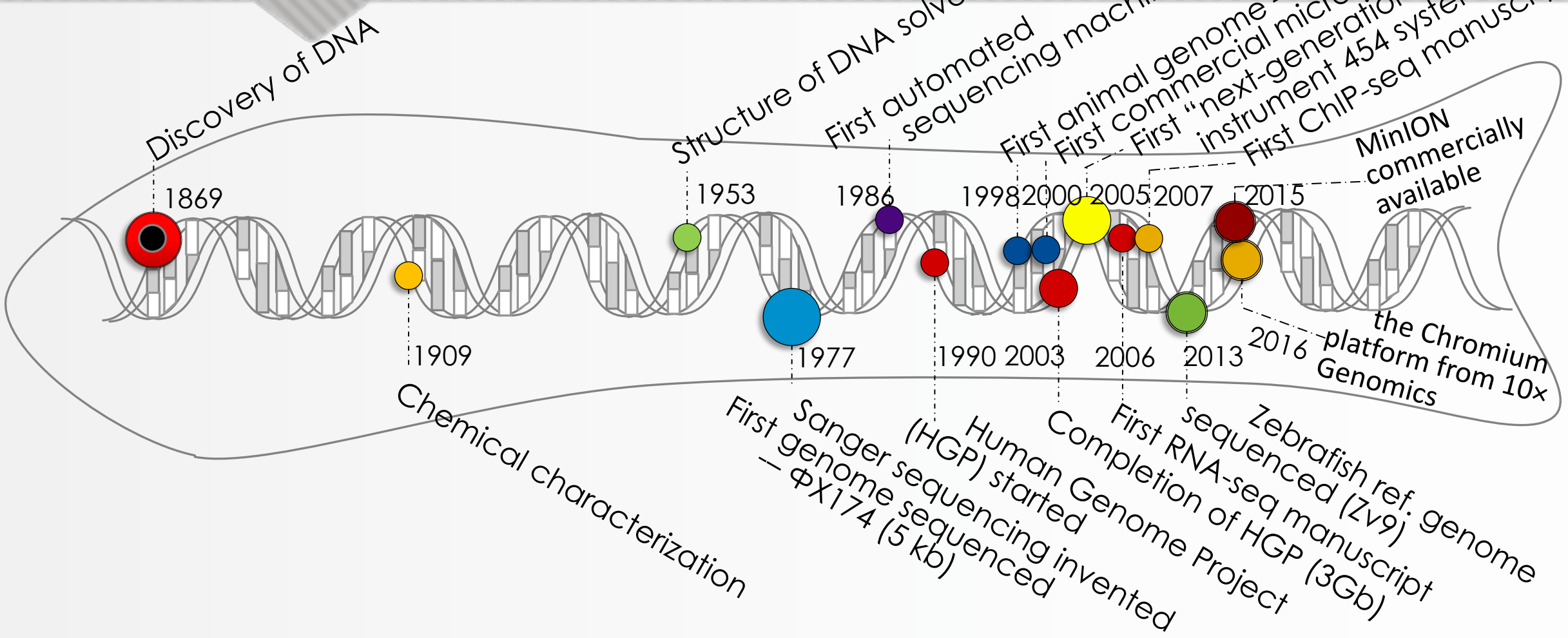
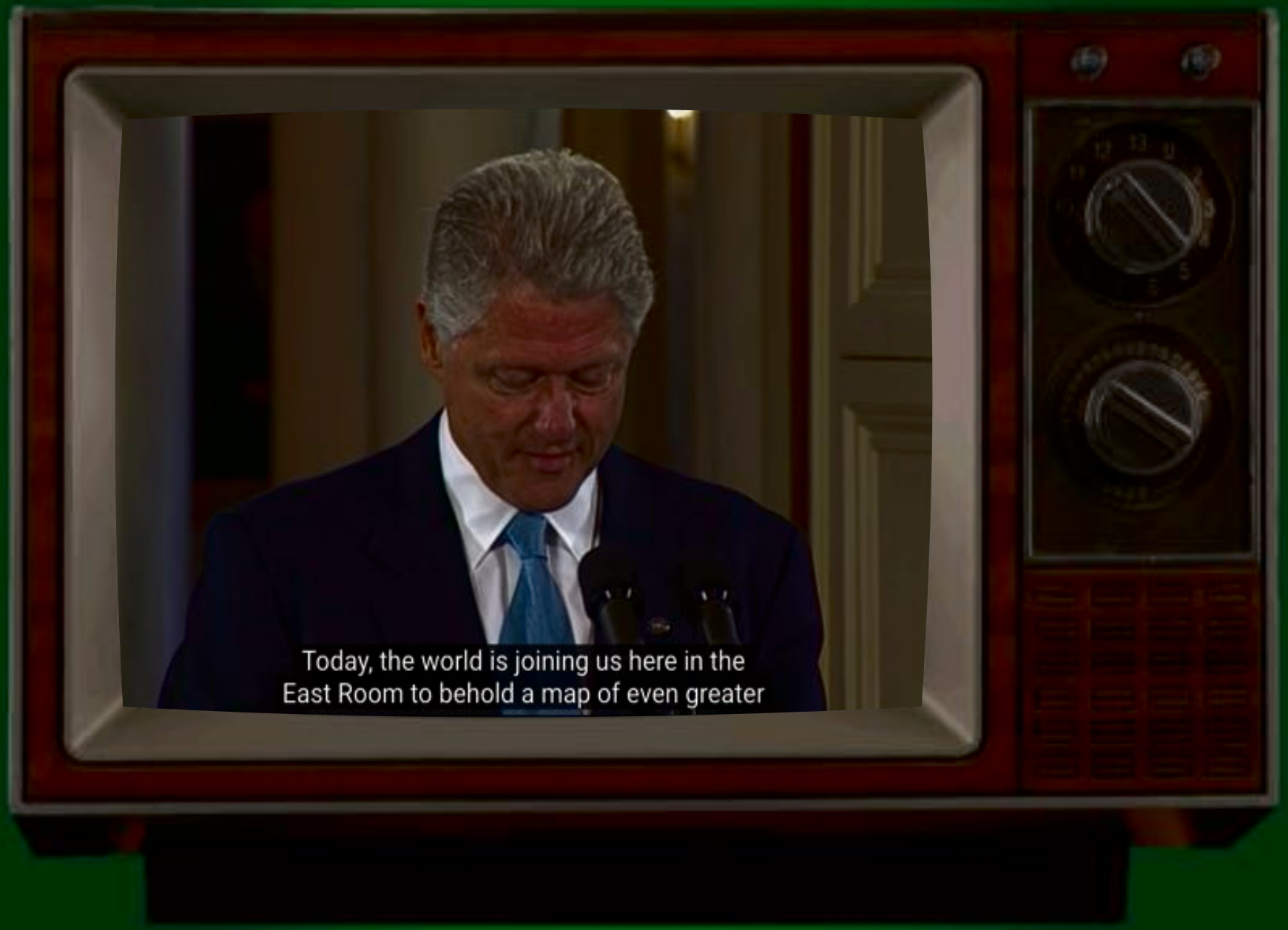
RNA-seq and ChIP-seq Analysis primer

Jianhong Ou @ DUKE, 2020

A QUICK HISTORY OF SEQUENCING





20 Years of the Human Genome

June 2000: International Human Genome Sequencing Consortium Announces "Working Draft" of Human Genome

https://www.genome.gov/news/news-release/NHGRI-commemorates-20th-anniversary-of-white-house-event-announcing-draft-human-genome-sequence

3 Main Steps for RNA-Seq and ChIP-Seq

01

Experimental design

Library type / strategy
Sequencing depth
Number of replicates
Batch effect

Consult a data analyst or bioinformatician to make sure robust experimental design

02

Bench work

RNA/DNA extraction
Library preparation
Sequencing & imaging

Double check

1. **Library prep batch**
2. **Layout of library on lane/ flow cell**

03

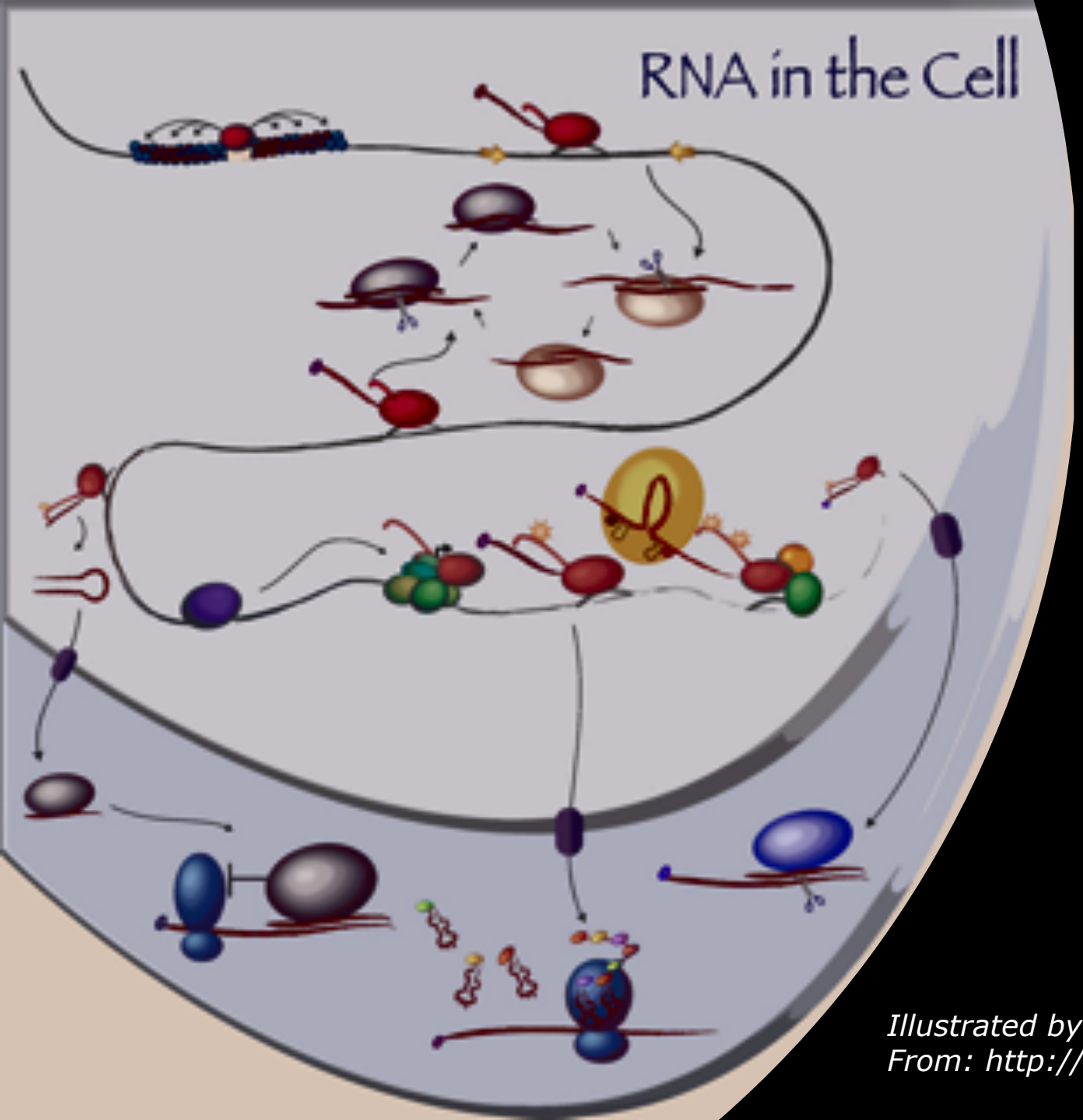
Data analysis

Quality control
Alignment & Quantification
Differential Expression test
Functional profiling

Discuss and optimize the analysis to meet you experiment design

1. **Blind analysis to avoid the bias**
2. **Discuss in time to optimize the analysis**

RNA-SEQ



The transcriptome is very complex

Illustrated by Mary Lindstrom; designed by M.L. and Amy White
From: http://web.mit.edu/sharplab/researchsummary.html

coding RNA

mRNA

Most are poly-adenylated

Non-coding RNA

Small RNAs

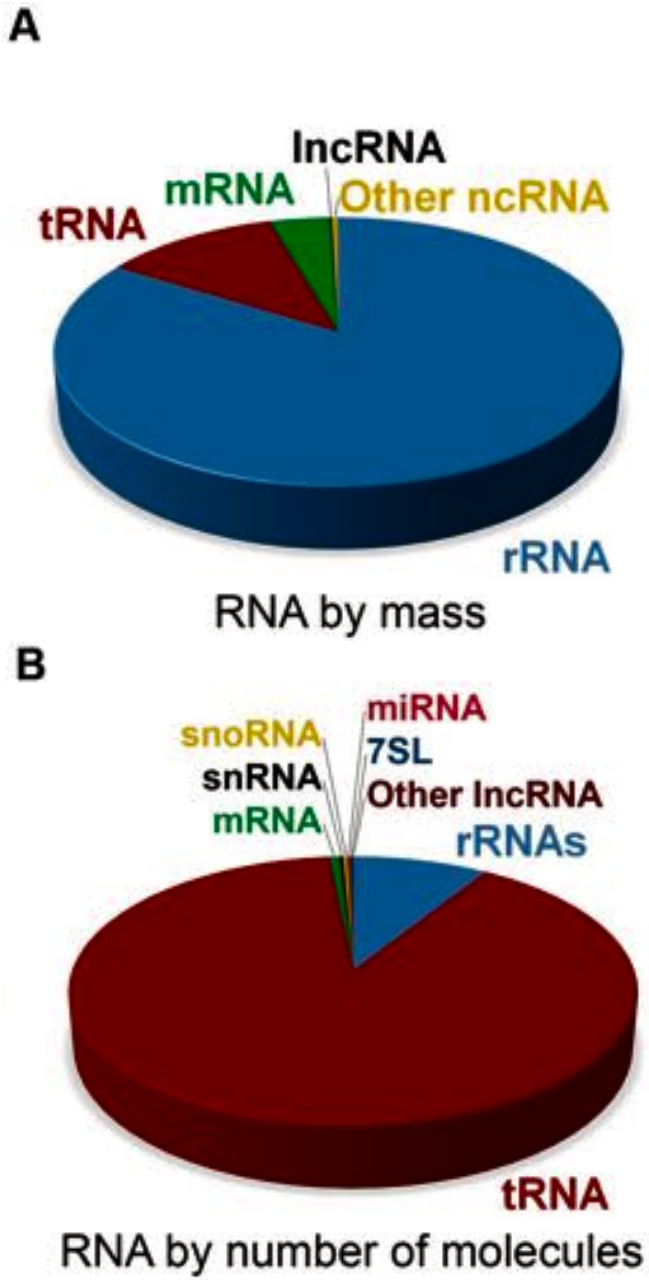
- siRNA
- miRNA
- snoRNA
- snRNA

Transcriptional RNAs

- rRNA
- tRNA

lncRNAs

Many are NOT poly-adenylated



Palazzo et.al., 2015. doi: 10.3389/fgene.2015.00002

coding RNA

mRNA

Most are poly-adenylated

Non-coding RNA

Small RNAs

- siRNA
- miRNA
- snoRNA
- snRNA

Transcriptional RNAs

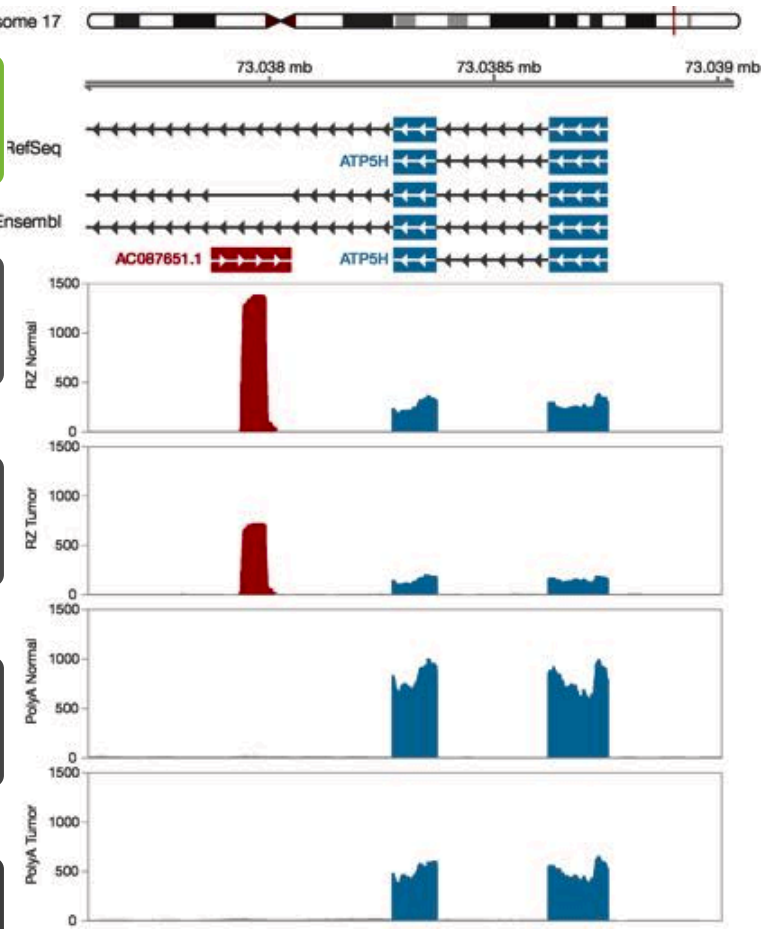
- rRNA
- tRNA

lncRNAs

Many are NOT poly-adenylated

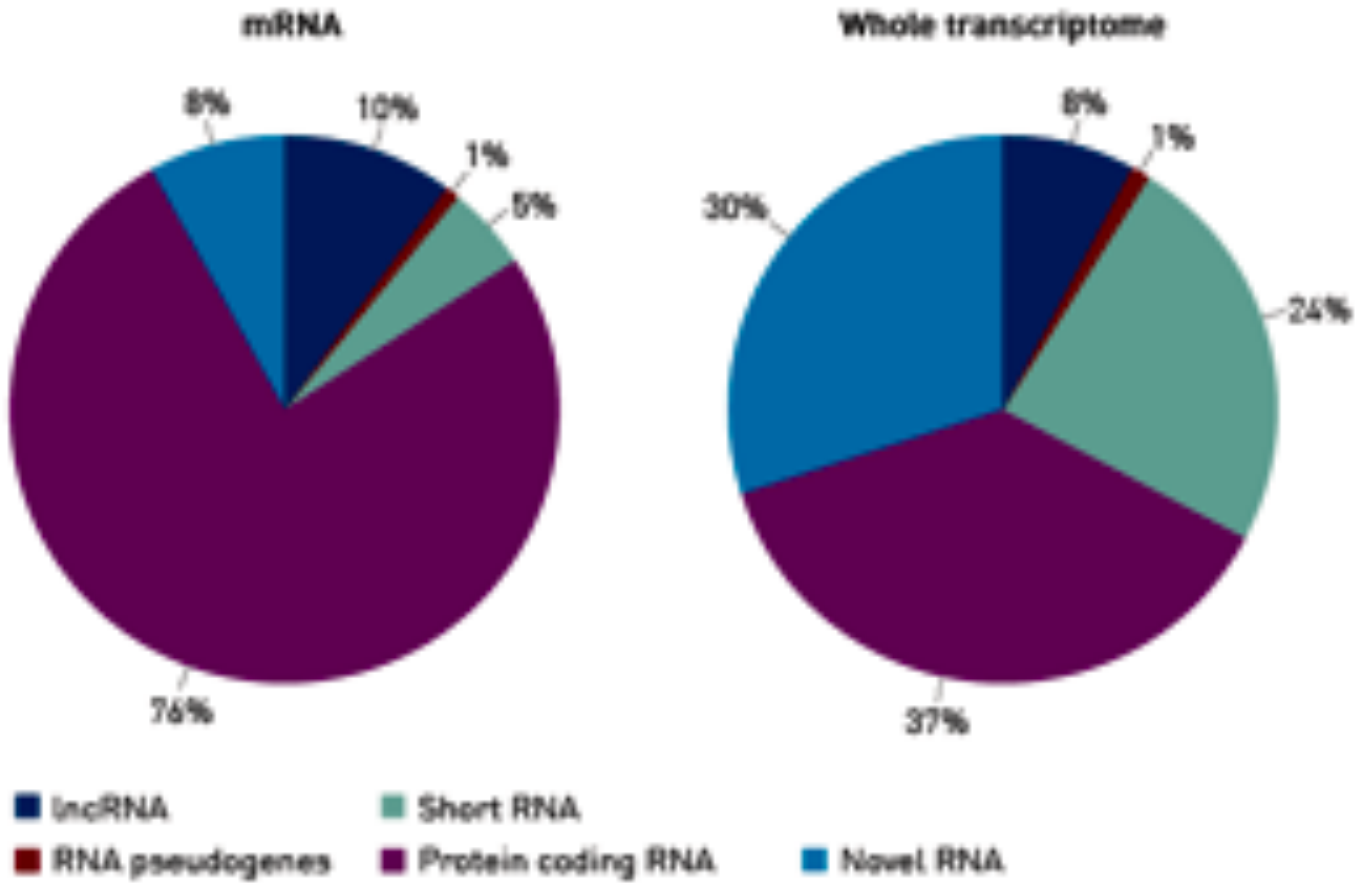
TruSeq mRNA → Capture mRNA → fragment → Make cDNA → add Adapters → Sequence

TruSeq Total RNA → Remove rRNA → fragment → Make cDNA → add Adapters → Sequence



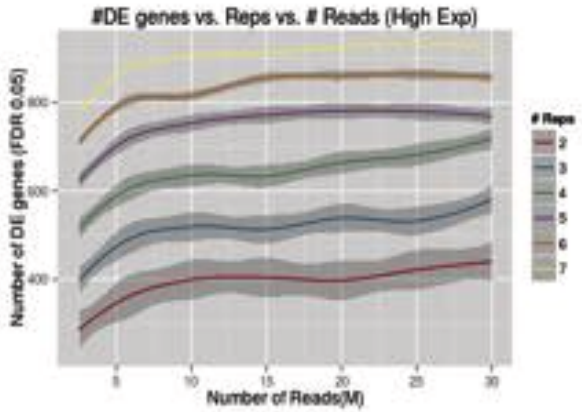
https://www.illumina.com/content/dam/illumina-marketing/documents/products/datasheets/datasheet_truseq_stranded_rna.pdf

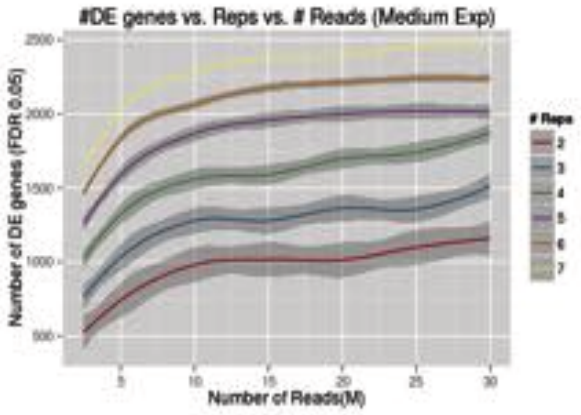
Distribution of RNA species in NGS samples after poly-A enrichment (mRNA) and rRNA depletion (whole transcriptome) sequencing

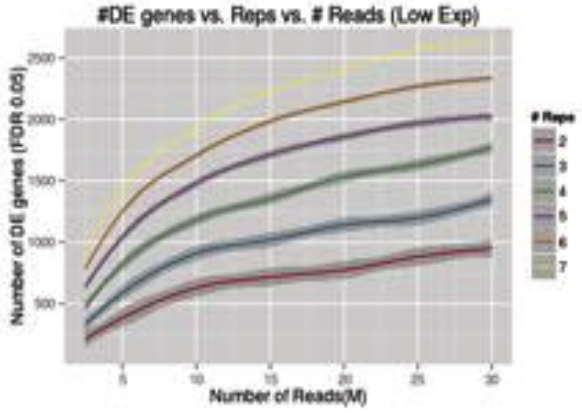


http://www.exiqon.com/whole-transcriptome-ngs

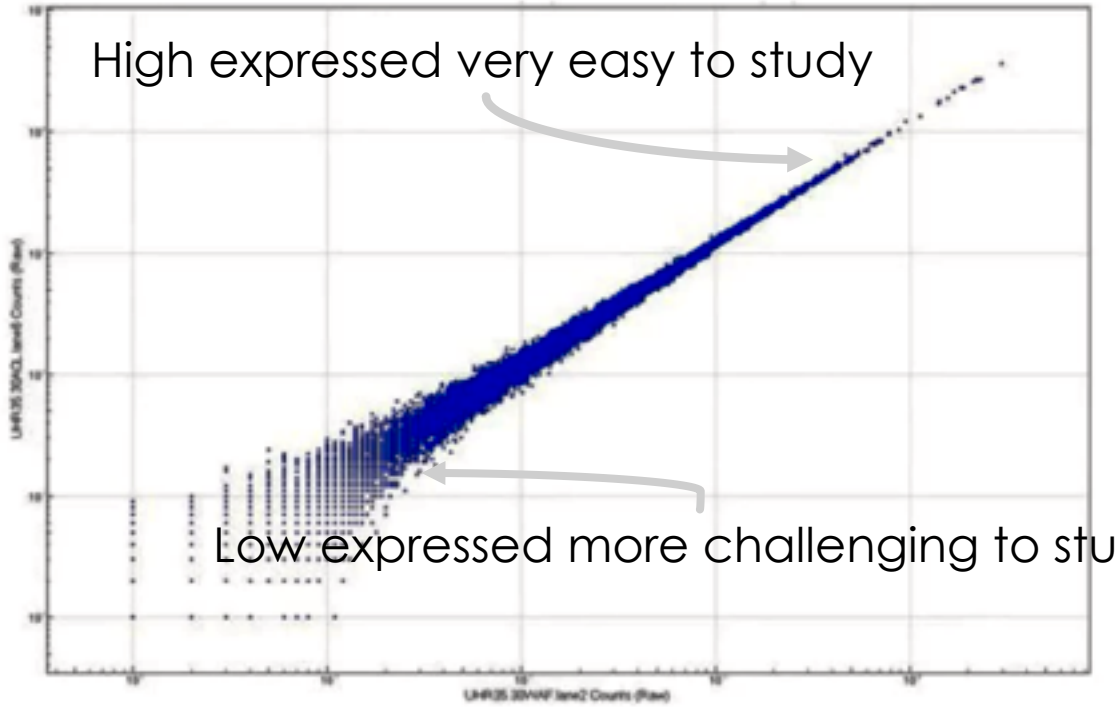
How many reads?

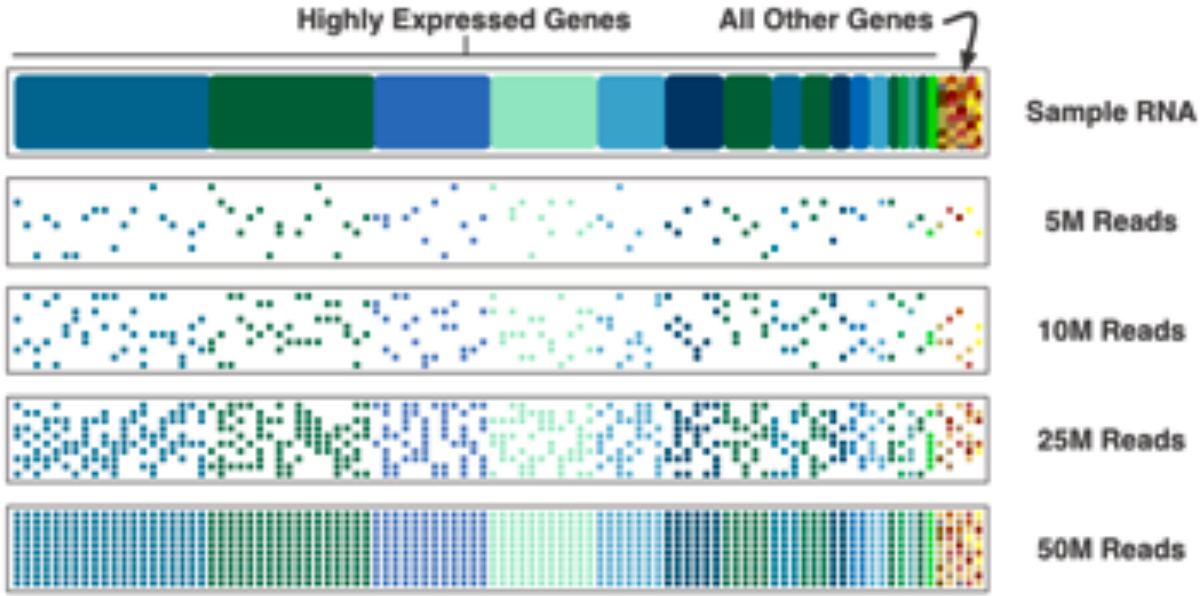






In most cases, increasing replicates is more beneficial than increasing sequencing depth.



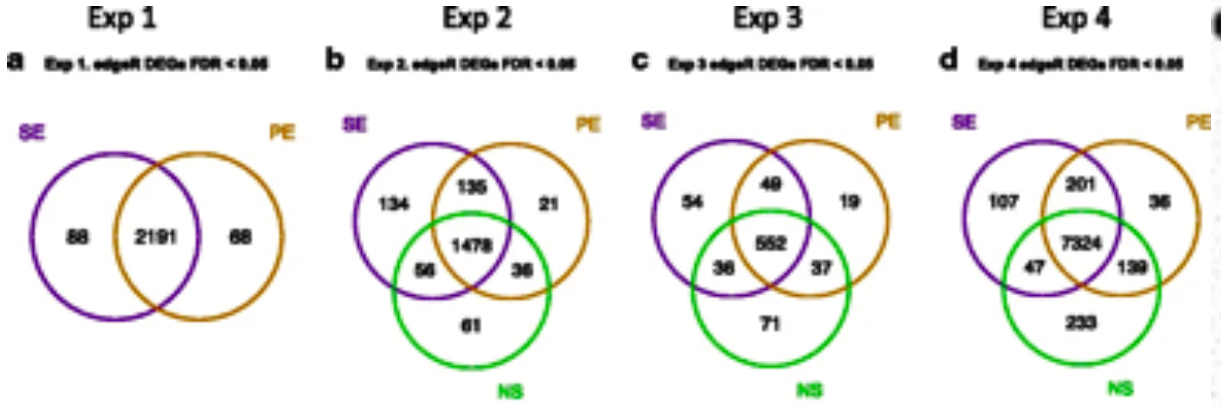


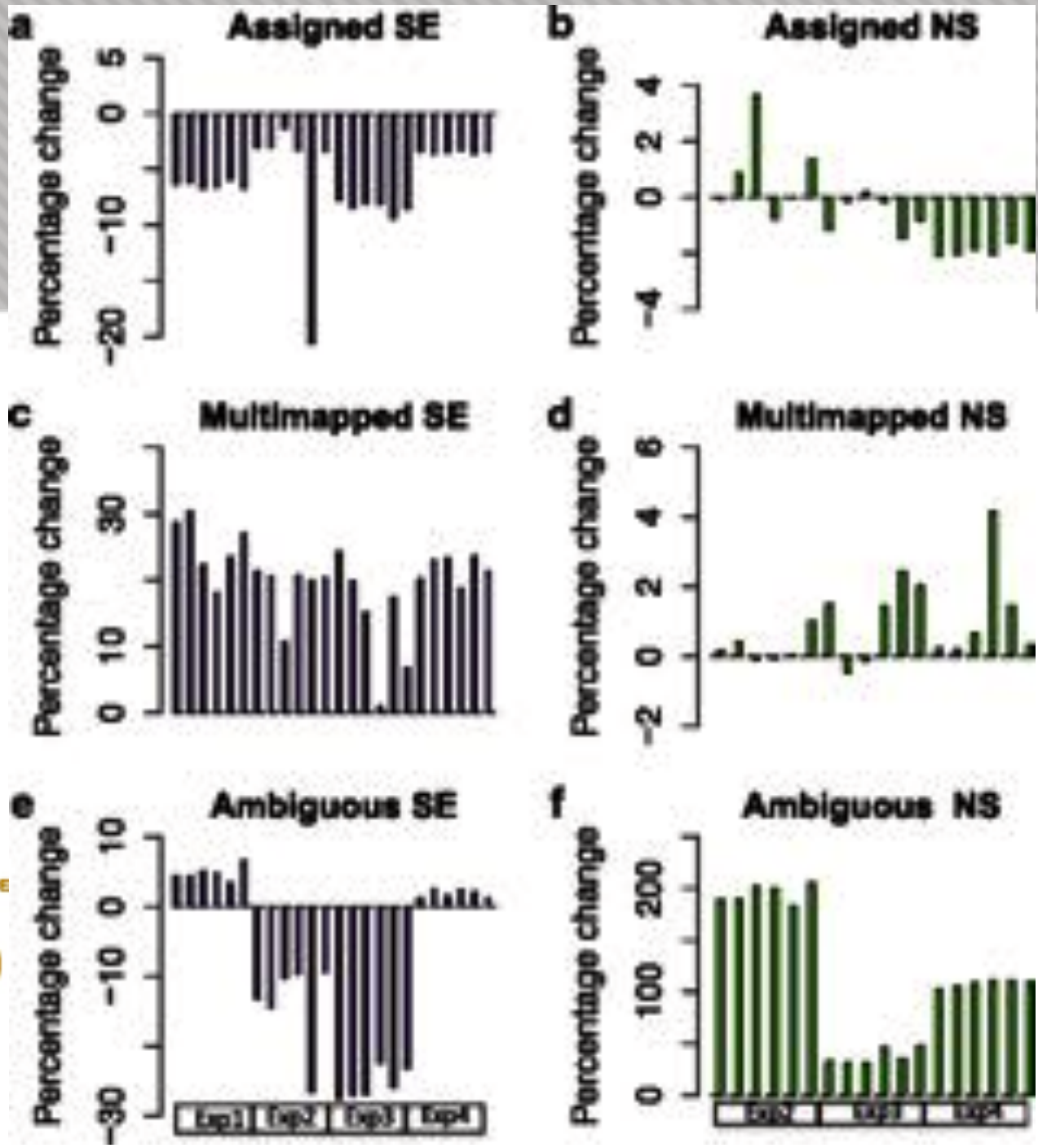
Liu et.al., 2014. doi: 10.1093/bioinformatics/btt688

http://info.l7informatics.com/blog/ngs-101-biology-is-a-big-data-problem

Single End vs Paired End

- Paired end (PE) sequencing improves mapping
  - to repeat sequences in the genome
  - across exon-exon junctions
- Single-end (SE) sequencing is cheaper than PE.





Corley et.al., 2017. doi: 10.1186/s12864-017-3797-0

Table 1: Recommended sequencing depths for typical RNA-seq experiments for different genome sizes (Genohub, 2015). DGE = differential gene expression, SR = single read, PE = paired-end.

	Small (bacteria)	Intermediate (fruit fly, worm	Large (mouse, human)
No. of reads for DGE ($x10^6$)	5 SR	10 SR	20–50 SR
No. of reads for *de novo* transcriptome assembly ($x10^6$)	30–65 PE	70–130 PE	100–200 PE
Read length (bp)	50	50–100	>100

For a basic RNA-seq differential expression experiment, 10M to 20M single end reads per sample is usually enough

Variance and blocking

Good experimental designs mitigate experimental error and the impact of factors not under study.

Krzywinski et.al., 2014 doi: 10.1038/nmeth.3005

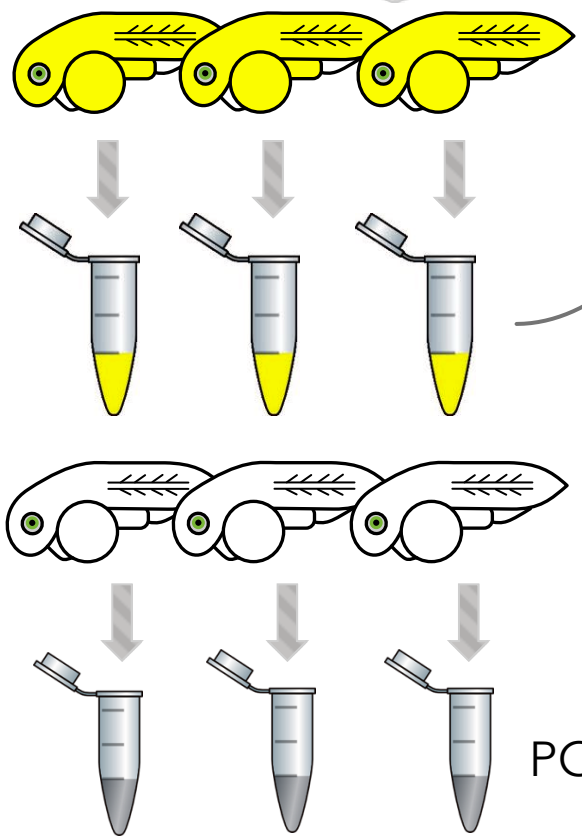
DO

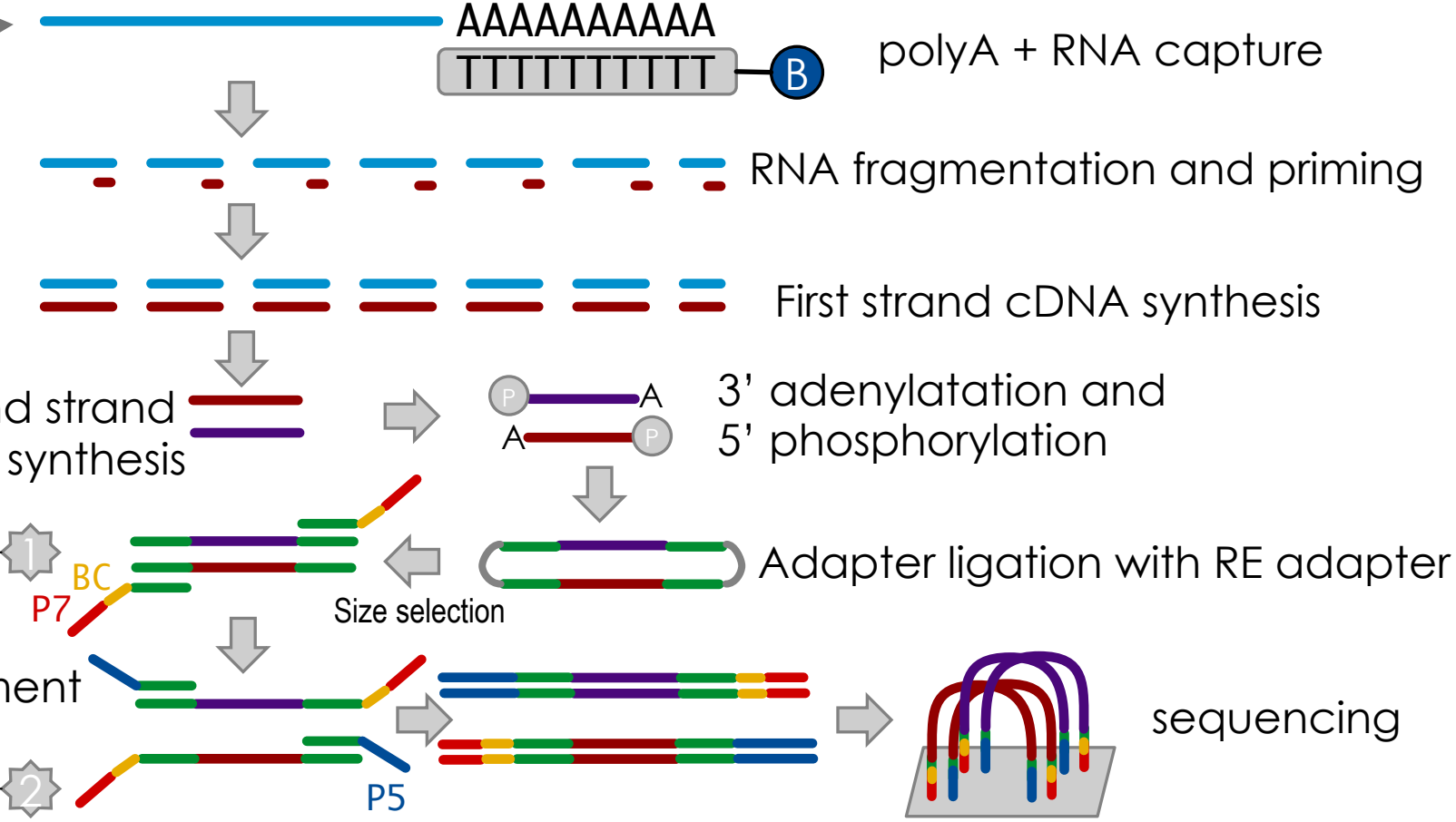
- Choosing organisms of **similar genetic background** (littermates)
- Choosing organisms of the **same sex** if possible
- Using a **constant sample collection time and sampling sites on tissues**.
- Having the **same laboratory technician** perform RNA prep and library prep, same **lots of reagents**, **limit processing time range**
- If variation between samples can not be removed, use **balanced blocking design** and **modeling the block effect** during analysis
- **Randomizing** samples to prevent a **confounding batch** effect if all samples can't be processed at one time.

DON'T

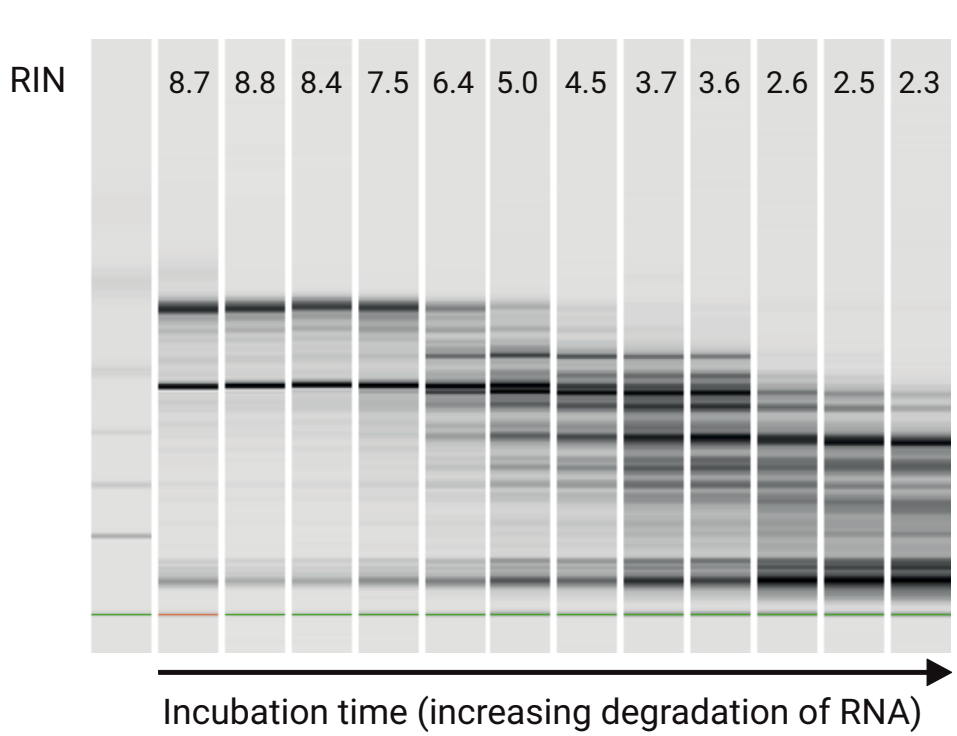
- Do all controls in one day and all exp another day.
- Use different reagents or kits within one experiment batch

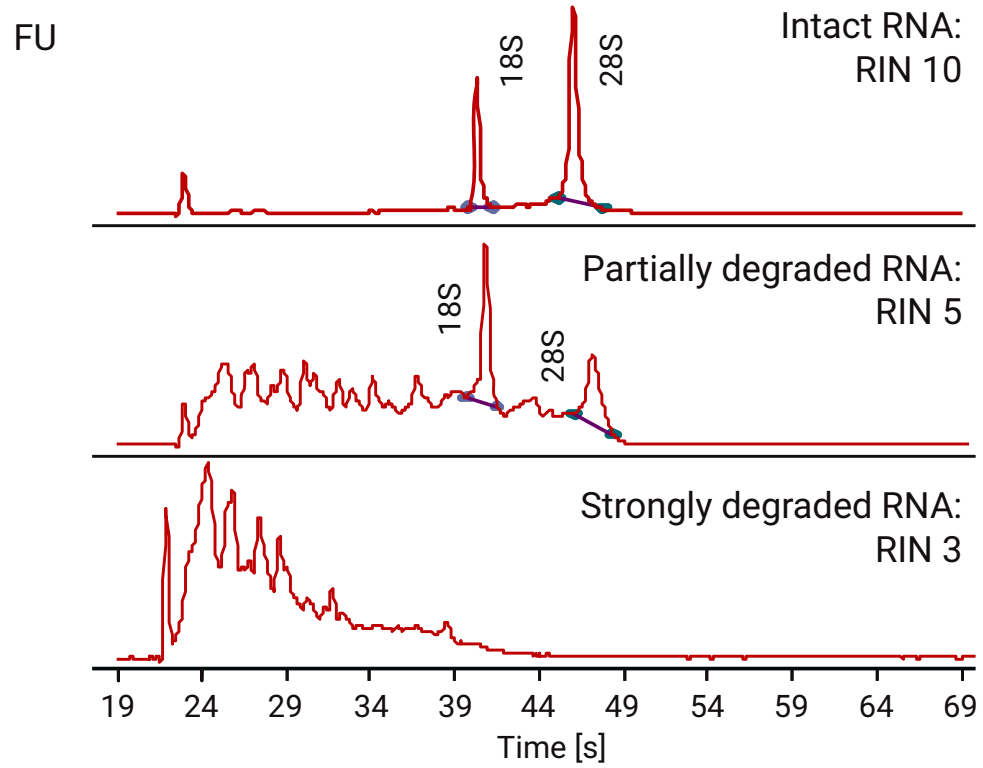
Illumina TruSeq RNA-seq protocol





RNA QUANTITY AND QUALITY (RIN)

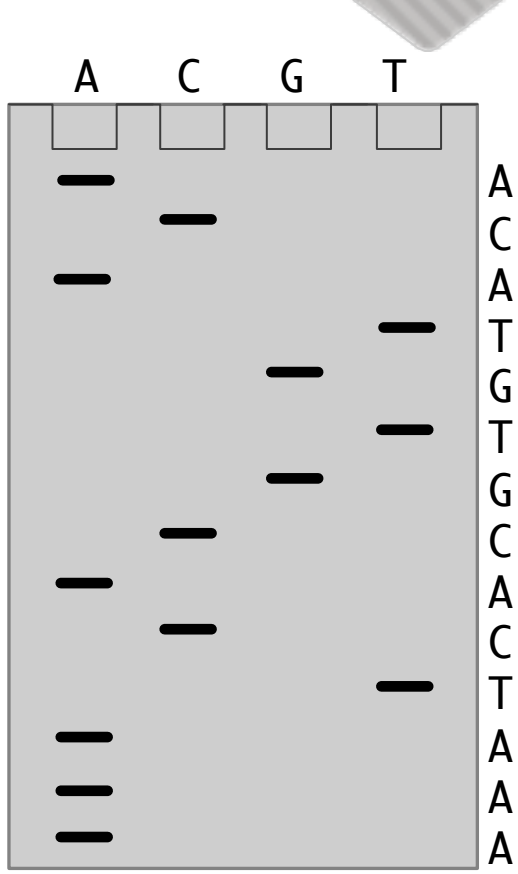




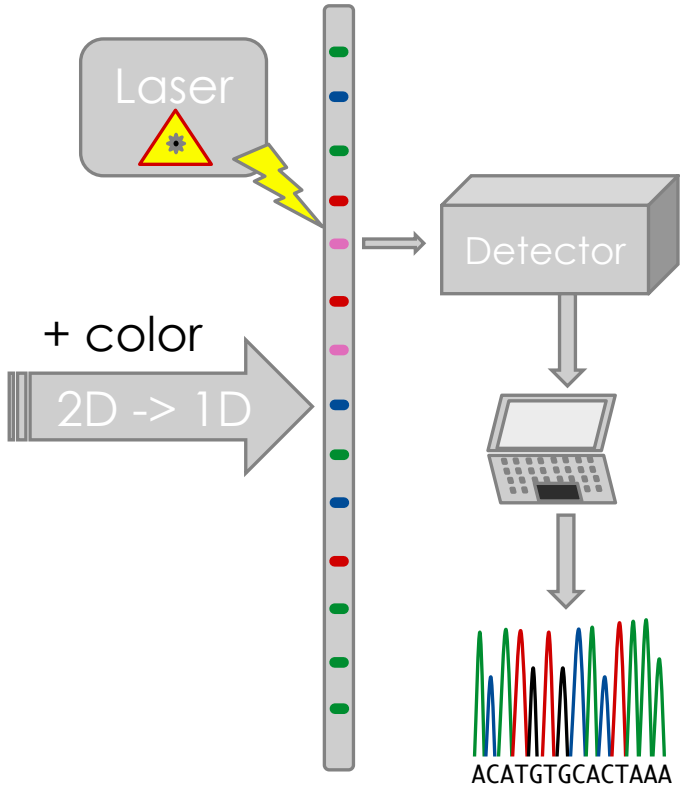
https://www.agilent.com/cs/library/applications/5991-8974EN_Bioanalyzer-TapeStation_appcompendium.pdf

Schroeder et al., 2006. doi:10.1186/1471-2199-7-3

Next Generation Sequencing (NGS)

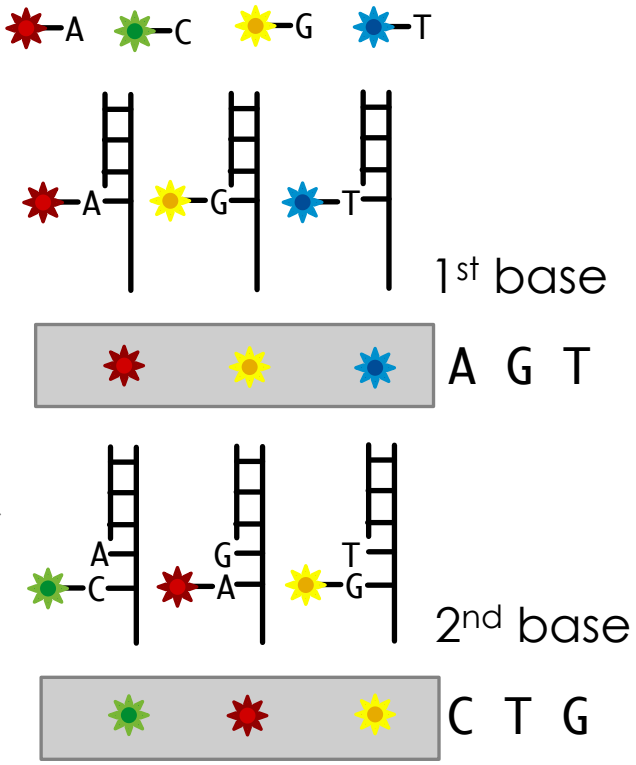


Sanger sequencing



parallel

Speed up



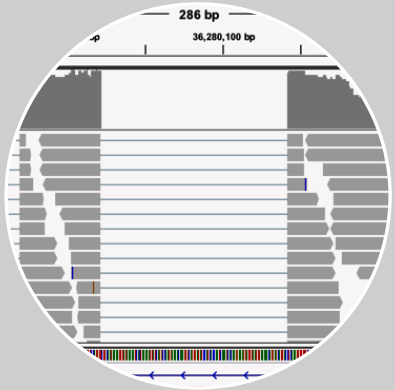
Illumina

nanopore

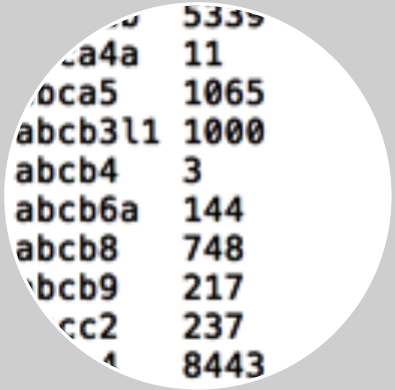
Next Generation

RNA-seq data analysis pipeline

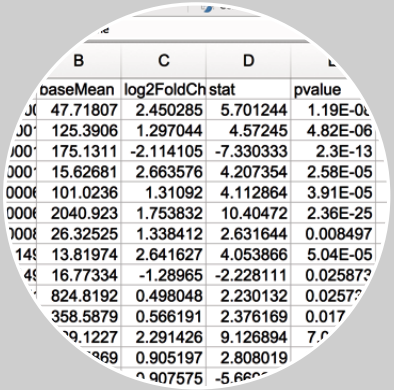
Reads



Alignments



Read counts



Differential Expression

PrePrOcess

Download files from seqServer

From: GCB Genome Sequencing Shared Resource
Sent: Monday, May 28, 2018 3:00 PM
To: Jianhong Ou, Ph.D. <jianhong.ou@duke.edu>;
Cc: GCB Genome Sequencing Shared Resource <sequencing@duke.edu>
Subject: Your HiSeq Order #xxxx

xxx,

Your run performed within specifications.

See your report at: http://seqweb.gcb.duke.edu/18/05/g2u4ouwjzjboh0w_4789_180525B1.html

See instruction below to retrieve your data.

You can download your data using unix command line (sftp) or a sftp client such as FileZilla or Cyberduck on an Apple computer.

The example of unix ftp command for downloading a directory recursively: get -r directory

****

Instruction for data download

****

sftp server name: dnaseq2.igsp.duke.edu

Username: ==========

Password: ==========

Your data is under path/to/your/data

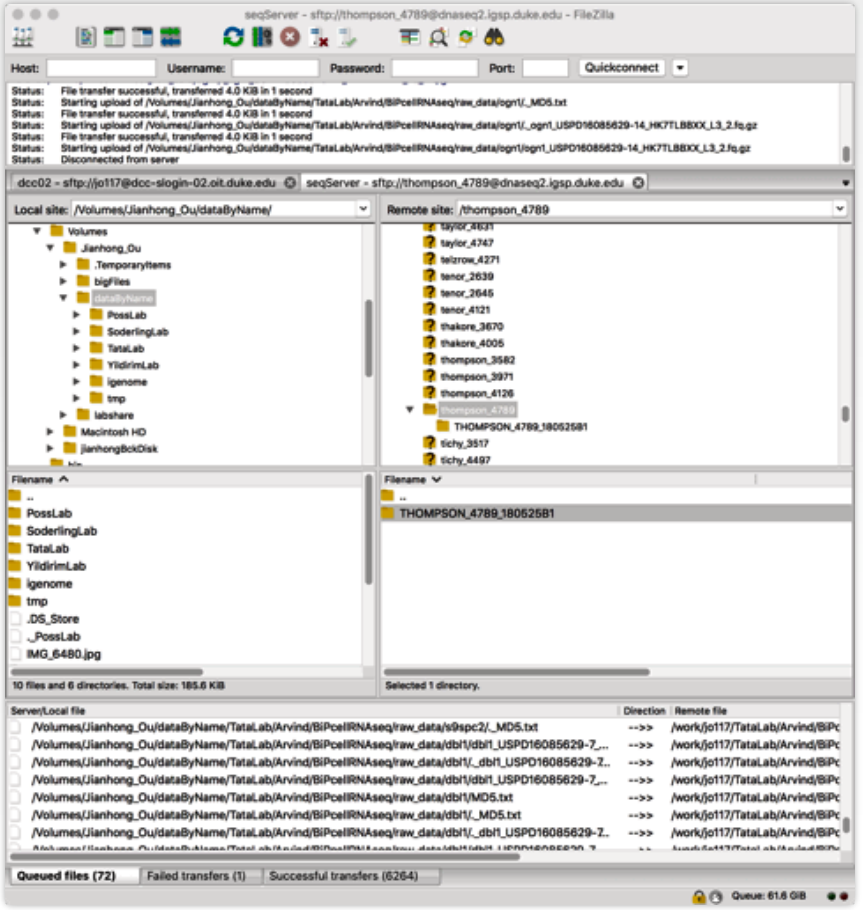
Reminder, your data will be available to download until 2018-06-27

Please, let us know if you have any questions or concerns.

Best regards and thank you for your business.

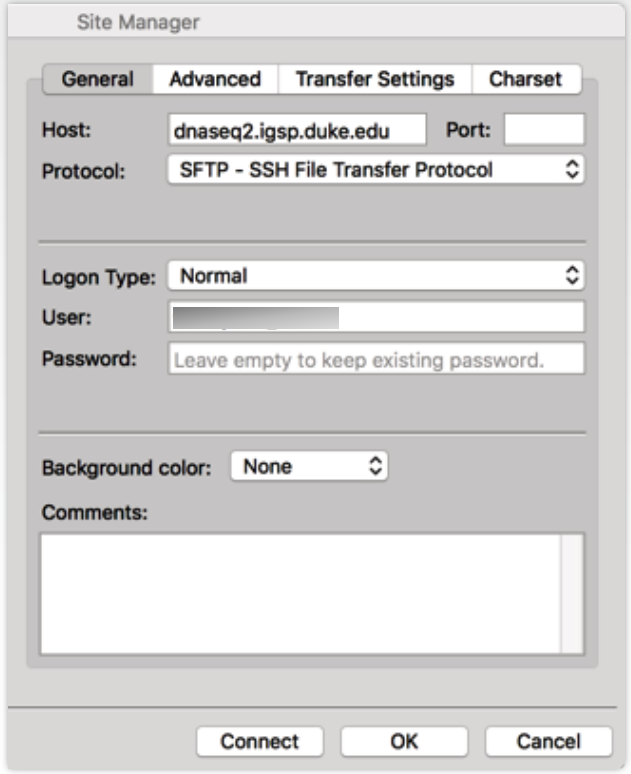
Nicolas

GCB Sequencing and Genomic Technology Shared Resource



Install FileZilla and download a file

- Goto https://software.duke.edu/
- NetID Login
- Search for software: FileZilla
- Click FileZilla
- Click download here
- Download FileZilla Client
- Install FileZilla Client



File formats: FASTA and FASTQ

- FASTA file:
  - Description line: each sequence started with a “>” symbol, and with or without sequence name
  - Following the initial line is the actual sequences
- FASTQ file:
  - Line1: begins with a '@' character and is followed by a sequence identifier and an optional description
  - Line2: is the raw sequence letters
  - Line3: begins with a '+' character and is *optionally* followed by the same sequence identifier (and any description) again
  - Line4: encodes the quality values for the sequence in Line 2, and must contain the same number of symbols as letters in the sequence

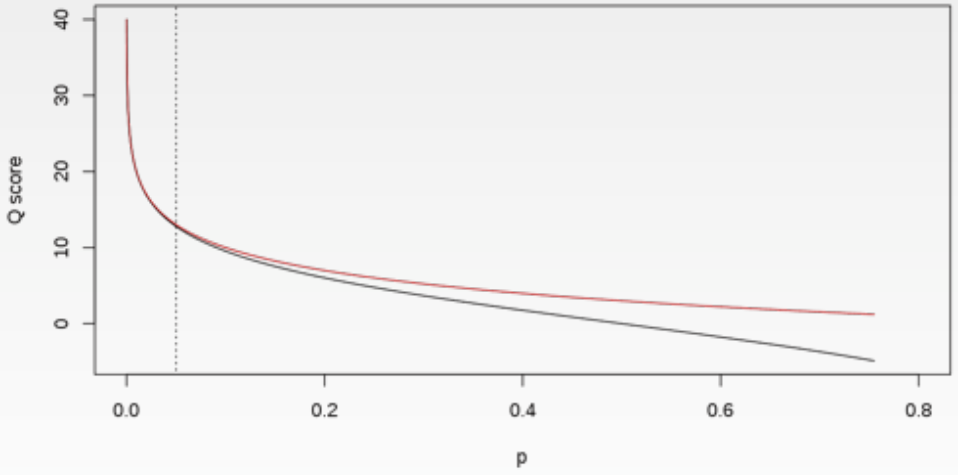
```
>seq1
ACGTACGTACGT
ACGTACGTACGT
>seq2
TGCATGCATGCA
```

```
@seq1
ACGTACGTACGTACGTACGTACGT
+
ABCDEFGHI!#%1234567890+-
@seq2
TGCATGCATGCA
+
ABCDEFGHI!#%
```

FASTQ: quality score

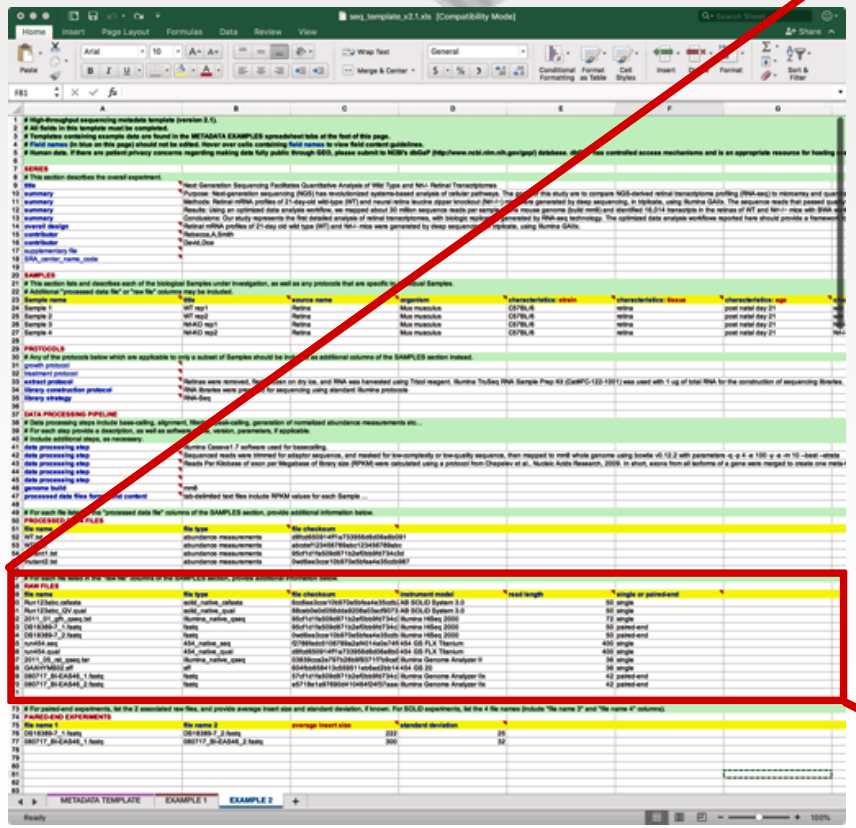
- **Quality**
  - $Q_{sanger} = -10 \log_{10} p \quad ; \quad Q_{solexa} = -10 \log_{10} \frac{p}{1-p}$
- **Encoding**

```
 SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS.....................................................
 ..........................XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX.....................
 ..............................IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII.....................
 ..............................JJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJ.....................
 LLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLL.....................................................
 !"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~
 |                         |    |        |                              |                     |
33                        59   64       73                            104                   126
  0........................26...31........40
                           -5....0........9.............................40
                                0........9.............................40
                                   3.....9.............................41
  0.2......................26...31........41

S - Sanger        Phred+33,  raw reads typically (0, 40)
X - Solexa        Solexa+64, raw reads typically (-5, 40)
I - Illumina 1.3+ Phred+64,  raw reads typically (0, 40)
J - Illumina 1.5+ Phred+64,  raw reads typically (3, 41)
    with 0=unused, 1=unused, 2=Read Segment Quality Control Indicator (bold)
    (Note: See discussion above).
L - Illumina 1.8+ Phred+33,  raw reads typically (0, 41)
```



Relationship between *Q* and *p* using the Sanger (red) and Solexa (black) equations (described above). The vertical dotted line indicates $p = 0.05$, or equivalently, $Q \approx 13$.

Submitting high-throughput sequence data to Gene Expression Omnibus (GEO)



RAW FILES

file name	file type	file checksum
Run123abc.csfasta	solid_native_csfasta	6cc6ee3cce10b970e
Run123abc_QV.qual	solid_native_qual	88ceb0e0d056dda92
2011_01_gfh_qseq.txt	Illumina_native_qseq	95cf1d1fa509d871b2
DS18389-7_1.fastq	fastq	95cf1d1fa509d871b2
DS18389-7_2.fastq	fastq	0wd6ee3cce10b970e

instrument model	read length	single or paired-end
AB SOLiD System 3.0	50	single
AB SOLiD System 3.0	50	single
Illumina HiSeq 2000	72	single
Illumina HiSeq 2000	50	paired-end
Illumina HiSeq 2000	50	paired-end

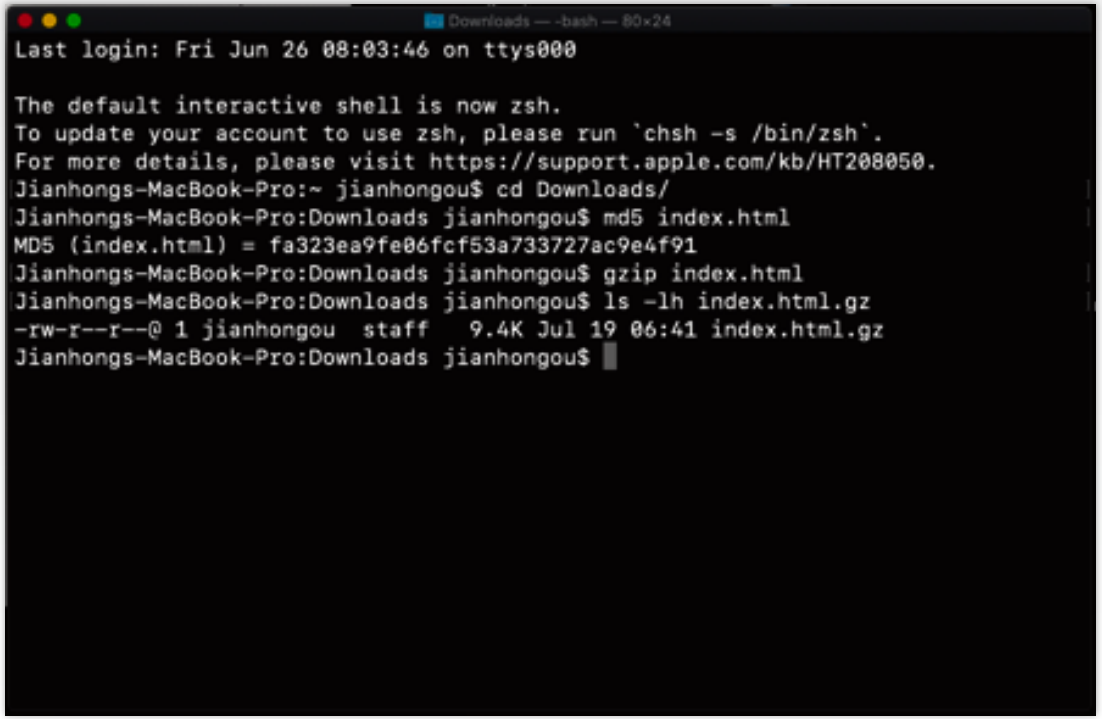
File formats: md5 and compressed files

- **md5**
  - **Unix**: md5sum <file>
  - **OS X**: md5 <file>
  - **Windows**: Application required. Many are available for free download.
    Eg: FCIV -md5 <file>; certUtil -hashfile <file> MD5

- **Data File Compression**: Individual files can be compressed to speed transfer, but this is not required. Acceptable compression formats are gzip and bzip2 (i.e. files ending with a .gz or .bz2 extension). Never compress binary files (e.g., BAM, bigWig, bigBed), and DO NOT upload ZIP archives (files with a .zip extension).

Exercises

- Get checksum for a file
- Compress and decompress a file

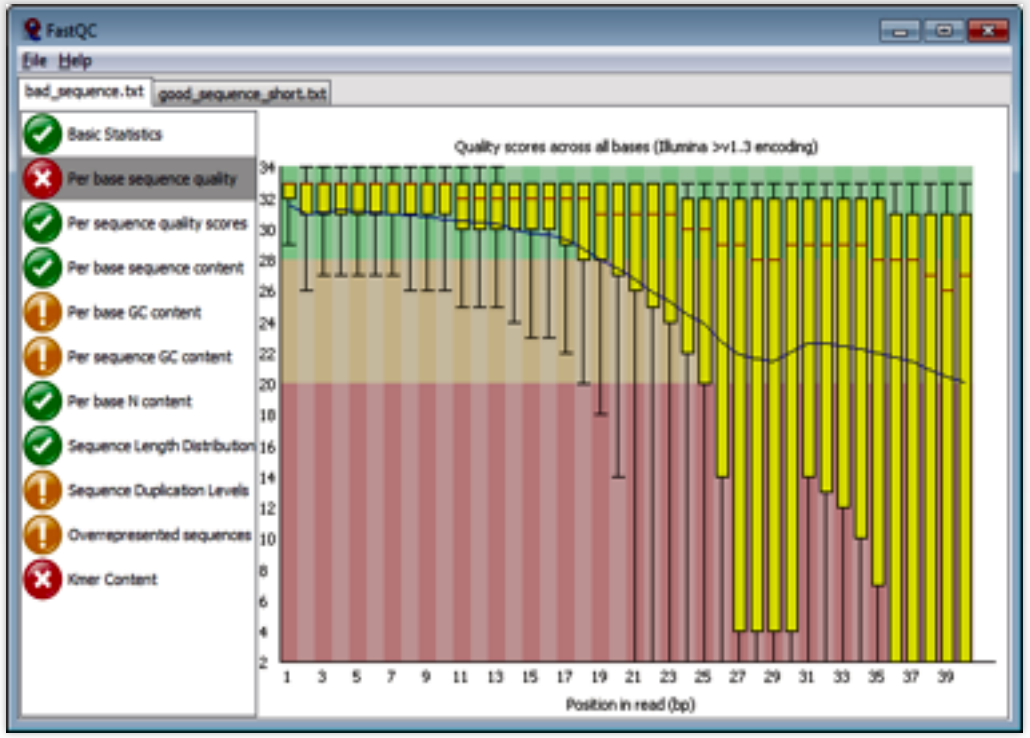


Backup your data

- Keep two copies in physically different storage spaces with their checksum.
  - Keep it in the cloud: Duke's Box (50G), OneDrive (1TB)
  - External Hard Drive
  - Burn it to a Blu-ray Disc
  - Put it on a USB Flash Drive
  - Save it to Network Attached Storage (NAS) Device

FASTQC

Function	A quality control tool for high throughput sequence data.
Language	Java
Requirements	A suitable Java Runtime Environment
Code Maturity	Stable.
Code Released	Yes, under GPL v3 or later.
Initial Contact	Simon Andrews

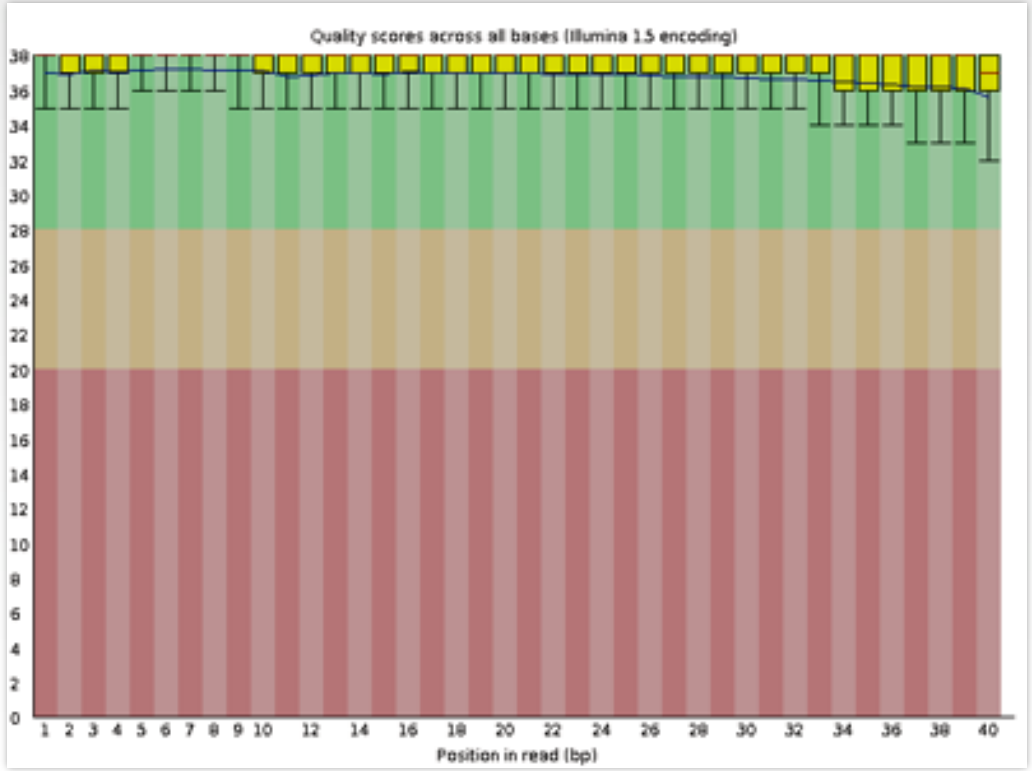


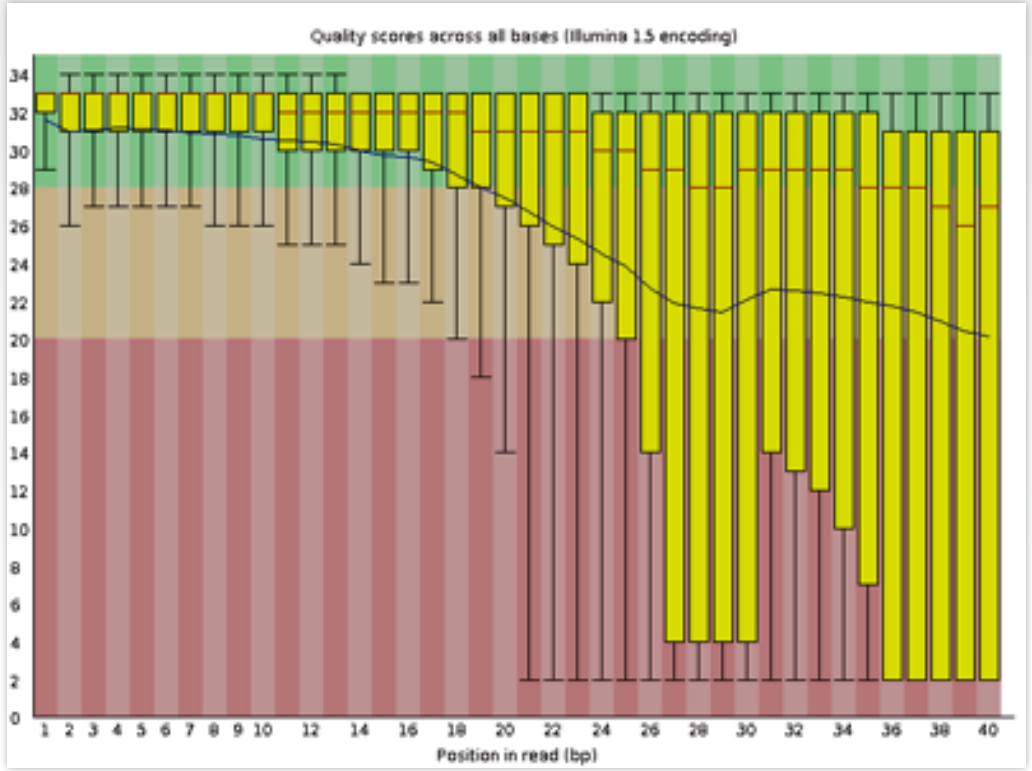
FASTQC: Basic Statistics

Measure	Value	Description
Filename	good_sequence_short.txt	The original filename of the file which was analysed
File type	Conventional base calls	Says whether the file appeared to contain actual base calls or colorspace data which had to be converted to base calls
Encoding	Illumina 1.5	Says which ASCII encoding of quality values was found in this file
Total Sequences	250000	A count of the total number of sequences processed
Sequences flagged as poor quality	0	A count of the number of sequences flagged as poor quality
Sequence length	40	Provides the length of the shortest and longest sequence in the set
%GC	45	The overall %GC of all bases in all sequences

FASTQC: Per base sequence quality

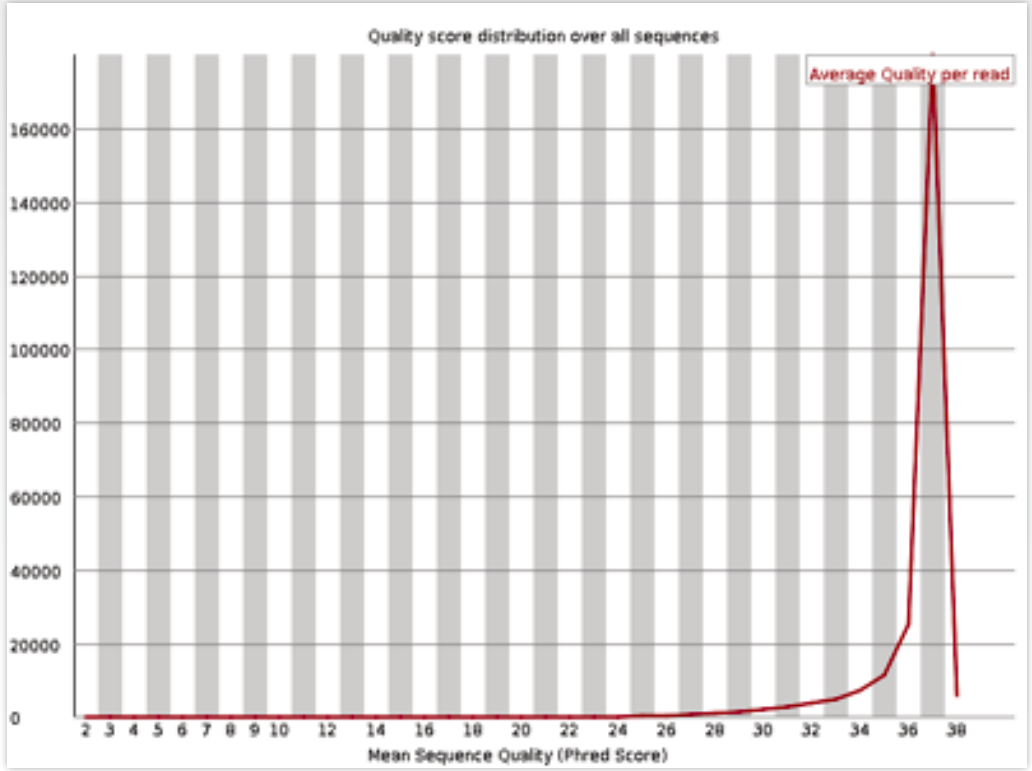
$$Q_{sanger} = -10\ \log_{10} p \quad ; \quad Q_{solexa} = -10\ \log_{10} \frac{p}{1-p}$$

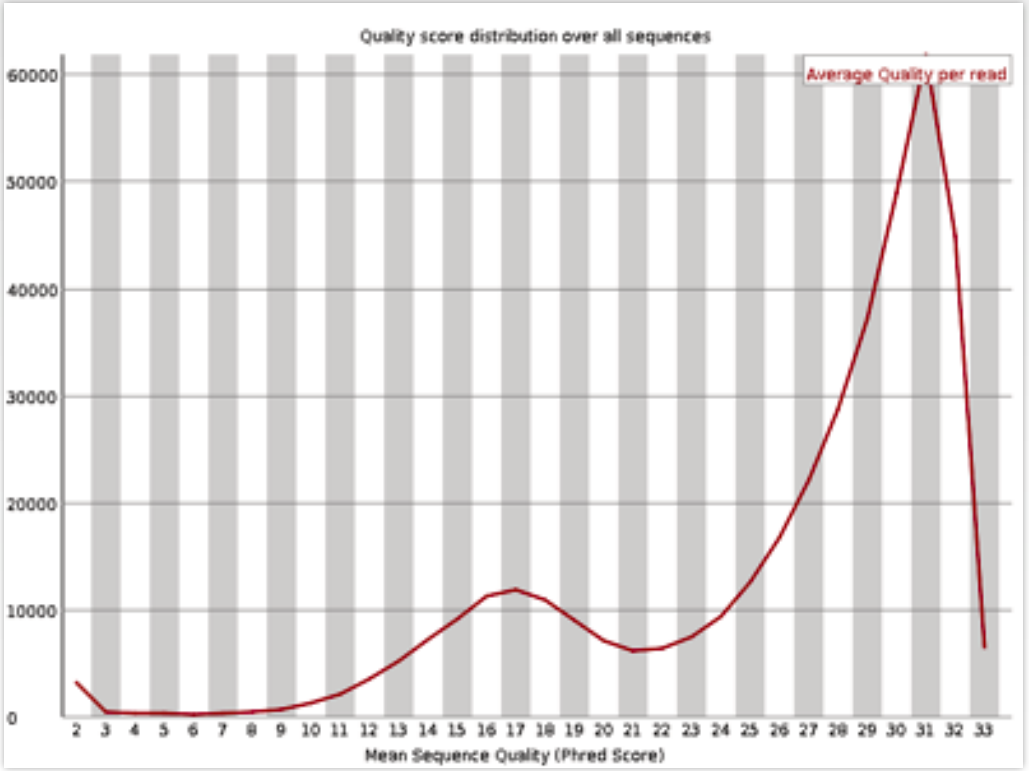




Warning: A warning will be issued if the lower quartile for any base is less than 10, or if the median for any base is less than 25.
Failure: This module will raise a failure if the lower quartile for any base is less than 5 or if the median for any base is less than 20.

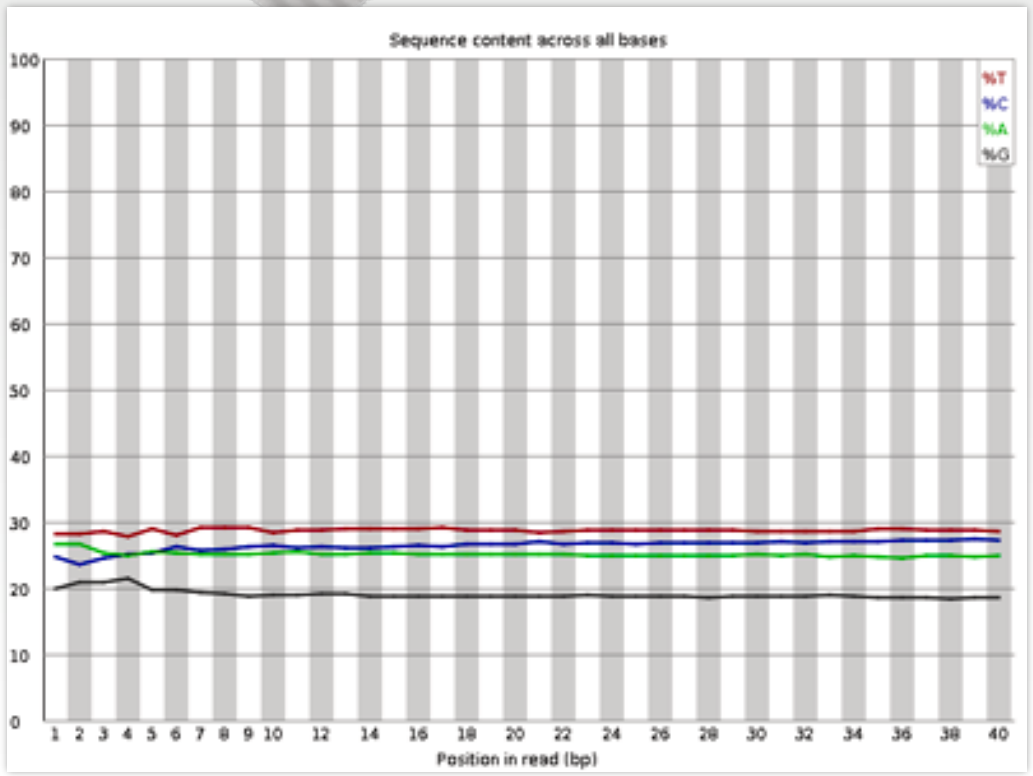
FASTQC: Per sequence quality scores

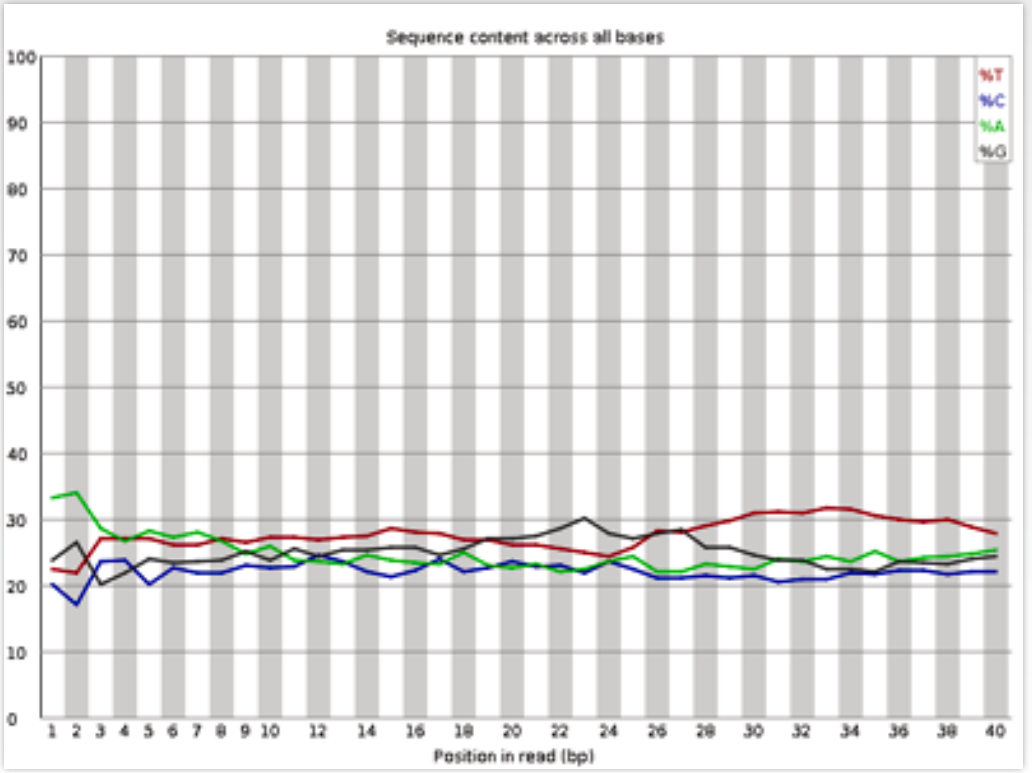




Warning: A warning is raised if the most frequently observed mean quality is below 27 - this equates to a 0.2% error rate.
Failure: An error is raised if the most frequently observed mean quality is below 20 - this equates to a 1% error rate.

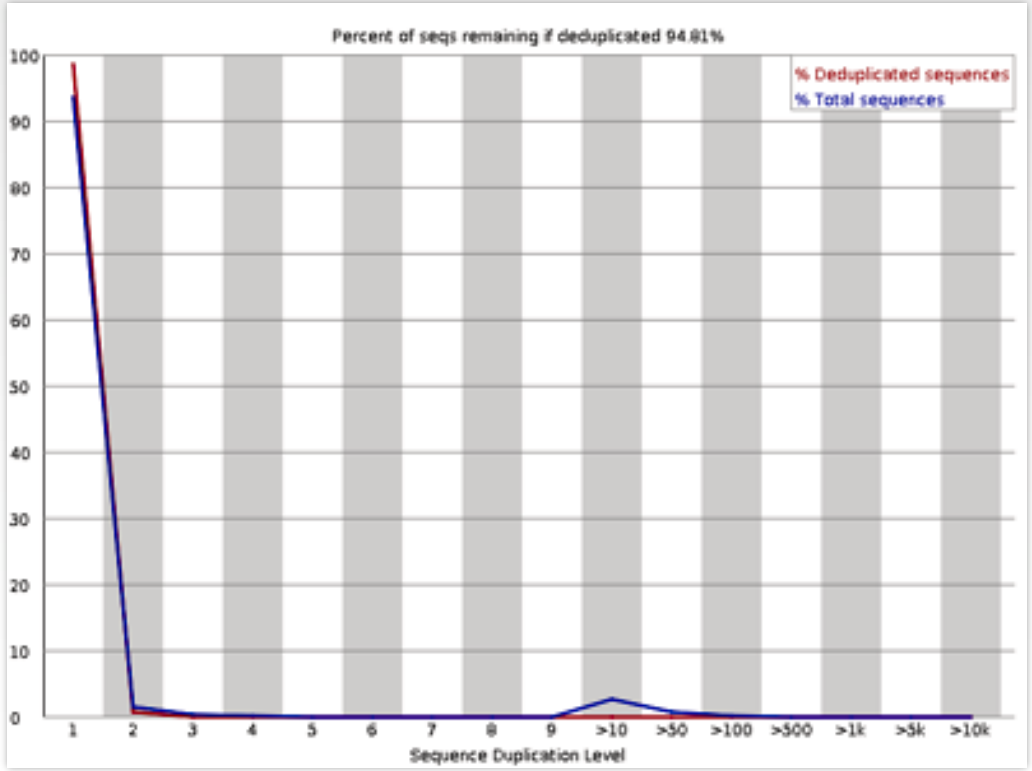
FASTQC: Per base sequence content

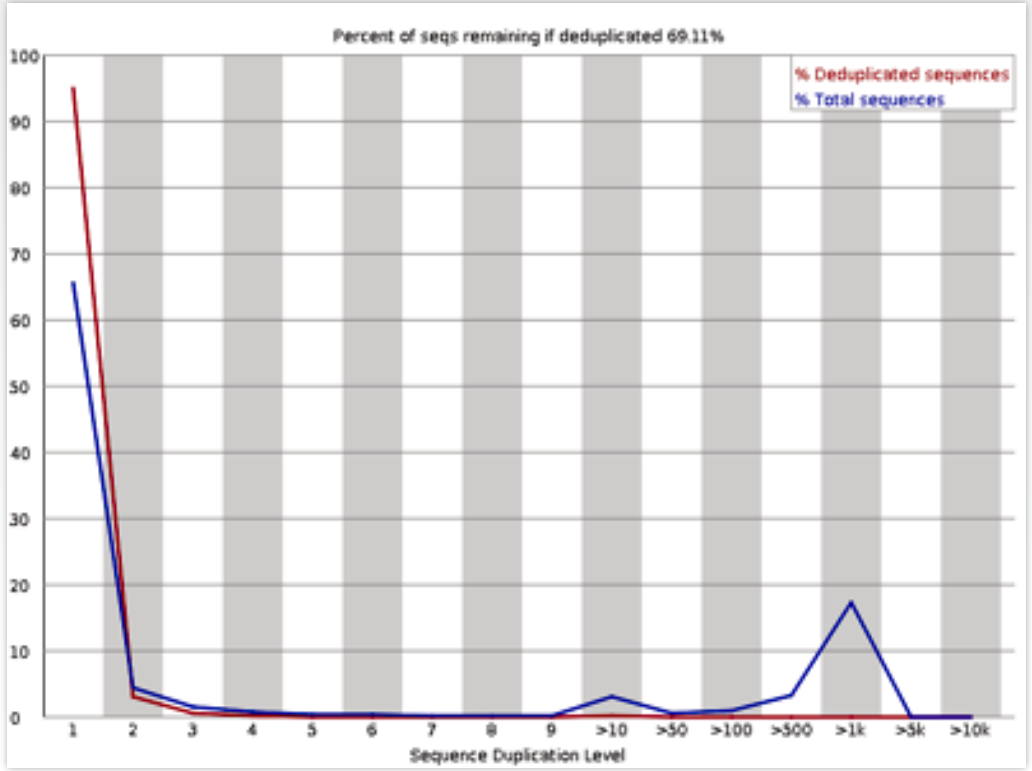




Warning: This module issues a warning if the difference between A and T, or G and C is greater than 10% in any position.
Failure: This module will fail if the difference between A and T, or G and C is greater than 20% in any position.

FASTQC: Sequence Duplication Levels

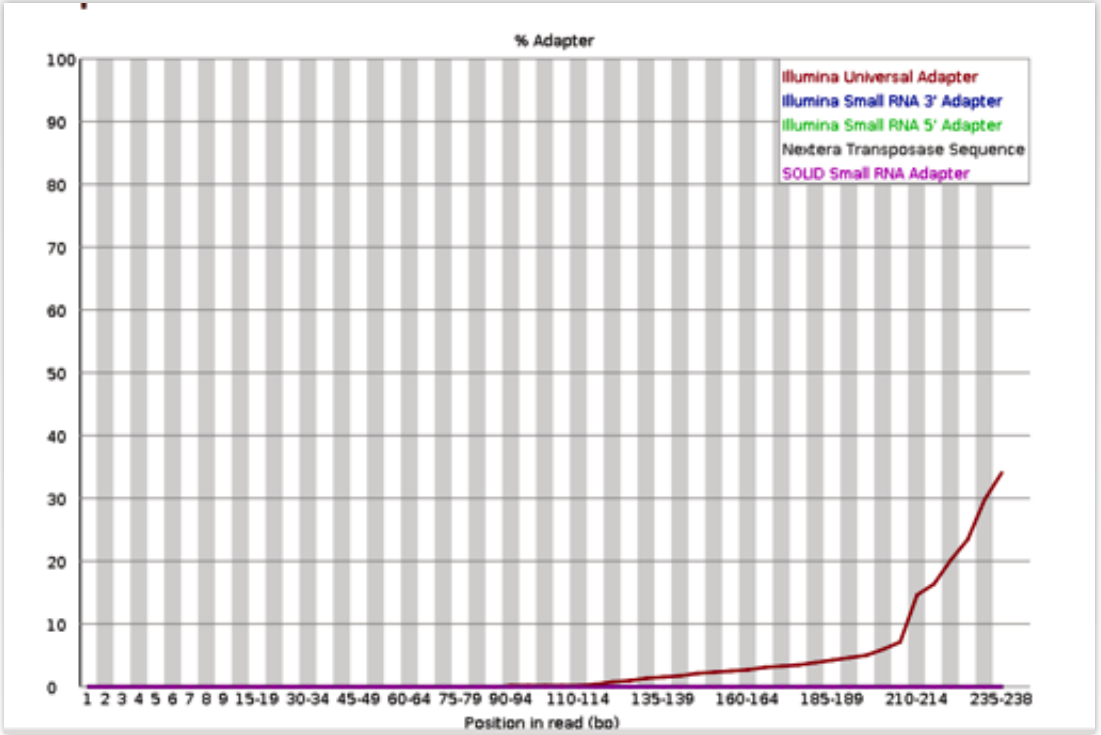




Warning: This module will issue a warning if non-unique sequences make up more than 20% of the total.
Failure: This module will issue a error if non-unique sequences make up more than 50% of the total.

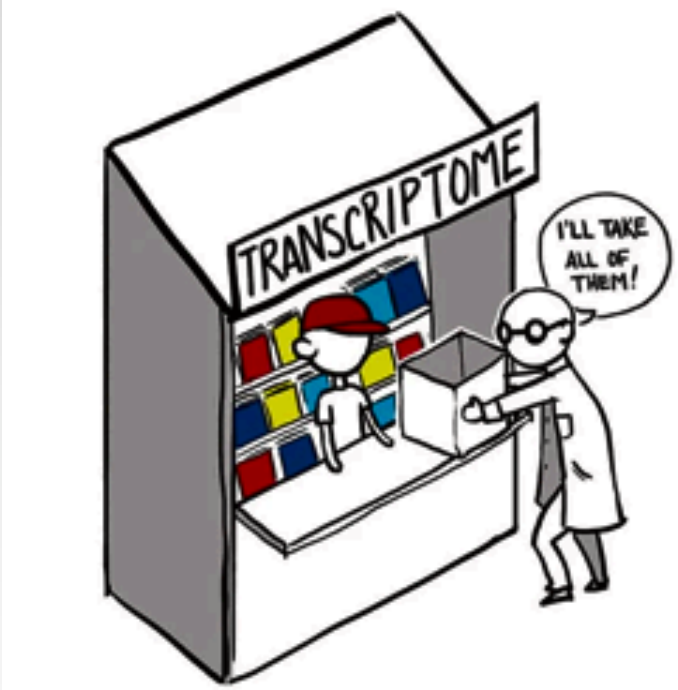
Trimming low-quality bases and adapters

- Cutadapt, Trim Galore!
- Trimmomatic
  - Using relaxed trimming thresholds
  - Improve mappability
  - Reduce mapping artifacts



Alignment and Quantification

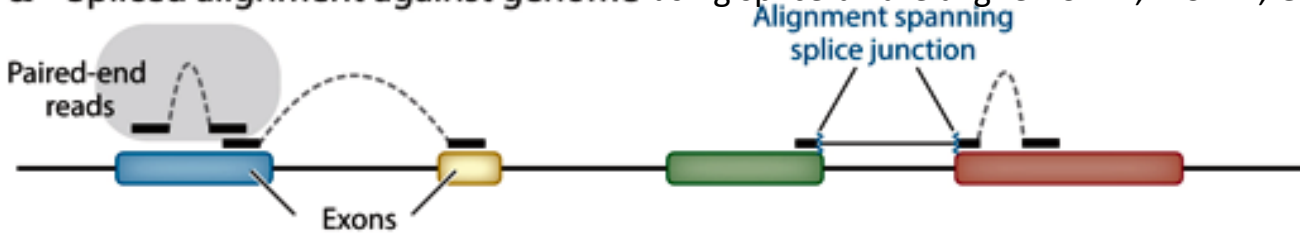
Genome reconstruction—akin to reassembling magazine articles after they have been through a paper shredder.



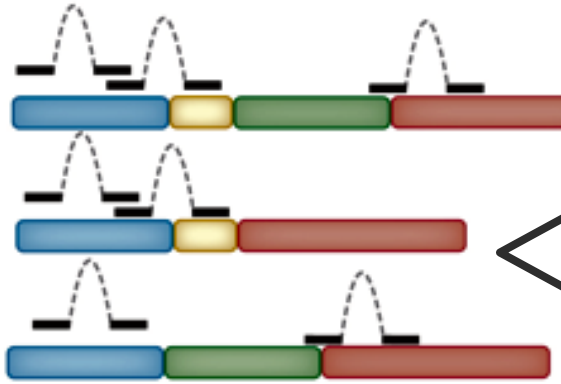
Abigail Yu

Lan Korf 2013. 10.1038/nmeth.2735

a Spliced alignment against genome using splice-aware aligner: STAR, HISAT2, GMAP, Tophat2

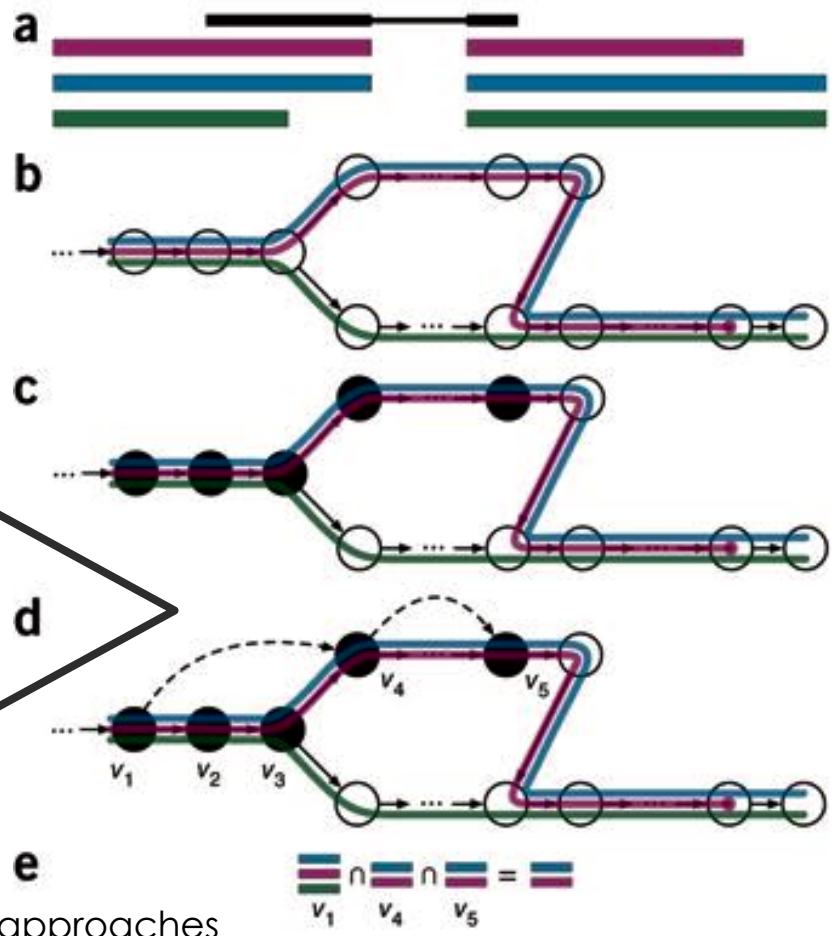


b Unspliced alignment against transcriptome using ungapped aligner: Bowtie, bwa



Van den Berge K, et al. 2019.
Annu. Rev. Biomed. Data Sci. 2:139–73

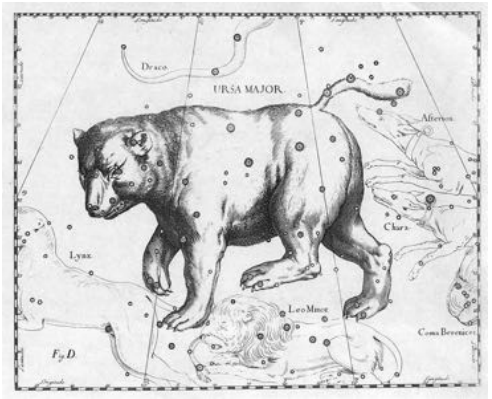
well annotated reference genome

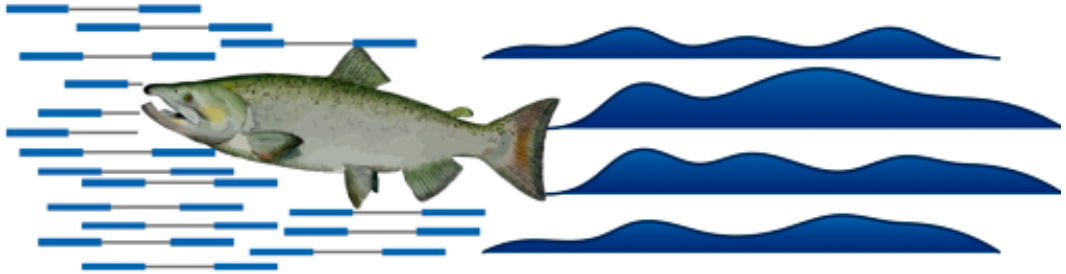


Bray et.al., 2016. doi: 10.1038/nbt.3519

Alignment free or Pseudo-alignment against transcriptome:
Salmon, Sailfish, Kallisto

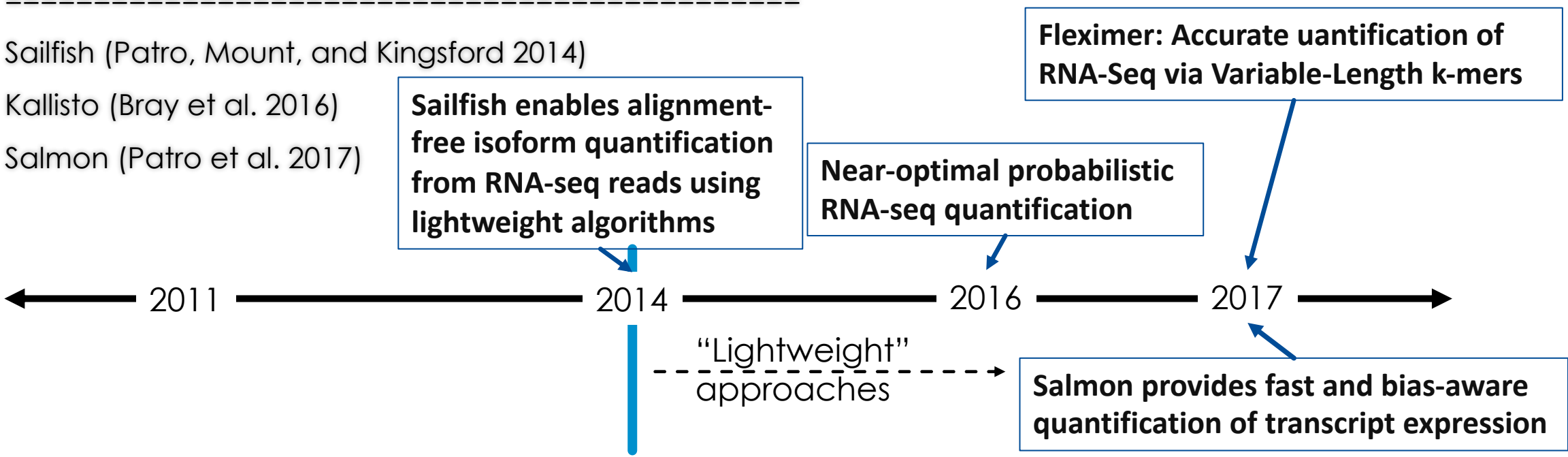
Sailfish: k-mer-based approaches
Kallisto: pseudo-alignment
Salmon: quasi-alignment, modeling GC-bias

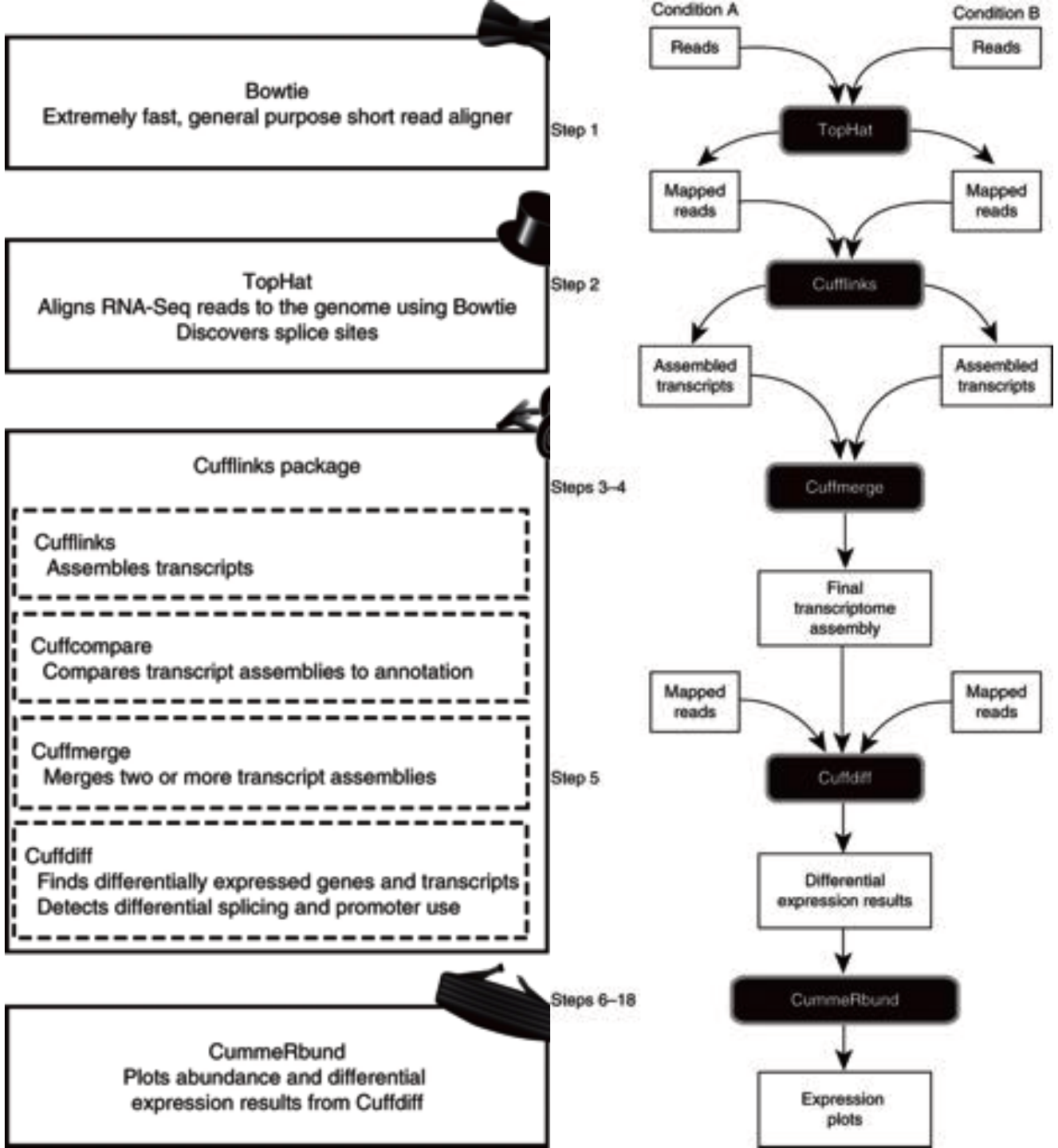




tximport + DESeq2/edgeR/limma-voom

- STAR (Spliced Transcripts Alignment to a Reference)
- RSEM(RNA-Seq by Expectation-Maximization)
- StringTie(downstream followed by Ballgown)
- ==========
- Sailfish (Patro, Mount, and Kingsford 2014)
- Kallisto (Bray et al. 2016)
- Salmon (Patro et al. 2017)





PROTOCOL

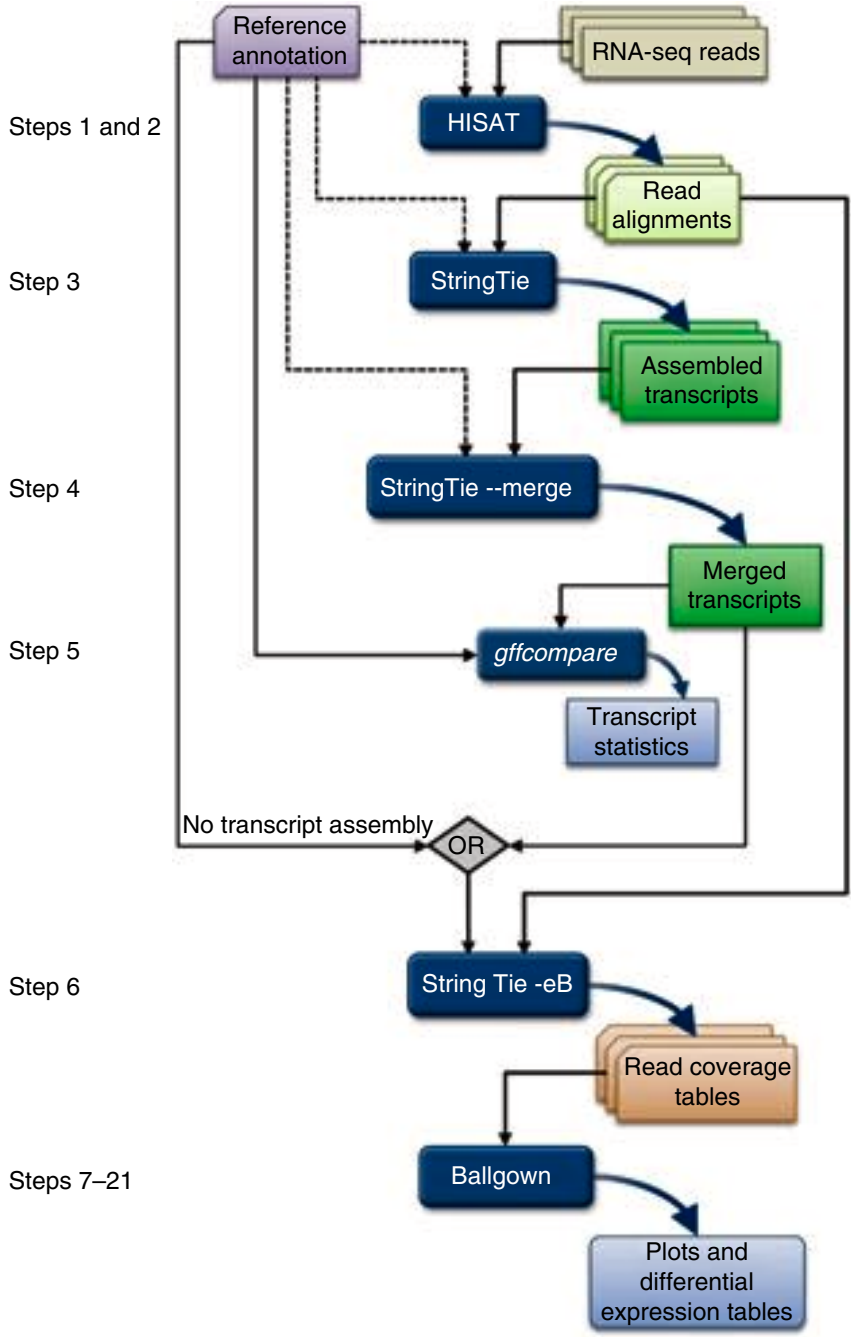
Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks

Cole Trapnell[1,2], Adam Roberts[3], Loyal Goff[1,2,4], Geo Pertea[5,6], Daehwan Kim[5,7], David R Kelley[1,2], Harold Pimentel[3], Steven L Salzberg[5,6], John L Rinn[1,2] & Lior Pachter[3,8,9]

[1]Broad Institute of MIT and Harvard, Cambridge, Massachusetts, USA. [2]Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, Massachusetts, USA. [3]Department of Computer Science, University of California, Berkeley, California, USA. [4]Computer Science and Artificial Intelligence Lab, Department of Electrical Engineering and Computer Science, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA. [5]Department of Medicine, McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. [6]Department of Biostatistics, Johns Hopkins University, Baltimore, Maryland, USA. [7]Center for Bioinformatics and Computational Biology, University of Maryland, College Park, Maryland, USA. [8]Department of Mathematics, University of California, Berkeley, California, USA. [9]Department of Molecular and Cell Biology, University of California, Berkeley, California, USA. Correspondence should be addressed to C.T. (cole@broadinstitute.org).

Published online 1 March 2012; corrected after print 7 August 2014; doi:10.1038/nprot.2012.016

- Reads are mapped directly to reference genome allowing for splice junctions across exons
- Methods require large amount of memory and CPU time
- Can only handle simple experiment design



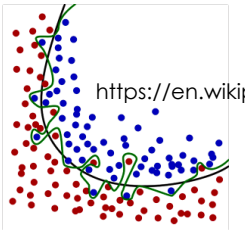
PROTOCOL

Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown

Mihaela Pertea[1,2], Daehwan Kim[1], Geo M Pertea[1], Jeffrey T Leek[3] & Steven L Salzberg[1–4]

[1]Center for Computational Biology, McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins School of Medicine, Baltimore, Maryland, USA. [2]Department of Computer Science, Whiting School of Engineering, Johns Hopkins University, Baltimore, Maryland, USA. [3]Department of Biostatistics, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland, USA. [4]Department of Biomedical Engineering, Johns Hopkins University, Baltimore, Maryland, USA. Correspondence should be addressed to S.L.S. (salzberg@jhu.edu).

Published online 11 August 2016; doi:10.1038/nprot.2016.095



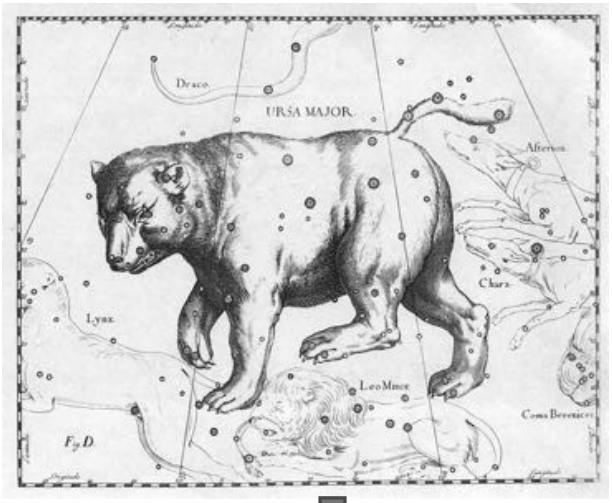
https://en.wikipedia.org/wiki/Overfitting

Note that Ballgown's statistical test is a standard linear model-based comparison. **For small sample sizes (n < 4 per group), it is often better to perform regularization.** This can be done using the limma[33] package in Bioconductor. Other regularized methods such as DESeq[23] and edgeR[20] can be applied to gene or exon counts, but they are not appropriate for direct application to FPKM abundance estimates. The statistical test uses a cumulative upper quartile normalization[34].

Kallisto, Sleuth pipeline

- considerably faster than traditional alignment + counting
- Transcripts level estimates rather than gene level. Transcripts level estimates can be aggregated to gene level, but abundance estimates for lowly expressed transcripts are highly variable and should be interpreted with caution.
- No precise alignments (no alignment files for visualization in genome browser)

https://scilifelab.github.io/courses/rnaseq/labs/kallisto



Near-optimal probabilistic RNA-seq quantification

Nicolas L Bray[1], Harold Pimentel[2], Páll Melsted[3] & Lior Pachter[2,4,5]

We present kallisto, an RNA-seq quantification program that is two orders of magnitude faster than previous approaches and achieves similar accuracy. Kallisto pseudoaligns reads to a reference, producing a list of transcripts that are compatible with each read while avoiding alignment of individual bases. We use kallisto to analyze 30 million unaligned paired-end RNA-seq reads in <10 min on a standard laptop computer. This removes a major computational bottleneck in RNA-seq analysis.

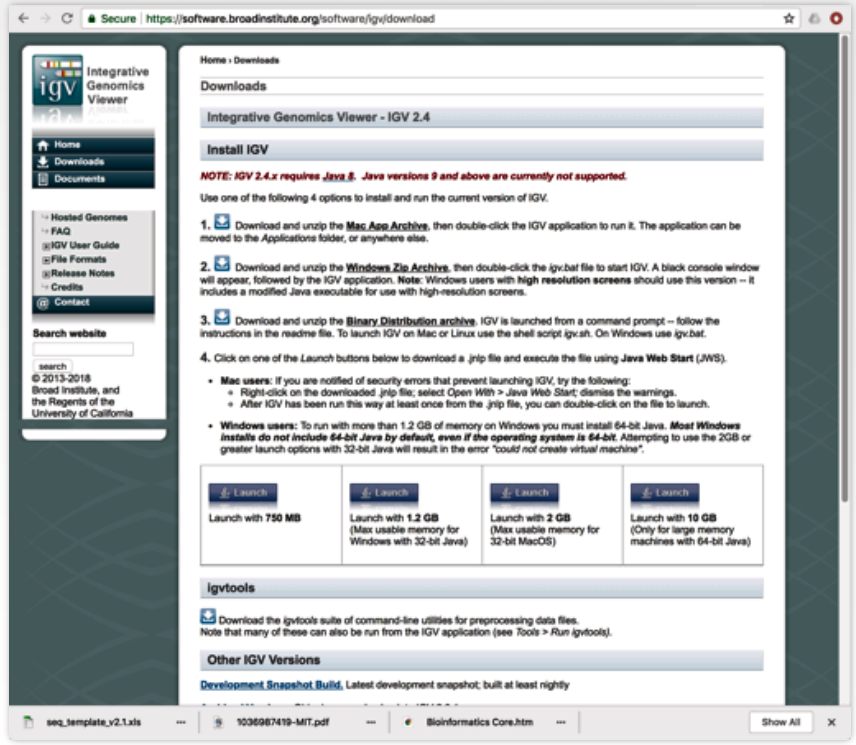
Differential analysis of RNA-seq incorporating quantification uncertainty

Harold Pimentel[1], Nicolas L Bray[2], Suzette Puente[3], Páll Melsted[4] & Lior Pachter[5]

We describe sleuth (http://pachterlab.github.io/sleuth), a method for the differential analysis of gene expression data that utilizes bootstrapping in conjunction with response error linear modeling to decouple biological variance from inferential variance. sleuth is implemented in an interactive shiny app that utilizes kallisto quantifications and bootstraps for fast and accurate analysis of data from RNA-seq experiments.

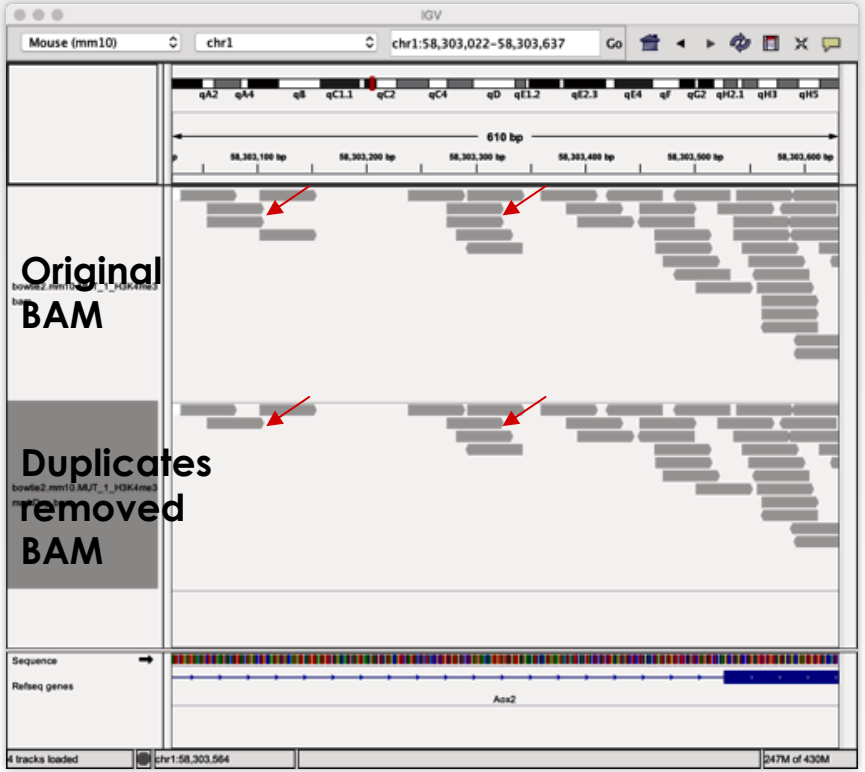
IGV: Integrative Genomics Viewer

- Goto https://software.broadinstitute.org/software/igv/
- Click Downloads icon
- Download IGV



File formats: sam/bam

- Sam/bam: alignment file contain mapping information.
- markDup: output of MarkDuplicates function of picard tools. Remove the duplicated reads for DNA-seq.
- Sort: sort the bam files by name or coordinates.
- Index: Bam index file for more efficiently access reads in a bam file.



Exercises

Visualizing data in Integrative Genomics Viewer

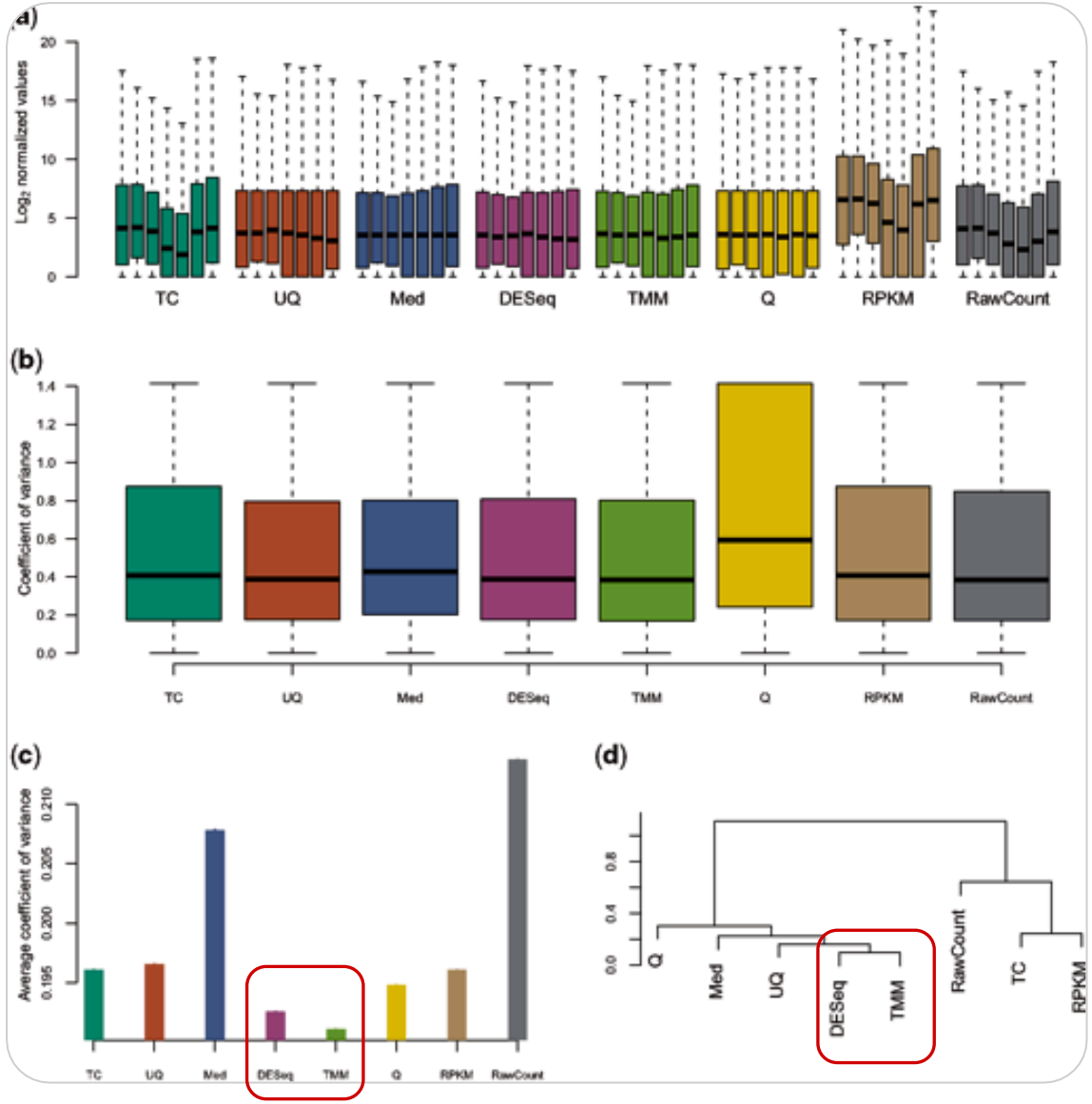
Visualizing data in UCSC genome browser

Count table

Gene Symbol	WT_rep1	WT_rep2	WT_rep3	KD_rep1	KD_rep2	KD_rep3
ackr4b	30	28	42	5	66	7
aclya	24	48	56	77	89	108
acot14	0	0	0	0	0	0
acsbg1	5	9	7	123	98	70
actr8	3536	3639	4586	225	545	898

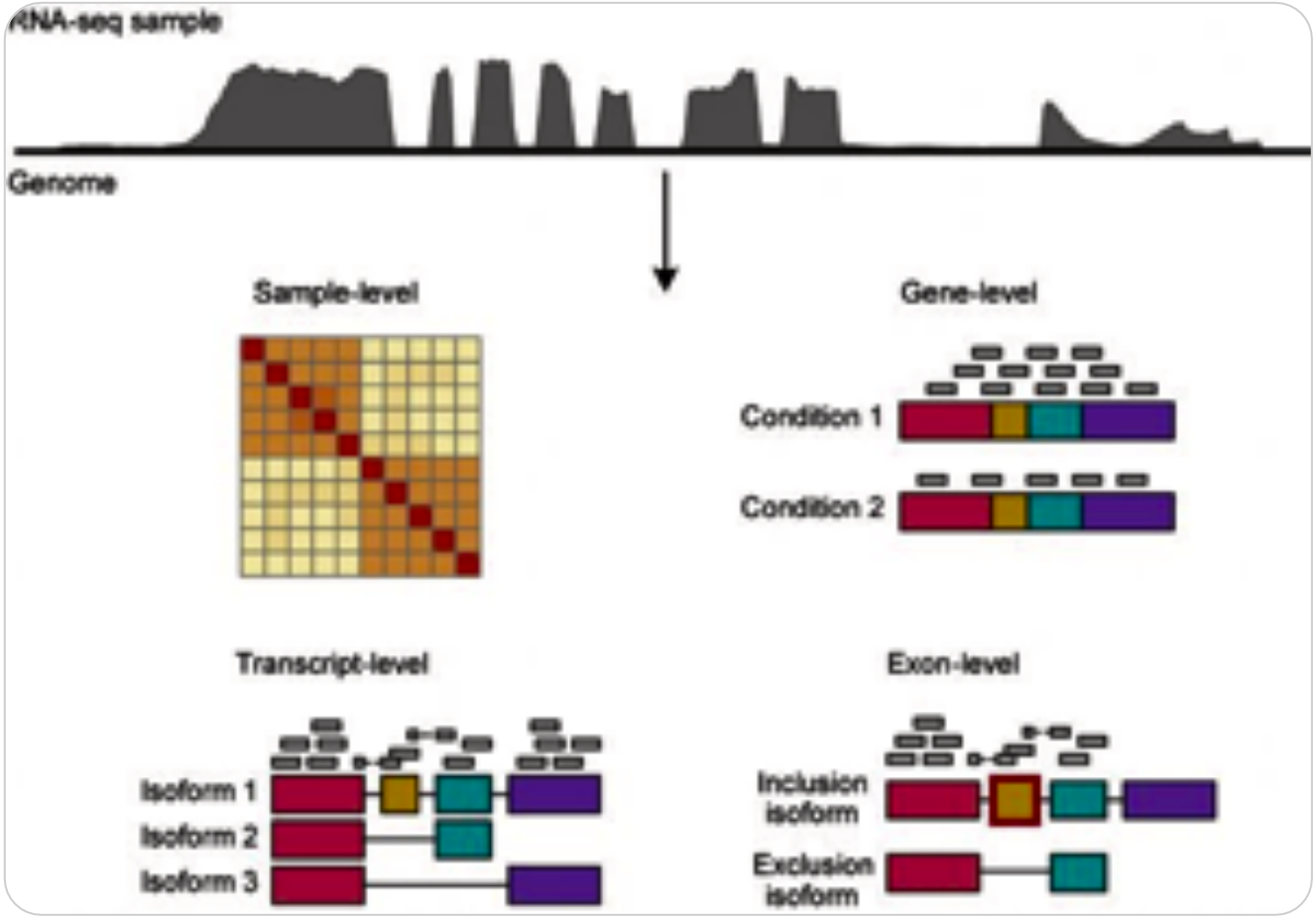
Normalization: most genes are not differential expressed

- TMM (Trimmed weighted mean)
- DESeq (median of log expression ratio)



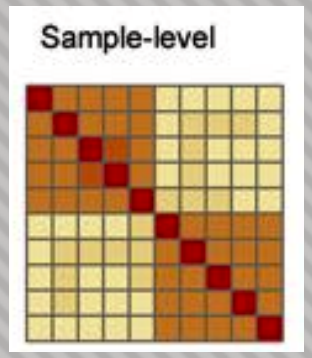
Dillies et.al., 2012. doi: 10.1093/bib/bbs046

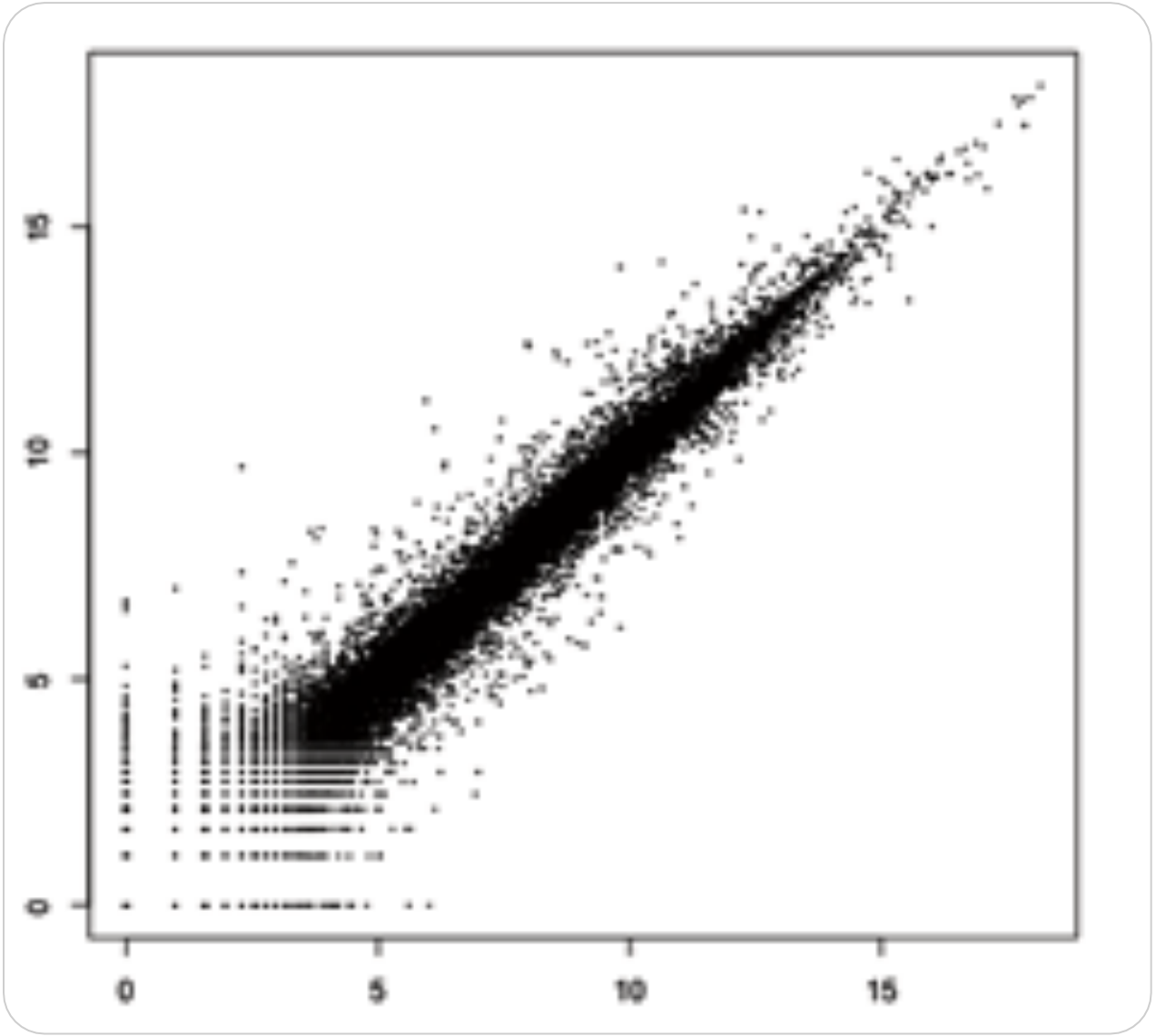
RNA-seq analyses at four different levels: sample-level, gene-level, transcript-level, and exon-level.



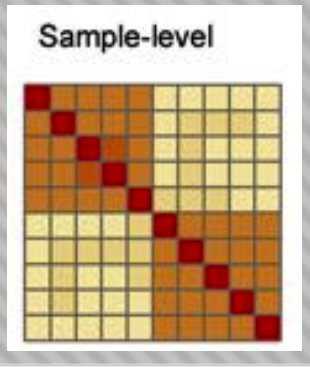
Li et.al., 2018. doi: 10.1007/s40484-018-0144-7

Scatter plot for replicates

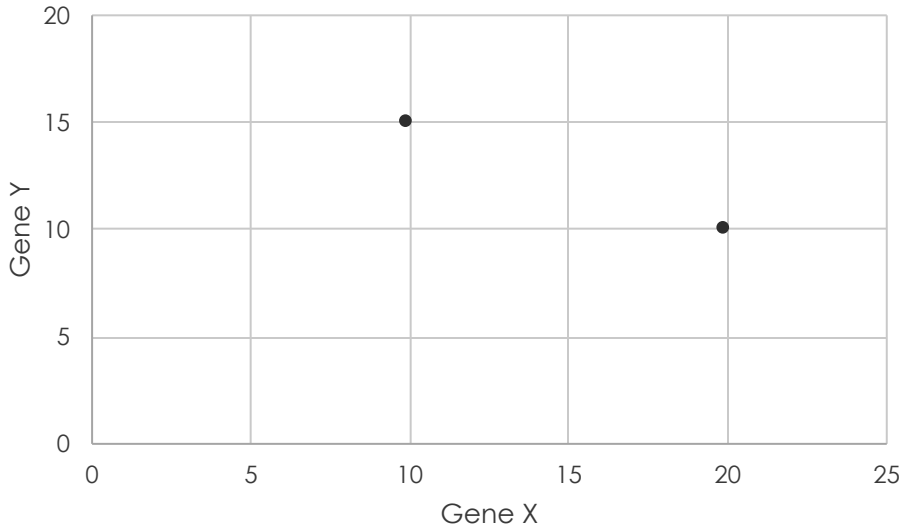


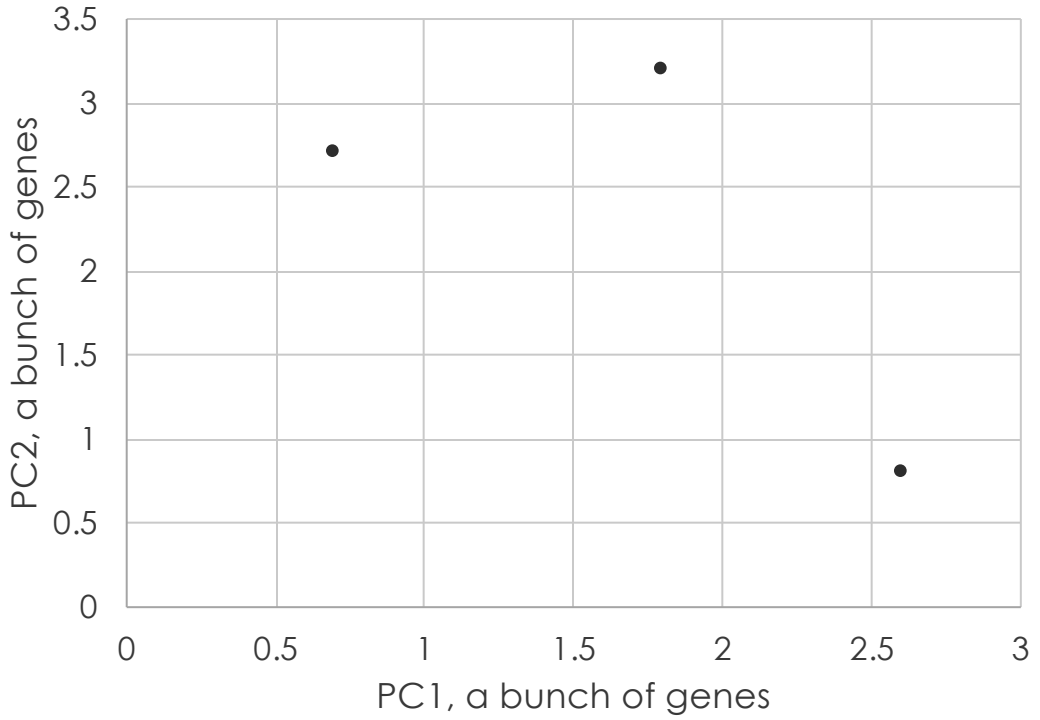


Principal component analysis (PCA)

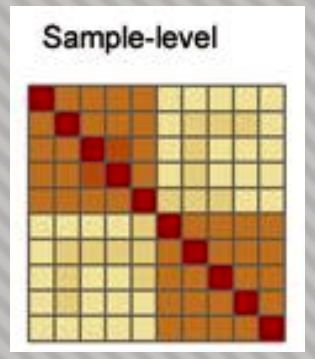


Gene	Sample1	Sample2
X	10	20
Y	15	10

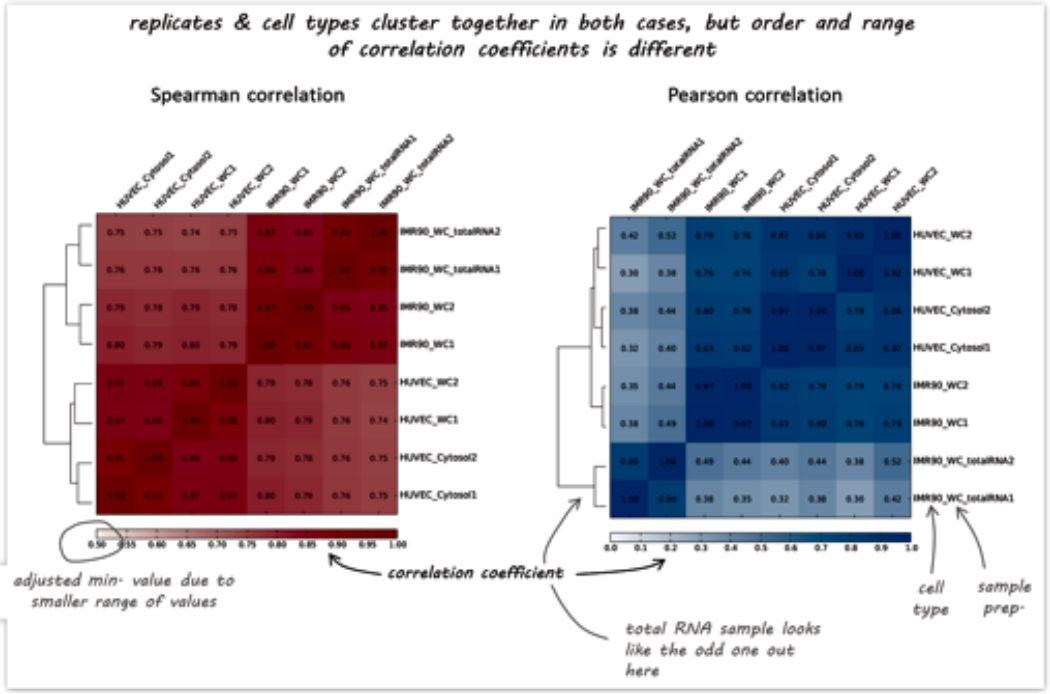




PCA reduces the complexity of expression data to show relationships on two axes

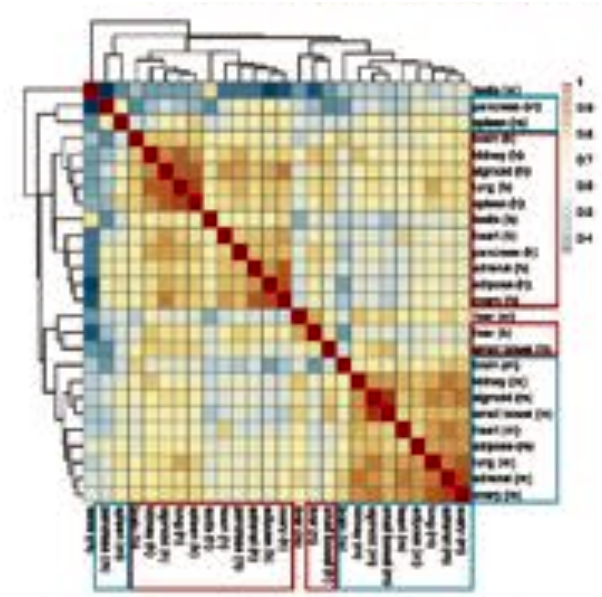


Sample-sample distance plot



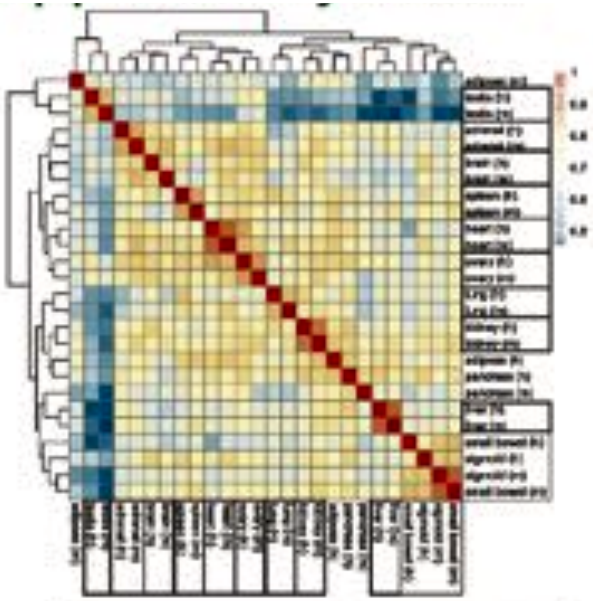
https://deeptools.readthedocs.io/en/develop/content/tools/plotCorrelation.html

Sample-level analysis is affected by batch effect



"Overall, our results indicate that there is **considerable RNA expression diversity between humans and mice**, well beyond what was described previously, likely reflecting the fundamental physiological differences between these two organisms."

Lin, Lin, and Snyder (2014), PNAS 111:48



"**Once we accounted for the batch effect** (...), the comparative gene expression data no longer clustered by species, and instead, we observed **a clear tendency for clustering by tissue**."

Gilad & Mizrahi-Man (2015), F1000Research 4:121

Summary of suitable analysis approaches for the three types of comparative analyses

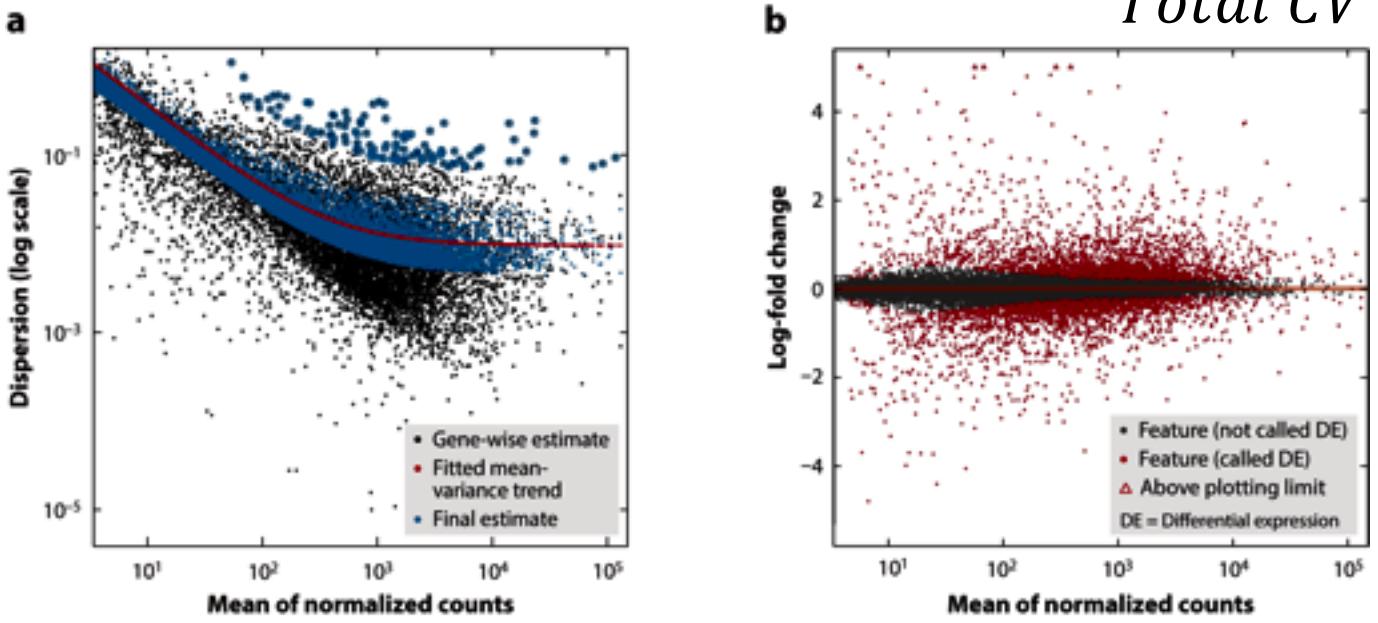
Task	Input data	Software (examples)
differential gene expression (DGE)	Gene counts	DESeq2, edgeR, voom/limma
differential transcript usage (DTU)	Transcript counts	DESeq2, edgeR, sleuth, voom/limma
differential exon usage (DEU)	Exon counts	DEXSeq, voom/limma, MISO, IsoformSwitchAnalyzeR

Differential Expression

Shared by all samples/events

Observed gene expr. variance = Biological variance + Technical variance

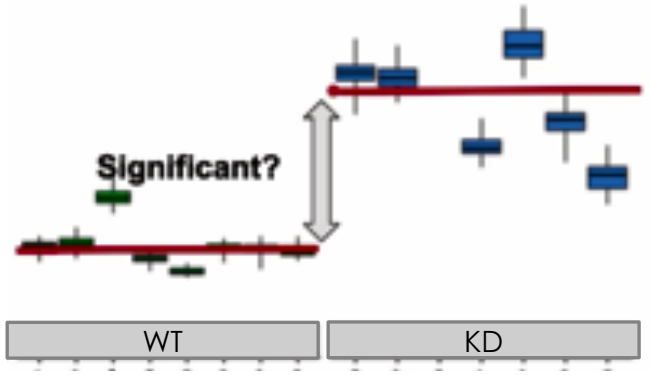
$$Total\ CV^2 = Biological\ CV^2 + Technical\ CV^2$$



Van den Berge K, et al. 2019. *Annu. Rev. Biomed. Data Sci.* 2:139–73

Berge et al. 2019. doi: 10.1146/annurev-biodatasci-072018-021255

Testing:
the variation between groups >
the variation within groups

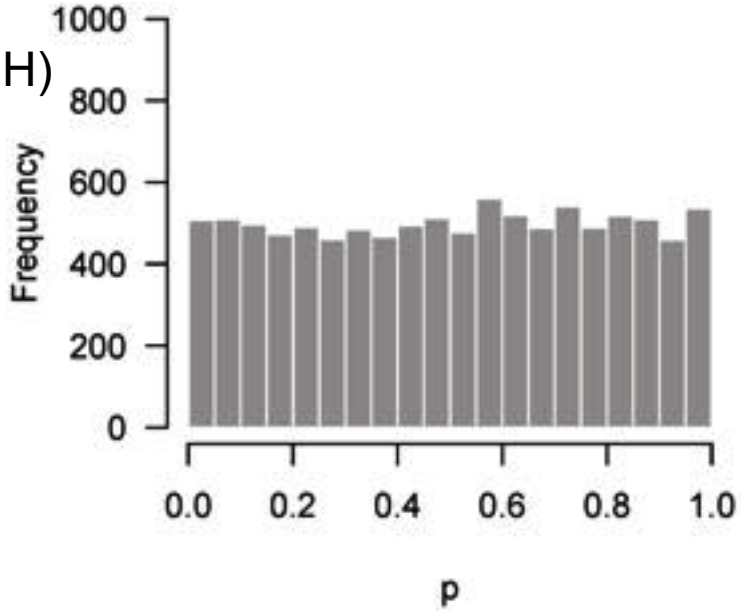


McCarthy et.al., 2012. doi: 10.1093/nar/gks042

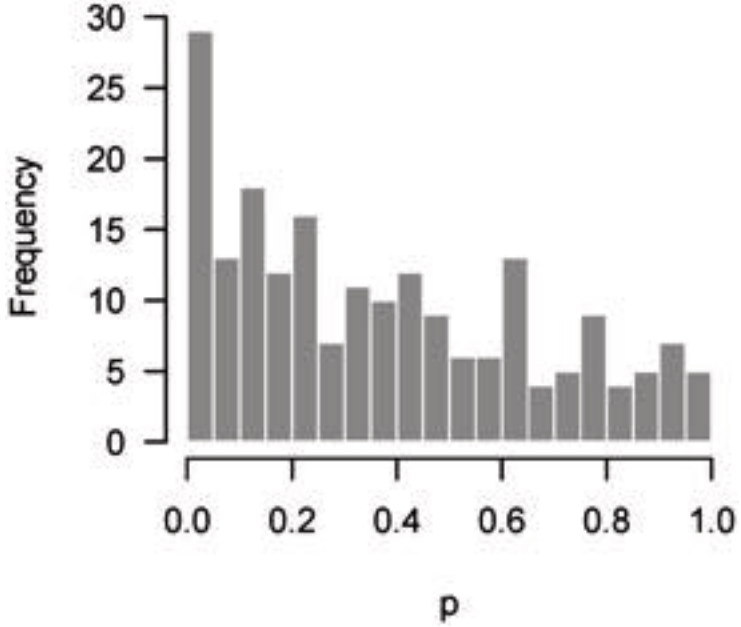
Multiple comparisons

Benjamini-Hochberg Correction (BH)
false-discovery rate (FDR)

From a uniform random number generator



From a sufficiently powered experiment



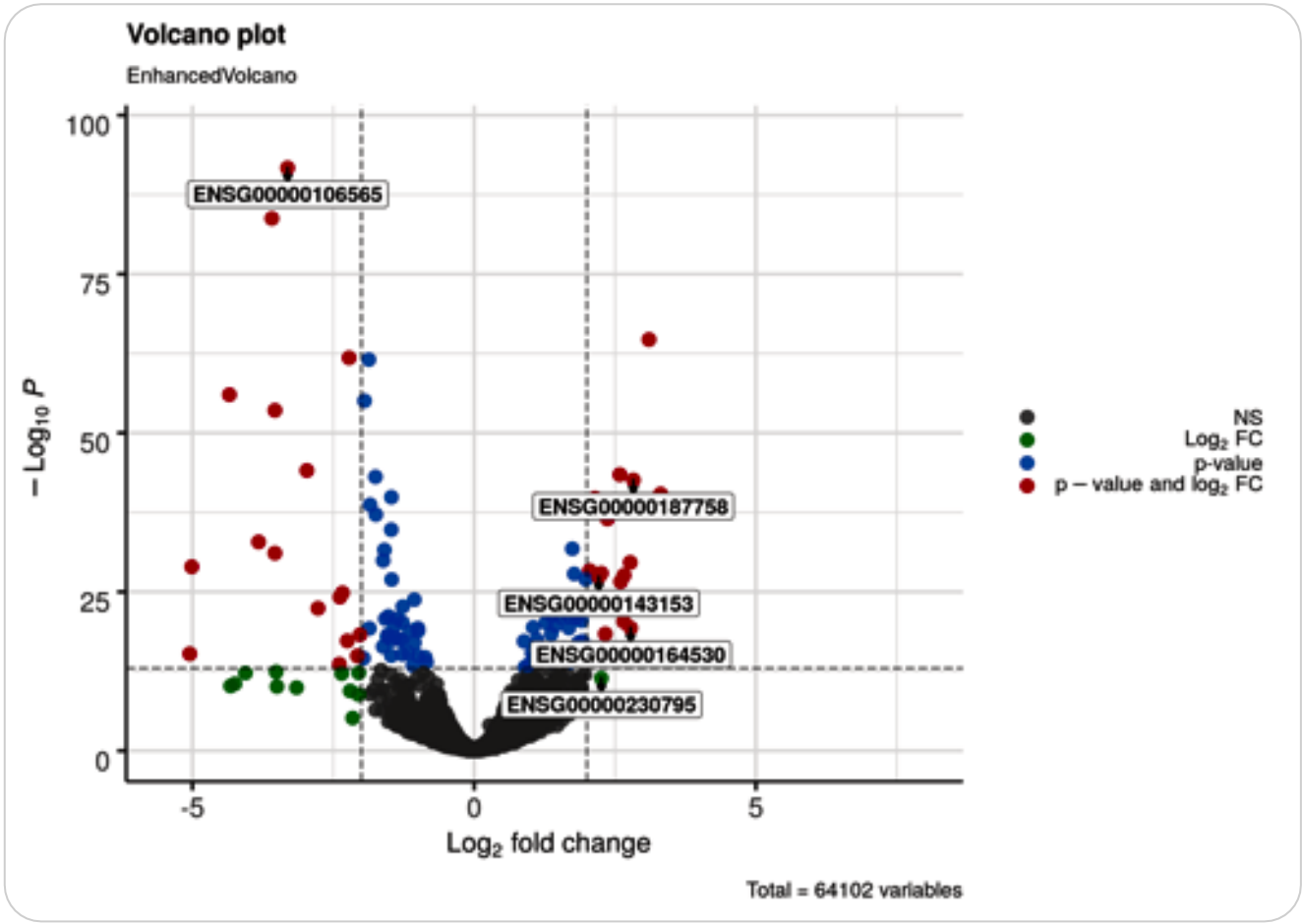
Breheny et al. 2018. doi:10.3390/ht7030023

Results

baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	gene_name	ensembl_id
569.7493	-2.51313	0.352548	-7.12847	1.01E-12	6.32E-12	aacs	ENSDARG00000012468
228.7958	-2.64457	0.241543	-10.9487	6.74E-28	1.41E-26	aamdc	ENSDARG00000103957
3299.677	-1.25607	0.237625	-5.28595	1.25E-07	4.81E-07	aamp	ENSDARG00000045019
151.558	1.228695	0.449503	2.733449	0.006267	0.012898	aarsd1	ENSDARG00000015747
423.9228	-1.38447	0.159835	-8.66182	4.64E-18	4.53E-17	aass	ENSDARG00000051816
2594.372	1.402565	0.149554	9.37834	6.7E-21	8.28E-20	abca1b	ENSDARG00000079009
1215.935	-1.7795	0.223888	-7.94816	1.89E-15	1.52E-14	abcb3l1	ENSDARG00000036787
208.7542	-2.28293	0.19053	-11.982	4.42E-33	1.35E-31	abcb6a	ENSDARG00000063297
206.3376	-1.2775	0.346485	-3.68701	0.000227	0.000587	abcb9	ENSDARG00000056200
223.5531	2.194671	0.430981	5.092271	3.54E-07	1.29E-06	abcc2	ENSDARG00000014031


Visualization of DEGs

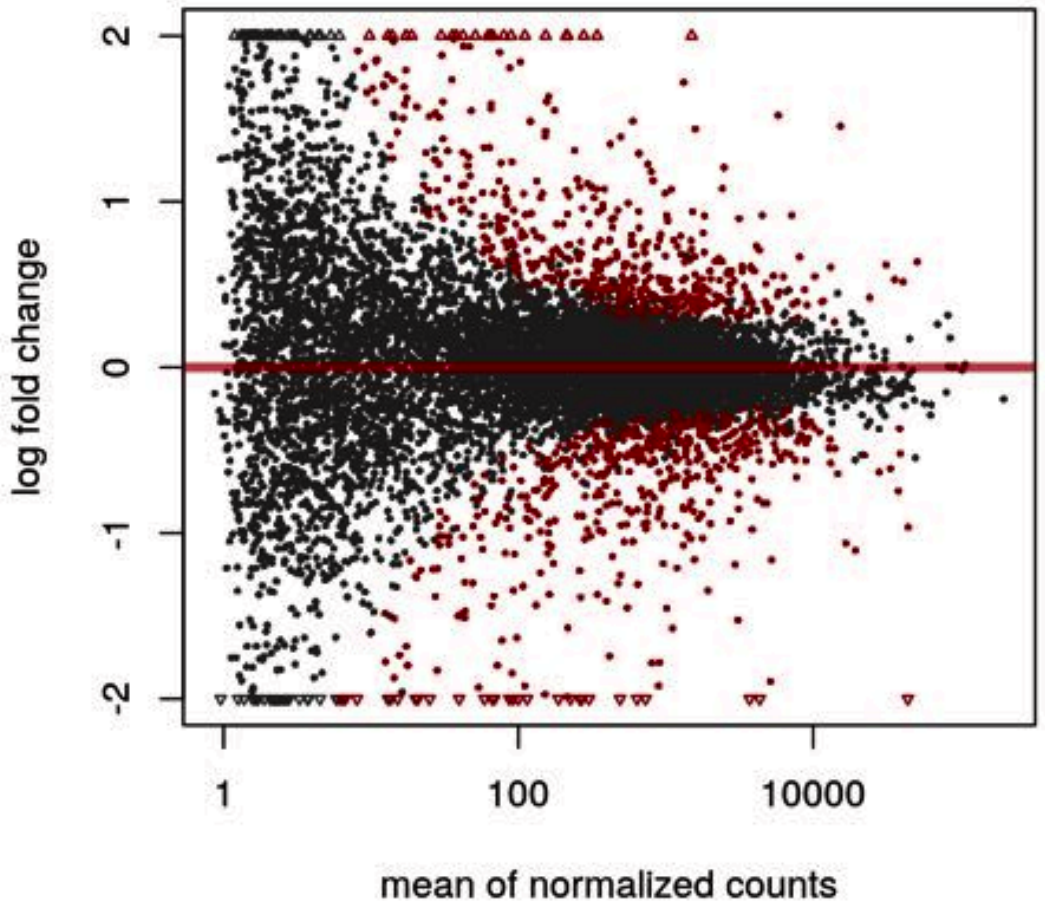
Volcano plot



https://bioconductor.org/packages/release/bioc/vignettes/EnhancedVolcano/inst/doc/EnhancedVolcano.html

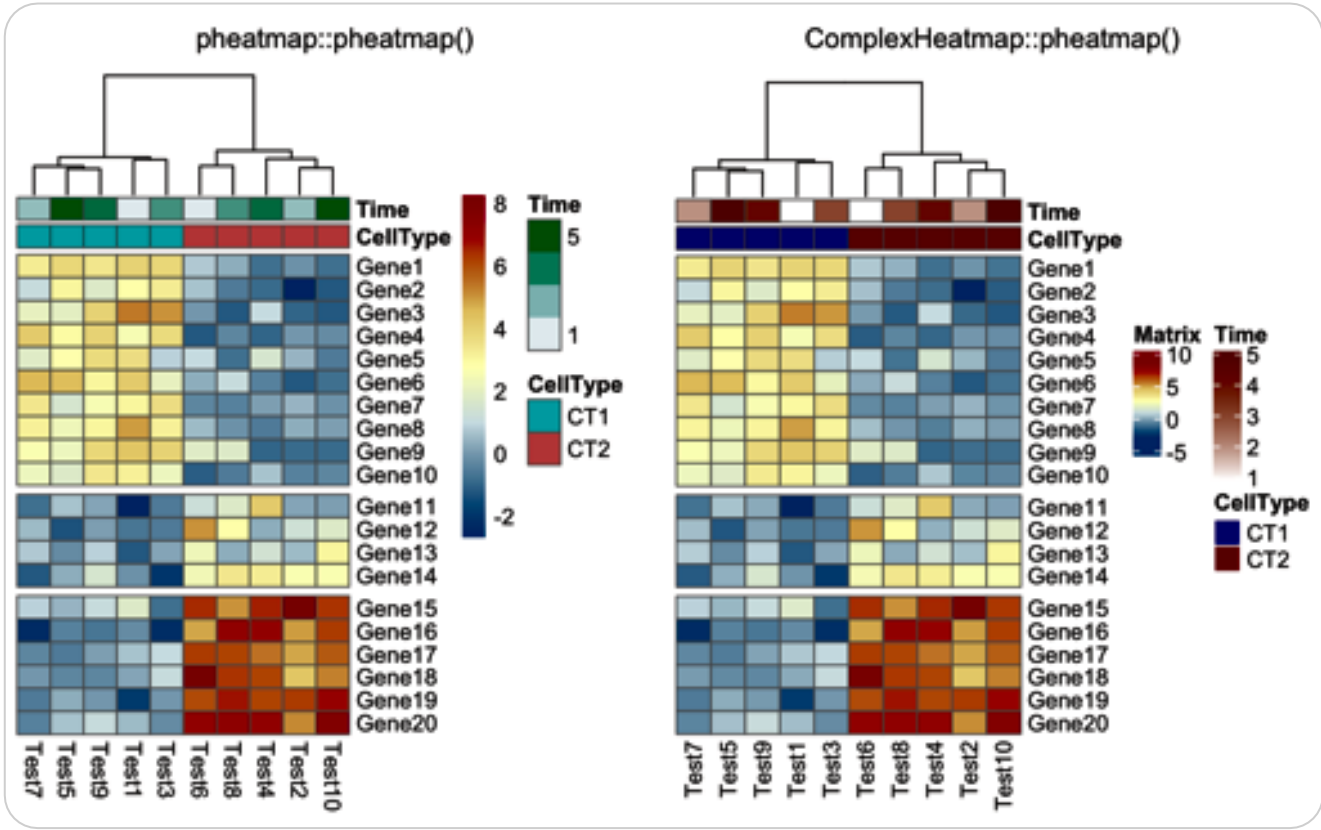
MA plot

M (log ratio) and A (mean average) scales



Heatmap

- Heatmap is a graphical reorientation of 3D data in 2D figure. It uses a system of color-coding to represent different values.
- Packages: pheatmap, ggplot2, ComplexHeatmap …



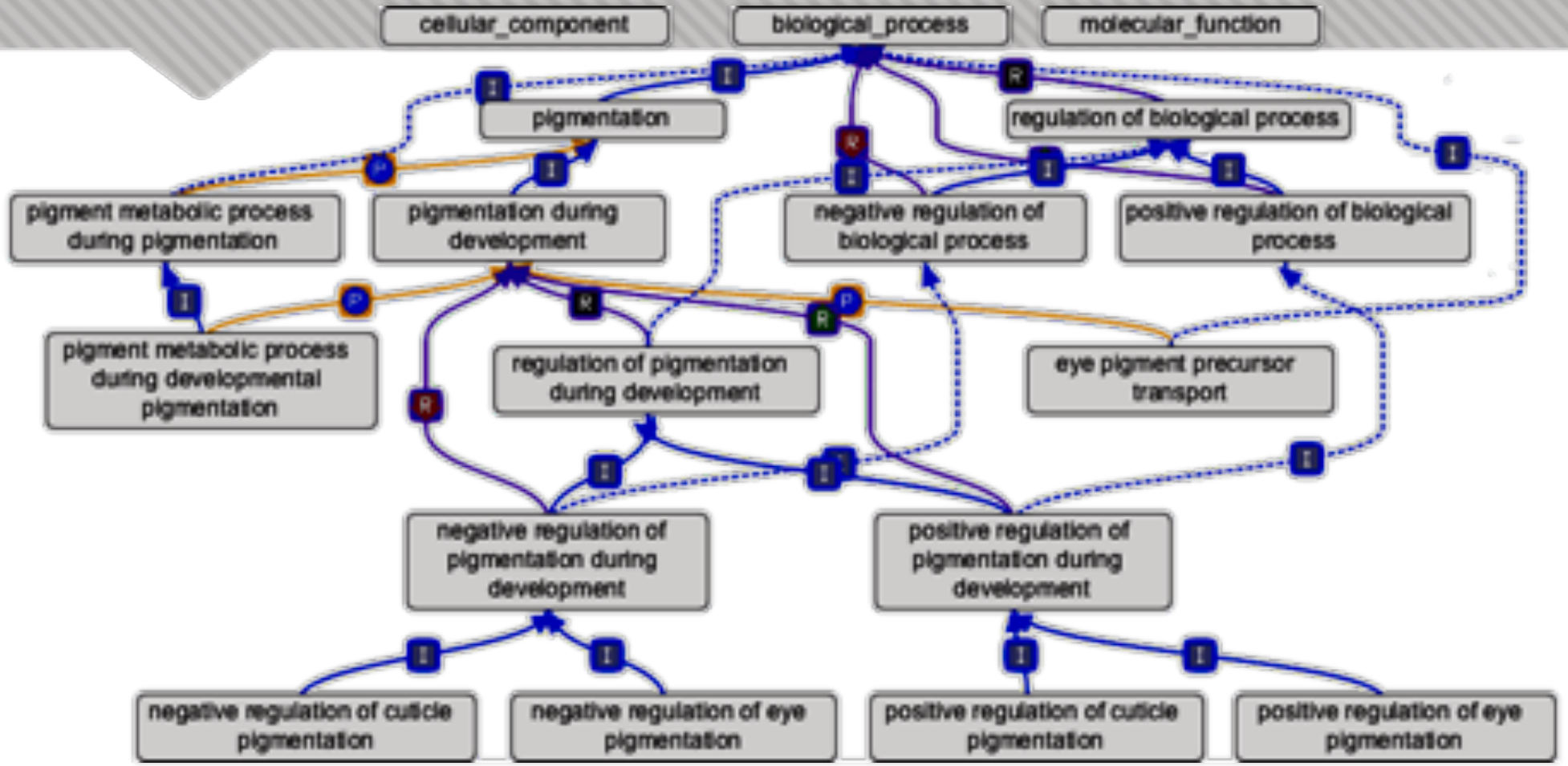
https://CRAN.R-project.org/package=pheatmap
https://bioconductor.org/packages/ComplexHeatmap/
https://jokergoo.github.io/2020/05/06/translate-from-pheatmap-to-complexheatmap/

Functional annotation of DE genes

- Gene ontology (GO) enrichment analysis
- Kyoty Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis
- Gene set enrichment analysis (GSEA)
- QIAGEN Ingenuity Pathway Analysis (IPA)

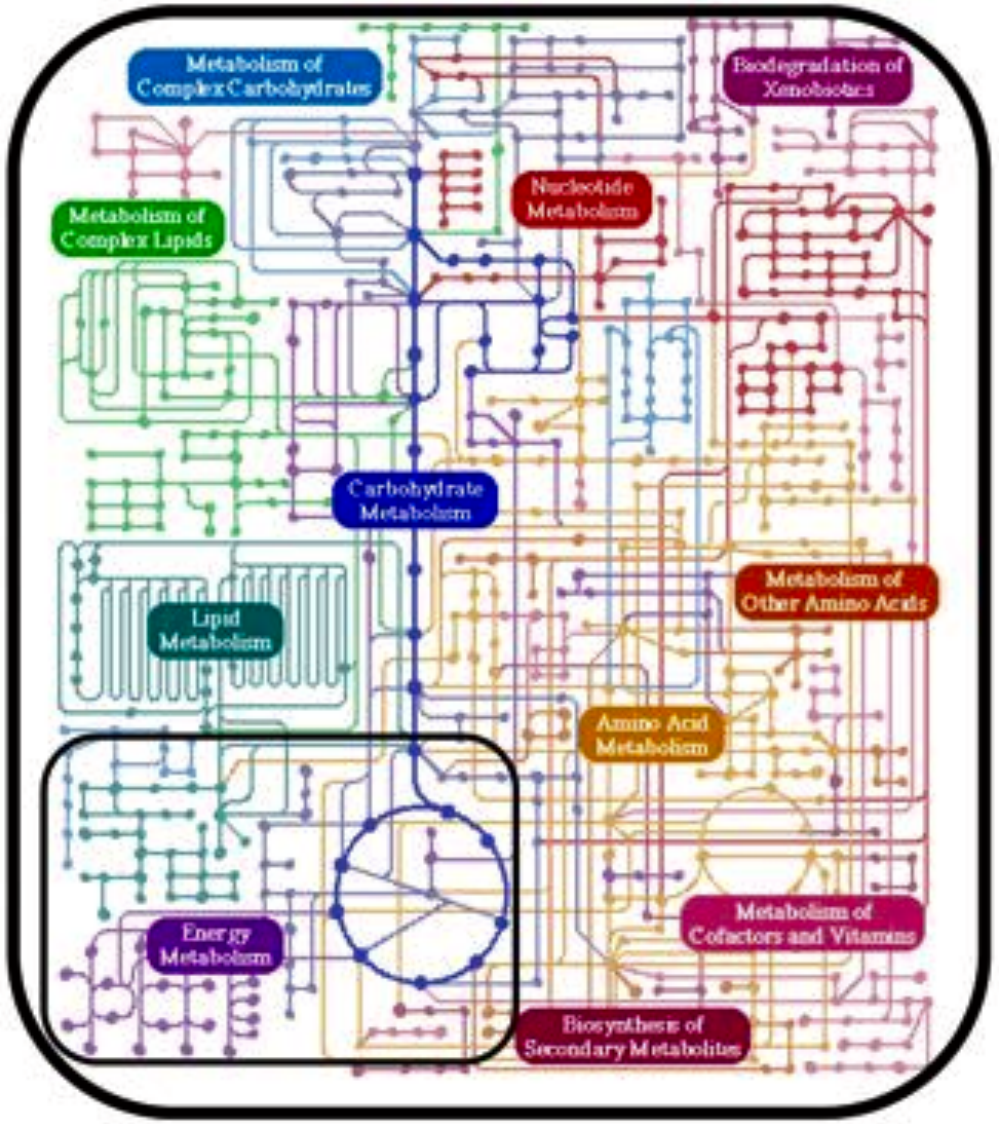
A gene set over-represented comparing to random sampling

Gene ontology



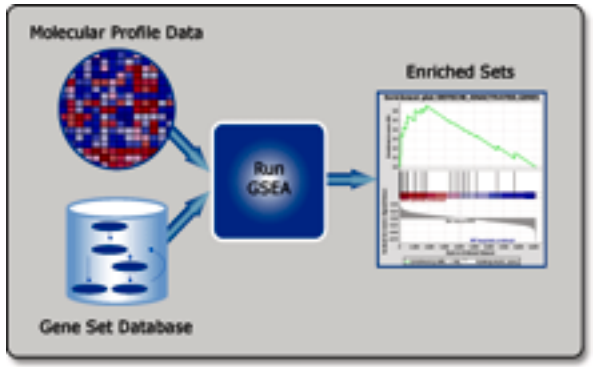
http://www-legacy.geneontology.org/GO.ontology.structure.shtml

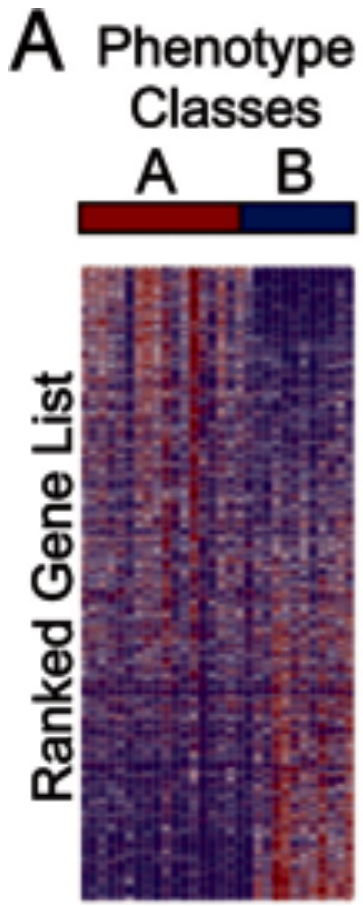
KEGG pathway enrichment analysis

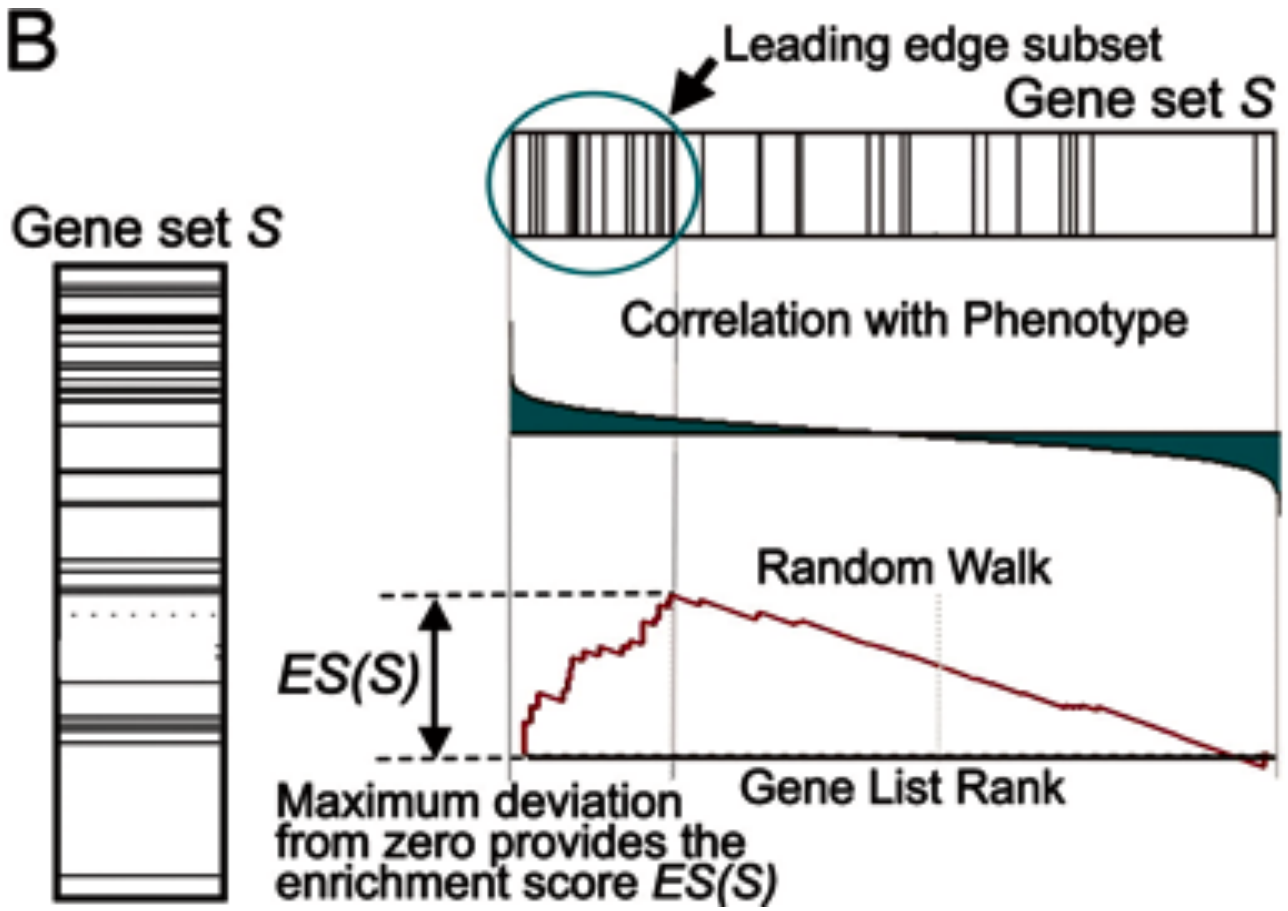


https://www.genome.jp/kegg/pathway.html

GSEA

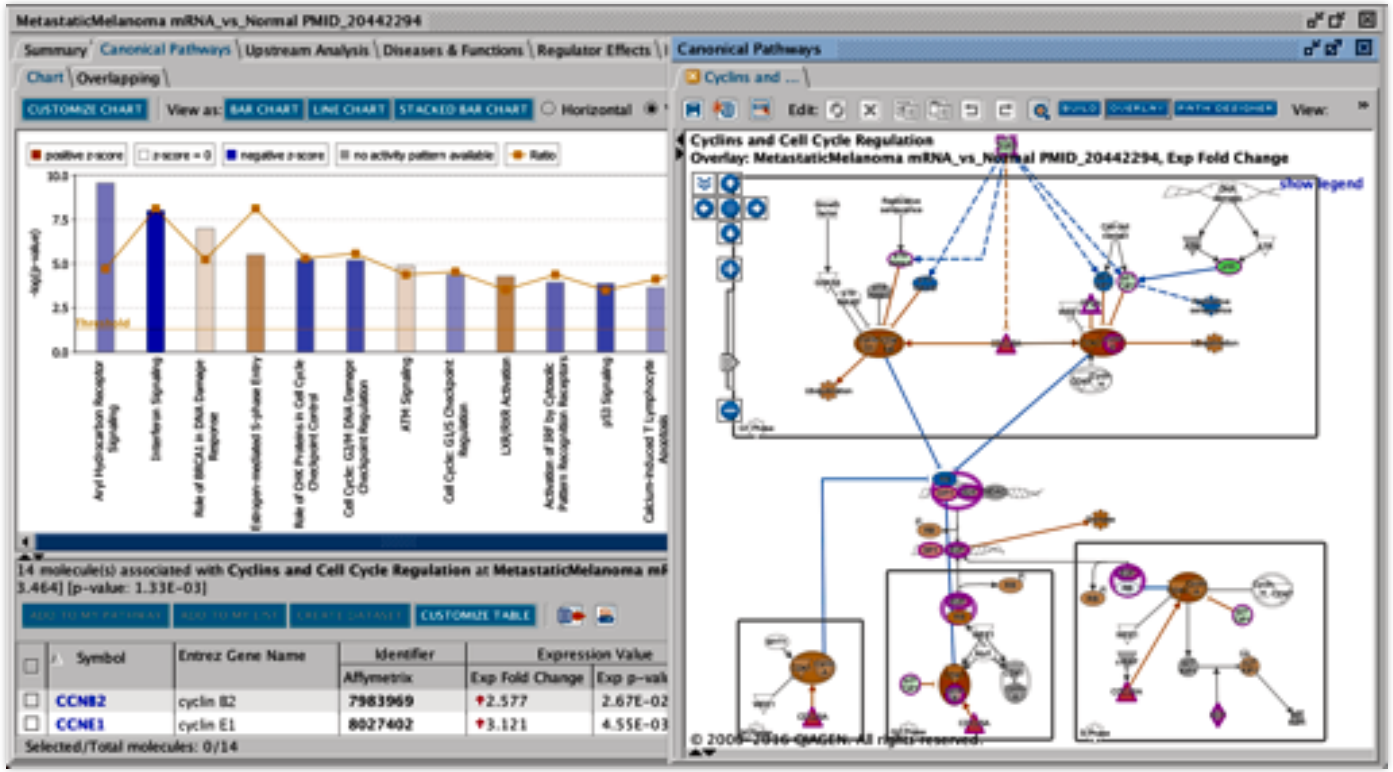






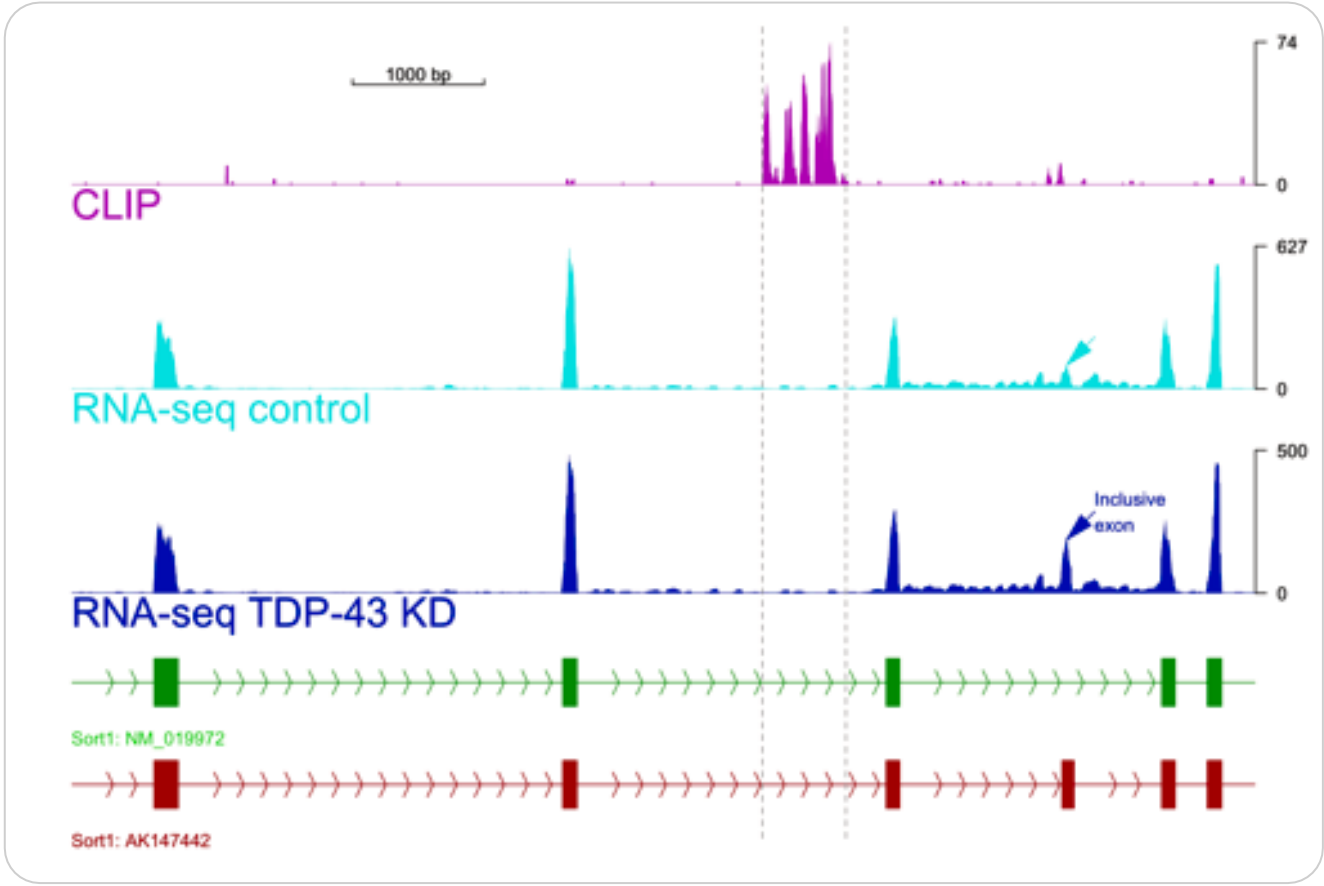
https://www.gsea-msigdb.org/gsea
Subramanian et.al., 2005. doi: 10.1073/pnas.0506580102

IPA



https://digitalinsights.qiagen.com/products/features/

View tracks by trackViewer

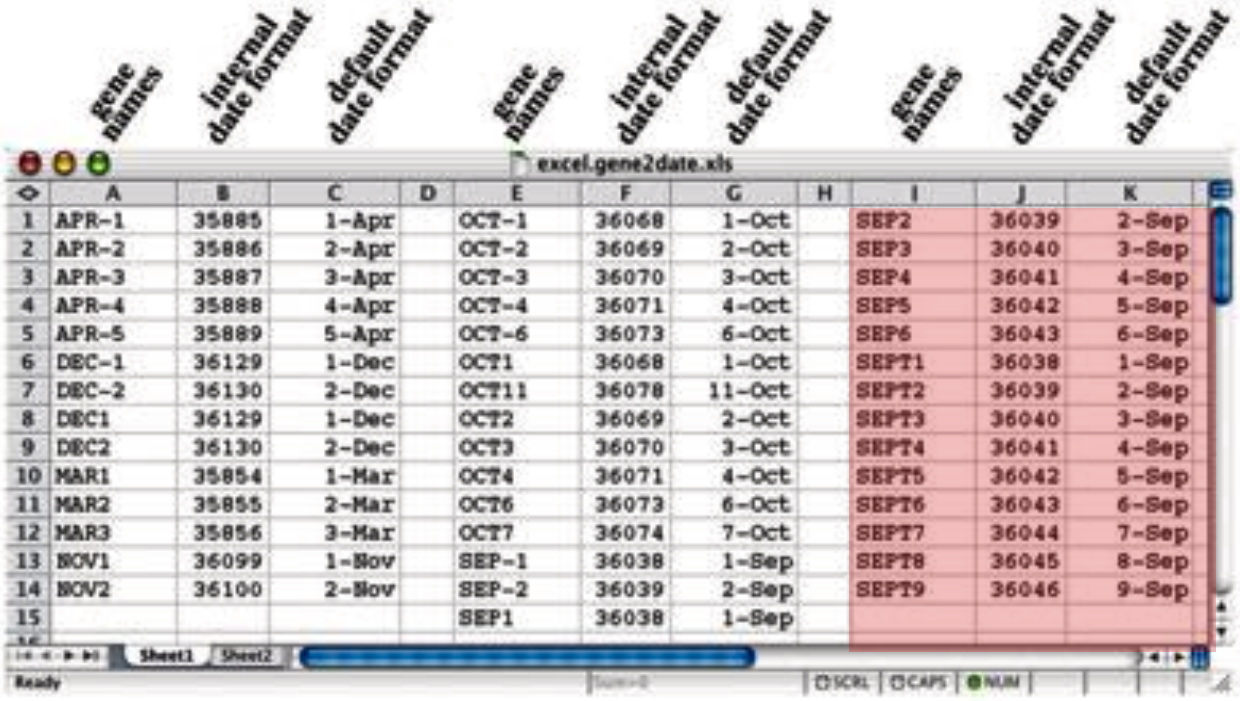


Ou et.al., 2019. doi: 10.1038/s41592-019-0430-y

Save results

The results can be saved in XLS file format using **WriteXLS** package to avoid the gene name errors that can be inadvertently introduced when opened by Excel.

```
library(WriteXLS)
WriteXLS(a_data_frame, "output.xls")
```



	A	B	C	D	E	F	G	H	I	J	K
1	APR-1	35885	1-Apr		OCT-1	36068	1-Oct		SEP2	36039	2-Sep
2	APR-2	35886	2-Apr		OCT-2	36069	2-Oct		SEP3	36040	3-Sep
3	APR-3	35887	3-Apr		OCT-3	36070	3-Oct		SEP4	36041	4-Sep
4	APR-4	35888	4-Apr		OCT-4	36071	4-Oct		SEP5	36042	5-Sep
5	APR-5	35889	5-Apr		OCT-6	36073	6-Oct		SEP6	36043	6-Sep
6	DEC-1	36129	1-Dec		OCT1	36068	1-Oct		SEPT1	36038	1-Sep
7	DEC-2	36130	2-Dec		OCT11	36078	11-Oct		SEPT2	36039	2-Sep
8	DEC1	36129	1-Dec		OCT2	36069	2-Oct		SEPT3	36040	3-Sep
9	DEC2	36130	2-Dec		OCT3	36070	3-Oct		SEPT4	36041	4-Sep
10	MAR1	35854	1-Mar		OCT4	36071	4-Oct		SEPT5	36042	5-Sep
11	MAR2	35855	2-Mar		OCT6	36073	6-Oct		SEPT6	36043	6-Sep
12	MAR3	35856	3-Mar		OCT7	36074	7-Oct		SEPT7	36044	7-Sep
13	NOV1	36099	1-Nov		SEP-1	36038	1-Sep		SEPT8	36045	8-Sep
14	NOV2	36100	2-Nov		SEP-2	36039	2-Sep		SEPT9	36046	9-Sep
15					SEP1	36038	1-Sep				

Zeeberg et.al., 2004. doi: 10.1186/1471-2105-5-80

Regeneromics Shared Resource can help your research!

Selected recent publications we have co-authored:

Identification and requirements of enhancers that direct gene expression during zebrafish fin regeneration. Thompson JD, Ou J, Lee N, Shin K, Cigliola V, Song L, Crawford GE, Kang J, Poss KD. Development. 2020 Jul 14:dev.191262. doi: 10.1242/dev.191262. Online ahead of print.

Persistence of a regeneration-associated, transitional alveolar epithelial cell state in pulmonary fibrosis. Kobayashi Y, Tata A, Konkimalla A, Katsura H, Lee RF, Ou J, Banovich NE, Kropski JA, Tata PR. Nat Cell Biol. 2020 Jul 13. doi: 10.1038/s41556-020-0542-8. Online ahead of print.

Persistence of a regeneration-associated, transitional alveolar epithelial cell state in pulmonary fibrosis. Kobayashi Y, Tata A, Konkimalla A, Katsura H, Lee RF, Ou J, Banovich NE, Kropski JA, Tata PR. Nat Cell Biol. 2020 Jul 13. doi: 10.1038/s41556-020-0542-8. Online ahead of print.

Nucleoporin 153 links nuclear pore complex to chromatin architecture by mediating CTCF and cohesin binding.Kadota S, Ou J, Shi Y, Lee JT, Sun J, Yildirim E. Nat Commun. 2020 May 25;11(1):2606. doi: 10.1038/s41467-020-16394-3.

Vitamin D Stimulates Cardiomyocyte Proliferation and Controls Organ Size and Regeneration in Zebrafish. Han Y, Chen A, Umansky KB, Oonk KA, Choi WY, Dickson AL, Ou J, Cigliola V, Yifa O, Cao J, Tornini VA, Cox BD, Tzahor E, Poss KD. Dev Cell. 2019 Mar 25;48(6):853-863.e5. doi: 10.1016/j.devcel.2019.01.001. Epub 2019 Jan 31.

We do: experimental design, bioinformatics analysis, manuscript preparation, grant applications

Jianhong Ou, Ph.D.

Email: rnirsr@duke.edu

https://sites.duke.edu/regenerationnext/jobsrni/

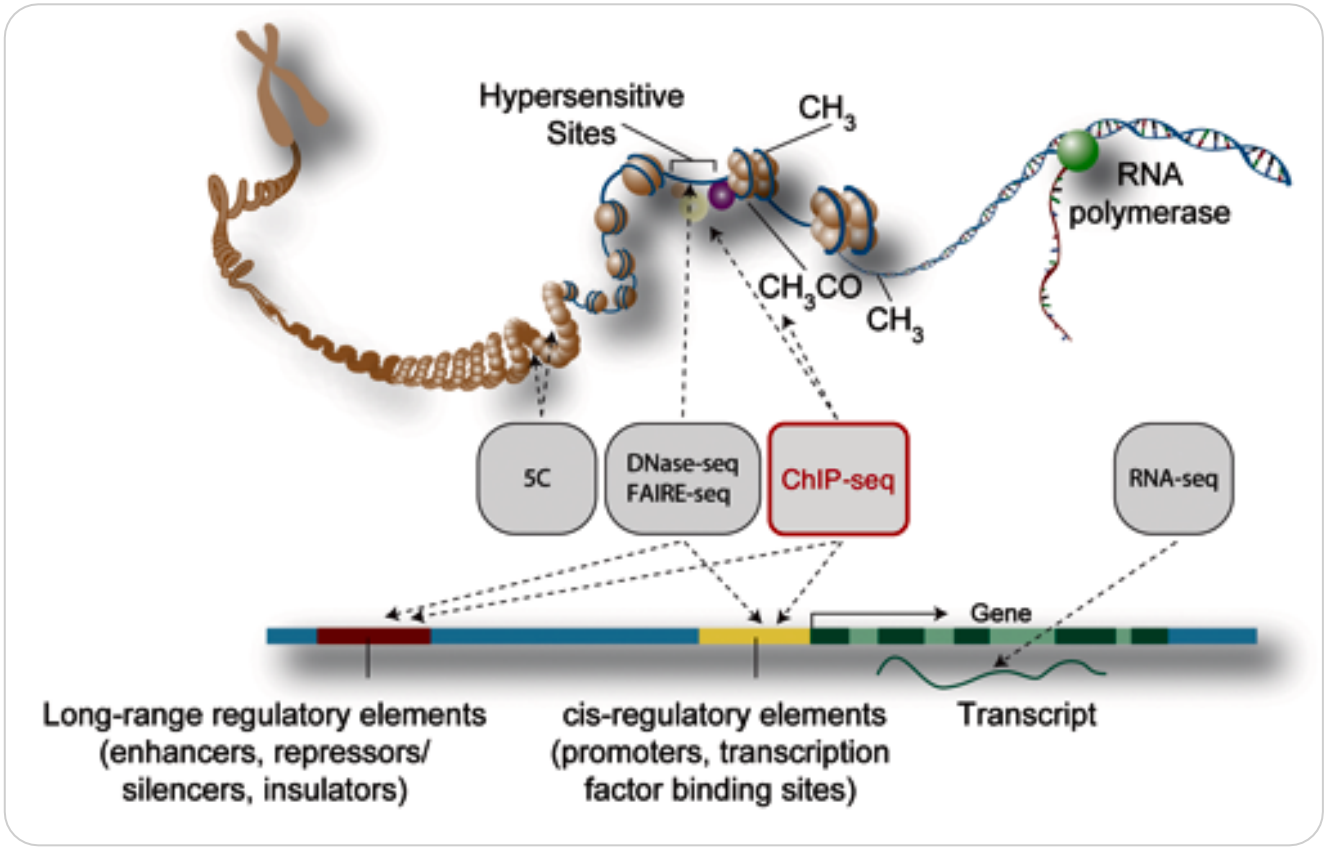
regenerationNEXT

ChIP-seq

ChIP-seq technology

Chromatin immunoprecipitation followed by sequencing (ChIP-seq) is a technique for genome-wide profiling of DNA-binding proteins, histone modifications or nucleosomes. – Peter J. Park

Park, 2009. doi:10.1038/nrg2641



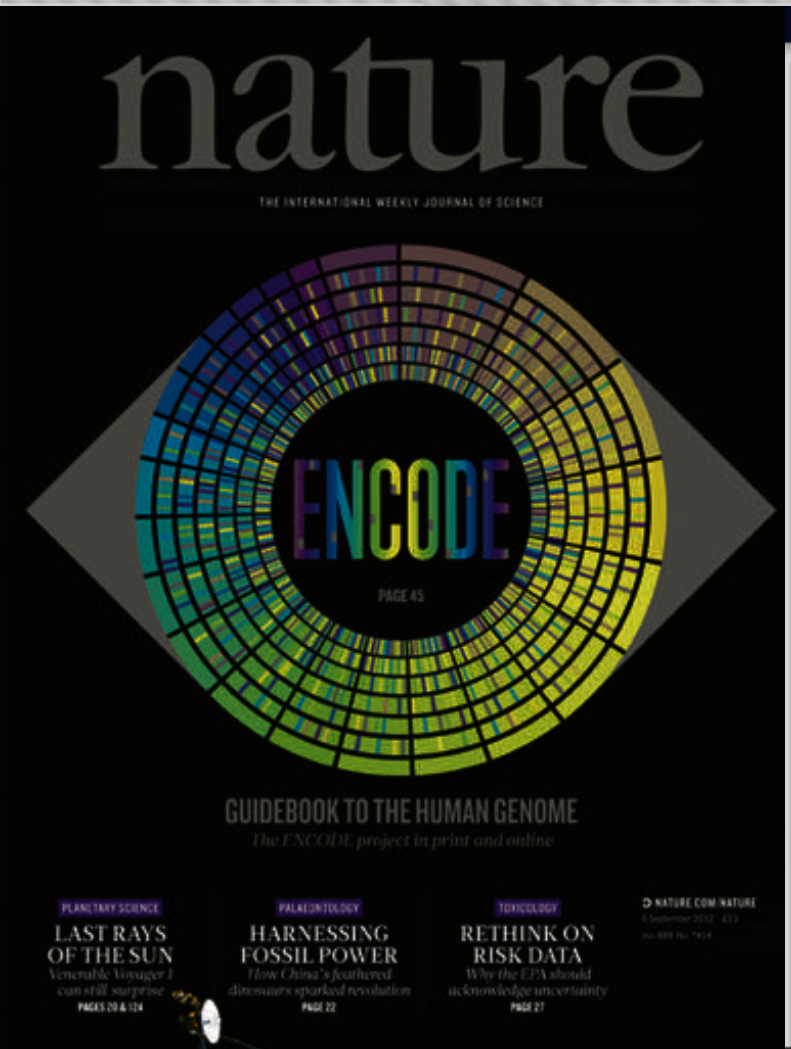
The ENCODE Project Consortium 2011. doi: 10.1371/journal.pbio.1001046

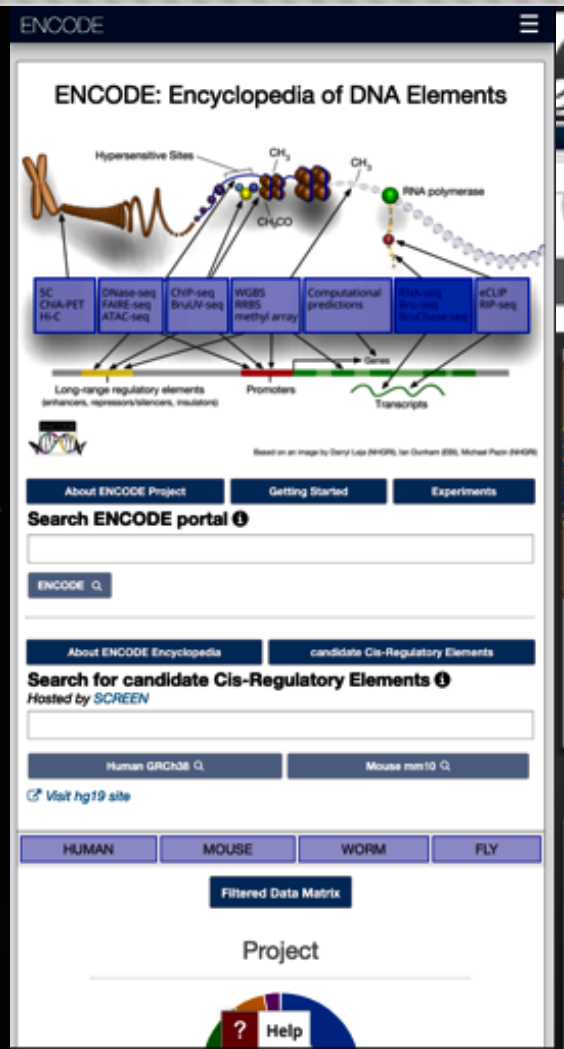
Uses

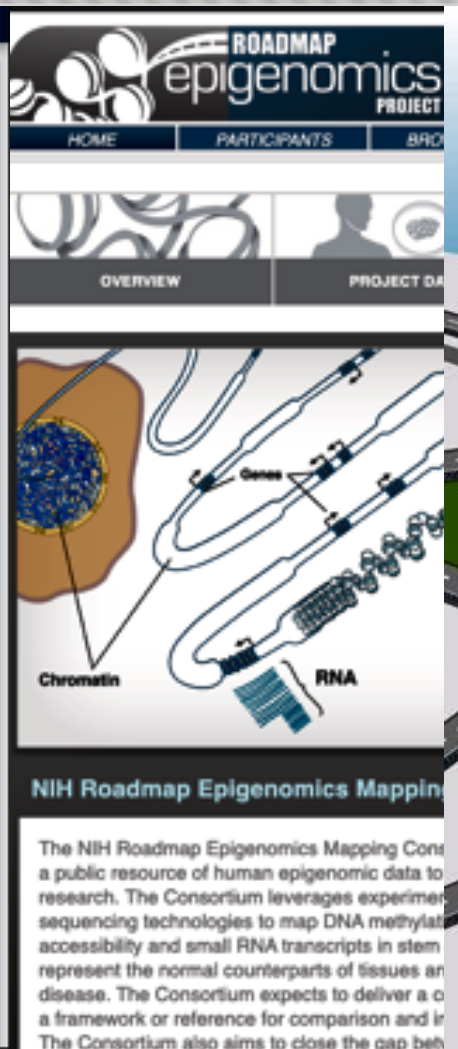
- Determine the DNA targets for transcription factors (TFs) in genome-wide
- Capture the histone modifications across the entire genome
- Define the TF binding motifs
- Reveal gene regulatory networks in combination with RNA sequencing and methylation analysis

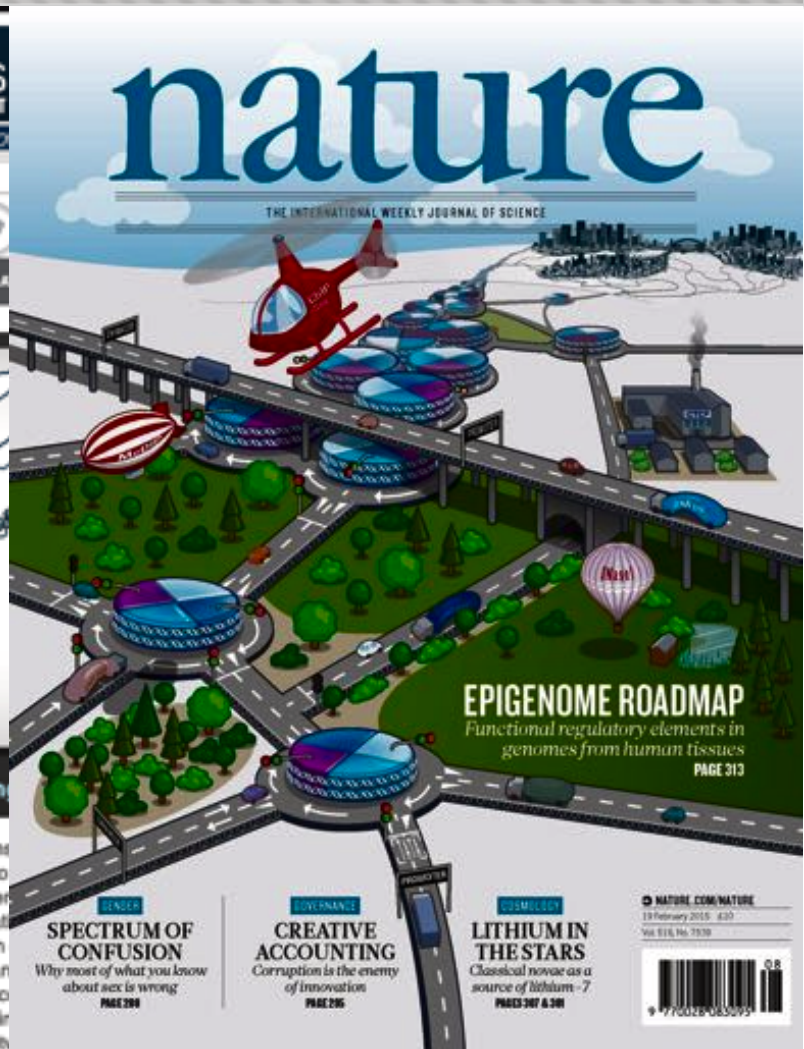
https://www.illumina.com/techniques/sequencing/dna-sequencing/chip-seq.html

ENCODE and Epigenomics Roadmap

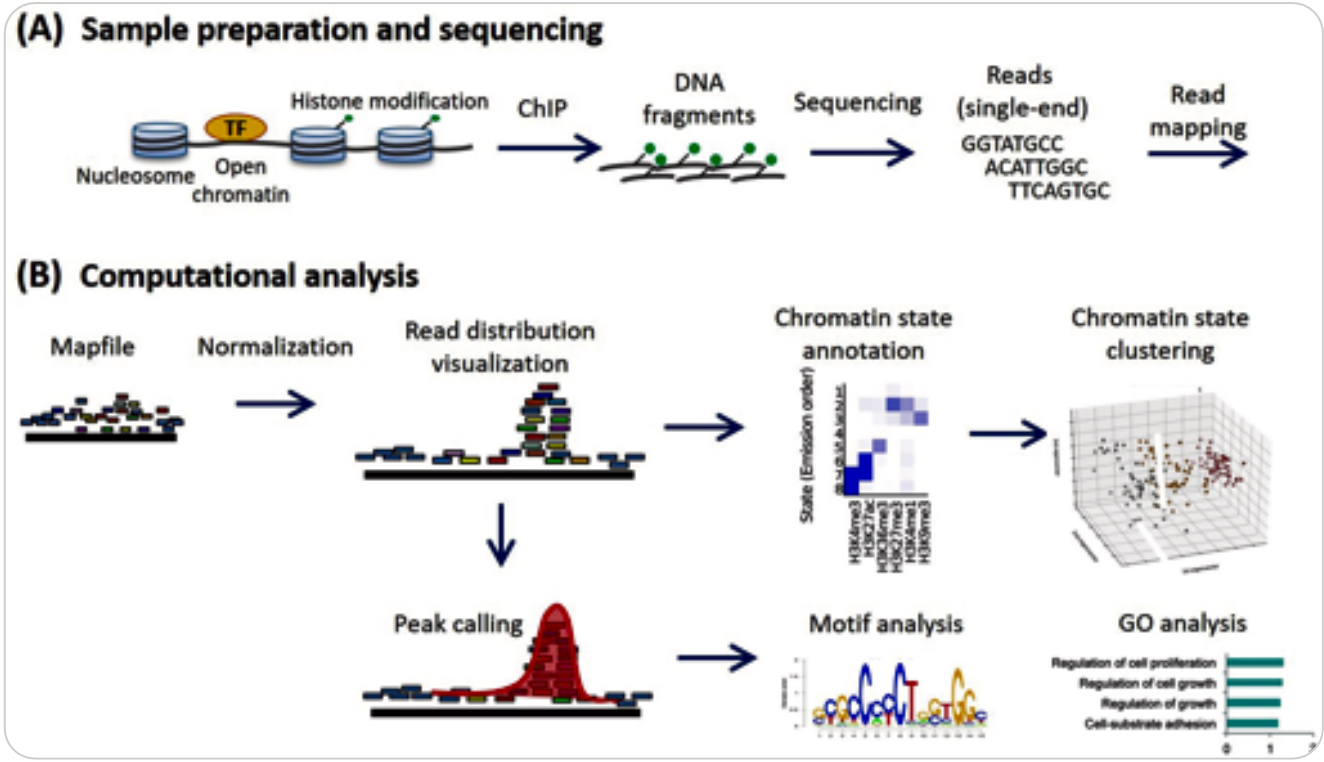






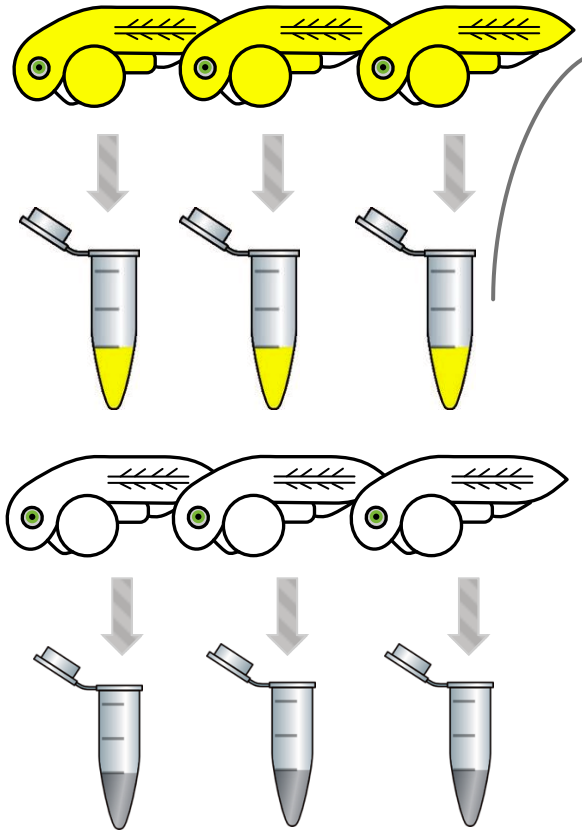


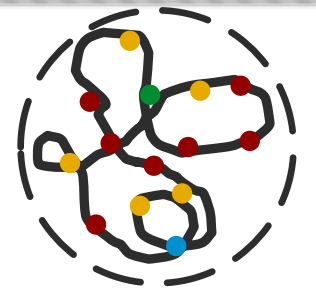
ChIP-seq analysis workflow



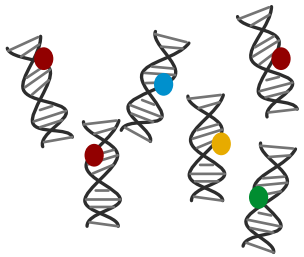
Nakato et.al., 2020. doi: 10.1016/j.ymeth.2020.03.005

ChIP-seq protocol

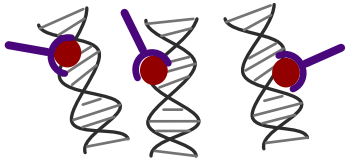




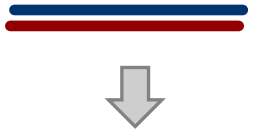
Cross-linked nucleus



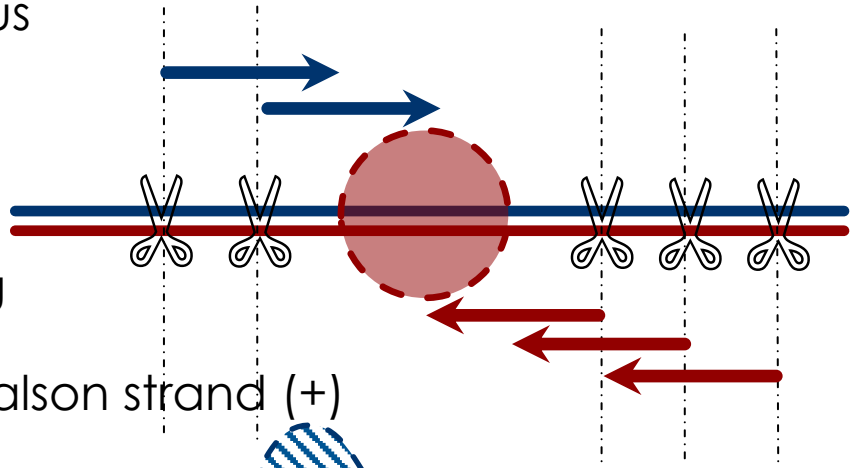
Cell lysis and
chromatin shearing



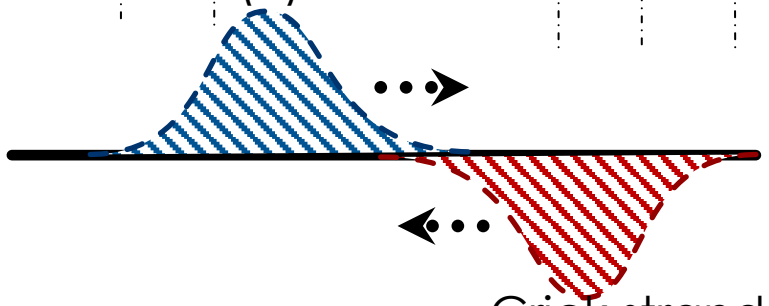
ChIP reaction



Crosslink reversal and DNA purification



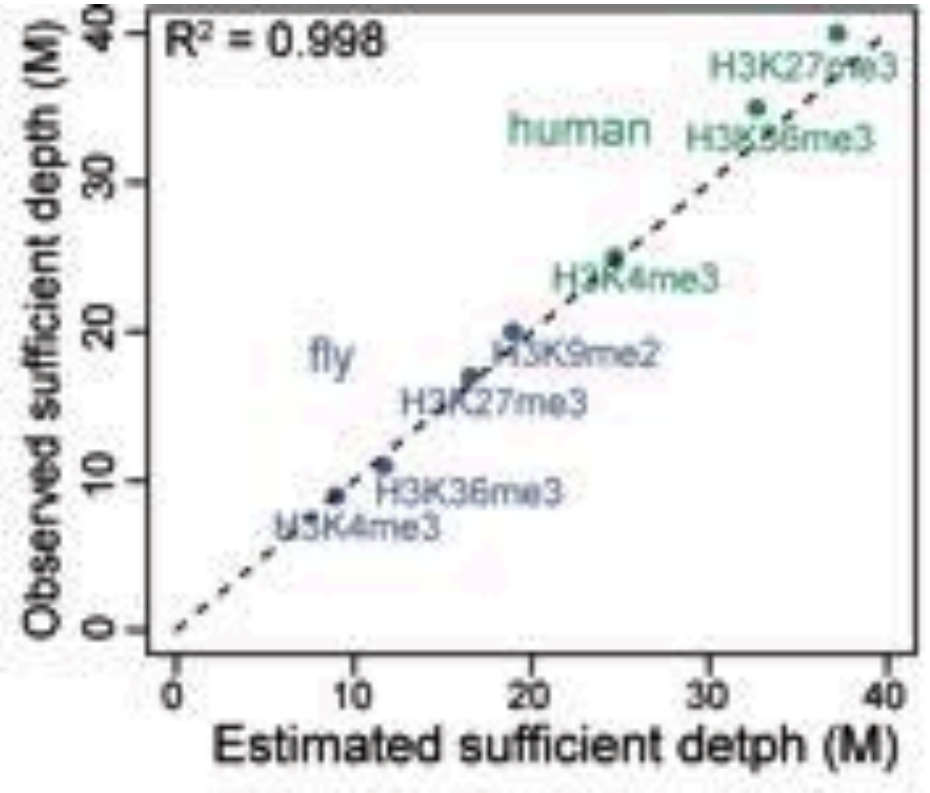
Walson strand (+)



Crick strand (-)

Sequencing Depth

- Sharp peaks (TFs)
  - > 10M reads human/mouse genomes
- Broad Peaks (Histones)
  - > 20M reads human/mouse genomes



Jung et.al., 2014. doi: 10.1093/nar/gku178

Control sample

Input DNA

- Controls for CNVs, sequencing biases, fragmentation and shearing biases

IgG

- As with input but also controls for non-specific binding

Replicates

- Biological replicate experiments are necessary.
- Use different antibodies are recommended.

Single end or paired end

- Paired end (PE) sequencing
  - improved efficiency of alignment to repetitive regions
  - Greater ability to detect fragment sizes
- Single-end (SE) sequencing is cheaper than PE.

Short reads alignment for ChIP-seq

Quality Assessment of sequencing:
FastQC

Mapping reads to reference genome:
bowtie/2, bwa, Rsubread, STAR

Remove potential PCR duplicates:
Picard software suite::MarkDuplicates,
Samtools::rmdup

Library complexity (Duplicates)

Landt et.al., 2012. doi: 10.1101/gr.136184.111

Duplication rates are a useful QC metric

(Duplicate reads/Total Mapped Reads) *100

Expected to be low

Non-Redundant Fraction (NRF)

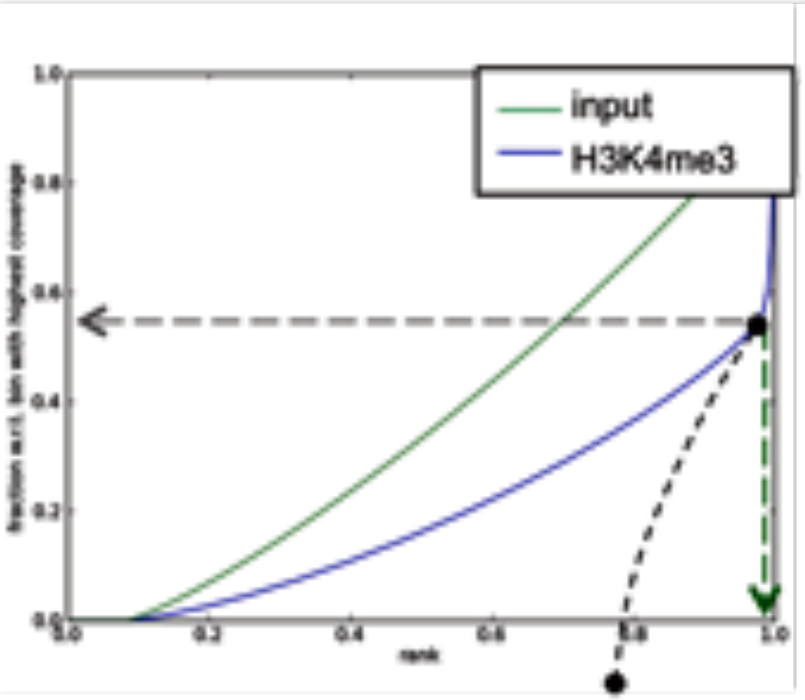
ENCODE guidelines: NRF >= 0.8 for 10M reads

PCR Bottleneck Coefficient (PBC)

A measure of library complexity. High-quality ChIP-seq data set should have PBC values >= 0.9

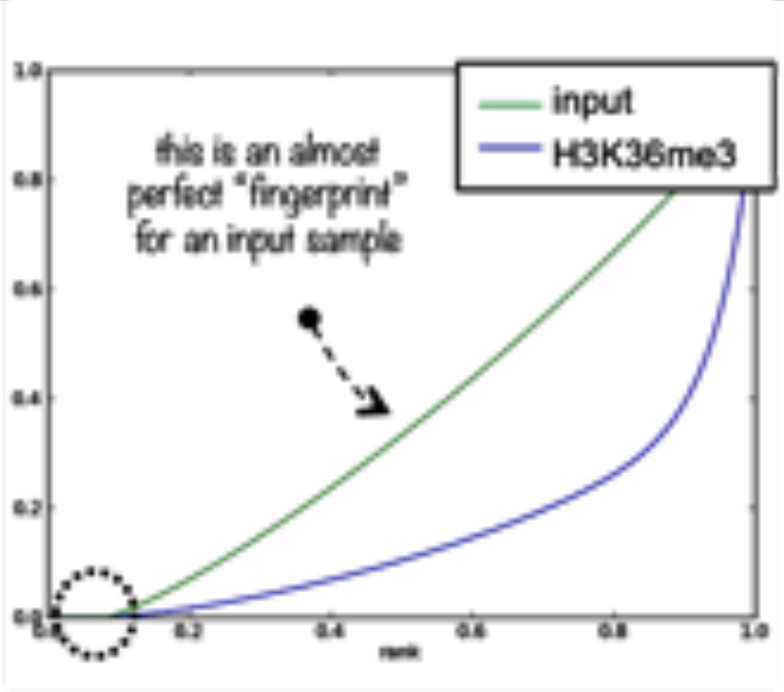
https://genome.ucsc.edu/ENCODE/qualityMetrics.html

Cumulative curve

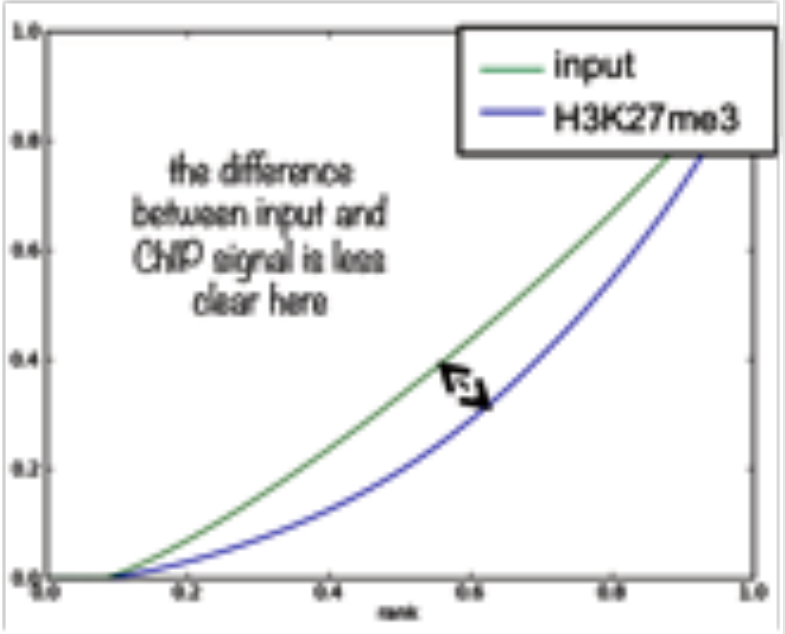


when counting the reads contained in **97%** of all genomic bins, only ca. 55% of the maximum number of reads are reached, i.e. 3% of the genome contain a very large fraction of reads!

this indicates very localized, very strong enrichments!
(as every biologist hopes for in a ChIP for H3K4me3)



pay attention to where the curves start to rise – this already gives you an assessment of how much of the genome you have not sequenced at all (i.e. bins containing zero reads – for this example, ca. 10% of the entire genome do not have any read)

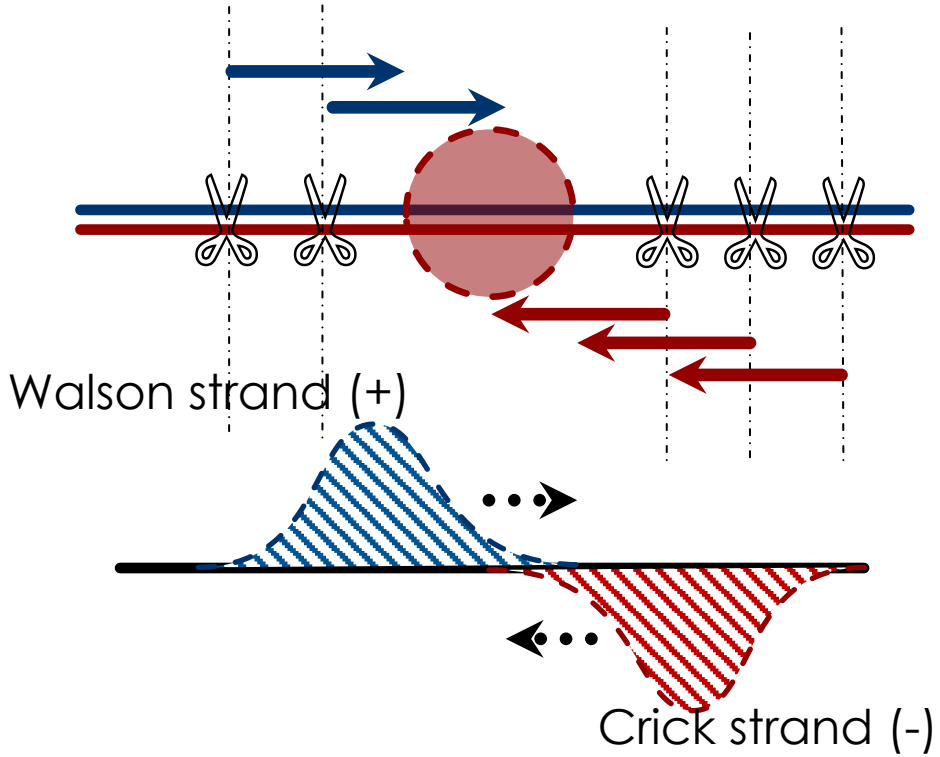


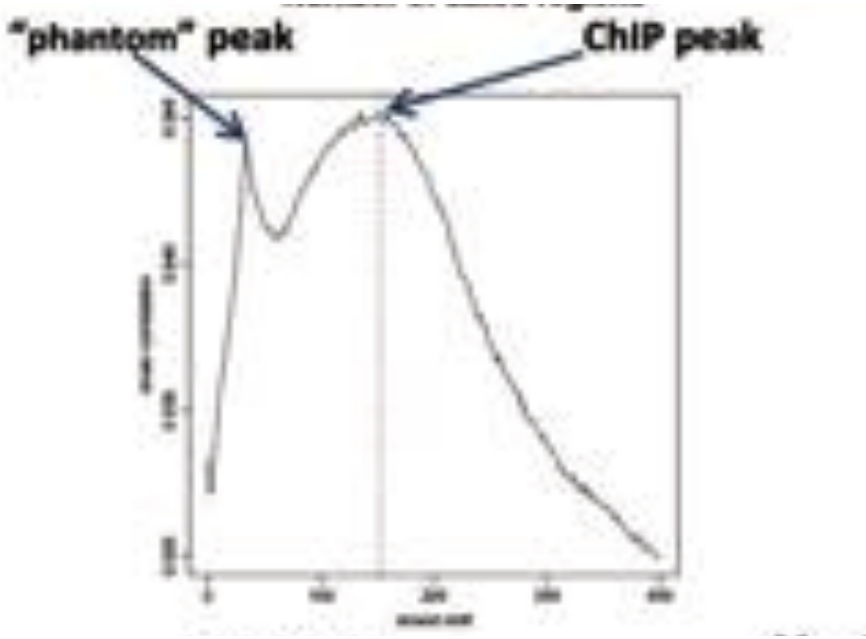
H3K27me3 is a mark that yields broad domains instead of narrow peaks

it is more difficult to distinguish input and ChIP, it does not mean, however, that this particular ChIP experiment failed

Diaz et.al. 2012. doi: 10.1515/1544-6115.1750
https://deeptools.readthedocs.io/en/develop/content/tools/plotFingerprint.html

Cross-correlation





Strand cross-correlation is computed as the Pearson correlation between the positive and the negative strand profiles at different strand shift distances

Kharchenko et.al., 2008. doi: 10.1038/nbt.1508

Quality Control

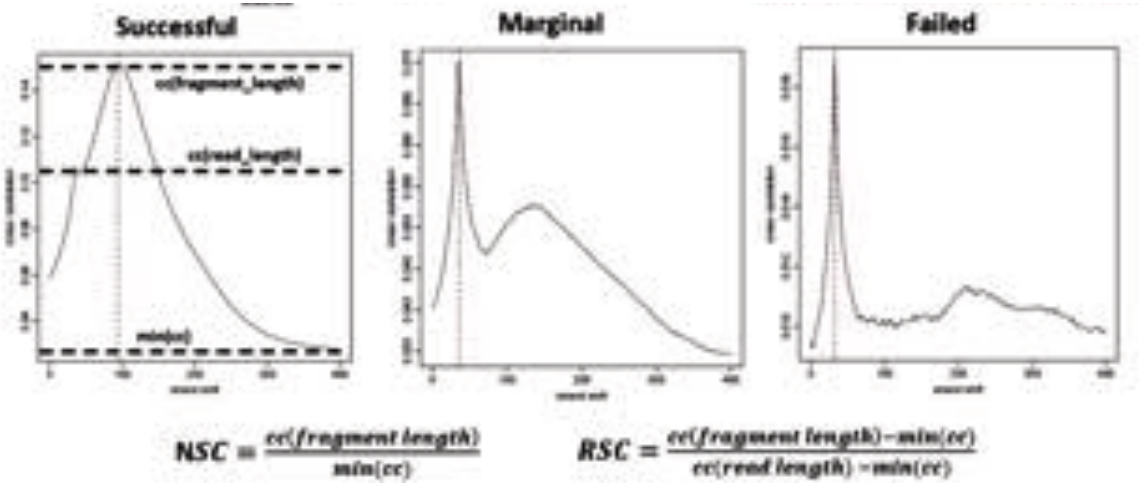
Normalized Strand Cross-correlation coefficient (NSC)

High-quality ChIP-seq data sets should have NSC values >= 1.05 .

Relative Strand Cross-correlation coefficient (RSC)

High-quality ChIP-seq data sets should have RSC values >= 0.8

https://genome.ucsc.edu/ENCODE/qualityMetrics.html



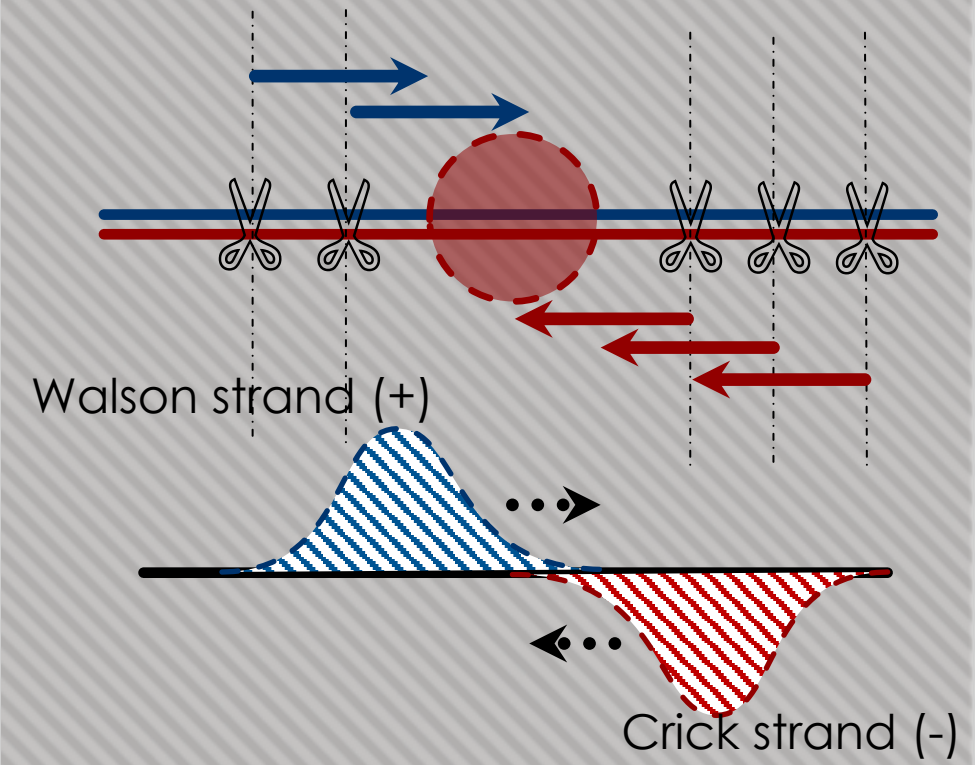
Landt et.al., 2012. doi: 10.1101/gr.136184.111

Calculate NSC and RSC by phantompeaktools

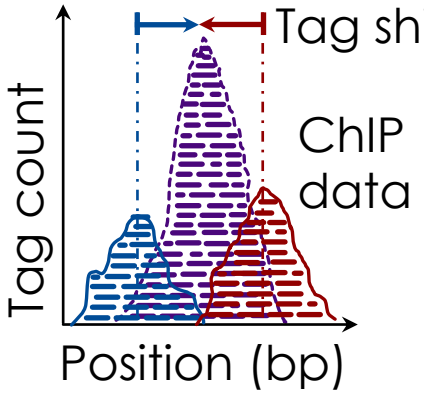
```
Rscript run_spp.R -c=<tagAlign/BAMfile> -savp -out=<outFile>
```

https://github.com/kundajelab/phantompeakqualtools

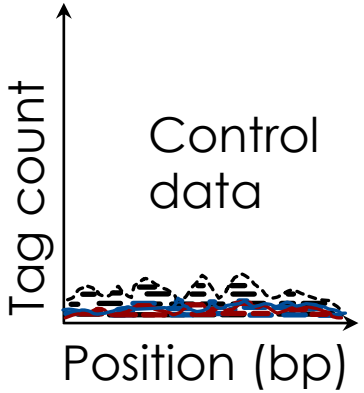
Call peaks



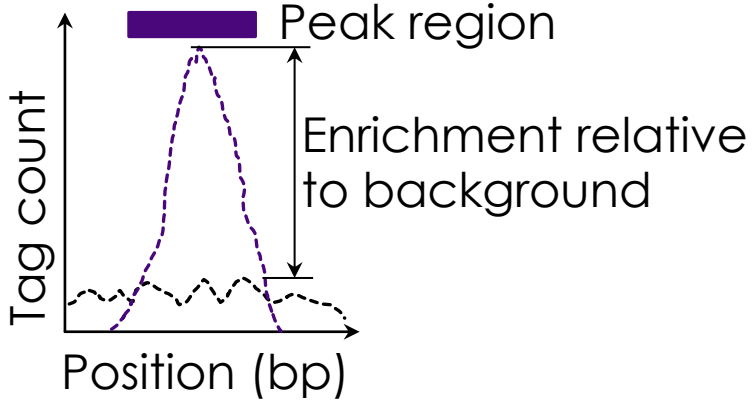
Generate signal profile along each chromosome



Define background (model or data)

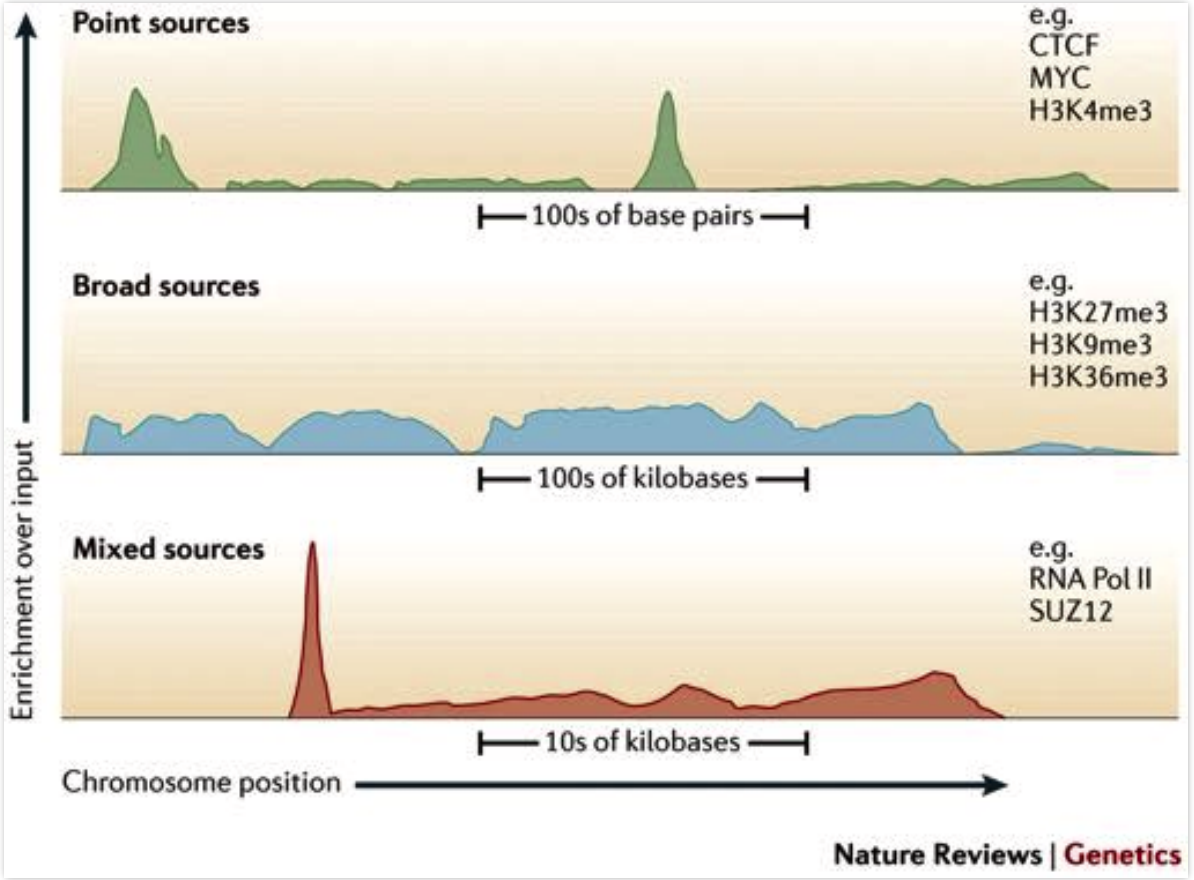


Identify peaks in ChIP signal

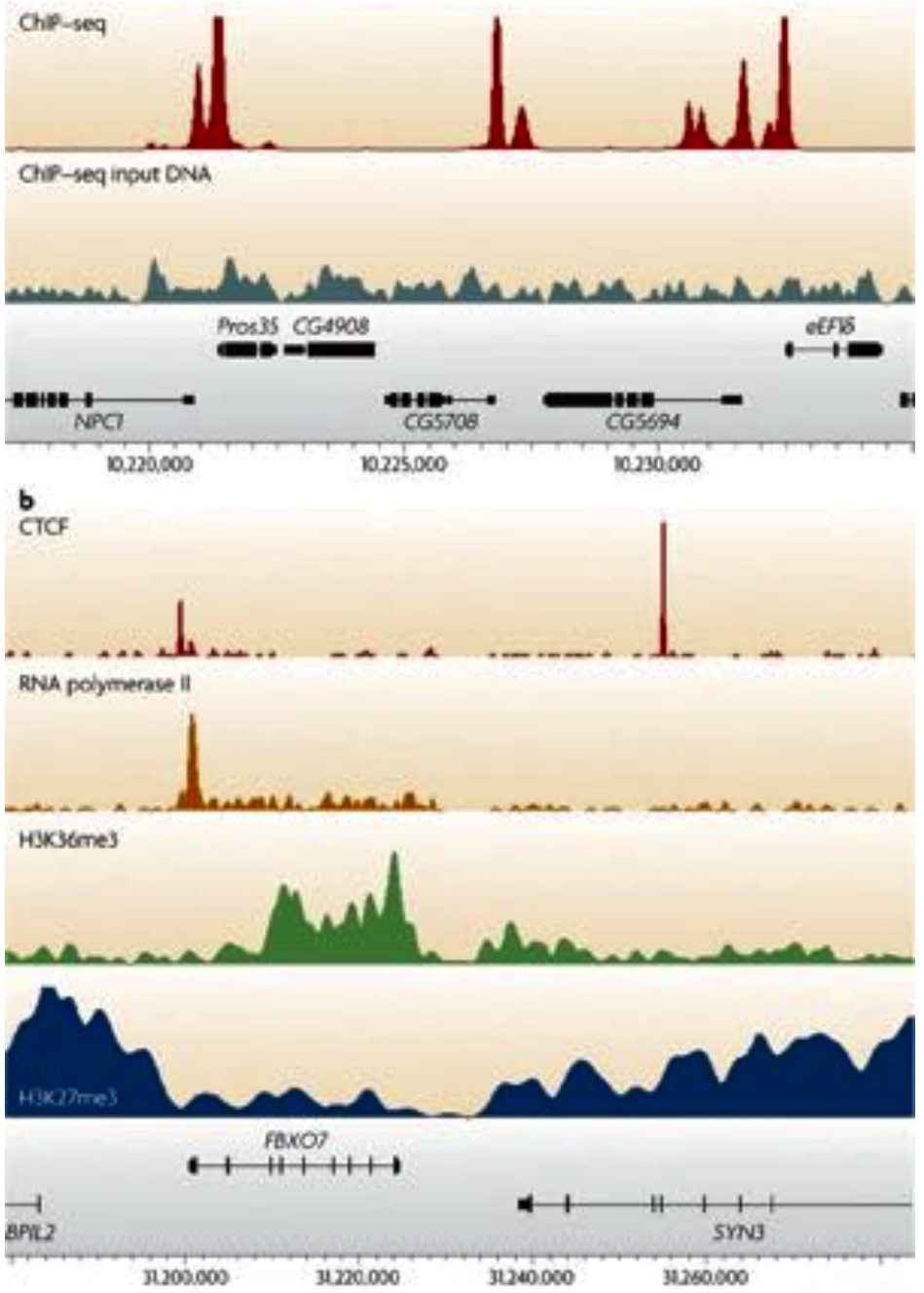


(Modified from Nature Methods doi:10.1038/nmeth.1371, S.Pepke)

Three different types of peaks

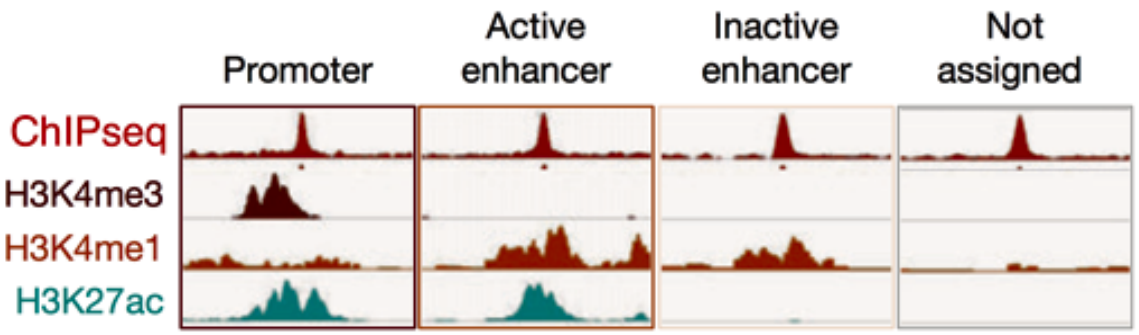


Sims et.al., 2014. doi: 10.1038/nrg3642



Park, 2009. doi:10.1038/nrg2641, P.Park

Histone modifications



- H3K27ac <--> active enhancers and promoters
- H3K4me3 <--> active promoter
- H3K4me1 <--> active enhancers
- H3K27me3 <--> silenced genes

Broad peaks	Narrow peaks
H3K27me3	H3ac
H3K36me3	H3K27ac
H3K4me1	H3K4me2
H3K79me2	H3K4me3
H3K79me3	H3K9ac
H3K9me1	
H3K9me2	
H4K20me1	

Zanconato et.al., 2015. doi:10.1038/ncb3216
https://www.cellsignal.com/contents/resources-reference-tables/histone-modification-table/science-tables-histone

Peak caller

Transcription factor peaks:

- MACS
- Homer

Histone marks:

- SICER2
- SPP+MACS
- Homer

Program	Reference	Version	Graphical user interface?	Window-based scan	Tag clustering	Gaussian kernel density estimator	Strand-specific scoring	Peak height or fold enrichment (FE)	Background subtraction	Compensates for genomic duplications or deletions	False Discovery Rate	Compare to normalized control data (FE)	Compare to statistical model fitted with control data	Statistical model or test
CisGenome	28	1.1	X*	X				X	X		X		X	conditional binomial model
Minimal ChipSeq Peak Finder	16	2.0.1			X			X				X		
E-RANGE	27	3.1			X			X				X	X	chromsome scale Poisson dist.
MACS	13	1.3.5		X				X			X		X	local Poisson dist.
QuEST	14	2.3				X		X			X**		X	chromsome scale Poisson dist.
HPeak	29	1.1		X				X					X	Hidden Markov Model
Sole-Search	23	1	X	X				X		X			X	One sample t-test
PeakSeq	21	1.01			X			X					X	conditional binomial model
SISSRS	32	1.4		X			X					X		
spp package (wtd & mtc)	31	1.7		X			X		X	X'	X			
				Generating density profiles			Peak assignment		Adjustments w. control data		Significance relative to control data			

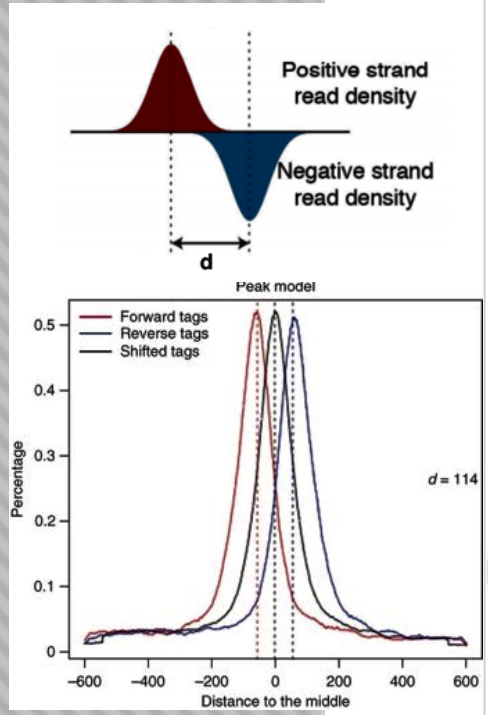
X* = Windows-only GUI or cross-platform command line interface

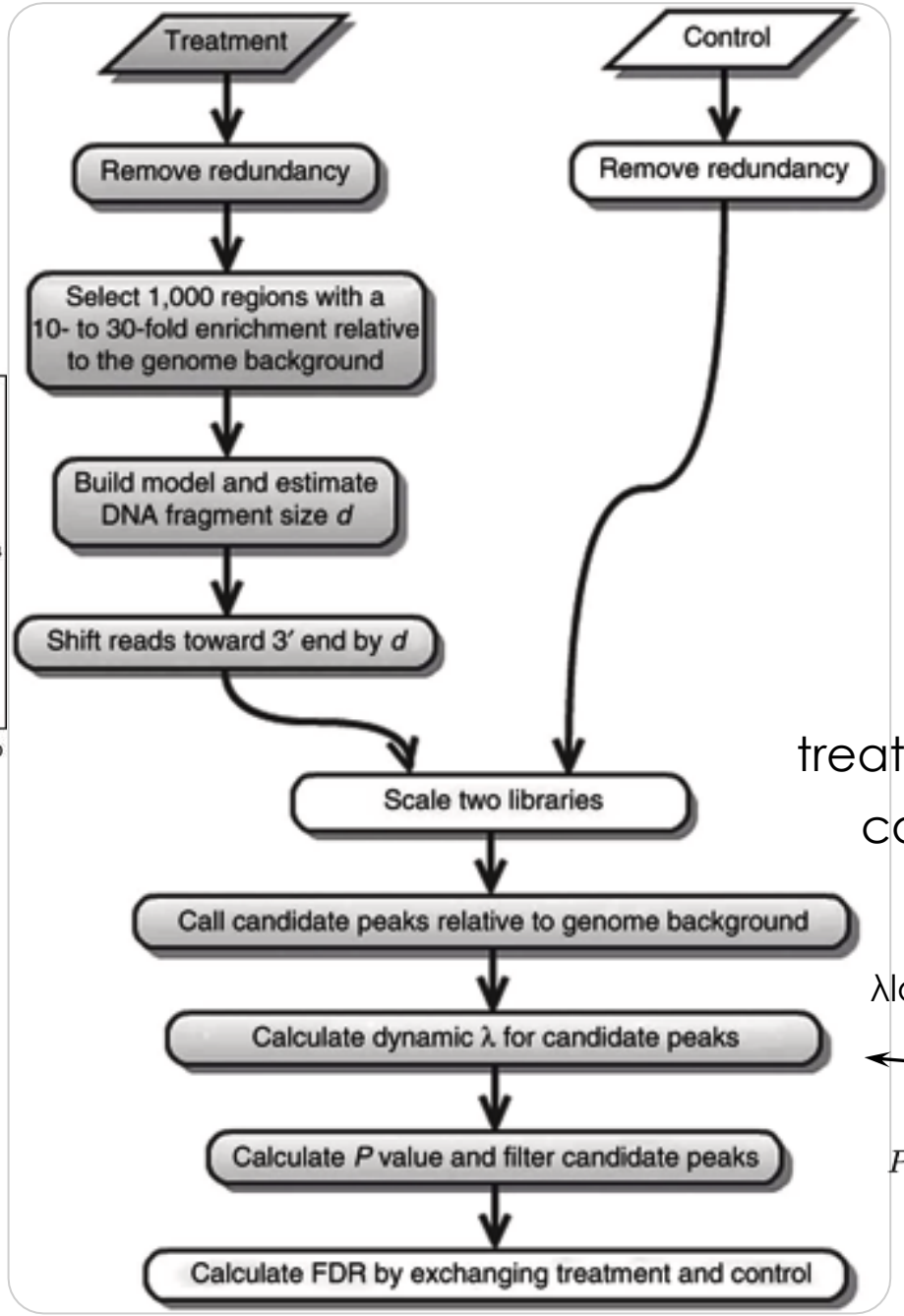
X** = optional if sufficient data is available to split control data

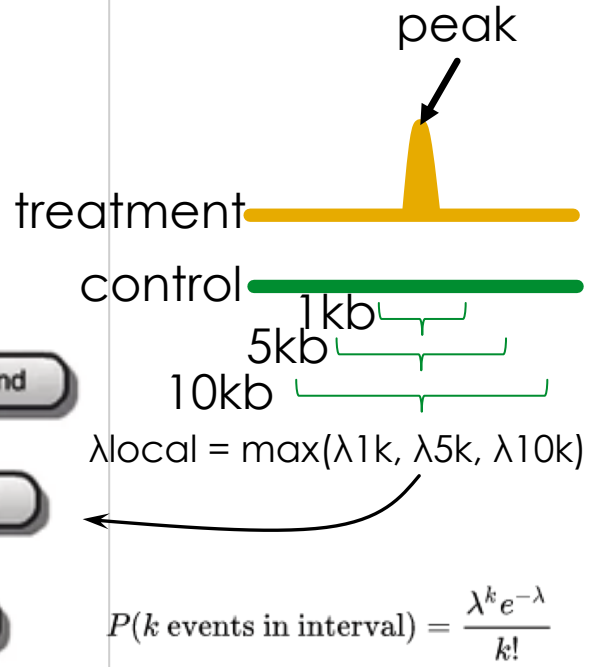
X' = method exludes putative duplicated regions, no treatment of deletions

Wilbanks et.al., 2010. doi: 10.1371/journal.pone.0011471

MACS/2 (Model-based Analysis of ChIP-seq)







$$P(k \text{ events in interval}) = \frac{\lambda^k e^{-\lambda}}{k!}$$

Feng et.al., 2012. doi: 10.1038/nprot.2012.101

Running MACS2

Sharp peaks:

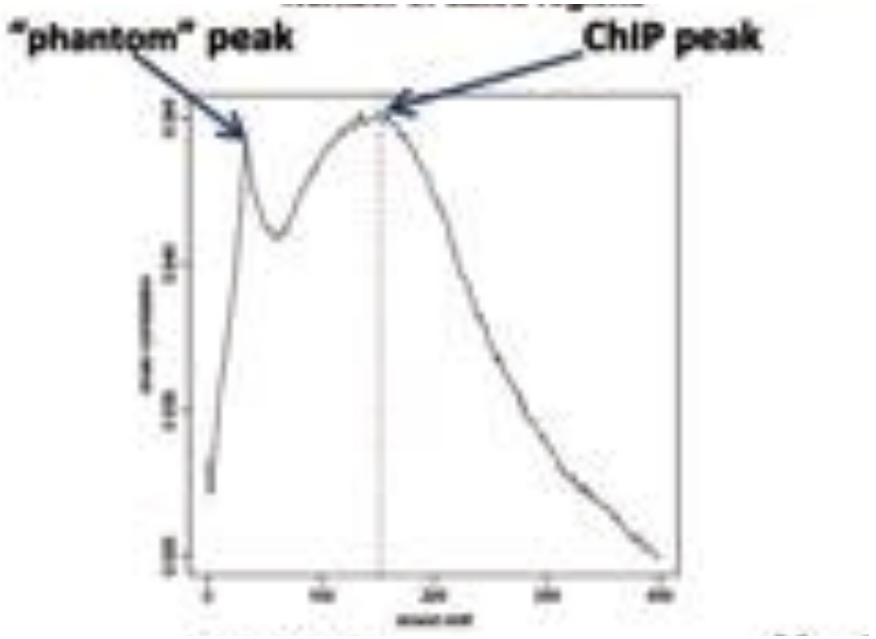
```
macs2 callpeak –t treamtment.bam –c input.bam \
       -f BAM –g hs –n sampleName --outdir outputFolder \
       -q 0.01
```

Broad peaks:

```
macs2 callpeak –t treamtment.bam –c input.bam \
       -f BAM –g hs –n sampleName --outdir outputFolder \
       --broad --broad-cutoff 0.1
```

Running MACS2 with user-defined model

```
macs2 callpeak –t treamtment.bam –c input.bam \
       -f BAM –g hs –n sampleName \
       --outdir outputFolder \
       --nomodel --shift 80 --extsize 160
```



Calculate cross-correlation by phantompeaktools
Rscript run_spp.R -c=<tagAlign/BAMfile> -savp -out=<outFile>

https://github.com/kundajelab/phantompeakqualtools

Output of MACS2

- NAME_peaks.xls
- NAME_peaks.narrowPeak
- NAME_summits.bed

- chromosome name
- start position of peak
- end position of peak
- length of peak region
- absolute peak summit position
- pileup height at peak summit
- -log10(pvalue) for the peak summit (e.g. pvalue =1e-10, then this value should be 10)
- fold enrichment for this peak summit against random Poisson distribution with local lambda,
- -log10(qvalue) at peak summit

File formats: bed, bedGraph, wig, bigwig, narrowPeak, broadPeak, …

- Help documentation: https://genome.ucsc.edu/FAQ/FAQformat.html

- BED format:
  - chrom chromStat chromEnd name score strand …
  - Sample file:

```
chr22 1000 5000 cloneA 960 +
chr22 2000 6000 cloneB 900 -
```

- WIG (Wiggle Track) format: (no strand information)
  - **variableStep format**

```
variableStep chrom=chrN
[span=windowSize]
chromStartA dataValueA
chromStartB dataValueB
... etc ... ... etc ...
```

  - **fixedStep format**

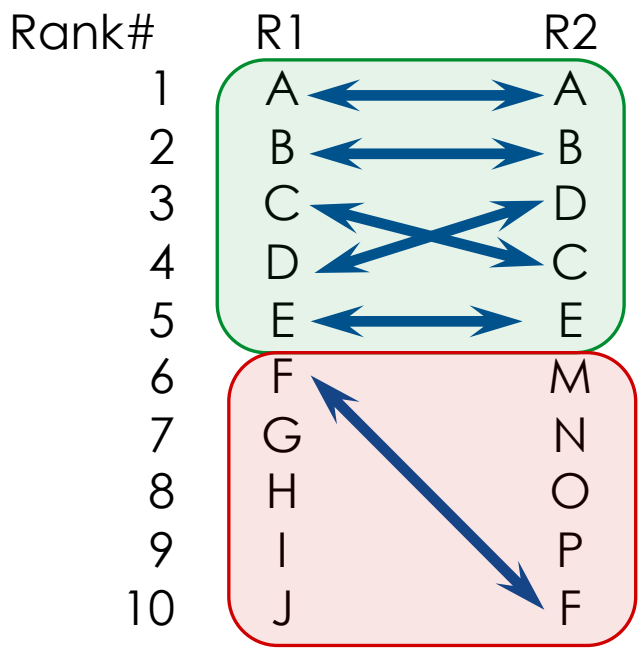
```
fixedStep chrom=chrN
start=position step=stepInterval
[span=windowSize]
dataValue1
dataValue2
... etc ...
```

Blacklist/Hotspots

- Blacklisted regions are genomic regions with anomalous, unstructured, high signal or read counts in NGS experiments, independent of cell type or experiment.
- Where to download: https://sites.google.com/site/anshulkundaje/projects/blacklists

Irreproducibility Discovery Rate (IDR) analysis

The IDR framework to filter the low reproducible peaks The IDR framework is a unified approach to measure the reproducibility of findings identified from replicate experiments and provide highly stable thresholds based on reproducibility. (Landt et al., 2012)

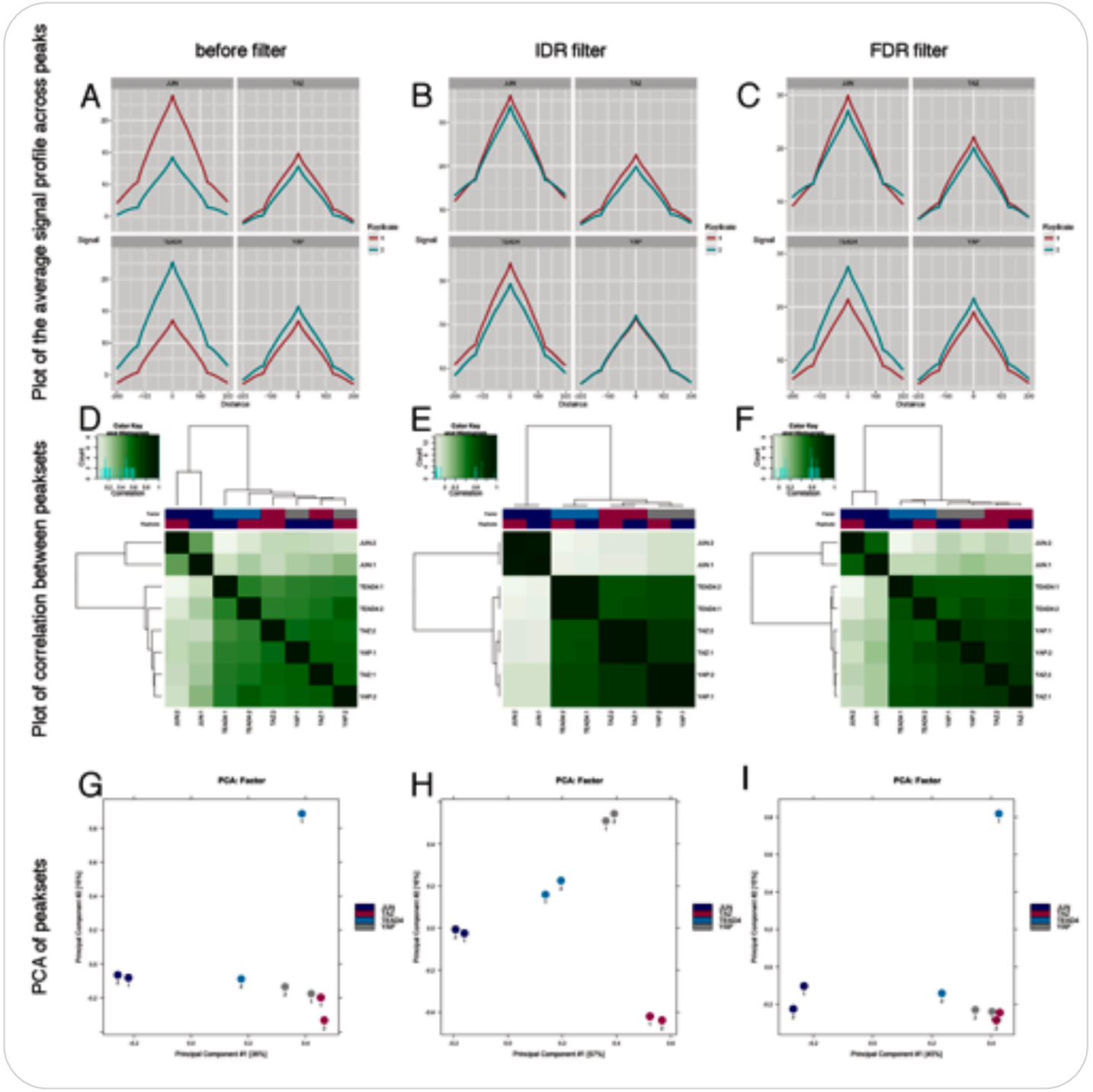


Good consistency

Bad consistency

https://arxiv.org/abs/1110.4705
https://github.com/nboley/idr

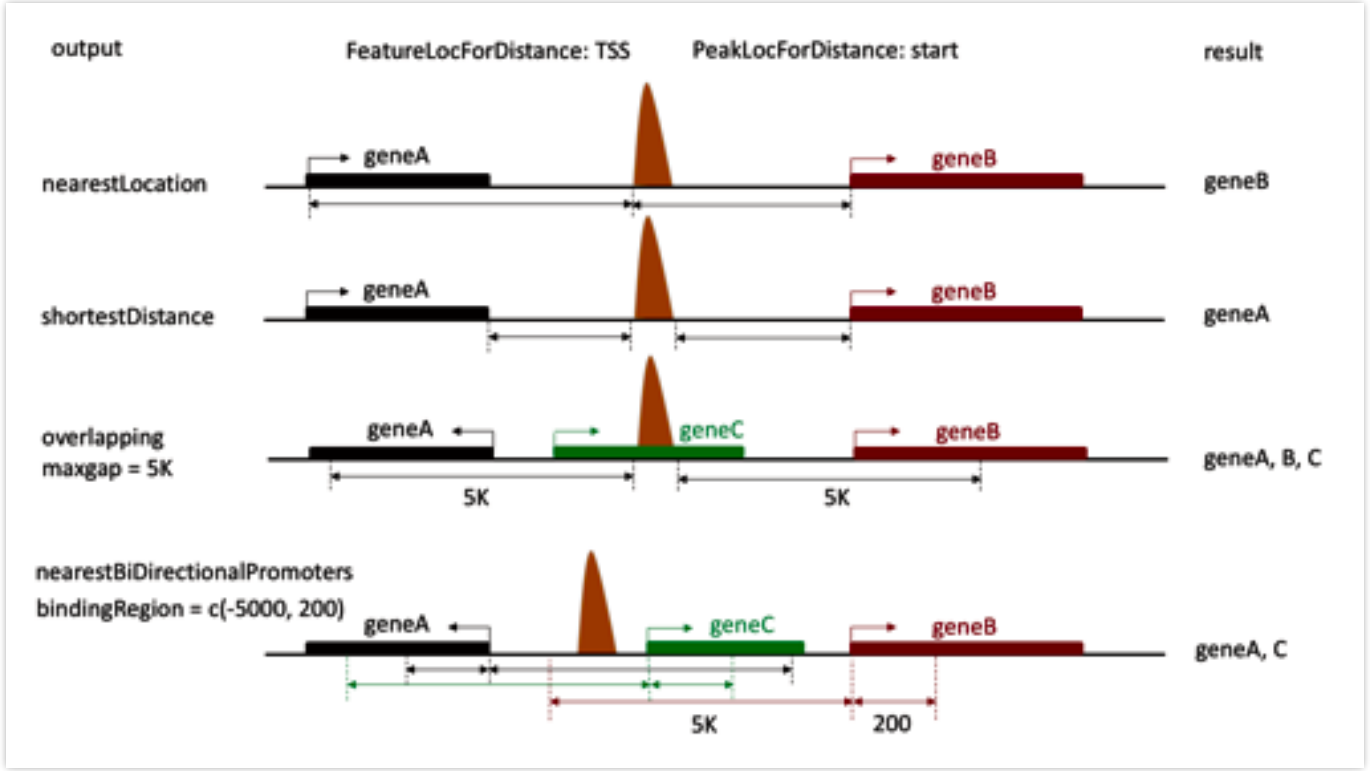
The effect of IDR



https://github.com/jianhong/ChIPseqStepByStep

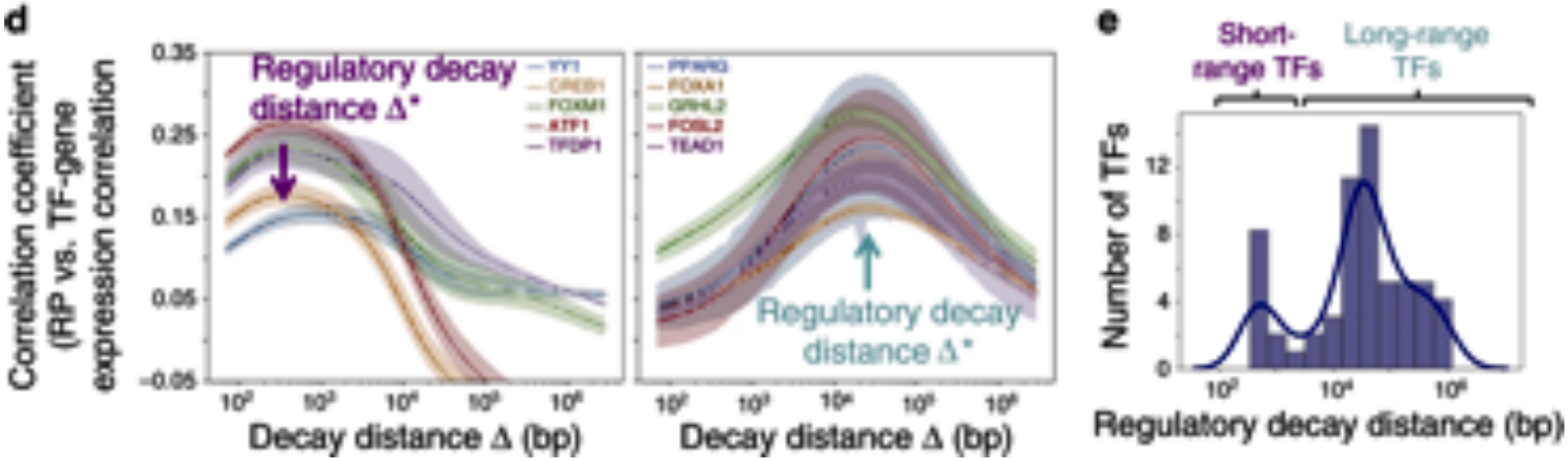
Annotation by ChIPpeakAnno

The *annotatePeakInBatch* and *annoPeaks* functions in **ChIPpeakAnno** package can be used to annotate the peaks. Depend on the experiment, we can annotate the peaks in multiple ways.



https://github.com/jianhong/workshop2020/blob/master/inst/extdata/ChIPpeakAnno_workshop2020.pdf

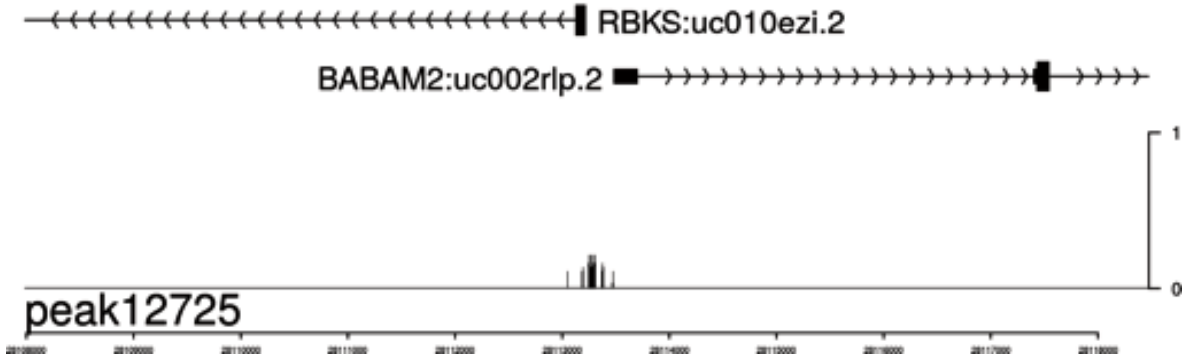
Two distinct TF classes: short-range and long-range



Chen et.al., 2020. doi: 10.1038/s41467-020-16106-x

Bidirectional promoters

- Bidirectional promoters are the DNA regions located between the 5' ends of two adjacent genes coded on opposite strands. The two adjacent genes are transcribed to the opposite directions, and often co-regulated by this shared promoter region(Robertson et al., 2007).



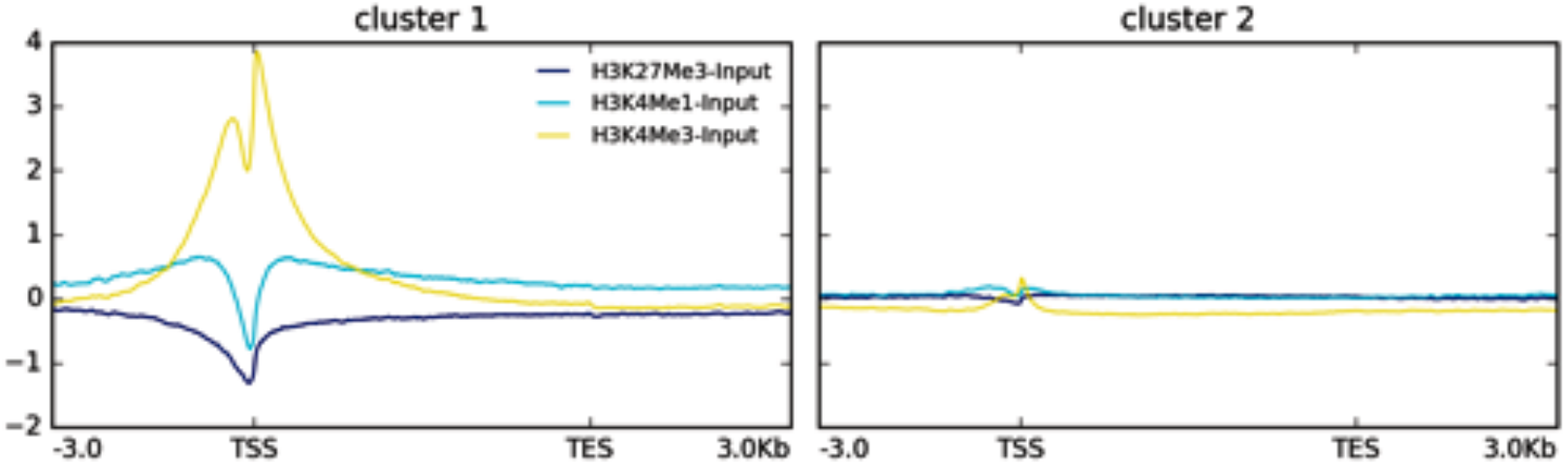
Functional annotation of DE genes

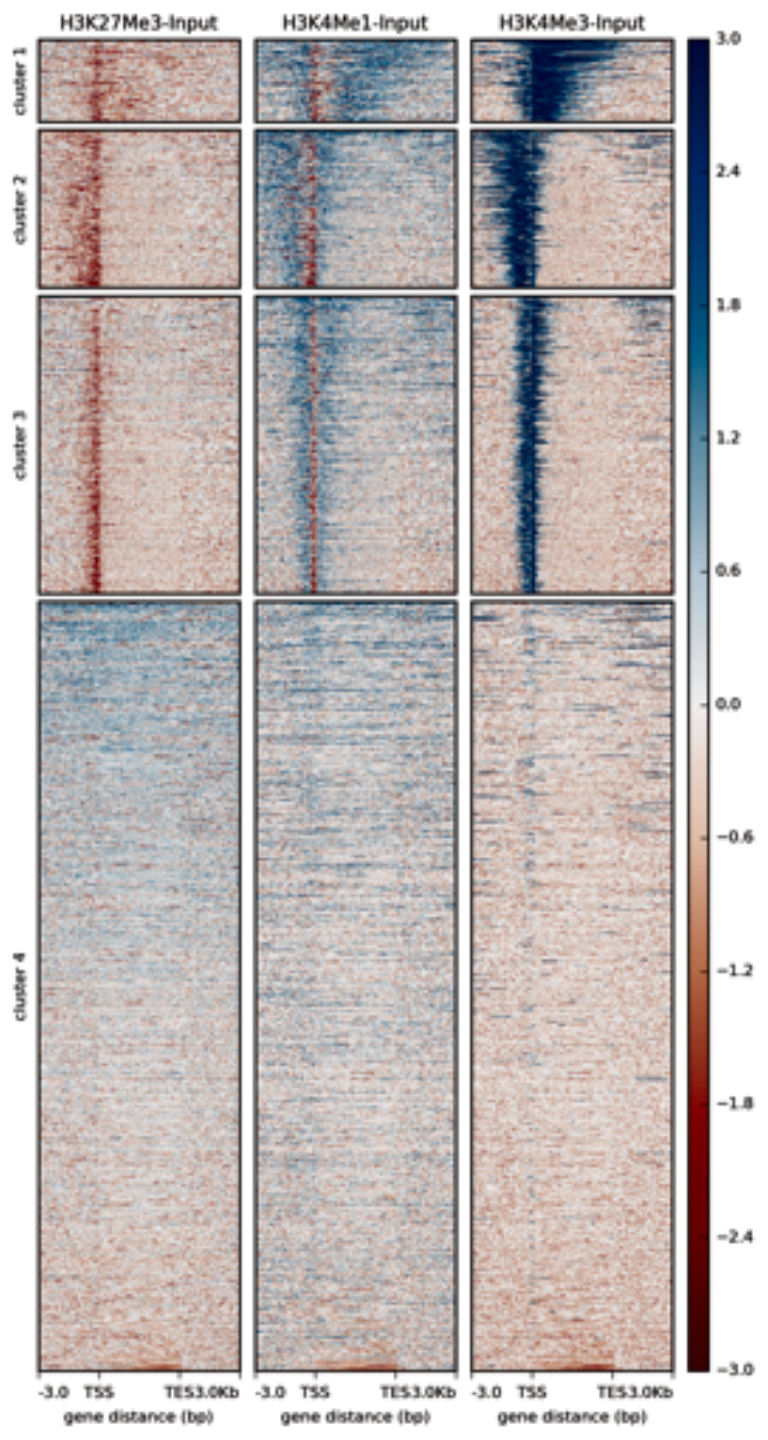
- Gene ontology (GO) enrichment analysis
- Kyoty Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis
- Gene set enrichment analysis (GSEA)
- QIAGEN Ingenuity Pathway Analysis (IPA)

A gene set over-represented comparing to random sampling

Metagene analysis

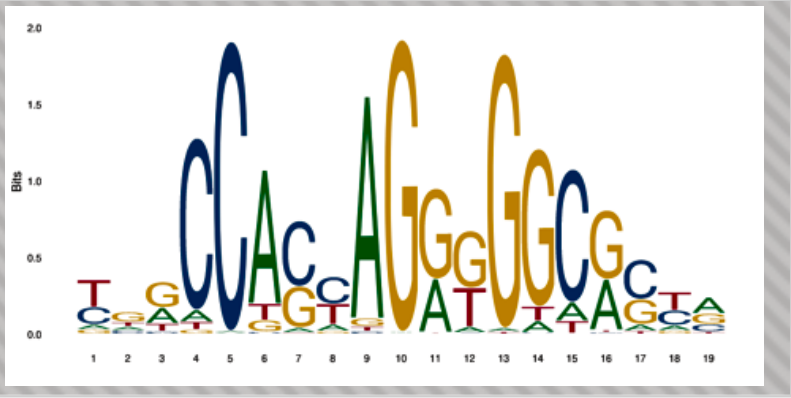
- A metagene analysis is an average of quantitative data over more than one genomic regions. The interested genomic regions include promoters, transcription factor binding sites, enhancers, etc.





https://deeptools.readthedocs.io/en/develop/content/tools/plotHeatmap.html

Motif search



- Sequence motif represents conserved characteristics such as DNA binding sites, where transcription factors bind, and catalytic sites in enzymes.
- A sequence logo is a graphical representation of sequence conservation in multiple sequences.
- To search an enriched motifs, multiple softwares could be used, such as the MEME Suite(Bailey et al., 1994), Consensus(Hertz et al., 1990), rGADEM, Homer, and et. al.

https://github.com/jianhong/workshop2020/blob/master/inst/extdata/motifStack_workshop2020.pdf

Build Regulation Network: Integrating TF binding with transcriptomic data

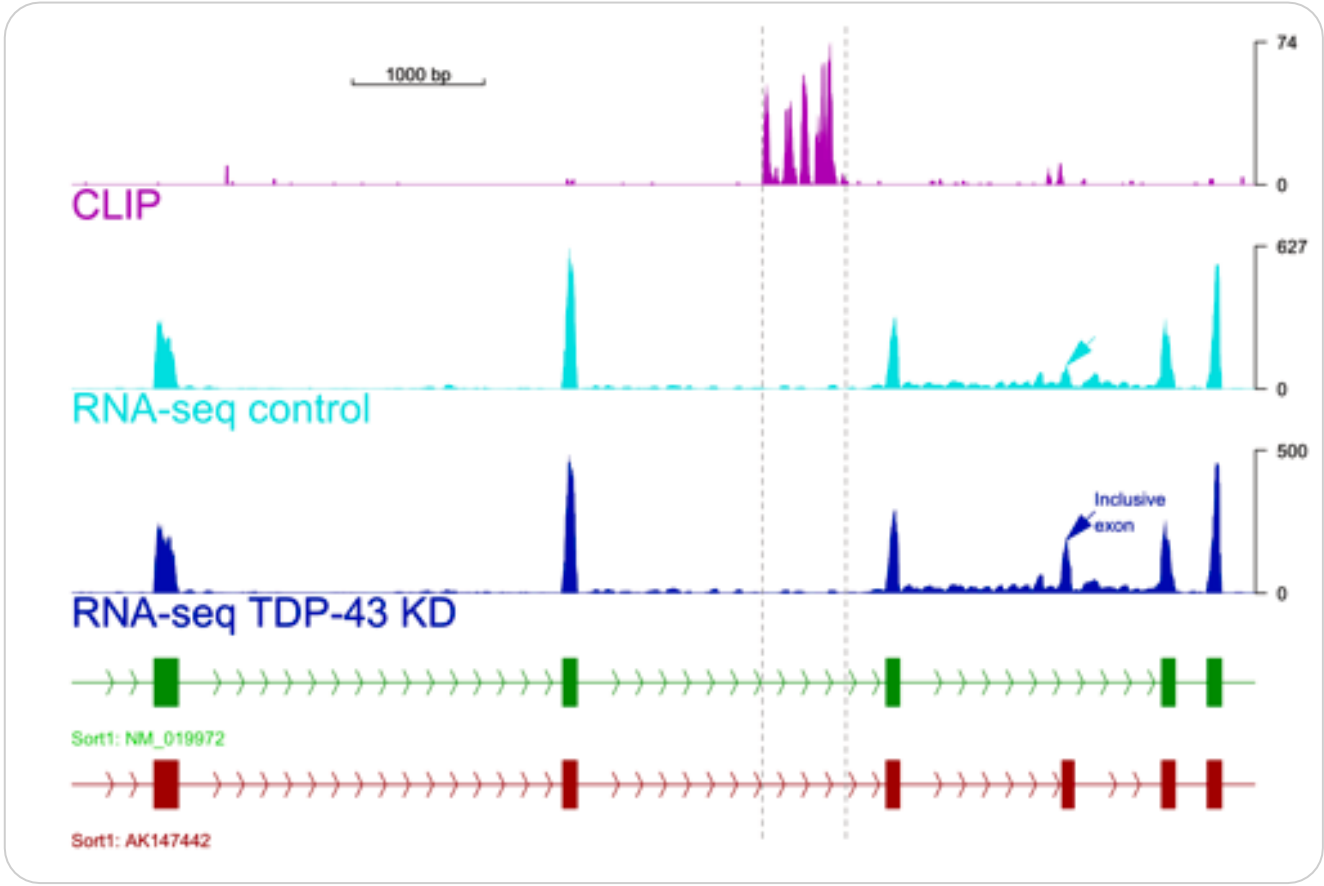
GeneNetworkBuilder (GNB) can be used to facilitate identification of the complex regulatory network of TFs and how indirect targets are inter-connected.

Issue: hairball – too much information

- filter data by enrichment analysis: ClueGO
- filter the interaction database by evidence.
- Focus on a subset network

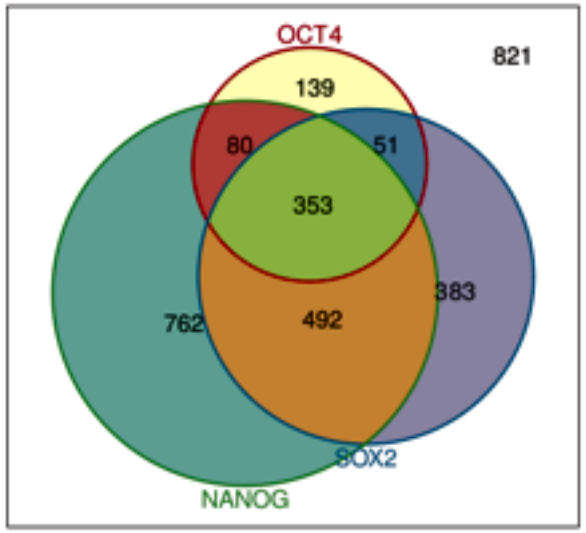
https://bioconductor.org/packages/GeneNetworkBuilder/

View tracks by trackViewer

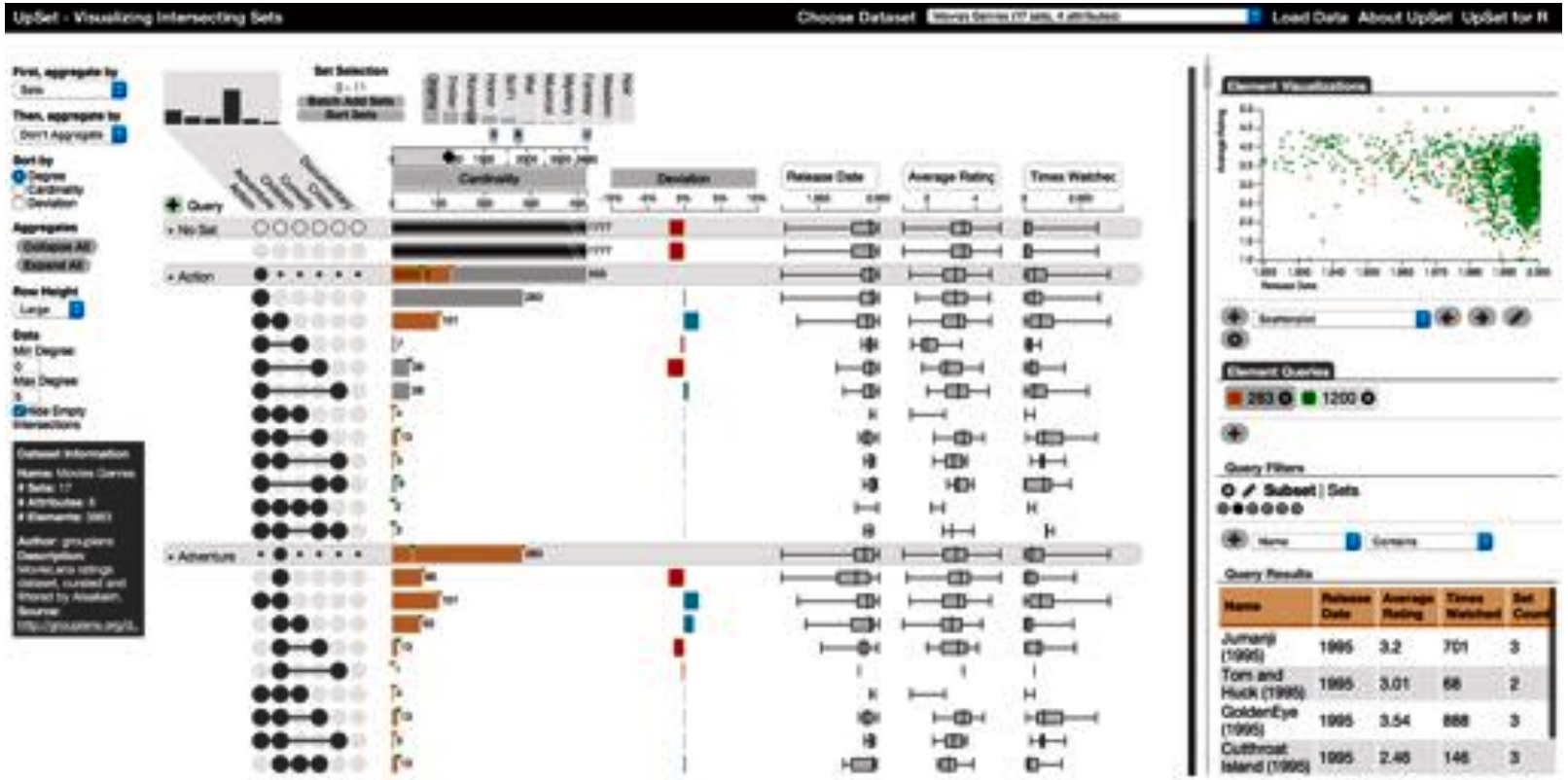


Ou et.al., 2019. doi: 10.1038/s41592-019-0430-y

Association among different sets of peaks
Venn diagram and upset plot



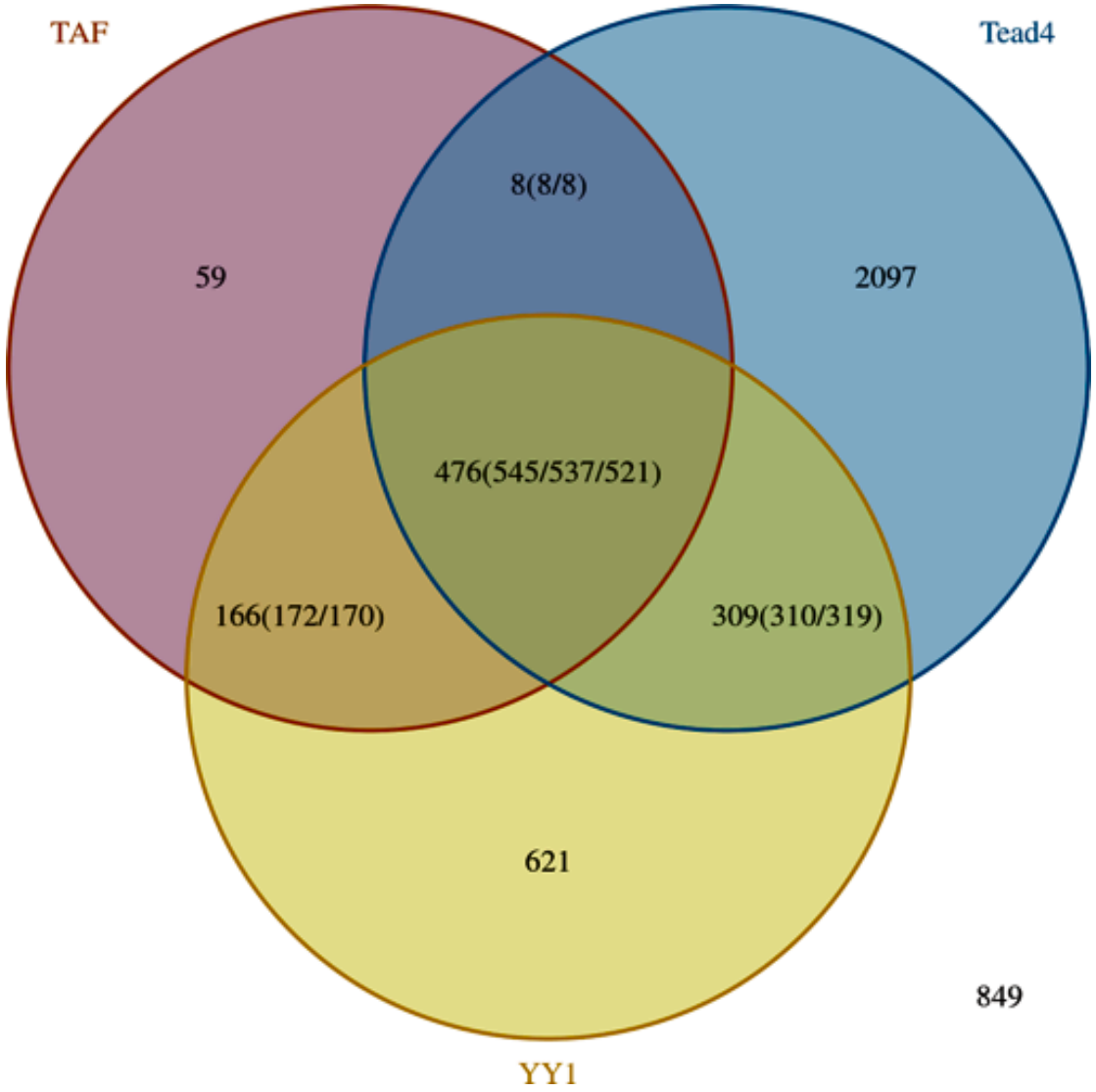
https://github.com/js229/Vennerable

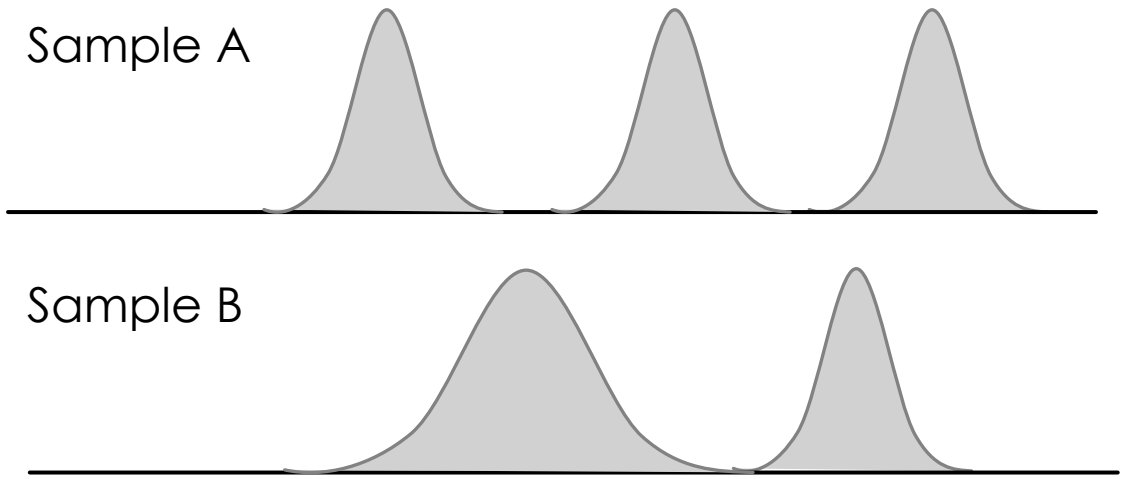


http://vcg.github.io/upset/

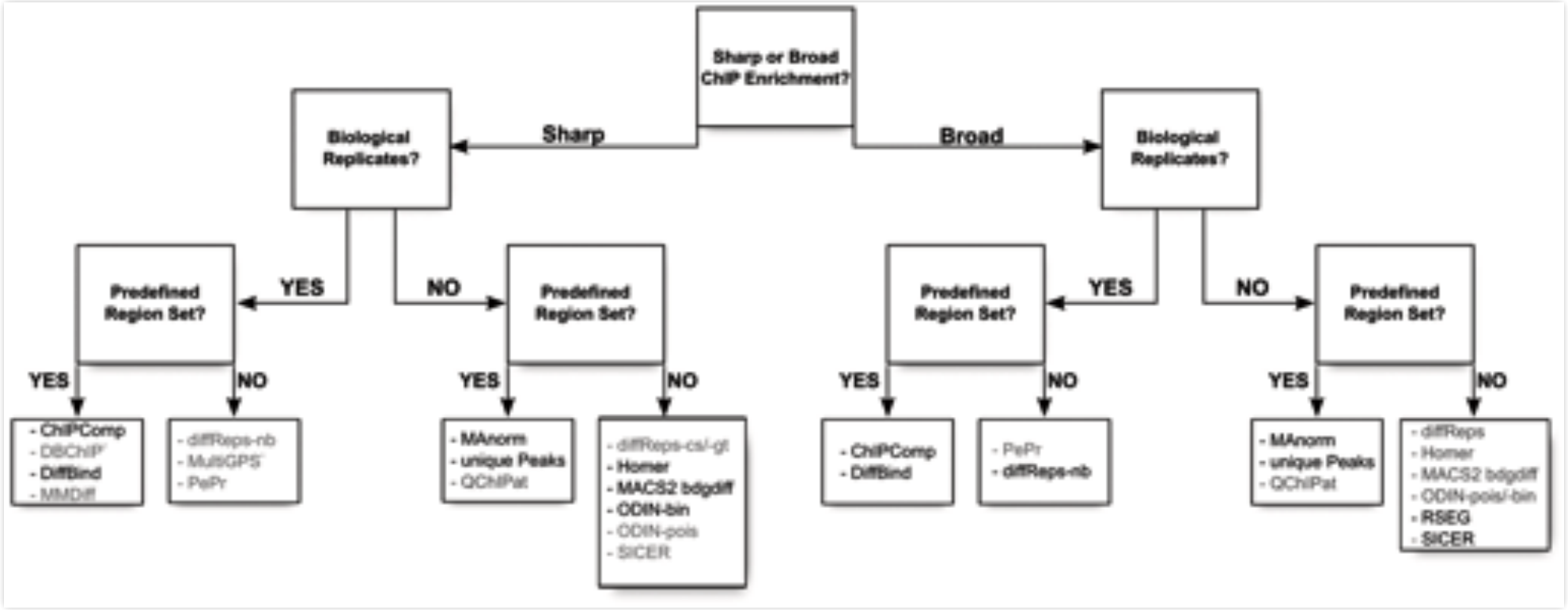
http://caleydo.org/tools/upset/

Find overlaps for replicates, *findOverlapsOfPeaks*





Differential binding sites analysis



Steinhauser et.al., 2016. doi: 10.1093/bib/bbv110

Differential binding sites analysis

DiffBind vs. csaw

Peak based vs. window based

Using control or not

Tools for ChIP-seq analysis

- Mapping: bowtie1/2, Rsubread, bwa
- Peak calling: CCAT, SICER, MACS, ZINBA, BayesPeak, chipseq, ChIPseqR, CSAR, csaw, GenoGAM, iSeq, PICS
- Quality assessment: ChIPQC
- Analysis of differential binding: DiffBind, csaw, ChIPDiff, DBChIP, MMDiff
- Peak annotation and pathway analysis: **ChIPpeakAnno**, Homer, PAVIS, GREAT, ChIPseeker, clusterProfiler, GOstats
- Gene Network Building: **GeneNetworkBuilder**, ChIPXpress
- Visualization: **trackViewer**, IGV, UCSC genome browser, rtracklayer
- Motif enrichment analysis: The MEME Suite, homer

Regeneromics Shared Resource can help your research!

Selected recent publications we have co-authored:

Identification and requirements of enhancers that direct gene expression during zebrafish fin regeneration. Thompson JD, Ou J, Lee N, Shin K, Cigliola V, Song L, Crawford GE, Kang J, Poss KD. Development. 2020 Jul 14:dev.191262. doi: 10.1242/dev.191262. Online ahead of print.

Persistence of a regeneration-associated, transitional alveolar epithelial cell state in pulmonary fibrosis. Kobayashi Y, Tata A, Konkimalla A, Katsura H, Lee RF, Ou J, Banovich NE, Kropski JA, Tata PR. Nat Cell Biol. 2020 Jul 13. doi: 10.1038/s41556-020-0542-8. Online ahead of print.

Persistence of a regeneration-associated, transitional alveolar epithelial cell state in pulmonary fibrosis. Kobayashi Y, Tata A, Konkimalla A, Katsura H, Lee RF, Ou J, Banovich NE, Kropski JA, Tata PR. Nat Cell Biol. 2020 Jul 13. doi: 10.1038/s41556-020-0542-8. Online ahead of print.

Nucleoporin 153 links nuclear pore complex to chromatin architecture by mediating CTCF and cohesin binding.Kadota S, Ou J, Shi Y, Lee JT, Sun J, Yildirim E. Nat Commun. 2020 May 25;11(1):2606. doi: 10.1038/s41467-020-16394-3.

Vitamin D Stimulates Cardiomyocyte Proliferation and Controls Organ Size and Regeneration in Zebrafish. Han Y, Chen A, Umansky KB, Oonk KA, Choi WY, Dickson AL, Ou J, Cigliola V, Yifa O, Cao J, Tornini VA, Cox BD, Tzahor E, Poss KD. Dev Cell. 2019 Mar 25;48(6):853-863.e5. doi: 10.1016/j.devcel.2019.01.001. Epub 2019 Jan 31.

We do: experimental design, bioinformatics analysis, manuscript preparation, grant applications

Jianhong Ou, Ph.D.

Email: rnirsr@duke.edu

https://sites.duke.edu/regenerationnext/jobsrni/

regenerationNEXT

Hands-on

Questions will be answered

- Prepare your system
- Basics of shell script
- Introduction of R programming language

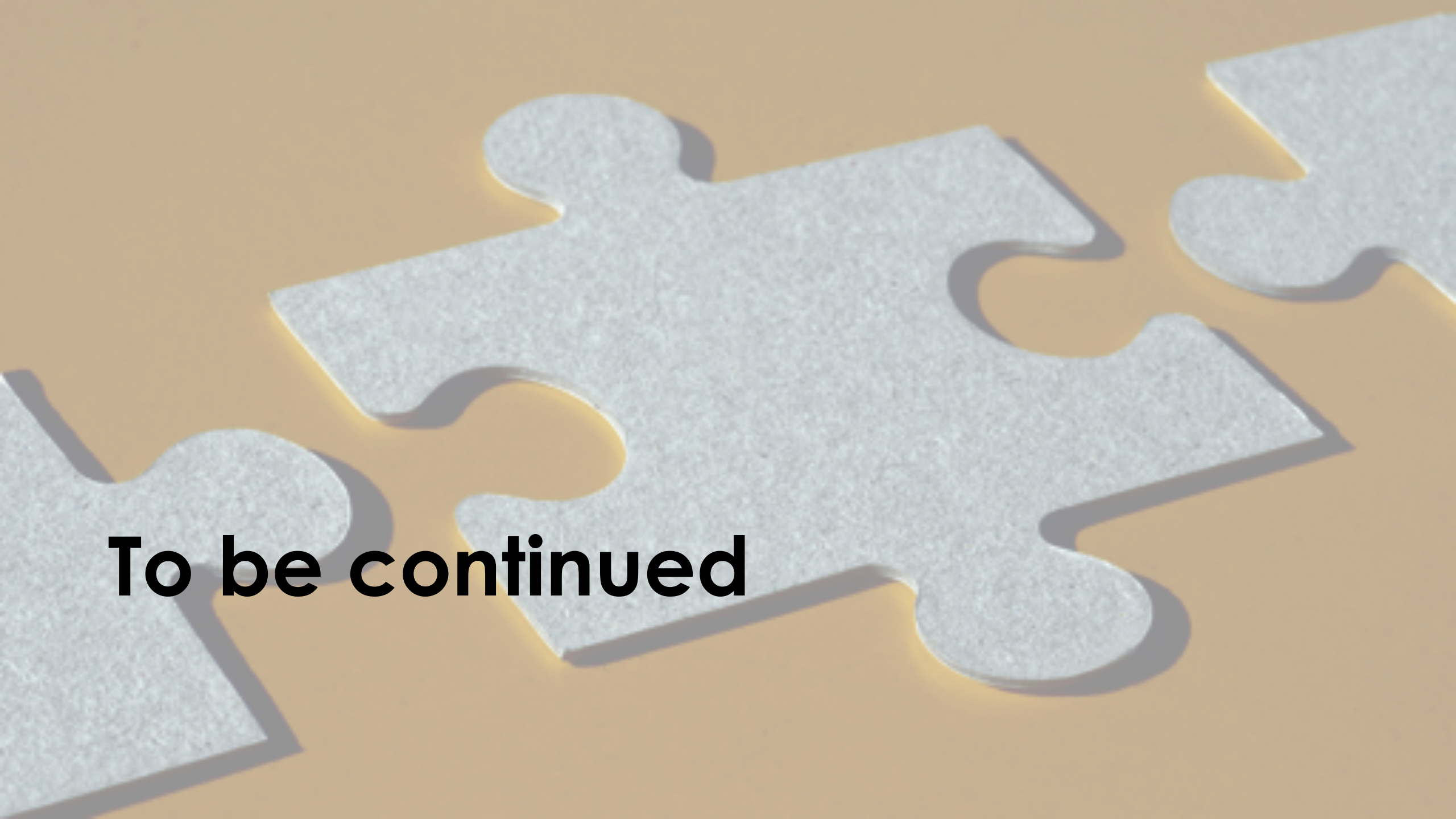
Set up your computing environment (m1)

- BEST Way: Install from source

Set up your computing environment (m2)

- Install Docker (https://www.docker.com/get-started)(30min)
- Check docker installed: docker --version
- docker pull jianhong/genomictools:latest
- cd ~
- mkdir tmp4genomictools
- docker run -e PASSWORD=123456 -p 8787:8787 -v ${PWD}/tmp4genomictools:/home/rstudio jianhong/genomictools:latest
  - localhost:8787 by username: rstudio password:123456

https://github.com/jianhong/genomictools


To be continued

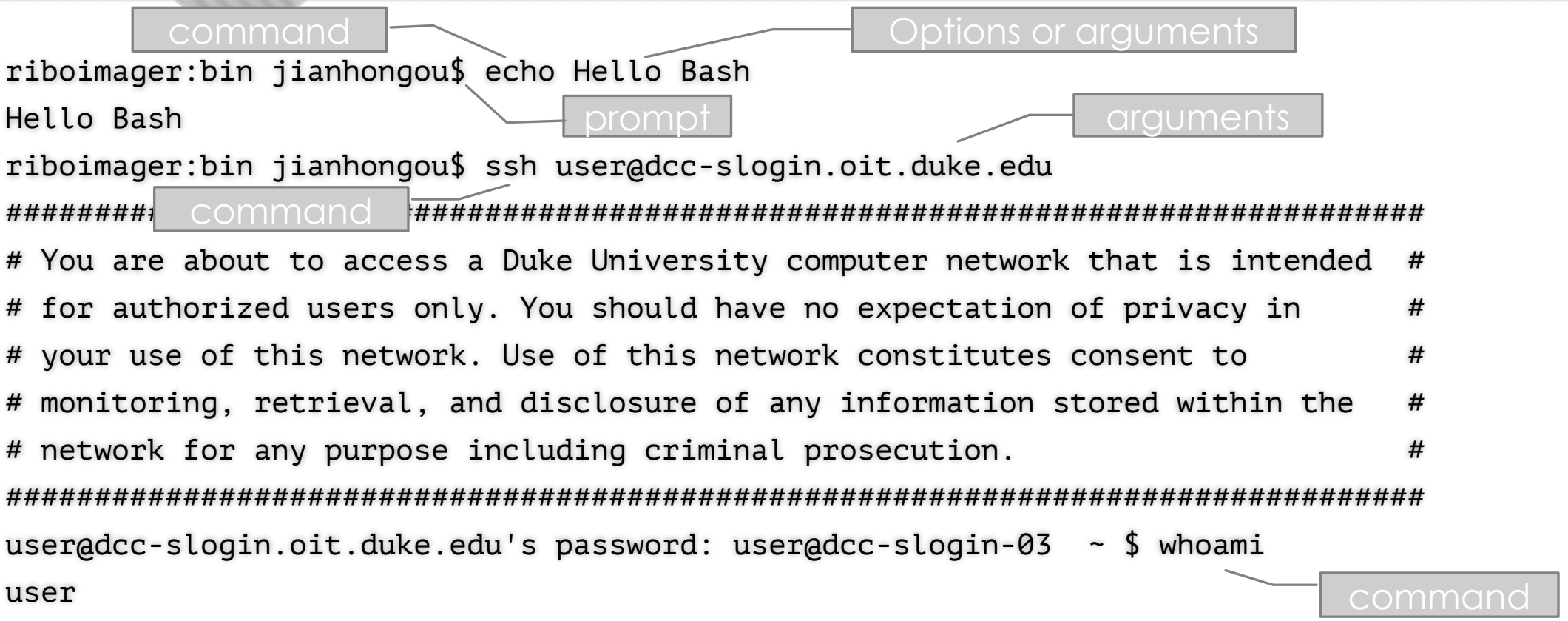
Start your docker

```
cd ~
mkdir tmp4genomictools
docker run -e PASSWORD=123456 -p 8787:8787 \
    -v ${PWD}/tmp4genomictools:/home/rstudio \
    jianhong/genomictools :latest
```

Basics of shell scripts

- Unix-like operating systems: “behave” like the original Unix operating system and comply(at leaset partially) with POSIX (portable operating system interface) standards.
  - Examples: Linux, OS X, Free BSD.
- A terminal: is a software that emulates a teletype writer terminal used in the early days of Unix.
- A shell: is a software that runs inside a terminal and interprets and executes user commands. One of the most popular shells being used today is called BASH (Bourne Again Shell)

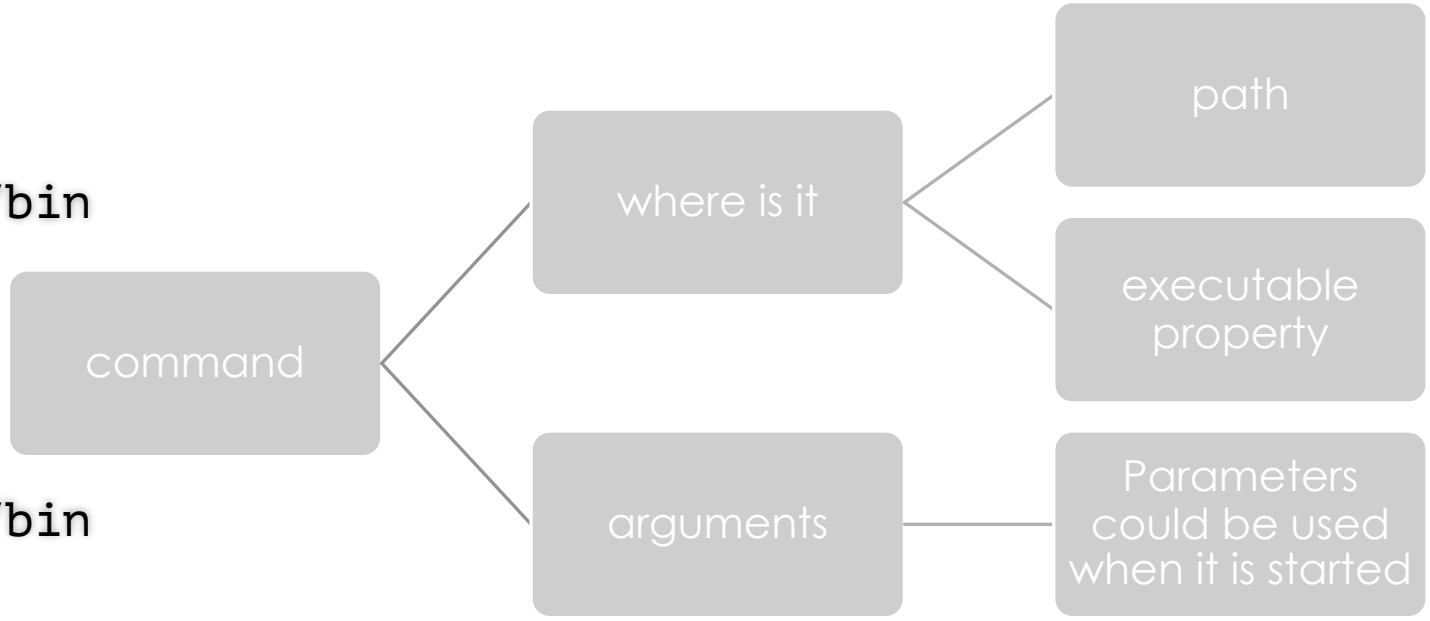
Hello Bash




```
riboimager:bin jianhongou$ echo Hello Bash
Hello Bash
riboimager:bin jianhongou$ ssh user@dcc-slogin.oit.duke.edu
##########################################################################
# You are about to access a Duke University computer network that is intended  #
# for authorized users only. You should have no expectation of privacy in      #
# your use of this network. Use of this network constitutes consent to         #
# monitoring, retrieval, and disclosure of any information stored within the   #
# network for any purpose including criminal prosecution.                      #
##########################################################################
user@dcc-slogin.oit.duke.edu's password: user@dcc-slogin-03  ~ $ whoami
user
```

command line

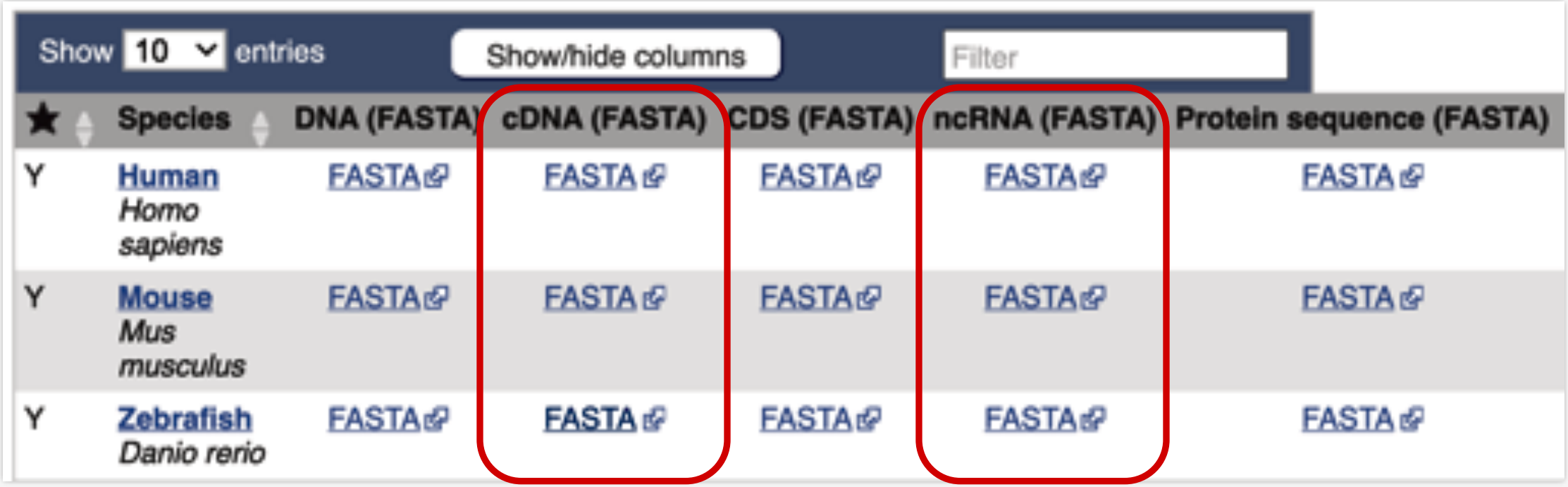
```
## to see working directory
$ pwd
/Users/jianhongou/miniconda3/bin
$ which pwd
/bin/pwd
$ /bin/pwd
/Users/jianhongou/miniconda3/bin
$ ls -lh bowtie2
-rwxrwxr-x  2 jianhongou  staff    18K Jan 22  2017 bowtie2
```



Getting help

```
## try to get documentation of bowtie
$ ./bowtie2 -h
## get help of `ls`
$ man ls
```

Download transcript fasta


Show 10 entries Show/hide columns Filter

★	Species	DNA (FASTA)	cDNA (FASTA)	CDS (FASTA)	ncRNA (FASTA)	Protein sequence (FASTA)
Y	Human *Homo sapiens*	FASTA	FASTA	FASTA	FASTA	FASTA
Y	Mouse *Mus musculus*	FASTA	FASTA	FASTA	FASTA	FASTA
Y	Zebrafish *Danio rerio*	FASTA	FASTA	FASTA	FASTA	FASTA

http://useast.ensembl.org/info/data/ftp/index.html

```
curl -O ftp://ftp.ensembl.org/pub/release-100/fasta/danio_rerio/cdna/Danio_rerio.GRCz11.cdna.all.fa.gz
```

Step1: build transcriptome index

kallisto

```
kallisto index -i danRer.GRCz11_transcrits.idx Danio_rerio.GRCz11.cdna.all.fa.gz
```

Salmon

```
salmon index -i danRer.GRCz11_transcrits.salmon.idx -t Danio_rerio.GRCz11.cdna.all.fa.gz
```

Step 2: quantify

kallisto

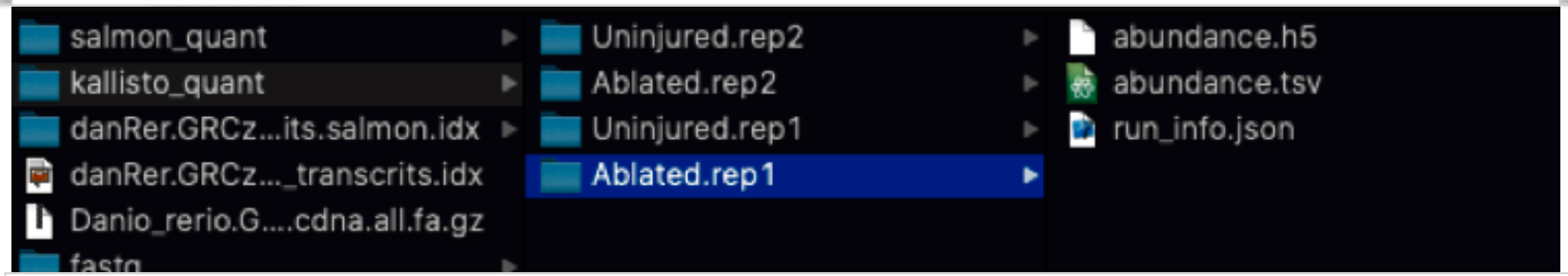
```
kallisto quant -i danRer.GRCz11_transcrits.idx \
               -o kallisto_quant/$cond.rep$rep \
               -b 30 -t 2 fastq/$cond.rep$rep.fastq.gz \
               --single -l 200 -s 50
```

Salmon

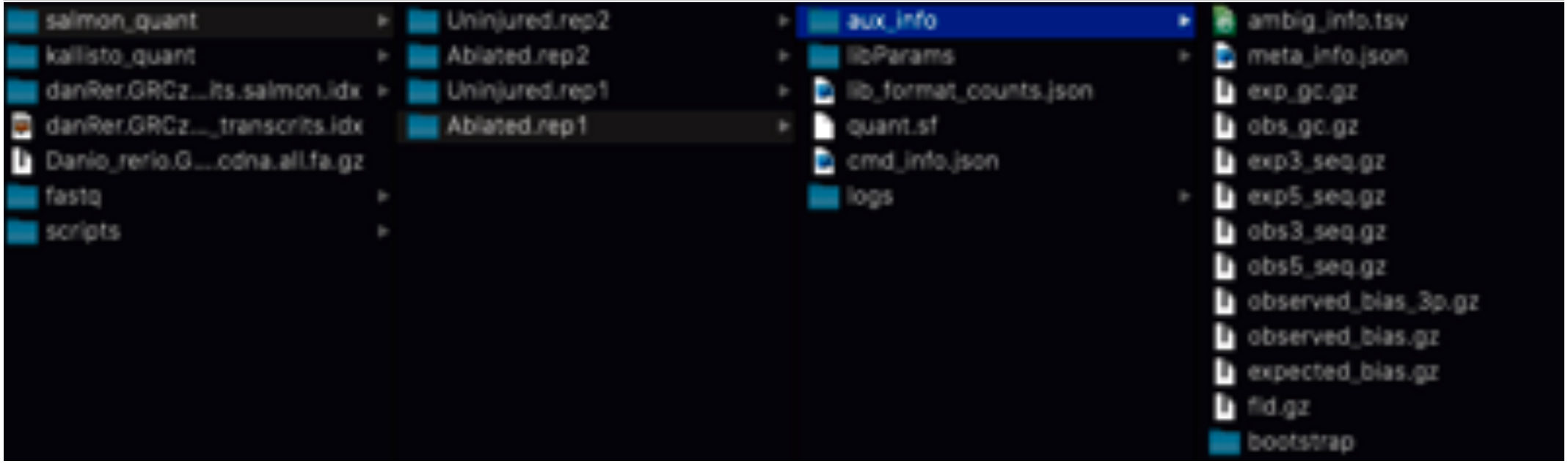
```
salmon quant -i danRer.GRCz11_transcrits.salmon.idx -l A \
             -r fastq/$cond.rep$rep.fastq.gz \
             --validateMappings -p 2 \
             -o salmon_quant/$cond.rep$rep \
             --numBootstraps 30 --seqBias --gcBias
```

output

kallisto



Salmon



output

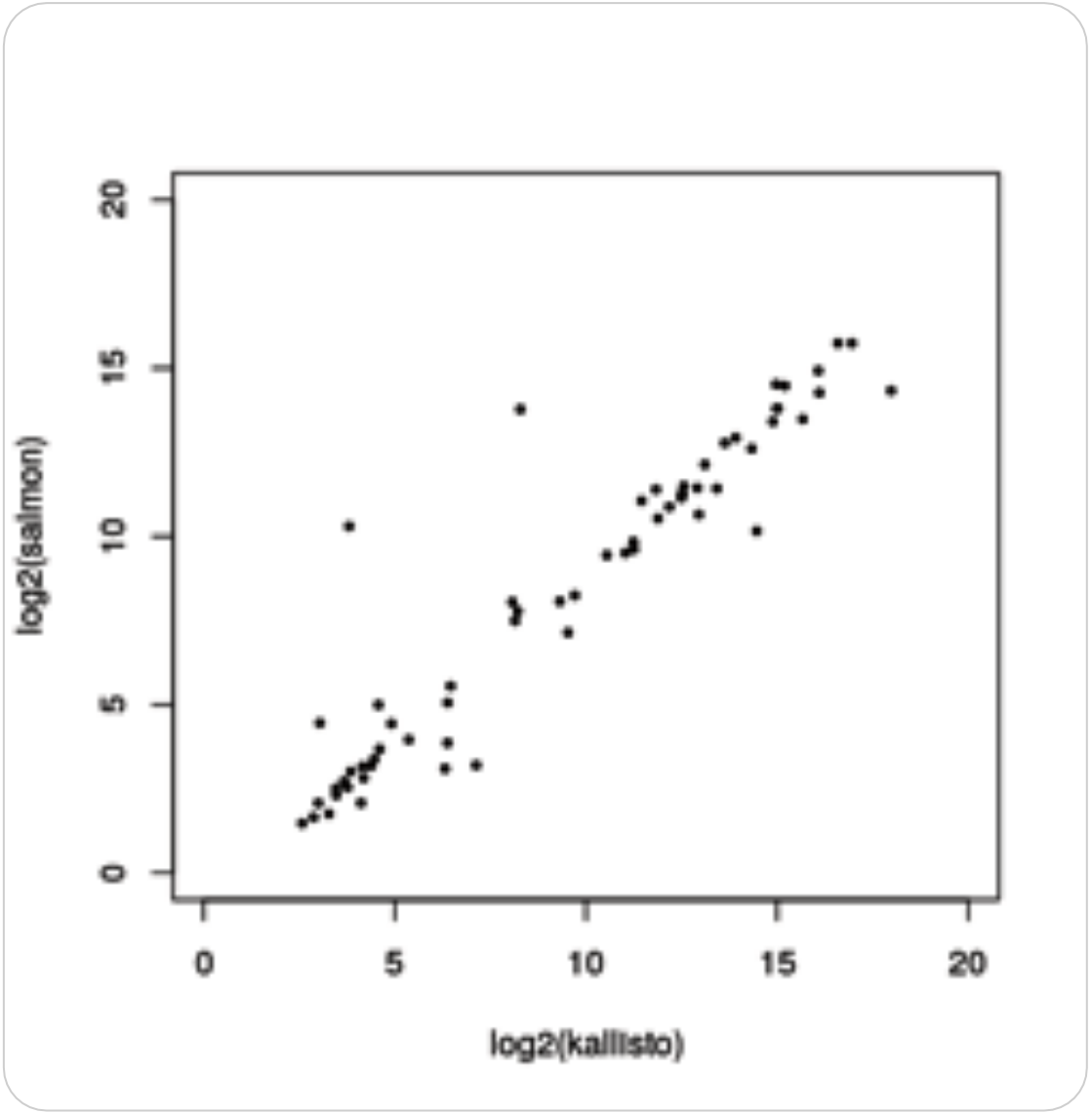
kallisto

	target_id	length	eff_length	est_counts	tpm
256	ENSDART00000146315.3	2622	2422.990	260.627000	5793.73000
257	ENSDART00000021121.8	1181	981.994	200.373000	10990.60000
279	ENSDART00000140081.2	6779	6579.990	1.760960	14.41500
281	ENSDART00000138182.3	6809	6609.990	0.239042	1.94789
330	ENSDART00000169072.2	8455	8255.990	1178.350000	7687.70000
331	ENSDART00000147947.2	8535	8335.990	719.649000	4650.01000

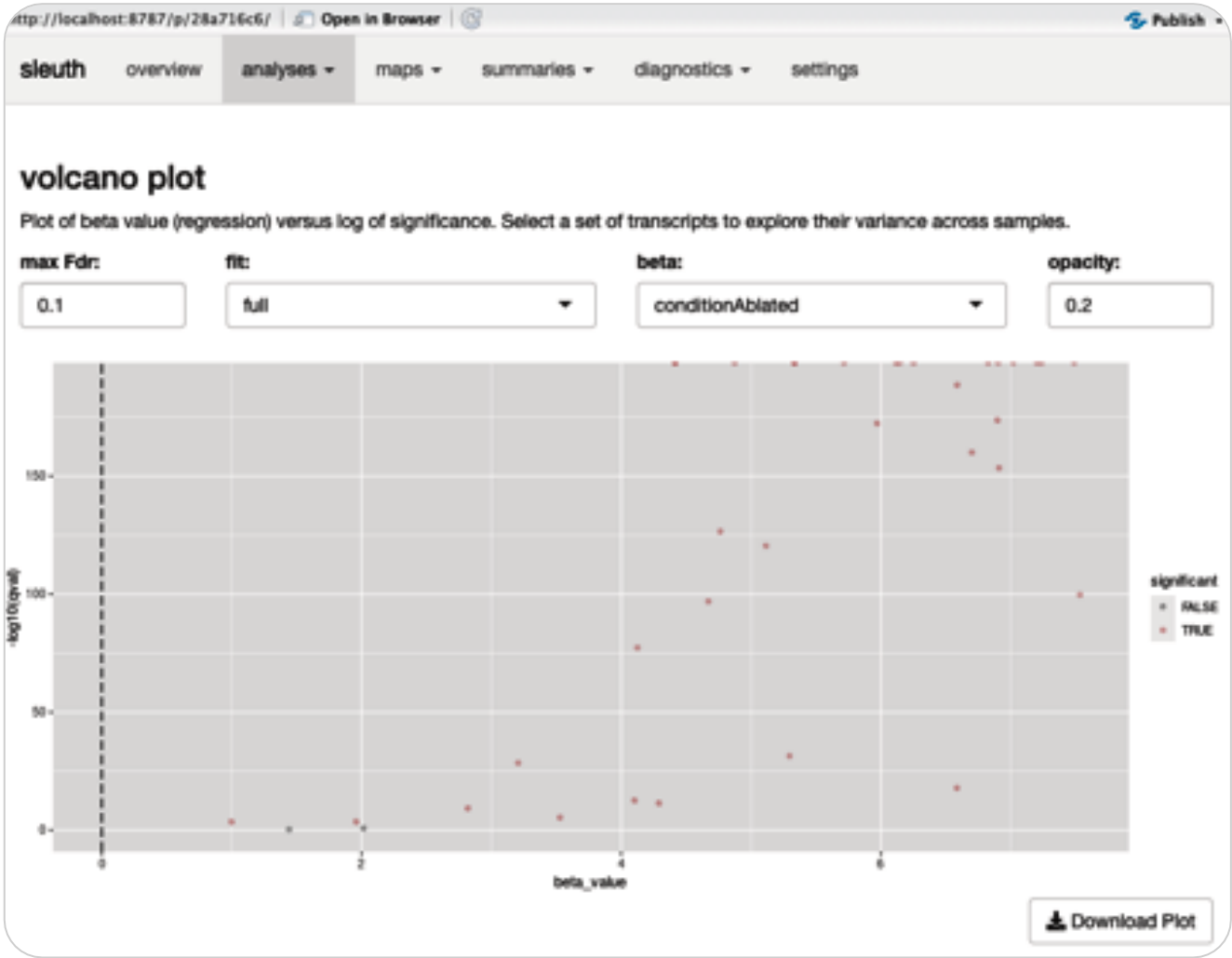
Salmon

	Name	Length	EffectiveLength	TPM	NumReads
256	ENSDART00000146315.3	2622	2838.809	2434.698251	292.406
257	ENSDART00000021121.8	1181	1526.830	2749.368836	177.594
279	ENSDART00000140081.2	6779	5857.133	8.071238	2.000
330	ENSDART00000169072.2	8455	9663.830	2781.720176	1137.280
331	ENSDART00000147947.2	8535	9769.232	1884.152025	778.720
339	ENSDART00000060594.6	2004	1621.171	14.579657	1.000

Kallisto and Salmon gene counts overall similar



Sleuth_live

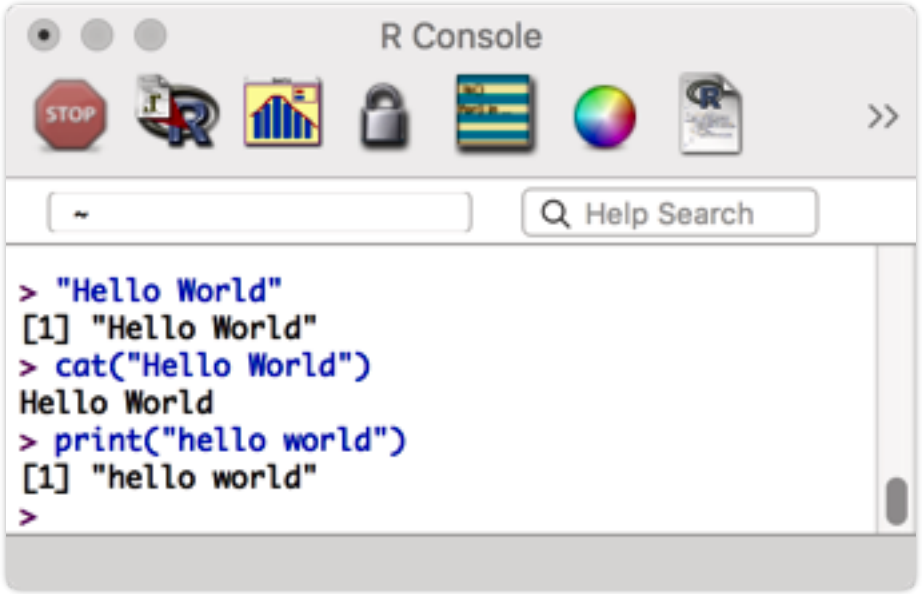

http://localhost:8787/p/28a716c6/
Open in Browser
Publish
sleuth
overview
analyses
maps
summaries
diagnostics
settings
volcano plot
Plot of beta value (regression) versus log of significance. Select a set of transcripts to explore their variance across samples.
max Fdr:
0.1
fit:
full
beta:
conditionAblated
opacity:
0.2
-log10(qval)
150
100
50
0
0
2
4
6
beta_value
significant
FALSE
TRUE
Download Plot

Introduction of R programming language

Install R/Bioconductor/Rstudio

- https://cloud.r-project.org/ 
- https://www.rstudio.com/products/rstudio/download/ 
- https://Bioconductor.org/ 

Exercise: Hello world



Where to start?

- From vignettes of a package https://bioconductor.org/packages/4.0/bioc/vignettes/trackViewer/inst/doc/trackViewer.html#lolliplot
- From help documents
- From a book

Grammar of R

```
hairLength <- 25
waistHeight <- 110
height <- 168
marryMe <- FALSE
wait <- 100 * 365
for(day in 1:wait){
  if(hairLength >= height - waistHeight){
      marryMe <- TRUE
      break
  }else{
      if(runif(1) < 0.5){
         next
      }
      hairLength <- hairLength + .1
  }
}
day
```

- 待我长发及腰，When my hair is longer enough to make hair bun
- 少年娶我可好？Will you give me your promise ring?
- 待你青丝绾正，When you get your driver license,
- 铺十里红妆可愿？Will you roll out the red carpet for me?
- 却怕长发及腰，I am afraid before my hair grew till my waist,
- 少年倾心他人。You will falling in love with another woman.
- 待你青丝绾正，I am afraid when you get your driver license,
- 笑看君怀她笑颜。I see another woman burying herself in your arms.
- ——何晓道 《十里红妆女儿梦》

Data structure of R

Dimension	Homogeneous	Heterogeneous
1d	Atomic vector	List
2d	Matrix	Data frame
Nd	Array	

Atomic vector: character, integer, logical, numeric, factor

Matrix, Array: atomic vector with dimension. Matrix is an array.

List: a chain for different kinds of data structures, including list.

Data frame: 2D version of List; All lists have same length of elements.

From Advanced R, http://adv-r.had.co.nz/Data-structures.html

Exercises

- What is the class of c('A', TRUE, 1L, 1, factor(letters[1:3]))
- What is the class of c(TRUE, 1L, 1, factor(letters[1:3]))
- What is the class of c(TRUE, 1L, factor(letters[1:3]))
- What is the class of c(TRUE, factor(letters[1:3]))
- m <- array(1:18, dim=c(3, 3, 2))
  i <- 1; j <- 3; k <- 2;
  m[i, j, k]
  m[(k-1)*(nrow(m)*ncol(m)) + (j-1)*nrow(m) + i]
- What is the difference between as.numeric(as.character(factor(c(3, 5, 1)))) and as.numeric(factor(c(3, 5, 1)))
- What is the difference between as.list(m) and as.list(as.data.frame(m))

How to get help

- help(functionName) or ?functionName or ??functionName : help(help); help("for")
- browseVignettes("grid")
- Google
- R mailing list: https://www.r-project.org/mail.html#instructions

How to install a package

- Install R packages: install.packages("BiocManager", repos="https://cloud.r-project.org")
- Install Bioconductor packages: BiocManager::install(c("org.Dr.eg.db", "TxDb.Drerio.UCSC.danRer10.refGene"))

Merge tables

- Question: how could we merge two excel sheet by gene symbols?
- Code:

```
m <- data.frame(symbol=letters[1:5], foldChange=runif(5))
n <- data.frame(gene=sample(letters[1:10], size=6), foldChange=runif(n=6))
z <- merge(m, n, by.x="symbol", by.y="gene", suffixes=c(".m", ".n"), all.x=TRUE, all.y=TRUE)
z
```

Set operations

- Question: how to find the shared/unique genes from 2 lists of genes?
- Code:

```
A <- sample(letters[1:10], size=8)
B <- sample(letters[1:10], size=5)
intersect(A, B) ## shared genes
setdiff(A, B) ## only in A
setdiff(B, A) ## only in B
```

How to read data from a file

- Tab-delimited file (tsv): read.delim("file.txt")
- Comma-delimited file (csv): read.csv("file.csv")
- Excel sheet (xls, xlsx): library(gdata); read.xls("file.xlsx", sheet=1)

How to save a table

- Save to a csv file: write.csv(data, "file.csv")
- Save to a xls file: library(WriteXLS); WriteXLS(dataframe, "file.xls")

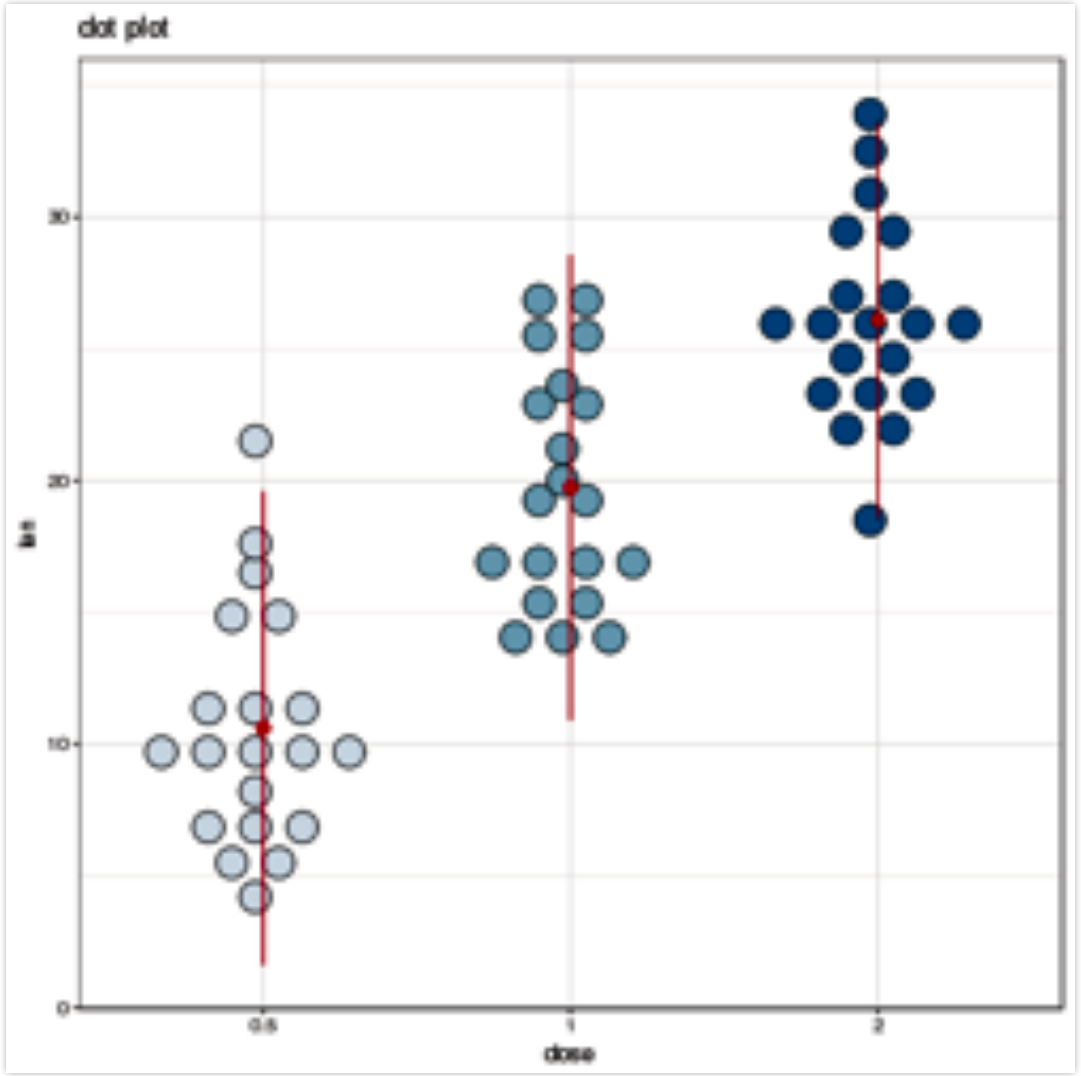
Basics of R plot

- Dots plot: plot(x=mtcars$wt, y=mtcars$mpg)
- Bar plot: counts <- table(mtcars$vs, mtcars$gear); barplot(counts)
- Histogram: hist(mtcars$vs)
- Pie: x<-table(mtcars$gear); pie(x)
- Boxplot: boxplot(mpg~cyl,data=mtcars)

ggplot2

Based on the grammer of graphics

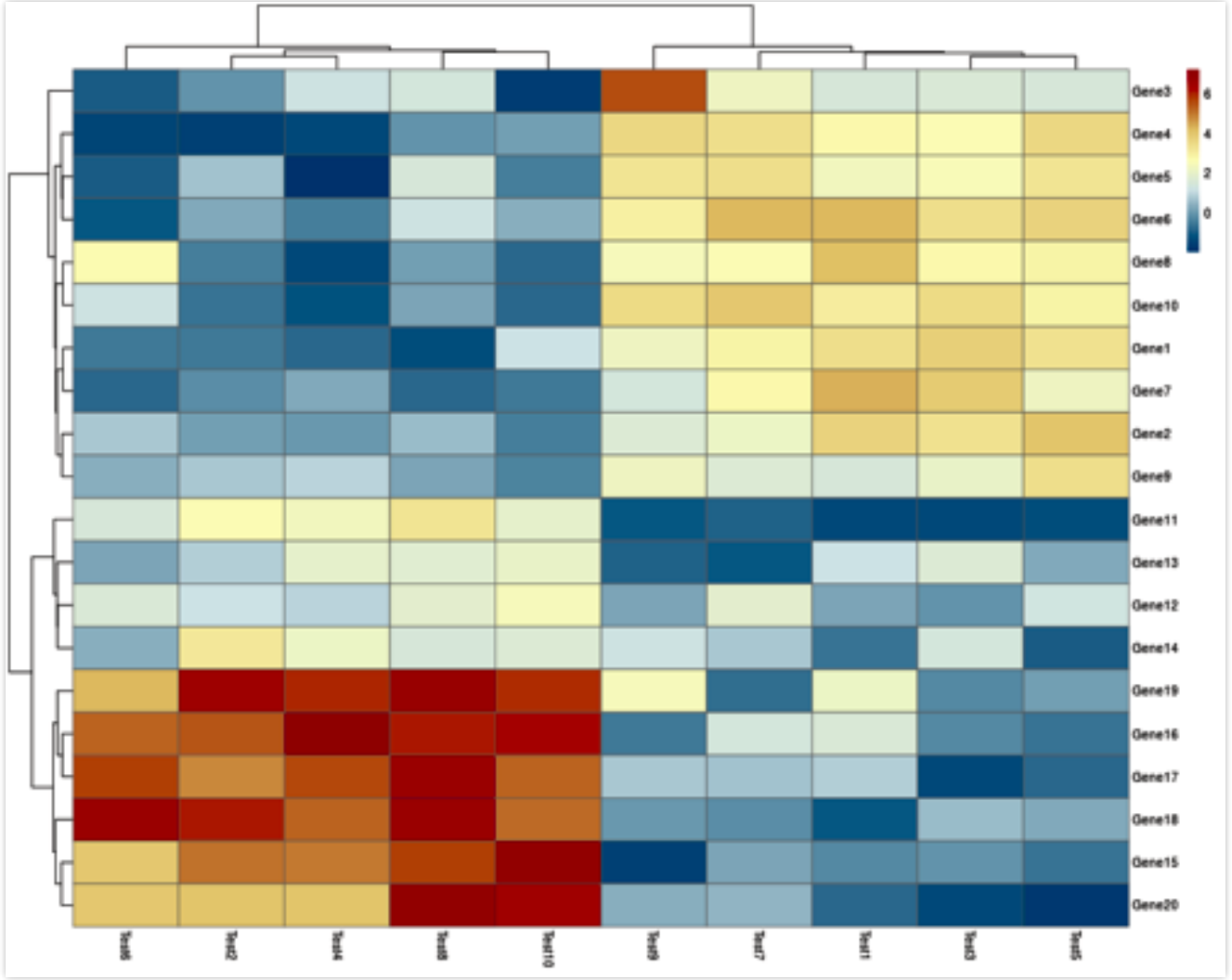
```
library(ggplot2)

data("ToothGrowth")

ToothGrowth$dose <- factor(ToothGrowth$dose)

ggplot(ToothGrowth, aes(x=dose, y=len, fill=dose)) +
geom_dotplot(binaxis='y', stackdir='center',
stackratio=1.5, dotsize=1.2) +
stat_summary(fun.data=mean_sdl, mult=1,
geom="pointrange", color="red") +
scale_fill_brewer(palette="Blues", guide=FALSE) +
theme_bw() + labs(title="dot plot")
```



pheatmap

Pretty heatmap

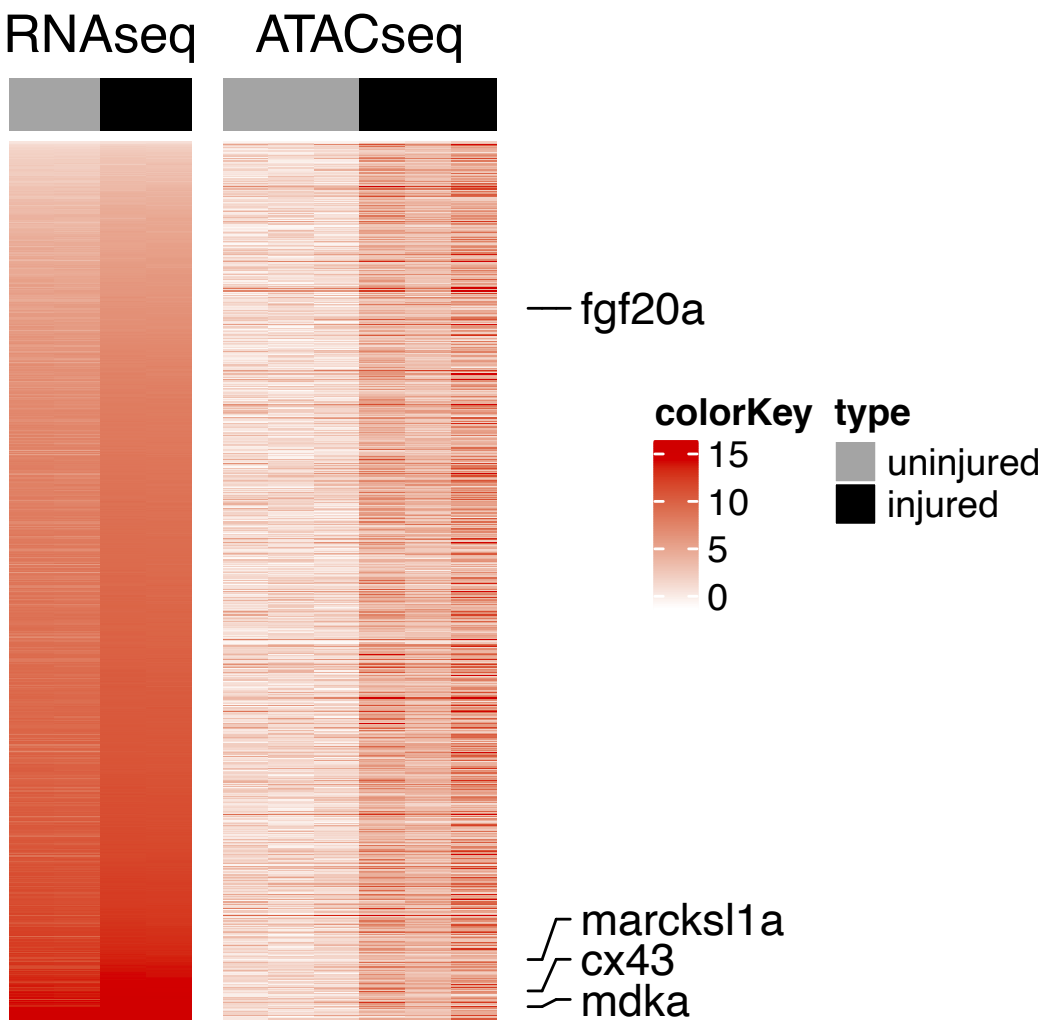
```
library(BiocManager)
install("pheatmap")
library(pheatmap)
test <- matrix(rnorm(200), 20, 10)
test[1:10, seq(1, 10, 2)] <- test[1:10, seq(1, 10, 2)] + 3
test[11:20, seq(2, 10, 2)] <- test[11:20, seq(2, 10, 2)] + 2
test[15:20, seq(2, 10, 2)] <- test[15:20, seq(2, 10, 2)] + 4
colnames(test) <- paste("Test", 1:10, sep = "")
rownames(test) <- paste("Gene", 1:20, sep = "")
pheatmap(test)
```



ComplexHeatmap

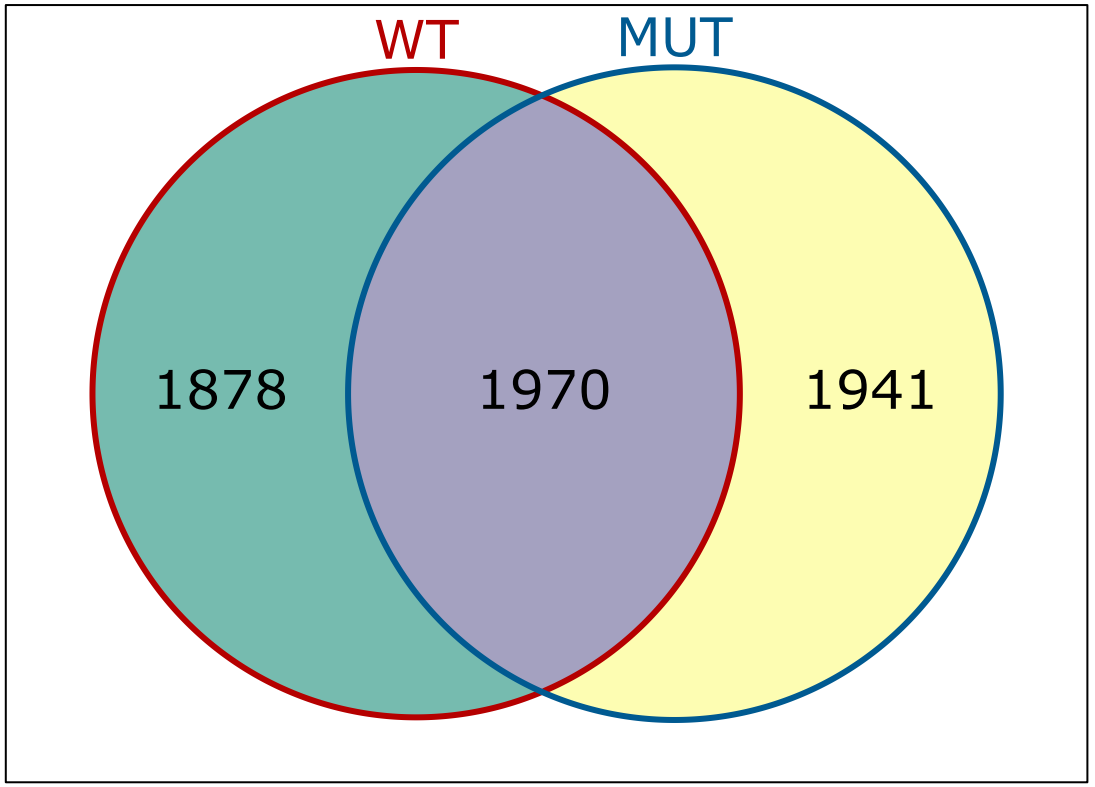
Plot multiple heatmaps in one canvas

rowAnnotation and colAnnotation



Venn Diagram: Vennerable

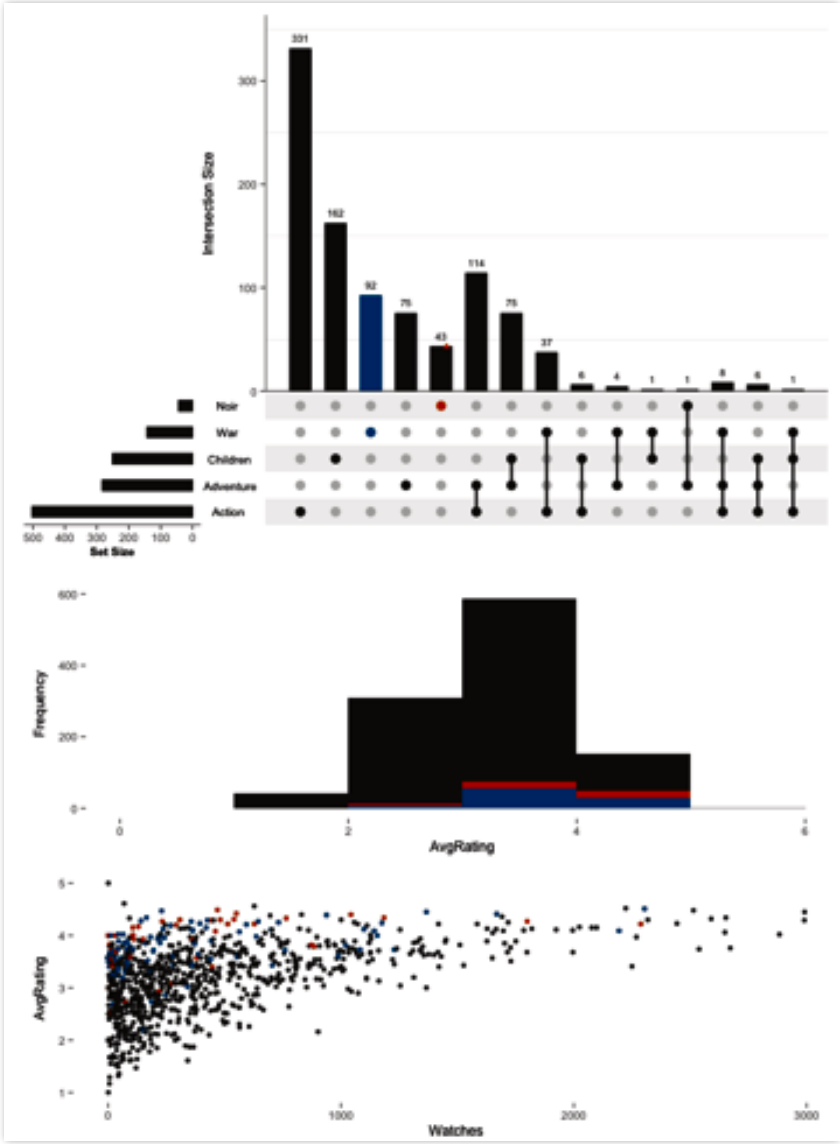
displays Venn and Euler diagrams



UpSetR

visualizes set intersections in a matrix layout

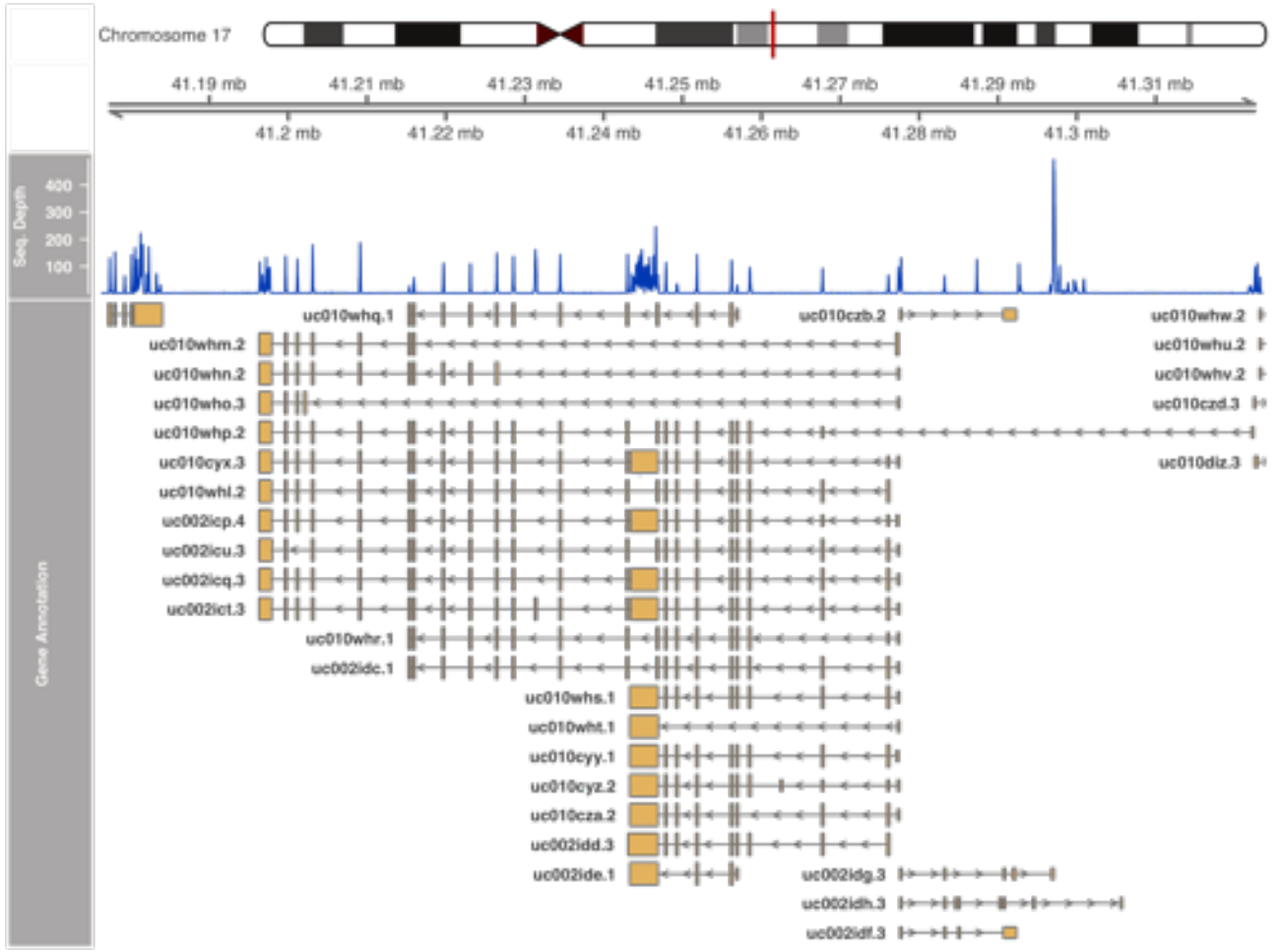
```
install("UpSetR")

library(UpSetR)

movies <- read.csv(
system.file("extdata", "movies.csv",
package = "UpSetR"), header=TRUE,
sep=";" )

upset(movies, nsets = 7, nintersects =
30, mb.ratio = c(0.5, 0.5), order.by =
c("freq", "degree"), decreasing =
c(TRUE,FALSE))
```



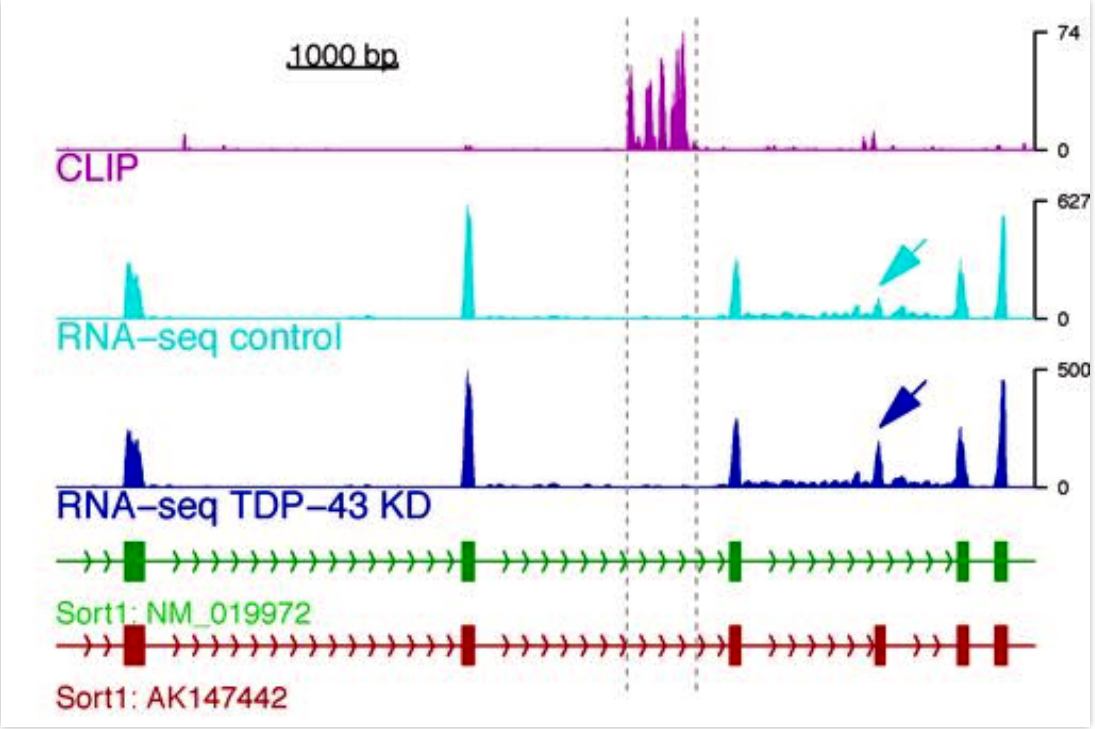
Gviz

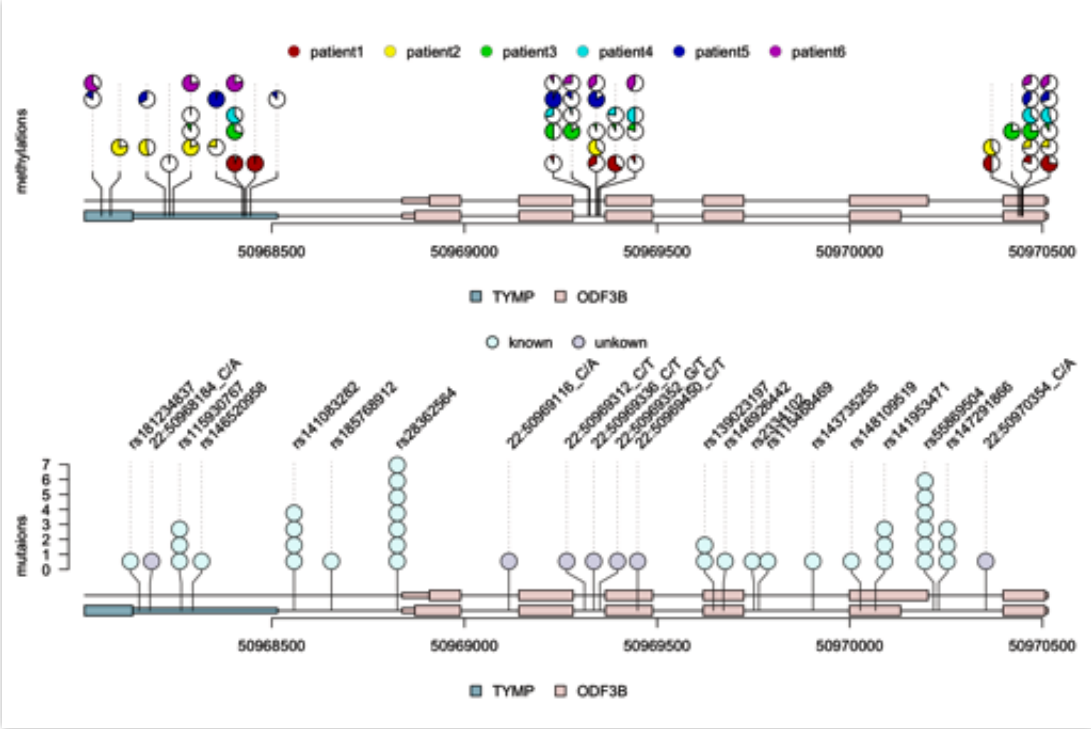
Plotting data and annotation information along genomic coordinates

```
library(Gviz)
library(GenomicRanges)
data(cpgIslands)
chr <- as.character(unique(seqnames(cpgIslands)))
gen <- genome(cpgIslands)
atrack <- AnnotationTrack(cpgIslands, name="CpG")
gtrack <- GenomeAxisTrack()
data(geneModels)
grtrack <- GeneRegionTrack(geneModels, genome=gen, chromosome=chr, name="Gene Model")
plotTracks(list(gtrack, atrack, grtrack))
```



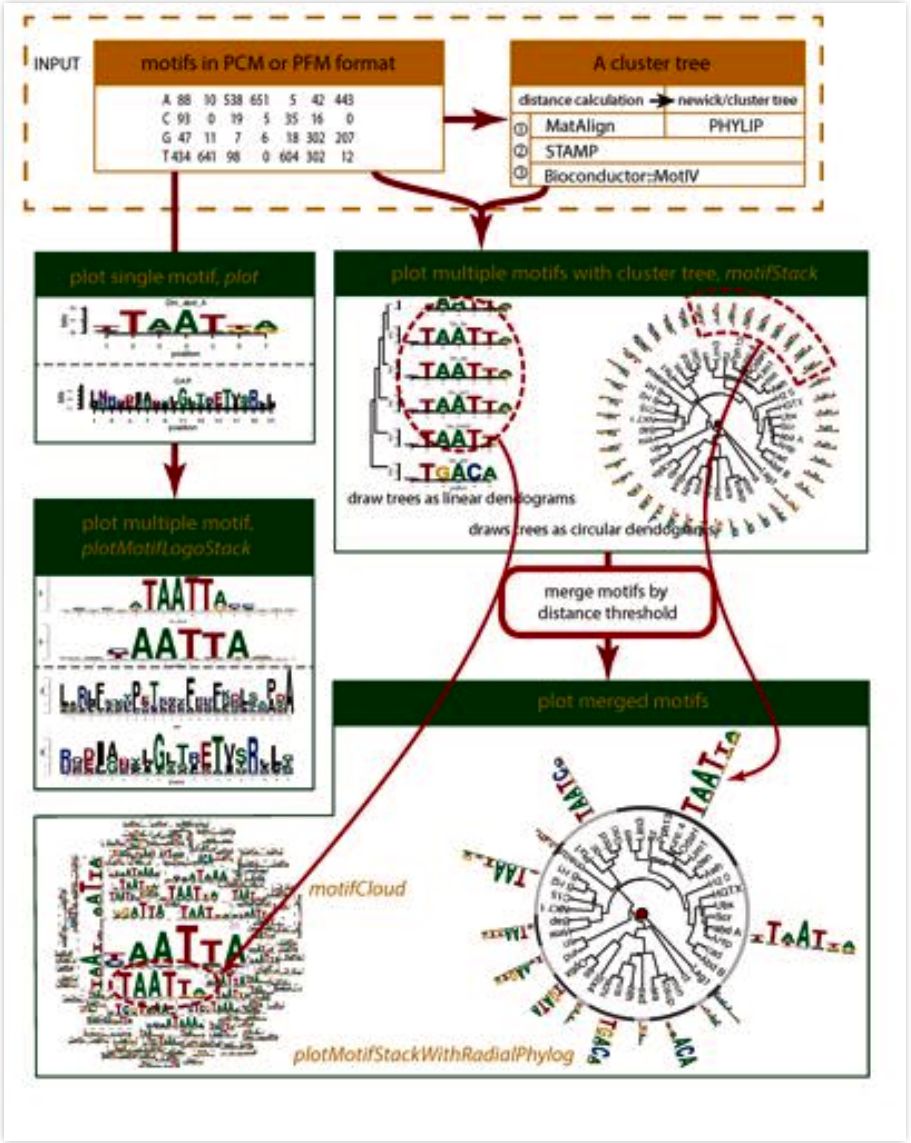
trackViewer





motifStack

Plot stacked logos for single or multiple DNA, RNA and amino acid sequence



Basics of Bioconductor objects

- GenomicRanges::GRanges:

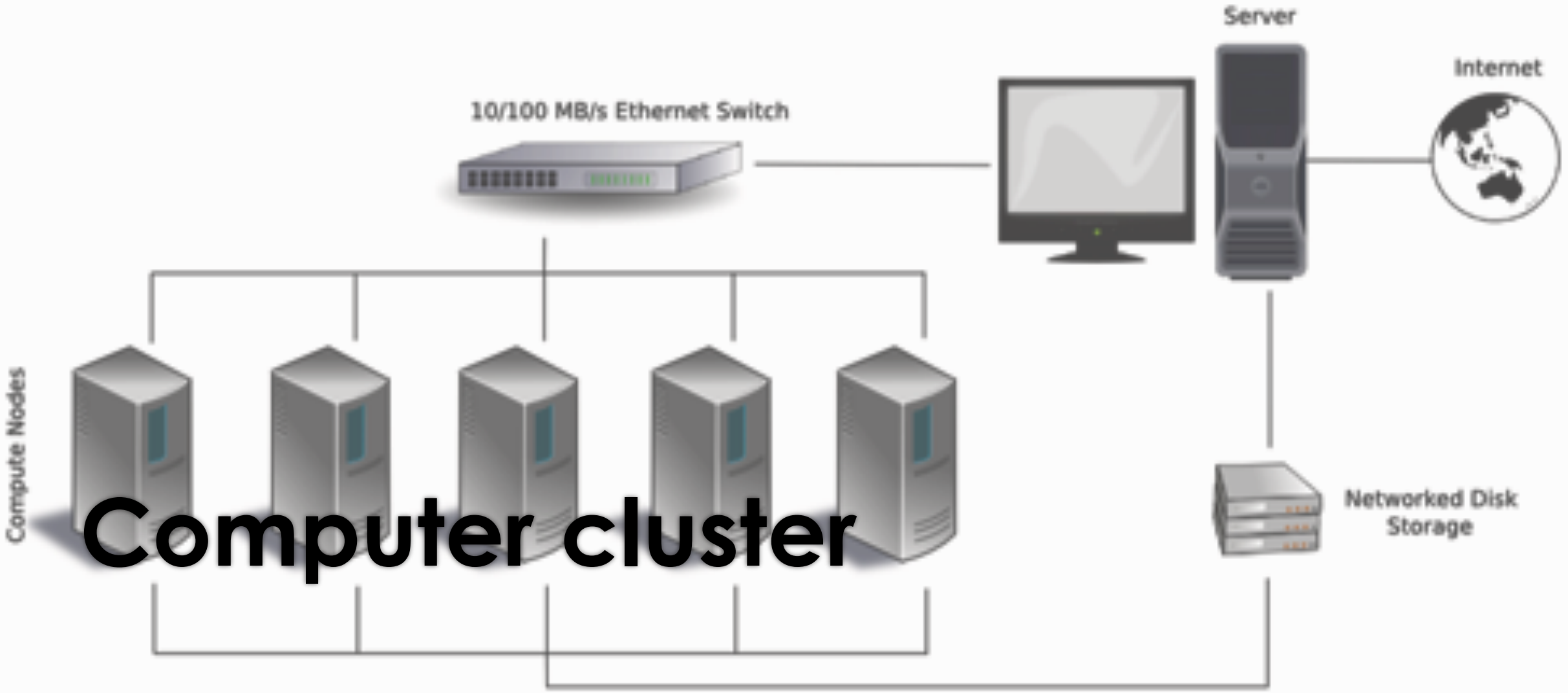
```
gr0 <- GRanges(Rle(c("chr2", "chr2", "chr1", "chr3"), c(1, 3, 2, 4)), IRanges(1:10, width=10:1))
gr0
```

- SummarizedExperiment::RangedSummarizedExperiment:

```
nrows <- 200; ncols <- 6
counts <- matrix(runif(nrows * ncols, 1, 1e4), nrows)
rowRanges <- GRanges(rep(c("chr1", "chr2"), c(50, 150)),
                     IRanges(floor(runif(200, 1e5, 1e6)), width=100),
                     strand=sample(c("+", "-"), 200, TRUE),
                     feature_id=sprintf("ID%03d", 1:200))
colData <- DataFrame(Treatment=rep(c("ChIP", "Input"), 3),
                     row.names=LETTERS[1:6])
rse <- SummarizedExperiment(assays=SimpleList(counts=counts),
                            rowRanges=rowRanges, colData=colData)
rse
```

Basics of annotation

- annotationDBi: include ChipDb, OrgDb, GODb, InparanoidDb and ReactomeDb
- BSgenome: The BSgenome class is a container for storing the full genome sequences of a given organism.
- GenomicFeatures: The GenomicFeatures package retrieves and manages transcript-related features from the UCSC Genome Bioinformatics and BioMart data resources.



Computer cluster

https://en.wikipedia.org/wiki/Computer_cluster

Cluster software

- Oracle Grid Engine, previously known as Sun Grid Engine (SGE) :qsub
- IBM Platform Load Sharing Facility (LSF): bsub
- Slurm workload manager: sbatch
- More: https://en.wikipedia.org/wiki/Comparison_of_cluster_software

The Duke compute cluster

- Wetsite: https://rc.duke.edu/dcc/
- Next class: **8/4 OR 9/1**, https://rc.duke.edu/dcc-training/
- DCC use slurm workload manager.

Accessing the DCC

- Login: ssh NetID@dcc-slogin.oit.duke.edu
- Copy files: scp, rsync
  - scp -r sourceFolder netid@dcc-slogin.oit.duke.edu:/work/netid/targetFolder
  - rsync -av sourceFolder netid@dcc-slogin.oit.duke.edu:/work/netid/targetFolder

DCC file systems

- /dscrhome: 250 GB group quota
- /work: temporary storage
- /datacommons: archival storage; available for $80/TB/year

module

- module avail
- module load
  - which STAR
  - module load STAR/2.5.3a
  - which STAR
  - echo $PATH

Submit a job

- srun:
  - Interactive mode: srun --pty bash -i
  - Run a script: srun --mem 1G /bin/hostname
- sbatch:
  - *#!/bin/bash*
  - *#SBATCH -J mapping #jobname*
  - *#SBATCH --array=1-2 # job array, can be 1,3,5,7 or 1-7:2 '%' will limit the number of job*
  - *#SBATCH -o pindel.out.%A_%a.txt # %A == $SLURM_ARRAY_JOB_ID #log folder must be there*
  - *#SBATCH -e pindel.err.%A_%a.txt # %a == $SLURM_ARRAY_TASK_ID*
  - *#SBATCH --mem-per-cpu=5G #5G*
  - *#SBATCH -c 1 # cpus-per-task*
  - *#SBATCH --partition=common*
  - i=$(($SLURM_ARRAY_TASK_ID-1))
  - echo $i > job.id.txt

DCC partitions

- **common**, for jobs that will run on the DCC core nodes (up to 64 GB RAM).
- **common-large**, for jobs that will run on the DCC core nodes (64-240 GB GB RAM).
- **gpu-common**, for jobs that will run on DCC GPU nodes.
- **Group partitions** (partition name varies), for jobs that will run on lab-owned nodes

Slurm commands

- sbatch – Submit a batch job
- squeue – show list of jobs
- scancel – delete one or more jobs

Additional information

Basic tools

- Bedtools
- Samtools
- Picard
- Ucsc genomic tools

Samtools

- SAM, BAM file convert:
  - samtools view -h file.bam > file.sam
  - samtools view -b -S file.sam > file.bam
- Sorting BAM
  - samtools sort file.bam -o outputbam
- Create BAM index
  - samtools index file.bam

Online resources

- cBio Cancer Genomics Portal (cBioPortal) & package cgdsr
- The Cancer Genome Atlas (TCGA) & package TCGAbiolinks, RTCGAToolbox
- Gene Expression Omnibus (GEO) & package GEOquery, SRAdb
- Encyclopedia of DNA Elements (ENCODE) & package ENCODExplorer
- recount2 & package recount

Use cgdsr package download data

- BiocManager::install("cgdsr")
- library(cgdsr)
- # Create CGDS object
- mycgds <- CGDS("https://www.cbioportal.org/")
- # Test the connection
- test(mycgds)
- # Get list of cancer studies at server
- getCancerStudies(mycgds)[, 1:2]
- # Get available case lists (collection of samples) for a given cancer study
- (mycancerstudy <- getCancerStudies(mycgds)[2,1])
- getCaseLists(mycgds, mycancerstudy)
- mycaselist <- getCaseLists(mycgds, mycancerstudy)[1, 1]
- # Get available genetic profiles
- getGeneticProfiles(mycgds,mycancerstudy)
- (mygeneticprofile <- getGeneticProfiles(mycgds,mycancerstudy)[2,1])
- # Get data slices for a specified list of genes, genetic profile and case list
- getProfileData(mycgds,c('BRCA1','BRCA2'),mygeneticprofile,mycaselist)
- # Get clinical data for the case list
- myclinicaldata <- getClinicalData(mycgds,mycaselist)
- dim(myclinicaldata)
- head(myclinicaldata)

Download expression data from recount2

```
## in shell apt-get update && apt-get install libxml2-dev
BiocManager::install("recount")
library('recount')
## Find a project of interest
project_info <- abstract_search('GSE32465')
## Explore info
project_info
## Download the gene-level RangedSummarizedExperiment data
download_study(project_info$project)
## Load the data
load(file.path(project_info$project, 'rse_gene.Rdata'))
## Delete it if you don't need it anymore
unlink(project_info$project, recursive = TRUE)
rse_gene
## the sample phenotype data provided by the recount project
head(colData(rse_gene))
## the sample feature data provided by the recount project
rowData(rse_gene)
```

```
## Scale counts by taking into account the total coverage per sample
rse <- scale_counts(rse_gene)
(title <- colData(rse)$title)
(group <- ifelse(grepl("uninduced", title), "uninduced", "induced"))
(target <- sub("^.*targeting (.*?) gene.*$", "\\1", title))
## Add sample information for DE analysis
colData(rse)$group <- factor(group)
colData(rse)$gene_target <- factor(target)
library(DESeq2)
## Specify design and switch to DESeq2 format
dds <- DESeqDataSet(rse, ~ gene_target + group)
## Perform DE analysis
dds <- DESeq(dds, test = 'LRT', reduced = ~ gene_target, fitType = 'local')
res <- results(dds)
## Explore results
head(res)
library(WriteXLS)
WriteXLS(as.data.frame(res), "DE.results.GSE32465.xls")
```

DESeq2

```
library(DESeq2)
load(url("http://qiubio.com/bioconductor/RNA-seq/ds1.Rdata"))
ls()
head(counts[, 1:7], 3)
grp <- as.factor(substr(colnames(counts), 1, 2)) ##assign group
dds <- DESeqDataSetFromMatrix(counts,
                              colData=data.frame(grp),
                              design=formula(~-1+grp))
design(dds)
dds <- DESeq(dds)
res <- results(dds)
res
mcols(res, use.names=TRUE)
plotMA(res)
plotDispEsts(dds)
## save results
library(WriteXLS)
WriteXLS(as.data.frame(res), "results.xls")
## try comparison of other groups
results(dds, contrast = c("grp", "PE", "DE"))
## question, how to compare group PE vs FE
```

Regeneromics Shared Resource can help your research!

Selected recent publications we have co-authored:

Identification and requirements of enhancers that direct gene expression during zebrafish fin regeneration. Thompson JD, Ou J, Lee N, Shin K, Cigliola V, Song L, Crawford GE, Kang J, Poss KD. Development. 2020 Jul 14:dev.191262. doi: 10.1242/dev.191262. Online ahead of print.

Persistence of a regeneration-associated, transitional alveolar epithelial cell state in pulmonary fibrosis. Kobayashi Y, Tata A, Konkimalla A, Katsura H, Lee RF, Ou J, Banovich NE, Kropski JA, Tata PR. Nat Cell Biol. 2020 Jul 13. doi: 10.1038/s41556-020-0542-8. Online ahead of print.

Persistence of a regeneration-associated, transitional alveolar epithelial cell state in pulmonary fibrosis. Kobayashi Y, Tata A, Konkimalla A, Katsura H, Lee RF, Ou J, Banovich NE, Kropski JA, Tata PR. Nat Cell Biol. 2020 Jul 13. doi: 10.1038/s41556-020-0542-8. Online ahead of print.

Nucleoporin 153 links nuclear pore complex to chromatin architecture by mediating CTCF and cohesin binding.Kadota S, Ou J, Shi Y, Lee JT, Sun J, Yildirim E. Nat Commun. 2020 May 25;11(1):2606. doi: 10.1038/s41467-020-16394-3.

Vitamin D Stimulates Cardiomyocyte Proliferation and Controls Organ Size and Regeneration in Zebrafish. Han Y, Chen A, Umansky KB, Oonk KA, Choi WY, Dickson AL, Ou J, Cigliola V, Yifa O, Cao J, Tornini VA, Cox BD, Tzahor E, Poss KD. Dev Cell. 2019 Mar 25;48(6):853-863.e5. doi: 10.1016/j.devcel.2019.01.001. Epub 2019 Jan 31.

We do: experimental design, bioinformatics analysis, manuscript preparation, grant applications

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