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Investigating the Decellularization of Plant Tissues Using at-home Kitchen Facilities During COVID

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ABSTRACT

To engineer transplants, donor tissues are decellularized to create acellular scaffolds for the patients' own cells to be seeded into. There is a need for alternative donor sources because the demand for tissue transplants exceeds the availability of human donors. Plants may offer a solution because of their accessibility, cellulose base, biocompatibility, natural vascularization, and variety of structures and properties available. In this paper, decellularization processes and procedures for plants are presented; and the decellularization of onion epithelial skins was performed using the detergent and detergent-free procedures modified to use at-home kitchen facilities because of school lockdowns during the COVID-19 pandemic. The results of decellularization were histologically analyzed using methylene blue, iodine, and pen ink. Promising results of cells with some debris, a mostly intact extracellular matrix (ECM), and an absence of nuclei, were obtained, which speak to the accessibility of plant scaffolds as a potential donor source.

Introduction

Tissue Engineering Methods and the Benefits of Plant Scaffolds

Tissue engineering began in the 1980s as a way to create living tissue substitutes (Adamski et al., 2018, p. 2). Decellularization is a method of tissue engineering where the native cell material is removed from a tissue to leave an acellular scaffold consisting of the extracellular matrix (ECM), which preserves the structure of the tissue and the vascular network (Gershlak et al., 2017, p. 3). Physical methods and chemical and biologic agents are used to decellularize by lysing cells, followed by rinsing to remove cell remnants (Crapo et al., 2011, p. 1). The goal of decellularization is to leave behind a "ghost tissue" scaffold, named for its white color, that retains the tissue structure while being non-immunogenic because the donor's cellular material is removed (Gershlak et al., 2017, p. 3; McNeill et al., 2018, p. 2). These decellularized tissue scaffolds can then be recellularized with the patient's own cells to create an autologous graft that guides the body to regenerate missing tissue and organs (Gershlak et al., 2017, p. 3; Adamski et al., 2018, p. 2).

The current scaffold types for tissue replacement – autologous, synthetic, and animal-derived grafts – are limited by low availability, cost, and biocompatibility (Adamski et al., 2018, p. 1-2). Animal and human tissues can be decellularized like plants to create animal-derived grafts, which are useful because they provide the most similar organ and vascular structures to what is desired in a transplant. However, animal and human tissue transplants are hindered by high cost, batch-to-batch variability, and limited availability, as well as environmental and ethical concerns (Adamski et al., 2018, p. 2; Gershlak et al., 2017, p. 3; Campuzano & Pelling, 2019, p. 2). These methods also have issues like inefficient vascularity, organ rejection, and biodegradability (McNeill et al., 2018, p. 2). Similarly, advanced manufacturing methods, like 3-D printing, can produce synthetic biomaterial scaffolds with unique physical

properties, but it is difficult to manufacture scaffolds with a functional vascular network (Adamski et al., 2018, p. 2). Synthetic biomaterials have undesirable environmental impacts because they are commonly generated from chemical processing of non-renewable resources like petroleum, and their production often yields toxic byproducts (Gershlak et al., 2017, p. 22).

Plants offer a possible solution to the challenges faced by other tissue engineering methods. Plants are affordable and readily available (McNeill et al., 2018, p. 2). Their chemical and physical composition have many benefits for creating tissue scaffolds: plant scaffolds benefit from their high surface area, excellent water transport and retention, interconnected porosity, preexisting vascular networks, and wide range of mechanical properties (Adamski et al., 2018, p. 1). Plant tissue scaffolds have been shown to be biocompatible and durable within the body because the ECM of plant tissues is composed primarily of cellulose, a common biomaterial (Adamski et al., 2018, p. 2). The accessibility and cost of plant materials facilitates more research at less cost and with less environmental and ethical impacts.

Successful decellularization leaves just the matrisome, composed of the ECM, a noncellular three-dimensional network, and its associated proteins (i.e. growth factors) (Gershlak et al., 2017, p. 3; Mendibil et al., 2020, p.1; Hillebrandt et al., 2019, p.572). Unlike collagen-based scaffolds, which are derived from mammalian tissues or artificially created, plant scaffolds are primarily composed of cellulose (Adamski et al., 2018, p. 2). Human cells do not produce the enzymes necessary to break down cellulose molecules, so cellulose scaffolds are more durable and better suited for long term application than mammalian and other collagen-based scaffolds (Adamski et al., 2018, p. 2). Additionally, cellulose has been extensively reviewed previously as a biomaterial and used in many clinical applications, where it has been shown to be biocompatible and promote wound healing (Gershlak et al., 2017, p. 3; Campuzano & Pelling, 2019, p. 2). Derived from plant and bacterial sources, it has been used to generate biomaterials for a wide range of applications like bone, cartilage, and wound healing, showing success *in vitro* and *in vivo* (Adamski et al., 2018, p. 2; Hickey et al., 2018, p. 3726).

Plant tissues offer the physical benefit of a naturally occurring vascular network when being used as scaffolds. Although plants and animals have fundamentally different approaches to transporting fluids, chemicals, and macromolecules, their vascular networks have surprisingly similar structures (Gershlak et al., 2017, p. 3). Plant vascular vessels act accordingly with Murray's law like mammalian vasculature, where they branch into smaller vessels to minimize hydraulic resistance (Adamski et al., 2018, p. 3). The topographical cues present in the vascularization of stems and leaves guide cell alignment (Campuzano & Pelling, 2019, p. 2). Even after decellularization, the plant network of vessels and interconnected pores is maintained (Adamski et al., 2018, p. 3).

The variety of existing plant structures can be utilized to fill the varied needs of tissue engineering and can be combined to mimic human tissue structures or to create two-dimensional cell cultures to study cell behavior (Adamski et al., 2018, p. 3; Campuzano & Pelling, 2019, p. 1). Plant species have a variety of mechanical properties even within each part; for example, rigid and tough components like stems, or more flexible and pliable components like leaves, which vary based on species in terms of shape, size, break strength, degree of vascularization, and degree of hydrophilicity (Adamski et al., 2018, p. 3). By using the natural architecture of plant tissue and matching the varied anatomical structures from the plant kingdom, it would be possible to find structures with mechanical properties to emulate what is needed for a human tissue engineered scaffold (Gershlak et al., 2017, p. 21).

The success of plant derived cellulose scaffolds has been demonstrated multiple times *in vivo* with mice (Adamski et al., 2018, p. 2). Cellulose based biomaterials remain stable *in vivo* and many cell types will attach and proliferate on plant-derived cellulose scaffolds (Hickey et al., 2018, p. 3731). For example, Modulevsky et al. demonstrated angiogenesis and cell migration occurred when a decellularized apple was implanted in a mouse, and Gershlak et al. showed that endothelial cells could be grown within the vasculature of decellularized leaves; further, Gershlak et al. showed in another experiment that cardiomyocytes could be grown on the surface of leaves that were able to contract (Adamski et al., 2018, p. 3). The work of Hickey et al., 2018 and other recent studies have shown that a multitude of plant-derived cellulose scaffolds are suitable *in vitro* and have shown successful decellularization and culture of mammalian cells *in vitro* and as implantable *in vivo* biomaterial (Hickey et al., 2018, p. 3726).

Acknowledging the success of plant scaffolds in creating viable, implantable tissues in previous research, the goal of this paper is to demonstrate the comparative effectiveness of several decellularization procedures so simple that they can be performed without access to a lab, due to school shutdowns over COVID. The procedures from Adamski et al. 2018 are adapted here to be performed without advanced lab materials, and the results are histologically analyzed. This exemplifies the positive benefits of using plants in tissue engineering: their accessibility, cost, low-toxicity, and relatively small ethical and environmental impacts.

Approaches to Tissue Engineering Plants

Decellularization aims to remove the cellular contents while preserving as much of the ECM as possible (McNeill et al., 2018, p. 2). Successful decellularization produces materials that are formed by the ECM components and similar in composition and architecture to the original tissue (Mendibil et al., 2020, p. 2).

Because of the variability in plant tissue types, alterations of the decellularization procedure may be necessary for each type, dependent upon the tissue's cellularity, density, lipid content, and thickness (McNeill et al., 2018, p. 2; Crapo et al., 2011, p. 4). Bigger tissues take longer to ensure complete decellularization, but increasing the treatment time increases the chance of damaging ECM components (Mendibil et al., 2020, p. 3). Some damage to the ECM is inevitable because all methods of decellularization will affect the structure and composition of the scaffold, so the aim is to minimize damage while still ensuring complete decellularization. (McNeill et al., 2018, p. 2; Crapo et al., 2011, p. 1). It is also important to consider the removal of the remnants of the decellularization detergents and chemicals that may affect cell invasion and proliferation during recellularization for the scaffold to be viable *in vivo* (Hickey et al., 2018, p. 3731).

In this paper, the detergent and detergent-free procedures from Adamski et al. were used (Adamski et al., 2018). The detergent-free method, a solution of bleach and baking soda, is adapted from a protocol that isolates leaf vasculature by removing soft tissue in order to clear the leaves and stems (Adamski et al., 2018, p. 3, 5). The detergent procedure baths were based on previously established methods to clear mammalian tissues (Adamski et al., 2018, p. 3). The detergent procedure involves first soaking samples in an SDS solution, then transferring samples to a solution of bleach and non-ionic surfactant (Triton X-100) (Adamski et al., 2018, p. 3). Adamski et al. found that both methods yield scaffolds with comparable mechanical properties and low cellular metabolic impact (Adamski et al., 2018, p. 3).

Explanation of Decellularization Procedure

I chose to adapt these methods from Adamski et al. beginning over lockdowns because of the availability and low toxicity of the solutions and the simplicity of the procedures. While I did not have access to a school lab over COVID, these procedures could be performed at home, except for heating the detergent-free solution without a fume hood. Instead, all the labs had to be performed at room temperature until I had access to the school lab again.

To evaluate the effectiveness of my decellularization, I used histological analysis because of the limitations of the situation. The stains that I used during testing, methylene blue, safranin, pen ink, and iodine, could all be easily accessed and were safe to use outside of the lab. While the conditions for successful decellularization heavily stress the quantification of remaining DNA, there was no way to perform these tests outside of the lab and without access to fluorescence. Therefore, I qualitatively analyzed the success of the decellularization using the nucleus as a benchmark for whether cellular components remained inside the cell since the nuclear material contained by the nucleus is the most important thing to remove from the scaffold to avoid immune responses *in vivo*. I also observed the structural stability of the samples throughout the decellularization process because the physical integrity of the scaffold is important to maintain for recellularization.

Below is a review of decellularization methods associated with the specific procedures used in this paper, along with their functions, benefits, and drawbacks. These methods are not specific to plant tissues and may be used in combination with one another.

Table 1: Outline of Different Decellularization Methods

Decellularization Method	How the Method Works	Benefits and Drawbacks of Method
Acid-Base (Associated with the detergent-free and detergent procedures)	- Solubilizes the cytoplasmic components of cells and disrupts the nucleic acids (Crapo et al., 2011, p. 5)	- Tends to denature proteins and may damage collagen, glycosaminoglycans (GAGs), and growth factors (Crapo et al., 2011, p. 5-6). - Bases can reduce the ECM mechanical properties more significantly than chemical and enzymatic agents through the cleavage of collagen fibrils and the disruption of collagen crosslinks (Crapo et al., 2011, p. 6). This is an issue for collagen-based scaffolds, but plants are cellulose based.
Osmosis (Associated with the detergent-free procedure)	- Hypertonic and hypotonic solutions make cells explode through osmotic shock (Mendibil et al., 2020, p. 5).	- Kills cells but does not remove the cell waste that it releases into the matrix, so cell remnants must be removed another way to complete decellularization (Mendibil et al., 2020, p. 5).
Surfactants (Associated with the detergent procedure)	- Solubilizes the cell membrane and dissociates the inner cell structure, such as DNA from proteins (Mendibil et al., 2020, p. 5; Crapo et al., 2011, p. 6). - Ionic surfactant is best at denaturing protein molecules and thus in dissolving membranes (Heikrujam et al., 2020, p. 2). - Removal of ECM proteins and DNA by detergents increases with exposure time (Crapo et al., 2011, p. 6).	- Disrupts and dissociates proteins in the ECM (Crapo et al., 2011, p. 6). - Combining multiple detergents allows for more complete detergent removal from ECM after decellularization, but also leads to more ECM protein loss (Crapo et al., 2011, p. 6).
SDS (an anionic surfactant) (Associated with detergent procedure)	- Solubilizes and creates pores in the plant cell and nuclear membranes that lead to the release of cellular components (Campuzano & Pelling, 2019, p. 2; Crapo et al., 2011, p. 5). - Efficient in removing nuclear remnants and cytoplasmic proteins/waste from dense tissues (Mendibil et al., 2020, p. 5; Crapo et al., 2011, p. 5).	- Tends to disrupt ultrastructure, remove GAG and growth factors, and damage collagen (Crapo et al., 2011, p. 5). - Short treatments done with SDS to minimize damage to proteins and overall matrix structure because ionic surfactants tend to denature proteins, which may alter the native structure of the matrix (Crapo et al., 2011, p. 5; Mendibil et al., 2020, p. 5)

Decellularization Method	How the Method Works	Benefits and Drawbacks of Method
		- More effective for removing cell residues from tissue compared to other detergents but is also more disruptive to ECM (Crapo et al., 2011, p. 6). - SDS appears more effective than Triton X-100 in removing nuclei from dense tissues while preserving tissue mechanics (Crapo et al., 2011, p. 6).
Triton X-100 (a nonionic surfactant) (Associated with detergent procedure)	- Disrupts DNA-protein, lipid-lipid, and lipid-protein interactions and to a lesser degree protein-protein interactions (Crapo et al., 2011, p. 5). - For tissue dilipidization, non-ionic detergents are more effective than ionic detergents (Crapo et al., 2011, p. 6).	- Leads to disruption of ultrastructure and removal of GAG (Crapo et al., 2011, p. 5). - Since Triton X-100 targets lipid-lipid and lipid-protein interactions while leaving protein-protein interactions intact, it is useful in tissues where key matrix components are primarily proteins but avoided when GAGs are key component of matrix (Mendibil et al., 2020, p. 5). - Causes ECM protein loss (Crapo et al., 2011, p. 6).

Assessing and Staining Decellularization

To determine if a tissue is successfully decellularized, these are the accepted conditions:

1. Less than 50 ng dsDNA per mg ECM dry weight
2. Less than 200 bp DNA fragment length
3. Lack of visible nuclear material in tissue sections stained with 4',6-diamidino-2-phenylindole (DAPI) or H&E (Crapo et al., 2011, p. 14)

The standard approach to assess decellularization is to use an absorbance-based DNA quantification. Commercially available kits measure DNA by digesting a piece of decellularized tissue, isolating and purifying the DNA, then using a spectrophotometer to measure the amount of DNA per mg of dry tissue. Then the tissue can be classified as successfully decellularized based on whether it passes the standard set by Crapo et al. in 2011 (Narciso et al., 2021, p. 2; Crapo et al., 2011, p. 14). It is also possible to quantitatively assay cell components like double stranded DNA, mitochondria, or membrane-associated molecules like phospholipids (Crapo et al., 2011, p. 14). Nucleic material is an important indicator of successful decellularization because DNA is directly correlated to host reactions when using the decellularized scaffold (Crapo et al., 2011, p. 15).

Decellularization can also be judged by visualizing the cell nuclei with histological stains like Hematoxylin and Eosin (H&E), DAPI, or Hoechst 33342 to confirm the absence of nuclei in comparison to the native tissue in a qualitative way (Narciso et al., 2021, p. 2). Histology is a relatively easy way to observe the decellularization and integrity of the remaining ECM, but even if there are no histologically visible nuclei in the tissue, it is still important to quantify DNA content by molecular techniques (Mendibil et al., 2020, p. 3). There are drawbacks to both of these methods: using the number of cell nuclei as a parameter for decellularization does not account for the DNA that is released from nucleus but still present in matrix which is detrimental to recellularization; and DNA quantification kits also are time consuming and require large amounts of tissue to be sampled from and assume that the tissue is uniformly decellularized which can be wasteful (Narciso et al., 2021, p. 2).

Methodology

(As adapted from Adamski et al. 2018)

A) Detergent-Free Heated (DFH)

1) Create solution of 5% (v/v) bleach and 3% (w/v) sodium bicarbonate
    a. *Referred to as DF solution
2) Submerge sample in DF solution heated to 60-70º C while stirring
3) Remove sample when it appears decellularized (white/translucent)

B) Detergent-Free Unheated (DFX)

1) Create solution of 5% (v/v) bleach and 3% (w/v) sodium bicarbonate
    a. *Referred to as DF solution
2) Submerge sample in DF solution at room temperature
3) Remove sample when it appears decellularized (white/translucent)

C) Detergent (D1-D2)

1) Create a solution of 10% (w/v) sodium dodecyl sulfate (SDS)
    a. *Referred to as D1 solution
    b. To avoid the SDS clumping, add gradually while stirring
2) Submerge sample in D1 solution at room temperature for a few hours or days depending on the size of the sample
3) Remove sample from D1 solution and wash in water to remove remnants of the SDS
4) Create a solution of 1% (v/v) non-ionic surfactant (Triton X-100) and 10% (v/v) bleach
    a. *Referred to as D2 solution
    b. To create solution more easily, mix Triton X-100 and bleach thoroughly before adding water
    c. Note: the D2 solution should be prepared fresh and used within 48 h of preparation
5) Submerge samples in D2 solution at room temperature until the samples appear fully clear
6) Remove sample when it appears decellularized (white/translucent)

D) Staining

To stain with iodine, methylene blue, and safranin:

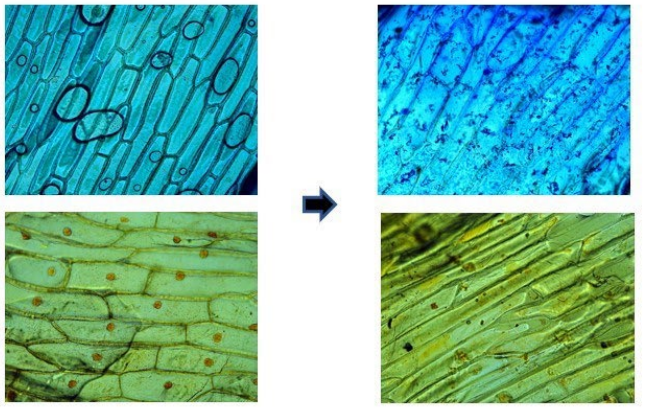
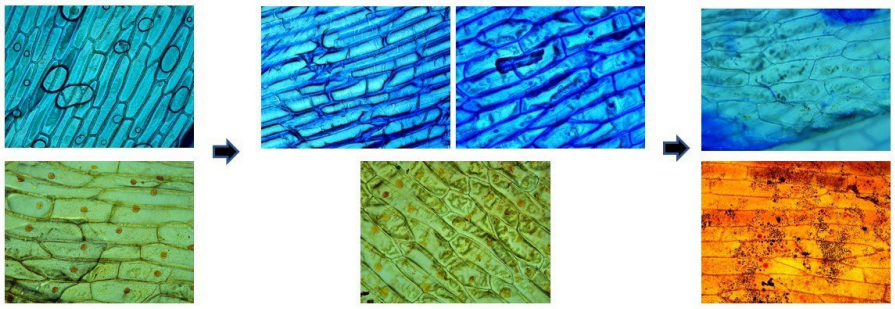
1) Wash the sample thoroughly to remove leftover decellularization solution
2) Add a few drops of stain to sample on microscope slide
3) Wait 30 sec to 1 min before washing the sample gently to remove excess dye
4) Place microscope slide over sample

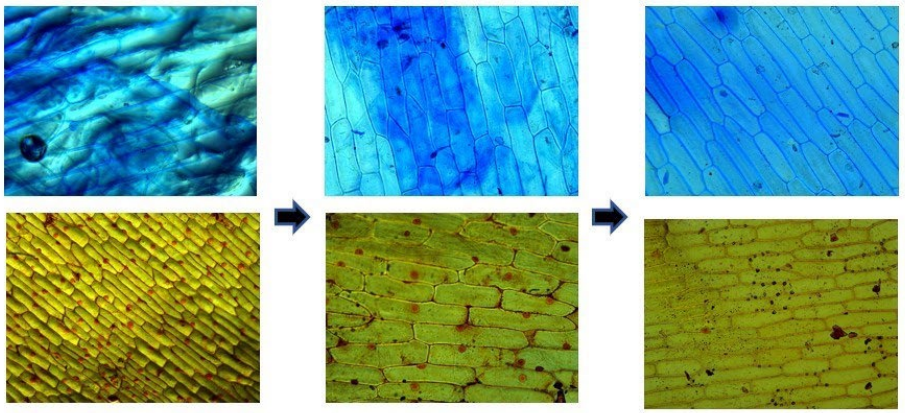
To stain with pen ink (Microbehunter, 2020):

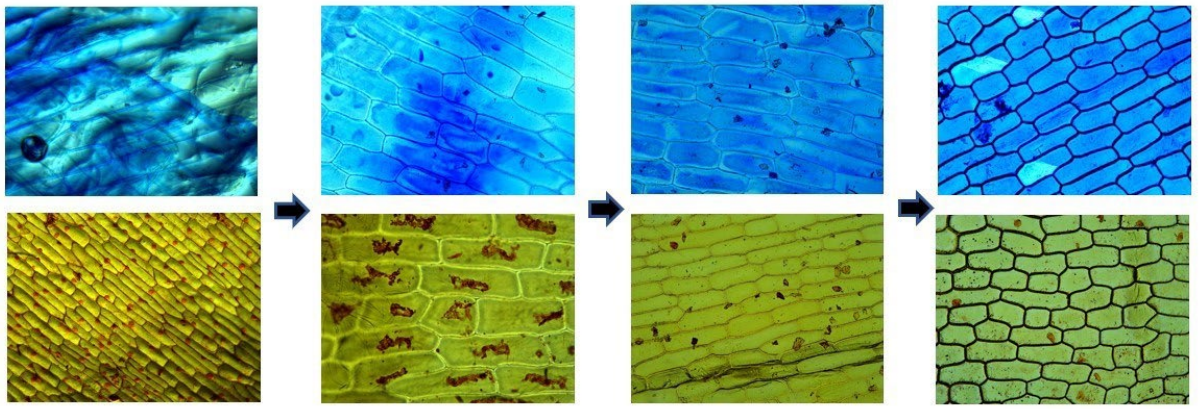
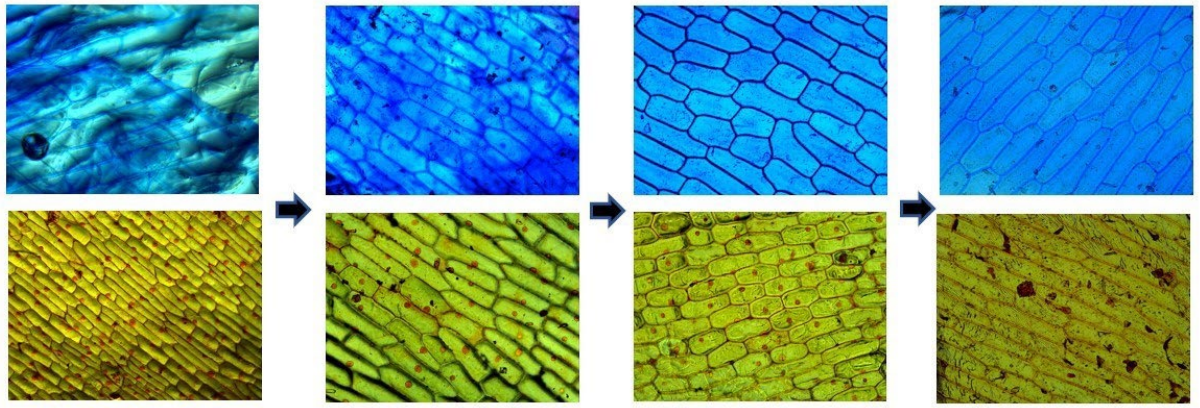
1) Wash the sample thoroughly to remove leftover decellularization solution
2) Quickly submerge the sample in 91% isopropyl alcohol
3) Quickly submerge samples in extracted pen ink
4) Wash excess stain from samples in water
5) Place microscope slide over sample

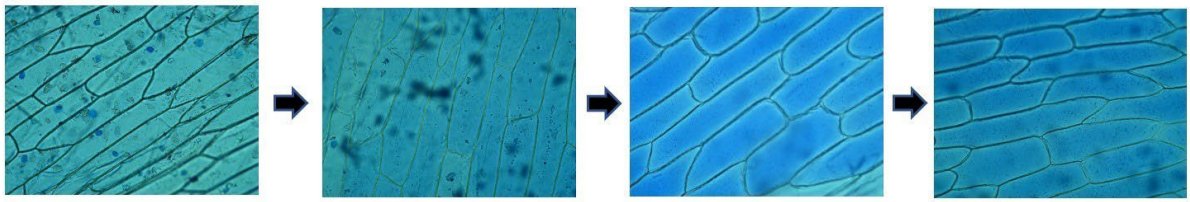
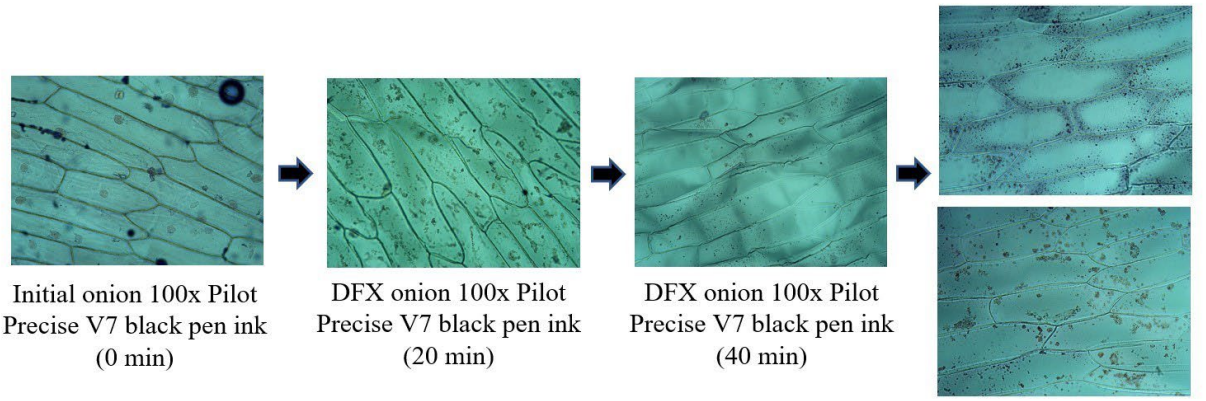
Results

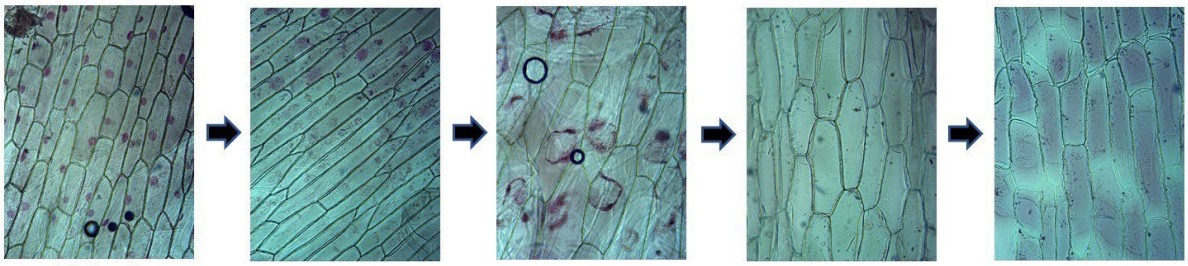
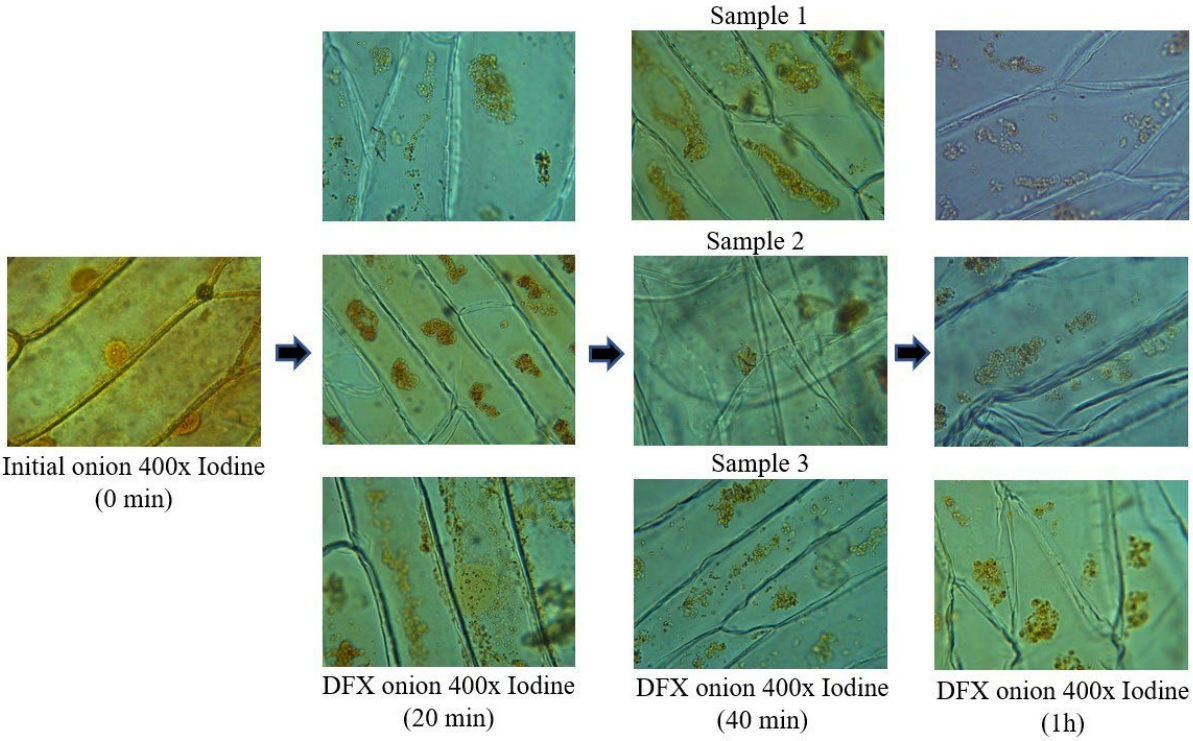
Table 2: Results of Decellularization Methods with Different Procedures, Samples, and Stains in Chronological Order

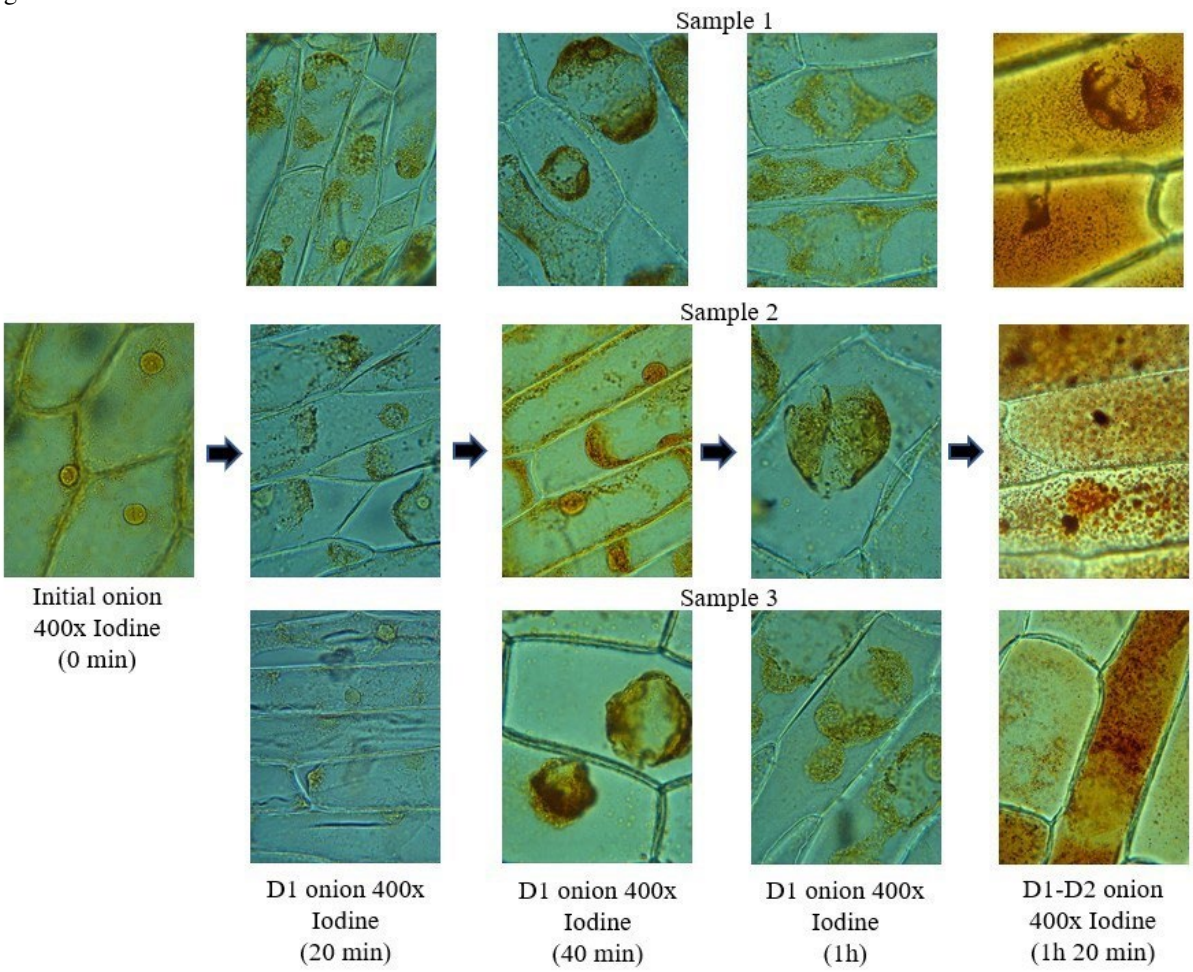
Procedure	Sample Type	Stain Type	Notes
Soak epithelial pieces in DFX solution for 80 min.	Onion epithelial skin	Methylene blue and iodine	After decellularization, there is debris left within cells but no more clear nuclei. This debris could be broken down cellular components.
Figure 1  Initial onion 100x methylene blue (T) iodine (B) (0 min) DFX onion 100x methylene blue (T) iodine (B) (1 h 20 min)			
Soak epithelial pieces in D1 solution for 5h 10min. Wash and transfer to D2 solution for 13h 45min.	Onion epithelial skin	Methylene blue and iodine	After treatment in the D1 solution, both the methylene blue and iodine-stained samples show increased texture and degradation of the cell membrane, though the iodine shows nuclei still present. With the D2 solution, the methylene blue samples show some debris but no nuclei. The D2 solution interacts strangely with the iodine stain, showing precipitate, but no nuclei as before.
Figure 2  Initial onion 100x methylene blue (T) iodine (B) (0 min) D1 onion 100x methylene blue (T) iodine (B) (5h 10 min) D1-D2 onion 100x methylene blue (T) iodine (B) (18h 55 min)			
Soak epithelial pieces in D1 solution for 1h 40 min. Wash	Onion epithelial skin	Methylene blue (with	The D1 solution results in some debris, but the nuclei are still intact. The samples spend less

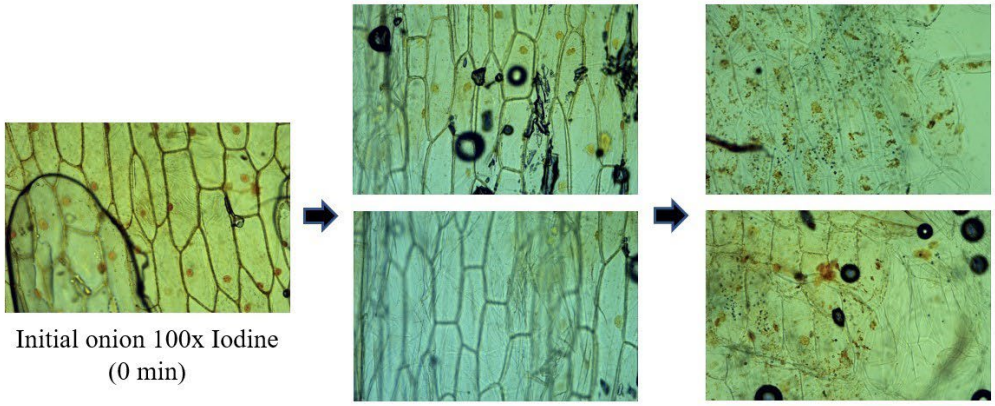
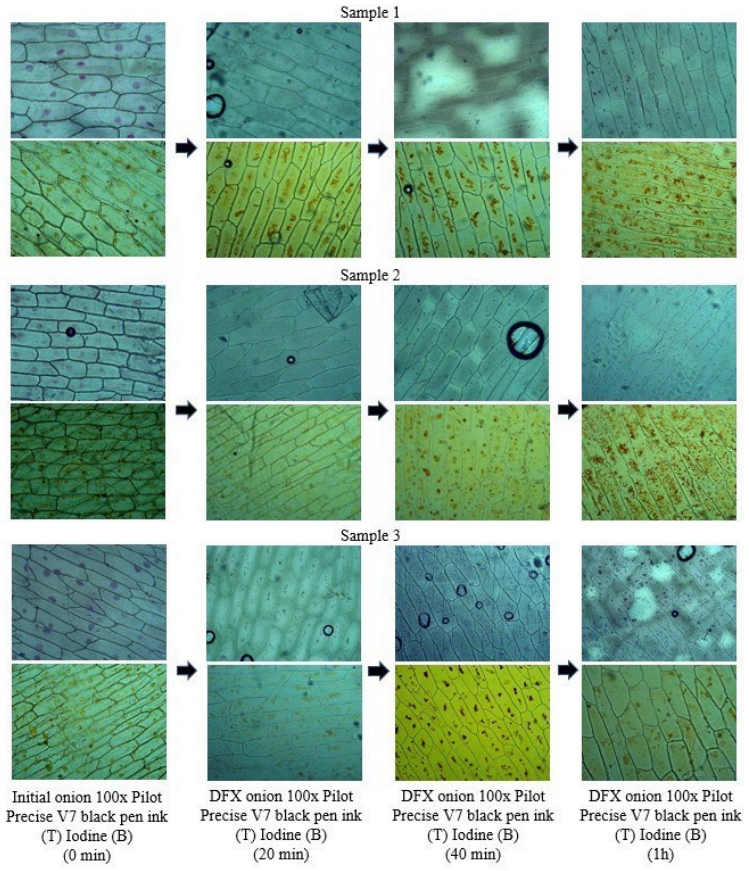
Procedure	Sample Type	Stain Type	Notes
and transfer to D2 solution for 1h 25 min. *When staining with methylene blue, use alcohol in staining procedure like with pen ink.		alcohol prep.) and iodine	time in the D1 solution than in Figure 2, and do not have the same affected cell membrane. By the end of the D1-D2 treatment, there are some crystallization and debris, but not many nuclei remaining. The effect of the D2 solution here is mitigated in comparison to Figure 2 because the time in the solution is much less. Despite spending far less time in treatment than the Figure 2 samples, the decellularization seems equally as thorough. *The alcohol staining step has insignificant effect on the methylene blue staining.
Figure 3  Initial onion 100x methylene blue (alc) (T) iodine (B) (0 min) D1 onion 100x methylene blue (alc) (T) iodine (B) (1h) D1-D2 onion 100x methylene blue (alc) (T) iodine (B) (3h 5 min)			
Soak epithelial pieces in DFX solution for 1h 40 min. Wash and transfer to D1 solution for 2h. Wash and transfer to D2 solution for 30 min.	Onion epithelial skin	Methylene blue and iodine	After 45 min in DFX solution, the nuclei appear to have ruptured, but they are still visible after two washes. There are some debris/organelles visible after 1h 40 min in DFX and 1h in D1 that are not dispersed within the cells, so it appears close to decellularization. At the end of DFX-D1-D2, there is still some debris, and there is darkening of cell walls (cause unknown) and jagged strips that may be the cell membrane breaking apart.

Procedure	Sample Type	Stain Type	Notes
Figure 4  Initial onion 100x methylene blue (alc) (T) iodine (B) (0 min) DFX onion 100x methylene blue (alc) (T) iodine (B) (45 min) DFX-D1 onion 100x methylene blue (alc) (T) iodine (B) (1h 40 min DFX + 1h D1) DFX-D1-D2 onion 100x methylene blue (alc) (T) iodine (B) (1h 40 min DFX + 2h D1+ 30 min D2)			
Soak epithelial pieces in room temperature distilled water for 4h 10 min.	Onion epithelial skin	Methylene blue and iodine	There is an absence of nuclei with iodine stain by 4h 10 min, perhaps because it is a hypotonic solution.
Figure 5  Initial onion 100x methylene blue (alc) (T) iodine (B) (0 min) dH2O onion 100x methylene blue (alc) (T) iodine (B) (1h 15 min) dH2O onion 100x methylene blue (alc) (T) iodine (B) (3h 20 min) dH2O onion 100x methylene blue (alc) (T) iodine (B) (4h 10 min)			
Soak epithelial pieces in DFX solution for 1h.	Onion epithelial skin	Sheaffer Classic Ink Cartridges (blue)	By 40 min, all the organelles and cellular structures are removed. There are small granules of debris within the cell that are almost unnoticeable. The Sheaffer ink dye takes better to the samples the longer that they have been in solution. By 1h 20 min, the samples are too delicate to continue decellularization.

Procedure	Sample Type	Stain Type	Notes
Figure 6  Initial onion 100x Sheaffer blue pen ink (0 min) DFX onion 100x Sheaffer blue pen ink (20 min) DFX onion 100x Sheaffer blue pen ink (40 min) DFX onion 100x Sheaffer blue pen ink (1h)			
Soak epithelial pieces in DFX solution for 1h. *Used DFX solution that was about 2 weeks old because Adamski et al. 2018 did not says that DF solution had an expiration like the D2 solution.	Onion epithelial skin	Pilot Precise V7 Black pen ink	The nuclei are more visible with the Pilot pen ink than the Sheaffer pen ink. The stain takes better to the samples as decellularization progresses. After 20 min, there are almost no intact nuclei visible, but there is a lot of debris. By 40 min, the amount of debris is decreased, and the cells appear empty. However, at 60 min, there is increased debris and variability between the results (cause unknown).
Figure 7  Initial onion 100x Pilot Precise V7 black pen ink (0 min) DFX onion 100x Pilot Precise V7 black pen ink (20 min) DFX onion 100x Pilot Precise V7 black pen ink (40 min) DFX onion 100x Pilot Precise V7 black pen ink (1h)			
Soak epithelial pieces in D1 solution for 1h. Wash and transfer to D2 solution for 20 min.	Onion epithelial skin	Pilot Precise V7 Black pen ink	After 20 min in D1 solution, there are cells with variable levels of decellularization – some nuclei are absent, degraded, or intact. After 40 min, there are structures that are either bloated nuclei or plasmolysis of the cell membrane. By 1h, the cells appear completely clear with less debris than Figure 7 at this point in the procedure. With the additional 20 min in the D2 solution, there is slightly more debris, and the dye took differently to the samples, which is probably due to an interaction with the D2 solution.

Procedure	Sample Type	Stain Type	Notes
Figure 8  Initial onion 100x Pilot Precise V7 black pen ink (0 min) D1 onion 100x Pilot Precise V7 black pen ink (20 min) D1 onion 100x Pilot Precise V7 black pen ink (40 min) D1 onion 100x Pilot Precise V7 black pen ink (1 h) D1-D2 onion 100x Pilot Precise V7 black pen ink (1h 20 min)			
Soak epithelial pieces in DFX solution for 1h.	Onion epithelial skin	Iodine	With iodine stain, the structures within the nuclei are not as clear as with the Pilot Precise V7 ink, but the nuclei are equally visible. Differentiating between three samples shows variation of results. After 20 min, there is a lot of debris in all samples and evidence of plasmolysis in sample 3. By 1h, there is less debris, but still a small amount present.
Figure 9  Sample 1 Sample 2 Sample 3 Initial onion 400x Iodine (0 min) DFX onion 400x Iodine (20 min) DFX onion 400x Iodine (40 min) DFX onion 400x Iodine (1h)			
Soak epithelial pieces in D1 solution for 1h. Wash and transfer to D2 solution for 20 min.	Onion epithelial skin	Iodine	There is plasmolysis and degradation of the cell wall during the 1h in D1, but the nuclei appear mostly intact (not as degraded as in the DFX

Procedure	Sample Type	Stain Type	Notes
			treatment). After 20 min in D2 solution, the nuclei are no longer visible. There is an extreme amount of debris and darkening, so it is possible that the iodine stain reacted with the D2 solution (likely the Triton X-100 because it did not react previously with the bleach).
Figure 10 			
Submerge epithelial pieces (weighted down by attaching to microscope slides) in DFH solution heated to 60-70º C with light stirring for 2 min.	Onion epithelial skin	Iodine	The samples degraded very quickly in the heated solution because they are so small. By 1 min 20 sec, there are still some nuclei visible, but the other cells are clear of nuclei and debris. By 2 min the samples were very delicate and had increased debris.

Procedure	Sample Type	Stain Type	Notes
Figure 11 			
Soak epithelial pieces in DFX solution for 1h. *In this lab the solution was cooler than room temperature	Onion epithelial skin	Pilot Precise V7 Black pen ink and Iodine	Most nuclei are eliminated after only 20 minutes in DFX solution. By 40 min and 1h, debris remains in the cells without much change.
Figure 12 			

Discussion

The goal of this study was to present a viable way to decellularize plant tissues without access to advanced lab materials. During the COVID pandemic with school lockdowns, my access to my school lab was restricted. Therefore, I reviewed the literature to find decellularization methods that could be possible in my kitchen at home without hazardous chemicals or fume hood requirements. I focused on the protocols of Adamski et al. because of the accessibility and safety of the procedures. Plant decellularization with bleach, baking soda, SDS, and Triton X-100 was possible to do in my kitchen because all these chemicals could be purchased online and were relatively non-toxic; additionally, I found that the original detergent-free procedure by Adamski et al. could be performed without heating so it would not require a fume hood when performed at home. These methods of decellularization are presented in Table 1, along with their effects, benefits, and drawbacks. The specific procedures for decellularization and staining used in this paper are also presented under Methodology.

With these limitations, I approached decellularization with fruits and vegetables from the supermarket. I began with cutting thin sections, then used a vegetable peeler. I preferred using green peppers, beets, carrots, string beans, cucumber, and celery because they had large cells for viewing and retained their texture well through treatment. Leafy vegetables like spinach, kale, beet leaves, and lettuce were not ideal because their cuticle prevented them from being decellularized in the DFX solution, and their cells were too small to see details; however, the DFH treatment successfully turned spinach into ghost tissue. Broccoli, cauliflower, and apples also made poor subjects because their cells were too small for viewing with this microscope.

To improve the quality of observations from the images, it was also important to make the samples as thin as possible so that overlapping cells did not muddle the image. The epithelial layer of an onion skin was the best sample because the single layer of cells made imaging much clearer in comparison to earlier labs where I was unable to produce a single layer of cells with a vegetable peeler. This choice of samples had an added benefit because the small samples could be decellularized quickly and more uniformly than samples with many layers of cells. This paper focuses on the results of the onion epithelial tissue samples because they provide the best visualization of decellularization.

To visualize results in early experiments, no dye was used, which made the ghost tissue effect noticeable in photos, but it was nearly impossible to see cellular details; only after decellularization could faint circular forms in cells be seen. When I began using methylene blue, as seen in Figures 1-5, these forms were clearly visible, but only after decellularization, and no cell structures could be seen before treatment. I originally mistook these forms for nuclei, which would suggest that decellularization was incomplete; however, Huang et al. explain that the nuclei cannot be seen initially because living cells take up methylene blue and reduce it to a colorless form, while only dead cells and cell debris stain blue (Huang et al., 1986, p. 80). Therefore, after treatment, the cells were dead, and these structures were only debris that remained in the cells after decellularization.

In early labs, I used safranin stain to compare to the methylene blue results because Imran et al. used the darkness of the safranin staining as an indication of how much nuclear material was in the cells. These lab results are not included in this paper because they were done before I began using onion epithelial skins. Using safranin did not show any cellular structure, and I could not determine if the lightness of the safranin towards the end of decellularization was due to a lack of nuclear material or interaction between the safranin and the remnants of decellularization solution in the cells. The safranin still stained the cells after decellularization the DFH labs, but it was less saturated and more rust-colored. In the detergent labs, the safranin stain looked identical throughout treatment in the D1 solution, but in D2 solution, it reacted to the samples similarly to the DFH results. Since safranin never showed cell details, it was not a helpful companion to the methylene blue, and I subsequently began using other stain types.

Pen ink and iodine proved to be the ideal stains of the options because they greatly improved cell visibility. Iodine, in Figures 1-5 and 9-12, brightly showed nuclei and cell structures before and after decellularization. Pen ink, in Figures 6-8 and 12, also showed the inner structures of the nuclei before decellularization. These two stains provided support to conclude that the forms seen after decellularization in the methylene blue stained samples, in Figures 1-5,

were not nuclei but the cell membrane during plasmolysis, and they showed in most samples of decellularization the rupture or absence of the nuclei. Since pen ink and iodine provided a clear view of the nuclei before decellularization, I began using them instead of methylene blue.

In DFX labs, Figures 1, 6, 7, 9, and 12, there was plasmolysis and breakdown of the cell membrane, accompanied by absence of the nucleus. Figure 9 showed the clearest visual of plasmolysis, while the other figures showed almost immediate elimination of the nucleus. By 20-40 minutes, the nuclei were removed in all DFX labs, but the degraded membrane remained in the cells. As time in the solution increased, the membrane and cell contents continued to degrade, leaving debris in the cells. More washes seemed to help remove the membrane debris because the pen ink samples, Figures 6, 7, and 12, which underwent more washes as part of the staining procedure, had less debris when compared to the iodine samples. This is especially clear in Figure 12, where the pen ink and iodine samples are shown side by side.

The DFH procedure decellularized samples much faster than the DFX labs, as seen in Figure 11, and there appeared to be less debris left after one minute and 20 seconds. While there were a few nuclei remaining, the empty cells were completely clear of debris. The results are variable, though, because the samples in solution for two minutes had debris and slightly degraded nuclei. This may be because when the images were taken, I would remove a section of the sample for staining and photographing, then return the other section to the solution. It was difficult to keep the heated samples fully submerged with the stirring of the solution, so different parts may have had more successful decellularization because of greater contact with the solution.

The detergent labs, Figures 2, 3, 8, and 10, had variable results with the different stains. Plasmolysis is clear within an hour in the D1 solution in Figures 8 and 10 and slightly in Figure 2, where the membrane peeled away from the cell wall and began to degrade, but the nuclei remained semi-intact. Figures 8 and 10 show the visible degradation of the cell and nuclear membranes during plasmolysis. In Figure 8, stained with pen ink, the cells appear clear after one hour in the D1 solution, but the iodine stained samples in Figure 10 still show large sections of the membrane in the cells at that same point. Unlike the other detergent labs, in Figure 2, the samples remained in the D1 solution for over five hours instead of one hour, and the nuclei still remained intact. In Figure 3, plasmolysis is not visible, and the nuclei remain present in the cells throughout treatment in the D1 solution. After the D2 treatment, there was a large increase in debris within cells stained with iodine, Figures 2 and 10, and some crystallization visible in Figures 2 and 3. There is a less severe increase in debris in Figures 8 and 12 stained with pen ink, and no increase in debris in the methylene blue stained samples in Figures 2 and 3. Since the majority of cell structures are removed, this increase in debris may be an interaction or precipitate formed by the D2 solution and the stains used, particularly iodine. It was likely the Triton X-100 that caused this reaction because the bleach in the DF solution did not form a precipitate with the iodine.

The lab with a combination of the DFX and detergent procedures, shown in Figure 4, had promising results. By doing the DFX treatment first, plasmolysis and degradation of the cell membrane and destruction of the nuclei occurred early, as opposed to the D1 treatment, which left most of the nuclei intact. This allowed the next D1 treatment to almost entirely clear the cells but for a small amount of cellular debris left outside of the cell plane. The D2 treatment seemed excessive since the cells were almost entirely clear already. It left completely clear cells, with a darkened cell wall and jagged pieces of cell membrane or wall. The DFX then D1 treatments together seemed to successfully decellularize, and using the D2 treatment afterwards should be avoided if the samples were going to be recellularized to avoid compromising the ECM.

Despite working in a kitchen lab, without access to a fume hood or advanced chemicals and tools, it was still possible to decellularize and analyze plant tissues. The ability to perform these labs under the limitations of COVID and still produce promising results speaks to the accessibility of plant decellularization. Early experimentation with different samples, stains, and solutions, lead to the more efficient procedure presented in this paper. In the future, as I attend university, I would like to continue this research with access to more advanced materials, like fluorescence microscopy and DNA quantification. I would also like to use other decellularization methods to compare to the procedures presented in this paper.

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