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I, Richard C Murdock, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Electrical Engineering.

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Development of Microfluidic Paper-based Analytical Devices for Point-of-Care Human Physiological and Performance Monitoring

Student’s name: Richard C Murdock

This work and its defense approved by:

Committee chair: Ian Papautsky, Ph.D.

Committee member: Joshua A Hagen, Ph.D

Committee member: Andrew Steckl, Ph.D.

Committee member: William Wee, Ph.D.

Committee member: Alison Weiss, Ph.D.

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DEVELOPMENT OF MICROFLUIDIC PAPER-BASED ANALYTICAL DEVICES FOR POINT-OF-CARE HUMAN PHYSIOLOGICAL AND PERFORMANCE MONITORING

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Richard Craig Murdock

B.S., Wright State University, Dayton, OH, 2006
M.S., Wright State University, Dayton, OH, 2010

Committee Chair: Ian Papautsky, Ph.D.
ABSTRACT

In this dissertation, two applications of microfluidic paper-based analytical devices (µPADs) as potential point-of-care (POC) devices were demonstrated. With their inexpensive fabrication, reduced reagent and sample volumes, compatibility with image analysis techniques, and usability outside of conventional laboratories, the µPADs provide a unique platform for performing a wide variety of bioassays. The first application is for the development of a paper-based enzyme-linked immunosorbent assay (P-ELISA) for the detection of a human performance biomarker, Neuropeptide Y (NPY). After optimizing the P-ELISA detection of rabbit IgG through the use of a novel combination of colorimetric image analysis and efficient enzyme – substrate systems, limits of detection were improved four orders of magnitude over previously reported values. Additionally, automated image analysis methods were developed to further simply quantitative measurements when using colorimetric substrates on paper-based platforms. The second application illustrates the transition of a clinical laboratory-based influenza assay to a one-step, POC-style µPAD which is capable of determining if a sample contains influenza and if an antiviral treatment would be effective. The µPAD was optimized to detect multiple strains of Influenza A and B, as well as determine if potential interferents, such as Streptococcus pneumoniae, are present in the sample. Operation of the µPAD simply involved adding a sample to the top port; the sample then transferred to four different reagent zones, and enzymatic reactions under different buffer conditions took place on the bottom of the device. Analysis can be performed by eye or through a colorimetric image analysis smartphone app. The µPAD
produced excellent results for all influenza types and sub-types tested. There is enormous potential for this type of device in not only limited resource environments, but in first world countries as well. Both of these applications demonstrate how µPADs can be used as versatile platforms for performing clinical-laboratory assays as POC devices.
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# TABLE OF CONTENTS

LIST OF FIGURES ................................................................................................................. viii

LIST OF TABLES .................................................................................................................... xii

CHAPTER I: INTRODUCTION ................................................................................................. 1
    Scope of Work .................................................................................................................. 5
    Significance and Innovations ......................................................................................... 7
    Chapter Summaries ......................................................................................................... 8

CHAPTER II: A MULTI-STEP IMMUNOASSAY FOR A HUMAN PERFORMANCE
BIOMARKER USING A PAPER-BASED PLATFORM WITH DIGITAL IMAGE
MEASUREMENT ANALYSIS .................................................................................................. 9
    Introduction ................................................................................................................... 9
    Materials and Methods ............................................................................................... 13
    Optimization of P-ELISA for NPY Detection ............................................................ 19
    Optimization of Image Analysis ............................................................................... 27
    Summary ..................................................................................................................... 36

CHAPTER III: A CLINICAL LABORATORY-BASED INFECTIOUS DISEASE
ASSAY IN A ONE-STEP, POINT-OF-CARE, MULTI-LAYER PAPER DEVICE
WITH PRE-LOADED REAGENTS ....................................................................................... 37
    Introduction ................................................................................................................ 37
    Materials and Methods .............................................................................................. 40
    Neuraminidase Activity Assay Optimization ............................................................. 44
    Variability of Influenza Type and Subtype Response to Neuraminidase Assay ...... 48
    Influenza Microfluidic Paper-based Analytical Device .............................................. 53
    Summary .................................................................................................................... 65

CHAPTER IV: CONCLUSIONS ............................................................................................... 66
    Future Work ................................................................................................................ 68

REFERENCES ....................................................................................................................... 70

APPENDIX A: MATLAB CODE FOR AUTOMATED IMAGE ANALYSIS ....................... 75
LIST OF FIGURES

Figure | Page
--- | ---
1. Evolution of Paper-based Assays. | 1
2. Overall Concept of Paper-based Point-of-Care Diagnostics. | 6
3. Schematic of P-ELISA Process. | 15
4. Effect of Enzyme Substrates and Sample Media Complexity on P-ELISA Function. 
   (A) Rabbit IgG in DI H2O using BCIP/NBT Substrate with a semi-log best fit (log x, y). 
   (B) Rabbit IgG in human saliva using BCIP/NBT substrate with a semi-log best fit (log x, y). 
   (C) Rabbit IgG in DI H2O using pNPP Substrate with a log-log best fit (log x, log y). 
   (D) Rabbit IgG in human saliva using pNPP Substrate with a log-log best fit (log x, log y). 
   Error bars indicate standard error of the mean (SEM) with n=4. | 20
5. Comparison of Enzyme Substrates and Media Complexity for the Detection of NPY using P-ELISA. 
   (A) Detection of NPY using BCIP/NBT substrate. 
   (B) Detection of NPY using pNPP substrate. 
   (C) Detection of NPY in human saliva using pNPP substrate. 
   Error bars indicate SEM with n=4. | 23
6. P-ELISA of NPY in DI H2O using HRP-labeled NPY Antibody and Colorimetric Substrate. 
   Semi-log best fit (log x, y). 
   Error bars indicate SEM with n=4. | 25
7. P-ELISA of NPY in DI H2O using HRP-labeled NPY Antibody and Chemiluminescent Substrate. 
   Semi-log best fit (log x, y). | 26
8. Enzyme-free P-ELISA of Rabbit IgG in DI H2O using IgG antibody conjugated Au NPs with silver enhancement stain. 
   (A) Plot of delta RGB vs. IgG concentration using Au NP – silver enhancement procedure. 
   Error bars indicate SEM with n=4. 
   (B) Schematic of Enzyme-free P-ELISA Assay. | 27
10. Comparison measurement of simulated pNPP ELISA substrate using delta RGB or grayscale analysis. 
    (Left image) Color image of simulated yellow substrate scale and white-black scale. 
    (Right image) Image after grayscale conversion. 
    Plot shows the comparison of the yellow scale (simulated substrate) to the white-black scale as a percentage of maximum value in both the RGB and Grayscale images. | 29

12. Comparison of Delta RGB and Delta CIE Image Change Quantification Methods for P-ELISA. (A) Delta RGB measurements from a digital image of a NPY P-ELISA. (B) Delta CIE measurements from the same digital image of the NPY P-ELISA. Error bars indicate SEM with n=4.

13. Automated MATLAB Image Analysis Process and Quantification of IgG P-ELISA. (A) Original image of IgG P-ELISA assay. (B) Thresholded Black & White Image conversion. (C) Identification of all circular white areas within specified parameters for creation of mask. (D) Selected circular white areas on mask are reduced in size to avoid edges of test spots. (E) Average RGB values for each test zone identified by the mask layer are processed by MATLAB to calculate the actual concentration of IgG present.


15. Comparison of Influenza Detection on Paper under Humid and Dry Assay Conditions. (A) Image of influenza neuraminidase activity assay under humidified and non-humidified (dry) assay conditions. Image analysis results (ΔCIE) of the influenza neuraminidase activity assay under (B) humid and (C) non-humid (dry) assay conditions. Specific sH1N1/07L-R neuraminidase activity is observed at pH of 7 with 0.1 mM Ca+ present (pH7+).

16. Comparison of Influenza Detection on Paper with Increasing Buffer Strength. (A) Image of influenza neuraminidase activity assay under 1X, 2X, and 3X buffer conditions. (B) Image analysis results (ΔCIE) of the sH1N1/07L-R influenza neuraminidase activity assay with 1X, 2X, and 3X buffers at pH of 4, 7, and 9. All buffers contained 0.1 mM Ca+2.

17. Comparison of Influenza Detection on Paper with Increasing Substrate and Increased Incubation Temperature. (A) Image of influenza neuraminidase activity assay after incubation at 20°C and 37°C, with substrate concentrations of 3.33X and 5X. (B) Image analysis results (ΔCIE) of the sH1N1/07L-R influenza neuraminidase activity assay after incubation at 20°C and 37°C, with substrate concentrations of 3.33X and 5X and buffer pH of 4, 7, and 9. All buffers contained 0.1 mM Ca+2.
18. Time Course Studies for Determination of Minimum Assay Time at Different Incubation Temperatures. Neuraminidase activity of sH1N1/07L-R, PIV, and S. pneumoniae at (A) 20°C and (B) 37°C. All samples were tested in pH 7 buffer with 0.1 mM Ca+2. .................................................................49

19. Previously Characterized Influence of pH and presence of Ca+2 on NA Activity. (A) NA activity was measured by incubating virus with fluorogenic substrate at different pH values. NA activity in acetate buffer pH 4, 5 and 6 is shown as closed squares (■), in PBS pH 6, 7 and 8 is shown as open circles (O), in Tris buffer pH 8 and 9 is shown as closed diamonds (♦), in CHES buffer pH 9 and 10 is shown as open triangles. (B) Calcium (+) indicates NA activity measured in the presence of 0.1 mM Ca2+; calcium (-) indicates NA activity measures in the presence of 25 mM of EDTA chelator. All plots show results of at least 3 independent experiments and the error bars indicate SD. ..................................................................................50

20. Strain Variation in NA Activity due to Different Buffer Conditions. (A) Image of neuraminidase activity assay with buffers at pH of 4, 7, and 9 and either 0.1 mM Ca+2 (+) or 5 mM EDTA. (B) Image analysis results (ΔCIE). (C) Summary of a positive or negative NA activity response based on threshold applied to the ΔCIE results (0.05). ..............................................................................................................51

21. Evaluation of NAI Effectiveness. (Left) Image of NA activity at pH 7+ compared to pH 7+ with oseltamivir (Tamiflu) added (350 nM). (Right) Corresponding image analysis results (ΔCIE). ........................................................................................................52

22. Design Iterations of the Influenza µPAD. (A) Two-layer device with reagent storage in bottom layer and lateral transport of sample. (B) Expanded two-layer device with additional buffer pathways. (C) Three-layer device with vertical sample transport. (D) Multi-layer device with outer lamination and glass fiber reagent storage pads. ........................................................................................................54

23. Influenza µPAD Design and Fabrication. (A) Exploded view of the influenza µPAD construction. (B) Image of the influenza µPADs after lamination and prior to individual device cutting. Nine devices can be manufactured at once. (C) Finished influenza µPAD, front and back. ........................................................................................................57

24. Evaluation of Enzyme Substrate Activity following Storage in Pullulan Matrix. (A) Pullulan was added at increasing concentrations to a non-dried, purified NA / X-NeuNAc reaction to test for potential assay interference. (B) X-NeuNAc was stored in Pullulan at increasing concentrations to determine optimal storage buffer concentration. ........................................................................................................59

25. Effect of Pre-Treatment of Distribution Layer on Influenza µPAD Operation. Treatment was done to prevent non-specific adherence of influenza particles to cellulose fibers. (A) No Treatment. (B) Treatment with 0.5% w/v BSA. (C) Treatment with 20 mM CTAB. ........................................................................................................60

26. Schematic for Interpretation of Influenza µPAD Assay ........................................................................................................62

27. Influenza µPADs Tested with Various Influenza Strains ........................................................................................................64
Image Analysis of the Influenza µPADs Tested with Various Influenza Strains. See Figure 26. In this case, the CIE X-coordinate change is being monitored for determination of a blue or non-blue result for each test spot.
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>53</td>
</tr>
</tbody>
</table>

1. Summary of previously characterized virus types and subtypes, as well as *S. pneumoniae* strains which are evaluated in this study. ..................................................53
CHAPTER I

INTRODUCTION

Paper-based materials have been incorporated into rapid diagnostic assays recently for a wide range of point-of-care (POC) applications, and in different forms, including dipsticks, lateral flow assays (LFAs), and microfluidic paper-based analytical devices (µPAD) [1]. Paper has been used as a substrate for diagnostics for quite some time, with urine dipsticks being introduced in 1850, followed by pH test strips in the 1920’s [2], the first FDA-approved LFA-based pregnancy test in 1976 [3], and the introduction of 2D and 3D µPADs in 2008 [4] (Figure 1).

Figure 1. Evolution of Paper-based Assays.
Each of these style devices have their niches in the realm of paper-based diagnostics, varying primarily due to the composition, or types, of paper used in the devices. Dipstick and \(\mu\)PAD assays primarily are composed of cellulose based materials, such as filter or chromatography paper. Cellulose has many advantageous qualities, namely that it is fibrous, hydrophilic, biodegradable, and insoluble in water and most organic solvents [5]. Surface chemistry and porosity of the cellulose can be modified through nitration, also making it hydrophobic, which can aid in tailoring the paper-based diagnostic to the specific application [6]. LFA’s primary substrate is nitrocellulose, or partially nitrated cellulose, which significantly reduces the amount of non-specific binding of proteins and other biomolecules to the surface of the cellulose fibers, effectively reducing background in bioassays and increasing sensitivity. Also, additives, such as polymers or surfactants, have been added to nitrocellulose membranes to modify their wetting properties [7].

Dipstick assays have generally been used for simple, one-step reagent assays where the analyte reacts directly with the substrate, such as pH detection, chemical level detection in water, or urinalysis. Strips of either filter or chromatography paper are soaked in pH indicator solutions, in the case of pH detection, or other reagents. The strips are then dried and either used as is or combined with multiple reagents on a single plastic strip for a multiplexed assay. Urinalysis test strips combine detection of multiple analytes onto one strip, detecting as many as 10-12 different substances such as glucose, protein, ketones, and bilirubin [8].

On the other hand, LFAs are used when more involved bioassays are needed, such as when attempting to determine the presence, qualitatively or quantitatively, of specific antigens or proteins in a sample. LFAs typically have 5 primary components: a sample pad, a conjugate pad, a nitrocellulose membrane, a wicking pad, and a plastic backing (Figure 1, middle, top row) [9].
These types of molecules may not react directly with a substrate and may require specific antibodies to act as capture molecules to trap them onto the surface of the paper-based diagnostic from the sample through the use of sandwich or competitive type assays. In either style assay, the sample is added to the sample pad, it then flows to the conjugate pad where the sample mixes with antibody-labelled particles (usually gold nanoparticles or fluorescent dye infused beads) to form complexes, and then finally flows to the control and test lines where the antigen-particle conjugates will either be captured on the test line by the corresponding capture antibodies (sandwich assay), or the antigen-particle conjugates will flow past the test line since the antibody on the particle is now blocked (competitive assay) [7]. Excess particles not bound to the antigen will bind on the control line and are used to indicate that the assay is functioning properly and that all antibodies are in good condition. The output of the sandwich assay is an increasingly darker test line with increasing analyte concentration, while the competitive assay exhibits a weakening test line with increasing concentration. A more complex version of an LFA has been developed, known as a two dimensional paper network (2DPN) card, which is able to perform an LFA with two separate flow paths, allowing for a multi-step assay to be completed on an LFA format [10].

The uniqueness of µPADs, pioneered by George M. Whitesides’ group at Harvard University, lies in their ability to act as inexpensive, easily fabricated microfluidic devices with passive pumping of fluid through hydrophobic-defined channels by utilizing capillary forces [4]. The hydrophobic barriers can be made through a variety of techniques, including physical separation, photoresist patterning, PDMS (polydimethylsiloxane) inkjet printing, and wax-based printing [5]. Additionally, µPADs can be formed as 2D and 3D devices, with 2D devices having a single plane of microfluidic channels and 3D devices having multiple layers of interconnected
channels and/or pass-through connections. The 2D µPADs allow for simple processing and
distribution of a sample to multiple analysis areas. To add additional processing steps, 3D
µPADs can store reagents in different layers to sequentially add reagents to an assay, process
multiple samples within a small footprint, and allow for programming of assays to be performed
through mechanical switching mechanisms, or perform filtration of samples within the µPAD
itself. Other versions of a µPAD are a microfluidic paper-based electrochemical device (µPED),
where electrodes are either printed or applied via photolithography techniques onto paper devices
for electrochemical detection methods [11], and paper origami devices, which are multi-layer
paper devices that are similar to µPADs; however, the origami devices rely on folding layers to
make connections at specific time intervals or sequence to produce multi-step processing or
reagent additions [12].

Monitoring aspects of human physiology and performance during various activities has
recently become a highly investigated research area. Many new commercial products are
available now to monitor human physical activity or responses while performing activities
ranging from playing sports, to driving, and even sleeping. Most of these devices are electronic
in nature, relying on accelerometers, skin-contact electrodes, and proprietary software to record
or calculate various physiological parameters. However, monitoring cognitive and physiological
performance biomarkers, such as neuropeptides, glucose, and lactate, is still an emerging field
due to the complicated sample collection and processing, as well as the need for a clinical lab to
perform analysis. Additionally, the ability to rapidly determine if the patient has an infectious
disease, if it is viral or bacterial in nature, and what type of treatment the infection will respond
to, would be invaluable. This would not only significantly reduce the treatment decision time for
the primary care provider, but it would also significantly shorten the impact of symptoms and
infection on the patient as well as potentially minimize infectious disease transfer to others. Considering the rapid course of viral action and severity of symptoms, early detection of influenza is critical, especially in certain subpopulations with preexisting medical conditions and in cases where individuals are in close daily contact with a small group of people, influenza can spread rapidly and cause substantial illness and loss of work days.

**Scope of Work**

Developing rapid, straightforward, and cost effective assays that can detect and track human physiological and performance biomarkers through the use of a portable imaging system, such as a smartphone, which would run an application to quickly process the image and provide immediate diagnostic feedback, is the vision of this research program. The long term goal of this research is to develop a microfluidic paper-based analytical device (µPAD) platform with the ability to perform a wide-variety of normally clinical laboratory-based assays as a point-of-care diagnostic in a quick, portable, inexpensive, and disposable form without any loss of assay sensitivity. The overall objective is to show the feasibility of using paper-based platforms or µPADs to replace commonly performed medical diagnostics, either in clinical laboratories or current point-of-care diagnostics that require expensive electronic devices and disposables. Therefore, the central hypothesis is that a wide range of bioassays, which are currently performed using expensive laboratory equipment or procedures, can be completed on a paper-based platform, either single or multi-layer (µPAD), through appropriate fluid control, sample processing, and reagent storage. The significance of this technology is reduced sample volumes, reduced user input, portability of the assay, reduced cost of the assay, and ease of disposability.
We started first with understanding the fundamental issues associated with performing biomolecular assays on a paper-based substrate. From there, we added another layer of complexity by shifting from a single paper layer device to a multi-layer device (µPAD) which was able to store reagents and perform ordered reagent reaction steps, reducing user input. Additionally, since the overall objective was to show the feasibility of using paper-based platforms or µPADs to replace commonly performed medical diagnostics without requiring expensive electronic devices and disposables, the end concept needed to be able to perform qualitative measurements by eye and quantitative measurements through the use of a camera-enabled smartphone or tablet in conjunction with a custom-designed image analysis app (Figure 2).

Figure 2. Overall Concept of Paper-based Point-of-Care Diagnostics.

To achieve the overall objective, the research was divided into two specific aims, with increasing application and device complexity. The first aim was to demonstrate a multi-step immunoassay for a human performance biomarker using a paper-based platform with digital image measurement analysis. The second aim was to transition a clinical laboratory-based infectious disease assay to a one-step, point-of-care, multi-layer paper device with pre-loaded reagents. Both of these aims allow us to understand the fundamentals of working with paper
substrates, and work towards the long-term goal of adapting multiple varieties of clinical-laboratory biomolecular assays to point-of-care diagnostics.

It is expected that valuable knowledge will be gained in the areas of development of point of care paper-based diagnostics which incorporate enzymatic and antibody assays, require stored reagents, and require sample processing and distribution steps prior to a diagnostic assay. Additionally, the direct technology outcomes of the proposed aims are: 1) a more cost-effective, time-saving paper-based immunoassay, with similar limit of detection capabilities and measurement via a smartphone or tablet, and 2) a point-of-care, infectious disease diagnostic with the ability to distinguish viral vs. bacterial and potential treatment options.

**Significance and Innovations**

The overall significance of this work is to better characterize paper-based platforms for detection of biomolecules related to human physiological and performance state, as well as to make these assays, and their associated measurement techniques, portable so that they can be truly point-of-care devices. The ability to measure biomarkers related to human physiological and performance state in near real-time, using inexpensive, disposable devices with mobile measurement techniques would provide a significant advancement in telemedicine, personal health monitoring, and athletic fields.

The key innovations resulting from this work include the first demonstration of using a paper-based ELISA for the detection of a cognitive biomarker (Neuropeptide Y) and the first µPAD for diagnosis of an infectious disease (influenza) with potential treatment identification (Tamiflu). Currently, devices or methods that can accomplish these tasks are either expensive, time-consuming, limited to laboratory conditions, not portable, or are not designed to assess
human performance metrics, or any combination of these factors. The innovations described here would advance the understanding of the application space for biomarker detection on future paper-based platforms. Additionally, combining these novel detection methods with mobile imaging and analysis provides a truly point-of-care type system.

**Chapter Summaries**

Following this introduction, Chapter II describes the transition of an ELISA for the detection of a human performance biomarker, NPY, to a paper-based platform. Many enzyme and enzyme substrate combinations were attempted to optimize the sensitivity of the assay as well as reduce replicate variation. Additionally, automated colorimetric image analysis methods were used to provide quantitative measurement capabilities. Next, Chapter III describes the development of a µPAD for detection of influenza and determination of antiviral treatment effectiveness. Optimization of multiple assay parameters on the paper-based platform are discussed, such as buffer strength, substrate concentration, and incubation temperature to name a few. Dried storage of the buffers and substrate in the µPAD is described as well. Design and fabrication of the µPAD is presented, along with the testing of multiple influenza strains on the completed µPAD design. Finally, Chapter IV summarizes the work in this dissertation and provides insight into the future direction of paper-based diagnostic work.
CHAPTER II

A MULTI-STEP IMMUNOASSAY FOR A HUMAN PERFORMANCE BIOMARKER USING A PAPER-BASED PLATFORM WITH DIGITAL IMAGE MEASUREMENT ANALYSIS

Introduction

The idea of monitoring human performance through biomarkers has recently become a highlighted topic in many fields. Assessing cognitive state, physiological state, and/or stress levels is of concern in a wide range of professions, including air traffic control settings [13], anesthesiologists [14], [15], professional athletes [16], commercial aviation pilots [17], [18] military unmanned combat aerial vehicles (UCAV) pilots [19], and ground troops [20]. One of the predominant biomarkers of interest is Neuropeptide Y (NPY) due to its association with the regulation of stress and anxiety [21], [22], fear, learning and memory [23], [24], blood pressure [25], [26], food intake [27], and sympathetic nervous system activity [28]. These markers are very relevant for diagnoses and treatment of post-traumatic stress disorder (PTSD) [29], which is of significant interest for returning deployed military personnel. Also, they are very relevant to military occupations which require high vigilance over long periods of time and the ability to adapt in a stressful environment. NPY is the most abundant neuropeptide in the human brain [30], with high levels of expression in the amygdala, hypothalamus, cortex, and hippocampus,
and is widely expressed throughout the central nervous system [30]-[32]. The role of NPY in the behavioral effects of stress in humans has been explored by studies where the plasma NPY concentrations of soldiers undergoing military survival training were measured following extreme interrogation stress [20]. High NPY levels were indicative of individuals with more stress resilience, or “stress hardiness”, and had better performance scores, whereas lower NPY was related to symptoms of dissociation [20]. Additionally, when comparing a group of special forces troops to a group of general troops after exposure to uncontrollable stress, the special forces troops had a higher average change of NPY levels between baseline and stressed states, indicating that NPY could help with “stress toughness” [33]. NPY levels have also been shown to increase significantly after a traumatic brain injury (TBI) event [34]. The ability to quickly and inexpensively assess levels of NPY could provide significant advantages in the early determination of personnel stress levels in mission-critical roles, early TBI diagnoses, as well as diagnoses and treatment of PTSD.

Paper microfluidic analytical devices have emerged in recent years [35], [36], leading to development of a number of point-of-care (POC) analyses, including HIV chips [37], [38], paper-based ELISA [39], [41], and low-cost colorimetric diagnostic assays [42]-[47]. A paper-based enzyme-linked immunosorbent assay (P-ELISA) combines the sensitivity and specificity of an ELISA with the convenience, low cost, and ease-of-use of paper-based platforms [39]. P-ELISAs are much faster to complete, with results in under an hour, whereas conventional ELISAs would require a minimum of 6 hours to complete. Costs are significantly reduced as well, since only 3 µL each of sample, blocking, antibody, and substrate solutions are needed for each test zone. This means that ~17 P-ELISA plates could be performed from one 96-well plate conventional ELISA kit, assuming the amount of secondary (or enzyme-labeled) antibody is the
Another significant advantage of using paper microzone plates is that it allows the user to print plates “on-demand” and opens opportunities for a wide range of non-standard formats for customized assays [48].

Other commercially available ELISA assays that are similar in nature have been recently developed. One of these devices is the SlipChip, developed by Dr. Ismagilov’s group. It is an ELISA platform that has the potential to be operated at POC, and that could be a direct comparison to a paper-ELISA device [49]-[51]. The SlipChip consists of two nanopatterned glass slides containing rows of either channels or nanoliter chambers. The slides are placed on top of each other, with hydrophobic treatment of the contact surfaces, to allow for microfluidic connections to be made as the two layers are slid over each other. The primary advantage of this setup over conventional microfluidics is the ability to perform the assay by merely slipping the slide layers in a sequential order to perform an ELISA. This eliminates the need for complicated control systems, and since it is magnetic bead-based, no assay-specific surface modifications are required.

However, there are some major drawbacks of the SlipChip when compared to the paper-based ELISA. First, the SlipChip uses magnetic beads as the capture element for the assay, which means that at each point before the device is slipped to the next step, a magnet has to be introduced by the user to aggregate the beads on the proper side of the chip prior to slipping. Second, from a fabrication point of view, the SlipChip still requires access to clean-room grade equipment, such as photolithography instrumentation and chemicals for glass etching. Even if the system was converted over for injection molding, a significant amount of design cost would be involved to produce the device. Loading of reagents into the SlipChip requires moderate user input and time as well. A paper-based ELISA can still be performed in less time without
incubation of the assay in a heated incubator, further reducing necessary accessory equipment needed compared to the SlipChip.

Colorimetric results of these assays can be viewed by naked eye, although it is difficult to precisely quantify the small changes in the analyte amount [52]. Promising colorimetric detection results have been demonstrated using video cameras [53], digital color analyzers [54], scanners [55] or custom portable readers [43]. Here we demonstrate the use of a digital camera to image the P-ELISA for detection of rabbit IgG, and use color-based image processing techniques to quantify substrate concentration changes. This technique not only allows for a wider range of colorimetric substrates to be used since it does not limit the imaging dynamic range through conversion to grayscale, it also means that a mobile-device camera could be used to perform measurements outside a laboratory setting. A key drawback of all these methods is the need for specialized instrumentation and for manual image analysis with a computer. Image measurement automation was achieved using MATLAB, for a Windows-based tablet, and an Android-based app (.APK) for an Android-based tablet.

To verify operation of the P-ELISA, we performed a standard 96-well plate-based ELISA procedure on the P-ELISA platform for detection of rabbit IgG with a colorimetric substrate. We used a wax-printed 96-microzone paper plate with a 12x8 array of circular test zones for running multiple P-ELISAs in parallel. This allowed us to use common microplate processing techniques with the P-ELISA format. For our wax-printed microzone plates, each test zone was 3 mm in diameter and required a minimum of 1.5 µL of solution to completely wet the test zone; however, 3 µL of solution were used to provide ample wetting without over-saturation. As reported in Cheng et al., smaller test zones could be used in this format with similar test results, but were kept in a 96-well similar configuration for ease of laboratory processing and imaging.
Additionally, we performed optimization tests for detection of IgG and NPY with different enzymes and enzyme substrates for use with the P-ELISA platform. We also examined the effect of physiological sample solutions on the P-ELISA operation and assay sensitivity.

**Materials and Methods**

**Chemicals and Solutions**

Rabbit IgG (1 mg/mL), goat anti-rabbit IgG antibody conjugated with Alkaline Phosphatase (AP) enzyme (1 mg/mL), goat anti-rabbit IgG antibody, and the p-Nitrophenyl Phosphate (pNPP) substrate for ELISA were purchased from Sigma Aldrich. Neuropeptide Y (NPY) was purchased from GenScript. Mouse anti-human Neuropeptide Y monoclonal antibody, 1-Step Ultra TMB-ELISA, SuperSignal ELISA Pico Chemiluminescent Substrate, and the QuantaBlu Fluorogenic Peroxidase Substrate Kit were purchased from Thermo-Pierce. The AP and horseradish peroxidase (HRP) Lightning Link Enzyme Labeling Kits were purchased from Innova Biosciences. The thiol- and carboxyl-modified poly (ethylene glycol) (PEG) was purchased from Prochimia. The blocking buffer consisted of 0.05% v/v Tween 20 and 1% w/v bovine serum albumin in 1X phosphate-buffered saline (PBS). The antibody incubation solution contained 0.05% v/v Tween 20 in 1X PBS with the conjugated antibody (1:1000 ratio). The NBT/BCIP colorimetric substrate consisted of 2.68 mM 5-bromo-4-chloro-3-indolylphosphate toluidine salt (BCIP), 1.8 mM nitro-blue tetrazolium chloride (NBT), 5 mM MgCl₂, 100 mM NaCl, and 0.05% v/v Tween 20 in 100 mM Tris Buffer (pH 9.5). Unfiltered human saliva was obtained from Innovative Research.
Printing of Paper 96-well Plates

A ‘negative’ image of a 96-well plate was designed using MS PowerPoint (or any computer-aided drawing program) where wax occupies the areas between wells and the inside of the wells are empty. The outside plate dimensions and well spacing were matched to a standard Costar 96-well microtiter plate. The printed well diameters prior to melting were ~5.56 mm, which was reduced from a normal well diameter to provide the optimal bed volume in each well after melting. Using a Xerox ColorQube 8570N solid wax ink printer, a wax image of the 96-well plate was printed onto Whatman #1 filter paper by attaching the filter paper to an 8.5”x11” sheet of paper for proper printer feeding. After allowing cooling for about 10 seconds, the filter paper was placed onto a hot plate (#3 setting) to allow the wax to fully melt through the wax paper evenly across the entire plate (~4 minutes). Plate was allowed to cool fully and could then be used immediately or stored for later use (Figure 3). Final well diameters after melting were ~3.8 mm.

Paper-Based ELISA

After creating the 96-well plate via the wax printing method, the plate was placed on a pipette tip box with the open side up. Initially, 3 µL of target solution was added at the desired concentration to each well (min 3 wells each). This was incubated at room temperature for 10 minutes and then each well was blocked with 3 µL of blocking buffer. Following an additional 10 minute incubation period, 3 µL of antibody incubation solution was added to each sample well and incubated for 2 minutes. Each well was then washed two times with 10 µL of 1X PBS while wicking away any excess solution in between washes with blotting paper. Finally, 3 µL of the enzymatic substrate was added to each well and allowed to incubate at room temperature for
the appropriate period of time. For the BCIP/NBT, pNPP, and TMB substrates, the incubation was allowed to continue until the samples dried completely (~30 min), and then the samples were imaged (Figure 3). The chemiluminescent substrate was incubated for 2 min and then imaging was performed due to the assay intensity degrading after 5 min. All assays were repeated in triplicate or quadruplicate.

Figure 3. Schematic of P-ELISA Process.

Enzyme-Free P-ELISA

Gold nanoparticles (Au NPs) with an average diameter of 16 nm were synthesized through previously published citrate-reduction techniques [56]-[59]. The Au NPs (3 mL, 10 nM, Millipore water) were then conjugated with poly (ethylene glycol) (PEG) (20 µL, 1mM) which contained a thiol group on one end and a carboxylic group on the other end. The mixtures were left in the dark overnight to allow for chemisorption of the thiol groups onto the surface of the Au NPs. The particles were washed multiple times via centrifugation with subsequent buffer exchanges to remove any unbound PEG molecules. Anti-rabbit IgG antibodies were then
conjugated to the carboxylic end of the PEG through EDC/NHS (N-(3-Dimethylaminopropyl)-
N'-ethylcarbodiimide hydrochloride/N-Hydroxysuccinimide) linkage chemistry. Briefly, freshly
prepared EDC (30 µL, 20 nM) and NHS (10 µL of 50 nM) were added to the Au NP-PEG-
COOH (30 µL, 10 nM) solution containing MES buffer (30 µL, pH 5.5) and mixed for 30 min.
Phosphate buffered saline (PBS) (250 µL, 1X, pH 7.55) was then added to the solution as well as
1.1 µL of the anti-rabbit IgG antibody stock. After mixing for 30 min, the particles were washed
multiple times via centrifugation with subsequent buffer exchanges, with a final resuspension
volume of 100 µL.

The P-ELISA was carried out as described earlier, with 3 µL of the Au NP-IgG Ab
solution replacing the conventional antibody step. Development was performed through a silver
enhancement staining kit (Ted Pella / BBInternational) where 3 drops of both the initiator and
enhancer were mixed together and then 3 µL of the solution was applied to each test zone and
were allowed to develop until dry.

**Image Analysis Methods**

Images were captured primarily using a Canon EPS/Rebel T3i/EOS 600D camera;
however, for the comparison of different imaging methods, an HTC Droid Eris smartphone and
an HP Color 4540 Scanner/Printer were used as well.

For manual processing of the P-ELISAs, the image captured by the camera was opened
using ImageJ (NIH). An average RGB (red, green, blue) value was obtained for each test spot by
selecting the test spot area, excluding the transition from color to gray-black wax at the edges,
and then selecting the color histogram function in ImageJ. These average RGB values were then
transferred into an Excel workbook which calculated the ΔRGB (Eq. 1) values relative to the
control well values and also converted each value into the corresponding CIE 1931 color space
coordinates (and ΔCIE) as described in our previous paper [60]. The ΔRGB calculation (Eq. 1)
determines the magnitude of the vector between the original (control) RGB value (R₀,G₀,B₀) and
the RGB value of interest (Rₙ,Gₙ,Bₙ). This was, at minimum, repeated in triplicate for each
concentration of antigen tested. Since all assays attempted use a single color shift (i.e. clear to
purple, clear to yellow, etc.), the ΔRGB values provided more consistent correlation to antigen
concentration than the ΔCIE values. However, if the assays had a two color shift (i.e. blue to
green, or clear to yellow to red), the ΔCIE values would be necessary to use for correlation as the
ΔRGB values would cause poor correlation due to the potential for overlapping values for
different concentrations of sample. The ΔRGB values were plotted against antigen concentration
to obtain a semi-log or log-log best fit equation using GraphPad Prism. Sensitivity and limit-of
detection values were determined by the 3σ method and by comparison of the 3σ value to the
background or control values.

\[ \Delta \text{RGB} = \sqrt{(Rₙ - R₀)^2 + (Gₙ - G₀)^2 + (Bₙ - B₀)^2} \]  \hspace{1cm} (1)

For automated image processing, image processing code was written into an M-file for
execution by MATLAB R2011a (The Mathworks, Inc.) which include the Image Processing
Toolbox. The code prompts the user to select an image to open from file which is then converted
into a 3-D array with red, green, and blue channel data in different layers. Next, a grey level
threshold is determined automatically for the image and is used to convert the RGB image to a
black and white (BW) image. Once converted, the program identifies the circular test zones,
which are now white spots, by excluding white spots with an eccentricity greater than 0.5 and/or
an area of less than 1000 pixels. This defines a mask for the original image, but since we want to exclude the edges of the white areas to prevent averaging any gray/black values into our RGB values, the white spot areas are reduced by 50% while maintaining the centroid position for each. The final mask is then applied to the original image where the average RGB intensity values are obtained for each circular area in the mask. The ΔRGB values are calculated from this data and concentrations are estimated by solving the semi-log best fit (log x, y) equation. Finally, the centroids and the concentrations for each area are output for display on the original image. Additionally, the average red, green, and blue channel values and corresponding calculated concentration for each well are output in an Excel spreadsheet (.xls).

The android application (.APK) implementation of the colorimetric algorithm is similar to the automated image processing MATLAB solution. Once an image is selected to analyze, either from device storage or captured from within the application, a calibration curve can be created. During calibration, the user selects a series of control tests at increasing concentrations of target and then assigns numeric concentration values to them. The app determines the “color” of each selection using an incremental average of the red, green, and blue pixel values from within the selection area on the screen. After the calibration is finished, the app converts all test spot RGB values to ΔRGB values (Eq. 1), along with a zero calibration point with an assigned concentration of 0. A regression is then created based on the equation: \( \log(y) = b \times \log(x) + a \), since a log-log response was observed for the pNPP substrate. The user can now return to the image of interest and begin selecting unknown test areas to analyze. For each unknown test area selected, the app calculates the average RGB, converts to ΔRGB values, and the corresponding concentration can be determined using the regression equation. The app also stores all data into
a text file which can be transferred via email attachment or Bluetooth file transfer to perform further data analysis.

**Optimization of P-ELISA for NPY Detection**

We began with verifying the P-ELISA operation on wax-printed Whatman filter paper using the colorimetric substrate (BCIP/NBT) and AP-labeled anti-rabbit IgG antibodies. We were able to measure rabbit IgG over a range of 0.1 to 5 µM using the ΔRGB image analysis method (Figure 4A), which allowed us to not only verify the P-ELISA procedure, but to confirm the ability to use the ΔRGB method of analysis against the black-and-white image conversion method used in Cheng et al. with similar antigen concentration ranges [39].

We then evaluated a different substrate with our P-ELISA to assess compatibility with the paper platform and any changes to the sensitivity, or LOD, of the assay. A common colorimetric substrate that is used in ELISAs in conjunction with the AP enzyme is p-nitrophenyl phosphate (pNPP). This substrate produces a clear to yellow color change in liquid as enzyme concentration increases and the change in absorption is monitored at 405 nm using a spectrophotometer. However, using a digital image and our image analysis process, we are able to track this change in color intensity through ΔRGB calculations. By not having to convert the image to grayscale, this allows a greater dynamic range of measurement with increased sensitivity, which is discussed in the image analysis optimization section. Even though it may be hard to visually identify minute differences between consecutive samples, we can visually detect the overall trend of increasing substrate conversion with increasing IgG concentration (Figure 4).
Figure 4. Effect of Enzyme Substrates and Sample Media Complexity on P-ELISA Function. (A) Rabbit IgG in DI H$_2$O using BCIP/NBT Substrate with a semi-log best fit (log x, y). (B) Rabbit IgG in human saliva using BCIP/NBT substrate with a semi-log best fit (log x, y). (C) Rabbit IgG in DI H$_2$O using pNPP Substrate with a log-log best fit (log x, log y). (D) Rabbit IgG in human saliva using pNPP Substrate with a log-log best fit (log x, log y). Error bars indicate standard error of the mean (SEM) with n=4.

Once the color image processing is applied, the relationship can be easily quantified via ΔRGB. While both substrates provide responses to increasing concentrations of IgG, the pNPP substrate provided significant improvement in the limit of detection of the assay (Figure 4C). We did find it interesting that the NBT/BCIP substrate produces a logarithmic response curve, while the pNPP substrate produces a linear response. Using the 3σ of the lowest concentration sample, we determined the limit of detection (LOD) of IgG with the pNPP substrate to be 7.5 pM (Figure 4C). This is 4 orders of magnitude improvement over what was reported in Cheng et al.
where the lowest IgG concentration attempted was 670 nM, which was lower than their reported LOD [39]. The conversion of the BCIP/NBT substrate is a two-step process, with the BCIP being directly hydrolyzed by the alkaline phosphatase enzyme and then the intermediate “indoxyl” product dimerizes with the NBT, reducing it to form the insoluble NBT-formazan indigo-colored dye. In contrast, the pNPP substrate is a one-step, direct hydrolysis of the pNPP to a soluble yellow end-product, p-nitrophenol. We suspect that the significant increase in sensitivity of the assay using the pNPP substrate is due primarily to the BCIP/NBT system relying on this two-step process, with the paper matrix potentially limiting the diffusion of the products and hindering the efficiency of the NBT to NBT-formazan conversion. It is also possible that the alkaline phosphatase enzyme exhibits differences in the rate of hydrolysis between the pNPP and BCIP substrates.

We compared the effects of sample media complexity on the performance of the P-ELISA by spiking similar concentrations of IgG into unfiltered human saliva. We tested the effect on the P-ELISA results using both the NBT/BCIP and pNPP substrates (Figures 4B and 4D). Variability increased between replicate samples for the NBT/BCIP substrate, with the LOD increasing to 250 nM from 160 nM without saliva. The saliva caused the pNPP substrate LOD to increase as well (1.72 nM), but sample-to-sample reproducibility was much better. Even though sensitivity is reduced from using a more purified sample, it was thought that using a more complex media would significantly hinder the assays function, even to the point of completely debilitating the P-ELISA. However, this does not appear to be the case, which provides a promising outlook for moving forward with paper-based diagnostics with more complex biological samples.
Our goal was to use the P-ELISA format to detect Neuropeptide Y, a human performance biomarker that indicates stress levels and potentially cognitive state. After conjugating the anti-human NPY antibody with the AP, the P-ELISA was performed with both the NBT/BCIP and pNPP enzyme substrates (Figure 5). While both substrates produced similar LODs, ~2.3 nM for NBT/BCIP and ~4.0 nM for pNPP, the replicate sample variability was significantly reduced for the pNPP substrate, as seen previously with the IgG experiments (Figure 5B). The tendency for the pNPP substrate to produce a log-log response curve was also observed with the NPY sample (Figure 5B). While the choice of enzyme substrate for the NPY P-ELISA does not appear to have a significant impact on the LOD, as observed in the rabbit IgG P-ELISA, the limiting factor here may not be the substrate itself. Multiple other factors could be affecting the LOD, such as antibody-enzyme labeling efficiency, anti-NPY affinity for NPY, and the accessibility of anti-NPY to bind NPY on the paper matrix due to the small size of NPY. Additionally, since plasma NPY concentrations can range from ~50-100 pM for non-stressed, healthy individuals [20], [33], [61], to ~400-1400 pM for individuals experiencing acute stress [20], [33], further optimization of the NPY P-ELISA is needed to extend the LOD into the pM range by addressing the factors listed previously.

For NPY in human saliva, we observed a similar decrease in LOD (~30 nM) as with IgG when compared to NPY dispersions in DI H₂O and sample-to-sample variability was unaffected (Figure 5C). The unfiltered saliva was primarily used as a mimic for a more complex biological fluid in this study; however, NPY has been found in salivary samples of resting subjects at ranges of 3.3 to 15.2 pM [61]. Even though this is an order of magnitude lower concentration than plasma NPY, it could provide a less intrusive method for the detection of cognitive biomarkers.
Figure 5. Comparison of Enzyme Substrates and Media Complexity for the Detection of NPY using P-ELISA. (A) Detection of NPY using BCIP/NBT substrate. (B) Detection of NPY using pNPP substrate. (C) Detection of NPY in human saliva using pNPP substrate. Error bars indicate SEM with n=4.
In addition to comparing two different enzyme substrates for the AP enzyme, we also tested substrates with the horseradish peroxidase conjugated NPY antibody to compare any detection limit or sensitivity enhancements/degradation. In a normal 96-well ELISA, HRP enzyme-labeled antibodies provide increased sensitivity due to higher reduction of their appropriate substrates per molecule of HRP compared to AP, and are compatible with a wider range of chemiluminescent and fluorometric substrates as well. HRP also has significant size advantage over AP, ~44 kDa for HRP and ~140 kDa for AP, which allows for quicker diffusion and higher enzyme to antibody ratios. Although the enzymes do require different colorimetric substrates, which complicate direct comparison of the enzyme functionalities on paper, both substrates are one-step solutions to minimize processing variation.

We found that the TMB colorimetric substrate showed significantly reduced sensitivity of NPY (Figure 6). The LOD was reduced two orders of magnitude to ~0.5 µM. Similarly, the performance of the chemiluminescent substrate, which was imaged ~2 minutes after the addition of the substrate, was very poor and produced erratic results (Figure 7). There does appear to be a general trend of increasing luminescence with increasing concentration; however, the sample-to-sample reproducibility had high variation. The most likely reason for this is that the paper substrate is interfering not only with the function of the HRP enzyme, but also with the diffusion of the chemiluminescent substrate through the paper therefore limiting the ability of the HRP enzyme to reduce the substrate. Wang et al. demonstrated a similar chemiluminescent assay with a chitosan-modified paper substrate, which appears to stabilize the response of the assay through minimizing the interaction of the antigen with the cellulose fibers [40]. However, the chemiluminescent assay used in that study was based on a sandwich ELISA, with capture
antibodies on the surface of the paper substrate, which could further reduce any unwanted interactions, but also increases cost and complexity of the assay.

One goal of the P-ELISA is to move this procedure out of the normal laboratory environment. A step toward achieving this would be to eliminate the enzyme altogether to have less stringent storage conditions of reagents and increase their longevity. Proteins are susceptible to degradation in solution during long-term storage at room temperature or at 4 °C primarily due to proteases, microbial contamination, or oxidation. Enzymatic activity is more likely to be degraded before antibody activity, due to their mostly globular structure, since any slight change in their conformation would more severely impact the enzyme’s function. Additionally, antibody (Ab) conjugation with gold nanoparticles (AuNPs) has been used quite extensively in lateral flow assay (LFA) applications where the Ab-AuNP conjugates are lyophilized on the devices with minimal decreases in Ab activity. This could provide a long-term storage solution where the Ab-AuNP conjugates could be resuspended when ready to be used.

![Image of ELISA results](image)

**Figure 6.** P-ELISA of NPY in DI H₂O using HRP-labeled NPY Antibody and Colorimetric Substrate. Semi-log best fit (log x, y). Error bars indicate SEM with n=4.
In place of an enzyme substrate, we chose to use a silver enhancement method where the Ag\(^+\) are precipitated onto the surface of the AuNPs and provide the same sort of signal amplification that the enzyme-enzyme substrate provides (Figure 8B). We found this technique to have very good sample-to-sample reproducibility and exhibit a high correlation coefficient to a log-log type response (Figure 8A). LOD for IgG with IgG Ab-AuNPs was ~10 nM using the silver enhancement reporter method. While this method does fall short of the LOD when using antibody-enzyme conjugates, it does provide an alternative method for signal amplification which would be more robust for long-term storage conditions.
Figure 8. Enzyme-free P-ELISA of Rabbit IgG in DI H₂O using IgG antibody conjugated Au NPs with silver enhancement stain. (A) Plot of delta RGB vs. IgG concentration using Au NP – silver enhancement procedure. Error bars indicate SEM with n=4. (B) Schematic of Enzyme-free P-ELISA Assay.

Optimization of Image Analysis

A common method for analyzing color images is to first convert the RGB image to a grayscale image, thereby simplifying analysis since each pixel corresponds to an intensity value. This works well for any sample where the color shift provides an increasingly opaque substance so that, when converted to a grayscale image, the pixel intensity values can be observed to decrease with increasing opacity. This was shown in Cheng et al., where the enzyme substrate chosen produced an increasingly darker blue/purple color with increasing amounts of antibody-enzyme conjugates and therefore antigen as well [41]. However, since we found the pNPP substrate to have better sample-to-sample consistency, increased sensitivity, and nearly identical
limits-of-detection, we determined that this grayscale conversion would significantly limit the measurement sensitivity.

To demonstrate this issue, we used a CMYK standard printer calibration scale which contained yellow and white-black scale standards (Figure 9). An image was taken of the calibration scales and the same image analysis process was performed as for the P-ELISA. The color image of the scales were analyzed using the ΔRGB method and then the image was converted to grayscale and analyzed by pixel intensity values since ΔRGB method is not possible. The white-black scale was assumed to be the “maximum” change possible in both conditions (i.e. 0 to 100 on the white-black scale is 0 to 100%). This allowed us to compare the change in response range of the yellow-scale for the color and grayscale images (Figure 10).

Figure 10. Comparison measurement of simulated pNPP ELISA substrate using delta RGB or grayscale analysis. (Left image) Color image of simulated yellow substrate scale and white-black scale. (Right image) Image after grayscale conversion. Plot shows the comparison of the yellow scale (simulated substrate) to the white-black scale as a percentage of maximum value in both the RGB and Grayscale images.

Even though the identical image was used for both analyses, a 30% reduction can be seen in the available response range when the image is converted to grayscale. This in turn leads to decreased measurement sensitivity since a larger antigen concentration shift is required to measure the same intensity shift. For example, in Figure 5, to measure the same percentage shift from 20 to 40 color units on the RGB plot, it would require a change of 35 to 75 color units on the grayscale plot which equates to roughly a 50% decrease in sensitivity.

Additionally, we tested the method of image capture to observe and quantify its effect on the measurement response range. We imaged the same P-ELISA using 3 different methods: a DSLR camera, a smartphone camera, and an RGB flatbed scanner (Figure 11). We found that the flatbed scanner and smartphone camera provided similar response ranges and slopes, while the DSLR camera provided the largest range and highest slope which indicates better
measurement sensitivity (Figure 11). All images in this manuscript which were used for analysis were captured using the DSLR camera.

As our group has previously reported [60], the CIE coordinate system can provide a better method of tracking color changes in an image than purely basing the response from either of the red, green, or blue channels. Interestingly, when we attempted to use the ΔCIE method for image analysis, which normally correlates color changes very efficiently, the results were not as accurate as the ΔRGB method. The ΔCIE method works excellently for assays which produce a wide range of color responses, such as pH testing; however, when applied to the P-ELISA method, which is a single color intensity shift, measuring the change in RGB values provides a more consistent correlation to changes in antigen concentration than using the changes in the CIE coordinates. Even when analyzing the same sample, the two methods produce quite different correlation curves when plotted against antigen concentration (Figure 12). We concluded that

![Figure 11. Comparison of P-ELISA Imaging Methods and the Effect on Measurement Sensitivity Range.](image)

Plot shows comparison of the measured delta RGB of the same sample via DSLR camera, RGB scanner, and smartphone camera. Visual comparison of the digital images shows minor color variations between the three imaging methods.
since the output of the P-ELISA assay is primarily an intensity change of the same color, the ΔRGB method is more appropriate in this instance.

Manually processing each test zone through ImageJ and Excel, as performed for the experiments in this work, is extremely tedious and time-consuming, and the process could be difficult to train new people how to use. Additionally, with the idea that P-ELISAs could be performed within a resource limited laboratory, we developed an automated image analysis program using MATLAB. Here, the user takes an image using a tablet capable of running MATLAB (in this case a Fujitsu Windows-based Tablet) and then runs the program (m-file) in MATLAB (Figure 14A, Appendix A). The user is prompted to select the image to analyze and the program automatically identifies the test zones on the paper-based 96-well microplate. The output of the program is a visual image of the analyzed plate with the corresponding calculated concentrations for each test zone.

**Figure 12. Comparison of Delta RGB and Delta CIE Image Change Quantification Methods for P-ELISA.** (A) Delta RGB measurements from a digital image of a NPY P-ELISA. (B) Delta CIE measurements from the same digital image of the NPY P-ELISA. Error bars indicate SEM with n=4.
The program accomplishes this automation through a series of unique steps. First, the image is converted to a purely black and white image using a threshold value (Figure 13B). Next, the white areas of the image are evaluated whether they are circular in nature and of sufficient pixel area to be a test spot, and their centroids are recorded for location in the image. From this information, an initial image mask is developed (Figure 13C). To make sure the edges of the test zones are excluded due to the possibility of wax color being incorporated into the RGB averages, the radius of each white area on the mask is decreased by 50% and a new mask is made (Figure 13D). The mask is applied to the original RGB image and the average RGB values for each white area of the mask are stored. The delta RGB values are calculated and input into the concentration correlation function to determine the corresponding antigen concentration, which is displayed on the final output image (Figures 13E). The program is able to output all results as an Excel file as well.

While the correlation function must be predetermined and input into the program, we are currently working on incorporating a built-in calibration method as well as allowing for the program to automatically perform image correction for uneven lighting conditions across the test zones, as was presented in our earlier work [60]. Simply printing a simulated pNPP scale onto the paper for standardized calibration is not feasible since multiple factors in the P-ELISA (i.e. ambient temperature and humidity, freshness of substrates, etc.) affect the amount of enzyme substrate color production. Therefore, a standard dilution series of a positive control would most likely still need to be performed in parallel with any assay as is done with normal ELISAs.
Figure 13. Automated MATLAB Image Analysis Process and Quantification of IgG P-ELISA. (A) Original image of P-ELISA assay. (B) Thresholded Black & White Image conversion. (C) Identification of all circular white areas within specified parameters for creation of mask. (D) Selected circular white areas on mask are reduced in size to avoid edges of test spots. (E) Average RGB values for each test zone identified by the mask layer are processed by MATLAB to calculate the actual concentration of IgG present.
For non-MATLAB capable platforms, we have developed an android-based application which can perform many of the same analysis features. The app allows a user to image a paper-based 96-well microplate, define a calibration series by selecting test zones and inputting the corresponding concentration, and then perform individual automated calculation of antigen levels in unknown samples (Figure 14B). The app performs concentration correlation through the same delta RGB calculations as performed for all experiments in this manuscript. However, since the system directly implements the regression algorithm, the main advantage is that the app can be further developed to evaluate different generalizations of linear regressions to determine which provides the best fits for the data when building a calibration curve. We are also working on incorporating the automated image analysis features described in the MATLAB version through implementation of Hough transform.

When comparing results from all three analysis methods, currently the automated MATLAB image analysis method most closely matches the calculated vs. actual concentration ratio, with an average difference of 24.9%, followed by the ImageJ-Excel method at 81.2% and the manual/automated Android app at 98.55% (Figure 14C). The correlation results of the Android app can most likely be improved through the use of the automated image processing, as in the MATLAB version, which would eliminate any user variability when selecting analysis areas as seen in the ImageJ-Excel method. Also, incorporating the lighting correction function should help reduce variability between columns of replicate samples with reference calibration points around each test area.
Figure 14. Image Analysis and Quantification of IgG P-ELISA Results with Windows- and Android-based Tablets. (A) Fujitsu Stylistic Q550 Tablet with Windows 7 running MATLAB. (B) Samsung Galaxy Tab 10.1 (GT-P7510) with Android 4.0.4 running a custom designed App (.APK). (C) Comparison of calculated IgG concentrations from the same IgG-pNPP P-ELISA via 3 different methods: 1) Manual image analysis using ImageJ-Excel on a PC, 2) Automated image analysis using MATLAB on a Windows tablet, and 3) Manual/automated image analysis using app on Android tablet. Error bars indicate SEM with n=4.
Summary

The ability to detect and monitor human performance biomarkers gives the potential to track an individual during a task and determine their physiological stress and cognitive load levels. In a military application, this could allow for field medics to assess a soldier’s stress level to help prevent PTSD, allow a field medic to determine if a soldier has suffered a traumatic brain injury (TBI) and begin treatment immediately to prevent further damage, or allow assessment of vigilance for soldiers in non-battlefield positions where inattentiveness could lead to mission failure. Tracking NPY and other relevant biomarkers (i.e. Orexin A, Cortisol, Glutamate) could help to prevent these situations.

Wax-printed paper-based diagnostics provide a unique and inexpensive platform to carry out biomolecular assays at very low sample and reagent volumes. Paper-based ELISAs were shown here to have pM to nM sensitivity using colorimetric substrates, even in a complex sample such as saliva, but could be enhanced further with assay optimization, use of alternate enzyme substrates, or nanoparticle-based enzyme-free assays. Additionally, the cost and time savings of performing a P-ELISA instead of a conventional ELISA would significantly increase not only the number of samples per dollar the laboratory would be able to process, but the number of patients in the same time period as well. The ability to use a smartphone or tablet platform to perform quick analysis of paper-based diagnostics can provide an easier alternative to expensive, time consuming laboratory procedures. This technology could be extremely beneficial in resource-limited laboratory environments, where diagnostic measurement equipment, such as spectrophotometers or multi-well plate readers, is not easily obtained due to funding constraints.
CHAPTER III

A CLINICAL LABORATORY-BASED INFECTIOUS DISEASE ASSAY IN A ONE-STEP, POINT-OF-CARE, MULTI-LAYER PAPER DEVICE WITH PRE-LOADED REAGENTS

Introduction

For individual care, from a primary care provider’s standpoint, the ability to rapidly determine if the patient has an infectious disease, if it is viral or bacterial in nature, and what type of treatment the infection will respond to, would be invaluable. This would not only significantly reduce the treatment decision time for the primary care provider, but it would also significantly shorten the impact of symptoms and infection on the patient as well as potentially minimize infectious disease transfer to others. Influenza accounts for nearly 200,000 hospitalizations and 36,000 deaths each year in the United States alone, with approximately 500,000 deaths worldwide [62]. Considering the rapid course of viral action and severity of symptoms, early detection of influenza is critical, especially in certain subpopulations. The elderly, young children, individuals with preexisting medical conditions, and pregnant women are particularly susceptible to severe cases of infection. Additionally, in cases where individuals are in close daily contact with a small group of people, influenza can spread rapidly and cause
substantial illness and loss of work days. In a military setting, such as a small base, this could significantly impact the preparedness of military units [63].

Influenza contains two antigenic surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), which are responsible for binding to a host cell and facilitating virus uptake and release of newly synthesized viruses. NA activity is essential for efficient replication of influenza; it increases access to the cell surface by removing decoy receptors on mucins, cilia and the cellular glycocalyx [64], [65]. NA facilitates the release of newly synthesized viruses from host cells, preventing self-aggregation of virus and guaranteeing virus spread [66], [67]. HA and NA constantly undergo mutations to their genetic sequences. During antigenic drift, influenza accumulates mutations in all gene segments. During antigenic shift, influenza exchanges HA or NA genome segments from different influenza viruses (e.g. pigs and birds) resulting in new virus subtypes. Antigenic drift and shift rapidly changes the influenza virus, making characterization and detection of influenza strains challenging. In reality, antibody-based influenza diagnostic tests often show deficient sensitivity for detection, particularly when new influenza strains emerge [68] as previously reported for H1N1pdm/09 pandemic and H3N2v [69]-[72].

Oseltamivir (Tamiflu®) and zanamivir (Relenza®), are currently the only anti-influenza drugs recommended for treatment in humans. NA-inhibitors (NAIs) halt influenza infection by limiting replication to only one infectious cycle [73]. NAI resistance has emerged in influenza virus, with resistance to oseltamivir more commonly seen than resistance to zanamivir. However, the currently available rapid diagnostic tests (RDTs) lack the ability to show if the strain of influenza detected is susceptible to these antiviral agents.
Currently, diagnosis of influenza is performed either through polymerase chain reaction (PCR) or through rapid antigen detection assays. PCR is a complicated laboratory procedure that must be performed by well-trained personnel, takes 2-4 hours to complete, and requires expensive laboratory equipment. Additionally, PCR requires the proper viral primers for the assay to work, which constantly need to be re-validated due to the constant change of the viral genome. The RDTs currently available are based on LFA platforms, meaning they are a point-of-care (POC) device, and are able to provide a result in 15-20 minutes. However, each device has different levels of differentiation of types of influenza (A only, A or B, A and B). While these tests are highly specific (>90%), they are not very sensitive and therefore can only provide the primary care provider with confirmation of influenza presence, not activity level.

Paper microfluidic analytical devices have emerged in recent years [35], [36], leading to development of a number of POC analyses, including HIV chips [37], [38], paper-based ELISA [39]-[41], [74] and low-cost colorimetric diagnostic assays [42]-[47]. Of specific interest are the microfluidic paper-based analytical devices (µPADs). These are paper devices which contain multiple hydrophobic patterned layer to guide a sample horizontally and vertically through a device while performing various processing steps, such as sample division, adjustment of sample pH, filtering, and even sequential reagent additions. Besides being inexpensive and relatively easy to manufacture, they can be quite robust with reagent storage for real-world POC use [75].

In this study, as an alternative to antibody-based diagnostic tests, we used a new approach to develop a theranostic, a device that is able to diagnose as well as determine appropriate therapeutic action, for influenza detection based on the properties of the viral NA. We confirmed NA activity at various pH levels, in the presence of cations or chelators, to determine a common buffer condition that would detect influenza A and B on a paper-based platform. We
also observed strain dependent pH responses that could be used to quickly “subtype” a suspected influenza sample. Additionally, NAIs were incorporated into the assay to determine strain-dependent NAI resistance. Once the optimal assay conditions were determined, along with optimization of reagent storage conditions, we incorporated these into a one-step, multi-layer, microfluidic paper-based analytical device for POC applications.

**Materials and Methods**

**Chemicals / Solutions**

5-Bromo-4-chloro-3-indoyl-alpha-D-N-acetylneuraminic acid (X-NeuNAc) was obtained from Santa Cruz Biosciences. Purified influenza A H1N1 Neuraminidase was obtained from Sino Biological. Pullulan from *Aureobasidium pullulans* was obtained from Sigma Aldrich. Sodium acetate, phosphate buffered saline (PBS), Tris(hydroxymethyl)aminomethane (Tris), calcium, EDTA, CTAB, and BSA were all sourced through Sigma Aldrich.

**Virus and Bacterial Stocks**

Live virus and bacterial stocks were used from frozen storage from Dr. Weiss’ group at the University of Cincinnati Medical Campus. Influenza viruses were initially obtained from the Centers for Disease Control. Viral samples were propagated in MDCK cells and the supernatants were harvested by centrifugation after cytopathogenic effects were observed (11, 12) and stored at -80°C. All viral and bacterial work was completed in a bio-safety level (BSL) 2 facility with proper PPE. Influenza A and B, as well as parainfluenza virus, samples were thawed and diluted 1:3 from frozen stocks into sterile DI H$_2$O. *Streptococcus pneumoniae* was thawed and cultured on a blood agar plate overnight at 37°C until colonies were observed. The colonies were
harvested and, after dilution, the final working solution absorption was 0.1 O.D. (600 nm) which is ~2x10^7 CFU/mL. This was then diluted 1:1500 into sterile DI H2O. Supernatants of mock infected MDCK cells were used as a negative control.

**Printing of Paper 96-well Plates**

A ‘negative’ image of a 96-well plate was designed using MS PowerPoint (or any computer-aided drawing program) where wax occupies the areas between wells and the inside of the wells are empty. The outside plate dimensions and well spacing were matched to a standard Costar 96-well microtiter plate. The printed well diameters prior to melting were ~5.56 mm, which was reduced from a normal well diameter to provide the optimal bed volume in each well after melting. Using a Xerox ColorQube 8570N solid wax ink printer, a wax image of the 96-well plate was printed onto Whatman #1 filter paper by attaching the filter paper to an 8.5”x11” sheet of paper for proper printer feeding. After allowing cooling for about 10 seconds, the filter paper was placed onto a hot plate (#3 setting) to allow the wax to fully melt through the wax paper evenly across the entire plate (~4 minutes). Plate was allowed to cool fully and could then be used immediately or stored for later use. Final well diameters after melting were ~3.8 mm.

**Neuraminidase Activity Assays Optimization**

After creating the 96-well paper plate via the wax printing method, the plate was trimmed to fit inside a large culture dish, with a small square culture plate inside the larger round culture plate to act as a support and suspend the paper plate. Initially, 10 µL of the appropriate buffer solution containing X-NeuNAc substrate was added to each well. This was immediately followed by the addition of 3 µL of the viral or bacterial diluted solution (1:3 for viral samples,
1:1500 for *S. pneumoniae* 4). Sodium acetate buffer (50 mM, 1X) was used for studies at pH 4, PBS (1X) was used for studies at pH 7, and Tris(hydroxymethyl)aminomethane (Tris) buffer (50 mM, 1X) was used for studies at pH 9. All buffers contained 200 mM NaCl and 14.82 mM X-NeuNAc substrate. The “+” after a buffer name indicates the presence of 0.1 mM Ca$^{2+}$, whereas the “-” indicates the presence of 5 mM EDTA. For all 96-well plate assays, a small amount of sterile DI H$_2$O was added to the bottom of the culture dish to prevent the assay from drying out too quickly by creating a humid environment unless otherwise noted. The cover was then placed on the culture dish and the assay was left to incubate in the BSL-2 hood overnight at either ambient temperature (18-20°C) or a heated, non-humidified incubator (37°C). After 10 hours of incubation, the samples were imaged with a digital camera.

**Reagent Preservation and Storage**

Using the previously described assay procedure for 96-well plate format, Pullulan was added at increasing concentrations (1, 2.5, 5, 7.5, and 10% w/v) to the various pH buffers and X-NeuNAc substrate with purified NA to determine if there was any potential interference with the assay performance. The samples were allowed to react until dried and were then imaged to compare performance.

For determination of the optimal storage buffer concentration, 6.5 µL the substrate and the appropriate buffer condition with increasing concentrations of Pullulan (1, 2.5, 5, 7.5, and 10% w/v) were dried onto the paper 96-well plate and allowed to sit in open air at room temperature for 3 days. Afterwards, 10 µL of DI H2O and 3 µL of purified NA (100 U/mL) were spotted onto the dried substrate areas and allowed to react for ~3 hours. The samples were imaged and image analysis was performed to determine relative NA activity levels.
**Influenza Microfluidic Paper-based Analytical Device (µPAD)**

Drawings for each layer were created using a computer aided drafting program (AutoCAD 2015). For the distribution and development layers, printable images which define the wax areas were made from the AutoCAD drawings and printed onto the Whatman #1 filter paper using the wax printing method described previously for the 96-well plate. The wax was subsequently heated to flow the wax through the entire cross-section of the filter paper. After printing, both the distribution and development layers were cut for assembly purposes to allow for 9 µPADs to be fabricated at one time. Additionally, the laser cutting printer was used to cut the sample inlet hole out of the top lamination layer (Staples 5 mil Lamination Sheets). The distribution layer was treated with 0.5% BSA (10 µL) and allowed to dry. Next, the development layer was loaded with 6.5 µL of the X-NeuNAc substrate in the appropriate buffer along with 7.5% w/v Pullulan. Finally, all layers were assembled and passed through a hot roll laminator (Western Magnum Model # XRL-120), followed by device cutting from the master using the laser cutting printer (Figure 23B). To evaluate samples, 30 µL of the previously discussed virus and bacterial sample dilutions were pipetted into the top sample port on the µPAD. Finally, the µPADs were both visually evaluated and imaged for image analysis.

**Image Analysis Methods**

Images were captured using a Panasonic Lumix DMC-ZS5 digital camera and processed using ImageJ (NIH). An average RGB value was obtained for each test spot by selecting the test spot area, excluding the transition from color to gray-black wax at the edges, and then selecting the color histogram function in ImageJ. These average RGB values were then transferred into an
Excel workbook which converted each value into the corresponding CIE 1931 color space coordinates (and ΔCIE) as described in our previous paper [60]. For the final device images, it was found that using only the x-axis coordinate of the ΔCIE value gave a very precise indication of the amount of color change. Therefore, any blue color shift due to the reduction of the substrate would be shown as a positive value (Figure 28).

**Neuraminidase Activity Assay Optimization**

In these experiments, an influenza neuraminidase activity assay is optimized for use on a wax-printed, Microfluidic Paper-Based Analytical Device (or µPAD). The initial optimization was performed on a wax-printed, 96-well plate format device, fabrication of which has been previously described [74]. The physical layout of the paper-based 96-well plate exactly matches the dimensions (well spacing) of a conventional 96-well microplate; however, where a conventional microplate would normally require >60 µL of fluid per well, the paper-based plate only requires a minimum of 3 µL to fill the test area. In the following experiments, a total of 13 µL (10 µL of buffer/substrate and 3 µL of analyte solution) per test area were used. The buffer/substrate solution (X-NeuNAc) was added to the device first, followed by the addition of the analyte. In most cases, the devices were imaged after 10 hours and image analysis was performed.

Four different strains of influenza A (pH1N1/09-S, sH1N1/07L-R, sH3N2/07L-S, and H3N2/07-R), one influenza B strain (Yamagata), and two potentially assay interfering upper respiratory infectious diseases (HPIV-2 and *Streptococcus pneumoniae*-4) were examined in this study. These infectious diseases were chosen to represent a cross-section of the potential influenza strains which could be encountered. Additionally, since NA is also produced by other
common human respiratory pathogens [76] that may be present in a mucosal sample, specifically parainfluenza virus [77] and bacteria such as *Streptococcus pneumoniae* [78]-[80], we incorporated these as potential false positive tests to determine if the assay could distinguish between influenza and other respiratory pathogens.

Previous work by Gallegos et al. [81] showed the feasibility of detecting influenza based purely on NA activity; however, in that study a fluorescent substrate (MUNANA) was used instead of the colorimetric substrate (X-NeuNAc) which was used for this study. The colorimetric substrate was chosen to make analysis easier, whether by visual interpretation or image analysis methods, since fluorescent substrates would require more complicated instrumentation for analysis. To verify operation of the colorimetric NA assay on the paper platform, we began by using just one influenza A subtype (sH1N1/07L-R) to determine a baseline response for comparison of detection performance. First, it was necessary to determine if the assay could develop in ambient (dry) conditions or if it was necessary to keep the assay in a humidified state to prevent drying prior to complete development. The “humid” condition was made by adding a small amount of DI H$_2$O to the bottom the culture dish prior to adding the cover. No water was added to the “dry” dish prior to covering. It was observed that while the “dry” assay did produce a visible response, it was very weak compared to the “humid” assay response (Figure 15). From this point on during the optimization, all experiments were performed under the “humid” conditions to maximize responses.

Next, we examined the buffer strengths for the 3 different pH conditions to verify that we had the optimal buffer conditions for the NA assay. Sodium acetate buffer (50 mM (1X), 100 mM (2x), and 150 mM (3X)) was used for studies at pH 4, PBS (1X, 2X, and 3X) was used for studies at pH 7, and Tris(hydroxymethyl)aminomethane (Tris) buffer (50 mM (1X), 100 mM
(2x), and 150 mM (3X)) was used for studies at pH 9. All buffers contained 200 mM NaCl and 14.82 mM (3.33X) X-NeuNAc substrate. The “+” after a buffer name indicates the presence of 0.1 mM Ca\(^{+2}\), whereas the “-” indicates the presence of 5 mM EDTA. From the results it was found that increasing buffer strength did not have a significant effect on assay response; however, a very minor increase was observed at the 2X PBS condition, but was not enough to justify changing buffer conditions (Figure 16). All buffer strengths were kept at their 1X concentration from this point forward.

![Image](image_url)

**Figure 15. Comparison of Influenza Detection on Paper under Humid and Dry Assay Conditions.** (A) Image of influenza neuraminidase activity assay under humidified and non-humidified (dry) assay conditions. Image analysis results (ΔCIE) of the influenza neuraminidase activity assay under (B) humid and (C) non-humid (dry) assay conditions. Specific sH1N1/07L-R neuraminidase activity is observed at pH of 7 with 0.1 mM Ca+ present (pH7+).
Figure 16. Comparison of Influenza Detection on Paper with Increasing Buffer Strength. (A) Image of influenza neuraminidase activity assay under 1X, 2X, and 3X buffer conditions. (B) Image analysis results (ΔCIE) of the sH1N1/07L-R influenza neuraminidase activity assay with 1X, 2X, and 3X buffers at pH of 4, 7, and 9. All contained 0.1 mM Ca\(^{2+}\).

We also examined optimization of assay incubation temperature along with increased substrate concentration to investigate any enhancements in assay development. Room temperature incubation (~20°C) and a non-humidified cell incubator incubation (37°C) were compared over 10 hours as well as increased X-NeuNAc concentrations (14.82 mM (3.33X) compared to 22.23 mM (5X)). It was expected to see enhanced assay response with both increased temperature and substrate concentration, which is confirmed by the results in Figure 17; however, it was not expected to see such an enhanced response at the 37°C temperature for the pH 9+ condition. While the elevated temperature will enhance most enzyme-substrate reaction rates due to higher system energy and reaction rates, the enhanced response at pH 9+, 37°C could also be due to buffer pH change since the H1N1 seems to respond similarly to the pH 7+ condition at 37°C. We decided to proceed with all experiments at room temperature (~20°C) due to a
more predictable assay response and the future POC use of the assay as a user would not want to have to have access to an incubator to develop the device.

Additionally, to determine if increased temperature resulted in decreased time to detection, a time course study was performed with the H1N1-S1R at 20°C and 37°C. The elevated temperature did significantly reduce the time to a positive result (i.e. enough blue development to detect significantly above background) by reducing assay time from ~400 minutes (20°C) to ~150 minutes (37°C) (Figure 18).

**Variability of Influenza Type and Subtype Response to Neuraminidase Assay**

From previous experiments [81], it was found that nearly all influenza A and B types have their maximum response in the pH 5-8 range (Figure 19A). Interestingly, the response profile in this pH range for each subtype was uniquely different. In addition, due to NA’s dependence on metal cations for proper protein folding and binding to sialic
Figure 18. Time Course Studies for Determination of Minimum Assay Time at Different Incubation Temperatures. Neuraminidase activity of sH1N1/07L-R, PIV, and *S. pneumoniae* at (A) 20°C and (B) 37°C. All samples were tested in pH 7 buffer with 0.1 mM Ca\(^{+2}\).

Acid, different responses were observed by each type and subtype of influenza when Ca\(^{+2}\) or EDTA was present (Figure 19B).

When these two tests were replicated on a paper-based platform, nearly identical results were observed for all influenza types and subtypes, and for the *S. pneumoniae*. These unique responses allow not only for determination as to whether the sample contains influenza or not, but also could potentially allow for initial determination of the type neuraminidase present (N1 mutation, N2 mutation, or Type B NA) (Figure 20A).

Surprisingly, the parainfluenza sample did not produce much response on the paper substrate, even at a near optimal pH of 4. The paper substrate is most likely interfering with the binding of the substrate to the PIV NA, which is actually beneficial since PIV is a potential interferent for influenza detection. The other potential false positive, *S. pneumoniae*, does show a strong response across all pH conditions. However, even though it responded at all buffer conditions, this can be used to identify/rule out a sample
Figure 19. Previously Characterized Influence of pH and presence of Ca\(^{2+}\) on NA Activity. (A) NA activity was measured by incubating virus with fluorogenic substrate at different pH values. NA activity in acetate buffer pH 4, 5 and 6 is shown as closed squares (■), in PBS pH 6, 7 and 8 is shown as open circles (O), in Tris buffer pH 8 and 9 is shown as closed diamonds (♦), in CHES buffer pH 9 and 10 is shown as open triangles. (B) Calcium (+) indicates NA activity measured in the presence of 0.1 mM Ca\(^{2+}\); calcium (-) indicates NA activity measures in the presence of 25 mM of EDTA chelator. All plots show results of at least 3 independent experiments and the error bars indicate SD.
containing high *S. pneumoniae* concentrations on a POC device since none of the influenza type A and B respond across such a broad range. Through the use of our image analysis methods, thresholds can be set for the amount of color change needed to be considered a positive response (Figure 20B). Finally, using the thresholds set previously, we can see the pattern each influenza type and subtype produce under the different buffer conditions (Figure 20C).

With the design goal for this POC device being a theranostic, a device that is able to diagnose as well as determine appropriate therapeutic action, we added physiologically relevant levels of the activated form of Tamiflu®, oseltamivir carboxylate, to the pH 7+ buffer condition which allowed for assessment of influenza NAI susceptibility. Since plasma levels of 180-350 nM have been reported when 75 mg oseltamivir is taken twice daily (14-18), we chose to use 350 nM as our initial test value. The pH1N1/09-S and

![Figure 20. Strain Variation in NA Activity due to Different Buffer Conditions.](image)

(A) Image of neuraminidase activity assay with buffers at pH of 4, 7, and 9 and either 0.1 mM Ca^{2+} (+) or 5 mM EDTA. (B) Image analysis results (ΔCIE). (C) Summary of a positive or negative NA activity response based on threshold applied to the ΔCIE results (0.05).
sH3N2/07L-S, the NA activity was reduced significantly in the presence of the NAI oseltamivir (Figure 21). This was expected since they were previously characterized as being “sensitive” strains to NAIs. The PIV did not demonstrate a significant change between non-NAI treated and NAI treated conditions; however, as previously discussed, the PIV does not produce a strong response on the paper platform, even in optimal pH conditions (Figure 21). Out of the known “resistant” strains, the sH1N1/07L-R demonstrated the highest resistance to the NAI (Figure 21). The other two resistant strains, influenza type B Yamagata and H3N2/07-R, both exhibited reduced NA activity in the presence of the NAI even though they contain the resistant mutation. Activity in both the Yamagata and H3N2/07-R were reduced to ~50% of their original NA activity with the NAI present (Figure 21). The *S. pneumoniae* was not affected by the NAI, which was expected since the NA on the surface is structurally different from influenza NA and the NAI Tamiflu specifically targets influenza NA. All of these findings very closely match the previous oseltamivir carboxylate IC$_{50}$ results for identical influenza strains (Table 1).

![Figure 21. Evaluation of NAI Effectiveness.](image)

*Figure 21. Evaluation of NAI Effectiveness.* (Left) Image of NA activity at pH 7+ compared to pH 7+ with oseltamivir (Tamiflu) added (350 nM). (Right) Corresponding image analysis results (ΔCIE).
Table 1. Summary of previously characterized virus types and subtypes, as well as *S. pneumoniae* strains which are evaluated in this study.

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Ct&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NA act. RFU&lt;sup&gt;b&lt;/sup&gt;</th>
<th>HA Titer</th>
<th>FFU per mL&lt;sup&gt;c&lt;/sup&gt;</th>
<th>% of max NA act. +EDTA (pH)&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Oseltamivir IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>GenBank ID #</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H1N1 strains</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/California/07/09 (H1N1pdm/09-S)</td>
<td>23</td>
<td>6600</td>
<td>320</td>
<td>7 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>44% (pH 6-7)</td>
<td>0.9</td>
<td>ACQ63272</td>
</tr>
<tr>
<td>A/Brisbane/59/07-Like (H1N1/07-R)</td>
<td>25</td>
<td>5000</td>
<td>160</td>
<td>3 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>54% (pH 6)</td>
<td>2220</td>
<td>ADE28752</td>
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<td><strong>H3N2 strains</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/Brisbane/10/07-Like (H3N2/07-S)</td>
<td>23</td>
<td>6700</td>
<td>40</td>
<td>3 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>50% (pH 7)</td>
<td>0.6</td>
<td>ACO95273</td>
</tr>
<tr>
<td>A/Texas/12/07 (H3N2/07-R)</td>
<td>25</td>
<td>6800</td>
<td>160</td>
<td>4 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>61% (pH 7)</td>
<td>15</td>
<td>ACA33536</td>
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<tr>
<td><strong>Type B strains</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B/Florida/04/06 (Yama/08-S)</td>
<td>27</td>
<td>5000</td>
<td>320</td>
<td>9 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>88% (pH 7)</td>
<td>19</td>
<td>ACA33351</td>
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<tr>
<td>Parainfluenza virus (PIV)</td>
<td>n/a</td>
<td>4800</td>
<td>ND</td>
<td>ND</td>
<td>27% (pH 5)</td>
<td>ND</td>
<td>AEQ39010</td>
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<tr>
<td><em>S. pneumoniae</em> ATCC 49619 (Strep 4)</td>
<td>n/a</td>
<td>5000</td>
<td>n/a</td>
<td>n/a</td>
<td>108% (pH 6-9)</td>
<td>ND</td>
<td>CAI94558</td>
</tr>
</tbody>
</table>

<sup>a</sup>: Rounded threshold cycle (Ct) values determined by RT-PCR in viral samples at 1 to 1000 dilution. <sup>b</sup>: Rounded average of RFU/h/μL in NA assay in PBS, pH 7 for all influenza and *S. pneumoniae* strains, and sodium acetate buffer, pH 5 for Parainfluenza type 2. <sup>c</sup>: Fluorescent Focus units were determined using the fluorescent focus-forming assay. <sup>d</sup>: % of maximal NA activity obtained in presence of 25 mM EDTA. All influenza strains tested at pH 7. Parainfluenza and *S. pneumoniae* tested at pH 5 and pH 7, respectively. n/a: Not applicable; ND: Not determined

**Influenza Microfluidic Paper-based Analytical Device**

To adapt this assay to a more user-friendly, point-of-care type test, a μPAD was designed and fabricated. Multiple iterations of the design were attempted and are briefly discussed. The first design consisted of two wax-patterned layers, with the top layer contained a sample addition area and two spots for viewing the colorimetric development. The bottom layer distributed the sample to two pathways, with each pathway containing a different buffer condition with X-NeuNAc substrate (Figure 22A). The two layers were joined together using a double-sided adhesive tape. Three different buffer conditions were attempted (pH 4+, 7+, and 9+) on two devices. While substrate color development was
observed, the results were not significant enough to distinguish response differences under the different buffer conditions.

Since we also knew that more than two buffer conditions would be needed to perform both the identification of influenza and antiviral susceptibility, we modified the initial design to accommodate 5 different test conditions (Figure 22B). Here, we observed poor distribution of the sample to all test zones, as well as the need for a large volume of sample (>100 µL). Primary flow was only seen in the channels immediately to the left and right of the sample area, with very little to no color development. It was

Figure 22. Design Iterations of the Influenza μPAD. (A) Two-layer device with reagent storage in bottom layer and lateral transport of sample. (B) Expanded two-layer device with additional buffer pathways. (C) Three-layer device with vertical sample transport. (D) Multi-layer device with outer lamination and glass fiber reagent storage pads.
determined that the lateral transport of the sample in the bottom layer was the primary reason for the poor performance.

In the third iteration, the device was modified to a vertical flow setup to not only reduce the total distance the sample had to be transported, which reduces the risk of sample loss through filtration and non-specific binding to the cellulose fibers, but also to significantly reduce, the amount of sample volume needed to perform multiple tests on a single device. The vertical flow design consisted of three wax-patterned paper layers with double-sided adhesive between each layer (Figure 22C). The top layer distributes the sample to four different test areas, the second layer contains stored buffer and/or substrate for the assay, and the final layer acts as the assay development layer which allows for the majority of the sample, buffer, and substrate to be combined in a single layer (Figure 22C). Each successive layer of the device has an increasing spot areas to drive flow from one layer to the next while minimizing volume loss in each transfer.

After loading the buffers and substrate into each appropriate spot, the devices were assembled and immediately tested. This designed did help reduce the sample volumes significantly; however, inconsistent transfer between layers was observed due to the fact that the paper was not in close enough contact to continue the capillary action of the sample. Also, it was noticed that the substrate did not leave the middle storage layer, with minimal color development. This could be due to a variety of issues, such as poor resuspension of the substrate after drying, poor transfer of the influenza through the device, or not enough flow rate to effectively transfer it to the distribution layer.

To address two of these issues, the middle paper layer was replaced with glass fiber conjugate pads, the same material used in LFAs to store Au NPs conjugated with
antibodies, as well as lamination of the entire device to help compress the device together for consistent contact of all layers of the device (Figure 22D). Introducing lamination into the fabrication means having to consider the stability of any enzymes or substrates which were stored in the device since the lamination process requires high levels of heat that could disrupt their function. As discussed later, we used an enzyme storage preservative to prolong their dry storage life as well as help mitigate any heat related issues during fabrication. Additionally, we used a laser cutter/printer to remove excess paper around the devices for proper lamination sealing and allow for 9 devices to be fabricated at a time. The buffer and substrates were dried onto the glass fiber pads, and then all layers were assembled in the hot roll laminator.

Liquid transfer in this design between layers was very good with quick wetting and transfer, and the total sample volume needed was reduced to 30 µL. Again, it was observed that there was minimal transfer of the substrate from the glass fiber pads to the distribution layer, making it difficult to visualize any color development. It became evident that the separate reagent storage layer in the device was not needed, and that the buffer/substrate combination could be stored directly in the development layer, thus leading us to our final design iteration.

The final version of the device consists of two wax-patterned filter paper layers and two outside layers of lamination. The construction and assembly of the µPAD is shown in Figure 23A. Briefly, AutoCAD drawings for each layer were created. For the distribution and development layers, printable images, which define the wax areas, were made from the AutoCAD drawings and printed onto the filter paper using the wax printing method described previously for the 96-well plate. Using a laser cutting printer
(Universal Laser Systems VLS3.50), the sample inlet was cut from the top lamination layer and both the distribution and development layers were cut for assembly purposes to allow for 9 µPADs to be fabricated at one time. The development layer was then loaded with the X-NeuNAc substrate in the appropriate buffer along with an enzyme storage agent (discussed later). Finally, all layers were assembled and placed into a carrier for hot roll lamination (Figure 23B) and then cut apart using the laser cutting printer. Initial fluid loading tests showed that 30-50 µL of a dye solution were adequate to fully wet the device (Figure 23C).

![Image](image-url)

**Figure 23. Influenza µPAD Design and Fabrication.** (A) Exploded view of the influenza µPAD construction. (B) Image of the influenza µPADs after lamination and prior to individual device cutting. Nine devices can be manufactured at once. (C) Finished influenza µPAD, front and back.
Multiple enzyme storage mixtures were attempted, with fair to poor results for most of the common reagent preservatives, such as BSA and trehalose. Pullulan, from *Aureobasidium pullulans*, is another sugar-based preservative storage method used previously by another group for enzyme preservation [82]. In their study, they showed very good retention of TaqDP enzyme activity, greater than 90%, even after 50 days of dried storage in Pullulan capsules [82]. We tested the pullulan with the enzyme substrate and purified neuraminidase enzyme in a non-dried assay to verify that there would not be any impact to assay performance (Figure 24A). Interestingly, at all concentrations of pullulan added to the system, an increase in NA activity was observed. One thought is that the sugar molecules cause a confinement of the NA and X-NeuNAc into close proximity which enhances reaction rates due to limited diffusion. Another thought is that since the reaction was allowed to proceed until the samples dried, the samples with lower pullulan concentrations dried faster than those with higher concentrations, thus allowing the reaction to proceed slightly longer. Both of these thoughts are supported by the fact that there appears to be a concentration dependent response at lower pullulan percentages (0-2.5% w/v), with it leveling off after 2.5% w/v and slightly declining at 10% w/v (Figure 24A). The decline at 10% w/v is most likely due to the high viscosity of the solution beginning to have a significant impact on the diffusion of the molecules. Additionally, to find the optimal pullulan concentration for storage of the X-NeuNAc, we examined the substrate performance after being dried with increasing concentrations of pullulan onto the paper 96-well plate. After 3 days at room temperature in open air conditions, the samples were resuspended with purified NA was added. The substrate which was stored in the 7.5% w/v pullulan (pH 7+ buffer) gave the highest activity
response when the fresh NA was added (Figure 24B). While 10% w/v was tested, and it did give second highest activity after dried storage, as mentioned previously, the solution is very viscous and is difficult to pipette accurately. Also, it is very slow to absorb into the filter paper, due to the high viscosity, for loading of the substrate onto the development layer. The 7.5% w/v pullulan concentration was selected as the optimum condition for substrate storage.

Following device assembly, 30 µL of DI H₂O containing the virus sample to be tested was added to the µPAD. Initially, very little to no color developed from the substrate in response to the virus sample (Figure 25, Left). Since we had already tested the ability of the dried substrate to resuspend and maintain its activity, we suspected the issue might be within the distribution layer. It was thought that the Whatman filter paper cellulose fibers were non-specifically binding the virus particles and not allowing them to travel to the development layer to react with the substrate. To mitigate this non-specific interaction, we pretreated the distribution layer with 0.5% w/v BSA and 20 mM CTAB to

![Figure 24. Evaluation of Enzyme Substrate Activity following Storage in Pullulan Matrix.](image)

(A) Pullulan was added at increasing concentrations to a non-dried, purified NA / X-NeuNAc reaction to test for potential assay interference. (B) X-NeuNAc was stored in Pullulan at increasing concentrations to determine optimal storage buffer concentration.
Figure 25. Effect of Pre-Treatment of Distribution Layer on Influenza µPAD Operation. Treatment was done to prevent non-specific adherence of influenza particles to cellulose fibers. (A) No Treatment. (B) Treatment with 0.5% w/v BSA. (C) Treatment with 20 mM CTAB.

see if either would limit the binding to the cellulose fibers. The BSA treated µPAD produced the best response, with a slight response from the CTAB treated µPAD, which is evident when observing the pH 7+ condition spot (top right spot in the images) (Figure 25, Middle and Right). All distribution layers for any new µPADs were subsequently pretreated with a 0.5% w/v BSA solution.

Once all parameters were optimized for influenza detection, reagent storage, and movement of sample through the µPAD, a final design was determined. The design still contains the distribution and development layers, as well as the top and bottom lamination layers. The four test areas in the development layer have an assay control and three different buffer/substrate conditions: pH 7+, pH 7+ with oseltamivir (Tamiflu), and pH 9- (Figure 26).

The assay control is used to verify that all reagents in the assay are still viable and that the sample flowed correctly through the µPAD. To accomplish this, purified NA (100 U/mL) was dried with 7.5% w/v Pullulan in the distribution layer directly above the control test spot on the development layer, which contained the dried pH 7+ buffer/substrate. When sample is added to the µPAD, the fluid resuspends the purified
NA in the distribution layer and then transfers it to the buffer/substrate in the development layer to produce the control reaction. This is similar to control lines on an LFA style assay to verify that the device and the assay has operated correctly.

The other buffer conditions for the μPAD were taken from the previous optimization work. The pH 7+ buffer/substrate condition was the buffer condition which all of the influenza types tested responded at and was therefore chosen to act as the “positive” condition for influenza. However, since other potential interferents, such as *S. pneumoniae*, are able to respond at the pH 7+ condition, this could create potential false positives for influenza. Thus, we chose the pH 9- buffer/substrate condition to act as a secondary verification for a positive influenza or positive for another NA-containing biological; however, sodium borate buffer was substituted for Tris as the pH 9 buffer due to better stability for storage on the paper. At pH 9-, *S. pneumoniae* responds very strongly while nearly all influenza strains have minimal response. For the pH 7+ with Tamiflu condition, we chose to lower the Tamiflu concentration from 350 nM to 150 nM to give a more accurate representation of NAI effectiveness if used for potential treatment.

To perform a test, 30 μL of DI H₂O containing the diluted virus sample to be tested is added to the μPAD via the sample port on top. The assay was allowed to sit for ~10 hours at room temperature prior to imaging. The schematic in Figure 26 describes the visual readout process of the influenza μPAD. First, the control test spot (top right of μPAD) is observed to confirm proper assay function via blue color development. Next, the pH 7+ and the pH 9- test areas (bottom right and top left of μPAD, respectively) are observed and compared to determine if the test is positive for influenza (pH 7+: blue, pH
9-: white), positive for another NA-containing biological or a potentially mixed sample (pH 7+: blue, pH 9-: blue; pH 7+: white, pH 9-: blue), or a negative response (pH 7+: white, pH 9-: white). If the assay shows a positive for influenza, the final step is to determine the NAI susceptibility of the influenza strain being detected for potential therapeutic treatment. By comparing the pH 7+ and pH 7+ with Tamiflu (Tami) test spots, the effectiveness of the NAI on the influenza can be determined. If the pH 7+ spot is blue and the pH 7+ with Tamiflu is blue, then the influenza strain will not respond to NAI treatment. Conversely, if the pH 7+ spot is blue and the pH 7+ with Tamiflu spot is white, the influenza strain is susceptible to NAIs and they could be used as a therapeutic treatment. Additionally, in the case of a blue response at the pH 7+ condition but a slightly less blue response at the pH 7+ with Tamiflu condition, image analysis can be used to determine an estimate of the NAI treatment effectiveness by comparing the intensity of the blue substrate signal.

Figure 26. Schematic for Interpretation of Influenza μPAD Assay.
When the virus and bacterial samples were tested, the µPAD responded ideally for all samples tested. All µPADs run indicated valid tests by the blue color development in the control test spot. The negative control media sample (Figure 27A) showed no response in all 3 test areas, indicating the assay functioned properly and that the assay was negative. The pH1N1/09-S sample produced a blue response for the pH 7+ condition only, indicating that the sample contains influenza and that the influenza strain detected is susceptible to NAIs (Figure 27B). The sH1N1/07L-R µPAD identified that the sample contained influenza (pH 7+: blue) and that the influenza strain detected is mostly NAI resistant. However, using the image analysis data (Figure 27C), we can conclude that 150 nM Tamiflu is able to reduce NA activity by ~40% for the sH1N1/07L-R strain. For the sH3N2/07L-S, H3N2/07-R, and Yamagata samples, all demonstrated a positive result at the pH 7+ condition only (Figure 27D, 27E, and 27F, respectively), even though H3N2/07-R and Yamagata are both considered resistant strains. As discussed earlier, this is due to the fact that even though their NA has the resistance mutation, their IC50’s are well below 150 nM for oseltamivir (Table 1).

During image analysis of the completed µPADs, it was discovered that by using only the change of the CIE x-coordinate, a much more accurate interpretation of a positive or negative result for each test spot could be made. Since the primary color development of the assay is from clear (white on the paper) to blue, this corresponds to a nearly linear shift on the CIE color space, which happens to be parallel to the CIE x-axis. Using this technique, any positive values indicate a blue color change while any negative values indicate non-blue color changes. These results match the results seen during the strain variation and NAI testing performed on the 96-well platform.
Figure 27. Influenza µPADs Tested with Various Influenza Strains.

Figure 28. Image Analysis of the Influenza µPADs Tested with Various Influenza Strains. See Figure 26. In this case, the CIE X-coordinate change is being monitored for determination of a blue or non-blue result for each test spot.
Summary

In this work, a novel use of a µPAD to develop a theranostic to not only detect influenza, but determine NAI susceptibility as well, was demonstrated. We used invariable characteristics of influenza biology (HA and NA) to detect and differentiate influenza virus from PIV and S. pneumoniae by measuring NA activity at different buffer conditions. The use of intrinsic viral characteristics for diagnostic provides a great advantage over antibody-recognition diagnostics, since binding to host factors is not subject to antigenic shift or drift. Moreover, assays performed in the presence and absence of NAI could direct the appropriate use of antiviral therapy.

Optimization of buffer and substrate concentrations for a NA activity assay for integration into a paper-based platform, determined proper incubation temperature for assay performance, and evaluated reagent storage in dried form were performed. Next, while not the primary goal of the work, we demonstrated the ability to differentiate strains of influenza, which could be useful as a clinical laboratory style assay instead of a POC style device. Finally, we designed a µPAD that would allow for simple, one-step addition of sample with the ability to both read the output visually or with the use of image-based analysis to determine if a sample contained influenza, and if it did, also determine the level of NAI susceptibility.

Future work in this area will primarily focus on reduction of assay time through investigation of alternate substrates, possibly fluorescent or chemiluminescent, or the incorporation of electrochemical detection capabilities into the µPAD to increase detection sensitivity. Most likely a voltammetry-type measurement will be used to determine amounts of NA activity. Additionally, real patient mucosal samples will be evaluated in future studies, possibly as part of a clinical trial.
CHAPTER IV

CONCLUSIONS

In this dissertation, the development and optimization of two paper-based platforms for detection of human performance and physiological biomarkers was described. The overall objective was to demonstrate that various current clinical laboratory-type assays could be accomplished on paper-based systems. The first assay that was demonstrated was a well-known, and highly used, clinical laboratory assay known as an ELISA. The second assay demonstrated was a novel influenza detection assay. The ability to successfully transition both the ELISA and influenza assays to paper-based platforms demonstrates the potential to transition many more clinical laboratory assays onto these inexpensive, disposable devices.

For the transition of an ELISA assay to a paper-based platform (P-ELISA), multiple assay parameters were optimized. A variety of enzymes and enzyme substrates were examined to find the optimum combination for detecting an immunoglobulin as well as a human performance biomarker, NPY. By simply changing out the substrate used, a 4 order of magnitude improvement in sensitivity was observed over previous studies. However, while the chosen substrate (pNPP) performed very well, with detection limits down into the pM (IgG) and nM (NPY) range, the image analysis methods needed to be optimized as well. Previously used grayscale analysis methods were sub-optimal due to the new substrate producing a yellow color,
which does not produce much color change when converted to grayscale. Colorimetric image analysis methods, specifically CIE 1931 color space conversion, provided nearly a 50% enhancement in measurement sensitivity over the grayscale method when used in conjunction with the yellow substrate. Finally, MATLAB- and Android-based programs were created to allow for non-laboratory equipment determination of analyte concentrations on the P-ELISA since the primary use of ELISAs are to precisely quantify analyte concentrations in a sample. In comparison with all image analysis methods, the automated MATLAB-based algorithm produced the most accurate results. The ability to detect large and small biomolecules on an inexpensive, disposable platform, and the ability to perform measurements using a tablet or smartphone, the possibility of using P-ELISAs in resource limited environments as well as potentially in conventional clinical laboratories to reduce sample volume, overall diagnostic costs, and equipment costs.

For the influenza detection assay, a novel, point-of-care style µPAD (microfluidic paper-based diagnostic) for influenza was developed with the ability to determine antiviral susceptibility of the strain for treatment decision. The assay exploits the enzymatic activity of NA surface proteins, present on all influenza strains, by using the NA to reduce a sialic acid-containing colorimetric substrate. Through the use of different pH buffers, as well as the presence of Ca$^{+2}$ or EDTA, variations in strain responses were seen which could lead to potential rapid strain typing without the use of PCR methods. Additionally, optimal buffer conditions were found such that all influenza A and B types would produce a response, but would also rule out potential false positive samples. In addition, an antiviral (NAI) was tested to determine if the influenza strain being detected would be susceptible to the NAI and could be used as a potential treatment option. The influenza µPAD, onto which the optimized buffer and substrates were
added, consisted of 4 layers, with the top and bottom consisting of lamination sheets, and the middle two layers consisting of wax-printed filter paper. The wax-printed layers first distributed the sample and then, second, provided confined areas for reagent storage and assay development. Dried storage of the enzyme substrate was accomplished through the addition of a unique polysaccharide from a fungus, Pullulan. Operation of the µPAD is simple: a sample is added to the µPAD through the top port, the sample is then distributed to 4 different reagent zones, and development of the enzymatic substrate under different buffer conditions takes place on bottom of the device. Analysis can be performed by eye or through a colorimetric image analysis smartphone app. The µPAD produced excellent results for all influenza types and sub-types tested. There is enormous potential for this type of device in not only limited resource environments, but in first world countries as well. The ability to not only detect influenza, but determine the effectiveness of an antiviral for treatment as well, is a significant advancement in medical care for influenza infected patients.

**FUTURE WORK**

While there are many paper-based diagnostic assays are currently available, it appears that many significant advancements are to come in the near future. One of the significant drivers of new breakthroughs in paper-based diagnostics is the ability to fabricate electronics onto paper, or combine thin-film electronics with paper substrates, which opens a vast number of new options for expanding the capabilities of paper-based diagnostics. Future work in this area will focus on these new opportunities to integrate electronics for increased functionality while maintaining an overall inexpensive and disposable platform. Enhancements such as microelectrodes for electrochemical detection, light emitting diodes (LEDs) and photodiodes for
on-device absorbance or fluorescence measurements, or integration of droplet microfluidics areas for complex sample processing could be possible. Ultimately, these improvements could not only lead to faster, more sensitive paper-based diagnostic, but they could also provide very inexpensive platforms which would require minimal external electronics to control, reducing costs even further. This is significant when taking into account that these devices were initially conceived for use in third world countries where resources are very limited.
REFERENCES


APPENDIX A

AUTOMATED IMAGE ANALYSIS - MATLAB CODE

clear all

path = imgetfile;

% load RGB intensities to ALLRGB then show image
ALLRGB = imread(path);
imshow(path)

% RedIntensity=[];
GreenIntensity=[];
BlueIntensity=[];
Area=[];
Concentration=zeros(1,96);

AutoLevel = graythresh(ALLRGB);
SetLevel = 0.6;
level = min([AutoLevel, SetLevel]);
BW = im2bw(ALLRGB,level);

CC = bwconncomp(BW);
stats = regionprops(CC, 'Eccentricity', 'Area');
% threshold
idx = find([stats.Eccentricity] < 0.5 & [stats.Area] > 1000);
BW2 = ismember(labelmatrix(CC), idx);
CC2 = bwconncomp(BW2);
stats2 = regionprops(CC2, 'centroid', 'Perimeter');
centroids = cat(1, stats2.Centroid);
radius = cat(1, stats2.Perimeter)/(2*pi);
count = nnz(centroids)/2;

% measure the image size and define the corresponding mask size
[Row, Col, Color] = size(ALLRGB);
Mask = zeros (Row, Col);

% shrink the size of auto-detected dots
[rr cc] = meshgrid(1:Col, 1:Row);
for S = 1:count
C = sqrt((rr - centroids(S,1)).^2 + (cc - centroids(S,2)).^2) <= (0.5*radius(S));
Mask = Mask + C;
end

CC3 = bwconncomp(Mask);
stats3 = regionprops(CC3, 'centroid', 'Area');
centroids2 = cat(1, stats3.Centroid);
if centroids2 > 0
    hold on
    plot(centroids(:,1), centroids(:,2), 'o')
    hold off
end

RedIntensity = [RedIntensity, cell2mat(struct2cell(regionprops(CC3, ALLRGB(:,:,1), 'MeanIntensity')))];
GreenIntensity = [GreenIntensity, cell2mat(struct2cell(regionprops(CC3, ALLRGB(:,:,2), 'MeanIntensity')))];
BlueIntensity = [BlueIntensity, cell2mat(struct2cell(regionprops(CC3, ALLRGB(:,:,3), 'MeanIntensity')))];
Area = [Area, cell2mat(struct2cell(regionprops(CC3, ALLRGB(:,:,1), 'Area')))];

% convert RGB to CIE coordinates, not necessary if use DeltaRGB
% [x,y,Y] = sRGBtoCIE(RedIntensity, GreenIntensity, BlueIntensity);

% calculate DeltaRGB
DeltaRGB = ((RedIntensity - 215.20).^2 + (GreenIntensity - 204.28).^2 + (BlueIntensity - 210.87).^2).^(0.5);
% DeltaRGB = ((RedIntensity - 199.38).^2 + (GreenIntensity - 202.4).^2 + (BlueIntensity - 187.09).^2).^(0.5);

% substitute DeltaRGB into calibration curve
% Concentration = zeros(1, CC2.NumObjects);
syms x;
for I = 1:count;
    Log10Concentration = solve(17.660536086*x + 46.177526081 - DeltaRGB(I), x);
    Conc = 10.^(Log10Concentration);
    Concentration(1,I) = Conc;
end

% display the delta values
for i=1:CC2.NumObjects
    DisplayConcentration = sprintf('%0.3g', Concentration(:,i));
    text(centroids2(i,1)-radius(i), centroids2(i,2)+(radius(i)+10),
        sprintf('%suM',DisplayConcentration), 'color', [0.1,1,0.1], 'Fontsize', 10);
end
Exporting Data to Excel (.XLS)

y0 = centroids2(:,2)';
[ys, IX] = sort(centroids2(:,2));
for j = 1:96
    x1(j) = centroids2(IX(j),1);
    SortedRedIntensity1(j) = RedIntensity(IX(j));
    SortedGreenIntensity1(j) = GreenIntensity(IX(j));
    SortedBlueIntensity1(j) = BlueIntensity(IX(j));
    SortedArea1(j) = Area(IX(j));
    SortedConcentration1(j) = Concentration(IX(j));
end
for j = 1:8
    [x2(1:12*(j-1):12*j), IX] = sort(x1(1:12*(j-1):12*j));
    for i = 1:12
        y2(i+12*(j-1)) = y1(IX(i)+12*(j-1));
        SortedRedIntensity2(i+12*(j-1)) = SortedRedIntensity1(IX(i)+12*(j-1));
        SortedGreenIntensity2(i+12*(j-1)) = SortedGreenIntensity1(IX(i)+12*(j-1));
        SortedBlueIntensity2(i+12*(j-1)) = SortedBlueIntensity1(IX(i)+12*(j-1));
        SortedArea2(i+12*(j-1)) = SortedArea1(IX(i)+12*(j-1));
        SortedConcentration2(i+12*(j-1)) = SortedConcentration1(IX(i)+12*(j-1));
    end
end
SortedConcentration3 = reshape(SortedConcentration2, 12, []);
SortedRedIntensity3 = reshape(SortedRedIntensity2, 12, []);
SortedGreenIntensity3 = reshape(SortedGreenIntensity2, 12, []);
SortedBlueIntensity3 = reshape(SortedBlueIntensity2, 12, []);
SortedArea3 = reshape(SortedArea2, 12, []);
SortedConcentration = SortedConcentration3';
SortedRedIntensity = SortedRedIntensity3';
SortedGreenIntensity = SortedGreenIntensity3';
SortedBlueIntensity = SortedBlueIntensity3';
SortedArea = SortedArea3';
xlswrite(name, SortedConcentration, 'raw data', 'A1');
xlswrite(name, SortedRedIntensity, 'raw data', 'A10');
xlswrite(name, SortedGreenIntensity, 'raw data', 'A19');
xlswrite(name, SortedBlueIntensity, 'raw data', 'A28');
xlswrite(name, SortedArea, 'raw data', 'A37');