# Graduate Research Plan Statement

Despite over 100 years dedicated to cancer research, nearly every single person alive today knows someone whose life has been directly impacted by this devastating disease. One relatively new area of cancer research is bacterial cancer therapies, in which bacteria are used as a living intratumoral drug delivery system. These bacterial based therapies have shown promise in treatment of solid cancerous tumors, specifically those of the colon[1]. This strategy leverages the bacterial trait of selectively colonizing tumors and surviving in the necrotic core of tumors, an area which current treatments fail to reach but is associated with the worst outcomes[2]. One of the most developed bacterial cancer therapies is the Synchronized Lysis Circuit (SLC) in which bacteria grow and lyse in cyclical patterns delivering continual therapeutic dosage at the tumor site. As the population of bacteria grow intratumorally, they form a biofilm, a collection of microorganisms held together by an extracellular polymeric substance. The SLC strategy struggles with efficacy and often only delivers dosage to the parts of the tumor in which the bacteria were originally seeded. By taking advantage of the role ion channels play in cell-type selective proliferation in a biofilm, dosage can reach a greater percentage of the tumor. *Bacillus subtilis* is a widely studied biofilm forming organism that have 2 distinct cell types, motile and biofilm forming. Recent studies have revealed that ion channels play a large role in the cell type specific proliferation of *B. subtilis*. An introduction of large concentrations of potassium ions or electric shocks to a biofilm has been shown to increase proliferation of motile cells, leading to global changes in the cell-type composition of biofilms. Successive exposures of potassium ions to the biofilm showed even greater increase of motile cells in cell-type composition[3]. By exposing a *B. subtilis* biofilm in a tumor with successive doses of potassium ions, the cancer therapy may be able to reach a greater percentage of the tumor via cell motility. By integrating the SLC system into *B. subtilis*, introducing doses of potassium ions or electric shocks to intratumoral biofilms, and controlling SLC activity, this cancer therapy can be more effective.

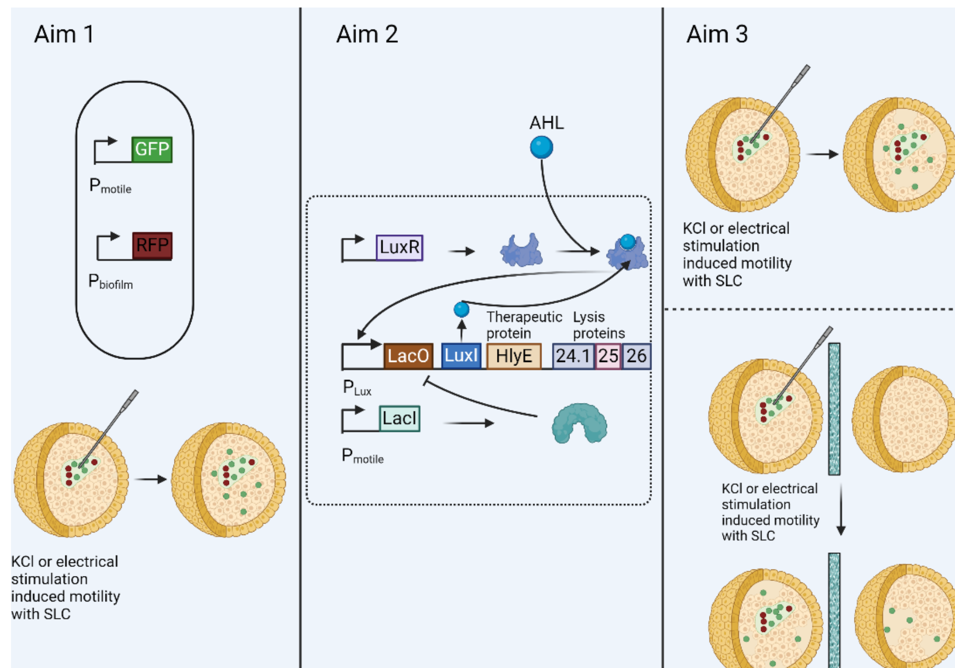


*Figure 1: Graphical abstract*

**Aim 1: Characterize B. subtilis motility in a CRC tumor spheroid to various stimulations.** To make dispersive intratumoral cancer therapy, motility and proliferation of motile *B. subtilis* must first be characterized. First, known promoters linked to the motile and matrix cell types will be paired with GFP and RFP respectively, and genomically integrated into a strain of B. subtilis. Then, colorectal cancer (CRC) tumor spheroids will be cultured with CT26 cells that are engineered to constitutively express luciferase and grown for 2 weeks. The engineered B. subtilis will then be inserted into the spheroids via microinjection. After overnight growth, the biofilms in the spheroids will be directly stimulated with either a -1 to -5V electric shock or 100-500 mM KCl solution, either once or 5 times 4 hours apart. Their cell type makeup and spread throughout the spheroid will be monitored daily for a week. Live fluorescence imaging will be used to gather dynamics data and measure

tumor cell death and radial distance travelled of motile cells. The treatment in which the cells exhibited the most motility will be used in AIM 3.

**Aim 2: Integrate the SLC system into *B. subtilis*.** While in the lab, bacteria are often kept planktonic, but bacteria in tumors are in a biofilm. *B. subtilis* is a model biofilm forming organism that is a clear first choice for developing as a biofilm-based cancer therapy. Here, we will be adapting the SLC to gram positive *B. subtilis*, however, many changes to the system must be made. The SLC was originally developed for gram negative bacteria using lysis proteins borrowed from an *E. coli* bacteriophage. We will take a similar approach by replacing those lysis genes in the SLC with lysis genes from a *B. subtilis* bacteriophage called SPP1. Genes 24. 1 and 26, components of the holin protein, which opens pores in the inner membrane, and gene 25, which is an endolysin that degrades peptidoglycan, will be used[4]. After the new lysis genes are put into the SLC, the B. subtilis genome must be modified to remove host circuit coupling. The SLC is induced by a class of quorum sensing molecules called AHLs. B. subtilis harbor a protein, AHL-lactonase, that degrades AHLs[5]. This protein will be knocked out of the genome using CRISPR Cas9 making cutes at both ends of the coding site and joined by non-homologous end joining. These changes will configure the SLC for use in *B. subtilis*, but to prevent motile cells from lysing before moving to a new part of the tumor a repressor must be added. A lacO operator will be placed in front of the lysis and therapy proteins and lacI will be put after the promoter activated by motility. Using this system, motile cells will suppress expression of the SLC but matrix producing cells won't. This genetic circuit will be constructed with standard plasmid cloning protocols and characterization will be performed in continuous culture using a microfluidics device. Continuous culture enables the observation of successive lysis events or lack thereof. Transient 300mM KCl shocks will be delivered to the bacteria in the microfluidics device and resulting lysis events will be documented. If there are still lysis events in motile cells, measured by GFP, directed mutagenesis will be performed on the promoter activated by motility and the motile mutant without lysis events will be selected for AIM 3.

**Aim 3: Characterize effectiveness of B. subtilis cancer therapy in single and paired spheroids.** After the SLC has been adapted to *B. subtilis*, the effectiveness of the SLC combined with the motility of the bacteria will be characterized. Methods will be replicated from AIM 1 for the culture of bacteria in the spheroid and application of the treatment that yielded the most motility. Dynamic time series of fluorescence will be recorded to measure tumor cell death and cell type specific proliferation and lysis events. Separately, one spheroid will be seeded with *B. subtilis*, grown overnight, washed with PBS, and put in the same well as another spheroid. The motility treatment from AIM 1 will be used on the infected spheroid and dynamic time series data will be collected to observe any infection of the originally uninfected spheroid. This will then be repeated with thicknesses 500-1500 uM of collagen hydrogel separating the spheroids, measuring how far the bacteria can travel to infect neighboring tumors.

**Intellectual Merit:** Bacterial based cancer therapy is a promising area of research but concerns regarding effectiveness remain. This proposal has the potential to increase the effectiveness of the therapy by introducing inducibly dispersive bacteria. This therapy even has the potential to effect neighboring tumors, so no more than one initial dose of bacteria is required. My experience working with both bacteria and mammalian cells, building genetic parts, and using microfluidic devices gives me a strong foundation to pursue this work. This work will be done in Jeff Hasty's lab at UCSD whose lab has developed and characterized many genetic circuits, built a repertoire of microfluidic technologies for multi-strain imaging of bacteria, and worked with bacterial-mammalian cell coculture in spheroids for many years.

**Broader Impacts**: Cancer is a devastating disease that is occurring at increasing rates in western society. Therefore, it is necessary to find treatments from any field that extends the healthspan of patients. The presented bacterial cancer therapy is a targeted platform that can be easily manipulated to deliver any cancer-specific drug able to be synthesized in a bacterium. I will also use my work as an opportunity to improve research accessibility by mentoring undergraduate students.

**References:** 1. Mills H et al. (2022) 2. Yamamoto A et al. (2023) 3. Comerci C et al. (2022) 4. Fernandes S. et al (2016) 5. Pan J. et al (2007)