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How Do You Like Them Apples? Cellulose Scaffolds Seeded with Mammalian Cells.

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3D scaffolds offer certain advantages over traditional 2D culturing techniques, notably an environment that more closely mimics the *in vivo* conditions. We include protocols that describe how to prepare and decellularize cellulose-rich scaffolds from different varieties of fruit and vegetables before re-seeding with a non-plant cell line, in this case, the mouse myoblast cell line, C_2C_{12}. Using a simple cell staining technique - the DNA stain, methyl green - and a transmitted light microscope, non-host derived cells were visible within the cellulose chambers of the apple scaffold. These protocols have been modified and tested over two iterations of a third year undergraduate Cell Biology lab course. The success of these experiments in the students' hands has meant the inexpensive addition of 3D cell culture techniques to the lab course curriculum.

Keywords: 3D, 2D, cell culture, apple, scaffold, animal cells, microscopy

Introduction

Traditionally, mammalian studies incorporating *in vitro* cell culture experiments use cells grown on a flat 2D surface (e.g. on the bottom of a tissue culture plate or flask). Yet, cell growth in tissue or in an organ involves processes that are influenced by a more complex 3D microenvironment. For example, unlike flat culture plates, tissue-bound cells are surrounded by an extra-cellular matrix (ECM) and, as reviewed by Haycock (2011) and Ravi et al. (2015), the raison-d'être for scaffolds is to reproduce those features found naturally within the ECM. The ECM plays an important role in cell support, cell differentiation, cell migration, and gene expression (as reviewed by Brizzi et al. 2012). Consequently, cellular responses measured in the context of a 3D microenvironment could provide a valid and reliable understanding of biological processes, more so than responses measured using traditional 2D cultures.

Commercially generated 3D scaffolds made of cellulose are available for cell experimentation; cellulose makes for an ideal surface and model for mimicking *in vivo* cellular processes as it is inexpensive to generate and highly stable. In 2014, Andrew Pelling's lab published a protocol that outlined a simple and cost-effective procedure to isolate and prepare apple-derived 3D scaffolds in which apple cells were replaced with mouse cells (Modulevsky *et al.* 2014). During the 2017-2018 school year, Dr. Alp Oran (A.O.) recruited an upper-year undergraduate student, Karine Loiselle (K.L), to modify and test the Pelling lab protocols in an undergraduate lab course setting.

This article shares student-tested protocols designed for the third year Cellular Biology laboratory (BIO 3152 and BIO 3552) at the University of Ottawa. A successful pilot trial of these protocols was completed in the Winter 2018 semester and repeated again, in expanded form, for the Winter of 2019. Each iteration of the lab course included two separate sections composed of up to twenty students per section meeting once per week for twelve weeks and for one lab of six hours per week.

The protocols, described herein as *Activities*, give students the tools and expertise to prepare their own cellulose-based 3D scaffolds. In short, students purge fruit and vegetable slices of their native plant cells and then re-seed them with a mouse myoblast cell line (C_2C_{12}). Scaffolds, once repopulated with the animal cells, are aldehyde fixed and stained with inexpensive chromogenic dyes to assess successful cell implantation by microscopy. Moreover, even though the protocols described in this report focus on the preparation and seeding of McIntosh apple scaffolds with C_2C_{12} cells, students in previous cohorts have tested these protocols with other fruits and vegetables. In summary, the protocols work with little to no modification for a variety of plants.

For those lab instructors seeking a relatively novel and cheap way to introduce 3D cell culture in their lab courses, this report is worth consideration. Moreover, the unusual idea of using fruit to grow animal cells can have a quirky appeal for students.

Student Outline
Seeding Bioscaffolds

Objectives

- Understand what and why we work with 3-Dimensional (3D) scaffolds
- Determine if, when, and where you can introduce plant-based scaffolds in your lab curricula

Activity 1 - Decellularization of McIntosh Apples

Imagine that you are in an accident in which you seriously damage or lose a limb. Alternatively, imagine a loved one needs a new liver. Replacement body parts and organs, especially those compatible with the host, are not easy to come by, at least not without serious immunosuppressive therapy to prevent rejection. Not surprisingly, developing an effective and inexpensive way to regrow these structures using the host's own cells is the focus of multiple labs around the world (as reviewed in Modulevsky *et al.* 2015). Over the past couple decades, the fields of tissue engineering and regenerative medicine have made some major in-roads, developing and demonstrating various substrates (both natural and artificial) capable of accepting and growing cells. Many of these biomaterials or "bioscaffolds" are of animal origin, opening ethical issues over their isolation and use; in the case of human sources, were these tissues obtained without coercion and with full consent? Moreover, commercial bioscaffolds (artificially generated or naturally isolated) vary in price from tens to thousands of US dollars per cm of material (Table 3-1, Modulevsky et al. 2015). Even on the lower end of the scale, these costs may be out-of-reach for those seeking tissue replacements in impoverished countries. Consequently, finding low-cost options that do not have the same ethical hang-ups as with animals becomes a worthwhile endeavor. As it turns out, Modulevsky et al. (2014) demonstrated that apples, once decelluarized, could act as a scaffold for mammalian cell growth in vitro, and at least at one ten thousandth of the cost as with the aforementioned commercial materials. More recently, the same group demonstrated that these cellulose-based scaffolds could be implanted into immunocompetent mice, avoid host rejection, and become integrated with host cells and vasculature (Modulevsky et al. 2016).

You will have the opportunity to repeat some of the work from these papers, taking apple slices, decellularizing them, and replacing the fruit cells with C_2C_{12} cells, a mouse myogenic cell line that is approved for use in teaching labs.

Timing:

This activity takes about 45-60 minutes to complete.

Materials:

- One (1) McIntosh apple, medium to large size, per group of two students
- 12 cm ruler
- Knife
- 0.5% (m/v) sodium dodecyl sulfate in dH_2O.
- 1.5 mL microfuge tubes
- Cutting board
- Fine forceps
- Sterile pipet tips and pipettes (micro, 5 mL and 10 mL)
- Tube rack for 1.5 mL tubes
- 3D rocking shaker (Cole-Parmer)
- Mandolin slicer (Börner V5)
- Phosphate Buffered Saline (PBS)
- Sterile PBS with 100 IU/mL of penicillin and streptomycin
- 2% glutaraldehyde in 0.1 M Phosphate Buffer
- Tissue Culture (TC) biosafety cabinet
- Tissue Culture (TC) incubator, 95% relative humidity, 37^{o}C, 5% CO_2

Protocol:

1. Each group of two students: Put your apple in the freezer at -20^{o}C to chill for 5 minutes. DO NOT LET THE FRUIT FREEZE. We want to make the apple slightly more rigid for intact sections with the mandolin. Freezing will expand or rupture the scaffold.
2. *Optional:* Using a knife, peel the apple and discard the skin.
3. On a cutting board, cut the chilled apple into 0.5 mm to 1 mm thick slices with the mandolin. Usually this is the

thinnest setting on the adjustable mandolin. Avoid pressing down on the mandolin as you slice across the blade. **Remember:** The blade is very sharp so be careful. **Watch your fingers!**

- With your knife, cut the hypanthium region of the slice into six 2.0 cm x 0.5 cm rectangles (Appendix A, Figure 1) parallel to the pedicle or stalk of the apple.
- Using a pair of fine forceps, place each rectangular piece into a separate 1.5 mL tube. So four 2.0 cm x 0.5 cm pieces in four tubes, one piece per tube.
- Add 1 mL of the 0.5% SDS solution to each 1.5 mL tube containing a slice.
- Place these 1.5 ml tubes into a tube rack. Be sure to label the tubes with your team number and slice number (1 to 6). Place the rack on a 3D rocking shaker so that the tubes are on their side in the rack and cannot fall out.
- Leave samples on a 3D rocking shaker at a speed and pitch so as to get good movement of the SDS over the pieces but not too vigorous so as to create many bubbles. Rock the tubes in their racks for a minimum of 12 hours and a maximum of 3 days. The detergent and rocking action help with plant cell lysis in the slices.
- At this point, maintaining sterility is important. Henceforth, we will manipulate the samples in a TC biosafety cabinet. After this decellularizaton period, the samples are washed 3 X 3 minutes per wash with sterile PBS. They are then treated with sterile PBS containing 100 IU/mL of penicillin and streptomycin (pen/str) antibiotics for three hours to minimize contamination, rinsed again 3 X 3, transferred to a solution of filter-sterilized 2% glutaraldehyde in 0.1 M phosphate buffer, and stored at 4^{o}C for a minimum of one hour. Thicker sections may need more time to be completely aldehyde-fixed.

Activity 2 - Preparation of Scaffolds

In *Activity 1*, you prepared apple slices, cut them into small pieces, and treated them with a detergent (SDS) in order to remove the native apple cells. From *Activity 1*, after decellularization, these "ghost" slices were then treated with antibiotics and then fixed in glutaraldehyde. You will now remove the fixative, wash, and treat with $NaBH_4$. This will then be followed by reconditioning of the scaffolds to mammalian growth media (GM) to prepare them to receive the mammalian cells in *Activity 3*. Sterility must be maintained; any solutions and equipment coming into contact with the biological samples (e.g. tubes and pipettes) must be pre-sterilized. This may involve autoclaving, filtering, or purchasing pre-sterilized items.

Timing:

This activity takes about 150 minutes to complete up to step 8.

Materials:

- Bioscaffolds from *Activity 1*
- Sterile Phosphate Buffered Saline (PBS)
- 1% $NaBH_4$ (m/v) in PBS; filter sterilized, 0.22 μm
- Fine-tip forceps
- 2x multiwell (MW) 6 well TC plates; 9.5 cm^2 per well; sterilized from manufacturer
- Sterilized glass coverslips, #1, 22 mm x 22 mm (Fisher)
- Sterile pipet tips and pipettes (micro, 5 mL and 10 mL)
- Growth Medium (GM): RPMI 1640 + 100 IU/ml penicillin and streptomycin + 20% fetal bovine serum
- Tissue Culture (TC) biosafety cabinet
- Tissue Culture (TC) incubator, 95% relative humidity, 37ºC, 5% CO_2

Protocol:

**BE VERY GENTLE WITH THE SCAFFOLDS AS THEY ARE FRAGILE.

1. In the TC cabinet, using a pipette tip, remove the glutaraldehyde solution from your scaffold samples in the 1.5 ml tubes and discard it in the approved waste beaker.
2. Wash the scaffolds 3 X 3 minutes per wash with 1 mL of sterile PBS. Add and remove the solutions gently. You can let the samples sit in a rack for each 3-minute duration.
3. After discarding your last PBS wash, using the forceps, carefully transfer scaffolds from the tubes into wells 2, 3, 5 and 6 of a MW6 plate, one scaffold per well. (Appendix B, Figure 2). Note: this plate is just temporary.
4. Add 3 mL of the 1% $NaBH_4$ solution to the wells containing scaffolds.
5. Incubate at room temperature for 1 hour. $NaBH_4$ will help stop aldehyde fixation; any free aldehyde groups will be reduced to non-reactive hydroxyl groups.
6. Transfer the scaffolds to a new six well plate and into the same wells as described in Step 3. But now, add one sterile glass coverslip (for 2D culture) to wells 1 and 4 (Appendix B, Figure 2).
7. Add 3 mL of growth medium (GM) to wells containing bioscaffolds. Replace the lid to the MW6 plate.
8. Place the plate in the TC incubator for a minimum of 12h at 37ºC, 5% CO_2.
9. Growth Medium will be changed every 48 hours until *Activity 3*. This will maintain scaffold saturation with GM.

Activity 3 - Adding C_2C_{12} cells

In the following exercise, you will seed the fixed "ghost" slices with the C_2C_{12} mouse myogenic cell line (Yaffe and Saxel 1977). Normally, this would involve students learning how to "split" cells in a tissue culture biosafety hood, count cells, and prepare them for seeding into the MW6 plates. We have included these steps in the protocol but for the sake of time, we will not be doing these steps for the ABLE workshop. Since C_2C_{12} cells are a popular model for examining myogenesis (development of muscle cells; Sabourin and Rudnicki 2000), the following weeks would include morphological examination and staining for myogenic markers. For example, we can investigate the expression and distribution of the early myogenic marker, myogenin, but this would be time consuming and expensive to accomplish. Instead, we will visualize the cells within the scaffolds by staining with the cationic DNA stain, methyl green (see *Activity 4*). For now, we wish to share with you our steps for seeding mammalian cells into the apple scaffolds. These same steps apply to other plant-based scaffolds tested over the past two years by the students.

Another point: 2D (coverslip) versus 3D (apple) scaffolds. One of the arguments for scaffolds is that they are closer mimics to the cellular environment experienced in vivo (as reviewed in Haycock 2011and Ravi *et al*. 2015). Unlike cells grown on a flat surface such as a tissue culture dish or a coverslip, cells cultured in a 3D scaffold will experience, mediate, and respond to their environment in what is arguably a closer approximation of natural development in their host organism. For this reason, we are sharing with you here our protocols for seeding both coverslips and apple scaffolds. Comparisons of 2D versus 3D cellular responses can be an additional concept to introduce in your lab curriculum.

Timing:

This activity takes about 180 minutes to complete up to step 19.

Materials:

- TC Multiwell (MW) 6 well plate from *Activity 2*
- 100 mm TC dish of C_2C_{12} cells cultured in GM at 60-80% confluency
- RPMI 1640 + 100 IU/ml pen/str + 20% fetal bovine serum (Growth Medium; GM; sterile)
- Phosphate Buffered Saline (PBS; sterile)
- 0.025% trypsin with 1 mM EDTA in sterile PBS; filter sterilized, 0.22 μm.
- Sterile 1.5 mL, 15 mL and 50 mL tubes (Falcon)
- Hemocytometer
- Centrifuge (for 5 mL to 50 mL tube sizes and capable of generating 500-600xg)
- Sterile Pipets (p2 to p1000 sizes, 5 mL and 10 mL) with pipettes
- Biosafety TC cabinet
- Tissue culture (TC) incubator, 95% relative humidity, 37ºC, 5% CO_2

Protocol:

In order to assess differences in our cells over time on either coverslips or in the apple scaffolds, you will need to start by preparing your cell cultures. By following *Activity 3*, you will learn how to seed C_2C_{12} cells onto glass coverslips and into bioscaffolds inside tissue culture dishes (Appendix B, Figure 2). This seeding must be done carefully and using good sterile technique. Competent pipetting and accurate cell counting are vital for the success of these experiments.

NOTE THESE STEPS ARE DONE IN A BIOSAFETY TC CABINET TO MINIMIZE MICROBIAL CONTAMINATION.

1. Obtain one lidded 100 mm TC dish of C_2C_{12} cells from the TC incubator (prepared in advance for you).
2. Examine the cells under the microscope. Note their shape, colour, and plating density.
3. While working in the biosafety cabinet, remove the culture medium from the dish with a 10 mL pipet and discard it into the provided waste beaker.
4. Carefully rinse the cells in the dish with 5 mL of sterile PBS, and then remove the wash.
5. Add 3 mL of a solution of 0.025% trypsin (w/v) with 1 mM EDTA in sterile PBS to the dish to facilitate detachment of the cells. Return the lid and gently swirl the solution to cover the cell monolayer with the solution. Incubate the cells in the TC incubator at 37ºC for 5 minutes or until the cells detach from themselves and the plate.
6. With the microscope, verify that the cells have detached from the plate and each other. If there are large clumps or 30%+ cells still attached to the plate, replace the plate back into the TC incubator for another couple minutes. Ideally, we wish to have unattached cells but clumps of two cells are normal and acceptable moving forward.
7. Add 4 mL of GM to the trypsinized (detached) cells floating in the dish. This will stop the trypsinization. To aid in detachment and dispersal of the cells, aspirate and discharge the cells with a sterile 5 mL pipette against the bottom of

the dish at least 5 times to break up the cell aggregates.

8. Transfer your cell suspension to a 50 mL centrifuge tube. This is your **stock cell suspension**.
9. Using a pipette set to 10 µL, load one side of a hemocytometer slide with a drop of cell suspension from step 8. Count the cells in the four corner regions of the hemocytometer (here is how to count: https://www.youtube.com/watch?v=pP0xERLUhyc). Calculate the concentration of your stock cell preparation.
10. To the wells that do NOT have a scaffold, only coverslips (1 and 4), you want to add around $7.5x10^5$ cells/coverslip in a volume of 3 mL of GM. This may require that you dilute your stock cell solution from step 8.
11. Make 10 mLof this $2.5x10^5$ cells/ml into a sterile 15 mL tube.
12. Mix the 10 mL cell-GM solution gently by pipetting or rocking the tube gently back and forth. Quickly transfer 3 mL ($7.5 x10^5$ cells) to wells 1 and 4.
13. To wells 2 and 5 WITH a scaffold, you want to add $5x10^5$ cells per scaffold in a 40 µL drop of GM.
14. This will require preparing another dilution of your stock cell solution (Step 8) in a sterile 15 ml tube. Prepare enough cells to seed three wells. Spin down for 5-10 minutes at 500xg.
15. Resuspend the cell pellet into 120 µL of GM and transfer your cell suspension into a labelled 1.5 mL tube.
16. Remove the medium from wells 2 and 5 of your MW6 plate and add 40 µL of your cell suspension to both scaffolds. Add it directly on top of the scaffolds so the cells can seed the scaffolds.
17. Place your MW6 well plate in the TC incubator for 1 hour at 37ºC, 5% CO_2.
18. Add 3 mL of GM to wells 2 and 5.
19. Incubate in the TC incubator for another 24 h at 37ºC, 5% CO_2.
20. After 24-48 hours, the scaffolds will be transferred to new MW6 TC plates, and fresh growth media will be added in each well. These will remain in culture for up to 7 days (enough time to have cell growth and spread in wells).

Activity 4 - Fixing and Staining of Cells with Methyl Green

Here you will carry out staining of the cells on both coverslips and in the scaffolds with the help of a dye. Methyl green will stain the nuclei and help visualize the cells against the background (APPENDIX C). One last point: the scaffolds will be examined before and after flattening with the help of another coverslip and a gentle application of pressure using your fingers. The flattening may help in visualization of cells in the scaffold. UPDATE 2020: We have tested a 0.02% (w/v) coomassie blue solution (dissolved in methanol, distilled water, and acetic acid at 4.6:4.6:0.8 ratio, respectively) in lieu of methyl green for superior staining of cells with minimal background.

Timing:

This activity normally takes around 120 minutes to complete.

Materials:

- Multiwell (MW) 6 well TC plate containing scaffolds + cells from *Activity 3*
- Phosphate Buffered Saline (PBS) in squeeze bottle
- 2% formaldehyde in PBS; filter sterilized, 0.22 μm
- 0.5% (w/v) methyl green in 0.1 M sodium acetate, pH 4.2; filter sterilized, 0.22 μm
- Fine-tip forceps
- Glass coverslips, 18 mm x 40 mm (Fisher)
- Glass microscope slides, frosted ends (Fisher)
- Nail polish
- Kimwipe™ disposable wipes
- Humid chamber (plastic container with lid – bread loaf size - and wet paper towel inside)
- Pipet tips and pipettes (micro, 5 mL and 10 mL)
- PBS-glycerol (50:50) mounting media

Protocol:

1. Recover your MW6 TC plate from *Activity 3*.
2. Remove and discard media from each well in the appropriate manner.
3. Gently rinse cells 3 X 3 minutes by adding 2-3 mL of PBS per well. Be sure to wash gently by adding PBS to the sides of the wells. DO NOT ADD YOUR WASHES DIRECTLY ONTO THE COVERSLIPS OR SCAFFOLDS.
4. After the last PBS wash, add 2 mL of 2% formaldehyde to each well.
5. Incubate at room temperature for 10 minutes.
6. Gently wash as before, 3 X 5 minutes with 2-3 mL of PBS per wash.
7. BEFORE REMOVING THE LAST WASH: Place two drops of 100 μL of methyl green onto a labelled sheet of Parafilm stretched over an empty pipette tip box.
8. FOR THE COVERSLIPS IN WELLS 1 & 4: Using the forceps, carefully retrieve the coverslips from their wells and then invert the coverslips face down onto the drop of methyl green. You want the cell side of the coverslip to touch the drops.
9. FOR THE SCAFFOLDS IN WELLS 2, 3, 5, & 6: Remove your last PBS wash from the wells in your MW6 plate, then add 100 μL of methyl green directly onto each scaffold in wells 2, 3, 5, and 6. Please do this carefully and reasonably quickly, as we do NOT want the cells in the scaffolds to dry out. Replace the lid to the MW6 plate.
10. Transfer both steps 8 and 9 to a humid chamber. This can be a large plastic Tupperware™ (large to accommodate a bread loaf) with some wet paper towel. Close the lid to trap the humidity. We do not want the cells to dry out as they are being stained.
11. Incubate with methyl green at room temperature for 20 minutes.
12. Using the forceps, replace your coverslips back into their corresponding wells of the MW6 plate, cell side up this time! Wash scaffolds and coverslips 2 X 5 minutes with 2-3 mL of PBS per wash. As before, be gentle with your washes.
13. During the 2nd wash from Step 12, prepare some glass microscope slides. Be sure to label the slides according to your MW6 plate (therefore, 1 to 6 and your team identity).
14. For glass slides 1 and 4, add 25 μL of PBS-glycerol per slide. With the help of your forceps, retrieve your coverslips from wells 1 and 4, remove the excess wash from the coverslips with a Kimwipe™, and then invert them (cell side down) onto the 25 μL drop of PBS-glycerol.
15. Using your fine forceps, gently transfer the bioscaffold from each well (2, 3, 5, and 6) onto the remaining glass slides, one scaffold per slide. Add carefully 25 μL of PBS-glycerol directly to the scaffold.

16. Take representative pictures of your cells on the 2D coverslips and in your 3D bioscaffolds. Await instructions from teaching assistants about proper use of the microscopes and cameras, what magnification to use, and software, etc. Be sure to note the settings and maintain the same settings for each photo where applicable.
17. Save your images as JPEGs and follow directions as to where to save and how to name your files.
18. Flattening your scaffolds - for slides 2, 3, 5, and 6: Place an 18 mm x 40 mm coverslip over each scaffold and press down lightly and evenly. The scaffold should flatten out under the pressure. Ideally, the coverslip should be flush with the slide.
19. Tack down the coverslip to the glass slide with the help of drops of nail polish at each corner.
20. After the nail polish has dried, take another set of images of your flattened scaffold. Is it easier to visualize the cells now?
21. Save your images as JPEGs in the indicated folder on the computer.

Materials

This section is a general summary of the equipment needed to carry out the activities outlined in the previous section.

For All Activities

For General Tissue Culture (TC)

- TC biosafety cabinet
- TC incubator, 95% relative humidity, 37^{O}C, 5% CO_2
- 37° C water bath (for warming solutions)
- Refrigerator (for TC media storage)
- Freezer (for TC serum, antibiotics, supplements)

For Each Lab Section

- light microscopes to visualize the cells
- centrifuges (1.5 mL, 15 mL, and 50 mL formats)
- vortex

For Each Pair of Students

- Hemocytometer
- Marker (to label tubes)
- Timer (for incubation time)
- Pipet tips: micro (p2, p20, p200, p1000), 5 mL, 10 mL (sterilized)
- Pipettes for pipet tips of above formats
- Waste containers
- Tube racks (1.5 mL, 5 mL, 10 mL, 50 mL formats)

Activity1 Setup

Each Pair of Students Needs

- One (1) McIntosh apple, medium to large size
- 12 cm ruler
- Knife
- 0.5% (m/v) sodium dode cyl sulfate in dH_2O.
- 1.5 mL tubes
- Cutting board
- Fine forceps
- Sterile pipet tips and pipettes (micro, 5 mL and 10 mL)
- Tube rack for 1.5 mL tubes
- 3D rocking shaker (VWR)
- Mandolin slicer (Börner V5)
- Phosphate Buffered Saline (PBS)
- Sterile PBS with 100 IU/ml of penicillin and streptomycin
- 2% glutaraldehyde in 0.1 M Phosphate buffer, filter sterilized, 0.22 μm

Activity 2 Setup

Each Pair of Students Needs

- Bioscaffolds from Activity 1
- Sterile Phosphate Buffered Saline (PBS)
- 1% $NaBH_4$ in PBS; filter sterilized, 0.22 μm
- Fine-tip forceps
- 2x multiwell (MW) 6 well TC plates; 9.5 cm_2 per well; sterilized from manufacturer
- Sterilized glass coverslips, #1 22 mm x 22 mm (Fisher)
- Sterile pipet tips and pipettes (micro, 5 mL and 10 mL)
- Growth medium (GM): RPMI 1640 + 100 IU/ml penicillin and streptomycin + 20% fetal bovine serum
- Tissue culture (TC) biosafety cabinet
- Tissue culture (TC) incubator, 95% relative humidity, 37^{O}C, 5% CO_2

Activity 3 Setup

Each Pair of Students Needs

- RPMI 1640 + 100 IU/mL pen/str + 20% fetal bovine serum (Growth Medium; GM; sterile)
- Phosphate Buffered Saline (PBS; sterile)
- 0.025% trypsin with 1 mM EDTA in sterile PBS; filter sterilized, 0.22 μm.
- Sterile 1.5 mL, 15 mL and 50 mL tubes (Falcon)
- 100 mm TC dish of C_2C_{12} cells cultured in GM at 60-80% confluence.
- Hemocytometer
- Centrifuge (for 5 mL to 50 mL tube size and capable of generating 500-600xg)
- Sterile pipet tips and pipettes (micro, 5 mL and 10 mL)
- TC Multiwell (MW) 6 well plate from Activity 2
- Biosafety TC cabinet
- Tissue culture (TC) incubator, 95% relative humidity, 37^{O}C, 5% CO_2

Activity 4 Setup

Each Pair of Students Needs

- Multiwell (MW) 6 well plate containing scaffolds + cells from Activity 3
- Phosphate Buffered Saline (PBS) in squeeze bottle
- 2% formaldehyde in PBS; 0.22 μm filter sterilized
- 0.5% (w/v) methyl green in 0.1 M sodium acetate, pH 4.2; filter sterilized, 0.22 μm
- Fine-tip forceps
- Kimwipes^{TM} disposable wipes
- Glass coverslips, 18 mm x 40 mm (Fisher)

- Glass microscope slides, frosted ends (Fisher)
- Nail polish
- Humid chamber (large – bread loaf size - plastic container with lid)
- (Sterile optional) pipet tips and pipettes (micro, 5 mL and 10 mL)
- PBS-glycerol (50:50) mounting media

Notes for the Instructor

As previously mentioned, these protocols were designed and first introduced into a 3rd year cell biology laboratory course during the Winter semester of 2018 at the University of Ottawa. Furthermore, they were based on the protocols described by Modulevsky *et al.* (2014).

The protocols described in this report would not have been possible without competent technical help. K.L., with technical help from C.L-P. and L.J., developed and tested various modifications to the 2014 protocols described in Modulevsky *et al.* (2014). This included the reduction and elimination of certain steps. In so doing, they developed the Activities described in this report that allow students the ability to repeat the published findings. For example, Modulevsky *et al.* (2014) specified incubation periods of a minimum of 12 hours for the decellularization of the apple scaffolds, a 6-hour incubation with antibiotics post-decellularization, and chemical-crosslinking of the naked scaffolds with glutaraldehyde for 12 hours. Shorter time periods of 1, 3, and 6 hours were tested but a minimum of 12 hours and a maximum of 3 days was determined to be the window for the decellularization of the apple scaffolds. Beyond 3 days, the scaffolds began to lose structural integrity in the SDS solution. An incubation period with antibiotics post-decellularization was initially removed for 2018 but has since been re-added for 2019 to reduce bacterial contamination of samples (a problem seen in 2018 with the students' samples but not in 2019 when reintroduced). The 6-hour chemical cross-linking with glutaraldehyde has been reduced to an incubation period of 1 hour. Similarly, a 12-hour incubation with sodium borohydride post-aldehyde fixation was reduced to 1 hour while another 6-hour incubation was reduced to 1 hour. Finally, the original paper called for the addition of six million cells per well loaded on to the scaffold. For the purposes of the lab course, this amount was logistically expensive and impractical to set up. Thankfully, K.L., L.J., and C.L-P. tested lower concentrations and were able to seed and visualize cells in their scaffolds with five hundred thousand to one million cells per scaffold. It should be noted that the seeding concentration of cells would be cell type dependent; conceivably, fewer cells would be needed as their proliferative capacity increases.

With respect to the scaffolds themselves, thin and even slices are important especially when the students begin taking pictures of cells in the scaffolds with the help of microscopes (see Appendices). From 2018, we noted that students, who did not practice good technique when using the mandolin, had thicker sections and, therefore, complicated images of their cells in the scaffolds. For this reason, it is important to i) purchase good quality (rigid) mandolins that can give very thin slices, and ii) ensure students are using the mandolins correctly. For example, some students tended to push down as they sliced, distorting the main body of the mandolin as the apple crossed the blade. This in turn created thicker slices. Unfortunately, the desirable thin slices have one important drawback – they are fragile. This fragility becomes worse with all the chemical treatments of the slice starting with decellularization.

After decellularization and fixation, it is imperative that instructors remind students that the apple scaffolds are very delicate, and that they can break easily if they are not handled properly. The best way to handle scaffolds is to use a pair of fine tip forceps and lift the scaffold slowly and gently. An alternative strategy is to place a cover slip under the scaffold and use the coverslip as platform to lift the scaffold in and out of the well. Moreover, we advise preparing some sample scaffolds in advance for the students to test and hone their skills before they work on their own scaffolds. In addition, different plants (other than McIntosh apples) will have differences in fragility so we recommend the preparation of additional slices of these samples for practice with the forceps. Another issue are structures within plant that may challenge the student's ability to distinguish cells from the scaffold. In the case of the apple, when selecting the regions of hypanthium to use for scaffolds, it is important to take note of the presence of vein-like structures – possibly sepal bundles or segmented vasculature (Herremans et al. 2015) - throughout the cortex region of the apple section. These structures should be avoided as they make it difficult to visualize the cells by microscopy; in 2018 and 2019, these structures were often confused with the C_2C_{12} cells. By extension, similar structures in other fruits and vegetables should be avoided. Interestingly, certain chromogenic dyes were trapped within these structures, providing higher background staining that masked regions of cell staining.

Currently, the scaffolds seeded with C_2C_{12} cells have been stained with trypan blue, methyl green (see Appendix C, Figures 3 and 4), hematoxylin, and Alexa488-labelled phalloidin (to visualize F-actin filaments in the cytosol). In hindsight, there is still work to be done to optimize the level of staining. UPDATE 2020: 0.02% (w/v) coomassie blue (CB) dissolved in a methanol: distilled water:acetic acid (4.6:4.6:0.8) mix will only stain cells after a 20-minute treatment (Appendix C, Figure 4b). So far, CB is the best stain at highlighting the cells against a colorless cellulose background.

For two semesters, students in BIO3152 and BIO3552 were able to experiment with the McIntosh apple as well as try other fruits and vegetables (easily sourced from the local grocer) to determine if they too could be sources of 3D cellulose scaffolds. So far, we have managed to re-apply these same protocols (with no modification) on Granny Smith apples, Bartlett pears, red mangoes, garden cucumbers, and garden radishes. We have tested potatoes (Yukon Gold) but the protocol, as described, does not work; decellularization produces an extremely viscous mix that points to limitations in the procedure and that not all plants are alike.

As of 2019, 3D scaffolds have become a major component of the lab course, with nearly 10 weeks of lab revolving around some aspect of 3D culture and experimentation. We have begun fluorescent staining for intracellular cell markers (e.g. actin staining with Alexa488-phalloidin) in addition to chromogenic cell staining with dyes (e.g. trypan blue, methyl green, and hematoxylin). In all past and future iterations of this lab, students are expected to work with McIntosh apples in order to maintain reproducibility and establish a baseline for student evaluation and fairness in assessment practices. Nonetheless, the option for the student to test new fruit and vegetables will continue to be an option as it provides the opportunity for novel findings in the course, and a level of ownership by the student with their experiments. Our only advice is to have more than one group work on the same novel fruit or vegetable to ensure reproducibility of the results. Other themes for consideration involves other cell lines or cell types and to see if, in the context of cellulose scaffolds, cells behave differently (e.g. versus traditional 2D culture).

In conclusion, we have developed a modified methodology inspired by the literature (Modulevsky *et al.* 2014) but adapted to work in an undergraduate teaching lab setting. The protocols allow lab instructors a cost-effective means to introduce current and relevant techniques in the field of 3D cell culture . Outside of the purchase of a good quality mandolin, the same reagents and equipment used to manipulate and examine cells using the traditional 2D platform can also be used to manipulate and examine cells in a 3D context. The only real challenge is the handling of the scaffolds but this can be learned with some carefully planned and timed practice sessions.

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Thank you to K.L for taking on this responsibility to develop this project from early test phase to a functional pilot for the Winter 2018 iteration of the lab. Thank you also to L.J. and C.L-P. who provided technical training to K.L. and who vetted the protocols developed by K.L. Thank you to ABLE Major Workshop selection committee and all the workshop participants for selecting and participating in the workshop.

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About the Authors

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Lucille Joly (L.J.) and Cindy Lesage-Pelletier (C.L-P.) are lab course technicians involved in the preparation of the Cell Biology lab at the University of Ottawa.

Appendix A: Preparation of Apple Slices

a b

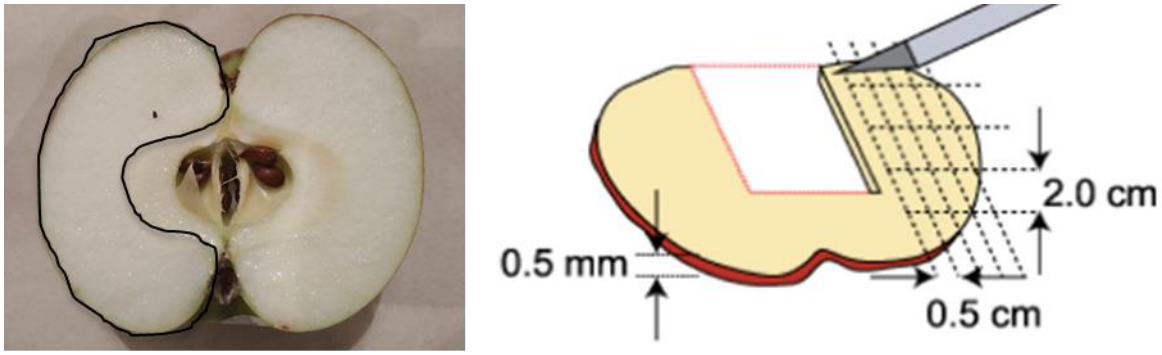

Figure 1. Apple slice and cartoon depicting the preparation of the apple sections from the hypanthium. a) McIntosh apple with the hypanthium region highlighted on one side (black outline on left). b) Using a mandolin, 0.5 mm to 1.0 mm thick slices are prepared and then cut with a knife as depicted. 0.5 cm X 2.0 cm segments from the hypanthium are prepared for decellularizaton. (Image b adapted from Modulevsky *et al.* 2014)

Appendix B: Setup of Tissue Culture Plates

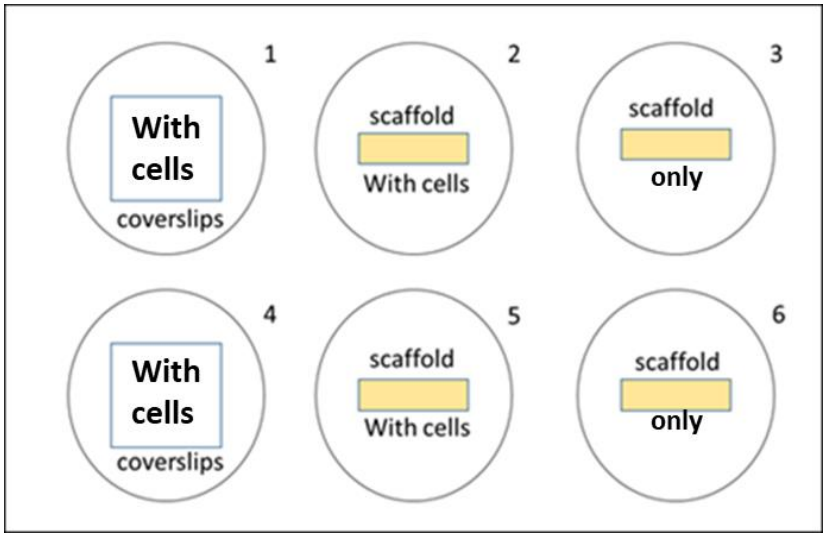

Figure 2. A cartoon of the plate setup for Activities 2 and 3. A 6-well tissue culture plate was loaded with sterilized glass coverslips (wells 1 and 4) or scaffolds from the apple (wells 2, 3, 5, and 6) isolated and processed as described in Activity 1. Wells 1, 2, 4, and 5 received C_2C_{12} cells.

Appendix C: Cell Cultures and Scaffolds Images

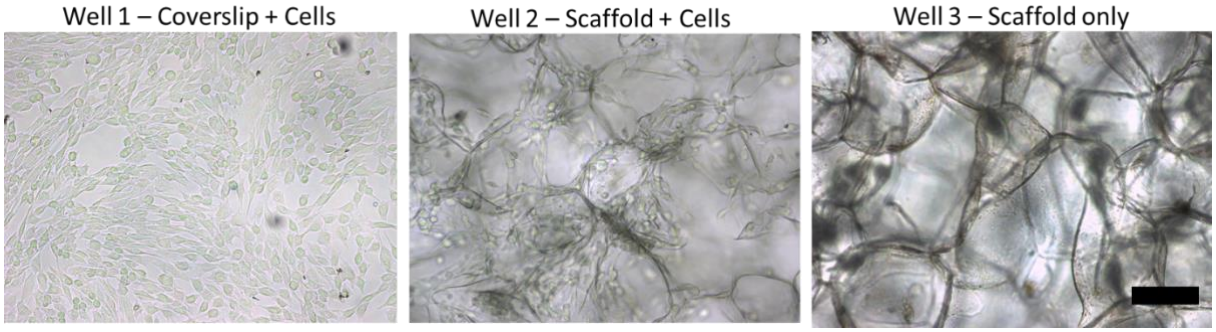

Figure 3. 2D and 3D cultures stained with methyl green. In keeping with Activity 3 and 4, cells were seeded into wells 1, 2, and 3 of a six-well plate. Well 1 represents C_2C_{12} cells seeded onto a glass coverslip; traditional 2D gel growth. Wells 2 and 3 are McIntosh apple scaffolds with well 2 receiving C_2C_{12} cells only; 3D cell culture. These samples were stained with methyl green as described in Activity 4. Different focal planes within the scaffolds reveal the existence of cells at multiple depths for Well 2 but empty chambers in Well 3. These are representative images taken by students registered in the 2019 Winter cohort of the Cell Biology lab. Visualized by brightfield microscopy. Scalebar, 120 μm.

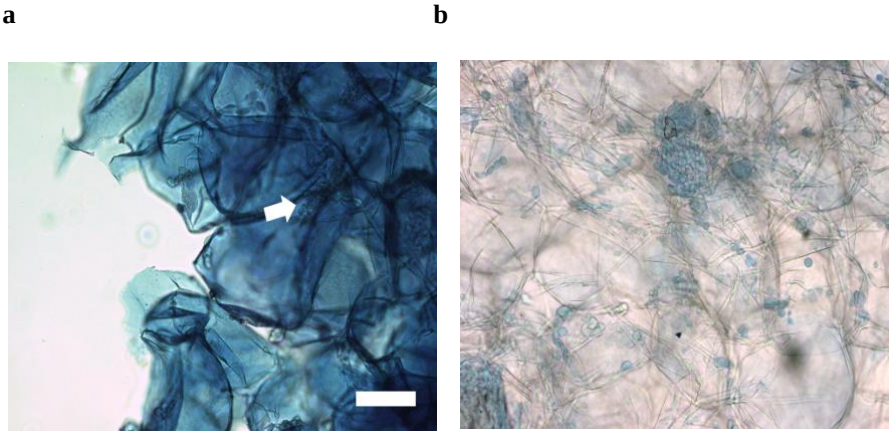

Figure 4. a) Trypan blue staining of cellulose scaffolds with cells results in high background coloring**.** From the 2018 cohort, a student showcases what was a typical staining outcome of scaffolds loaded with cells (arrow). Unlike methyl green, trypan blue appeared to stain the cellulose chambers as well as the cells. b) UPDATE 2020: Cells stained with 0.02% (w/v) coomassie blue gave clear staining of cells only, seen in blue. Both visualized by brightfield microscopy at same magnification. Scalebar, 100 μm.

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