University of Zurich[UZH]

# BIO634 - Day 2: RNA sequencing technologies

**Carla Bello**, carla.bello@ieu.uzh.ch

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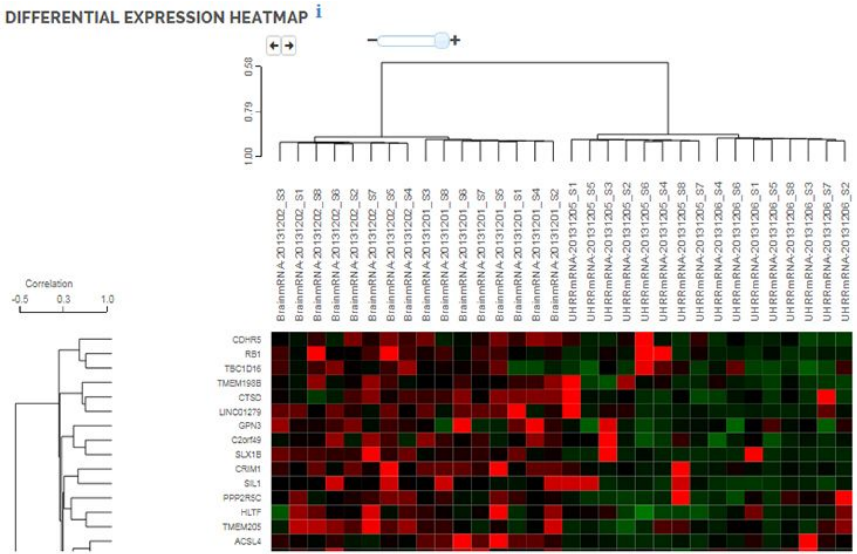
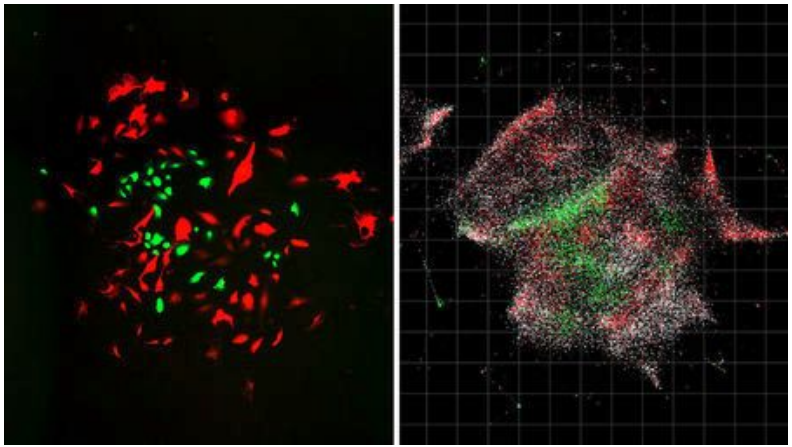
# Overview

- **Introduction** to RNA sequencing
- **RNA sequencing workflow**: steps to analyze the data
- **Important considerations**: technical, biological replicates, etc
- **Abundance quantification:** Gene, exons, transcripts
- **Differential expression analyses:** DEseq, edgeR, etc

# RNAseq reveals the presence and quantity of RNA

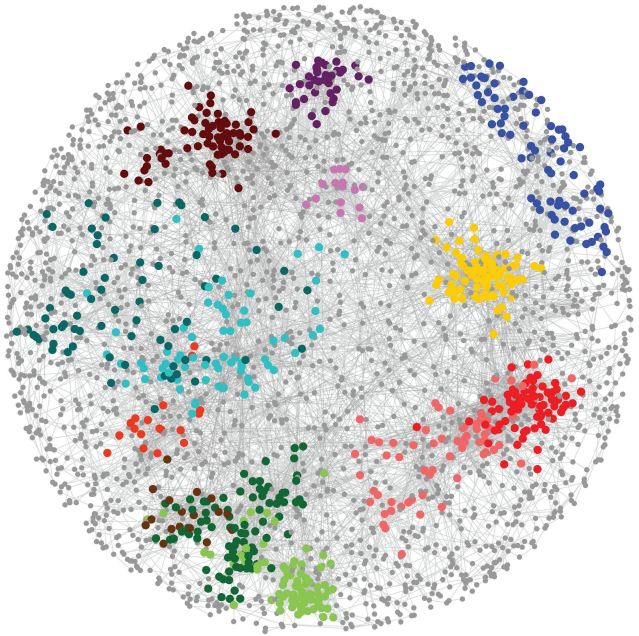


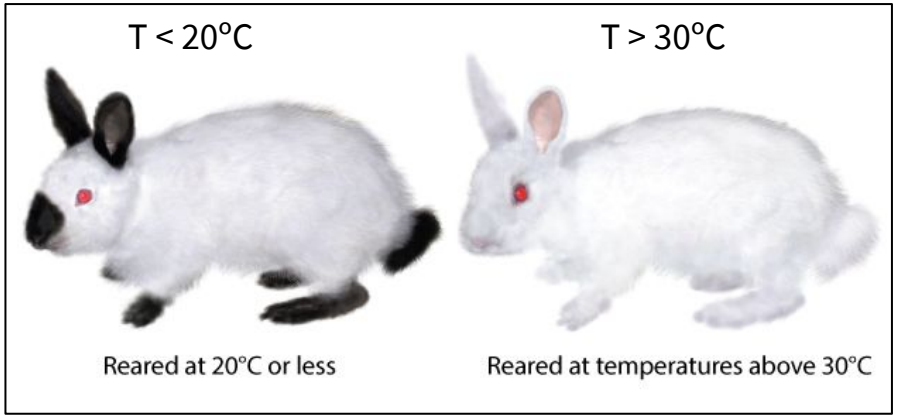
NGS technology that **quantifies the transcription / expression of the genome**

# Gene expression and phenotype

Image from Wikipedia



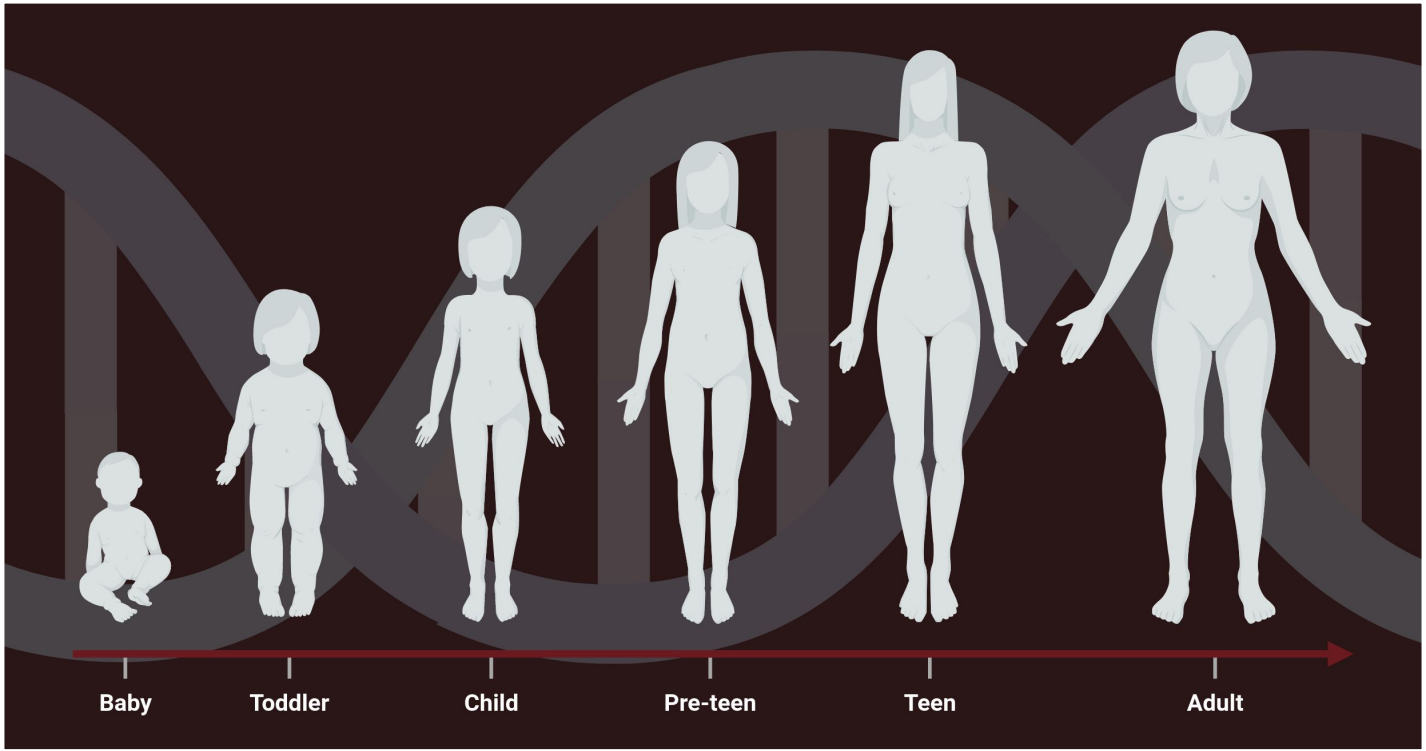
Himalayan rabbit




**Example**: A pigment gene is influenced by temperature.
When the temperature is **< 20°C the gene is inactive**

Nothing in the genome makes sense except in the light of the transcriptome

# RNA sequencing of whole genomes

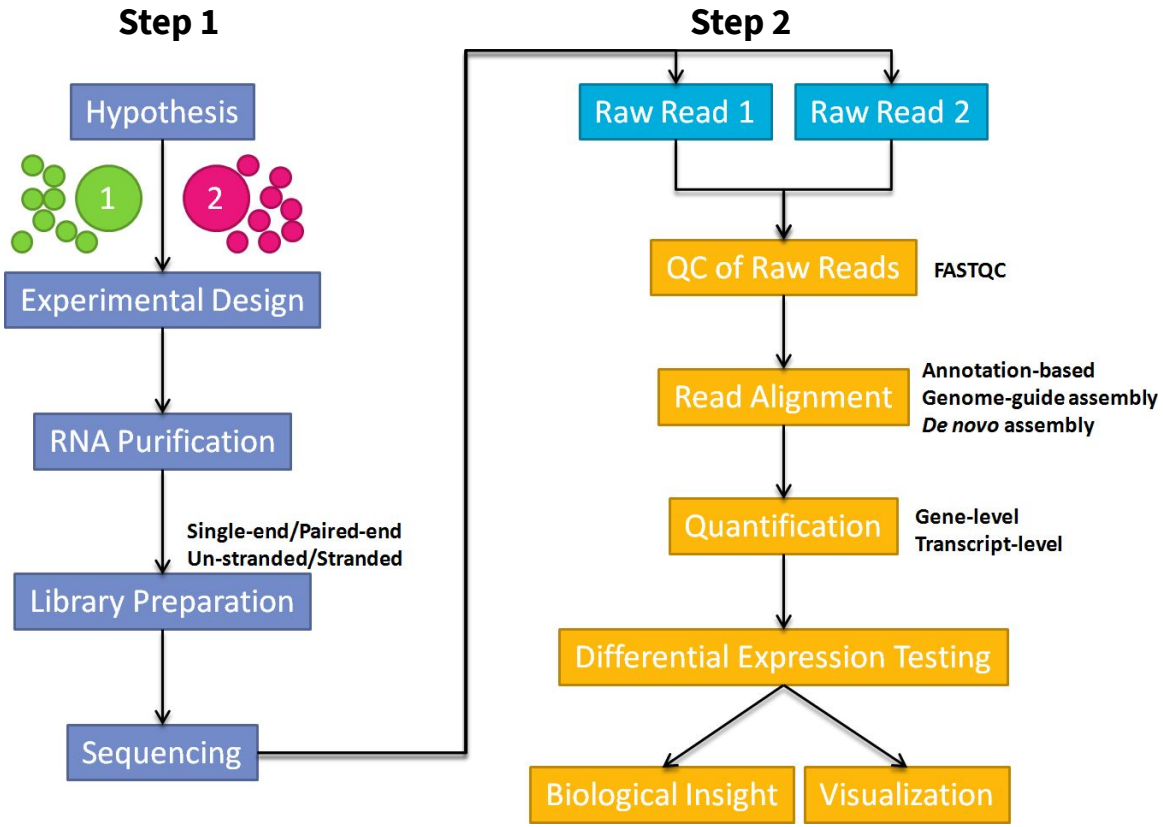



Expression of all the genes in the genome at different stages of aging

# Applications of RNA sequencing

- **Gene** expression/**differential** gene expression
- **Detecting novel** or **alternative** transcripts
- **De-novo transcriptome** assembly
- **SNP analysis,** e.g disease association studies
- **Allele-specific expression**
- **RNA studies**: miRNA, tRNAs, snRNA, lncRNAs, etc.

# RNA sequencing workflow



# Abundance quantification

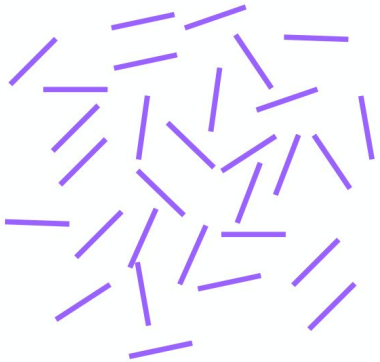


Image from Dr. Charlotte Soneson

# Abundance quantification

Genome alignment of RNA-seq requires a splice-aware aligner (STAR, HISAT2)

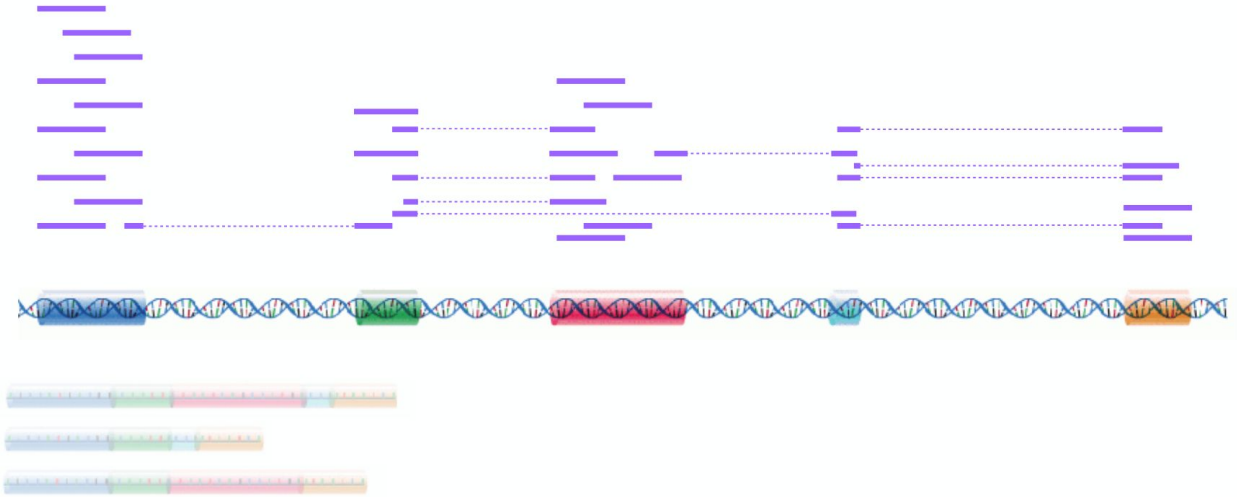
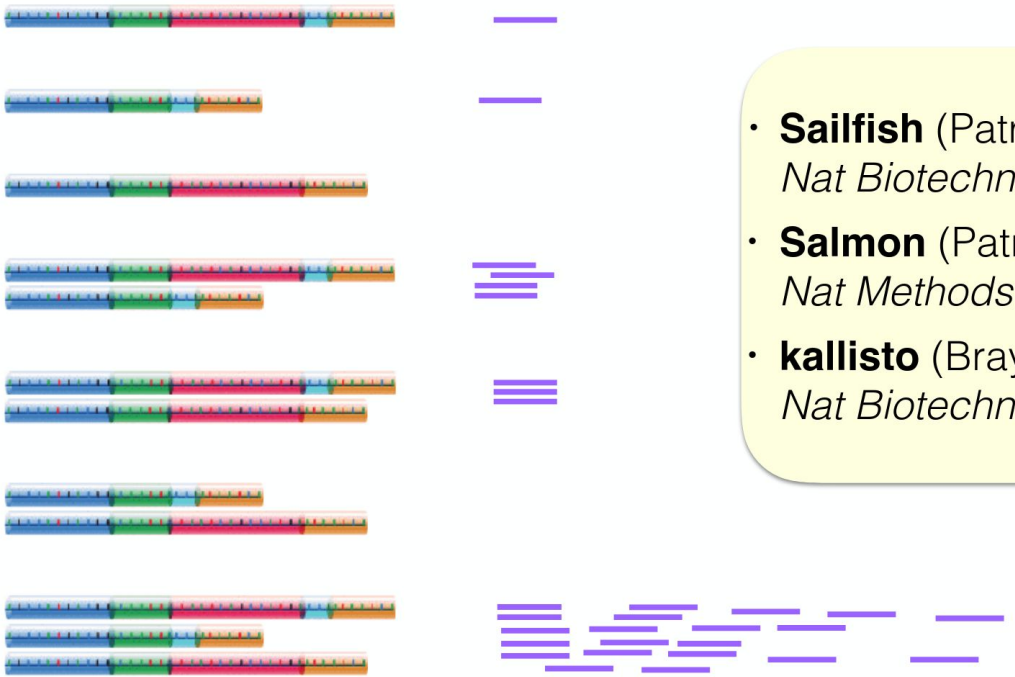


Image from Dr. Charlotte Soneson

# Abundance quantification

## Transcript-level counts, e.g. obtained by "alignment-free" estimation methods

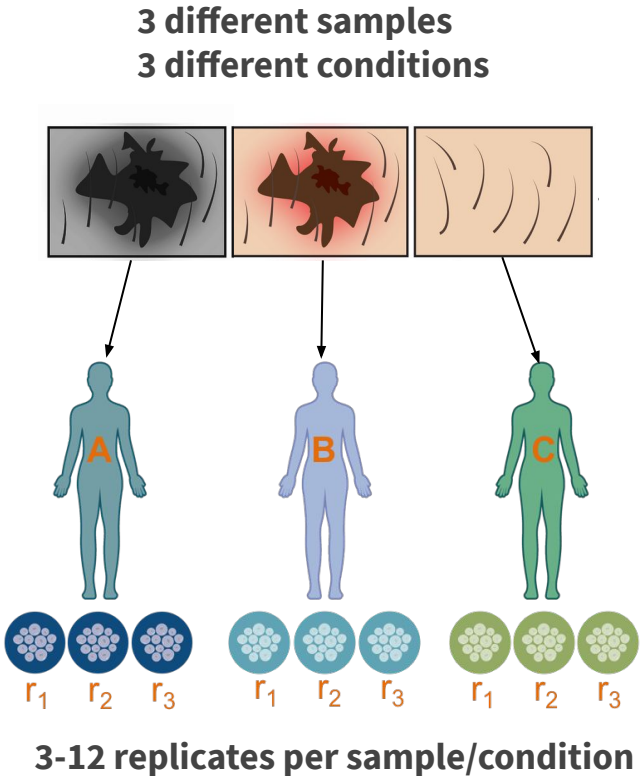


- **Sailfish** (Patro et al, *Nat Biotechnol* 2014)
- **Salmon** (Patro et al, *Nat Methods* 2017)
- **kallisto** (Bray et al, *Nat Biotechnol* 2016)

Pre-indexed datasets of transcripts

Image from Dr. Charlotte Soneson

# Important considerations before sequencing




**Technical and biological replicates are important** and should be taken into consideration **when planning the experiments**

# Technical replicates

1. **<u>Technical replicates:</u> Biological material is the same** but the **technical steps used to measure gene expression are repeated.**

In particular **RNA-seq library preparation** (RNA fragmentation, cDNA synthesis and PCR amplification) **may introduce biases in the data**.

# Biological replicates and statistical power

2. **Biological replicates**: Are different biological samples that are processed separately. They are **required if inference on the population is to be made**, with **three** biological replicates **being the minimum for any inferential analysis.**

3. **Desired statistical power**, that is the capacity for detecting statistically significant differences in gene expression between experimental groups.

# Different methods to analyze RNA-seq data

- There are different packages for **differential expression analysis**, such as **edgeR** and **DESeq** based on negative binomial (NB) distributions or **baySeq** and **EBSeq** which are **Bayesian** approaches based on a **negative binomial model.**

- These packages work mostly by **estimating the variance mean dependance** in **count** data.

# Factors to consider for RNAseq analysis

1) **Within sample**
   - Gene/transcript length
   - Relative expression (a few highly expressed genes)

2) **Between samples**
   - Sequencing depth (library size)
   - Sequencing biases

**3)** Raw read counts **are <u>NOT</u> directly** comparable **between** samples:
**Solution**: Normalize read counts

# RNA-Seq Read Count Normalization

- **RPKM**: **Reads per kilobase of transcript** per million reads of library (single-end reads)

- **FPKM**: **Fragments per kilobase of transcripts** per million reads of library (paired-end reads)

- **TPM**: **Transcripts per million** reads of library

# RNA-Seq Read Count Normalization

## RPKM:
## Reads Per Kilobase and Million mapped reads

- Unit of measurement

$$RPKM = \#MappedReads * \frac{1000bases * 10^6}{length\ of\ transcript * Total\ number\ of\ mapped\ reads}$$

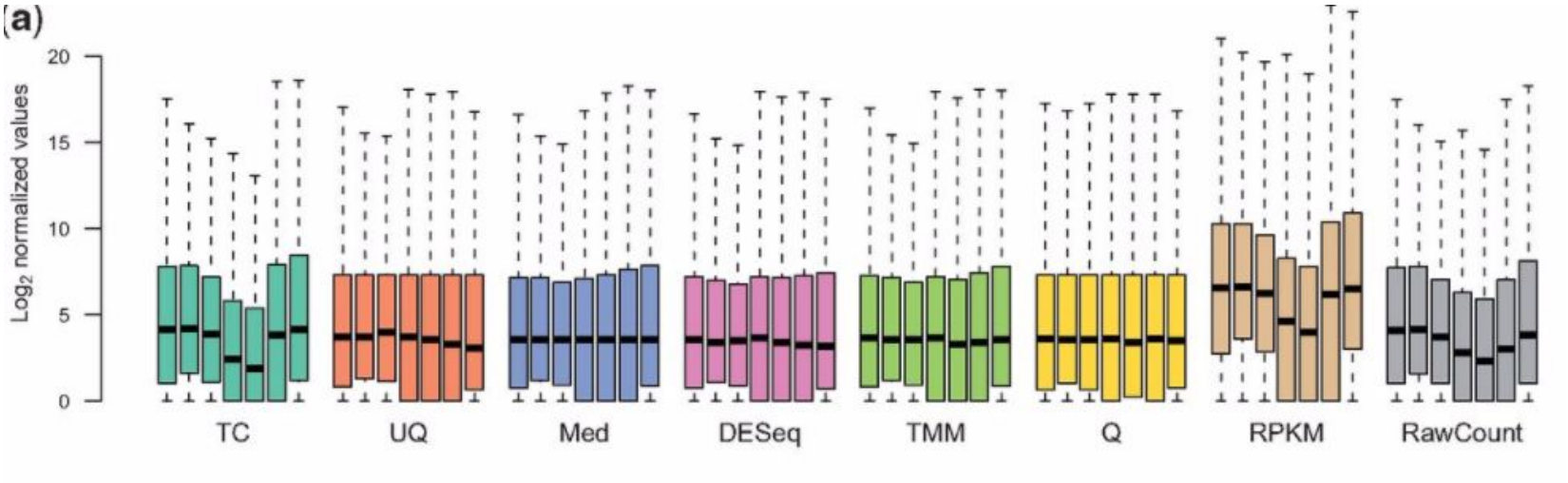
- RPKM reflects the molar concentration of a transcript in the starting sample by normalizing for
  - RNA length
  - Total read number in the measurement

- This facilitates transparent comparison of transcript levels within and between samples

Mortazavi et al (2008) Nature Methods, 5(7), 621

# RNA-Seq Read count normalization

RPKM/FPKM are normalized counts. **DESeq/edgeR** requires **raw counts** as **input** as they have their **own normalisation methods**



# Differential expression analyses

- **Many statistical methods available**
  - T-test
  - Poisson Distribution
  - Negative binomial

- **No clear consensus yet.**

- **Tools shown to perform well (under certain circumstances):**
  - LIMMA (TMM)
  - DESeq (RLE)
  - edgeR (TMM)
  - Cuffdiff (FPKM)
  - RSEM (EM)
  - Trinity

**Identify genes that show differences in expression level between conditions (samples)**

# Differential expression analyses

1. Mapping **RNAseq** data with **Salmon**
2. Importing **transcript abundance** with the ***tximport*** **library**
3. **Differential analysis**: A comparison between **DEseq** and **edgeR**

# Later in the afternoon

1. List of differentially expressed genes
2. Biological context
3. Pathway Analysis (differentially expressed biological pathways)
4. Gene Set Enrichment Analysis (GSEA) (functional enrichment between two biological groups)
5. Co-expression analysis

# Hands-on session - Part II: RNA-seq

**Please, go here and follow the instructions:**

https://github.com/carlalbc

https://github.com/carlalbc/BIO634_2021/

# ~~ Lunch break from 12:00 to 13:30 ~~



## Enjoy! See you in a bit! :)