Next Generation Sequencing

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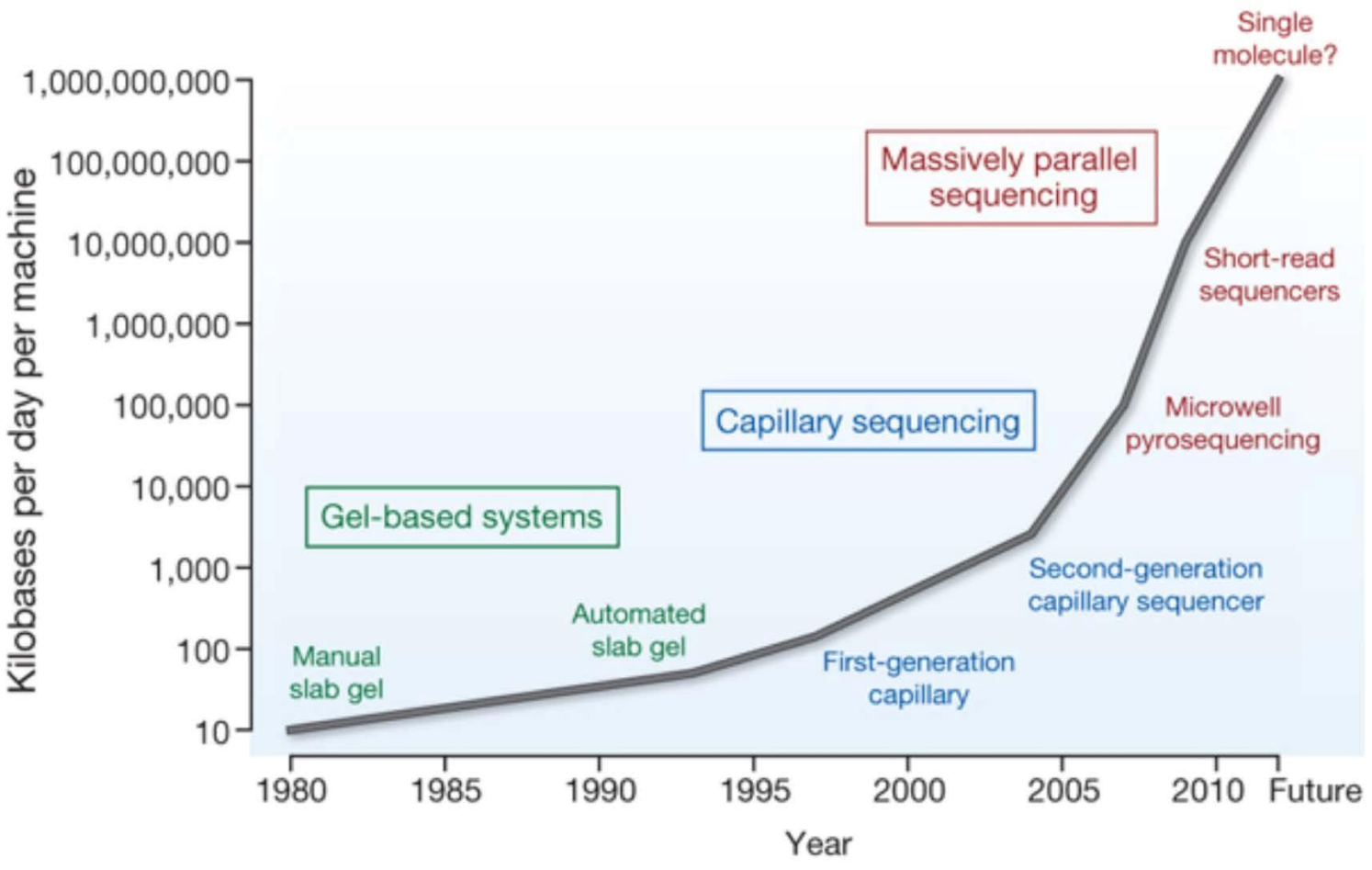
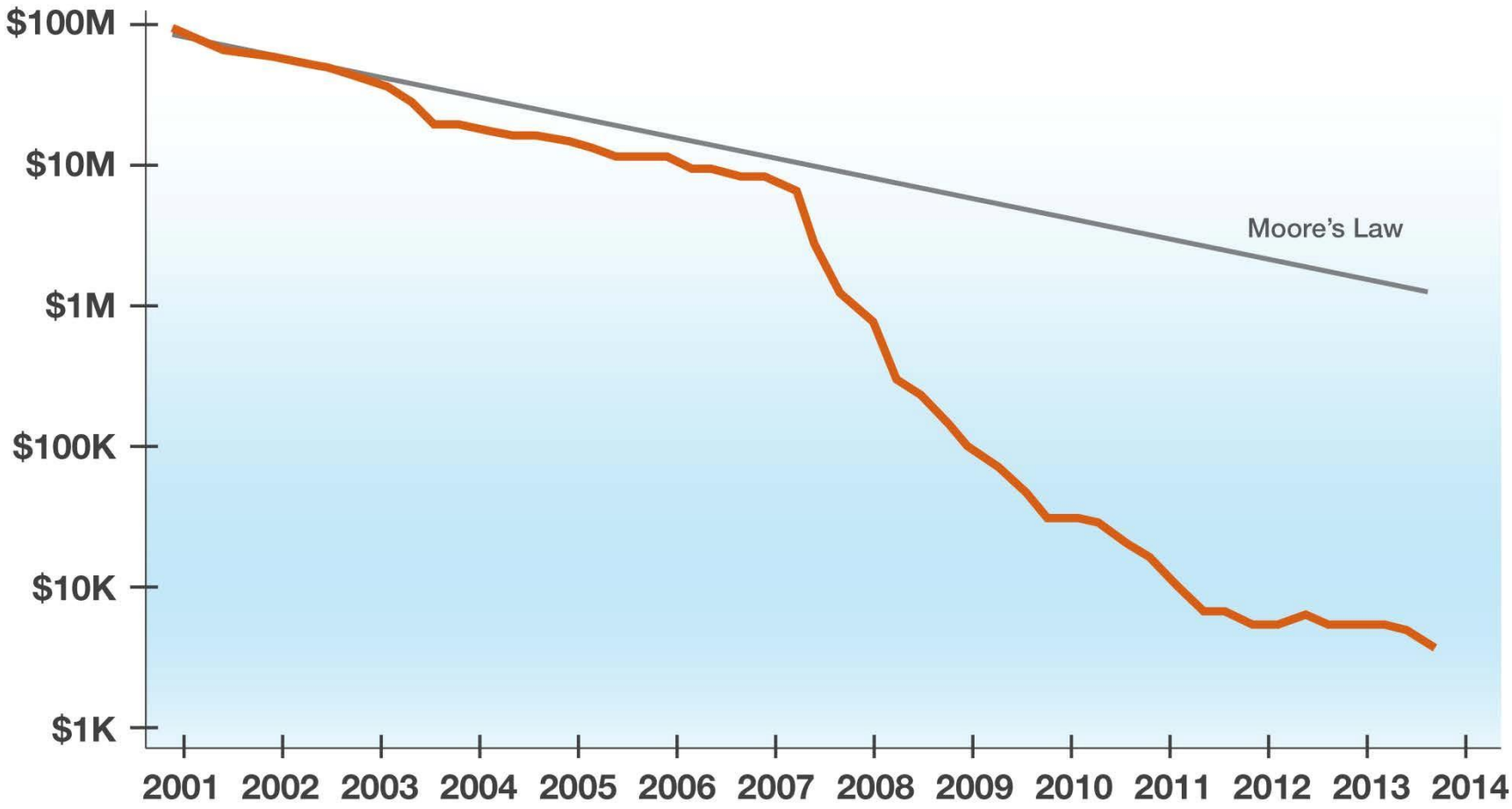
NGS part of the course

Week 4	Friday 10/2	15.15-17.00	NGS lecture 1: Introduction to NGS, alignment, assembly
Week 6	Thursday 23/2	08.00-09.45	NGS lecture 2: RNA-seq, metagenomics
Week 6	Thursday 23/2	10.00-11.45	NGS computer lab: Resequencing analysis
Week 7	Thursday 2/3	10.00-11.45	Marcela Davila: Exome sequencing
Week 8	Thursday 9/3	08.00-09.45	Alexander Schliep: HMM applications

History of sequencing



Cost Per Genome



A paradigm shift

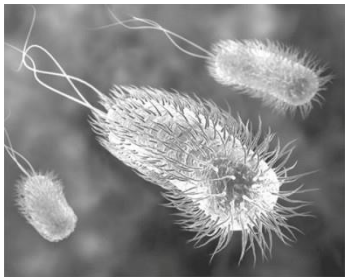
Thanks to NGS:

- From single genes to complete genomes
- From single transcripts to whole transcriptomes
- From microarrays to RNA-seq
- From single organisms to complex pools (e.g. metagenomes)
- From model organisms to the species you are actually studying

Generates huge amounts of data

- A bioinformatics challenge

NGS Applications



- Whole genome sequencing
  - De novo sequencing
    - No reference genome available
    - *De novo* assembly
  - Resequencing
    - High quality reference sequence available
    - SNP/Indel detection (whole genome)
    - Genomic rearrangements
  - Application examples:
    - Biodiversity, epidemiology, pathogen detection, evolution

NGS Applications

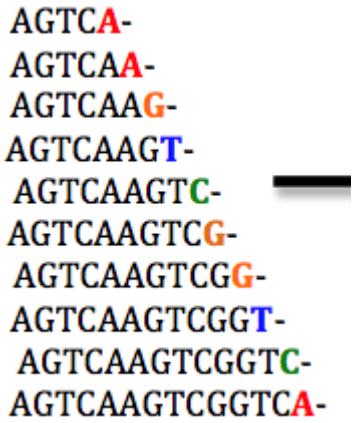
- Transcriptome sequencing (RNA-seq)
  - Gene expression (differential gene expression)
  - Novel splice sites/splice variants
  - *De novo* transcriptome assembly (find new genes)
  - Non-coding RNA
- Exome sequencing (amplicon sequencing)
  - SNP/Indel detection (only exons)
  - Genomic rearrangements
  - Examples: Medicine, human genetics, cancer research
- Metagenomics
  - Microbial communities (human gut, environment etc.)
  - Species composition
  - Genes/functions

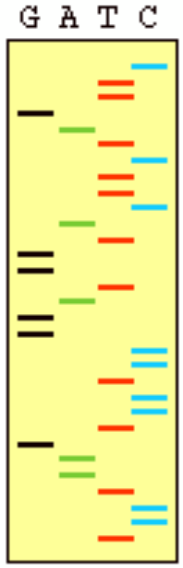
Sequencing techniques

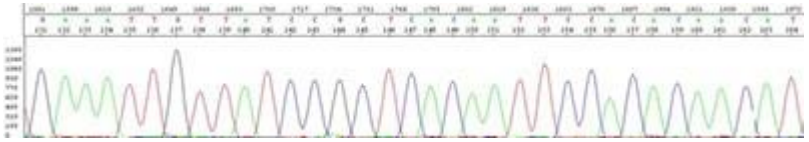
- First generation sequencing
  - Sanger sequencing
- Next generation sequencing (massively parallel sequencing)
  - Illumina sequencing
  - 454 pyrosequencing
  - SOLiD sequencing
- Single molecule sequencing (3rd generation sequencing)
  - PacBio
  - Oxford Nanopore

Sanger sequencing (1st generation sequencing)

- Frederick Sanger 1977
- First sequence: Bacteriophage Phi X 174
- Shearing of DNA
- Cloning in bacteria
- To each sequence reaction dNTP's (dATP,dGTP,dTTP, dCTP) and one of the four ddNTP's are added
- The ddNTP's are incorporated randomly by the DNA polymerase.
- Determine the sequence by gel electrophoresis or fluorescence.







Sanger sequencing (1st generation sequencing)

The ”Gold standard” sequencing for single genes

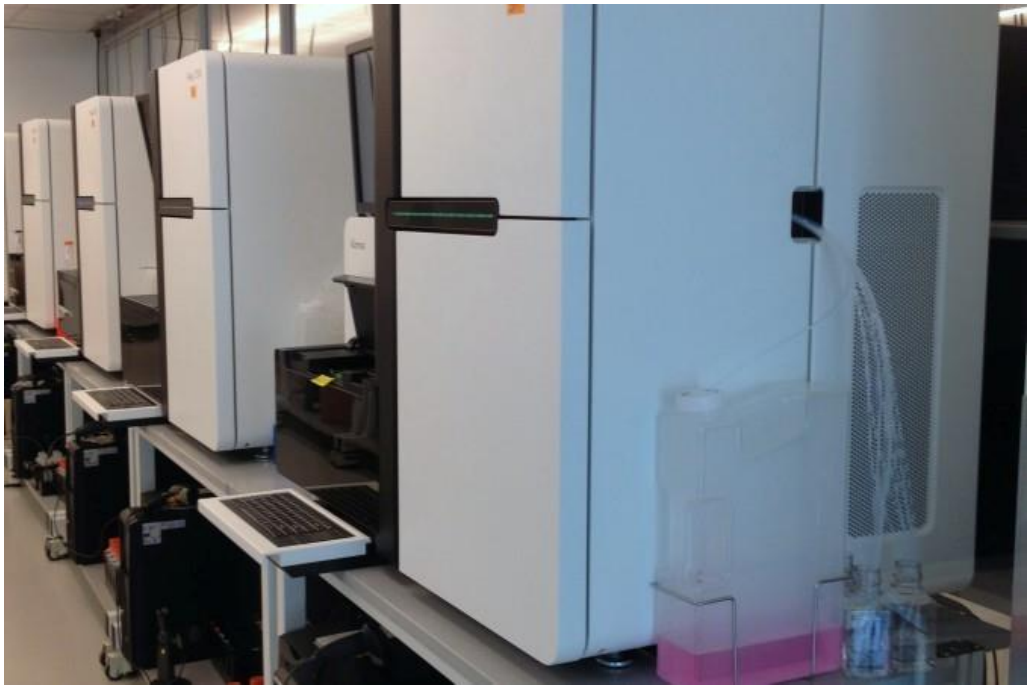
+ High accuracy

+ Long fragments (800-1000 bp)

- Low throughput

Illumina sequencing

- High throughput sequencing
- Sequencing by synthesis (SBS)
- HiSeq 2500 (high output mode)
  - 2x100 bp reads
  - 2x125 bp reads
  - Paired end sequencing
  - ~50 Gbp per lane
  - 8 lanes per flowcell
  - Run time 5-7 days



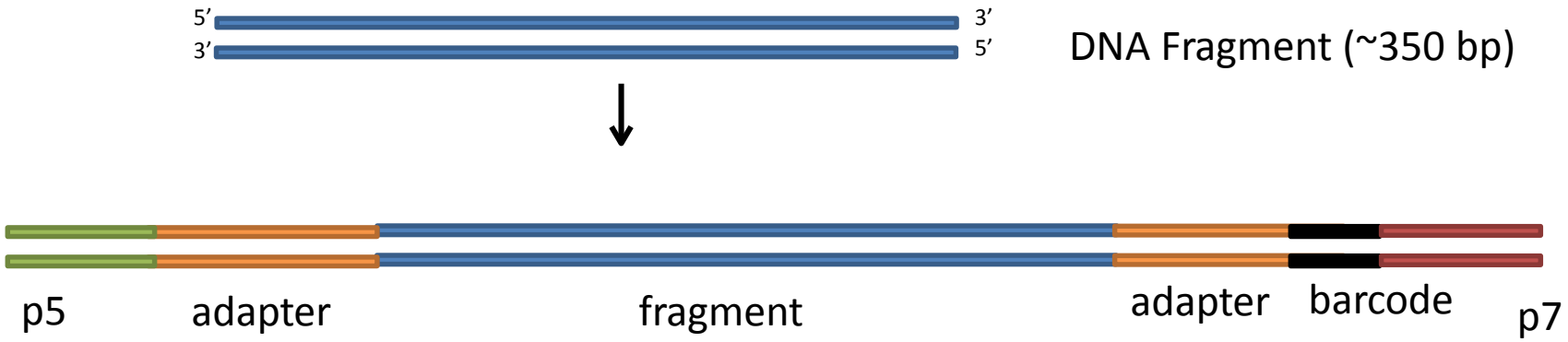
Sample preparation (Illumina)

- DNA extraction (or RNA extraction)
- 1-2 µg of total DNA per sample as starting material

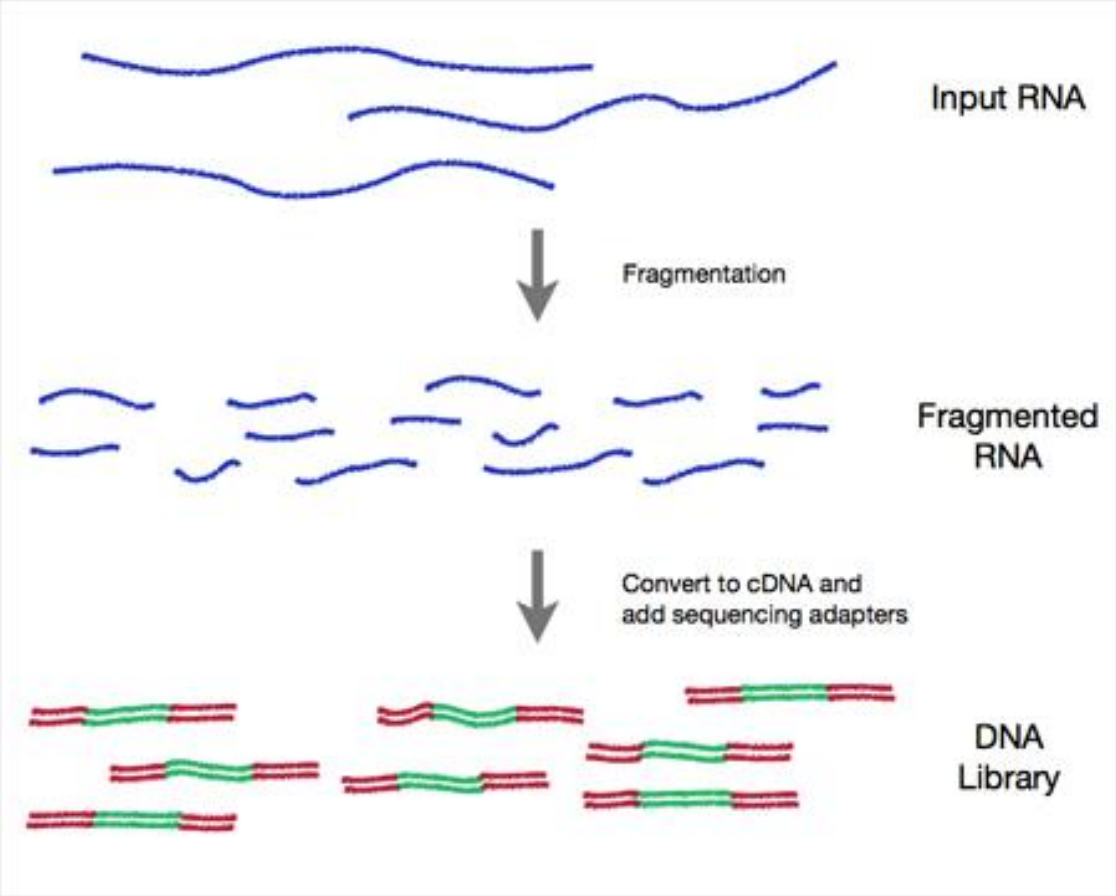
- Shear the DNA to 300-600 bp long fragments (typically 350 bp)
  - Sonication (ultrasound)
  - Random enzymatic digestion
  - Nebulization

Library preparation

- Ligation of adapter sequences to the fragments.
- Barcoding possible (makes it possible to run several samples in the same lane)

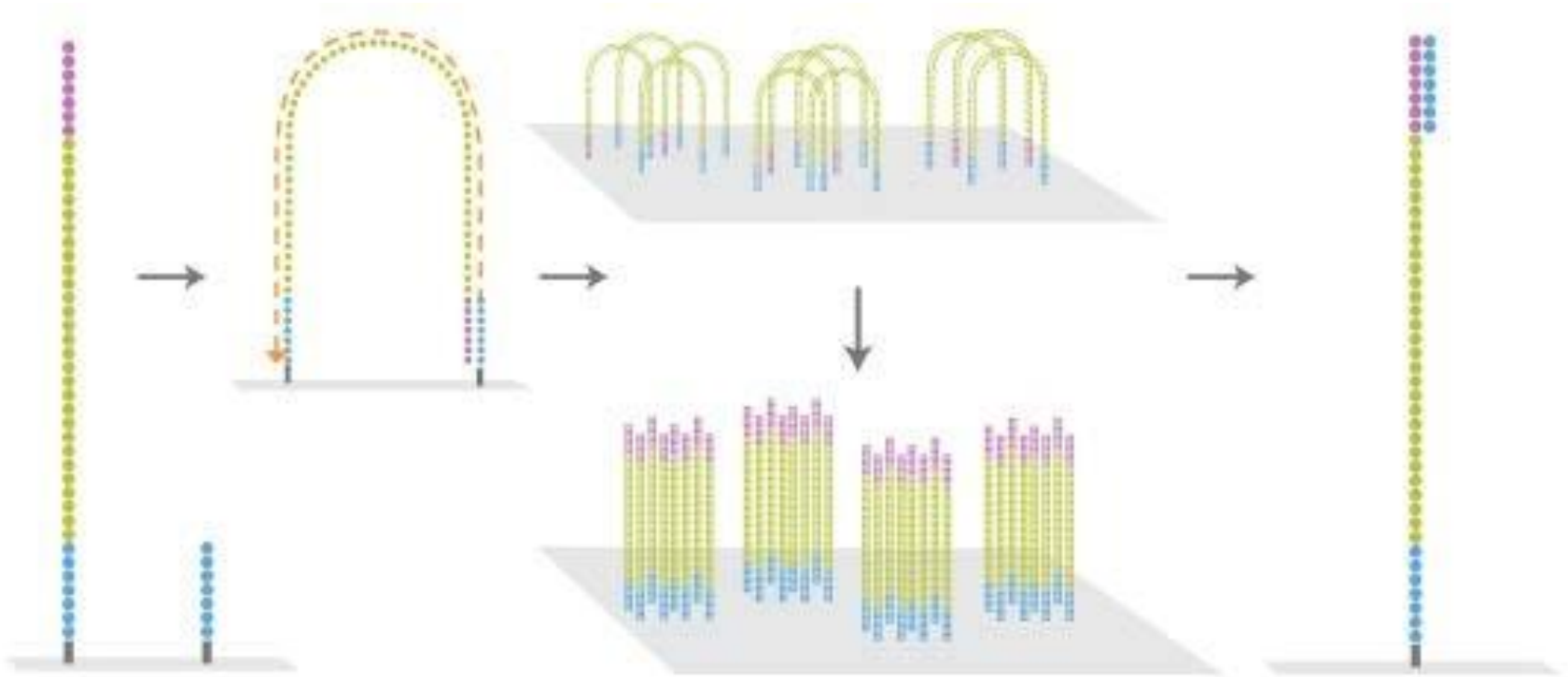


Library preparation for RNA-seq

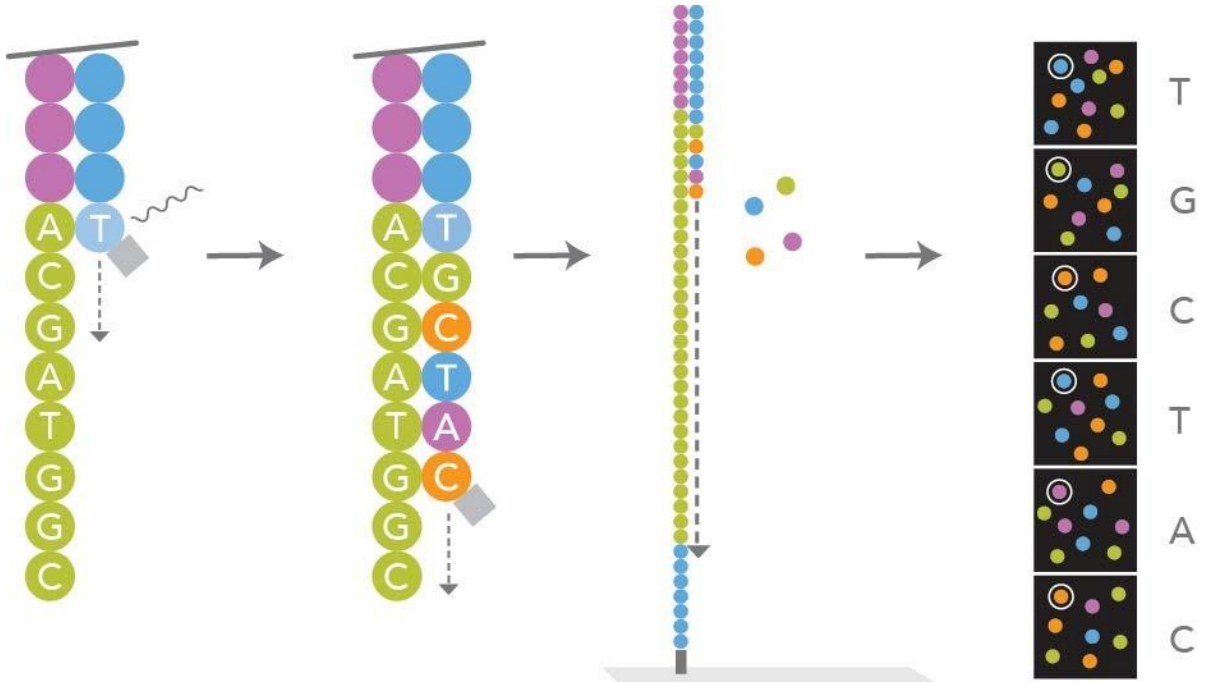


Cluster generation

- PCR amplification step (bridge amplification)

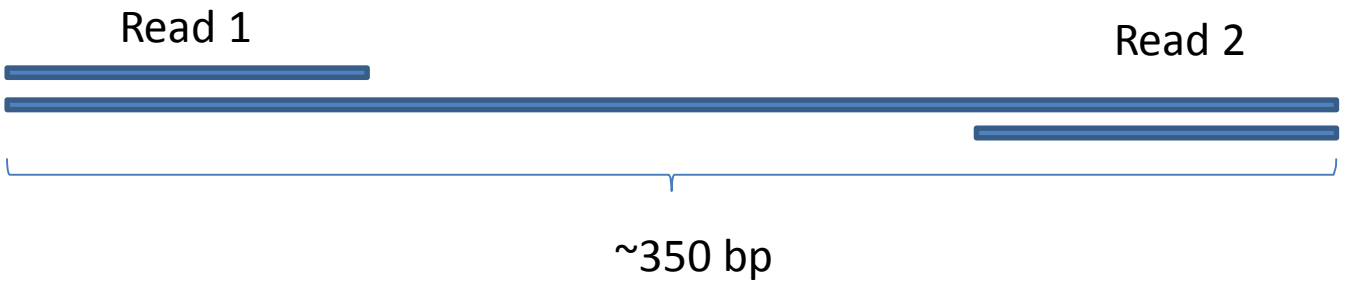


Sequencing by synthesis

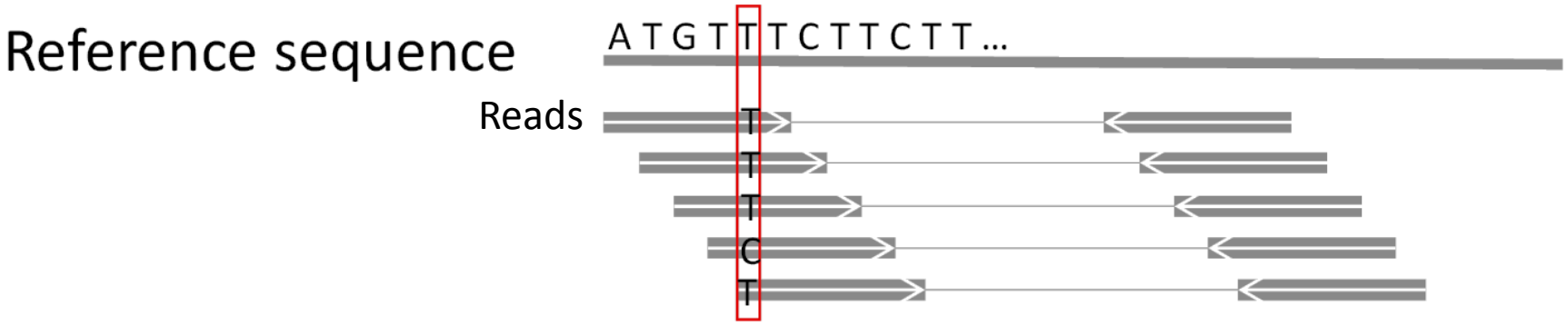


Paired end sequencing

- Information about pairs can help both in alignment and assembly



General idea

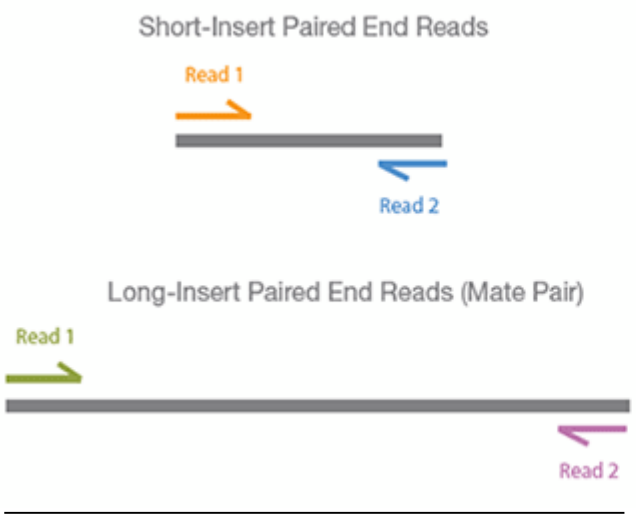


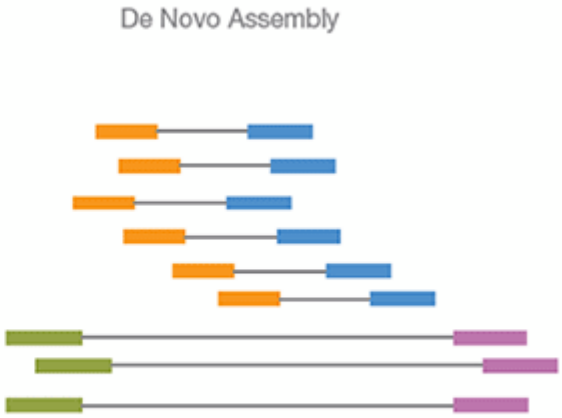
- Each nucleotide is sequenced multiple times
- Short but many reads
- Error rate up to 1 % - compensated by high coverage

- *Coverage* – The average number of times a nucleotide is sequenced

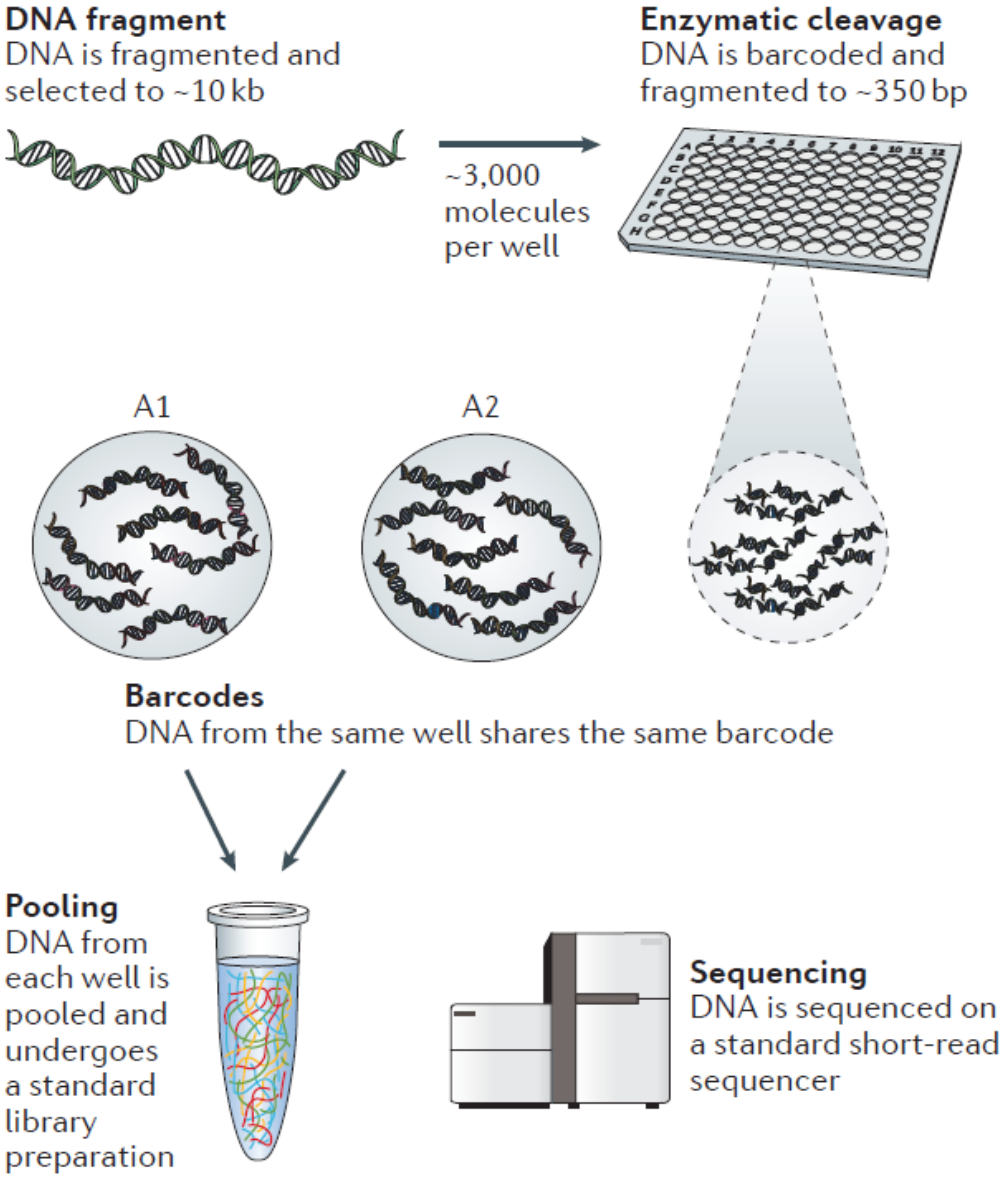
Mate pair reads

- Paired end reads with long (1000-10000 bp) insert size
- Can help in *de novo* assembly





Synthetic long reads



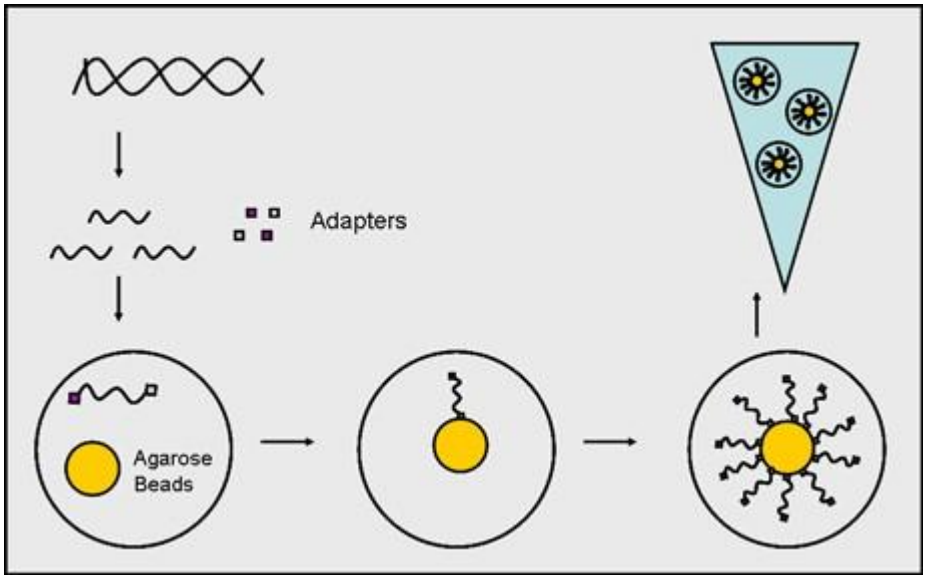
- Moleculo illumina sequencing (random long ~10bp fragments)
- combine with normal illumina (short reads)
- Can help in *de novo* assembly

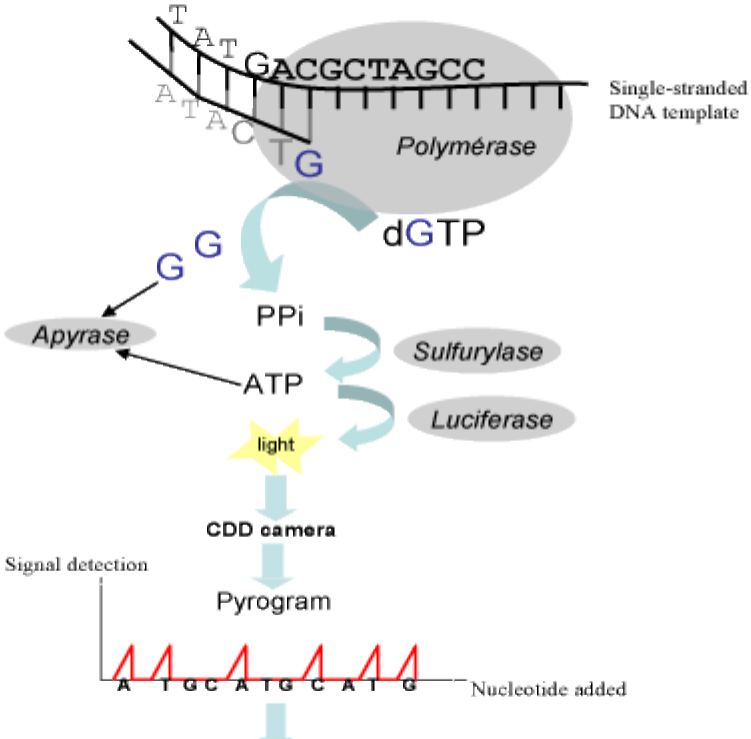
Illumina sequencing summary

- Read length ~ 2 x 100 bp
- Advantages
  - High Throughput (~400 gigabases per flowcell)
  - Low cost per base
- Disadvantages
  - Error rate up to 1% (Phred score 20) (only substitutions)
  - Problems with AT- and GC-rich regions
  - Long sequencing times (depending on the read length)

454 sequencing

- Massively parallel pyrosequencing
- Introduced by Roche in 2005
- Read length at least 400 bp
- 1 sequencing round ~500 million bases, takes 4 hours



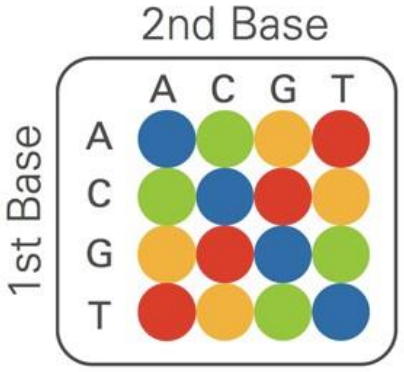


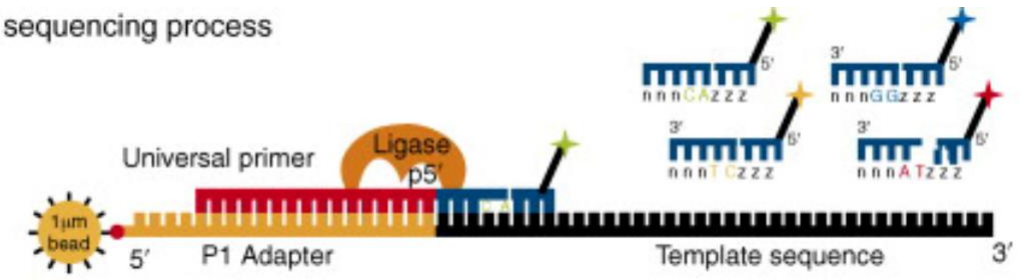
454 sequencing

- Advantages
  - Fast run time
  - Long reads 400-750 bp
- Disadvantages
  - Low throughput (compared to Illumina)
  - Error rate at 1%. Type of errors: Indels
  - Problematic at homopolymeric regions, e.g. TAAAAAAA
  - Relatively high running costs

SOLiD sequencing

- Life technologies/ABI
- Sequencing by ligation (SBL)
- ”Color space” dinucleotides
- Paired end, read length 75 + 35 bp



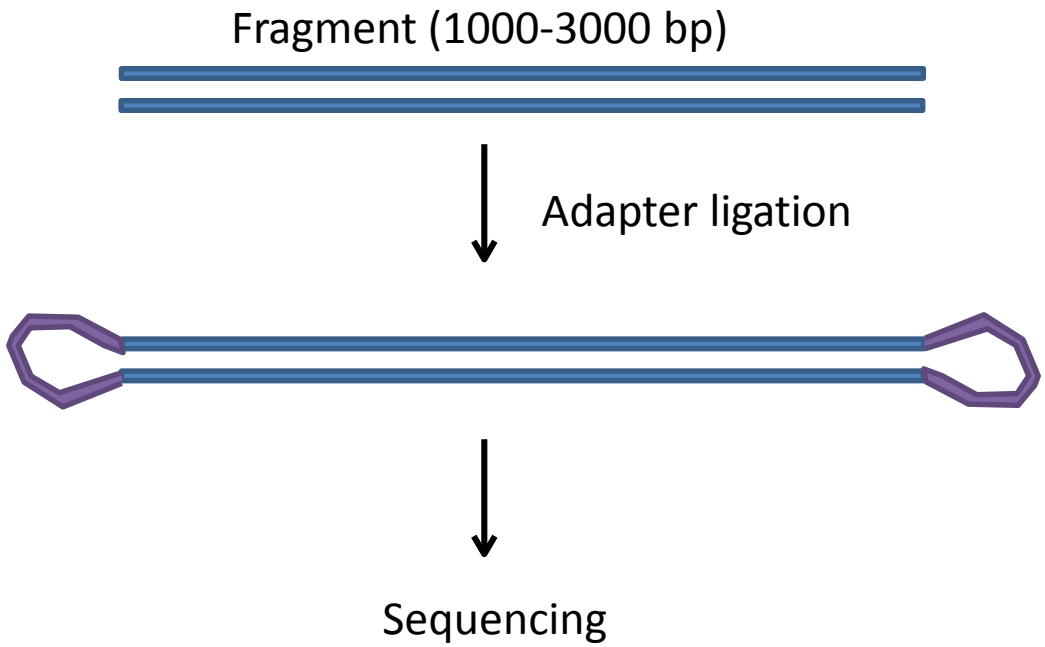


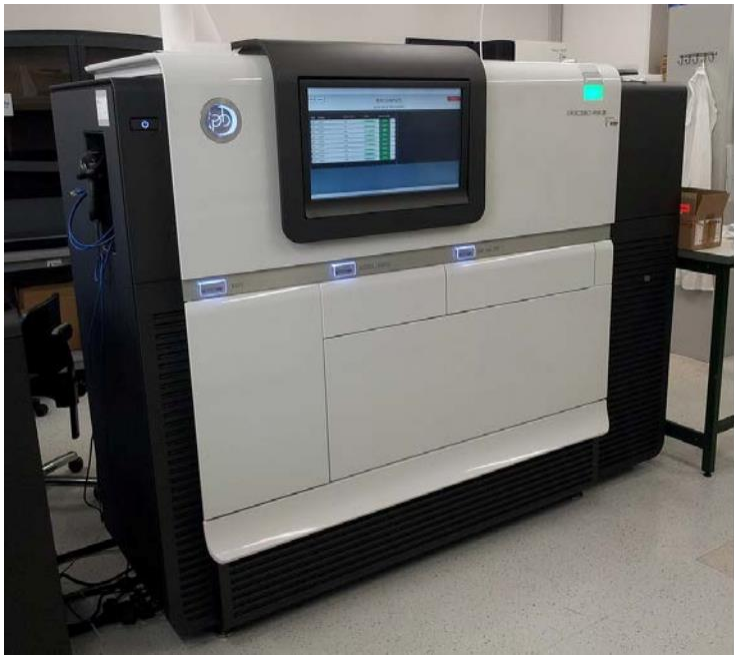
SOLiD sequencing

- Advantages
  - High throughput
  - Low cost per base
  - High accuracy when reference genome is available (resequencing)
- Disadvantages
  - Few software working with color space
  - Problems with AT- and GC-rich regions
  - Long sequencing times
  - Short read lengths

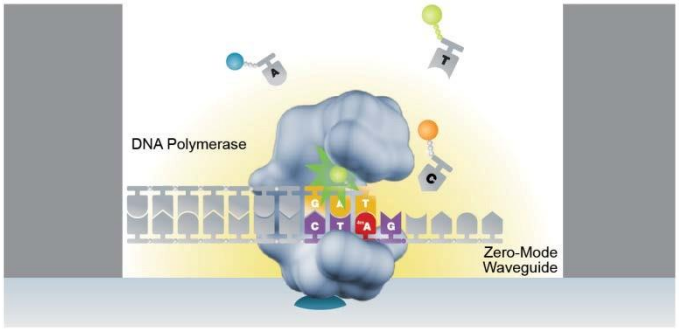
Pacific Biosciences (PacBio)

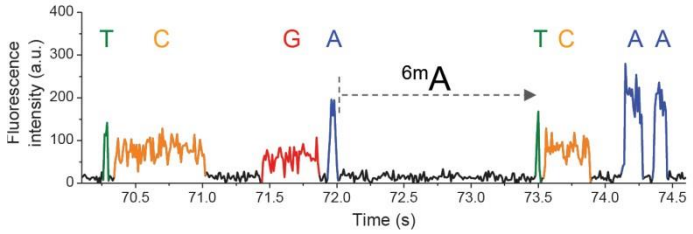
- Single molecule sequencing (no PCR amplification step)





PacBio sequencing





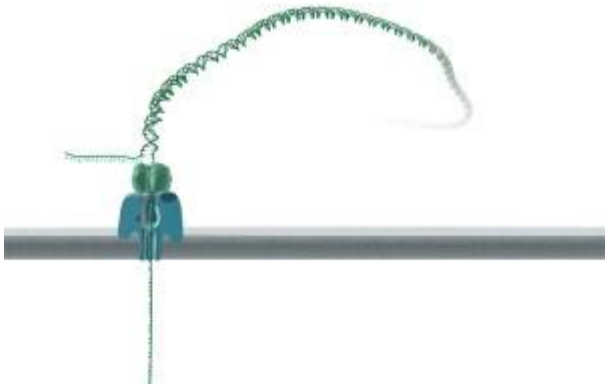
- SMRT – Single Molecule Real Time
- High error rate (up to 10 %)
- Random errors
- Sequence ~10,000 bases – Same fragment sequenced multiple times

PacBio sequencing

- Advantages:
  - Long fragments – average length 1000 bases
  - No PCR amplification step (no PCR bias)
- Disadvantages:
  - High error rate (but random errors)
  - Expensive
  - Low throughput

Can be used together with Illumina for hybrid approaches

Oxford Nanopore



“Technique promises it will produce a human genome in 15 minutes”

NGS data analysis

- Pre-processing of raw sequencing reads
  - Remove bad quality data
- For resequencing:
  - Alignment to reference genome
  - Variant detection
- For *de novo* DNA sequencing
  - Assembly of the reads
  - Genome annotation
- RNA-seq

NGS data processing

- reference genome

Genome resequencing	Read filtering and trimming	Read mapping against reference genome followed by realignment		Calling of polymorphisms and structural variants
RNA-seq	Read filtering and trimming	Splice-aware mapping against reference followed by read counting	Within and between sample normalization	Identification of differentially abundant transcripts
Metagenomic sequencing	Read filtering and trimming	Sensitive mapping against a reference followed by read counting	Within and between sample normalization	Identification of differentially abundant genes/species

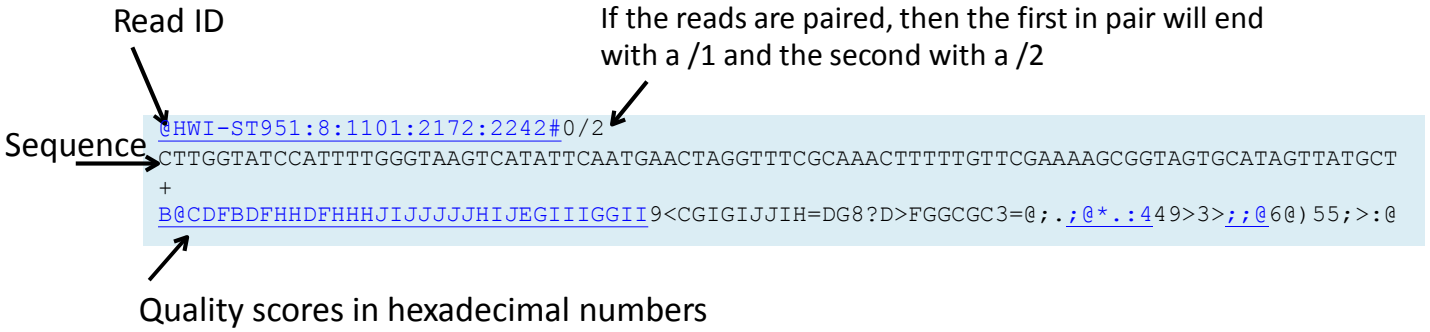
Data analysis

- Huge amount of data to handle
- Many tools are command-based tools
- Working in a Linux environment
- Make use of a bioinformatics server or a computer cluster
- Run several CPUs in parallel

Pre-processing

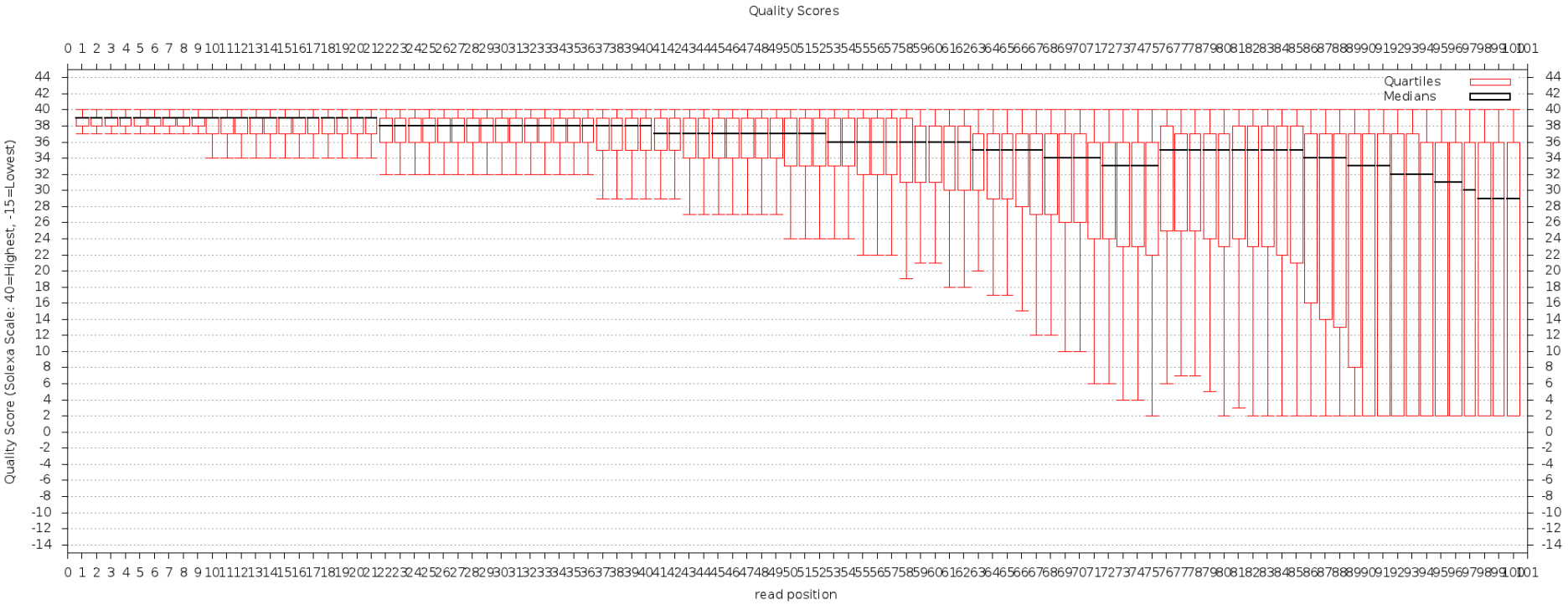
What do the sequence files look like?

- Sample1_2.fastq
- Each sequence file can contain ~20 million reads
- For paired end sequencing: 2 files per sample
- Many samples



Read quality per base

Example: Using fastx toolkit



Command line:

```
~/fastq_quality_boxplot_graph.sh –i R1_output.txt –o R1_box_plot.png
```

Alignment and quantification

Example of alignment

Read:

TCAACTCTGCCAACACCTTCCTCCTCCAGGAAGCACTCCTGGATTTCCCTCTTGCCAACAAGATTCTGGGAGGGCA

Genome:

ATAAAATGGCCAAAATTAACTAGAAGGTGAGTAGAAACTTAAATAAACTAATTACCATTGATGAGAAAAAAAATC
TGCCACTGAAAAAGGCACCCGGTCCAGAGGGTTTCATGAGCGGGAACTGTAGAAACCTTTCGAATTCAACTCTGC
CAACACCTTCCTCCTCCAGGAAGCACTCCTGGATTTCCCTCTTGCCAACAAGATTCTGGGAGGGCAGCTCCTCCA
ACATGCCCCCAACAGCTCTCTGCAGACATATCATATCATATCATATCTTCCATACCATAACTGCCATGCCATACA

How would you find that?

- Brute force

  TCGATCC

  GACCTCATCGATCCCACTG

- Smith-Waterman alignment
- Blast (local alignment)
- Suffix tree
- Burrows-Wheeler transform

Burrows wheeler transform

Read: GATC Reference: TCGATCC

- Let $ represent the end of the reference string
  - TCGATCC$
- Perform all possible rotations of the string
- Sort the rotations in alphabetical order
- Save only the last column and back-transform when needed

TCGATCC$
$TCGATCC
C$TCGATC
CC$TCGAT
TCC$TCGA
ATCC$TCG
GATCC$TC
CGATCC$T

$TCGATCC
ATCC$TCG
C$TCGATC
CC$TCGAT
CGATCC$T
GATCC$TC
TCC$TCGA
TCGATCC$

Alignment of reads

- Smith-Waterman
- BLAST (100 times faster)
- Vmatch (suffix trees) 100 times faster
- Bwa, bowtie etc. (based on Burrows-Wheeler transform) fast alignment

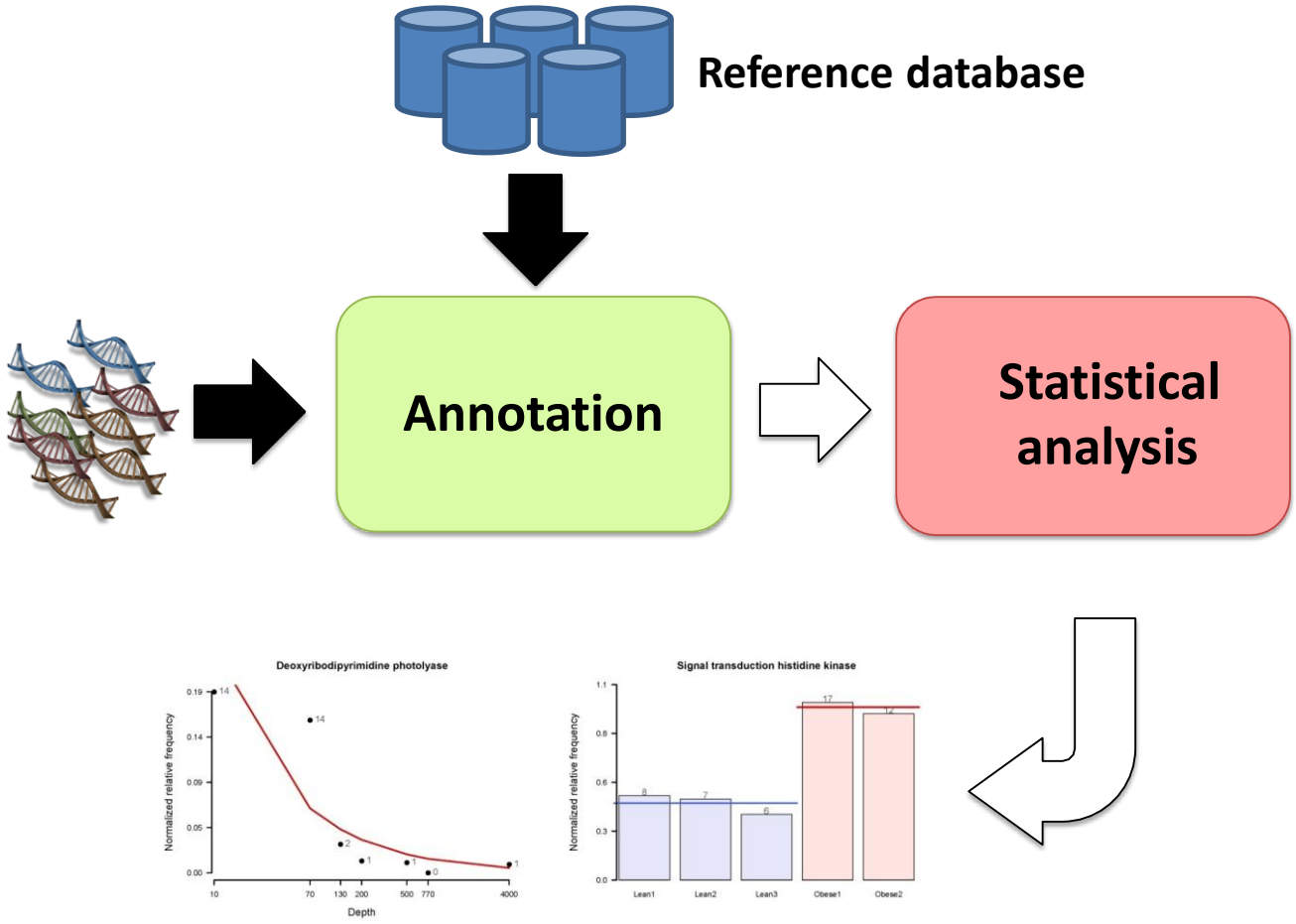
Alignment of reads to protein reference

Fast and sensitive protein alignment using DIAMOND

Benjamin Buchfink[1], Chao Xie[2,3] &
Daniel H Huson[1,2]

Nature Methods, January 2015

Claimed by the authors to be 20,000 times faster than blastx in mapping short reads to a protein database.



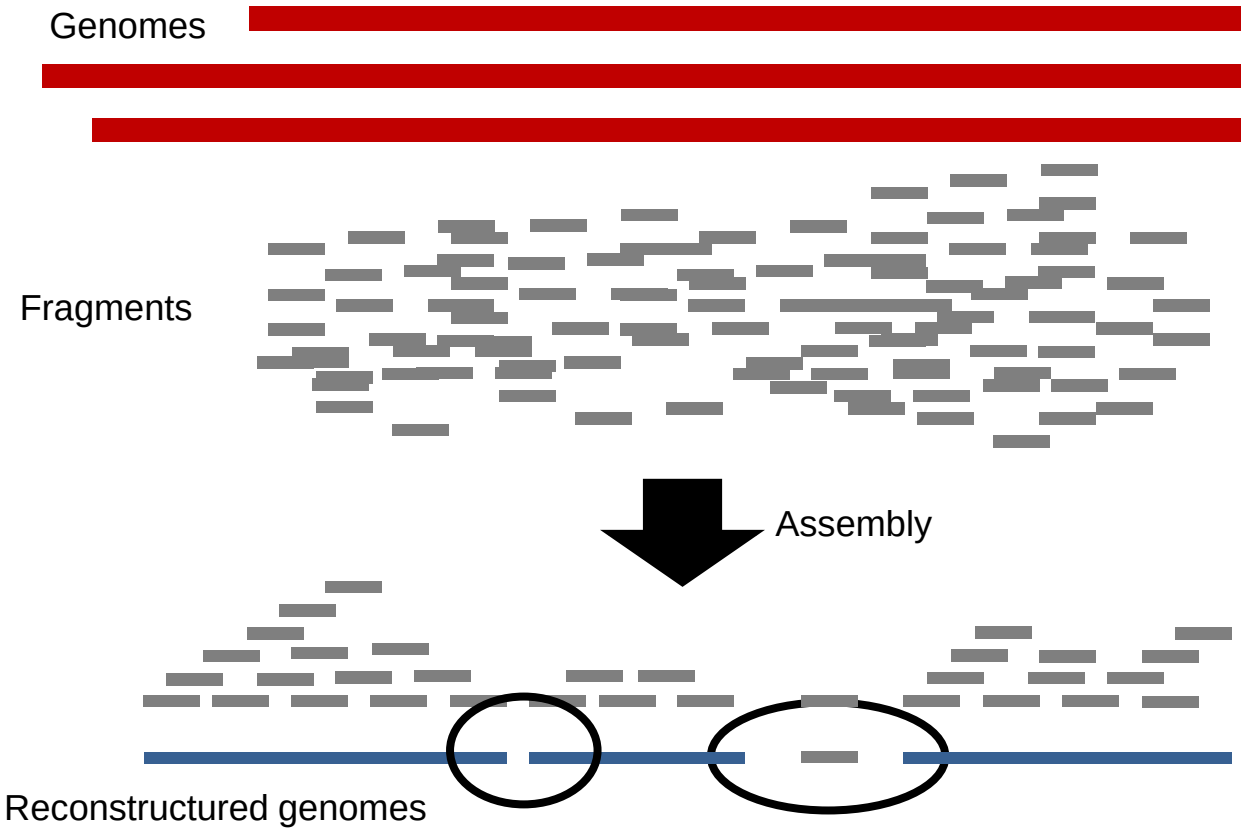
Kristiansson, E., Hugenholtz, P., Dalevi, D. (2009). ShotgunFunctionalizeR – an R-package for functional analysis of metagenomes. Bioinformatics 25(20). http://shotgun.zool.gu.se

No reference genome

De novo assembly

- Puzzle reads together to longer fragments (contigs)

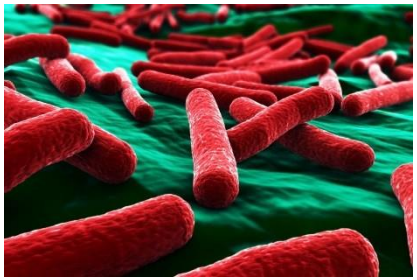
Genome assembly



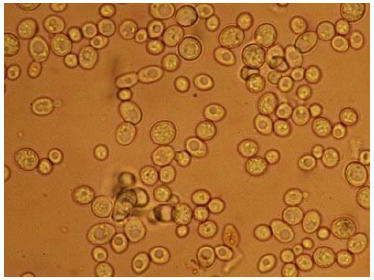
Genome assembly – challenges

- Computationally heavy
  - Computational complexity: $o(n^2)$
  - Memory complexity: $o(n^2)$
- Sequencing errors
- Repetitive regions

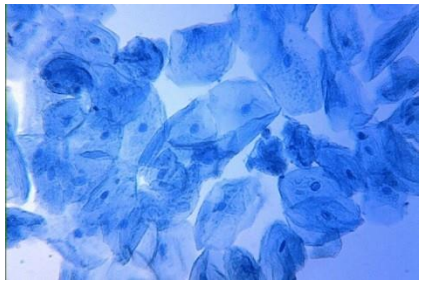
Genome complexity



E. coli
~4.5 Mbases
~4300 genes



S.cerevisiae
~12 Mbases
~6000 genes



Human
~3 Gbases
~20000 genes

Spruce
~20 Gbases
~30000 genes

Increased genome complexity

Assembly of the spruce genome

- Large and complex genome
  - 20 gigabases (6 times as big as the human genome)
  - Many repetitive regions
- Assembly statistics
  - 1 terabases sequenced (mainly Illumina)
  - 3 million contigs longer than 1000 bases
  - 30 % of the genome
  - Assembly had to be done on a supercomputer with 1 TB RAM.

Summary – Next generation sequencing

- Next generation sequencing enables sequencing of billions of DNA fragments simultaneously
- Huge amount of sequence data in a short time
- Highly applicability in many areas of biology and medicine
- Needs bioinformatics to handle and analyze the produced data