Practical Example: NGS – data handling and single cell differentiation

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Useful URL

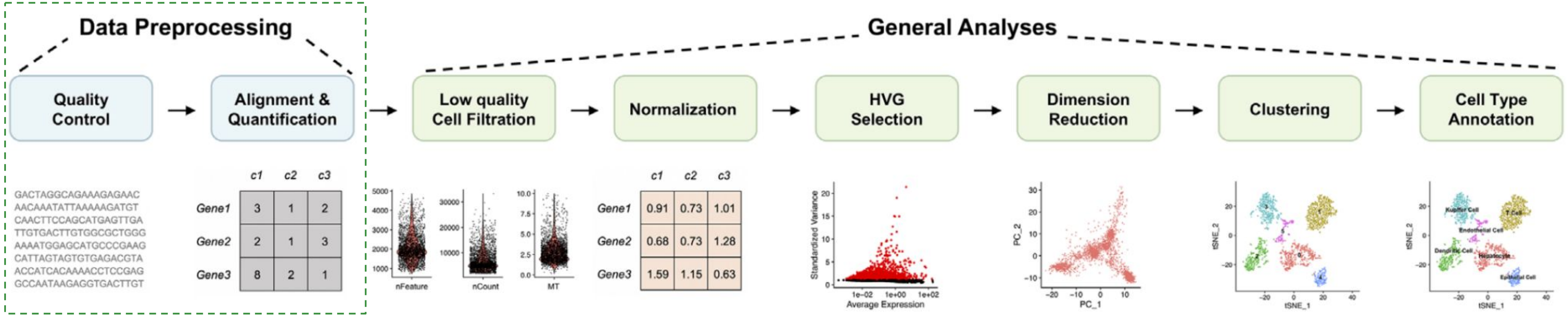
- Course material: https://www.costalab.org/software-lab-in-bioinformatics-2023/
- Single Cell Broad Institute Workshop: https://broadinstitute.github.io/2020_scWorkshop/
- Best Practices in Single Cell Analysis: https://www.embopress.org/doi/10.15252/msb.20188746

Overview

- DNA Sequencing:
  - New Generation Sequencing Methods
  - Sequencing Quality Check
  - Alignment of Sequenced Reads
- Single Cell Sequencing
  - Demultiplexing Reads
  - Single Cell Expression Matrix
  - From the Expression Matrix to Information Retrieval

Overview

DNA Sequencing

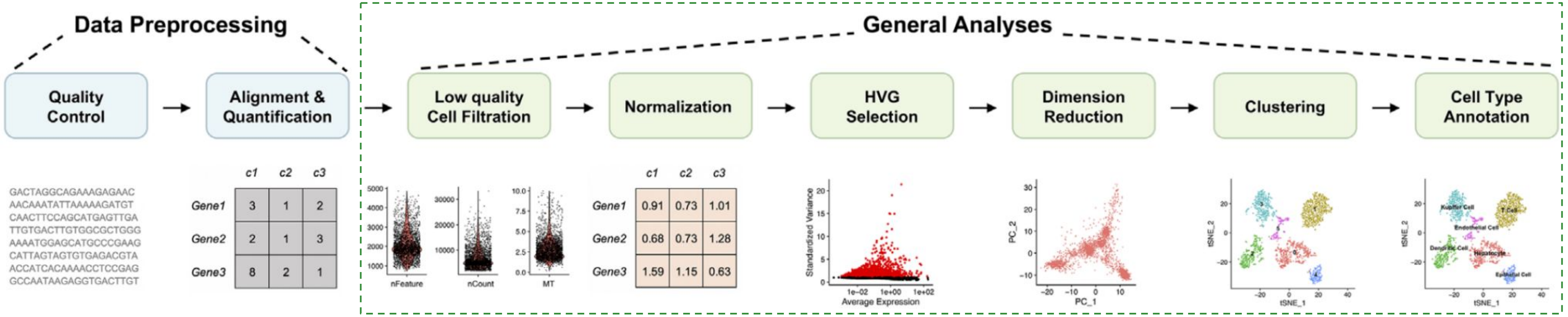


	c1	c2	c3
Gene1	3	1	2
Gene2	2	1	3
Gene3	8	2	1

	c1	c2	c3
Gene1	0.91	0.73	1.01
Gene2	0.68	0.73	1.28
Gene3	1.59	1.15	0.63

Overview

Single Cell Pipeline



```
GACTAGGCAGAAAGAGAAC
AACAAATATTAAAAAGATGT
CAACTTCCAGCATGAGTTGA
TTGTGACTTGTGGCGCTGGG
AAAATGGAGCATGCCCGAAG
CATTAGTAGTGTGAGACGTA
ACCATCACAAAACCTCCGAG
GCCAATAAGAGGTGACTTGT
```

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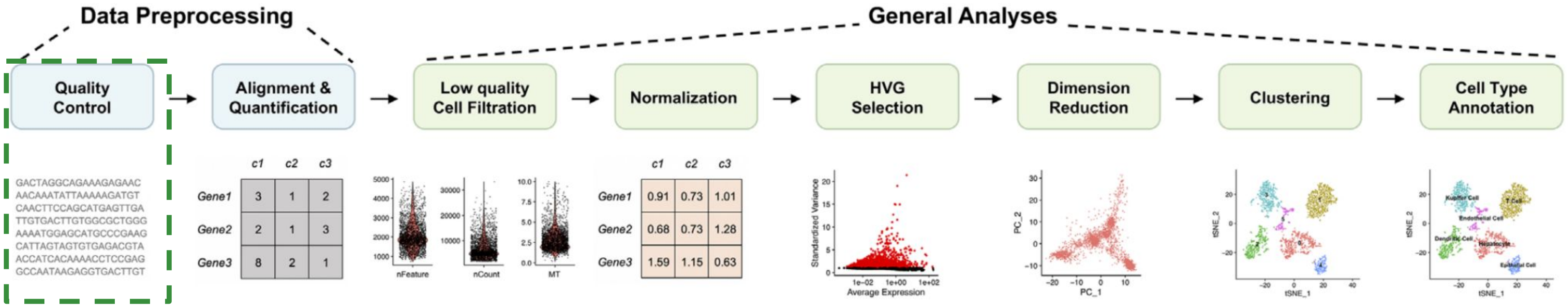
Overview

DNA Sequencing



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DNA Sequencing

- Problem: Converting a DNA molecule to a string
  - string: sequence of bases (A, T, C, G)

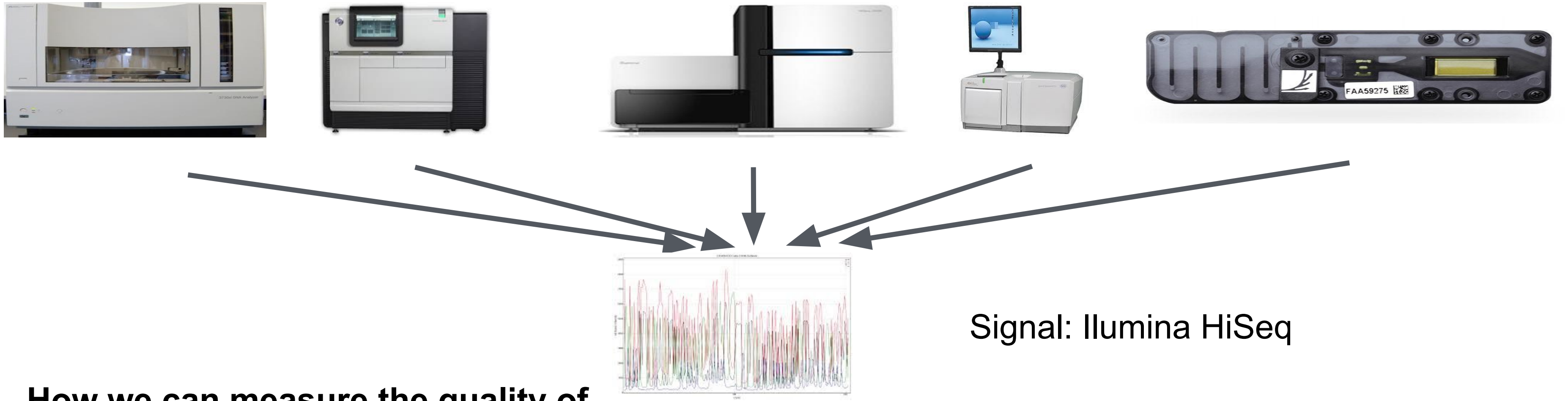
- Many possible sequencing techniques exist:

FASTA file

- Can be used to store DNA/protein sequence in a text-based file
- Mainly used to store large genomic sequences
- Header (lines that start with '>') + DNA sequence
- Alphabet: A, C, G, T, N

```
>NC_005248.1:3950-4810 Escherichia coli plasmid pIGAL1, complete sequence
ATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTC
ACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACT
GGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTT
AAAGTTCTGCTATGTGGCGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATAC
ACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGT
AAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATC
GGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGG
AACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGCAGCAATGGCAACAAC
GTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAG
```

From signal to string data



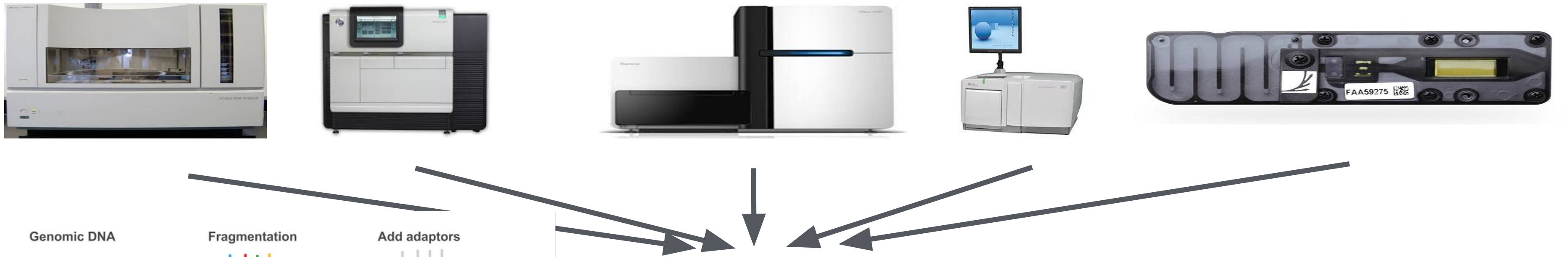
Signal: Ilumina HiSeq

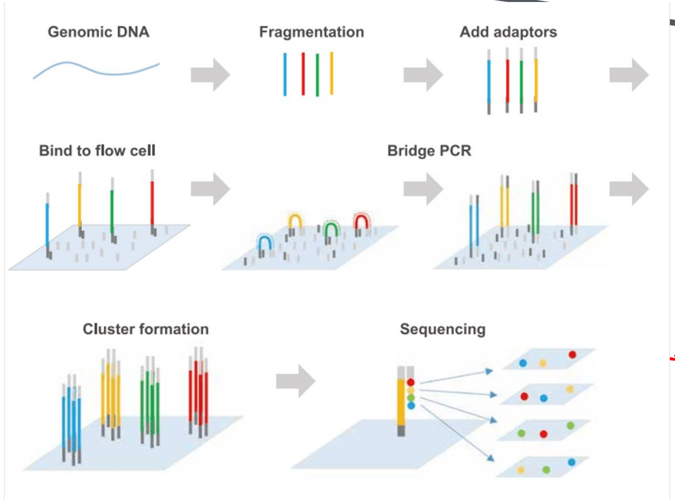
How we can measure the quality of these algorithms?

Base detection algorithms

ACCTTCGAACGGCGGGGGGTTACAA

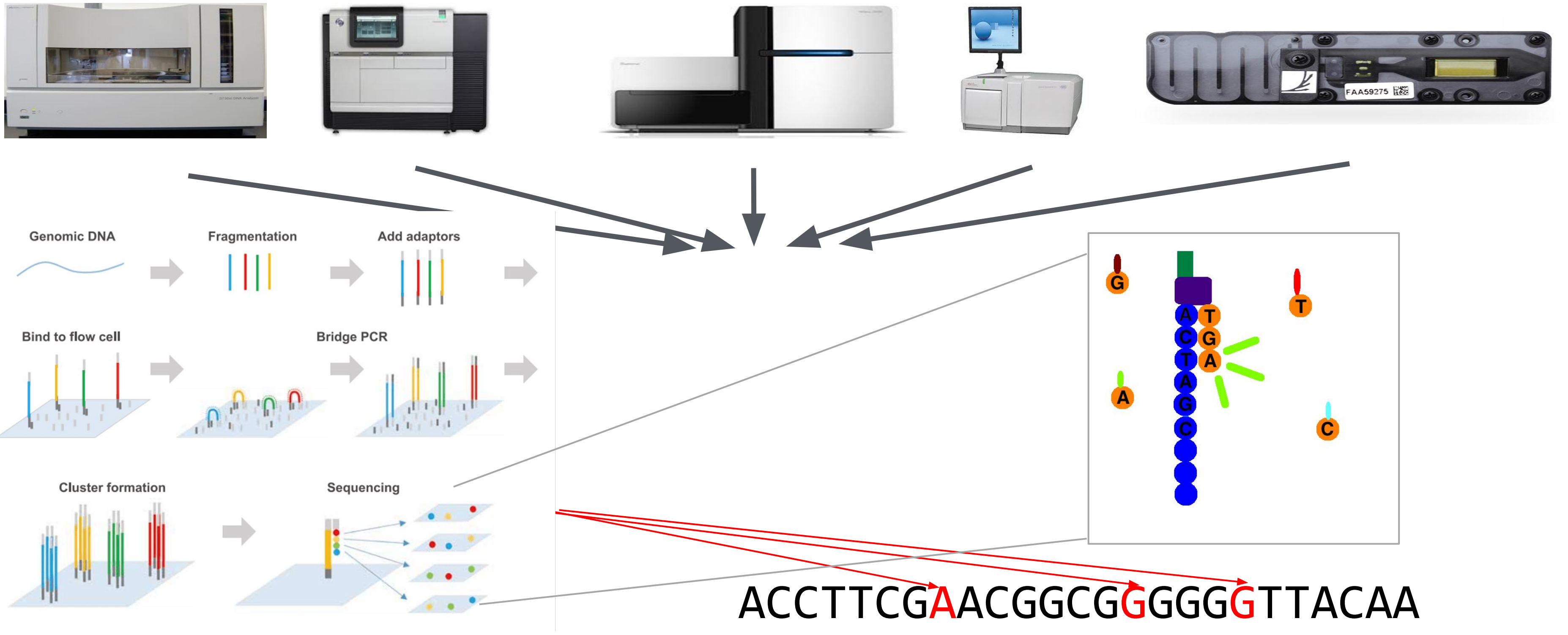
From signal to string data





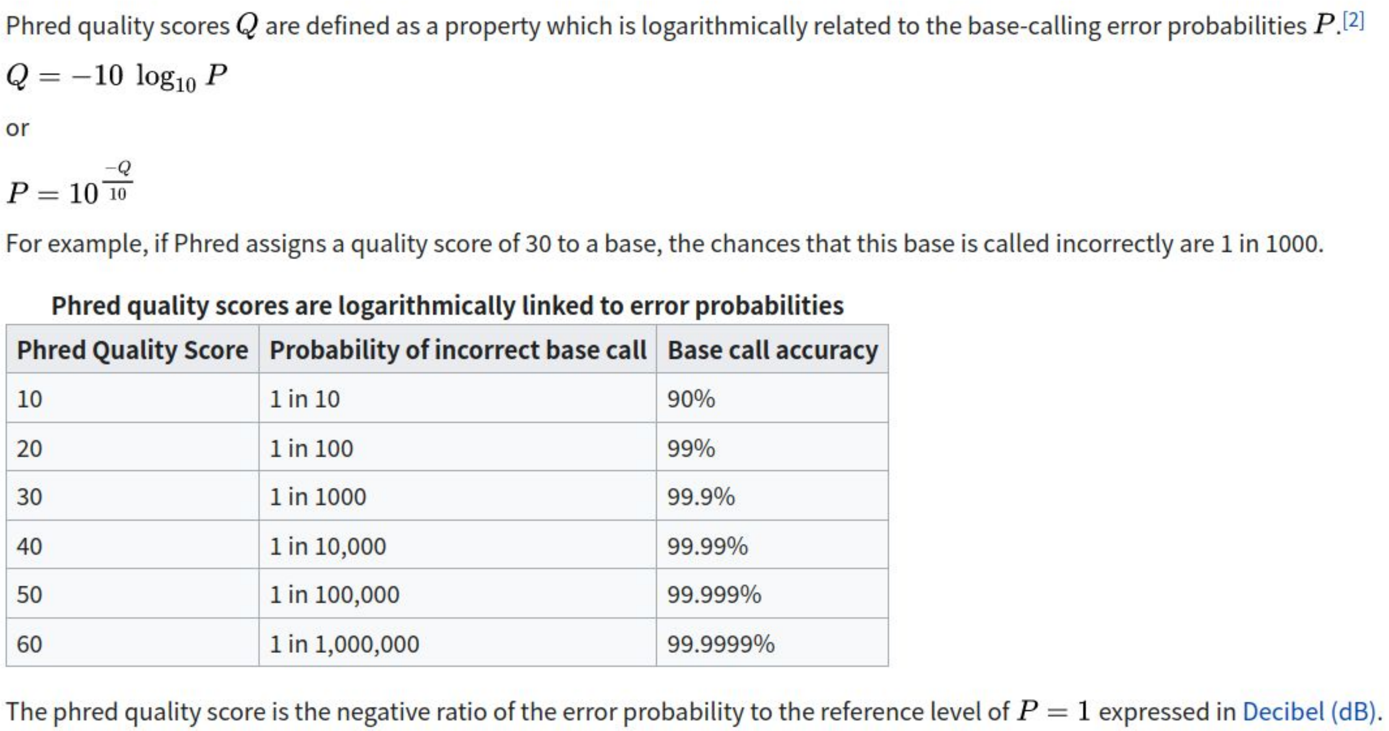
ACCTTCGAACGGCGGGGGGTTACAA

From signal to string data



ACCTTCGAACGGCGGGGGGTTACAA

FASTQ



Phred quality scores Q are defined as a property which is logarithmically related to the base-calling error probabilities P.[2]

$$Q = -10\ \log_{10} P$$

or

$$P = 10^{\frac{-Q}{10}}$$

For example, if Phred assigns a quality score of 30 to a base, the chances that this base is called incorrectly are 1 in 1000.

Phred quality scores are logarithmically linked to error probabilities

Phred Quality Score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%
40	1 in 10,000	99.99%
50	1 in 100,000	99.999%
60	1 in 1,000,000	99.9999%

The phred quality score is the negative ratio of the error probability to the reference level of $P = 1$ expressed in Decibel (dB).

https://en.wikipedia.org/wiki/Phred_quality_score

FASTQ

- Also text-based. Mainly used to store short DNA sequences (reads) from NGS-based experiments.
- Line 1: Begins with '@' and is followed by a an identifier.
- Line 2: DNA sequence.
- Line 3: Begins with '+' and is optionally followed by the same sequence identifier (and any description) again.
- Line 4: Quality values for the sequence in Line 2, and must contain the same number of symbols as the sequence.

```
@NS500746:56:HLY2MAFXX:1:11101:10096:1016 1:N:0:1
GCCTGNCGCATTGCATTCATCAAACGCTGAATAGCAAAGCCTCTACGCGATTTCATAGTGGAGGCCTCCAGCAATCTTGAACACTC
ATCCTTAATACCTTTCTTTTTGGGGTAATTATACTCATCGCGAATATCCTTAAGAGGGCGTTCAGCAGCCAGCTTGCGG
+
AAAAA#EEEEEEEEEEAEE/AEEEAEEEEEEEEEEEAEE/EEEEEEEEEEEA/EE<AEEEEEEEEEEEEEAEAEAE/EEEEEE/E<AEE
<EE/EEE//E/AEEEEE<AAEEEEEEEEEEEAEE/EAAA/AEAEEEEEE/EEEAA<AAEA<EEAEAEEE</AE<A/A<<<
```

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GCCTGNCGCATTGCATTCATCAAACGCTGAATAGCAAAGCCTCTACGCGATTTCATAGTGGAGGCCTCCAGCAATCTTGAACACTC
ATCCTTAATACCTTTCTTTTTGGGTAATTATACTCATCGCGAATATCCTTAAGAGGGCGTTCAGCAGCCAGCTTGCGG
+
AAAAA#EEEEEEEEEAEE/AEEEAEEEEEEEEEEAEE/EEEEEEEEEEEA/EE<AEEEEEEEEEEEEEAEAEAE/EEEEEE/E<AEE
<EE/EEE//E/AEEEEE<AAEEEEEEEEEEAEE/EAAA/AEAEEEEEE/EEEAA<AAEA<EEAEAEEE</AE<A/A<<<
```

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ATCCTTAATACCTTTCTTTTTGGGGTAATTATACTCATCGCGAATATCCTTAAGAGGGCGTTCAGCAGCCAGCTTGCGG
+
AAAAA#EEEEEEEEEAEE/AEEEAEEEEEEEEEEAEE/EEEEEEEEEEEA/EE<AEEEEEEEEEEEEAEAEAE/EEEEEE/E<AEE
<EE/EEE//E/AEEEEE<AAEEEEEEEEEEAEE/EAAA/AEAEEEEEE/EEEAA<AAEA<EEAEAEEE</AE<A/A<<<
```

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GCCTGNCGCATTGCATTCATCAAACGCTGAATAGCAAAGCCTCTACGCGATTTCATAGTGGAGGCCTCCAGCAATCTTGAACACTC
ATCCTTAATACCTTTCTTTTTGGGGTAATTATACTCATCGCGAATATCCTTAAGAGGGCGTTCAGCAGCCAGCTTGCGG
+
AAAAA#EEEEEEEEEEAEE/AEEEAEEEEEEEEEEEEAEE/EEEEEEEEEEEEA/EE<AEEEEEEEEEEEEEEAEAEAE/EEEEEE/E<AEE
<EE/EEE//E/AEEEEE<AAEEEEEEEEEEEEAEE/EAAA/AEAEEEEEEE/EEEAA<AAEA<EEAEAEEE</AE<A/A<<<
```

FASTQ Evaluation – FastQC

Fastq files can be very big with millions of (long) reads. Infeasible to investigate.

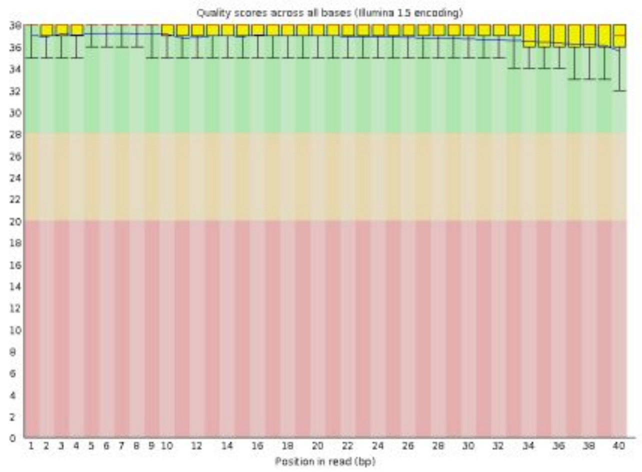
- Phred-Score hard to read in ASCII form.
- FastQC (usually provided by NGS core facilities)
- Tool to analyse quality of reads from sequencing.
- Indicate problems in library preparation or sequencing steps.

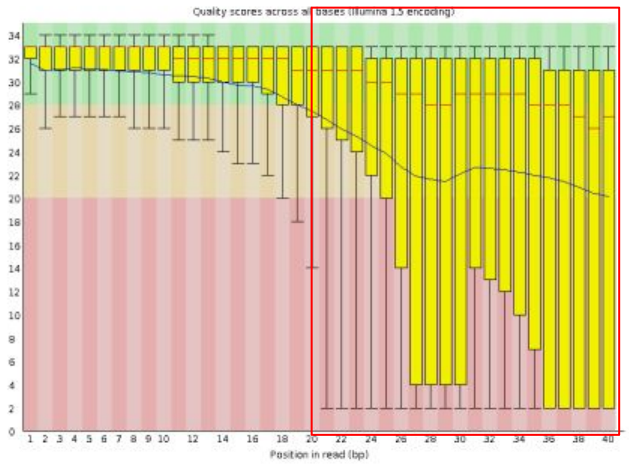
Example – **good quality sequences**
http://www.bioinformatics.babraham.ac.uk/projects/fastqc/good_sequence_short_fastqc.html
Example – **bad quality sequences**
http://www.bioinformatics.babraham.ac.uk/projects/fastqc/bad_sequence_fastqc.html

Help Documents:
https://www.bioinformatics.babraham.ac.uk/projects/fastqc/Help/

FASTQ Evaluation – FastQC

Sequencing quality decreases with size.

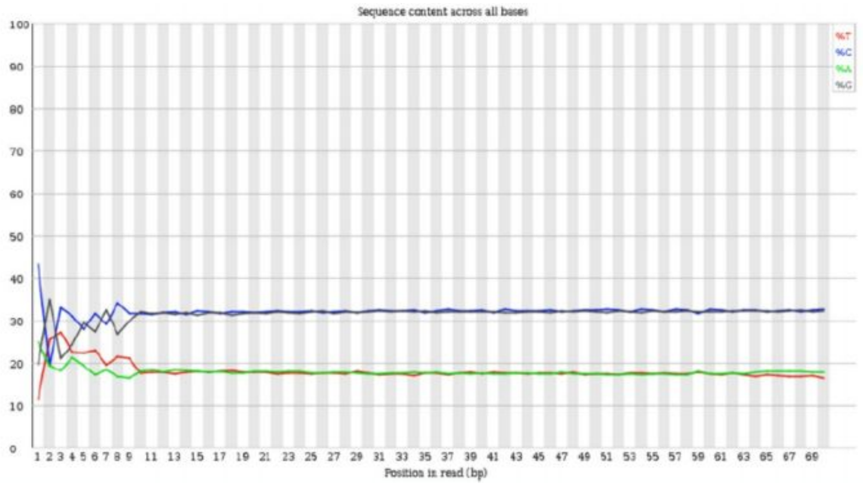




Solution: trim ends of reads, if quality is low.

FASTQ Evaluation – FastQC

Read position sequence bias.



Solution: Trim starts of reads.

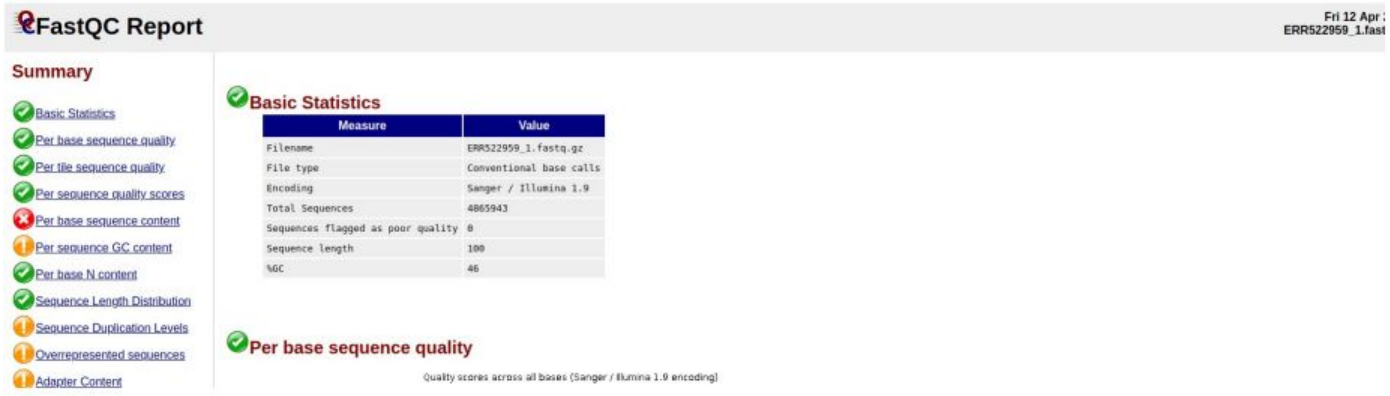
Hands-on time

- Download **data1.zip** from the lecture website.
- Use FastQC to analyze the data:
    - **create** new directory “**fastqc_results**”
    - read the documentation of FastQC to understand how to export the files to the new directory:
    - fastqc -h
    - What do you see?
      What is the overall quality?
      Do we have any adapters?
- Trim the reads from the identified adapter using **trim_galore** (trim_galore –help) in a new folder “**trimmed_results**”. Again analyze the fastq. What do you see? Are the adapters gone?

trim_galore guide:
[TrimGalore/Trim_Galore_User_Guide.md at master]

Hands-on time

1. ● fastqc -o fastqc_results/ ERR522959_1.fastq.gz ERR522959_2.fastq.gz



FastQC Report

Fri 12 Apr
ERR522959_1.fast

Summary

- ✓ Basic Statistics
- ✓ Per base sequence quality
- ✓ Per tile sequence quality
- ✓ Per sequence quality scores
- ✗ Per base sequence content
- ! Per sequence GC content
- ✓ Per base N content
- ✓ Sequence Length Distribution
- ! Sequence Duplication Levels
- ! Overrepresented sequences
- ! Adapter Content

Basic Statistics

Measure	Value
Filename	ERR522959_1.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	4865943
Sequences flagged as poor quality	0
Sequence length	100
%GC	46

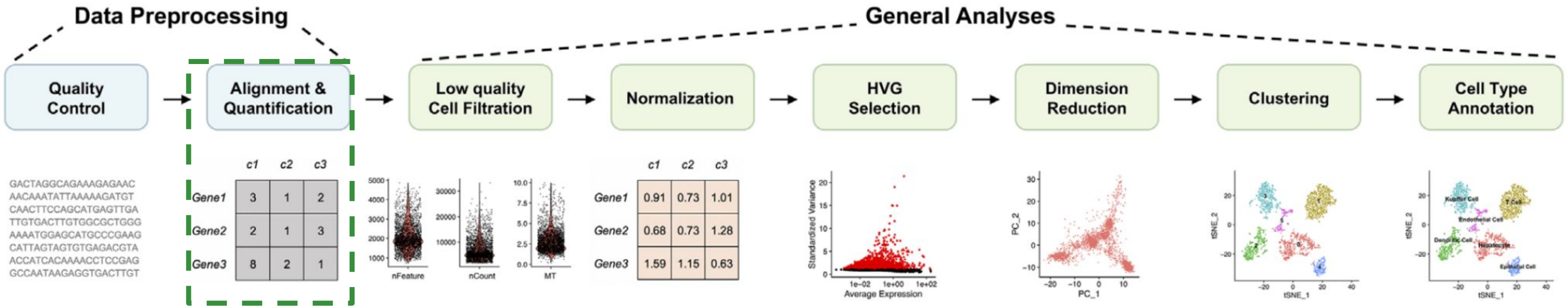
Per base sequence quality

Quality scores across all bases (Sanger / Illumina 1.9 encoding)

2. ● trim_galore –nextera -o trimmed_results/ ERR522959_1.fastq.gz ERR522959_2.fastq.gz
3. ● fastqc -o trimmed_results/ trimmed_results/ERR522959_1_trimmed.fq.gztrimmed_results/ERR522959_2_trimmed.fq.gz

Overview

DNA Sequencing



GACTAGGCAGAAAGAGAAC
AACAAATATTAAAAAGATGT
CAACTTCCAGCATGAGTTGA
TTGTGACTTGTGGCGCTGGG
AAAATGGAGCATGCCCGAAG
CATTAGTAGTGTGAGACGTA
ACCATCACAAAACCTCCGAG
GCCAATAAGAGGTGACTTGT

	c1	c2	c3
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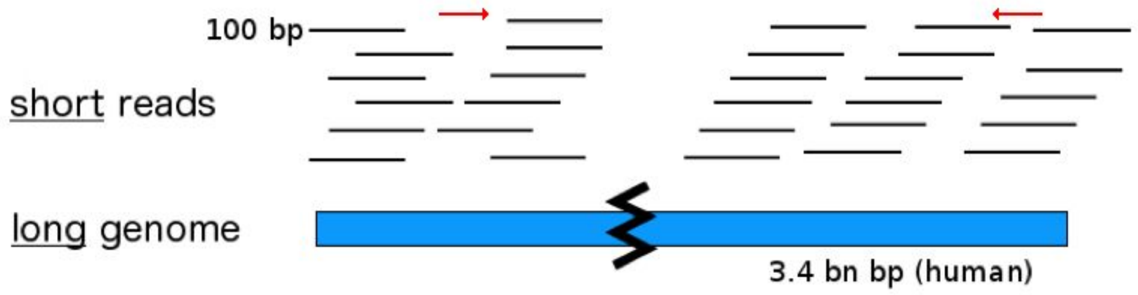
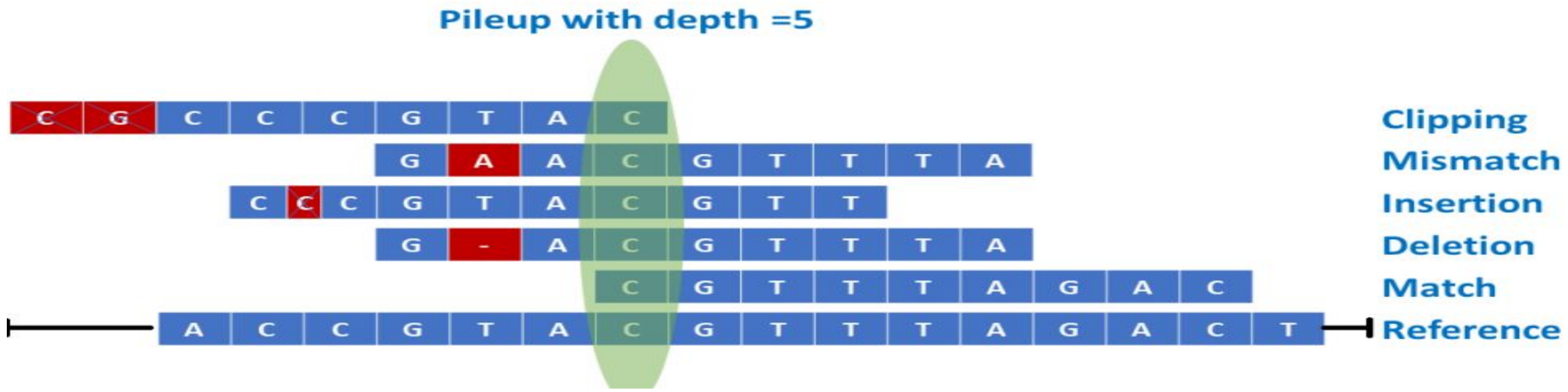
Alignment

Usually very large genomes (with repetitive regions) and very small reads.



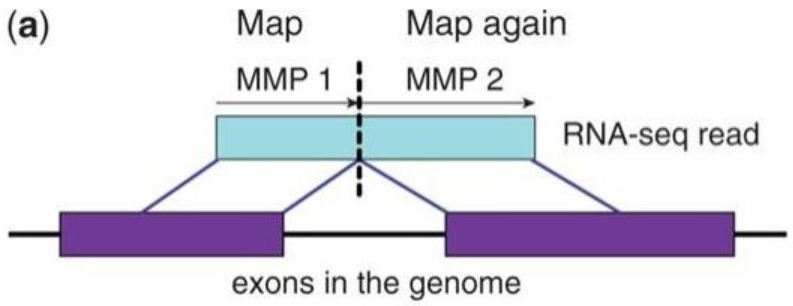
Alignment

Problem: aligning DNA sequence to a reference genome.



STAR

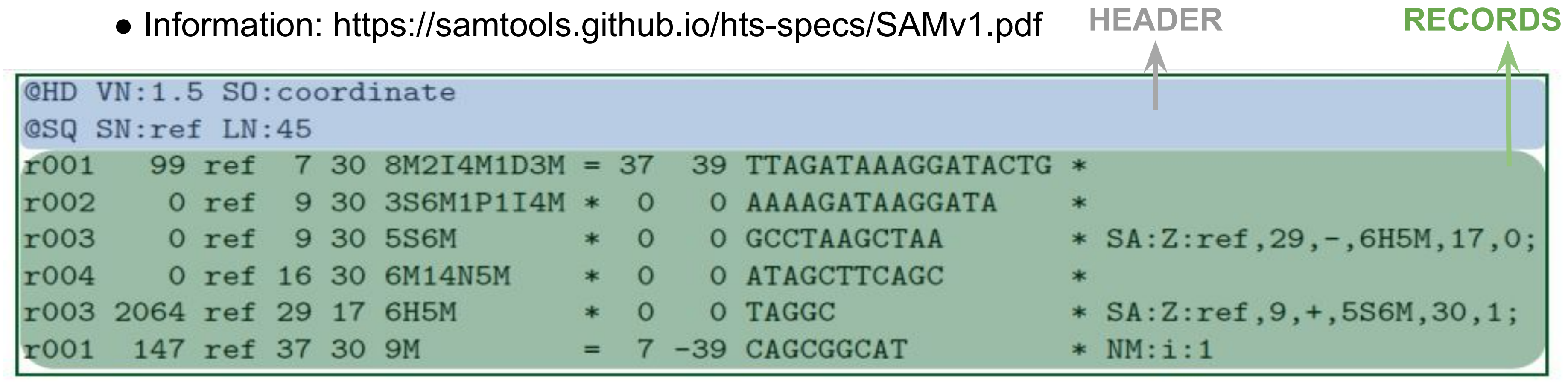
STAR allows a sequence to be split and aligned to different exons



Source: Dobin et al. (2013), Bioinformatics.

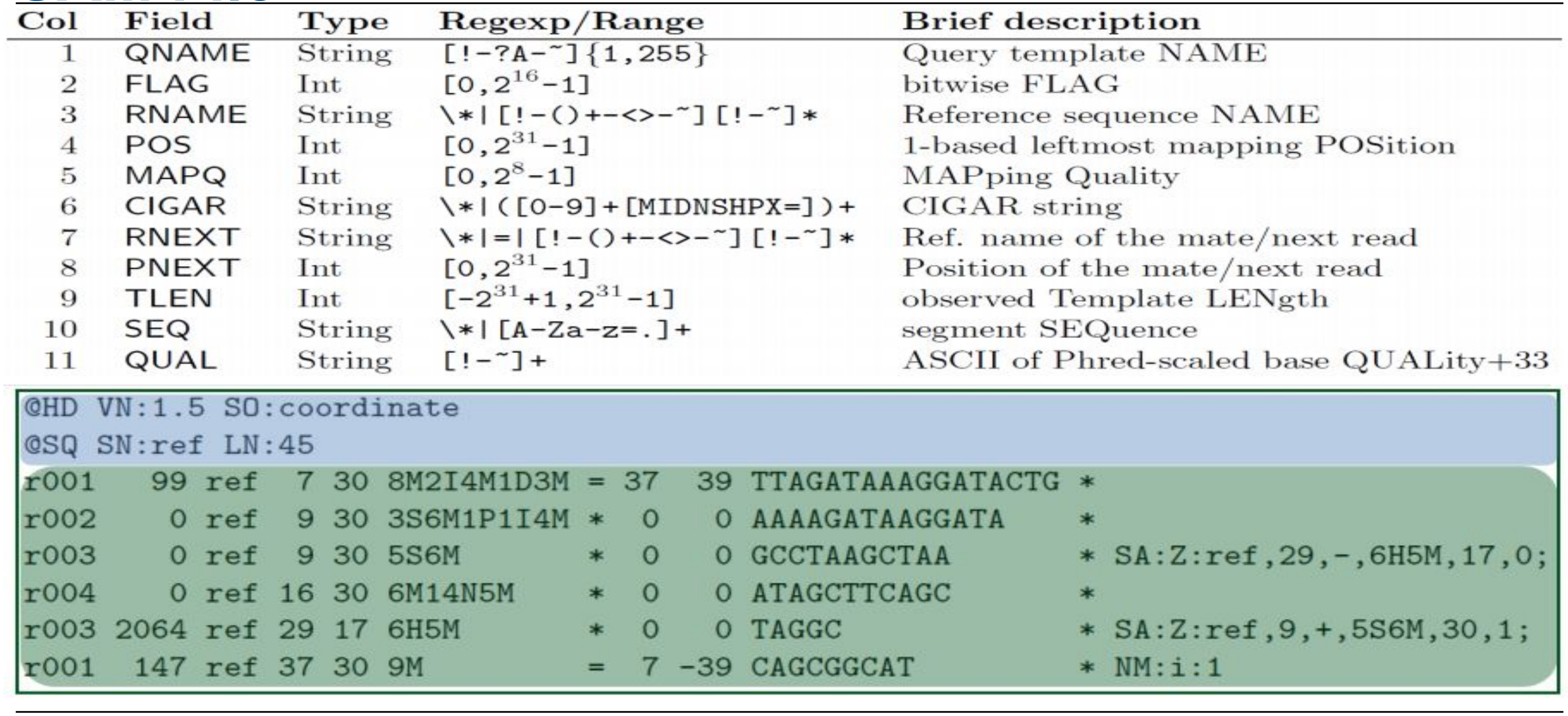
SAM File

- Sequence Alignment/Map format.
- Text-based tab-delimited file.
- Header + records (aligned reads)
  - Information: https://samtools.github.io/hts-specs/SAMv1.pdf


HEADER
RECORDS

```
@HD VN:1.5 SO:coordinate
@SQ SN:ref LN:45
r001    99 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG *
r002     0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA    *
r003     0 ref  9 30 5S6M       *  0   0 GCCTAAGCTAA       * SA:Z:ref,29,-,6H5M,17,0;
r004     0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003  2064 ref 29 17 6H5M       *  0   0 TAGGC             * SA:Z:ref,9,+,5S6M,30,1;
r001   147 ref 37 30 9M         =  7 -39 CAGCGGCAT         * NM:i:1
```

SAM File



Col	Field	Type	Regexp/Range	Brief description
1	QNAME	String	`[!-?A-~]{1,255}`	Query template NAME
2	FLAG	Int	$[0,2^{16}-1]$	bitwise FLAG
3	RNAME	String	`\*|[!-()+-<>-~][!-~]*`	Reference sequence NAME
4	POS	Int	$[0,2^{31}-1]$	1-based leftmost mapping POSition
5	MAPQ	Int	$[0,2^{8}-1]$	MAPping Quality
6	CIGAR	String	`\*|([0-9]+[MIDNSHPX=])+`	CIGAR string
7	RNEXT	String	`\*|=|[!-()+-<>-~][!-~]*`	Ref. name of the mate/next read
8	PNEXT	Int	$[0,2^{31}-1]$	Position of the mate/next read
9	TLEN	Int	$[-2^{31}+1,2^{31}-1]$	observed Template LENgth
10	SEQ	String	`\*|[A-Za-z=.]+`	segment SEQuence
11	QUAL	String	`[!-~]+`	ASCII of Phred-scaled base QUALity+33

```
@HD VN:1.5 SO:coordinate
@SQ SN:ref LN:45
r001   99 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG *
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r003    0 ref  9 30 5S6M       *  0   0 GCCTAAGCTAA       * SA:Z:ref,29,-,6H5M,17,0;
r004    0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003 2064 ref 29 17 6H5M       *  0   0 TAGGC             * SA:Z:ref,9,+,5S6M,30,1;
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r004    0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
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r001  147 ref 37 30 9M         =  7 -39 CAGCGGCAT         * NM:i:1
```

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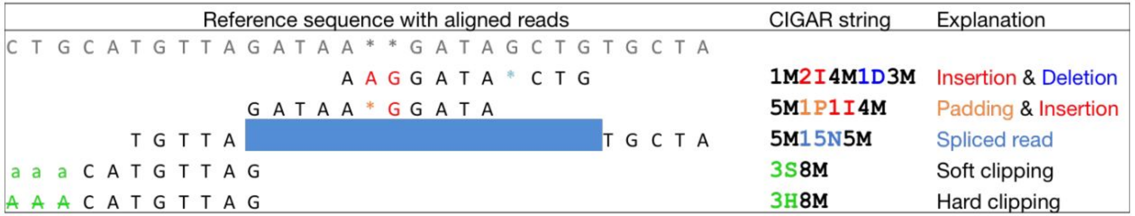
SAM File

CIGAR (Concise idiosyncratic gapped alignment report string)

Operator	Description
M	alignment match(can be a sequence match or mismatch
I	inserting to the reference
D	deletion from the reference
N	kkip region from the reference
S	soft clipping(clipped sequence present in SEQ)
H	hard clipping(clipped sequence NOT present in SEQ)
P	padding(silence from the reference)
=	sequence match
X	sequence mismatch



```
Reference sequence with aligned reads                          CIGAR string   Explanation
C T G C A T G T T A G A T A A * * G A T A G C T G T G C T A
                            A A G G A T A * C T G              1M2I4M1D3M     Insertion & Deletion
                    G A T A A * G G A T A                      5M1P1I4M       Padding & Insertion
          T G T T A                               T G C T A    5M15N5M        Spliced read
a a a C A T G T T A G                                          3S8M           Soft clipping
~~A~~ ~~A~~ ~~A~~ C A T G T T A G                              3H8M           Hard clipping
```

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BAM File

- Binary Alignment/Map format – compressed version of SAM.
- Compression: BGZF block compression.
- Efficient random access: UCSC bin/chunk scheme.
- BAI index files.
- More Information:
  http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2723002/
  http://www.ncbi.nlm.nih.gov/pmc/articles/PMC186604/

In summary

Reference sequence (FASTA)

```
>chr1
TACCTCCAGGGGGCATCCTCCCCCCCAATTCG
AAACACAATCGTAGCCCCTGGCACTACCTATG
TGTGTCAATTCGGAGAGAGAGATTCACGAA
AAAAAAGTCTGGACTCAACTAGGATACACACA
TTCGGCTACAGATACCAAAAAAAAAAAAAAAA
AAATTTTCACCATTGAGGCACCACCTTCTCGT
CGCTGCGTCGCTCTGCTCGCTTCGGCTAAAAA
TTCGCGCAATACATTCGGCTACAGATACCAAA
```

Unaligned reads

```
@seq1
ATTCGAAACA...
+
DDED88(999...
@seq2
CCCCGTTTCA...
+
AAC887BBAC...
```

Alignment/ Mapping

SAM/BAM format aligned reads

```
seq1   99     1      3666901      60
149M   =      3666935      185
ATTCGAAACA...DDED88(999    MC:Z:151M
MD:Z:149      RG:Z:15-0017315_1   NM:i:0
MQ:i:60       AS:i:149      XS:i:44
seq2   147    1      3666935      60
151M   =      3666901      -185
CCCCGTTTCA...AAC887BBAC...MC:Z:149M
MD:Z:151      RG:Z:15-0017315_1   NM:i:0
MQ:i:60       AS:i:151      XS:i:59
```

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Samtools

- Provides various utilities for manipulating alignments in the SAM format.

- Tools useful for quality check and bias correction.

- More Information:
  Paper: http://www.ncbi.nlm.nih.gov/pubmed/19505943
  Website: http://samtools.sourceforge.net/

Hands-on time

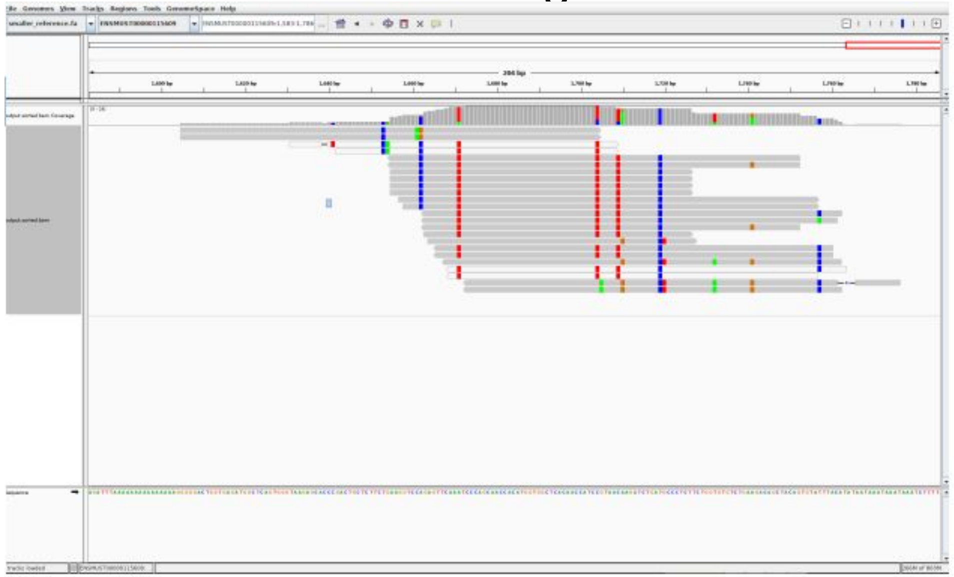
- Download data2.zip from the lecture website.
- Use STAR to align the reads to the supplied small reference genome (smaller_reference.fa) and output sam file
- FIRST! Index the genome: STAR --runThreadN 4 --runMode genomeGenerate --genomeDir output_dir/ --genomeFastaFiles smaller_reference.fa
    - STAR --help # for manuals
- Convert the SAM file to BAM (samtools view --help)
- Sort and index (samtools sort; samtools index)

IGV

● Tool for visualising sequences, reads and/or variants at genomic locations

● Open IGV. From menu: Genomes → Load genomes from file. → Navigate to genome fasta file

- File →
- Load from File →
- Navigate to indexed Bam file.



Single Cell Sequencing

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010110110101
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Single Cell Analysis

● Extract sequences from a specific cell for the purpose of discovering differences in gene expression level

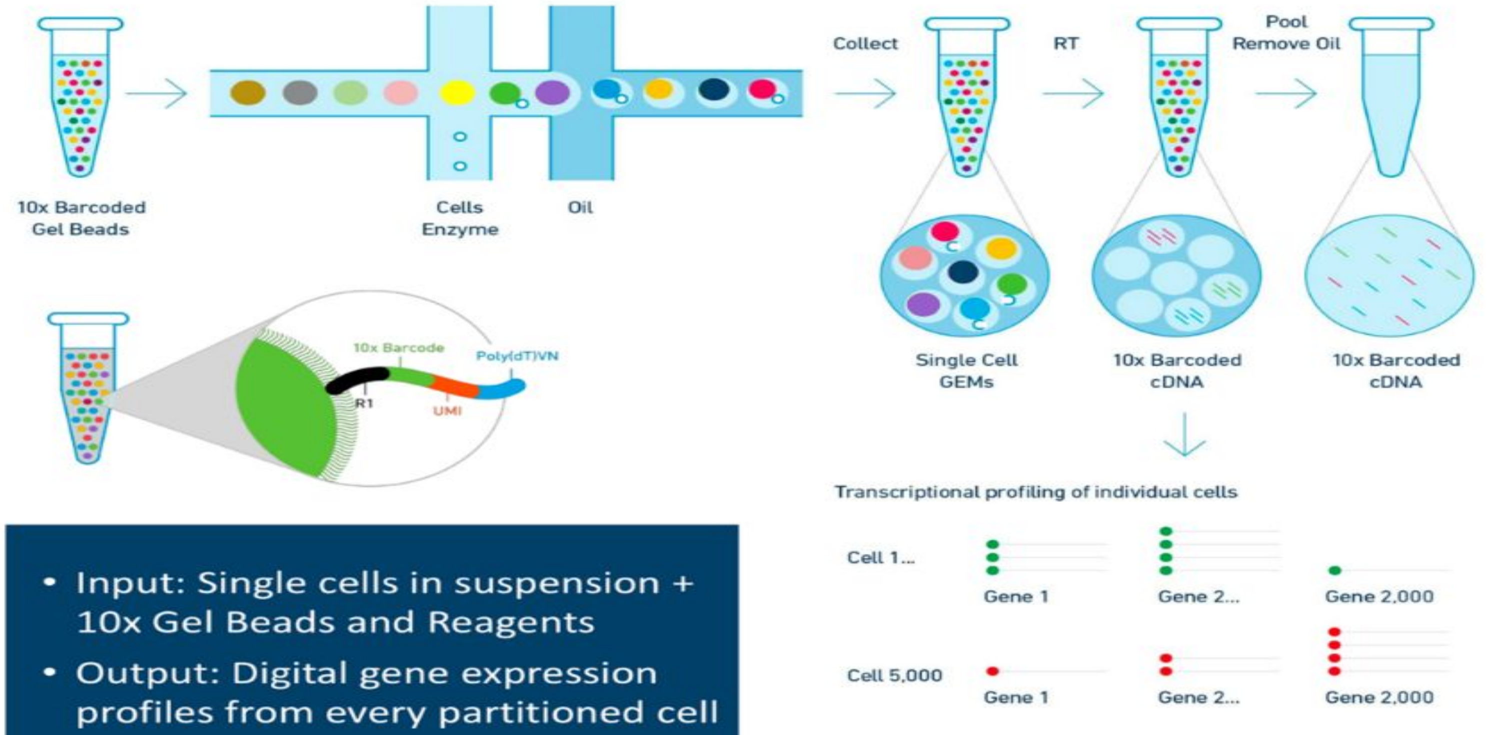
● Every sample is prepared by artificially adding a barcode and (preferably) Unique Molecule Identifier (UMI)

● All molecules from the same batch have the same barcode

● Every individual molecule has a separate UMI

● Because of sequencing errors, we need to make sure that we can correct small amount of bases (1-2) and still have the same barcode – by maximizing the Hamming distance

Single Cell Analysis



- Input: Single cells in suspension + 10x Gel Beads and Reagents
- Output: Digital gene expression profiles from every partitioned cell

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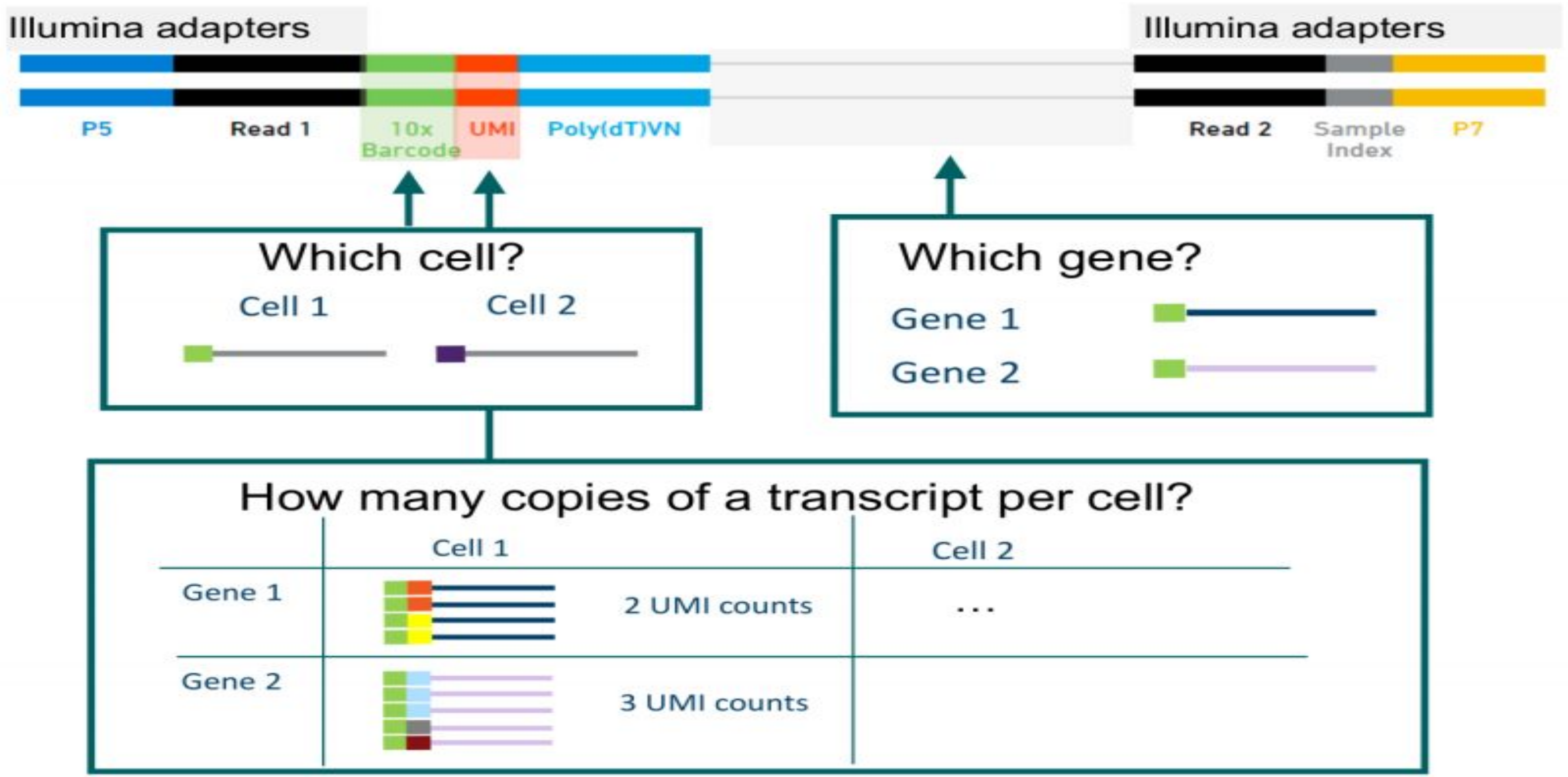
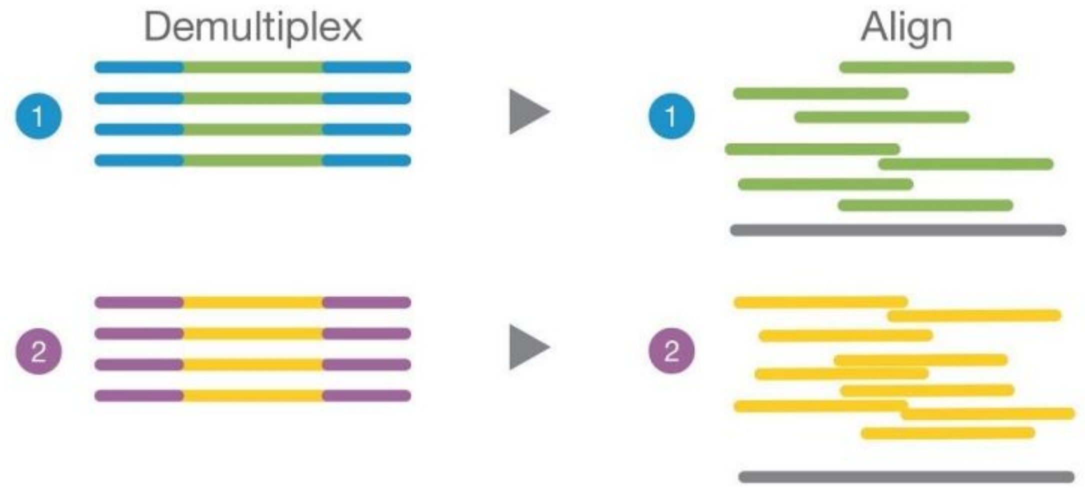
Demultiplexing



Source: 10x genomics

Demultiplexing

Distinguishing different DNA samples based on added barcode

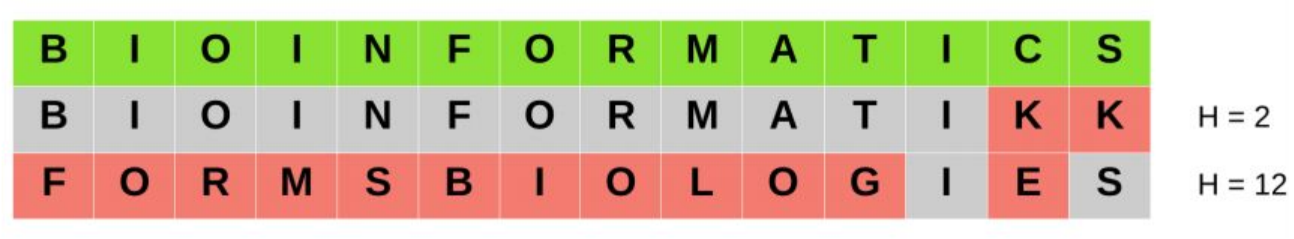


Source: Illumina webinar

Hamming Distance

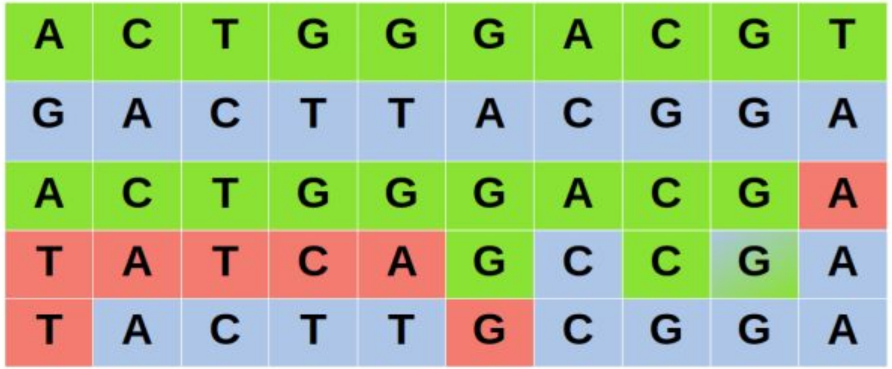
● A measure of similarity between two strings of equal length

● Measured by the amount substitutions needed to derive the second string from the first



B	I	O	I	N	F	O	R	M	A	T	I	C	S	
B	I	O	I	N	F	O	R	M	A	T	I	K	K	H = 2
F	O	R	M	S	B	I	O	L	O	G	I	E	S	H = 12

Hamming Distance - Example



A	C	T	G	G	G	A	C	G	T	Barcode 1
G	A	C	T	T	A	C	G	G	A	Barcode 2
A	C	T	G	G	G	A	C	G	A	Read 1 - H(1) = 1; H(2) = 9
T	A	T	C	A	G	C	C	G	A	Read 2 - H(1) = 6; H(2) = 6
T	A	C	T	T	G	C	G	G	A	Read 3 - H(1) = 7; H(2) = 2

Designing a set of equidistant barcodes for optimal error correction is NP-complete problem

Demultiplexing

- Demultiplexing both:
    - Barcode
    - UMI (Unique Molecule Identifier)

- Usually UMI is added to read of the paired read.

- This results in one Fastq File per barcode

Demultiplexing - Example

- For simplicity a demultiplexing script is provided as well as sample data - data3.zip.

Use it to extract demultiplexed reads and get familiar with the inputs and outputs.

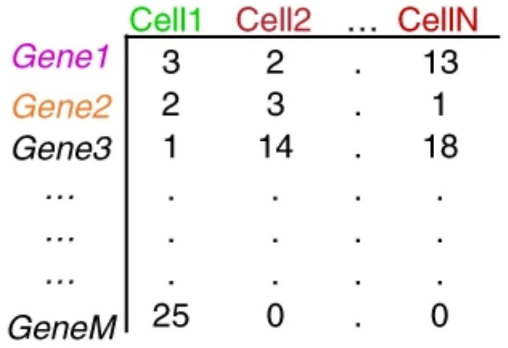
```
mkdir data3/results
```

```
python3 ../script/demultiplexing.py -b data3/10cells_barcodes.txt -f
data3/10cells_read1.fastq -r data3/10cells_read2.fastq -o data3/results/
```

Expression Matrix

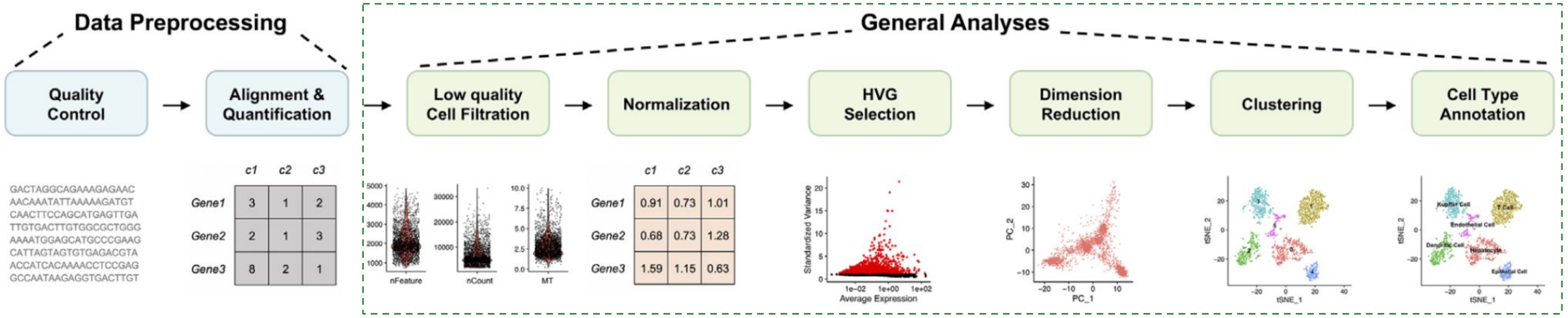
- After performing QC we align the reads and count UMIs for specific barcodes and positions to create an Expression Matrix (mxn).
  n can vary to ~100 to ~ 1M

- Columns represent a cell
- Rows represent a gene (transpose used by some authors)



	Cell1	Cell2	...	CellN
Gene1	3	2	.	13
Gene2	2	3	.	1
Gene3	1	14	.	18
...	.	.	.	.
...	.	.	.	.
...	.	.	.	.
GeneM	25	0	.	0

Data preprocessing



	c1	c2	c3
Gene1	3	1	2
Gene2	2	1	3
Gene3	8	2	1

	c1	c2	c3
Gene1	0.91	0.73	1.01
Gene2	0.68	0.73	1.28
Gene3	1.59	1.15	0.63

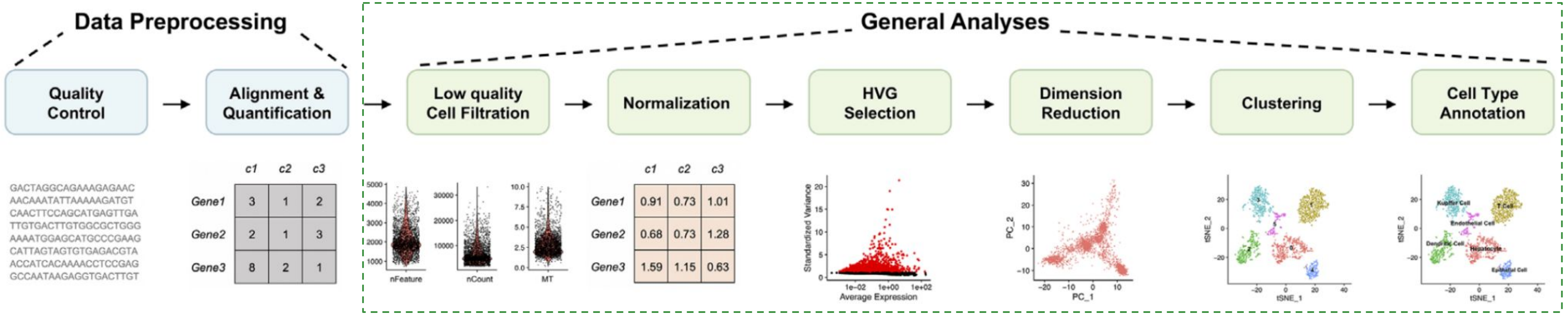
Scanpy

● A python package designed for higher level analysis and exploration of single-cell RNA-seq data.

● Current version: 1.9.3

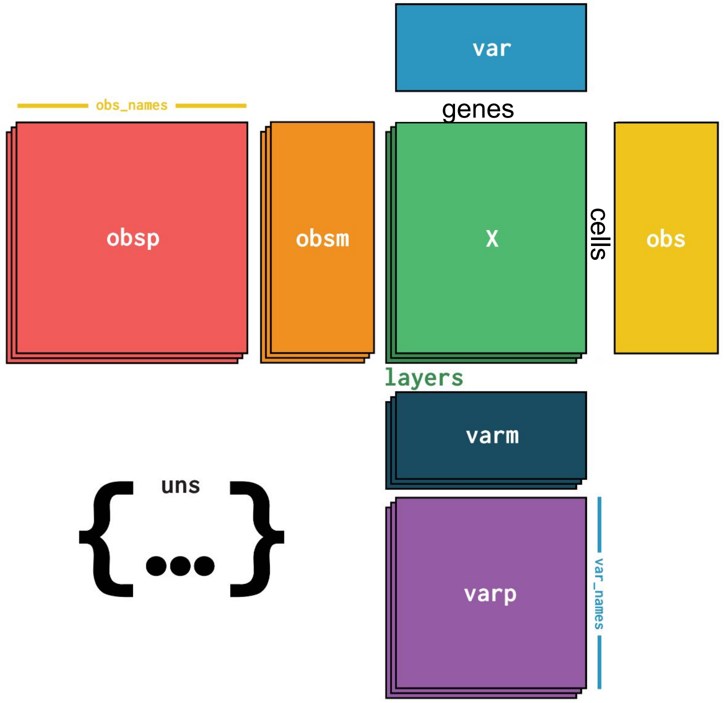
● Allows various functions like PCA and clustering and supports an array of different plotting capabilities.

Scanpy-pipeline



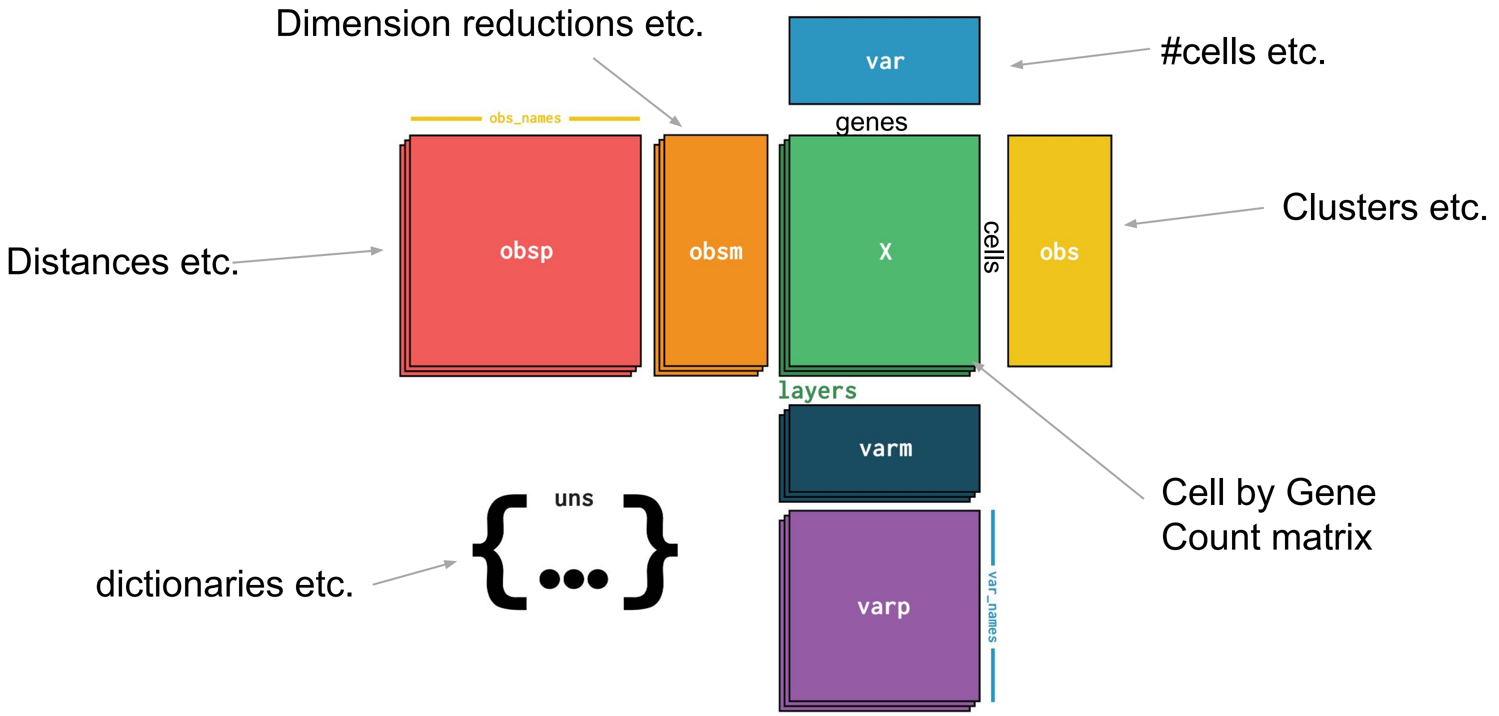
D Jovic, X Liang, H Zeng, L Lin, F Xu, Y Luo, 2022

Scanpy-anndata



https://anndata.readthedocs.io/en/latest/

Scanpy-anndata



Scanpy-load data

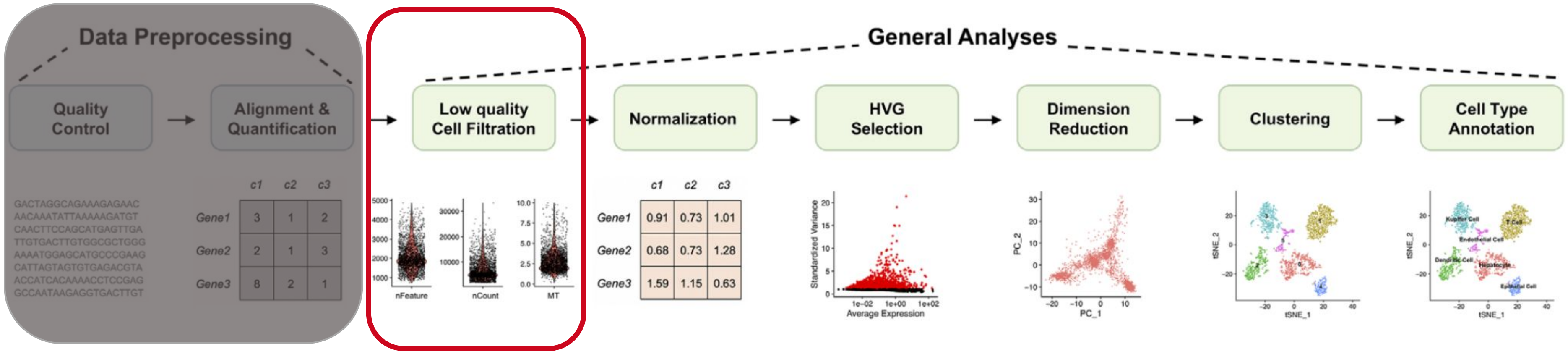
```
import numpy as np
import pandas as pd
import scanpy as sc
import scanpy.external as sce
sc.settings.verbosity = 3             # verbosity: errors (0), warnings (1), info (2), hints (3)
sc.logging.print_header()
sc.settings.set_figure_params(dpi=100, facecolor='white')
```

Scanpy-load data

```
import numpy as np
import pandas as pd
import scanpy as sc
import scanpy.external as sce
sc.settings.verbosity = 3             # verbosity: errors (0), warnings (1), info (2), hints (3)
sc.logging.print_header()
sc.settings.set_figure_params(dpi=100, facecolor='white')


## load anndata from h5ad file
adata =  sc.read_h5ad("ifnb.h5ad")  # Reading the h5ad containing the data
adata.var_names_make_unique() # Making the data unique
```

Scanpy-pipeline



D Jovic, X Liang, H Zeng, L Lin, F Xu, Y Luo, 2022

Scanpy-preprocessing

```
sc.pp.filter_cells(adata, min_genes=200) ## min_genes: Minimal feature per cell
sc.pp.filter_genes(adata, min_cells=3)   ## min_cells: Minimal cells per genes

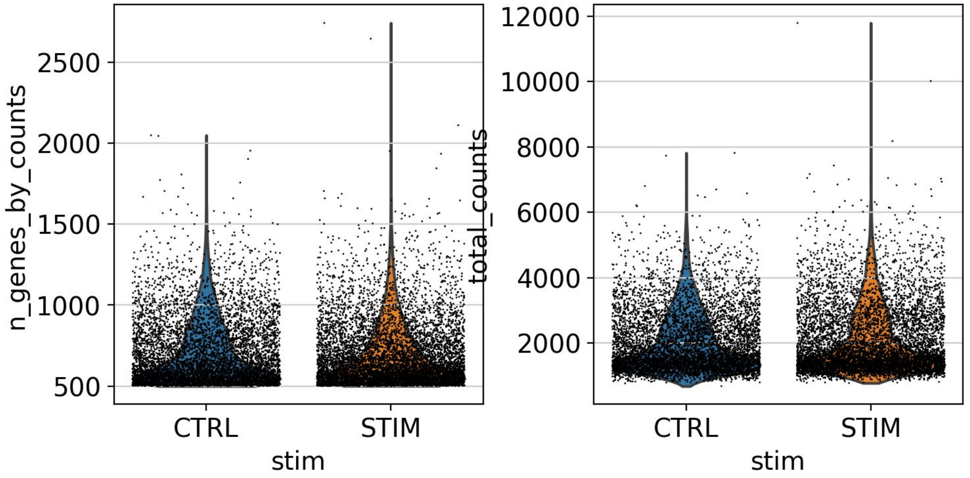

sc.pp.calculate_qc_metrics(adata,
                           percent_top=None,
                           log1p=False,
                           inplace=True)
sc.pl.violin(adata, ['n_genes_by_counts', 'total_counts'],
                     groupby="stim", jitter=0.4, multi_panel=True)
```

Cells with too high (doublets) or low counts (not viable cells) can be artifacts!

Scanpy-preprocessing

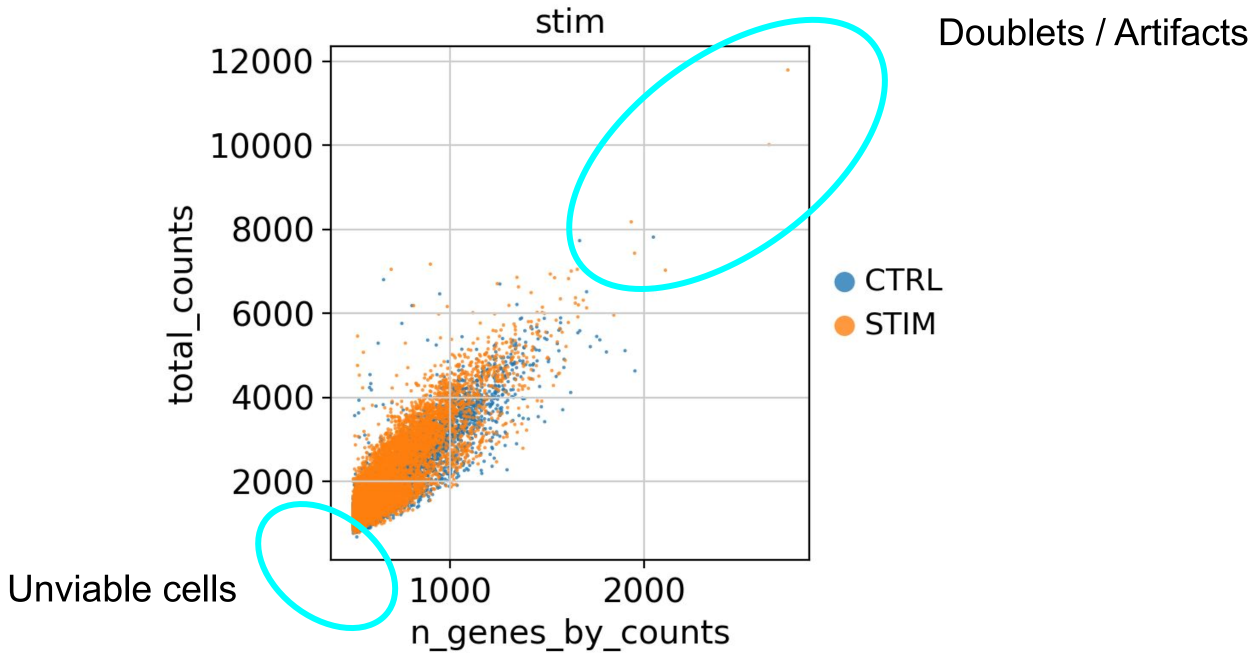
```
sc.pp.filter_cells(adata, min_genes=200) ## min_genes: Minimal feature per cell
sc.pp.filter_genes(adata, min_cells=3)   ## min_cells: Minimal cells per genes


sc.pp.calculate_qc_metrics(adata, percent_top=None, log1p=False, inplace=True)
sc.pl.violin(adata, ['n_genes_by_counts', 'total_counts'],
                    groupby="stim", jitter=0.4, multi_panel=True)
```



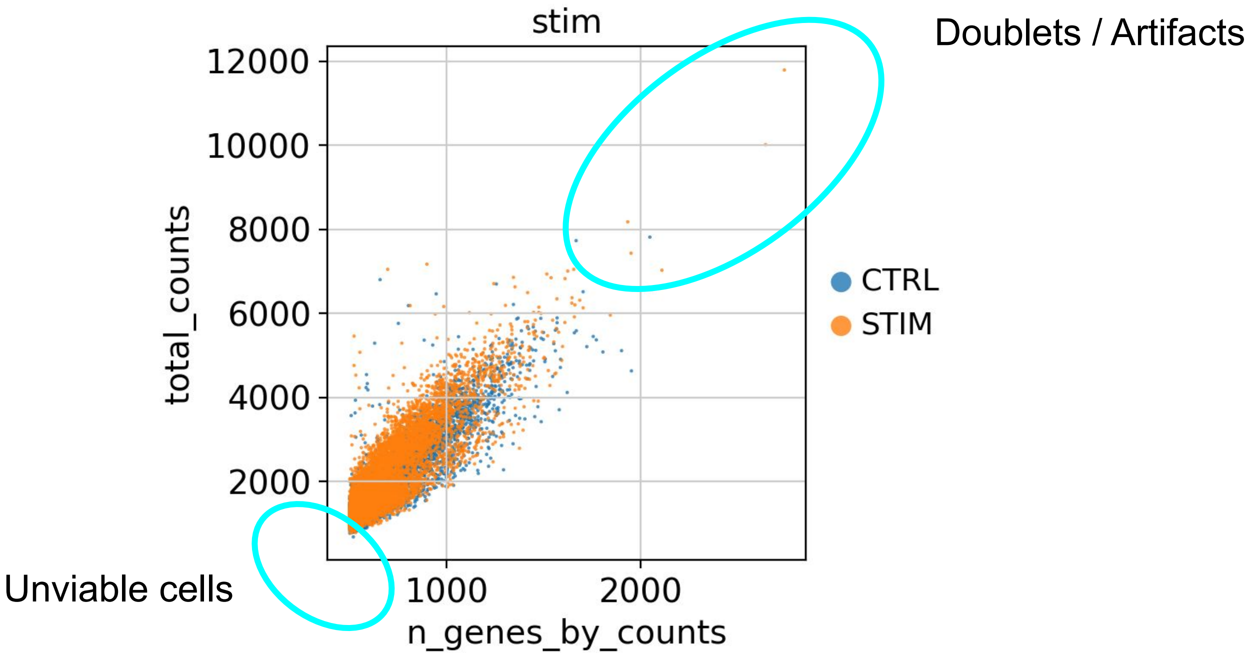
Scanpy-preprocessing

```
sc.pl.scatter(adata, x="n_genes_by_counts", y="total_counts", color="stim",alpha=0.8)
```

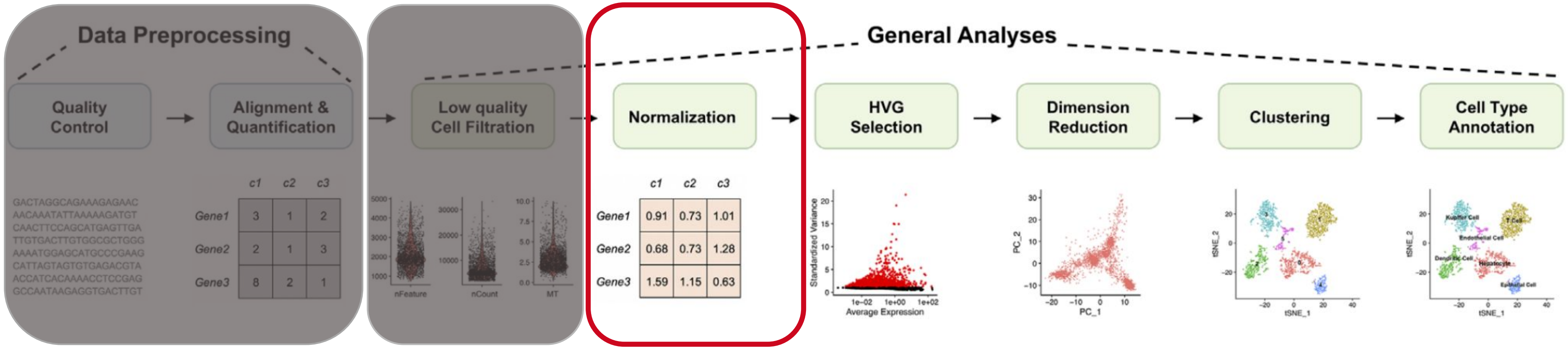


Scanpy-preprocessing

```
sc.pl.scatter(adata, x="n_genes_by_counts", y="total_counts", color="stim",alpha=0.8)
adata = adata[adata.obs.n_genes_by_counts < 2500, :]
```



Scanpy-pipeline



	c1	c2	c3
Gene1	3	1	2
Gene2	2	1	3
Gene3	8	2	1

	c1	c2	c3
Gene1	0.91	0.73	1.01
Gene2	0.68	0.73	1.28
Gene3	1.59	1.15	0.63

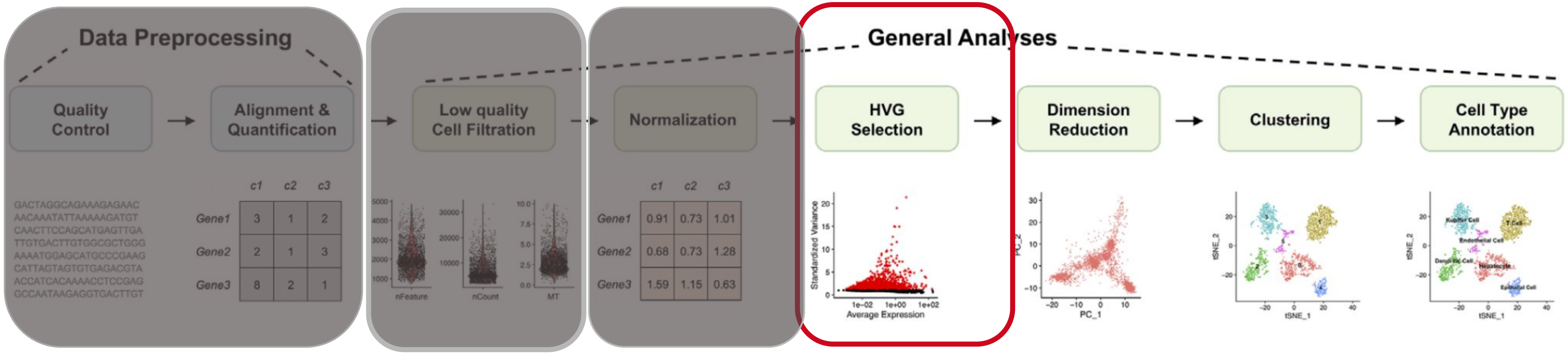
```
GACTAGGCAGAAAGAGAAC
AACAAATATTAAAAAGATGT
CAACTTCCAGCATGAGTTGA
TTGTGACTTGTGGCGCTGGG
AAAATGGAGCATGCCCGAAG
CATTAGTAGTGTGAGACGTA
ACCATCACAAAACCTCCGAG
GCCAATAAGAGGTGACTTGT
```

D Jovic, X Liang, H Zeng, L Lin, F Xu, Y Luo, 2022

Scanpy-Cell Normalization

```
# Normalization
sc.pp.normalize_total(adata,
                      target_sum=1e4)
sc.pp.log1p(adata)
```

Scanpy-pipeline



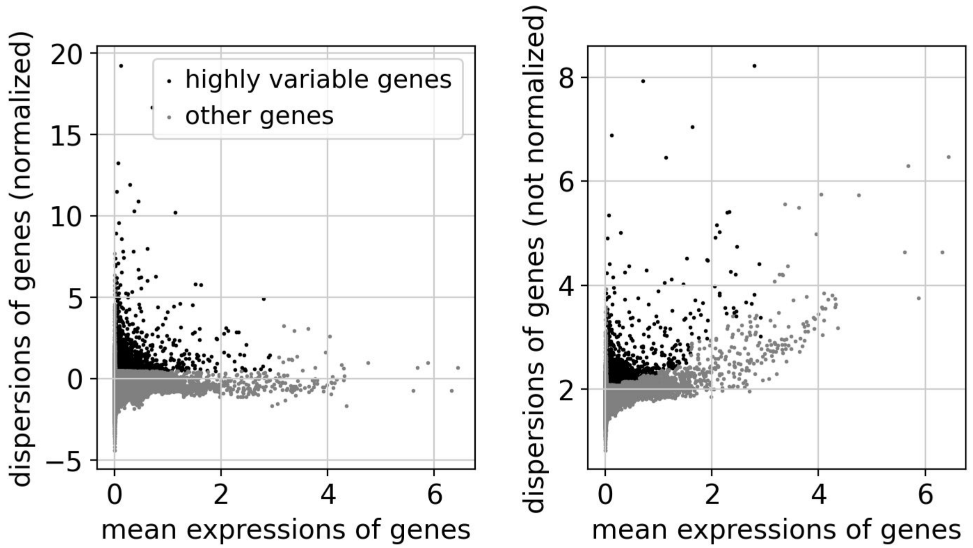
D Jovic, X Liang, H Zeng, L Lin, F Xu, Y Luo, 2022

Scanpy-Features

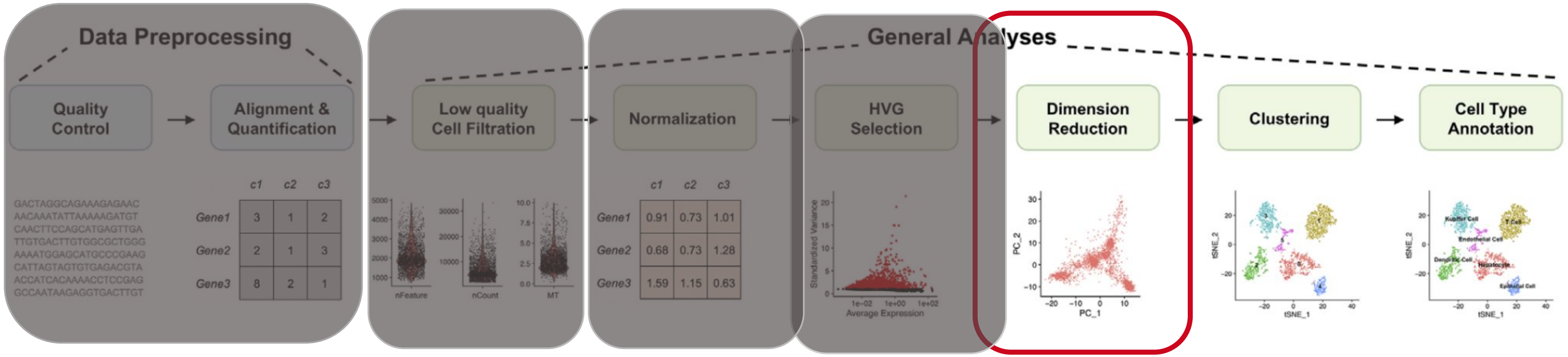
```
sc.pp.highly_variable_genes(adata,
                            min_mean=0.0125,
                            max_mean=3,
                            min_disp=0.5)
```

Scanpy-Features

```
sc.pp.highly_variable_genes(adata, min_mean=0.0125, max_mean=3, min_disp=0.5)
sc.pl.highly_variable_genes(adata)
```



Scanpy-pipeline

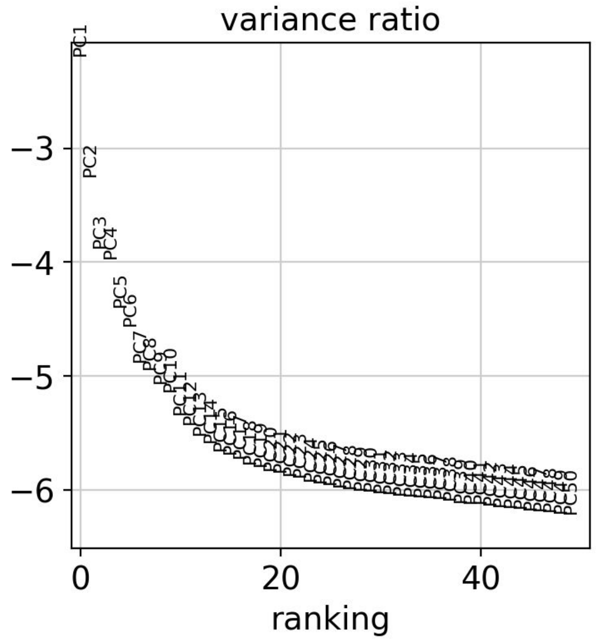


Scanpy-Dimension reduction

```
sc.tl.pca(adata,
          svd_solver='arpack')
```

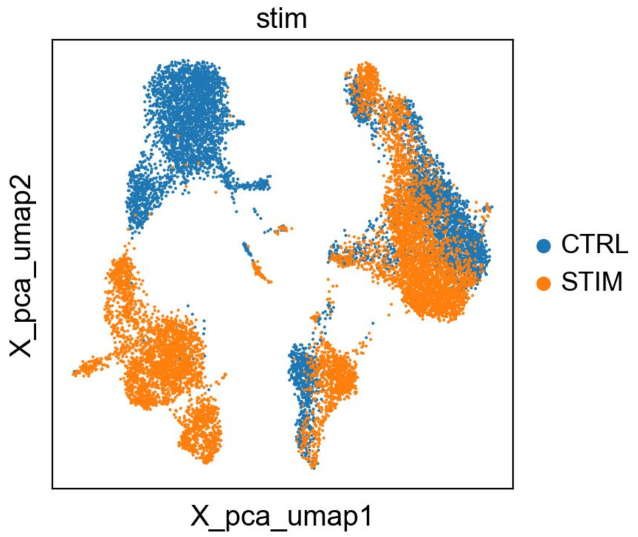
Scanpy-Dimension reduction

```
sc.tl.pca(adata, svd_solver='arpack')
sc.pl.pca_variance_ratio(adata, log=True, n_pcs=50)
```

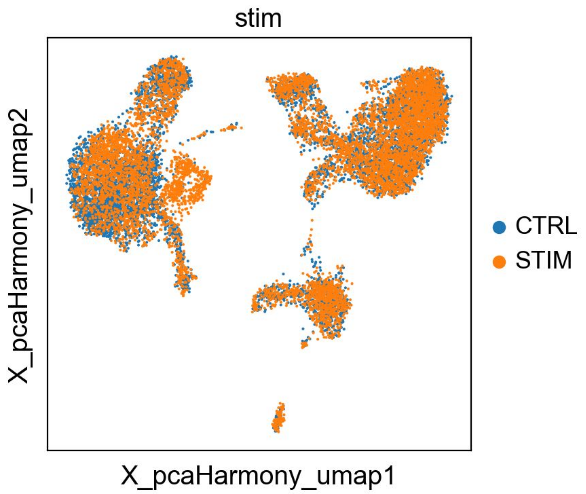


Scanpy-Data integration

Before data integration



After data integration



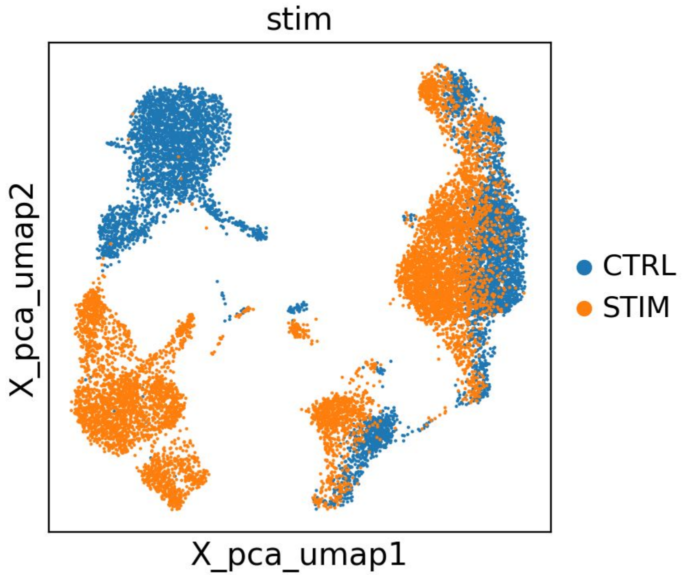
Scanpy-Uncorrected

```
sc.pp.neighbors(adata,
                n_neighbors=10,
                n_pcs=40,
                use_rep="X_pca")
sc.tl.umap(adata)
adata.obsm['X_pca_umap'] = adata.obsm['X_umap']
```

Scanpy-Uncorrected

```
sc.pp.neighbors(adata, n_neighbors=10, n_pcs=40, use_rep="X_pca")
sc.tl.umap(adata)
adata.obsm['X_pca_umap'] = adata.obsm['X_umap']
```

```
sc.pl.embedding(adata, color='stim', basis="X_pca_umap")
```



Scanpy-Data integration

```
import harmonypy as hm
harmony_out = hm.run_harmony(data_mat=adata.obsm['X_pca'][:, 0:40],
                             meta_data=adata.obs,
                             vars_use="stim" )
adata.obsm['X_pca_harmony'] = harmony_out.Z_corr.T
```

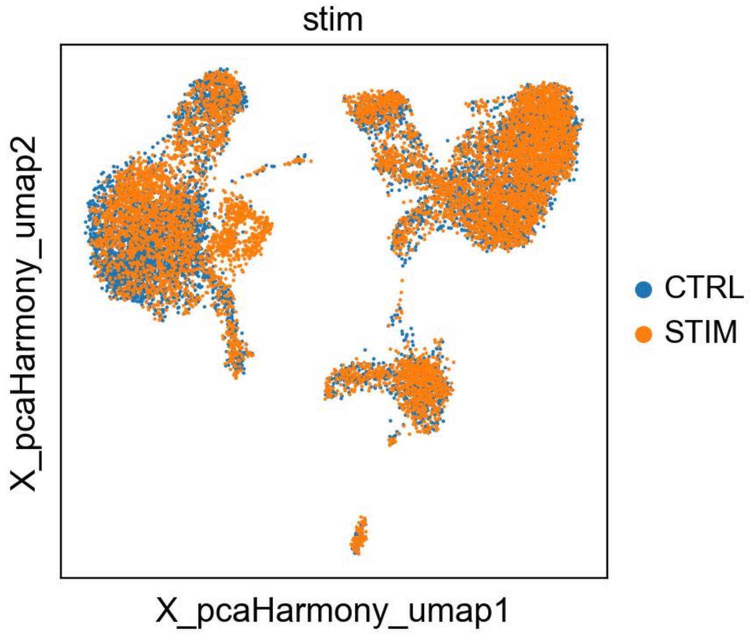
Scanpy-Data integration

```
import harmonypy as hm
harmony_out = hm.run_harmony(data_mat=adata.obsm['X_pca'][:, 0:40],
                             meta_data=adata.obs,
                             vars_use="stim" )
adata.obsm['X_pca_harmony'] = harmony_out.Z_corr.T


sc.pp.neighbors(adata, n_neighbors=10, n_pcs=40, use_rep="X_pca_harmony")
sc.tl.umap(adata)
adata.obsm['X_pcaHarmony_umap'] = adata.obsm['X_umap']
```

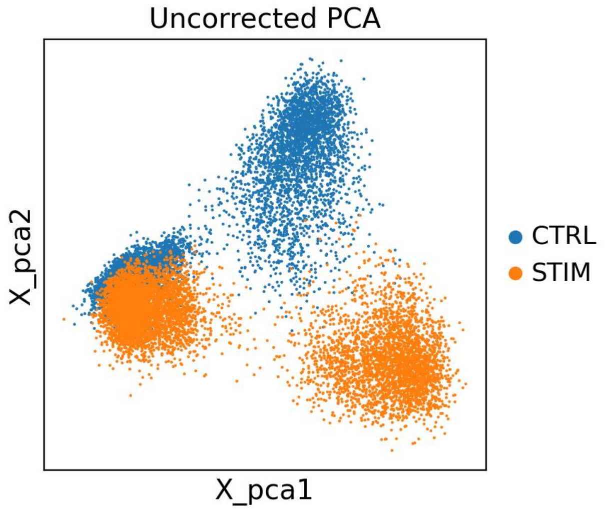
Scanpy-Data integration

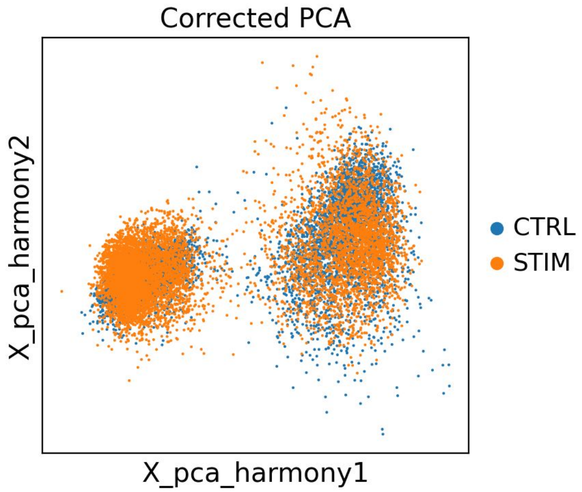
```
sc.pl.embedding(adata, color='stim', basis="X_pcaHarmony_umap")
```



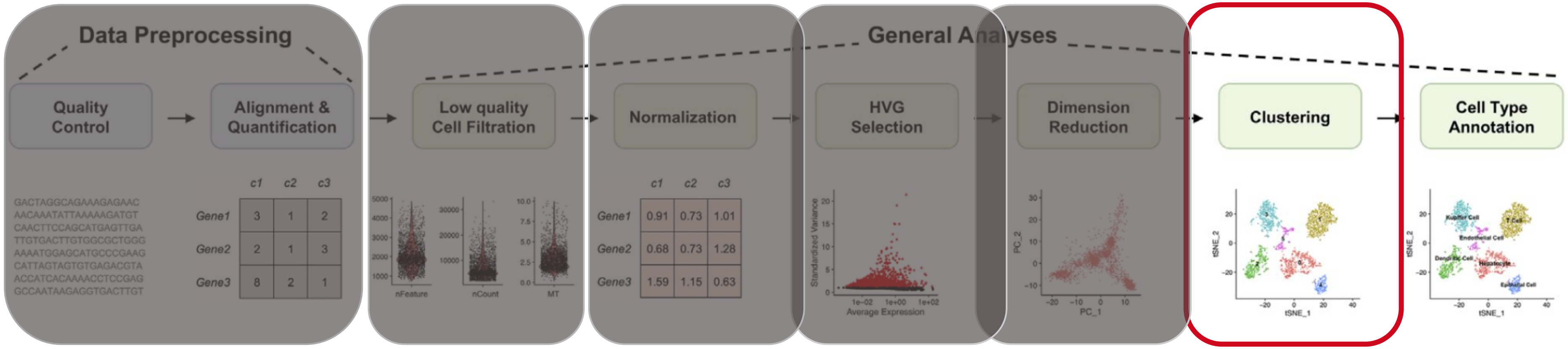
Scanpy-Data integration

```
sc.pl.embedding(adata, color='stim', basis='X_pca',title='Uncorrected PCA')
sc.pl.embedding(adata, color='stim', basis='X_pca_harmony',title='Corrected PCA')
```





Scanpy-pipeline



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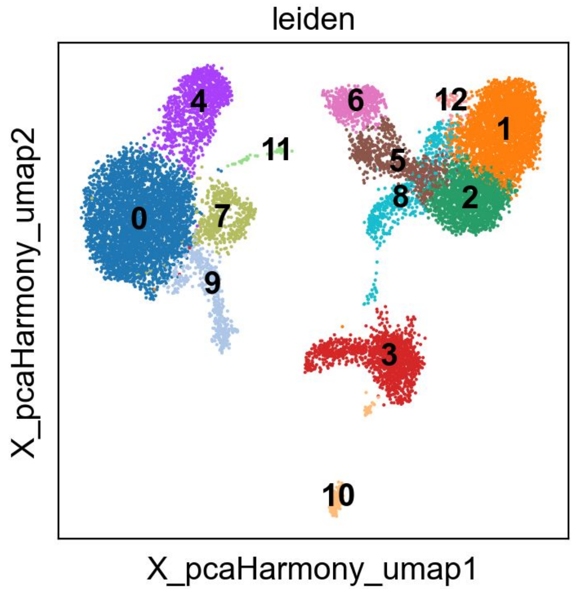
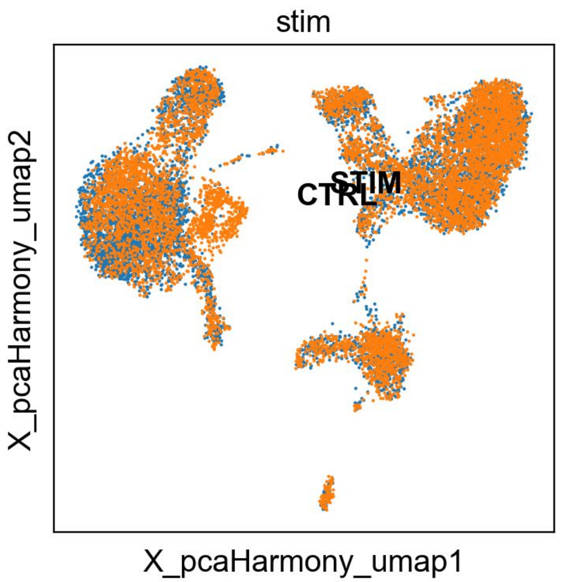
Scanpy-Cluster cells

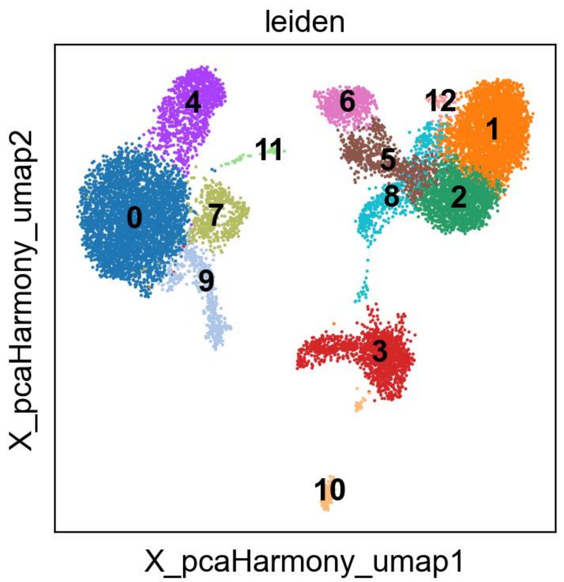
```
sc.tl.leiden(adata, resolution=0.7)
sc.pl.embedding(adata, color='leiden', basis='X_pcaHarmony_umap', legend_loc="on data")
```



Scanpy-Cluster cells

```
sc.pl.embedding(adata, color=('stim','leiden'),
                basis='X_pcaHarmony_umap',legend_loc="on data")
```



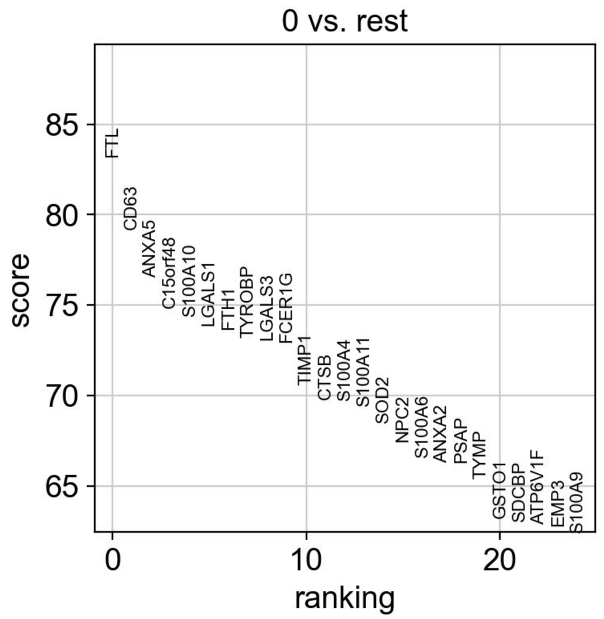


Scanpy-Identify markers for cells

```
sc.tl.rank_genes_groups(adata,
                        'leiden',
                         method='wilcoxon',
                         corr_method='bonferroni')
```

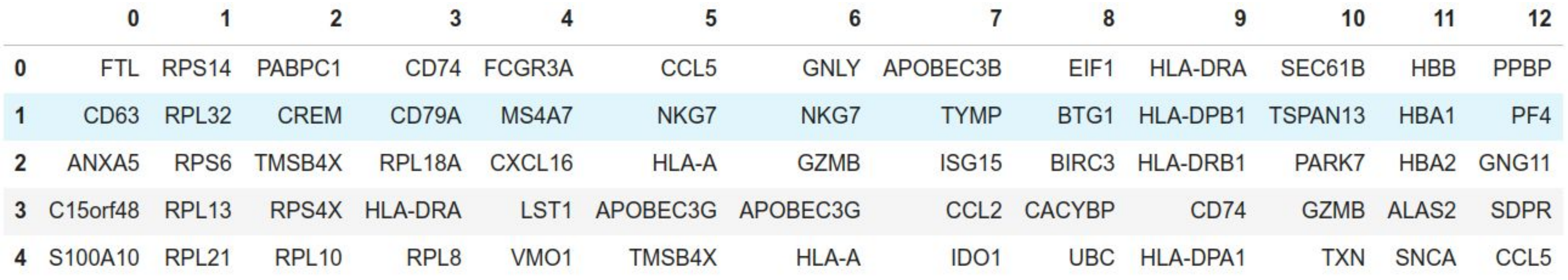
Scanpy-Identify markers for cells

```
sc.tl.rank_genes_groups(adata, 'leiden', method='wilcoxon',corr_method='bonferroni')
sc.pl.rank_genes_groups(adata, n_genes=25, sharey=False, groups='0')
```



Scanpy-Identify markers for cells

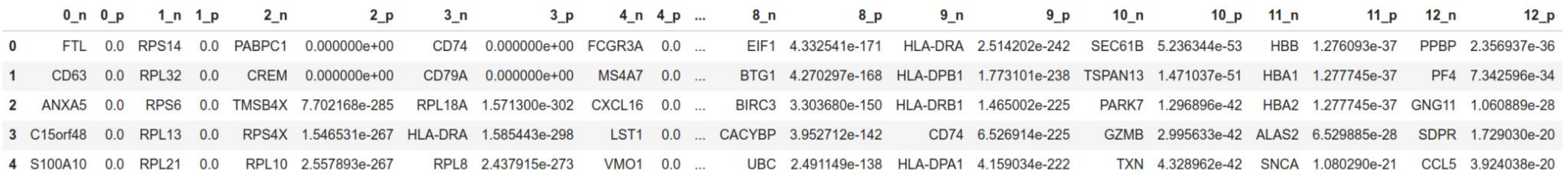
```
pd.DataFrame(adata.uns['rank_genes_groups']['names']).head()
```



	0	1	2	3	4	5	6	7	8	9	10	11	12
0	FTL	RPS14	PABPC1	CD74	FCGR3A	CCL5	GNLY	APOBEC3B	EIF1	HLA-DRA	SEC61B	HBB	PPBP
1	CD63	RPL32	CREM	CD79A	MS4A7	NKG7	NKG7	TYMP	BTG1	HLA-DPB1	TSPAN13	HBA1	PF4
2	ANXA5	RPS6	TMSB4X	RPL18A	CXCL16	HLA-A	GZMB	ISG15	BIRC3	HLA-DRB1	PARK7	HBA2	GNG11
3	C15orf48	RPL13	RPS4X	HLA-DRA	LST1	APOBEC3G	APOBEC3G	CCL2	CACYBP	CD74	GZMB	ALAS2	SDPR
4	S100A10	RPL21	RPL10	RPL8	VMO1	TMSB4X	HLA-A	IDO1	UBC	HLA-DPA1	TXN	SNCA	CCL5

Scanpy-Identify markers for cells

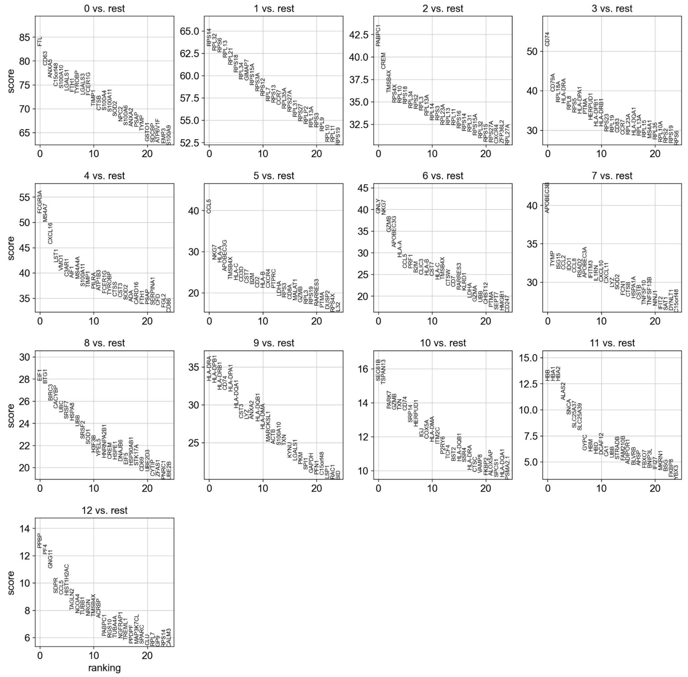
```python
result = adata.uns['rank_genes_groups']
groups = result['names'].dtype.names
pd.DataFrame(
    {group + '_' + key[:1]: result[key][group]
    for group in groups for key in ['names', 'pvals']}).head(5)
```



	0_n	0_p	1_n	1_p	2_n	2_p	3_n	3_p	4_n	4_p	...	8_n	8_p	9_n	9_p	10_n	10_p	11_n	11_p	12_n	12_p
0	FTL	0.0	RPS14	0.0	PABPC1	0.000000e+00	CD74	0.000000e+00	FCGR3A	0.0	...	EIF1	4.332541e-171	HLA-DRA	2.514202e-242	SEC61B	5.236344e-53	HBB	1.276093e-37	PPBP	2.356937e-36
1	CD63	0.0	RPL32	0.0	CREM	0.000000e+00	CD79A	0.000000e+00	MS4A7	0.0	...	BTG1	4.270297e-168	HLA-DPB1	1.773101e-238	TSPAN13	1.471037e-51	HBA1	1.277745e-37	PF4	7.342596e-34
2	ANXA5	0.0	RPS6	0.0	TMSB4X	7.702168e-285	RPL18A	1.571300e-302	CXCL16	0.0	...	BIRC3	3.303680e-150	HLA-DRB1	1.465002e-225	PARK7	1.296896e-42	HBA2	1.277745e-37	GNG11	1.060889e-28
3	C15orf48	0.0	RPL13	0.0	RPS4X	1.546531e-267	HLA-DRA	1.585443e-298	LST1	0.0	...	CACYBP	3.952712e-142	CD74	6.526914e-225	GZMB	2.995633e-42	ALAS2	6.529885e-28	SDPR	1.729030e-20
4	S100A10	0.0	RPL21	0.0	RPL10	2.557893e-267	RPL8	2.437915e-273	VMO1	0.0	...	UBC	2.491149e-138	HLA-DPA1	4.159034e-222	TXN	4.328962e-42	SNCA	1.080290e-21	CCL5	3.924038e-20

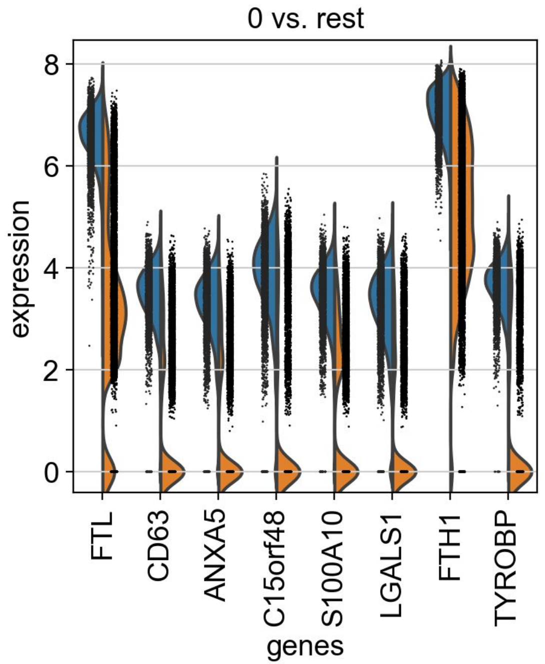
Scanpy-Identify markers for cells

```
sc.pl.rank_genes_groups(adata, n_genes=25, sharey=False)
```



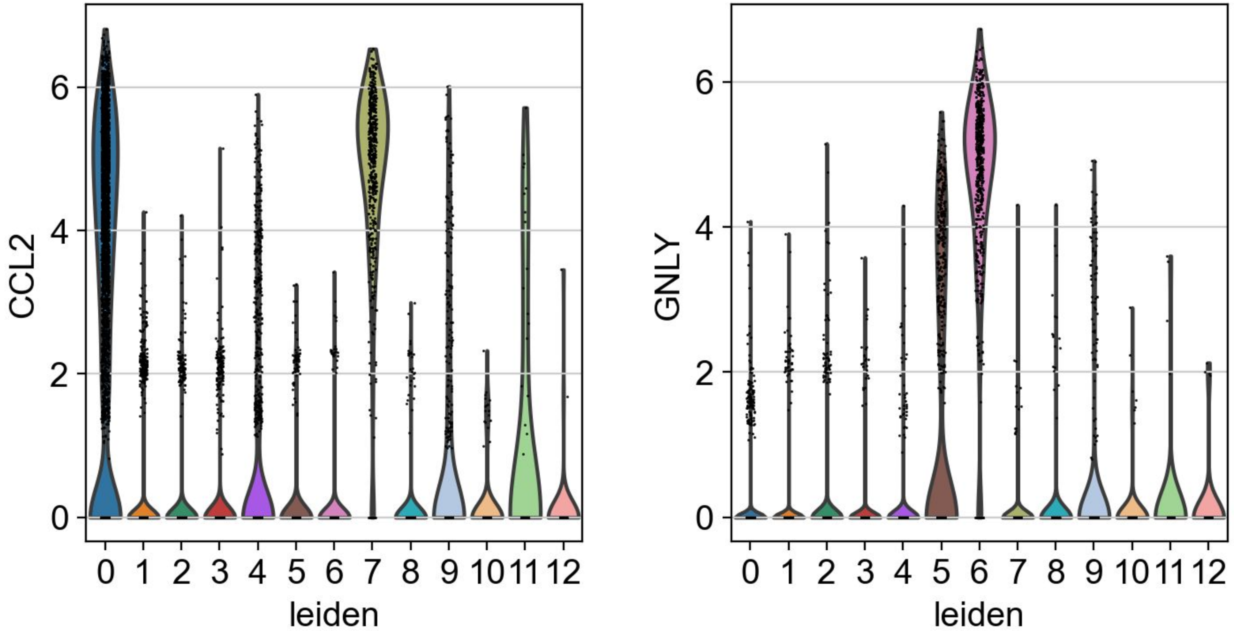
Scanpy-Identify markers for cells

```
sc.pl.rank_genes_groups_violin(adata, groups='0', n_genes=8)
```



Scanpy-Identify markers for cells

```
sc.pl.violin(adata, ["CCL2", "GNLY"], groupby='leiden')
```



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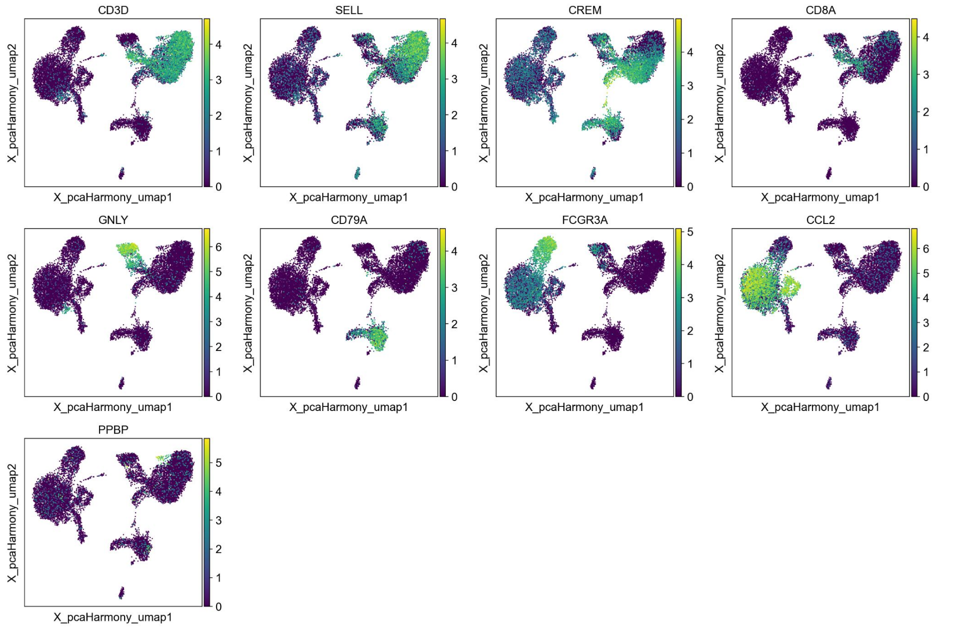
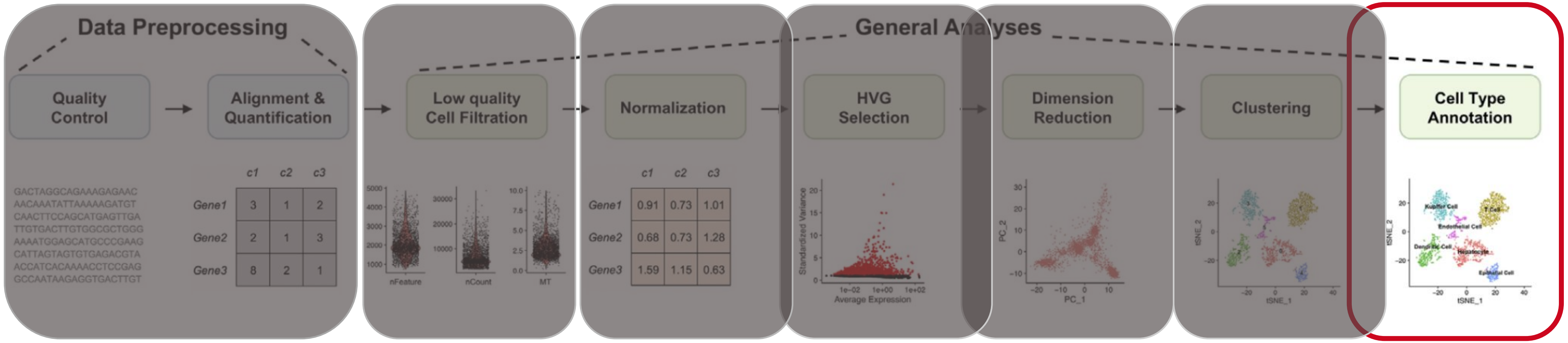
Scanpy-Identify markers for cells

```
genes = ["CD3D", "SELL", "CREM", "CD8A", "GNLY","CD79A", "FCGR3A","CCL2", "PPBP"]
sc.pl.embedding(adata, color=genes, basis='X_pcaHarmony_umap')
```



Scanpy-pipeline

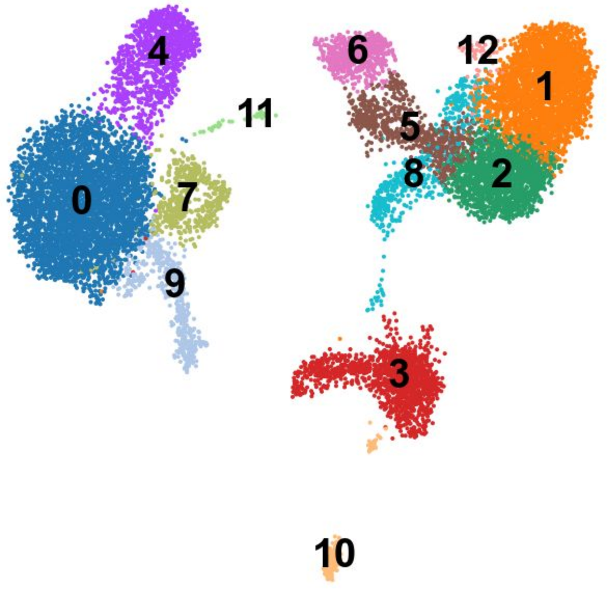


Scanpy-Identify markers for cells

```
new_cluster_names ={"0" :  "CD14 Mono", "1" : "CD4 Naive T", "2" : "CD4 Memory T",
                    "3" : "B", "4" : "CD16 Mono", "5" : "CD8 T",
                    "6" : "NK", "7" : "CD14 Mono", "8" : "T activated",
                    "9" : "DC", "10" : "pDC", "11" : "Eryth",
                    "12" : "Mk"}
adata.obs['annotation'] = (adata.obs['leiden'].map(new_cluster_names).astype('category'))
```

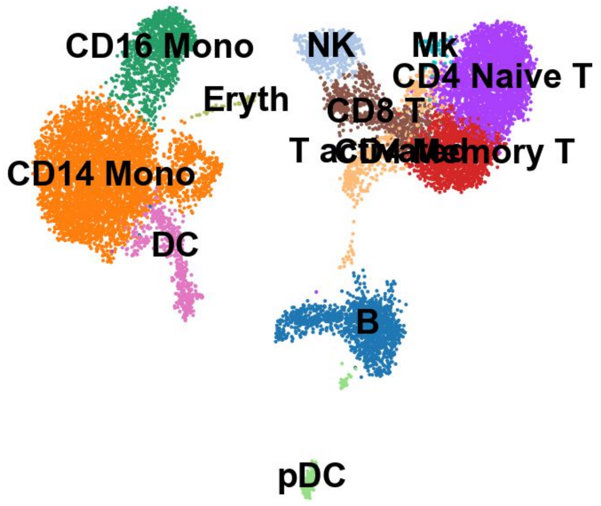
Scanpy-Identify markers for cells

```
sc.pl.umap(adata, color='leiden',
           legend_loc='on data', title='', frameon=False, save='.pdf')
```

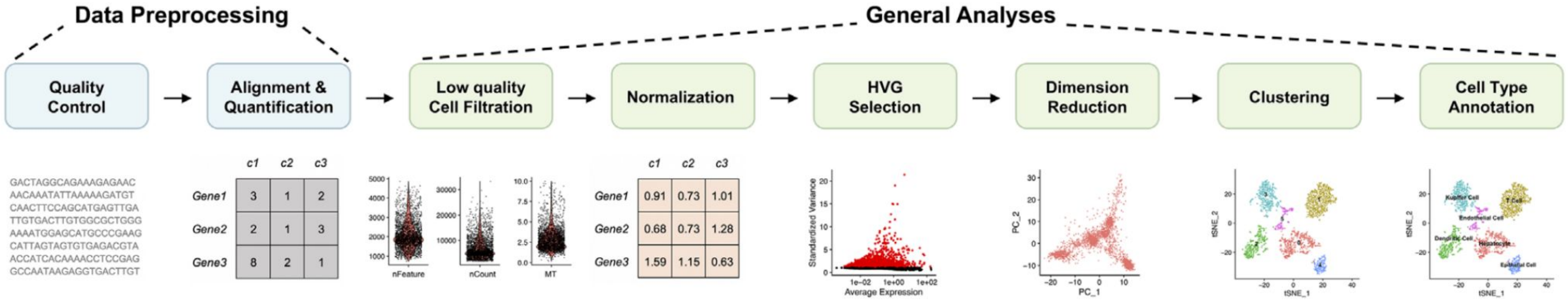


Scanpy-Expert-based cluster annotation

```
sc.pl.umap(adata, color='annotation',
           legend_loc='on data', title='', frameon=False, save='.pdf')
```



Scanpy-pipeline



D Jovic, X Liang, H Zeng, L Lin, F Xu, Y Luo, 2022

Alternative in Python - seurat

Very similar analysis can be made in R using Seurat:

https://costalab.ukaachen.de/open_data/SOSE_2021/lecture_2_singlecell_practice.pdf

Thanks for your attention

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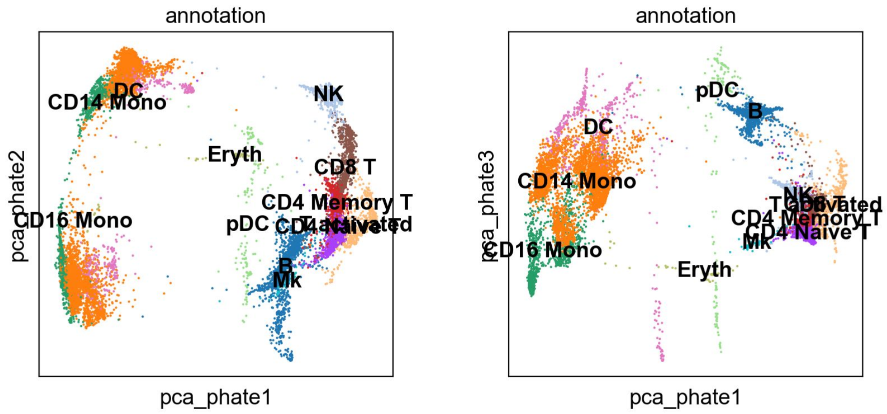
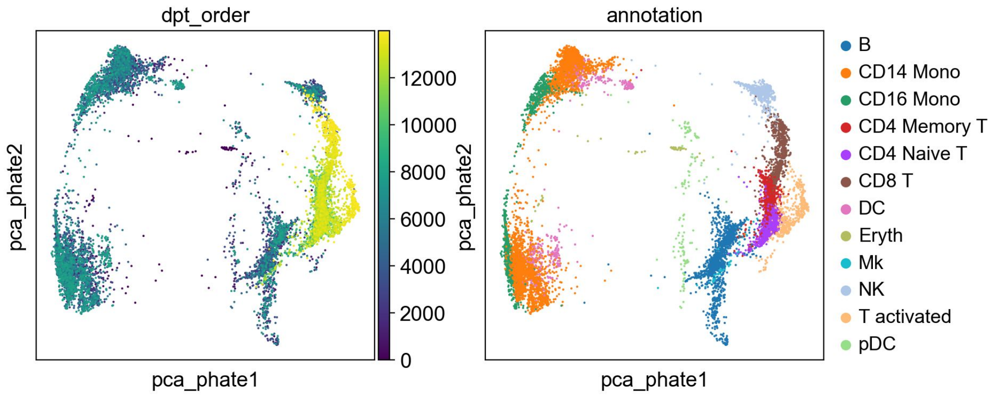
Phate trajectory embedding

```
import phate
phate_op = phate.PHATE(n_components=3)
data_phate = phate_op.fit_transform(adata.obsm['X_pca'])
adata.obsm['X_pca_phate'] = data_phate
sc.pl.embedding(adata, basis ='pca_phate', color=['annotation'],
                      legend_loc='on data', dimensions=[(0,1), (0,2)])
```



Scanpy inferring pseudotime of the embeddings

```
sc.tl.diffmap(adata, n_comps=10)
adata.uns['iroot'] = np.flatnonzero(adata.obs['annotation'] == 'CD4 Naive T')[0]
sc.tl.dpt(adata, n_branchings=1, n_dcs=10)
sc.pl.embedding(adata, basis='pca_phate', color=['dpt_order', 'annotation'])
```



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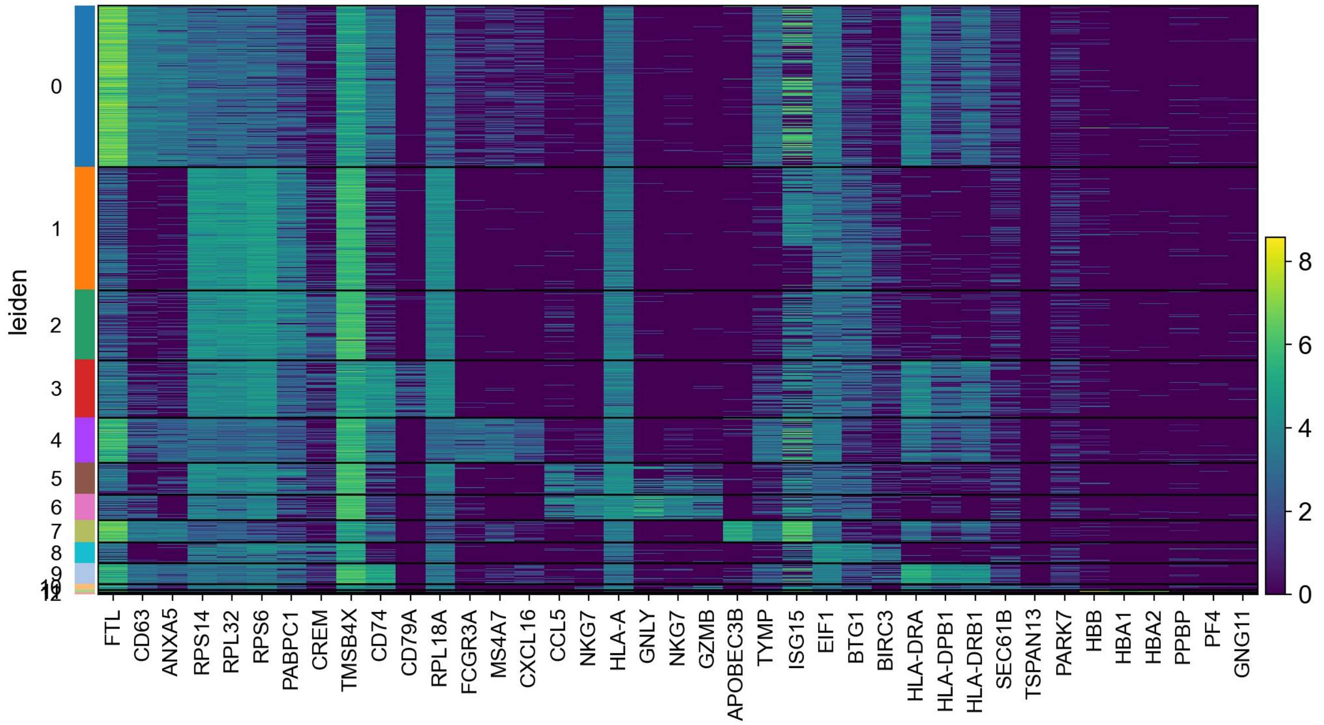
Scanpy-Identify markers for cells

```
sc.pl.heatmap(adata, genes, groupby='leiden')
```



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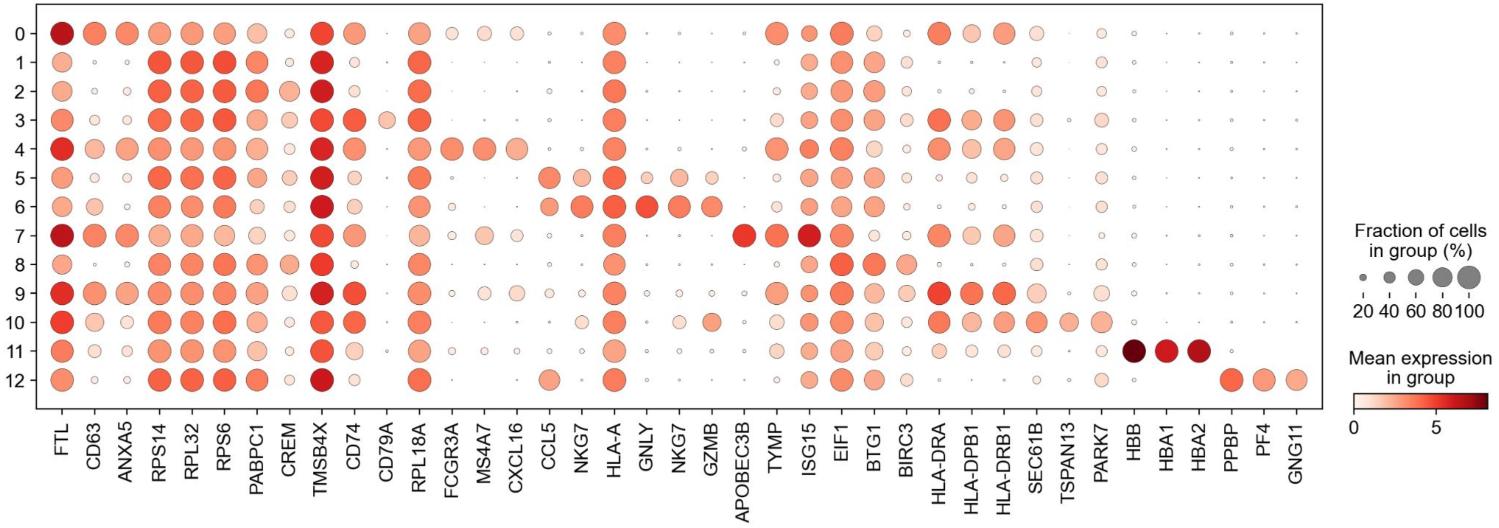
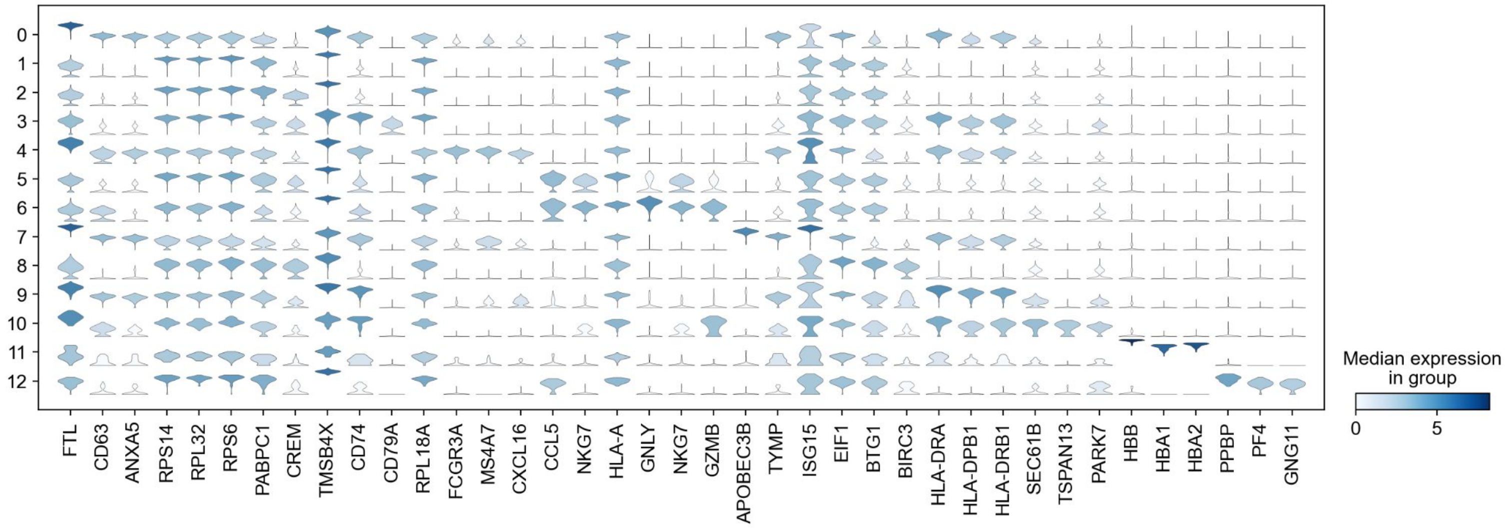
Scanpy-Identify markers for cells

```
sc.pl.dotplot(adata, genes, groupby='leiden')
```



Scanpy-Identify markers for cells

```
sc.pl.stacked_violin(adata, genes, groupby='leiden', rotation=90);
```



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