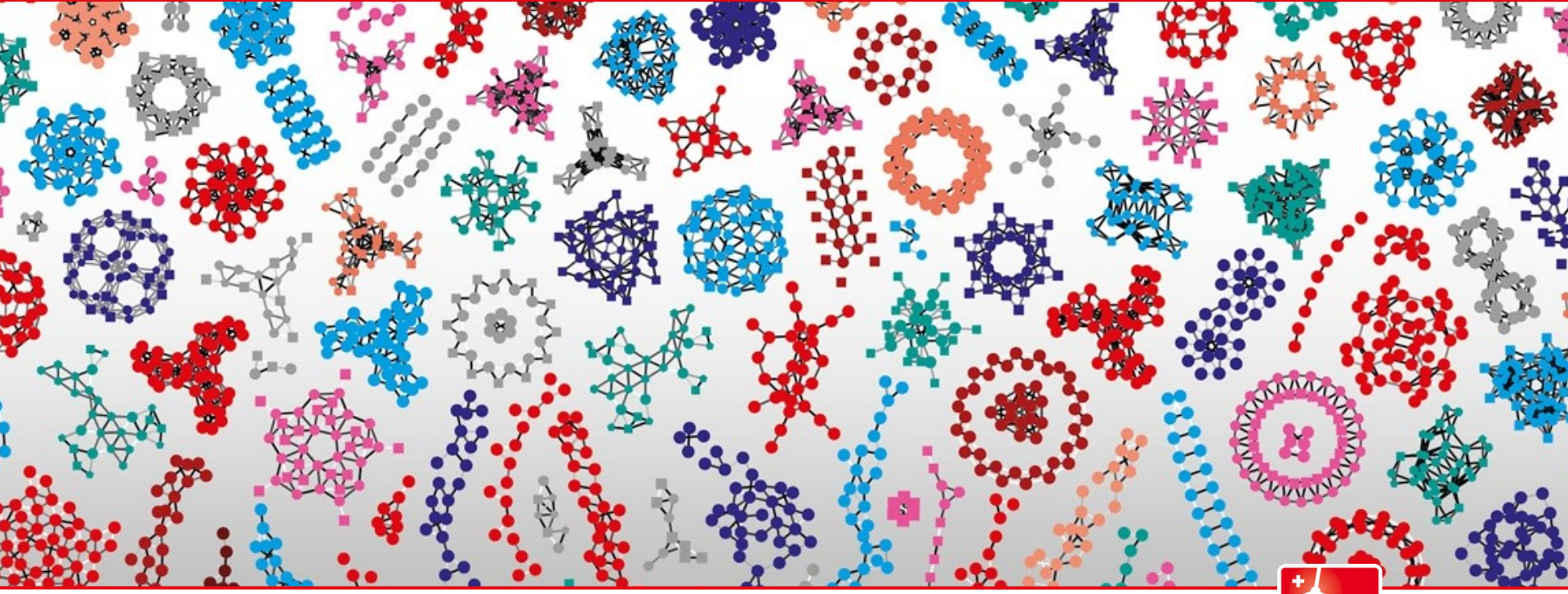
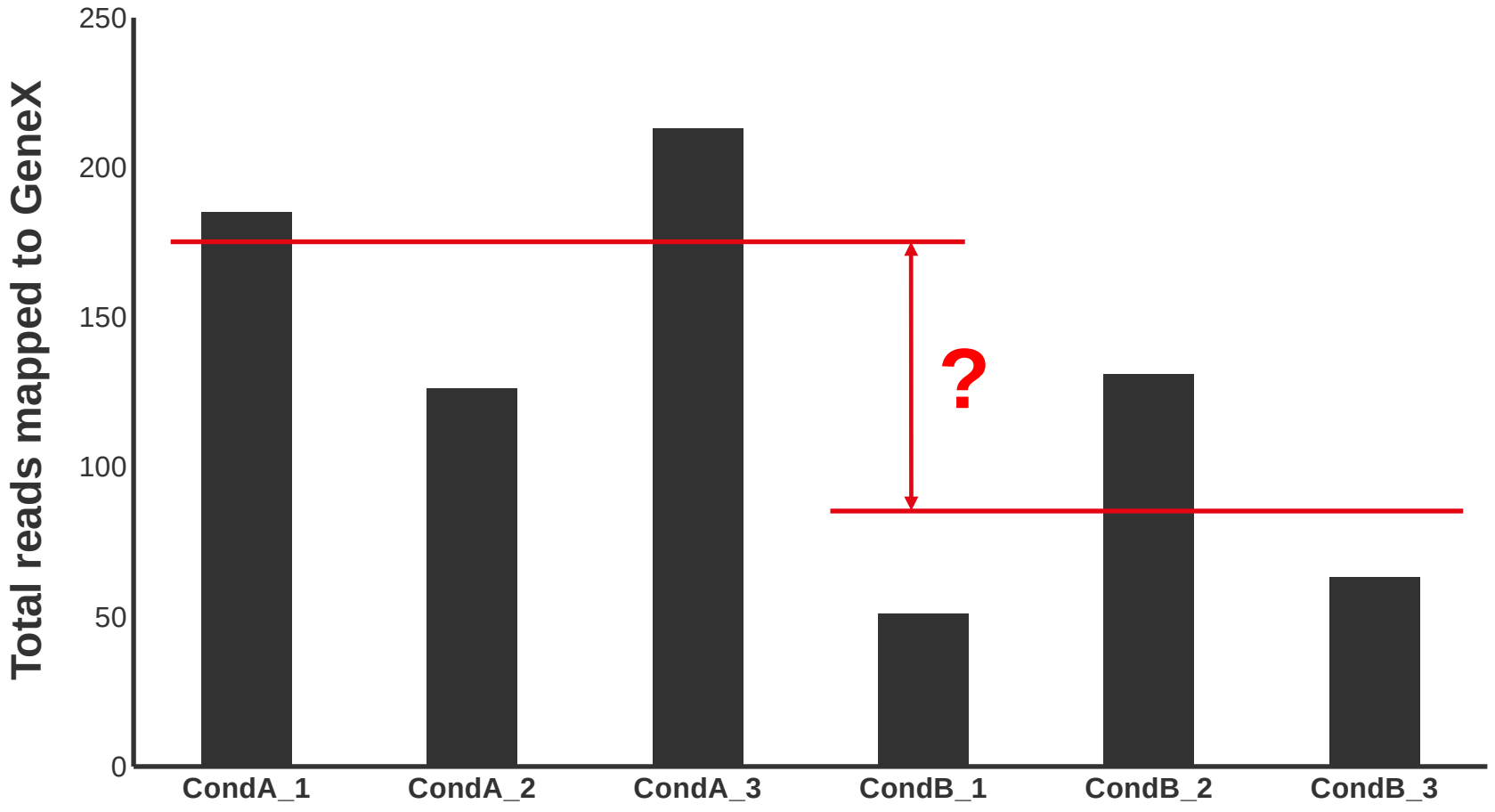


Swiss Institute of Bioinformatics

Introduction to RNA-Seq – Differential Expression

Wandrille Duchemin

How to define statistical significance?



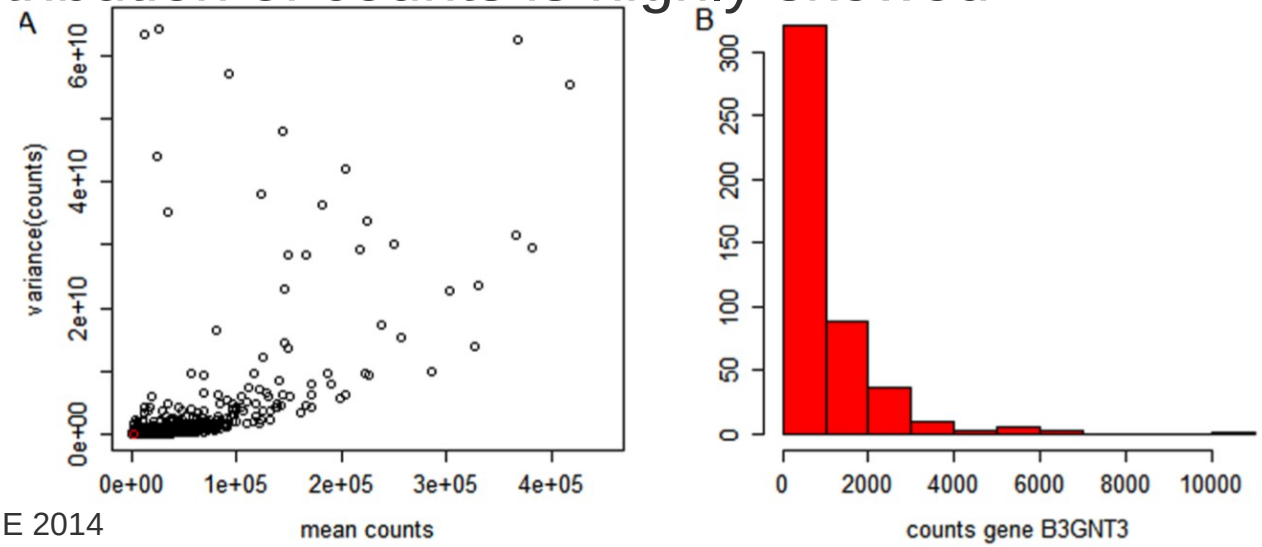
Statistical modeling of RNA-Seq data

- **Essentially, two approaches**
  - Non-parametric: *eg* voom (based on limma)
  - Parametric: *eg* edgeR and DESeq2

- **We will be working with the parametric approaches packaged in edgeR and DESeq2**
  - Important assumptions:
    - most genes are not differentially expressed
    - probability of a read mapping to geneX is the same for all samples in a class

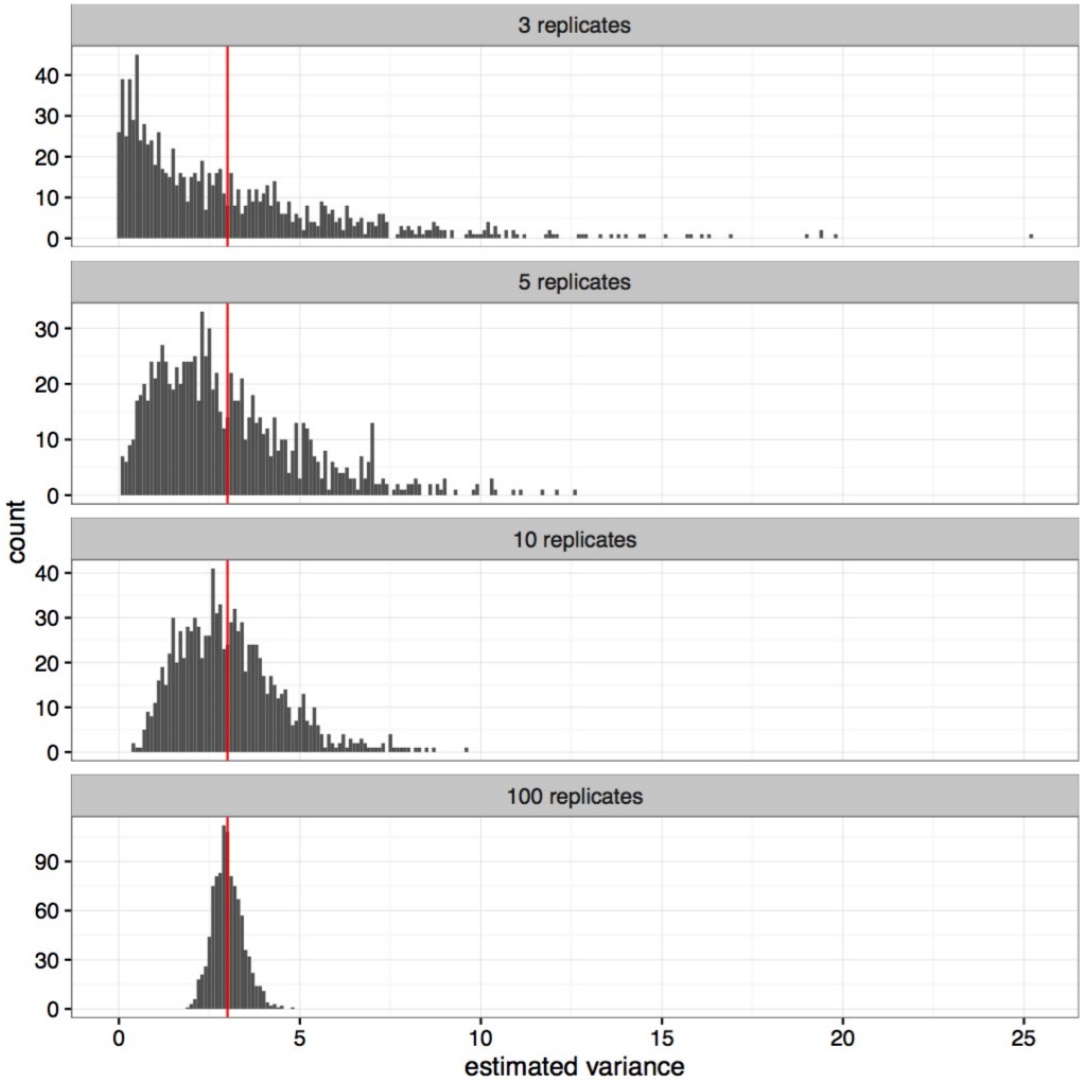
Challenges for RNA-Seq

- **Which statistical distribution is most appropriate?**
- **How to normalize read counts between samples?**
- **Estimating variance is difficult**
  - Typically, very few replicates
  - The variance depends on the mean count
  - Distribution of counts is highly skewed



Zweiner *et al*, PLoS ONE 2014

Estimating variance of a normally distributed variable



True variance = 3

What does this tell you about the ideal number of replicates for your RNA-Seq experiment?

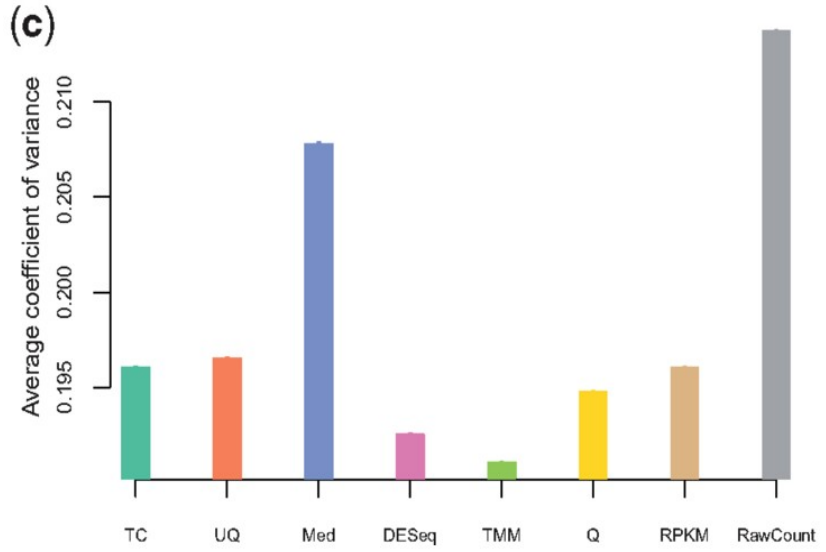
The negative binomial distribution

- Used by both edgeR and DESeq2, essentially a generalized Poisson distribution
- Variance is modeled as:
  - $var(X) = \mu + \theta\mu^2$
- θ = dispersion
- $\sqrt{\theta}$ = "biological coefficient of variation"
- Allows mRNA proportions to vary across samples, accurately capturing variability across biological replicates
- Implemented using a "generalized linear model"

Normalization of RNA-Seq data

- **Raw read counts are not directly comparable across samples. They depend on:**
  - Abundance in source material
  - Gene length
  - Sequencing depth
  - Sequencing biases
  - …
- **edgeR uses the “Trimmed Mean of M-Values” (TMM) method**
- **DESeq2 uses the “Relative Log Expression” (RLE) method**

TMM and RLE normalization yield comparable results



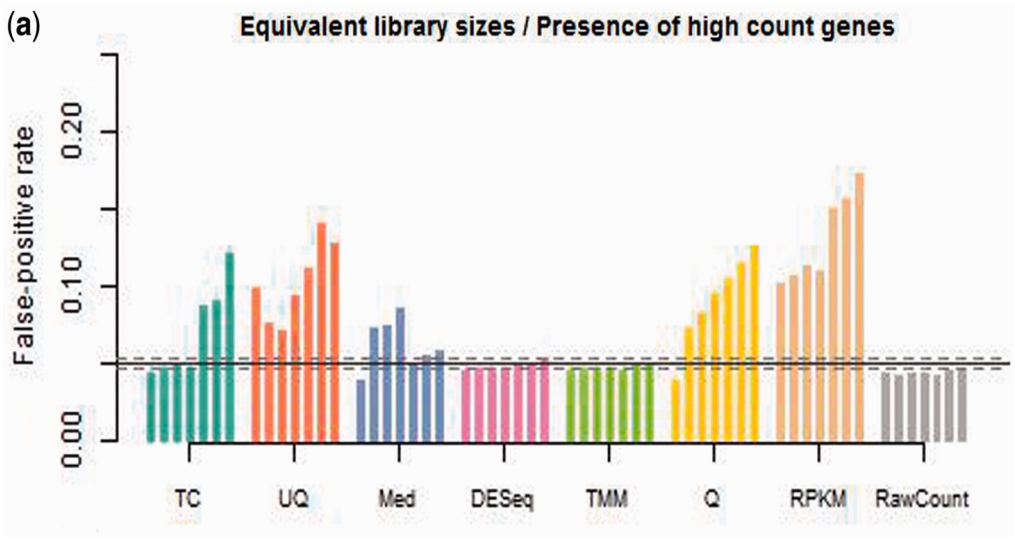


Table 3: Summary of comparison results for the seven normalization methods under consideration

Method	Distribution	Intra-Variance	Housekeeping	Clustering	False-positive rate
TC	−	+	+	−	−
UQ	++	++	+	++	−
Med	++	++	−	++	−
DESeq	++	++	++	++	++
TMM	++	++	++	++	++
Q	++	−	+	++	−
RPKM	−	+	+	−	−

A '−' indicates that the method provided unsatisfactory results for the given criterion, while a '+' and '++' indicate satisfactory and very satisfactory results for the given criterion.

Dillies *et al* 2013

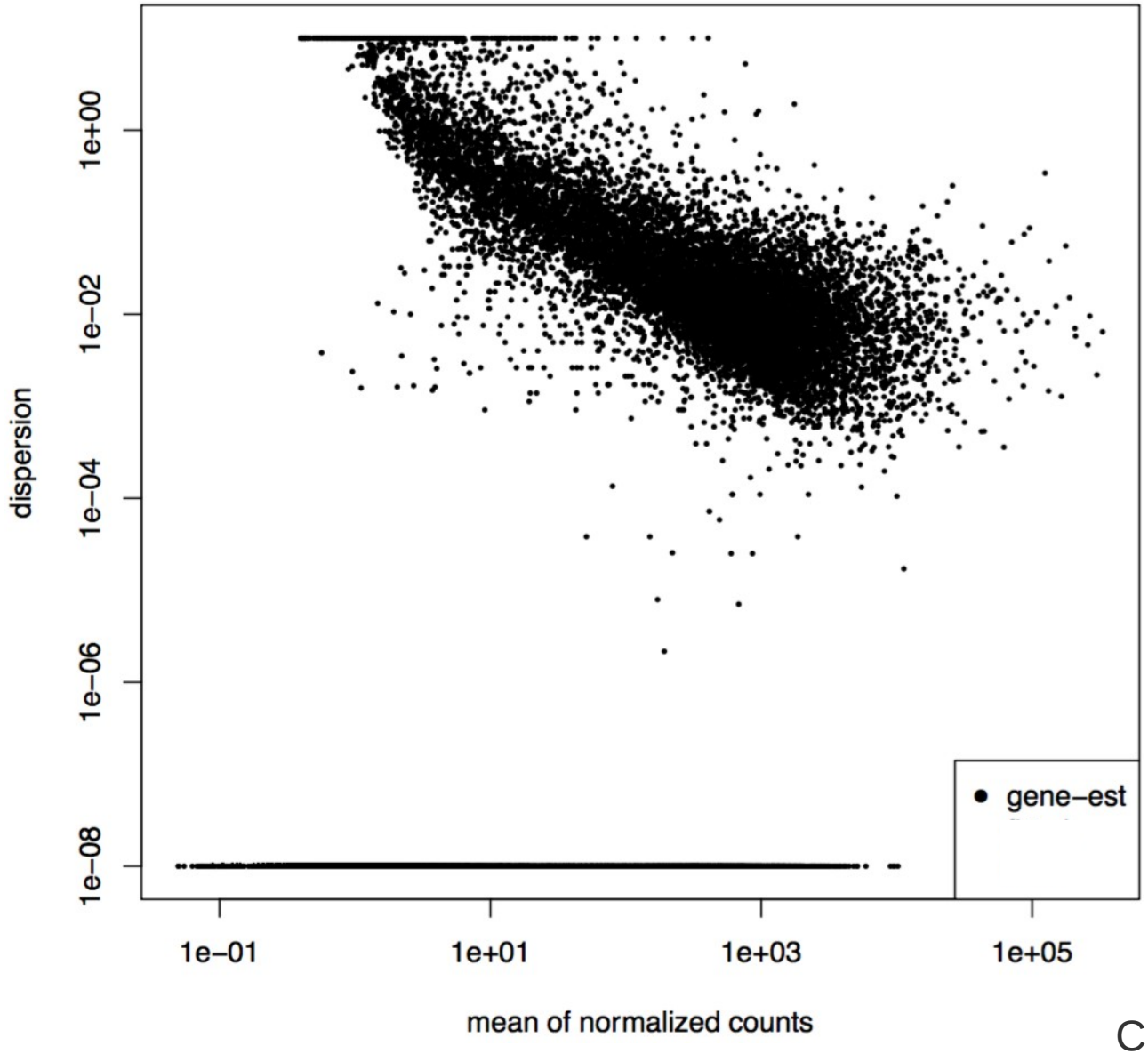
TMM and RLE normalization yield comparable results

- **Keep in mind that “normalization factors” from edgeR and “size factors” from DESeq2 are not equivalent theoretical parameters**
  - For a more detailed discussion, see Maza 2016

Shrinkage of dispersion estimates

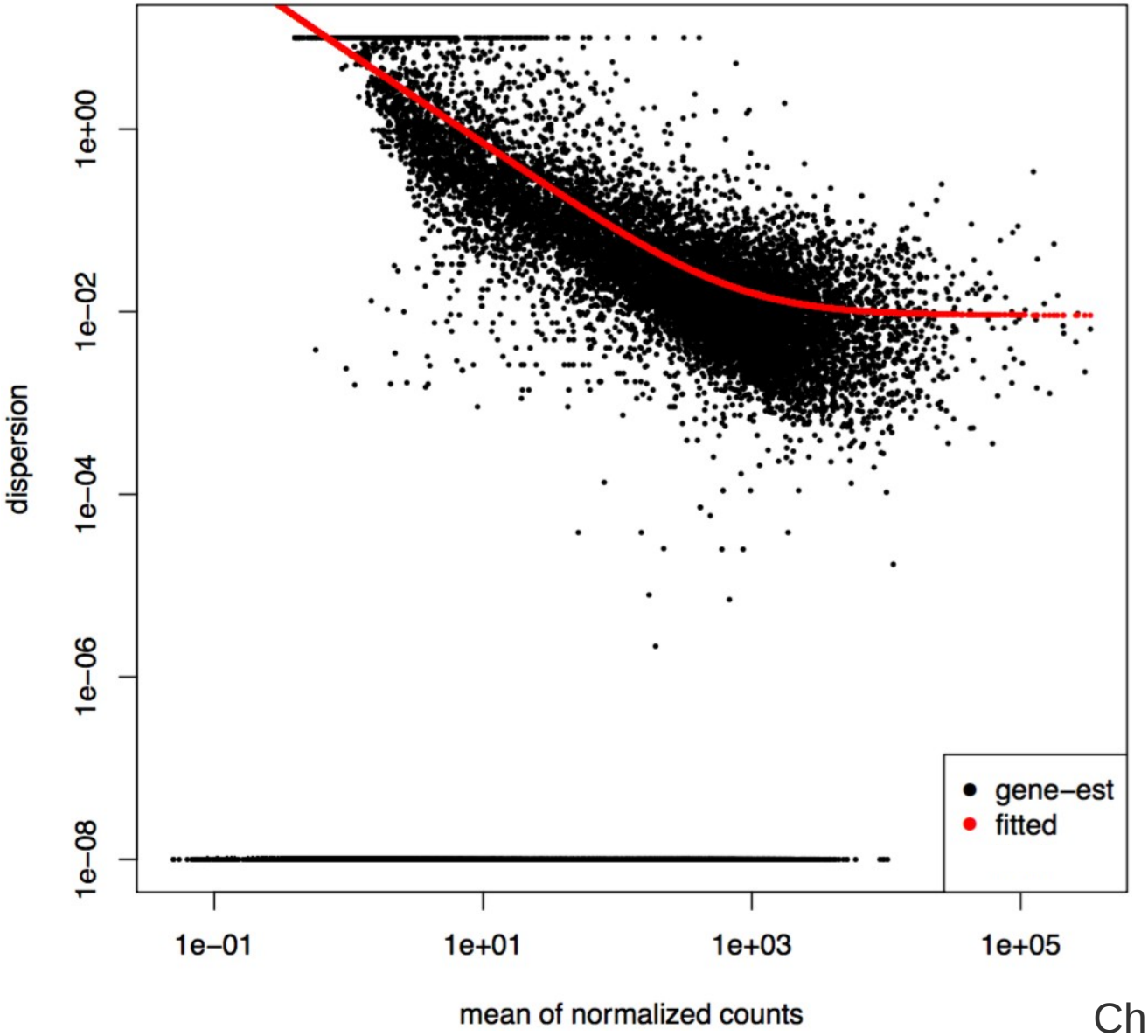
- **Problem: we often have few replicates**
- **Solution: take advantage of the large number of genes, shrink the gene-wise estimates towards a center value defined by the observed distribution of dispersions across:**
  - All genes (“common” dispersion estimate)
  - Genes with similar expression (“trended” dispersion estimate)

Shrinkage dispersion estimation



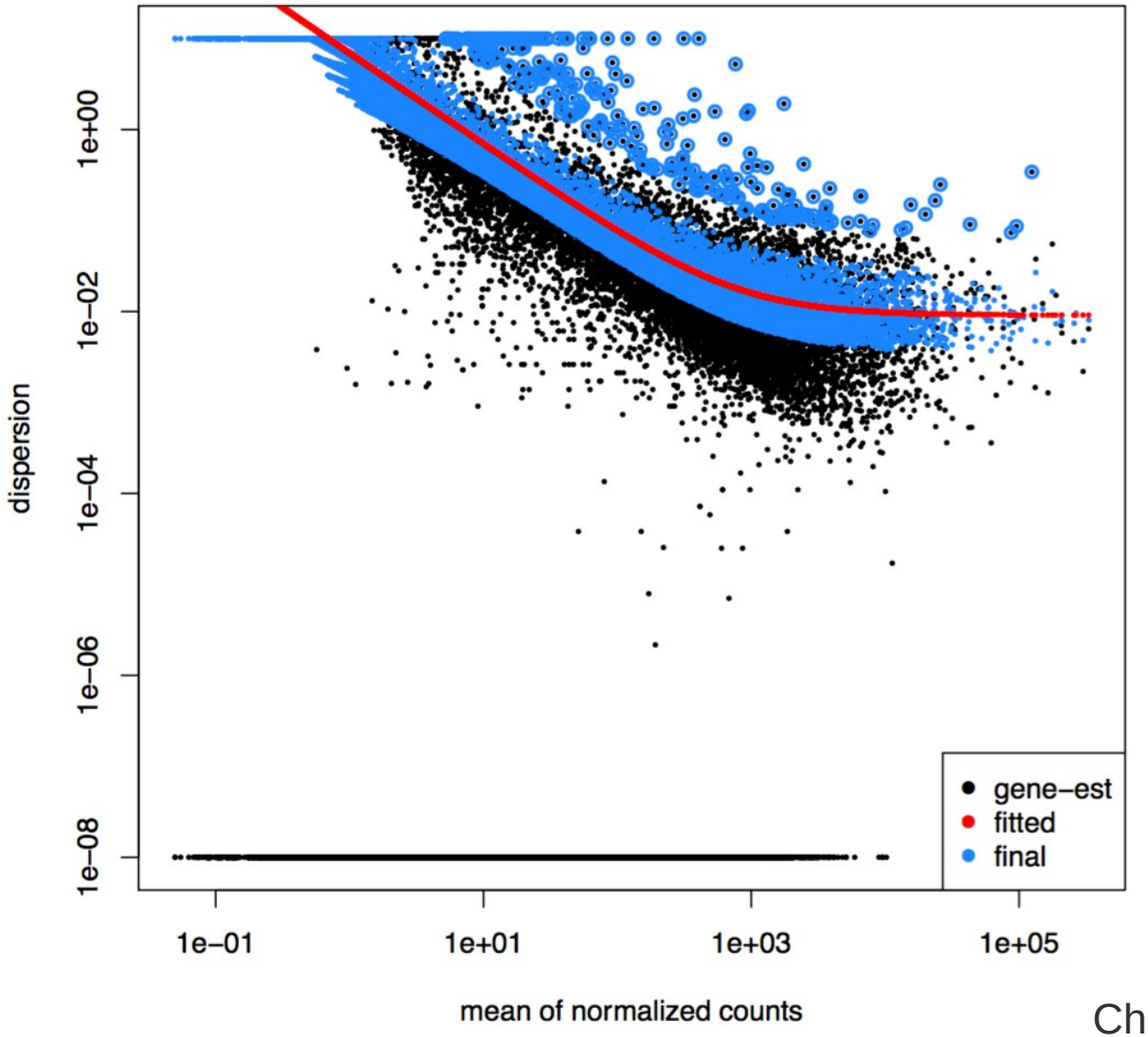
Charlotte Soneson

Shrinkage dispersion estimation



Charlotte Soneson

Shrinkage dispersion estimation

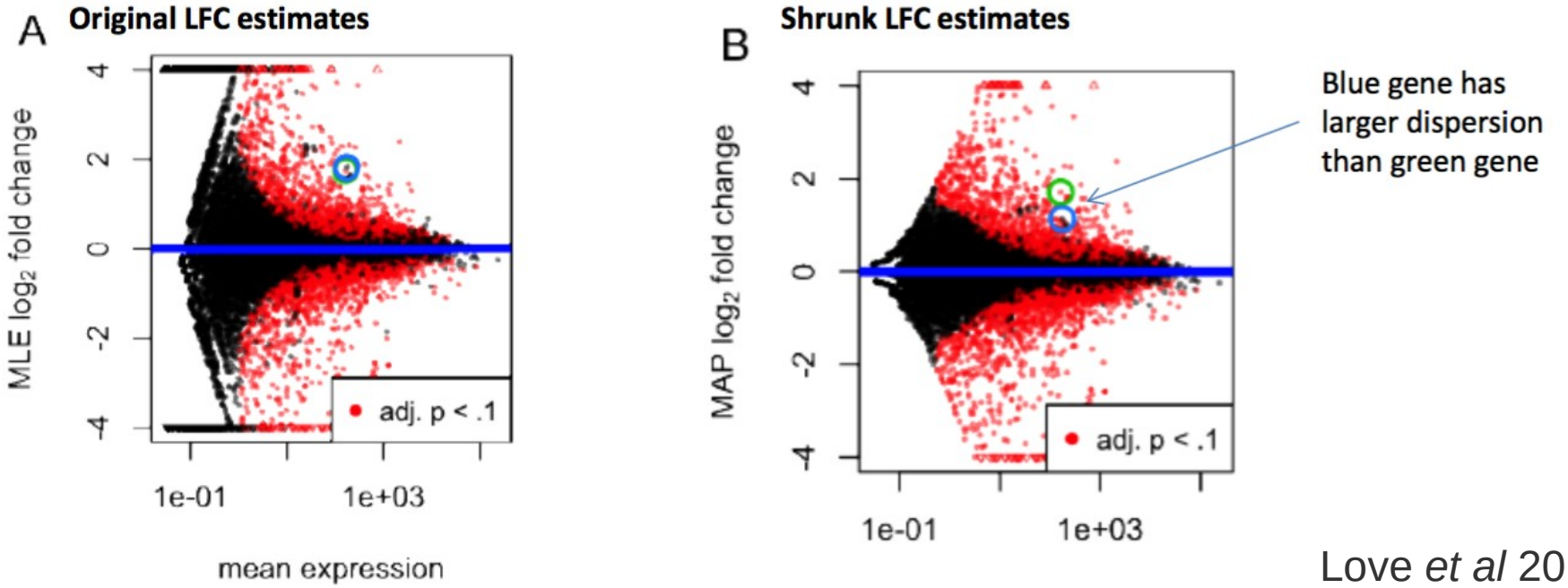


Charlotte Soneson

Shrinkage of log-fold change

- **Problem: weakly expressed genes tend to show much stronger differences between conditions, because count data are very noisy when counts are low**

- **Solution: shrink LFC estimates toward 0 such that shrinkage is stronger when less info is available (*eg* low counts, high dispersion, low replicates)**



Love *et al* 2014

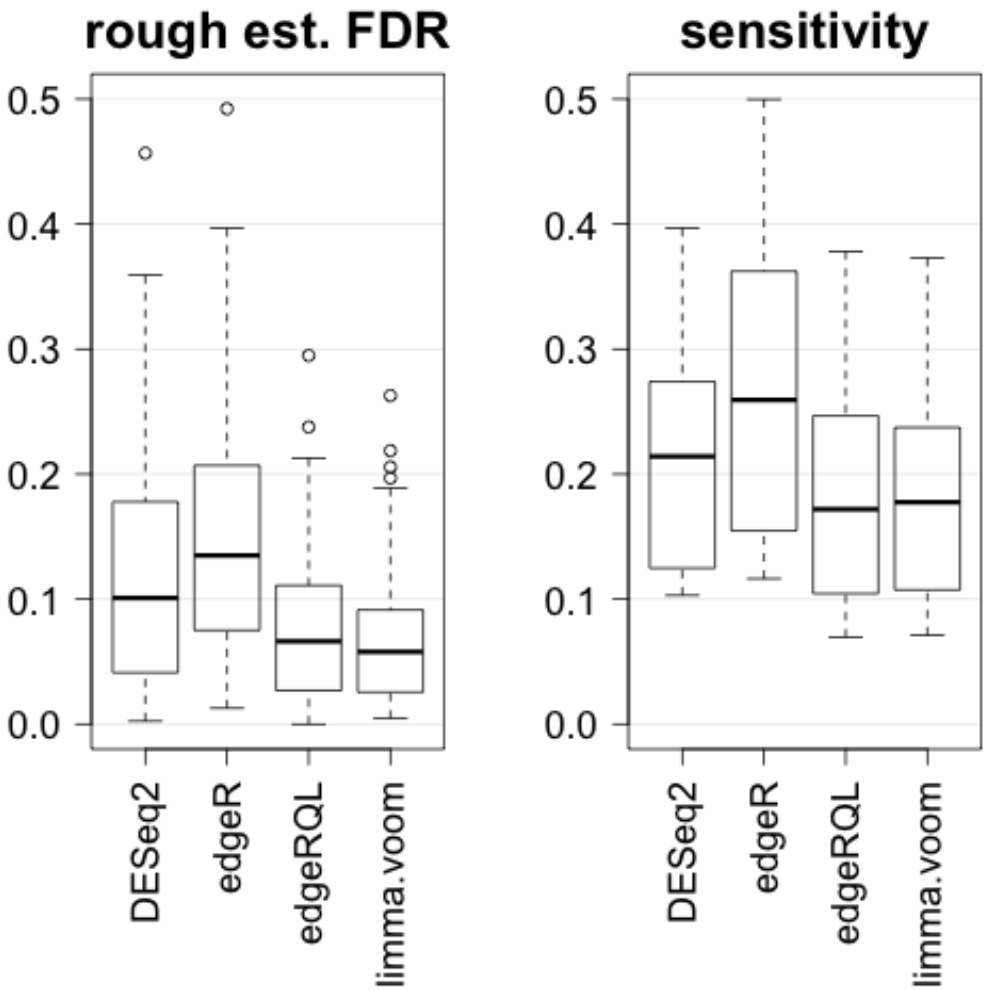
Tests for differential expression – DESeq2

- **A Z-score is calculated for each gene by dividing the shrunken LFC estimate by the standard error of the estimate**
- **The Z-score is compared to a standard normal distribution to obtain a P-value (Wald test)**
- **To correct for multiple testing, adjusted p-values are calculated using the Benjamin-Hochberg procedure**
  - Consider a genome with 20,000 genes
  - At a threshold of p = 0.05, we would expect 1000 significant tests even if there is NO differential expression

Tests for differential expression – edgeR

- **Different methods for different cases :**
- **“simple” 1 factor : exactTest()** , using the computed conditional distribution for the sum of counts in a group
- **Otherwise a GLM framework in used :**
- **QL F-test :** is preferred because it gives “stricter error rate control by accounting for the uncertainty in dispersion estimation… (which can be considerable when you have few replicates and/or the amount of shrinkage is low), whereas the other methods do not”
- **LRT :** when “the dispersions are very large and the counts are very small, whereby some of the approximations in the QL framework seem to fail”

edgeR vs DESeq2



https://mikelove.wordpress.com/2016/09/28/deseq2-or-edger/

edgeR “Robust” Mode

Dispersion estimates can be sensitive to outliers. If this is a significant aspect of your dataset, edgeR has a more robust implementation to estimate dispersions:

https://www.rdocumentation.org/packages/edgeR/versions/3.14.0/topics/estimateGLMRobustDisp

Practical

- Go to the website and follow the Differential Expression Inference practical

Enrichment analysis

- **What to do with your list of differentially expressed gene ?**
- **Interpretation can be difficult, especially when many genes are DE**

Enrichment analysis

- What to do with your list of differentially expressed gene ?
- Interpretation can be difficult, especially when many genes are DE
- We facilitate this by “translating” this from a list of genes to a more readily interpretable set : biological functions, pathways, transcription factors, …

Enrichment analysis

- What to do with your list of differentially expressed gene ?
- Interpretation can be difficult, especially when many genes are DE
- We facilitate this by “translating” this from a list of genes to a more readily interpretable set : biological functions, pathways, transcription factors, …
- 2 main aspects:
  - Mapping from genes to interpretable objects
  - Method to determine significant enrichment

Enrichment analysis - mapping

The goal is to regroup certain genes together in meaningful sets

- **Genes involved in the same pathway (eg. DNA repair)**
- **Genes located in the same biological compartment**
- **Genes with a similar molecular function**
- **Genes regulated by the same transcription factor**

…

Enrichment analysis - mapping

A few of the possibilities :

- Gene Ontology
- Reactome
- KEGG
- MSigDB
- Custom set

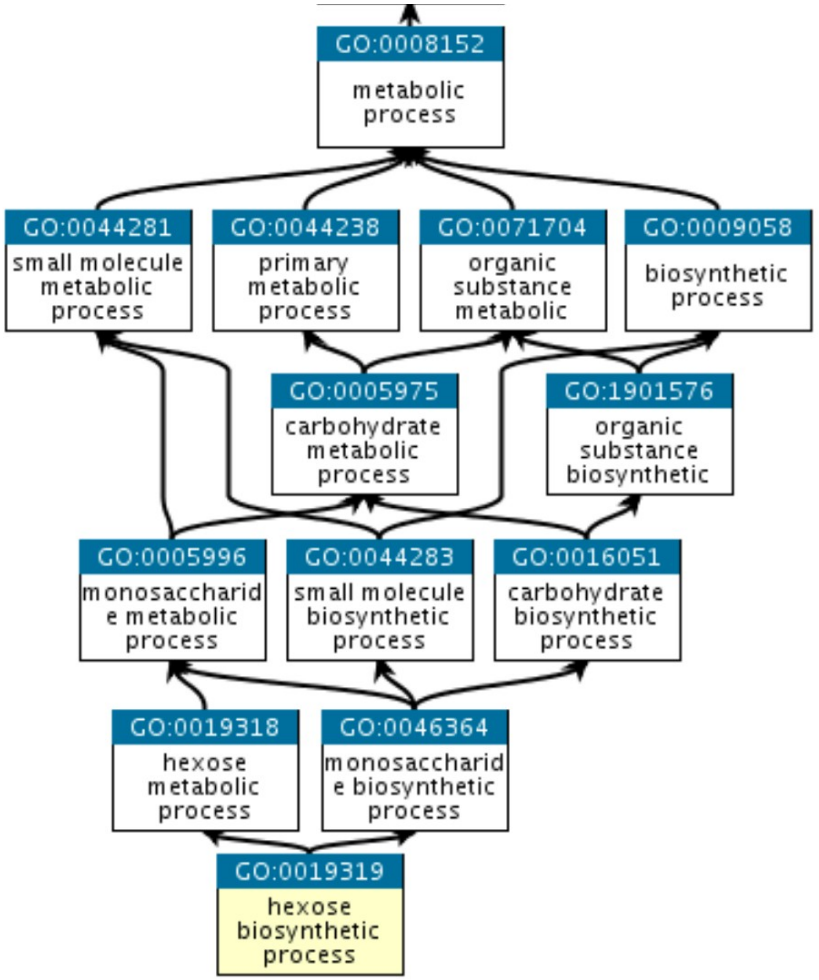
...

Enrichment analysis - mapping

Gene ontology : http://geneontology.org

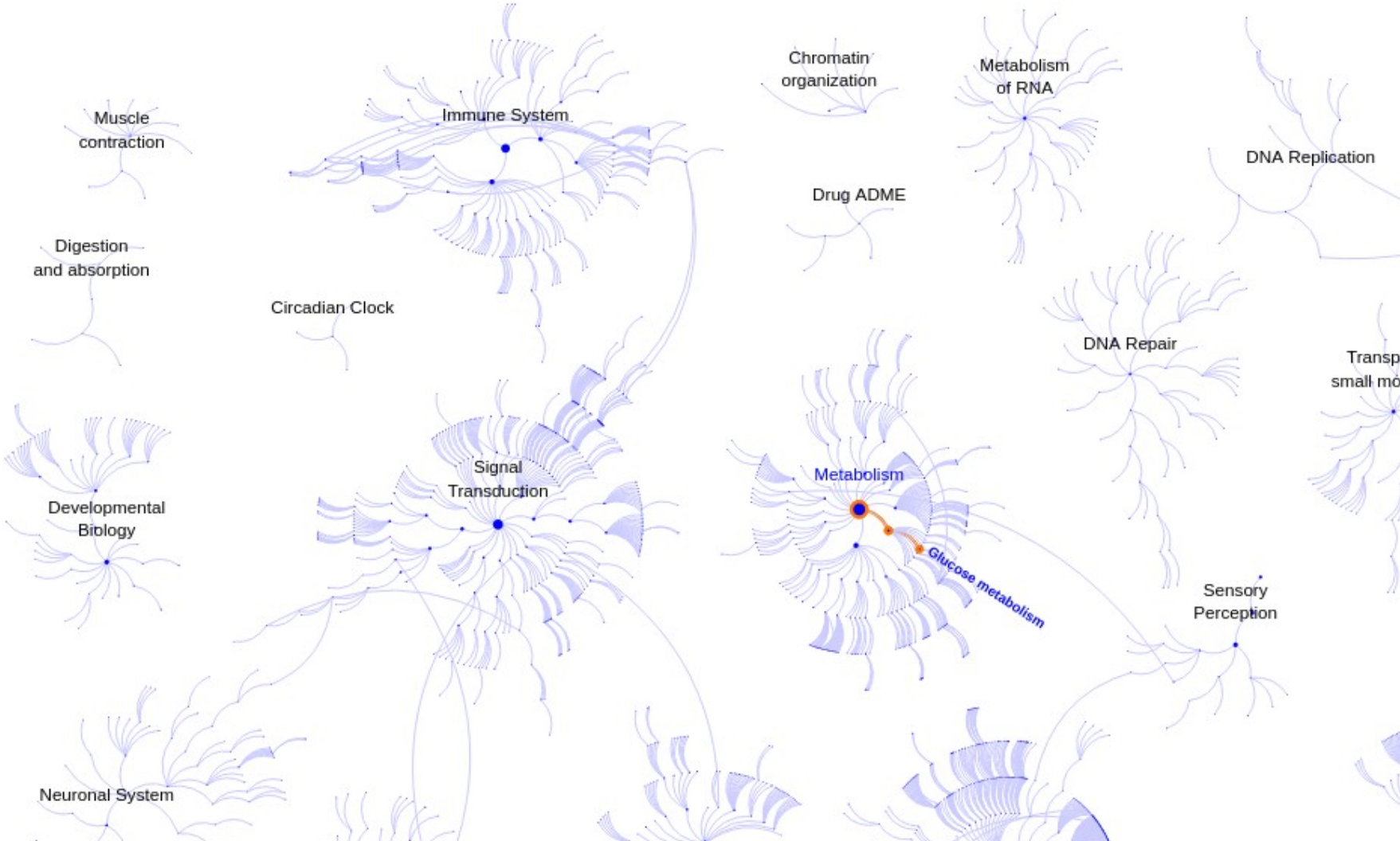
3 domains of nested terms:

- **Molecular Function**
- **Cellular Component**
- **Biological Process**



Enrichment analysis - mapping

Reactome : https://reactome.org



Enrichment analysis - mapping

MSigDB : http://www.gsea-msigdb.org/gsea/msigdb/index.jsp

Human mouse and rat only

H **hallmark gene sets** are coherently expressed signatures derived by aggregating many MSigDB gene sets to represent well-defined biological states or processes.

C1 **positional gene sets** for each human chromosome and cytogenetic band.

C2 **curated gene sets** from online pathway databases, publications in PubMed, and knowledge of domain experts.

C3 **regulatory target gene sets** based on gene target predictions for microRNA seed sequences and predicted transcription factor binding sites.

C4 **computational gene sets** defined by mining large collections of cancer-oriented microarray data.

C5 **ontology gene sets** consist of genes annotated by the same ontology term.

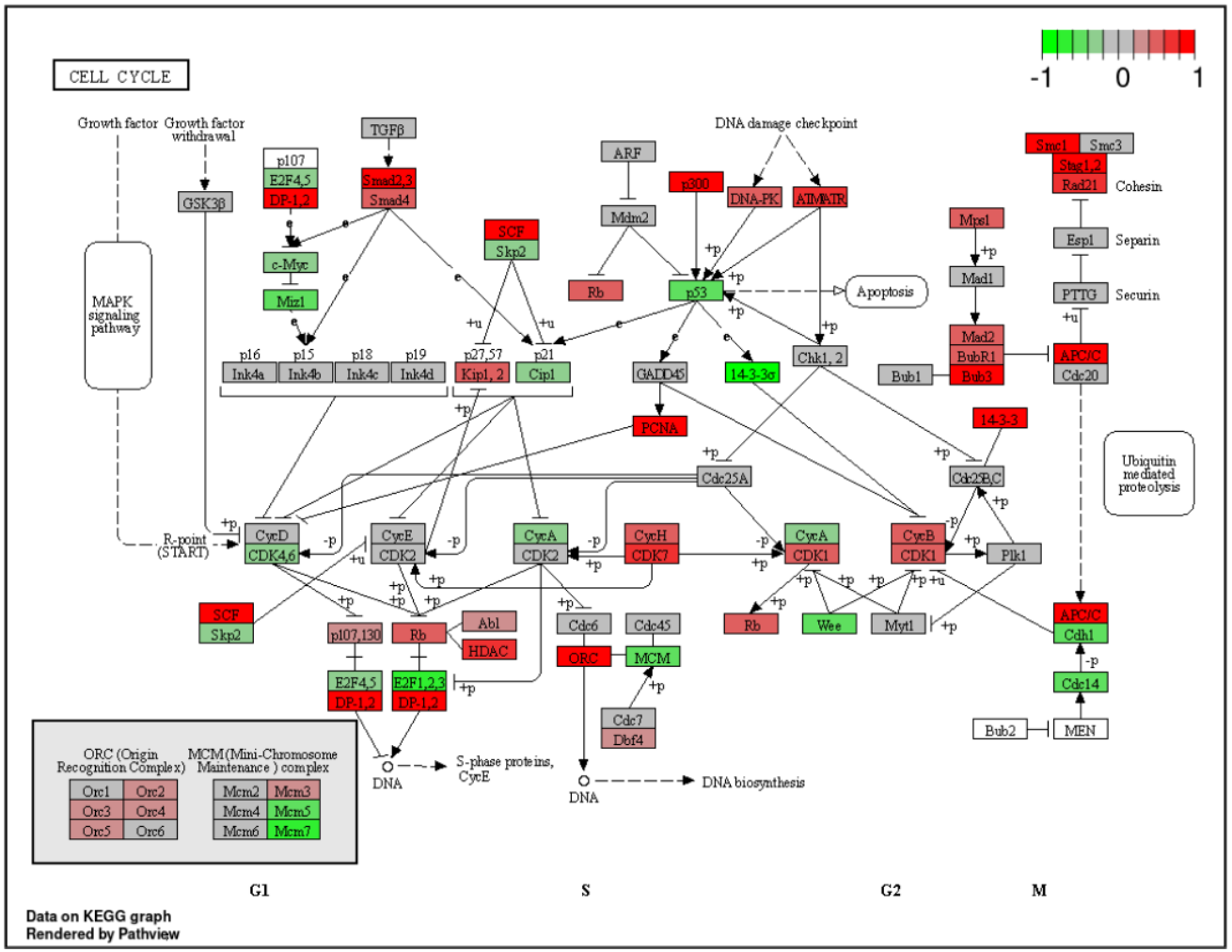
C6 **oncogenic signature gene sets** defined directly from microarray gene expression data from cancer gene perturbations.

C7 **immunologic signature gene sets** represent cell states and perturbations within the immune system.

C8 **cell type signature gene sets** curated from cluster markers identified in single-cell sequencing studies of human tissue.

Enrichment analysis - mapping

KEGG : https://www.genome.jp/kegg/ - KEGG PATHWAY



Projection of DE data
onto a KEGG pathway map
With R package pathview

https://bioconductor.org/packages/release/bioc/html/pathview.html

Enrichment analysis - mapping

Custom gene sets :

- **Derived from specialized litterature**
- **Tentative annotation**
- **Understudied organisms**
- **...**

Enrichment analysis – computing enrichment

2 possible approaches (among many!)

- Over-representation analysis
- Gene Set Enrichment Analysis

Enrichment analysis – computing enrichment

2 possible approaches (among many!)

- **Over-representation analysis**
  - **Fisher's exact test (with p-val correction)**

	DE	Not DE
in gene set	A	B
not in gene set	C	D

N = A+B+C+D **# total genes**
M = A+B **# genes in set**

n = A+C **# DE genes**
k = A **# DE genes in set**

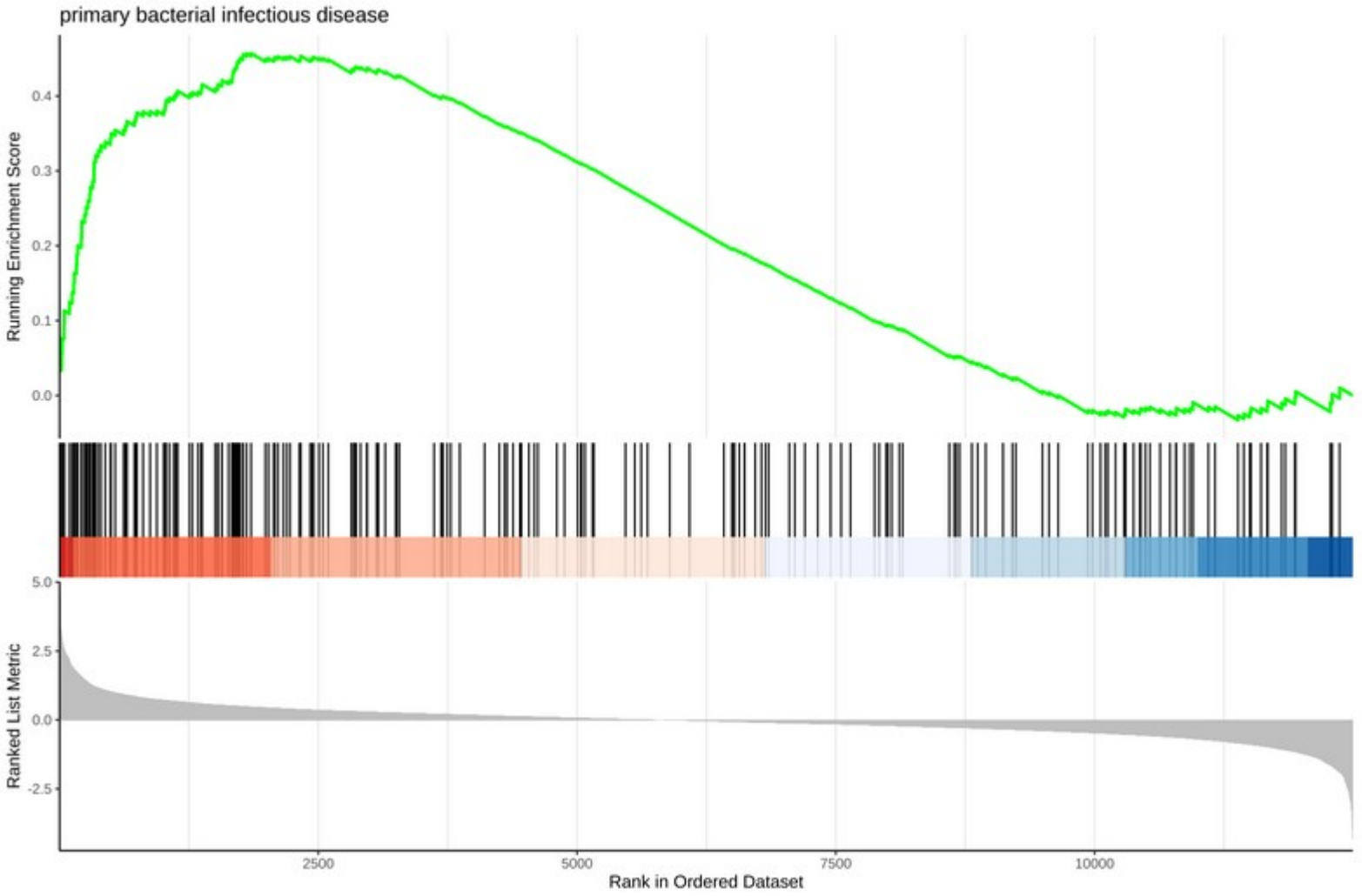
$$p = 1 - \sum_{i=0}^{k-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

Enrichment analysis – computing enrichment

2 possible approaches (among many!)

- Gene Set Enrichment Analysis
  - Do not rely on 0/1 DE status, but on a continuous measurement (eg. log2FC)
  - Computes Enrichment Score from list of ranked genes
  - Estimates significance using permutations

Enrichment analysis – computing enrichment



Enrichment Score visualized using functions from the R package enrichplot
http://www.bioconductor.org/packages/release/bioc/html/enrichplot.html

Enrichment analysis – computing enrichment

Many more methods or implementations:

- **Signaling Pathway Impact Analysis**
  - **https://bioconductor.org/packages/release/bioc/html/SPIA.html**
- **ISMARA**
  - **https://ismara.unibas.ch/mara/**

...

Practical

- Go to the website and follow the Enrichment practical

- Also, maybe give a try to gProfiler, and ORA tool developed and maintained in Estonia:

  https://biit.cs.ut.ee/gprofiler/gost

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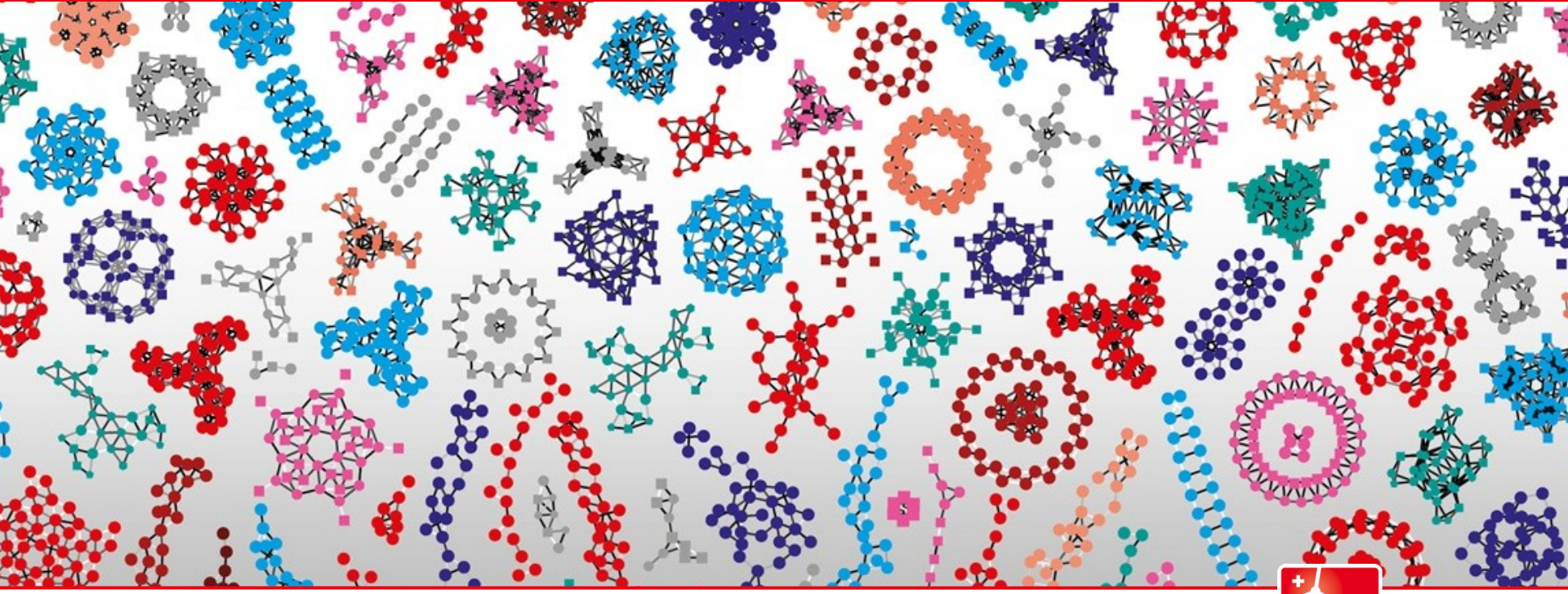

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