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An Inquiry-Based Laboratory Curriculum Investigating Cell Viability Using Mammalian Cell Culture and Fluorescence Microscopy

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Inquiry-based research experiences in an undergraduate laboratory lead to an increase in critical thinking and improvement in student learning gains. In this new curriculum, students work in groups to design experiments to investigate the effects of a chosen factor (e.g., acetaminophen, green tea) on cultured mammalian cells. The goal of this curriculum is to advance important student research skills, including graphing and experimental design, critical thinking skills, and scientific communication. Students apply a rigorous scientific approach to the interpretation of the data generated, including analysis of the generated data and its relationship of the data to previously published work. The focus of this paper is to discuss the curriculum model, the pedagogical goals, the logistics of implementing authentic research experiences in undergraduate courses, and the potential modifications for institutions with varying instrument availability.

Keywords: cell biology, cell culture, fluorescence microscopy, inquiry-based learning; CURE

Introduction

Semester-long, course-embedded undergraduate research experiences (CUREs) have been shown to improve student content learning gains, attitudes towards science, and understanding of experimental design (Goyette and DeLuca, 2007; Murthy, 2014). Here, we present our curriculum for such a CURE for a cell biology course, using mammalian tissue culture and fluorescence microscopy laboratory techniques.

Cell Biology with Laboratory (BIOL3400K) is sophomore/junior-level course at Georgia Gwinnett College. This course is required for all Biology majors and Clinical-track Exercise Science majors. Previously, BIOL3400K students participated in two, unrelated laboratory modules. By developing and implementing a semester-long CURE, we aimed to improve student research skills (e.g., literature searches, experimental design) and introduce students to modern cell biology laboratory techniques (cell culture, fluorescence microscopy).

In this CURE, students work in groups to design and conduct a research project investigating the effect of a chosen factor (e.g., lemon juice, coffee) on the viability of cultured PtK2 cells (male rat-kangaroo epithelial kidney cells). Before beginning their experiments, groups will be responsible for performing background literature searches to determine if the effects of their selected item on cell viability are known and for submitting a formal research proposal. During the first phase of the module, students will assess the effect of their chosen factor on cell viability using trypan blue exclusion assays. While conducting these experiments, students will gain skills in aseptic technique, maintenance of mammalian cell cultures, cell dilutions, use of hemocytometers, cell viability assays, graphing, experimental design, and data analysis. Student groups will have at least two attempts at their cell viability experiments, allowing them the opportunity to improve and expand upon their initial experimental designs.

During the second phase of the project, students will investigate the mechanism by which their chosen factor alters cell viability using fluorescent microscopy to evaluate potential changes in apoptotic cells, the cytoskeleton, and cell cycle distribution. Throughout these experiments, students will acquire skills in using tissue culture chamber slides, fixing and fluorescently labeling cells, the use of fluorescent microscopes, and identification of apoptotic cells and cells in various stages of the cell cycle. At the end of the semester, students will orally present their projects to the class and submit a manuscript-

style, formal lab report, synthesizing their findings from both phases of the project.

Preparation and Design of the Research Experience

This semester-long research project spans 15 consecutive weeks with laboratory time of 2.75 hours each week. In an academic year, approximately 12-14 cell biology sections participate, with 5-6 lab sections per Fall and Spring semesters and two sections each summer semester. In each lab section students are broken into six groups which generate approximately 30-36 lab groups each Fall and Spring semesters and 12 lab groups each summer semester. Faculty members teaching this course may vary each semester. However, coordinated materials, equipment, and supplies maintained the consistency of the research experience.

Implementation of the Research Experience

Students use cultured mammalian cells to investigate the effects of a chosen factor on those cells using two major techniques, cell culture with the trypan exclusion assay (7 weeks) and fluorescence microscopy (5 weeks). In this CURE, PtK2 rat kangaroo kidney epithelial cells were used; however, other mammalian cell lines can be used with similar results. Before experimentation, students must first research a potential experimental factor and its impact on cell growth or cell death in mammalian cells. Students are directed to websites such as PubMed and Galileo to find primary literature using the suggested search term phrasing. Each student returns to the lab, and their lab group members, with a primary research article in support of their factor suggestion. Student groups then discuss each student's findings before selecting the final factor to investigate for the semester and generate a hypothesis based on the previous studies found in the literature. The only limitation set by the instructions is that the factor must be polar due to the nature of the experimental process. Examples of some factors previously chosen by students include the juice of crushed pomegranate seeds, monosodium glutamate powder (MSG), Tylenol cold and flu medication, creatine monohydrate supplement, and caffeine pills.

The cell culture module encompasses the first seven weeks of the semester and includes an investigative, training and experimental phase. The purpose of this module was to determine the impact of the experimental factor on cells, specifically if it impacted cell growth or apoptosis. Early in the term (Weeks 2 and 3), student training includes the use of equipment and micro- and serological pipetting. Student technicians prepare a T-25 flask seeded with PtK2 cells for each student group. Cell students learn sterile technique and passaging of cells using that initial flask. In Weeks 4-5, students train to complete the experimental technique of of passaging into two flasks (control and experimental) and the trypan blue exclusion assay. This practice assay provides students an opportunity to make errors, and learn from them, in a low stakes environment. The practice factor used by all students is lemon juice at a 1:1000 final flask dilution, with an equal volume of Phosphate Buffer Solution (PBS), added to the control flask. During these practice labs, student groups decide the factor to study for the semester. In Weeks 6-8, students complete the experiment of treating their cells with their chosen factor in two trials. The first trial, students are encouraged to treat their PtK2 cells with a final flask concentration of 1:1000. Based on those results, students then determine the final flask concentration of the second trial. Students also use the previous research findings found during the investigative stage. Using this information as a guideline informs students if their factor concentration is reflective of the previous findings. Trypan blue exclusion assay, a hemocytometer, and either a phase objective in the fluorescent microscope or a compound light microscope are used to determine numbers of viable and non-viable cells. Student calculations of the data collected include total cells per ml, total viable cells per ml, total nonviable cells per ml, and percent viable cells.

The microscopy module comprises the final five weeks of the semester. The purpose of this module was to determine the impact of the experimental factor on mitosis or apoptosis. Student technicians seeded one 4-well chambered slide per student group. For convenience in the preparation phase, these cells did not originate from the student's flasks, but rather new PtK2 cells are grown in large quantities and used for this purpose. A few days before the first microscopy lab, the cells must treat with the student factor and incubate. Faculty members had previously completed this step for the student groups, but in recent semesters one student in each student group is delegated to come in during the non-lab time to treat the cells on their chamber slide. This change increased student ownership in their project as a continuous project with one hypothesis and not two distinct, independent modules. Treatment of two wells with the final factor concentration selected by the group and an equal volume of PBS in the other two wells generates control and experimental flasks. After 48-72 hours of incubation with the factor, students prepare the slides for staining by fixing, permeabilizing and blocking the cells during Week 9. The following lab, students stain the cells with 1) Anti-tubulin (microtubule stain), 2) Phalloidin (actin stain), and DAPI mounting media (DNA stain). For the next three lab sessions, student groups rotate through the inverted fluorescence microscopes to collect ten images of each well, five field of views (FOV) each with a DAPI only image and three-color overlay image of all fluorescent stains, for a total of 40 images. The DAPI (blue) the only image generates reliable data on the stage of mitosis or apoptosis as the

DNA is key to determining this stage. The three-color overlay of all stains reinforces the information in the DAPI image and shows the impact to the microtubules (green) and actin filaments (red). Student calculations include percent mitotic cells, percent apoptotic cells and percent viable cells.

Student Materials

Each week students are responsible for completing the steps stated in the weekly protocol and a related worksheet. These documents are designed to provide basic content and explanation of scientific importance, reinforce the protocol steps of current and future project labs, and prepare solutions or calculations for next week's lab. The protocol specifically includes a written section that includes the experimental steps as well as helpful technique suggestions in comment bubbles such as how to avoid bubbles. Additionally, the protocol contains a work-flow chart demonstrating the protocol in visual form. The associated worksheet contains content questions based on protocol narrative, critical thinking questions about the experimental process, scientific method reinforcement, quantitation practice for the upcoming lab, math calculations specific to their project, graphing practice. institutions.

The following presents a selected student protocol from an upcoming, published curriculum lab manual (Barnes, et al), instructor notes, and suggestions on how to modify curriculum for different courses and institutions.

Student Outline
Expanding PtK2 Cultures and Treating Cells with Lemon Juice

This week you will expand your group PtK2 culture to two T-25 flaks and treat cells for a "practice" trypan blue exclusion assay examining the effect of lemon juice on PtK2 cell viability.

Objectives

- Passage PtK2 cells and expand cultures to two flasks
- Treat cells with Phosphate Buffer Solution (PBS)** (control) or lemon juice (experimental) for next week's trypan blue assay
- Prepare experimental factor (will vary among lab groups) for the upcoming semester project

Introduction

This semester, students will be given a population of mammalian cells (PtK2 cells) to maintain throughout the semester. These cells are the sample population that will make up the **Experimental** group and **Control** group. Student groups are using those cells to test how an experimental factor (aka **independent variable**) impacts those cells. The cells treated with the independent variable are the Experimental group, and the cells treated with the **placebo** (**Phosphate-Buffered Saline** (PBS)) are the Control Group. Today students are testing cells with lemon juice to practice the cell techniques. In Lab 4, students will collect the data from this experiment using a **Hemocytometer**. The hemocytometer measures numbers of cells (live vs. dead); and the equations associated are calculating cells per ml (total, live, and dead) and percentages (viable). This means that the type of data collected limits the effects that we can see/measure (aka **dependent variable**). When graphing the collected data, the dependent variable is placed on the Y-axis.

The **hypothesis/prediction** statements are educated guesses. These are guesses of how the experimental factor will impact the cell population. These are also limited by the type of experiment and data collected. In the upcoming semester the analysis of the provided protocols limit student hypotheses to the experimental factor increasing cell growth, decreasing cell growth, increasing apoptosis, or decreasing apoptosis.

Phosphate Buffer Solution*:* Most of the solutions you will be using in lab are prepared in phosphate buffer solution (PBS). You will also use PBS to wash your cells to remove excess buffer/solution. This buffer maintains both the physiological pH (7.4) and osmotic balance of our cells.

Materials

Cell culture media supplemented with FBS
Trypsin
T-25 flasks
PtK2 cells
Automatic pipettes
Serological pipettes
Waste beaker
Industrial Sharpie™ marker
Paper towels and/or Kimwipes™,
Spray bottles with 70% ethanol
Sterile PBS
Sterile diluted lemon juice in PBS (1:10 dilution)

Protocol

Part I. Expanding PtK2 Cultures

Preparing Materials

1. Put on gloves.
2. Warm media and trypsin in 37°C water bath for approximately 5 minutes.

***Notes**

Use the remainder of your trypsin and media from last week first.

You will need a minimum of 20 ml of media and 3 ml of trypsin for this week's lab. If you do not have enough media and/or trypsin in your tubes from last week, obtain new tubes from your instructor. Make sure to label these new tubes well with group ID, date, and section.

3. Use 70% ethanol to clean the working area.
4. Remove the media and trypsin from the 37ºC CO_2 incubator. Use a paper towel to dry each bottle, making sure that the liquid does not drip onto any surfaces. Use ethanol to sterilize your own gloves (Make sure to wipe in between fingers!), and then ethanol each bottle, wiping from the top downwards.
5. Transfer media and trypsin to the cell culture hood.

Determining Cell Confluence

1. Remove a 25 cm2 flask (i.e. a T-25 flask) from the CO_2 incubator.
2. Look at the cells with the inverted microscope to determine the confluence. They will most likely be covering the bottom surface of the flask, at about 80-90% confluence (aka with few open, non-cell covered spaces).
3. Spray a Paper towel with ethanol and use it to wipe down the T-25, as you are now going to place it in the hood.

Trypsinizing the Cells

1. Loosen the lids on the trypsin, the media, and your T-25 flask.
2. Use a 10 ml serological pipette to remove the media in the T-25 and dispose of this liquid into the waste receptacle (a labelled bottle in the tissue culture hood).
3. Mix the trypsin by triturating (pipetting up and down with a serological pipette) 3-5 times.

   ***Notes**:
   Triturating the trypsin is very important. If you skip this step, the trypsin will not work as well.
   Check the color of your trypsin after triturating. It should be medium pink to red in color. If your trypsin is light pink or clear, tell your instructor.
4. Add 3 ml of trypsin to your T-25 flask.
5. Transfer the T-25 flask to the 37ºC CO_2 incubator. The trypsinization will take approximately 7-8 minutes to complete; however, you should check your flask to see if the cells have detached every 2-3 minutes.

   ***Note**: If the monolayer of cells is not lifting after the 7-8 minute incubation, you can gently tap the side of the flask to help detach the cells.

What's going during this step? Trypsin is a protease, meaning it hydrolyzes peptide bonds to break down proteins. During the incubation step, the enzyme trypsin will degrade extracellular matrix proteins surrounding the PtK2 cells. This will cause the normally adherent PtK2 cells to detach from the bottom of the flask.

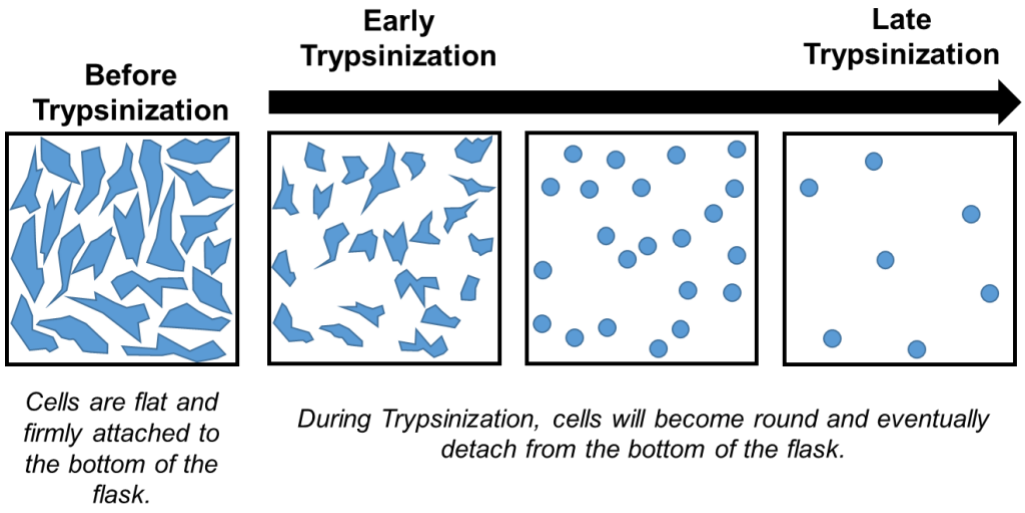


Figure 1. Trypsinization.

Inactivating the Trypsin and Passaging the Cells

1. Remove your T-25 flask from the CO_2 incubator and return it to the tissue culture hood using aseptic technique.
2. Get a new 15 ml conical tube and label it with your group name.
3. Loosen the caps on the bottle of media, on your T-25, and your 15 ml conical tube.
4. Using a new pipette, transfer 6 ml of media to the flask. Pipette the cells up and down in the flask in order to break up clumps. You should also wash the bottom of the flask to make sure all the cells are collected.

   ***Notes**:
   Minimize bubbles when pipetting.
   Be careful not to suction the solution up into the automatic pipette aid.

What's going during this step? FBS, a component of the PtK2 cell culture media, contains many different proteins. Some of these proteins are protease inhibitors, which will inhibit the enzymatic activity trypsin. The specific timing of the inactivation of trypsin when passaging cells is very important. Inactivating the trypsin the too soon will result incomplete detachment of the PtK2 cells from the bottom of the flask. If you wait too long to inactivate the trypsin, the trypsin will completely break down extracellular matrix proteins and will begin to degrade cell surface proteins, resulting in cell death.

5. Transfer all of this solution (9 mls) to the labeled 15 ml conical tube that is inside the tissue culture hood.
   **Notes: Check the empty flask in the microscope to verify that the majority of cells have been removed. If there are too many cells still in the flask, put back 4-5 mls of the 9 ml solution back to the flask and continue to wash the bottom of the flask for a few more minutes. Then return that solution back to the conical tube before moving to the next step.
6. Centrifuge the cell suspension for 5 minutes at 500 rcf (not rpm!).
   ***Notes:**
   On the centrifuge we are using in lab, this is entered as "0.5 x 1000" rcf.
   Make sure the centrifuge is balanced.
   You may need to create a "Blank" (aka "Dummy" tube) 15 ml tube that contains water at the same volume as your conical tube.
7. During the centrifugation, prepare your **TWO** new T-25 flasks:
   - Get **TWO** new T-25 flasks. Label these new T-25 flask with the cell type (PtK2), your lab section, the passage number the date, and a group identifier, and lab section number. Also, label one flask "control" and the other "experimental."
   - **Add 5 ml of media to each new T-25.**
8. After centrifugation, return your 15 ml conical tube to the tissue culture hood.
9. Using a new serological pipette, remove the supernatant from the top of the tube and transfer it to the waste beaker. **DO NOT TOUCH THE CELL PELLET**. It's OK, to leave a small amount of supernatant in the conical tube rather than risking pipetting up your cell pellet.
10. Add 3 ml of media to the conical tube and resuspend the cell pellet by triturating 10-20 times. Again, pipette slowly to minimize bubbles.
    ***Notes:**
    Again, pipette carefully to avoid bubbles.
    PtK2 cell produce a lot of extracellular matrix proteins. In order to completely resuspend the cells pellet, you may need to triturate the pellet more. Keep checking your pellet to make sure it has gone into solution.
11. Add 1 ml of the cell suspension to each of the new T-25s.
    ***Notes**:
    If your cell suspension has been sitting for a while and the cells have settled to the bottom, triturate the cell suspension 3-5 times to mix before transferring cells to the flask
    Your new flasks should contain 1 ml of cell suspension AND 5 ml of media (see step 7 above).
12. Cap the new T-25 flask, lay them on their sides, and gently swirl it to evenly distribute the cells.
13. Examine the new flasks with the microscope and confirm that cells are present. You can also examine your old T-25 flask to verify that you removed most of the cells during the trypsinization step.

Part II. Adding Cell Treatments

1. Obtain one tube sterile PBS and one tube sterile diluted lemon juice in PBS (1:10 working stock) from your instructor

2. Add 60 µl of PBS to your control T-25 flask.
   ***Note:** Your experimental factor, lemon juice, is diluted in PBS. To ensure that this experiment only has one independent variable, PBS is added to the control flask.

3. Add 60 µl of diluted lemon juice (1:10 working stock) to your experimental T-25 flask.
4. Note: Your lemon juice working stock has been diluted 10-fold in PBS (1:10 dilution). By adding 60 µl of this stock to your flask containing 6 ml of media, you are diluting the lemon juice 100-fold more (1:100 dilution). This means your overall dilution is 1000-fold (1:1000 dilution). See Worksheet 5 for more explanation and practice problems.
5. Cap both T-25 flasks, lay them on their sides, and gently swirl them to evenly distribute the treatments.
6. Place both flasks in the CO_2 incubator.

Clean Up

1. Cap your old T-25 and place it in biohazard waste.
2. Pour your leftover cell suspension (~2 ml) into the liquid cell culture waste container in the hood. Dispose of the 15 ml conical tube in the biohazard waste.
3. Cap your media and trypsin tubes and return them to the refrigerator in your designated class tube racks.
4. Wipe down your work area inside of the hood with ethanol.

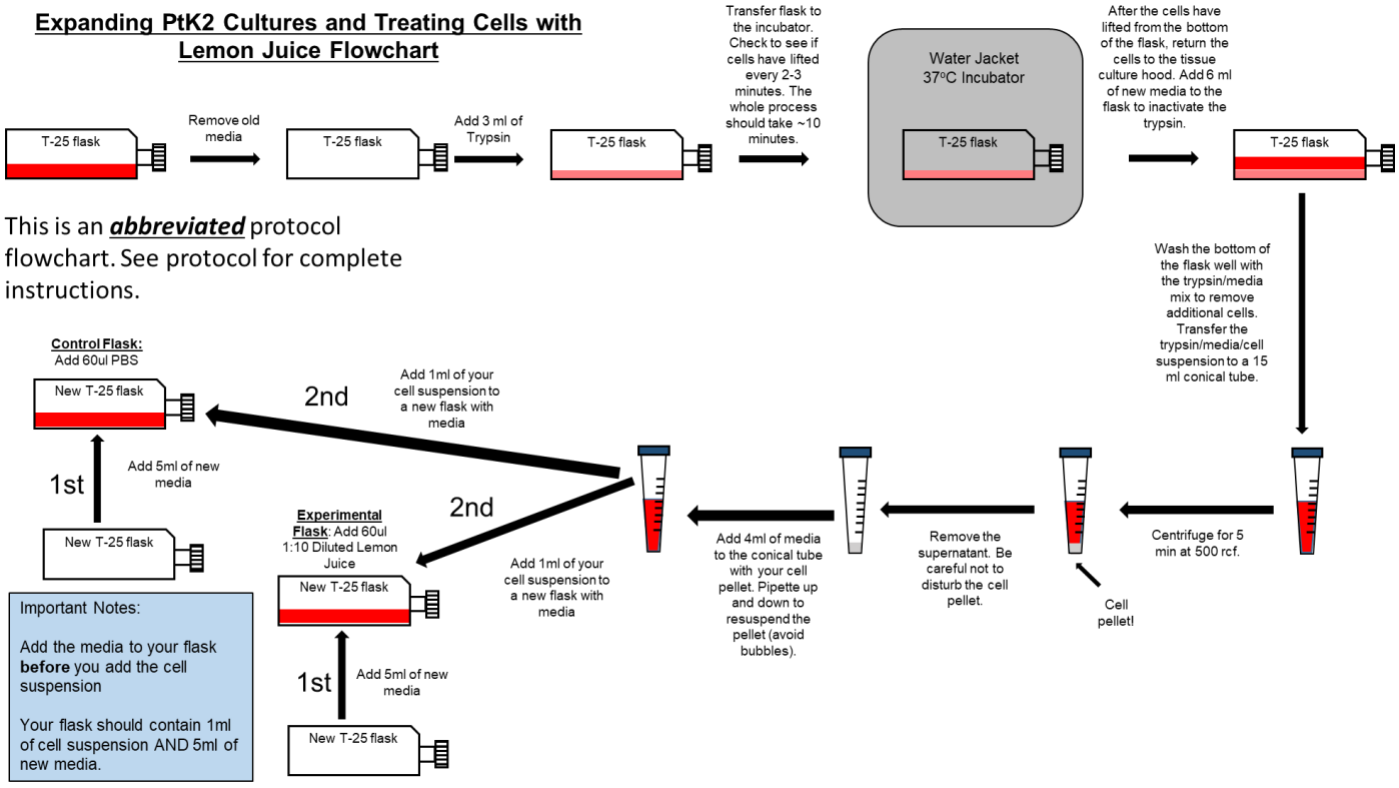


Figure 2. Laboratory protocol flowchart.

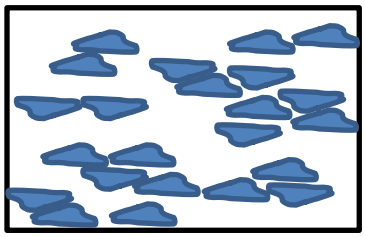
Worksheet **Name**_____

Instructions: Use the content provided in the Lab 2&3 protocols to answer the following questions.

IMPORTANT DUE NEXT WEEK: Each student must bring in a potential factor to study.

I. **Purpose of Protocol Steps**: Students at the 3000-4000 level should not just follow a protocol, but understand the purpose for each step. This information provides an important foundation to ensure successful troubleshooting when the experimental process produces unexpected results.

1. Checking for Confluence: Confluence is the percentage of cells that have grown on the bottom of your flask compared to the areas without cells. Below are graphical representations of what is visualized when looking at a culture flask in the microscope. Identify which flask is: A) 80%-90% confluent, B) 50%-60% confluent, or C) 10%-20% confluent.



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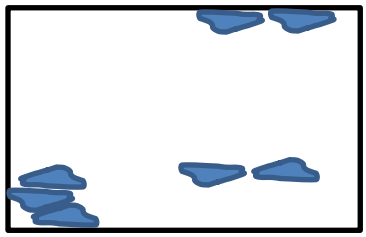

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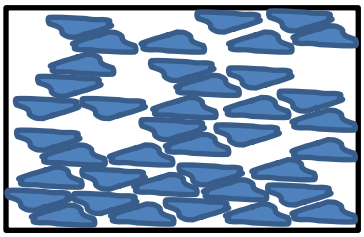

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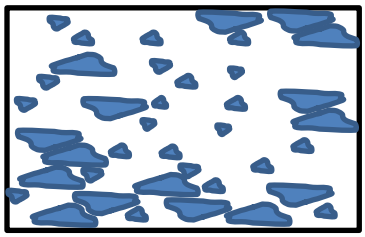
2. Which one of the above flasks (A, B or C) is a viable option for passaging cells? Explain why you chose this flask in 1-2 sentences.

3. Trypsin Reaction: PtK2 cells strongly attach to the bottom of the flask using anchor proteins embedded in the cellular membrane. Trypsin can interact and enzymatically cleave the part membrane proteins located on the outside of the plasma membrane, thus releasing the cells from the bottom of the flask. Below are graphical representations of what is visualized when looking at a culture flask in the microscope. Identify which flask contains cells that are: A) completely detached from the bottom of the flasks and ready to move onto the next step, or B) not completely detached therefore more incubation time is needed (or more trypsin might be needed).



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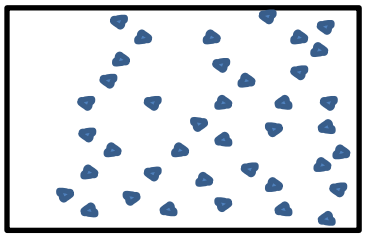

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4. Trypsin can kill cells if used at a high concentration or if left on the cells for an excessive amount of time. Based on what you know about the function of Trypsin, how does trypsin kill the cells? Explain your answer in 1-2 sentences.

II. **Dilution Ratios**: Concentration of factor within the flask: In this course, students will be expected to mathematically determine how much reagent to add to the cells to reach a specific concentration. As you know, when you add a small volume of concentrated solution to a large volume of liquid, that solution becomes diluted. In biology, knowing the exact concentration of your final solution within your microfuge tube or cell culture flask is imperative to maintaining the correct reaction concentration or homeostatic environment.

Use the Dilution Equation to solve. Mi X Vi = Mf X Vf

Example Question: In the protocol, it states to add Solution A to a $1/1000^{th}$ dilution into your flask. You know that the stock solution of Solution A is already a $1/10^{th}$ dilution and that you have 5ml of media in your flask. What volume of the Solution A stock do you need to add to create a $1/1000^{th}$ dilution final volume in the flask?

- Step 1: Convert or simplify variables
    - 5mls is converted to 5000 µl
    - 1/10 is simplified to 0.1
    - 1/1000 is simplified to 0.001
- Step 2: Solve for "X" using the Dilution equation:

$$Mi \times Vi = Mf \times Vf$$

$$0.1 * \mathbf{X\ \mu l} = 0.001 * 5000\ \mu l$$

$$\frac{\cancel{0.1} * \mathbf{X\ \mu l}}{\cancel{0.1}} = \frac{0.001 * 5000\ \mu l}{0.1}$$

$$\mathbf{X\ \mu l} = 50\ \mu l$$

Answer: To achieve the final concentration of $1/1000^{th}$ Solution A within your flask, you need to add 50µl of the $1/10^{th}$ stock solution of Solution A.

Practice Exercises

1. If you wanted to test the effect of a 1:2000 dilution (2,000-fold) of lemon juice on PtK2 cells, how much of the 1:10 diluted lemon juice would you add to the 6mls in the experimental flask?

2. If you wanted to test the effect of a 1:500 dilution (500-fold) of coffee on PtK2 cells, how much of the 1:10 diluted coffee would you add to the 9mls in the experimental flask?

III. Applying the math to your **PROJECT**

1. The first experiment of the student experimental factor is next week (Lab 5). Students will test the effect of 1:1000 dilution of your chosen experimental factor on PtK2 cells. Today you students will make a 1/10 stock dilution of their factor. What volume of the 1:10 diluted experimental factor are you going to add to the 6 ml in the experimental flask to achieve a 1:1000 final dilution ratio?
2. Based on your answer, what volume PBS are you going to add to the 6 ml in the control flask?

Materials

For the cell culture module, the equipment and supplies needed are a tissue culture hood, compound light microscope, CO_2 water jacket incubator, water bath, and mammalian cells.

For the microscopy module, the equipment and supplies needed are a fluorescence microscope, CO_2 water jacket incubator, fluorescence stains, and mammalian cells.

Notes for the Instructor

This semester-long research project can be scaled up or down as needed by each institution or lab course. For example, in a shorter quarter-term, some labs can be eliminated or condensed; such as the initial training lemon juice experiment can be eliminated, and sterile technique and passaging training. Other options for condensing labs include combining Week 9 and Week 10 by eliminating the traditional fluorescent stains and using multiple mounting media to achieve the same results. Equipment limitations, such as no access to a fluorescence microscope, are alleviated by cutting the microscopy module and using a compound light microscope in the cell culture module. Varied research questions offer the option of different fluroscence stains. An option to scale up is to purchase an automated cell counter to decrease the lab time for manual counting. Furthermore, this curriculum is not specific to PtK2 cells; the use ofother cell lines demonstrates similar results.

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