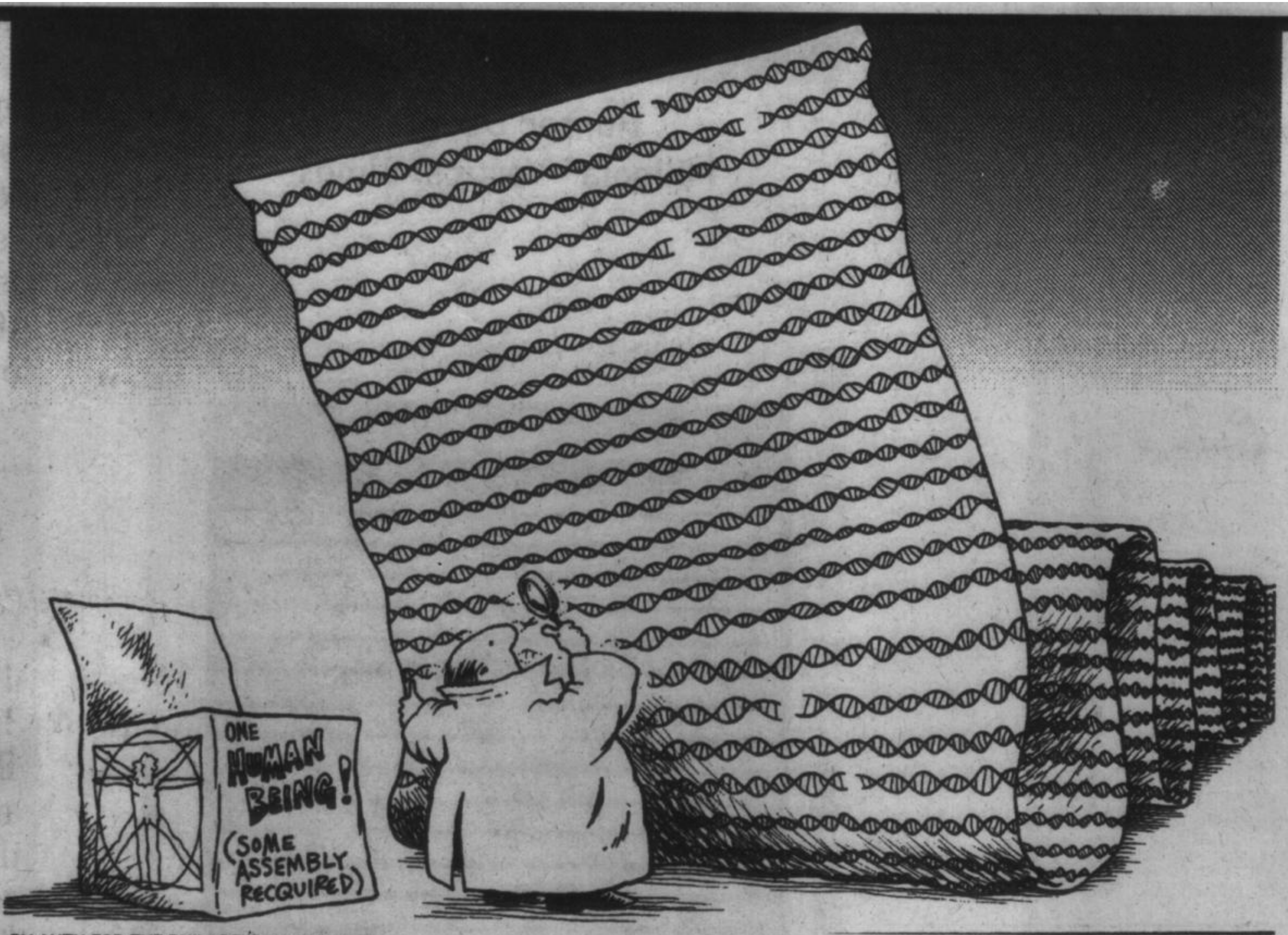


BY AUTH FOR THE PHILADELPHIA INQUIRER

UNIVERSITY OF EXETER

Modern Approaches to Sequencing

Dr Konrad Paszkiewicz, Head, Exeter Sequencing Service,

Director Wellcome Trust Biomedical Informatics Hub,

January 2014

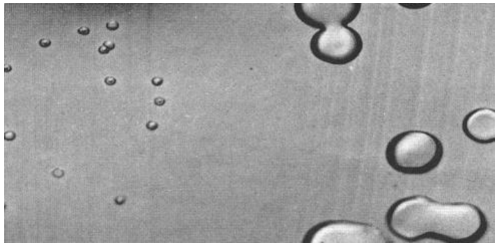
Contents

- Review of Sanger Sequencing
- Timeline and impact of human genome project
- Second generation sequencing technologies
- Third generation sequencing technologies
- Nanopore sequencing technologies

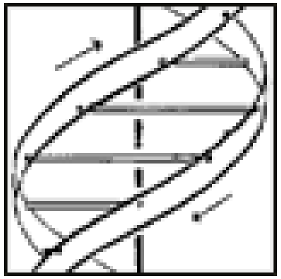
USB Nanopore sequencer

Timeline

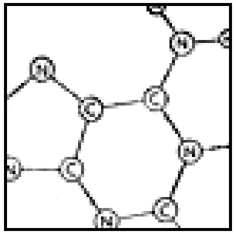
1944: Avery, O.T., et al “Studies on the chemical nature of the substance inducing transformation of Pneumococcal types”



1953: Watson J.D. and Crick F.H.C. “A Structure for Deoxyribose Nucleic Acid“



1953: Watson J.D. and Crick F.H.C. “Genetical Implications of the structure of Deoxyribonucleic Acid“



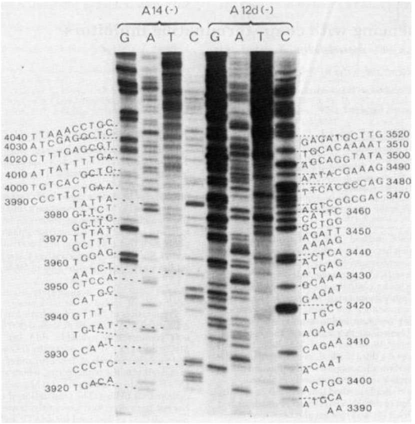
1959 – First homeogenous DNA purified

Timeline

1970 – First discovery of type II restriction enzymes

1972: sequencing of the first gene from RNA by Walter Fiers

1976: sequencing of the first complete genome by Fiers (Bacteriophage MS2 which infects *E.coli*)



1977: Maxam AM, Gilbert W. "A new method for sequencing DNA".

1977: Sanger F, Nicklen S, Coulson AR. "DNA sequencing with chain-terminating inhibitors"

http://1.usa.gov/noyyPOhttp://bit.ly/nHn8Tw

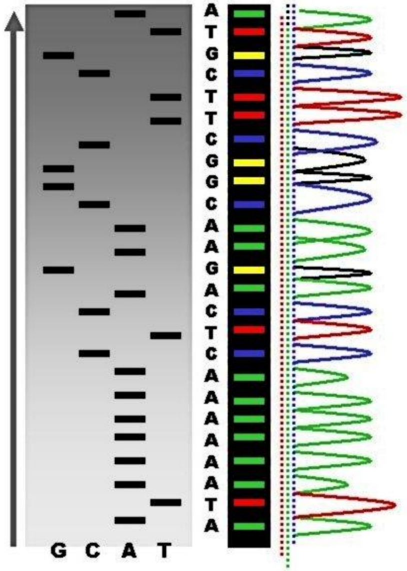
Timeline

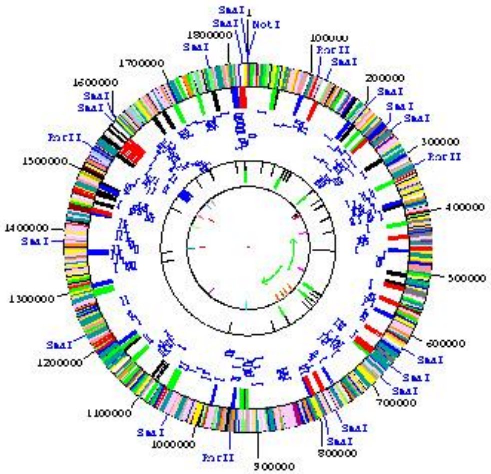
1985-86: Leroy Hood use fluorescently labeled ddNTPs, set the stage for automated sequencing

1987: Applied Biosystems markets first automated sequencing machine (ABI 370)

1990: National Institutes of Health (NIH) begins large-scale sequencing trials ($0.75/base) Human Genome Project (HGP) begins, $3-billion and 15 years

1995: Craig Venter at TIGR published the Haemophilus influenzae genome. First use of whole-genome shotgun sequencing





http://bit.ly/2KrFp0 http://bit.ly/qlQD18

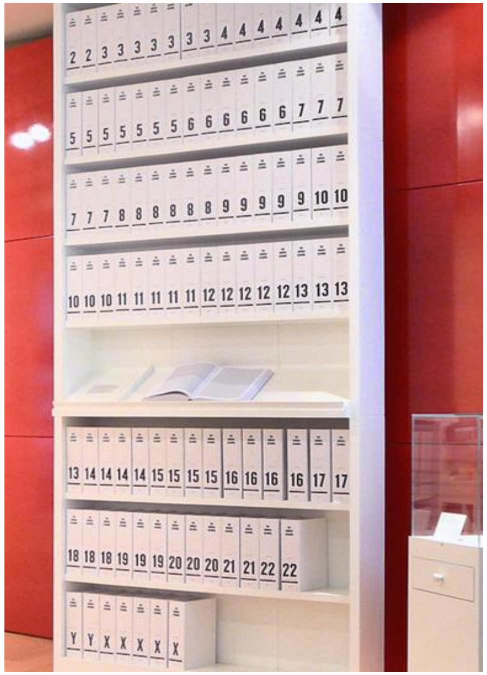
Timeline

1998: Green & Ewing publish "phred" base caller/scorer

2000: Sydney Brenner and Lynx Therapeutics publishes "MPSS", parallelized bead-base sequencing tech, launches "Next-Gen"

2001: HGP/Celera draft assembly published in Nature/Science

2003: HGP "complete" genome released. ENCODE project launched



2004: 454 releases pyrosequencer, costs 6-fold less than automated Sanger sequencing

http://bit.ly/pNKUDJ

Illumina-era Timeline

1998: Shankar Balasubramanian and David Klenerman patent ”A method for reproducing molecular arrays” and found Solexa

2001: £12 million series A funding

2003: Mercier, J.-F., et al. “Solid phase DNA amplification”

2004: Solexa acquires Solid phase DNA amplification method

Illumina-era Timeline

2006: Solexa release Genome Analyser I

2007: Illumina acquires Solexa

2008: Illumina releases GAII

2008: Human microbiome project launched

2010: Illumina HiSeq 2000 released

2011: MiSeq launched

2012: ENCODE publications

2012: Illumina HiSeq 2500 released

2013-2015: RIP 454

Review of Sanger Sequencing

Fred Sanger 1918-2013

Double Nobel laureate and developer of the dideoxy sequencing method, first published in December 1977. [Credit: Wellcome Images]

"Fred Sanger is a quiet giant, whose discoveries and inventions transformed our research world.” (A.Bradley, WTSI.)

The challenge of DNA sequencing

- 1953 – Double helix discovered
- 1971 - First DNA sequence determined (12bp!)
- 1977 – Sanger sequencing method published

- Why did it take so long?

Some possible reasons

- The chemical properties of different DNA molecules were so similar that it appeared difficult to separate them
- The chain length of naturally occurring DNA molecules is much greater than for proteins and made complete sequencing seem unapproachable.
- The 20 amino acid residues found in proteins have widely varying properties that had proven useful in the separation of peptides.
  - Only four bases in DNA made sequencing a more difficult problem for DNA than for protein.
- No base-specific DNAases were known.
  - Protein sequencing had depended upon proteases that cleave adjacent to certain amino acids
- DNA was considered boring compared to proteins

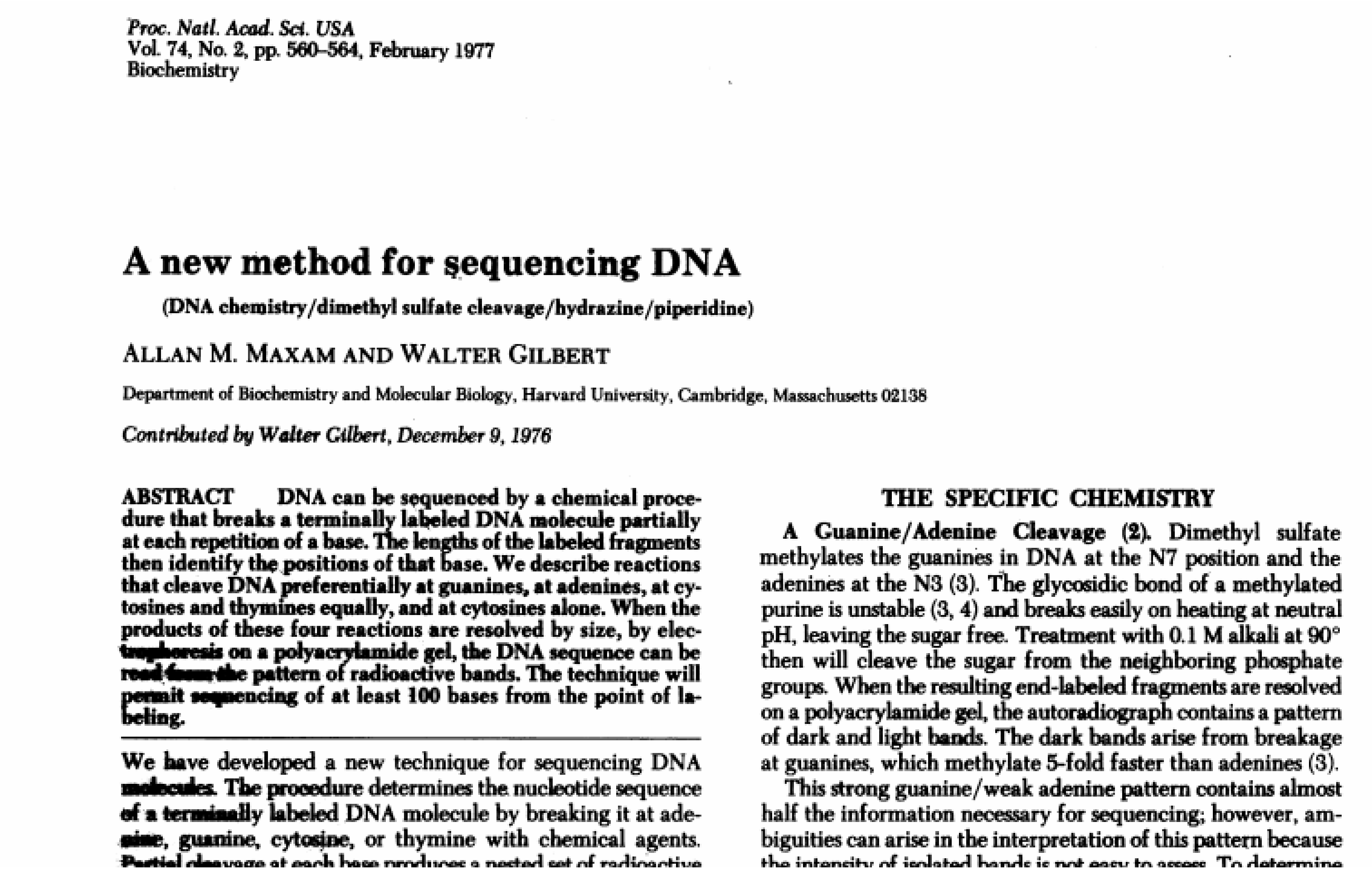
Clyde A. Hutchison III
DNA sequencing: bench to bedside and beyond Nucl. Acids Res. (2007) 35 (18): 6227-6237 a

Steps on the road to sequencing

- 1959 – First homeogenous DNA purified
- 1970 – First discovery of type II restriction enzymes
- 1972 – First RNA gene sequence published (lac operon)
- 1975 – Sanger first publishes his plus/minus method of sequencing (unable to distinguish homopolymers)
- 1977 – Maxam & Gilbert publish their method (could distinguish homopolymers)
- 1977 – Sanger publishes Dideoxy sequencing method

Clyde A. Hutchison III
DNA sequencing: bench to bedside and beyond Nucl. Acids Res. (2007) 35 (18): 6227-6237 a

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.

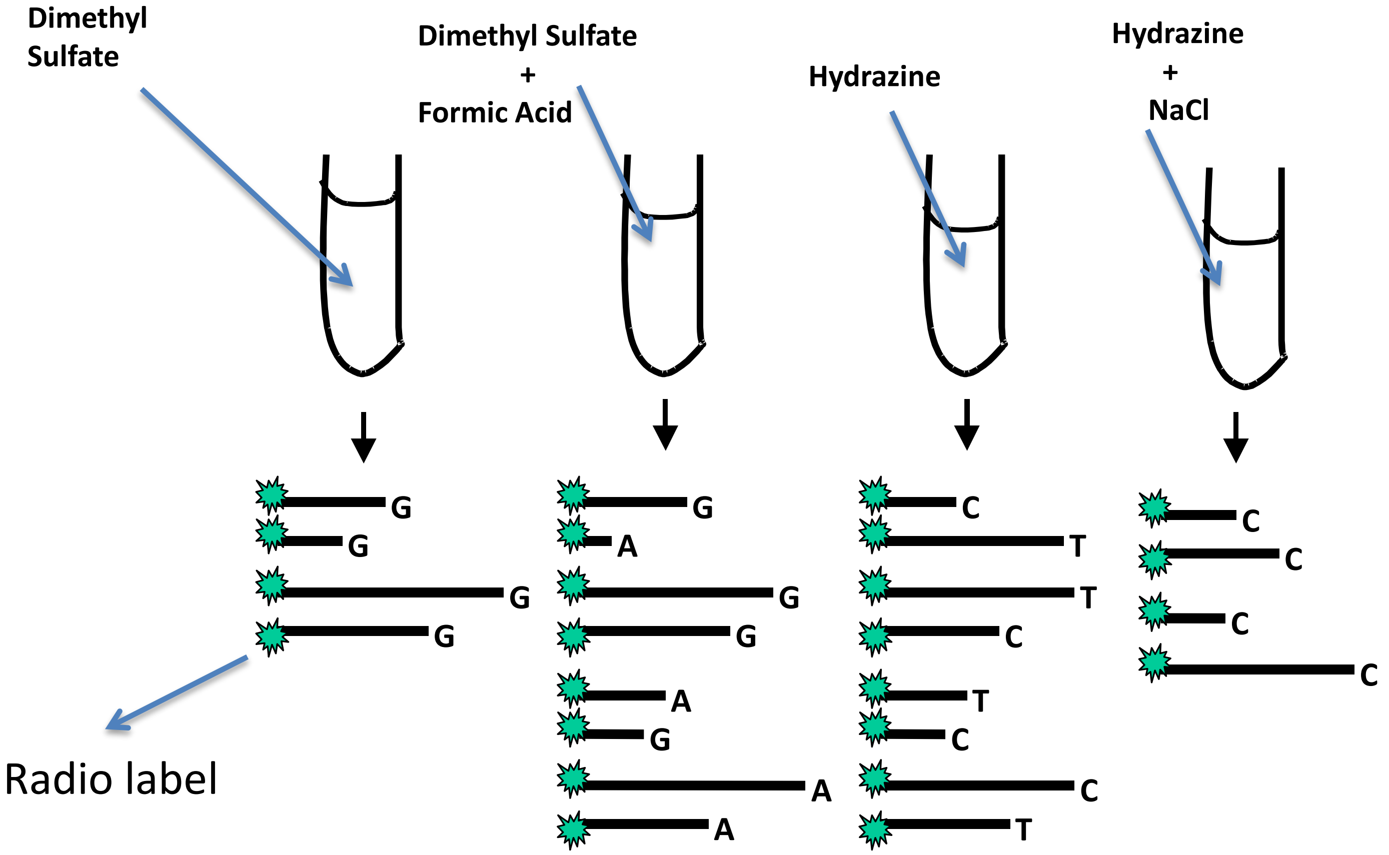
We have developed a new technique for sequencing DNA molecules. The procedure determines the nucleotide sequence of a terminally labeled DNA molecule by breaking it at adenine, guanine, cytosine, or thymine with chemical agents. Partial cleavage at each base produces a nested set of radioactive

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



Dimethyl Sulfate

Dimethyl Sulfate
+
Formic Acid

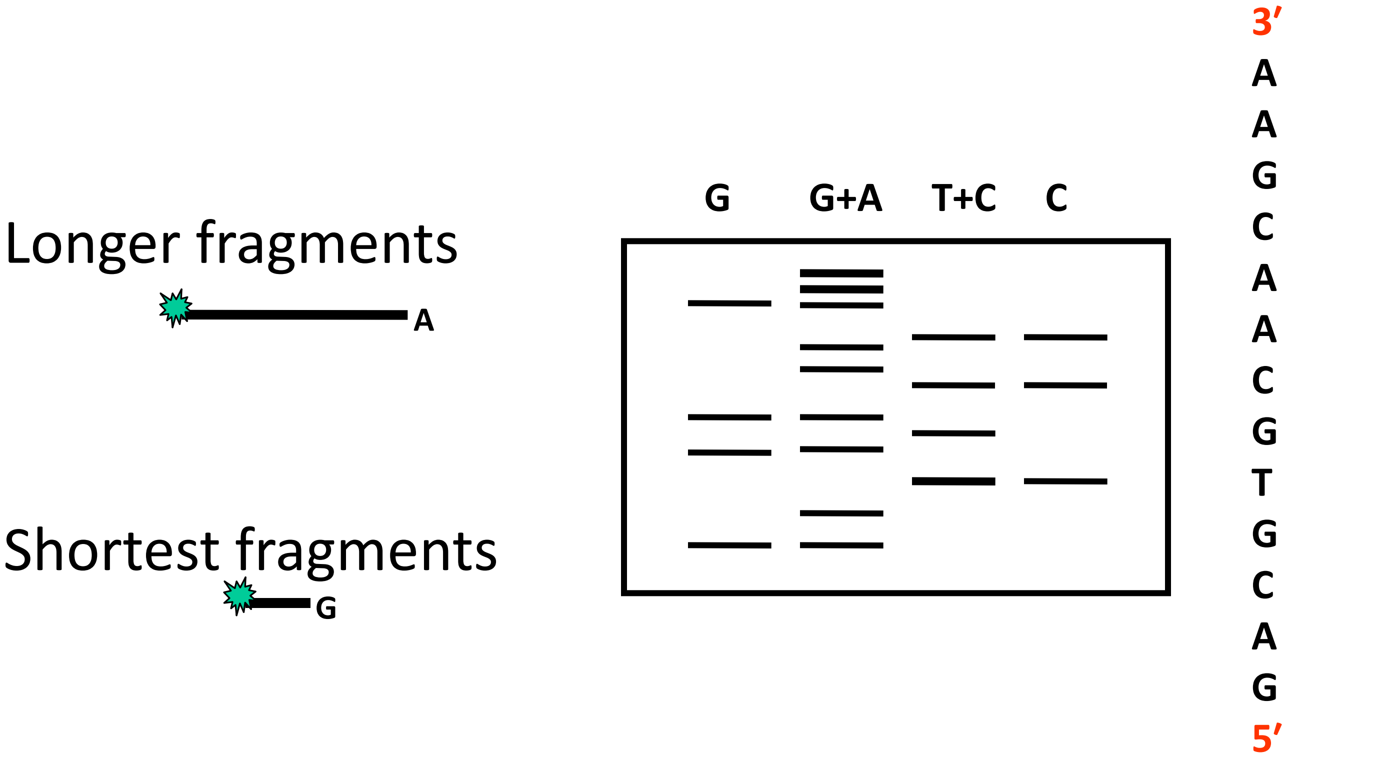
Hydrazine

Hydrazine
+
NaCl

Radio label

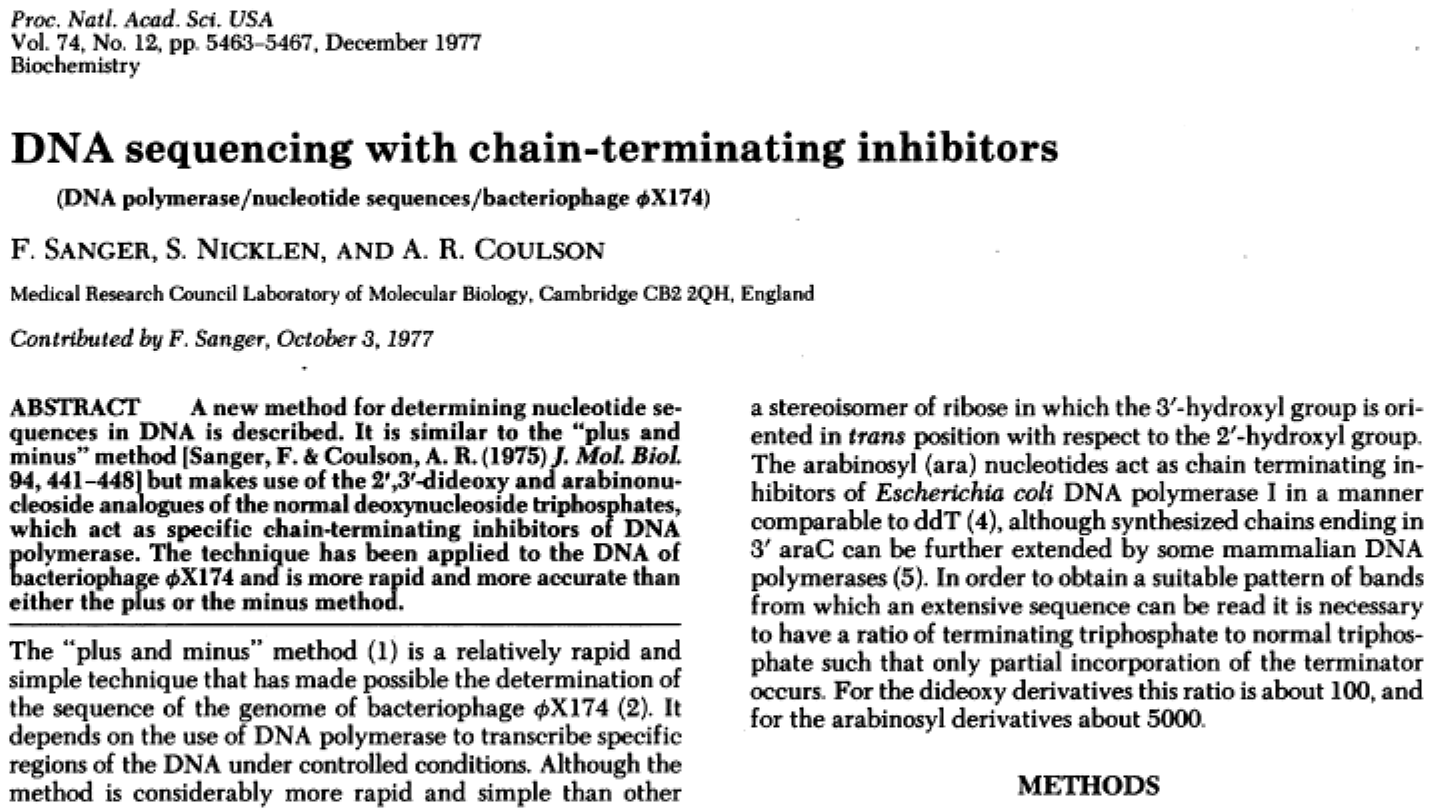
Maxam-Gilbert sequencing is performed by chain breakage at specific nucleotides.

Maxam-Gilbert Sequencing



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

AGCTGCCCG

A

ddATP + **four dNTPs**	**ddA** **dAdGdCdTdGdCdCdCdG**

C

ddCTP + **four dNTPs**	**dAdGddC** **dAdGdCdTdGddC** **dAdGdCdTdGdCddC** **dAdGdCdTdGdCdCddC**

G

ddGTP + **four dNTPs**	**dAddG** **dAdGdCdTddG** **dAdGdCdTdGdCdCdCddG**

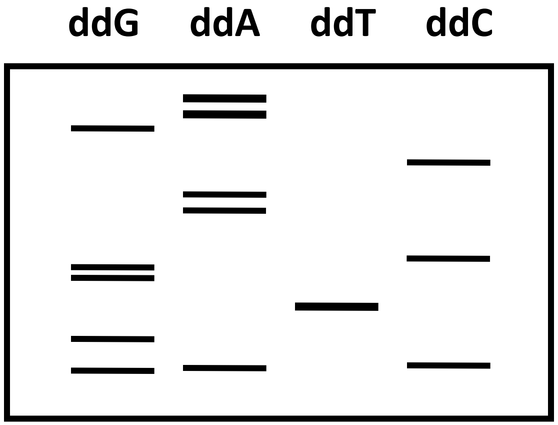
T

ddTTP + **four dNTPs**	**dAdGdCddT** **dAdGdCdTdGdCdCdCdG**

Sanger di-deoxy method

Longer fragments

Shortest fragments



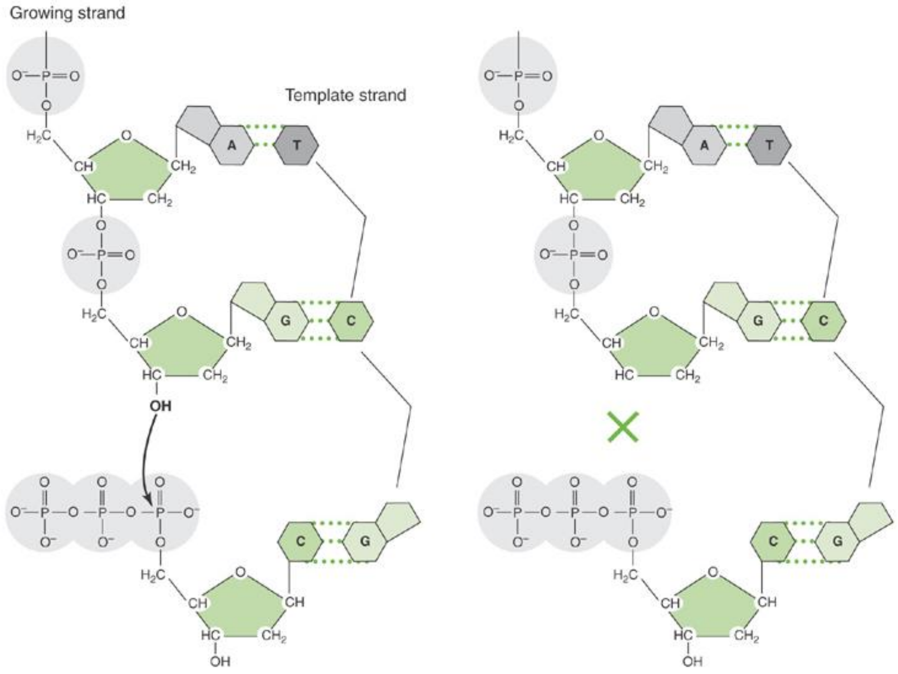
3′
A
A
G
C
A
A
C
G
T
G
C
A
G
5′

Sanger Sequencing

- 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, 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?

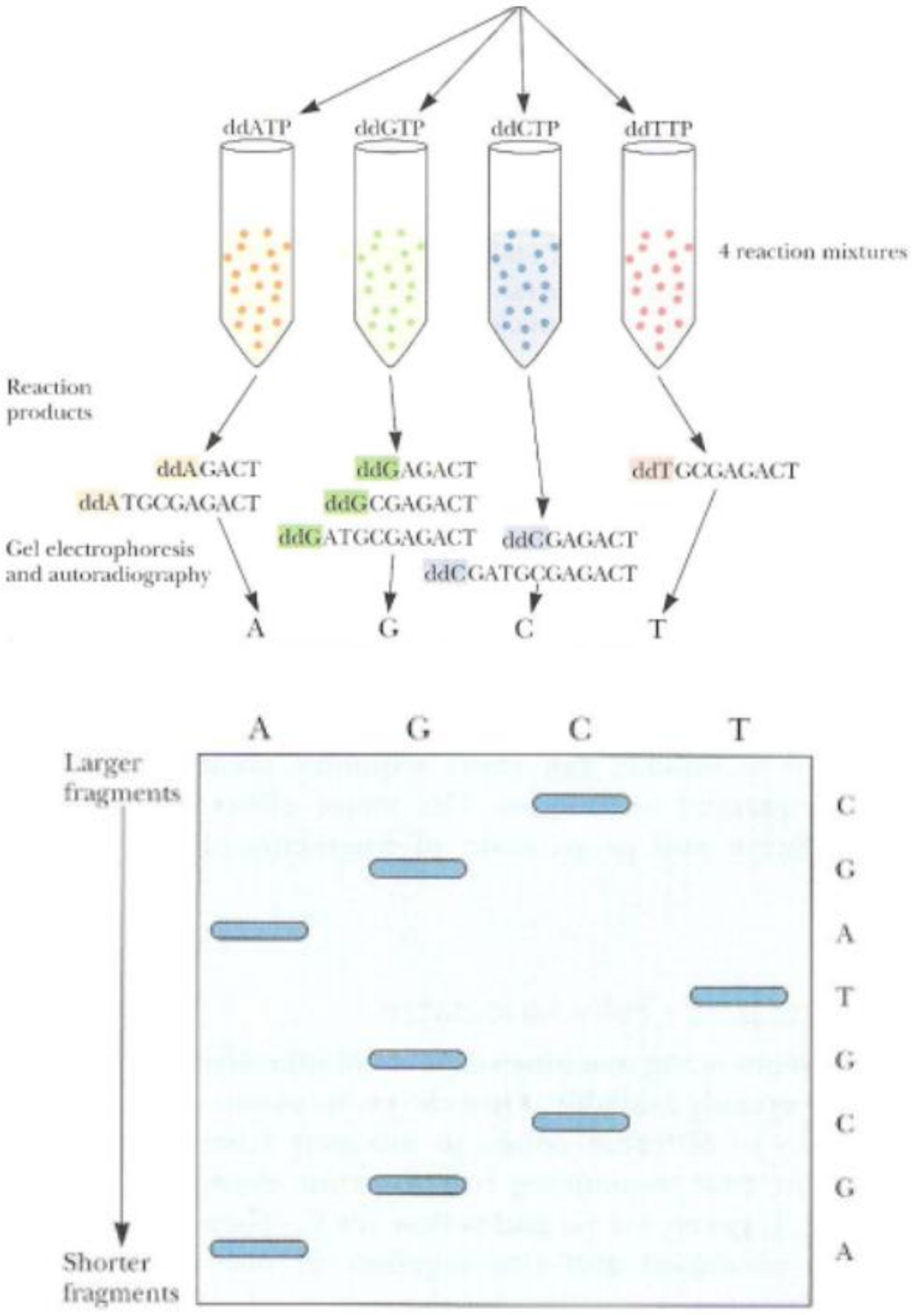
The 3′-OH group necessary for formation of the phosphodiester bond is missing in ddNTPs



Chain terminates at ddG

Dideoxy Method

- Run four separate reactions each with different ddNTPs
- Run on a gel in four separate lanes
- Read the gel from the bottom up

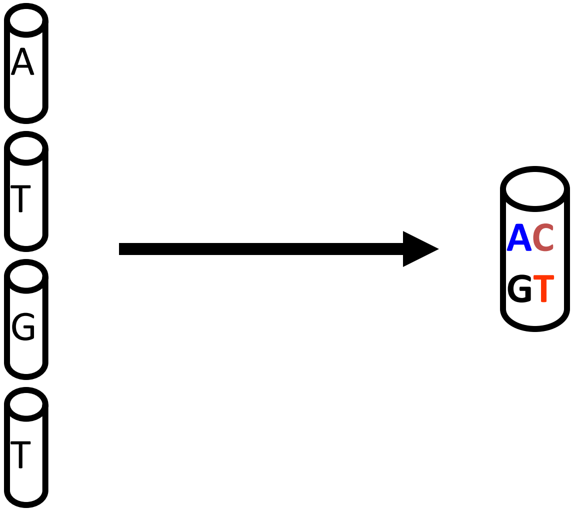


Improvements to Sanger’s original method

- Cycle sequencing is chain termination sequencing performed in a thermal cycler.
  - Requires a heat-stable DNA polymerase.
- Fluorescent dyes are multicyclic molecules that absorb and emit fluorescent light at specific wavelengths.
  - Examples are fluorescein and rhodamine derivatives.
  - For sequencing applications, these molecules can be covalently attached to nucleotides.

Dye Terminator Sequencing

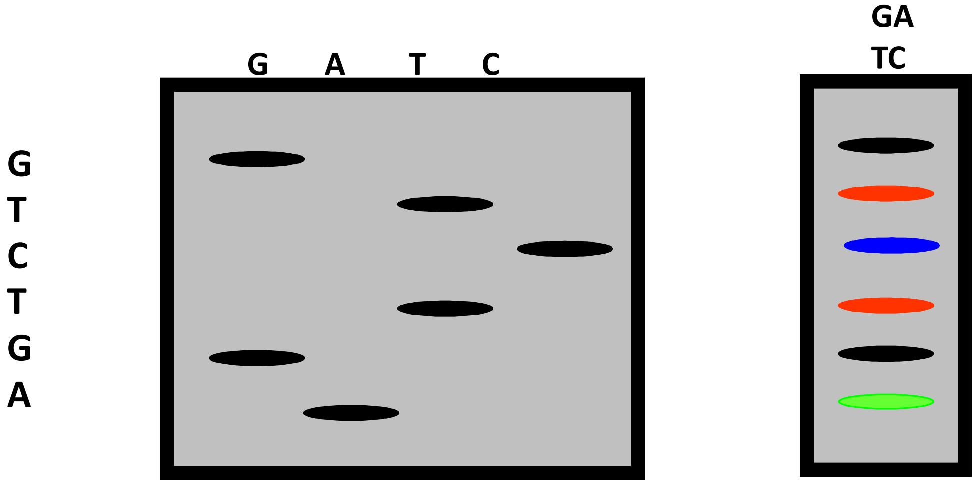
- A distinct dye or “color” is used for each of the four ddNTP.
- Since the terminating nucleotides can be distinguished by color, all four reactions can be performed in a single tube.



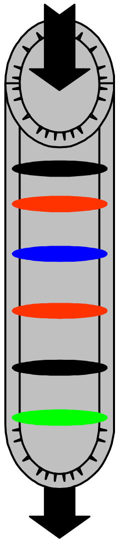
The fragments are distinguished by size and “color.”

Dye Terminator Sequencing

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



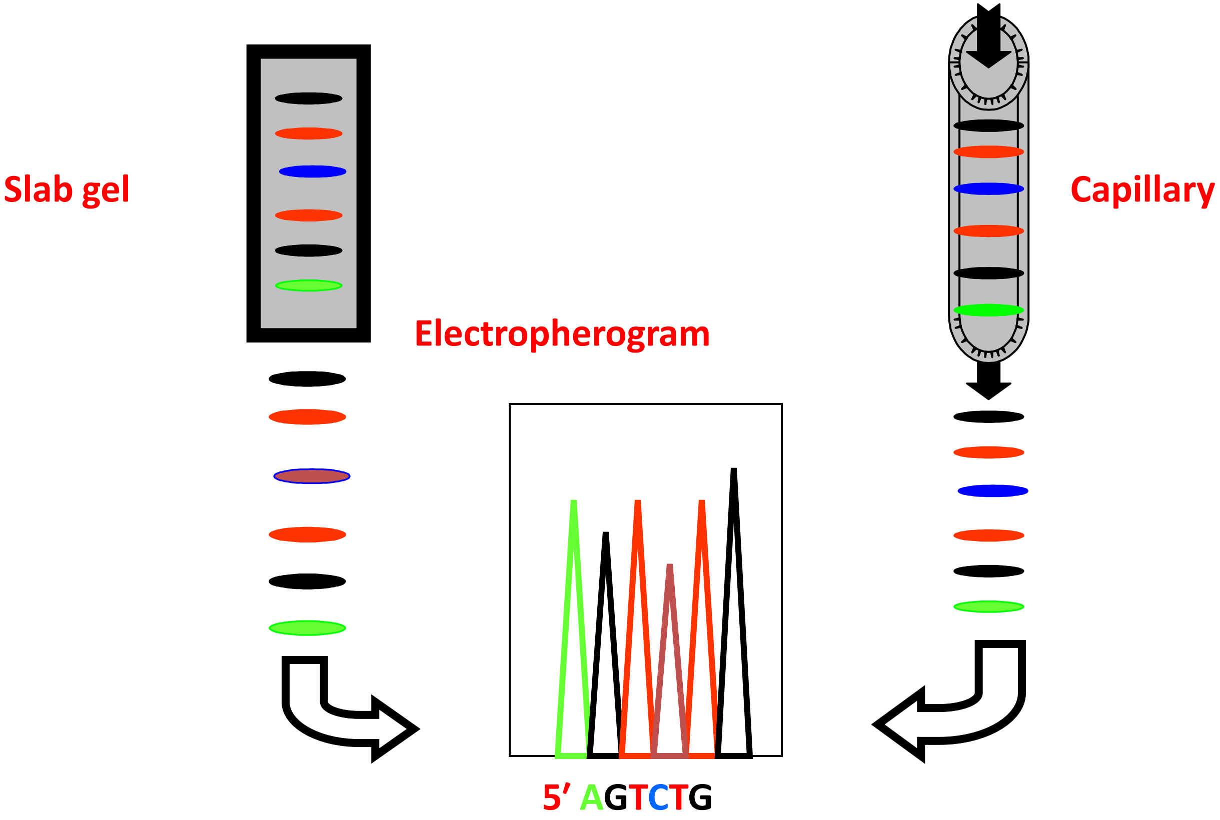
Slab gel



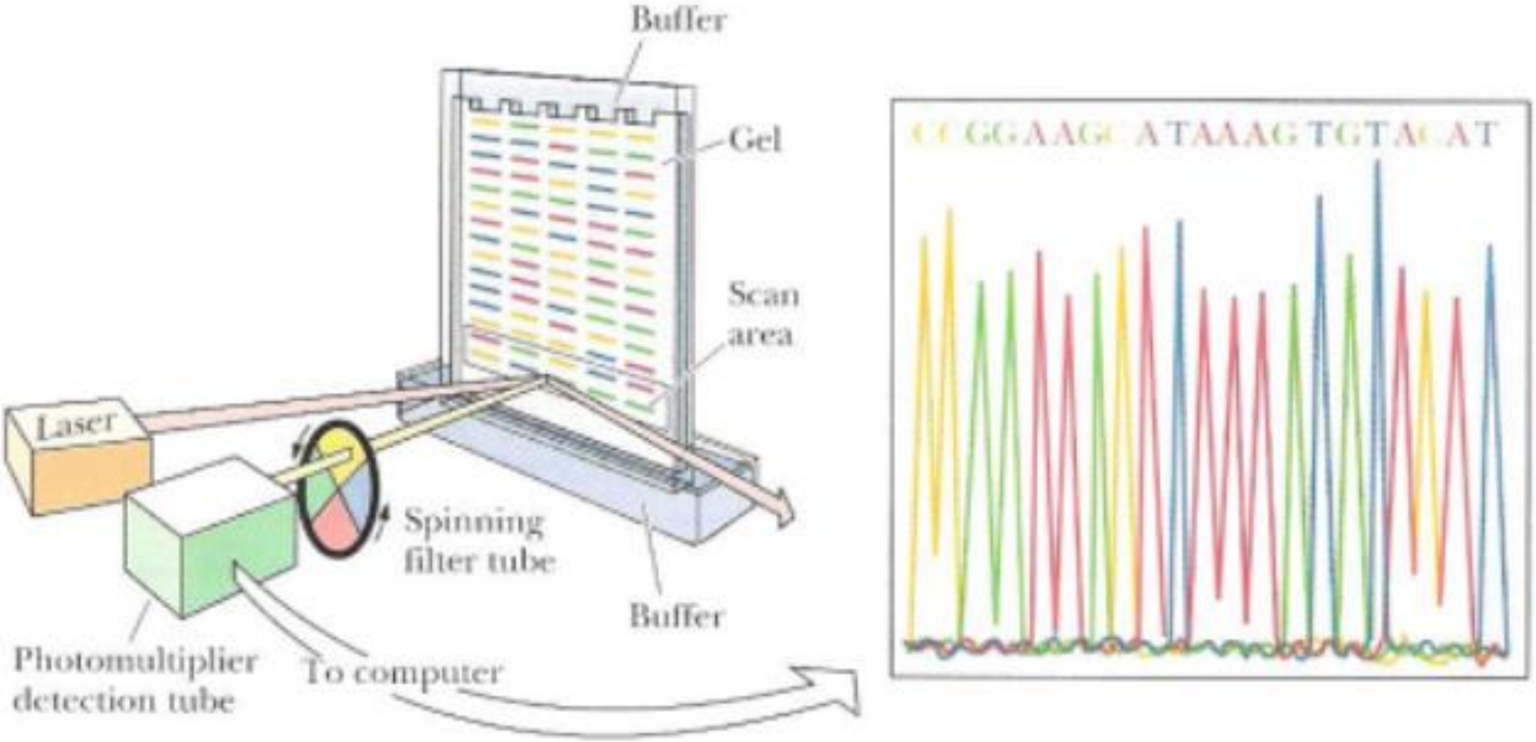
Capillary

Dye Terminator Sequencing

- The DNA ladder is read on an electropherogram.



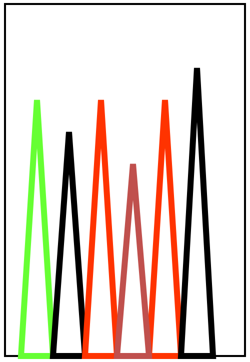
Automated Version of the Dideoxy Method



Automated Sequencing

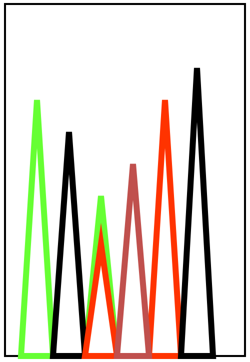
- Dye primer or dye terminator sequencing on capillary instruments.
- Sequence analysis software provides analyzed sequence in text and electropherogram form.
- Peak patterns reflect mutations or sequence changes.

T/T



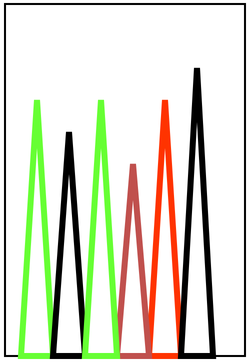
5’ A G T C T G

T/A



5’ AG(T/A)CTG

A/A



5’ A G A C T G

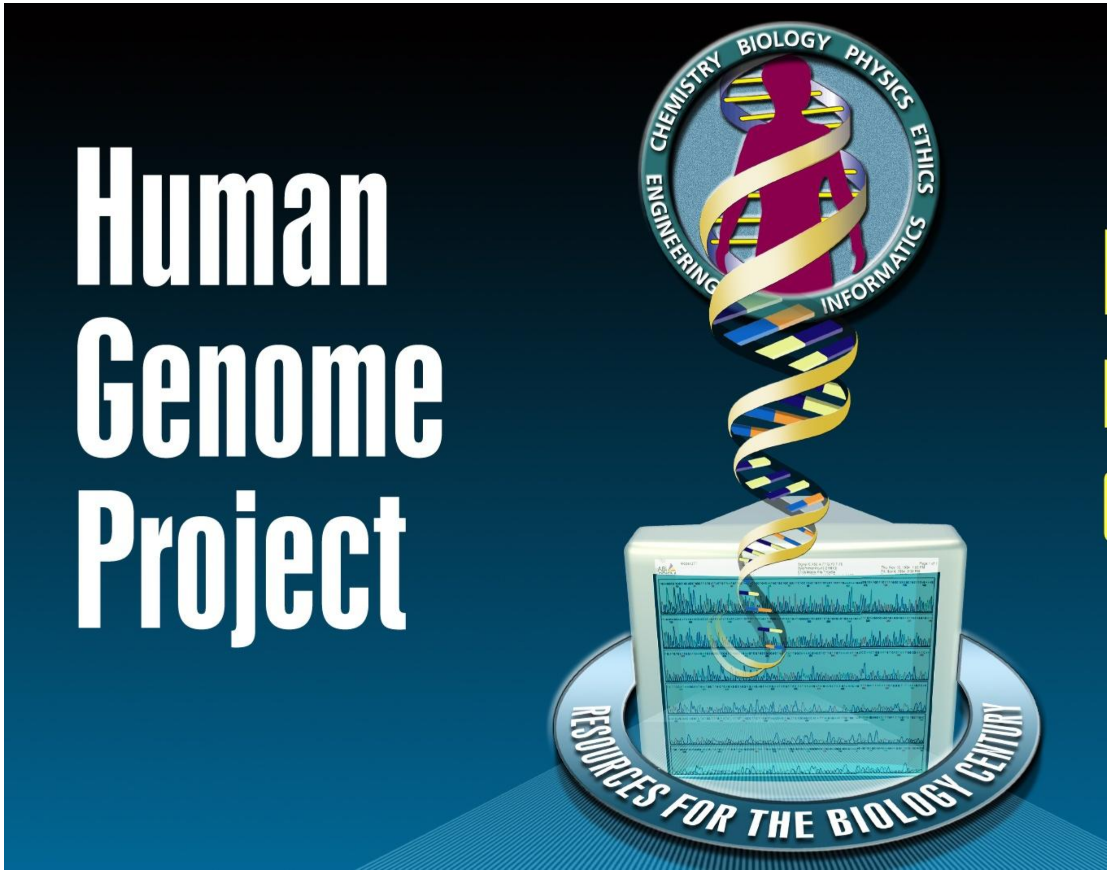
First generation (Sanger) sequencing

Throughput	50-100kb, 96 sequences per run
Read length	0.5-2kbp
Accuracy	high quality bases - 99%: ~900bp very high quality bases - 99.9%: ~600bp 99.999%: 400-500bp
Price per raw base	~$200,000/Gb

Sanger Sequencing
Useful videos

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

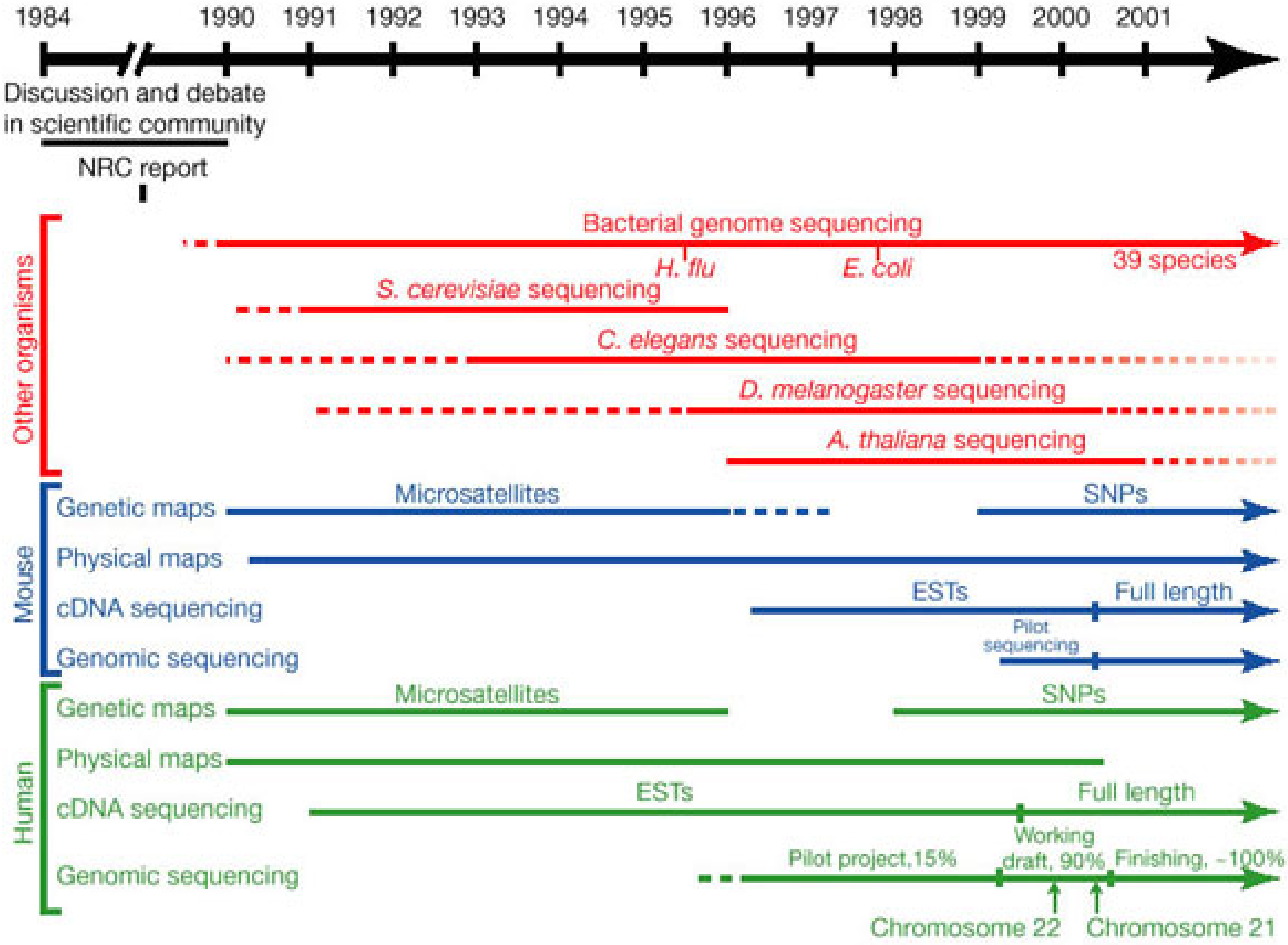
Human genome project



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

Human Genome Project



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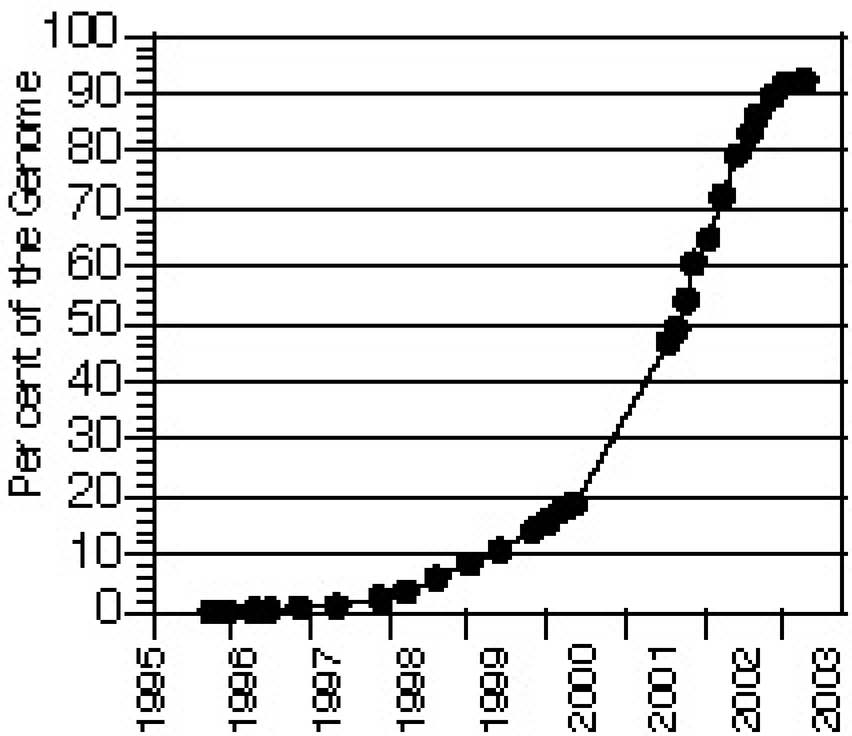
Human Genome Project



This blog post indicates ~2.86Gbase/3.1Gbase of the non-redundant genome has been sequenced in hg18 or ~**92%** centromeres, telomeres, and highly repetitive regions left

http://bit.ly/qML5Uq

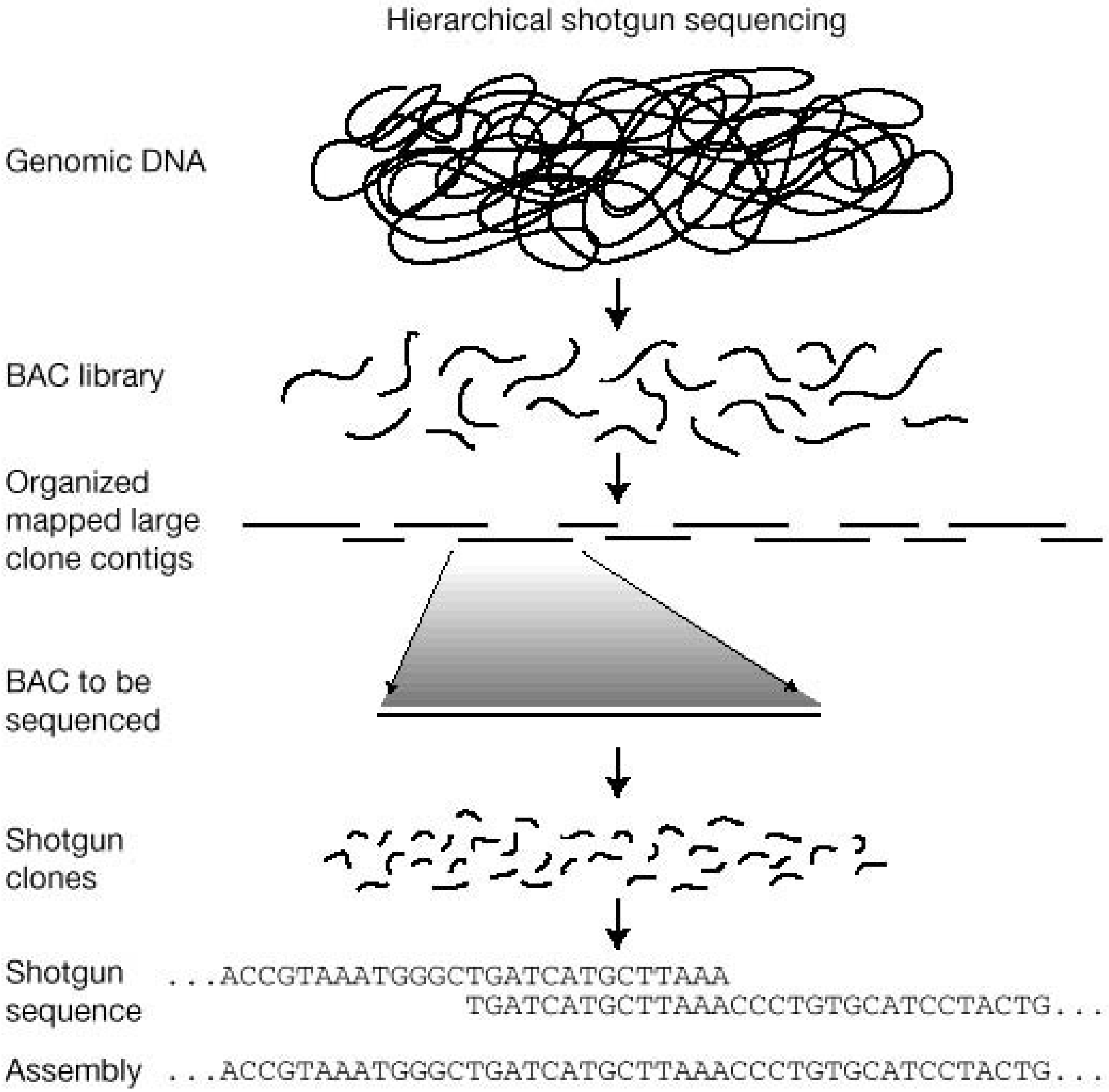
How it was Accomplished

- 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

Shotgun Sequencing

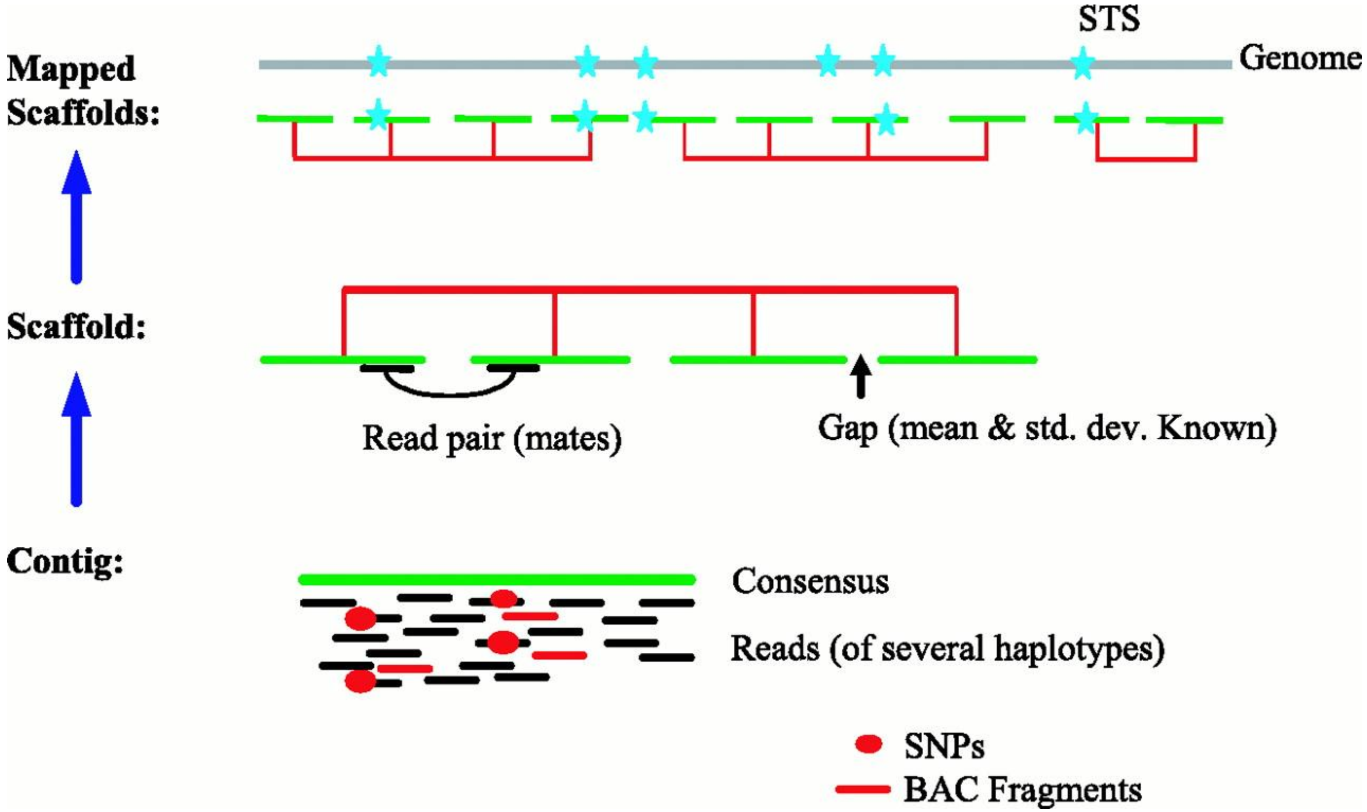
- Celera
  - Started in Sept 1999, goal was to do in \$300M and 3 years what the public project was doing for \$3B and 15 years!
  - Whole-genome shotgun sequencing
  - Used both whole-genome assembly and regional chromosome assembly
  - Incorporated data from the public project
  - Raised ethical concerns about the ownership of the human genome and patentability of genes

Hierarchical Sequencing


Hierarchical shotgun sequencing
Genomic DNA
BAC library
Organized mapped large clone contigs
BAC to be sequenced
Shotgun clones
Shotgun sequence
...ACCGTAAATGGGCTGATCATGCTTAAA
TGATCATGCTTAAACCCTGTGCATCCTACTG...
Assembly
...ACCGTAAATGGGCTGATCATGCTTAAACCCTGTGCATCCTACTG...

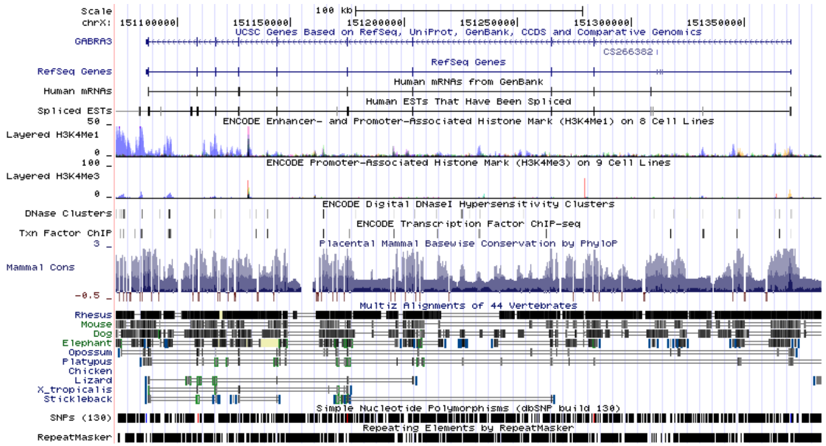
http://bit.ly/qM3Qbk

Celera Shotgun Sequencing



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

HGP Data Access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100 kb
chrX: 151100000| 151150000| 151200000| 151250000| 151300000| 151350000|
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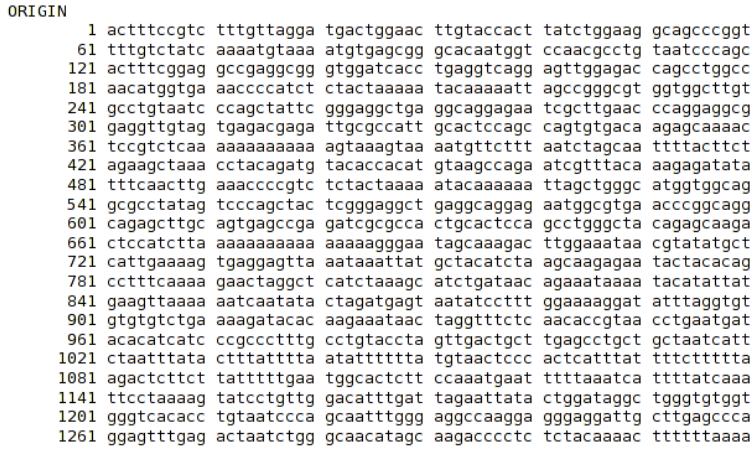
GABRA3
CS266382
RefSeq Genes
RefSeq Genes
Human mRNAs from GenBank
Human mRNAs
Human ESTs That Have Been Spliced
Spliced ESTs
50 _
ENCODE Enhancer- and Promoter-Associated Histone Mark (H3K4Me1) on 8 Cell Lines
Layered H3K4Me1
0 _
100 _
ENCODE Promoter-Associated Histone Mark (H3K4Me3) on 9 Cell Lines
Layered H3K4Me3
0 _
ENCODE Digital DNaseI Hypersensitivity Clusters
DNase Clusters
ENCODE Transcription Factor ChIP-seq
Txn Factor ChIP
3 _
Placental Mammal Basewise Conservation by PhyloP
Mammal Cons
-0.5 _
Multiz Alignments of 44 Vertebrates
Rhesus
Mouse
Dog
Elephant
Opossum
Platypus
Chicken
Lizard
X_tropicalis
Stickleback
Simple Nucleotide Polymorphisms (dbSNP build 130)
SNPs (130)
Repeating Elements by RepeatMasker
RepeatMasker



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       61 tttgtctatc aaaatgtaaa atgtgagcgg gcacaatggt ccaacgcctg taatcccagc
      121 actttcggag gccgaggcgg gtggatcacc tgaggtcagg agttggagac cagcctggcc
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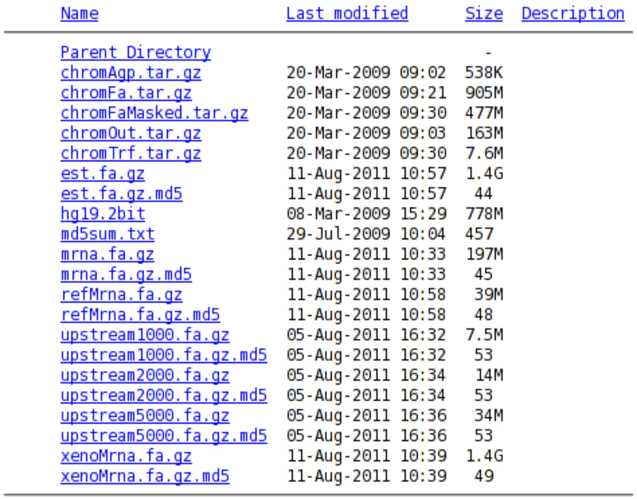

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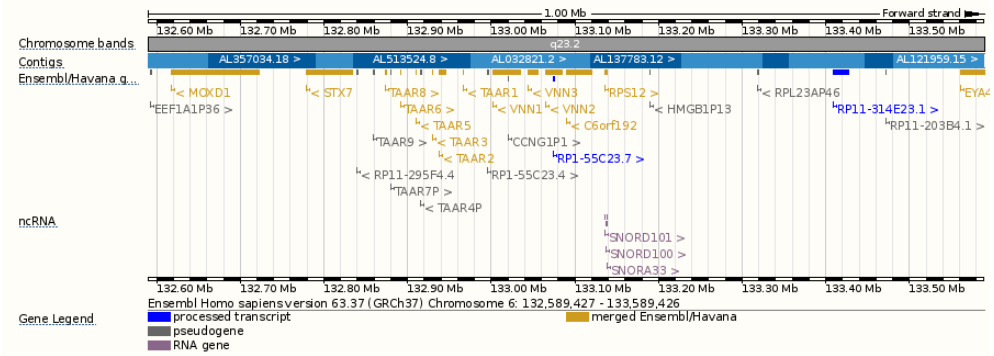
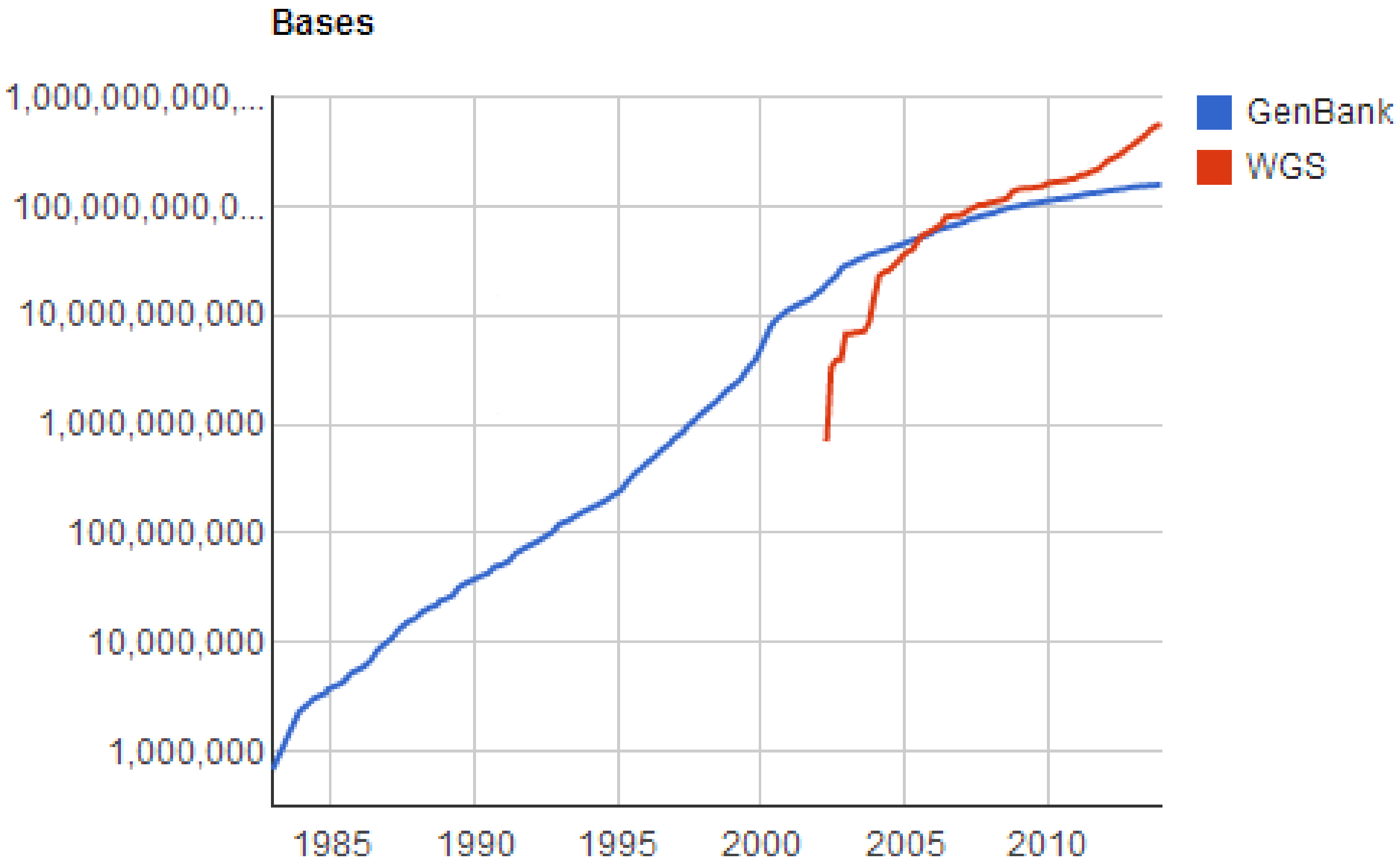
chromAgp.tar.gz	20-Mar-2009 09:02	538K	
chromFa.tar.gz	20-Mar-2009 09:21	905M	
chromFaMasked.tar.gz	20-Mar-2009 09:30	477M	
chromOut.tar.gz	20-Mar-2009 09:03	163M	
chromTrf.tar.gz	20-Mar-2009 09:30	7.6M	
est.fa.gz	11-Aug-2011 10:57	1.4G	
est.fa.gz.md5	11-Aug-2011 10:57	44	
hg19.2bit	08-Mar-2009 15:29	778M	
md5sum.txt	29-Jul-2009 10:04	457	
mrna.fa.gz	11-Aug-2011 10:33	197M	
mrna.fa.gz.md5	11-Aug-2011 10:33	45	
refMrna.fa.gz	11-Aug-2011 10:58	39M	
refMrna.fa.gz.md5	11-Aug-2011 10:58	48	
upstream1000.fa.gz	05-Aug-2011 16:32	7.5M	
upstream1000.fa.gz.md5	05-Aug-2011 16:32	53	
upstream2000.fa.gz	05-Aug-2011 16:34	14M	
upstream2000.fa.gz.md5	05-Aug-2011 16:34	53	
upstream5000.fa.gz	05-Aug-2011 16:36	34M	
upstream5000.fa.gz.md5	05-Aug-2011 16:36	53	
xenoMrna.fa.gz	11-Aug-2011 10:39	1.4G	
xenoMrna.fa.gz.md5	11-Aug-2011 10:39	49	



Results in GenBank, UCSC, Ensembl & others

Growth of Genbank



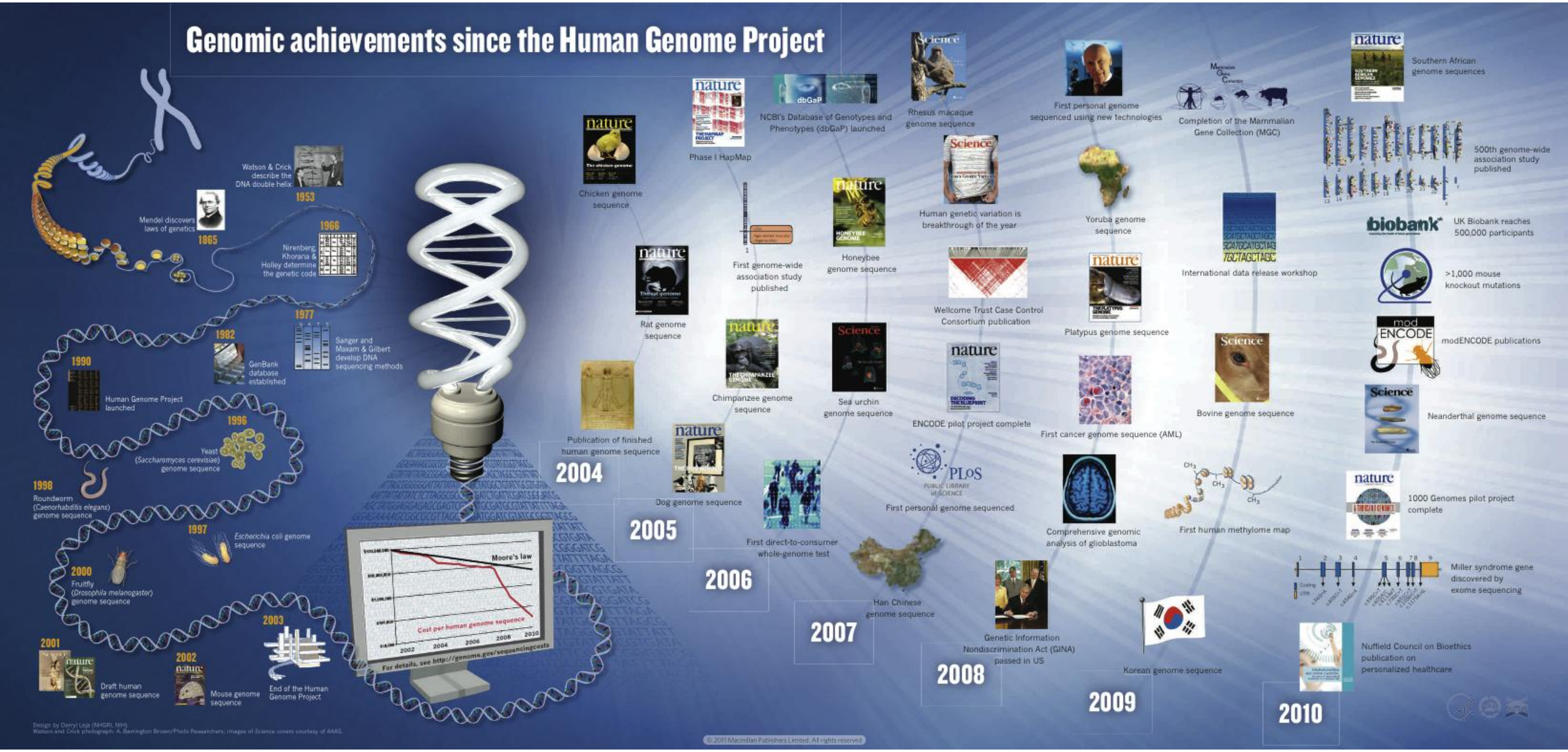
December 2013   156,230,531,562 bases

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

20110813 http://www.ncbi.nlm.nih.gov/sites/genome

Achievements Since the HGP


Genomic achievements since the Human Genome Project
1865
Mendel discovers laws of genetics
1953
Watson & Crick describe the DNA double helix
1966
Nirenberg, Khorana & Holley determine the genetic code
1977
Sanger and Maxam & Gilbert develop DNA sequencing methods
1982
GenBank database established
1990
Human Genome Project launched
1996
Yeast (Saccharomyces cerevisiae) genome sequence
1998
Roundworm (Caenorhabditis elegans) genome sequence
1997
Escherichia coli genome sequence
2000
Fruitfly (Drosophila melanogaster) genome sequence
2001
Draft human genome sequence
2002
Mouse genome sequence
2003
End of the Human Genome Project
Moore's law
Cost per human genome sequence
$100,000,000
$10,000,000
$1,000,000
$100,000
$10,000
2002
2004
2006
2008
2010
For details, see http://genome.gov/sequencingcosts
Design by Darryl Leja (NHGRI, NIH).
Watson and Crick photograph: A. Barrington Brown/Photo Researchers; images of Science covers courtesy of AAAS.
2004
Publication of finished human genome sequence
Chicken genome sequence
Rat genome sequence
Phase I HapMap
2005
Dog genome sequence
Chimpanzee genome sequence
NCBI's Database of Genotypes and Phenotypes (dbGaP) launched
First genome-wide association study published
Honeybee genome sequence
Sea urchin genome sequence
2006
First direct-to-consumer whole-genome test
Rhesus macaque genome sequence
Human genetic variation is breakthrough of the year
Wellcome Trust Case Control Consortium publication
ENCODE pilot project complete
First personal genome sequenced
2007
Han Chinese genome sequence
Genetic Information Nondiscrimination Act (GINA) passed in US
2008
First personal genome sequenced using new technologies
Yoruba genome sequence
Platypus genome sequence
First cancer genome sequence (AML)
Comprehensive genomic analysis of glioblastoma
2009
Korean genome sequence
Completion of the Mammalian Gene Collection (MGC)
International data release workshop
Bovine genome sequence
First human methylome map
Southern African genome sequences
500th genome-wide association study published
UK Biobank reaches 500,000 participants
>1,000 mouse knockout mutations
modENCODE publications
Neanderthal genome sequence
1000 Genomes pilot project complete
Miller syndrome gene discovered by exome sequencing
Nuffield Council on Bioethics publication on personalized healthcare
2010
© 2011 Macmillan Publishers Limited. All rights reserved

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

Second generation sequencing tech

Second generation sequencing definition

“Synchronized reagent wash of nucleotide triphosphates followed by optical imaging” – *Niedringhaus, T. et al, Reviews Analytical Chemistry, 2011, 83 4327-4341*

Illumina HiSeq

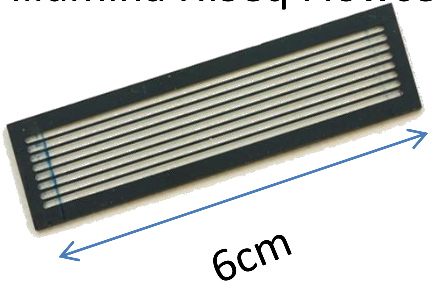
Illumina HiSeq Key Features

- Advantages
  - Large volume of data (300Gb per run)
  - Short run time (< 1 day)
  - Straightforward sample prep
  - Well established open source software community
- Disadvantages
  - Requires pooling of large numbers of samples to achieve lowest costs
  - Short reads (36-150bp)

Illumina Sequence By Synthesis

- Produces approximately 1.6 billion short reads (18bp-150bp) per flowcell
- Each run takes 2-9 days depending on the configuration
- Each flowcell is divided into either 2 or 8 separate lanes (channels)

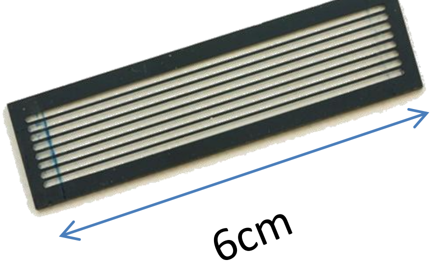
Illumina HiSeq Flowcell



Illumina HiSeq setup

Automated sample preparation

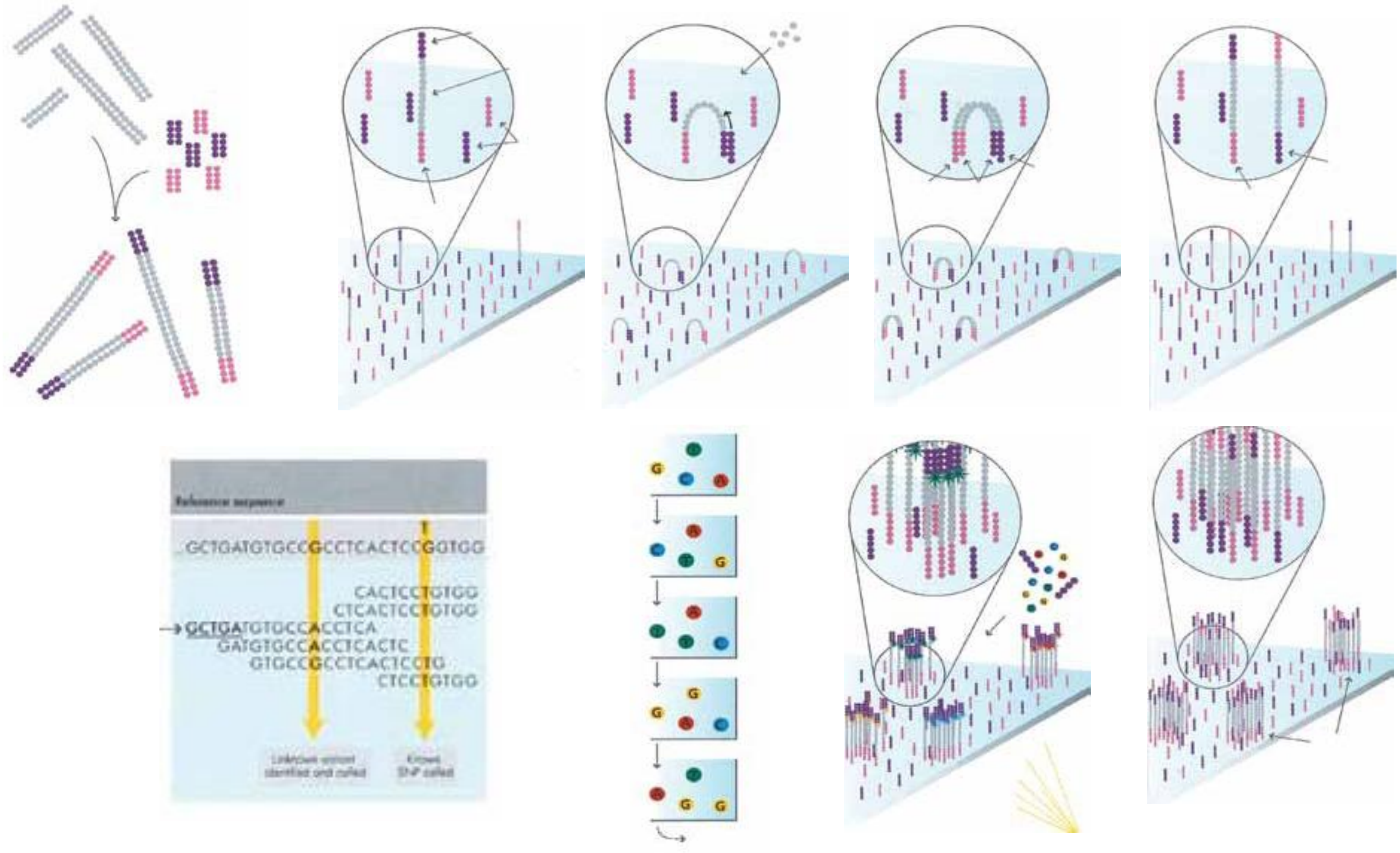
Illumina HiSeq Flowcell



cBot Cluster generation

HiSeq 2500

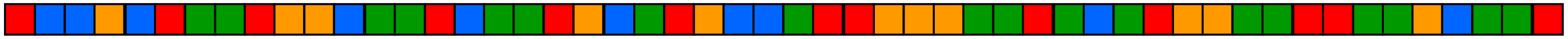
Illumina Sequencing



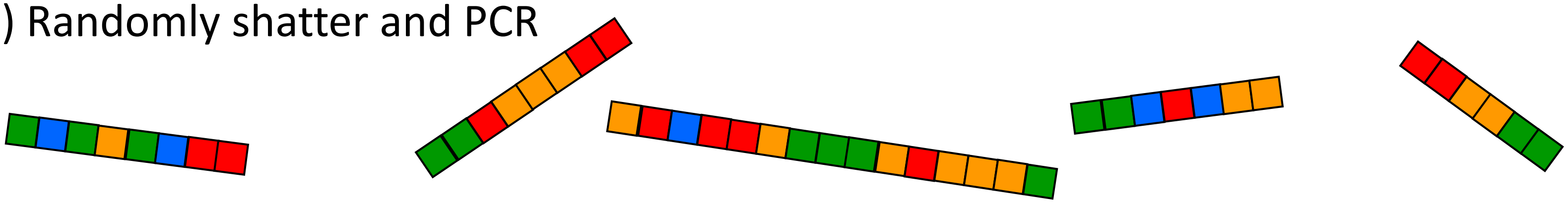
http://www.illumina.com/Documents/products/techspotlights/techspotlight_sequencing.pdf

DNA sample preparation (over-simplified)

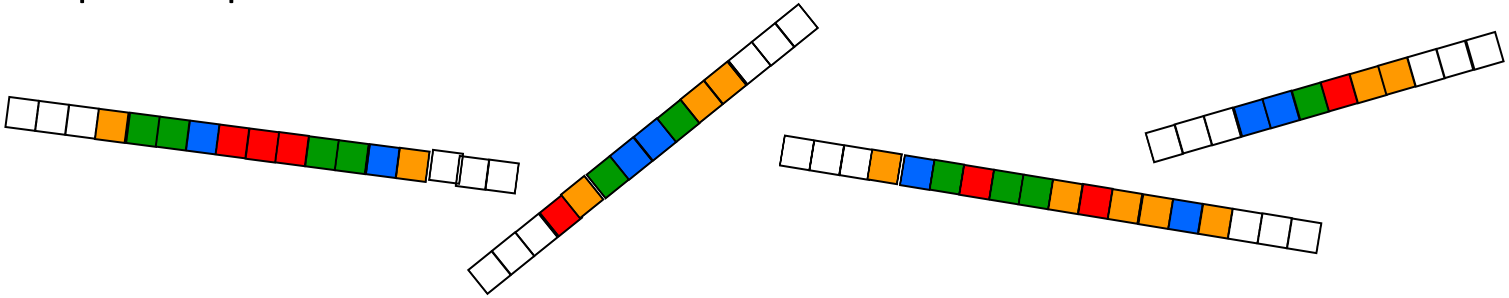
1) Extract DNA



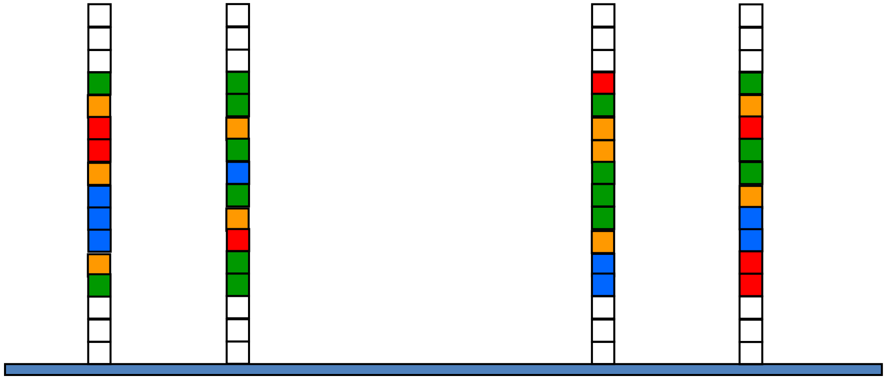
2) Randomly shatter and PCR



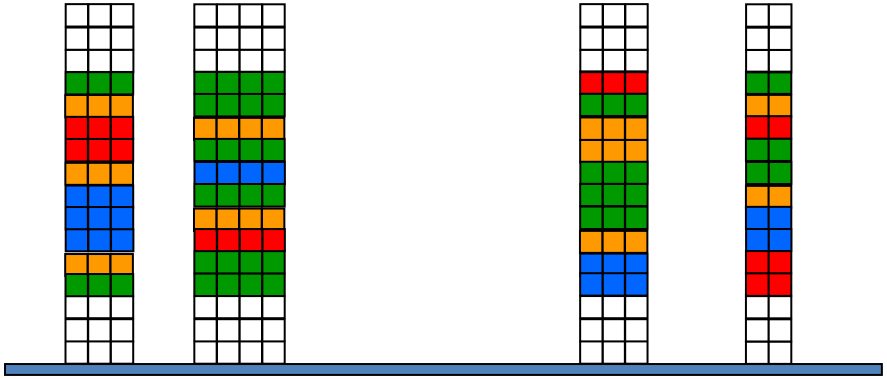
3) Attach adapter sequence □□□



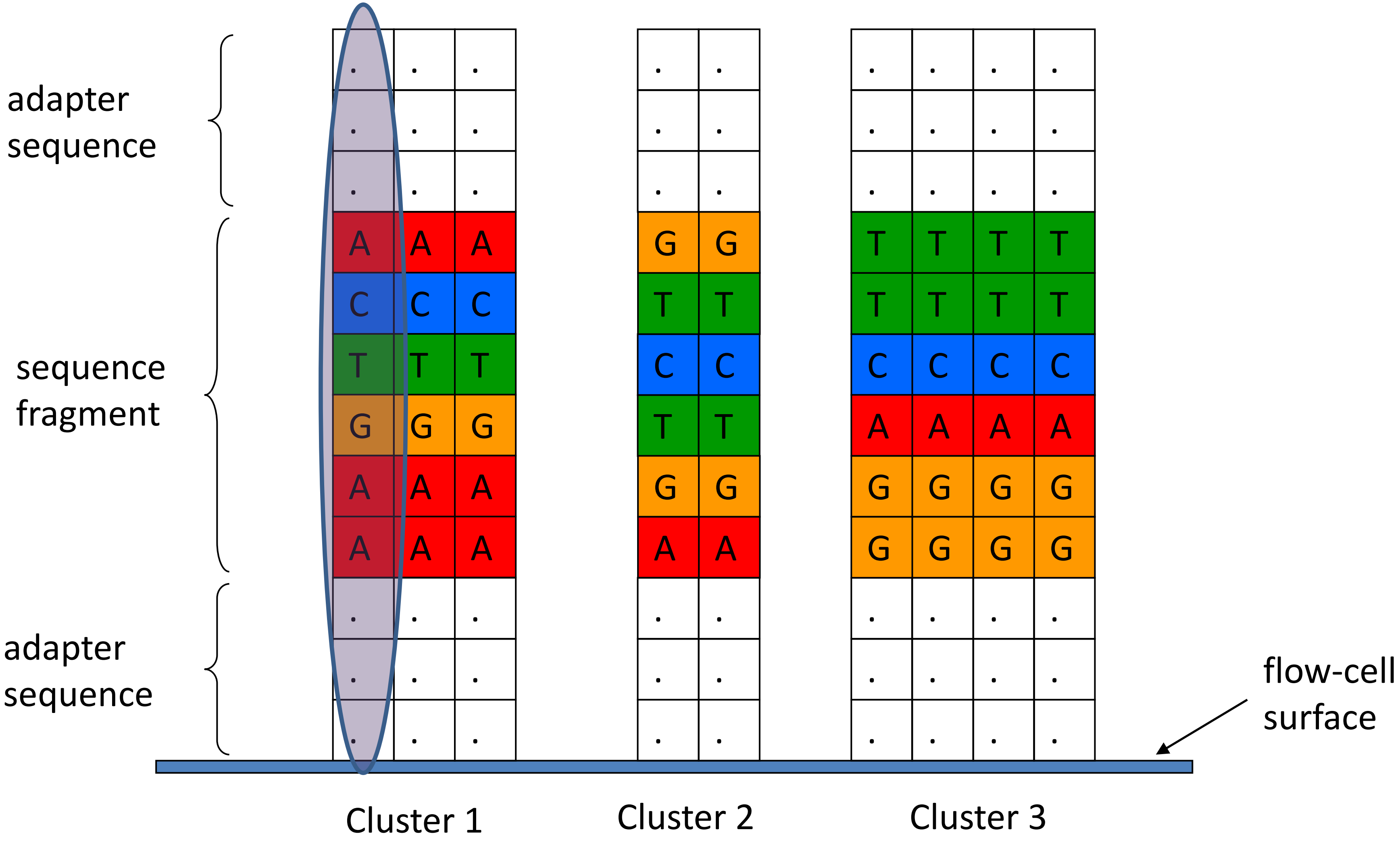
4) Attach to flow-cell surface



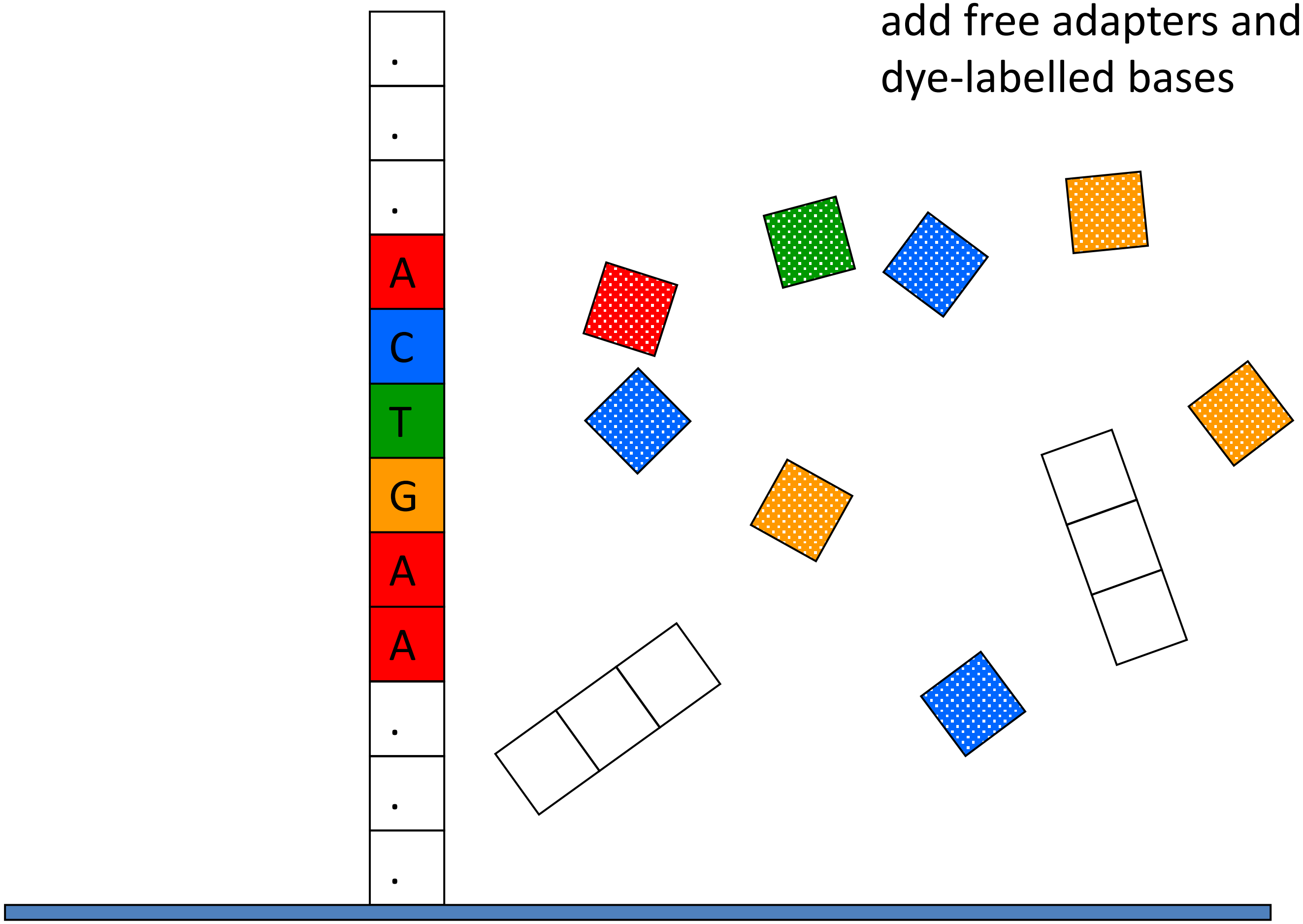
5) PCR-amplify into clusters



Sequence clusters on the flow cell

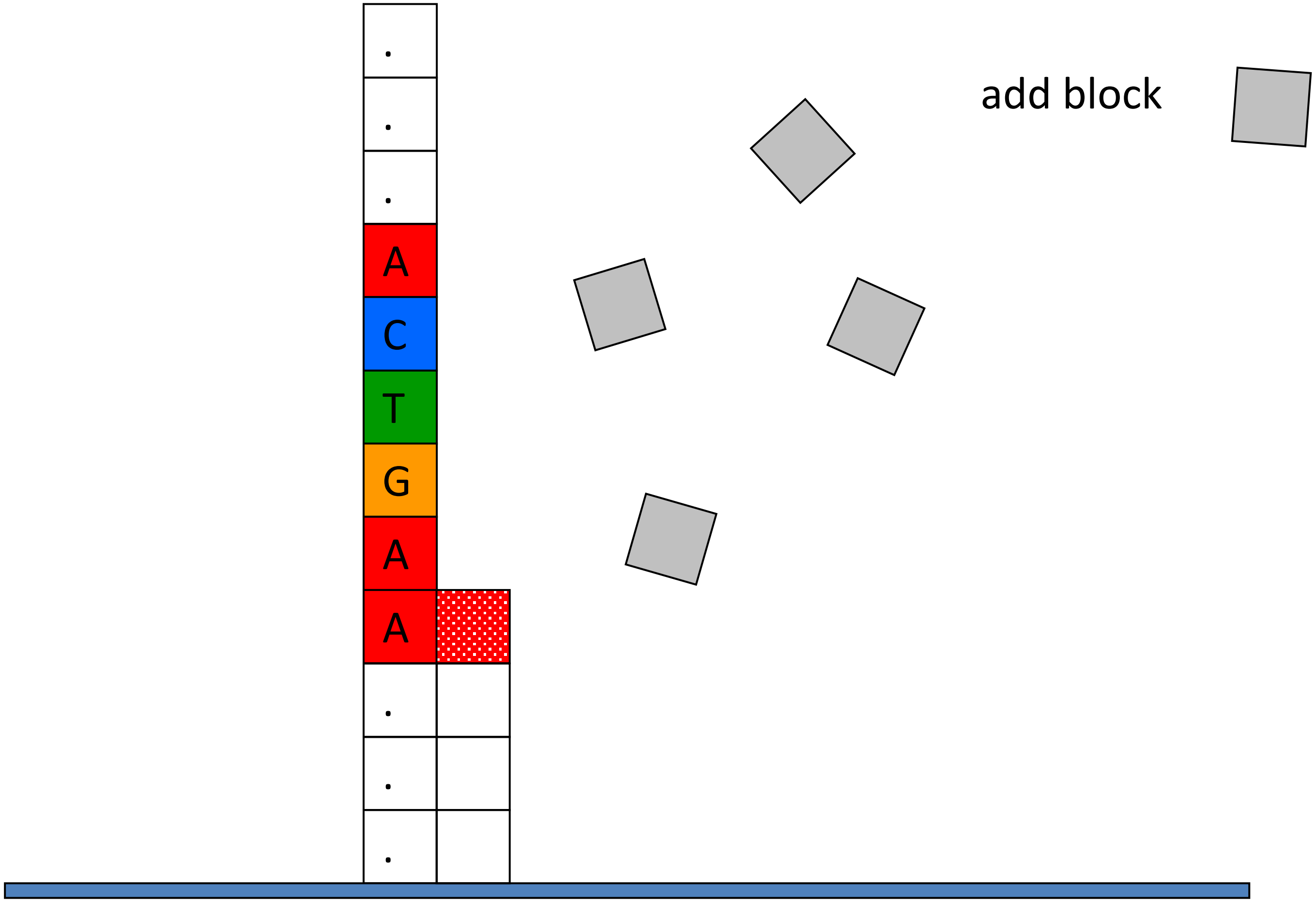


Sequencing cycle 1



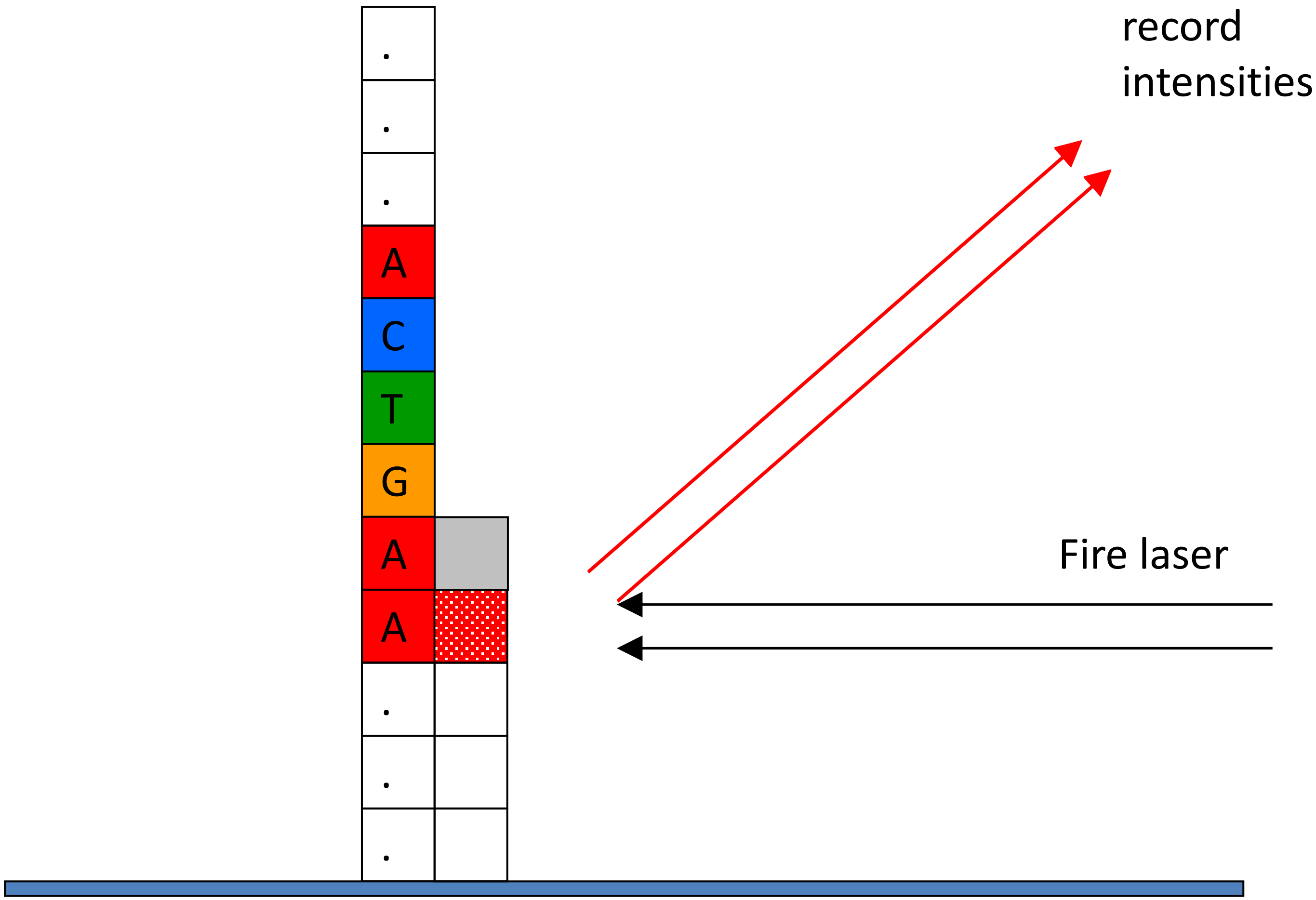
add free adapters and dye-labelled bases

Sequencing cycle 1

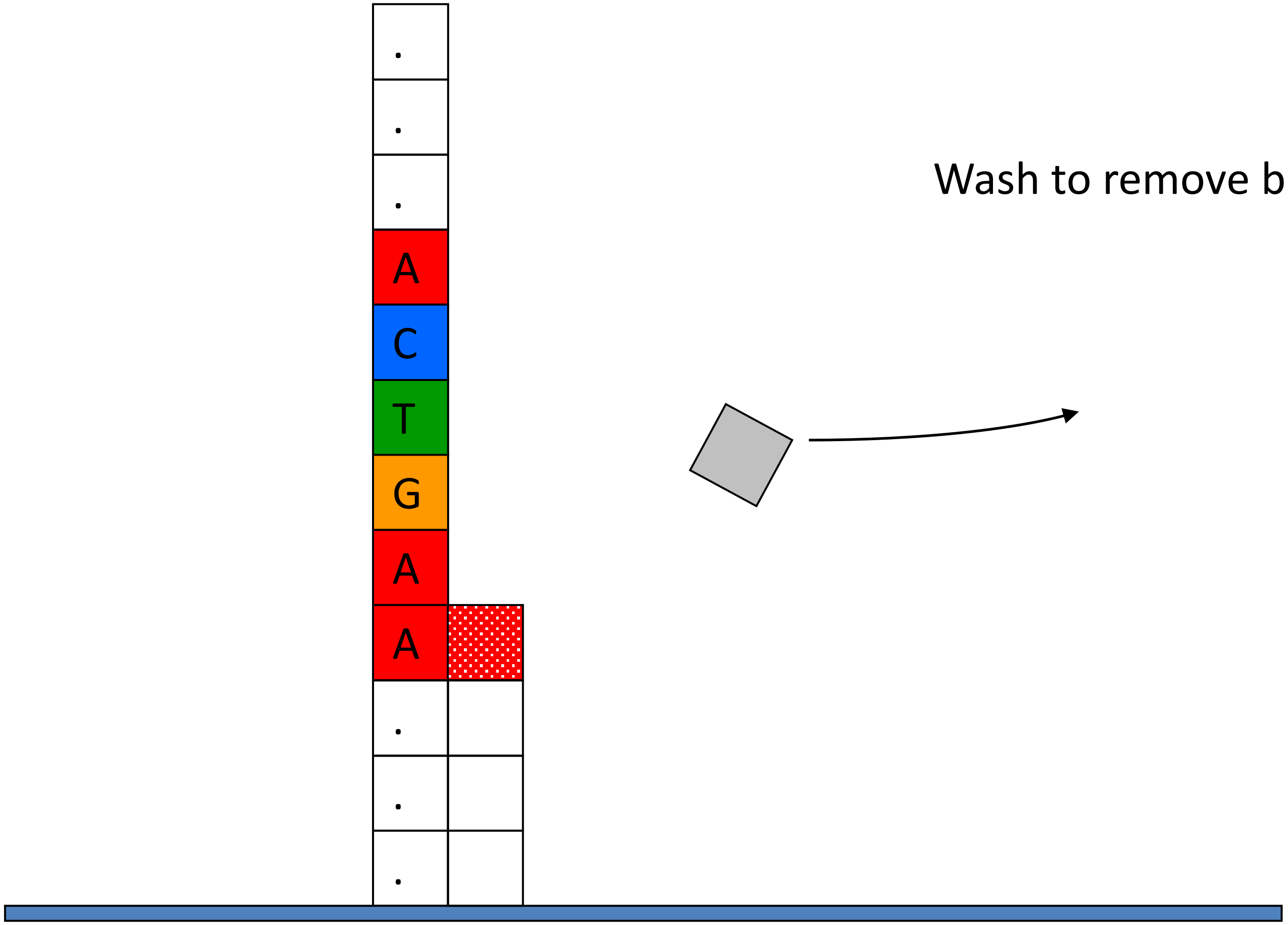


add block

Sequencing cycle 1

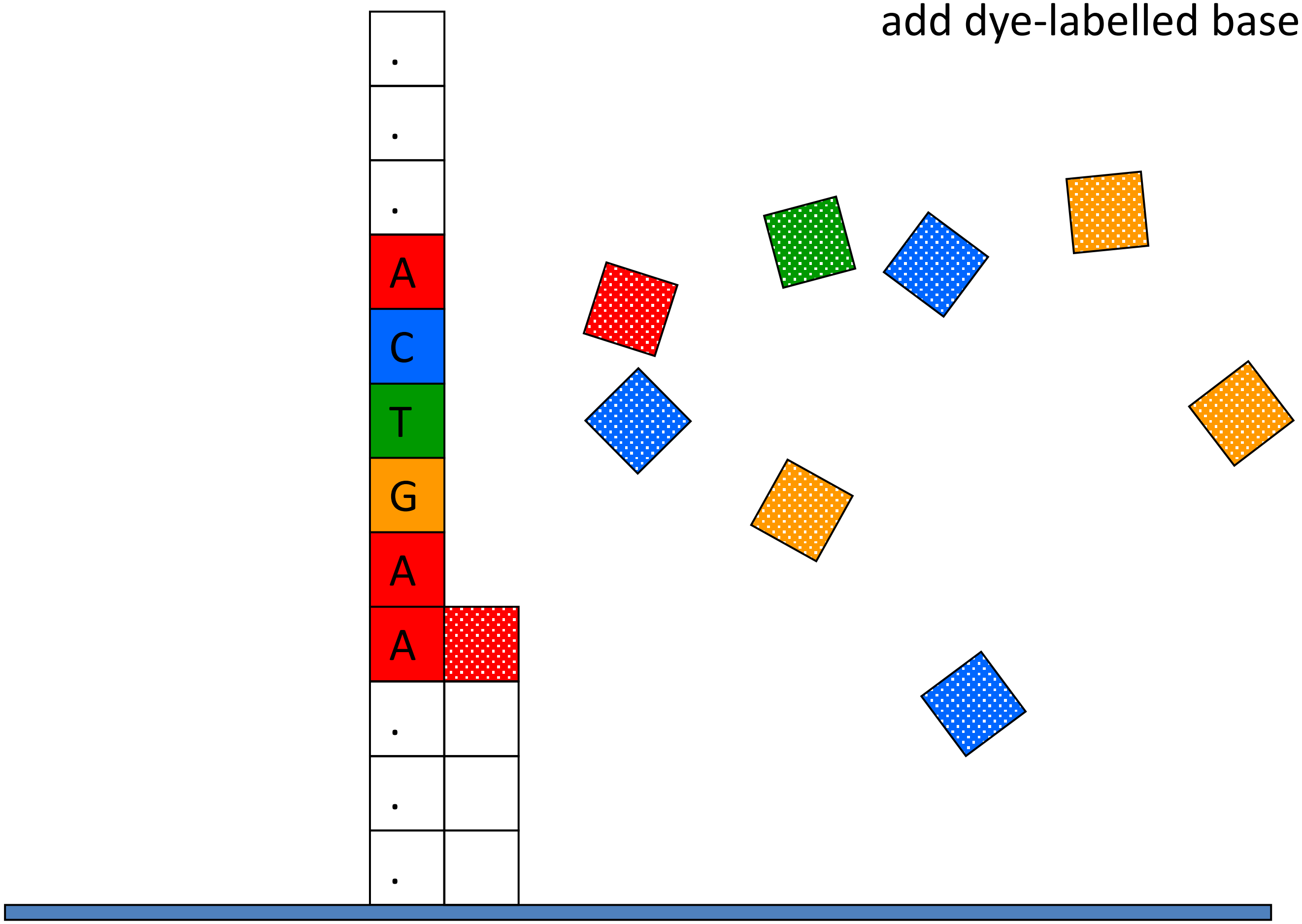


Sequencing cycle 1

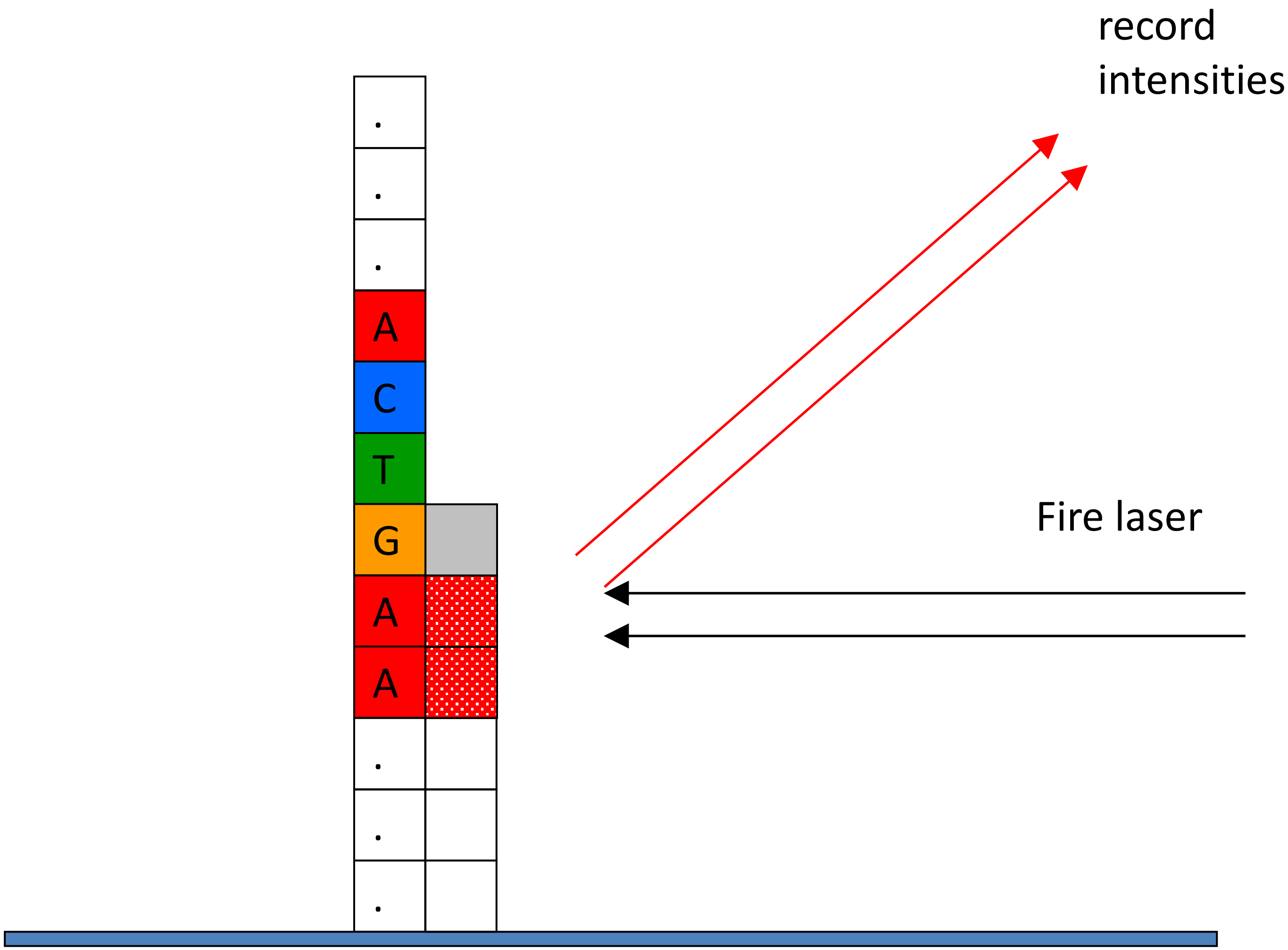


Wash to remove block

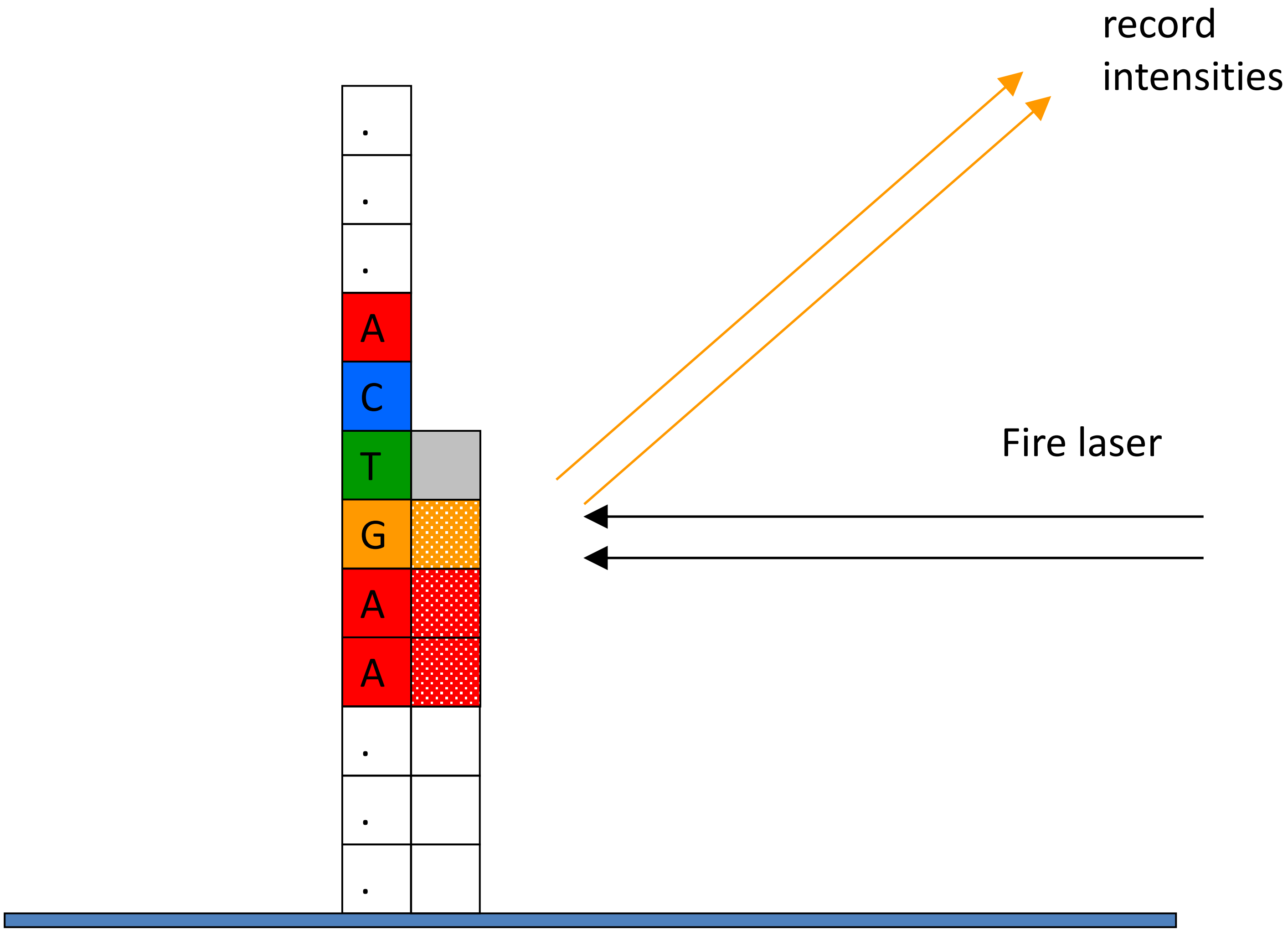
Sequencing cycle 2



Sequencing cycle 2



Sequencing cycle 3



Note

- The schema is over-simplified

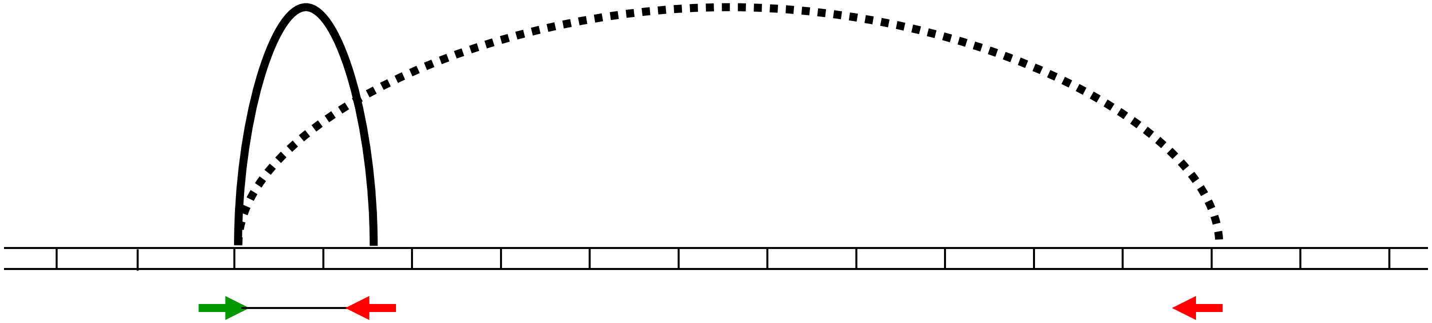
- In reality
  - Sequencing is done from the top of the strand down towards the bottom of the flowcell
  - Reversible blocking agents are part of the modified dNTP fluorophores

Paired-End Sequencing

- Provides distance relationship between two reads
- Important for many applications
  - Characterise insertions, deletions, copy number variants, rearrangements
  - Required for *De novo* assembly
  - Enables sequencing across repeats
  - Useful for Di-Tags, cDNA sequencing, etc.
- Enables sample multiplexing (identifier tags)
- Increases output per flowcell

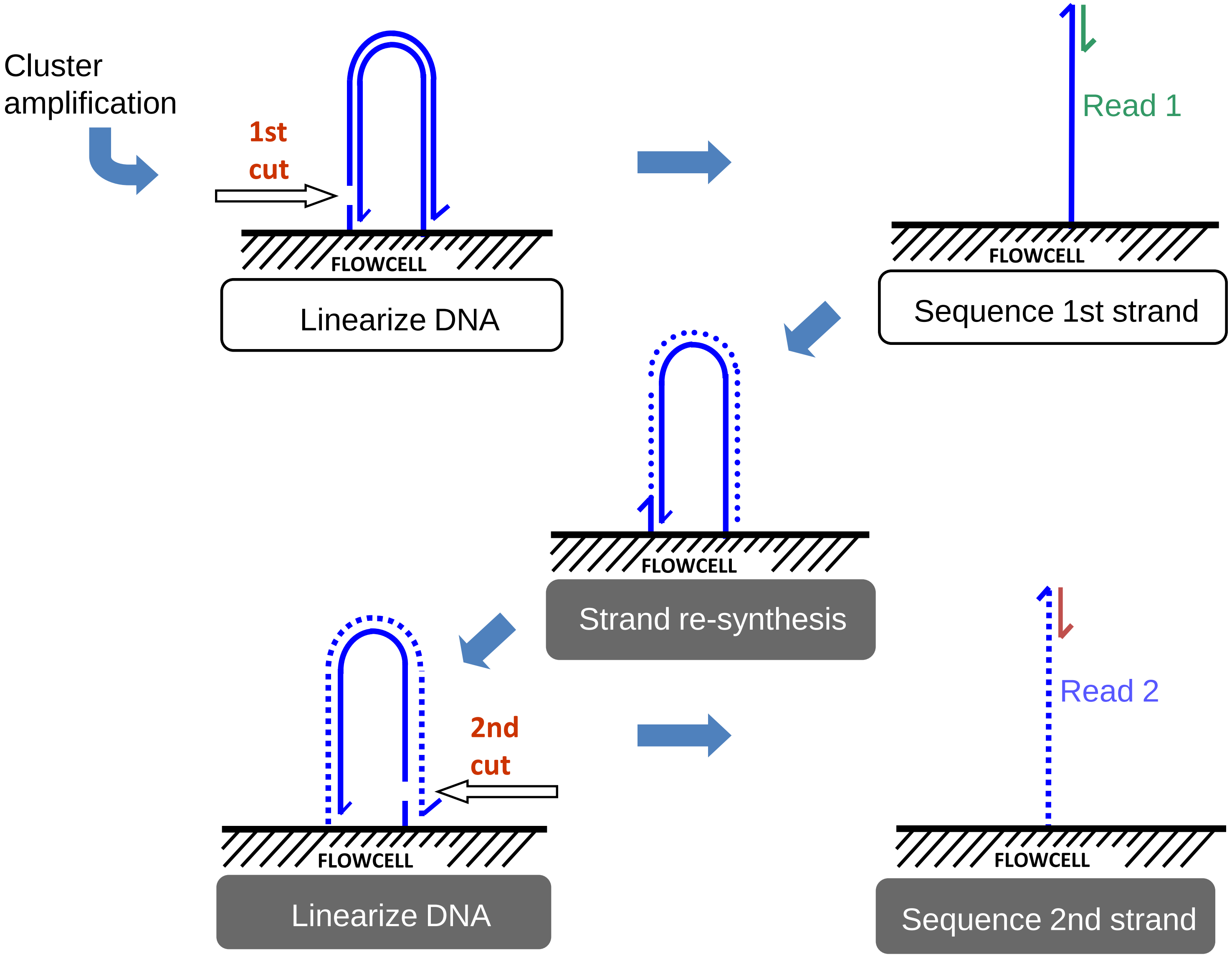
Working with Paired Reads

- Applicable to different fragment size ranges
  - up to ~600 bp for standard libraries
  - 2 - 20kb mate-pair libraries



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

Illumina Paired-End Sequencing



Cluster amplification

1st cut

Linearize DNA

Read 1

Sequence 1st strand

Strand re-synthesis

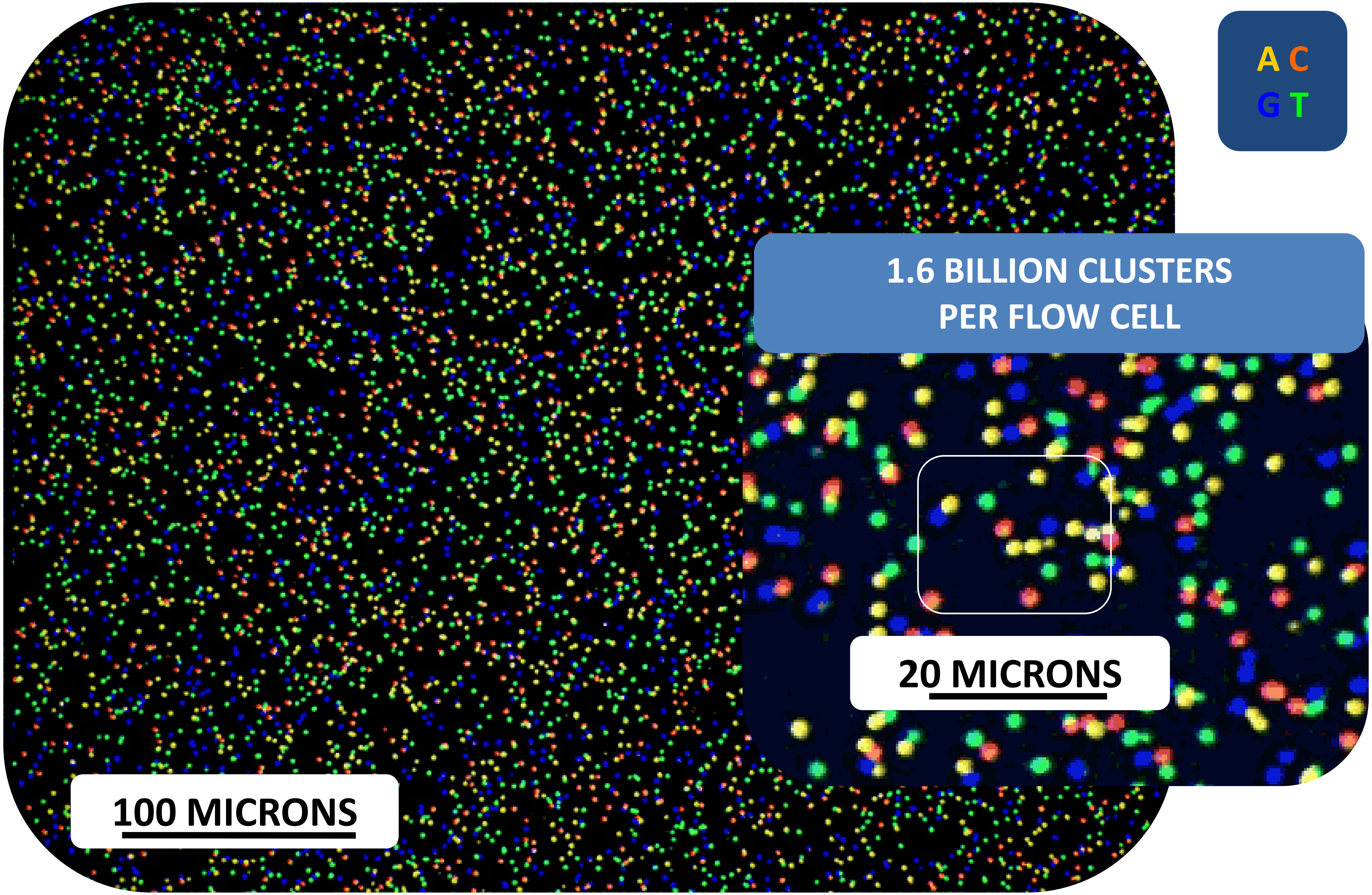
2nd cut

Linearize DNA

Read 2

Sequence 2nd strand

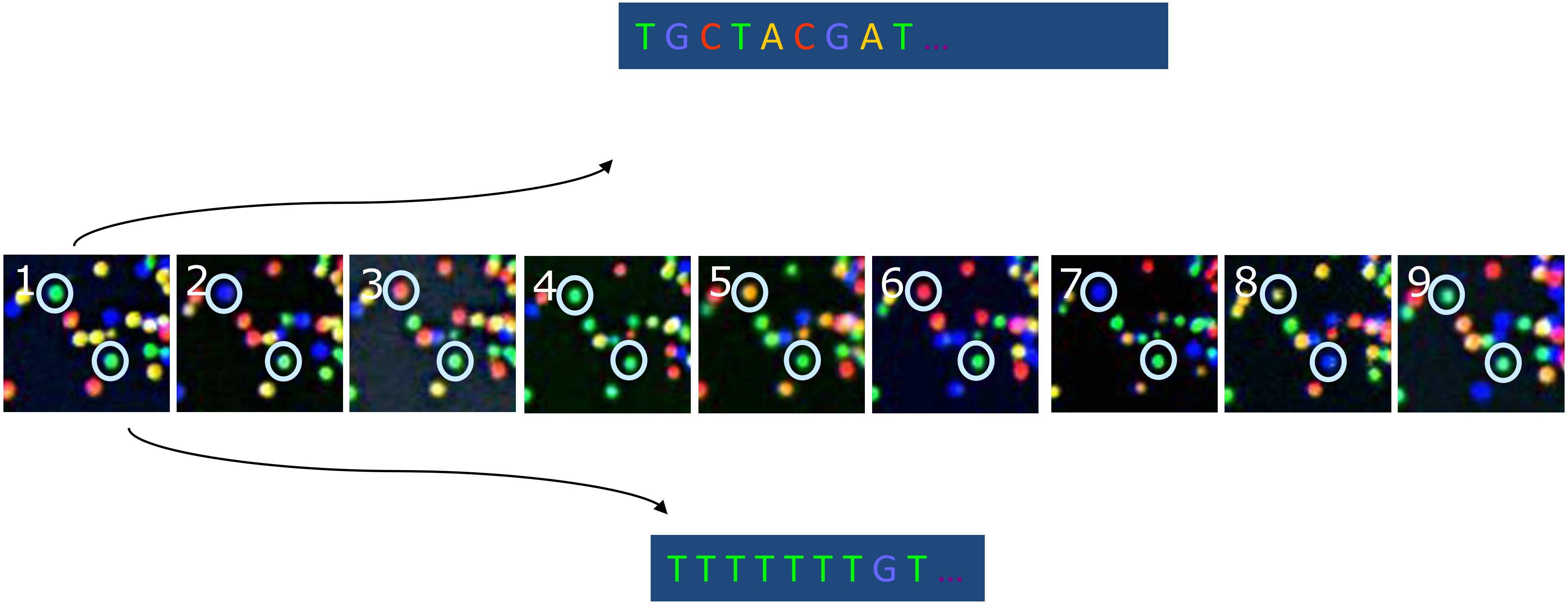
Illumina Sequencing : How it looks



1.6 BILLION CLUSTERS PER FLOW CELL

A C
G T

Base calling from raw data



TGCTACGAT...

TTTTTTTGT...

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

Current read lengths = 36-150 nt
Total sequence data for 1 paired-end run with 100bp = 300Gb!

HiSeq 2000 vs 2500 flowcells

HiSeq 2000
8 lanes

12 day run time

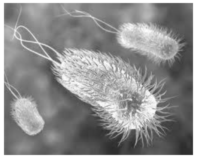
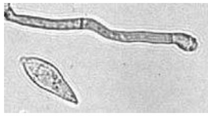
HiSeq 2500
2 lanes

2 day run time

Comparison

APPLICATION	RAPID RUN MODE	HIGH OUTPUT MODE
ChIP-Seq Transcription Factor 1 x 36 bp	40 Samples 7 Hours	200 Samples 2 Days
mRNA-Seq 2 x 50 bp	24 Samples 16 Hours	120 Samples 5 Days
TruSeq Exome Seq 62 MB Region 100x Coverage 2 x 100 bp	15 Samples 27 Hours	85 Samples 12 Days
Human Whole Genome >30x Coverage 2 x 100 bp	1 Sample 27 Hours	5 Samples 12 Days

What does this mean?

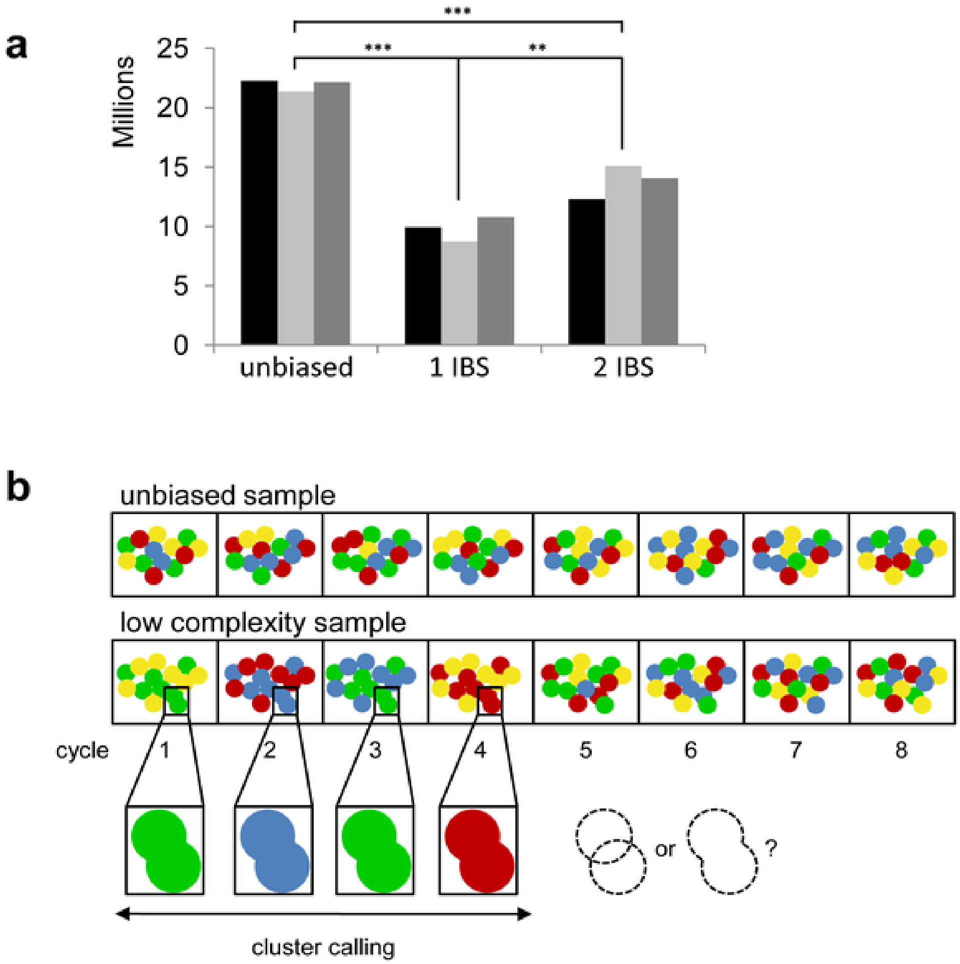
	Rapid run	Slow run
	48 genomes (£250 per sample)	48 genomes/lane (£210 per sample)
	10 genomes (£510 per sample)	10 genomes/lane (£350 per sample)
	8 genomes (£590 per sample)	8 genomes/lane (£400 per sample)
	1 genome (£3400)	1 genome (£4000)

Potential issues with Illumina sequencing

- Low diversity sequences
  - 16S/amplicon sequences
  - Custom adaptors with barcodes at 5’ end
- GC/AT bias
  - GC clusters are smaller than AT
  - (less of a problem post June 2011)
- Specific motifs which are difficult to sequence
  - GGC motif
  - Inverted repeats

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

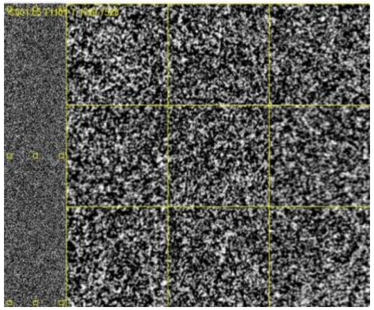
Low Diversity samples



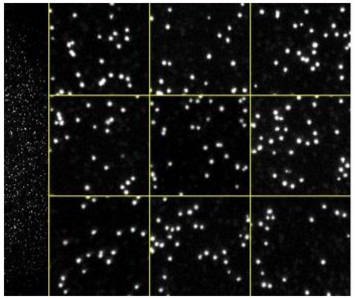
Krueger F, Andrews SR, Osborne CS (2011) Large Scale Loss of Data in Low-Diversity Illumina Sequencing Libraries Can Be Recovered by Deferred Cluster Calling. PLoS ONE 6(1): e16607. doi:10.1371/journal.pone.0016607
http://www.plosone.org/article/info:doi/10.1371/journal.pone.0016607

Low Diversity Example

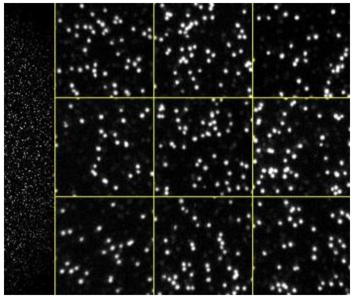
- Cycle 1



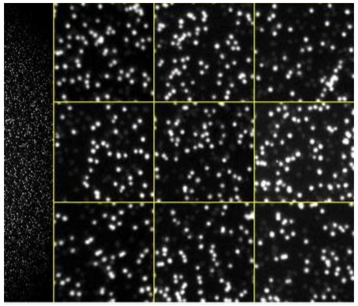
T



C



A



G

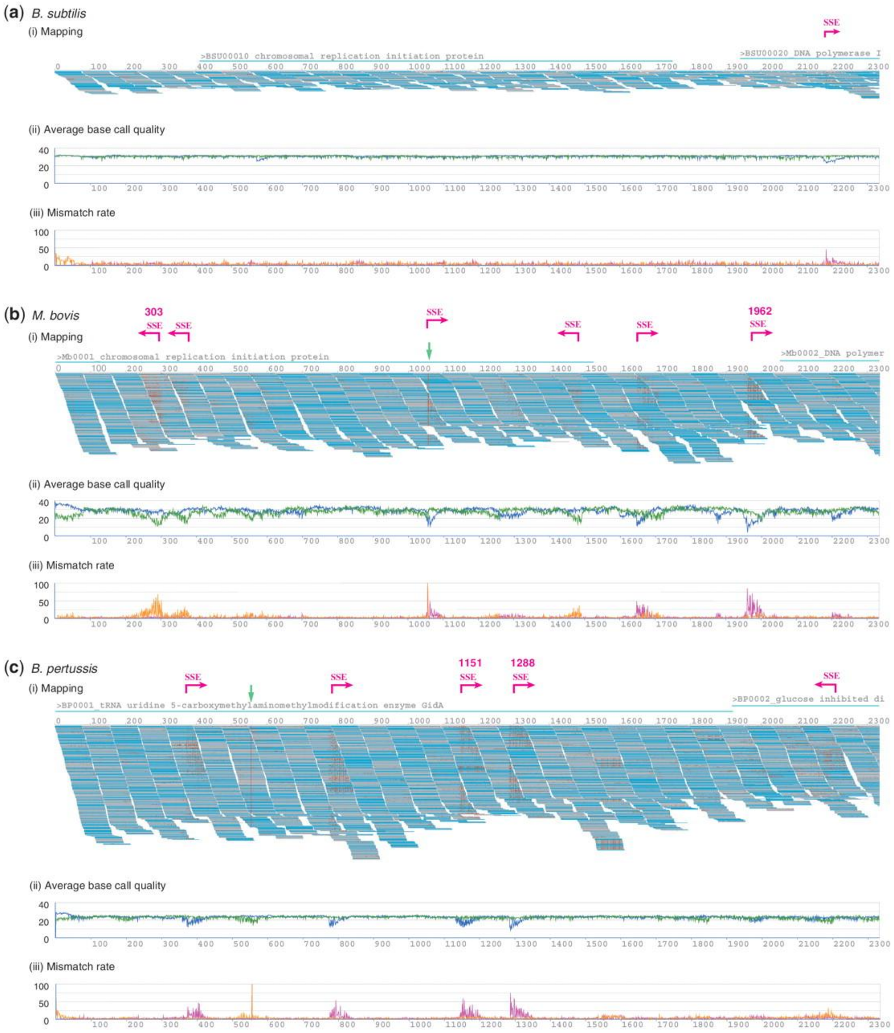
Low diversity workarounds

- Multiplex with diverse samples and sequence across multiple lanes/runs
- Add PhiX control
- If dealing with amplicon, TraDIS or RAD-seq material, design multiple offset primers

Low diversity issue ‘fixed’

- Illumina are bringing out two improvements which are claimed to alleviate the problem
  - Software fix to prevent quality fall off due to incorrect phasing estimates. Available on MiSeq now.
    - Possibility of implementing those same changes on the HiSeq is uncertain
  - Ordered flowcells – base-caller will know apriori where clusters should be

Sequence-specific error motifs (GCC and inverted repeats)



Nakamura K et al. Nucl. Acids Res. 2011;nar.gkr344

Illumina MiSeq

- Same technology and chemistry as HiSeq
- 2X300bp reads
- 15 Gbase/run
- Run 48-72 hours
- **$800-$1000 / run**
- **$100K instrument**
- **$50k for additional 2 year service contract**
- Capable of sequencing 48 5Mb bacterial genomes per run
- Libraries compatible with HiSeq

Future Illumina developments

- 2x250bp reads (HiSeq fast run mode)
- Ordered flowcells
- 2x400bp reads (MiSeq)
- 10kb synthetic reads (approx. 5-6 million per lane)
    - Useful for phasing of haplotypes
    - Formed from short reads so repeat spanning is still problematic

Other equipment (optional)

Agilent Bravo liquid handling robot
£85k

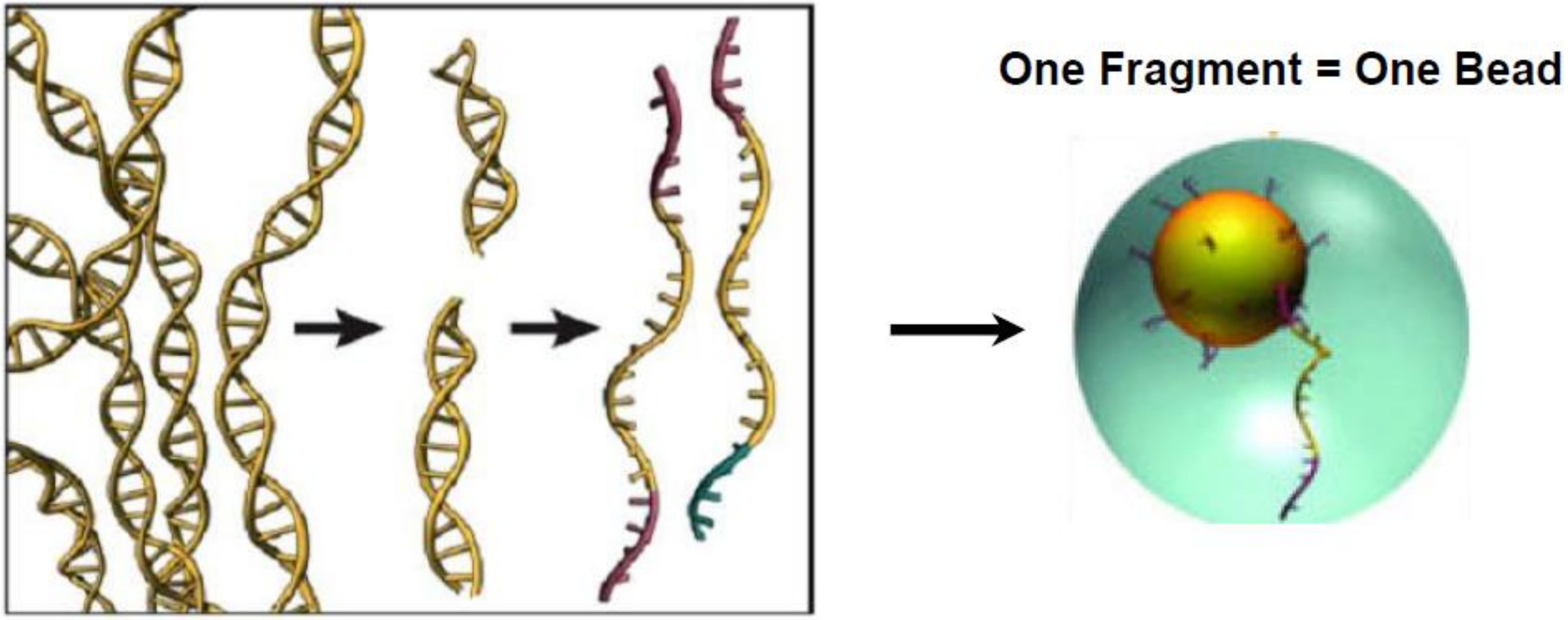
Agilent Tapestation
£30k

Covaris 96-well sonicator
£90k

Roche 454 Key Features

- Advantages
  - Long read lengths (200-1000bp)
  - Multiple samples possible
  - Short run time (< 1 day)
- Disadvantages
  - Relatively expensive (~£8k per run)
  - Low volume of sequence data (100Mb-1Gb)
  - Complex sample prep
  - Roche discontinuing support from 2015

454 Step 1: Sample preparation

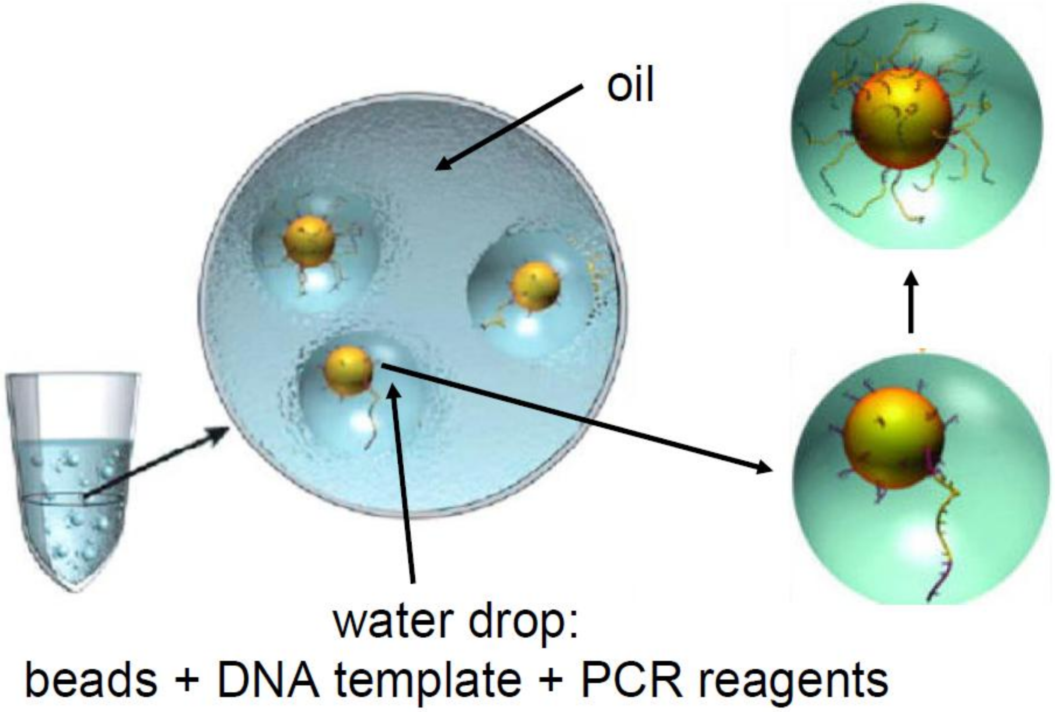


1. Genomic DNA is isolated and fragmented.
2. Adaptors are ligated to single stranded DNA
3. This forms a library

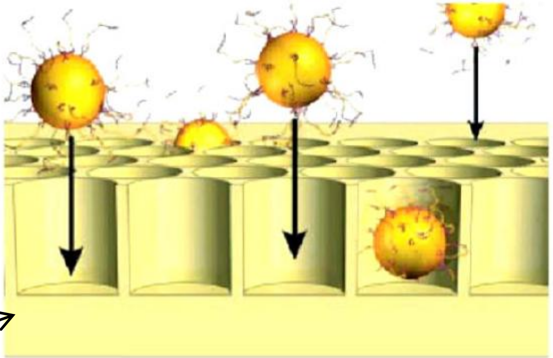
4. The single stranded DNA library is immobilised onto proprietary DNA capture beads

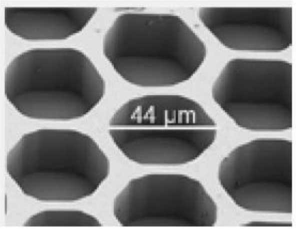
454 Step 2: Amplification

Water-based emulsion PCR



454 Step 3: Load emPCR products

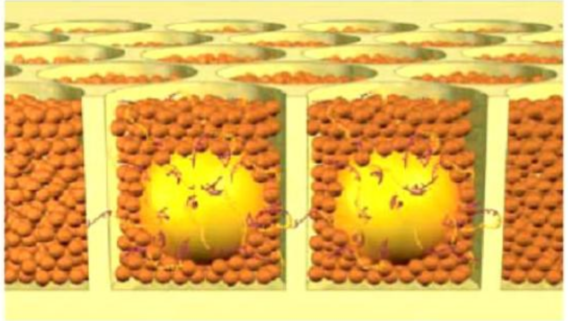


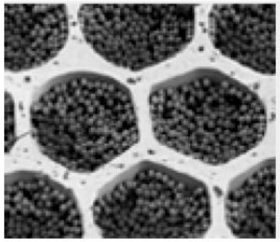


Picotitre plate

- enrich for DNA + beads

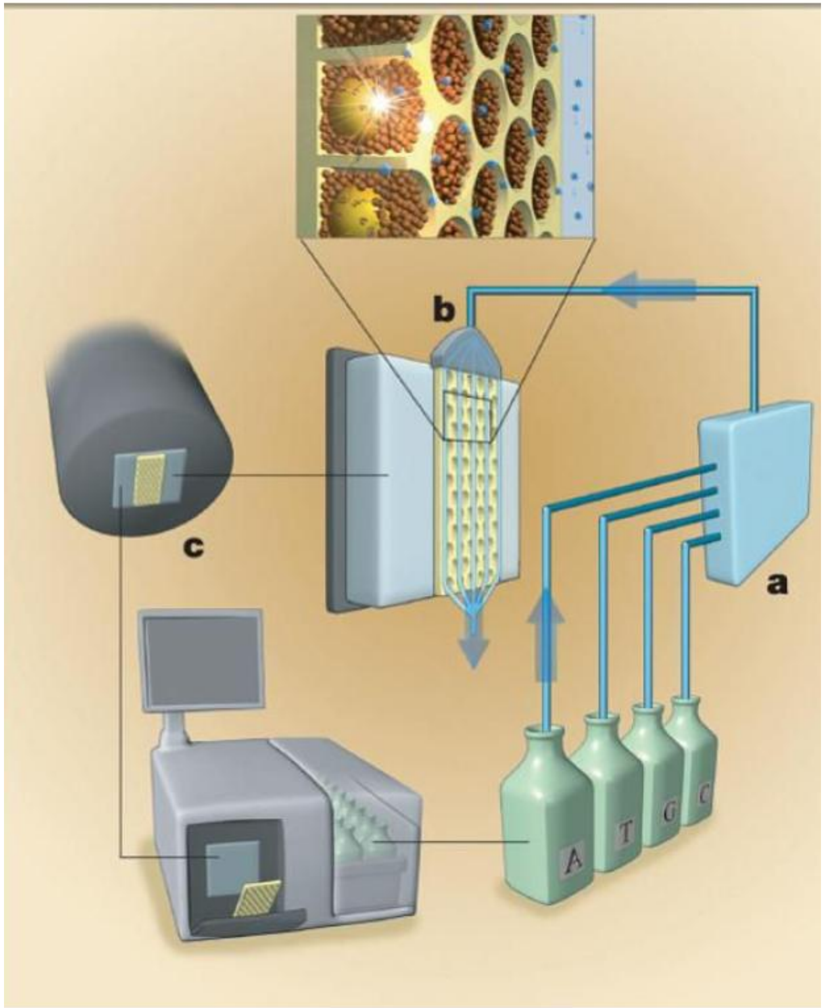
- diameter of the wells allows for only 1 bead/well





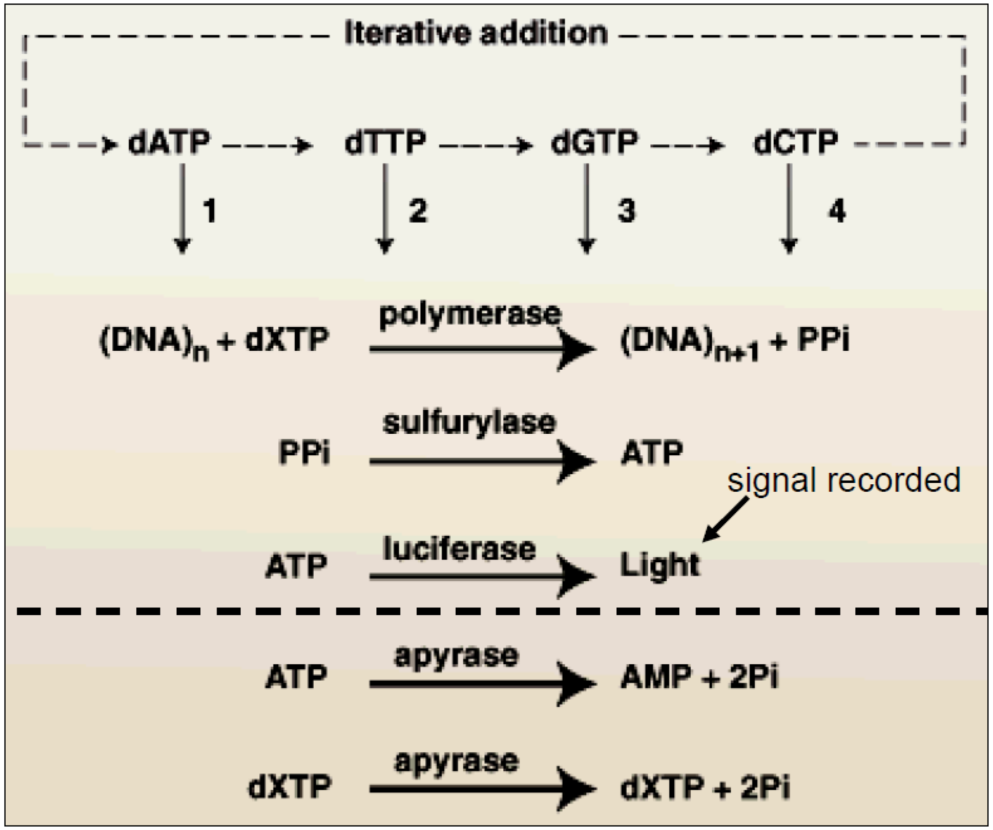
Smaller beads (red) carrying immobilized enzymes required for pyrophosphate sequencing are deposited into each well.

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



Iterative addition

dATP → dTTP → dGTP → dCTP

1 2 3 4

$(DNA)_n + dXTP \xrightarrow{\text{polymerase}} (DNA)_{n+1} + PPi$

$PPi \xrightarrow{\text{sulfurylase}} ATP$

$ATP \xrightarrow{\text{luciferase}} Light$

signal recorded

$ATP \xrightarrow{\text{apyrase}} AMP + 2Pi$

$dXTP \xrightarrow{\text{apyrase}} dXTP + 2Pi$

SOLiD

- Differs from Illumina and 454
  - No dXTP reagents are used
  - Oligonucleotide primer-based sequencing is used
  - Two bases are read at a time
  - High accuracy

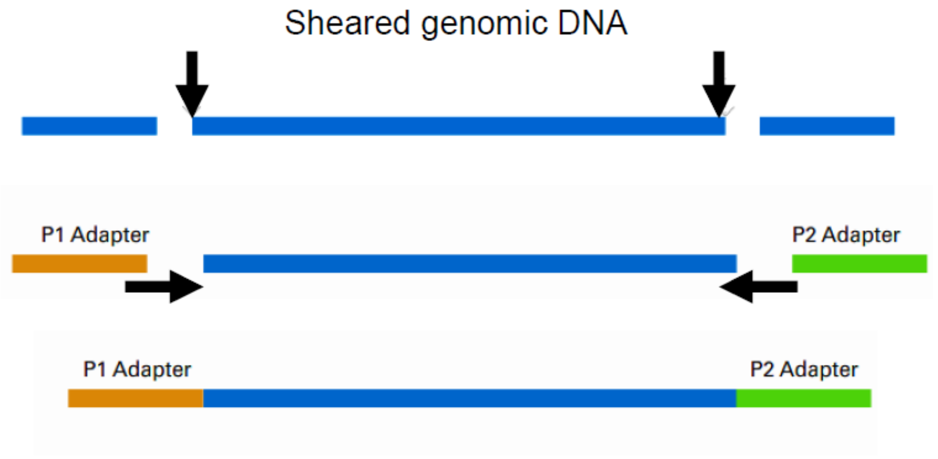
BUT – Only one colour is emitted

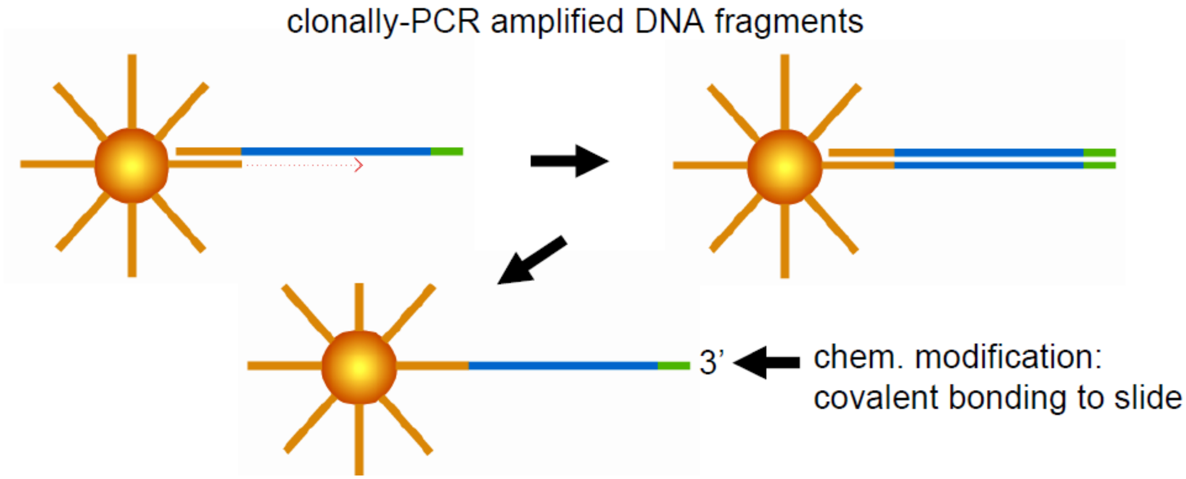
Need several sequencing steps to convert colour to a sequence

Life Technologies SOLiD

- Advantages
  - Two base encoding system
  - Every base read twice
  - Large volume of sequence data (270Gb per run possible)
- Disadvantages
  - Short read lengths (30-80bp)
  - Complex sample prep
  - Bioinformatics support less comprehensive
  - Paired-end reads more complex than Illumina or 454

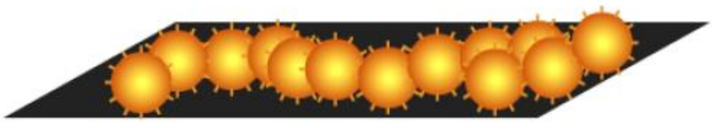
SOLiD: Step 1 Sample Prep



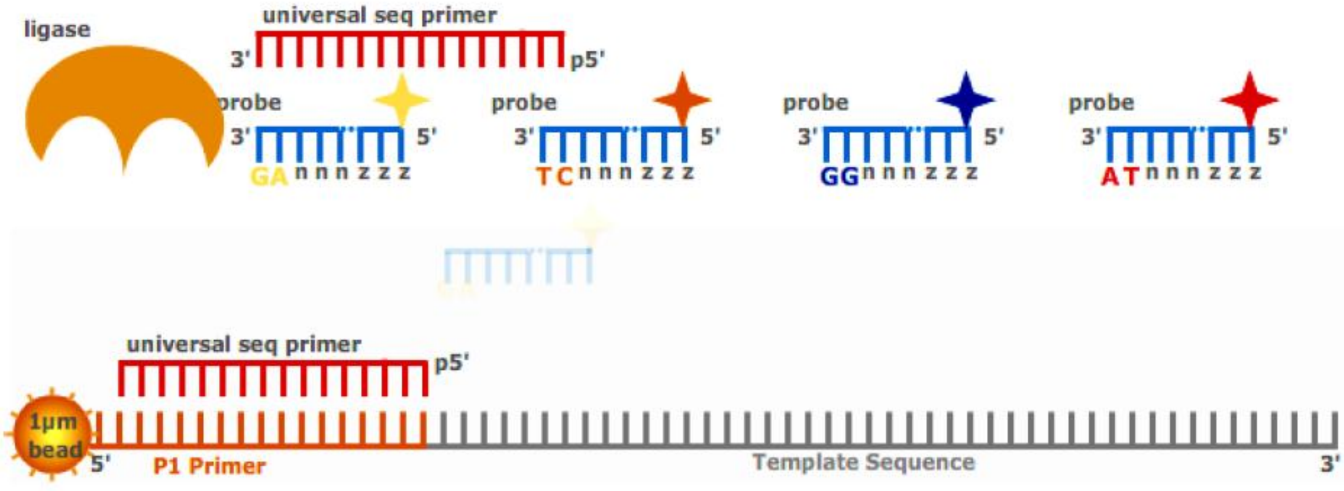

clonally-PCR amplified DNA fragments
3’
chem. modification:
covalent bonding to slide

SOLiD: Step 2 Attach beads

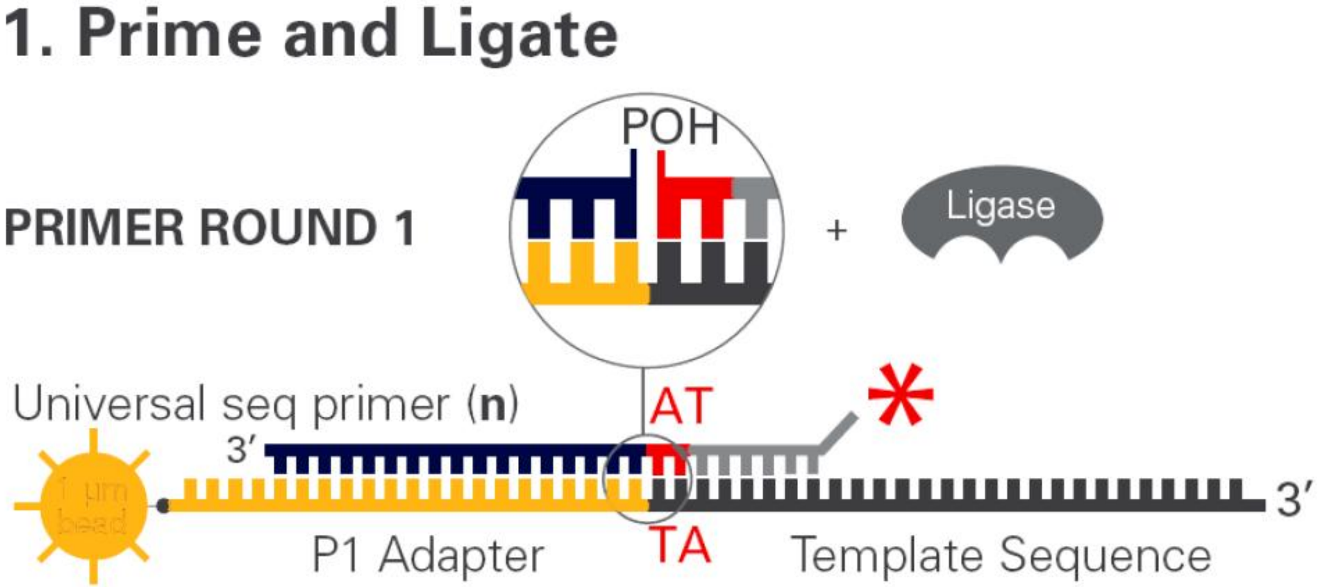
3’-modified beads
deposited onto glass slide



Sequential ligation with dye-labeled **oligonucleotides**

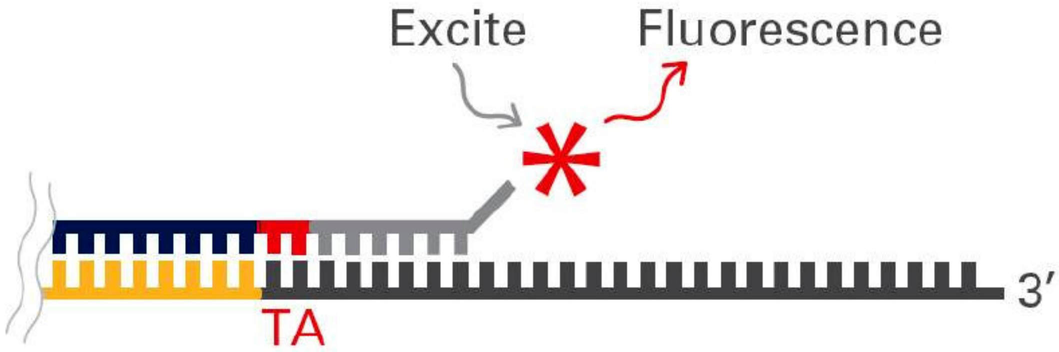


SOLiD: Step 3 Sequencing 1

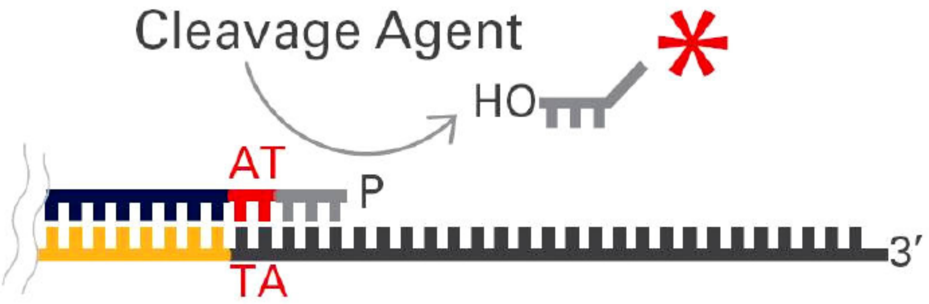


SOLiD: Step 3 Sequencing 2

2. Image

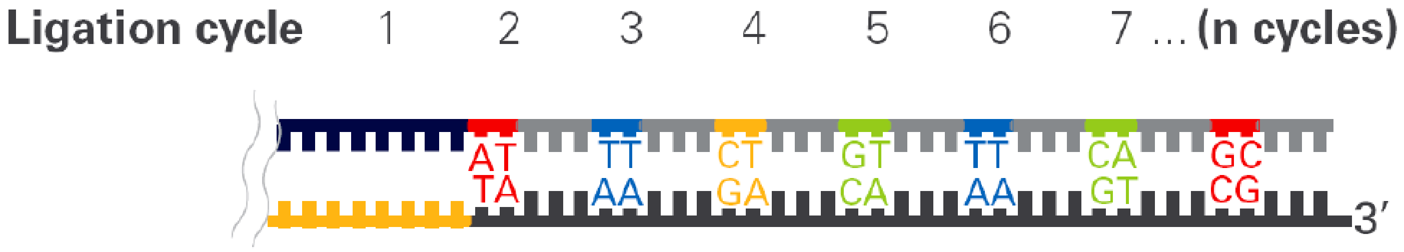


3. Cleave off Fluor



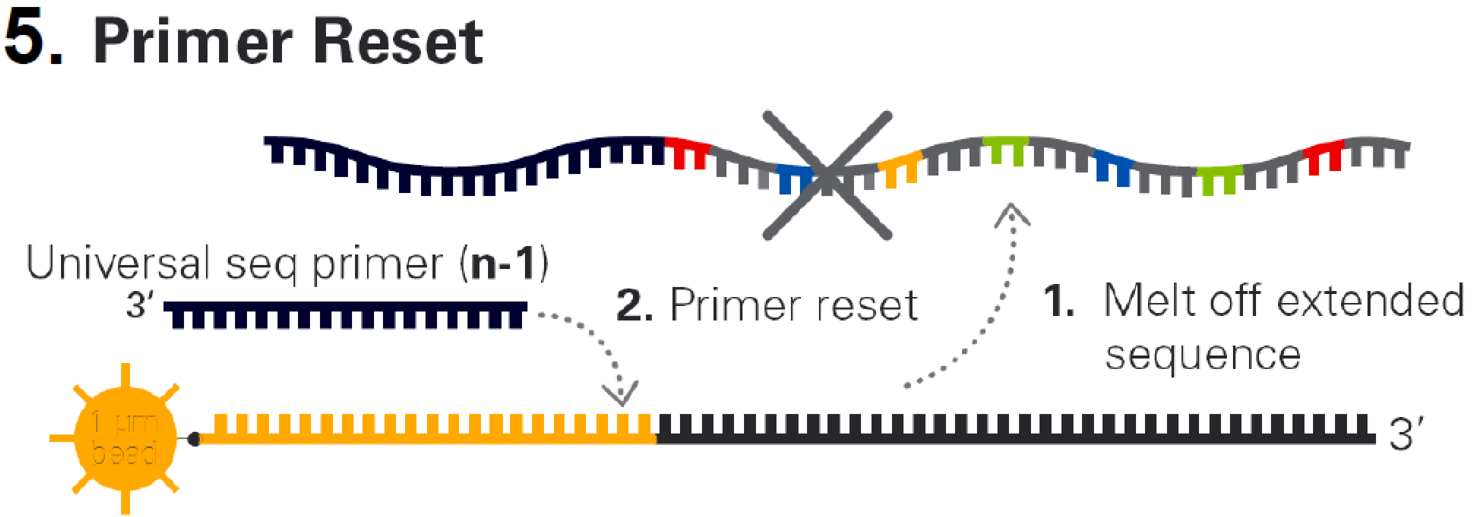
SOLiD Step 3 Sequencing 3

4. Repeat steps 1-4 to Extend Sequence



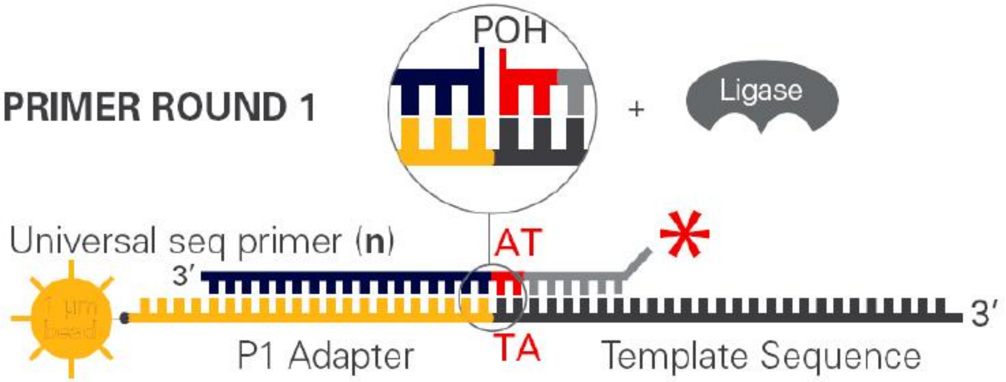
A random primer is ligated to the template only when the labeled nucleotide complements the fifth nucleotide on the template, counting from the end of the previously ligated primer.

SOLiD Step 3 Sequencing 4

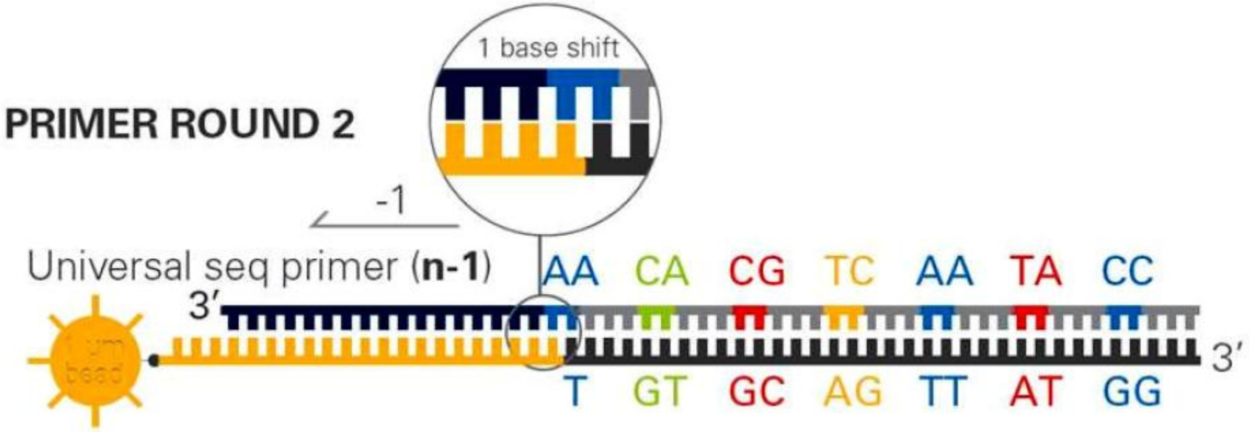


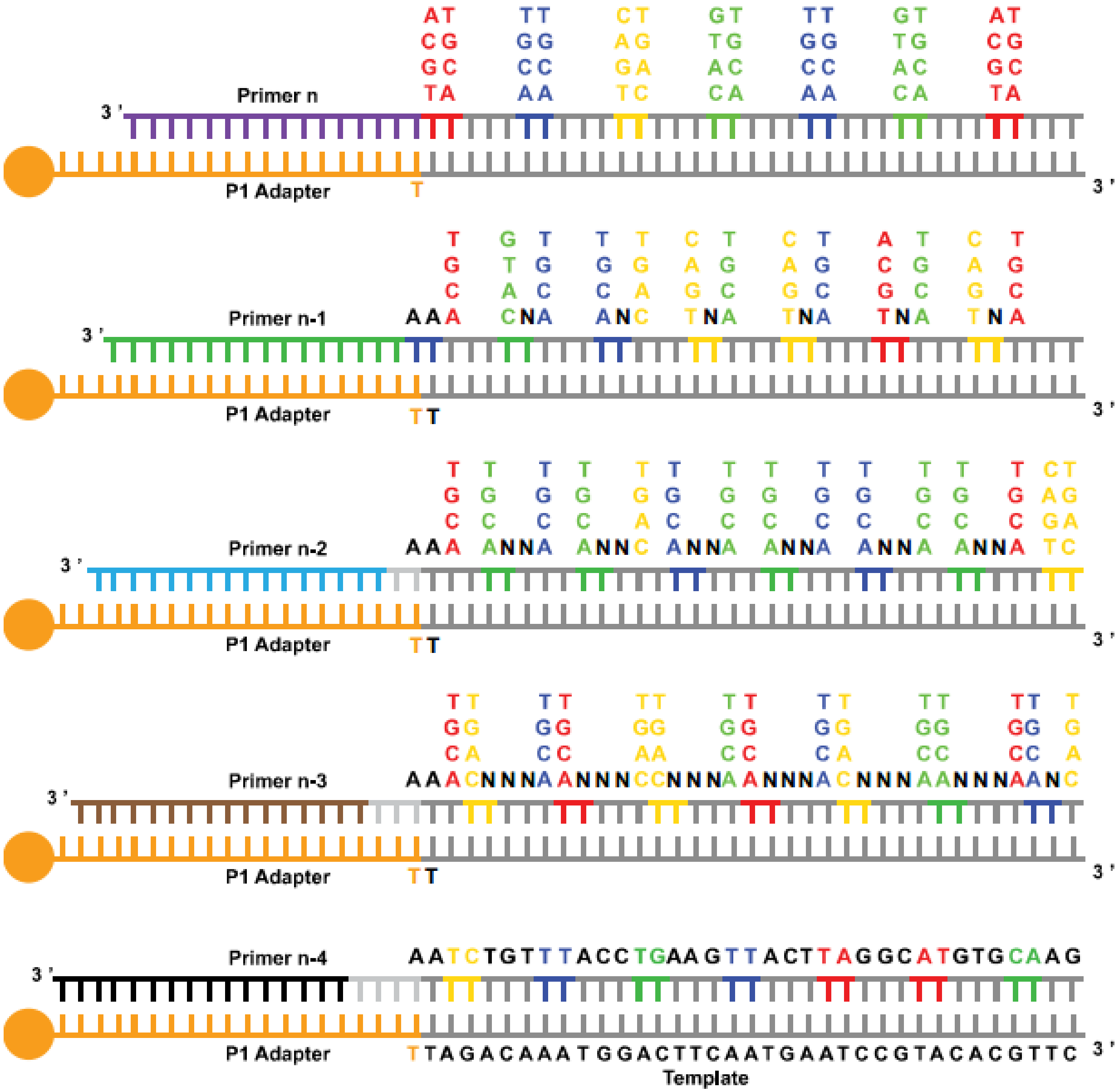
SOLiD Step 3 Sequencing 5

1. Prime and Ligate

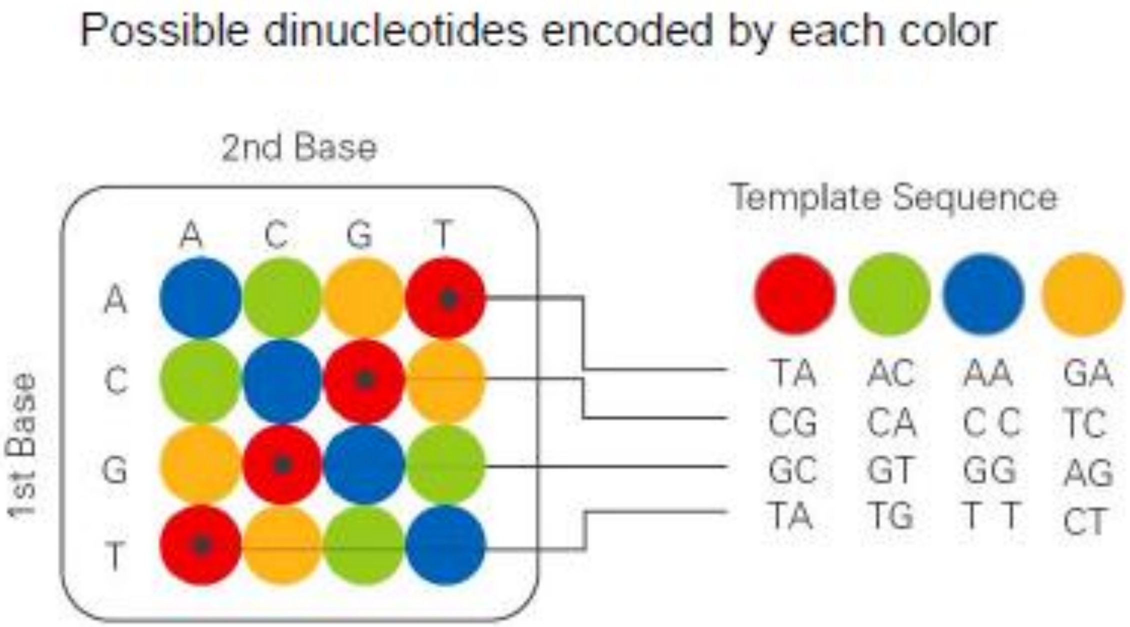


6. Repeat steps 1-5 with new primer




Primer n
3 '
P1 Adapter
T
3 '
Primer n-1
3 '
P1 Adapter
TT
3 '
Primer n-2
3 '
P1 Adapter
TT
3 '
Primer n-3
3 '
P1 Adapter
TT
3 '
Primer n-4
3 '
AATCTGTTTACCTGAAGTTACTTAGGCATGTGCAAG
P1 Adapter
TTAGACAAATGGACTTCAATGAATCCGTACACGTTC
3 '
Template

SoLID Colour space



Possible dinucleotides encoded by each color

2nd Base

1st Base

	A	C	G	T
A	🟦	🟩	🟧	🟥
C	🟩	🟦	🟥	🟧
G	🟧	🟥	🟦	🟩
T	🟥	🟧	🟩	🟦

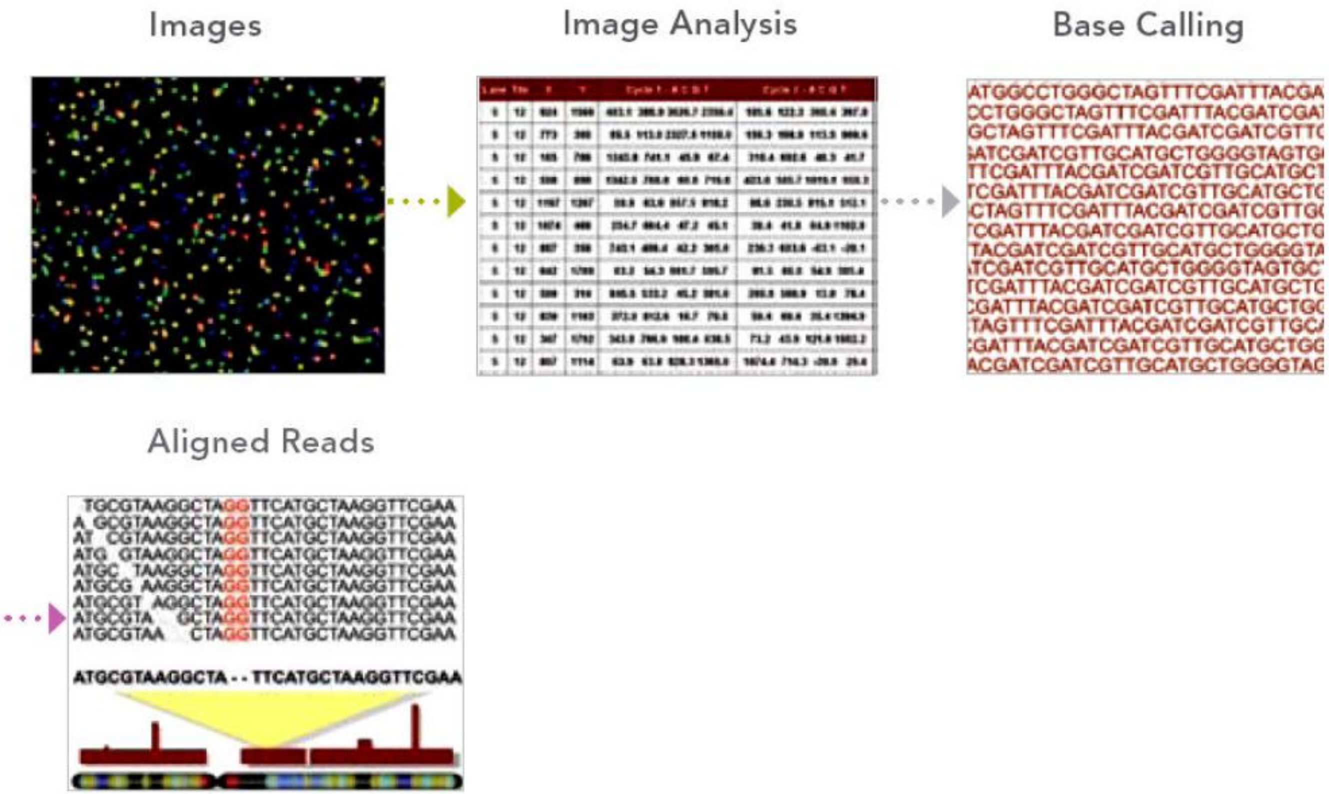
Template Sequence

🟥	🟩	🟦	🟧
TA	AC	AA	GA
CG	CA	C C	TC
GC	GT	GG	AG
TA	TG	T T	CT

Common features

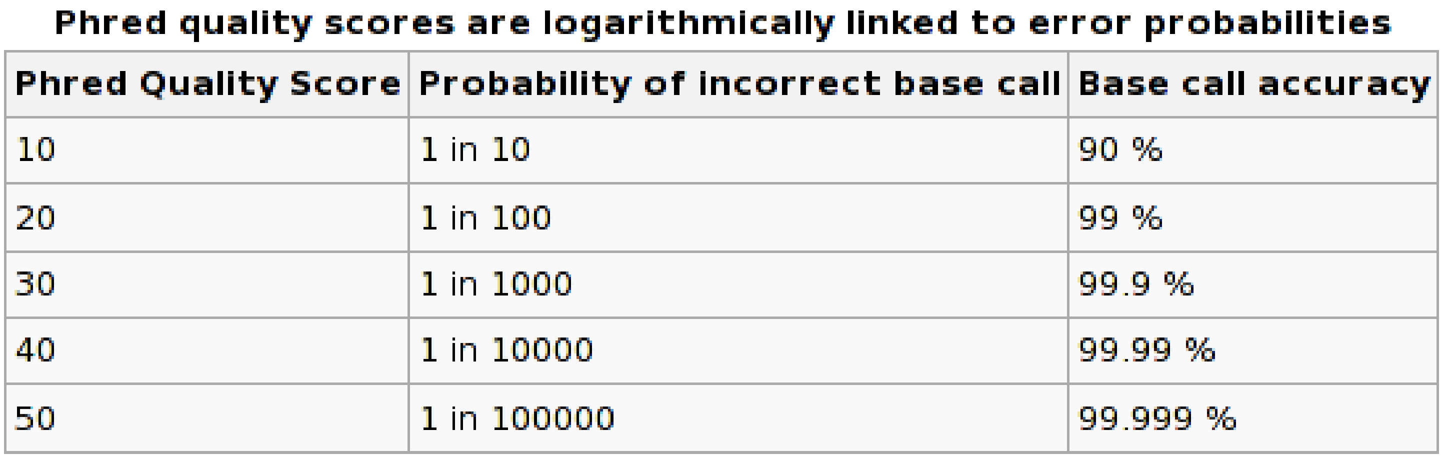
- Most 2nd generation platforms share the following:
  - Adaptor sequences to fix probes to a surface/bead
  - Amplification
  - Use of fluorescent probes/CCD devices or pH sensors
  - Capable of paired-end reads
  - Post-processing software to determine image quality
  - Shorter read lengths compared to traditional capillary based sequencers
  - Much higher data volumes (~Gb)
  - Sequence a human genome in a matter of days

Common features



Phred Score

- Phred program: http://en.wikipedia.org/wiki/Phred_base_calling
- Q = -10 log10(P)
- P = 10^(-Q/10)



Phred quality scores are logarithmically linked to error probabilities

Phred Quality Score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90 %
20	1 in 100	99 %
30	1 in 1000	99.9 %
40	1 in 10000	99.99 %
50	1 in 100000	99.999 %

Bioinformatics implications

- 100-10,000 fold increase in data volumes
- Tool development
- Data quality is poorer
- Less bioinformatics manpower available per sequencing project
- Finished genomes are usually of poorer quality than Sanger ‘gold-standard’ genomes
- Due to data volume, other applications have become feasible
- E.g. RNA-seq, ChIP-seq, Meth-Seq.

Benchtop sequencers

The NGS Market

- Currently dominated by Illumina (70% instruments)
- Market split into:
  - Low throughput but fast: clinical applications and sequencer for individual labs
  - Very high throughput: genome centers and large-scale projects
- E.g Illumina HiSeq 2000 vs. MiSeq
  - 300Gbase per 10 day run vs 7 Gbase in 48 hours

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

Benchtop sequencers

- Roche 454 Junior, Illumina Miseq are essentially miniature versions of the 454 and HiSeq
- Life Technologies Ion Torrent and Ion Proton are benchtop sequencers derived from 454 pyrosequencing
- Designed for individual groups
- Typical instrument cost is $150k (inc 3 year service contract)
- Typical run cost in consumables: $1000/run (at maximum output)

Illumina MiSeq

- Same technology and chemistry as HiSeq
- 2X250bp
- 7.5 Gbase/run
- Run 48 hours
- **$800 / run**
- **$100K instrument**
- **$50k for additional 2 year service contract**
- No additional wet-lab equipment required
- Capable of sequencing 20-30 bacterial genomes per run
- RNA-seq of up to 6 samples
- Libraries compatible with HiSeq

Roche 454 Junior

- Same chemistry
- 100K reads, 700bp
- 70 Mbases/run
- Focus on clinical, 510K validated assays
- **$1000 per run**
- **$100K instrument**

- Now uncompetitive – Roche reviewing future

Life Technology Ion Torrent

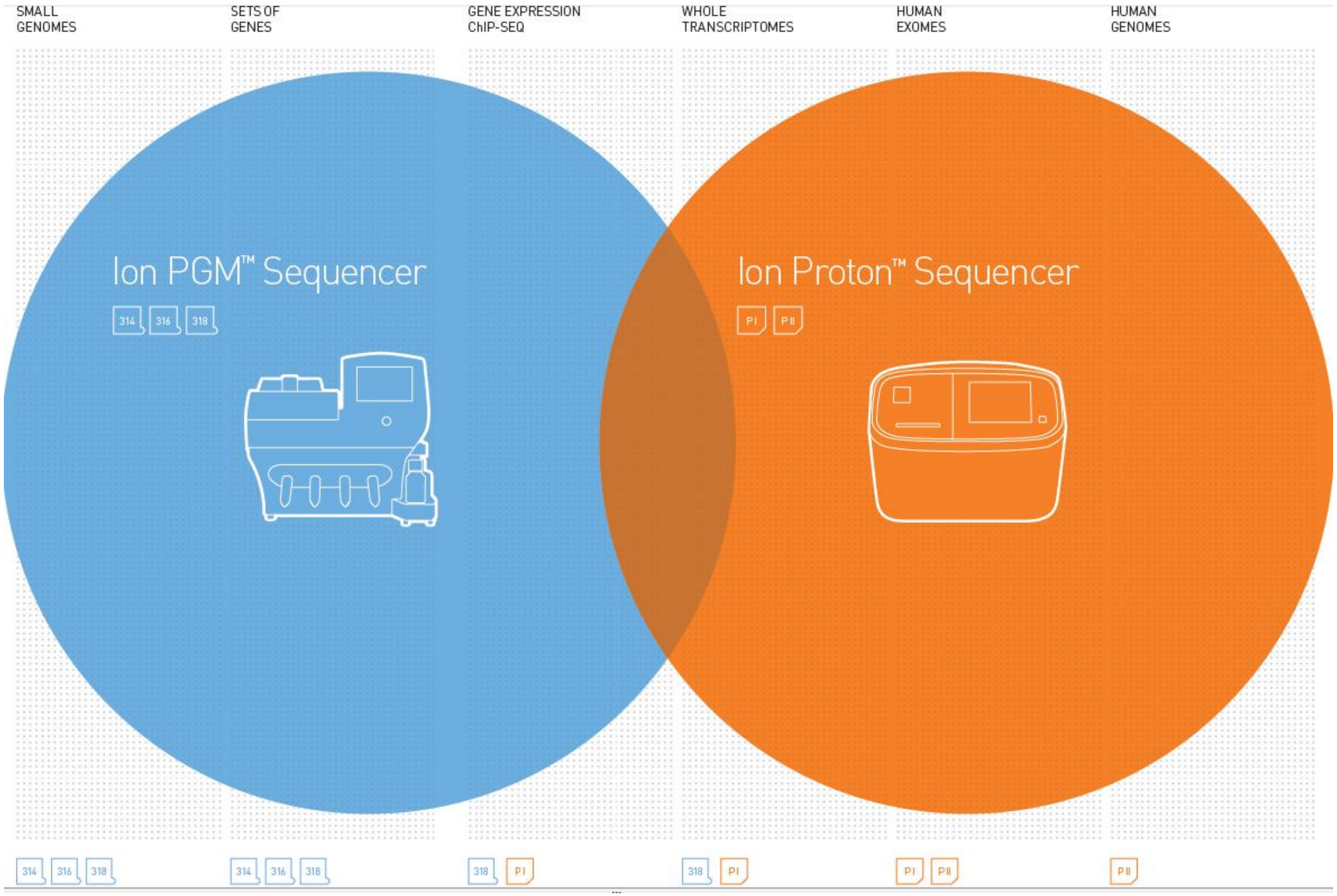
454-like chemistry without dye-labelled nucleotides

- 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**

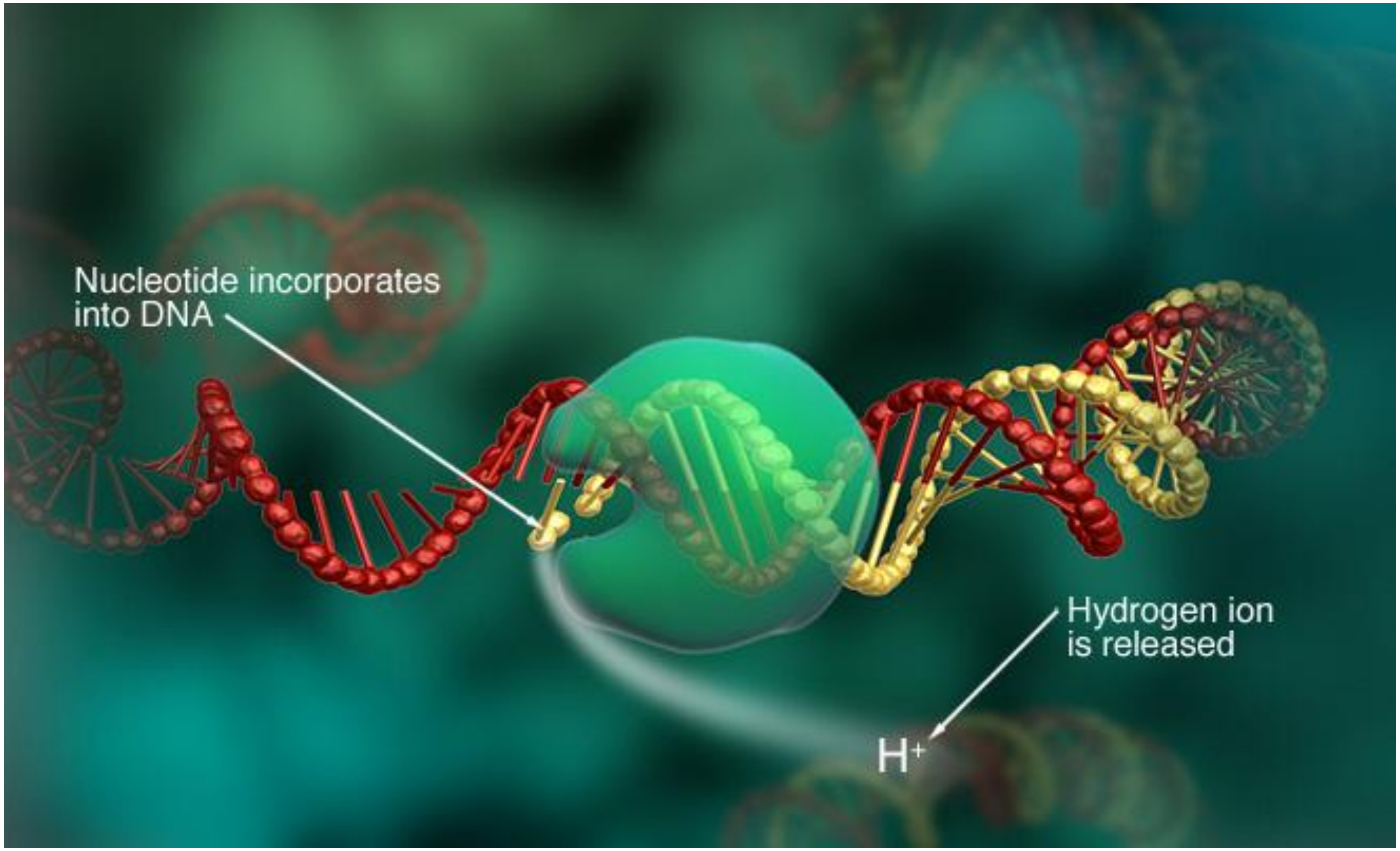
Life Technology Ion Proton

- 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

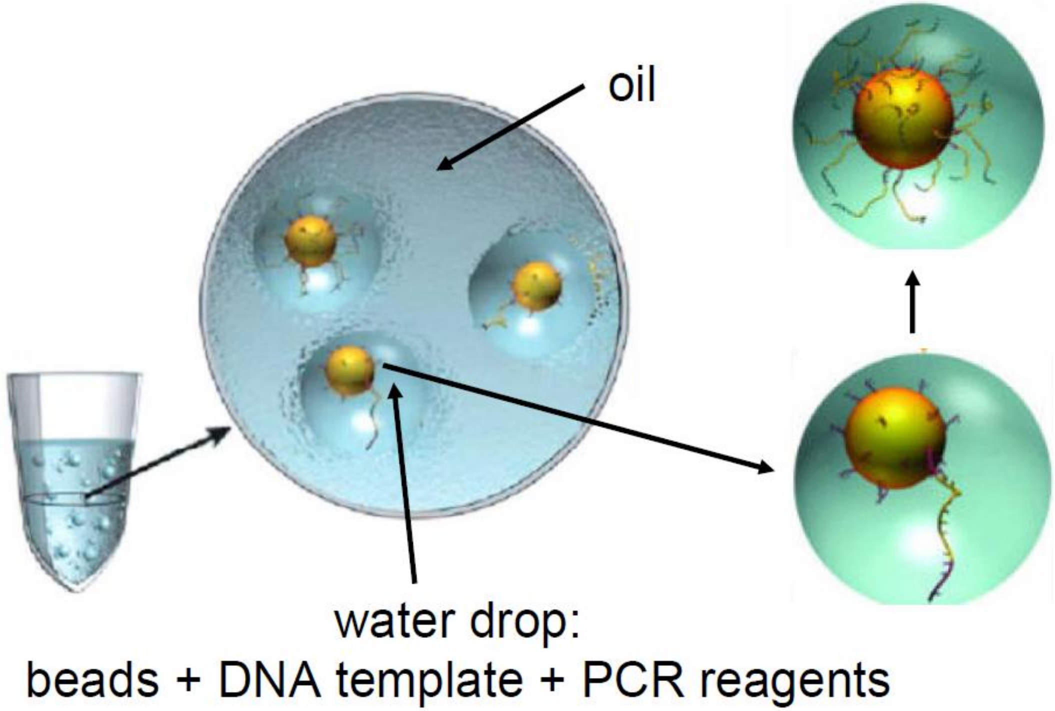


Ion Torrent

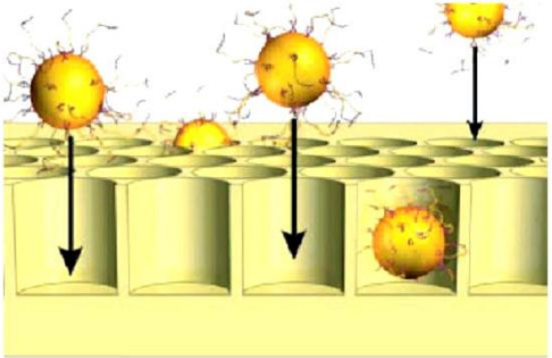

Nucleotide incorporates into DNA
Hydrogen ion is released
H^+

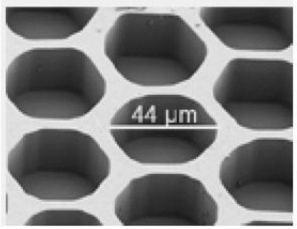
Library prep

- 454 style library using emulsion PCR

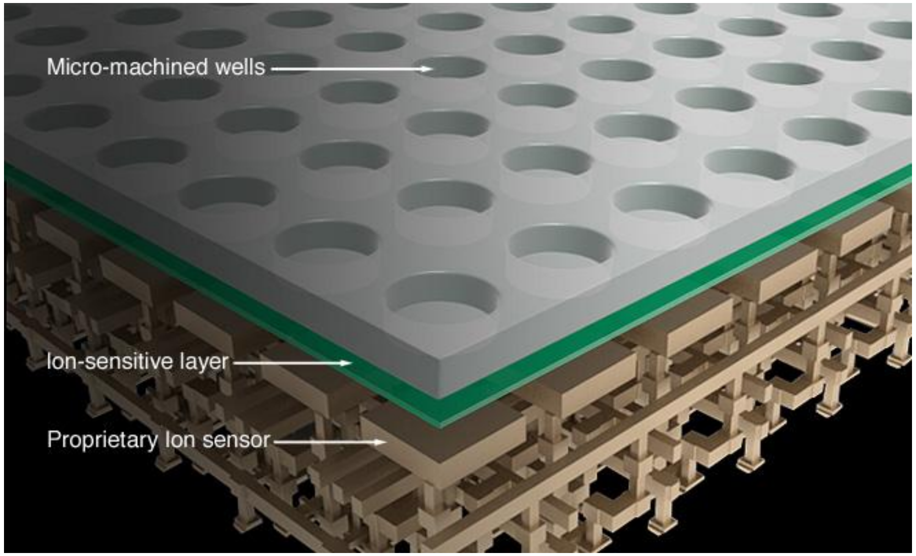


Ion Torrent

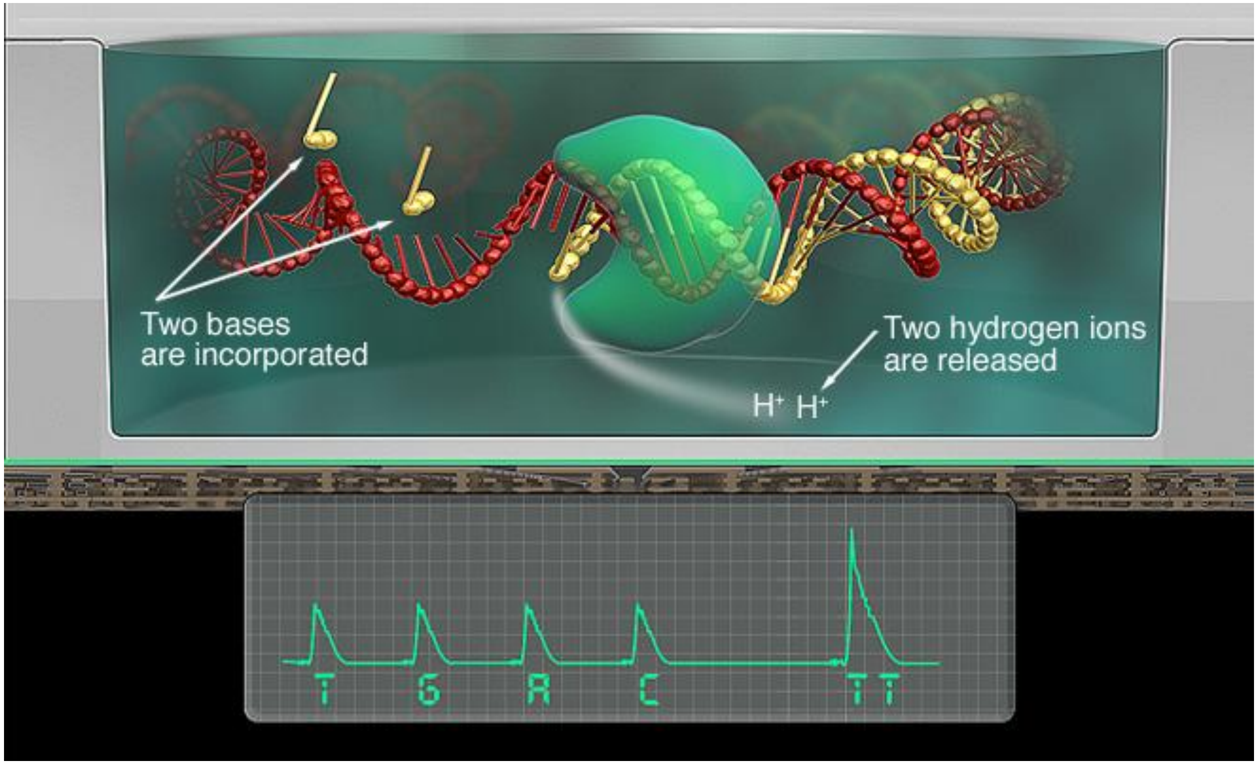




- enrich for DNA + beads
- diameter of the wells allows for only 1 bead/well



Ion System


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

Benchtop sequencers

Ion Proton (P1 chip)

- 60-80M reads
- Up to single-end 200 base pair runs
- 16Gb/run
- 4 hour run time
- **$1500/run**
- **$150K instrument**

- **One touch system required**

Illumina MiSeq

- 30M reads
- 2X300bp
- 15 Gbase/run
- Run 48-72 hours
- **$1000 / run**
- **$100K instrument**

- **No additional equipment required**

Roche 454 Junior

- Same chemistry
- 100K reads, 700bp
- 70 Mbases/run
- Focus on clinical, 510K validated assays
- **$1000 per run**
- **$100K instrument**

Useful benchtop review paper

- Loman, N. J., Misra, R. V, Dallman, T. J., Constantinidou, C., Gharbia, S. E., Wain, J., & Pallen, M. J. (2012). Performance comparison of benchtop high-throughput sequencing platforms. *Nature biotechnology*, *30*(5), 434–9. doi:10.1038/nbt.2198

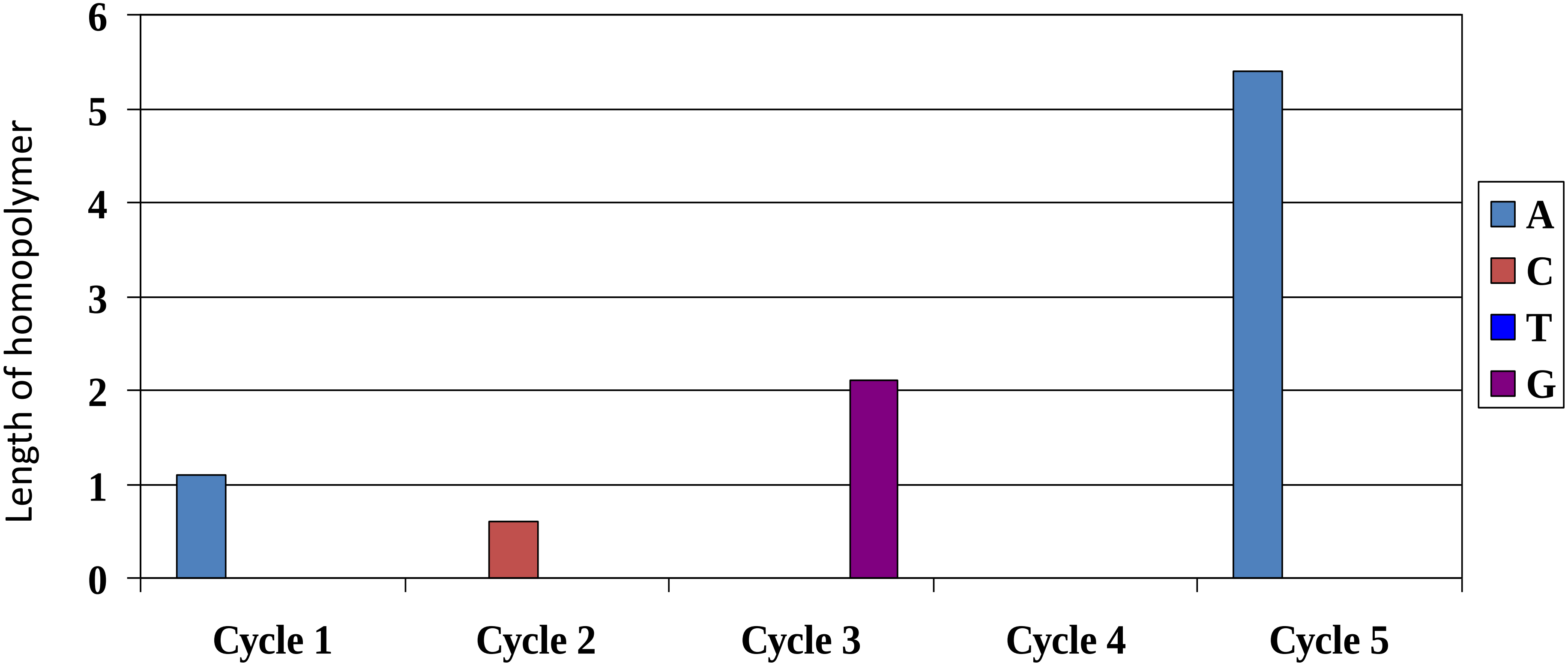
Possible problems

- These are common to all platforms
  - Biases introduced by sample preparation
  - Errors in base-calling
  - High GC/AT biases can cause difficulties

- 454 and Ion Torrent have difficulty sequencing homopolymeric tracts accurately
- Latest Ion Torrent reagent upgrades claim to reduce these
- Illumina also has specific motifs which are difficult to sequence

  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

Homopolymer errors



A **?c** **TT** **-** **AAAAA?a**

- Different between signal of 1 and signal of 2 = **100%.**
- Different between signal of 5 and 6 is **20%**
- More difficult to decide if we have AAAAA or AAAAAA
- Is the final sequence: ACTTNAAAAA or ANTTAAAAAAA or ATTAAAA…. Etc

Third generation sequencers

Third generation sequencers

- My definition: Single-molecule sequencing
- Currently only PacBio RS is commercially available

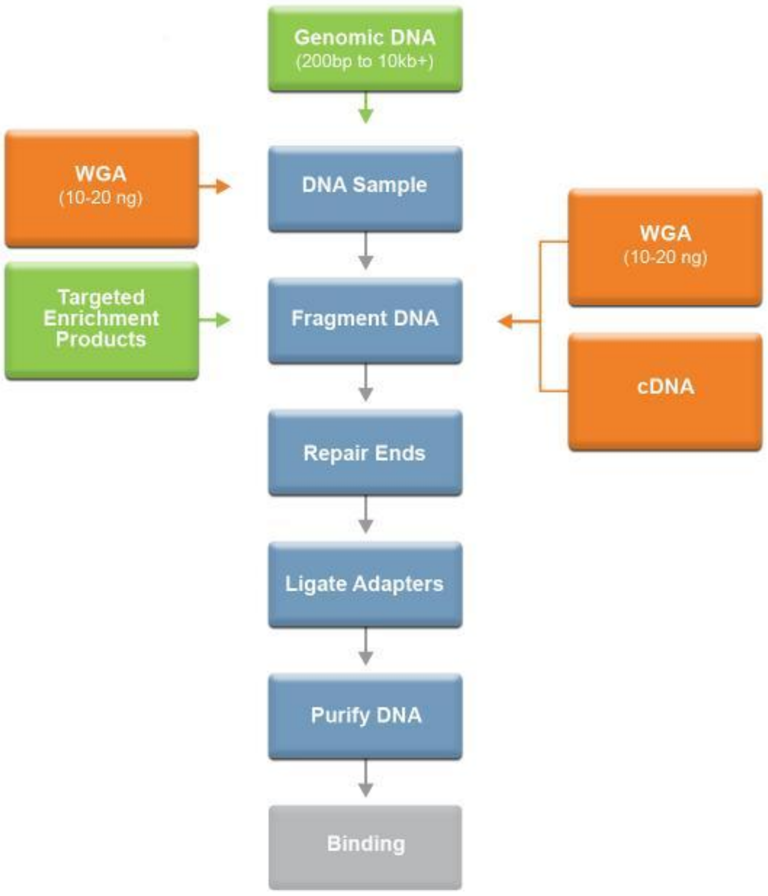
Pacific Biosciences RS II

Introduction

- Based on monitoring a single molecule of DNA polymerase within a zero mode waveguide (ZMW)
  - 150,000 ZMWs on a SMRT flowcell on PacBio RSII
- 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 flurorescent label for milliseconds -> nucleotide recorded

Library prep

Sample Preparation



Genomic DNA (200bp to 10kb+)

WGA (10-20 ng)

DNA Sample

Targeted Enrichment Products

Fragment DNA

WGA (10-20 ng)

cDNA

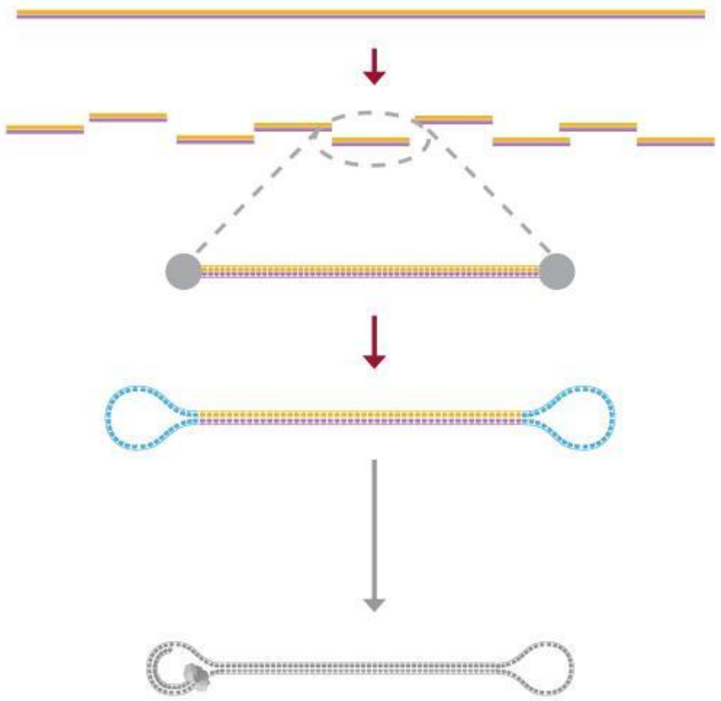
Repair Ends

Ligate Adapters

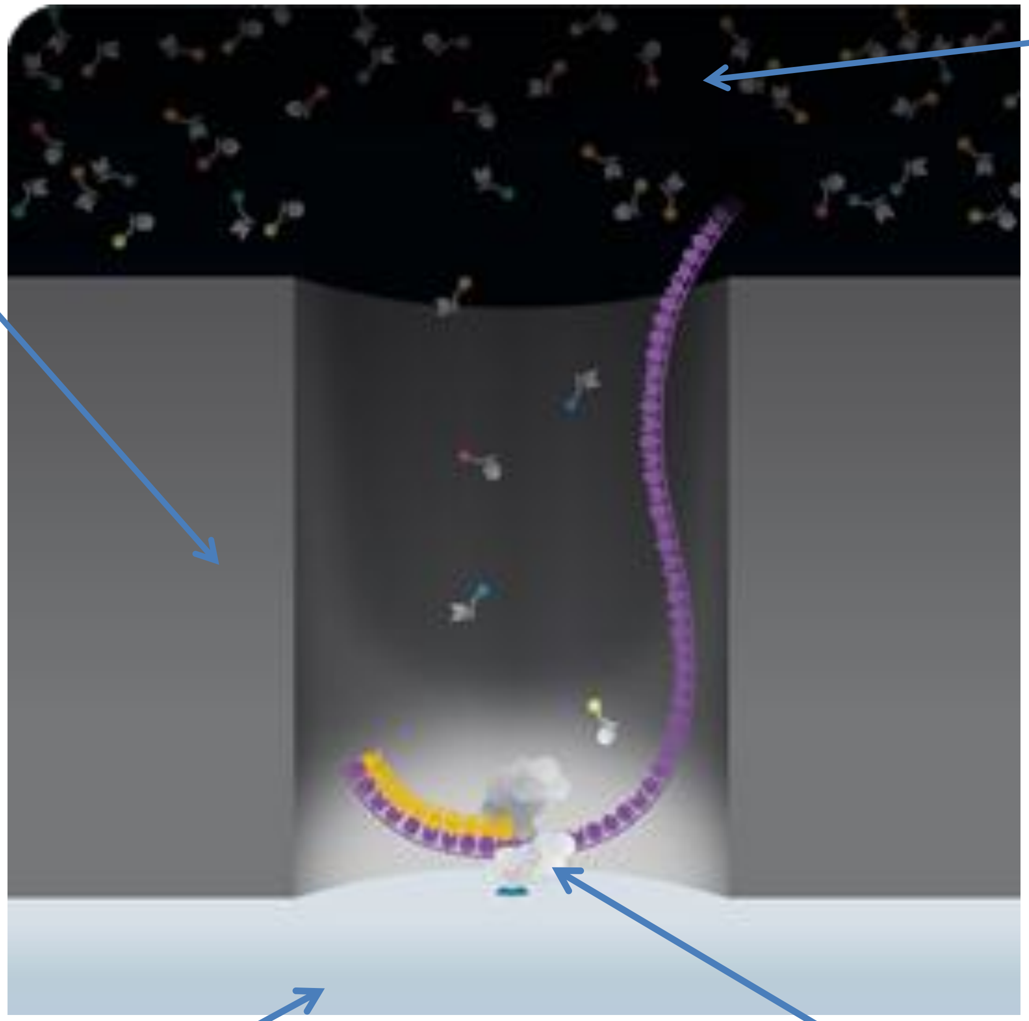
Purify DNA

Binding

Building of SMRTbell



SMRT Cell

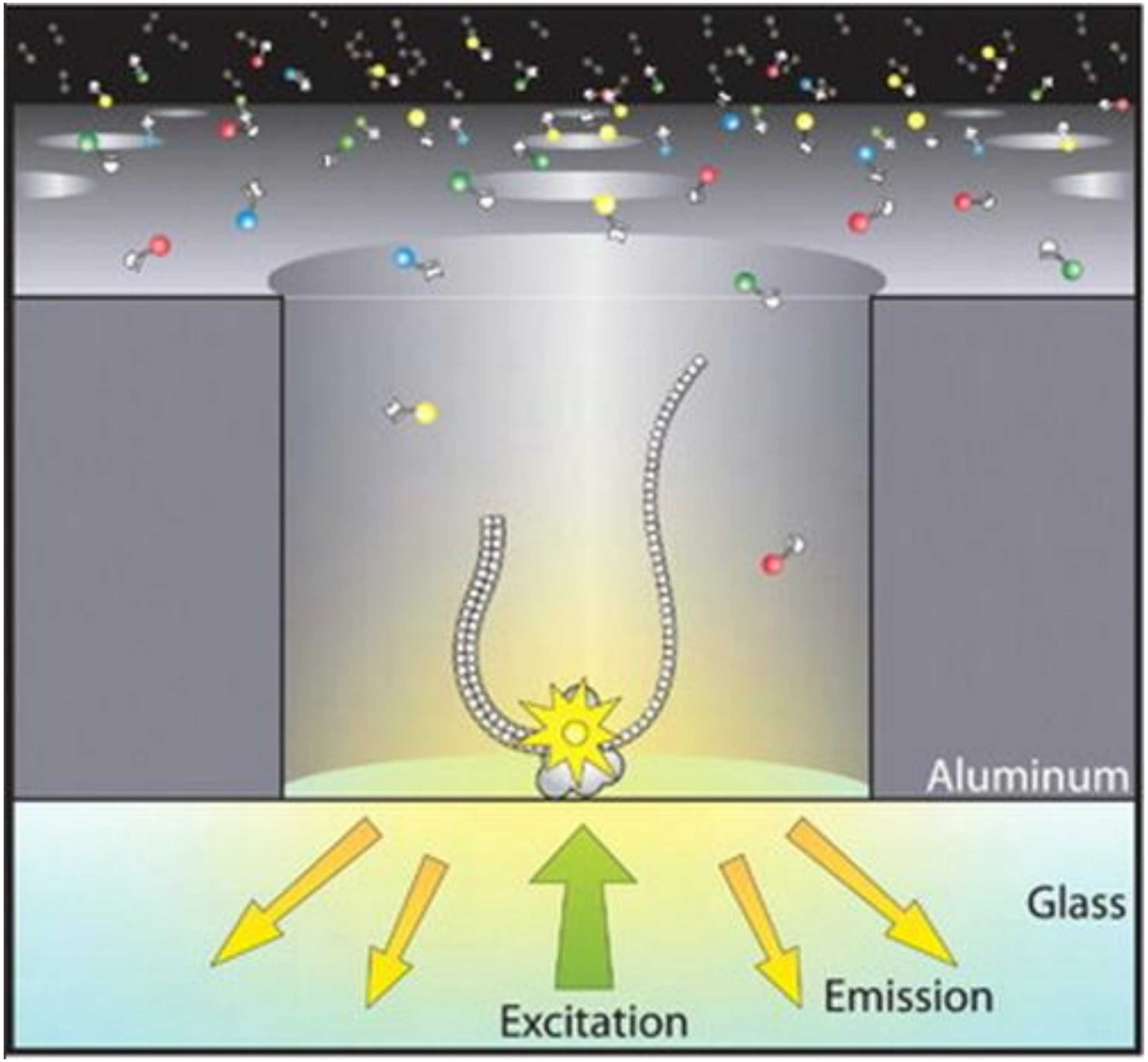


Zero mode waveguide

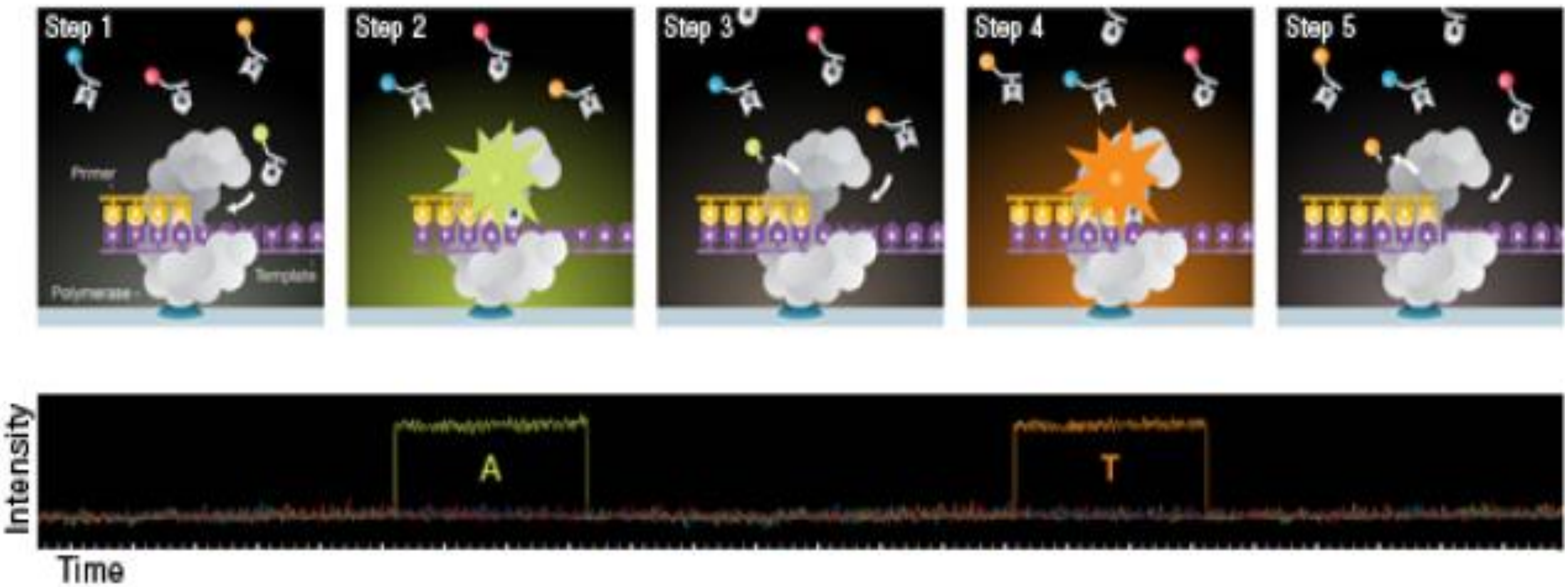
Free nucleotides

Laser and detector

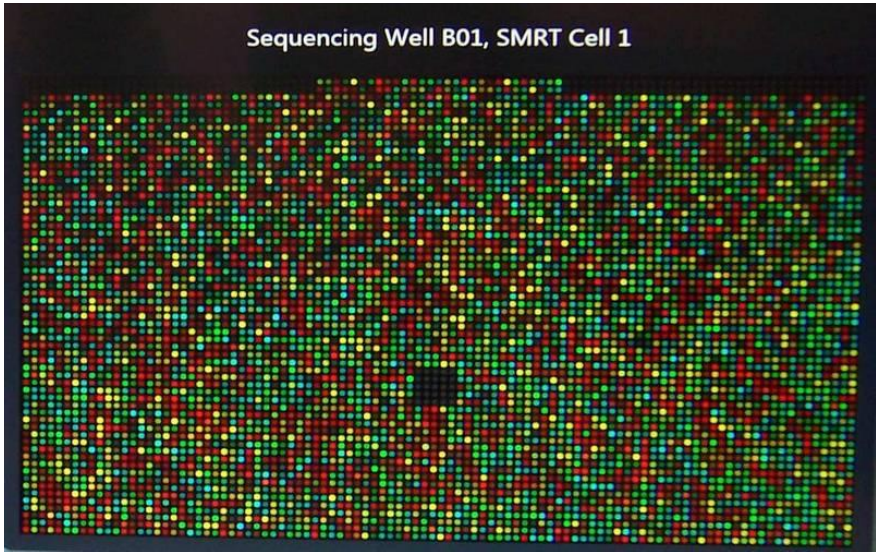
Immobilised DNA polymerase


Aluminum
Glass
Emission
Excitation

Observing a single polymerase

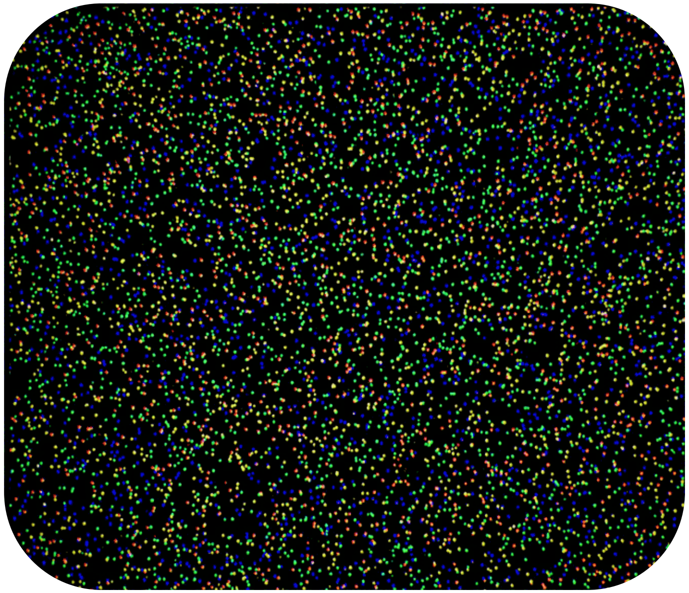


What it looks like



PacBio ZMWs with single DNA strand

Ordered



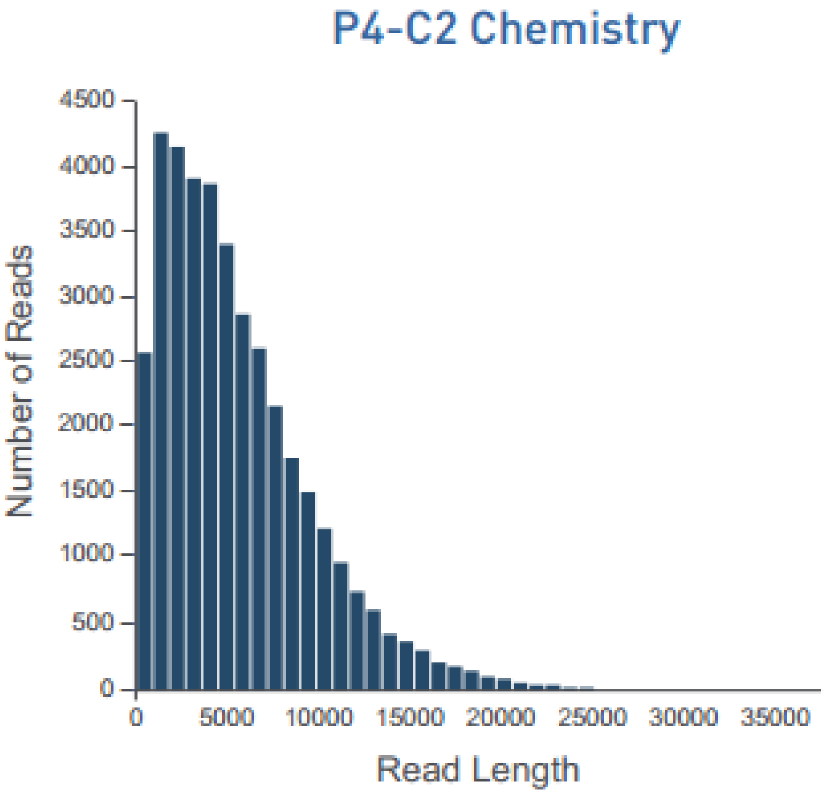
Illumina DNA mono-colonal clusters

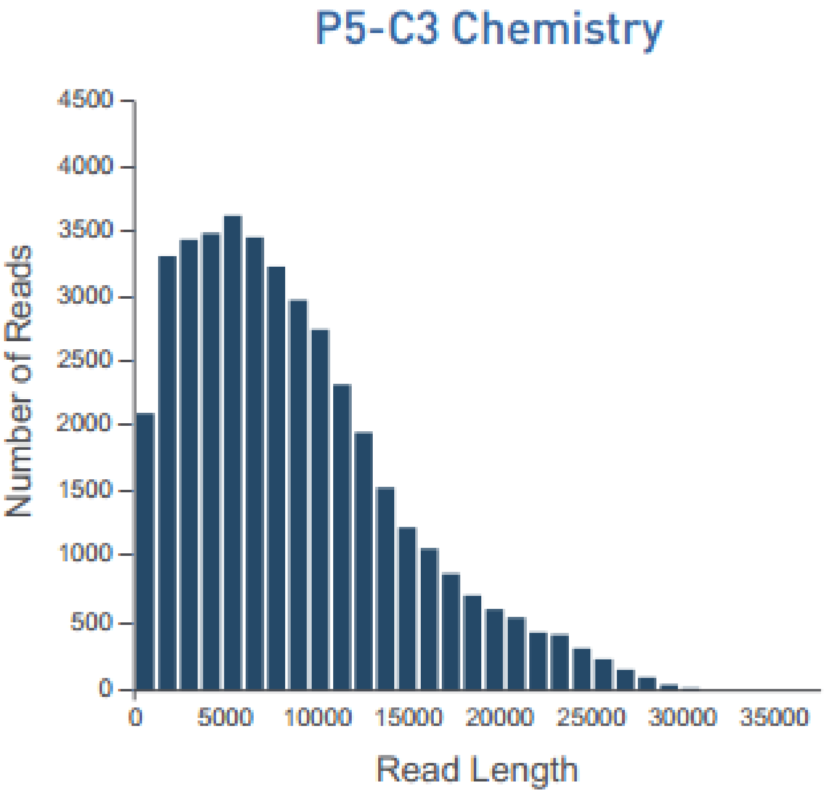
Unordered

Output statistics

- Approximately 100,000-150,000 sequences per SMRT flowcell
- 300-500Mb output per SMRT flowcell
  - $500 per run
- Library prep required
  - ~$500 per sample
  - ~0.5ug per sample
- Size selection required to get the longest reads
- Read lengths
  - Distribution
  - Mean 8.5kb up to 20-25kb

Read lengths



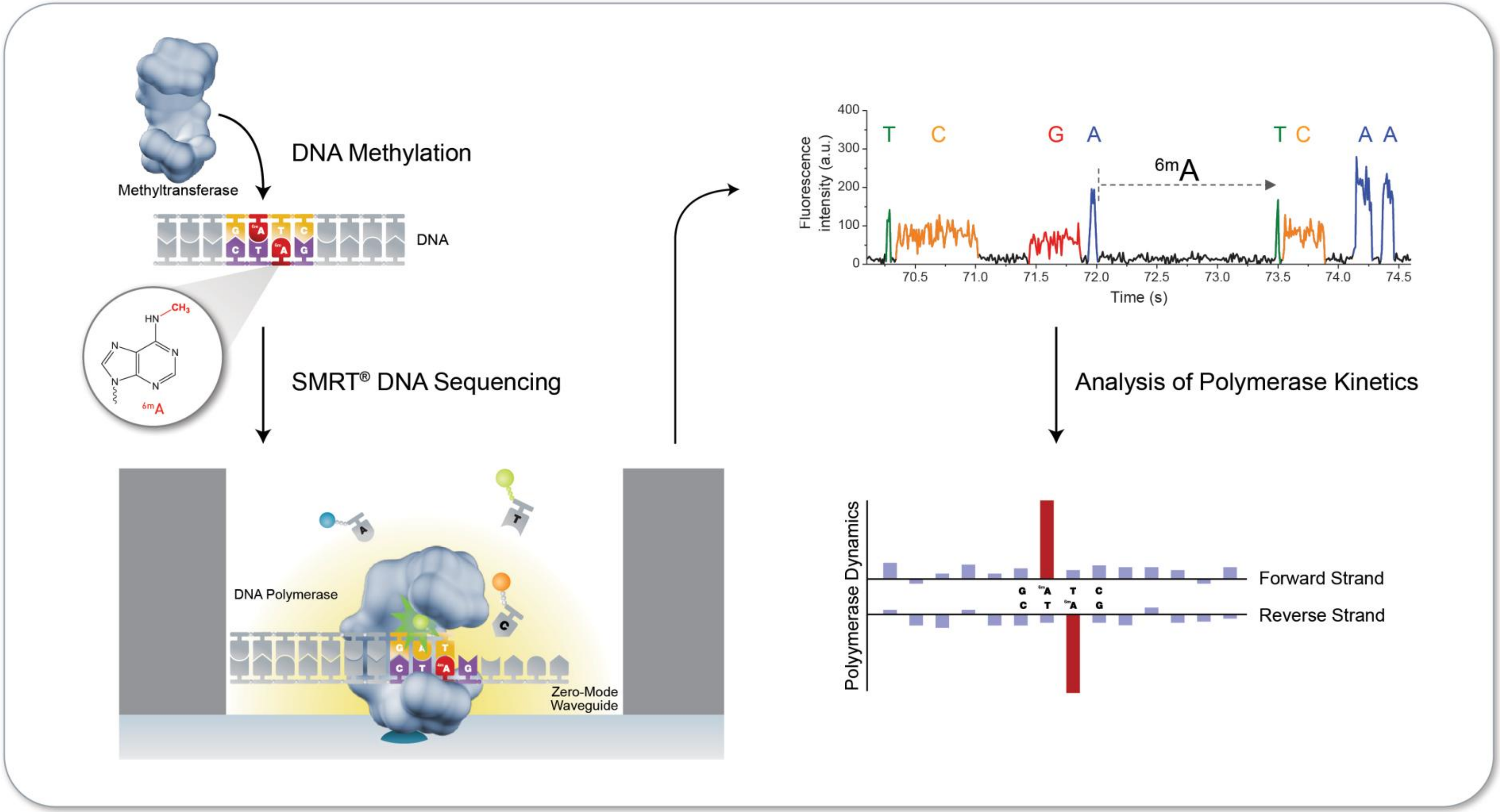


Novel applications

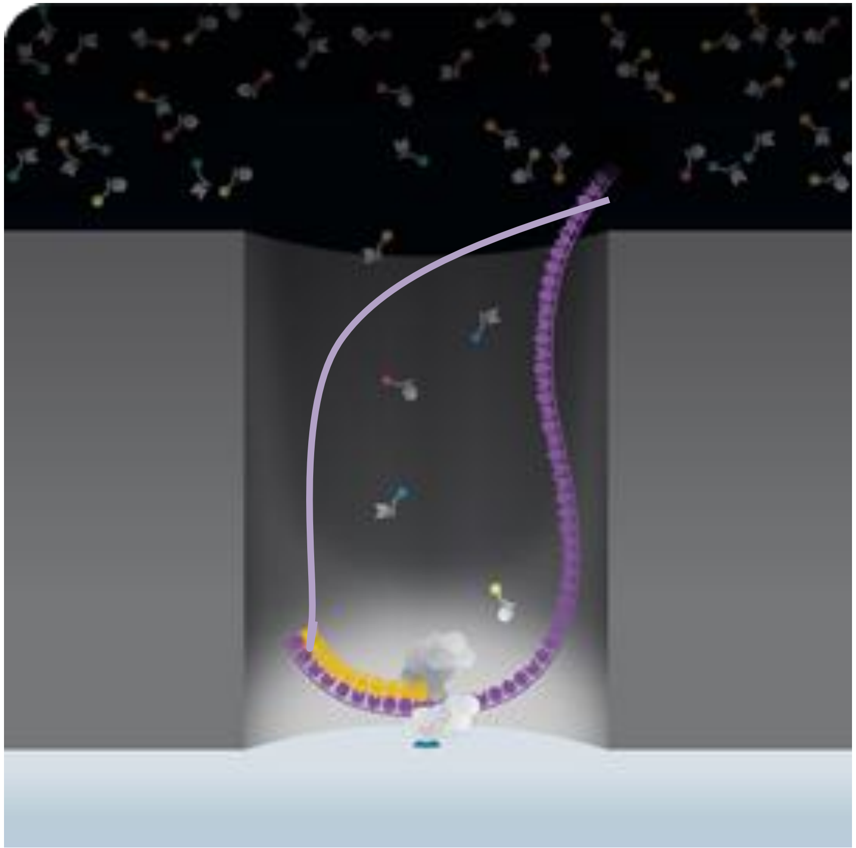
- Epigenetic changes (e.g. Methylation) affect the amount of time a fluorophore is held by the polymerase

- Circularise each DNA fragment and sequence continuously

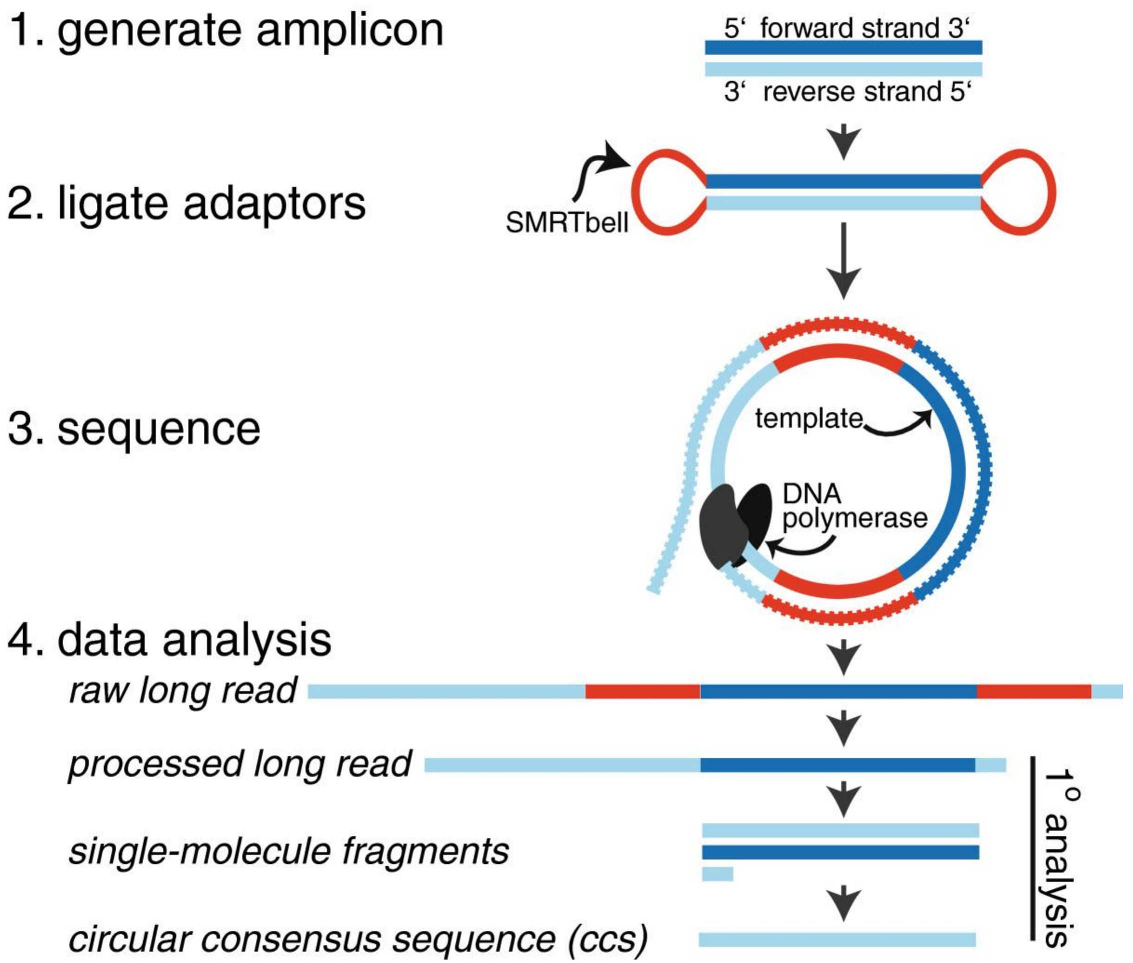
Epigenetic changes



Circular consensus sequencing



Circular consensus sequencing



1. generate amplicon

2. ligate adaptors

3. sequence

4. data analysis

raw long read

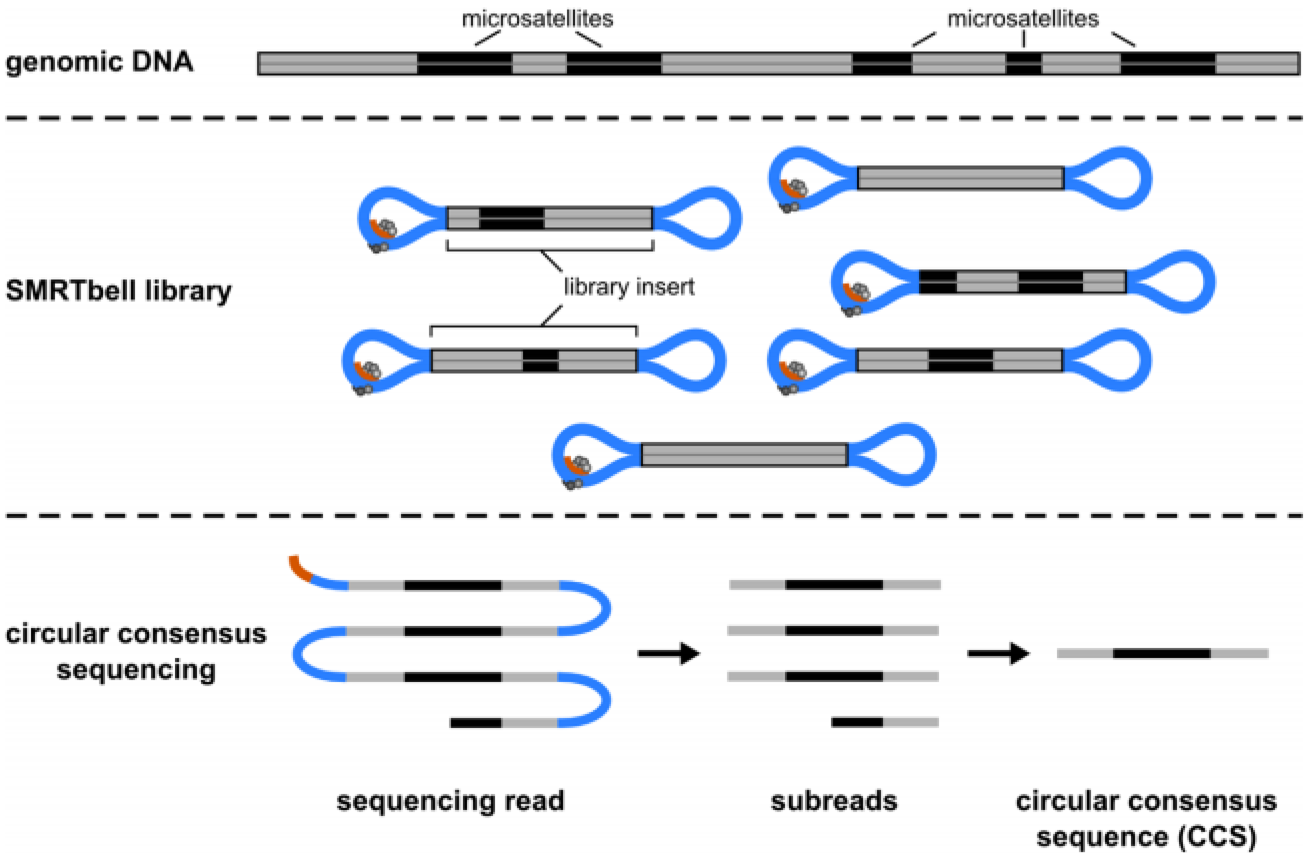
processed long read

single-molecule fragments

circular consensus sequence (ccs)

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 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

Issues to be aware of

- PCR chimeras (affects all PCR amplicon methods)
- Chimeric sequences can be generated during library preparation
- Shorter sequences can be loaded preferentially
  - Uniform amplicon size reduces this
  - PacBio Magbead loading system

Circular consensus sequencing (CCS)

- Raw error rates of a single pass read is high (10-15%)
- It is possible to read the same molecule repeatedly using CCS mode sequencing
- Can do this up to seven times to reduce error rates to around 0.1-0.5%
- Disadvantages
  - Reduction in read length proportional to number of passes (e.g 7 passes – max read length 3kb).
  - Reduction of total number of reads as some ZMW polymerases will fail

Pacific Biosciences

- Advantages
  - Longer reads lengths (median 8.5kb up to 25kb with P5-C3 chemistry)
  - 40 minute run time
  - Cost per run is low ($400 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 required (micrograms needed)
  - If you use PCR based methods – it is NO LONGER single molecule
  - Enzyme based
  - Only 50,000 reads/run. 400-500Mb yield
  - High (10-15%) error rate per run (but CCS can reduce this to <~1%)
  - $750k machine
  - Lab requirements very stringent

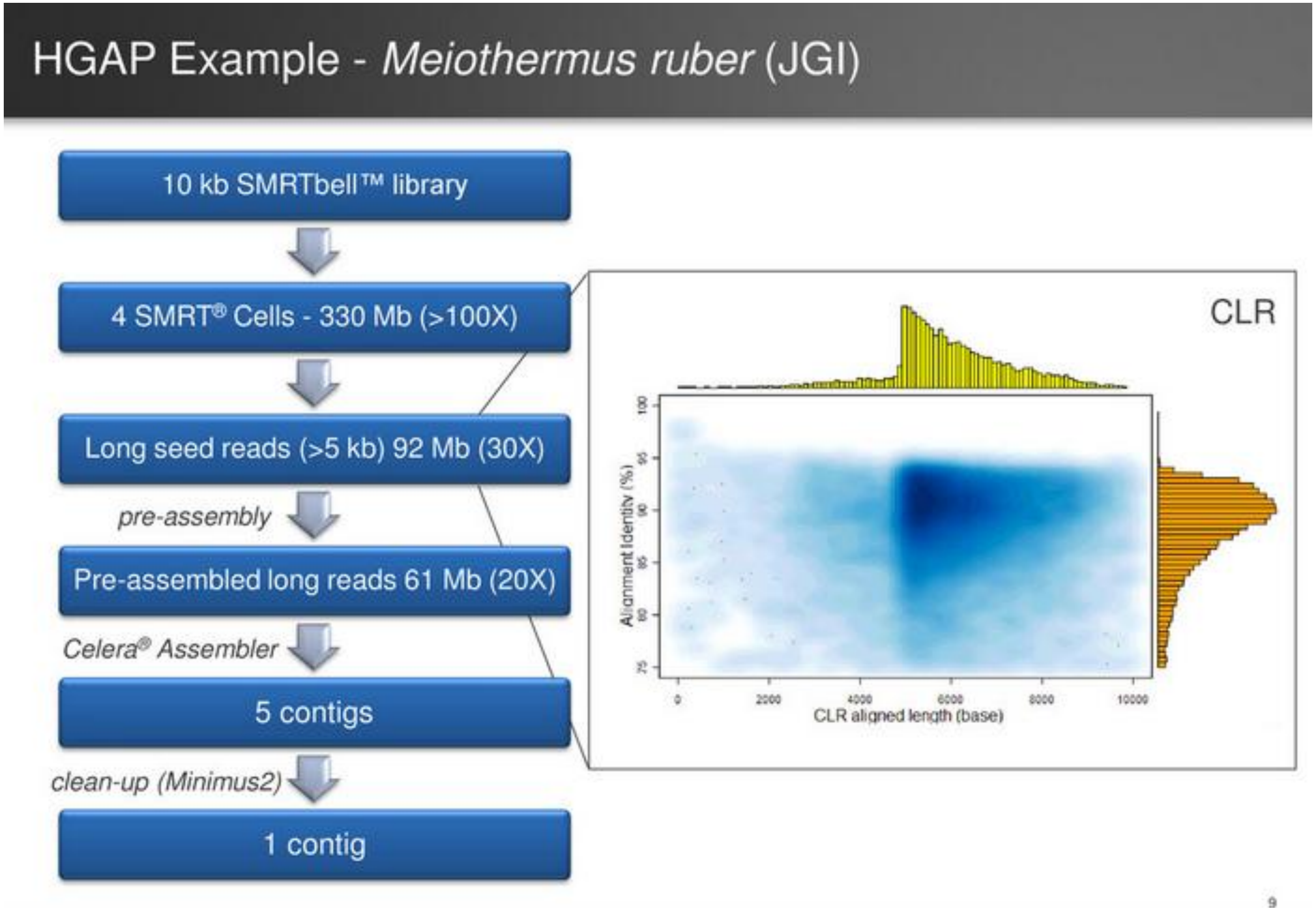
Bioinformatics Implications

- Relatively low data and high per base cost limits practical widespread use
- Can obtain useful 20-25kb fragments (C5 chemistry)
- Best used in conjunction with error correction algorithms utilising shorter PacBio reads or Illumina data
- Excellent to help scaffold genomes
- Able to generate complete bacterial genomes

.
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

Hierichical genome assembly



HGAP Example - *Meiothermus ruber* (JGI)

10 kb SMRTbell™ library

4 SMRT® Cells - 330 Mb (>100X)

Long seed reads (>5 kb) 92 Mb (30X)

pre-assembly

Pre-assembled long reads 61 Mb (20X)

Celera® Assembler

5 contigs

clean-up (Minimus2)

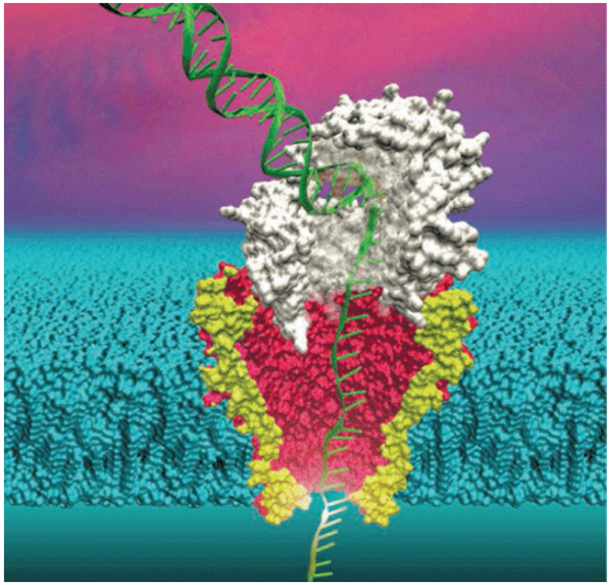
1 contig

CLR
Alignment Identity (%)
100
95
90
85
80
75
0
2000
4000
6000
8000
10000
CLR aligned length (base)

PacBio training resources

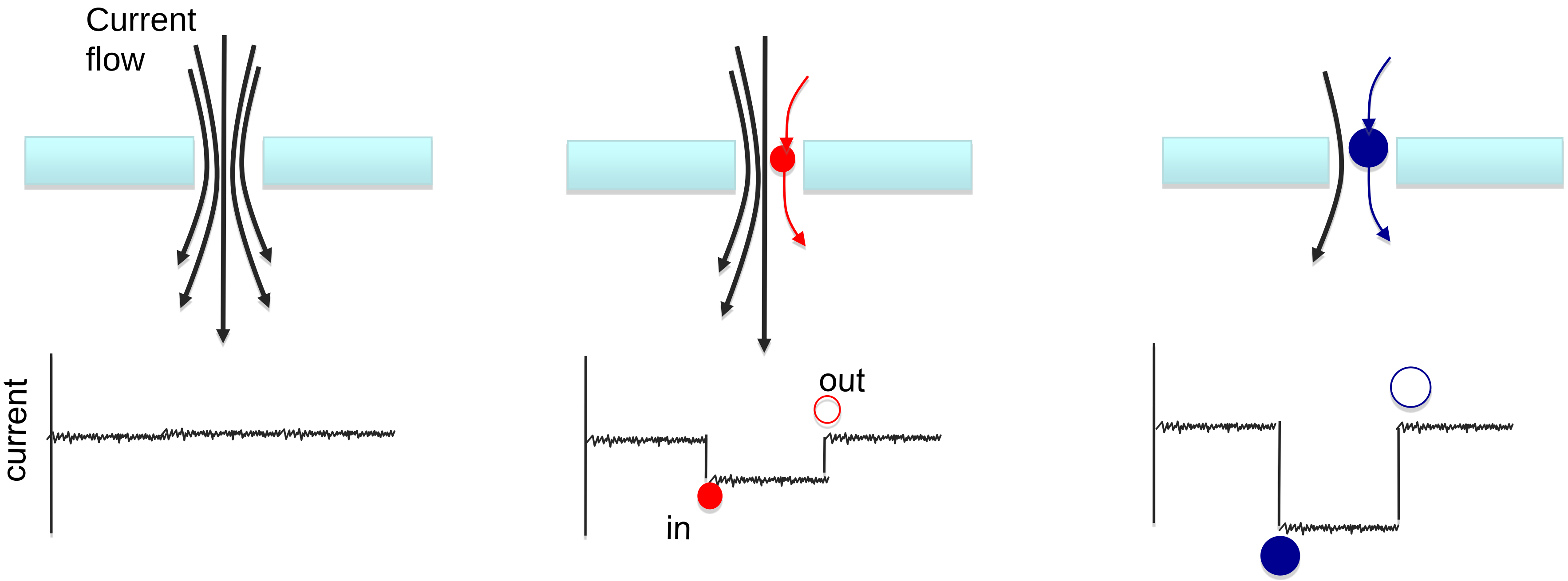
- https://github.com/PacificBiosciences/Bioinformatics-Training/wiki

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



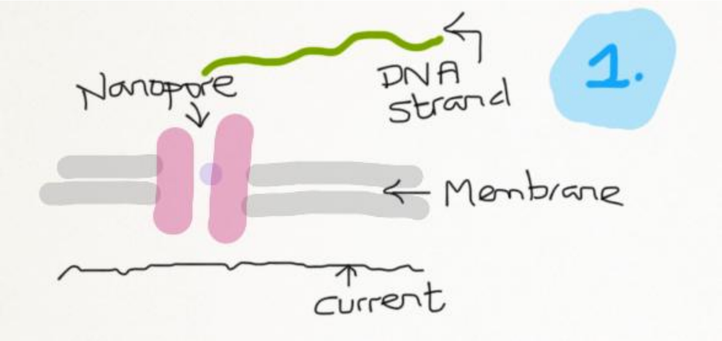
What is a nanopore?

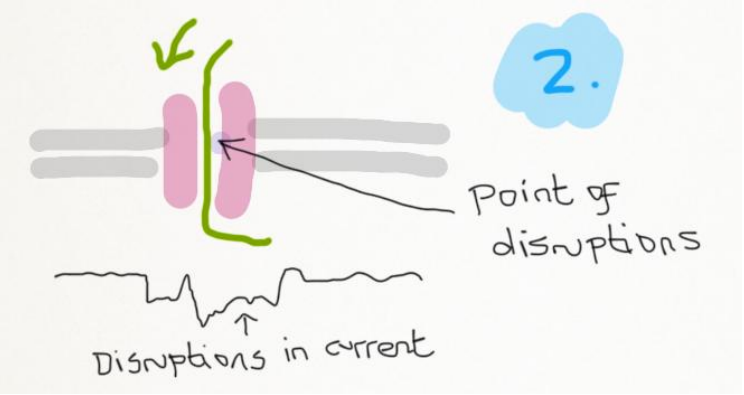
- Either biological or synthetic

- 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 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

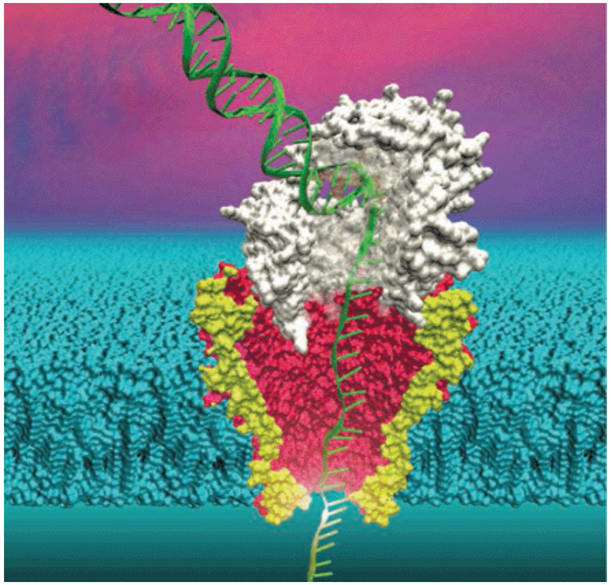




http://thenerdyvet.com/category/tech/

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



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 capacitative 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

genia

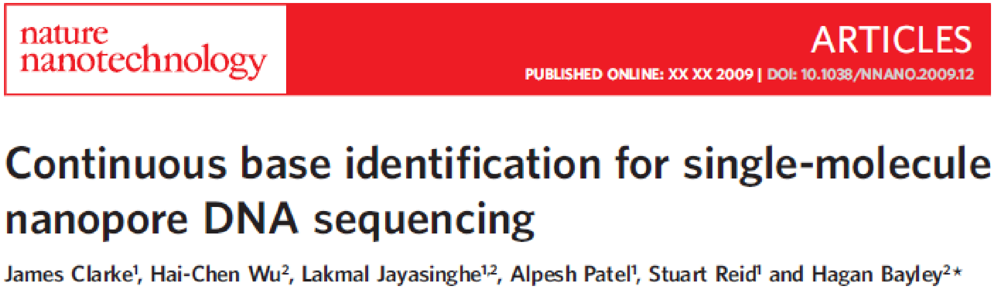
Nabsys

Oxford NANOPORE Technologies®

- Company which appears closest to commercialisation
- Two approaches to sequencing
  - Exo-nuclease sequencing (originally part of a co-marketing agreement with Illumina)
  - Strand sequencing

- Both use synthetic membranes compatible with alpha-haemolysin derived pores
- Strand sequencing method is being commercialised

Nucleotide Recognition



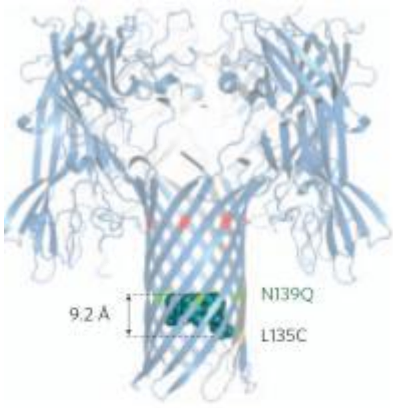
nature nanotechnology

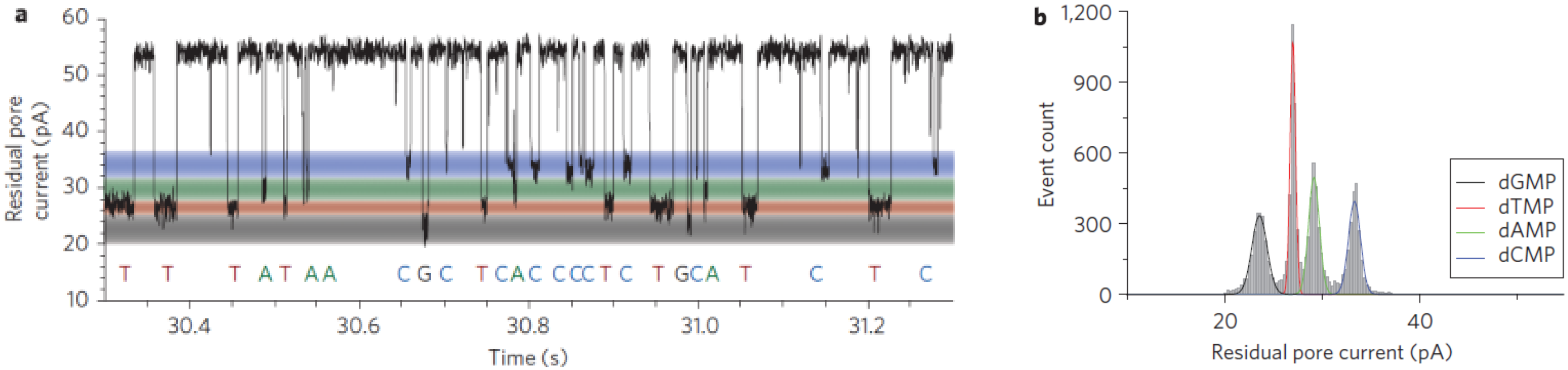
ARTICLES

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

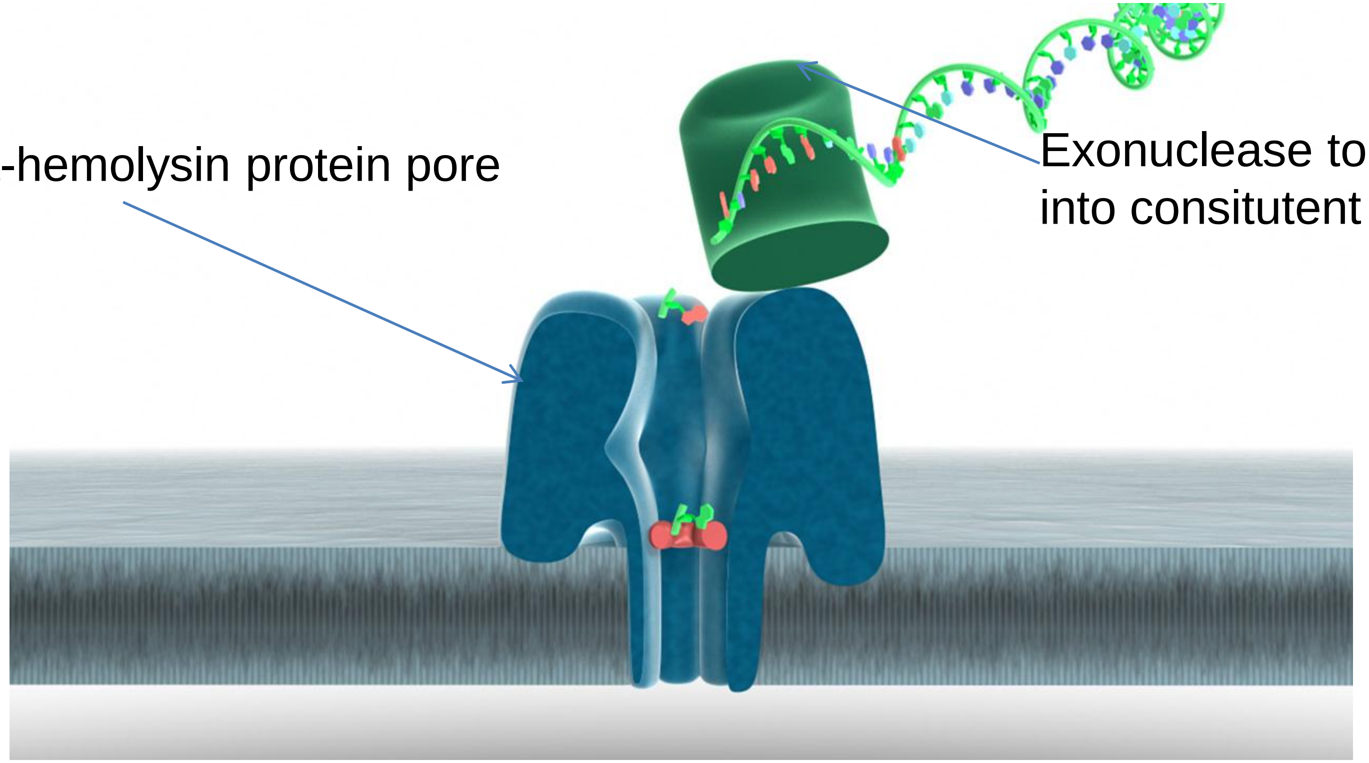
Continuous base identification for single-molecule nanopore DNA sequencing

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





Exonuclease sequencing

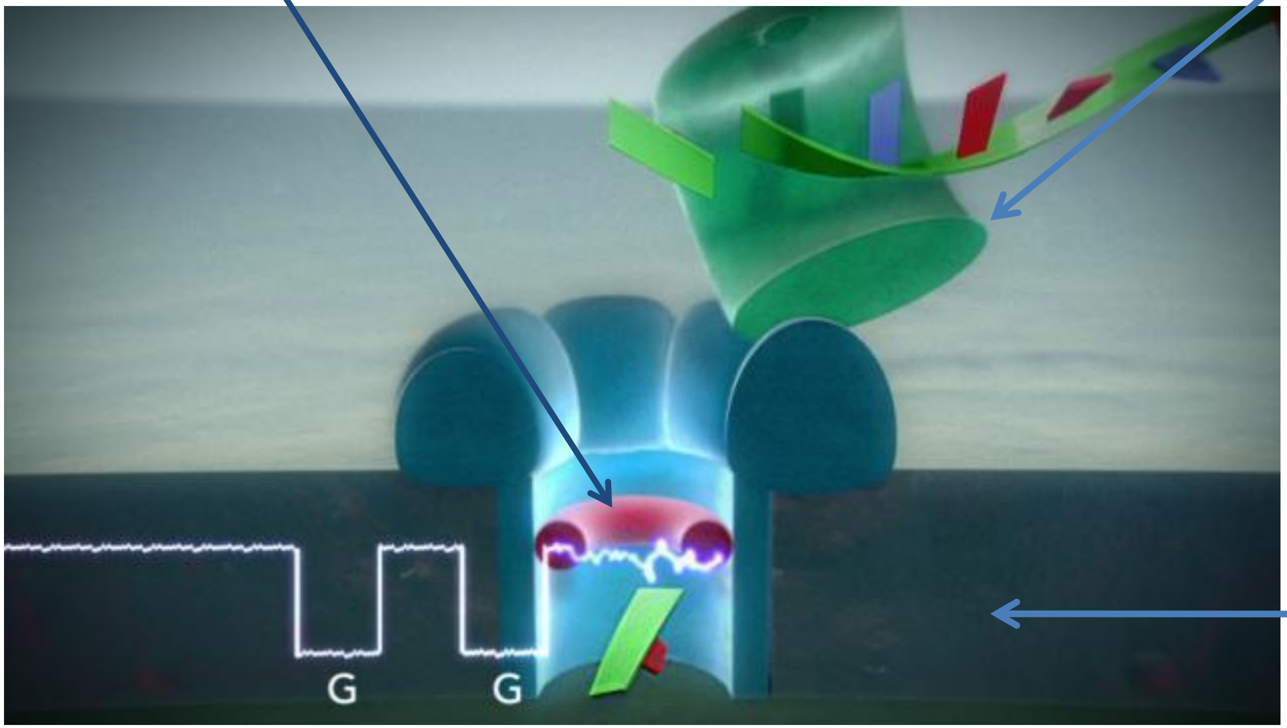


Alpha-hemolysin protein pore

Exonuclease to chop DNA into consitutent nucleotides

Cyclodextrin molecule inside alpha hemolysin

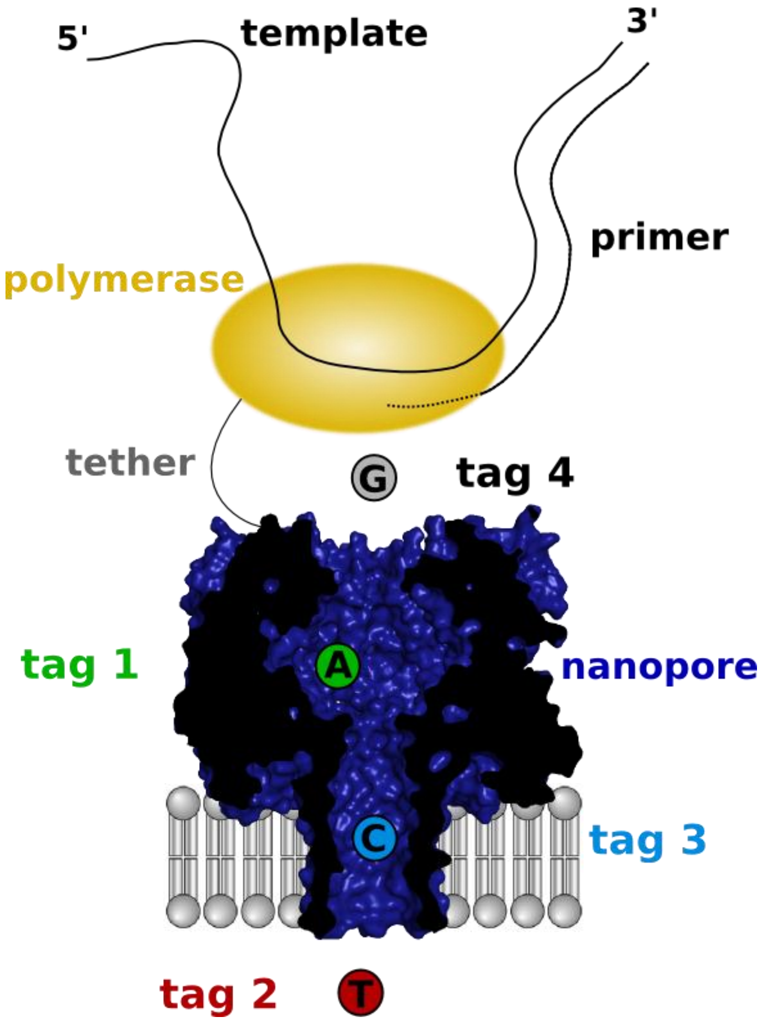
Exonuclease



Lipid bilayer

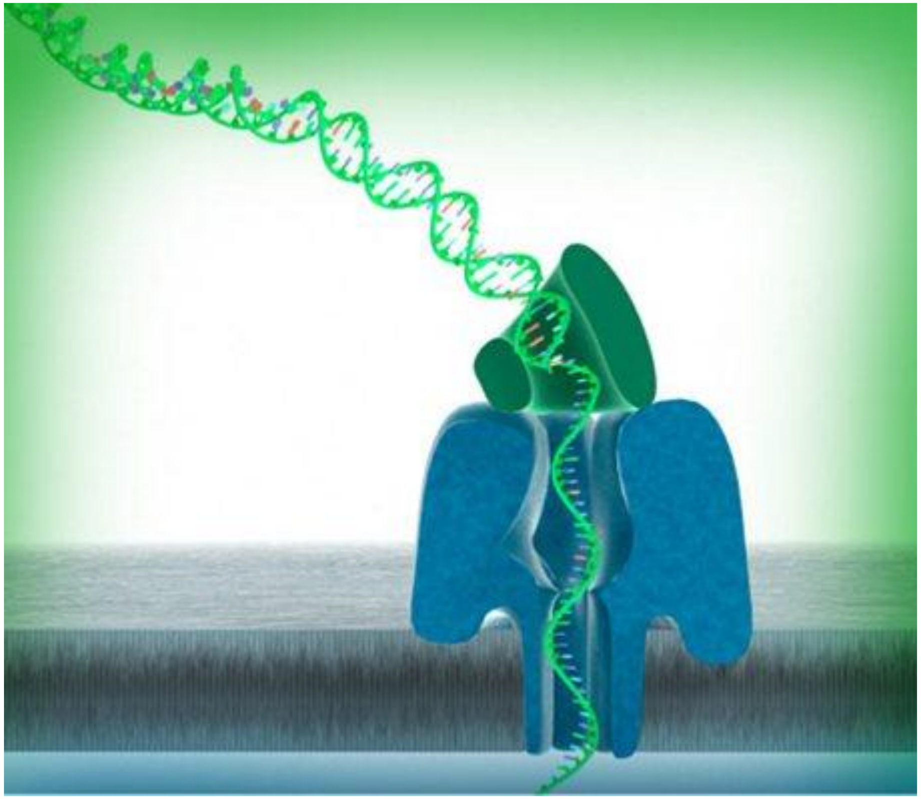
- Cyclodextrin inside alpha-hemolysin transiently binds to DNA base
- Interrupts the current through the pore
- Signal is indicative of base

Similar to Genia approach



Strand-sequencing

- Used in the recently advertised GRIDIon and MinIon systems

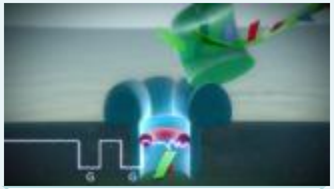


Novel applications

Application Specific

Adaptable protein nanopore:

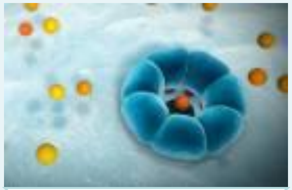
DNA Sequencing



Proteins

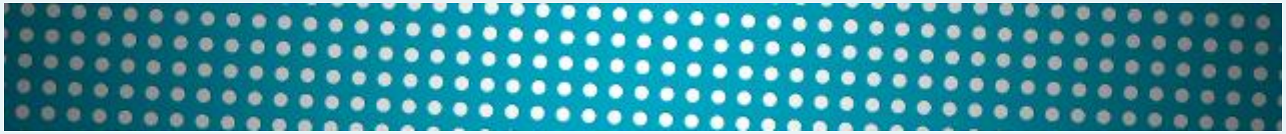
Polymers

Small Molecules



Generic Platform

Sensor array chip: many nanopores in parallel



Electronic read-out system

Oxford Nanopore Platforms

- GridION for sequencing centres
  - Promise of human genome in a few hours for around $1000
  - 2000 and 5000 pore instruments
  - No estimated pricing of instrument

- MinIon for individual researchers
  - $900 for 512 pore chip
  - 100Mb-1Gb per MinIon
  - Disposable after 6 hour run
  - 4% error rate in trials (mostly deletions)

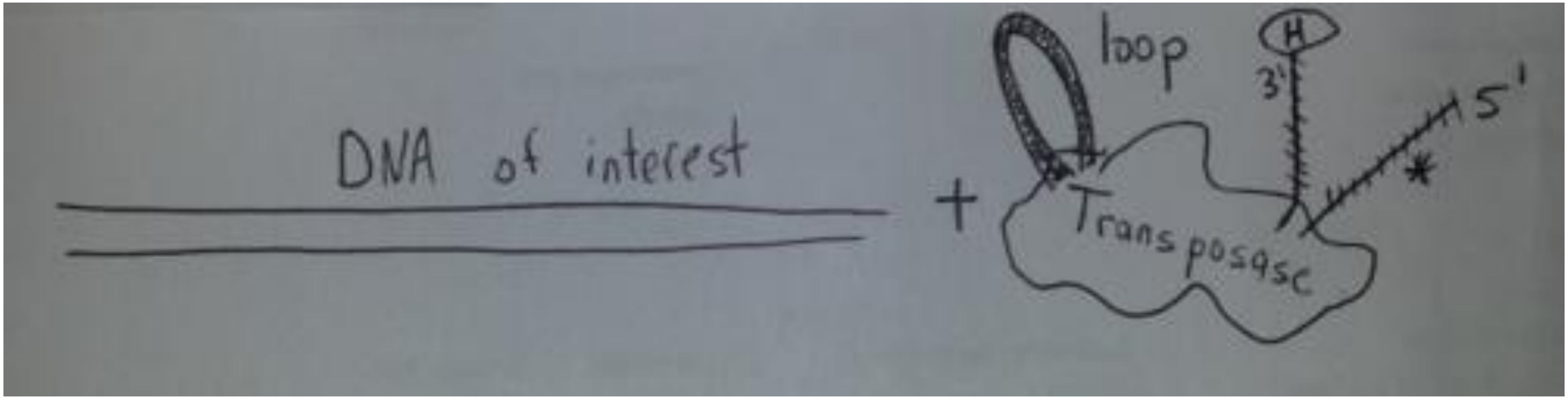
MinIon Details

- Uses the strand sequencing technique
- Requires library preparation
- ‘Run –until’ technology
- More of a ‘sequencing sensor’ than a direct competitor to 2nd generation sequencing
- Very useful for detection
- Likely to become as ubiquitous as a PCR-machine

Library preparation

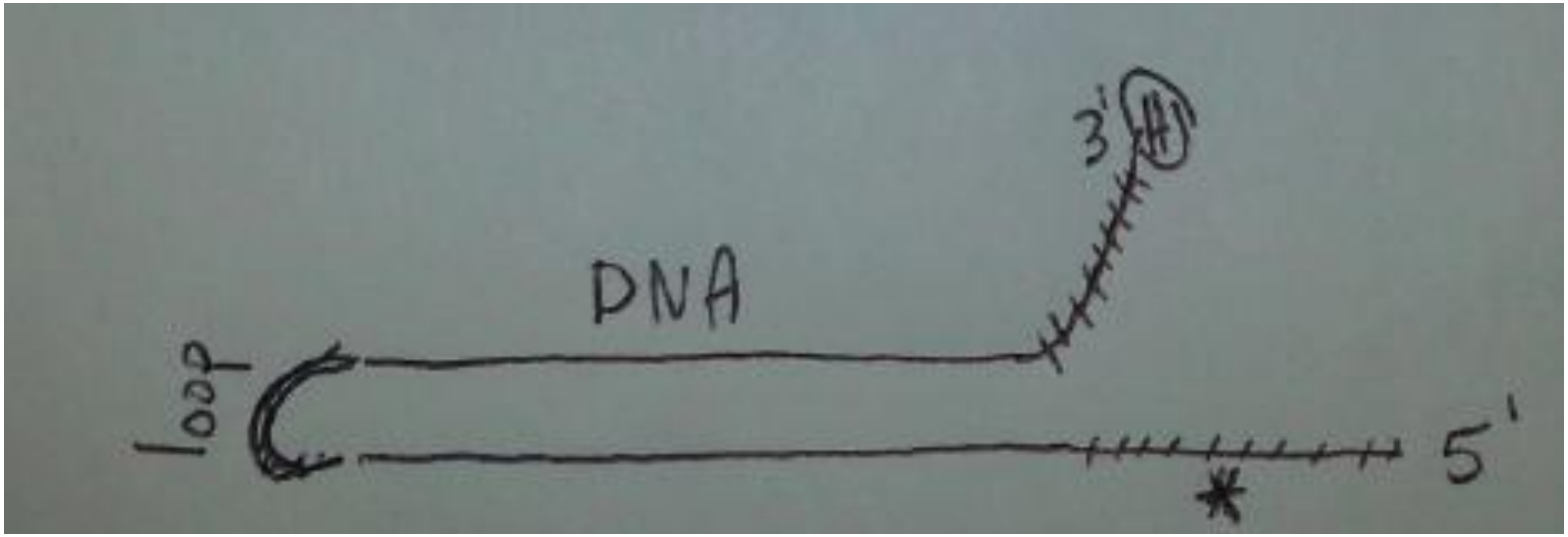
- Transposase-based library preparation is still required
- Enzymatic biases will still be present *and may be more difficult to detect with lower number of reads*
- Efficiency of transposase may limit maximum read lengths
- Unclear whether system can be washed effectively part-way through a run to load different samples

Library preparation – Step 1



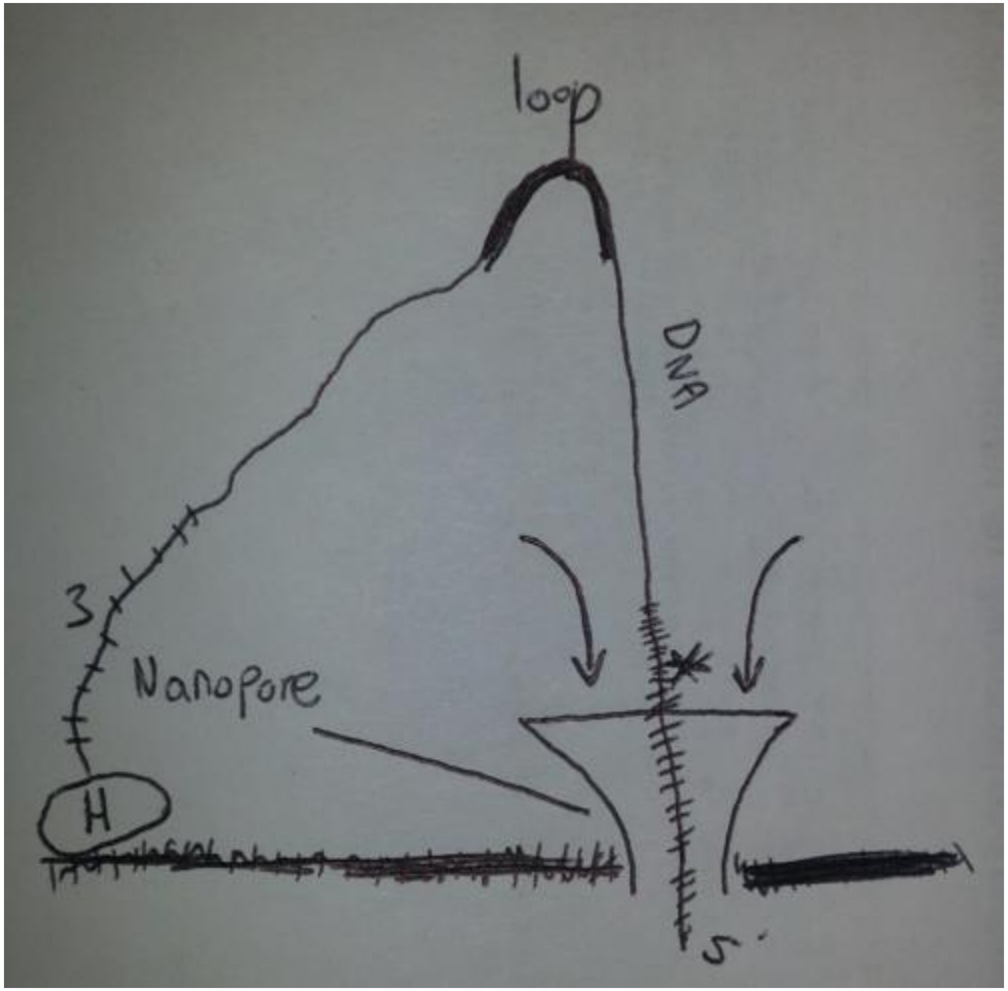
http://erlichya.tumblr.com/

Library preparation – Step 2



http://erlichya.tumblr.com/

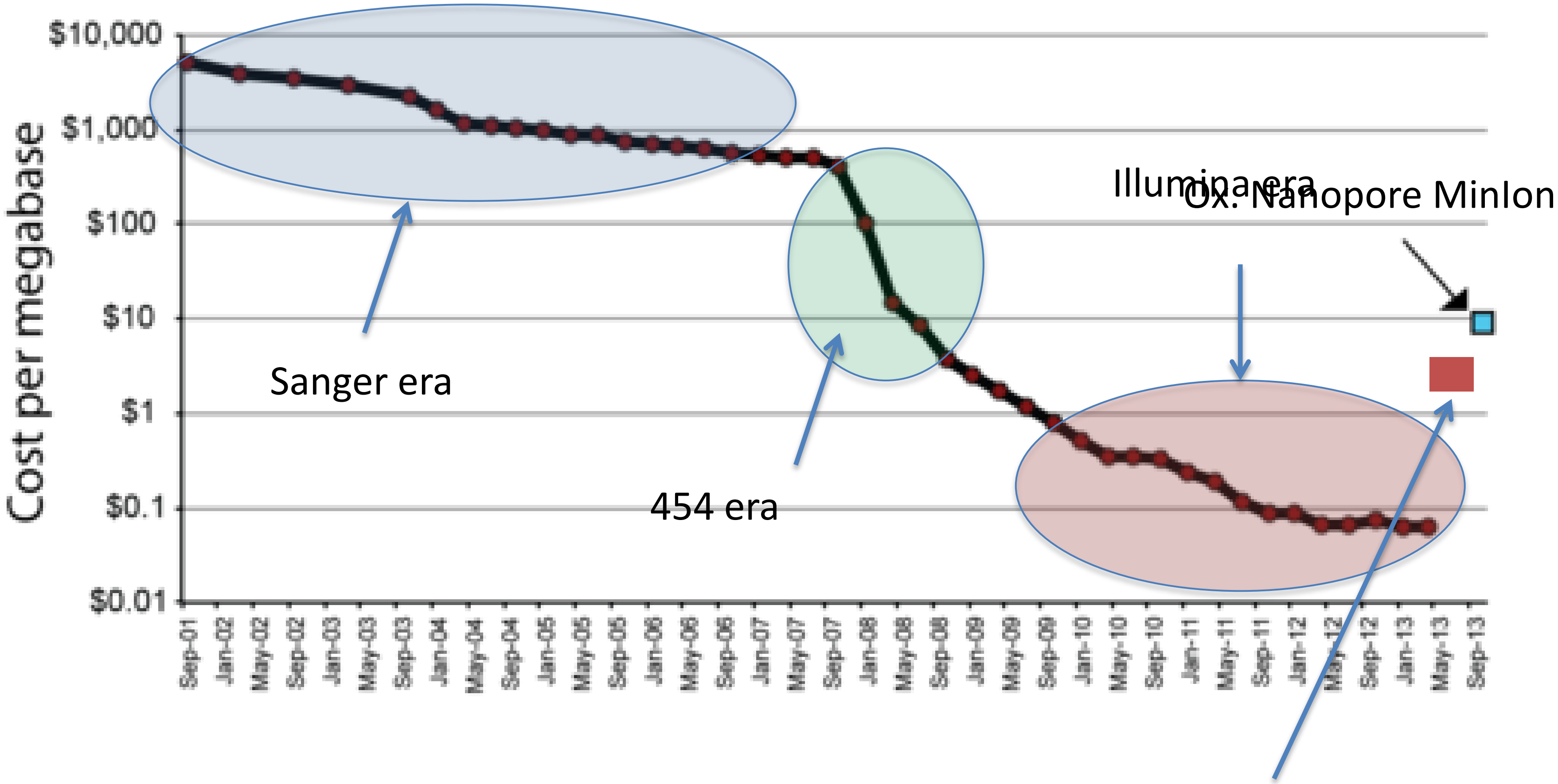
Library preparation – Step 3



http://erlichya.tumblr.com/

Caveats

Cost per megabase



Sanger era

454 era

Illumina era

Ox. Nanopore MinIon

PacBio

Oxford Nanopore is not single molecule

- The lipid bi-layers contain different types of nanopore
- Each has a different error profile
- It will still be necessary to over-sample and use sequences determined from complementary nanopores to reduce the overall error rate
- Will still likely need minimum of 5-10x coverage per genome (5-10 bacterial genomes per run)

Oxford nanopore

- Potential Advantages
  - Long reads lengths (10s – 100s kb)
  - Protein –> solid-state upgrades may eliminate reagent costs (3-5 years)
  - Fast turn around
  - Could measure epigenetic modifications and other molecules
- Potential Disadvantages
  - Potentially non-stochastic errors (i.e. some sequences harder to sequence accurately)
  - Library prep required
  - Not single molecule
  - Cost per base is ~$10

Bioinformatics Implications

- Will prove to be yet another step change as with 2nd generation sequencing
- Could obtain >100kb fragments
- Denovo assembly and phasing will be made easier
- Low number of reads per run and high per base cost may not make it useful for standard RNA-seq
- Burden will shift even further towards data management and downstream annotation
- …it will lead to different bottlenecks

Min Ion Access Programme

MinION™ Access Programme

In late November 2013, Oxford Nanopore opened registration for a MinION Access Programme (MAP - product preview). This is a substantial but initially controlled programme designed to give life science researchers access to nanopore sequencing technology at no risk and minimal cost.

MAP participants will be at the forefront of applying a completely novel, long-read, real-time sequencing system to existing and new application areas. MAP participants will gain hands-on understanding of the MinION technology, its capabilities and features. They will also play an active role in assessing and developing the system over time. Oxford Nanopore believes that any life science researcher can and should be able to exploit MinION in their own work. Accordingly, Oxford Nanopore is accepting applications for MAP participation from all[1, 2].

About the programme

A substantial number of selected participants will receive a MinION Access programme package. This will include:

- At least one complete MinION system (device, flowcells and software tools).
- MAP participants will be asked to pay a refundable $1,000 deposit on the MinION USB device, plus shipping.
- Oxford Nanopore will provide a regular baseline supply of flowcells sufficient to allow frequent usage of the system. MAP participants will ONLY pay shipping costs on these flowcells. Any additional flowcells required at the participants' discretion may be available for purchase at a MAP-only price of $999 each plus shipping and taxes.
- Oxford Nanopore will provide Sequencing Preparation Kits. MAP participants may choose to develop their own sample preparation and analysis methods; however, at this stage on an unsupported basis.

https://www.nanoporetech.com/technology/the-minion-device-a-miniaturised-sensing-system/map-application-form

Useful papers/videos

- http://www.nanoporetech.com/technology/analytes-and-applications-dna-rna-proteins/dna-an-introduction-to-nanopore-sequencing

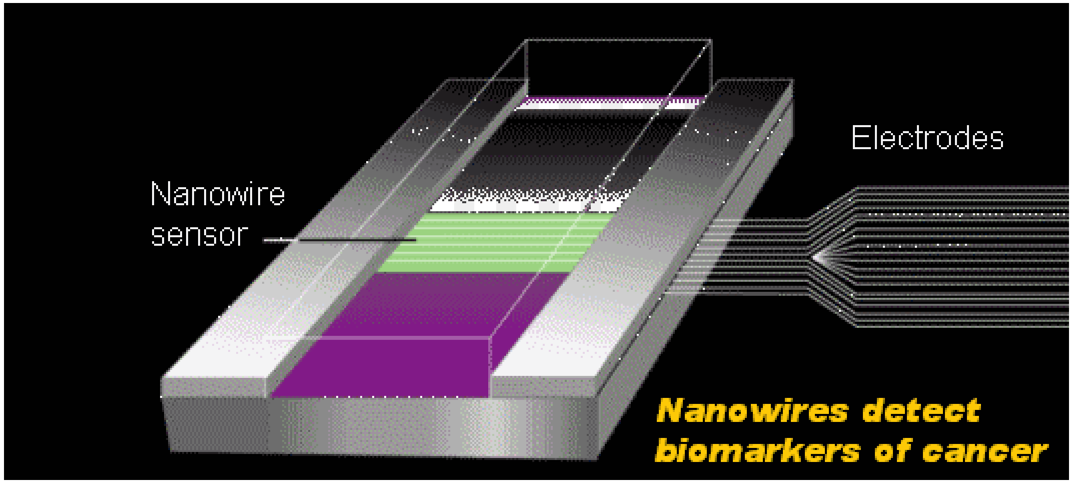
Beyond nanopores

General issues with nanopores

- Single base-pair resolution is not available
  - Typically 3-4 nucleotides fit into a nanopore
- Only one detector per DNA strand
- Fast translocation of DNA through pore
- Small signal and high noise
- Unstable lipid bilayers

Nanowire alternatives

- QuantumDx QSEQ

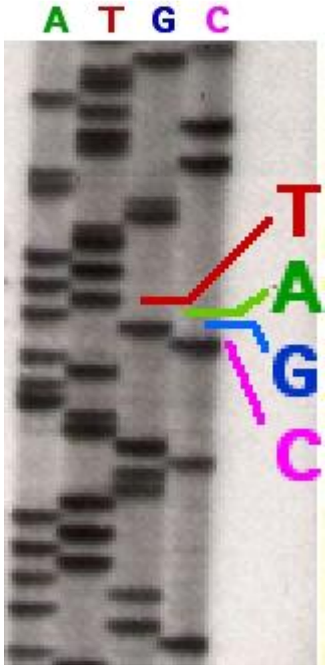


Many others in development

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

Sequencing – back on the benchtop

1980



2000

2015?

Thanks to:

Audrey Farbos

Karen Moore

Wellcome Trust

Contact me:

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Supported by wellcome trust