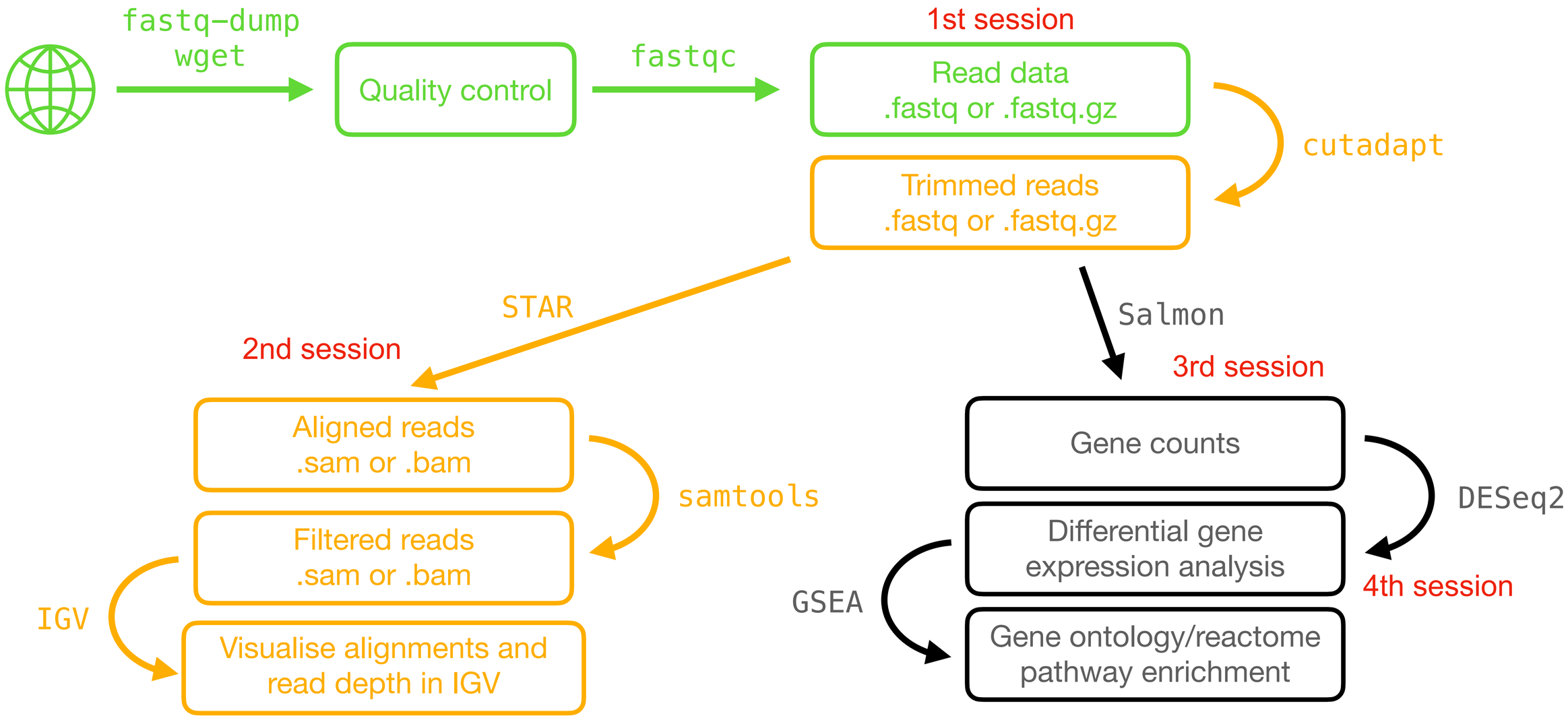
NGS workshop

Gene count using Salmon
3rd session
10th May 2023

Mirvat Surakhy and James Carrington

Overview of the course



Quantification of Gene expression

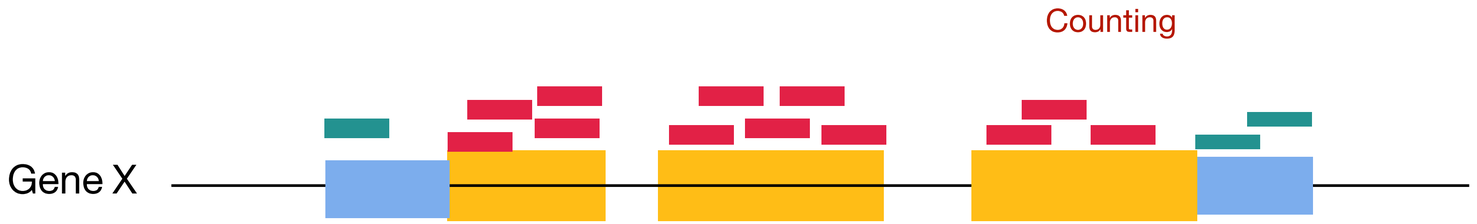
1. Gene level counting

- Reads aligned to the reference genome using transcriptome annotation (GTF) as guidance
  The goal is to identify the genomic location where these reads originated from.
- Tools:
  - FeatureCounts from Subread
  - Htseq-count
- output: raw count of reads that map to a single location
  i.e (the sum of reads associated with each of the exons (feature) that belong to that gene

Mapping to reference genome

Ref: AATCCTGGGAATTCGCGTTTAATTACGTTCCAA
Read: TTCGCGTTATTACGTTGCAA

Ref: AATCCTGGGAATTCGCGTTTAATTACGTTCCAA
Read: TTCGCGTT——TTACGTTGCAA



- Different transcripts of the same gene and alternative splicing are not accounted for.

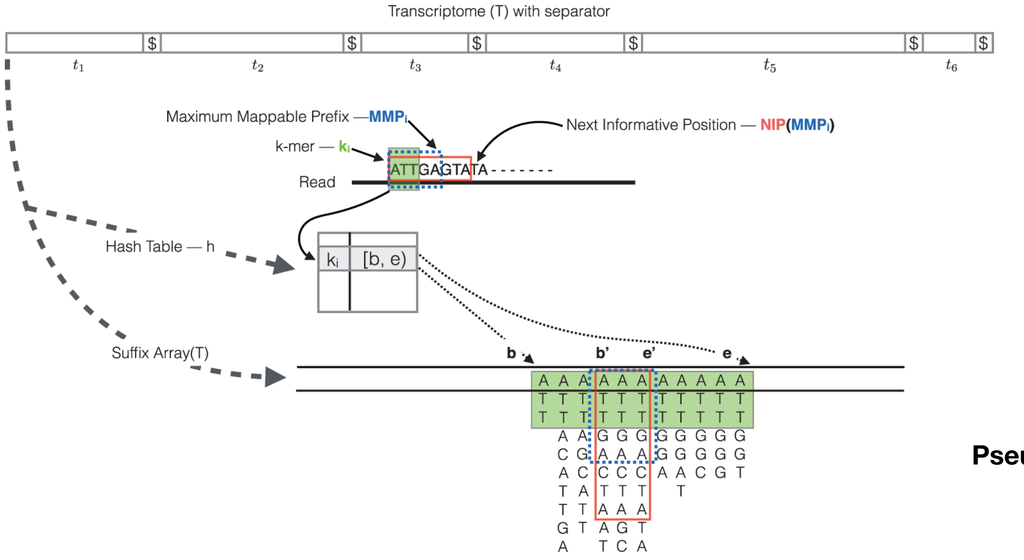
Quantification of Gene expression

2. Transcriptome mapping and counting

- Lightweight alignment tools (**Salmon**, Kallisto)
- Quasi-mapping or pseudo-alignment

  Index

  - Suffix array
  - A hash table (mapping each transcript to its location in the SA)



Pseudo-alignment

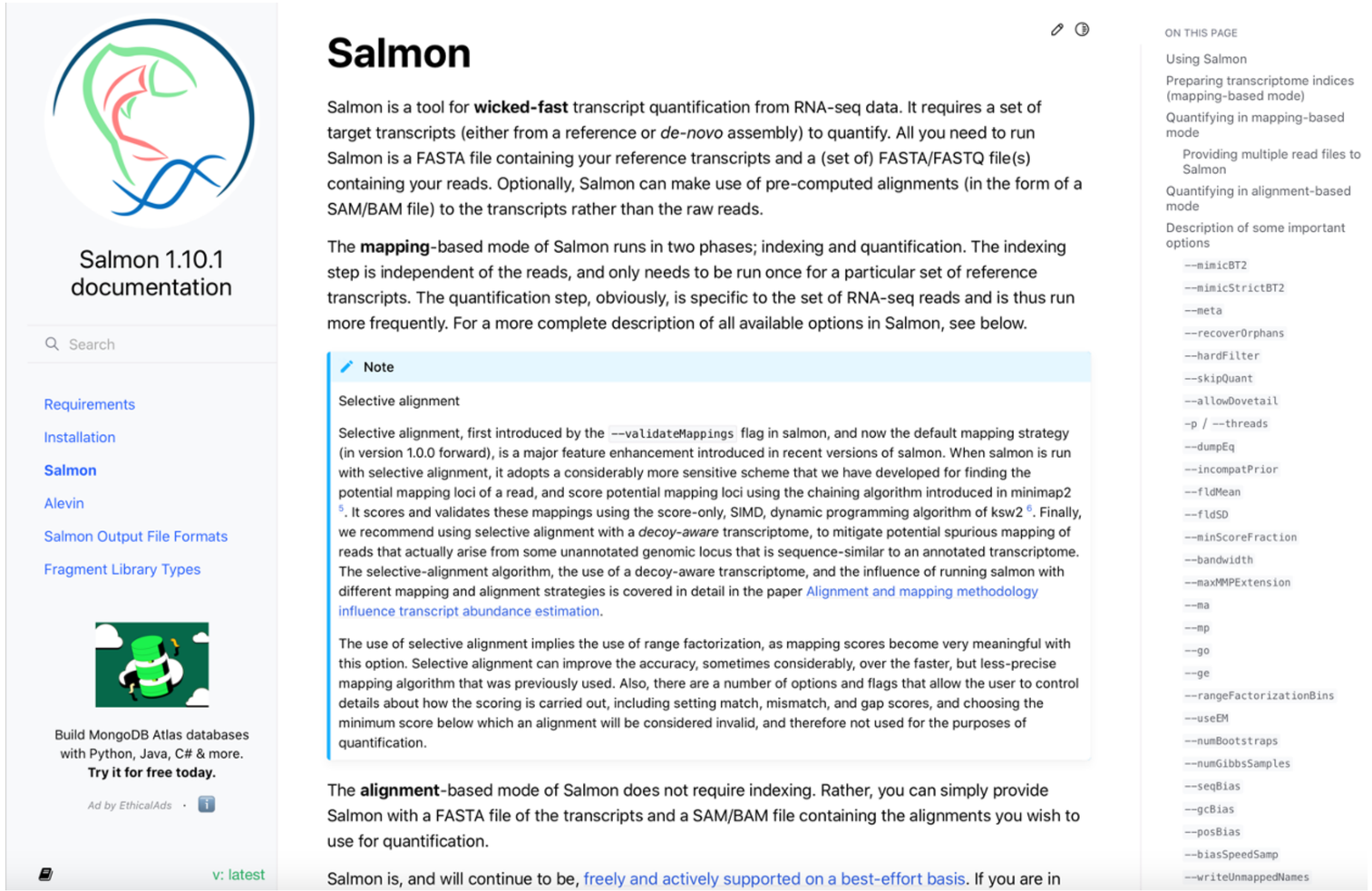
Srivastava A., Sarkar H., Gupta N., Patro R. Bioinformatics (2016) 32 (12): i192-i200.

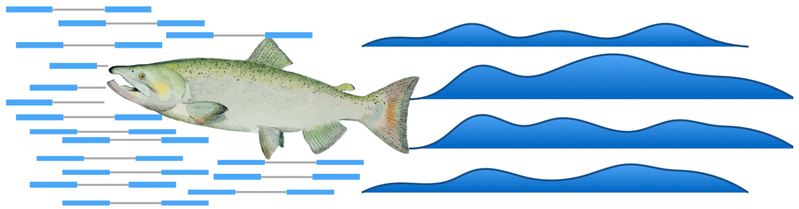
Quantification of Gene expression

2. Transcriptome mapping and counting

- Salmon utilizes sample-specific bias models for abundance estimation
- Improve the accuracy of gene expression
- Faster
- Output is a pseudo-count showing transcript expression estimates (abundance)
- Can be converted to raw gene counts for differential expression
- Cannot be used for variant calling and fusion detection

Salmon





https://combine-lab.github.io/salmon/

https://salmon.readthedocs.io/en/latest/salmon.html#using-salmon

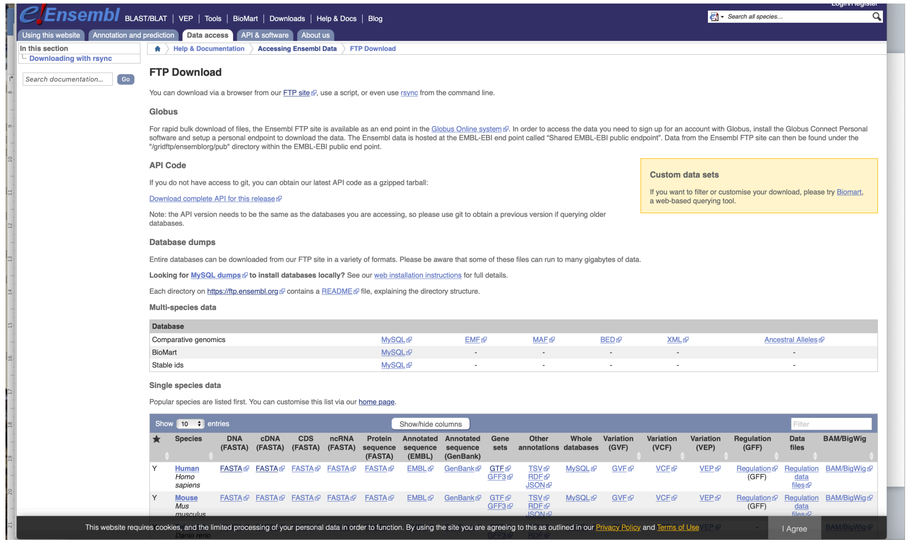
Patro, R., Duggal, G., Love, M. I., Irizarry, R. A., & Kingsford, C. (2017). Salmon provides fast and bias-aware quantification of transcript expression. Nature Methods.

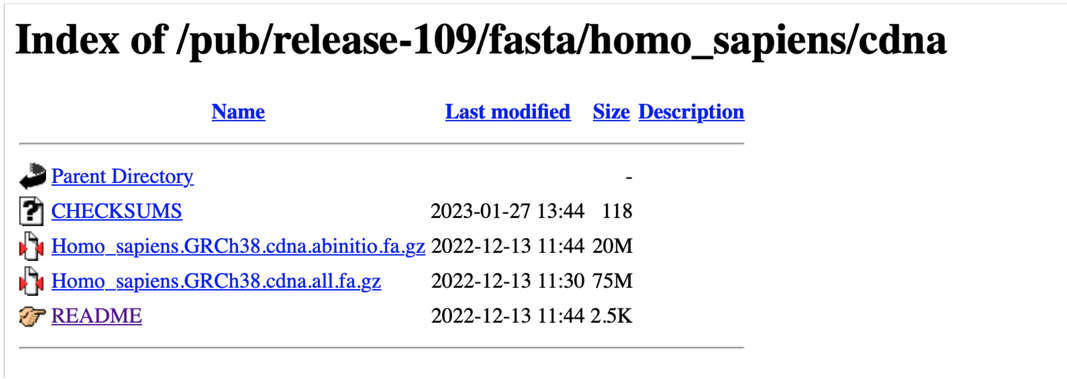
Practical

- Create an index to the transcriptome with Salmon (demo)
- Quantify transcript expression using Salmon
- Extract raw gene count in R using tximport

Salmon index… required files

- Files are from the Ensembl website https://www.ensembl.org/info/data/ftp/index.html





- wget ftp://ftp.ensembl.org/pub/release-109/fasta/homo_sapiens/cdna/Homo_sapiens.GRCh38.cdna.all.fa.gz

- wget ftp://ftp.ensembl.org/pub/release-109/fasta/homo_sapiens/dna/Homo_sapiens.GRCh38.dna.primary_assembly.fa.gz

Salmon Index …. Don’t run

Ready to use for the workshop: /mnt/beegfs/workshop/A_reference_files/salmon_index

```
  GNU nano 4.8                                New Buffer                                Modified
#!/bin/bash
#SBATCH --job-name=Salmon_Index
#SBATCH --cpus-per-task=6
#SBATCH --mem=10000
#SBATCH --output=outfile.%j
#SBATCH --error=errfile.%j

#this script generates Salmon Index

salmon index \
-t Homo_sapiens.GRCh38.cdna_dna.fa \
-i salmon_index \
--decoys decoys.txt

##-t: the path to the transcriptome and dna combined files (in FASTA format)
#-i: the path to the folder to store the indices generated
#-k: the length of kmer to use to create the indices. -k 31 is the default value.
#It is optimized for 75bp or longer reads
#--decoys: text file that lists the names of the chromosomes


^G Get Help  ^O Write Out  ^W Where Is  ^K Cut Text  ^J Justify  ^C Cur Pos  M-U Undo
^X Exit      ^R Read File  ^\ Replace   ^U Paste Text ^T To Spell ^_ Go To Line M-E Redo
```

Fastq files for DGE

```
$cd /mnt/beegfs/workshop/A_demo_files/
files_for_differentialExpression/fastq_files$ ls -lh
total 723M
-rw-rw-r-- 1 path1126 proudfoot 90M Apr 17 14:21 5aza_rep1_sample_1.fastq.gz
-rw-rw-r-- 1 path1126 proudfoot 91M Apr 17 14:21 5aza_rep1_sample_2.fastq.gz
-rw-rw-r-- 1 path1126 proudfoot 90M Apr 17 14:21 5aza_rep2_sample_1.fastq.gz
-rw-rw-r-- 1 path1126 proudfoot 91M Apr 17 14:21 5aza_rep2_sample_2.fastq.gz
-rw-rw-r-- 1 path1126 proudfoot 91M Apr 17 14:21 DMSO_rep1_sample_1.fastq.gz
-rw-rw-r-- 1 path1126 proudfoot 91M Apr 17 14:21 DMSO_rep1_sample_2.fastq.gz
-rw-rw-r-- 1 path1126 proudfoot 91M Apr 17 14:21 DMSO_rep2_sample_1.fastq.gz
-rw-r-w-r-- 1 path1126 proudfoot 91M Apr 1714:21 DMSO_rep2_sample_2.fastq.gz
```

Salmon quants for abundance estimation

```
 salmon quant -i location of the salmon index folder \
 -l A To allow Salmon to automatically infer the library type \
 -1 R1.fastq.gz \
 -2 R2.fastq.gz \
 -o specify the output directory \
 --seqBias \
 --gcBias \
 -p specifies the number of processors or cores we would like to use for
multithreading


#to optimize abundance estimates (more accurate)
#--seqBias --gcBias to correct for seq bias and gc bias
```

Salmon quant

```
$ cd /mnt/beegfs/workshop/<SSO>
$ mkdir -p results/salmon_quants
$ nano
```

```
  GNU nano 4.8                                New Buffer                                Modified
#!/bin/bash
#SBATCH --job-name=Salmon_quant
#SBATCH --cpus-per-task=8
#SBATCH --mem=24000
#SBATCH --output=outfile_salmon.%j
#SBATCH --error=errfile_salmon.%j

cd /mnt/beegfs/workshop/A_demo_files/files_for_differentialExpression/fastq_files

for file in *_1.fastq.gz; do

filename=$(basename "$file" _1.fastq.gz)  ###create a prefix called filename

 salmon quant -i /mnt/beegfs/workshop/A_reference_files/salmon_index \
 -l A \
 -1 ${filename}_1.fastq.gz \
 -2 ${filename}_2.fastq.gz \
 -o /mnt/beegfs/workshop/<SSO>/results/salmon_quants/${filename}_quant \
 --seqBias \
 --gcBias \
 -p 8

done

^G Get Help   ^O Write Out  ^W Where Is   ^K Cut Text   ^J Justify    ^C Cur Pos    M-U Undo
^X Exit       ^R Read File  ^\ Replace    ^U Paste Text ^T To Spell   ^_ Go To Line M-E Redo
```

- Save your script as salmon_quant.bash
- Run the script and check the status of the job
  - What module to use?
  - What command do you use to run the job?
  - What command do you use to check the status of your job?

- What module to use?
  - module avail ###optional for now
  - module load SALMON/1.9.0
- Run the script
  - sbatch salmon_quant.bash
- Check if the script is running
  - squeue -u **<SSO>**

Output of salmon quant

- When your job has finished navigate to the output directory

  cd /mnt/beegfs/workshop/<SSO>/results/salmon_quants

- View the content of the folder (what command do we use to list the files?)

quants file

```
$ ls -lh
drwxrwxr-x 5 jesu2166 hassan    6 May  5 12:10 5aza_rep1_sample_quant
drwxrwxr-x 5 jesu2166 hassan    6 May  5 12:15 5aza_rep2_sample_quant
drwxrwxr-x 5 jesu2166 hassan    6 May  5 12:19 DMSO_rep1_sample_quant
drwxrwxr-x 5 jesu2166 hassan    6 May  5 12:24 DMSO_rep2_sample_quant
```

Navigate to one of the folders

```
$ cd 5aza_rep1_sample_quant
$ ls -lh
total 12M
drwxrwxr-x 2 jesu2166 hassan  12 May  5 12:10 aux_info
-rw-rw-r-- 1 jesu2166 hassan 393 May  5 12:06 cmd_info.json
-rw-rw-r-- 1 jesu2166 hassan 575 May  5 12:10 lib_format_counts.json
drwxrwxr-x 2 jesu2166 hassan   1 May  5 12:10 libParams
drwxrwxr-x 2 jesu2166 hassan   1 May  5 10:59 logs
-rw-rw-r-- 1 jesu2166 hassan 12M May  5 12:10 quant.sf
```

View the content of the quant.sf

```
$less quant.sf
```

Name	Length	EffectiveLength	TPM	NumReads	
ENST00000631435.1		12	12.000	0.000000	0.000
ENST00000415118.1		8	7.000	0.000000	0.000
ENST00000448914.1		13	13.000	0.000000	0.000
ENST00000434970.2		9	9.000	0.000000	0.000
ENST00000632524.1		11	11.000	0.000000	0.000
ENST00000633009.1		20	19.000	0.000000	0.000
ENST00000634070.1		18	17.000	0.000000	0.000
ENST00000632963.1		20	19.000	0.000000	0.000
ENST00000633030.1		19	18.000	0.000000	0.000
.					
.					
.					
.					
ENST00000620516.4		1179	1145.496	12.961913	5.427

TPM: Transcripts Per Million

Press q to exit the less command

Extract the raw counts from the quants file

We will be using R and R studio

- **R**: programming language and environment for data manipulation, statistical computing, and graphical display
- **R:** Free and open source https://www.r-project.org/

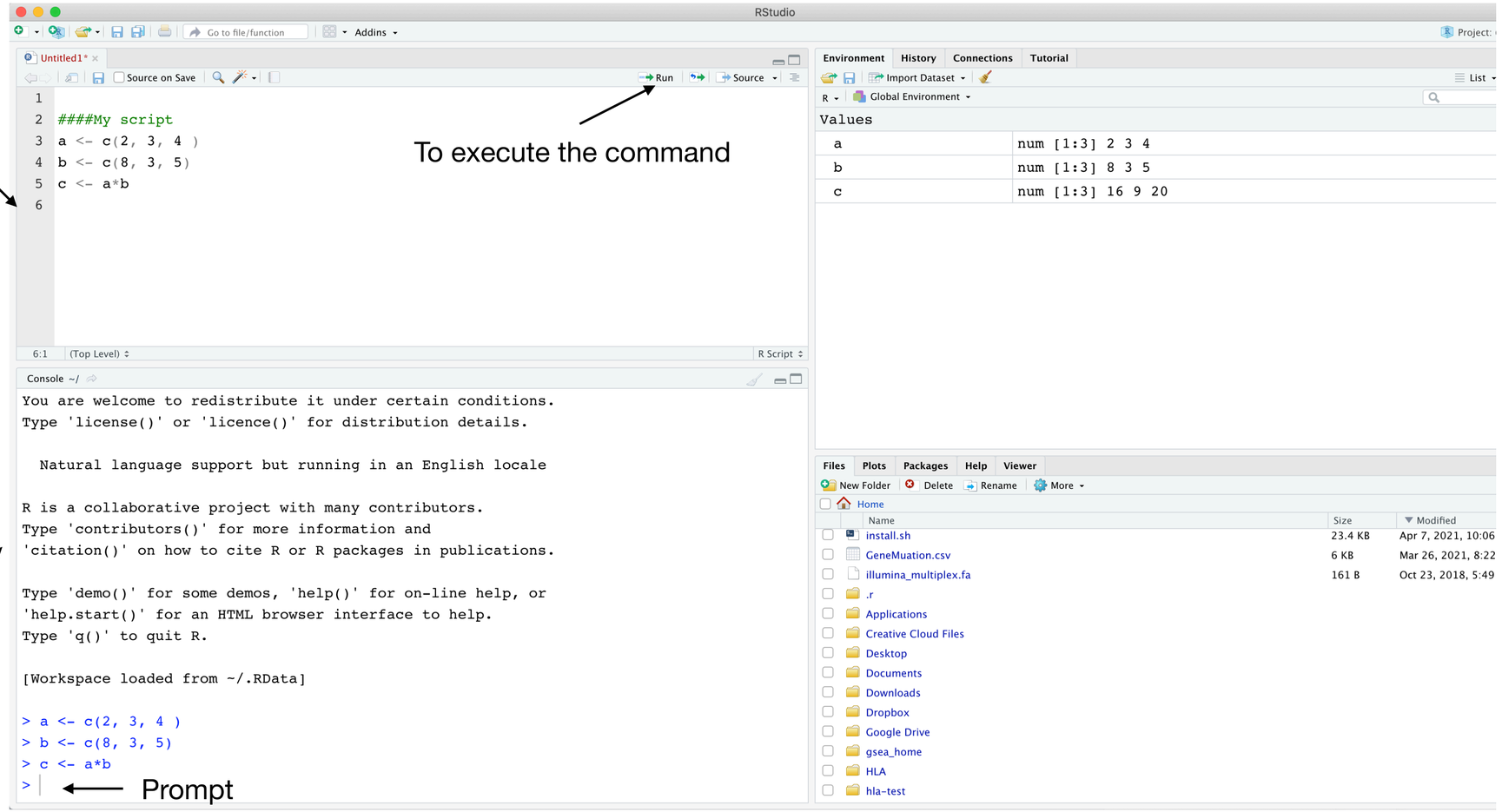
- **R studio:** Free and open source IDE (Integrated Development Environment) for R. Available for Mac, Windows and Linux

Rstudio interface

Script editor

To execute the command

Object, history and Enviroment



R Console

Tree of folders, graph window, Packages, help and Viewrt

Best practices when using R

- Document everything you do so your code is reproducible.
- Use the # sign to comment. In R anything after the # is ignored.
- Write the code in the script editor rather than R console.
- Keep track of all the libraries used in the analysis together with the R version.

R script to extract the raw count and save it

- Before running R we need to create an output directory to save the results
- cd /mnt/beegfs/workshop/**<SSO>**/
- mkdir /mnt/beegfs/workshop/**<SSO>**/results/counts
- Use the NoMachine
- $ srun -c 8 --mem 8000 --X11 --pty bash
- Are you sure you want to continue connecting (yes/no/[fingerprint])? yes
- SSO@linux020's password: Type your SSO password
- $ module load RSTUDIO TXIMETA/3.15 R_NGS_ANALYSIS/1.0
- $ rstudio

R script…. tximport.r

Open an R script

File > New_File> R Script

Save it in your working directory: File > save > tximport.r

```
###To install libraries in R, demo only, don't run  all done for this course ###########
#if (!require("BiocManager", quietly = TRUE))
# install.packages("BiocManager")
#BiocManager::install("tximport")
#install.packages("dplyr")
#Load the required libraries
library("tximport")
library("dplyr")
## assign your working directory
setwd ("/mnt/beegfs/workshop/<SSO>/results")
## List all directories containing data
samples <- list.files(path = "./salmon_quants", full.names = T, pattern="_sample_quant$")
## Obtain a vector of all filenames including the path
files <- file.path(samples, "quant.sf")
## list all the files to the console
files
##assign a shorter name for each element
names(files) <- list.files("salmon_quants")
```

R script…. tximport.r

```
## Read the annotation file

tx2gene <- read.csv("/mnt/beegfs/workshop/A_reference_files/tx2gene_ens109.csv")

# Run tximport

txi <- tximport(files, type="salmon", tx2gene=tx2gene[,c("TXNAME", "GENEID")], countsFromAbundance="lengthScaledTPM",
ignoreTxVersion = TRUE)

## Check the output

head(txi[["counts"]])

## Extract the counts, round the values and change the list to a data frame

counts <- txi$counts %>% round() %>% data.frame()

## Change the column names

colnames (counts) <- c("5aza_rep1", "5aza_rep2", "DMSO_rep1", "DMSO_rep2" )

View(counts)

### Save the results for the next session using the write.table command

write.table(counts, "counts/Count_fromtximport_Salmon.txt", sep ="\t", quote = F)

####keep a note of the packages and their versions

sessionInfo()
```

Before the end of this session

- Save your code
- Quite Rstudio
- cancel your job for the Rstudio session otherwise, your group will be charged as long as the job is running
- scancel job ID
- or use: scancel -u <SSO> #this will cancel all your jobs