Using Perfringolysin O (PFO) to Create a Cell-Free *in vitro* System to Study Mouse Malaria (*Plasmodium yoelii*)

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Background

Malaria (*Plasmodium spp.)* is a parasitic infection spread through the *Anopheles* mosquito. This disease is one of the most prevalent globally, infecting over 240 million in 2020 alone.[1] The lifecycle is highly complex, and all known symptoms of disease stem back to the blood-stage that these parasites go through inside a host (Figure 1). The liver-stage of the life cycle is incredibly difficult to study due to its particular growth requirements and unfortunately, the field lacks a successful *in vitro* culturing method that would facilitate studying the transition from early-to-late liver-stage development. Today, only a select number of hepatoma cell lines support the *in vitro* culturing of liver-stage mouse malaria (*P. yoelii*) parasites, and a few primary human hepatocyte cultures support the growth and development of human malaria (*P. falciparum*) parasites. However, many readily available mammalian hepatocyte cell lines result in cell death and do not support the development of sporozoites into liver-stage parasites after 24-48 hours. Thus, we aim to establish an *in vitro* cell-free culture system that researchers can use to study late liver-stage development. We focused on culturing mouse malaria (*P. yoelii*) parasites for our investigation, utilizing a cholesterol-dependent cytolysin, called perfringolysin O (PFO), to selectively permeabilize the hepatocyte plasma membrane and separate the parasitophorous vacuole so it could be cultured in a cell-free *in vitro* system.

PFO is an exotoxin produced by the bacterium *Clostridium perfringes* (Figure 2). When in contact with a cell, PFO monomers will bind to cholesterol molecules within the membrane and at 37°C, will oligomerize to form pores of ~300 Å or (30 nm) in diameter.[2] Once the membrane has been permeabilized, we can separate the plasma membrane to isolate portions of interest (e.g., the cytosolic fraction or the parasitophorous vacuole). Previous investigations have used PFO to isolate the host cytosolic compartment to identify secreted proteins from chlamydial inclusions.[2] Another study utilized PFO to separate the bacterial vacuole of *Listeria monocytogenes* to investigate the function of membrane proteins from inside the host cytosolic compartment.[3] However, our investigation is the first of its kind, as we are attempting to not only separate the *P. yoelii*- containing parasitophorous vacuoles using PFO, but to isolate these vacuoles and grow them in a separate *in vitro* culturing medium.

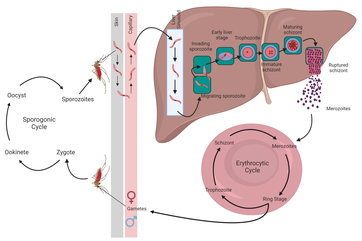


Figure 1. The life cycle of the *Plasmodium* parasite. This image focuses on liver-stage development, which is the are of interest for our investigation. We will replicate liver-stage development *in vitro* using a new culturing medium that does not rely on hepatic cells. Created with BioRender.

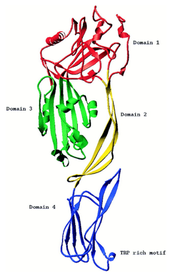


Figure 2. Structure of Perfringolysin O (PFO). PFO is an exotoxin that will selectively permeabilize the hepatocyte plasma membrane so we can separate the parasitophorous vacuole and culture the parasites in a cell-free *in vitro* system. Image from Johnson and Heuck's (2016) investigation. [4]

Goals

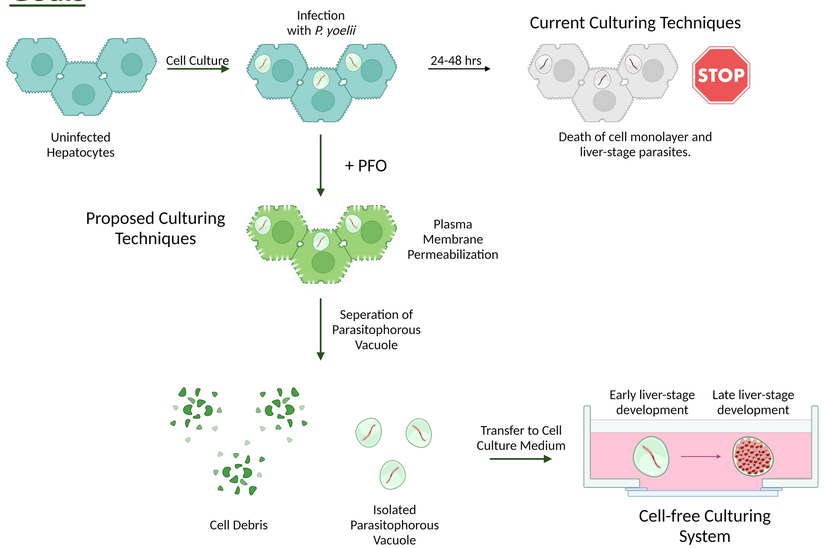
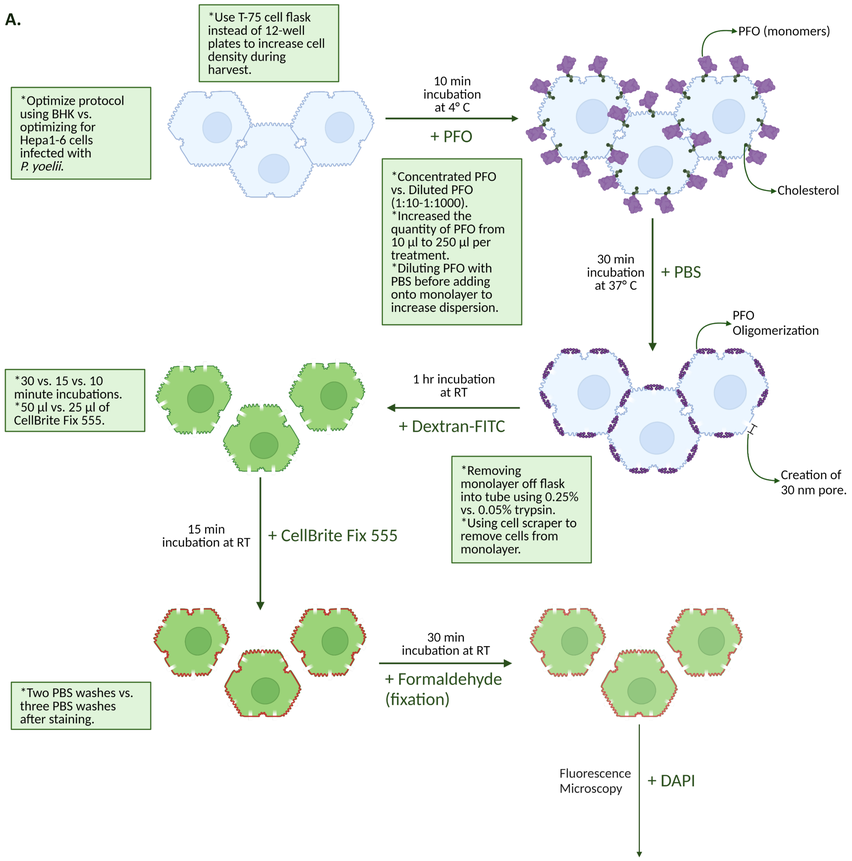


Figure 3. Our goal is to use PFO to separate the parasitophorous vacuole from infected (*P. yoelii)* hepatocytes. The diagram shows the current culturing techniques used to study these parasites, which results in the death of both the parasite and the host cells. Ultimately, the permeabilization of the plasma membrane and separation of the parasitophorous vacuole before eventually transferring it to a culture medium, allows for the study from early-to-late liver-stage development. Created with BioRender.

Results



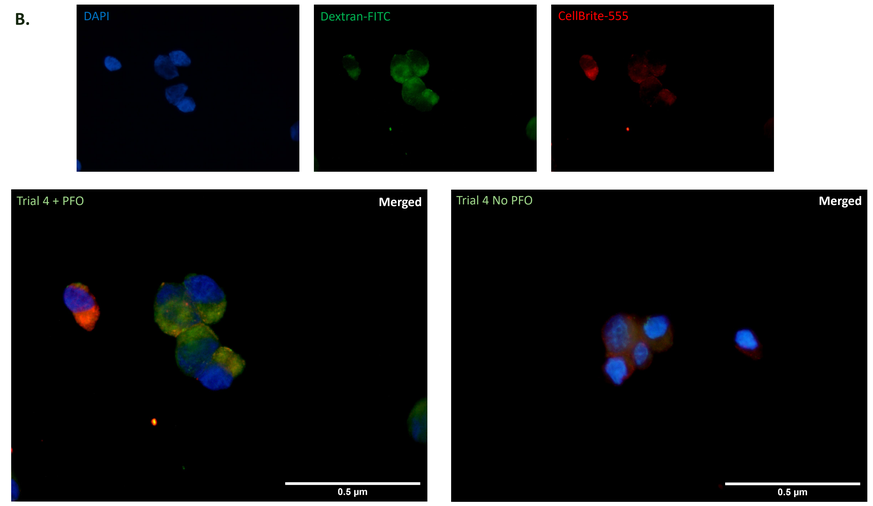


Figure 4. A. PFO treatment protocol and fluorescent staining assay. Green notes (next to assay steps) indicate changes made to the protocol over eight PFO trials. **B.** Fluorescent images of BHK cells from the fourth PFO trial. Images on the top row correspond to the DAPI nuclei stain (blue), Dextran-FITC reporter molecule (green), CellBrite-555 membrane stain (red) for the treatment group. From the composite images in the bottom row, we can see that the BHK cells treated with PFO (left) have stronger intracellular concentrations of Dextran-FITC compared to the control group (right) that did not receive the PFO treatment. This supports our findings that PFO will selectively permeabilize the cellular membrane of treated cells. Created with BioRender.

Future Directions

During our investigations, we were able to successfully establish that baby hamster kidney (BHK) cells can be selectively permeabilized using PFO (Figure 4A) as intracellular concentrations of our reporter molecule (Dextran-FITC) were strongest within PFO-treated cells compared to non-PFO treated cells. Unfortunately, the first two trials using BHK cells were unsuccessful due to an inability to harvest whole cells from the 12-well culture dish. By the end of the assay, any whole cells, had been damaged beyond repair. However, by our third trial, we determined that using a T-75 flask to culture and harvest our cells would increase our yields dramatically. We can see in Figure 5 that the third trial was a success and produced viable cells; however, the staining from the CellBrite-555 membrane stain was overblown and needed modification. The fourth trial (Figure 4B) was by far the most successful application, yielding accurate staining patterns that allow us to directly compare the Dextran-FITC signal between our PFO treatment and control group. After this, there was one more trial (Figure 5) with BHK cells to troubleshoot inconsistencies with the membrane dye. However, we were unsatisfied with the results and proceeded with adapting the protocol to a hepatocyte cell line.

Adapting this protocol (Figure 5) to a murine hepatocyte cell line (Hepa1-6 cells) has raised several issues stemming from differences in membrane cholesterol content between BHK and Hepa1-6 cells. Unfortunately, during the harvesting step (after PFO treatment) in subsequent trials, the smaller, more cholesterol-rich Hepa1-6 cells have lysed. We have since adapted the protocol to use a flexible cell scraper to remove the cell monolayer off the T-75 flask, with positive results (Figure 5). Though this trial was more successful, we are still facing issues adapting the protocol and establishing whether the intracellular concentrations of Dextran-FITC will be stronger in the PFO-treated Hepa1-6 cells as expected. After this proof-of-concept, we will then move on to infecting the Hepa1-6 cells using *P. yoelii* sporozoites harvested from infected *Anopheles* mosquitoes. After infection, we will proceed with our PFO permeabilization protocol but using immunofluorescence. We hope these efforts will allow us to begin the isolation process to extract the parasitophorous vacuole so we can start determining the best cell culture media conditions to support the *in vitro* development of late liver-stage parasites.

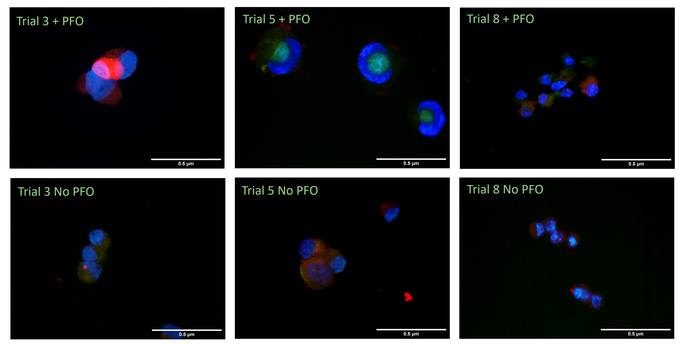


Figure 5. Florescent images of BHK (left and center columns) and Hepa1-6 cells (right columns) cells from trials 3, 5, and 8. DAPI (blue) is a nuclei stain, CellBrite-555 (red) is a cell membrane stain, and Dextran-FITC (green) is our reporter molecule to detect the permeability of a cell. Treatment groups were treated with PFO and can be seen in the top row, while their respective control group can be seen in the bottom row. These trials were variably successful, stemming from an inability to get consistent staining of both the cell membrane and differentiation between trial groups for the reporter molecule.

Acknowledgements

I would like to thank Dr. Anna Groat Carmona for all her support and mentorship, as well as the UWT lab staff for their kindness and encouragement.

References

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