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Alignment and feature counting

Ashley Sawle

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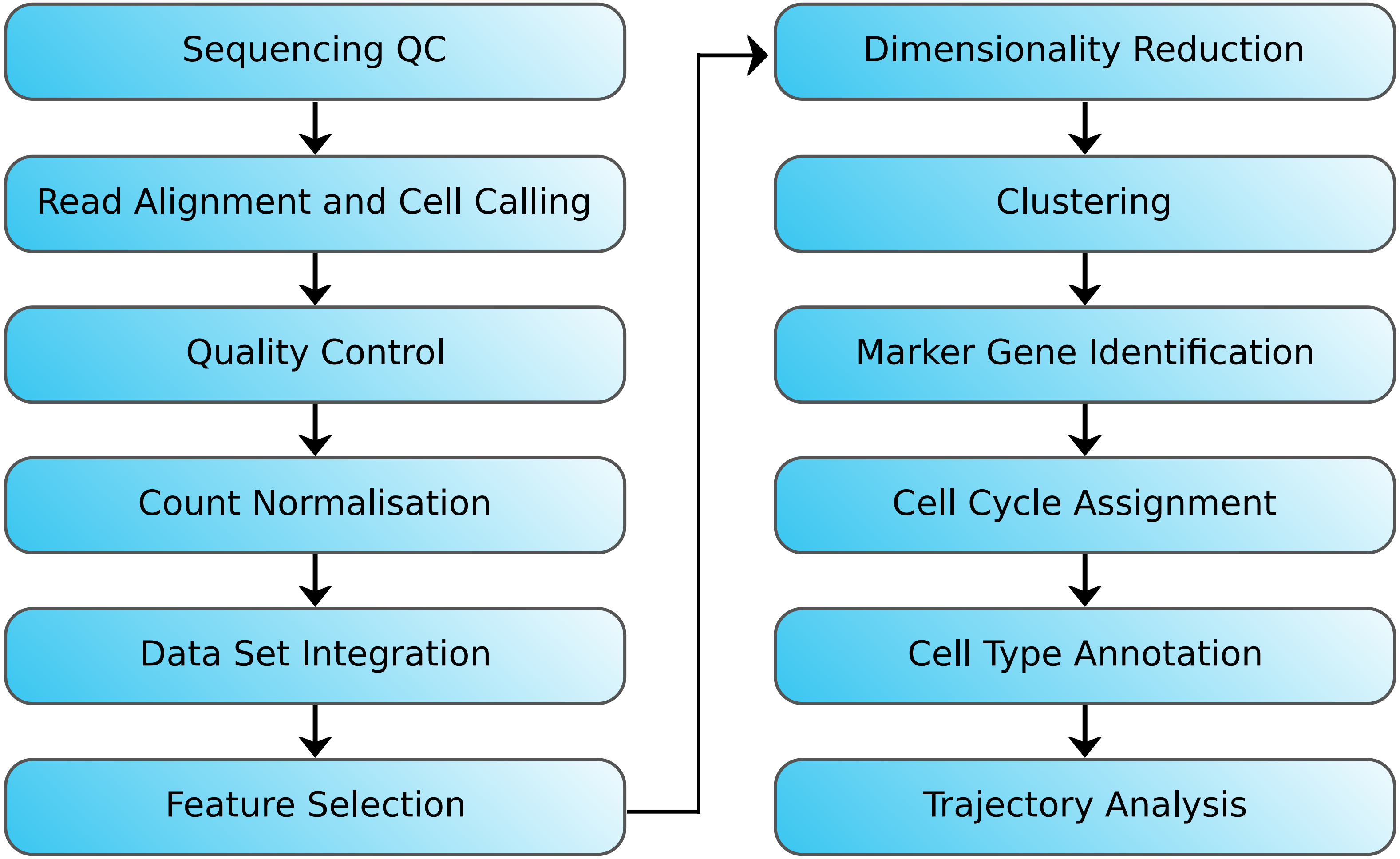
Single Cell RNAseq Analysis Workflow



Sequencing QC

Read Alignment and Cell Calling

Quality Control

Count Normalisation

Data Set Integration

Feature Selection

Dimensionality Reduction

Clustering

Marker Gene Identification

Cell Cycle Assignment

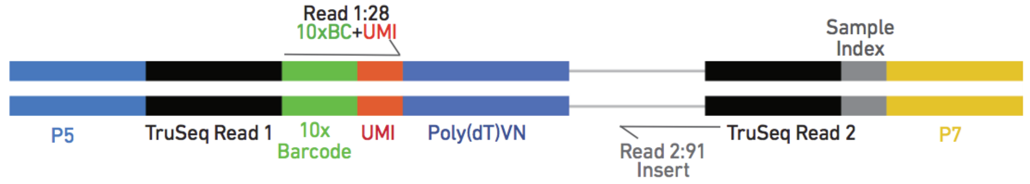
Cell Type Annotation

Trajectory Analysis

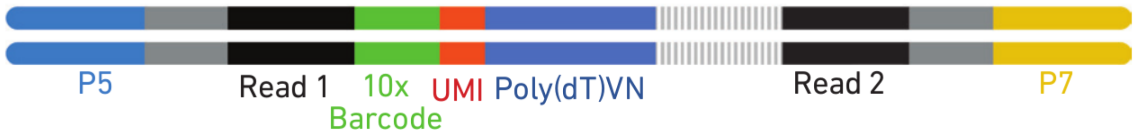
10x library file structure

The 10x library contains four pieces of information, in the form of DNA sequences, for each “read”.

- **sample index** - identifies the library, with one or two indexes per sample
- **10x barcode** - identifies the droplet in the library
- **UMI** - identifies the transcript molecule within a cell and gene
- **insert** - the transcript molecule



Chromium Single Cell 3' Gene Expression Library



Raw fastq files

The sequences for any given fragment will generally be delivered in 3 or 4 files:

- **I1**: I7 sample index
- **I2**: I5 sample index if present (dual indexing only)
- **R1**: 10x barcode + UMI
- **R2**: insert sequence

QC of Raw Reads

- FASTQC:

Alignment and counting

The first steps in the analysis of single cell RNAseq data:

- Align reads to genome
- Annotate reads with feature (gene)
- Quantify gene expression

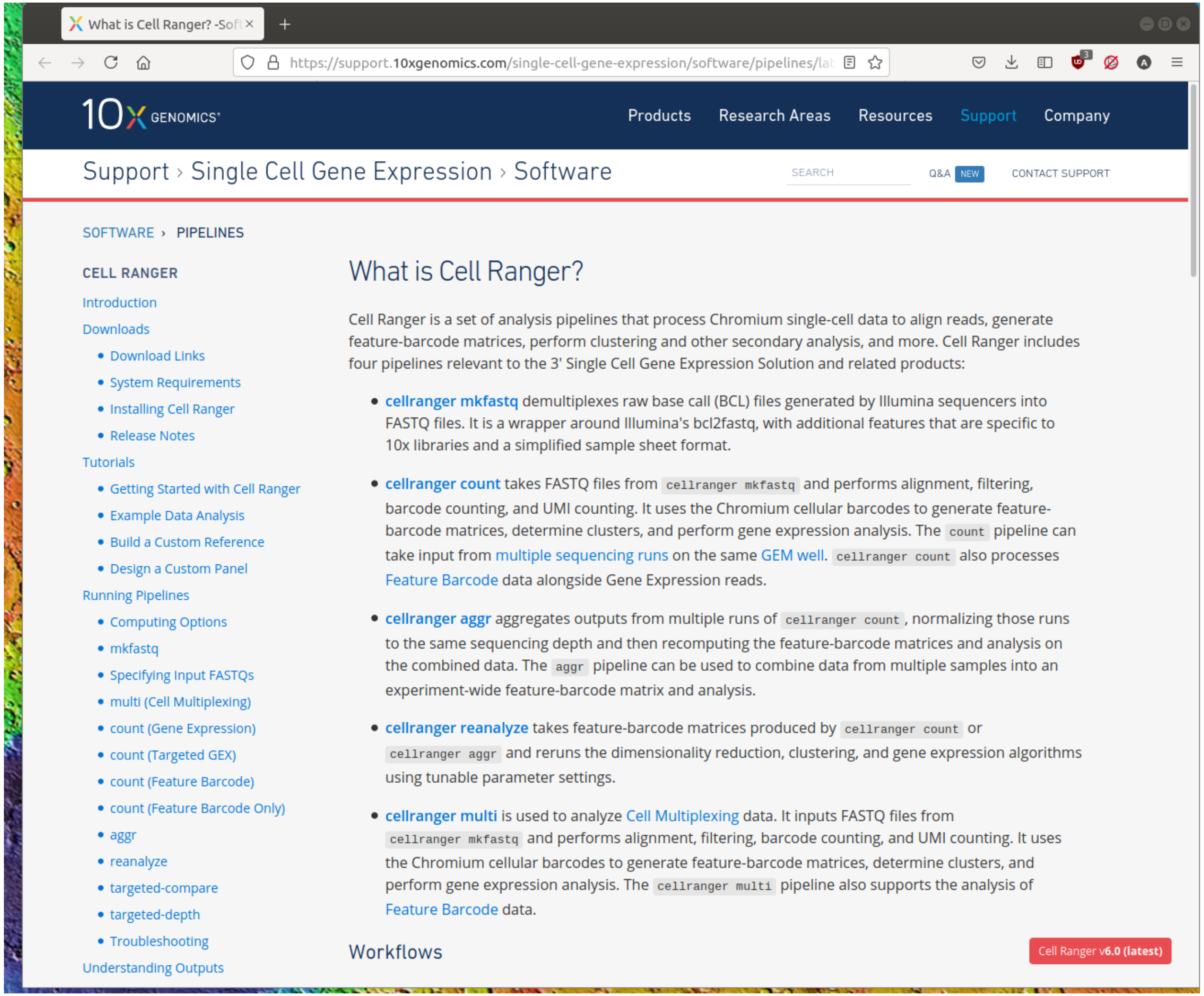
Cell Ranger

- 10x Cell Ranger - This not only carries out the alignment and feature counting, but will also:
  - Call cells
  - Generate a summary report in html format
  - Generate a “cloupe” file

Alternative methods include:

- STAR solo:
  - Generates outputs very similar to CellRanger minus the cloupe file and the QC report
  - Will run with lower memory requirements in a shorter time than Cell Ranger
- Alevin:
  - Based on the popular Salmon tool for bulk RNAseq feature counting
  - Alevin supports both 10x-Chromium and Drop-seq derived data

Obtaining Cell Ranger



What is Cell Ranger? -Sof
https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/lat
10x GENOMICS
Products
Research Areas
Resources
Support
Company
Support › Single Cell Gene Expression › Software
SEARCH
Q&A NEW
CONTACT SUPPORT
SOFTWARE › PIPELINES

CELL RANGER

- Introduction
- Downloads
  - Download Links
  - System Requirements
  - Installing Cell Ranger
  - Release Notes
- Tutorials
  - Getting Started with Cell Ranger
  - Example Data Analysis
  - Build a Custom Reference
  - Design a Custom Panel
- Running Pipelines
  - Computing Options
  - mkfastq
  - Specifying Input FASTQs
  - multi (Cell Multiplexing)
  - count (Gene Expression)
  - count (Targeted GEX)
  - count (Feature Barcode)
  - count (Feature Barcode Only)
  - aggr
  - reanalyze
  - targeted-compare
  - targeted-depth
  - Troubleshooting
- Understanding Outputs

What is Cell Ranger?

Cell Ranger is a set of analysis pipelines that process Chromium single-cell data to align reads, generate feature-barcode matrices, perform clustering and other secondary analysis, and more. Cell Ranger includes four pipelines relevant to the 3' Single Cell Gene Expression Solution and related products:

- **cellranger mkfastq** demultiplexes raw base call (BCL) files generated by Illumina sequencers into FASTQ files. It is a wrapper around Illumina's bcl2fastq, with additional features that are specific to 10x libraries and a simplified sample sheet format.
- **cellranger count** takes FASTQ files from `cellranger mkfastq` and performs alignment, filtering, barcode counting, and UMI counting. It uses the Chromium cellular barcodes to generate feature-barcode matrices, determine clusters, and perform gene expression analysis. The `count` pipeline can take input from multiple sequencing runs on the same GEM well. `cellranger count` also processes Feature Barcode data alongside Gene Expression reads.
- **cellranger aggr** aggregates outputs from multiple runs of `cellranger count`, normalizing those runs to the same sequencing depth and then recomputing the feature-barcode matrices and analysis on the combined data. The `aggr` pipeline can be used to combine data from multiple samples into an experiment-wide feature-barcode matrix and analysis.
- **cellranger reanalyze** takes feature-barcode matrices produced by `cellranger count` or `cellranger aggr` and reruns the dimensionality reduction, clustering, and gene expression algorithms using tunable parameter settings.
- **cellranger multi** is used to analyze Cell Multiplexing data. It inputs FASTQ files from `cellranger mkfastq` and performs alignment, filtering, barcode counting, and UMI counting. It uses the Chromium cellular barcodes to generate feature-barcode matrices, determine clusters, and perform gene expression analysis. The `cellranger multi` pipeline also supports the analysis of Feature Barcode data.

Workflows

Cell Ranger tools

Cell Ranger includes a number of different tools for analysing scRNAseq data, including:

- `cellranger mkref` - for making custom references
- `cellranger count` - for aligning reads and generating a count matrix
- `cellranger aggr` - for combining multiple samples and normalising the counts

Preparing the raw fastq files

Cell Ranger requires the fastq file names to follow a convention:

```
<SampleName>_S<SampleNumber>_L00<Lane>_<Read>_001.fastq.gz
```

e.g. for a single sample in the Caron data set we have:

```
    SRR9264343_S0_L001_I1_001.fastq.gz
    SRR9264343_S0_L001_R1_001.fastq.gz
    SRR9264343_S0_L001_R2_001.fastq.gz
```

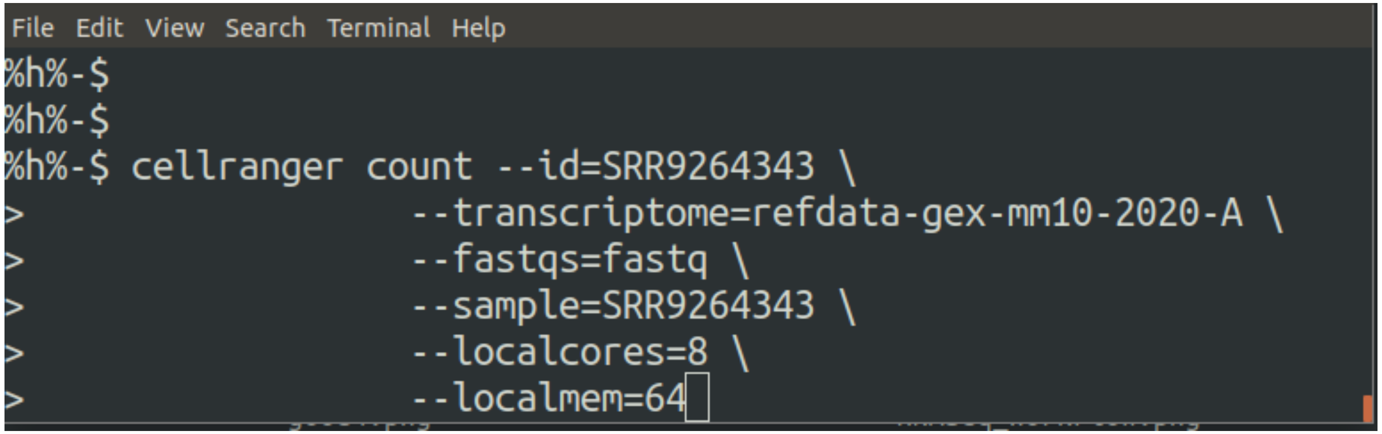
Genome/Transcriptome Reference

As with other aligners Cell Ranger requires the information about the genome and transcriptome of interest to be provided in a specific format.

- Obtain from the 10x website for human or mouse (or both - PDX)
- Build a custom reference with `cellranger mkref`

Running `cellranger count`

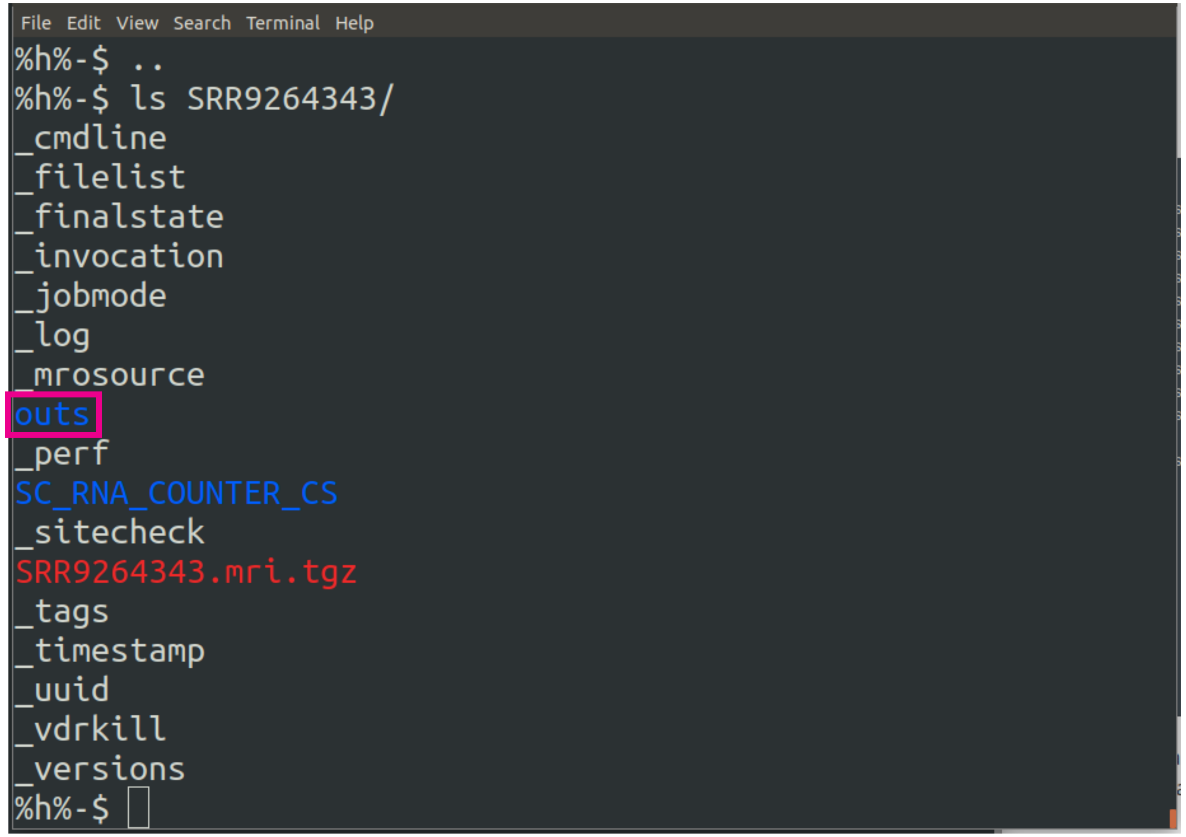
- Computationally very intensive
- High memory requirements



```
%h%-$
%h%-$
%h%-$ cellranger count --id=SRR9264343 \
>                  --transcriptome=refdata-gex-mm10-2020-A \
>                  --fastqs=fastq \
>                  --sample=SRR9264343 \
>                  --localcores=8 \
>                  --localmem=64
```

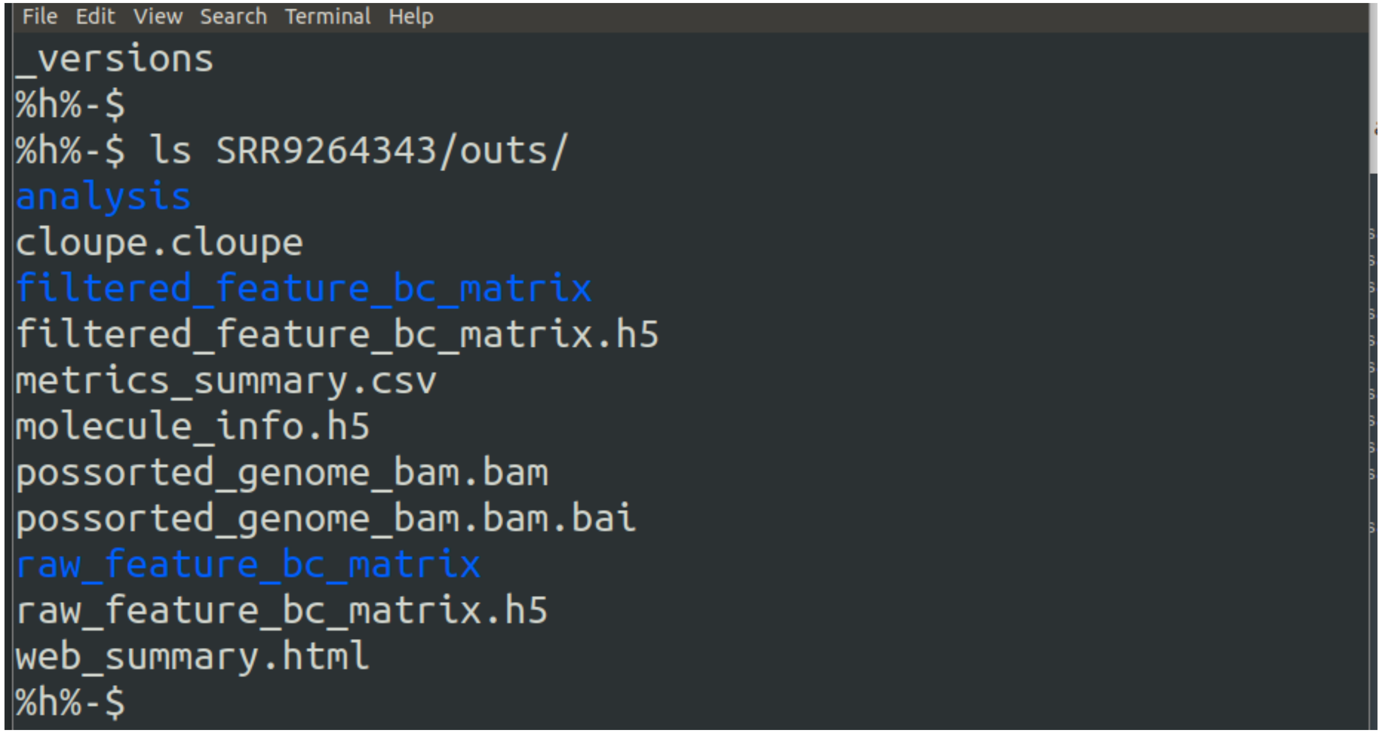
Cell Ranger outputs

- One directory per sample



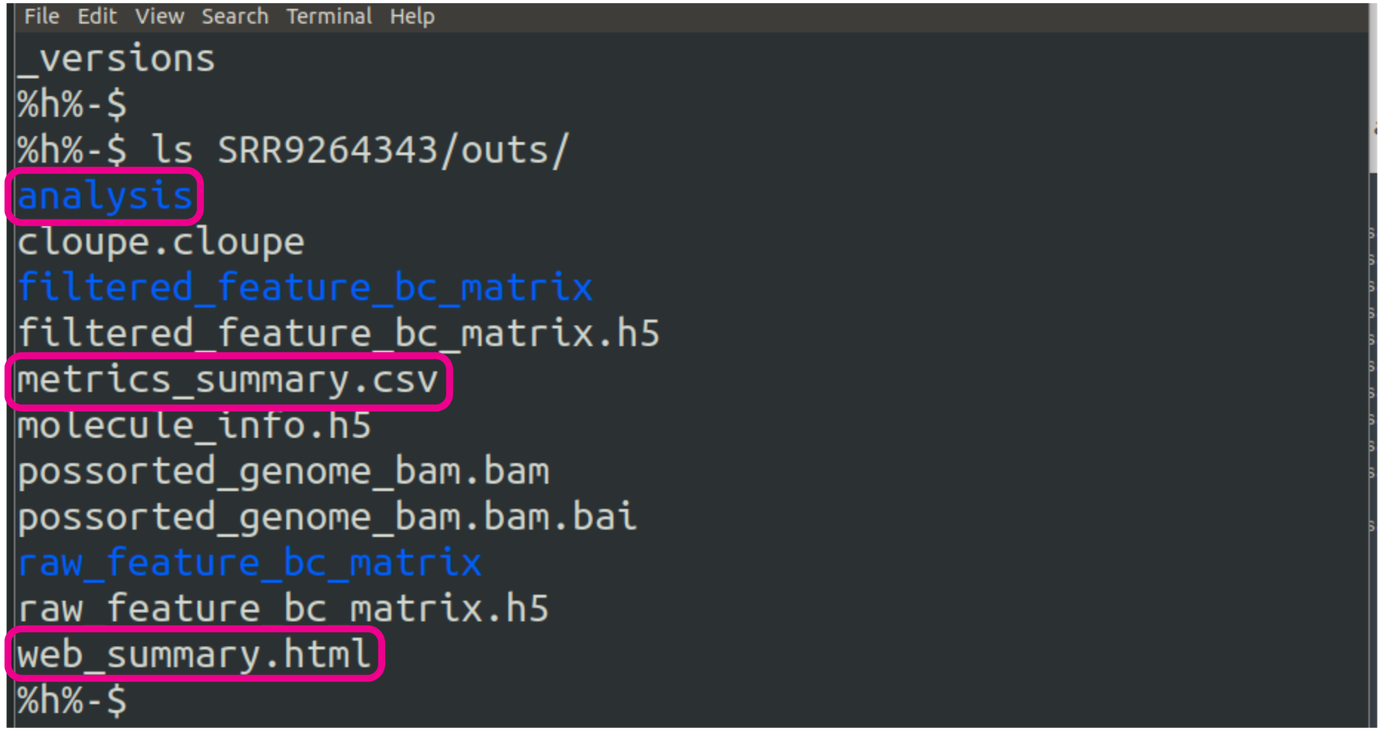
```
%h%-$ ..
%h%-$ ls SRR9264343/
_cmdline
_filelist
_finalstate
_invocation
_jobmode
_log
_mrosource
outs
_perf
SC_RNA_COUNTER_CS
_sitecheck
SRR9264343.mri.tgz
_tags
_timestamp
_uuid
_vdrkill
_versions
%h%-$
```

Cell Ranger outputs



```
_versions
%h%-$
%h%-$ ls SRR9264343/outs/
analysis
cloupe.cloupe
filtered_feature_bc_matrix
filtered_feature_bc_matrix.h5
metrics_summary.csv
molecule_info.h5
possorted_genome_bam.bam
possorted_genome_bam.bam.bai
raw_feature_bc_matrix
raw_feature_bc_matrix.h5
web_summary.html
%h%-$
```

Cell Ranger outputs



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raw_feature_bc_matrix
raw_feature_bc_matrix.h5
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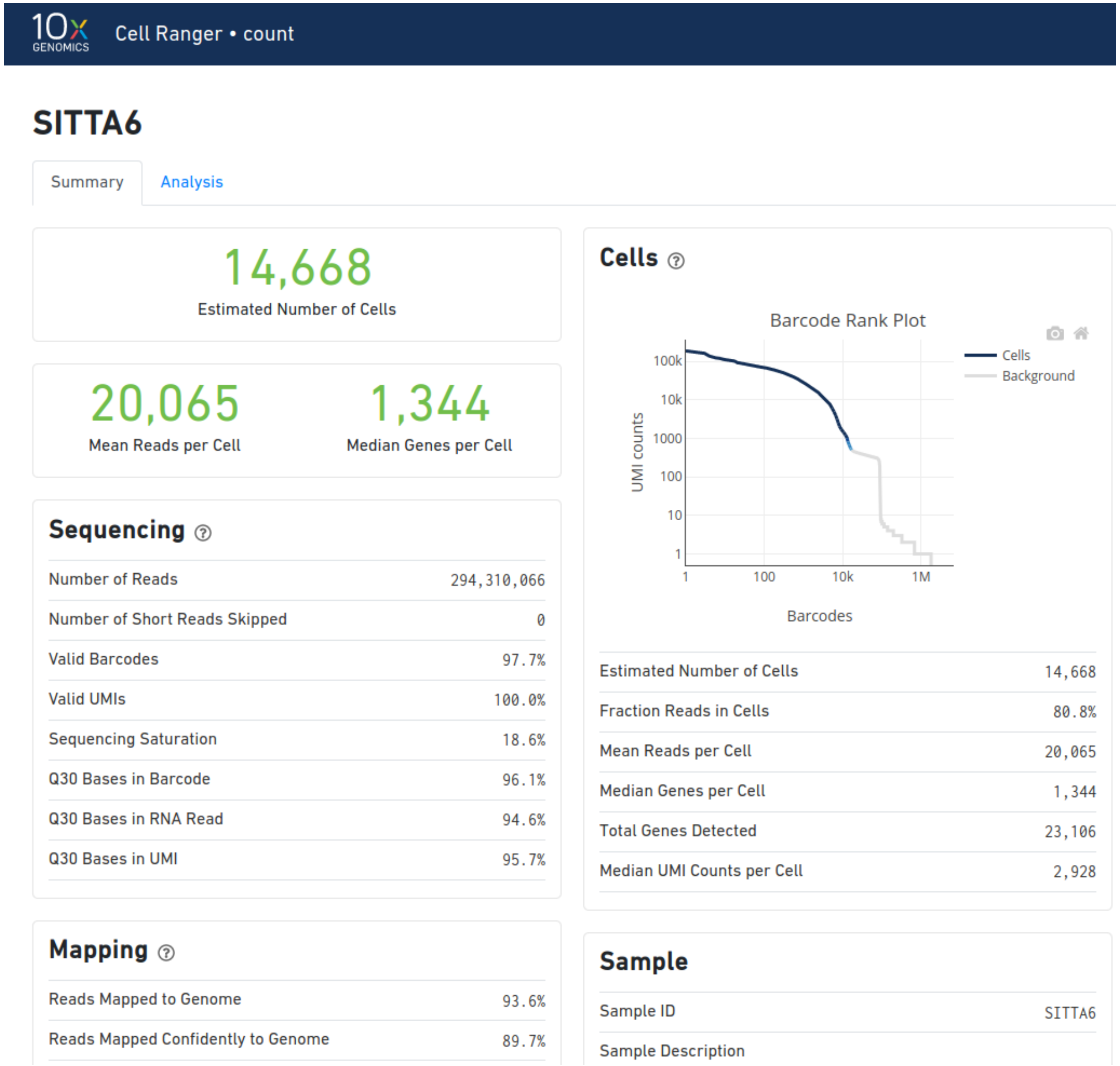
%h%-$
```

Cell Ranger report



Cell Ranger • count

SITTA6

Summary Analysis

14,668
Estimated Number of Cells

20,065
Mean Reads per Cell

1,344
Median Genes per Cell

Sequencing

Number of Reads	294,310,066
Number of Short Reads Skipped	0
Valid Barcodes	97.7%
Valid UMIs	100.0%
Sequencing Saturation	18.6%
Q30 Bases in Barcode	96.1%
Q30 Bases in RNA Read	94.6%
Q30 Bases in UMI	95.7%

Mapping

Reads Mapped to Genome	93.6%
Reads Mapped Confidently to Genome	89.7%

Cells

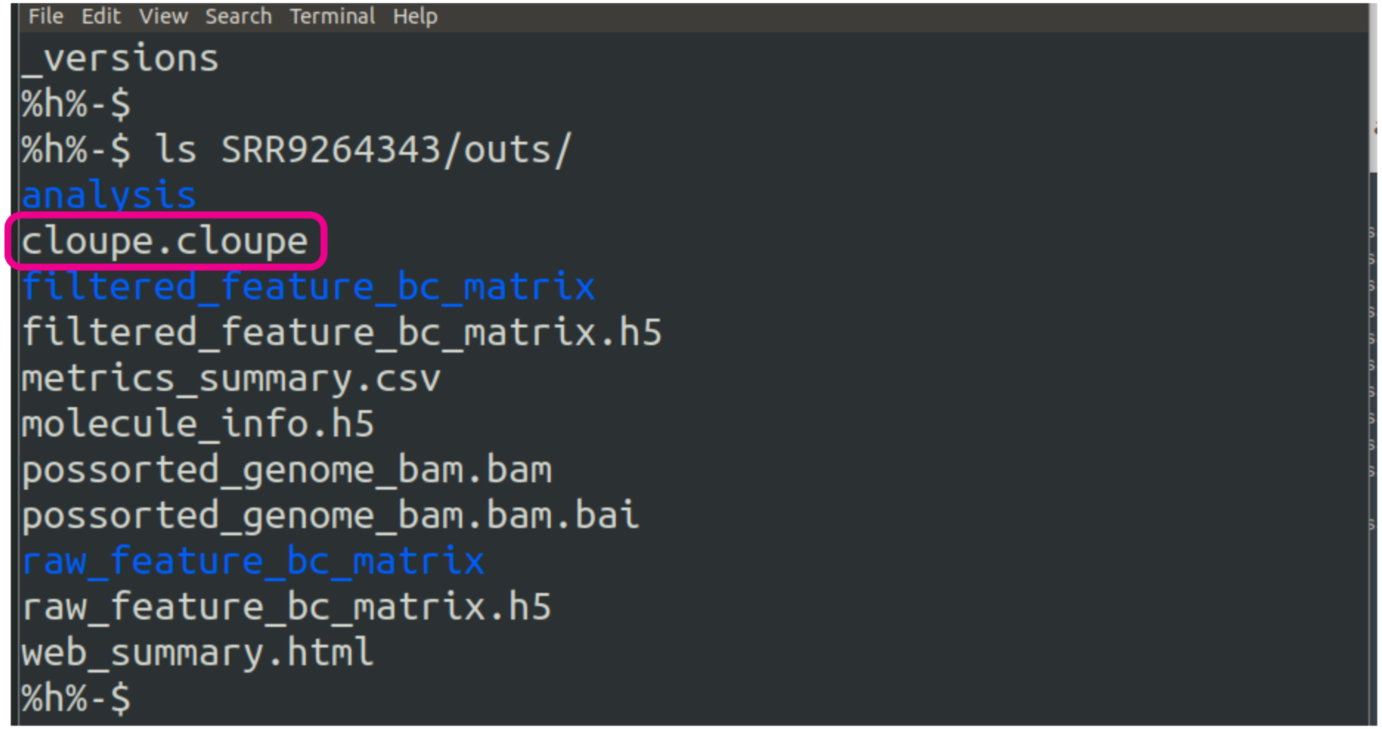
Barcode Rank Plot

Estimated Number of Cells	14,668
Fraction Reads in Cells	80.8%
Mean Reads per Cell	20,065
Median Genes per Cell	1,344
Total Genes Detected	23,106
Median UMI Counts per Cell	2,928

Sample

Sample ID	SITTA6
Sample Description	

Cell Ranger outputs



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web_summary.html
%h%-$
```
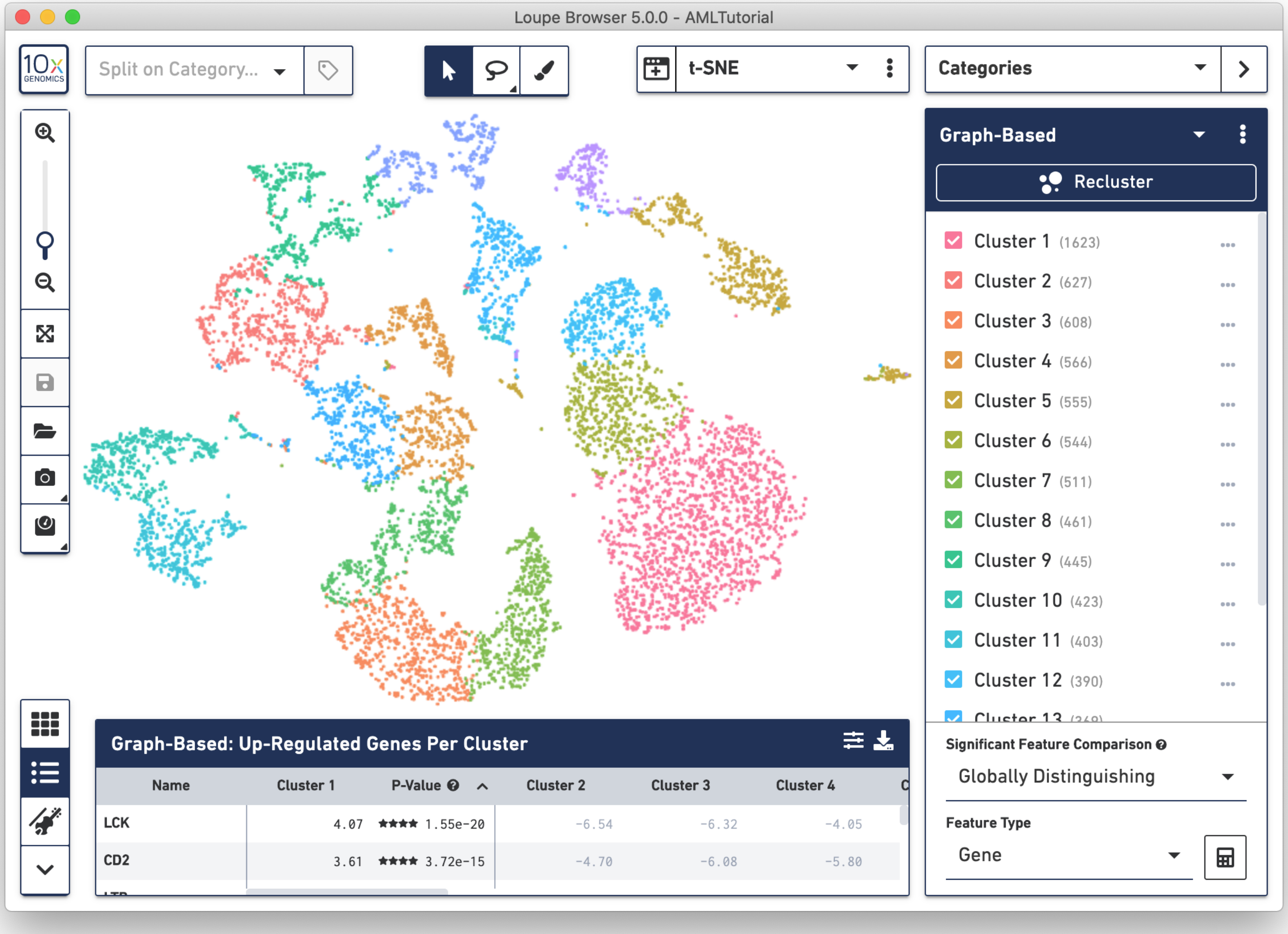

Loupe Browser



Cluster 1 (1623)
Cluster 2 (627)
Cluster 3 (608)
Cluster 4 (566)
Cluster 5 (555)
Cluster 6 (544)
Cluster 7 (511)
Cluster 8 (461)
Cluster 9 (445)
Cluster 10 (423)
Cluster 11 (403)
Cluster 12 (390)

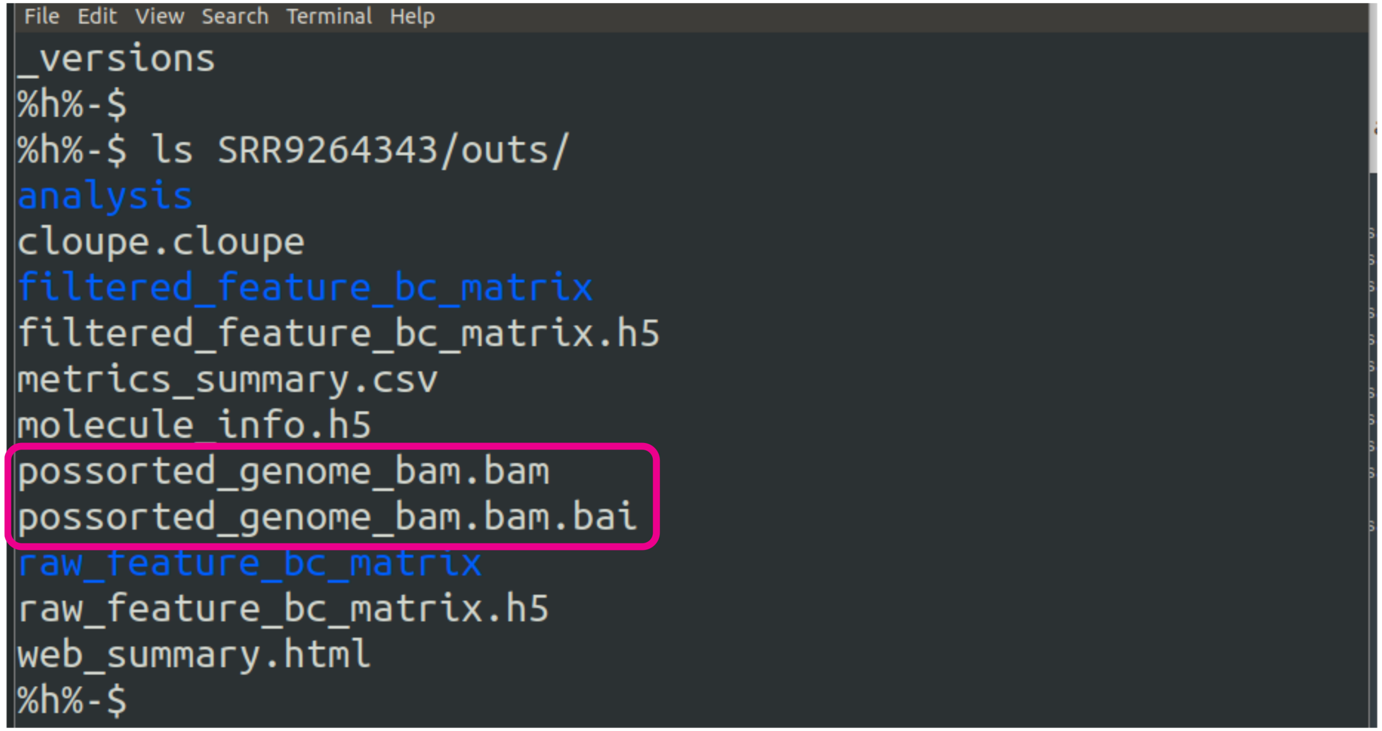
Significant Feature Comparison: Globally Distinguishing

Feature Type: Gene

Graph-Based: Up-Regulated Genes Per Cluster

Name	Cluster 1	P-Value	Cluster 2	Cluster 3	Cluster 4
LCK	4.07	★★★★ 1.55e-20	-6.54	-6.32	-4.05
CD2	3.61	★★★★ 3.72e-15	-4.70	-6.08	-5.80

Cell Ranger outputs



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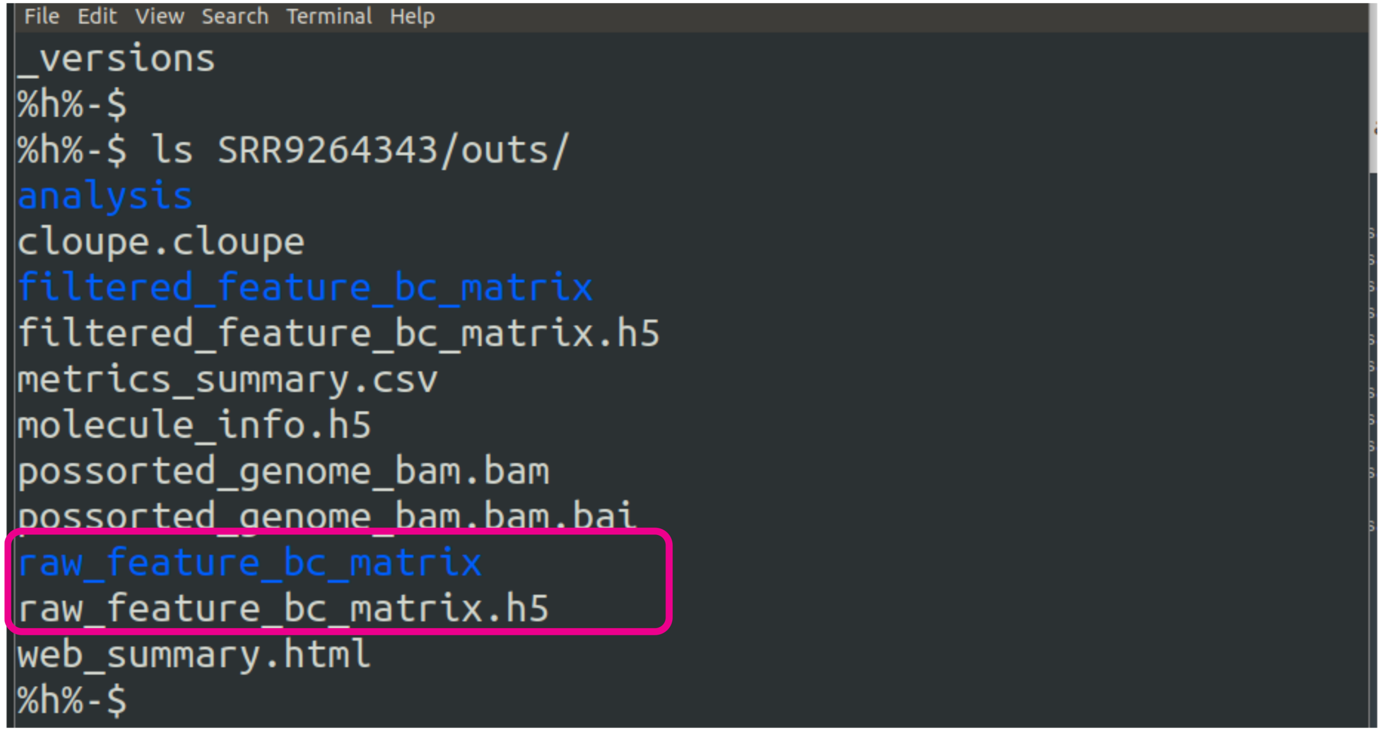

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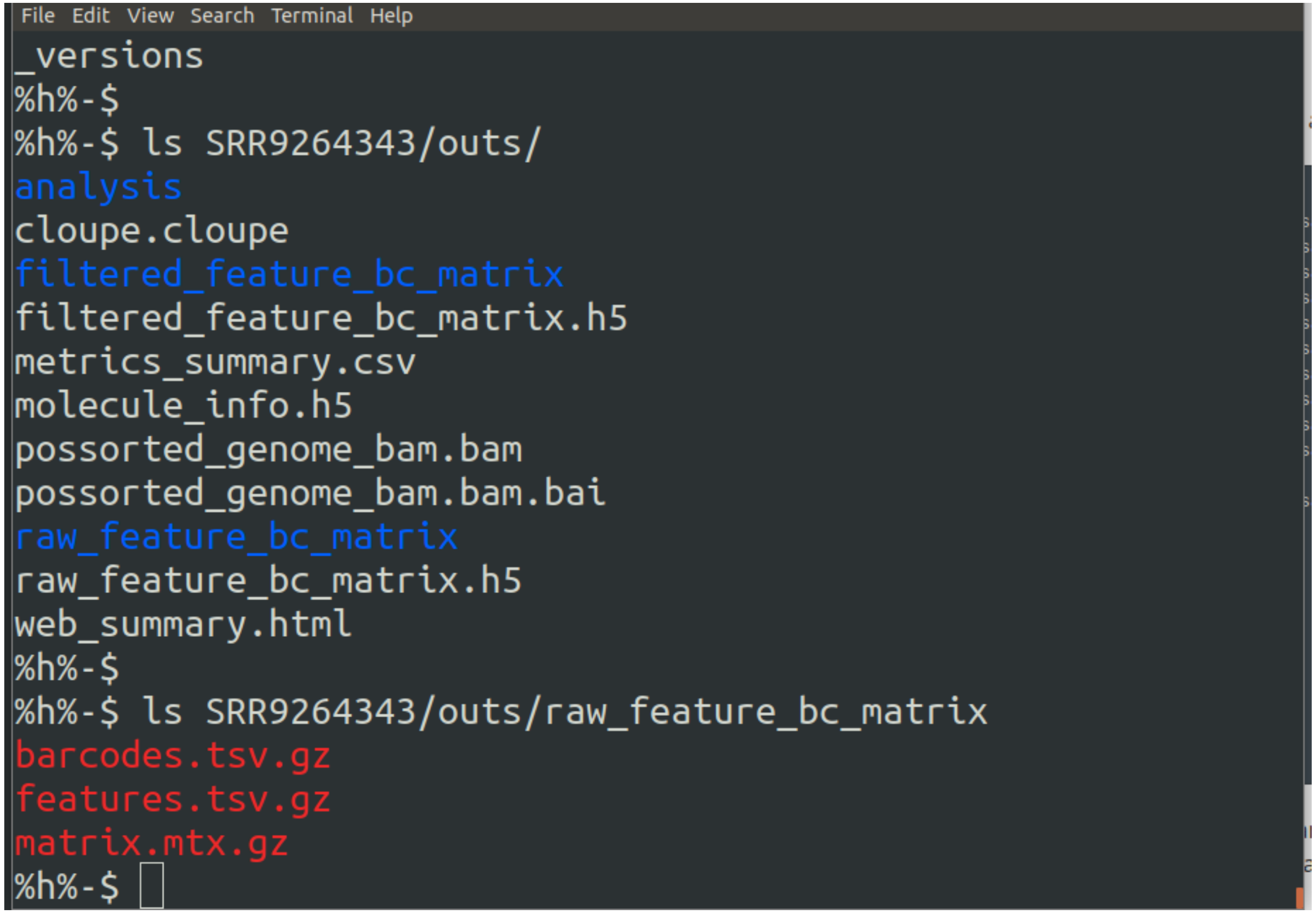

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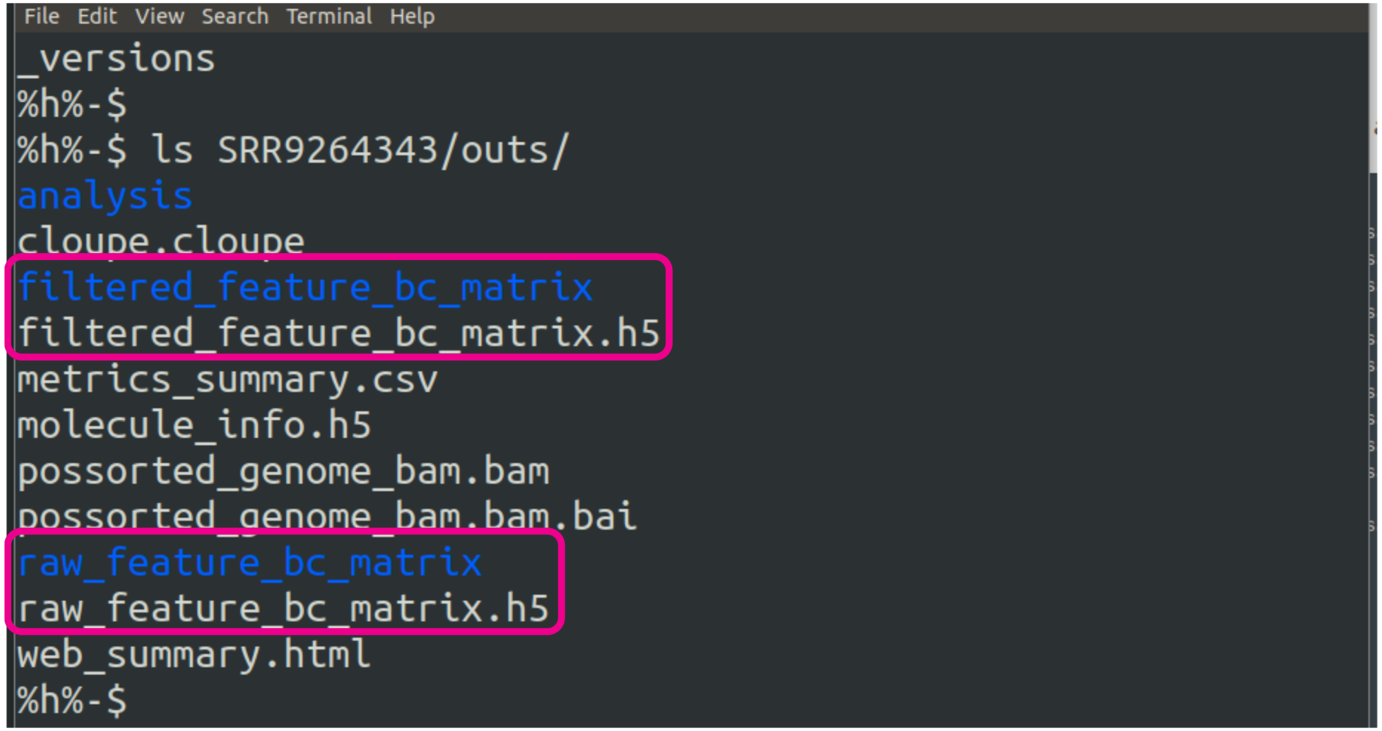
possorted_genome_bam.bam.bai
raw_feature_bc_matrix
raw_feature_bc_matrix.h5
web_summary.html
%h%-$
%h%-$ ls SRR9264343/outs/raw_feature_bc_matrix
barcodes.tsv.gz
features.tsv.gz
matrix.mtx.gz
%h%-$
```

Cell Ranger outputs



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raw_feature_bc_matrix.h5
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```
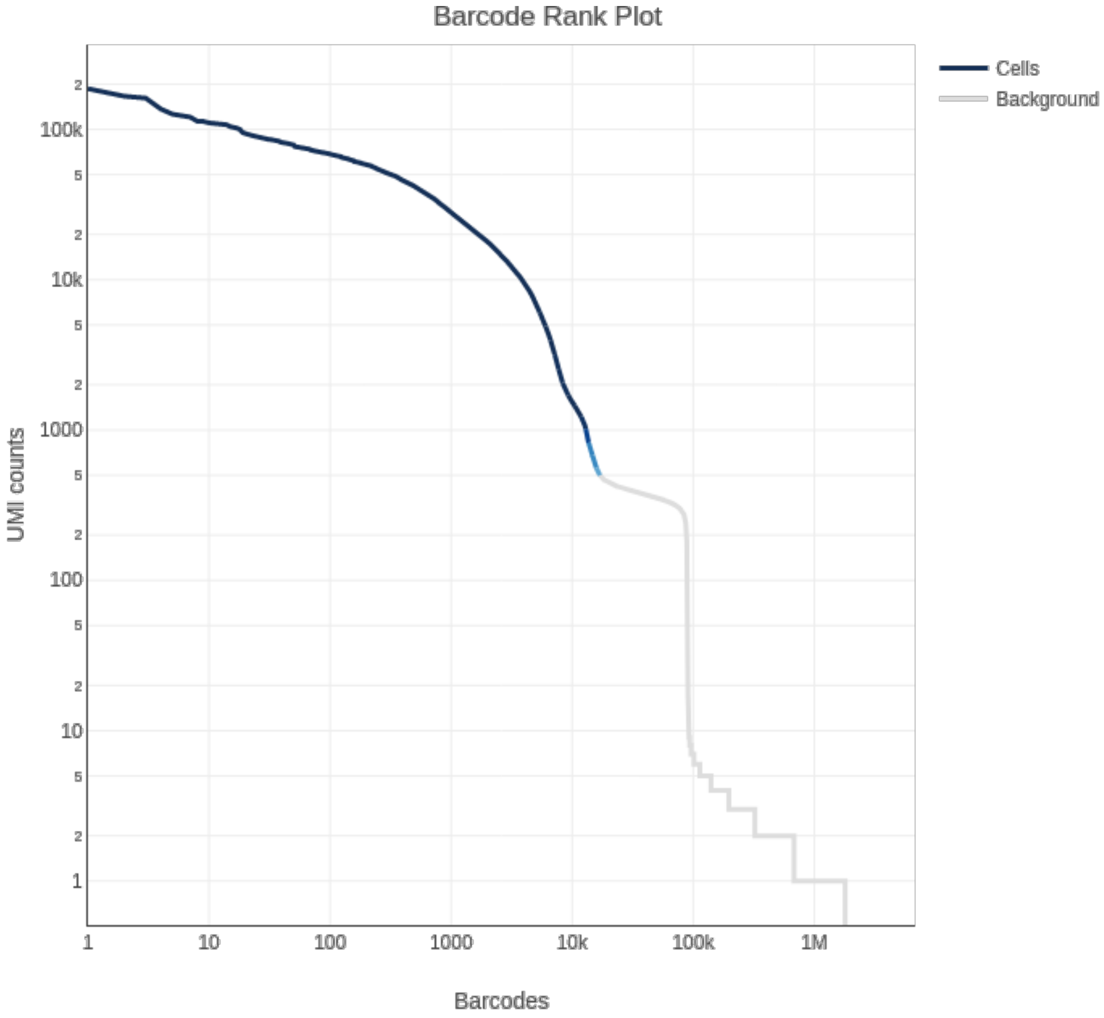
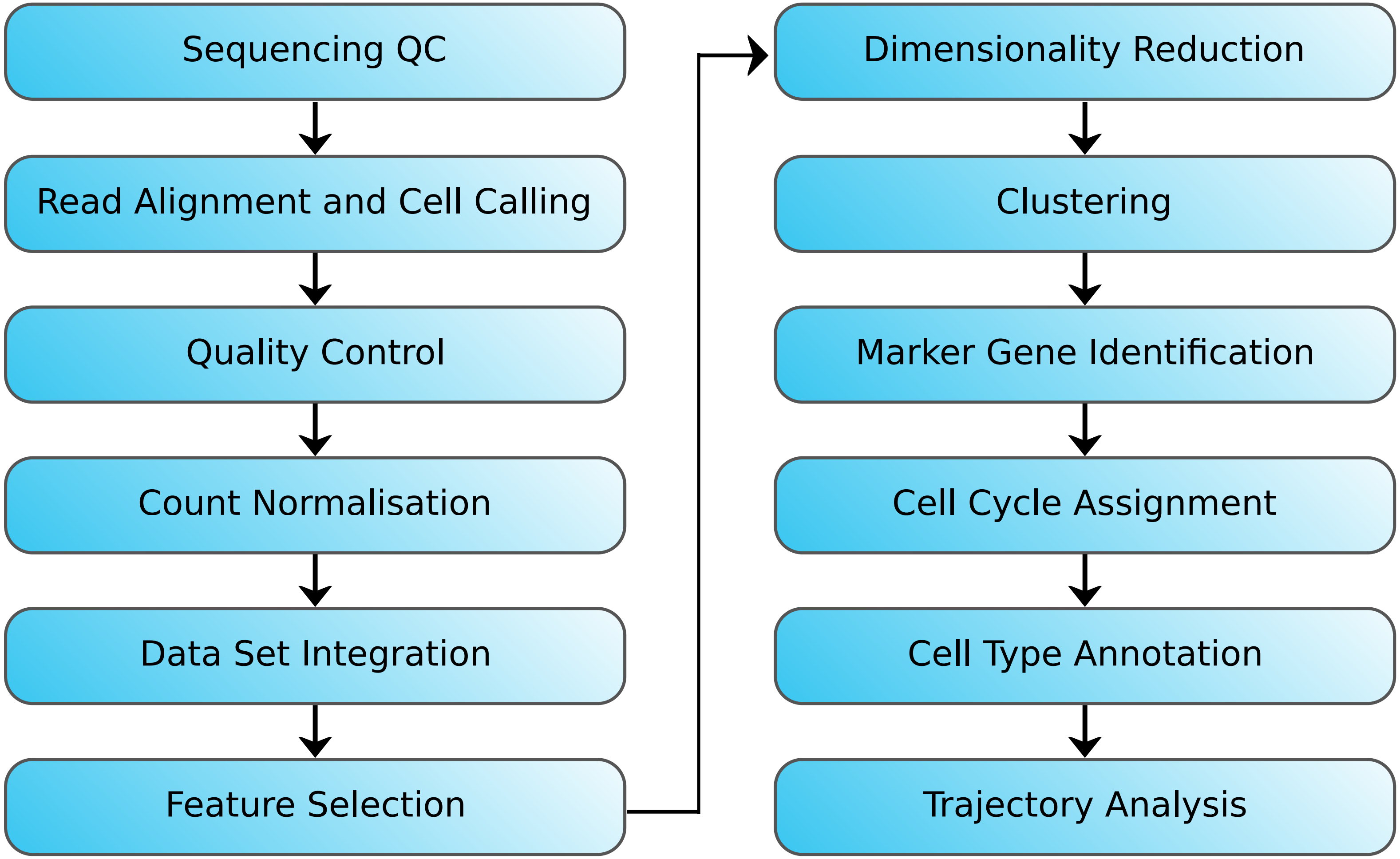

Cell Ranger cell calling



Single Cell RNAseq Analysis Workflow



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