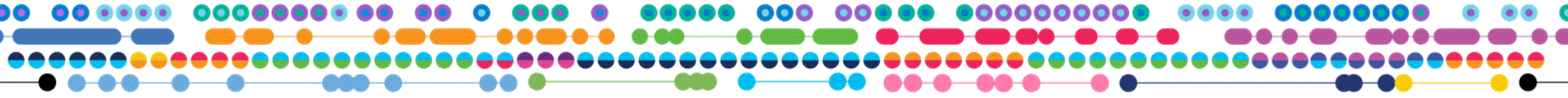
10x GENOMICS

Sample Preparation for 10x Genomics

A How To Guide: Considerations and Best Practices

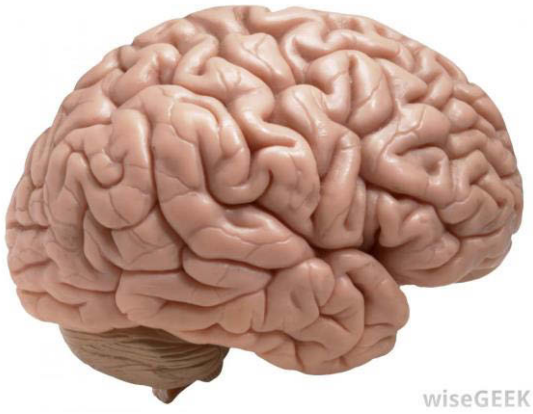
Agnieszka Ciesielska PhD, STA 10x Genomics



From averages to high resolution

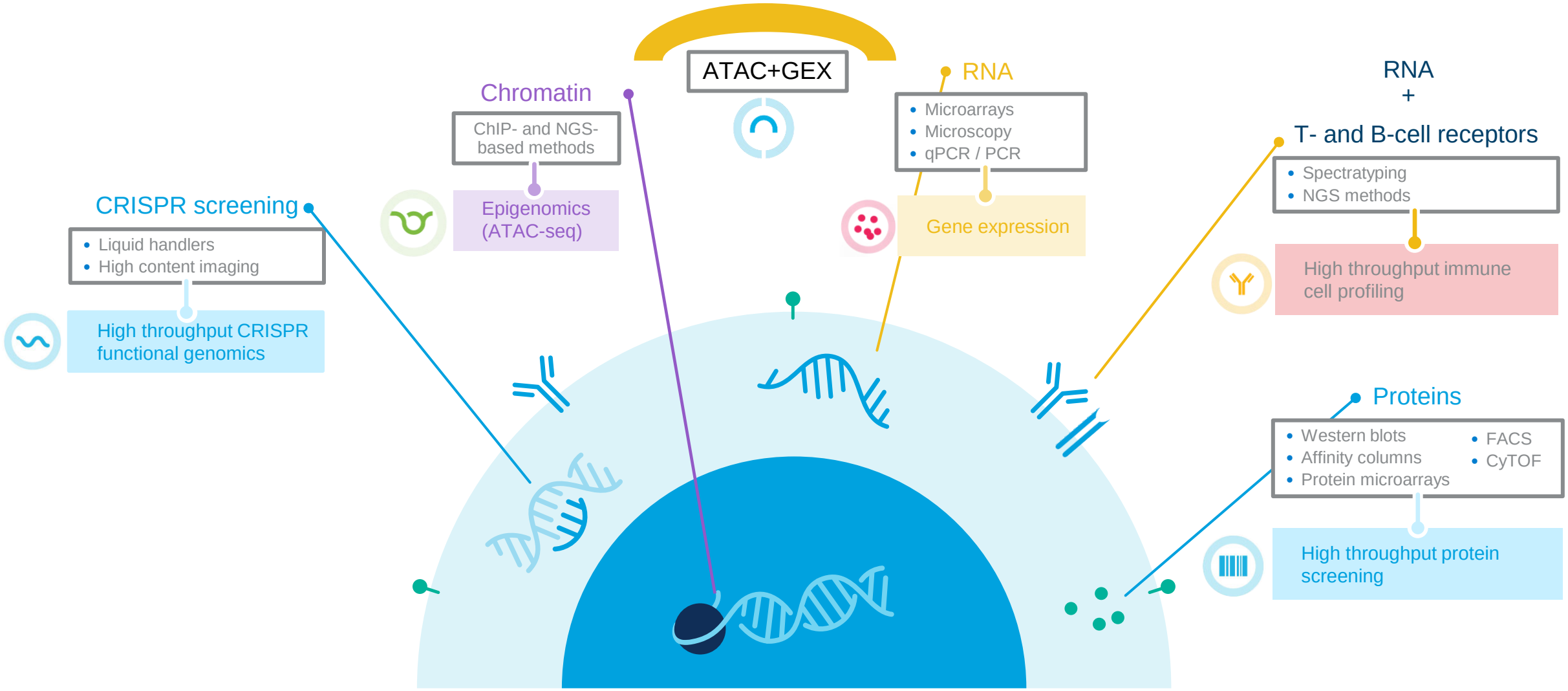
Whole Tissue/Organs

(Genetic) Disease Model



Resolve Cell Type-Specific Data

Next generation molecular profiling solutions



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How We Think About Sample Preparation

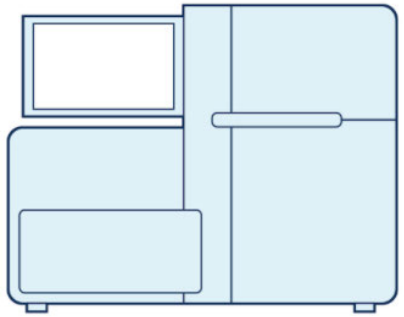
It’s what you bring to the experiment.

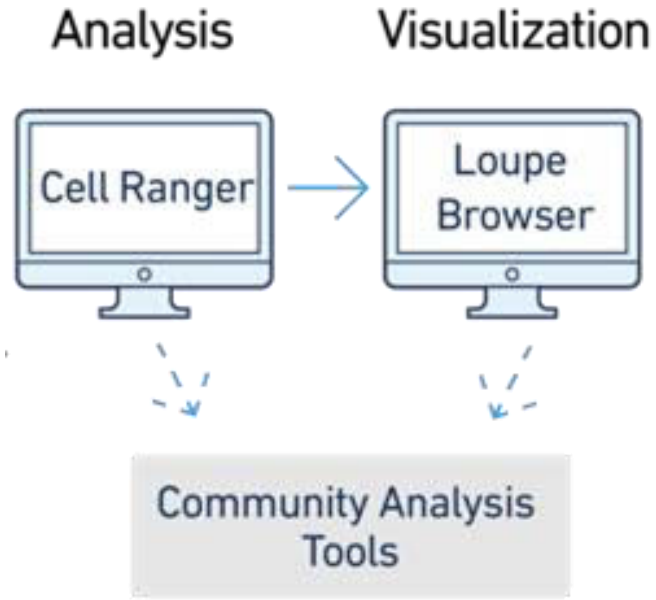
It’s a workflow. A set of decisions.

Quality is critical.

Chromium Single Cell Gene Expression Workflow

Input | **Library Creation** | **Sequencing** | **Data Analysis and Visualization**





Suspension of dissociated single cell/nuclei

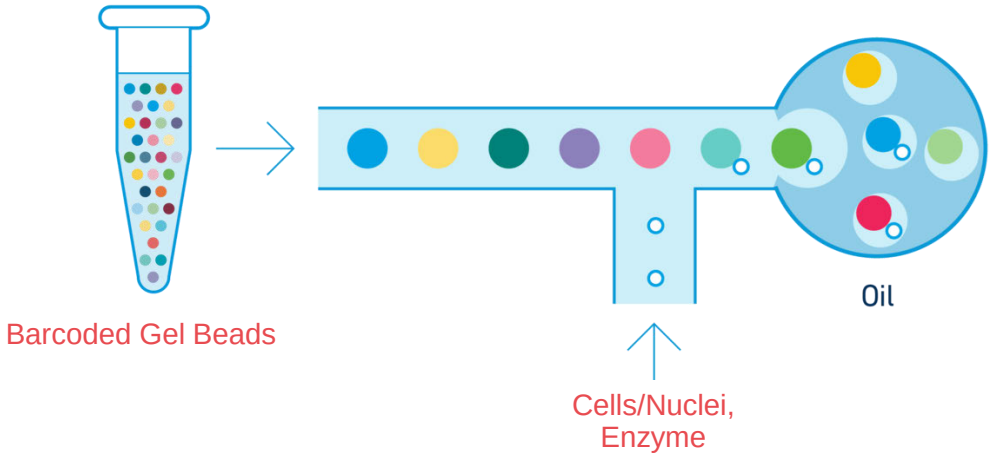
Cell partitioning and molecular barcoding

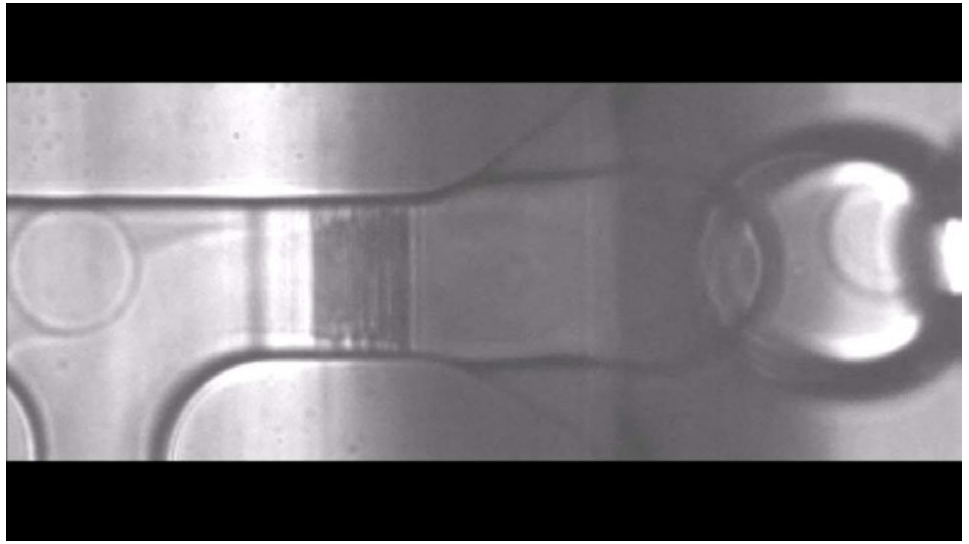
Sequencing

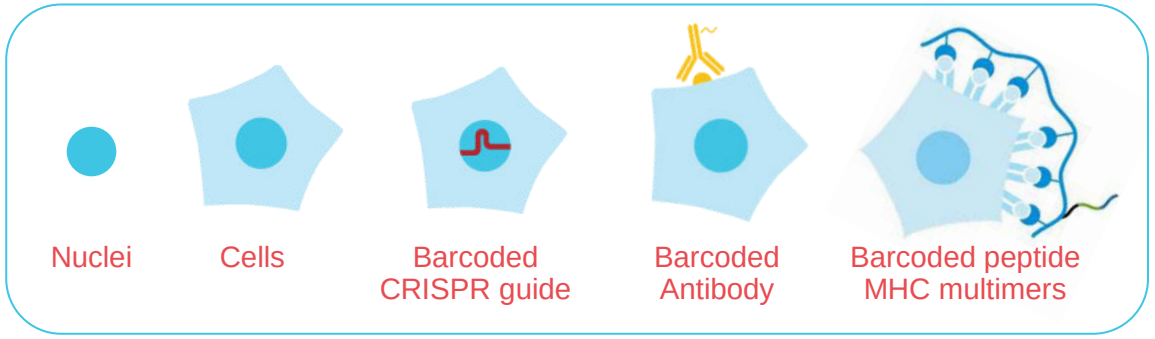
- **8 channels/chip**
- **500-10 000 cells recovered per channel**
- **40-65% cells recovered**

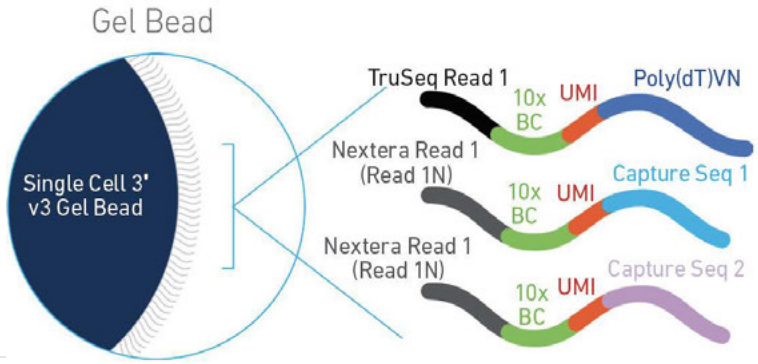
Technology

Partitioning and molecular barcoding millions of parallel reactions









Single cell sample prep resources from 10x Genomics

- https://support.10xgenomics.com/
- Protocols are free to download

General sample preparation guidelines

- Guidelines for optimal sample preparation
- Guidelines for accurate target cell counts
- General cell preparation guide
- Preparation of single cell suspensions from cultured cell lines
- Isolation of nuclei

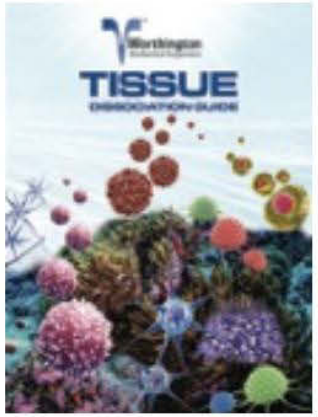
Preparation of specific sample types

- Fresh frozen human-mouse cell line mixtures
- Fresh frozen human peripheral blood mononuclear cells
- Dissociation of mouse embryonic neural tissue
- Tumor dissociation
- Methanol fixation of cells
- Moss protoplast suspensions

Sample improvement

- Enrichment of CD3+ T cells from dissociated tissues
- Removal of dead cells from single cell suspensions

General Cell Handling Recommendations



Worthington Tissue Dissociation Guide

Introduction

Tissue dissociation/primary cell isolation and cell harvesting are principal applications for enzymes in tissue culture research and cell biology studies. Despite the widespread use of enzymes for these applications over the years, their mechanisms of action in dissociation and harvesting are not well understood. As a result, the choice of one technique over another is often arbitrary and based more on past experience than on an understanding of why the method works and what modifications could lead to even better results.

Tissue Tables (references, grouped by tissue type and species)

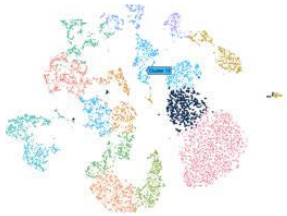
Adipose/Fat	Adrenal	Bone	Brain
Cartilage	Colon	Endothelial	Epithelial
Eye	Heart	Intestine	Kidney
Liver	Lung	Lymph nodes	Mammary
Miscellaneous	Muscle	Neural	Pancreas
Parotid	Pituitary	Prostate	Reproductive
Scales	Skin	Spleen	Stem
Thymus	Thyroid/Parathyroid	Tonsil	Tumor

https://www.worthington-biochem.com/tissuedissociation/

General Cell Handling Recommendations

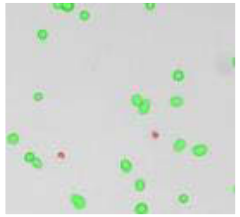
Analysis of Single Cell Transcriptomes

- Requires a fully dissociated, **single cell suspension.**
- Minimizing the presence of cellular aggregates, dead cells, non-cellular nucleic acids and potential inhibitors of reverse transcription is critical to obtaining high quality data.
- Suspension cell lines, bead-enriched and flow-sorted cells can be used directly after washing.



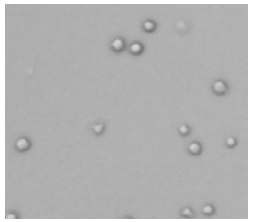
Importance of Input Cell Quality

- Ideally, input cell suspensions should contain more than **90% viable cells**.
- **The presence of a high fraction of non-viable or dying cells may decrease recovery.**
  - **The presence of ambient RNA and cellular debris may impact application performance and negatively impact quality metrics reported by Cell Ranger.**



Cell Handling

- It is important to **treat cells *gently*** to minimize cell lysis and loss:
  - **When cells lyse, the released ambient mRNA will contaminate other GEMs**
  - **Wash cells twice** using a wide-bore pipette tip to remove ambient RNA and contaminants.
  - **Wash and resuspend in PBS + 0.04% non-acetylated BSA to minimize cell loss during handling.**



General Cell Handling Recommendations

Debris/Aggregate Removal

- **Use a cell strainer to remove aggregates or debris from washed cells**
- **The presence of cell aggregates, debris and/or fibers can result in inaccurate cell counts**
- GEM generation occurs in microfluidic channels that are narrower than the typical human hair (i.e. < 100 µm) and the presence of cell debris or large aggregates **may clog or wet the chip**

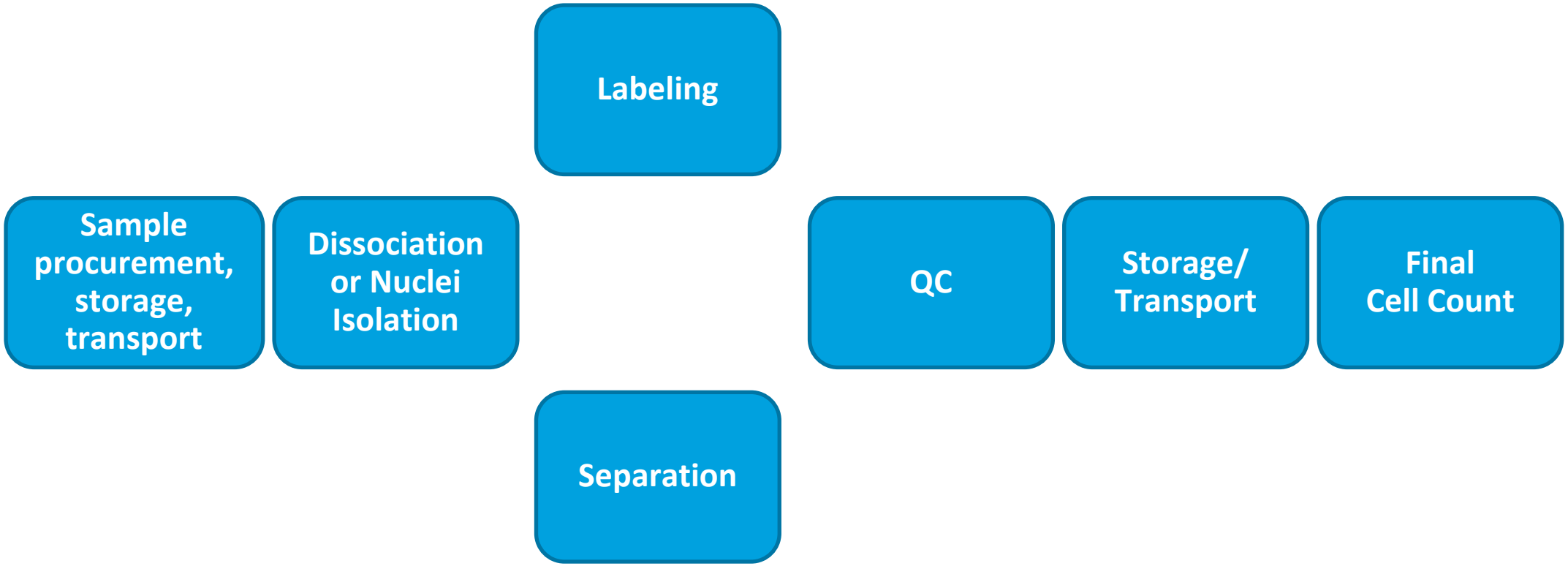
Cell Counting

- Quantitate cells accurately before loading into the system
  - **Approximately 65% loaded cells will be recovered**
  - To maximize the likelihood of achieving the desired recovery target, the optimal input **cell concentration is 700-1200 cells/µl**
  - **Recommended range: 500 to 10,000 recovered cells**
  - **Under- or over-loading may impact application performance**

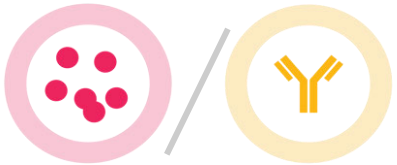
Storage of Single Cell Suspensions

- **Cell suspensions should always be kept on ice and where possible proceed with cell loading immediately after sample preparation**
  - Ideally incubation time should be kept to a minimum (< 30 min)
- **Some cell types are more fragile and cell viability may decrease significantly if not processed and loaded immediately**

It’s a Workflow. A Set of Decisions.



Choosing a Single Cell Assay



Gene Expression

Provides **transcriptome +/- immune receptor profiling**

Considerations:

- Interested in **cellular mRNA**?
- Interested in **feature barcode** or **cellplexing**?
- Interested in **targeted gene** expression?
- Interested in **automation**?
- High sensitivity

Multiome ATAC + Gene Expression

Provides nuclear transcriptome with **paired** chromatin accessibility profiling

Considerations:

- Interested in multimodal cell phenotyping?
- Pair gene expression with regulatory activity?
- **Limited sample** type?
- Interested in **nuclear** mRNA only?

ATAC

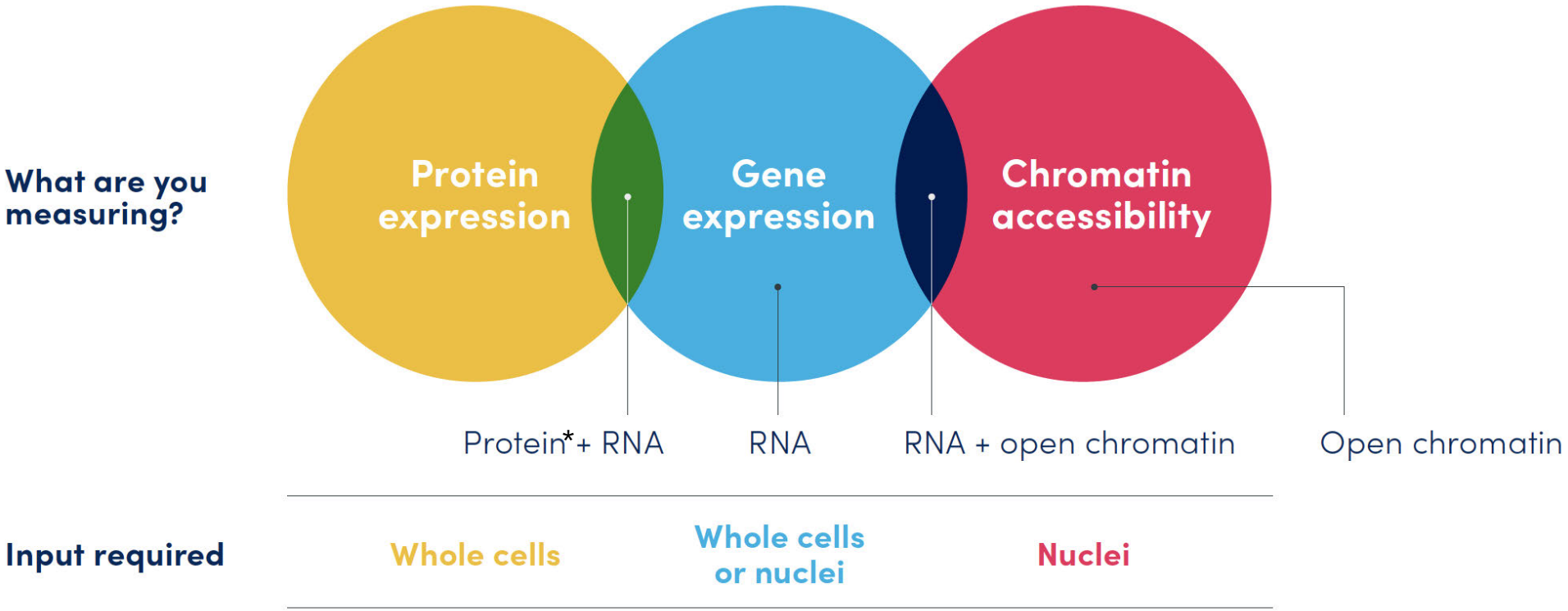
Provides **chromatin** accessibility profiling

Considerations:

- Interested in **open chromatin & TF binding** only?
- Sample types with **unknown or low mRNA integrity**?
- Cost sensitive?

Integrate data with third party tools using Single Cell Multiome ATAC + Gene Expression as bridge

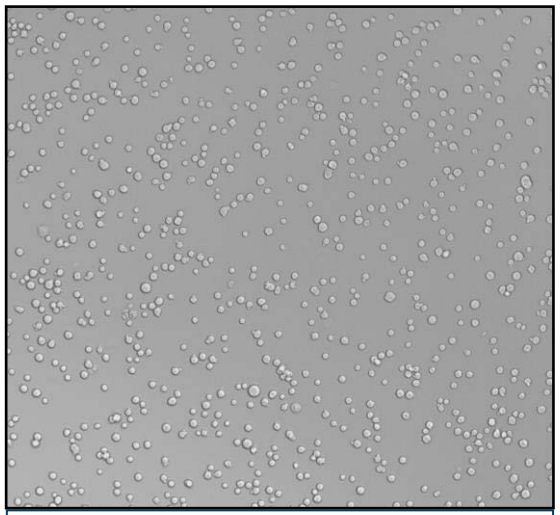
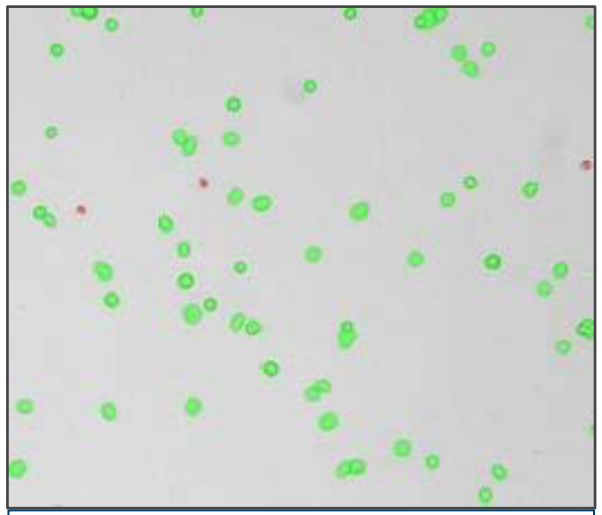
Different assays require different input materials



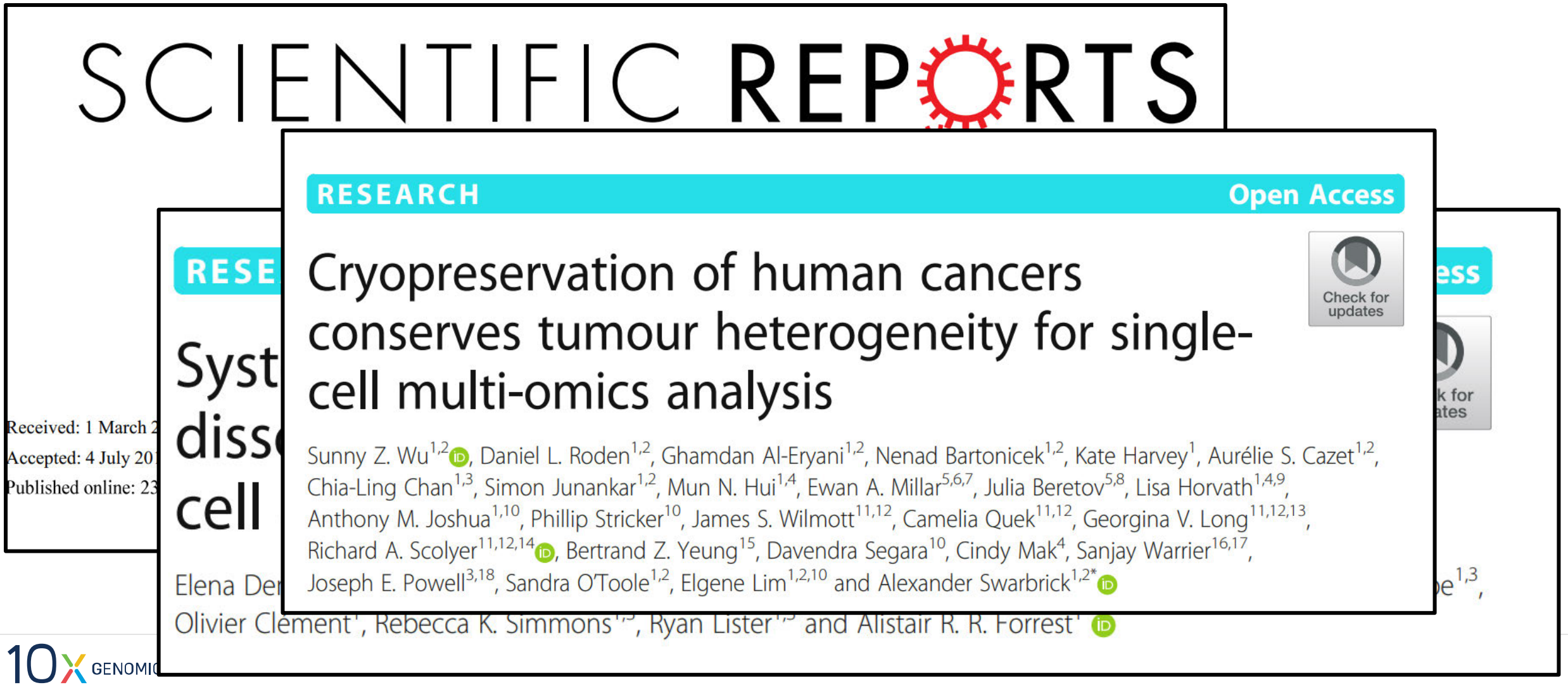
*Cell Surface protein

Quality is Critical

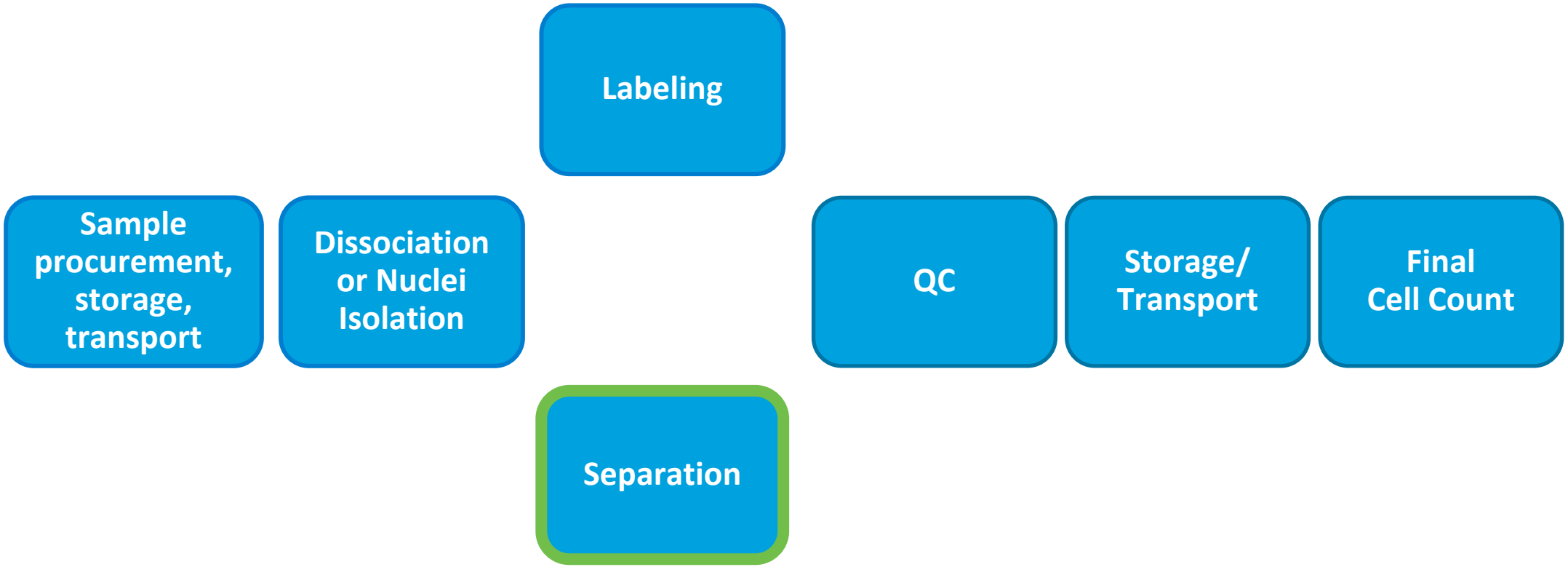
Goal is to minimize

Clean	Healthy	Intact
		
• Aggregates/clumps • Subcellular debris • Free-floating RNA/DNA	• Biological decomposition • RNA leakage (background) • RNA degradation (signal)	• Physical decomposition • RNA leakage (background) • RNA degradation (signal)

Sample Procurement, Storage, and Transport



Detailed decisions: Single Cell GEX sample separation



Sample Separation

Separate intact cells and nuclei from

- Aggregates/clumps
- Debris
- Free-floating mRNA
- Dead Cells
- Enrichment/Depletion

Challenges with separation

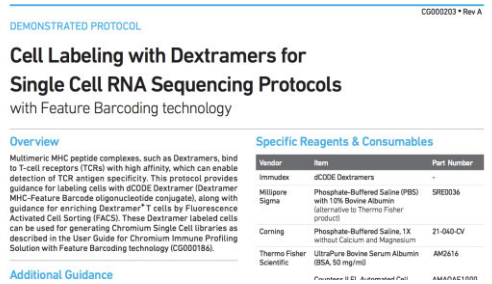
- Samples are fragile
- Physical stress
- Buffers
- Time
- Yield

Want the minimum handling necessary. Maintain sample integrity.

Basic Methods for Sample Separation

Method	Thorough centrifugation (e.g. 3x with PBS + 0.04% BSA)	Gentle centrifugation (e.g. 1x with media)	Magnetic beads	Density Gradient	FACS
10x Protocol Example	PBMC (CG000039)	Cell Prep Guide (CG000053)	Dead Cell Removal (CG000093)	Nuclei Isolation (CG000124)	Customer Developed Protocol (Martelotto)
Sample Size	Abundant	Limited	Abundant	Abundant	Limited
Benefits	Thorough	Gentle	Specific, easily accessible, scalable	Removes Debris	Versatile, quick
Possible Challenges	Yield, Harsh	Less thorough	Yield	Yield, Harsh, Time	Expensive, Harsh

Sample Prep Workflow: Rare Cell Population



CG000203

TotalSeq-C Abs

Flow Abs

Dextramer panel

Dead cell marker

Starting material
(PBMCs, DTC, CD8 T Cells, etc)

7AAD

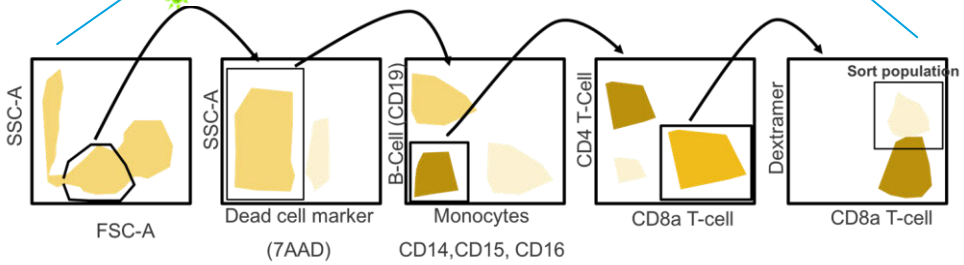
Cell staining

Cell sorting

PBS + 2% FBS:
cell viability

Optional bulk depletion to increase sorting speed

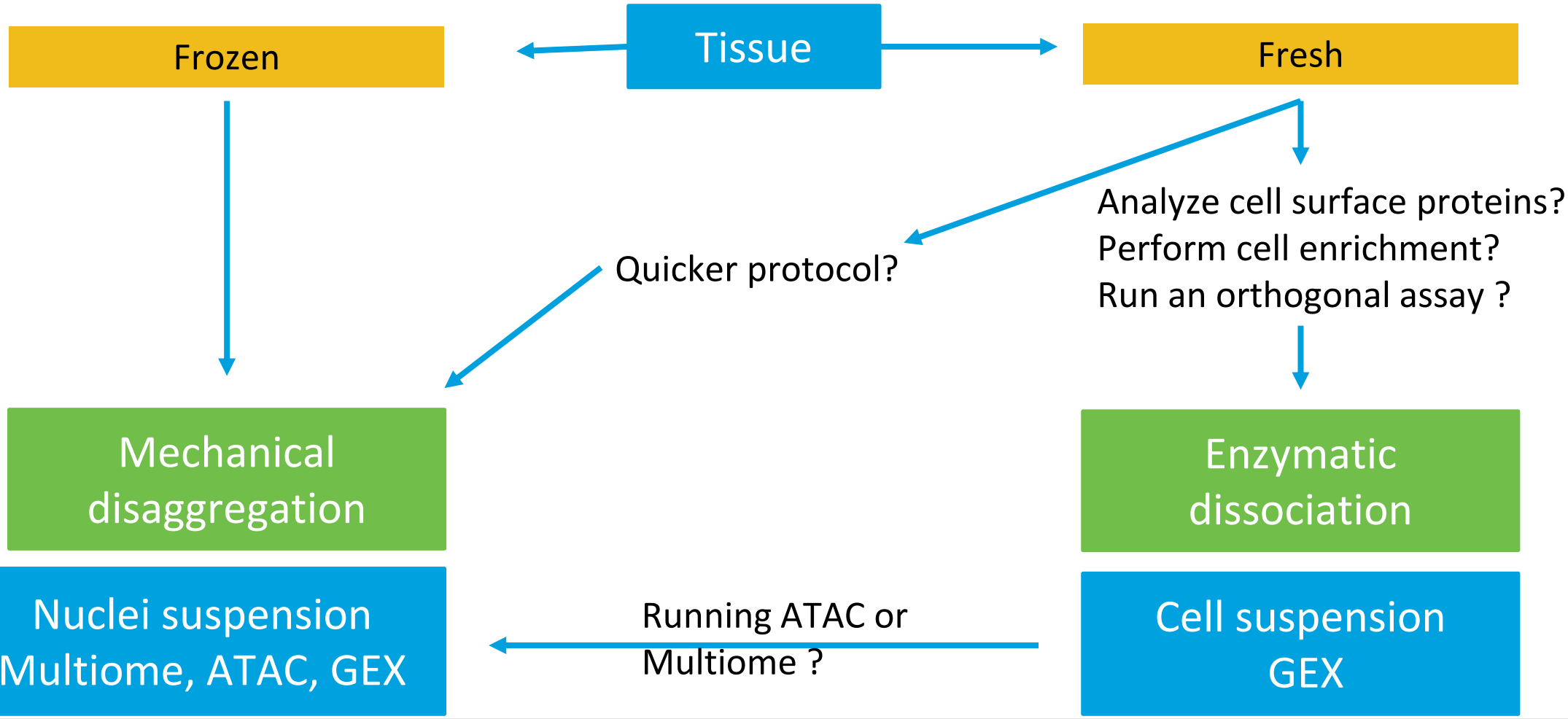
Gating:
sorting specificity/ clean-up



10X GENOMICS

Isolation of Nuclei for Single Cell Sequencing

Prepare a Cell or Nuclei Suspension



Why Use Nuclei?

A clean, viable single cell suspension is necessary for optimal results in scRNA sequencing. However, there are times when getting a good cell suspension is difficult and nuclei is an alternative option.

- **When cells are large and exceed the limits for the microfluidic chip**
  - Hepatocytes
  - Neurons with significant extensions
- **When cells are of a challenging shape**
  - Cardiomyocytes
- **When cells are difficult to get into a single cell suspension**
  - Sample contains a lot of debris
  - Neurons are highly interconnected and may not efficiently dissociate into single cells after enzymatic treatment
  - Dissociation-resistant tissue samples such as complex tissues/ organs where nuclei (but not whole intact cells) can be isolated

Why Use Nuclei?

- ***Possible* solution for archival (cryopreserved) or damaged samples in which the cell wall is breaking down**
  - Laser capture microdissection will physically damage whole cells (cell wall)
  - Nuclei isolation will not rescue damaged cells that are already dying or undergoing apoptosis
- ***Possible* solution for experiments aiming to reveal molecular genetic regulatory mechanisms specific to the nucleus**
- **Sample types that have a cell wall that does not lyse in our assay**
  - Various plants, yeast
- **For ATAC and Multiome**

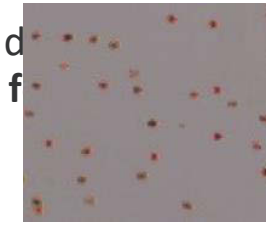
General Handling Recommendations

Starting Sample Requirements

- Tissues or cell suspensions
- Dissociate tissues when possible, some tissues will require going straight into nuclei isolation
- If starting with low viability cell suspension, sorting prior to nuclei isolation may help reduce ambient DNA and cellular debris
  - **Sorting after nuclei isolation is not recommended as it may damage nuclear membrane**

Nuclei Isolation

- Refer to Demonstrated Protocols for Nuclei Isolation for Single Cell ATAC Sequencing:
  - Nuclei Isolation from mouse brain tissue
  - Nuclei Isolation from cell lines and PBMCs
  - **Isolation of Nuclei for Single Cell RNA Sequencing demonstrated protocol decreases single cell ATAC assay performance**
- Resuspend nuclei in d Buffer (1X)—**critical f performance**



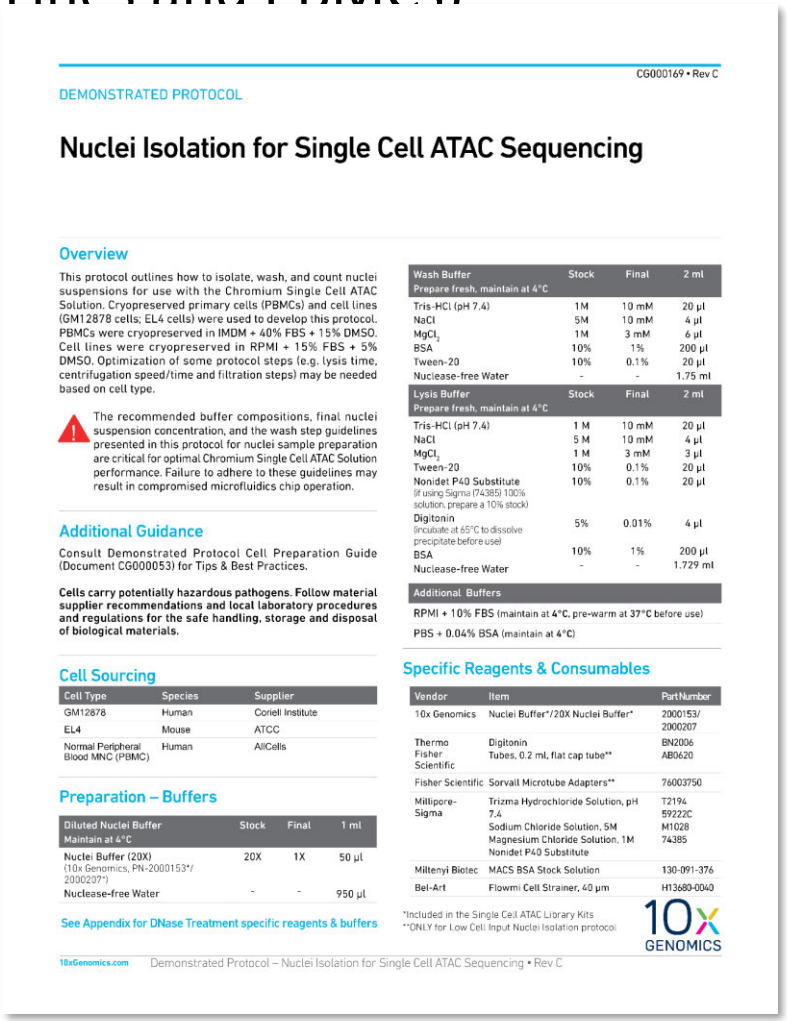
Nuclei Isolation

- It is important to **treat nuclei gently** to minimize lysis and loss
- Count Nuclei using Countess and trypan blue, ideal viability should be <5% live
  - Counting may also be done using ethidium homodimer and fluorescence microscope or Countess II FL.
- Visualization under the microscope may give further inc nuclear membrane

Demonstrated Protocol Available from 10x Genomics

Nuclei Isolation for Single Cell ATAC Sequencing (From Cell Lines and PBMCs)

- Demonstrated protocol includes recommendations/tips for preparing nuclei from PBMCs and from cell lines (GM12878:EL4 mix), fresh and cryopreserved
- Low sample input protocol in appendix for limited samples
- Protocol can be adapted for other cell types with optimization
- Demonstrated Protocol is available on 10x support website
- **Note: Not all demonstrated protocols on our website will be compatible with the Chromium Single Cell ATAC Solution**



CG000169 • Rev C

DEMONSTRATED PROTOCOL

Nuclei Isolation for Single Cell ATAC Sequencing

Overview

This protocol outlines how to isolate, wash, and count nuclei suspensions for use with the Chromium Single Cell ATAC Solution. Cryopreserved primary cells (PBMCs) and cell lines (GM12878 cells; EL4 cells) were used to develop this protocol. PBMCs were cryopreserved in IMDM + 40% FBS + 15% DMSO. Cell lines were cryopreserved in RPMI + 15% FBS + 5% DMSO. Optimization of some protocol steps (e.g. lysis time, centrifugation speed/time and filtration steps) may be needed based on cell type.

The recommended buffer compositions, final nuclei suspension concentration, and the wash step guidelines presented in this protocol for nuclei sample preparation are critical for optimal Chromium Single Cell ATAC Solution performance. Failure to adhere to these guidelines may result in compromised microfluidics chip operation.

Additional Guidance

Consult Demonstrated Protocol Cell Preparation Guide (Document CG000053) for Tips & Best Practices.

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

Cell Sourcing

Cell Type	Species	Supplier
GM12878	Human	Coriell Institute
EL4	Mouse	ATCC
Normal Peripheral Blood MNC (PBMC)	Human	AllCells

Preparation – Buffers

Diluted Nuclei Buffer Maintain at 4°C	Stock	Final	1 ml
Nuclei Buffer (20X) (10x Genomics, PN-2000153*/ 2000207*)	20X	1X	50 µl
Nuclease-free Water	-	-	950 µl

See Appendix for DNase Treatment specific reagents & buffers

Wash Buffer Prepare fresh, maintain at 4°C	Stock	Final	2 ml
Tris-HCl (pH 7.4)	1M	10 mM	20 µl
NaCl	5M	10 mM	4 µl
$MgCl_2$	1M	3 mM	6 µl
BSA	10%	1%	200 µl
Tween-20	10%	0.1%	20 µl
Nuclease-free Water	-	-	1.75 ml

Lysis Buffer Prepare fresh, maintain at 4°C	Stock	Final	2 ml
Tris-HCl (pH 7.4)	1 M	10 mM	20 µl
NaCl	5 M	10 mM	4 µl
$MgCl_2$	1 M	3 mM	3 µl
Tween-20	10%	0.1%	20 µl
Nonidet P40 Substitute (if using Sigma (74385) 100% solution, prepare a 10% stock)	10%	0.1%	20 µl
Digitonin (incubate at 65°C to dissolve precipitate before use)	5%	0.01%	4 µl
BSA	10%	1%	200 µl
Nuclease-free Water	-	-	1.729 ml

Additional Buffers
RPMI + 10% FBS (maintain at 4°C, pre-warm at 37°C before use)
PBS + 0.04% BSA (maintain at 4°C)

Specific Reagents & Consumables

Vendor	Item	Part Number
10x Genomics	Nuclei Buffer*/20X Nuclei Buffer*	2000153/ 2000207
Thermo Fisher Scientific	Digitonin Tubes, 0.2 ml, flat cap tube**	BN2006 AB0620
Fisher Scientific	Sorvall Microtube Adapters**	76003750
Millipore-Sigma	Trizma Hydrochloride Solution, pH 7.4 Sodium Chloride Solution, 5M Magnesium Chloride Solution, 1M Nonidet P40 Substitute	T2194 59222C M1028 74385
Miltenyi Biotec	MACS BSA Stock Solution	130-091-376
Bel-Art	Flowmi Cell Strainer, 40 µm	H13680-0040

*Included in the Single Cell ATAC Library Kits
**ONLY for Low Cell Input Nuclei Isolation protocol

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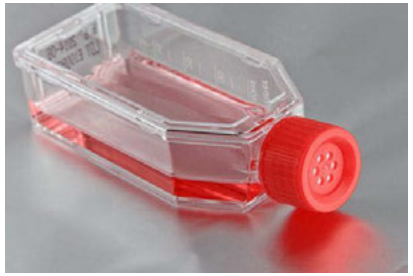
10xGenomics.com Demonstrated Protocol – Nuclei Isolation for Single Cell ATAC Sequencing • Rev C

Validated with Nuclei Isolated from Multiple Sample Types

Cell Lines

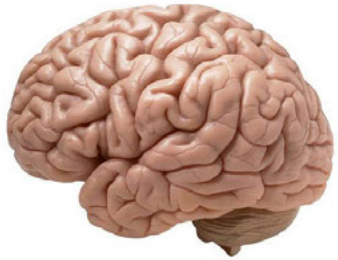


- Suspension: GM12878, A20, EL4, K562
- Adherent: A549

Primary Immune Cells

- Human Peripheral Blood Mononuclear Cells
- Human Bone Marrow Mononuclear Cells

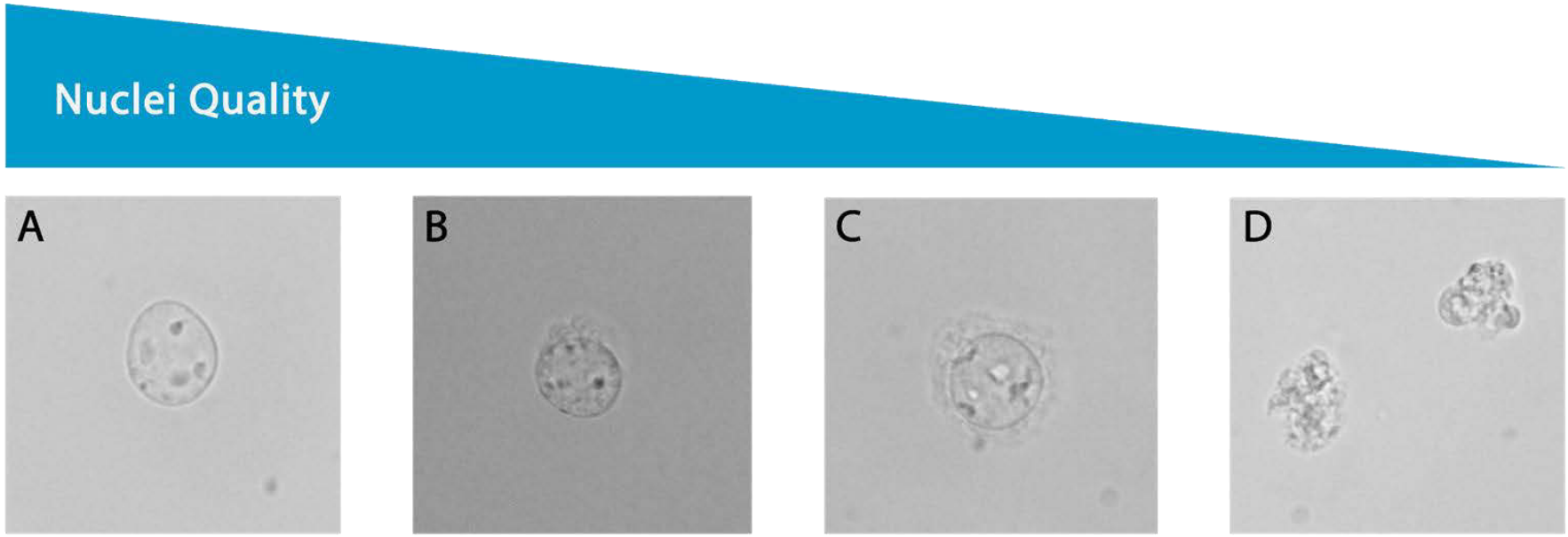
Dissociated Primary Tissues



- Embryonic Mouse Brain Tissue
- Adult Mouse Brain Tissue
- Mouse Splenocytes

Nuclei isolation for Single Cell ATAC sequencing

Nuclear morphology can indicate nuclei quality



A: High-quality nuclei have well-resolved edges. Optimal quality for single cell ATAC libraries.
B: Mostly intact nuclei with minor evidence of blebbing. Quality single cell ATAC libraries can still be produced.
C: Nuclei with strong evidence of blebbing. ***Proceed at your own risk.***
D: Nuclei are no longer intact. ***Do not proceed!***

Data Review – Cells vs Nuclei

Gene Expression Levels Are Well Correlated Between Cells and Nuclei

nature biotechnology

ANALYSIS

https://doi.org/10.1038/s41587-020-0465-8

Check for updates

Systematic comparison of single-cell and single-nucleus RNA-sequencing methods

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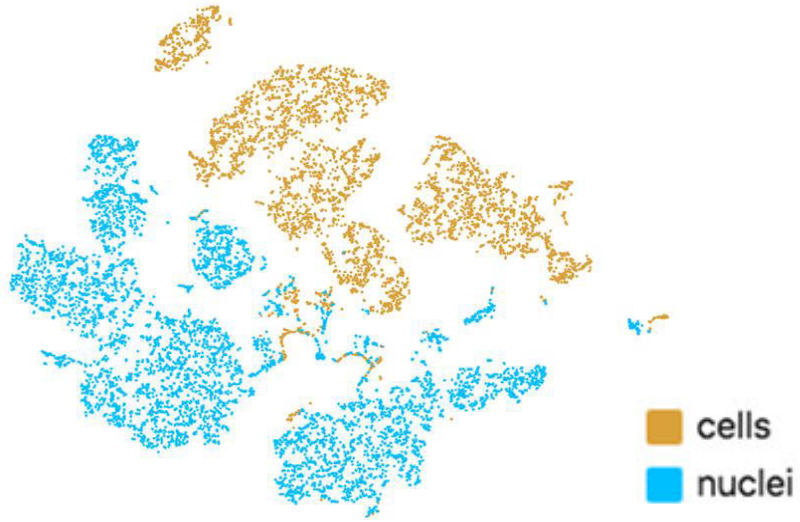
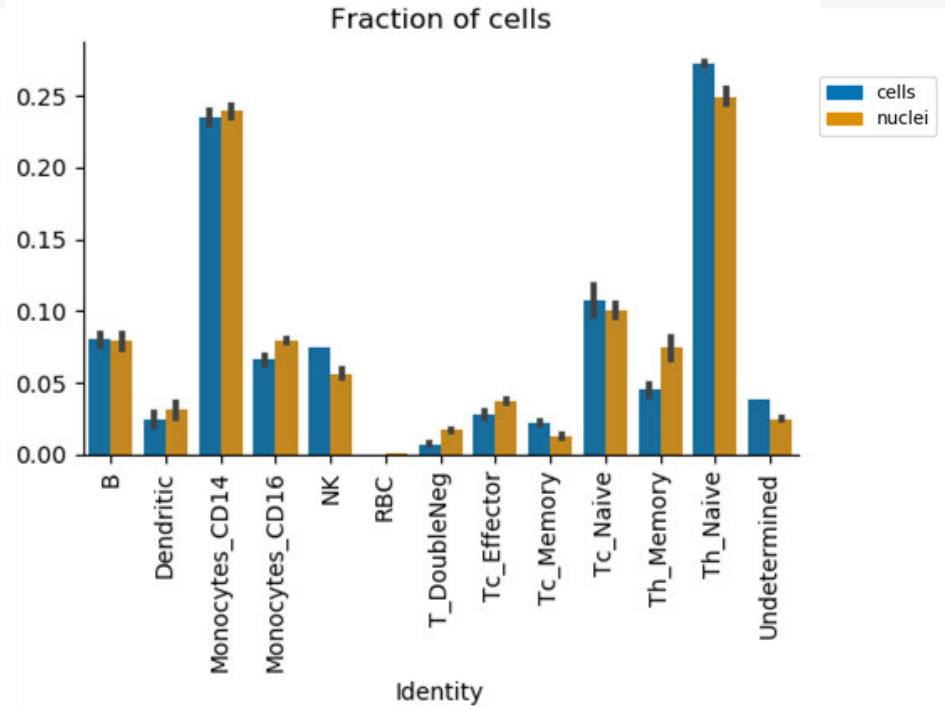
How does information from nuclei compare to cells?

Clusters do not overlap but biological information is conserved

PBMCs



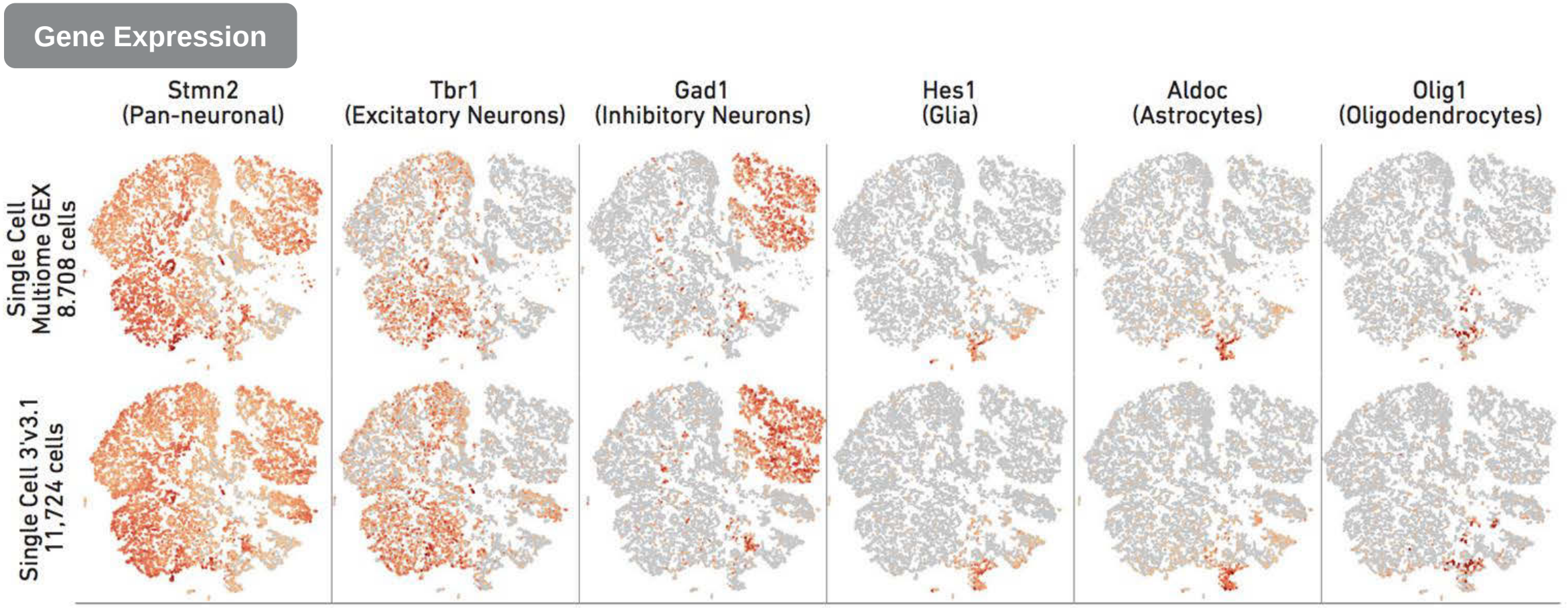
- Cells run on SC3'v3.1 capture mostly mRNA
- **Nuclei run on Multiome ATAC+GEX capture mostly pre-mRNA (unspliced mRNA)**



- **Cell populations can still be identified**

Cell type specific markers are conserved

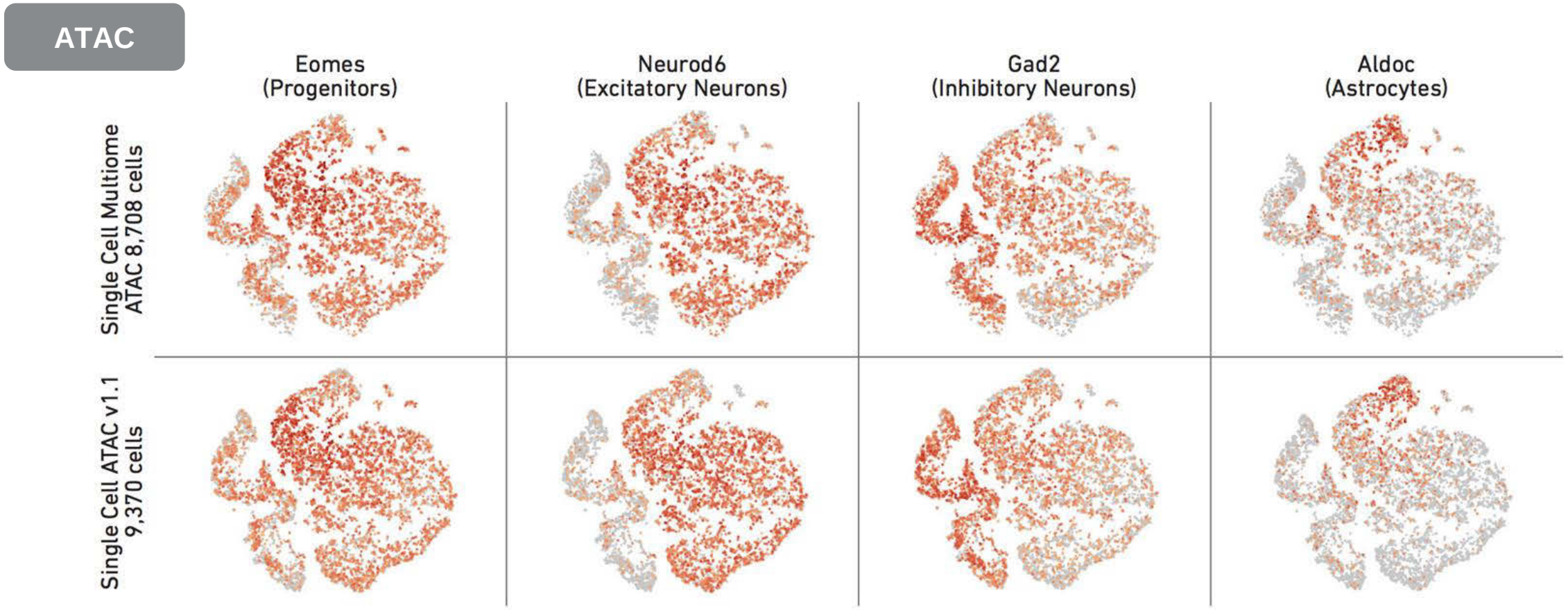
Between Single Cell Multiome Gene Expression and 3’ v3.1



Mouse embryonic E18.5 brain nuclei

Cell type specific markers are conserved

Between Single Cell Multiome ATAC and ATAC v1.1

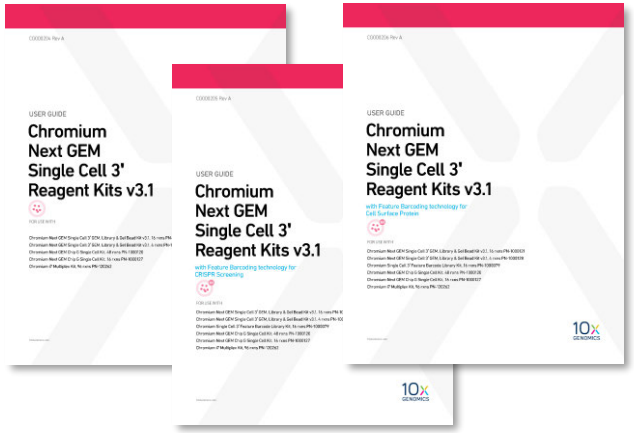


Mouse embryonic E18.5 brain nuclei

Resources

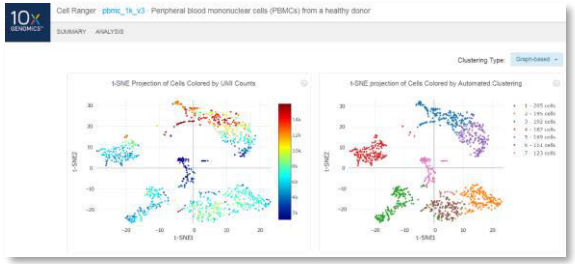
Demonstrated Protocols

User Guides



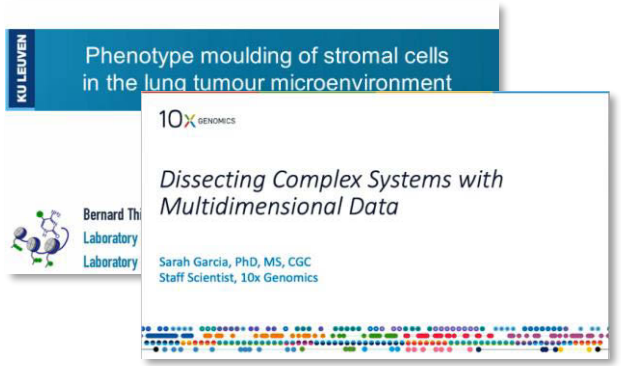
Application Notes

Public Data Sets



How-To Videos

Scientific Seminars



https://support.10xgenomics.com/
https://www.10xgenomics.com/10x-university/

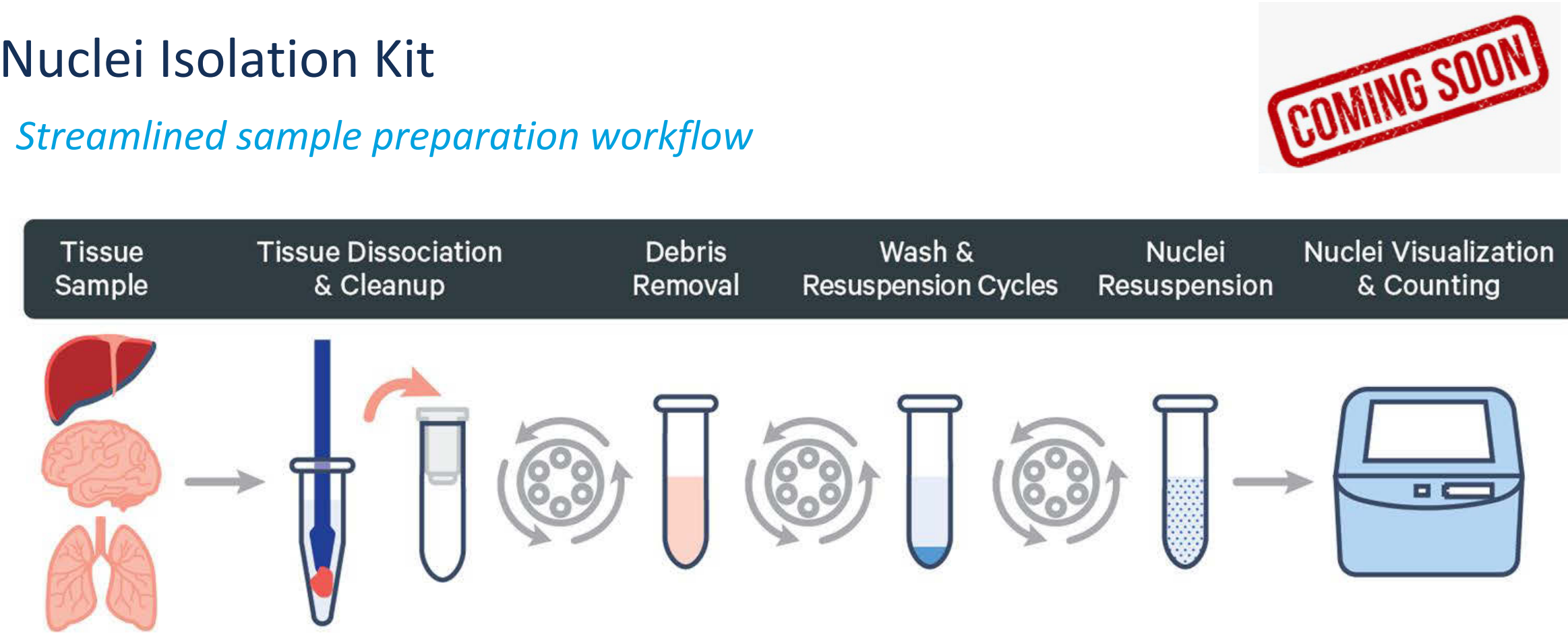
Customer Developed Protocols

https://community.10xgenomics.com/t5/Customer-Developed-Protocols/ct-p/customer-protocols

1. High Molecular Weight Genomic DNA Extraction from Grape LeavesContributed by: Xia Xu,Lance Cadle-Davidson USDA-ARS, GGRU
2. CTAB Protocol for Isolating DNA from Plant TissueContributed by: Allen Van Deynze, Van Deynze Lab, UC Davis
3. Cell dissociation and crypt isolation of the mouse small intestineContributed by: Aviv Regev, Regev Lab, Broad Institute
4. Tissue dissociation and single cell preparation of breast cancer patient-derived xenograftsContributed by: Ioannis Ragoussis and Morag Park
5. Isolation of single cell suspensions from epidermisContributed by: Samuel Lukowski
6. Generation of single cell suspension from E8.25 mouse embryosContributed by: Bertie Gottgens
7. Preparation of non-myocyte cardiac single cell suspensionsContributed by: Galen Squiers & Alex Pinto, Pinto Lab, The Jackson Laboratory, Bar Harbor
8. 'Frankenstein' protocol for nuclei isolation from fresh and frozen tissueContributed by: Luciano Martelotto, Ph.D., Melbourne, Centre for Cancer Research, Victorian Comprehensive Cancer Centre

Nuclei Isolation Kit

Streamlined sample preparation workflow

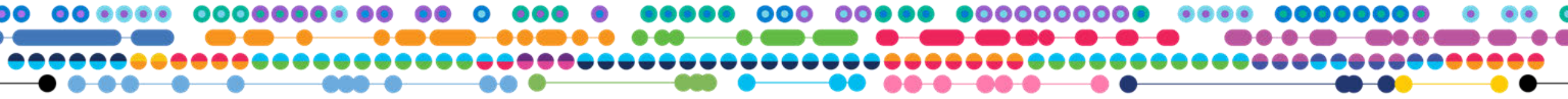


All you need is an hour of lab time, a benchtop centrifuge, and an interesting frozen sample!

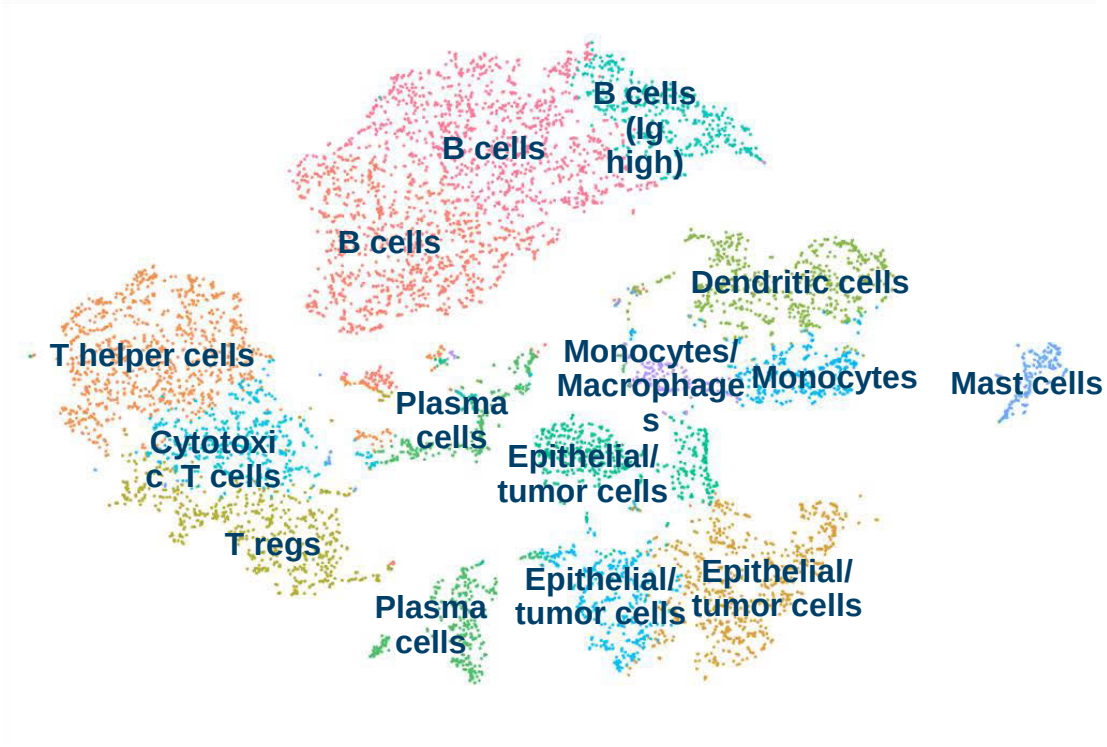
10x GENOMICS

Thank you

Agnieszka.Ciesielska@10xgenomics.com



Separation Method: Gentle Centrifugation



Sample Attributes

- Small sample (<200k cells)
- Fragile cells: dissociated, frozen, and thawed
- Poor viability (<40%)

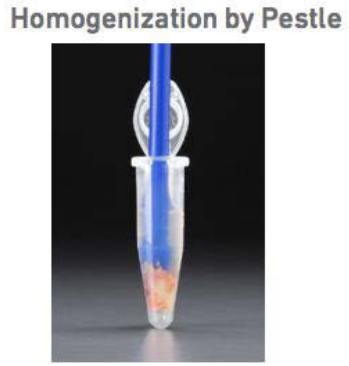
Protocol Decision – Maintain integrity by treating cells gently:

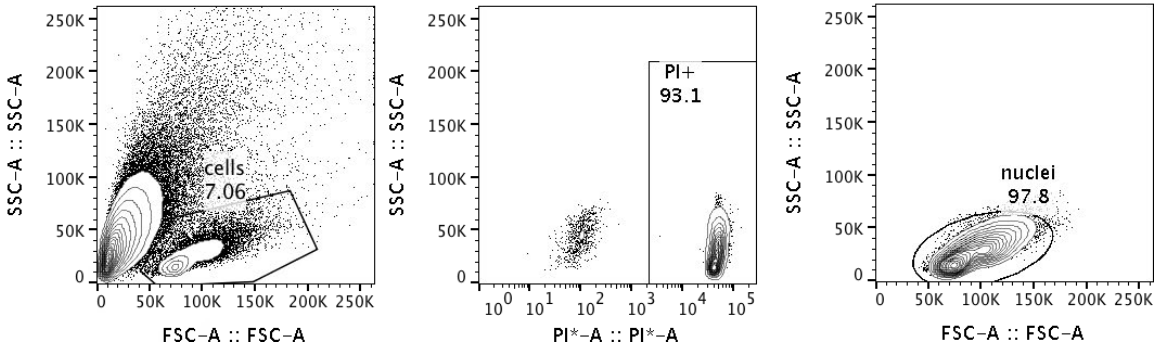
- Keep cells in media + 10%FBS
- Use a swinging bucket centrifuge
- Remove dead cells by centrifugation

Results and Further Reading

- 10x Application Note (LIT000022)
- 10x-pert Webinar – Tumor Microenvironment

Example: Frozen Tissue GEX





Protocol goal –
Maintain integrity by working quickly:

- Mechanical dissociation
- **Use FACS to remove debris and ambient mRNA**
- Collect nuclei in GEM-RT mix and process immediately

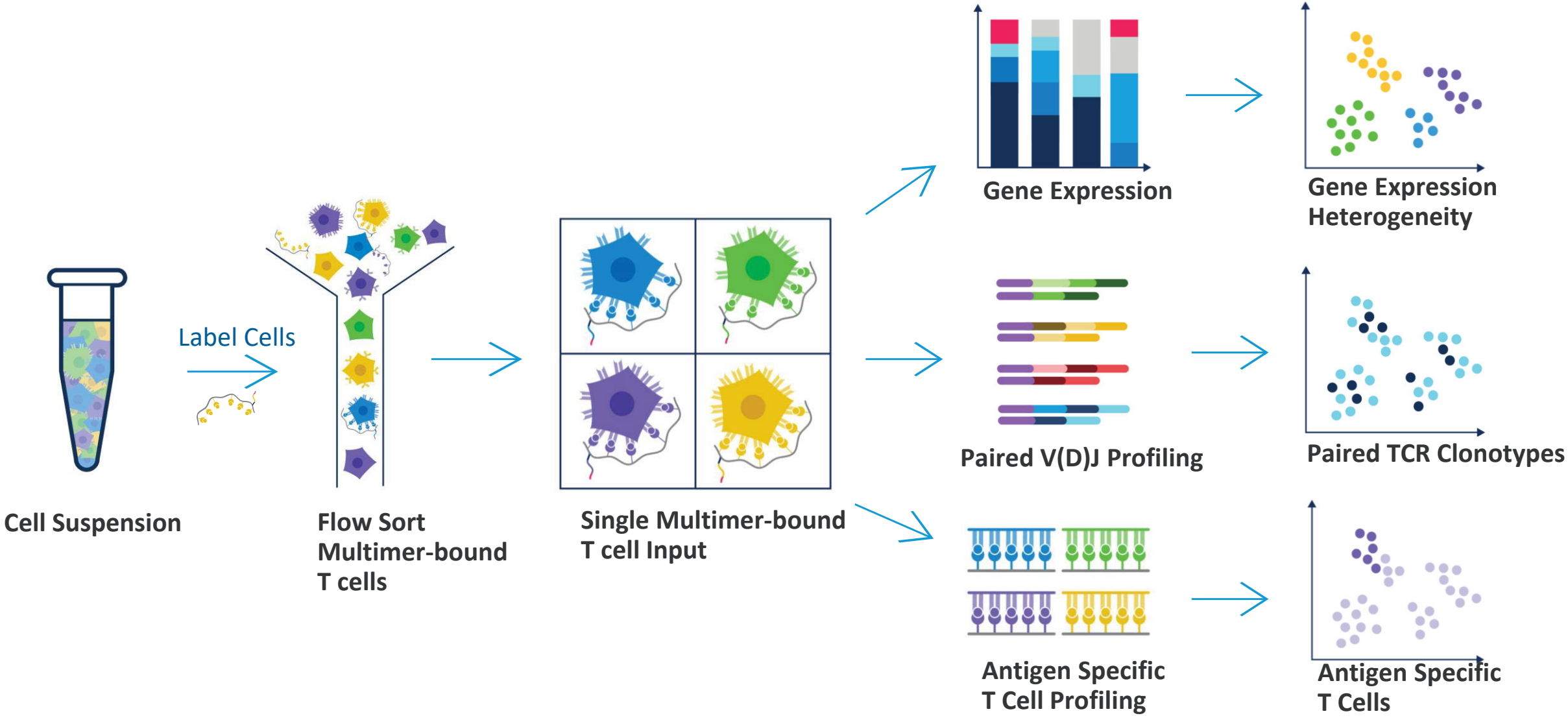
Why is this sample challenging?

- Small piece of tissue
- Frozen tissue

References

- **10x Genomics Customer Developed Protocol from Luciano Marletotto**

Reveal Antigen Specificity with Feature Barcoding Technology



Summary of Key Lessons

When our standard guidance isn't applicable:

- Treat cells gently and minimize decomposition
  - Use gentl(er) lysis conditions
  - Reduce wash steps*
  - Use a swinging bucket centrifuge
  - Keep cells in media + FBS instead of PBS**
- Work quickly
  - Consider sorting, it is a versatile tool for sample prep
  - Minimize unnecessary handling steps
- *Consider the benefits and drawbacks of every different technique*

*Cell surface protein analysis requires thorough washing
**ATAC has specific buffer formulation

Nuclei Isolation for Single Cell ATAC Sequencing

Required Reagents and Buffer Composition

Reagents

Vendor	Item	Part Number
10x Genomics	Nuclei Buffer*/20X Nuclei Buffer*	2000153/ 2000207
Thermo Fisher Scientific	Digitonin Tubes, 0.2 ml, flat cap tube**	BN2006 AB0620
Fisher Scientific	Sorvall Microtube Adapters**	76003750
Millipore-Sigma	Trizma Hydrochloride Solution, pH 7.4 Sodium Chloride Solution, 5M Magnesium Chloride Solution, 1M Nonidet P40 Substitute	T2194 59222C M1028 74385
Miltenyi Biotec	MACS BSA Stock Solution	130-091-376
Bel-Art	Flowmi Cell Strainer, 40 µm	H13680-0040

*Included in the Single Cell ATAC Library Kits
**ONLY for the Low Cell Input Nuclei Isolation protocol

Buffers

Diluted Nuclei Buffer Maintain at 4°C	Stock	Final	1 ml
Nuclei Buffer (20X) (10x Genomics, PN-2000153*/ 2000207*)	20X	1X	50 µl
Nuclease-free Water	-	-	950 µl

Wash Buffer Prepare fresh, maintain at 4°C	Stock	Final	2 ml
Tris-HCl (pH 7.4)	1M	10 mM	20 µl
NaCl	5M	10 mM	4 µl
$MgCl_2$	1M	3 mM	6 µl
BSA	10%	1%	200 µl
Tween-20	10%	0.1%	20 µl
Nuclease-free Water	-	-	1.75 ml

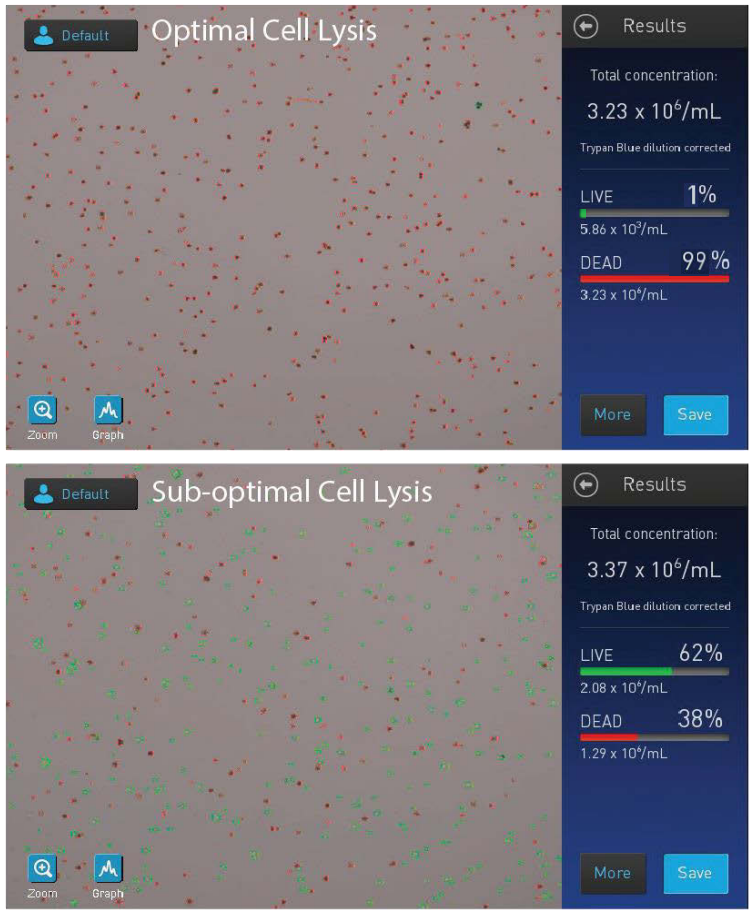
Lysis Buffer Prepare fresh, maintain at 4°C	Stock	Final	2 ml
Tris-HCl (pH 7.4)	1 M	10 mM	20 µl
NaCl	5 M	10 mM	4 µl
$MgCl_2$	1 M	3 mM	3 µl
Tween-20	10%	0.1%	20 µl
Nonidet P40 Substitute (if using Sigma (74385) 100% solution, prepare a 10% stock)	10%	0.1%	20 µl
Digitonin (incubate at 65°C to dissolve precipitate before use)	5%	0.01%	4 µl
BSA	10%	1%	200 µl
Nuclease-free Water	-	-	1.729 ml

Additional Buffers
RPMI + 10% FBS (maintain at **4°C**, pre-warm at **37°C** before use)
PBS + 0.04% BSA (maintain at **4°C**)

Supplied in Chromium Single Cell ATAC Library Kits. The tube contains enough to make ~32 ml of working dilution for final nuclei resuspension. Typical usage is up to 1 ml per sample. **Once prepared, maintain diluted Nuclei Buffer on ice while isolating nuclei.**

Nuclei isolation for Single Cell Multiome ATAC + Gene Expression sequencing

Lysis can be assessed using a cell viability stain



When counting look for:

- <5% Live Cells (or >95% dead)
  - Nuclei will stain as dead
  - Lysis time will be cell-type dependent
  - Lysis time course may be required to determine optimal lysis time
- Clean, clump free nuclei
  - Filtering may help break clumps and remove debris

This provides a yes/no answer as to whether the cell membrane was lysed. Resolution is enough to assess clumping and debris but may not be enough to evaluate nuclear membrane integrity

For hard to count cells (small size, lots of debris), use a fluorescent stain like Ethidium Homodimer-1. An automated counter with fluorescent capability is needed.