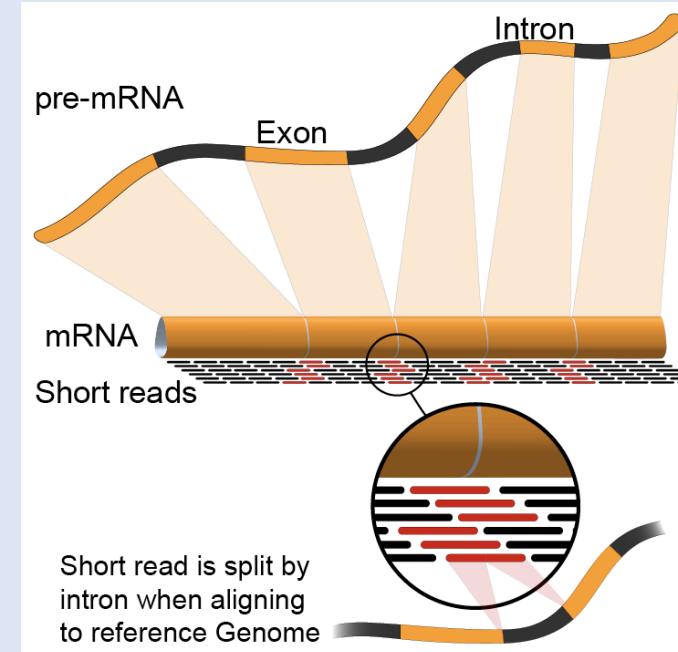
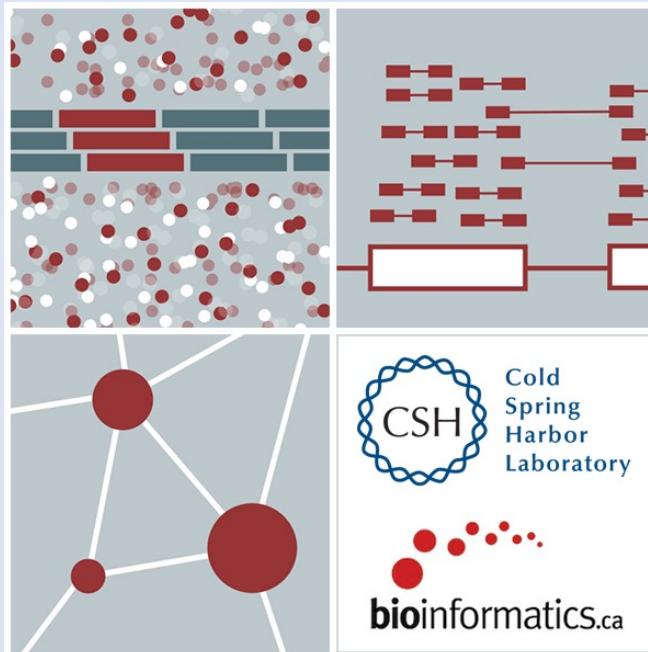




Cancer
Research
Institute™

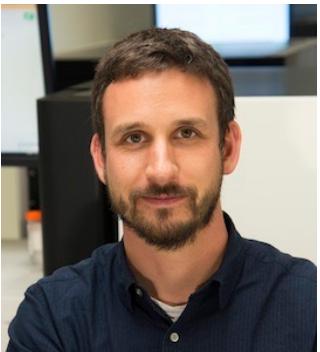
Introduction to bulk DNA/RNA sequencing

Kelsy Cotto, Malachi Griffith, Obi Griffith, Charles Hayfron-Benjamin, Evelyn Schmidt, Kartik Singhal, Zach Skidmore
CRI Bioinformatics Workshop. Apr 27-May 2, 2024



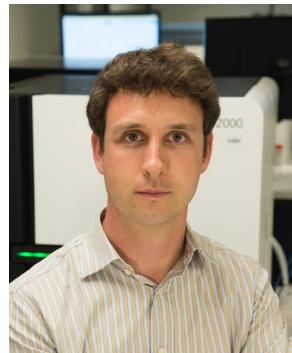
Washington University in St. Louis
SCHOOL OF MEDICINE

Introductions to Bioinformatics instructors (WashU team)



Malachi Griffith

Associate Professor of Medicine
Associate Professor of Genetics
Assistant Director, MGI



Obi Griffith

Associate Professor of Medicine
Associate Professor of Genetics
Assistant Director, MGI



griffithlab.org

rnabio.org genviz.org pmbio.org



Kelsy Cotto
Staff Scientist



Charles Hayfron-Benjamin
Visiting Scholar



Evelyn Schmidt
Analyst

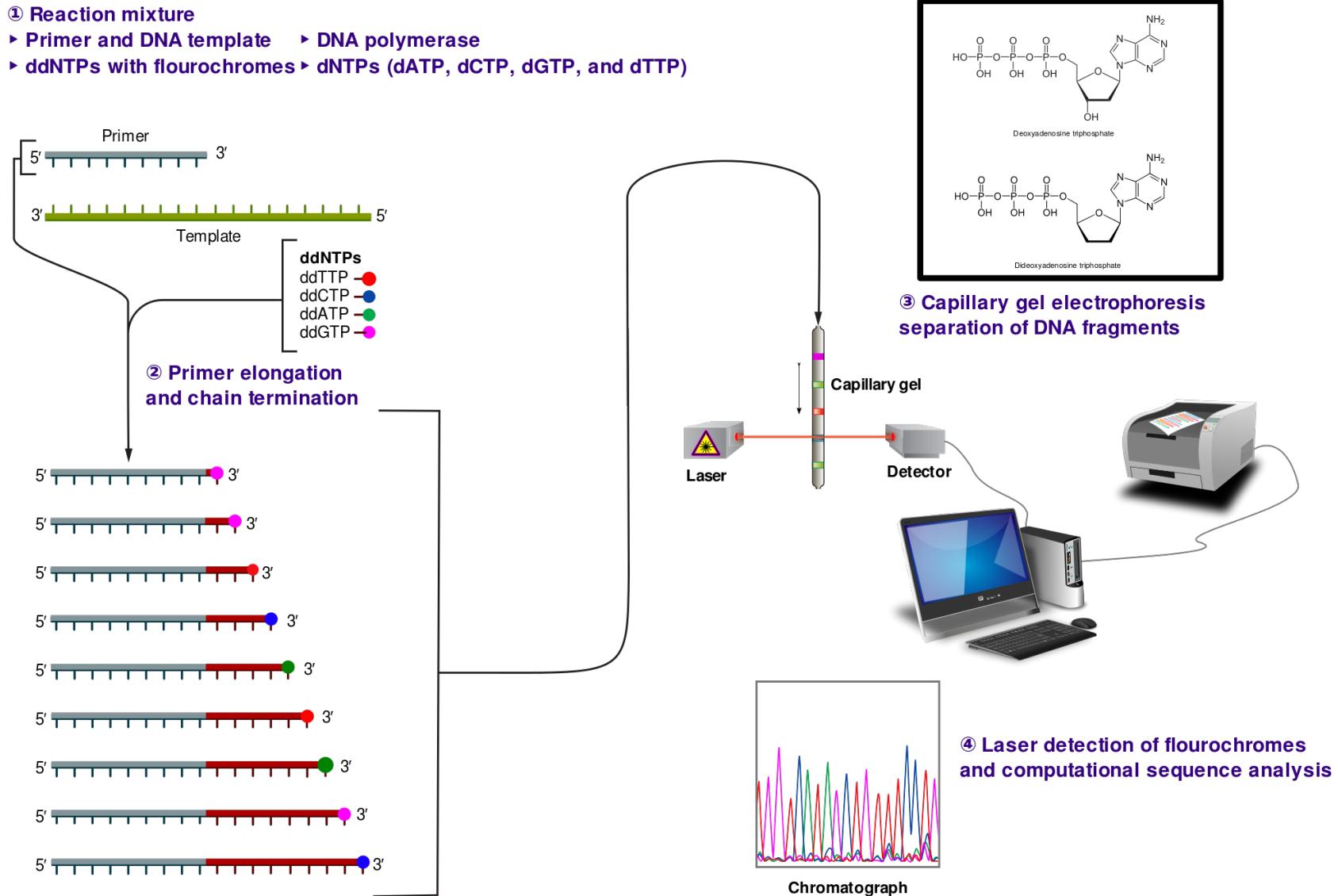


Zach Skidmore
Staff Scientist



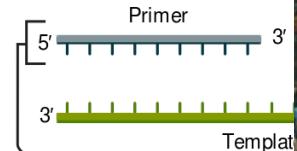
Kartik Singhal
Graduate Student

DNA/RNA analysis began with (Sanger) sequencing

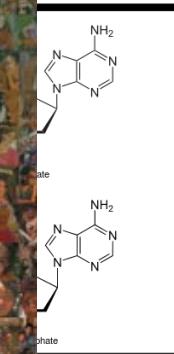
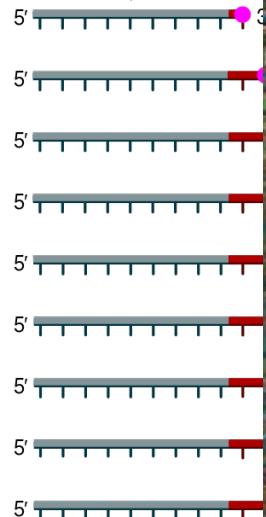


DNA/RNA analysis sequencing

- ① Reaction mixture
- Primer and DNA template
- ddNTPs with flourochromes



- ② Primer extension

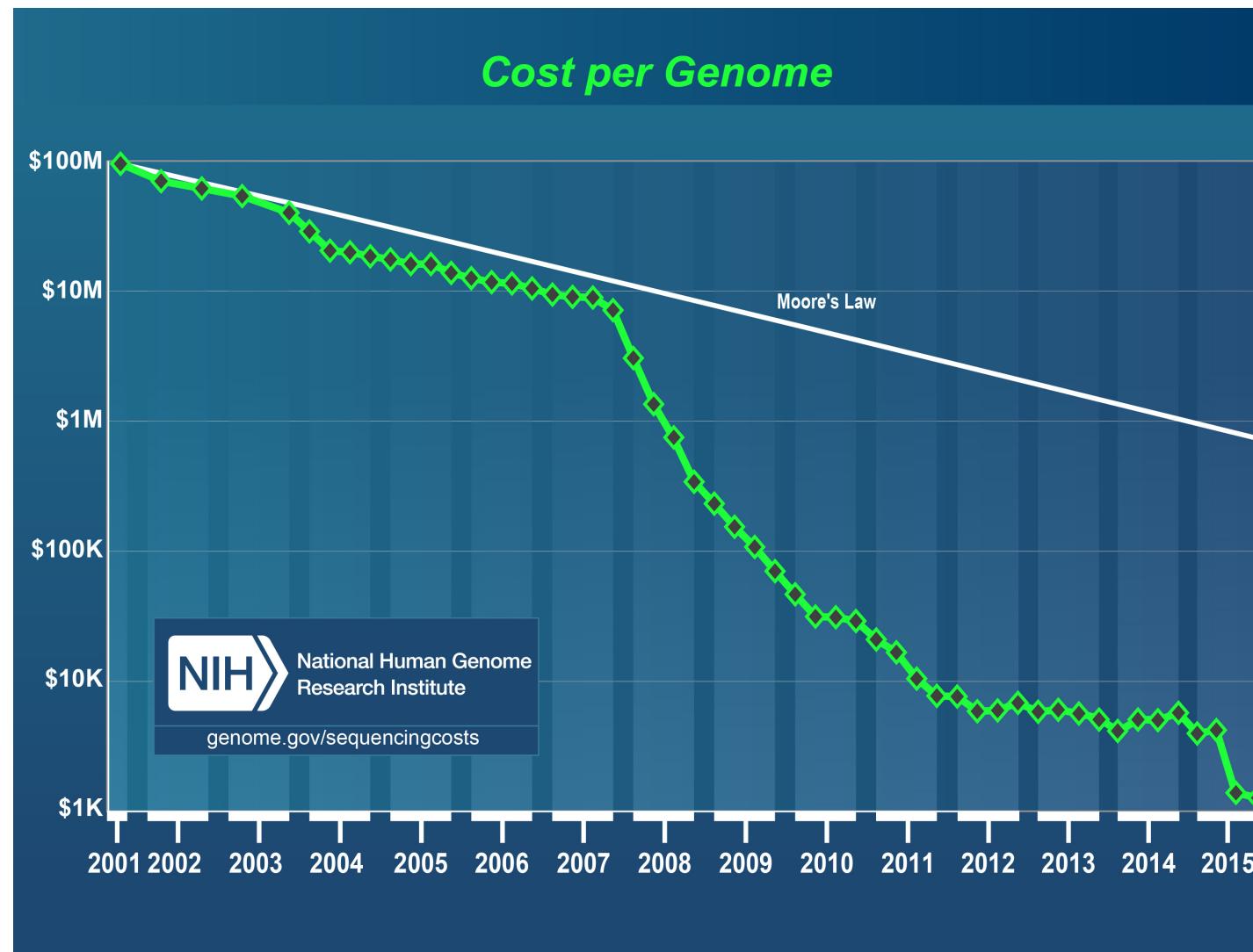


- Fluorophores
- Phosphates



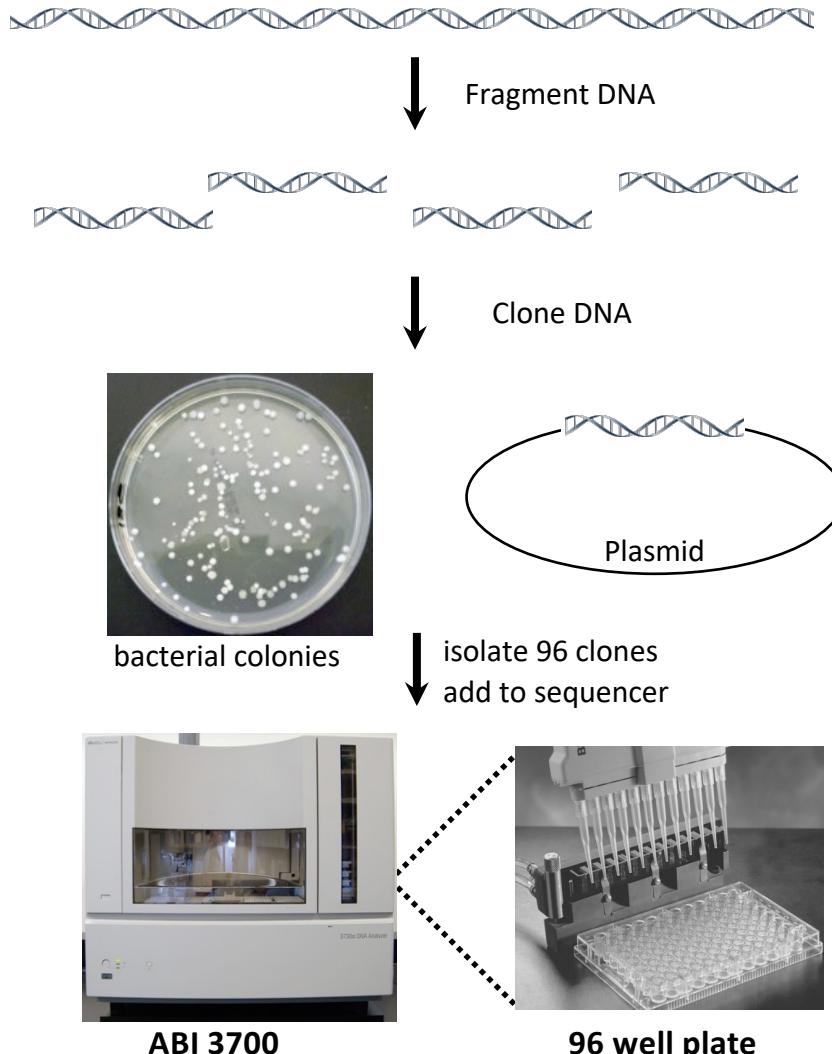
- Resolution of fluorochromes
- Automated sequence analysis

Costs of sequencing have plummeted from ~\$100M to ~\$1000 per human genome

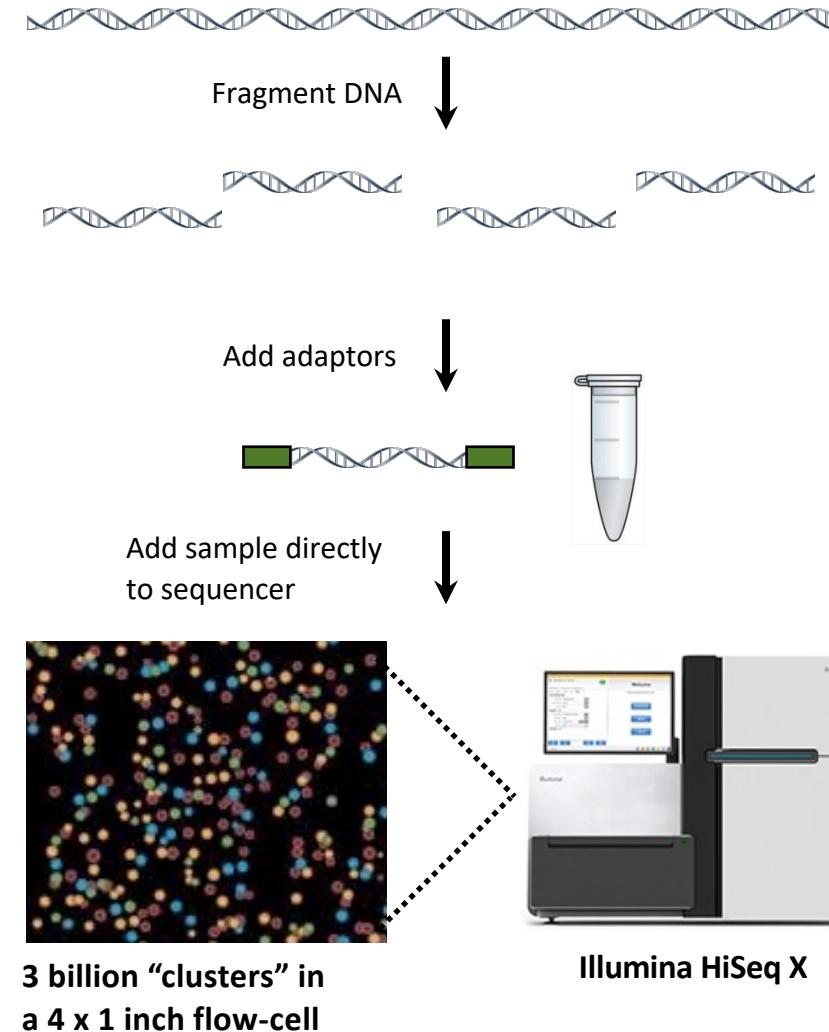


What changed? Level of parallelism

First Generation:



Second/next Generation:

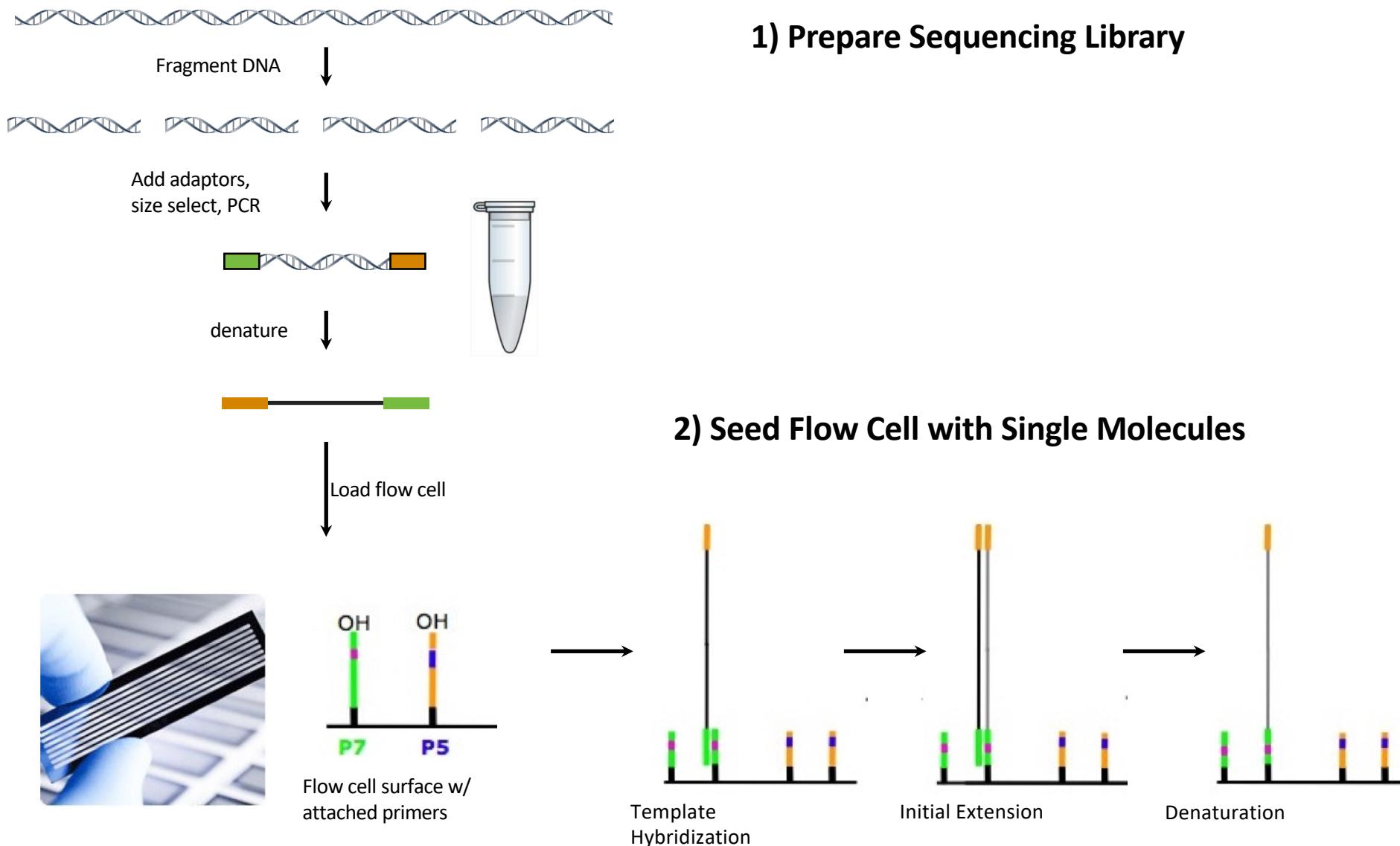


MPS (NGS) Platforms: Illumina is currently dominant

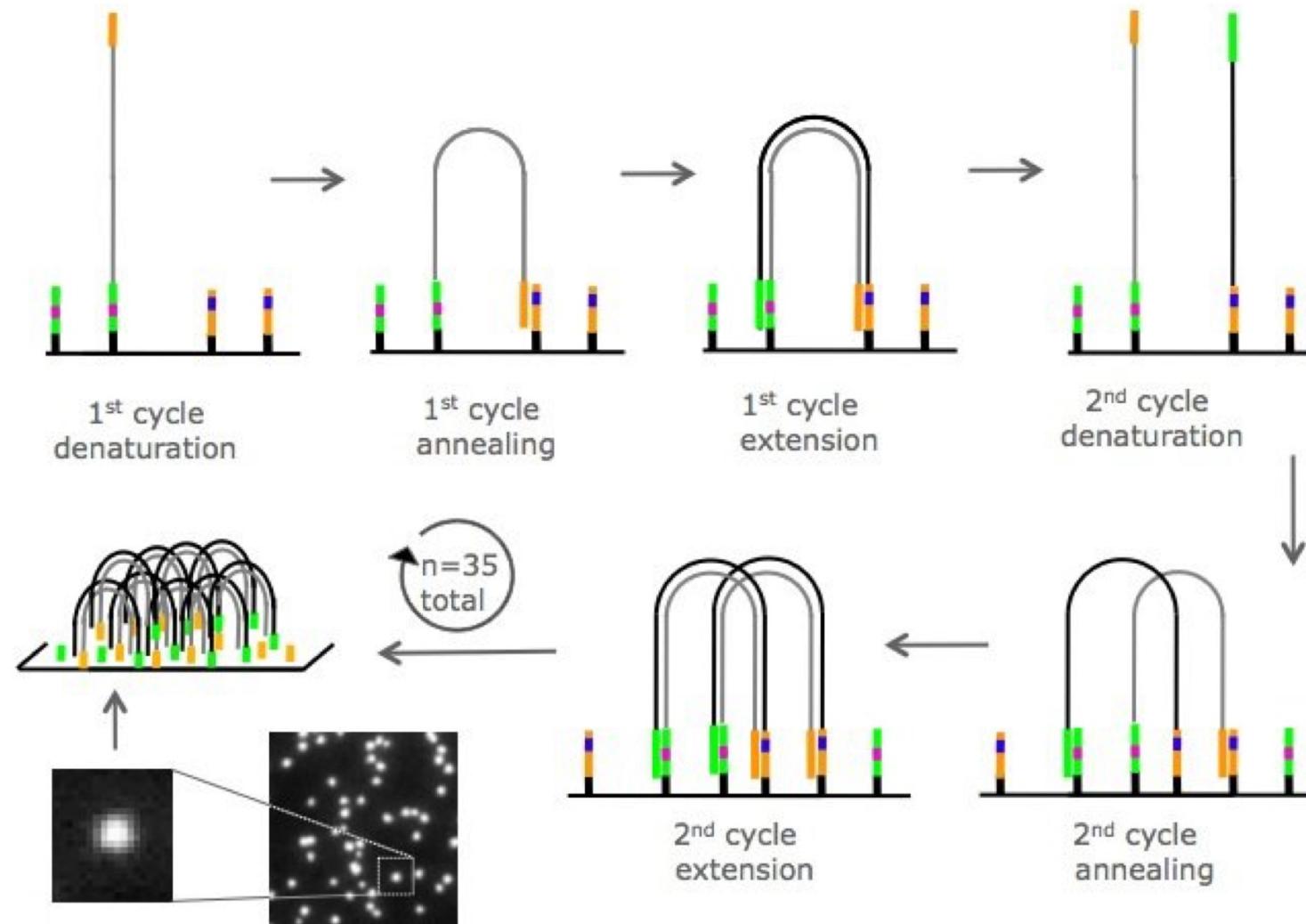
	NextSeq Series [⊕]	HiSeq 4000 System	HiSeq X Series [‡]	NovaSeq 6000 System
Popular Applications & Methods	Key Application	Key Application	Key Application	Key Application
Large Whole-Genome Sequencing (human, plant, animal)				
Small Whole-Genome Sequencing (microbe, virus)				
Exome Sequencing				
Targeted Gene Sequencing (amplicon, gene panel)				
Whole-Transcriptome Sequencing				
Gene Expression Profiling with mRNA-Seq				
miRNA & Small RNA Analysis				
DNA-Protein Interaction Analysis				
Methylation Sequencing				
Shotgun Metagenomics				
Run Time	12–30 hours	< 1–3.5 days	< 3 days	 ~13 - 38 hours (dual SP flow cells) ~13–25 hours (dual S1 flow cells) ~16–36 hours (dual S2 flow cells) ~44 hours (dual S4 flow cells)
Maximum Output	120 Gb	1500 Gb	1800 Gb	6000 Gb
Maximum Reads Per Run	400 million	5 billion	6 billion	20 billion
Maximum Read Length	2 × 150 bp	2 × 150 bp	2 × 150 bp	2 × 250**

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

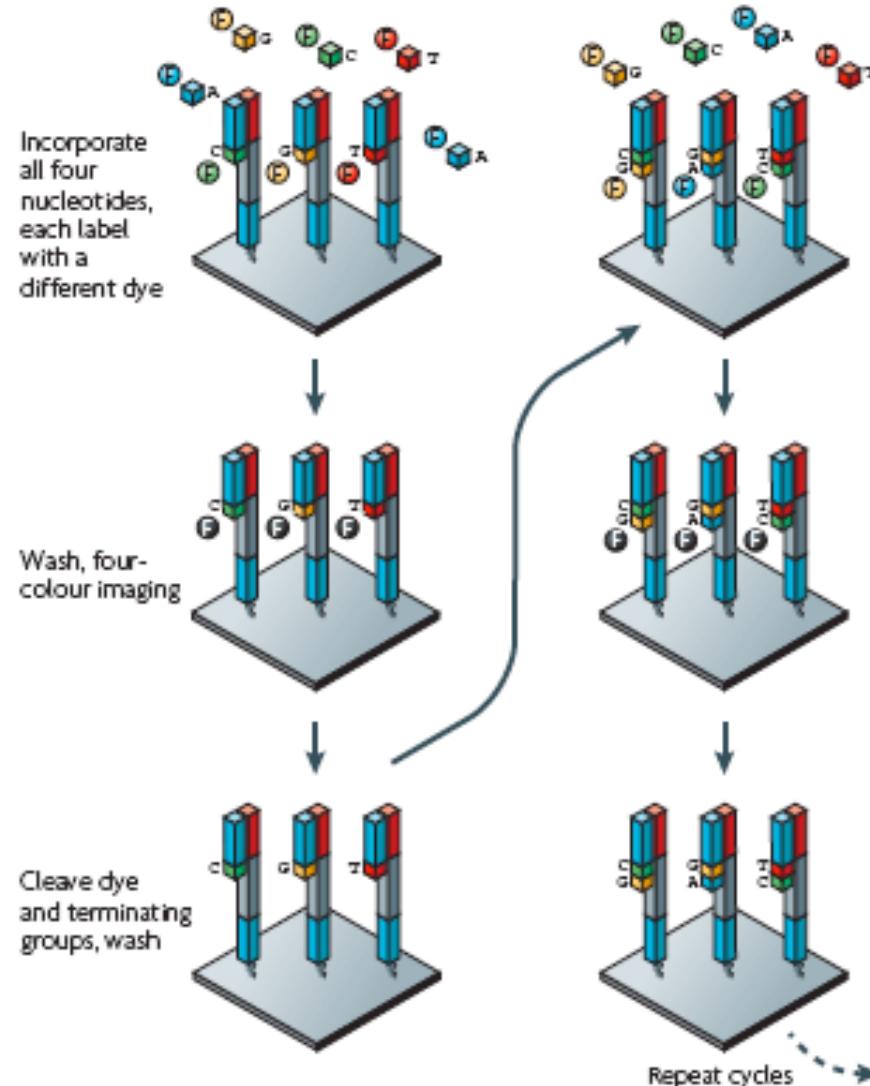
Illumina sequencing trick #1: immobilizing single molecules on a solid surface



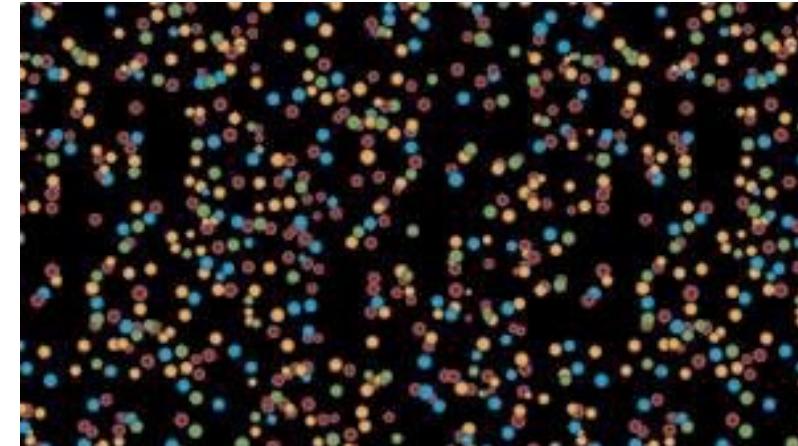
Illumina sequencing trick #2: cluster amplification by solid-phase “bridge” PCR



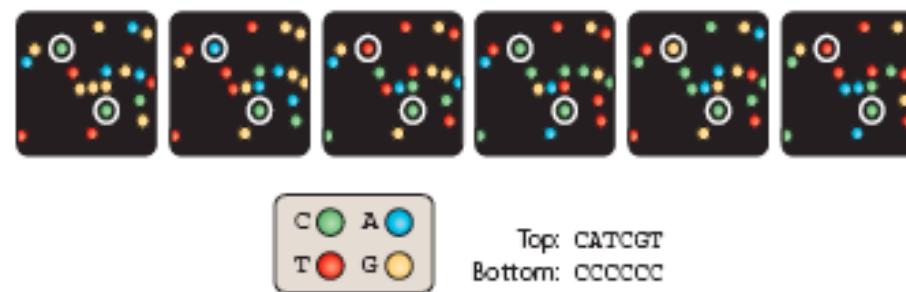
Illumina sequencing trick #3: base-by-base DNA synthesis with 4 dyes & reversible terminators



4 images merged



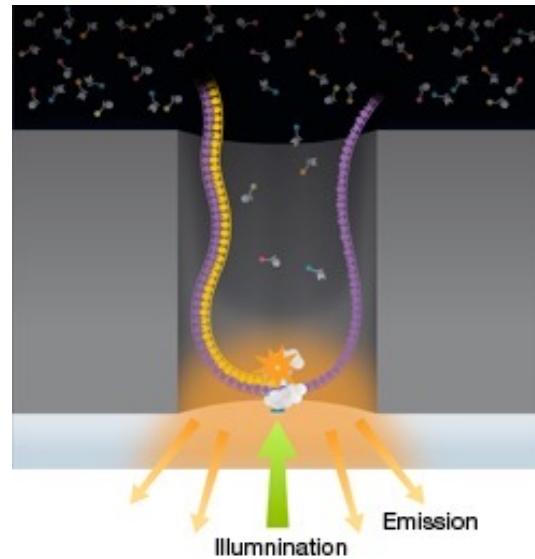
6 cycles w/ base-calling



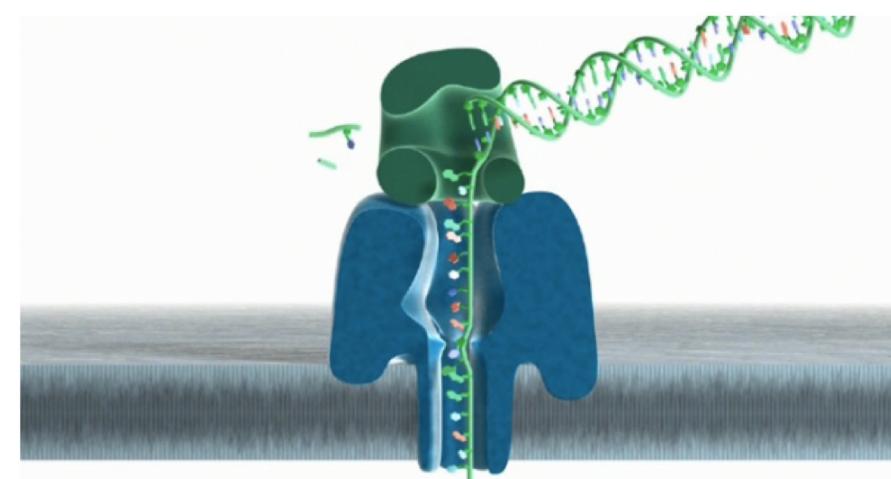
Next-next (3rd) generation sequencing platforms

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

Pacific Biosciences: watching a polymerase synthesize DNA in real time



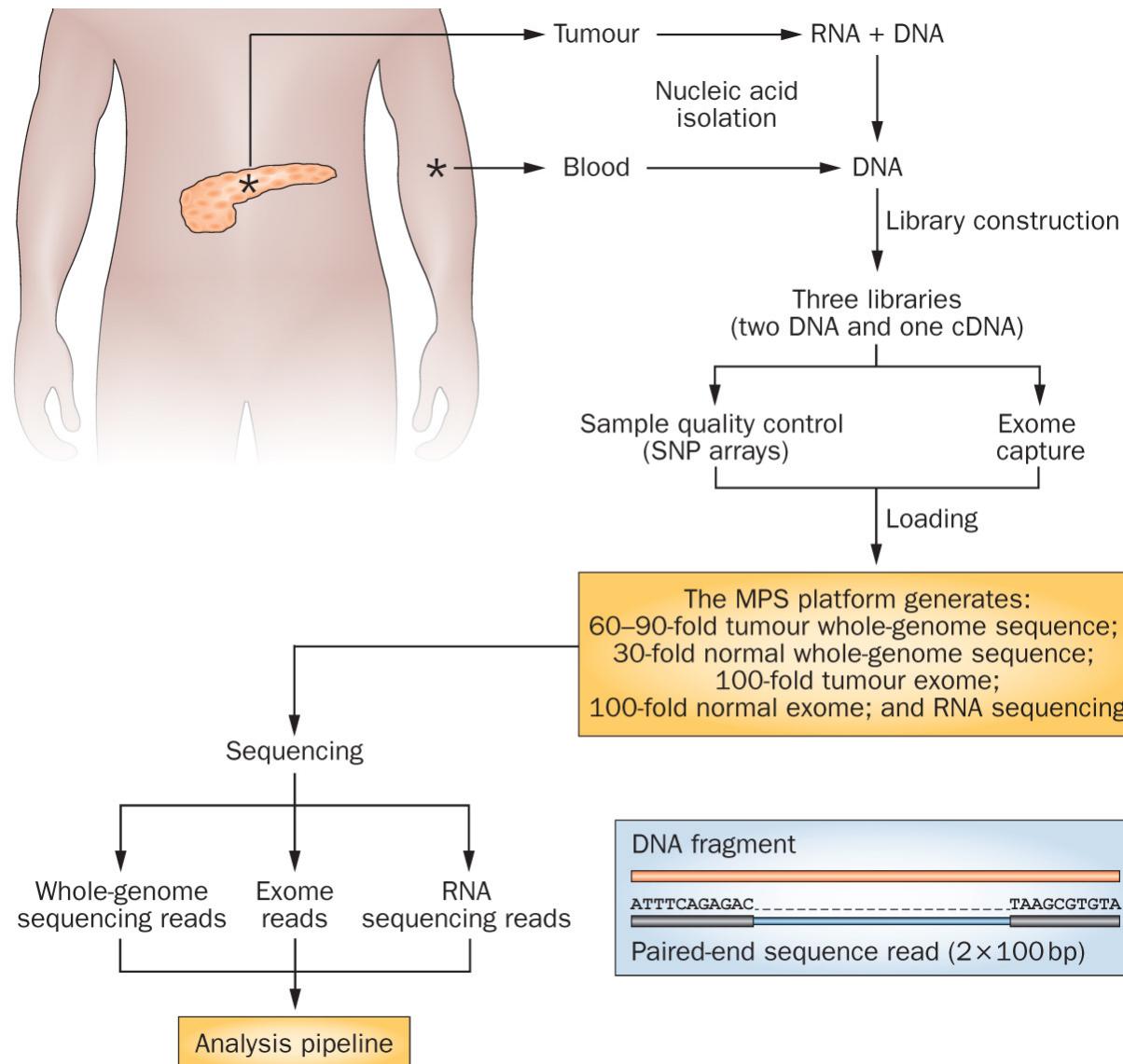
Oxford Nanopore: Translocating DNA through a nanopore with electrode-based detection



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

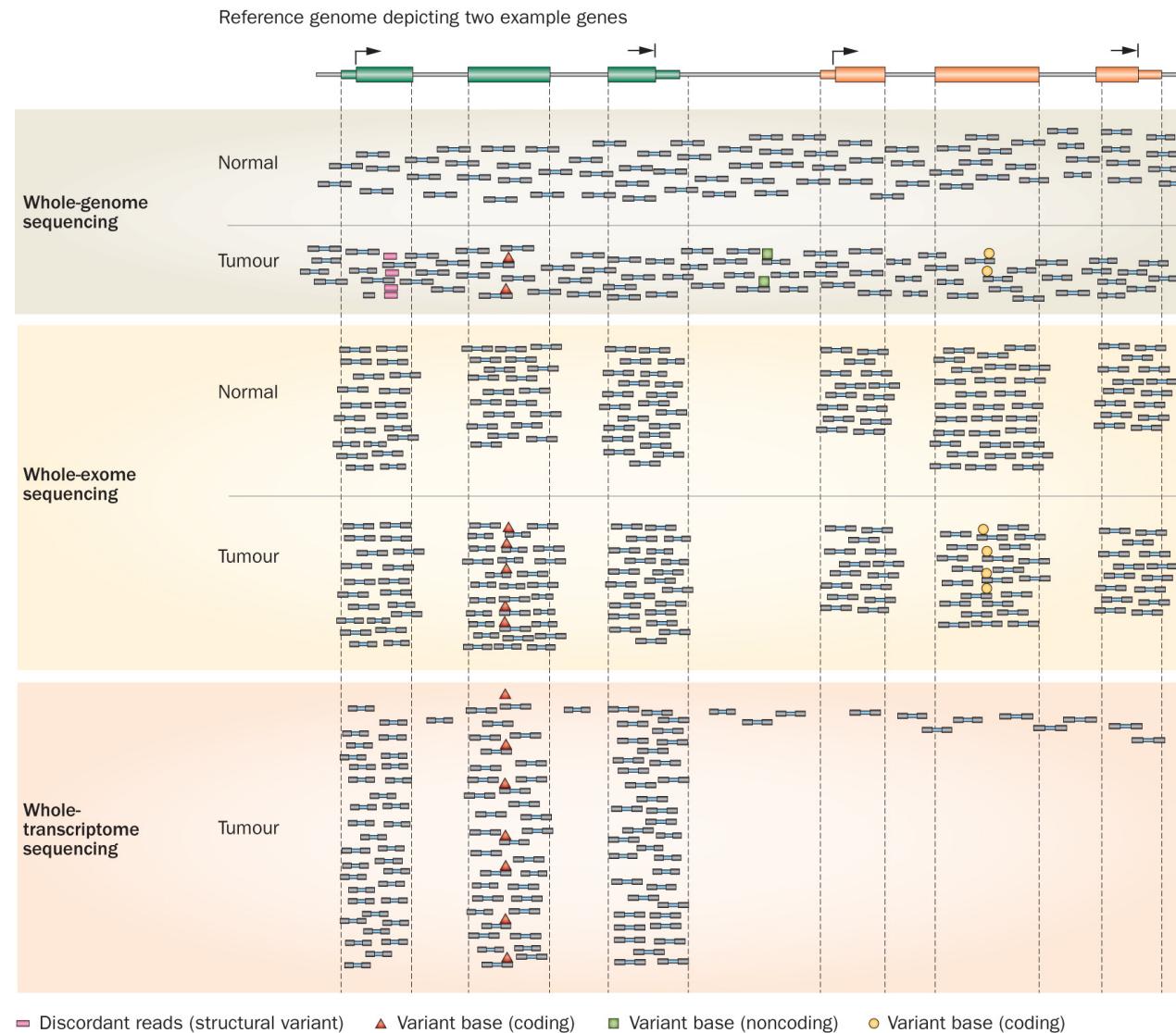
Status: Currently limited by low throughput, high error rate (10-15%) and high cost (>\$50,000 per human genome). 3rd generation technologies have proven useful, but generally for niche applications so far.

Cancer genomics research has exploded with the rapid advances in DNA sequencing technologies



Whole genome, exome and transcriptome allow us to detect and confirm many molecular alteration types

'WGS'
'Exome'
'RNA-seq'



Many other omic approaches exist

Envirome	Enviromics	Gene related environment factors (envirome)		
Environmental DNA	Environmental omics	Sequencing of ambient DNA		
Epigenome	Epigenomics	Epigenetic modifications	Molecular genetics	Epigenomics is the study of the complete set of epigenetic modifications on the genetic material of a cell, collectively known as the epigenome
Exposome (2005)	Exposomics	An individual's environmental exposures, including in the prenatal environment	Molecular genetics	A proposed term and field of study of the disease-causing effects of environmental factors (the "nurture" component of "nature vs. nurture"). ^[5]
Exposome (2009)		Composite occupational exposures and occupational health problems	Occupational safety and health	The proposers of this term were aware of the previous term as used above but proposed to apply the term to a new field. ^{[6][7]}
Exome	Exomics	Exons in a genome	Molecular Genetics	
Foodome	Foodomics	Food and Nutrition issues related to bioactivity, quality, safety and traceability of foods through the application and integration of advanced omics technologies to improve consumer's well-being, health, and confidence.	Nutrition	The term was first defined in 2009 ^[8]
Genome	Genomics (Classical genetics)	Genes (DNA sequences/Chromosomes)	Genetics	"Genome" refers to the set of all genes in an organism. However, "genome" was coined decades before it was discovered that most DNA is "non-coding" and not part of a gene; thus, "genome" originally referred to the entire collection of DNA within an organism. Today, both definitions are used, depending on the

Nearly 50 “omics” topics in biology defined:

https://en.wikipedia.org/wiki/List_of_omics_topics_in_biology

How does it work? Short read alignments are the currency of DNA/RNA analysis



- Alignment is about fitting individual pieces (reads) into the correct part of the puzzle
- The human genome project gave us the picture on the box cover (the reference genome)
- Imperfections in how the pieces fit can indicate damage or variation in picture

Reference:

AGCCTGAGACCGTAAAAAA**AGTCAAG**

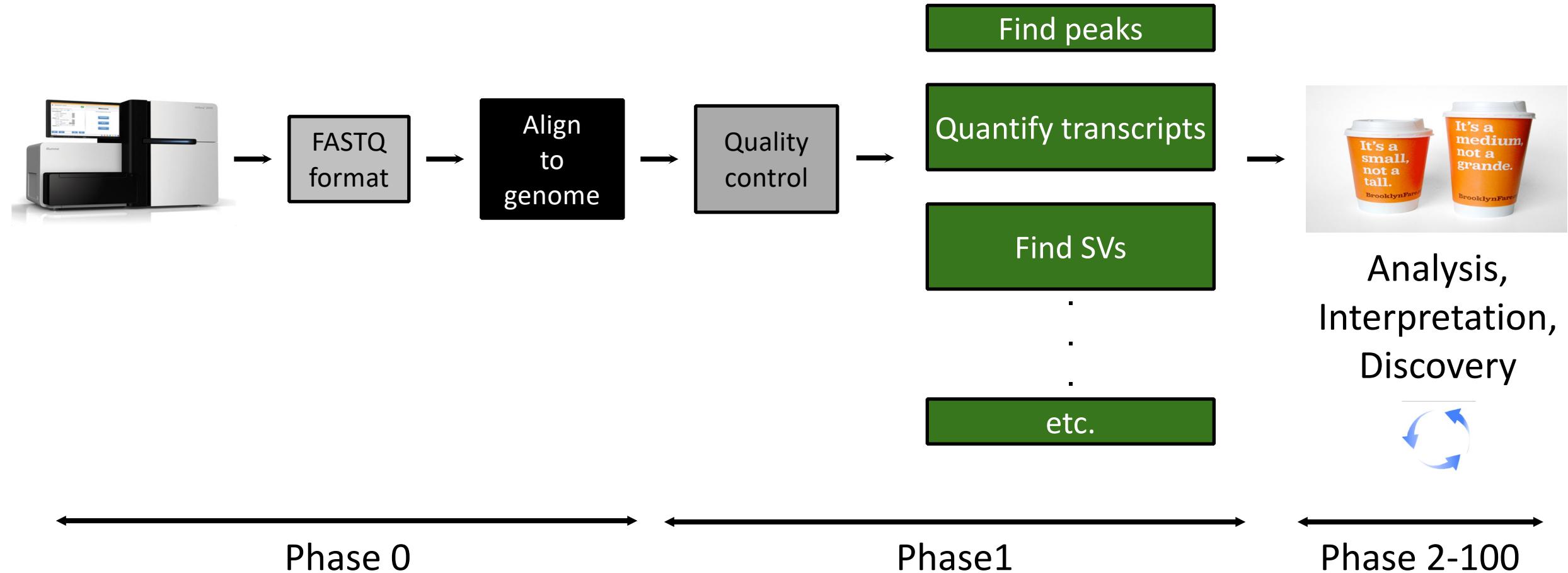
|||||||||||||||||

A read sequence:

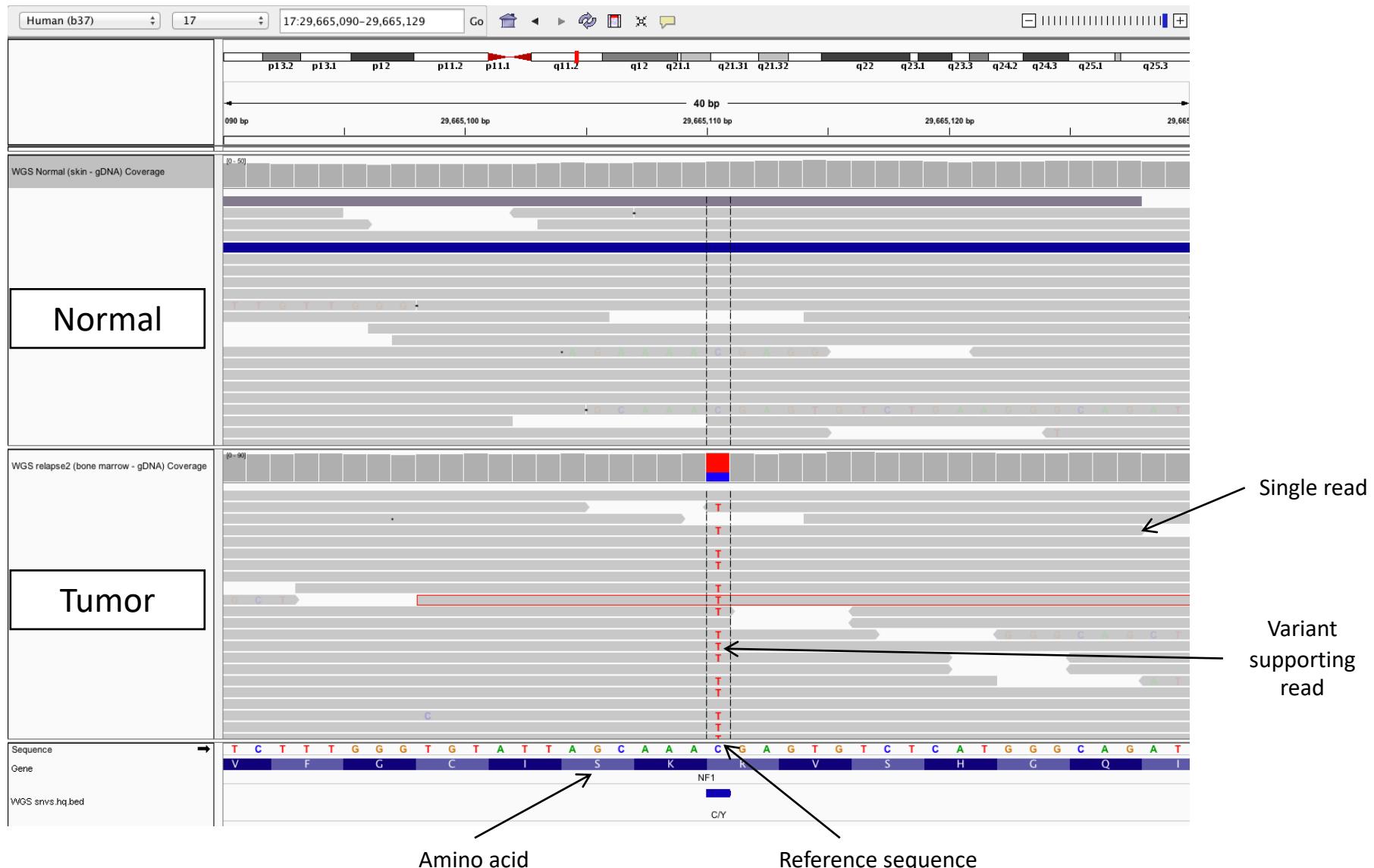
GAGACCGTAAAAAA**CGTC**



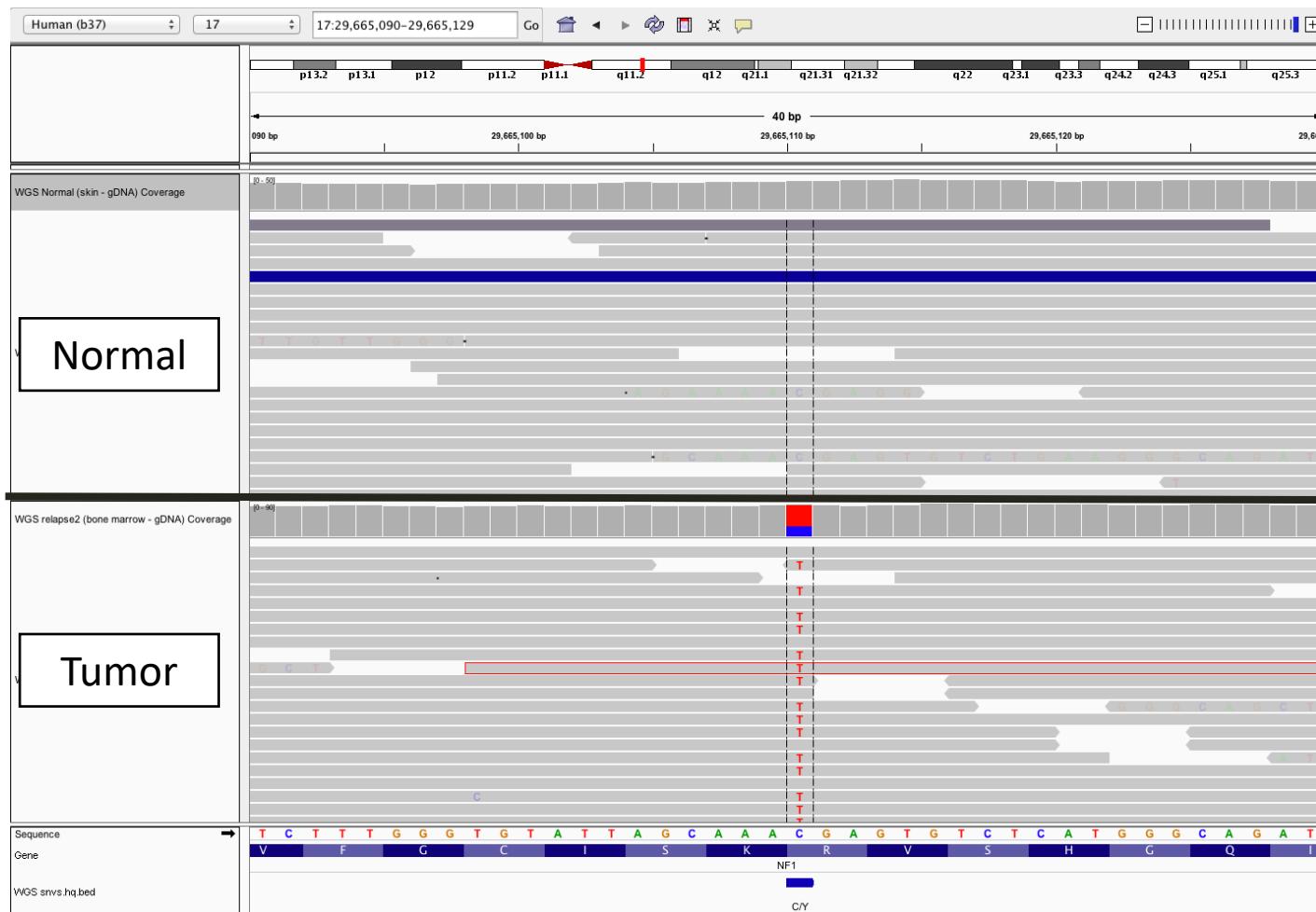
Alignment is central to most genomics research



Single nucleotide variants (SNVs) and insertions/deletions (indels) appear as short alignment discrepancies from reference genome



Variant allele fraction (VAF) and coverage



VAF = Variant reads / Total reads

Coverage track

VAF = 0/20 = 0%

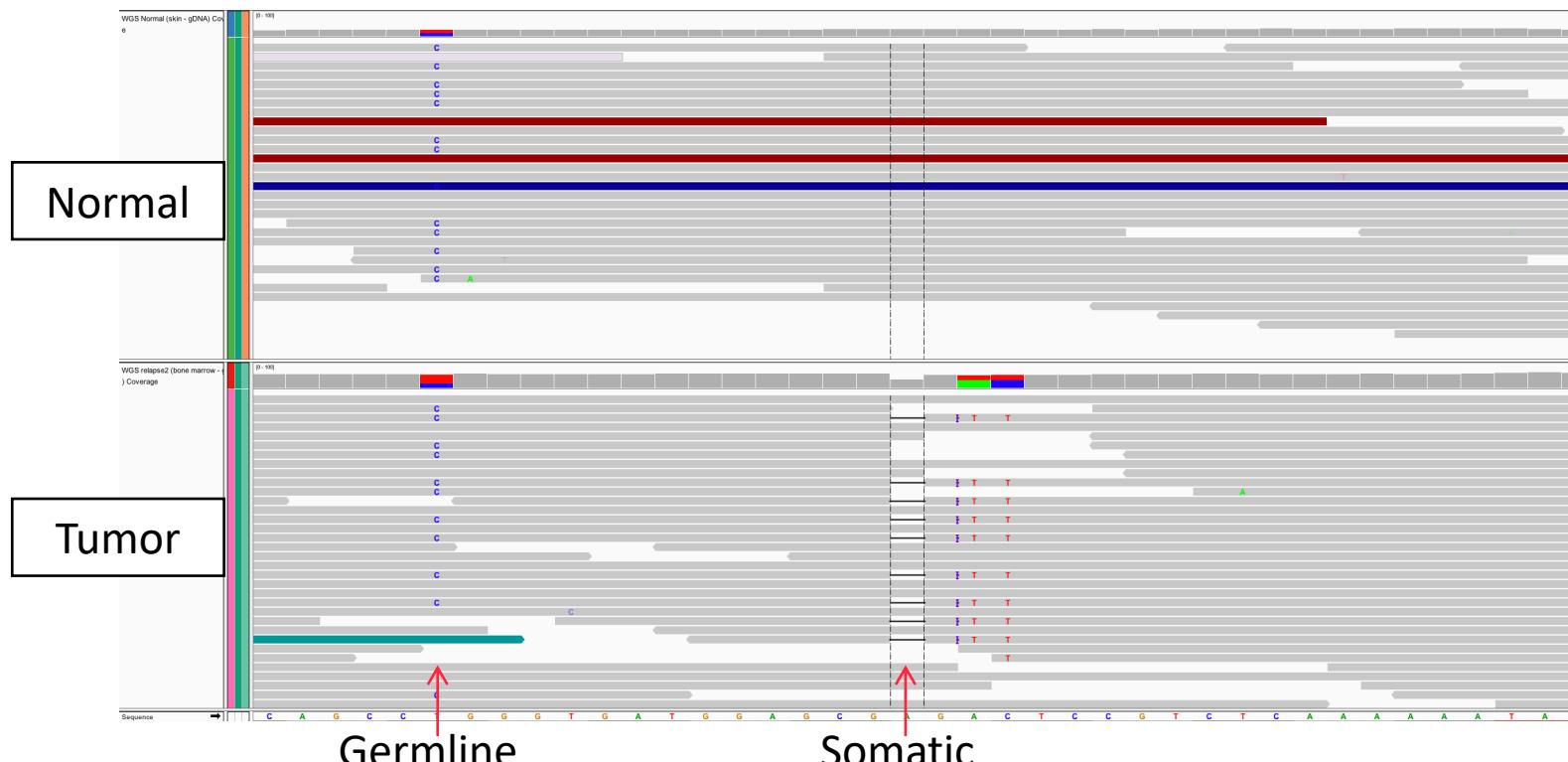
VAF = 14/20 = 70%

Total coverage=20X

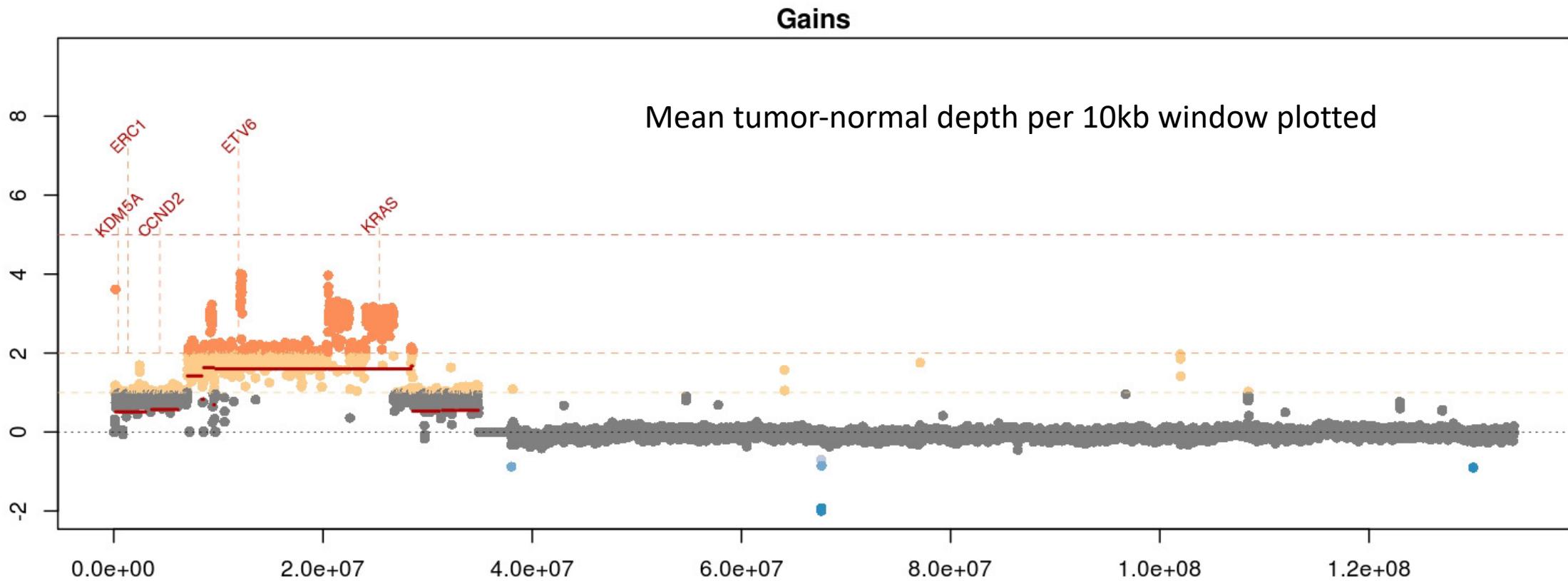
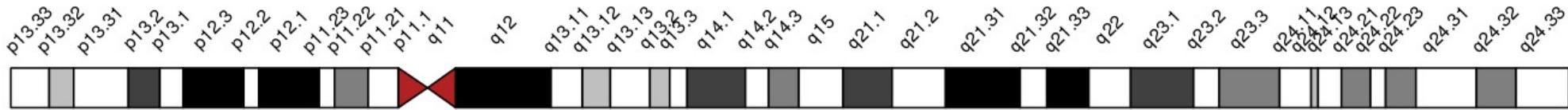
Recall that we have two copies of our genome ($2n$). Mutations are typically in one copy. A heterozygous variant (one copy) is expected to have VAF = 50%. Often not true due to sample purity, tumor heterogeneity, sampling error, alignment issues, copy number variation, etc.

Both somatic and germline mutations are important

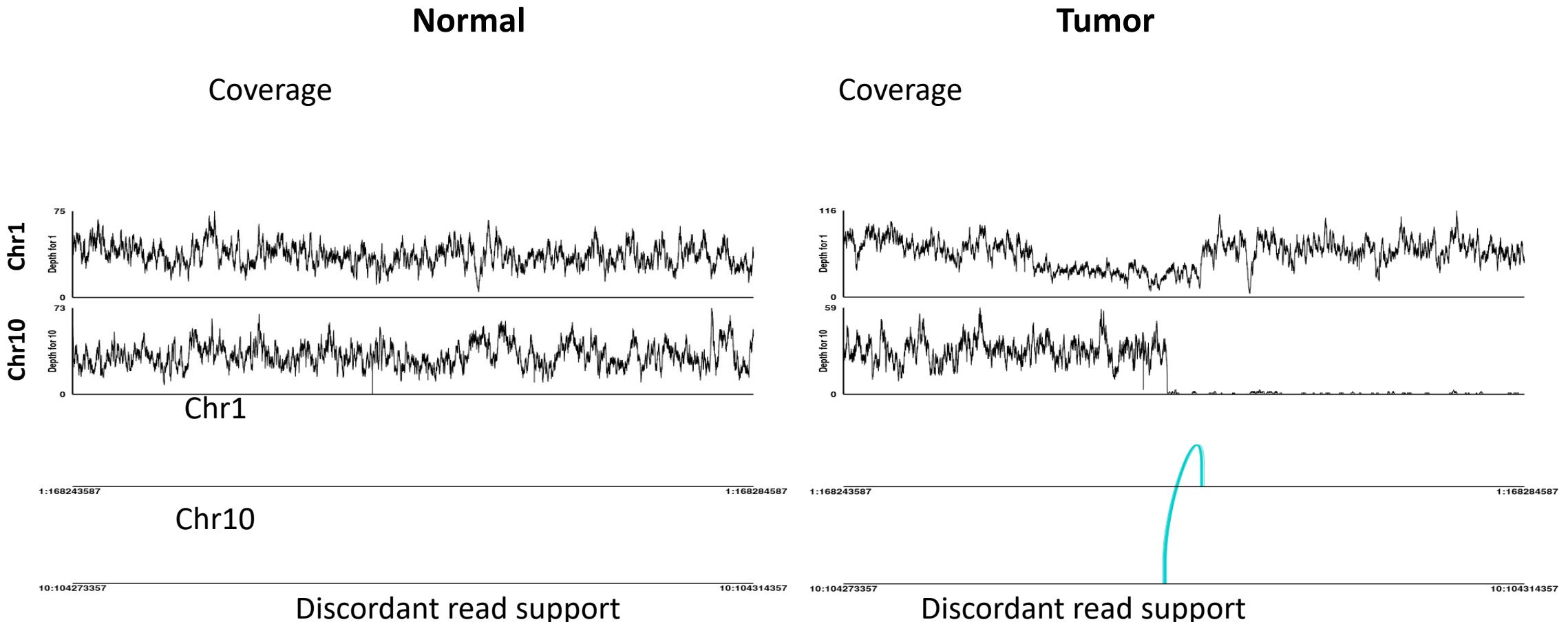
- Germline mutations
 - Present in egg or sperm
 - All cells of affected offspring
 - Heritable
 - Cause of familial cancers
- Somatic mutations
 - Occur in non-germline tissues
 - Only tumor cells
 - Non-heritable
 - Cause of sporadic cancers



Copy number variants (CNVs) appear as deviations from in alignment “depth” or “coverage”

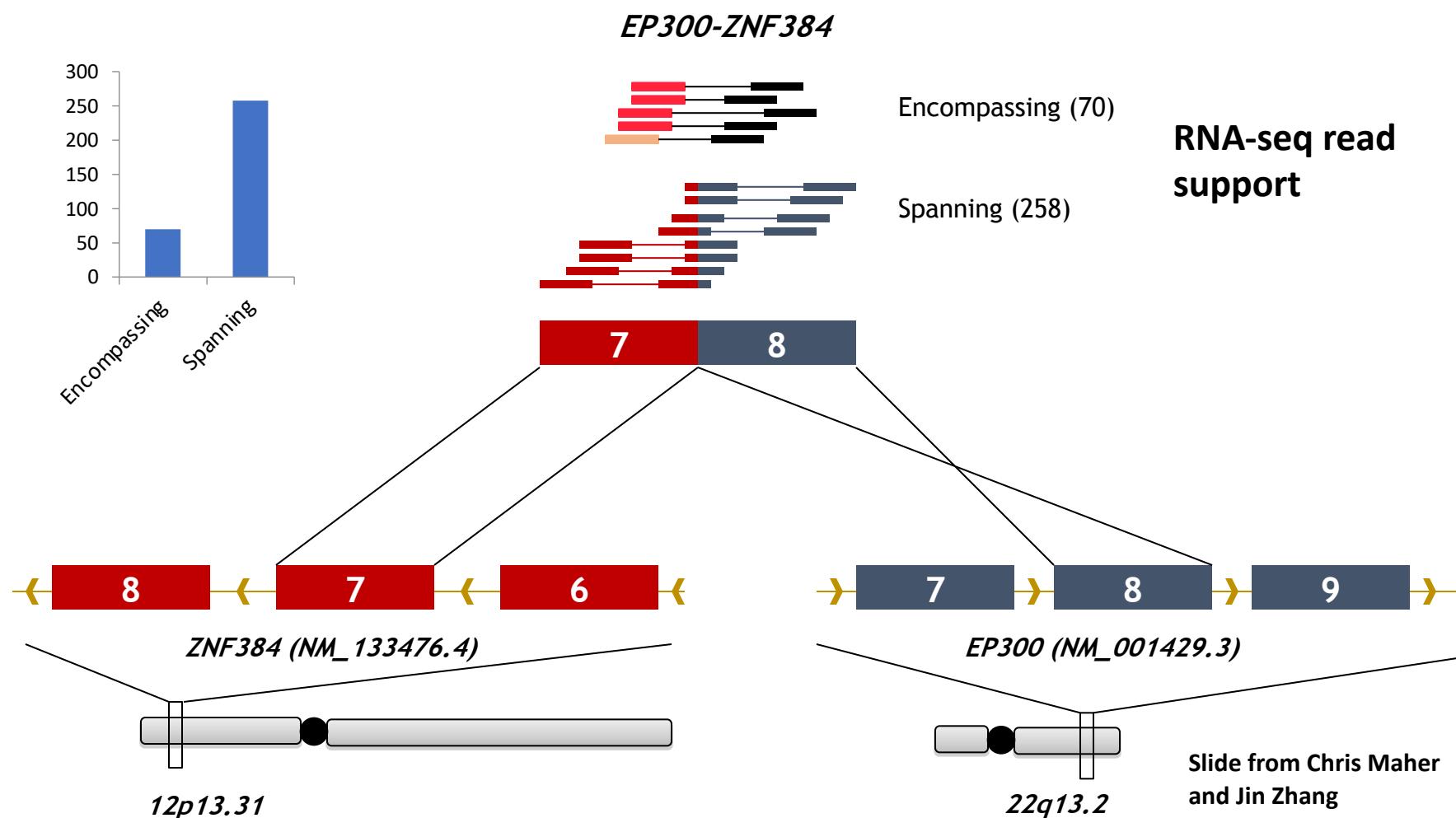


Structural variants (SVs) can be identified using a combination of coverage and discordant read alignments



A Chr1-Chr10 (TBX19-SUFU) unbalanced translocation identified in an adult acute lymphocytic leukemia.

Expressed gene fusions can be identified by discordant read alignments spanning known exons from RNA-seq data



Exons 1-8 of EP300 fused to exons 7-10 of ZNF384 in head-to-tail fashion.

Viral expression and integrations can be identified by competitive alignment with human and viral genome databases

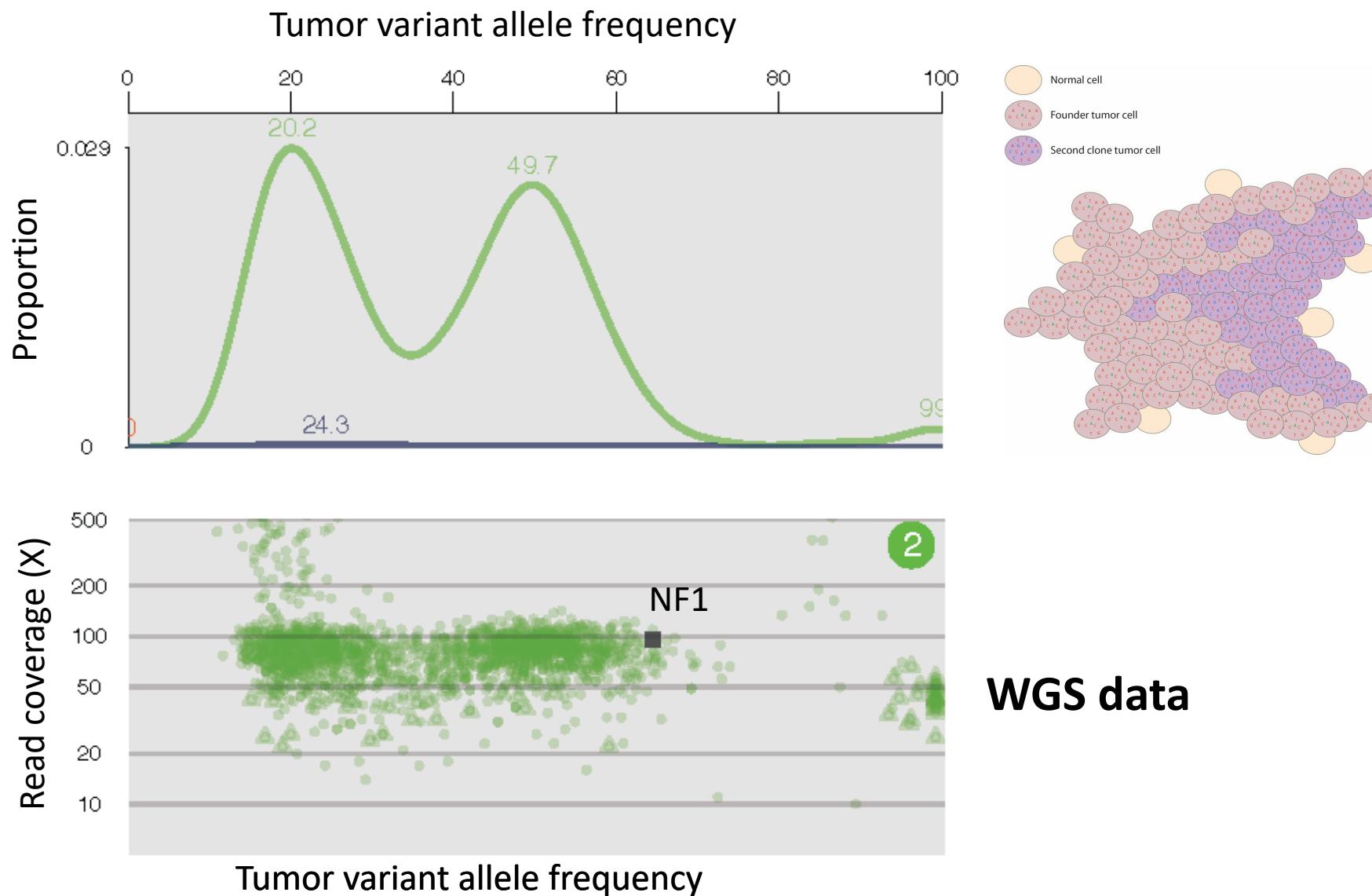


- Many reads align directly to viral genome (Hepatitis B)
- Soft-clipped reads in human reference help identify integration site

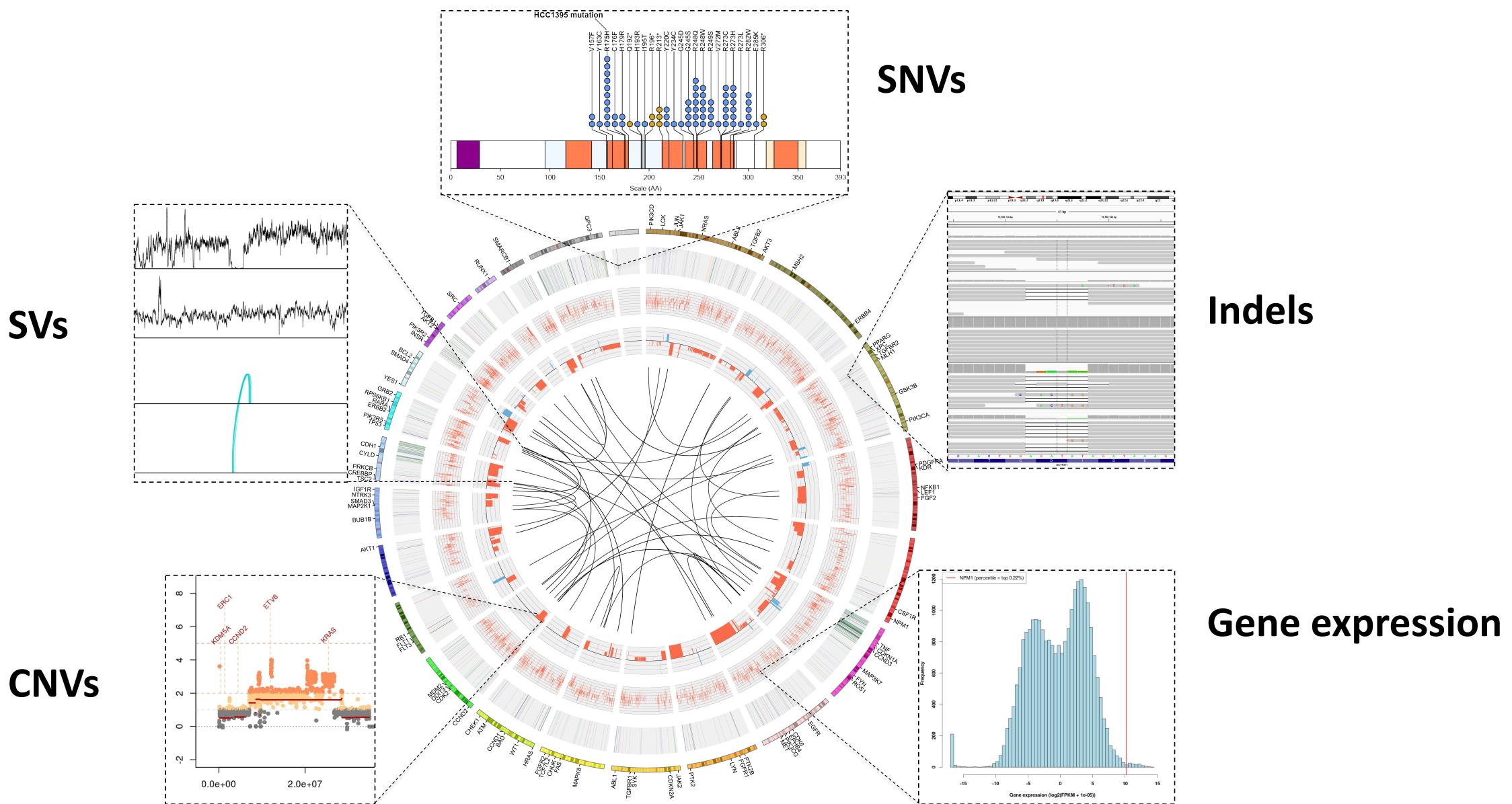


Example from HCC case thought to be virus negative

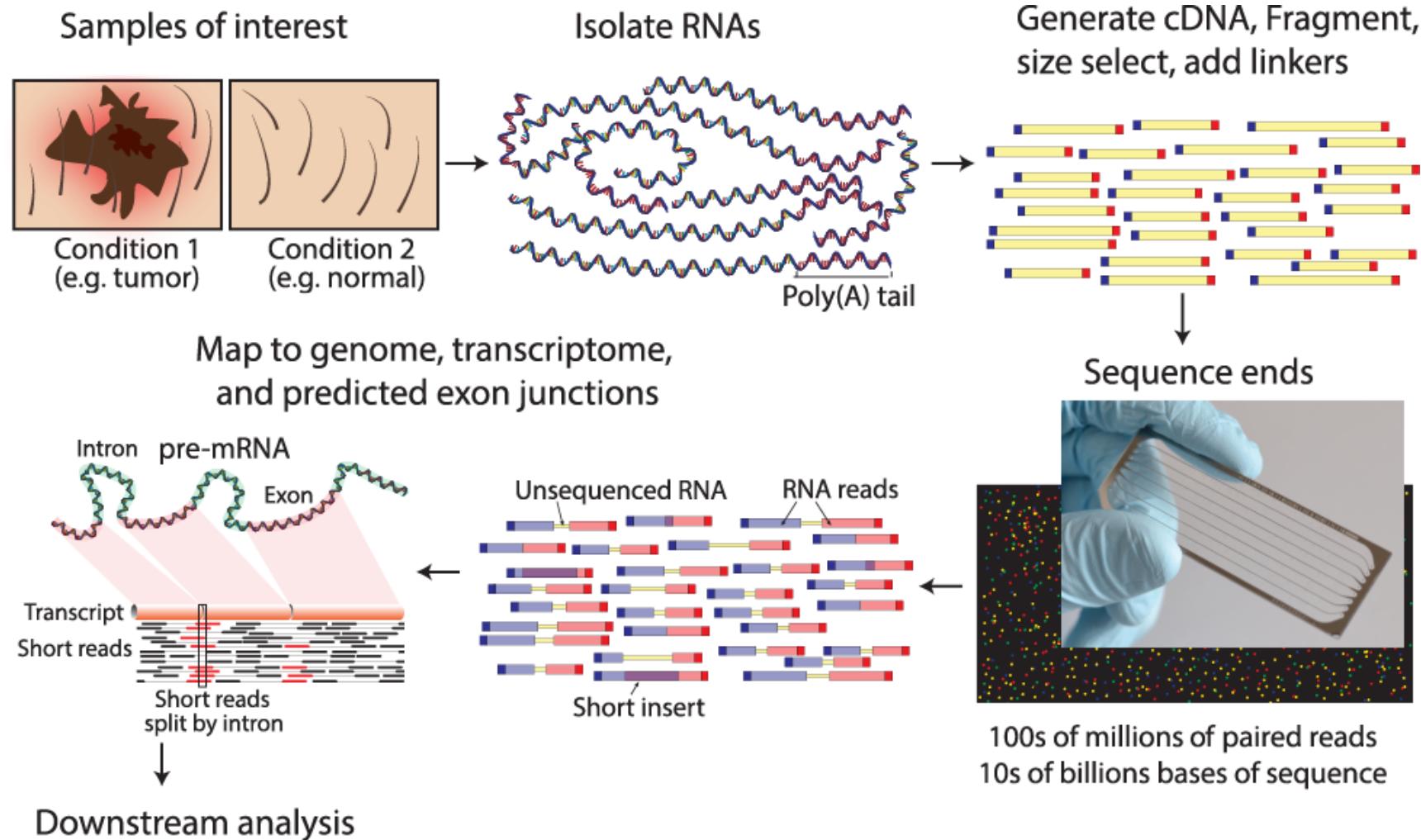
Tumor clonal architecture can be inferred from distributions of VAFs



Tumor genome analysis can reveal dozens to thousands of alterations per patient



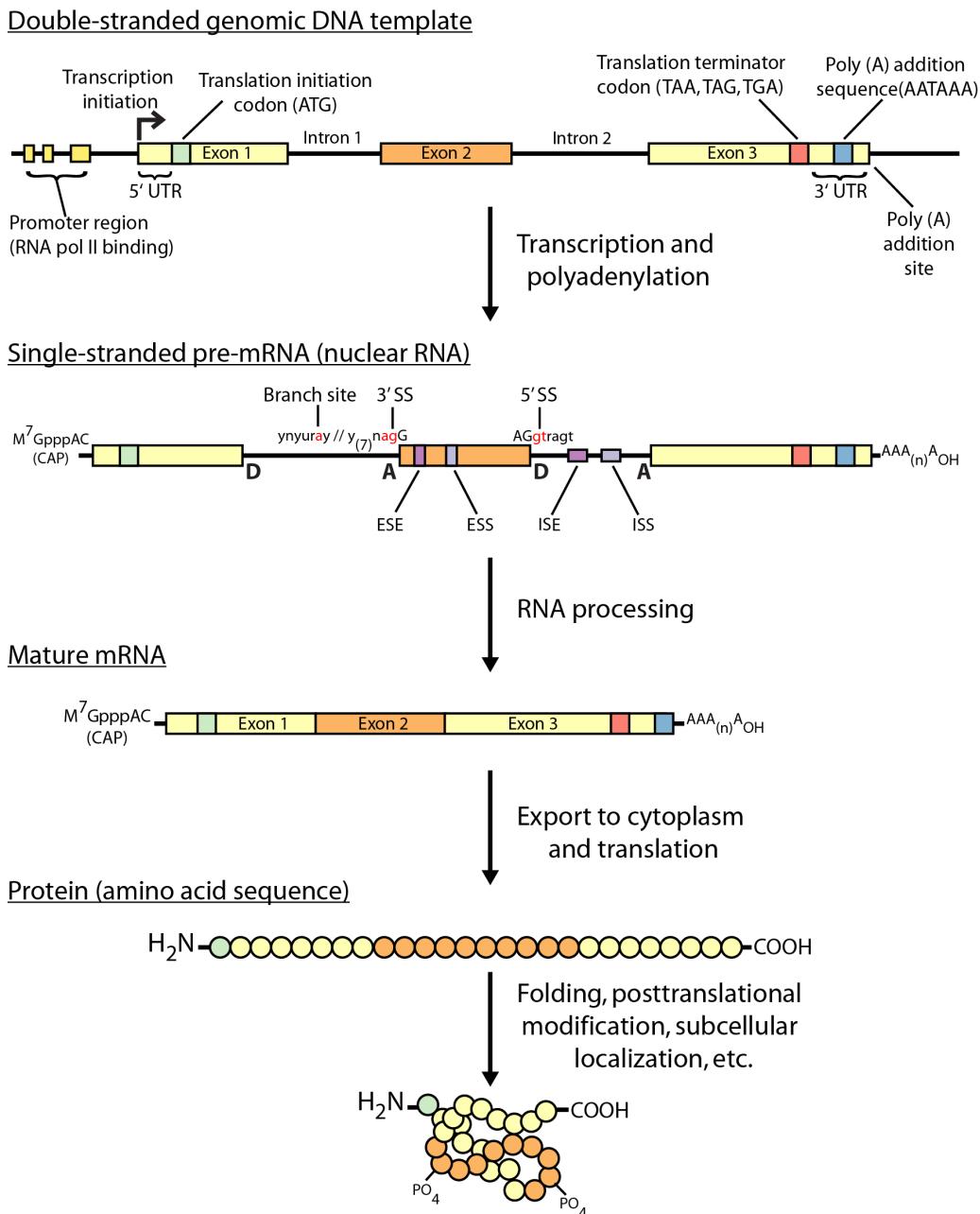
RNA sequencing



Gene expression

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

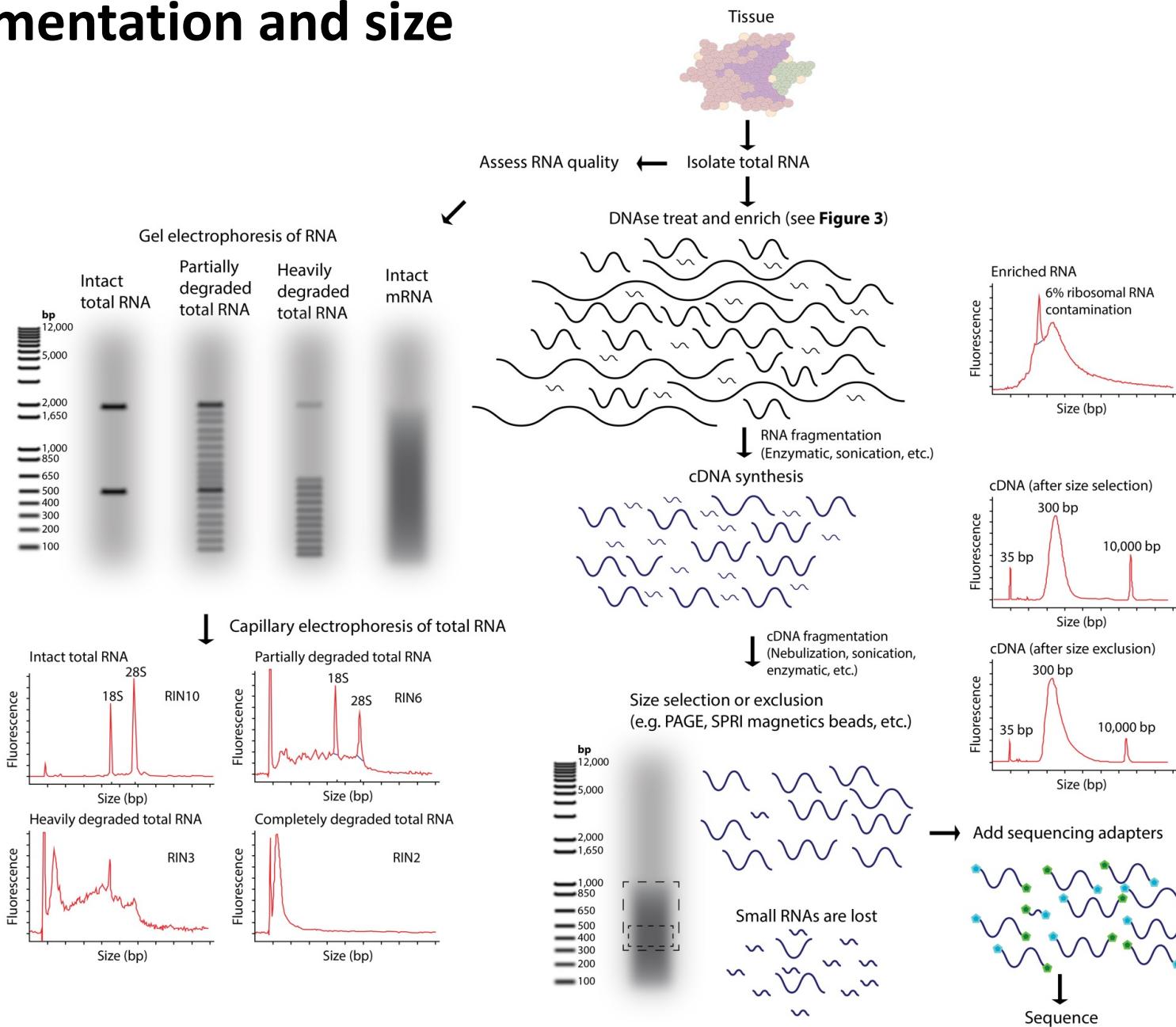
Does it differ depending on the sequencing platform? Or for bulk vs single cell sequencing?



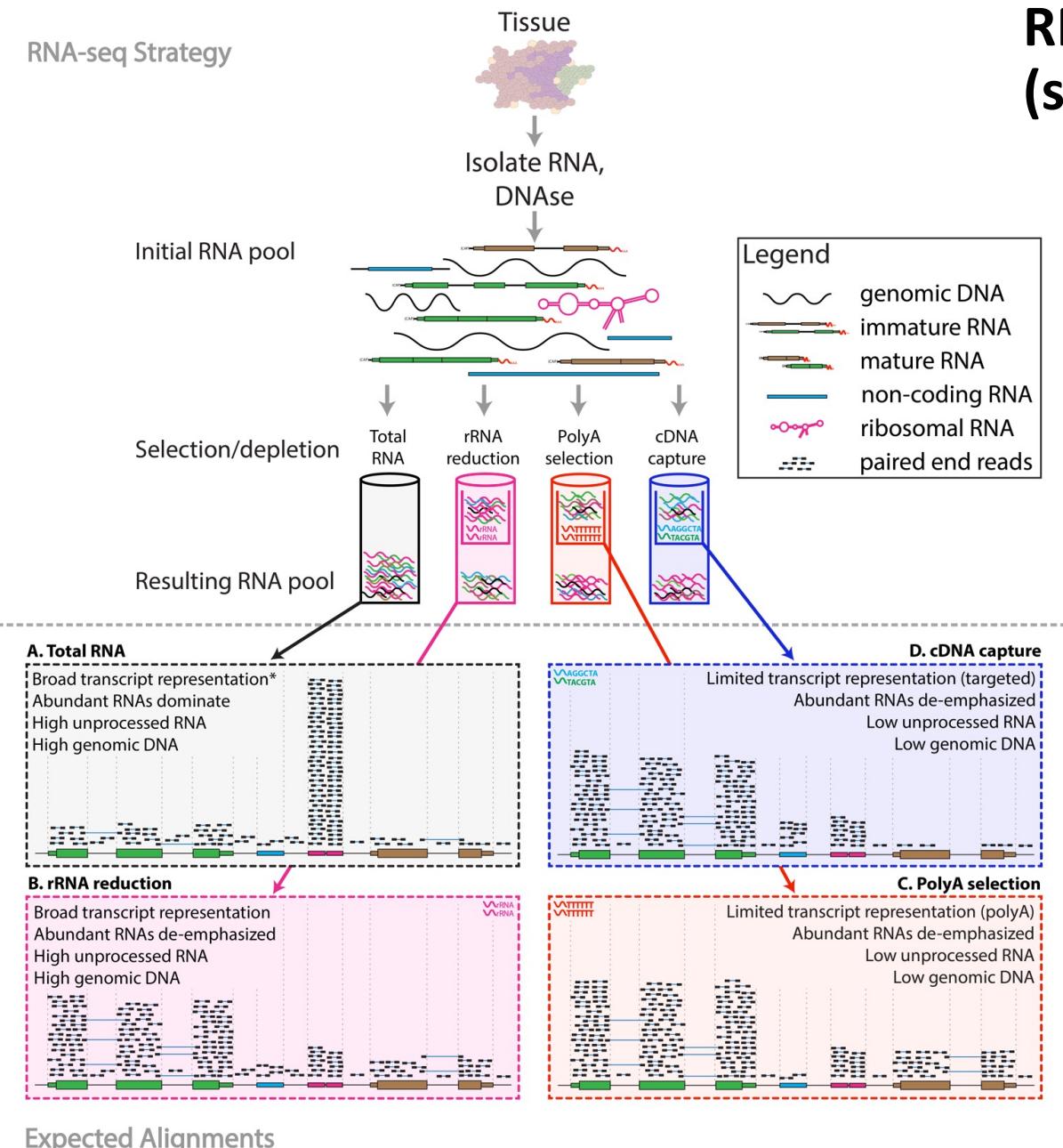
Challenges

- 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)

RNA-seq - Fragmentation and size selection



RNA-seq - sequence enrichment (selection/depletion)



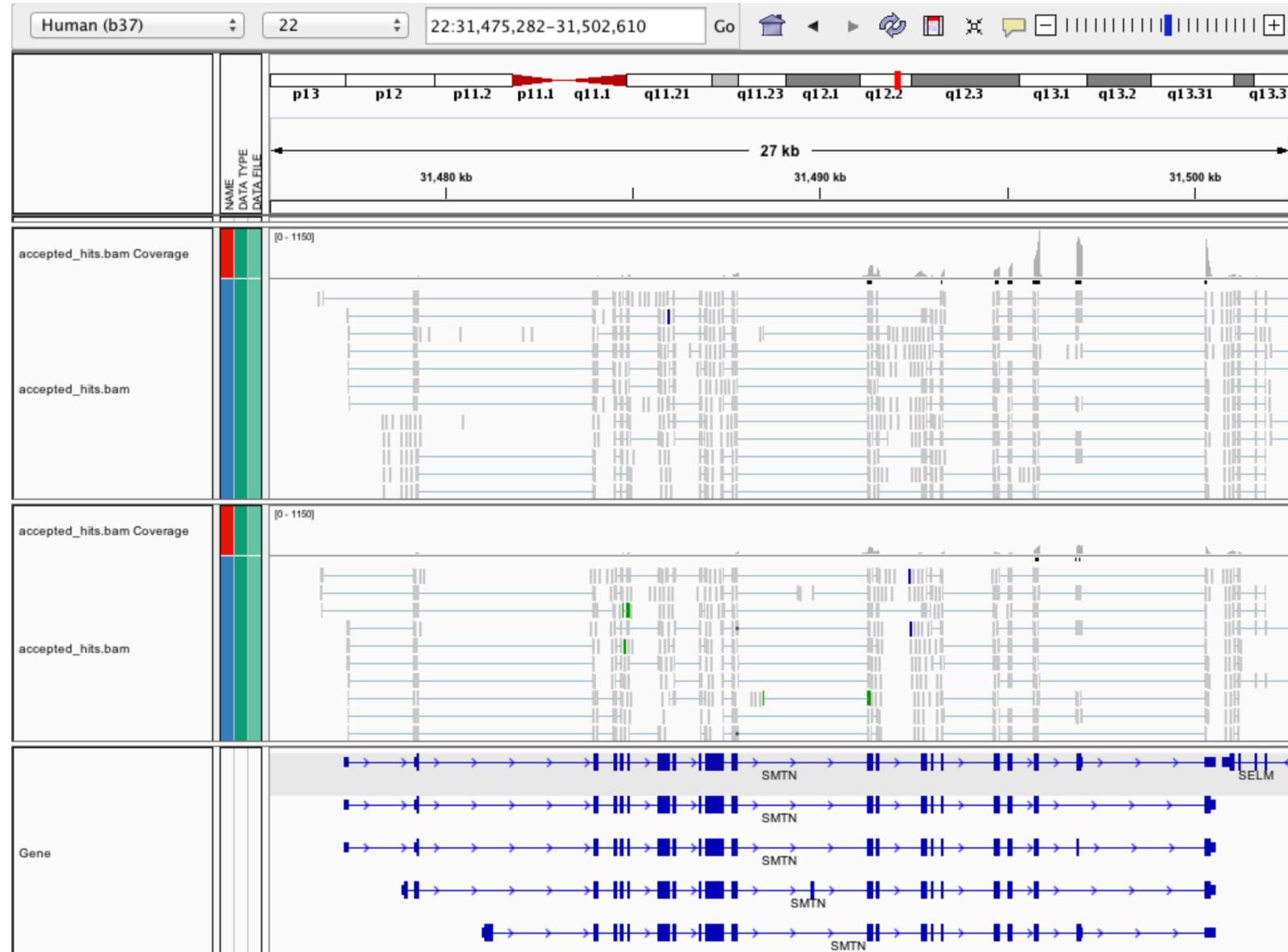
Common analysis goals of RNA-Seq analysis (what can you ask of the data?)

- 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, ‘STARfusion’ for fusion detection, etc.
 4. Post process
 - Import into downstream software (R, Python, etc.)
 5. Summarize and visualize
 - Create gene lists, prioritize candidates for validation, etc.

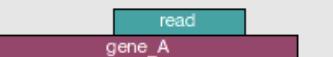
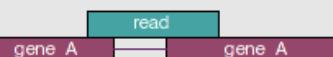
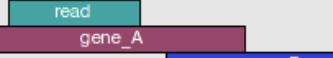
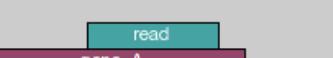
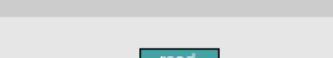
Expression estimation for known genes and transcripts



3' bias
→

Down-regulated
↓

HTSeq-count basically counts reads supporting a feature (exon, gene) by assessing overlapping coordinates

	union	intersection _strict	intersection _nonempty
 read gene_A	gene_A	gene_A	gene_A
 gene_A read	gene_A	no_feature	gene_A
 read gene_A gene_A	gene_A	no_feature	gene_A
 read gene_A gene_A	gene_A	gene_A	gene_A
 read gene_A gene_B	gene_A	gene_A	gene_A
 read gene_A gene_B	ambiguous	gene_A	gene_A
 read gene_A gene_B	ambiguous	ambiguous	ambiguous

Note, if gene_A and gene_B on opposite strands, sequence data is stranded, and correct HTSeq parameter set then this read may not be ambiguous

Whether a read is counted depends on the nature of overlap and “mode” selected

What is FPKM?

- Why not just count reads in my RNAseq data? → **Fragments**
- The relative expression of a transcript is proportional to the number of cDNA fragments that originate from it. However:
 - # fragments is biased towards larger genes → **Per Kilobase of transcript**
 - # fragments is related to total library depth → **per Million mapped reads.**

What is FPKM?

- FPKM attempts to normalize for gene size and library depth
 - remember – RPKM is essentially the same!
- C = number of mappable fragments for a gene (transcript)
- N = total number of mappable fragments in the library
- L = number of base pairs in the gene (transcript)
 - $\text{FPKM} = (\text{C} / (\text{N} \times \text{L})) \times 1,000 \times 1,000,000$
 - $\text{FPKM} = (1,000,000,000 \times \text{C}) / (\text{N} \times \text{L})$
 - $\text{FPKM} = (\text{C} / (\text{N} / 1,000,000)) / (\text{L}/1000)$
- More reading:
 - <http://www.biostars.org/p/11378/>
 - <http://www.biostars.org/p/68126/>

How do FPKM and TPM differ?

- TPM: Transcript per Kilobase Million
- The difference is in the order of operations:

FPKM

- 1) Determine total fragment count, divide by 1,000,000 (per Million)
- 2) Divide each gene/transcript fragment count by #1 (Fragments Per Million)
- 3) Divide each FPM by length of each gene/transcript in kilobases (FPKM)

TPM

- 1) Divide each gene/transcript fragment count by length of the transcript in kilobases (Fragments Per Kilobase)
- 2) Sum all FPK values for the sample and divide by 1,000,000 (per Million)
- 3) Divide #1 by #2 (TPM)

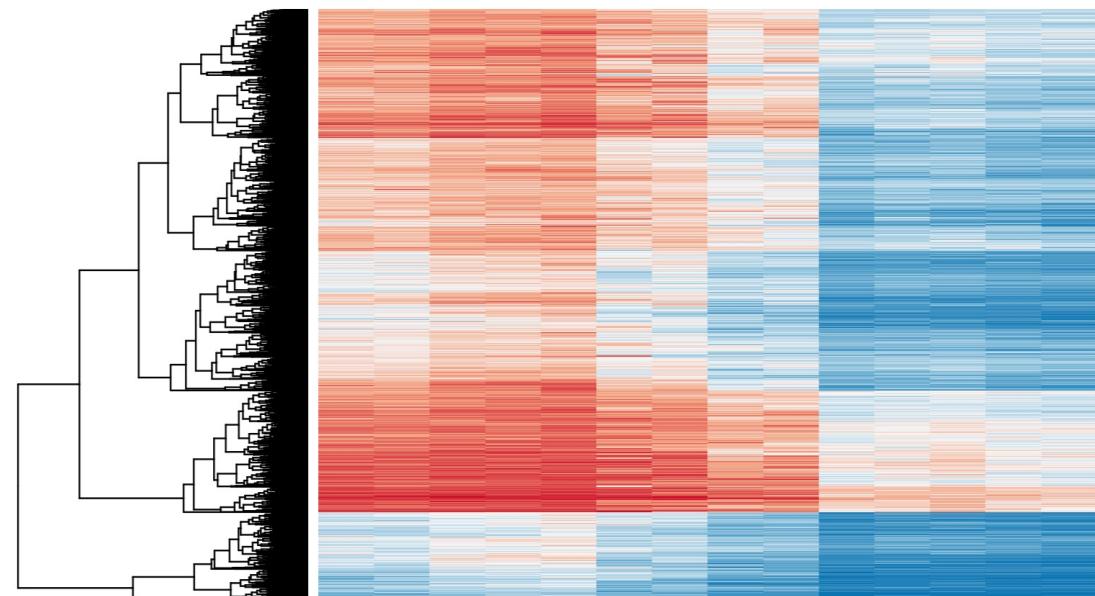
- The sum of all TPMs in each sample is the same. Easier to compare across samples!
- <http://www.rna-seqblog.com/rpkm-fpkm-and-tpm-clearly-explained/>
- <https://www.ncbi.nlm.nih.gov/pubmed/22872506>

'FPKM/TPM' expression estimates vs. 'raw' counts

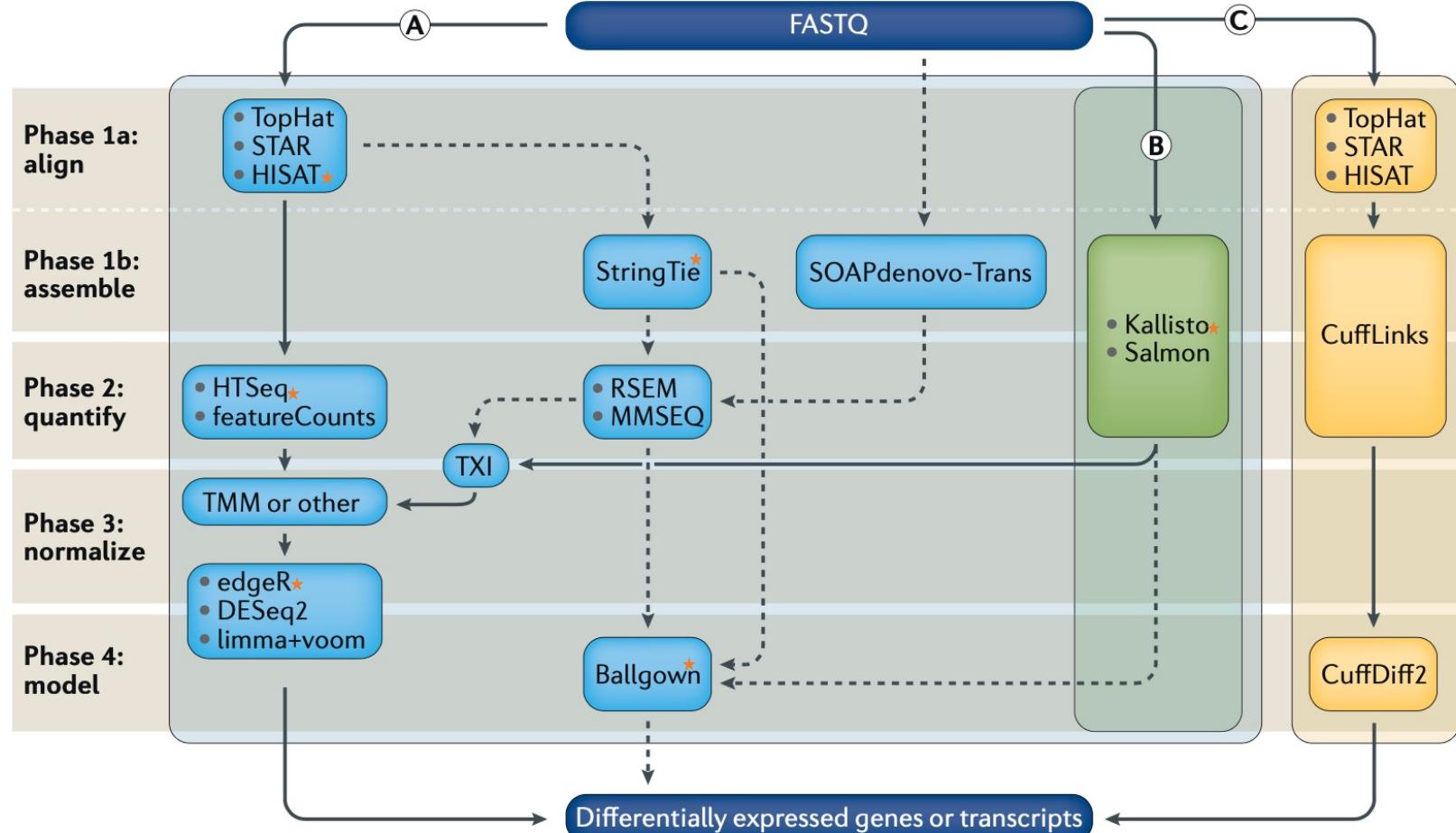
- Which should I use?
 - Long running debate, but the general consensus:
- FPKM/TPM
 - When you want to leverage more holistic approaches
 - Transcript/Isoform deconvolution
 - Good for visualization (e.g., heatmaps)
 - Calculating fold changes, etc.
- Counts
 - "More robust" statistical methods for differential expression
 - Accommodates more sophisticated experimental designs with appropriate statistical tests
 - Not suitable for transcript/isoform level estimation

Differential Expression

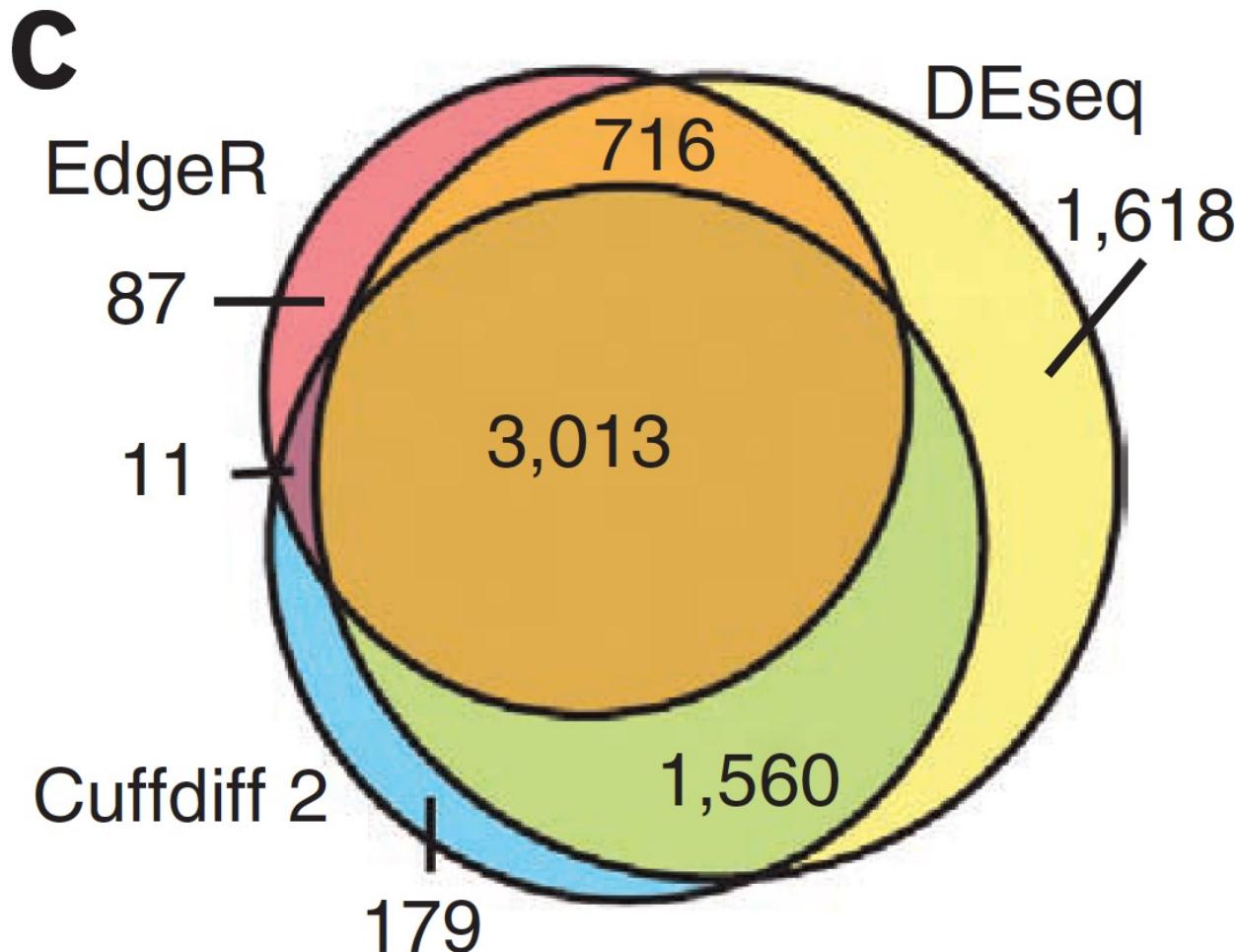
- Tying gene expression back to genotype/phenotype
- What genes/transcripts are being expressed at higher/lower levels in different groups of samples?
 - Are these differences ‘significant’, accounting for variance/noise?



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



Multiple approaches advisable



Multiple testing correction

- As more attributes are compared, differences due solely to chance become more likely!
- Well known from array studies
 - 10,000s genes/transcripts
 - 100,000s exons
- With RNA-seq, more of a problem than ever
 - All the complexity of the transcriptome gives huge numbers of potential features
 - Genes, transcripts, exons, junctions, retained introns, microRNAs, lncRNAs, etc
- Bioconductor multtest
 - <http://www.bioconductor.org/packages/release/bioc/html/multtest.html>

Discussion of bulk vs single cell RNA-seq

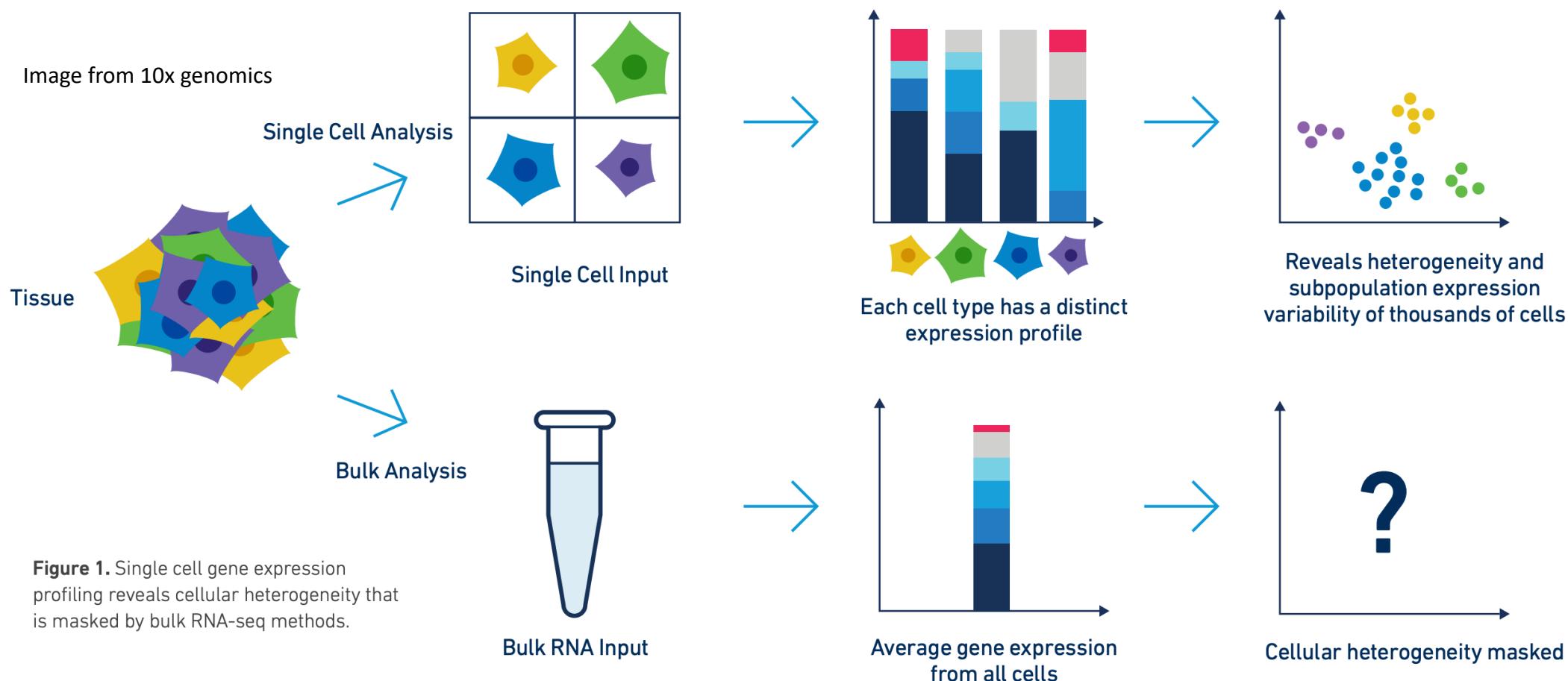
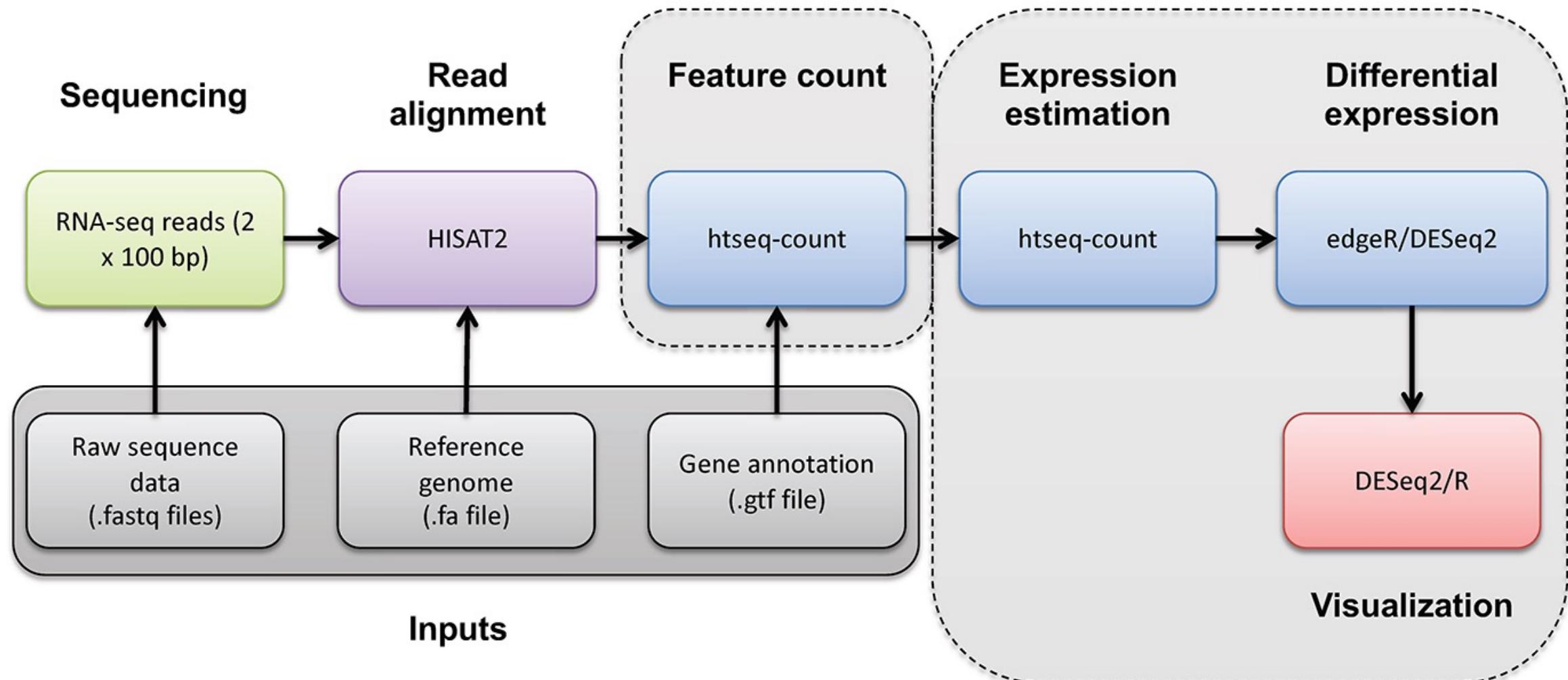


Figure 1. Single cell gene expression profiling reveals cellular heterogeneity that is masked by bulk RNA-seq methods.

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

Steps upstream of the hands-on exercises



Introduction to rnabio.org

We are on a Coffee Break & Networking Session