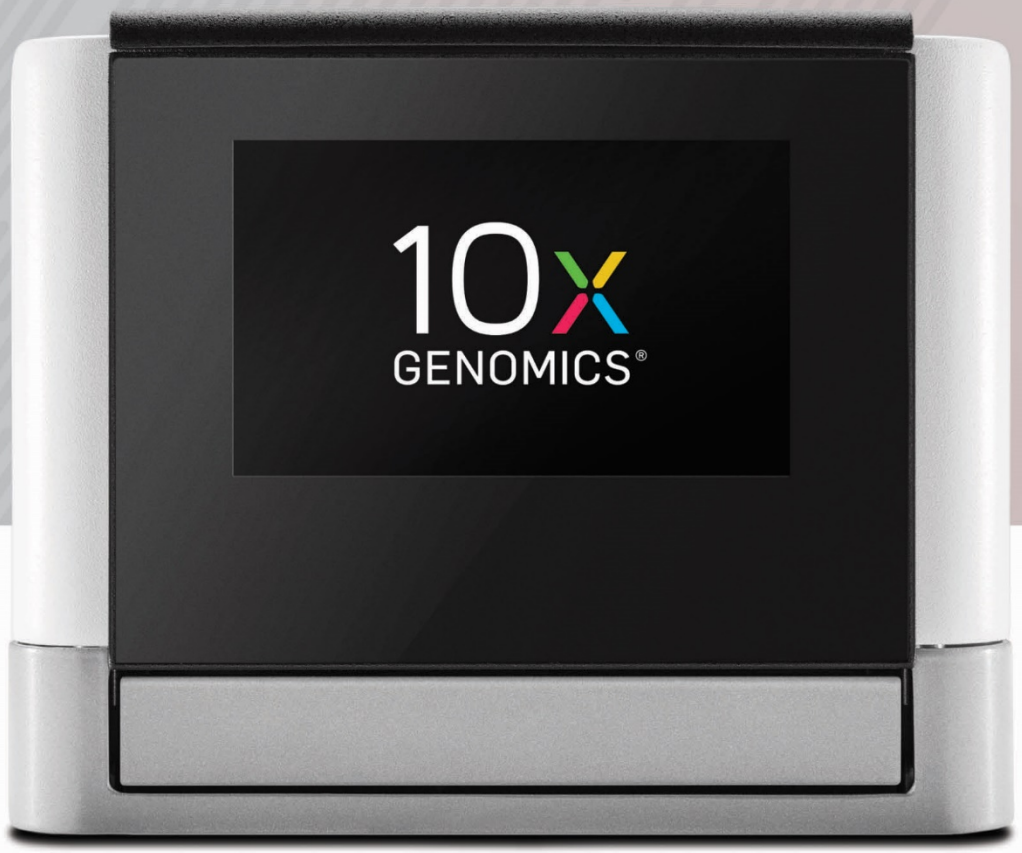
10x Genomics®

Sample Preparation Demonstrated Protocol

Methanol Fixation of Cells for Single Cell RNA Sequencing



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Demonstrated Protocol

Methanol Fixation of Cells for Single Cell RNA Sequencing

1. Overview

This Demonstrated Protocol describes best practices and general protocols for methanol fixation of cells, storing, rehydrating and recovering fixed cells from single cell suspensions in preparation for use in 10x Genomics® Single Cell Protocols. Minimizing the presence of cellular aggregates, dead cells, cellular debris, cytoplasmic nucleic acids, and potential inhibitors of reverse transcription is critical to obtaining high quality data.

The Protocols described here are expected to be compatible with many, but not all, cell or tissue types. Additional optimization may be required for the preparation of cell or tissue types that are particularly sensitive to suspension composition or handling techniques. Preparation of single cells direct from solid tissues or cryopreserved samples may also require additional optimization during dissociation and/or cell handling not covered here. For additional information on preparation of specific sample types, consult 10x Genomics Demonstrated Protocols available on the Support site support.10xgenomics.com.

2. Getting Started

2.1. Tips & Safety

Best practices for handling any cell type includes using sterile techniques, nuclease-free reagents and consumables, minimizing pipetting steps, and using wide-bore pipette tips when possible to minimize cell damage. Transfer pipettes may be used to remove supernatant after centrifugation to minimize disturbance of the cell pellet.

To determine accurate cell counts, best practices include sampling the cell suspension at least twice and at least two counts on each sample (*i.e.* a minimum of four counts in total, based on two independent draws from the cell suspension). Consult Technical Note *Guidelines on Accurate Target Cell Counts* (Document CG000091) for more information.

CRITICAL!

Human and animal 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.

2.2. Literature

a) This Protocol is an adaptation of Alles J, Karaiskos N, Samantha D, Praktiknjo SD, Grosswendt S, Wahle P, Ruffault PL, Ayoub. S, Schreyer L, Boltengagen A, Birchmeier C, Zinzen R, Kocks C, and Rajewsky N. "Cell fixation and preservation for droplet-based single-cell transcriptomics" *BMC Biology* **2017** 15:44.

2.3. General Materials

Supplier	Description	Part Number (US)
	Microcentrifuge for 2 ml LoBind tubes	-
	Vortex Mixer	-
Rainin	Tips LTS 200UL Filter RT-L200FLR	17007961
	Tips LTS 1ML Filter RT-L1000FLR	17007954
	Tips LTS W-O 200UL Fltr RT-L200WFLR	17014294
	Tips LTS W-O 1ML Fltr RT-L1000WFLR	17014297
	Pipet-Lite LTS Pipette L-200XLS+	17014391
	Pipet-Lite LTS Pipette L-1000XLS+	17014382
Bel-Art	Flowmi™ Cell Strainer, 40 µm	H13680-0040
Thermo Fisher Scientific	Dulbecco's Phosphate-Buffered Saline (DPBS), No Calcium, No Magnesium	14190144
	Trypan Blue Stain (0.4%)	15250061
	UltraPure™ Bovine Serum Albumin (BSA, 50 mg/ml)	AM2616
	Countess® II FL Automated Cell Counter	AMAQAF1000
	Countess® II FL Automated Cell Counting Chamber Slides	C10228
Eppendorf	DNA LoBind Tubes, 2.0 ml*	022431048
Sigma-Aldrich	Methanol (for HPLC, ≥99.9%)	34860-100ML-
	Protector RNAse Inhibitor (40 U/µL)	3335399001
	Nuclei PURE 2M Sucrose Cushion Solution *(component of Nuclei PURE Prep Isolation Kit)*	NUC201-1KT
	Nuclei PURE Sucrose Cushion Buffer *(component of Nuclei PURE Prep Isolation Kit)*	NUC201-1KT
VWR	10 ml Serological Pipette	89130-898
	Sterile Polypropylene Centrifuge Tubes with Flat Caps, 15 ml	21008-103
Integra	PIPETBOY acu 2	155018

*No substitutions are allowed. Items have been validated by 10x Genomics® and are required for Single Cell workflow, training and system operations.

2.4. Preparation – Buffers

a) Prepare **10 ml** chilled (4°C) Rehydration Buffer: 1X DPBS containing 1.0 % BSA and 0.5U/µl RNAse Inhibitor.

b) Place 100% methanol at –20°C. **2 ml** methanol is needed for each sample.

c) Place 1X DPBS at 4°C. **5 ml** 1X DPBS is needed for each sample.

d) Prepare Sucrose Cushion Buffer I: Mix **700 µl** Nuclei PURE 2M Sucrose Cushion Solution with **100 µl** Nuclei PURE Sucrose Cushion Buffer for each sample.

2.5. Cell Preparation and Sourcing

NOTE

This Protocol was demonstrated with the cells outlined below, including cells dissociated from fresh E18 Mouse Combined Cortex, Hippocampus, and Ventricular Zone tissue from BrainBits® (www.brainbitsllc.com). Materials were stored according to manufacturer's recommendations prior to starting the Protocol. Fresh embryonic mouse brain tissue was shipped on cold packs and used immediately upon receipt. Consult Demonstrated Protocol Dissociation of Mouse Embryonic Neural Tissue for Single Cell RNA Sequencing (Document CG00055) to obtain a single cell suspension from embryonic mouse brain tissue.

Cell Type	Description	Supplier	Part Number
Jurkat, Clone E6-1	Human Peripheral Blood T Lymphocytes	ATCC	ATCC® TIB-152™
Neuronal Tissue	E18 Mouse Combined Cortex, Hippocampus, and Ventricular Zone	BrainBits®	C57EHCV

3. Methanol Fixation & Rehydration Protocol

This Protocol assumes that the input single cell suspensions are prepared, washed and counted as described in the applicable 10x Genomics® Demonstrated Protocol:

- *Single Cell Suspensions from Cultured Cell Lines for Single Cell RNA Sequencing* (Document CG00054)
- *Dissociation of Mouse Embryonic Neural Tissue for Single Cell RNA Sequencing* (Document CG00055)

3.1. Methanol Fixation

NOTE

This Protocol was demonstrated using a sample size of ~5 x 10^6 total cells.

Fixation time and number of washes may need to be optimized depending on the cell type.

a) Centrifuge **5 x 10^6 cells** in a 2ml Eppendorf tube at **300 rcf** for **3 min**.

b) Remove the supernatant without disrupting the cell pellet.

c) Using a **wide-bore** pipette tip, add **1 ml** chilled 1X DPBS and gently pipette mix 10 times or until cells are completely suspended.

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

Repeat

e) Repeat steps b – d.

f) Remove the supernatant without disrupting the cell pellet.

g) Using a 200 µl **wide-bore** pipette tip, add **100 µl** chilled 1X DPBS and gently pipette mix 10 times or until cells are completely suspended.

h) Add **900 µl** chilled 100% methanol drop by drop to the cells in the tube while gently vortexing the tube (at the lowest speed setting) to prevent the cells from clumping.

i) Fix the cells on ice for **15 min**.

CRITICAL!

When working with different cell/tissue types, it is recommended to optimize fixation time. At this point, fixation efficacy should be assessed via staining the cells with trypan blue and viability should be assessed using the Countess® II FL Automated Cell Counter/microscopy. If a high fraction of viable cells is still present, incrementally increase the fixation time and monitor efficacy via microscopy.

NOTE

If cell debris and large clumps are present, filter the fixed cells through a cell strainer. For low volume, a 40 µm Flowmi™ Tip Strainer is recommended to minimize loss of sample volume. To increase the efficiency of debris removal, the cell suspension may be filtered twice.

j) Count the fixed cells using a Countess II FL Automated Cell Counter or a hemocytometer.

NOTE

If working with cell suspensions with excessive debris and aggregates, proceed to step 3.2 for Density Gradient Centrifugation. If cell suspension does not have debris and aggregates, proceed to step 3.3 for Rehydration.

k) Store the fixed cells at **4°C**, **–20°C** or at **–80°C** for up to **6 days** before proceeding to either step 3.2 or 3.3.

NOTE

Storage temperature and time may need to be optimized for different cell types.

3.2. Density Gradient Centrifugation

NOTE

This is an optional step, recommended only for cell suspensions with excessive cell debris and aggregates. This step assumes that >3 x 10^6 methanol fixed cells were obtained in step 3.1j. Fixed cells stored at –20°C or at –80°C should be placed on ice to equilibrate to 4°C before proceeding to the next step.

a) Centrifuge the fixed cells at **3000 rcf** for **5 min** at **4°C**.

b) Remove the supernatant without disrupting the fixed cell pellet.

c) Using a **regular-bore** pipette tip, add **500 µl** chilled Rehydration Buffer and gently pipette mix 5 times or until cells are completely suspended.

d) Using a **regular-bore** pipette tip, add **900 µl** Sucrose Cushion Buffer I to each tube containing **500 µl** resuspended fixed cells from step c and pipette mix 10 times.

e) Prepare sucrose gradient by adding **500 µl** Sucrose Cushion Buffer I to a 2 ml Eppendorf tube.

f) Carefully layer the **1400 µl** cell suspension from step d on top of the Sucrose Cushion Buffer I to form two separate layers. Do not mix the layers.

g) Centrifuge the sucrose gradient containing the fixed cells at **13000 x g** for **45 min** at **4°C**.

h) Carefully remove supernatant, leaving **100 µl** in each tube. Using a **regular-bore** pipette tip, resuspend the fixed cell pellets.

i) Using a **regular-bore** pipette tip, add **900 µl** chilled Rehydration Buffer and gently pipette mix 10 times or until cells are completely suspended.

j) Centrifuge the fixed cells at **3000 rcf** for **5 min** at **4°C**.

k) Remove supernatant without disrupting the cell pellet.

l) Using a **regular-bore** pipette tip, add **1 ml** chilled Rehydration Buffer or an appropriate volume to the pelleted fixed cells to achieve the target cell concentration of **700 – 1200 cells/µl** (7 x 10^5 – 1.2 x 10^6 cells/ml). Gently pipette mix 8 – 10 times or until cells are completely suspended.

CRITICAL!

Maintaining the rehydrated cells on ice for a prolonged period results in aggregation and clumping of the cells.

If clumping of the methanol fixed cells is observed after step l, pass the cell suspension through the 40 µm Flowmi™ Cell Strainer to remove larger debris. Determine cell concentration after straining the cell suspension.

m) Proceed directly with the 10x Genomics® Single Cell Protocol **immediately** after Density Gradient Centrifugation (without proceeding to step 3.3).

NOTE

Alternatively, the cell pellet at step k may be resuspended in 100 µl Rehydration Buffer followed by addition of 900 µl chilled 100% methanol. Add the methanol drop by drop to the cells while gently vortexing the tube (at the lowest speed) to avoid clumping of cells. Store the fixed cells at 4°C, –20°C or at –80°C for up to 6 days before proceeding to step 3.3 for Rehydration. Storage temperature and time may need to be optimized for different cell types.

3.3. Rehydration

NOTE

The RNAse Inhibitor concentration in the Rehydration buffer may need to be optimized for different cell types.

Fixed cells stored at –20°C or at –80°C should be placed on ice to equilibrate to 4°C before proceeding to the next step.

a) Centrifuge fixed cells at **3000 rcf** for **10 min** at **4°C**.

b) Remove supernatant without disrupting the cell pellet.

c) Using a **wide-bore** pipette tip, add **1 ml** chilled Rehydration Buffer and gently pipette mix 10 times or until cells are completely suspended.

d) Centrifuge at **3000 rcf** for **10 min** at **4°C**.

e) Remove the supernatant without disrupting the cell pellet.

Repeat

f) Repeat steps b – d.

g) Using a **regular-bore** pipette tip, add **1 ml** chilled Rehydration Buffer or an appropriate volume to the pelleted fixed cells to achieve the target cell concentration of **700 – 1200 cells/µl** (7×10^5 – 1.2×10^6 cells/ml). Gently pipette mix 8 – 10 times or until cells are completely suspended.

CRITICAL!

Maintaining the rehydrated cells on ice for a prolonged period results in aggregation and clumping of the cells.

If clumping of the methanol fixed cells is observed after step f, pass the cell suspension through the 40 µm Flowmi™ Cell Strainer to remove larger debris. Determine cell concentration after straining the cell suspension.

h) Proceed **immediately** with the 10x Genomics® Single Cell Protocol using the rehydrated methanol fixed cell suspension.

4. Results

When starting with 5 x 10^6 total cells, the recovery rate of cells after methanol fixation, storage, and rehydration was ~50% for both Jurkat T Lymphocytes and Mouse Embryonic Neuronal cells. After methanol fixation and rehydration of the cells, the cells appeared well singulated with no cellular debris. Trypan Blue staining of the methanol fixed cells showed that 100% of the cells were dead, indicating that all cells were effectively fixed and permeabilized. No viable cells were observed after following this Protocol. Results may vary between different cell types.

5. Troubleshooting

5.1. Troubleshooting Sample Preparation

Problem	Possible Reason	Solution
High fraction of viable cells post methanol fixation	• Incomplete cell fixation • Inefficient resuspension of cell pellet prior to methanol fixation	• Incrementally increase fixation time and monitor efficacy using microscopy • Gently pipette mix cell pellet using a P200 pipette and a wide-bore pipette tip set to 100 µl until no cell clumps are visible in suspension
High fraction of visible debris post methanol fixation	• Insufficient removal of debris	• Filter fixed cell suspension with the appropriate strainer • Use flow cytometry to sort sample • Run the samples through Density Gradient Centrifugation
<700 fixed cells/µl after final resuspension	• Low input cell number prior to cell fixation • Overly dilute fixed cell suspension • Cell loss during washing steps	• Increase sample/tissue input to dissociate single cells • Concentrate fixed cell suspension to achieve target concentration of 700 – 1200 fixed cells/µl • After centrifugation, aspirate supernatant from surface of the tube that is opposite to the surface where the cell pellet forms

5.2. Troubleshooting Partitioning & Library Preparation

Problem	Possible Reason	Solution
Low/no cDNA yield	• Low quality input material	• Reduce fraction of dead cells and debris following Demonstrated Protocol *Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing* (Document CG000093) • Obtain intact tissue and avoid freeze-thaw cycles
	• Overly dilute fixed cell suspension	• Concentrate fixed cell suspension to achieve target concentration of 700 – 1200 fixed cells/µl
	• Aggregation of fixed cells and/or possible clog during partitioning	• Confirm the use of the correct Rehydration Buffer (1X DPBS + 1% BSA + 0.5U/µl RNAse Inhibitor) to reduce fixed cell aggregation • Fixed cell suspensions should always be kept on ice • Use regular-bore pipette tips during final resuspension of fixed cells • Filter fixed cell suspension with the appropriate strainer • Fixed cells combined with the Single Cell Master Mix should be gently pipette mixed 5 – 10 times with a regular-bore pipette tip and samples immediately loaded with the same pipette tip into the chip
	• Low number of cycles during cDNA amplification	• Increase the recommended number of cycles during cDNA Amplification by 1 – 2 cycles • Run sample aliquot undiluted on the Bioanalyzer for QC
Low final library yield	• Low number of cycles during SI-PCR	• Increase the recommended number of cycles during SI-PCR by 1 – 2 cycles

5.3. Troubleshooting Data Analysis

Problem	Possible Reason	Solution
Low (<50%) "Fraction Reads in Cells"	• High fraction of ambient RNA in fixed cell suspension	• Reduce fraction of dead cells and debris in the input material following Demonstrated Protocol *Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing* (Document CG000093) • Add 1 – 2 additional wash steps when preparing the input material • Run the sample through additional Density centrifugation steps
Low library complexity (low number of genes/UMI's per cell)	• Low cDNA yield • Low quality input material	• Reduce fraction of dead cells and debris in the input material following Demonstrated Protocol *Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing* (Document CG000093) • Obtain intact tissue and avoid freeze-thaw of cycles • Optimize cell/tissue dissociation protocol for improved sample quality • Filter fixed cell suspension with the appropriate strainer • Use flow cytometry to sort sample • Increase/optimize the concentration of RNAse inhibitor in Rehydration Buffer to prevent RNA degradation