Implementation and Optimization of Multimodal mRNA and Protein Single-Cell Analysis

Arjun Jain, Andrew J. Martins, Neha Bansal, Matthew Mule, John S. Tsang
Systems Genomics and Bioinformatics Unit, Laboratory of Immune System Biology
National Institutes of Allergy and Infectious Diseases, National Institutes of Health

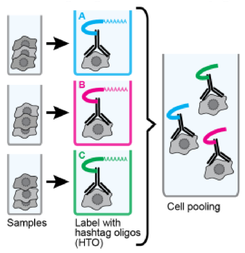
Introduction

- New high-throughput single cell mRNA-sequencing (scRNAseq) techniques have proven invaluable for
  - Understanding the behavior of individual immune cells
  - Describing heterogenous immune cell populations
- However, limitations of mRNA detection efficiency has made it difficult to relate these data to the large body of flow cytometry protein-based analysis that has been done for different immune cell types
- To achieve a comprehensive understanding of immune behavior, we need a technique for relating the cells identified by scRNAseq to those we already know about from flow
- This project describes
  - Implementation and optimization of CITEseq, a method that combines highly multiplexed protein marker detection with unbiased transcriptome profiling for thousands of single cells
  - Low-volume optimization of Plate-based scRNAseq for deeper analyses

CITE-seq Methodology

1. Cell Sequential Staining Protocol

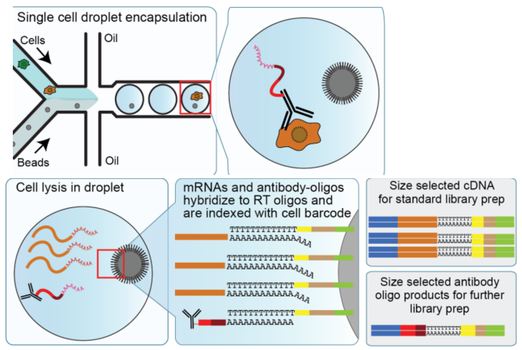
A. Stain cells with Hashtag-oligonucleotide-conjugated (HTO) antibodies and pool



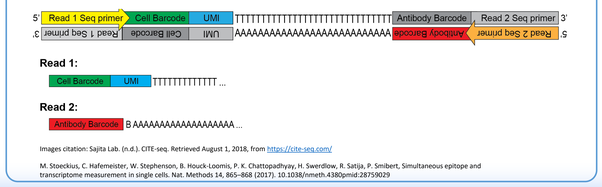
HTOs allow for sample multiplexing

B. Stain pooled cells with CITE-seq DNA-barcoded antibodies:

2. Drop-seq or 10x Genomics 3' assay to prepare libraries



3. Sequencing ADT, HTO, and cDNA libraries



Images citation: Sajita Lab. (n.d.). CITE-seq. Retrieved August 1, 2018, from https://cite-seq.com/

M. Stoeckius, C. Hafemeister, W. Stephenson, B. Houck-Loomis, P. K. Chattopadhyay, H. Swerdlow, R. Satija, P. Smibert, Simultaneous epitope and transcriptome measurement in single cells. Nat. Methods 14, 865–868 (2017). 10.1038/nmeth.4380pmid:28759029

Viability Experiment

Purpose

To determine the optimal condition for achieving high cell viability during CITE-seq cell staining protocol.

Hypothesis

Tween in the staining buffer (2% BSA, 0.02% Tween in PBS) and sodium azide in antibody buffer lead to decreased cell viability.

Procedure

1. Prepare Tween (2% BSA, 0.02% Tween in PBS) and NO Tween (2% BSA in PBS) in separate test tubes
2. Thaw out cells and add to WB(PBS + 2%FBS)
3. Wash 3X in FBS to remove DMSO from cells
4. Separate cells into four vials with two vials and resuspend two in No Tween buffer and two in Tween buffer
5. Conduct baseline viability count on 2µL of No Tween sample
6. Incubate cells for 30 min on ice
7. Conduct viability count #2 with no tween sample
8. Wash the four vials 3X in their respective buffers
9. Conduct viability count #3 to compare Tween vs No Tween
10. Add antibody cocktail (sodium azide) to only two of the test tubes
11. Incubate cells with antibodies for 30 min on ice
12. Wash 3 times to remove unbound antibodies (2X Tween buffer, 1X only PBS)
13. Conduct viability count #4 to compare both Tween vs No Tween and Sodium Azide vs NO Sodium Azide
14. Incubated cells on ice for 1 hour 15 mins (mimic time to 10X run) in PBS only
15. Conduct viability count #5 with Automated Hemocytometer

Results

- Baseline viability (post DMSO wash, No Tween): 83%
- Count #2 (Post incubation pre wash timepoint): 91%
- Count #3 (Tween vs No Tween):

	Sample 1	Sample 2	Sample3	Sample 4
Tween	+	+	-	-
Live	45	79	152	189
Dead	4	19	43	25
Cell Viability (%)	92%	80%	78%	88%
Cells/mL	900000	1580000	3040000	3780000

- Count #4 (No Tween vs Tween, No Sodium Azide vs Sodium Azide)

	Sample 1	Sample 2	Sample3	Sample 4
Tween	+	+	-	-
Antibody	-	+	-	+
Live	101	104	84	144
Dead	56	48	45	53
Cell Viability (%)	64%	68%	65%	73%
Cells/mL	2020000	2080000	1680000	2880000

- Count #5 (Simulate time to 10x/Drop-seq run)

	Sample 1	Sample 2	Sample3	Sample 4
Tween	+	+	-	-
Antibody	-	+	-	+
Live	91	43	24	33
Dead	27	13	7	23
Cell Viability (%)	77%	68%	65%	73%
Cells/mL	10000	860000	480000	660000

Conclusion

Tween and Sodium Azide have NO effect on the cell viability, although observed high variability between replicates. Future hypothesis to test if whether CD20 triggers cell apoptosis.

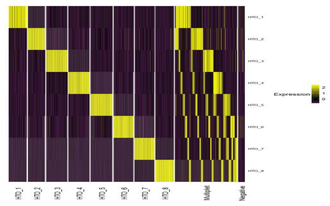
Seurat Analysis of CITE-seq Antibody Dilution Data

Purpose: Determine the lowest amount of each antibody which can effectively separate cell populations. The results of this experiment can make future CITE-seq experiments more cost-effective.

Methodology: Seurat is an R package designed for QC, analysis, and exploration of multi-modal data. CITE-seq staining was run with 8 million healthy PBMCs (peripheral blood mononuclear cells), with 4 pairs of hashtag-oligonucleotides (HTOs) to label 4 different antibody amounts.

HTO	Antibody Amount (µg)
HTO 1, HTO 2	1µg
HTO 3, HTO 4	0.5µg
HTO 5, HTO 6	0.25µg
HTO 7, HTO 8	0.125µg

Demultiplexing the samples based on HTOs (remove doublets, negatives):

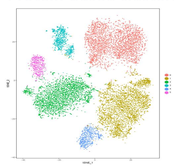


Number of cells detected:

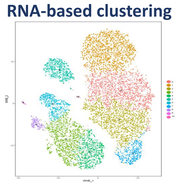
Singlets	Doublets	Negatives
13185	5044	469

Clustering the cells based on mRNA and Protein levels:

Protein-based clustering

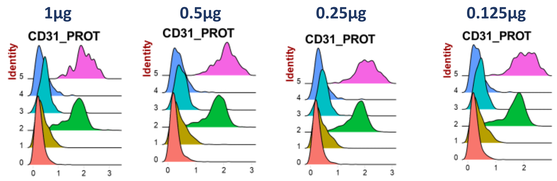


RNA-based clustering



Separating data into dilutions:

Example: RidgePlots of CD31 for each antibody amount

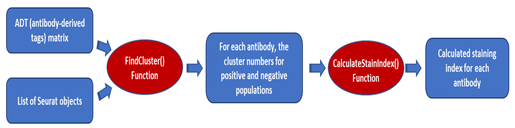


Calculating Staining Index:

Measurement of separation between positive and negative populations

$$\frac{[MF1 - MF2]}{2xSD}$$

Development of reproducible function for calculation of staining index from CITE-seq experiment:



Staining Index Plot:

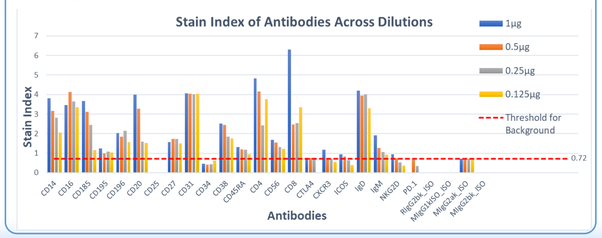


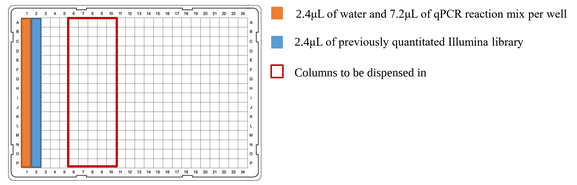
Plate-based scRNAseq Optimization with Nanoliter Dispensing

Overview

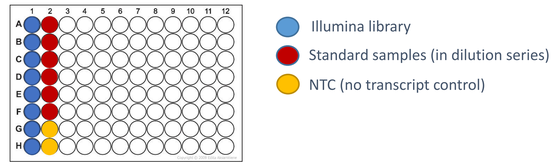
- 10x/Drop-seq is advantageous for analyzing the transcriptome of many cells, but as a tradeoff it has lower quality data per cell
- In general, plate-based scRNAseq methods produce higher quality data per cell by detecting more transcripts per cell and capturing the entire transcript
- Therefore, we plan on implementing plate-based scRNAseq to look closer into the specific cell subsets identified by CITE-seq
- High Quality ⟶ High Cost
- **To make plate-based scRNAseq more cost-effective and efficient in capturing transcripts, we propose optimizing it with nanoliter dispensing to scale down the reaction sizes**
- **Experimentally verified that the TTP Labtech Mosquito Nanoliter Dispenser dispenses equal volumes in the wells of a 384-well plate**

Procedure

1. Prepare 384-well plate

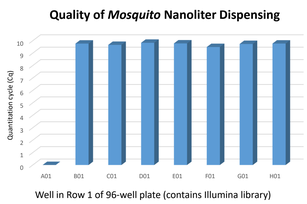


2. Using Mosquito nanoliter dispenser, aliquot from column 1 into columns 7-11 and from column 2 into columns 7-11
   - Each well in columns 7-11 should contain 0.72µL of qPCR reaction mix, 0.24µL of water, and 0.24µL of quantitated Illumina library



3. Run 96-well plate on qPCR and obtain Cq values for each well
   - Cq value: cycle number at which fluorescent signal crosses the threshold/marks timepoint at which target sequence is being exponentially amplified

Results



- Cq values consistent across the wells containing the previously quantitated Illumina library
- However, the first well has a Ct value of 0 and thus did not contain any liquid.
- **Overall, these results indicate that the Mosquito TTP Labtech nanoliter dispenser successfully dispenses equally into each well**
- **Additional investigation needs to be done for dispensing that in first well**

Conclusion

This project has demonstrated the optimization and implementation of a technique for high-throughput, multimodal, single-cell analysis. This technique can be utilized to explore the behavior of individual immune cells in responding populations (i.e. vaccine) and consequently how that shapes immune response.

Acknowledgements

This study was funded by the Intramural Research Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health.