CBC Data Therapy

RNA-Seq Discussion
RNA-Seq versus Microarrays

- Correlation of fold change between arrays and RNAseq is similar to correlation between array platforms
- Technical replicates have less variation
- Extra analysis: prediction of alternative splicing, SNPs
- Low- and high-expressed genes do not match

**RNA-seq: An assessment of technical reproducibility and comparison with gene expression arrays**

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RNA-Seq workflow

Samples from two conditions

Isolate RNA

Generate cDNA

Create sequencing library by fragmenting, size selection and adding adaptors

Run sequencer

Generate short reads

Identify differentially expressed genes

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Designing the Study

• Final Goals?
  • Transcriptome assembly (characterization of the gene space)
  • Differential gene expression?
  • Identify rare isoforms?
  • Identify variants?

• Characteristics of the System?
  • Genome?
    • Quality of the reference
    • Availability of an annotation?
    • Introns?
  • Close relative? How close?
  • Other transcriptomic resources?
Designing the Study

• Experimental design
  • Biological replicates
  • Technical replicates
  • Minimize lane effects

• Appropriate Sequencing Technologies
  • HiSeq 3000/4000/TenX

• Read depth

• Barcoding (multiplex -> how much?)

• Read length

• Paired vs. single-end
Paired versus Single-End

**single-end**
- independent reads

**paired-end**
- two inwardly oriented reads separated by ~200 nt

**mate-paired**
- two outwardly oriented reads separated by ~3000 nt
Analysis Challenges

- Biases may mean what we are seeing is not reflective of true state of the transcriptome.
- Alternative splicing!
- Gene level, exon level?
- Multimapping, partial mapping, not mapping
- Normalization issues
  - Size (depth) of datasets
  - Gene length differences
One Gene: Two Isoforms: Opposite Functions

Example in Apoptosis:

For complete biological understanding, you need to know which isoforms are expressed.

Bcl-x

- Bcl-x_L: Inhibits Apoptosis
- Bcl-x_S: Activates Cell Death
Quality Control

- Quality Control:
  - FASTQ Files
    - FASTQC (Preview)
      - Barcodes and adaptors should be removed by Illumina Casava/BaseSpace
      - Examine lane effects, quality issues, library depth considerations
    - Trimmomatic or Sickle
      - Trim poor quality bases
      - Remove short reads
      - Identify proper pairs
Read Mapping or Assembly

• Read Mapping
  • Genome reference available?
    • Annotation available?
      • Bowtie2/TopHAT
      • HiSAT2
      • STAR
      • StringTie
  • No Genome Available
    • De novo transcriptome assembly
      • Reference Guided (StringTie)
      • No Reference or very fragmented (Trinity)
Read Mapping or Assembly

• Mapping to genome vs transcriptome?

• Is your reference the right version?
• Does your annotation match your reference?
Generating Raw Reads

• Generate an alignment file
  • SAM or BAM file format
    • Detailed information on how each read aligns to the genome
  • Interrogate file to convert to raw reads
    • Read counts across each gene (not normalized)
      • HTSeq
    • FPKM/RPKM -> TopHat/Cufflinks -> Full Solution for Differential Expression
• From de novo assembly
  • Non-splice aware aligner (Bowtie2 or BWA)
    • Convert to raw reads via Express
Differential Expression

- TopHat/Cufflinks
- Alternatives mostly exist in R
  - TopHat/Cufflinks also interfaces with Cummerbund for visualization
  - DESeq2 – more conservative – ideal for proper replication
  - EdgeR – slightly more permissive – similar normalization
  - G-FOLD - poor replication – preliminary view of DE candidates