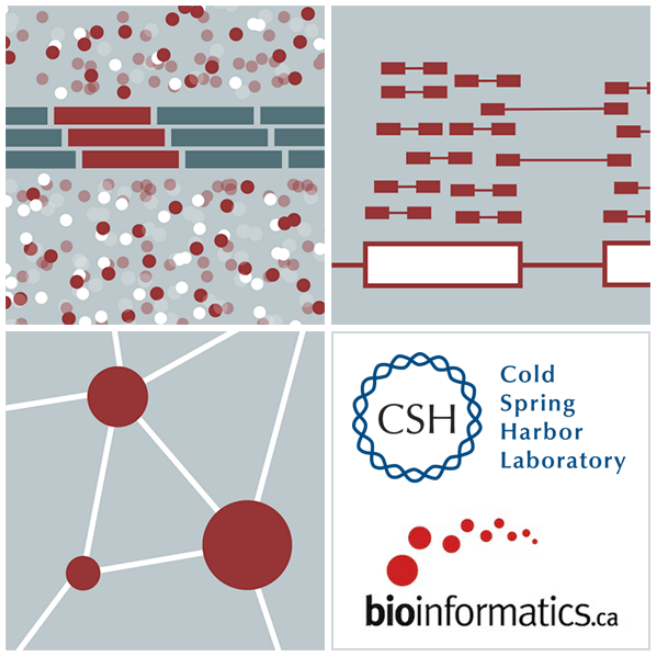
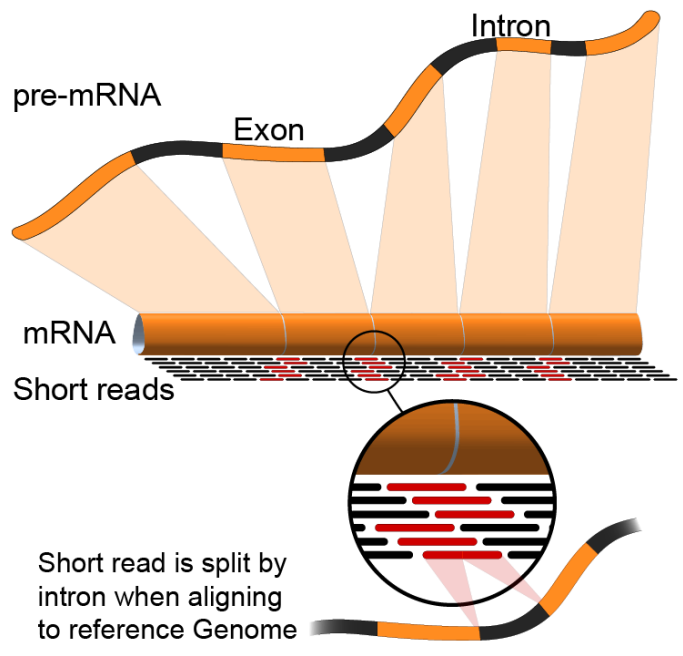
Cold Spring Harbor Laboratory

Module 1:
Intro to RNA-seq

Arpad Danos, Felicia Gomez, Obi Griffith, Malachi Griffith,
My Hoang, Mariam Khanfar, Chris Miller, Kartik Singhal

Advanced Sequencing Technologies & Bioinformatics Analysis November 10-23, 2024





Washington University in St. Louis
School of Medicine

Learning objectives of the course

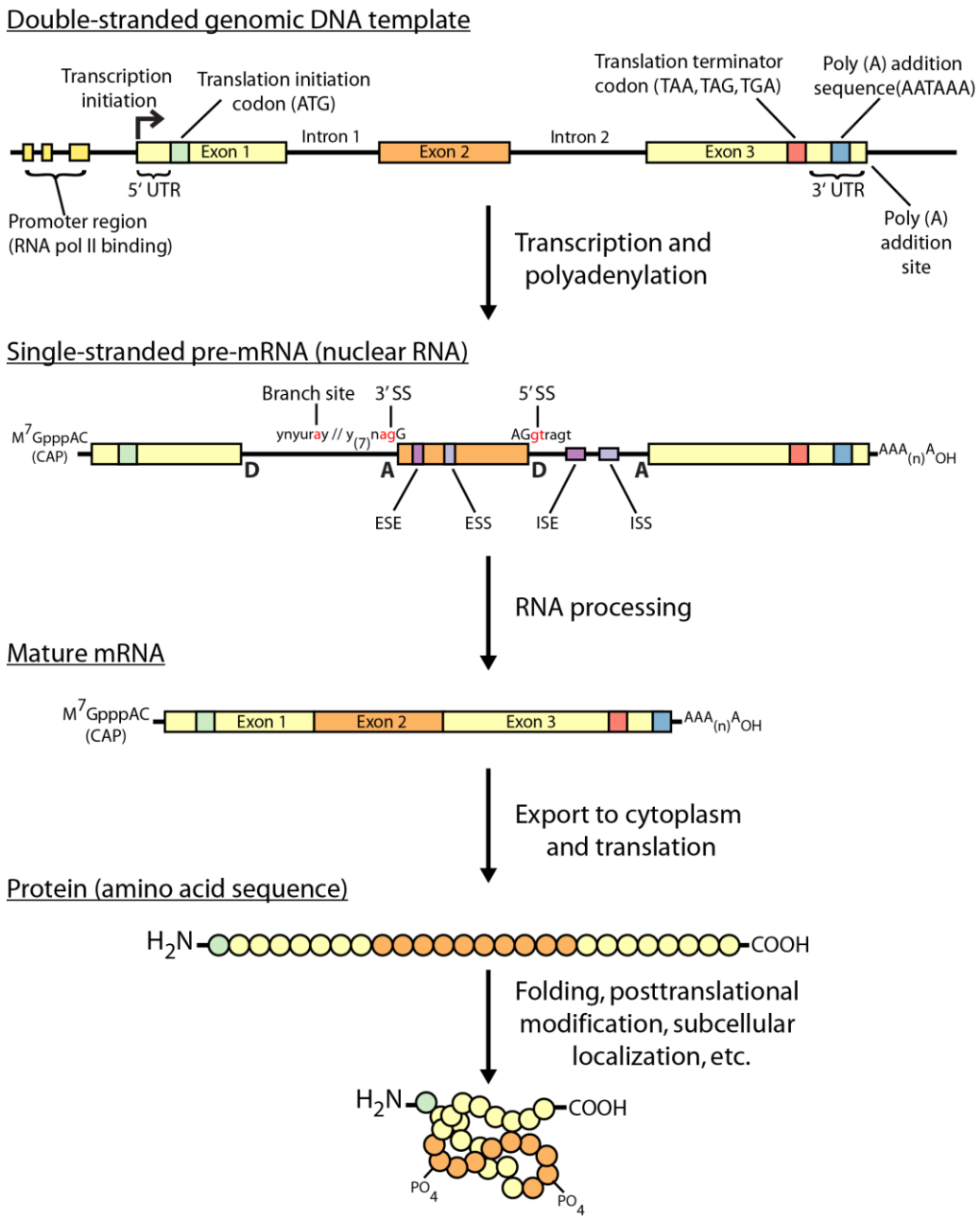
- **Module 1: Introduction to RNA Sequencing**
- Module 2: Alignment and Visualization
- Module 3: Expression and Differential Expression
- Module 4: Alignment Free Expression Estimation

- 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

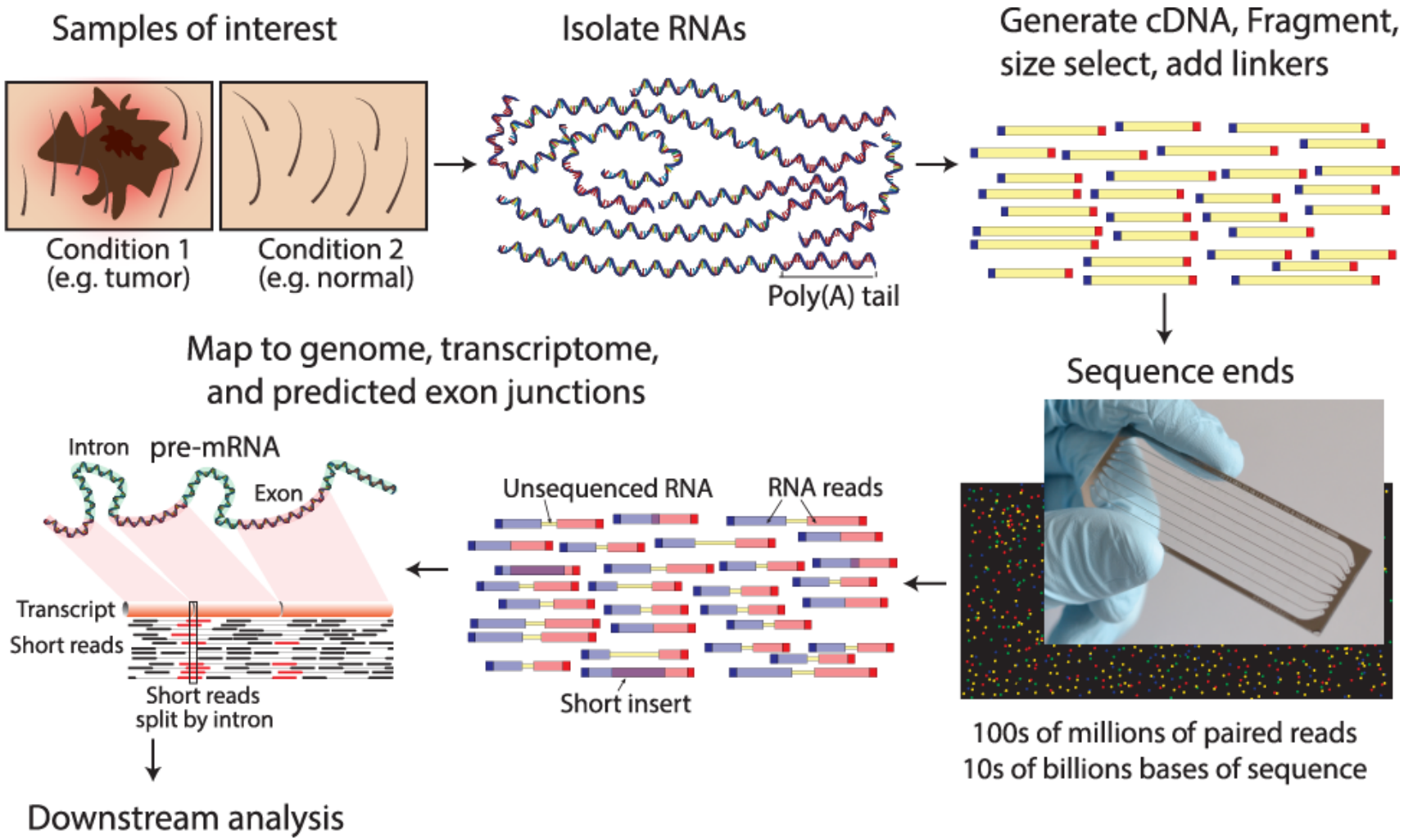
Learning objectives of module 1

- Introduction to the theory and practice of RNA sequencing (RNA-seq) analysis
  - Background molecular biology
  - Challenges specific to RNA-seq
  - General goals and themes of RNA-seq analysis workflows
  - Common technical questions related to RNA-seq analysis
  - Introduction to the RNA-seq hands on tutorial

Gene expression



RNA sequencing

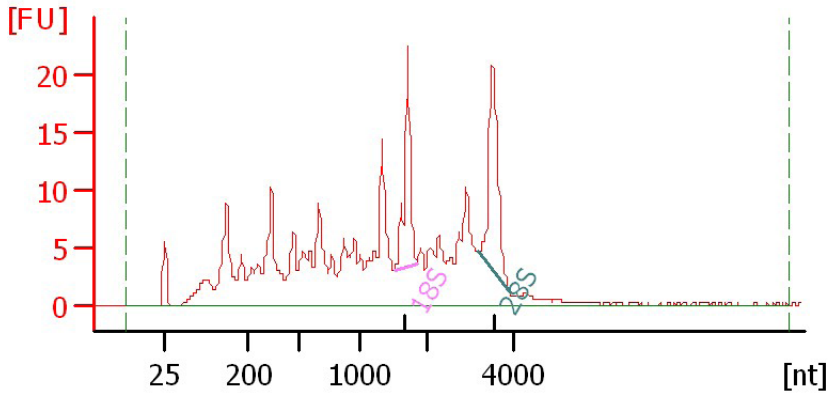


Challenges

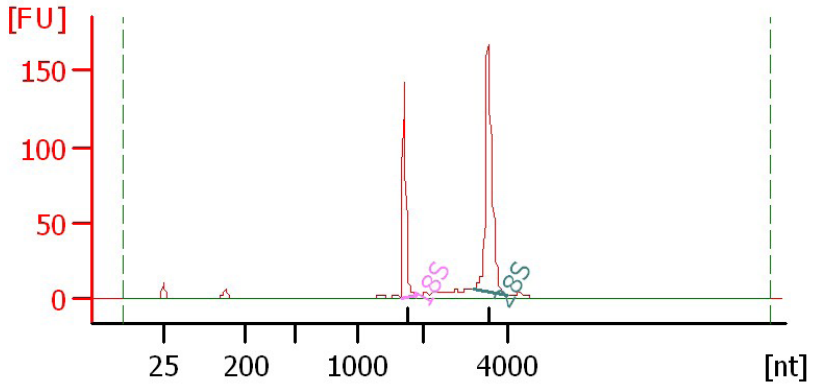
- Sample
  - Purity?, quantity?, quality?
- RNAs consist of small exons that may be separated by large introns
  - Mapping reads to genome is challenging
- The relative abundance of RNAs vary wildly
  - 10^5 – 10^7 orders of magnitude
  - Since RNA sequencing works by random sampling, a small fraction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

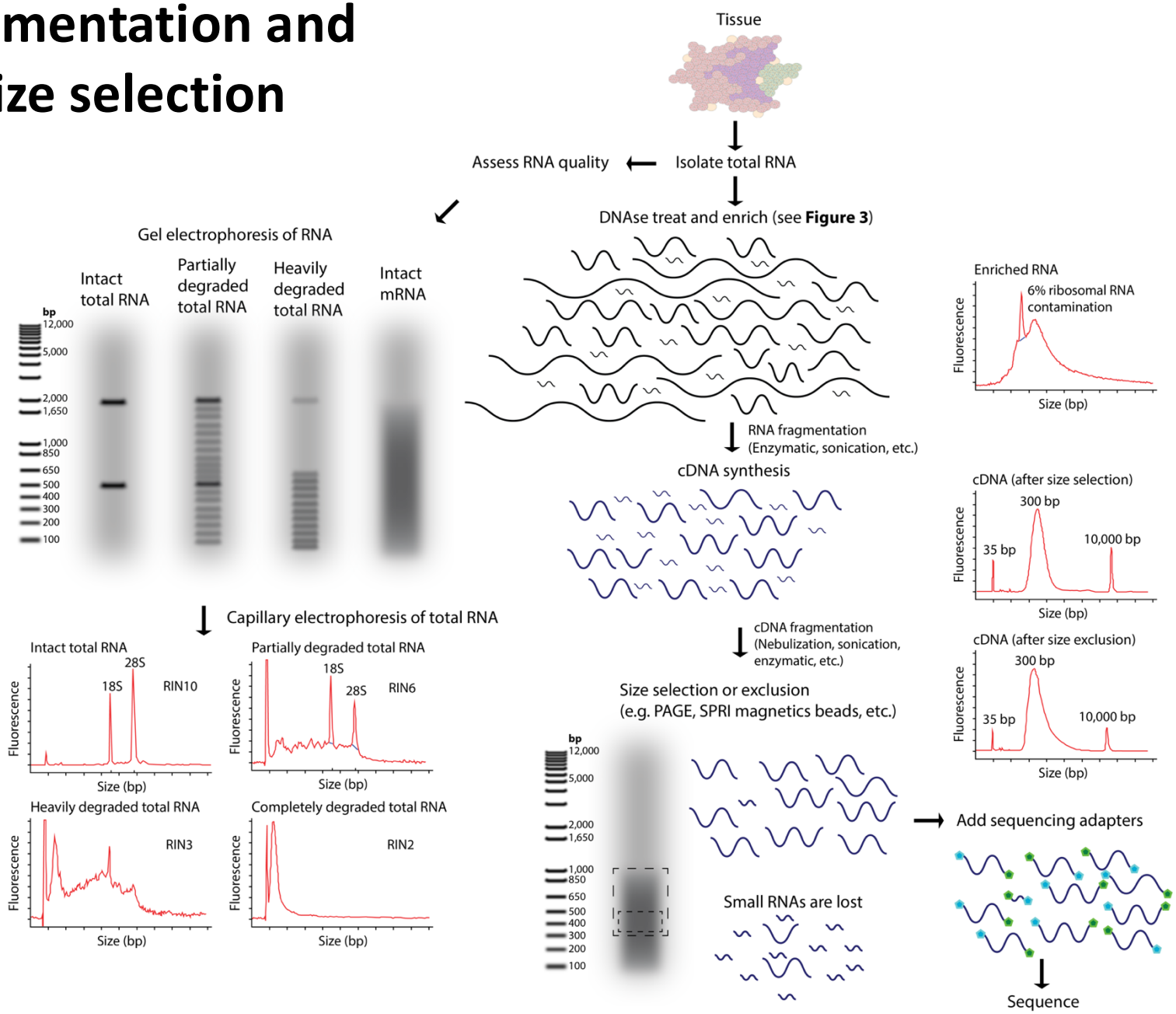
- Several additional initiatives are underway to develop standards and best practices that cover many of these concepts. These include: the Sequencing Quality Control (SEQC) consortium, the Roadmap Epigenomics Mapping Consortium (REMC), and the Beta Cell Biology Consortium (BCBC).

There are many RNA-seq library construction strategies

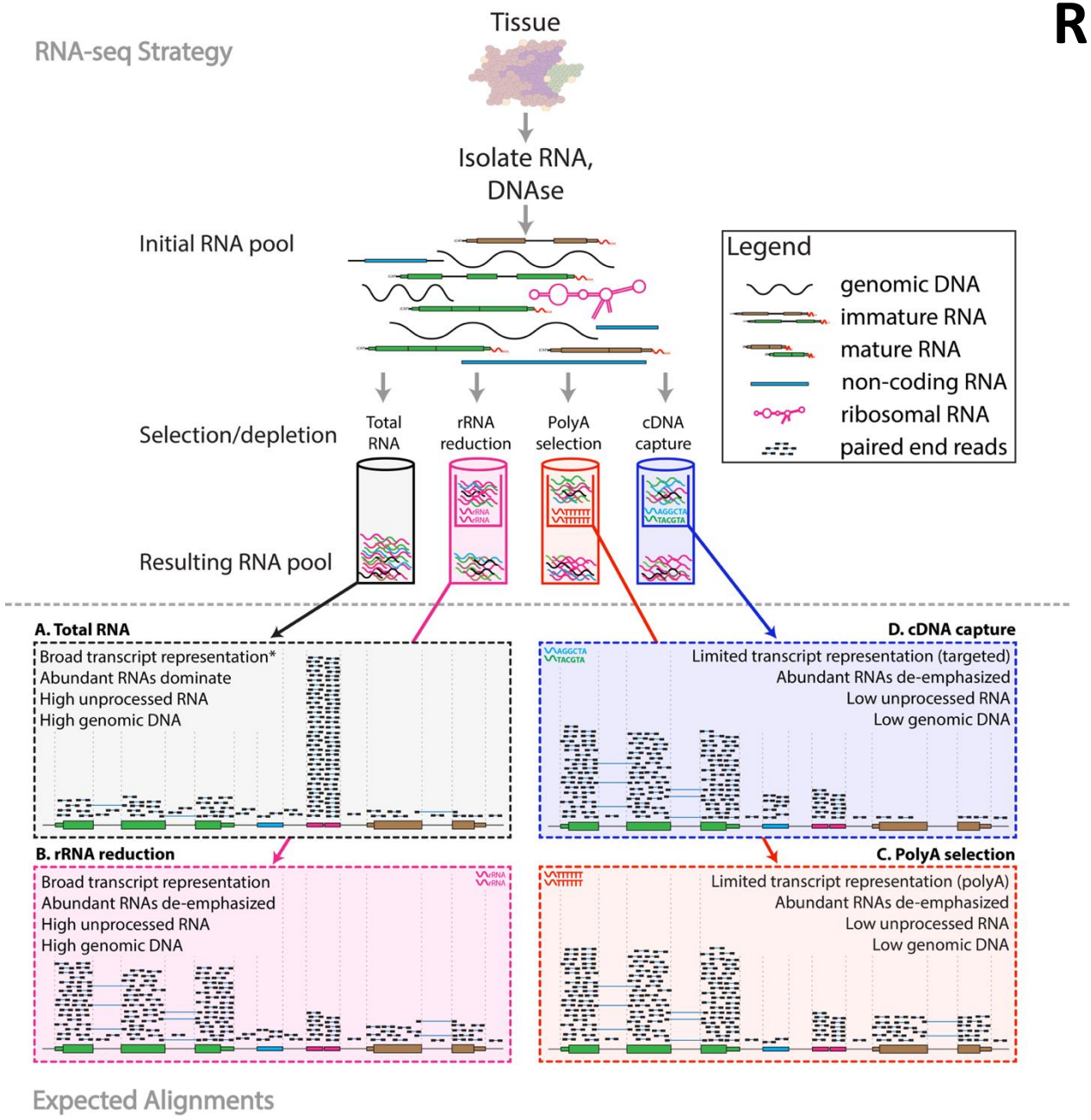
- Total RNA versus polyA+ RNA?
- Ribo-reduction?
- Size selection (before and/or after cDNA synthesis)
  - Small RNAs (microRNAs) vs. large RNAs?
  - A narrow fragment size distribution vs. a broad one?
- Linear amplification?
- Stranded vs. un-stranded libraries
- Exome captured vs. un-captured
- Library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

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

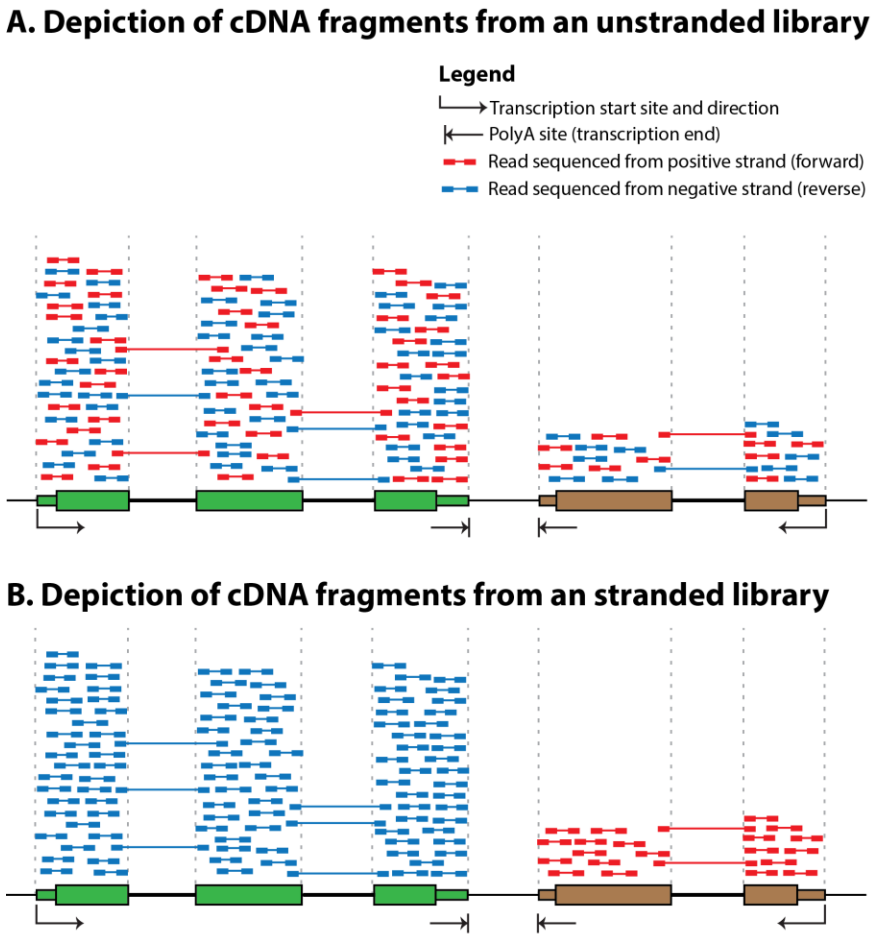
Fragmentation and size selection

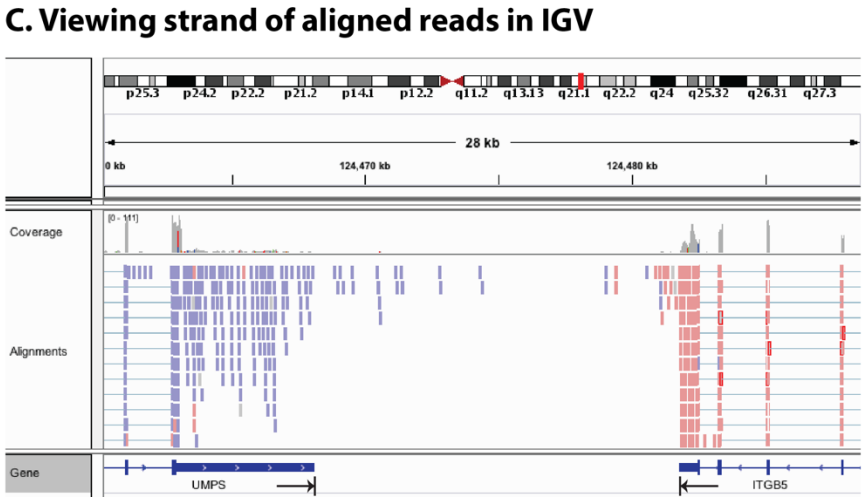


RNA sequence enrichment (selection/depletion)



Stranded vs. unstranded



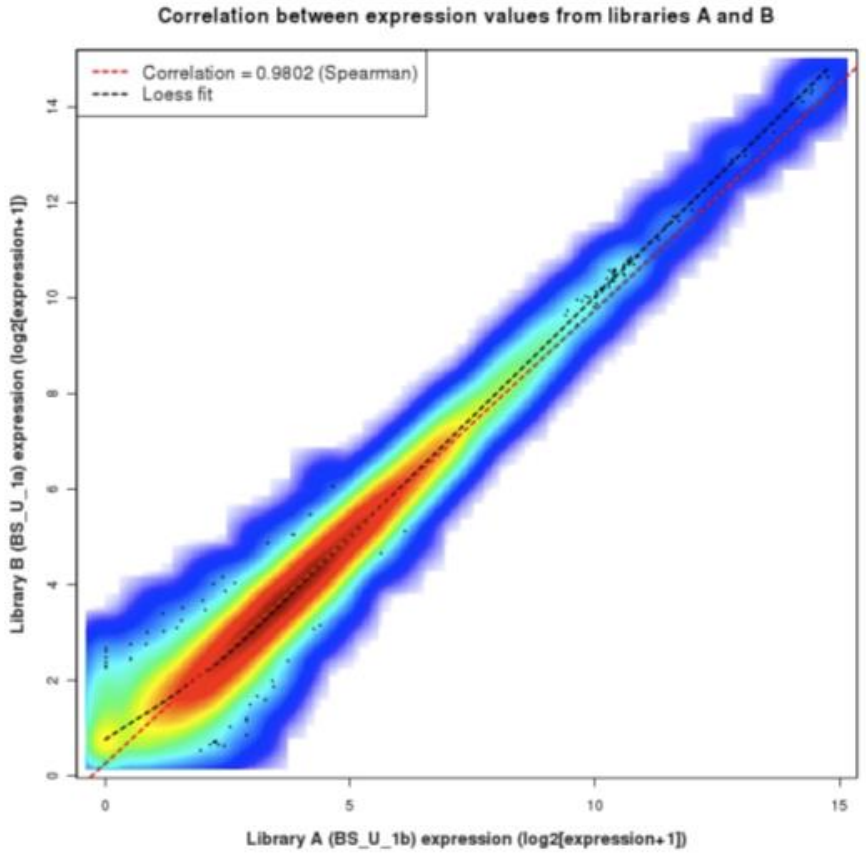


https://rnabio.org/module-09-appendix/0009/12/01/StrandSettings/

(detailed discussion and cheat sheet)

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?)

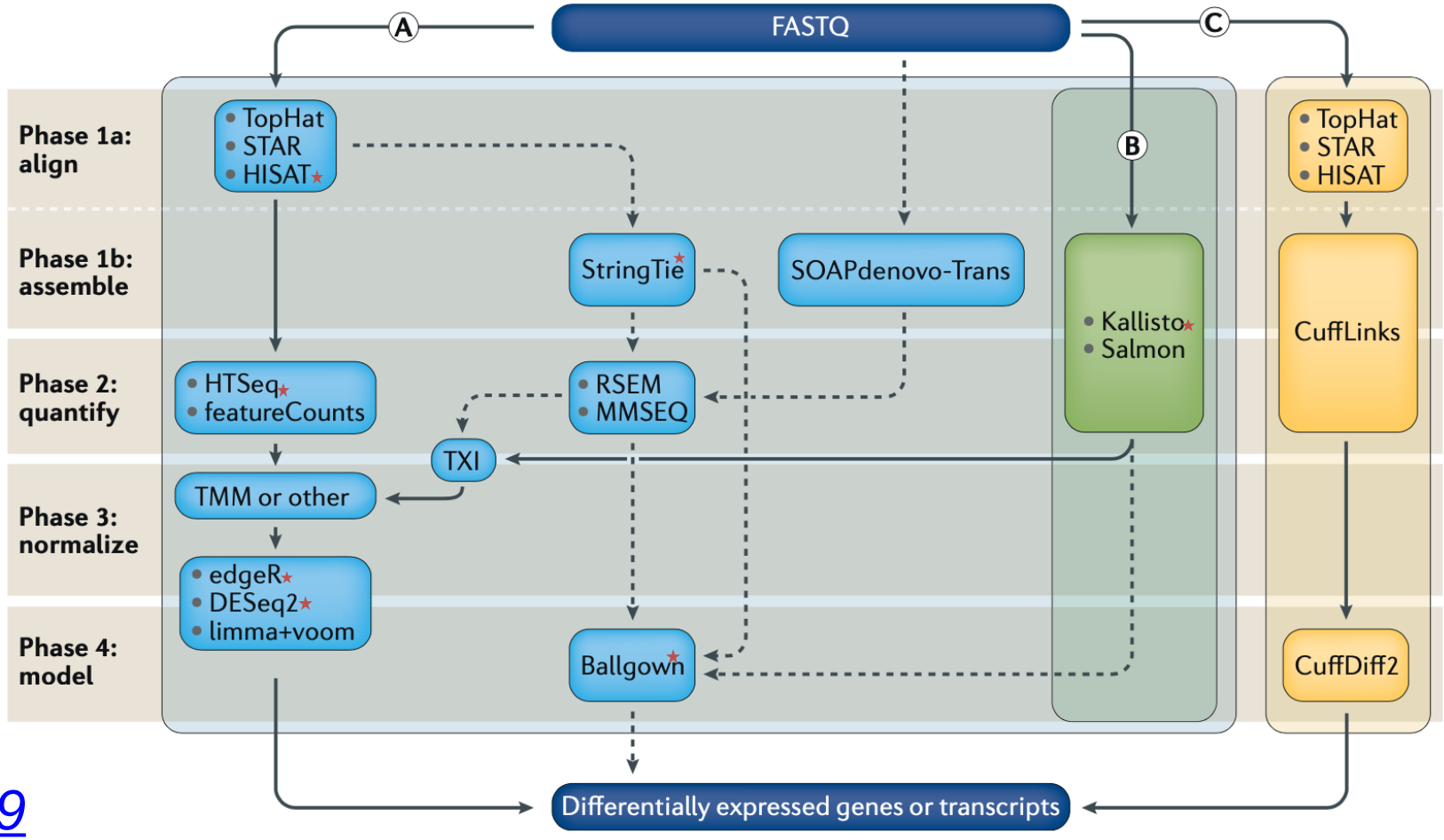
- Gene expression and differential expression
- Alternative expression analysis
- Transcript discovery and annotation
- Allele specific expression
  - Relating to SNPs or mutations
- Mutation discovery
- Fusion detection
- RNA editing

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]

★Covered in this course

Discussion of bulk vs single cell RNA-seq

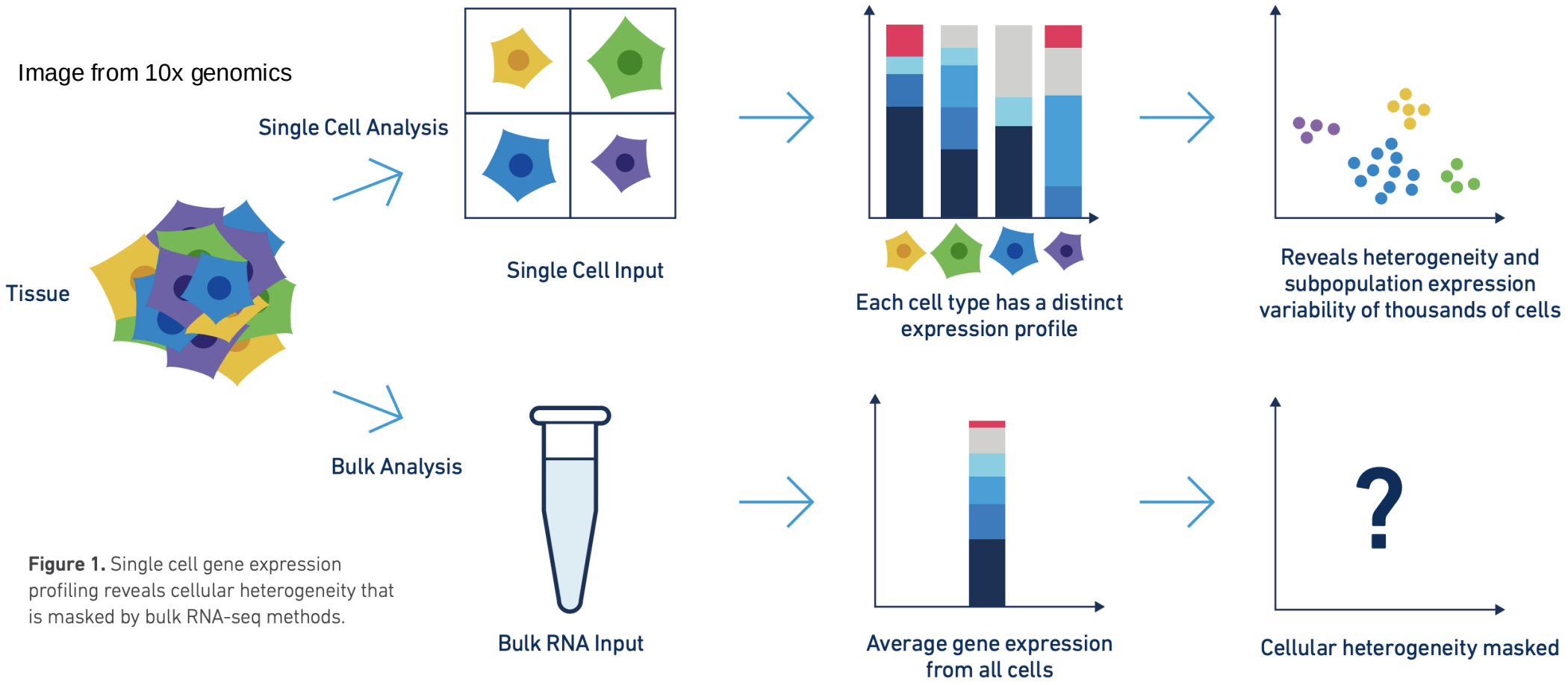


Figure 1. Single cell gene expression profiling reveals cellular heterogeneity that is masked by bulk RNA-seq methods.

Factors to compare: Cost, complexity of library prep, complexity of analysis, qualitative and quantitative differences in richness of information obtained.

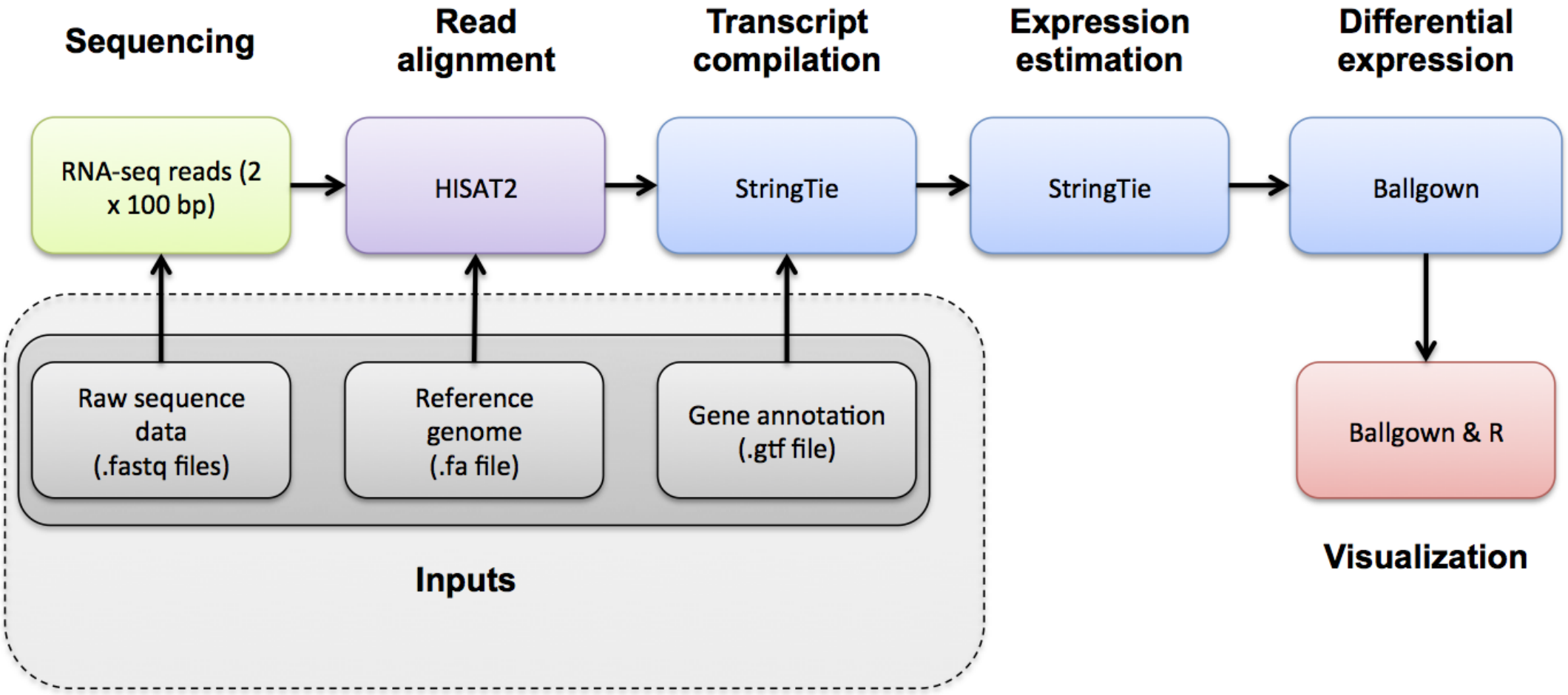
Common questions (and answers)

- Supplementary Table 7

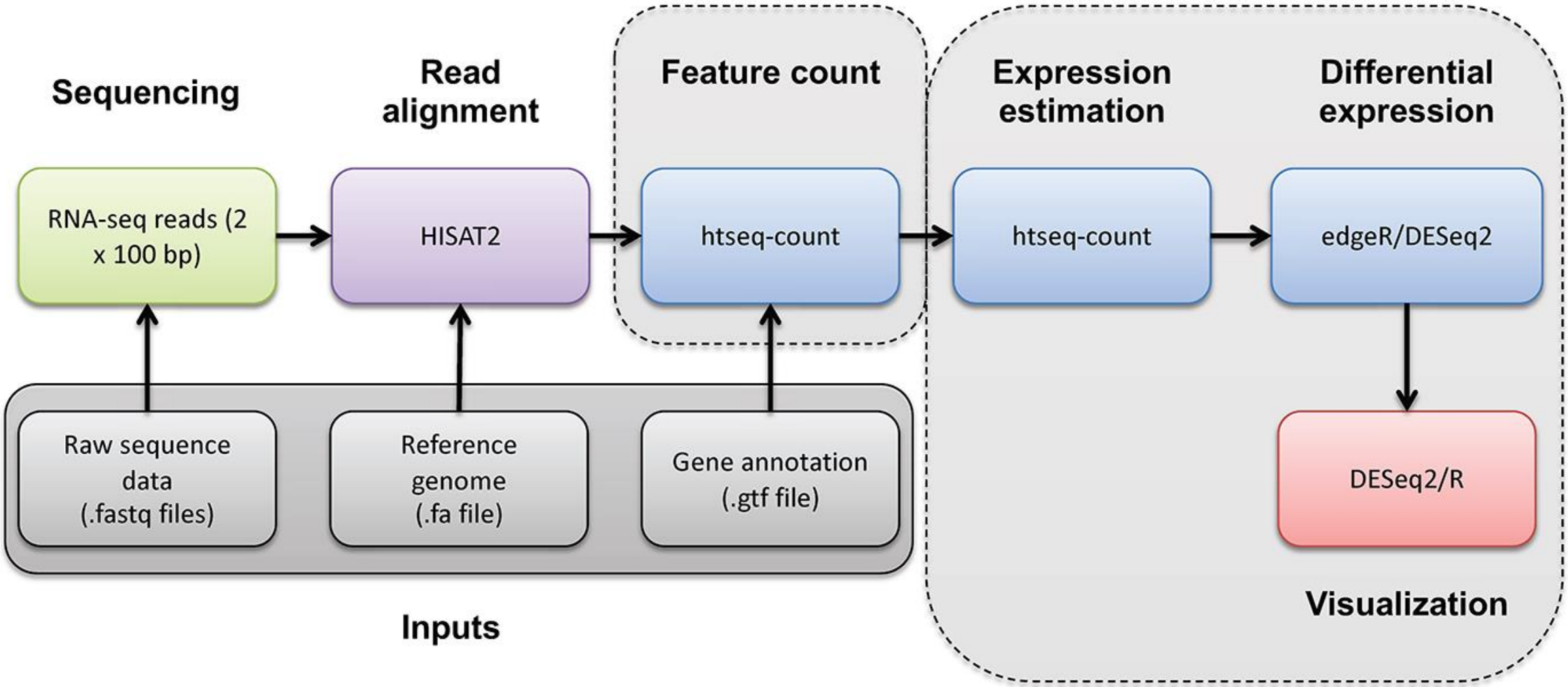
- Malachi Griffith*, Jason R. Walker, Nicholas C. Spies, Benjamin J. Ainscough, Obi L. Griffith*. 2015. Informatics for RNA-seq: A web resource for analysis on the cloud. 11(8):e1004393. 2015.
  - http://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1004393

Introduction to tutorial (Module 1)

HISAT2/StringTie/Ballgown RNA-seq Pipeline



HISAT2/htseq-count/edgeR/DEseq2 Pipeline



We are on a Coffee Break & Networking Session

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