Chapter 5

CONCLUDING REMARKS AND LESSONS LEARNED

5.1 Working with burdensome genetic circuits

After years working with the *pop cap*, *cap and release*, and *A=B* circuits, it is no longer a surprise to me that relatively few projects have been published in the space of explicit bacterial population control. It is extremely difficult—and at some level a matter of luck—to generate bacteria that contain functional versions of a population control circuit, and again difficult to prepare these bacteria for experiments in which they must demonstrate their population control circuit functions.

Genetic circuits that burden bacteria work against evolution by natural selection and are quickly lost [64], overtaken by mutants that reduce circuit burden. In a sense, population control circuits like ours are the *ultimate* burdensome circuit in that they are designed to burden cells *to death* when performing their control functions. Consequently, the threat of circuit mutation is around every corner and demands very precise, mutation-aware experimental procedures. Much of our experimentation with population control circuits resulted in "failed" experiments in which a cell line designed to perform some population control function would not demonstrate that function, presumably because it had acquired an inactivating mutation along the way to the experiment. Experiments like these do not generate quantitative data that help us make progress on lines of inquiry, but rather send us back to the drawing board, where we must grasp at possible reasons for their "failure" and hope to address these reasons with some tweak to the experiment preparation.

For experimenters working with highly burdensome genetic circuits, especially those incorporating lethal toxins, we suggest making tweaks to minimize two things: intensity of circuit burden and duration of growth under burden.

The stronger the fitness penalty imposed by a genetic circuit, the more quickly a population of bacteria expressing that circuit will be overtaken by unburdened mutants. In our case of toxin containing genetic circuits, direct addition of toxin-expressing DNA to cells is *guaranteed* to result in some amount of toxin expression in cells due to leaky expression from that DNA. Due to the potency of the most bacterial toxins, even this very weak expression represents a strong fitness penalty that predisposes that population of cells to takeover by mutant bacteria that have

inactivated the toxin. If possible, minimize the intensity of that fitness penalty by expressing an antitoxin in the cells *before* toxin-expressing DNA is ever introduced.

In preparing our cell lines for experimentation, we changed the conventional practice of two plasmid co-transformation into two plasmid transformation by *sequential* single plasmid transformation. In most cases, concurrent co-transformation of toxin and antitoxin containing plasmids would not generate functional cell lines; somehow all resultant colonies from this transformation were without the desired functions. Instead, first transforming antitoxin expressing plasmid, then transforming toxin expressing plasmid into that singly transformed cell line would yield the desired results. We hypothesize that the antitoxin-expressing DNA, arriving first, produces enough antitoxin to sequester any toxin basally expressed off the plasmid that arrives second, minimizing the burden of that toxin.

The second quantity to minimize is the duration of time a cell line is allowed to grow containing a burdensome genetic circuit. Even cell lines prepared carefully by transforming antotoxin and toxin-containing plasmids in the right order are subject to loss of function. The standard practice in preparing bacterial cells for experimentation is an overnight outgrowth to high density followed by dilution to low density for experimentation. We find that this overnight growth step gives our cell lines entirely too much time to acquire inactivating mutations; cell lines grown overnight *usually* do not demonstrate the desired circuit functions. We specify "*usually*" because the acquisition of mutations is not a deterministic process; some overnight cultures may acquire mutations early during outgrowth and lose function, some may not. We would expect the distribution of functional vs non-functional overnight cultures to follow that outlines in Luria and Delbrück's famous experiment [105].

Thus, in our preparations for experimentation, we minimize outgrowth duration. Instead of overnight outgrowth to densities around OD600 2-3, we only briefly outgrow cells from fresh transformations for a few hours to around OD600 0.3. The combination of correct transformation order and minimal outgrowth helped us reliably set up experiments that demonstrated *some* circuit behavior, though the reproducibility of each dataset remains to be seen.

Minimizing these two quantities, burden intensity and duration of growth under burden, precludes normal storage conventions for bacterial cell strains. Generating glycerol stocks of genetic circuit containing bacteria requires too much growth under circuit burden to ensure the stocked cell line will be functional. Instead, store

plasmids and freshly transform cell lines before each experiment.

5.2 Choosing appropriate measurements

Determining characteristics of bacterial cocultures can be done using a number of different measurement methods. In our work, we used optical measurements made by an incubator/plate reader, flow cytometry measurements and viable cell counting to measure our two coculture quantities of interes: total population size and coculture composition.

Optical measurements can be taken with the highest time resolution thanks to laboratory robots like incubator/plate readers that can culture cells and take optical measurements at the same time. This time resolution is incredibly valuable when working with systems that are expected to show characteristic dynamics. However, optical measurements sacrifice accuracy for time resolution. In our experiments, we found that optical density measurements severely misrepresent the total size of a population that is undergoing active cell death due to toxin expression. Additionally, determining the composition of a coculture of cells, even if they are distinctly labeled with fluorescent makers, is also inaccurate.

In theory, the most accurate way to measure the composition of a coculture is to perform flow cytometry. Flow cytometry is a technique designed to resolve different populations of label-expressing cells and as such, should be the perfect tool for determining coculture composition. It turns out this is generally true. We were able to make confident measurements of coculture composition using flow cytometry, but the trade off of accuracy vs time resolution is again true with this technique. Measuring cocultures with flow cytometry requires periodic sampling of the coculture and lengthly preparation for the cytometry process. This greatly limits the realistic time resolution possible with this technique. Additionally, flow cytometers are operating at the limit of their particle size resolution when measuring bacteria, meaning dust, debris and noise are often easily confused for a bacterium during cytometry. This makes estimates of total population size very innacurate.

The gold standard technique for determining total population size is CFU counting: estimating viable cell density by counting colonies of bacteria on agar plates. This technique again requires sampling and preparation, limiting time resolution, but this time the trade of time resolution for measurement accuracy may be worth it. Combining CFU counting with fluorescent imaging allows the measurement of labeled subpopulation densities and thus, the composition of a coculture. The high

accuracy of population size and composition measurement makes this technique desirable for experiments where accuracy matters.

Taken together, methods like high throughput screening, which we use extensively in our work generating population control circuits, may be better conducted using easy to acquire, but inaccurate measurements like optical density or fluorescence measurement. Later, during more detailed testing, CFU counting would be the preferred measurement type. In our particular work, we do not believe flow cytometry is a viable measurement type and suggest CFU counting as an alternative.