



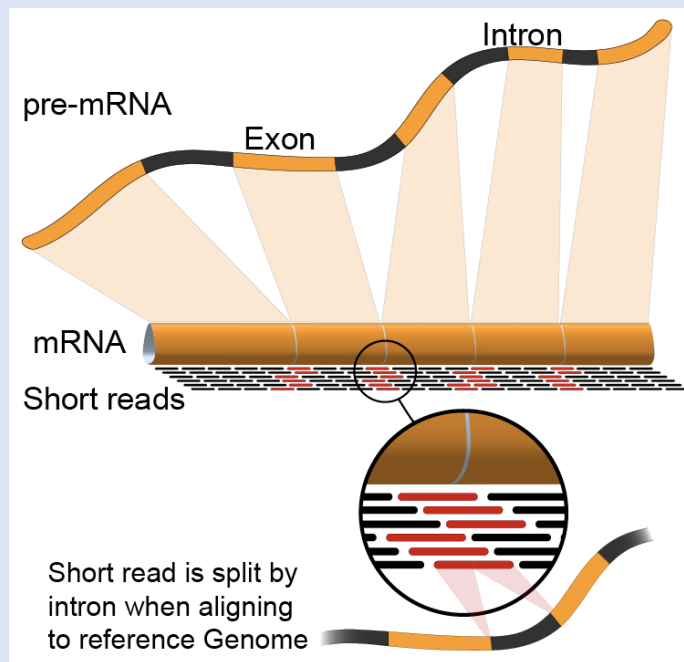
Cold  
Spring  
Harbor  
Laboratory

# RNA-Seq Module 3

## Abundance Estimation and Differential Expression

Arpad Danos, Felicia Gomez, Obi Griffith, Malachi Griffith,  
My Hoang, Mariam Khanfar, Chris Miller, Kartik Singhal

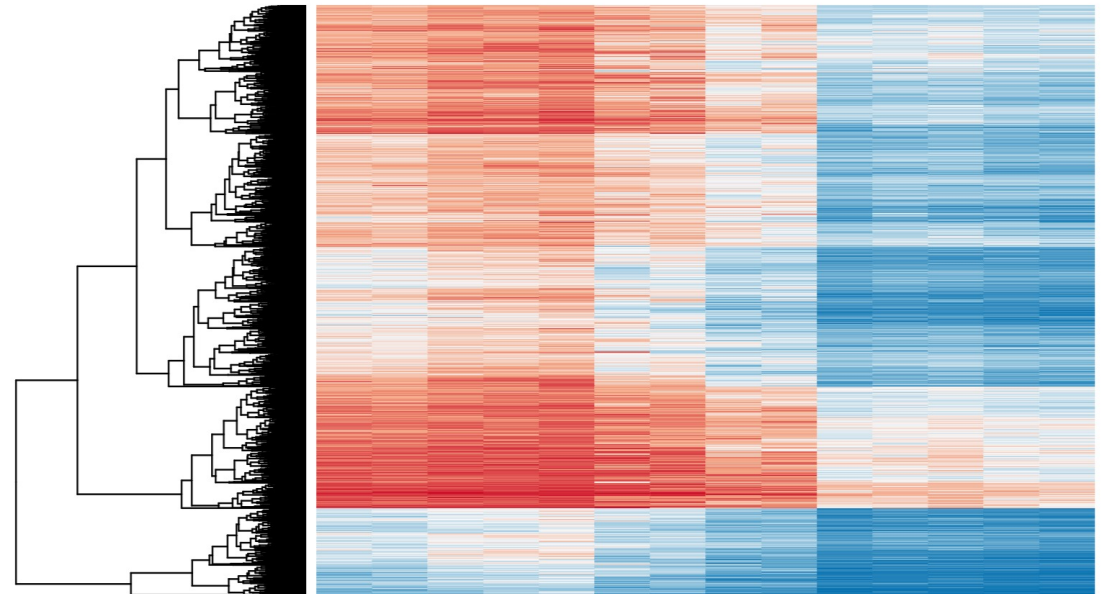
Advanced Sequencing Technologies & Bioinformatics Analysis November 5-19, 2023



Washington University in St. Louis  
SCHOOL OF MEDICINE

# Differential Expression

- Tying gene expression back to genotype/phenotype
- What genes/transcripts are being expressed at higher/lower levels in different groups of samples?
  - Are these differences ‘significant’, accounting for variance/noise?
- Examples (used in course):
  - UHR cells vs HBR brain
  - Tumor vs Normal tissue
  - Wild-type vs gene KO cells



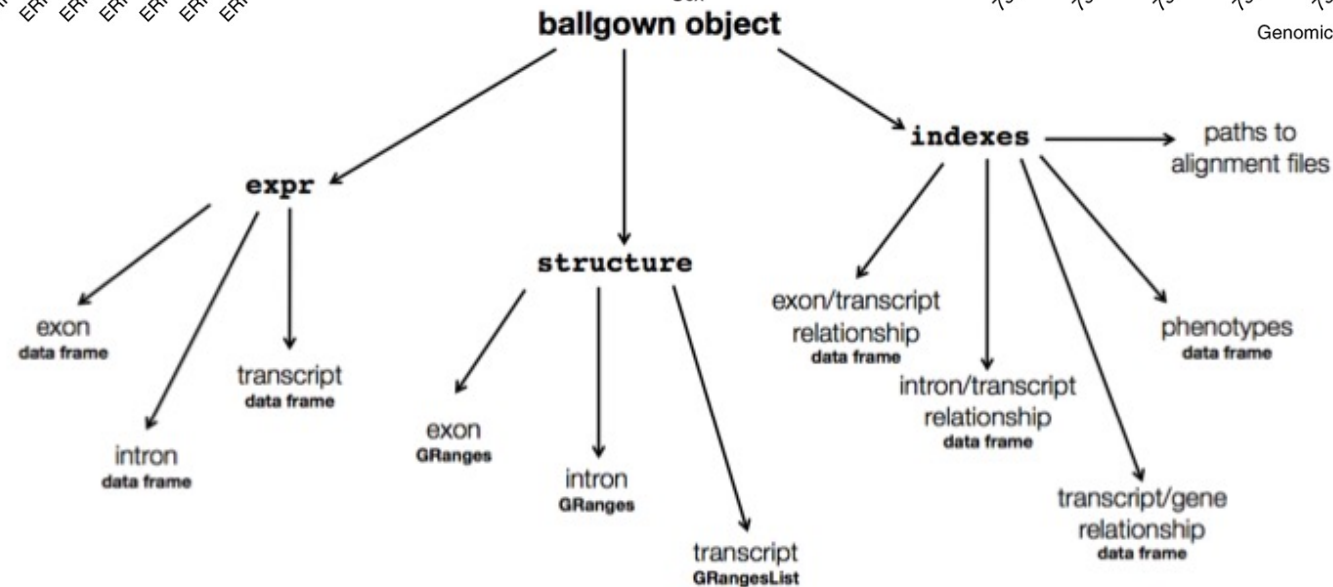
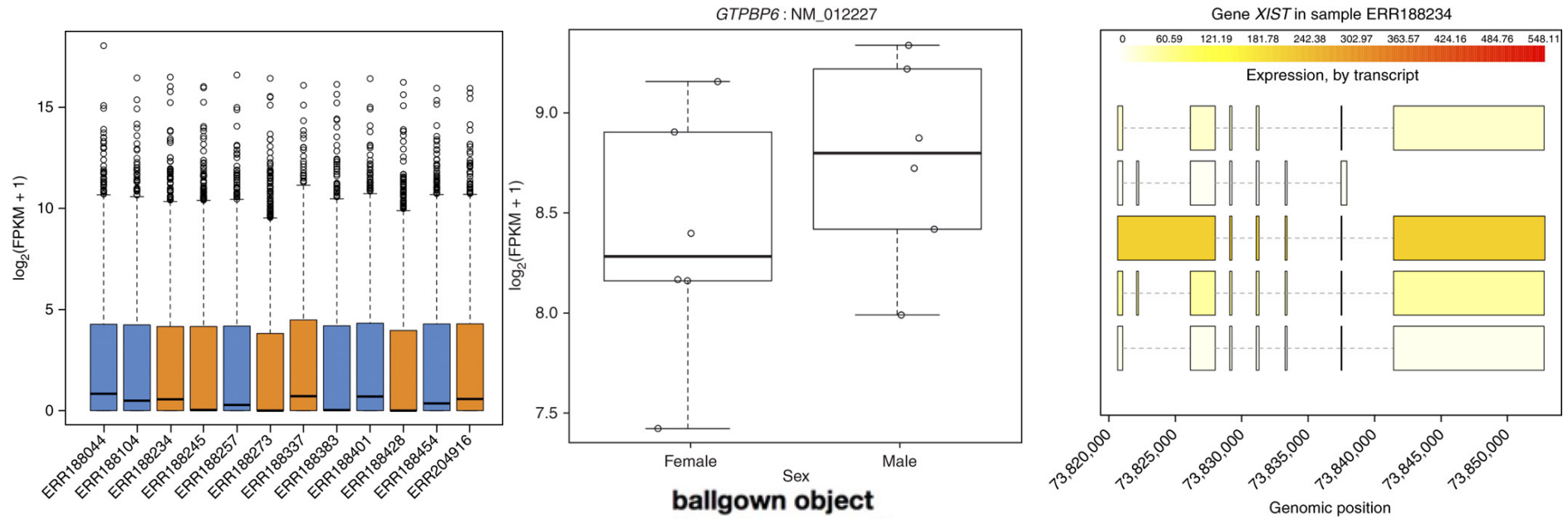
# Differential Expression with Ballgown

Parametric F-test comparing nested linear models

- Two models are fit to each feature, using expression as the outcome
  - one including the covariate of interest (e.g., case/control status or time) and one not including that covariate.
- An F statistic and p-value are calculated using the fits of the two models.
  - A significant p-value means the model including the covariate of interest fits significantly better than the model without that covariate, indicating differential expression.
- Adjust for multiple testing by reporting q-values:
  - $q < 0.05$  the false discovery rate should be controlled at ~5%.

[Frazee et al. \(2014\)](#)

# Ballgown for Visualization with R



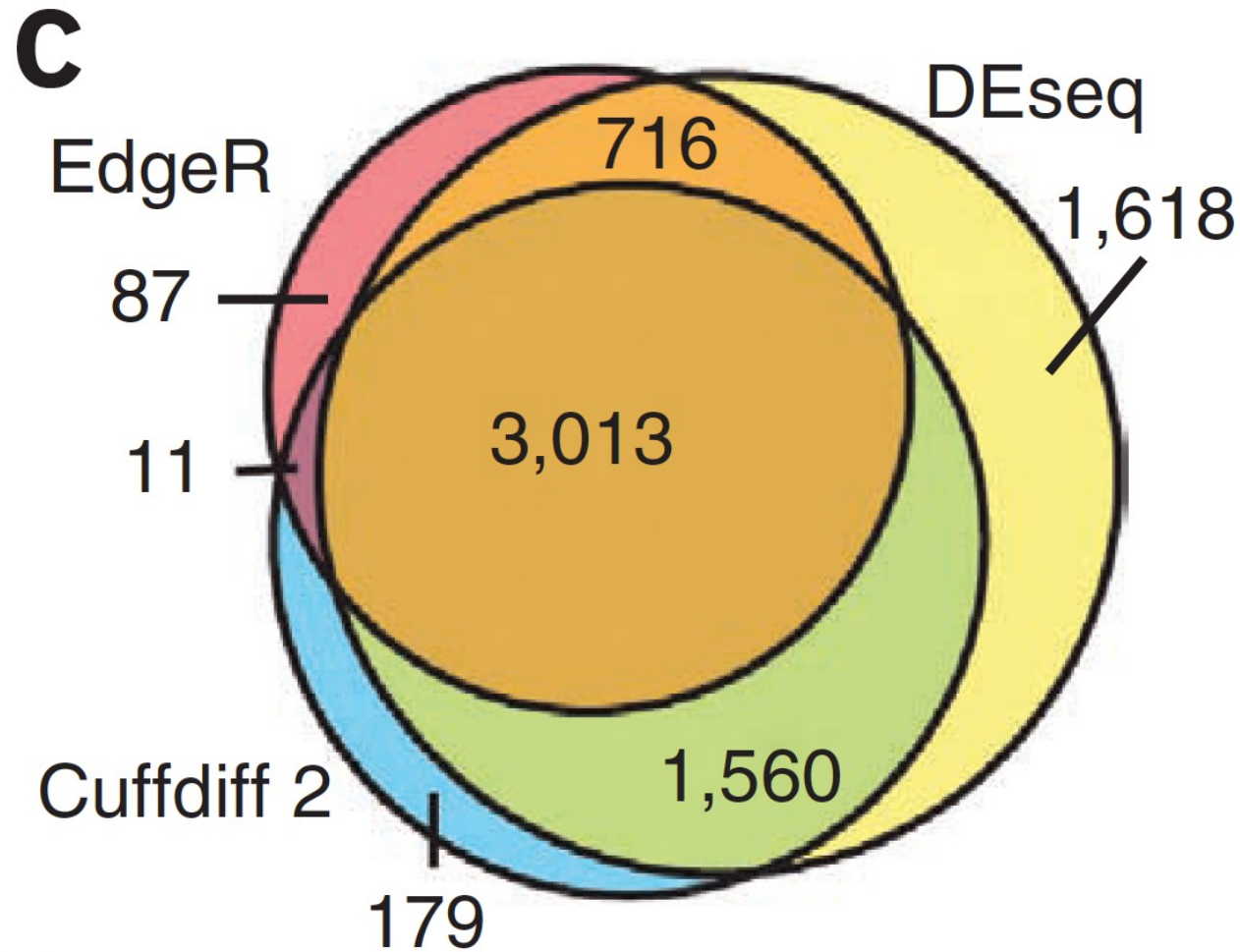
# Alternative differential expression methods

- Raw count approaches
  - DESeq2 - <http://www-huber.embl.de/users/anders/DESeq/>
  - edgeR - <http://www.bioconductor.org/packages/release/bioc/html/edgeR.html>
  - Others...

# ‘FPKM/TPM’ expression estimates vs. ‘raw’ counts

- Which should I use?
  - Long running debate, but the general consensus:
- FPKM/TPM
  - When you want to leverage benefits of tuxedo suite
    - Isoform deconvolution
  - Good for visualization (e.g., heatmaps)
  - Calculating fold changes, etc.
- Counts
  - “More robust” statistical methods for differential expression
    - Stringtie/Balgon approach is also robust
  - Accommodates more sophisticated experimental designs with appropriate statistical tests

# Multiple approaches advisable



# Lessons learned from microarray days

- Hansen et al. “Sequencing Technology Does Not Eliminate Biological Variability.” Nature Biotechnology 29, no. 7 (2011): 572–573.
- Power analysis for RNA-seq experiments
  - <http://scotty.genetics.utah.edu/>
- RNA-seq need for biological replicates
  - <http://www.biostars.org/p/1161/>
- RNA-seq study design
  - <http://www.biostars.org/p/68885/>



# Multiple testing correction

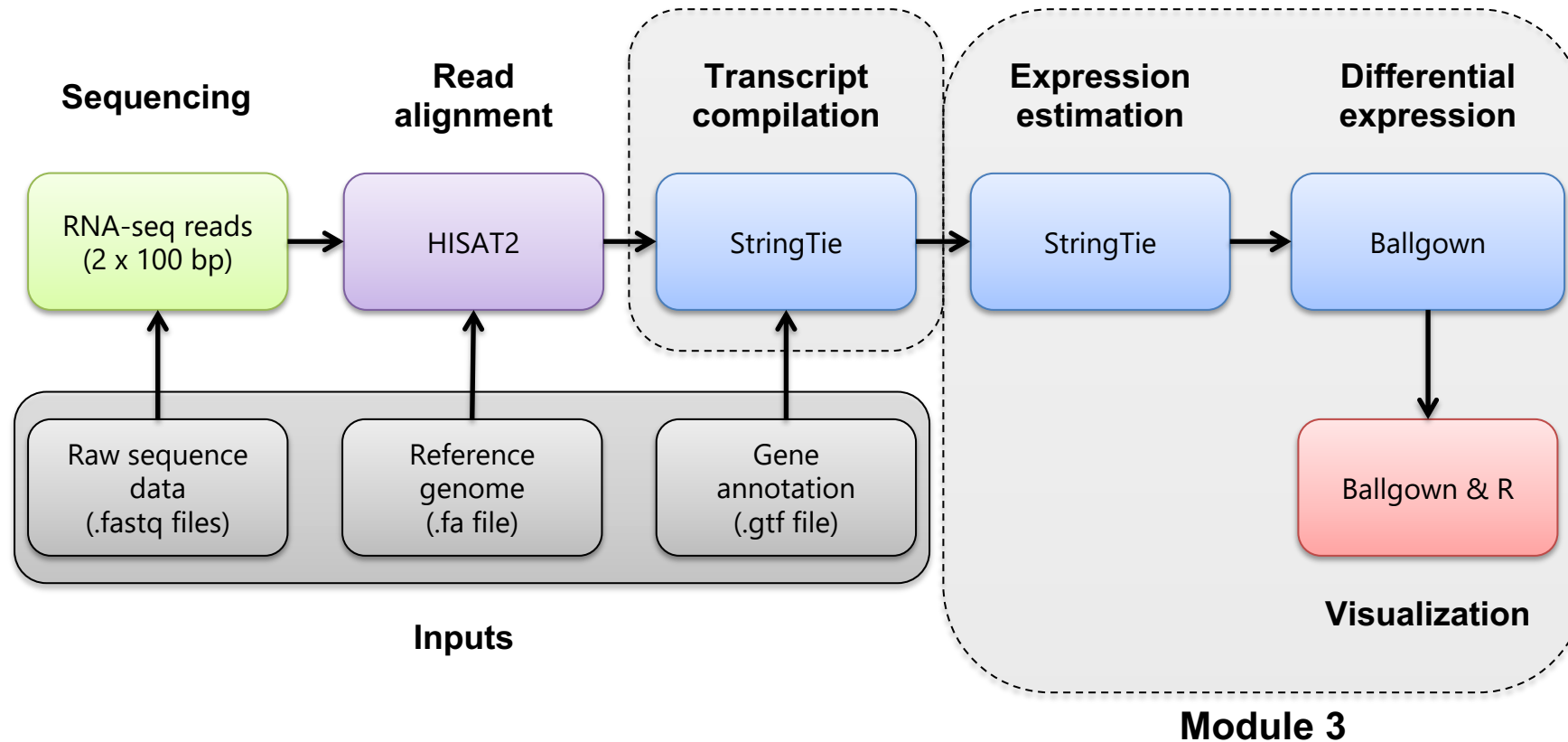
- As more attributes are compared, differences due solely to chance become more likely!
- Well known from array studies
  - 10,000s genes/transcripts
  - 100,000s exons
- With RNA-seq, more of a problem than ever
  - All the complexity of the transcriptome gives huge numbers of potential features
    - Genes, transcripts, exons, junctions, retained introns, microRNAs, lncRNAs, etc
- Bioconductor multtest
  - <http://www.bioconductor.org/packages/release/bioc/html/multtest.html>

# Downstream interpretation of expression analysis

- Topic for an entire course
- Expression estimates and differential expression lists from StringTie, Ballgown or other alternatives can be fed into many analysis pipelines
- See supplemental R tutorial for how to format expression data and start manipulating in R
- Clustering/Heatmaps
  - Provided by Ballgown
  - For more customized analysis various R packages exist:
    - hclust, heatmap.2, plotrix, ggplot2, etc.
- Classification
  - For RNA-seq data we still rarely have sufficient sample size and clinical details but this is changing
    - Weka is a good learning tool
    - RandomForests R package (biostar tutorial being developed)
- Pathway analysis
  - GSEA, IPA, Cytoscape, many R/BioConductor packages:  
<http://www.bioconductor.org/help/search/index.html?q=pathway>

[https://genviz.org/module-04-expression/0004/01/01/Expression Profiling and Visualization/](https://genviz.org/module-04-expression/0004/01/01/Expression%20Profiling%20and%20Visualization/)

# HISAT2/StringTie/Ballgown RNA-seq Pipeline



We are on a Coffee Break & Networking  
Session