Lab 0: HGEN 47900

Sequencing data processing & R basics

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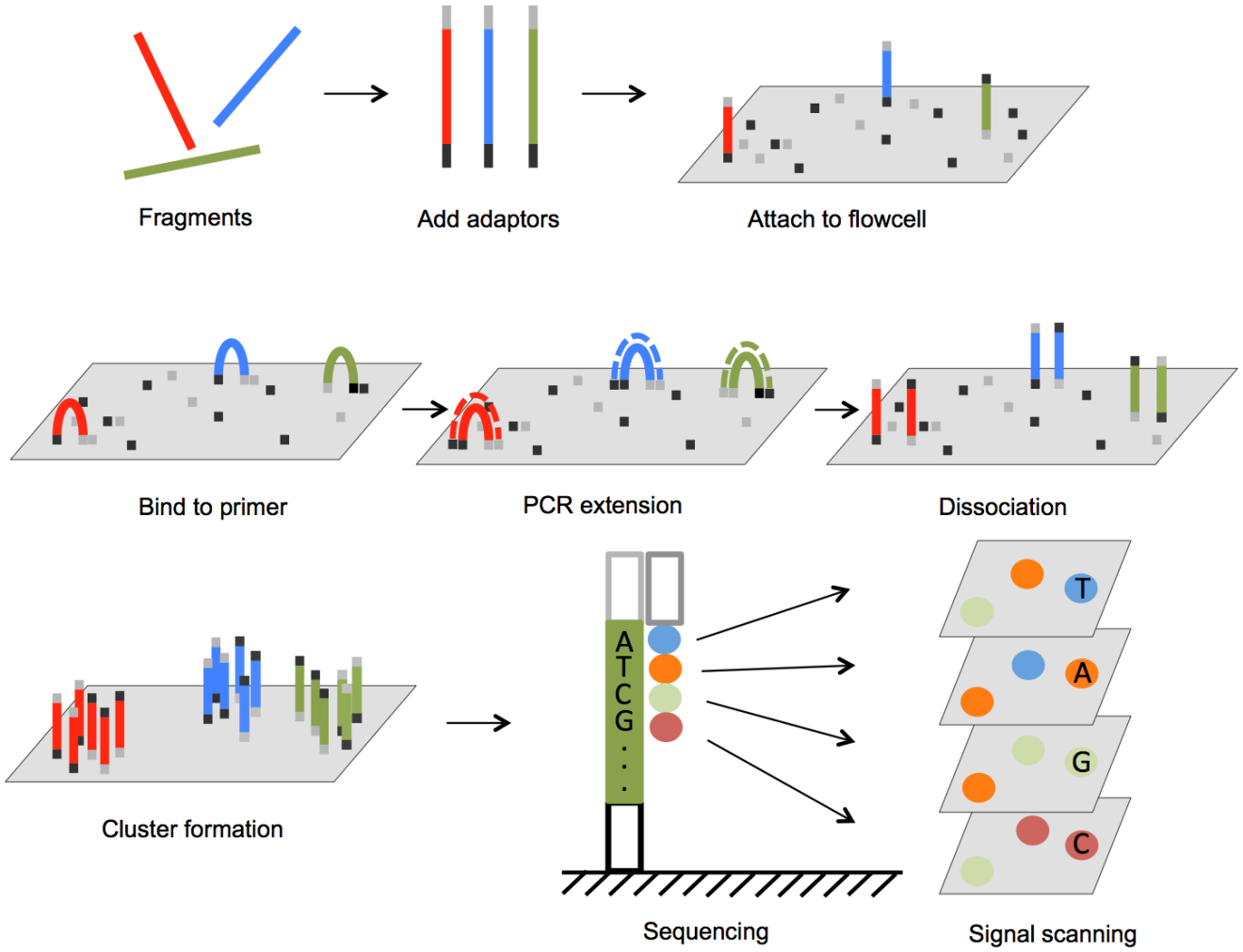
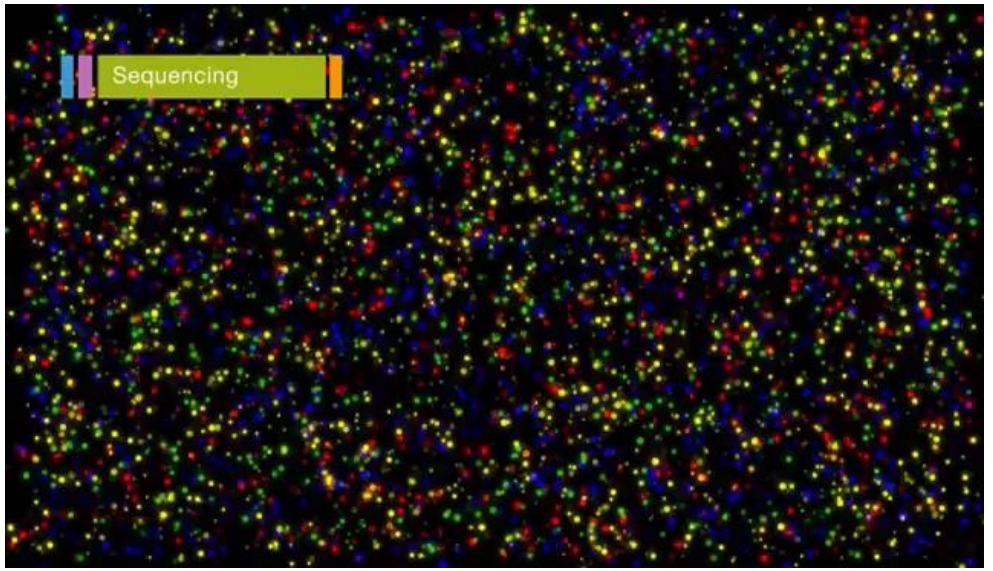
Next Generation Sequencing (Illumina)





Adaptors (p5 and p7) are added to the ends of the DNA fragments to be sequenced.
Those are used to attach to the flow cell and allow to perform the bridge amplification.
This is a simple solid phase PCR where individual fragments are locally amplified and form **clusters**.

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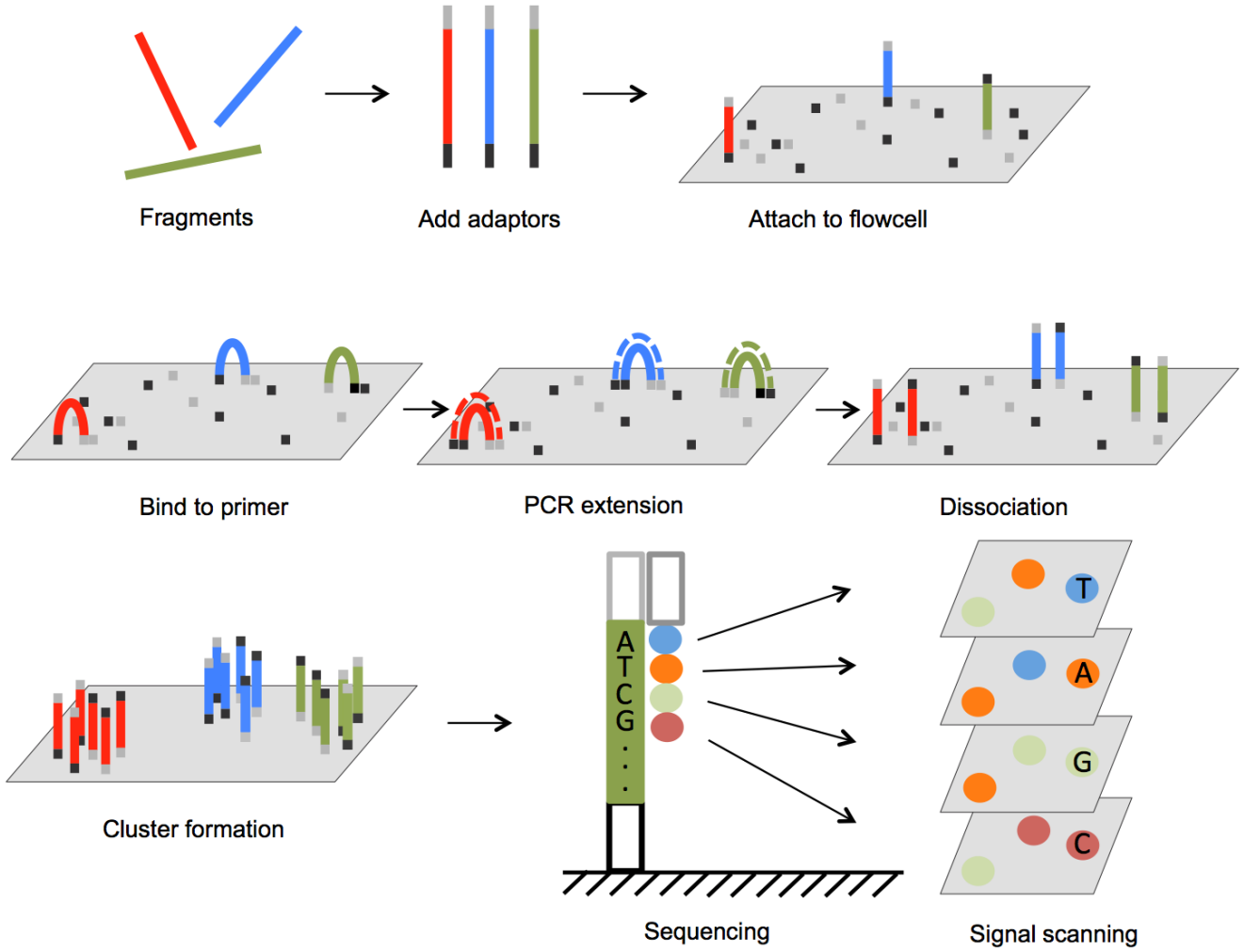
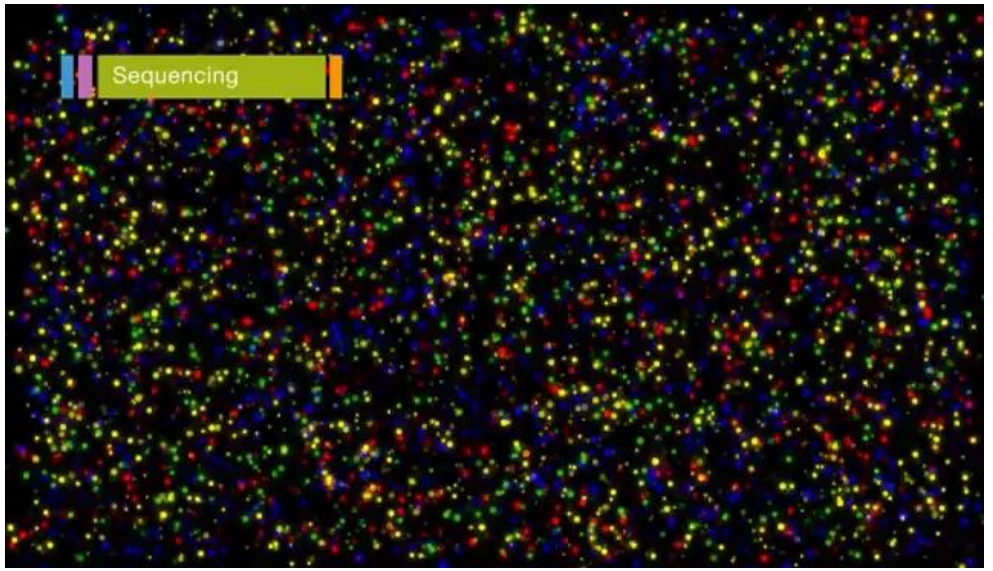




Each cycle, all four 3'-modified nucleotides are added to the flow cell are the extension happens for 1 bp. Further extension being blocked by the 3' modification.
After washing and imaging, the 3' modification is removed and the next cycle begins.

Next Generation Sequencing (Illumina): more details

Illumina sequencing kits come in different flavors depending on:

- The machine (MySeq, NextSeq, NovaSeq): this mostly changes the total number of reads,
- The output of the kit (mid vs high): this also changes the total number of reads,
- The number of cycles (up to 2x250 bp): this changes the length of the reads.

Cluster calling:

The detection of amplicon clusters on the flow cell is called **cluster calling**. On most Illumina machines, it happens only once at the beginning of the sequencing run (usually at cycle 5). This step is dependent on the diversity of nucleotides in the first few cycles of read 1.

In older, 2-channel systems (T is green, C is red, A is green + red and G has no color), having more than 2-3 Gs in the first bp of read 1 could prevent the detection of the cluster. Check what chemistry you use and how diverse you expect your library to be. Similarly, if all your DNA fragments start with the exact same sequence, then the flow cell could be uniformly colored, which makes it difficult to detect clusters.

A potential solution to increase the diversity is to add some Phi X174 phage DNA to your library.

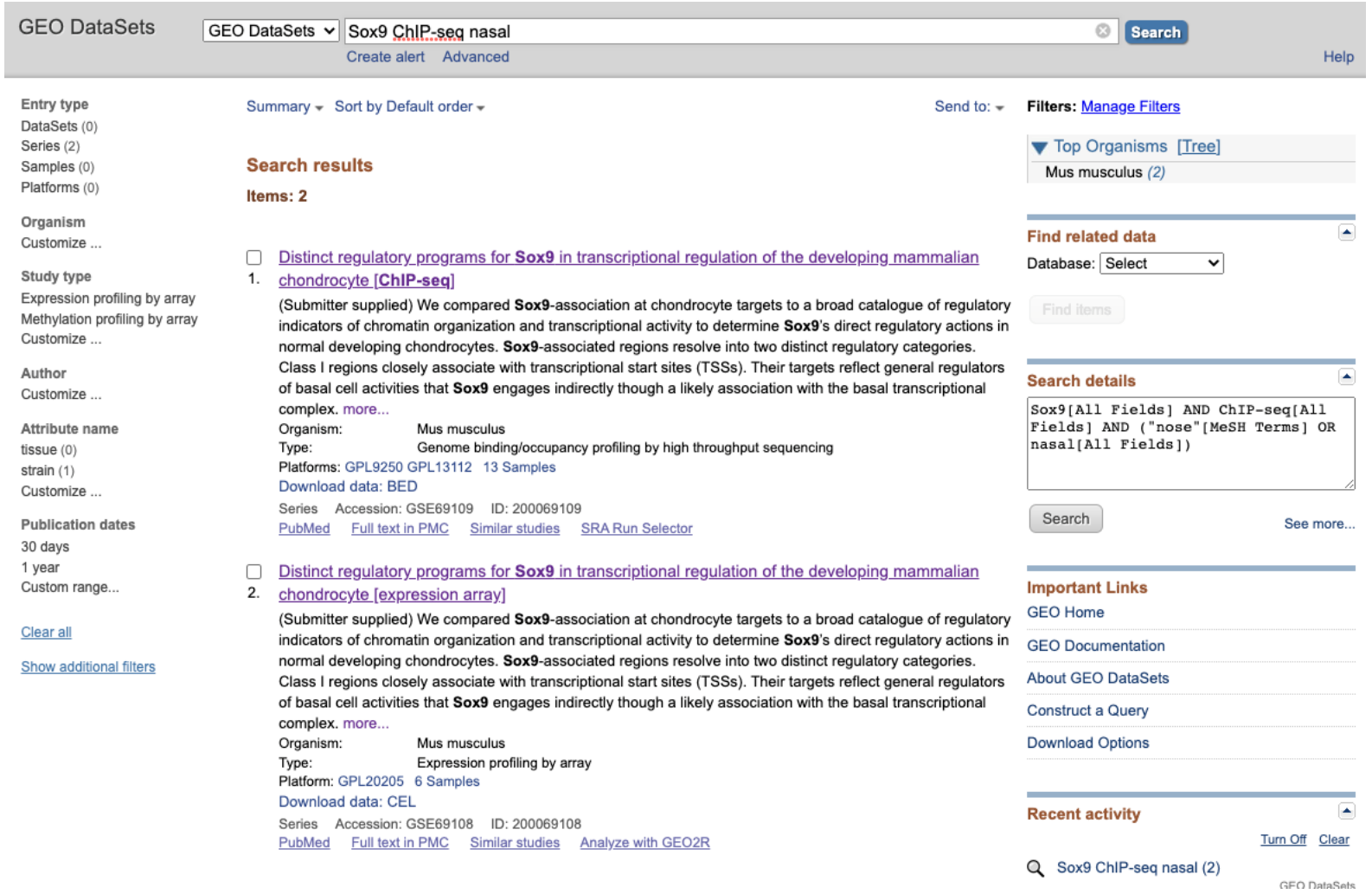
The expected **cluster density** on a NextSeq 500/550 High output kit is ~ 170k – 220k clusters/mm^2

Cluster filtering:

After some number of cycles (e.g., 25 on a NextSeq), the clusters are filtered to guarantee the quality of the base call. If clusters overlap too much, they will be discarded. If they are not homogeneous, they will be discarded. The risk of this happening increases with the concentration of DNA in your library.

A good sequencing run has over 90% clusters passing filter.

Finding data on NCBI (Gene Expression Omnibus)



(Has all types of data, not only gene expression)

https://www.ncbi.nlm.nih.gov/geo/

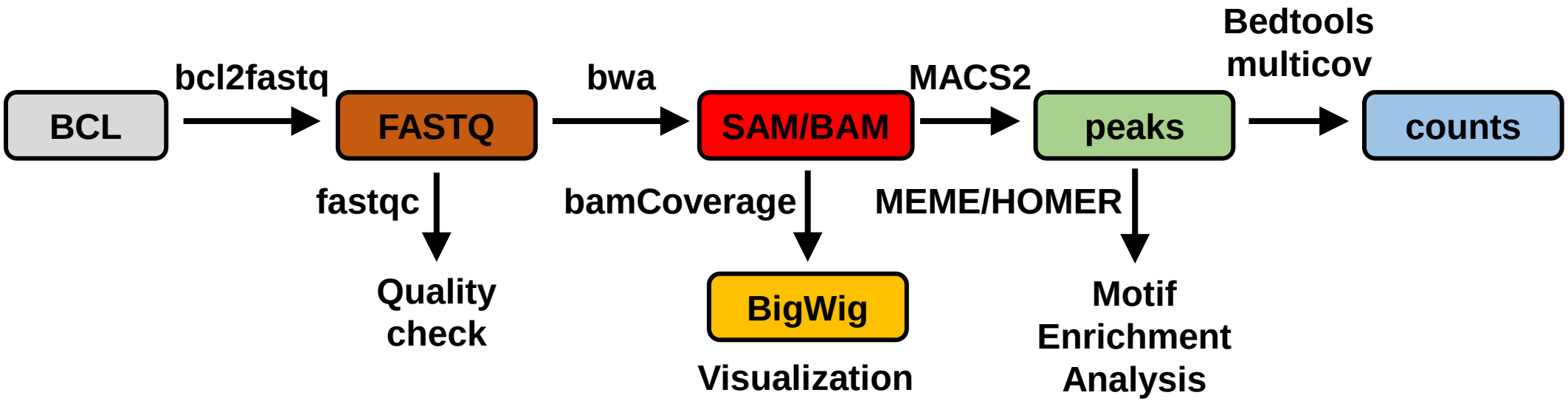
Fetching data on Sequence Read Archive (SRA)

Using **sra-tools** (https://github.com/ncbi/sra-tools/wiki/) to fetch the data

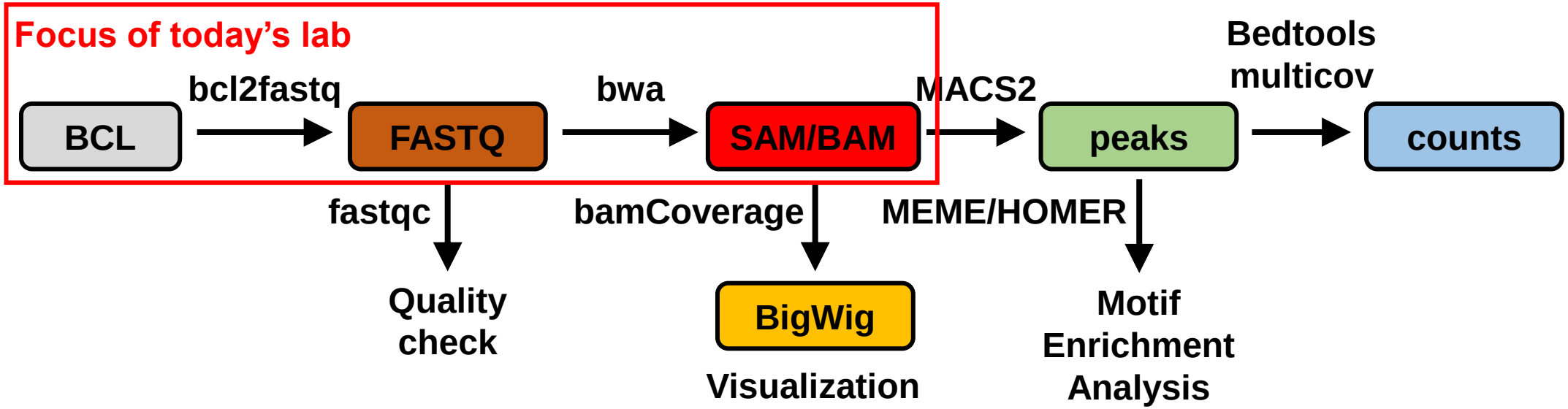
`fastq-dump -split-files SRRnumber`

```
reads read      : 40,527,423
reads written   : 40,527,423
sbastide@nobel:/data/personal_folders/sbastide/sequencing_data_tutorial$ ls
SRR2034943.fastq
sbastide@nobel:/data/personal_folders/sbastide/sequencing_data_tutorial$
```

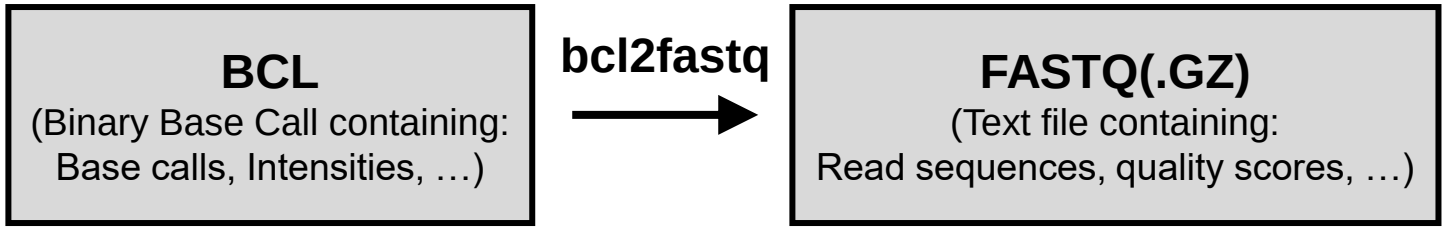
Full pipeline for ATAC-seq/ChIP-seq



Full pipeline for ATAC-seq/ChIP-seq



Fastq files




```
sbastide@nobel:/data/personal_folders/sbastide/sequencing_data_tutorial/Fastqs$ head -n 12 Sample10.fq
@SRR2034943.8532554 ILLUMINA-3AB384:61:FC:8:21:8047:3232 length=42
ACTAGGCAATATTTTTTACCAATGACAAACTTAAGTGTCTTC
+
EHIIIIIIIIIIIIHIIIIIIGIGIIIIIIIIIIIIIIIII
@SRR2034943.13764671 ILLUMINA-3AB384:61:FC:8:34:8719:5489 length=42
GACAAGGGCATGCTCTTGTGTGTATGCATGTGGGGCCATAGG
+
##########################################
@SRR2034943.31253095 ILLUMINA-3AB384:61:FC:8:79:13486:4287 length=42
TGCATCTGATCTGTGGCCAGACAGCCTAGACTACTGTGTCCA
+
IIIIIIIIIIIIHIIHIIIIIIIEIHIIIIIHIFFHGIIII
```

Fastq files contain records for all individual reads (i.e. sequences of nucleotides derived from a single cluster)
Each record has 4 lines

- Line 1 (**header**): starts with a @ followed by the sequence label
- Line 2: DNA sequence (may contain Ns when the base calling is bad)
- Line 3: Line break (contains a **+** by convention)
- Line 4: Quality (Phred) scores in ASCII

```
fastqc SRR2034943.fastq
```

Creates a .html file that contains a bunch of information about the sequencing run

Optional: read trimming using Cutadapt (for ATAC-seq)

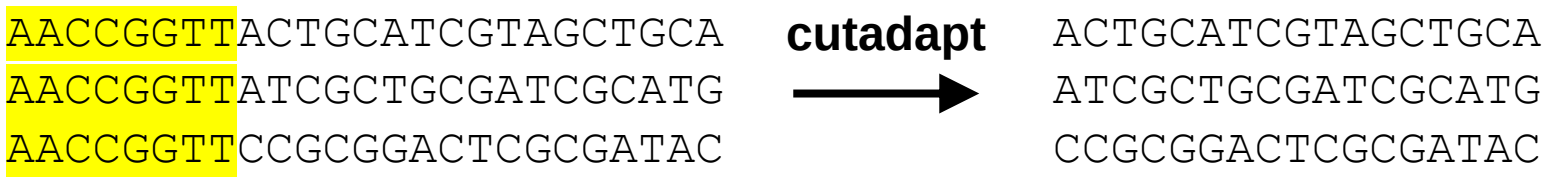
https://cutadapt.readthedocs.io/en/stable/guide.html

Not necessary if using modern aligners (STAR, BWA-MEM, …)
Be careful, by default, Bowtie2 uses end-to-end alignment which does not allow soft-clipping of the beginning or end of the read sequences

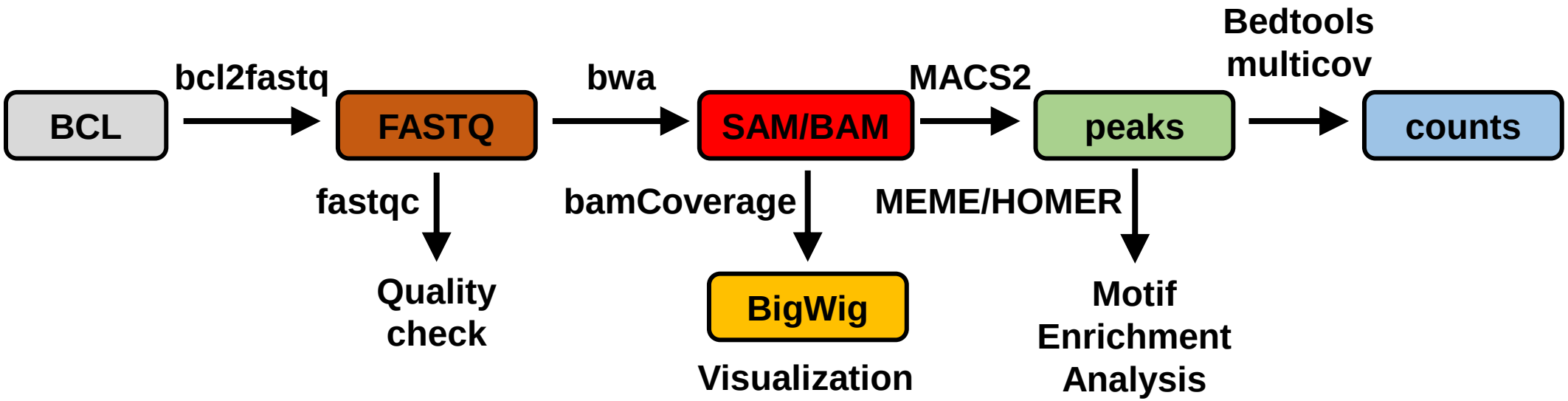
Other tools: **Trimmomatic**, **Trim Galore**

(Tn5 from the Nextera kit introduces CTGTCTCTTATACACATCT)

```
cutadapt -a AACCGGTT -o output.fastq input.fastq
```



Full pipeline for ATAC-seq/ChIP-seq



Mapping reads to a Genome using BWA-MEM

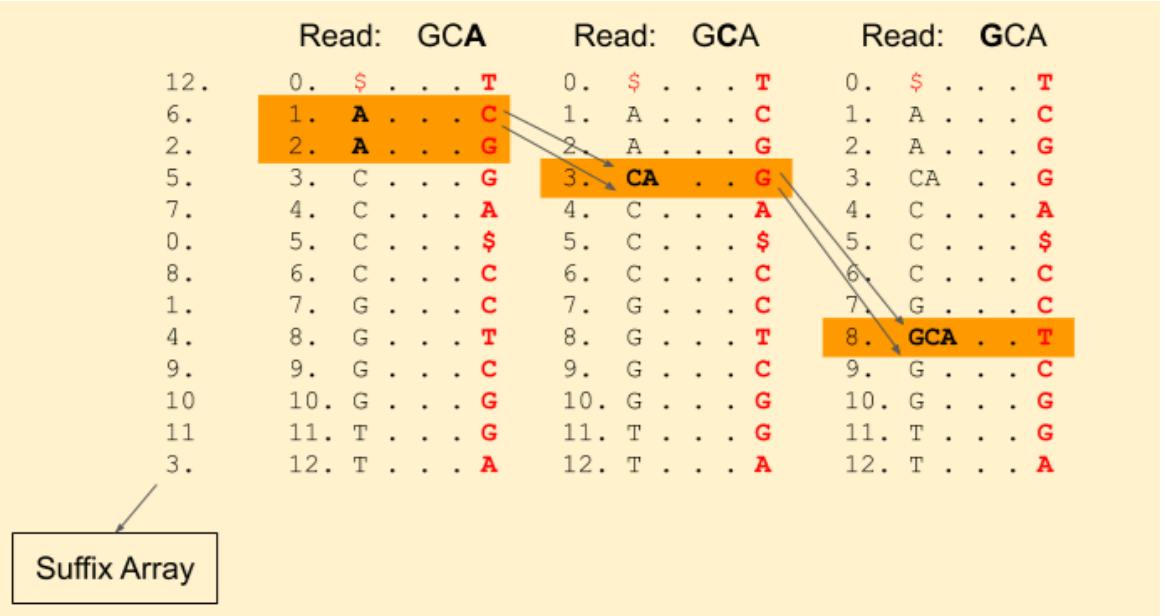
```
sbastide@nobel:/data/personal_folders/sbastide/sequencing_data_tutorial/FastQC$ bwa mem

Usage: bwa mem [options] <idxbase> <in1.fq> [in2.fq]
```

`<idxbase>` corresponds to the BW transformed index (reference genome)

Burrows-Wheeler Transform (BWT) is useful for compression:

- Completely reversible without loss of information
- Only the output is required to uncompress
- Same characters tend to cluster together (e.g. BWT(ACAACG$) = GC$AAAC = GC$3AC)

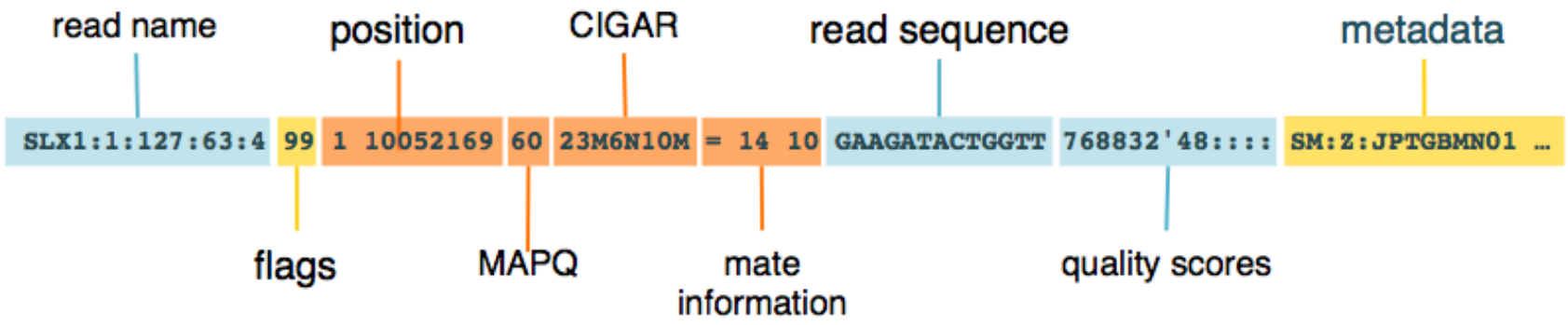
Transformation				
1. Input	2. All rotations	3. Sort into lexical order	4. Take the last column	5. Output
^BANANA|	^BANANA| |^BANANA A|^BANAN NA|^BANA ANA|^BAN NANA|^BA ANANA|^B BANANA|^	ANANA|^B ANA|^BAN A|^BANAN BANANA|^ NANA|^BA NA|^BANA ^BANANA| |^BANANA	ANANA|^B ANA|^BAN A|^BANAN BANANA|^ NANA|^BA NA|^BANA ^BANANA| |^BANANA	BNN^AA|A



SAM file format

SAM files contain a lot of information about reads and their mapping stats
When reads map to multiple locations, all of those are indicated in separate lines

Thus, SAM files are big files (1.6 Mb for 10,000 reads).
They can be compressed to a binary format called BAM using samtools (475 Kb for 10,000 reads).




```
bwa mem /data/genomes/mm10.fa input.fastq| samtools sort -O bam -T output.tmp -o output.bam
```

`samtools sort` will sort the alignments: first by chromosome, then by position

`|` is called a “pipe” and allows to use the output of a first command as the input of a second command

Alignment statistics and indexing

```
samtools stats -in ./Bams/SRR2034943.bam
```

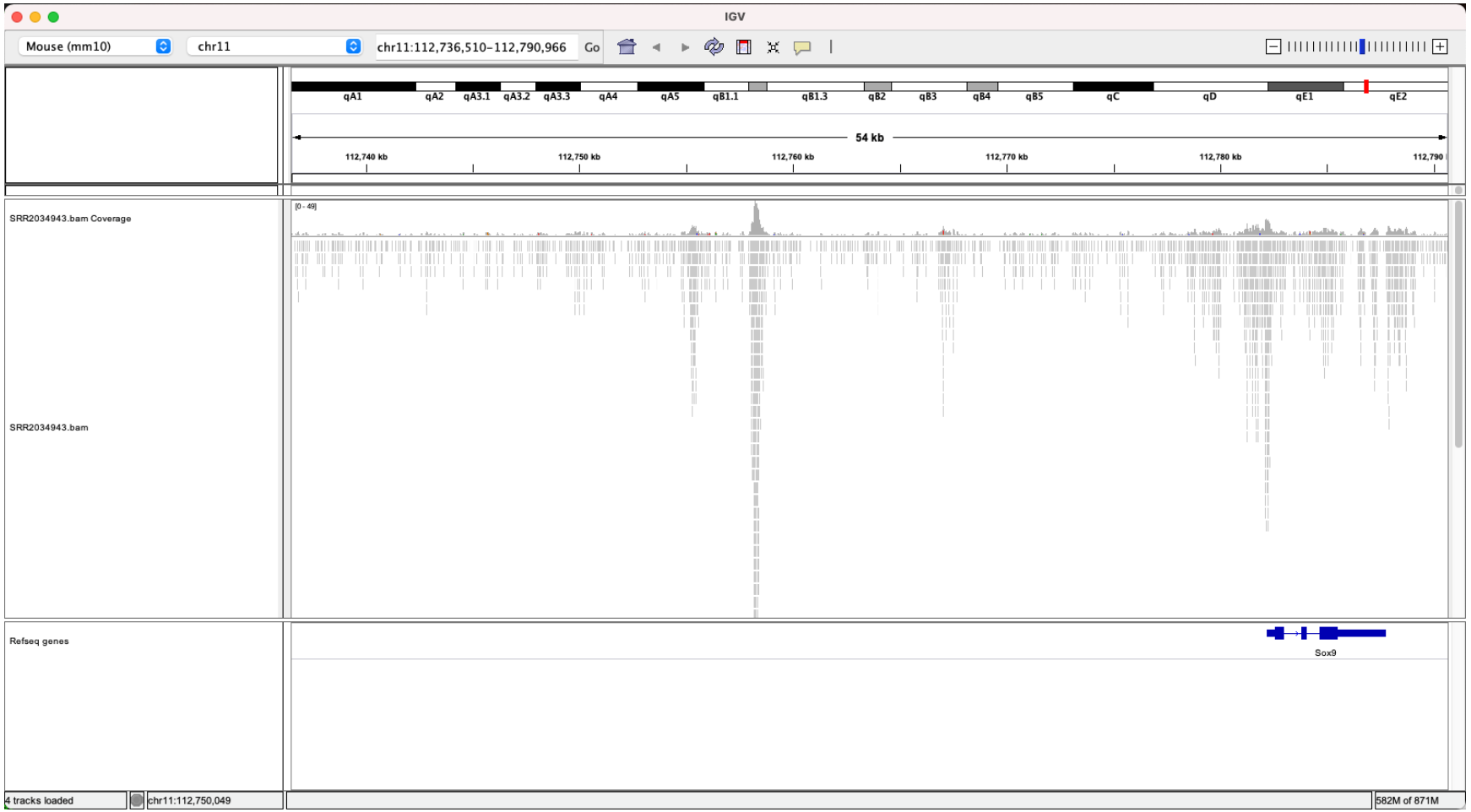
```
sbastide@nobel:/data/personal_folders/sbastide/sequencing_data_tutorial$ bamtools stats -in ./Bams/SRR2034943.bam

**********************************************
Stats for BAM file(s):
**********************************************

Total reads:       40527423
Mapped reads:      33973845     (83.8293%)
Forward strand:    23575568     (58.1719%)
Reverse strand:    16951855     (41.8281%)
Failed QC:         0    (0%)
Duplicates:        0    (0%)
Paired-end reads:  0    (0%)
```

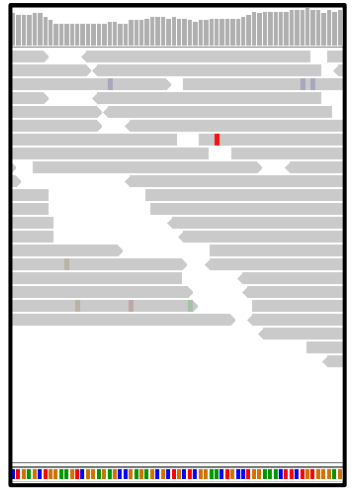
```
samtools index ./Bams/SRR2034943.bam
```

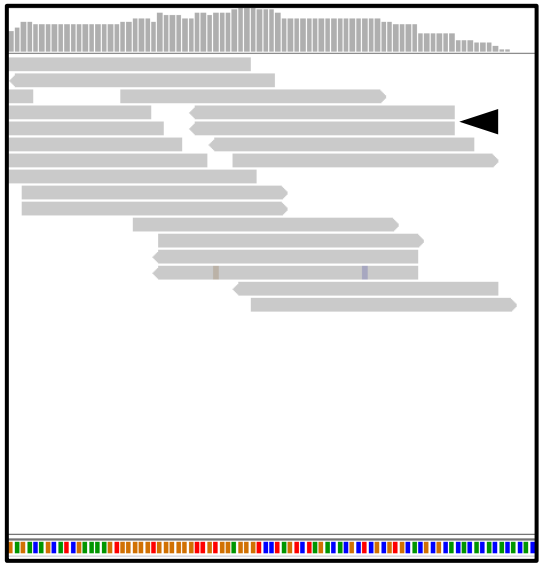
Creates a .bam.bai file that allows to quickly and efficiently search the BAM file without having to read through every line from the top

Visualization using BAM files in IGV

Change to Mm10 →







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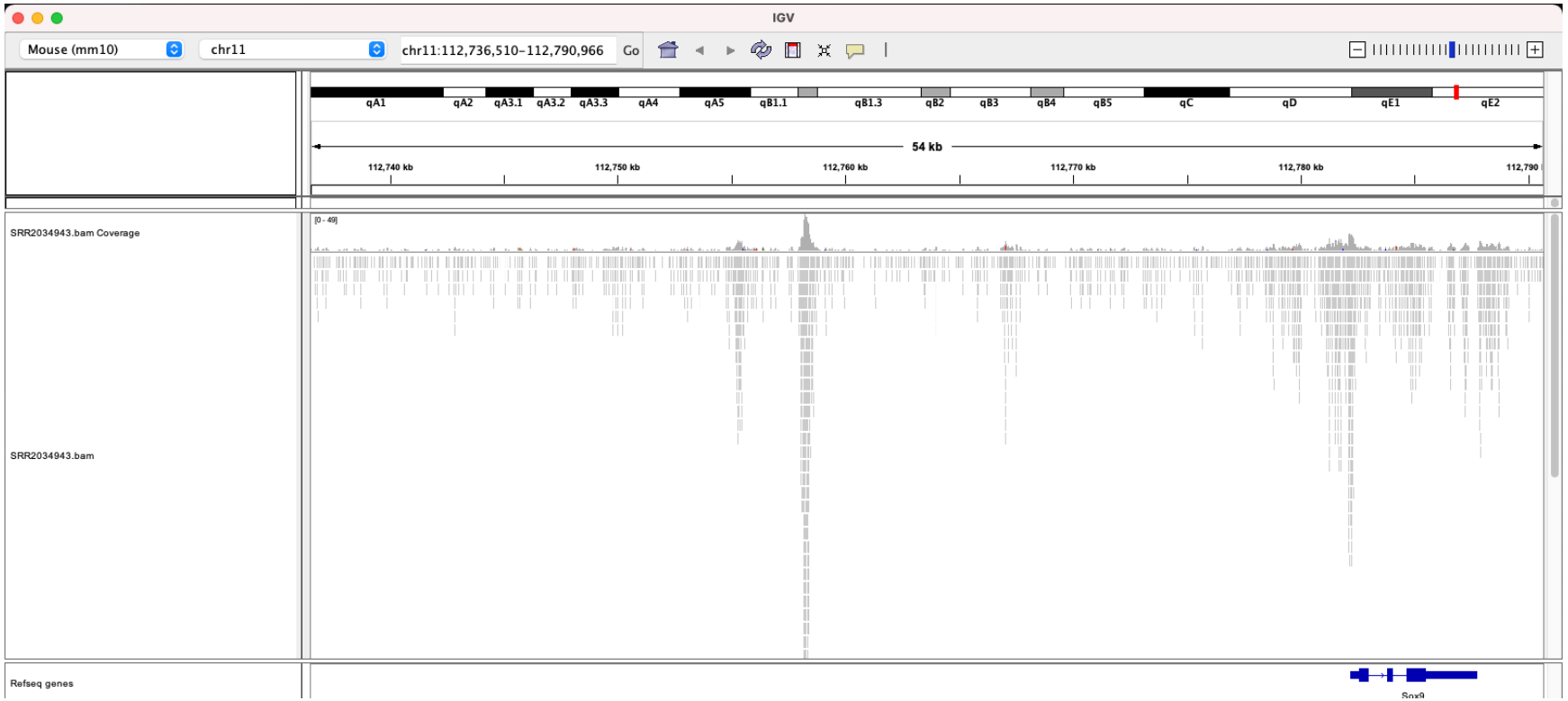
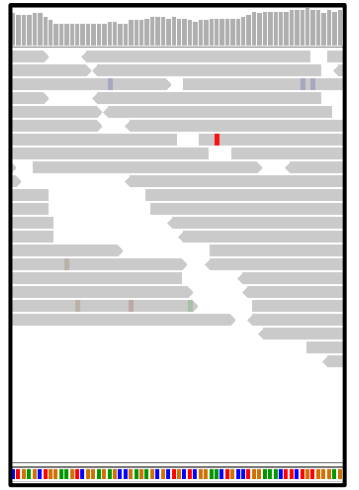
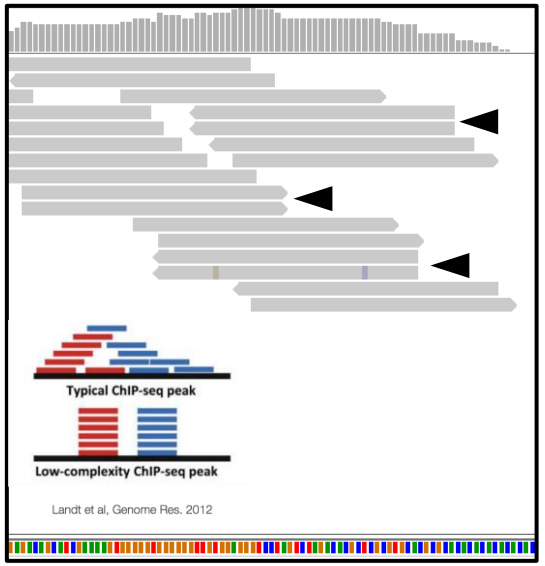
Change to Mm10





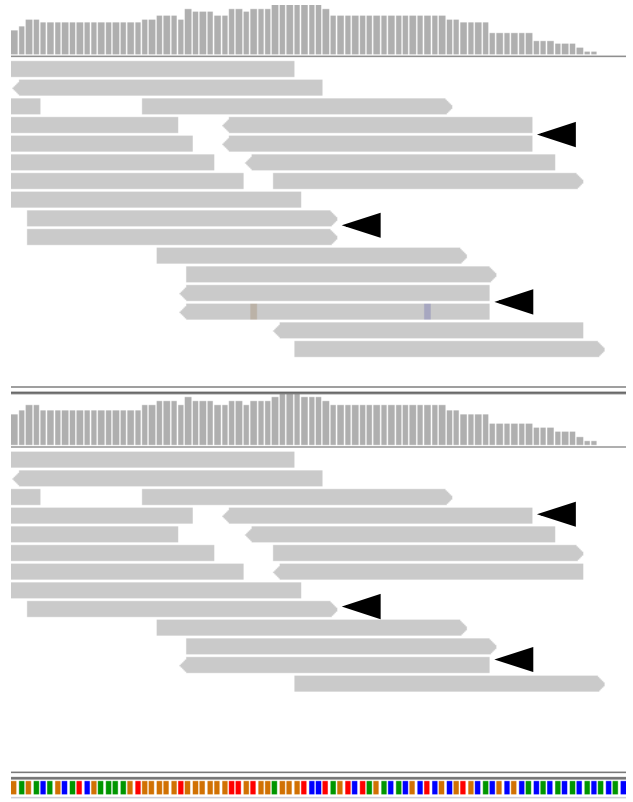


PCR duplicates originate from the library amplification. Performing large numbers of cycles to get enough material to sequence massively increases the number of those duplicates. The expected number of duplicates depends on the method (for instance, a typical run of ATAC-seq has 25-30% of duplicates)

Optional: removing duplicates using Picard

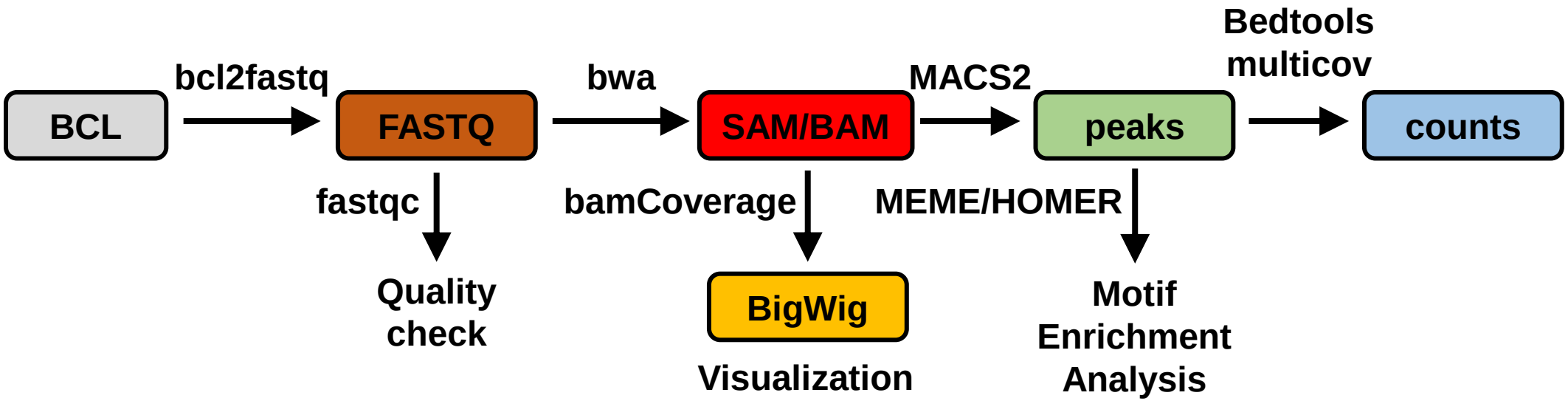
```
cd Bams
java -jar  picard.jar MarkDuplicates \
 I=bam_file.bam \
 O=bam_file_rmdup.bam \
 M=bam_file_marked_dup_metrics.txt \
 REMOVE_DUPLICATES=True
```

LIBRARY	UNPAIRED_READS_EXAMINED	UNMAPPED_READS	UNPAIRED_READ_DUPLICATES	PERCENT_DUPLICATION
Library	33973845	6553578	6809555	0.200435



Removing PCR duplicates allows a more accurate quantification of the features you are interested in (gene expression, DNA binding, chromatin accessibility, …).

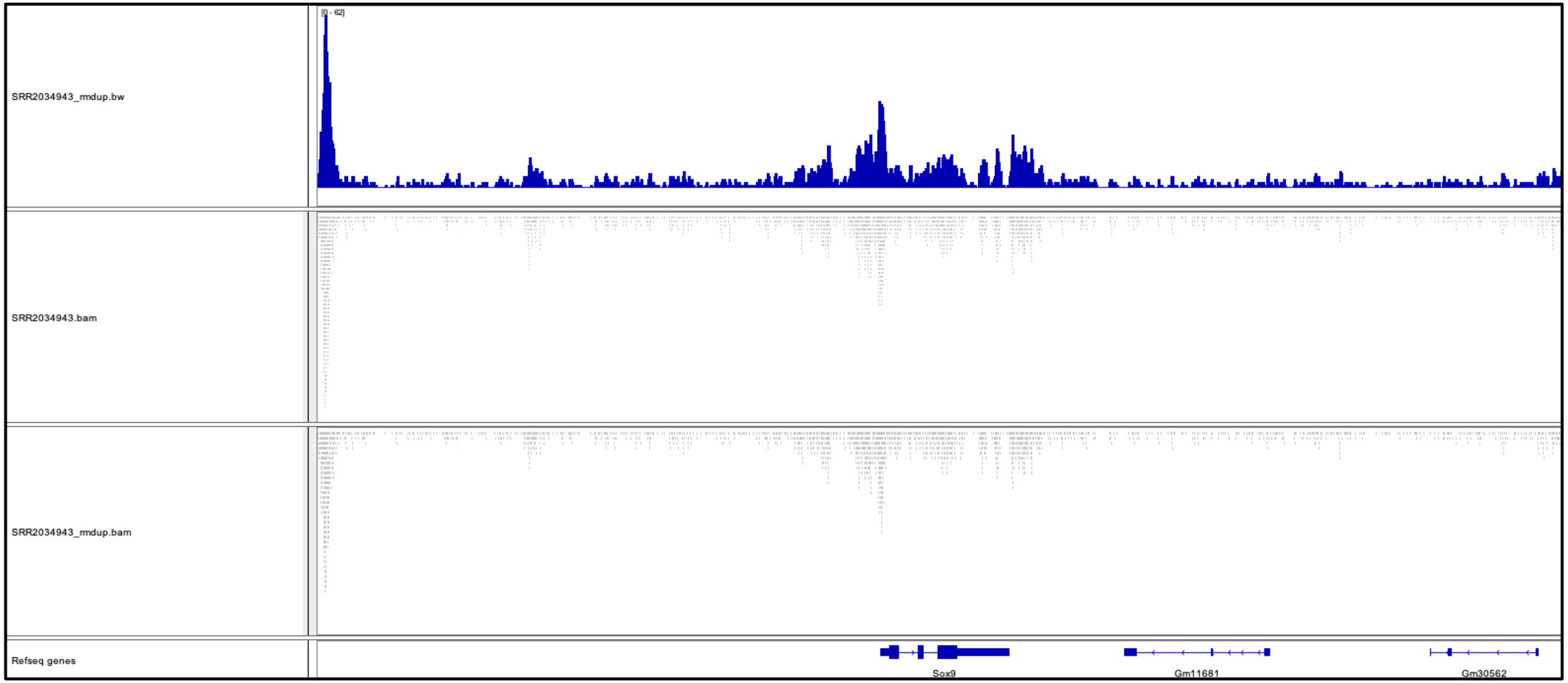
Full pipeline for ATAC-seq/ChIP-seq



Visualization using BigWig files

```
sbastide@nobel:/data/personal_folders/sbastide/sequencing_data_tutorial/Bams$ bamCoverage
usage: An example usage is:$ bamCoverage -b reads.bam -o coverage.bw
```

```
bamCoverage -b ./Bams/SRR2034943_rmdup.bam -o BigWigs/SRR2034943_rmdup.bw
```



Full pipeline for ATAC-seq/ChIP-seq