Canadian Bioinformatics Workshops

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In collaboration with
Cold Spring Harbor Laboratory
&
New York Genome Center
Module 1
Introduction to RNA sequencing (lecture)

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High-throughput Biology: From Sequence to Networks
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Learning objectives of the course

- Module 0: Introduction to cloud computing
- **Module 1: Introduction to RNA sequencing**
- Module 2: RNA-seq 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

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Learning objectives of module 1

- Introduction to the theory and practice of RNA sequencing (RNA-seq) analysis
  - Rationale for sequencing RNA
  - Challenges specific to RNA-seq
  - General goals and themes of RNA-seq analysis work flows
  - Common technical questions related to RNA-seq analysis
  - Getting help outside of this course
  - Introduction to the RNA-seq hands on tutorial
RNA sequencing and analysis

Gene expression

RNA sequencing

Samples of interest

Isolate RNAs

Generate cDNA, Fragment, size select, add linkers

Sequence ends

Map to genome, transcriptome, and predicted exon junctions

Downstream analysis

100s of millions fp paired reads
10s of billions bases of sequence

RNA sequencing and analysis

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Why sequence RNA (versus DNA)?

• Functional studies
  – Genome may be constant but an experimental condition has a pronounced effect on gene expression
    • e.g. Drug treated vs. untreated cell line
    • e.g. Wild type versus knock out mice
• Predicting transcript sequence from genome sequence is difficult
  – Gene annotation is revolutionized by RNA-seq
• Some molecular features can only be observed at the RNA level
  – Alternative isoforms, fusion transcripts, RNA editing

Why sequence RNA (versus DNA)?

• Interpreting mutations that do not have an obvious effect on protein sequence
  – ‘Regulatory’ mutations that affect what mRNA isoform is expressed and how much
    • e.g. splice sites, promoters, exonic/intronic splicing motifs, etc.
• Prioritizing protein coding somatic mutations (often heterozygous)
  – If the gene is not expressed, a mutation in that gene would be less interesting
  – If the gene is expressed but only from the wild type allele, this might suggest loss-of-function (haploinsufficiency)
  – If the mutant allele itself is expressed, this might suggest a candidate drug target
Challenges

• Sample
  – Purity?, quantity?, quality?
• RNAs consist of small exons that may be separated by large introns
  – Mapping reads to genome is challenging
• The relative abundance of RNAs vary wildly
  – $10^5 - 10^7$ orders of magnitude
  – Since RNA sequencing works by random sampling, a small fraction of highly expressed genes may consume the majority of reads
  – Ribosomal and mitochondrial genes
• RNAs come in a wide range of sizes
  – Small RNAs must be captured separately
  – PolyA selection of large RNAs may result in 3’ end bias
• RNA is fragile compared to DNA (easily degraded)

Agilent example / interpretation

• https://github.com/griffithlab/rnaseq_tutorial/wiki/Resources/Agilent_Trace_Examples.pdf
• ‘RIN’ = RNA integrity number
  – 0 (bad) to 10 (good)

RIN = 6.0  RIN = 10
Design considerations

- Standards, Guidelines and Best Practices for RNA-seq
  - The ENCODE Consortium
  - Download from the Course Wiki
  - Meta data to supply, replicates, sequencing depth, control experiments, reporting standards, etc.


There are many RNA-seq library construction strategies

- Total RNA versus polyA+ RNA?
- Ribo-reduction?
- Size selection (before and/or after cDNA synthesis)
  - Small RNAs (microRNAs) vs. large RNAs?
  - A narrow fragment size distribution vs. a broad one?
- Linear amplification?
- Stranded vs. un-stranded libraries
- Exome captured vs. un-captured
- Library normalization?

- These details can affect analysis strategy
  - Especially comparisons between libraries
RNA sequencing and analysis

**Fragmentation and size selection**

- Gel electrophoresis of RNA
  - INTACT total RNA
  - Partially degraded total RNA
  - Heavily degraded total RNA
  - INTACT rRNA

- Capillary electrophoresis of total RNA
  - INTACT total RNA
  - Partially degraded total RNA
  - Completely degraded total RNA

Size selection or exclusion (e.g., PAGE, SPRI magnetic beads, etc.)

- Add sequencing adaptors

**Expected Alignments**

RNA-seq Strategy

- A. Total RNA
- B. rRNA Reduction
- C. PolyA Selection
- D. cDNA Capture

Legend:
- genomic DNA
- immature RNA
- mature RNA
- non-coding RNA
- ribosomal RNA
- paired end reads

**RNA sequence selection/depletion**

- Initial RNA pool
- Selection/depletion
- Resulting RNA pool

- Broad transcript representation
  - Abundant RNAs emphasized
  - High unprocessed RNA
  - High genomic DNA

- Limited transcript representation (targeted)
  - Abundant RNAs de-emphasized
  - Low unprocessed RNA
  - Low genomic DNA

- Limited transcript representation (polyA)
  - Abundant RNAs de-emphasized
  - Low unprocessed RNA
  - Low genomic DNA

**Expected Alignments**
**Replicates**

- Technical Replicate
  - Multiple instances of sequence generation
    - Flow Cells, Lanes, Indexes
- Biological Replicate
  - Multiple isolations of cells showing the same phenotype, stage or other experimental condition
  - Some example concerns/challenges:
    - Environmental Factors, Growth Conditions, Time
  - Correlation Coefficient 0.92-0.98
Common analysis goals of RNA-Seq analysis (what can you ask of the data?)

- Gene expression and differential expression
- Alternative expression analysis
- Transcript discovery and annotation
- Allele specific expression
  - Relating to SNPs or mutations
- Mutation discovery
- Fusion detection
- RNA editing

General themes of RNA-seq workflows

- Each type of RNA-seq analysis has distinct requirements and challenges but also a common theme:
  1. Obtain raw data (convert format)
  2. Align/assemble reads
  3. Process alignment with a tool specific to the goal
     - e.g. ‘cufflinks’ for expression analysis, ‘defuse’ for fusion detection, etc.
  4. Post process
     - Import into downstream software (R, Matlab, Cytoscape, Ingenuity, etc.)
  5. Summarize and visualize
     - Create gene lists, prioritize candidates for validation, etc.
Tool recommendations

• Alignment
  – BWA (PMID: 20080505)
    • Align to genome + junction database
  – Tophat (PMID: 19289445), STAR (PMID: 23104886), MapSplice (PMID: 20802226), hmmSplicer
    (PMID: 21079731)
    • Spliced alignment to genome

• Expression, differential expression alternative expression
  – Cufflinks/Cuffdiff (PMID: 20436464), ALEXA-seq (PMID: 20835245), RUM (PMID: 21775302)

• Fusion detection
  – Tophat-fusion (PMID: 21835007), ChimeraScan (PMID: 21840877), Defuse (PMID: 21625565), Comrad
    (PMID: 21478487)

• Transcript assembly
  – Trinity (PMID: 21572440), Oases (PMID: 22368243), Trans-ABySS (PMID: 20935650)

• Visit the ‘SeqAnswers’ or ‘BioStar’ forums for more recommendations and discussion
  – http://seqanswers.com/
  – http://www.biostars.org/

SeqAnswers exercise

• Go to:
  – http://seqanswers.com/

• Click the ‘Wiki’ link
  – http://seqanswers.com/wiki/SEQAnswers

• Visit the ‘Software Hub’

• Browse the software that has been added
  – http://seqanswers.com/wiki/Special:BrowseData

• Use the tag cloud to identify tools related to your area of interest. e.g. RNA-seq alignment
Common questions: Should I remove duplicates for RNA-seq?

• Maybe... more complicated question than for DNA
• Concern.
  – Duplicates may correspond to biased PCR amplification of particular fragments
  – For highly expressed, short genes, duplicates are expected even if there is no amplification bias
  – Removing them may reduce the dynamic range of expression estimates
• Assess library complexity and decide...
• If you do remove them, assess duplicates at the level of paired-end reads (fragments) not single end reads

Common questions: How much library depth is needed for RNA-seq?

• Depends on a number of factors:
  – Question being asked of the data. Gene expression? Alternative expression? Mutation calling?
  – Tissue type, RNA preparation, quality of input RNA, library construction method, etc.
  – Sequencing type: read length, paired vs. unpaired, etc.
  – Computational approach and resources
• Identify publications with similar goals
• Pilot experiment
• Good news: 1-2 lanes of recent Illumina HiSeq data should be enough for most purposes
Common questions: What mapping strategy should I use for RNA-seq?

• Depends on read length
• < 50 bp reads
  – Use aligner like BWA and a genome + junction database
  – Junction database needs to be tailored to read length
    • Or you can use a standard junction database for all read lengths and an aligner that allows substring alignments for the junctions only (e.g. BLAST ... slow).
  – Assembly strategy may also work (e.g. Trans-ABySS)
• > 50 bp reads
  – Spliced aligner such as Bowtie/TopHat

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Visualization of spliced alignment of RNA-seq data

Normal WGS

Tumor WGS

Tumor RNA-seq

Acceptor site mutation

IGV screenshot
Common questions: how reliable are expression predictions from RNA-seq?

- Are novel exon-exon junctions real?
  - What proportion validate by RT-PCR and Sanger sequencing?
- Are differential/alternative expression changes observed between tissues accurate?
  - How well do DE values correlate with qPCR?
- 384 validations
  - qPCR, RT-PCR, Sanger sequencing
- See ALEXA-Seq publication for details:
  - Also includes comparison to microarrays

Validation (qualitative)

33 of 192 assays shown. Overall validation rate = 85%
Validation (quantitative)

qPCR of 192 exons identified as alternatively expressed by ALEXA-Seq

Validation rate = 88%

Common questions: What if I don’t have a reference genome for my species?

• Have you considered sequencing the genome of your species?

• If that is not practical or you simply prefer a transcript discovery approach that does not rely on prior knowledge of the genome or transcriptome there are some tools available ...
  – Unfortunately de novo transcriptome assembly is beyond the scope of this workshop
  – The good news is that the skills you learn here will help you figure out how to install and run those tools yourself
BioStar exercise

• Go to the BioStar website:
  – http://www.biostars.org/
  – If you do not already have an OpenID (e.g. Google, Yahoo, etc.)
  – Login -> ‘get one’
• Login and set up your user profile
• Tasks:
  – Find a question that seems useful and ‘vote it up’
  – Answer a question [optional]
  – Search for a topic area of interest and ask a question that has not already been asked [optional]
Bowtie/Tophat/Cufflinks/Cuffdiff
RNA-seq Pipeline

**Inputs**
- RNA-seq reads (2 x 100 bp)
- Raw sequence data (.fastq files)
- Reference genome (.fa file)
- Gene annotation (.gtf file)

**Sequencing**
- Bowtie/Tophat alignment (genome)

**Read alignment**
- Cufflinks

**Transcript compilation**
- Cufflinks (cuffmerge)

**Gene identification**
- Cuffdiff (A:B comparison)

**Differential expression**
- CummRbund

**Visualization**

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**Module 2**

**RNA sequencing and analysis**

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**We are on a Coffee Break & Networking Session**

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