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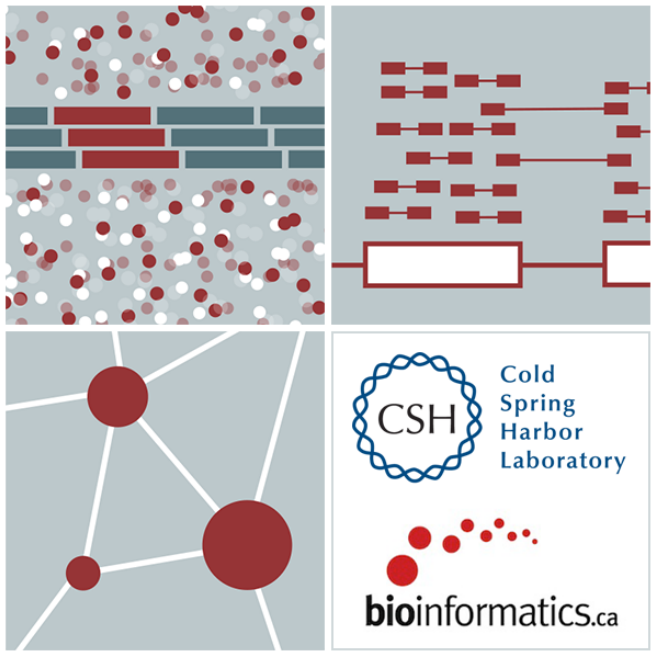
Introduction to RNA sequencing

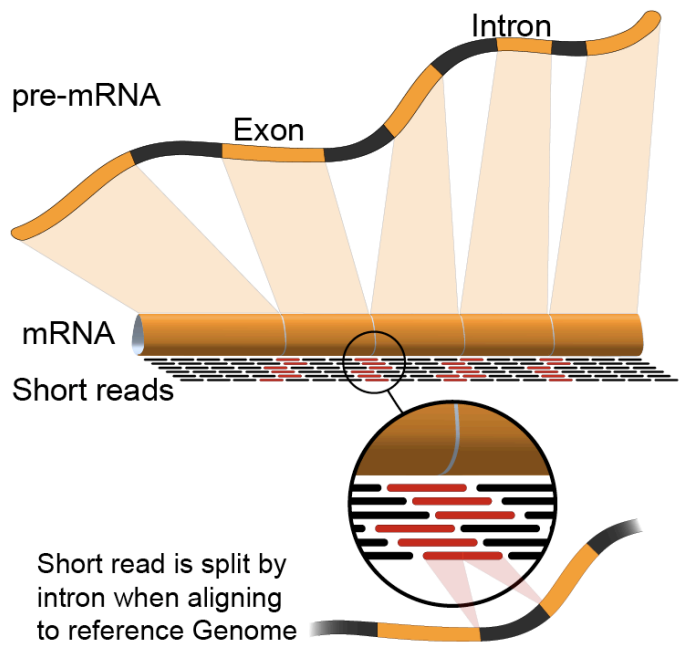
Kelsy Cotto, Obi Griffith, Malachi Griffith, Saad Khan, Allegra Petti, Huiming Xia

Informatics for RNA-Seq Analysis

June 17-19, 2020

bioinformatics.ca





Washington University in St. Louis
School of Medicine

Learning objectives of the course

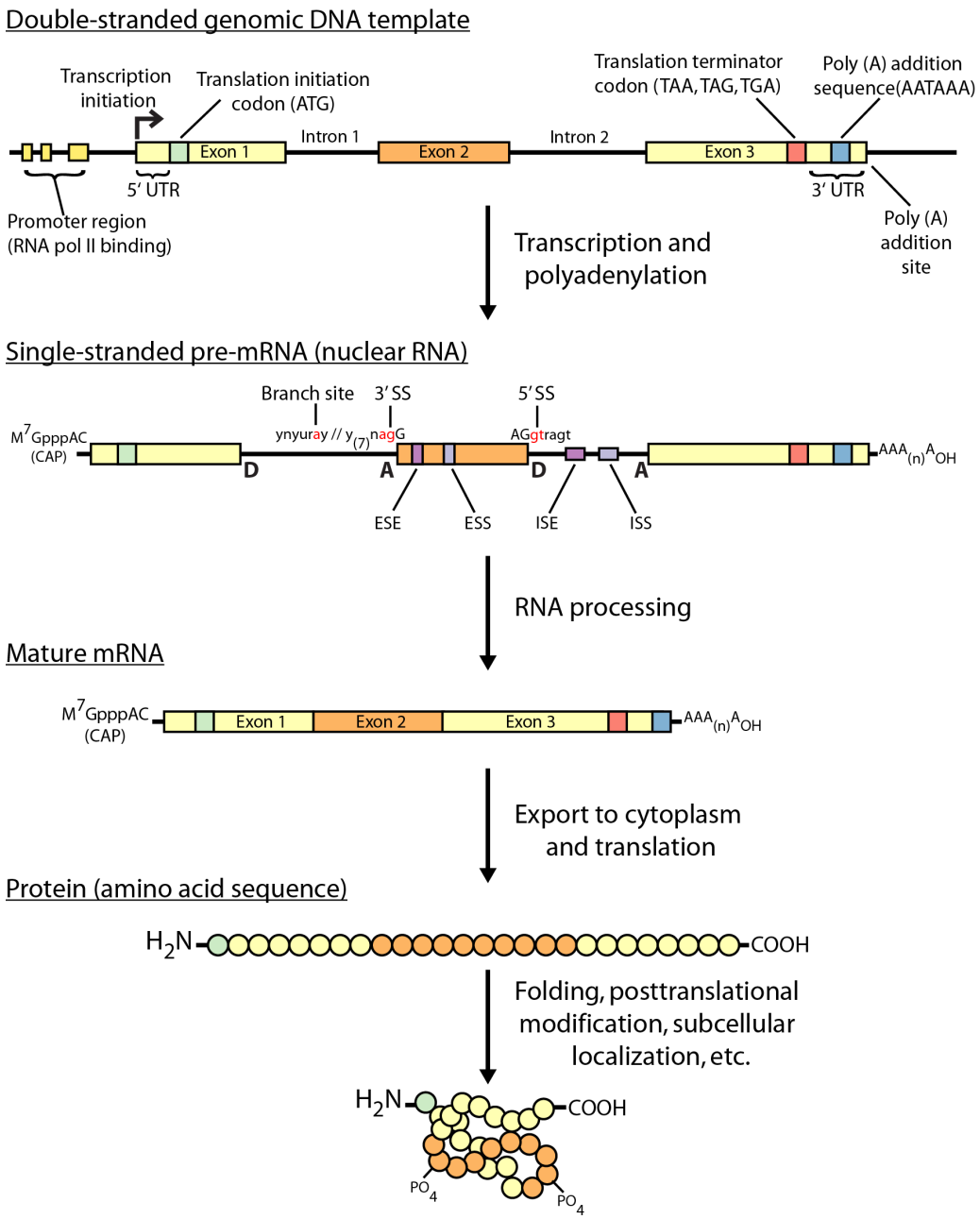
- **Module 1: Introduction to RNA Sequencing**
- Module 2: Alignment and Visualization
- Module 3: Expression and Differential Expression
- Module 4: Alignment Free Expression Estimation
- Module 5: Single Cell RNA-Seq

- 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

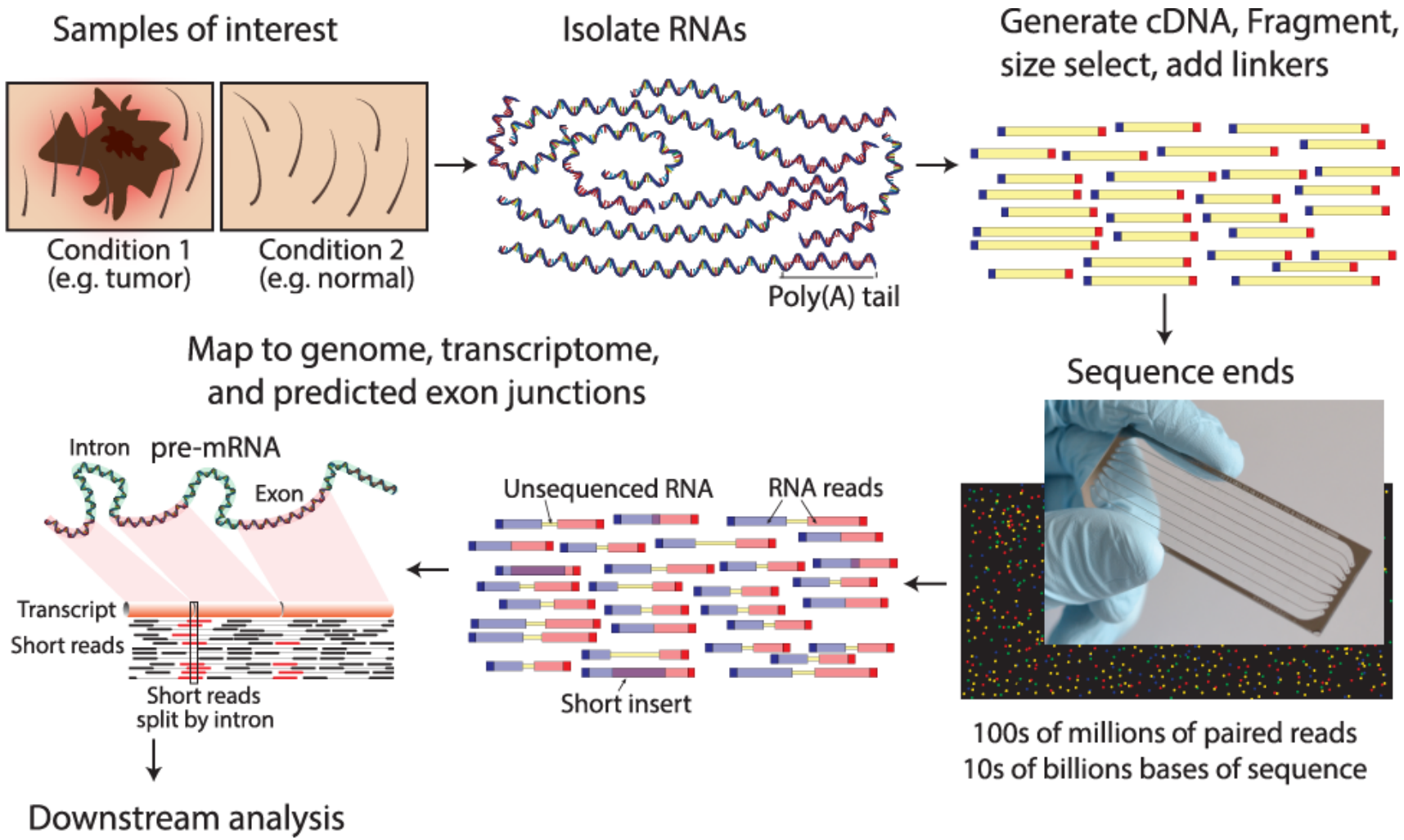
Learning objectives of module 1

- 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
  - Introduction to the RNA-seq hands on tutorial

Gene expression



RNA sequencing



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

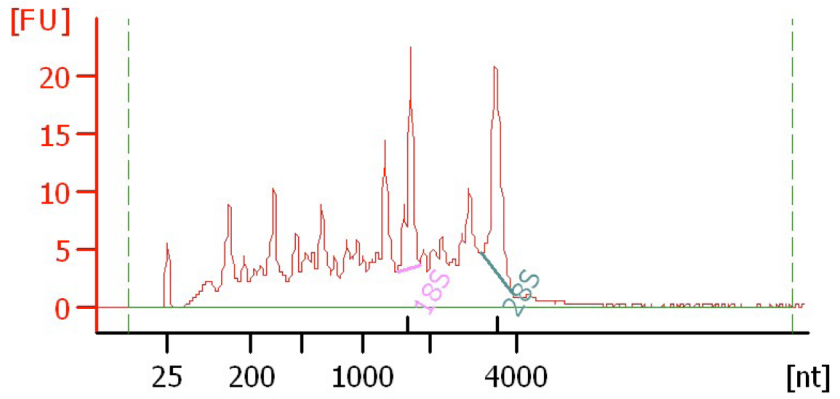
- Prioritizing protein coding somatic mutations (often heterozygous)
  - If the gene is not expressed, a mutation in that gene would be less interesting
  - If the gene is expressed but only from the wild type allele, this might suggest loss-of-function (haploinsufficiency)
  - If the mutant allele itself is expressed, this might suggest a candidate drug target

Challenges

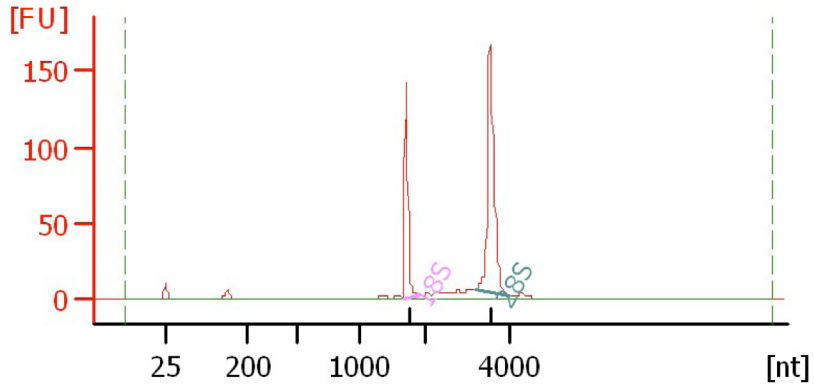
- Sample
  - Purity?, quantity?, quality?
- RNAs consist of small exons that may be separated by large introns
  - Mapping reads to genome is challenging
- The relative abundance of RNAs vary wildly
  - 10^5 – 10^7 orders of magnitude
  - Since RNA sequencing works by random sampling, a small fraction 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://goo.gl/uC5a3C
- ‘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.
- https://goo.gl/6LePBW

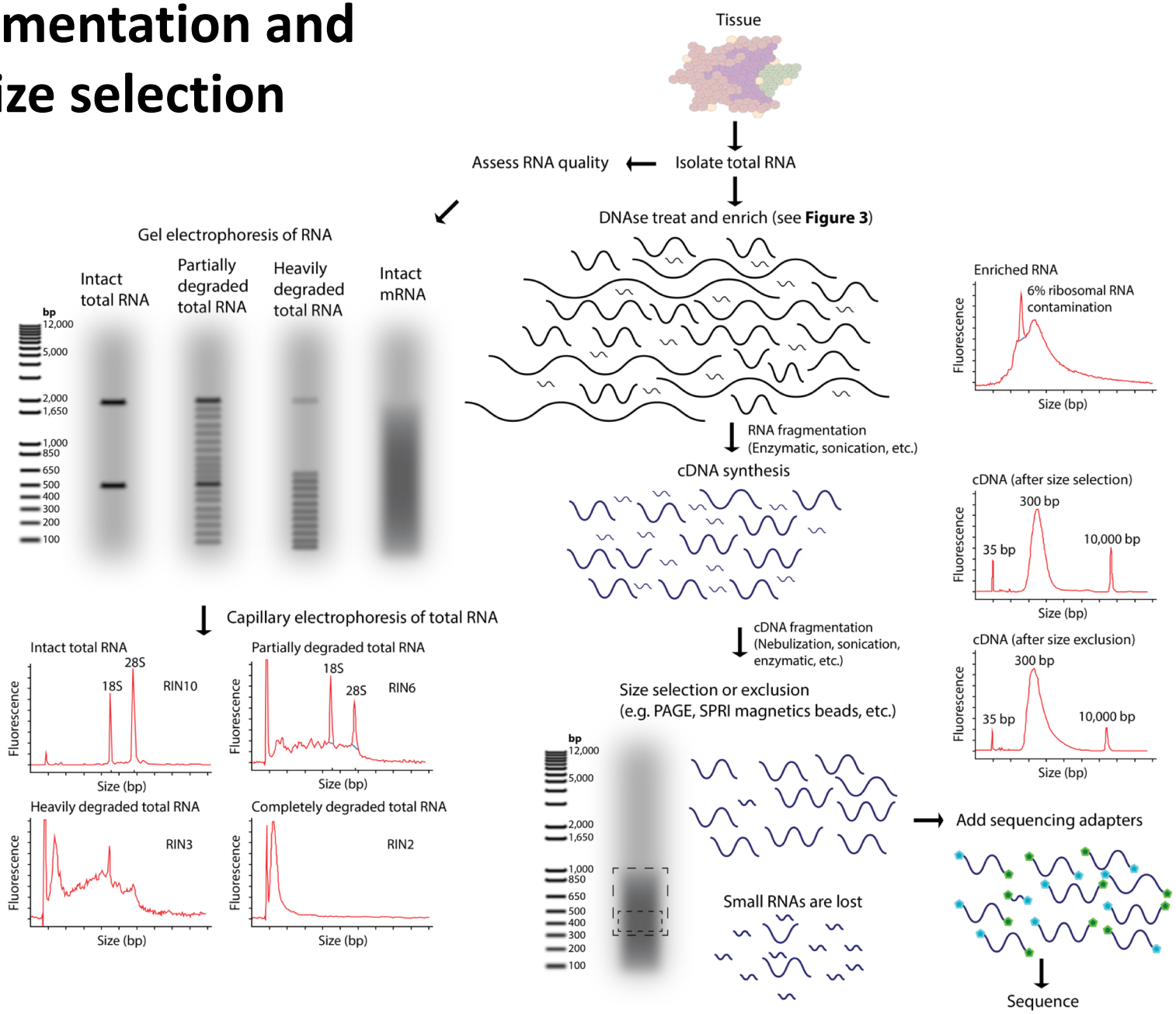
- Several additional initiatives are underway to develop standards and best practices that cover many of these concepts. These include: the Sequencing Quality Control (SEQC) consortium, the Roadmap Epigenomics Mapping Consortium (REMC), and the Beta Cell Biology Consortium (BCBC).

There are many RNA-seq library construction strategies

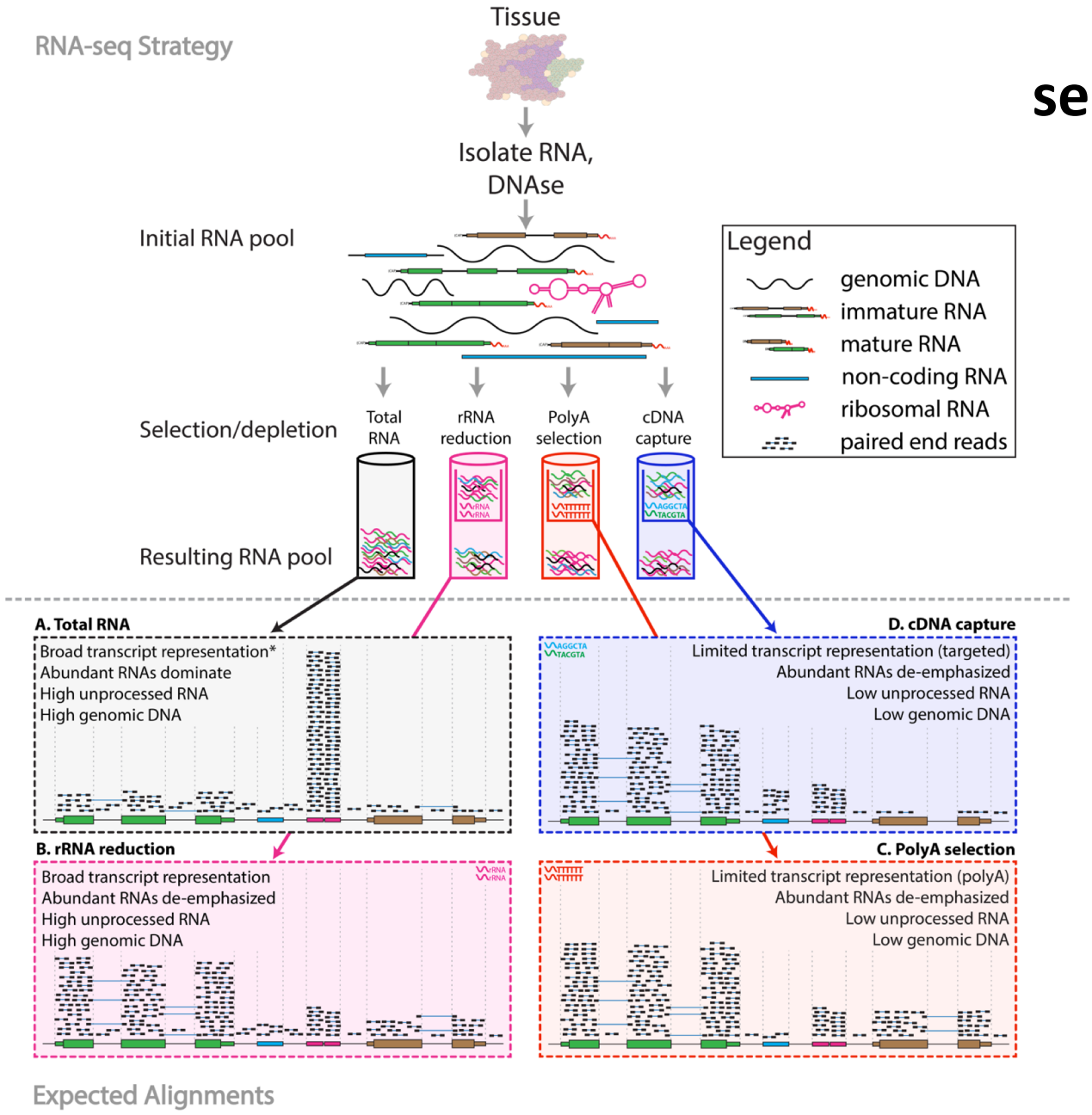
- Total RNA versus polyA+ RNA?
- Ribo-reduction?
- Size selection (before and/or after cDNA synthesis)
  - Small RNAs (microRNAs) vs. large RNAs?
  - A narrow fragment size distribution vs. a broad one?
- Linear amplification?
- Stranded vs. un-stranded libraries
- Exome captured vs. un-captured
- Library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

- These details can affect analysis strategy
  - Especially comparisons between libraries

Fragmentation and size selection

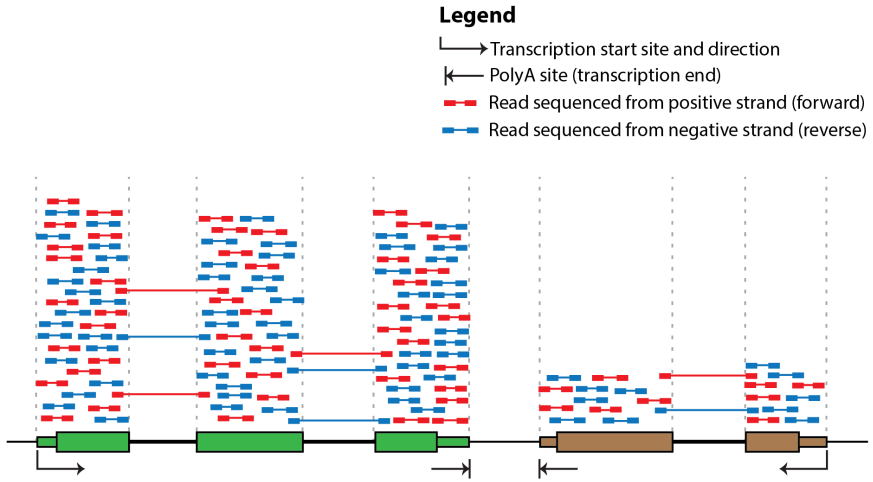


RNA sequence selection/depletion

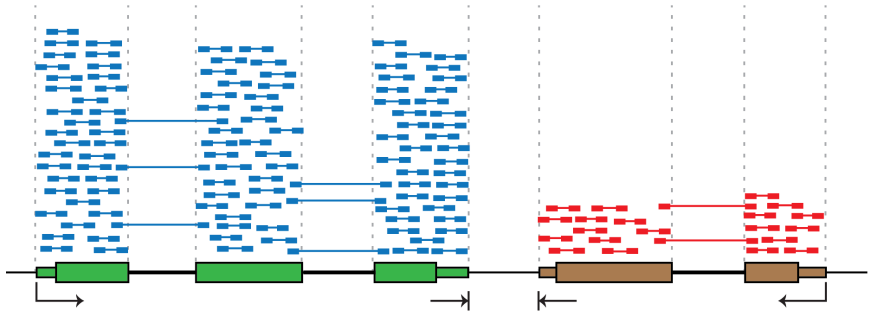


Stranded vs. unstranded

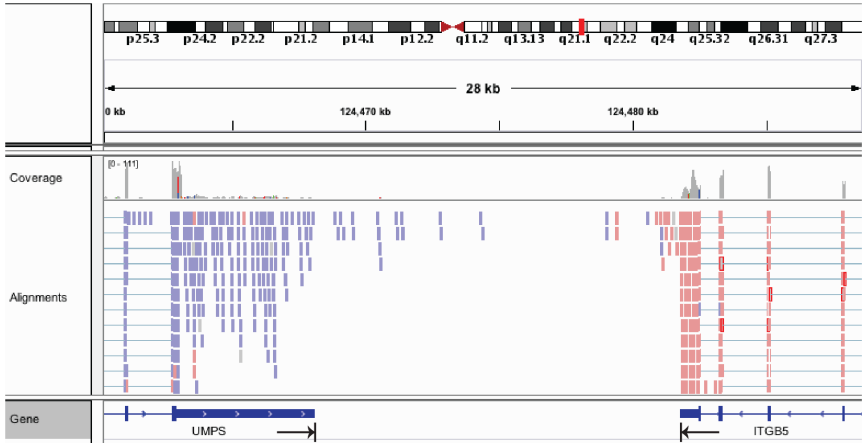
A. Depiction of cDNA fragments from an unstranded library



B. Depiction of cDNA fragments from an stranded library

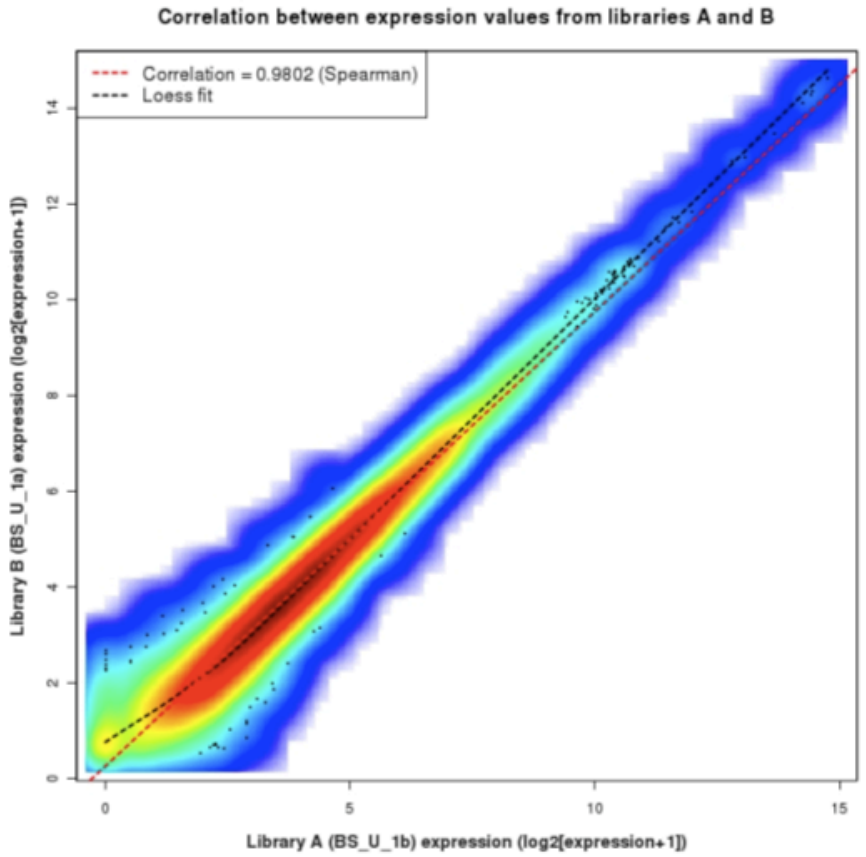


C. Viewing strand of aligned reads in IGV



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?)

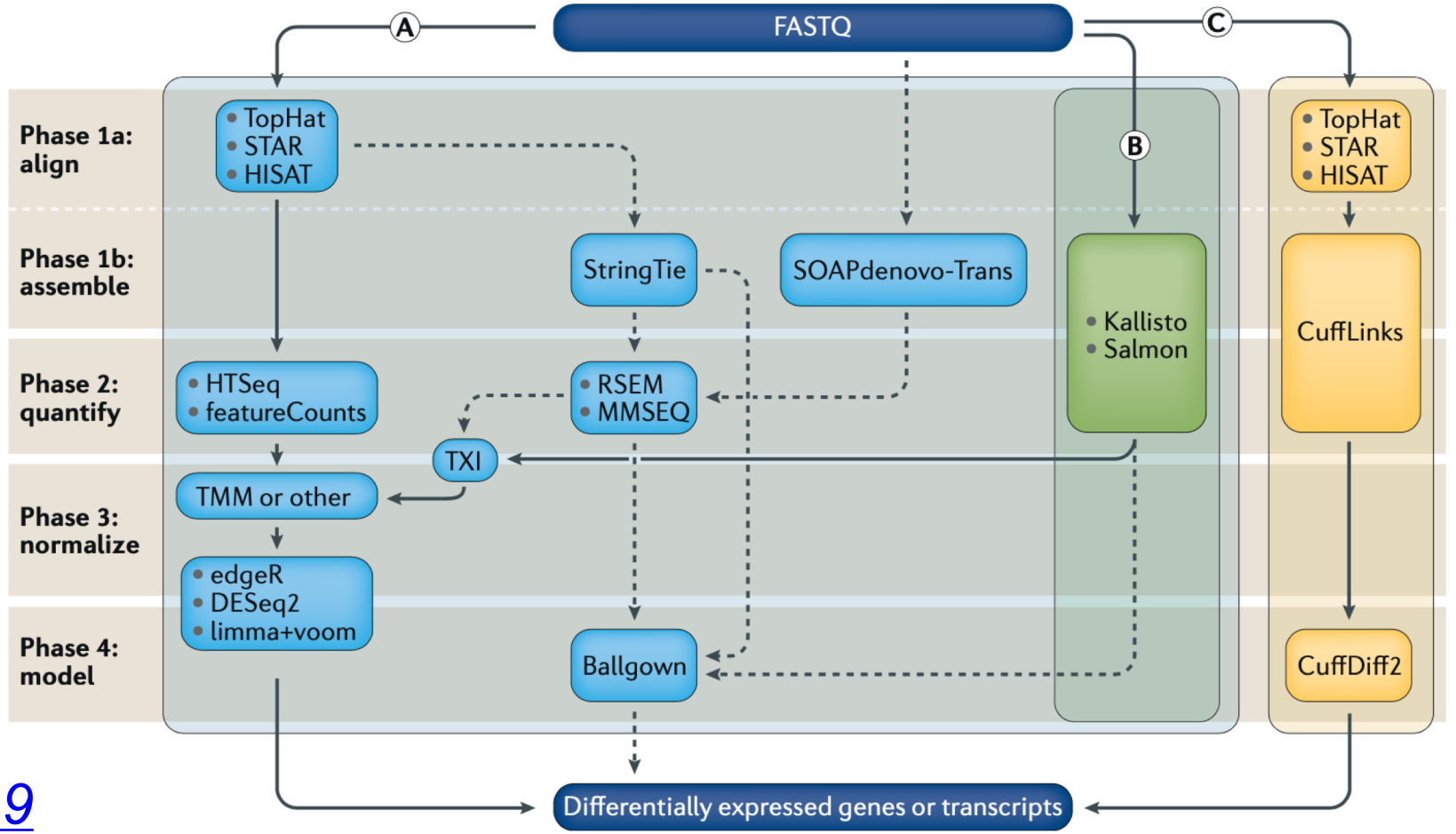
- Gene expression and differential expression
- Alternative expression analysis
- Transcript discovery and annotation
- Allele specific expression
  - Relating to SNPs or mutations
- Mutation discovery
- Fusion detection
- RNA editing

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.

Examples of RNA-seq data analysis workflows for differential gene expression



Stark et al. 2019

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

- 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

- For DGE analysis only, 30-40 million reads is a common recommendation. More replicates can be more valuable than deeper libraries. Short (e.g. 50-75bp), single-end reads may be used to drive cost down as low as possible.

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, STAR, HISAT, etc.

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 currently 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
  - Also we provide example tools in Supplementary Table 2.
  - https://github.com/griffithlab/rnaseq_tutorial/wiki/Kallisto

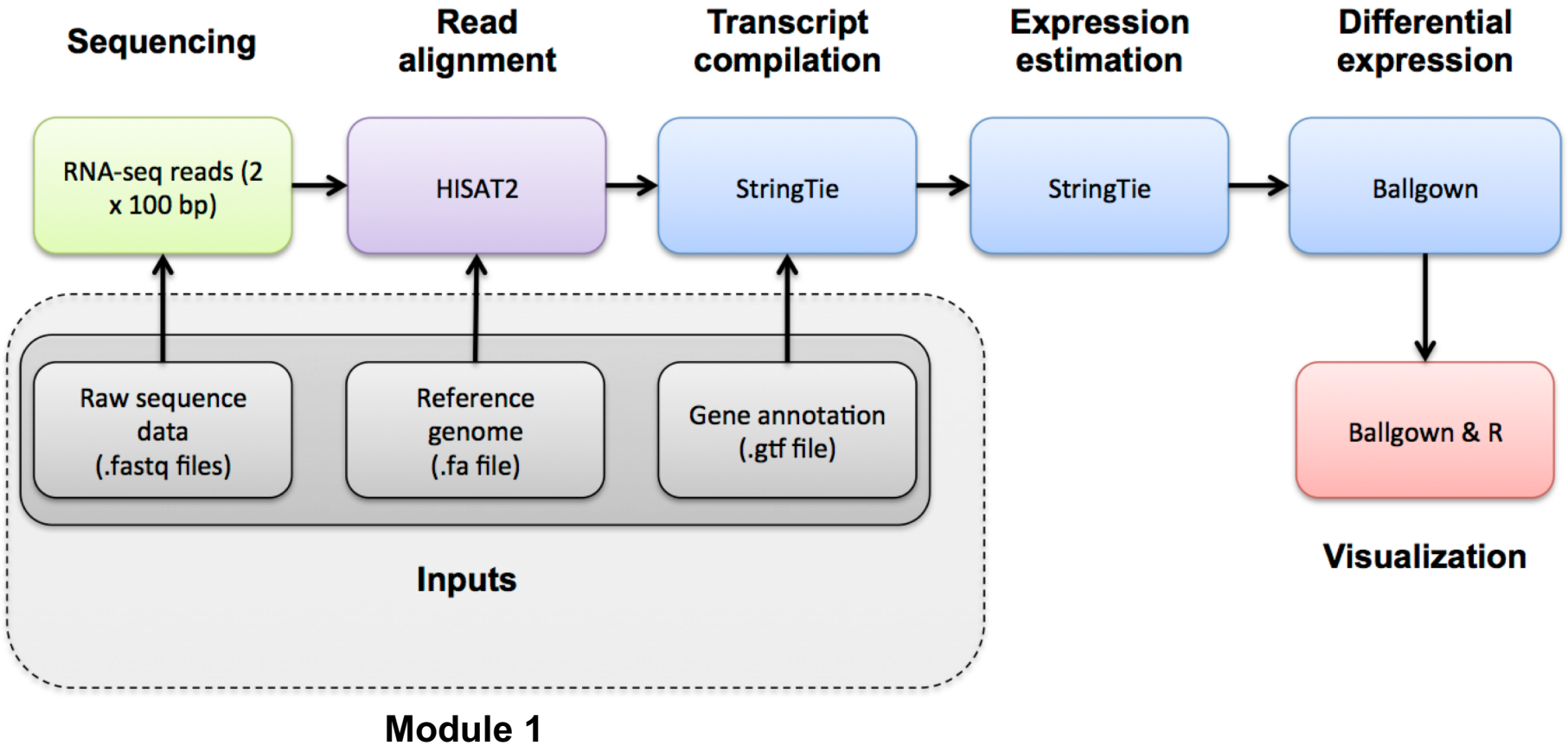
More common questions (and answers)

- Supplementary Table 7

- Malachi Griffith*, Jason R. Walker, Nicholas C. Spies, Benjamin J. Ainscough, Obi L. Griffith*. 2015. Informatics for RNA-seq: A web resource for analysis on the cloud. 11(8):e1004393. 2015.
  - http://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1004393

Introduction to tutorial (Module 1)

HISAT2/StringTie/Ballgown RNA-seq Pipeline



We are on a Coffee Break & Networking Session

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