RNA-Seq: GTF/GFF and Splice Aligner

BCB 5250 Introduction to Bioinformatics II

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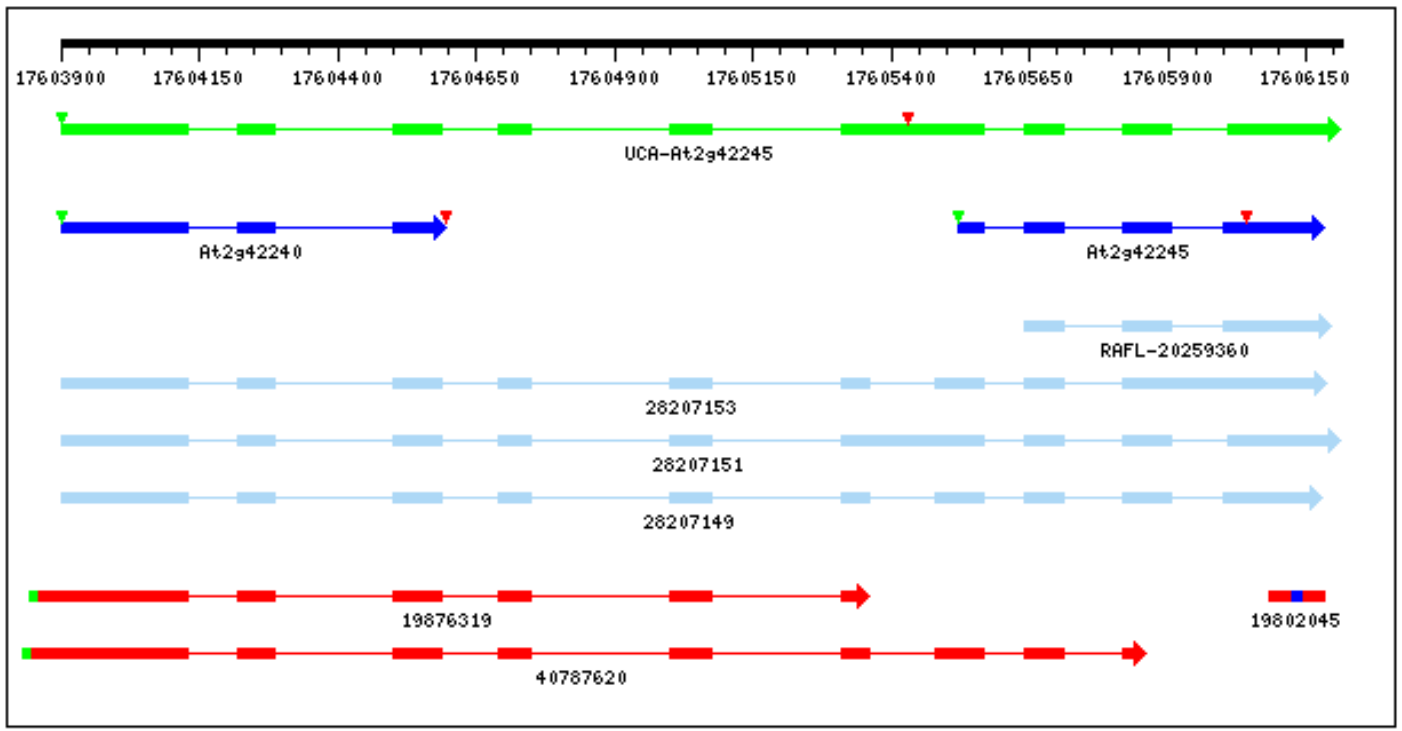
Genome sequence file: where to get?

- Illumina iGenomes (recommended)
  - http://support.illumina.com/sequencing/sequencing_software/igenome.html
- Ensembl
  - http://ensemblgenomes.org/info/access/ftp

- NCBI genome
  - http://www.ncbi.nlm.nih.gov/genome/
- Organism specific databases/websites.

Genome annotation file



GFF/GTF File Format - Definition

- The GFF (General Feature Format) format
  - one line per feature
  - each containing 9 columns of data
  - plus optional track definition lines.
- GFF has many versions (GFF, GFF2, GFF3)
- GTF (General Transfer Format) identical to GFF2.
- Most spliced aligner supports both GTF and GFF3 (mostly GTF)

GTF/GTF2 format

9 columns:

```
<seqname> <source> <feature> <start> <end> <score> <strand> <frame> [attributes]
```

- seqname - name of the chromosome or scaffold
- source – program or database that generated this feature.
- feature – Examples: "CDS", “gene", “transcript”, and "exon".
- start - The starting position of the feature in the sequence.
- end - The ending position of the feature (inclusive).
- score - A score between 0 and 1000.
- strand – ‘+ ‘(forward) or ‘-’ (reverse) or '.' (don't know/don't care).
- Frame – reading frame '0', '1' or '2'
- attribute – A semicolon-separated list of tag-value pairs, providing additional information about each feature.

Example of GTF2 format

```
AB000381 Twinscan  CDS          380   401   .   +   0  gene_id "001"; transcript_id "001.1";
AB000381 Twinscan  CDS          501   650   .   +   2  gene_id "001"; transcript_id "001.1";
AB000381 Twinscan  CDS          700   707   .   +   2  gene_id "001"; transcript_id "001.1";
AB000381 Twinscan  start_codon  380   382   .   +   0  gene_id "001"; transcript_id "001.1";
AB000381 Twinscan  stop_codon   708   710   .   +   0  gene_id "001"; transcript_id "001.1";
```

A simple example with 3 translated exons. Order of rows is not important.

Some annotation sources (e.g. Ensembl) add the gene_name attribute

```
gene_id "ENSBTAG00000020601"; transcript_id "ENSBTAT00000027448"; gene_name "ZNF366";
```

http://mblab.wustl.edu/GTF2.html

Generic Feature Format Version 3 (GFF3)

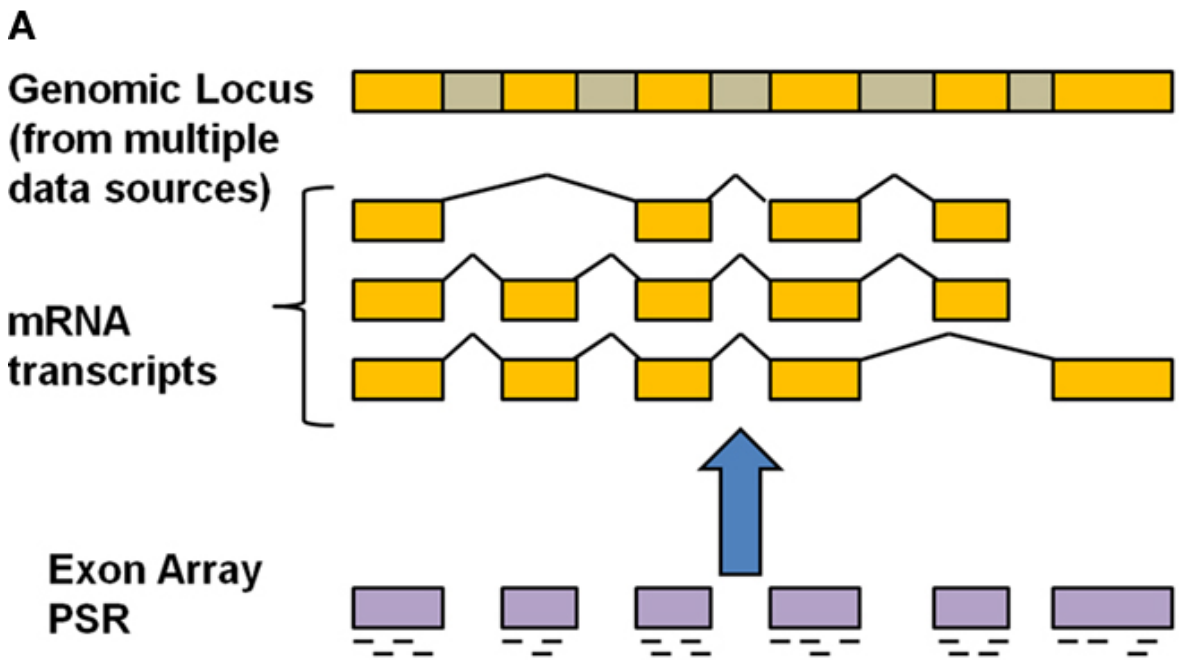
GFF3 adds parent feature

```
##gff-version 3.2.1
##sequence-region   ctg123 1 1497228
ctg123 . gene            1000  9000  .  +  .  ID=gene00001;Name=EDEN

ctg123 . TF_binding_site 1000  1012  .  +  .  ID=tfbs00001;Parent=gene00001

ctg123 . mRNA            1050  9000  .  +  .  ID=mRNA00001;Parent=gene00001;Name=E
ctg123 . mRNA            1050  9000  .  +  .  ID=mRNA00002;Parent=gene00001;Name=E
ctg123 . mRNA            1300  9000  .  +  .  ID=mRNA00003;Parent=gene00001;Name=E

ctg123 . exon            1300  1500  .  +  .  ID=exon00001;Parent=mRNA00003
ctg123 . exon            1050  1500  .  +  .  ID=exon00002;Parent=mRNA00001,mRNA00
ctg123 . exon            3000  3902  .  +  .  ID=exon00003;Parent=mRNA00001,mRNA00
ctg123 . exon            5000  5500  .  +  .  ID=exon00004;Parent=mRNA00001,mRNA00
ctg123 . exon            7000  9000  .  +  .  ID=exon00005;Parent=mRNA00001,mRNA00

ctg123 . CDS             1201  1500  .  +  0  ID=cds00001;Parent=mRNA00001;Name=ed
ctg123 . CDS             3000  3902  .  +  0  ID=cds00001;Parent=mRNA00001;Name=ed
ctg123 . CDS             5000  5500  .  +  0  ID=cds00001;Parent=mRNA00001;Name=ed
ctg123 . CDS             7000  7600  .  +  0  ID=cds00001;Parent=mRNA00001;Name=ed

ctg123 . CDS             1201  1500  .  +  0  ID=cds00002;Parent=mRNA00002;Name=ed
ctg123 . CDS             5000  5500  .  +  0  ID=cds00002;Parent=mRNA00002;Name=ed
ctg123 . CDS             7000  7600  .  +  0  ID=cds00002;Parent=mRNA00002;Name=ed

ctg123 . CDS             3301  3902  .  +  0  ID=cds00003;Parent=mRNA00003;Name=ed
ctg123 . CDS             5000  5500  .  +  1  ID=cds00003;Parent=mRNA00003;Name=ed
ctg123 . CDS             7000  7600  .  +  1  ID=cds00003;Parent=mRNA00003;Name=ed

ctg123 . CDS             3391  3902  .  +  0  ID=cds00004;Parent=mRNA00003;Name=ed
ctg123 . CDS             5000  5500  .  +  1  ID=cds00004;Parent=mRNA00003;Name=ed
ctg123 . CDS             7000  7600  .  +  1  ID=cds00004;Parent=mRNA00003;Name=ed
```

http://www.sequenceontology.org/gff3.shtml

GFF2 vs GFF3

- GFF2
  - two-level hierarchies *transcript* → *exon*
- GFF3
  - three-level hierarchy of *gene* → *transcript* → *exon*



http://gmod.org/wiki/GFF2

Conversion GFF3 To GTF

- Optional
- Use `gffread` (comes with the Cufflinks software suite)

```
$ gffread my.gff3 -T -o my.gtf
```

- See `gffread -h` for more information

Download Prepare GTF/GFF file

- Download it from Illumina's iGenomes project (for model species)
  - http://support.illumina.com/sequencing/sequencing_software/igenome.html

Or

- Download gff3 files from genome database,
  - Ensembl gnome
  - http://ensemblgenomes.org/

Splice Aligner

- The alignment process consists of choosing an appropriate reference genome to map our reads against and performing the read alignment using one of several splice-aware alignment tools such as STAR or HISAT2.
- The choice of aligner is often a personal preference and also dependent on the computational resources that are available to you.

One Critique from a Student

A critique of this paper, which must do more with the field of bioinformatics itself, is that there are so many methods and tools available to basically do the same thing. How does one reconcile this? What I usually do is to see which tools have been highly cited. This gives me a good baseline as to whether I can trust the output of a program. I also like to use well-documented tools. Using a 'black box' is something I really don't like to do, because I have no way of analyzing the results or understanding how the results were derived. I can only use another similar tool, and compare those results, but then, I might as well have used the other tool to begin with. In sum, I think this field, while very mature, is still exciting and growing. Especially with the advent of third-generation sequencing.

HISAT2 V.S. STAR

- https://www.biostars.org/p/288726/

The main benefit of hisat2 is that is uses fewer resources than STAR and that it can better handler known SNPs if you make the aligner aware of them. Aside from that, I essentially always get better results from STAR, which is why we use it in our standard pipelines instead of hisat2 (this also bears out in published comparisons).

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ADD COMMENT • link written 2.2 years ago by Devon Ryan ♦ 94k

2.2 years ago by
Devon Ryan ♦ 94k
Freiburg, Germany

thanks! I will then stick to STAR and DESEP2 method. One more question is if i use DESEQ2, but i actually want to know the abundance of my transcript, in this case would you still recommend using RPKM from Cuffdiff? or other methods?

ADD REPLY • link written 2.2 years ago by langya • 60

You can get normalized counts from DESeq2, so either use them directly or convert them to FPKMs or just divide by transcript length if you want some sort of length-normalized value.

ADD REPLY • link written 2.2 years ago by Devon Ryan ♦ 94k

For reference, here is a published comparison: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5792058/

ADD REPLY • link written 11 months ago by takeshi • 0

- Based on their latest respective releases, do you still consider STAR better than HISAT2?
- What are the qualifications behind this assertion, are they different from before?

ADD REPLY • link written 7 months ago by Anand Rao • 250

- yes
- At least STAR has added a plethora of new features

1

ADD REPLY • link written 7 months ago by Devon Ryan ♦ 94k

Another Question from a Student

I do not fully understand how to resolve the difficulty posed by various splicing alternative and the resultant isoforms when using short Illumina reads. If the reads are not long enough to span across various exon boundaries, how can such splicing be deduced? PE reads may help eliminate some ambiguity, but I don't understand how PE reads will map back to the transcriptome with alternative splicings.

Also, with regard to single-cell, I would be interested to know how much variation exists in the RNA content among different cells of a single tissue sample.

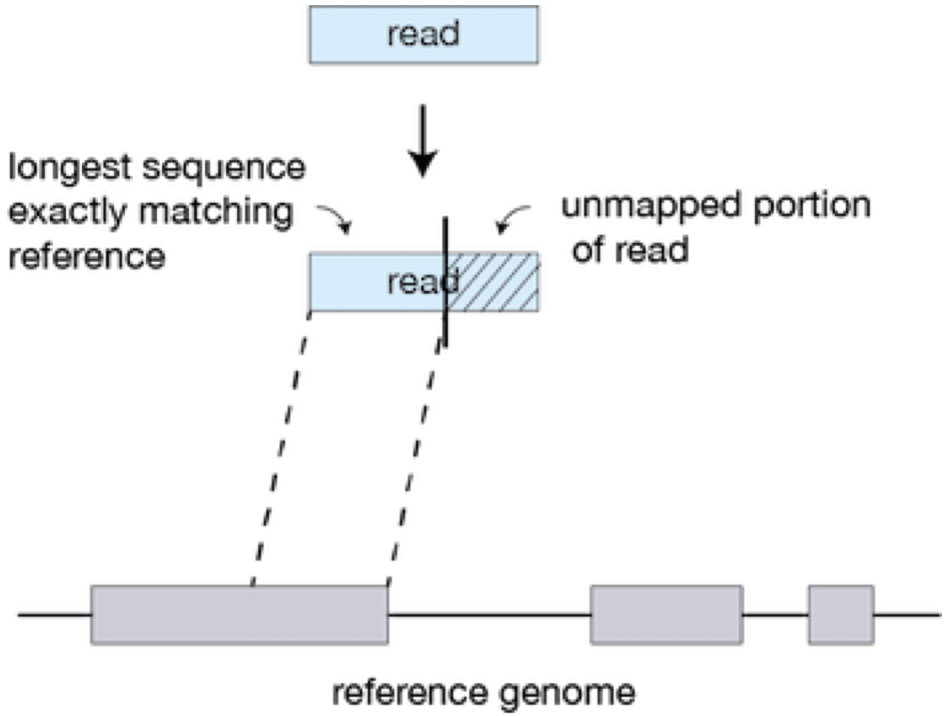
STAR Alignment Strategy

STAR is shown to have high accuracy and outperforms other aligners by more than a factor of 50 in mapping speed, but it is memory intensive. The algorithm achieves this highly efficient mapping by performing a two-step process:

- Seed searching
- Clustering, stitching, and scoring

Seed searching

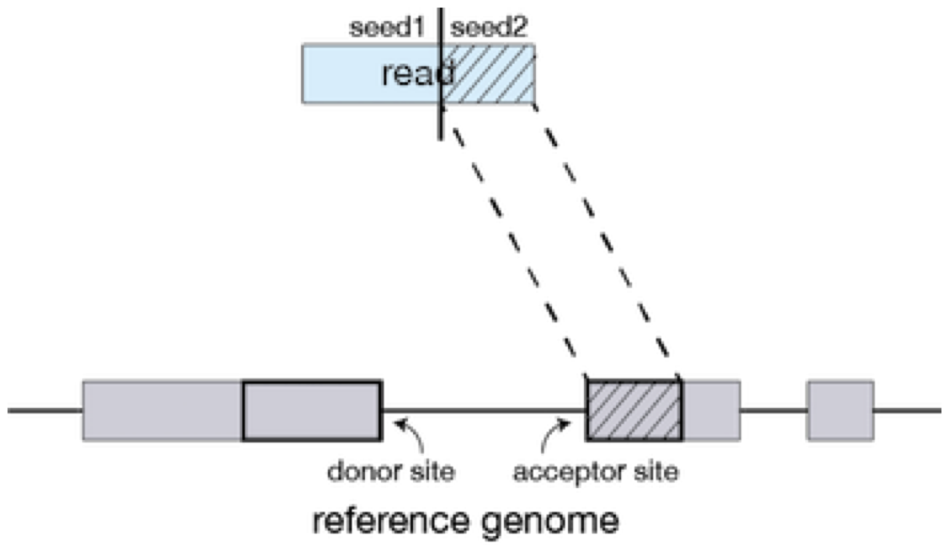
- For every read that STAR aligns, STAR will search for the longest sequence that exactly matches one or more locations on the reference genome. These longest matching sequences are called the Maximal Mappable Prefixes (MMPs):



https://hbctraining.github.io/Intro-to-rnaseq-hpc-O2/lessons/03_alignment.html

Seed searching

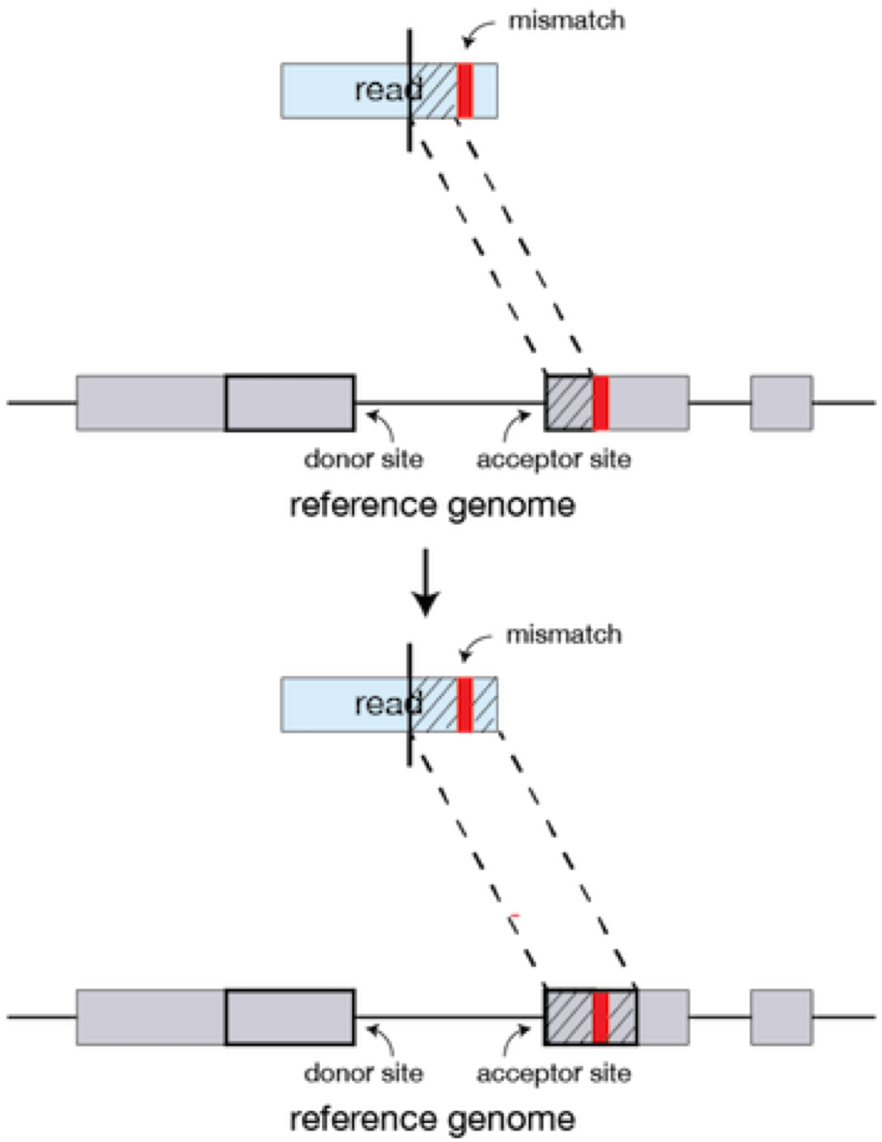
- The different parts of the read that are mapped separately are called ‘seeds’. So the first MMP that is mapped to the genome is called *seed1*.
- STAR will then search again for only the unmapped portion of the read to find the next longest sequence that exactly matches the reference genome, or the next MMP, which will be *seed2*.



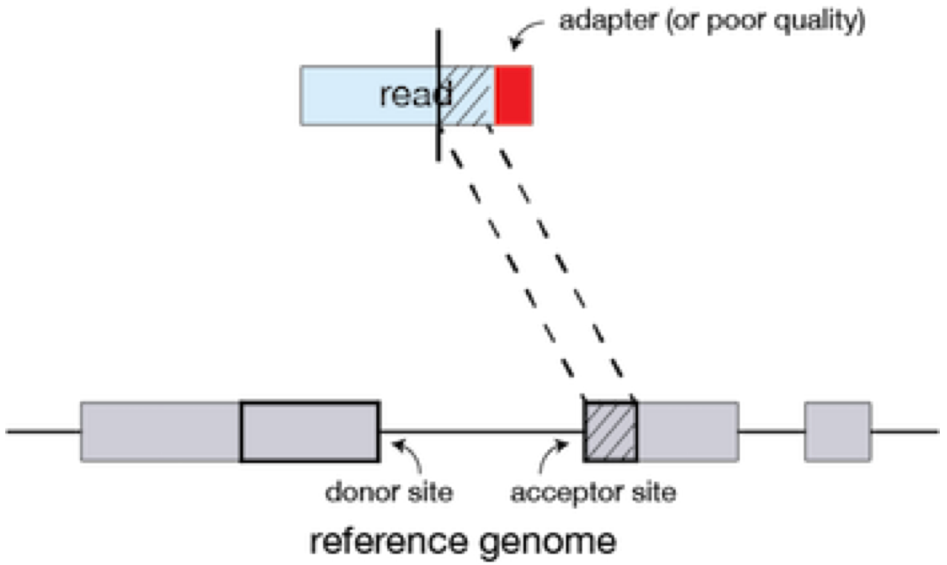
This sequential searching of only the unmapped portions of reads underlies the efficiency of the STAR algorithm. STAR uses an uncompressed suffix array (SA) to efficiently search for the MMPs, this allows for quick searching against even the largest reference genomes. Other slower aligners use algorithms that often search for the entire read sequence before splitting reads and performing iterative rounds of mapping.

https://hbctraining.github.io/Intro-to-rnaseq-hpc-O2/lessons/03_alignment.html

Best Mapping



- If STAR does not find an exact matching sequence for each part of the read due to mismatches or indels, the previous MMPs will be extended.
- If extension does not give a good alignment, then the poor quality or adapter sequence (or other contaminating sequence) will be soft clipped.



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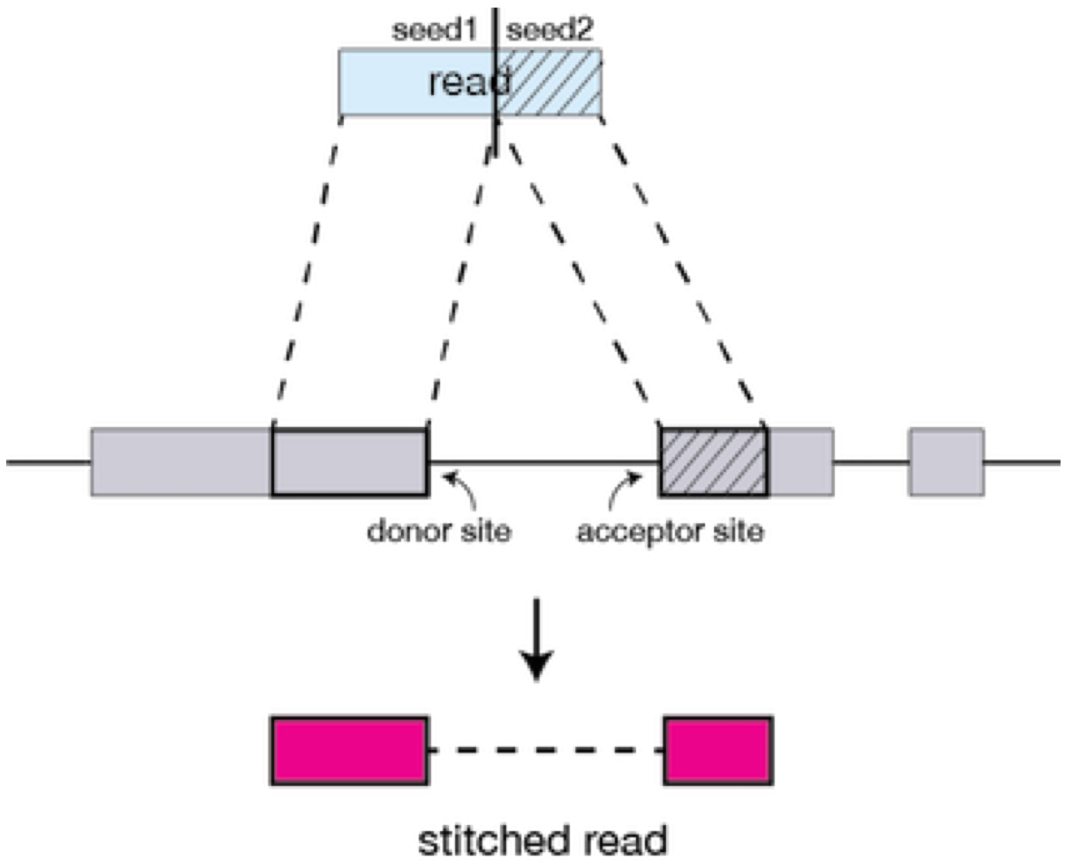
Clustering, stitching, and scoring



- The separate seeds are stitched together to create a complete read by first clustering the seeds together based on proximity to a set of 'anchor' seeds, or seeds that are not multi-mapping.
- Then the seeds are stitched together based on the best alignment for the read (scoring based on mismatches, indels, gaps, etc.).

https://hbctraining.github.io/Intro-to-rnaseq-hpc-O2/lessons/03_alignment.html

Additional Resources

- Additional Resources

Useful resources about Samtools

Good Tutorial

- https://davetang.org/wiki/tiki-index.php?page=SAMTools
- http://quinlanlab.org/tutorials/samtools/samtools.html

Decoding SAM flags

- https://broadinstitute.github.io/picard/explain-flags.html

Biological and technical replicates

- Biological replicates
  - RNA from independent growth of cells and tissues
  - Account for biological variations
- Technical replicates
  - Different library preparations of the same RNA-Seq sample
  - Account for **batch effects** from library preparations
    - Sample loading, cluster amplifications, etc

Recommended RNA-Seq sequencing depth based on genome size

Recommended RNA-Seq Parameters

Optimal sequencing depth for RNA-Seq will vary based on the scientific objective of study but here are some general recommendations based on sample type and application:

Sample Type	Reads Needed for Differential Expression (millions)	Reads Needed for Rare Transcript or De Novo Assembly (millions)	Read Length
Small Genomes (i.e. Bacteria / Fungi)	5	30 - 65	50 SR or PE for positional info
Intermediate Genomes (i.e. Drosophila / C. Elegans)	10	70 - 130	50 – 100 SR or PE for positional info
Large Genomes (i.e. Human / Mouse)	15 - 25	100 - 200	>100 SR or PE for positional info

https://genohub.com/ngs/

https://genohub.com/next-generation-sequencing-guide/#depth2

How many biological replicates?

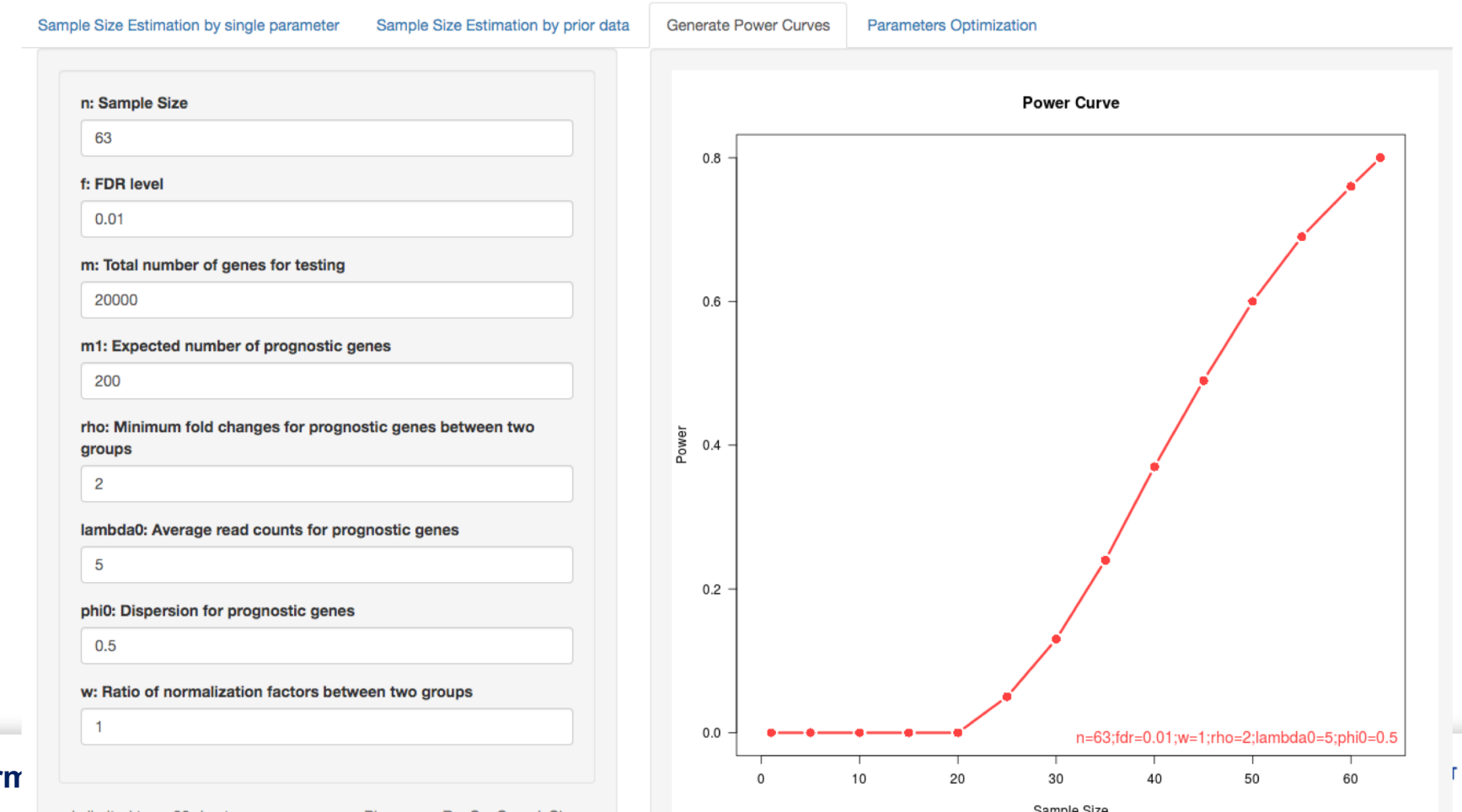
- As many as possible…

- Analysis of 48 biological replicates in two conditions
  - Requires 20 biological replicates to detect > 85% of all differentially expressed genes
- Recommend at least **six** biological replicates per condition
  - **Twelve** biological replicates needed to detect smaller fold changes (≥ 0.3-fold difference in expression)
- Three biological replicates per condition can usually detect genes with ≥ 2-fold difference in expression

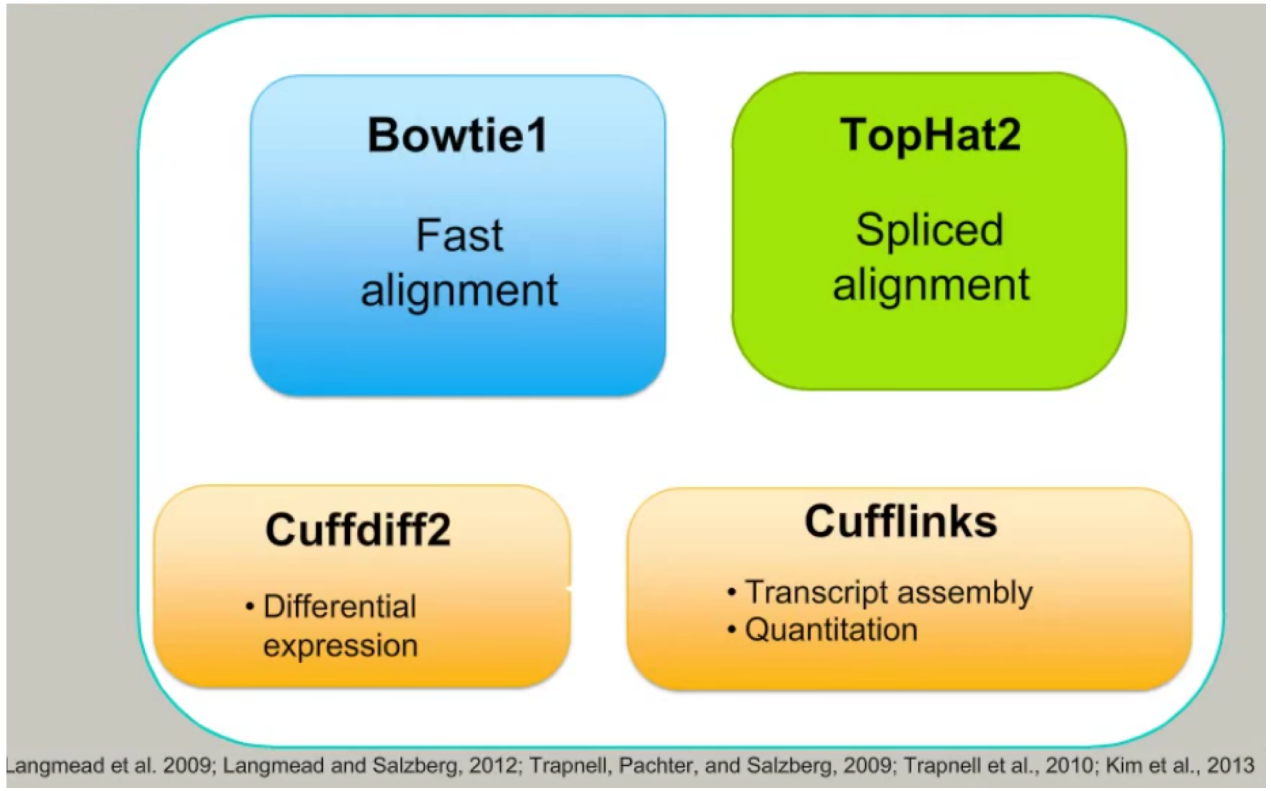
Schurch NJ *et al.* How many biological replicates are needed in an RNA-Seq experiment and which differential expression tool should you use? RNA. 2016 Jun;22(6):839-51.

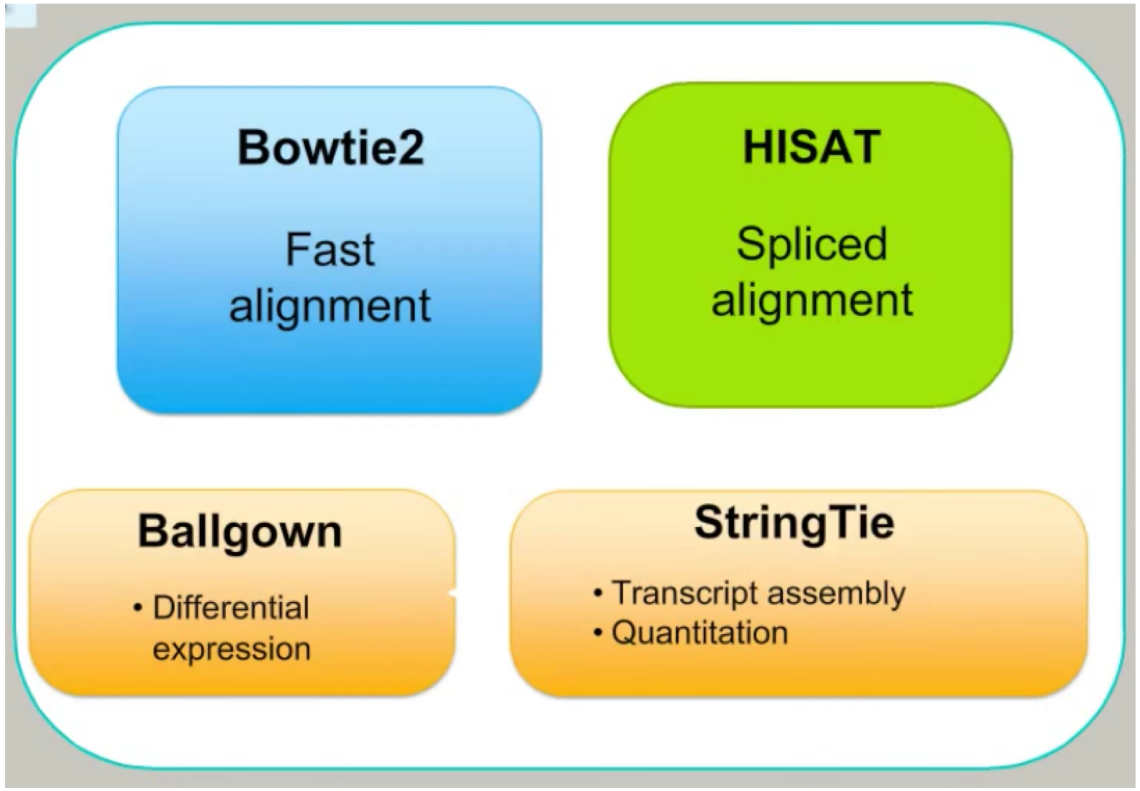
Power curves for number of biological replicates in each condition

- https://www.ncbi.nlm.nih.gov/pubmed/23961961
- https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5975570/
- https://cqs-vumc.shinyapps.io/rnaseqsamplesizeweb/



Tuxedo vs Next Tuxedo





Steven Salzberg - Transcriptome Assembly Computational Challenges of Next Generation Sequence Data (https://www.youtube.com/watch?v=2qGiw4MRK3c)

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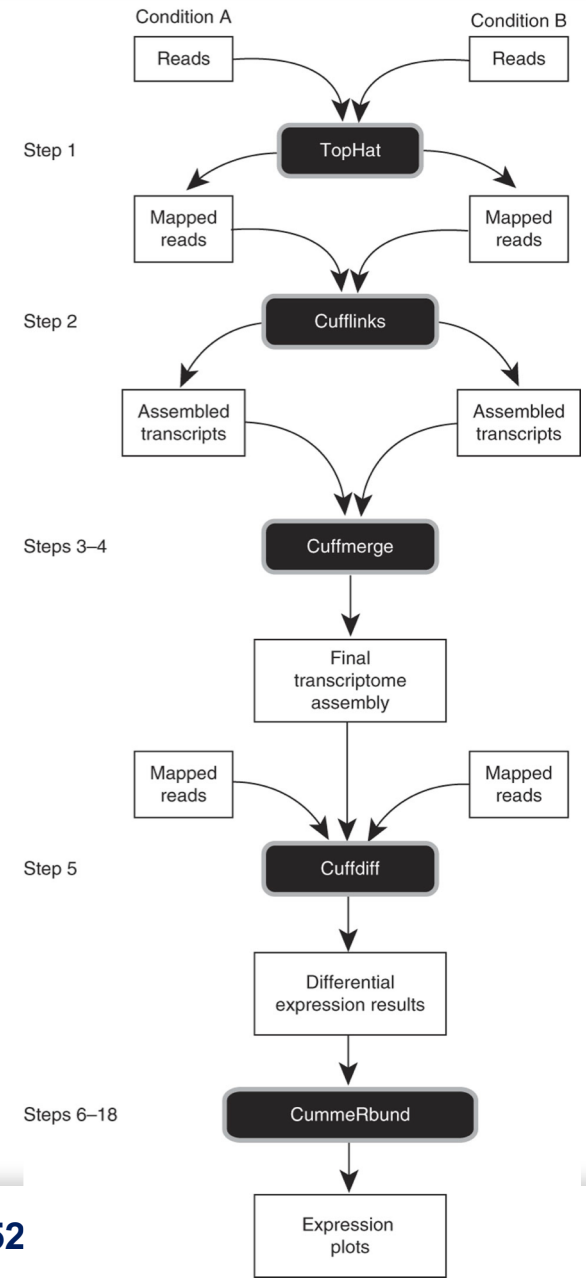
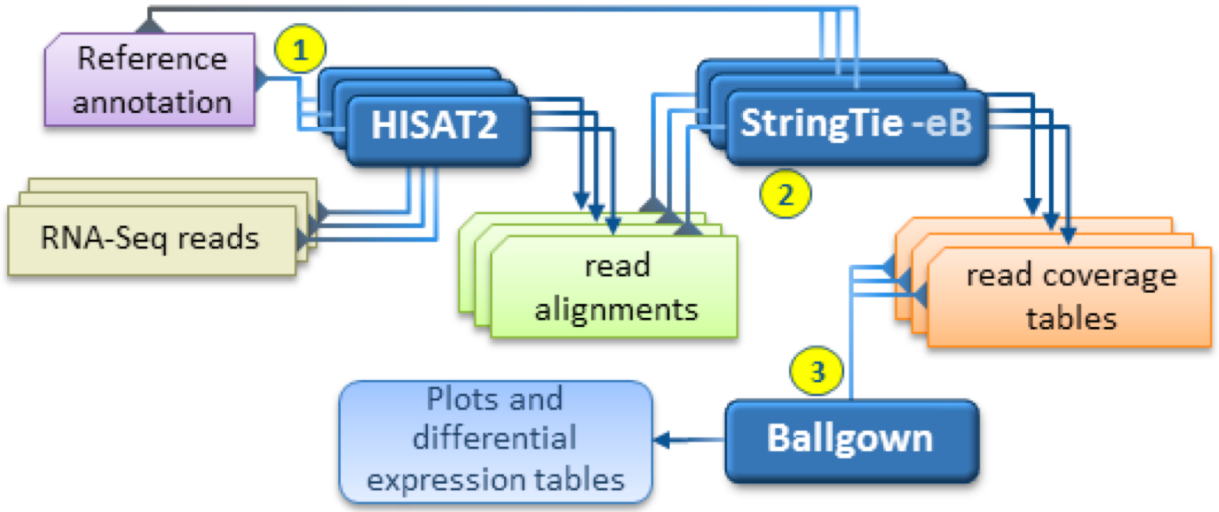
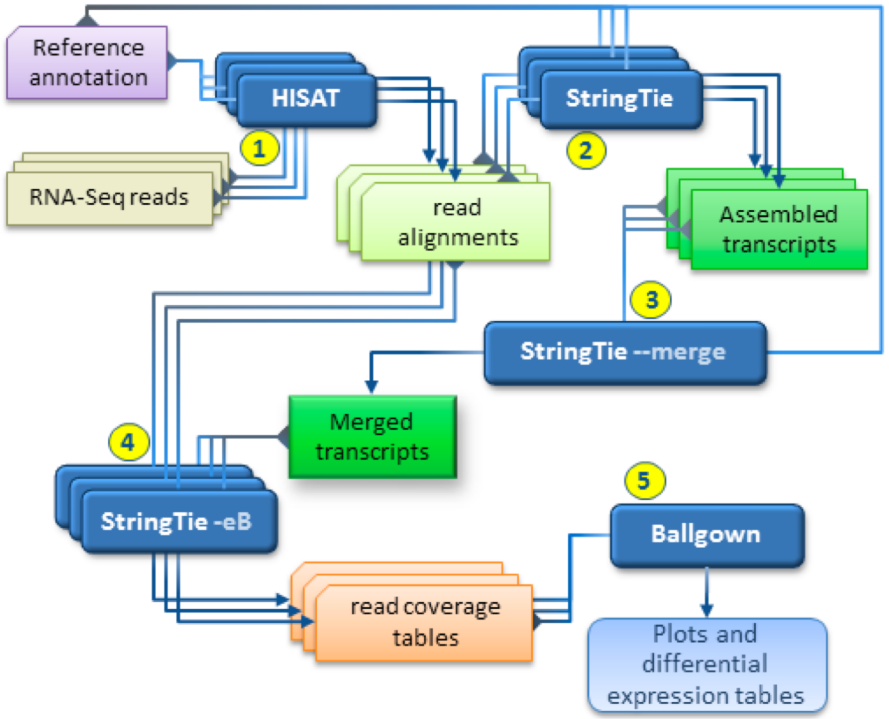


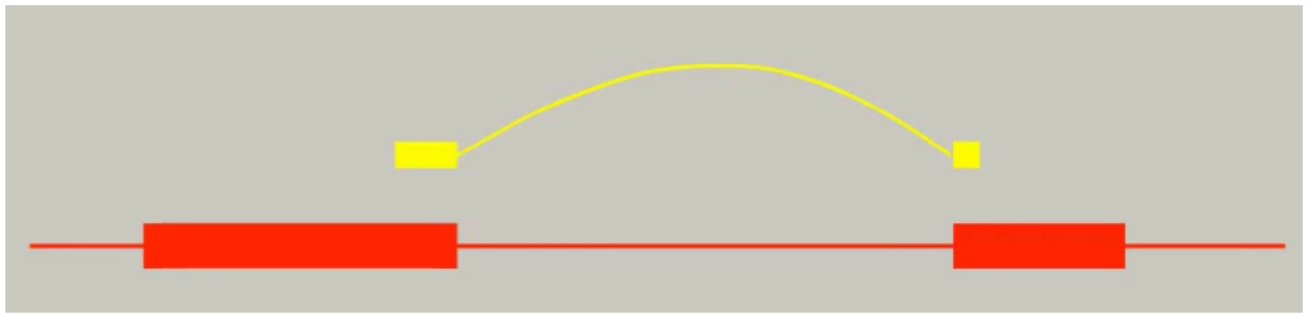




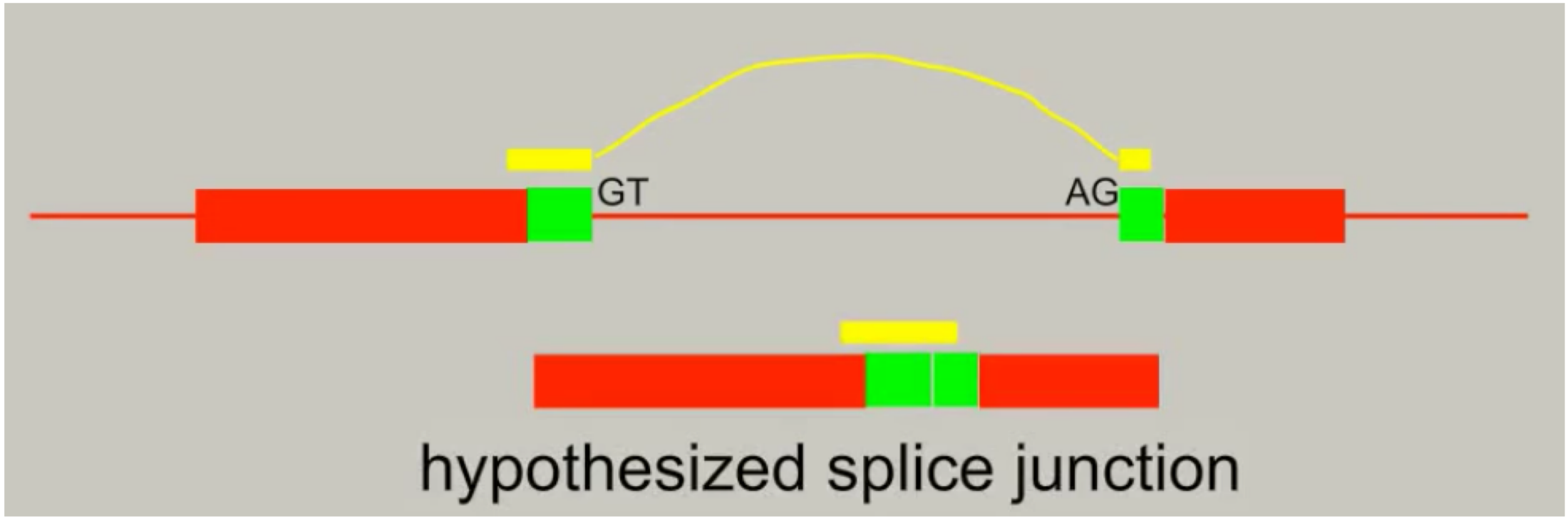
How most spliced-aligner works

- Two Steps
  1. Uses unspliced aligner (e.g., Bowtie) to map reads to reference genome
  2. For unmapped reads in step 1
     1. It detects potential splice sites for introns
     2. It uses these candidate splice sites to correctly align multiexon-spanning reads

- Spliced alignment challenge:
  - Aligning short reads across introns



Concatenate and try to map reads



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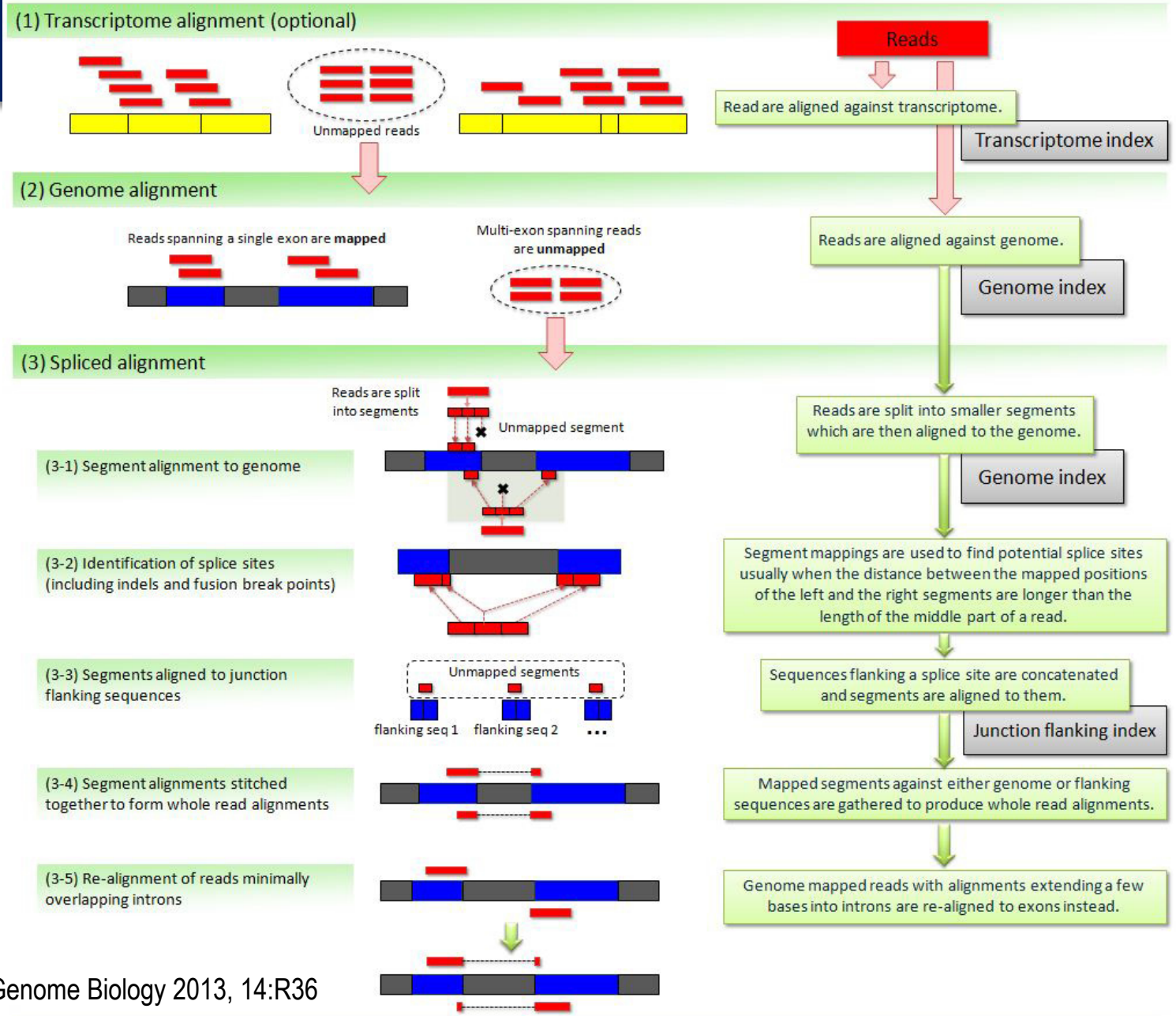
Tophat 2



Kim et al. Genome Biology 2013, 14:R36

How tophat works: junctions

- From supplied annotation file (GFF) or list of junction coordinates
- Find splice junctions without a reference annotation:
  - By first mapping RNA-Seq reads to the genome, TopHat identifies potential exons
  - Using this initial mapping information, TopHat builds a database of possible splice junctions
  - then maps the reads against these junctions to confirm them

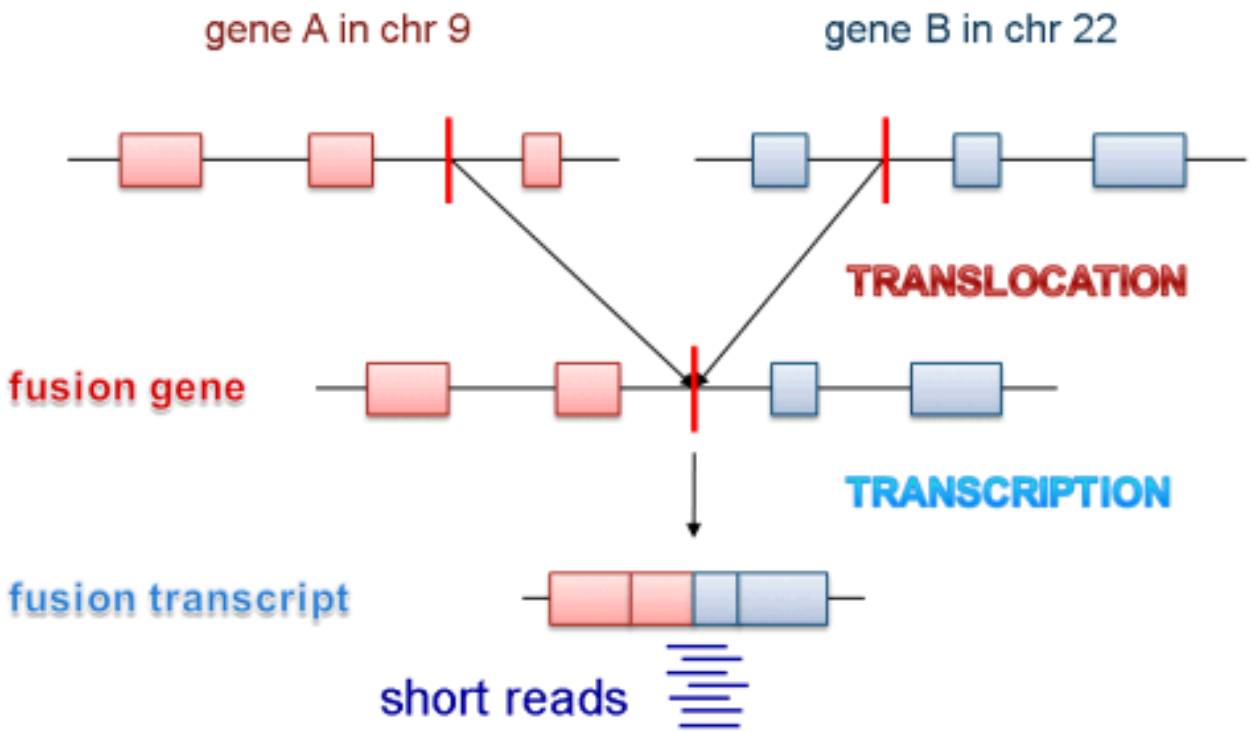
https://ccb.jhu.edu/software/tophat/manual.shtml

Tophat 1 vs Tophat 2

- TopHat2 can align longer reads (optimized for reads 75bp or longer)
- TopHat2 allowing for variable-length indels with respect to the reference genome.
- TopHat2 can align reads across fusion breaks

Kim et al. Genome Biology 2013, 14:R36

Gene fusion



Fusion genes are chimeric genes formed by two previously separated genes. They may be the products of chromosome structure changes such as insertion, deletion, inversion and translocation.

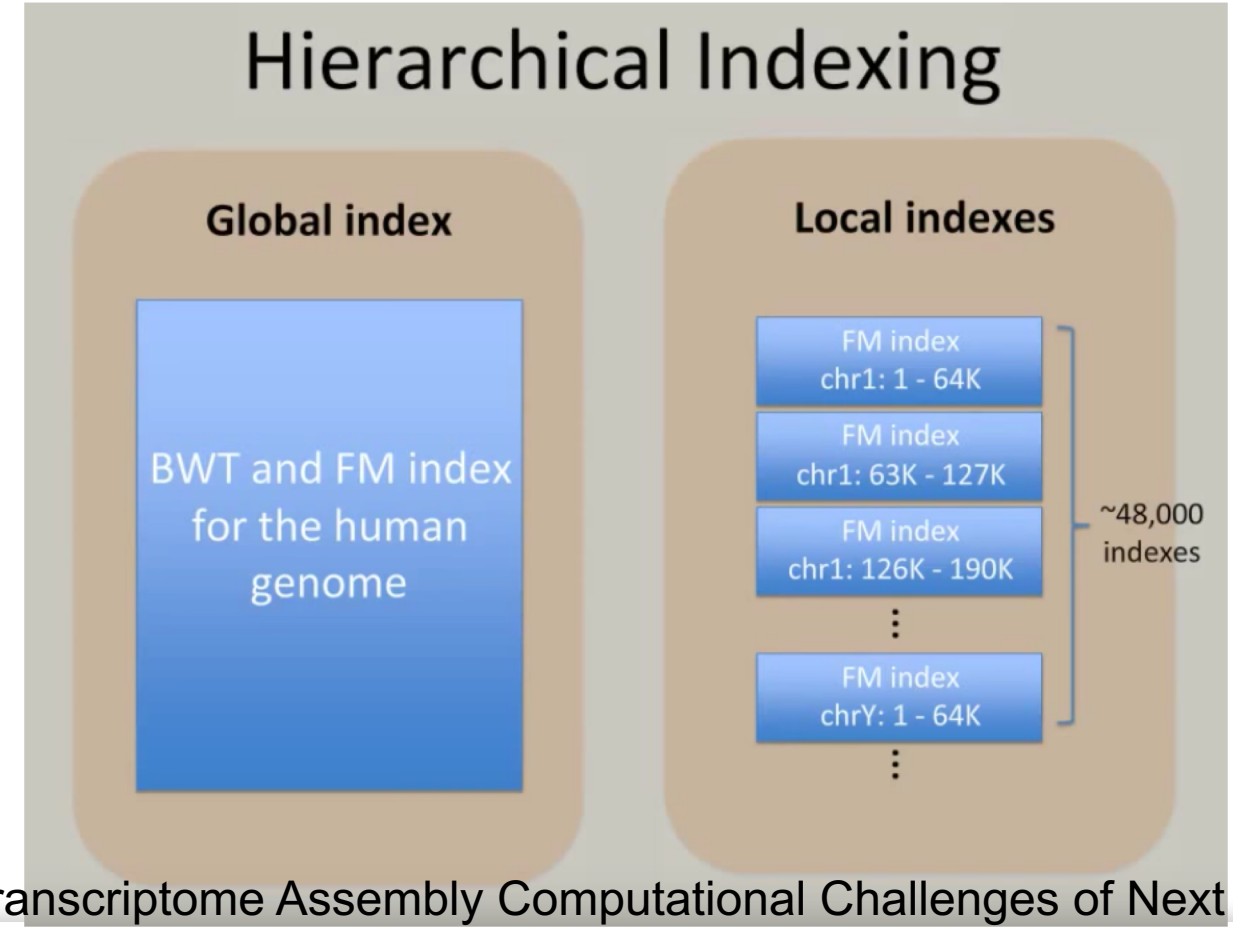
http://donglab.ecnu.edu.cn/databases/FusionCancer/

Tophat2 usage: input files

- Required input files
  - read file (fastq)
  - genome_index_base: indexed genome sequence
    - Download genome sequence
    - Generate genome index
- Optional input file
  - Genome annotation file (gff or gtf format)

HISAT

- Hierarchical Indexing for Spliced Alignment of Transcripts (HISAT)
- More speed: use Bowtie's BWT for everything → 38 faster than TopHat2



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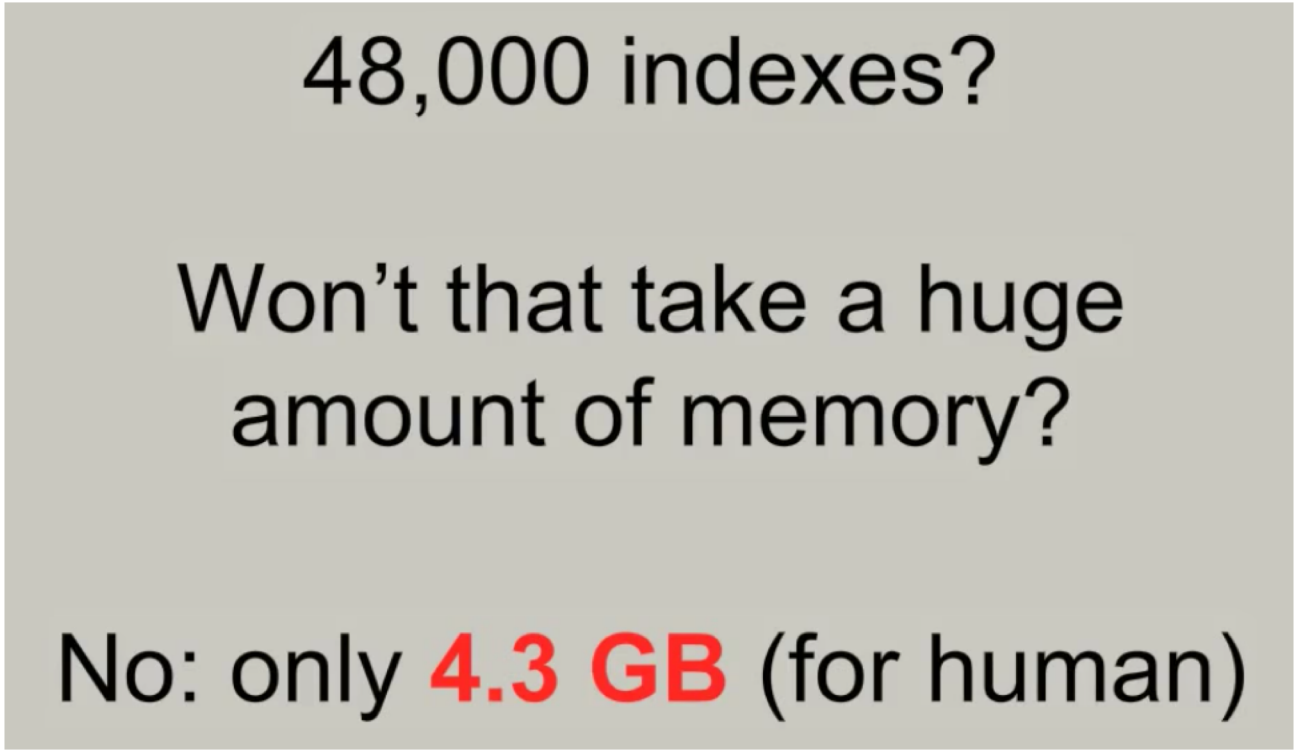

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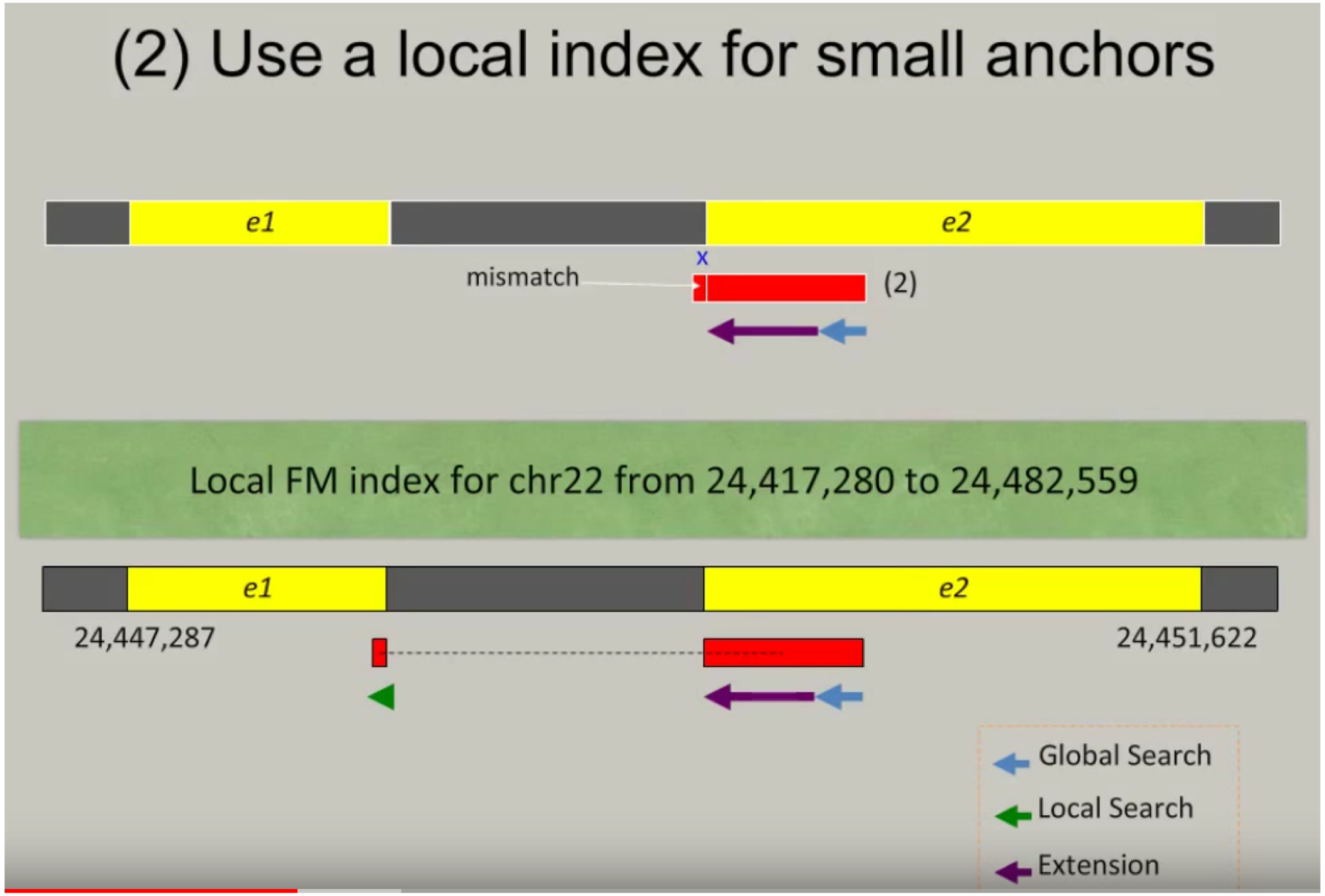

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