History of sequencing, overview & comparison of NGS technologies

Introduction to Linux for Bioinformatics Workshop

Moi University Bioinformatics Hub

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24th March 2023

Overview of Sequencing

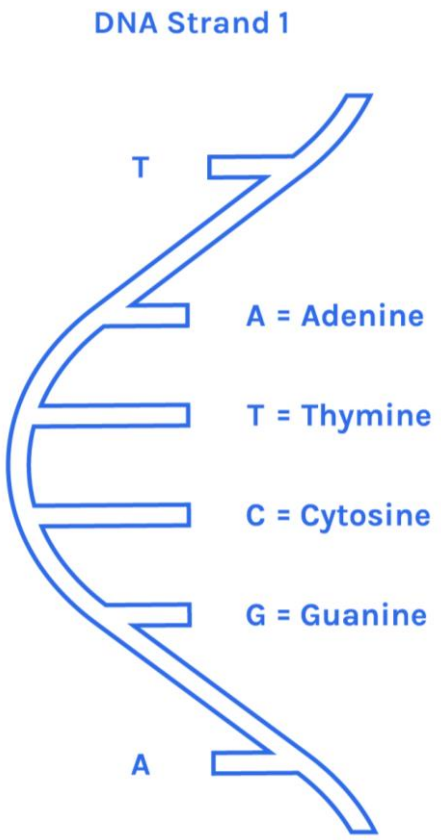
Definition of sequencing

What is DNA sequencing?

- Determining the order of the 4 nucleotides that make up a DNA strand or oligonucleotide

What is Next Generation Sequencing - NGS?

- Technology used for sequencing many DNA strands **at the same** time instead of **one at a time** as with traditional Sanger sequencing by capillary electrophoresis.
- Also called "**massively-parallel sequencing**".
- Enabled by use of unique **barcodes** or **indexes** to label DNA libraries of individual samples.



https://medium.com/prime-movers-lab/how-dna-sequencing-started-the-microbiome-revolution-and-what-it-means-for-the-future-e660006e0d98

General steps of sequencing

Qubit

NanoDrop

DNA quantification

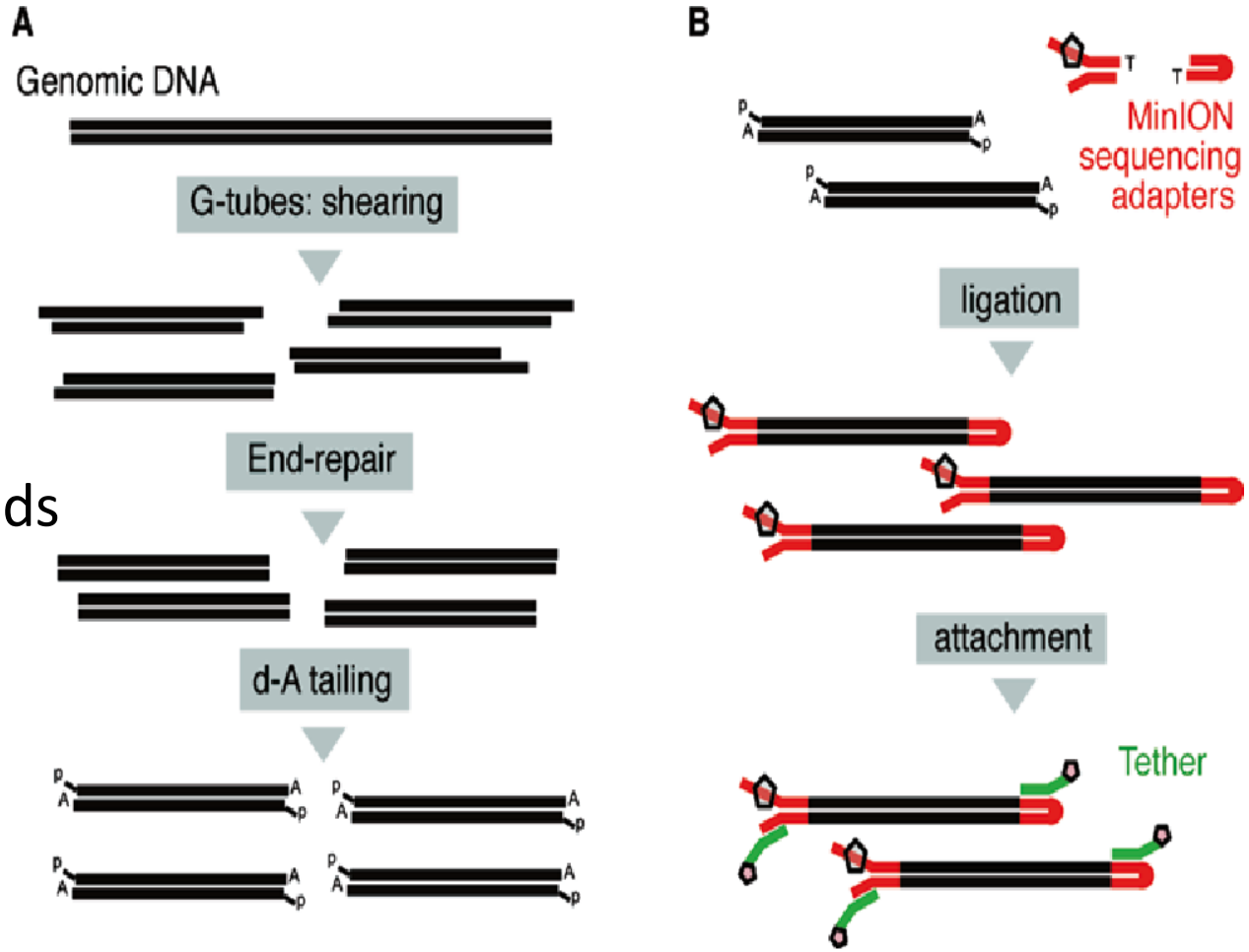
Data acquisition & analysis

https://medium.com/prime-movers-lab/how-dna-sequencing-started-the-microbiome-revolution-and-what-it-means-for-the-future-e660006e0d98

General steps of sequencing

Library preparation

1. DNA fragmentation (A)
   a. Covaris tubes
   b. Insulin Needles
2. End-repair (A)
   a. Converting stick ends to blunt ends
   b. Adding short oligo A tail
3. Barcoding (B)
4. Adapter ligation (B)



History of Sequencing

History of sequencing

1869 — First DNA isolation by Friedrich Mietscher

1953 — Discovery of DNA structure by Watson, Crick and Franklin

1965 — Robert Holley sequenced the first tRNA

1972 — Sequenced the 1st complete protein coding gene – coat protein of bacteriophage MS2

1977 — Fredrick Sanger discovered the first sequencing method – Chain Termination method

1977 — Sanger sequenced the first complete genome - bacteriophage PhiX174

History of sequencing

- First Generation sequencing methods

Year of Discovery		Technology	Maximum read length
1977	Fredrick Sanger	Chain Termination Method	~50bp
1977	Maxam & Gilbert	Chemical modification of DNA	~50bp
1984	Fritz Pohl	Direct blotting electrophoresis system GATC1500	N/A
1987	Leroy Hood & Michael Hunkapiller	ABI 370 – Applied Biosystems - First automated Sanger sequencer	20 – 30 bp

History of sequencing

- Second generation sequencing

Year of Discovery	Developers/ manufacturers	Technology	Maximum read length
1996	Mostafa Ronaghi, Mathias Uhlen & Pȧl Nyŕen	Pyrosequencing - sequencing by synthesis	1000bp
2005	Jonathan Rothberg & colleagues	Roche 454 Sequencing System - Automated pyrosequencing	1000bp
2006	Applied Biosystems	Life Technologies SOLiD system – sequencing by ligation	60bp
2007	Illumina Inc.	Illumina sequencing – sequencing by synthesis	150 – 500bp
2010	Ion Torrent Systems Inc	Ion Torrent - pH-mediated sequencer	100bp

History of sequencing

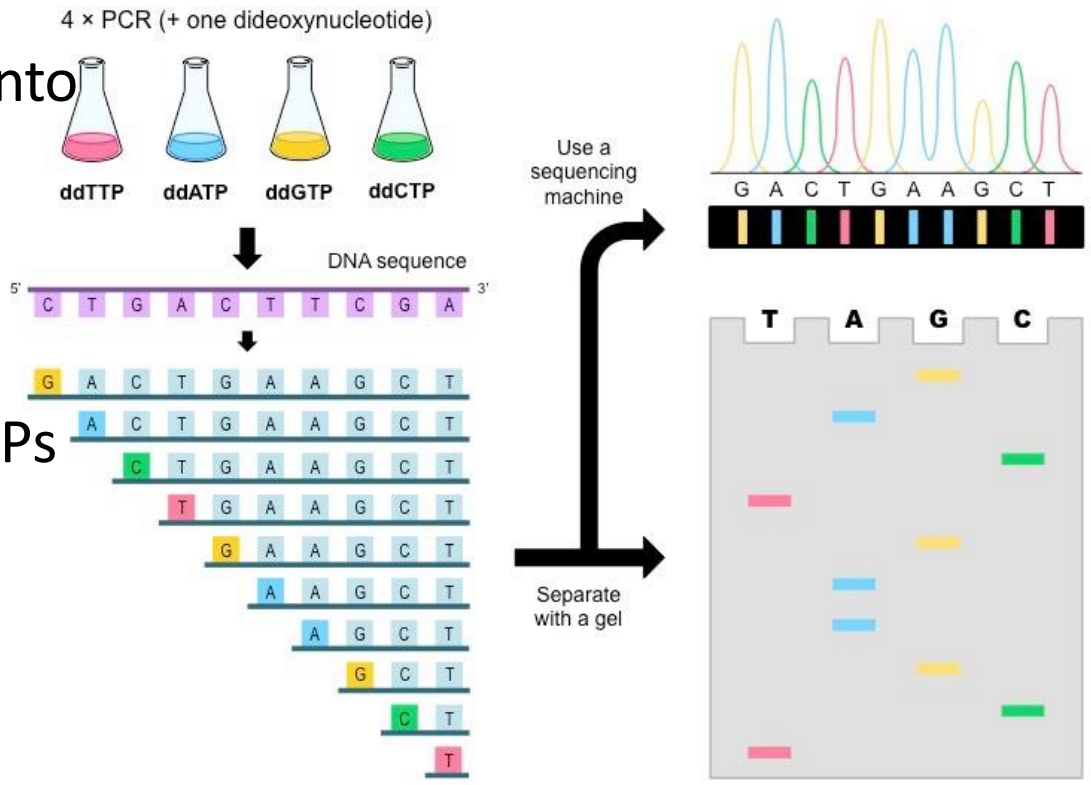
- Third Generation Sequencing

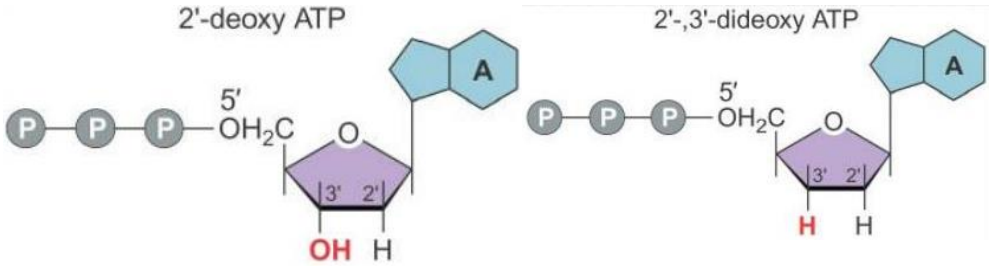
Year of Discovery	Manufacturer	Technology	Average read length
1996	Oxford Nanopore Tech Limited	Nanopore sequencing	Long 10 – 100kb Ultralong - 2Mb
2005	Pacific Biosciences SMRT	PacBio sequencing	13 - 20kb

Principles of various Sequencing Technologies

Sanger sequencing - principle

- The double-stranded DNA (dsDNA) is denatured into two single-stranded DNA (ssDNA).
- A primer that corresponds to one end of the sequence is attached.
- Four polymerase solutions with four types of dNTPs and only one type of ddNTP are added.
- The DNA synthesis reaction initiates and the chain extends until a termination nucleotide is randomly incorporated.
- The resulting DNA fragments are denatured into ssDNA. The denatured fragments are separated by gel electrophoresis and the sequence is determined.

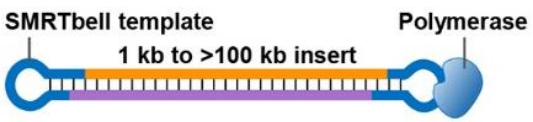




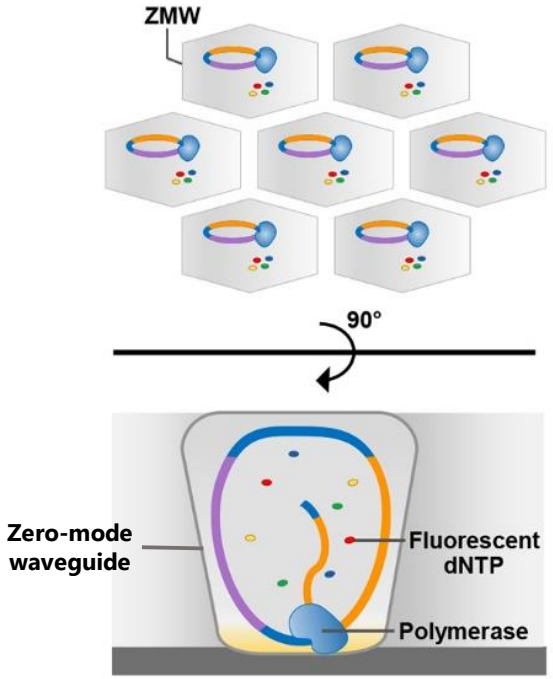
https://www.slideserve.com/dahlia-hendrix/molecular-biology-techniques-a-primer-powerpoint-ppt-presentation

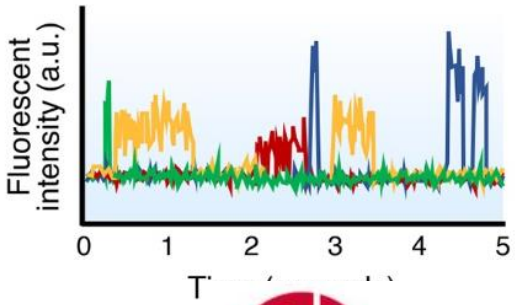
https://microbenotes.com/dna-sequencing-maxam-gilbert-and-sanger-dideoxy-method/

PacBio sequencing SMRT- principle



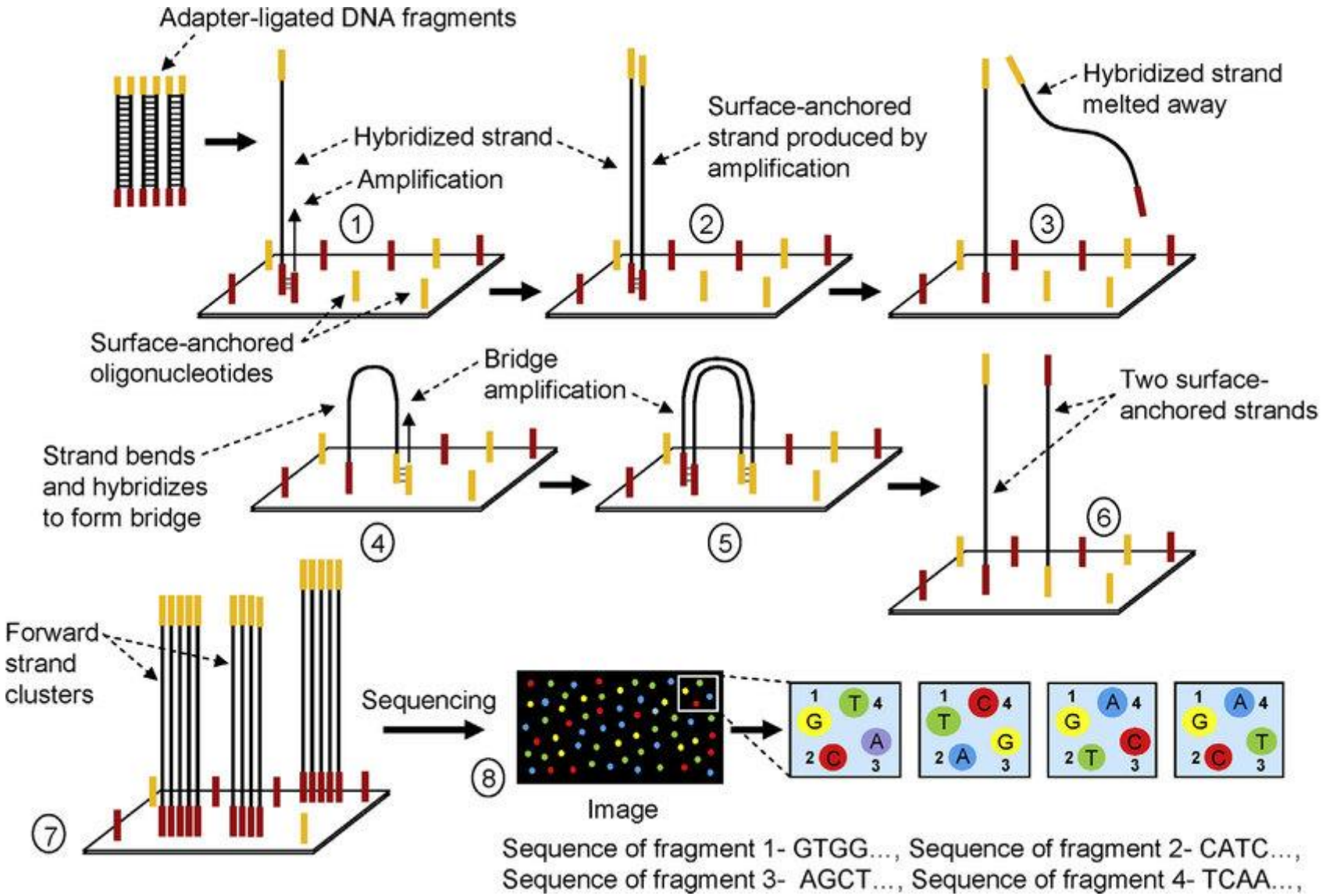
- SMRT – single molecule real time
- ZMW – zero mode waveguide
- SMRT bell generated during DNA lib prep
- Its bound by DNA polymerase & loaded into a SMRT cell with >8 million ZMW chambers
- Elongation occurs through addition of one flourescent labelled dNTP to SMRTbell template at a time
- A light pulse excites the fluorophore; emission detected by camera and converted to corresponding base
- Fluorophore cleaved and released into sequencing buffer to complete one cycle



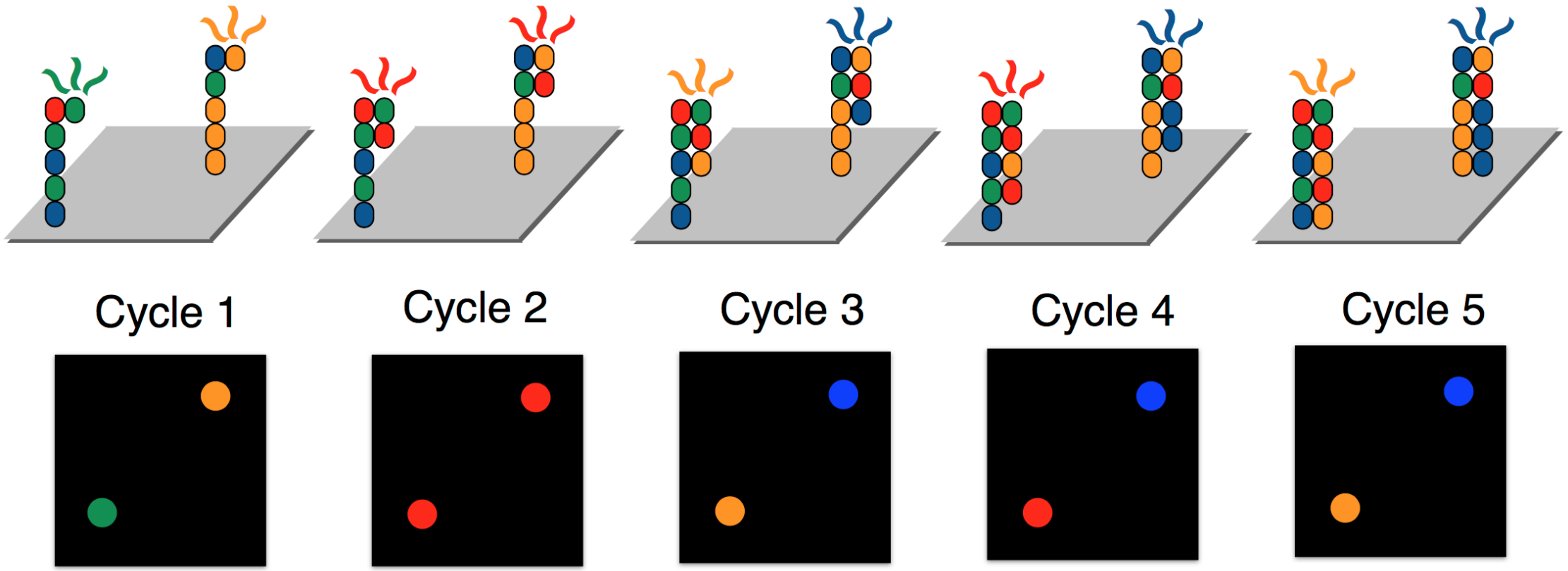


PACBIO®

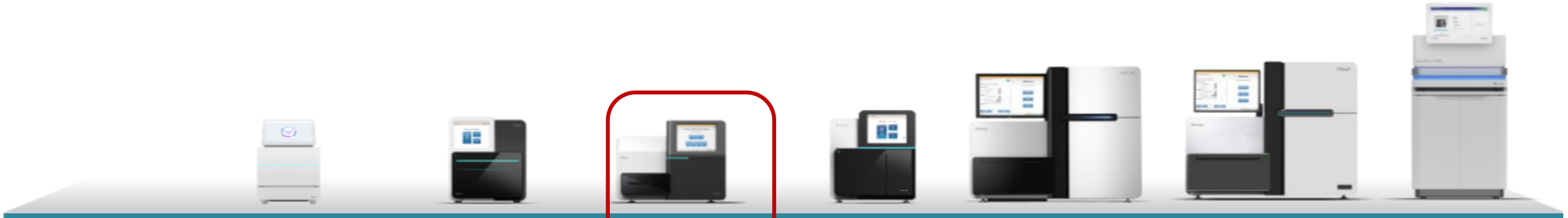
Illumina sequencing - principle



Illumina sequencing - principle

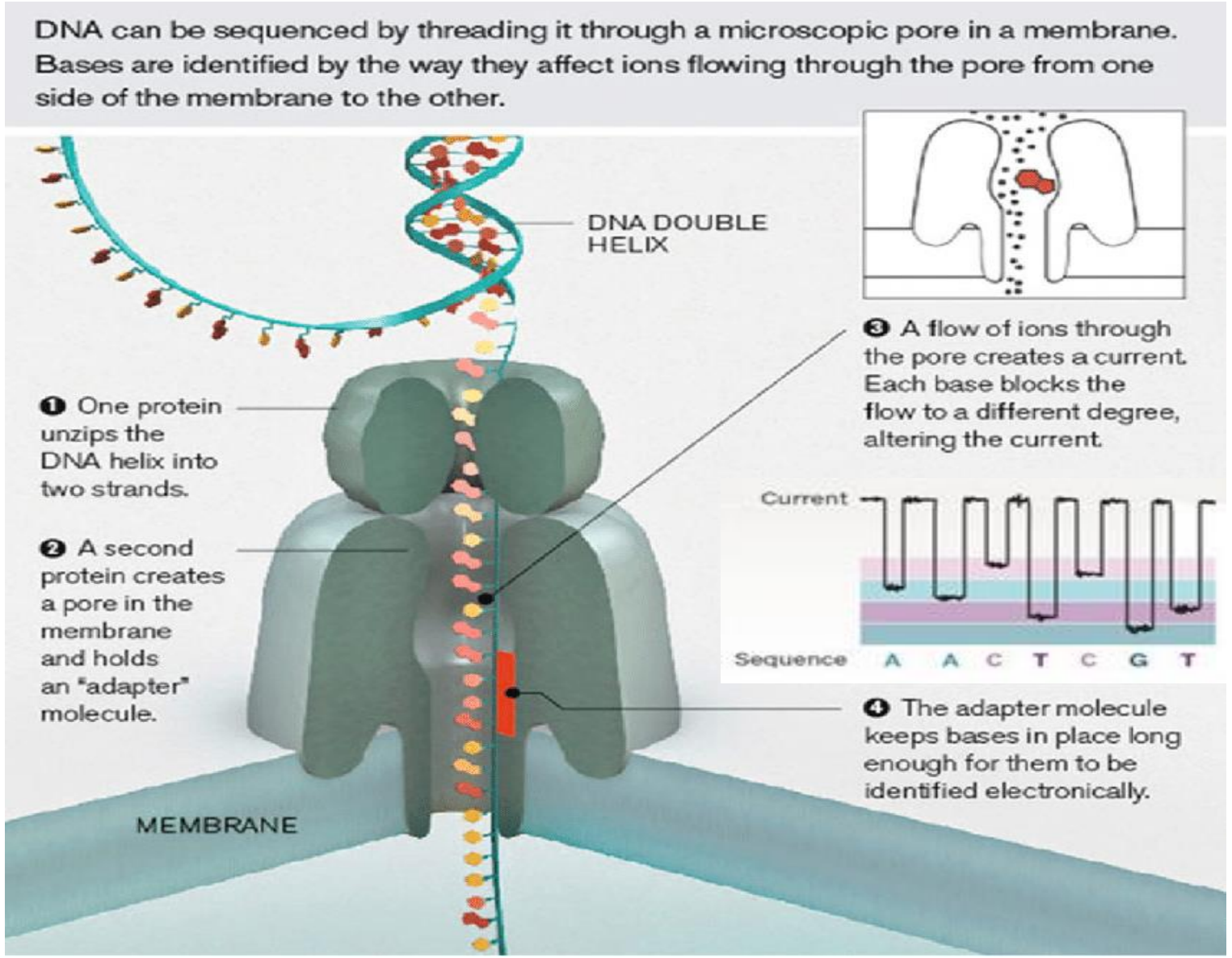


Illumina sequencing - devices



Sequencing System	iSeq™	MiniSeq™	MiSeq®	NextSeq®	HiSeq® 4000	HiSeq® X Five/Ten	NovaSeq® 6000
Output per run	1.2 Gb	7.5 Gb	15 Gb	120 Gb	1.5 Tb	1.8 Tb	1 Tb - 6 Tb[1]
Instrument price	$19.9K	$49.5K	$99K	$275K	$900K	$6M[2]/$10M[2]	$985K
Installed base[3]	NA	~600	~6,000	~2,400	~2,300[4]		~285

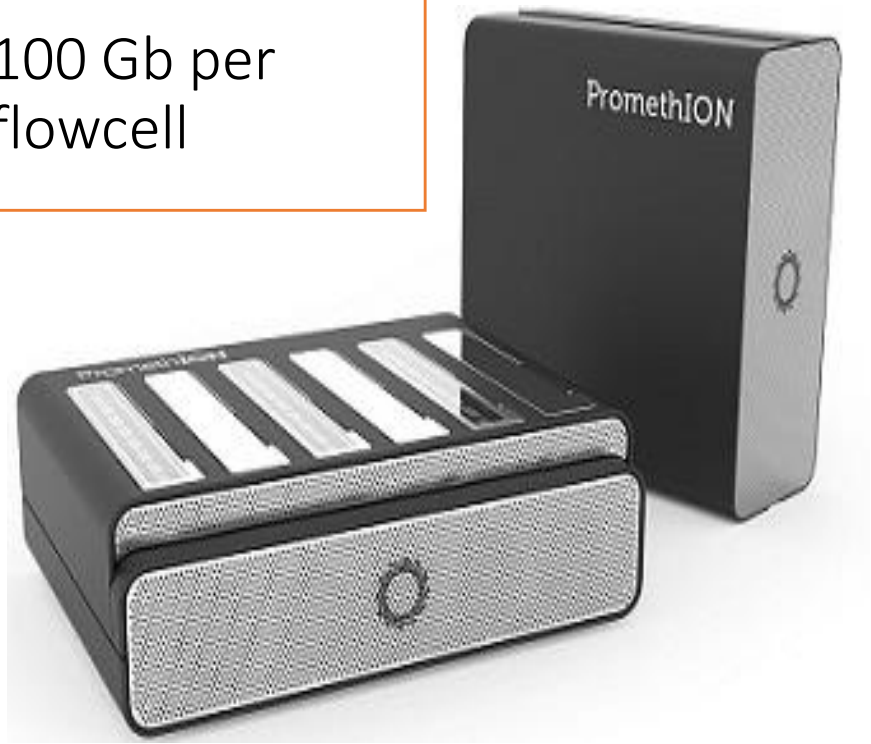
Oxford Nanopore sequencing - principle



Nanopore sequencing - devices

1. MinION
   - 1 flowcell:
   - 2048 pores
   - 512 pores are used at a time
   - ~ 400 bases/sec are read
   - 10 -25 Gb data

3. PromethION
   - 48 flowcells
   - 1200 pores per cell
   - 100 Gb per flowcell



2. GridION
   - 5 flowcells
   - 2048 x 5 pores
   - 100 Gb data

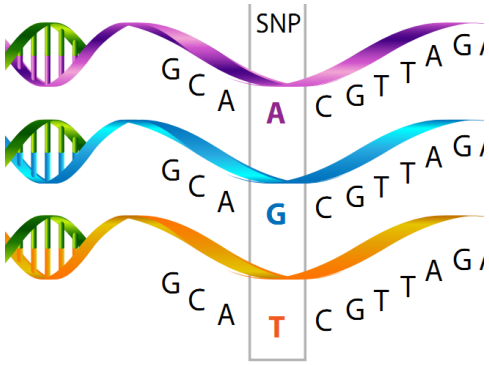
Comparison of Sequencing Technologies

Long versus Short sequencing technologies

Feature	Sanger	Oxford Nanopore	Illumina	PacBio HiFi
Read length	Short reads: 200 - 600bp	Long reads 10 – 100kbp Ultra-long ~3Mb	Short reads 150 – 250bp	Long reads 10 – 20kbp
Read type	Paired end reads	Single reads	Paired-end reads: forward & reverse	Single reads
Accuracy	100% Gold Standard	R.9.4.1 89 – 93% R10.4.1 99%	99.9%	99.9%
Applications	Amplicon sequencing, Clone checking ChIP-Sequencing RNA Sequencing	Whole genome sequencing, Plasmid reccnstruction	Small genome sequencing, amplicon sequencing, clone checking & ChIP-Seq, RNA Seq	Whole genome sequencing

Advantages of Illumina sequencing

- High accuracy of ~99.9% - SNP analysis, allele identification
- High sequence coverage – good sequence depth >100
- High throughput data - sequence billions of template strands simultaneously
- Parallel sequencing – use of unique indexes to label diff samples



Disadvantages of Illumina sequencing

- Initially expensive to install
- Non-contiguous or incomplete assemblies
- Poor resolution of regions rich in repeat sequences & AT rich genomes

illumina®

Advantages of Nanopore sequencing

- Ultra-long read lengths – longest reads up to date is >4 Mb in length; Resolve plasmid sequences & repetitive regions; completion of small genomes (viruses, bacterial)
- Real-time data analysis: no fixed run-time; stop run when data is sufficient; sequence new genome of unknown length
- Direct molecular analysis - Sequencing native DNA (and RNA) avoids amplification bias
- Portable – fits in adult palm – Flongle, Minion and Mrk1c

Disadvantages of Nanopore sequencing

- Sequencing error of ~5% - early chemistries R9.4.1, R10.3

  New chemistry R10.4.1 promises higher accuracy of 99.9%

Long reads versus short reads – Human Genome

Human Genome Project (1990-2003)

Generated first draft of human genome – 92% complete

Used Bacterial Artificial Chromosome cloning and Sanger short read sequencing

Telomere-to-Telomere (T2T) consortium 31st March 2022

Completed the remaining 8% (Nurk et al 2022) that were complex regions:

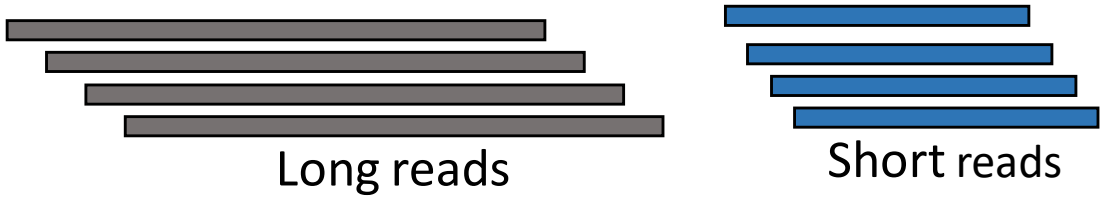
centromeric satellite arrays, subtelomeric regions, rDNA arrays, short arms of acrocentric chromosomes – rich in long repeat sequences

Used using PacBio HiFi and ONT:

- ONT produced ultra-long reads - >100kb
- PacBio HiFi produced high accurate reads - ~20kb

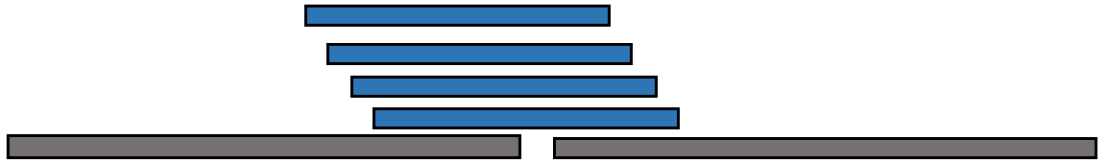
Hybrid genome assembling

Use of long and short reads to polish an assembly

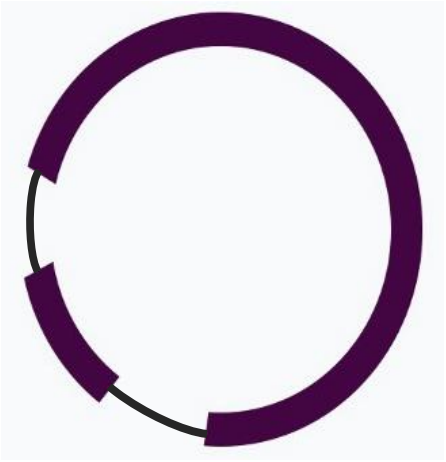


Two strategies of generating a contiguous sequence:

1. Short reads to polish long read assembly



2. Use long reads to polish a short read assembly



Question & Answer session