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# Passive Mechanical Lysis of Bioinspired Systems: Computational Modeling and Microfluidic Experiments

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To my parents who will always love and support me To my colleagues who continuously inspire me to reach higher To my family who are always proud of me regardless To my brother whom I hope will be inspired by this endeavor To my friends who made the journey an enjoyable experience

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### Abstract

Many developed nations depend on oil for the production of gasoline, diesel, and natural gas. Meanwhile, oil shortages progress and bottlenecks in oil productions continue to materialize. These and other factors result in an energy crisis, which cause detrimental social and economic effects. Because of the impending energy crisis, various potential energy sources have developed including solar, wind, hydroelectric, nuclear, and biomass.

Within the biomass sector for renewable energy sources, algae-based biofuels have become one of the most exciting, new feedstocks. Of the potential plant biofuel feedstocks, microalgae is attractive in comparison to other crops because it is versatile and doesn't pose a threat to food sources. Despite its many advantages, the process to convert the microalgae into a biofuel is very complex and inefficient. All steps within the algae to biofuel production line must be optimized for microalgal biofuel to be sustainable. The production of biofuels from algae begins with selecting and cultivating an algae strain and giving it all the necessities to grow. The algae is then harvested and processed for specific uses. It is the harvesting or lysing step, which includes the extraction of the algal lipids, which is the biggest hindrance of algae being used as a cost effective energy source. The lysing step within the microalgal biofuel processing is of particular interest and will be the focus of this work.

This work discusses the optimization of the biofuel production from microalgae biomass through computational and experimental approaches. With atomic force microscopy (AFM), a key mechanical property that would aid in the computational modeling of mechanical lysis in the in-house computational fluid dynamics (CFD) code, Particle-Surface Analysis Code (P-STAC), was determined. In P-STAC, various flow patterns were modeled that would most effectively lyse microalgal cells based on the shear stresses placed on the cells, which will be compared against microfluidic experiments using lipid specific dyes. These results would be influential in developing an energy-efficient method of processing microalgae for biofuel.

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### 1. Introduction and Background

Presently, two-thirds of America's petroleum is imported, 60% of which is used for transportation fuels. Mandated by the Energy Independence Act of 2007, the United States must have at least 36 billion gallons of renewable fuels incorporated into the transportation fuel industry by 2022 [1]. To meet the new requirement, energy independence, and cleaner fuel sources, multiple avenues of renewable energy sources have been explored including biomass, solar, hydro, wind, and nuclear.

Of the biomass options to create biofuel, microalgae is attractive in comparison to other crops because (1) it has a very high oil/lipid yield, over 500 times that of corn as seen in Table 1-1; (2) it does not compete with American food supply; (3) it does not compete for water or land resources because it can grow in uninhabitable, unfertile areas; (4) it has a higher productivity than any of the other crops; (5) it can be used in the sanitation industry to capture carbon dioxide, treat waste water, and release clean oxygen as a byproduct; and (6) the algae by-products – aside from its lipids and the other energy portion of the algae cell – can be used for other purposes[2].

Сгор	Oil Yield (Gallons/acre)
Corn	18
Cotton	35
Soybean	48
Mustard Seed	61
Sunflower	102
Rapeseed/Canola	127
Jathropha	202
Oil Palm	635
Algae (10 g/m <sup>2</sup> /day at 15% TAG)	1,200
Algae (50 g/m <sup>2</sup> /day at 50% TAG)	10,000

Table 1- 1 A comparison of the different feedstocks used to create fuel from plant based oils. Algae out produces all the other crops and does not compete with food sources. Adapted from [3].

Microalgae are photosynthetic microorganisms that are not only critical to aquatic ecosystems, but are increasingly becoming more essential in commercial applications [3]. As microalgae contributes to the purification of water, they also aid in the sequestration of carbon dioxide and metals produced in industrial areas. These organisms produce a diversity of macromolecules and vitamins that are used in dietary supplements, agriculture and aquaculture feed, cosmetics, dyeing agents, pharmaceuticals, biofertilizers, and biofuel (see Figure 1-1).

The process to convert algae into a biofuel is complex and costly. Increases in efficiencies could make algae biofuel a viable form of renewable fuel. The production of biofuels from algae begins with selecting and growing an algae strain. The algae is then harvested and processed for specific uses, including biofuel synthesis. Although, the concept of an algae drop-in fuel has been validated, the steps for converting algae to biofuels i635n the production line must be optimized to minimize the energy-input needed to mass-produce alg1200al biofuel [3]. The harvesting step, which includes the extraction of the algal lipids, is the biggest limitation for algae to be used as a cost effective energy source [4]. In addition, while there is a plethora of dewatering (algae-water separation) and lysing (cell membrane rupture) techniques that have been evaluated based on their cost effectiveness, none of those technologies has been deemed energy efficient in large-scale production. To progress, the field needs efficient dewatering and lysis techniques for the sustainability of microalgal biofuels.



Figure 1-1 A schematic drawing of how biodiesel/biofuel is created from microalgae. The microalgae is grown and processed based on the type of end product desired.

Often, to obtain the metabolites needed to produce microalgal products, cell lysis, or disruption, is required. Some disruption methods include chemical lysis, osmotic shock, enzymatic lysis and mechanical lysis. Of these disruption methods, associated mechanical lysis techniques include high pressure homogenizers, ultrasonicators, bead mills, freeze presses, autoclaves, microwaves, and lyophilizators [5]. Although these methods are effective, they are energy intensive, which limits there utility for industrial applications. To this point, a study was conducted where we performed various lysing techniques and compared the effects of the disruption methods. These results will be discussed in section 2.2.

### **1.1 Algae and Biomechanics**

Microalgae are unicellular algae that exist on the microscopic levels in freshwater or marine systems. They can exist independently or in groups, and can be grown in open systems such as ponds or closed system such as photobioreactors[5]. Through photosynthesis, the microalgae release roughly 99% of all the atmosphere's oxygen making it an important part of the ecosystem and human health in general [6]. Consisting of over 30,000 known species, these photosynthetic microorganisms are diverse [7], and not only do they aid in carbon dioxide sequestration, which improves air quality, but they also are very useful in wastewater treatment [4, 8]. Microalgal applications span from increasing nutritional value of food and manufacturing cosmetics to the production of pharmaceuticals and renewable energy sources [9, 10].

One very important facet in microalgae potential industries is the ability to mechanically extract components from the microalgae. This extraction is particularly interesting because there are 2D and 3D linked responses involved in the biomechanics. In the case of macroalgae (multicellular, macroscopic algae) and many other plants, the general morphogenic rules for 2D are equivalent to those in 3D structures [11]. Ultimately, plant organs are not dictated by the cells themselves as their shape and overall form in a 3D manner are independent. This is opposite than what is seen in biofilms since plant structures are not dictated by aggregation of cells but the partitioning of the entire structure into cells. Furthermore, biomechanics has not been shown to affect shapes of planar structures as they do in cellular morphogenesis. However, studying biomechanics in microalgae needs to be further developed. Because of the potential of microalgae in a

plethora of industries, investigators are taking a deeper look into its biomechanics. Some of the recent mechanical characterization of microalgae has been in determining its elastic modulus [12], force and energy requirements for lysis [13], tensile strengths [14, 15], and rheological properties [16]. However, due to the vastness of the microalgae family, there often exists a range of mechanical properties for microalgae across species. Another example of mechanics with microalgae is in the biofuels community, where mechanical lysis can potentially have some benefits from a chemical and environmental standpoint [17]. Currently, one of the most energy inefficient steps of biofuel production involves cell lysis, via bead milling, mechanical pressing, osmotic shock, or sonication [18, 19]. Many of these approaches rely on shearing of the membrane to lyse the cell and thus understanding the mechanical properties of microalgae may be essential in developing efficient microalgae biofuel extraction processes [4, 12, 20].

#### **1.2 Microalgal Biofuel: Challenges and Research Efforts**

Microalgal biofuels is a very promising renewable energy source since 1-3% of the United States crop area could potentially be used to produce 50% of the US's transportation needs (this section's 73). However, to harvest, process, and produce biofuel from microalgae is a very expensive and energy intensive process. These steps have efficiency issues and can be broken down into three common themes: algae cultivation, oil recovery, and fuel production as shown in Figure 1-2. Within the algae cultivation list of issues concerning efficiency, genetic modifications of microalgae have been investigated for the manipulation of gene expression regarding algal metabolism [21]. By altering the metabolism of the cell, one could utilize the lipids for biofuel, starches for alcohols, and hydrogen produces for fuel cells. Selecting the proper microalgal strain varies depending on the desired product to make. For example, there are many by-products of microalgae within the realm of renewable energy, two of which are biofuels and ethanol. For each of these products different microalgal cell components are used, lipids, and carbohydrates, respectively [22].

As shown in Figure 1-2, another approach within the algae cultivation efforts include the design of photobioreactors. Next, one must then decide whether the microalgae will be grown in a closed system, called a photobioreactor, or an open system, like an open pond system. Both of these growing systems have benefits and risks that need to be taken into consideration. In the photobioreactors, the system is more controlled and allows easily regulating air circulation and decreasing the potential for contamination. A downfall to the photobioreactor is that these systems can be relatively expensive. In open pond systems, however, the algae are exposed to the environment, which increases the contamination

probability. Aside from the probability of contamination, the pond could be in an area that would otherwise be unusable, which could make every piece of land one of purpose. Advancements such as internally lit air-lift environments and reducing environmental impacts through optimization of depth and hydraulic retention time have been conducted to increase the productivity and scalability for photobioreactors and open pond systems, respectively. [23, 24]

The second theme is the oil recovery phase. Within this area is the harvesting or processing of the microalgae. This step has great variation depending on the desired product and process used. Some processes require the cells to be *dewatered* [4, 25]. Dewatering is the separation of the microalgal cells from the media or growing fluid. Current practices within the dewatering component of oil recovery have been made in many areas along with their reliability and limitations, as described by Uduman et al. Advances in this area are necessary and include using forward osmosis as a partial means of dewatering microalgae [26], electrically dewatering [27], and the use of cationically modified cellulose polymers [28]. In addition to dewatering, some processes require the cells be lysed, or for the membrane to be disrupted. Others require the fermentation of the cells in order to them to produce ethanol. Ultimately, the processing of the microalgae greatly depends on the final desired product.

Afterward dewatering, transesterification occurs – the third theme to algal inefficiencies, fuel production. Transesterification is a set of chemical reactions creating biofuels from lipids. The main biological functions of lipids such as serving as structural components of cell membranes, signaling molecules, and energy storage. Lipids constitute a broad group of naturally occurring molecules including fats, waxes, sterols, phospholipids, fat-soluble vitamins (such as vitamins A, D, E, and K), monoglycerides, diglycerides, and triglycerides. Within transesterification, triglycerides from the lipids are transformed into biofuel using alcohol with the aid of catalysts. Common alcohols used in transesterification are ethanol and methanol. The catalysts commonly used include heat, potassium hydroxide, sodium hydroxide, and sodium methoxide to speed up the reaction. This reaction transforms the fatty acids to crude glycerol and mono-alkyl esters, or the biofuel. Advances in this area include a transesterification process that does not rely on catalysts to speed up the reaction, but in situ lipid hydrolysis and supercritical transesterification [29]. The independence of this process from catalysts reduces the overall cost of the biofuel.


Figure 1-2 Creating biofuel from microalgae is very cost intensive. By optimizing in algae cultivation, oil recovery, and fuel production, microalgal biofuels would be more sustainable.

#### **1.3 The Cost of Microalgal Biofuel**

The competitiveness of biofuels to petroleum greatly depends on the production cost associated with the cultivation, oil recovery, and fuel production. In order to estimate its cost and compare it to petroleum, the amount of algal biomass that has an equivalent energy potential to a given quantity of petroleum is calculated using equation 1-1.

$$M = \frac{E_{petroleum}}{q(1-w)E_{biogas} + ywE_{biodiesel}}$$
<sup>1-1</sup>

*M* represents the quantity of algal biomass in tons.  $E_{petroleum}$  is the amount of energy within a barrel of crude petroleum, which is approximately 6100 MJ [5]. *q* is the volume of biogas produced from the residual algal biomass through anaerobic digestion and is estimated to be around 400 m<sup>3</sup>/ton. *w* is the oil content of the biomass in percent by weight. This is determined by many factors including the algal strain selected, growing conditions, etc; the percent weight of the oil is an important factor as discussed in the previous section.  $E_{biogas}$ is the energy content of the biogas and is approximately 23.4 MJ/m<sup>3</sup>. *y* is the yield of biodiesel produced from the algal oil – typically 80%. *E<sub>biodiesel</sub>* is the biodiesel's average energy content, which is around 37800 MJ/ton.

Using these factors and working under the assumption that transportation costs are equivalent for biofuel and petroleum based fuel, an acceptable price can be calculated for biomass per ton. As of March 2016, WTI Crude Oil costs ~\$40 per barrel [30]. At this rate,

for algal biomass to be competitive with petroleum diesel, it must cost \$136.68/ton. Currently, it costs nearly \$3000/ton; therefore, the price of biomass must decrease by 95%.

Cost estimates can be improved based on empirical data for system performance. Three aspects of production – energy and carbon balance, environmental impacts, and production costs – have been investigated [31]. With this great amount of advancement necessary for competitiveness of algal biofuel, innovation in all aspects of production are necessary.

# 2. Methodology

In this section, the methodology for both experiments and computational modeling will be discussed.

## 2.1 Strain Selection

The selected microalgal strain was *Scenedesmus dimorphus*, which has many applications including its use in biomass and wastewater treatment applications [32]. *S. dimorphus* was selected because of its high lipid percentage compared to other microalgal strains and its relatively manageable cultivation as shown in Table 2-1. While present in freshwater, *S. dimorphus* can also grow in domestic sewage or in areas deemed unusable. These variations of growing conditions can cause phenotypical changes allowing these 10µm long microorganisms to grow in colonies or alone in crescent, bi-convex, rounded tetrad, or circular shapes [33]. The molecular composition of *S. dimorphus* varies as well such as for protein, carbohydrates, and lipids are 8 - 18%, 21 - 52%, and 16 - 40% by dry weight, respectively [7]. The microalgae was obtained from the University of Texas in Austin (UTEX) Culture Collection of Algae under strain 1237. This database was chosen due to their expertise and plethora of algae strains, which include those from the freshwater diatom, extreme environment, salt plains, and snow algal varieties.

Strain	M/F	Protein	Carbohydrates	Lipids	Nucleic acid
Scenedesmus obliquus	F	50 - 56	10-17	12 - 14	3-6
Scenedesmus quadricauda	F	47	-	1.9	-
Scenedesmus dimorphus	F	8-18	21 - 52	16 - 40	-
Chlamydomonas rheinhardii	F	48	17	21	-
Chlorella vulgaris	F	51 - 58	12-17	14-22	4-5
Chlorella pyrenoidosa	F	57	26	2	-
Spirogy ra sp.	F	6-20	33 - 64	11 - 21	-
Dunaliella bioculata	М	49	4	8	-
Dunaliella salina	М	57	32	6	-
Euglena gracilis	F	39-61	14 - 18	14 - 20	-
Prymnesium parvum	М	28 - 45	25 - 33	22-38	1-2
Tetraselmis maculata	М	52	15	3	-
Porphyridium cruentum	м	28 - 39	40 - 57	9-14	-
Spirulina platensis	F	46 - 63	8 - 14	4-9	2 – 5
Spirulina maxima	F	60 - 71	13-16	6-7	3 - 4.5
Synechoccus sp.	М	63	15	11	5
Anabaena cylindrica	F	43 - 56	25 - 30	4-7	-

Table 2-1 Chemical Composition of Selected Microalgae Expressed on a Percent Dry Matter Basis. Scenedesmus dimorphus has a large percentage of lipids by dry weight in comparison to other microalgal strains. The M and F denote marine or freshwater species. [8]

## 2.2 Cultivation

The microalgae arrives in an agar slant from the University of Texas Culture Collection and is transferred to test tubes containing growth media. Once the algae sample is received from UTEX, 9mL sterile screw-capped test tubes filled with proteose media were inoculated with 2 scoops of the algae from the agar slant. The proteose media recipe, which is made in-house for the first stage of the growth cycle, can be found in the Appendix 10.1.1. The microalgae within the test tubes are placed on the shaker table apparatus. A diagram of the growth setup is shown in Figure 2-1. The growth setup consists of the following: 125 Watt, 6500 Kelvin growth lamp connected to a timer to allow the lamp to be on 12 hours and off for the next 12 hours to simulate day and night cycles; a shaker table to keep the samples in constant movement that would keep the algae constantly agitated to avoid the cells settling to the bottom and inhibiting their growth; and an aluminum reflector insulation board to redirect the growth lamp's light back onto the algae while shielding the algae samples from ambient light during the dark hours. The algae are allowed to grow in those test tubes for five days within the growth environment. Subsequently, the algae is then transferred to 25 mL sterile flasks containing the proteose media and grown for another five days.



Figure 2-1 The growth set up for the Scenedesmus dimorphus within the Cellular Biomechanics Laboratory. The set up consists of a shaker, timer, reflective boards, and full spectrum growth bulb. (Image: compliments of Nateé Johnson.)

Next, the microalgae were transferred to flasks and additional media is added to promote growth. In this stage, Modified Bold 3M media was used which was also made in-house. Modified Bold has vitamins B<sub>12</sub>, Thiamine, and Biotin, which keep the algae healthy. Formulation for the Modified Bold 3N media can be found in Appendix10.1.3. Monitoring via a Zeiss Axiovert 200 microscope was done to verify the maturation of the cells. From this, a growth curve (Fig. 2-2) was generated using a combination of hemocytometry and photospectrometry. During this time, the algae-media suspension is stirred on a stir plate until a stationary phase is met.



Figure 2-2 Growth curve of S. dimorphus. Growth is evident between days 15 and 35. Growth indicates that there are more cells growing than dying. Stationary phase is reached once the growth and death rate of the cells are in equilibrium.

#### 2.3 Monitoring Growth

To monitor the growth of the cells, a two-step process must be executed, hemocytometery and photospectrometry. Hemocytometry is the process of counting cells with a hemocytometer. The generic name of this device is a *counting chamber*, but because of its initial use in performing blood counts, the most widely used type of chamber is the hemocytometer. There are many applications that require cell suspensions and its cell concentration. Those applications include, but are not limited to, cell culture and microbiology. In order to use the hemocyometer, one must ensure that the sample solution is diluted enough taking note of the dilution rate as this is important in calculating the total number of cells in the media of interest. Additionally, the counting grid and a coverslip are properly cleaned.



To begin, place the coverslip onto the hemocytometer. The coverslip must be heavy enough to withstand the surface tension of the fluid. Next, pipette into the introduction point of the hemocytometer enough media to cover the counting area (approximately 0.1uL). Place the hemocytometer on the microscope allowing the cells to settle, and choose a counting pattern. Common counting patterns include counting squares on the diagonal and counting the four corners and center of the counting grid as shown below. For microalgae, the latter was used. Within the 5x5, 1/25 sq. mm.



Figure 2-4 Counting regimes for hemocytometry. In order to properly count cells within a given sample, consistency when counting is necessary. Scheme B was used in the microalgae calculations.

Choosing a counting regime also includes the provisions whether a cell is "in" or "out" of the counting area. This comes about when a cell is on the edge or border of a counting grid square. One can say the cell is "in" if it is on the top and right borders of the counting square and "out" if it's on the bottom or left border. Using the selected counting regime, count the cells and determine the mean number of cells within each chamber. Multiply that number by 10,000 and your dilution factor to yield the number of cells per milliliter. This process was repeated at various dilutions in order to correlate algae concentrations at various dilution rates, which would be combined with optical density readings from photospectometry.



Figure 2-5 Photospectrometer Schematic. A light source is diffracted to the desired wavelength, which shines through the sample. The remaining light is captured with the detector and quantified.

Photospectrometry is widely used in clinical chemistry in determining the concentration of substances within a solution. In photospectrometry, a solution is examined to determine the amount of color that exists within it using the amount of absorbed light. The light source can be ultraviolet, infrared, or in the visible spectrum as shown in Figure 2-4. When the light energy is absorbed by the material, it is considered absorption. First, the light source's intensity is measured through a blank and then through the sample. Next, experimental data is used to calculate the transmittance and absorbance.

Photospectrometers can report an absorbed, transmitted, or reflected value to quantify the concentration of the desired molecule within the solution. For this photospectrometry work, a Tecan Safire II photospectrometer was used. Using Bodipy 505/515, a green lipophilic fluorescent dye, the lipid components of the microalgal cells were stained and detected with the photospectrometer.



Figure 2-6 Sample serial dilution plate for photospectometry in order to determine algae concentration.

Determining the dilution vs cell concentration equation, absorption measurement mode was used to obtain the optical density readings. This means a 96-well plate was used, and each row represented a different dilution rate (see Fig. 2-6). Each row had at least 6 wells filled with algal solution. The remaining rows were of just algal media without cells, deionized water, and blank wells. These would serve as controls that would help us identify any optical density values seem inaccurate. After the plate was read, optical density measurements were correlated to the respective concentrations. These results were used to develop a graph that shows their correlation. This is important because we could use the equation generated from the graph to determine the amount of cells within a certain solution without using a hemocytometer in the future. The procedure for this experiment and other photospectrometry experiments associated with this work are located in the appendix. The resulting correlation graph is shown below in Figure 2-7. The corresponding equation is y = 24,342,000x - 656,900 with an R<sup>2</sup> value of 0.97.



Figure 2-7 Correlation graph for algal cell concentration and optical density as determined by the Tecan Safire 2 photospectometer.

# 2.4 Shearing tests

In the lysing techniques tests, *S. dimorphus* cells underwent various modes of lysing to determine the effectiveness of the Bodipy lipophilic marker and help gain a better understanding of the current lysing processes. Various cell disruption techniques, including microwave, sonication, and shearing between glass and aluminum surfaces, were performed. Once the disruption was concluded, the solutions were filtered and the photospectrometer was used to determine the absorbance Bodipy 505/515 remaining in the precipitate. This revealed that shearing worked significantly better than the other methods employed.

#### **2.4.1 Preparation**

Algae were allowed to absorb the Bodipy dye for approximately 1 week. Then the viability of the dye was checked by examining the fluorescence. The algae and dye were spun down and the dye solution was replaced with dH<sub>2</sub>O. *S. dimorphus* with Bodipy 505/515 dye was imaged under bright field (Fig. 2-5a) and fluorescence (Fig. 2-5b) microscopy are shown. The same exposure (0.3) was kept throughout for imaging for consistency. The magnification was 150 times.



Figure 2-8 Microscopic images of *S. dimorphus* at 150x. a) Bright field image showing healthy cells. b) Fluorescent image of the same cells in 2-8 (a) stained with Bodipy 505/515.

#### **2.4.2 Disruption Methods**

There were four disruption methods investigated to compare their effectiveness: sonication, frothing, microwave, and manual shearing via metal flat punch. The microalgae slurry was placed in a sonicator for 14 minutes. The algae solution is poured into 80 mL beaker for the frothing disruption test. A frother was used on the microalgae slurry for 7 minutes. The microwave was utilized for a total of 7 minutes as well. However, due to the evaporation of the dH2O, this disruption method required 3 times more microalgal slurry to obtain enough of a sample to be used in the analysis via photospectrometry. The fourth disruption method used was shear via a metal flat punch whose diameter was approximately 1.5 inches. The slurry was sheared between the flat punch and a glass petri dish for 7 minutes.

The microalgae solutions were removed from their respective disruption tests with needle and syringe and a drop of each sample was placed on a coverslip. A 0.22  $\mu$ m filter was used for the remainder of the tested microalgal slurry to remove intact cells and debris before measurement. The microalgal slurry obtained from the flat punch shear test was filtered twice to ensure only subcellular parts were used in the following optical density tests. Optical density measurements were undertaken with a Tecan Safire 2 photospectrometer. The Bodipy dye used to stain the algal lipids becomes excited between 450-490 nm. Three controls were included: 1.5ml dH<sub>2</sub>O to 0.5  $\mu$ l Bodipy; dH<sub>2</sub>O; and blank wells within 96-well microplate. The results show that shearing is a good candidate for algal cell lysing (Fig 2-9).



**Bodipy Absorbance** 

Figure 2-9 In the shear test, various lysis methods were tested. The photospectrometer quantified the amount of Bodipy 505/515 dye left in the solution. This would help in approximating the amount of cells that were lysed. This data suggests that the metal shear technique technique produced the most lysing. OD stands for optical density.

# 2.5 Atomic Force Microscopy

Atomic force microscopy has been increasingly used to investigate microbial surfaces at high resolution [34]. The technique provides three-dimensional images of the surface ultrastructure with molecular resolution, in real time, under physiological conditions, and with minimal sample preparation. AFM is more than a surface-imaging tool in that force measurements can be used to probe the physical properties of the specimen, such as molecular interactions, surface hydrophobicity, surface charges, and mechanical properties. These measurements provide new insight into the structure-function relationships of microbial surfaces.



Figure 2-10 This image depicts how the Atomic Force Microscope works to record the topography of a surface.

The atomic force microscope utilizes a laser that is pointed onto a cantilever specific to the application of the user. This means, for softer samples, using a stiff tip, compared to the sample, could damage the sample. Likewise, having a stiff sample and soft cantilever could damage the cantilever. For more information on selection of cantilevers, see Appendix 10.3. The laser is then reflected from the cantilever to a mirror, then to a position sensitive photodetector (PSPD). The PSPD keeps analyzes where the cantilever is on the sample. The vertical bending of the cantilever will be determined by the change in the top and bottom halves, while the lateral motion is determined by the left and right halves. It is imperative that the cantilevers are carefully selected to ensure that its spring constant is compatible with the sample. This ensures that the cantilever won't damage your sample while not being damaged itself from the rigidity of the sample. With the data collected from the PSPD, the microscope sends the location data to the computer's software where it is compiled to make an image. In the case shown in Figure 2-10, a topography plot is generated. Afterwards, the data is fed back into the scanner to continue imaging. Two AFM instruments were used, Park Systems XE-70 and an Asylum Research MFP-3D atomic force microscope. Images from the Park AFM are shown in Figure 2-11. The topography images are shown in the top row and the respective error signal is shown below. The error signal shown here is to give a raw depiction of what the surface of the cell looks like before post processing. Although it looks to be the topography image, the error signal is the difference between the trace and retrace scans, as each image is a compilation of two traces.





A similar concept of imaging is applied for taking force measurements of a sample. However, instead of scanning across the sample, the cantilever is pressed straight down into the sample. Figure 2-12 is a depiction of the events that take place while performing a force measurement. First, the cantilever approaches the sample and snaps into contact with the surface due to van der Waals forces. Then, the cantilever continues to press into the sample until a certain threshold is reached. This threshold is set at the beginning of the experiment and can be dependent on the amount of force applied or the depth pressed into the sample, among other threshold types. After the set point is reached, the cantilever begins to retract from the sample and abruptly pulls off from the sample due to molecular adhesion. Material/mechanical properties can determined from the force measurements including ultimate strength and Young's modulus which can be gathered by the contact portion of the force distance curve shown above. Values of Young's modulus for *S*. *dimorphus* will be discussed in the section 3.



Figure 2-12 This is a schematic of a force/distance curve produced by an atomic force microscope force measurement. The data collected from this test can be used to quantify a subject's moduli values, strength values, magnetic capabilities, et cetera.

## **2.6 Microfluidics**

Microfluidics encompasses the designs of small volume systems which are typical in biochemical laboratory operations on a microscale. Generally, the size for a microfluidic device ranges from about 10 to 500 micrometers. Many industries utilize microfluidic devices including pharmaceutical and life science research, point of care diagnostics, as well as drug delivery [35]. As the applications of microfluidics evolve, microfabrication techniques develop. Some of the prominent techniques include lamination, hot embossing, injection molding, laser micromachining, ultrasonic technologies, as well as lithography.

Soft lithography belongs to a family of lithography fabrication techniques of microfluidic devices. Its cost effectiveness, good resolution at small scales, and ability to recycle the molds make it the most popular technique of the group. Soft lithography uses elastomeric materials, mainly polydimethysiloxane (PDMS), contributing to its low cost. PDMS is biocompatible, permeable to gas, and is transparent. Soft lithography begins with the design of the microfluidic channel where the mask is printed onto the transparency with a high resolution printer. Second, the master template is transferred onto a silicon wafer using ultraviolet light to cure a photoresist like SU-8, a negative photoresist. The unexposed SU-8 is washed away and the desired design remains. Third, a mold is made from the wafer template using PDMS. Once the PDMS has cured, the PDMS mold can be removed from the wafer and placed on a substrate, usually glass, to run the microfluidic experiments. This process is shown below in Figure 2-13.



Figure 2-13 Soft photolithography is conducted by (A) designing the channel and making a transparency mask. (B) A master template is made by pouring a photoresist, here SU-8 (blue), on a silicon wafer (orange) with the mask on top of the SU-8. The wafer is then exposed to ultraviolet light, which creates the channels. (C) The PDMS is poured on the developed wafer and allowed to cure, or harden, which molds the channel into the polymer. (D) Once the PDMS is cured, the channels are ready to be utilized for experimentation. The desired channels are cut from the polymer/wafer and plasma cleaned or oxidized. Then, they are placed on a substrate, glass (grey), and tubing is inserted.

#### 2.7 Computational Modeling Approach

The Particle Surface Tribology Analysis Code (P-STAC) is a multiphase, computational modeling algorithm developed in the Particle Flow Tribology Laboratory (PFTL) at Carnegie Mellon University incorporating solid mechanics, fluid dynamics, and particle dynamics. P-STAC has many applications and has been shown to be a robust algorithm for multiphase modeling [36]. All of the simulations within this work will be using P-STAC.

The approach taken for modeling biological cells within a liquid substrate was Eulerian-Lagrangian. In computational modeling, there are two approaches that must be considered when modeling various mediums. The first is where the medium is modeled as a continuum, known as Eulerian. It is commonly described by the Cauchy equation (Eq. 2-1) where the conservation of momentum is applied.

$$\rho (DV/DT) = \nabla \cdot \sigma + f \qquad 2-1$$

The second is where the medium is modeled as discrete elements. Unlike in the Eulerian approach, the momentum is applied using Newton's second law as shown in Equation 2-2.

$$\mathbf{F} = \mathbf{m}\mathbf{a}$$
 2-2

It is imperative to accurately model a multiphase flow. Therefore, combining both approaches are used to simulate solid particles (Lagrangian) in a liquid body (Eulerian). In order to couple these two phases, particle and fluid, Stokes drag correlation was employed using the Equation 2-3 where  $\eta$  is the dynamic viscosity of the fluid, *a* is the particle radius and *U* is the relative velocity between the fluid and the particle.

$$\mathbf{F}_{\mathbf{drag}} = 6\pi\eta a \mathbf{U}$$
 2-3

By combining these two approaches, it is possible to incorporate more physics modes including stress and particle collisions. These particle collisions are modeled using discrete element modeling using the classic spring and dashpot model shown in Equation 2-4. [37]

$$\mathbf{F} = \mathbf{K}_{\text{part}} \times \mathbf{U} - \mathbf{B}_{\text{part}} \times \mathbf{V}_{\mathbf{n}}$$
 2-4

The stress on the solid particles was calculated by using the von Mises criterion (Eq. 2-5). This stress value will be used to understand cell lysis discussed in section 6.

$$\sigma_{v} = \sqrt{\frac{1}{2}} \left[ (\sigma_{11} - \sigma_{22})^{2} + (\sigma_{22} - \sigma_{33})^{2} + (\sigma_{11} - \sigma_{33})^{2} + 6(\sigma_{12}^{2} + \sigma_{23}^{2} + \sigma_{13}^{2}) \right]$$
 2-5

# **3. Elastic Response of Microalgae** *Scenedesmus dimorphus* in **Dry and Aqueous Environments**

A version of this chapter has been published in Applied Physics Letters in 2014.

With the re-emergence of microalgae as a replacement feedstock for petroleumderived oils, researchers are working to understand its chemical and mechanical behavior. In this work, the mechanical properties of microalgae, *Scenedesmus dimorphus*, were investigated at the subcellular level to determine the elastic response of cells that were in an aqueous and dried state using nano-scale indentation through Atomic Force Microscopy (AFM). The elastic modulus of single-celled *S. dimorphus* cells increased over tenfold from an aqueous state to a dried state, which allows us to better understand the biophysical response of microalgae to stress.

# **3.1 Introduction**

Microalgae are photosynthetic microorganisms that are not only critical to aquatic ecosystems, but are increasingly being studied in numerous application areas including biofuels, water purification, and the sequestration of carbon dioxide and metals produced in industrial areas. [4, 8, 38] These organisms also produce a diversity of macromolecules and vitamins that are used in dietary supplements, agriculture and aquaculture feed, cosmetics, dyeing agents, pharmaceuticals, and biofertilizers. [10] Often though, there is a challenge with obtaining the microalgal products as they are encapsulated within the microalgae and thus approaches addressing cell lysis or disruption are required. [39] There are a variety of disruption methods including chemical lysis, osmotic shock, enzymatic lysis and mechanical lysis, yet there is not one approach that is used for all applications. [40] One growing area for disruption methods is mechanical lysis, which include highpressure homogenizers, ultrasonicators, microwaves, and lyophilizators. The most common mechanism of mechanical disruption is shearing whether this is through solid or liquid shearing. [41] Solid shearing occurs in processes like bead beating or milling. However liquid shearing occurs in homogenizers, French presses, etc. [40] Although these methods are useful, they are energy intensive, which limits their utility for larger scale applications. [18] By better understanding the microalgal biophysical mechanical response in both the aqueous and dried states, more efficient techniques for cell lysing may be developed. [13]

#### **3.2 Methods**

The microalgal strain Scenedesmus dimorphus (S. dimorphus), which has many applications including its use in biomass and wastewater treatment applications, was used for this investigation. [32, 42] (see Appendix 10.4 - S1) Variations of growing conditions can cause phenotypical changes allowing these microorganisms, approximately 10µm in length, to grow in colonies or alone in crescent, bi-convex, rounded tetrad, or circular shapes. [33] S. dimorphus cells' molecular composition, namely the protein, carbohydrate, and lipid content, vary as well having dry weight percentages of 8 - 18%, 21 - 52%, and 16 - 40 %, respectively. [Burton, 2009 #34] To probe the mechanical properties of the S. dimorphus microalgae, AFM was used because it is considered one of the best tools to study the mechanical properties of cells. AFM enables consistent biological investigations in both dry and aqueous environments while enabling high-resolution imaging and force measurements for obtaining mechanical properties. [34] Mechanical properties, like the elastic modulus of various biological materials have been obtained with AFM previously including that for mouse embryonic stem cells and yeast cells, which have ranging elastic moduli of 17.87 kPa and 0.6 MPa, respectively. [43-45] Here, we report the mechanical properties for microalgae in an aqueous and dried state.

We cultured *S. dimorphus* (UTEX algae culture collection) in modified bold 3N and proteose media. For AFM imaging, glass slides were plasma cleaned and for the aqueous experiments, they were treated with 0.01% poly-1-lysine solution to enhance cell adhesion. The dried samples were inoculated from a culture grown on proteose agar medium and allowed to dry on the slides. Both imaging and nanoindentation on the *S. dimorphus* cells were performed on an Asylum Research MFP-3D atomic force microscope

in a vibration isolation chamber under ambient air at room temperature. Two types of cantilevers were selected for probing the aqueous and dried *S. dimorphus* cells, which had spring constants of 0.08 - 0.26 N/m and 0.5 - 4.4 N/m. (see Appendix 10.4 –S2) The specific spring constants for the cantilevers used were calculated using the Sader method [46] implemented with the Asylum Research software.

# **3.3 Results and Discussion**

Before nanoindentation, a single, dried cell was imaged in air using the stiffer cantilever to obtain a representative 3D height image shown in Figure 3-1(a). Next, the nanoindentation was initiated at select subcellular locations to determine the mechanical properties [Figure 3-1(b)]. A minimum of ten measurements was obtained at each location. Utilizing the Asylum software, data from the approach portion of the force vs. indentation curves were analyzed using relationships for a Hertzian contact model. A similar technique was used to mechanically characterize embryonic mouse stem cells. [44]



Figure 3-1 (a) A representative AFM 3D height image showing the surface topography of a dried S. dimorphus cell. (b) An AFM topographical image of a representative single, dried S. dimorphus cell for the determination of the Young's Modulus at subcellular points 1, 2, and 3 through nanoindentation.

A representative force vs. indentation plot with the Hertzian contact model approximation applied is shown in Figure 3-2a. After accumulating at least ten force-indentation data sets from the three points of the microalgae, histogram plots were determined to project the variation in the data [Figure 3-2(b)]. The distribution of the Young's modulus values approximate a normal distribution with a mean value of 36.72 MPa and a standard deviation of 10.71 MPa for one cell.

For aqueous cells, a representative force versus indentation curve is shown in Figure 3-3(a), which result in a Young's modulus of approximately 3.95 MPa with a Hertzian approximation. Similar to the dried cell results [Fig. 3-2(b)], a histogram was generated from multiple nanoindentations [Figure 3-3(b)] to analyze the distribution of the results, which, for many biophysical measurements, are inherently variable in biology. The dried cell Young's moduli values are more broadly distributed on the histogram, which may be due to the differences in cell properties that are likely more significant when the cells are in solution versus dried.



Figure 3-2 (a) A representative AFM force vs. indentation approach curve from a defined nanoindentation location on single, dried *S. dimorphus* cell. In this experiment, the Young's modulus was calculated to be 26.85 MPa using a Hertzian contact model. (b) A histogram of binned Young's Modulus values from the designated locations on the same cell.



Figure 3-3 (a) A representative experimental AFM force versus indentation approach curve for a S. dimorphus cell in solution. The Young's modulus here was calculated to be 3.95 MPa using a Hertzian contact model assumption. Because of the very close fit, the red Hertzian fit curve overlaps the blue measurement line. (b) A histogram of binned Young's Modulus values from representative locations on the same cell.

After analyzing the results of multiple cells, from both cell types (N = 8 cells with 236 measurements), the Young's moduli values were calculated with 95% confidence intervals (p  $\leq$  0.05) for *S. dimorphus*. For the dried cells, the Young's modulus was estimated to be 57.96  $\pm$  7.20 MPa (mean  $\pm$  standard deviation of the mean). The Young's modulus value for aqueous *S. dimorphus* cells was estimated to be 2.21  $\pm$  0.40 MPa (Fig. 3-4).



Figure 3-4 Comparison of dried versus aqueous Young's moduli measurements of Scenedesmus dimorphus cells. The error bars represent standard deviation of the mean with  $p \le 0.05$ .

With this data, one can compare the elastic moduli of *S. dimorphus* cells, in aqueous and dried forms, to the moduli of other biological materials. The bud scar on a yeast cell was investigated with AFM as well and was found to have an elastic modulus of 6.10 MPa, which is slightly higher than the value observed for the aqueous *S. dimorphus* cell. [45]

The bud scar's modulus value was attributed to the difference in the architecture of the cell wall when it was compared to other mammalian cells whose modulus were significantly lower, in the 100 Pa to 0.1 MPa range. However, using a micromanipulation technique, the cell wall of S. cerevisiae at stationary phase was found to have a Young's modulus value of  $107 \pm 4$  MPa. [47] Another well-studied microorganism that is interesting to compare to our microalgae is *Escherichia coli*. The elastic moduli of a variety of *E. coli* strains range from 0.05 - 221 MPa [48] and is within the range of other biological materials as well (see Appendix 10.4 - S3). The findings of significant difference between aqueous and dry moduli values is important in biology since the variation can be very broad under different environmental conditions. For example, dry and hydrated murein sacculi of E coli has a tenfold increase between dry and hydrated environments (300 MPa and 25 MPa, respectively). [49] Additionally, hydrated and dehydrated human dentin has a small difference in magnitude (22 GPa and 16 GPa, respectively) while hydrated and dehydrated horse hoof keratin has a 100-fold increase (410 MPa and 14.6 GPa, respectively). [50, 51] Mechanical response is specific to each cell type and thus understanding these differences for each cell type is essential.
### **3.4 Conclusion**

In conclusion, this work reports the elastic properties of single cell microalgae. We were able to both image and determine the elastic properties for *Scenedesmus dimorphus* cells using AFM under both dry and aqueous conditions. Through our approach, the biophysical response of their elastic moduli was  $57.96 \pm 7.20$  MPa and  $2.21 \pm 0.40$  MPa for dry and aqueous microalgae, respectively. In addition, comparing the dry and aqueous states for algae reveals a significantly greater difference than tenfold in their elastic properties. These findings may be particularly important for cell lysis due to the diversity of algae-based industries where this data may be applicable including dietary supplements, cosmetics, pharmaceuticals, and biofuels. We believe that these findings will be important to researchers in applied physics, biology, and engineering.

# 4. Modeling the Mechanical Stresses on Red Blood Cells in Microfluidics

Due to the lack of data in literature about the material properties of microalgal cells, and more specifically *S. dimorphus*, erythrocytes (red blood cells) were considered for the initial advancement of the P-STAC model.

Hemolysis, the lysing of red blood cells, is of a great importance when developing cardiac assist equipment to increase the potential of viable cells after the device. In this chapter, an in-house, multiphase, computational model called Particle-Surface Tribology Analysis Code (P-STAC) was custom developed in the Particle Flow and Tribology Laboratory at Carnegie Mellon University. P-STAC incorporates the physics of the particle dynamics, fluid dynamics and solid mechanics, which allows us to study a range of cell sizes and fluid domains while understanding how stresses in various conditions affect the cells. The framework was compared with published work to examine the accuracy of the computational framework. Additionally, this framework was applied to human red blood cells.

#### **4.1 Introduction**

Microfluidics has been used to both study and manipulate biological systems through shear flows. [52] It is used to investigate cell lysis through various means including electrically with electroporation [53], enzymatically [54], and mechanically [55], among others. Mechanical lysing is important for both understanding diseases and developing commercial products in a diversity of areas including therapeutics, nutraceuticals, and renewable energy. [9]

The mechanical mechanism that many lysis methods utilize is shearing.[12] Shearing the cells to induce lysis has been studied for various cell types [56, 57] and numerous mathematical models have been developed to try to quantify the percentage of cells that were lysed. [58, 59] The mathematical models to be discussed herein are specific to red blood cells and can only predict the percentage of cells that are lysed based on the amount of hemoglobin that are released from the erythrocytes into the plasma, making it difficult to evaluate lysis on a per cell basis, for both red blood cells and other cell types. [60]

From an engineering point of view, lysis relies more on a threshold, or yield strength, to determine whether a material fails. This concept has been applied to hemolysis and mammalian cell lysis. [61, 62] Lysis was determined based on the amount of cells whose membranes were stressed past its threshold of surface tension.

Understanding the mechanical response of individual cells from stresses, including normal and shear forces, plays an important role in future technologies. In this work, an in-house computational fluid dynamics code was developed to determine, from a tribology perspective, microfluidic flows and configurations that will increase the individual stresses on red blood cells. These stresses would aid their lysis and we will compare our computational results to the two mathematical modelling principles, hemoglobin release and exceeding membrane surface tension. This is unique compared to other lysis techniques because we are able to computationally evaluate how stresses affect each individual cell.

#### **4.2 Mathematical Models**

The two mathematical models to be discussed were developed to determine hemolysis, or lysis of red blood cells.

#### 4.2.1 Mathematical model based on hemoglobin released

Shear stress and its effect on red blood cells have been investigated in many experimental setups including, cone and plate viscometers [63], capillaries [64], cup and piston apparatuses [65], and turbulent jets [66]. Understanding these shear stresses and their effect on hemolysis led to the development of a mathematical model based on the amount of hemoglobin released from the erythrocytes in to the blood plasma. [60] When developing this model, a couette system with axial flow was created to deliver repeatable shear stresses during each operation. Hemolysis was then measured using the Equation 4-1 where  $\Delta$ Hb is the amount of hemoglobin released, Hb is the total amount of hemoglobin, Ht is the haematocrit, VHb is the whole blood hemoglobin concentration, PHb0 is the plasma hemoglobin at the beginning of the experiment, and PHb is the plasma hemoglobin at the end of the experiment.

$$\Delta$$
Hb/Hb [%] = ((1-Ht/100) \* (PHb - PHb<sub>0</sub>))/VHb×100 4-1

From the experimental data by Heuser and Opitz in 1980, a power-law equation that takes into account the stresses applied to the amount of time the stress was applied as shown in equation 4-2 where t is exposure time and  $\tau$  is the shear stress applied.

$$\Delta Hb/Hb = 1.8 \times 10^{-6} * t^{0.765} * \tau^{1.991}$$
 4-2

Herein, equation 4-2 will be implemented into the P-STAC framework and compared to the computational results.

#### 4.2.2 Mathematical model based on exceeded threshold of surface tension

In determining the viscoelasticity of red blood cells, Jay (1973) conducted experiments to squeeze erythrocytes through the micropipettes of various sizes using pressure gradients. [61] Deformation, volume loss, and rupture of the red blood cells were observed and an equation was developed that determined the breaking tension (T) of the cell membranes as shown in equation 4-3.

$$T = (\Delta P * \rho * g) / [(4 (1/D_p - 1/D_s)) * 10,000]$$
 4-3

Here, T is the breaking tension,  $\rho$  is the specific gravity of mercury,  $\Delta P$  is the final pressure (cm of Hg), Dp is the micropipette diameter, and Ds is the diameter of the spherical cap.

## 4.3 Computational Modeling Framework Applied to Red Blood Cell Lysing

The mechanical response of cells to forces including stretching, shear, etc. are known to be important in many diseases. The understanding of mechanics in biological systems also may play important roles in future technologies. For example, the mechanics of algae and red blood cells in terms of mechanical lysing is important for commercial products as well. Both of these biological systems can be manipulated with shear flow, yet control over the mechanical perturbations can be very important as it pertains to energy inefficiencies associated with mechanically lysing cells. In this section, the P-STAC framework is compared to hemolysis experiments. We implement this computational approach to examine the lysis of cells in various shear stress regimes.

First, in order to simulate red blood cells in a concentric cylinder viscometer with varying shear stress, as done by Leverett, et al 1972, we model a channel with a Couette flow to achieve a constant shear stress throughout the fluid while at steady state. To determine the lid velocity for a couette flow, Equation 4-4 was used.

$$\boldsymbol{\tau} = \boldsymbol{\gamma} \boldsymbol{\mu}$$
 4-4

Given the experiment done by Leverett, et al was over 2 minutes in duration with 20-second acceleration and deceleration to the desired set point, the velocity of the fluid was initialized to achieve steady state to quickly decreasing computational time. Once a constant shear stress is achieved within the flow, the Equation 4-2 was used to determine the percent of red blood cells lysed within the flow (Fig. 4-1). The properties of the red

blood cells are shown in Table 4-1. With the percent of cells lysed, the data was plotted as shown in Figure 4-2 which follows the general trend set by Leverett et al., 1972.

Table 4-1 The model parameters of the red blood cells that was used in the computational framework. The hematocrit does not change the percentage of red blood cells hemolyzed; Cell–cell interaction is not an important mechanism of normal erythrocyte destruction in the concentric cylinder viscometer. [63, 67]

Model Parameters								
Density of human erythrocytes	$1.100 \text{ g/cm}^3$ [9]							
Plasma density	$1.030 \text{ g/cm}^3$ [9]							
Plasma viscosity	0.0035 Pa*s [52]							
Gap height	0.100 mm [9]							
RBC diameter	8 μm [52]							
Hematocrit	2%							





Figure 4-2 a) Percent lysed in experiments done by Leverett et al 1972. b) Percent lysed using P-STAC. Comparing this data shows that we have good qualitative agreement between the model and experiments.

#### 4.4 Cell Lysis Heterogeneity

Here, two mathematical models of cell lysis were introduced. The first was based on the amount of hemoglobin released from a red blood cell where no matter the stress condition, the cell is gradually being lysed. The second was based on a threshold of surface tension in the membrane being exceeded. This method only considers the cell to be lysed after a certain value is reached. A visual depiction is shown in Figure 4.3.



Figure 4-3 The two mathematical approaches to cell lysis, cell lysis heterogeneity. a) The hemoglobin release approach shows that if 50% of the cells are lysed, all of the cells are lysed by 50%. b) The surface tension threshold relies on a ultimate strength of the membrane to be surpassed. In the event that 50% of the cells are lysed, only 2 out of 4 cells are lysed.

In the hemoglobin release model, the graph of the percentage of cells lysed versus stress applied to the cells would be similar to what was seen in section 4.2.1. However, in the threshold model, that same graph would look like a step function. When comparing the percent lysis histograms of these two models, the hemoglobin release approach would have bins of similar heights. This would show that many of the cells experienced the same amount of stress and were lysed the same amount. On the other hand, the bins of the

threshold approach histogram could be described as some being lysed and the others intact. This would mean that the cells experienced either high or low amounts of stress.

Thus far, a Poiseuille flow simulation was run using P-STAC to see which approach best fit computational data. The histogram is shown below in Figure 4-4. The histogram shows that the threshold approach best fits the P-STAC simulation where some cells experienced large amounts of stress and others did not.



Stress on Cells within a Simulated Microfluidic Channel

Figure 4-4 A histogram from the P-STAC simulation suggests that the cell lysis heterogeneity approach that more accurately applies to P-STAC would be the surface tension threshold approach because all of the cells do not see the same stress but rather there is a split between cells who experience high stress and those who experience low stress.

## 5. Studying Effects on Fluid Velocity and Particle Stress

In this section, some preliminary results from using P-STAC in microfluidic channels will be discussed. First, parametric studies were conducted investigating the effects of percent closures, pressure drops, and viscosity of the fluid on the fluid velocity and particles within the fluid. Then the stresses will be applied to the particles (or cells) to see how they are affected by various obstruction shapes.

#### **5.1 Parametric Studies**

The Particle Surface Tribology Analysis Code (PSTAC) is a multiphase, computational modeling algorithm developed in the Particle Flow Tribology Laboratory (PFTL) at Carnegie Mellon incorporating solid mechanics, fluid dynamics, and particle dynamics. For all of the experiments in computational study, the channels are all three-dimensional with lengths of 100µm x 25µm x 25µm and all particles start from the same location as shown in Figure 5-1. All of the cells within the parametric study are for microalgal cells. Since many of the properties of microalgae are unknown, so we are assuming red blood cell properties as described in section 4.3 with the exception of the size of the cells and hematocrit. Microalgae are significantly smaller than erythrocytes and the radius used is 3µm. The solid fraction, hematocrit in hematology terms, of the microalgal cells within the fluid is 5%. The boundary conditions are no-slip on the top and bottom surfaces and pressure inlet/outlet on the left and right surfaces, respectively.



Figure 5-1 A computational model of a straight channel with randomized particles. The channel dimensions are  $100\mu m \ge 25\mu m \ge 25\mu m$ . The boundary conditions are no-slip on the top and bottom surfaces and pressure inlet/outlet on the left and right surfaces, respectively.

### 5.1.1 Effect of Channel Percent Closure on Velocity

Here a computational parametric study was performed to observe how velocity is affected by the closure of the microfluidic chamber. Below, in Figure 5-2 the channels with their velocity contours have varying closures from zero to one hundred percent. The maximum velocity of each closed percentage is shown in the graph in Figure 5-3. The flow is driven by pressure where the inlet pressure is 2000 Pa and the outlet pressure is 0 Pa. The maximum velocity occurs when the channel is 25% closed. This can be attributed to the fluid speeding up in the area of where the obstruction is. Figure 5-2 Particle and fluid velocity simulation images from PSTAC at the same time step for various channel closures with 2000MPa pressure drop. (a) 0% closed (fully open), (b) 12.5% closed, (c) 25% closed, (d) 37.5% closed, (e) 50% closed, (f) 65% closed, (g) 70% closed, (h) 75% closed, and (i) 100% closed (fully closed).







Figure 5-3 The maximum velocity for each channel is plotted. As the channel becomes more blocked by the obstruction, the velocity diminishes.

#### **5.1.2 Effect of Pressure Difference on Velocity**

Here a computational parametric study was performed to observe how velocity is affected by the pressure drop across the microfluidic chamber. In Figure 5-4, the channels with their velocity contours have varying pressures from 1 to 10,000 Pa. As the pressure drop increases, the velocity increases, as expected. We can see that the particles also move quicker as the pressure difference increases across the microfluidic channel.



Figure 5-4 These images depict the flow of particles driven by a pressure difference across the channel with the higher pressure on the left and causing the particles to flow towards the right. The pressure drops are indicated on the left column while the time steps are shown horizontally. From the model, it is evident that the higher the pressure drop, the faster the particles will travel.

#### **5.1.3 Effect of Fluid Viscosity on Velocity**

Here a computational parametric study was performed to observe how velocity is affected by the fluid viscosity within the microfluidic chamber. The fluid viscosities were input to that of water, ethylene glycol, castor oil, and glycerol whose viscosities are 0.001Pa-s, 0.0162 Pa-s, 0.650 Pa-s, and 0.950 Pa-s, respectively. In Figure 5-5, the channels with their velocity contours are shown as the fluid viscosities vary. In addition, graphs of the particle velocities are shown underneath each channel contour plot. These figures show that the velocities of the particles and fluid decreases as the fluid viscosity increases.



Figure 5-5 A simulation of the effect that fluid viscosity has on fluid and particle velocity in microfluidic channels is shown. As viscosity increases, velocities in the particles and fluid decreases.

#### 5.1.4 Effect of the Obstruction Shapes on Stress within Microfluidic Channels

In hematology, any method of lysis is avoided. However, in studying microalgae, maximizing the amount of lysis that occurs within the system while limiting the amount of energy input is desired. The computational model will help in developing a microfluidic system that will increase lysis of microalgal cells in a passive manner.

Here are some of the obstructions, shown in Figure 5-6, which are placed in the microfluidic channels to increase stagnation and cavitation and cause rapid expansion and compression which would in turn increase the amount of stress on the cells. All of the microfluidic channels have the same dimensions of 0.100 mm by 0.025 mm by 0.025 mm and a velocity gradient of 10 mm/s.



Figure 5-6 These are SolidWorks designs of the obstructions to be used in the computational microfluidic channels. They will be described as a) "stepped", b)" rectangular", and c) "multiple" or "staggered" obstructions.

In the contour plots (Fig. 5-7), the von Mises stress is applied to the particles only and the legend depicts the stresses on each of the cells flowing through the channel. Its units are Pascals. The velocity magnitude legend is only applicable to the fluid domain. The visualized 3D fluid domain is clipped at 25% of the width for each of the channels so that the particles can be seen. However, the velocity can be seen as the fluid particles (or nodes) correspond to the velocity magnitude as well. The velocity units are mm/s. The values shown on each of the legend are over the entire time period that the particular simulation was run.

We can see in Figure 5-7f the microfluidic channel with no obstructions in the flow. This serves as the standard for the flow. In these computational models, the fluid is driven by a velocity gradient on the inlet of the channel, the left boundary, at 10mm/s. As one would expect, we have higher velocities in the configurations that have obstructions blocking the flow. As the channel is constricted, the fluid travels faster in areas of constriction. Although this is true, since the constrictions impede into the flow the same distance, the velocity of the fluid between the top and bottom obstructions of each configuration are similar. This leads one to conclude that the shape of obstructions have little effect on the stress of the particles, or cells, flowing through the microfluidic channel. A comparison chart in Figure 5-8 demonstrates their similarities in von Mises stress on particles within the fluid flow.



Figure 5-7 The von Mises stress on the particles and the fluid velocity magnitude are shown in each flow configuration obtaining obstructions. All of these simulations are velocity driven (V = 10 mm/s). a) Single obstruction, b) Stepped obstruction, c) Multiple similar obstructions, d) Multiple stepped obstructions, e) Staggered similar obstructions, and f) No obstructions are the cases studied thus far.



Figure 5-8 Comparing the configurations. The maximum stress levels on the configuration geometries are similar. This would indicate that the shape of the obstruction has little effect on the stress on the particles when the constriction distance is the same for all configurations.

# 6. Geometric Effects in Microfluidics on Heterogeneous Cell Stress using an Eulerian-Lagrangian Approach

A version of this chapter has been published in Lab on a Chip in 2016.

The response of individual cells at the micro-scale in cell mechanics is important in understanding how they are affected by changing environments. To control cell stresses, microfluidics can be implemented since there is tremendous control over the geometry of the devices. Designing microfluidic devices to induce and manipulate stress levels on biological cells can be aided by computational modeling approaches. Such approaches serve as an efficient precursor to fabricating various microfluidic geometries that induce predictable levels of stress on biological cells, based on their mechanical properties. Here, a three-dimensional, multiphase computational fluid dynamics (CFD) modeling approach was implemented for soft biological materials. The computational model incorporates the physics of the particle dynamics, fluid dynamics and solid mechanics, which allows us to study how stresses affect the cells. By using an Eulerian-Lagrangian approach to treat the fluid domain as a continuum in the microfluidics, we are conducting studies of the cells' movement and the stresses applied to the cell. As a result of our studies, we were able to determine that a channel with periodically alternating columns of obstacles was capable of stressing cells at the highest rate, and that microfluidic systems can be engineered to impose heterogeneous cell stresses through geometric configuring. We found that when using controlled geometries of the microfluidics channels with staggered obstructions, we could increase the maximum cell stress by nearly 200 times over cells flowing through microfluidic channels with no obstructions. Incorporating computational modeling in the design of microfluidic configurations for controllable cell stressing could help in the design of microfluidic devices for stressing cells such as cell homogenizers.

#### **6.1 Introduction**

Microfluidics has been used in a variety of lab-on-a-chip applications to study the response of cells to different stimuli including chemical, electrical, and mechanical inputs [1-4]. Variations in the fluid flow in microfluidic systems affect the behavior of cells within the fluid domain and applications in this area include cell cultivation, adhesion, lysis, and stressing [5-8]. Mechanically stimulating cells can result in an array of cell signaling responses including those for proliferation, differentiation, and apoptosis [9-13]. Here we focus on examining the fluid stress applied to individual cells in microfluidic systems with controlled geometries. To understand this, we developed a computational fluid dynamics (CFD) approach to determine microfluidic flows and configurations that will increase stresses experienced by individual cells. We were able to computationally evaluate how stresses affect individual cells and correlate their heterogeneous response to the differences in geometric microfluidic configuration. This approach will help develop a greater understanding of the mechanical response of individual cells from fluid stresses in microfluidics, but also can play an important role in future technologies such as in protein extraction and cell lysis in areas such as pharmaceuticals and biofuels.



Figure 6-1 Geometrically-controlled microfluidics for inducing heterogeneous cell stress through an Eulerian-Lagrangian approach. a) The mesh for the microfluidic channel is used to calculate pressure at the center of each fluid cell (mesh cube). The velocities, u, v, and w, are calculated on the perimeter of each mesh cell. b) Pressure drop along the length of the channel. c) The velocity streamlines for this microfluidic configuration. d) The stress on each particle, or fibroblast cell, is calculated based on Equation 6-12. The dimensions of this microfluidic channel are 100um x 25 um x 25um

#### **6.2 Microfluidic Computational Modeling Framework**

Modelling stress on cells in fluid flow is important in many areas such as hematology. Although models have been developed, the ability to use multiphase approaches that allow for combined solid domains to be dictated by viscous flows while enabling the cell to be tracked throughout the fluid domain [14, 15] have not been readily available and transparent. This type of model would provide a robust approach for understanding cell stresses in microfluidic systems. To accomplish this, one assumes the particles follow the fluid velocity streamlines [16] or that cells act as the fluid in the system, and thus are accounted for in the fluid domain for computational modelling [17]. Our approach focuses on the computational incorporation of cells within fluid flow to increase the amount of stress applied to cells through geometric changes using an Eulerian-Lagrangian approach. With an Eulerian approach, the primary media, the fluid, is treated as a continuum. Alternatively, in a Lagrangian approach, the secondary media, the biological cells, are treated as discrete particles.

In our approach, the fluid domain is treated with an Eulerian approach and the cells with a Lagrangian approach. We first modified the existing Eulerian-Lagrangian approach to account for the fluid stress acting on the individual cells, and then we vary the overall microfluidic geometries to assess their impact on stress levels; a fibroblast was used as a model cell here. Figure 6-1 shows a microfluidic device with cell stresses under a controlled geometry.

#### **6.2.1 Eulerian Approach**

The software used in this study was the custom Particle-Surface Tribology Analysis Code (P-STAC), a computational tool developed to create multiphysics CFD simulations of multiphase (fluid-particle) systems [37, 68-70]. To model the fluid phase in the domain, the Navier-Stokes momentum equations, Eq. 6-1 through 6-4, were approximated using the Chorin projection method [71-73]. In the Navier Stokes equation, *g* is the gravitational acceleration, *t* is time,  $\rho$  is density,  $\mu$  is the fluid viscosity, *u*, *v*, and *w* are the fluid velocities in the *x*, *y*, and *z* directions, respectively. In the momentum equations, Eq. 6-1 through 6-3, velocity components are solved using an Euler time-stepping algorithm. In the continuity equation (Eq. 6-4), pressure is solved using successive over-relaxation (SOR) and the pressure and velocity are coupled together.

$$\rho\left(\frac{\partial u}{\partial t} + u\frac{\partial u}{\partial x} + v\frac{\partial u}{\partial y} + w\frac{\partial u}{\partial z}\right) = -\frac{\partial p}{\partial x} + \mu\left(\frac{\partial^2 u}{\partial x^2} + \frac{\partial^2 u}{\partial y^2} + \frac{\partial^2 u}{\partial z^2}\right) + \rho g_x \qquad 6-1$$

$$\rho\left(\frac{\partial v}{\partial t} + u\frac{\partial v}{\partial x} + v\frac{\partial v}{\partial y} + w\frac{\partial v}{\partial z}\right) = -\frac{\partial p}{\partial y} + \mu\left(\frac{\partial^2 v}{\partial x^2} + \frac{\partial^2 v}{\partial y^2} + \frac{\partial^2 v}{\partial z^2}\right) + \rho g_y \qquad 6-2$$

$$\rho\left(\frac{\partial w}{\partial t} + u\frac{\partial w}{\partial x} + v\frac{\partial w}{\partial y} + w\frac{\partial w}{\partial z}\right) = -\frac{\partial p}{\partial z} + \mu\left(\frac{\partial^2 w}{\partial x^2} + \frac{\partial^2 w}{\partial y^2} + \frac{\partial^2 w}{\partial z^2}\right) + \rho g_z \qquad 6-3$$

$$\frac{\partial u}{\partial x} + \frac{\partial v}{\partial y} + \frac{\partial w}{\partial z} = 0$$
<sup>6-4</sup>

The fluid domain is discretised using a finite-difference method. Rectangular mesh geometries that were smaller than the particle size were chosen for the fluid domain (Fig. 6-1a). The boundary conditions consist of no-slip on the top and bottom walls (y-direction), forward and backward walls (z-direction); velocity inlet on the left wall (x-direction); and a pressure outlet on the right wall (x-direction). All simulations have an inlet velocity of 10mm/s and maintain a Reynolds number less than 1 with consistent fluid and cell properties (Table 6-1) with microfluidic dimensions of 100  $\mu$ m x 25  $\mu$ m x 25  $\mu$ m. The right wall's pressure was set to 0 Pa resulting in a pressure distribution across the length of the microfluidic system (Fig. 6-1b). The velocity streamlines (Fig. 6-1c) and cell stresses (Fig. 6-1d) reveal heterogeneous responses through our microfluidic systems.

Table 6-1 Properties of the Cells, Fluid, and Channel Walls within P-STAC for Studying Geometric Effects in Microfluidic Channels

<u>Cell Properties</u>					Fluid Properties		<u>Channel</u> <u>Walls</u>
Radius	Solid	Mass	Density	Elastic	Density	Viscosity	Spring
	Fraction			Modulus			Coefficient
4.22.000	50/	27, 10-12 ~	0.0011	7500 Pa	9.8	0.4 mDa.a	100 N/mm
4.22 μm	570	27×10 - g	g/mm <sup>3</sup>	[74]	kg/mm <sup>3</sup>	0.4 111 a's	100 N/IIIII

#### 6.2.2 Lagrangian Approach

The cells are modelled as spheres within this framework based on their tendency to become spherical when not attached to a substrate [75]. The Lagrangian phase of the microfluidic channel consists of only the cells and the discrete element method was used to computationally model collisions between cells and the walls of the channel using a spring-dashpot model (Eq. 6-5) where F is the force applied to the cell,  $K_{spring}$  is the spring constant of the cell, d is the spring compression distance,  $V_n$  is the relative normal velocity of the colliding cells, and  $B_{dashpot}$  is the damping coefficient. The spring constant was calculated using Equation 6-6 based on the assumption of Hertzian contact where only small deflections (d = 10%) are experienced. Here,  $E^*$  represents the Young's Modulus (or elastic modulus) and R' is the reduced radius.

$$\vec{F} = K_{spring} \times \vec{d} - \overrightarrow{V_n} \times B_{dashpot}$$
 6-5

$$K_{spring} = \frac{4E^*}{3\sqrt{R'}} \times d^{3/2}$$
 6-6

$$\frac{1}{E^*} = \frac{1 - v_1^2}{E_1} + \frac{1 - v_2^2}{E_2}$$
 6-7

$$\frac{1}{R'} = \frac{1}{R_1} + \frac{1}{R_2}$$
 6-8

$$B_{dashpot} = \sqrt{\frac{K_{spring}}{mass}}$$
6-9

$$dT = C \sqrt{(mass/K_{spring})}$$
 6-10

The P-STAC framework checks for collisions at each time step, which is set before the initiation of the simulation. To determine the time step (dT), a general spring-mass system is used as shown in Equation 6-10 where the constant *C* is 0.2 [76].

#### 6.2.3 Stokes Approach to Coupling

Calculating each cell's stresses uses the coupling of the fluid and solid domains through a Stokes assumption. The Stokes assumption is used for domains where the flow is laminar, which is the case in our microfluidics approach. The Stokes drag force (Eq. 6-11) represents the effect of the fluid on the fibroblasts and is calculated for x, y, and zdirections. Here  $\mu$  is viscosity, r is the radius of the cell, and v is the cell velocity in relation to the fluid velocity. The cell's new position is calculated from the Stokes drag and using Newton's 2nd law [37].

$$F_{Stokes Drag} = 6\pi\mu r\nu \qquad \qquad 6-11$$

In the calculation of the stress on the biological cells, the comparative stress theory allows us to utilize the fluid viscous stresses and apply them to the cells using von Mises yield criterion, which results in the overall stress as shown in Equation 6-14 [71, 77]. Here, the viscous stresses and the normal stresses are maximized. In these equations *p* is pressure,  $\lambda$  is a thermodynamic material constant of viscosity,  $\tau$  is the viscous part of the stress tensor  $\sigma$ , and  $\delta$  is the strain tensor. This stress is applied to the cells resulting in stress on individual cells (Fig. 6-1d).

The current model predicts the stress experienced by cells in microfluidic channels, although the model does not have two way particle-fluid coupling. As a result, the fluid affects the cells but the cells do not affect the fluid in this model. However, this model provides insight for understanding how the physics-based movement of cells to different locations of the microfluidic channel can affect the stress that the cells experience.

$$\boldsymbol{\sigma} := -\boldsymbol{p}\boldsymbol{I} + \boldsymbol{\tau} := (-\boldsymbol{p} + \lambda d\boldsymbol{i}\boldsymbol{v}\boldsymbol{\vec{u}})\boldsymbol{I} + 2\boldsymbol{\mu}\boldsymbol{\delta}$$
 6-12

$$\boldsymbol{\delta} := \frac{1}{2} \left[ \left( \frac{\partial u_i}{\partial x_j} + \frac{\partial u_j}{\partial x_i} \right) \right]_{i,j=1,2,3}$$
 6-13

$$\boldsymbol{\sigma}_{scalar} = \left[\frac{1}{6}\sum \left(\boldsymbol{\sigma}_{ii} - \boldsymbol{\sigma}_{jj}\right)^2 + \sum \boldsymbol{\sigma}_{ij}^2\right]^{\frac{1}{2}}$$
 6-14



Figure 6-2 The stresses on the cells as they move through the channel geometry. Cells moving through microfluidic channels with a controlled geometry due to a constriction (grey) have different stresses at each time point: a)  $t=5.1 \times 10^{-6}$  s, b)  $t=6.4 \times 10^{-6}$  s, c)  $t=7.71\times 10^{-6}$  s, and d)  $t=9.0\times 10^{-6}$  s. e) The maximum stress experienced at a given time for the cells.

#### **6.3 Results and Discussion**

To study how cells are stressed when altering the geometric environment of the microfluidics, controlled configurations were implemented and evaluated using P-STAC. The fluid field was allowed to reach quasi-steady state before the particle dynamics of the cells were simulated. Additionally, the CFD solver was suspended once steady state was reached in order to decrease computational time. Figure 6-2 shows how the von Mises stresses on the cells altered over time as the cells individually pass through the constricted channel geometry. The von Mises stress levels (whose magnitude in Pa) is indicated by the color of the cells themselves. A snapshot was taken at designated times to compare the maximum amount of stress experienced by the cells within that time domain. As the cells approach areas of constriction, their stress levels increase. This is attributed to the fluid velocity (whose magnitude in mm/s is indicated by channel color) increasing relative to the cell's velocity, which induces high shear stress on the cells.

The stress on each cell was directly correlated to the geometry in the microfluidic system. To investigate this, we designed configurations of the microfluidics and computationally analysed the cell stresses that resulted (Fig. 6-3). As each grey obstruction block is 25% of the channel height, geometries in 6-3c, 6-3d, and 6-3e have flow that is constricted by 50%.

The stresses of each of the cells are shown in the histogram plots for each respective geometry (Fig. 6-3-right side panels). Each cell is individually tracked as labelled in Figure 3 and maintain the same identifying number in each histogram. These cells were started at
the same point of each simulation. The two cells that are at the end of the channel were not accounted for in these plots and are discarded to the right of the channel because the time step utilized was too large for the discrete element modelling to capture their movement. By doing this, we were able to decrease the computational time while accurately simulating the other particles in the flow [22].

From the computational model, there is the least amount of stress on the cells when there is no obstruction present. Although the obstruction free microfluidic design has a high fluid velocity of 16.5 mm/s throughout most of the channel, the cells' velocities are near that of the fluid as discussed previously (equation 6-11). All of the obstructioncontaining geometries allowed for the majority of the cells to be stressed above 80% of the maximum stress experienced within the simulation. This is important when a configuration with the maximum amount of cells stressed is desired.



Figure 6-3 Controlled geometries for our microfluidic systems were designed and evaluated to understand cell stress response. The geometries included a) No obstruction, b) Single square obstructions blocking 25% of the fluid flow, c) Mirror square obstructions on opposite walls blocking 50% of the fluid flow, d) Mirror square obstructions spaced across the channel width blocking 50% of the fluid flow, and e) periodically alternating columns of obstacles spaced across the channel width blocking 50% of the fluid flow, and e) maximum stress experienced throughout the simulation for each cell.

The periodically alternating columns of obstacles configuration (shown in Fig 6-3e) produced the greatest amount of stress on the cells with greater than 175 times increase over the no obstruction case (Fig. 6-4). This seems to be due to both the constriction size and the repeated offset locations of the obstructions in the flow. Additionally, adding an offset row of these spaced obstructions increases the stress values.



Figure 6-4 The maximum stress experienced by cells in each microfluidic configuration studied.

Along with studying the obstructions' effects on stress within a microfluidic channel, we examined how other parameters would affect the amount of stress on the cells. The concentration of the cells within the media, fluid velocity, fluid density, and fluid viscosity were varied (Fig. 6-5). The velocity and viscosity are the only parameters that seem to dramatically affect the cell's stress level. Because of the no-slip boundary conditions (i.e., the fluid velocity at the walls is zero), there are greater velocity gradients

within the channel as the input velocity increases, and the stresses on the cells will increase as either the velocity gradients increase or the fluid viscosity. While increases in the concentration of cells has a moderately proportional impact on the cell stress, the changes in fluid density seems to affect all the viscous stresses the same so that there is no net change.



Figure 6-5 Results of a parametric study of various parameters on cell stress. The effects of a) Cell concentration, b) Fluid velocity, c) Fluid density, and d) Fluid viscosity were examined.

#### **6.4 Conclusions**

To investigate the stress effects on cells by changing the geometrical configurations of a microfluidic channel, an in-house computational tool, which includes fluid dynamics, particle dynamics, and solid mechanics, was modified to incorporate a modified von Mises stress. The modified von Mises stress enables one to quantify and track the effect of the surrounding fluid flow field on each moving cells. After testing various geometries, the flow configuration that produced the most amount of stress on the cells was the periodically alternating columns of obstacles geometry, which showed a tremendous increase in the maximum stress. Having multiple obstructions even of the same configuration also increased the stress. This work is advantageous because we can begin to characterize effects of stress on cell-like material in microfluidic environments while virtually testing the system before conducting expensive and time-consuming experimentation. In addition to studying the resulting stress on the cell, we can change the fluid environment and type of cells within the computational domain to optimize experiments within the laboratory. The results of these findings will be of great interest to researchers and industry working in lab-on-a-chip, cell mechanics, and fluid flow arenas.

# 7. Correlation of Microfluidic Modeling to Experiments for Passive Mechanical Lysing

As discussed in previous sections, microfluidic devices have many applications in a variety of fields, including studying the flow of cells in various flow conditions. In this section, the P-STAC model will be used to aid in the design of a microfluidic device that will lyse microalgal cells. The Bodipy lipophilic marker and photospectrometry will be used to help determine the percentage of synthetic cells that are lysed. Prior to utilizing microalgae, fluorescent microbeads and liposomes, containing calcein, were used to validate the flow patterns and photospectrometry analysis capabilities for the various configurations. Additionally, quantification of obstructions within flow was achieved in this chapter through the k-factor.

#### 7.1 Introduction

Microfluidics has been used to manipulate biological systems through shear flows [52] and to study lysis electrically with electroporation [53], enzymatically [54], and mechanically [55], among others. Mechanical lysing is important for both understanding diseases and developing commercial products in a diversity of areas. Biological systems can be manipulated with shear flow yet control over the mechanical perturbations can be very important as it pertains to energy inefficiencies associated with mechanically lysing cells. Some work has been done on lysis of microalgae in microfluidics [78], but the majority of the works do not combine microfluidics for the purpose of lysing, but for environmentally stressing them to produce more lipids. [79, 80] A review by Schaap et al 2012 shows lab on a chip technologies for microalgae. [81] Most of the technologies focus on three key areas, which include detection of algae, growth and manipulation of algae, and products that can be created from algae.

#### 7.2 Fabrication of Microfluidic Channels

Microfluidic devices have been fabricated using two techniques within the scope of this work, 3D printing and soft lithography. Soft lithography, as described in section 2, is a set of lithographic techniques, which utilize elastomeric materials, most notably, polydimethylsiloxane (PDMS). Within the family of soft lithography, photolithography and deep reactive-ion etching (DRIE) were utilized to fabricate the microfluidic channels.

The first technique utilized was 3D printing. The advantage of using the 3D printer for making master molds to perform the soft lithography is that the devices can rapidly be fabricated on campus. However, the issue with using 3D printed molds is that the lowest resolution available is 100  $\mu$ m. For features that would effectively stress the cells, I would need a device with greater than 100  $\mu$ m resolution. The 3D printed microfluidic device is shown below in Figure 7-1.



Figure 7-1 a) Microfluidic channels fabricated using a 3D printer to create the master mold for soft lithography. The blue channel had a measurement of  $300\mu m$  constriction and its brighfield image at the constriction is shown in in (b). The green channel had a measurement of  $100\mu m$  constriction and its brighfield image at the constriction is shown in in (c). The pink channel had a measurement of  $200\mu m$  constriction and its brighfield image at the constriction is shown in in (c).

The second technique used was soft lithography. The soft lithography technique utilized for the first set of channels was photolithography. Photolithography is a microfabrication method which uses light, namely UV, and light sensitive photoresist on a silicon wafer to create a desired pattern. This process also requires masks to be made using transparencies. The transparencies were designed using AutoCAD and featured rectangular channels with the smallest constriction being 25µm and elements of 15µm. The channel types include a straight channel, stepped channels, staggered channels, and a channel with

a rapidly expanding outlet. The first channel to be fabricated was the straight channel in the MEMS lab at CMU. After exposing the SU-8 covered silicon wafer while the mask was applied, the master mold for the channels were created for the next step of PDMS curing. The resulting wafer is shown in Figure 7-2. It can be seen that the first channels fabricated did not turn out as expected. The waviness of the channel is attributed to too aggressive residual rinsing of the SU-8 developer and drying with air. The white residue is an indication of underdevelopment of the SU-8.

The next set of channels fabricated with photolithography was made in the NanoFab lab at CMU's ECE department. The wafer had the desired channel molds to move forward with creating the PDMS device. The wafer, device and microscopic image are below (Fig. 6-3). The channel dimensions are 50mm x  $25\mu$ m x  $13\mu$ m. For devices with such long x-dimensions, or in this case, high aspect rations, large pressures to force the fluid forward are required to overcome the flow resistance. Manual pumping, via a syringe, is difficult and, therefore, a syringe pump must be utilized.



Figure 7- 2 The first wafer to be used as a master mold was not successful. The channel was not straight due to the amount of force used in rinsing and drying the wafer after the SU-8 was exposed. Also, the white residue denotes the wafer SU-8 was underdeveloped.



Figure 7-3 a) The wafer with the straight channel master mold is pictured. b) The microscopic image of the straight channel. The red arrows show where the channel is on the wafer and microscopic image. c) The completed device was not successful in flowing fluid through the channel manually with a syringe because of the great amount of constant, high pressure required to flow through a channel of that length.

This can be explained with the following Darcy-Weisbach equation shown below. It states that as the length of the channel increases, the greater the pressure must become to achieve flow.

$$\Delta p = \frac{\rho f L v^2}{2D}$$
 7-1

where  $\Delta p$  is the pressure drop across the pipe;  $\rho$  is density; *f* is the friction coefficient; *L* is the length of the pipe; *v* is the velocity; and *D* is the internal channel diameter. Knowing this and the results from Chapter 5 with the configuration shape and spacing parametric studies, a new mask was created as shown below in Figure 7-4. Each channel is 0.5 inches (12700 µm) long with a 100 µm middle section resembling those simulated in Chapter 6. Each letter represents a different configuration. The defining feature of each channel is shown in Figure 7-5.



Figure 7-4 A mask was designed using AutoCAD to fabricate a master mold from a silicon wafer.



Figure 7-5 Microfluidic configurations designed in AutoCAD to lyse cells. These configurations were chosen based on preliminary studies conducted in Chapter 5.

In order to achieve these fluidic designs on this scale, experimentally, deep reactive ion etching (DRIE) – another soft lithography method – was used. DRIE, also known as plasma etching, is a desired technique due to its narrow gap, high aspect ratios, and shape freedom capabilities. To begin using this process a new mask was created using the designs shown in Figure 7-5. The mask was first drawn in AutoCAD then transferred from virtual to physical form on a Heidelberg DWL 66FS using soda lime glass as the substrate. After the mask was created, photolithography was used to transfer the design. This was done first by applying AZ 4110 positive photoresist onto the wafer and spinning it down to the desired thickness. Then a Karl Suss MA6 Contact Aligner was used to press and align the wafer against the mask. The photoresist covered wafer was exposed to UV light for 50 seconds and immediately developed in AZ 400K developer and water at a one to four ratio.

After the photolithography phase of DRIE, the Bosch process was utilized using a STS Multiplex ICP RIE. Here, sulfur hexafluoride (SF<sub>6</sub>) and oxygen (O<sub>2</sub>) etch the silicon surface for 12 seconds and octafluorocyclobutane (C<sub>4</sub>F<sub>8</sub>) passivized the surface for 8 seconds. The passivation step is essential in etching as it prevents undesired lateral etching, since vertical etching occurs in a dishing, or bowl-shaped, manner as shown in Figure 7-6. Similar DRIE processes are more detailed in the literature. [82, 83] With the aforementioned etching/passivation recipe, the etch rate is about 1.2 microns per minute and was verified using a KLA Tencor P-15 Profilometer.



Figure 7-6 Deep Reactive Ion Etching (DRIE) process steps. In order to etch a custom pattern from a mask into a silicon wafer, DRIE was used. The masking layer protects certain areas of the Si wafer from being etched. SF<sub>6</sub> is used to etch the wafer and it does so in a dishing fashion. Therefore, a passivation step with  $C_4F_8$  is needed to prevent lateral etching. This Bosch process continues until the desired etch distance (*z*) is achieved.

#### 7.3 Microfluidic Flow Configurations with Fluorescent Beads and Liposomes

#### Fluorescent Beads

The microfluidic channels with the flow configurations shown in Chapter 6 were utilized to study microscopic fluorescent beads, liposomes, and microalgae. The 1 $\mu$ m fluorescent beads were the starting point for the investigation of the fabrication of the microfluidic channels. Not only was there flow within the channel, but the streamlines were visible at proper exposure rates on the Zeiss Axiovert 200 microscope's Andor Zyla 5.5 camera and complementary software (Fig. 7-7).



Figure 7-7 Streamlines using fluorescent beads in a microfluidic channel. The geometry shown is configuration E (see Fig.7-5). These streamlines show that the obstruction is not the same z-distance over its height allowing these  $1\mu$ m particles to squeeze through the gaps.

The streamlines produced in this configuration (E) appear to pass over the obstruction instead of going around it. Some possible reasons for this include an uneven etch within the DRIE process; PDMS may not have been able to penetrate the small etched area of the wafer to create the obstruction properly; and a great amount of force pressing against the PDMS obstruction, making it difficult for the structure to withstand it. Potential solutions include using profilometry to endure the constant etching of the wafer and refabricating the wafer if necessary; and choosing a different PDMS curing agent to polymer ratio.

Flow testing with the fluorescent beads in the microfluidic channels occurred on all configurations. Initially some clogging did occur due to the coagulation of the beads, but the addition of TritonX and sonication of the mixture proved to alleviate any clumping prior to configuration constriction. Once testing with the beads was completed, liposome testing commenced.

#### Liposomes

Liposomes are spherical vesicles composed of lipids, or fats, which can be used in a variety of applications including drug delivery, wound healing, and gene transfer. [84-86] These "fat bodies" can be tailored for specific applications as they have tunable properties such as size, permeability, and rigidity. [87] [88] Therefore, liposomes are of interest in this work because of the amount of control one has over their formation, making building a computational model around them more all-inclusive in comparison to more dynamic cell types.

Dipalmitoylphosphatidylcholine (DPPC) lipids (Fig. 7-8A) were utilized to create liposomes for analysis of the microfluidic configurations. The liposomes were created with a simple 2-step process depicted in Figure 7-8B. The first step is dehydration of the lipids. The liposomes are suspended in chloroform and must be dehydrated using a desiccator and fume hood. Doing this creates an ordered film arrangement where the tails are attracted to each other and the heads are attracted to one another [89, 90]. Next is the dispersion step in which deionized water is introduced to the lipid films creating the lipid vesicles. The polarity of the water causes the lipid layers to separate in an orderly fashion from the layer and encapsulate water droplets. The vesicles formed vary in size, typically between 2 and  $10\mu$ m. For these experiments, calcein – a cell-permeant, viability, assay stain – was added to the dH<sub>2</sub>O to cause them to fluoresce (Fig. 7-8D) for the microfluidic testing and rupture analysis.



Figure 7-8 DPPC liposome creation and imaging. A) Molecular structure of DPPC lipids featuring the two hydrophobic tails and a hydrophilic head. B) The liposomes are created in a 2-step process which includes dehydrating the liposomes to form an ordered layer and rehydrating them with deionized water containing calcein. (Fig. 7-8B compliments of Kyle Justus). The calcein allows liposomes (brightfield image - C) to fluoresce during imaging as shown in D.

After the liposomes have flown through the channel, the solution is collected and is filtered using a Millipore 0.22µm filter. Then, it is plated for spectrophotometry. Similar to what was done in chapter 2, spectrophotometry was used to determine the percentage of liposomes that were lysed, as calcein would remain after liposome rupture and filtering. Instead of measuring the absorbance as what was done before, fluorescence intensity was measured. This is because the absorbance of the solution was too small to differentiate any differences between them at the emission and excitation wavelengths. Intensity, like absorbance, correlates to the concentration of the emitting species as shown in the Beer-Lambert law shown in Equation 7-2.

$$A = \log_{10}\left(\frac{l_0}{I}\right) = \varepsilon lc$$
<sup>7-2</sup>

Here, *A* is absorbance, *I* is transmitted intensity,  $I_0$  is the incident intensity,  $\varepsilon$  is the molar absorptivity (or molar extinction coefficient), *l* is absorption path length, and *c* is the concentration of the emitting species. In order to measure the intensity of the calcein remaining in the solution, the emission and excitation wavelengths must be known, around 515 nm and 480 nm respectively as shown in Figure 7-9. [91]

The first group of liposomes were part of a dilution series, similar to which was done in Chapter 2. The liposomes were diluted with deionized water (dH<sub>2</sub>O). Figure 7-10 shows that the solution with 100% liposomes has the highest relative fluorescence units (RFU) which indicates that it has the most fluorescence compared to the other diluted samples. Here our controls were the blank well and a solution that had no liposomes but was only dH<sub>2</sub>O.



Figure 7-9 Fluorescence spectra for calcein as reported by supplier, ThermoFischer Scientific. Here the excitation curve is in blue, while the emission curve is shown as red. Recreated from ThermoFischer Scientific Data Sheet.



## **Liposome Dilution Intensities**

Figure 7-10 Liposome dilution series. DPPC liposomes were diluted and a photospetrometer was used to measure the intensity of the calcein intensity indicating the presence of the stain. This aided in the validation that method of measuring lysis later on will be captured using the plate reader.

The second group of liposomes went through the respective microfluidic channel configurations followed by the 0.22µm filter. The results are shown in Figure 7-11. The controls for this experiment are similar to those done in the dilution series: dH<sub>2</sub>O, blank well, and 100% liposome solution that did not go through any channel. Based on the RFU values, it appears that of the channel configurations, configuration F disrupted the most cells. Meanwhile the configuration without any obstructions (A) and the single obstruction configuration (B) both had similar, low RFU values at 44709 and 40449 respectively. As anticipated, the liposomes that experienced "no shear" did not flow through any microfluidic channel and therefore had a lower RFU value than any set of liposomes that experienced shear.



Figure 7-11 Liposomes were subjected to stress within the different microfluidic configurations. The resulting fluorescence intensities were captured using the photospectrometer. Configuration F had the greatest RFU value indicating that those liposomes experienced the greatest amount of lysis. Error bars represent standard deviation (n=3). This chart is organized by increasing RFU value.

To ensure that the filter did not affect our results, an additional study was conducted with calcein, liposomes containing calcein, and  $dH_2O$ . The ratio of calcein to  $dH_2O$  is approximately the same as the ratio used to fill the liposomes. As seen in Figure 7-12, the filter has little effect on the RFU values. It is also apparent that the filter is able to capture the liposomes that have not been ruptured allowing only the media to pass.



Filter has little effect on RFU values

Figure 7- 12 Chart showing the effect of filtering liposomes after flowing through the microfluidic channels.

#### 7.4 P-STAC and Microfluidic Experiment Correlation

Similar to Chapter 6, P-STAC was used to determine the effect of flow geometries on the stress on the cells. This stress ultimately could be used to be an indicator of cell lysis. From the computational model (n=5), configuration G exerted the greatest amount of stress on the particles flowing through the microfluidic channel, with configuration H, a close second with 11.46 Pa and 11.23 Pa, respectively. As expected, the configuration with no obstructions exerted very little stress on the particles (0.05 Pa).



**Computational Effects of Configurations** 

Figure 7-13 Results of P-STAC testing of configurations. Configuration G had the greatest amount of stress. The error bars represent standard deviation (n=5). This chart is organized by increasing k-factor.

In order to quantify the shapes within the channel, a parameter – the k-factor – was created. The k-factor takes into account the frontal aspect ratio of the obstructions  $(\lambda_f)$  [92], the total area of the channel  $(A_t)$ , percentages of constriction  $(c_p)$  and the frequency (f), and length (l) of the obstructions. The equation to calculate the k-factor  $(k_f)$  is shown below. Using the k-factor to quantify the configurations, we can use this as a tool to compare the computational and experimental models.

$$k_f = \lambda_f * \sum c_p * \left(\frac{f}{c_p}\right) * l$$
7-3



Figure 7-14 Schematic to determine the k-factor for a configuration. As the Poiseuille flow travels through the channel from left to right, so does the determination of f. An "occurrence" (f) happens each time the dotted line passes a group of obstructions. Other geometry parameters are shown.

Configuration	$\lambda_{f}$	k-factor
А	0	0
D	0.0625	0.0208
С	0.125	0.125
В	0.125	0.25
F	0.125	0.5
E	0.1875	0.5625
G	0.25	1
Н	0.375	1.5
I	0.5625	1.6875

Table 7-1 Table of k-factors for each configuration listed in increasing order.



# Comparing P-STAC and Experiments using the k-factor

Figure 7- 15 Comparison of microfluidic experiments and computational simulations measured in intensity and stress, respectively. The configuration is listed on top of the chart for the respective k-factor values.

When comparing these experimental results from the liposomes to those from the computational models, we can see a general upward trend, as seen in Figure 7-15. As the k-factor increases, the stress or lysis level increases, which is what one would expect. The correlation coefficient for the experimental and computational data is 0.78 as described by

the Pearson product moment correlation coefficient or Pearson's r. To determine this value Equation 7-5 was used where  $\bar{x}$  and  $\bar{y}$  are the sample means for the respective data sets. R-values fall between -1 and +1 where a 1 is a total positive correlation, -1 is a total negative correlation, and zero is no correlation at all. This means that lysis (determined by the experiment) and stress (determined from the computational model) have a positive correlation as determined by Pearson's r.

$$r = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}}$$
7-5

The k-factor positively correlated with R-values of 0.63 and 0.93 for the experiment and computational model respectively. This suggests that the k-factor is a good agent to quantify the configurations in cell stress and lysis.

Given the 0.78 R-value, there are some discrepancies between the qualitative trends of the computational model and the experimental results. For example, the model shows configurations E and F ( $k_f = 0.56$  and 0.50, respectively) are less effective when compared to the other configurations when evaluated against the experimental results. Also, configuration D ( $k_f = 0.02$ ) seems to be much higher in fluorescence intensity, or RFU, than its computational model. Possible modes of error in the experiment include rubber tubing expanding and leakage in the tubing due to the high pressure.

#### 7.5 Conclusions

The geometries and resolution required greatly dictated the fabrication techniques utilized. 3D printing, photolithography, and deep reactive ion etching (DRIE) were explored. Initially, the channels designed were either too long or wide to lyse the cells. Ultimately, DRIE was chosen to fabricate channels for the exploration of lysis in microfluidic channels because of its high resolution and fabrication costs. The channels designed for experiments were chosen as a result of the parametric studies within Chapter 5.

Within this chapter, fluorescent beads and liposomes assisted in understanding how different flow configurations affect the stress and lysis of the particles in computational model and experimental results, respectively. During experimentation, some leakage at the syringe-hose interface occurred towards the end of the various runs due to the buildup of high pressures within the channel. However, I do not believe that this affected the effect of the configurations in rupturing the liposomes.

Using photospectometry in combination with the microfluidics, I was able to quantify lysis via fluorescence intensity of calcein, a fluorescent dye within the liposomes. In comparing the model with experiments, the k-factor was created to quantify the complexity of the configurations and their obstructions. For the liposomes, as the k-factor increased, so did the lysis levels of the particles, resulting in a Pearson's R-value of 0.75.

### 8. Conclusions and Future Work

The goal of this work was to design microfluidic devices that passively lyse microalgal cells mechanically which would be comparable across computational and experimental scales. By understanding microalgal lysis at small scales, the concept could be applied for large scale production of biofuel.

First, mechanical properties of microalgal strain *Scenedesmus dimorphus* was explored using atomic force microscopy. Properly selecting the cantilever was crucial to the accuracy of the AFM measurements. I was able to obtain the Young's modulus, or elastic modulus, in its hydrated and dehydrated states (2.21 MPa and 57.96 MPa respectively). This modulus value was used within P-STAC to model microalgal cells. Additional work in this area would include the elucidation of other microalgal properties, namely the strength of the cell in its hydrated and dehydrated states. These properties (elastic modulus and strength) would also be useful if obtained for liposomes.

Secondly, shear tests were conducted to reveal shearing the cells is an effective method of lysing microalgal cells. This compared well with the literature and pressed the work in a direction that capitalized on stressing the cells via microfluidics. This is the opposite of what is done in hematology. In hematology, reduction of stress to eliminate hemolysis is the primary goal. Additionally, in section 4.2, the two mathematical models were introduced and were expounded upon in section 5.2 with the computational model preliminary results. An investigation into these models with P-STAC beginning with a couette flow chamber should be conducted along with a parametric study of channel configurations for microfluidic devices. This will help to understand which lysis approach is more accurate.

Thirdly, computational parametric studies were conducted to aid in the preliminary microfluidic channel design of the experimental studies. These studies varied size, shape, and spacing of the obstruction. Additionally, various velocities, viscosities, concentrations, and densities of the fluid within the channel were evaluated. Ultimately, it was determined that the fluid velocity has the greatest impact of the parameters within the parametric study. Also, the constriction of the flow path plays a great part in the stress experienced by the particles within the flow – not the shape of the obstruction. By increasing the stress on the particles, we increase the amount of lysis within the device.

With the computational model guiding the experimental microfluidic designs, specific flow configurations were fabricated. After exploring various fabrication techniques, photolithography and deep reactive ion etching (DRIE) were utilized to create the channels on a silicon wafer, which would be used to mold PDMS. To test the viability of the channels, fluorescent microbeads were used. Afterwards, liposomes treated with calcein were created and flown through the channels to monitor the lysing ability of the channels. Tunable properties of liposomes were beneficial to this work as this relieved

much of the variability when making the computational model. To quantify the amount of liposome rupture, spectrophotometry was used to measure the fluorescence intensity of the expelled calcein. At the conclusion of the spectrophotometry, the flow geometry with the greatest lysing effect was configuration F – mirror square obstructions spaced across the channel width blocking 50% of the fluid flow. Although configuration F was not the most intricate design, it may have been more effective experimentally due to effects not captured in the computational model, like clogging or clumping of the liposomes.

To better understand the channel geometries, a k-factor was developed to quantify characteristics of the obstructions. Those characteristics include constriction percentages, frequency, and density of the obstructions. The k-factor positively correlated to both the experiments and computation data having Pearson's R-values of 0.63 and 0.93, respectively.

A few of the simulation and experimental results did not qualitatively correlate perfectly but had a positive Pearson's R-value was 0.78. With these findings, greater improvements could be made to the computational code that would assist in capturing the elastic behavior of biological samples. Additionally, computational time must be drastically improved for the model to be efficient for more complex designs while having the resolution required to understand all of the phenomena occurring to the fluid and the particles within the flow. Also, as the liposomes vary in size from 2 to  $10\mu m$ , a computational model taking that into account would also aid in more accurate results.

The microfluidic channels were specifically designed to lyse microalgal cells for biofuel applications. However, some clogging of the channel did occur unparalleled to that experienced with the fluorescent beads or liposomes. This created a greater buildup of pressure and significant leakage from the rubber tubing, making testing of the complicated channels more difficult. The non-spherical nature of the microalgae played a large role in the clogging of of the channels, and ultimately, decreased the lysis potential. Similar to Chapter 2, Bodipy 505/515 was used to stain the lipids of the microalgae. The excitation and emission wavelengths were 480 nm and 590 nm, respectively. [93] For the geometries tested (A, B, C, D, and F), fluorescence intensities were obtained, but only for one test. Therefore, no conclusions can be drawn at this time for that data. Additional testing is necessary in order to make any claims on microfluidic lysis for microalgae.

Another phase of this work would include conducting additional microfluidic experiments with microalgae for comparison to the liposomes. Doing this, one could compare the results to the liposome configuration results and to the computational model. I expect the liposome and microalgae experimental results to be similar, if not the same qualitatively. With P-STAC, I was able to simulate spherical particles making the the correlation to the experiments more plausible. Further advancement of P-STAC is required to capture the shape of the microalgae for better correlation data.

In addition to accounting for cell shape and simulation time, P-STAC could be greatly improved by a replicating the exact materials used in the experiements. This would include the material and dimensions of the channels, substrate, and tubing. Additionally, being able to input and account for all of the cell properties, like its viscoelasticity/shape deformation, would allow for a better chance of a quantitative agreement. Quantitatively comparing the experimental and computational models was difficult given the parameters we were able to gather from our analysis tools. Stress and fluorescence intensity are good measures for comparing the two, however, another quantitative method could be explored in order to obtain more information. In regards to quantification of the channel geometries, k-factor could be improved. Although it correlates well with the computational model with spherical particles (r = 0.93), it may not correlate well with the results from microalgae experiments based on their abnormal shape. I believe the particle shape, along with the various cell properties, dictate the overall lysing potential of the cell. This can be accomodated within the k-value for further exploration.

Furthermore, greater advancements could be made in the DRIE recipe for creating consistent obstruction geometries. As shown in Chapter 7, microfluidic beads were seen flowing on top of the obstruction instead of around it. This would indicate that the obstruction did not evenly spread across the channel. This could mean the wafer was not etched at a constant rate. Careful investigation on the DRIE recipe is necessary to fabricate obstructions on such a small scale with features on the size of 6µm.
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10. Appendix

# **10.1 Media Formulations**

These formulations were used for *Scenedesmus dimorphus* microalgae strain for use in biofuels.

They are adaptations from those from the University of Texas-Austin, Culture Collection

of Algae.

# **10.1.1 Proteose-Peptone Medium**

General purpose freshwater medium suitable for axenic cultures.

Directions:

For 1 L total pH ~6.8

1. Add proteose-peptone to Bristol Medium.

\*For 1.5% agar medium, add 15 g of agar into the flask; do not mix.

2. Cover and autoclave medium.

#	Component	Amo	unt Stock Solution Concentration	Final Concentration
1	Bristol Medium	1 I	L	
2	Proteose Peptone 211684)	(BD 1 g/	/L	

# **10.1.2 Bristol Medium**

H.C. Bold's modification of Bristol's recipe (Bold 1949). General purpose freshwater medium and as Bristol's solution, an essential component of other media.

### Directions:

For 1 L total

- To approximately 900 mL of dH<sub>2</sub>O add each of the components in the order specified while stirring continuously.
- 2. Bring total volume to 1 L with  $dH_2O$ .

\*For 1.5% agar medium, add 15 g of agar into the flask; do not mix.

- 3. Cover and autoclave medium.
- 4. Store at refrigerator temperature.

#	Component	Amount	Stock Solution Concentration	Final Concentration
1	NaNO <sub>3</sub> (Fisher BP360- 500)	10 mL/L	10 g/400mL dH2O 2.5g/100ml	2.94 mM
2	CaCl <sub>2</sub> •2H <sub>2</sub> O (Sigma C- 3881)	10 mL/L	1 g/400mL dH2O .25g/100ml	0.17 mM
3	MgSO <sub>4</sub> •7H <sub>2</sub> O (Sigma 230391)	10 mL/L	3 g/400mL dH2O .75g/100ml	0.3 mM
4	K <sub>2</sub> HPO <sub>4</sub> (Sigma P 3786)	10 mL/L	3 g/400mL dH2O .75g/100ml	0.43 mM
5	KH <sub>2</sub> PO <sub>4</sub> (Sigma P 0662)	10 mL/L	7 g/400mL dH2O 1.25g/100ml	1.29 mM
6	NaCl (Fisher S271-500)	10 mL/L	1 g/400mL dH2O .25g/100ml	0.43 mM

### 10.1.3 Modified Bold 3N Medium

Modification of Bold's recipe. General purpose freshwater medium used for axenic cultures, especially blue-greens and reds.

Directions:

For 1 L total; pH 6.2

- To approximately 850 mL of dH<sub>2</sub>O, add each of the components in the order specified (except vitamins) while stirring continuously.
- 2. Bring the total volume to 1 L with  $dH_2O$ .

\*For 1.5% agar medium, add 15 g of agar into the flask; do not mix.

- 3. Cover and autoclave medium.
- 4. When cooled add vitamins.

\*For agar medium, add vitamins, mix, and dispense before agar solidifies.

5. Store at refrigerator temperature.

#	Component	Amount	Stock Solution Concentration	<b>Final</b> Concentration		
1	NaNO <sub>3</sub> (Fisher BP360- 500)	30 mL/L	10 g/400mL dH2O	8.82 mM		
2	CaCl <sub>2</sub> •2H <sub>2</sub> O (Sigma C- 3881)	10 mL/L	1 g/400mL dH2O	0.17 mM		
3	MgSO4•7H2O (Sigma 230391)	10 mL/L	3 g/400mL dH2O	0.3 mM		
4	K <sub>2</sub> HPO <sub>4</sub> (Sigma P 3786)	10 mL/L	3 g/400mL dH2O	0.43 mM		
5	KH <sub>2</sub> PO <sub>4</sub> (Sigma P 0662)	10 mL/L	7 g/400mL dH2O	1.29 mM		
6	NaCl (Fisher S271- 500)	10 mL/L	1 g/400mL dH2O	0.43 mM		
7	P-IV Metal Solution	6 mL/L				
8	Soilwater: GR+ Medium	40 mL/L				
9	Vitamin B <sub>12</sub>	1 mL/L				
10	Biotin Vitamin Solution	1 mL/L				
11	Thiamine Vitamin Solution	1 mL/L				

# **10.1.4 P-IV Metal Solution**

This solution is an ingredient to make the Modified Bold solution.

Directions:

For 1 L total Note: Final concentration listed is for the stock solution.

1. To approximately 950 mL of  $dH_2O$ , add the nutrients in the order listed while stirring continuously.

Note: The Na<sub>2</sub>EDTA should be fully dissolved before adding other components.

- 2. Bring total volume to 1 L with dH<sub>2</sub>O.
- 3. Store at refrigerator temperature.

#	Component	Amount	Stock Solution Concentration	Final Concentration
1	Na <sub>2</sub> EDTA·2H <sub>2</sub> O (Sigma ED255)	0.75 g/L		2 mM
2	FeCl <sub>3</sub> •6H <sub>2</sub> O (Sigma 1513)	0.097 g/L		0.36 mM
3	MnCl <sub>2</sub> •4H <sub>2</sub> O (Baker 2540)	0.041 g/L		0.21 mM
4	ZnCl <sub>2</sub> (Sigma Z- 0152)	0.005 g/L		0.037 mM
5	CoCl <sub>2</sub> •6H <sub>2</sub> O (Sigma C-3169)	0.002 g/L		0.0084 mM
6	Na <sub>2</sub> MoO <sub>4</sub> •2H <sub>2</sub> O (J.T. Baker 3764)	0.004 g/L		0.017 mM

#### 10.1.5 Soilwater: GR+ Medium

The basic garden-type soilwater; includes a pinch of CaCO<sub>3</sub>, which is added to the soil and water prior to steaming; suitable for most phototrophic freshwater algae. Optional ingredients: add vitamin B<sub>12</sub> to cultures of *Volvox*; a pinch of NH<sub>4</sub>MgPO<sub>4</sub>. 6H<sub>2</sub>O added to soilwater cultures of *Botryococcus*, *Synechococcus* and some *Euglenoids* enhances growth and to LB 826 Gonium pectorale increases the numbers of 16-celled colonies formed.

### Directions:

For 200 mL total

- 1. Combine all components listed.
- Cover the medium container and steam for 2 consecutive days, 3 hours on each day. Pasteurization is a gradual rising of temperature to approximately 95°C in 15 minutes. Then increased just over 98°C for the 3 hour duration. Cooling occurs gradually at room temperature.
- 3. Refrigerate 24 hours or more and bring to room temperature before using.

#	Component	Amount	Final			
			Concentration	Concentration		
1	Green House Soil	1 tsp/200 mL dH <sub>2</sub> 0				
2	CaCO <sub>3</sub> (optional) (Fisher C 64)	1 mg/200 mL dH2O		0.05 mM		

#### **10.1.6 Green House Soil**

Prior to its use in soil-water media, treat soil in batches by placing it in a heat-resistant pan lined with aluminum foil, fill the soil to a so depth of <sup>1</sup>/<sub>4</sub> inch, and bake at 150°C for 2 hours. After it cools, cover the pan with aluminum foil and store in darkness at room temperature. Avoid excessive moisture during storage.

#### Considerations:

- 1. The soil should be a loam, with a mixture of particle sizes (sand, silt, clay).
- It should contain a moderate amount (15 20%) of very-well-decomposed organic matter.
- 3. It must not contain pesticides, especially herbicides.
- 4. It should be soil that has been aged (preferably for 6 months or more) under moist conditions and not, for example, fresh potting soil, soil that contains fresh manure, or soil to which a commercial fertilizer was recently applied.
- 5. A slightly acidic soil derived from granite or other igneous rock is preferable to soil obtained from calcareous soils. Calcium carbonate can be added to the soilwater medium when it is prepared if a slightly alkaline medium is required.
- 6. Particulate matter in the soil such as gravel, Perlite, or vermiculite are not necessarily damaging but can be of considerable nuisance when wishing to quantitate the amount of soil used in the medium or when handling algae that are physically associated with the soil. Particulate organic matter, such as compost that is only partially degraded, should be avoided altogether.

### **10.1.7 Vitamin B**<sub>12</sub>

The vitamin  $B_{12}$  formulation is to be used in the Modified Bold solution. Make sure to add this AFTER the autoclave step to ensure the vitamins are not denatured.

### Directions:

For 200 mL total

- 1. Prepare 200 mL of HEPES buffer (50 mM).
- 2. Adjust the pH to 7.8.
- 3. Add Vitamin  $B_{12}$  (0.1 mM) wait until fully dissolved.
- 4. Sterilize by 0.45 μm Millipore filter. Store in dark at freezer temperature.
  \*\* The amount of vitamins added can vary from medium to medium so the final concentration is not listed.

#	Component	Amount	Stock Solution	Final
			Concentration	Concentration
1	HEPES buffer pH 7.8 (Sigma H-3375)	2.4 g/200 mL dH2O		
2	Vitamin B <sub>12</sub> (cyanocobalamin, (Sigma V-6629)	0.027 g/200 mL dH2O		

# **10.1.8 Biotin Vitamin Solution**

The Biotin vitamin formulation is to be used in the Modified Bold solution. Make sure to add this AFTER the autoclave step to ensure the vitamins are not denatured.

Directions:

For 200 mL total

- 1. Prepare 200 mL of HEPES buffer (50 mM).
- 2. Adjust the pH to 7.8.
- 3. Add biotin (0.1 mM) wait until fully dissolved.
- 4. Sterilize by 0.45 μm Millipore filter. Store in dark at freezer temperature.
  \*\* The amount of vitamins added can vary from medium to medium so the final concentration is not listed.

#	Component	Amount	Stock Solution	Final
			Concentration	Concentration
1	HEPES buffer pH 7.8 (Sigma H- 3375)	2.4 g/200 mL dH2O		
2	Biotin (Sigma B-4639)	0.005 g/200 mL dH2O		

# **10.1.9 Thiamine Vitamin Solution**

The Thiamine vitamin formulation is to be used in the Modified Bold solution. Make sure to add this AFTER the autoclave step to ensure the vitamins are not denatured.

Directions:

For 50 mL total

- 1. Prepare 50 mL of HEPES buffer (50 mM).
- 2. Adjust the pH to 7.8.
- 3. Add Thiamine (6.5 mM) wait until fully dissolved.
- 4. Sterilize by 0.45 μm Millipore filter. Store in dark at freezer temperature.
  \*\* The amount of vitamins added can vary from medium to medium so the final concentration is not listed.

#	Component	Amount	Stock Solution	Final
			Concentration	Concentration
1	HEPES buffer pH 7.8 (Sigma H-3375)	1.2 g/100 mL dH20		
2	Thiamine (Sigma T- 1270)	0.11 g/100 mL dH20		

### **10.2 Photospectography**

In section 2.3, photospectography was introduced as a method to monitor the growth of the microalgal cells. In this section, details directions on how to take these measurements are listed.

### Preparation for Optical Density reading on the Tecan Safire 2

### How To for ALGAE

### 1. Prepare Hood

- a. Spray the fume hood with alcohol.
- b. Turn on the UV light.

# 2. Obtain Materials

- a. Get a 96 well clear plate with top from the shelf where all the supplies are.
- b. Get the 100-1000 um micropipette from the bench. They are by the stir plate.
- c. Get a test tube holder. They are lying randomly around the lab. These are the colored blocks with the different sized holes in them.
- 3. Obtain Sample
  - a. Turn off the UV light on the hood and place your materials there.
  - b. Spray the micropipette with alcohol and sit it on one of the tip boxes so that the white part of the pipet does not touch anything.

- c. From the shaker, obtain the respective day test tube. Ie. "Day 3".
- d. By this point make sure you have gloves on and spray them with alcohol.
- e. Spray the test tube with alcohol and place it in the test tube holder.
- f. After it has dried some, move on to the next step.
- 4. Prepare 96 Well Plate
  - a. Place a blue tip on the micropipette. These tips are in the largest box.
  - b. Make sure the pipet is set for 200. And open the 96 well plate.
  - c. Shake and then open the algae sample and pipet 200 mirons into at least 6 consecutive wells for two rows. Take note/write down the rows you put them in.
    - i. You can do A1-A6 and C1-C6.
    - ii. If you suspect contamination, change the tip.
  - d. Close the plate and sample when complete and dispose of used tip.
- 5. Put away materials.
  - a. Put the sample back on the shaker and the pipet back on its home bench.
  - b. Clear the hood of any used material.
  - c. Before taking the plate out of the hood, be sure it is closed.

# 6. WE ARE READY FOR THE TECAN PLATE READER!! $\textcircled{\odot}$

### **Operation of the Tecan Safire 2**

How-To Instructions for ALGAE

The Equipment is Room 278 of Mellon

- 1. Turn on Tecan
  - a. Turn on the Tecan Microplate reader by pressing the triangle button on the right side near the bottom. You can tell that it is on if the light illuminates.
- 2. Log in
  - a. The computer to the left of the machine should already be logged in. If not, just choose "Tecan User".
- 3. Begin Software
  - a. On the desktop, click on XFLOUR4
  - b. Click "Ok" on the pop up menu.
  - c. An Excel spreadsheet should open with a blue background a picture of the Tecan machine. You will see the mouse turn to a sand timer while it is loading.
  - d. Wait for it to finish.
- 4. Link Tecan to Excel
  - a. Under the tab labeled XFLOUR4SafireII, click "Connect".
  - b. The door will open. Insert the plate with the A1 in the top left corner.
  - c. Close the door by clicking on -> "Movements" -> "In" -> Ok. The window will close.
- 5. Load Parameters

- under the same tab labeled XFLOUR4SafireII, select "Load Measurement Parameter"
- b. A pop up box will appear. Click on "Documents" -> "LeDuc" -> "Kristin"
- c. Double click on "Algae22MAY2012" for the set to load.
- 6. Double Check Parameters
  - a. Under the XFLOUR4 tab, click on "Edit Measurement Parameters".
  - b. Go through each tab to make sure that the following are true.
    - i. Measurement mode: Absorbance
    - ii. Wavelength: 680 nm
    - iii. Shake time: 5 seconds
    - iv. Settle time: 5 seconds
  - c. Close panel
- 7. Run Experiment
  - a. Under the XFLOUR4SafireII tab, click on "Start Measurement".
  - b. You will hear some beeping noises until it is finished.
  - c. When it is done, you will see a filled out Excel spreadsheet similar to the following. You will save this file later.
  - d. Close the door by clicking on -> "Movements" -> "In" -> Ok. The window will close.

	A	В	C	D	E	F	G	Н	1	J	K	L	M	N
1	SAFIRE II;	Serial nurr	nber: 60500	0006; Firm	ware: V 2.1	0 12/2007 5	Safire2; XF	LUOR4SAF	IREII Versio	on: V 4.62b				
2	Date:					23/5/12								
3	Time:					17:03								
4														
5	Measurem	ent mode:				Absorbanc	e							
6	Measurem	ent wavelen	gth:			680	nm							
7	Number of	reads:				10								
8	Plate defin	ition file:				COS96fw.p	odf							
9	Time betwe	een move ar	nd read:			10	ms							
10	Shake dura	ation (Orbita	I Medium):			5	S							
11	Shake sett	le time:				5	S							
12	Target Tem	perature:				37	°C							
13	Current Ter	mperature:				27.8	°C							
14														
15	Rawdata (	OD)				Temperatu	re:		27.7	°C				
16														
17		1	2	3	4	5	6	7	8	9	10	11	12	
18	A	0.4647	0.4929	0.4594	0.4735	0.4968	0.4752	0.0412	0.0477	0.0414	0.0411	0.0393	0.0589	
19	В	0.2320	0.2254	0.2315	0.2450	0.2260	0.2334	0.0382	0.0540	0.0429	0.0429	0.0408	0.0518	
20	С	0.1209	0.1352	0.1331	0.1387	0.1279	0.1356	0.0397	0.0552	0.0414	0.0415	0.0406	0.0624	
21	D	0.0884	0.0842	0.0787	0.0919	0.0878	0.0822	0.0404	0.0703	0.0431	0.0435	0.0433	0.0792	
22	E	0.0603	0.0595	0.0577	0.0588	0.0547	0.0579	0.0399	0.0643	0.0402	0.0428	0.0431	0.0529	
23	F	0.0491	0.0521	0.0497	0.0488	0.0525	0.0486	0.0414	0.0625	0.0411	0.0419	0.0424	0.0553	
24	G	0.0419	0.0396	0.0396	0.0321	0.0396	0.0391	0.0368	0.0528	0.0406	0.0400	0.0411	0.0544	
25	н	0.0405	0.0485	0.0505	0.0407	0.0412	0.0392	0.0426	0.0605	0.0443	0.0433	0.0443	0.0555	
26		L												
27														
28	1													

- 8. Rerun experiment at 600nm.
  - a. Under the XFLOUR4SafireII tab, click on "Edit Measurement

Parameters".

- b. Change the Wavelength to 600nm.
- 9. Run Experiment Again
  - a. Under the XFLOUR4SafireII tab, click on "Run Experiment".
  - b. You will hear some beeping noises until it is finished.
  - c. When it is done, you will see a filled out Excel spreadsheet similar to the following.
- 10. Save the file.
  - a. After the second experiment is run, there will be two worksheets in the Excel file. One for 600nm and one for 680nm.
  - b. Click on "File" -> "Save As" -> "Documents" -> "LeDuc" -> "Kristin" ->
     "Time Lapse" STOP!!

- c. Click ONE TIME on a previous saved file.
- d. Change the day to the corresponding day and change the date to the current date.
- e. Click "Save"
- 11. Back Up File
  - a. If you have a jump drive, follow the same procedure to have your own copy.
  - b. If you do not have a jump drive, email it to me and/or yourself.
- 12. Close Program
  - a. Take out the plate and place the top back on.
  - b. Under the XFLOUR4SafireII tab, click on "Disconnect".
  - c. Close the Excel program by clicking on "X" in the top right corner of the window.

### 13. Shut off Tecan

- a. Press the lighted triangle. (The same button used to turn it on.)
- b. You can tell that it is off when the light is no longer illuminated.

#### **10.3 AFM Cantilever Selection and Calibration**

In section 2.5, atomic force microscopy and the importance of selecting appropriate cantilevers for experimentation was introduced. Within this section, the cantilever selection and calibration will briefly be discussed.

# **Cantilever Selection**

Selecting the right cantilever for an application or sample is imperative. Cantilevers are generally made from silicon nitride (Si<sub>3</sub>N<sub>4</sub>) or silicon (Si) and vary in length and shape to achieve a variety of spring constants and resonant frequencies. Additionally, the probes can be coated in other material to increases electrostatic, magnetic, or chemical forces. The imaging mode, conductivity required, performance in liquid, and stiffness are just a few of the parameters that must be determined prior to choosing a cantilever.

As Figure 10-1 denotes, there are three main imaging modes with AFM: contact, tapping, and non-contact mode. In contact mode, also known as repulsive mode, there is less than 0.5 nm separation between the probe and the surface and remains constant throughout the imaging process. This separation can be considered *soft physical contact*. The spring constant should be less than that of the surface to allow the cantilever to bend in order for it to deform as it scans the sample, gathering information on the sample's topography. Although contact method of imaging can sometimes damage soft samples, this mode is good for fast scanning, rough samples, and friction analysis.

More information about contact mode and the "repulsive region" is described below in an excerpt from Park Systems, a manufacturer of AFM equipment.

The following is an excerpt from Park Systems (2016) regarding contact mode imaging.

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At the right side of the [f/d] curve the atoms are separated by a large distance. As the atoms are gradually brought together, they first weakly attract each other. This attraction increases until the atoms are so close together that their electron clouds begin to repel each other electrostatically. This electrostatic repulsion progressively weakens the attractive force as the inter-atomic separation continues to decrease. The force goes to zero when the distance between the atoms reaches a couple of angstroms, about the length of a chemical bond. When the total van der Waals force becomes positive (repulsive), the atoms are in contact.

The slope of the van der Waals curve is very steep in the repulsive or contact region. As a result, the repulsive van der Waals force balances almost any force that attempts to push the atoms closer together. In AFM this means that when the cantilever pushes the tip against the sample, the cantilever bends rather than forcing the tip atoms closer to the sample atoms. Even if you design a very stiff cantilever to exert large forces on the sample, the inter-atomic separation between the tip and sample atoms is unlikely to decrease much. Instead, the sample surface is likely to deform (as in Nanolithography). In addition to the repulsive van der Waals force described above, two other forces are generally present during Contact mode operation of the Park AFM: a capillary force exerted by the thin water layer often present in an ambient environment as well as the force exerted by the cantilever itself. The capillary force arises when water wicks its way around the tip, applying a strong attractive force (about 8-10 N) that holds the tip in contact with the surface. The magnitude of the capillary force depends upon the tip-to-sample separation. The force exerted by the cantilever is like the force of a compressed spring. The magnitude and sign (repulsive or attractive) of the cantilever force depends upon the deflection of the cantilever and upon its spring constant.

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The second imaging mode in AFM is tapping mode where the separation distance between the sample and cantilever is between 0.5 and 2nm. This type of imaging is similar to the contact mode, but differs bin the fact that the cantilever oscillates at its resonant frequency causing the cantilever probe to lightly *tap* on the sample surface. With a constant oscillation amplitude, the tip-sample interaction is maintained at a level that the surface topography is acquired. Sometimes referred to as Dynamic Force Microscopy (DFM), intermittent contact, or AC mode, tapping mode has the advantages of allowing a high resolution of samples for soft or loosely adhered samples. This is an advantage for biological samples. A drawback to this type of imaging is that a slower scan speed is required and imaging can become difficult in aqueous environments.

The third most used imaging mode in AFM is non-contact mode. In non-contact mode, the cantilever is separated from the surface at 0.1-10nm and is controlled by attractive Van der Waals forces, opposite to contact mode's repulsive forces, and does not touch the sample's surface. A view of these repulsive and attractive forces, from contact and non-contact modes, respectively, are shown below in Figure ##. In non-contact mode, the cantilever oscillates above the surface during the scan. Topography is acquired by monitoring the changes of the amplitude using a feedback loop. This mode of imaging extends the life of the probe because it exerts an extremely low force on the sample on the order of 10<sup>-12</sup> N. Some of its disadvantages include lower resolution images and occasionally the complementary use of ultra-high vacuum (UHV) for better quality images. Additionally, contaminant layer on the surface can interfere with the cantilever oscillation, which affects the image quality.



Figure 10-1 A graph showing the three primary imaging modes and the interactive forces that occur in AFM.

Stiffness, or the spring constant, of the cantilever is an important feature of a cantilever. The spring constant, k, is determined from solid mechanic's beam theory exemplified in the following equation and Figure 10-1.

$$\delta_{max} = \frac{FL^3}{3EI}$$
 10-1

$$\theta = \frac{FL^2}{2EI}$$
 10-2

Equation 10-1 calculates the maximum deflection,  $\delta_{\text{max}}$ , and equation 10-2 calculates the slope at the free end of the cantilever,  $\theta$ . The slope and deflection are proportional to the force at the end of the cantilever, and therefore can be used to determine *F*. Herein, *F* is force applied, *L* is the length of the cantilever, *E* is the Young's modulus of the cantilever, and *I* is the moment of inertia which can be calculated using Equation 10-3.

$$I = \frac{bh^3}{12}$$
 10-3

Here, *b* is the thickness of the cantilever and *h* is the height of the cantilever. Utilizing these equations in combination with Hooke's law ( $F=-k\delta$ ), one can determine the spring constant of an AFM cantilever. Generally, this is supplied by the cantilever manufacturer within a given range. It is then verified using AFM and further testing will commence.



Figure 10- 2 Schematic to calculate the spring constant of an AFM cantilever.

### **Cantilever Calibration**

Calibrating the software for each cantilever can be a difficult process. It requires a stiff substrate to press the cantilever into in order to determine its spring constant and cantilever sensitivity. Afterwards its thermal noise is calculated by vibrating the cantilever to find its inviers optical lever sensitivity (InvOLS). Calculating these properties in this manner, exposes the cantilever to unnecessary risk of damage.

With the Asylum AFM used for the determination of the microalgal Young's modulus values, an automatied probe calibration feature, called GetReal, was utilized. GetReal uses two well-established calibration techniques to determine the spring constant and InvOLS during one calculation. The two calibration techniques, thermal noise method and Sader method, are basis from the automated calibration.

10.4 Supplementary figures from "Probing the Elastic Response of Microalga Scenedesmus dimorphus in Dry and Aqueous Environments though Atomic Force Microscopy"



Figure 8-1 Bright-field microscopic image of microalgal strain *Scenedesmus dimorphus*. This image shows their unicellular nature as well as colony formation.


Figure 8-2 A thermal noise spectrum where the spring constant is calculated using the Sader Method. The resonance frequency is approximately 74 Hz for this cantilever and the spring constant is 2.14 N/m.

Table 10- 1 A comparison of the Young's Modulus of *S. dimorophus* cell with other biological materials. The asterisk (\*) denotes that the value is the mean  $\pm$  standard deviation of the mean, where  $p \le 0.05$ . Sources: [1] Radmacher, M. 1997; [94]Eaton, et al 2008; [3] H. Tuson, et al. 2012; [4] A. Pelling, et al. 2004; [5] Touhami, et al. 2003.

Material	Young's Modulus (MPa)
Staphylococcus aureus	95.40 <sup>2</sup>
Collagen	1000 1
Murein Sacculi of Gram-negative bacteria	25 <sup>5</sup>
Rubber	1.4 <sup>1</sup>
Living Animal Cells	$0.001 - 0.1^1$
Escherichia coli	$0.05 - 221^3$
Yeast Cell	$0.72-0.75$ $^4$
Bud Scar on Yeast Cell	$6.1 \pm 2.4$ <sup>5</sup>
S. dimorphus in Aqueous Environment	$2.21 \pm 0.40 *$
S. dimorphus in Dry Environment	$57.96 \pm 7.20*$