Reproducible Workflows with R and Sequencing Data

A Beginner's Guide to transcriptomic analysis

Adrian Sven Geissler

7th of February 2024

Diverse target audience

Photo by Amy Elting on Unsplash

Presentation takeaways

- Define "*transcriptomic*" sequencing and its relevance
- Recognize structure of sequencing data
- Outline a simple analysis pipeline with bash
- Identify concept and advantages of a Snakemake workflow
  (How I avoid using bash as much as possible)
- Connect a workflow with R analysis scripts
- Introduction to software management across platforms
- Fun with 📦packages and R tweaks

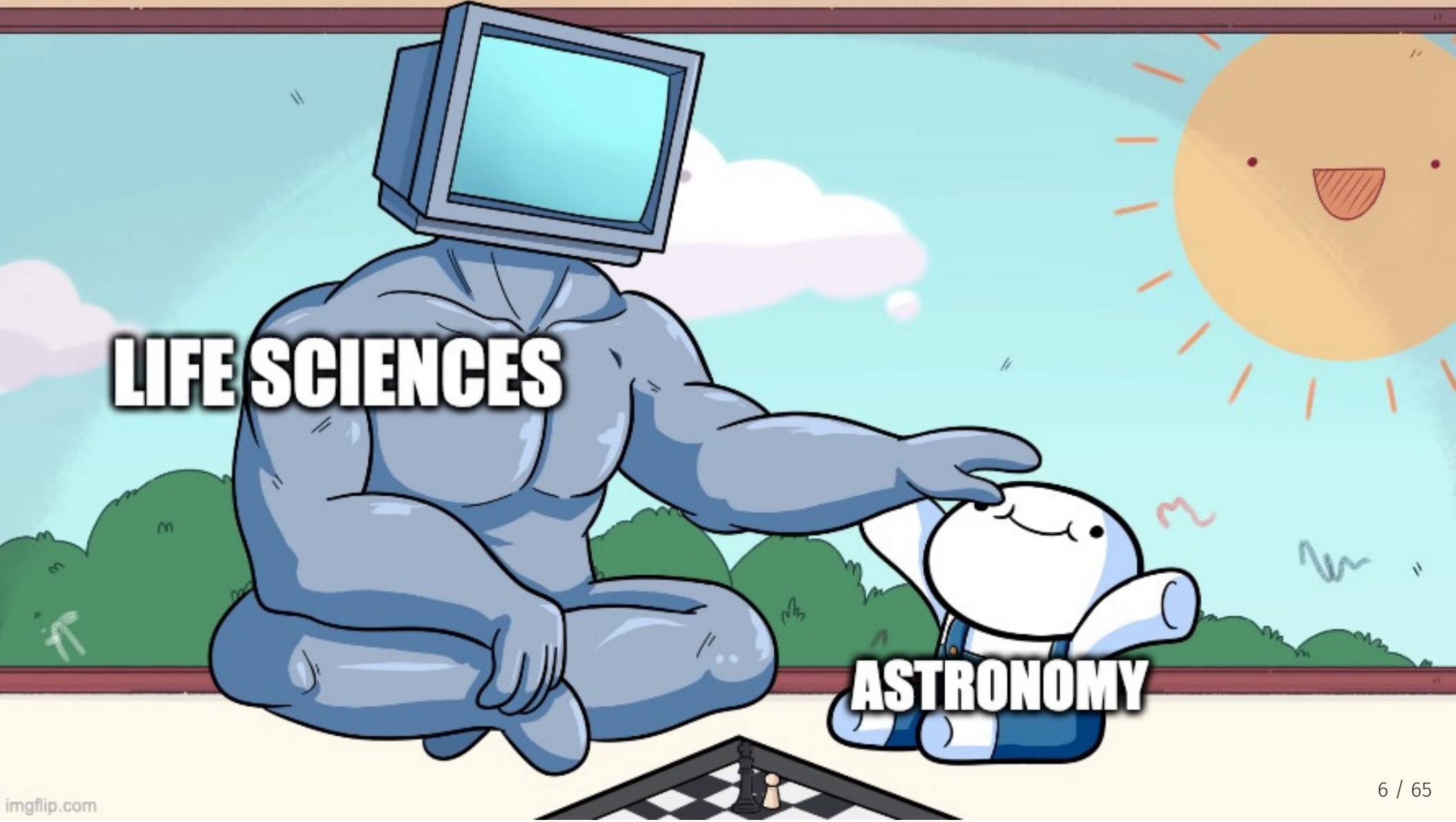
Slides:

https://github.com/asgeissler/2024-CopenhagenR-Seminar

Snakemake tutorial workflow:

https://github.com/asgeissler/2024-Snakemake-Intro

Photo by Greg Rakozy on Unsplash


LIFE SCIENCES
ASTRONOMY
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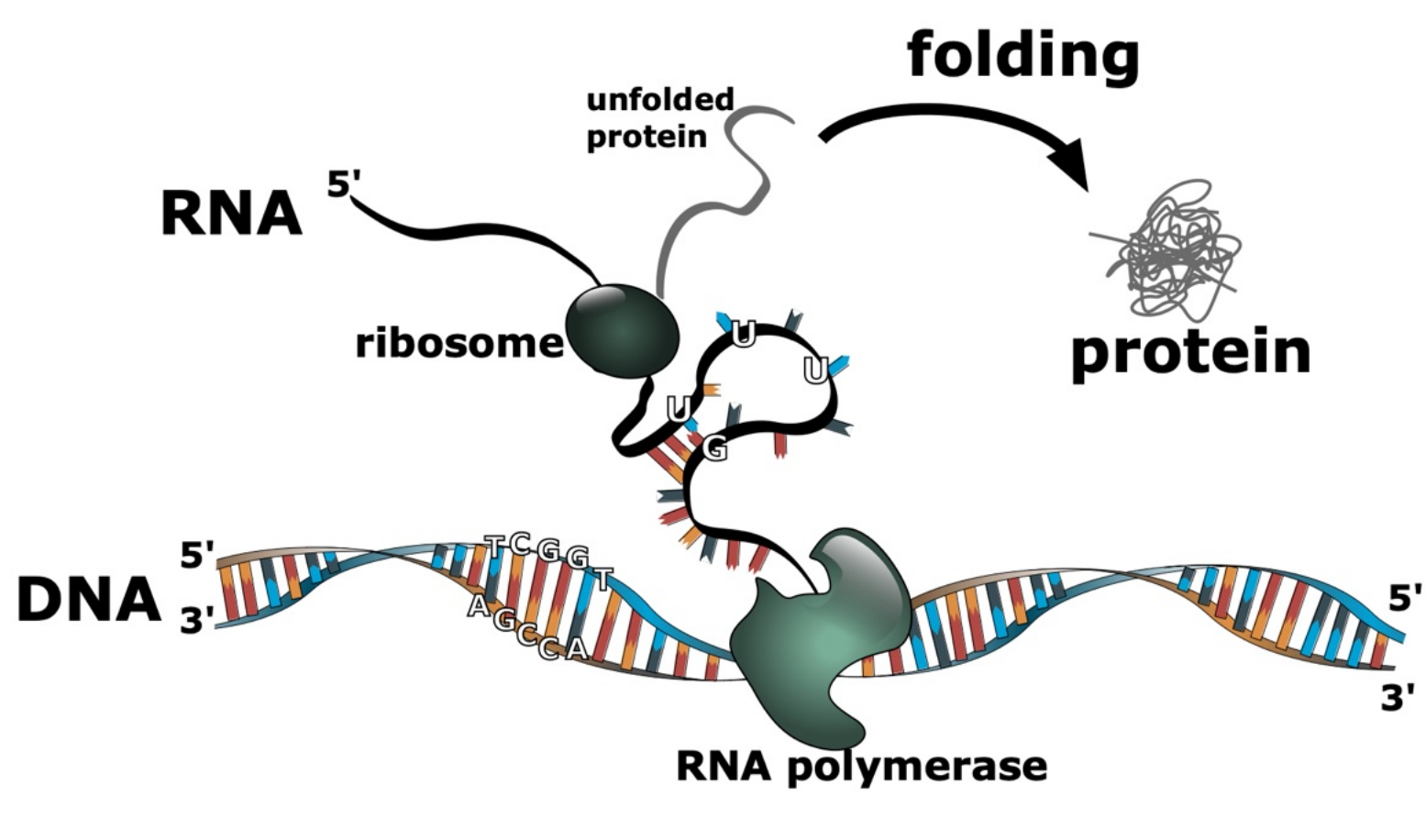
Big Data: Astronomical or Genomical?

Data Phase	Astronomy	Twitter	YouTube	Genomics
Acquisition	25 zetta-bytes/year	0.5–15 billion tweets/year	500–900 million hours/year	1 zetta-bases/year
Storage	1 EB/year	1–17 PB/year	1–2 EB/year	2–40 EB/year
Analysis	In situ data reduction	Topic and sentiment mining	Limited requirements	Heterogeneous data and analysis
	Real-time processing	Metadata analysis		Variant calling, ~2 trillion central processing unit (CPU) hours
	Massive volumes			All-pairs genome alignments, ~10,000 trillion CPU hours
Distribution	Dedicated lines from antennae to server (600 TB/s)	Small units of distribution	Major component of modern user's bandwidth (10 MB/s)	Many small (10 MB/s) and fewer massive (10 TB/s) data movement

doi:10.1371/journal.pbio.1002195.t001

Stephens, et al. PLOS Biology 2015

Gene expression: From genome, over RNA, to proteins



-ome suffix: *A totality of some sort*

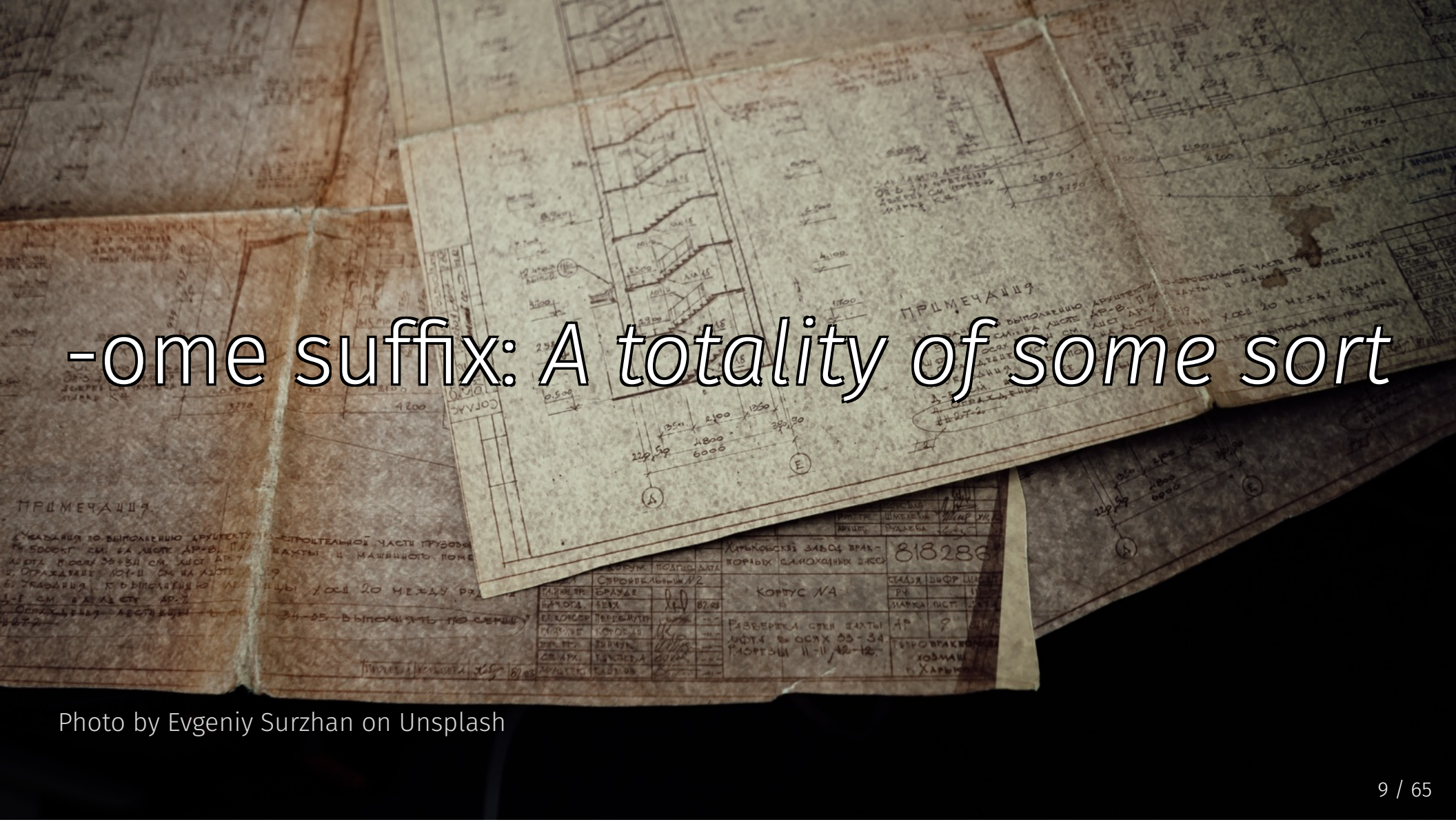


Photo by Evgeniy Surzhan on Unsplash

Differences in gene expression drive phenotypes

- ⚙️Cell tissue
- 🧬Genome
- 🌲Environment & 💊Exposure
- ☠️Disease associate factors

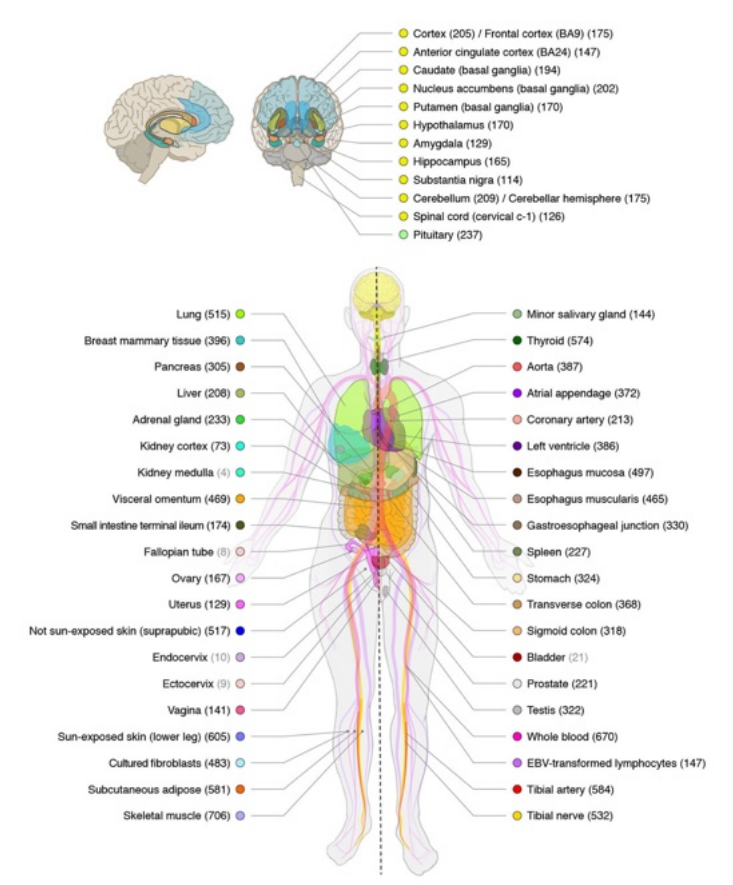


Figure from the GTEx Consortium, Science 2020

Transcriptomic analysis: What is happening in the cells?

Photo by Evgeniy Surzhan on Unsplash

Photo by Dave Weatherall on Unsplash

Photo by Barn Images on Unsplash

Biologist's preferred tool

Photo by Stephen Baker on Unsplash

The *pre*-R fun bottleneck

Photo by Nathan Dumlao on Unsplash

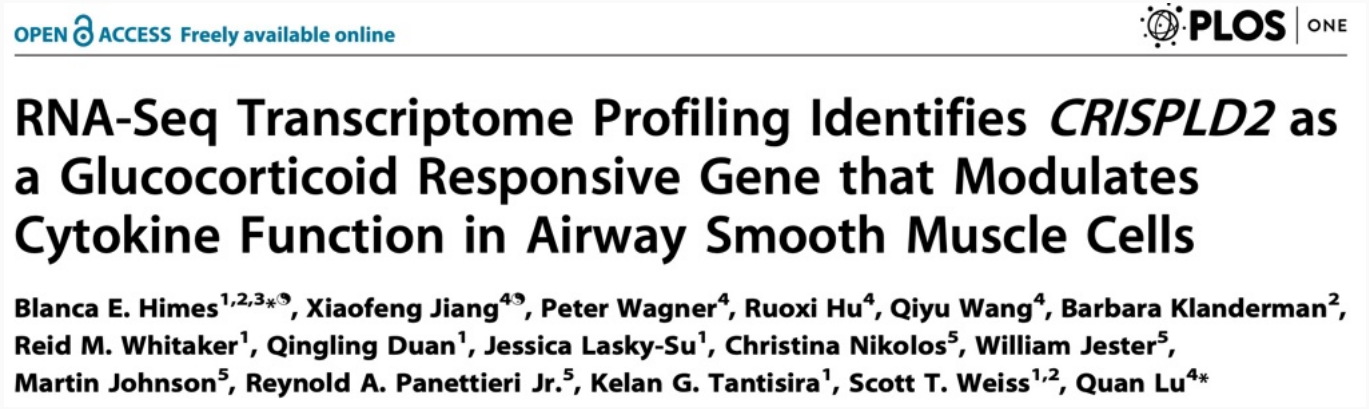
Biological *high-throughput* measurements

- RNA Sequencing data
- Terabytes of > 100 compressed txt's
- Specialized tools (bash scripts)
- Long running times

Intermediate result:
Count Matrix < 100 MB
Rows: Genes, Columns: Samples/Patients

Data exploration and analysis
(R scripts / notebooks)

Transcriptomic "airway" dataset from 2014



OPEN ACCESS Freely available online

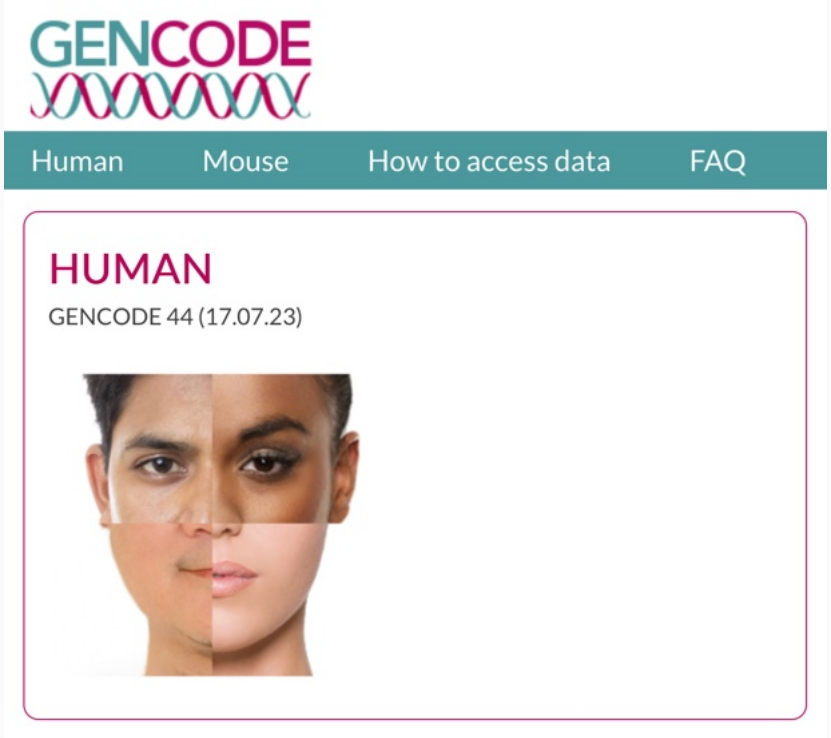
PLOS ONE

RNA-Seq Transcriptome Profiling Identifies *CRISPLD2* as a Glucocorticoid Responsive Gene that Modulates Cytokine Function in Airway Smooth Muscle Cells

Blanca E. Himes[1,2,3*], Xiaofeng Jiang[4], Peter Wagner[4], Ruoxi Hu[4], Qiyu Wang[4], Barbara Klanderman[2], Reid M. Whitaker[1], Qingling Duan[1], Jessica Lasky-Su[1], Christina Nikolos[5], William Jester[5], Martin Johnson[5], Reynold A. Panettieri Jr.[5], Kelan G. Tantisira[1], Scott T. Weiss[1,2], Quan Lu[4*]

- Freely and openly available (without ethics board)
- RNA sequencing datasets for Asthma drug treatment vs control
- 4 human cell lines (genotypes)

Downloading the human genome



https://www.gencodegenes.org/

Copy and paste links from website:

```
$ URL="https://ftp.ebi.ac.uk/pub/\
  databases/gencode/\
  Gencode_human/release_44/"

$ wget $URL/GRCh38.primary_assembly.genome.fa.gz \
  -O genome.fna.gz

$ wget $URL/gencode.v44.basic.annotation.gff3.gz \
  -O genome.gff.gz
```

🧬🕺 🧍‍♀️👶

806 MBytes compressed text; 3 GBytes uncompressed

```
$ gunzip -c genome.fna.gz| less
>chr1 1
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
 ...
TGCTCATGAAGTGTGAGTTAATGCACTCAAATCAATGGTTGTGCACGGTTTATATGAATA
TTAGTGATTACAAAATATTATCAATAGACCTTGTCACAACTGTTATTGAAGAACTAATCA
TCTATTGCTTATTTAGGTCTTTCTCTCCTGCCAGAATGTGCGCTCCAGGTGGAGAGGTAT
GTTGCCTTATCCGTGGCTGGATATATAGAGATTCCCACACTGCCTTGCACACGAGCACTG
CTGGGTAAATATTTGTTGGCTGCAGGAAAACGTGAAGGAATAGGCCCTCCAATGGGAGGA
 ...
>chr2 2
 ...
```

Gene annotations

34 MBytes compressed, 902 MBytes uncompressed

```
$ gunzip -c genome.gff.gz | less
##gff-version 3
#description: evidence-based annotation of the human genome (GRCh38), version 44 (Ensembl 110)
[ ... ]
chr1    HAVANA    gene    11869    14409    .    +    .    ID=ENSG00000290825.1;[ ... ];gene_name=DDX11L2;[ ... ]
...
```

Chromosome 1 has a gene at positions $11,869$ to $14,409$ on the $+1$ strand.

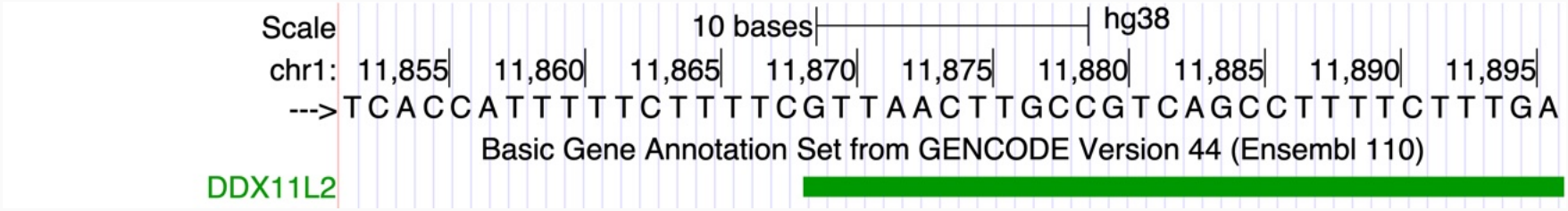


Figure from https://genome-euro.ucsc.edu

Dataset access: Sequence Read Archive

From publication text: *"The RNA-Seq data is available at..."*

https://trace.ncbi.nlm.nih.gov/Traces/?view=study&acc=SRP033351

Downloaded sample files

- Option 1: Using the custom `SRA Toolkit` (bash...)
- Option 2: Clicking on website

```
$ ls
raw-data    genome.fna.gz   genome.gff.gz

$ ls -1 raw-data
SRR1039508_1.fastq.gz   # (control sample)
SRR1039508_2.fastq.gz
SRR1039509_1.fastq.gz   # (asthma treated case)
SRR1039509_2.fastq.gz
...
```

In this dataset, the files are $3.3 - 7.3$ GBytes large
($0.9 - 1.7$ GBytes compressed)

Paired-end sequencing data

```
> ls -1 raw-data
SRR1039508_1.fastq.gz
SRR1039508_2.fastq.gz
...
```

The data contains the "*front*" and the "*back*" of the sequenced read

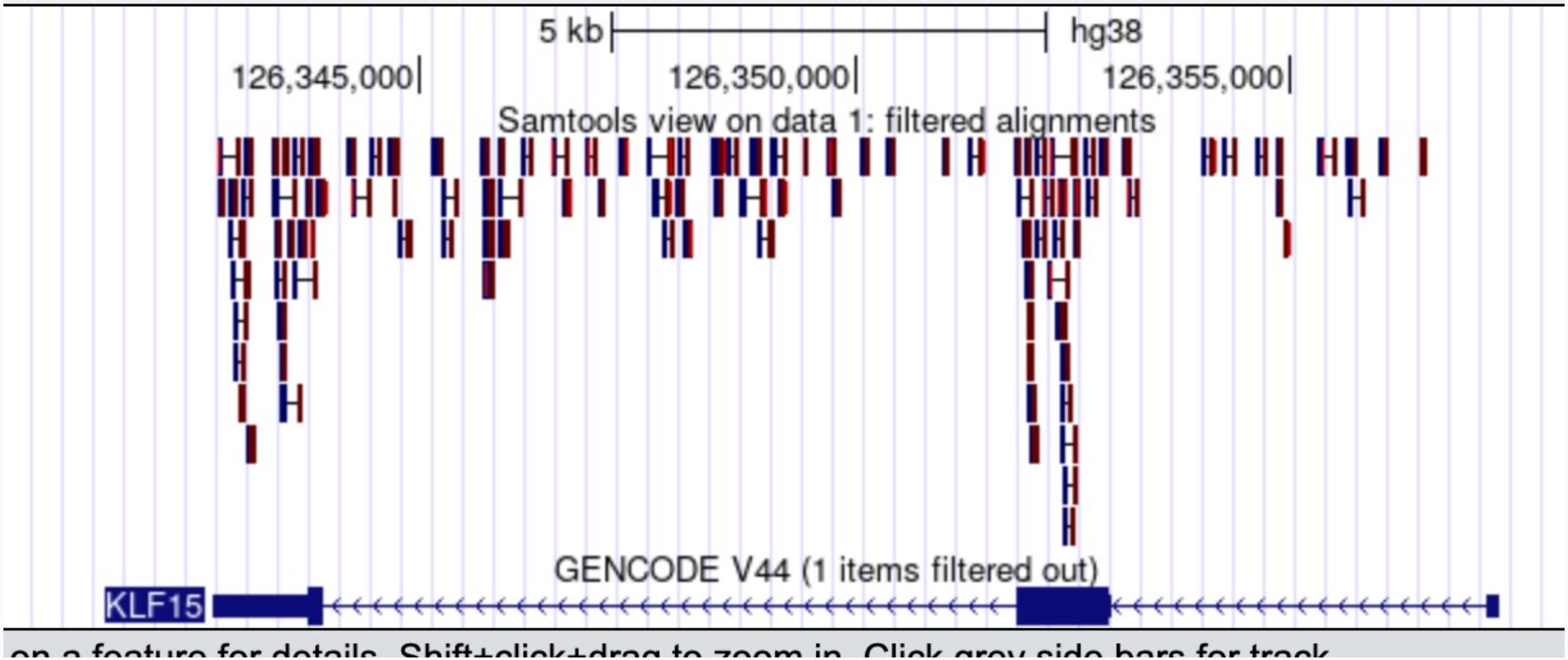
Photo by Taylor Smith on Unsplash

Lots of compressed text files

```
> gunzip -c data/SRR1039508_1.fastq.gz | head
@SRR1039508.1 HWI-ST177:290:C0TECACXX:1:1101:1225:2130 length=63
CATTGCTGATACCAANNNNNNNNGCATTCCTCAAGGTCTTCCTCCTTCCCTTACGGAATTACA
+SRR1039508.1 HWI-ST177:290:C0TECACXX:1:1101:1225:2130 length=63
HJJJJJJJJJJJJJJ########00?GHIJJJJJJJIJJJJJJJJJJJJJJJJJJHHHFFFFFD
 ...
```

That look like one of those ACTG genome thingies!

Goal: Number of reads mapping to a gene



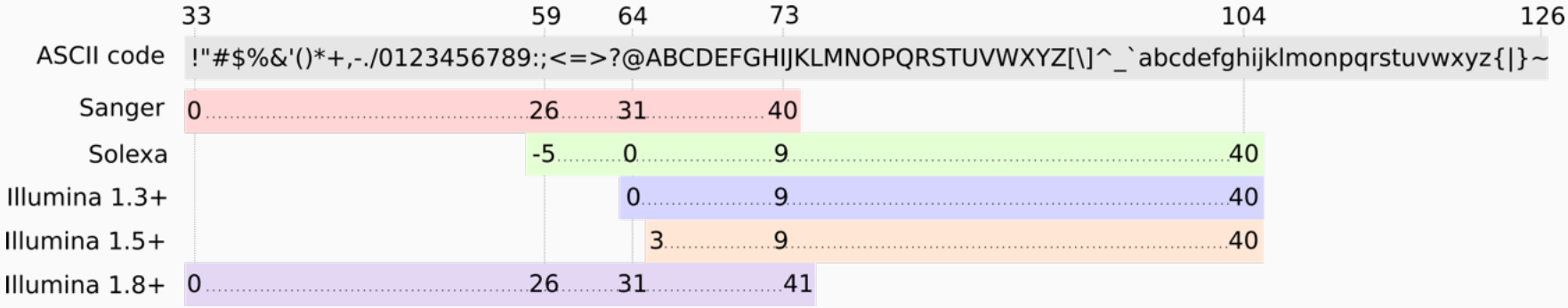

HERE ARE YOUR SEQUENCES
OH JUST
SIMPLE TEXT LOOKUP RIGHT?
imgflip.com
RIGHT?

Sequencing quality filtering is needed

$-10 \cdot log_{10}($ Probability of sequencing error $)$

Encoded as single, *printable ASCII* character

(details depend on the sequencing platform manufacturer)



https://usegalaxy.be/training-material//topics/metagenomics/tutorials/metatranscriptomics/slides-plain.html

Efficient sequence mapping algorithm is needed

- Short reads: < 100 nucleotides (characters)
- This dataset: $13 - 34$ million read pairs, per sample
- Human genome: > 3.1 billion base pairs (characters)

~~Naive string lookup~~

Efficient sequence mapping algorithms

1. Search via a phonebook-like index structure
   (*e.g.*, Burrows–Wheeler Transformation)
2. *Approximate* via sequence spectrum
   (*k*-mer frequency)

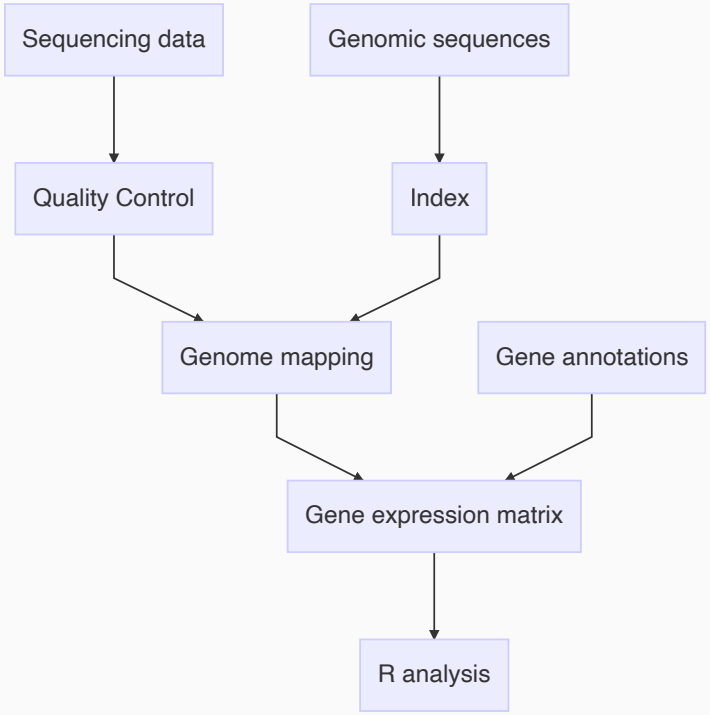
Photo by Johnny Briggs on Unsplash

Suggested reading:

Li, Durbin. Bioinformatics 2009 https://doi.org/10.1093/bioinformatics/btp324

Patro, *et al.* Nature Methods 2017 https://doi.org/10.1038/nmeth.4197

Outline processing pipeline



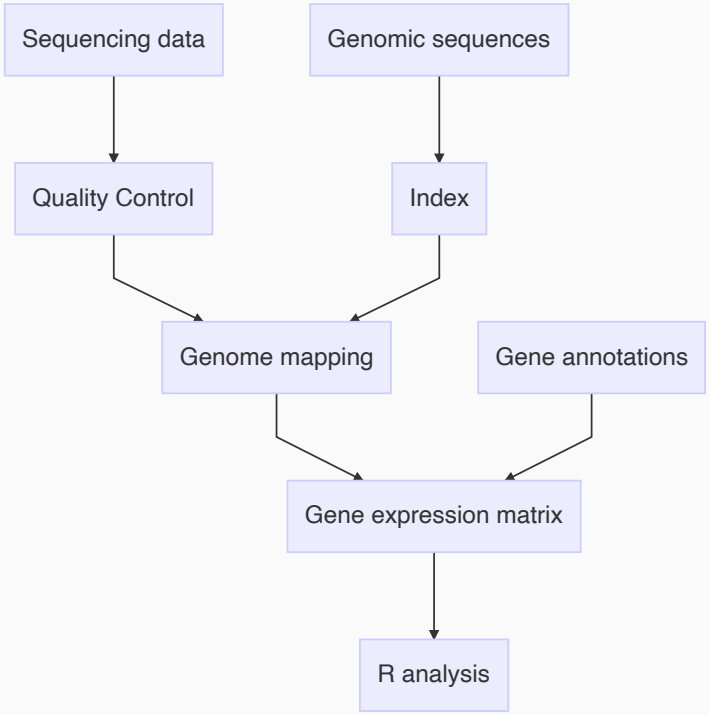
Bash programming primer

```
qcTool raw-data/sample1.fastq.gz > clean-data/sample1.fastq.gz
qcTool raw-data/sample2.fastq.gz > clean-data/sample2.fastq.gz
qcTool raw-data/sample3.fastq.gz > clean-data/sample3.fastq.gz
qcTool raw-data/sample4.fastq.gz > clean-data/sample4.fastq.gz
...
```

Slightly better solution

```
for i in raw-data/*fastq.gz ; do
  filename=$(basename $i)
  qcTool $i > clean-data/$filename
done
```

Conceptual bash pipeline



A file `concept-pipeline.bash`

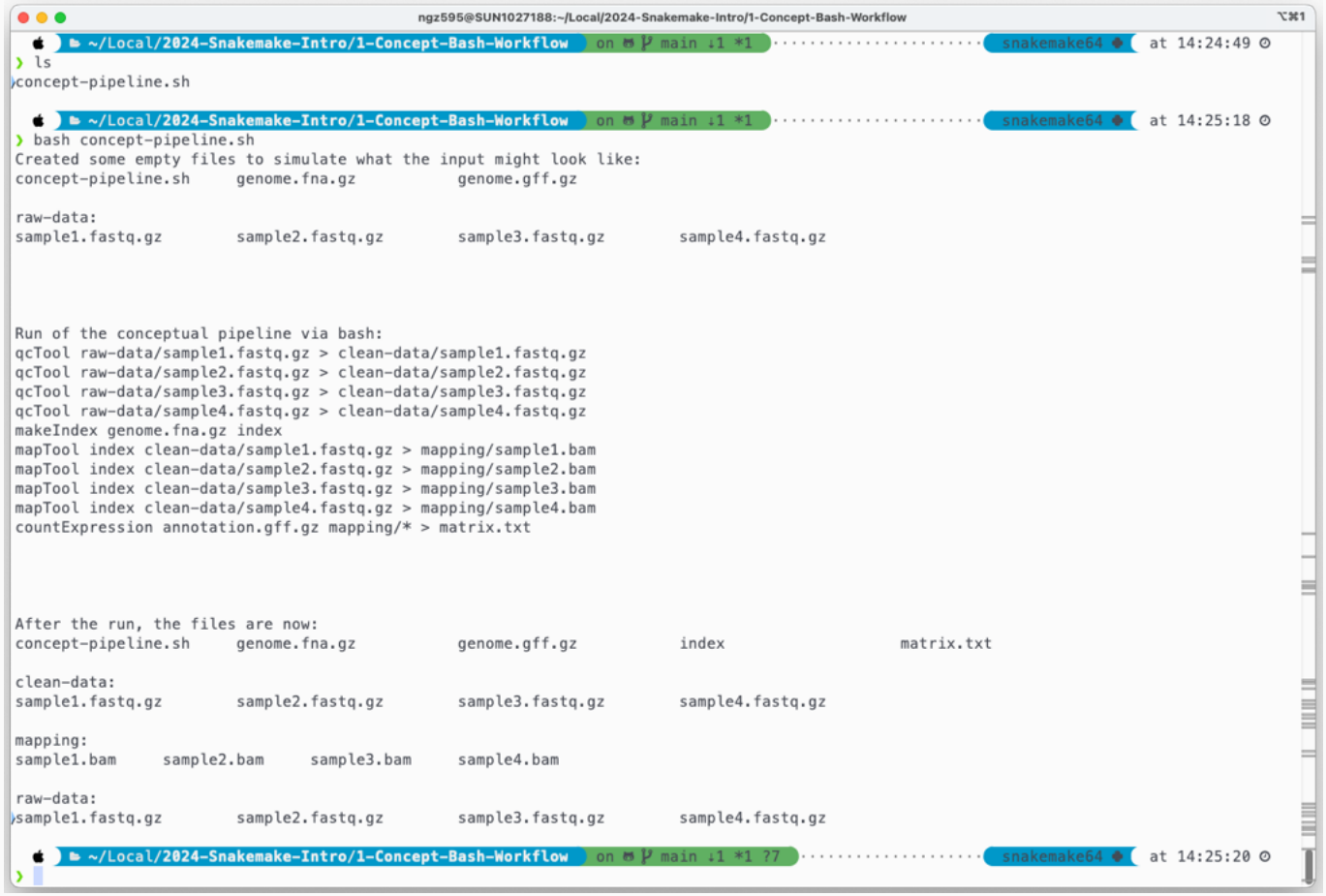
```
for i in raw-data/*.fastq.gz ; do
  filename=$(basename $i)
  qcTool $i > clean-data/$filename
done

makeIndex genome.fna.gz index

for i in clean-data/*.fastq.gz ; do
  filename=$(basename $i .fastq.gz)
  mapTool index $i > mapping/$filename.bam
done

countExpression annotation.gff.gz mapping/*  \
  > matrix.txt
```

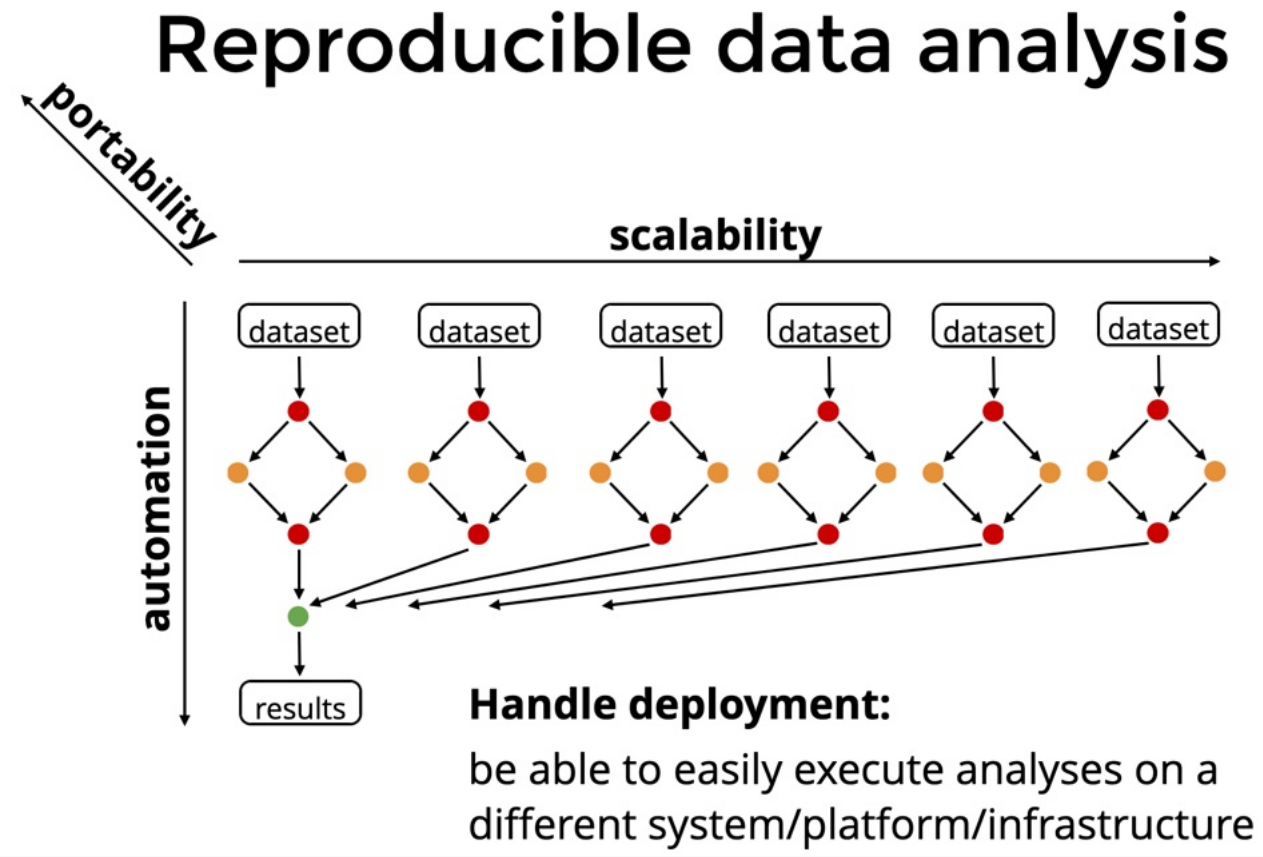
https://github.com/asgeissler/2024-Snakemake-Intro/



Bash concerns

- Adding new samples? Re-run pipeline & over-write files?
- Running steps in parallel?
- Consistency when changing parameters?
- Software management?

Snakemake to the rescue



Slide by Johannes Köster https://slides.com/johanneskoester/snakemake-tutorial

Towards Snakemake 👨‍🍳"cooking" rules 📚🍳

```
$ cat Snakefile

rule make_my_index:
    input:
        'genome.fna.gz'
    output:
        'index'
    shell:
        """
        makeIndex {input} {output}
        """
```

`snakemake --cores all make_my_index`

or

`snakemake --cores all index`

Wildcard placeholders

```
rule qc_cleaning:
    input:
        'raw-data/{sample}.fastq.gz'
    output:
        'clean-data/{sample}.fastq.gz'
    shell:
        """
        qcTool {input} > {output}
        """
```

```
$ snakemake --cores all clean-data/sample1.fastq.gz
Select jobs to execute...
rule qc_cleaning:
    input: raw-data/sample1.fastq.gz
    output: clean-data/sample1.fastq.gz
    jobid: 1
    reason: Missing output files: clean-data/sample1.fastq.gz
    wildcards: sample=sample1
```

Rules with list and named parameters

```
xs = ['sample1', 'sample2', 'sample3', 'sample4']

rule count_expression:
    input:
        genome = 'genome.gff.gz',
        mapping = expand('mapping/{sample}.bam', sample = xs)
    output:
        'matrix.txt'
    shell:
        """
        echo "I am the named genome input: {input.genome}"
        echo "We are the input mappings: {input.mapping}"
        echo "I am the first mapping: {input.mapping[0]}"

        countExpression {input} > {output}
        """
```

```
$ snakemake --cores all matrix.txt
...
I am the named genome input: genome.gff.gz
We are the input mappings: mapping/sample1.bam mapping/sample2.bam mapping/sample3.bam ...
I am the first mapping: mapping/sample1.bam
```

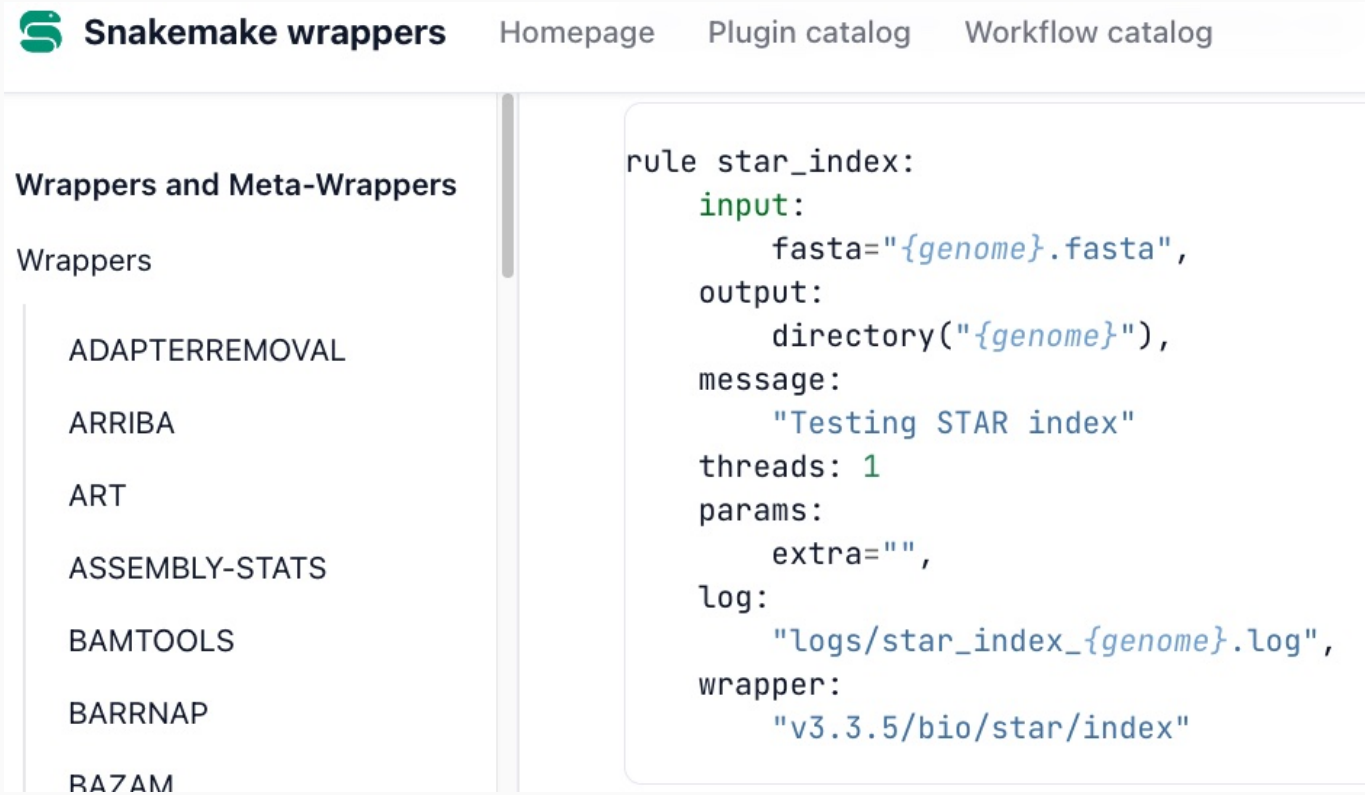
```
$ qcTool
bash: qcTool: command not found
```

What are the actual tools, and how to use them?

Thank you for this short intro to bash...

But: Can we do with less bash coding, please?

Snakemake wrappers



https://snakemake-wrappers.readthedocs.io/en/stable/wrappers/star/index.html

📦 Automatic dependencies resolution

When running with

```
snakemake --use-conda
```

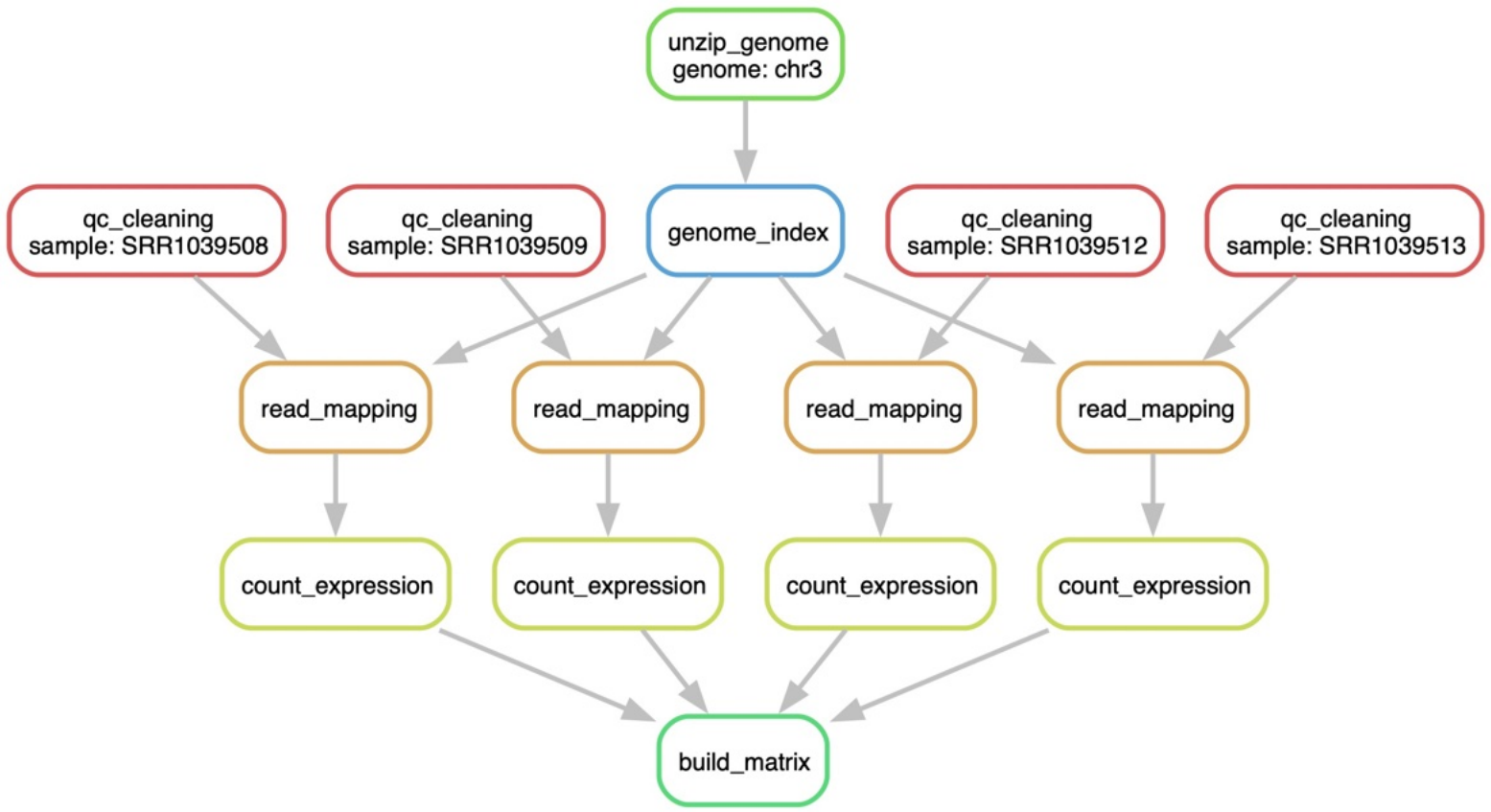
the software dependencies will be automatically deployed into an isolated environment before execution.

Software dependencies

- `star=2.7.11a`

https://snakemake-wrappers.readthedocs.io/en/stable/wrappers/star/index.html

Tutorial workflow



Tutorial workflow

```
$ git clone https://github.com/asgeissler/2024-Snakemake-Intro/
$ cd 3-Tutorial-Workflow
$ snakemake --cores all --use-conda --conda-frontend=mamba \
    gene-expression-matrix.tsv
```

Should run in < 15 min on a notebook

```
$ head -n 3 gene-expression-matrix.tsv
Geneid            Chr  Start End        Strand Length SRR1039508 SRR1039509 SRR1039512 SRR1039513
ENSG00000223587.2 chr3 11745 24849          +   13105  0           1         0          0
ENSG00000224918.1 chr3 53348 54346          -   999    0           0         0          0
```

```
$  grep ENSG00000163884.4 gene-expression-matrix.tsv
ENSG00000163884.4  chr3 126342635 126357408 - 14774    220         3384      128        1518
```

The end of the *pre*-R fun bottleneck

Intermediate result:

Count Matrix < 100 MB

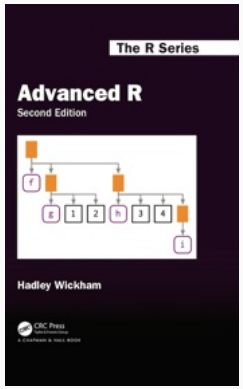
Rows: Genes, Columns: Samples/Patients

Data and software from Bioconductor

https://bioconductor.org/

"The mission of the Bioconductor project is [...] **open source** software [...] rigorous and **reproducible** analysis [...] welcoming **community** of developers and data scientists."

*"The Bioconductor community is a long-term user of **S4** and has produced much of the best material about its **effective use**. "*
— Hadley Wickham



{airway} 

```
install.packages("BiocManager")
BiocManager::install("airway")
```

```
library(airway)
data(airway)
airway
```

```
## class: RangedSummarizedExperiment
## dim: 63677 8
## metadata(1): ''
## assays(1): counts
## rownames(63677): ENSG00000000003 ENSG00000000005 ... ENSG00000273492
##   ENSG00000273493
## rowData names(10): gene_id gene_name ... seq_coord_system symbol
## colnames(8): SRR1039508 SRR1039509 ... SRR1039520 SRR1039521
## colData names(9): SampleName cell ... Sample BioSample
```

{airway} 

Memo R pipes: `g(f(x))` is `x ▷ f() ▷ g()`
(`▷` ligature, typed `|>`)

```
airway ▷
  assay() ▷
  as.matrix() ▷
  _[1:5, 1:5]
```

```
##                 SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516
## ENSG00000000003        679        448        873        408       1138
## ENSG00000000005          0          0          0          0          0
## ENSG00000000419        467        515        621        365        587
## ENSG00000000457        260        211        263        164        245
## ENSG00000000460         60         55         40         35         78
```

The full matrix of gene (row) expression levels per sample (columns) from the *pre-R* fun step.

Sample meta information

`colData`: Column meta information data

```
airway ▷ colData() ▷ as_tibble() ▷ head(n = 3) ▷ kable()
```

SampleName	cell	dex	albut	Run	avgLength	Experiment	Sample	BioSample
GSM1275862	N61311	untrt	untrt	SRR1039508	126	SRX384345	SRS508568	SAMN02422669
GSM1275863	N61311	trt	untrt	SRR1039509	126	SRX384346	SRS508567	SAMN02422675
GSM1275866	N052611	untrt	untrt	SRR1039512	126	SRX384349	SRS508571	SAMN02422678

Asthma drug treated (`trt`) or control (`untrt`)

`kable()` from {knitr} 📦makes pretty tables in these slides
S4 class: `colData(airway)` accessed the object's slot `airway@colData`

🧬🙋 Our Genes

```
airway ▷
  rowData() ▷ as_tibble() ▷
  select(gene_id, gene_name, chromosome = seq_name,
         gene_seq_start, gene_seq_end, seq_strand) ▷
  filter(chromosome == 'X') ▷
  arrange(gene_seq_start) ▷
  mutate_if(is.numeric, prettyNum, big.mark = ',') ▷
  head(n = 3) ▷ kable()
```

gene_id	gene_name	chromosome	gene_seq_start	gene_seq_end	seq_strand
ENSG00000228572	LL0YNC03-29C1.1	X	170,410	171,758	1
ENSG00000182378	PLCXD1	X	192,989	220,023	1
ENSG00000178605	GTPBP6	X	220,025	230,886	-1

tibbles are the better dataframes {tidyverse} 📦🧹

Tidy data exploration

```
# One  dexamethasone drug treated library
my.data ←
  airway ▷
  assay() ▷
  as_tibble(rownames = 'gene_id') ▷
  select(gene_id, expression = SRR1039509)
my.data ▷ head(n = 3) ▷ kable()
```

gene_id	expression
ENSG00000000003	448
ENSG00000000005	0
ENSG00000000419	515

```
my.genes ←
  airway ▷
  rowData() ▷
  as_tibble() ▷
  select(gene_id, gene_name, chromosome = sec
         gene_seq_start, gene_seq_end, seq_st
my.genes ▷ str()
```

```
## tibble [63,677 × 6] (S3: tbl_df/tbl/data.frame)
##  $ gene_id       : chr [1:63677] "ENSG00000000
##  $ gene_name     : chr [1:63677] "TSPAN6" "TNMD
##  $ chromosome    : chr [1:63677] "X" "X" "20" "
##  $ gene_seq_start: int [1:63677] 99883667 99839
##  $ gene_seq_end  : int [1:63677] 99894988 99854
##  $ seq_strand    : int [1:63677] -1 1 -1 -1 1 -
```

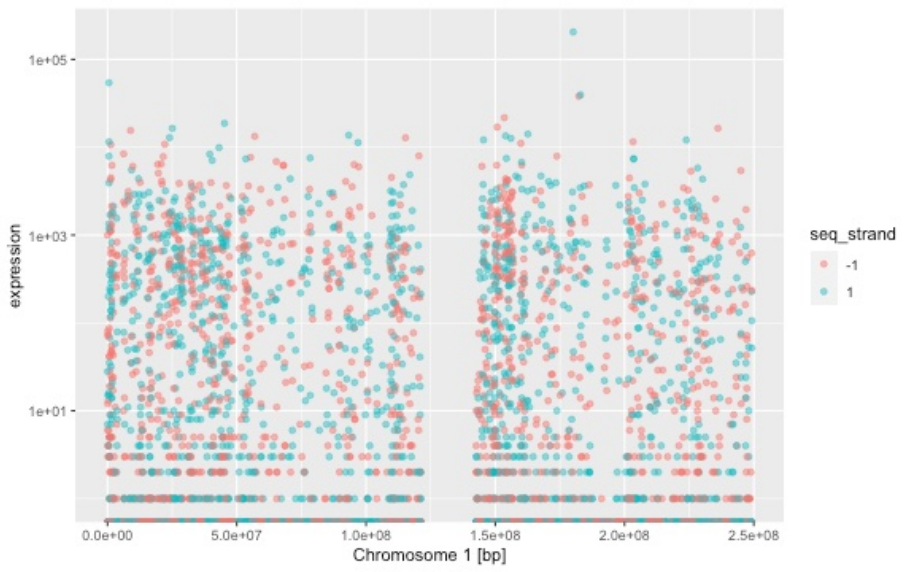
Use the join!

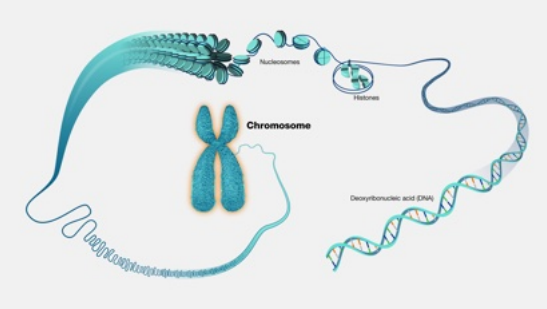
```
my.genes.data ← full_join(
  my.genes,
  my.data,
  by = 'gene_id'
)
```

gene_id	gene_name	chromosome	gene_seq_start	gene_seq_end	seq_strand	expression
ENSG00000000003	TSPAN6	X	99883667	99894988	-1	448
ENSG00000000005	TNMD	X	99839799	99854882	1	0
ENSG00000000419	DPM1	20	49551404	49575092	-1	515

A first genomic expression figure with {ggplot2}

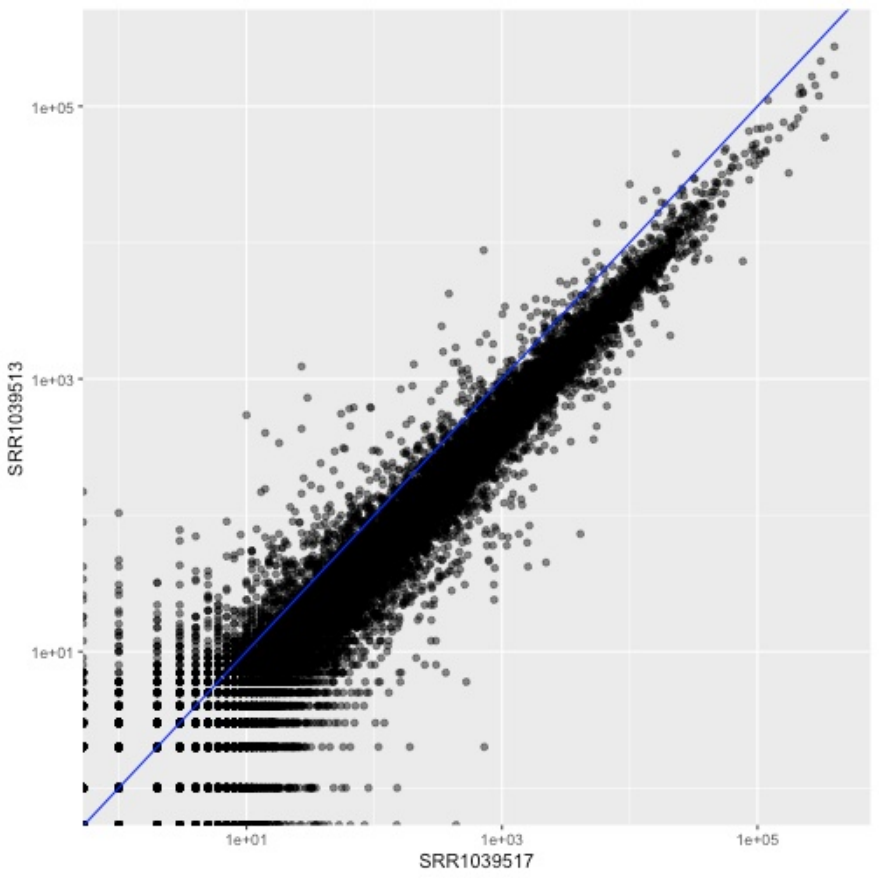
```
plot1 ←
  my.genes.data ▷
  filter(chromosome == '1') ▷
  mutate_at('seq_strand',
            as.character) ▷
  ggplot(aes(
    x = gene_seq_start,
    y = expression,
    color = seq_strand),
  ) +
  geom_point(alpha = .5) +
  scale_y_log10() +
  xlab('Chromosome 1 [bp]')
```





Motivation normalization

```
scatter.plot ←
  airway ▷
  assay() ▷
  as_tibble() ▷
  ggplot(aes(x = SRR1039517,
             y = SRR1039513)) +
  geom_point(alpha = 0.5) +
  geom_abline(slope = 1,
              color = 'blue') +
  scale_x_log10() +
  scale_y_log10()
```



Which waterway has more fish?

Photos by Mega Caesaria and Rick Wallace on Unsplash

RNA-seq data normalization

Potential method, size-factor normalization:

Per library (column) compute the median ratio of gene counts relative to geometric mean per gene (row)

Suggested reading:

Anders S and Huber W. Differential expression analysis for sequence count data. Nature Preceding 2010

RNA-seq analysis workflow tutorial on Bioconductor:

https://bioconductor.org/packages/release/workflows/vignettes/rnaseqGene/inst/doc/rnaseqGene.html

Differential expression analysisw with {DESeq2}

```
library(DESeq2)

des ←
  airway ▷
  # convert to DataSet for DESeq
  # analyse expression by cell line and dex treatment
  DESeqDataSet(design = ~ cell + dex) ▷
  # Run DESeq2 normalization and regression models
  DESeq()

# Statistical results
res ← results(des, tidy = TRUE)
```

RNA-seq analysis workflow tutorial on Bioconductor:

https://bioconductor.org/packages/release/workflows/vignettes/rnaseqGene/inst/doc/rnaseqGene.html

Statistical analysis of expression changes

$$\text{Expression Fold Change} = \frac{\text{Expression in condition A}}{\text{Expression in condition B}}$$

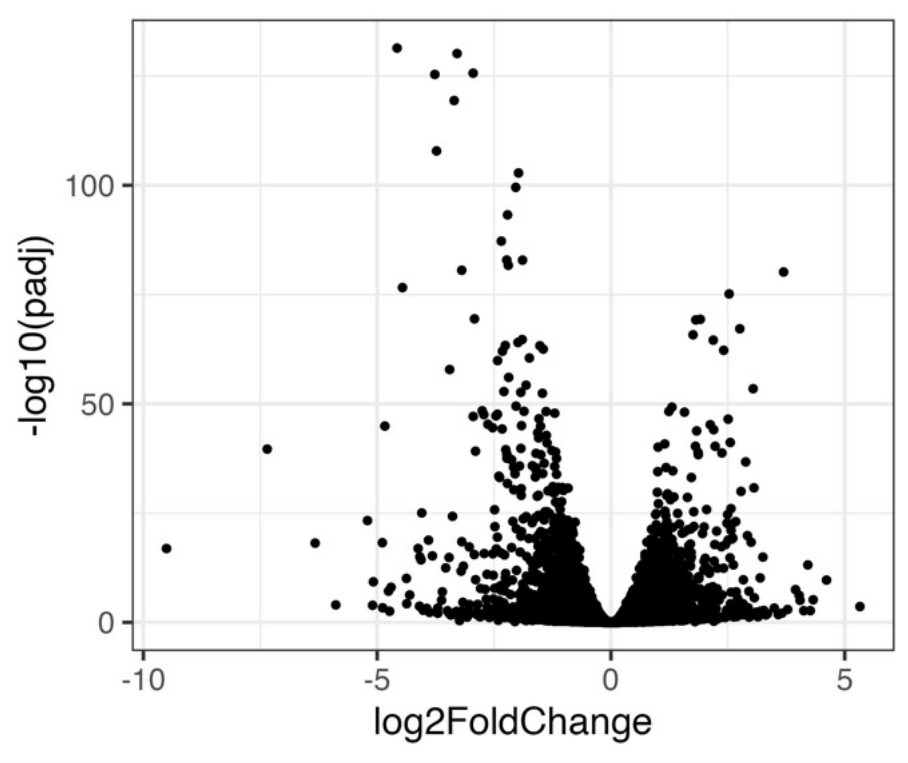
A	B	Fold Change	log2
100	100	1.0	0
100	50	2.0	1
100	200	0.5	-1

Testing for statistical significance and false discovery rate (FDR) adjustment

Analysis results

```
res ▷
  ggplot(aes(log2FoldChange, - log10(padj)))
  geom_point() +
  theme_bw(18)

ggsave('volcano.jpeg')
```



Hello academic paper writing phase

```
my.analysis ←
  res ▷
  filter(padj ⩽ 0.05, abs(log2FoldChange) ⩾ 1) ▷
  left_join(airway ▷ rowData() ▷ as_tibble(), c('row' = 'gene_id')) ▷
  select(gene_id = row, gene_name, log2FoldChange, FDR = padj) ▷
  arrange(FDR)
```

gene_id	gene_name	log2FoldChange	FDR
ENSG00000152583	SPARCL1	-4.57	< 1e-100
ENSG00000165995	CACNB2	-3.29	< 1e-100
ENSG00000120129	DUSP1	-2.94	< 1e-100
ENSG00000101347	SAMHD1	-3.76	< 1e-100
ENSG00000189221	MAOA	-3.35	< 1e-100
ENSG00000211445	GPX3	-3.73	< 1e-100

Dealing with the office devil {openxlsx}

```
library(openxlsx)

my.analysis ▷
  write.xlsx('my-analysis.xlsx')
```

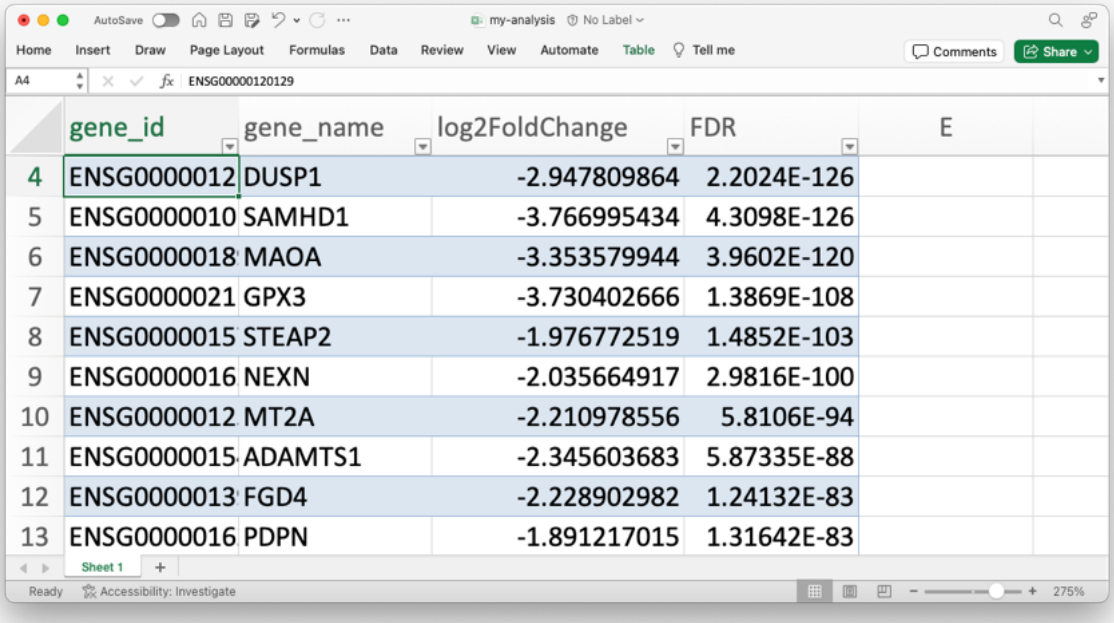


Photo by Stephen Baker on Unsplash

🏴‍☠️R and 🐍

```
rule my_analysis:
    input:
        script = 'analysis.R',
        data = 'gene-expression-matrix.tsv'
    output:
      'volcano.jpeg',
      'my-analysis.xlsx'
    shell:
        "RScript {input.script}"
```

- Pro tip: Define script as input (triggers rerun on changes)
- Explicitly list data input, such that script gets executed at the right time
- Increase reproducibility by declaring software environment with conda

Snakemake Handbook:

https://snakemake.readthedocs.io/en/stable/snakefiles/deployment.html#integrated-package-management

There is no "one size fits all" expression analysis workflow

Photo by Julian Hochgesang on Unsplash

Presentation takeaways

- Define "*transcriptomic*" sequencing and its relevance
- Recognize structure of sequencing data
- Outline a simple analysis pipeline with bash
- Identify concept and advantages of a Snakemake workflow
  (How I avoid using bash as much as possible)
- Connect a workflow with R analysis scripts
- Introduction to software management across platforms
- Fun with 📦packages and R tweaks

Photo by Peter Lloyd on Unsplash

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Independent Research Fund Denmark

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