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Low-cost methods of on-demand fluid flow, nematode egg separation and counting

Matthew Philip Weinstein
Iowa State University

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Low-cost methods of on-demand fluid flow, nematode egg separation and counting

by

Matthew Weinstein

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Electrical Engineering

Program of Study Committee:
Santosh Pandey, Major Professor
Gregory Tylka
Long Que

The student author and the program of study committee are solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

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ABSTRACT

There is an increasing interest to re-invent device technologies for use in actual field applications where resources may be limited. Numerous examples exist in the medical and agricultural industry where the demand and benefits for on-the-spot methods of sample collection, preparation, and characterization is being recognized by the scientific community. Some key attributes of devices that cater to this demand of on-the-spot testing are portability, low cost, disposability, easy fabrication and storage, simple operational procedure, and reasonable sensitivity and selectivity.

Within the abovementioned realm of device technologies, the thesis attempts to provide low-cost alternative methods for two applications: (i) fluid flow in paper microfluidic devices and (ii) nematode egg separation and counting.

Chapter 1 presents a technique to control and operate fluid flow between multiple paper substrates by employing a folded paper cantilever strip. The paper strip is mechanically actuated by a small fluid droplet. The technique can be scaled up to accomplish multiple colorimetric tests, and is demonstrated for the parallel detection of three analytes (glucose, nitrite, and protein). This actuator is intended for use in paper microfluidic devices to quantify levels of disease biomarkers.

Chapter 2 describes a method to separate nematode eggs from soil particles followed by counting the number of eggs within a fluid suspension. Nematode eggs with similar-sized soil particles are collected from a series of soil processing steps. Conventionally, through a staining procedure and sucrose centrifugation, a relatively cleaner soil suspension with eggs is obtained that is later counted manually on a

microscopic slide. Here we present a method of density-based centrifugation that seems to provide better efficiency in separating the eggs from the soil particles. Egg counting from large-volume fluids is accomplished on a scanner with a custom imaging software that recognizes and counts the number of eggs.

In summary, the use of paper substrates is advocated in the thesis because they are considerably cheaper than polymeric substrates and can be easily fabricated, molded, stored, transported, and imaged. For both applications, the methods presented here could be adopted in field settings and integrated with other current procedures of sample preparation and characterization.

CHAPTER 1

FOLDED PAPER-BASED CANTILEVER ACTUATOR

FOR ON-DEMAND FLUID FLOW BETWEEN PAPER STRIPS

1.1 Introduction

In recent years, the concept of fabricating devices on paper substrates has gained momentum where the flow of fluids is simply controlled by capillary forces of the paper, and thus minimizing or eliminating the use of conventional microfluidic flow instruments [1,3,5]. In comparison to their counterparts (i.e. polymeric microfluidic devices), paper-based microfluidic devices are more user-friendly, cheaper, disposable, portable, and compatible with a range of chemical and biological samples [4,6-10]. This makes them particularly attractive for applications in resource-limited settings where there is scarcity of equipment, power or trained personnel to perform the necessary experiments [2,24,25]. Other application scenarios may arise where there is a need for quick, on-the-spot diagnostic test prior to a detailed study of the data in a laboratory setting. We can imagine applications of paper microfluidic devices in a number of sectors where quality and safety control check of machines or human health is of utmost importance such as the healthcare, automotive, aviation, food and agriculture industry.

Amongst the various methods of conducting analytical tests in paper microfluidics, the colorimetric scheme is the simplest and most straightforward where a specific chemical reaction (triggered by the presence of certain target molecules) produces a distinct color change in the paper strip [1,2,11-17]. The chosen chemical reaction may occur in a single step or in a sequence of multiple steps. Single-step chemical reactions are often enzymatic

in nature and are easy to conduct using one or two test paper strips [26,27]. A multi-step chemical reaction requires several rounds of mixing the sample and reagent solutions that is often performed sequentially by manual pipetting and controlled incubation times at each step (e.g. enzyme-linked immunosorbent assays and DNA-based tests) [14]. Performing multi-step chemical reactions on paper microfluidic devices is challenging because the distinct incubation times for each chemical reaction almost necessitates the presence of a human operator to trigger each step.

Some efforts towards creating automated valve systems to regulate a multi-step chemical reaction in paper microfluidics have been reported in the literature [14,15]. While mechanical or electronic valves can adequately control the fluid flow between two paper strips, they are impractical to integrate in paper microfluidic devices in terms of cost, efficiency, and space requirements. Hence, simple schemes have been proposed in the literature to modify or manipulate the flow rate of fluids in paper test strips. Some examples are as follows. Fluid flow rate in the paper test strips is altered by changing the channel width [15]. Different lengths of paper strips access a shared reservoir system, thereby allowing a fixed volume of fluid through each channel [15,18-20,23]. Specific water-soluble solids pre-deposited on the test strip control the time delay in the passage of fluid through a section of the channel. [13,21,22]. A dissolvable sugar bridge is used to stop additional fluid flow after delivering a desired volume of fluid [14]. Instead of using water-soluble solutes, a sponge pad can be used to absorb a certain amount of fluid, expand thereafter, and thus connect two separate test strips [15].

Here we introduce a new valve method to start and control fluid flow between two or more paper test strips for applications to paper-based microfluidic devices. The valve is

primarily made of the same paper substrate but is uniquely folded and shaped as a cantilever beam. We call it the *paper cantilever actuator* (PCA) and it does not require any external instruments or forces (e.g. electricity, pressure and chemical) to be activated. The PCA is activated simply by dropping a small amount of the fluid ($< 8 \mu\text{L}$) at its fold to connect or disconnect the microfluidic channels. Through a series of experiments, we demonstrate its basic operating principle and the range of configurations for its functioning. To show its applicability in parallel testing of multiple analytes, the PCA microfluidic valve system is applied to a programmable colorimetric diagnostic platform to detect the presence of glucose, nitrite and protein in a sample solution.

1.2 Methods and Procedure

1.2.1 Materials and chemicals

Pieces of 200×200 mm. chromatography paper (Whatman) were cut to form the reservoirs, actuators and receiver channels using a paper trimmer (Fiskars SureCut). Single-sided tape was used at non-flow connection points. Glass slides and plastic pads supported each reservoir and receiver channels. Three different colorimetric chemical assays were produced using our device to detect glucose, nitrite and protein respectively. The glucose reagent is composed of glucose oxidase, horseradish peroxidase (Sigma Aldrich), potassium iodide (Sigma Aldrich), trehalose (Sigma Aldrich) and phosphate buffer with pH of 6.0. The nitrite assay's reagent contains citric acid monohydrate (Fisher Scientific), methanol (Sigma Aldrich), sulfanilamide (ACROS), N-(1-Naphthyl) Ethylenediamine (Sigma Aldrich). For the protein assay, two solutions are prepared for testing: a priming and reagent solution. The priming solution contains Citrate buffer

(ACROS), Ethanol (Sigma Aldrich), hydrochloric acid (Fisher Scientific) and water. The reagent solution is a mixture of tetrabromophenol blue dye (Sigma Aldrich), ethanol and water. The fluid flow and color change are recorded and tracked by a webcam (Logitech).

1.2.2 Device actuation principle

The basic principle behind the mechanical motion of a paper cantilever actuator is by straightening out a folded area by wetting the folded area with water. The chromatography paper, made from cellulose fibers, is manually pressed flat and packed together evenly. When the paper is folded, the cellulose molecules in the inner part of the folded area are packed more densely than when laid flat. Upon dropping water to the folded area, the tension between the cellulose fibers is relaxed that, in turn, expands the volume of the paper in all dimensions, causing the folded area to straighten. Based on preliminary testing, we identified the nature and number of folds needed for consistent relaxation of the cellulose fibers and the production of maximum desired actuation. In the device setup, the paper cantilever actuator is folded into four equal lengths and 4 - 8 μL water is applied to the tip of the actuator.

1.2.3 Colorimetric assay for analyte detection

The two chemicals required to induce the reaction of glucose into hydrogen peroxide are glucose oxidase and horseradish peroxidase [29]. For every 1 mL of reagent solution, 120 units of Glucose Oxidase and 30 units of Horseradish Peroxidase are mixed into Phosphate Buffer Saline (PBS, pH = 6.0). The PBS is prepared to a 6.0 pH solution by adding two chemicals, 33mg of Sodium Phosphate Dibasic Heptahydrate and 105.25

mg of Potassium Phosphate Monobasic Anhydrous for every 1 mL of distilled water. The PBS mixture is stirred at room temperature for around 5 minutes or until all the solids have been dissolved in the water. Once the two chemicals are added to PBS, 2.27 mg of Trehalose is added that serves to stabilize the performance and stability of the enzymes in the solution [30]. Thereafter, 99.6 mg/ml of Potassium Iodide is added to the solution which is then mixed at room temperature for around 10 minutes or until all of the solutes were fully dissolved in the solution. For every cm^2 unit area of paper, about 15.28 μL of reagent is applied to form the testing area. The chemical reaction process is as follows: glucose is oxidized to gamma-gluconolactone in the presence of glucose oxidase (flavin adenine dinucleotide, FAD) which is reduced to FADH_2 . After that, FADH_2 is re-oxidized to produce FAD and hydrogen peroxide. With horseradish peroxidase serving as a catalyst, the hydrogen peroxide reacts with the potassium iodide to produce a yellow-brown color compound [31]. In reagent solution, trehalose is added to stabilize the oxidase that improves reaction efficiency [30].

For the nitrite colorimetric assay, a diazo compound needs to be formed from a nitrous acid. This compound reacts with the dye material to form the color change for colorimetric analysis. The chemical to turn nitrite into nitrous acid is 69.3 mg/mL Citric Acid Monohydrate mixed into Methanol. The resulting acid reacts with 8.61mg/mL Sulfanilamide to form a diazo compound. The dye chemical that the compound reacts with is N-(1-Naphthyl) Ethylenediamine, turning it from a colorless solution into a magenta color [32]. The components are mixed at room temperature for around 10 minutes or until the solutes all completely dissolve. For every cm^2 unit area of paper, about 15.28 μL of reagent is applied to form the testing area.

In the protein assay, a protein needs to bind to a dye chemical in an ionic bond. In an acidic environment, ionic bonds form between the dye and the protein. The resulting complex with the physical change in size produces an absorbance shift, creating a visible color change [33]. Two solutions are used to prepare the testing area: a priming solution and the dye reagent solution. The priming solution is formed by adding 51 mg/mL of 4.0 pH Citrate Buffer to a solution made up of 92 % distilled water and 8 % Ethanol. In order to lower the pH of the solution to a more acidic level, in this case 1.8 pH, we add around 1300 μ L of 1 M Hydrochloric Acid and mix the solution together for about 5 minutes at room temperature. For every cm^2 unit area of paper, about 15.28 μ L of primer is applied to form the testing area. The second solution is the dye reagent, which consists of 8.87 mg/mL tetrabromophenol blue mixed into a combination of 95% Ethanol and 5% distilled water. This solution is mixed at room temperature for 5 minutes or until the solute fully dissolves. For every cm^2 unit area of paper, about 15.28 μ L of reagent is applied to form the testing area. An acidic surface causes the tetrabromophenol blue to bind with protein, which changes the color from orange to blue [34].

1.3 Results and Discussion

1.3.1 Switch actuator application

The first application we demonstrate here using paper-based cantilever actuator (PCA) is the switching of fluid flow. There are two types of switches that can be implemented: ON switch and OFF switch. The paper strip is cut into 30mm lengths and 7 mm widths. The resulting strip is folded into four pieces at intervals of 7.5 mm. A 200 mg weight is applied to the folded actuators for 5 seconds each as a way to standardize fold

pressure. Once folded, single-sided tape is applied to the end of the actuator to prevent the sample fluid from flowing into the actuator. The reservoir strip is made of a $20 \text{ mm} \times 13 \text{ mm}$ area to serve as a reservoir and a $30 \text{ mm} \times 3 \text{ mm}$ initial channel. The receiver channels are cut in 30 mm lengths and 3 mm widths. For ON switch operation, the receiver channel is suspended 2mm above the reservoir strip (Figure 1.1a-i). The reservoir strip is soaked with $225 \mu\text{L}$ of blue dyed water until saturation (Figure 1.1a-ii). Before activating the actuator, the fluid is unable to flow to the receiver strip due to the physical separation of the reservoir strip and the receiver channel. Then, $4 \mu\text{L}$ of red dyed water is introduced to the tip of the actuator. This causes the activation of the PCA, which lifts the reservoir strip

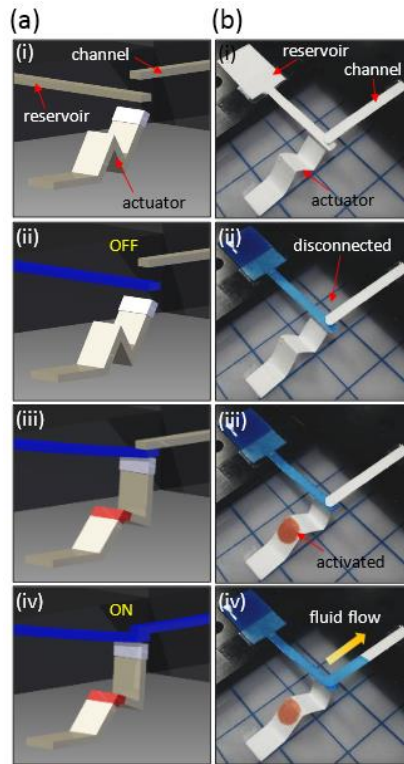


Figure 1.1: Paper-based actuation switch from OFF to ON position. **(a)** CAD simulation of actuation procedure: Reservoir strip begins disconnected from receiver channel, hanging between channel and primed PCA, the OFF position, then filled with blue dyed water (i, ii). PCA is activated by wetting the fold with red dyed water, pushing the end beneath the reservoir strip up vertically to make contact (iii). Actuator continues to push the reservoir strip end up into receiver channel, providing a new avenue for fluid flow, the ON position (iv). **(b)** Practical example of OFF to ON paper actuation.

upwards and connects to the receiver channel (Figure 1.1a-iii). The blue dyed water transfers from the reservoir strip to the receiver channel and flows to the end of the channel (Figure 1.1a-iv). As the actuator is activated once the folded area is wet, a user does not need to provide an excess volume of fluid. The operation of the ON switch in the actual system follows the process depicted by the CAD simulation (Figure 1.1b). For OFF switch operation, a reservoir strip is attached on top of the receiver strip (Figure 1.2a-i). Since both reservoir and receiver channels are initially attached, when the reservoir strip is saturated with the blue dyed liquid, the fluid begins flowing into the receiver channel without activating the actuator (Figure 1.2a-ii and iii). Once the actuator absorbs the liquid, without activating the actuator (Figure 1.2a-ii and iii). Once the actuator absorbs the liquid,

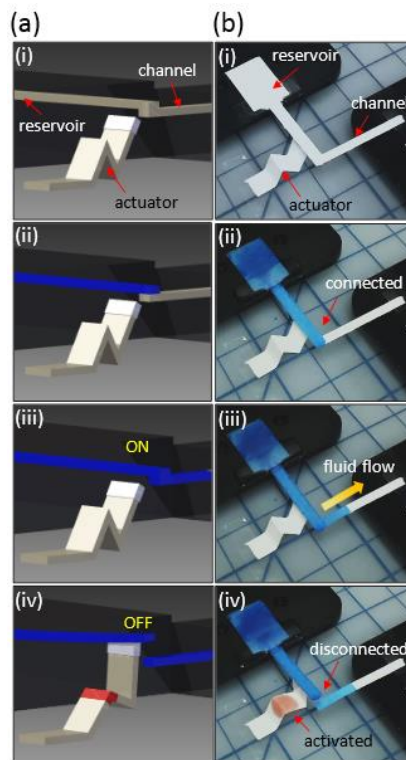


Figure 1.2: Paper based actuation switch from ON to OFF position. **(a)** CAD simulation of actuation procedure: Reservoir strip begins connected from receiver channel, the primed PCA placed beneath the reservoir strip (i). Reservoir strip filled with blue dyed water (ii). Liquid travels from reservoir strip to receiver channel to maintain fluid flow, the ON position (iii). PCA is activated by wetting the fold with red dyed water, pushing reservoir strip up vertically, disconnecting the reservoir strip from receiver channel and ceasing fluid flow, the OFF position (iv). **(b)** Practical example of ON to OFF paper actuation.

it is activated and disconnects the reservoir strip from the receiver, thereby stopping flow (Figure 1.2a-iv). Similar to the ON switch, operation of the system in reality successfully follows this procedure (Figure 1.2b).

1.3.2 Actuation of change in flow path

The paper-based cantilever actuator (PCA) can be used to switch the flow path from one channel to another as opposed to just starting and stopping flow. In the example operation the reservoir strip is connected to receiver channel I similar to the OFF switch configuration (Figure 1.3b). Receiver channel II is positioned 2 mm vertically above the

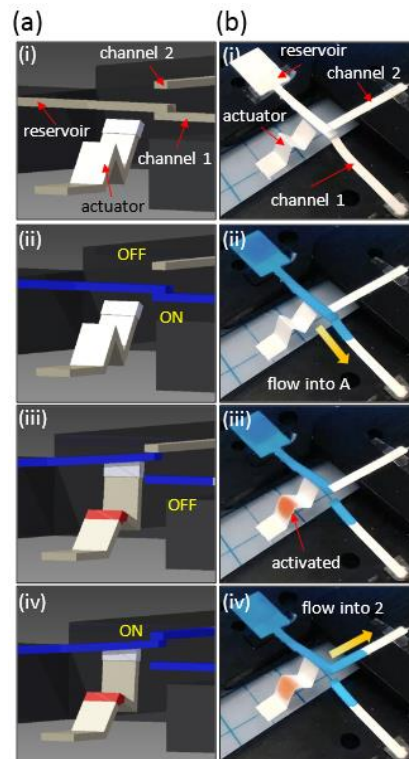


Figure 1.3: Paper-based junction actuation, starting from channel I to channel II. **(a)** CAD simulation of actuation procedure: Reservoir strip placed on top of channel I and below channel II, with PCA primed beneath reservoir strip (i). Reservoir strip filled with blue dyed water, fluid flows from reservoir strip to channel I, the ON position, while remaining disconnected from channel II, OFF position (ii). Paper actuator activated with red dyed water, pushing reservoir strip up vertically away from channel I and in contact with channel II, turning channel I OFF (iii). The liquid from reservoir strip travels down the newly connected channel II, turning channel I ON (iv). **(b)** Practical example of switch from ON to OFF to ON.

connection of those two paper strips (Figure 1.3a-i). The materials, volumes and dimensions of the paper strips are kept same as those in the switch actuation system. Blue dyed water flows into channel I from the reservoir strip without wetting channel II (Figure 1.3a-ii and iii). When the tip of the PCA absorbs a small amount of water, the actuator lifts up the reservoir strip and connects it to channel II. Therefore, fluid stops flowing into channel I and begins to flow into channel II (Figure 1.3a-iv). The next example operation is to switch the path twice by activating the PCA twice. The reservoir strip is initially positioned between channel I and channel II (Figure 1.4a-i). When the reservoir strip is

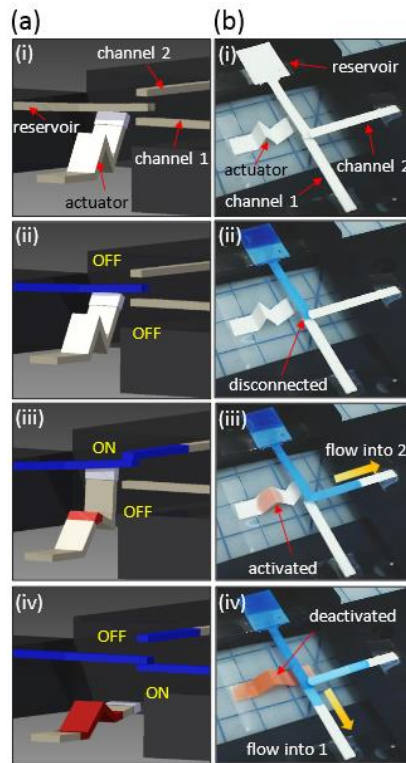


Figure 1.4: Paper-based junction actuation, starting from neither channel I or II. (a) CAD simulation of actuation procedure: Reservoir strip held aloft by primed PCA between channels I and II (i). Reservoir strip filled with blue dyed water, but cannot travel into either channel, leaving both in the OFF position (ii). PCA activated by a small amount of red dyed water, pushing reservoir strip up to connect to channel II. Fluid flows into channel II, turning ON, while channel I remains OFF (iii). More red water is applied to the paper actuator, deactivating and letting reservoir strip fall onto channel I and away from channel II. The fluid ceases flowing in channel II, turning OFF, while it begins flowing into channel I, turning ON (iv). (b) Practical example of actuation procedure.

saturated, 4 μL of red dyed water is introduced to the tip of the PCA. The top surface of the reservoir strip is attached to the bottom of channel II and fluid flows into channel II (Figure 1.4a-ii and iii). While a fluid keeps flowing into channel II, 8 μL of red dyed water is introduced to the tip of PCA. The reservoir strip is disconnected from channel II and falls to the top surface of channel I. The liquid then continues to flow into channel I (Figure 1.4a-iv). The physical example of the system correctly follows the procedure (Figure 1.4b).

1.3.3 Employing multiple folds in the actuator

The paper-based cantilever actuator (PCA) can have multiple folds to perform complex fluid flow operations. Using a two-fold PCA, a paper strip can be lifted up twice to make fluid connections to multiple channels placed at an identical height. In the first example, a reservoir strip is attached to the top surface of channel I while channel II is 4 mm above the interface (Figure 1.5). As the blue dyed liquid flows into the channel I from the reservoir (Figure 1.5a-i), the first fold of PCA is activated that lifts up the reservoir strip. The reservoir strip disconnects from channel I and remains stationary between both testing strips (Figure 1.5a-ii). After that, another red dyed droplet is pipetted to the second fold of PCA that lifts the reservoir strip further and helps to reach the bottom of channel II (Figure 1.5a-iii). Once the reservoir strip is attached to channel II, fluid flows into the strip (Figure 1.5a-iv). A side view of the actual system demonstrates the capability of the vertical two-fold actuator (Figure 1.5b). In addition, two-fold PCA can perform multiple lifting operations horizontally. An example to depict this actuation scheme involves two reservoir strips placed above channel I and channel II (Figure 1.6). Underneath channel I, the two-fold PCA is located (Figure 1.6a-i). The first fold of the PCA is activated and lifts channel I (Figure 1.6a-ii), which causes the blue fluid to flow from the first reservoir into channel

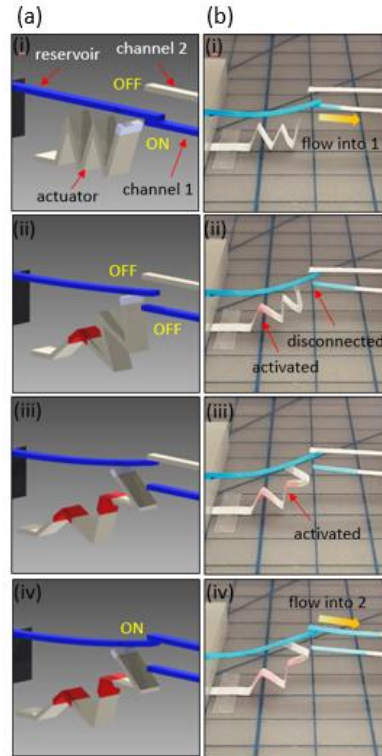


Figure 1.5: Two-fold vertical actuation from channel I to channel II. **(a)** CAD simulation of actuation procedure: Reservoir strip filled with blue dyed water is placed in contact with channel I, allowing fluid flow into the channel to turn it ON. The two folded PCA is positioned beneath reservoir strip parallel to its pathway (i). The actuator is activated by wetting the first fold with red dye liquid, raising reservoir strip away from channel I and kept between the two channels, leaving both in the OFF position (ii). The second fold of the actuator is wetted, raising reservoir strip and making contact with channel II (iii). Contact with channel II permits fluid flow, turning channel II ON while channel I remains OFF (iv). **(b)** Practical example of actuation procedure.

I. While flowing, the PCA is deactivated by wetting a folded area forming the obtuse angle, which causes the channel I strip to descend and the PCA to step forward to the position underneath channel II (Figure 1.6a-iii). After moving, PCA is re-activated by wetting the second fold which causes the green fluid to flow into channel II from the second reservoir (Figure 1.6a-iv). The top view of the physical system demonstrates the viability of the horizontal system (Figure 1.6b).

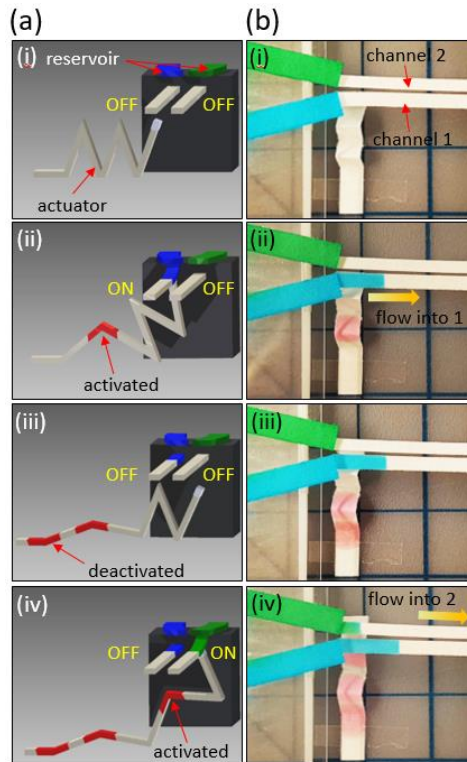


Figure 1.6: Two-fold horizontal actuation from channel I to channel II. **(a)** CAD simulation of actuation procedure: Two reservoir strips are filled with different colored water and placed above separate channels I and II, with PCA primed beneath channel I perpendicularly (i). First fold of the actuator is wetted with red dyed water, pushing channel I up into first reservoir strip and beginning fluid flow, turning channel I ON (ii). The actuator is deactivated with more red water, moving the end horizontally beneath channel II, and channel I strip falls away from the reservoir to turn OFF (iii). The second fold of PCA is wetted, pushing channel II up into the second reservoir strip, turning channel II ON while channel I remains OFF (iv). **(b)** Practical example of actuation procedure. The process matches the expectations in (a), turning channel I ON before moving horizontally to turn channel II ON while channel I turns OFF (i-iv).

1.3.4 Programmable PCA system

A programmable PCA device consists of a bottom platform (11 cm × 13.5 cm) and cover (Figure 1.7). The bottom part contains two reservoir strip holders, two timer strip holders and three reagent strip spaces (Figure 1.7a). A top cover contains six PCA inlets, reservoir inlets and timer strip inlets (Figure 1.7b). The reservoirs, which carry the fluid to be analyzed, are placed into the reservoir strip holders and extend across the gap over the actuators onto the platform on the other side of the device. The midpoints of the reservoirs

are 4.3 cm apart. Actuators, which lift the reservoir strips into the bottom of the reagent strips, are fixed in place by the grooves on the lowest level of the device. The midpoints of the actuators are 1.3 cm apart. Timer strips extend out over the actuators and provide actuator fluid to the peak point of each actuator. The actuator timer strips are taped into the slots opposite the reservoir strips. The reagent strips are placed into the respective grooves and have the same spacing as the actuators. A total of ten inlets are on the cover: two reservoir inlets, two timer strip inlets and six manual actuation inlets. As the water flows

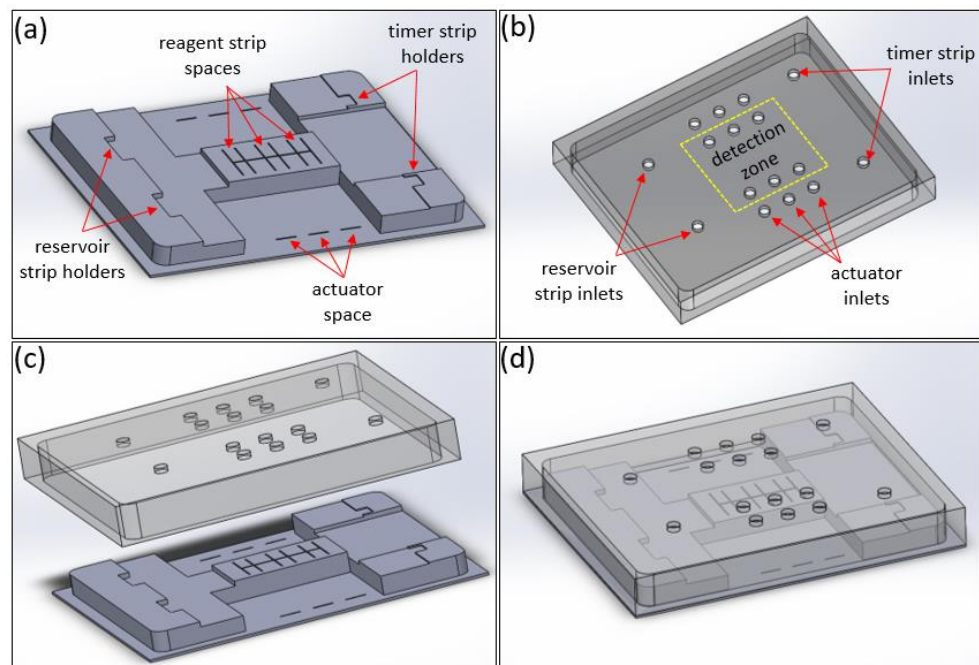


Figure 1.7: The two-part mold design for the system comprised of a base and a cover. The overall dimensions of the mold are 13.5 cm x 11 cm x 2.38 cm. **(a)** The base of the system is the platform for the experiment. The notches in the base serve as alignment aids for the paper strips. The notches that house the reservoir and timing strip are raised on three edges to aid in fluid containment. This is not necessary on the actuator notches and the receiver strip notches due to the low volumes of fluids flowing through them. **(b)** Top cover serves as protection for the parts. Its hollow body rests over the top of the experiment, with a clearance height of .59 cm. The 12 holes in the middle of the cover serve as viewing ports, placed at the initial point of contact between the reservoir and receiver strips, and near the center of the receiver strip where the contrast in colors can be seen more clearly. The cover has overall dimensions of 13.5 cm x 11 cm x 1.59 cm. Wall thicknesses are between .2 cm and .45 cm. **(c)** The cover is placed centered over the base once all the paper is in place. The holes in the cover line up over the viewing ports and fluid distribution points. **(d)** The full assembly fits together as shown.

through the timer strips, the actuators are activated in pre-defined sequence which lifts the reservoir strips up to and making contact with the reagent strips. As a proof-of-concept, the paper cantilever device is implemented to detect three different biological agents from a single source of artificial saliva: glucose, nitrite and protein (Figure 1.8). Saliva contains known quantities of glucose, nitrite and protein and can be used a non-invasive bodily fluid for certain disease tests. As the artificial saliva travels down the reservoir strip, the timer strips are wetted in order to control the actuation of different areas of the reservoir strip.

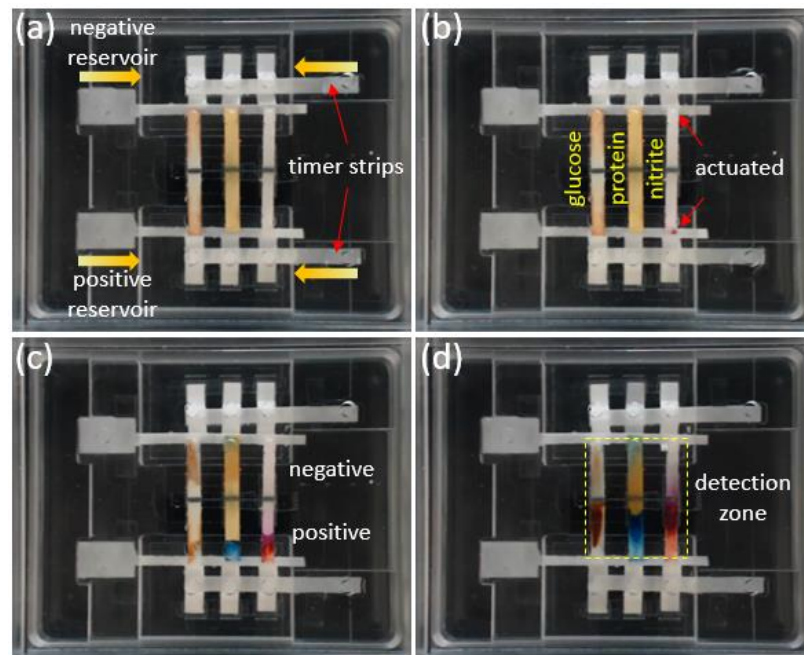


Figure 1.8: The verification of the biological test system. The test determines the presence of glucose, protein, and nitrite using a positive control of artificial saliva containing each of the test substances, and a negative control of artificial saliva that does not contain any of the test substances. **(a)** Once the strips are positioned onto the base, the positive and negative controls were deposited through the ports onto the reservoir strips. 250 μ L of each were used for the test. When the fluid in each reservoir has nearly reached the end of its respective strip, actuation fluid was deposited onto the timer strips and flows opposite the direction of the reservoir flow. **(b)** When the actuation fluid flows down the timer strips and reaches the actuators, the fluid triggers the actuators. This lifts the reservoirs upwards to make contact with the receiver strips. Fluid from the reservoirs begins to flow into the receiver strips, stimulating a reaction. **(c)** As flow continues, the color difference becomes more evident. Fluid continues to flow and the reaction continues to take place. **(d)** Once fluid from all six connections has reached the detection zone, conclusions may be drawn about the presence of glucose, protein, and nitrite.

An actuation raises a portion of the reservoir strip into contact with the corresponding reagent strip. Each reagent strip was prepared beforehand to perform one of the three colorimetric assays so that all three are done in parallel (Figure 1.8a-c). After chemical reactions occur in the reagent strips, color changes are observed through the detection zones (Figure 1.8d).

1.4 Conclusion

As paper-based microfluidic devices are being adopted for multi-step biochemical assays, there is an important need of valves that switch or regulate the introduction of certain fluids at specific times and locations. Here a method has been proposed to connect and disconnect fluid flow between paper strips using a paper-based valve mechanism. The valve comprises a strip of paper that is folded at pre-defined lengths and is wetted when the actuation step is desired. The folded area of the paper relaxes upon wetting, thereby unfolding or levelling the folds. This basic actuation mechanism has been used to demonstrate a number of single or repeated switching operations using one or more paper-based actuators. We demonstrated the application of the actuators to control fluid flow by making or removing physical contacts between multiple paper strips placed in different three-dimensional topologies. As an example, we illustrated a system-level design of the actuator where three analytes (glucose, nitrite and protein) are simultaneously detected from a sample of artificial saliva. The presented method of switching fluid flow is simple and void of any external hardware that makes it easier for integration with current paper microfluidic devices and systems.

CHAPTER 2

METHOD OF NEMATODE EGG SEPARATION AND COUNTING

2.1 Introduction

Routine diagnostics of soil samples from farmlands is vital in maintaining healthy crop yields as it can help discover sources of plant disease. One common source of low crop yields are nematodes or plant-parasitic roundworms that hatch in the soil and find their way into the plant host system during its later growth stages [35]. Among the many nematode species available in nature, the nematode *Heterodera glycines* or soybean cyst nematodes (SCN) is particularly important because of its relevance to the multi-billion dollar soybean crop industry and the mammoth annual losses it causes to this staple crop worldwide [35]. In order to reduce the effects of nematodes on crop yield, farmers often resort to methods such as crop rotation and use of seed treatments and nematicides. However, the effectiveness of any adopted method of preventing nematode infestation is debatable, especially in the field where the type and degree of variables in nature is largely unpredictable.

In order to gauge the level of future infestation, farmers usually send their field samples to soil diagnostic clinics where the eggs of SCN are extracted and counted to get an approximate number of egg population. The process starts by passing the soil sample core through a coarse roller system to loosen the soil and remove any large pebbles or rocks. The dried soil sample is mixed with adequate amount of water, stirred rigorously, and passed through a set of sieves. Each sieve has a characteristic mesh size to allow particles smaller than a specific size to pass through. After the sieving steps, the collected

sample is grinded to mechanically dislodge the eggs from the ruptured cysts. Finally, the collected sample comprises SCN eggs and similar-or smaller-sized soil particles from which the eggs need to be separated and the egg count needs to be estimated.

Currently, the most popular method of egg separation from soil particles involves centrifugation with 40% sucrose serving as a density gradient medium to separate the eggs from soil [36]. Any organic particle (e.g. nematodes) appears to float in the sucrose suspension and most soil particles would sediment at the bottom. Even though the sucrose method works good for the extraction of larger organic bodies such as whole worms and cysts, its efficiency is moderate while separating nematode eggs [37]. There is a greater abundance of tiny debris with shapes and sizes similar to the eggs. The small size of the eggs also makes it easier for them to get dragged and trapped among soil particles into the soil pellet that forms during centrifugation. Because of the issues in separating SCN eggs from similar-sized soil particles, the egg counting is performed manually where a small volume of egg and soil suspension (with eggs stained with a specific purple dye) is spread on a microscopic counting slide and counted for the number of eggs. The total egg count is extrapolated based on the results from the small sample which could lead to potential misrepresentation of the total egg count along with human bias. The sucrose centrifugation method also kills any living samples due to the osmotic pressure of the solution.

We explored a number of methods to separate the SCN eggs from the suspension of eggs and similar-sized soil particles. If we could reliably separate the SCN eggs from the soil particles, the egg counting could be automated by a machine and thereby avoid the need for a human eye to identify and count individual eggs from a background of soil particles. We started by researching methods and devices built for particle separation.

Commercial flow cytometers are accurate but impractical in terms of cost and portability. Microfluidic particle-sorting devices have been primarily designed to separate objects of pre-specified size and shape (e.g. white and red blood cells from liquid biopsy, glass or silica beads) from fluid samples based on inertial flow, magnetic bead affinity or electrophoresis. We found that the soil and egg suspension tend to clog the microfluidic inlet ports or the channels and the range of sizes of the soil particles varied greatly from sample to sample, thus eliminating the use of any microfluidic device for the separation purpose. Our initial tests indicated that centrifugation method needs to be revisited. Upon centrifugation in water, the soil and eggs sediment to the bottom of the test tube to form a pellet and it is difficult to see the eggs even when the pellet is mechanically dislodged on a glass slide.

Based on the initial attempts, we decided to try a version of centrifugation called density gradient centrifugation where the density gradient of the solvent is created upon spinning the tube at a certain speed in a centrifuge. We utilized a commercially available density gradient medium Opti-Prep™. This chemical has been used in order to increase extraction efficiency of nematode worms from soil, and we believe it may serve the same purpose to separate worm eggs for counting [37]. The chemical is also isosmotic, allowing living samples to be centrifuged. We conducted experiments to demonstrate how the presence of Opti-Prep (in varying concentrations) within the whole soil sample affects the extraction efficiency of eggs from soil. Our initial results suggest that the separation efficiency of eggs from soil particles is significantly better using Opti-Prep based density gradient centrifugation than those by sucrose method. Additionally, we present a paper-based imaging method for counting eggs after the Opti-Prep separation process from large

volumes of egg suspensions. The use of chromatography paper provides a cheap avenue to perform large-volume egg counting as opposed to that on conventional glass slides. In typical egg counting techniques, about 1mL of the solution is manually counted on a glass slide and is extrapolated to predict the egg count of the whole solution. Our method, using a paper substrate to absorb the fluid and a flatbed scanner to scan the large area, allows us to count the eggs within the entire solution. This counting can provide a direct count of the number of SCN eggs within a given sample to better determine the nematode population in the soil.

2.2 Materials and Procedure

2.2.1 Materials and chemicals

Soil samples with eggs for analysis were provided by the laboratory of Dr. Gregory Tylka in the Department of Plant Pathology and Microbiology. The *H. glycines* populations were maintained in greenhouse culture pots on *H. glycines*-susceptible soybeans, *Glycine max* cv Williams 82, grown in sandy soil naturally infested with *H. glycines* (HG Type 2.5.7). The eggs were released from female nematodes crushed by a motorized rubber stopper against a 250- μ m- pore sieve [38]. The solution containing released eggs and other debris was stained using a combination of 1M HCl and acid fuchsin stain heated to near boiling to assist in making nematode eggs visually distinct from non-organic debris. Sheets of 200 mm \times 200 mm chromatography paper (WhatmanTM) served as sample collection sites, the bounded area formed by melting wax printed onto the paper by a wax printer (Xerox ColorQube 8570) [7]. Transparency sheets (Apollo) were used to protect the collection area during handling and scanning. Opti-Prep (chemical name:

iodixanol) served as the density gradient medium for the centrifugation that separates the eggs from the soil (Sigma Aldrich). Collection areas are scanned using a commercial scanner (EPSON Perfection V750 Pro) to form images from which the count of eggs is estimated.

2.2.2 Egg centrifugation and counting

Four different concentrations of Opti-Prep were studied in order to calibrate egg extraction efficiency, including a control sample with no Opti-Prep. This control solution contained 10 mL of just the soil sample. The other three concentrations were set to be 20%, 40% and 50% of the density gradient medium in relation to the whole solution. To form these concentrations, three tubes held 2 mL, 4 mL, and 5 mL of Opti-Prep followed by the soil sample layered on top until it reached 10 mL in total (Figure 2.1). All four solutions were provided an additional 150 μ L from a clean solution containing only eggs to ensure the presence of eggs in each solution. After the tubes containing the solutions



Figure 2.3: 5 mL sample of centrifuged solution is poured onto a 5 in \times 5 in square chromatography paper to spread the eggs and any remaining soil for imaging.

were prepared they were centrifuged at a maximum relative centrifugal force of $1442 \times g$ for approximately 1 minute.

To prepare the centrifuged solutions for counting, three sample collection areas were prepared on sheets of chromatography paper for each concentration (Figure 2.2). Each sheet corresponds to one of three sections of the solution; the top 5 mL, the bottom 5 mL, and the pellet. The top 5 mL of the solution was poured onto the first sheet, and allowed

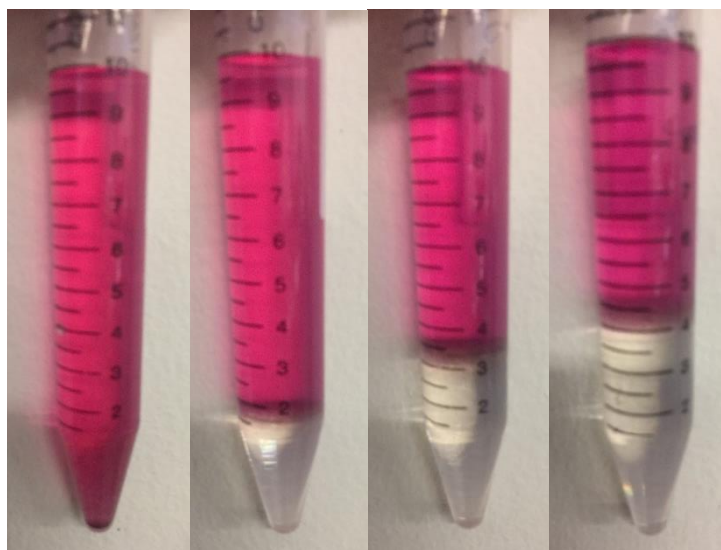


Figure 2.1: Images of the four different concentrations of Opti-Prep with egg sample before centrifugation. From left to right: Control, 20%, 40%, 50%

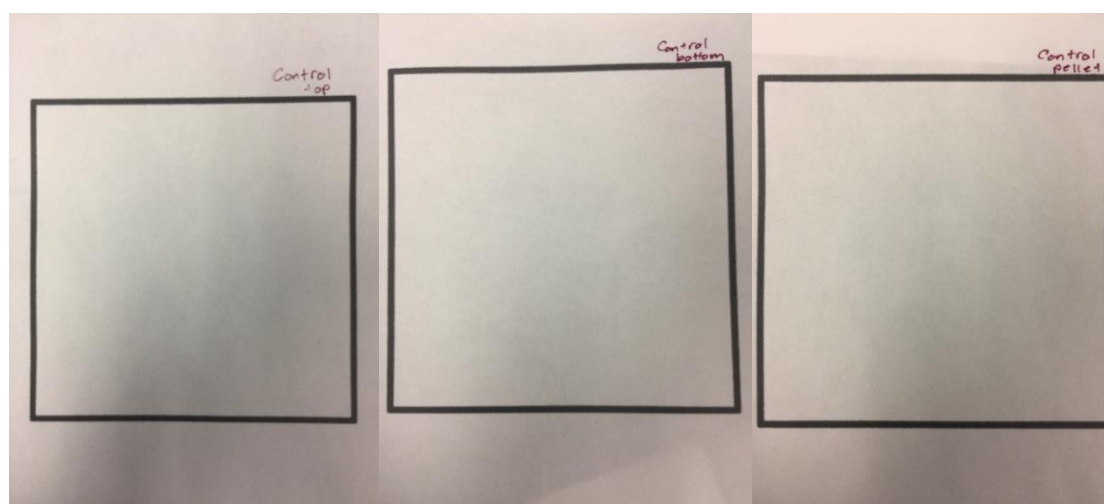


Figure 2.2: Three bounded areas of chromatography paper are used to capture the three collection sections of each solution. Each one forms a 5 in \times 5 in area.

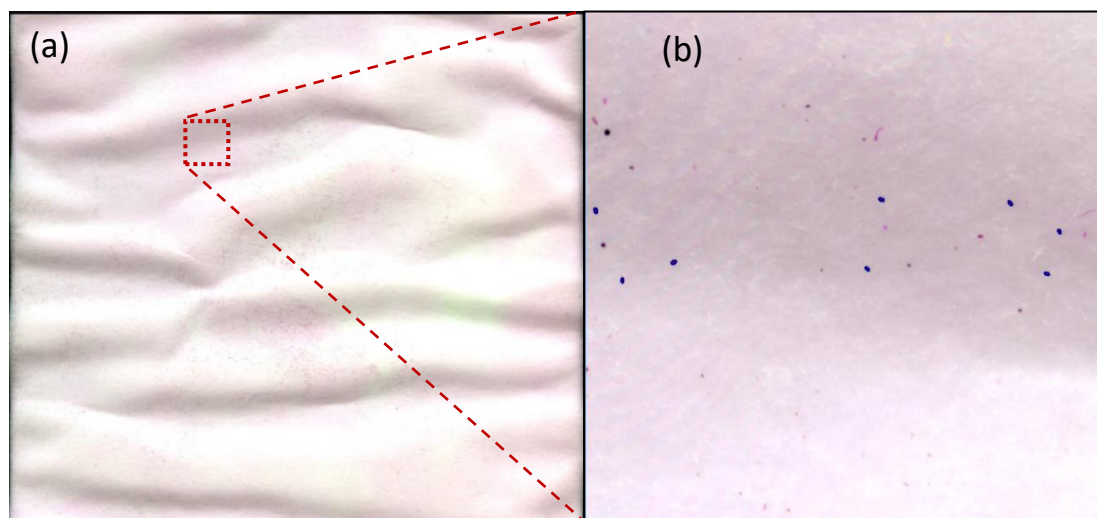


Figure 2.4: The final images of the paper collection areas of the poured solution. **(a)** The paper is scanned into a 16000×16000 dot bitmap, allowing objects as small as the eggs to be resolved. **(b)** The Matlab program divides the original bitmap into 256 squares, so that the eggs may be visible for counting by the program.

to dry for about 3 minutes (Figure 2.3). Then a transparency sheet was placed on top, before being moved onto the scanner. After scanning the top 5 mL, the bottom 5 mL of the solution was poured onto its paper sheet to dry, and scanned. The pellet was diluted to 5 mL using water and put through the same process as the rest of the solution. Each collection area is scanned into a 5 in \times 5 in square with a resolution of 3200 dpi in order to make the eggs visible. A Matlab program segmented the final image into 256 squares and counted the eggs within each square, providing a total number of eggs within the whole 5 mL sample (Figure 2.4). On average, the time for the program to get an egg count was around 10 seconds, taking a few seconds less for cleaner images and more for dirtier images. By doing this for all three sections, the number of eggs present in the 10 mL solution can be determined, rather than an approximation based on a small sample size.

Table 2.1 Egg extraction in the Supernatant		
Opti-Prep Concentration	Average Extraction Efficiency	Standard Deviation
0% [Control]	44%	14%
20%	72%	4%
40%	53%	6%
50%	56%	5%

Table 2.1: Comparison of egg extraction efficiency of four different concentrations of Opti-Prep in a 10 mL dirty egg solution. Each Opti-Prep concentration in the above table is tested in three separate experimental runs.

2.3 Results & Discussion

The procedure to get the egg counts from the Matlab program was performed three times for each Opti-Prep concentration to help determine the consistency of our extraction experiment. From these counts, we can determine what percentage of eggs counted were separated from the main pellet (Table 2.1). Overall, the extraction efficiency with a 20% concentration of Opti-Prep performed the best out of the three non-control solution,

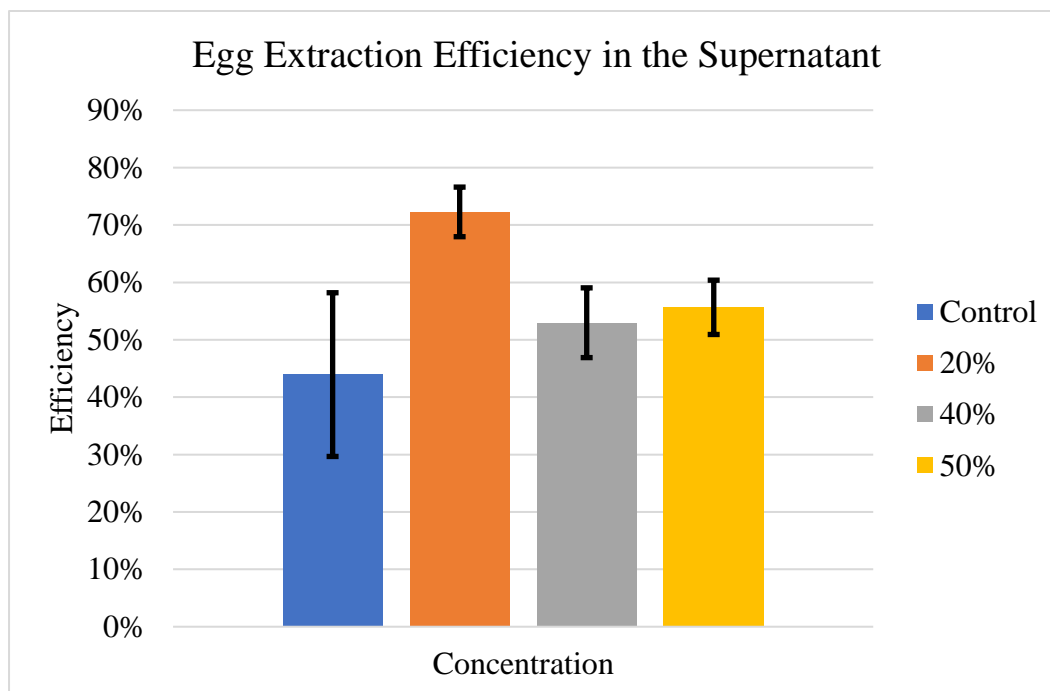


Figure 2.5: Chart comparing the extraction efficiencies of four different concentrations of Opti-prep. Efficiency percentage refers to number of eggs found in the supernatant compared to the total egg count within both supernatant and pellet. Control indicates 0%

although they all demonstrated a marked improvement compared to the sample without Opti-Prep. The interface between the stained egg sample liquid and the Opti-Prep likely forms the most suitable density gradient for the eggs at this concentration during centrifugation. For all three samples with Opti-Prep, the chemical forms a density barrier where the majority of eggs are prevented from following the soil particles into the pellet formed at the bottom. The 40% concentration performing the worst of the three is unusual given the assumption that if 20% performs the best, the greater the concentration in the sample the lower the efficiency. However, the difference between 40% and 50% falls within their standard deviation so this pattern may right itself upon further iteration (Figure 2.5). Another major advantage of all three Opti-Prep solutions versus the control is that the standard deviations are much closer to zero, indicating a greater consistency in the egg counts.

When counting the samples, there was some degree of error due to one variable between sample images, that being the lighting conditions. The Matlab code scans for eggs in the cut-up squares of the images by converting the RGB color code of the scanned image

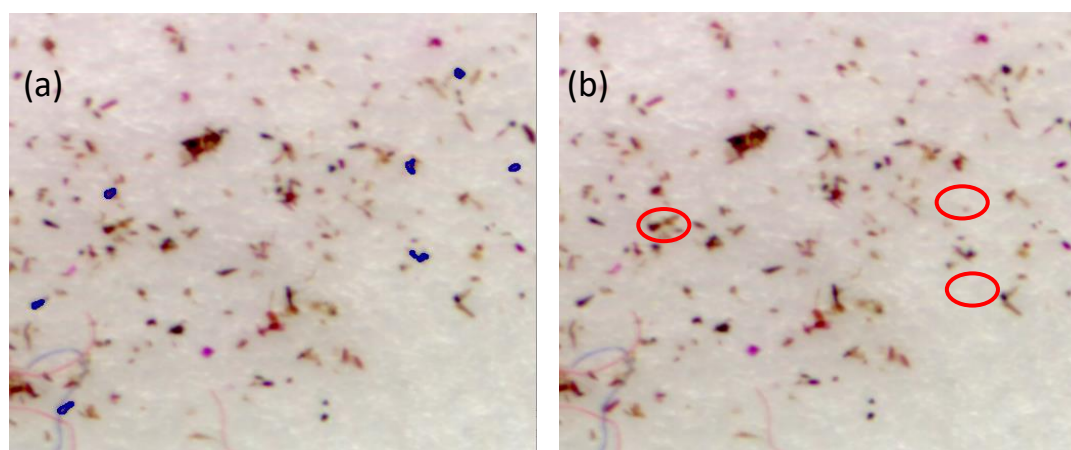


Figure 2.6: Example of Matlab program miscounting egg numbers in image. **(a)** Matlab program gets an egg count number, drawing a blue contour around the objects it recognizes as eggs. **(b)** A manual inspection shows certain objects labeled as eggs to be falsely identified, circled in red.

into the HSV color code. From there, it eliminates objects found in the images by comparing the values of objects found to what we believe the HSV values of the eggs should be. In a uniform lighting condition, this range would be easily determined through normalization over several samples. However, as can be seen in the scanned images, ridges form in the paper during the time it soaks the stained liquid to leave behind eggs and debris (Figure 2.4). This leads to a change in the background lighting of the image areas even within the same image, leading to potential errors in the counting process. In order to get a more accurate count of the eggs, we run the code at different filter ranges in order to observe how the egg count number changes, and specifically determine which frames of the image seem to have a disproportionate amount of eggs (Figure 2.6). By manually cross-checking the frames which seem most likely to have false positives, we can determine how the HSV numbers of those objects factor into potential changes for the filter ranges. Overall, manual review of each 5 mL sample tended to present about a 2% average difference in the number of eggs counted. The largest difference was found in the samples or image blocks that contained the largest amount of debris, expected considering more objects lead to an increased chance of false classification. Future steps to improve the efficiency of the counting method involve adjusting settings with both the scanner and the paper. More manual control of the scanner's settings, such as exposure and focal length would potentially improve the images taken of the paper. A method to keep the paper flat even when wet could make the resulting images less varied as well. By making the images taken by the scanner be more uniform, we would be able to better rely on the code and fine tune it to more accurately determine the number of eggs in the sample.

2.4 Conclusion

Laboratory soil processing techniques are routinely employed to infer the count of nematode eggs in soil samples collected from farmlands. However, the collected egg suspensions from soil extracts often have varying amounts of soil particles that are difficult to remove completely. As such, nematode egg counting is typically conducted under a microscope and is a labor-intensive task. In this chapter, we presented a method to extract eggs from a dirty sample using density gradient centrifugation. The Opti-prep chemical significantly helps to isolate the soil particles from nematode eggs in just one round of centrifugation. Repeated centrifugation of the supernatant solution have shown to improve this egg extraction efficiency. Furthermore, high-throughput method of egg counting is accomplished by imaging the suspension poured on a filter paper, and using a Matlab program to automatically identify and count the eggs. Based on the results presented here, there is hope to improve the method by using better lighting conditions, minimizing ripples in the filter paper, and using smarter algorithms to accurately identify the nematode eggs. Because the proposed method is based on centrifugation and portable scanner, there is hope of developing a field-based egg counting apparatus and thus eliminating the need of sending egg samples to a microscope facility.

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