Single-cell Multiomics analysis at FGCZ with 10x Genomics Platform

Author: Qin Zhang
Functional Genomics Center Zurich

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Section 1: How to prepare single-cell suspension for 10x standard gene expression profiling

Important Note: Acquiring a high-quality single-cell suspension is crucial for the success of the entire 10x experiment. Hence, we highly recommend optimizing the dissociation procedure for your cells/tissues. This optimization should encompass various factors such as cell viability, yield, presence of debris, clumps, as well as evaluating any steps that require cells to wait on ice (e.g., waiting for other samples to be dissociated or during transportation to our facility) to assess their potential impact on cell quality.

1. Resuspension buffer

To prepare single cells for Chromium/X Single Cell applications, please refer to the Cell Preparation Guide from 10x. Demonstrated protocols for single cell or nuclei isolation from different tissues are provided by 10x (link attached at the end of this section).

It is recommended to use 1X PBS (calcium and magnesium-free) containing 0.04% weight/volume BSA (400 µg/ml) for washing and resuspension of cells. Fresh and frozen/thawed PBMC samples and cell lines have been tested with this buffer.

Primary cells, stem cells, and other sensitive cell types may require washing and resuspension in alternative buffers to maximize viability. For other buffers that have been tested, please refer to page 36 of the Cell Preparation Guide. It is also possible to use most cell culture media with up to 10% FBS or up to 2% BSA to maintain cell health with little to no adverse downstream effects.

Media should not contain excessive amounts of EDTA (> 0.1mM) or magnesium (> 3mM), as those components will inhibit the reverse transcription reaction. Any surfactants (Tween-20, etc.) should also be avoided as they may interfere with GEM generation.

2. Targeted cell number

Depending on your specific biological question, you have the flexibility to determine the number of cells you wish to recover during the experiment. For standard 10x gene expression analysis, please consult the table provided below. It is important to note that a higher number of cells to be recovered will result in an increased likelihood of obtaining multiplets. Consequently, the sequencing cost will also rise accordingly.
Please be aware that multiplets are sequenced during the experiment; however, the data from these multiplets are subsequently excluded from further bioinformatic analysis. This exclusion results in a waste of sequencing costs. Therefore, it is crucial to consider this trade-off when deciding on the number of cells to recover for your experiment.

Multiplet Rate (%)	# of Cells Loaded	# of Cells Recovered
~0.4%	~825	~500
~0.8%	~1,650	~1,000
~1.6%	~3,300	~2,000
~2.4%	~4,950	~3,000
~3.2%	~6,600	~4,000
~4.0%	~8,250	~5,000
~4.8%	~9,900	~6,000
~5.6%	~11,550	~7,000
~6.4%	~13,200	~8,000
~7.2%	~14,850	~9,000
~8.0%	~16,500	~10,000

If the goal is to recover 10,000 cells, we request bringing a minimum of 50,000 single cells to our facility. This count includes cells required for concentration measurement using an automated cell counter and quality assessment under a microscope. Please be aware that due to technical limitations, there is a possibility of a

small but notable percentage of well/run failures. To accommodate such situations, having 50,000 cells will allow us to repeat the 10x chip loading process once, increasing the likelihood of achieving the desired cell recovery.

Furthermore, when using FACS for cell sorting, it is common for the estimated cell number to be overestimated. Therefore, it is advisable to add some margin (50%) when performing cell sorting to ensure an adequate number of cells are obtained.

The actual cell recovery rate can vary depending on the physical characteristics of the cells and experiment-specific factors, such as cell concentration (how accurate it is), cell viability, presence of debris, aggregates, and other variables. Therefore, the targeted cell number serves as an indication, and the actual number of cells recovered can differ significantly from the target.

3. Cell concentration and viability

For optimal results, it is recommended to maintain an input cell concentration ranging from **700 to 1,200 /µl**. Additionally, it is crucial to ensure that the cell viability exceeds 90% and that the single-cell suspension is free of debris and clumps.

Some useful links for single-cell suspension preparation

How many cells do I need?

Cell Thawing protocols: four different protocols to thaw cryopreserved cell suspensions

Demonstrated Protocols: isolate single cells/nuclei from different sources

Customer Developed Protocols: different tissue dissociation protocols development by customers

Publications: check the related publications for your cell type

How to handle RNAase rich tissue (e.g. pancreas, spleen, lung).

Best practice for working with nuclei.

Isolate nuclei for 3' gene expression profiling.

Optimize nuclei isolation for 3' gene expression profiling.

Recommendations for working with organoid.

How to process neutrophils?

What are the best practices for flow sorting cells.

How to dissociate tissues of interest?

Worthington tissue dissociation table.

MASC tissue dissociation kits.

Section 2: Single-cell RNA-seq with cell surface protein detection

If you are performing single-cell RNA-seq and simultaneously detecting cell surface proteins, it is advisable to refer to the guidelines provided by 10x Genomics or BioLegend. For 10x Genomics, they recommend using a cell input range of 0.2-2 x 10^6 cells for the experiment. Additionally, they provide a comprehensive wash strategy that takes into account cell viability. Alternatively, BioLegend suggests using 1 x 10^6 cells for the procedure.

When using this combined approach, please note that TotalSeq™-B is designed for the 10x Genomics 3' protocol, while TotalSeq™-C is intended for the 5' protocol with Feature Barcode technology. The specific usage of each reagent is outlined in the following table:

Format-Specific Reagents

	TotalSeq™-B	TotalSeq™-C
Antibodies	TotalSeq™-B antibodies and/or hashtag reagents	TotalSeq™-C antibodies and/or hashtag reagents
	(For use only with the Single Cell 3' v3 Feature Barcoding kit)	(For use only with the Single Cell V(D)J Feature Barcoding kit)
Biotin (*optional*)	A biotinylated antibody and TotalSeq™-B barcoded streptavidin	A biotinylated antibody and TotalSeq™-C barcoded streptavidin
Single Index Kit	Chromium Single Cell 3' Feature Barcode Library Kit 16 rxns 1000079	Chromium Single Cell 5' Library Construction Kit 16 rxns 1000020
OR **Dual Index Kit**	Dual Index Kit NT Set A (for Feature Barcode Libraries) 96 rxns 1000242	Dual Index Kit TN Set A (for Feature Barcode Libraries) 96 rxns 1000250

NOTE: The TotalSeq™ antibodies used will vary based on the nature of the experiment. **DO NOT** combine TotalSeq™-B and TotalSeq™-C antibodies in a single experiment.

Section 3: Cell Multiplexing

Cell multiplexing refers to a technique used to label cells (or nuclei) from separate samples with unique molecular tags before pooling them together for single-cell RNA sequencing (scRNA-seq). This allows for the simultaneous analysis of multiple samples within a single scRNA-seq experiment.

Cell multiplexing offers several advantages, including enhanced sample throughput and the ability to analyze a larger number of cells within a single experiment. Additionally, it aids in the detection and removal of multiplets, which are cells that inadvertently get captured together, prior to data analysis. There are two primary strategies for cell multiplexing. One approach involves using antibody-based hashtags, as offered by BioLegend. The second strategy involves lipid-based tags, as provided by 10x Genomics.

BioLegend's hashtags are designed to target cell surface markers that are ubiquitously expressed on cells. These hashtags are conjugated to unique barcodes, allowing for the identification and tracking of individual cells during the scRNA-seq process. For human samples, the hashtag reagents recognize CD298 and β2-Microglobulin, while for mouse samples, the hashtag antibodies target CD45 and H-2 MHC Class I. To utilize these hashtags, it is recommended to follow the staining protocol provided. The protocol has been demonstrated using a cell input of $1x10^6$ cells.

Additionally, the nuclear hashtag antibodies are capable of recognizing a family of nuclear pore complex proteins. They may exhibit cross-reactivity with various vertebrate organisms and even other invertebrate species, such as Xenopus and yeast.

The 3' CellPlex from 10x is designed to be applicable across different species, as it is species agnostic. It consists of 12 oligo-conjugated lipid tags that can incorporate into the cell and nuclear membranes, allowing for the labelling of cells from individual samples. To label your samples, it is recommended to follow the demonstrated protocol provided by 10x. This protocol is supported for cell or nuclei inputs of at least 0.1×10^6 per sample. If sample quantity is not a limitation, it is recommended to use a range of $0.5\text{-}2 \times 10^6$ cells or nuclei per sample. Using an insufficient number of cells or nuclei may result in high background in the Cell Multiplexing data. This can occur due to incomplete removal of supernatant during the wash steps, which may be caused by poor visibility of the cell or nuclei pellet and an inadequate number of cells or nuclei remaining for accurate counting and pooling. It is expected that 30-70% of cells or nuclei may be lost after labelling and washing steps. It is important to note that the assay is time-sensitive. Cells or nuclei should be pooled within 30 minutes after labelling and washing. After pooling, the samples should be immediately loaded onto the 10x chip. Prolonged periods of time, either post-labelling or post-pooling, can have a severe impact on the cell multiplexing data, even if the single-cell behaviour of the gene expression data is only mildly affected.

It's important to consider that loading a higher number of cells can result in an increase in multiplets, as illustrated in the following table. Therefore, it's essential to strike a balance between the desired number of cells to be recovered and the anticipated level of multiplets, considering the associated sequencing costs.

Targeted Cell Recovery	10,000 cells	30,000 cells
Cells Loaded	16,500	49,500
Cell Barcodes Detected	~9,200	~23,400
Singlets	~8,400	~17,700
Multiplets	~780	~5,600

The number of tags utilized in cell multiplexing is determined by the desired cell recovery rate. If you aim to recover a higher number of cells, it is recommended to use a greater number of tags. This is because the detection rate of multiplets is influenced by the number of tags employed.

Targeted Cell Recovery	Number of Tags
500-2,500	2
2,500-10,000	2-4
10,000-20,000	4-8
20,000-30,000	8-12

Number of Tags	Multiplets Detectable
2	50.0%
4	75.0%
8	87.5%
12	91.5%

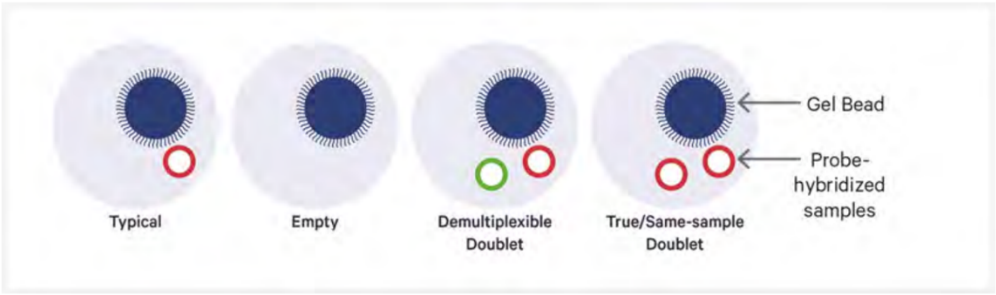
The choice of technology for multiplexing depends on factors such as whether you are starting with cells or nuclei and the specific cell type you are analysing. To explore and compare antibody-based and lipid-based multiplexing techniques for single-cell RNA sequencing (scRNA-seq), we recommend referring to a publication that examines this topic in detail.

Section 4: Single-cell Gene Expression Flex protocol

Chromium Fixed RNA Profiling provides scalable solutions for measuring gene expression in single-cell and nuclei suspensions that have been fixed with formaldehyde. After cell fixation, the cells can be stored at 4 °C for up to one week or at -80 °C for up to six months. This makes the kit suitable for time course experiments, reduces logistical risks, and helps preserve fragile cells by locking the samples in their biological states at the point of collection.

This kit differs from the standard 10x 3' or 5' kits as it is probe-based. Instead of sequencing specific ends of the transcripts, gene expression is measured using probe pairs specifically designed to hybridize to mRNA. Currently, only Human and Mouse probes are available. However, you can create a custom probe using this guide.

There are two types of protocols available for this kit:
1. The Singleplexed sample kit allows for efficient partitioning of 500-10,000 cells per channel. It also enables the detection of cell surface proteins using Biolegend TotalSeq™-B or Biolegend TotalSeq™-C antibodies. It's important to perform antibody staining before cell fixation with this protocol.
2. The Multiplexed sample kit increases the experiment size and cell number by allowing up to 4 or 16 samples to be run within a single GEM reaction. The kit contains 4 or 16 probe sets, each with a Probe Barcode. The Probe Barcode enables sample multiplexing and downstream demultiplexing. By including a Probe Barcode in each probe pair, GEMs containing more than one cell can be identified, and the data generated from those cells can be demultiplexed, provided the cells have unique Probe Barcodes.



With the Chromium Fixed RNA Kit, you can recover and demultiplex up to 10,000 cells per sample when using 4 reactions and 4 Probe Barcodes (BC). This results in a total of 40,000 cells recovered per GEM reaction.
If you use more than four Probe Barcodes, you can recover and demultiplex up to 8,000 cells per sample. For instance, when using 4 reactions and 16 Probe Barcodes, you can recover and demultiplex a total of 128,000 cells (16 * 8,000 cells) per GEM reaction.

Undetectable Multiplet Rate (%)	Cells Loaded/ Probe Barcode	Cells Recovered/ Probe Barcode	Cells Equally Distributed on 4 Probe Barcodes		Cells Equally Distributed on 16 Probe Barcodes	
			Cells Loaded/ Well	Cells Recovered/ Well	Cells Loaded/ Well	Cells Recovered/ Well
~0.4	825	500	3,300	2,000	13,200	8,000
~0.8	1,650	1,000	6,600	4,000	26,400	16,000
~1.6	3,300	2,000	13,200	8,000	52,800	32,000
~2.4	4,950	3,000	19,800	12,000	79,200	48,000
~3.2	6,600	4,000	26,400	16,000	105,600	64,000
~4.0	8,250	5,000	33,000	20,000	132,000	80,000
~4.8	9,900	6,000	39,600	24,000	158,400	96,000
~5.6	11,550	7,000	46,200	28,000	184,800	112,000
~6.4	13,200	8,000	52,800	32,000	211,200	128,000
~7.2	14,850	9,000	59,400	36,000	n/a*	n/a*
~8.0	16,500	10,000	66,000	40,000	n/a*	n/a*

* These cell numbers are not supported.

The Multiplexed kit also enables the detection of cell surface proteins using Biolegend TotalSeq™-C antibodies. It's important to perform antibody staining before cell fixation with this protocol.

Please follow the provided protocols to fix your samples, whether they are cells or tissue. Additionally, this kit is compatible with FFPE (formalin-fixed, paraffin-embedded) samples. For isolating cells from FFPE tissue sections, you can refer to the specific protocol provided. It's worth noting that this kit has been extensively tested with various tissues, and you can find more detailed information in the provided documentation.

For this kit, we require a minimum of 1 million cells to be submitted. Prior to fixation, it is important to assess the viability and cleanliness of your cell sample. Highly viable single-cell or nuclei suspensions with a viability of over 80% will yield the best sensitivity and cell recovery. To achieve optimal results, ensure that your samples have minimal debris. Debris may contain RNA that is not derived from cells and can contribute to non-cellular background noise. Therefore, it is advisable to minimize debris for the best possible outcomes.

Some useful links for single-cell suspension preparation

Can post-fixation samples be sorted?

Intracullular Staining with Flex kit.

How to design custom probes to detect EGFP and RFP?

How to design custom probes for multiplexed samples?

How do I order and use custom panel?

Can OCT embedded tissue be used?

Can formalin fixed tissue be used?