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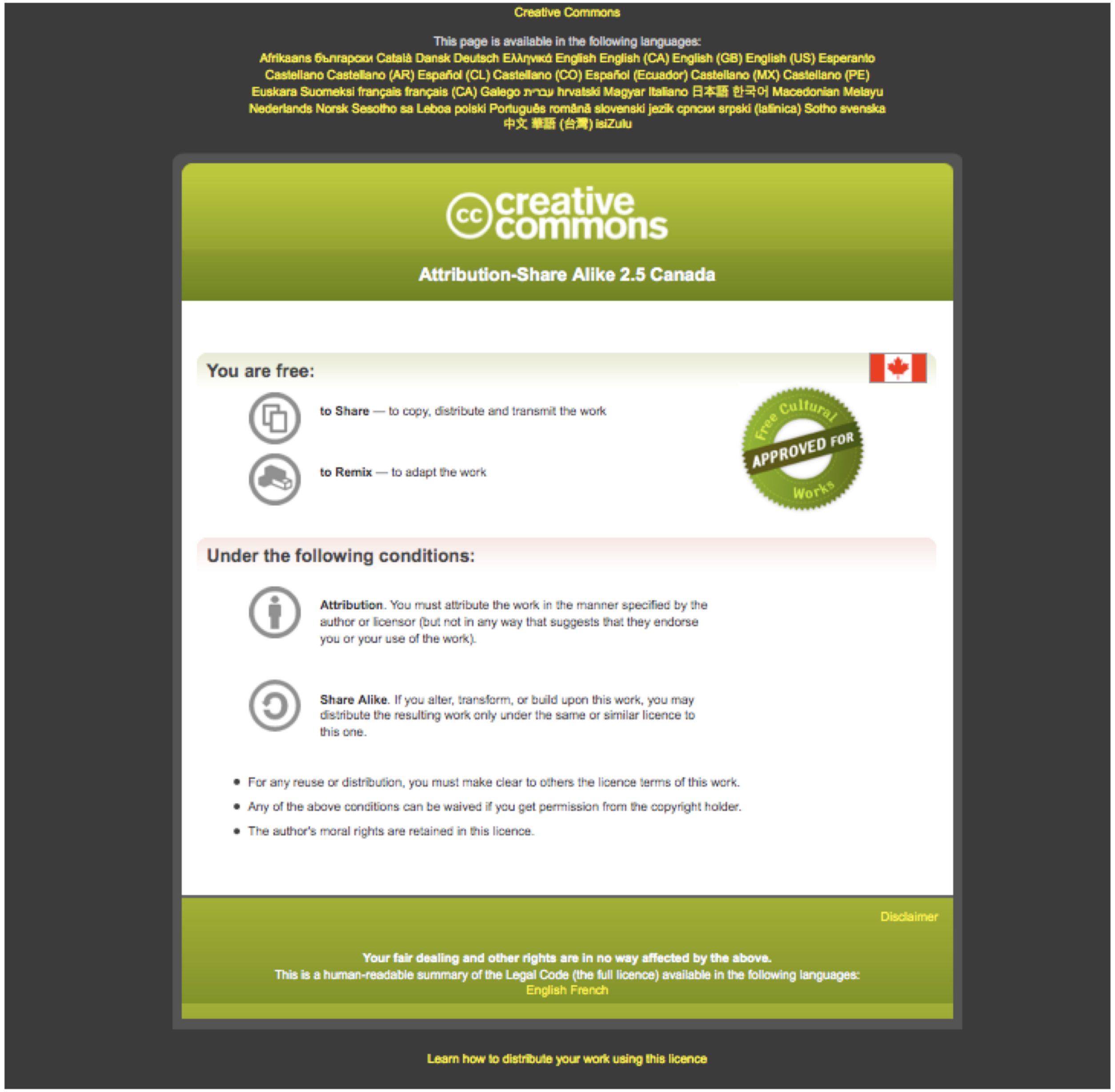

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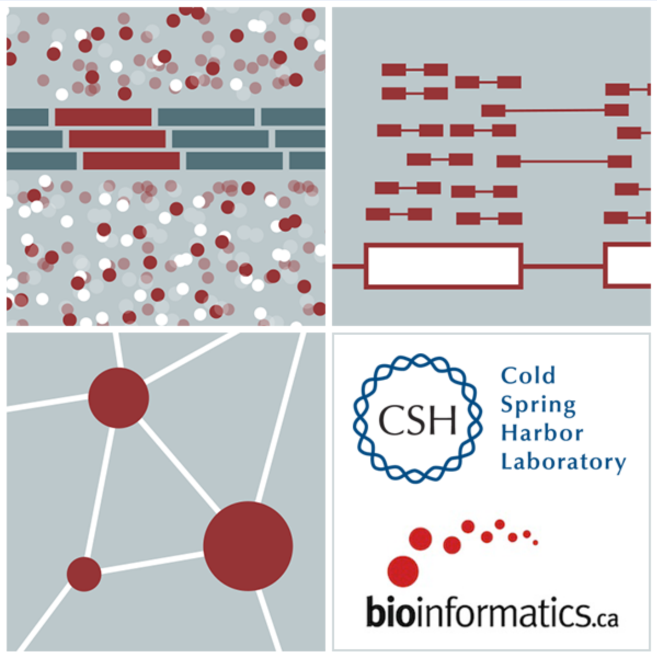
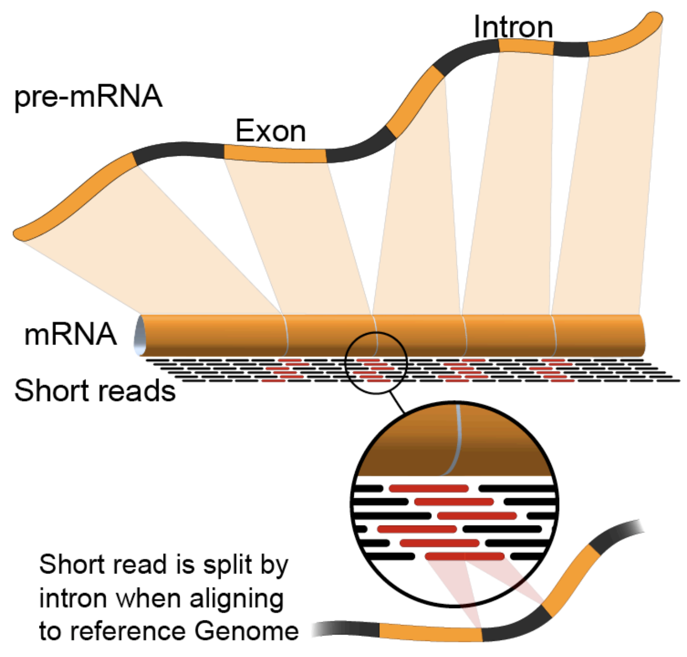
RNA-Seq Module 3: Differential Expression

Obi Griffith and Malachi Griffith

RNA-seq Analysis 2023. July 17-19, 2023

bioinformatics.ca




Intron
pre-mRNA
Exon
mRNA
Short reads
Short read is split by intron when aligning to reference Genome

Washington University in St. Louis

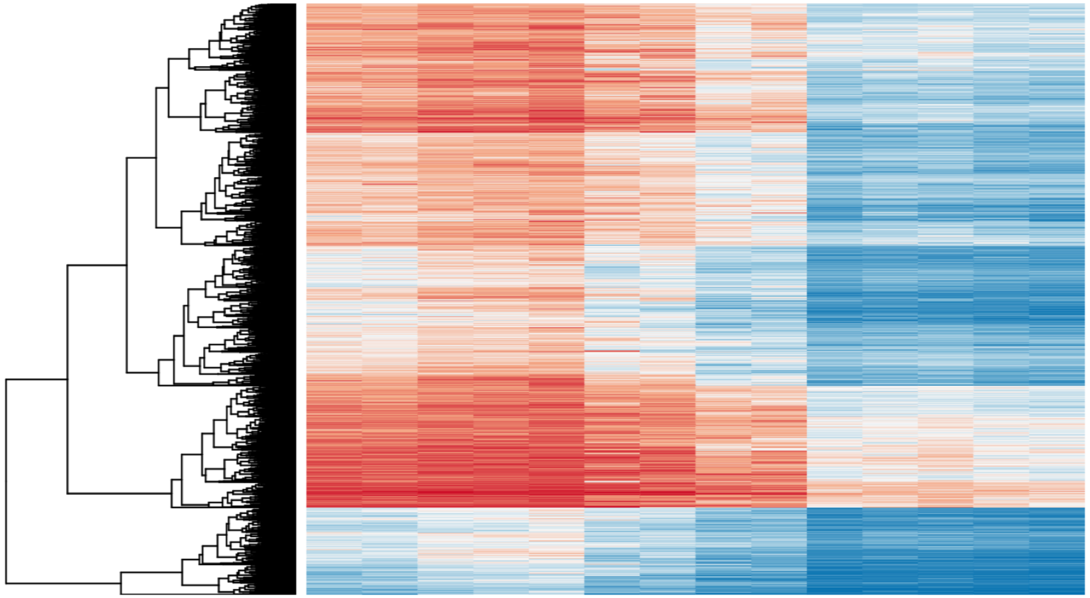
School of Medicine

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

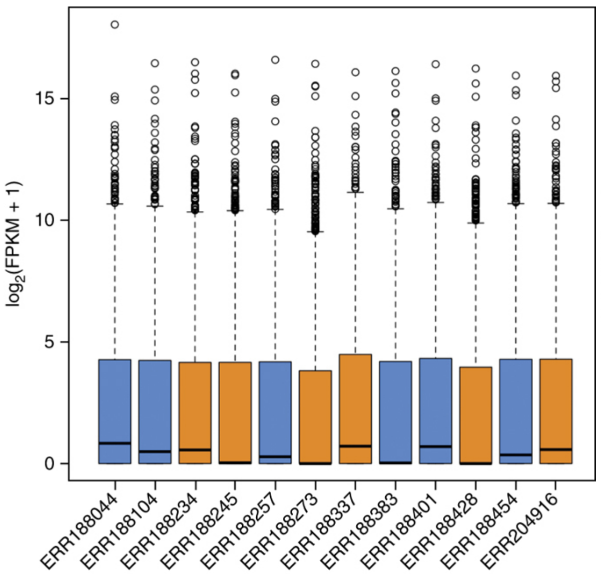
- Two models are fit to each feature, using expression as the outcome
  - one including the covariate of interest (e.g., case/control status or time) and one not including that covariate.

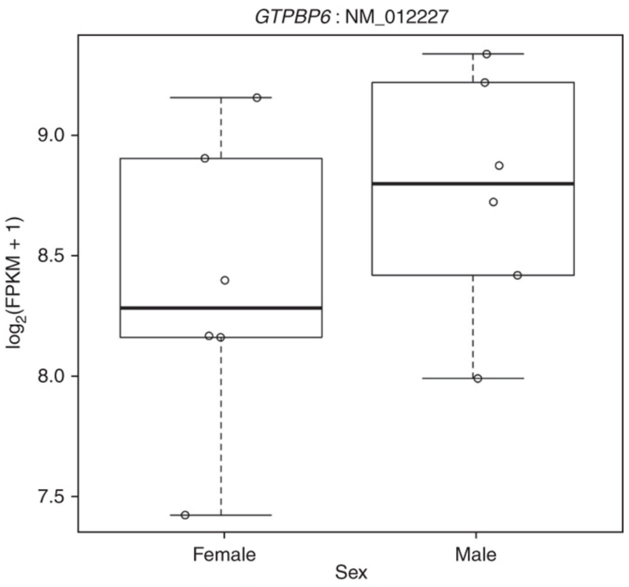
- An F statistic and p-value are calculated using the fits of the two models.
  - A significant p-value means the model including the covariate of interest fits significantly better than the model without that covariate, indicating differential expression.

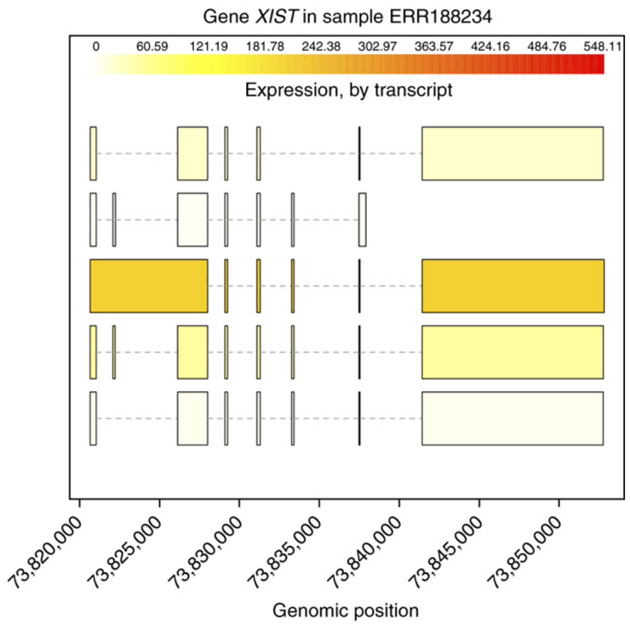
- We adjust for multiple testing by reporting q-values:
  - $q < 0.05$ the false discovery rate should be controlled at ~5%.

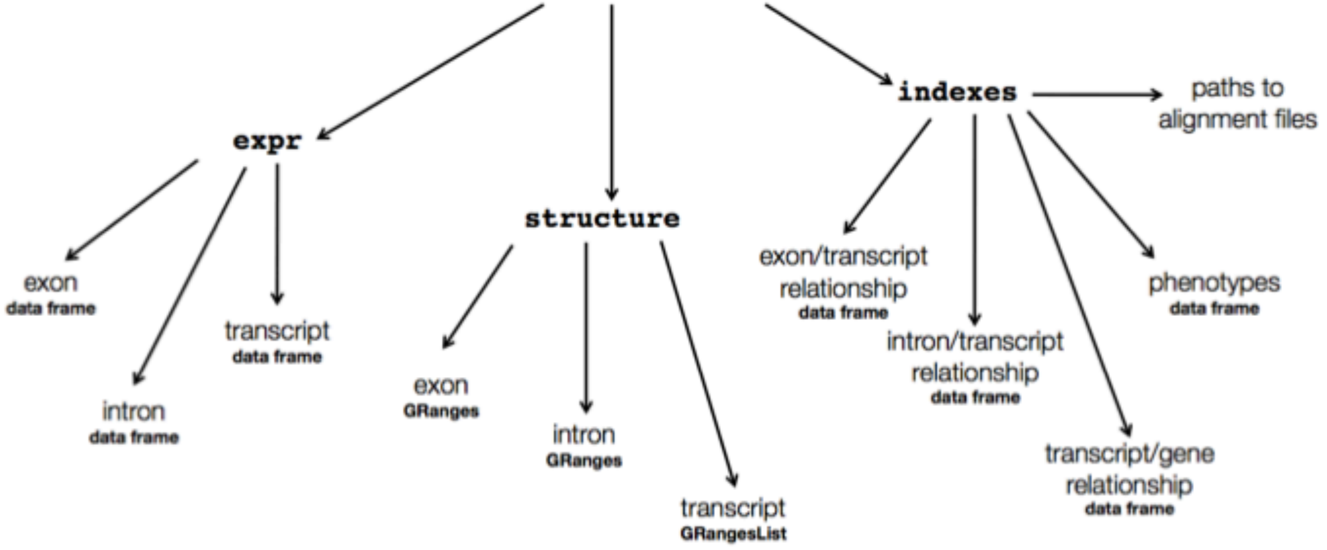
Frazee et al. (2014)

Ballgown for Visualization with R









ballgown object

expr

exon
data frame

intron
data frame

transcript
data frame

structure

exon
GRanges

intron
GRanges

transcript
GRangesList

indexes

paths to
alignment files

exon/transcript
relationship
data frame

intron/transcript
relationship
data frame

transcript/gene
relationship
data frame

phenotypes
data frame

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…

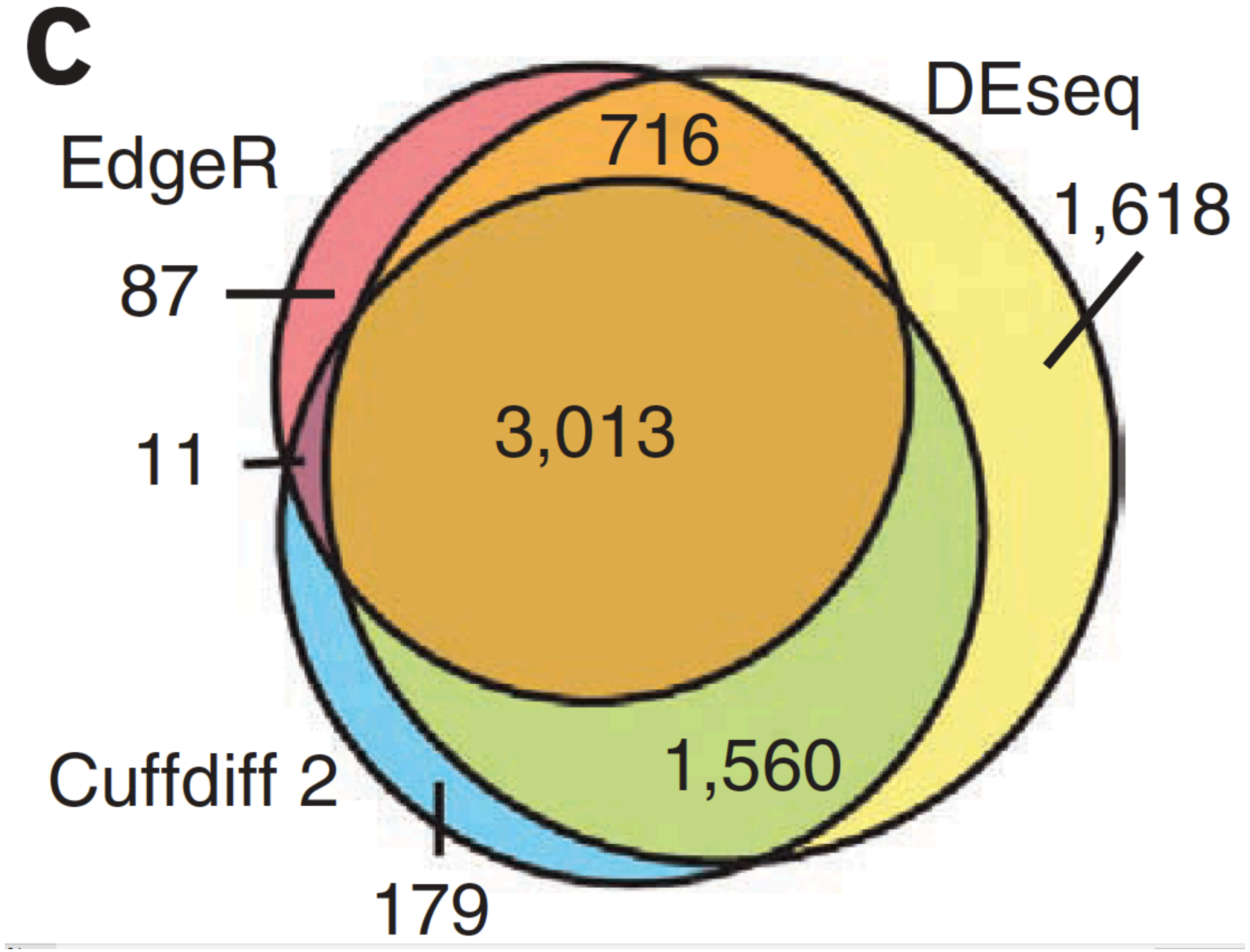
‘FPKM/TPM’ expression estimates vs. ‘raw’ counts

- 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
  - 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

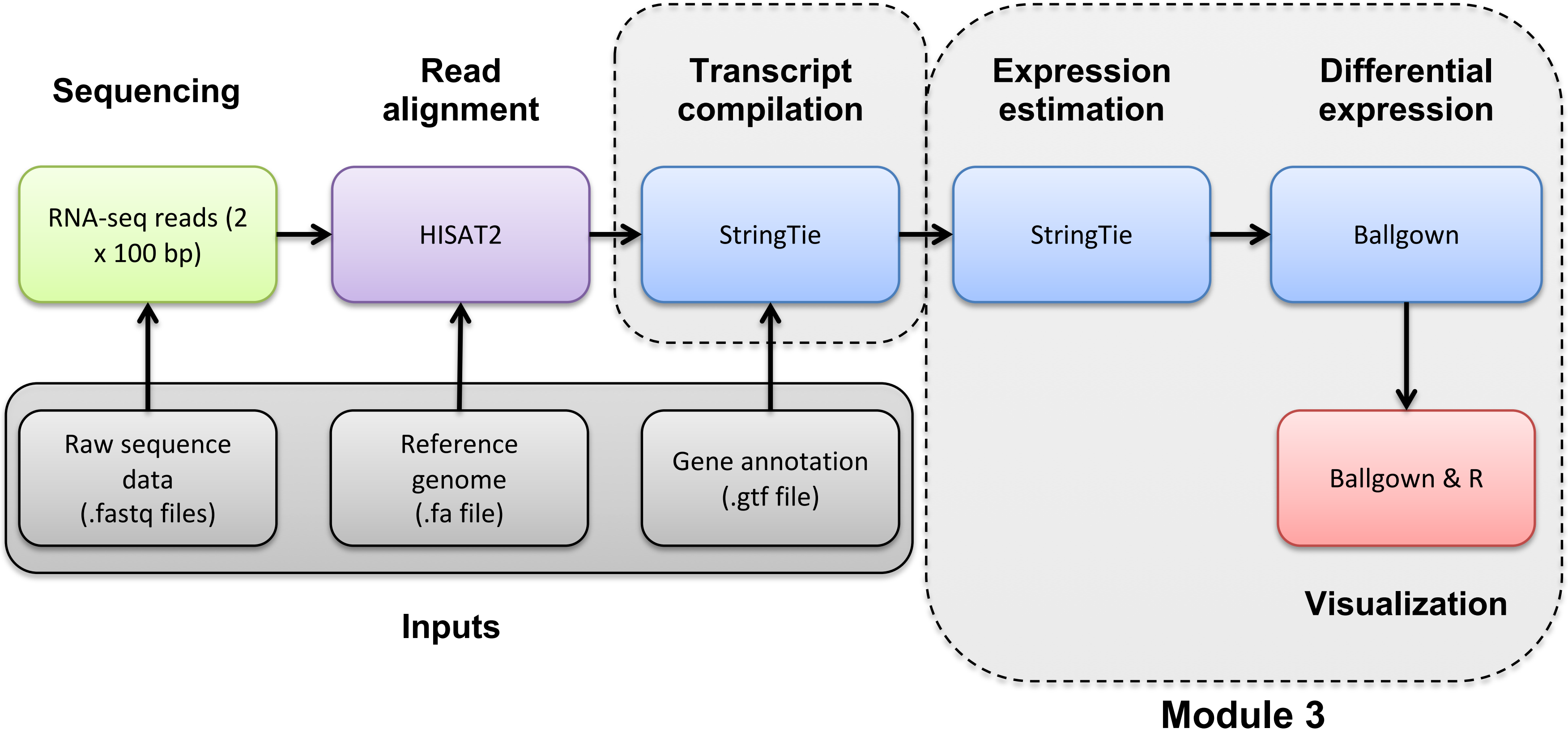
Downstream interpretation of expression analysis

- 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/

HISAT2/StringTie/Ballgown RNA-seq Pipeline



We are on a Coffee Break & Networking Session

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