Fixation of Cells for Chromium Fixed RNA Profiling

Introduction

Chromium Fixed RNA Profiling offers comprehensive scalable solutions to measure gene expression in single cell that are fixed with formaldehyde. This protocol outlines how to perform fixation on single cell for use with Chromium Fixed RNA Profiling workflow.

Sample Quality

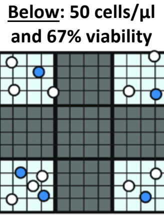
- Use high quality single cell that can withstand the fixation steps.
- This fixation protocol requires ≥300,000 cells.
- Highly viable single cell (>80%) will have the greatest sensitivity and cell recovery.
- Samples should have minimal debris for best results; debris can have associated RNA that can contribute to non-cell background.
- If possible, please sort for live cells via flow cytometry or perform bead clean-up to remove dead cells prior to beginning the cell fixation protocol.

Buffer Preparation – Prepare fresh

Day 1 - Fixation Buffer (Maintain at Room Temp.)	Stock	Final	Per Sample (µl)
Nuclease-free Water	-	-	435
Concentrated Fixation Buffer	10X	1X	55
Formaldehyde	37%	4%	60
Day 2 - Quenching Buffer (Maintain at 4°C)	Stock	Final	Per Sample (µl)
Nuclease-free Water	-	-	938
Concentrated Quench Buffer	8X	1X	134

Cell Fixation Protocol

<u>Day 1</u>



a. Mix 5 µl of your **cells** and 5 µl of **trypan blue**. Load 6 µl of the mixture onto a **hemocytometer** and look under a **light microscope** to assess viability and cell count. Only proceed with the rest of this protocol if cells exhibit **>80% viability** and **≥300,000 cell count**. Please **take a picture** of the cells through the microscope's eyepiece and, when the images for all 16 samples are collected, **email the images** to Zhenghui.Liu@CUAnschutz.edu.
   - *COUNT transparent cells (live) and IGNORE blue cells (dead).* ***Cells/µl = [cells in all 4 large squares] x 5***

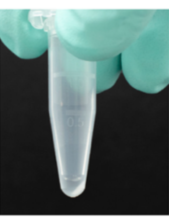
b. Centrifuge sample at **300-400 rcf** for **5 min** (PBMCs/cell lines) or **150 rcf** for **10 min** (dissociated tumor cells) at **4°C**.

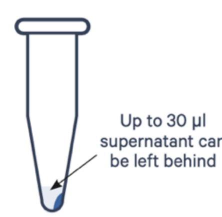
c. Remove the supernatant without disturbing the pellet. Up to 30 µl supernatant may be left behind to optimize cell recovery without significantly impacting assay performance.

d. Add **500 µl** Fixation Buffer (●) to the sample pellet and pipette mix 5x.

e. Incubate for ***exactly*** **20 h** at **4°C.**

f. *DO NOT agitate or mix the sample during incubation.*





<u>Day 2</u>

a. Add **500 µl** Additive C (●) to the sample in the Fixation Buffer and pipette mix 5x.

b. Centrifuge at **850 rcf** for **5 min** at **room temperature**.

c. Remove the supernatant without disturbing the pellet.

d. Add **1 ml chilled** Quenching Buffer (●) to the sample pellet and pipette mix 5x and keep on ice.

e. Thaw Enhancer (●) for **10 min** at **65°C**. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use.

f. *DO NOT keep the thawed reagent on ice, or the solution will precipitate. Once thawed, Enhancer (●) can be kept at 42°C for up to 10 min.*

g. Add 100 µl pre-warmed Enhancer (●) to 1,000 µl fixed sample in Quenching Buffer (●). Pipette mix.

h. Add 275 µl 50% glycerol (●) to 1,100 µl fixed sample in Quenching Buffer (●) and Enhancer (●). Pipette mix.

i. Store fixed sample in **-80°C** for up to 12 months.

j. Email Nicole.Manning@CUAnschutz.edu to schedule your sample drop off.

k. Drop off your **samples** and **'Sample Information Form'** at the **Genomics Core** located at **RC2-P15-9400A**.

Sample Information Form

Instructions: Please accompany this form when you drop-off your samples at the Genomics Core located at RC2-P15-9400A.

Principal Investigator: ______________________

Sample Drop-off Date: ______________________

	Sample Name		Group
1.	________________________	/	______________
2.	________________________	/	______________
3.	________________________	/	______________
4.	________________________	/	______________
5.	________________________	/	______________
6.	________________________	/	______________
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8.	________________________	/	______________
9.	________________________	/	______________
10.	________________________	/	______________
11.	________________________	/	______________
12.	________________________	/	______________
13.	________________________	/	______________
14.	________________________	/	______________
15.	________________________	/	______________
16.	________________________	/	______________

Materials and Methods

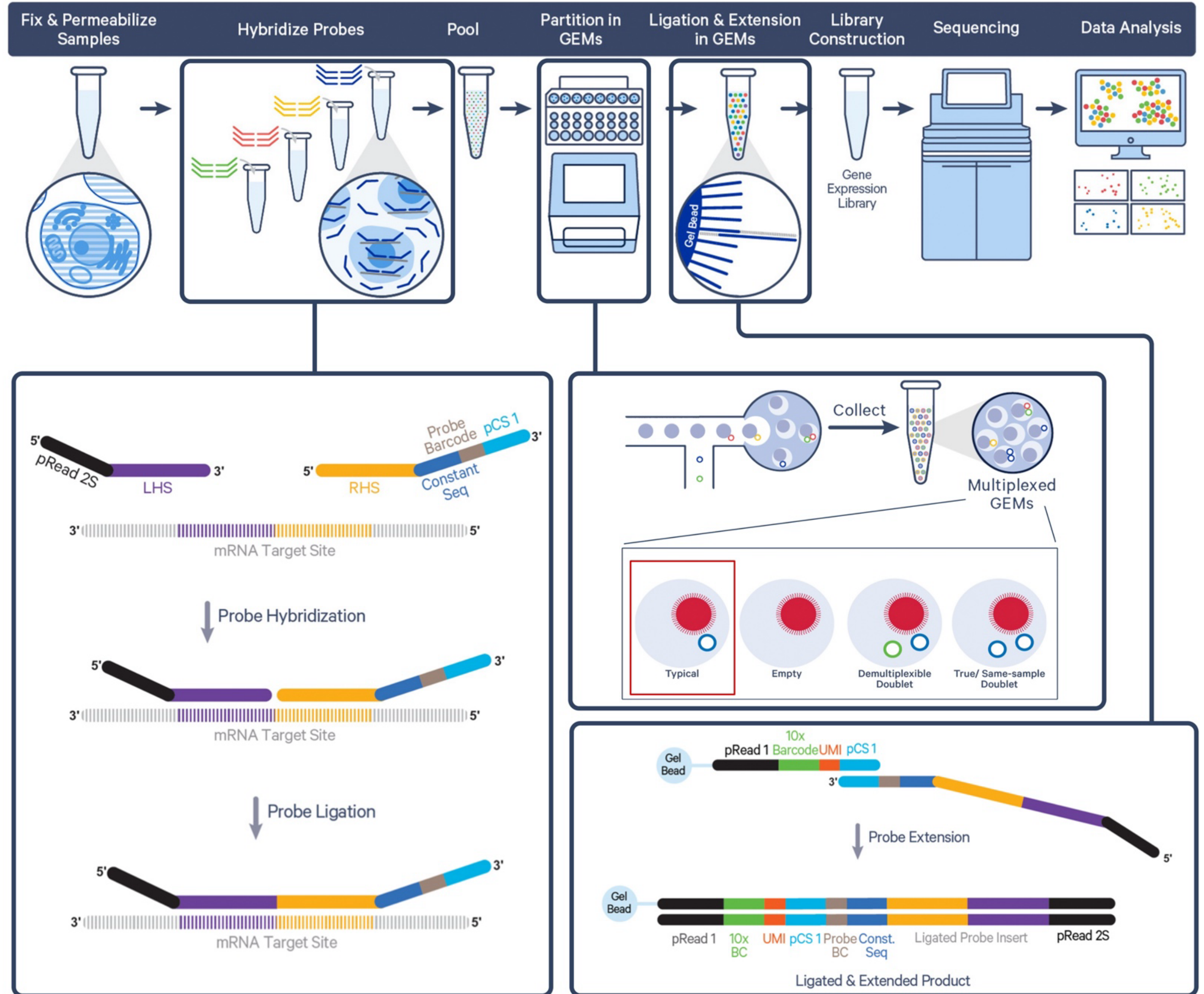
Fixed Single Cell RNA-seq

Cells exhibiting >80% viability are fixed in a 4% formaldehyde fixative solution and using Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit (10x Genomics). The whole transcriptome probe pairs (10X Genomics) are added to the fixed single cell suspensions to hybridize to their complementary target RNA in an overnight incubation. After hybridization, the unbound probes are washed off. The fixed and probe-hybridized single cell suspensions are loaded onto Chromium X (10X Genomics) microfluidics instrument to generate partitioned nanoliter-scale droplets in oil emulsion. Each droplet contains a barcoded gel bead, a single cell, and enzyme Master Mix (10X Genomics) for probe pair ligation and gel bead primer barcode extension. The droplets in oil emulsion are place in a thermal cycler for 60 min at 25°C, 45 min at 60°C, and 20 min at 80°C. The single cell-barcoded, ligated probe product undergo library preparation to be compatible with Illumina next-generation sequencing.

Sequencing

The gene expression library derived from single cell-barcoded, ligated probe product were sequenced as paired-end 150 bp reads on the Illumina NovaSeq X (Illumina) at the University of Colorado - Genomics Shared Resource (Aurora, CO, USA) at a depth of 20,000 reads per cell.

What is Fixed Single Cell RNA-seq?



10X Genomics' ***Fixed Single Cell RNA-seq*** offers comprehensive scalable solutions to measure gene expression in single cell that are **fixed with formaldehyde**. Over 18,000 coding genes are detected using >55,000 **probe-pair sets** (available for human or mouse only) that hybridize to mRNA target sites with maximum specificity, sensitivity, and sequencing efficiency. Each set of probes contains a unique *sample-specific barcode* that can be assigned back to its sample of origin. Owing to these *sample-specific barcodes*, we pool 16 samples (**targeting 5,000 – 8,000 cells/sample**) to **reduce cost per sample** by splitting the reagent costs across 16 samples. These 16 samples can be provided by one investigator or multiple investigators.