UC DAVIS HEALTH

Bulk RNA Sequencing in Clinical and Translational Research

CLINICAL AND TRANSLATIONAL SCIENCE CENTER

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Outline

- What is RNA sequencing and what can it tell you?
- Designing an RNASeq experiment
- Analyzing your data
- Resources

RNA Sequencing

RNA Sequencing

- RNA sequencing measures gene expression by directly sequencing reverse-transcribed mRNA

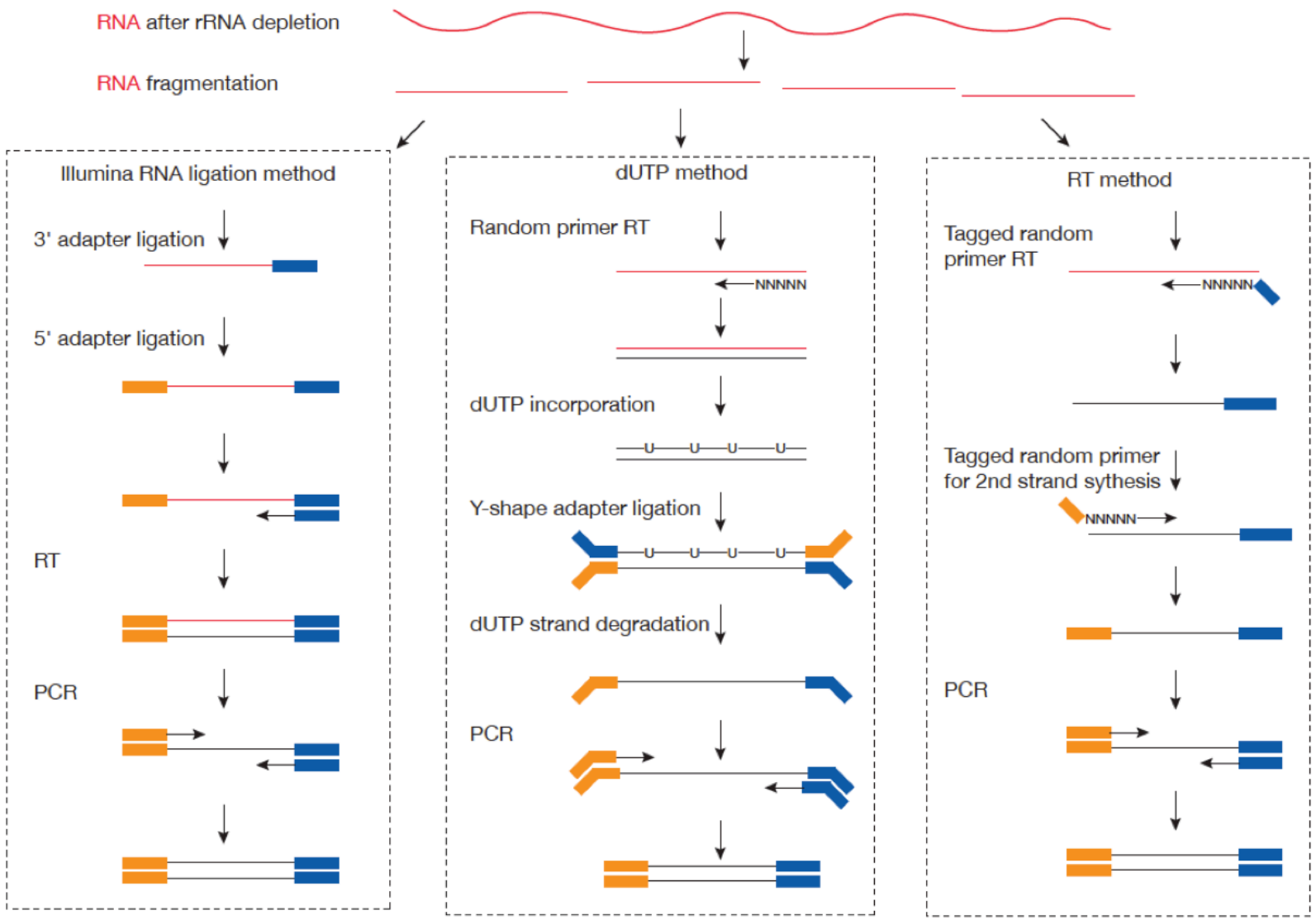
- Bulk RNASeq can tell you:
  - Gene expression
  - Transcript expression (with appropriate protocol)

- Limitations
  - Expression measured will be average of that in whole sample
  - Heterogeneity of cell types can add noise
  - Quantification of gene expression is relative

RNA Sequencing

- Steps in RNASequencing (short read)
  1. RNA extraction
  2. Enrichment for mRNA
  3. Fragment RNA
  4. Reverse transcribe, add adapters
  5. PCR amplification
  6. Sequence

RNA Sequencing



Experimental Design

Experimental Design

- Principles of good experimental design in non-omics experiments still apply

- Generally, a minimum of 3 samples/group is required to be able to even conduct any statistical analysis
  - Continuous covariates and very large multifactorial designs are exceptions
  - Adequate power will often require more samples

Experimental Design

- RNA extraction batch tends to be the largest source of technical variability
- If possible, have same person do all RNA extraction in a single session
- Otherwise, **RANDOMIZE** RNA extraction batches so that they are independent of variables of interest, as much as possible
  - Can adjust for RNA extraction batch in statistical model after the fact, if not completely confounded with variables of interest

Experimental Design

- **NEVER, EVER DO THIS:**
  - Day 1: Extract RNA for all treated samples
  - Day 2: Extract RNA for all control samples
- **Your experiment will be unable to distinguish batch and treatment effects**
- **This is UNFIXABLE with statistics**

Experimental Design

- Sample pooling is sometimes necessary to have enough RNA
- In this case, your unit of replication is pool rather than sample
- Still need replicates
  - Don’t pool all of your control samples into one pool and all of your treated samples into another pool
- Each pool should consist of distinct samples
  - Don’t e.g. pool all of your control samples together then split into groups for library prep

Experimental Design

- If you are using more than one lane of sequencing, lane-to-lane variability in sequencing can be mitigated by doing the following:
  - Prepare barcoded libraries—allows samples to be distinguished
  - Pool all libraries
  - Split pool across all lanes being used
- DNA Tech Core at the Genome Center does this
- Make sure your sequencing provider is doing something similar
- If you have more samples than unique barcodes, will need to randomize samples into lanes

Experimental Design

- Power and required sample size depends on:

1. Amount of variability
   - Human >> mouse >> cell line
2. Size of effect to be detected
   - Subtle effects require more samples than large effects
3. Analysis used

Experimental Design

- Resampling of **pilot data** gives best estimates of power
- Other approaches can be unreliable
  - Different methods give very different estimates
  - Too many unverifiable assumptions

Poplawski A, Binder H. Feasibility of sample size calculation for RNA-seq studies. Brief Bioinform. 2018 Jul 20;19(4):713-720. doi: 10.1093/bib/bbw144. PMID: 28100468.

- Experience shows, however:
  - 3 replicates/group is typically adequate for cell line studies
  - Human studies require 1 or 2 orders of magnitude above that

Experimental Design

- Matching groups by sex, age, comorbidities, smoking status, etc. reduces bias
- Covariate adjustment in analysis can reduce variability
- However, isolated subjects that are very different from the group can't be accounted for in analysis:
  - E.g. only male or only smoker in group of women/nonsmokers
  - Try to avoid this situation
  - Throwing a subject from an unreplicated group in "just to see" is a waste of money
- Using subjects as their own control (e.g. pre-post design) can increase power

Analyzing your data

Preprocessing

- You will get fastq files from your sequencing provider
  - Contains sequences for every read + quality scores
  - Side note: sequencing providers only keep your data for a limited time, plan to download data to your storage **ASAP** or risk losing it!

Preprocessing

- Raw sequence data need to be preprocessed into a form that can be analyzed readily
- Requires access to a compute cluster + knowledge of linux command line
- Or hire someone to do preprocessing
  - Statisticians, even with ‘omics experience, will generally **not** do this for you
  - Need to plan specifically for bioinformatics support

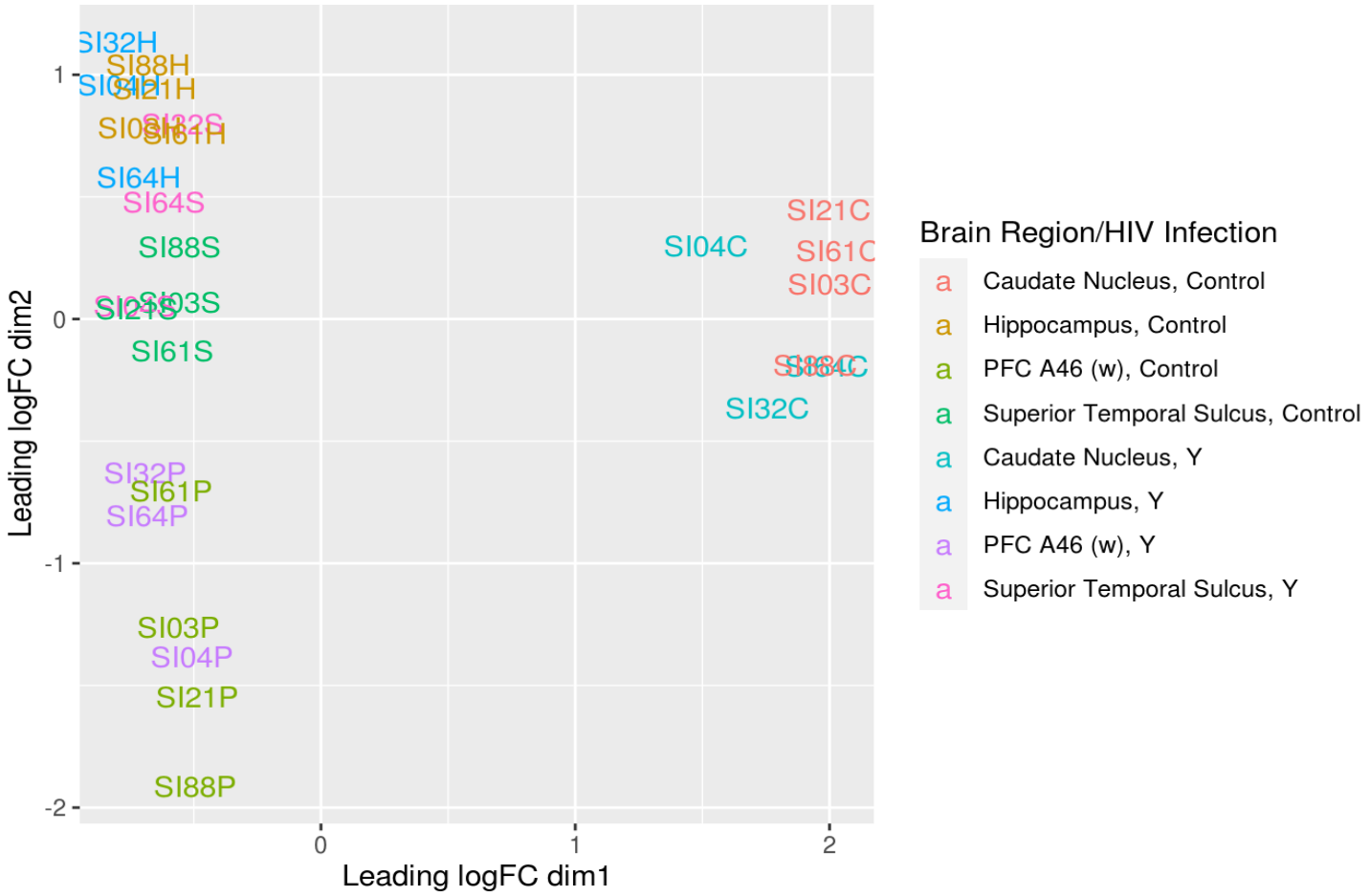
Preprocessing

- Data preprocessing includes:
  - Removing bases of unwanted sequence (Ex. vectors, adapter, primer sequence, polyA tails)
  - Merge/join short overlapping paired-end reads
  - Remove low quality bases or N characters
  - Remove reads originating from PCR duplication
  - Remove reads that are not of primary interest (contamination)
  - Remove too short reads
- Cleaned sequence data aligned to genome
- Reads belonging to each gene (exon, transcript) counted/quantified

Statistical Analysis

- Some common analyses of RNASeq data:
  - Visual summaries
  - Differential expression analysis
  - Pathway enrichment analyses
  - Weighted Gene Coexpression Network Analysis (WGCNA)

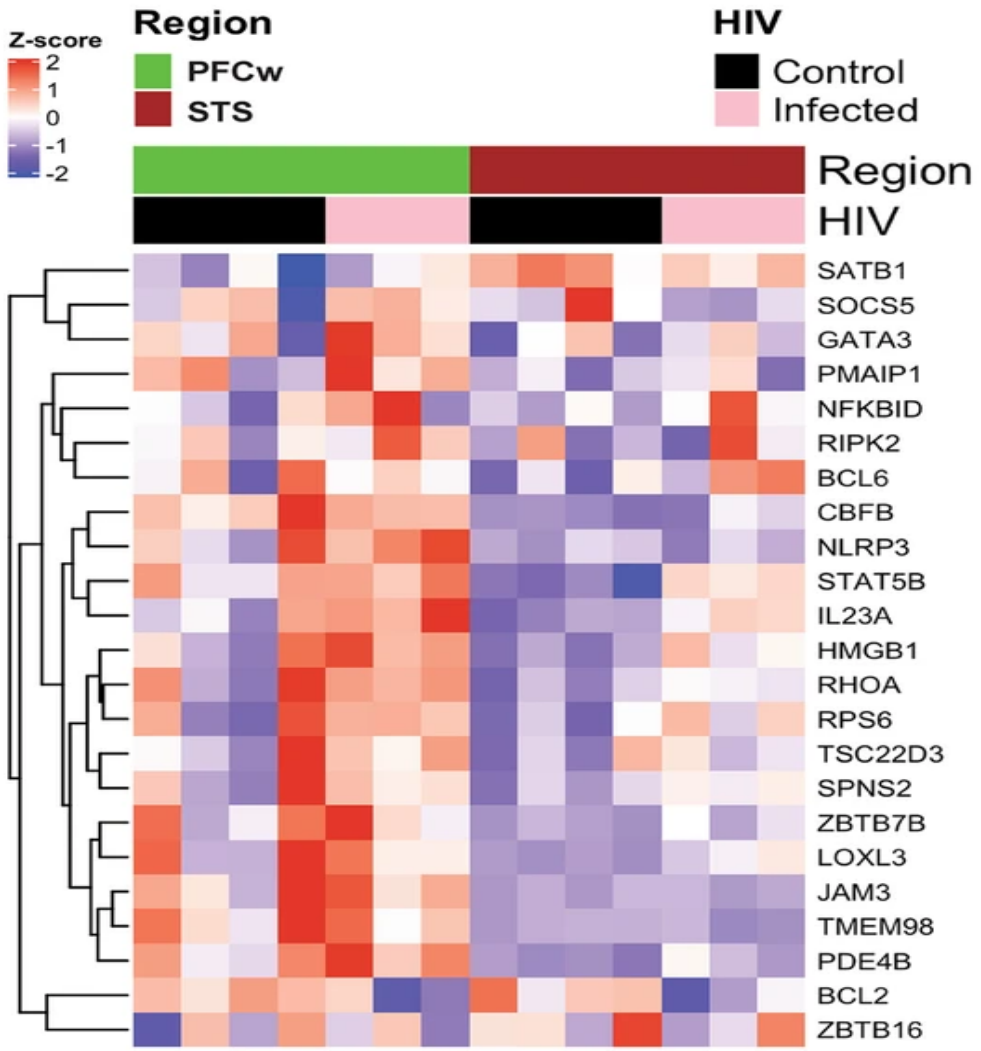
Visualizations



Multidimensional Scaling Plot

- Shows relative distances between whole transcriptomic profile
- Useful for identifying unusual samples
- More distance between groups often means more DE genes in later analysis

Visualizations

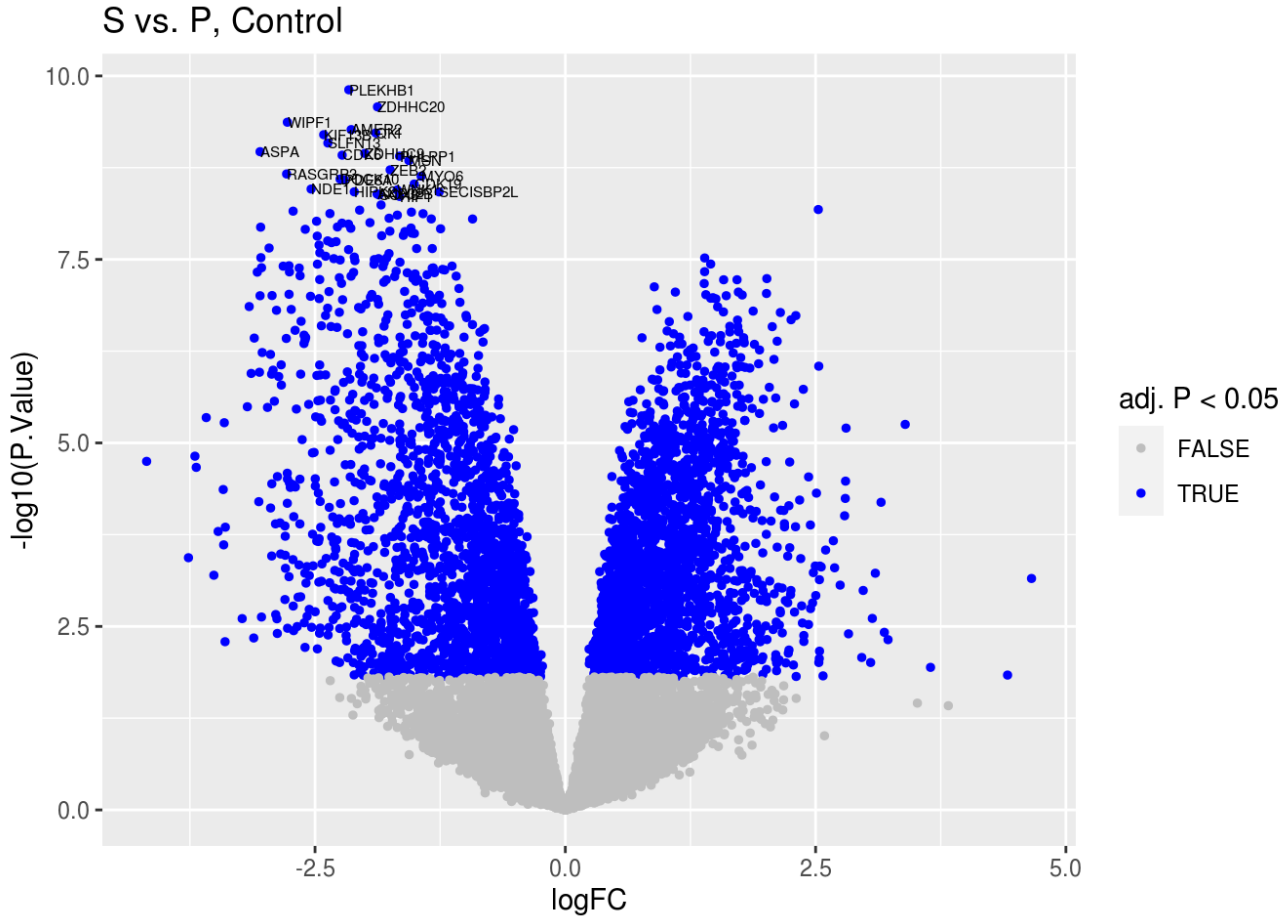


Heatmap

- Shows expression level of individual samples for selected genes
- Most useful when limited to focused set of genes
  - Can't show gene names for >50 genes
- Genes typically clustered based on hierarchical clustering dendrogram
- Samples often clustered as well

Hawes, C.E., Elizaldi, S.R., Beckman, D. *et al.* Neuroinflammatory transcriptional programs induced in rhesus pre-frontal cortex white matter during acute SHIV infection. *J Neuroinflammation* **19**, 250 (2022). https://doi.org/10.1186/s12974-022-02610-y

Visualizations



Volcano Plot

- Displays differential expression results
- Plot of –log10 p-value by log fold change
- Can quickly show if DE genes are predominantly up- or downregulated
- Top genes are labelled

Differential Expression Analysis

- What genes differ in expression between groups?
- Or, what genes are correlated with a continuous outcome?

- Steps in DE:
  - Filter low expressed/uninteresting genes
  - Normalize data to account for library size differences
  - Transform/weight data if required by model
  - Fit statistical model to each gene
  - **Adjust p-values for multiple testing**
    "Significant" means adjusted $P < 0.05$

Differential Expression Analysis

- Popular Bioconductor packages for DE include:
  - DESeq, based on negative binomial model fitted to gene counts
  - edgeR, based on negative binomial model fitted to gene counts
  - limma-voom, based on weighted linear models fitted to log-transformed counts per million reads
- All of these can accommodate complicated study designs
- limma allows for random effects
- Comparison papers show limma-voom better controls the false discovery rate at the nominal rate

Soneson C, Delorenzi M. A comparison of methods for differential expression analysis of RNA-seq data. BMC Bioinformatics. 2013 Mar 9;14:91. doi: 10.1186/1471-2105-14-91. PMID: 23497356; PMCID: PMC3608160.

Differential Expression Analysis

- A table of DE results might look like this (~10K rows not shown):

Gene.stable.ID	Gene.name	logFC	AveExpr	P.Value	adj.P.Val
ENSMUSG00000103477	5930409G06Rik	1.664192137	1.837293153	0.001950398	0.999161216
ENSMUSG00000020721	Helz	-0.363050365	7.501167612	0.003238648	0.999161216
ENSMUSG00000026051	Ecrg4	-1.255273365	2.499099269	0.003247058	0.999161216
ENSMUSG00000029798	Herc6	-1.445749184	2.050030151	0.003562989	0.999161216
ENSMUSG00000038872	Zfhx3	-0.378363542	8.239061179	0.003879801	0.999161216
ENSMUSG00000052675	Zfp112	1.11791828	3.113930496	0.004248763	0.999161216
ENSMUSG00000037108	Zcwpw1	0.591054065	4.725995765	0.004378957	0.999161216
ENSMUSG00000038010	Ccdc138	-0.840359112	4.178710837	0.004715516	0.999161216
ENSMUSG00000014905	Dnajb9	-0.501504798	5.243296442	0.0050183	0.999161216
ENSMUSG00000022311	Csmd3	-0.877654845	3.42558905	0.005062491	0.999161216
ENSMUSG00000090272	Mndal	1.371770654	2.262543233	0.005264431	0.999161216
ENSMUSG00000044968	Napepld	-1.666212304	2.06202902	0.005863092	0.999161216
ENSMUSG00000025507	Pidd1	1.268521656	2.077240363	0.006642031	0.999161216
ENSMUSG00000034912	Mdga2	-0.53890529	5.107036276	0.006768523	0.999161216
ENSMUSG00000067336	Bmpr2	-0.303627268	7.83187622	0.006771913	0.999161216

Pathway Analysis

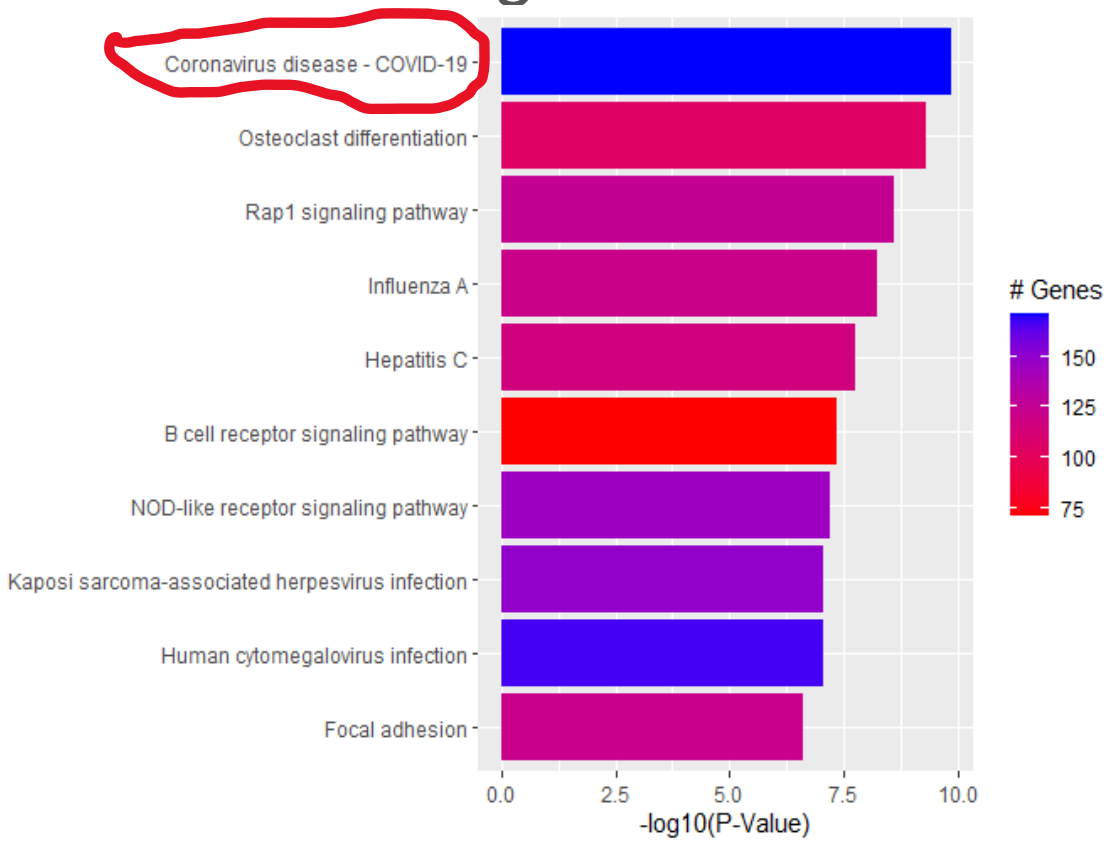
- DE analyses can be difficult to interpret
- Pathway or gene ontology enrichment analyses can summarize DE results into a more manageable form
- What pathways/gene sets are overrepresented among significant genes, or at the top of the DE results?
- Common databases:
  - KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways
  - GO (Gene Ontology), a controlled vocabulary for describing gene products
  - Reactome pathways
  - MSigDB (Molecular Signatures Database), used by GSEA
  - Ingenuity Pathway Analysis (requires license for use)

Pathway Analysis

- Enrichment analyses often take one of two approaches:
  1. Is a given gene set overrepresented in my gene list (e.g. significant genes)?
     - Uses Fisher's Exact Test or hypergeometric test
     - Approach taken by DAVID (https://david.ncifcrf.gov/helps/tutorial.pdf)
  2. Is a given gene set ranked higher in my DE analysis results (or other ranked list) than would be expected by chance
     - Kolmogorov-Smirnov test, GSEA's leading edge analysis
- Gene ontology is complicated by directed acyclic graph structure of GO terms
  - R package topGO applies either of the above approaches in a way that preferentially tests more specific terms (e.g. "positive regulation of granzyme B production") over less specific ones ("immune system process").

Pathway Analysis

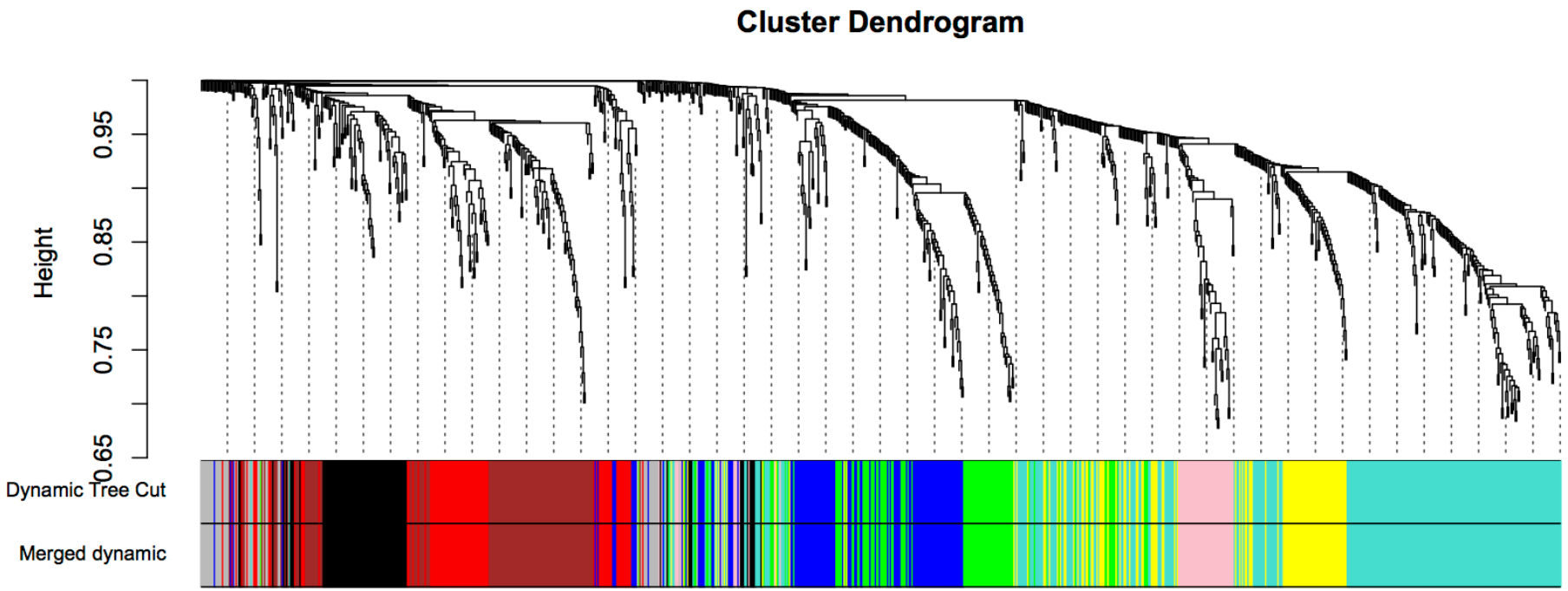
- Enrichment analysis results are a descriptive tool, not a smoking gun
- Top KEGG enrichment results using data from *2018* paper:



Reanalysis of data from Dorothée Selimoglu-Buet, et al. "A miR-150/TET3 pathway regulates the generation of mouse and human non-classical monocyte subset." Nature Communications volume 9, Article number: 5455 (2018)

WGCNA

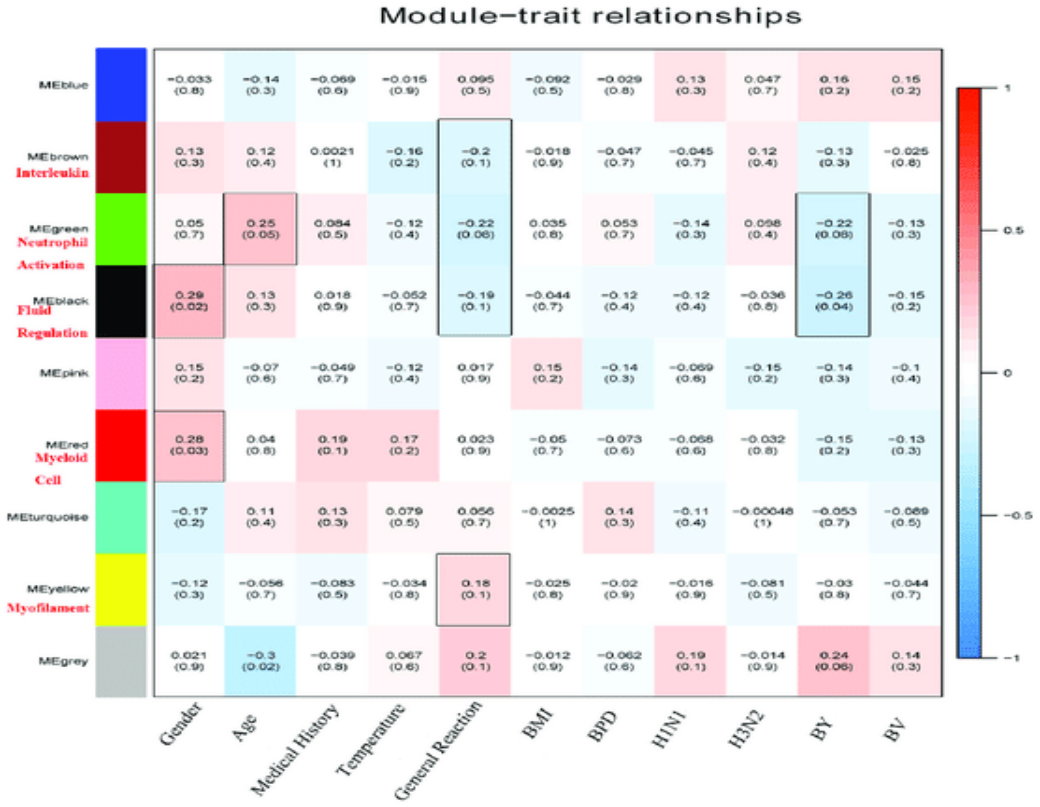
- Weighted Gene Coexpression Network Analysis (Langfelder and Horvath, 2008) identifies modules of coexpressed genes:



Langfelder, P., Horvath, S. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics* **9**, 559 (2008). https://doi.org/10.1186/1471-2105-9-559

WGCNA

- "Eigengenes" of modules (first principal components of gene expression) provide useful summary
  - ~10 eigengenes vs. 10K genes
  - Easier to calculate/interpret correlations given large quantities of metadata
  - Great for integrating matched data from multiple omics methods



Yang, J., Zhang, J., Fan, R., Zhao, W., Han, T., Duan, K., ... & Yang, X. (2020). Identifying Potential Candidate Hub Genes and Functionally Enriched Pathways in the Immune Responses to Quadrivalent Inactivated Influenza Vaccines in the Elderly Through Co-Expression Network Analysis. *Frontiers in immunology*, *11*, 603337.

Resources

- Available to anyone on fee-for-service basis:
  - UCD Genome Center DNA Technologies Core
    Library preparation, sequencing, and other services
    https://dnatech.genomecenter.ucdavis.edu/
  - UCD Genome Center Bioinformatics Core
    Wide range of analysis services including everything mentioned in this talk
    https://bioinformatics.ucdavis.edu/
- For IDDRC projects:
  - IDDRC BBRD Core
    Statistical analysis including gene expression data
    https://health.ucdavis.edu/mindinstitute/centers/intellectual-developmental-disabilities-research/cores/bbrd.html
- Cancer Center Genomics Shared Resource:
  https://health.ucdavis.edu/cancer/research/sharedresources/ger.html

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