Cold Spring Harbor Laboratory

Advanced Sequencing Technologies & Applications

http://meetings.cshl.edu/courses.html

Cold Spring Harbor Laboratory

RNA-Seq Module 3
Expression and Differential Expression (lecture)

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Advanced Sequencing Technologies & Applications

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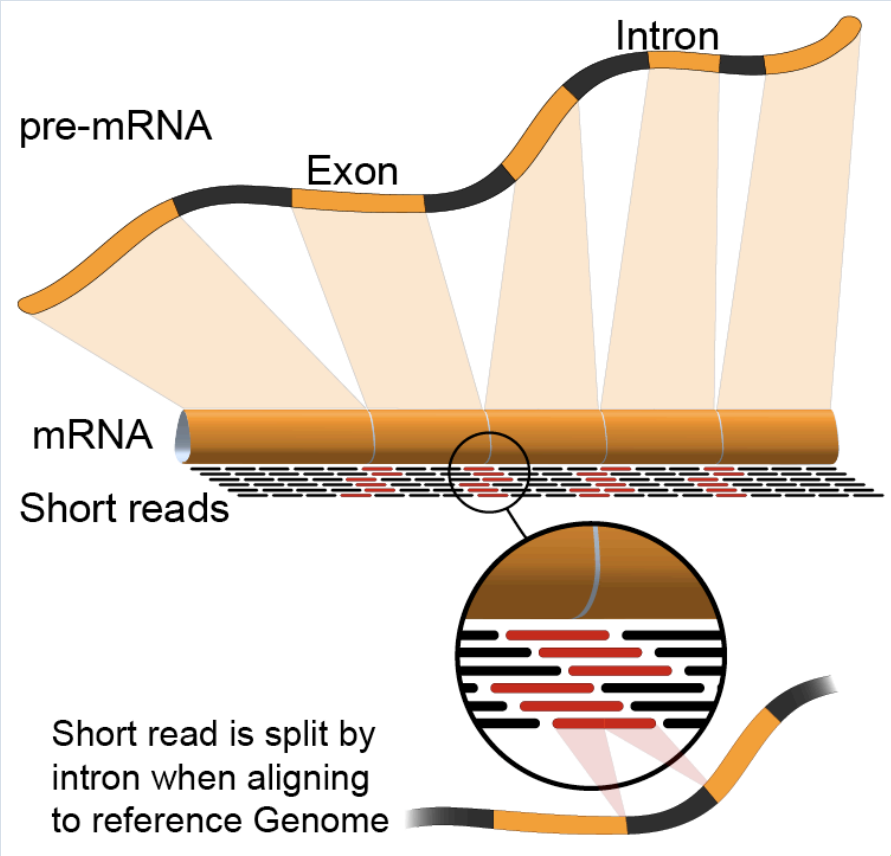


THE GENOME INSTITUTE at Washington University

Learning objectives of the course

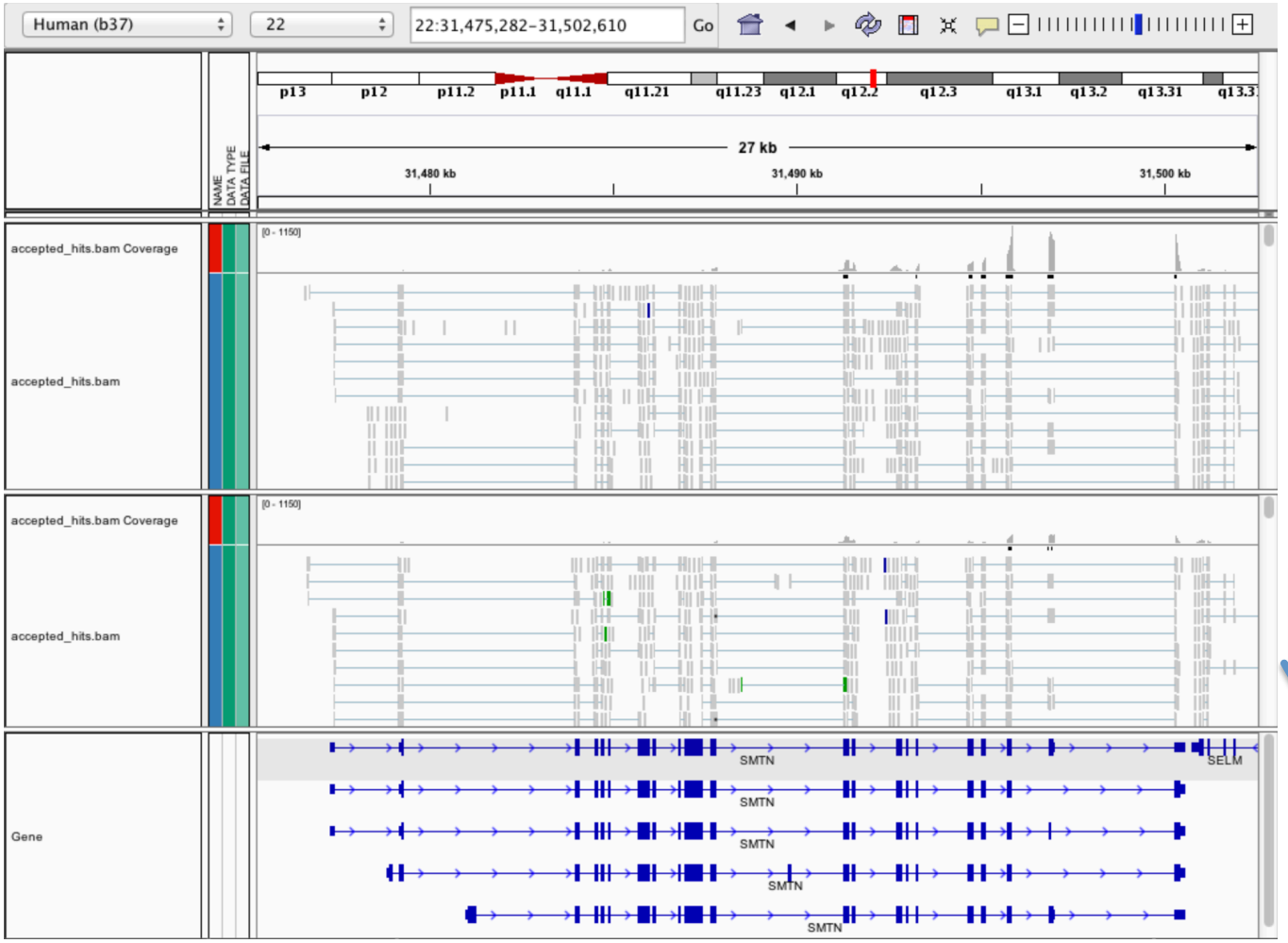
- Module 1: Introduction to RNA Sequencing
- Module 2: Alignment and Visualization
- **Module 3: Expression and Differential Expression**
- Module 4: Isoform Discovery and Alternative Expression

- Tutorials
  - Provide a working example of an RNA-seq analysis pipeline
  - Run in a ‘reasonable’ amount of time with modest computer resources
  - Self contained, self explanatory, portable

Learning Objectives of Module

- Expression estimation for known genes and transcripts
- 'FPKM' expression estimates vs. 'raw' counts
- Differential expression methods
- Downstream interpretation of expression and differential estimates
  - multiple testing, clustering, heatmaps, classification, pathway analysis, etc.

Expression estimation for known genes and transcripts



3' bias

Down-regulated

What is FPKM (RPKM)

- RPKM: Reads Per Kilobase of transcript per Million mapped reads.
- FPKM: Fragments Per Kilobase of transcript per Million mapped reads.
- In RNA-Seq, the relative expression of a transcript is proportional to the number of cDNA fragments that originate from it. However:
  - The number of fragments is also biased towards larger genes
  - The total number of fragments is related to total library depth
- FPKM (or RPKM) attempt to normalize for gene size and library depth

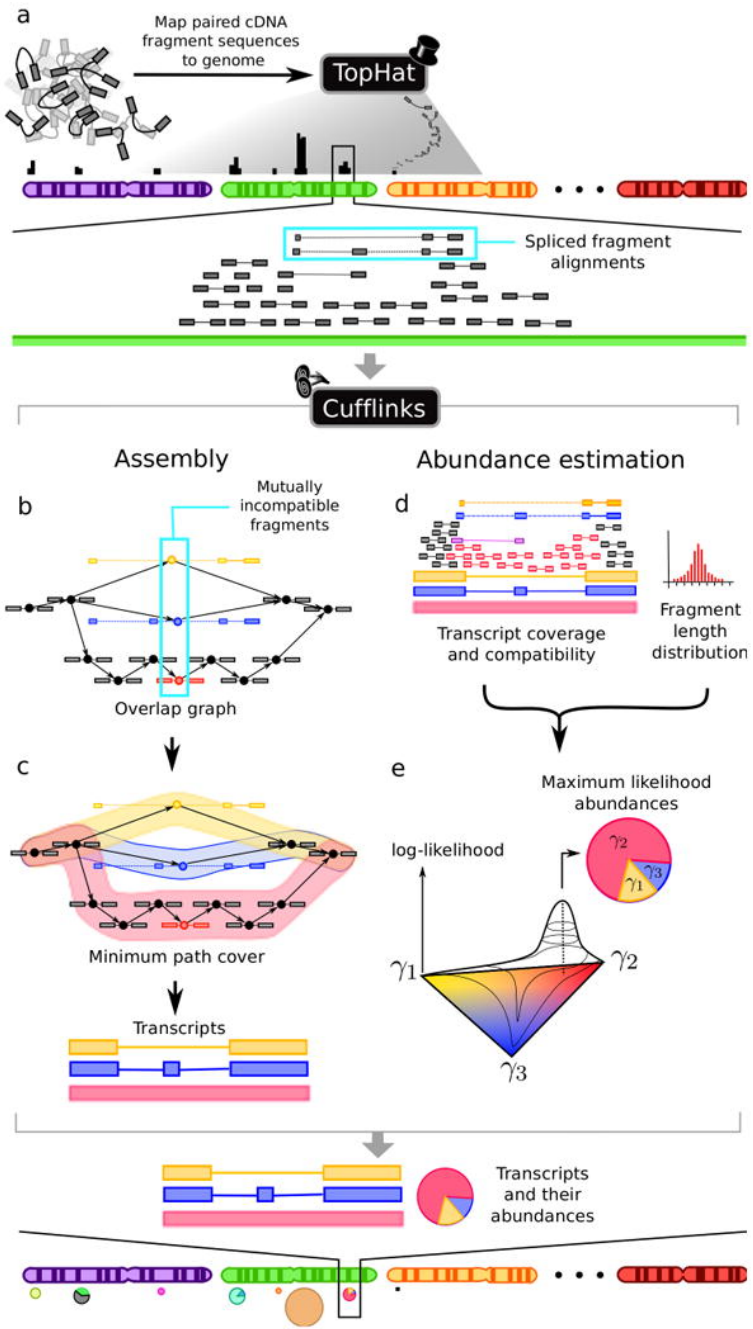
- RPKM (or FPKM) = (10^9 * C) / (N * L)
  - C = number of mappable reads/fragments for a gene/transcript/exon/etc
  - N = total number of mappable reads/fragments in the library
  - L = number of base pairs in the gene/transcript/exon/etc

- http://www.biostars.org/p/11378/
- http://www.biostars.org/p/68126/

How does cufflinks work?

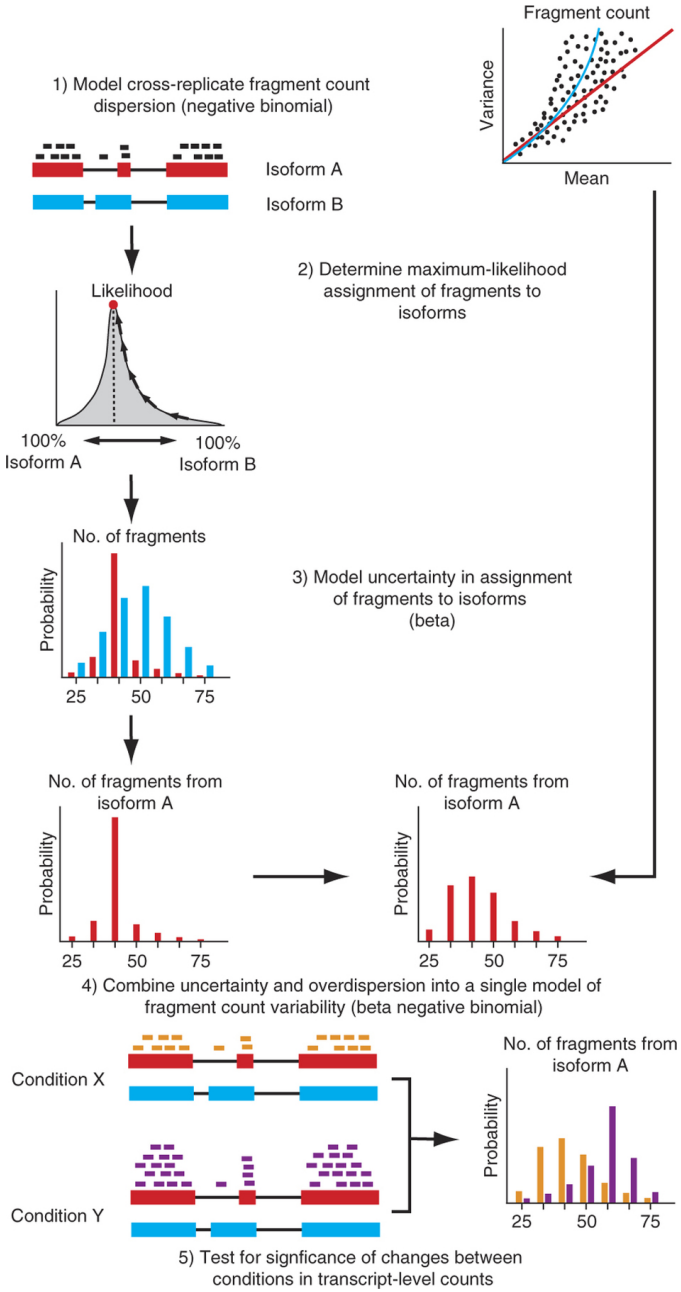
- Overlapping 'bundles' of fragment alignments are assembled, fragments are connected in an overlap graph, transcript isoforms are inferred from the minimum paths required to cover the graph
- Abundance of each isoform is estimated with a maximum likelihood probabilistic model
  - makes use of information such as fragment length distribution

http://cole-trapnell-lab.github.io/cufflinks/papers/



How does cuffdiff work?

- The variability in fragment count for each gene across replicates is modeled.
- The fragment count for each isoform is estimated in each replicate (as before), along with a measure of uncertainty in this estimate arising from ambiguously mapped reads
  - transcripts with more shared exons and few uniquely assigned fragments will have greater uncertainty
- The algorithm combines estimates of uncertainty and cross-replicate variability under a beta negative binomial model of fragment count variability to estimate count variances for each transcript in each library
- These variance estimates are used during statistical testing to report significantly differentially expressed genes and transcripts.



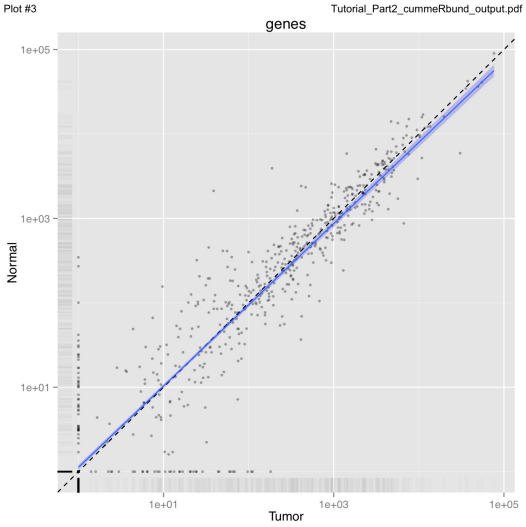
Why is cuffmerge necessary?

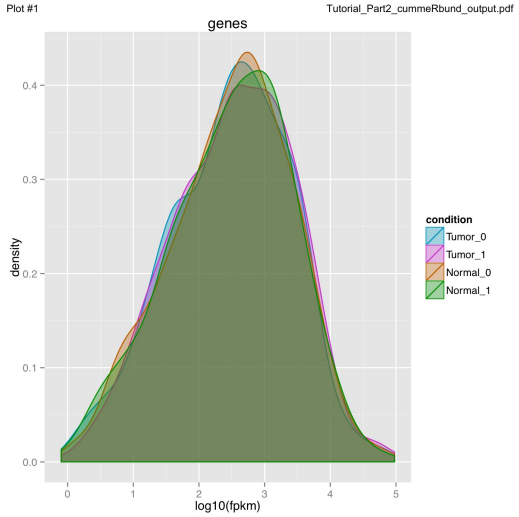
- Cuffmerge
  - Allows merge of several Cufflinks assemblies together
    - Necessary because even with replicates cufflinks will not necessarily assemble the same numbers and structures of transcripts
  - Filters a number of transfrags that are probably artifacts.
  - Optional: provide reference GTF to merge novel isoforms and known isoforms and maximize overall assembly quality.
  - Make an assembly GTF file suitable for use with Cuffdiff
    - Compare apples to apples

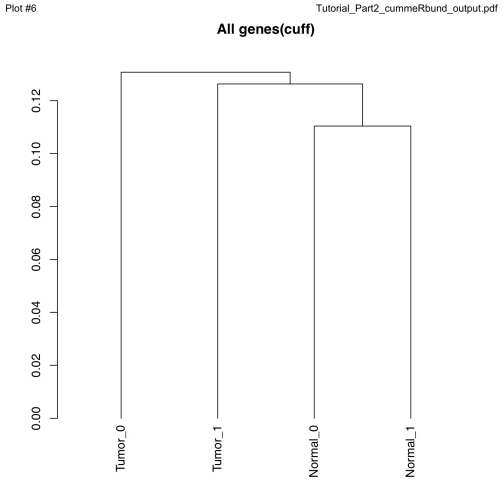
What do we get from cummeRbund?

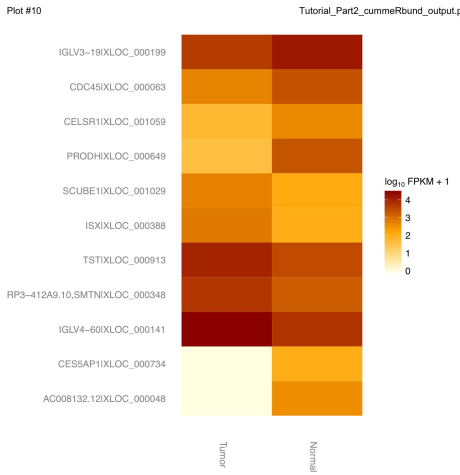
- Automatically generates many of the commonly used data visualizations
- Distribution plots
- Overall correlations plots
- MA plots
- Volcano plots
- Clustering, PCA and MDS plots to assess global relationships between conditions
- Heatmaps
- Gene/transcript-level plots showing transcript structures and expression levels

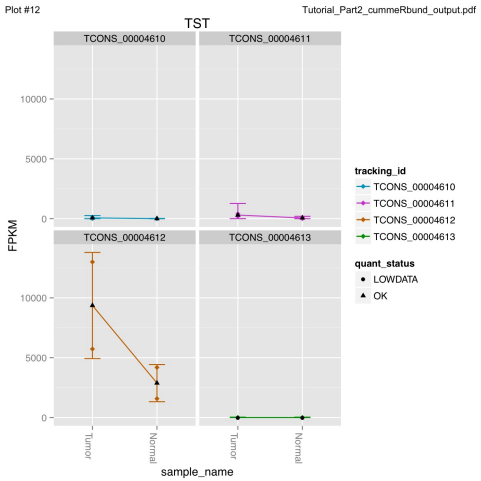
What do we get from cummeRbund?

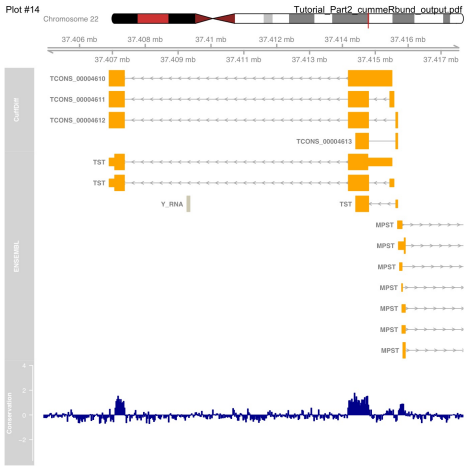












Alternatives to FPKM

- Raw read counts as an alternate for differential expression analysis
  - Instead of calculating FPKM, simply assign reads/fragments to a defined set of genes/transcripts and determine "raw counts"
    - Transcript structures could still be defined by something like cufflinks
- HTSeq (htseq-count)
  - http://www-huber.embl.de/users/anders/HTSeq/doc/count.html
  - htseq-count --mode intersection-strict --stranded no --minaqual 1 --type exon --idattr transcript_id accepted_hits.sam chr22.gff > transcript_read_counts_table.tsv
  - Important caveat of 'transcript' analysis by htseq-count:
    - http://seqanswers.com/forums/showthread.php?t=18068

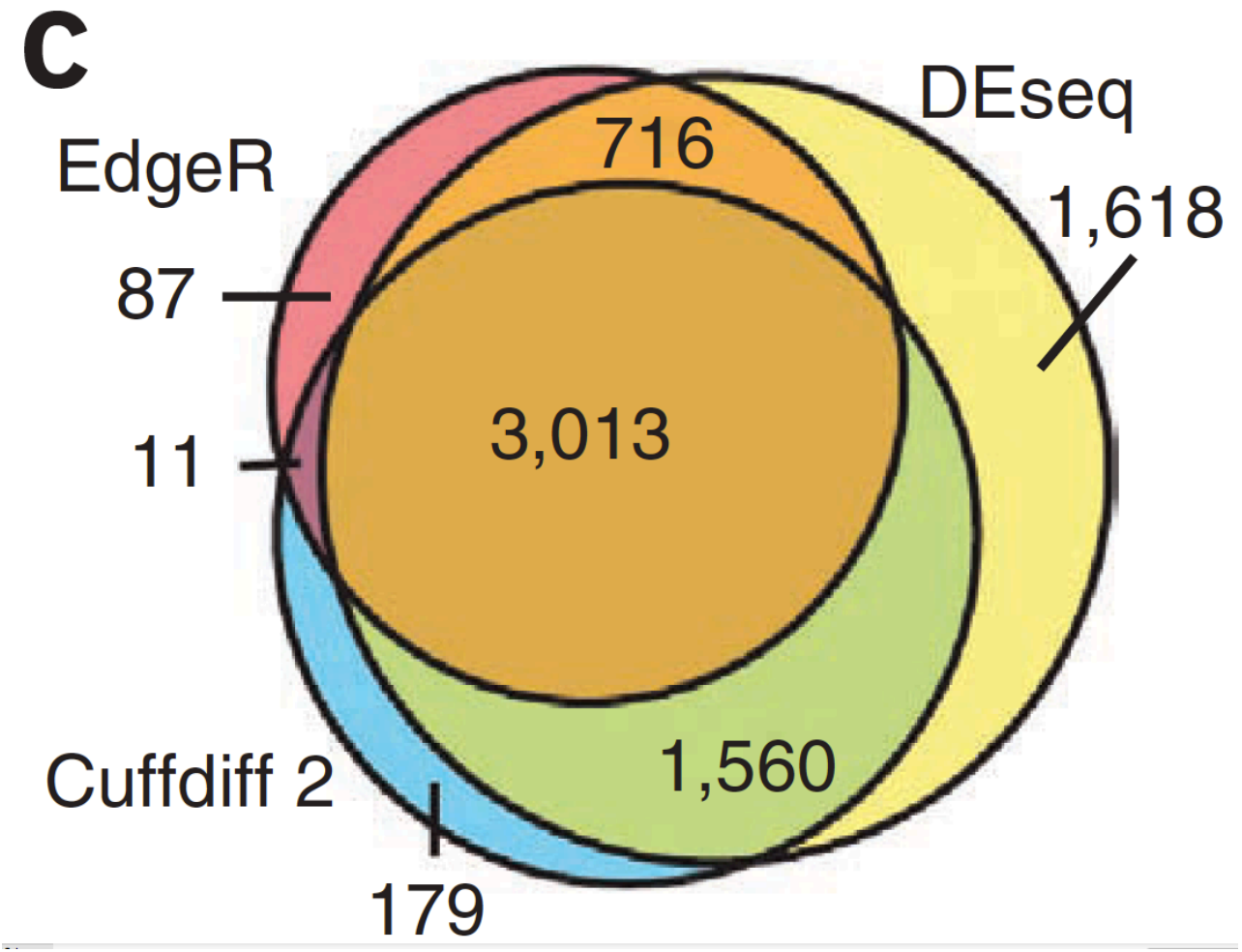
‘FPKM’ expression estimates vs. ‘raw’ counts

- Which should I use?
- FPKM
  - When you want to leverage benefits of tuxedo suite
  - 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

Alternative differential expression methods

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

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://euler.bc.edu/marthlab/scotty/scotty.php
- 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

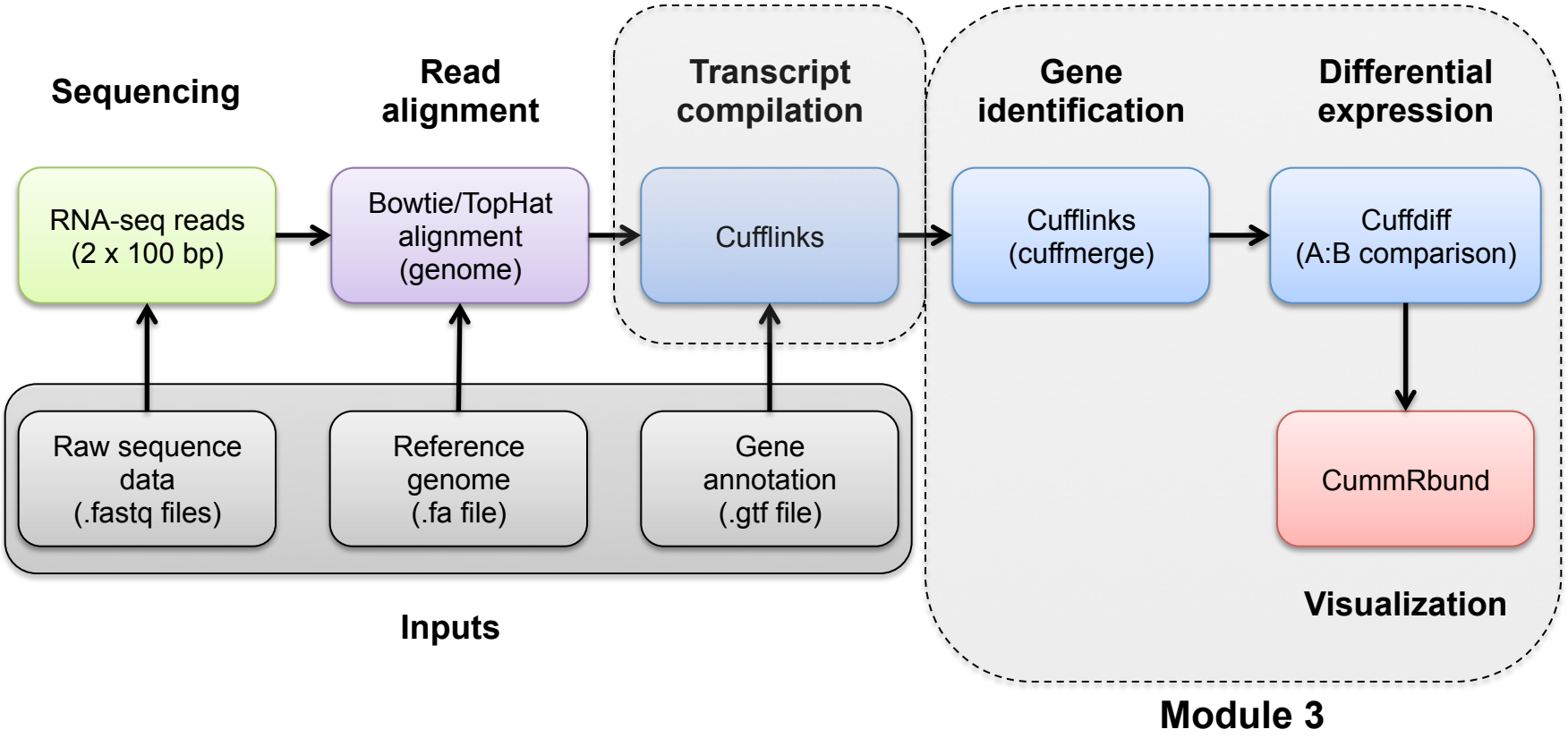
- As more attributes are compared, it becomes more likely that the treatment and control groups will appear to differ on at least one attribute by random chance alone.
- Well known from array studies
  - 10,000s genes/transcripts
  - 100,000s exons
- With RNA-seq, more of a problem than ever
  - All the complexity of the transcriptome
  - Almost infinite number 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 cufflinks/cuffdiff (or alternative) can be fed into many analysis pipelines
- See supplemental R tutorial for how to format cufflinks data and start manipulating in R
- Clustering/Heatmaps
  - Provided by cummeRbund
  - 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
  - IPA
  - Cytoscape
  - Many R/BioConductor packages: http://www.bioconductor.org/help/search/index.html?q=pathway

Introduction to tutorial (Module 3)

Bowtie/Tophat/Cufflinks/Cuffdiff RNA-seq Pipeline



Break