A Study of Chemotaxis: Evaluations of Established Methods, Microfluidic Devices, and the Role of *Dictyostelium discoideum*

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Keywords: **Chemotaxis, Microfluidics, *Dictystelium discoideum,* Oncology, Immunology, Polydimetylsiloxaine.**

Abstract: Chemotaxis plays a key role in intercellular communication and interaction with the surrounding environment; it is particularly relevant to oncological and immunological research. Over the years various assay methods have been developed to observe chemotactic behavior. This paper reviews and compares the main assay methods and then reviews the roles that the *Dictyostelium discoideum* amoeba and microfluidic structures have recently played in this body of work. After exploring established chemotaxis techniques, we outline a method for evaluating these various techniques and then introduce our own proposed design. This includes a sample y-shaped device that will be used in actual trials. We also present recent results of device fabrication and chemotaxis experimentation.

Introduction:

Cells respond to one another and their surrounding environments mainly by reacting to light, temperature, and chemicals. When microbes follow the trail of a particular chemical towards the source of a concentration gradient, it is known as chemotaxis[1-4]. This form of communication plays a key role in understanding basic cell function, immunity, and cancer research[1-4]. Different chemical messengers interact with receptors on the cell membrane and in the cytoplasm that trigger various processes within cells—including moving towards potential food sources[5-9]. In particular, such chemicals called chemoattractants stimulate the cytoskeleton to initiate cell movement[9]. This type of response to chemical stimulus is believed to be how immune cells find their way to infection sites and injuries; it also appears to be part of the same mechanism that enables cancer cells to metastasize[1& 4]. Over the years, various assay methods were developed to study this cell behavior—dating back to the Boyden method of the 1960's up to the more recent use of microfluidic devices[5-10].

Various types of organisms ranging from lymphocytes to small worms have been used in chemotaxis research[2-5, 7, & 9]. *Dictyostelium discoideum* is an amoeba studied in various related experiments because it mimics many of the same life processes such as chemotactic responses, phagocytosis, and pseudopodia-based locomotion found in immune and disease cells[11-13]. *Dictyostelium discoideum* also exhibits a particular survival behavior when faced by starvation conditions; they merge together to form slugs and that eventually unify into a tree-like structure so as to deploy spores[13-16]. A chemoattractant called cyclic adenosine monophosphate (cAMP) initiates this aggregation[14-16]. Portions of the *Dictyostelium* population are genetically preprogrammed to expire in the formation of the stalk while the other portion is similarly instructed to form the bulbous structure containing spores that ascends to the top of the stalk for their release[14 & 15]. Recent work has been to observe the effects of DNA alteration with respect to slug, stalk and bulb formation; in mutant strains that lack the copine A gene; they tended to merge into larger slugs but couldn't form the stalks with fruiting bodies unless mixed with the wild type *Dictyostelium*[14 & 15].

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Microfluidics is a field of study that makes use of devices comprised of micron-scale structures such as channels, valves, and chambers. Fluids at this size behave much differently than at the macro-scale. For example, water behaves much more like honey in channels ranging from 50-100μm in width and from 10-100μm in height[10, 17, & 18]. This property also allows for different fluids to be put through the same channel without mixing[17 & 18]. Additionally, microfluidic devices have made it possible to conduct experiments that weren't previously possible in the macro range such as chemically treating specific portions of a cell or surgery on microscopic worms[10, 17-19]. Microfluidic devices offer benefits such as greater accuracy and reduced amounts of sample sizes by conducting various reactions in microscopic scale chambers[17-19]. They also provide greater safety by reducing likely exposure to dangerous chemicals due to the smaller samples required. Multiple reactions may be observed and compared on the same array[17-19].

Fabrication methods for creating microfluidic devices have changed over the years. Early methods for forming microfluidic structures required the use of chemical etchants such as Hydrofluoric Acid and Potassium Hydroxide to trace out wells and channels onto crystal, glass, silicon, or plastic substrates[10, 17-19]. More recently, a rubbery polymer called poly-dimethylsiloxaine (PDMS) has gained much popularity in microfabrication. This is because PDMS is non-toxic, biocompatible, and very easy to mold[20 & 21]. Molding is commonly achieved through the use of photoresists from the SU-8 series for creating the desired device shapes[20-22]. The recent work of Stephen Quake demonstrated that sophisticated devices comprised of air driven valves, pumps, chambers, and channels can effectively control the directional flow of various forms of chemical and biological samples[23].

Microfluidic channels have proven useful to chemotaxis studies because complex channel patterns create effective chemoattractant gradients for observing cell movement[10, 24-26]. In recent developments, *Dictyostelium discoideum* research has incorporated the use of microfluidic arrays for benefits that include improved control of chemoattractants and cell motility[27-29]. This natural progression serves as a spring board for this project. In this paper the established methods of chemotaxis experimentation will be described--including the strengths and weaknesses of each method. From there we will explain our proposed plan for evaluating several of these techniques so that their performance can eventually be compared to that of our own device.

Summary of Assay Techniques:

Boyden method:

Stephen Boyden published his methods in 1961[5, 11 & 13]. He developed a simple, gravity-based device consisting of two chambers; one chamber was set above another with a thin membrane separating them[11 & 13]. A leukocyte serum, grown in the peritoneal cavities of rabbits, was introduced into the top chamber and a chemo-attractant, tuberculin, pipetted into the bottom chamber[11 & 13]. After a short incubation period, the two chambers would be separated and the diffusion of leukocytes through the membrane would be observed[11, 13]. Boyden's technique allows post-assay analysis by encouraging a large number of leukocytes to cross the membrane but this same benefit also demonstrates the design's shortcomings. It requires the sacrifice of large amounts of cells and chemoattractant[5, 11 & 13]. Additionally, this method lacks any fine control over the reagent's concentration gradient.

Zigmund/Dunn method:

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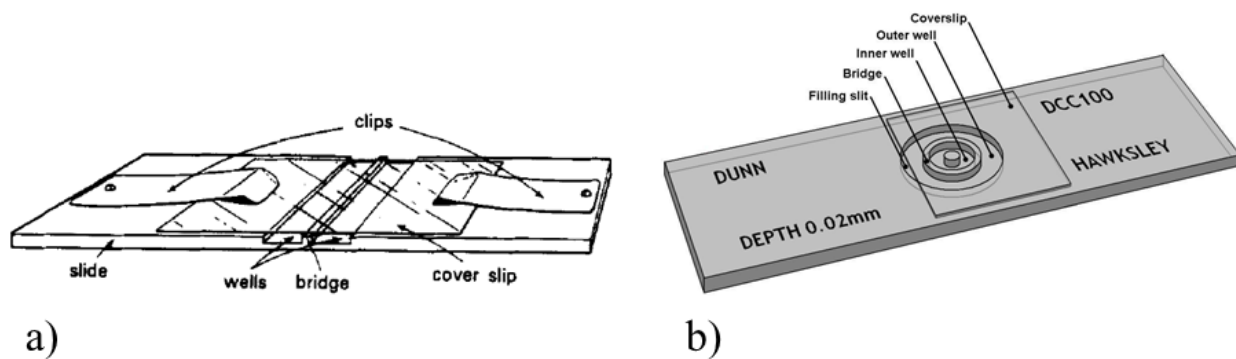

Figure 1: Images of the a) Zigmund and b) Dunn chambers[15 &16]

Fig. 1 shows both the Zigmund and Dunn assay chambers designed for the observation of cell movement and slightly improved control of the chemoattractant gradient. Both devices employ wells for containing the reagent as shown in Fig. 1a & b. The cells are placed on the slide and then observed to see how they respond to the chemoattractant[5, 11, 15, & 16]. The Dunn design differs from the Zigmund on two points. First, it uses circular wells. Second, it employs a wax seal between the inner and outer wells (see Fig. 1-b). This helps reduce the waste of reagent. The main benefit of this assay type is that allows better linear direction of the chemical gradient. This means that cells can be observed and recorded as they move towards the chemical sources[11, 15 & 16]. Two major failings of these designs are that they still both offer relatively poor control over gradients and require the use of much chemoattractant[5, 11, 15, & 16.]

micropipette method:

Micropipetting involves the use of specialized pipettes with diameters ranging from 1-2 microns[5 & 19]. They can deliver volumes of reagents as small as 0.3 pl[19]. Repeated pulses from a pump help to disperse the chemoattractant and entice cells to move towards the point of dispersion. Researchers can move individual cells by selectively positioning the micropipette tip and releasing the reagent[19].Where this method fails is that it doesn't allow for control of the chemical gradient as it spreads out across the culture substrate[5].

scratch-wound-assay:

The scratch-wound assay is performed by growing a monolayer of cells over a glass slide section[5, 18 & 21]. Then either a needle or pipette tip creates a "wound" in the single layer of cells[5 18, & 21]. Time-lapsed footage is taken to observe the patterns and rate of regrowth. This method was included because chemotaxis appears plays a role in the manner that cells are directed towards one another during wound healing[5, 18 & 21]. Growth usually begins between 3-24 hours after the scratch is created[18]. This method doesn't accommodate fine control of cell movement as the cells move towards one another as the "wound" gradually closes[5, 18 & 21].

under-agar assay:

Under-Agar assays function simply. They consist of at least two wells cut into agarose-gel in a petri dish[5, 11, & 20]. In one well, chemoattractant is placed and in the other, cell samples are

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deposited. The specimens respond by crawling under the agar towards the trace amounts of chemattractant that they sense[5, 11, & 20]. This is particularly useful for recording image captures with a bottom objective microscope and camera software. The two major short comings of the under-agar method is that it doesn't allow for control of gradient dispersion –much like micropipetting-- and it doesn't work for some cells that can't elongate themselves under the agar[5& 11].

microfluidic devices:

Microfluidic devices offer the benefits described above. Two of the major assets to chemotactic research are their ability to give better control of chemical gradients and the means to direct the movement of cell samples based on device geometry[3, 5, 7, 12, 21, 24-26, & 29]. This means that microfluidic devices provide efficiency as well as a better simulation of the environments in which cells such as lymphocytes are most active. These designs also accommodate observation and digital recording of cell movement[3, 5, 7, 12, 21 & 24]. This is particularly the case when molded PDMS constitutes the body of the channels. Simple glass cover slips can easily serve as the floor for the devices in a bottom objective microscope or the roof in a top objective model[5 & 26].

Despite the advantages such platforms offer, the very properties that give them their strengths also give them their weaknesses. To begin, they are dependent on a molding technique to fabricate them; this means, if the mold is improperly designed or damaged, the device isn't likely to function correctly[5, 24 & 26]. This also means that there is a limited 1D mobility for the chemoattractant as and a reliance on external pumps to supply the needed sheer force required to stimulate the proper fluid movement.[5, 12 &24]. One exception to the latter condition occurs when the PDMS channels and glass substrate are exposed to oxygen plasma. After exposure to the plasma the glass substrate temporarily becomes hydrophilic due to the plasma causing changes in the surface charges of both PDMS and glass substrate[25]. It encourages fluid flow through the channels without the need for external aid and also bonds the PDMS and glass irreversably[25].

Experimental protocol and proposed design:

Time, legal requirements, and resources won't allow for every possible chemotaxis assay method to be explored. The best choices for our project will be the under agar, Zigmund/Dunn technique, and microfluidic devices. The latter includes our proposed design.

The above-mentioned methods were also selected because they would best accommodate the use of time-lapsed image capturing. This will allow chemotactic behavior to be recorded and measured for later comparison. From the gathered image data, it would be possible to plot the movement and speed of the samples. *Dictyostelium discoideum* will be the main subject of our trials. The two chemoattractants that will be used are cAMP and Folic Acid (FA). This will require slightly different preparations for *Dictyostelium* in each case. Testing cAMP, will mean exposing the *Dictyostelium* to starvation conditions. This is done by removing them from their nutrient medium and placing them into a buffer solution of potassium phosphate. They will be kept cold for several hours before use to slow down their metabolic processes. After being sufficiently starved, they can be placed into the test environments for observation. The use of FA requires that *Dictyostelium* must be grown from spores on FA-fed bacteria. "Raising them on it" increases their likelihood to respond to the FA as needed. This will provide a means to evaluate which method offers the best results.

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Initial trials would begin with the under-agar assay. This appears to be the simplest and most direct means to observe cellular chemotactic responses. Following this, a Zigmund/Dunn device would be used. This will be a commercially fabricated device from **ibidi, llc** made to accommodate a bottom objective microscope. Finally, microfluidic devices would be tested.

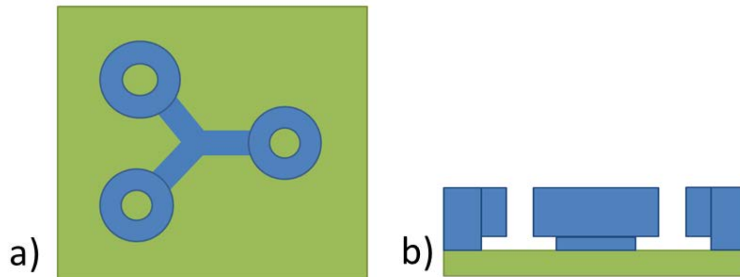

Figure 2: The view of the microfluidic devices from a) the top and b) a cut-away view.

Fig. 2 demonstrates the basic concept of the device. In a), the green area is the substrate like a glass coverslip and the blue areas are the PDMS-based structures. In part a) only the shape of the channels and wells were highlighted to emphasize the concept. Our design is a simple 3-chamber/3-channel set-up. This design will work well with a bottom objective microscope when mounted on a thin cover slip. One well would be for *Dictyostelium* samples and the other two would be for chemical attractants.

An in-depth description of our fabrication process can be found in a previous work[26]. The method for creating the chemical gradient is based on this process, it will be described briefly. Using photolithography and PDMS molding, it is possible to create gradient by pouring PDMS onto a previously designed mold. Complex, serpentine channels effectively create the proper chemical gradient due to their shape[3].

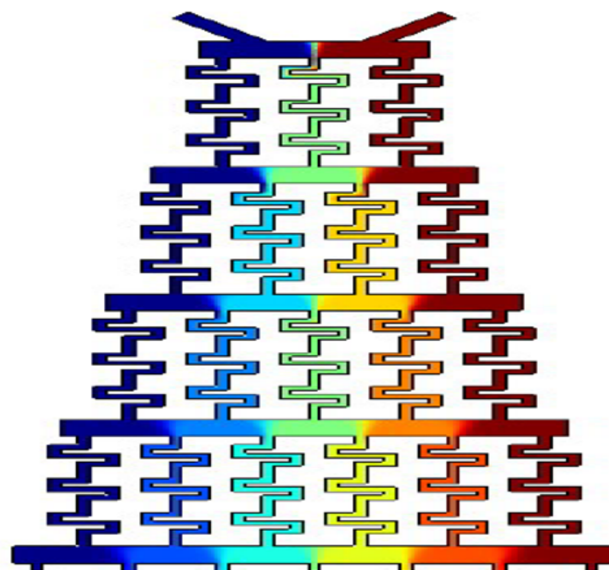

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Figure 3: An example of a serpentine microfluidic channels structure with a varied gradient[3].

Fig. 3 provides a clearer example of the type of structure that can be created with this style of fabrication. The dark blue and dark red represent two separate samples introduced into the channels through the upper left and right entry channels[3]. The shift in hues represents the diffusion of the two reagents throughout the device. Our device will operate on this same basic principle, but will be the simple "y-shape." This shape fit the needs of this project because the simpler design will make it easier to locate and record chemotactic responses during the trials. Fig. 4 a-c illustrates several stages of mold fabrication. Fig. 4 c shows a completed channel structure that is ready to be for the insertion of tubs and application to a substrate such as a glass cover slip. The dimensions of the channels are 100µm x 1.5 mm. The circular wells have a radius of 1mm and a height of 100µm.

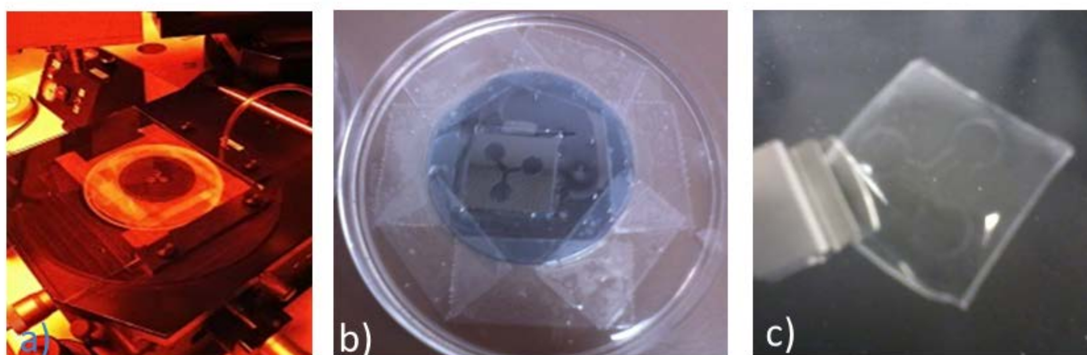

Figure 4: Several stages of the mold and channel fabrication at a) the mold is formed on the mask aligner for exposure, b) the completed mold after removing the PDMS channels and c) the finished channels themselves.

The testing protocol will be as follows: Microchannels will be tested with clear isopropanol and then isopropanol mixed with food coloring to verify basic functionality of the devices. Next, a standard fluorescent solution will serve to further observe the dynamics of fluid flow in the channels. After this, a solution containing polystyrene microbeads will be employed to observe the interaction between the bead solution and the channel under fluorescent light. Such trials will culminate with live tests of *Dictyostelium discoideum*. Video software will help create time-lapsed video images to measure the average movement in the channels, compared with other devices.

under-agar assay:

Time allowed us to attempt an under-agar assay trial. The basic protocol followed Dave Knecht's method from dictBase.org[30]. Nutrient agarose was poured into P60 petri dishes and allowed to solidify. The agarose consisted of a nutrient media (SM) mixed with agar powder. The specific recipe can be found in the document at the dictyBase website[30]. 25mL of the SM was blended to 0.375g of agarose and then heated in a microwave until boiling. Agarose generally melts around 100°C and cools at 45°C. The agar was divided amongst three separate plates (8 mL each) and allowed to cool after pouring. Once cooled, several wells were cut into each plate with a razor blade and small spatula. Fig.5a&b) show how the plates appeared before addition of *Dictyostelium* and chemoattractant.

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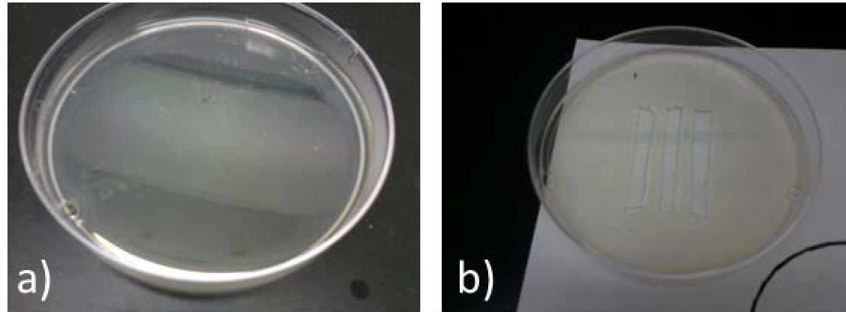

Figure 5: An agar plate a) after pouring and b) with the wells cut into it after cooling.

The under-agar technique offered two possible methods for employing chemoattractant. The first required FA to be diluted in SM and mixed with agar powder. The second method precluded the addition of agar and was directly deposited into the central well. Fig. 6a) shows the finished plate prior to incubation.

Figure 6: An agar plate a) with cells and chemoattracted added, b) being stored in a humid chamber for a short time, and c) looking for chemotactic movement on a microscope.

The filled plates were placed into a container with damp towels, as illustrated in Fig. 6b) and incubated at 20° for an hour. After this, the first potential signs of chemotaxis could be examined under a microscope as shown in Fig. 6c). If signs of movement towards the gradient were present, the next step would be to start time-lapsed recording of images. The results from the chemotaxis trial and device testing will be described next.

Results:

chemotaxis:

The results for the chemotaxis trial were inconclusive. Upon examination, it appeared that the *Dictyostelium discoideum* spread out in all directions. This included in the opposite direction of the FA. It is difficult to determine whether the FA had any effect at all or whether the *Dictyostelium* diffused under the agar randomly.

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fabrication:

This study has yielded some results from device fabrication. Fig.5a provides an example of a simple bar-shaped microfluidic device and Fig. 5 b & c of the y-shaped device. The bar shaped device functioned properly initially, but the y-shaped channels were initially pulled from the substrate by the micro-tubing attached to the syringe pumps as shown in Fig. 5c and 6b. Plasma bonding was one possible solution to sealing the chamber to the substrate, however time constraints and resources didn’t allow for this currently.

Burgoyne offered a possible means of bonding the channel structures to the glass wafer[31]. This method required the spinning of a 10 micron thick film to the glass slide. Spin profiling on the spin coater revealed that a spin cycle of 6000 rpm for 30 seconds produced this approximate thickness. This method called for a hot plate set to between 70°C -80°C. A partial bake for several minutes served to make the PDMS film tacky so that a tight seal could form after a full baking time of 30 minutes. The original version listed 20-gauge syringe needles to be cut and then imbedded into the channel structure. They could then function as attachment points for the microfluidic tubing. The needle segments would then be reinforced with the addition of cut pipette tips. Burgoyne recommended coating the bottom of the pipettes with PDMS to firmly attach them to the channel structure; the channel would then be replaced on the hot plate for 20-30 minutes to bake[31]. The final step would be to fill the pipette bases with PDMS and bake them again for 30 minutes to form a firm support[31]. Further testing found this method to be effective and eliminated the separation of the y-shaped channel from the glass slide bottom.

Fig.5b) shows our variation on this method. Instead of the metal needle tip, we found that 20-gauge microtubing worked very well as a substitute. This reduced the need for added materials and costs related to the needle tips and also offered an added degree of safety by eliminating the need to cut metal needle tips and risk potential puncture/laceration wounds.

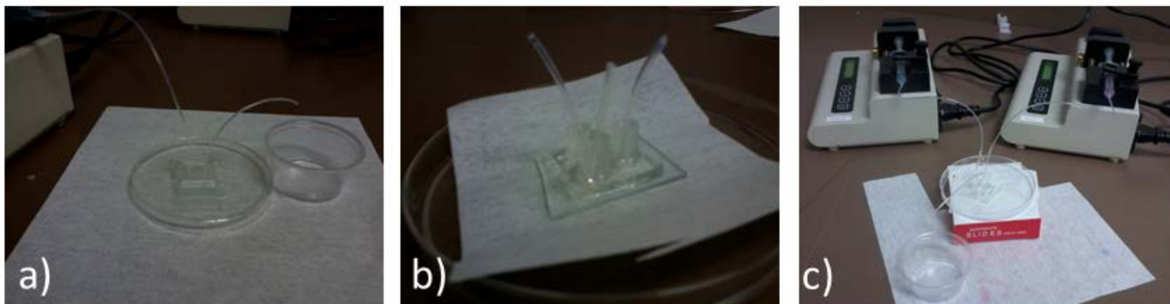

Figure 5: The devices used for initial functionality test including a) a simple bar-shape chamber with an inlet and outlet, b) the y-shaped device before connection to the syringe pumps, and c) the y-shaped chamber connected to syringe pumps before testing.

Fig.6 provides a better view of the tests conducted with food coloring. As can been seen in Fig.6a), the basic bar device functioned temporarily, but the seal between PDMS and untreated glass bottom failed. In Fig.6b & c) two views of the modified fabrication are shown. Testing revealed that the seal was far more stable and the supports for the channel inlets were quite strong. In particular, Fig.6c) shows the device functioning as intended; the fluids are passing

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through the y-shape without mixing until the outlet tube. The major drawback of the pipette supports was that it obstructed a clear view of the fluids entering the channels from the top. Additionally, it appeared that there was an issue with liquid PDMS leakage from under the pipette supports after pouring. This further distorted the view of fluid flow from the top. An uneven top on the PDMS combined with the narrow area (approximate 2.5cm x 2.5cm) still made this problematic for a design with the dimensions listed above. However, it seems this method appears to work well for devices with of a standard glass slide[31].

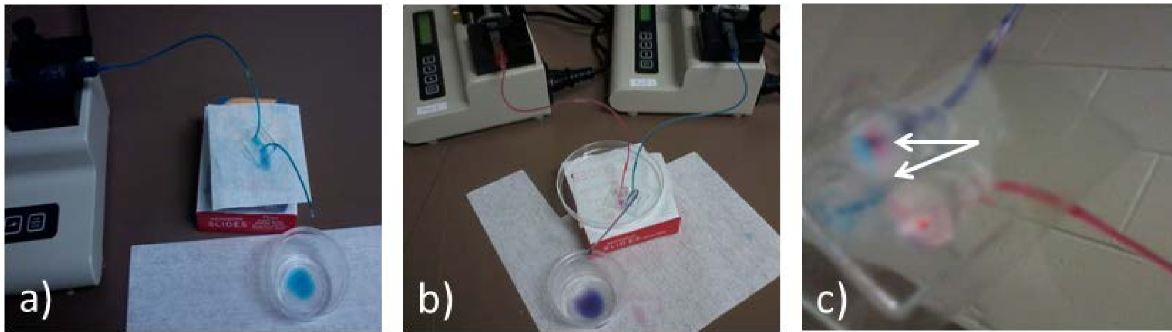

Figure 6: The devices during successful tests for a) the bar-shaped chamber and b) the y-shape; c) shows the correct function of the y-shape with the fluids coexisting in the channel without mixing(see arrows).

An attempt was made to remedy this issue by simply pouring the PDMS thicker than before. Fig. 7 offers examples of the thinner and thicker devices. The channel PDMS in Fig.7a) measured to a thickness of 1.7mm. A thickness of 5.68mm appeared to be much more stable and eliminated the need for the pipette supports (Fig.7b & c). To further bolster the strength of the seal and the stability of the channel, PDMS was spread around the top inlet holes for the tubing and around the base where it connected with the substrate. Additionally, 16-gauge microtubing worked well to form adaptors for connecting tubing from syringes--Fig.7c). Fabrication revealed that after 9mm, the PDMS became more difficult to separate from the mold. Fig.7c) offers an image of the most recent completed device; the PDMS measured a thickness of 9.42mm.

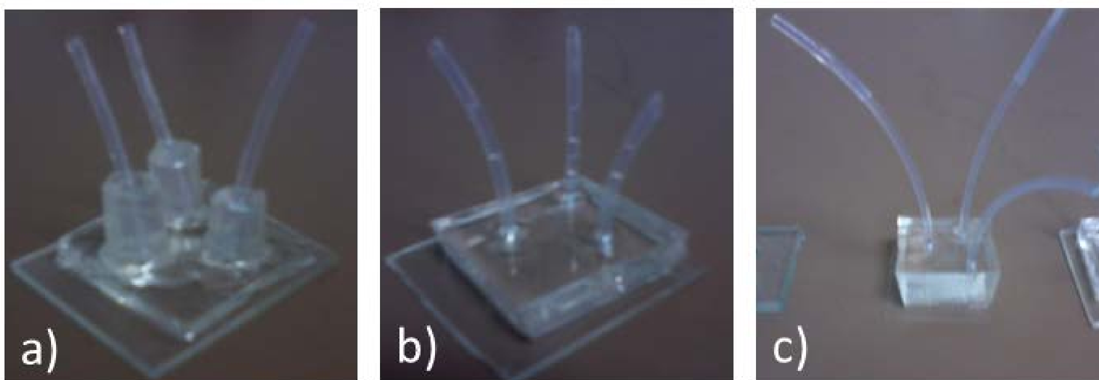

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Figure 7: The devices a) employing pipette-tip bases, b) the 5.68mm thickness without the pipette bases, and c) the 9.42mm thick device using a glass coverslip for a substrate.

Figure.7c) also showcases another fabrication success. Bottom objective microscopes are commonly used in various forms of scientific study—including cell related studies such as chemotaxis. We wanted to see if a 0.13-0.17mm thick glass coverslip could be spun with PDMS to make a microfluidic array that would accommodate a bottom objective. Experimentation revealed that double-sided tape was strong enough to sufficiently hold a cover-slip to a glass slide section during spin coating--6000 rpm for 30 seconds as mentioned earlier. Afterwards, the coverslip was carefully separated from the slide and placed on the hotplate at the same temperature range described above for a couple of minutes to partially bake it. Once baking enough to become tacky, the y-shaped channels could be attached to the device and then placed back on the hotplate for approximately 20-30 minutes. After cooling, the tubes were carefully inserted into PDMS and the reinforcement of the seals around the bottom and inlets with more PDMS was done before putting back onto the hotplate for an additional 30 minutes.

During the assembly of the final device it was observed that the thicker PDMS helped add resiliency to the overall array despite the thin and brittle nature of the glass cover slip. Care was necessary but the device proved stronger than initially expected.

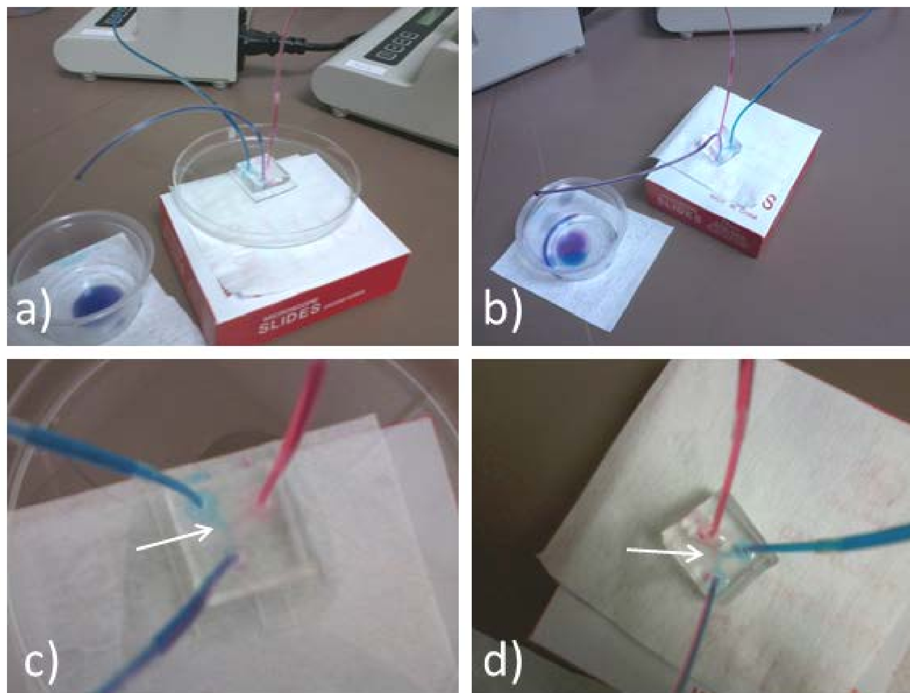

Figure 8: ¾ views of the a) 5.68mm thick device, b) 9.42mm device and then overhead views of c) the 5.68mm device and d) the 9.42mm device with fluid flowing through them.

Fig.8 demonstrates the functionality of the devices. Fig.8a & b) provide views from farther away, while Fig.8c & d) show the fluid correctly flowing through both devices with no leaks from above. Both performed as required and the thicker PDMS managed the stresses exerted on the

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microfluidic from the tubing channels very well. It eliminated the need for the thicker supports that obstructed a clear view of the device during operation. This method also produced a device that can be further adapted to work with a bottom objective microscope.

Discussion:

Chemotaxis research holds much potential for advancing medicine and biology. The various assay techniques have been reviewed so as to help set the stage for our own work. Microfluidic devices offer a means to conduct such studies while reducing the need for large sample sizes. We have introduced the concept and model of the device that will be tested in the coming work to compare with these established methods. Once this basic, preliminary research is complete, more complex channel designs of channels may be explored—including the incorporation of air-driven pumps and valves. Trials meant to further understanding of fluids of varying degrees of viscosity may be carried out. Additionally, it would open up the possibility of gaining a deeper understanding and a greater capacity of simulating the common *in vivo* environments that host microbes subject to this field of research. An eventual goal of this work is the development of devices that will offer efficient, inexpensive means to researchers to conduct their own experiments.

The main success of this research came from the fabrication and testing of devices. The net results appeared to not only be devices that passed the initial tests for basic function, but also improved upon another fabrication method by eliminating the need for metal syringe tips and pipette columns for support. This means an increased safety benefit, fewer materials needed, and a reduced cost related to materials. We also demonstrated how glass cover slips ranging in thickness from 0.13mm-0.17mm can be effectively spun with PDMS to serve as substrates. The devices described in this paper are ready to be tested with fluorescent solutions—the next phase in evaluating them.

Despite the lack of conclusive results from the under-agar experiment, it provided an opportunity to begin working with agarose and FA. It is currently undetermined why the *Dictyostelium discoideum* failed to react to the FA as expected. This will require further discussion and experimentation to find the cause. It is most likely that new trials will be conducted with freshly-ordered FA to see if there is a difference. Chemotaxis trials will also incorporate the Zigmond/Dunn-style assays recently purchased from **ibidi, llc** as they allow for several chemotaxis trials to be conducted on one slide. This latter assay offers the possibility of experimenting with several concentrations of chemoattractants at one time or a normal assay next to a well containing only buffer solution. Chemotactic trials will be conducted with the y-shaped device described here.

This work has also offered opportunities to further learning related to biology, photolithography, microfabrication—including soft lithography, MEMS devices, and lab-on a-chip technology. It has involved working with chemical processing (as in exposing photoresist to UV radiation for fabricating molds), learning proper laboratory procedure for handling biological samples, networking with faculty on campus, writing grant proposals to gain funding, improving research skills, participating in summer education programs, and presenting research at events on campus, at Michigan's state capital, and at conferences such as A.SE.E.

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