Introduction to RNA-seq for differential gene expression (DGE) analysis

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07/18/22

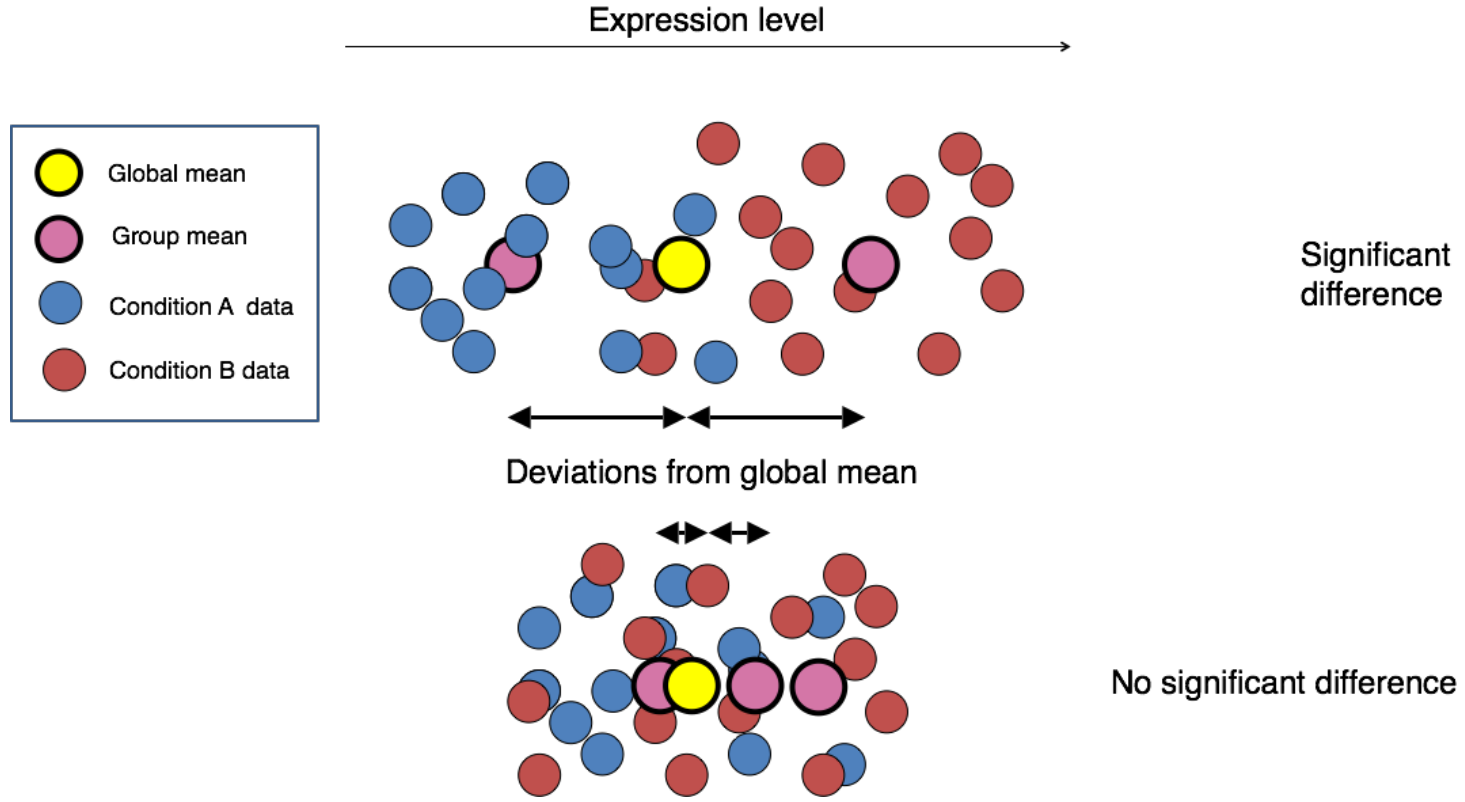
Differential expression analysis

- **Aim: Test quantitative expression changes between two or more experimental groups**

- Perform separate statistical test for read counts of each gene

Sample metadata:

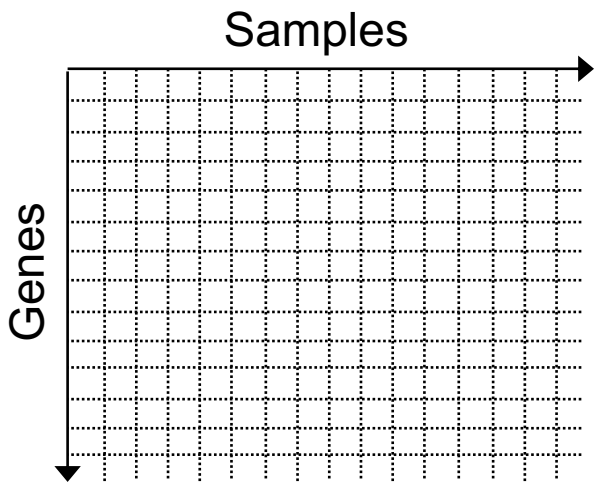
	Sex	Age	Tx-group
Sample_1	F	33	vehicle
Sample_1	F	21	vehicle
Sample_1	M	22	tx
Sample_1	F	29	tx
….	….	….	….
Sample_X	M	35	tx



https://hbctraining.github.io/DGE_workshop/lessons/04_DGE_DESeq2_analysis.html

Differential expression analysis

Gene expression matrix
(read counts)



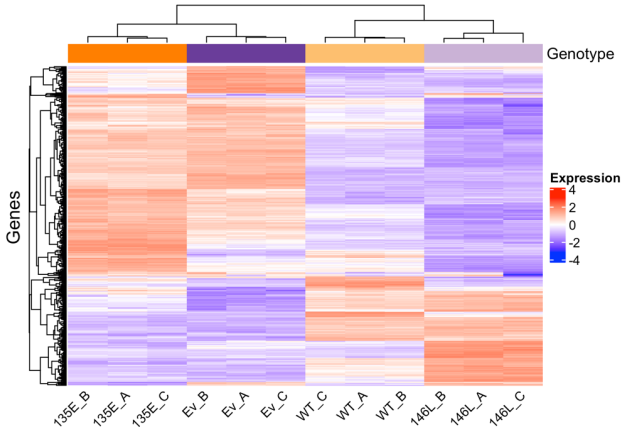
+

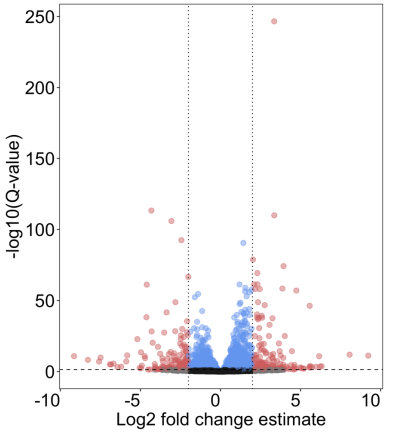
Sample metadata

- Normalization
- Exploratory analysis
- Statistical modeling
- Results prioritization
- Visualization

Differential expression results

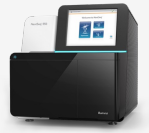
ID	lfc	lfcSE	stat	pvalue	padj	Gene
ENSG032219	2.89	0.12	-25.4	1.6E-251	1.7E-249	ARID4A
ENSG012951	-3.44	0.15	-24.5	1.2E-132	1.4E-122	KLK10
ENSG016754	-3.14	0.12	-24.1	2.9E-123	1.4E-118	KLK5
ENSG080097	-2.78	0.11	-20.7	5.7E-115	2.4E-109	LGALS1
ENSG006277	5.92	0.28	19.8	1.9E-80	3.5E-92	UCHL1
…..	…..	…..	…..	…..	…..	…..





Overview of a typical RNA-seq experiment

Sample/library preparation



Experimental Design
Sample groups
Replicate #
Sequencing strategy

Conduct experiment

Library preparation
Choose library type
RNA isolation procedure
Spike-ins

Sequencing
Paired or single-end
Choose read length
Read depth

Reads in FASTQ format

Data Pre-processing

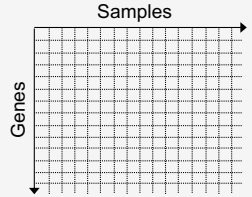
Quality control
Sequence quality
Adapter content
GC content

Read trimming
PolyA
Low quality bases
Adapters

Read mapping
Mapped %
rRNA %
Duplicate rate

Feature quantification
Read strand
Select genome annotation

Read counts



Downstream analysis

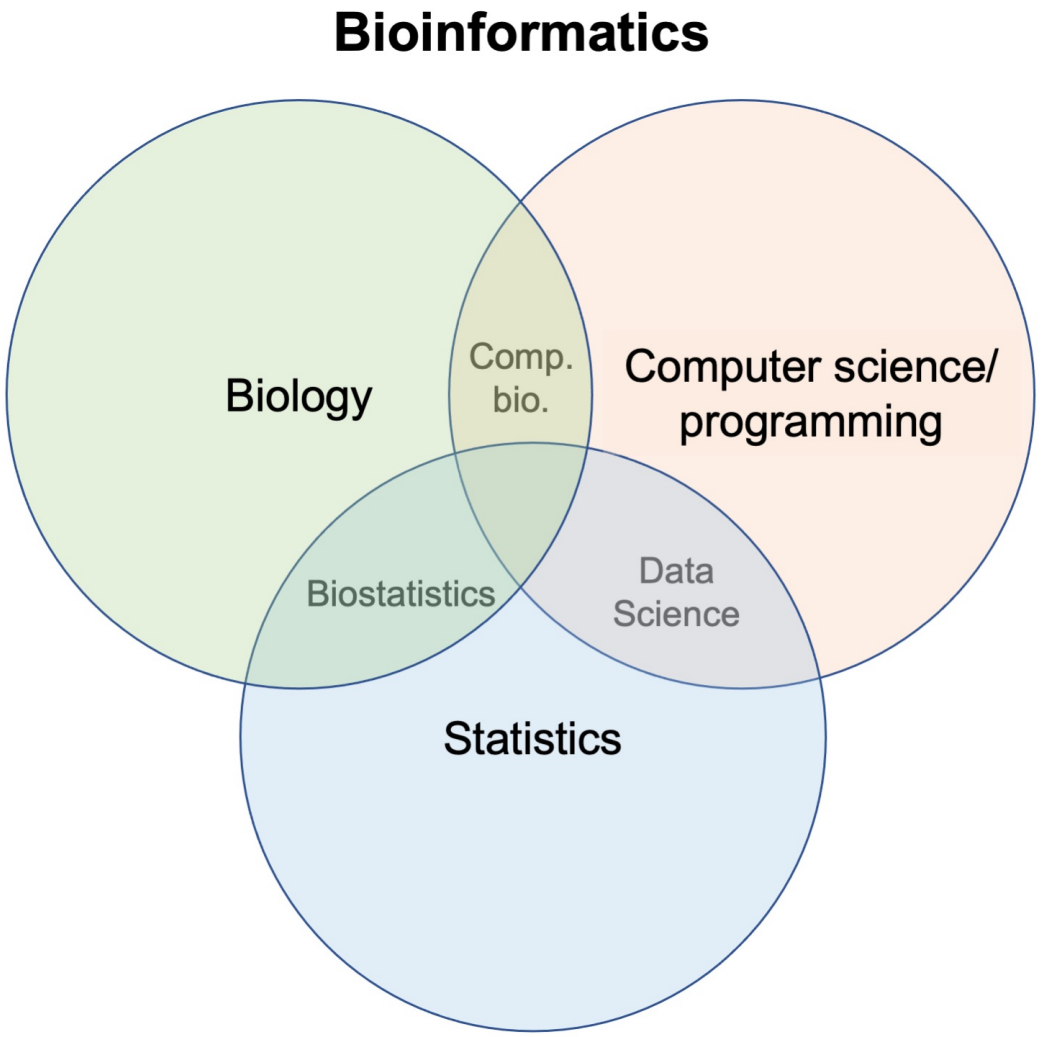
Normalization
RPKM/FPKM
TPM
Size factors/TMM

Differential expression
Statistical modeling
Visualization

Functional analysis
Pathway analysis
Gene ontology analysis

Integrative genomics
RNA-seq + ChIP-seq
RNA-seq + WGS/WES

Technical skills for RNA-seq analysis



Data pre-processing:

- Requires understanding of library preparation and sequencing technology
- More dependent on programming & computational skill set
- Often performed using established pipelines

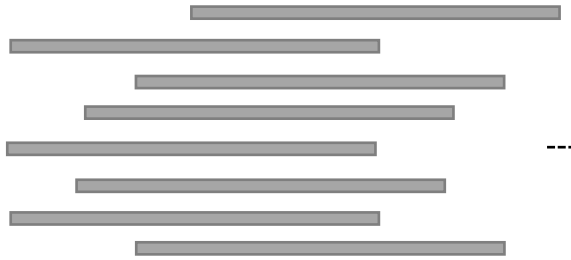
Down-stream analysis

- Requires basic knowledge of statistical concepts
- More dependent on statistical programming & data visualization skill set

Talk outline:

- **Library preparation**
  - Basic protocol
  - Types of libraries
- **Data pre-processing**
  - Quality control
  - Read trimming
  - Read mapping
  - Read counting/expression quantification
- **Sample preparation & experimental design**
  - Replicates
  - Sequencing depth
  - Sequencing configuration (read type & length)

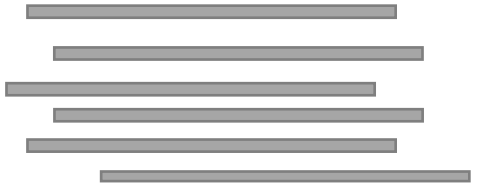
Library preparation for RNA-seq



Total RNA

Enrichment

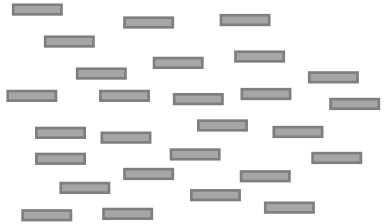
(e.g. PolyA capture, ribodepletion)



RNAs of interest

(e.g. mRNAs)

Fragmentation



Fragmented RNA

Reverse transcription (RT)

cDNA:RNA hybrid

5’

First strand synthesis

Special DNA polymerase used to RT cDNA using RNA as the template

Double stranded cDNA (dscDNA)

Second strand synthesis

3’ ends adenylated & 5’ ends repaired before adapter ligation

Index 1 SP1 DNA insert SP2 Index 2

Adapter ligation

Sequencing adapters & barcodes for sample multiplexing

PCR amplification

Amplified libraries

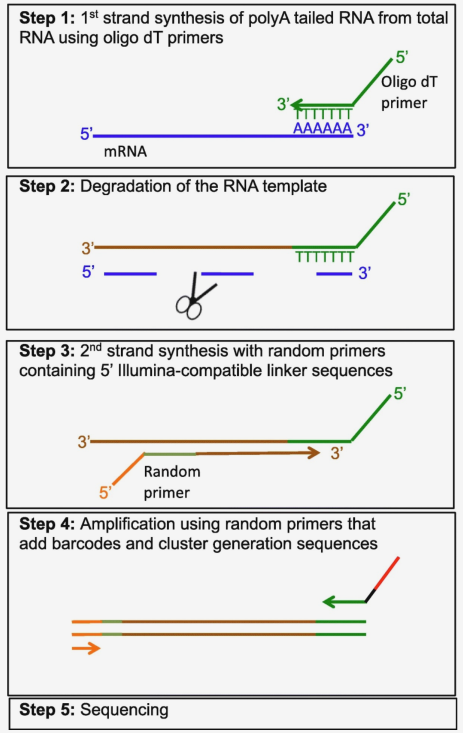
1 set for each sample

Sequencing ready cDNA pool

Applications of different library types

3’ End

3’ method (LEXO)

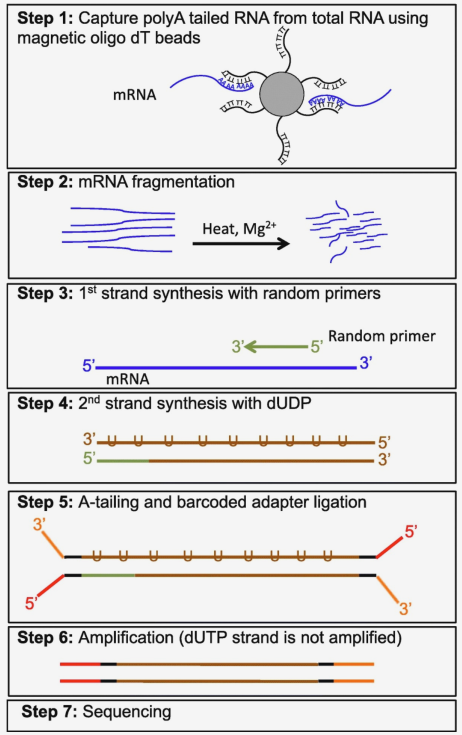


Adapted from Fukua *et al*, 2019. *Genom. Biol*

Differential Expression
Lower cost/High Throughput
Low Input and Low-Quality Samples
FFPE

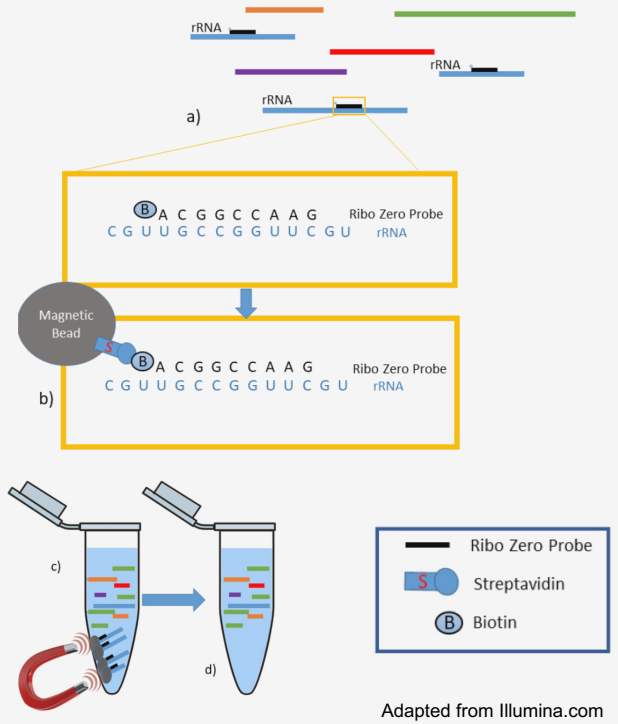
Full-length - PolyA

Traditional method (KAPA)



Full Length mRNA
Differential Expression
Splice Variants
SNV Detection
Low Input with Amplification

Ribodepletion



Adapted from Illumina.com

Full length mRNA + lincRNAs
Differential Expression
Splice Variants
SNVs
FFPE

Throughput

Cost / Data Richness

Image Credit:
Lexogen Inc

Sample preparation

- **Be consistent with sample prep**
  - Practice protocol 1st, don't get better over course of collecting more samples
- **Minimize batches**
  - 1 batch is ideal, otherwise, smallest number possible
  - MUST randomly distribute samples from experimental conditions across batches
  - Treat each batch EXACTLY the same
- **Collect replicates**
  - Statistics cannot be done on one sample! (statistics is study of populations)
  - The more you collect, the more power you have to discover DEGs
  - Make each replicate as similar as possible e.g. same passage number of cell line
- **Work with your genomics core (they do this a lot)**
- **Pilot experiments can be valuable**

Spend time to creating a high-quality dataset upfront, rather than trying to improve & rescue it later

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Low quality bases
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Mapped %
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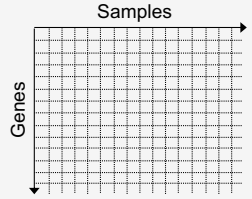
Duplicate rate

Feature quantification
Read strand
Select genome annotation

Read counts



Downstream analysis

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Size factors/TMM

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Visualization

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Gene ontology analysis

Integrative genomics
RNA-seq + ChIP-seq
RNA-seq + WGS/WES

FASTQ files

- 4 lines per record
- Single file or 1 per direction for paired-end reads (R1.fastq vs. R2.fastq)

Single FASTQ entry:

```
@SRR1039508.1 HWI-ST177:290:C0TECACXX:1:1101:1225:2130 length=63
CATTGCTGATACCAANNNNNNNNGCATTCCTCAAGGTCTTCCTCCTTCCCTTACGGAATTACA
+
HJJJJJJJJJJJJJ########00?GHIJJJJJJJIJJJJJJJJJJJJJJJJJHHHFFFFFD
```

- Row 1: Header line (starts with @)
- Row 2: Base calls
- Row 3: '+' or line 1
- Row 4: Base qualities

In bash:

```
> zcat $sample.fastq.gz | head –n 12
```

```
[d41294d@discovery7 ATACseq_6-4-19]$ zcat 648-PKA-Cre-C3Tag_S4_R2_001.fastq.gz | head -n12
@NB501031:325:HK52YBGXB:1:11101:9675:1055 2:N:0:ACCACTGT
CTGGGCAGCTGGGGGGTGGGGTGGGGAAGACACAGGAGCCACACAGAGAGAGACATCCCATCCTGTCTCCATTGCT
+
AAAAAEEEEAEEEEEE/E/EE6EEEEEEEAEEEEEEA//EEEEEAEEEAEEE/E/EEEA<EEEEEEEEEEE/EAEE
@NB501031:325:HK52YBGXB:1:11101:7613:1055 2:N:0:ACCACTGT
GTGTTAGGGACTTCTCAAGGAAGTTCTATAGATAGAGCCCAGTACTCTTGAGTGACAGGGGGAACATCTGTGTAAA
+
6AA6AEAAE/EEEEEEEEEEEE/AEAEEEEEE6EEEAE/EEEE//EE/AEE/E/<EEEEEEE/EEEAEE<EAAEE
@NB501031:325:HK52YBGXB:1:11101:16664:1055 2:N:0:ACCACTGT
ATGCTGGAGTTCTGTGGCCACCACCTTCTCAAACACTCATTCATCCATTGAATGGGACATAGGGGACAATAGTGAT
+
AAAAAEEAEAEEEEEEEA</A<AEAEEEEEEA/E//EEEEEAEEEEEA/EE/EEEEEEEAA6/EA/EE/</EEE/E
```

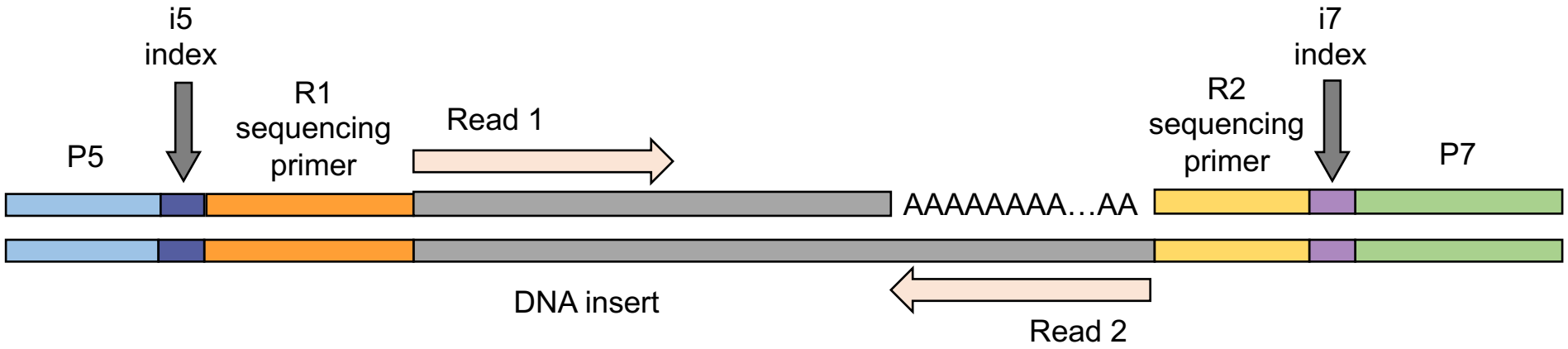
Phred Score	Probability of incorrect call	Accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%
40	1 in 10,000	99.99%
50	1 in 100,000	99.999%
60	1 in 1,000,000	99.9999%

Read trimming (pre-processing)

Commonly trimmed sequences:
- Sequencing adapters
- PolyA tails
- Low quality bases
- 'N' base calls (no call)

- Process of 'cleaning' NGS reads to improve **speed** & **quality** of downstream analysis
- NGS reads contain sequences not wanted for downstream steps:
- Trimming algorithms search reads for specified sequences and remove matches based on user specified behavior

Typical Illumina RNA-seq library

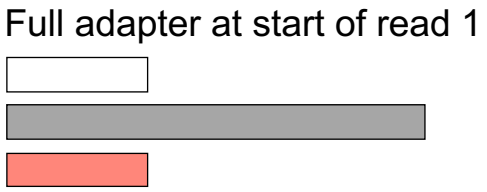


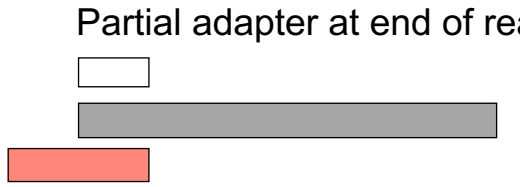
Legend:

DNA insert

Adapter

Trimmed



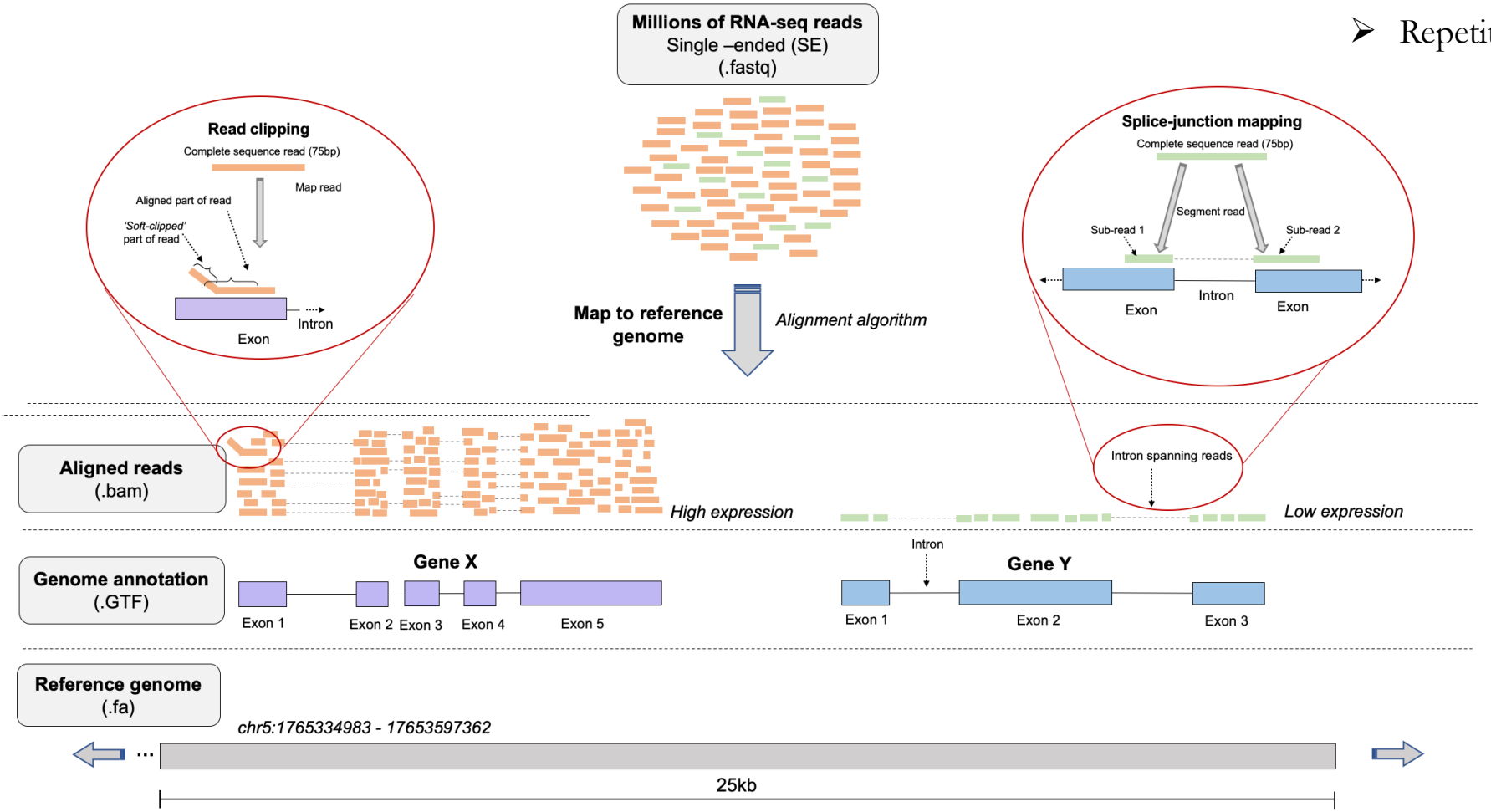


Read mapping to a reference genome

- Reads can be aligned to a reference genome for well studied organisms. e.g. hg38, mm10
- **Where is the most likely origin for a read in a reference genome?**

Challenges:

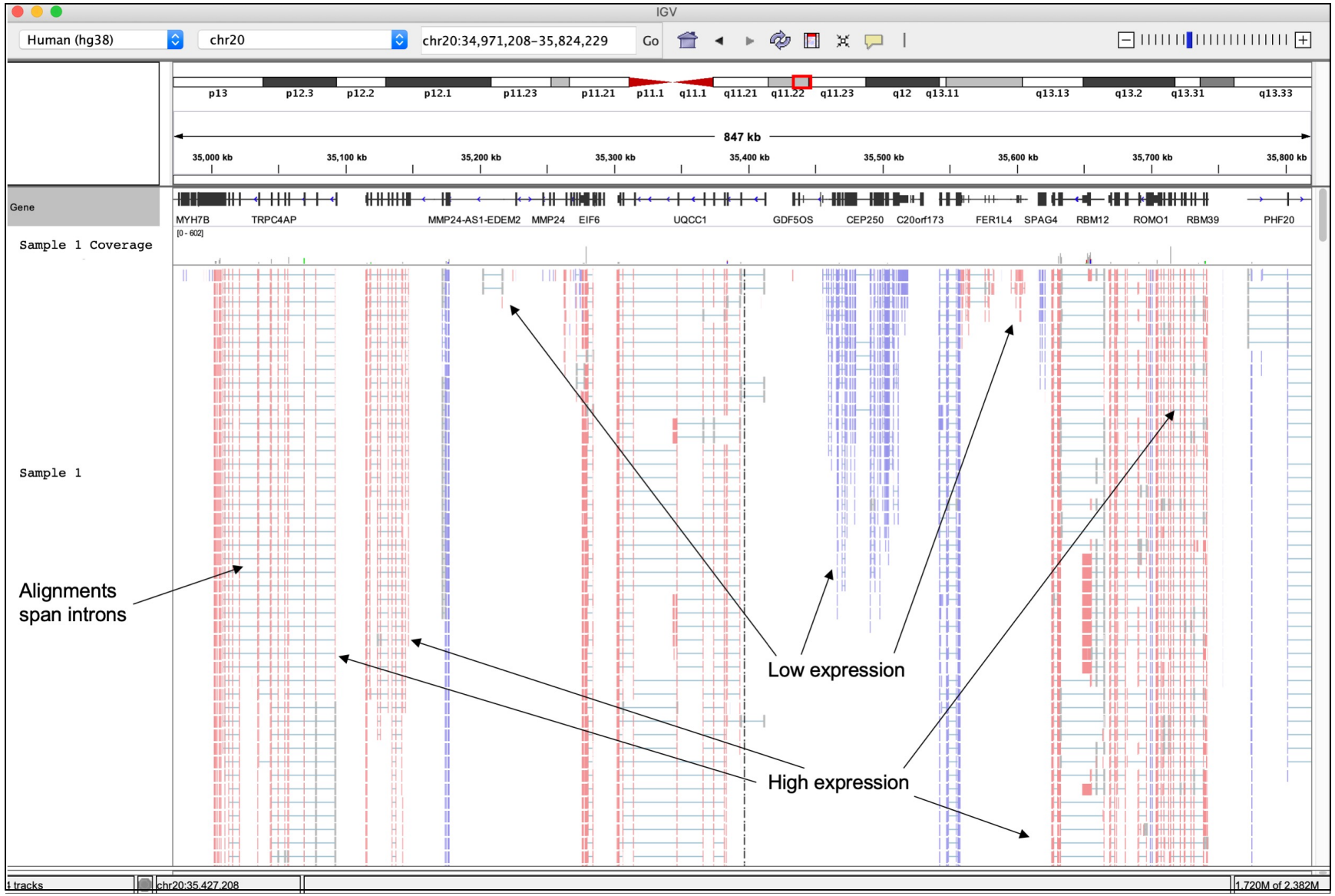
- Enormous search space
- Computationally intensive
- Genome vs transcriptome
- Genetic variation
- Repetitive sequences



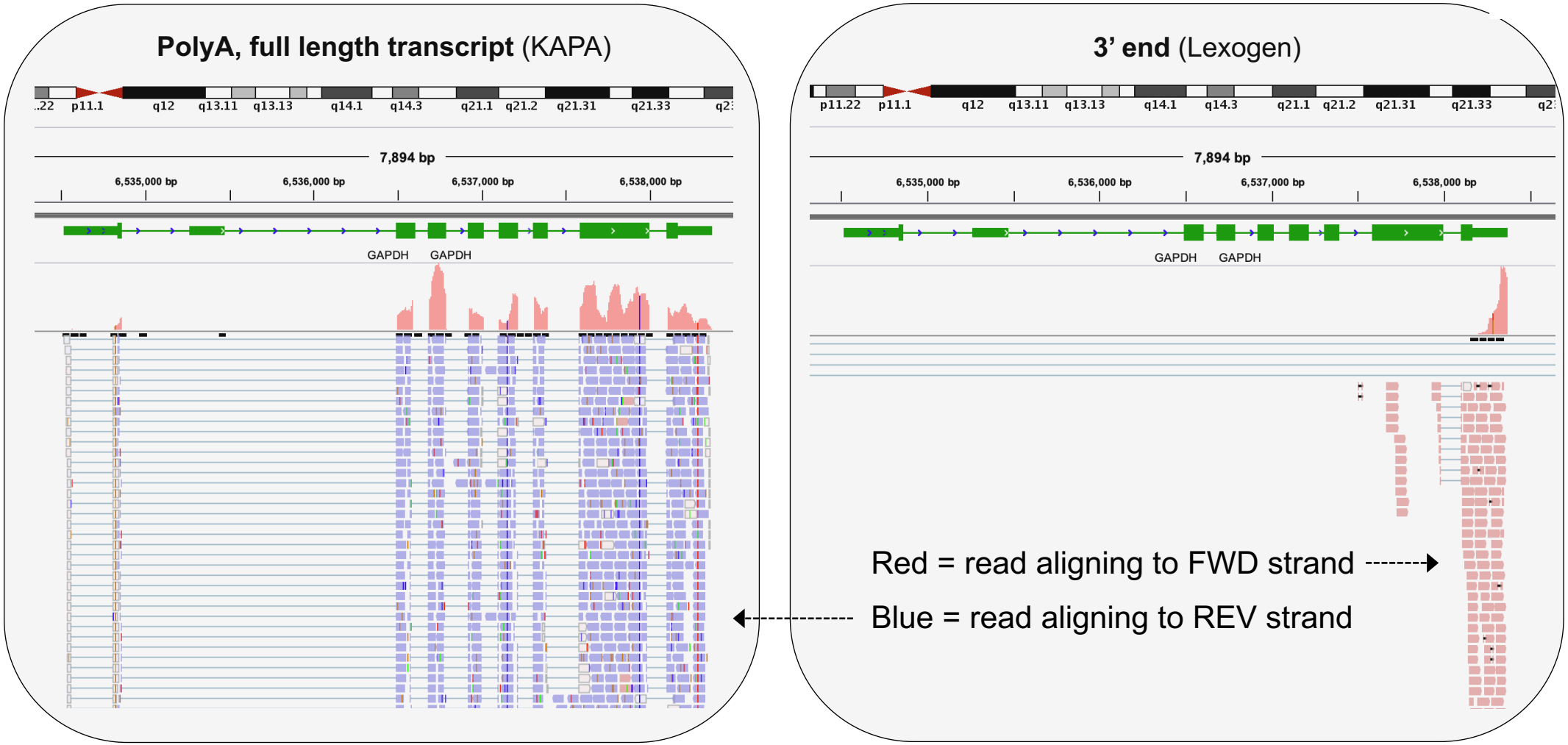
Conesa et al, Genome Biol, 2016, DOI 10.1186/s13059-016-0881-8

Aligned reads in browser

- Real data example using Integrative Genomic Viewer (IGV)
- This is just <u>1 sample</u>



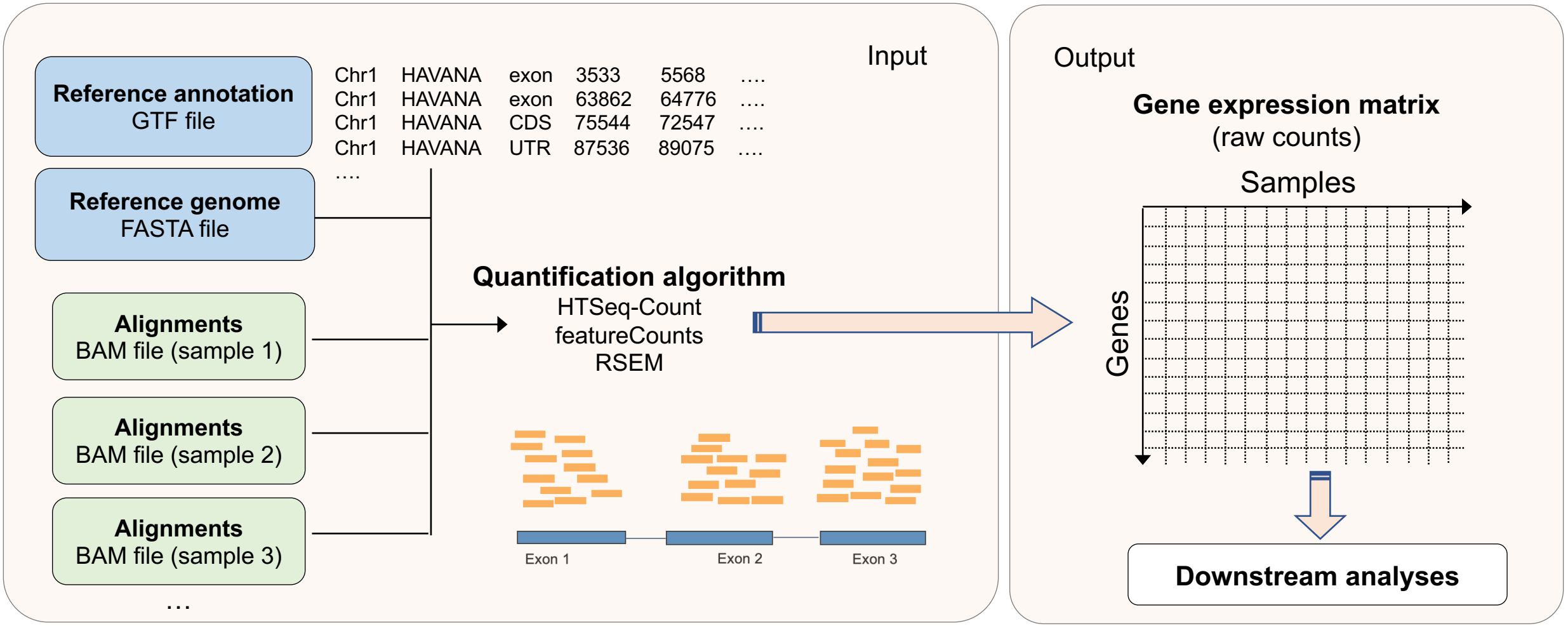
Data from different library types looks different



Quality control, analysis pipelines, and possible hypotheses therefore inherently differ

Quantification (read counting)

- Count reads at each genomic position for desired feature of interest (usually exons for RNA-seq)



Replicates

- Arguably more important than read depth or length for DE

How many biological replicates are needed in an RNA-seq experiment and which differential expression tool should you use?

NICHOLAS J. SCHURCH,[1,6] PIETÀ SCHOFIELD,[1,2,6] MAREK GIERLIŃSKI,[1,2,6] CHRISTIAN COLE,[1,6] ALEXANDER SHERSTNEV,[1,6] VIJENDER SINGH,[2] NICOLA WROBEL,[3] KARIM GHARBI,[3] GORDON G. SIMPSON,[4] TOM OWEN-HUGHES,[2] MARK BLAXTER,[3] and GEOFFREY J. BARTON[1,2,5]

[1]Division of Computational Biology, College of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom
[2]Division of Gene Regulation and Expression, College of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom
[3]Edinburgh Genomics, University of Edinburgh, Edinburgh EH9 3JT, United Kingdom
[4]Division of Plant Sciences, College of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom
[5]Division of Biological Chemistry and Drug Discovery, College of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom

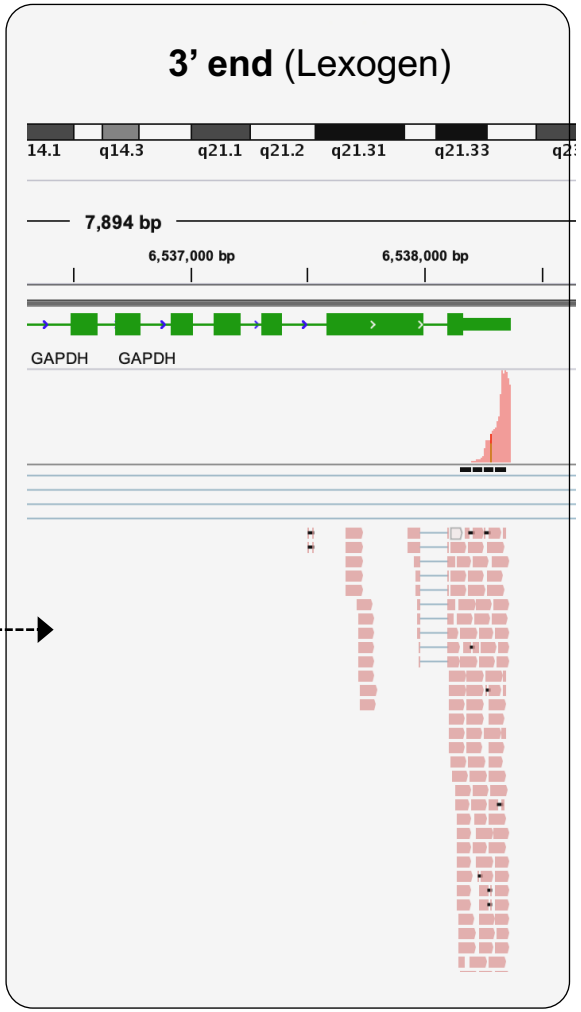
"With 3 biological replicates, 9 of the 11 tools evaluated found only 20%–40% of the significantly differentially expressed (SDE) genes"

- Schurch *et al,* RNA, 2016

- Suggested minimum no. of replicates should = 6
- More heterogeneity = more replicates needed (e.g. human tissues vs. cultured cell lines)

Sequencing depth (or coverage?)

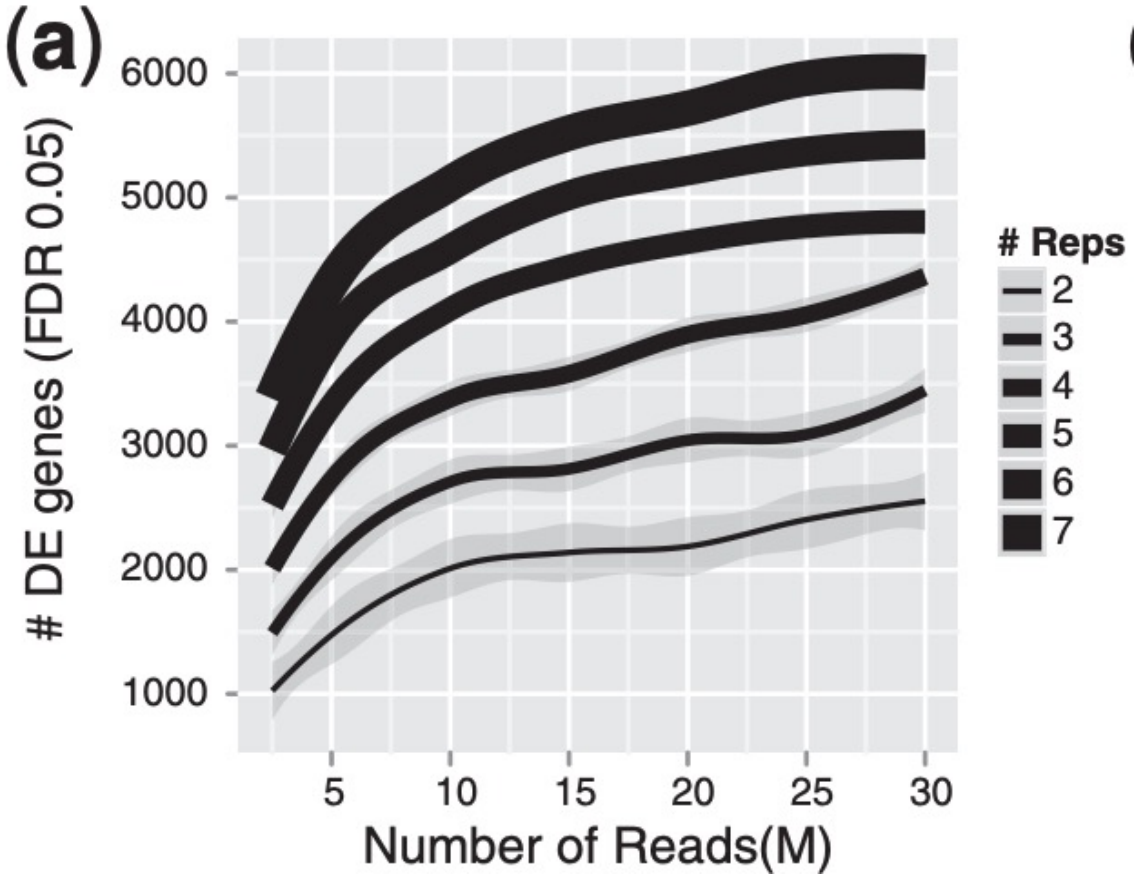
- ‘*Coverage*’ doesn’t have much meaning for transcriptome data
  - We are less concerned with how many times we cover a specific locus w/ a read..
  - More meaningful for WES, WGS, Genome Assembly
- Generally total of <u>10-30 million reads</u> for DGE of eukaryotic genomes
- Some species require many fewer than this
- Technology also affects required read number (3’-end data needs fewer)
- Checking saturation can help you assess if you’ve sequenced enough (next slide)
- Try to avoid generating libraries of differing complexity (vastly different reads nos.)



Depth vs. Replicates

Which is more important?

- No. of DEGs increases w/ replicate no.
- Diminishing returns after 10-15M reads (for this dataset)
- Additional replicates are more valuable than sequencing really deeply (for DE analysis)



Liu *et al*, 2014, *Bioinformatics*

Replicates & Statistical power

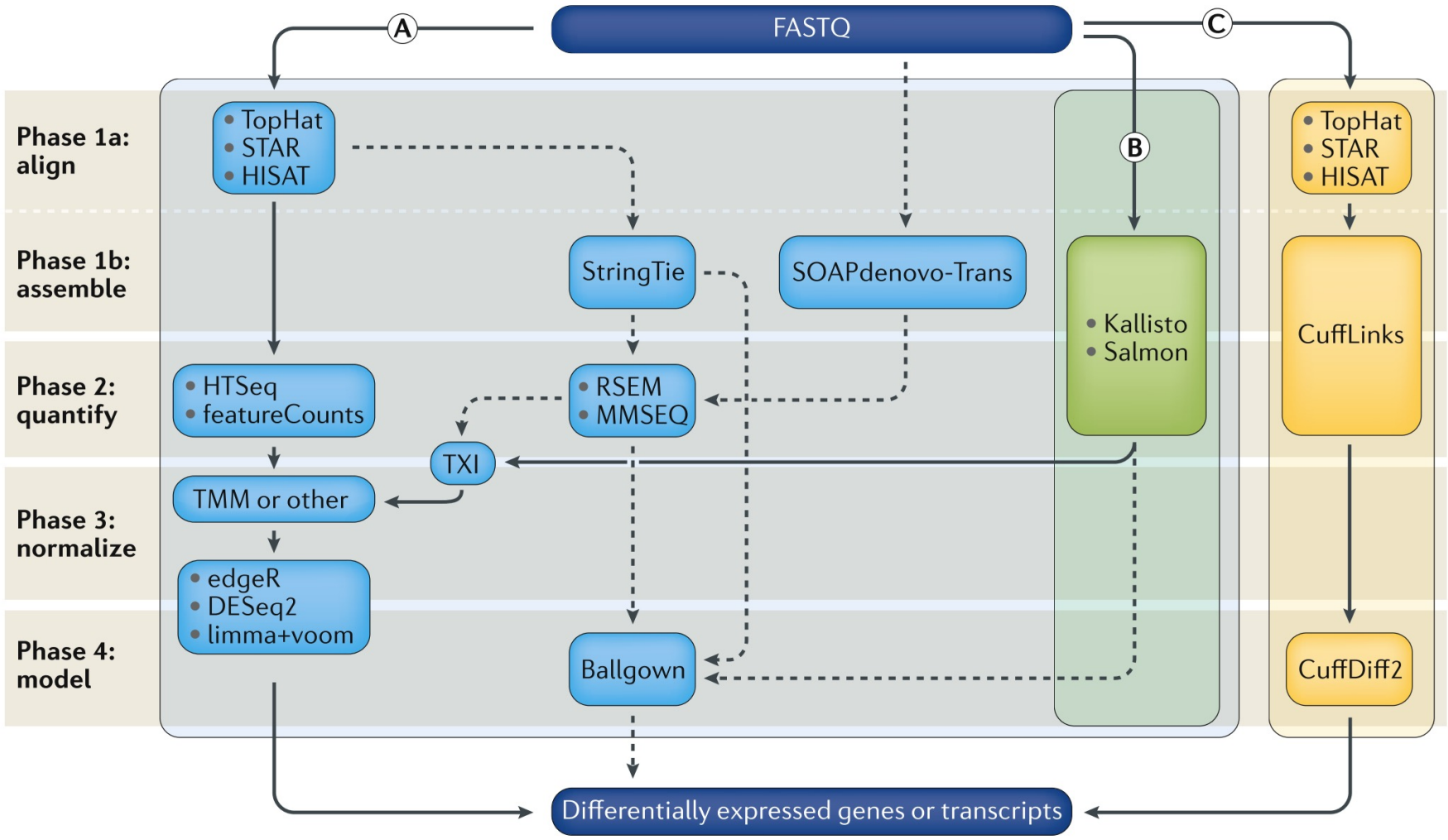
- More replicates improve power to detect smaller fold changes (effect size)
- More replicates improve power at the same sequencing depth

Table 1 Statistical power to detect differential expression varies with effect size, sequencing depth and number of replicates

	Replicates per group		
	3	5	10
Effect size (fold change)			
1.25	17 %	25 %	44 %
1.5	43 %	64 %	91 %
2	87 %	98 %	100 %
Sequencing depth (millions of reads)			
3	19 %	29 %	52 %
10	33 %	51 %	80 %
15	38 %	57 %	85 %

Conesa *et al*, 2016, *Genome Biol.*

Differential expression workflow(s)



- Several tools available for each step
- Each have various strengths, weaknesses, applications

Stark *et al,* 2019, *Nat Reviews genetics*

Differential analysis methods

Table 1: Software packages for detecting differential expression

Method	Version	Reference	Normalization[a]	Read count distribution assumption	Differential expression test
edgeR	3.0.8	[4]	TMM/Upper quartile/RLE (DESeq-like)/None (all scaling factors are set to be one)	Negative binomial distribution	Exact test
DESeq	1.10.1	[5]	DESeq sizeFactors	Negative binomial distribution	Exact test
baySeq	1.12.0	[6]	Scaling factors (quantile/TMM/total)	Negative binomial distribution	Assesses the posterior probabilities of models for differentially and non-differentially expressed genes via empirical Bayesian methods and then compares these posterior likelihoods
NOIseq	1.1.4	[7]	RPKM/TMM/Upper quartile	Nonparametric method	Contrasts fold changes and absolute differences within a condition to determine the null distribution and then compares the observed differences to this null
SAMseq (samr)	2.0	[8]	SAMseq specialized method based on the mean read count over the null features of the data set	Nonparametric method	Wilcoxon rank statistic and a resampling strategy
Limma	3.14.4	[9]	TMM	voom transformation of counts	Empirical Bayes method
Cuffdiff 2 (Cufflinks)	2.0.2-beta	[10]	Geometric (DESeq-like)/quartile/classic-fpkm	Beta negative binomial distribution	*t*-test
EBSeq	1.1.7	[11]	DESeq median normalization	Negative binomial distribution	Evaluates the posterior probability of differentially and non-differentially expressed entities (genes or isoforms) via empirical Bayesian methods

Seyednasrollah *et al*, *Brief. In Bioinfo*. 2013

Differential analysis methods

scientific reports

Check for updates

OPEN

Systematic comparison and assessment of RNA-seq procedures for gene expression quantitative analysis

Luis A. Corchete[1,2,3,4,5]✉, Elizabeta A. Rojas[1,2,3], Diego Alonso-López[2], Javier De Las Rivas[2,3], Norma C. Gutiérrez[1,2,3,5] & Francisco J. Burguillo[4]

Soneson and Delorenzi *BMC Bioinformatics* 2013, **14**:91
http://www.biomedcentral.com/1471-2105/14/91

BMC Bioinformatics

RESEARCH ARTICLE Open Access

A comparison of methods for differential expression analysis of RNA-seq data

Charlotte Soneson[1*] and Mauro Delorenzi[1,2]

Rapaport *et al. Genome Biology* 2013, **14**:R95
http://genomebiology.com/2013/14/9/R95

Genome Biology

METHOD Open Access

Comprehensive evaluation of differential gene expression analysis methods for RNA-seq data

Franck Rapaport[1], Raya Khanin[1], Yupu Liang[1], Mono Pirun[1], Azra Krek[1], Paul Zumbo[2,3], Christopher E Mason[2,3], Nicholas D Socci[1] and Doron Betel[3,4*]

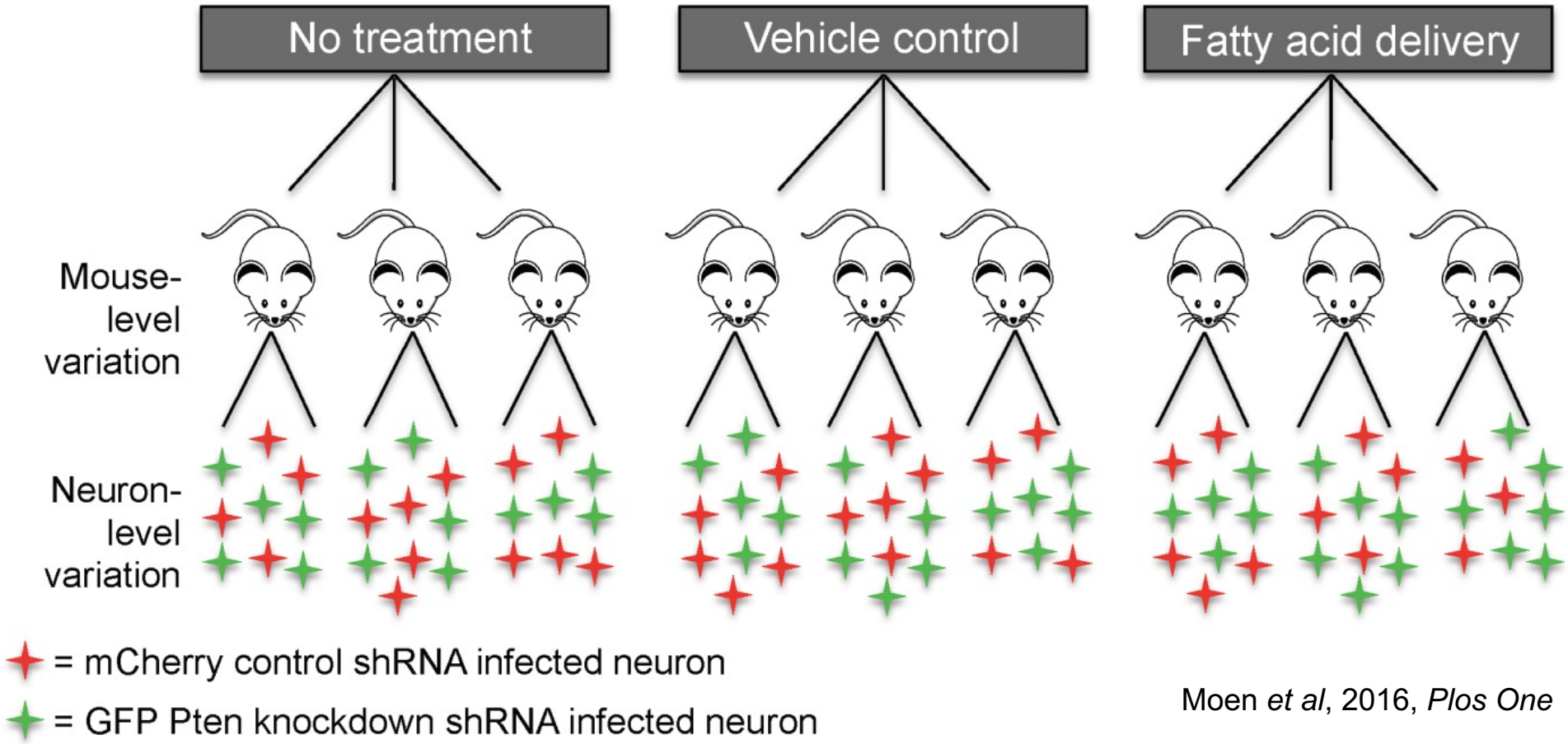
Comparison of software packages for detecting differential expression in RNA-seq studies

Fatemeh Seyednasrollah, Asta Laiho and Laura L. Elo

Submitted: 20th August 2013; Received (in revised form): 9th October 2013

➢ See *'Useful links'* folder on GitHub

Hierarchical study designs



Moen *et al*, 2016, *Plos One*

- Within-sample correlation must be accounted for
- Applies to time-course experiments also
- Limma can handle hierarchical study designs

Take home messages

- Work with your genomics core to design an experiment that will address your hypothesis
- You should always know and understand the type of library used to generate your data
- If you don’t perform data pre-processing, you should understand the basics steps involved
- Replicates are critical for differential expression analysis
- Various DE analysis tools are available. Experimental design can affect which one is most suitable

Questions?