Spring 5-2015

Investigating The Optimization of Zebrafish (Danio rerio) Dechorionation For High-Throughput Applications In Influenza Research

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INVESTIGATING THE OPTIMIZATION OF ZEBRAFISH (*DANIO RERIO)* DECHORIONATION FOR HIGH-THROUGHPUT APPLICATIONS IN INFLUENZA RESEARCH

by

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A Thesis Submitted in Partial Fulfillment
of the Requirements for a Degree with Honors
(Bioengineering)

The Honors College

University of Maine

May 2015

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Abstract

Seasonal influenza A virus infections present a serious problem to our society every year. The rapid evolutionary time of this pathogen, due to its high mutation rate, makes the potential for pandemic outbreak a constant threat. As a result, researchers must continuously focus their efforts on developing new and unique treatments to combat the emergence of novel strains. The zebrafish, which has recently been shown to recapitulate mammalian influenza infection and respond positively when treated with a known anti-viral, is the ideal animal model for optimizing these laborious drug screenings into a high-throughput process. Unfortunately, there are several bottlenecks in the process of preparing zebrafish for infection that currently limit their potential. One such bottleneck is the tedious procedure of removing the chorion, the protective shell of a zebrafish egg, from each embryo. Here, the optimization of this dechorionation process is described. In this investigation, a novel dechorionation method was discovered, which uses hydraulic pressure to extrude individual eggs through small diameter tubing to induce dechorionation. Some initial characterization of this method was performed, and its relative capability was compared with the two other prominent dechorionation techniques, the forceps method and the protease method. While these two methods easily outperformed the extrusion method, they respectively lack the usability and statistical evidence necessary to be currently considered for such an application. The hydraulic extrusion method described herein, while still in the early stages of its development, demonstrated much promise as a rapid dechorionation method, and warrants continued research into its improvements.

Acknowledgements

Over the past year, I have been lucky enough to receive a great amount of support and encouragement from a number of individuals. Paul Millard has been a better advisor than I could have ever hoped for. His guidance and advice proved invaluable throughout this entire process, and his constant availability, during an especially busy year, was truly appreciated. During the design process Wilson Adams gave far too much of his valuable time helping me find materials and build devices, for which I am exceedingly grateful. Thank you also to Meghan Breitbach and everyone else in the Kim lab for supplying me with zebrafish, and always being so kind and supportive of my work.

To my fellow engineers in honors, Aoife Ryle, Nick Carter, and Andres Velez, thank you for listening to and sympathizing with my frequent struggles. Enduring this process alongside all of you has helped me to maintain my focus and find solutions to the many problems I faced. Lastly, as always, thank you to my friends and family for always acting interested in my research, and never letting me doubt my abilities

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1. Introduction

1.1 Zebrafish background

Over the past decade, the zebrafish (*Danio rerio*) has quickly become an important animal model for an impressive variety of research applications. There are a number of characteristics possessed by this organism that make its use amenable to a broad range of disciplines. Adult zebrafish (Fig. 1) are about 3 cm long, with eggs

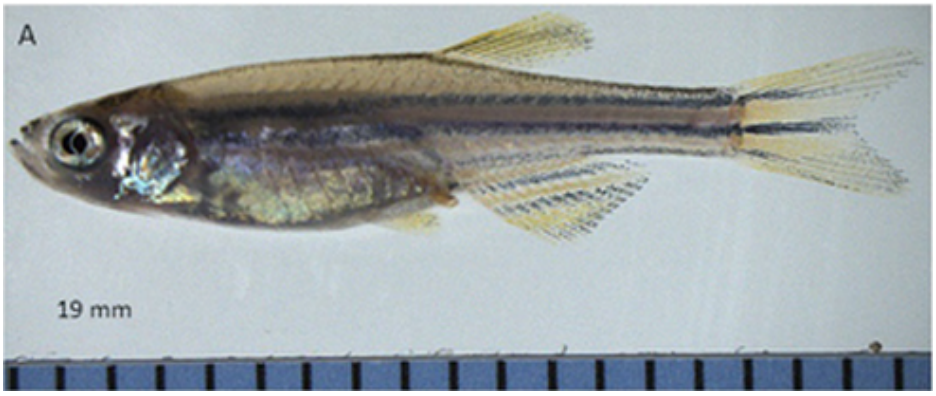


Figure 1: A normally developed young adult zebrafish [21]

spanning only about 1.1 mm in diameter [1] [2]. The small size of the fish renders it relatively inexpensive to house and propagate. Zebrafish mature sexually relatively quickly in two to three months, and are capable of producing, in a single spawning, upwards of 200 eggs per week [3]. Their external fertilization and development, in addition to almost fully transparent embryos, allows for many developmental and physiological processes to be monitored easily with a simple stereo microscope. The genome of the zebrafish, while only about half the size of the human genome, has been found to have a remarkable similarity to our own [3]. It is now known that approximately 70% of human genes have at least one clear zebrafish orthologue, a functionally equivalent gene having evolved from a common ancestral gene [4].

Therefore, zebrafish have been found to be excellent models in investigating vertebrate gene function, and are used increasingly in research into human genetic diseases [4]. In fact, the National Institutes of Health (NIH) has recently recognized the zebrafish as an acceptable alternative model for research pertaining to human disease and physiology [1].

1.2 Influenza background

For many of us, the influenza virus, or the "Flu" as it is most commonly known, is little more than an occasional inconvenience. However, on a national scale, its effects are much more grave. Every few years, localized influenza epidemics across the United States cost the government billions of dollars in additional health care expenses, and result in a significant drop in productivity (Fig. 2) (Lowen, Mubareka, Tumpey, Garcia-Sastre, & Palese, 2006). Tens of thousands of individuals infected by the influenza virus ultimately die as a result of the disease and even more are hospitalized (Lowen, Mubareka, Tumpey, Garcia-Sastre, & Palese, 2006). To the healthy individual, influenza is usually nothing more than a brief upper respiratory tract infection (Thangavel & Bouvier, 2014). However, for the elderly, pregnant women, or immunocompromised individuals, more serious and potentially lethal complications may arise (Thangavel & Bouvier, 2014). Although less common, pandemic outbreaks of the influenza virus have historically occurred every 10 to 40 years, and these have been much more deadly (Lowen, Mubareka, Tumpey, Garcia-Sastre, & Palese, 2006). In fact, influenza pandemics in 1918, 1957, and 1968 collectively resulted in a total of more than 50 million deaths worldwide (Lowen, Mubareka, Tumpey, Garcia-Sastre, & Palese, 2006).

While our proficiency in combating these viruses has seen dramatic improvement over the past century, influenza's ability to undergo antigenic drift, as well as antigenic shift,

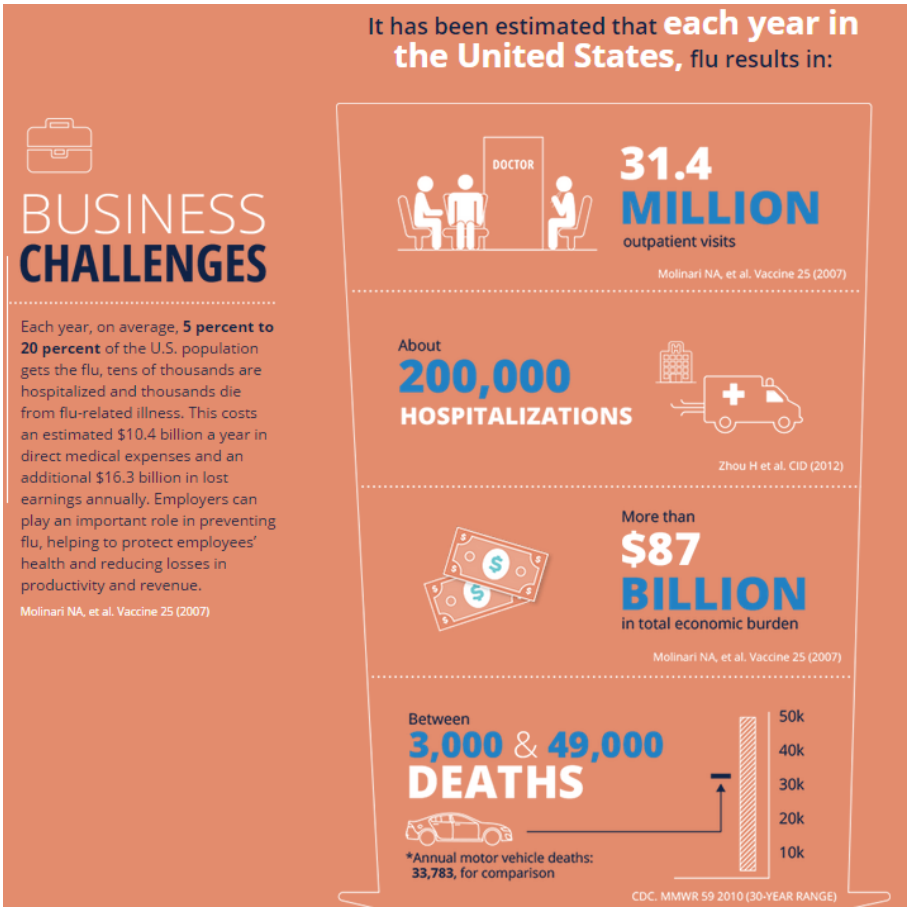


Figure 2: CDC infographic highlighting Influenza's impact on productivity in the United States [20]

allows for the development of many new strains in a relatively short time [7]. The resulting rapid evolutionary cycles of this virus make it problematic for us to maintain effective anti-influenza treatments [8]. Thus, the need for modern medicine to keep pace with this continually evolving pathogen is of increasing importance, especially with the lingering threat of pandemic outbreak.

1.3 Zebrafish use in influenza research

1.3.1 Animal model

Although our primary concern is largely with viral infection in humans, the majority of influenza research is necessarily conducted using a variety of alternative

animal models. Such alternatives consist of mice, Syrian hamsters, guinea pigs, dogs, cats, ferrets, domestic swine, cotton rats, non-human primates such as pigtailed macaques, and zebrafish [6]. While there are pros and cons associated with each model system, the zebrafish model will be of primary interest to influenza researchers for several reasons. Recently, zebrafish have been shown to be infected by influenza virus, to mimic mammalian immune response to influenza A virus infection, and even to respond positively to anti-influenza drugs (Zanamivir) [8]. Zebrafish rely on just their innate immunity for the first 4-6 weeks of development, a unique benefit of the zebrafish model that permits the investigation of innate immunity and its effect on influenza infection [8]. This offers the intriguing opportunity to observe how just the innate immune system, independent of the adaptive immune system, responds to viral infection. Additionally, given this organism's small size and short maturation period, it has a significant potential for high-throughput applications [9]. Therefore, when considering the future of the zebrafish model in influenza research, one logical next step is to begin modifying their use for the screening of potential antiviral drugs.

1.3.2 Potential for rapid drug screenings

In preparing zebrafish eggs for influenza A virus infection and eventual treatment with the anti-influenza compounds such as Zanamivir [8], three major bottlenecks can be identified. The first pertains to the protective acellular envelope that surrounds the embryo during the first 48 to 72 hours of its development, known as the chorion. The chorion could be considered functionally equivalent to the shell of a chicken's egg, although significantly smaller, fully transparent, and far less rigid. On the third day post fertilization, and sometimes not until the following day, zebrafish embryos naturally

hatch from this outer chorion shell [9]. The time of hatching between individual batches, and even between individuals within a single batch is sporadic and, in general, is not closely indicative of developmental progress [9]. For a range of experimental studies the chorion is removed in a process called dechorionation, e.g. just before the embryo is infected with the influenza virus at about 48 hours post-fertilization (hpf) [8]. The chorion is typically removed manually using a pair of fine watchmaker's forceps [8]. Unfortunately, this process can be tedious when working with 50 eggs or more. Even though individuals can become highly proficient in these manual dechorionation skills, the process is not compatible with high-throughput procedures.

The second major bottleneck that is encountered is the process of infecting each zebrafish embryo with the influenza A virus. After being dechorionated, the embryo must first be anesthetized in a tricaine solution [8]. Each individual must then be correctly aligned on an agarose gel to permit microinjections in the appropriate anatomic location [8]. The microinjection process is a learned skill, and requires a great deal of time and practice to develop fully. An individual must be able to inject at exactly the same position and depth in each fish, a task requiring extreme precision and consistency over many repetitions. These procedures are tedious, and are typically performed manually.

The final bottleneck in potentially using zebrafish for high-throughput drug screening is the process of *in vivo* fluorescence imaging of each fish in order to evaluate levels of infection and the efficacy of anti-viral therapies. A genetically modified strain of influenza A virus, which expresses green fluorescent protein upon infection and replication, can be used to infect the zebrafish [8]. The transparency of the early-stage

permits evaluation of infection via fluorescence microscopy, allowing the progression of infection to be monitored in real time [8]. Therefore, the effects of anti-influenza treatments, such as Zanamivir, can also be monitored in real time. This process again will involve repetitive manual positioning of individual embryos to ensure the consistency of imaging. Thus, the zebrafish model has great potential for use in rapid identification of novel anti-influenza therapies, but a number of barriers must be circumvented in order to optimize it as a high-throughput process. The work presented here is directed toward the optimization of the dechorionation process.

1.4 Current dechorionation methods

1.4.1 Natural hatching

Zebrafish normally hatch within 48 to 72 hpf. Hatching is achieved through a process which is known as "chorion softening," which results from the embryo's release of proteolytic hatching enzymes that degrade the chorion from the inside [10]. As it is weakened, the chorion is eventually torn open by the wiggling movements of the developing embryo inside, releasing it into the surrounding medium [11]. The closer an individual egg is to hatching, the weaker its chorion usually is as a protective barrier [10].

1.4.2 Forceps method

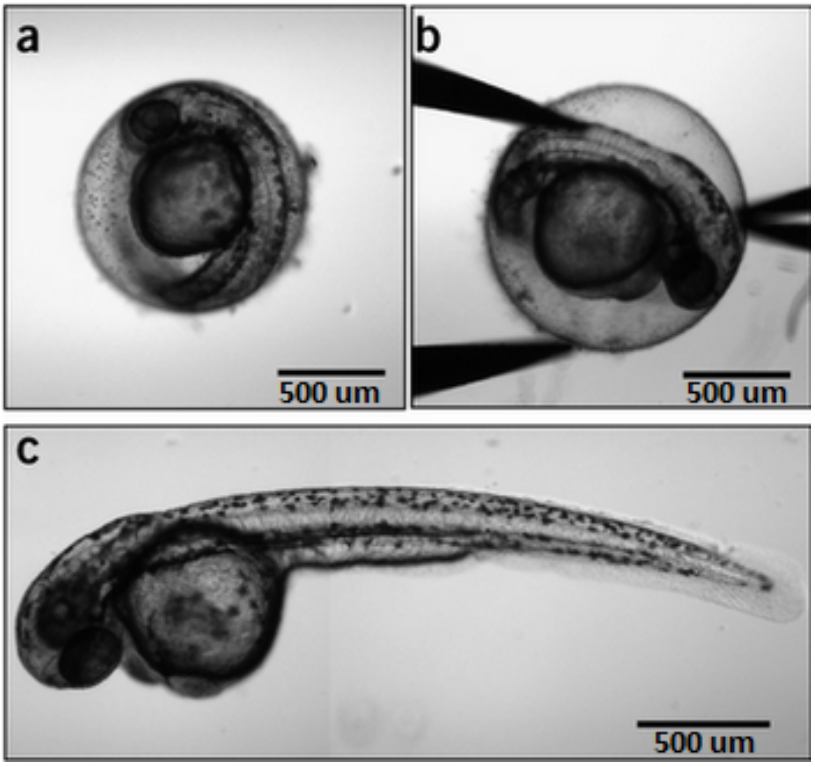


Figure 3: Typical forceps dechorionation process. (a) 48 hpf zebrafish embryo within its chorion. (b) Forceps positioning egg and grasping chorion in preparation to remove it. (c) Successfully dechorionated embryo [22]

The dechorionation of zebrafish is a common practice in research, and is performed over a range of developmental ages, depending on the nature of the study [12] [13] [14]. As discussed previously, the most prominent current technique for dechorionation is the forceps method (Fig. 3). Most procedures involve the use of Dumont #5 forceps, but similar alternatives also suffice [9]. This procedure is performed by first lightly pinching the chorion with one forceps in order to establish a grip on the egg. Another forceps is used to pinch the chorion directly adjacent to the first forceps. The two forceps are then moved apart while continuing to grip the chorion, gently tearing the chorion open. This initial opening may not always be large enough to permit the embryo to exit, so the process is often repeated in order to enlarge the tear. Depending

on the developmental age of the embryo, it will either swim out of the chorion, or the egg must be inverted to allow gravity to carry the embryo out.

The most significant benefit of using this method is the degree of control that is given to the operator. As the embryos are necessarily dechorionated individually, care is taken in the removal of every chorion. A trained individual can dechorionate as many as 50 eggs in under 10 minutes. This method requires precision on a sub-millimeter scale. Embryos at younger developmental ages tend to be much more fragile, and as a result are even harder to remove with forceps without being damaged. This requires exceptionally smooth and gentle movements, which is increasingly difficult as the number of embryos increases. While working with smaller samples of fewer than 50 eggs, this is an ideal method to use as it is quick, simple, and has a high rate of success. However, with larger sample sizes comes greater fatigue and ultimately slower dechorionation speeds. Additionally, while mechanizing this method is certainly feasible, the required complexity would likely make it exceedingly expensive. Research efforts should be focused on developing other more simplified techniques before the automation of the forceps method is seriously considered. Therefore, the forceps method is a great way to dechorionate small batches of eggs, but appears to have little potential for use in rapid drug screenings.

1.4.3 Protease method

Another technique currently used for dechorionation involves a biochemical-based approach using a protease enzyme [15] [16] [2]. The method involves incubating zebrafish eggs in a solution of protease, at a concentration and length of time appropriate for the embryo's developmental age. Protease collected from *Streptomyces griseus* is

most commonly used with this technique. Over time the protease will degrade the chorion, weakening it. In effect, this method simulates the natural hatching process, but hydrolysis is initiated from the outside and at a much higher rate than the natural process. The chorion eventually is weakened to the extent that gentle manual pipetting ruptures the chorion, which falls away from the embryo. After this treatment, the protease solution is discarded and the embryos are typically washed several times to remove bound protease. This method has also been used in conjunction with the forceps method, as the protease solution weakens the chorion and it becomes much easier to tear open with forceps, thereby simplifying the recovery of embryos. There are a number of pros and cons to this method as well, however.

The most obvious positive feature of this method is its ability to dechorionate many embryos simultaneously. While the forceps method is restricted to dechorionating in series, the protease method can operate on a relatively large number of eggs in parallel. This method also requires minimal effort from the operator, in comparison to manual dechorionation with forceps, and as such can be repeated many times without regard for fatigue. A third benefit is that the speed of protease dechorionations is independent of the sample size. As the forceps method must dechorionate sequentially, the number of embryos being dechorionated directly affects how long the entire procedure takes. However, since the protease method can dechorionate embryos in parallel, the sample volume has no effect on the dechorionation speed.

One of the biggest drawbacks to this method is its inherent lack of control. Because the time of dechorionation is unique from egg to egg during a protease treatment, those embryos that hatch early on in the incubation period will be directly

exposed to protease enzymes for the rest of the treatment. Therefore, depending on the exposure time and the protease concentration, embryos may be injured or even killed by the end of these treatments. Although proteolytic enzymes are also involved in the natural hatching process, they are specific to zebrafish and exist at much lower concentrations. As of yet, no conclusive studies have been conducted as to what non-lethal effects these protease treatments may have on zebrafish embryos. A study in 2010 investigated the survival rates of zebrafish embryos dechorionated using this protease method [17]. It reported a dechorionation success rate on embryos 6 hpf of no more than 25% per sample, and an overall survival rate at 2 days post-fertilization (dpf) never exceeding 5% of the total 50 eggs per sample [17]. It strongly indicated that this treatment is detrimental to the embryos, although it may not initially appear so [17]. More recently, an automated device was described which dechorionates zebrafish embryos using this protease method in a high-throughput fashion [18]. Interestingly, it reported a ≥95% dechorionation success rate on embryos 4 hpf, with only 2% embryo mortality by 24 hpf [18]. It should be noted that the former study used protease with an activity of 4 U/mL at a final concentration of .001 mg/mL, while the latter used protease with an activity of 6 U/mL at a concentration of .1 mg/mL [17] [18]. Unfortunately, other than the conflicting results of these two articles, no other conclusive investigations have been conducted on the survival rates of zebrafish embryos dechorionated using the protease method. Thus, while this method has potential for high-throughput applications, it is difficult to control and is plagued by a lack of statistically conclusive survivability data.

2. Preliminary experiments

2.1 Design process:

In this investigation an attempt was made to develop a procedure to automate the physical process of zebrafish dechorionation, and to compare this procedure with the two most widely used methods for dechorionation. While a chemical approach, such as the protease method, offers the benefit of parallel dechorionation, the drawbacks associated with chemical methods described earlier directed the investigation toward mechanical methods. This decision was based on the uncertainty surrounding the potential perturbation of the embryo resulting from chemical dechorionation, and the fact that such an optimized method has already been described [18]. In comparison, mechanical injuries are generally more readily identifiable.

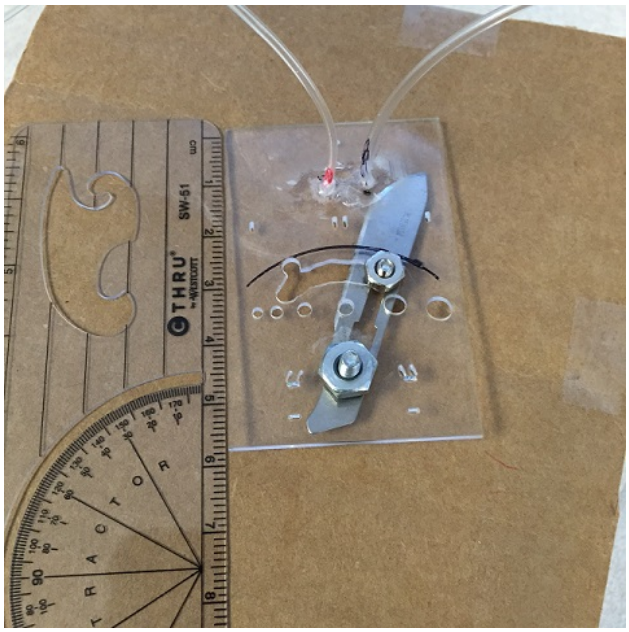


Figure 4: First version of chorion slicing device. A dissecting blade was attached to the acrylic frame at two positions, allowing it to be swiveled back and forth across bottom face of the device. Two segments of tubing, with different inner diameters, were attached through the top of the acrylic. These allowed the eggs to be vacuum stabilized on the bottom of the device so that dechorionation with the blade could be attempted.

In approaching mechanical dechorionation, there are many potential options for physically penetrating the chorion that may be explored. While the ideal method can dechorionate eggs in parallel, this may not be realistic for all mechanical options. Additionally, the amount of time and materials available to this study was limited, and

partially dictated which methods were chosen for further investigation. Therefore, initial experiments focused on the use of a surgical blade or razor blade to manually slice open the chorion, as it was decided that this was the most immediately feasible option. These experiments proved unsuccessful, as the size of the eggs and their malleability made it virtually impossible to get a clean cut by hand. Additionally, the use of forceps to stabilize each egg against the slicing force of the blade was not sufficient, and the egg would often slide out from under the blade before it could be penetrated. To avoid this problem the use of suction to position the egg was investigated subsequently. Tubing having the optimal inner diameter and a sufficient pressure drop to secure the egg were identified. The resulting tests of this stabilization method were promising. A small device (Fig. 4) was constructed which combined this vacuum stabilization with the surgical blade, in an attempt to slice off the top of the chorion while it was held in place by the vacuum. This method was considered viable because gravitational force caused the embryos to rest in the bottom half of the chorion. Therefore, if the egg was held stable at its apex, a blade could theoretically slice through this upper section without damaging the embryo. This device failed because either the blade still failed to cut consistently into the chorion, or once the chorion was breached it lost its original shape and the exposed embryo would be destroyed by the applied vacuum.

Several variations of this slicing method were investigated. Another device (Fig. 5) was constructed which followed the same dechorionation principles as the previous design, but used a variety of small sized wells to stabilize the eggs rather than a vacuum. Specifically, the base of this device was 3D printed so that 6 square wells existed on its top face (Fig. 5.B). The first two wells were both 1.0 mm wide, and their depths were 0.60 and 0.80 mm, respectively. The second two wells were both 1.1 mm wide, and their depths were 0.66 and 0.88 mm, respectively. The third pair of wells were both 1.2 mm wide, and their depths were 0.72 and 0.96 mm, respectively. This device was unsuccessful because, depending on the well size, the blade would either pass over the egg without slicing it, or push it out of the well without penetrating the chorion. In an attempt to further stabilize the eggs within these wells, drops of 1% agar were placed on them and allowed to solidify. While this helped greatly in immobilizing the eggs, it made them much harder to position correctly and also tended to deflate the chorion slightly, making them even more difficult to slice. Several other alternative methods were also investigated, such as drawing the eggs out of solidified agar using a vacuum

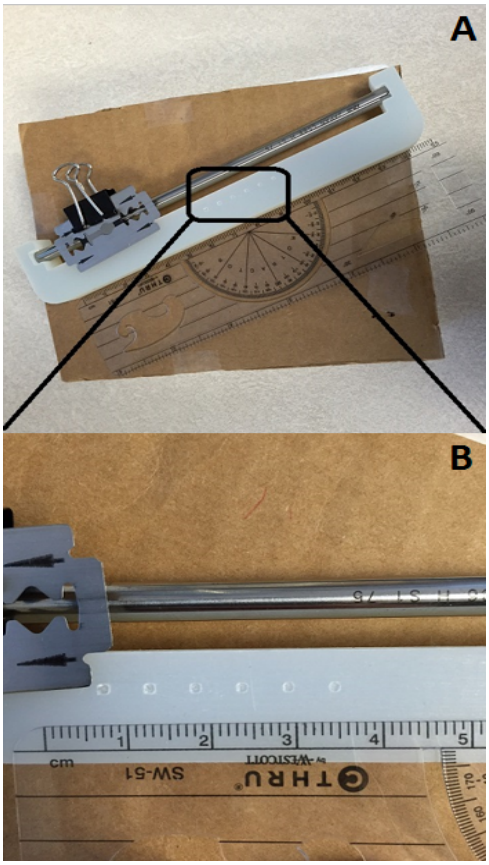


Figure 5: Second version of chorion slicing device. (A) The main body was 3D-printed to allow a linear ball spline to be attached, with a razor blade clamped on to its sliding stage. (B) A series of wells, decreasing in size, were printed into the device to hold the eggs and allow the blade to pass by and slice through them.

with a large pressure drop, and attempting to permanently deform the chorion using suction. These methods were largely unsuccessful as well, and while the viability of these methods might have improved with further experimentation, investigation of other alternatives was deemed to be the best course of action.

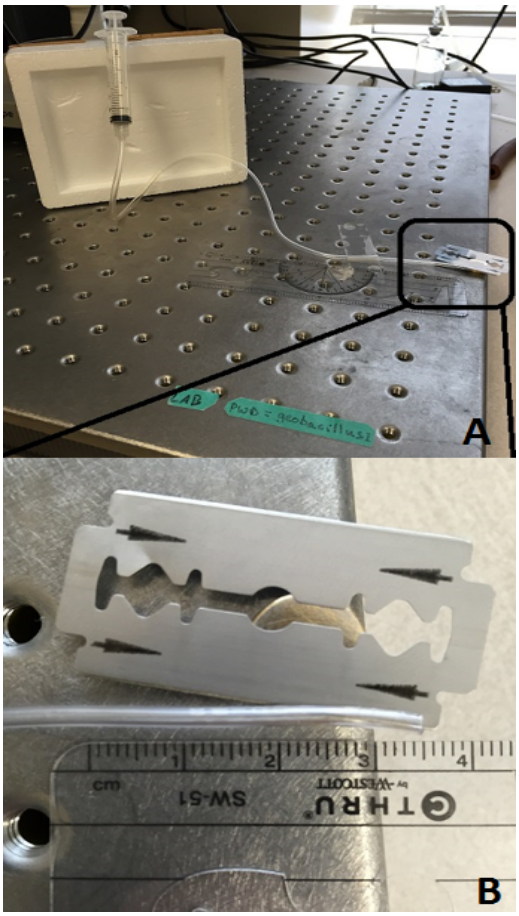


Figure 6: Third version of chorion slicing device. (A) Tubing was connected to a syringe and held steady adjacent to a razor blade. (B) The razor blade was held still via a magnet and allowed to slice approximately half-way into the end of the tubing.

Thus far, the main problem with the slicing method was the difficulty in finding a reliable method to hold the egg firmly enough to permit the blade to slice through it without killing the embryo. To avoid this problem an alternative method was tested. Perhaps more comparable to the mechanics of a sawmill, a method was tested in which the blade was stationary, while the eggs were moved sequentially past it (Fig. 6). Using tubing (Tygon MicroBore 0.04" ID Tubing; Component Supply, Co., Fort Meade, FL) with an inner diameter that was slightly smaller (1.0 mm) than the average egg diameter (1.1 mm), a blade (platinum chrome double edge razor blade; Personna) was placed at the end of the tubing and eggs were manually pumped through it and forced past the blade. Initially, as successful dechorionations were identified for the first time, the interpretation was that this slicing method was effective. However, after further

investigation it was discovered that the dechorionations were actually occurring as the eggs were being forced through tubing sections that were smaller than the diameter of the chorion. In response, the chorions would rupture and the embryo would frequently be fully separated from the chorion. Indeed, the blade was actually having no effect, as the eggs squeezed around it without damage. This serendipitous observation of what might have been a hydraulic extrusion dechorionation method served as the basis for a new technique that was further characterized and evaluated.

2.2 Evaluation process:

In order to evaluate and compare the current success of each dechorionation method for use in rapid drug-screening, a design matrix was constructed using several key design parameters. Specifically, five parameters were chosen, based on features deemed essential to a high-throughput process that would potentially be applicable to influenza research. The first parameter was the dechorionation success rate for each method. The second parameter was the survivability of successfully dechorionated embryos. The third parameter was the speed at which each method could dechorionate embryos. The fourth parameter was the usability of each method. The fifth and final parameter was the cost requirements associated with each dechorionation method. Details on how each parameter was measured and evaluated are described in the following section.

3. Materials and methods

3.1 Zebrafish

All zebrafish (*Danio rerio*) used in this study were handled in accordance with the University of Maine IACUC Guidelines for Use of Zebrafish in Research, Teaching, and Testing (Appendix A), and never allowed to develop past 72 hpf. Zebrafish were maintained in the Zebrafish facility at the University of Maine, Orono, ME. The facility was run in accordance with IACUC standards. IACUC approved guidelines for zebrafish maintenance and care adhered to standard procedures of a 14-hour light, 10-hour dark cycle at 28ºC. Embryos were collected from natural spawnings of adult AB zebrafish, and raised in egg water (60 µg/ml Instant Ocean sea salts; Aquarium systems, Mentor, OH) and incubated at 28.5 ºC. All dechorionation procedures were conducted on zebrafish eggs at 48±2 hpf. Before any dechorionations were performed, all dead, unfertilized, or clearly abnormal eggs were removed from each batch.

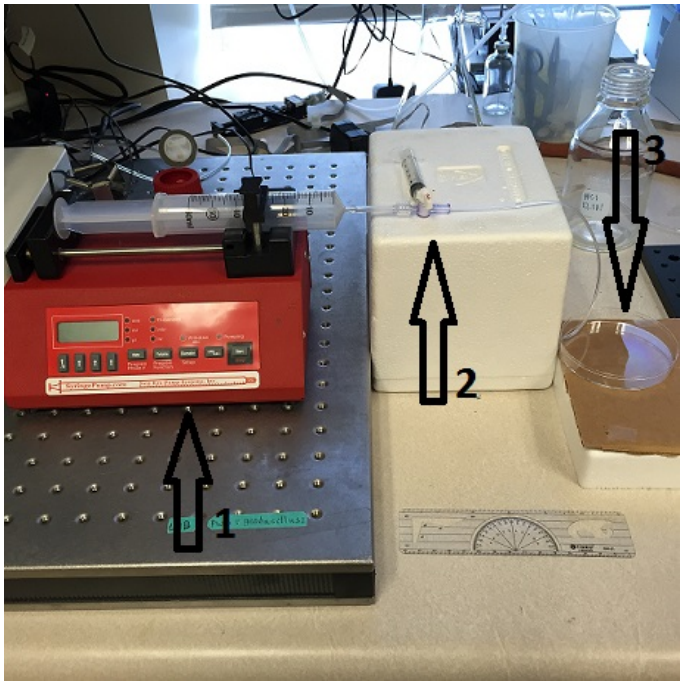


Figure 7: General set-up of extrusion dechorionation apparatus. (1) Syringe pump. (2) 4-way luer stopcock. (3) Collection dish.

3.2 Extrusion method set-up

In order to characterize and evaluate this novel dechorionation method in a reproducible fashion, a simple apparatus was set up as shown

(Fig. 7). In conjunction with a syringe pump (Fig. 8.A2) (NE-300 Just Infusion Syringe

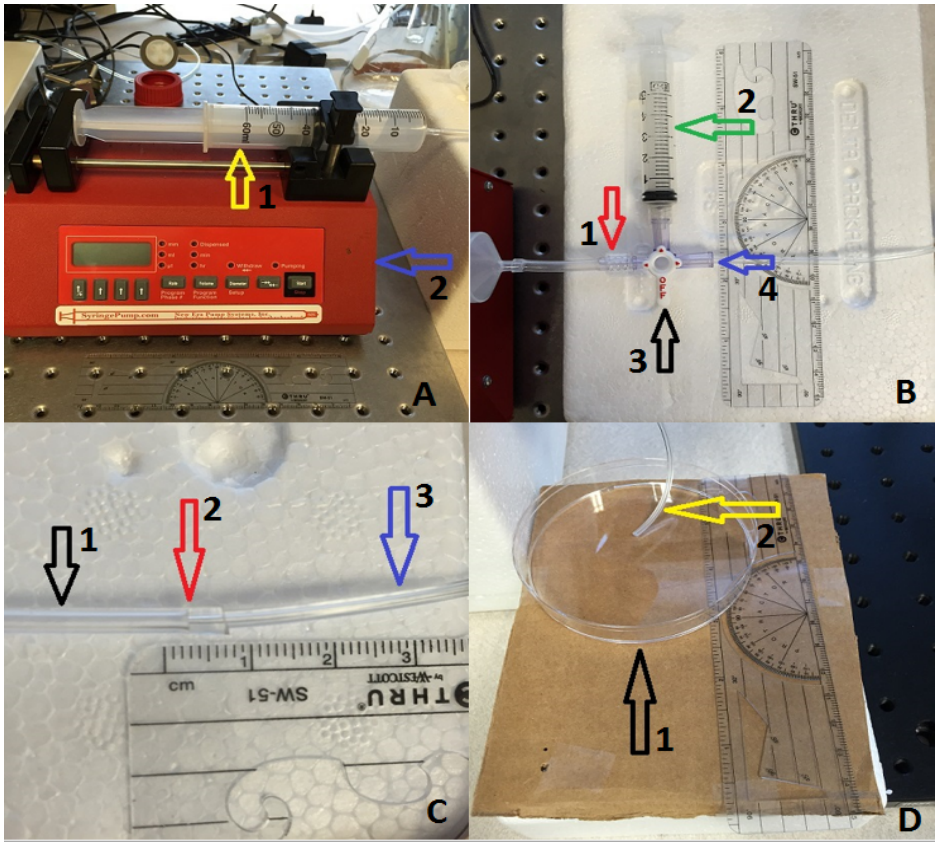


Figure 8: Components of extrusion dechorionation apparatus. (A) The 60 mL syringe (1) attached to the syringe pump (2). (B) 4-way stopcock (3) with the syringe pump port (1), injection syringe port (2) and 0.06" tubing port (4). (C) Site of shear dechorionation (2) at the junction of the 0.06" tubing (1) and the .03" tubing (3). (D) End of the .03" tubing (2) leading to collection dish (2).

Pump; New Era Pump Systems, Inc., Farmingdale, NY), a 60 mL syringe (Fig. 8.A1) (60 mL slip-tip syringe; BD, Inc., Franklin Lakes, NJ) was attached to a 4-way Luer stopcock (Fig. 8.B3) (WPI, Inc., Sarasota, FL), with a 5 mL injection syringe (BD Luek-Lok™; BD, Inc., Franklin Lakes, NJ) attached to the port perpendicular to the syringe pump (Fig. 8.B2), and 0.06" ID tubing (Tygon R-3603 Lab Tubing; Component Supply, Co., Fort Meade, FL) inserted into the port opposite the pump (Fig. 8.B4). From the stopcock, the 0.06" ID tubing extended approximately 2". The end of this tubing (Fig. 8.C1) overlapped with the end of a 7" segment of 0.03" ID tubing (Fig. 8.C3) (Tygon

MicroBore 0.03” ID Tubing; Component Supply, Co., Fort Meade, FL) which was inserted approximately 0.5 cm into the 0.06” ID tubing This was the location at which dechorionation occurred (Fig. 8.C2). The 0.03” ID tubing led to a plastic petri dish bottom for collection (Fig. 8.D1) (100 x 15 mm; Fisherbrand, Loughborough, Leicestershire, England).

3.3 Dechorionation procedures

To ensure reproducible results, a specific dechorionation procedure for each method was followed any time dechorionations were conducted.

3.3.1 Extrusion method

Before beginning each experiment, the syringe pump was allowed to run for 5-10 seconds, in order to wet the tubing and facilitate the positioning of eggs. To start, the stopcock was adjusted to block all flow through the syringe pump port. The 5 mL injection syringe was then removed from the stopcock and used to aspirate a single egg, along with approximately 2 mL of egg water, into its chamber. Next, the syringe was reattached to the stopcock and the egg was carefully injected approximately 1 cm into the 0.06” tubing. With the egg positioned, the stopcock was then adjusted to block all flow through the injection syringe port. At this point, the pump was turned on and run at a flow rate of 20 mL/min, and the resulting dechorionated embryo was collected in the petri dish at the end of the 0.03” tubing. The syringe pump was then turned off and the stopcock was readjusted to block all flow through the syringe pump port. This process was repeated for each egg being dechorionated.

3.3.2 Forceps method

All dechorionations using this method were conducted under a stereoscope (series SZ-STS; Olympus, Melville, NY). As previously described, this method began by first grasping the chorion of an egg with both forceps (#5 Dumont 72700-D; EMS, Hatfield, PA) adjacent to each other. The forceps were then gently pulled apart from one another, slowly tearing the chorion. If the embryo did not immediately swim out of this opening, this procedure was repeated to enlarge the tear. If the embryo could not escape the chorion, the chorion would be inverted with the forceps so that the embryo would fall out. This process was repeated for each egg being dechorionated.

3.3.3 Protease method

All dechorionations using this method were also conducted under the same stereoscope. This method began by first draining the water out of the plastic petri dish (100 x 15 mm; Fisherbrand, Loughborough, Leicestershire, England) holding the eggs. Next, 25 mL of a protease solution at a final concentration of 0.5 mg/mL (*Streptomyces griseus*, ≥3.5 U/mg activity; Sigma-Aldrich, St. Louis, MO) was poured into the petri dish. The eggs were allowed to incubate until all eggs were dechorionated (Fig. 9). These eggs were agitated frequently (~ every 2 minutes) via manual pipetting (Disposable Polyethylene Transfer Pipet 13-711-7; Fisher Scientific, Pittsburgh, PA) to facilitate dechorionation. After the incubation was complete, the protease solution was carefully decanted from the petri dish. The dish was then refilled with 50 mL of egg water, and the embryos were washed in this fresh water via manual pipetting.

The egg water was then drained, and this washing procedure was repeated two more times to ensure that all remaining enzyme was removed. After the third wash, the dish was refilled with egg water for the last time. This process was repeated for each batch of eggs being dechorionated.

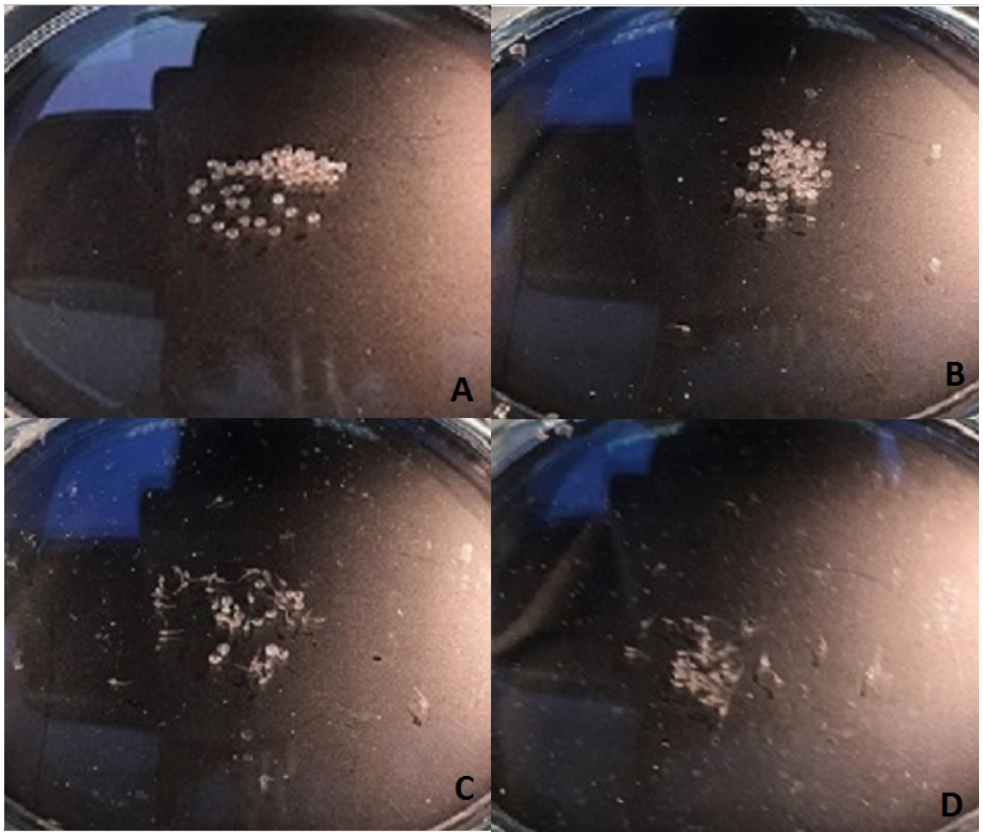


Figure 9: Progress of a typical protease dechorionation procedure. More eggs become dechorionated over time. (A) 0 minutes. (B) 5 minutes. (C) 10 minutes. (D) 15 minutes, dechorionation fully complete.

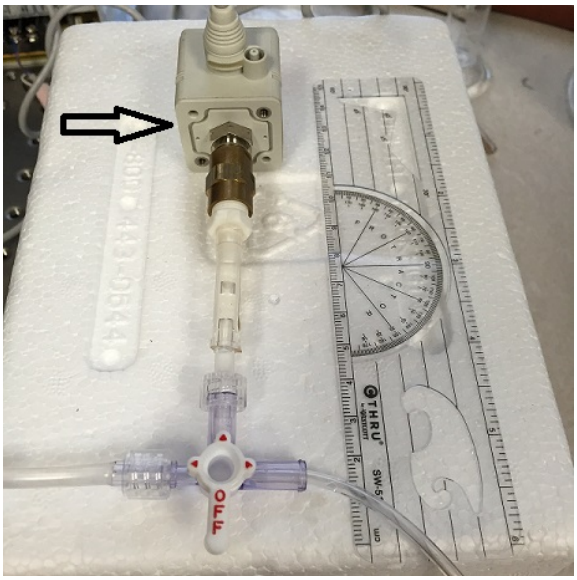


Figure 10: Set-up used to monitor average pressure drop required to induce extrusion dechorionation. Digital pressure switch (arrow) replaced the injection syringe after each egg was positioned.

3.4 Initial characterization of extrusion method

3.4.1 Effects of variable flow rate on dechorionation success

In order to gain a better understanding of the mechanism by which the extrusion method worked, some initial characterization was performed. As this method is intended to be used in high-throughput screening, the rate at which eggs could be passed through this system without degrading the success rate was of critical importance. Five different flow rates were tested in order to determine if the flow rate of the suspending medium had an effect on dechorionation success. Flow rates of 3, 7, 12, 16, and 20 mL/min were evaluated, using a sample size of 20 eggs at each flow rate.

3.4.2 Average pressure drop required for extrusion dechorionation

Additionally, a second investigation was conducted in order to measure the average pressure drop necessary to extrude an egg through the .03" ID tubing and induce dechorionation. In order to do this, the 5 mL injection syringe connected to the stopcock was switched out, after injecting the egg, with a High Precision, Digital Pressure Switch

(Fig. 10) (series ISE40; SMC, Yorba Linda, CA). The stopcock was then adjusted to permit flow through all three ports, so that the pressure behind the egg could be monitored, and the syringe pump was allowed to run. A maximum pressure could be identified just before the egg entered the 0.03" tubing, and this maximum pressure drop

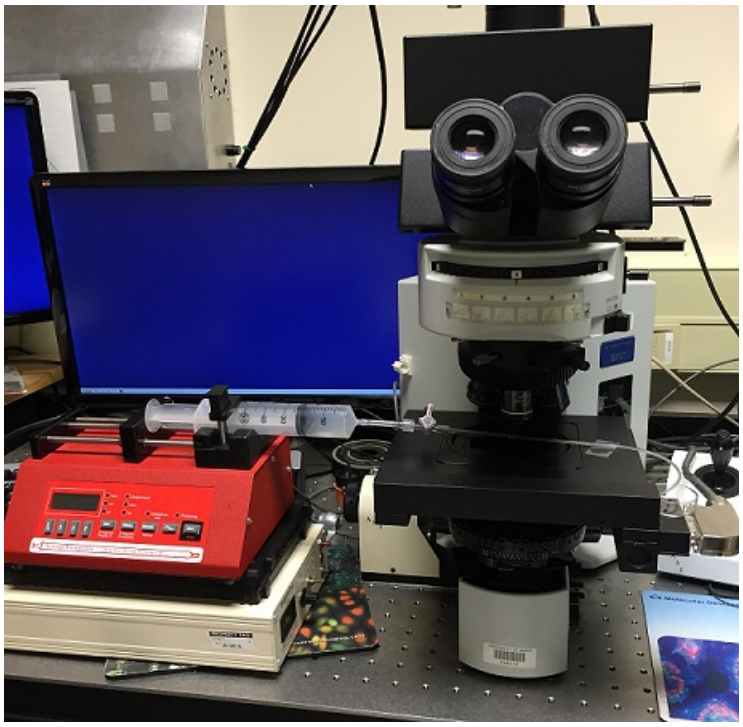


Figure 11: Set-up used to visualize extrusion dechorionations.

was measured and averaged over 20 dechorionations. Two trials were performed, with respective flow rates of 3 and 20 mL/min, in order to further elucidate the relationship between required pressure drop and flow rate.

3.4.3 Visualization of extrusion dechorionation

Lastly, using the image analysis software Metamorph (version 7.7.2.0; Universal Imaging, Bedford Hills, NY) in conjunction with a system microscope (series BX51; Olympus, Melville, NY), a series of time lapse digital video recordings were taken during the extrusion dechorionation process (Fig. 11). Using a 4x magnification objective lens,

a time lapse interval of 0.5 seconds between frames, and a flow rate of 3 ml/min, the dechorionation of individual eggs was observed and recorded as they moved from the 0.06" ID tubing into the 0.03" ID tubing. Interest was primarily focused on better understanding how the chorion is actually removed, but also in determining what parameters dictate if a dechorionation will be successful or not.

3.5 Evaluation of design parameters

3.5.1 Dechorionation success rate

The dechorionation success rate for each method was measured over the course of three trials. In each trial, 50 eggs were dechorionated using each method, respectively. For the purposes of this study, embryos were only considered successfully dechorionated if they were fully separated from their chorion and sustained no visibly obvious injuries, e.g. yolk sac partially torn off. After each set of dechorionations, the number of embryos that were considered successfully dechorionated was determined and recorded.

3.5.2 Survivability

Within the same three trials, the survivability of successfully dechorionated embryos was also evaluated. After each dechorionation procedure, the successfully dechorionated embryos from each method were placed into plastic petri dishes. Additionally, in each trial 50 non-dechorionated eggs were set aside in a separate petri dish to act as a control group. All four petri dishes were then allowed to incubate for the next 24 hours, or until they were approximately 71 hpf. At this point, the number of embryos that had developed normally after dechorionation was determined for each method. The number of embryos from the control group that had developed normally

was also determined. The survivability for each method was then measured as the ratio of the percentage of successfully dechorionated embryos that developed normally to the percentage of control group embryos that developed normally. Determination of normal development was based on descriptions and images of normally developed zebrafish between 48 and 72 hpf [9]. In evaluating normal development for the control group, whether an embryo had hatched yet or not was disregarded, as it is known that time of hatching is generally not indicative of normal development.

3.5.3 Dechorionation speed

The speed at which embryos could be dechorionated by each method was also evaluated over the same three trials. This was measured by determining the time required to attempt dechorionation by each method, using a batch of 50 eggs. Timing was accomplished using a standard iPhone stop watch. For the forceps method, timing began as soon as the first dechorionation was begun, and timing was stopped just as the last dechorionation was finished. For the shear method, timing began as soon as the first egg was aspirated into the 5 mL injection syringe, and timing was stopped as soon as the final egg was delivered to the collection dish. For the protease method, timing began as soon as the initial egg water draining process was started, and timing was stopped as soon as the third wash was completed. All preparatory procedures or other actions not immediately essential to the actual dechorionation procedure, such as preparing the protease solution, were not included in the timing process.

3.5.4 Usability

In evaluating the ease of use of each method, the required operator effort and skill were considered as primary factors. Rankings were based on experience using each method in evaluating the three previous design parameters, dechorionation success rate, survivability, and dechorionation speed.

3.5.5 Cost

In evaluating the costs associated with each dechorionation method, only components deemed absolutely necessary to the proper application of the three previously outlined dechorionation procedures were taken into consideration. For each method, both the initial costs and the continuous costs were evaluated.

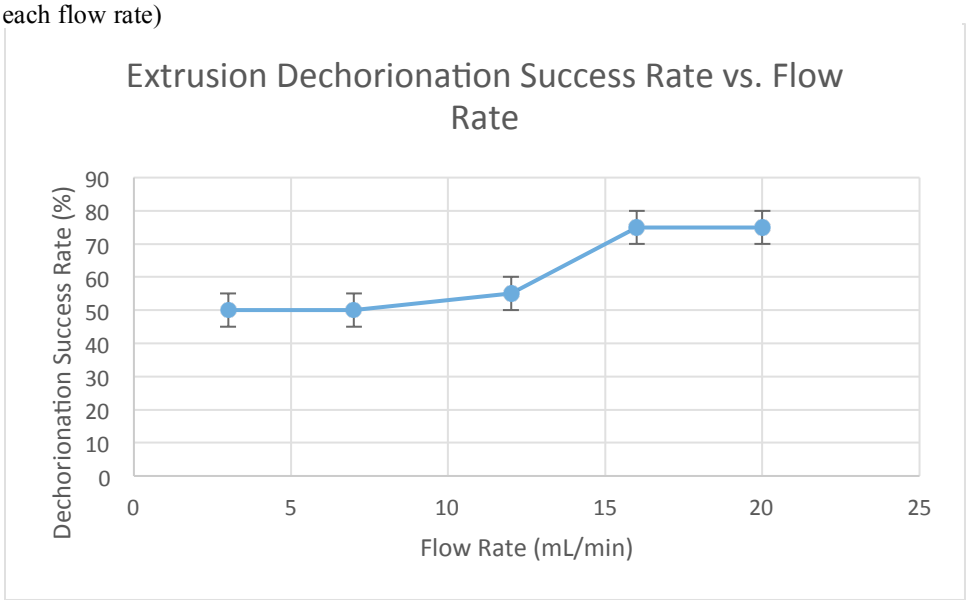
4. Results

4.1 Initial characterization of extrusion method

4.1.1 Effects of variable flow rate on dechorionation success

As is shown (Fig. 12), the resulting extrusion dechorionation success rate was greater at higher flow rates. At 3 and 7 mL/min the success rate remained at 50%, increasing only slightly to 55% at 12 mL/min, and then increasing to 75% for flow rates of 16 and 20 mL/min, respectively.

Figure 12: The relationship between the applied flow rate and the extrusion dechorionation success rate. At five respective flow rates, eggs were dechorionated using the extrusion method and the relative success of each trial was recorded. (n=20 for each flow rate)



4.1.2 Average pressure drop required for extrusion dechorionation

Table 1: Relationship between applied flow rate and average pressure drop required to induce extrusion dechorionation. The average maximum pressure drop required to induce extrusion dechorionation, at flow rates of 3 and 20 mL/min, with their respective standard deviations. (n=20 for each flow rate) * $p = .86$, $\alpha = .05$

Flow Rate (mL/min)	Average ΔP (psi)	Standard Deviation (psi)
3	3.2*	1.1
20	3.3*	1.1

The pressure drop required to induce extrusion dechorionation, at flow rates of 3 and 20 mL/min, was 3.2 and 3.3 psi, respectively (Table 1). The standard deviations of both values were approximately 1.1 psi. The difference between these two observed average pressure drops was not statistically significant.

4.1.3 Visualization of extrusion dechorionation

Several images from time lapse imaging of a successful extrusion dechorionation are shown (Fig. 13). What should be emphasized is the position of the yolk sac (red

arrows) over time. As the chorion is slowly pushed farther into the 0.03" tubing, the yolk sac also appears to be slowly pulled closer toward it. The relative orientation of the yolk sac does not appear to change over the course of the last three images. An image of the embryo post-dechorionation is shown (Fig. 14). It should be noted that the embryo is completely unharmed and successfully removed from its chorion.

Several images from the time lapse of an unsuccessful extrusion dechorionation are shown (Fig. 15). Again, the position of the yolk sac (red arrows) over time should be emphasized. The yolk sac appears to remain just at the outer edge of the 0.03" tubing throughout all four images. Simultaneously, the rest of the egg appears to be getting pulled in closer to the 0.03" tubing as well, although its movement is less obvious than in Figure 14. An image of the embryo post-dechorionation is shown (Fig. 16). embryo is also fully removed from its chorion.

It should also be reemphasized that both Figure 13.D and Figure 15.D are the final time lapse images from each respective dechorionation, before the maximum pressure was reached and each egg was fully extruded through the 0.03" tubing.

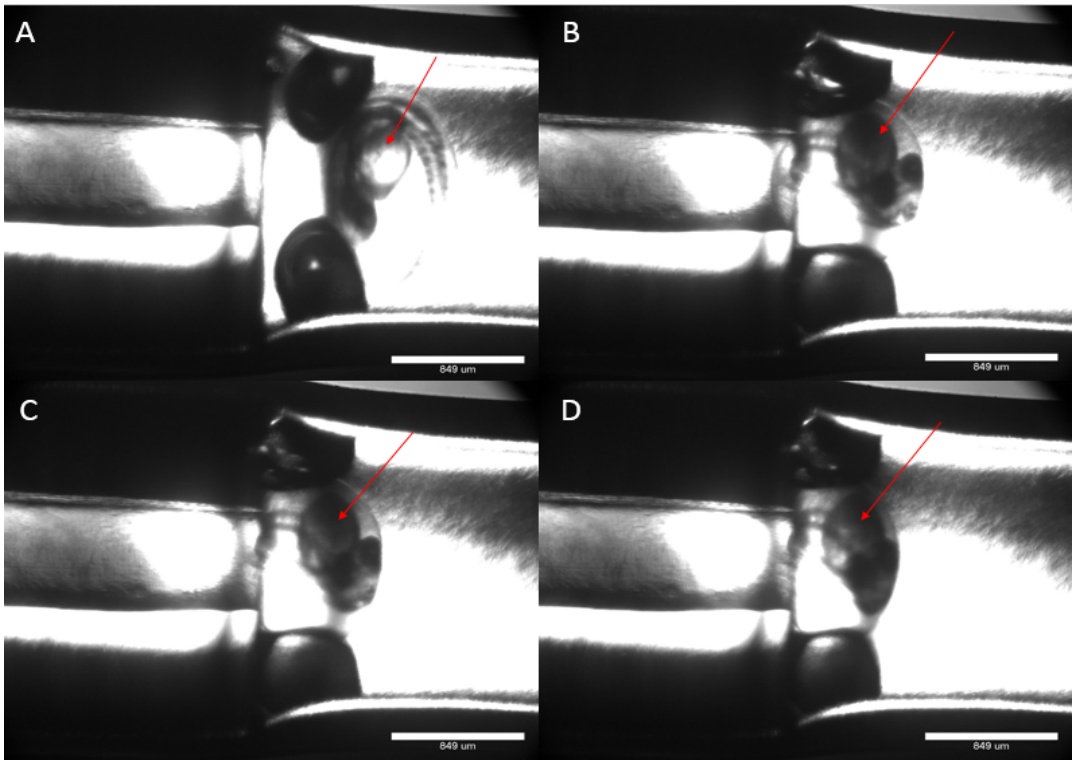


Figure 13: Images from time lapse of successful extrusion dechorionation. 0.03" tubing is on the left, and the 0.06" tubing is on the right in each image. (A) 0 seconds. (B) 7.5 seconds. (C) 15 seconds. (D) 22.5 seconds. (Arrows) Yolk sac of embryo.

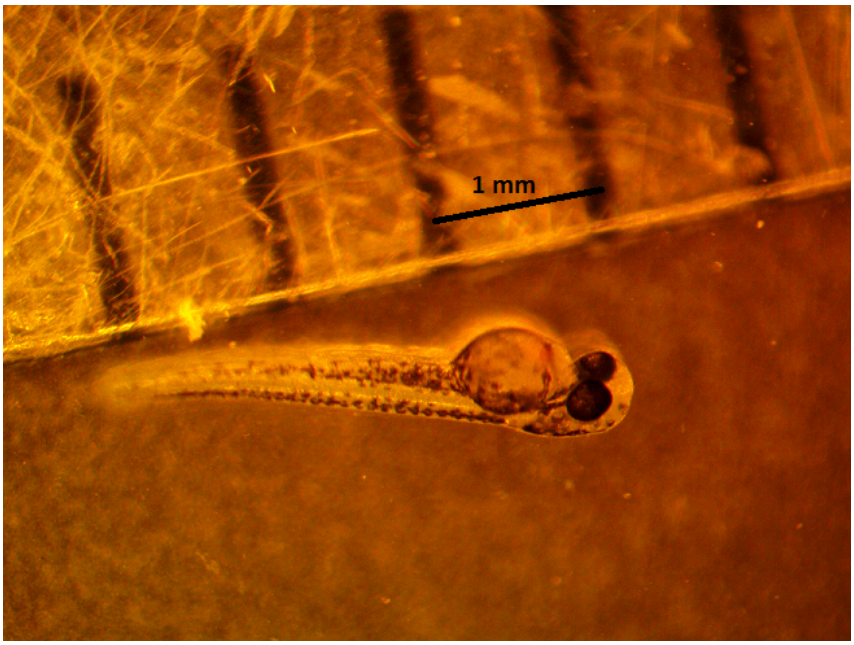


Figure 14: Embryo successfully dechorionated using extrusion method.

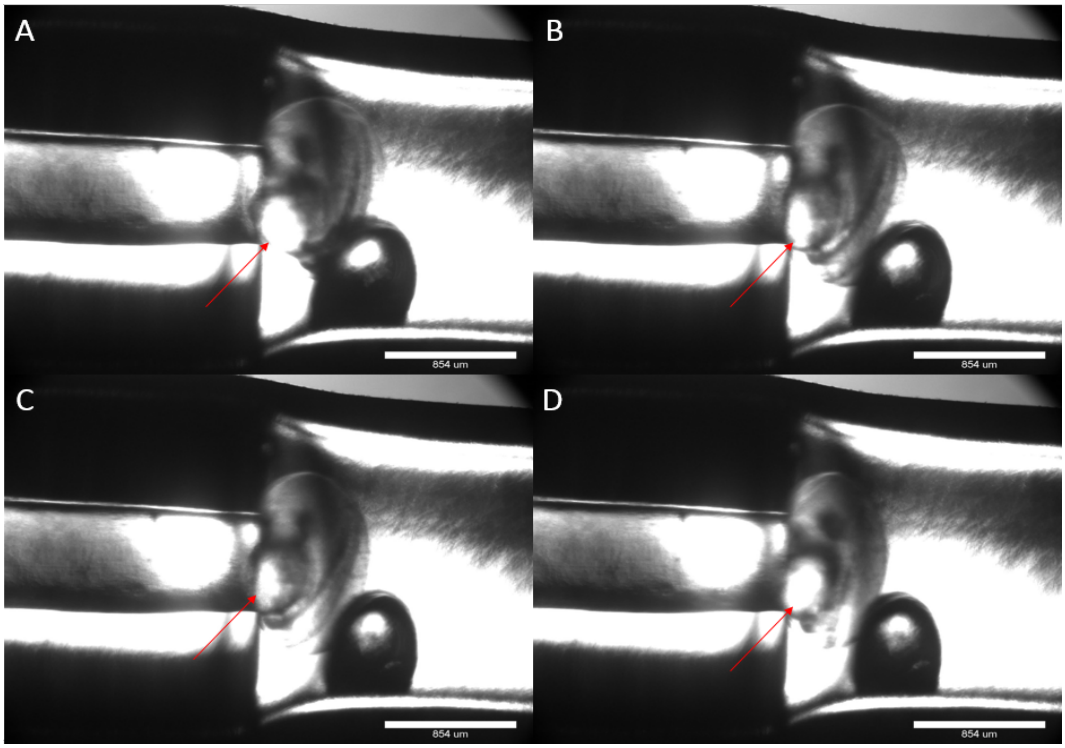


Figure 15: Images from time lapse of unsuccessful extrusion dechorionation. 0.03" tubing is on the left, and the 0.06" tubing is on the right in each image.(A) 0 seconds. (B) 6.5 seconds. (C) 13 seconds. (D) 19.5 seconds. (Arrows) Yolk sac of embryo.

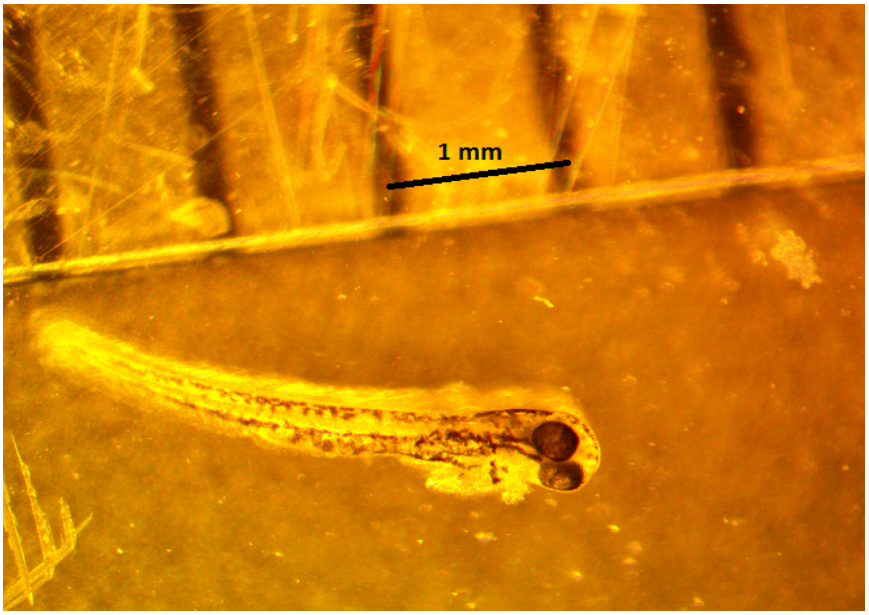


Figure 16: Embryo unsuccessfully dechorionated using extrusion method

4.2 Design parameters

4.2.1 Dechorionation success rate

Table 2: Results for dechorionation success rate trials. The dechorionation success rate for each method over three trials, and their respective averages. (n=50 per trial)

Trial #	Forceps (%)	Protease (%)	Extrusion (%)
1	100	100	36
2	96	100	40
3	96	100	38
Average	**97**	**100**	**38**

Over the three trials performed, the protease method was found to have the highest average dechorionation success rate of 100% (Table 2). The forceps method had the next highest average success rate of 97.33%, and the extrusion method had the lowest average success rate of just 38%. It should be noted that the vast majority of failed extrusion dechorionations were due exclusively to damage sustained by the yolk sac of the embryo.

4.2.2 Survivability

Table 3: Results for survivability trials. The survival rates of embryos dechorionated using each method over three trials, relative to the control group, and their respective averages. (n=50 per trial)

Trial #	Forceps (%)	Protease (%)	Extrusion (%)	Control (%)
1	102	91.8	85	98
2	100	100	85	100
3	93.8	100	84.2	100
Average	**98.6**	**97.3**	**84.7**	**99.3**

It was found that those embryos successfully dechorionated using the forceps method ultimately had the highest survivability of 98.6%, relative to the control group (Table 3). The protease method produced the next best survivability of 97.3%, and the extrusion method produced the worst survivability of 84.7%, relative to the control

group. The control group had an average survivability of 99.3%, which was the highest average survivability observed, relative to each dechorionation method.

4.2.3 Dechorionation speed

Table 4: Results for the dechorionation speed trials. The dechorionation speed of each method over three trials, and their respective averages. (n=50 per trial)

Trial #	Forceps (seconds)	Protease (seconds)	Extrusion (seconds)
1	524	1288	1130
2	529	1243	1113
3	454	1574	1113
Average	**502.3**	**1368.3**	**1118.7**

In evaluating dechorionation speed (Table 4), it was found that using the forceps method dechorionation on a batch of 50 eggs was the fastest, finishing in just over 8 min on average (502.3 sec). The extrusion method was the second fastest, with an average time of about 18 min and 30 sec (1118.7 sec). The protease method was the slowest dechorionation method, taking just under 23 min on average (1368.3 sec).

4.2.4 Usability

Evaluating the usability, which was defined as how easily each method could be learned and properly used, was strictly a qualitative assessment. It was determined that the protease method currently has the best usability. Because the operator does not need any real skill to perform it, and the only effort required is the occasional mixing of the eggs to help induce dechorionation, and the brief washing procedures. The extrusion dechorionation method had the next best usability. Besides the initial set up, the operator's only task was to manually position each egg in the tubing with the injection syringe and turn the pump on and off. While this relatively easy task required minimal skill, it could become quite tedious over time as it must be performed individually for

every egg being dechorionated. The forceps method was ultimately chosen as the least usable dechorionation method. This was due to the level of dexterity required of the operator in manually dechorionating each embryo, without causing damage. While practice can significantly improve operator stamina, fatigue is still a legitimate concern in applying this method to large batches of eggs (>50).

4.2.5 Cost

Table 5: Costs associated with dechorionation methods. Both the initial costs and continuous costs for each method were considered. For the continuous costs, the price is broken down as cost per dechorionation (DC).

Methods	Initial Costs ($)		Continuous Costs ($)		Total ($)
	Component	*Price ($)*	*Component*	*Price($/DC)*	
Forceps	Dumont #5 Forceps (72700-D)	32	Labor	0.11	32 + .11/DC
Protease	Protease from *Streptomyces griseus*	48	Protease from Streptomyces griseus	0.12	48 + .12/DC
Extrusion	Tygon Lab Tubing 1/16" ID	0.32	NE-300 Just Infusion Syringe Pump	2.70E-06	278.16 + 2.7E-6/DC
	Tygon MicroBore Tubing .03" ID	0.83			
	NE-300 Just Infusion Syringe Pump	275			
	BD Luer-Lok Tip 5 mL Syringe	0.33			
	BD Slip-Tip 60 mL Syringe	1.68			
	4-Way Luer Stopcock	4.33			

In determining the cost to use each method, two factors had to be considered: the initial costs, and the continuous costs (Table 5). In analyzing initial cost, the extrusion method was clearly the most expensive ($278.16), as it required a syringe pump which was by far the most costly component out of the three methods ($275). Next was the protease method, which only required the protease from *Streptomyces griseus* ($48). This left the forceps method as the cheapest method initially, as only the forceps were required ($32). However, when continuous costs were factored in, it became clear that

the protease method was ultimately the most expensive. This is because it had the most significant continuous cost ($.12/DC), as the protease must be consistently repurchased. When factoring in the continuous costs for labor ($0.11/DC), the forceps method became the second most costly. Labor costs were based on an operator who can dechorionate an average of 1400 eggs per day, while working 8 hours a day at a pay rate of $20 an hour. While there is a continuous energy cost for running the syringe pump, it was marginal ($2.7E-6/DC) in comparison to the protease and forceps long term cost requirements. Therefore, the extrusion method was determined to be the cheapest dechorionation method overall, followed by the forceps method as the second most costly, and lastly the protease method as the most expensive option.

4.3 Design matrix

Table 6: Design matrix comparing the relative capabilities of each dechorionation method. Each parameter was given its own weight based on perceived importance. The method that produced the best results for each parameter received a 3, the next best a 2, and the worst received a 1.

Methods	Cost (x1)	Speed (x2)	Usability (x2)	Survivability (x3)	DC Success Rate (x3)	Totals
Forceps	2	3	1	3	2	**25**
Protease	1	1	3	2	3	**24**
Extrusion	3	2	2	1	1	**17**

In comparing the three dechorionation methods via the above design matrix, the method with the best result for each design parameter was given a 3, the second best method was given a 2, and the least effective method a 1. Each design parameter was given its own weight (x1, x2, or x3), based on how important each was considered to be in the dechorionation process. The forceps method received the highest score (25), having achieved the best results for the dechorionation speed and survivability parameters. The protease method received the second highest score (24), having achieved

the best results for the usability and dechorionation success rate parameters. Lastly, the extrusion method received the lowest score (17), with the best results for the cost parameter.

5. Discussion

5.1 Extrusion characterization

In an effort to optimize the process of dechorionating zebrafish embryos for high-

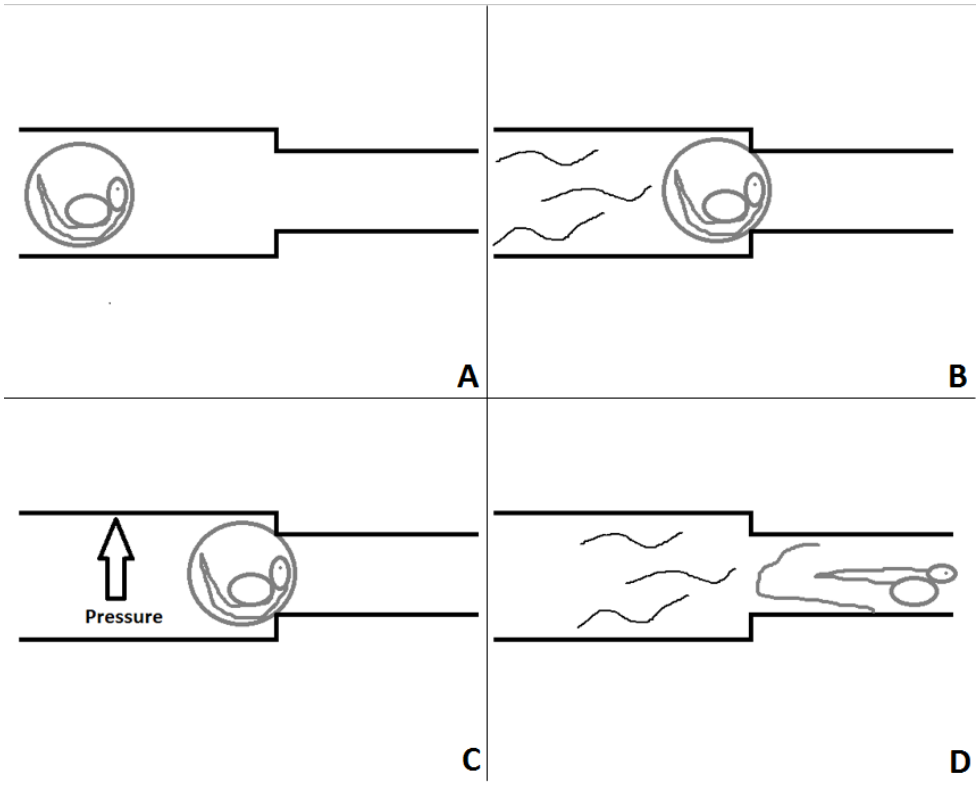


Figure 17: Cartoon depicting extrusion dechorionation process. (A) Zebrafish egg moving through larger diameter tubing. (B) Egg contacts smaller diameter tubing, stopping all flow. (C) Pressure begins to build behind egg as it continues to occlude the flow path. (D) Chorion yields to pressure build up and tears open, dechorionating the embryo and allowing normal flow to resume.

throughput applications in influenza research, a novel hydraulic extrusion dechorionation method was discovered (Fig. 17). As previously described, this extrusion method consists of pumping a zebrafish egg through a series of tubing segments with decreasing

inner diameter, until it eventually contacts tubing with an inner diameter approximately 30% smaller than that of an average egg. As the egg reaches the opening of this tubing, the malleable nature of the chorion allows it to act as a plug, temporarily stopping all flow within the system. As the flow rate of water entering this system is constant, hydraulic pressure begins to build up behind this plugged egg. Once the pressure is great enough, the egg is suddenly extruded through this smaller tubing and both the embryo and the chorion are quickly ejected from the system. Depending on a variety of factors, the chorion may be ripped off during this event, resulting in a successfully dechorionated and unharmed embryo. Alternatively, the dechorionated embryo may be damaged in this process, or the chorion may not be removed at all. To gain a better understanding of this unique dechorionation method, some initial characterization was performed.

As one goal of this study was to optimize the dechorionation process to be more high-throughput, therefore the speed at which these eggs could be pumped through this system was of primary interest. To address this, the relationship between flow rate and dechorionation success rate was investigated. The results of this experiment initially seemed to indicate that higher flow rates resulted in higher dechorionation success rates, as the success rate resulting from the highest flow rate used (75% at 20 mL/min) was 25% higher than that for the lowest rate used (50% at 3 mL/min). However, upon conducting many more dechorionations (150) using a maximum flow rate of 20 mL/min, an average dechorionation success rate of only 38% was observed. This conflicted with the initial results from the variable flow rate investigation, suggesting that many more trials need to be conducted at all previously tested flow rates in order to achieve a more

accurate picture of this relationship, or that experimental parameters that had not been identified were confounding the results of these tests.

Another feature of interest in this process was the amount of pressure required to induce extrusion dechorionation. As the significance that the flow rate has in this process was unknown, the average maximum hydraulic pressure was measured at the highest (20 mL/min) and lowest (3 mL/min) flow rates tested. The average pressure drops for these two flow rates (3.3 and 3.2 psi, respectively) were not found to be significantly different, and both flow rates produced a standard deviation of approximately 1.1 psi. This indicated that, while there was substantial variability in the required hydraulic pressure from egg to egg, the applied flow rate did not appear to have any effect on this factor. Rather, the flow rate only affected how quickly this maximum pressure was reached and, therefore, how fast the eggs were dechorionated. The time it took to dechorionate individual eggs at each flow rate was not determined, but the difference in speed was evident. This suggests that, rather than using a constant flow rate, a pulsed flow might be used instead to limit any excess shear stress a naked embryo may experience downstream from the site of dechorionation.

Since the actual extrusion dechorionation process happens so quickly and at such a small scale, a series of time lapses images of embryos being dechorionated using this method were recorded in the Olympus system microscope. Specifically, one successful and one unsuccessful dechorionation were recorded and compared. The time lapse images are consistent with the hypothesized mechanism for dechorionation. Essentially, once the egg has occluded flow into the smaller diameter tubing, the hydraulic pressure behind the egg begins to slowly build, forcing it farther into the tube. The pressure

continues to rise until, what is thought to be a shear stress experienced by the chorion induced by the outer walls of the 0.03" tubing, reaches a maximum and the chorion is torn open, freeing the embryo and allowing normal flow to resume. Unfortunately, this process does not appear to guarantee the safety of the embryo.

As noted in the results for the dechorionation success rate, the extrusion method seemed to specifically damage the yolk sac of the embryos whenever it would fail. The time lapse images help to explain why this was occurring. In the time lapse images of the unsuccessful extrusion dechorionation, the yolk sac of the embryo appeared to be pinned behind the outer edge of the 0.03" tubing, the edge closest to the camera, throughout all four images. The post-dechorionation picture showed the yolk sac of the embryo was torn off, suggesting this occurred as it was pinned on the edge of the tubing. Alternatively, in time lapse images of the successful extrusion dechorionation, the yolk sac can be seen positioned away from this edge, almost in line with the center of the tubing. The post-dechorionation image showed that its yolk sac was intact, suggesting that it avoided this edge and remained unharmed. Therefore, it is believed that the orientation of the embryo, as it is carried to the 0.03" tubing and eventually extruded through it, may be a critical factor in determining if the dechorionation will be successful.

While the chorion normally acts as a protective barrier, in this process it appears to behave more like a net that is wrapped around the embryo. As the embryo is curled up in its normally inflated chorion, it cannot fit through the 0.03" tubing. However, since the chorion is so flexible, it can be deformed slowly and pushed into the smaller tubing as the hydraulic pressure continues to build, also deflating it. As a result, the embryo's space within its chorion is slowly diminished, as it is reeled in closer to the opening of the

tubing. Depending on the orientation of the embryo, the yolk sac may be pinned behind the edge of the end tubing wall as the chorion continues to pull the embryo in. If this happens when the maximum pressure is reached, the yolk sac will likely be damaged as the chorion finally yields and the remainder of the embryo is instantaneously forced through the smaller tubing. Unfortunately, there are often bubbles present in the system (as is seen in both time lapse image series) that interfere with the eggs' orientation. Additionally, the embryos tend to wiggle around within the chorion during the dechorionation process. Therefore, it seems that controlling the position and orientation of individual embryos as they approach the extrusion dechorionation site may not be realistic for this particular model.

5.2 Evaluation and comparison of dechorionation methods

In order to compare the three dechorionation methods outlined in this study, a design matrix was constructed which focused on several design parameters, all determined to be crucial aspects of a successful dechorionation process. The results of testing the parameters identified in this matrix seemed to indicate that, currently, the forceps method is the best method for dechorionating 48 hpf zebrafish embryos. The protease method was deemed slightly less capable by just two points, and the extrusion method proved to be the lowest performing dechorionation method by far. What gave the forceps method its primary advantage were its dominant speed and the high survival rate of dechorionated embryos. However, even with these benefits, the forceps method has no legitimate potential for use in rapid drug screenings, or any high-throughput process in which embryos numbering in the hundreds to thousands might be required. Its lack of usability and fatigue constraints present too great a barrier to make its use realistic in this

context. It is, however, the ideal method when working with smaller sample sizes, but when given a large volume of eggs the required operator effort can quickly jump to unreasonable levels.

Based on the design matrix, the next best option to consider would be the protease method. This method yielded a 100% dechorionation success rate and was considered the easiest procedure to use. Interestingly, it also produced very high and consistent survivability results, almost surpassing the survival rates of the forceps method. This was particularly surprising, as the final protease concentration used (0.5 mg/mL) was much higher than those used in either of the other two studies (.001 and 0.1 mg/mL, respectively) focusing on the survivability of protease dechorionated zebrafish embryos [17] [18]. A higher concentration was used here in an attempt to speed up the overall dechorionation process. While these results certainly do not prove that the protease method has negligible side-effects on the development of zebrafish embryos, or on their susceptibility to pathogens or environmental toxicants, it does suggest that the embryo's age at the time of dechorionation is an important factor. Regardless, the costs associated with this method still pose a potential problem to its implementation in high-throughput drug screenings. While the concentration of protease can be reduced to conserve enzyme, this will also decrease its potency and result in longer incubation times, slower rates of dechorionation, and potentially higher mortality. Overall, there still may not be enough known about this method with regards to proper enzyme concentration, exposure duration, applicable developmental age, and potential side-effects, to permit its use in such high volume applications.

Lastly, the extrusion dechorionation method appeared to perform quite poorly relative to the other methods, especially with respect to the two most important parameters, survivability and dechorionation success rate. It was preferable to the protease method in terms of cost and speed parameters, and to the forceps method in usability and cost again, but was still easily the least capable method overall. However, the design matrix fails to address this method's potential for improvement. As discussed in the characterization of this method, if these extrusion dechorionations still are effective with more of a gradual decrease in tubing diameter, rather than the immediate jump from 0.06" to 0.03", then a significant improvement in the survival and dechorionation success rates may be observed. Additionally, if a more streamlined mechanism for positioning individual eggs for extrusion dechorionation existed, it is likely that both the usability and dechorionation speed of this method would also be improved dramatically. Hypothetically, the combined effects of these improvements could vault this method from worst to best dechorionation method, relative to the other two. Thus, while the capability of this method is currently quite limited, its potential for further development is significant and warrants continued research.

6. Conclusions

6.1 Current work

While most individuals are familiar with seasonal influenza infections, many do not realize the serious threat they pose to our health and global economy. The annual reoccurrence of this pathogen forces the continual development of new and effective antivirals to combat their rapid evolution. Additionally, the potential for a truly novel strain to develop and trigger a pandemic outbreak is a constant global threat. The use of

animal models in research has been an invaluable tool in the fight against influenza. Not only do they help us gain a better understanding of the pathogenesis of this disease, they can also aid in the development of new drugs and therapies. The prominence of the zebrafish as an animal model has already been established in a variety of fields. The zebrafish has recently been successfully infected with two strains of human influenza A virus, and shown to mimic clinical symptoms of influenza infections in humans [8]. In addition, upon treatment with the anti-influenza drug, Zanamivir, infected fish displayed legitimate signs of improvement [8]. Thus, as the zebrafish model has demonstrated uses in studying viral infection, it also has potential for the screening of novel antiviral therapies.

While there are several bottlenecks that currently stand in the way of fully optimizing the use of zebrafish in rapid anti-influenza drug screenings, this investigation focused specifically on improving the process of dechorionating zebrafish embryos. As there are currently only two commonly used methods of dechorionation, the forceps and protease method, it was the goal of this study to discover a third technique that would be applicable to use in high-throughput assays. In doing so, a novel hydraulic extrusion method was identified and described. Initial characterization of this method suggested that more research and development will be necessary before it can be used in such high-throughput applications. While the applied flow rate does not seem to have an effect on the pressure drop required to induce dechorionation, its effects on the dechorionation success rate are still unclear. However, based on images taken of actual extrusion dechorionations, the resulting success seems to depend on the position of the embryo in the chorion at the time of dechorionation, relative to the end wall of the 0.03” tubing.

This positioning appears to be random in nature, however, and thus may not be controllable in the current method.

In an effort to compare the relative capabilities of these three dechorionation methods, a series of experiments were conducted in order to evaluate each method via several key design parameters. Each parameter was given a weight, and the relative performance of each method for each parameter was ultimately compared through a design matrix. Based on these result, the forceps method was considered the best current dechorionation method, followed by the protease and extrusion methods, respectively. However, while the use of forceps in dechorionation is ideal when working with small samples, it would likely never be applied in a rapid drug screening, due to its lack of usability. While the protease method produced very promising results as well, more needs to be understood about its use, in terms of potential side effects and applicability, before it should be legitimately considered. Extrusion dechorionation was clearly the least capable method at this point in time, but it is at an early stage of development. Given its potential, with continued research it is expected that the extrusion dechorionation method could be the method of choice for high-throughput dechorionation. However, much work will be required before that point is reached.

6.2 Limitations

As with any research, certain limitations existed in the evaluation of the relative capabilities of these three dechorionation methods. Probably the most significant limitation to the results presented here was the fact that only zebrafish embryos approximately 48 hpf were used in evaluating each method. This point in development was chosen because it is the age at which zebrafish are dechorionated and infected for

influenza research [8]. As discussed, the embryo's developmental age is thought to be a crucial factor in determining how successful a certain dechorionation method will be. For example, the main reason the forceps method produced superior results was because, at 48 hpf, embryos are able to swim out of a penetrated chorion and are much more resistant to mechanical handling. If 24 hpf or younger embryos were used, the results are likely to have been far less impressive.

A second limitation that is worthy of note is the definition of a successful dechorionation. For simplicity, it was limited to only those embryos that had been physically unharmed and fully removed from their chorion. While data related to this was not tabulated, there was a fraction of the embryos which, when run through the extrusion dechorionation process, emerged completely unharmed but still remained within the chorion, which was quite deflated. These dechorionations were considered unsuccessful, although the embryos were completely unharmed. As such, the resulting analysis does not differentiate embryos damaged in the extrusion process described from those that simply failed to have their chorion removed.

The last significant limitation to this study was the fact that survival rates were only monitored up to 1 day post dechorionation. This was because, due to the IACUC guidelines to which this investigation adhered, zebrafish could not be allowed to develop past 72 hpf (Appendix A). As all dechorionations were performed at about 48 hpf, survival rates could only be monitored for 24 hours at most. With respect to a realistic drug screening process, the survival rates of dechorionated embryos would need to be monitored up to at least 5 days post dechorionation, as this is the length of time that mortality is monitored in influenza infected fish [8]. In order to allow the collection of

these crucial long-term survivability results future experiments would need to be extended.

6.3 Future work

Looking forward, there is still much to do before the zebrafish model can be used in the rapid identification of novel anti-influenza treatments. Specific to the optimization of the dechorionation process, there are several different research paths that can be taken. First, with respect to the novel extrusion method described in this paper, a much more in depth characterization of this method needs to be conducted in order to understand the relationship between parameters such as the flow rate and tubing diameter and the effect that they have on the dechorionation success rate. As the vast majority of failed dechorionations appeared to be due to the yolk sac being torn off as it passed the lip of the smaller tubing, potentially using a more gradual decrease in diameter, such as with a glass pipette, should be investigated. In such a device, there would be no significant edge for the yolk sac to be pinned against, and ideally, the chorion would receive all of the force as it is increasingly deformed, up until it ruptures and safely ejects the embryo downstream. Also, as the vast majority of time using the extrusion dechorionation method was spent manually positioning individual eggs, and so the development of a mechanism to automate this process would greatly increase the dechorionation rate. Ideally, an entire batch of eggs could be poured into this device, and the eggs would be released sequentially into the tubing for dechorionation. Multiple dechorionation paths, using parallel pump systems could further expedite this process and allow handling of larger numbers of eggs. Last, using suction rather than a pumping mechanism to move eggs through the tubing and to induce dechorionation should be investigated.

The second path that could be taken is further investigating the potential optimization of the other two dechorionation methods. Even while the forceps method is relatively limited in its potential for further development, the protease method has shown legitimate promise for use in rapid dechorionations. If there was more statistical evidence supporting its use, especially with respect to its potential side effects, it could easily become another prominent option for these high-throughput applications. Additionally, determining the final protease concentrations and incubation times that work best at each developmental stage is key to further development of this method for a broader range of applications.

Finally, as with any process, there are sure to be many more methods for dechorionating embryos other than the three discussed in this paper. Therefore, more effort could be focused on discovering other novel dechorionation methods. Considering how quickly zebrafish become a central animal model in such a broad range of research disciplines, the development of many other high-throughput applications for this model, each with their own unique requirements, is likely in the coming years. A single method that is effective in all applications would be ideal, but it is more likely that many methods will exist simultaneously, each with their own advantages and disadvantages, allowing researchers to select the one that meets the requirements of their research.

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8. Appendices

8.1 Appendix A

University of Maine
Institutional Animal Care and Use Committee
Guidelines for Use of Zebrafish in Research, Teaching, and Testing

General Guidelines

This document is intended to assist researchers/instructors working with Zebrafish in determining when Institutional Animal Care and Use Committee (IACUC) review is required. These guidelines were adapted from the "Guidelines for Use of Zebrafish in the NIH Intramural Research Program."

Current Office of Laboratory Animal Welfare (OLAW) interpretation of Public Health Service (PHS) policy considers aquatic species as "live, vertebrate animals" at hatching (as does the University of Maine's Policies and Procedures for the Humane Care and Use of Animals). Although this is an imprecise stage for Zebrafish, it can be approximated at 72 hours post fertilization. The IACUC has agreed on the following guidelines for all research, teaching, or testing activities involving Zebrafish:

1) 0-3 days post fertilization (dpf), IACUC protocol submission for approval IS NOT required; however, euthanasia guidelines must be followed, (see below).

2) 4+ dpf, IACUC protocol submission for approval IS required. Since early stages (4-7dpf) do not feel pain or distress, the researcher/instructor may check "no" for the pain category (question 7d of the Protocol Review Form) when working at those stages. The pain and distress categorization of the ≥8dpf fish should be determined by the investigator based on the specific procedures described in the protocol.

3) If proposed studies involve fish at both stages 1 and 2 above, the protocol should mention the use of Zebrafish at 0-3 dpf, but only descriptions of procedures at the 4+ dpf stage are required.

Scientific Background

These guidelines are predicated on the need to minimize suffering and distress in Zebrafish. Suffering requires that the animal have both the neural apparatus for detecting noxious stimuli as well as the mental ability to interpret such stimuli as aversive (1). Many studies have demonstrated that adult Zebrafish show evidence of higher order cognition, being responsive to a variety of learning protocols (e.g. 2, 3, 4, 5), including learning to avoid aversive stimuli (6, 7, 8, 9). Thus while the ability of adult fish to experience suffering remains controversial in the scientific literature [for recent reviews reaching conflicting opinions see (10) and (11)], there is sufficient evidence to take a cautious approach in adult Zebrafish by instituting guidelines that ensure rapid euthanasia.

In contrast, there is no evidence of higher order cognition in Zebrafish during the first week of development although this may change as research techniques in pain perception science improve (12). Developmental studies examining learning (13), reward (14), social (15, 16) and fright (17) behaviors have found that these functions become operational only in older fish. During the first week of development, embryonic movements are simple reflexes that do not provide evidence for a capacity for suffering. Thus during the first week, Zebrafish larvae can respond to simple stimuli but are assumed not to have reached the point in brain development where stimuli can be experienced as aversive.

Zebrafish larvae during the first week resemble early mouse embryos in that they are chiefly sustained by nutrients derived from the yolk. The criterion of nutritional independence for developmentally immature animals is subject to empirical verification and has found support in international regulations for the welfare of immature vertebrates (18). While the capacity for suffering is the primary criterion for establishing a threshold for 8 days post fertilization (dpf) for euthanasia in Zebrafish, the criterion of independent feeding also supports this age.

Hatching occurs at approximately 72 hours (which would be at the end of day 3 post fertilization), although hatching is not an accepted staging index in Zebrafish (19). Zebrafish larvae are not able to feed upon hatching and are sustained by nutrients derived from the yolk, which is not depleted until 7 dpf (20). Only after 7 dpf do Zebrafish larvae manifest signs of ill health in the absence of external feeding (21). Active feeding cannot commence at hatching because brain structures required for detecting and catching prey have not developed and the mouth and gut are occluded. At hatching, larvae lack taste buds (22, 23), have poor visual acuity (15), and cannot swim effectively as they lack a

swim bladder and have deficient motor control (24, 25). Therefore, in Zebrafish the period between hatching and nutritional independence at 8 dpf is essentially an extension of the early embryonic stage during which the fish continues to develop sensory and motor functions required for the independent larval stage.

Euthanasia Guidelines

The acceptable method of euthanasia of Zebrafish at all stages is by overdose of tricaine methane sulfonate (MS222, 200-300 mg/l) by prolonged immersion. Fish should be left in the solution for at least 10 minutes following cessation of opercular movement. A request for an exception to use any other method must be submitted to the IACUC for review/approval.

Zebrafish carcasses should be disposed of as according to University policies.

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Approved 06/27/2011

9. Author's biography

Thomas R. Hoffmann was born in Bangor, Maine on September 22, 1993. He was raised in Hampden, Maine and graduated from Hampden Academy in 2011. Thomas is currently majoring in Bioengineering, with a minor in pre-medical studies. He was the historian for the Sophomore Owls honor society, member of the Student Portfolio Investment Fund (SPIFFY), and just recently became a certified Emergency Medical Technician (EMT) in the state of Maine.

Upon graduation, Thomas' immediate plans are to find a job in the biotechnology industry. Long term, he would like attend graduate school, but is currently undecided between pursuing medicine or some other field of study.