Introducing Mammalian Cell Colony Formation in the Undergraduate Biology Laboratory

Ashley Robinson, Mackenzie M. Crow, Austin Kratz, Taylor Ritts, Yewseok K. Suh[#], and Verónica A. Segarra[#]

Department of Biology, High Point University, High Point, NC 27268

[#]Co-corresponding Authors

ksuh@highpoint.edu

vsegarra@highpoint.edu

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ABSTRACT

Clonogenic assays are a simple and robust method that allow researchers to characterize mammalian cell line features, including the ability of a single cell to grow into a colony. We have used this assay as a tool in the undergraduate biology laboratory, exposing students to a more specialized form of mammalian cell culture and helping them refine scientific research skills and knowledge. In this article, we share an easy and undergraduate-friendly method of using HeLa cells to carry out clonogenic assays. The methods described include the introduction of different treatments to assess their effect in HeLa cell colony formation. In this laboratory exercise, undergraduate students utilize different cell culture techniques such as growing, harvesting, counting, diluting, staining, and imaging cells.

KEY WORDS: Clonogenic assay, Cytotoxic agents, HeLa cells, Mammalian cell colony formation, undergraduate laboratory, Open Inquiry-Based Curriculum

INTRODUCTION

In the year 1956, two researchers, Puck and Marcus, published a seminal paper detailing a new method of assessing the colony formation ability of mammalian cells (1). This type of culture analysis would later be deemed the “clonogenic assay.” The clonogenic assay is an *in vitro* cell survival assay that helps us characterize mammalian cell line features like the ability to grow in a colony. The simplicity and robustness of this method have allowed researchers to discover key differences between cellular behaviors found in tumors versus normal tissues, as well as how these behaviors can be altered when cells are exposed to potential drug therapies (2).

The simplicity and robustness that make the clonogenic assay a great tool in the research laboratory, also makes it a great technique to introduce in the undergraduate laboratory classroom. Moreover, the clonogenic assay’s relatedness and amenability to answering interesting research questions in cancer biology makes it an ideal hands-on method for undergraduates to gain familiarity with a research topic that has universal impact. Due to its prevalence, cancer biology is an effective way to engage students with life sciences research (3). While there are many didactic resources available to learn about cancer, direct access to undergraduate research experiences in the classroom lab regarding the topic is limited. In this paper, we describe how HeLa cells can be used as a mammalian model to teach undergraduate students to carry out clonogenic assays and implement techniques such as growing, harvesting, counting, diluting, fixing, staining, and imaging mammalian cells. These experiments also easily allow undergraduates to test the effect of different treatments on HeLa cell colony formation. Overall, this approach allows for students to experience how changing the cellular environment can lead to differences in cancer cell behaviors. Furthermore, this activity allows students to acquire skills that can spark their interest in biomedical research.

PROCEDURE

Safety Considerations and Precautions

Prior to the first laboratory session, students were required to complete relevant online CITI Training, Hepatitis B vaccine declination form, on-site blood-borne pathogen and laboratory safety guidelines training, and sign a document confirming that they agreed to comply by laboratory regulations. These requirements follow standard safety BSL-2 recommendations which include use of a laminar flow hood or biosafety cabinet, protective eyewear, lab coats, closed-toe shoes, and gloves at all times, as well as proper disposal of BSL-2 materials (such as HeLa cells) and reagents (4). Students were also required to watch videos on laboratory safety (NC Community Colleges

BioNetwork Lab Safety Video Series:

<https://www.youtube.com/playlist?list=PL4qaj9envIYnBaQSPpcOMUqWiQUAgPoMq>) and complete a quiz with a passing grade to demonstrate their understanding. More details about the safety considerations and precautions are provided in Appendix 1.

Materials and Methods

Instructor Preparation

Detailed information on how instructors can best prepare to teach/implement using HeLa cells in the undergraduate lab can be found on our previous publication (5).

Implementation of experiments in the undergraduate laboratory

In preparation to perform clonogenic assays, students were required to complete safety training (Appendix 1), given a lab skills/aseptic technique review, and tissue culture introduction. Groups of two to three students discussed the video, watched the instructor passage HeLa cells onto new plates, and mirrored the technique independently (Appendix 2), ultimately generating their own HeLa cell starter plate to care for. Students grew a population of HeLa cells on this 100 mm plate to around 50-80% confluency and used the cells harvested to set up their clonogenic assay (Appendix 3). In short, the spent media from the plate were aspirated and the plate was washed with 3 mL of phosphate-buffered saline (PBS) twice. 1 mL of warm 0.25% Trypsin/EDTA solution was then added, and the plate was set to rest in the 37°C incubator for 5 minutes. After 5 minutes, 1 mL of warm media was added to the plate to neutralize Trypsin/EDTA and the resulting cell suspension was pipetted approximately 10-15 times in order to break up cell clumps and make a homogeneous cell suspension. Students transferred 2 mL of this cell suspension to a 15 mL conical tube and a 10 μ L aliquot was taken from this to be used in a counting slide. Using a microscope, students calculated the average cell concentration per square with the counting slide. This average was then used to calculate the number of cells per microliter of the cell suspension. Using this calculated cell concentration, students adjusted the volume (or generated a new more dilute suspension) to 10 cells/ μ L. Finally, six-well plates were inoculated with 10 microliters of the cell suspension, or 100 cells, per well for the clonogenic assay. Students then selected the conditions to test the clonogenic capacity of HeLa cells. One option included different concentrations of fetal bovine serum (FBS), either 5% FBS or 10% FBS. Another option included different types of anticancer agents, either fisetin or cycloheximide. When one of the anticancer agents was chosen, the effect of the agent was compared to the control treated with dimethyl sulfoxide (DMSO).

If the students selected the FBS, 3 mL of Dulbecco's Modified Eagle Medium (DMEM) with 5% FBS was pipetted into each well in top row of the plate (wells A1-A3). Then, 3 mL of 10% FBS containing DMEM was pipetted into the bottom row of the plate (A4-A6). If the students selected one of the anticancer agents, 3 mL of DMSO containing media was added to the top rows of the plate (C1-C3, E1-E3) whereas the media containing the anticancer agent (60 μ M fisetin or 50 μ g/mL cycloheximide) were added to the bottom rows of the plate (C4-D6, E4-E6). Finally, the plates were labeled and incubated at 37°C in a humidified atmosphere consisting 5% CO₂ for one week. After one week, students removed the assay plates from the incubator and the spent media were carefully aspirated. Students then used the Richard-Allan Scientific Three-Step Stain Set (Thermo Fisher Cat. No. 3300) to fix and stain cell colonies. Briefly, wells were filled with 0.5 mL of methanol fixative (Fixative solution of the kit) and left to incubate at room temperature for 2 minutes. After removal of the fixative solution, 0.5 mL of eosin Y dye (Solution A of the kit) was added to each well and incubated for 2 minutes at room temperature. Eosin dye was then removed and the plate was incubated with methylene blue/azure A solution (Solution B of the kit) for 2 minutes also at room temperature. Finally, all wells were aspirated and gently rinsed with deionized water. The resulting plates were analyzed under a microscope and the number of colonies were counted and recorded. Statistical analysis of the data was performed using Microsoft Excel software. Two-tailed t-test analyses were used to compare the means of the groups and *P* values < 0.01 were considered statistically significant.

Intended Audience

This laboratory exercise is intended for undergraduate students in college/university. At High Point University (HPU), this exercise was implemented in an upper level cancer biology class to explore concepts related to tumor growth and characteristics of human cancer cells in culture.

Alternative Applications

The simplicity and robustness of the experiments described above also make it appropriate for implementation in lower level biology courses as long as the class size and cell culture facilities can accommodate the students enrolled in the courses of interest.

CONCLUSION

This laboratory activity is an effective way for undergraduate students to explore mammalian cell colony formation, reinforcing student understanding of cell biology and cancer biology. Furthermore, students have opportunities to formulate hypotheses, draw inquiry-based conclusions, and utilize the scientific process.

SUPPLEMENTAL MATERIALS

Appendix 1: Safety Considerations and Precautions

Appendix 2: Passaging HeLa Cells Student Protocol

Appendix 3: Clonogenic Assay Student Protocols

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Appendix 1: Safety Considerations and Precautions

Work with mammalian cells in culture such as HeLa cells, requires the use of a biosafety level 2 (BSL-2) cell culture lab that is equipped with specialized equipment such as biosafety cabinets. These are often shared-use facilities available to students and instructors. All individuals using this facility should be properly trained in the safe use of materials and facilities.

Safety Training Requirements for Students and Instructors:

1) Online CITI training. (<https://www.citiprogram.org/index.cfm?pageID=154&icat=0&ac=0>)

When registering for an account, select your institution as the *Organization Affiliation*.

When asked if you would like Continuing Education Credits, choose no.

Select the curriculum under “Human Subjects Research” called

“Biomedical Research Investigators” under Question 1.

Select “Biosafety Complete Training” under Question 5.

Select “Not at this time” or “No” for the other Questions.

Complete the following modules in the Biosafety Complete Training series.

- OSHA Bloodborne Pathogens Standard,
- Hepatitis B Virus (HBV) Vaccination,
- Labels and Engineering Controls,
- Universal Precautions and Work Practices

Once you complete these modules, send your completion certificate to your instructor.

2) Hepatitis B Vaccine or Declination Form

Vaccination against the Hepatitis B virus (HBV) is provided free of charge to students and instructors who have exposure to human blood, blood products, certain human body fluids, tissues, organs, and primary cell lines. The HBV vaccination is a series of 3 shots given over a period of 6 months (0, 1-2, 4-6 months).

Individuals with occupational exposure to blood or other potentially infectious materials (OPIM) may be at risk of acquiring Hepatitis B virus (HBV) infection. These individuals are asked to sign a form (institution specific) to certify that they have been given the opportunity to be vaccinated against the Hepatitis B virus at no charge, if they have not already. It is important to note that many individuals receive this vaccine as part of their routine vaccinations.

3) On-site blood-borne pathogen training checklist

Students and instructors are provided with training inside the cell culture facility so that they can be familiar with specific work practices, the location of personal protective equipment (PPE) and eyewash facilities, biohazards waste handling, and disinfection/spill responses. At the end of the training, trainees complete and sign a verification of training certifying that the site-specific information was reviewed and understood.

Appendix 2: Passaging HeLa Cells Student Protocol

Protocol:

1. As starting material, use HeLa cells growing on a 100 mm dish at ~50-80% confluency. [Be sure to label your plates with date, initials, cell type, and passage number]
2. Aspirate spent media.
3. Rinse cells gently and carefully two times with room temperature PBS (~3 mL/rinse).
4. Add 1 mL warm 0.25% Trypsin/EDTA to cells
5. Incubate for 5 min at 37°C incubator
6. Add warm media (1 mL) to cells and pipette up and down to break up cell clumps.
7. Transfer the desired amount of cell suspension to seed growth in new 100 mm dish (1/10) recommended
8. Add 10 mL of DMEM 10% FBS to the new plate, gently swirl to mix and spread contents.
9. Place in 37°C incubator, track growth daily using the inverted microscope, you will need to passage again when confluence reaches ~50-80%.

Appendix 3: Clonogenic Assay Student Protocols

HeLa Cell Colony Formation (Clonogenic) Assay on 6-well plates (Part I)

Protocol:

1. As a starting material, use HeLa cells growing on a 100 mm dish at ~50-80% confluency.
[Note: your group probably has more than one plate to use for this, select the plate that is closest to 80% confluency; **preparing one plate per group** should be enough for all members of the group to ultimately set up one colony formation assay (one 6-well plate per student)]
2. Aspirate spent media.
3. Rinse cells gently and carefully two times with PBS at room temperature (~3 mL each wash).
4. Add 1 mL warm 0.25% Trypsin/EDTA to cells.
5. Incubate for 5 min at 37°C incubator.
6. Add warm media (1 mL) to cells and pipette up and down to break up cell clumps.
7. Transfer ~2 mL of cell suspension into a 15 mL conical tube.
8. Measure the cell concentration (cell/microliter) using one of the chambers in a Kova Glasstic Slide (Kova International Cat. No. 87144) and a microscope.

To count cells:

Step 1- Using a micropipette, place 10 μ L (undiluted harvested sample) into a blank counting chamber

Step 2- Using a microscope and using the grid as a focusing guide, find cells on small squares to count.

Step 3- Determine the average number of cells per small grid or square (count at least 7-10 small squares).

Step 4- Multiply the calculated average by 90. That is your cell concentration in cells/microliter.

9. Dilute your original stock cell suspension to a final cell concentration of 10 cells per microliter.
10. Into each of the wells of a 6-well plate, pipette 10 μ L (100 cells) of your dilute cell suspension.
11. Using a pipette aid, pipette 3 mL DMEM with 5% FBS into each of the wells in top row.

Note: Make sure the bottoms of the wells are completely covered with media by gently rocking the plate. Be sure not to get media splatters on the lid during the process.

12. Using a pipette aid, pipette 3 mL DMEM with 10% FBS into each of the wells in bottom row.

Note: Make sure the bottoms of the wells are completely covered with media by gently rocking the plate. Be sure not to get media splatters on the lid during the process.

13. Place your plate in the 37°C incubator and leave undisturbed for a whole week, until our next lab period.

Note: Place the plate deep inside the incubator to prevent disturbing of the plates which can happen when people grab their dishes or plates to check their cells. You do not need to check on your 6-well plate, just let the colonies grow undisturbed for a full week until our next lab period.

Appendix 3 continued

HeLa Cell Colony Formation (Clonogenic) Assay on 6-well plates (Part II)

As a reminder, identify the experimental conditions of each of the wells on your 6-well plate:

Protocol:

1. Aspirate the media out of each well (you do not have to switch pipette tips in between).
2. You will be using Richard-Allan Scientific Three-Step Stain Set (Thermo Fisher Cat. No. 3300) to fix and stain cell colonies.
3. Add 0.5 mL of **Fixative Solution** into each well of your 6-well plate.
4. Incubate at room temperature for 2 minutes.
5. Aspirate the solution out of each well (you do not have to switch pipette tips in between).
6. Add 0.5 mL of **Solution A (eosin Y dye, stains cytoplasm)** into each well of your 6-well plate.
7. Incubate at room temperature for 2 minutes.
8. Aspirate the solution out of each well (you do not have to switch pipette tips in between).
9. Add 0.5 mL of **Solution B (methylene blue/azure A dye, stains DNA i.e. nucleus)** into each well of your 6-well plate.
10. Incubate at room temperature for 2 minutes.
11. Aspirate the solution out of each of the wells (you do not have to switch pipette tips in between).
12. Rinse each well gently with deionized water using squirt bottle.
13. View colonies and take images as desired to record your results.
14. Record colony counts and perform appropriate statistics as desired.

Figure 1.

Figure 1. *Micrographs of HeLa cell colonies grown under different conditions.* Clonogenic assays were performed using 6-well plates. Cells were grown in DMEM media supplemented with 10% FBS (A), 5% FBS (B), 10% FBS and DMSO (C), or 10% FBS and 60 μ M Fisetin (D). All images were acquired after HeLa cell colonies were fixed, stained with eosin dye, methylene dye, and rinsed with water. (scale bar, 40 microns)

Figure 2.

Figure 2. Representative student results for the clonogenic assays. Clonogenic assays were performed in 6-well plates with HeLa cells. Pictures of 6-well plates were shown on the left whereas the quantitative analyses were shown on the right. **(A)** Cells were grown in media supplemented with 5% FBS (1-3) or 10% FBS (4-6). On average, 16.7 and 19.3 colonies were formed under 5% and 10% FBS conditions, respectively. **(B)** The data are presented as mean $\pm$ standard error of the mean (SEM). $P > 0.01$ versus control. **(C)** Cells were grown in 10% FBS containing DMEM media with DMSO (control, 1-3) or 60 μ M Fisetin (4-6). On average, 20.3 and 8.3 colonies were formed under control and Fisetin treatment, respectively. **(D)** The data are presented as mean $\pm$ SEM. $P > 0.01$

versus control. **(E)** Cells were grown in 10% FBS containing DMEM media with DMSO (control, 1-3) or 50 µg/mL Cycloheximide (4-6). On average, 17.7 colonies were formed in control group. No colonies were formed when treated with Cycloheximide. **(F)** The data are presented as mean ± standard error of the mean (SEM). * $P < 0.01$ versus control. All assays were performed in triplicates. The asterisk indicates statistically significant difference.