“The double helix is indeed a remarkable molecule. Modern man is perhaps 50,000 years old, civilization has existed for scarcely 10,000 years and the United States for only just over 200 years; but DNA and RNA have been around for at least several billion years.

All that time the double helix has been there, and active, and yet we are the first creatures on Earth to become aware of its existence.”

Francis Crick (1916–2004)

History of DNA and modern approaches to sequencing

Konrad Paszkiewicz

January 2016

Contents

- A short history of DNA
- Review of first generation sequencing techniques
- Short-read second generation sequencing technology
  - Illumina
  - Life Tech Ion Torrent
- Third generation single molecule sequencing
  - PacBio
  - Oxford Nanopore

A short history of DNA

“The double helix is indeed a remarkable molecule. Modern man is perhaps 50,000 years old, civilization has existed for scarcely 10,000 years and the United States for only just over 200 years; but DNA and RNA have been around for at least several billion years.

All that time the double helix has been there, and active, and yet we are the first creatures on Earth to become aware of its existence.”

Francis Crick (1916–2004)

The first person to isolate DNA

- Friedrich Miescher
  - Born with poor hearing
  - Father was a doctor and refused to allow Freidrich to become a priest
- Graduated as a doctor in 1868
  - Persuaded by his uncle not to become a practising doctor and instead pursue natural science
  - But he was reluctant…

Friedrich Miescher

Biology PhD angst in the 1800s

"I already had cause to regret that I had so little experience with mathematics and physics… For this reason many facts still remained obscure to me."

His uncle counselled:

"I believe you overestimate the importance of special training…"

Friedrich Miescher

1869 - First isolation of DNA

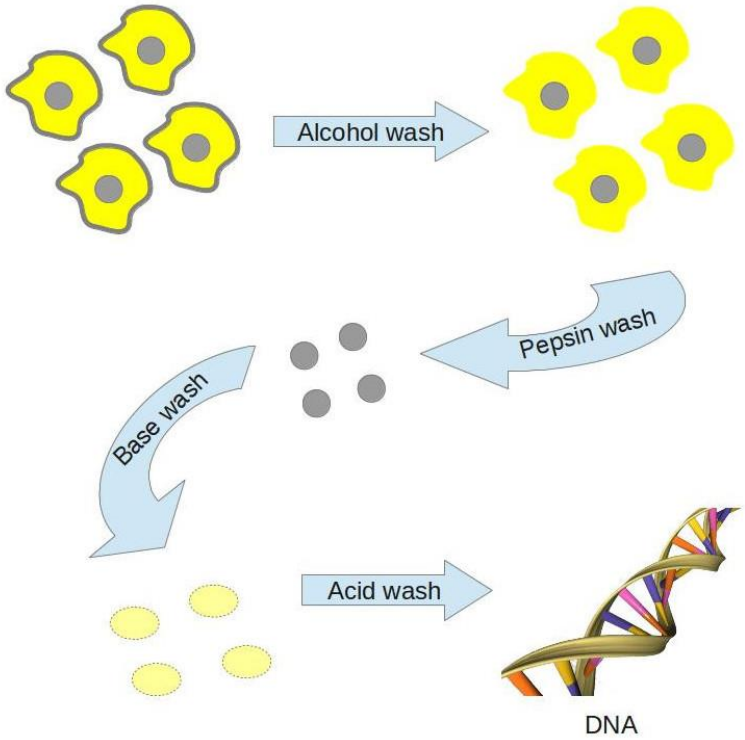
- Worked in Felix Hoppe-Seyler's laboratory in Tubingen, Germany
  - The founding father of biochemistry
- The lab was one of the first to crystallise haemoglobin and describe the interaction between haemoglobin and oxygen
- Friedrich was interested in the chemistry of the nucleus
- Freidrich extracted 'nuclein' on cold winter nights
  - Initially from human leukocytes extracted from bandage pus from the local hospital
  - Later from salmon sperm

Felix Hoppe-Seyler

Friedrich Miescher

Meischer's isolation technique

- Cells from surgical bandages or salmon sperm
- Alcohol to remove outer cell membrane
- Pepsin from pig stomachs
- Basic solution to dissolve nuclein in the nucleus
- Acid solution to precipitate the nuclein
- Difficult to do without also precipitating bound protein



http://www.howdoweknowit.com/2013/07/03/how-do-we-know-the-genetic-code-part-2/

Biology PhD angst in the 1800s

His student remembered*:*

“Friedrich failed to turn up for his own wedding. We went off to look for him. We found him quietly working in his laboratory.“

“I go at 5am to the laboratory and work in an unheated room. No solution can be left standing for more than 5 minutes… Often it goes on until late into the night.”

Friedrich Miescher

1874 - First hints to composition

- By 1874 Meischer had determined that nuclein was
  - A basic acid
  - High molecular weight
  - Nuclein was bound to ‘protamin’
- Came close to guessing its function
  - “If one wants to assume that a single substance is the specific cause of fertilisation, the one should undoubtedly first and foremost consider nuclein”
  - Discarded the idea because he thought it unlikely that nuclein could encode sufficient information

Friedrich Miescher

1881 - Discovering the composition of nuclein

- Kossel worked in the same lab as Freidrich Miescher
- Wanted to relate chemical composition to biological function

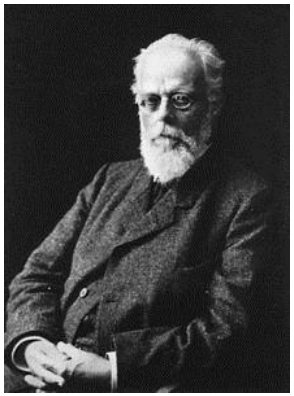
- Discovered fundamental building blocks of nuclein
  - Adenine, Cytosine, Guanine, Thymine, and Uracil
  - Identified histone proteins and that nuclein was bound to histone in the nucleus
  - Inferred that nuclein was not used for energy storage but was linked to cell growth

Albrecht Kossel

1890s - Molecular basis of heredity

- How are characteristics transmitted between generations?

- Lots of theories
  - Stereo-isomers
  - Asymmetric atoms
  - Complex molecules
- Realisation that hereditary information is transmitted by one or more molecules
- 1893 August Weismann – germ plasm theory
- 1894 Eduard Strasburger- “nuclei from nuclei”



August Weismann

Eduard Strasburger

1900 - What we knew

Known

- Distinction between proteins and nucleic acids
- Somehow nuclein was involved in cell growth
- Somehow the nucleus was involved in cell division
- Rediscovery of Mendel's laws

Unknown

- Mendel's lost laws
- Base composition of nucleic acids
- Role of the nucleus
- Distinction between RNA and DNA
- Significance of chromosomes
- That enzymes were proteins
- Most of biochemistry

1902 – Linking chromosome count to somatic and gametic cells

- Walter Sutton using grasshopper gametes
- Theodore Boveri using sea urchins

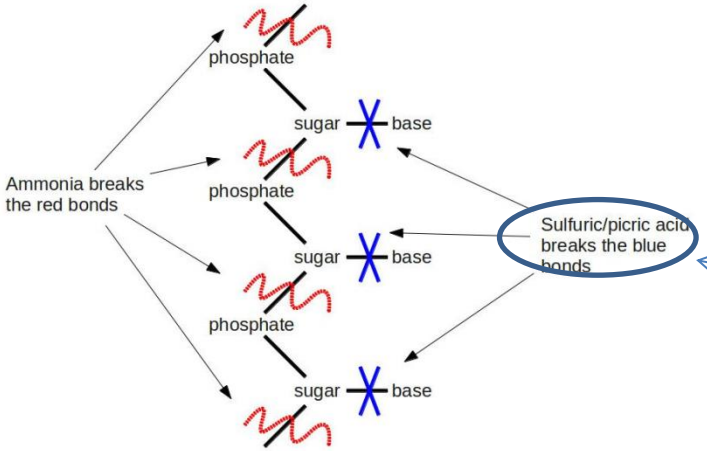
Walter Sutton and Theodore Boveri

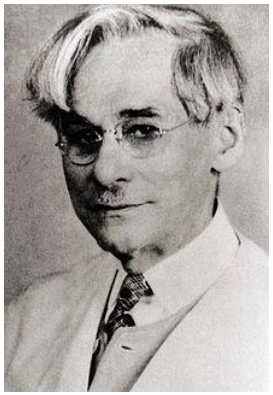
"I may finally call attention to the probability that the association of paternal and maternal chromosomes in pairs and their subsequent separation during the reducing division as indicated above may constitute the physical basis of the Mendelian law of heredity."

- Theodore Boveri *Sutton, W. S. 1903. The chromosomes in heredity. Biological Bulletin, 4:231-251.*

1910s - More on the composition of DNA

- Determined relative composition of sugars, phosphate and sugars by hydrolysis of nucleic acid





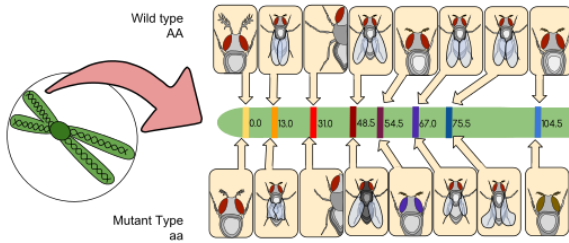
Phoebus Levene

Close relative of TNT explosive

- Enabled the discovery of DNA and RNA bases
- Unfortunately, this method can destroy bases and bias results
- Made it impossible to compare composition between species
- Phoebus Levene proposed the tetranucleotide hypothesis
  - DNA consisted of repeating units of thymine, guanine adenine and cytosine
  - E.g. GACT GACT GACT
  - Convinced many that DNA could not be a carrier of hereditary information
  - Led to the assumption that DNA was just a structural component of cells

1910-30s - Chromosome theory of heredity

- Chromosome as a unit of heritability confirmed by Thomas Morgan and his student Alfred Sturtevant creates the first genetic map in 1913-15 in Drosophila



Thomas Morgan

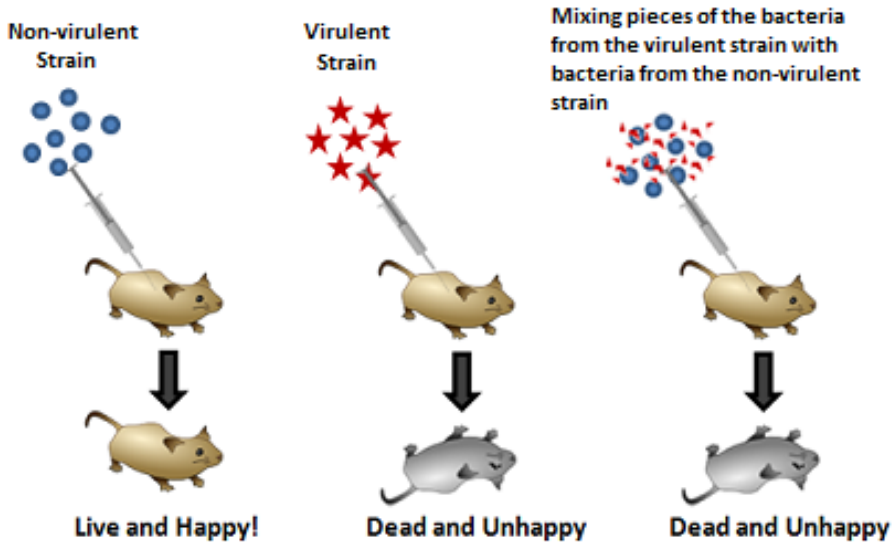
- Hypothesized that crossing-over during meiosis could explain variations in progeny phenotype after crossing over

- Genetic recombination shown to be caused by physical recombination of chromosomes by Barbara McClintock & Harriet Creighton in 1930

Barbara McClintock

1928 - Inheritance of virulence

- Established that non-virulent pneumococci bacteria could be converted be made virulent by exposure to lysed virulent bacteria



- What was the 'transforming principle' which underlay this observation?

Frederick Griffiths

"Could do more with a kerosene tin and a primus stove than most men could do with a palace"

Hedley Wright

http://mic.sgmjournals.org/content/73/1/1.full.pdf

1944 – What is life?

- An ‘aperiodic solid crystal’ could code for an organism
- “A well-ordered association of atoms endowed with sufficient resistivity to keep its order permanently”
- Also placed living systems into a thermodynamic framework

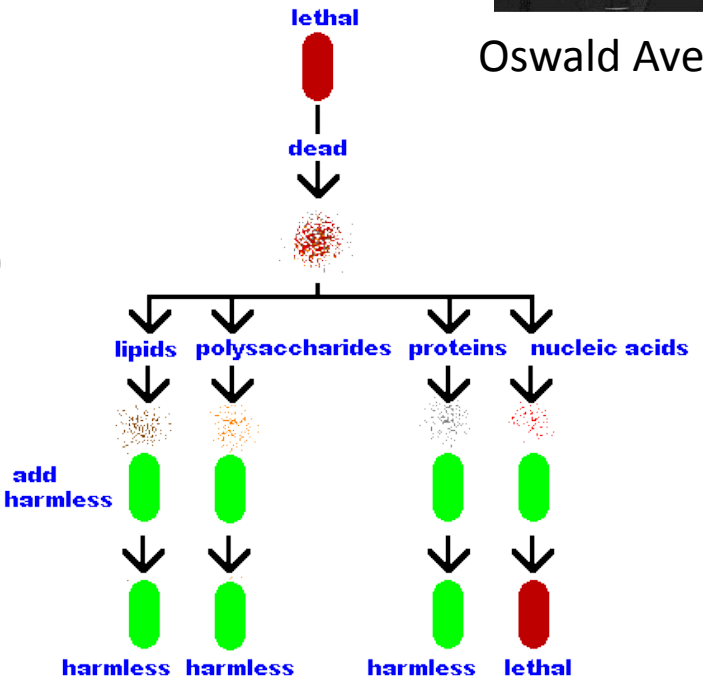
- Served as inspiration for Watson & Crick

Erwin Schrodinger

1944 – Establishing DNA as the transforming principle

- Separated cellular components and repeated Griffiths experiments
- Enabled by new 'ultra-centrifugation' technology
- Extended Griffiths work to prove that nucleic acids were the 'transforming principle'
- Also demonstrated that DNA, not RNA was the genetic material
- Incredibly small amounts – 1 in 600 million were sufficient to induce transformation

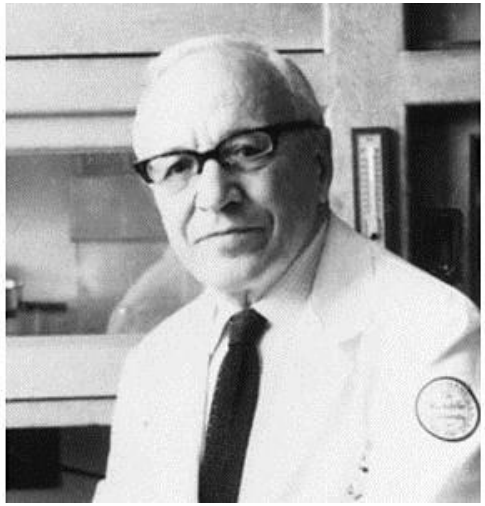
Oswald Avery



http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2135445/

1945 – 1952 Critique

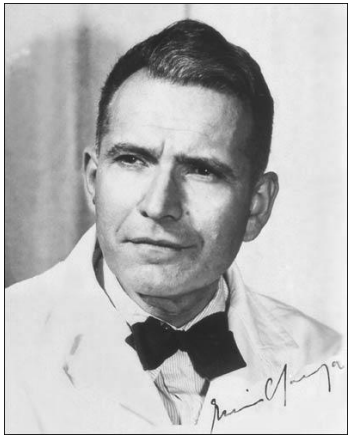
- Alfred Mirksy was a pioneer of molecular biology
- Isolated chromatin from a wide variety of cells
- He was concerned that Avery's results could be the result of protein contamination
- Fought a battle against Avery
- Convinced the Nobel panel not to award a prize to Avery
- Later, Mirsky would actually demonstrate the 'Constancy' of DNA throughout somatic cells



Alfred Mirsky

1950 – Base composition between organisms

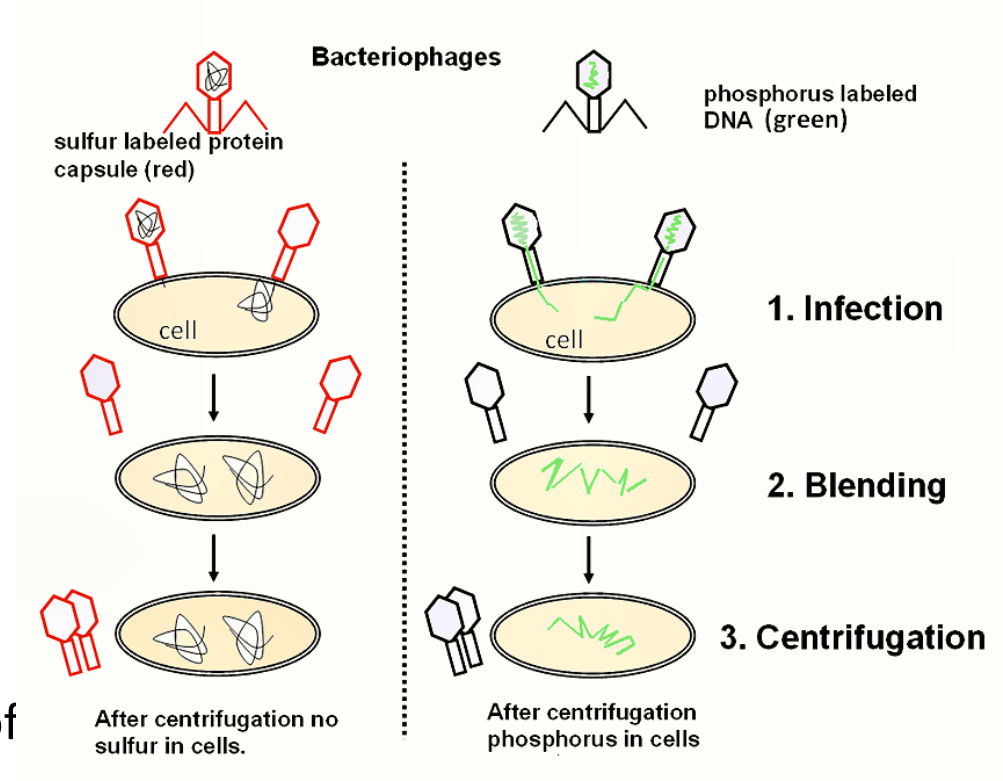
- Erwin Chargaff hit back at Mirsky and developed the base complementarity hypothesis with Masson Gulland
- Determined that the molar ratio of A/T and G/C were always very close to 1
- Relative proportions of bases varied between species but was the same within species
- Refuted Levene's 30 year-old tetranucleotide hypothesis



Erwin Chargaff

1952- Confirmation of Avery's experiment

- Grow bacteriophage using radioactive substrates
  - Protein with radioactive sulphur
  - DNA with radioactive phosphorous
- Bacteriophages infected bacteria by injecting DNA, not protein
- Was taken as confirmation of the role of DNA as genetic material
- Yet there was still the possibility of protein contamination here



Hershey Chase experiment

http://en.wikipedia.org/wiki/Hershey%E2%80%93Chase_experiment

1952 – X-ray diffraction patterns of DNA

- Wilkins, Franklin and Gosling
- Much improved X-ray diffraction patterns of the B-form of DNA
- Wilkins developed a method to obtain improved diffraction patterns using sodium thymonucleate to draw out long thin strands of DNA

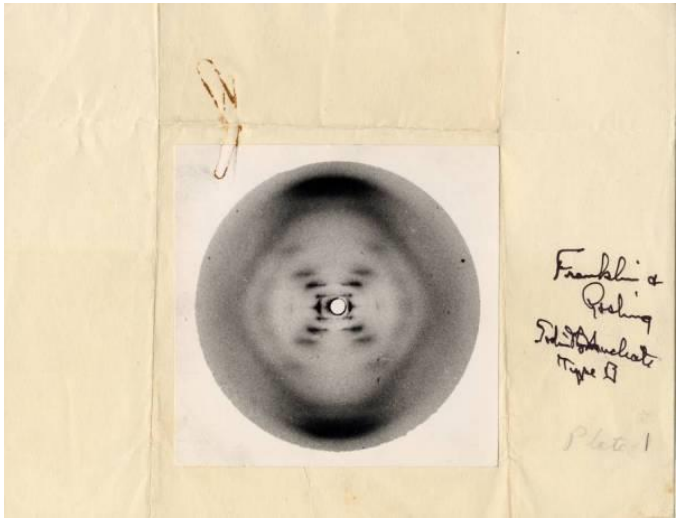
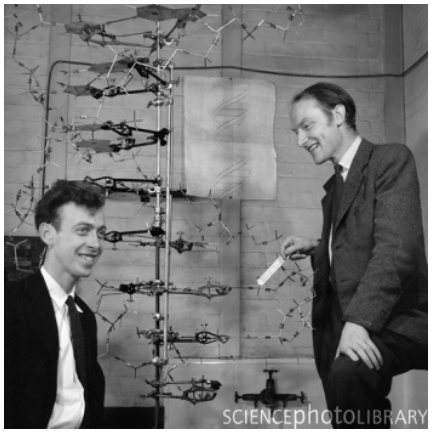


Photo Number 51

http://paulingblog.wordpress.com/2009/07/09/the-x-ray-crystallography-that-propelled-the-race-for-dna-astburys-pictures-vs-franklins-photo-51/

1953 – Watson & Crick obtain a structure for DNA

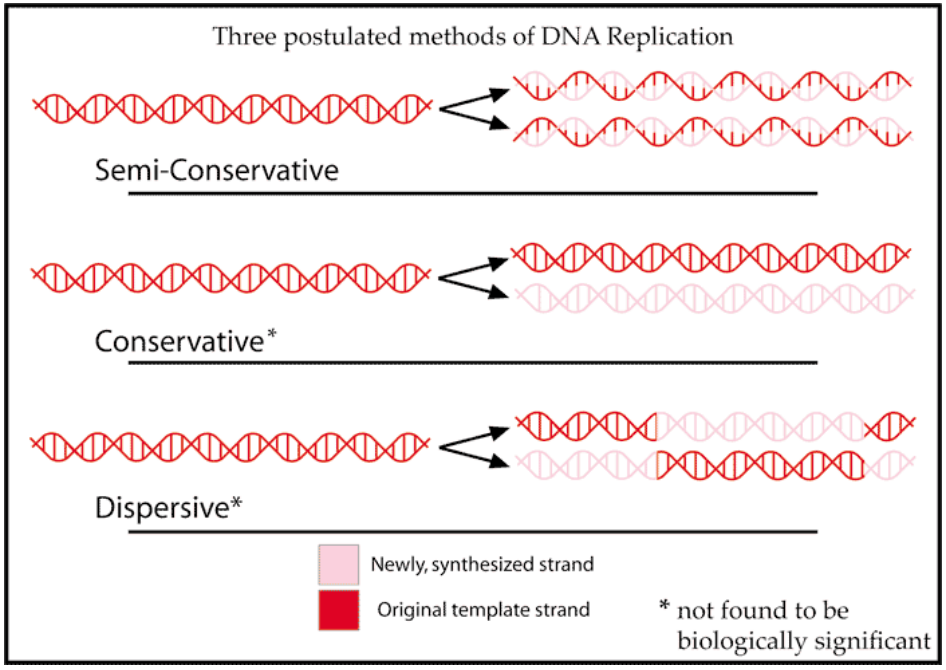
- B-model of DNA
- Relied upon data from Maurice Wilkins and Rosalind Franklin via Maz Perutz
- *"It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material.“*
- Broad acceptance of the structure did not occur until around 1960



Francis Crick & James Watson

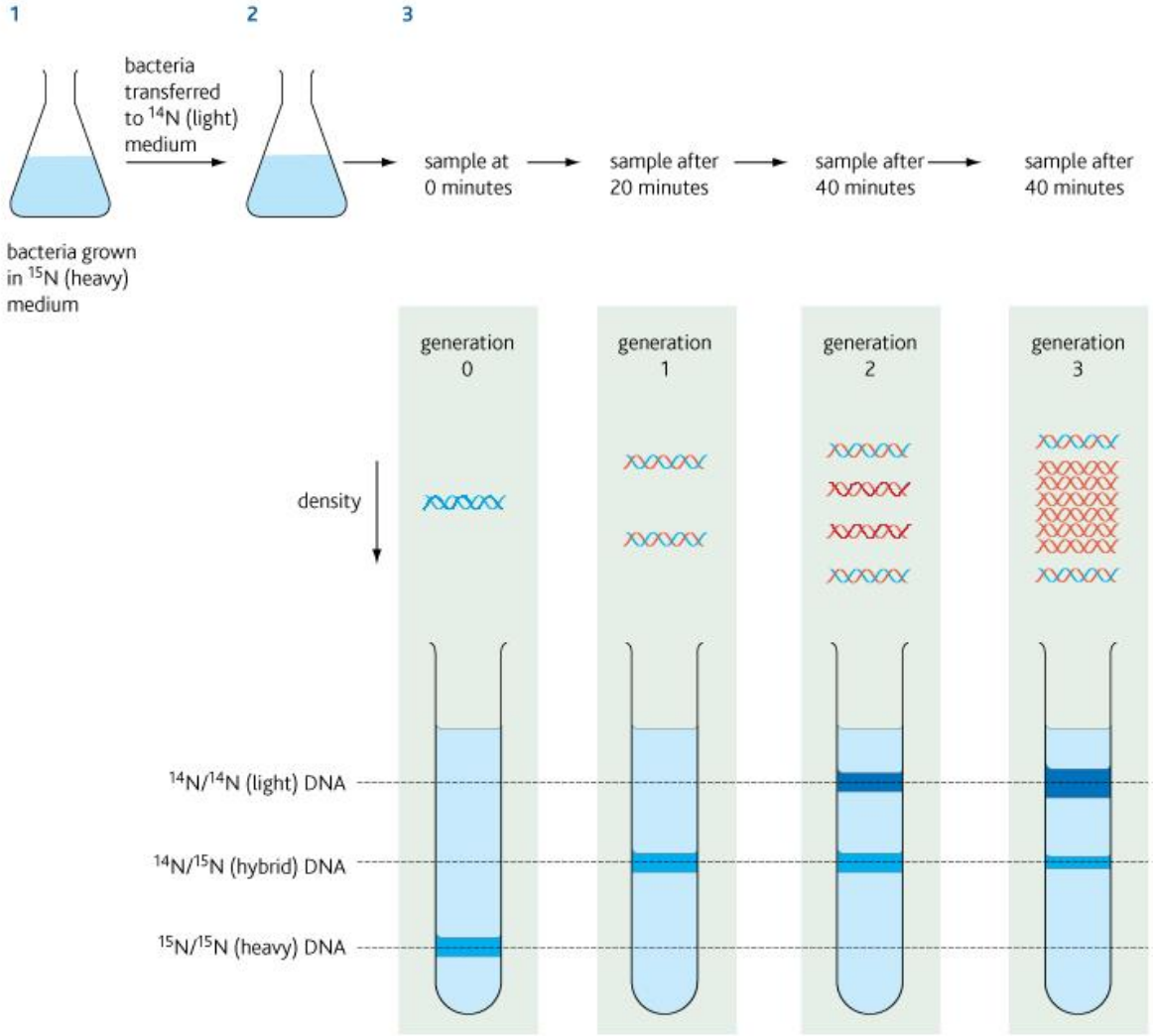
1958 – Evidence for the mechanism of DNA replication

- Meselson & Stahl
- Supported Watson & Crick's hypothesis of semi-conservative DNA replication



http://en.wikipedia.org/wiki/Meselson%E2%80%93Stahl_experiment

1958 – Evidence for the semi-conservative mechanism of DNA replication



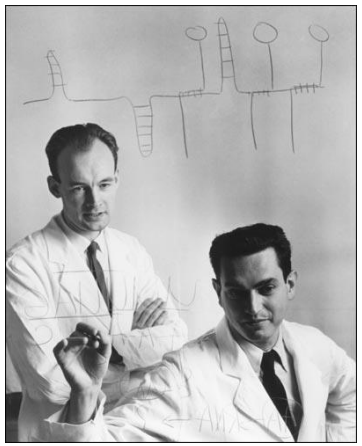
Other developments in molecular biology

- 1954 - George Gamow proposed a 3-letter code
- 1955 – Polynucleotide phosphorylase discovered
  - Enabled synthesis of homogeneous nucleotide polymers
- 1957 – Crick lays out ‘central dogma’
- 1957-1963
  - RNA structure
  - Work on DNA-RNA hybridization
- 1960s
  - Crystal structures of tRNAs
  - Role in protein synthesis
  - Role of ribosomes

- Set the stage for…

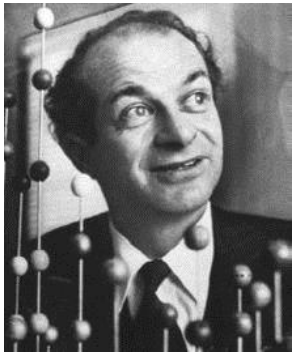
1961 - Deciphering the genetic code

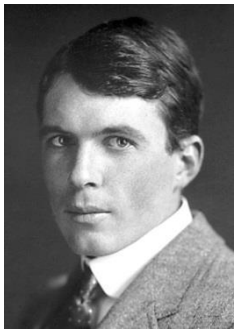
- How did DNA code for proteins?
- Nirenberg and Matthaei
- Used polynucleotide phophorylase to construct a poly-uracil polymer
- Added to a cell-free system containing ribosomes, nucleotides, amino acids, energy
- This produced an amino acid chain of phenylalanine
- Completed in mid 1960s by Har Gobind Khohrana



Other key figures

- Max Delbruck
  - Physicist who helped found molecular biology
- Salvador Luria
  - James Watson's PhD supervisor
  - Demonstrated with Delbruck that inheritance in bacteria was Darwinian and not Lamarkian
- Linus Pauling
  - Proposed triple helix model for DNA
- Lawrence Bragg
  - Hosted Watson & Crick
  - Rival of Pauling's
- Jerry Donohue, William Astbury, Raymond Gosling, John Randall, Fred Neufield, Herbert Wilson…





1962

- Nobel Prize awarded for Physiology or Medicine to Watson, Crick and Wilkins
- Rosalind Franklin died in 1958 of suspected radiation induced cancer

Russia's Usmanov to give back Watson's auctioned Nobel medal

The medal sold at auction for £3m

Russia's richest man has revealed that he bought US scientist James Watson's Nobel Prize gold medal, and intends to return it to him.

Related Stories

Oswald Avery

- Avery died in 1955
- It is unknown whether he learned of Watson & Crick's structure of DNA
- He never received a Nobel for his work
- However his 1944 paper is cited around 40 times a year and has cited over 2000 times in the past 20 years

Oswald Avery

Further reading

- Eighth day of creation – Horace Freeland Judson
- Life's Greatest Secret – Matthew Cobb
- Oswald Avery, DNA, and the transformation of biology. Cobb, M. Current Biology. Volume 24, Issue 2, 20 January 2014, Pages R55–R60

First generation sequencing

The development of sequencing methodologies

- What do we mean by ‘sequencing’?

- Determining the order and identity of chemical units in a polymer chain
  - Amino acids in the case of proteins
  - Nucleotides in the case of RNA and DNA

- Why do we do it?
  - 3D structure and function is dependent on sequence

1949 – Amino acids

- Sequenced bovine insulin
- Developed a method to label N-terminal amino acids
  - Enabled him to count four polypeptide chains
- Used hydrolysis and chromatography to identify fragments

Fred Sanger

1965 - RNA sequencing and structure

- Sequenced transfer RNA of alanine
- Used 2 ribonuclease enzymes to cleave the enzyme at specific motifs
- Chromatography
- 1968 Nobel prize

Robert Holley

May 1965 *R. W. Holley, G. A. Everett, J. T. Madison, and A. Zamir* 2127

Pancreatic RNase fragments: pG–G–G–C | G–U | G–U | G–G–C | G–U | A–G–U | A–G–C |

G–G–U | C | G–C | G–C | C | C | U | C | U | U | I–G–C |

Taka-Diastase T1 fragments: pG | G | G | C–G | U–G | U–G | G | C–G | U–A–G | U–A–G | C–G | G | U–C–G | C–G | C(C,C,U)C–U–U–I | G | C–

RNase (*continued*) G–G–T | ψ | C | G–C | I–ψ | G–G–G–A–G–A–G–U* | C | U | C | C |

G–A–U | U | C | C | G–G–A–C | U | C | G–U | C | C | A–C | C | AOH

T1 (*continued*) G | G | T–ψ–C–G | C–I–ψ–G | G | G | A–G | A–G | U*(C,U)C–C–G |

A–U–U–C–C–G | G | A–C–U–C–G | U–C–C–A–C–C–AOH

FIG. 7. One of many possible arrangements of the pancreatic RNase and RNase T1 digest fragments that shows the overlaps between the two digests. The RNA molecule is accounted for by the 16 oligonucleotide sequences indicated by the *solid lines*. Only the positions of the two terminal sequences are known. *Vertical lines* indicate the position of enzymatic attack. The *asterisk* indicates that the uridine may be partially substituted by DiHU.

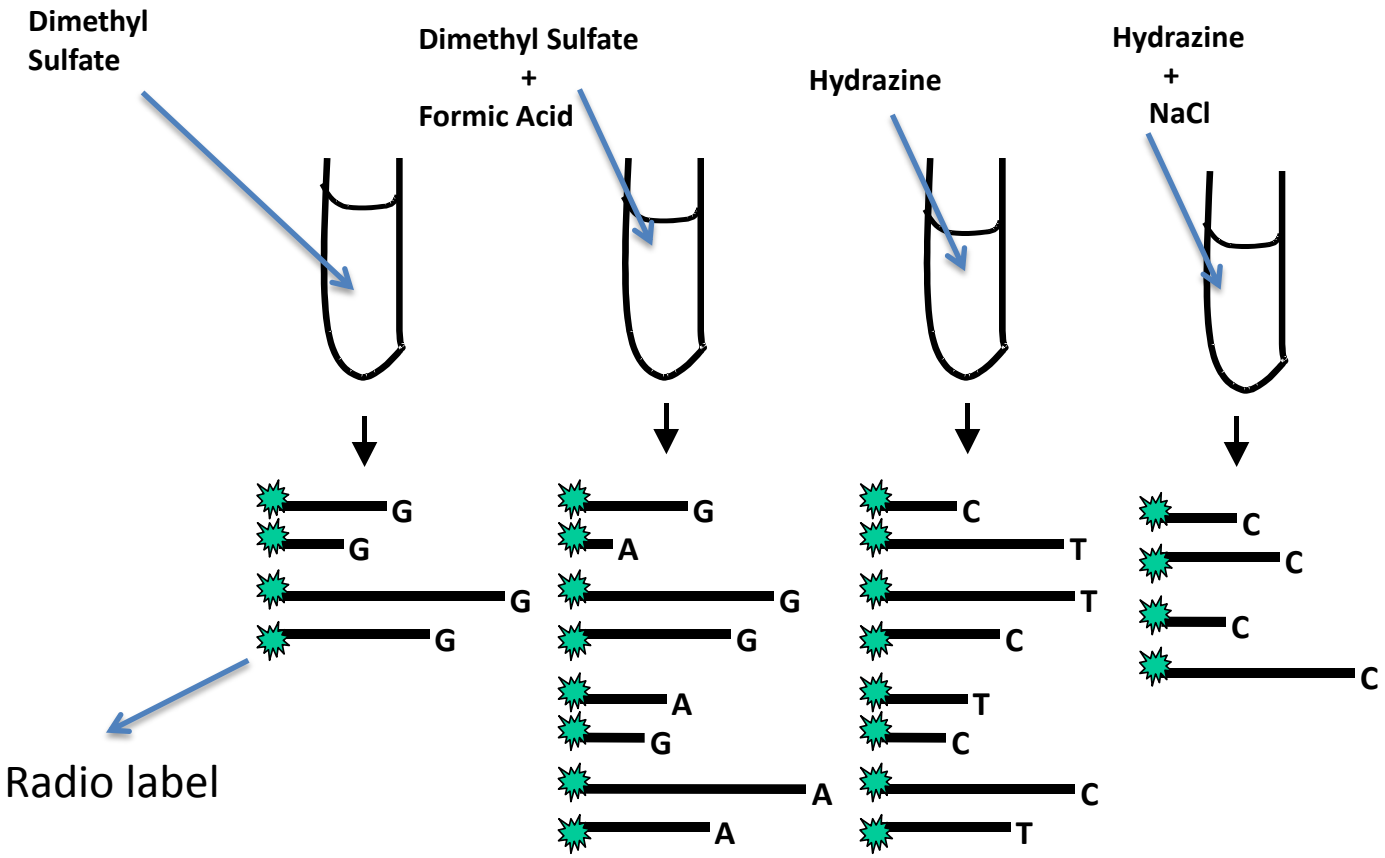
http://www.sciencemag.org/content/147/3664/1462

1975 - The dawn DNA sequencing

- Between 1975-1977 three methods of DNA sequencing were published

- Fred Sanger's Plus/Minus method
- Maxam-Gilbert
- Fred Sanger's chain termination method

Maxam-Gilbert Sequencing



Maxam-Gilbert sequencing is performed by chain breakage at specific nucleotides.

Maxam-Gilbert Sequencing

Proc. Natl. Acad. Sci. USA
Vol. 74, No. 2, pp. 560–564, February 1977
Biochemistry

A new method for sequencing DNA

(DNA chemistry/dimethyl sulfate cleavage/hydrazine/piperidine)

ALLAN M. MAXAM AND WALTER GILBERT

Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138

Contributed by Walter Gilbert, December 9, 1976

ABSTRACT **DNA can be sequenced by a chemical procedure that breaks a terminally labeled DNA molecule partially at each repetition of a base. The lengths of the labeled fragments then identify the positions of that base. We describe reactions that cleave DNA preferentially at guanines, at adenines, at cytosines and thymines equally, and at cytosines alone. When the products of these four reactions are resolved by size, by electrophoresis on a polyacrylamide gel, the DNA sequence can be read from the pattern of radioactive bands. The technique will permit sequencing of at least 100 bases from the point of labeling.**

We have developed a new technique for sequencing DNA molecules. The procedure determines the nucleotide sequence of a terminally labeled DNA molecule by breaking it at adenine, guanine, cytosine, or thymine with chemical agents. Partial cleavage at each base produces a nested set of radioactive

THE SPECIFIC CHEMISTRY

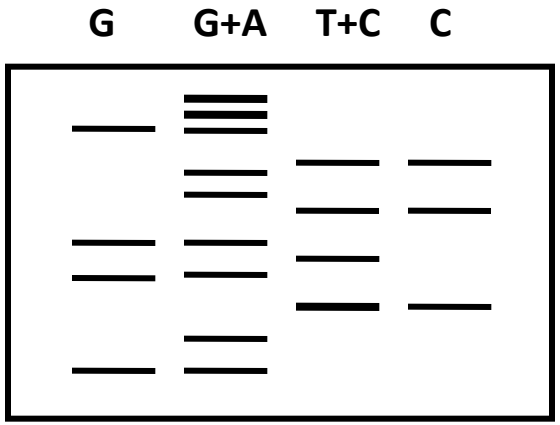
A Guanine/Adenine Cleavage (2). Dimethyl sulfate methylates the guanines in DNA at the N7 position and the adenines at the N3 (3). The glycosidic bond of a methylated purine is unstable (3, 4) and breaks easily on heating at neutral pH, leaving the sugar free. Treatment with 0.1 M alkali at 90° then will cleave the sugar from the neighboring phosphate groups. When the resulting end-labeled fragments are resolved on a polyacrylamide gel, the autoradiograph contains a pattern of dark and light bands. The dark bands arise from breakage at guanines, which methylate 5-fold faster than adenines (3).

This strong guanine/weak adenine pattern contains almost half the information necessary for sequencing; however, ambiguities can arise in the interpretation of this pattern because the intensity of isolated bands is not easy to assess. To determine

Maxam-Gilbert Sequencing

Longer fragments

Shortest fragments



3′
A
A
G
C
A
A
C
G
T
G
C
A
G
5′

Sequencing gels are read from bottom to top (5′ to 3′).

Sanger di-deoxy sequencing method

Proc. Natl. Acad. Sci. USA
Vol. 74, No. 12, pp. 5463–5467, December 1977
Biochemistry

DNA sequencing with chain-terminating inhibitors

(DNA polymerase/nucleotide sequences/bacteriophage φX174)

F. SANGER, S. NICKLEN, AND A. R. COULSON

Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, England

Contributed by F. Sanger, October 3, 1977

ABSTRACT **A new method for determining nucleotide sequences in DNA is described. It is similar to the "plus and minus" method [Sanger, F. & Coulson, A. R. (1975) *J. Mol. Biol.* 94, 441–448] but makes use of the 2′,3′-dideoxy and arabinonucleoside analogues of the normal deoxynucleoside triphosphates, which act as specific chain-terminating inhibitors of DNA polymerase. The technique has been applied to the DNA of bacteriophage φX174 and is more rapid and more accurate than either the plus or the minus method.**

The "plus and minus" method (1) is a relatively rapid and simple technique that has made possible the determination of the sequence of the genome of bacteriophage φX174 (2). It depends on the use of DNA polymerase to transcribe specific regions of the DNA under controlled conditions. Although the method is considerably more rapid and simple than other

a stereoisomer of ribose in which the 3′-hydroxyl group is oriented in *trans* position with respect to the 2′-hydroxyl group. The arabinosyl (ara) nucleotides act as chain terminating inhibitors of *Escherichia coli* DNA polymerase I in a manner comparable to ddT (4), although synthesized chains ending in 3′ araC can be further extended by some mammalian DNA polymerases (5). In order to obtain a suitable pattern of bands from which an extensive sequence can be read it is necessary to have a ratio of terminating triphosphate to normal triphosphate such that only partial incorporation of the terminator occurs. For the dideoxy derivatives this ratio is about 100, and for the arabinosyl derivatives about 5000.

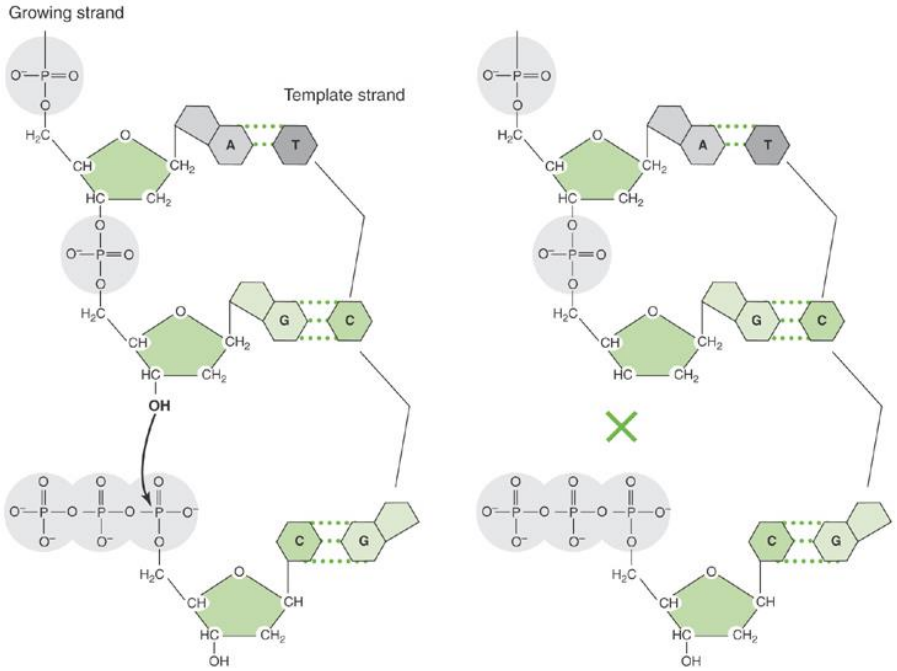
METHODS

Sanger Sequencing

- Uses a mixture of radio-labelled di-deoxy (ddNTP) and dexoy (dNTP) nucleotides to terminate base incorporation as soon as a ddNTP is encountered
- With addition of enzyme (DNA polymerase), the primer is extended until a ddNTP is encountered.
- The chain will end with the incorporation of the ddNTP
- With the proper dNTP:ddNTP ratio (about 100:1), the chain will terminate throughout the length of the template.
- All terminated chains will end in the ddNTP added to that reaction

How is sequencing terminated at each of the 4 bases?

The 3’-OH group necessary for formation of the phosphodiester bond is missing in ddNTPs



Chain terminates at ddG

Sanger sequencing

AGCTGCCCG

Possible fragment lengths

A

ddATP + four dNTPs

ddA
dAdGdCdTdGdCdCdCdG

2

C

ddCTP + four dNTPs

dAdGddC
dAdGdCdTdGddC
dAdGdCdTdGdCddC
dAdGdCdTdGdCdCddC

4

G

ddGTP + four dNTPs

dAddG
dAdGdCdTddG
dAdGdCdTdGdCdCdCddG

3

T

ddTTP + four dNTPs

dAdGdCddT
dAdGdCdTdGdCdCdCdG

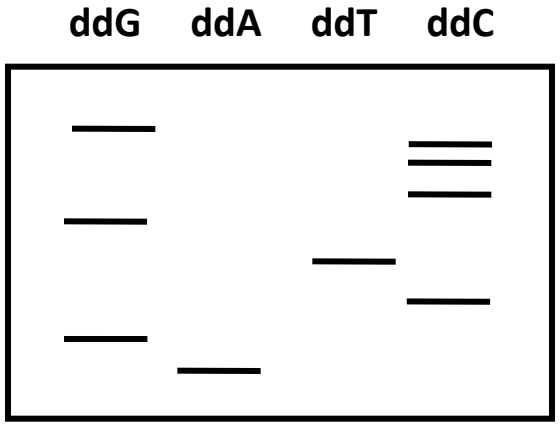
2

Sanger di-deoxy method

AGCTGCCCG

Longer fragments

Shortest fragments



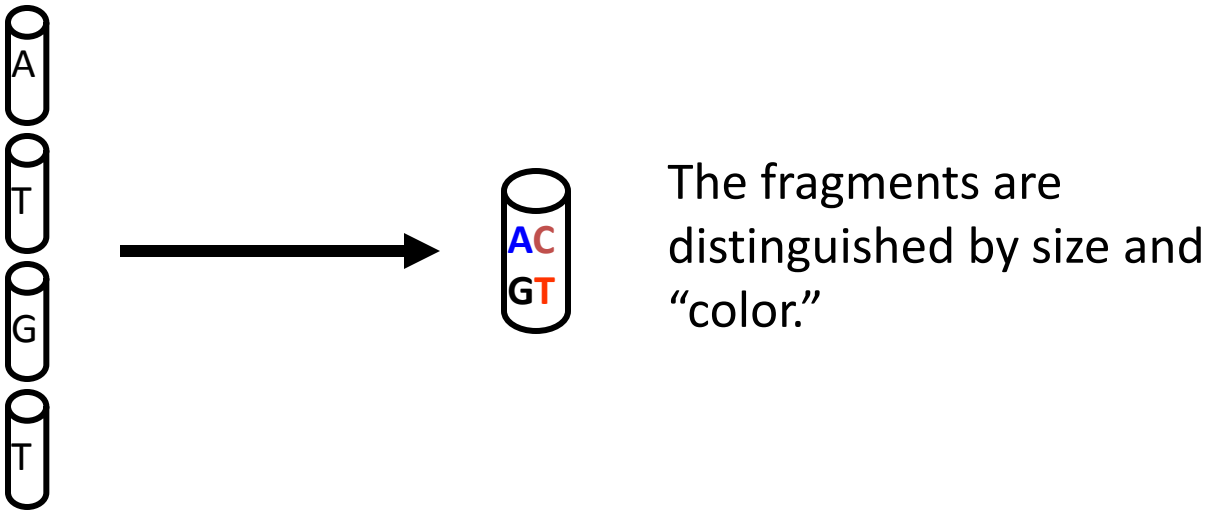
3′
G
C
C
C
G
T
C
G
A
5′

1985: Automating Sanger Sequencing

- Disadvantages of Sanger sequencing
  - Labour intensive
  - Used radioactive labels
  - Interpretation/analysis was subjective
- Difficult to scale up
- Leroy Hood, Michael Hunkapiller developed an automated method utilising:
  - Fluorescent labels instead of radioactivity
  - Utilise computerised algorithms to analyse data
  - Robotics
- Development of PCR by Kary Mullis (NGS would be impossible without it)

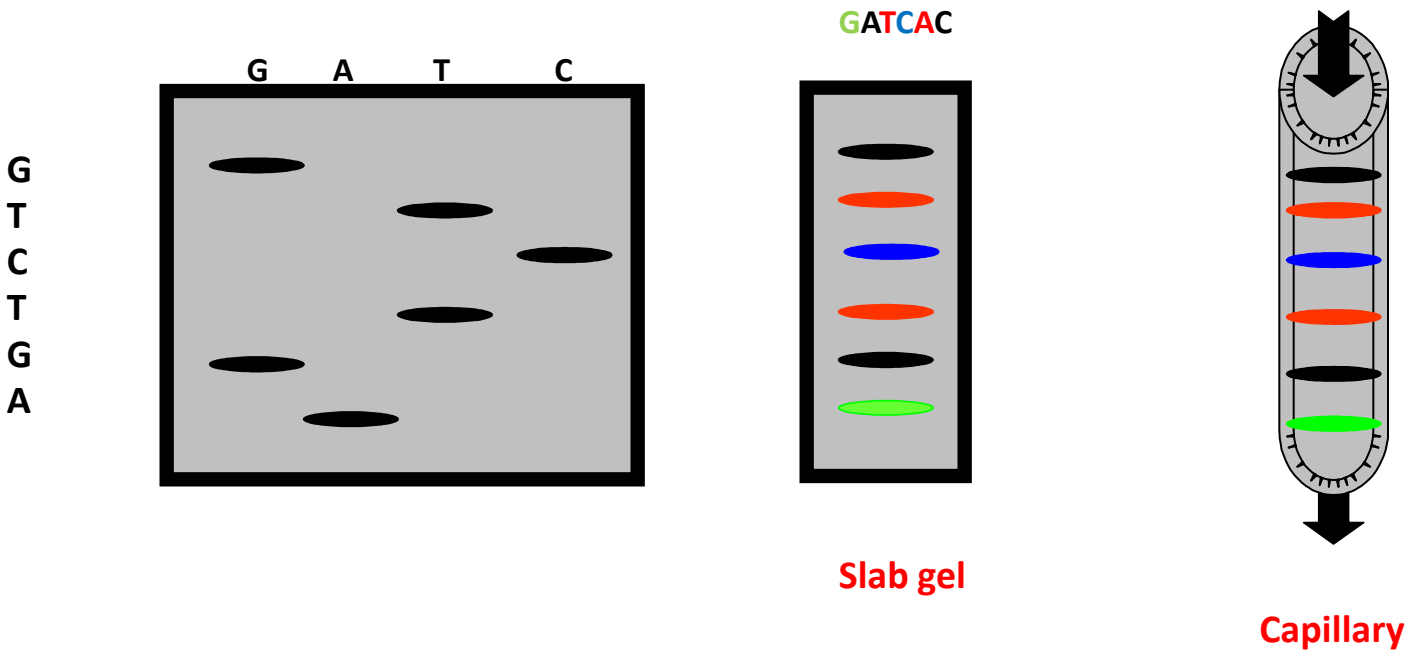
Dye Terminator Sequencing

- A distinct dye or “color” is used for each of the four ddNTP.
- Since the terminating nucleotides can be distinguished by color, all four reactions can be performed in a single tube.



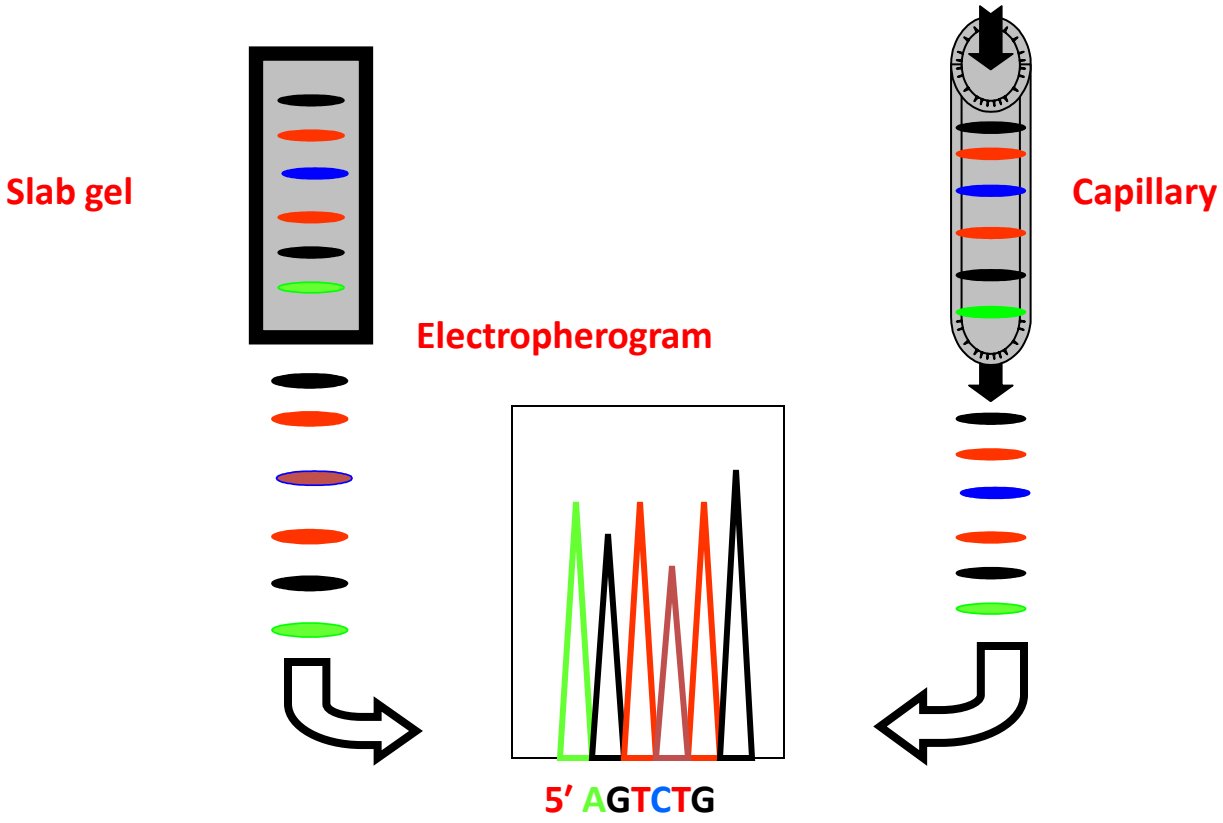
Dye Terminator Sequencing

The DNA ladder is resolved in one gel lane or in a capillary



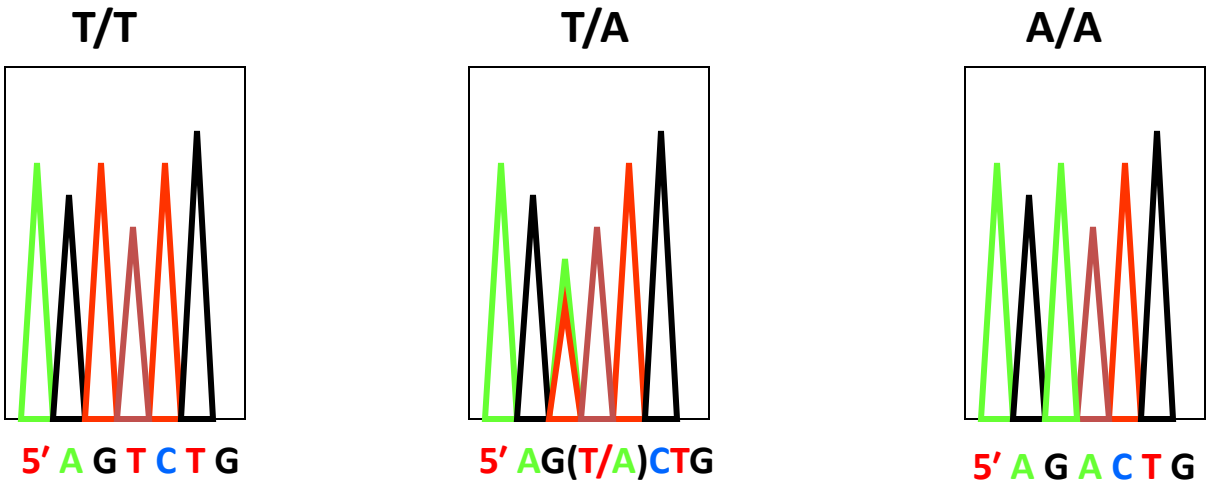
Dye Terminator Sequencing

- The DNA ladder is read on an electropherogram.



Automated Sequencing

- Dye primer or dye terminator sequencing on capillary instruments.
- Sequence analysis software provides analyzed sequence in text and electropherogram form.
- Peak patterns reflect mutations or sequence changes.



Sanger Sequencing
Useful videos

- http://www.youtube.com/watch?v=91294ZAG2hg&feature=related
- http://www.youtube.com/watch?v=bEFLBf5WEtc&feature=fvwrel

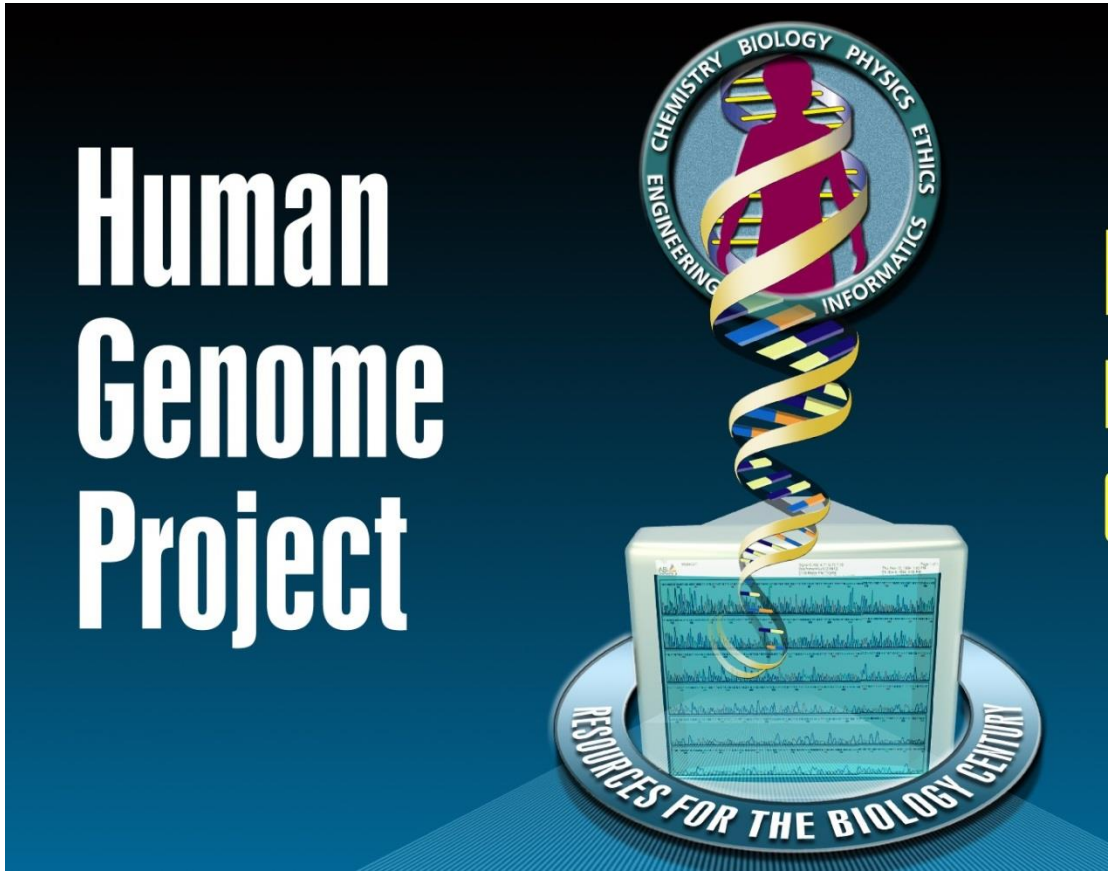
Features of Sanger Sequencing

- 96-384 sequences per run
- 500bp-1kb read lengths
- $100 per megabase
- Accuracy decreases with length (99.999% at 500bp down to 99% at 900bp)
- Still the most accurate technique for sequencing

Limitations of Sanger Sequencing

- Cloning/Sub-cloning
  - DNA must be compatible with biological machinery of host cells and can introduce bias
  - Labour and/or machines to prepare clones requires significant capital
- Difficult to distinguish allele frequency
  - Especially if not in 1:1 ratio
- Cost
  - $10,000,000 to sequence a 1Gbase genome to 10x coverage

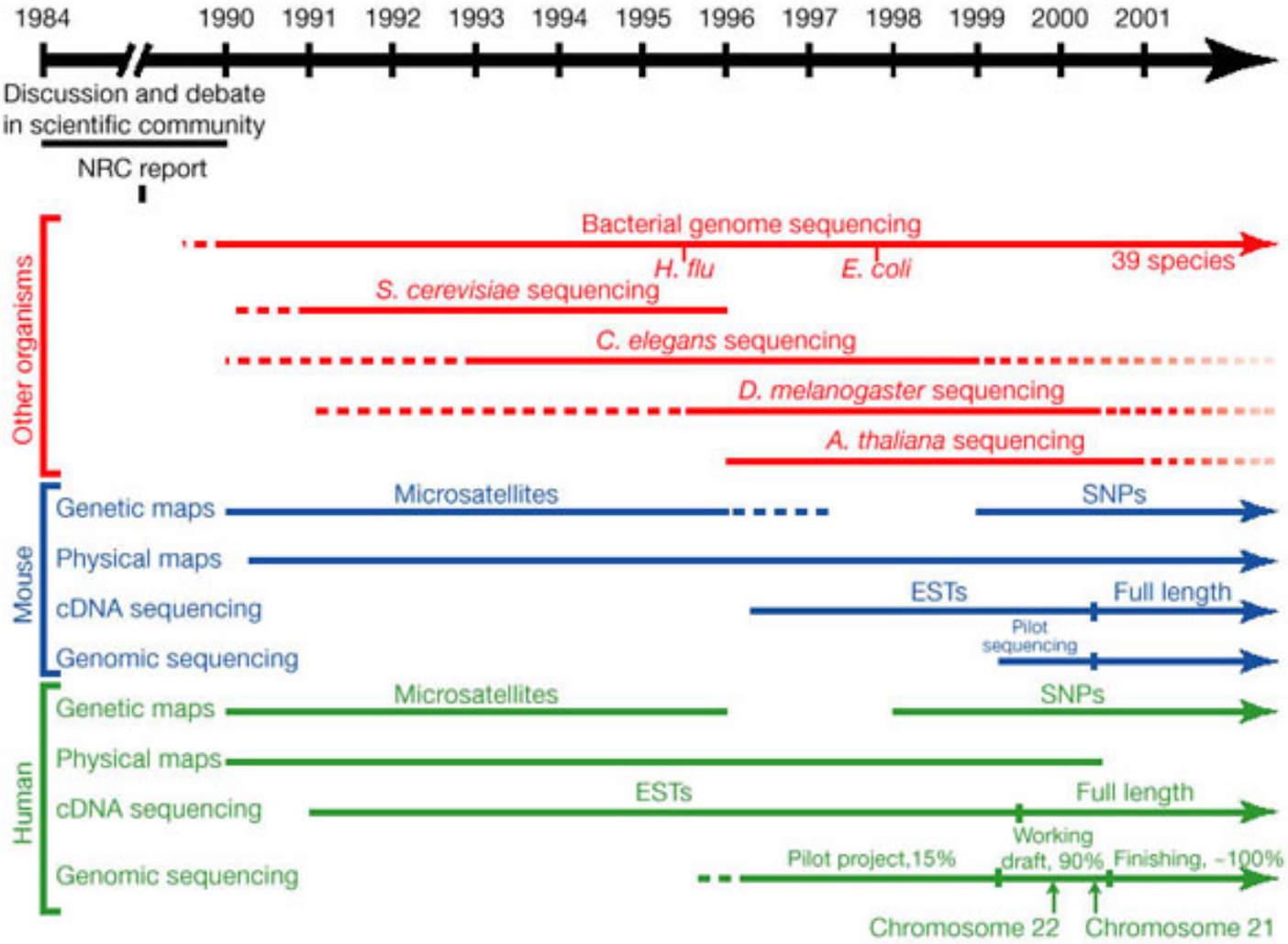
Human genome project



Human Genome Project

- One of the largest scientific endeavors
  - Target accuracy 1:10,000 bases
  - Started in 1990 by DoE and NIH
  - $3Billion and 15 years
  - Goal was to identify 25K genes and 3 billion bases
- Used the Sanger sequencing method
- Draft assembly done in 2000, complete genome by 2003, last chromosome published in 2006
- Still being improved

Human Genome Project

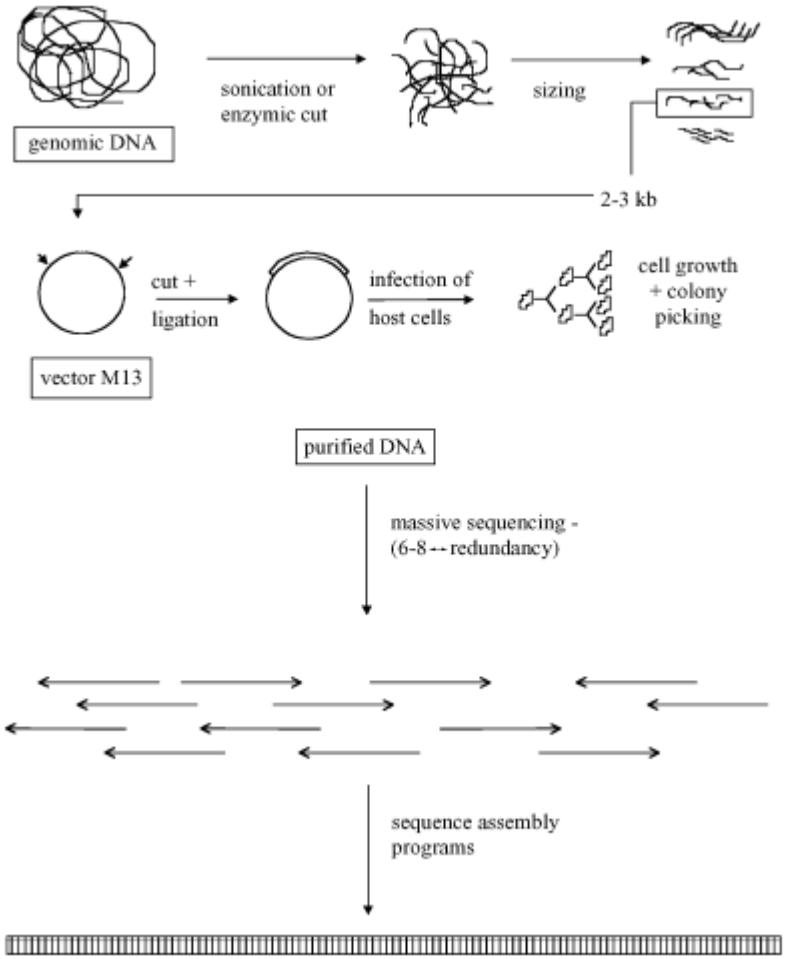


http://bit.ly/q3Qsd5

How it was Accomplished

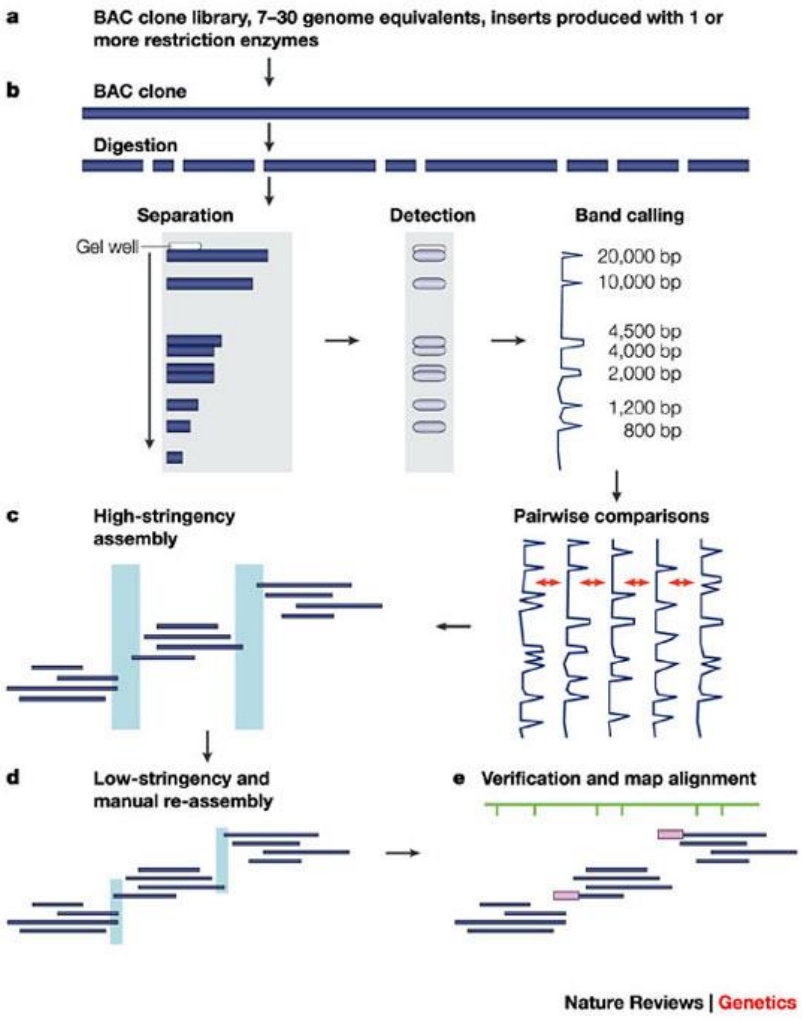
- Public Project
  - Hierarchical shotgun approach
  - Large segments of DNA were cloned via BACs and located along the chromosome
  - These BACs where shotgun sequenced
- Celera
  - Pure shotgun sequencing
  - Used public data (released daily) to help with assembly

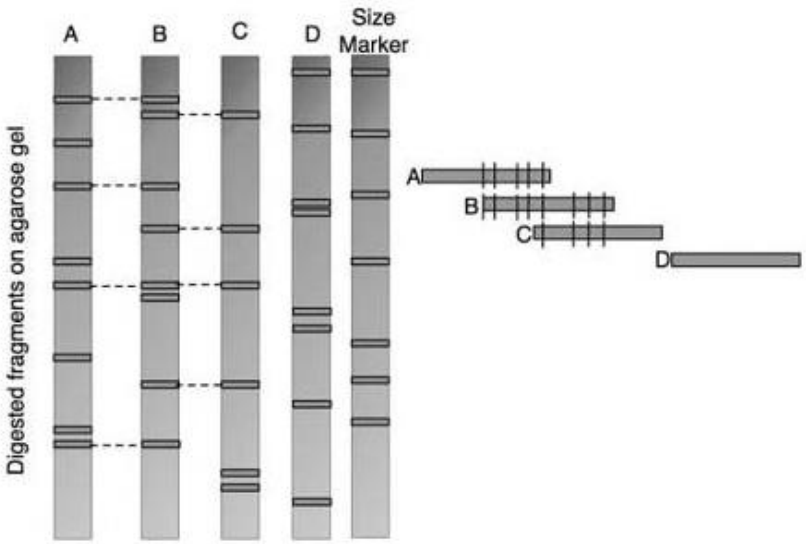
Method 1: Hierarchical Sequencing



http://www.icb.uncu.edu.ar/upload/dnasequencing.pdf

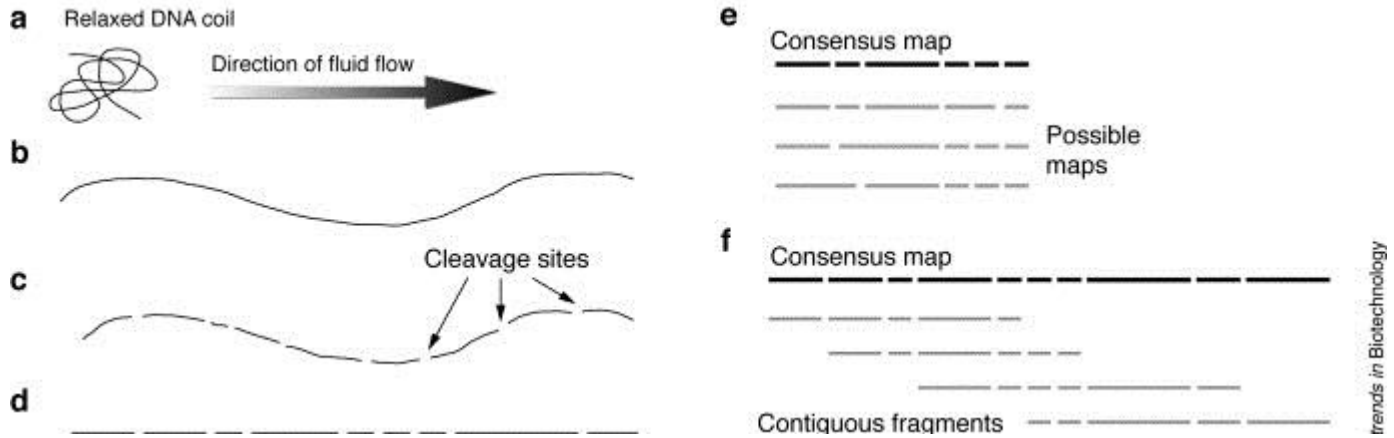
Using Bacterial artificial chromosomes (BACs) to aid assembly





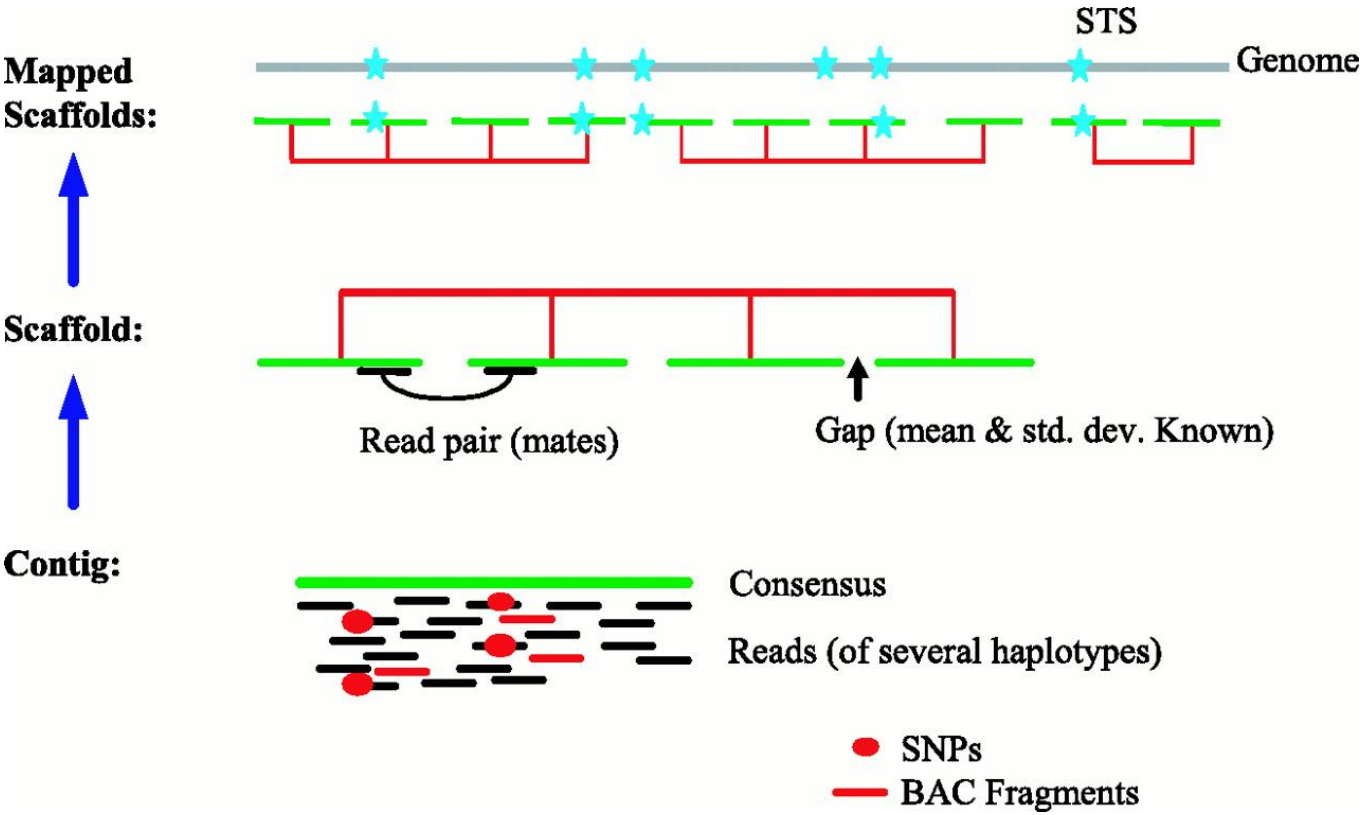
Nature Reviews Genetics **5**, 578-588 (August 2004) | doi:10.1038/nrg1404

Using optical mapping approaches to aid assembly



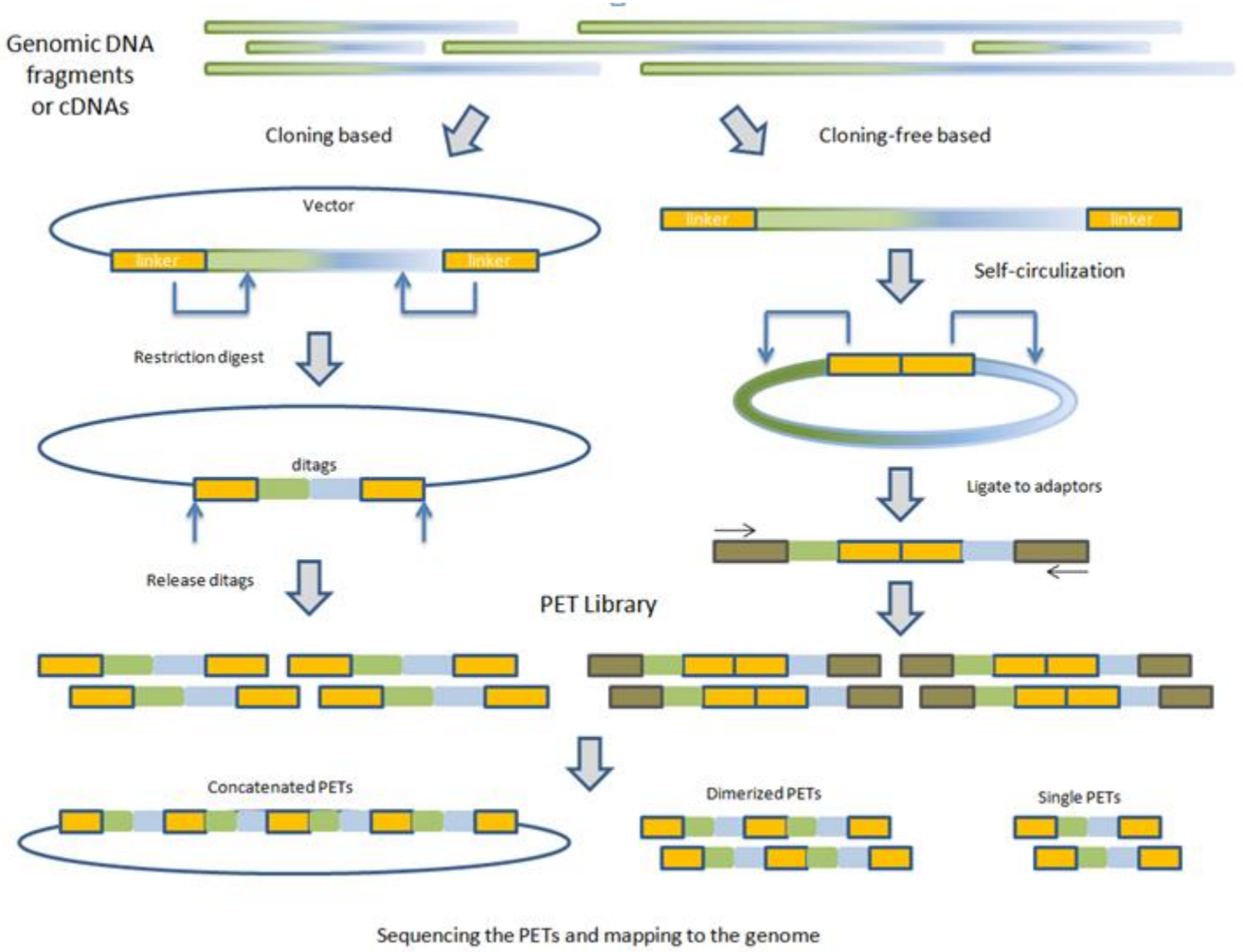
Christopher Aston, Bud Mishra, David C Schwartz, Optical mapping and its potential for large-scale sequencing projects, Trends in Biotechnology, Volume 17, Issue 7, 1 July 1999, Pages 297-302, ISSN 0167-7799, http://dx.doi.org/10.1016/S0167-7799(99)01326-8

Method 2: Celera Shotgun Sequencing

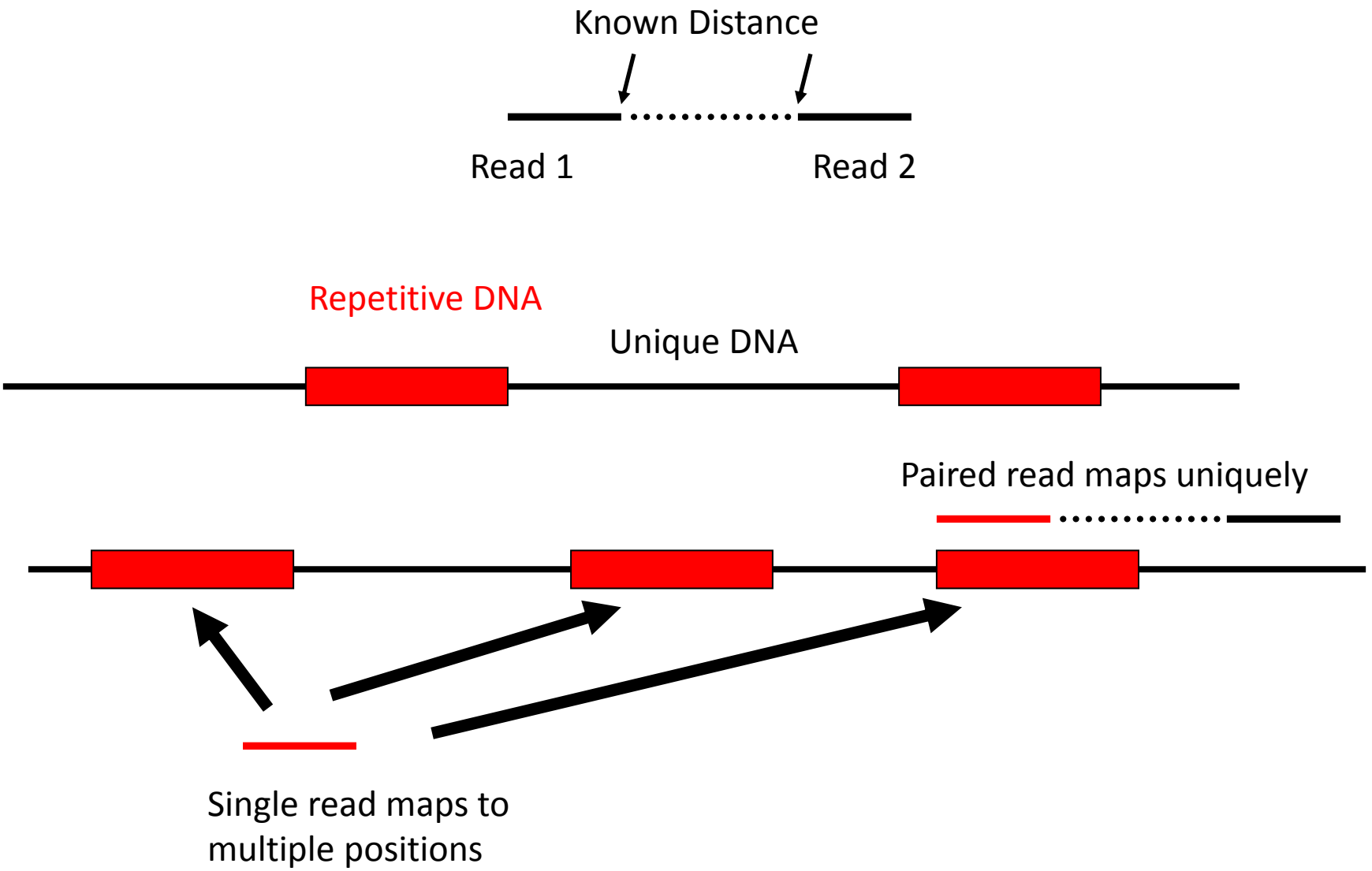


- Used paired-end strategy with variable insert size: 2, 10, and 50kbp

Paired-end sequencing

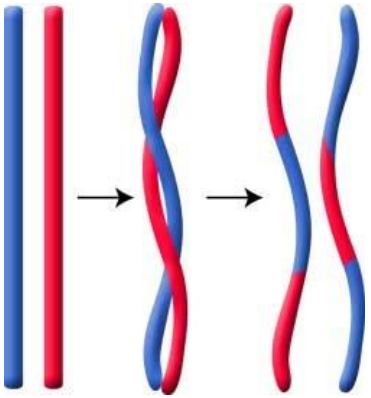


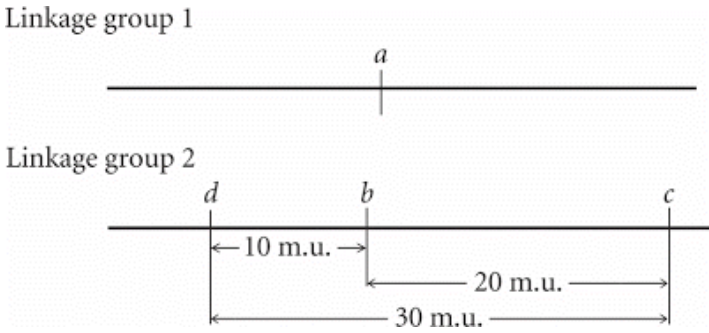
Paired-end reads are important



Using genetic maps to aid assembly

- Calculate recombination frequencies for microsatellites or sequence-tag-sites
- Can help to order assembled sequence scaffolds





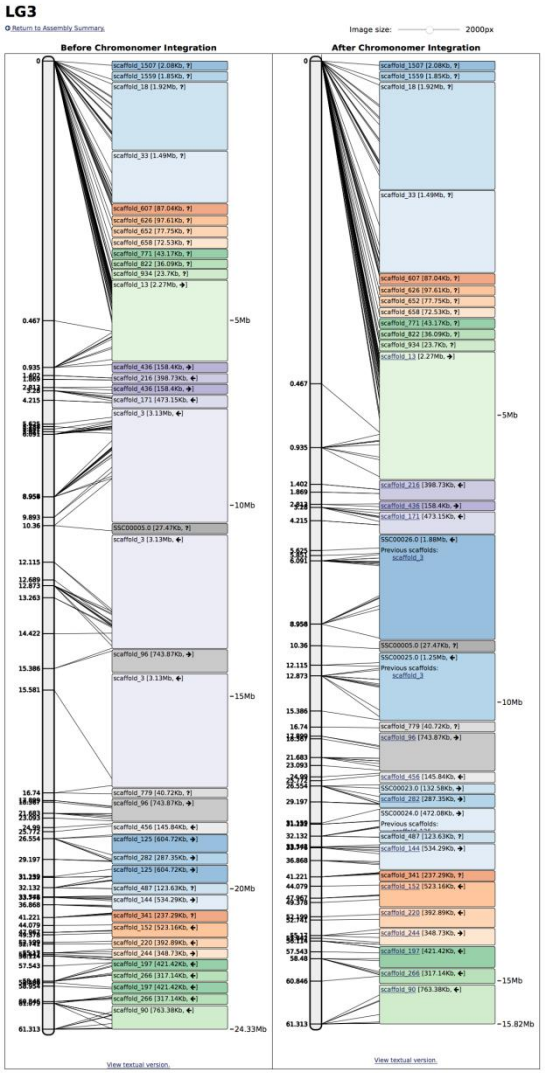
https://sites.google.com/site/geneticmappingwiki/linkage_maps

Dib et al. *Nature* **380**, 152 - 154 (14 March 1996); doi:10.1038/380152a0

Chromonomer – Improving genetic maps for the NGS era

- Takes RAD and/or other marker data
- Assembly AGP file
- Alignment of markers to assembly (SAM/BAM)

- Re-order and if necessary break scaffolds to ensure assemblies are consistent with genetic map

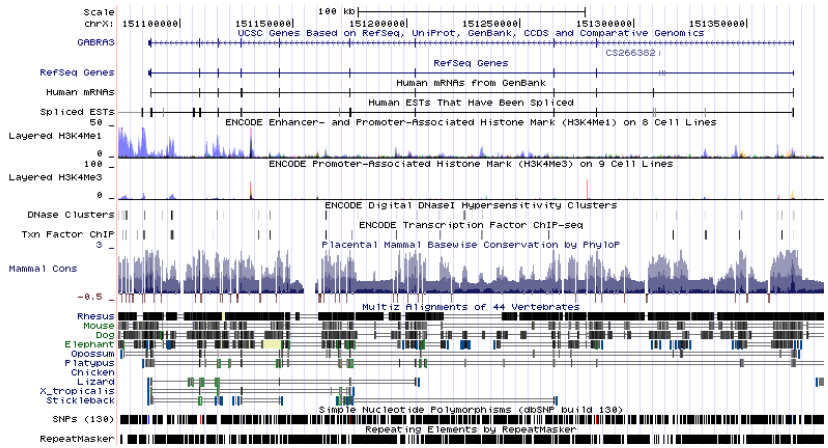


Amores, A., Catchen, J., Nanda, I., Warren, W., Walter, R., Schartl, M., & Postlethwait, J. H. (2014). *A RAD-Tag Genetic Map for the Platyfish (Xiphophorus maculatus) Reveals Mechanisms of Karyotype Evolution Among Teleost Fish*. **Genetics**, 197(2), 625–641.

Outcome of the HGP

- Spurred the sequencing of other organisms
  - 36 “complete” eukaryotes (~250 in various stages)
  - 1704 “complete” microbial genomes
  - 2685 “complete” viral genomes
- Enabled a multitude of related projects:
  - Encode, modEncode
  - HapMap, dbGAP, dbSNP, 1000 Genomes
  - Genome-Wide Association Studies, WTCCC
  - Medical testing, GeneTests, 23AndMe, personal genomes
  - Cancer sequencing, COSMIC, TCGA, ICGC
- Provided a context to organize diverse datasets

HGP Data Access

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       61 tttgtctatc aaaatgtaaa atgtgagcgg gcacaatggt ccaacgcctg taatcccagc
      121 actttcggag gccgaggcgg gtggatcacc tgaggtcagg agttggagac cagcctggcc
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chromFa.tar.gz	20-Mar-2009 09:21	905M	
chromFaMasked.tar.gz	20-Mar-2009 09:30	477M	
chromOut.tar.gz	20-Mar-2009 09:03	163M	
chromTrf.tar.gz	20-Mar-2009 09:30	7.6M	
est.fa.gz	11-Aug-2011 10:57	1.4G	
est.fa.gz.md5	11-Aug-2011 10:57	44	
hg19.2bit	08-Mar-2009 15:29	778M	
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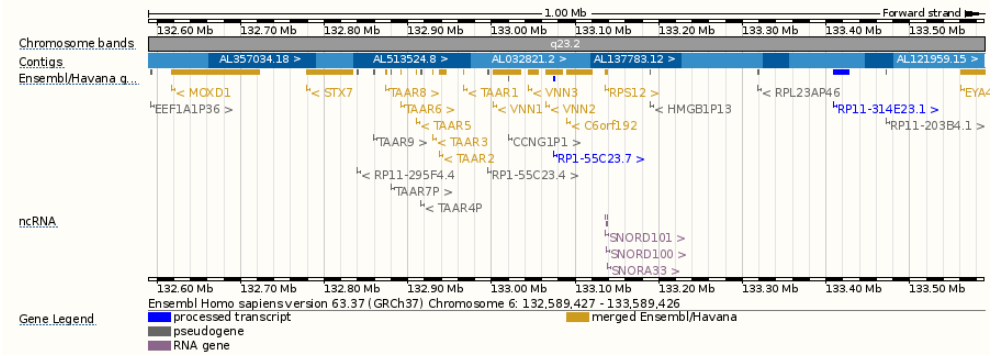
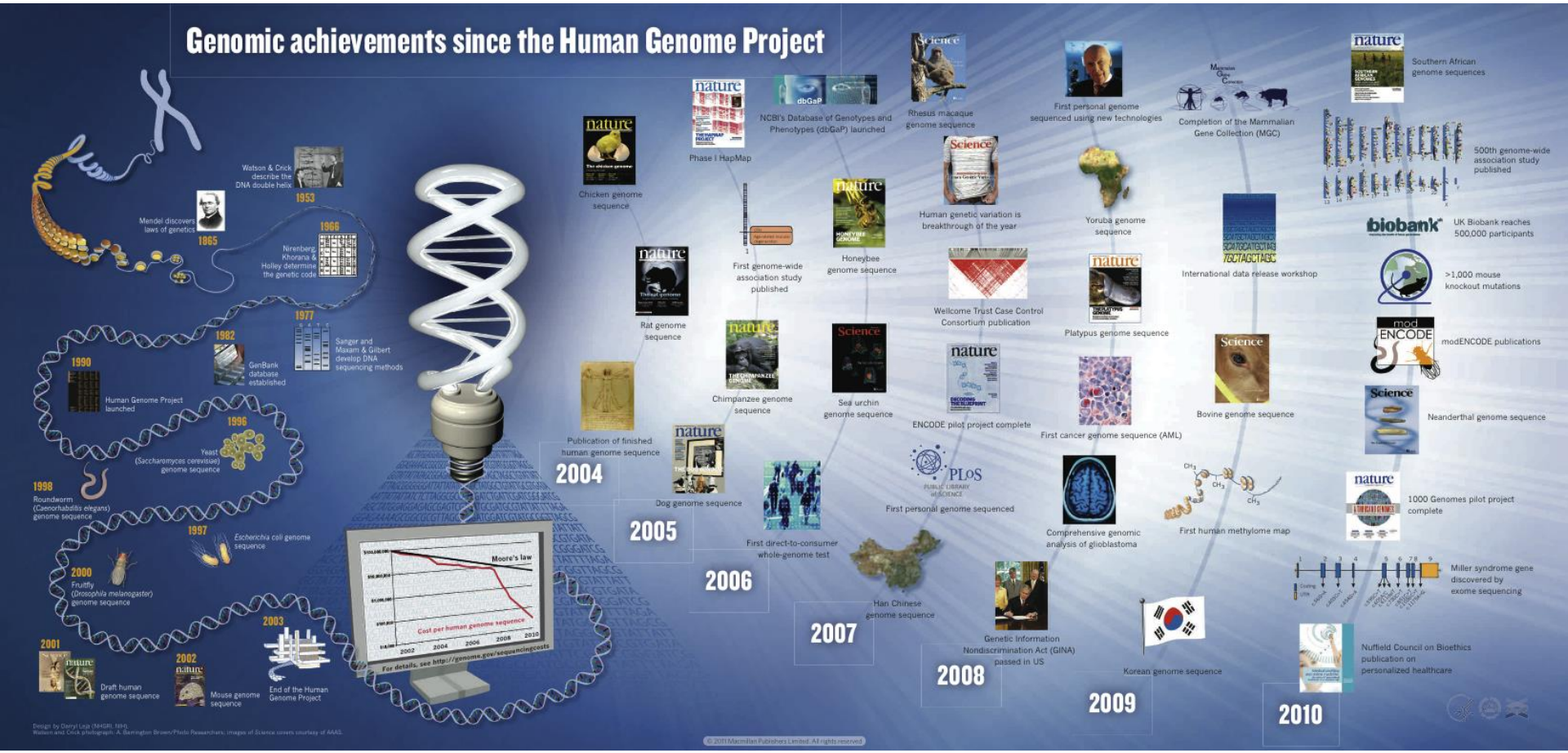


Results in GenBank, UCSC, Ensembl & others

Achievements Since the HGP



ED Green *et al. Nature* 470, 204-213 (2011) doi:10.1038/nature09764

Economic Impact of the Project

- Battelle Technology Partnership Practice released a study in May 2011 that quantifies the economic impact of the HGP was **$796 billion!**
- Genomics supports:
  - >51,000 jobs
  - Indirectly, 310,000 jobs
  - Adds at least $67 billion to the US economy

http://www.genome.gov/27544383

2004 onwards:
Beyond 1 species, 1 genome

- Cost of producing a single genome could vary from $100,000s to $10s of millions using capillary sequencers
- Labour intensive methodology

- New methods were required to lower the overall cost per genome

Second generation short read technologies

Large scale sequencing 2006

PRODUCTION

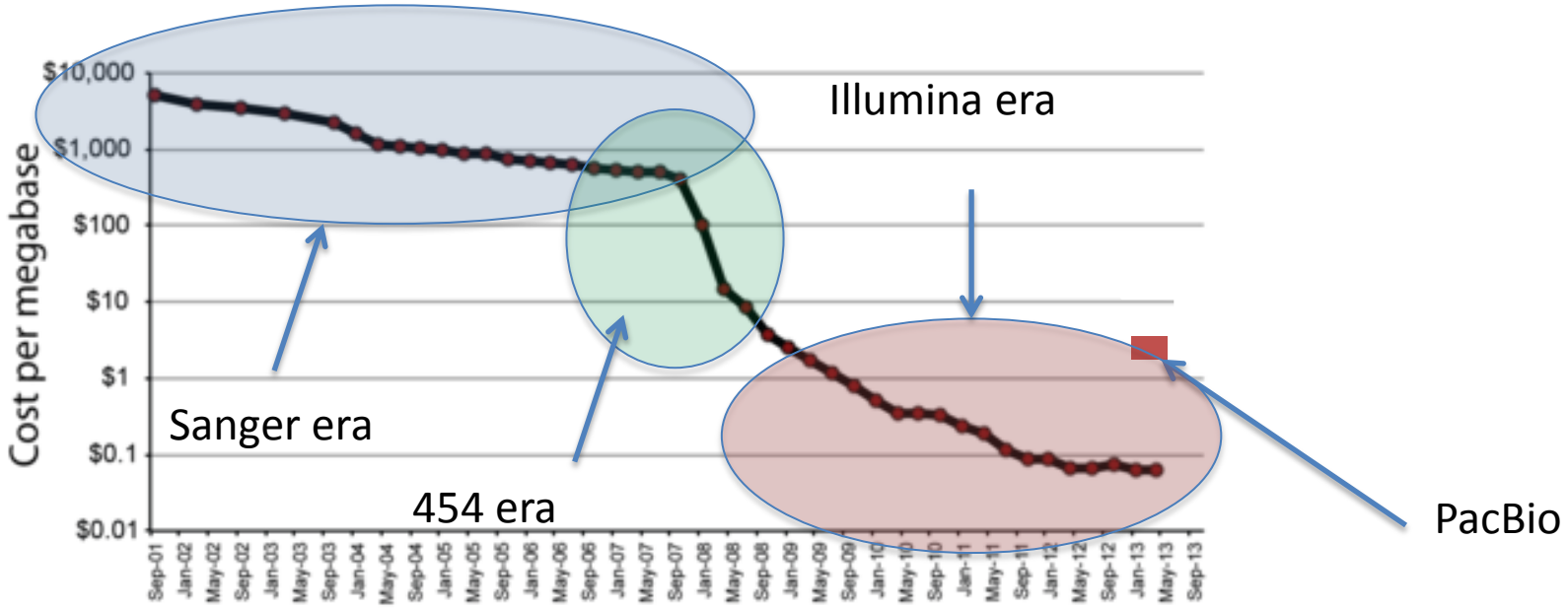
Rooms of equipment
Subcloning > picking > prepping
35 FTEs
3-4 weeks

SEQUENCING

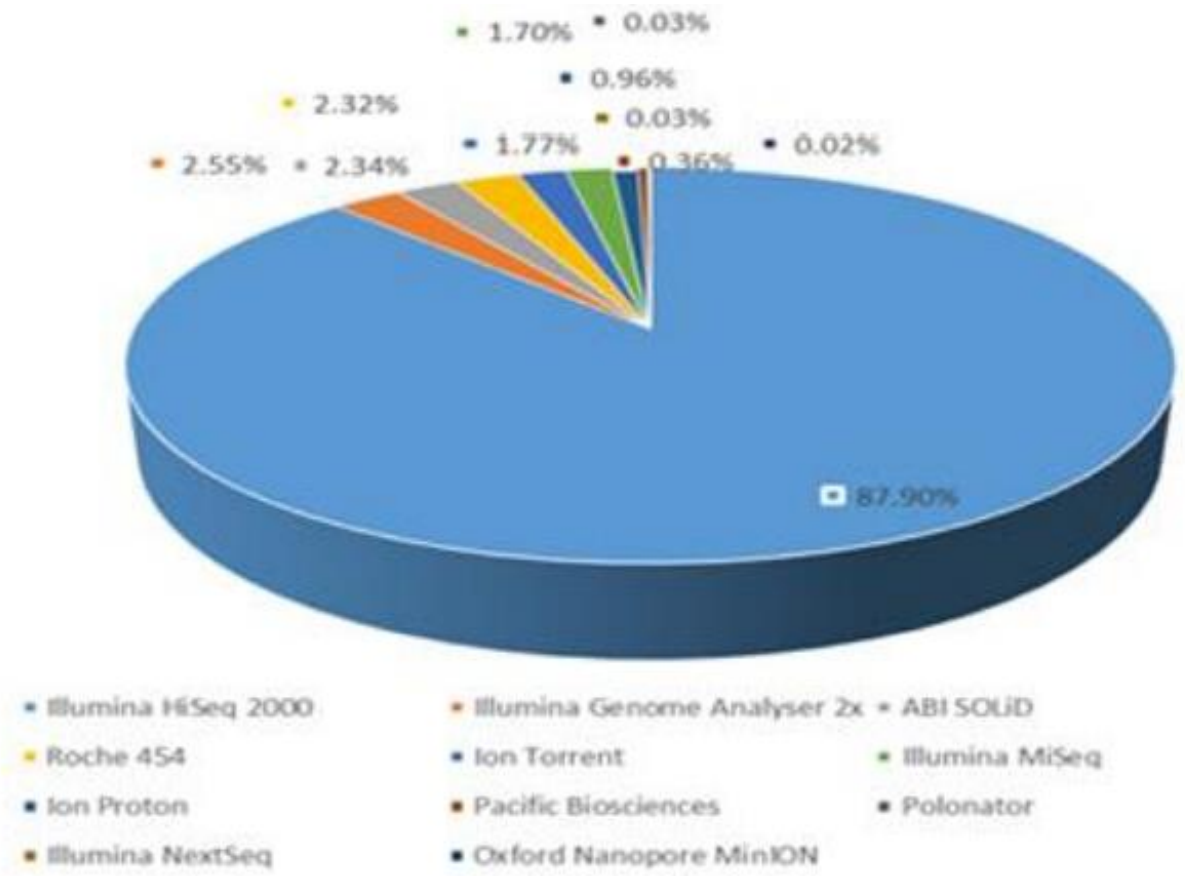
74x Capillary Sequencers
10 FTEs
15-40 runs per day
1-2Mb per instrument per day
120Mb total capacity per day

Key advantages over Sanger Sequencing

- Hugely reduced labour requirements
  - No need to perform cloning
- Reduced Cost per sequence
- Reduced time to result
- Decentralisation



Worldwide sequencers



http://www.gosreports.com/global-sequencing-instrument-industry-2015-market-research-report/

Fun fact

- Clive Brown
- Formerly director of Computational Biology at Solexa (Illumina)
- Chief Technology Officer at Oxford Nanopore

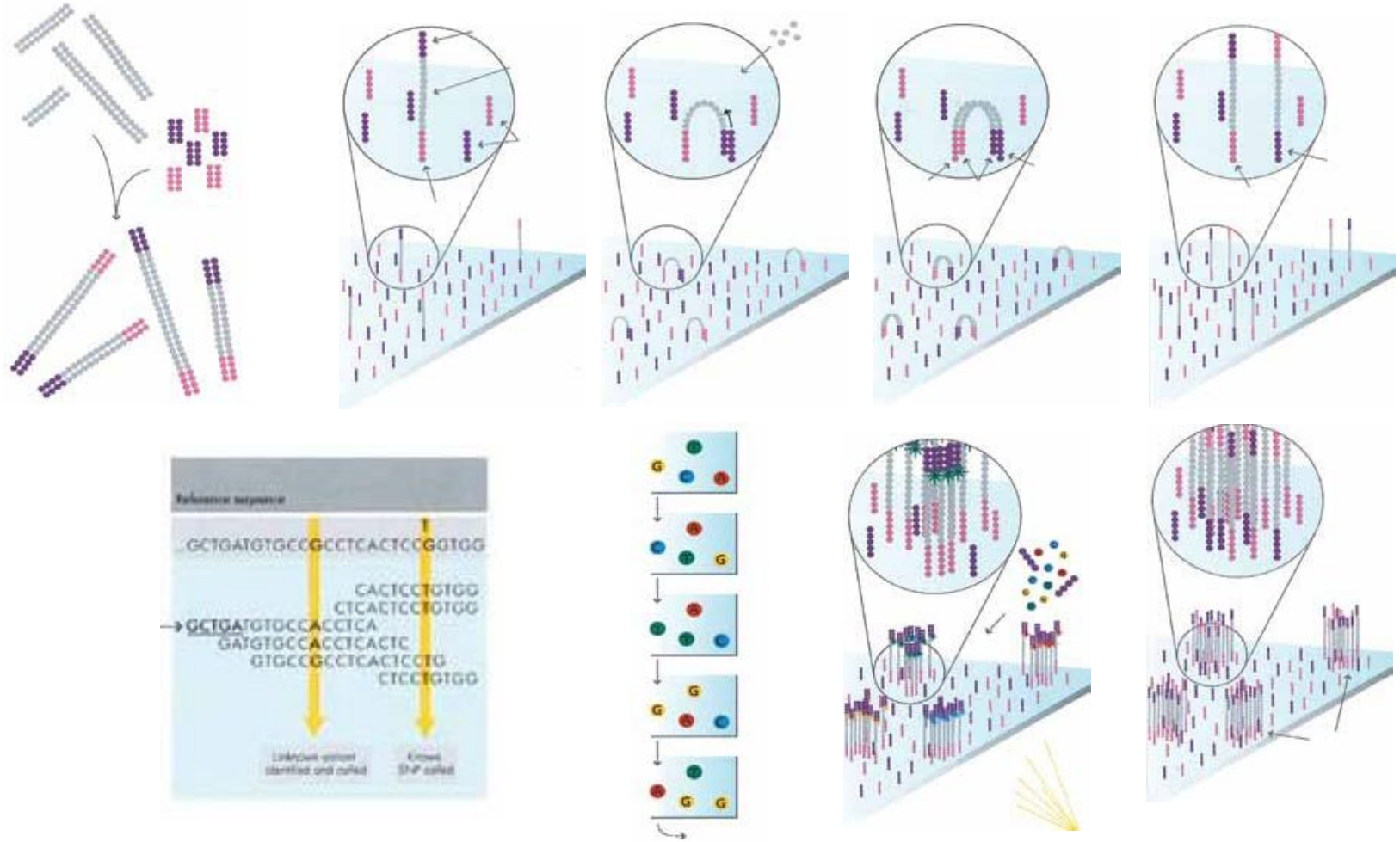
Illumina Sequencing By Synthesis

Illumina HiSeq

Illumina NextSeq

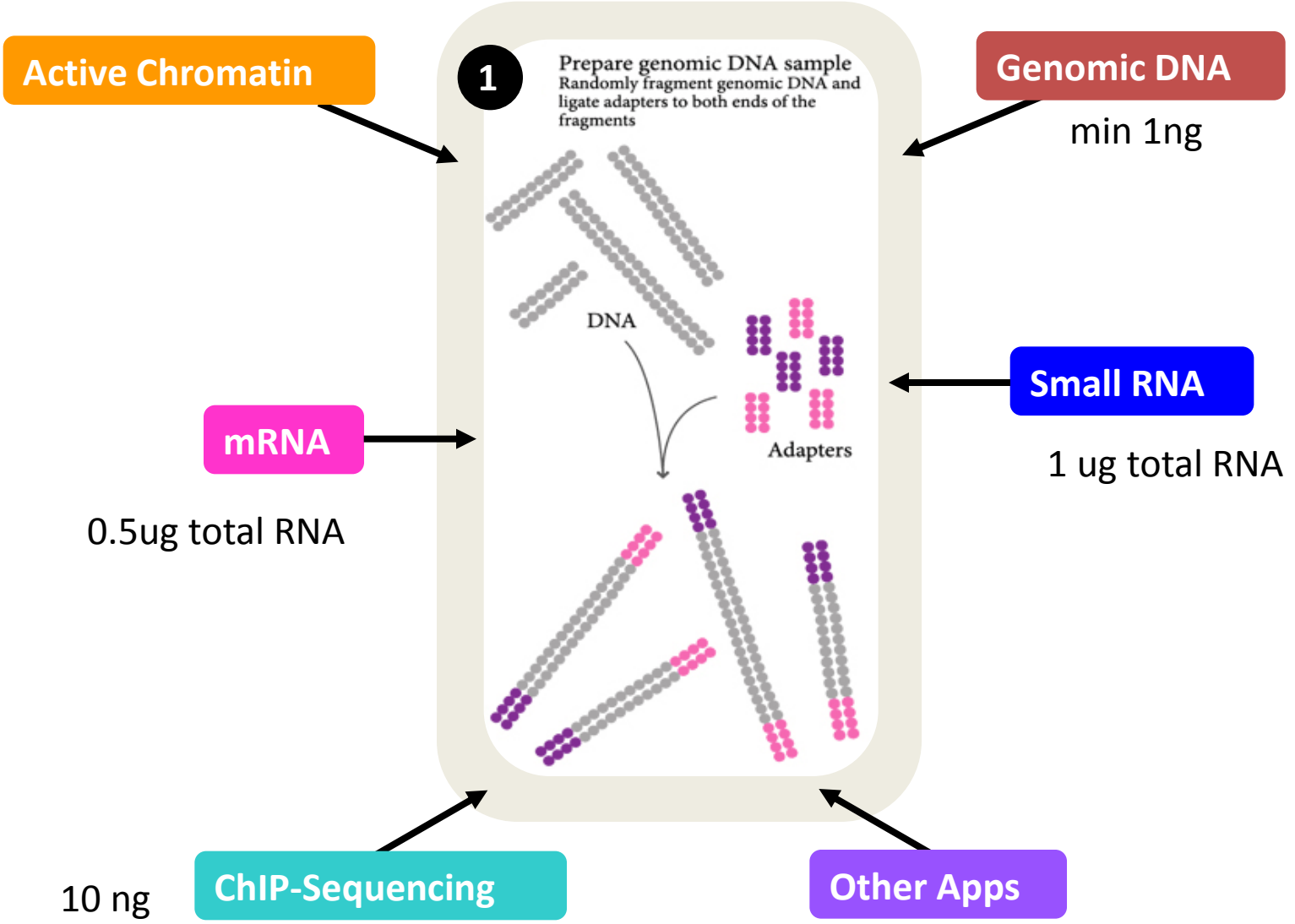
Illumina MiSeq

Illumina Sequencing

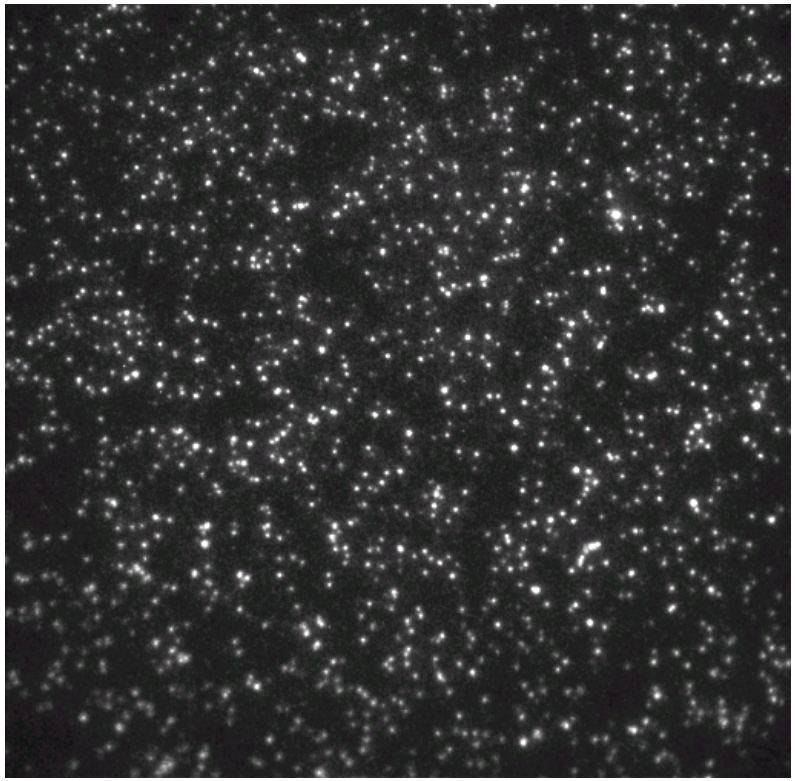


http://www.illumina.com/Documents/products/techspotlights/techspotlight_sequencing.pdf

Step 1: Sample Preparation



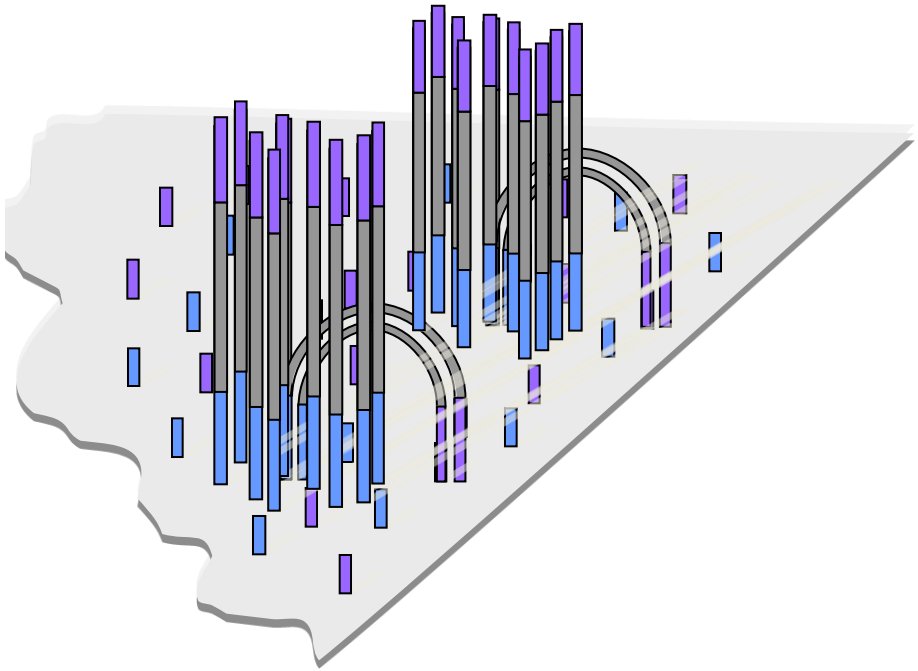
Step 2: Clonal Single Molecule Arrays



100um

Random array of clusters

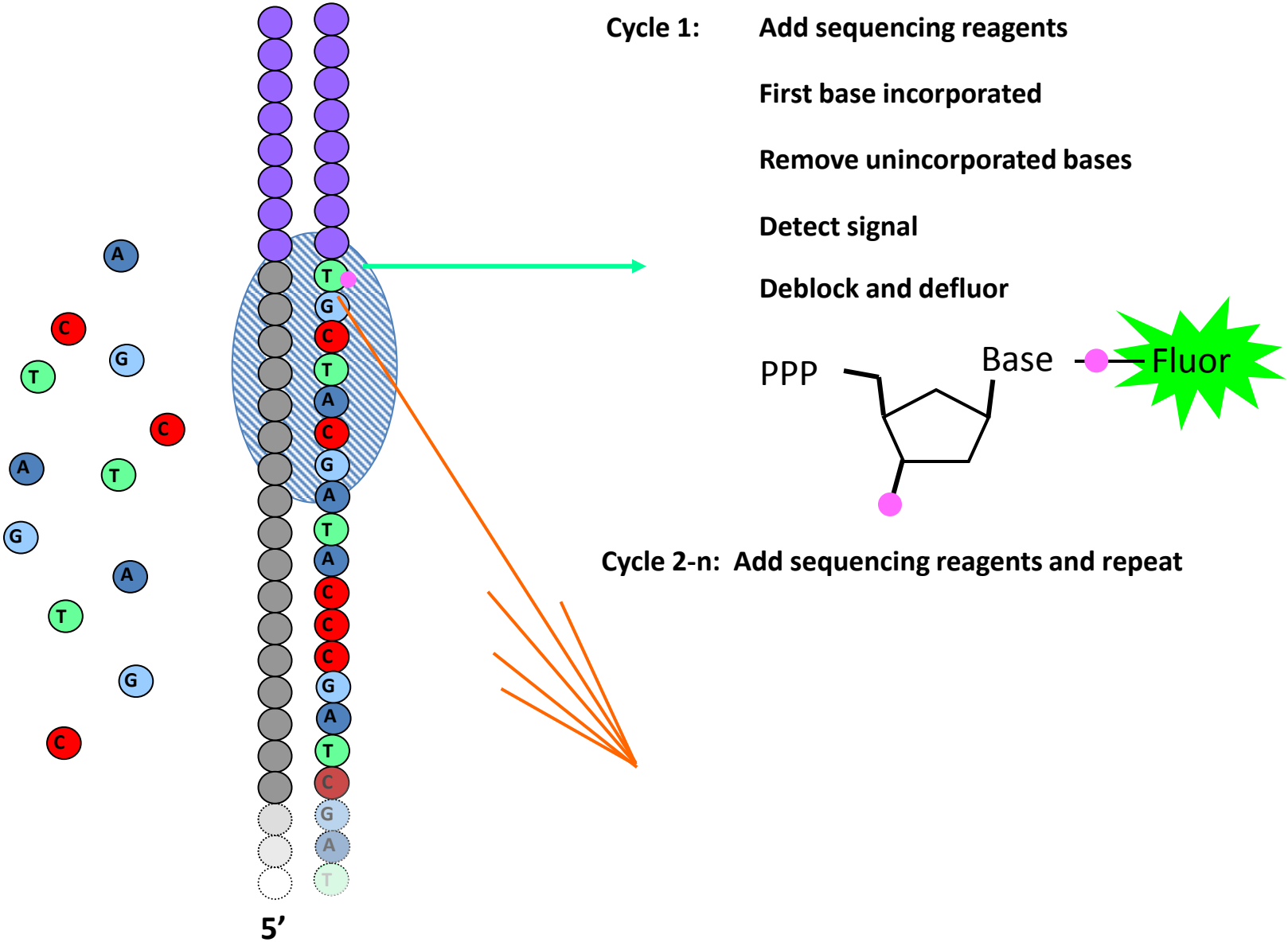
Attach single molecules to surface
Amplify to form clusters



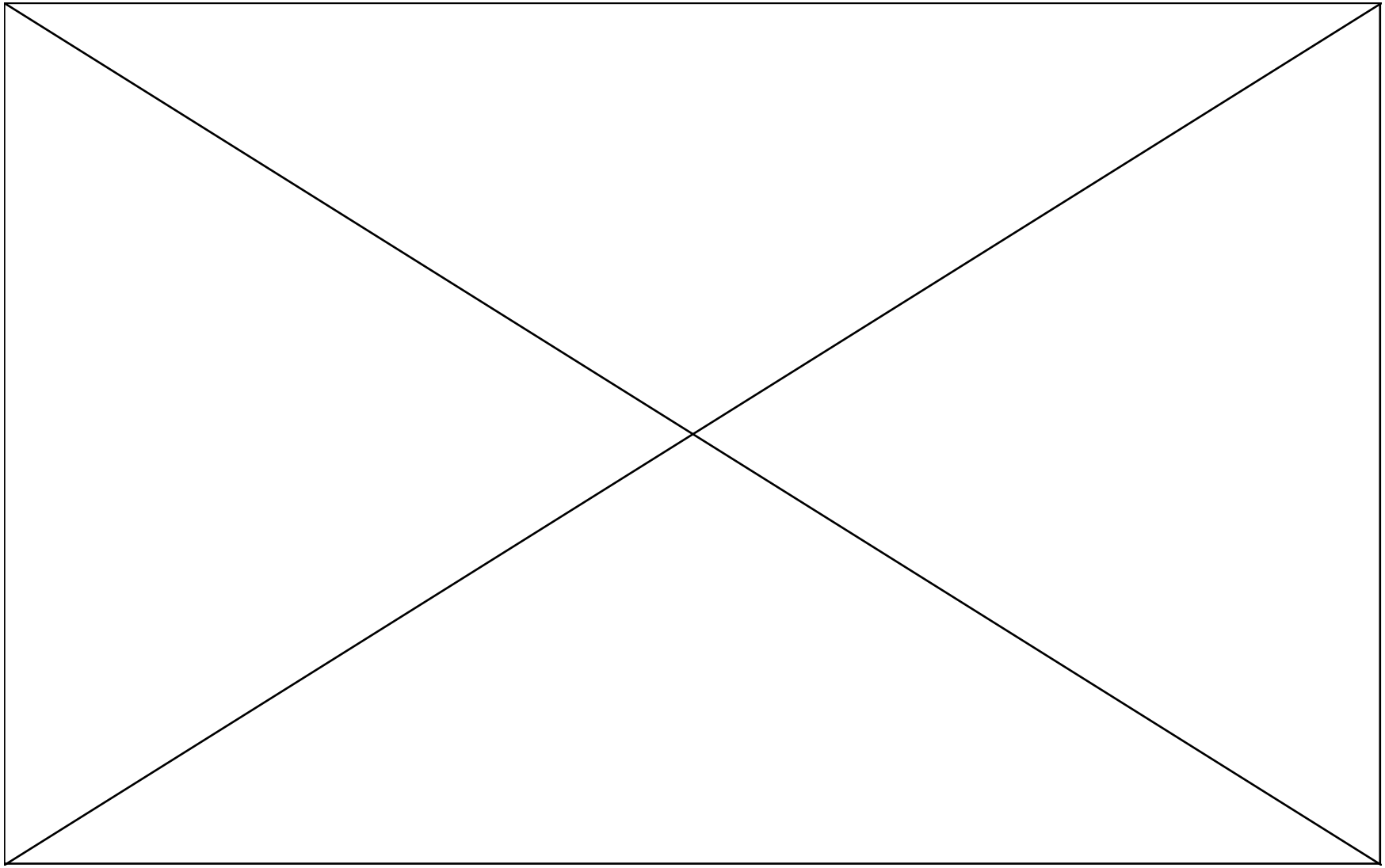
~1000 molecules per ~ 1 um cluster
~2 billion clusters per flowcell

1 cluster = 1 sequence

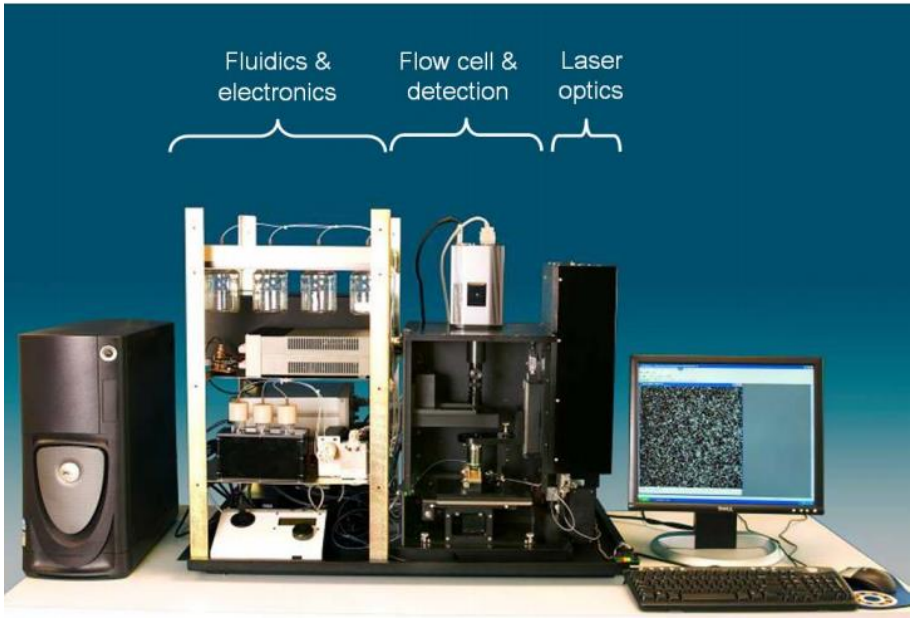
Step 3: Sequencing By Synthesis (SBS)

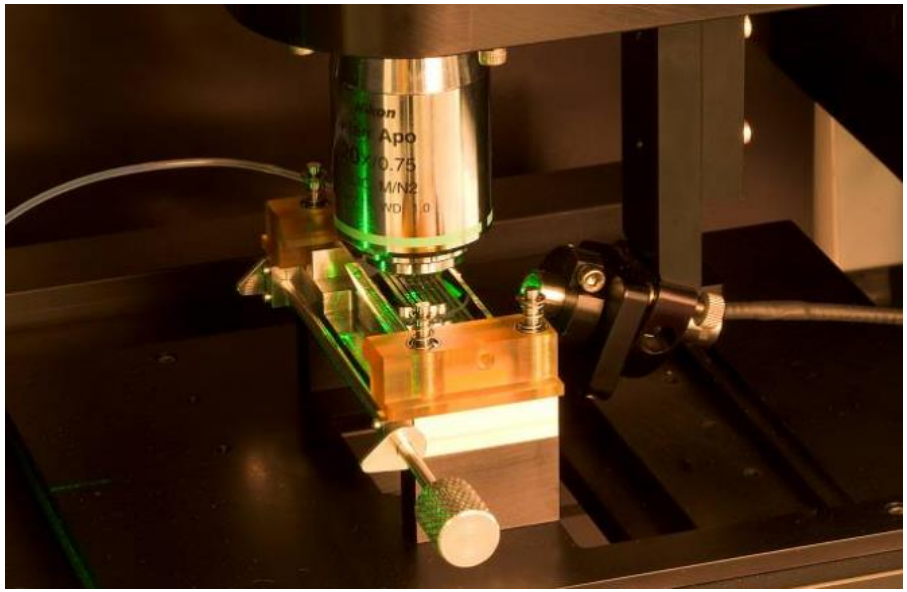


Illumina video

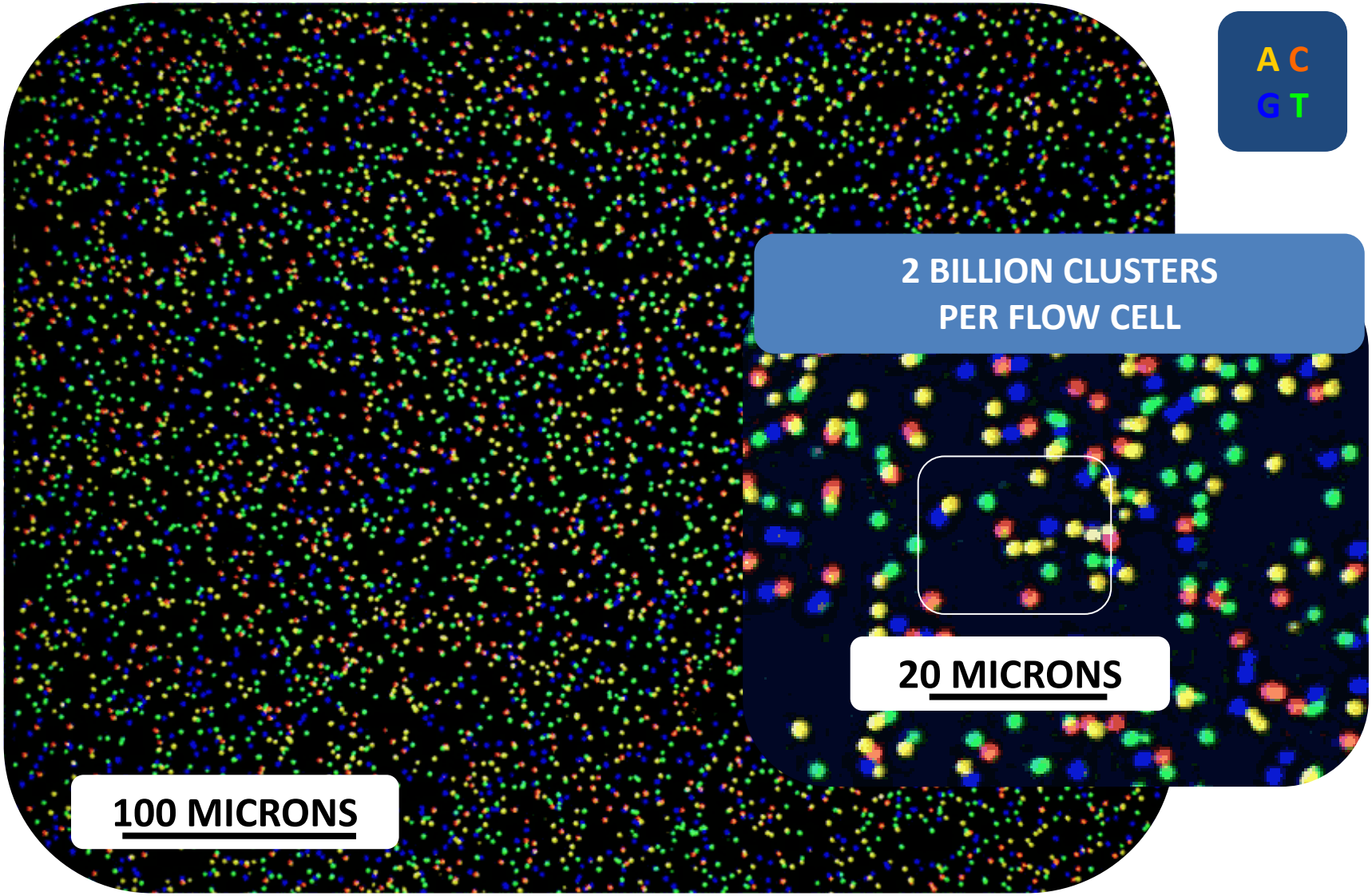


Under the hood:

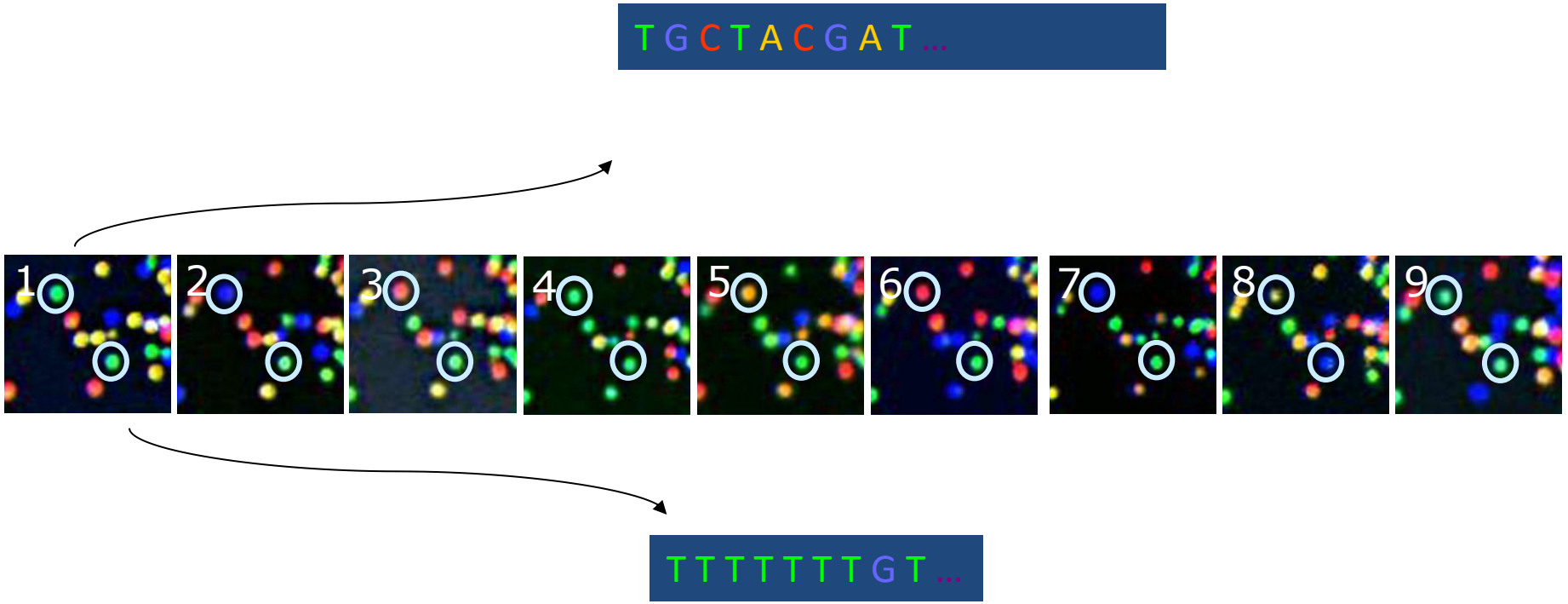




Illumina Sequencing : How it looks

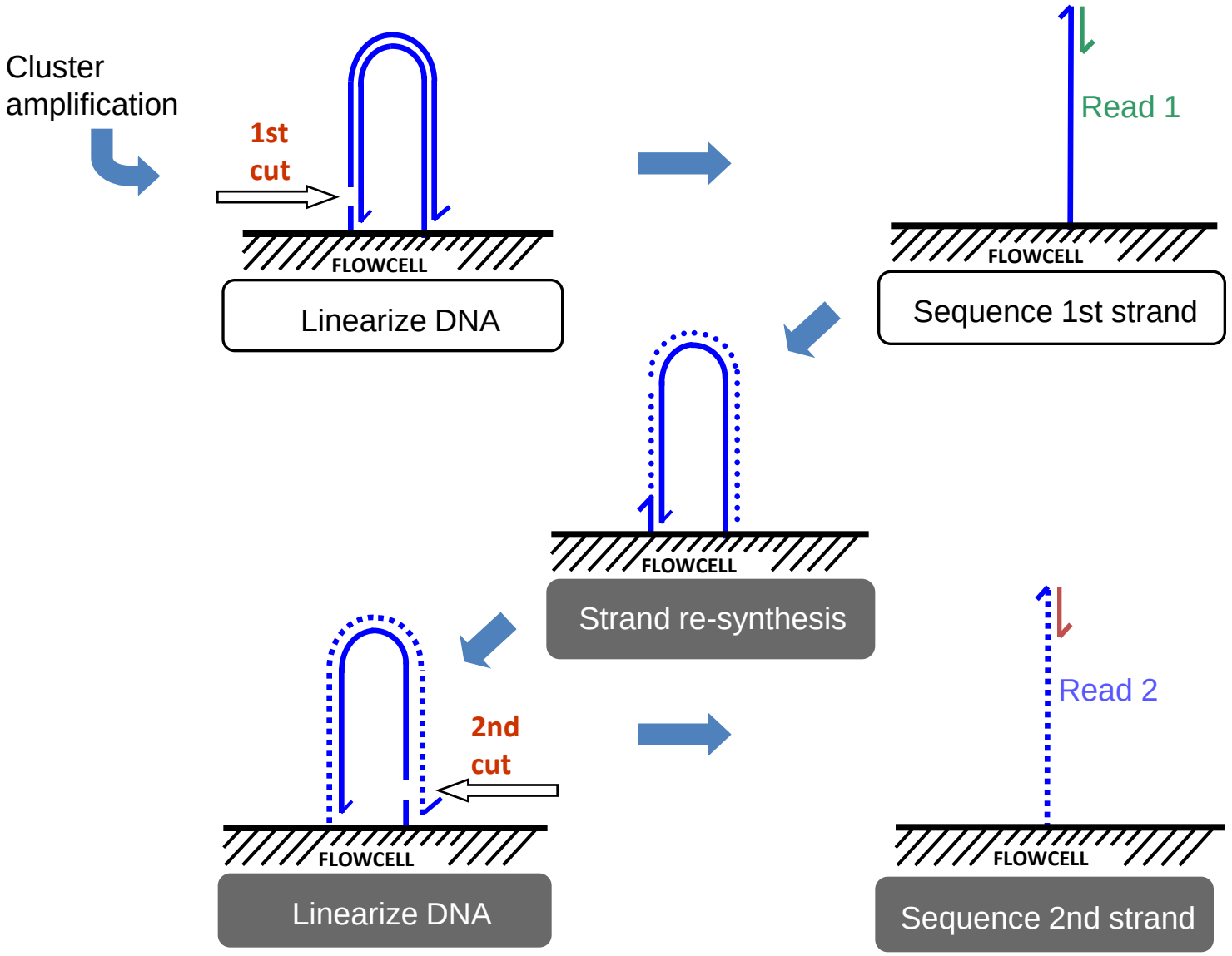


Base calling from raw data



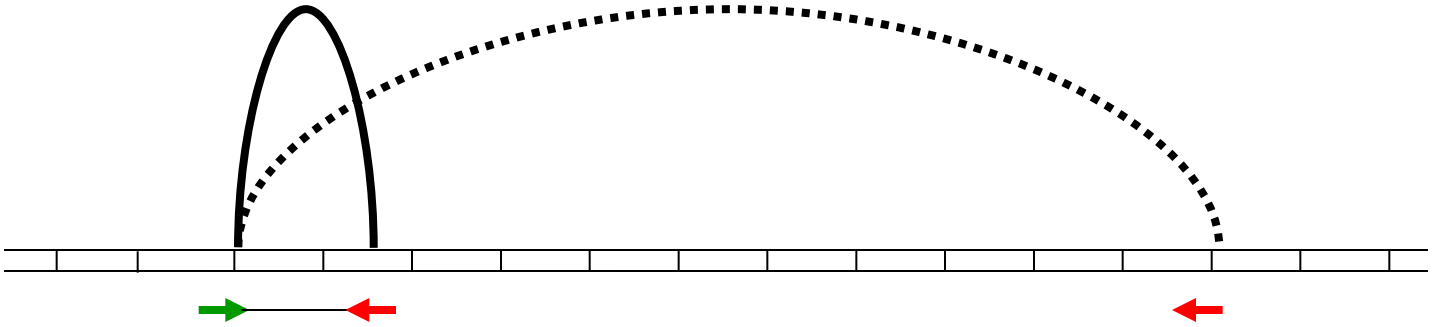
The identity of each base of a cluster is read off from sequential images.

Illumina Paired-End Sequencing



Working with Paired Reads

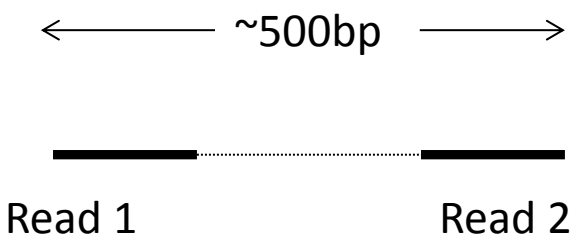
- Applicable to different fragment size ranges
  - up to ~800 bp for standard paired-libraries
  - 2 - 20kb mate-pair libraries



Enables alignment software to assign unique positions to previously *non-unique* reads

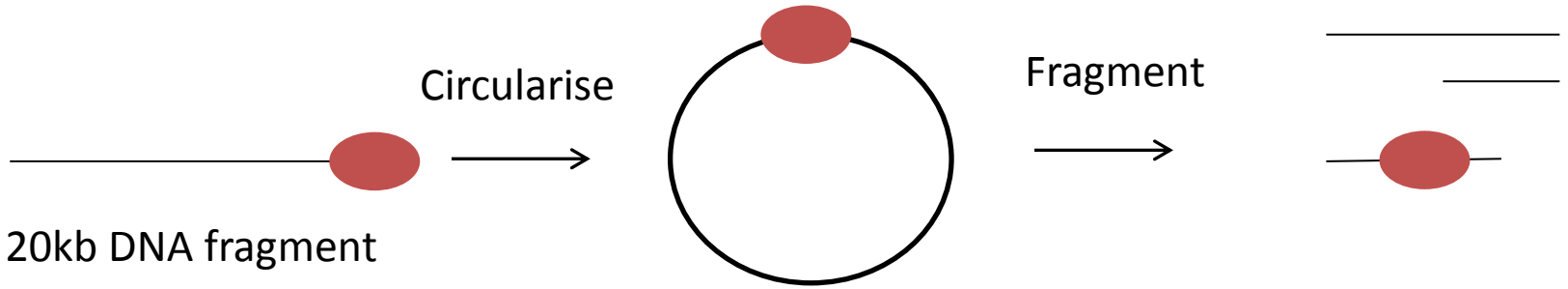
Mate pair vs paired-end reads

- Paired end reads



Mate pair vs paired-end reads on

- Mate pair libraries



- Purify fragments containing biotin moiety using Streptavidin beads
- Create a standard Illumina library and sequence using paired-end reads
- Physical fragment size is 500
- Genomic distance between read 1 and read 2 is 19.5kb

= Biotin moiety

Illumina platforms

Illumina HiSeq

- 500Gbase/flowcell
- 8 human genomes
- 6 day run time
- High output or rapid run mode
- Read lengths up to 250bp
- Requires large numbers of samples (or large genomes) to obtain lowest cost

- 4 colour chemsitry
- £750,000 incl 3 year servicing

Illumina NextSeq 500

- 90Gbase/flowcell
- 1 human genome
- 2 day run time
- High output or rapid run mode
- Read lengths up to 150bp

- 2-colour chemistry

- £250,000 incl 3 year servicing

Illumina MiSeq

- 15Gbase/flowcell
- 2 day run time
- Read lengths up to 300bp

- 4 colour chemsitry

- £90,000 incl 3 year servicing

Illumina Developments

	 HiSeq 2500		 HiSeq 3000	 HiSeq 4000
Run Mode	Rapid Run	High-Output	N/A	N/A
Flow Cells per Run	1 or 2	1 or 2	1	1 or 2
Output Range	10-300 Gb	50-1000 Gb	125-750 Gb	125-1500 Gb
Run Time	7-60 hours	<1-6 days	<1-3.5 days	<1-3.5 days
Reads per Flow Cell†	300 million	2 billion	2.5 billion	2.5 billion
Maximum Read Length	2 x 250 bp	2 x 125 bp	2 x 150 bp	2 x 150 bp

Illumina Miniseq

- $50,000
- 25 million reads per run
- 2 colour SBS chemistry

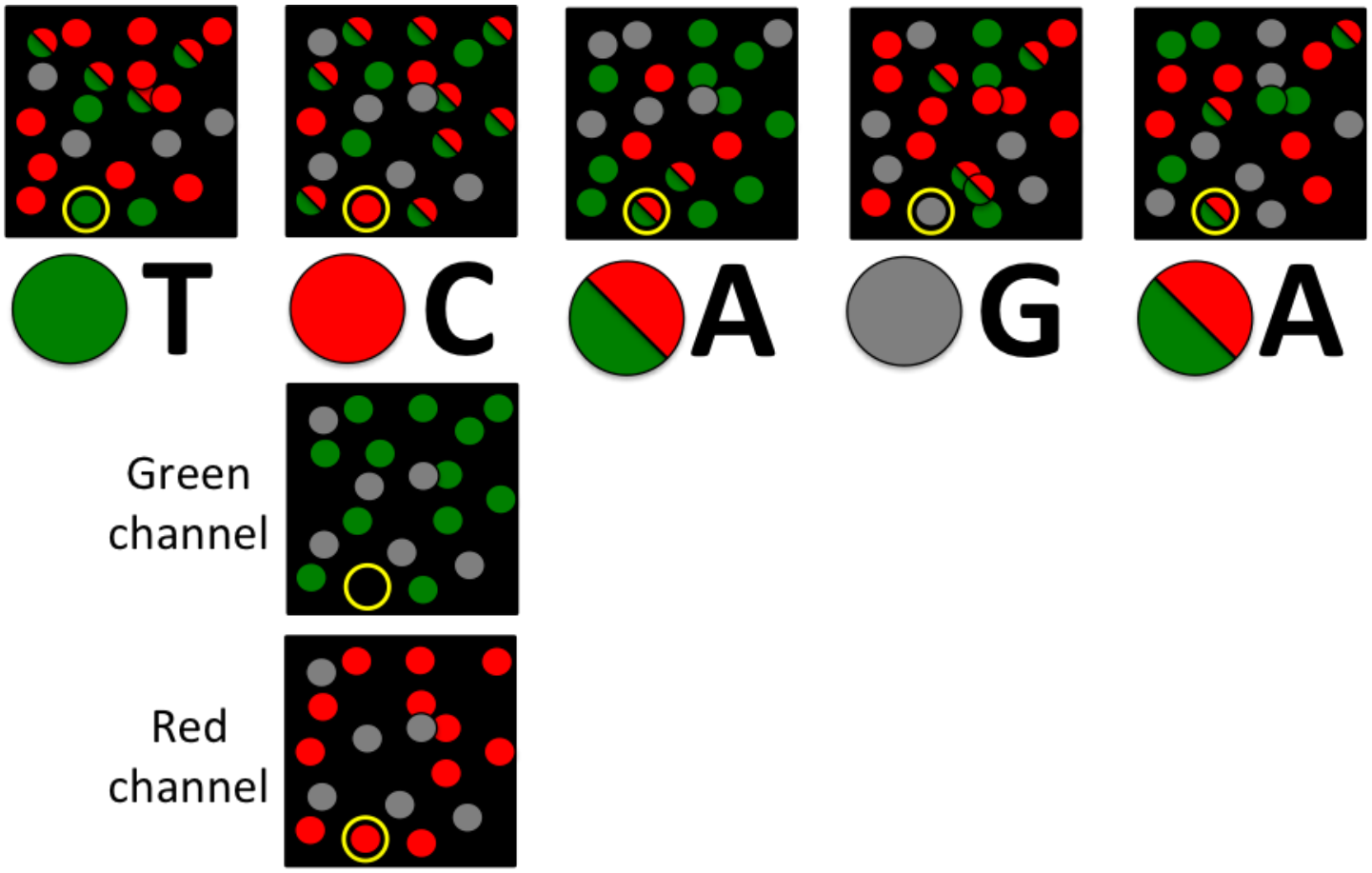
Increasing throughput

- NextSeq
  - Utilises 2-colour instead of 4-colour chemistry to reduce sequencing time
- HiSeq 2500, NextSeq and MiSeq
  - Clusters formed randomly on the surface of the flowcell
- HiSeq 3000, 4000
  - Clusters only form within nanowells
  - Patterned flowcells

2-colour chemistry

- Instead of using 4 different dyes for each nucleotide, use 2
- Label T as green and C as red
- Label A as green and red
- Label G with no dye
- Rely on cluster position to call G bases

2-colour chemistry

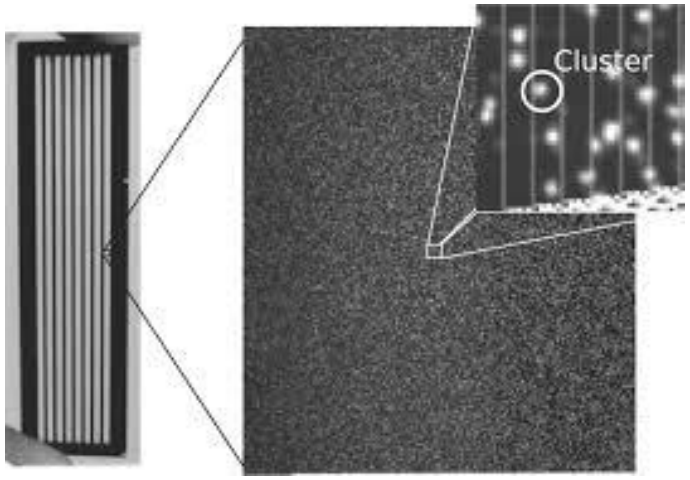


http://core-genomics.blogspot.cz/2014/01/nextseq-500s-new-chemistry-described.html

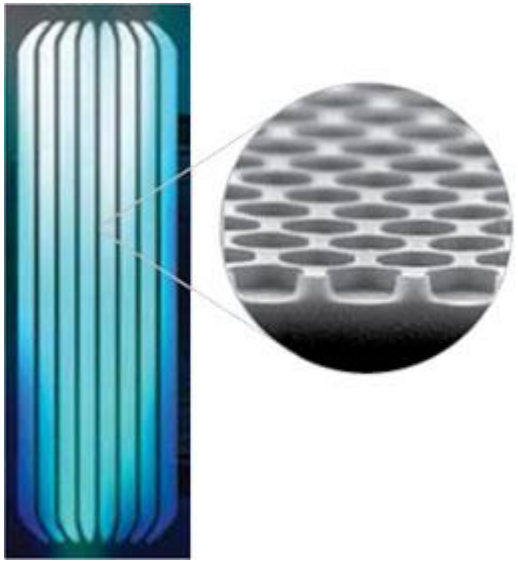
Advantages/disadvantages

- Advantages
  - Speed
  - Only two pictures need to be taken each cycle instead of four
- Disadvantages
  - Higher likelihood of errors
  - Difficult to calibrate guanine quality scores
  - With fragments shorter than read length, tendency is to call G with high quality scores

Patterned flowcells

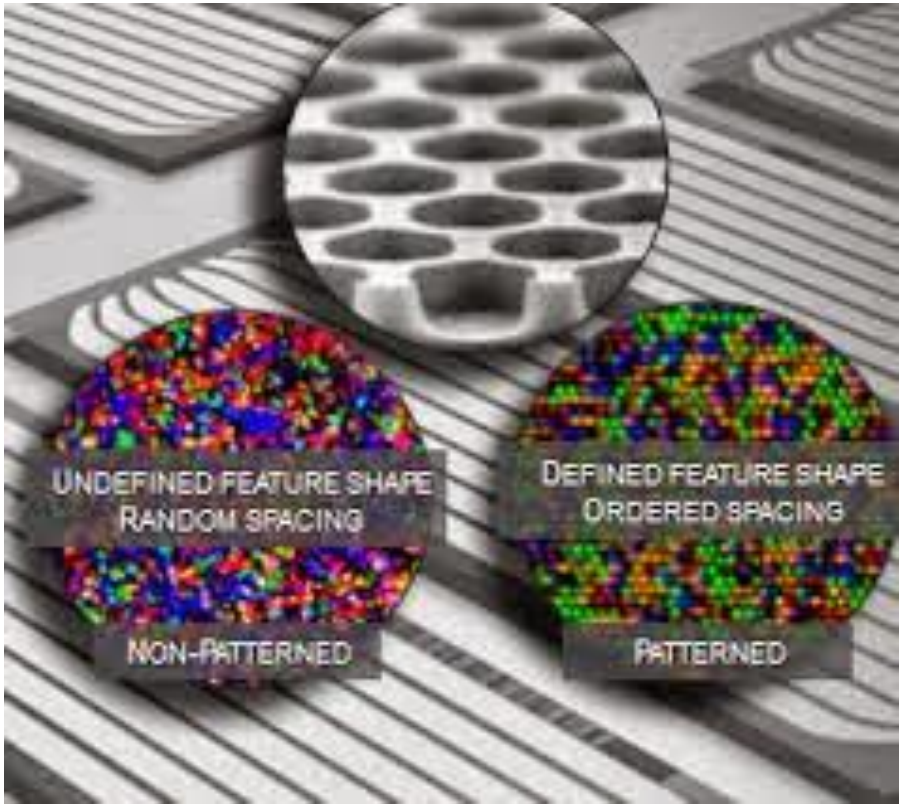


Randomly clustered flowcell
(2500)



Patterned flowcell
(3000/4000)

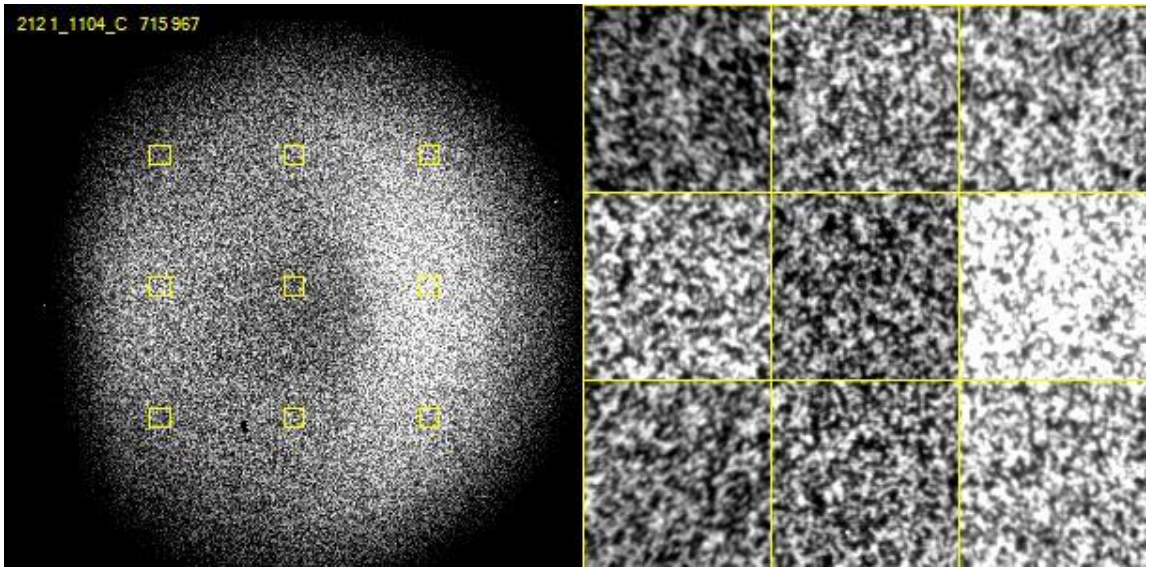
Comparison


UNDEFINED FEATURE SHAPE
RANDOM SPACING
NON-PATTERNED
DEFINED FEATURE SHAPE
ORDERED SPACING
PATTERNED

Advantages

- Removes the need to detect cluster location during first 4 cycles of sequencing

- Lower sensitivity to over-clustering



Advantages

- Allows for exclusion amplification to reduce the number of polyclonal clusters
- Utilises an electric field to transport labelled dNTPs to wells faster than amplicons can diffuse between wells
- 1 sequence per well
- Whichever sequence starts replicating first within a well will rapidly out-compete other sequences
- Removes upper poisson-limit on random flowcell clustering (~37%)

http://www.google.com/patents/WO2013188582A1?cl=en

Disadvantages

- Possibility to obtain large number of duplicated sequences across wells
  - Caused by seeding of adjacent wells
  - Can be caused by under-clustering
  - Still important to load correct concentrations
- Limits on DNA fragment length

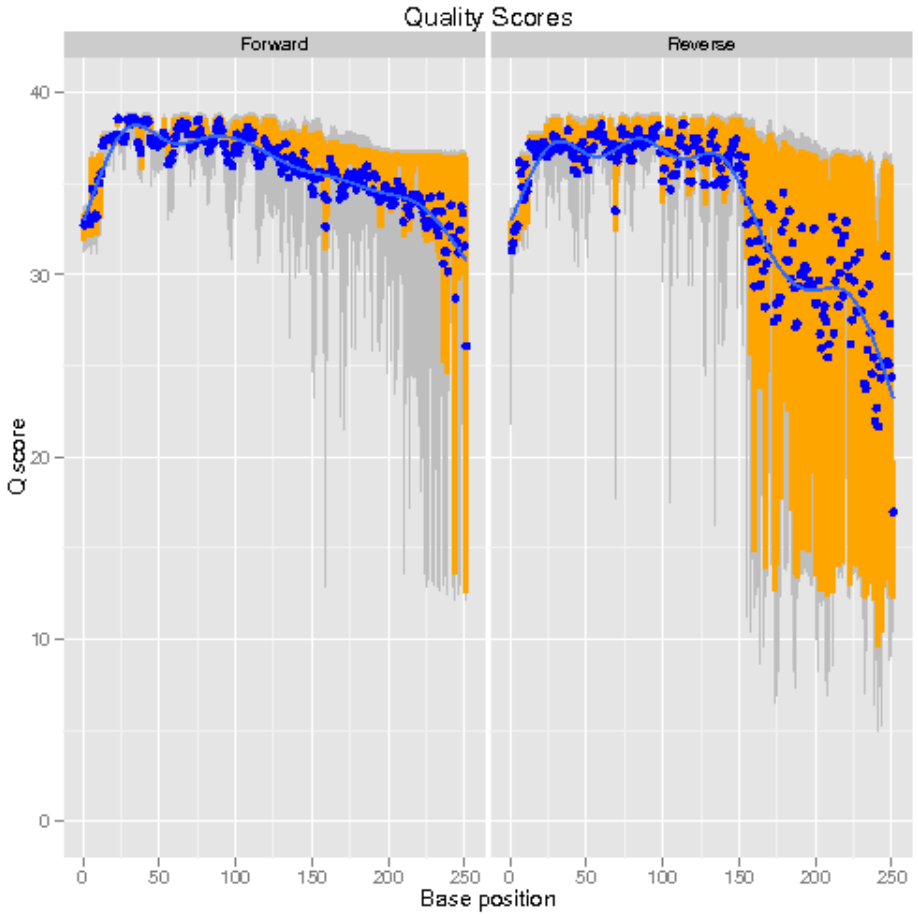
Potential issues with Illumina sequencing

- Specific motifs which are difficult to sequence
  - GGC motif
  - Inverted repeats

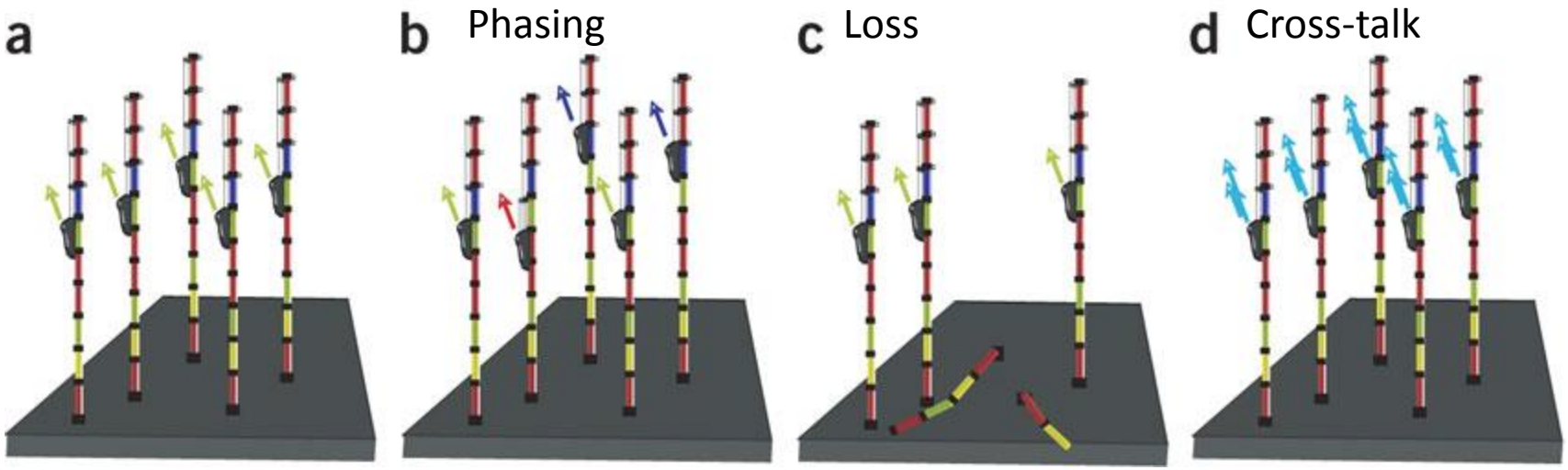
- Now mostly resolved
  - Low diversity sequences
    - 16S/amplicon sequences
    - Custom adaptors with barcodes at 5' end
    - Now a much reduced problem thanks to software updates
  - GC/AT bias
    - GC clusters are smaller than AT
    - (less of a problem post June 2011)

Nakamura, K., Oshima, T., Morimoto, T., Ikeda, S., Yoshikawa, H., Shiwa, Y., Ishikawa, S., et al. (2011). Sequence-specific error profile of Illumina sequencers. Nucleic acids research, gkr344–. Retrieved from http://nar.oxfordjournals.org/cgi/content/abstract/gkr344v1

Why do quality scores drop towards the end of a read?



3 main factors



Schematic representation of main Illumina noise factors.
(a–d) A DNA cluster comprises identical DNA templates (colored boxes) that are attached to the flow cell. Nascent strands (black boxes) and DNA polymerase (black ovals) are depicted.
(a) In the ideal situation, after several cycles the signal (green arrows) is strong, coherent and corresponds to the interrogated position.
(b) Phasing noise introduces lagging (blue arrows) and leading (red arrow) nascent strands, which transmit a mixture of signals.
(c) Fading is attributed to loss of material that reduces the signal intensity (c).
(d) Changes in the fluorophore cross-talk cause misinterpretation of the received signal (blue arrows; d). For simplicity, the noise factors are presented separately from each other.

http://arep.med.harvard.edu/pdf/Fuller_09.pdf

Erlich et al. Nature Methods 5: 679-682 (2008)

Limits to Illumina technology

- Limitations:
  - Reagent degradation
  - Dephasing
    - Leads to higher error rates
    - A 1% loss of signal or polymerase error every cycle leads to only 35% correct signal after 100 cycles
  - Sequencing time is always governed by the cyclic nature of the instrument (one base at a time)
    - Ideally dispense with incorporate, image, wash cycles
  - Size of fragments which can be clustered on the flowcell
    - Read lengths beyond the size of the DNA fragment are useless
    - Inefficient clustering >800bp
  - PCR steps during library preparation or clustering will result in bias

Features of Illumina Sequencing

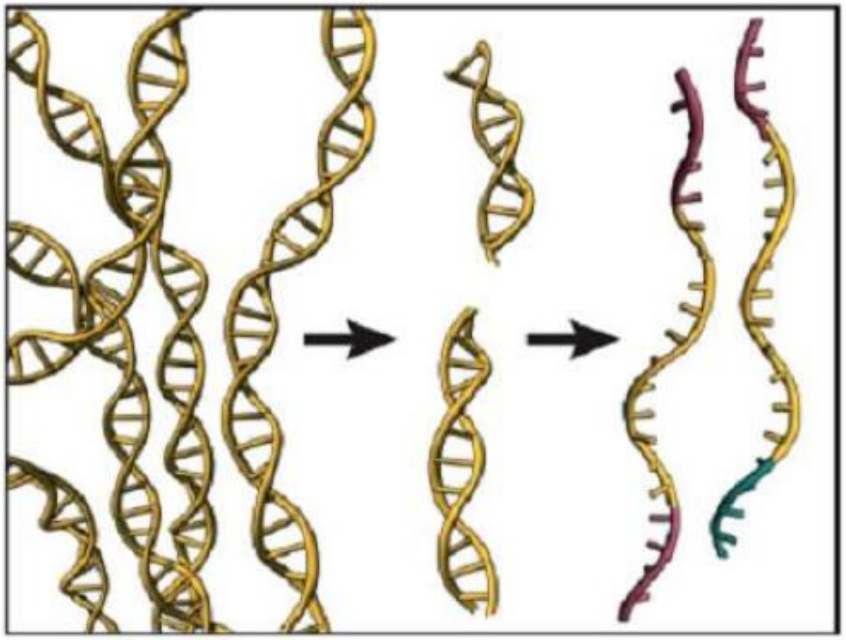
- 1 – 300 million sequences per run/lane (depending on platform and configuration)
- 36-300bp read lengths
- $0.01 - $0.1 per megabase
- Accuracy decreases along read length but ~0.5%

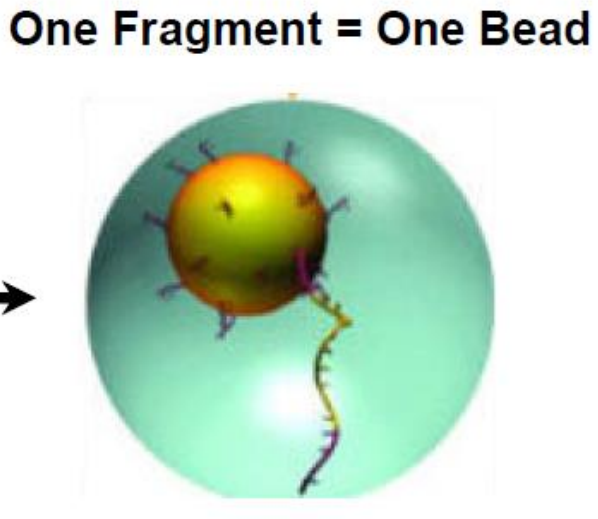
454 and Ion Torrent

Fun fact

- Jonathan Rothberg
- Set up 454 in late 90s
- Sold to Roche in 2007
- Founded Ion Torrent in 2007
- Superseded 454
- Sold to Life Tech in 2010

454 Step 1: Sample preparation



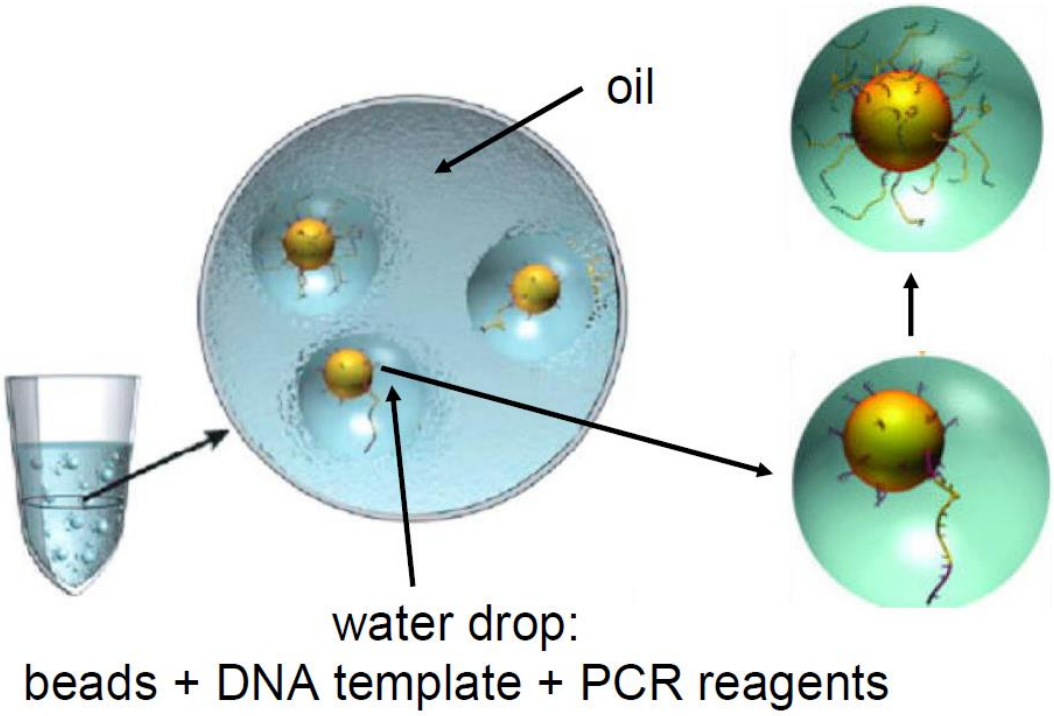


1. Genomic DNA is isolated and fragmented.
2. Adaptors are ligated to single stranded DNA
3. This forms a library

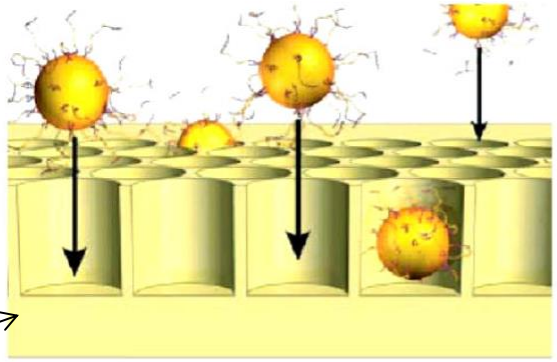
4. The single stranded DNA library is immobilised onto proprietary DNA capture beads

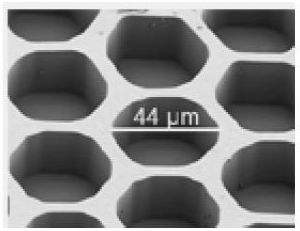
454 Step 2: Amplification

Water-based emulsion PCR



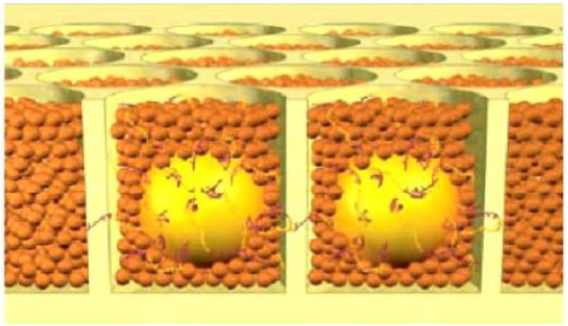
454 Step 3: Load emPCR products

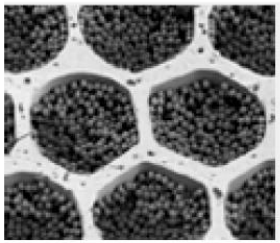




Picotitre plate

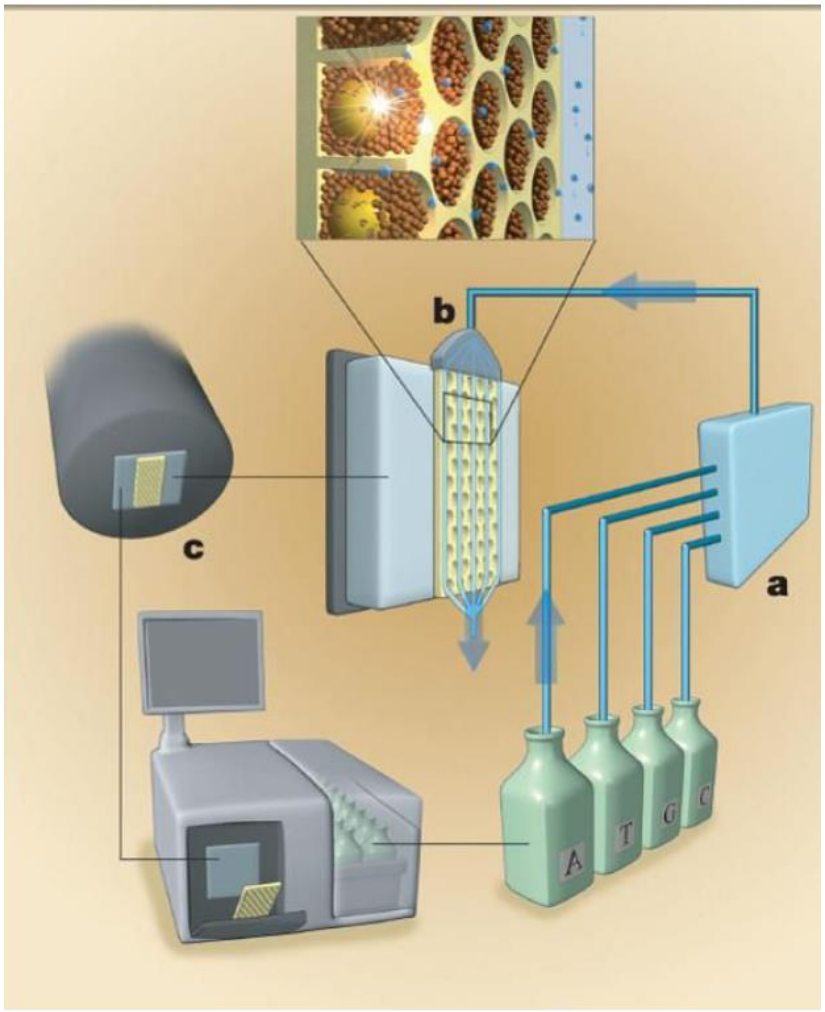
- enrich for DNA + beads
- diameter of the wells allows for only 1 bead/well





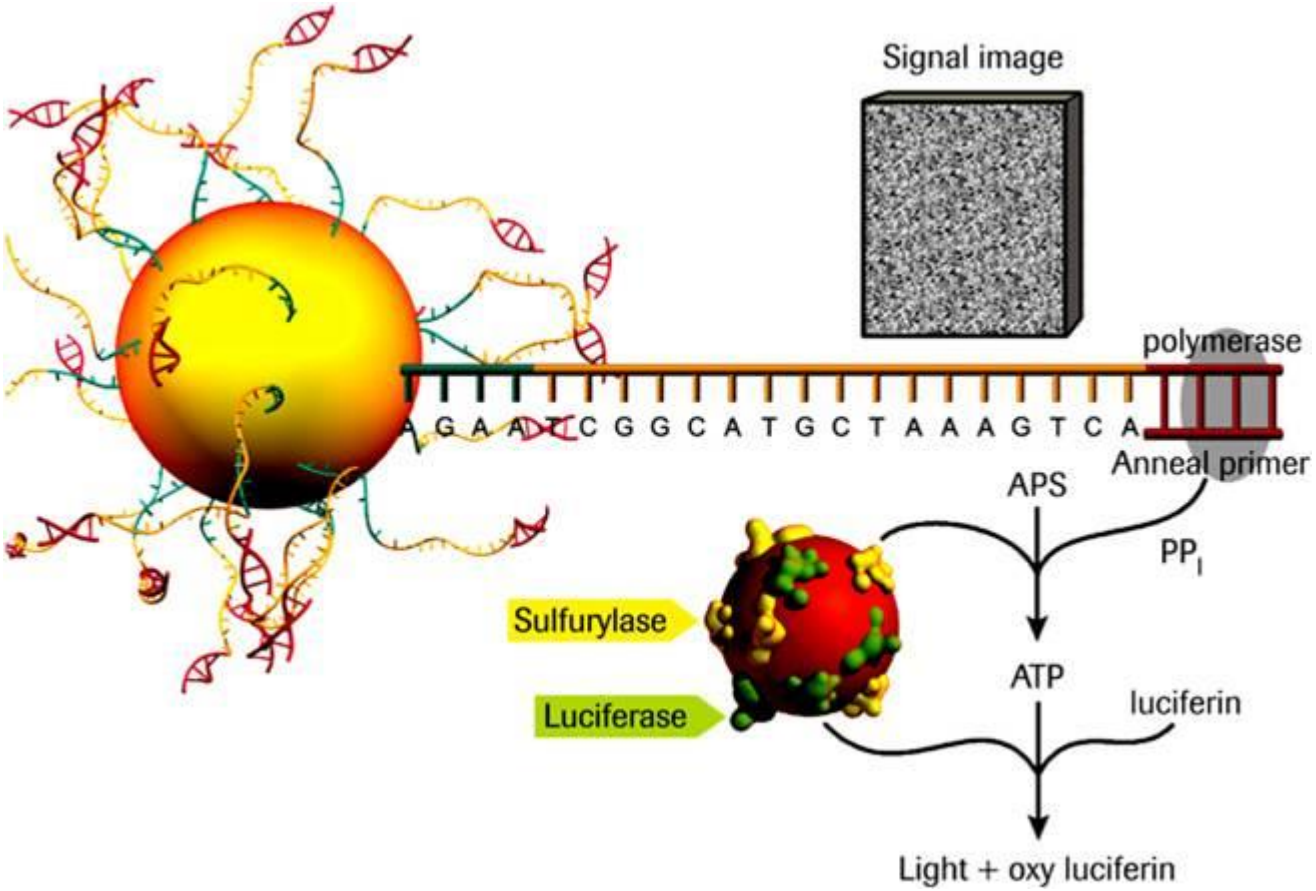
Smaller beads (red) carrying immobilized enzymes required for pyrophosphate sequencing are deposited into each well.

454 Step 4: Pyro-sequencing



1. Nucleotides are pumped sequentially across the plate
2. ~ 1 million reads obtained during 1 run
3. Addition of nucleotides to DNA on a particular bead generates a light signal

454 Chemistry



Life Technology Ion Torrent

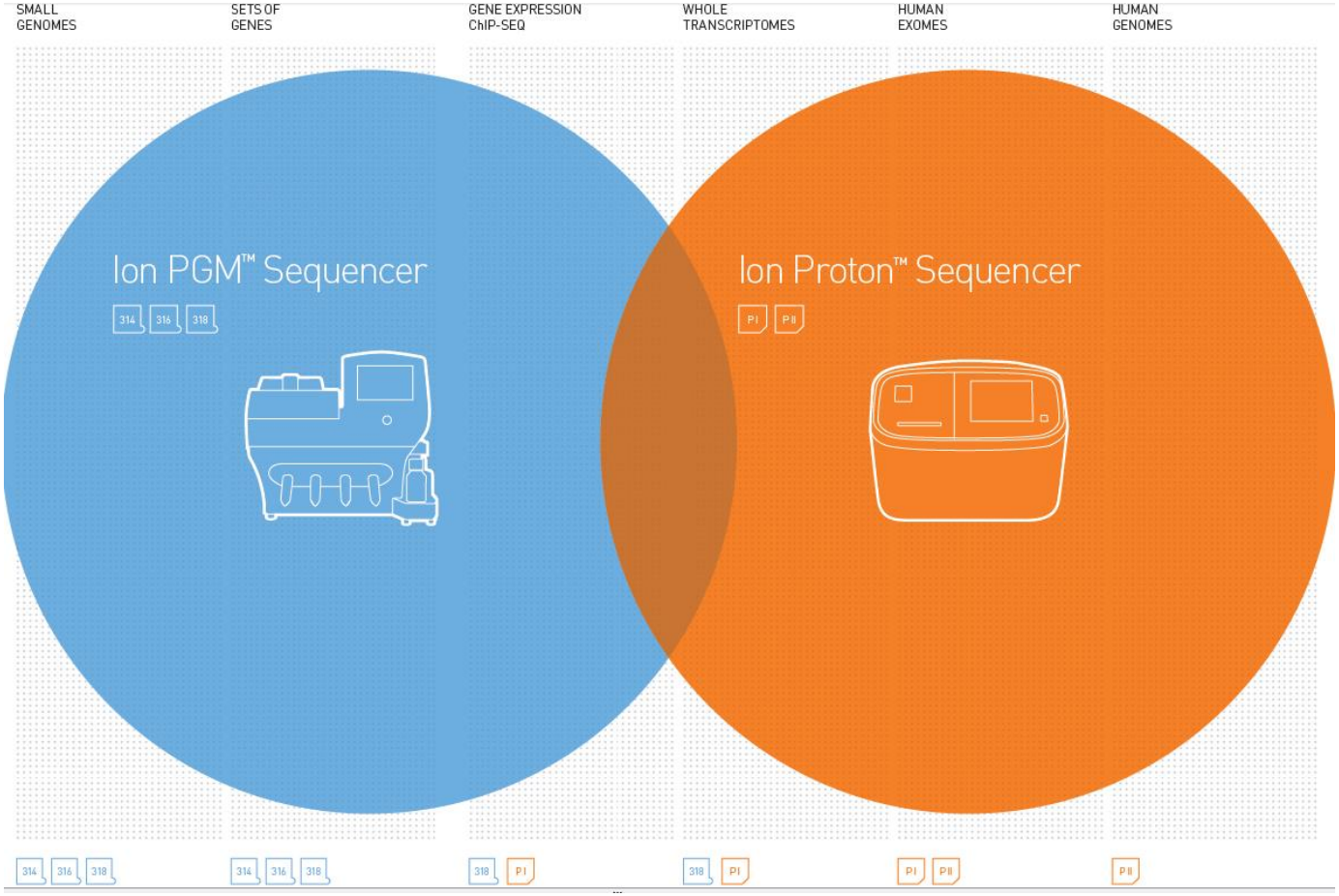
454-like chemistry without dye-labelled nucleotides

- No optics, CMOS chip sensor
- Up to 400bp reads (single-end)
- 2 hour run-time (+5 hours on One Touch)
- Output is dependent on chip type (314, 316 or 318)
- 318 (11M wells) >1Gbase in 3 hours
- **$700 per run**
- **$50K for the instrument, plus $75k for additional One Touch station and Server**
- **Libraries not compatible with Ion Proton**

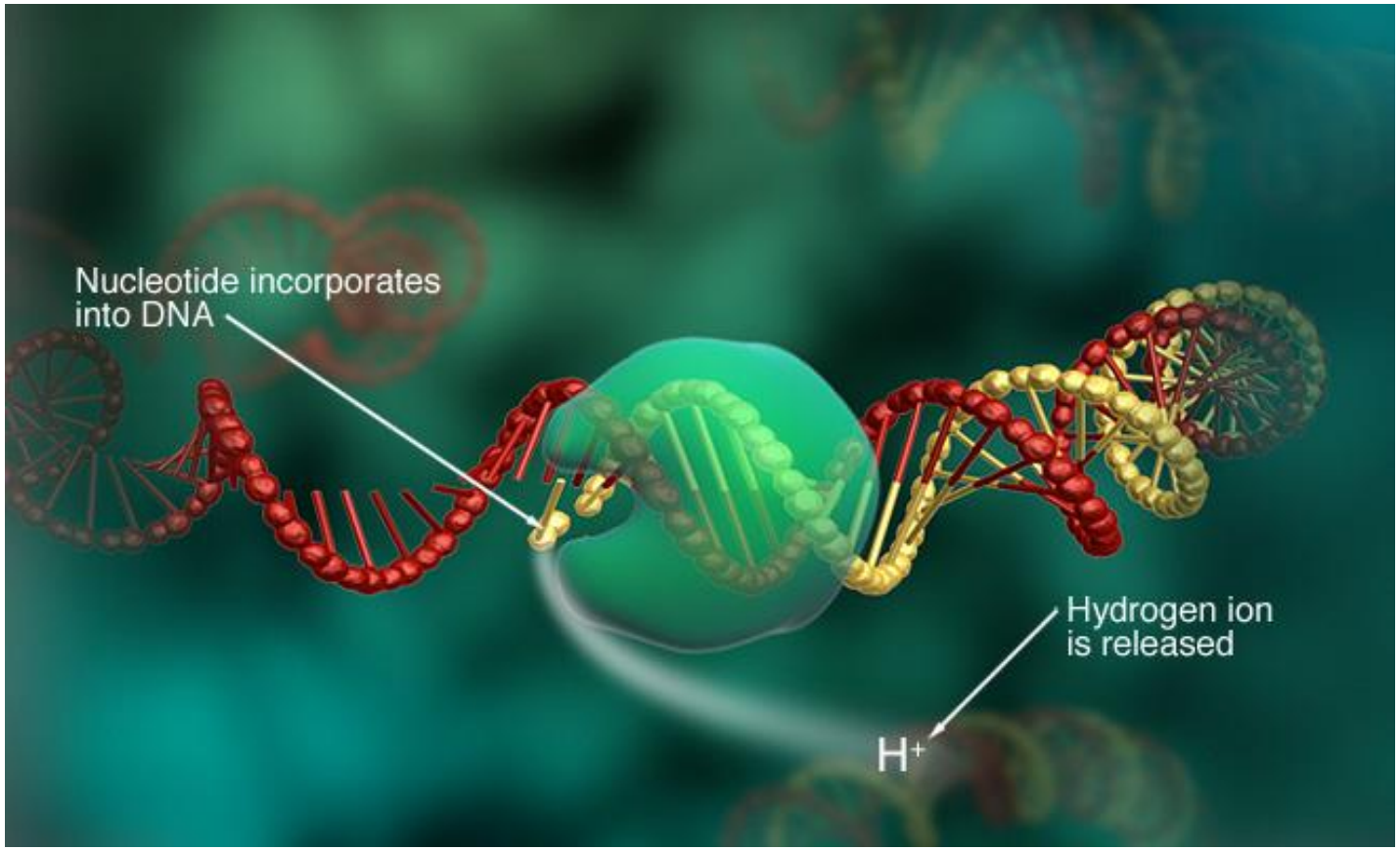
Life Technology Ion Proton

- 454-like chemistry without dye-labelled nucleotides
- No optics, CMOS chip sensor
- Up to 200bp reads (single-end)
- 2 hour run-time (+8 hours on One Touch)
- Output is dependent on chip type (P1 or P2 coming soon)
- 60-80 million reads (P1)
- **$1500 per run**
- **$150K for the instrument, plus $75k for additional One Touch station and Server**
- **Libraries not compatible with Ion Torrent**

Ion Torrent vs Ion Proton

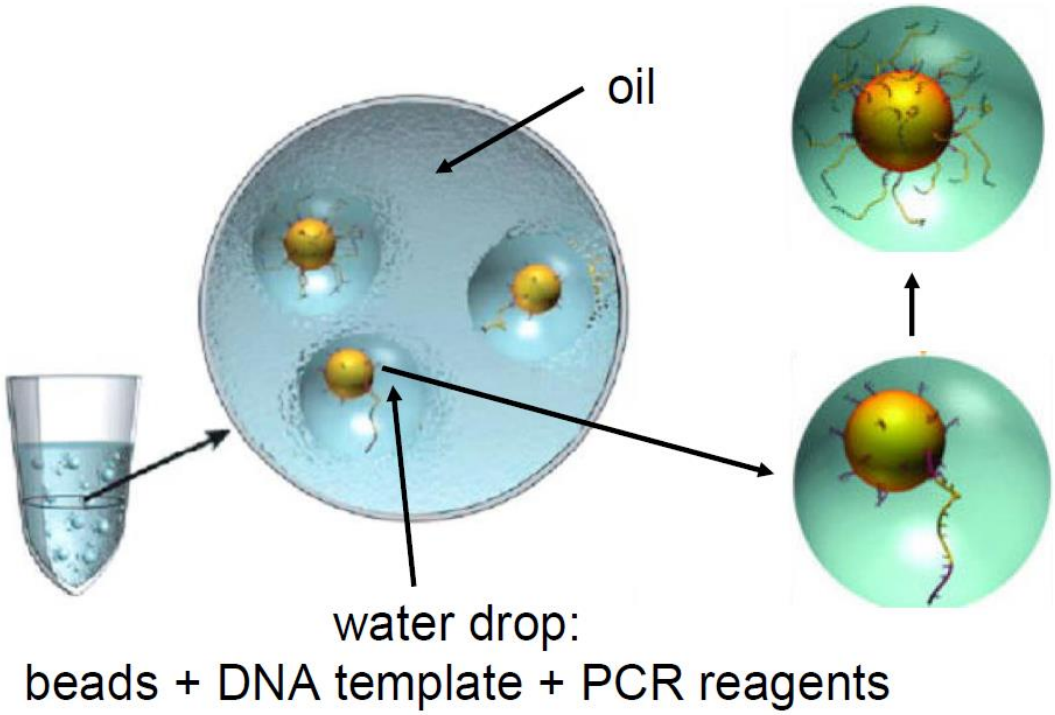


Ion Torrent

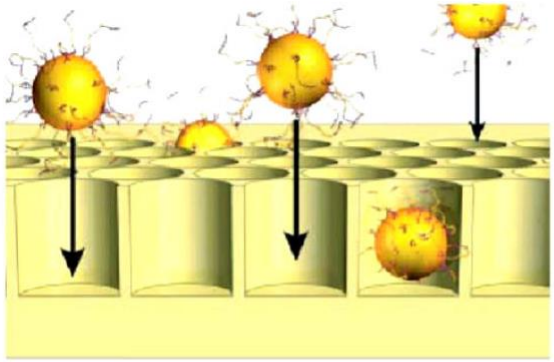

Nucleotide incorporates into DNA
Hydrogen ion is released
H+

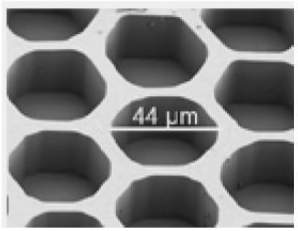
Library prep

- 454 style library using emulsion PCR

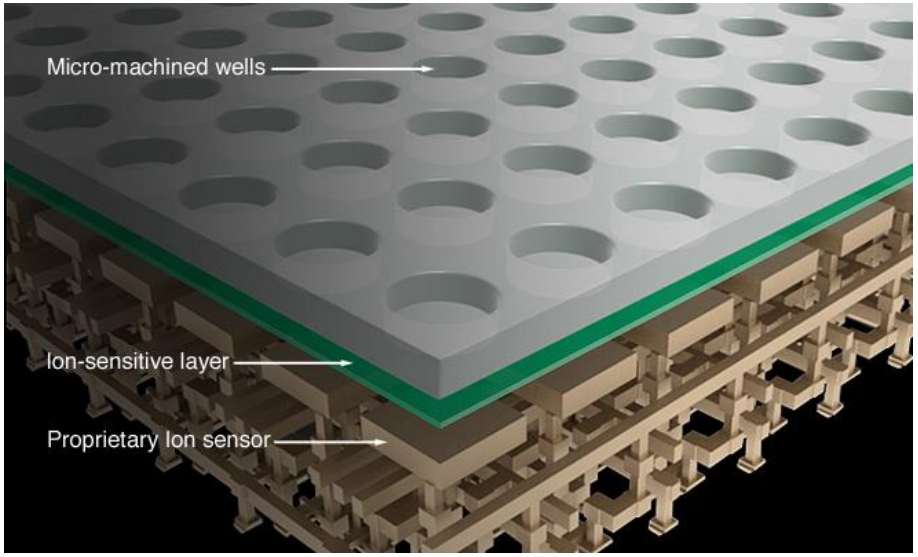


Ion Torrent

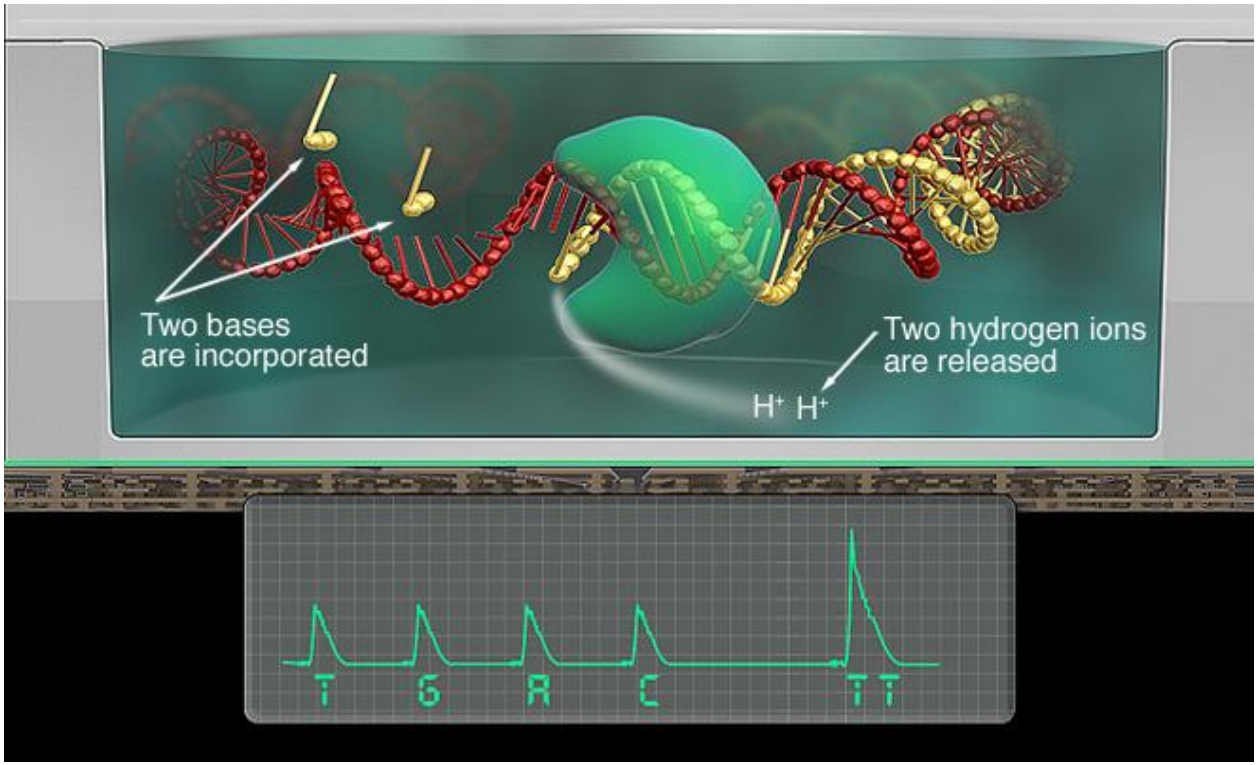




- enrich for DNA + beads
- diameter of the wells allows for only 1 bead/well



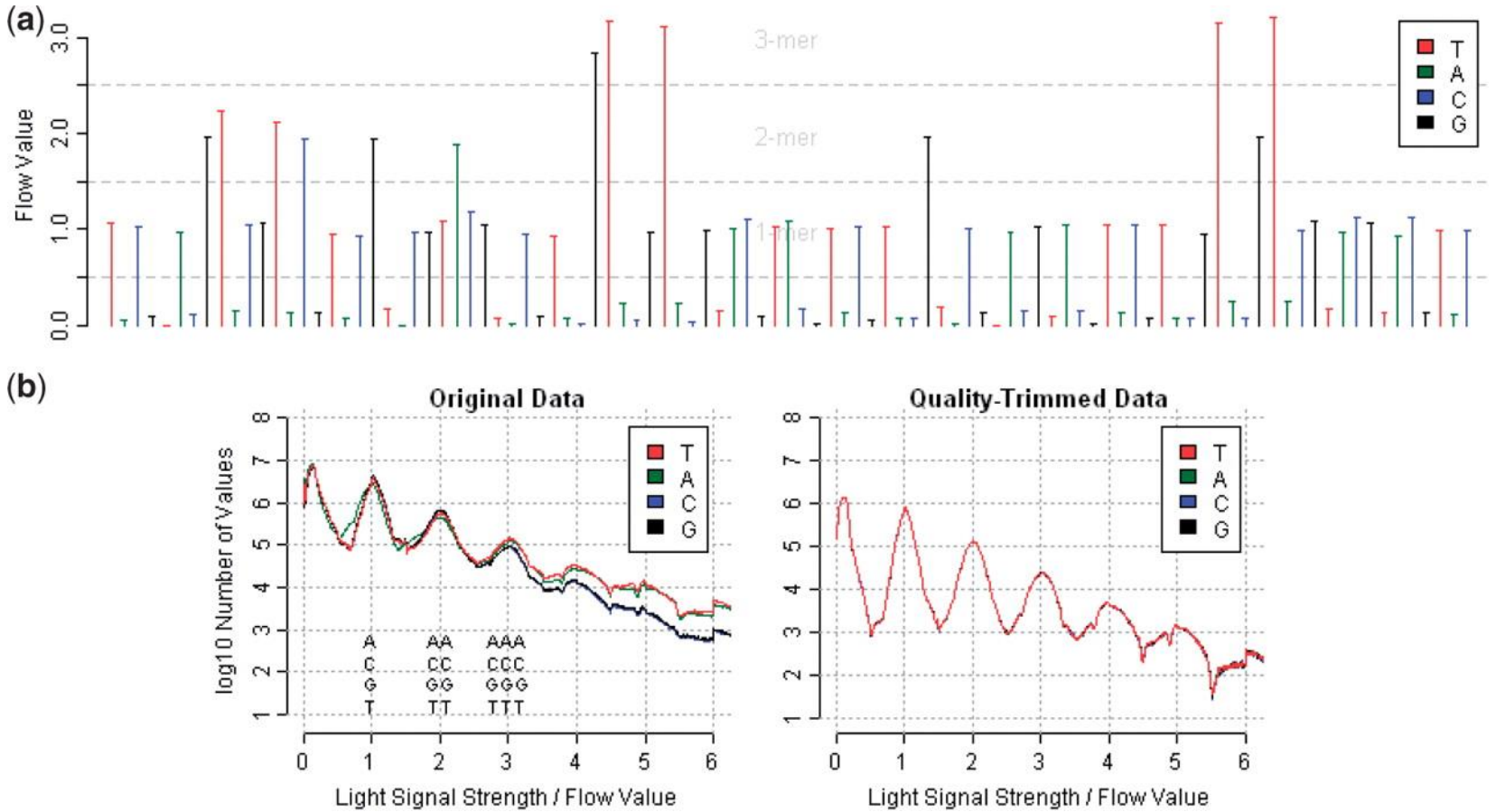
Ion System


Two bases
are incorporated
Two hydrogen ions
are released
H+ H+
T G A C T T

Homopolymer issues

- Unlike Illumina platforms 454/Ion Torrent does not contain a blocking agent
- At each cycle multiple bases of the same type can be incorporated
- E.g.
  - Sequence is AGTCCCCT
  - The CCCC will be incorporated in a single cycle

Homopolymer issues



Susanne Balzer et al. Bioinformatics 2010;26:i420-i425

Limits to Ion technology

- Limitations:
  - Reagent degradation
  - Dephasing
    - Leads to higher error rates
    - A 1% loss of signal or polymerase error every cycle leads to only 35% correct signal after 100 cycles
  - Sequencing time is always governed by the cyclic nature of the instrument
    - Ideally dispense with incorporate, image, wash cycles
  - Size of fragments which can be amplified on beads
    - Read lengths beyond the size of the DNA fragment are useless
  - PCR steps during library preparation or clustering will result in bias
  - Inaccurate homopolymer calls

Features of Ion Torrent Sequencing

- 5-80 million sequences per run (depending on platform and configuration)
- 100-400 bp read lengths
- ~$0.1 per megabase
- Accuracy decreases along read length but ~0.5%-1%
- Note increased propensity for homopolymers (although much work has gone in to reduce the impact)

Third generation sequencers

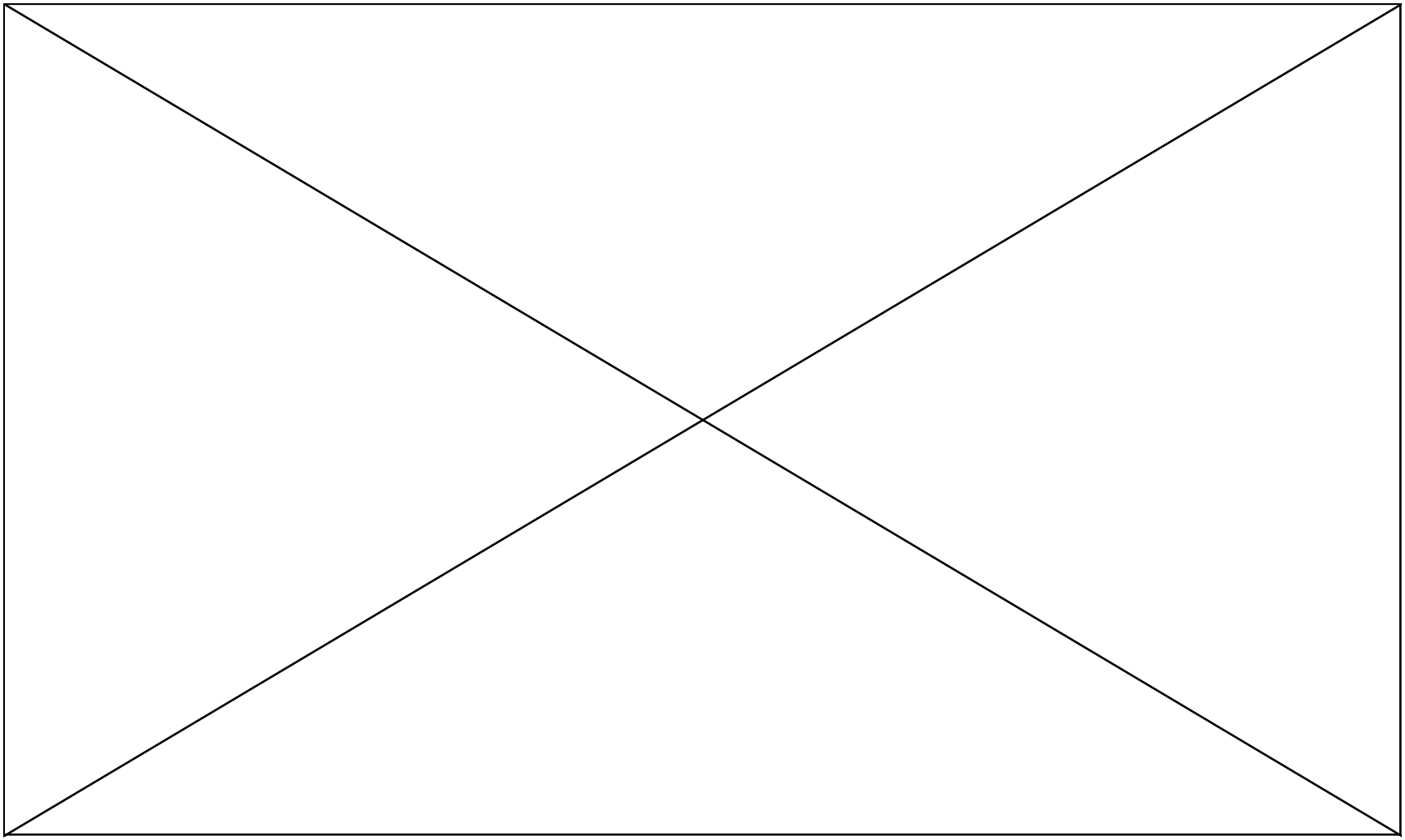
- My definition: single-molecule DNA sequencing
- Currently only PacBio RS II is commercially available

Pacific Biosciences RS II

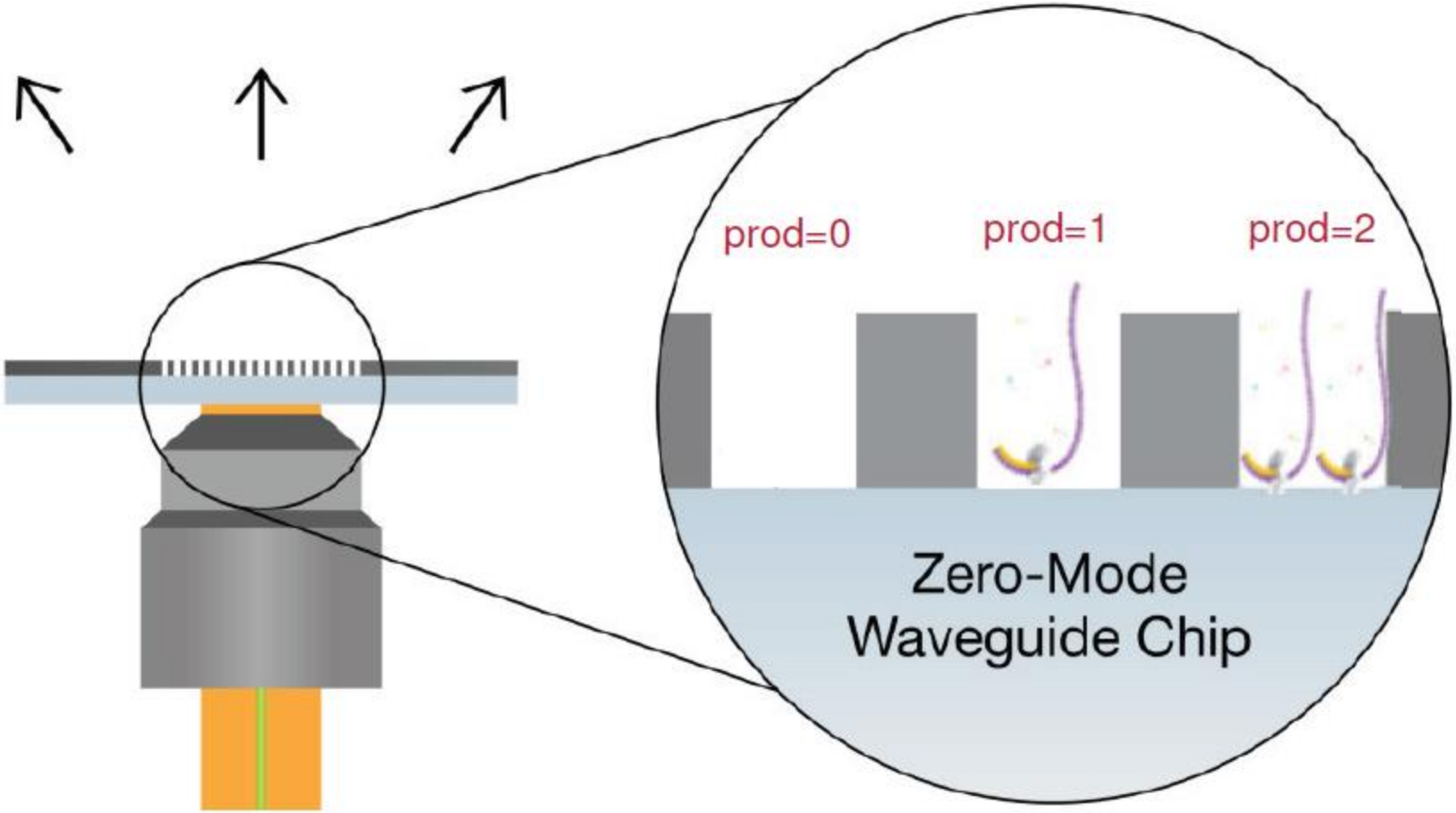
Introduction

- Based on monitoring a single molecule of DNA polymerase within a zero mode waveguide (ZMW)
- Nucleotides with fluorophore attached to phosphate (rather than base) diffuse in and out of ZMW (microseconds)
- As polymerase attaches complementary nucleotide, fluorescent label is cleaved off
- Incorporation excites fluorescent label for milliseconds -> nucleotide recorded

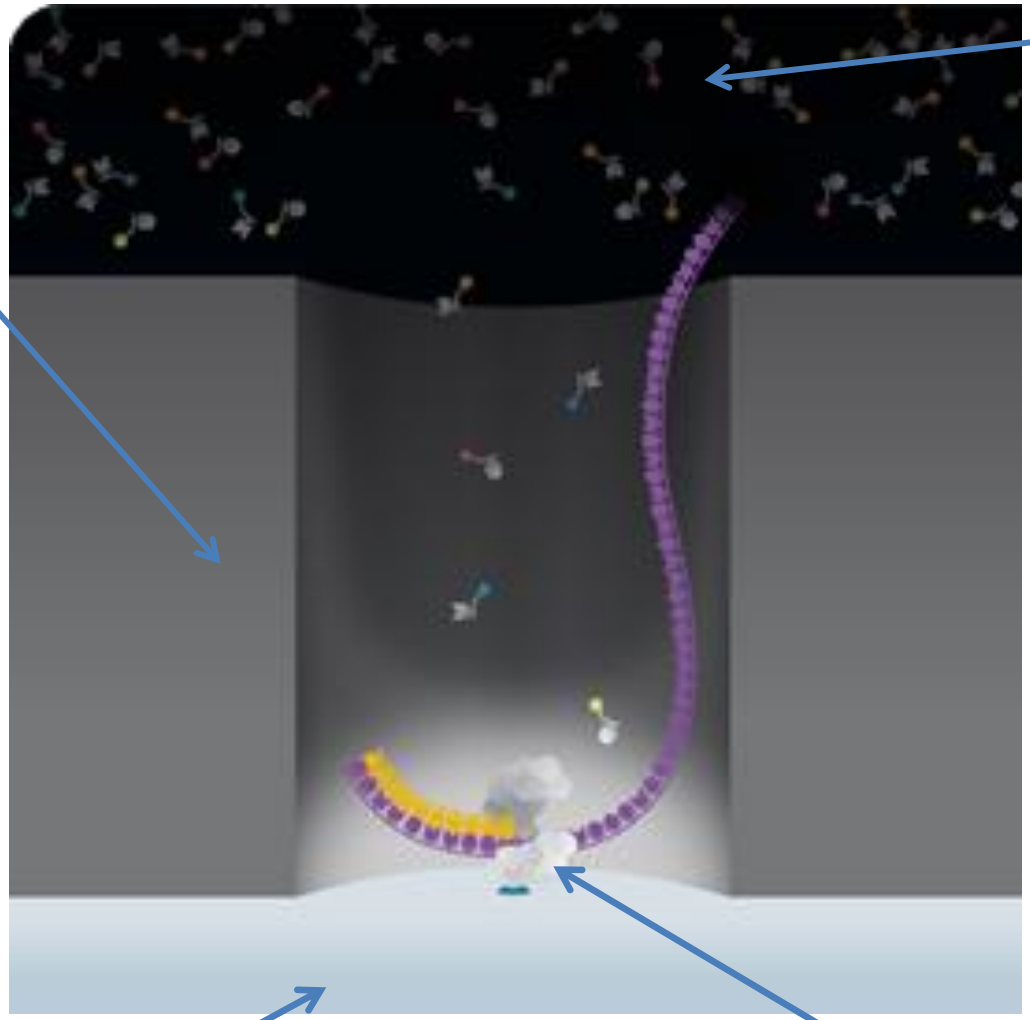
PacBio video



SMRT Cell

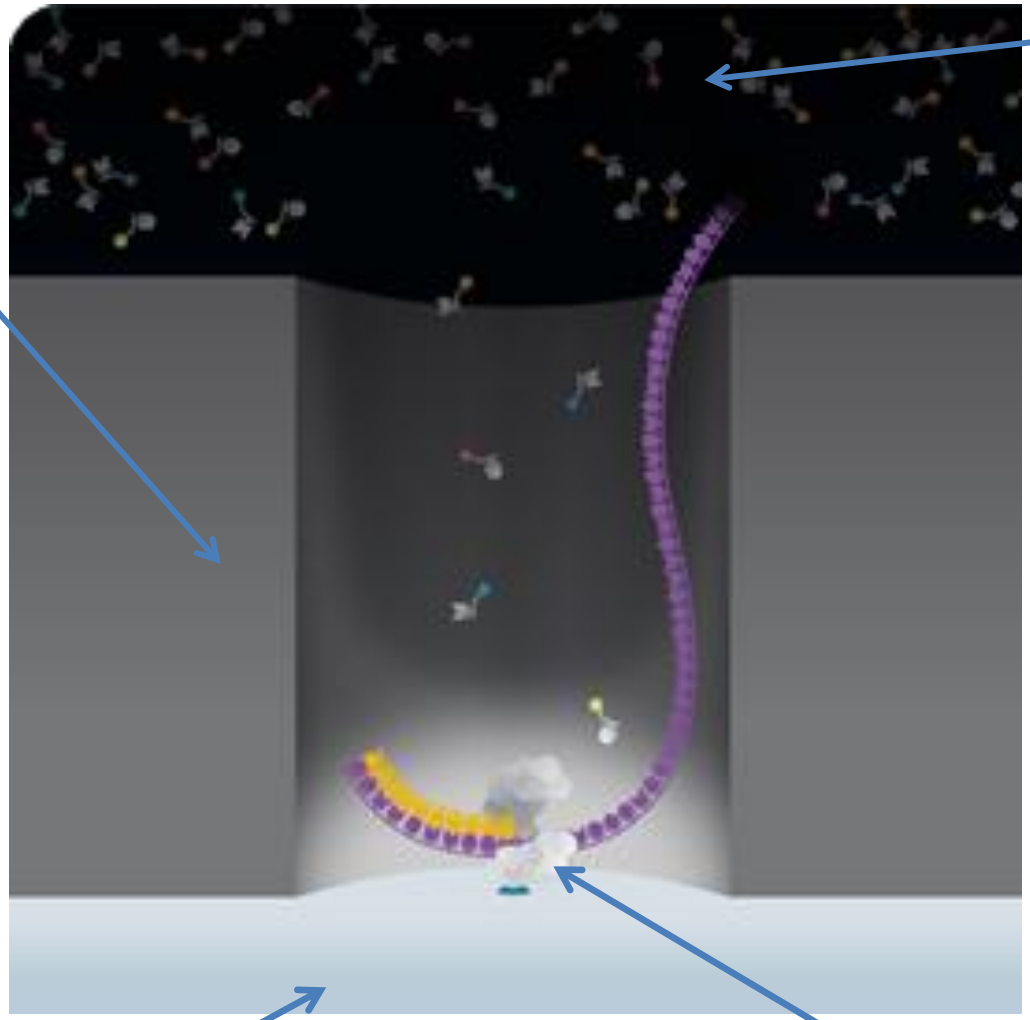

prod=0
prod=1
prod=2
Zero-Mode
Waveguide Chip


Prism
Lens
ZMW 5
Light Source
Monochrome Detector

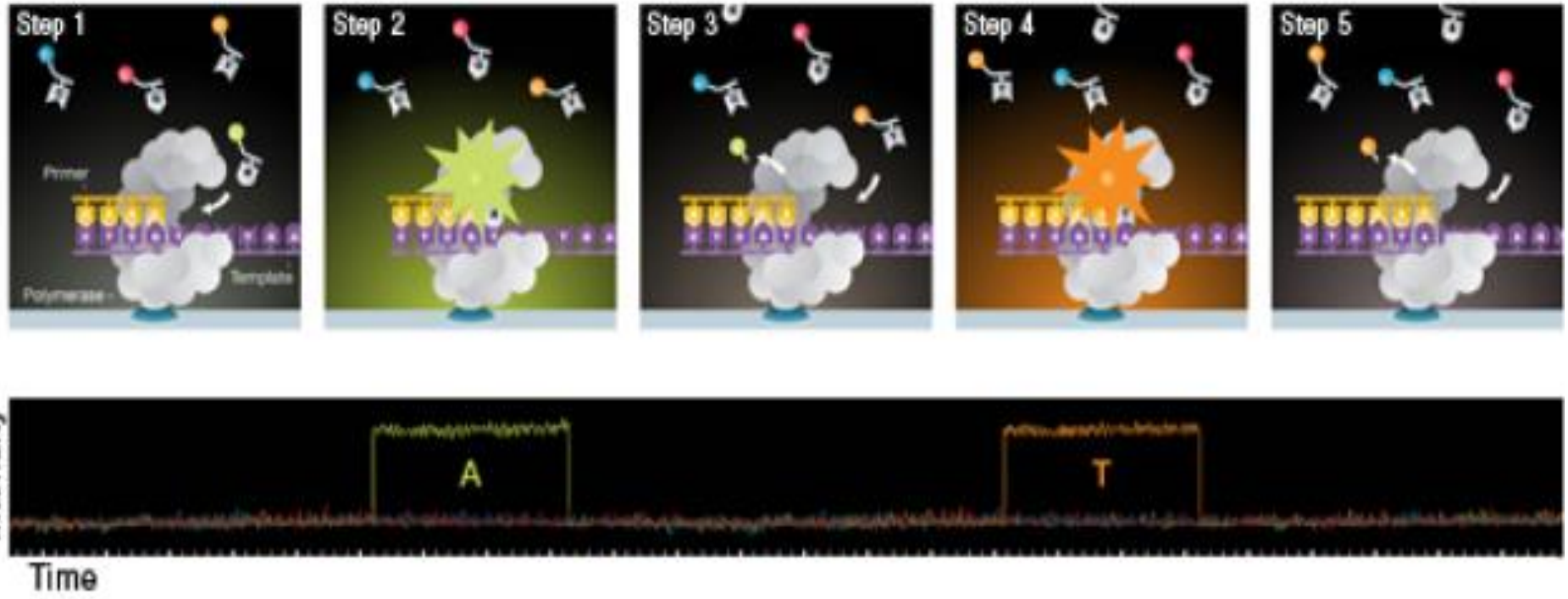

Zero mode waveguide
Free nucleotides
Laser and detector
Immobilised DNA polymerase

Reducing noise in the Zero Mode Waveguide (ZMW)

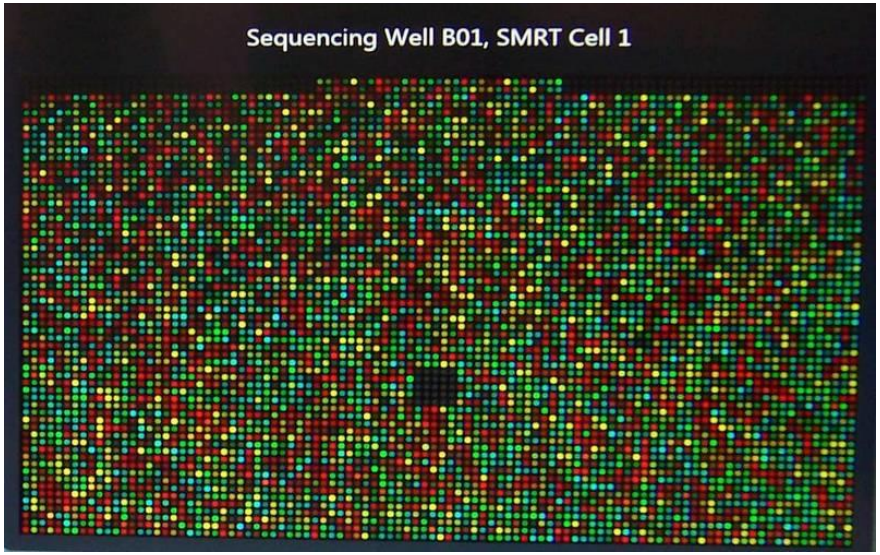
- Sequencing takes place in the ZMW
- Sequencing can only take place if one and only one DNA/polymerase complex is present in each ZMW
- Each ZMW is just 70nm wide
- Wavelength of laser light used to illuminate ZMW ~500nm
- Therefore light incident on ZMW will act as an *evanescent wave* and only penetrate the first ~30nm
- This reduces the amount of noise from fluorescence of non-incorporated fluorophores


Zero mode waveguide
Free nucleotides
Laser and detector
Immobilised DNA polymerase

Observing a single polymerase

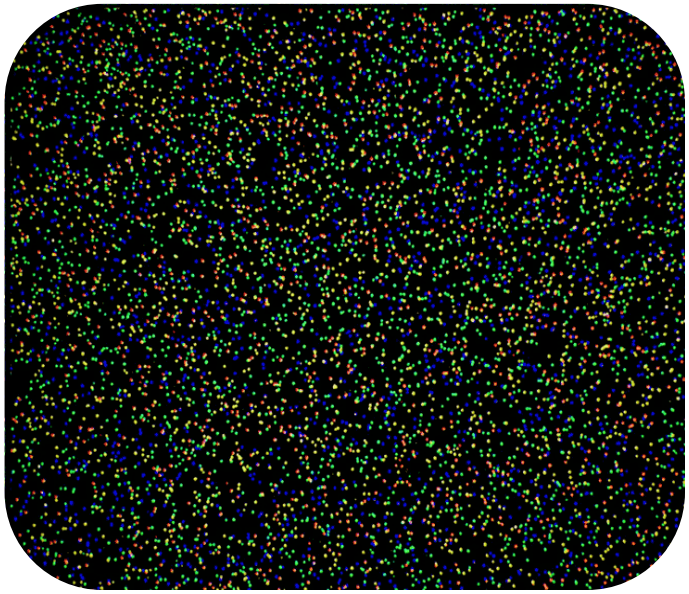


What it looks like



PacBio ZMWs with single DNA strand

Ordered



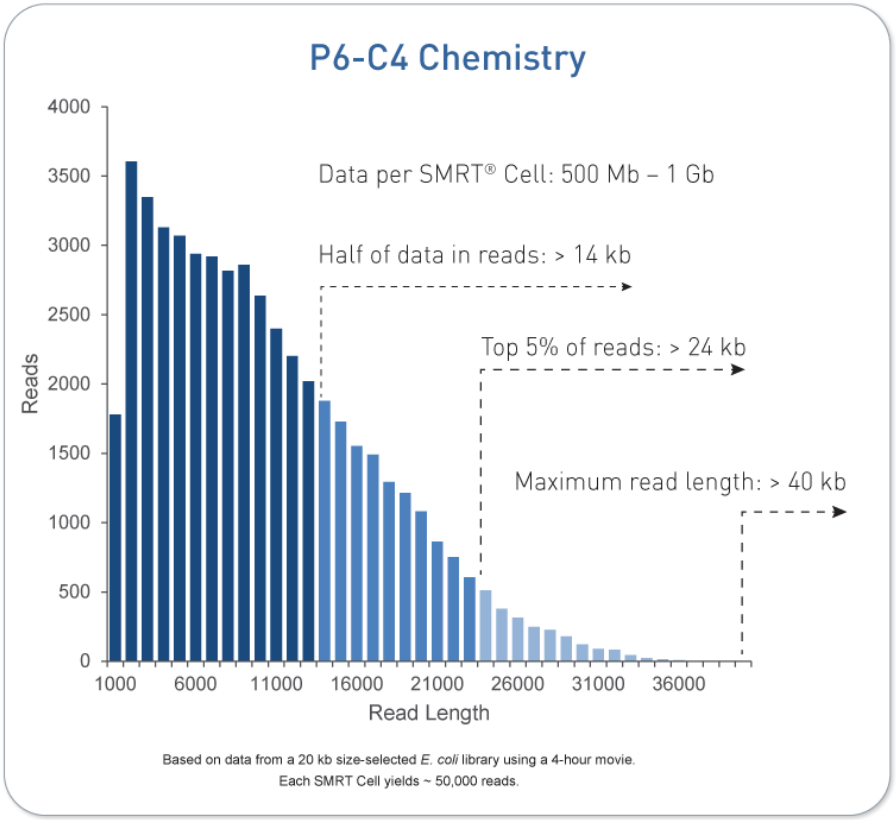
Illumina DNA mono-colonal clusters

Unordered

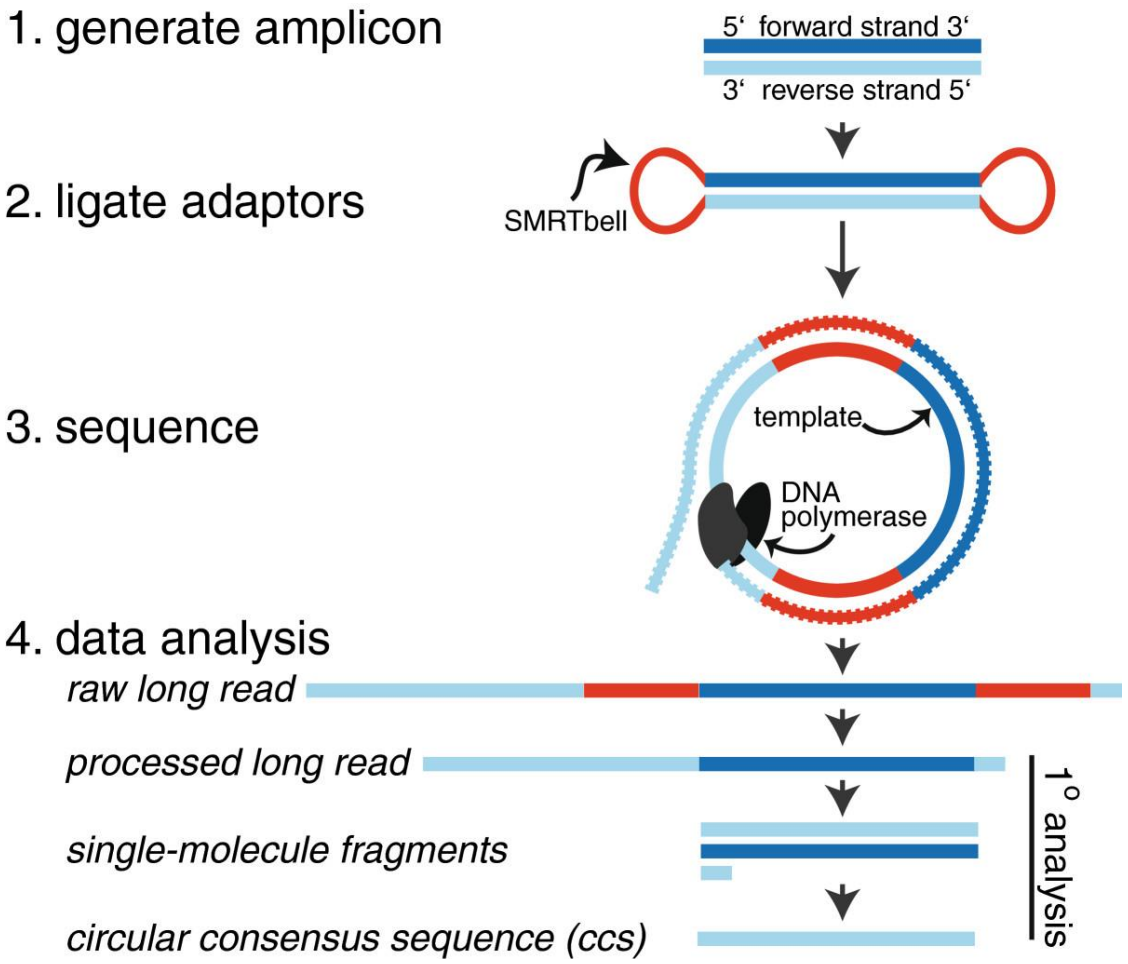
Output statistics

- Approximately 50,000 sequences per SMRT flowcell
- 500Mb-1Gbase output per SMRT flowcell
  - $200 per run
- Library prep required
  - ~$500 per sample
  - ~1ug per sample for short 1-2kb reads
  - 20-50ug for 20-30kb reads
- Size selection required to get the longest reads
- Read lengths
  - Distribution
  - Mean 12kb up to 20-40kb

Polymerase read lengths



Circular consensus sequencing



Fichot, E. B., & Norman, R. S. (2013). Microbial phylogenetic profiling with the Pacific Biosciences sequencing platform. *Microbiome*, *1*(1), 10. doi:10.1186/2049-2618-1-10

Circular consensus sequencing

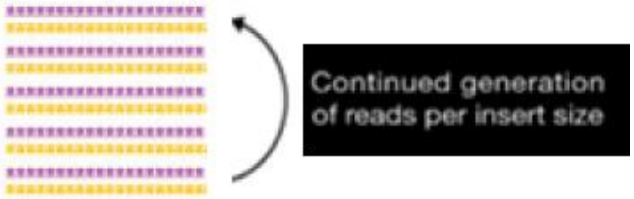
Standard Sequencing for Continuous Long Reads (CLR)

Large Insert Sizes

Generates one pass on each molecule sequenced

Circular Consensus Sequencing (CCS)

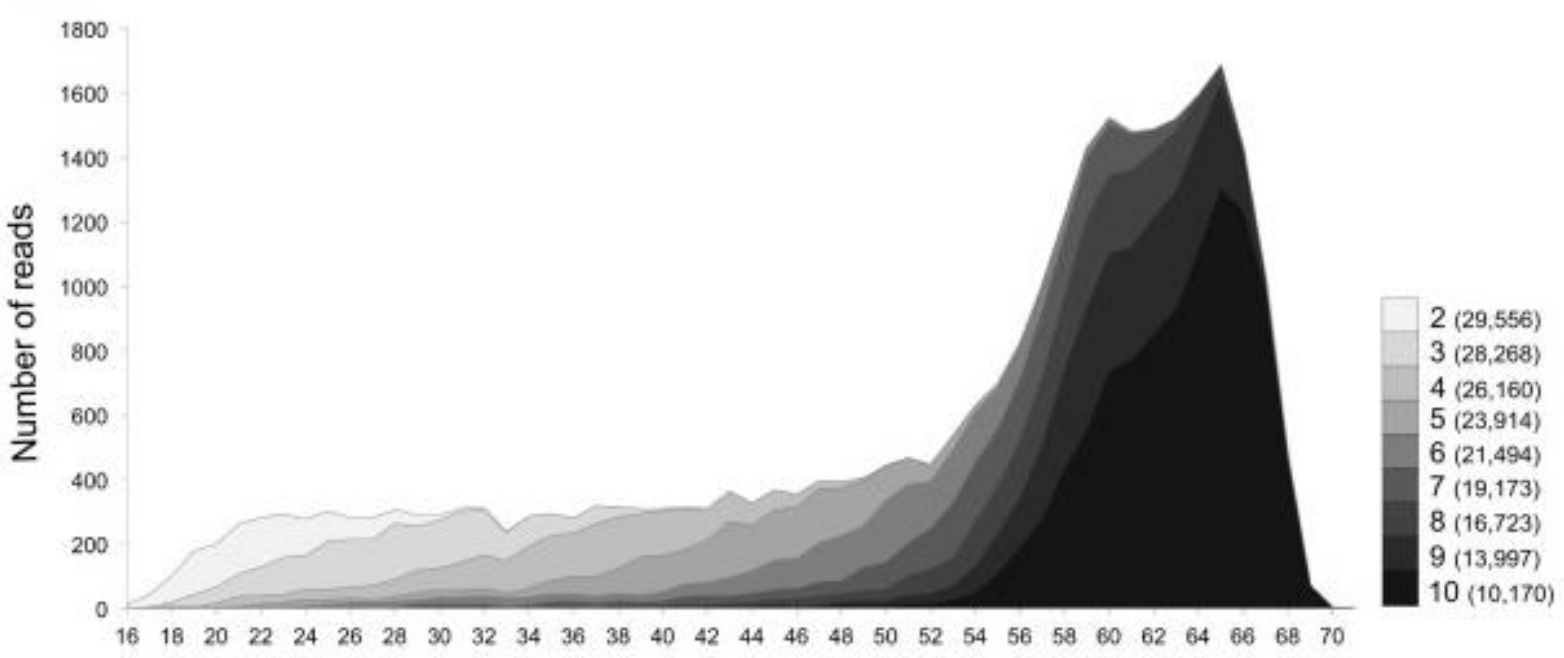
Small Insert Sizes



Generates multiple passes on each molecule sequenced

Note on read length and accuracy

- Error rate of a single read has a phred quality score of approximately 9 (~12% error rate)
- Therefore to obtain a phred quality above 30 we need to have at least 4 passes of the molecule
- With a median polymerase read length of 12kb, this means fragments with a size of approximately 3kb will be high quality



http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4152597/

Note on read length and accuracy

- In short
  - Shorter reads will tend to be higher quality
  - Longer reads will tend to be lower quality

- Depending on the application this may or may not be a problem
  - Long, error prone fragments are OK for scaffolding genome assemblies
  - May not be so good if you have a 20kb amplicon

Issues to be aware of

- PCR chimeras and artefacts (affects all PCR amplicon methods)
- Partial adaptor ligation

Normal library

Single stranded overhang

Fragment concatermerization

Issues to be aware of

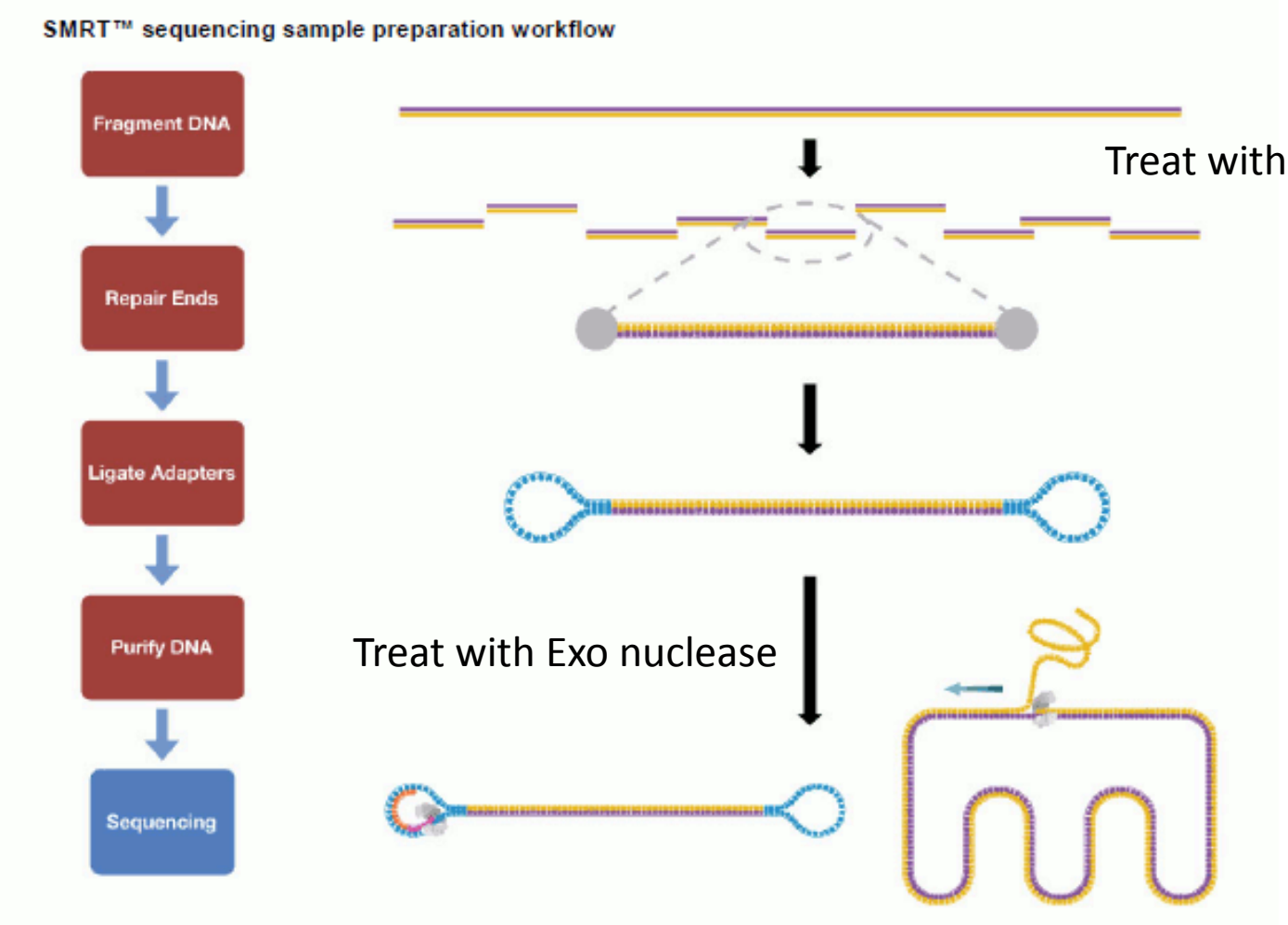
- Shorter sequences will be loaded preferentially
  - Need to enrich for long fragments using gel extraction (hence large starting material)

Issues to be aware of

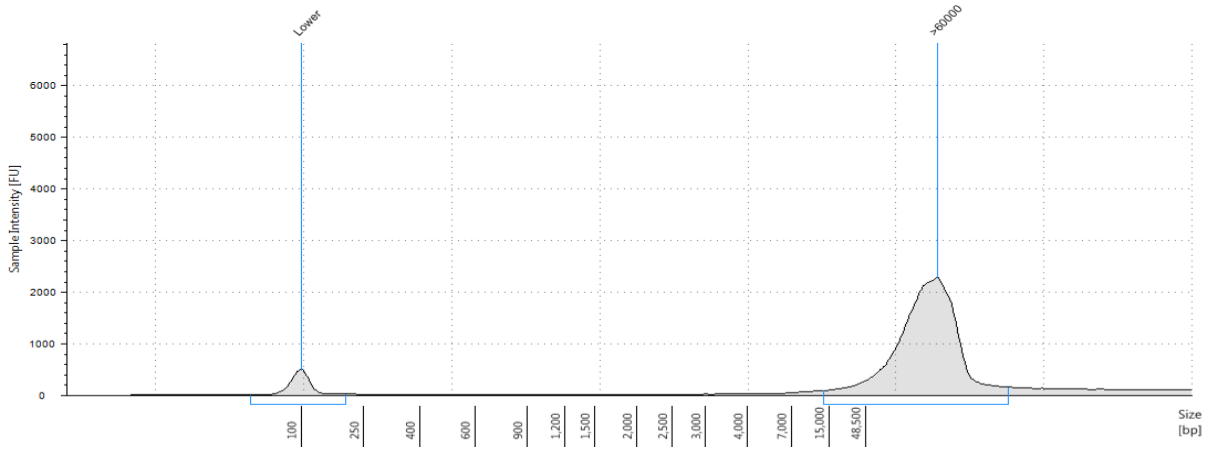
- DNA quality
  - This is absolutely crucial as we are sequencing native DNA
  - You need to be aware of several things
  - What is the source of your DNA (plants, fungi, etc)
    - Fungi often have carbohydrates bound to DNA which are hard to remove
  - Method of DNA isolation
    - Any carry over of CTAB, phenol/cholorform etc will interfere with sequencing
  - Quantification of contaminants
    - Dye-based (e.g. Qubit)
    - Nanodrop absorbance at 260/230 (2.0-2.2) and 260/280 (1.8)
  - Run a to look at your material and check for degradation
    - Don’t be tempted to run degraded material if you want long and representative reads

Library prep notes from the field

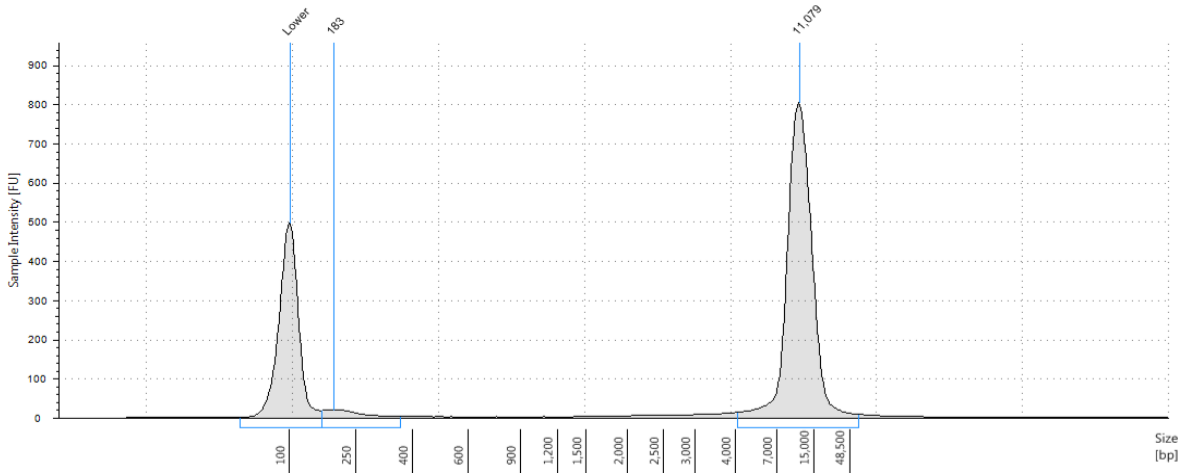
(Courtesy Jeremie Poschmann)



Fragment DNA



Shearing: hydro-shear, g-tube, needle, sonication etc.



Repair DNA

- Exonuclease treatment to remove single stranded DNA
- Make blunt ends for ligation
- Beware of:
  - DNA damage: alkylation, oxydation, UV-crosslinks, AP-sites, intercalating agents
  - DNA binders: polyphenols, secondary metabolites , pigments, polysaccharides
  - Polymerase inhibitors: salts (EDTA), phenol, alcohols

Example nanodrop curves

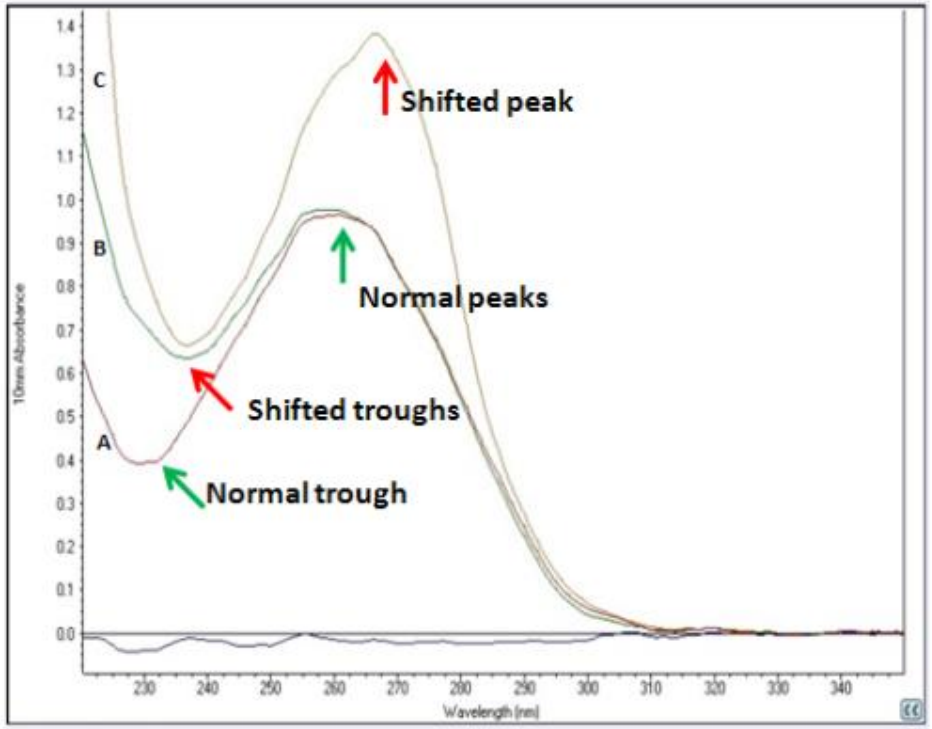
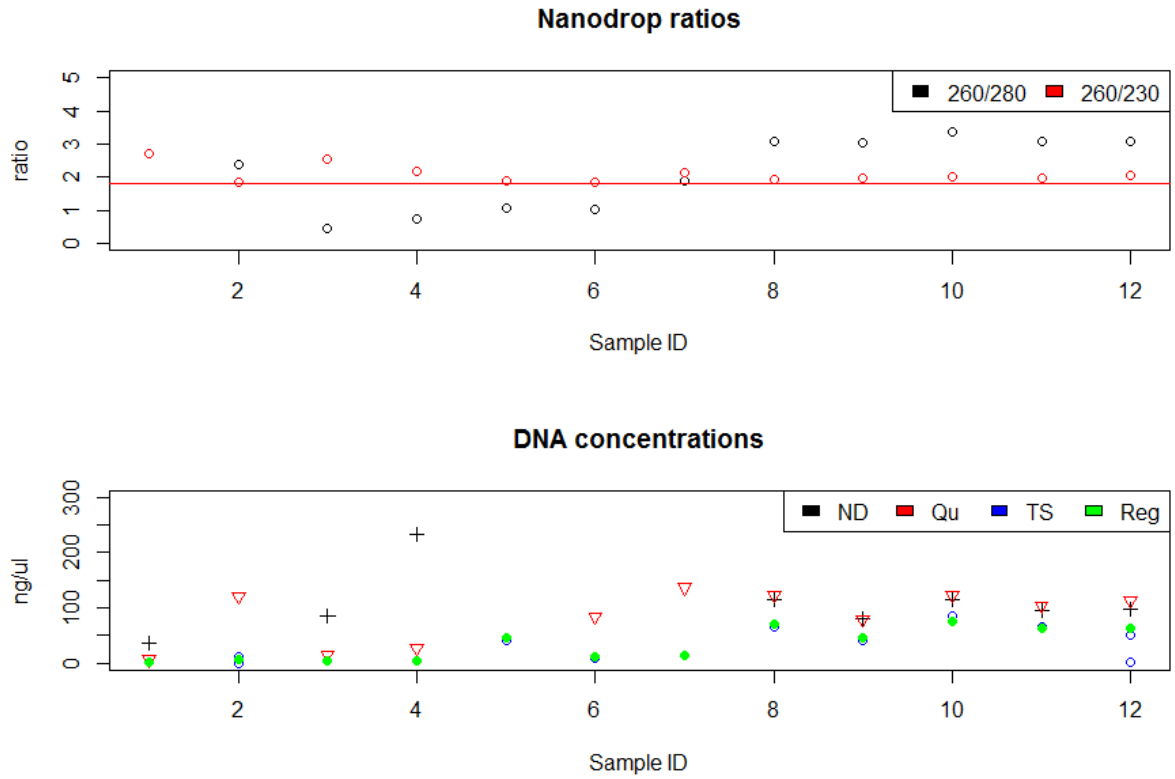


FIGURE 2. Spectra of purified DNA without contamination (A), and of the same DNA sample contaminated with guanidine (B) and phenol (C).

http://www.nanodrop.com/Library/T042-NanoDrop-Spectrophotometers-Nucleic-Acid-Purity-Ratios.pdf

Estimating the quality of DNA



Different methods will often give different results – particularly when DNA is damaged or contaminated

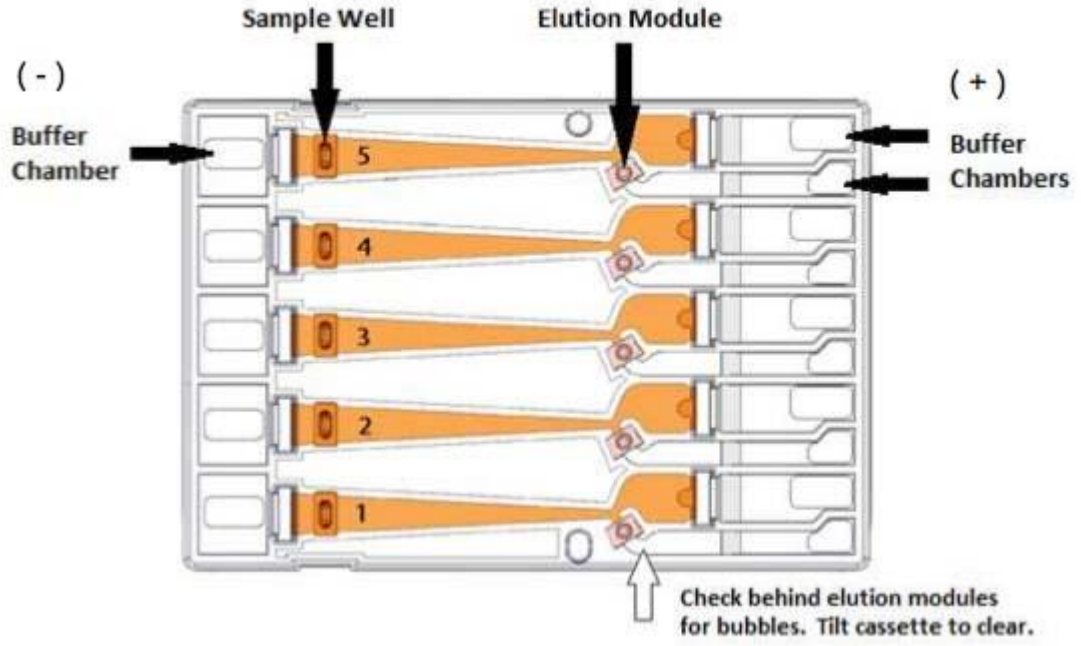
Ligate adapters

- Blunt end ligation:

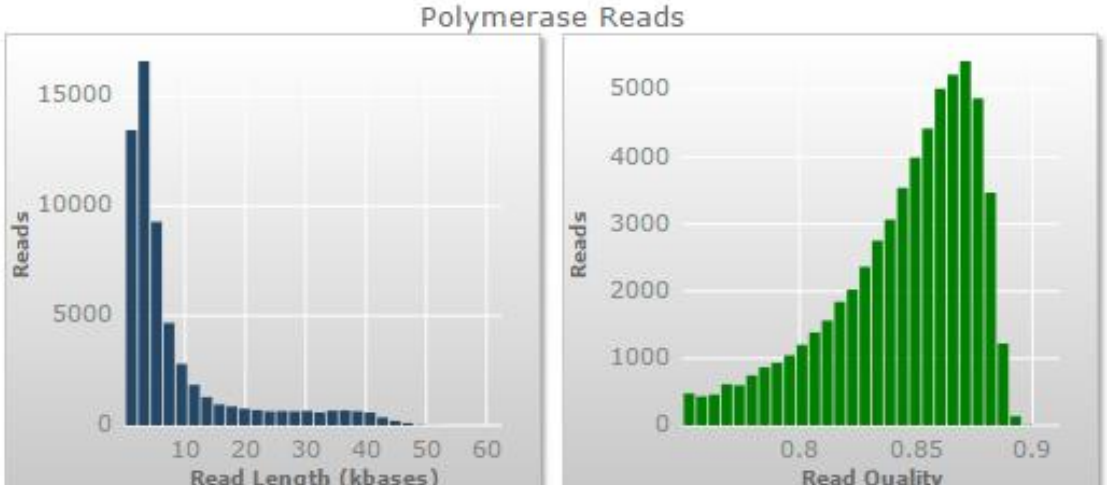
Beware of chimeras:

Purify DNA

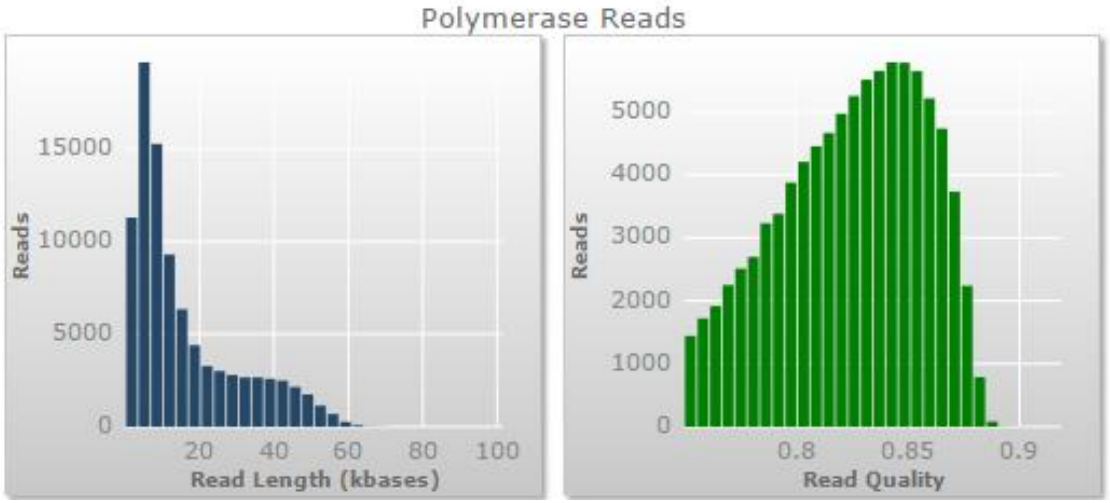
- Do size selection and multiple clean ups in order to remove smaller fragments and adapter chimeras
- Remove non circular DNA using Exo VII and III
- Size select with Blue Pippin



Take home message 1
You want nice and clean DNA



0.5Gb output

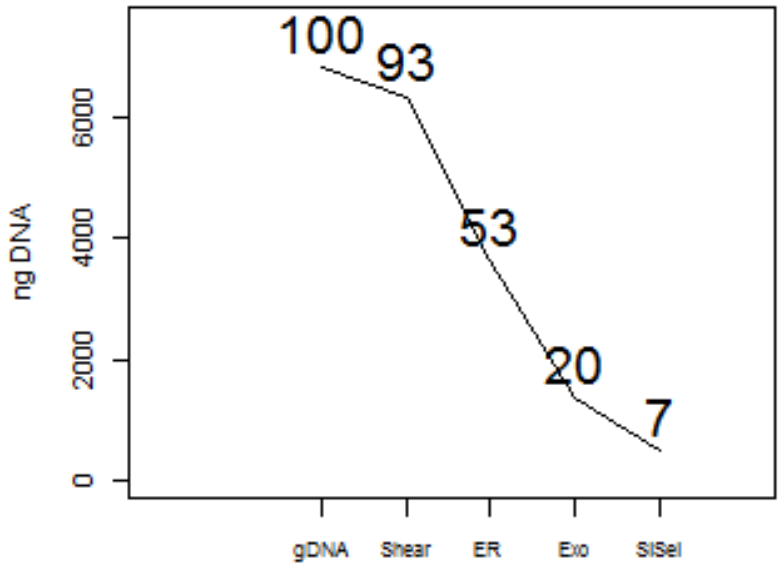


1.5Gb output

Take home message 2

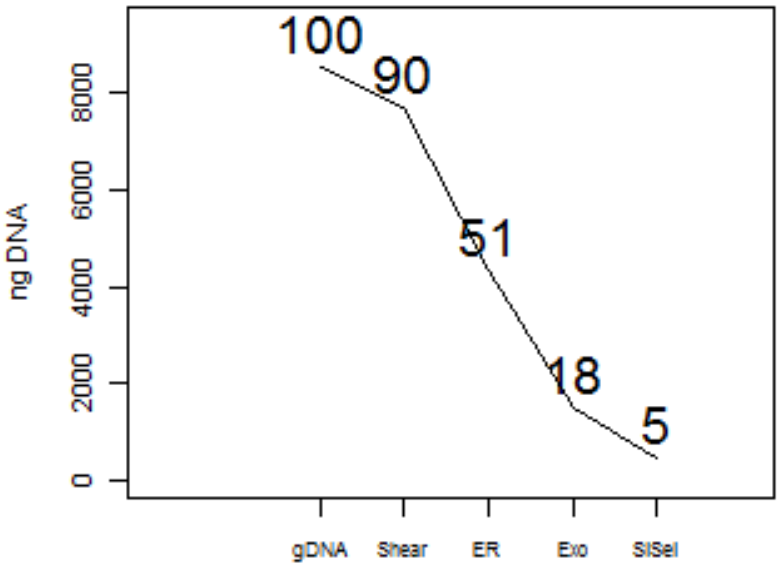
You need a lot of it

6.8ug C34



0.475 ug

8.5ug C39



0.46 ug

Minimum requirements is 0.1 ug

Pacific Biosciences

- Advantages
  - Longer reads lengths (median 12kb up to 40kb with P6-C4 chemistry)
  - Cost per run is low ($250 per run plus $400 per library prep)
  - Same molecule can be sequenced repeatedly
  - Epigenetic modifications can be detected
  - Long reads enable haplotype resolution
- Disadvantages
  - Library prep still required (micrograms needed)
  - If you use PCR based methods – you are no longer sequencing true single molecules
  - Still enzyme based
  - Only 50,000 reads/run. 400-500Mb yield
  - High (12%) error rate per read (but consensus can reduce <1%)
  - $800k machine
  - Run time is anywhere between 40 mins-6 hours per SMRT cell

Bioinformatics Implications

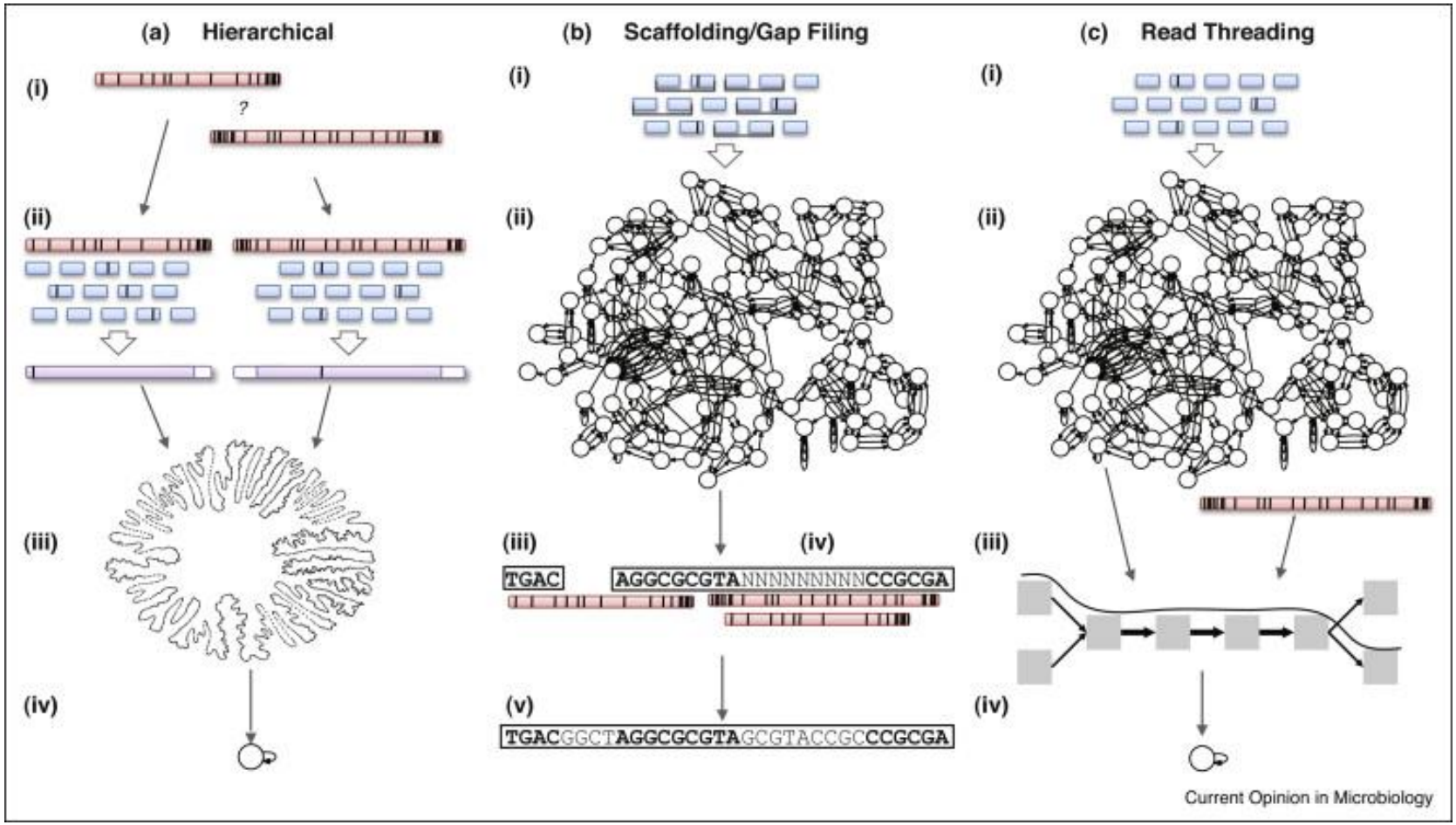
- Relatively low data and high per base cost limits widespread use
- Can obtain useful 20-40kb fragments (P4-C6 chemistry)
- Best used in conjunction with error correction algorithms utilising shorter PacBio reads (or Illumina data)– e.g. Wheat D genome
- Excellent to assist scaffolding of genomes
- Able to generate complete bacterial genomes
- Has been used to generate higher eukaryote genomes (e.g. Drosophila, Human) but cost can be prohibitive
- Ability to sequence entire

Sergey Koren, Adam M Phillippy, One chromosome, one contig: complete microbial genomes from long-read sequencing and assembly, Current Opinion in Microbiology, Volume 23, February 2015, Pages 110-120, ISSN 1369-5274, http://dx.doi.org/10.1016/j.mib.2014.11.014. (http://www.sciencedirect.com/science/article/pii/S1369527414001817)

Koren, Sergey; Schatz, Michael C; Walenz, Brian P; Martin, Jeffrey; Howard, Jason T et al. (2012)
Hybrid error correction and de novo assembly of single-molecule sequencing reads
Nature biotechnology vol. 30 (7) p. 693-700

Chin, C.-S., Alexander, D. H., Marks, P., Klammer, A. A., Drake, J., Heiner, C., … Korlach, J. (2013). Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data. *Nature methods*, *10*(6), 563–9. doi:10.1038/nmeth.2474

Genome assembly methods



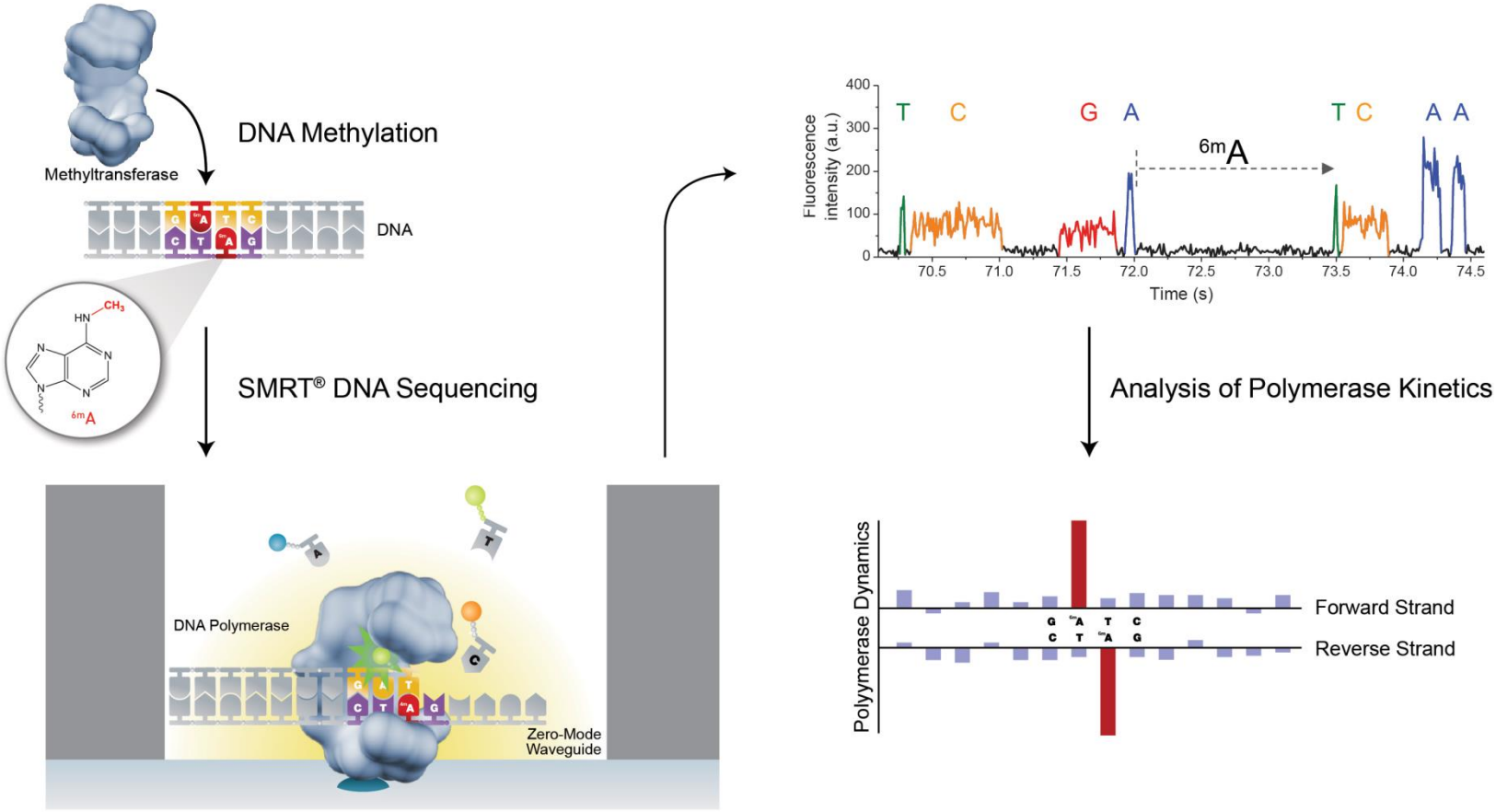
http://www.sciencedirect.com/science/article/pii/S1369527414001817

Novel applications

- Epigenetic changes (e.g. Methylation) affect the amount of time a fluorophore is held by the polymerase

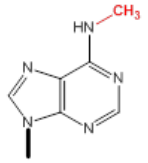
- Circularise each DNA fragment and sequence continuously

Epigenetic changes

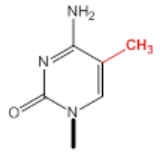


Types of modifications

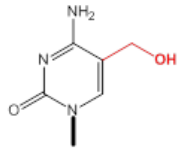
Eukaryotes:



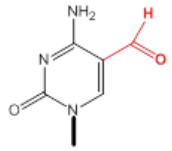
6-mA



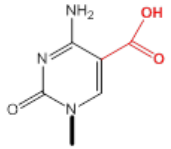
5-mC



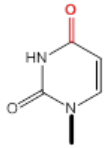
5-hmC



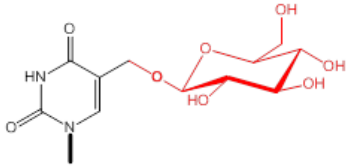
5-fC



5-caC

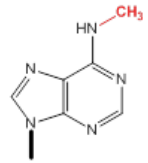


U

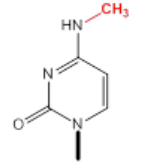


Base J

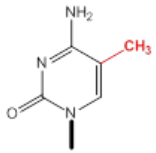
Prokaryotes:



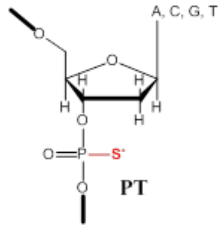
6-mA



4-mC



5-mC

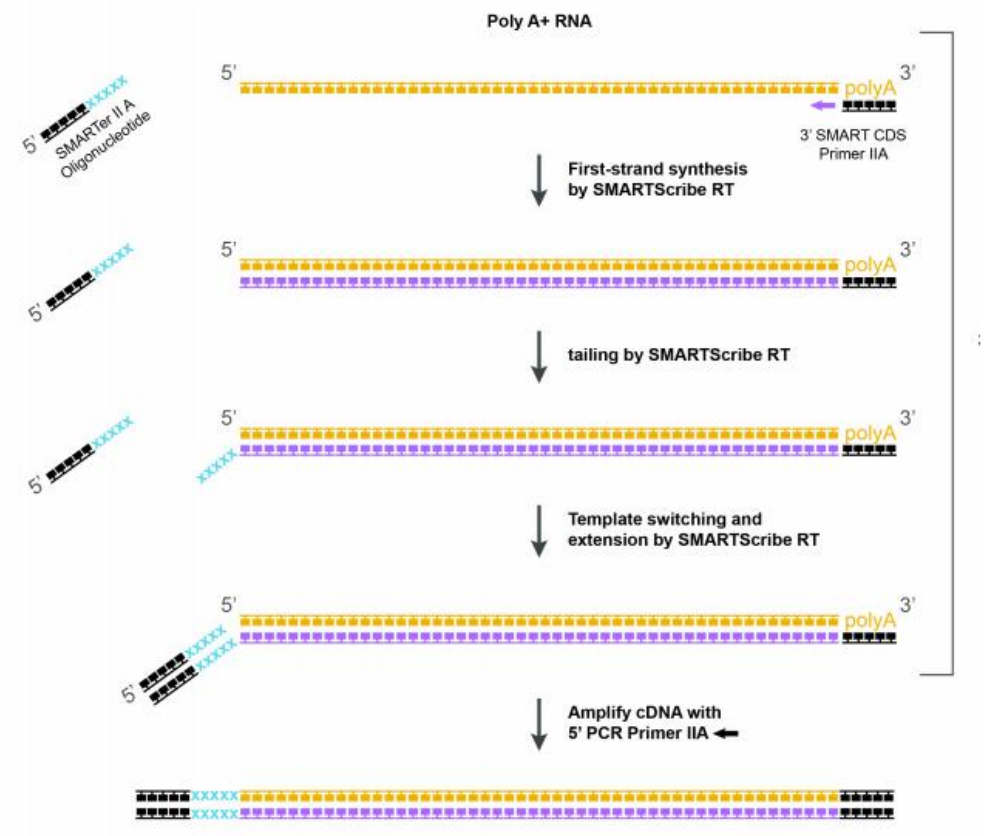


PT

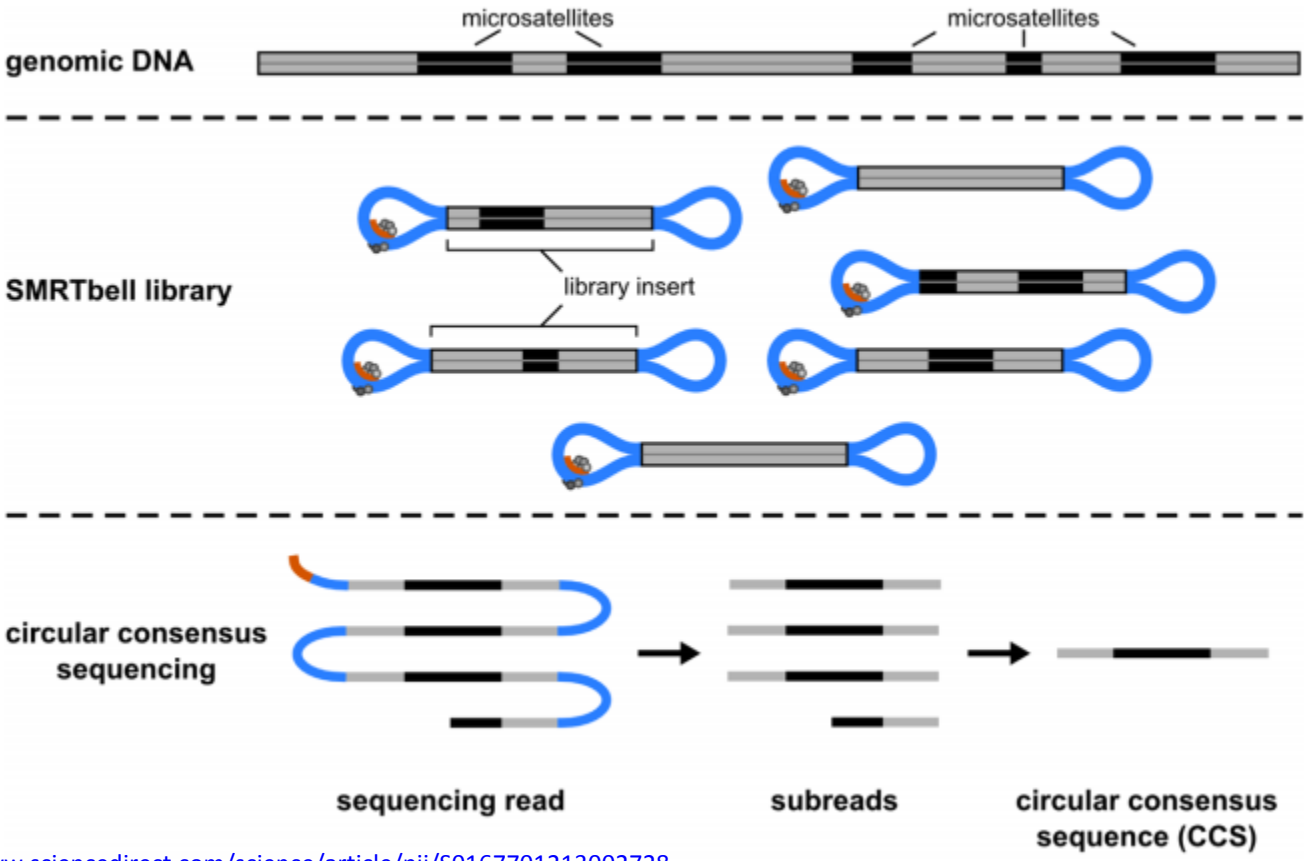
http://www.pacb.com/applications/epigenetics/

Full length transcript sequencing

- Requires polyA RNA
- Uses SMRTer approach
- Ability to sequence full length transcripts with no need for assembly

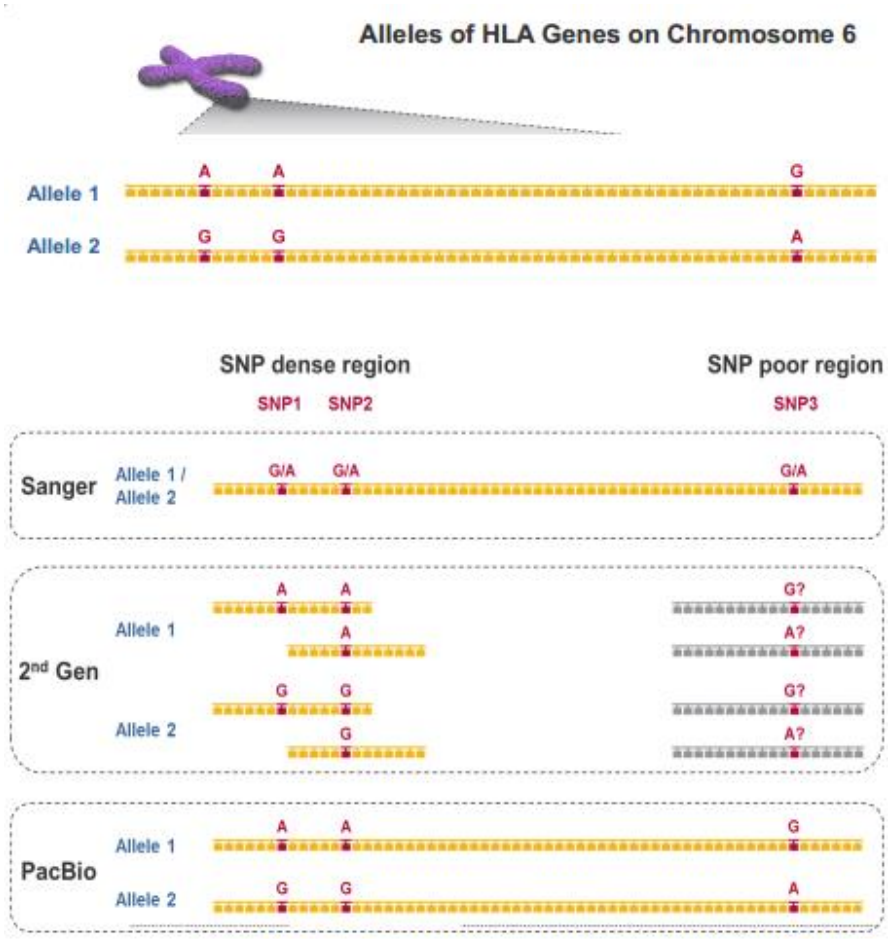


Circular consensus sequencing for rRNA or microsatellites



http://www.sciencedirect.com/science/article/pii/S0167701213002728
http://www.biotechniques.com/multimedia/archive/00230/BTN_A_000114104_O_230651a.pdf
http://www.microbiomejournal.com/content/1/1/10

Long reads to resolve haplotypes



http://www.pacificbiosciences.com/pdf/Poster_AlleleLevelSequencing_PhasingFulllengthHLAClassIandIIGenes.pdf

PacBio developments

- Sequel system due to ship in the coming months
- Generate approximately 7Gbase data per cell
- $350,000 (half the cost of an RSII)
- Chemistry and analysis should be similar to that described above

PacBio training resources

- https://github.com/PacificBiosciences/Bioinformatics-Training/wiki

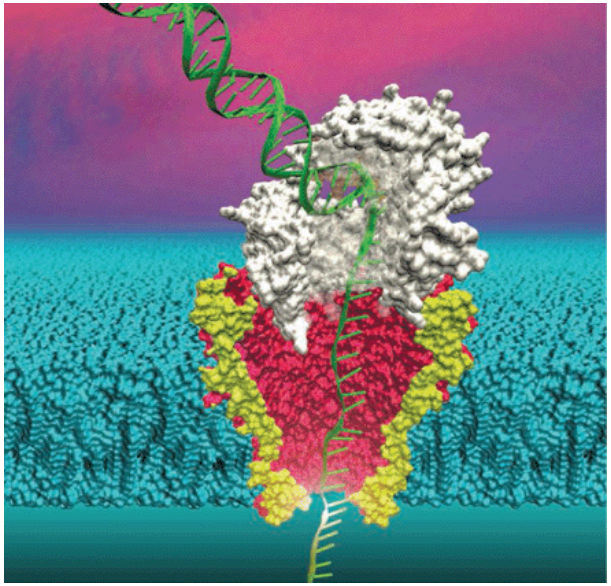
Useful PacBio papers

- Resolving the complexity of the human genome using single-molecule sequencing
- Defining a personal, allele-specific, and single-molecule long-read transcriptome
- Heyn, Holger et al. (2015) An adenine code for DNA: A second life for N6-methyladenine. *Cell*

Features of PacBio sequencing

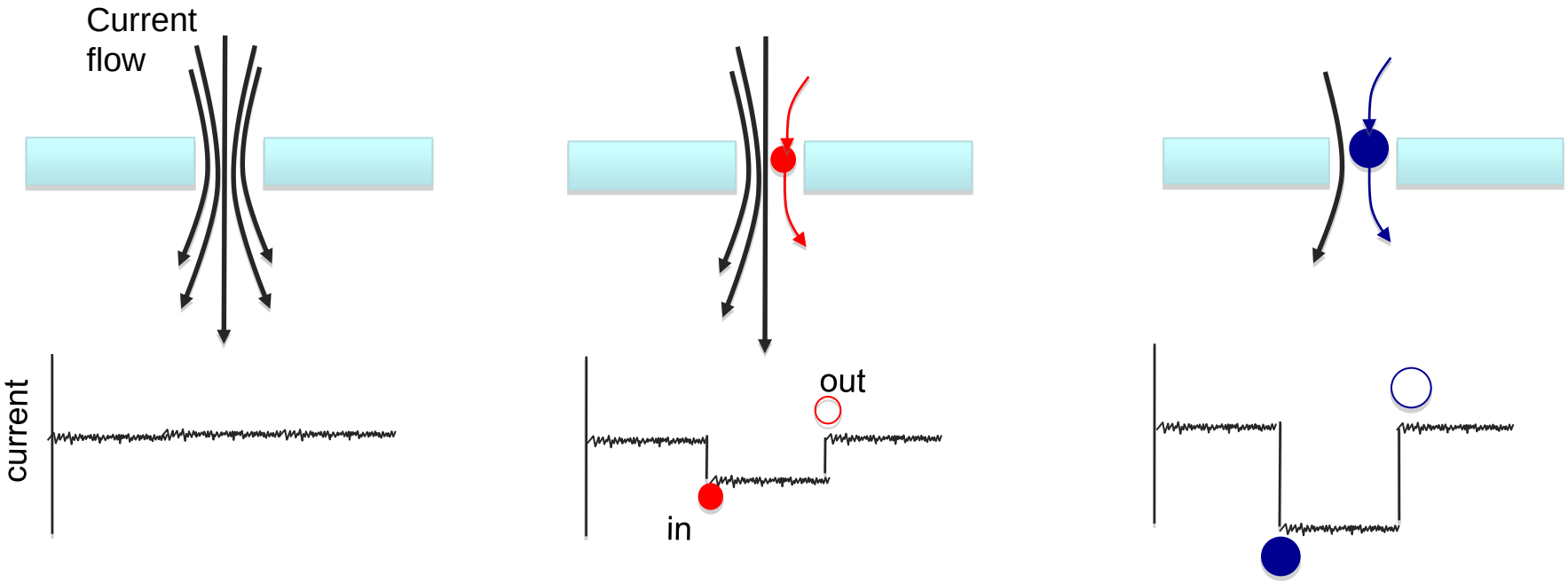
- ~50,000 sequences per SMRT-cell
- Up to 40kb read lengths
- ~$5 per megabase
- Accuracy depends on DNA length ~0.5%-12% error rate
- Ability to sequence native DNA – relieving PCR biases
- Claimed random error profile

Nanopore sequencing



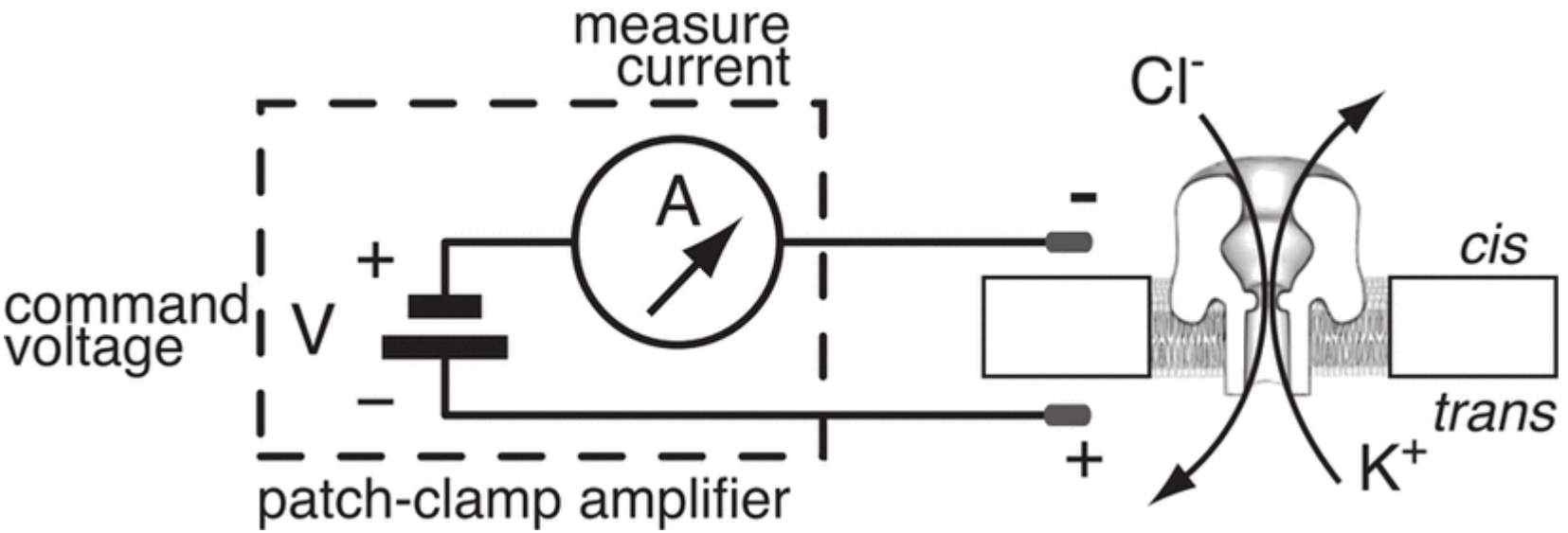
What is a nanopore?

- Nanopore = ‘very small hole’
- Electrical current flows through the hole
- Introduce analyte of interest into the hole ➔ identify “analyte” by the disruption or block to the electrical current



https://nanoporetech.com/science-technology/introduction-to-nanopore-sensing/introduction-to-nanopore-sensing

Detection



http://ieeexplore.ieee.org/xpls/icp.jsp?arnumber=5626570

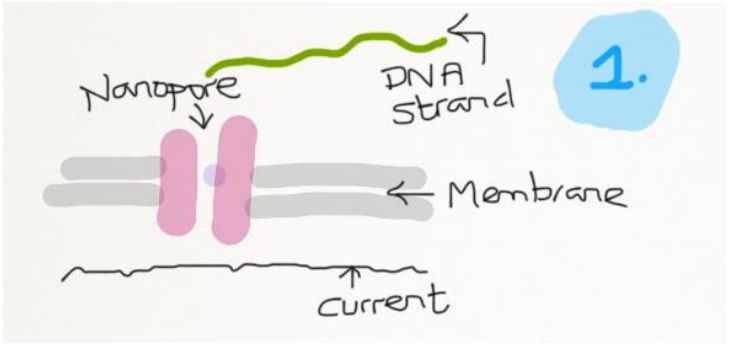
Types of pore

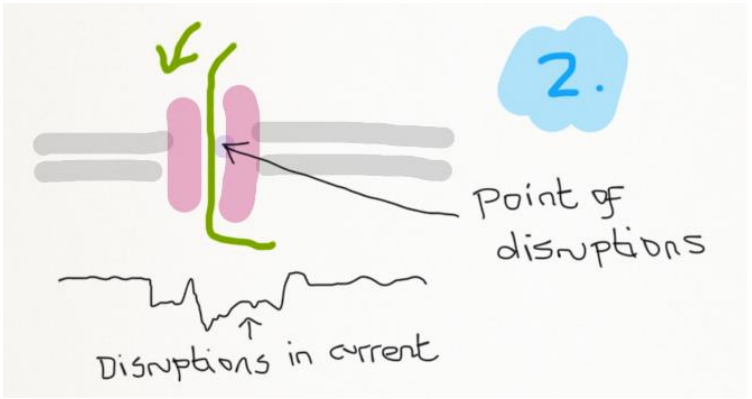
- Either biological or synthetic

- Biological
  - Lipid bilayers with alpha-haemlolysin pores
  - Best developed
  - Pores are stable but bilayers are difficult to maintain
- Synthetic
  - Graphene, or titanium nitride layer with solid-state pores
  - Less developed
  - Theoretically much more robust

Nanopore DNA sequencing

- Theory is quite simple
- Feed a 4nm wide DNA molecule through a 5nm wide hole
- As DNA passes through the hole, measure some property to determine which base is present
- Holds the promise of no library prep and enormously parallel sequencing
- In practice this is not easy to achieve

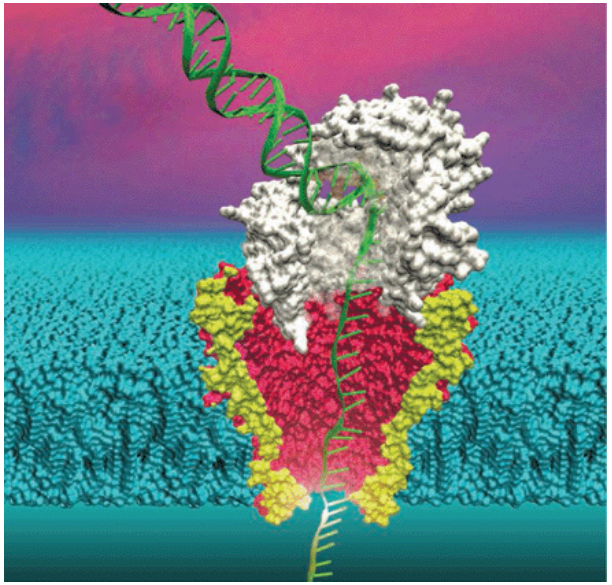




http://thenerdyvet.com/category/tech/

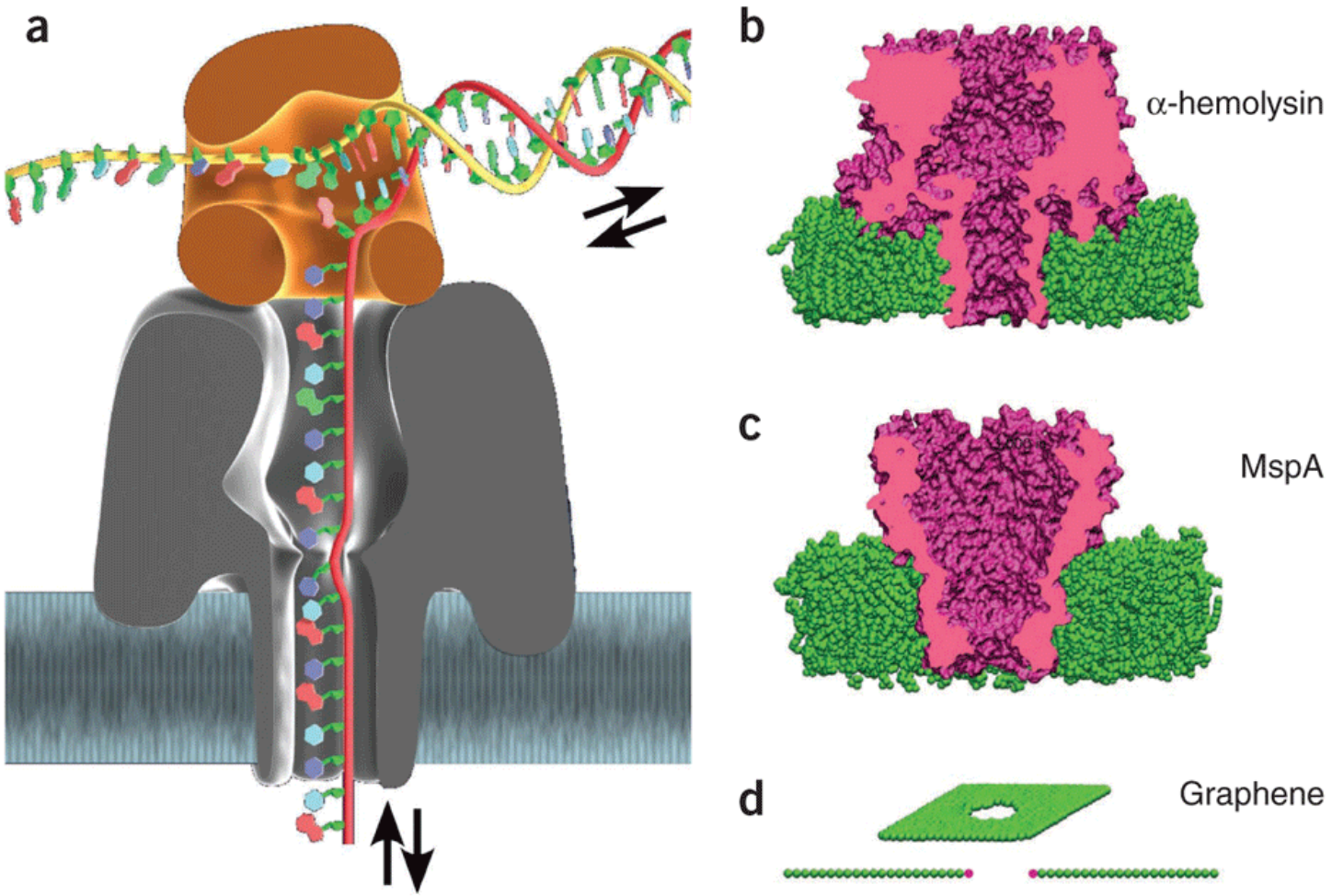
Nanopore sequencing

- In practice, it is much harder
- Problems:
  - DNA moves through the pore quickly
  - Holes are difficult/impossible to design to be thin enough so that only one base is physically located within the hole
  - DNA bases are difficult to distinguish from each other without some form of labelling
  - Electrical noise and quantum effects make signal to noise ratios very low
  - Search space for DNA to find a pore is large



http://omicfrontiers.com/2014/04/10/nanopore

Nanopore sequencing



http://www.nature.com/nbt/journal/v30/n4/full/nbt.2181.html

Nucleotide Recognition

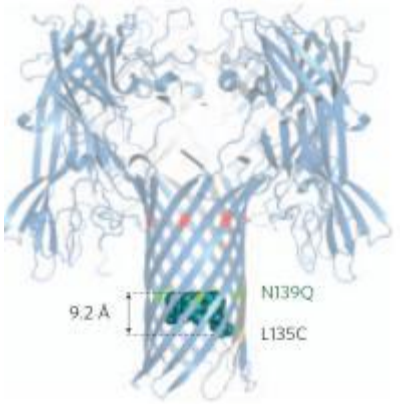
nature nanotechnology

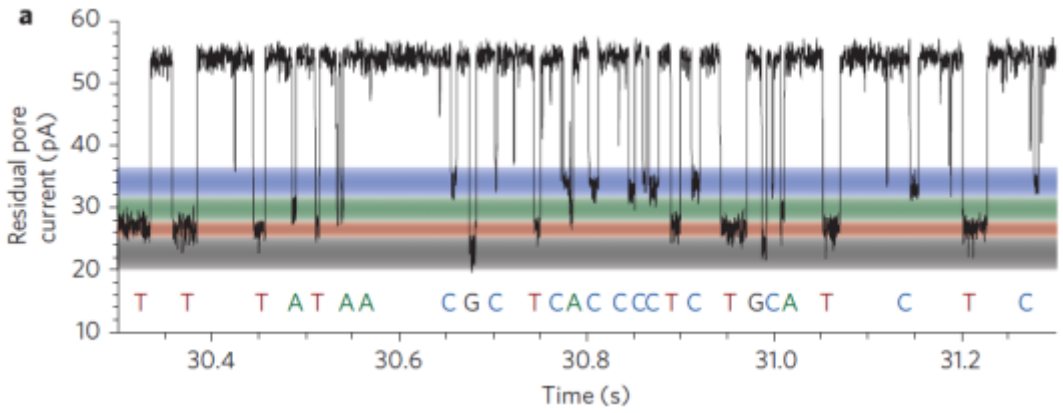
ARTICLES

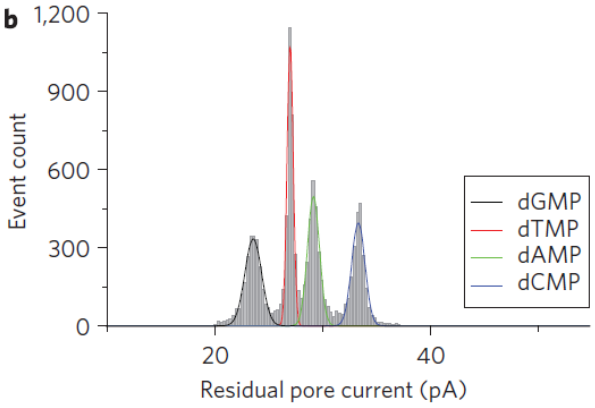
PUBLISHED ONLINE: XX XX 2009 | DOI: 10.1038/NNANO.2009.12

Continuous base identification for single-molecule nanopore DNA sequencing

James Clarke[1], Hai-Chen Wu[2], Lakmal Jayasinghe[1,2], Alpesh Patel[1], Stuart Reid[1] and Hagan Bayley[2]*







Approaches to simplify nanopore sequencing

- Slow down movement of bases through nanopore
  - Use an enzyme to chop DNA up and sequence individual bases as they pass through a pore
  - And/or use an enzyme to slow the progress of DNA through a pore
  - Monitor capacitive changes in the bilayer
- Hybridize labels to single stranded DNA
  - Force the labels to disassociate as they pass through the pore
  - Detect the labels

Niedringhaus, T. P., Milanova, D., Kerby, M. B., Snyder, M. P., & Barron, A. E. (2011). Landscape of next-generation sequencing technologies. *Analytical chemistry*, *83*(12), 4327–41. doi:10.1021/ac2010857

Companies involved

- ONT is closest to commercialisation

Oxford Nanopore MAP programme

- MinIon Access Programme
- Provides access to several flowcells and reagents for library preparation
- Beta testing programme – open to all
- Registration fee of $1000
  - Access to a MinIon system and starter pack of reagents
- Additional flowcells for $500-$900 each

Oxford Nanopore

Oxford Nanopore platforms

MinION Mk 1

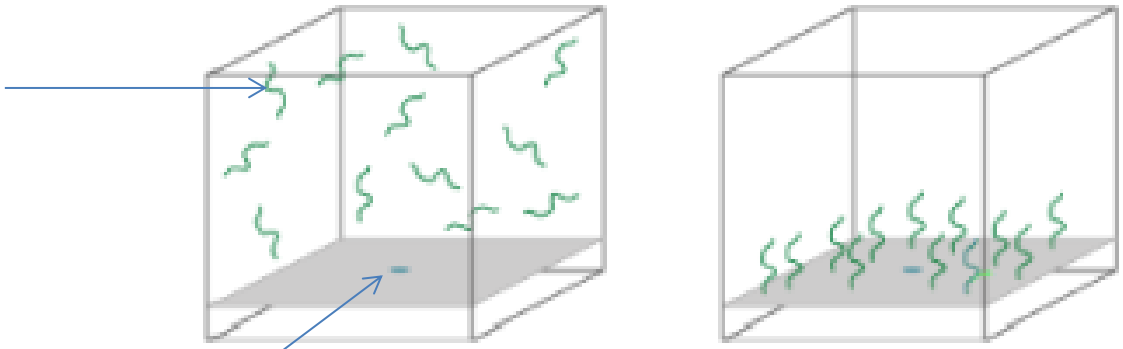
Up to 1-2Gbases/run

PromethION

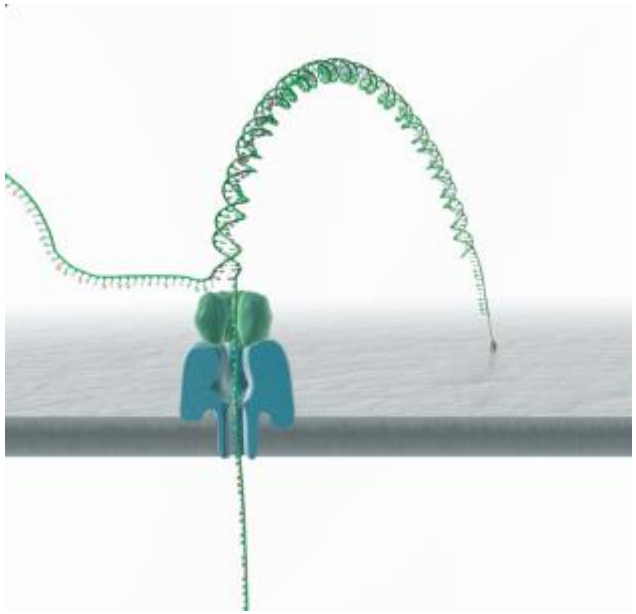
Up to 20-30Gbase/run

DNA binding to membrane

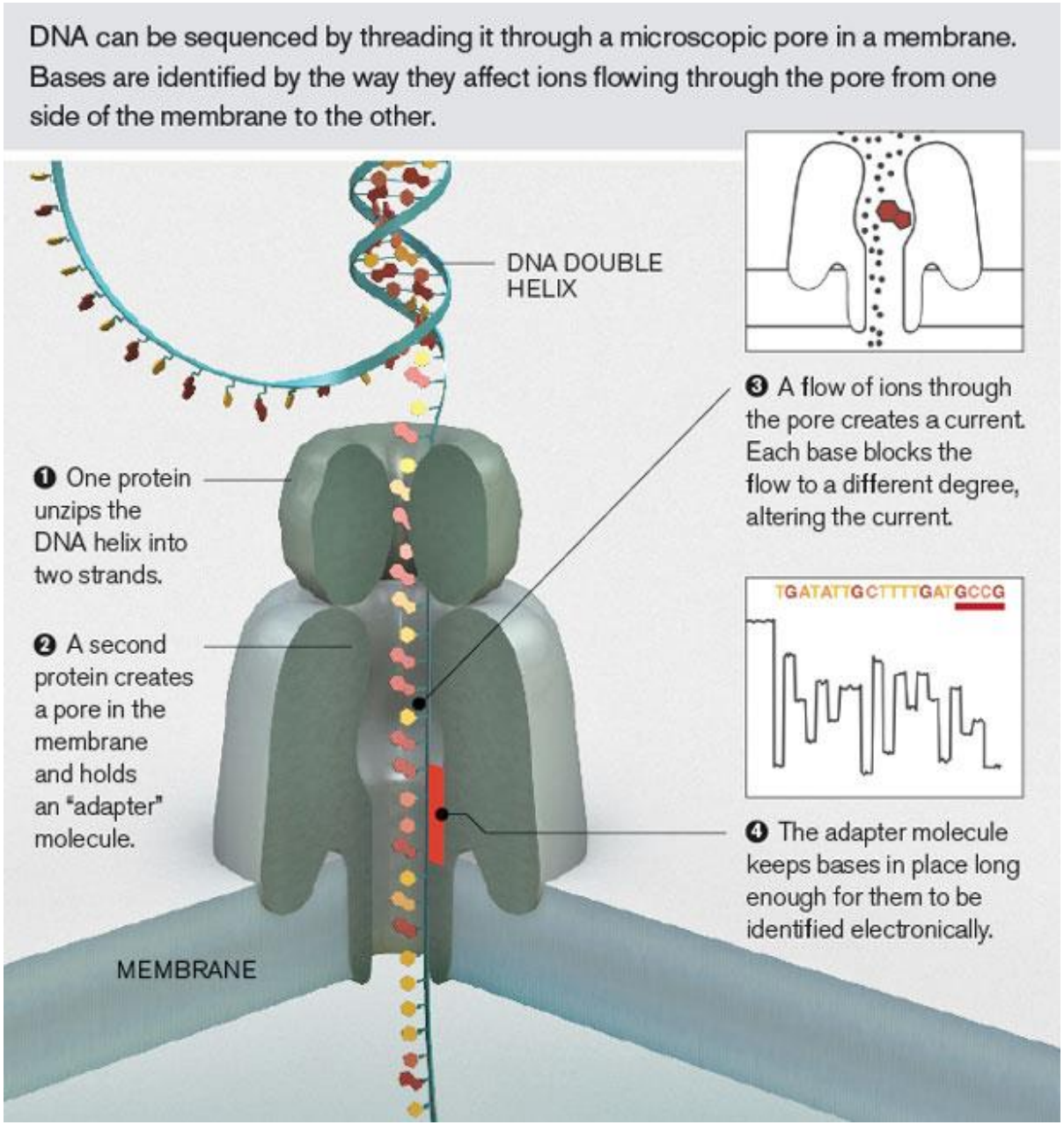
Double stranded DNA fragment



Pore



Oxford Nanopore principle



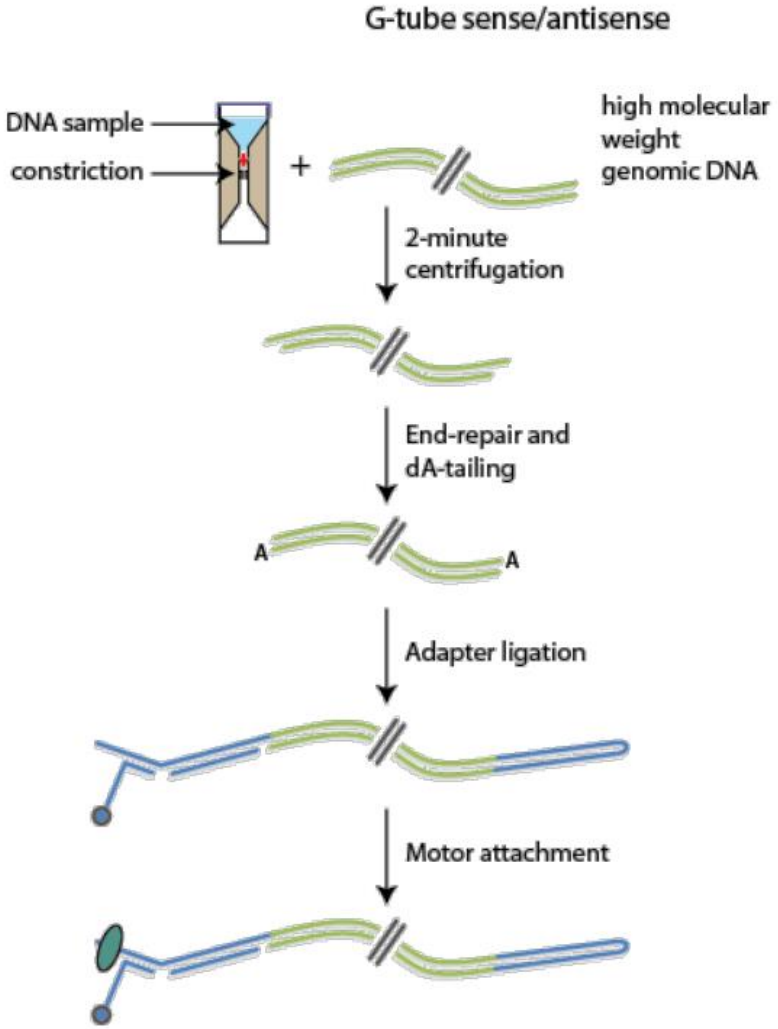
http://www2.technologyreview.com/article/427677/nanopore-sequencing/

MinIon features

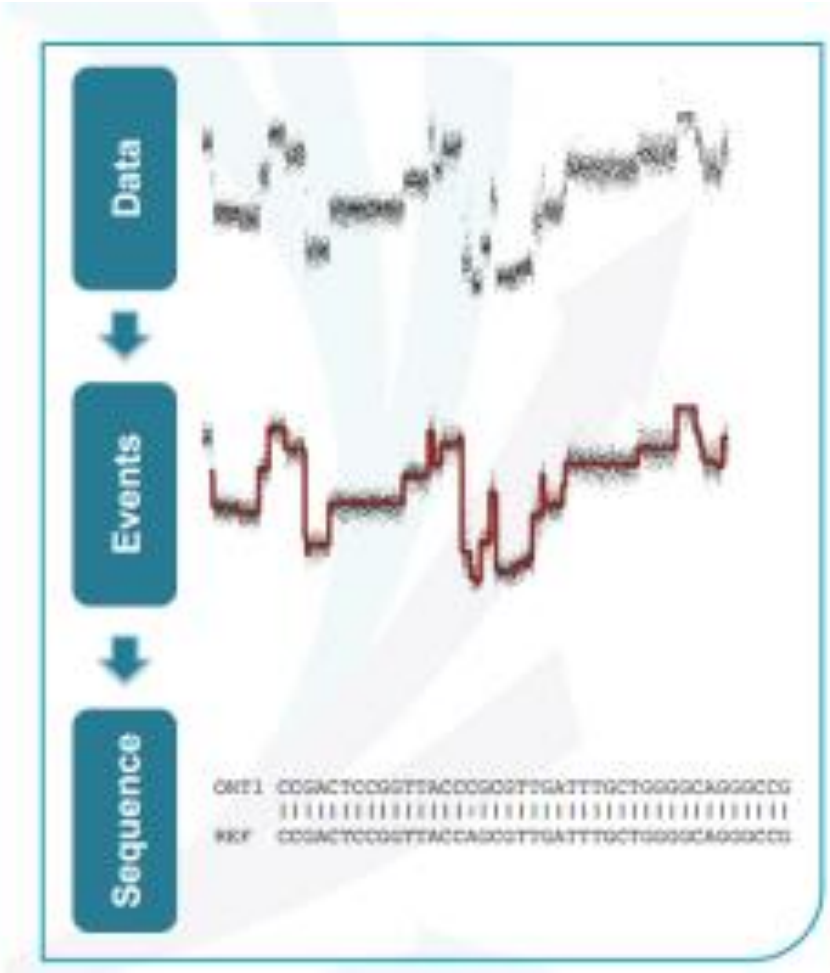
- 512 pores
- Library preparation is required
- Read lengths up to 40-50kb
  - Limited by input DNA
- Up to 2Gbase output
  - Complex flowcell manufacturing
  - Improved in the past few months

Library preparation

- Input requirements
  - Depends on fragment length required
  - Ideally upwards of 1ug of DNA
  - Low input option available – 20ng
- Issues – keeping long DNA fragments
- New kits attempting to minimise input requirements



Challenge of basecalling

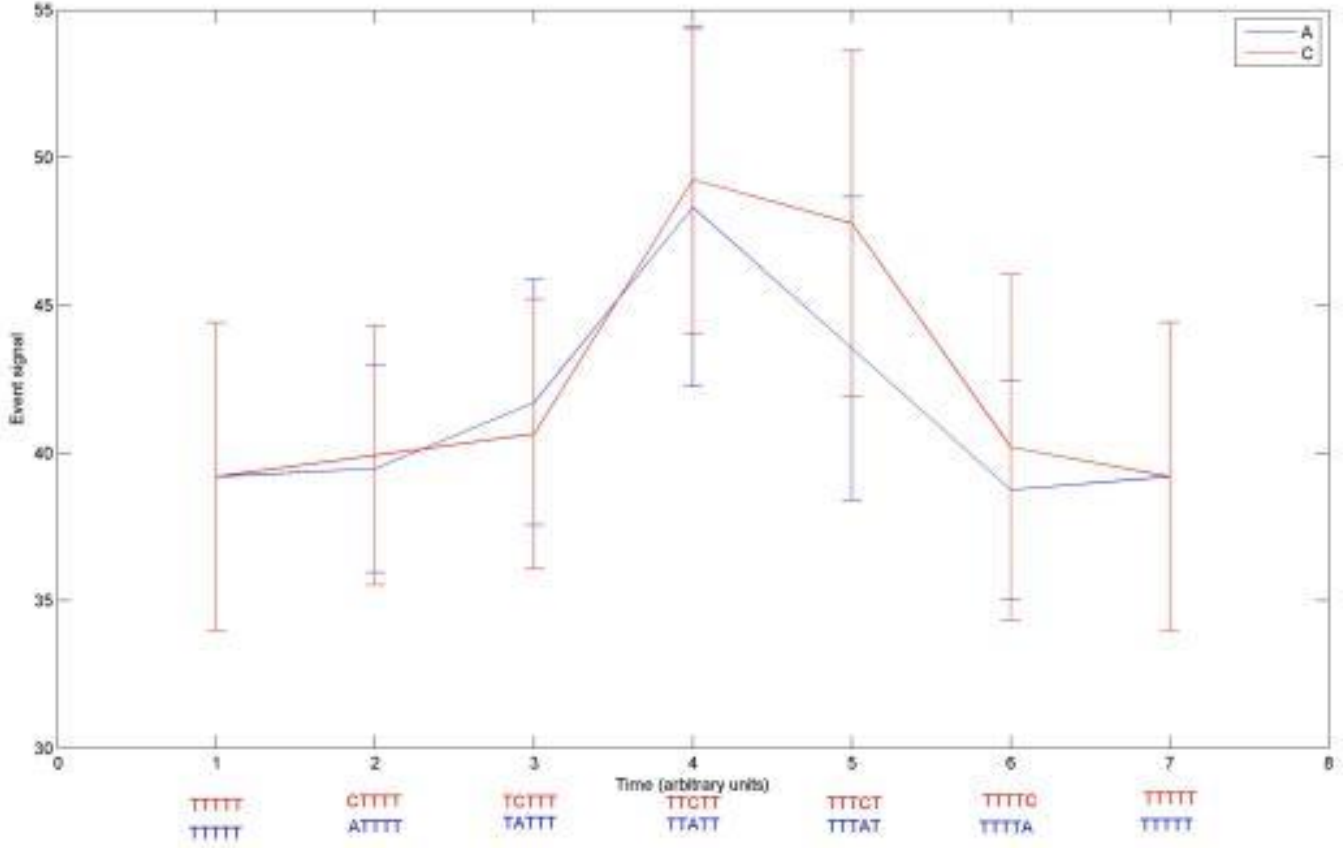


- Hidden Markov model
- Only four options per transition
- Pore type = distinct kmer length

- Form probabilistic path through measured states currents and transitions
  - e.g. Viterbi algorithm

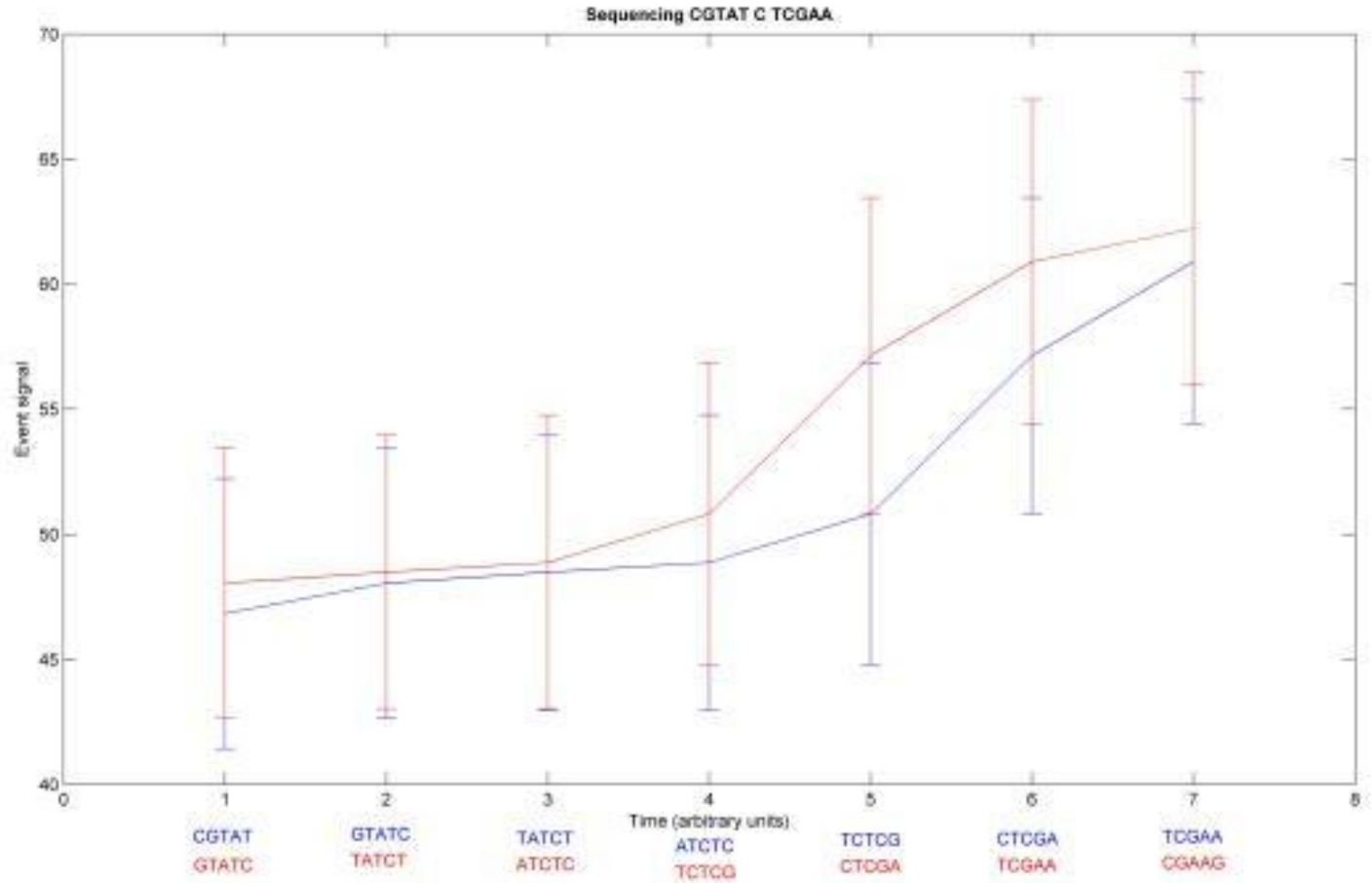
Challenge of 5-mer basecalling

- TTTTTATTTTT vs TTTTTCTTTTT



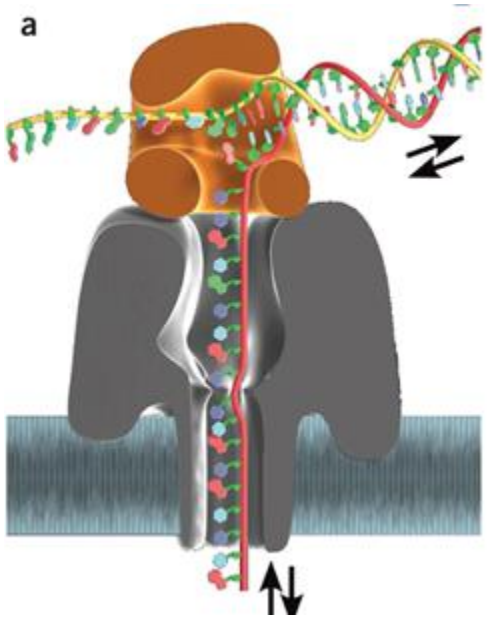
Challenge of 5-mer basecalling

- CGTATTCGAA vs CGTATCTCGAA

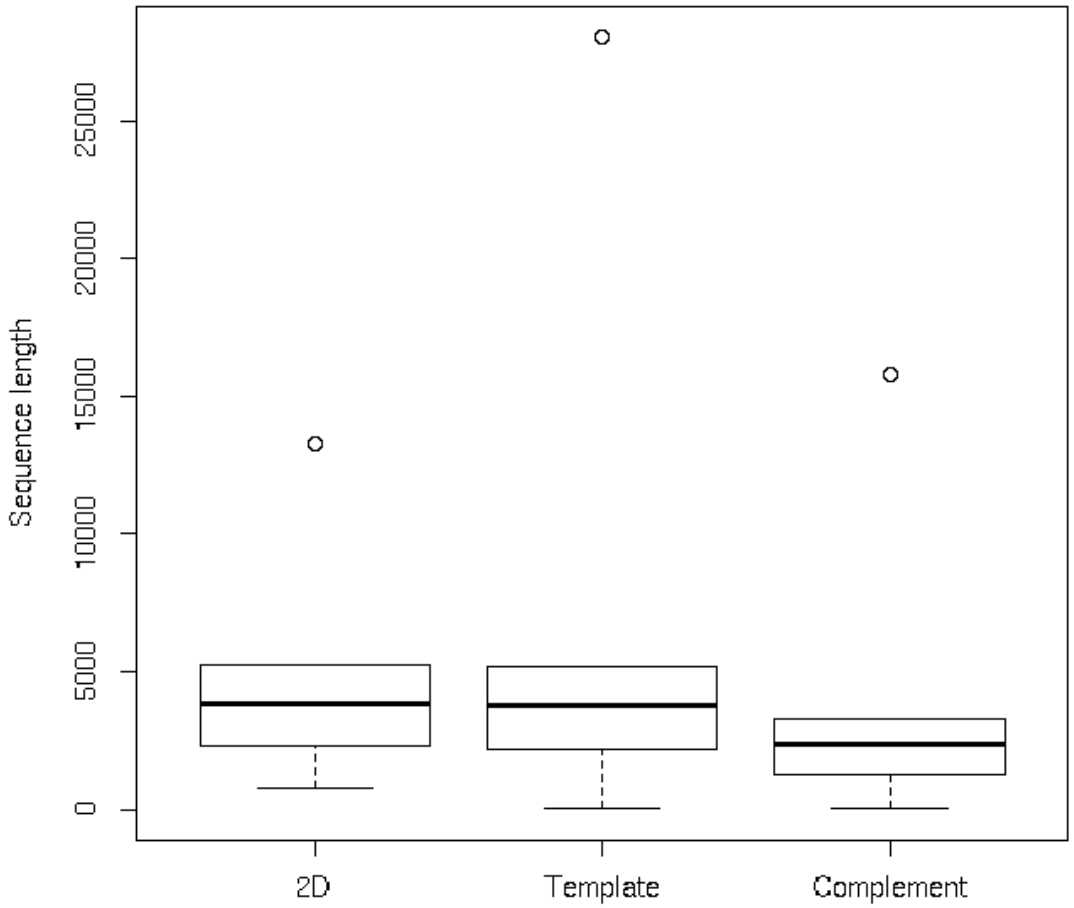


Basecalling 1D vs 2D reads

- Both the template and complementary strand can be sequenced
- This doesn't always work
- If it does, the base-calling can be improved
- Different kmers at the same locus can improve basecalling
- Up to 40% of reads using latest chemistry are 2D
- Attempts are being made to modify the library preparation to increase the proportion of 2D reads

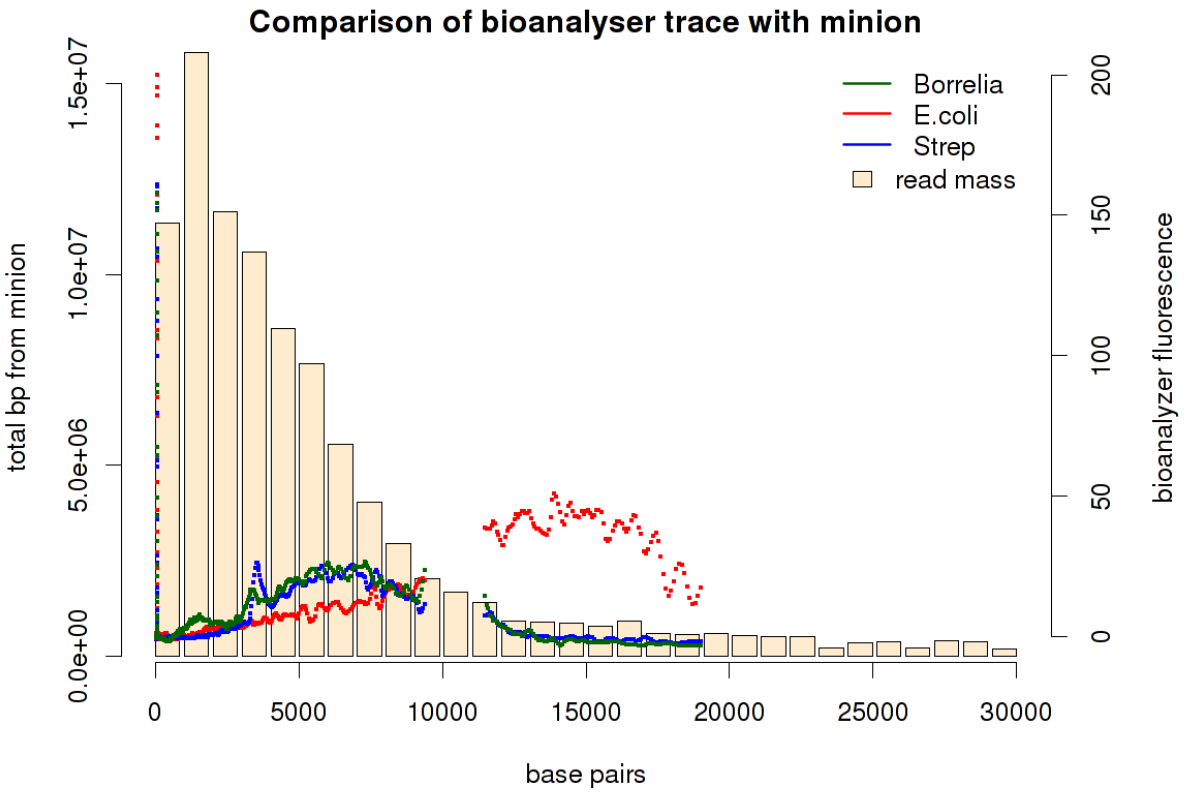


Lengths of 2D vs 1D reads

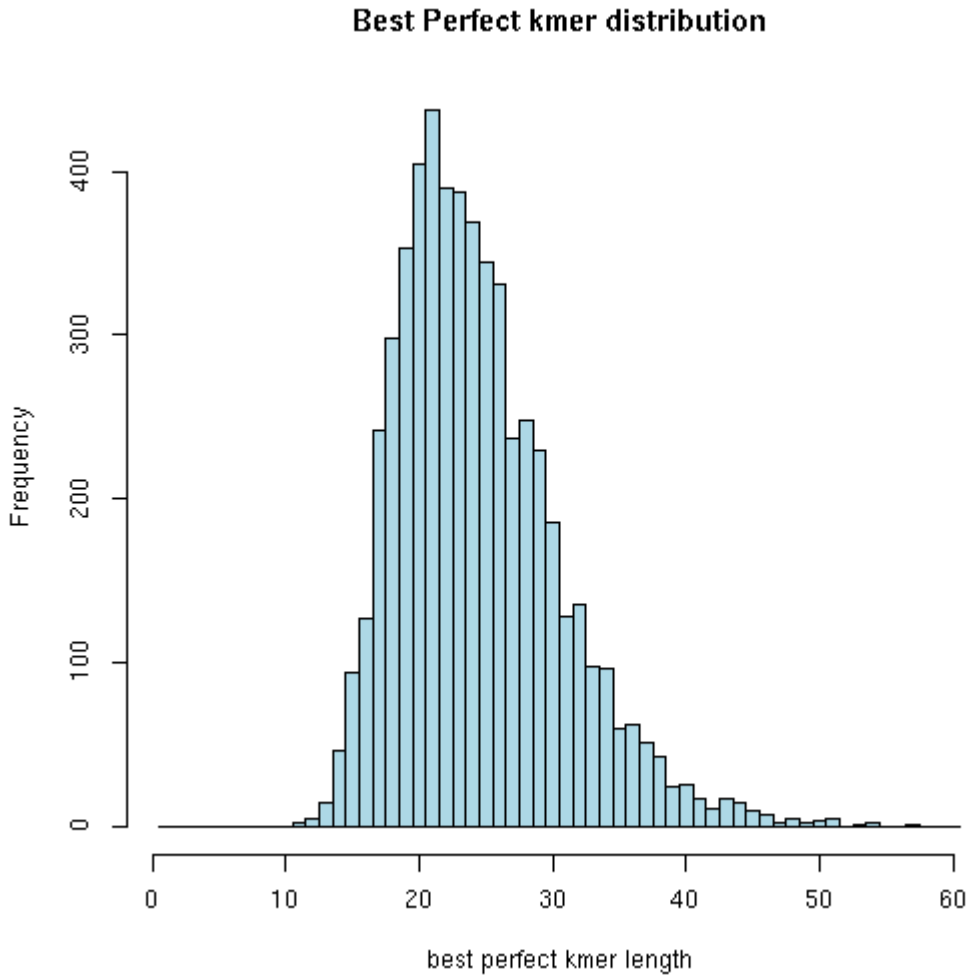


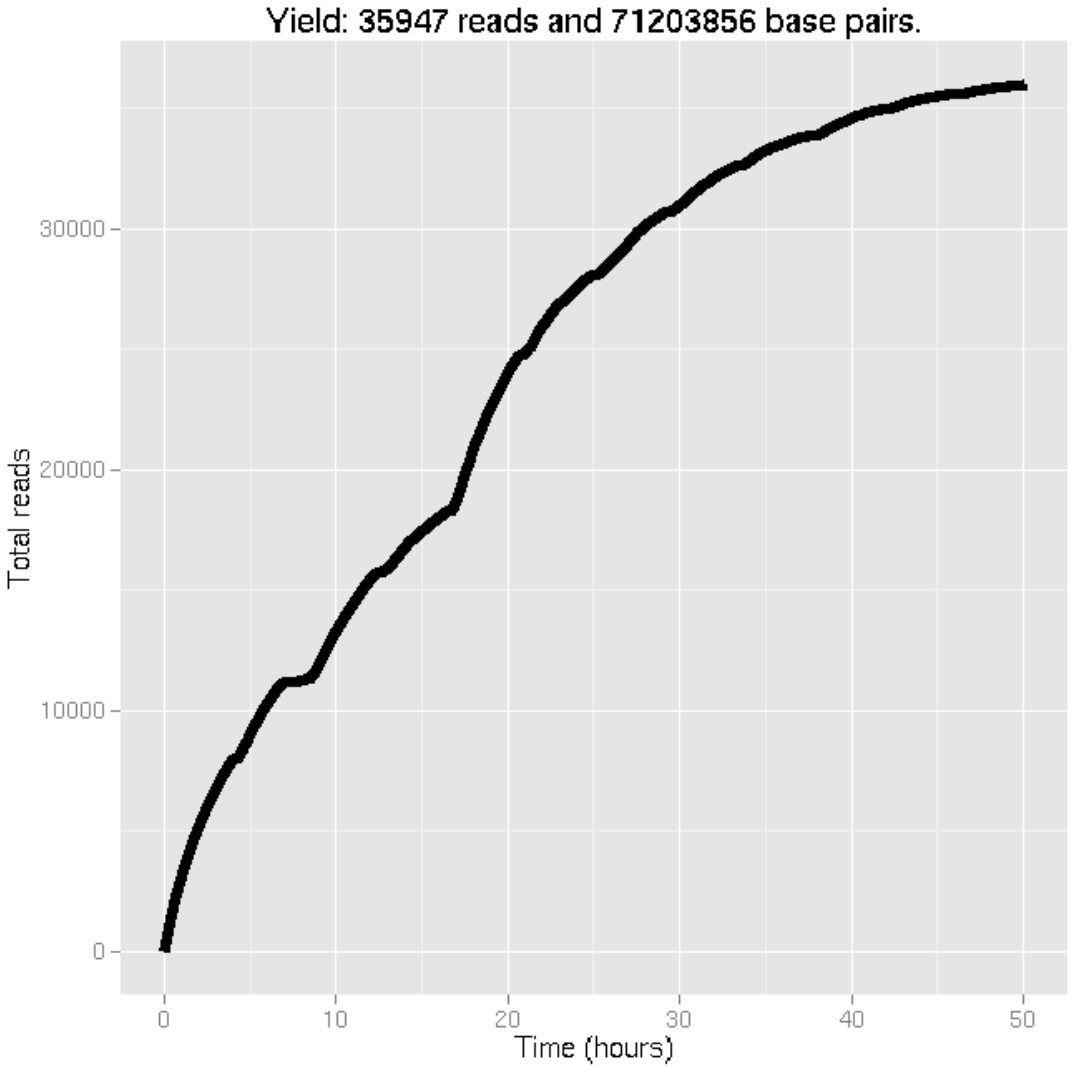
Read lengths

- Highly dependent on input DNA length
- Difficult to preserve DNA lengths

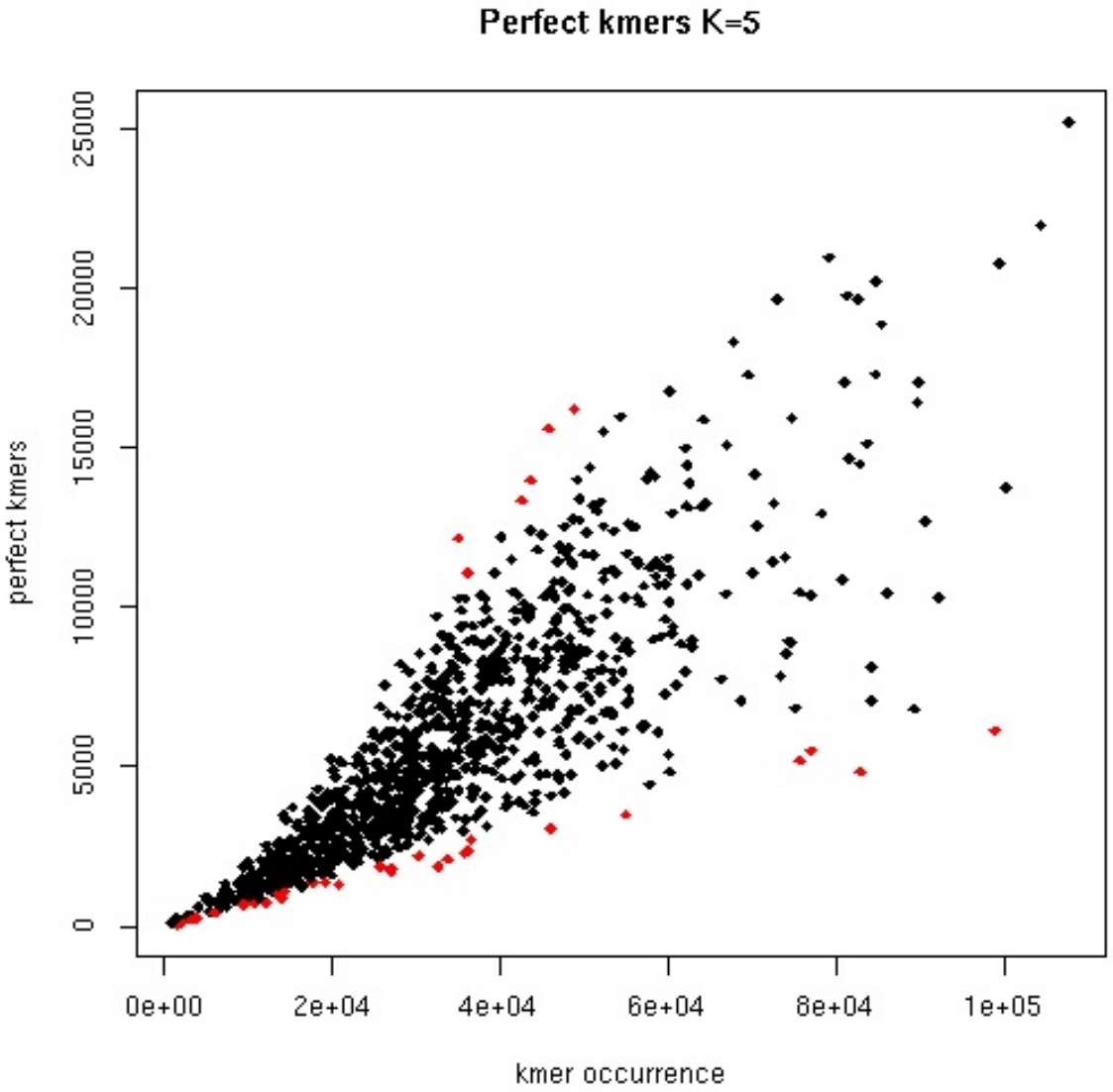


Longest perfect stretches




Yield: 35947 reads and 71203856 base pairs.
Total reads
0
10000
20000
30000
0
10
20
30
40
50
Time (hours)

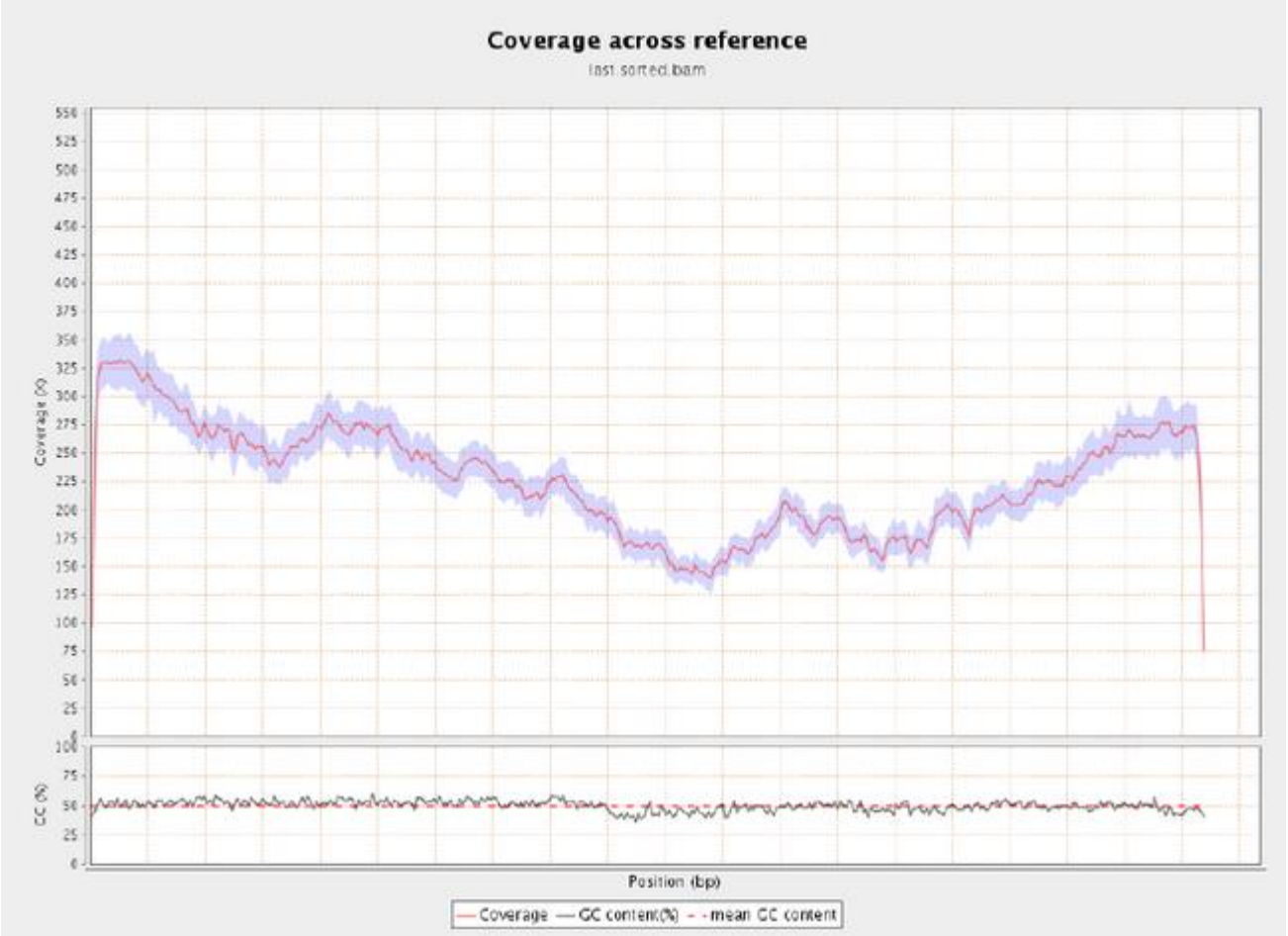
Hard to read motifs



Hard Kmers
AAAAA
ACCTA
ACCTC
AGCGC
AGCTA
AGGTC

Easy Kmers
AAGAA
ACGAA
CGTTC
CTTTC
GAACG

Coverage of an E.coli genome

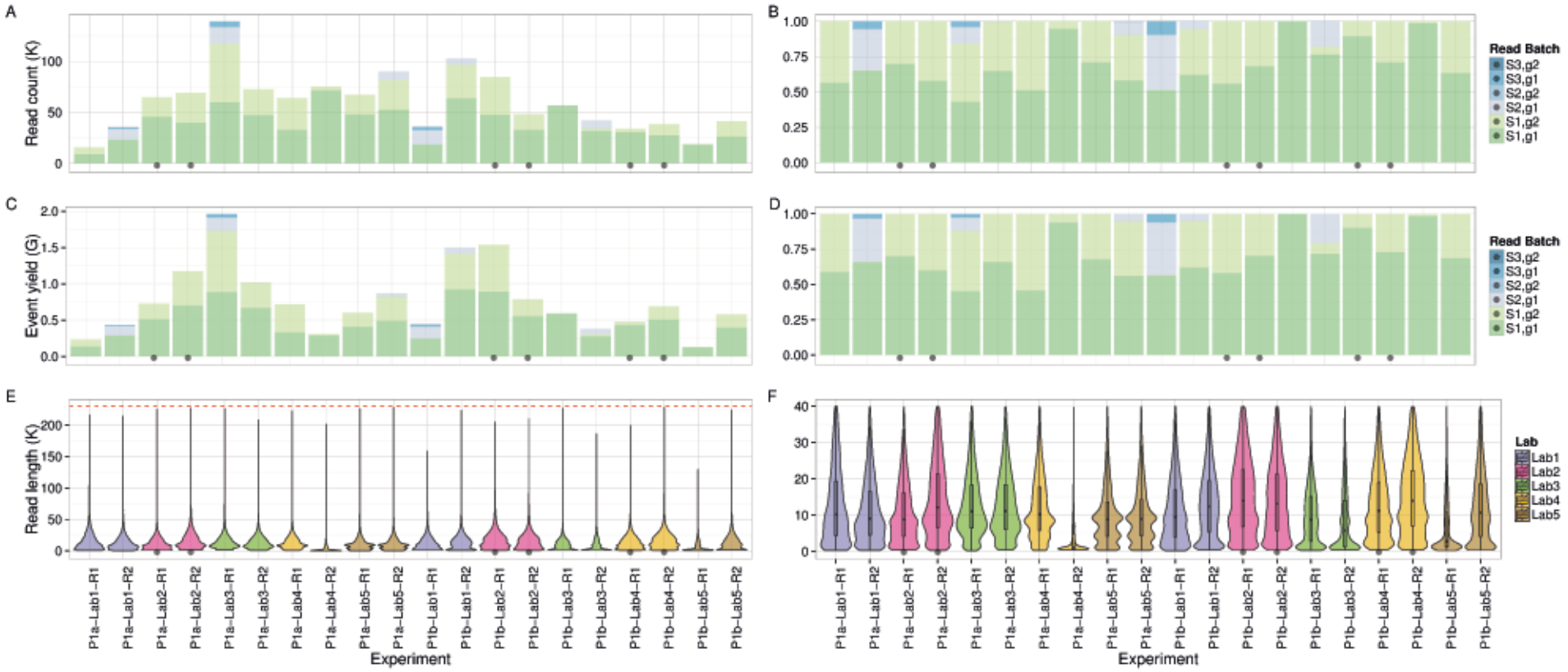



12 kb
511 bp

MinIon Analysis Reference Consortiom (MARC)

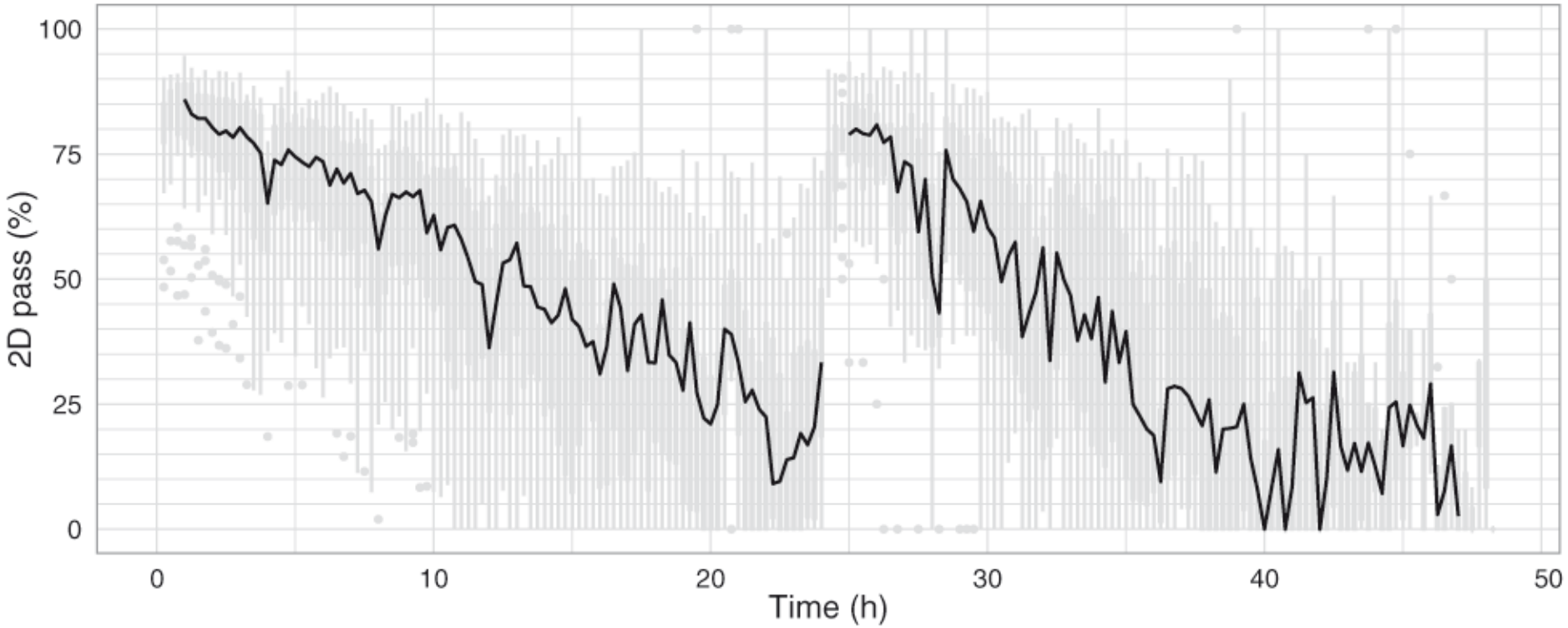
- Group of 20 labs evaluating MinIon performance using E.coli
- http://f1000research.com/articles/4-1075/v1

Yield of MinIon flowcells



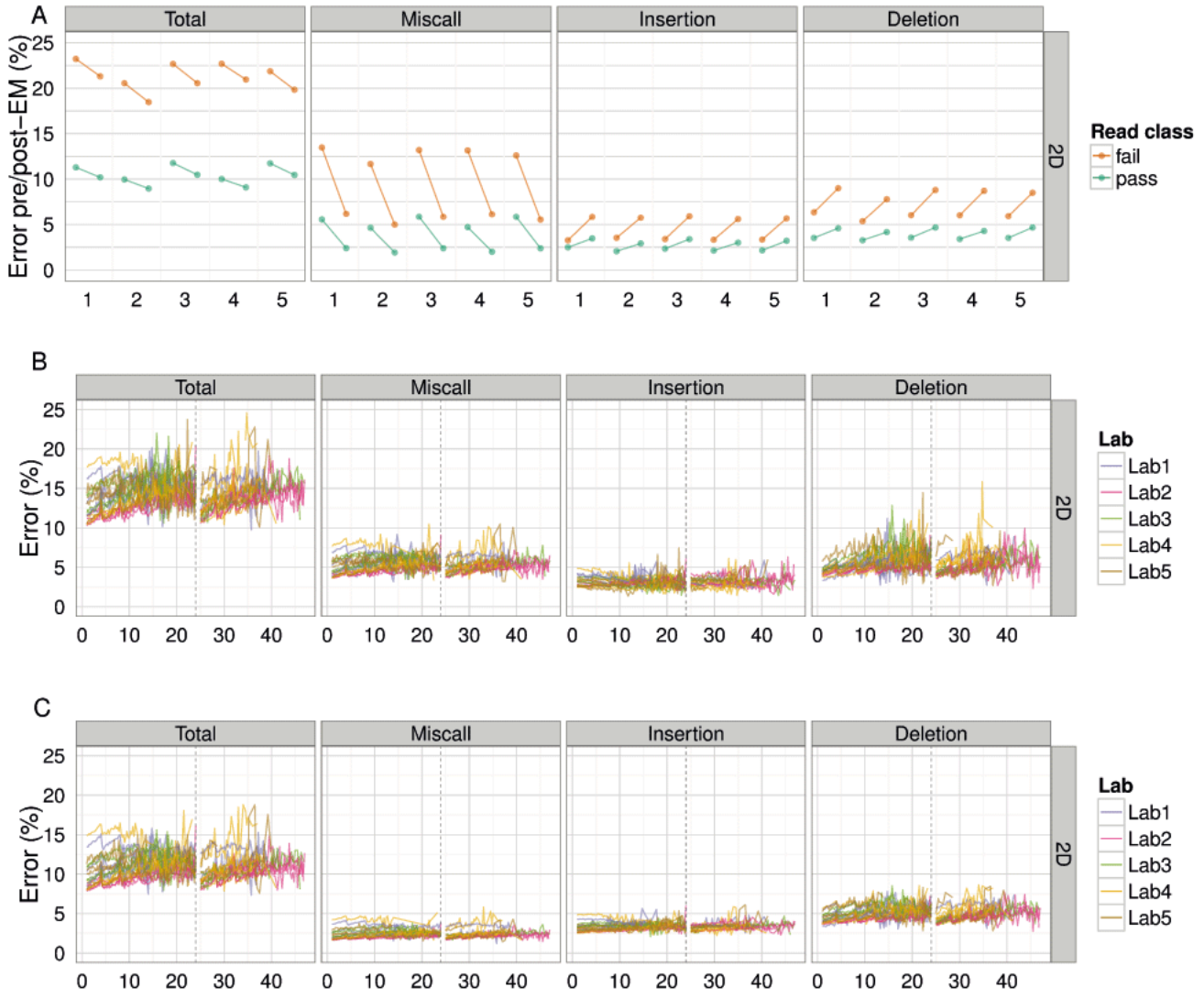
Ip CLC, Loose M, Tyson JR et al. 2015 [version 1; referees: 2 approved] F1000Research 2015, 4:1075 (doi: 10.12688/f1000research.7201.1)

Percentage of 2D pass reads produced over time.



Ip CLC, Loose M, Tyson JR et al. 2015 [version 1; referees: 2 approved] F1000Research 2015, 4:1075 (doi: 10.12688/f1000research.7201.1)

Error rates of BWA-MEM EM-corrected alignments of target 2D base-calls.

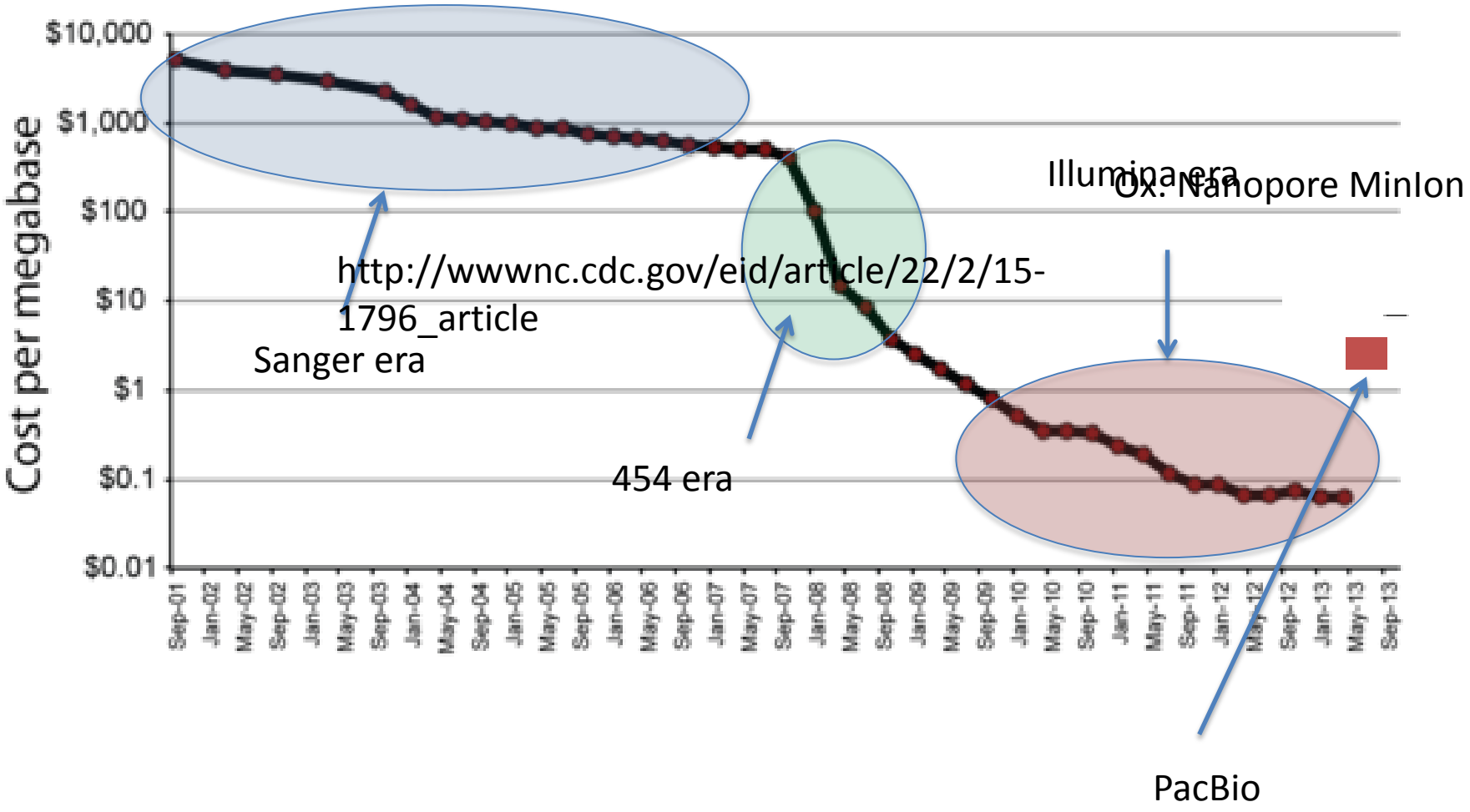


Ip CLC, Loose M, Tyson JR et al. 2015 [version 1; referees: 2 approved] F1000Research 2015, 4:1075 (doi: 10.12688/f1000research.7201.1)

Improvements

- FASTQ – per base quality values don't make much sense
- Move towards 6mer basecalling
- Other types of model taking into account effects of bases sitting outside the pore
- Improvements to pore types
  - Utilise multiple pore types on a single flowcell
  - This would not make it a true single molecule sequencer since we would rely upon consensus (probably does not matter)
- Library preparation
- Flowcell reliability
- Access to basecalling API

Cost per megabase



MinIon for denovo assembly and variant calling

- *De novo* sequencing and variant calling with nanopores using PoreSeq

- Oxford Nanopore sequencing, hybrid error correction, and de novo assembly of a eukaryotic genome

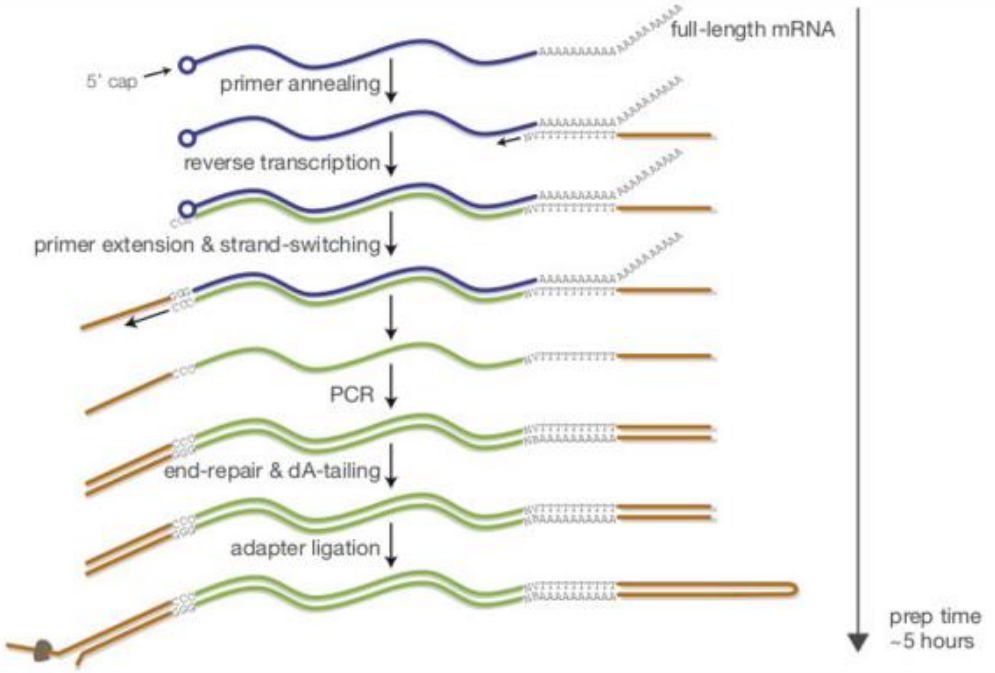
Novel applications

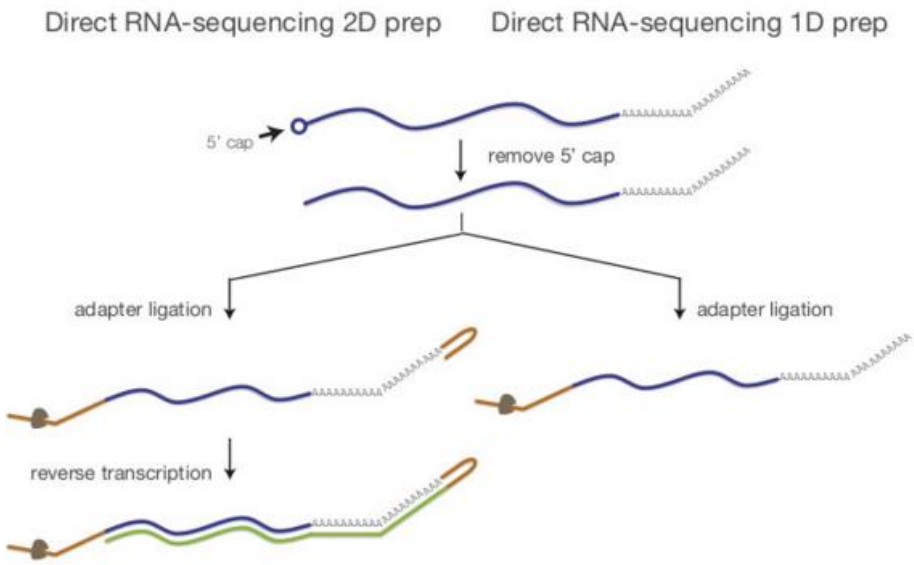
- Portable in-situ sequencing

- Nanopore sequencing as a rapidly deployable Ebola outbreak tool
- Nanopore sequencing in microgravity

Novel applications

- Direct RNA sequencing





Novel applications

- Rapid pathogen detection in clinical settings

- Rapid identification of viral pathogens
- Rapid draft sequencing of Salmonella during a hospital outbreak

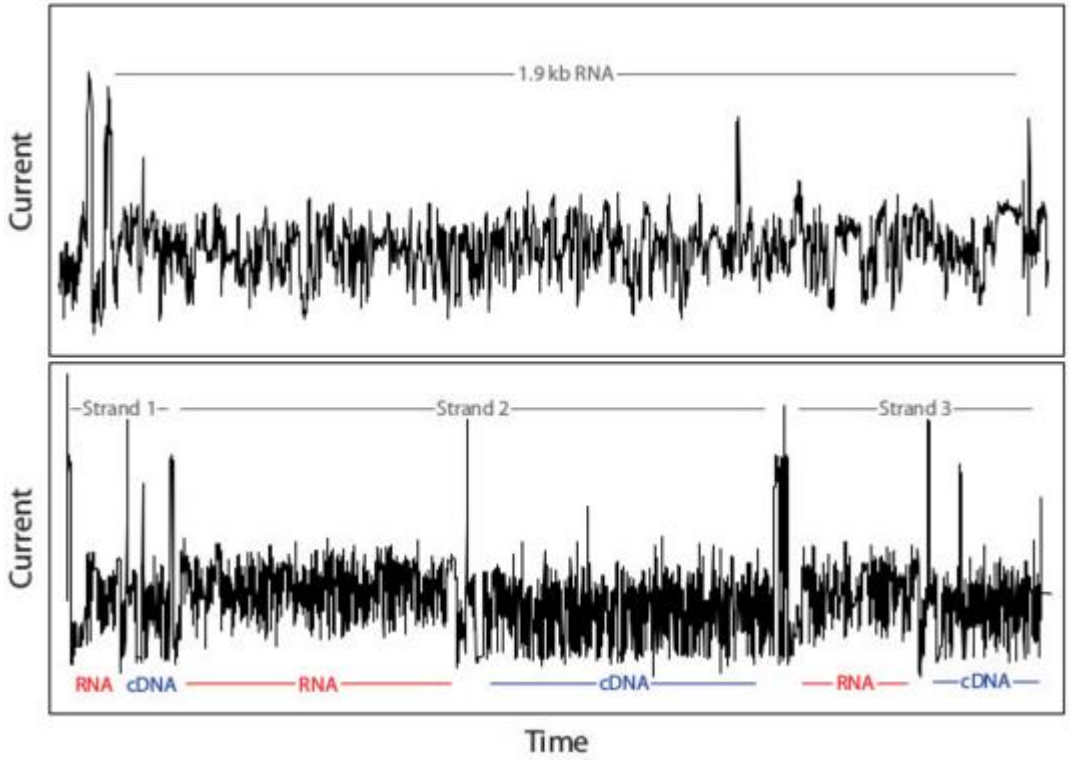
Novel applications

- Teaching aids

- Integration of mobile sequencers into a classroom

Novel applications

- Direct RNA sequencing



Software packages

- Tracking and managing MinIon data
  - MinoTour - http://minotour.github.io/minoTour

- Processing ONT data
  - Poretools https://github.com/arq5x/poretools
  - poRe http://sourceforge.net/projects/rpore/
  - NanoCorr http://schatzlab.cshl.edu/data/nanocorr/
  - Nanopolish https://github.com/jts/nanopolish/
  - PoreSeq - http://www.nature.com/nbt/journal/v33/n10/full/nbt.3360.html
  - MarginAlign - https://github.com/benedictpaten/marginAlign
  - Lordec https://www.gatb.fr/software/lordec/

- Alignment

  Sensitive but slow aligners
  - BLAST
  - BLAT
  - LAST
  - BWA with the right parameters
- Assembly
  - Possible to obtain ONT-only assembly
  - Error correction with other data types is also possible
  - offer an alternative to PacBio or Illumina synthetic long reads

Summary

- Very encouraging
- Portable applications have been demonstrated
- Error rate is ~12-15% for R8 2D reads
  - Some success with completing bacterial genomes using this data
- Probably 12 months before full commercial launch (I said this last year as well…)
- Fast-mode promises 2-3x increase in data output (at the probable cost of accuracy)
- Highly recommended review paper by MARC consortium
  - http://f1000research.com/articles/4-1075/v1

Opportunities

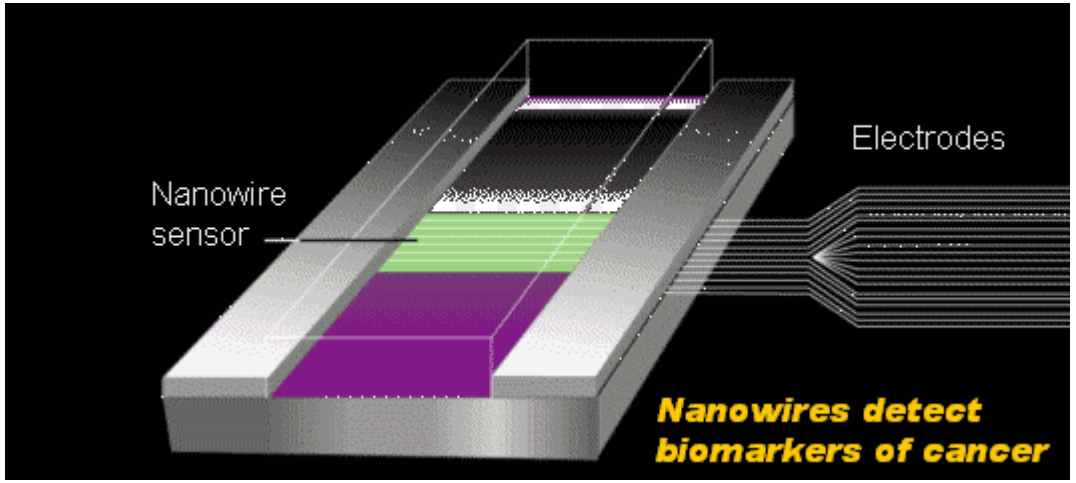
- Converting reference genomes to ‘wiggle-space’
- Developing ‘wiggle-space’ aligners
- Online ‘streaming’ bioinformatics
  - Analytics - one read at a time
- Developing complex sample -> sequencer ready protocols for use in the field
- Identifying sources of bias

General issues with nanopores

- Single base-pair resolution is not available
  - Typically 4-5 nucleotides fit into a nanopore
- Only one detector per DNA strand
  - One or two shots at detection
- Fast translocation of DNA through pore
- Small signal and high noise
- Bilayer stability

Nanowire alternatives

- QuantumDx QSEQ



Many others in development

- http://www.allseq.com/knowledgebank/sequencing-platforms

In conclusion

- We are mastering reading DNA (at least some of it)
- Now we are in a position to precisely edit and engineer biological systems

Thanks to:

Karen Moore

Jeremie Poschmann

Audrey Farbos

Paul O'Neill

Wellcome Trust

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http://sequencing.exeter.ac.uk

Supported by wellcome trust

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