both a contrived a natural setting was used in order to verify the diagnostic capabilities of the device in a controlled (artificial incubation) and real-life setting (the human eye). For the contrived setting, Acuvue Oasys contact lenses were artificially incubated using a polydimethylsiloxane (PDMS) microfluidic chamber (because of its durability and modularity as a rubber, PDMS can be molded into any form to facilitate microfluidics) and a syringe pump [26]. Artificially created tear fluid with a controlled concentration of lactoferrin was injected into the microfluidic chamber at a flow rate of 1 µL/min (mimicking the tear flow rate of the eye) [24]. The concentration of lysozyme in solution varied by the following values: 0µg/mL, 50 µg/mL, 100 µg/mL, 250µg/mL, 500µg/mL, and 1,000µg/mL (mimicking the mean lactoferrin concentration in the eye: 1,768 µg/mL) [24]. Lactoferrin was tested at the following concentrations: 0mg/mL, 1mg/mL, 2mg/mL, and 4mg/mL. Higher concentrations of lactoferrin were used in order to test the dynamic range of the assay [15]. In order to obtain these concentrations a serial 1:10 dilution between the commercially purchased protein solution and phosphate-buffered saline (PBS) was used. Contact lenses were incubated in the microfluidic chamber for 12 hours, and data was collected within 15 minutes of contact lens removal to ensure a maximum yield of quantifiable lysozyme and lactoferrin [27].

Incubated contact lenses were used in order to understand how the contact lenses interacted with the assays (lysozyme and lactoferrin) themselves and to see if a signal could be read from the tear fluid deposited onto the contact lenses [17]. The following figure represents how both contact lenses were incubated and which reagents were placed for both assay tests.

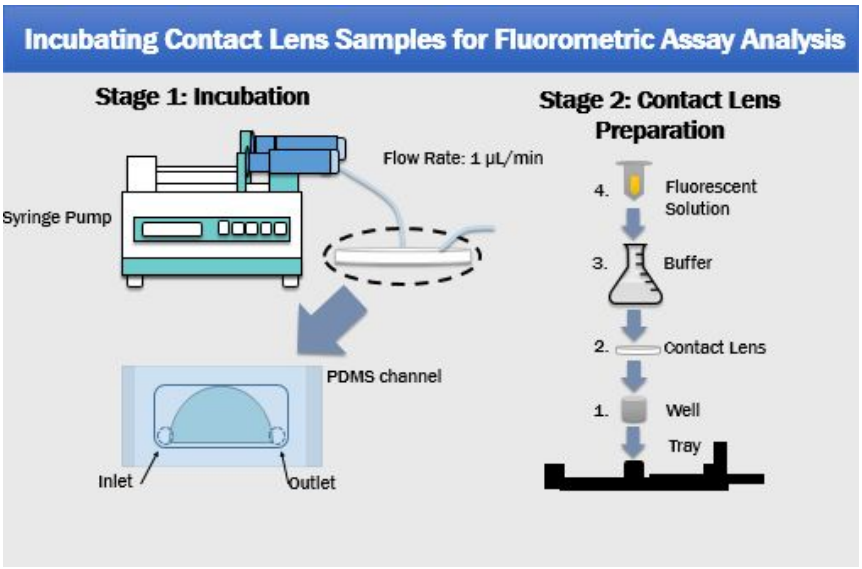
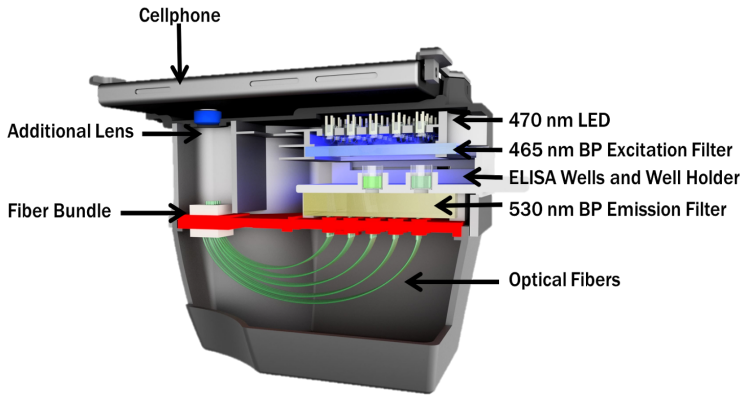


Figure 10:Artificial Incubation: For understanding the contact lens interactions with the tear fluid protein assays, an artificial incubation was done. The contact lenses were subjected to a specified concentration of either lysozyme or lactoferrin (concentrations would vary given the test being done). Furthermore, they were subjected to conditions that mimicked that of the eye. For example, they were subjected to the flow rate stated in Gachon's et al. study [25]. Stage two represents the preparation of contact lenses for analysis.

Fluorescence spectra in solution (and with contact lenses) was measured in two separate devices for lysozyme and lactoferrin. For lysozyme, a 3D printed optomechanical fluorescent ELISA reader was created and is shown below in figure 2. The device houses a 470 nm LED, a 465 nm BP excitation filter, ELISA wells, a 530 n, BP emission filter, and optical fibers to transmit the light to the cellphone image sensor [3]. These specific optical components were chosen to measure this specific assay. Future studies must be done to see how this device can be multiplexed to measure a panel of proteins. The fluorescent light was recorded with a Nokia Lumia cell-phone (also shown in figure 1).



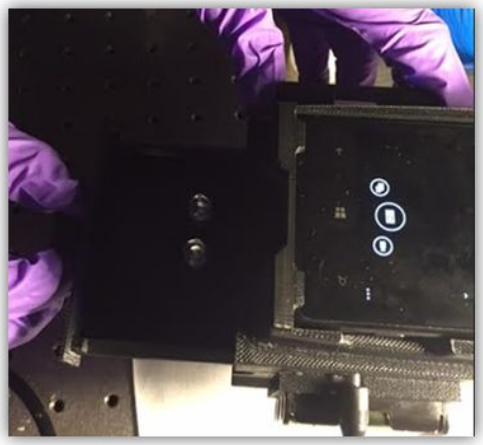
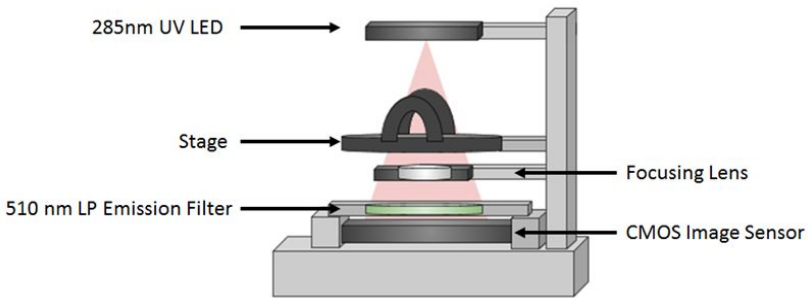


Figure 2: Lysozyme Assay Mobile Phone-Based Device- This device was calibrated specifically to measure lysozyme in solution. It uses a series of optomechanical parts in order to measure the fluorescent assay.

To measure lactoferrin, a 285 nm UV LED was used as an excitation light source; an additional lens was placed before the 510 nm LP emission filter; and a CMOS Image sensor was used to calibrate the assay before integrating the device with a cell-phone (refer to figure 3 below). These specific optomechanical components were used in order to specifically measure lactoferrin in solution following the parameters set by Daniel Citterio [15].



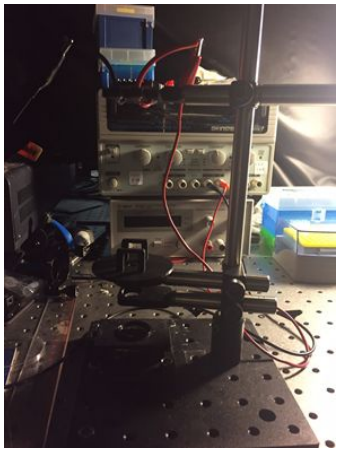


Figure 3: Lactoferrin Set Up- Apparatus for collecting data using optomechanical parts and a UV LED.

Following Gachon's study, adults ages 30-45 who regularly use contact lenses were sampled [24]. According to Gachon, adults naturally have a higher and more stable/less fluctuating concentration of tear proteins in comparison to children [24]. As a result, this device was calibrated with a higher dynamic range and limit of detection that may not be viable for use on children [23]. Adults were used as a dependent variable to see the effects of lens wear and deposition in comparison to artificially incubated contact lenses. The sample size consisted of a convenience sample of 3-5 adult males from the University of California Los Angeles (a large public research institute located in Southern California). Adult males were used for this study on the basis of availability and as a proxy for the population of healthy adult males.

A machine learning algorithm was created using Matlab code for image registration in order to image features within the wells and gather light intensity from the series of photons taken every minute for twelve minutes. Paralleling Berg's data analysis procedure, from the raw images, a scaled intensity map is created by the machine learning algorithm by normalizing the intensity values to a control [3]. Next, a graphical representation of the change in the intensity values over time was interpolated against a standard curve to attain protein concentration values [3]. The concentration values were further analyzed using a machine learning algorithm to generate a diagnoses or indicate clinically relevant lysozyme concentrations [3]. On the other hand, for sampling lactoferrin, an image processing software, ImageJ, was used along with mechanical analysis with Microsoft Excel. ImageJ was used to analyze the lactoferrin because the assay was not integrated with a cell-phone at the time of data collection.

V. Findings[4]

Lysozyme has a natural affinity to lyse bacteria cell walls by breaking the glycosidic linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine [12]. As a result of this affinity, it is possible to measure lysozyme concentration via a fluorescent assay [10]. A serial dilution titration using various concentrations of lysozyme was performed to understand how the device reads a fluorescent signal for varying concentrations of lysozyme. This experiment was performed not only to calibrate the device, but also to see if the device yielded comparable results to a gold-standard ELISA well-plate reader as recording in the commercial assay literature [18]. Using lysozyme concentrations of 0 mg/mL, 0.005 mg/mL, 0.01 mg/mL, 0.015 mg/mL, 0.02 mg/mL, 0.025 mg/mL, and 0.03mg/mL a serial dilution titration was achieved and compared to the data from an ELISA Well-Plate reader (refer to figure 11). While this graph does have comparable shape to the graph from the literature, further statistical analysis between my study's findings and my academic literature's findings could not be done because my academic literature did not release its data. Furthermore, the units of fluorescent intensity are arbitrary because each reader has a different dynamic range, thereby reading photons differently [3]. Therefore, it would not be of merit to do a comparative statistical analysis between both sources of data since the values for fluorescent intensity are arbitrary. In order to understand the

[4] All analysis was analyzed using comparative descriptive statistics.

dynamic range of the reader, a comparable shape between the foundational literature and the mobile reader is sufficient enough to merit a titration.

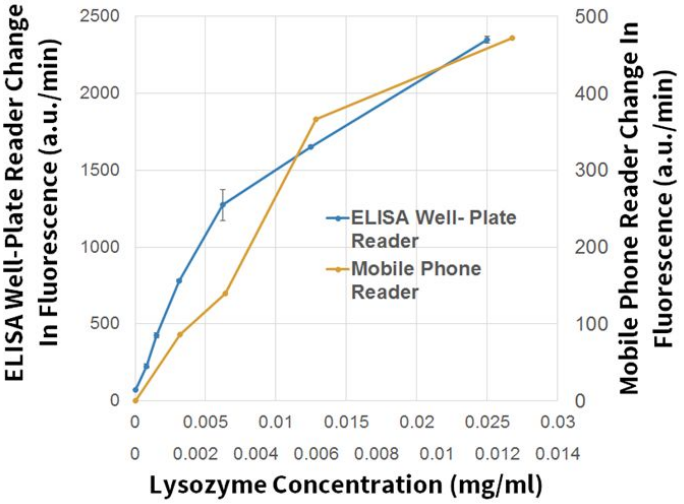


Figure 11: Lysozyme Serial Dilution Titration- This graph relates the fluorescent intensity of the lysozyme assay to the concentration of the assay. It also compares the fluorescent intensity values of the mobile reader with the gold-standard reader.

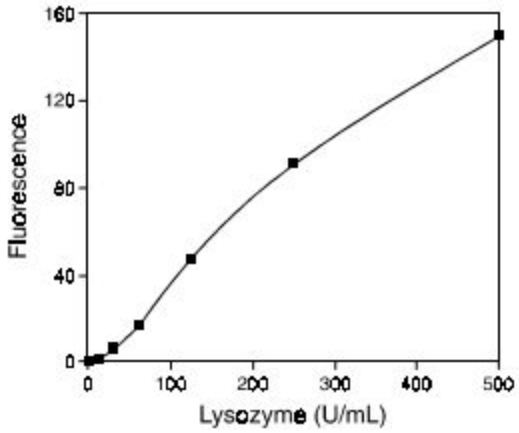


Figure 1. Detection of lysozyme activity using the EnzChek Lysozyme Assay Kit. Increasing amounts of lysozyme were incubated with the DQ lysozyme substrate for 60 minutes at 37°C. The fluorescence was measured in a fluorescence microplate reader using excitation/ emission of ~485/530 nm. A background fluorescence of 20 fluorescence units was subtracted from each value.

Figure 12: Literature Titration Curve: Source: Molecular Probes (2001). Description available from source [19].

Given the comparable relationship between the mobile phone reader, the gold-standard ELISA reader, and the gold-standard literature, a study was conducted to understand how adding a contact lens to the titration would affect the dynamic range of the lysozyme assay. The same procedure was used as the first titration, the only difference was that incubated contact lenses were used as the substrate for lysozyme samples. The following data was collected using contact lenses incubated in 0 mg/mL, 0.5 mg/mL, 1 mg/mL, and 2 mg/mL lysozyme concentration (refer to figure 13).

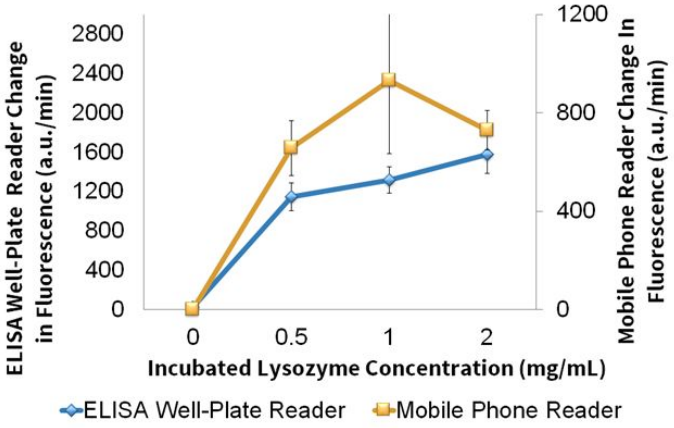


Figure 13: Incubated Contact Lens Lysozyme Serial Dilution Titration- This graph relates the fluorescent intensity of the lysozyme assay to the concentration of the assay. It also compares the fluorescent intensity values of the mobile reader with the gold-standard reader.

Given the comparable shape of the titration generated between the gold-standard reader and the mobile-phone reader, the graph indicates the titration for various lysozyme concentrations yielded comparable results to the gold-standard ELISA-well-plate reader. However, according to Charles Leahy in his study "Initial in Vivo Tear Protein Deposition on Individual Hydrogel Contact Lenses", contact lenses do not absorb all of the lysozyme they are incubated in [17]. In fact, there exists a protein transfer factor show below for a 2 mg/mL incubated lysozyme concentration. It was found that the standard deviation for the sample between the gold-standard ELISA Well-Plate Reader and the Mobile Reader was ±0.035 mg/mL indicating that both readers yield comparable results of diagnostic value.

	Incubation Concentration (mg/mL)	Final Lysozyme Concentration (mg/mL)	Protein Transfer Factor ($x10^{-3}$)
Mobile Reader	2	0.019	9.5
ELISA Well-Plate Reader	2	0.014	6.8

Figure 14: Incubated Contact Lens Lysozyme Protein Transfer Factor. This data relates the protein transfer factor to lysozyme deposition on contact lenses [35].

As the relationship between contact lens deposition within the lysozyme assay system was established, a human participant's tear fluid was sampled over a three day period under IRB approval. According to Gachon et al. the human participant's tear fluid should not fluctuate if the participant is healthy because abnormal tear secretion only occurs in the advent of a bacterial infection or other autoimmune system disorders [24]. Therefore, it was fitting that the data taken over the three day period revealed that the participant's lysozyme concentration remained at around 3 mg/mL with an average concentration of 2.71± 0.13 mg/mL. This value is very close to the reported value in Gachon's research of 2.39±0.65 mg/mL (refer to figure 15) [24]. The percent error between the two readings 13.38% mg/mL. The percent difference can be attributed to protein denaturation. According to a study conducted by Mann and Tighe at Aston University, denaturation occurs as a result of a protein unfolding and losing its tertiary structure [24]. This process could occur as a result of lens drying and solution interaction [24]. As a result, the

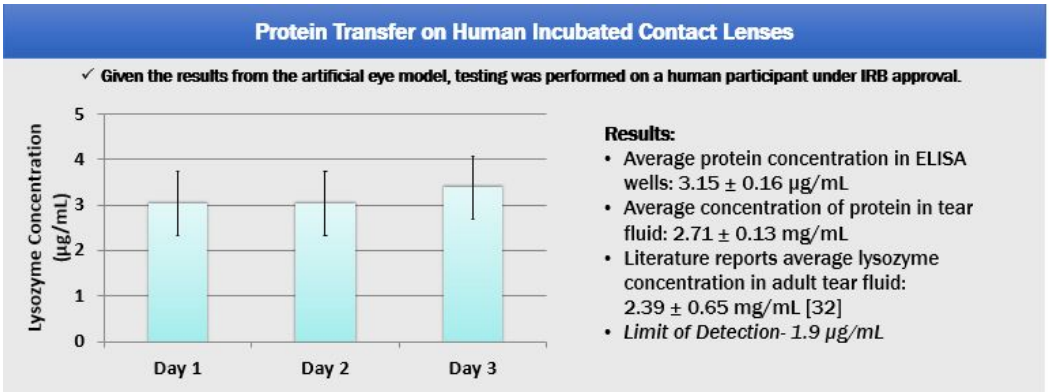


Figure 15: Human Incubated Contact Lenses: This data represents a three day study to measure human tears conducted under IRB approval.

contact lenses tested in both readers should have had different properties (dryness, temperature,etc.) that contributed to the percent error in the measurement. Further studies would have to be conducted to understand the root of the error.

A similar study was conducted to measure lactoferrin deposited on a contact lens. In a comparable study to Daniel Citterio's work with a paper based assay for analyzing lactoferrin in human tear fluid, my study focused on implementing his same chemical principles but in a liquid solution in order to reduce the need for sample preparation. In order to calibrate the optomechanical setup, a fluorescence emission spectra was measured using a gold-standard spectrofluorometer .

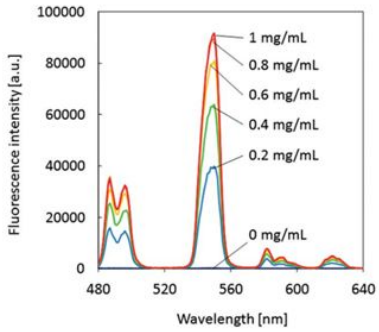


Fig. 3 Fluorescence emission spectra of 100 µM $TbCl_3$ solutions (50 mM HEPES, 3.75 mM $NaHCO_3$, pH 7.4) in the absence and presence of human lactoferrin at various concentrations; $\lambda_{ex} = 290$ nm.

Figure 7: Source: Daniel Citterio et al. 2014. Description provided by the source.

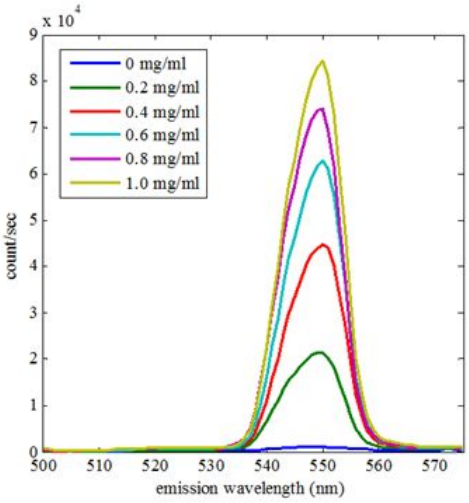


Figure 16: Fluorescence Emission Spectra for Lactoferrin Assay in Solution.

Both fluorescence spectra have comparable shapes for their respective lactoferrin concentrations indicating that it is possible to measure lactoferrin using a liquid based solution setup. Furthermore, a direct statistical comparison to understand the accuracy of the measurements in comparison to Citterio's study could not be done because Citterio has not disclosed the data he gathered. Additionally, the units for light intensity are arbitrary indicating that statistically comparing the fluorescent intensity of the system would not be a viable analysis for determining accuracy [3].

Since the overall goal of this study is to see if it is possible to measure lactoferrin on contact lenses, a second test was conducted to determine if the CMOS image sensor could read a signal from the assay just as well as the gold-standard spectrofluorometer (refer to figure 17). It was found that the CMOS image sensor could read a signal from a 4 mg/mL lactoferrin solution. However, when compared to the control of a 0 mg/mL lactoferrin solution the signal is very weak. The weak signal could be a result of autofluorescence from the external environment as the device was not made into a closed system [32]. Likewise, a second test was conducted to understand the amount of autofluorescence within the signal when a contact lens was placed into the system. Shown in figure 18, it was possible to attain a signal from the system using a gold-standard spectrofluorometer . However, the contact lens still yielded a considerable amount of autofluorescence such that the CMOS reader would not be able to distinguish the fluorescent signal from the contact lens autofluorescence. Due to the unique nature of the lactoferrin assay, Knight and Billiton have studied methods to increase the signal to noise ratio of biological assays and imaging systems [32]. Knight et al. reveal that autofluorescence tends to plague assays that are excited in UV light. Specialized optical filters can be used to increase the signal-to-noise ratio of the system [32].

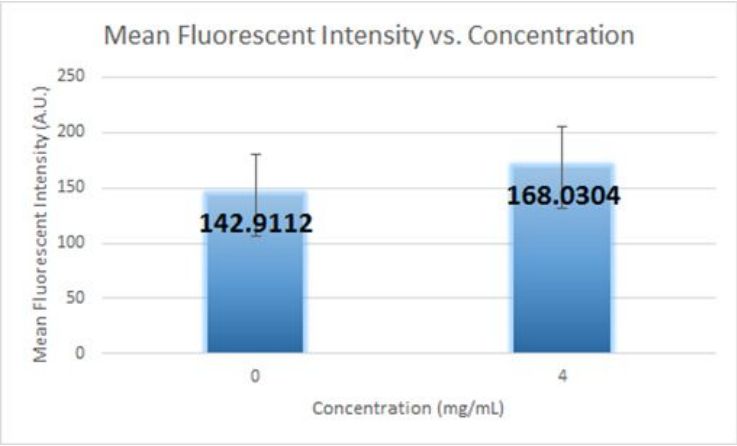


Figure 17: Fluorescence intensity of lactoferrin sample measured with the CMOS imaging system.

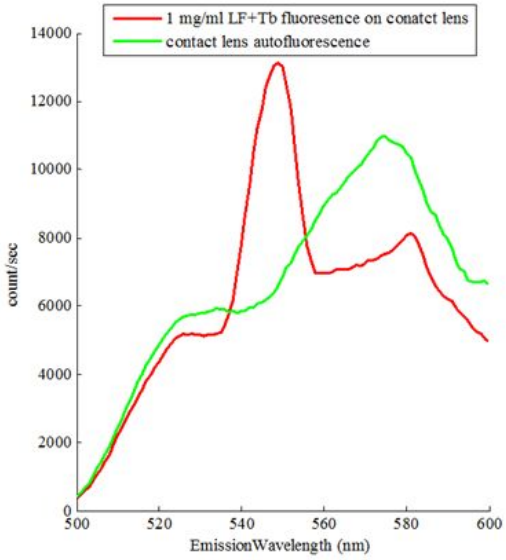


Figure 18: Fluorescent emission of lactoferrin-terbium complex versus the autofluorescent emission of contact lenses.

VI.Conclusion

Overall, my study found that it is possible to measure lysozyme and lactoferrin on contact lenses. The lysozyme assay mobile-phone reader allows for a shortened turnaround time such that gold-standard results can be gathered within ~5 minutes. The lysozyme assay mobile-phone reader yielded lysozyme protein measurements within a 13.38% mg/mL sampling error [18]. Further tests to improve the machine learning algorithm developed by Brandon Berg has not been studied; however, doing so will improve the calibration of the device in order to attain better diagnostic results [3]. Additionally, the serial dilution titration between the lysozyme assay and the same assay measured in a gold-standard ELISA reader exhibited a comparable titration curve shape, meaning that both devices yield comparable measurements with a comparable dynamic range. The same graphical behavior was found in the incubated lysozyme contact lens titration, indicating that the mobile-phone reader was calibrated to read at a comparable dynamic range as the ELISA reader. The lactoferrin assay on the other hand could not yield a signal strong enough to measure clinically relevant lactoferrin levels [18]. Through troubleshooting the assay, it was found that the chemical complexation between lactoferrin and terbium did occur as a signal was measured with a gold-standard spectrofluorometer . However, when the same assay was placed in the CMOS image sensing system, the signal was not strong enough to yield viable results in comparison to Citterio's work due to an excess autofluorescence signal [15]. Therefore, further studies with the lactoferrin assay is of interest in order to multiplex the lysozyme mobile-reader.

My study improves upon the current academic studies of Brandon Berg and Daniel Citterio because it presents a new and novel method of measuring proteins in tear fluid: specifically lysozyme and lactoferrin. This method of measuring lysozyme and lactoferrin using a mobile phone has never been studied before. Therefore, by improving upon Citterio's paper-based device, with the limitation of a turnaround time of ~15 min and needing to recreate each paper strip for testing, my study allows for high-throughput testing without the need for a laboratory setting [15]. Furthermore, by modifying Brandon Berg's device to specifically measure lysozyme in tear fluid, my study reveals that the modular design of Berg's device can allow for the mobile-phone-reader to be multiplexed to measure a panel of proteins and biological substances in order to improve disease diagnostics [3]. Nevertheless, the assays could be enhanced through modification of the machine learning algorithm in order to obtain more accurate and clinically relevant lysozyme and lactoferrin measurements. More specifically, the lactoferrin assay has a low signal-to-noise ratio. There exists multiple studies that describe techniques to increase the signal-to-noise ratio in bio-photonic analyses, such as Knight's work with dual-wavelength fluorescence spectra [32]. The use of dual-wavelength fluorescence spectra analysis has not been implemented with either a lysozyme or lactoferrin assay.

Lastly, the purpose of this work is to create a portable device to improve the field of medical diagnostics. The portability, clinical relevance, and simplicity of the device allows for individuals with minimal medical training to perform gold-standard diagnostic testing.

Furthermore, the connectivity of cellular devices allows for results to be shared on the cloud which can connect low-resource and rural areas to laboratory and medical centers throughout the world.

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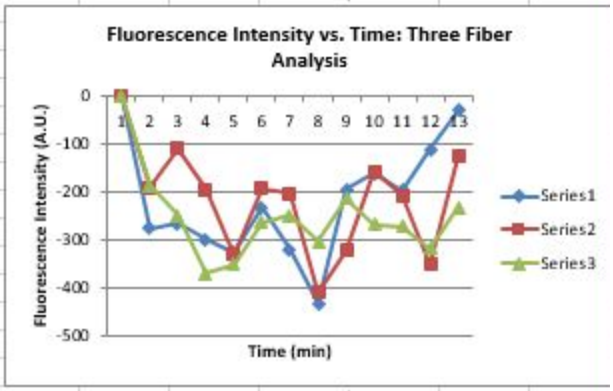
Appendix

Lysozyme Data Collected for Mobile Phone Reader Analysis

500 u/mL Analysis

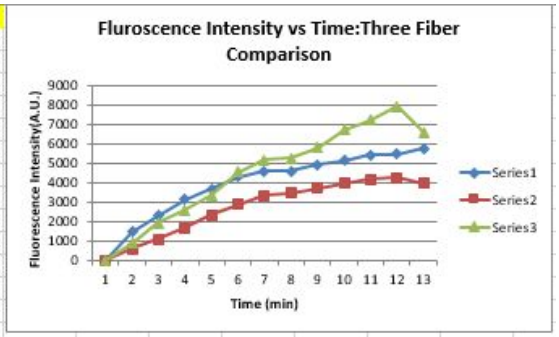
Minutes	Intensity	Yzeroed	Intensity 1	Y1zeroec	Intensity 2	Y2zeroed
1	11452	0	8391.816	0	7769.856	0
2	11175	-276.5	8201.148	-190.7	7583.447	-186.4
3	11187	-264.9	8283.265	-108.6	7520.789	-249.1
4	11153	-298.6	8197.969	-193.8	7399.835	-370
5	11125	-326.9	8061.683	-330.1	7418.411	-351.4
6	11220	-231.4	8198.705	-193.1	7505.275	-264.6
7	11131	-320.3	8189.138	-202.7	7521.472	-248.4
8	11018	-434	7982.187	-409.6	7465.6	-304.3
9	11256	-195.1	8068.743	-323.1	7555.539	-214.3
10	11291	-160.6	8235.268	-156.5	7501.359	-268.5
11	11256	-196	8184.955	-206.9	7497.187	-272.7
12	11340	-111.2	8040.57	-351.2	7452.103	-317.8
13	11421	-30.43	8266.542	-125.3	7537.01	-232.8

x	Yzeroed	Y1zeroec	Y2zeroed	Average
1	0	0	0	0
2	-276.5	-190.7	-186.409	-217.8
3	-264.9	-108.6	-249.067	-207.5
4	-298.6	-193.8	-370.021	-287.5
5	-326.9	-330.1	-351.445	-336.2
6	-231.4	-193.1	-264.581	-229.7
7	-320.3	-202.7	-248.384	-257.1
8	-434	-409.6	-304.256	-382.6
9	-195.1	-323.1	-214.317	-244.2
10	-160.6	-156.5	-268.497	-195.2
11	-196	-206.9	-272.669	-225.2
12	-111.2	-351.2	-317.753	-260.1
13	-30.43	-125.3	-232.846	-129.5

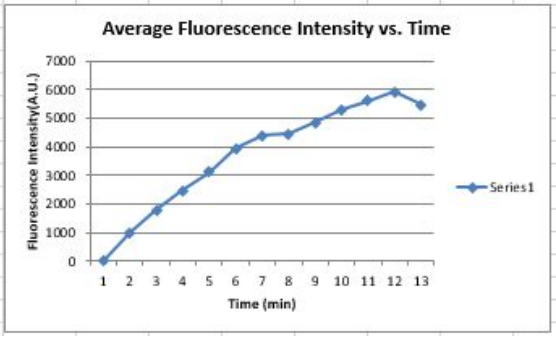


1000 u/mL Analysis

Time	Intensity	Yzeroed	Intensity 1	Y1zeroed	Intensity 2	Y2zeroed
1	48828.781	0	50290.379	0	42003.535	0
2	50306.746	1477.965	50893.805	603.426	42907.777	904.242
3	51138.582	2309.801	51401.574	1111.195	43929.613	1926.078
4	51966.602	3137.821	51959.742	1669.363	44622.875	2619.34
5	52518.93	3690.149	52624.152	2333.773	45353.656	3350.121
6	53128.125	4299.344	53206.797	2916.418	46566.504	4562.969
7	53442.207	4613.426	53637.469	3347.09	47181.934	5178.399
8	53445.828	4617.047	53765.969	3475.59	47261.875	5258.34
9	53765.027	4936.246	54037.078	3746.699	47835.695	5832.16
10	53985.602	5156.821	54291.105	4000.726	48742.422	6738.887
11	54257.41	5428.629	54475.195	4184.816	49237.316	7233.781
12	54329.559	5500.778	54570.453	4280.074	49944.98	7941.445
13	54625.414	5796.633	54292.059	4001.68	48595.73	6592.195



Time	Yzeroed	Y1zeroed	Y2zeroed	Average
1	0	0	0	0
2	1477.965	603.426	904.242	995.211
3	2309.801	1111.195	1926.078	1782.358
4	3137.821	1669.363	2619.34	2475.508
5	3690.149	2333.773	3350.121	3124.681
6	4299.344	2916.418	4562.969	3926.243667
7	4613.426	3347.09	5178.399	4379.638333
8	4617.047	3475.59	5258.34	4450.325667
9	4936.246	3746.699	5832.16	4838.368333
10	5156.821	4000.726	6738.887	5298.811333
11	5428.629	4184.816	7233.781	5615.742
12	5500.778	4280.074	7941.445	5907.432333
13	5796.633	4001.68	6592.195	5463.502667



Synthesis of lysozyme data: Process Analysis

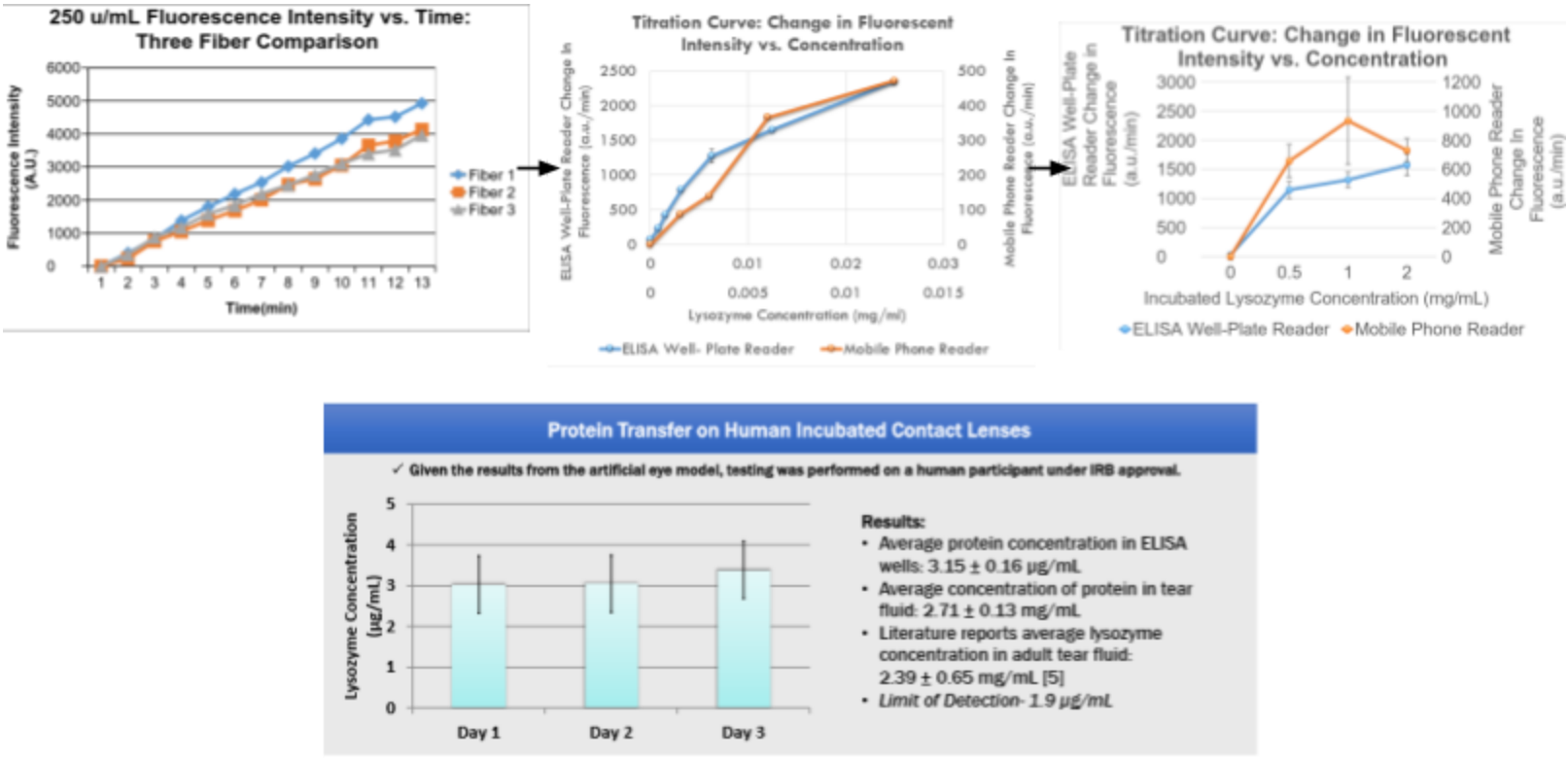
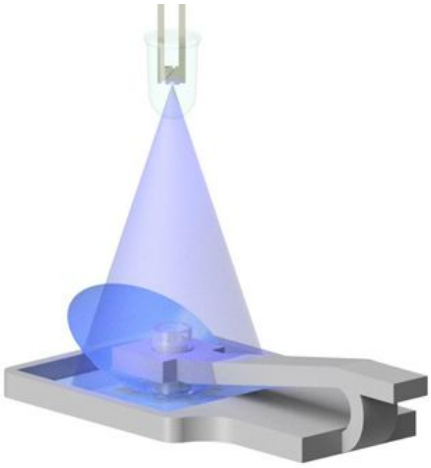
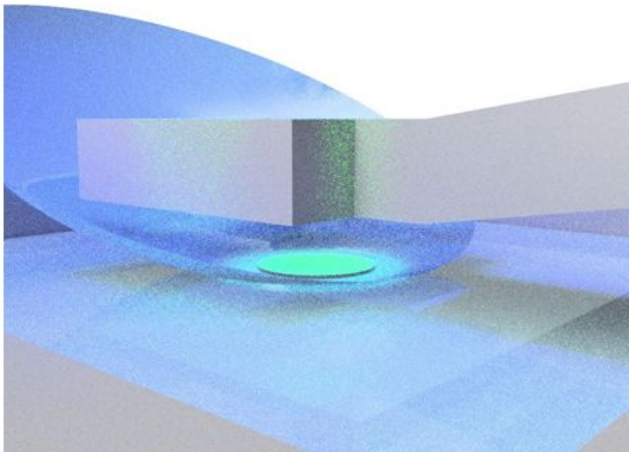


Figure: Titration through Contact Lens Incubation Data Processing- In order to process the data and compare the serial dilution titration with the contact lens titration, first a single sample with a known concentration of lysozyme was placed in the cell-phone based device and the fluorescent intensity of the assay was gathered. Since this device was adapted to measure the lysozyme assay specifically, three optical fibers were placed below each well sampling area to gather a large field of view and minimize optical aberrations. The data gathered from one known concentration of lysozyme was plotted (as shown in the first graph). A serial dilution was performed in both the gold-standard Elisa Well- Plate Reader and the Mobile Phone Reader to generate the second graph. Finally, a serial dilution titration was performed using contact lenses with known concentrations of lysozyme to generate the third graph. In order to test contact lenses with unknown concentrations of lysozyme, a human participant wore contact lenses for twelve hours. The contact lenses were analyzed in both the mobile reader and the gold-standard ELISA Well-Plate Reader and concentration values were deduced from interpolating the fluorescent intensity data gathered from the human worn contact lenses with the incubated contact lens titration curve. This data was gathered over a three day period and plotted on the bar graph shown above.

Lactoferrin Data Collected for Preliminary Titration Analysis

Artist Rendering of Assay Fluorescence

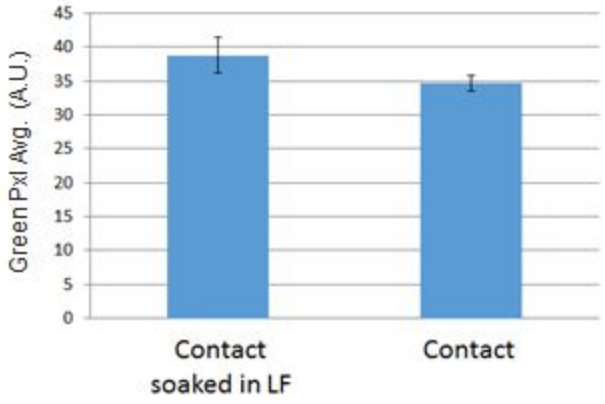




Sample of the Spectrofluorometer Lactoferrin Titration Data

Wavelength	0 mg/ml	0.2 mg/ml	0.4 mg/ml	0.6 mg/ml	0.8 mg/ml	1.0 mg/ml
499.95	46.6411	143.257	333.166	361.487	424.793	439.786
500.95	57.398	108.044	209.339	346.092	312.325	369.729
501.95	63.2908	112.898	189.876	311.334	265.145	316.466
502.95	65.852	154.235	209.691	244.352	298.078	376.068
503.95	80.7455	121.119	228.199	222.933	317.728	373.904
504.95	85.3308	161.775	243.554	293.334	321.779	384.006
505.95	88.2054	181.814	268.224	300.628	327.632	385.24
506.95	107.528	154.914	271.559	267.914	328.061	386.386
507.95	121.763	221.39	293.345	381.906	374.526	357.921
508.95	141.913	227.811	349.192	364.131	422.022	464.974
509.95	171.96	198.416	302.353	355.268	423.302	476.218
510.95	221.704	233.171	353.585	424.307	479.738	563.842
511.95	220.333	309.243	425.216	402.021	552.79	492.868
512.95	271.643	306.821	381.087	478.808	568.714	517.897
513.95	219.309	373.426	499.885	407.016	644.133	620.421
514.95	259.646	391.472	491.344	551.269	611.194	655.14
515.95	320.989	357.329	545.091	563.262	646.042	621.814
516.95	320.327	430.51	634.56	552.939	767.198	801.889
517.95	375.252	449.482	637.125	630.939	752.602	849.523
518.95	316.664	412.501	687.526	654.189	762.536	770.871
519.95	345.192	465.174	681.993	681.993	797.775	871.457
520.95	402.881	539.315	690.678	726.92	801.538	842.046
521.95	399.364	451.176	753.428	690.817	870.016	960.699
522.95	430.605	555.205	765.066	716.972	872.186	985.868
523.95	471.349	513.397	728.068	772.332	865.287	956.031
524.95	400.954	616.007	734.738	801.946	936.366	1014.78
525.95	501.023	485.153	813.909	791.236	945.419	1061.06
526.95	438.18	529.952	724.972	706.617	1050.79	1007.19

Incubated Contact Lens: CMOS Reader Emission



- Lens soaked overnight in 2 mg/mL Lf soln.
- Signal very weak
- Autofluorescence