Cell Preparation for Single Cell Protocols

Introduction

10x Genomics Single Cell protocols require a suspension of viable single cells or nuclei as input. Minimizing the presence of cellular aggregates, dead cells, noncellular nucleic acids, and potential biochemical inhibitors of reverse transcription is critical to obtaining high-quality data.

This Cell Preparation Handbook describes best practices to help maintain cell viability and maximize sample quality during sample preparation. General protocols for sample handling, purifying, and counting for both abundant and limited cell suspensions are also provided here.

The general protocols described here are expected to be compatible with many, but not all cell and sample types. Additional optimization may be required for sensitive samples and solid tissues. For additional information on preparation of specific sample types, consult the Demonstrated Protocols available on the 10x Genomics Support website.

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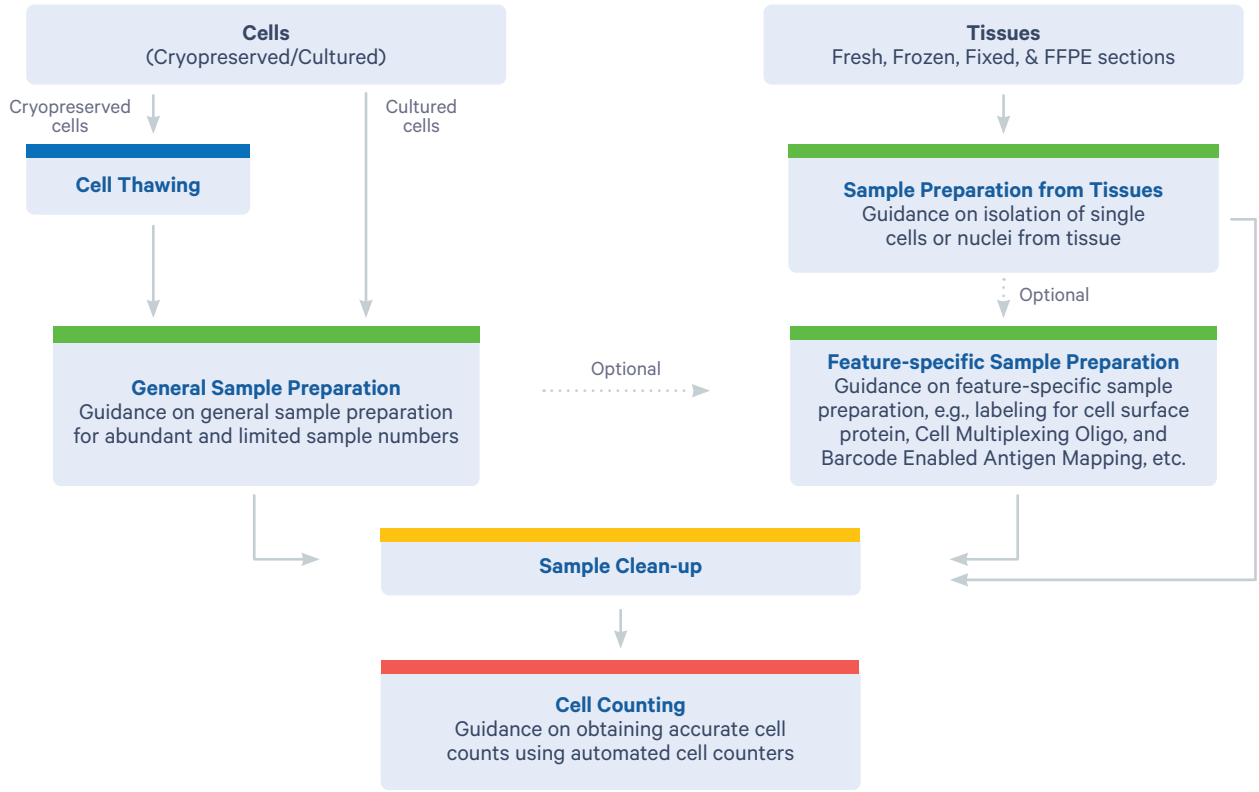
Getting Started with Sample Preparation



Before starting, it is recommended to refer to Tips & Best Practices for detailed guidance on proper sample handling techniques to ensure high sample quality during preparation.

Sample Input Types for Various 10x Genomics Assays

Products	Sample Input	Sample Prep Documents on 10x Genomics Support Website
Single Cell Gene Expression	Cells & nuclei*	https://www.10xgenomics.com/support/single-cell-gene-expression/documentation/steps/sample-prep
Single Cell Immune Profiling	Cells & nuclei*	https://www.10xgenomics.com/support/single-cell-immune-profiling/documentation/steps/sample-prep
Single Cell ATAC	Nuclei	https://www.10xgenomics.com/support/single-cell-atac/documentation/steps/sample-prep
Single Cell Multiome ATAC + Gene Expression	Nuclei	https://www.10xgenomics.com/support/single-cell-multiome-atac-plus-gene-expression/documentation/steps/sample-prep
Single Cell Gene Expression Flex	Fixed cells and nuclei*	https://www.10xgenomics.com/support/single-cell-gene-expression-flex/documentation/steps/sample-prep

** Nuclei are not supported with V(D)J or with Feature Barcode technology for cell surface protein or CRISPR.*

The protocol details provided in this Handbook are based on the Demonstrated Protocols and Sample Prep User Guides available on the 10x Genomics support website. Consult the applicable documents for the most updated information.

Tips & Best Practices

Tips & Best Practices section includes additional guidance

Signifies critical step requiring accurate execution

Troubleshooting section includes additional guidance

Proper cell preparation and handling are required to fully maximize sample quantity and quality for successful downstream 10x Genomics experiments.

Sample Quality for Cell Suspensions

The key considerations for preparing high-quality single cell suspensions are:

- Minimal cell debris
- Low levels of cell aggregates
- High cell viability (at least 70%)
  - *Non-viable and dying cells increase ambient RNA contamination and cell clumping leading to elevated microfluidic chip clogs and wetting failures.*

Sample Quality for Nuclei Suspensions

The key considerations for preparing high-quality nuclei suspensions are:

- Minimal cell debris
- Low levels of nuclei aggregates
- High-quality nuclear membranes
- Low cell viability
  - *Automated cell counters label nuclei as dead cells. The ratio of nuclei to intact cells will be quantified as viability (% viable cells) and should be less than 5%.*

The following sections describe tips & best practices for sample handling that should be applied throughout all steps of the cell preparation process to minimize sample loss and preserve sample quality and viability.

Consult the Technical Note Best Practices to Minimize Chromium Next GEM Chip Clogs and Wetting Failures (CG000479) for more information.

Sample Handling

Improper or prolonged sample handling can negatively impact sample quality. Samples left in inappropriate suspension buffers for too long may show increased cell loss, or aggregate formation. These aggregates (as well as other impurities including debris and fibers) can interfere with accurate cell or nuclei counting and lead to microfluidic chip failures when generating Gel Beads-in-emulsion (GEMs).

*Once samples are prepared, proceed **immediately** to the relevant User Guide.*

Place samples on ice after resuspension to avoid temperature shock and load as soon as possible. Some sample types should be placed at room temperature instead of ice. For example, room temperature is recommended for granulocytes & neutrophils.

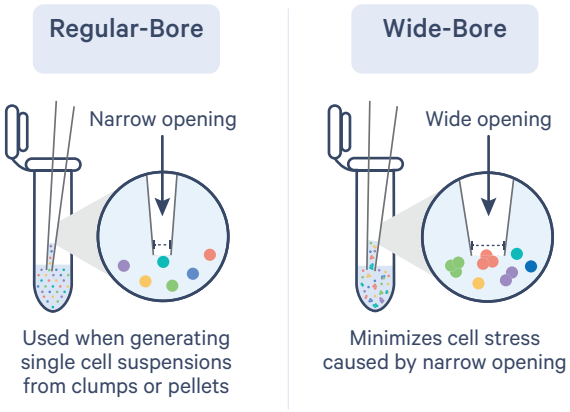
Pipetting Guidance

Pipette Tips

Sample preparation requires both regular-bore and wide-bore pipette tips. Regular-bore tips are narrower than wide-bore tips and are useful for generating single cell suspensions from pellets or clumps.

Wide-bore tips help minimize cell stress caused by narrow opening and can be used to maintain a single cell suspension.

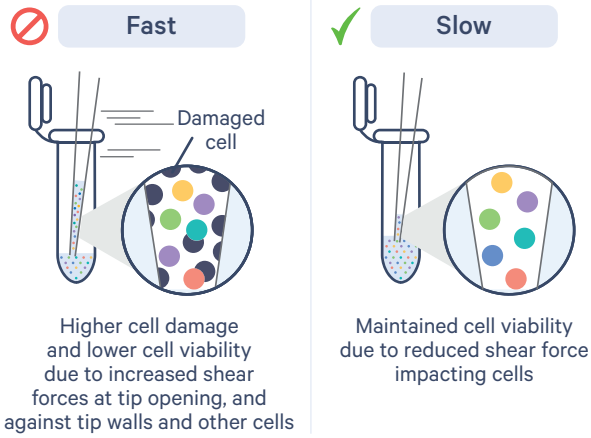
Pipette Tip Options



Pipetting Speed

Pipette cell suspensions slowly and gently to minimize physical damage to cells from shearing forces. Rough pipetting, regardless of pipette tip size, can negatively impact sample quality metrics such as fraction reads in cells and median genes per cell.

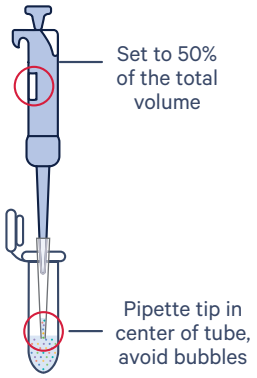
Proper Pipetting Speed



Pipetting to Mix

Use a wide-bore tip to maintain a single cell suspension during mixing before loading. Set the pipette to 50% of the suspension volume. For example, set the pipette to ~350 µl for a 700 µl cell suspension. Place the pipette tip in the middle of the tube with the tip always partly submerged in the liquid when cells are aspirated.

Pipetting to Mix



Centrifuging Samples

Centrifugation Conditions

Optimize centrifugation conditions for specific sample types to achieve the ideal pellet.

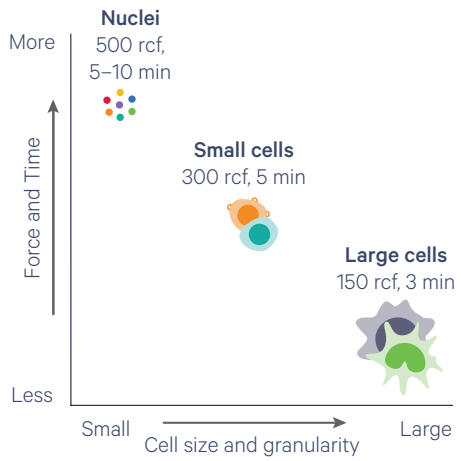
Ideal Pellet

- Solid, not too tight
- Minimal sample in supernatant
- Minimal debris
- Representative of sample (important for heterogeneous samples)
- Nuclei pellets are less solid and may be easily disrupted leading to sample loss

Cell number, concentration, size, and density all influence pelleting efficiency at a given centrifugation speed, time, and temperature.

TIPS *Excessive centrifugation is not recommended. Fully resuspend the pellet while taking care to limit pipetting to minimize damage to cells.*

Centrifugation Conditions by Sample Type



When working with a new sample type, save and count cells in supernatant to ensure cell loss is low.

TIPS *For centrifugation conditions of fixed samples, consult the Demonstrated Protocol Fixation of Cells and Nuclei for Fixed RNA Profiling (CG000478).*

Types of Centrifuges

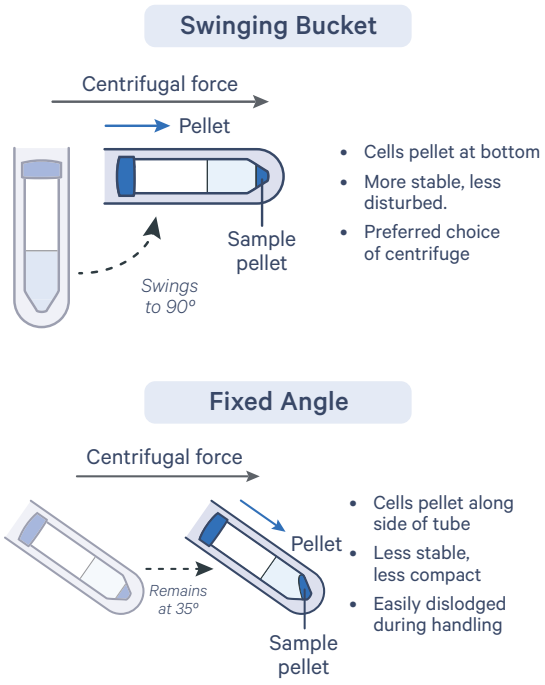
Two types of centrifuges exist characterized by their rotor: swinging bucket and fixed angle.

Due to differences in mechanics, each rotor produces a different type of pellet. These differences can interfere with successful sample recovery in later steps.

Anticipate pellet location depending on the type of the centrifuge used. Remove the supernatant without disturbing the pellet.

A swinging bucket centrifuge is preferred, especially when working with nuclei or lower cell numbers to help minimize cell loss. Consult product-specific protocols for recommendations regarding compatible centrifuge tubes.

Centrifuges Classified by Rotor Type



Washing and Resuspension

Wash and resuspension should be performed to minimize cell clumping, cell loss, and debris while also to maximize cell health. Stressed or dying cells will negatively impact data quality.

Wash and Resuspension

- Choose appropriate wash & resuspension buffers (sample type and assay dependent)
- Wash with concentration of <5,000 cells/µl to avoid clumping
- Optimize buffer composition and volume, centrifugation conditions, and the number of washes to reduce cell loss and debris

Buffer Composition

Media and buffer composition are critical for ensuring sample quality. Bovine Serum Albumin or BSA is added to PBS to minimize cell loss and aggregation and to maximize cell health (see Table below). Primary cells, stem cells, and other sensitive cell types like dissociated tumor cells may require alternative buffers to maximize viability.

Considerations for Cells

PBS: 1X PBS (calcium and magnesium free) containing 0.04% weight/volume BSA (400 µg/ml) is recommended for most general sample preparation protocols to help minimize cell loss and aggregation.

Increase BSA/FBS concentration in wash and resuspension buffers (0.04% to 1% BSA or up to 10% FBS) to maintain cell viability. Consult product-specific Demonstrated Protocols for detailed guidance.

Cell culture media: If cell viability cannot be maintained in a PBS-based buffer, it is possible to wash and resuspend in common cell culture media.

Wash and/or resuspend in cell culture media plus BSA/FBS (1% BSA or up to 10% FBS) to maintain cell viability.

Buffers/media should not contain excessive amounts of EDTA (>0.1 mM) or magnesium (>3 mM), and should be free of surfactants (i.e. Tween-20) as these components will interfere with downstream reverse transcription and GEM generation.

Consult product-specific protocols for guidance on compatible buffers and Appendix for Alternative Buffers & Cell Culture Media.

Overview of the Recommended Wash & Resuspension Buffers for Fresh Cells

Fresh Cells	General Sample Preparation	Feature-specific Sample Preparation Protocols	
		Cell Surface Protein and/or Cell Multiplexing Oligo (CMO) Labeling	Barcode Enabled Antigen Mapping Labeling
Human and mouse cell lines (cryopreserved or freshly cultured)	PBS + 0.04% BSA	PBS + 1% BSA *Alternate buffers: PBS + 10% FBS, Cell Culture Media + 1% BSA*	*Not tested*
Human blood cells (PBMCs, BMMCs)	PBS + 0.04% BSA	PBS + 1% BSA *Alternate buffers: PBS + 10% FBS*	PBS + 2% FBS
Mouse blood cells (PBMCs, BMMCs, splenocytes)	PBS + 1% BSA	PBS + 1% BSA *Alternate buffers: PBS + 10% FBS*	PBS + 2% FBS
Dissociated tumor cells (DTCs)	Cell Culture Media + 10% FBS	PBS + 1% BSA *Alternate buffers: PBS + 10% FBS*	*Not tested*
Dissociated brain tissue	NbActiv-1	NbActiv-1 + 1% BSA (only tested for CMO protocol)	*Not tested*

This table provides an overview of recommended buffers for sample types that have been tested for general and feature-specific sample preparation protocols. All the recommended buffers are not provided here. Refer to the specific Sample Prep User Guides or Demonstrated Protocols for the most updated information.

Considerations for Nuclei

For gene expression-based assays, RNase Inhibitor must be added to resuspension buffers to preserve RNA quality. Use the appropriate buffer and concentration of RNase Inhibitor as indicated by the applicable product-specific protocol when resuspending nuclei. However, for standalone Single Cell ATAC only assay, additional RNase Inhibitor is not required.

The table on the right provides an overview of the recommended resuspension buffer for nuclei for various 10x Genomics Single Cell assays. Consult the appropriate product-specific User Guide or Demonstrated Protocol for details.

Overview of the Recommended Wash & Resuspension Buffers for Nuclei

Product	Resuspension Buffer
Single Cell Gene Expression	1X PBS containing BSA* with RNase inhibitor
Single Cell Multiome ATAC + Gene Expression	1X Nuclei Buffer** with RNase inhibitor
Single Cell ATAC	1X Nuclei Buffer** without RNase inhibitor

**Consult the appropriate Demonstrated Protocol or Sample Prep User Guide for full buffer composition.*

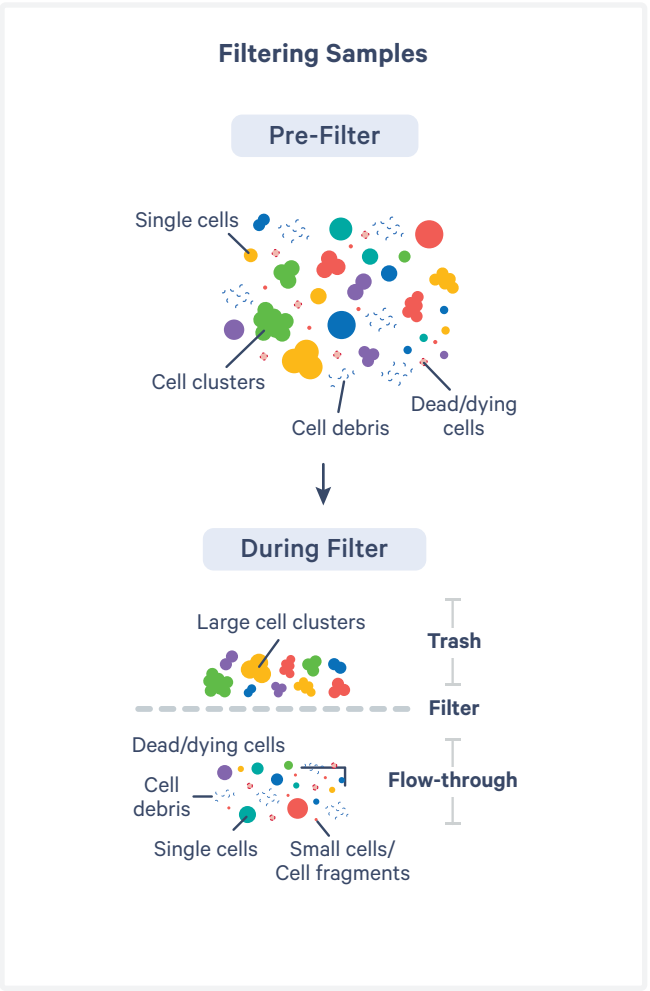
***Prepared using 10x Genomics 20X Nuclei Buffer (PN-2000207). Not all RNase Inhibitors are compatible with all 10x Genomics Single Cell assays.*

Straining and Filtering

GEM generation relies on microfluidic channels that are <100 µm wide. Filtering cell suspensions with an appropriate cell strainer is helpful for removing large cell clumps and/or aggregated debris, ensuring optimal performance.

Use strainer with a pore size that is larger than the maximum cell diameter in the sample, but small enough to catch larger clumps.

TIPS *When filtering, use large volumes and filter at the last wash step (versus the final cell suspension) to minimize sample loss.*

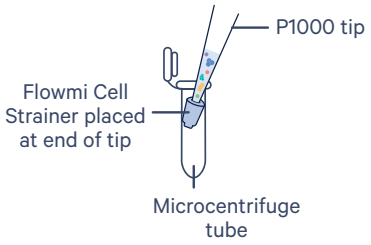


For general use, the MACS SmartStrainer (30 µm) is recommended. It generally causes minimal changes to cell concentration. A minimum sample volume of >600 µl is required due to volume loss that can occur. For low cell suspension volumes, the Flowmi Cell Strainer (40 µm) is recommended.

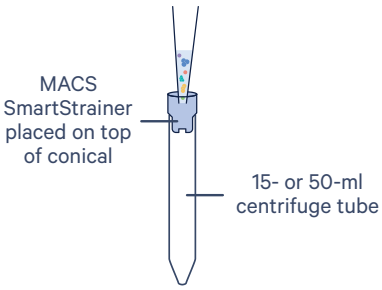
	Strainer	Size & Bore	Average Volume Loss	Average Change in Conc.
	MACS SmartStrainer *(recommended for general use)*	30 µm, Wide	130.0 µl ± 36.0 µl	3% ± 11%
	Flowmi Cell Strainer *(recommended for low cell suspension volumes)*	40 µm, Regular	20.0 µl ± 9.4 µl	21% ± 6%

TIPS *Due to some volume loss during filtering, always recount the cell suspension after straining.*

Using the Flowmi Cell Strainer



Using the MACS SmartStrainer



Cell Stock Concentration

The total number of cells required is determined by the cell recovery target defined by the user. Consult the applicable 10x Genomics Single Cell assay to determine this relationship.

Always count the final cell suspension that will be added to the Single Cell Master Mix.

Below are some guidelines on determining cell concentrations.

- **Counting accuracy**: Optimize cell suspension concentration according to the recommended dynamic range of counting technique used. The concentration should always be determined based on the total number of cells, not just viable cells.
- **Reproducible counts**: Count final cell suspension at least twice, ideally 3–4x. Ensure that individual counts are within 25% of each other.
- **Adding cells to Single Cell Master Mix**: Cell volume added should be <40% and ideally <20% of the total reaction volume (<15 µl for standard throughput assays and <30 µl for high throughput assays) to reduce debris or inhibitory substance (e.g. magnesium and EDTA) carryover into the GEM reaction.
- **Maximizing accuracy and precision**: Do not pipette cell suspension volumes <2 µl. Select the appropriate pipette for the targeted transfer volume. Ensure pipettes are calibrated and properly maintained.
- **Transfer volume:** Cell Suspension Volume Calculator tables are included in each of the product User Guides. Consult the applicable User Guide to determine the appropriate cell suspension volumes and concentrations.

Reagents & Consumables

The items in the tables below have been tested by 10x Genomics and perform optimally with the Chromium Single Cell protocols. Substituting materials may adversely affect system performance. This list does not include standard laboratory equipment, such as water baths, pH meters, freezers, etc.

Item	Description		Supplier	Part Number (US)
Plastics				
0.2 ml PCR 8-tube strips	PCR Tubes 0.2 ml 8-tube strips	Choose either Eppendorf, USA Scientific or Thermo Fisher Scientific PCR 8-tube strips.	Eppendorf	951010022
	TempAssure PCR 8-tube strip		USA Scientific	1402-4700
	MicroAmp 8-Tube Strip, 0.2 ml MicroAmp 8 -Cap Strip, clear		Thermo Fisher Scientific	N8010580 N8010535
1.5-ml tubes	DNA LoBind Tubes 1.5 ml		Eppendorf	22431005
2-ml tubes	DNA LoBind Tubes 2.0 ml			22431048
15-ml tubes	Corning 15 ml centrifuge tubes	Choose either tubes for reagent & buffer preparation.	Corning	CLS430791
50-ml tubes	Corning 50 ml centrifuge tubes		Corning	CLS430829
Pipette Tips	*Refer to Pipette Tip Recommendations for 10x Genomics Single Cell Protocols (CG000492) for a list of recommended pipette tips.*			
Kits & Reagents				
Sterile 1X PBS	Phosphate-Buffered Saline without Calcium & Magnesium *Or any equivalent sterile PBS*		Corning	21-040-CM
Nuclease-free water	Molecular Grade Nuclease-free Water		Thermo Fisher Scientific	AM9937
FBS	Fetal Bovine Serum, qualified, heat inactivated		Thermo Fisher Scientific	16140071
	Avantor Seradigm Premium Grade Fetal Bovine Serum		VWR	97068-085
Viability dye	Invitrogen eBioscience 7-AAD Viability Staining Solution		Invitrogen	00699350
BSA	UltraPure Bovine Serum Albumin (BSA, 50 mg/ml)		Thermo Fisher Scientific	AM2616
	MACS BSA Stock Solution		Miltenyi Biotec	130-091-376
	Albumin, Bovine Serum, 10% Aqueous Solution, Nuclease-Free *For Isolation of Cells from FFPE Tissue Sections for Chromium Fixed RNA Profiling*		Millipore Sigma	126615
	Bovine Serum Albumin In DPBS (10%)		Millipore Sigma	A1595
Filtration				
Filters	MACS SmartStrainers, 30 µm		Miltenyi Biotec	130-098-458
	Flowmi Cell Strainer, 40 µm		Bel-Art Flowmi	H13680-0040
	Flowmi Cell Strainer, 70 µm			H13680-0070

Cell Counting			
Label	Trypan Blue Label (0.4%)	Thermo Fisher Scientific	T10282
	Live/DEAD Viability/Cytotoxicity Kit for mammalian cells		L3224
	Acridine Orange/Propidium Iodide Stain	Logos Biosystems	F23001
Cell counter & slides – Countess	Countess II FL Automated Cell Counter	Thermo Fisher Scientific	AMQAF1000
	Countess 3 FL Automated Cell Counter		A49866
	Countess Cell Counting Chamber Slides		C10228
Cell counter & slides – Luna	LUNA-FL Automated Fluorescence Cell Counter	Logos Biosystems	L20001
	LUNA Cell Counting Slides, 50 Slides		T13001
Cell counter & slides – Cellaca	Cellaca MX High-throughput Automated Cell Counter (MX-SYS1 FL 5)	Nexcelom Bioscience	MX-SYS1
	Nexcelom High-throughput Counting Plates		CHM24-A100-001
	ViaStain AO/PI (acridine orange/ propidium iodide)		CS2-0106-5mL
Cell counter & slides – Cellometer	Cellometer K2 Bundle w/ Matrix Software	Nexcelom Bioscience	CMT-K2-MX-150
	VS Cellometer AOPI Staining Solution		CS2-0106-25ML
	PD100 Counting Chambers 1 case		CHT4-PD100-003
Cell counter & slides – NucleoCounter	NucleoCounter NC-202 Instrument	ChemoMetec	970-2020
	Via2-Cassette		941-0024
Cell counter & slides – Vi-CELL BLU	Vi-CELL BLU System	Beckman Coulter	C19201
	Vi-CELL BLU single reagent kit		C06019
	Sample vials		C24843
	0.5M single-use concentration control (20 vials of 0.5 x 10^6 beads/mL)		C09147
	2.0M single-use concentration control (20 vials of 2 x 10^6 beads/mL)		C09148
	4.0M single-use concentration control (20 vials of 4 x 10^6 beads/mL)		C09149
	10.0M single-use concentration control (20 vials of 10 x 10^6 beads/mL)		C09150
	50% single-use viability control (20 vials of 50% viability beads)		C09145
Equipment			
Vortex	Vortex Mixer	VWR	10153-838
Centrifuge	Refrigerated Eppendorf Centrifuge Or any equivalent centrifuge	Millipore-Sigma	5427R or 5424R
Sorter	MA900 Multi-Application Cell Sorter Or any equivalent cell sorter	Sony	MA900
-	Inverted tissue culture microscope with 10X/20X magnification and fluorescence imaging capability	-	-

Choose counter based on availability & preference.

For some items, a number of vendor options are listed. Choose item based on availability and preference. Refer to the manufacturer's website for regional part numbers.

1. Cryopreservation and Cell Thawing

For 10x Genomics Single Cell assays, freshly prepared samples will generate high-quality data. However, fresh samples might not always be available, and in such instances, high-quality data can also be generated from cryopreserved samples stored over extended periods of time.

1.1 Cryopreservation

When cryopreserving samples, it is important that the sample is of the highest quality, and is handled appropriately in order to ensure minimal cell loss during freezing and thawing.

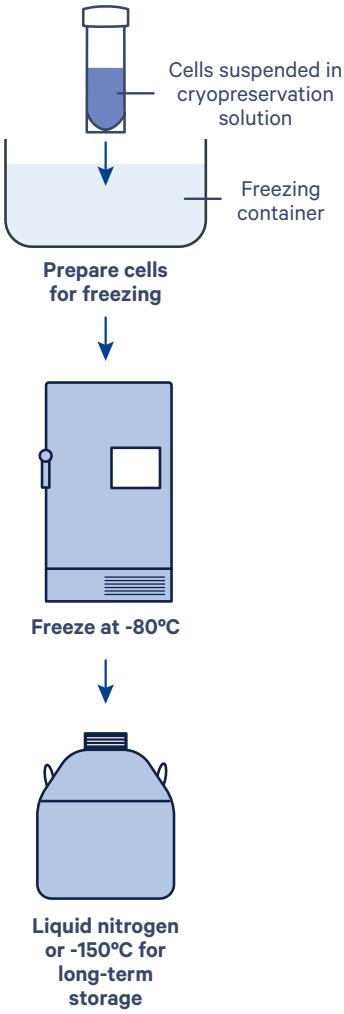
Recommended best practices include the following:

- >90% cell viability is recommended.

TIPS *If viability is <80%, consider dead cell removal.*

- Freeze ≥1 million cells/ml to minimize loss due to cell stress and death. Avoid freezing diluted cells.
- Use a cryoprotectant or specialized cryopreservation solution. 10% DMSO in media or serum is recommended. Commercially available media (i.e. Cryostor) can also be used. Ensure sample type compatibility when choosing a solution.
- Use a freezing container to control the rate of freezing to minimize cell loss.
- Hold cell suspensions at -80ºC, then move to liquid nitrogen or -150ºC freezer for long-term storage. The longer the cells are kept at -80ºC, the higher the risk of a cell viability drop post-thawing.

Cryopreserving nuclei is not recommended.



1.2 Cell Thawing

When thawing cryopreserved samples, use the correct protocol for each sample type to minimize cell loss, unintentional changes in population frequency, or drop in viability post thawing. For example, fragile cells such as primary or tumor cells require more gentle thawing methods compared to robust cultured cell lines.

Using appropriate thawing buffers, media/buffers that are added to the cryopreserved cells to dilute the DMSO, will also minimize cell loss (see Table below).

TIPS *If low cell viability is observed after thawing, sample cleanup may be performed (see Sample Cleanup).*

10x Genomics offers four thawing protocols that are compatible with all Single Cell products.

- PBMCs/Cell Lines Direct Media Thawing
- PBMCs/Cell Lines Dropwise Media Thawing
- Tissue Derived Tumor Cells Thawing
- 293T/3T3 Cell Lines Mixture Thawing

Consult the Handbook Cell Thawing Protocols for Single Cell Assays (CG000447) for details.

Overview of the Recommended Thawing Buffers Based on Sample Type

Sample Type	Thawing Buffer
Human and mouse cell lines	*Cell Culture Media + 10% FBS
Human blood cells (PBMC, BMMC)	
Mouse blood cells (PBMCs, BMMC, splenocytes)	
Dissociated tumor cells (DTCs)	

**Consult appropriate Demonstrated Protocol for cell culture media and alternative buffers compatible with Single Cell Gene Expression and Immune Profiling products.*

Data Highlight: Comparing Thawing Protocols

Objective: Compare thawing protocols and their effect on cell viability and population frequency.

Methods: Fresh bone marrow mononuclear cells (BMMCs) were isolated from an adult C57BL/6 mouse. A fraction of the fresh cells was used to generate Gene Expression data using Chromium Single Cell 3' workflow, and in parallel the rest of the cells were cryopreserved. The cryopreserved cells were later thawed using the three different thaw protocols and used to generate 3' Gene Expression data as well. Cells were thawed by four independent users with varying experience levels using Direct Media Thawing and Dropwise Media Thawing protocols. Additionally, one of the four users also thawed using the Cell Lines Mixture Thawing protocol.

The data from freshly isolated cells were compared with those of cryopreserved cells thawed using the three different protocols. Viability was measured using flow cytometry for both fresh and cryopreserved cells.

Results: Flow data indicate that thawing BMMCs with the thaw protocol designed for more robust cell lines resulted in fewer viable cells (Figure 1A).

Cell type annotation via Single Cell Gene Expression analysis showed different cell type abundance when cryopreserved cells were thawed using the Cell Lines Mixture Thawing protocol (Figure 1B). The annotation was consistent between fresh cells and cryopreserved cells that were thawed using either the Direct or Dropwise Media Thawing protocols.

Global gene expression was highly correlated between fresh and cryopreserved BMMCs that were thawed using either the direct or dropwise thawing protocols. Thawing BMMCs using the Cell Lines Mixture Thawing protocol resulted in reduced fraction reads in cells and genes detected. (Figure 1C-D). Lower viability and increased cell stress result in more apoptotic and dead cells and may also increase overall cell loss. This leads to increased ambient RNA and lower fraction read in cells.

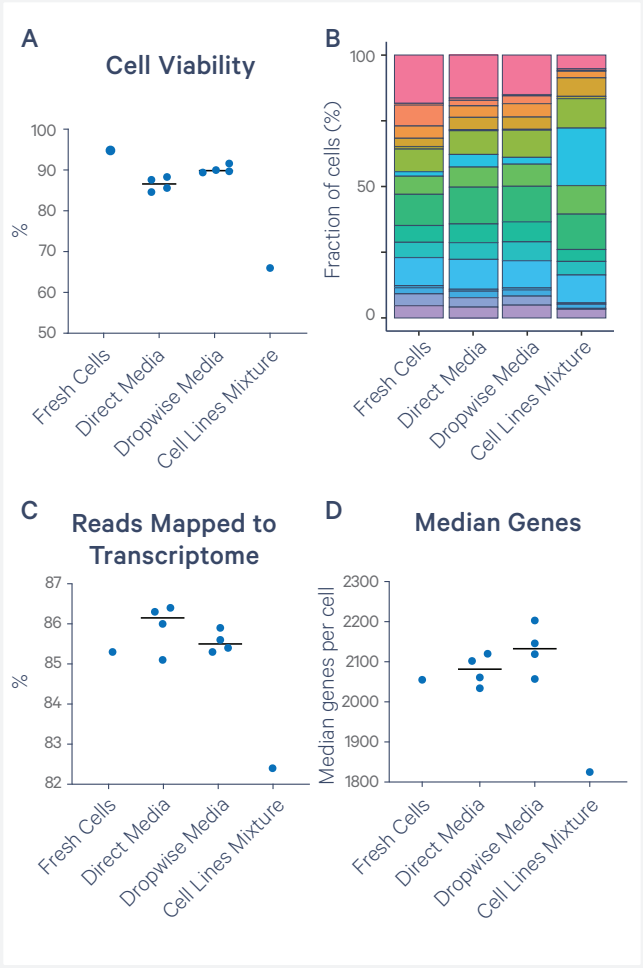


Figure 1. Mouse bone marrow mononuclear cell thawing comparison with three different thaw techniques compared to fresh cells. Viability was measured using 7-AAD. (A) Frequency of 7-AAD negative singlet cells are shown here. (B) Relative abundance of each cell type for each thaw method. (C) Fraction reads in cells and (D) median genes per cell were compared across thawing protocols.

Conclusions: Direct and Dropwise Media Thawing protocols resulted in similar cell population distributions and global gene expression compared to fresh cells. For BMMCs, direct or dropwise media thawing is the appropriate protocol to use. In conclusion, not all thaw protocols are suitable for all samples. Choosing the most-appropriate thaw protocol requires an understanding of the sample type.

2. Sample Preparation

2.1 Overview

This chapter provides guidance on general and advanced sample preparation for 10x Genomics Single Cell assays. Further optimization on some steps may be needed when working with unfamiliar or uncharacterized samples such as solid tissues, fragile primary cells, etc.

2.2 General Cell Preparation Protocols

These protocols assume that cells are already in a single cell suspension from cell culture, dissociated tissues, cell sorting, or other cell isolation methods.

Preparation of Sample with Abundant Cells

The following protocols are best suited for abundant samples for which avoiding cell losses during washing and straining is not critical.

a. Obtain the cells of interest in suspension.

b. Using a **wide-bore** pipette tip, gently and thoroughly mix the cells.

c. Determine the cell concentration using an automated cell counter or a hemocytometer.

d. Calculate the cell resuspension volume required for the desired target concentration.
To calculate cell resuspension volume, assume ~50% cell loss.

e. If significant amounts of cell clumps or debris are observed, gently mix cells by pipetting up and down 10–15x and filter cells using an appropriate cell strainer.

f. Centrifuge cells at **300 rcf** for **5 min** at **room temperature** and nuclei at **500 rcf** for **5–10 min** at **4°C**.

- *Smaller or larger cells may require optimization of centrifugation conditions. Speed and time may be adjusted to minimize cell loss and preserve cell integrity. Depending on the rotor type, the cell pellet forms on the side or on the bottom of the tube. Know the expected position of the pellet, especially when working with small or limited cells, as the pellet can be difficult to see.*

g. Remove the supernatant without disrupting the cell/nuclei pellet. Save the removed supernatant on ice in another tube until the protocol is complete.

h. Add **1 ml** PBS with 0.04%–1% BSA or 10% FBS, and repeat steps f-g.

i. Add the appropriate volume (as determined at step 2.2 d) of 1X PBS with 0.04% BSA–1% BSA or 10% FBS to the tube. Addition of RNase Inhibitor may be required for nuclei, refer to the section Resuspension Buffers – Considerations for Nuclei for more information. Gently pipette 5–10x or until the cells are completely suspended.

j. Determine the cell concentration using a manual/automated cell counter. If cell aggregates/clumps are observed, filter the sample using a cell strainer and recount.

- *If cell concentration is significantly lower than expected, the sample may not have formed efficient cell pellets during centrifugation. Check the concentration of the saved supernatants and re-pellet at a higher centrifugation speed.*
- *Cell loss may also be the result of non-optimized cell/nuclei buffer resulting in lysis or death. Cell loss may also occur during filtering steps.*

k. Adjust the volume to obtain the target cell concentration, if necessary and recount.

l. Once the target cell concentration is obtained, place the cells on ice.

m. Proceed with the appropriate 10x Genomics Single Cell protocol.

Protocol for Samples with Limited Cells

The protocol described here is best suited if the initial cell suspension has <200,000 cells in total (<200,000 suspension or <100,000 adherent cells). For such samples, plate-based sample preparation guidelines can also be followed (CG000426).

When preparing limited samples (<200,000 for suspension cells or <100,000 for adherent cells), it is important to maximize yield and minimize cell damage and loss. Minimize cell handling and save all the supernatants until the protocol is complete. One cell wash is recommended to remove any ambient RNA and contaminants, but may be skipped if the number of cells is low (<100,000) and there is little visible debris in the suspension. If cells are unstable in PBS, the buffer can be replaced with most common cell culture buffers and media with minimal reduction in performance.

Cells may be centrifuged in either tubes or in a 96-well plate.

a. Using a **wide-bore** pipette tip, gently and thoroughly mix the single cell suspension. If feasible, determine the cell concentration using an automated cell counter or a hemocytometer (see Cell Counting & Quality Control for more information on cell counting).

- *Quantification at this point enables an approximation of the appropriate volume for the subsequent resuspension in order to obtain target concentrations. When determining the volume for resuspension, assume ~25% of cells will be lost during wash steps.*

b. If cell clumps or cell debris is observed, gently mix cells by pipetting up and down 10–15x and filter cells using an appropriate cell strainer.

c. Centrifuge cells at **300 rcf** for **5 min**.

- *Some cells need to be centrifuged at higher speed (e.g. up to 400 rcf) or for longer time (e.g. up to 10 min) to minimize cell loss due to inefficient pelleting. However, do not over-centrifuge as increased centrifugation speed can compromise the cell integrity and viability.*
- *Depending on the rotor type, the cell pellet forms on the side or on the bottom of the tube. Know the expected position of the pellet, especially when working with small or limited cells, as the pellet can be difficult to see.*

d. Remove the supernatant without disrupting the cell pellet, leaving ~50 µl or an appropriate volume to achieve the target cell concentration. Save the removed supernatant in another tube until the protocol is complete.

- *If using a 96-well plate: Remove the supernatant without disturbing the pellet. A multichannel vacuum pump is recommended. When using the vacuum pump, place the 96-well plate at a 45° angle and touch the side walls with the pipette tip. DO NOT touch the pellet. Remove the entire supernatant.*

e. If performing a wash step, add **1 ml** PBS with 0.04%–1% BSA or 10% FBS, and repeat steps c-d. One cell wash is recommended to remove any ambient RNA and contaminants.

f. Using a regular-bore pipette tip, resuspend the cell pellet in the leftover supernatant by gently pipetting up and down 10–15x.

g. If using a 96-well plate: add appropriate volume PBS with 0.04%–1% BSA or 10% FBS and resuspend as above.

h. Determine the cell concentration using an automated cell counter or a hemocytometer.

- *If cell concentration is lower than expected, check the concentration of saved supernatants and re-pellet the supernatant at a higher centrifugation speed. It is possible that cells were not efficiently pelleted at the initial speed.*

i. If necessary, adjust the volume to obtain the target cell concentration.

j. Once the target cell concentration is obtained, place the cells on ice.

k. Proceed with the appropriate 10x Genomics Single Cell protocol.

- *Low-input protocol for ATAC (CG000169) and Multiome (CG000365) may only be used for these products.*

2.3 Sample Preparation from Tissues

Isolating fresh single cell suspensions from tissue has not been tested extensively for 10x Genomics. However, the following resources can be used as a starting point:

- 10x Genomics Demonstrated Protocols for dissociating mouse embryonic neural tissue (CG00055) and mouse tumors (CG000147).
- Protocols from the Worthington Tissue Dissociation Guide, 10x Genomics Publications, and Customer Developed Protocols.
  - *These protocols are not directly supported or certified by 10x Genomics.*
- Commercially available tissue dissociation solutions and some cell enrichment products have also been validated in some cases. Consult the product-specific documents for guidance.
  - *The specific conditions may differ depending on tissue type (soft, solid, necrotic, etc.), and may need optimization.*

Key Considerations

- Cell viability should be >80% (although 70% is acceptable). Perform sample cleanup for lower viability samples.
- Optimize the sample preparation procedure and evaluate yield, viability, and levels of aggregation and debris before running a specific cell or tissue type for the first time.

Fixed Samples

- Fixed samples may be stored up to 7 days at 4ºC or longer at lower temperatures (up to 6 months at -80ºC) before using for Fixed RNA Profiling assay.
- Fixed samples are only compatible with the Chromium Fixed RNA Profiling workflow. Use Demonstrated Protocol Tissue Fixation & Dissociation for Chromium Fixed RNA Profiling (CG000553) for isolating single cells from fixed tissues.

Frozen Tissues:

- Do not use frozen tissue samples to generate single cell suspensions; nuclei should be isolated instead. Consult the Chromium Nuclei Isolation Kit User Guide (CG000505) for guidance.

2.4 Feature-Specific Sample Preparation Protocols

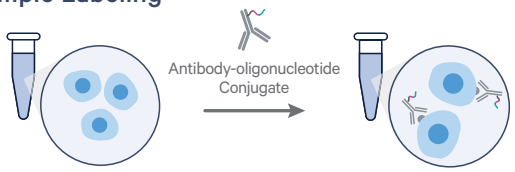
The following sections provide general guidance on some feature-specific sample preparation protocols. For detailed guidance, consult the specific Demonstrated Protocols or Sample Prep User Guides available on the 10x Genomics Support website.

Cell Surface Protein Labeling

Cell surface proteins can be labeled using a specific protein binding molecule, such as an antibody conjugated to a Feature Barcode oligonucleotide. The Demonstrated Protocol Cell Surface Protein Labeling for Single Cell RNA Sequencing Protocols with Feature Barcode technology (CG000149) provides general guidance for antibody-oligonucleotide conjugation and outlines cell surface protein labeling and for enriching labeled cells using flow sorting.

Cell Surface Protein

Sample Labeling



General Workflow

- Obtain a single cell suspension
- Label the suspension with antibody-oligonucleotide conjugates and antibodies (if enriching cells through flow sorting)
- Wash cell suspension
- Resuspend and count
- Proceed to 10x Genomics Single Cell protocols with Feature Barcode technology

10x Genomics supports oligonucleotide-conjugated TotalSeq™-B and C antibodies from Biolegend, but other antibodies can be linked with a 10x Genomics Feature Barcode using the Abcam Oligonucleotide Conjugation Kit. Consult Barcode Whitelist for Custom Feature Barcode conjugates (Document CG000193) for more information.

Key Considerations:

Co-label with antibodies: If performing flow sorting, prepare flow sorting and cell surface protein labeling antibody pools separately, before labeling cells with both antibody pools at the same time. When using antibodies to enrich labeled cells, use distinct antibody clones for flow sorting and cell surface protein labeling.

Optimize antibody concentrations: Always perform an initial titration of each antibody to determine the optimal concentration. Consult the Technical Note Quality Control of Cell Surface Protein Labeling using Flow Cytometry (CG000231) for guidance. Omitting optimization may lead to an excess of unbound antibodies, resulting in increased antibody aggregates, antibody sequencing, and cell staining background.

Remove antibody aggregates: Over time, antibodies clump together and form aggregates, which if not removed, carry over to final libraries and will result in wasted sequencing. Centrifuge antibody pools to pellet the antibody aggregates and transfer the supernatant for use in subsequent workflow steps.

Wash samples thoroughly: Thorough sample washing is critical to obtain high-quality data. Failure to fully remove the unbound antibody will result in high background and wasted antibody reads. Choose the appropriate washing protocol for labeled cells depending upon the starting cell viability.

Labeling with Barcode Enabled Antigen Mapping (BEAM) Reagents

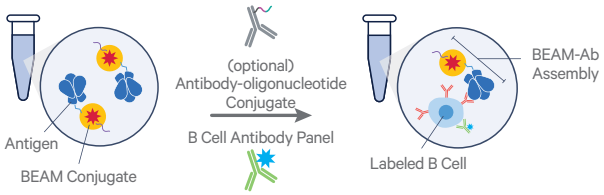
Chromium Single Cell 5' Barcode Enabled Antigen Mapping (BEAM) enables multiplexed screening of antigen targets to match unique antigens with their corresponding B-cell receptors (BCRs) and T-cell receptors (TCRs), allowing rapid discovery of antigen-specific BCR (BEAM-Ab) and TCR (BEAM-T). BEAM-Ab and BEAM-T are simple add-ons to the 10x Genomics Single Cell Immune Profiling workflow. In addition, samples can be labeled with antibody-oligonucleotide conjugates before flow sorting to enable detection of cell surface proteins.

BEAM-Ab

In this assay, user-supplied biotinylated antigens are assembled with 10x Genomics BEAM reagents to prepare BEAM-Ab Assemblies. The assemblies are used to label cells followed by washing and flow cell sorting of antigen-specific B cells.

BEAM-Ab

Reagent Assembly and Labeling



General Workflow

- Obtain a single cell suspension
- Prepare BEAM-Ab Assemblies
- Label the suspension with BEAM-Ab Assemblies and antibodies of interest
- Wash cell suspension
- Flow sort to enrich for BEAM-Ab specific (PE+) CD19+ B cells
- Proceed to 10x Genomics Immune Profiling protocols with Feature Barcode technology for BEAM

Key Considerations for BEAM-Ab:

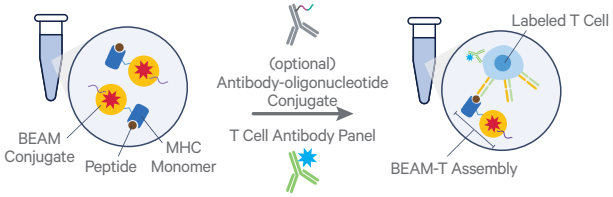
- **Antigen pre-screening:** Each antigen should be pre-screened to assess the nonspecific binding of that antigen
- **Negative controls:** For each experiment, one Negative Control Assembly is required.
- **Flow sorting:** Enriching for both BEAM-Ab positive interactions and B cell-specific cell surface markers is highly recommended to increase the frequency of antigen-specific cells in the sample.

BEAM-T

In this assay, user-supplied peptides are assembled with uniquely barcoded 10x Genomics BEAM Conjugates and loadable MHC monomers to prepare BEAM-T Assemblies. The assemblies are used to label cells. This is followed by washing and flow sorting of antigen-specific T cells.

BEAM-T

Reagent Assembly and Labeling



General Workflow

- Obtain a single cell suspension
- Prepare BEAM-T Assemblies
- Label the suspension with BEAM-T Assemblies and antibodies of interest
- Wash cell suspension
- Flow sort to enrich for BEAM-T specific (PE+) CD8+ T cells
- Proceed to 10x Genomics Immune Profiling protocols with Feature Barcode technology for BEAM

Key Considerations for BEAM-T:

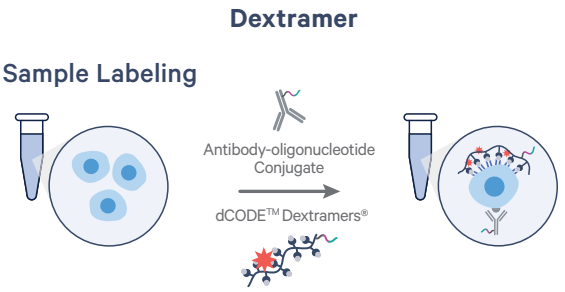
- **MHC allele compatibility:** The MHC allele displayed on the BEAM-T should match the donor's MHC.
- **Peptide pre-screening:** Each peptide that will be used in a multiplex BEAM-T experiment needs to be pre-screened to ensure proper and optimal loading in the MHC.
- **Cell labeling:** When staining cells with both BEAM-T reagents and antibodies, always perform the BEAM-T staining before antibody staining. Antibodies bound to their cell surface markers can sterically hinder the binding of BEAM-T Assemblies.
- **Controls:** One negative control per MHC-type is required in the BEAM-T Assembly pool. Negative controls are used to evaluate the background threshold during data analysis.
- **Flow sorting:** Enriching for both BEAM-T positive interactions and T cell-specific cell surface markers is highly recommended to increase the frequency of antigen-specific cells in the sample. The percentage of T cells in a diverse cell population that interacts with a specific antigen is generally very low (frequently less than 1%).

The above recommendations may not be applicable for all sample types and experiments and additional optimization may be required.

Consult the Demonstrated Protocol Reagent Assembly, Sample Labeling & Flow Sorting For Barcode Enabled Antigen Mapping (BEAM) (CG000595) or the Technical Note Chromium Single Cell 5' Barcode Enabled Antigen Mapping (BEAM) – Flow Sorting Guidance (CG000598) for more information.

Labeling with Dextramer Reagents

Multimeric MHC peptide complexes, such as dCODE™ Dextramer® reagents, bind to T-cell receptors (TCRs) with high affinity and enable detection of TCR antigen specificity.



General Workflow

- Obtain a single cell suspension.
- Label the suspension with dCODE™ Dextramer® and antibodies of interest
- Wash cell suspension
- Flow sort to enrich for dCODE™ Dextramer® specific T cells (highly recommended)
- Proceed to 10x Genomics Immune Profiling protocols with Feature Barcode technology

Key Considerations for Dextramer Labeling:

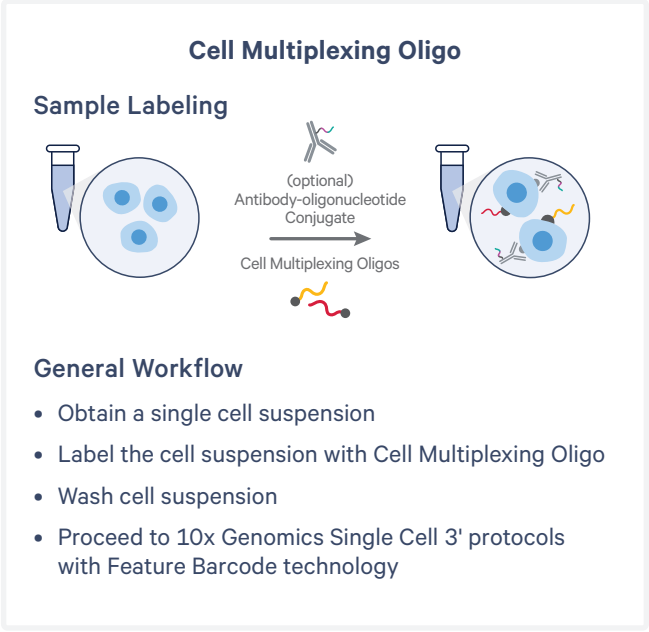
- **MHC allele compatibility:** The MHC allele displayed on the dCODE™ Dextramer® should match the donor's MHC.
- **Cell labeling:** To obtain more accurate cell calling, cells should be labeled with both antibody-oligonucleotide conjugates and dCODE™ Dextramer® reagents. dCODE™ Dextramer® reagent only types of analyses are not supported currently. dCODE™ Dextramer® labeling is performed before antibody labeling as antibodies bound to their cell surface markers can sterically hinder the binding of dCODE™ Dextramer®.
- **Controls:** Include more than one negative control in the dCODE™ Dextramer® pool. Negative controls are used to evaluate the background threshold during data analysis.
- **Flow sorting:** Enriching for both dCODE™ Dextramer® positive interactions and T cell-specific cell surface markers is highly recommended to increase the frequency of antigen-specific cells in the cell sample. The percentage of T cells in a diverse cell population that interacts with a specific antigen is generally very low (frequently less than 1%).

The above recommendations may not be applicable for all sample types and experiments and additional optimization may be required. To learn more about use of dCODE™ Dextramer® and the appropriate selection of compatible negative controls, contact Immudex.

Consult the Demonstrated Protocol Cell Labeling with dCODE™ Dextramer® for Single Cell RNA Sequencing Protocols (CG000203) for more information.

Labeling with Cell Multiplexing Oligo

Cell multiplexing allows users to label cells or nuclei (from cell lines or dissociated primary cells) with a molecular tag (Cell Multiplexing Oligo or CMO), before running pooled samples together on a 10x Genomics chip. Cell multiplexing using 10x Genomics CellPlex technology enables increased throughput by allowing for multiple labeled samples to be mixed and processed in a single GEM well. Molecular tag information assigned to cells can be used to identify and filter droplets containing more than one cell.



Key Considerations:

Follow the general guidelines below to ensure high-quality cell multiplexing data when performed alone or in combination with cell surface protein labeling.

- **Input quantity:** If the number of cells/nuclei is >200,000 (>500,000 is ideal), then perform tube-based CMO labeling (Demonstrated Protocol CG000391). If the number of cells/nuclei is <100,000 (adherent cell lines) or <200,000 (suspension cells), then perform plate-based CMO labeling (Demonstrated Protocol CG000426).
- **Cell quality:** Start with high-quality cell suspensions/nuclei (from fresh cultured cells or dissociated (primary cells) or cryopreserved single cell suspension. Poor quality samples may lead to high levels of background in cell multiplexing data.
- **Nuclei:** The CellPlex assay has only been tested on nuclei isolated using the Demonstrated Protocol Isolation of Nuclei for Single Cell RNA Sequencing & Tissues for Single Cell RNA Sequencing (CG000124). Nuclei isolated using alternative protocols or nuclei isolated from frozen tissues have not been tested for compatibility with the 3' CellPlex assay and are not supported.
- **Cell viability:** Cell viability should be >80% (>90% is ideal). Alternate washing protocols are provided in the Demonstrated Protocol CG000391 for samples with <80% viability. Choose appropriate wash protocols based on the starting sample viability.
- **Debris removal:** It is important to remove debris, as CMO lipids may bind to cell/nuclei debris fragments and can lead to high background levels if not removed. Remove debris before or after CMO labeling. Flow sorting is recommended to remove debris and dead/dying cells, leading to improved signal-to-noise ratios in CellPlex data.
- **Wash steps and supernatant removal:** Post labeling, it is critical to thoroughly wash cells and fully remove the supernatant to obtain high-quality data. Carryover of unbound CMOs will lead to high background in the final cell multiplexing data and a decrease in usable CMO reads.
- **Wash buffer**: Select an appropriate wash buffer to maintain sample viability.
- **Sample handling**: It is critical to work efficiently and avoid letting cells/nuclei sit for extended periods of time, as this can increase background noise in cell multiplexing data. Pool cells/nuclei as soon as possible after CMO labeling and washing (within 30 minutes). After pooling, load cells/nuclei onto the 10x Genomics chip as soon as possible (within 30 minutes).

For detailed guidance, consult:

- *Technical Note Chromium Next GEM Single Cell 3' v3.1: Cell Multiplexing (CG000383)*
- *Demonstrated Protocol Cell Multiplexing Oligo Labeling for Single Cell RNA Sequencing Protocols with Feature Barcode technology (CG000391)*
- *Demonstrated Protocol Plate-based Sample Preparation for Single Cell RNA Sequencing (CG000426)*

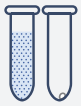
3. Sample Cleanup

A clean sample is critical for proper microfluidic performance. Dead and dying cells tend to stick together and cause cell aggregation, and therefore should be removed. While wash steps may be sufficient for sample cleanup, additional cleanup methods may be necessary to remove debris and cell aggregates.

Sample cleanup steps will result in cell loss. Follow best practices to maintain cell viability.

For limited samples (<100,000 for adherent cells or <200,000 for suspension cells), minimize number of cleanup steps performed. If cleanup is required, filtering and flow sorting are the recommended methods to use to maintain high cell recovery (>50%).

The table below outlines common sample cleanup methods and their compatibility with specific cell populations.

	Standard Cleanup Methods				Advanced Cleanup Methods	
	Filtering	Washes	Slow Centrifugation	Density Gradient	Dead Cell Removal	Cell Sorting
						
Large Debris (>30 µm)	✓	✓	⊘	✓	⊘	✓
Small Debris (<30 µm)	⊘	✓	✓	⊘	⊘	✓
Dead Cells	⊘	⊘	⊘	⊘	✓	✓
Cell Aggregates	✓	⊘	⊘	✓	⊘	✓

3.1 Standard Cleanup Methods

Some standard cleanup methods that can be used alone or in combination to remove debris and aggregates include filtration, cell washes, slow centrifugation, and density gradient centrifugation. Refer to Tips & Best Practices for more guidance on filtering, cell washes, and cell centrifugation.

Density Gradient

Density gradient centrifugation allows the separation of cells based on their shape and size and may be used to isolate specific cell populations through centrifugation with a gradient media. There are a variety of density media and kits, each with their own properties and use cases. The heterogeneous cell mixture is carefully layered on top of the density gradient media and centrifuged (with or without a brake depending on the protocol), separating by cell density. The desired populations can then be carefully isolated. Density gradient centrifugation is commonly used in fractionating PBMCs or isolating plasma from blood cells, but may also be used for removing large debris and cell aggregates.

3.2 Advanced Cleanup Methods

The following sections provide a brief overview of the two most common advanced cleanup methods, flow sorting and bead-based dead cell removal. When possible, flow sorting is the preferred method.

Flow Sorting

Flow sorting is a powerful tool that allows selection and purification of a specific population of interest and ensure a uniform single cell suspension compatible with 10x Genomics products. It is strongly recommended to consult a core facility or an experienced flow user before initiating a flow sorting experiment.

Below are some general guidelines for optimizing sorting conditions before running a 10x Genomics experiment. These guidelines emphasize gentle cell handling to preserve cell health and viability.

Cell loss during flow sorting is common. Optimize the protocol steps accordingly.

Pre-sorting guidelines

Before sorting, it is important to properly prepare sample, collection tube, and instrument for a successful run.

Instrument guidelines

Instrument settings and gating parameters require adjustment based on the sample type and the instrument.

Instrument settings

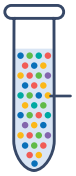
- Low to medium sample pressure or flow rate is recommended.

TIPS *Increase flow rate if population of interest is very small (<0.01%) or if sorting a large number of cells.*

- Sorting efficiency should be high
- Low nozzle pressure is required to achieve the high sample quality necessary for single cell and nuclei applications. Avoid high to low-pressure changes.

Sorting Guidelines

Sample

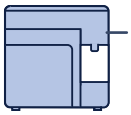


- Add live/dead cell marker 1–5 min before sorting
- 5–10 x 10^6 cells/ml
- Temperature: 4ºC

Collection Tube/Buffer

- Tube coated & filled with collection buffer before sorting
- Composition: optimal for sample with increased concentration of serum/BSA to account for dilution during sorting process
- Compatible with 10x Genomics workflow (no EDTA or excessive magnesium)
- Volume: depends on sorter, nozzle, sort mode, and desired # of sorted cells
- Temperature: 4ºC

Cell Sorter



- Adjust sort stream for each sort container
- Ensure sheath fluid is compatible with 10x Genomics workflow (no EDTA or excessive magnesium)

- Use a larger nozzle size to ensure lower sheath pressure. This helps minimize shear stress.
- Optimize PMT (Photomultiplier tube) voltage. Contact flow core for assistance
- Use the Purity setting as opposed to Yield setting for flow sorting.

Adjusting voltage

Good

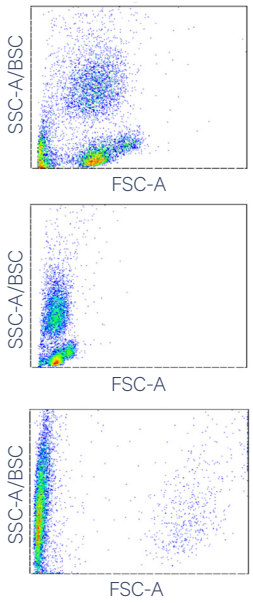
- Clear separation of cells of interest and cell debris, dead cells, etc.

Too Low

- Loss of resolution of small events.

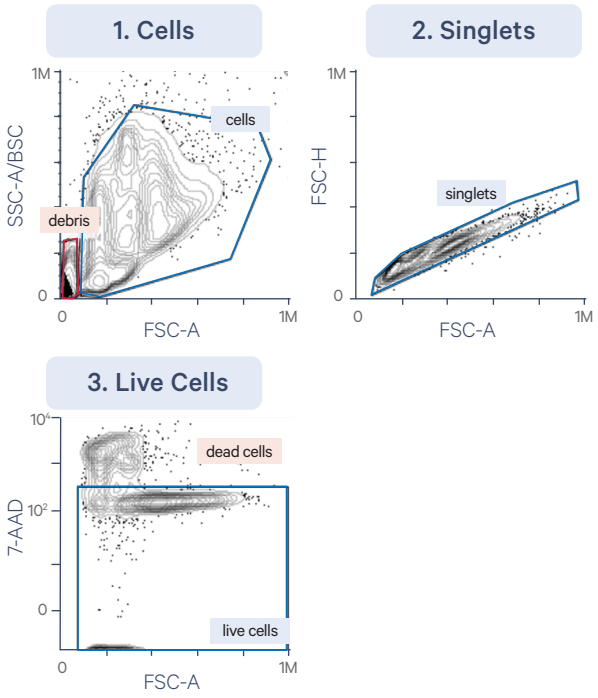
Too High

- Loss of resolution of large events.



FSC-A: forward scatter-area
SSC-A: side scatter-area
BSC: back scatter

Gating parameters



Gating parameters

Gating out unwanted cell populations will help enrich for the population of interest.

- **Debris:** Gate on forward scatter-area (FSC-A) vs. side scatter-area/back scatter (SSC-A/BSC). Gating for granularity will exclude very small debris particles
- **Doublets:** Plot the height (FSC-H) or width against the area for FSC or SSC to identify and exclude doublets.
- **Dead cells:** Use FSC-A vs. viability dye to gate out positive cells.
- Refer to Appendix A3 for example gating considerations for nuclei.

Sorting guidelines

- Use a collection buffer that is compatible with 10x Genomics Single Cell workflows. It should not contain EDTA or excessive magnesium.
- Collection buffer should be optimal for the sample and should contain a higher than normal concentration of serum or BSA, as flow sorting will dilute the collection buffer as cells are sorted.
- Maximize sort efficiency during sorting.

Post-sorting guidelines

- For ≤10,000 cells, sort into low volume of collection buffer.
- For >10,000 cells, the sample can be concentrated by centrifugation and removing supernatant. If no pellet is visible, leave 20–50 µl supernatant behind.
  Select a speed compatible with sample type. For low yield and fragile cells, a longer, slower speed is recommended.
- Cells/nuclei are often more fragile after sorting. Minimize additional sources of cell stress like handling, temperature, and time. Keep samples on ice before, during, and after sorting
- Count cells/nuclei after sorting or after concentration.

  Numbers reported by flow cytometers can vary by up to 50% and must be confirmed before loading. If yield is very low, additional counting should be avoided.

- Promptly load cells into 10x Genomics chips after sorting/post-sorting concentration to avoid a decrease in viability

  Avoid excessive time (>30 min) on ice if sorting and loading more than one sample.

Dead Cell Removal and Bead Enrichment Cleanup

Bead-based enrichment methods rely on beads that recognize specific moieties, sequestering those cells so the flow through is depleted of that population. It can also serve as sample cleanup method.

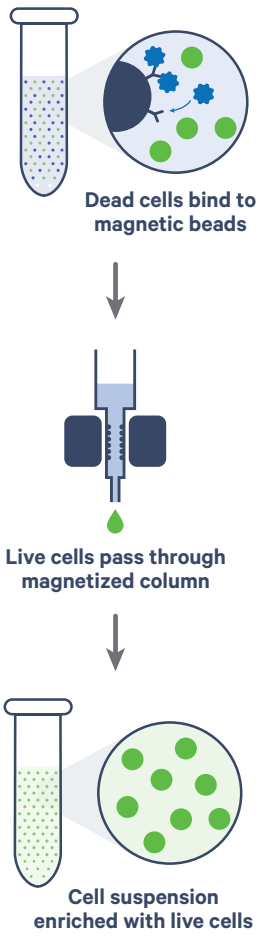
	Flow Sorting	Beads
Purity	>90%	Varies by sample type, typically <90%
Throughput	One at a time	Multiple samples in parallel
Starting sample input	As low as 100,000	~1 x 10^6 recommended
Temperature	Regulated	Benchtop (on ice/ room temperature)
# of populations that can be purified	Multiple (2-6; sorter dependent)	1 (positive or negative selection)

While flow sorting is preferred, Miltenyi Biotec offers column-based MACS MicroBeads for dead cell removal and other cell depletion or enrichment methods.

Consult the Demonstrated Protocol Removal of Dead Cells for Single Cell RNA Sequencing (CG00093) or the Demonstrated Protocol Enrichment of CD3+ T Cells from Dissociated Tissues for Single Cell RNA Sequencing and Immune Repertoire Profiling (CG000123) for more information.

Bead-based methods typically require large starting sample volumes and may result in cell loss. Stressed or early apoptotic cells may be marked as dead cells, therefore minimizing stress during sample preparation is important.

TIPS *Filter cell suspensions (30 µm) before performing the Dead Cell Removal protocol. Dead cells can clog pores in magnetic column. Refer to manufacturer's guidelines for recommendations to reduce cell loss.*



Other magnetic beads, such as EasySep from STEMCELL Technologies and Dynabeads from Invitrogen, have been used to successfully enrich cells of interest for Barcode Enabled Antigen Mapping (BEAM) and are likely to be successful for other applications with 10x Genomics Single Cell Gene Expression and Single Cell Immune Profiling assays.

4. Cell Counting and Quality Control

4.1 Overview

Cell suspension visualization and quantification is critical for determining accurate concentration, viability, suspension quality, and cell sizes before use in 10x Genomics Single Cell assays.

Using an automated cell counter is recommended for most applications.

Cell Counting Best Practices

- Check for debris or cell aggregates. They may cause microfluidic chip clogs. Additional pipetting or filtering may be required.
- Pipette gently to avoid damaging cells.
- Pipette mix sample immediately before counting to ensure accurate representation.
- Two independent draws per sample are recommended. Counts should be within 25% of each other. For counters capable of taking multiple counts per sample, count at least twice from one draw (4 total counts recommended).
- Optimize instrument settings and counting protocol based on sample type. Samples types with very small size (such as isolated nuclei), highly variable cell size, or high levels of debris may require optimizing instrument settings, performing multiple counts, or manual counting via hemocytometer.
- The cell concentration should always be determined based on the total number of cells, not just viable cells.

4.2 Automated Counter Overview

Note: Refer to the manufacturer's user guide for details and advice on operation and usage.

A range of automated counters are available for purchase for use in counting single cell suspensions. Choosing the appropriate counter can depend on a number of important factors (see Table 1 and 2).

TIPS *Cell size is directly correlated to RNA content, relative cDNA yields, and transcript counts. Visual estimation of cell size can provide relative expectations in yield. Knowing the approximate cell diameter may help in choosing an appropriate automated cell counter.*

The following automated counters were evaluated and found to have comparable accuracy when counting single cell suspensions:

- Nexcelom Cellaca MX FL5
- Nexcelom Cellometer K2
- Thermo Fisher Countess II FL
- Thermo Fisher Countess 3 FL
- Logos Biosystems LUNA-FL
- ChemoMetec NucleoCounter NC-202
- Beckman Coulter Vi-CELL BLU

TIPS *Users may consider additional counters with comparable features and should prioritize features most important for their own use cases.*

Advantages and considerations between automated counters evaluated as compared to manual counting are described in Table 1.

	Advantages	Considerations
Cellaca MX FL5	• Fully automated sample focusing and counting • Robust and accurate for challenging debris-rich samples, nuclei are officially supported	• Custom protocol creation may require additional training/understanding of the instrument software
Cellometer K2	• Straightforward ability to fine-tune focus, brightness, and exposure • Robust and accurate for challenging debris-rich samples, nuclei are officially supported	• Requires manual focus for all counts
Countess II FL	• Auto and user-adjusted focus • Straightforward ability to fine-tune focus, brightness, and exposure	• Older device, being replaced by Countess 3 FL
Countess 3 FL	• Auto and user-adjusted focus • Straightforward ability to fine-tune focus, brightness, and exposure	• Very small cells <4 µm not officially supported • Not compatible with nuclei counting • Slightly slower counting speed compared to Countess 2 FL (~30 sec vs ~15 sec)
LUNA-FL	• Nuclei are officially supported	• Requires manual focus for all initial counts
NucleoCounter NC-202	• Fully automated dye mixing, focusing, and counting • Robust and accurate for more challenging debris-rich samples (provides debris metric)	• Users limited to pre-set counting protocols, may limit flexibility or customization for challenging samples
Vi-CELL BLU	• Fully automated sample mixing, loading, dye mixing, focusing, and counting to reduce user variability • Ability to reanalyze previous samples with custom protocols • Robust and accurate for more challenging debris-rich samples	• Custom protocol creation and data manipulation may require expert users • Daily calibration is recommended
Hemocytometer	• Highly accurate for common sample types (cell lines and PBMCs) and challenging samples (nuclei and debris rich)	• Low throughput and time intensive • Subjective counting/viability by user • Limited range in cell concentration for accurate counting

Table 1. Advantages and considerations of manual and automated cell counters

	Cellaca MX FL5	Cellometer K2	Countess 2FL	Countess 3FL	LUNA-FL	Nucleocounter NC-202	ViCell Blue	Hemocytometer
Instrument Cost	High	Medium	Low	Low	Low	Medium	High	N/A
Fluorescence	Yes	Yes	Yes	Yes	Yes	Yes	No	Microscope-dependent
Requires additional fluorescent filter	No	No	Yes	Yes	No	No	N/A	N/A
Throughput	Medium	Low	Low	Low	Low	Low	High	Low
Time per brightfield count	~2 sec	~60 sec	~ 15 sec	~30 sec	~7 sec	~30 sec	~80–120 sec	~10 min
Sample volume	25 µl	10 µl	10 µl	10 µl	Trypan Blue: 10 µl Fluorescence: 18 µl	100 µl	Fast: 170 µl Regular: 200 µl	10 µl
***Concentration (cells/ml)**	1×10^5–1×10^7	1×10^5–1×10^7	1×10^4–1×10^7	1×10^4–1×10^7	1×10^4–1×10^7 (5×10^4–1×10^7 preferred)	5×10^4–1×10^7	1×10^4–1.5×10^7	2.5×10^5–2.5×10^6
***Cell Diameter**	5–80 µm	4–90 µm	7–60 µm	~4–60 µm	1–90 µm (5–60 µm preferred)	5–30 µm	2–60 µm	Microscope-dependent
Allow adjustment of focus and light intensity	Yes	Yes	Yes	Yes	Yes	No	Yes	N/A
Custom cell calling protocols	Yes	Yes	Yes	Yes	Yes	No	Yes	N/A
21 CFR Part 11 compliant software	Yes	Yes	No	Yes	No	Yes	Yes	N/A
Reports and manual data transfer	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Data transfer via networking	Yes	Yes	No	Yes	No	Yes	Yes	N/A

*Based in manufacturer's documents/resources

Table 2. Comparison of key characteristics of automated and manual counters

4.3 Considerations when using Automated and Manual Cell Counters

Refer to Table 2 for full comparison of all considerations between counters evaluated.

Cost

Instruments that require a higher investment typically offer advanced features such as higher throughput and decreased hands-on time. In addition to the instrument, users can also incur costs for consumables, additional features such as fluorescent capabilities, and ongoing service or software contracts.

Sample Type

Sample type may impact accuracy when using any counting method. Factors to consider include small or irregular sizes, aggregates or clumps, and high levels of debris. Automated counting algorithms are generally robust, but some sample types may result in greater inaccuracies (Figure 2). Examples include isolated nuclei or dissociated tumor cells (DTCs). It is recommended to take a minimum of two counts per sample. More challenging samples may benefit from additional counting.

Small Cells and Isolated Nuclei

Small cells and isolated nuclei (<10 µm) may be difficult to distinguish from debris or other particles. Using a fluorescent viability stain (e.g. AO/PI or Ethidium Homodimer-1) is strongly recommended. Trypan blue may result in an overestimated cell or nuclei count.

TIPS *If small cells or nuclei are routinely analyzed, consider an appropriate automated counter. Refer to the manufacturer for official guidance.*

Counters supporting nuclei counting are Cellaca MX FL5, Cellometer K2, and LUNA-FL.

Countess II FL and NucleoCounter NC-202 have also been used for nuclei counting. Manual counting using hemocytometer is also recommended. Countess 3 is currently incompatible with isolated nuclei counting.

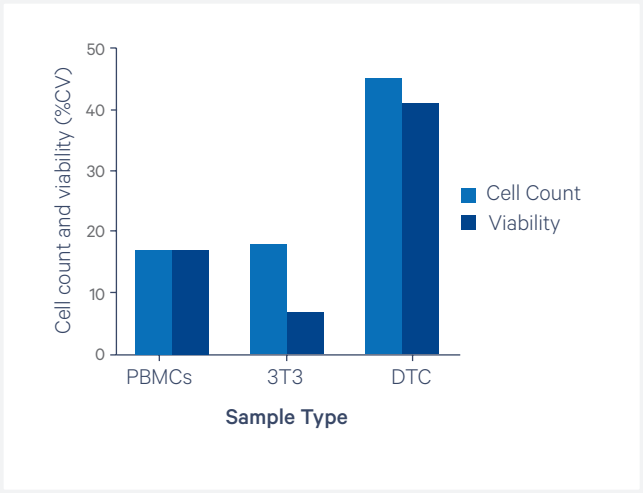


Figure 2. Counts and viabilities of three sample types were evaluated across several automated counters: Countess II FL, Countess 3 FL, LUNA-FL, Cellaca MX FL5, and ViCELL BLU. Greater coefficients of variation (standard deviation/mean) were seen across automated counters for both cell count and viability determination for samples with high amounts of debris (DTC), while samples with lower amounts of debris (3T3 and PBMC) show more consistency across counters.

Cell Aggregates and Debris

Automated cell counter software may struggle to accurately count samples with high amounts of aggregation or debris. Optimizing sample preparation procedures can improve sample quality and counting accuracy.

TIPS *Manual counting by hemocytometer is also recommended for challenging samples, e.g., debris-rich cell/nuclei suspensions.*

If possible, creating custom protocols that gate out debris and cell aggregates is recommended. The Cellaca MX FL5 and Vi-CELL BLU can differentiate cell aggregates, while the NucleoCounter NC-202 can quantify debris particles.

Figure 3 shows representative images of samples with high cell debris/aggregates compared to an ideal sample. Figure 4 shows the impact of adjusting aggregated cell calling parameters on cell detection.

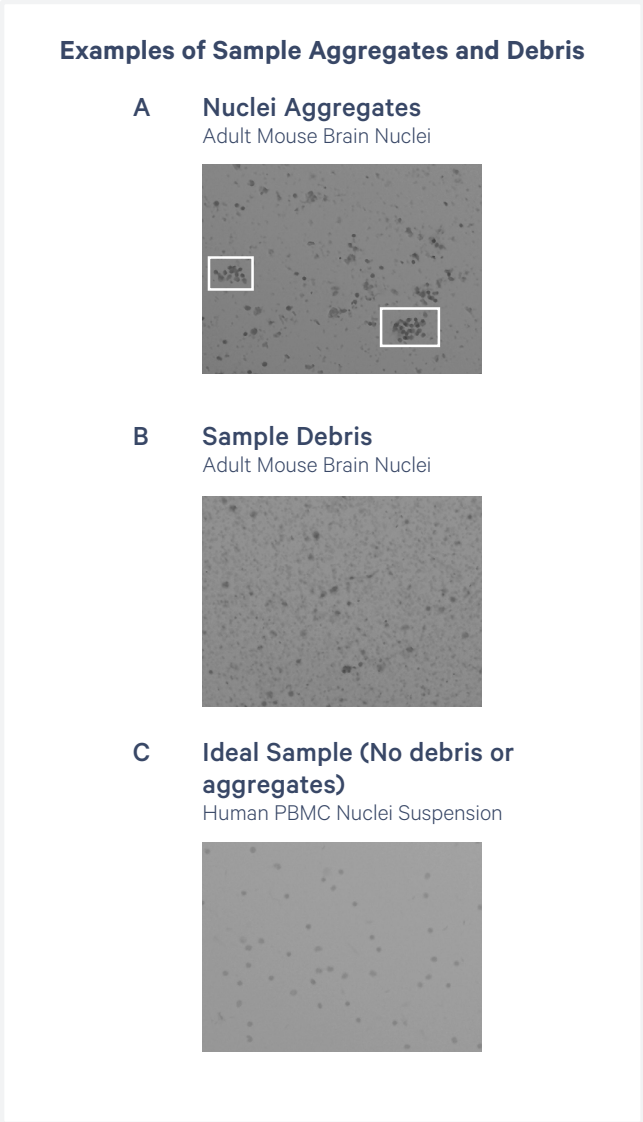


Figure 3. Representative images showing (A) adult mouse brain nuclei with elevated nuclei aggregates, (B) adult mouse brain nuclei with high levels of debris, and (C) an ideal human PBMC nuclei suspension lacking cell aggregates and debris.

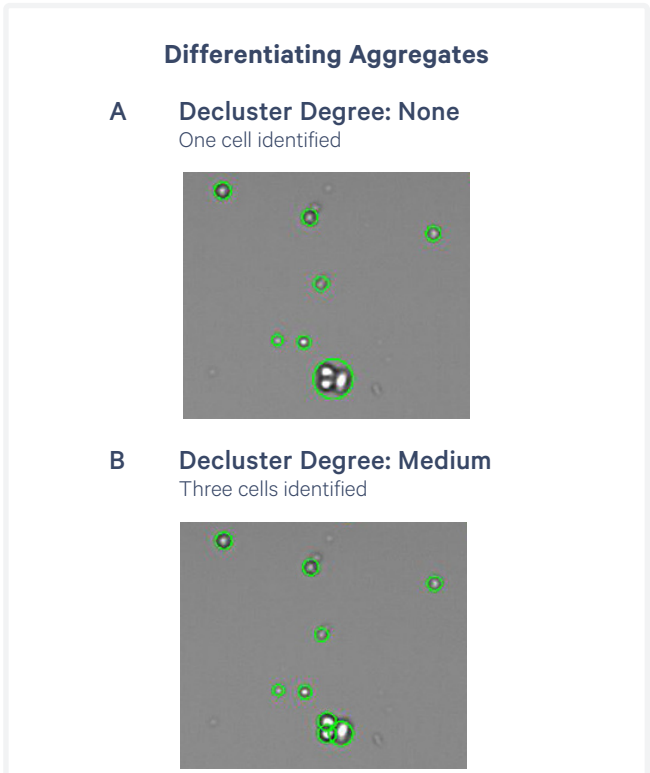


Figure 4. Lung cancer DTC sample stained with trypan blue and imaged on the Vi-CELL BLU cell counter shows presence of cell aggregates. Adjusting the cell calling parameter "decluster degree" allows this software to detect cells that are clustered. (A) When the "decluster degree" is set to "none" the cluster of cells are counted as one cell. (B) Adjusting the "decluster degree" to "medium" allows the Vi-CELL BLU software to call individual cells within the aggregate.

Fluorescent Viability Counting

Determining cell viability using staining is critical for accurate counting. For isolated nuclei, fixed cells, or cell suspensions with high amounts of debris, using a fluorescent nucleic acid dye and capable automated counter or microscope is strongly recommended. Avoid trypan blue for such samples, as it can lead to overestimated counts (Figure 5). It is recommended to use a dual fluorescent stain, such as AO/PI to mark viability and eliminate apoptotic or necrotic cells and debris.

For further information on counting fixed cells or isolated nuclei, consult the Demonstrated Protocol Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling (CG000478).

Follow manufacturer's guidance on proper storage and preparation of viability stains.

Comparison of fluorescent and non-fluorescent staining dyes

Dye	Mechanism of Action	Useful for
Trypan Blue	Cell impermeable, stain dead cells or nuclei but may also stain debris	Counting cell or nuclei suspensions free of debris
AO/PI*	AO: Cell permeable, stains all nucleated cells green PI: Cell impermeable, stain dead cells or nuclei red	Live and dead cell calling in suspensions with debris
Ethidium Homodimer-1*	Cell impermeable, stain dead cells or nuclei	Counting nuclei and fixed cells, measuring cell viability

* Fluorescent dye

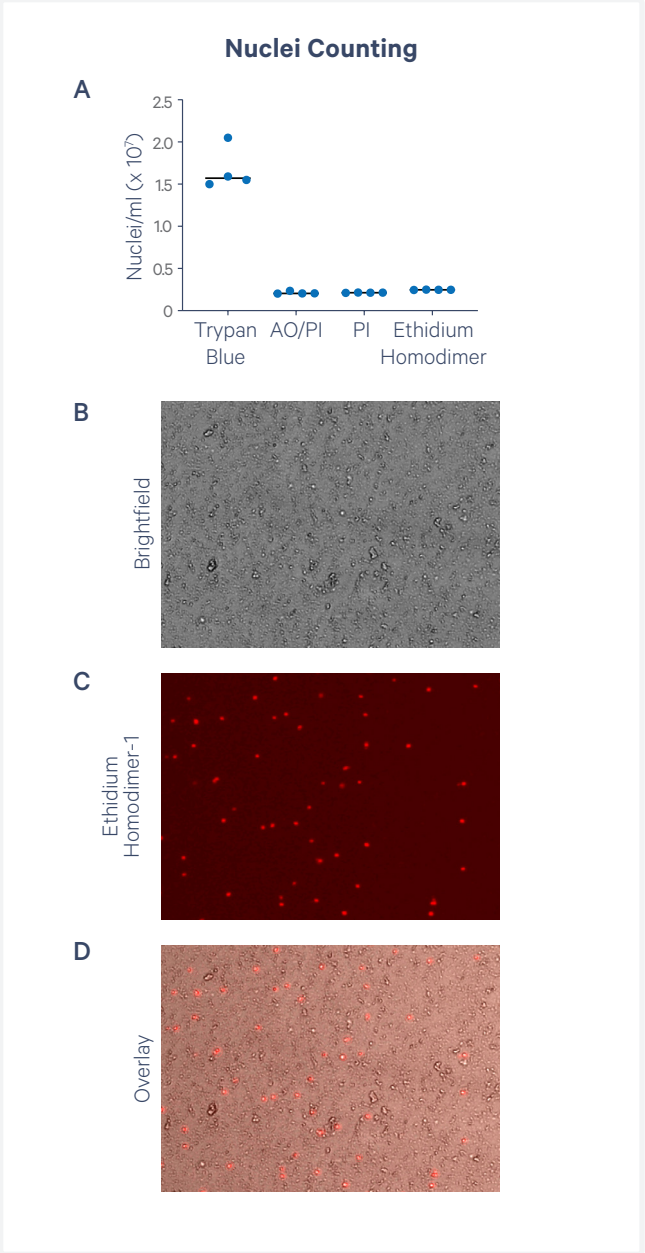


Figure 5. Adult mouse brain nuclei stained with trypan blue, AO/PI, Propidium Iodide, and Ethidium Homodimer-1 before counting on Luna FL. Trypan blue staining resulted in significantly higher counts than the fluorescent dyes due to stained debris included in the nuclei count (A).

Representative images on Luna FL viewed in brightfield (B), with Ethidium homodimer-1 (C), or overlay of brightfield and Ethidium Homodimer-1 (D).

Optimize fluorescent signal and instrument settings (for each sample):

- Visually evaluate cell counting images for strong fluorescent signal and minimal to low background.
- Assess if the cell resuspension buffer contributes to background fluorescence. Diluting the cells and buffer solution in PBS, while staying within the automated counter's optimal cell counting concentration, may improve fluorescent signal to noise and improve counting accuracy.
- Analyze image to ensure majority of live and dead cells are called appropriately. Adjust and recount as necessary.
- Typical adjustments include focus, fluorescence exposure time, and fluorescence intensity.

Fluorescence exposure time: Visually check image and adjust exposure time to find the optimal range.

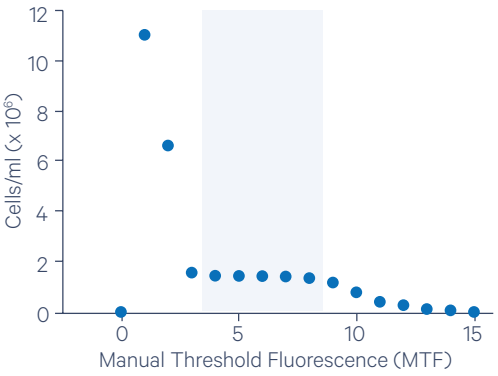
- Too low = too dim for detection
- Too high = increased background and reduced contrast

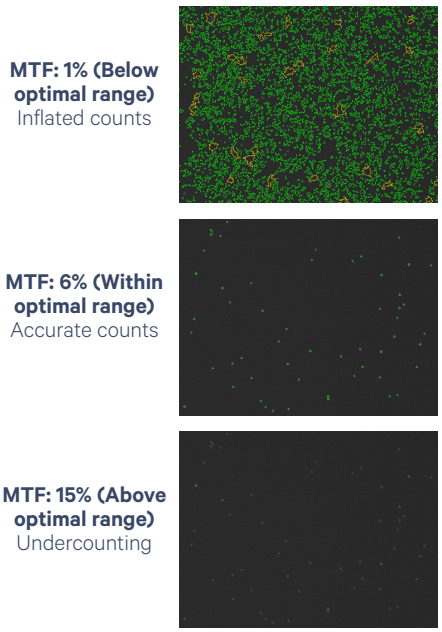
Fluorescence thresholding: Set optimal fluorescence threshold to ensure software is accurately distinguishing live and dead cells from background debris. This reduces false positives and negatives.

- Too low = overly permissive counting and inflated cell counts.
- Too high = overly stringent and under counting.
- Visually check live and dead cell calling and adjust as necessary.

Example – Impact of Optimal Fluorescence Thresholding

Fixed human PBMCs prepared according to the Single Cell Gene Expression Flex workflow → Stained with AO/PI & quantified using the Cellaca MX FL5 → Manual Threshold Fluorescence (MTF), a fluorescence threshold parameter, adjusted from 0-15% to determine an optimal fluorescence intensity threshold for cell counting.





MTF: 1% (Below optimal range)
Inflated counts

MTF: 6% (Within optimal range)
Accurate counts

MTF: 15% (Above optimal range)
Undercounting

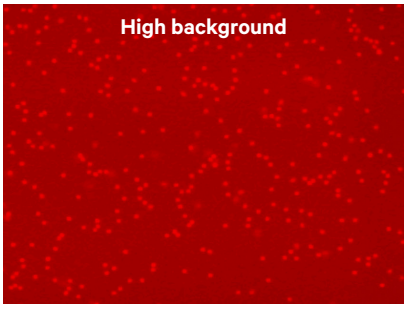
Impact of sample dilution on fluorescent signal

Some sample or cell resuspension buffers may have background autofluorescence that impacts counting accuracy; changing the fluorescence threshold/gating might not fix the background issue. In such cases, diluting sample leads to stable counts over a broader range of fluorescence thresholds.

Example – Impact of Sample Dilution

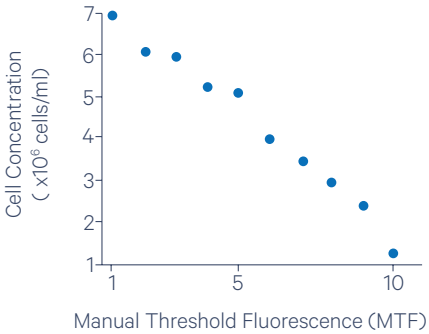
Fixed human PBMCs prepared according to the Single Cell Gene Expression Flex workflow

Undiluted (~2,000 cells/ml)
Stained with AO/PI & counted on LUNA-FL



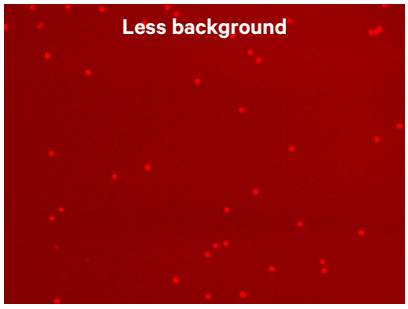
High background (signal to noise issue) may lead to variable and inaccurate cell counting. Automated counting software may struggle with reduced signal to noise, resulting in difficulty in differentiating cells from the background. Users may struggle in selecting an appropriate fluorescence threshold setting to take an accurate cell count.

Highly variable cell counts



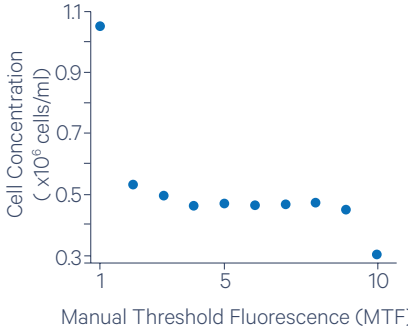
Highly variable cell counts for the undiluted sample upon varying the Fluorescent Threshold setting from 1–10 and capturing a cell count at each setting

Diluted 1:10 in PBS (~200 cells/ml)
Stained with AO/PI & counted on LUNA-FL



By diluting the sample, the high fluorescence background is reduced (improved signal to noise), which leads to more consistent counting across a wider range of fluorescence thresholds, and less dependency that the user selects an exact fluorescence threshold to determine an accurate cell count.

More consistent cell counts



More consistent cell counts for the diluted sample upon varying the Fluorescent Threshold setting from 1–10 and capturing a cell count at each setting

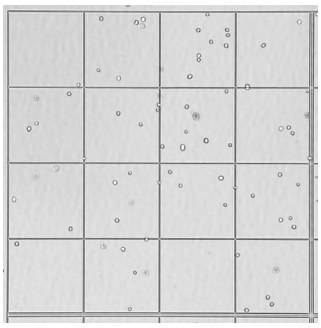
User Variability

User variability can be introduced in both manual and automated cell counting protocols. Manual counting by hemocytometer is subjective and numbers can vary from user to user. Using an automated counter helps reduce variability, however some still remain. Sources of user variability may include:

- Pipetting and cell solution handling steps
- Manual adjustments to achieve proper focus and light intensity
- Modifications to cell determination parameters such as circularity, brightness, and size
- Manual calculations for dilution factor, cell count, and viability

Efforts to eliminate user variability will give users more reliable counting numbers to ensure accuracy in downstream single cell experiments.

Example of User Variability & Impact on Downstream Experiments



- 3T3 cells stained with 0.4% trypan blue and imaged via hemocytometer.
- Live cell counting performed by two users on a 1 mm^2 area.

User 1	User 2	Variability in Cell Counts
63 cells	69 cells	6 cells (~9%)

A user targeting 10,000 recovered cells may instead only recover 9,000 total cells if their initial cell count is incorrect and overestimated by 10%.

Pipetting

Follow good pipetting practices, minimize sources of pipetting error, and ensure pipettes are routinely calibrated.

Ensure homogeneous mixture by mixing cell suspensions thoroughly before counting as cells settle over time. Insufficient or no mixing may result in high variability and inaccuracy in counts and viability readout.

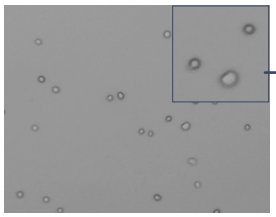
Some automated counters eliminate error from manual pipetting steps on board the instrument. The Vi-CELL BLU performs automated sample mixing and cell staining while the NucleoCounter NC-202 performs automated cell staining.

Focus and Light Intensity

Achieving accurate focus and light intensity is critical to accurately count cells and calculate viability. When proper focus and light intensity is used for cells stained with trypan blue, live cells will appear round, with light, bright centers and distinct dark borders while dead cells will appear uniformly blue or dark, and may lack distinct edges.

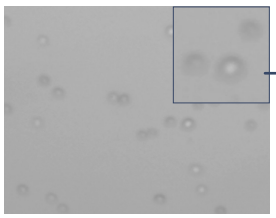
Impact of Focus on Counting

Optimally Focused



Appearance of live cells
- Round with light, bright centers
- Distinct dark borders

Incorrectly Focused



Appearance of live cells
- Broad, non-distinct dark borders
- Absent/minimal light-colored centers
- Blurry, lack sufficient visual contrast between borders and centers
- Dark and uniform in color

However, achieving proper focus and light intensity can be subjective, and modifying focus and amount of light can shift perception of what a user or cell calling algorithm may perceive as a live or dead cell (Figure 6). Using an automated cell counter that can perform autofocus and auto-light adjustment will ensure accurate cell calling and consistency between users, samples, and over time.

Users should not rely on automatic settings to always be correct and should still perform a visual check to ensure live and dead cells are being called accurately.

Challenging samples that contain high amounts of debris or are small in size like isolated nuclei, may benefit from visual verification performed by an experienced user. Manual adjustments of focus and lighting may help differentiate between live and dead cells in these challenging cases.

Automated counters that allow for adjustments of focus and light intensity are listed in Table 2.

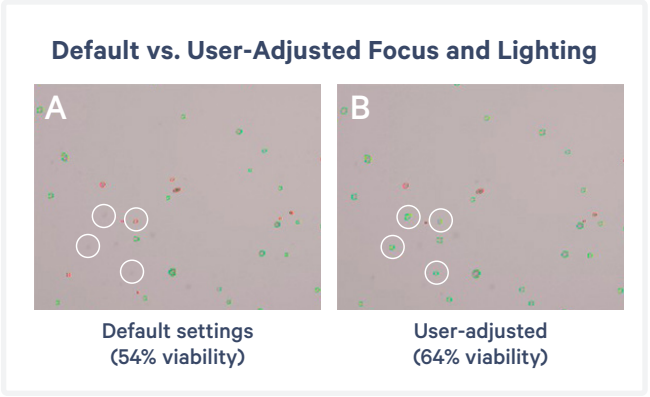


Figure 6. Lung cancer Dissociated Tumor Cells (DTCs) stained with trypan blue were imaged on Countess 3 FL. Default focus parameters (A) reported 54% viability, whereas fine tuning brightness and focus by an expert user led to 64% viability (B), which is in close alignment with manual analysis via hemocytometer (69% viable).

Cell Determination Parameters

Automated counters offer additional advantages allowing automated determination or gating of live versus dead cells. Users should familiarize themselves with these settings for their automated counter, as custom creation of cell calling protocols offer greater accuracy and consistency.

Adjustable cell gating parameters may include cell size, circularity/roundedness, and brightness or fluorescent intensity. See Figure 7 for an example of the impact of adjusting the cell determination parameters in cell calling. Each automated cell counter will have unique cell calling algorithms for determining live versus dead cells and thus results may vary depending on cell size, shape, and presence of aggregates or debris. Any manual adjustments to cell determination parameters should be saved as set protocols for consistency between samples, users, and over time. Automated counters that allow custom protocol creation are listed in Table 2.

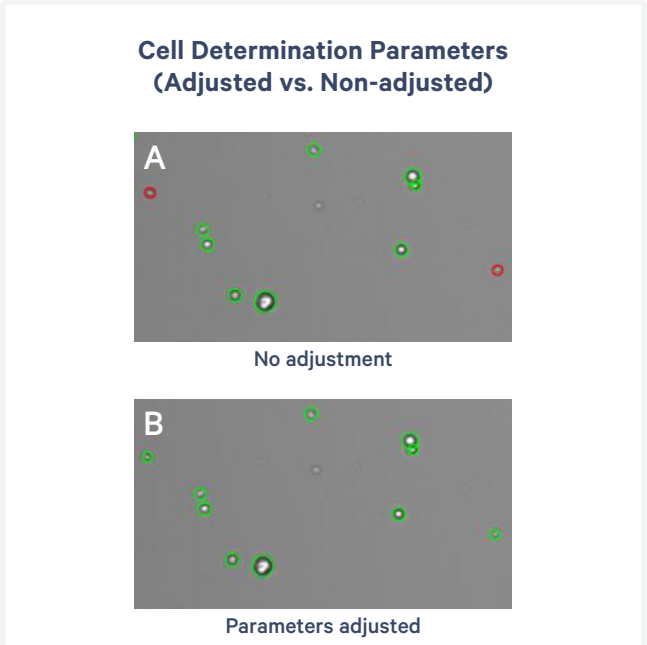


Figure 7. Lung cancer DTCs when stained with trypan blue and imaged on the Vi-CELL BLU without adjustment (A). On adjusting the cell calling parameters for cell sharpness (B), viable spot brightness and viable spot area, the software is able to more accurately call viable cells.

Ease of Use

When considering an automated counter, consider each platform's ease of use including number of counts taken per day, number of users trained on the instrument, and if additional training is required to create cell counting protocols. Different counters offer different capabilities that may impact their ease of use. For example, some counters offer advanced features but may have a less straightforward interface.

Counting time

The time required to count each sample varies based on the counter and its capabilities. Brightfield counting is typically faster than fluorescent counting. Some counters take both brightfield and fluorescent images, which increase overall counting time. Counter software may cross-reference populations between the two images in order to determine cell calling. Some sophisticated counters capture multiple images per sample to determine concentration.

TIPS *If possible, creating and using pre-set cell calling protocols can decrease counting time and increase throughput for all automated cell counters.*

Sample throughput

If a high number of samples will be counted on a regular basis, consider selecting an automated counter that offers higher sample throughput capabilities. Higher throughput counters offer the ability to prepare and load several samples at a time to be analyzed automatically without requiring hands-on manipulation by the user for each individual sample.

Data Analysis and Report Generation

Some automated counters can capture and save field-of-view images, as well as generate reports stating cell count (live, dead, and total cell count) and cell viability details. Networking capabilities allow for streamlined data transfer and storage. This provides the option to save large data files, while eliminating the need for manual file export.

Some automated counters offer the additional feature of 21 CFR, part 11 FDA compliance for electronic record keeping. These software features support compliance with FDA guidelines for user access, security, and audit trails. In addition, they provide tracking protocol changes and lock final versions and review and approve data electronically.

Appendix

A1. Alternative Buffers and Cell Culture Media for Washing and Resuspending Samples

This section describes buffers and media that are compatible with Single Cell Gene Expression and Immune Profiling assays.

Cell Culture Media

- Eagle's Minimum Essential Medium (EMEM) + 10% FBS
- Dulbecco's Modified Eagle Medium (DMEM) + 10% FBS
- Iscove's Modified Eagle Medium (IMEM) + 10% FBS
- Roswell Park Memorial Institute (RPMI) + 10% FBS
- Ham's F12 + 10% FBS
- 1:1 DMEM/F12 +10% FBS
- M199
- NbActiv +/- 1% BSA

Alternative Buffers

- Dulbecco's Phosphate-Buffered Saline (DPBS) with serum
- Hank's Balanced Salt Solution (HBSS) with serum
- 1X PBS + up to 10% FBS
- 1X PBS + up to 2% BSA

If using a buffer that is not listed, minimize the cell suspension volume added to the Single Cell Master Mix. Buffers not listed here have not been tested by 10x Genomics.

If using a proprietary buffer, incorporate a wash step and reduce the resuspension volume to minimize carryover of any potentially inhibitory substances.

A2. Dead Cell Markers Compatible with 10x Genomics Single Cell Workflows

- 7-aminoactinomycin D (7-AAD): 7-AAD associates with DNA in cells with compromised cell membranes and can be used to label and remove dead (non-viable) and early apoptotic cells (7-AAD+)
- DAPI/Hoechst
- Other live/dead stains may also be used to enrich viable cell populations of interest in single cell suspensions.

For ATAC and Multiome ATAC + Gene Expression Assays

The choice of sorting dye is critical for both ATAC and Multiome ATAC + gene expression assays. Several intercalating or minor-groove binding dyes can disrupt chromatin structure and negatively impact data quality.

The following dyes are **not recommended**:

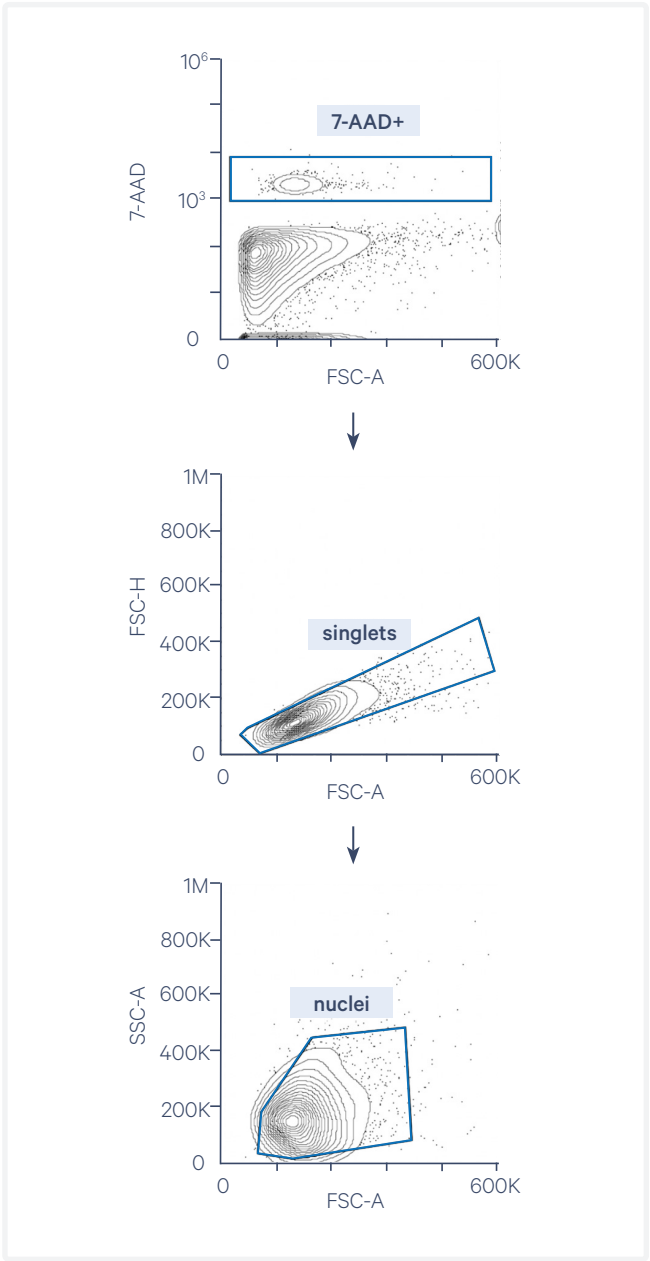
- Propidium Iodide (PI)
- Diamidino-2-phenylindole (DAPI)
- Ethidium Homodimer
- Vybrant DyeCycle Green
- Zombie Violet

7-AAD has shown reproducible success for both ATAC and Multiome ATAC + gene expression assays. This dye maintains high sensitivity and targeting, while also reducing non-cell/background reads.

A3. Gating Strategy for Sorting Nuclei

Gating Considerations for Nuclei:

- Gate for nuclei dye positive events using 7-AAD+ or other DNA intercalating dye.
- Gate on singlets using FSC-A and FSC-H.
- Gate on FSC and SSC/BSC (back scatter) to select nuclei. Nuclei are smaller than cells, but slightly larger than debris. Carefully set FSC and SSC/BSC to ensure the population is not lost.



Document Revision Summary

Document Number	CG00053
Title	Cell Preparation for Single Cell Protocols
Revision	Rev C to Rev D
Revision Date	June 2023

Specific Changes:

Update in organization and format along with additional guidance on:

- Best practices
- Sample preparation
- Sample cleanup
- Automated cell counters

General Changes:

Updated for general minor consistency of language, terms, and format throughout

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