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A Microfluidic System for Preparation and Digestion of Leaves to Isolate Protoplasts

THESIS

submitted in partial satisfaction of the requirements
for the degree of

MASTER OF SCIENCE

in Biomedical Engineering

by

Justin Ronald Stovner

Thesis Committee:
Associate Professor Jered Haun Irvine, Chair
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Associate Professor Elliot Hui

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DEDICATION

To my family for supporting me through everything,

To my friends for providing me with time away,

and to Angelica for always being there with me and accepting me for who I am.

Nature has music for those who listen.

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Abstract of the Thesis

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Justin Ronald Stovner

Master of Science in Biomedical Engineering

University of California, Irvine, 2021

Associate Professor Jered B. Haun, Irvine, Chair

Protoplasts are a prominent cell type for conducting gene transfection in the agricultural space in order to produce modified crops that can mature quicker or survive droughts. Traditional methods for isolation of protoplasts involve manually preparing leaves that are digested in an enzymatic solution containing cellulase and macerozyme for as long as six to eight hours. Understanding current techniques for isolation of protoplasts demonstrated there is prevailing need for a platform that can streamline this process, with quicker isolation times and less user interaction. Our lab has previously performed mammalian tissue digestion to isolate single cells with a microfluidic platform that utilizes mechanical shear forces in combination with enzymatic digestion. Initial tests were performed with the previously developed digestion device on tomato leaves, before being redesigned to suit leaf digestion by optimizing channel design and chamber holding volume. Tomato plants were used as our model because of their quick growth cycles, abundance and previous literature research. Multiple flow rates were tested to determine the ideal conditions for obtaining the highest yield and viability of protoplasts, through Trypan Blue staining. Protoplast yield and viability showed an increase over the digestion period, but a decrease with higher flow rates through the digestion device. Under optimal conditions the device yielded double the number of protoplasts in 2/3 the time compared to the traditional method, while

maintaining equivalent viability. In order to streamline the preparation steps, a second preparation device was developed to prepare leaf samples in a quicker manner than the traditional method. The preparation device was then tested in conjunction with the digestion device. Cell yield and viability from using the leaf preparation device were shown to be equivalent to traditional preparation method with Trypan Blue staining. Cells were then cultured and tagged with calcein blue and calcofluor white, to demonstrate continued viability and cell wall formation.

Introduction

Improving Protoplast Isolation

In the developed world an estimated 800 million people experience some form of food shortage, and this number is even higher in undeveloped countries (1). This number is expected to grow as population increases and the "green revolution", in the past century, is expected to hit a plateau. The shortage in the global food supply is not only related to consumption by people, but also livestock and new biofuels that are in development (2). These sectors are putting strain on the market and shortening the supply that is available for human consumption. An added strain to the global food supply is the perception there is a surplus in crops specifically with maize, rice, wheat and soy; which is influencing a decline in spending on agricultural research. As a result, it is predicted food supplies will fall short of the global demand by the year 2050 (3). A key solution to maintain the growth of the global food supply is the modification of plants cells, protoplasts, to increase crop yield, become drought resistance and pest resistance.

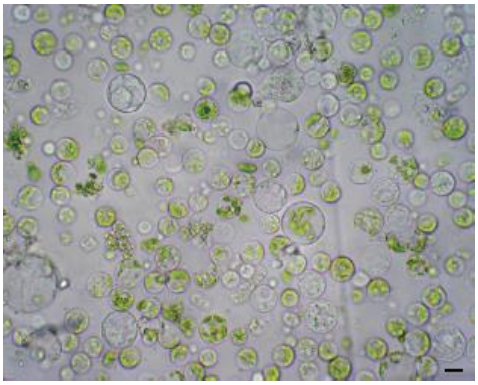

Figure 1 *– Rice Protoplasts*

Image of protoplasts obtain through a Nikon digital camera under an Olympus microscope using a 40x objective. Scale bar = 40 μm (4).

Protoplasts are plant cells liberated of their cell wall and are a versatile experimental system for gene editing. The first isolation of protoplasts was reported roughly 50 years ago, and since then have been used to observe and understand cellular processes, such as cell wall formation, cell division, photosynthesis, and embryogenesis (5). Recently genetic studies have been conducted on plants to observe gene integration and expression after transfection. Many techniques have been researched and implemented to modify genes within protoplasts such as PEG-mediated transfection, biolistic bombardment and *Agrobacterium*-mediated transient transformation (4). All of which have been used successfully to produce crops with faster growth cycles and drought resistance.

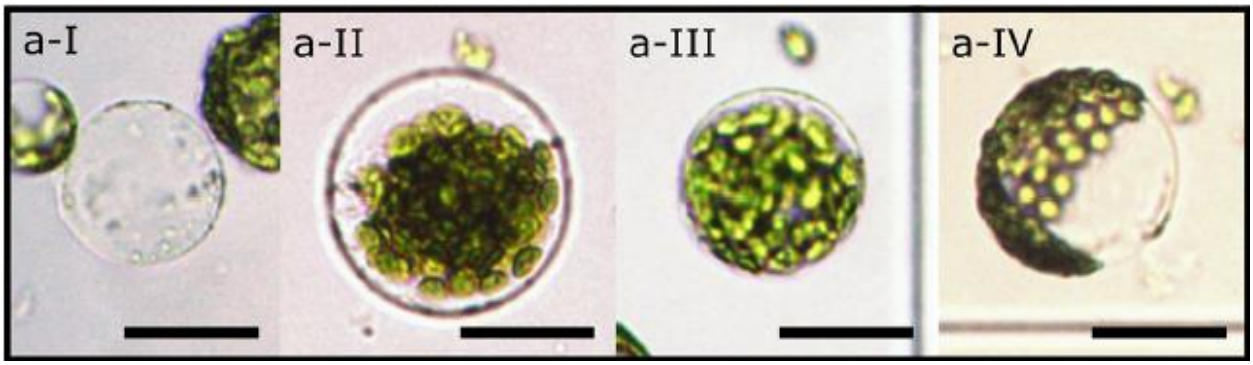

Figure 2 *– K. blossfeldiana 'Charming Red Meadow' Protoplasts*

Protoplasts from K. blossfelddiana 'Charming Red Meadow'. ***a:*** *Different types of protoplasts* ***a-I****: protoplast without chloroplasts;* ***a-II:*** *protoplast with chloroplasts concentrated in the middle of the cell;* ***a-III:*** *protoplast with chloroplasts distributed homogeneously throughout the cell;* ***a-IV****: vacuolated protoplast with chloroplasts concentrated in one area of the cell. Scale bar = 25 µm (6).*

Protoplasts have a unique morphology as they can look green to the naked eye. However, under a microscope the green hue is produced by chloroplasts which are small organelles that perform photosynthesis. Chloroplasts are typically oval shaped and are about 1-2 micrometers thick and 5-7 micrometers in diameter. Typical protoplast morphology can contain chloroplasts in many areas of the cell as shown by figure 2. Protoplasts are roughly 30-50 micrometers in diameter, spherical in shape, and translucent without their cell wall. These features can help identify intact protoplasts, fully released protoplasts (6).

Even with developments in the last 50 years, protoplast isolation is still considered a manual technique with multiple laborious steps. The steps typically involved with protoplast isolation are preparation of enzymatic and WI solutions, preparation of the leaves, desiccation of leaves, digestion of leaves, washing and filtering of leaves, and then cell counting and gene transfection. The enzymatic solution, containing cellulase and macerozyme, targets and breaks down pectin, cellulose and hemicellulose microfibril that hold the cell wall components together around the protoplasts (7, 8). The WI solution contains osmotic stabilizing compounds, such as mannitol, to preserve the isolated protoplasts after digestion. Leaves are prepared by cutting them with a scalpel or razor blade, into 1-millimeter strips; if larger leaves are used the central midrib is removed. The leaf strips are suspended in enzymatic solution, in a conical tube or cell plate; then the leaves must be completely submerged before being desiccated for 30 minutes. After desiccation the leaves are digested for a minimum of three hours, before being filtered through 70-micrometer mesh. The cells are concentrated and resuspended in WI solution before finally being counted and used for gene editing (4, 5, 9, 10, 11). The entirety of these experiment can take eight to ten hours and can have numerous errors based on human factors and interactions. There is a

need for devices that can streamline this process and reduce human interaction to reduce potential errors.

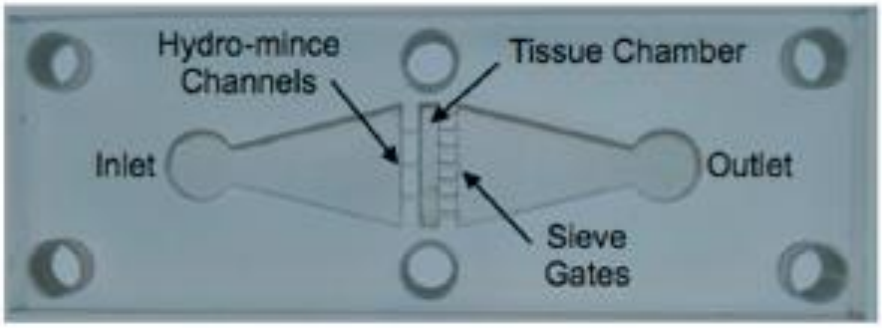

Figure 3 *– Digestion Device Schematic*

Image of laser-etched acrylic sheet containing the tissue holding chamber, hydro-mince channels (left) and Sieve Gates (right) (12).

Our research group has previously developed a device that can break down mammalian tissue into aggregates and single cells using fluidic shear forces in combination with enzymatic digestion. As shown in figure 3, the device was developed around processing a standard core needle biopsy from a mammal, roughly 1 centimeter x 1 millimeter, into a cell suspension without the need for manual processing. The initial design contained a holding chamber for the sample with channels on the left and right side. The channels on the left are called hydro-mince channels because of the hydrodynamic shear forces applied from the high velocity fluid flow into the holding chamber. On the right are sieve gates that hold the bulk of the tissue in the holding chamber, while releasing digested cells into the system. The initial channel designs were evaluated using COMSOL Multiphysics software and it was determined the optimal design channel design was three hydro-mince channels, 200 micrometers in width, with seven sieve gates. The height of

the channel was determined to optimally function at 1 millimeter. The evaluation of the channels was performed using 1 milliliter per minute flow rate, but the results can translate and scale according to higher flow rates. Device testing was initially conducted using beef liver tissue and returned cell viability between 70% and 80% with a cell yield higher than the control digestion (12).

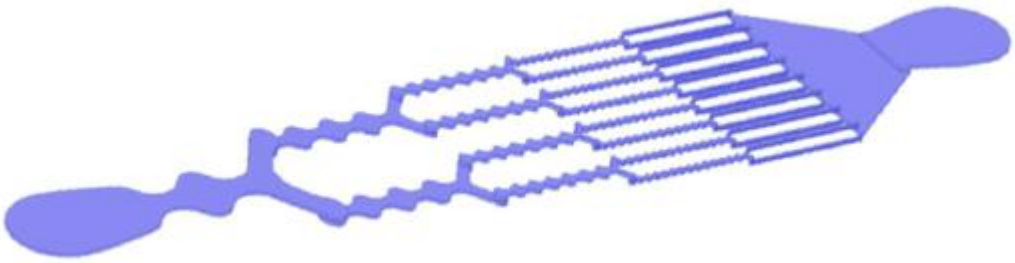

Figure 4 *– Dissociation Device Schematic*

Schematic showing the branching channel design containing alternating constriction and expansion regions (13, 14).

A second device developed by our research group utilized fluidic jets and high shear force zones in an oscillating channel structure to break down aggregate cells into single cells, as shown in figure 4. The dissociation device was designed to be downstream of the digestion device. As cells and aggregates break off from the bulk tissue in the digestion device, they proceed through the dissociation device to break down into single cells. The oscillating structure causes high velocity zones that create high shear force zones that break apart these large aggregates of cells that can then be used for analysis. The size of the channel is reduced by half, but double in quantity with every branch in order to maintain the same width across each stage and remain constant throughout the device. The reduction in channel size produces an effect referred to as

"hydrodynamic micro-scalpels" that precisely cut away cells from aggregates as the channel size becomes smaller. The device was first tested on colon cancer cell lines HCT 116 and LS 174T, and lung cancer cell line NCI-H1650 obtained from ATCC (Manassas, VA) (13, 14). The results showed a higher yield of single cells than the control, demonstrating the oscillating channel structure can be utilized to break aggregates into single cells.

Figure 5 *– Minced Digestion Device*

Image of the fabricated minced digestion device containing hose barb inlet/outlet and a central luer connector. (15)

A combination of the digestion and dissociation device was developed recently by our research group and found to fully digest and break down aggregates into single cells, as shown in figure 5. This device has a similar holding chamber as the initial digestion device, but has hydro-mincing channels on either side. This design iteration eliminates the asymmetrical layout and replaces it with a symmetrical layout to allow for bidirectional fluid flow. In addition, the hydro-mince channels were widened from 200 micrometers to 250 micrometers to reduce the shear force on recirculating cell. Outside of the hydro-mince channels are the dissociation channels that contain the oscillating structure for providing hydrodynamic micro-scalpels to break down

aggregates into single cells. A luer connector was incorporated above the holding chamber of the device for easy loading and unloading of the sample, and sealing of the device for processing. Murine kidney was initially characterized with this device and demonstrated an overall high yield of single cells compared to control (15).

Overview

Increasing the number of transfections for furthering the expansion of viable crops is reliant on how quickly protoplasts can be isolated. Using the previously developed minced digestion device, constructed from the digestion and dissociation devices, we can accelerate the speed at which digestion of leaves can occur while increasing the yield of protoplasts. The initial stage would be development of a leaf preparation device to quickly prepare samples of the appropriate size for digestion. We will need to load the leaves quickly, hold the leaves in place, and allow for quick slicing into proper sizes, without crushing. The protoplast viability from this device will need be maintained to allow for future use with culturing and gene editing techniques.

The second stage of the device is the minced digestion device that will digest samples and provide protoplasts. This device must be able to digest the leaves reliably without lowering the protoplast yield and viability. The channel design can be adjusted to accommodate for the protoplasts' susceptibility to damage from recirculation, and the large size of the protoplasts in comparison to mammalian cells. A scaled-up design is envisioned for digesting a larger sample size, that will encompass the modified channel design for protoplasts.

By eliminating the need for numerous human interaction steps and combining mechanical shear forces with enzymatic digestion. We ideally want the device to perform the entire digestion process to obtain protoplasts for downstream processes such as culturing or gene editing. My

project will explore and seek to create a suitable leaf preparation device that can be utilized with a modified and tested version of the minced digestion device. The next chapter outlines the design and fabrication methods used for creating the devices and the envisioned integration of these devices; while the following chapter characterize the constructed devices by evaluating flow rates, cell yield and cell viability.

Chapter 1

Design and Fabrication of Devices

In this section I will discuss the initial stages of this project, which include designing, fabricating and verifying a device for preparing leaves into 1-millimeter strips. Initial verification testing with the minced digestion device previously developed, and designing, fabricating, and verifying an expanded version of the minced digestion device.

Overview

Each device will be created and tested separately before being combined to create an integrated microfluidic system for digestion of protoplasts. The first phase, the leaf preparation device, was built based on the design criteria to prepare leaves quicker and consistently for the following sections. Numerous criteria directed the development of the leaf preparation device that align with our successes and drawbacks.

To reduce the sample preparation time of leaves for digestion, the device would need to cut a minimum of six leaves into 1-millimeter strips in a single step. As a result, our first design criterion for our device is to load and remove samples with minimal steps. The device was designed with two pieces that would combine, and split, to be able to access the cutting area. A second criterion is when the two parts of the device combine, they must not crush the leaves in order to prevent loss of viable protoplasts. Therefore, a gap was designed between the two halves of the device, to provide a snug fit for the leaves that would prevent crushing and sliding when being cut.

Slots were designed into the bottom half of the device with a 1-millimeter spacing to guide a cutting blade to consistently cut leaves into 1-millimeter strips.

Designing to reduce digestion time and combine mechanical digestion with chemical digestion was our second design criteria. Traditional methods for protoplast isolation focus on digestion in a chemical solution for multiple hours to break down the cell wall. A previously developed minced digestion device from our laboratory, that utilizes hydrodynamic fluid forces in the micro domain, can be combined with chemical digestion to shorten digestion times and increase protoplast yield. The hydrodynamic fluid forces would provide a mechanical means of breaking down the cell wall; in addition to removing digested components from the bulk of the leaves to allow for deeper enzymatic penetration.

Our third design criterion is scaling of the minced digestion device to verify the possibly of digesting larger samples of leaves for industry applications. To achieve this feat the digestion device was parallelized to enlarge the digestion chamber to three times the original amount. However, parallelizing the device would reduce the velocity across the tissue if a single channel input was implemented due to conservation of mass, or continuity, resulting in a need for higher initial flow rate through the entire system. As a result, the device was outfitted with three separate hose barb inlets that would all feed into the same tissue holding chamber. The original minced digestion device was designed for digesting mammalian tissue; as a result, the hydro-mince channel dimensions were widened to double the size to compensate for the size of the protoplasts. Maintaining the same flow rate as the original digestion device and increasing the width of the hydro-mince channels, will help minimize damage to cells during processing and recirculation.

Our device designs were guided by the design criteria to reduce the time needed for leaf preparation and reduce the time needed to digest leaf samples by combining mechanical digestion

with chemical digestion in order to increase protoplast yield and maintain protoplast viability. As a result, two separate devices were developed, manufactured and tested; that can be integrated for future testing with other downstream devices.

Leaf Preparation Device

Design 1

The initial design for our leaf preparation device was inspired by large fruit choppers and mammalian biopsy punches currently on the market. My device derives the quick single action preparation for chopping leaves; although, is scaled down to accommodate the size of leaves in order to produce the 1-millimeter required width. I created multiple iteration of the device utilizing SolidWorks – a computer-aided design software. Instead of having wires that chop leaves, like a fruit chopper, I utilize scalpel and razor blades to ensure a clean-cut edge on the leaf strips. A clean-cut edge on the leaf strips is necessary for allowing better infiltration of the enzymatic solution in order to obtain a higher yield of protoplasts.

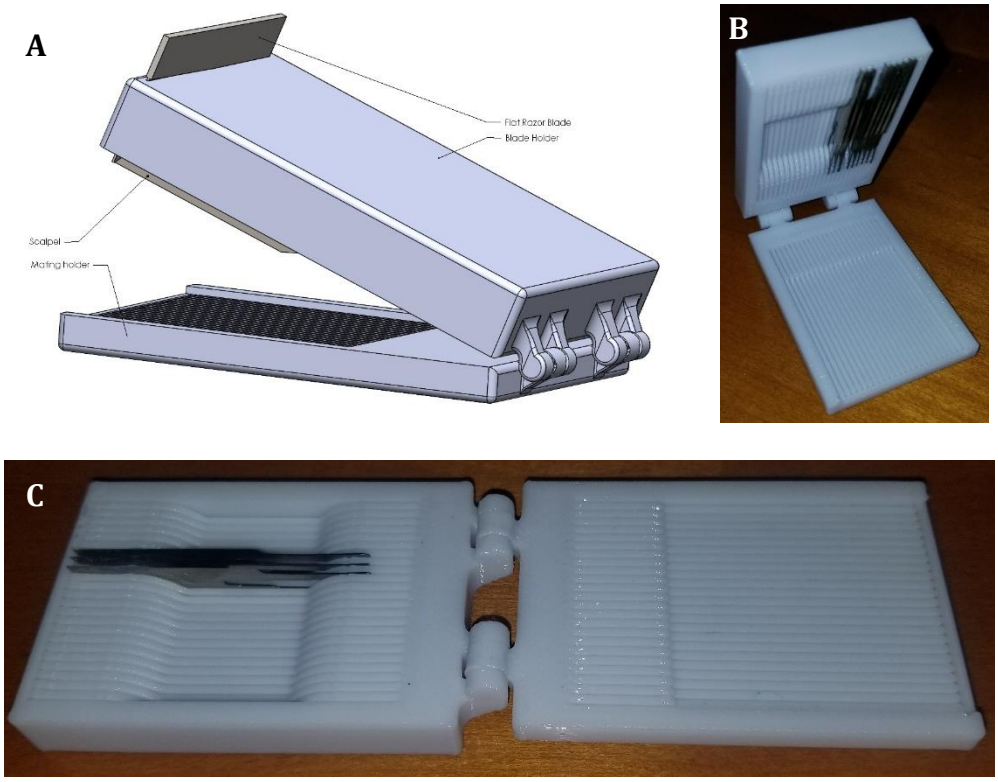

Figure 6 *– Leaf Preparation Device – Design 1*

Design one of the lead preparation device. ***A*** *CAD model of the first design of the leaf preparation device, containing scalpel and razor blades for preparing leaves.* ***B*** *Image of a fabricated leaf preparation device.* ***C*** *Image of the device fully opened, with the hinges snapped together.*

Figure 6 depicts the design that I created that encompasses two halves that mate together with snap fit hinges. Using a press fit, scalpel blades are embedded in the top part of the device, spacing them 1 millimeter apart. The bottom half is designed with mating shallow grooves with a depth of up to 1.75 millimeters. The shallow grooves provide a space for chopping into the leaves and also prevent crushing in order to create cleaner cut edges. A razor blade is embedded at one end of the device in order to remove the midrib of larger leaves. Leaves are oriented perpendicular

to the scalpel blades and the grooves, then the lid is closed to chop the leaves into consistent 1-millimeter strips.

Fabrication

There were several options to fabricate the first design of the leaf preparation device. We needed to consider a fabrication method that could produce thin walls for separating the scalpel blades and holding them in place. We also wanted the device to be made of a semi-rigid material to be able to hold its shape when being utilized, but snap fit together during assembly. The device also needed to be made of a biocompatible material to prevent cell death.

A preferred method for rapid fabrication of prototypes is 3D printing. This method allows for quick turn around and fast iterating of devices for prototyping. It also avoids the issues with spending large quantities of money on CNC machining or molds for plastic parts. Our first design was manufactured through 3D printing using Acrylonitrile Butadiene Styrene (ABS) on a Flashforge from RapidTech at the Institute for Design and Manufacturing Innovation (IDMI) facilities at the University of California, Irvine. Our second iteration of this design was also fabrication by RapidTech, but created using polycarbonate on a Fortus 3D printer.

As depicted in Figure 6-C the device was fabricated in two parts that could be snapped together. For an initial fabrication the device held the scalpels in place and could close completely. However, during our initial testing we found after the device was closed to cut the leaves, the scalpels did not fully chop through them. This could be a result of slight flexibility in the leaves causing them to slip into the bottom grooves and thus not be fully cut by the blades. From these tests a second design was developed.

Design 2

The second design retained the two-piece design with the hinges and snap assembly, as shown in figure 7. However, the top portion of the device was redesigned to remove the embedded scalpels and them allow to slide through 1-millimeter spaced slots to slice the leaves, instead of chopping. The design of the bottom half of the device was maintained with shallow grooves that mate with the slots on the upper half of the device. The bottom half of the device also has a small groove on the front end that is used to help clamp the lid shut to hold the leaves in place. This second design slices the leaves into strips, which is equivalent to the standard preparation technique for protoplast isolation – hand cutting. The leaves are placed onto the bottom half of the device and then the top half is closed, holding the leaves in place. Then a scalpel is slid through each slot on the top half of the device to slice the leaves.

Figure 7 *– Leaf Preparation Device – Design 2*

Design two of the leaf preparation device. ***A*** *CAD model of the second design of the leaf preparation device, with the two halves opened.* ***B*** *CAD model of the device with the two halves closed.* ***C*** *Image of the fully fabricated device.*

Fabrication

This device was also fabricated out of polycarbonate at RapidTech utilizing the Fortus 3D printer, as shown in figure 7. The preference for polycarbonate is because of its biocompatibility, durability and slight elastic properties. The two halves were printed separately and then assembled by snapping the hinges together. Results were obtained in combination with the digestion device, as presented later in chapter 4.

Digestion Device

Design

A microfluidic digestion device previously developed in our research group utilizes high velocity fluid flow to deliver high shear forces to tissue within a holding chamber. This was initially utilized for combining mechanical forces and enzymatic activity to digest mammalian tissue in order to obtain single cells; but has now been adapted for obtaining protoplasts from leaves. Initial designs of the device were previously created through Onshape; then later recreated in AutoCad and SolidWorks. Fluid dynamic properties were optimized previously through COMSOL Multiphysics software.

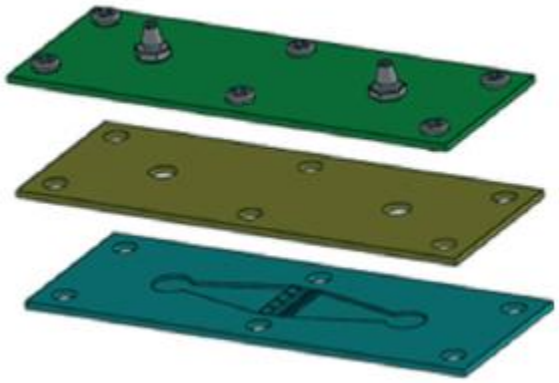

Figure 8 *– CAD Assembly of Digestion Device*

CAD assembly model of the digestion device: a PDMS gasket is sandwiched between two layers of acrylic, with the microfluidic channels laser etched into the bottom layer (12).

As shown in figure 8, the device has a 1.5 millimeter by 1 centimeter holding chamber size to hold tissue according to the size of a standard Tru-Cut core biopsy needle. Upstream of the tissue chamber are three hydro-mince channels with a width of 200 micrometers, that focus fluid flow into a high velocity jet that apply hydrodynamic shear forces on the tissue. Downstream of the tissue chamber are channels that act as a sieve gates with a width of 500 micrometers to hold the tissue in place, but allow smaller aggregates and cells to move out of the tissue chamber (12). The overall goal of this device is to mimic the standard methods for sample preparation of single cells, by scalpel mincing, through an automated microfluidic platform.

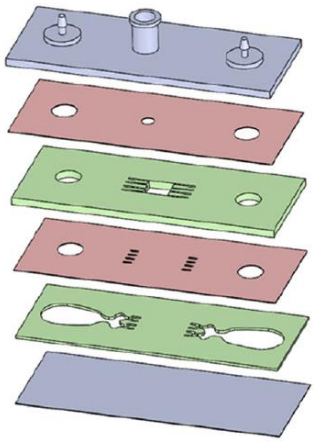

Figure 9 *– CAD Assembly of Minced Digestion Device*

CAD assembly model of the minced digestion device, containing PET, acrylic and PSA layers (15).

A second iteration of the device was developed later by our research group that compacted the original design and increased the number of hydro-mince channels from three to four, while also adding a luer connector inlet over the tissue holding chamber for rapid loading and unloading of a sample, as shown in figure 9. The holding chamber is 1 millimeter by 1 millimeter and can be sealed during processing by utilizing the luer connector and a stopcock. The device also contains a luer connector that the sample can be loaded through and then sealed with a stopcock to prevent leaking.

This second iteration also incorporated dissociation channels upstream of the hydro-mince channels and downstream of the sieve channels. These channels are from a separate design, the dissociation device, previously developed by our research group that is used for dissociation of aggregates into single cells, as by figure 4. The design of this device was modeled after a microvascular capillary network with a branching structure. The branching channel design maintains the same velocity throughout each channel, while also reducing the sizing of the channels by half for each branch, to increase the shear stress. To add a second design feature to

these channels and increase the shear force an oscillation in the channel structure was implemented. The oscillation was designed with smooth curves in order to avoid mixing from microvortices and maintain laminar flow (13, 14).

Fabrication

A VLS 4.60 60W CO_2 laser (Universal Laser Systems, Scottsdale, AZ) was used to fabricate the fluidic channels, of the first iteration of the device, out of acrylic. Polydimethylsiloxane (PDMS) was used to fabricate a gasket that was placed between a top and bottom acrylic layer. The entire assembly was held together by nylon screws, as shown in figure 10 (12). The device was made of acrylic and PDMS because it is biocompatible and can be easily fabricated to create multiple iterations of the device in a short period of time.

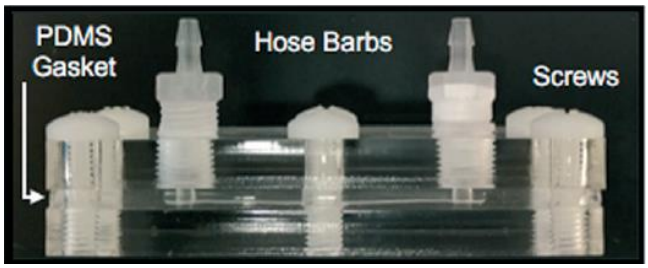

Figure 10 *– Fabricated Digestion Device*

Image of a fabricated digestion device (12).

The second iteration of the digestion device, the minced digestion device, was manufactured by ALine, Inc. (Rancho Dominguez, CA) using layer lamination technology. Each layer is made from hard plastic sheets with the pattern cut into the plastic using a laser etching device. The layers are then bonded together using pressure sensitive adhesive (PSA) to create the

final device, as shown in figure 5 (14, 15) The components of the device are known to be biocompatible and easy to fabricate on a commercial level.

Scaled-Up Digestion Device

Design

The scaled-up digestion device was designed as a parallelized version of the minced digestion device, as shown in figure 11. Similar to the minced digestion device, AutoCAD and SolidWorks were used for the designing process. It contains over three times the number of channels and three times the sample holding volume of the previous device. After parallelization two extra sets of inlets were added to maintain the same velocity flow through the hydro-mince channels of the device.

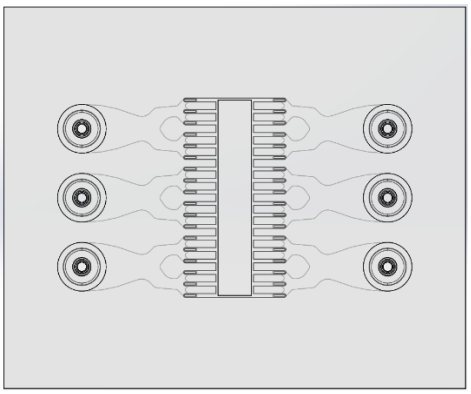

Figure 11 *– CAD Model of Scaled-Up Digestion Device*

CAD model of a scaled-up digestion device, with over triple the number of channels and inlets/outlets.

In response to the data collected from using the minced digestion device (presented in chapter 2), the dissociation channels were removed from the scaled-up device, because the

dissociation channels were concluded to induce enough shear force to reduce the viability of the protoplasts. This was concluded after understanding that protoplasts are vulnerable without a cell wall. A second design modification resulting from the size of the protoplasts, ranging from 30 to 70 micrometers in comparison to mammalian cells ranging between 5 to 30 micrometers previously tested with our device, was increasing the size of the hydro-mince and sieve channels from 250 micrometers to 500 micrometers in width; this would reduce the shear force on already released cells (15, 16). Instead of using a luer connector to load the sample into the device, an adhesive capping system was developed to allow for loading of sample into the entire tissue holding chamber. The adhesive capping layer was made of a single layer of each, PSA and polyethylene terephthalate (PET) that contained a small cutout in the PSA above the holding chamber; this would prevent PSA contact with the sample. After loading the sample into the tissue holding chamber, the capping layer was added to seal off the device. The scaled-up digestion device was designed to maintain cell integrity while still applying sufficient shear force on the leaf samples for mechanical digestion.

Fabrication

The device was fabricated using the same technique as the minced digestion device from ALine, Inc. Their laser etching and layer bonding technique with PSA, allowed for quick fabrication and assembly, while also guaranteeing biocompatibility.

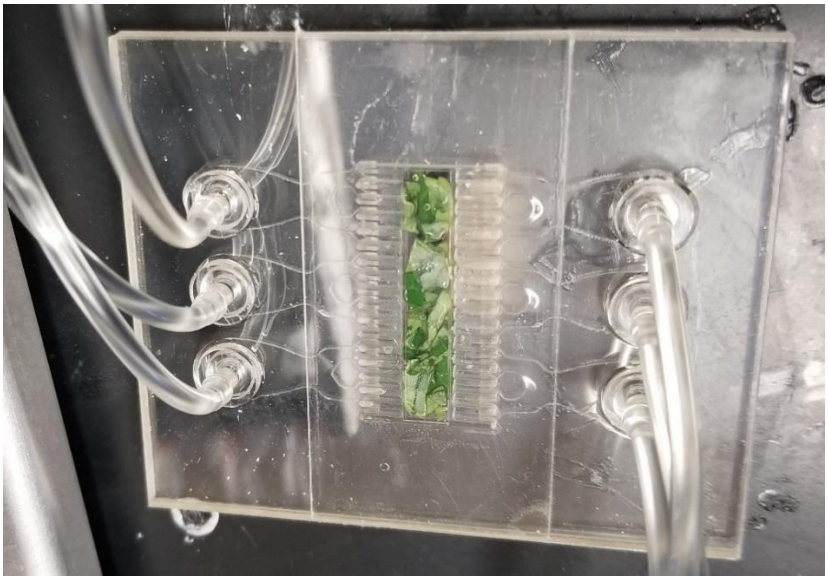

Figure 12 *– Scaled-Up Digestion Device*

Image of a fabricated scaled-up digestion device in use with leave digestion.

The final fabricated device was created with 7 fluidic layers bonded together with 6 PSA layers and 6 inlet/outlet hose barb ports for attaching to a fluid pump, as shown in figure 12. Layers 1, 3, 7, and 9 are made using PET to provide semi-rigid and thin layers for channel designs that range in thickness from 0.025 to 0.254 millimeters. Layers 5 and 11 are fabricated using acrylic to provide rigidity and stability for the device, that range in thickness from 0.508 to 1.524 millimeters. Layers 2, 4, 6, 8, 10, and 12 are made with double-sided PSA to bond all other layers together.

Theoretical Device Integration

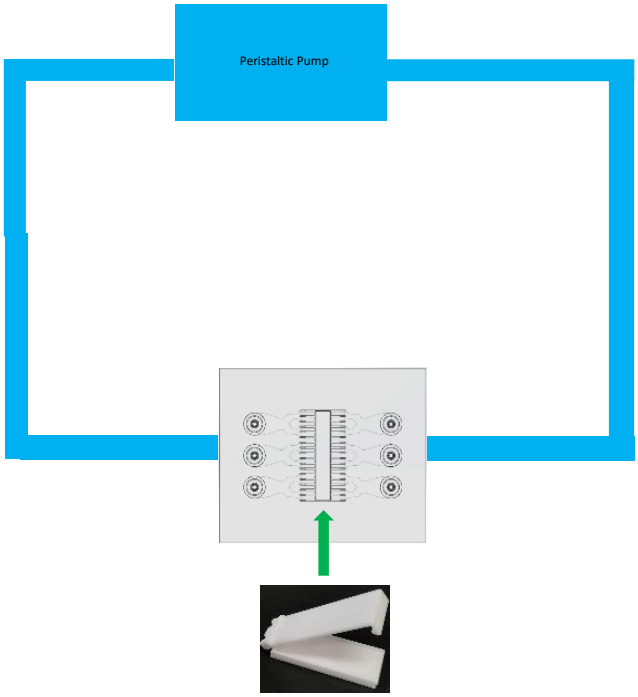

Figure 13 *– Diagram of Fully Integrated Digestion Platform*

Displayed is the envisioned integration of the two devices for the isolation of protoplasts.

After the leaf preparation and the scaled-up digestion device are optimized, they can be integrated as shown in figure 13. Leaf samples will first be sliced into 1-millimeter strips using the leaf preparation device. Prepared leaf samples will then be loaded into the scaled-up digestion device, then the device will be sealed off using a capping layer with PSA. The scaled-up digestion device will then be hooked up to a peristaltic pump and processed for the required amount of time.

Chapter 2

Minced Digestion Device Characterization with Plant Leaves

In this chapter, I will discuss tests for characterizing the mined digestion device with regards to optimal flow rates, cell yield and cell viability using leaf samples from tomato (solanum lycopersicum) plants.

Introduction

We wanted to determine if the minced digestion design could be utilized to digest leaves to obtain protoplasts. It has been previously demonstrated that the minced digestion device can be utilized to obtain single cells from mammalian tissue. Compared to plant leaves mammalian tissue is softer and less rigid, due to the absence of a cell wall. The cell wall of plants provides protection; thus, it can be determined that the leaves will respond to high velocity fluid forces in a different manner than mammalian tissue. In addition, partially digested leaves could potentially clog the channels and reduce the yield of protoplasts. The flow rates that return the highest protoplast yield and viability will be the ground work for further tests.

We first had to determine the sample size that can fit comfortably into the digestion device without blocking or clogging the channels. Since previous mammalian data showed positive results, we determined we would use a similar mass of leaves as our starting point. Once we determined a reliable amount of tissue that could fit into the digestion device, we could then test for protoplast yield and viability. Protoplast yield and viability was obtained by using Trypan Blue staining on a hemocytometry. Trypan blue staining shows if a cell membrane is compromised and

can help initially determine if a cell is alive or dead; and thus, provide initial feedback on the efficacy of the minced digestion device with leaves.

Materials and Methods

Tomato Plants

Device performance was determined by using tomato plant lines, due to their simple growth cycles and ease of maintenance in the laboratory. We chose to utilize Husky Cherry Red tomato plants due to their broad flat leaf structure and obtainability; however, we expect the results to be similar for other tomato plants species. The Husky Cherry Red tomato plant is a hybrid tomato plant designed to be indoors and sprout in a short period of time: in as little as three days.

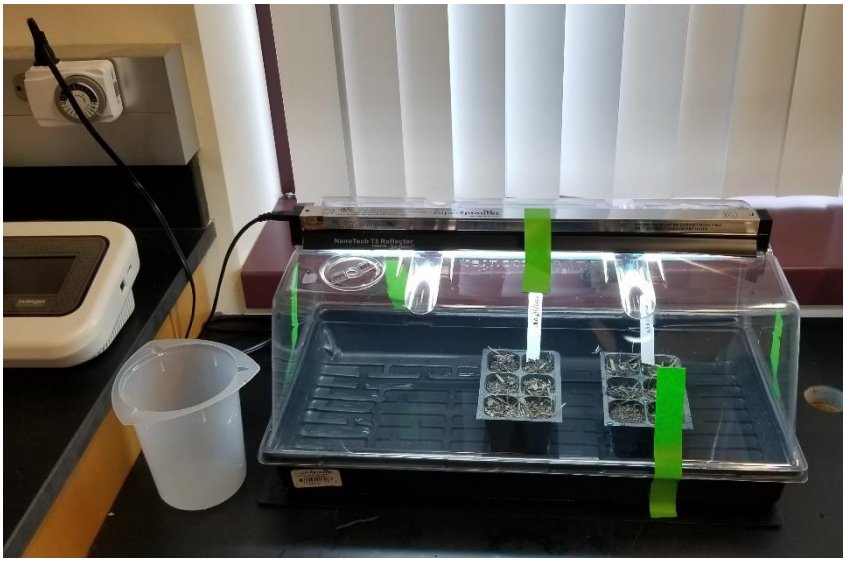

Figure 14 *– Green House Setup*

Image of a greenhouse setup in our laboratory, with grow light, heating pad, and a timer switch.

To control the growth of the tomato plants, a greenhouse was installed in our laboratory, as shown in figure 14. The greenhouse setup is controlled using a timer switch and runs on a 10-hour photocycle period to mimic environmental lighting conditions. Underneath the greenhouse is a heating pad to maintain the correct environmental temperature throughout the day, and cause evaporation of water placed in the bottom of the greenhouse to provide water to the plants. Tomato seeds were planted in six-well seed starters using Miracle-Gro Raised Bed Soil. Harvesting of the tomato plant leaves occurred between 2-4 weeks, and typically provided for more than 20-30 leaves.

Control Digestion

In order to quantify device performance a control digestion was implemented. About 4 to 7 leaves were prepared from 2–4-week-old tomato plants for each control group of two-hour and three-hour digestion times. The leaves were prepared by cutting them into 1-millimeter strips using a No. 11 scalpel and placed inside a 15-milliliter conical tube. Two solutions were prepared, Enzymatic for digesting of the leaves and WI for resuspending the protoplasts. Enzymatic solution was created using 20 mM of MES, 20 mM potassium chloride, 10 mM calcium chloride, 0.4 M mannitol, 1.5% (wt/vol) cellulase R10 (Yakult), 0.4% (wt/vol) maceroenzyme R10 (Yakult), and 0.1% bovine serum albumin (BSA) in ultra-filtered deionized water. WI solution was prepared using 4 mM MES, 20 mM potassium chloride, 0.4 M mannitol and 1% BSA in ultra-filtered deionized water.

Five milliliters of enzymatic solution were added to each conical tube and the leaves were shifted using tweezers to ensure they were fully soaked/submerged. The conical tubes were then partially capped (i.e the cap of conical tube is screwed on halfway) and were then wrapped in

aluminum foil to block any light. All conical tubes were then desiccated for 30 minutes; and then left to digest for the specified times.

After the specified digestion times, 1:1 ratio of WI to enzymatic solution was added to each conical tube; then the contents of each conical tube were strained through a 70-micrometer cell strainer into a new conical tube. The filtered solution was then centrifuged at 100 *g* for 2 minutes to pellet the cells. The supernatant was aspirated away and the cell pellet was resuspended in 100-150 microliters of WI solution. The control parameters used were 7 milligrams of leaves and 100 milligram of leaves using roughly 4-7 leaves for both two-hour and three-hour digestion time points.

Device Preparation and Digestion

The device was initially connected using 3-5 inches of tubing (ID: 1/32", OD: 3/32"), on the inlet and outlet of the device; and then the tubing was connected to a peristaltic pump. Leaves were harvested from 2–4-week-old husky cherry red tomato plants and were hand cut into 1-millimeter strips using a No. 11 scalpel; the stems of the leaves were also removed. About 7 milligrams of sample were loaded into the digestion device through the central luer connector using tweezers, and then a 2-way stopcock was connected to the luer connector. A syringe was filled with enzymatic solution and connected to the stopcock; then internal channels of the device were filled with the enzymatic solution until they reach the inlet and outlet of the device. The stopcock was then closed and the syringe was removed. A syringe was filled with enzymatic solution and a 20- or 22-gauge needle was attached to the syringe. One of the hoses connected to the peristaltic pump was then disconnected and the needle was inserted into the hose; then the enzymatic solution was loaded into the device, ensuring all air bubbles were removed. Once filled, the needle and syringe were disconnected and the hose was reconnected to peristaltic pump. The

device was run for a total of 2 hours with extraction every 30 minutes. The extractions involved flushing out the device into a 15- or 60-ml conical tube and then the device was reloaded with enzymatic solution; the leaf strips were not removed during this process.

A 1:1 ratio of WI to enzymatic solution was added to each conical tube and then strained through a 70-micrometer cell strainer into a new conical tube. The filtered solution was then centrifuged at 100 g for 2 minutes to pellet the cells. The supernatant was aspirated away and the cell pellet was resuspended in 70 microliters of WI solution. The data points that were analyzed from the device were 30, 60, 90 and 120 minutes, for 5, 10 and 20 milliliters per minute flow rates.

Protoplast Counting and Imaging

A hemocytometer was utilized to obtain the cell yield and calculate the cell viability. 10 microliters of each sample were taken and mixed with a 1:1 ratio of trypan blue and pipetted gently to mix. Trypan blue stains dead cells blue by infiltrating compromised cell membranes. 10 microliters of the mixture was added to the hemocytometer and protoplasts were counted using an inverted light microscope. Protoplast concentration is determined by using the following equation:

$$cell\ per\ ml = \frac{total\ cells\ counted * \ dilution\ factor}{number\ of\ squares\ counted} * 10{,}000$$

Protoplasts were then plated in 96 well flat bottom culture plates for imaging with an inverted fluorescence microscope to check for cell morphology.

Results

Flow Rate Analysis

Our tests were to determine the optimal flow rate for digesting leaves to obtain protoplasts. The flow rates that were used with the minced digestion device during this experiment were 5, 10 and 20 milliliter per minute. After each 30-minute interval, during a two-hour digestion, the protoplasts were extracted and a hemocytometer was used to obtain the cell yield and viability. Control samples were also used to compare to the digestion device: a 7-milligram control was used to compare directly to the sample size of the device and a 100-milligram control was used to compare to previously published protocols; both controls had a two-hour and three-hour digestion end point.

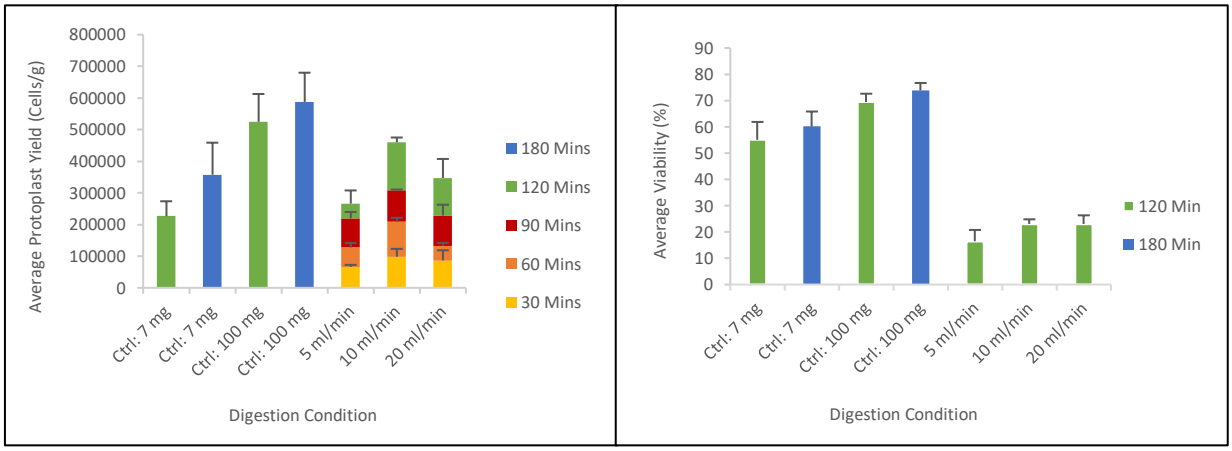

Figure 15 *– Average Protoplast Yield and Viability for Minced Digestion Device*

Protoplast yield and viability obtained from using the minced digestion device. Graph on the left depicts average protoplast yield from different time intervals based on digestion condition. Graph on the right depicts average protoplast viability from different time intervals based on digestion condition.

As figure 15 demonstrates, 10 milliliters per minute obtained the highest yield of cells with around 445,000 protoplasts; in contrast, 5 milliliters per minute had the lowest yield of cells with about 265,000 protoplasts. The 7-milligram controls obtained about 226,000 and 357,000 protoplasts for the two-hour and three-hour digestion times, respectively. The 100-milligram controls obtained about 524,000 and 587,000 protoplasts for the two-hour and three-hour digestion times, respectively. The viability for the controls were around 50-75%, versus the digestion device that had a significantly lower viability between 10-25%.

Phenotypic Analysis

Images of the protoplasts were taken using a Nikon TE200 inverted fluorescence Hoffman contrast microscope. Isolated protoplasts from the control and device groups were imaged using brightfield.

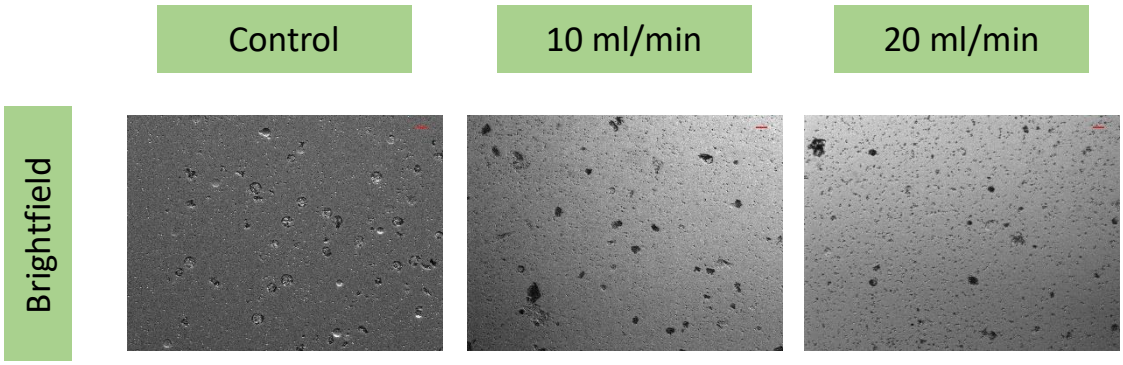

Figure 16 *– Brightfield Images of Protoplasts After Minced Digestion Device*

Brightfield images of protoplasts taken after digestion at 3 hours for the control and with the minced digestion device for 10 ml/min and 20 ml/min flow rates at 2 hours. Scale bar is equal to 50 μm, with 10x objective.

Phenotypic analysis demonstrates that protoplasts obtained from the control and device digestion have a similar morphology as protoplasts shown from previous papers. The protoplasts are spherical in shape, and contain an abundance of chloroplasts. Using a micro-ruler, we were able to estimate the size of the protoplasts to be between 30 and 70 micrometers in diameter.

Discussion

This initial trial of the minced device provided an excellent understanding of how protoplast isolation is possible on a microfluidic device. We were able to obtain consistent data for the three flow rates that showed an overall trend. The 5 milliliter per minute flow rate showed a low yield and viability of protoplasts as compared to the other flow rates, concluding that this flow rate could be too low to provide the necessary shear force to assist with the breakdown of the cell wall. In contrast, the 20 milliliter per minute flow rate demonstrated as you progress to higher flow rates the cell yield becomes lower, this could mean that some of the released protoplasts are dying due to recirculation damage; as shown by the higher viability. As a result, this could mean the higher flow rates could provide selectivity for stronger protoplasts. The 10 milliliter per minute flow rate provided the highest yield of protoplasts, demonstrating that there is a balance between the applied shear force to digest the cell wall versus recirculation damage.

The viability for each device condition was low compared to the control conditions. We determined this could be a result of the sample size of 7-milligrams. The small sample size does not provide enough released protoplasts for running viability testing. However, a trend is still present as the 5 milliliter per minute flow rate showed the lowest viability, supporting the idea that this parameter is too low to obtain protoplasts compared to the control. The highest cell viability at 20 milliliters per minute also demonstrated that the device is possibly destroying weaker

protoplasts, because the cell numbers were still less, but comparable, to the 10 milliliter per minute flow rate.

Images taken of protoplasts obtained from the minced digestion device showed a spherical shape and the presence of chloroplasts. This morphology is comparable to the control digestion and previous papers, indicating the protoplasts are alive and fully liberated of their cell wall. However, the density of protoplasts is low for the 10 milliliter per minute and 20 milliliter per minute flow rates because of the small sample size.

These results helped us redesign the digestion device into the scaled-up digestion device to obtain reliable protoplast yield and viability, while also reducing recirculation damage.

Chapter 3

Scaled-Up Digestion Device Characterization with Plant Leaves

In this chapter, I will discuss tests for characterizing the scaled-up digestion device with regards to optimal flow rates, cell yield and cell viability using leaf samples from tomato (solanum lycopersicum) plants.

Introduction

After initial tests with the minced digestion device, we determined that a sample size of 7 milligrams may be too small to obtain reliable viability data. Thus, a larger digestion device was fabricated to hold three times the amount of the original device. In addition, it was determined that the protoplasts may be more fragile than mammalian cells after being digested, so the dissociation style channels were altered to remove the oscillating design. This eliminated the varying flow leading into the digestion chamber, and would reduce the amount of shear force applied on the protoplasts as they recirculate through the system. The hydro-mince channels were also altered to increase their size from 250 micrometers to 500 micrometers in width. This was changed to reduce the amount of damage caused by recirculating already free protoplasts, but still provide fluidic shear force on the sample for digestion.

Tripling the size of the digestion device resulted in triple the amount of sample that could be digested at once, offering better reliability for analyzing cell yield and viability. Due to the increased number of hydro-mince channels we had to connect the device to three channels on the peristaltic pump and program each one with an identical flow rate, to equate to the same flow rate

as the original digestion device. Unlike chapter two where we tested 5, 10 and 20 milliliters per minute, we dropped the 5 milliliter per minute flow condition and added a 30 milliliter per minute flow condition to verify our expectations that higher flow rates resulted in a lower cell yield. After digestion, cell yield and viability were determined by using Trypan Blue staining on a hemocytometry.

Materials and Methods

Tomato Plants

To keep consistent with chapter two we used the same species of tomato plants: Husky Cherry Red. All growth parameters were maintained from chapter two for the following tests.

Control Digestion

The enzymatic and WI solutions used in chapter two were also utilized in this chapter and the following experiments.

The same set up and operations as mentioned in chapter two were also utilized in this chapter for digestion of the control samples; except the 7-milligram control sample was removed due to the larger amount of leaf tissue required in the scaled-up digestion device. In addition, the 100-milligram control sample was replaced with a control sample size of about 21 milligrams to equate to the scaled-up digestion device.

Device Preparation and Digestion

Similar to chapter two, the device was initially connected using 3-5 inches of tubing (ID: 1/32”, OD: 3/32”), on the inlets and outlets of the device; and then the tubing was connected to each channel of the peristaltic pump. Roughly 2–4-week-old husky cherry red tomato plant leaves were harvested and hand cut into 1-millimeter strips using a No. 11 scalpel; the stems of the leaves

were also removed. About 21 milligrams of sample was loaded into the digestion device through the central loading channel and then capped off with a capping layer using PSA. A syringe was filled with enzymatic solution and a 20- or 22-gauge needle was attached to the syringe. One at a time, each tubing attached to each channel of the peristaltic pump was removed and enzymatic solution was loaded in using the syringe with the needle. Once filled, the needle and syringe were disconnected and the tubing was reconnected to peristaltic pump. The device was run for a total of 2 hours with extraction every 30 minutes. The extractions involved flushing each channel of the device into a 15- or 60-ml conical tube and then the device was reloaded with enzymatic solution; the leaf strips were not removed during this process.

Analogous to chapter two, 1:1 ratio of WI to enzymatic solution was added to each conical tube and then strained through a 70-micrometer cell strainer into a new conical tube. The filtered solution was then centrifuged at 100 *g* for 2 minutes to pellet the cells; then supernatant was then aspirated away. A second wash step is performed by adding 5 milliliters of WI solution to resuspend the pellet. The conical tube was centrifuged again to pellet the cells and the supernatant was aspirated away; to ensure no enzymatic solution is left behind. The cell pellet was resuspended in 100-150 microliters of WI solution. The data points that were analyzed from the device were 30, 60, 90 and 120 minutes, for 10, 20 and 30 milliliters per minute flow rates.

Protoplast Counting and Imaging

Equivalent to chapter two, a hemocytometer was utilized to obtain the cell yield and calculate the cell viability. 10 microliters of each sample were taken and mixed with a 1:1 ratio of trypan blue and pipetted gently to mix. 10 microliters of the mixture were added to the hemocytometer and was counted using an inverted light microscope. Cell concentration is determined by using the following equation:

$$cell\ per\ ml = \frac{total\ cells\ counted * \ dilution\ factor}{number\ of\ squares\ counted} * 10{,}000$$

Protoplasts were then plated in 96 well flat bottom culture plates for imaging with an inverted fluorescence microscope to check for cell morphology.

Results

Flow Rate Analysis

Our tests with the scaled-up digestion device were designed to obtain and verify protoplast yield and viability with a larger sample size. We tested flow rates of 10, 20, and 30 milliliters per minute, for a duration of two-hours with extractions at 30-minute intervals. After extraction, protoplasts were counted using a hemocytometer and cell viability was obtained. Two-hour and three-hour control samples were tested and compared to the scaled-up digestion device conditions.

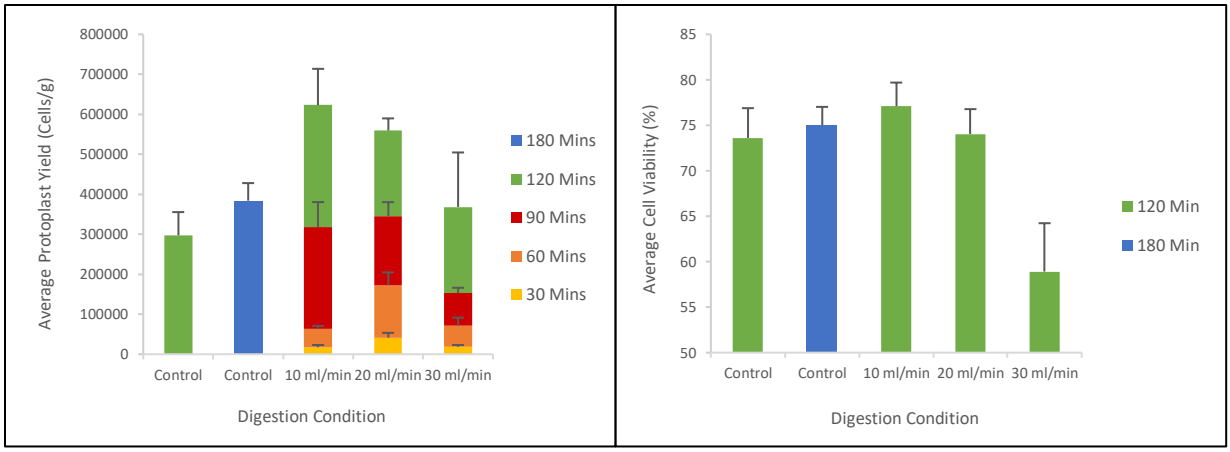

Figure 17 *– Average Protoplast Yield and Viability for the Scaled-up Digestion Device*

Protoplast yield and viability obtained from using the scaled-up digestion device. Graph on the left depicts average protoplast yield from different time intervals based on digestion condition. Graph on the right depicts average protoplast viability from different time intervals based on digestion condition.

As shown in figure 17, 10 milliliters per minute obtained the highest protoplast yield around 622,000 cells per gram; while 30 milliliters per minute obtained the lowest protoplast yield of about 368,000 cells per gram, amongst the device conditions. The 20 milliliters per minute condition fell between the two other conditions with a yield of about 559,000 cells per gram. The controls obtained about 297,000 and 383,000 cells per gram for the two-hour and three-hour time points, respectively. Cell viability for each device condition fell between 59% and 77%, with 10 milliliters per minute having the highest at 77% and 30 milliliters per minute having the lowest at 59%. The controls had a cell viability of 74% and 75% for the two-hour and three-hour respectively.

Phenotypic Analysis

Images of protoplasts were taken using an Olympus IX83 inverted fluorescence microscope. Isolated protoplasts from the control and device groups were imaged using brightfield.

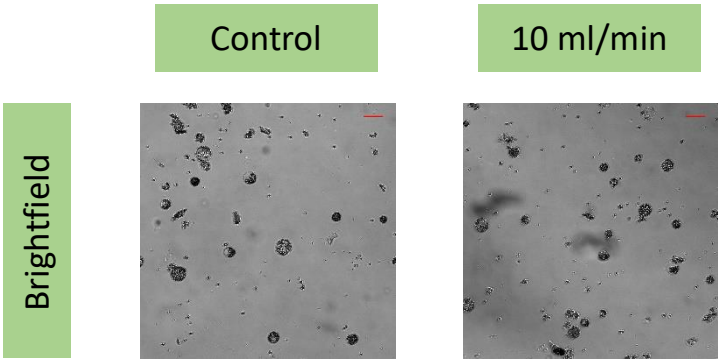

Figure 18 *– Brightfield Images of Protoplasts After the Scaled-up Digestion Device*

Brightfield images of protoplasts taken after three-hour control digestion and with the scaled-up digestion device at 10 ml/min flow rate after two hours. Scale bar is equal to 50 µm, with 20x objective.

Phenotypic analysis demonstrates that protoplasts obtained from the control digestion and device digestion at 10 milliliters per minute, still have a similar morphology as protoplasts obtained from the minced digestion device and literature; as shown by their spherical shape, and abundance of chloroplasts. Using a micro-ruler, we were able to estimate the size of the protoplasts to be similar to the previously obtained images in chapter 2: between 30 and 70 micrometers in diameter.

Discussion

The data obtained from using the scaled-up digestion device provided a better understanding of protoplast yield and viability. We were able to obtain consistent data and

optimize the flow rate by demonstrating 10 milliliters per minute produced the highest yield and viability of cells. We believe at this flow rate there is enough fluid flow to provide adequate shear force without damaging the protoplasts during recirculation. The trend that is shown by the data indicates that higher flow rates damage or destroy protoplasts during recirculation; as demonstrated by the falling cell yield and viability for 20 and 30 milliliters per minute.

A second trend was observed in later digestion time points, around 90 minutes and 120 minutes, which resulted in a larger protoplast yield than the early time points of 30 and 60 minutes. As time progresses more protoplasts are released, hinting that the rigid cell wall provides protection early on in digestion and requires time before enzymatic penetration deeper into the bulk of the leaves. As the cell walls become digested the fluidic shear force assist with removing hanging components and opens up new areas for enzymatic digestion. In addition, the digestion device did demonstrate a reduction in required digestion time, from three hours to two hours, and doubled the yield of cells.

Similar to the images taken in chapter 2, protoplasts obtained from the scaled-up digestion device showed the presence chloroplasts and a spherical shape. This morphology is comparable to the control digestion and previous papers, indicating the protoplasts are alive and fully liberated of their cell wall. Further phenotypic studies with 20 and 30 milliliters per minute will need to be conducted in the future.

Chapter 4

Leaf Preparation Device Characterization

In this chapter, I will discuss tests for characterizing the leaf preparation device in combination with the scaled-up digestion device with regards to cell yield and cell viability using leaf samples from tomato (solanum lycopersicum) plants.

Introduction

We found that the preparation of leaves for digestion was long, laborious and inconsistent in the size of the leaf strips produced. In response, we created a device to prepare leaves samples quickly and consistently. We went through two iterations of the device before we found a suitable design to cleanly cut the leaves. The first design was created after an egg chopper; leaves were placed in between two halves of the device and the lid of the device would be closed to chop the leaves into 1-millimeter sized strips, to fit into the digestion device. This design was initially tested with cutting leaf samples and was determined that it crushed the leaves rather than sliced them cleanly.

The leaf preparation device was redesigned to hold the leaves and allow for scalpel to slice through slots in the device to cut the leaves into 1-millimeter strips. After initial tests it was determined the leaf strip edges were cut cleanly and the device was then tested with the scaled-up digestion device. The leaves were cut using the leaf preparation device and then placed into the scaled-up digestion device. After digestion, protoplast yield and viability were determined by using Trypan Blue staining on a hemocytometry. To ensure viability, we cultured the protoplasts

and then stained them with calcein blue for cytoplasmic activity, and calcofluor white for cell wall growth.

Materials and Methods

Tomato Plants

To keep consistent with chapter two and three we used the same species of tomato plants: Husky Cherry Red. All growth parameters were maintained from chapter two for the following tests.

Control Digestion

The enzymatic and WI solutions used in chapter two were also utilized in this chapter and the following experiments.

The same set up and operations as mentioned in chapter three were also utilized in this chapter for digestion of the control samples; except a three-hour control using leaves prepared with the leaf preparation device was also evaluated.

Device Preparation and Digestion

Similar to chapter three, the device was initially connected using 3-5 inches of tubing (ID: 1/32”, OD: 3/32”), on the inlets and outlets of the device; and then the tubing was connected to each channel of the peristaltic pump. Roughly 2–4-week-old husky cherry red tomato plant leaves were harvested and were cut with the leaf preparation device into 1-millimeter strips using a No. 11 scalpel; the stems of the leaves were also removed. About 21 milligrams of sample were loaded into the digestion device through the central loading channel and then sealed off with a capping layer using PSA. A syringe was filled with enzymatic solution and a 20- or 22-gauge needle was attached to the syringe. One at a time, each tubing attached to each channel of the peristaltic pump

was removed and enzymatic solution was loaded in using the syringe with the needle. Once filled, the needle and syringe were disconnected and the tubing was reconnected to peristaltic pump. The device was run for a total of 2 hours with extractions every 30 minutes. The extractions involved flushing each channel of the device into a 15- or 60-ml conical tube and then the device was reloaded with enzymatic solution; the leaf strips were not removed during this process.

Following the steps in chapter three, 1:1 ratio of WI to enzymatic solution was added to each conical tube and strained through a 70-micrometer cell strainer into a new conical tube. The filtered solution was then centrifuged at 100 *g* for 2 minutes to pellet the cells; the supernatant was then aspirated away. A second wash step was performed by adding 5 milliliters of WI solution to resuspend the pellet. The conical tube was centrifuged again, at 100 *g* for 2 minutes, to pellet the cells and the supernatant was aspirated away; in order to ensure no enzymatic solution is left behind. The cell pellet was resuspended in 100-150 microliters of WI solution. The data points that were analyzed from the device were 30, 60, 90 and 120 minutes for 10 milliliters per minute.

Protoplast Counting

Following the same steps as chapter three, a hemocytometer was utilized to obtain the cell yield and calculate the cell viability. 10 microliters of each sample were taken and mixed with a 1:1 ratio of trypan blue and pipetted gently to mix. 10 microliters of the mixture were added to the hemocytometer and was counted using an inverted light microscope. Cell concentration is determined by using the following equation:

$$cell\ per\ ml = \frac{total\ cells\ counted * \ dilution\ factor}{number\ of\ squares\ counted} * 10{,}000$$

Fluorescence Staining and Imaging – Pre-Culture

Protoplasts were plated in 96 well flat-bottom culture plates for staining with calcein blue and calcofluor white before being imaged with an Olympus IX83 inverted fluorescence microscope to check for cell morphology.

Calcein blue was prepared by mixing it with DMSO at a concentration between 1-10 mM and stored as a stock solution in the dark at -20 °C, that would be used later for making a working solution. A working solution was created by combining calcein blue stock solution to WI solution at a concentration between 1-25 μM; this solution was stored on ice in the dark, until used. A calcofluor white working solution was also prepared in a similar manner by combining calcofluor white to WI solution at a concentration between 1-25 μM and was stored in the dark at room temperature until used.

Both stains were added to separate wells, containing protoplasts in roughly 150-200 microliters of WI solution, at a ratio of 4 microliters of stain to 200 microliters of WI. Calcein blue stained samples were stored in the dark at room temperature for 15 minutes before imaging. Calcofluor white strained samples were stored in the dark at room temperature for 5 minutes before imaging. Samples were then imaged using an Olympus IX83 inverted fluorescence microscope with bright-field and DAPI filter at 50 millisecond exposure.

Protoplast Culturing

Protoplast culture media was created by adding 4.4 grams of Murashige and Skoog Basal Medium to 1 liter of culture water, supplemented with 4.6 micromolar zeatin, 10.8 micromolar of naphthaleneacetic acid (NAA), 0.66 molar mannitol, 0.075% plant preservative mixture (PPM), 1% penicillin and streptomycin. A PH of 7 is optimal for culturing with this culture media. Finished media was then sterile filtered before being stored at 4 °C.

In a culture hood, protoplasts were plated in 96 well plates at equivalent concentrations between the control and the experimental groups, based on the counts obtained from the hemocytometer in the previous section. Roughly 200 microliters of culture media was added to each culture well containing protoplasts. The well plate was then sealed with a parafilm and placed in a culture box with a grow light on a 15-hour photoperiod, for 7 days. At day 3 culture media was replaced.

Fluorescence Staining and Imaging – Post-Culturing

Similar to the previous sections, a working solution was created by combining calcein blue stock solution, created in the previous section, to WI solution at a concentration between 1-25 μM; this solution was stored on ice and in the dark until used. A calcofluor white working solution was also prepared in a similar manner by combining calcofluor white to WI solution at a concentration between 1-25 μM and was stored in the dark at room temperature until used.

Both stains were added to separate wells containing cultured protoplasts and culture media at a concentration of 2%. Calcein blue stained samples were stored in the dark at room temperature for 15 minutes before imaging. Calcofluor white strained samples were stored in the dark at room temperature for 5 minutes before imaging. Samples were then imaged using an Olympus IX83 inverted fluorescence microscope with bright-field and DAPI filter at 50 millisecond exposure.

Results

Flow Rate Analysis

The tests we performed with the leaf preparation device was in conjunction with the scaled-up digestion device, in order to demonstrate cell yield and viability were maintained in comparison to the traditional preparation method. We tested the prepared leaves at 10 milliliters per minute for

a duration of two hours, with extractions at 30-minute intervals. A control sample at three hours was also prepared using the leaf preparation device and compared to the combination of the leaf preparation device and the scaled-up digestion device; data obtained previously in chapter three was also compared to the collected data.

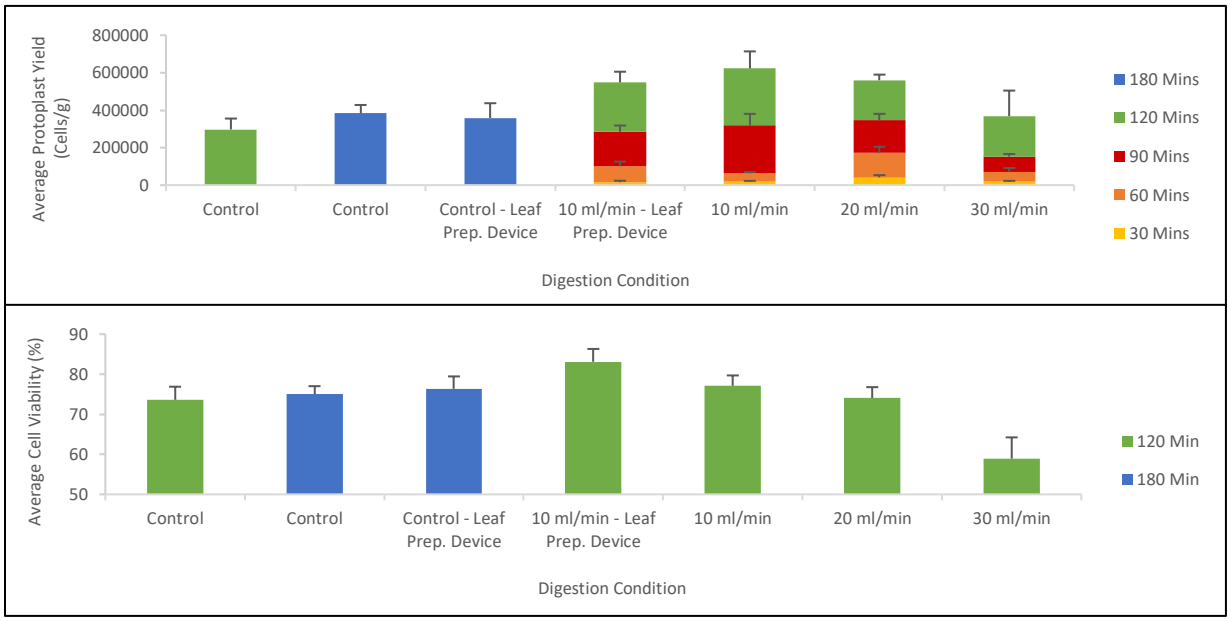

Figure 19 *– Average Protoplast Yield and Viability for the Leaf Preparation Device*

Protoplast yield and viability obtained from using the leaf preparation device in combination with the scaled-up digestion device. The upper graph depicts average protoplast yield from different time intervals based on digestion condition. The lower graph depicts average protoplast viability from different time intervals based on digestion condition. Data from figure 17 was included for comparison.

As shown in figure 19, the 10 milliliters per minute obtained roughly 548,000 protoplasts with an average viability around 83%, from using the leaf preparation device. The control condition obtained roughly 358,000 protoplasts with an average viability of 76%. The data from

chapter three was also included in figure 19 as a comparison to the using the leaf preparation device.

Protoplast Morphology and Fluorescence Staining Analysis

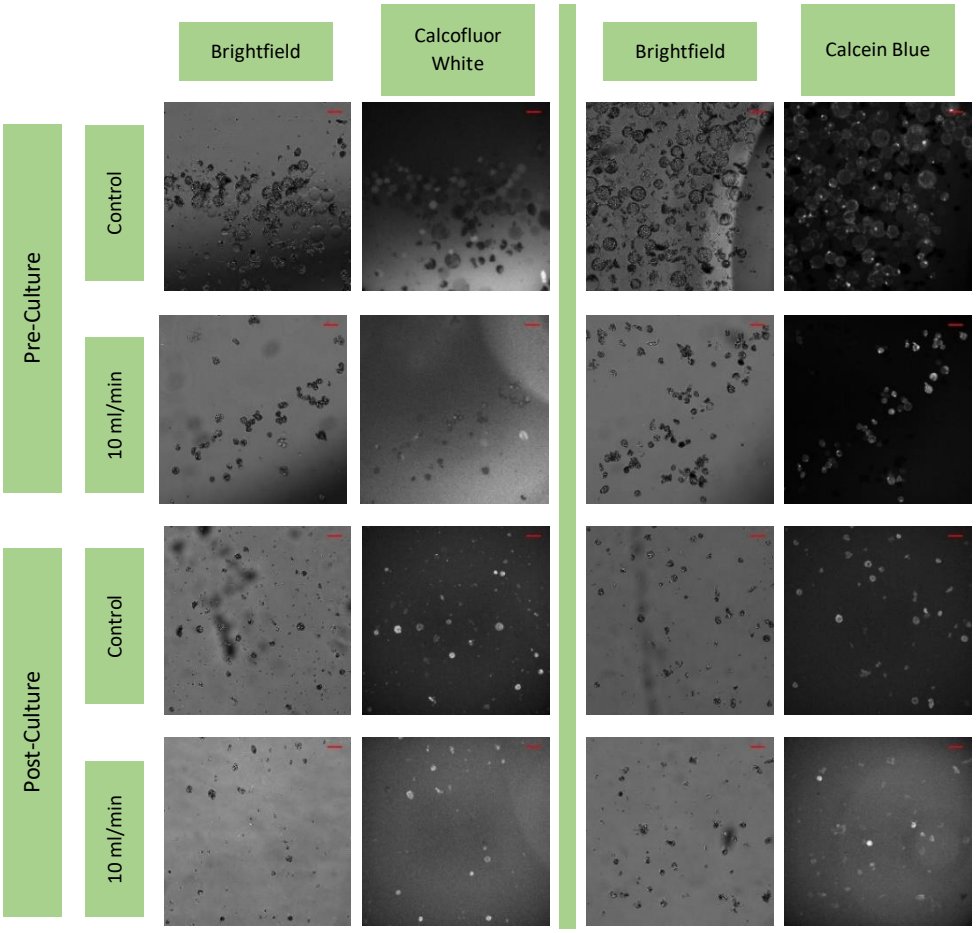

Figure 20 *– Fluorescence Images of Protoplast Before and After Culture*

Fluorescence images of protoplasts taken, before and after culturing, after three-hour control digestion and with the leaf preparation device combined with the scaled-up digestion device at 10 ml/min flow rate after two hours. Protoplasts were stained with Calcein Blue or Calcofluor White Scale bar is equal to 50 µm, with 20x objective.

Images of the protoplasts before and after culturing were taken using an Olympus IX83 inverted fluorescence microscope. Phenotypic analysis demonstrates that protoplasts from before and after culture have similar morphology. The protoplasts are spherical in shape, and contain an abundance of chloroplasts. We were able to estimate the size of the protoplasts based on pixel size to be similar to previously obtained images in chapter 2: between 30 and 70 micrometers in diameter. A noted difference between freshly isolated protoplasts and cultured protoplasts is their transparency. Freshly isolated protoplasts have a transparency equivalent to protoplasts isolated in chapter 2 and in literature. However, protoplasts that have been cultured appear to be opaque and have a slightly rougher edge, as shown in figure 20.

DAPI images of protoplasts show fluorescence signal with both the calcein blue and calcofluor white stain. The freshly isolated protoplasts are shown to be alive and active because of the fluorescence from the calcein blue in the cytoplasm, indicating enzymatic activity. In contrast, there is little-to-no calcofluor white staining occurring, indicating the cell wall is completely removed.

The cells imaged after culturing show continued enzymatic activity from the calcien blue fluorescence, indicating protoplast viability. In contrast to freshly isolated protoplasts, cultured protoplast fluoresced with the presence of calcofluor white, indicating the cells have regrown their cell wall.

Discussion

The data obtained from using the leaf preparation device, in conjunction with the scaled-up digestion device, was used to evaluate if streamlining and simplifying the preparation steps would reduce protoplast yield or viability. From the data that was obtained, the device has shown

that it does not reduce the viability of the protoplasts, because it produced a viability of about 86% while the scaled-up device presented in chapter 3, produced a viability around 77% using the standard preparation method. This difference in viability could be the result of some destruction of protoplasts that are released and recirculated through the device; as shown by the lower cell yield compared to the scaled-up device in chapter 3. This is a possible result of the slots on the bottom of the leaf preparation device causing slight crushing of the leaves when being cut. A slight modification of this device to make the slots shallower will fix this issue. The results are still promising, because the control for the leaf preparation device obtained the same yield of protoplasts as the standard control method, around 375,000 cells. In addition, the leaf preparation device in combination with the scaled-up digestion device still produced about 1.5 times more protoplasts than the control samples.

Phenotypic analysis of protoplast obtained using the leaf preparation device in combination with the scaled-digestion device showed the presence of chloroplasts, a spherical shape, and transparency before culturing. This morphology is comparable to the control digestions and literature, indicating the protoplasts are alive. Calcein blue fluorescence imaging results also demonstrated there was still a presence of enzymatic activity within the cell, indicating they were still active before culturing. In addition, the protoplasts did not fluoresce in the presence of calcofluor white staining demonstrating the absence of a cell wall, indicating the cell wall was fully digested by using the devices.

Bright-field images of the protoplasts after culturing showed a slight change in morphology from transparent to opaque and roughing of the edges of the cells, indicating the possible presence of a cell wall. Calcein blue straining confirmed the protoplasts were still viable and active; while

calcofluor staining confirmed a formation of a cell wall around the protoplasts. These results indicate the ability of the devices to isolate viable cells for culturing.

Chapter 5

Conclusion and Future Directions

The leaf preparation device has the potential to streamline the initial steps for preparing leaves for our scaled-up digestion device. Future work with this device will include a redesign to have scalpel blades assembled in parallel to cut all the leaves at once. The device can also be redesigned to act as a rectangular biopsy punch that can prepare samples from more than five leaves at once. This design can be envisioned as an integrated piece that is attached to the scaled-up digestion device, and when the leaves are punched out, they are immediately inserted into the tissue holding chamber. This will prevent cross contamination and reduce the latency of having to transfer leaves into the scaled-up digestion device by hand, which can prevent drying of the leaf samples.

Our scaled-up digestion device has shown to have the potential to be employed as the central step for isolating protoplasts. Future work with this device will include testing the device with alternating fluid flow direction on the peristaltic pump to potentially increase cell yield even further. The device can also be redesigned to replace the three inlets/outlets with a single inlet/outlet, in order to run multiple devices in parallel. Calcein blue and calcofluor white staining on protoplasts, after digestion with this device, can be utilized to determine cell yield and viability. Future work with these stains includes developing an algorithm to evaluate cell yield and viability from fluorescence imaging. In addition, culturing protoplasts obtained from this digestion device is a viable measure for determining cell viability and proliferation potential. Future work will include slight modifications to culturing parameters in order to optimize protoplast culturing, and further validation of cell yield and viability with calcein blue and calcofluor white.

Future directions with these devices are testing with other plant species such as maze and soy beans. Further expectations after testing will be combining this system to post processing devices that perform genetic modifications and cell biomarker analysis.

References

1. M. Parry, "Climate change, global food supply and risk of hunger," *The Royal Society,* 2005.
2. P. D'ordorico, "Feeding humanity through global food trade," *Earth's Future,* 2014.
3. S. Long, "Meeting the Global Food Demand of the Future by Engineering Crop Photosynthesis and Yield Potential," *Cell,* 2015.
4. Y. Zhang, "A highly efficient rice green tissue protoplast system for transient gene expression and studying light/chloroplast-related processes," *Plant Methods,* 2011.
5. S.-D. Yoo, "Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis," *Nature Protocols,* 2007.
6. J. Cui, "Protoplast isolation and culture from Kalanchoë species: optimization of plant growth regulator concentration for efficient callus production," *Plant Cell, Tissue and Organ Culture,* 2019.
7. S.-Y. Ding, "How Does Plant Cell Wall Nanoscale Architechture Correlate with Enzymatic Digestibility?," *Science,* 2012.
8. Z.-H. He, "A Cell Wall-associated, Receptor-like Protein Kinase," *The Journal of Biological Chemistry,* 1996.
9. S.-D. Yoo, T.-H. Cho and a. J. Sheen, "Arabidopsis mesophyll protoplasts: A versatile cell system for transient gene expression analysis," *Nature Protocols,* pp. 2: 1565-1575, 2007.
10. Z. Li, D. Zhang, X. Xiong and et.al, "A potent Cas9-derived gene activator for plant and mammalian cells," *Nature Plants,* 2017.
11. J.-F. Li, D. Zhang and J. Sheen, "Epitope-tagged protein-based artificial miRNA screens for optimized gene silencing in plants," *Nature Protocols,* 2014.

12. X. Qiu, T. Westerof and et.al., "Microfluidic device for rapid digestion of tissues into cellular suspensions," *Lab on a Chip,* 2017.

13. X. Qiu, J. D. Jesus and et.al., "Microfluidic device for mechanical dissociation of cancer cell aggregates into single cells," *Lab Chip,* 2016.

14. X. Qiu, J.-H. Huang and et.al., "Microfluidic channel optimization to improve hydrodynamic dissociation of cell aggregates and tissue," *Scientific Reports,* 2018.

15. J. Lombardo, M. Aliaghaei and e. al., "Microfluidic platform accelerates tissue processing into single cells for molecular analysis and primary culture models," *Nature Communications,* 2021.

16. D. Rouan, M.-H. Montane and e. al., "Relationship between protoplast size and critical field strength in protoplast electropulsing and application to reliable DNA uptake in Brassica," *Plant Cell Reports,* 1991.

17. "Viability and Cytotoxicity Assay Reagents—Section 15.2," ThermoFisher Scientific, [Online]. Available: https://www.thermofisher.com/us/en/home/references/molecular-probes-the- hand book/assays-for-cell-viability-proliferation-and-function/viability-and-cytotoxicity-assay-reagents. html#head1. [Accessed 2021].

18. Millipore, "18909 Calcofluor White Stain," [Online]. Available: https://www.sigmaaldrich.com/ content/dam/sigma-aldrich/docs/Sigma-Aldrich/Datasheet/1/18909dat.pdf. [Accessed 2021].

Appendix

Standard Operating Procedures

Tomato Plant Growth Protocol: Husky Cherry Red

Materials

Super Sprouter Premium Propagation Kit with Heat Mat	Amazon
6-Cell Seedling Starter Tray	Amazon
2-Outlet Plug-In Countdown Lighting Timer	Lowe's
Miracle-Gro – Organic Raised Bed Soil	Lowe's
Husky Cherry Red Hybrid Tomato	Totally Tomato

Methods

1. Plug in outlet timer and set the photocycle required for the greenhouse.
2. Set up heat pad on a flat heat resistant surface, and plug heat pad into outlet timer.
3. Place greenhouse on heating pad and place the grow light on top of the greenhouse.
    - Ensure the greenhouse is in room temperature.
4. Plug in greenhouse to the outlet timer.
5. Take a 6-cell seedling starter tray and fill each cell with roughly 2/3 to 3/4 of the miracle-gro soil.
6. Place 2 tomato seeds in each cell, and then fill the rest of the cell with soil.
7. Fill each cell with water.
    - Ensure the water soaks into the soil.
8. Place the full cell tray into the greenhouse.
9. Fill the bottom of the greenhouse with 1-2 inches of water.
10. Put top on greenhouse.
    - Every 3-4 days refill greenhouse.
    - Every 2-3 weeks clean out greenhouse.

Leaf Preparation Device Protocol

Materials

Leaf Preparation Device
Roughly 3-week-old tomato leaves

Methods

1. Choose well-expanded leaves from about 3-week-old plants.
    - Note: The selection of healthy leaves at the proper developmental stage is a very important factor in protoplast experiments. Protoplasts prepared from leaves recovered from stress conditions (e.g., drought, flooding, extreme temperature and constant mechanical perturbation) may look similar to those from healthy leaves. However, we have often experienced low transfection efficiency with the protoplasts from stressed leaves.
2. Ensure the leaf preparation device is clean before use.
3. Open the device by pulling outward on the front of the device to unlock the top piece.
4. Place chosen leaves inside the device.
    - Lay the leaves perpendicular to the channels on the bottom piece of the device.
    - Stacking 2-3 leaves on top of each other can help prevent sliding of the leaves.
5. Closed the device and ensure its locked.
6. Take a scalpel and run it through the slots on the top piece of the device.
    - Ensure to run it through every slot to get 1-millimeter strips.
7. Open the device by pulling outward on the front of the device to unlock the top piece.
8. Remove the leaf strips from the device.

Manual Digestion Protocol

Materials

Cellulase Onozuka™ R-10	Yakult (Kanematsu)
Maceronzyme™ R-10	Yakult (Kanematsu)
Morpholineethanesulfonic acid (MES)	Sigma Aldrich (M8250)
Mannitol	Sigma Aldrich (M4125)
Potassium Chloride	Sigma Aldrich (P3911)
Calcium Chloride	Sigma Aldrich (C7902)
Bovine Serum Albumin	Fisher Scientific (BP1600-100)
70-micrometer Sterile Cell Strainers	Fisher Scientific (50-105-0135)
Roughly 3-week-old Tomato Leaves	
15- or 50-ml Conical Tube	

Solutions

All Solutions are made with ultra-purified deionized water.

Enzymatic Solution

0.2 M 4-morpholineethanesulfonic acid, PH 5.7	20 mM
Cellulase R10	1.5% (wt/vol)
Maceroenzyme R10	0.4% (wt/vol)
Mannitol	0.4 M
KCl	20 mM
CaCl2	10 mM
B-Mercaptoethanol	1-5 mM
BSA	0.1% BSA

W1 Solution

0.2 M 4-morpholineethanesulfonic acid, PH 5.7	4 mM
Mannitol	0.4 M
KCl	20 mM
BSA	1% BSA

Methods

1. Choose well-expanded leaves from about 3-week-old plants.
    - Note: The selection of healthy leaves at the proper developmental stage is a very important factor in protoplast experiments. Protoplasts prepared from leaves recovered from stress conditions (e.g., drought, flooding, extreme temperature and constant mechanical perturbation) may look similar to those from healthy leaves. However, we have often experienced low transfection efficiency with the protoplasts from stressed leaves.
2. Hand cut 1-mm leaf strips from the middle part of a leaf (remove the midrib of the leaf) using a fresh sharp scalpel without tissue crushing at the cutting site.
    - We typically use around 4-7 leaves for each control.
    - Use a clean white piece of paper to inspect for wounded/crushed tissue (juicy and dark green stains); redo cutting if this happens.
    - Weigh the minced leaf samples and record, in grams, the used sample.
3. Transfer leaf strips quickly and gently into the prepared enzyme solution, in a conical tube, by completely submerging them using a pair of flat-tip forceps.
    - Immediate dipping and submerging of leaf strips are very critical for protoplast yield.
    - When leaf strips are dried out on the paper during cutting, the enzyme solution cannot penetrate and protoplast yield is decreased significantly.
    - For our experiments typically use 5 mls of enzymatic solution
    - Leave the cap of the conical tube unscrewed slightly for the next step.
4. Wrap the conical tube in aluminum foil to block any light.
5. Vacuum infiltrate leaf strips for 30 min in the dark using a desiccator.
6. Continue the digestion, without shaking, in the dark for 3 hours at room temperature.
    - After the digestion, the enzyme solution should turn green after a gentle swirling motion, which indicates the release of protoplasts.
    - Extended incubation of leaves (16–18 h) in the dark is stressful and might eliminate physiological responses of leaf cells.
7. Prepare a 2-hour control digestion as per steps 1 through 6.
8. Dilute the enzyme/protoplast solution with an equal volume of W1 solution before filtration to remove undigested leaf tissues.
    - For our experiment we use 5 ml of W1.
9. Prepare a 70-μm nylon mesh, then filter the enzyme solution containing protoplasts through the mesh into a 50 ml conical tube.
10. Centrifuge the resulting solution at 100 g to pellet the protoplasts in a 50-ml round-bottomed tube for 2 min.
    - A higher speed (200g) of centrifugation may help to increase protoplast recovery.
11. Remove as much supernatant as possible.
    - For culturing or fluorescence staining add 5-10 mls of WI solution to cell pellet and repeat steps 10 and 11.
12. Resuspend the protoplast pellet in W1 solution.
    - For our experiment we will use 0.1-0.15 ml of W1 solution.
13. Count cells with the hemacytometer.
14. Image cells with a microscope and take pictures.
    - If necessary, stain cells with calcein blue or calcofluor white before imaging.

Minced Digestion Device Protocol

Materials

Cellulase Onozuka™ R-10	Yakult (Kanematsu)
Maceronzyme™ R-10	Yakult (Kanematsu)
Morpholineethanesulfonic acid (MES)	Sigma Aldrich (M8250)
Mannitol	Sigma Aldrich (M4125)
Potassium Chloride	Sigma Aldrich (P3911)
Calcium Chloride	Sigma Aldrich (C7902)
Bovine Serum Albumin	Fisher Scientific (BP1600-100)
70-micrometer Sterile Cell Strainers	Fisher Scientific (50-105-0135)

Roughly 3-week-old Tomato Leaves
Minced Digestion Device
2-way Stopcock
Peristaltic Pump
Tubing (ID: 1/32", OD: 3/32")
20- or 22- Gauge Blunt Tip Needle
15- or 50-ml Conical Tube

Solutions

All Solutions are made with ultra-purified deionized water.

Enzymatic Solution

0.2 M 4-morpholineethanesulfonic acid, PH 5.7	20 mM
Cellulase R10	1.5% (wt/vol)
Maceroenzyme R10	0.4% (wt/vol)
Mannitol	0.4 M
KCl	20 mM
CaCl2	10 mM
B-Mercaptoethanol	1-5 mM
BSA	0.1% BSA

W1 Solution

0.2 M 4-morpholineethanesulfonic acid, PH 5.7	4 mM
Mannitol	0.4 M
KCl	20 mM
BSA	1% BSA

Methods

1. Choose well-expanded leaves from about 3-week-old plants.
    - Note: The selection of healthy leaves at the proper developmental stage is a very important factor in protoplast experiments. Protoplasts prepared from leaves recovered from stress conditions (e.g., drought, flooding, extreme temperature and constant mechanical perturbation) may look similar to those from healthy leaves. However, we have often experienced low transfection efficiency with the protoplasts from stressed leaves.
2. Hand cut 1-mm leaf strips from the middle part of a leaf (remove the midrib of the leaf) using a fresh sharp scalpel without tissue crushing at the cutting site.
    - We typically use around 4-7 leaves for each control.
    - Use a clean white piece of paper to inspect for wounded/crushed tissue (juicy and dark green stains); redo cutting if this happens.
    - Weigh the leaf strips and record, in grams, the used sample.
3. Insert roughly 7 milligrams of leaf strips into the minced digestion device through the luer connector.
4. Attach a 2-way stopcock to the luer connector.
5. Fill a 3 ml syringe with enzymatic solution
6. Attach the syringe to the stopcock and fill the device.
7. Close the stopcock and then detach the syringe.
8. Attach a 3–5 inch length of tubing (ID: 1/32”, OD: 3/32”) to each hose barb on the peristaltic pump carriage.
    - Careful to not force the tubing too far onto the hose barb, in order to prevent it from becoming stuck.
9. Fill the tubing length and peristaltic pump carriage with enzymatic solution.
10. Attach the carriage to the peristaltic pump.
11. Attached each open end of the tubing to each hose barb on the minced digestion device to complete a loop.
    - Careful to not force the tubing too far onto the hose barb, in order to prevent it from becoming stuck.
12. Set the peristaltic pump to the proper RPM that corresponds to the flow rate being tested.
    - A calibration step may be needed to determine the RPM that equates to the flow rate required.
13. Run peristaltic pump for allotted interval time.
14. Turn off peristaltic pump and prepare a 3 ml syringe with enzymatic solution.
15. Attach a 20- or 22-gauge needle to the syringe.
16. Detach one end of the tubing and insert the syringe with the needle and slowly extract the solution into a conical tube.
17. Remove the syringe with the needle and reattach the tubing.
18. Run the device for the next allotted interval.
19. Dilute the enzyme/protoplast solution in the conical tube with an equal volume of W1 solution before filtration to remove undigested leaf tissues.
20. Prepare a 70-μm nylon mesh, then filter the enzyme solution containing protoplasts through the mesh into a 50 ml conical tube.
21. Centrifuge the resulting solution at 100 g to pellet the protoplasts in a 50-ml round-bottomed tube for 2 min.

- A higher speed (200g) of centrifugation may help to increase protoplast recovery.

22. Remove as much supernatant as possible.
    - For culturing or fluorescence staining add 5-10 mls of WI solution to cell pellet and repeat steps 10 and 11.
23. Resuspend the protoplast pellet in W1 solution.
    - For our experiment we will use 0.07 ml of W1 solution.
24. Count cells with the hemacytometer.
25. Image cells with a microscope and take pictures.
26. If necessary, stain cells with calcein blue or calcofluor white before imaging.

Scaled-up Digestion Device Protocol

Materials

Cellulase Onozuka™ R-10	Yakult (Kanematsu)
Maceronzyme™ R-10	Yakult (Kanematsu)
Morpholineethanesulfonic acid (MES)	Sigma Aldrich (M8250)
Mannitol	Sigma Aldrich (M4125)
Potassium Chloride	Sigma Aldrich (P3911)
Calcium Chloride	Sigma Aldrich (C7902)
Bovine Serum Albumin	Fisher Scientific (BP1600-100)
70-micrometer Sterile Cell Strainers	Fisher Scientific (50-105-0135)

Roughly 3-week-old Tomato Leaves
Scaled-up Digestion Device
Peristaltic Pump
Tubing (ID: 1/32", OD: 3/32")
20- or 22- Gauge Blunt Tip Needle
15- or 50-ml Conical Tube

Solutions

All Solutions are made with ultra-purified deionized water.

Enzymatic Solution

0.2 M 4-morpholineethanesulfonic acid, PH 5.7	20 mM
Cellulase R10	1.5% (wt/vol)
Maceroenzyme R10	0.4% (wt/vol)
Mannitol	0.4 M
KCl	20 mM
CaCl2	10 mM
B-Mercaptoethanol	1-5 mM
BSA	0.1% BSA

W1 Solution

0.2 M 4-morpholineethanesulfonic acid, PH 5.7	4 mM
Mannitol	0.4 M
KCl	20 mM
BSA	1% BSA

Methods

1. Choose well-expanded leaves from about 3-week-old plants.
    - Note: The selection of healthy leaves at the proper developmental stage is a very important factor in protoplast experiments. Protoplasts prepared from leaves recovered from stress conditions (e.g., drought, flooding, extreme temperature and constant mechanical perturbation) may look similar to those from healthy leaves. However, we have often experienced low transfection efficiency with the protoplasts from stressed leaves.
2. Hand cut 1-mm leaf strips from the middle part of a leaf (remove the midrib of the leaf) using a fresh sharp scalpel without tissue crushing at the cutting site.
    - If using the Leaf Preparation Device, see previous protocol for preparation technique.
    - We typically use around 4-7 leaves for each control.
    - Use a clean white piece of paper to inspect for wounded/crushed tissue (juicy and dark green stains); redo cutting if this happens.
    - Weigh the leaf strips and record, in grams, the used sample.
3. Fill the holding chamber with roughly 21 grams of leaf strips, or an amount that can fit comfortably.
4. Cap the holding chamber of the device with the PSA capping layer.
5. Fill a 10 ml syringe with enzymatic solution.
6. Attach a 20- or 22-gauge needle to the syringe.
7. Attach a 3–5 inch length of tubing (ID: 1/32”, OD: 3/32”) to each hose barb on the peristaltic pump carriage.
    - Careful to not force the tubing too far onto the hose barb, in order to prevent it from becoming stuck.
8. Attach the other ends to the scaled-up digestion device’s hose barbs.
    - When connecting them to the device, ensure each channel is connected in a single loop and not cross connected with another channel.
    - Careful to not force the tubing too far onto the hose barb, in order to prevent it from becoming stuck.
9. Disconnect one end of tubing from the carriage and attach the syringe with the needle.
10. Fill the channel with enzymatic solution.
11. Attach the carriage to the peristaltic pump.
12. Remove the syringe and reattach the tube to the carriage.
    - Careful to not force the tubing too far onto the hose barb, in order to prevent it from becoming stuck.
13. Repeat Steps 9-12 for each channels of the device.
14. Set the peristaltic pump to the proper RPM that corresponds to the flow rate being tested.
    - A calibration step may be needed to determine the RPM that equates to the flow rate required.
15. Run peristaltic pump for allotted interval time.
16. Turn off peristaltic pump and prepare a 10 ml syringe with 6 ml of enzymatic solution.
17. Attach a 20- or 22-gauge needle to the syringe.
18. Detach one end of the tubing from one channel and insert the syringe with the needle
19. Slowly extract the solution into a conical tube, by injecting 2 ml through the channel.
20. Remove the syringe with the needle and reattach the tubing.

21. Repeat steps 17-19 for each channel and collect the sample in the same conical tube.
22. Dilute the enzyme/protoplast solution in the conical tube with an equal volume of W1 solution before filtration to remove undigested leaf tissues.
23. Prepare a 70-μm nylon mesh, then filter the enzyme solution containing protoplasts through the mesh into a 50 ml conical tube.
24. Centrifuge the resulting solution at 100 g to pellet the protoplasts in a 50-ml round-bottomed tube for 2 min.
    - A higher speed (200g) of centrifugation may help to increase protoplast recovery.
25. Remove as much supernatant as possible.
    - For culturing or fluorescence staining add 5-10 mls of WI solution to cell pellet and repeat steps 23 and 24 once to ensure removal of enzymes.
26. Resuspend the protoplast pellet in W1 solution.
    - For our experiment we will use 0.07 ml of W1 solution.
27. Count cells with the hemacytometer.
28. Image cells with a microscope and take pictures.
29. If necessary, stain cells with calcein blue or calcofluor white before imaging.

Trypan Blue Staining Protocol

Materials

Trypan Blue 0.4% Solution
Bright-Line Reichert Hemocytometer

Methods

1. Dilute the cell suspension sample in ratio 1:1 with Trypan Blue 0.4% Solution.
    - NOTE: Trypan Blue binds to serum proteins.
2. Place the coverslip on hemocytometer chambers and carefully fill them with Trypan Blue treated cells (10 µl).
    - NOTE: Do not over-or under-fill the chamber.
3. Incubate the cells with hemocytometer for 1-2 minutes at room temperature.
    - NOTE: Do not exceed incubation time of more than 30 minutes as viable cells will also pick up the stain after 30 minutes.
4. Place the hemocytometer under an inverted light microscope and count the cells in four 1 x 1 mm squares of one chamber and determine the average number of cells per square.
    - NOTE: For accurate cell count, the number of cells in a 1 x 1 mm square should range 5-30. If > 30 cells are observed the dilute the cell suspension further. If less than 5 cells are seen use undiluted sample.
5. Determine cell count and cell viability.

$$\boldsymbol{Cell\ Per\ ml} = \frac{total\ cells\ counted * \ dilution\ factor}{number\ of\ squares\ counted} * 10{,}000\ cell/ml$$

$$\boldsymbol{Cell\ Viability}\ (\%) = \frac{Total\ Viable\ Cells\ (unstained)}{Total\ Cells\ (unstained + stained)} * 100$$

Protoplast Culturing Protocol

Materials

Murashige and Skoog Basal Medium (MS Basal Medium)	Sigma Aldrich (M5519)
Zeatin	Sigma Aldrich (Z0164)
1-Naphthaleneacetic acid	Sigma Aldrich (N0640)
Mannitol	Sigma Aldrich (M4125)
Plant Preservative Mixture	Plant Cell Technology
Penicillin and Streptomycin	
Water, Sterile-Filtered, BioReagent, Suitable for Cell Culture	
96-Well, Cell Culture-Treated, Flat-Bottom Microplate	Fisher Scientific (FB012931)

Culture Media

Murashige and Skoog Basal Medium	4.4 grams per 1L of culture water
Water sterile-filtered, BioReagent, suitable for cell culture	1 L
Zeatin	4.6 μM
1-Naphthaleneacetic acid	10.8 μM
Mannitol	0.66 M
Plant Preservative Mixture	0.05%-0.075%
Penicillin and Streptomycin	1%

Methods

Note: Perform these steps in the culture hood:

1. Put required sample into as many wells as needed of a sterile 96-well culture plate
    - Calculate an equivalent concentration of cells for each experimental and control group.
2. Record the volume of sample used for each well.
3. Add 200 μl of MS Basal Medium to each well containing a sample.
4. Seal edges of well plate with parafilm.
5. Incubate in the dark at 25C with an 8.6 W grow light, with a 15-hour photoperiod.
6. After 4 days replace the miedum in each well.
7. Remove roughly 150 μl from each well and then add 150 μl of new MS Basal Medium to each well.
    - Reseal with parafilm after this step is done.
8. After 7 days check cultures for viability and cell wall formation.

Calcein Blue Staining Protocol

Materials

Calcein Blue AM	Fisher Scientific (C1429)
DMSO	Sigma Aldrich (D8418)
96-Well, Cell Culture-Treated, Flat-Bottom Microplate	Fisher Scientific (FB012931)
2 milliliter Microcentrifuge Tubes	

Methods

Stain Preparation:

Stock Stain:

1. Remove Calcein Blue from -20C freezer and mix with ice-cold DMSO to create a stock solution between 1-10 mM.
2. Vortex to mix
3. Store stain at -20C in the dark until needed for making working solution.

Working Solution:

1. Add stock Calcein Blue solution with selected cell medium, in a microcentrifuge tube, to make a concentration between 1-25 μM.
2. Mix solution by flicking tube
3. Store working solution on ice in the dark until needed.
    - Note: If stored properly, Calcein Blue can be prepared several hours in advance with little degradation.

Staining Samples and Observations:

1. Add Calcein Blue working solution to each sample at a concentration of 2%.
2. Carefully pipette sample to mix.
3. Store sample at RT in the dark for 15min before observing.
4. Observe through a fluorescence inverted compound microscope.
    - Use a DAPI filter cube.
    - Specific exposure settings will have to be tested in-house, but once defined, should remain consistent.
5. Quantify viability by comparing living (stained) cell counts to total cells.
    - Depending on population size, multiple observations may provide better estimates.

Calcofluor White Staining Protocol

Materials

Calcofluor White	Sigma Aldrich (18909)
96-Well, Cell Culture-Treated, Flat-Bottom Microplate	Fisher Scientific (FB012931)
2 milliliter microcentrifuge tubes	

Methods

Stain Preparation:

Working Solution:

1. Add stock Calcofluor White solution with selected cell medium, in a microcentrifuge tube, at a concentration between 1-25 μM.
2. Mix solution by flicking tube
3. Store working solution in the dark until needed.
    - Note: If stored properly, Calcofluor White can be prepared several hours in advance with little degradation.

Staining Samples and Observations:

1. Add Calcofluor White working solution to each sample at a concentration of 2%.
2. Carefully pipette sample to mix.
3. Store sample at RT in the dark for 5min before observing.
4. Observe through a fluorescence inverted compound microscope.
    - Use a DAPI filter cube.
    - Specific exposure settings will have to be tested in-house, but once defined, should remain consistent.