Analysing 10X Single Cell RNA-Seq Data

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Course Outline

• How 10X single cell RNA-Seq works

• Evaluating CellRanger QC
  – [Exercise] Looking at CellRanger QC reports

• Dimensionality Reduction (PCA and tSNE)
  – [Exercise] Using the Loupe cell browser
  – [Exercise] Analysing data in R using Seurat
How 10X RNA-Seq Works

- Cells
- RT Reagents
- Oil
- Barcoded Beads
- Gel Beads in Emulsion (GEMs)
How 10X RNA-Seq Works

- Oligo dT
- Cell barcode (same within GEM)
- UMI (all different)
- Priming site
How 10X RNA-Seq Works

- **Oligo dT**
- **Cell barcode (same within GEM)**
- **UMI (all different)**
- **Priming site**

**Reverse Transcription**

**Mix RNAs and Cells**

**Illumina Library Prep**
How 10X RNA-Seq Works

- **Illumina Adapter**
- **UMI**
- **Cell Barcode**
- **3’ RNA Insert**
- **Sample Barcode**

**Legend**:
- **Sample level barcode** – same for all cells and RNAs in a library
- **Cell level barcode (16bp)** – same for all RNAs in a cell
- **UMI (10bp)** – unique for one RNA in one cell
10X Produces Barcode Counts

UMIs are finally related to genes to get per-gene counts
The 10X Software Suite

Chromium Controller  
Runs the chromium system for creating GEMs

Cell Ranger  
Pipeline for mapping, filtering, QC and quantitation of libraries

Loupe Browser  
Desktop software for visualisation and analysis of single cell data.
Cell Ranger

- Barcode Extraction and filtering
  - Identifies cell level barcodes
- Mapping to reference
  - Uses STAR aligner
- Generate count table
  - UMIs per gene in each cell
- Dimensionality Reduction
  - PCA and tSNE
- Clustering
  - K-means and Graph Based
CellRanger Commands

- **I1**
  - Index file. All identical (or one of 4) at Babraham

- **R1**
  - Barcode reads
    - 16bp cell level barcode
    - 10bp UMI

- **R2**
  - 3’ RNA-seq read

```
scrALI001_S1_L001_I1_001.fastq.gz
scrALI001_S1_L001_R1_001.fastq.gz
scrALI001_S1_L001_R2_001.fastq.gz
```
CellRanger Commands

• **CellRanger Count** (quantitates a single run)

```bash
$ cellranger count --id=COURSE \\
    --transcriptome=/bi/apps/cellranger/references/GRCh38/ \\
    --fastqs=/bi/home/andrewss/10X/ \\
    --localcores=8 \\
    --localmem=32
```

• **CellRanger aggr** (merges multiple runs)

```bash
$ cellranger aggr --id=MERGED \\
    --csv=merge_me.csv \\
    --normalize=mapped
```
Output files generated

- **web_summary.html** - Web format QC report

- **filtered_features_bc_matrix**
  - **barcodes.tsv.gz** - cell level barcodes seen in this sample
  - **features.tsv.gz** - list of quantitated features (usually Ensembl genes)
  - **matrix.mtx.gz** - (sparse) matrix of counts for cells and features

- **possorted_genome_bam.bam** - BAM file of mapped reads

- **molecule_info.h5** – Details of the cell barcodes – used for merging

- **cloupe.cloupe** - Analysis data for Loupe Cell browser
Evaluating CellRanger Output

• Look at barcode splitting report
  – Check sample level barcodes

• Look at `web_summary.html` file
  – Check number of cells
  – Check quality of data
  – Check coverage per cell
  – Check library diversity
Sample Level Barcodes

- Only present if multiple libraries mixed in a lane
- Get standard barcode split report, but with 4 barcodes used per sample
- Even coverage within and between libraries
CellRanger Reports

- HTML report – comes with each sample and aggregated group of samples

- Gives some basic metrics to judge the quality of the samples and spot any issues in the data or processing
Estimated Number of Cells

15,894

Mean Reads per Cell

11,380

Median Genes per Cell

2,174

Sequencing

Number of Reads

180,876,636

Valid Barcodes

98.1%

Sequencing Saturation

10.3%

Q30 Bases in Barcode

98.4%

Q30 Bases in RNA Read

82.7%

Q30 Bases in UMI

98.7%

Mapping

Reads Mapped to Genome

95.4%

Reads Mapped Confidently to Genome

90.2%

Reads Mapped Confidently to Intergenic Regions

3.0%

Reads Mapped Confidently to Intronic Regions

12.8%

Reads Mapped Confidently to Exonic Regions

74.4%

Reads Mapped Confidently to Transcriptome

71.9%

Reads Mapped Antisense to Gene

0.9%

Sample

Name

embryoid_d4

Description

Transcriptome

mm10

Chemistry

Single Cell 3' v3

Cell Ranger Version

3.0.2
# Errors and Warnings

## Analysis Detected Some Issues with Your Sequencing Run

<table>
<thead>
<tr>
<th>Alert</th>
<th>Value</th>
<th>Detail</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Fraction Reads Confidently Mapped To Transcriptome</td>
<td>51.5%</td>
<td>Ideal &gt; 60%. This can indicate use of the wrong reference transcriptome, poor library quality, or poor sequencing quality. Application performance may be affected.</td>
</tr>
</tbody>
</table>

## Analysis Detected Some Serious Issues with Your Sequencing Run

<table>
<thead>
<tr>
<th>Alert</th>
<th>Value</th>
<th>Detail</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Cells Detected</td>
<td>0</td>
<td>No valid sequencing data was detected. Please check the sequencing data.</td>
</tr>
<tr>
<td>Low Fraction Valid UMI</td>
<td>0.0%</td>
<td>Ideal &gt; 75%. This usually indicates a quality issue with the Illumina R2 read. Application performance is likely to be affected.</td>
</tr>
<tr>
<td>Low Barcode Q30 Fraction (Illumina I7 Read)</td>
<td>67.5%</td>
<td>Ideal &gt; 70%. Application performance may be affected.</td>
</tr>
<tr>
<td>Low UMI Q30 Fraction (Illumina R2 Read)</td>
<td>29.2%</td>
<td>Ideal &gt; 80%. Application performance is likely to be affected.</td>
</tr>
</tbody>
</table>
How many cells do you have?

• Cell number is determined from the number of cell barcodes with ‘reasonable’ numbers of observations

• Need to separate signal from background – real cell associated barcodes vs noise from empty GEMs and mis-called sequences

• Changing the thresholds used can give very different predictions for cell numbers
How many cells do you have?

- Start by looking at the quality of the base calls in the barcodes
- Bad calls will lead to inaccurate cell assignments
How many cells do you have?

- Start by looking at the quality of the base calls in the barcodes
- Bad calls will lead to inaccurate cell assignments
How many cells do you have

- Plot of UMIs (reads) per cell vs number of cells
- Blue region was called as valid cells
- Grey region is considered noise
- Both axes are log scale!!!
How many cells do you have

CellRanger v3 uses a liberal cutoff to define cells. This was designed to accommodate (normally cancer) samples where cells might have wildly different amounts of RNA. It will include large numbers of cells with small numbers of UMIs. If this doesn’t apply to your sample then this will over-predict valid cells.
How much data do you have per cell?

- Mean Reads per Cell: 11,380
- Median Genes per Cell: 2,174

### Mapping

- Reads Mapped to Genome: 95.4%
- Reads Mapped Confidence to Genome: 90.2%
- Reads Mapped Confidence to Intergenic Regions: 3.0%
- Reads Mapped Confidence to Intronic Regions: 12.8%
- Reads Mapped Confidence to Exonic Regions: 74.4%
- Reads Mapped Confidence to Transcriptome: 71.0%
- Reads Mapped Antisense to Gene: 0.9%

### Summary

- Estimated Number of Cells: 15,894
- Fraction Reads in Cells: 88.1%
- Mean Reads per Cell: 11,380
- Median Genes per Cell: 2,174
- Total Genes Detected: 20,185
- Median UMI Counts per Cell: 5,742

- Reads should map well
- Check reads are mostly in transcripts
- Means and medians can be misleading when cells are variable
How much data do you have per cell?

• Some details about mapping
  – Reads should map to the 3’ end of transcripts (oligo dT selection)
  – Reads count as exonic if 50% of them overlaps an exon
  – Multi-mapped reads which only hit one exon are considered to be uniquely mapped
  – Reads associate with genes based on overlap and direction
  – Only confident (unique) transcriptome reads are used for analysis
How much data do you have per cell?

• Difficult to generalise how much data to create/expect
  – Depends on cell type, genome and other factors

• In general though, sensible numbers would be:
  – Reads per cell ~10,000
  – Genes per cell 2000 – 3000

• Be careful that different versions of Cellranger have reported raw and deduplicated counts – they can be *very* different
How deeply sequenced is your library

![Sequencing Saturation Graph](image1)

![Median Genes per Cell Graph](image2)
How deeply sequenced is your library
Is coverage variation affecting your data?
Exercise – Evaluating CellRanger Reports

• Look at the selection of CellRanger reports to get an idea for the metrics they provide

• The data we’re going to use for the rest of the day is in “course_web_summary.html”, do you see any problems which would concern us with this data at this stage?
## Course Data CellRanger QC

The analysis detected some issues. [Details »](#)

<table>
<thead>
<tr>
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<th>Detail</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Alert Icon" /> <img src="image" alt="Low Fraction Reads Confidently Mapped To Transcriptome" /></td>
<td>26.2%</td>
<td>Ideal &gt; 30%. This can indicate use of the wrong reference transcriptome, a reference transcriptome with overlapping genes, poor library quality, poor sequencing quality, or reads shorter than the recommended minimum. Application performance may be affected.</td>
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</tbody>
</table>

### Mapping

<table>
<thead>
<tr>
<th>Mapping Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reads Mapped to Genome</td>
<td>47.5%</td>
</tr>
<tr>
<td>Reads Mapped Confidently to Genome</td>
<td>46.1%</td>
</tr>
<tr>
<td>Reads Mapped Confidently to Intergenic Regions</td>
<td>2.0%</td>
</tr>
<tr>
<td>Reads Mapped Confidently to Intronic Regions</td>
<td>14.2%</td>
</tr>
<tr>
<td>Reads Mapped Confidently Exonic Regions</td>
<td>29.9%</td>
</tr>
<tr>
<td>Reads Mapped Confidently to Transcriptome</td>
<td>28.2%</td>
</tr>
<tr>
<td>Reads Mapped Antisense to Gene</td>
<td>0.6%</td>
</tr>
</tbody>
</table>

- **Actual Problem**: [Mapping](image)
- **Value Reported**: [Low Fraction Reads Confidently Mapped To Transcriptome](image)
Course Data QC – Read1 (Barcodes)
Course Data QC – Read2 (RNA)