Video Article

Using Mouse Mammary Tumor Cells to Teach Core Biology Concepts: A Simple Lab Module

Victoria McIlrath¹, Alice Trye¹, Ann Aguanno¹

¹Department of Natural Sciences, Marymount Manhattan College

Correspondence to: Ann Aguanno at aaguanno@mmm.edu

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Abstract

Undergraduate biology students are required to learn, understand and apply a variety of cellular and molecular biology concepts and techniques in preparation for biomedical, graduate and professional programs or careers in science. To address this, a simple laboratory module was devised to teach the concepts of cell division, cellular communication and cancer through the application of animal cell culture techniques. Here the mouse mammary tumor (MMT) cell line is used to model for breast cancer. Students learn to grow and characterize these animal cells in culture and test the effects of traditional and non-traditional chemotherapy agents on cell proliferation. Specifically, students determine the optimal cell concentration for plating and growing cells, learn how to prepare and dilute drug solutions, identify the best dosage and treatment time course of the antiproliferative agents, and ascertain the rate of cell death in response to various treatments. The module employs both a standard cell counting technique using a hemocytometer and a novel cell counting method using microscopy software. The experimental procedure lends to open-ended inquiry as students can modify critical steps of the protocol, including testing homeopathic agents and over-the-counter drugs. In short, this lab module requires students to use the scientific process to apply their knowledge of the cell cycle, cellular signaling pathways, cancer and modes of treatment, all while developing an array of laboratory skills including cell culture and analysis of experimental data not routinely taught in the undergraduate classroom.

Video Link

The video component of this article can be found at <http://www.jove.com/video/52528/>

Introduction

Often in undergraduate general biology courses, the topics of cell cycle regulation and cancer are touched upon but not explored in detail because the breadth of content in these courses leaves little time for depth. In addition, undergraduate biology students are not typically exposed to the advanced techniques associated with animal cell culture. To help students develop a deeper understanding of these concepts, while applying and analyzing what they have learned, a laboratory activity was developed as a modification of the *Walter Reed Army Institute of Research* (WRAIR) extended laboratory activity¹. The lab module uses a step-wise, experimental strategy that includes growing and characterizing a cancer cell model, developing and executing cell counting methods, establishing optimal time course and dosages for treating cells with anti-proliferative agents, and identifying aberrant cell-signaling pathways. The experiment also allows for open-ended inquiry.

Most of the techniques required for this activity can be accomplished in a typical biology-teaching laboratory. The activity starts with students characterizing the morphology and growth rate of the mouse mammary tumor (MMT) cell line, a model for human breast cancer². Breast cancer was chosen as the model cancer because of its prevalence in the population, its familiarity to college-aged students, and the widespread data available. The MMT cell line was specifically selected because it is easily obtainable, well characterized, has a short doubling time and is easy to grow. In addition, MMT cells are estrogen-dependent which is consistent with most female breast cancers. Students then identify aberrant cell-signaling pathways in the MMT cells by treating the cells with chemotherapy drugs whose mechanism of action is well established. The concentration of the drugs and length of the treatments are varied allowing students to evaluate the effect of these variables on the rate of cell division. The key assay for this activity is the determination of cell viability, which simply requires cell counting, using one of two methods. Each method depends on strong microscopy skills. Students determine cell viability by using a standard, hemocytometer method and a novel photomicroscopy method and propose. Based on their findings, they can propose and test modifications to the activity. Students then represent their data and interpret the results to refine their hypothesis and devise new experimental strategies.

This laboratory activity is suited for freshman or sophomore level students majoring in the biological sciences. It is condensed into a one-week lab module that can be completed in a first year, general biology or second year, cellular/molecular biology course. Skills needed for proper completion of the activity include basic arithmetic and algebra, familiarity with an array of core laboratory skills (e.g., pipetting, solution making, sterile technique), data analysis, basic light microscopy and time management, along with instructor knowledge of cell culture and spreadsheet software. Reagents required include an animal cell line model for cancer (e.g., mouse mammary tumor cells, MMT²), chemotherapy agents (e.g., tamoxifen, curcumin, metformin, and aspirin), trypan blue and cell culture media (e.g., *Eagle's Minimum Essential Medium*; EMEM) with

appropriate supplements (e.g., donor horse and fetal bovine serum). Instruments needed include an inverted light microscope with digital camera attachment, computer, 100 mm and 24 well tissue culture plates, CO₂ incubator (or equivalent), biosafety cabinet (BSC; Class II), hemocytometer, and digital microscopy software.

There are good examples of specific lab activities that rely on animal cell culture to teach undergraduate students about concepts in cell biology³. However many require supplies or techniques that are not easily accessible (e.g., radioactive isotopes, live animal tissue, advanced imaging equipment^{1,4,5}), describe protocols that are quite advanced (e.g., suitable for a 400 level course⁶), or require multi-week or semester long projects^{6,7}. The lab activity described here is straightforward and can be conducted in a single week with common lab equipment.

In summary, this lab module effectively introduces or reinforces the concepts of cell cycle, cellular signaling pathways and cancer while teaching basic and advanced lab skills, experimental data analysis, the method of animal cell culture and the scientific process. The laboratory module is simple and economically accessible and provides both flexibility and opportunity for open-ended inquiry. The activity encourages student creativity by providing a template experimental strategy that acts as a guide but not a recipe. Most importantly, the activity satisfies all learning domains of Blooms Taxonomy⁸ as it requires remembering, understanding, applying, analyzing, evaluating and creating by engaging students in a process that pulls them out of the textbook and into the world of scientific research.

Protocol

Notes: Conduct all work with cells and cell culture reagents in a Class II biosafety cabinet (BSC)⁹. MMT cells are classified as Biosafety Level I, as they pose low to moderate biological risk. Apply proper cleaning and decontamination procedures to the BSC between uses (e.g., ultraviolet light, 70% ethanol wipe down).

1. Grow MMT cells

1. Grow cells in 10 cm tissue culture dishes containing 10 ml of nutrient rich media that consists of *Eagles Minimum Essential Medium* (EMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 1% Antimycotic/Antibiotic (10 units penicillin, 100 µg streptomycin, 0.25 µg amphotericin B). Cultivate cells in a humidified 5% CO₂ chamber, at 37 °C. Plate cells at a density of 3.6×10^6 cells/cm² (see Counting Cells in step 2).
2. Replace half of the cell culture media with fresh media every 48 hrs unless otherwise indicated. Check cell viability and morphology by examination with an inverted phase contrast light microscope.
 1. Note and characterize cells for size, structure, shape, organization and estimated number under at 100 - 200X magnification. At this density, the cells should appear in one plane, not clumped atop each other and in close proximity. Each cell consists of a thick, spherical core with thin, long, branch-like extensions expanding from it that come to a point (see **Figure 1**).
3. Subdivide cells when they reach a cell density of 7.2×10^6 cells/cm². Cells exhibit a doubling rate of 1.8×10^6 cells/cm² per 24 hrs. Designate cells *Passage X* (Px) to denote the number of times they have been split.
 1. At generation 3 (i.e., after three passages, P3), harvest, count, and plate the cells onto a 24 well tissue culture plate at a density of 3.6×10^6 cells/cm².
4. View cells every day and record digital photomicrographs. Note and describe cell morphology (i.e., size, shape, light reflective properties). Determine cell doubling time by counting cells regularly and relating elapsed time to cell number.

2. Count MMT Cells

Note: Count cells to determine if cells need to be subcultured, to set up for an experiment or to determine cell viability. There are two methods presented here.

1. Use of a hemocytometer, a standard technique to count cells.
 1. Obtain a bright-line hemocytometer, a 0.2% solution of trypan blue dye, a compound light microscope, sterile test tubes, pipettes and a manual cell counter.
 2. Under the BSC, use a 10 ml pipette to remove cells from a 10 cm tissue culture dish. If cells are grown on a 24 well plate use a 1 ml pipette or 1,000 µl micropipette to remove the media.
 3. Draw cell media into the pipette, place the tip of the pipette against the bottom of the dish and expel the media while sliding the pipette tip across the plate. Repeat 4 - 5 times to help dislodge cells, dissociate clumps and lead to a more accurate cell count.
 4. Place the resulting cell suspension in a 15 ml sterile tube (or 1 ml micro centrifuge or other small volume tube). Otherwise, leave the cells in the original tissue culture plate/well.
 5. Under the BSC, combine 10 µl of the cell suspension with 10 µl of the trypan blue solution (1:1 ratio) in a non-sterile micro centrifuge or other small volume tube.
Note: Trypan blue is a vital stain that is not absorbed by healthy viable cells. When cells are damaged or dead, trypan blue can enter the cell allowing dead cells to be counted (aka *dye exclusion method*). Therefore under a microscope, dead cells appear dark purple while viable cells are a bright and light in color.
 6. Incubate at RT for 5 min.
 7. Place the cover slip on the hemocytometer. Apply 10 µl of the trypan blue-cell suspension mixture into the groove. View the hemocytometer at 100 - 200X magnification. A four-corner grid is apparent (see **Figure 2**).
 8. Count the number of viable cells (unstained) versus total cells in each gridded area. Average the four grids and multiply by 1×10^4 to obtain cells/ml.

1. Extrapolate the total number of cells per dish (or cells/cm²). Use this number to either determine the correct volume of the cell suspension to use to seed a new tissue culture plate at the desired density of 3.6×10^6 cells/cm² or determine total number of viable cells on the plate or in the well.
Note: For additional guidance on the use of a hemocytometer, see¹⁰.
2. Use software and a digital camera to count viable cells.
 1. Obtain a digital camera, its accompanying software, a 0.4% solution of trypan blue dye, a compound light microscope, sterile test tubes, pipettes and a computer.
Note: A Moticam 2,000 digital camera with accompanying Motic Software is employed here. Any comparable camera and software should be sufficient. Ensure that the digital camera software has been calibrated in advance (according to manufacturer's protocol).
 2. Under the BSC, use a 1 ml pipet or 1,000 μ l micropipette to remove the media from the MMT cells growing in a 24 well tissue culture dish. Wash the adherent cells by gently applying a small amount (approximately 500 μ l) of un-supplemented (*i.e.*, EMEM without any supplements) fresh media to the plate then removing it. Apply 10 μ l of 0.2% trypan blue (made by diluting 1:1 with un-supplemented EMEM) directly to the well.
 3. Incubate the plate at RT for 5 min.
 4. View the plate under the microscope at 100 - 200X magnification with a digital camera attached. Plate may be washed with un-supplemented media if staining with 2% trypan blue is too dark.
 5. Open the software on the computer and verify that the software is calibrated to the objective used via the settings tab. An image of the FOV should appear.
 6. Select the *Grid* from the *Measure* tab. A grid of squares approximately 0.005 cm in width will appear. Select *Grid Info* to confirm the area of each square.
 7. Choose a specific area of the grid to count cells (*i.e.*, 5 squares x 9 squares). Select *Rectangle* from the *Measure* tab. Click the corner of a square and drag your cursor to encompass the squares of choice.
Note: A shade of green will cover the squares of choice and a white box will appear in the corner that provides the width, height, area, and perimeter of the section of the grid chosen (see **Figure 3**).
 8. Count the number of viable cells within the determined area. Do the same for two other locations in the same well or plate by moving the plate under the microscope.
 9. Be sure that the measured area is the same and the magnification has not changed. Calculate the average number of viable cells within the area. Using the area of the well (for a 24 well plate, one well has an area of 2 cm², for a 100 mM dish the area is 78.6 cm²), extrapolate the number of viable cells from the area delineated in the grid to the total number of cells within the well (cells/cm²).

3. Treat MMT Cells with anti-proliferative Agents

1. Prepare solutions of the selected anti-proliferative therapeutic agents (tamoxifen, curcumin and metformin) and optional drug, aspirin under the BSC.
 1. Dissolve curcumin and tamoxifen in 100% ethanol to generate a stock concentration of 27 mM. Dissolve metformin and aspirin in un-supplemented EMEM to generate a stock concentration of 500 mM and 15 mM, respectively.
2. Establish a Dose Response.
 1. Treat MMT cells with the three anti-proliferative therapeutic agents (tamoxifen, curcumin and metformin) and optional drug (aspirin) at varying concentrations for 96 hrs to generate a dose response curve. Initially administer all drugs at a range of concentrations based on published reports^{1,11-16} and then at concentrations larger or smaller than those published.
Note: A dose response determines the minimum concentration of a drug necessary to produce the desired results. Here the desired result is a reduction in cell proliferation as compared to the control.
 1. For tamoxifen and curcumin, use concentrations (and corresponding volumes) of 0.054 mM (1 μ l), 0.108 mM (2 μ l), 0.162 mM (3 μ l) and 0.216 mM (4 μ l).
 2. For metformin, use concentrations (and corresponding volumes) of 2 mM (2 μ l), 4 mM (4 μ l), 6 mM (6 μ l), 8 mM (8 μ l) and 10 mM (10 μ l).
 3. For aspirin, use concentrations (and corresponding volumes) of 0.030 mM (1 μ l), 0.060 mM (2 μ l), 0.099 mM (3.3 μ l), 0.150 mM (5 μ l), and 0.216 mM (6.7 μ l).
 2. Split MMT cells from the 10 cm dish onto a 24 well plate at a concentration of 3.6×10^6 cells/cm². Determine initial cell concentration by both cell-counting methods (Step 2). Call this new 24 well plate of cells "*Day Split*".
 3. 24 hrs after cell plating, treat the MMT cells with each of the anti-proliferative therapeutic agents, tamoxifen, curcumin, metformin and aspirin, at the concentrations described in Step 3.2.1. Refer to this as "*Day 0*".
 1. As each well can hold a maximum of 500 μ l of media, use micropipettes with sterile micropipette tips and a new tip each time a new well is treated to avoid cross contamination. Use two wells as controls: cells grown in the absence of any drug treatment (negative control) and cells grown in the presence 100% ethanol (solvent control).
 4. Grow cells and re-administer drugs at the described concentrations when the cells are fed every other day (see Step 1.2) for 96 hrs (until *Day 4*). On Days 1 - 4 of treatment, observe the cells under the microscope and count using the method in Step 2.2 (see **Figure 4**).
 5. Repeat the experiment at least three times.
 6. Determine optimal concentration of each drug by graphing the relationship between cell viability and drug dosage over the length of the experiment (see **Figure 5**).
3. Establish a Time Course.

1. Treat MMT cells with the three anti-proliferative therapeutic agents (tamoxifen, curcumin and metformin) at a fixed concentration for varying time periods. Use the optimal concentration identified through the Dose Response experiments (see Step 3.2).
 1. Use the following concentrations: 0.216 mM tamoxifen, 0.216 mM curcumin and 10 mM metformin.
Note: A time course determines the amount of time necessary for a drug to produce its optimal desired result. Here, the desired result is a reduction in cell proliferation as compared to the control.
2. Split MMT cells from the 10 cm dish onto a 24 well plate at a concentration of 3.6×10^6 cells/cm². Determine initial cell concentration by both cell-counting methods (Step 2). Call this new 24 well plate of cells "*Day Split*".
3. 24 hrs after cell plating, treat the MMT cells with each of the anti-proliferative therapeutic agents, tamoxifen, curcumin, metformin and aspirin, at the optimal concentrations identified in Step 3.2. Refer to this as "*Day 0*".
 1. As each well can hold a maximum of 500 µl of media, use micropipettes with sterile micropipette tips and a new tip each time a new well is treated to avoid cross contamination.
 2. Use two wells as controls: cells grown in the absence of any drug treatment (negative control) and cells grown in the presence 100% ethanol (solvent control).
4. Grow cells and re-administer drugs at the selected concentration when the cells are fed every other day (see Step 1.2) for 96 hrs (until Day 4). On Days 1 - 4 of treatment, observe the cells under the microscope and count using the method in Step 2.2 (see **Figure 4**).
5. Repeat the experiment at least three times.
6. Determine optimal exposure time of MMT cells to each drug by graphing the relationship between cell viability and length (time) of drug treatment at the selected concentration (see **Figure 6**).

4. The Lab Module

Note: The following is a 5-day lab schedule for the lab module. **Figure 7** is a flow chart of what the schedule would entail within the 5 days. For this activity to be completed in five days either a time course or a dose response curve is generated. There is not sufficient time to generate both curves. A dose response experiment is described below. A time course experiment can be easily interchanged

1. Split the class into groups: have one group establish a dose response and the other a time course choosing a concentration in the middle of the proposed dose response concentrations.
 1. Maintain sufficient cultures and drug stocks at appropriate concentrations. Unless otherwise noted, perform all work under the BSC.
2. Day 1
 1. Direct students to make media and grow cells (as in step 1).
 2. As students obtain a stock of MMT cells on a 10 cm plate, direct the students to prepare cell culture media and give a tutorial in the use of the hemocytometer, digital microscopy and the digital camera microscopy software (as in step 2).
 3. Calibrate the software to the objectives used on the microscope (e.g., 4X, 10X, 20X) now using manufacturer's protocol.
3. Day 2
 1. Have students confirm total cell number and cell viability (as in step 2).
 2. Have students count cells on the 100 mm dish using both the microscopy software and the hemocytometer methods, to confirm similar results are obtained.
 3. Direct students to obtain a 24 well plate and split cells from the 100 mm dish to the 24 well plates to the desired starting cell plating density (as in step 1). This is considered *Day Split*. Place the 24 well plate in a cell culture incubator for 24 hrs.
4. Day 3
 1. Grow MMT cells for 24 hrs on a 24 well plate. Have students observe cells under microscope and count using microscopy software (as in step 2).
 2. Give the student/team samples of cancer treatment drug stocks. Have them prepare and dilute the original stock solution (as in step 3). Add the various concentrations of the drugs to their cells to establish a dose response curve. This is called *Day 0*.
 3. Incubate cells (step 1.2) with the drugs for maximum of 48 hrs.
5. Day 4
 1. Have students observe treated cells after 24 hrs. This is *Day 1*.
 2. Count each well using microscopy software. Record photographs of each well so that counting can be conducted outside the lab (as in step 2).
 3. Incubate cells (step 1.2) with the drugs for another 24 hrs.
Note: Instructor provides additional tutorials and reviews of data analysis and graphical presentation. Students learn to create figures, plots, and statistical analysis for the following day of data collection.
6. Day 5
 1. Have students treated cells after 48 hrs, *Day 2*.
 2. Count each well using microscopy software. Record photographs of each well so that counting can be conducted outside the lab (as in step 2).
 3. Harvest and collect cells for counting by the traditional hemocytometer method as a means of verifying student ability to effectively use the microscopy software method (as in step 2).
Note: Collect data and draw conclusions or revise hypotheses, accordingly.

Representative Results

Growing MMT cells and comparing counting methods.

Mouse mammary tumor cells were successfully grown and characterized (**Figure 1**) and a novel cell counting method developed using Motic Software, a digital camera-associated software program for a microscope. This new cell counting method was compared to a traditional counting method employing a hemocytometer (**Figure 2**) and was shown to be equally accurate in determining cell number (**Table 1**). To control for any effects on cell number due to different growth conditions or cell type, the comparison between the two cell counting methods was conducted in the presence and absence of anti-proliferative agents and with a different cell line, the neuronal model PC12¹⁷.

Figure 3 shows the delineation of the area for counting cells with this method using a superimposed grid of defined dimensions. The grid is laid over the field of view (FOV) and a green frame is then applied to a designated length and width of the grid (i.e., 9x5 squares). The cells are counted within the green frame and the number of cells is extrapolated, as discussed in the protocol.

Treating MMT cells: Determining the dose response of MMT cells to anti-proliferative agents.

Dose response studies (**Figure 4**) were conducted for each anti-proliferative agent. Throughout these experiments data was continually gathered, reviewed and analyzed to see if possible revisions to the protocol were warranted. The experiment was conducted three times, with each study producing similar results. Experiments were continued until all cells were dead or the effects of the drugs had plateaued. Digital photomicrographs of the results are presented in **Figure 4**, and a graphical analysis of those findings is presented in **Figure 5**. Effective concentration ranges are reported in the legend of **Figure 4**, on the X-axis of **Figure 5** and in the Protocol section.

At tamoxifen's lowest concentration, 0.054 mM, there was robust inhibition of cell growth, approximately 83% when compared with untreated cells (**Figure 4A**). Cell viability continued to decrease with increasing concentrations of tamoxifen (**Figure 5A**). The optimal dosage of tamoxifen was determined to be 0.216 mM because after 48 hr complete cell death occurred at that concentration (**Figure 4A**). Interestingly, these reductions in cell propagation reveal no indication of a cellular resistance to tamoxifen, as would be expected based on the literature^{18,19} underscoring that *in vitro* systems model but do not always fully represent *in vivo* events. Furthermore, when contrasted with the lowest treatment concentrations of curcumin (**Figure 4B and 5A**) and metformin (**Figure 4C and 5B**), tamoxifen's lowest concentration was 9% and 50% more effective in stopping cell division, respectively.

Curcumin exhibited a concentration dependent effect on the inhibition of cell division as well. With increasing concentrations, there was a significant decrease in cell viability (**Figure 4D and 5A**). The optimal dosage of curcumin was determined to be 0.216 mM because after 48 hr, like tamoxifen, there was complete cell death at that concentration.

Metformin treatment yielded the least dramatic decrease in MMT cell viability, leading to 33% reduction at lowest concentration. A concomitant decrease in cell viability with increasing metformin concentration was observed (**Figure 4C and 5B**). The optimal dosage of metformin was determined to be the highest concentration tested (10 mM). Compared to tamoxifen and curcumin, both routinely used as chemotherapy agents, metformin was 30-40% less effective in inhibiting the cell cycle. Interestingly, the effects of metformin on cell division were consistent with those obtained by administration of aspirin (**Figure 4D and 5A**), a salicylic drug with no clearly identified mechanism of cell growth inhibition.

Treating MMT cells: Determining the time course of the MMT cellular response to anti-proliferative agents.

A time course study was also conducted for each anti-proliferative agent because the effective dose of an administered drug may be impacted by time of exposure. As this module assays for cell viability, it is important to first identify how quickly the cells divide (doubling time) so a logical window for the length of time the cells are exposed to the drugs can be chosen. It is essential, therefore, that MMT cell doubling time be ascertained when initially characterizing the cells. Throughout these experiments, data was continually gathered, reviewed and analyzed to see if possible revisions to the protocol were warranted. The experiment was conducted three times, with each study producing similar results. Experiments were continued until all cells were dead or the effects had plateaued.

Time course studies revealed that treatment of MMT cells with optimized concentrations of each anti-proliferative drug (tamoxifen 0.216 mM, curcumin 0.216 mM, metformin 10 mM) for 96 hr resulted in complete cell death or a leveling off of the effects of the drug (**Figure 6**). Although aspirin, the "optional" drug, did affect cell viability in our dose response studies, the impact was minor and therefore was not included in time course studies.

Figure 1. MMT cells. Digital photomicrograph of "healthy", untreated mouse mammary tumor (MMT) cells grown at 3.6×10^6 cells/cm² in modified *Eagles Minimum Essential Medium* (EMEM).100X, scale=0.2 mm, indicated by white bar. [Please click here to view a larger version of this figure.](#)

Figure 2. Hemocytometer grid. Photomicrograph of hemocytometer grid, 100x. Entire grid section shown in circle (FOV). [Please click here to view a larger version of this figure.](#)

Figure 3. Designating the counting area with Motic software. Image of grid overlay on FOV and defined area for counting MMT cells established with the Motic software as seen through microscope. 100X, scale= 0.2 mm, indicated by white bar. [Please click here to view a larger version of this figure.](#)

Tamoxifen

Curcumin

Metformin

Aspirin

Figure 4. Dose response of MMT cells to anti-proliferative agents. Digital photomicrographs of MMT cells grown in the presence of anti-proliferative agents at varying concentrations for 96 hrs. Tamoxifen: (A1) .054 mM (A2) .108 mM (A3) .162 mM (A4) .216 mM; Curcumin: (B1) .054 mM (B2) .108 mM (B3) .162 mM (B4) .216 mM; Metformin: (C1) 2 mM (C2) 4 mM (C3) 6 mM (C4) 8 mM (C5) 10 mM; Aspirin: (D1) .030 mM (D2) .060 mM (D3) .099 mM (D4) .150 mM (D5) .216 mM; Ethanol (100%) and Untreated are both controls. 100X, scale=0.1 mm, indicated by blue lines making up each square within the grid. [Please click here to view a larger version of this figure.](#)

Figure 5. Graphical representation of dose response experiments of MMT cells to anti-proliferative agents. Effects of increasing concentrations of anti-proliferative agents (A) tamoxifen, curcumin, aspirin, and (B) metformin on MMT cell viability. Results are expressed as a percentage of control after 48 hrs of treatment, although cell treatment continued for 96 hrs. $n=3$, STD is indicated.

Figure 6. Graphical representation of time course analysis of MMT cellular response to anti-proliferative agents. Time course study of the effects of tamoxifen (0.216mM), curcumin (0.216mM), and metformin (10 mM) on MMT cell viability over 96 hrs. Results are expressed as a percentage of control after 48 hrs of treatment. Shown here are results of a representative experiment, $n=1$.

Figure 7. Flow chart of 5-day lab module.

Cell Type	Drug Administered	Day of Experiment	Concentration of Drug	Cell Count with Motic	Cell Count with Hemocytometer
MMT	Untreated	Day 2	-	7.8×10^4 cells/cm ²	8.0×10^4 cells/cm ²
MMT	Ethanol	Day 2	10 mM	7.1×10^4 cells/cm ²	7.2×10^4 cells/cm ²
MMT	Tamoxifen	Day 2	.216 mM	0 cells/cm ²	0 cells/cm ²
MMT	Tamoxifen	Day 2	.054 mM	1.3×10^4 cells/cm ²	1.5×10^4 cells/cm ²
MMT	Curcumin	Day 2	.054 mM	1.9×10^4 cells/cm ²	2.0×10^4 cells/cm ²
MMT	Curcumin	Day 2	.216 mM	0 cells/cm ²	0 cells/cm ²
MMT	Metformin	Day 2	2 mM	5.1×10^4 cells/cm ²	5.2×10^4 cells/cm ²
MMT	Metformin	Day 2	6 mM	3.4×10^4 cells/cm ²	3.5×10^4 cells/cm ²
MMT	Aspirin	Day 2	.099 mM	2.8×10^4 cells/cm ²	3.0×10^4 cells/cm ²
PC12	Untreated	Split	-	2.5×10^4 cells/cm ²	2.1×10^4 cells/cm ²

Table 1. Comparison of hemocytometer and Motic software counting methods. Results of cell counting of MMT cells, using either Motic software or the hemocytometer methods. Both untreated and treated cells were counted, as well as a different cell line, PC12, as a control.

Discussion

A lab module is presented that aims to teach a variety of topics in cell biology through the advanced techniques of animal cell culture. The module achieves this by analyzing the effects of a number of anti-proliferative chemicals on the replication of cells that model human breast cancer. The primary assay relies on the fundamental technique of cell counting and introduces a novel way to count cells using microscopy software. The activities comprising the module can be conducted with instruments and equipment available in most biology programs. The module can be implemented in a 5-day schedule with supplies that are inexpensive and easily obtained. Although a particular brand of digital microscope camera and microscopy software is used here (Motic Software), any comparable camera and software should suffice.

This lab module utilizes MMT cells as a model for human breast cancer. Students are first taught to grow and characterize these cells in culture. It is important to underscore that successful completion of this laboratory module requires the investigators (aka students) be well acquainted with the appearance and behavior of healthy MMT cells, particularly since untreated MMT cells serve as controls. Therefore thorough characterization of the cell growth patterns, doubling time and general morphology of MMT cells is essential if proper dose response and time course data are to be generated.

Once characterized, the MMT cells were then tested for their response to various anti-proliferative agents as a means of teaching the topics of cell division, cell cycle regulation, cell signaling and cancer. Cell viability is used to understand the effect of these drugs on the cells. A novel method of counting cells, using software associated with a microscope-mounted digital camera, is conducted and compared to a traditional counting hemocytometer-based procedure to verify its accuracy. This novel method provides two advantages, which are particularly noteworthy because it is used in undergraduate teaching labs. First, since the cells do not need to be removed from the tissue culture plate for counting, less time is needed to perform the main experimental assay. This allows for a large number of experimental variables to be tested at once and in a limited time period. Second, the method provides a digital record of the cellular response to the experimental treatment allowing the students to evaluate the results and refine their experimental strategy outside of the laboratory.

There are several issues to note when implementing both of these counting methods. First, prior to counting the cells with either method, the cells are incubated with trypan blue to distinguish between a viable cell and a dead cell. Dead cells are penetrated by the dye and appear dark blue, whereas viable cells are bright white. However, if the cells are incubated with dye beyond the recommended incubation period, they will be over-stained and hard to distinguish. Second, MMT cells may clump on the hemocytometer or the tissue culture plate and be difficult to count individually. Therefore, a "clump" is designated to contain a certain number of cells (e.g., 10 cells/clump) and every clump present on the grid is then multiplied by that number during the count. Finally, MMT cells adhere to the surface of dishes in which they are grown. It is therefore necessary to touch the tip of the pipet directly to the plate when removing the cells and apply force to break up cell clumps and remove as many cells from the plate as possible. As a result it may take several attempts to obtain an accurate count when using the traditional hemocytometer method.

With the cells well characterized and counting methods verified, two different experiments are conducted; a dose response study of the effects of varying concentrations of anti-proliferative drugs on MMT cell division and a time course experiment to elucidate the optimal time of exposure to these drugs. These are highly instructive experiments as trial and error is necessary and application of the primary literature is required for a successful experiment. For example, initial attempts at elucidating a dose response of MMT cells to these anti-proliferative agents revealed that the concentrations tested were too high, as there was 100% cell death within 24 hr of drug administration. An expanded review of the literature led to a revised range of treatment concentrations. This effectively teaches students how to use literature databases, read, analyze and critique primary literature articles and underscores how those skills are essential for the scientific process.

Results of the dose response studies showed that tamoxifen exhibits the strongest anti-proliferative effect at all concentrations tested. Tamoxifen, an anti-mitotic drug, is used specifically as an anti-estrogen therapy for estrogen sensitive cancers, typically in postmenopausal, hormone-sensitive patients¹⁸⁻²⁰. The drug is currently used as a breast cancer treatment. Tamoxifen competes with estrogen for the intracellular estrogen receptor and when bound, down regulates expression of cyclins D and B, two important regulators of the cell cycle. Results obtained in the

activity suggest that tamoxifen successfully bonded to the estrogen receptor and inhibited the cell's progression through the cell cycle, resulting in the decreased cell proliferation observed. Interestingly, the reduction in MMT cell propagation rates across all concentrations of tamoxifen administered reveal no indication of cellular resistance to tamoxifen, as would be expected based on the literature (see for example¹⁹). This serves as a good reminder that the *in vitro* conditions in which the experiment was conducted do not fully model *in vivo* circumstances.

Curcumin treatment of MMT cells also resulted in a reduction of cell viability. Curcumin is a compound derived from the tumeric root and acts as a cell growth inhibitor^{12,16}. It works by down-regulating the NF-kappa B pathway and ornithine decarboxylase (ODC) activity. NF-kappa B is a protein complex that controls transcription of anti-apoptotic genes such TRAF1 and TRAF2, which code for proteins that inhibit apoptosis. ODC is an enzyme that catalyzes the production of polyamines, proteins that provide cancer cells with heightened sources of nutrition leading to enhanced cell growth. The drug is in current use as a breast cancer treatment. The anti-proliferative effects of curcumin clearly shows the effect that down regulation of the cell signaling pathway involving NF-kappa B and ornithine decarboxylase has on cell division.

Reduced cell viability was similarly observed with metformin treatment. Metformin, a medication typically administered to Type II diabetics, was chosen because published reports suggest a correlation between patients who take metformin and a lower incidence of breast cancer¹⁵. It is believed that the agent modulates the c-MYC gene and thereby down-regulates the activity of cyclin D. Up-regulation of cyclins, molecules that serve as checkpoint within the cell cycle, allows cells to progress through the cycle at a faster rate resulting in increased cell division and growth. The effects of metformin treatment on MMT cells shows how modulation of the c-MYC gene also leads to down-regulation of cyclin D and a reduction in cell division. The metformin data further exemplifies how a medication designed for one condition- in this case Diabetes- may have a mechanism of action suitable for other aberrant physiological events. In addition, it is important to underscore that although tamoxifen, curcumin, and metformin all retard abnormal cellular proliferation through different mechanisms, each successfully decreased cell populations and interfered with cell division *in vitro*.

This module also allows for open-ended inquiry in that another medication, herbal remedy, or agent can be tested. Aspirin, an over-the-counter analgesic, antipyretic, and anti-inflammation medication used to relieve mild pain and reduce fever was chosen in this module. Aspirin's ability to reduce inflammation may inhibit tumor growth by slowing development of new blood vessels that nourish them and interfering with stem cells that fuel their growth^{11,13,14}. Interestingly, administration of aspirin to MMT cells did result in some reduction in cell viability. Little is known about the role of aspirin in cancer prevention and treatment although many correlative studies are reported^{13,14}, providing an excellent example of correlative versus causative relationships.

The time course studies revealed that, when administered at optimized doses, the anti-proliferative drugs are highly effective at interfering with cell division within 4 days of initial exposure. These studies also provide an opportunity for the students to use what they learned about MMT cell growth characteristics when devising an experimental strategy for conducting these experiments. In fact, thoroughly characterizing the cell growth patterns, doubling time and general cell morphology of MMT cells is essential for both accurate dose response and time course data.

Both dose response and time course studies determine the relationship between drug concentration and time of exposure, respectively, and the effect a drug has in a biological system. This is an excellent way to get students thinking about intermolecular interactions and other foundational concepts in biochemistry while conducting inquiry driven, research activities. It is worth noting that since many biology majors plan to attend medical school, this lab module aligns well with the newly instituted changes in medical school requirements²¹.

This lab module can be implemented exactly as described or can readily serve as a template. A host of variations can be incorporated into this activity, such as the choice of anti-proliferative agent, cell type or nutrient modification in the growth media. As long as a hypothesis is developed that tests fundamental and advanced concepts in cell or general biology, the permutations of this module are numerous. Regardless of the permutation, the implementation of this activity naturally directs student to learn a large array of laboratory techniques and become familiar with a variety of equipment and instrumentation.

In summary, the lab module described here allows students to fully participate in the process of science, develop their abilities to acquire, analyze and graphically represent data, and hone their time management and collaborative skills. The module is designed to facilitate open-ended inquiry and is easily adaptable to various course schedules and skill levels. It should be noted that the first two authors of this paper are undergraduate biology majors, clearly demonstrating that this lab module is suitable for that population.

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