Design and Creation of a Novel Live-Cell Barcoding Method

Consequences of Use of Method on Cellular Research

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

Opening Statement:

Time of flight cytometry, or CyTOF, is the most common laboratory method used in analysis of single cell contents. This type of analysis is useful especially in the biomedical field as while CyTOF allows you to understand cellular compositions and molecular dynamics within the cell, it also allows research to detail the effects of drug/other medical treatment on certain cell types (Santra, 2020). Tumor treatments can be compared directly by number of surviving cancer cells or functioning cancer cells, and medications can be tested to see whether they inactivate the desired cell processes to treat a specific condition. The machines required for these analyses cost tens of thousands of dollars, and upwards of eighty thousand dollars a year for maintenance and consumables not including personnel and test kit costs **(**Leipold, 2015**)**. Because of this price, most lab buildings only have access to one facility of CyTOF machines, and so must share time on them with multiple other labs. This sharing means that any time allotted to a lab that week must be spent as frugally as possible to maximize productivity and keep projects running within their timelines. Currently, cell samples are run either individually as is or are barcoded to allow for up to 20 samples to be pooled together for one run (Gadalla, 2022). This barcoding is accomplished through tagging individual samples with a "barcode" of three out of six different antibodies (6 choose 3 = 20 samples) (Arnett, 2023). The current barcoding methods fall short of accurately measuring all the contents of a cell, either dropping certain cell types or chunks of cell count, as the conjugates used to tag cells are unstable or require killing the cells prior to them being fixed, leading to loss of surface targets. The 20-sample upper limit for analysis also impedes progress and allows for less sample-sample variation, as the more samples that are run at the same time means that all are influenced by less pooling/loading error that could skew results (Fernandez-Zapata, 2020).

Technical Discussion:

The proposed novel method to be created by this capstone group and the Sturek Lab within the UVA Pulmonary and Critical Care division of the Medicine School will allow for up to seventy total samples to be pooled together and will allow for live-cell fixation and greatly reduced target loss when running samples through the CyTOF machine. This project has been ongoing within the Sturek Lab for over a year, and while novel use of a conjugated protein has been identified as a strong candidate for use in barcoding, a panel of eight heavy metal isotopes must still be identified to test efficiency and longevity within a 70-sample protocol. The protein in question is wheat germ agglutinin (WGA), an inexpensive heterodimeric lectin with a high binding affinity to residues commonly found on glycoproteins, which exist attached to the outside of cell membranes. The ability of WGA to easily bind to the membranes of virtually all mammalian cells allows for cells to be labeled with a barcode before being fixed and permeabilized, processes that "freeze" but also kill cells prior to incubation with antibodies, but also significantly reduce cell density and induce damage to the integrity of membranes (Cheng, 2019). This allows WGA to tag cells and their extracellular proteins while they are still alive, preserving fixation-sensitive epitopes for later binding with antibodies. Once working isotopes have been identified and tested, a significant amount of CyTOF data analysis must be done to compare the new method with those of other labs and existing kits to demonstrate increased efficacy (Van Gassen, 2019). This data analysis will proceed as follows. Time on the UVA CyTOF core's machines will be donated by the Sturek Lab, with money for isotopes being given as a grant by UVA Biomedical Engineering in support of their student's capstone project. Wet lab work will be done in the Sturek Lab, and data analysis will be done with the license of the lab

remotely by team members. This analysis consists of 4 main task groupings, normalization, cleanup, debarcoding, and review. Normalization begins with retrieving the CyTOF .fcs files from the sample run and inputting them into a normalizer application. This process involves equalizing retrieved sample data to user added beads with known masses in order to flatten the mass curve that comes with extended machine use, once the resultant cell masses have been matched to correct masses, the beads are then removed from the data. In the cleanup portion of analysis, the normalized file is uploaded into a cloud CyTOF analysis software called OMIC, where the file goes through a series of steps to isolate the cell singlets, or individual cell artifacts, from doublets (larger artifacts created by multiple cells being considered by the machine as one) and junk volume. The user draws multiple gates over the singlet volume, with each gate refining the area selected further, and finally dead cells are removed from the file with a live vs. dead gate. Debarcoding involves a MATLAB application that takes the cleaned file and a debarcoding key, a spreadsheet with a list of all the samples and their respective barcodes, which allow the program to separate pooled cells by their barcode tags. Finally, the file can be uploaded back into OMIC for a final cleanup task to separate cells that were able to be debarcoded from those that were not, either due to barcode mixing or lost antibody tags. The debarcoded, live cell, un-debarcoded, and dead cell percentages in relation to initial total cells are all saved as metrics to compare the results of our method with the same metrics as taken from other barcoding methods.

STS Discussion:

While this method does not involve human or animal trials, it has far-reaching applications in novel drug and therapy research. The ability to accurately test far more samples

than is currently possible, with a greater accuracy and less cell type loss will help labs and pharma companies to better test the efficacy of their treatments. With a method that does not drop certain cell components, the effect a drug has upon a cell type can be more acutely determined, leading to factual assumptions on effects of treatments on different regions of the body. The time and monetary savings will also allow smaller labs that fight for use of their funding/continued funding to test more samples and forward their research over three times as fast. This helps equalize the playing field between university and biomedical giants in terms of research output and advancement capabilities, which is a large issue in the current research environment. Large pharmaceutical companies are of course, built towards the end goal of profiting as much as possible from their efforts, meaning that they will choose to R&D for conditions that affect many people or will be exorbitantly expensive to treat (Perkins, 2001). They do not see a long run value in developing cures for a vast number of conditions that affect small subsets of the population, as they will not return a profit or provide a compensatory amount of goodwill, it is the much smaller university and grant-funded research labs that investigate cures for these types of conditions. These small labs are in a constant battle to stay funded, with historic increases to annual NIH grant funding becoming lowered in recent years, the huge time commitment of writing grant proposals, as well as disparities in grants being given to new principal investigators, small labs face a whole host of issues when trying to find funding for their projects (Abkowitz, 2018). In my experience in a research lab here at UVA, grant-writing takes a considerable amount of time out of a researcher's time spent on furthering their project goals, with dozens of applications being sent out at times before one is approved. These grants are also exceedingly strict on what they can be spent on, leading to needing to manage a multitude of different accounts for purchase of both basic materials and more advanced

reagents for fear of having funding revoked if something outside the scope of that grant is bought with the money. The stress and strict nature of what these grants allow researchers to work on is not a problem in large pharma company labs, where funding is constant, and grants need not be applied for. This creates a great barrier to the research and development of cures and treatments for conditions that are not on the radar of large biomedical corporations in terms of profit value, and small labs have to struggle constantly, making slow progress on these projects that can save lives and improve the quality of life of potentially large subsets of the population.

Conclusion:

With the biomedical applications of cytometry by time of flight being so far-reaching and essential to reviewing effects of treatments and drugs on specified conditions, it only goes to say that any improvements to the process that can be made should be made. Our proposed method of using wheat germ agglutinin as a protein conjugate to tag live-cells both preserves cell populations and structure over current methods and allows for ubiquitous tagging of all mammalian cells. Through creating an eight metal barcode, up to 70 samples will be able to be pooled together, 3.5x the amount that current barcoding methods accurately allow for, along with the low price point of WGA, our method, if it is proven to have a low debarcoded cell rate after analysis will surely become the standard for live-cell barcoding within CyTOF. Through experiments testing the efficacy of different metals within a barcode panel, and analysis of our method vs. data from other barcoding methods, we hope to prove that our method provides better metric scores and will create the most value for laboratories. This method will also help to bridge the widening gap between the capabilities of small university labs and big pharma labs, which

will allow for more time and care to be taken on creating cures and treatments for lesser-known conditions that typically get passed over by companies purely motivated by profit.

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