Cell Fixation Protocol for GEM-X Single Cell 3' & 5' Assays

Introduction

GEM-X Single Cell 3' and 5' assays with the advanced GEM-X microfluidics offer unmatched sensitivity and cell recovery efficiency for transcriptomic profiling. This document provides a fixation protocol for both freshly isolated and cryopreserved peripheral blood mononuclear cells (PBMCs) that can be used as input for compatible GEM-X 3' and 5' workflows. Fixation offers opportunities to streamline workflow processes and enhance workflow consistency.

Storage recommendations for the fixed cells and post-storage processing conditions are also provided. An overview of data derived from fixed human PBMCs processed using the GEM-X Single Cell 5' v3 workflow is shown in the Data Highlights section. See the Reference section for compatible user guides.

Additional Guidance

Isolate PBMCs as described in Demonstrated Protocol for Isolation of Leukocytes, Bone Marrow, and Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing (CG000392 Rev B).

Consult the Cell Preparation Guide (CG000053) for Tips & Best Practices during sample preparation and guidance of on determining accurate cell counts.

Tissue and cells carry potentially hazardous pathogens. Follow material supplier recommendations and local laboratory procedures and regulations for the safe handling, storage, and disposal of biological materials.

Specific Reagents & Consumables

Vendor	Item	Part Number
For Fixation		
Thermo Fisher Scientific	DSP *Premium grade*	PG82081
	Sucrose	S5-500
	10X DPBS	14-200-075
	1M MgCl2	AM9530G
	Nuclease-free Water	AM9906
Millipore Sigma	Methanol *≥99.9%*	34860-1L-R
Thermo Fisher Scientific	Methyl sulfoxide *99.7+%, Extra Dry*	127790025
	Tris-HCL pH 7.5	BP1757-100
For Rehydration		
Thermo Fisher Scientific	Sodium Citrate pH 6	J61815-AK
	Nuclease-free Water	AM9906
Millipore Sigma	Albumin, Bovine Serum, *10% Aqueous Solution, Nuclease-Free*	126615
	Sodium Chloride Solution	71386
	RNAse inhibitor	3335402001
For Cell Counting		
Nexcelom Biosciences	ViaStain PI Staining Solution	CS1-0109-5mL
	ViaStain AOPI Staining Solution	CS2-0106-5mL
-	Automated Cell Counter *See recommendations in Appendix*	-
Biotium	NucSpot 470	40083

Additional Materials		
Eppendorf	DNA LoBind Tubes 2.0 ml	022431048
VWR	Vortex Mixer *(or equivalent)*	10153-838
Thermo Fisher Scientific	Syringes	14-955-459
	Needles (18-21 gauge) *(or equivalent for aspirating Methyl Sulfoxide from bottle)*	14-817-209
-	Refrigerated centrifuge	-
-	Pipette tips *(including wide-bore tips)*	-
-	Disposable transfer pipette *(optional)*	-

This list may not include some standard reagents and laboratory equipment.

Buffer Preparation

Stock Solutions

DSP Stock Solution
(scale volume up or down based on numbers provided below)

- Add 104.25 mg DSP to ~2 ml DMSO and pipette mix. Add DMSO for a final volume of 2.5 ml
- Aliquot 65 µl/tube & store -80°C (each aliquot sufficient for 4 samples).
- The prepared stock solution can be stored at -80°C for up to 6 months.
- Thaw at room temperature before use. Once thawed, DO NOT refreeze.

Reagents	Stock	Final	Volume* (1 rxn)
Dehydration Buffer *Maintain on ice* *Prepared buffer can be stored at 4°C for up to 1 week*			
Sucrose	1,000 mM	300 mM	300 µl
10X PBS	10X	1X	100 µl
MgCl2	1,000 mM	3 mM	3 µl
Water (RNase free)	-	-	597 µl
Total			**1,000 µl**
Rehydration Buffer/Wash Buffer *Maintain on ice***			
Sodium Citrate PH6	500 mM	45 mM	135 µl
Sodium Chloride	5,000 mM	450 mM	135 µl
BSA	10 %	1 %	150 µl
RNAse inhibitor	40 U/µl	0.20 U/µl	3 µl
Water	-	-	1,077 µl
Total			**1,500 µl**
Resuspension Buffer *Maintain on ice***			
10x PBS	10X	2X	100 µl
BSA	10 %	1 %	50 µl
RNAse inhibitor	40 U/µl	1 U/µl	12.5 µl
Water (RNase free)	-	-	337.5 µl
Total			**500 µl**

**When calculating volumes for >1 rxn, add 10% overage.*
***Buffer prepared without RNAse inhibitor can be stored at 4°C for 1 week. Add RNAse Inhibitor when ready to use.*

Tips & Best Practices

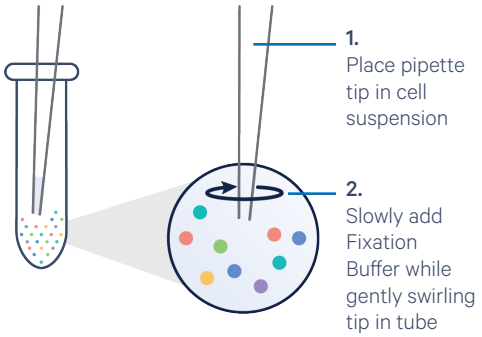
The recommendations are critical for optimal performance.

- Use wide-bore pipette tips when indicated. A wide-bore pipette tip should be used when mixing cells in suspension.
- A regular-bore pipette tip should be used when resuspending a cell pellet.

Fixation

- Prepare fresh Fixation Buffer and use within 5 min for fixing cells.

Adding Fixation Buffer to Cells



- When fixing cells, dispense Fixation Buffer by placing the pipette tip in the cell suspension and dispensing directly into the cell suspension while gently swirling the tip in the tube to mix.
- Excess residual BSA can increase precipitate formation during fixation, which can impact cell counting. After fixation, remove as much supernatant as possible without disturbing the pellet. If precipitate is observed after rehydration, filter the sample.

  Recommended filters
  Sysmex: Sysmex Sterile Single-Pack CellTrics Filters (30 µm), 04-004-2326
  OR
  Miltenyi Biotec: MACS SmartStrainers (30 µm) 130-098-458/ Pre-Separation Filters (30 µm) 130-041-407

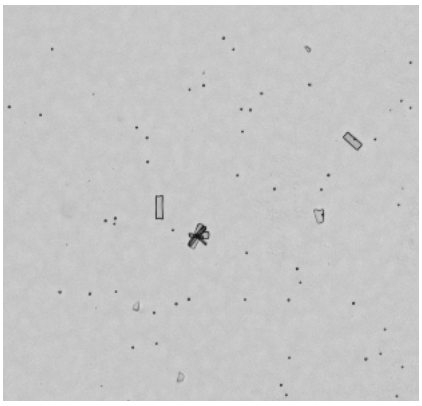
Centrifugation

- Use a swinging-bucket rotor for higher cell recovery.
- If pellet is not clearly visible while removing the supernatant, initially draw supernatant from the center of the liquid volume without touching the pipette tip to the tube wall.
- When ~100 µl volume is left, tilt the tube at an angle and touch the tip to the side of the tube where no visible particulate material is present and remove the remaining supernatant.

Cell Counting

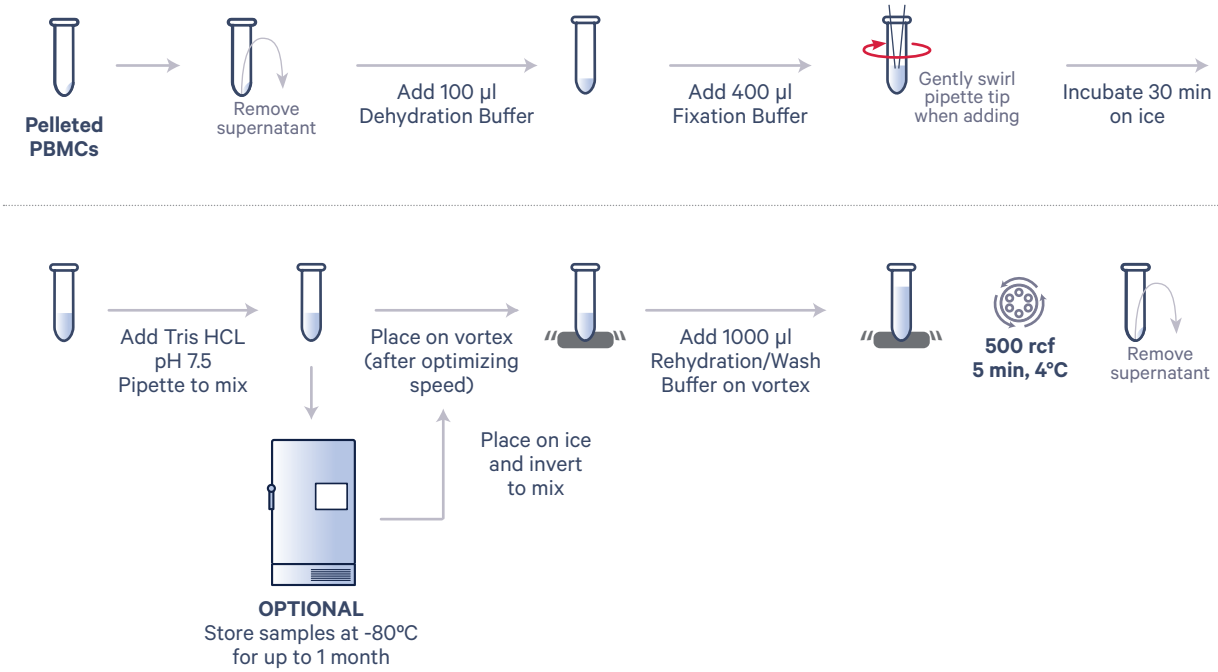
- Samples should have minimal debris for best results; debris can have associated RNA that can contribute to non-cell background.
- Accurate sample counting is critical for optimal assay performance.
- Sample should be stained with a fluorescent nucleic acid dye and counted using an automated cell counter. See Appendix for details.
- When counting fixed cells, small crystals may be visible but that will not impact the downstream assay performance (see image below).
- DO NOT use trypan blue for counting (not supported).

Example of Small Crystals



Protocol Overview

Fixation Protocol



Cell Fixation & Rehydration Protocol

This protocol used both freshly isolated and cryopreserved PBMCs, isolated as described in CG000392 (Rev B & subsequent revisions).

- Prepare the buffers specified in the Buffer Preparation section.
- Pre-chill Methanol on ice or at 4°C prior to Fixation Buffer preparation (step c).

Prepare Cells

TIPS *Use wide-bore pipette tips where indicated.*

a. Prepare PBMC single cell suspension (as described in CG000392 Rev B) and determine cell concentration. Transfer 100,000 - 1x10^6 cells to a 2-ml Eppendorf tube.

b. Centrifuge cell suspension at **300-400 rcf** for **5 min** at **4°C.**

c. Using a pipette tip (or disposable transfer pipette), remove the supernatant without disturbing the pellet. Maintain pellet on ice.

d. Prepare Fixation Buffer and maintain on ice.

Fixation Buffer should be prepared right before cell fixation and should be used within 5 min. Processing more than 8 samples at one time is not recommended.

Fixation Buffer- Prepare fresh *Prepare fresh. Use within 5 min.*				
Reagents	**Stock**	**Final***	**1 Sample (µl)**	**8 Samples + 10% (µl)**
DSP Stock Solution	100 mM	3.125 mM	12.5	110
Methanol *Pre-chilled*	100%	-	387.5	3,410
Total			**400**	**3,520**

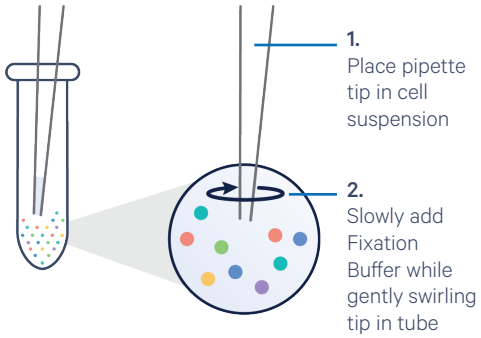
e. Resuspend the cell pellet in **100 µl** Dehydration Buffer (1x10^5 - 1x10^6 cells/100 µl recommended) and **immediately** proceed to Fixation.

If processing multiple samples, process one cell pellet at a time. Resuspend the cell pellet from the first sample and immediately add Fixation Buffer. Then process the pellet from the second sample and add Fixation Buffer and so on. Leaving cells in Dehydration Buffer for longer can result in precipitation.

Fixation

f. Using a pipette, slowly dispense **400 µl** Fixation Buffer (over ~10-15 sec) by placing the pipette tip in the cell suspension and dispensing directly into the cell suspension while gently swirling the pipette tip in the tube to mix.

Adding Fixation Buffer to Cells



TIPS *Ensure that the buffer is dispensed directly on to the cells and not to the tube walls.*

g. Using a wide-bore pipette tip (pipette set at 400 µl), gently pipette mix until the suspension looks uniform. Once the buffer is fully mixed, the solution will appear clear.

h. Incubate for **30 min** on **ice**.

OPTIONAL: After ~15 min, invert/flick the tube to mix, then continue with the incubation.

i. Add **10 µl** 1M Tris-HCl (pH 7.5) per 500 µl reaction volume. Pipette mix.

j. Proceed either directly to Rehydration or store cells at **−80°C.**

STOP *Samples can be stored at −80°C for up to 1 month.*

If storing cells at **−80°C**, when ready to use, retrieve cells and place on ice. Invert/flick the tube a few times to mix and proceed directly to Rehydration.

Rehydration

k. Set a vortex mixer to an optimal low speed setting based on the guidance below.

i. Add **500 µl** Methanol (80%) to a 2-ml Eppendorf tube. This will be used **only for optimizing vortex speed**.

ii. Turn the vortex mixer "ON" and set the speed to 1 (lowest setting).

iii. Firmly grasp the top of the 2-ml tube (cap open) with Methanol and set on the vortex mixer. Slowly increase the speed of the vortex until the liquid reaches the 0.75 - 1.0 ml line of the tube (typically speed level 2-3 setting or ~700 rpm for most vortex mixers). Use this vortex setting for the next step.

l. With one hand, firmly hold the top of the tube with the fixed cells from step j (cap open) on top of the vortex mixer that has been turned on at the setting described above. With the other hand, using a pipette, slowly (over ~10-15 sec) add **1 ml** Rehydration/Wash Buffer directly to the fixed cells while vortexing the tube dropwise.

TIPS *Ensure that the buffer is dispensed directly on to the cells and not to the tube walls.*

m. Centrifuge at **500 rcf** for **5 min** at **4°C**.

TIPS *Use of swinging-bucket rotor is recommended for higher cell recovery.*

n. Using a pipette (or disposable transfer pipette), remove the supernatant without disturbing the pellet.

o. Add **500 µl** Rehydration/Wash buffer and gently pipette mix to resuspend the pellet.

TIPS *The pellet may not be fully resuspended at this stage. A wide-bore tip may be used to gently mix the suspension. If precipitate is observed after rehydration, filter sample.*

p. Centrifuge at **500 rcf** for **5 min** at **4°C**.

q. Using a pipette (or disposable transfer pipette), remove the supernatant without disturbing the pellet.

r. Based on starting cell concentration and assuming ~40-50% cell loss, add an appropriate volume (**~100*-500 µl)** of chilled Resuspension Buffer. Pipette mix to resuspend the pellet, aiming for a final cell concentration of 700-1,200 cells/µl.

TIPS *See Appendix for cell counting guidance.*

s. Proceed **immediately** to compatible GEM-X Single Cell 3' or 5' protocol (see References).

**If less than 100 µl is used for resuspension, use 12.5 µl instead of 25 µl for counting.*

Data Highlights

The representative Data Highlights demonstrate that fixed human PBMCs (with or without storage at −80°C prior to processing), retain the single cell transcriptome information when processed using the GEM-X Single Cell 5' v3 workflow (Figs. 1-2). It is important to note that while there are differences in data quality for fixed samples compared to fresh samples, both gene expression and V(D)J information is maintained in fixed samples.

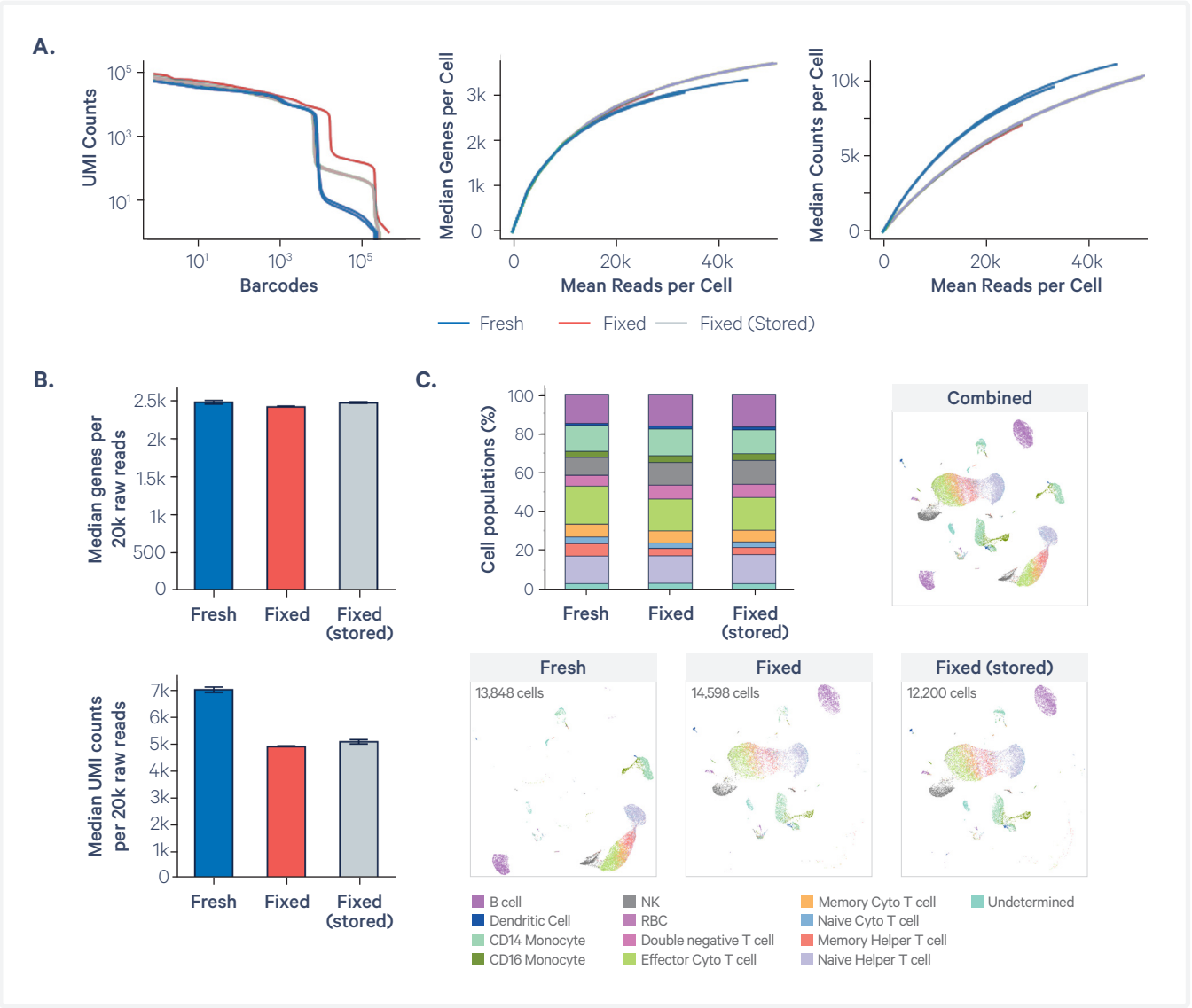


Figure 1. Analysis of fixed human PBMCs using the GEM-X Single Cell 5' v3 workflow demonstrates that the transcriptome is preserved post fixation. **(A)** Barcode rank plot & assay complexity or sensitivity plots of PBMCs isolated from whole blood which were either directly processed (fresh) or fixed and processed or fixed and stored for 1 month and then processed. 10,000 cells were processed directly while 5,000 fixed cells were stored and then processed. **(B)** Quantification of assay complexity and sensitivity at 20k raw reads per cell (RRPC). **(C)** Cell population frequency and representative UMAP plots (no batch correction) across the three conditions.

Data Highlights

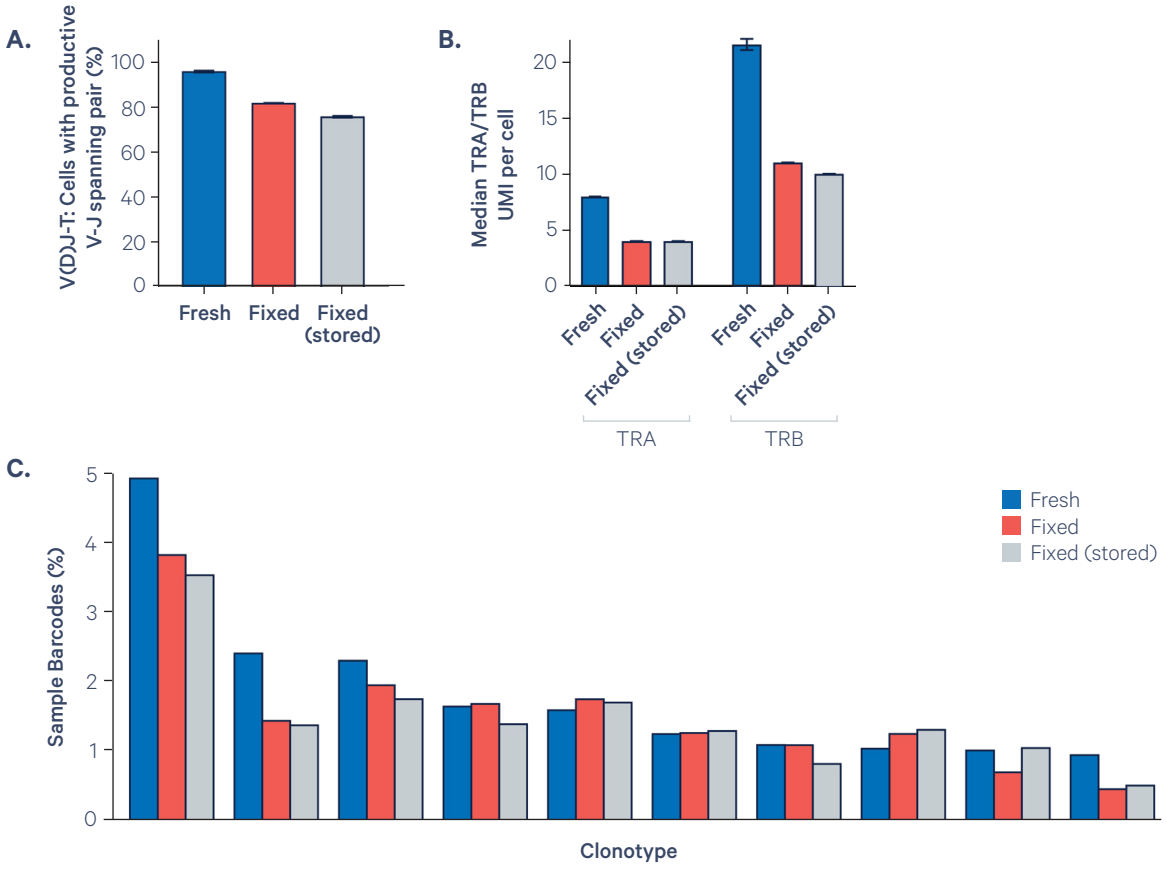


Figure 2. Analysis of fixed human PBMCs using the GEM-X Single Cell 5' v3 workflow demonstrates that V(D)J transcripts are preserved post fixation. **(A-B)** V(D)J paired sequences and T-cell receptor distribution observed in PBMCs isolated from whole blood which were either directly processed (fresh) or fixed and processed or fixed and stored for 1 month and then processed. **(C)** Sample barcode frequency for each clonotype across the three conditions.

Appendix

Post-Storage Processing of Fixed Cells

- When ready to use samples stored at **−80°C**, retrieve the cells from **−80°C** and place on ice.
- Invert/flick the tube a few times to mix and proceed to Rehydration.

Cell Counting

- Accurate sample counting is critical for optimal assay performance.
- The cells should be stained with an appropriate dye and counted using an automated cell counter. See below for the dye recommendation for a specific counter.

Counter	Dye Recommended
Cellaca MX	AO/PI staining solution
Countess 3 FL	PI staining solution
Cellometer K2	**Nucspot 470

*** Dilute the stock to 1:100 and mix 1:1 with the sample. For example, add 10 µl diluted dye to 10 µl sample.*

The following section provides counting guidance using AO/PI staining solution and the Cellaca counter. For counting guidance using other dyes/ counters, refer to manufacturer's instructions.

- **Counting using AO/PI Staining Solution:** This protocol provides instructions for counting samples using AO/PI staining solution and the Cellaca counter to enable accurate quantification even in the presence of subcellular debris. The optimal cell concentration for the Cellaca counter is 100-10,000 cells/µl. Refer to manufacturer's instructions for details on operations.
  - Add **25 µl** AO/PI staining solution into Mixing Row of Cellaca plate
  - Gently mix the sample. If the sample is too concentrated, a 1:1 dilution in PBS can also be prepared. For example, add 15 µl fixed cell suspension to 15 µl PBS.

    If starting cell input is 100,000 cells, dilute sample prior to counting (For example, dilute the single cell suspension with 20 µl Resuspension Buffer, mix with 25 µl AO/PI)

  - Add **25 µl** sample to Mixing Row of plate containing AO/PI staining solution. Gently pipette mix 8x.
  - Transfer stained sample to Loading Row of Cellaca plate.
  - For counting fixed samples, **only** use the PI (Propidium Iodide) channel.

    Refer to manufacturer's instructions for details.

References

1. Chromium GEM-X Single Cell 3' v4 Gene Expression (CG000731)
2. Chromium GEM-X Single Cell 3' v4 Gene Expression with Feature Barcoding technology for Cell Surface Protein User Guide (CG000732)
3. Chromium GEM-X Single Cell 5' v3 Gene Expression User Guide (CG000733)
4. Chromium GEM-X Single Cell 5' v3 Gene Expression with Feature Barcoding technology for Cell Surface Protein User Guide (CG000734)
5. Chromium GEM-X Single Cell 5' v3 Gene Expression with Feature Barcoding technology for CRISPR Screening User Guide (CG000735)

Consult the 10x Genomics Support Website for the most current information regarding compatible user guides and associated datasets.

Document Revision Summary

Document Number	CG000776
Title	Cell Fixation Protocol for GEM-X Single Cell 3' & 5' Assays
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Description of Changes	N/A

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