DEMONSTRATED PROTOCOL

Methanol Fixation of Cells for Single Cell RNA Sequencing

Overview

This protocol outlines methanol fixation and rehydration of single cell suspensions for use with 10x Genomics Single Cell protocols. The protocol was demonstrated with Jurkat T lymphocytes, embryonic brain cells, and human peripheral blood mononuclear cells (PMBCs). Additional optimization may be required when working with other cell types (e.g. media type, resuspension buffer, centrifugation speed, and time).

Preparation of single cell suspensions directly from solid tissues or cryopreserved samples may also require additional optimization during dissociation and/or cell handling, which is not covered here.

Additional Guidance

Consult Demonstrated Protocol Cell Preparation Guide (Document CG00053) for Tips & Best Practices on handling cells. Input single cell suspensions for this protocol were prepared, washed, and counted as described in:

- Demonstrated Protocol – Single Cell Suspensions from Cultured Cell Lines for Single Cell RNA Sequencing (Document CG00054)
- Demonstrated Protocol – Fresh Frozen Human Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing (Document CG00039)
- Demonstrated Protocol – Dissociation of Mouse Embryonic Neural Tissue for Single Cell RNA Sequencing (Document CG00055)

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.

Cell Sourcing

Cell Type Used/Species	Supplier	Part Number
Peripheral Blood T Lymphocytes Jurkat, Clone E6-1/Human	ATCC	ATCC TIB-152
Combined Cortex, Hippocampus, and Ventricular Zone/E18 Mouse	BrainBits	C57EHCV
Peripheral Blood Mononuclear Cells (PBMC)/Human	AllCells	PB001

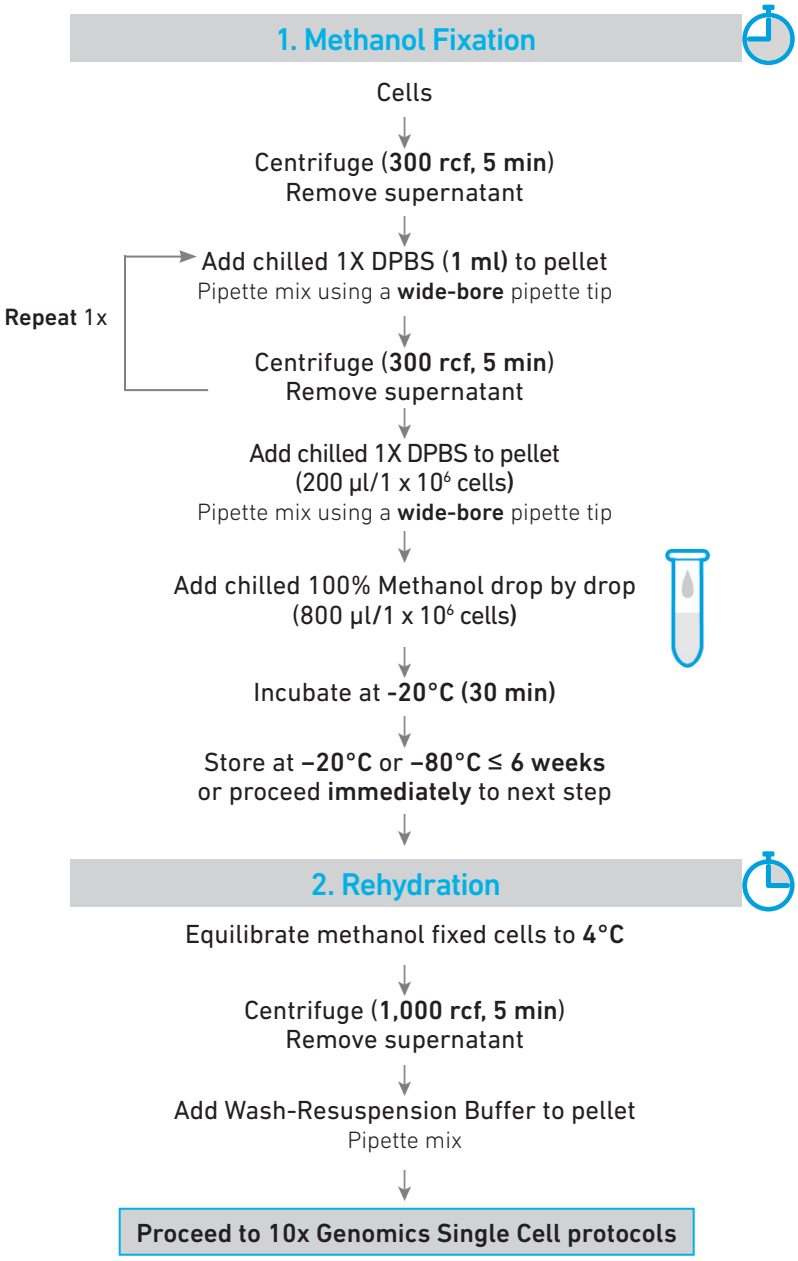
Preparation – Buffers

Buffers Maintain at 4°C	Composition
3X SSC Buffer	3X SSC in Nuclease-free Water
Wash-Resuspension Buffer	0.04% BSA + 1mM DTT + 0.2 U/µl RNase Inhibitor in 3X SSC Buffer
Additional Buffers/Reagents	
1X DPBS (maintain at 4°C)	
100% Methanol (maintain at –20°C)	

Specific Reagents & Consumables

Vendor	Item	Part Number
Thermo Fisher Scientific	Dulbecco's Phosphate-Buffered Saline (DPBS), No Calcium, No Magnesium	14190144
	Trypan Blue Stain (0.4%)	T10282
	UltraPure Bovine Serum Albumin (BSA, 50 mg/ml)	AM2616
	Countess II FL Automated Cell Counter	AMQAF1000
	Countess II FL Automated Cell Counting Chamber Slides	C10228
Millipore Sigma	Methanol (for HPLC, ≥99.9%)	34860-100ML
	Protector RNAse Inhibitor (40 U/µL)	3335399001
	SSC Buffer 20X Concentrate	S6639-1L
	DL-Dithiothreitol Solution BioUltra, for molecular biology	43816
Bel-Art	Flowmi Cell Strainer, 40 µm	H13680-0040
Eppendorf	DNA LoBind Tubes, 2.0 ml	022431048

Protocol Overview



Protocol

1. Methanol Fixation

This protocol was demonstrated using 1-2 x 10^6 cells.

a. Harvest or thaw the cells in appropriate culture medium as per manufacturer's instructions. Transfer cells to a 2-ml microcentrifuge tube.

b. Centrifuge at **300 rcf** for **5 min** at **4°C**.

c. Remove the supernatant without disrupting the cell pellet.

d. Using a **wide-bore** pipette tip, add **1 ml** chilled 1X DPBS and gently pipette mix 10x or until cells are resuspended. Each tube should have 1-2 x10^6 cells.

e. Centrifuge at **300 rcf** for **5 min** at **4°C**.

f. **Repeat c-e.**

g. Remove the supernatant without disrupting the cell pellet.

h. Using a **wide-bore** pipette tip, add **200 µl** chilled 1X DPBS/1 x 10^6 cells and gently pipette mix 10x or until cells are resuspended.

i. Add **800 µl** chilled 100% methanol/1 x 10^6 cells. To avoid clumping of cells, add methanol **drop by drop** while gently stirring the cell suspension with the pipette tip in the microcentrifuge tube. Scale up the volumes of 1X DPBS and methanol if using >1 x 10^6 cells.

j. Incubate for **30 min** at **–20°C**.

k. Store fixed cells at **–20°C** or **–80°C** for up to **6 weeks** or **immediately** proceed to step 2.

2. Rehydration

a. Place the microcentrifuge tube containing the methanol-fixed cells on ice to equilibrate to **4°C** for **5 min**.

b. Centrifuge fixed cells at **1,000 rcf** for **5 min** at **4°C**.

c. Remove the supernatant without disrupting the cell pellet.

d. Based on starting cell concentration and assuming ~50% cell loss, add an appropriate volume Wash-Resuspension Buffer to obtain desired cell concentration. See Cell Suspension Volume Calculator Table of the relevant User Guide. Gently pipette mix using a **regular-bore** pipette tip.

e. If cell debris and large clumps are present, pass the sample through a 40 µm Flowmi Cell Strainer.

f. Determine cell concentration using a Countess II Automated Cell Counter or a hemocytometer. Proceed **immediately** with the 10x Genomics Single Cell protocols. Delay in proceeding may result in RNA loss.

To minimize Wash-Resuspension Buffer carryover, it is recommended to use ≤4 µl cell suspension stock when preparing cell suspension for GEM Generation and Barcoding Step of the 10x Genomics Single Cell protocols. If necessary, concentrate the sample to achieve higher cell recovery.

Results

When starting with 2 x 10^6 total cells, the recovery rate of cells after methanol fixation, storage, and rehydration was approximately 90%. After methanol fixation and rehydration, the cells appeared well singulated with no cellular debris under the microscope. Trypan blue staining of the methanol-fixed and rehydrated cells resulted in all cells staining positively, indicating that the cells have been effectively fixed and permeabilized.

Troubleshooting

Problem	Possible Solution
High fraction of viable cells post methanol fixation	Incrementally increase fixation time and monitor efficacy using microscopy Gently pipette mix cell pellet using a 200-µl pipette (set to 100 µl) and a wide-bore pipette tip until no cell clumps are visible in suspension
High fraction of visible debris post methanol fixation	Filter fixed cell suspension with the appropriate strainer Use flow cytometry to sort sample
<700 fixed cells/µl after final resuspension	Concentrate fixed cell suspension to achieve target concentration After centrifugation, aspirate supernatant from surface of the tube that is opposite to the surface where the cell pellet forms

References

J. Chen, F. Cheung, R. Shi, H. Zhou, W. Lu, CHI Consortium, PBMC fixation and processing for Chromium Single-Cell RNA sequencing. *J. Transl. Med.* 16, 198 (2018).

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