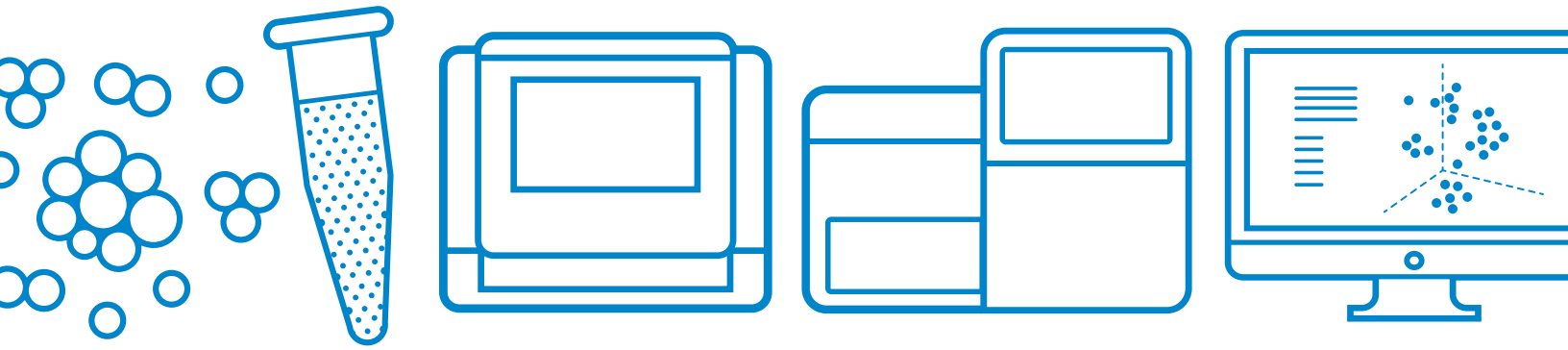
10X GENOMICS

Experiment Planning Guide

Getting Started with Single Cell Gene Expression



Advantages of Single Cell Gene Expression Profiling

Go beyond traditional gene expression analysis to characterize cell populations, cell types, cell states, and more on a cell-by-cell basis

Differences in gene expression in organisms, tissue, and disease states have historically been quantified using a number of approaches such as microarrays and bulk RNA sequencing (RNA-seq), to name a few. These typically require hundreds to millions of cells as input, resulting in only an average reading across cell populations. Complex biological processes in developmental biology, cancer, neuroscience, immunology, and infectious disease usually involve multiple individual cells, with different cell fates, states, and functions. In these dynamic cellular events, bulk measurements provide limited information, as individual cellular measurements are lost (1)(Figure 1).

Recently, single cell transcriptomic technologies, including our high throughput Chromium Single Cell Gene Expression Solution, allow the direct measurement of gene expression at the single cell level to quantify intracellular population heterogeneity and characterize cell types, cell states, and dynamic cellular transitions cell by cell. In addition to potentially identifying new cell subtypes and rare cell populations, single cell technologies enable a better understanding of transcription dynamics and gene regulatory relationships.

While the number of transcripts sequenced per sample are similar between single cell RNA-seq and bulk expression experiments, single cell gene expression studies allow you to extend beyond traditional global marker gene analysis to the characterization of cell types or cell states and the concomitant dynamic changes in regulatory pathways, which are driven by many genes. Importantly, single cell gene expression allows for an unbiased characterization of cell populations independently of any prior knowledge of cell subtypes or cell markers. In order to take full advantage of the rich information enabled by single cell transcriptomic technologies, a few dedicated steps with regard to experimental design, sample preparation, and downstream data analysis should be considered prior to starting your first experiments.

This guide helps you get started with your single cell gene expression experiments and serves as a roadmap to help design your experiments, optimize experimental parameters, and identify the computational/analytical tools to best analyze your single cell gene expression data.

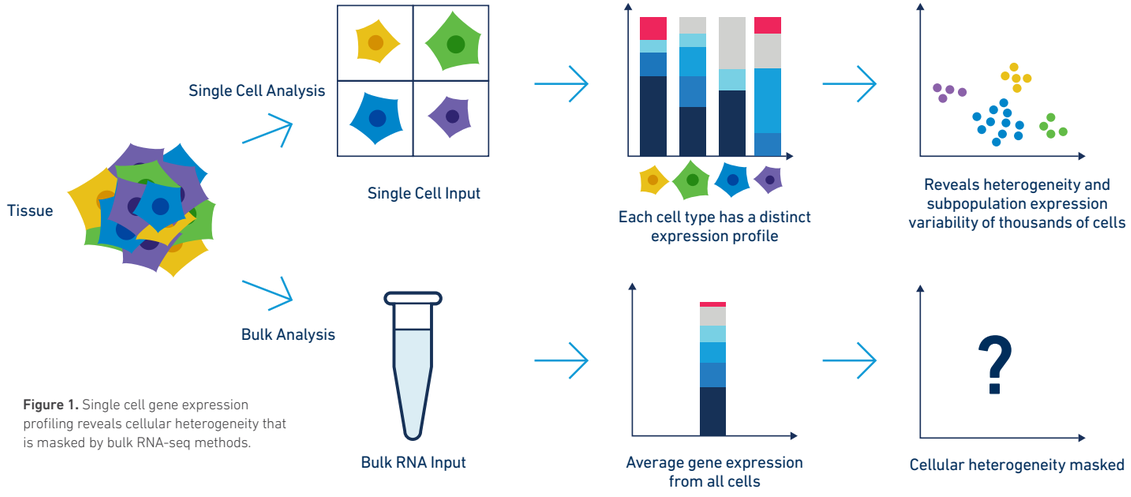


Figure 1. Single cell gene expression profiling reveals cellular heterogeneity that is masked by bulk RNA-seq methods.

Research Considerations Overview

STEP 1

What scientific questions do I want to answer?

Do I want to characterize, identify, atlas or catalog mixed cell populations?

Do I want to identify cell markers and regulatory pathways involved in cellular processes?

Am I characterizing or identifying novel cell types or states?

STEP 2

What are best practices for preparing and processing my sample?

Sample type and preparation

Sample processing

STEP 3

How many cells and replicates do my experiments require?

Number of cells

Sequencing depth

Number of replicates

Batch effects

STEP 4

How do I analyze and visualize my data?

Process and analyze sequencing data

Visualization

Preparing for Single Cell Gene Expression Experiments

Before starting your single cell experiments, we recommend that you walk through a four step process to help guide your experimental design and determine how to best answer your research questions.

STEP 1 What scientific questions do I want to answer?

Single cell gene expression analysis can provide answers to many different types of research questions. These questions include but are not limited to:

Do I want to characterize, identify, atlas, or catalog mixed cell populations from tissue or organs?

SEE SELECTED PUBLICATIONS

25, 26, 27, 29, 32, 51, 58, 59, 60, 61

Do I want to characterize or identify novel biomarkers and regulatory pathways involved in complex cellular processes?

SEE SELECTED PUBLICATIONS

13, 14, 15, 16, 35, 37, 38, 39, 41, 42, 43, 45, 46, 47, 50, 51, 52, 53, 54, 55

Do I want to characterize or identify novel cell types or states involved in complex cellular processes?

SEE SELECTED PUBLICATIONS

12, 17, 18, 19, 20, 21, 22, 23, 24, 28, 30, 31, 33, 34, 36, 40, 44, 48, 49, 56

STEP 2 What are best practices for preparing and processing my sample?

Sample Type & Preparation

It is critical that you obtain a well-singulated cell suspension free of cell debris, with minimal cell aggregates and high viability (>70%). It is also important to know the size range of the cells studied. The cell size is usually correlated with the quantity of transcripts expressed in the cell. A wide range of cell sizes (up to >30 µm) are compatible with the Chromium Single Cell Chips used in our Gene Expression Solutions. In general, cell preparation protocols will vary depending on the tissue of origin and the cell types studied. Each tissue type is unique and thus, it is critical to optimize sample preparation before starting any single cell experiment (see technical note on optimal sample preparation: **go.10xgenomics.com/scRNA-3/optimal-sample-prep**). Cryopreservation, fixation (see demonstrated protocol for methanol fixation: **go.10xgenomics.com/scRNA-3/methanol-fixation**), and nuclei isolation from archival samples (see demonstrated protocol for nuclei isolation: **go.10xgenomics.com/scRNA-3/nuclei-isolation**) are alternative preparation methods that are compatible with our system.

Sample Processing

The ability to process samples quickly after isolation or tissue dissociation is critical in maintaining cell integrity and preserving each cell's transcriptome. Be aware that any sample manipulations may adversely affect gene expression profiles, cell states, or cell viability and introduce bias in the study (2).

STEP 3 How many cells and replicates do my experiments require?

Number of Cells

Deciding on the number of cells required depends on the expected heterogeneity of the cells in the sample, the number of cells available in the sample, the minimum frequency expected of a subpopulation type, and the minimum number of cells of each cell type desired for data analysis (see online tool: **satijalab.org/howmanycells**). If the sample diversity is not known, a high number of cells at low sequencing depth may be the most flexible option to obtain a representative proportion of the cell population and meaningful biological information. The Chromium System can recover up to ~65% of the cells loaded with a low doublet rate (0.9% per 1000 cells). The high throughput capability of the Chromium System enables the processing of highly heterogeneous samples, which may require thousands of cells to fully resolve each subpopulation. In contrast, the high cell recovery rate of our system makes it suitable for samples that are limited in cell numbers.

Sequencing Depth

The sequencing depth per experiment is dependent on both the total mRNA content in individual cells and the diversity of mRNA species in those cells. In general, at the same transcript diversity, cells expressing a low amount of mRNA will require much lower sequencing depth than cells expressing a large amount of mRNA. When sequencing costs or capacity are limiting, there is often a trade-off between sequencing a higher number of cells (breadth) and sequencing a lower number of cells with more reads (depth). (see the technical note for more information: **go.10xgenomics.com/scRNA-3/number-and-depth**). 10x Genomics single cell gene expression libraries are compatible with short-read sequencers. Additionally, our protocol uses unique molecular identifiers (UMIs) to barcode each transcript molecule before amplification takes place, resulting in a digital gene expression profile while accounting for PCR amplification bias.

Number of Replicates

Determining the number of replicates depends on the research project, the type of sample, and the number of cells required in the study. The matter of biological replicates is still an open question in the field. In some studies, one sample alone can be seen as sufficient, where each cell represents a biological replicate and different samples from different individuals account for the variability of a particular biological process. In other studies, to mitigate biological variability occurring in small cell populations across time, it can be beneficial to pool cells from different samples to cover all aspects of the cell population being studied. Other cases may require the use of multiple replicates derived from one sample to increase the total number of cells in the study.

Batch Effects

Batch effects can be introduced at any stage of the workflow and are mostly due to logistical constraints that result in different preparation times, operators, and handling protocols. The 10x Genomics Chromium System demonstrates minimal technical variability across a variety of technical replicates (see the technical note: **go.10xgenomics.com/scRNA-3/technical-replicates**). When combining data from multiple libraries, we recommend equalizing the read depth (depth normalization) between libraries before merging to reduce batch effects introduced by sequencing (see: **go.10xgenomics.com/scRNA-3/depth-normalization**). In addition, a number of computational tools including Seurat (3), scran (4), and scrone (5) can correct batch effects.

STEP 4 How do I analyze and visualize my data?

Process and Analyze Sequencing Data

After sequencing, you will process your raw data through a set of analysis pipelines (Cell Ranger) that will align reads, filter, count barcodes and UMIs, generate Feature-Barcode matrices, and perform clustering and gene expression analysis. Cell Ranger can aggregate outputs from multiple experiments, normalize to the same sequencing depth, and re-analyze the combined data. Cell Ranger pipelines run on Linux systems, and most software dependencies come bundled in the Cell Ranger package (see system requirements: **go.10xgenomics.com/scRNA-3/system-requirements**).

Visualization

Loupe Cell Browser is a desktop application designed for quick, interactive single cell data visualization and analysis. Built to accelerate the discovery of new marker genes, you can identify rare cell types and explore novel substructures within your data, with no prior knowledge of programming required (see online tutorial **go.10xgenomics.com/scRNA-3/visualization-tutorial**).

The Cell Ranger pipeline produces output files that most open source packages developed in R or python can interpret for analysis. Some of the most popular software packages used for single cell gene expression analysis are Seurat (3) and Monocle (6). If you do have prior programming knowledge, both R packages perform QC checks, secondary analysis, and exploration of single cell gene expression data (see extensive list of packages **github.com/seandavi/awesome-single-cell** or **www.scrna-tools.org**).

Use Case Examples

Browse this short collection of use case examples to help give you further guidance from the literature about how to set up your single cell gene expression experiments using our technology

EXPERIMENT SNAPSHOT	AIM	RESULT	SAMPLE PREP	LIBRARY PREP	SEQUENCING	ANALYSIS TOOLS
RESEARCH AREA Developmental Biology **ORGANISM** *Mus musculus* **SAMPLE TYPE** Flow-sorted intestinal stem cells	*To study the priming and self-renewal mechanisms of intestinal stem cells*	The renewal and differentiation of Lgr5+ intestinal stem cells is critical to the continuous regeneration of the epithelial lining of the gut, and Wnt and R-spondin ligands are both required to maintain this stem cell population. In a recent Nature publication, Yan and colleagues used single cell gene expression analysis to show that Lgr5+ cells consisted of 3 cellular subpopulations (cycling, non-cycling, and transit amplifying cells). Yan K.S. et al., *Nature*, 2017, **doi.org/10.1038/nature22313**	• 6x Lgr5-eGFP-IRES-CreER mice treated in vivo with adenovirus Fc control, Fc-FZD8-CRD, Fc-RSPO1, Fc-RSPO2, scFc-DKK1, Fc-LGR5-ECD • Harvest jejunum, sort gfp+ cells for all 6 conditions, (gfp+ cells) and for Fc control condition. Sort gfp- cells. • Number of cells available per condition (~1000 cells)	• 1x Chromium Single Cell 3' Library & Gel Bead Kit v2, 16 rxns • Chromium Single Cell A Chip Kit, 16 rxns • Chromium i7 Multiplex Kit, 96 rxns • Chromium Single Cell Controller • Duplicate libraries • 14 libraries (2 libraries per sample) • 1000 cells targeted per library	• 50,000 reads per cell • 700 million reads total • 2x Illumina NextSeq runs (2x 75 cycles)	• Cell Ranger • Secondary analysis with R code
RESEARCH AREA Neuroscience, Developmental Biology **ORGANISM** *Drosophila melanogaster* **SAMPLE TYPE** Brain tissue	*To catalog the diversity of cell types and regulatory states in the brain, and how these change during ageing*	In a recent Cell paper, Davie and colleagues characterized the entire adult *Drosophila melanogaster* brain sampled across its lifespan. Using single cell RNA sequencing, they identified more than 50 cell populations by specific transcription factors and their downstream gene regulatory networks. Finally, they identified a novel neuronal cell state driven by two specific marker genes. Davie K. et al., *Cell*, 2018, **doi.org/10.1016/j.cell.2018.05.057**	• 2 different *D. melanogaster* strains • 13 different time points (from newly-eclosed to 50-days old) • Combined 20 male and 20 female brain	• 2x Chromium Single Cell 3' Library & Gel Bead Kit v2, 16 rxns • Chromium Single Cell A Chip Kit, 16 rxns • Chromium i7 Multiplex Kit, 96 rxns • Chromium Single Cell Controller • Biological duplicates • 26 libraries (1 library per sample) • 5000 cells targeted per library	• 50,000 reads per cell • 6500 million reads total • 2x flow cell HiSeq 4000 runs with Illumina HiSeq 3000/4000 series kit (150 cycles)	• Cell Ranger • Scater R • Seurat • SCENIC
RESEARCH AREA Cardiology, Developmental Biology **ORGANISM** *Mus musculus* **SAMPLE TYPE** Heart tissue	*To characterize the transcriptional profiles of non-myocyte cardiac lineages in the mouse heart*	Skelly and colleagues characterized the murine non-myocyte cardiac cellular landscape using single cell RNA sequencing. Detailed molecular analysis revealed the diversity of cell populations composing the heart, uncovered an extensive network of intercellular communication, and suggested a prevalent sexual dimorphism in cardiac gene expression. Skelly D.A. et al., *Cell Rep.*, 2018, **doi.org/10.1016/j.celrep.2017.12.072**	• Pool 2 female mouse hearts • Pool 2 male mouse hearts • Flow sorting to remove endothelial cells, dead cells, and debris	• 1x Chromium Single Cell 3' Library & Gel Bead Kit v2, 4 rxns • Chromium Single Cell A Chip Kit, 16 rxns • Chromium i7 Multiplex Kit, 96 rxns • Chromium Single Cell Controller • Biological duplicate • 2 libraries (1 library per sample) • 7000 cells targeted per library	• 50,000 reads per cell • 700 million reads total • 2x lane of Illumina HiSeq 4000 runs with Illumina HiSeq 3000/4000 series kit (150 cycles)	• Cell Ranger • Seurat • Tidyverse
RESEARCH AREA Immunology **ORGANISM** *Homo sapiens* **SAMPLE TYPE** Peripheral blood and ascites from cancer patients	*To study monocyte heterogeneity and their potential to differentiate into distinct lineages*	Goudot and colleagues used single cell gene expression analysis to determine that the CD14+/CD16- monocytes are a homogenous population. The monocytes did not express any monocyte-derived dendritic cells (mo-DCs) signature genes, suggesting that they were not primed to the mo-DC differentiation. Monocytes all expressed a partial monocyte-derived macrophage (mo-Macs) gene signature, which suggests that the cells were pre-committed to a default mo-Macs differentiation pathway without the presence of any mo-DC environmental triggers. Goudot C. et al., *Immunity*, 2017, **doi.org/10.1016/j.immuni.2017.08.016**	• CD14+ monocytes enriched from PBMC and cultured 5 days +/- M-CSF, IL-34, GM-CSF, IL-4 and TNF-a • 2 different patients (PBMC)	• 1x Chromium Single Cell 3' Library & Gel Bead Kit v2, 4 rxns • Chromium Single Cell A Chip Kit, 16 rxns • Chromium i7 Multiplex Kit, 96 rxns • Chromium Single Cell Controller • 2 libraries (1 library per sample) • 500 cells targeted per library	• 100,000 reads per cell • 100 million reads total • 1x rapid flow cell Illumina HiSeq 2500 runs with Illumina HiSeq 2000 series kit (2x 100 cycles)	• Cell Ranger • Seurat
RESEARCH AREA Neuroscience **ORGANISM** *Homo sapiens* **SAMPLE TYPE** Nuclei preparation of post-mortem frozen adult brain tissue	*To interrogate archived brain samples at single cell resolution*	Nagy and colleagues used single cell gene expression analysis on nuclei derived from dorsolateral prefrontal cortex of individual with major depressive disorder or healthy controls. Almost 80,000 nuclei from 34 frozen brain samples were analyzed and this approach allowed a sensitive, efficient, and unbiased classification of cell types in the brain. The results show that this high-resolution approach can reveal previously undetectable changes in specific cell types in the context of complex phenotypes and heterogeneous tissues. Nagy et al., *bioRxiv*, 2018, **doi.org/10.1101/384479**	• 34 samples (dorsolateral prefrontal cortex) from recently deceased 17 major depressive disorder and 17 control individuals • Nuclei extraction for each sample	• 2x Chromium Single Cell 3' Library & Gel Bead Kit v2, 16 rxns • 1x Chromium Single Cell 3' Library & Gel Bead Kit v2, 4 rxns • Chromium i7 Multiplex Kit, 96 rxns • Chromium Single Cell Controller • Biological replicates (17 ea) • 34 libraries • ~3000 nuclei targeted per library	• ~70,000 reads per nuclei • ~7 billion reads total • 20x lanes of Illumina HiSeq 4000 runs with Illumina HiSeq 3000/4000 series kit (150 cycles)	• Cell Ranger • Seurat • Monocle

CITATIONS

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SELECTED PUBLICATIONS

We have gathered a number of useful references and peer-reviewed manuscripts to provide you more in-depth information about single cell transcriptomics as it relates to your research interests.

General Reviews

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Linking Data, Developers and Discovery

Below you will find a number of useful online tools to maximize the success of your experiments, including links to 10x Genomics Support documentation, datasets, as well as other available resources outlining best practices.

Documentation (sample preparation, library preparation, instrument and sequencing)

go.10xgenomics.com/scRNA-3/sample-prep
go.10xgenomics.com/scRNA-3/library-prep
go.10xgenomics.com/scRNA-3/sequencing
go.10xgenomics.com/scRNA-3/instrument
go.10xgenomics.com/scRNA-3/support

Software

go.10xgenomics.com/scRNA-3/software

Single cell analysis tools

Seurat tutorial: **satijalab.org/seurat/**
How many cells?: **satijalab.org/howmanycells**
Monocle tutorial: **cole-trapnell-lab.github.io/monocle-release/docs/**
Scanpy: **scanpy.readthedocs.io/en/latest/**
Phenograph: **github.com/jacoblevine/PhenoGraph**
Wishbone: **github.com/ManuSetty/wishbone**
Cellrouter: **github.com/edroaldo/cellrouter**

For a complete list of single cell analysis software packages, see:
www.scrna-tools.org/

Datasets

go.10xgenomics.com/scRNA-3/datasets

Resources from 10x Genomics

We are dedicated to helping you get the most out of your 10x Genomics system by offering multiple helpful resources:

Solutions and Products

Along with our suite of complete solutions, we offer an ever-growing catalogue of services to help you find the answers to your research questions.

10x Compatible Products

Access our list of key partner products that have been certified compatible to work with our various solutions.

10x University

Immerse yourself in 10x University, a comprehensive step-by-step learning and training environment containing video tutorials and trainings.

Blog

Keep up to date with the 10x Genomics Blog, where you'll find everything from tips and tricks to the latest 10x news.

10x Library

Easy access to our complete library of product literature, customer publications, application notes, protocols, and many other useful resources.

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Visit the support site for documentation, software, and datasets that will help you get the most out of your 10x Genomics products.

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