Cold Spring Harbor Laboratory

Introduction to RNA sequencing (lecture)

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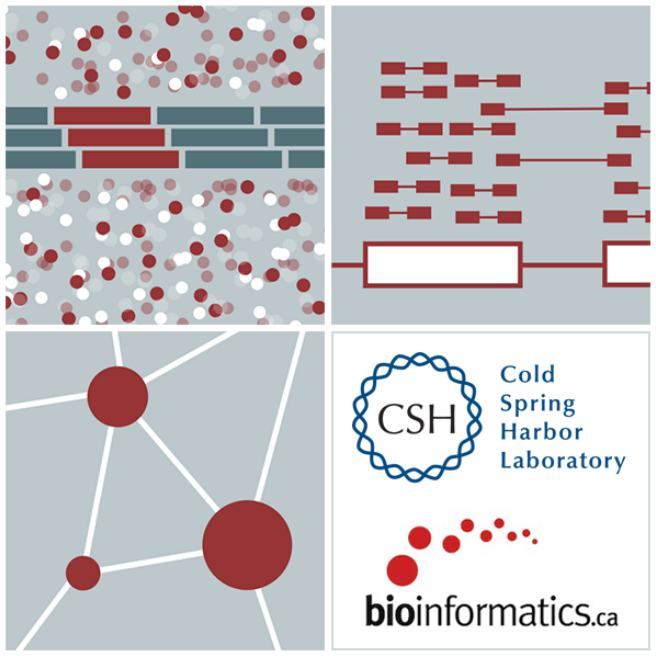
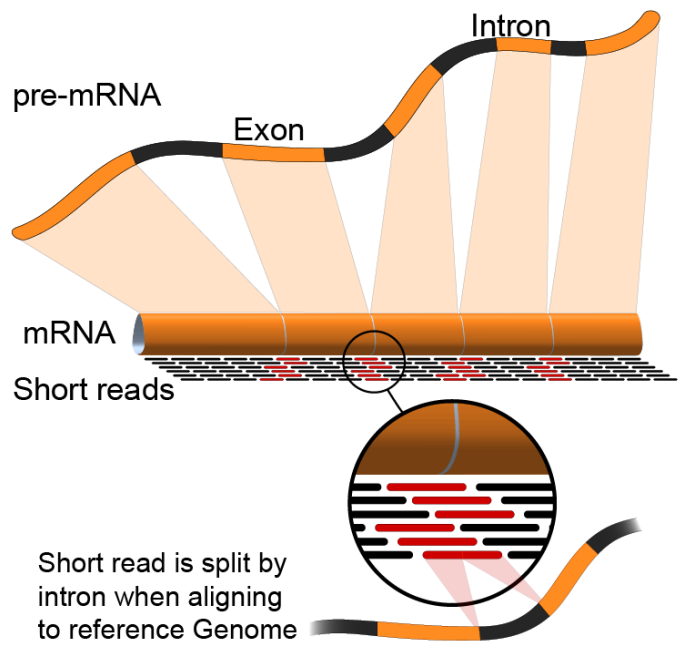
My Hoang, Mariam Khanfar, Chris Miller, Kartik Singhal, Jennie Yao

Advanced Sequencing Technologies & Bioinformatics Analysis November 10-21, 2025





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Introduction to course – philosophy and goals

Do "the bioinformatics" for someone, and you help them for a day.
Teach someone to do bioinformatics, and you help them for a lifetime.

- Ancient Chinese proverb

- Course goals
  - Learn concepts and develop skills for sequence analysis
  - Build the foundation for tackling your own analysis challenges
  - Learn to think like a bioinformatician
  - Have fun

Introductions to Bioinformatics instructors (WashU team)

Malachi Griffith
Professor of Medicine
Professor of Genetics
Assistant Director, MGI

Obi Griffith
Professor of Medicine
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Chris Miller
Associate Professor of Medicine

Felicia Gomez
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Student poll (respond in slack)

Not counting the pre-requisites and materials for this course:

- Do you consider yourself a bioinformatician? Computational biologist?
- Are you familiar with linux/command line?
  - Intermediate?
  - Expert?
- Do you sometimes write code?
- Are you familiar with R?
  - Intermediate?
  - Expert?
- Do you use git/github?
- What organism do you work with? (Put an animal emoji in slack)
- Are you interested in bulk RNAseq, scRNAseq, or both?

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

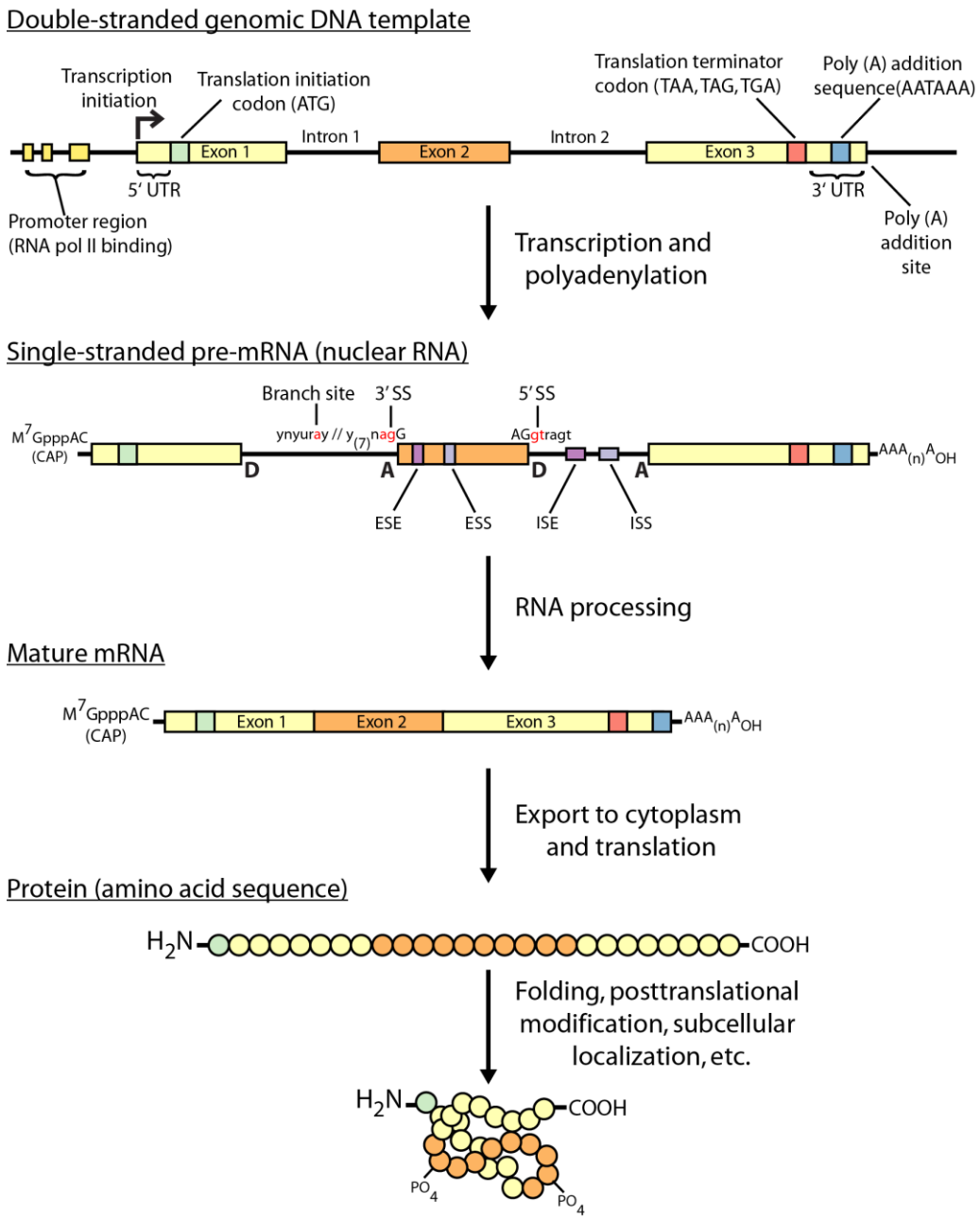
Learning objectives of module 1

- 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

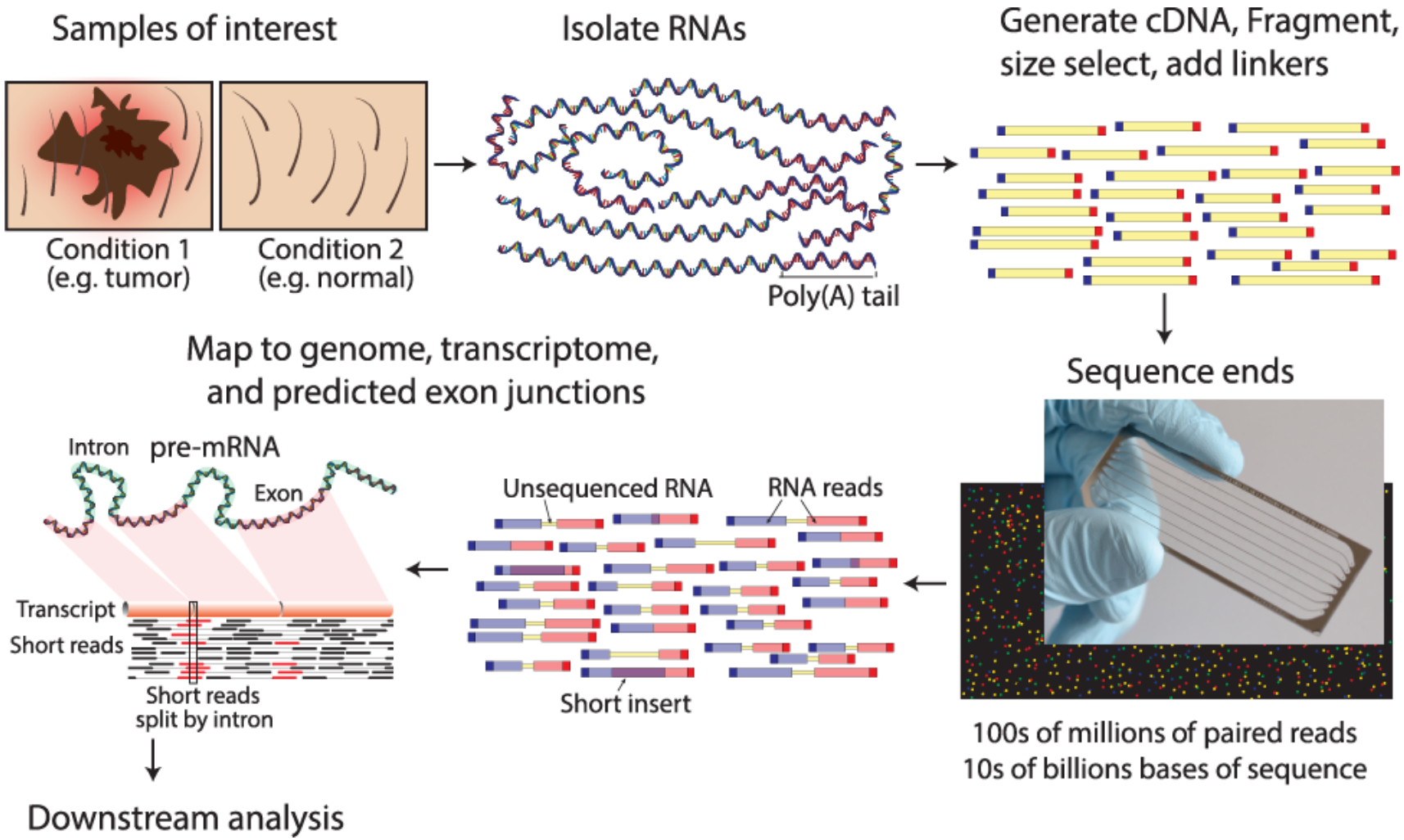
Gene expression (brief review of central dogma)

Thinking about the molecular biology here, what is actually being sequenced in an RNA-seq experiment?

Does it differ depending on the sequencing platform? Or for bulk vs single cell sequencing?



RNA sequencing overview



MPS (NGS) Platforms: Illumina is currently dominant

Production-scale sequencers

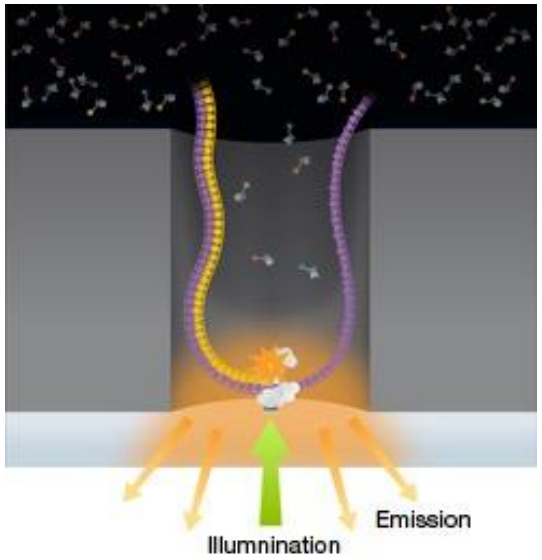
Key specifications	 NextSeq 1000 and 2000 Systems	 NovaSeq 6000 System	 NovaSeq X Series
Max output per flow cell	540 Gb[a]	3 Tb[b]	8 Tb[c]
Run time (range)[d]	~8–44 hr	~13–44 hr	~17–48 hr
Max reads per run (single reads)	1.8B[a]	10B (single flow cell)[b] 20B (dual flow cells)	26B (single flow cell)[c] 52B (dual flow cells)[c,e]
Max read length	2 × 300 bp	2 × 250 bp	2 × 150 bp

- Higher accuracy, range of capacity and throughput
- Slightly longer read lengths on some platforms

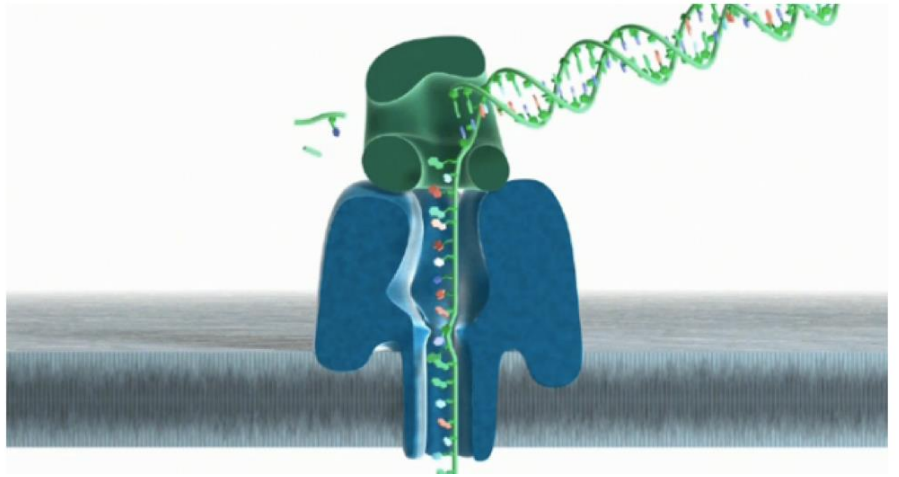
Next-next (3rd) generation sequencing platforms

Defining Characteristics: Long reads (10-100 kb) from single molecules.

Pacific Biosciences: watching a polymerase synthesize DNA/cDNA in real time



Oxford Nanopore: Translocating DNA/RNA through a nanopore with electrode-based detection



The promise: Long reads will allow us to accurately sequence and assemble whole human genomes, from scratch, without using the reference genome.

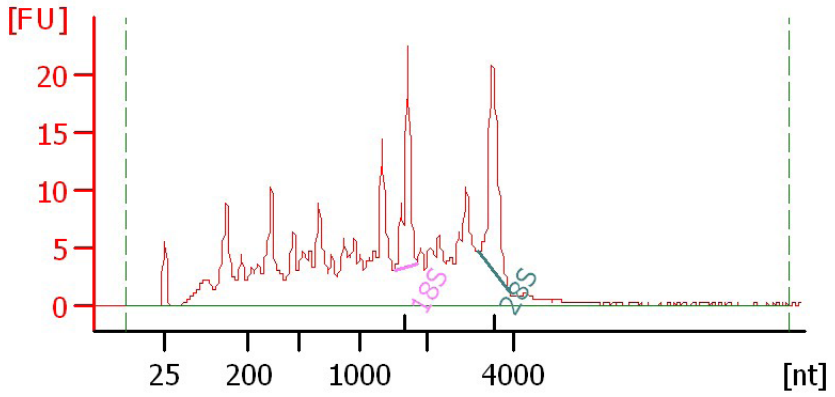
Status: Currently limited by lower throughput, higher base error rate and higher cost. 3rd generation technologies have proven useful, but generally for niche applications so far.

Challenges specific to RNA sequencing

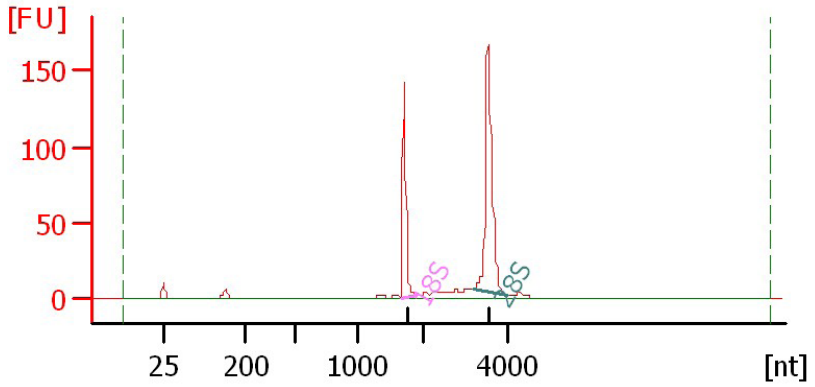
- Sample
  - Purity?, quantity?, quality?
- RNAs consist of small exons that may be separated by large introns
  - Mapping reads to genome is challenging
- The relative abundance of RNAs vary wildly
  - 10^5 – 10^7 orders of magnitude
  - Since RNA sequencing works by random sampling, a small fraction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



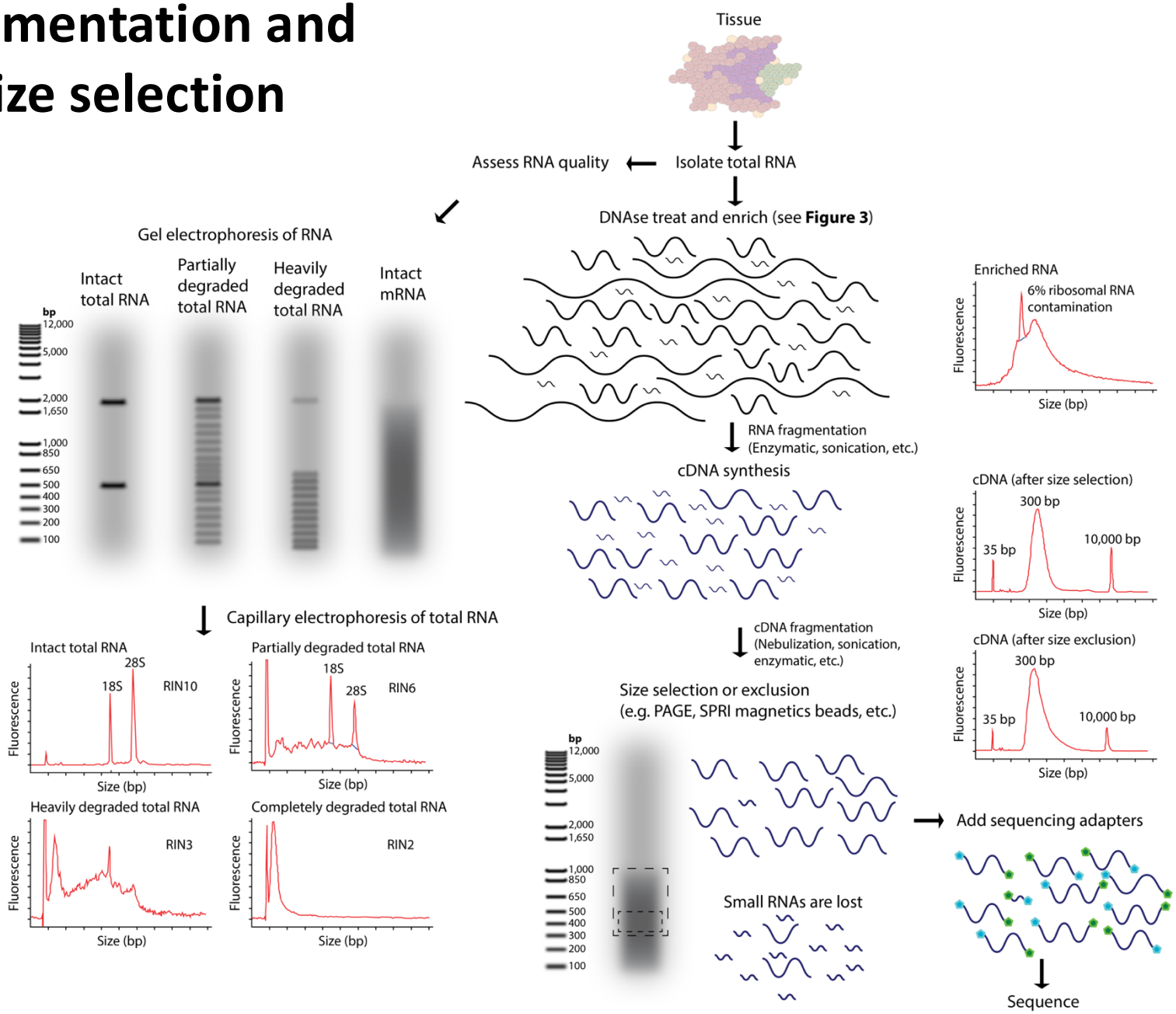
RIN = 10

There are many RNA-seq library construction strategies

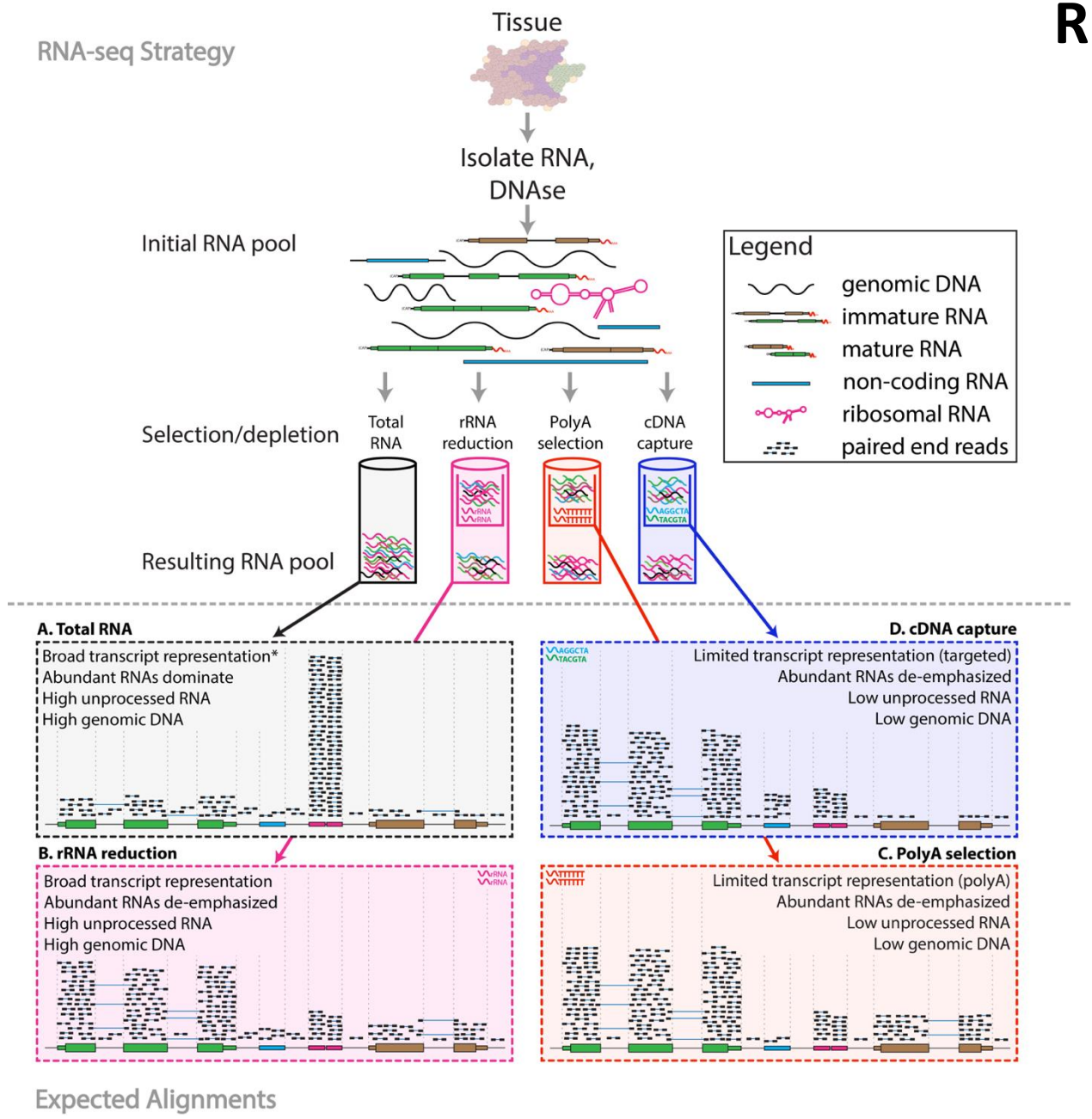
- Total RNA versus polyA+ RNA?
- Ribo-reduction?
- Size selection (before and/or after cDNA synthesis)
  - Small RNAs (microRNAs) vs. large RNAs?
  - A narrow fragment size distribution vs. a broad one?
- Linear amplification?
- Stranded vs. un-stranded libraries
- Library normalization?
  - Exome captured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

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

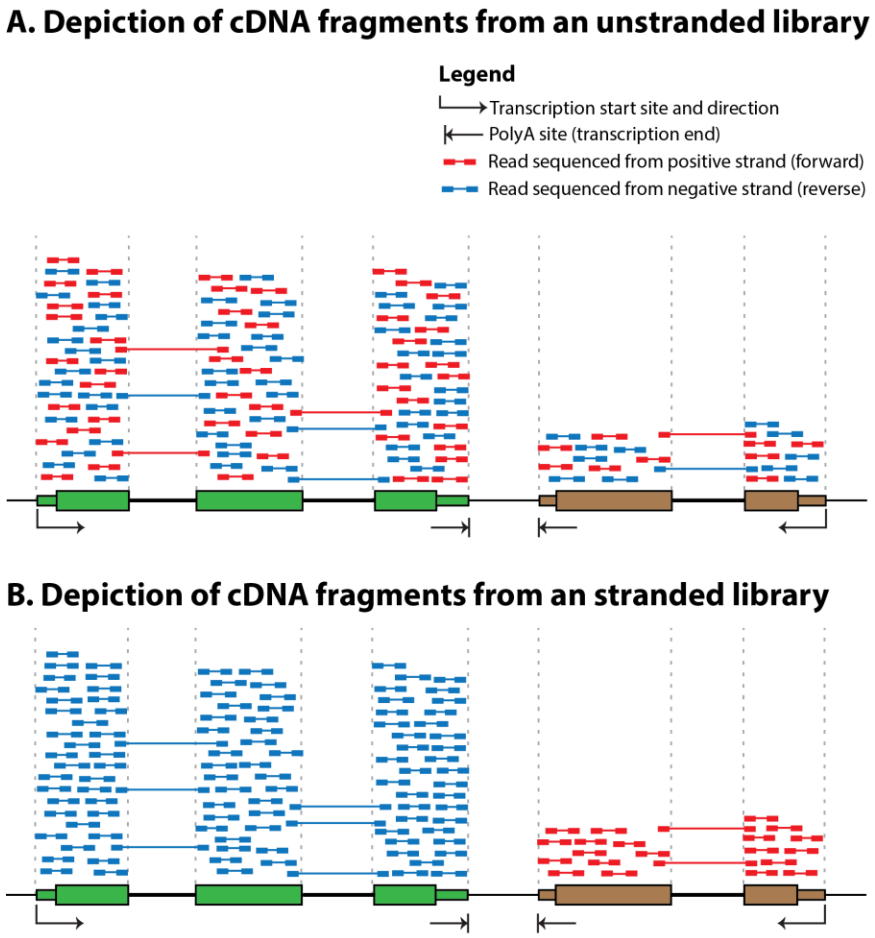
Fragmentation and size selection

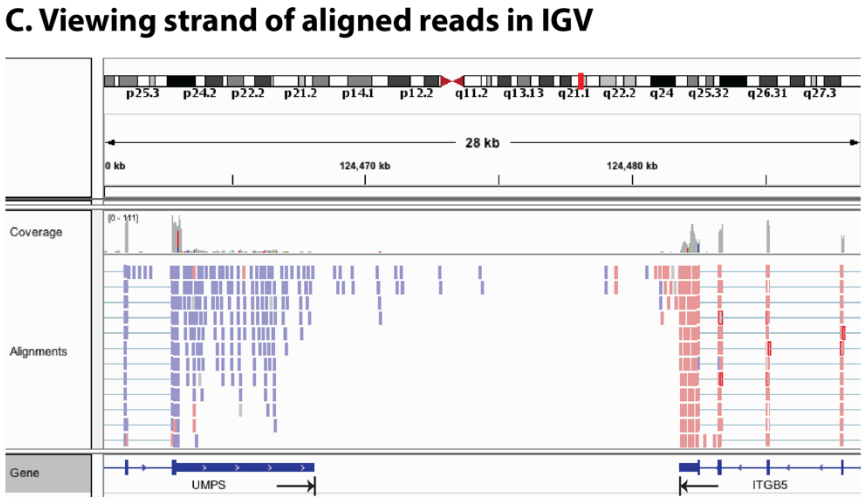


RNA sequence enrichment (selection/depletion)



Stranded vs. unstranded





https://rnabio.org/module-09-appendix/0009/12/01/StrandSettings/
(detailed discussion and cheat sheet)

Ordering RNA-seq data, “coverage”, and cost?

RNA-seq full service, cost per sample[a]	< 12 Samples	≥ 2500 Samples*	~Targeted Coverage
PolyA selection	$287	$215	30M reads
Ribosomal depletion, RiboErase (H/M/R)	$297	$226	30M reads
Ribosomal depletion, FastSelect (H/M/R)	$268	$205	30M reads
Ribosomal depletion, FastSelect (H/M/R+Globin)	$272	$213	30M reads
Ribosomal depletion, Watchmaker (H/M/R+Globin)	$291	NA	30M reads
Low input - Takara SMARTseq mRNA	$267	$203	30M reads
Low input - Sigma Seqplex	$273	$202	30M reads

- An example menu from a sequencing core facility (circa 2024)
- Options primarily relate to method of enrichment and input amounts
- “Coverage” is a non-intuitive concept in bulk-RNAseq.
  - 30M reads is sufficient for gene abundance estimation (increase for other applications)

Common analysis goals of RNA-Seq analysis (what can you ask of the data?)

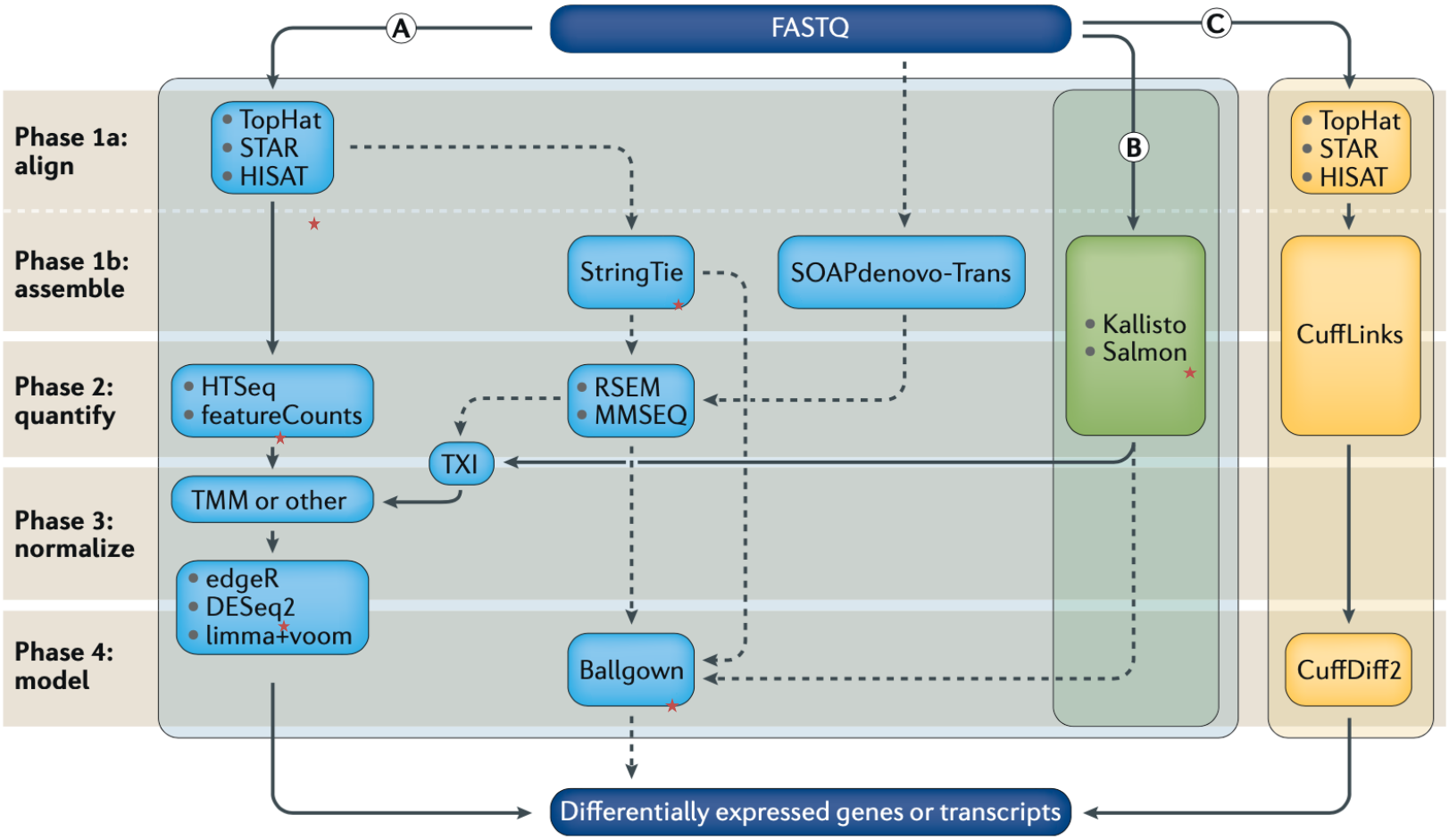
- Gene expression and differential expression
- Alternative expression analysis
- Transcript discovery and annotation
- Allele specific expression
  - Relating to SNPs or mutations
- Mutation discovery
- Fusion detection
- RNA editing

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. ‘stringtie’ for expression analysis, ‘star-fusion’ for fusion detection, etc.
4. Post process
   - Import into downstream software (R, python, WebGestalt, etc.)
5. Summarize and visualize
   - Create gene lists, prioritize candidates for validation, etc.

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



[RNA sequencing: the teenage years]

★ Covered in rnabio.org

Discussion of bulk vs single cell RNA-seq

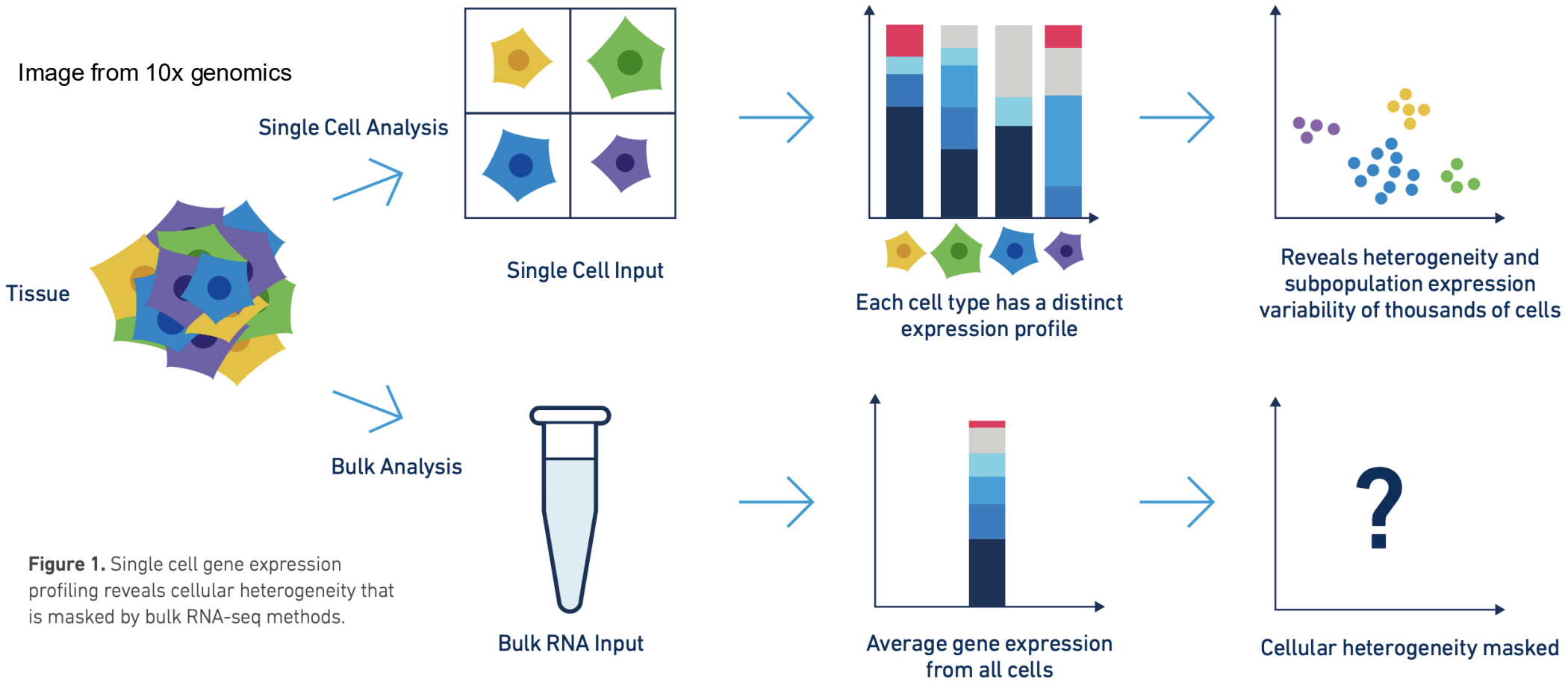
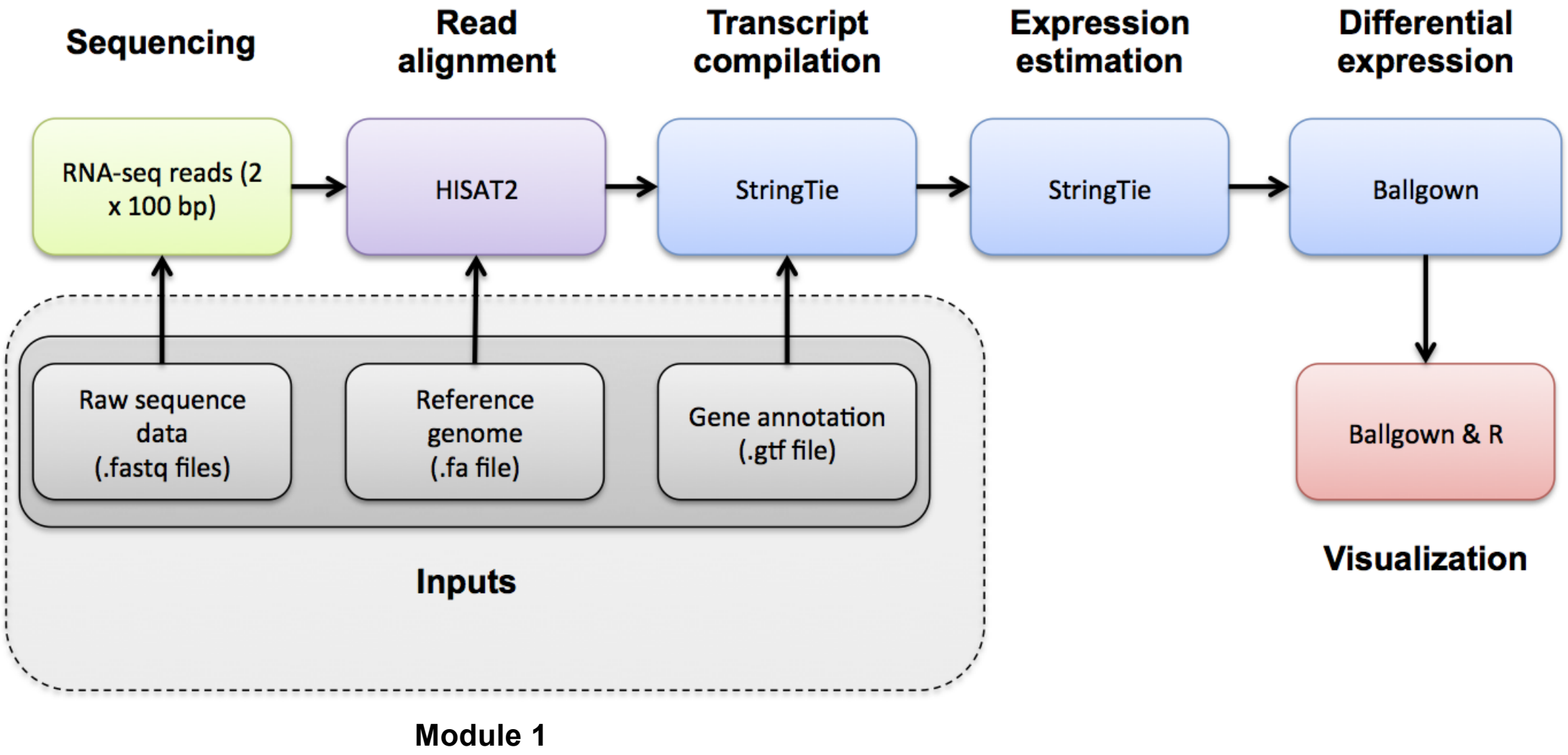


Figure 1. Single cell gene expression profiling reveals cellular heterogeneity that is masked by bulk RNA-seq methods.

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

Introduction to tutorial (Module 1)

HISAT2/StringTie/Ballgown RNA-seq Pipeline



Bioinformatics troubleshooting cheat sheet

- ❑ Check your inputs!
- ❑ Mix of incompatible reference genomes used (see this tutorial)
- ❑ Mix of incompatible gene/transcript identifiers
- ❑ Reference sequence names (e.g. “1” vs “chr1”)
- ❑ 1-based vs 0-based coordinates (see this tutorial)
- ❑ Computational tasks fail due to resource limitations (memory and storage)
- ❑ Dependency hell for bioinformatics tools. Learn to use containers (e.g. docker) or environment managers (e.g. conda)

We are on a Coffee Break & Networking Session

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