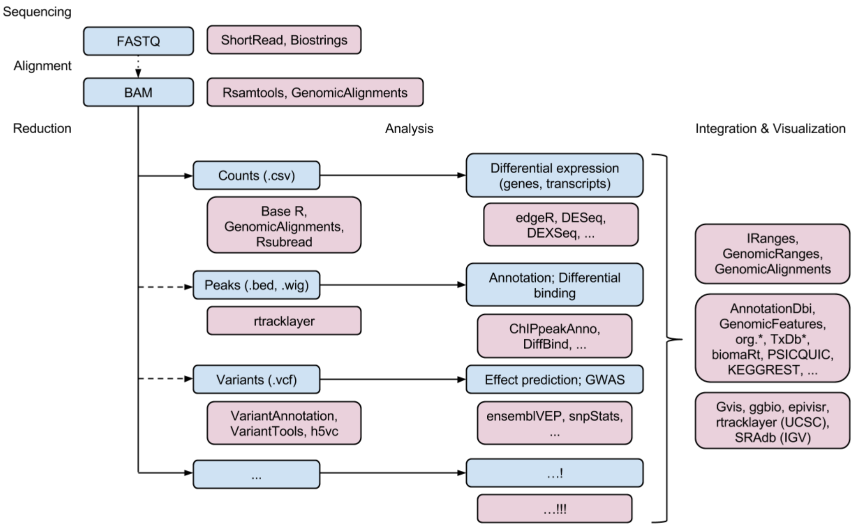
RNA-seq preprocessing

Mikhail Dozmorov

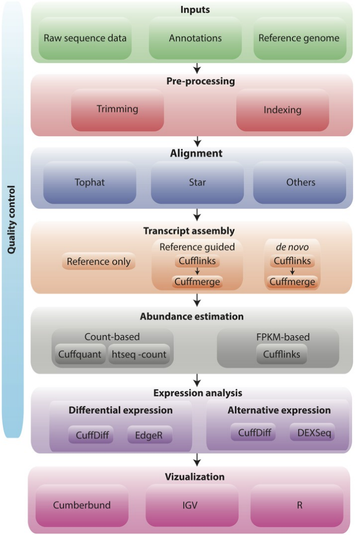
2021-03-03

Computational ecosystem of sequencing


Sequencing
FASTQ
ShortRead, Biostrings
Alignment
BAM
Rsamtools, GenomicAlignments
Reduction
Analysis
Integration & Visualization
Counts (.csv)
Base R, GenomicAlignments, Rsubread
Differential expression (genes, transcripts)
edgeR, DESeq, DEXSeq, ...
Peaks (.bed, .wig)
rtracklayer
Annotation; Differential binding
ChIPpeakAnno, DiffBind, ...
Variants (.vcf)
VariantAnnotation, VariantTools, h5vc
Effect prediction; GWAS
ensemblVEP, snpStats, ...
...
...!
...!!!
IRanges, GenomicRanges, GenomicAlignments
AnnotationDbi, GenomicFeatures, org.*, TxDb*, biomaRt, PSICQUIC, KEGGREST, ...
Gvis, ggbio, epivisr, rtracklayer (UCSC), SRAdb (IGV)

http://genomebiology.biomedcentral.com/articles/10.1186/s13059-016-0881-8

RNA-seq analysis workflow



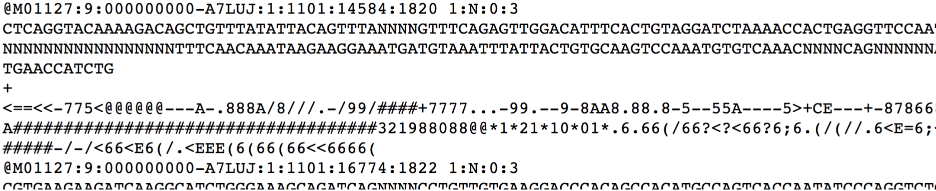
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4900641/

FASTA/FASTQ format

FASTA: text-based representation of nucleotide sequence

```
>Human mitochondrion
GATCACAGGTCTATCACCCTATTAACCACTCACGGGAGCTCTCCATGCATTTGGTATTTTCGTCTGGGGG
GTATGCACGCGATAGCATTGCGAGACGCTGGAGCCGGAGCACCCTATGTCGCAGTATCTGTCTTTGATTC
CTGCCTCATCCTATTATTTATCGCACCTACGTTCAATATTACAGGCGAACATACTTACTAAAGTGTGTTA
```

FASTQ: sequence and quality info



```
@M01127:9:000000000-A7LUJ:1:1101:14584:1820 1:N:0:3
CTCAGGTACAAAAGACAGCTGTTTATATTACAGTTTANNNNGTTTCAGAGTTGGACATTTCACTGTAGGATCTAAAACCACTGAGGTTCCAA
NNNNNNNNNNNNNNNNNTTTCAACAAATAAGAAGGAAATGATGTAAATTTATTACTGTGCAAGTCCAAATGTGTCAAACNNNNCAGNNNNNN
TGAACCATCTG
+
<==<<-775<@@@@@@---A-.888A/8///.-/99/####+7777...-99.--9-8AA8.88.8-5--55A----5>+CE---+-87866
A####################################321988088@@*1*21*10*01*.6.66(/66?<?<66?6;6.(/(//.6<E=6;
#####-/-/<66<E6(/.<EEE(6(66(66<<6666(
@M01127:9:000000000-A7LUJ:1:1101:16774:1822 1:N:0:3
CGTGAAGAAGATCAAGGCATCTGGGAAAGCAGATCAGNNNNCCTGTTGTGAAGGACCCACAGCCACATGCCAGTCACCAATATCCCAGGTCT
```

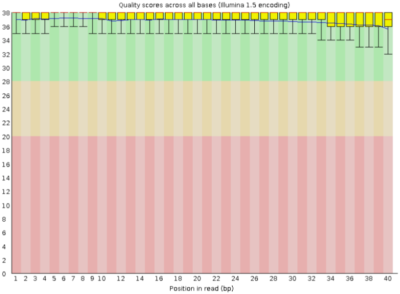
http://zhanglab.ccmb.med.umich.edu/FASTA/

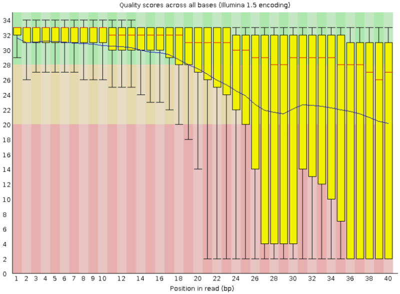
Quality of base calling

- **Phred quality score** is widely used to characterize the quality of base calling
- Phred quality score $= -10 * log_{10}(P)$, where P is probability that base-calling is wrong
- Phred score of 30 means there is $1/1000$ chance that the base-calling is wrong
- The quality of the bases tend to drop at the end of the read, a pattern observed in sequencing-by-synthesis techniques

Quality control

- **FASTQC** - Quality of raw and aligned sequencing data
    - Base quality per position
    - Nucleotide per position
    - GC content
    - K-mer enrichment





http://www.bioinformatics.babraham.ac.uk/projects/fastqc/, Video tutorial how to interpret, https://www.youtube.com/watch?v=bz93ReOv87Y

RNA-seq-specific quality control

- **RNASeQC** - quality of mapped (aligned) data
- **RSeQC** - Python-based table and graph QC reports
- **MultiQC** - Summarization and visualization QC results for multiple samples in one report. Recognizes multiple QC tools

http://www.broadinstitute.org/cancer/cga/rna-seqc, Deluca DS, Levin JZ, Sivachenko A, Fennell T, Nazaire MD, Williams C, Reich M, Winckler W, Getz G. (2012) RNA- SeQC: RNA-seq metrics for quality control and process optimization. Bioinformatics

http://rseqc.sourceforge.net/, Wang L, Wang S, Li W* RSeQC: quality control of RNA- seq experiments Bioinformatics (2012) 28 (16): 2184-2185. doi: 10.1093/bioinformatics/bts356

http://multiqc.info/

Adapter trimming

- **Cutadapt** - full control over adapter trimming
- **FASTX-Toolkit** - set of tools for low-level sequence trimming/cutting
- **Trimmomatic** - well-documented and easy-to-use adapter trimmer using multiple algorithms. Handles single- and paired-end reads, accountss for read quality
- **Flexbar**: similar to Trimmomatic by functionality

https://cutadapt.readthedocs.io/en/latest/guide.html

http://hannonlab.cshl.edu/fastx_toolkit/

http://www.usadellab.org/cms/?page=trimmomatic

https://github.com/seqan/flexbar/wiki/Manual

Duplicates removal

- Duplicates may correspond to biased PCR amplification of particular fragments
- For highly expressed, short genes, duplicates are expected even if there is no amplification bias
- Removing them may reduce the dynamic range of expression estimates

Generally, do not remove duplicates from RNA-seq data

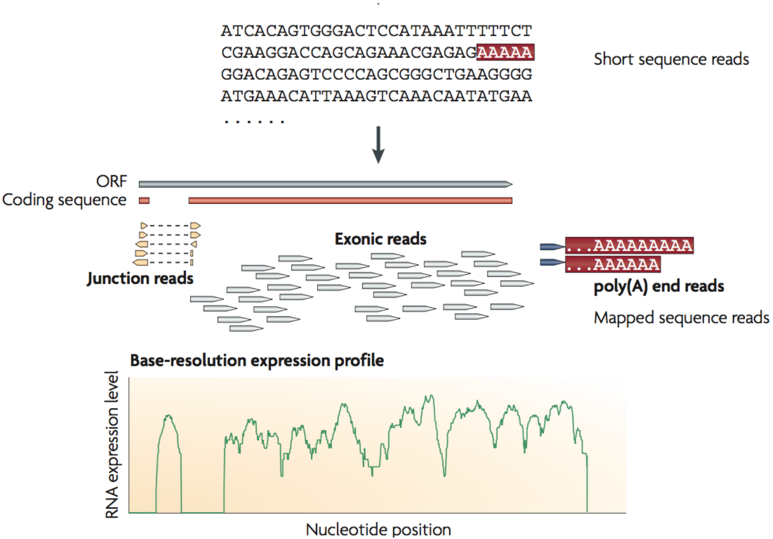
- If you ultimately want to remove duplicates, use Picard tools' `MarkDuplicates` command

https://broadinstitute.github.io/picard/command-line-overview.html#MarkDuplicates

Alignment

- RNA-seq aligners face an additional problem, not encountered in DNA-only alignment: many RNA-seq reads will span introns
- The average human intron length is $>$6,000 bp (some are $>$1 Mbp in length)
- In a typical human RNA-seq experiment using 100-bp reads, $>$35% of the reads will span multiple exons - align over splice junctions
- Aligners must be splice-aware, especially when aligning longer ($>$50bp) reads

Three types of reads: exonic-, junction- and poly(A) end-reads.



Strategies for gapped alignments of RNA-seq reads

Exon-first method

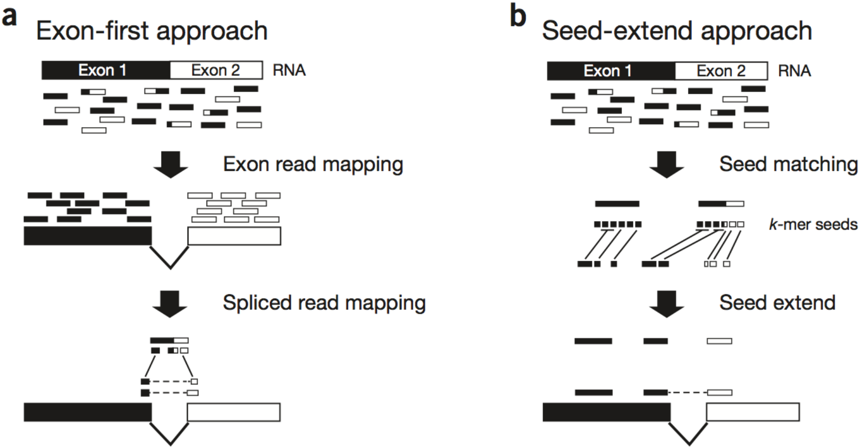
- Map full, unspliced reads (reads originating from a single exon) to exons
- Divide the remaining reads into smaller pieces and map them to the genome
- An extension process extends mapped smaller pieces to find candidate splice sites to support a spliced alignment.

Strategies for gapped alignments of RNA-seq reads

Seed-and-extend methods

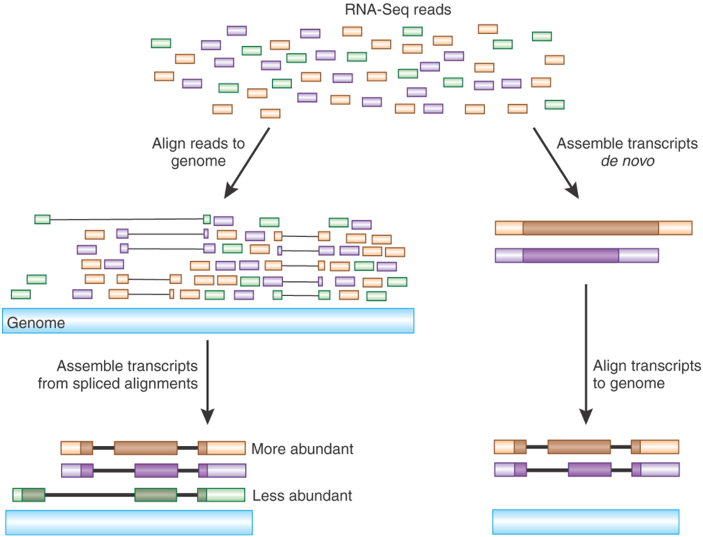
- Divide each RNA-seq read in small words (k-mers) of similar size
- Store a map of all k-mers in the genome in an efficient lookup data structure
- Map k-mers to the genome via the lookup structure
- Mapped k-mers are extended into larger alignments, which may include gaps flanked by splice sites.

Strategies for gapped alignments of RNA-seq reads



https://www.nature.com/articles/nmeth.1613

Alignment to the reference genome is the most frequently used for transcript quantification


RNA-Seq reads
Align reads to genome
Assemble transcripts *de novo*
Genome
Assemble transcripts from spliced alignments
Align transcripts to genome
More abundant
Less abundant

Alignment - Mapping RNA-seq reads to the genome

- **BWA** - general purpose algorithms based on Burrouws-Wheeler Transform
- **STAR** - fast and accurate aligner
- **HISAT**: (hierarchical indexing for spliced alignment of transcripts) uses two types of indexes for alignment: a global, whole-genome index and tens of thousands of small local indexes. Can detect novel splice sites, transcription initiation and termination sites. A part of the new "Tuxedo suite", including `StringTie` and `Ballgown`
- **subread**: a fast and accurate aligner, R and command line. The whole package includes `subjunc` for junction detection, and `featureCounts` for extracting read counts per gene from aligned SAM/BAM files

http://bio-bwa.sourceforge.net/

https://github.com/alexdobin/STAR

http://ccb.jhu.edu/software/hisat2/index.shtml

http://subread.sourceforge.net/

Timeline and extensive comparison of aligners https://www.ebi.ac.uk/~nf/hts_mappers/

De novo assembly

- **Trans-ABySS** - De novo assembly of RNA-Seq data
- **Velvet-Oases** - De novo transcriptome assembler for very short reads
- **SOAPdenovo-trans** - De novo transcriptome assembler accounting for alternative splicing and different expression level among transcripts
- **Trinity** - RNA-Seq De novo Assembly Using Trinity set of tools

http://www.bcgsc.ca/platform/bioinfo/software/trans-abyss

https://www.ebi.ac.uk/~zerbino/oases/

http://soap.genomics.org.cn/SOAPdenovo-Trans.html

https://github.com/trinityrnaseq/trinityrnaseq/wiki