Cold
Spring
Harbor
Laboratory

Advanced Sequencing Technologies & Applications

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

Cold Spring Harbor Laboratory

RNA-Seq Module 1
Introduction to RNA sequencing (lecture)

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Advanced Sequencing Technologies & Applications
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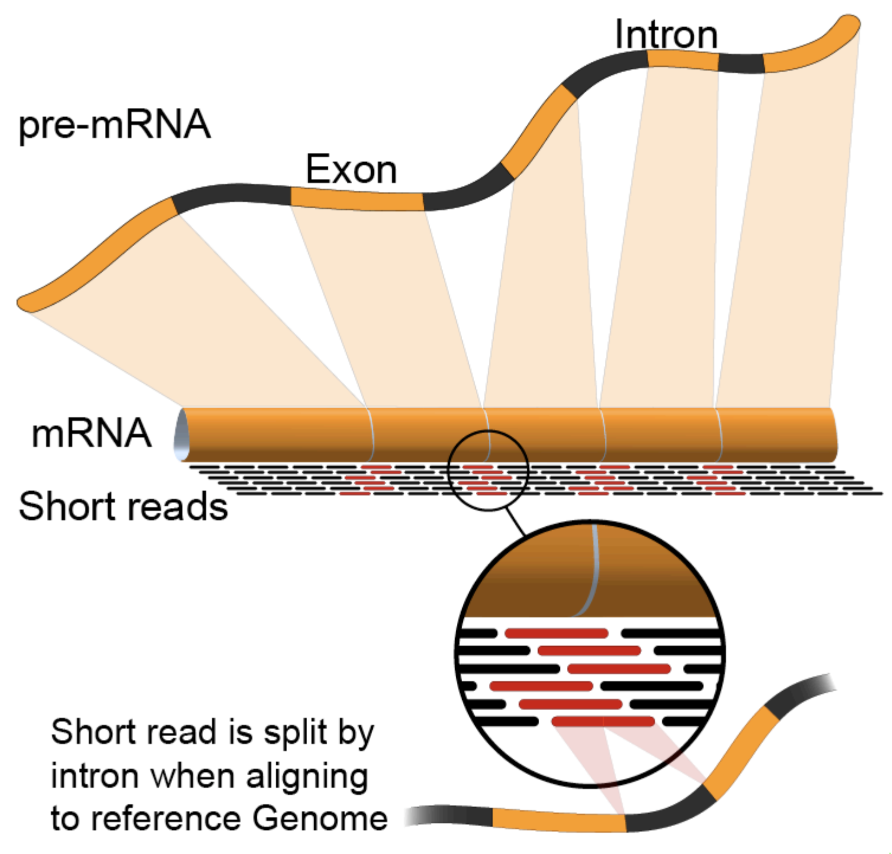

Intron
pre-mRNA
Exon
mRNA
Short reads
Short read is split by intron when aligning to reference Genome

THE GENOME INSTITUTE at Washington University

Learning objectives of the course

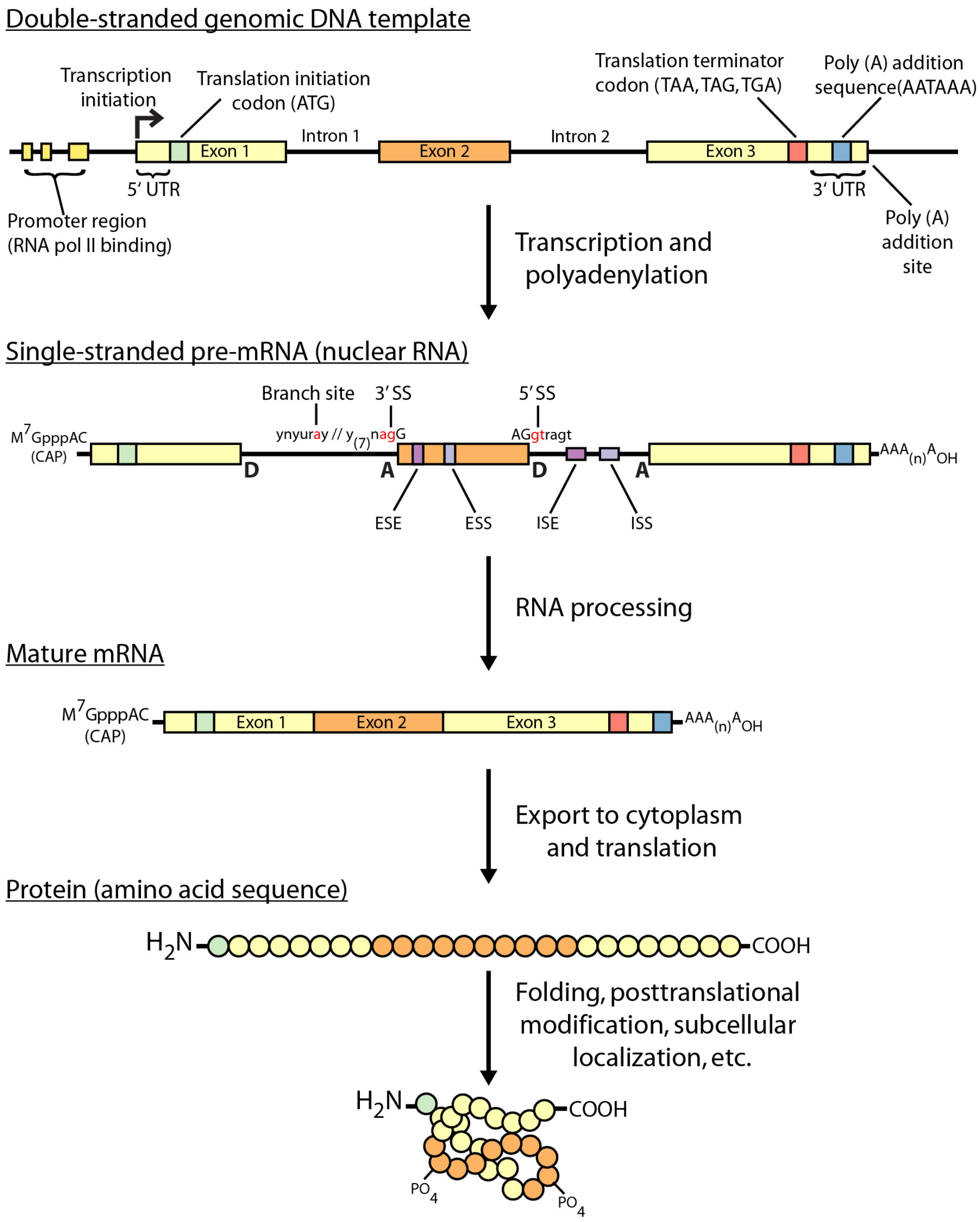
- **Module 1: Introduction to RNA Sequencing**
- Module 2: Alignment and Visualization
- Module 3: Expression and Differential Expression
- Module 4: Isoform Discovery and Alternative Expression

- 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

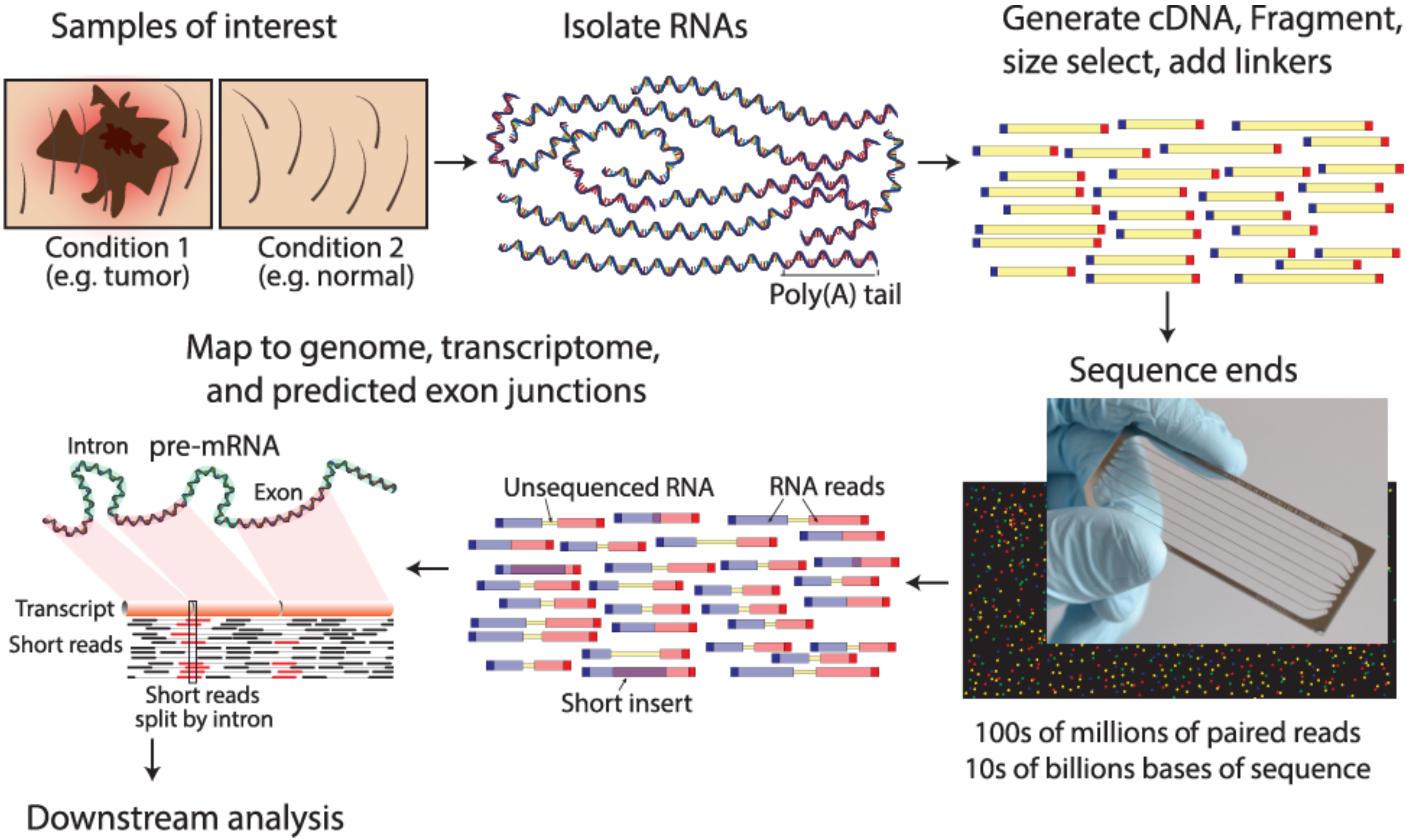
Learning objectives of module 1

- 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

Gene expression


Double-stranded genomic DNA template
Transcription initiation
Translation initiation codon (ATG)
Translation terminator codon (TAA, TAG, TGA)
Poly (A) addition sequence(AATAAA)
Exon 1
Intron 1
Exon 2
Intron 2
Exon 3
5′ UTR
3′ UTR
Promoter region (RNA pol II binding)
Poly (A) addition site
Transcription and polyadenylation
Single-stranded pre-mRNA (nuclear RNA)
Branch site
3′ SS
5′ SS
M7GpppAC (CAP)
ynyuray // y(7)nagG
AGgtragt
AAA(n)AOH
D
A
D
A
ESE
ESS
ISE
ISS
RNA processing
Mature mRNA
M7GpppAC (CAP)
Exon 1
Exon 2
Exon 3
AAA(n)AOH
Export to cytoplasm and translation
Protein (amino acid sequence)
H2N
COOH
Folding, posttranslational modification, subcellular localization, etc.
H2N
COOH
PO4
PO4

RNA sequencing



Samples of interest

Isolate RNAs

Generate cDNA, Fragment, size select, add linkers

Sequence ends

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

Map to genome, transcriptome, and predicted exon junctions

Downstream analysis

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)?

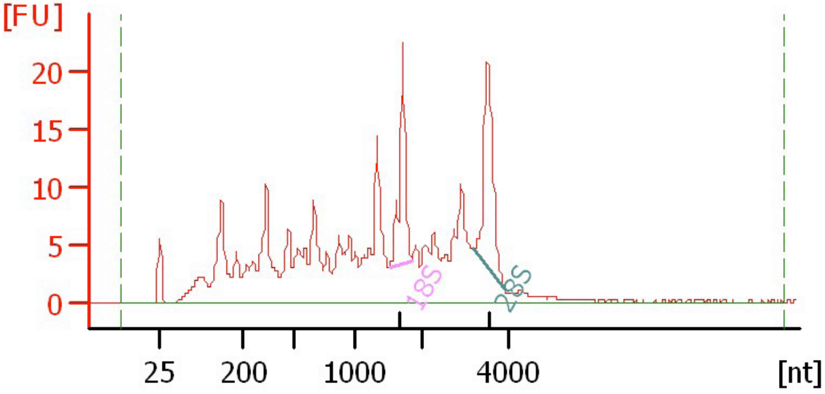
- Interpreting mutations that do not have an obvious effect on protein sequence
  - 'Regulatory' mutations that affect what mRNA isoform is expressed and how much
- Prioritizing protein coding somatic mutations (often heterozygous)
  - If the gene is not expressed, a mutation in that gene would be less 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

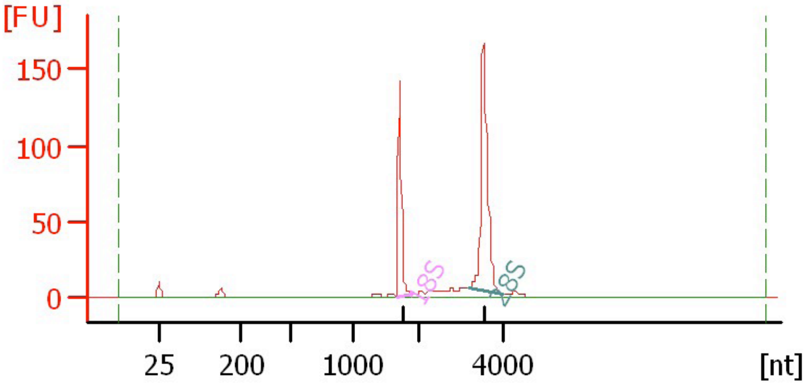
- Sample
  - Purity?, quantity?, quality?
- RNAs consist of small exons that may be separated by large introns
  - Mapping reads to genome is challenging
- The relative abundance of RNAs vary wildly
  - 10^5 – 10^7 orders of magnitude
  - Since RNA sequencing works by random sampling, a small fraction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

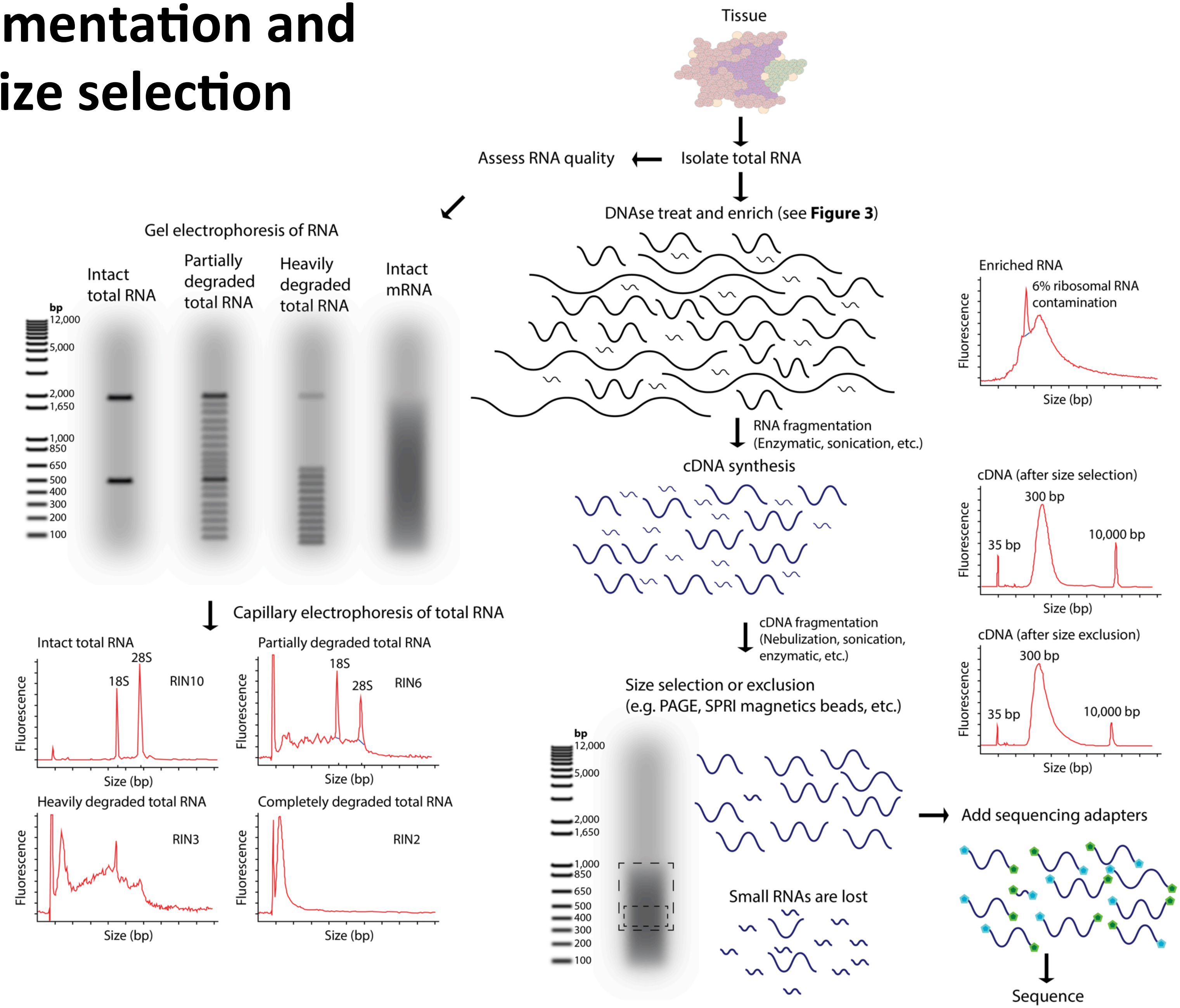
- Several additional initiatives are underway to develop standards and best practices that cover many of these concepts. These include: the Sequencing Quality Control (SEQC) consortium, the Roadmap Epigenomics Mapping Consortium (REMC), and the Beta Cell Biology Consortium (BCBC).

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?

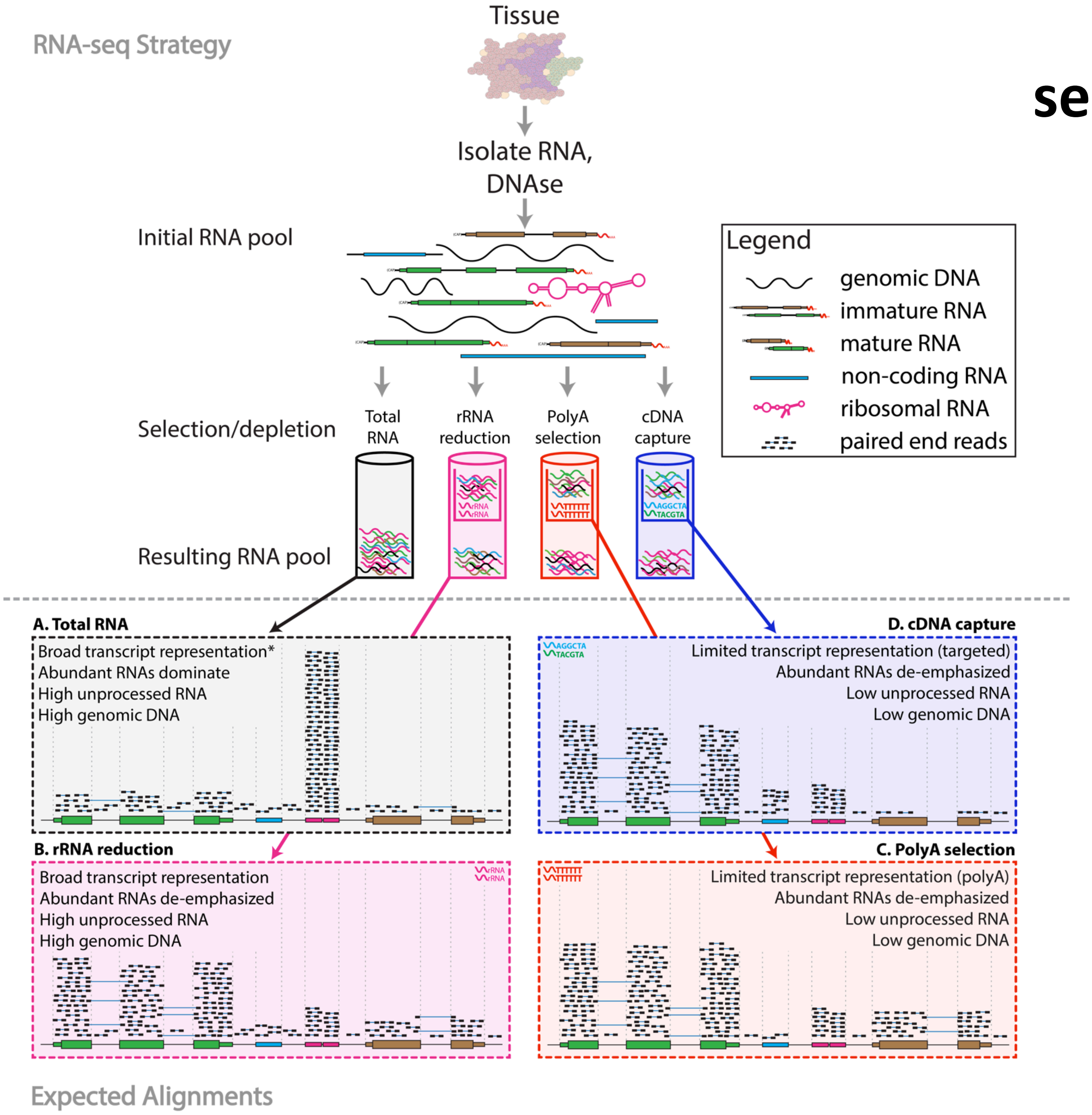
- These details can affect analysis strategy
  - Especially comparisons between libraries

Fragmentation and size selection



Tissue
Isolate total RNA
Assess RNA quality
DNAse treat and enrich (see **Figure 3**)
Gel electrophoresis of RNA
Intact total RNA
Partially degraded total RNA
Heavily degraded total RNA
Intact mRNA
bp
12,000
5,000
2,000
1,650
1,000
850
650
500
400
300
200
100
Capillary electrophoresis of total RNA
Intact total RNA
28S
18S
RIN10
Fluorescence
Size (bp)
Partially degraded total RNA
18S
28S
RIN6
Fluorescence
Size (bp)
Heavily degraded total RNA
RIN3
Fluorescence
Size (bp)
Completely degraded total RNA
RIN2
Fluorescence
Size (bp)
Enriched RNA
6% ribosomal RNA contamination
Fluorescence
Size (bp)
RNA fragmentation (Enzymatic, sonication, etc.)
cDNA synthesis
cDNA (after size selection)
300 bp
35 bp
10,000 bp
Fluorescence
Size (bp)
cDNA fragmentation (Nebulization, sonication, enzymatic, etc.)
cDNA (after size exclusion)
300 bp
35 bp
10,000 bp
Fluorescence
Size (bp)
Size selection or exclusion (e.g. PAGE, SPRI magnetics beads, etc.)
bp
12,000
5,000
2,000
1,650
1,000
850
650
500
400
300
200
100
Small RNAs are lost
Add sequencing adapters
Sequence

RNA sequence selection/depletion



RNA-seq Strategy

Tissue

Isolate RNA, DNAse

Initial RNA pool

Selection/depletion

Total RNA

rRNA reduction

PolyA selection

cDNA capture

Resulting RNA pool

Legend

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

A. Total RNA

Broad transcript representation*
Abundant RNAs dominate
High unprocessed RNA
High genomic DNA

D. cDNA capture

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

B. rRNA reduction

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

C. PolyA selection

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

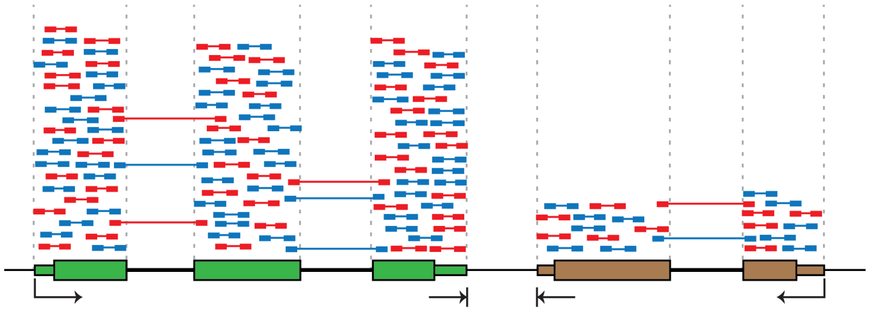
Expected Alignments

Stranded vs. unstranded

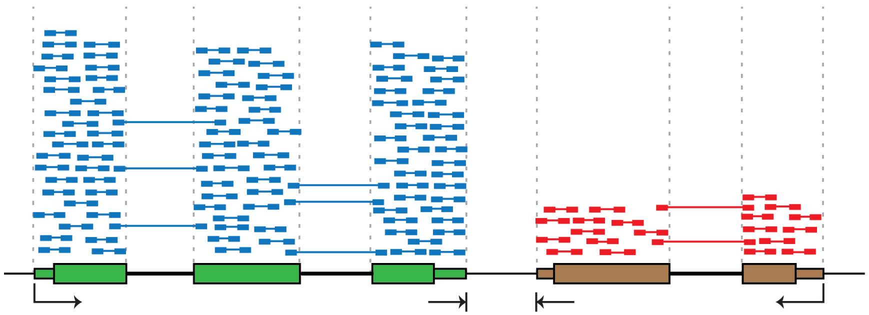
A. Depiction of cDNA fragments from an unstranded library

Legend

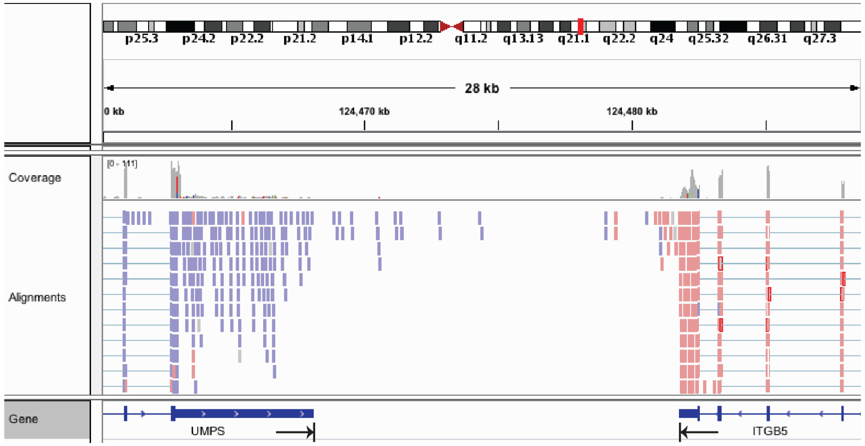
- └→ Transcription start site and direction
- |← PolyA site (transcription end)
- 🟥 Read sequenced from positive strand (forward)
- 🟦 Read sequenced from negative strand (reverse)



B. Depiction of cDNA fragments from an stranded library

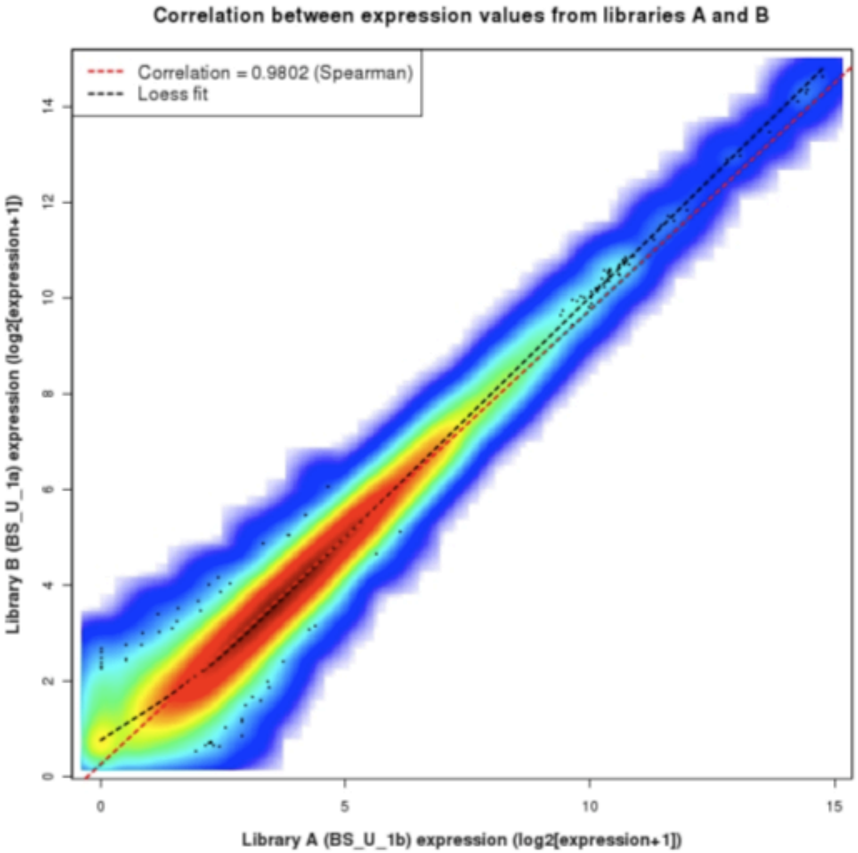


C. Viewing strand of aligned reads in IGV



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.

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]

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
- 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, 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

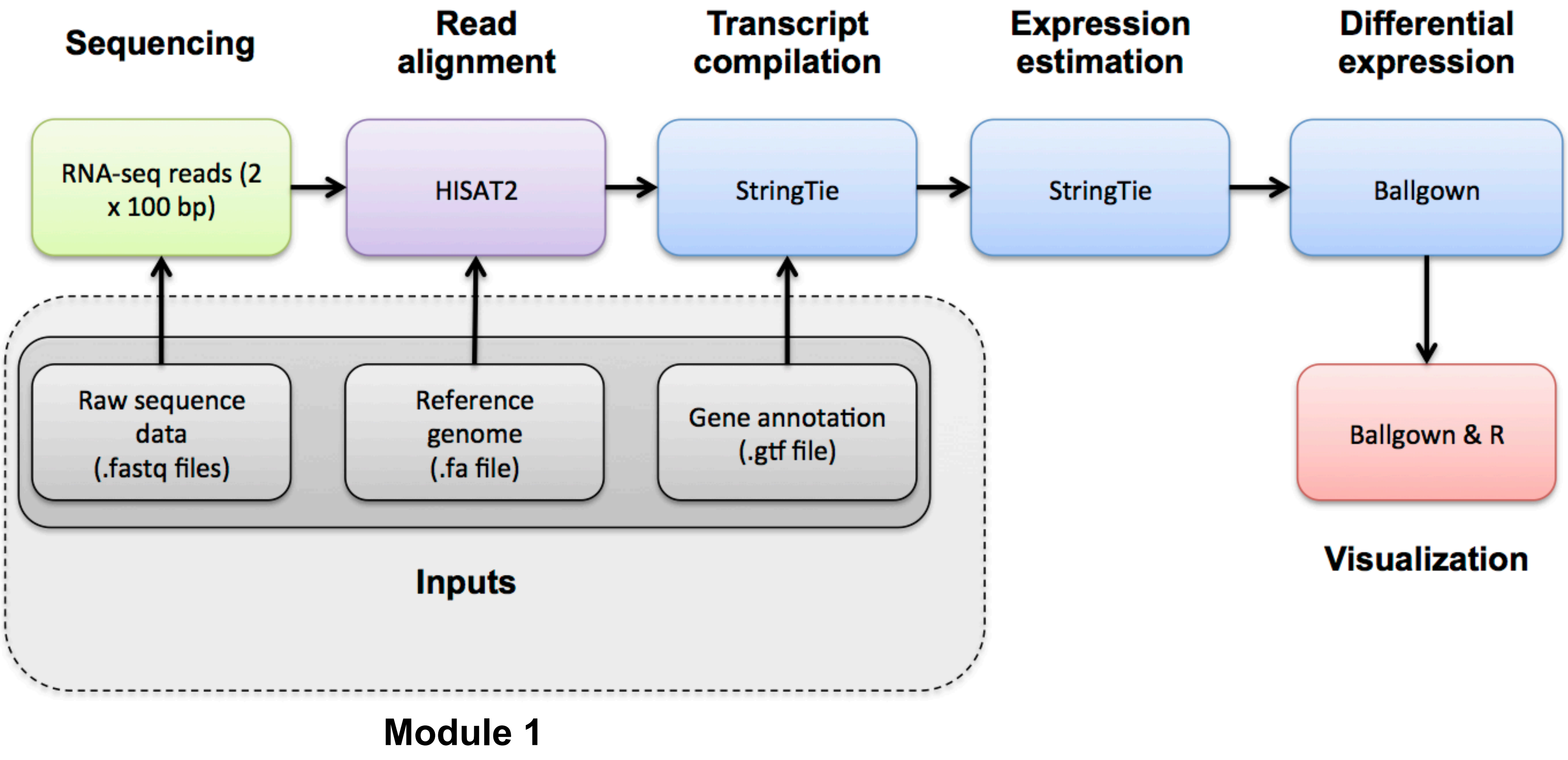
More common questions (and answers)

- 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