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Molecular parasitology CURE: Understanding the apoptosis pathway in kinetoplastid parasites

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Embedding inquiry driven research in undergraduate courses allows integration of core concepts and competencies necessary to developing scientific thinking and lab skills. These are critical skills for undergraduates to be successful in science careers and for admission into graduate school. However, there are only a handful of examples of collaborative CUREs in Biology where students have an opportunity to connect with a network of researchers outside of their own institution, and none in the field of parasitology. In Spring 2021, we piloted a mini-CURE where student groups from University of Mary Washington and Georgia State University collaboratively completed research projects as part of a research-intensive course on Molecular Parasitology. The benefits of this approach were immediately obvious as students interacted across institutions, learned from each other's disciplinary expertise, and informed their own research with data collected by their collaborators. It provided enrichment to the course by adding networking opportunities as well as cross-disciplinary knowledge sharing. We present here our CURE model as a way for other educators to design and implement similar cross-institutional interdisciplinary CUREs that can be modified to align with their research expertise.

Keywords: CURE, Graphical Abstract, Bioinformatics, Molecular parasitology, interdisciplinary, cross-institutional

Introduction

The benefits of Course-based Undergraduate Research (CURE) are numerous and well documented. For students at Primarily Undergraduate Institutions (PUI), these provide high-impact research experiences that can culminate in retention in STEM careers and motivation to pursue graduate level education. They provide opportunities for students to make discoveries, collaborate, engage in meaningful research and develop a sense of ownership of their lab work. For faculty, especially at PUI, these provide tractable models of modern, collaborative science and move toward the complex, interdisciplinary nature of scientific investigation as an effective platform for integrating the goals of research and education. A wide variety of successful CUREs have been developed with different research themes, however only a handful of CUREs currently prioritize on the benefits of collaborative research across institutions. Two collaborative CUREs that are widely reported and highly successful are the malate dehydrogenase CURE (Bell et al. 2020) and the HHMI SEA- PHAGES CURE (Staub et al. 2016). Our CURE, Experiential Collaborative Parasite Research across institutions (ECoPaR) provided students an opportunity to engage in a cross-institutional, cross-disciplinary research experience and effectively contribute to ongoing Kinetoplastid research. This collaboration was between students and faculty of University of Mary Washington, Georgia State University and Albright University.

A large number of students at the University of Mary Washington (UMW) express an interest in pursuing careers in medicine and/or biomedical research. It is imperative that these students have authentic hands-on research experience beyond typical lab courses that prepare them for their careers. Working on an important problem using

a surrogate for a pathogen allows the collaborative research team (students and professors) to safely explore the biology of these parasites and improve our knowledge to find a treatment for three important pathogenic organisms (*C. fasciculata* is non-pathogenic but biologically close representative of all pathogenic kinetoplastids) . This project serves as a great hook to engage students in authentic research and increases opportunities to use inquiry driven teaching designed methods.

Goals and Specific Objectives for the CURE

Research objectives

To better understand the biology of kinetoplastid pathogens by creating and expanding a new tool kit for understanding of the apoptosis pathway in *C. fasciculata* parasites. The two focus questions for this project are:

Specific Aim/Project 1:

Understanding the biological relevance of programmed cell death in kinetoplastid parasites. It has been speculated in previous studies on Leishmania that the programmed cell death in the parasite is a mechanism to avoid hyper-activation of immune reaction from the host (Kaczanowski et al. 2011). Apoptosis of parasites in the insect vector creates a bottleneck so that the fittest parasites are selected for transmission into the human host. However, it is unclear as to what triggers apoptosis versus cell replication. Students were tasked with research projects that aimed at investigating environmental triggers like nutrients, starvation, temperature changes, osmotic changes, and hypoxia to see which of these serve as signal for apoptosis. Students used cellular, molecular and biochemical assays to better characterize the triggers of apoptosis and expand the current tool kit for identifying morphological changes such as change in cell shape, motility and metabolic health.

Specific Aim/Project 2:

Identification and characterization of the genes in activation and regulation of apoptosis in kinetoplastid parasites. This project aims to identify genes involved in the apoptosis pathway. Protozoan parasites seem to have very few overlapping genes in the apoptosis pathway when compared to their mammalian or insect hosts. This characteristic is particularly attractive when looking for drug targets, as parasitic drugs will not affect the host, because they target parasite-specific pathways. A common approach in the field for identification of gene function is creating parasite mutants where genes have been removed, and replaced with a tagged gene to study gene expression. Although we couldn't complete this objective in Spring 2021 course due to COVID related disruptions, an essential part of our courses going forward will be establishing a CRISPR-cas9 system to study putative apoptosis gene localization and function in the parasite (Beneke et al. 2017; Beneke and Gluenz 2019).

Teaching objectives

The main objective of this course is to highlight how students can lead the development of novel cellular, molecular, and biochemical tools for characterization of a little-studied non-pathogenic parasite (*C. fasciculata)* for use as a parasite model. This course will be available to Biology and Biomedical majors in the department and satisfies the Research Intensive requirement. The Vision and Change committee recommended the incorporation of research experiences as an integral component of biology over a decade ago. Embedding inquiry-driven research in undergraduate courses allows integration of core concepts and competencies which are necessary to developing scientific thinking and lab skills. These are critical skills for undergraduates to be successful in science careers and for admission into graduate school. Below are two areas that I plan develop for this collaborative CURE.

Specific Aim/Project 3:

Building a framework to educate students on cutting edge molecular, cellular and biochemical techniques that dissect the molecular players of a poorly characterized pathway in kinetoplastid parasites. The emphasis was understanding the scientific process rather than mastery of individual labs/ techniques. Data was shared using a shared drive and will be shared with future students to ensure that students contribute to authentic publishable research.

Specific Aim/Project 4:

This specific aim was not accomplished during Spring 2021, but for future courses, we will develop assessment to measure student attitudes about research, career goals, previous collaboration experiences, and

their beliefs about the importance of collaboration in scientific research. The assessment will focus on whether ECoPaR increases students' appreciation for and experience with scientific collaboration. Such an assessment is not available at the moment, because of lack of collaborative CUREs. The assessment data will be used to improve the course and make changes to make it more applicable to students. It will also demonstrate growth in collaboration over successive years and show result from a collaborative CURE versus one without collaboration.

How to incorporate cross-institutional collaborative work throughout the CURE

In our course we had 5 project groups. Each group consisted of two- three students from both institutions. The central theme of the research was decided by the instructors. For our CURE, this theme was assessing effects of environmental stressors on morphology, biochemistry, and gene expression of *C. fasciculata*. Below is a list of activities we developed and incorporated in our CUREs that ensured continued collaboration and joint research with feedback from peers and faculty from home institution as well as partner institution.

a) In the beginning of the course, we had a Zoom session where students and faculty from both institutions gave introductions with ice breakers. Students were asked to form groups and pick project partners.
b) To familiarize students with the areas of molecular parasitology, neglected tropical diseases, and techniques, we invited speakers from top parasitology labs and held zoom seminars. Our students were required to participate by asking questions and engaging in meaningful discussions. We also had joint journal club via Zoom on papers related to the research idea that students chose to pursue in the class.
c) Each group was tasked with developing a collaborative hypothesis and experimental plan in the form of a graphical abstract and sharing on google drive for comments from peers. An example of this assignment is provided in the student outline.
d) To make sure students based their research questions on known evidence in literature for the above assignment, they were asked to research at least 3 primary papers and 2 review articles. They were asked to develop an annotated bibliography together for developing their experimental plan.

Throughout the semester, data sharing and analysis was done by maintaining an online joint lab notebook with periodic instructor evaluation from both institutions.

Student Outline

Assignment I : Survey of protozoan parasites via light and fluorescence and Microscopy

Introduction to Protozoan parasites

Protozoa are a very diverse group of unicellular, heterotrophic eukaryotes that are members of the kingdom Protista (photosynthetic algae are also in this kingdom). They are found in all types of habitats, including soil, freshwater and saltwater. Some live in symbiosis with other organisms, others are free-living consumers, and a few are parasitic to humans. Protozoans do not have cell walls, but many are surrounded by a proteinaceous outer covering called a pellicle. Both sexual and asexual modes of reproduction occur in this group. Protozoan pathogens vary in their mode of transmission, or method of gaining access to a new host. Some are transmitted by vector (an insect or arthropod that transmits a microbial pathogen), by ingestion of contaminated food or water, or even by sexual contact.

Many species form a dormant stage called a cyst that is resistant to adverse environmental conditions. This allows them to exist outside of a host cell for some time and is often the stage that is transmitted to a new host. The feeding (metabolically active) form of these organisms is known as a trophozoite.

Protozoa are often grouped based on the type of structures they use for locomotion (motility).

Amoebozoans use cytoplasmic projections called pseudopodia

- ex: *Entamoeba histolytica*

Flagellates use flagella

- *Trypanosoma gambiense*
- *Trypanosoma cruzi*
- *Giardia lamblia (intestinalis)*
- *Trichomonas vaginalis*

Ciliates use cilia.

- Balantidium coli
- Paramecium spp. (non-pathogenic)

Apicomplexans have no means of locomotion in their mature form

- *Plasmodium falciparum*
- *Toxoplasma gondii*

Microscopic examination of sexual and asexual stages of *Plasmodium falciparum*

Malaria has been a major disease of humankind for thousands of years. It is referred to in numerous biblical passages and in the writings of Hippocrates. Although drugs are available for treatment, malaria is still considered by many to be the most important infectious disease of humans: there are approximately 200 million to 500 million new cases each year in the world, and the disease is the direct cause of 1 million to 2.5 million deaths per year.

Malaria is caused by protozoa of the genus *Plasmodium.* Four species cause disease in humans: *P. falciparum, P. vivax, P. ovale and P. malariae*. Malaria is spread to humans by the bite of female mosquitoes of the genus *Anopheles*. Like many protozoa, plasmodia pass through a number of stages in the course of their two-host life cycle. Refer to the CDC website for the life cycle of malaria and different stages (Prevention 2020 Jul 16).

Malaria parasites can be identified by examining under the microscope a drop of the patient's blood, spread out as a “blood smear” on a microscope slide. Prior to examination, the specimen are stained with the Giemsa stain to give the parasites a distinctive appearance. Observe the slides provided and try to find each of the asexual and sexual stages of parasites on slides labeled 1-3 under the slide under 100x oil immersion.

Fluorescence Microscopy

Fluorescence Microscopy is a type of light microscopy (fluorescence microscopy) in which a specific molecule within a specimen is detected through a fluorescent tag. The specificity of fluorescent microscopy is achieved through illumination of the specimen with light of a defined wavelength designed to excite the fluorescent tag. The primary benefit of fluorescence microscopy is the specificity it provides, but the intensity of the fluorescent signal also enhances the sensitivity of detection.

Fluorescent molecules absorb energy from light of a short, high-energy wavelength and emit a portion of that energy at a longer wavelength of less energy. Some fluorescent molecules that are commonly used by cell biologists are the DNA-specific DAPI stain (absorbs at 358 nm; emits at 461 nm), fluorescein (green) (absorbs at 494 nm; emits at 521 nm), and Texas red (absorbs at 589 nm; emits at 615 nm).

A) What color will a DAPI-stained molecule appear?

B) What color will a fluorescein-labeled molecule appear?

C) What color will a Texas red-labeled molecule appear?

The fluorescence microscope achieves this specificity through a set of filters that selectively allow the absorption wavelength of light which excites the fluorescent tag to reach the specimen and the emission wavelength for that tag to reach the ocular lens. The pair of filters is arranged in a filter cube such that a light path from a high energy mercury light source passes through a selective excitation filter before being diffracted off a dichromatic mirror and reaching the specimen. The light diffracted from the specimen then must pass through a barrier or emission filter, selectively allowing only the emission wavelength of the fluorescent tag to reach the ocular lens.

Methods for labeling molecules with fluorescent tags

Four commonly used methods for attaching a fluorescent tag to a molecule of interest for fluorescence microscopy are described below. Methods 1, 2, and 4 will be used in today’s exercise.

1) Direct attachment through non-covalent binding of fluorescent stain. This type of labeling is done through fluorescent molecules that display natural binding specificity for a molecule of interest. Although this method is less universally useful, it is used routinely to label DNA through DAPI, Hoechst staining, and propidium iodide staining. DAPI fluoresces blue only when bound to the minor groove of DNA.

2) Immunofluorescence: indirect attachment through a fluorescently labeled antibody. This commonly used method uses an antibody to target a fluorescent tag to a protein of interest. Antibodies are produced by cells of vertebrate

immune systems to specifically recognize and bring about the destruction of foreign molecules. Cell biologists make use of antibody-specificity as a means for targeting a fluorescent tag to the protein it recognizes. Figure 3 shows that the fluorescent tag can either be directly (A) attached to the antibody recognizing the protein or interest or it can be indirectly (B) attached to it through a fluorescently tagged secondary antibody that recognizes that antibody (called the primary antibody). The indirect method B is more commonly used, because fluorescently labeled secondary antibodies that recognize any primary antibody produced in a particular vertebrate (e.g., mouse or rabbit) are widely available through commercial sources. The wide availability of these secondary antibodies makes this method more convenient, in practice.

3) Direct attachment through covalent linkage. Although it requires more work, it is also possible to chemically link a fluorescent tag to any protein of interest. Fluorescent molecules with chemically reactive side chains are commercially available for catalyzing the covalent linkage of a fluorescent tag to a purified protein in a test tube reaction. The in vitro labeled protein must then be injected into a living cell, in which it can be monitored by fluorescence microscopy.

4) In vivo expression of GFP-fusion proteins. This method also allows the behavior of a labeled protein to be monitored in a living cell. However, this method allows the cell to do the work of producing the fluorescently labeled protein. This is done by producing a recombinant gene in which the gene encoding the protein of interest is fused to a gene for the naturally fluorescing Green Fluorescent Protein (GFP) from the jellyfish *Aequorea victoria*. The recombinant gene is then transferred into the genome of a living organism, where it is transcribed and translated into a GFP-tagged version of the protein of interest.

Methods 1 and 2 require that the cells be treated with a fixative before staining. Methods 3 or 4 require a living specimen. The researcher chooses which of these methods to use on the basis of what he/she wants to learn from an experiment and the time and cost required. In today's exercise we will use Methods 1 and 2.

Toxoplasma gondii cellular structure under Fluorescence microscope

Toxoplasmosis is a disease that results from infection with the *Toxoplasma gondii* parasite, one of the world's most common parasites. Infection usually occurs by eating undercooked contaminated meat, exposure from infected cat feces, or mother-to-child transmission during pregnancy. Members of the cat family (Felidae) are the definitive hosts; many mammals and birds serve as intermediate hosts. Organisms enter the intestinal epithelium and can spread to many host tissues. Individual organisms are lunate, about 6 × 2 μm, and multiply within host cells. Tissue cysts containing hundreds of quiescent organisms may form as infection wanes. *Toxoplasma* reproduces sexually only in cats. Organisms infecting the intestinal epithelium produce oocysts which are shed in the feces.

Toxoplasma gondii Life cycle

Slides 4- 8 show intracellular *Toxoplasma gondii* parasites expressing a cytosolic red fluorescent protein. The nucleus of the host cell and the parasite are stained blue with DAPI. The parasites grow inside a "bubble" called parasitophorous vacuole. Visualize the parasite at various stages of division indicated by the varying number of parasites per parasitophorous vacuole, divides by a binary process consisting of internal budding that results in only two daughter cells per round of division. Below is a schematic showing the parasites within the host cell and position of various organelles in the parasite. Observe the provided slides under fluorescence microscope. Collect images in different channels and prepare a figure. More instructions about image acquisition and figure composition will be provided during the class.

Chagas disease is caused by the parasite *Trypanosoma cruzi,* which is transmitted to animals and people by insect vectors and is found only in the Americas (mainly, in rural areas of Latin America where poverty is widespread). The vector triatomine insect (or "kissing bug") transmits the parasites while taking a blood meal near the site of the bite wound. Trypomastigotes enter the host through the wound or through intact mucosal membranes,

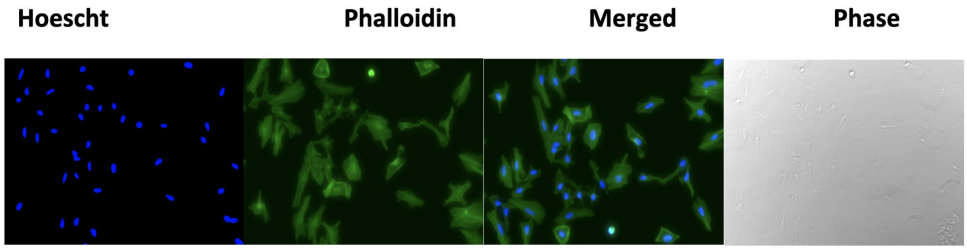
such as the conjunctiva. Chagas disease (*T. cruzi* infection) is also referred to as American trypanosomiasis. Refer to CDC website for detailed life cycle of *T. cruzi.* Observe slides 9-10 for cellular features of the parasite. Like other members of the Kinetoplastid groups, the parasite possesses a flagellum that emerges from the flagellar pocket which is located near the single mitochondrion called Kinetoplast. In the epimastigotes stages of the parasite, the Kinetoplast and the flagellar pocket are found in a position anterior to the nucleus (Prevention 2019 Apr 12).

Post-lab assignment

Each lab partner should do this assignment independently. The program you will use to edit images is Image J. Download image J here

https://imagej.nih.gov/ij/download.html (Links to an external site.)

The final image you generate will be 8 panel figure from the images you took on the microscope. You will show two images each in all 3 channels and the overlay image. Your figure should have at least two representative frames Remember each frame will have 4 images one in each channel. See example below as to what your final figure should look like. For editing the images you will have to use an image editing tool called image J.



You will use Image J to adjust brightness, contrast, background, add color appropriate color and finally develop an overlay image. To edit images, use the tutorials below and you must use image J program (please don't use any automatic online image adjuster, points will be taken off for that, image J is specifically meant for microscopy and this assignment aims to give you practice using the program).

For coloring the images, watch these instructions

https://www.youtube.com/watch?v=qPjtOpvNbZU

https://www.youtube.com/watch?v=i4GkiX_UTBg

For making a Merged / overlay image watch these instructions

https://www.youtube.com/watch?v=LnRhCG5AC2I

https://www.youtube.com/watch?v=qOfKfMNLMiM

Once you edit the images use Powerpoint to arrange images as panels like the example shown above.

Assignment II : Bioinformatics analysis of gene of interest

Background The *Trypanosomatidae* are a group of unicellular, flagellated, obligate parasites, including many important pathogens of humans and animals. African trypanosomes (*Trypanosoma brucei, T. congolense and T.*

vivax) are endemic in rural areas of sub-Saharan Africa, where they cause sleeping sickness in humans, and wasting disease (nagana) in cattle. These diseases are invariably fatal if left untreated. TriTrypDB is a functional genomic resource for the *Trypanosomatidae*. The genomes of multiple (46 *Trypanosomatidae* species) have been sequenced and are available and integrated with other 'omics' datasets on TriTrypDB. Viewing a gene page can be achieved by either entering a specific gene ID in the ID search
window in the figure below interactive banner.

1. Type CFAC1_210021700 in the search bar at the left hand corner of the front page. The gene page contains all available information for a gene displayed on a single page, including synteny maps, information on gene structure (number of exons), number of transcripts. orthologs and paralogs the actual sequence (amino acid and nucleotide) of the displayed gene. In addition, links to User Comments (and access to the gene-specific
comment form; and links outs to the gene record on GeneDB are available through the gene page. You will now explore some of the tools will show you some of the tools that we are going to use to research about our gene of interest.

2. Pick your gene of interest from the excel sheet
On canvas and carry out the following steps on the TriTrypDb database.
3. Enter the gene id on the search bar at the left hand corner of the front page. Research your gene and note down the following regarding the gene

i) How big is the gene?

ii) How big is the protein?

iii) Does it have transmembrane domains (TMHMM)?

iv) Does it have a mitochondrial localization signal (Mitoprot)?

v) Does it have a signal peptide?

vi) Does it have a homolog in all three trypanosmatids (T. brucei, T.cruzi, L. Mexicana)?

vii) Perform a Clustal W alignment of the C. fasiculata gene with the three trypansomatids?

viii) Go to NCBI Blast and Blast the gene against Yeast
(taxid:4932)https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=Blast Search&LINK_LOC=blasthome

a. Write down the Gene id of the yeast homolog.
b. Write down % sequence alignment.

ix) Repeat the steps above with Human (humans (taxid:9606))

a. Write down the Gene id of the yeast homolog
b. Perecentage sequence alignment

x) Look around the gene page for any other additional information you may find on the gene. Record your findings below.

4. If your proteins was predicted to be a membrane protein, explore protein topology in membrane (number of TM domains, location of N and C terminal) using Protter https://wlab.ethz.ch/protter/#
Enter the protein sequence of your protein and run the program. Paste the output from Protter below. How well did Protter predictions match TriTrypDB predictions?

5 .Now run your proteins through HHPred and BLAST domain to predict functional domains of the protein. What domains were identified?
Was there a consensus between HHPred and BLAST domain?
What is the top hit on HHpred and what was the probability value?

6. If you are looking to expand the activity for an upper level class consider running them through Alphafold https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb
Or Lomets https://zhanggroup.org/LOMETS/

Assignment III: Experimental Outline and Graphical abstract Assignment

An abstract of a scientific journal or a conference presentation helps familiarize authors with the outline of the project, convey important findings of the study, stimulates interests and sparks collaboration. In the past decade an increasing number of journals have encouraged the use of visual abstracts, also known as graphical abstracts, to disseminate the summary of scientific research. As one can imagine, the use of visuals in the form of diagrams, pictures, or infographics can allow readers to gain a quick overview, facilitating browsing and increasing visibility of the paper to both researchers and social media. Currently more than 50 journals and institutions have adopted its use in their social media strategy to disseminate scientific research. For this assignment you will design a graphical abstract with your project partner. You will modify your graphical abstract later in the semester to encompass the changes you make after the first iteration of the experiment.

Version 1: Graphical abstract for hypothesis and experimental design

Below are some required readings before you start your graphical abstract.

Required reading:

•"Attract readers at a glance with your Graphical Abstract," http://crosstalk.cell.com/blog/attract-readers-at-a-glance-with-your-graphical-abstract

•Cell Press Graphical Abstract Guidelines.
http://www.cell.com/pb/assets/raw/shared/figureguidelines/GA_guide.pdf

https://www.cell.com/fulltext/S0092-8674(15)01570-6

I also recommend browsing articles to get a sense of good design and essential elements of a good graphical abstract. A good graphical abstract has all necessary components of the experimental process without clutter or too many distractions. Once you get a sense of what graphical abstracts are and you start designing yours, I suggest you draft out one on pen and paper and discuss with your project partner and peers in the class. Consider the following platforms to start building yours. Feel free to explore other tools as you like.

1. Biorender: https://biorender.com/

2. Blender: http://www.blender.org/

3. Adobe Illustrator: https://www.adobe.com/

4. 3d Max: https://www.autodesk.com/

5. GIMP: https://www.gimp.org/

Version 2:Graphical abstract for revised experimental design

For this assignment review my comments on your previous assignment and incorporate changes accordingly. Pay special attention to spelling mistakes, replicates, controls, organization that help convey "bottom line" of the experimentation process.

Notes for the Instructor

The lessons provided here lend to modular implementations. Instructors can pick and choose between microscopy, bioinformatics or graphical abstract as suitable for their classes. For the microscopy activity, if you don't have access to antibodies and parasite strains expressing certain markers, Light microscopy is a good alternative to expose students to cellular organization and organellar complexity in protozoan organisms. Carolina Biologicals is a great resource for buying pre-stained slides.

A few examples of graphical abstracts from our class are provided in the appendix. We recommend giving this assignments where students receive feedback from instructors and have a chance to modify and re-submit the graphical abstracts.

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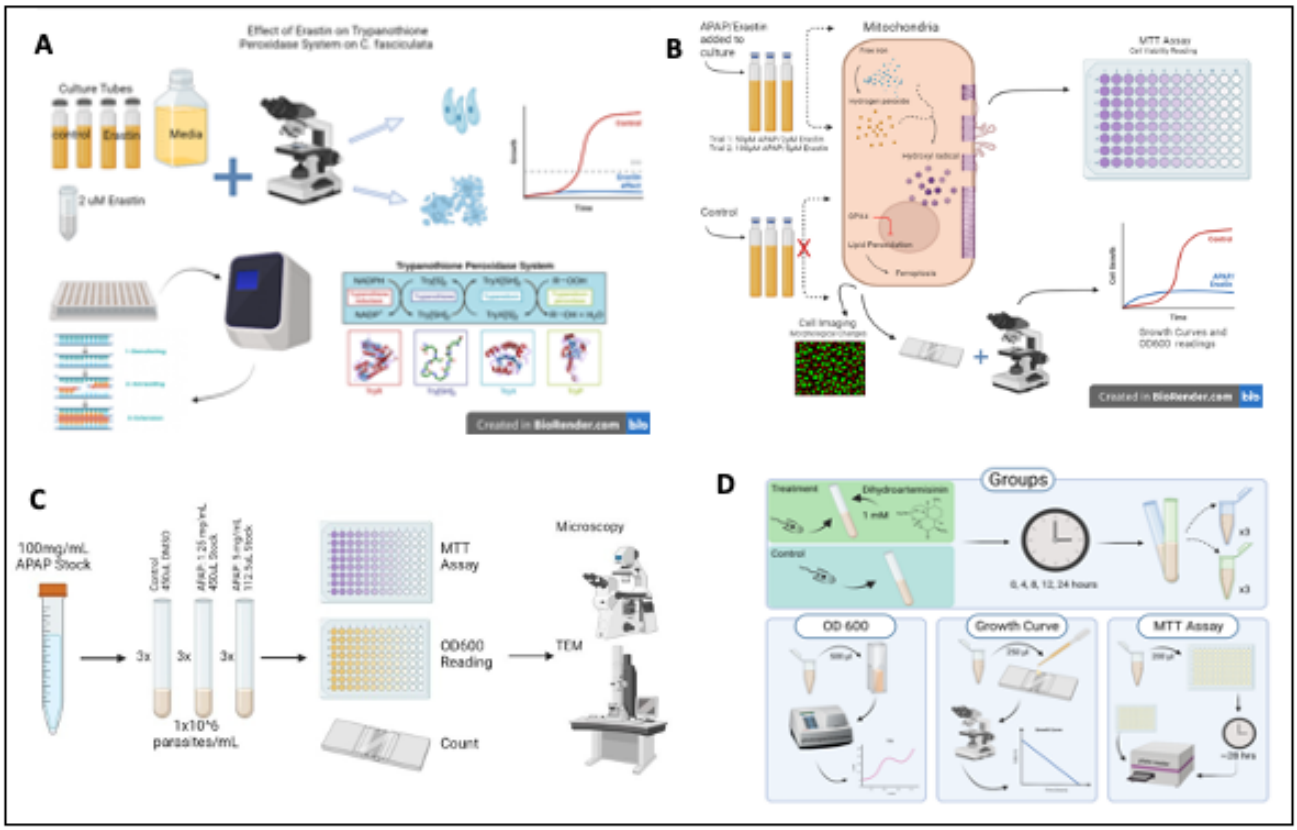
About the Authors

Swati Agrawal is currently an Assistant Professor at University of Mary Washington where she teaches Introductory Biology and Cell Biology. These lessons were developed for a Molecular Parasitology course, which is a research-intensive course for senior Biology and Biomedical students.

Appendix A



Example of student work: Students in the Molecular Parasitology course (collaborative CURE with Dr. Paul Ulrich's class) presented their hypothesis and experimental design through Graphical abstracts.

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