

# Canadian Bioinformatics Workshops

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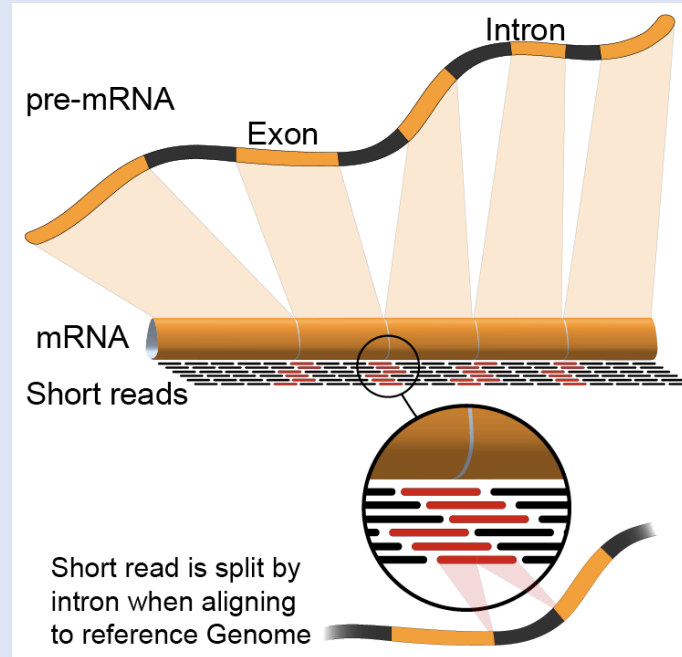
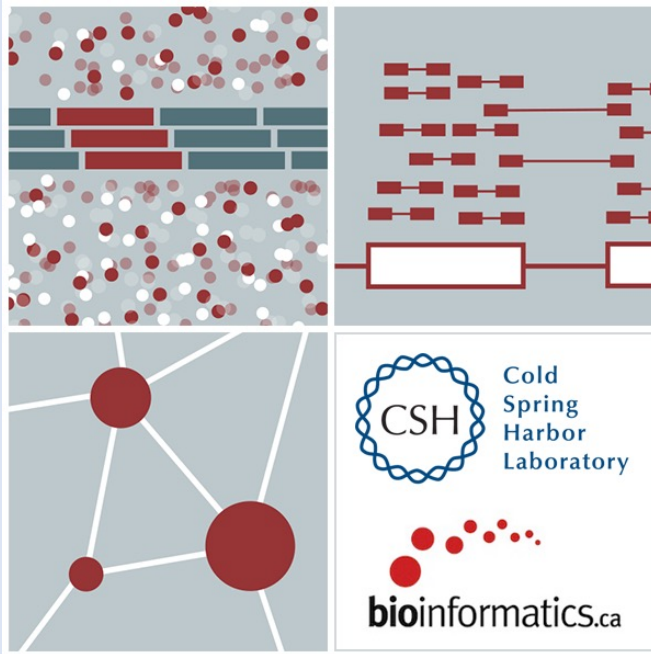
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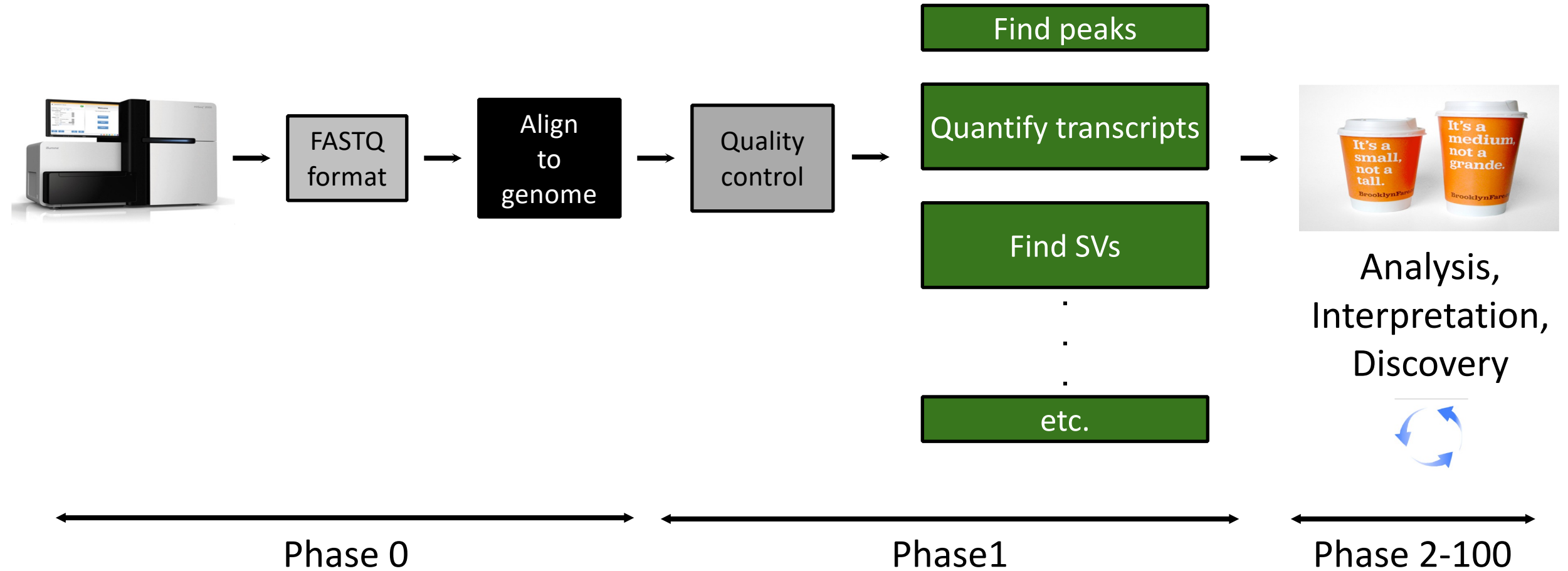
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# RNA-Seq Module 2: Alignment

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RNA-seq Analysis 2025. July 7-9, 2025



# Alignment is central to most genomic research



# Alignment - How does it work?



- 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 changes to a copy of the picture

Reference: AGCCTGAGACCGTAAAAAA**A**GTCAAG

|||||

A read sequence:

GAGACCGTAAAAAA**C**GTC

↑  
A variant!

# RNA-seq alignment challenges

- Computational cost
  - 100's of millions of reads
- Introns!
  - Align to a transcriptome or align to a genome?
    - Spliced vs. unspliced alignments
- Can I just align my data once using one approach and be done with it?
  - Unfortunately, probably not

# Three RNA-seq mapping strategies

## De novo assembly

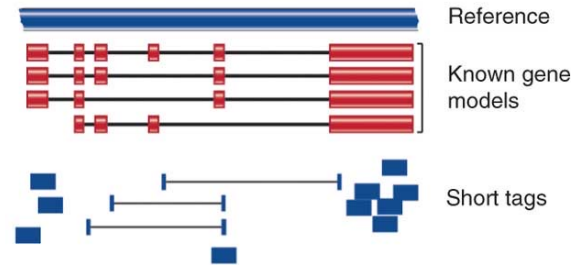


Assemble transcripts from overlapping tags



Optional: align to genome to get exon structure

## Align to transcriptome



Use known and/or predicted gene models to examine individual features

## Align to reference genome



Infer possible transcripts and abundance

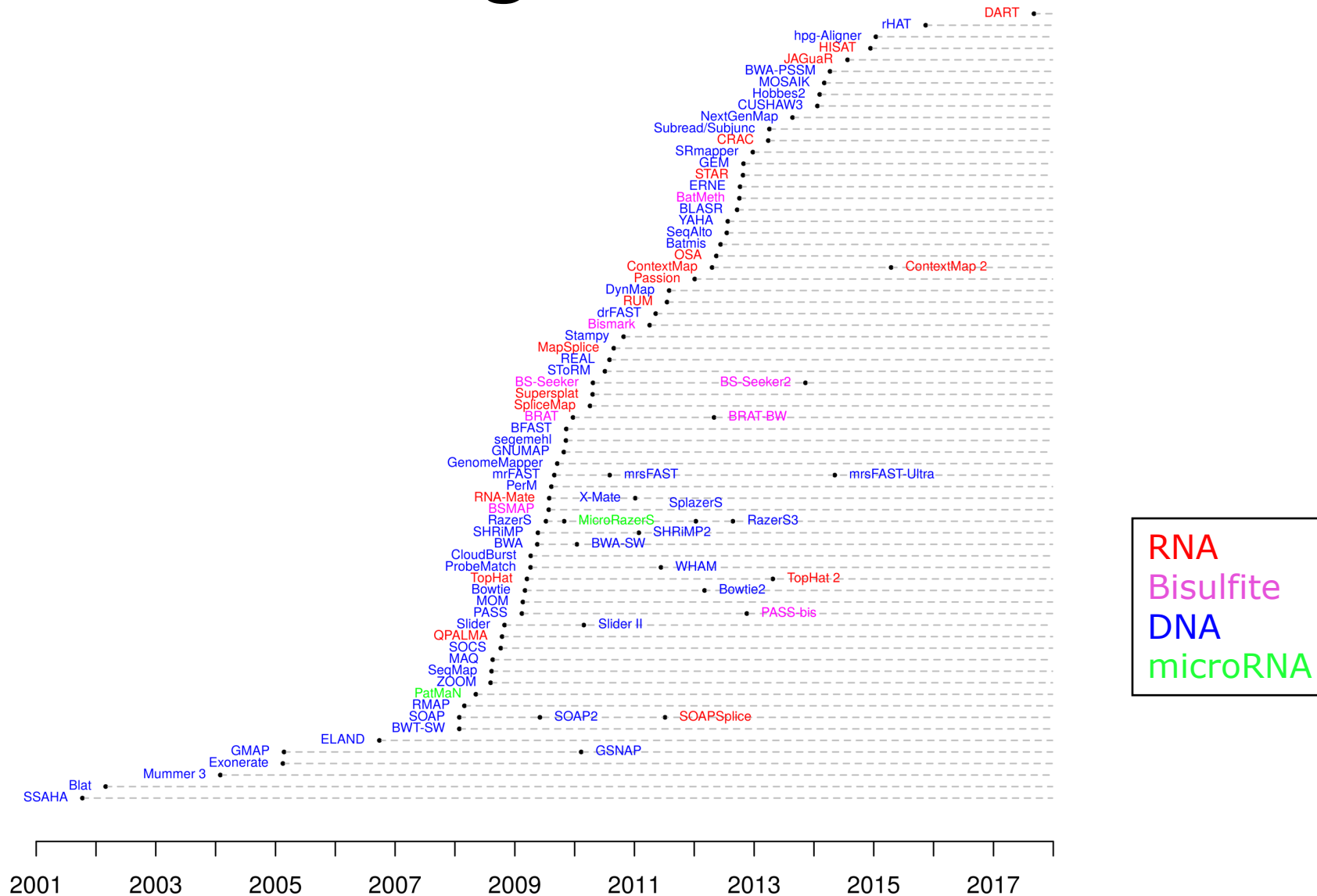
Diagrams from Cloonan & Grimmond, Nature Methods 2010

# Which alignment strategy is best?

- De novo assembly
  - If a reference genome does not exist for the species being studied
  - If complex polymorphisms/mutations/haplotypes might be missed by comparing to the reference genome
- Align to transcriptome
  - If you have short reads (< 50bp)
  - Relies on known transcripts
- Align to reference genome
  - All other cases
  - Does not rely on known transcripts – allows for discovery
- Each strategy involves different alignment/assembly tools



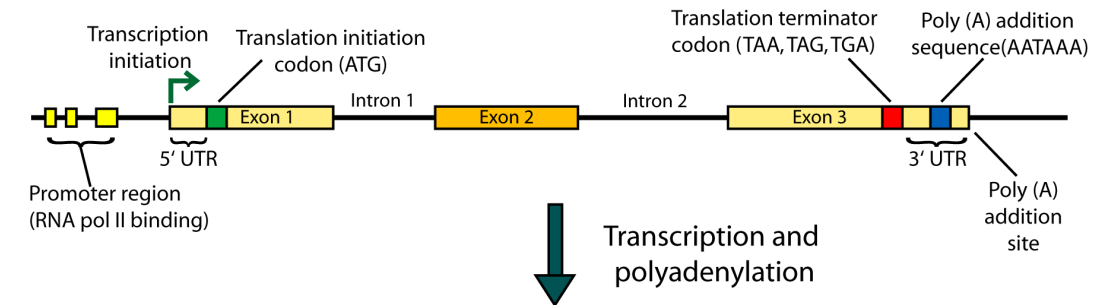
# Which read aligner should I use?



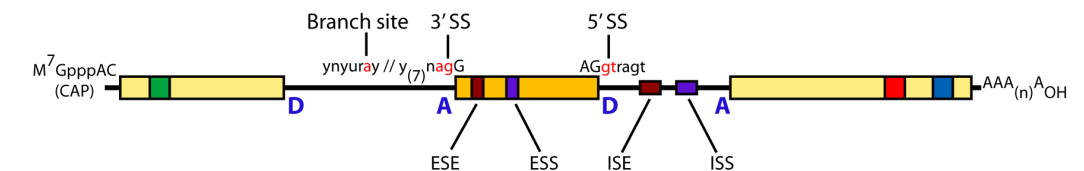
# Should I use a splice-aware or unspliced mapper?

- The fragments being sequenced in RNA-seq represent mRNA - introns are removed
- But we are usually aligning these reads back to the reference genome
- Unless your reads are short (<50bp) you should use a splice-aware aligner
  - HISAT2, STAR, MapSplice, etc.

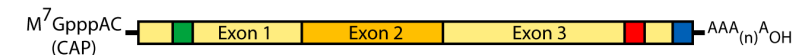
## Double-stranded genomic DNA template



## Single-stranded pre-mRNA (nuclear RNA)



## Mature mRNA



# HISAT/HISAT2

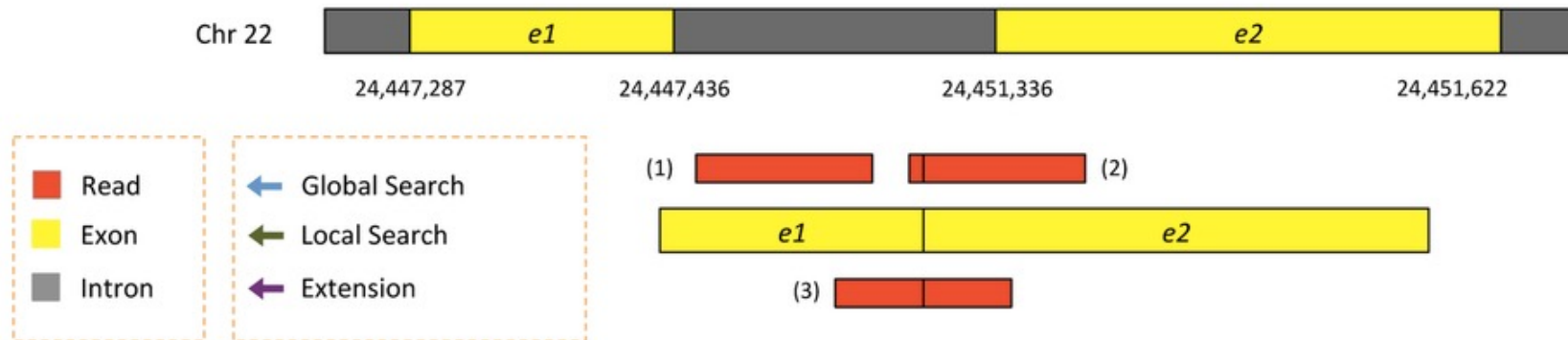
- HISAT is a ‘splice-aware’ RNA-seq read aligner
  - HISAT = **H**ierarchical **I**ndexing for **S**pliced **A**lignments of **T**ranscripts
- Requires a reference genome
- Very fast
- Uses an indexing scheme based on the Burrows-Wheeler transform and the Ferragina-Manzini (FM) index
- Multiple types of indexes for alignment
  - a whole-genome FM index to anchor each alignment
  - numerous local FM indexes for very rapid extensions of these alignments.
  - Whole-genome indices with SNPs and known transcript structures accounted for

Kim et al. 2015. Nat Methods 12:357–360

# HISAT/HISAT2 algorithm

- Uses a hierarchical indexing algorithm + several adaptive strategies
  - based on the position of a read with respect to splice sites
- 1) Find candidate locations across the whole genome first
  - mapping part of each read using the global FM index
  - Generally identifies one or a small number of candidates.
- 2) Do local alignment
  - selects one of ~48,000 local indexes for each candidate
  - uses it to align the remainder of the read.
- For paired reads, each mate is separately aligned
  - If a read fails to align, then the alignments of its mate are used as anchors to map the unaligned mate

# HISAT2 Alignment



- Two exons from chr22
- Three reads

Kim et al. 2015. Nat Methods 12:357–360

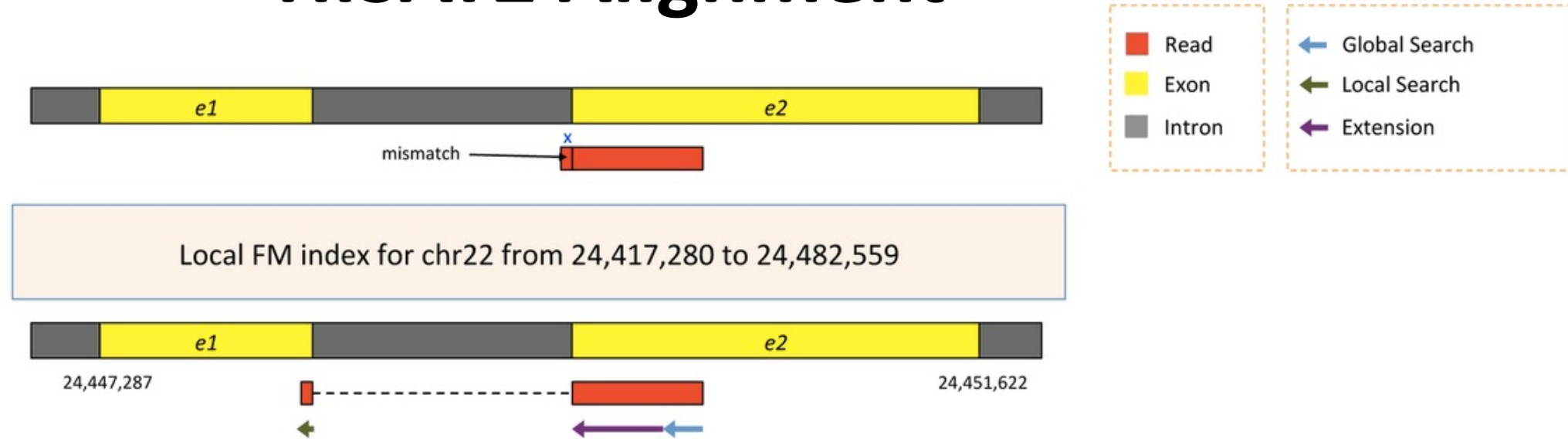
# HISAT2 Alignment



- 1) Search for read position with global FM index (slower)
- 2) Once at least 28bp and exactly one location switch to extension mode against reference genome (faster)

Kim et al. 2015. Nat Methods 12:357–360

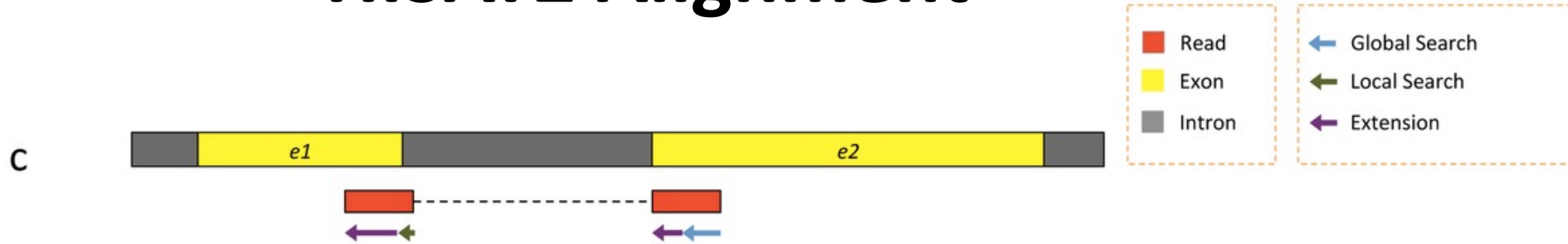
# HISAT2 Alignment



- 1) Search for read position with global FM index (slower)
- 2) Extend until mismatch at 93bp (faster)
- 3) Switch to local FM index to align remaining 8bp
  - index covers only a small region, so we find just one match
- 4) Check for compatibility and combine into single spliced alignment

Kim et al. 2015. Nat Methods 12:357–360

# HISAT2 Alignment



- 1) global search until exactly one match of at least 28bp (slower)
- 2) Extend until mismatch at 51bp (faster)
- 3) switch to local FM index to align first 8bp of remaining read
  - If too many matches increase prefix size
- 4) Extend again
- 5) Check for compatibility and combine into single spliced alignment

Kim et al. 2015. Nat Methods 12:357–360



# What is the output of HISAT2?

- A SAM/BAM file
  - SAM stands for Sequence Alignment/Map format
  - BAM is the binary version of a SAM file
- Remember, compressed files require special handling compared to plain text files
- How can I convert BAM to SAM?
  - <http://www.biostars.org/p/1701/>
- Is HISAT2 the only mapper to consider for RNA-seq data?
  - <http://www.biostars.org/p/60478/>

# We are on a Coffee Break & Networking Session

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