Whole-Genome Sequencing: Technology and Data

Bioinformatics Workshop for *M. tuberculosis*
Genomics and Phylogenomics

July 9-14, 2018 @The Philippine Genome Center

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advancing health worldwide

PHILIPPINE GENOME CENTER

BERKELEY LAB
Lawrence Berkeley National Laboratory
Bringing Science Solutions to the World

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DNA Sequencing Technologies: Past and Present

Illumina MiSeq

Illumina HiSeq

454 Sequencer

SOLiD system

PacBio

Ion Proton

Nanopore

DNA Sequencing: Why do we care?

"... [A] knowledge of sequences could contribute much to our understanding of living matter."
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First Generation DNA Sequencing: Sanger dideoxy sequencing (~1975)

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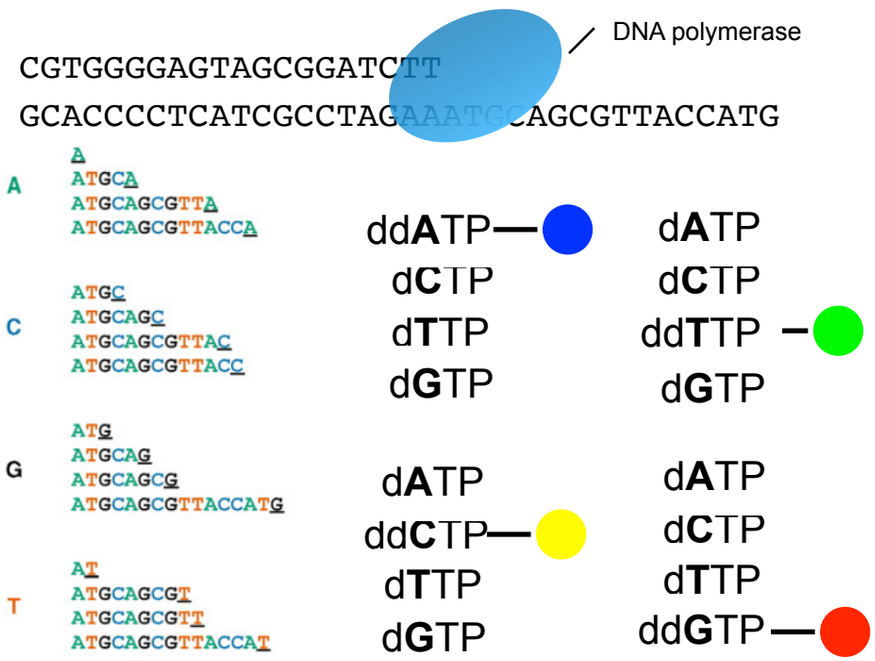
I. DNA Synthesis with dideoxynucleotides



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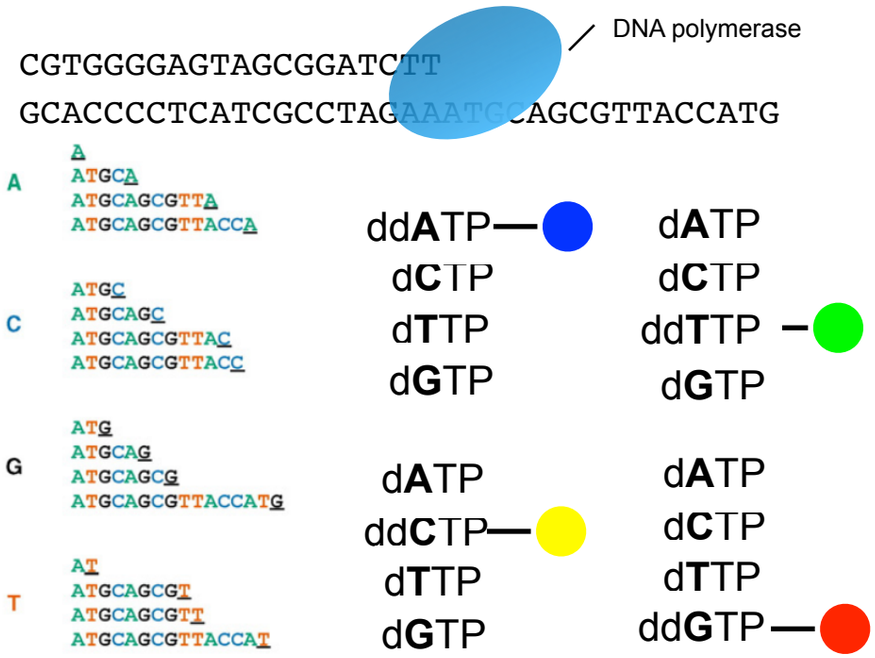

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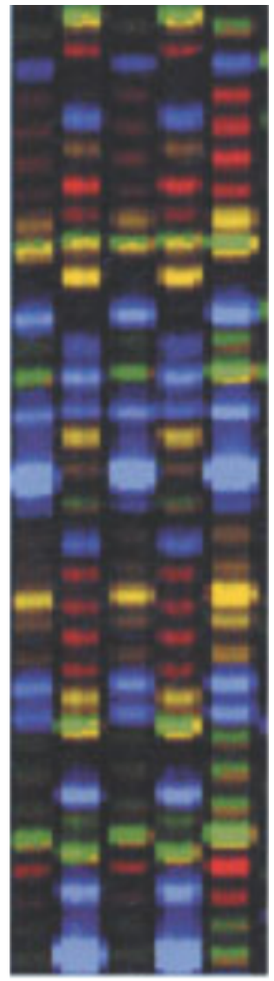
II. Electrophoresis



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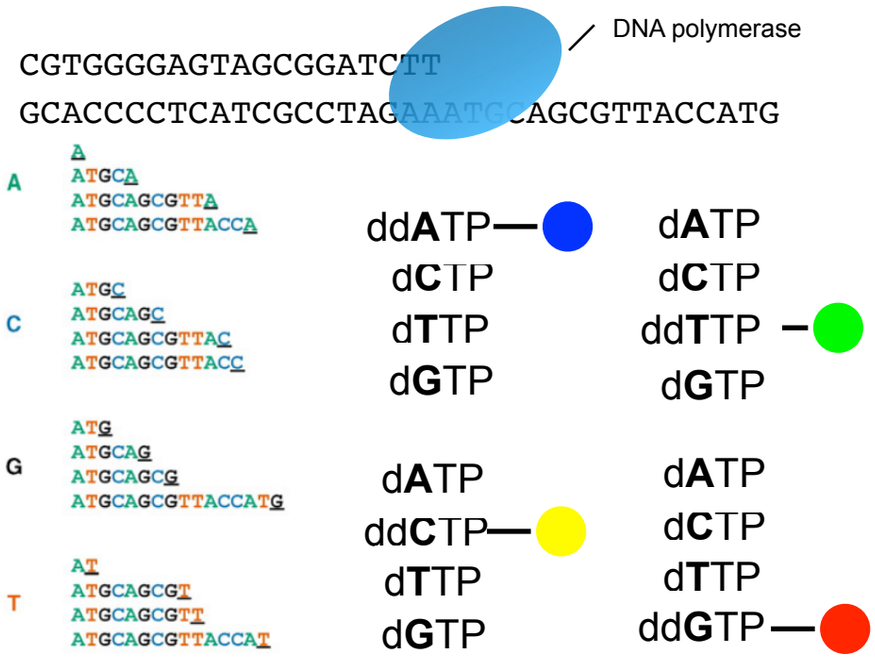

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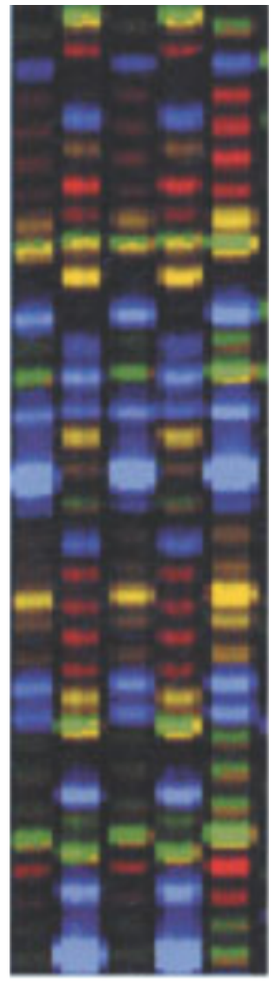

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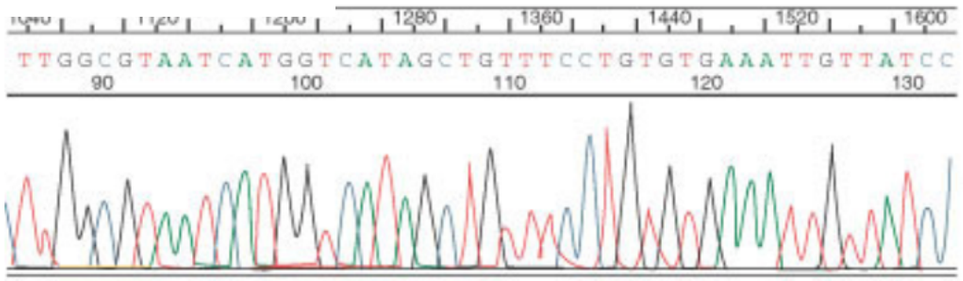
III. Electropherogram



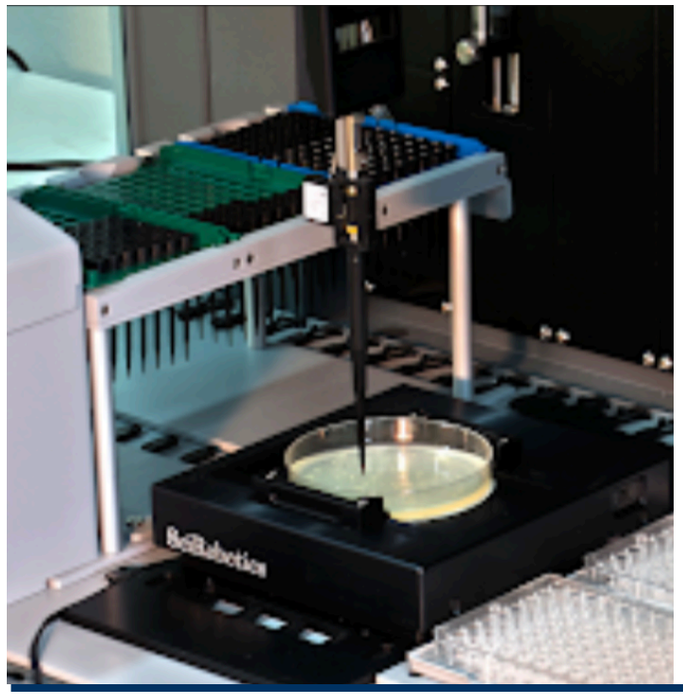
Automation of Sanger Sequencing

ABI 3730xl: 96/384 well capillary system

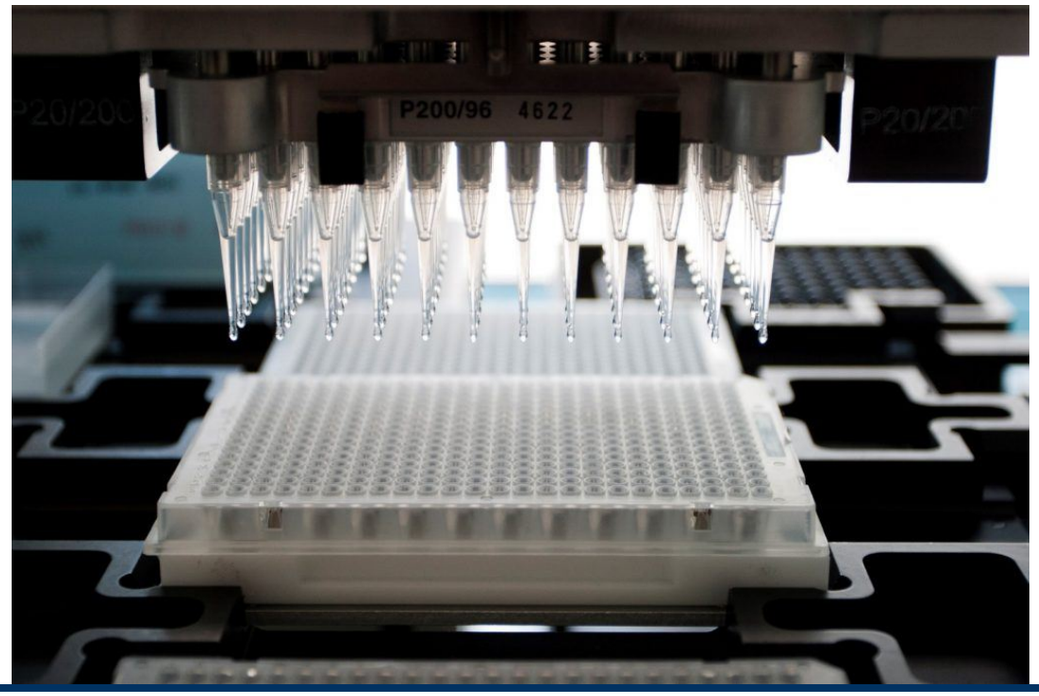
2001

ABI Sequencers, Venter Institute

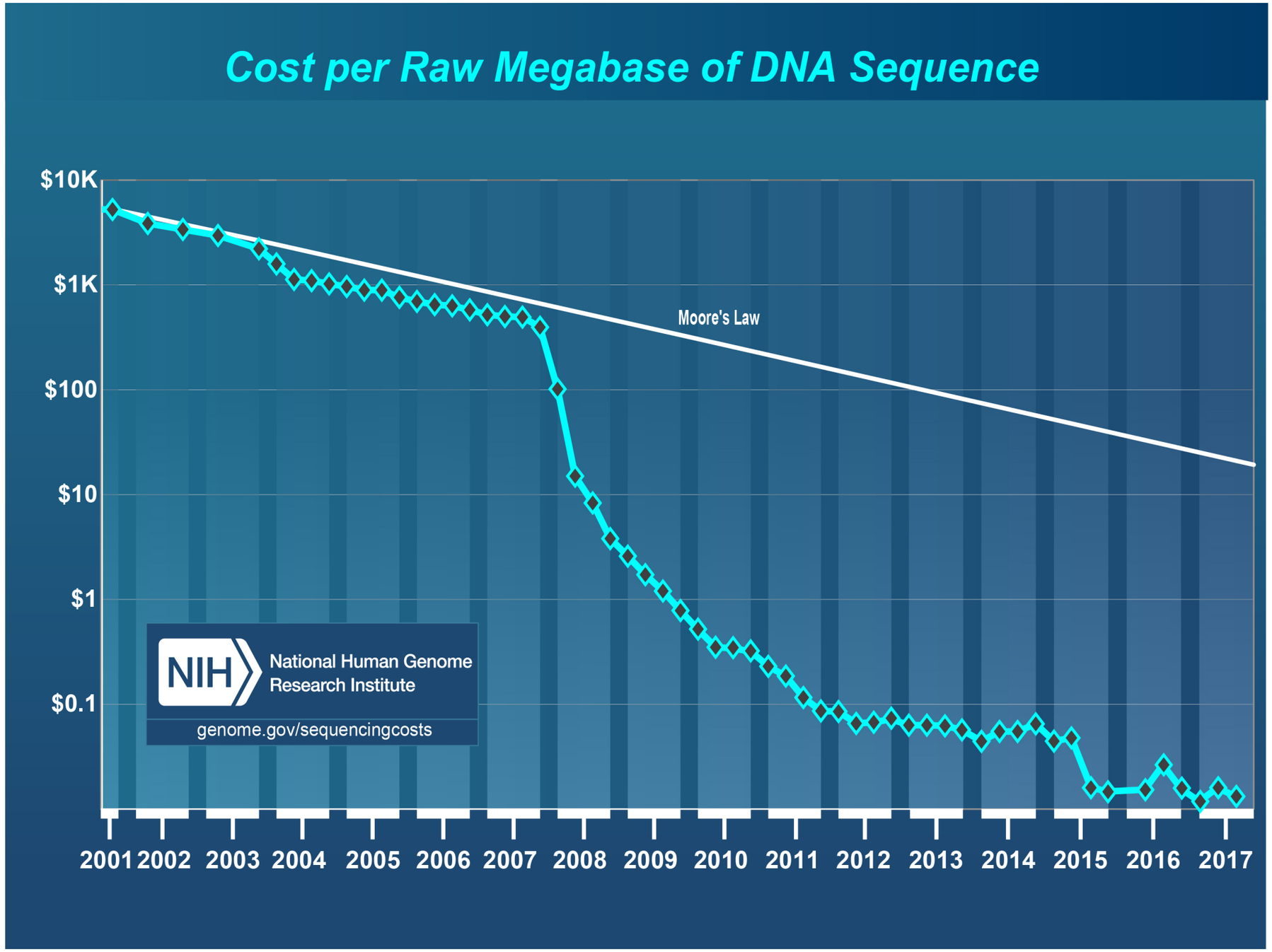
Automated colony picking



Automated plating



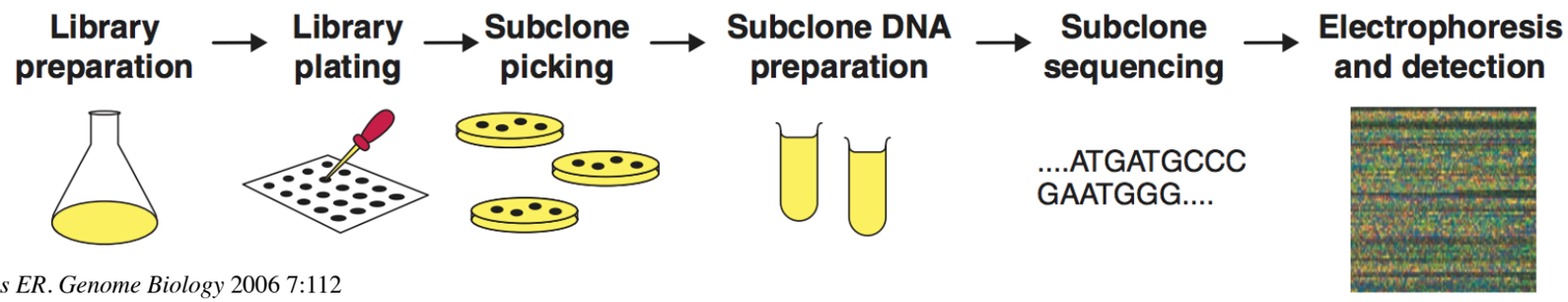
2004-2007: from *colonies* to *clusters*



Next-Generation Sequencing (NGS)

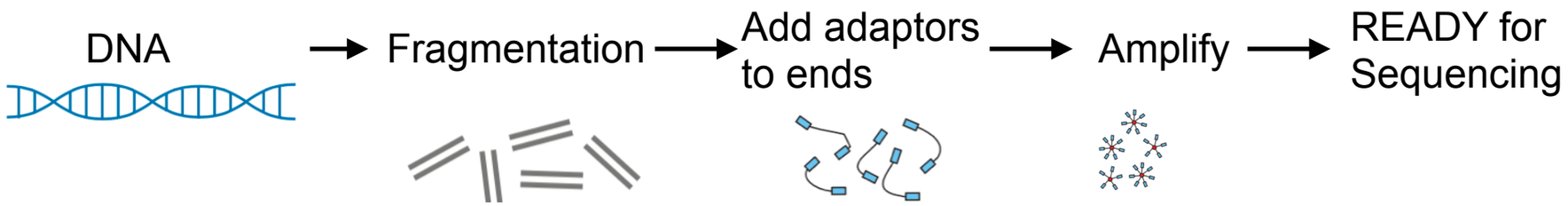
Next-generation Sequencing = **Second-generation Sequencing** = **Massively Parallel Sequencing**

Conventional Sequencing



Mardis ER. Genome Biology 2006 7:112

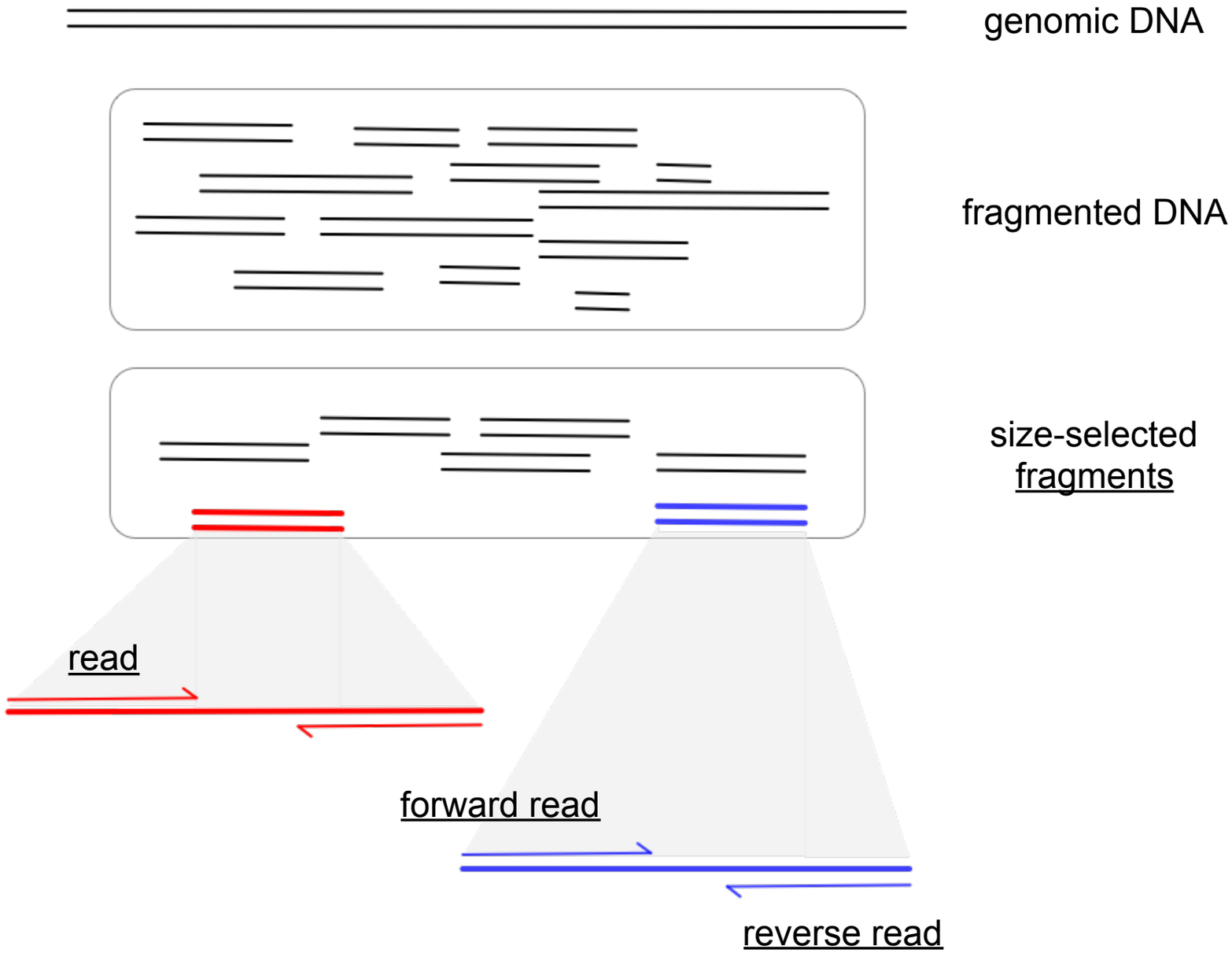
NGS



NGS Commonalities

- Sequencing by synthesis: coupling of molecular biology and detection
- Library construction: easier, faster, cheaper
- randomly fragmented DNA + "adapter" sequences (platform-specific)
- Amplification needed before sequencing

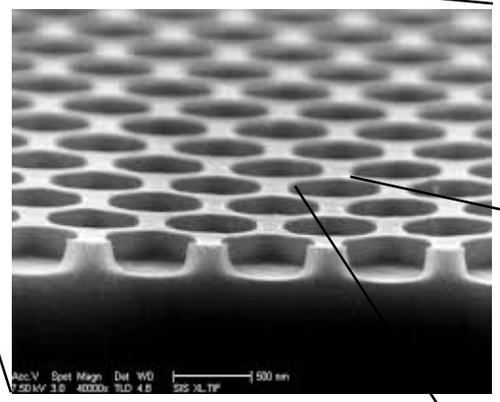
Next-Generation Sequencing (NGS)



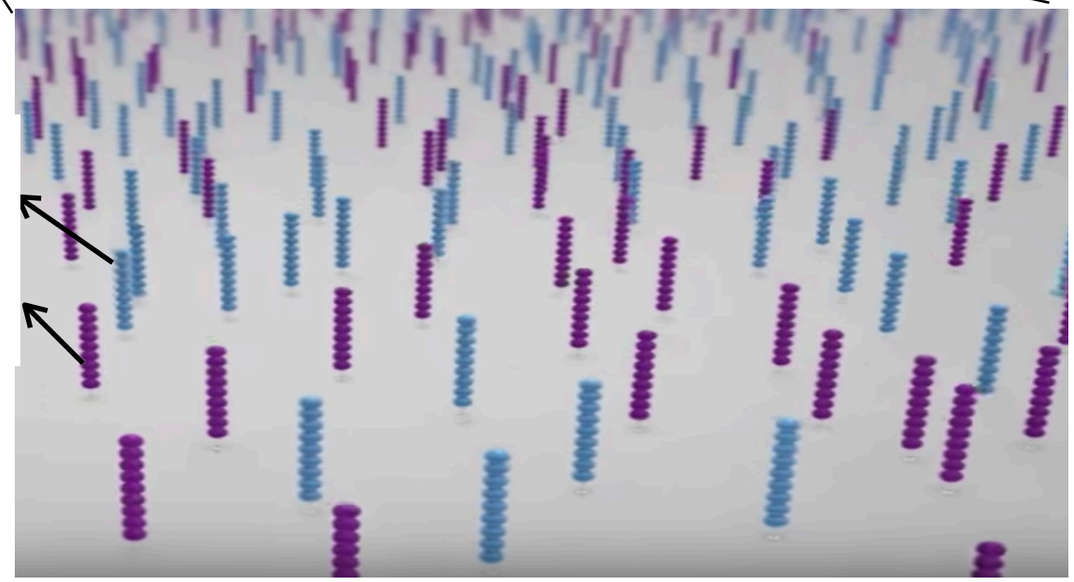
Illumina Sequencing: Flow cell

Illumina flow cell: glass slide where sequencing chemistry occurs

lane

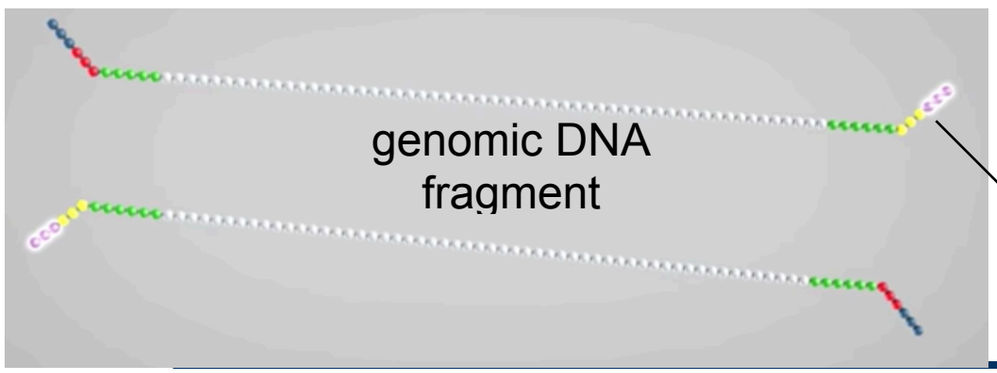


"Lawn" of oligos on the flow cell surface



2 types of flow cell oligos

DNA fragments with adapters complementary to these oligos at ends



region complementary to flow cell oligo

Illumina Sequencing: Amplification

Clustering: Isothermal amplification of the DNA fragments on the solid surface

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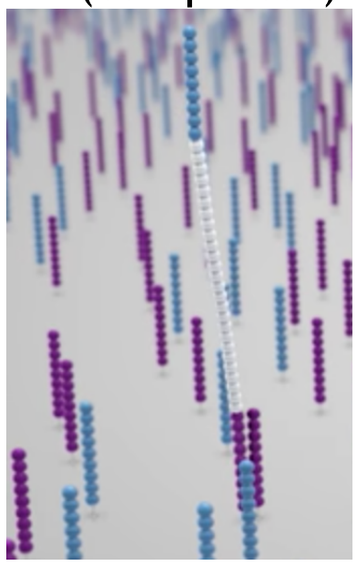
Hybridization of fragments (templates)



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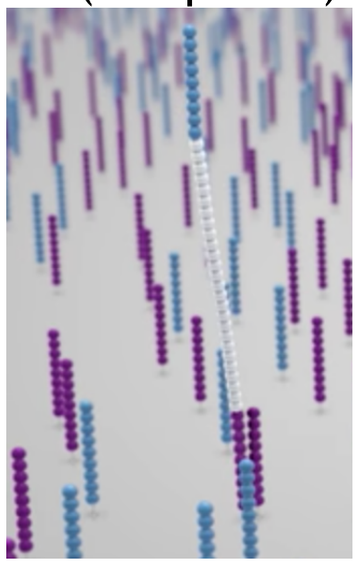
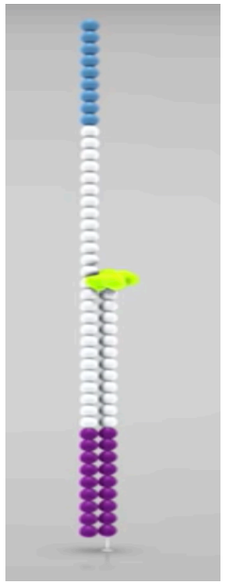


Synthesis of dsDNA



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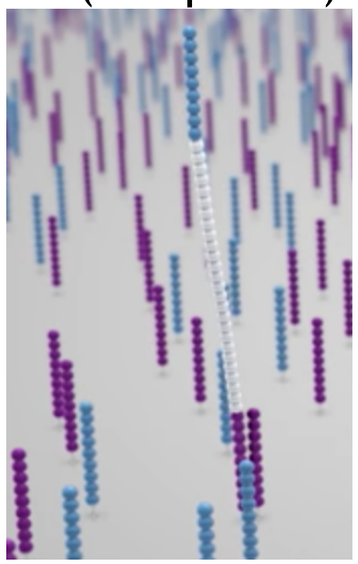

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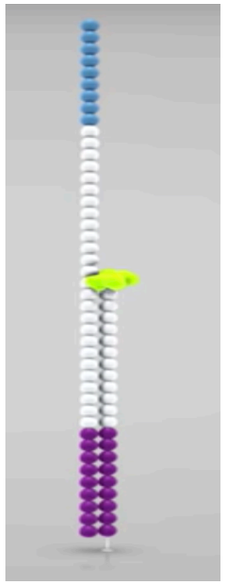

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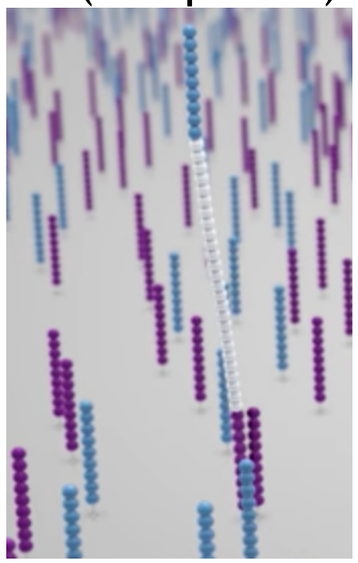

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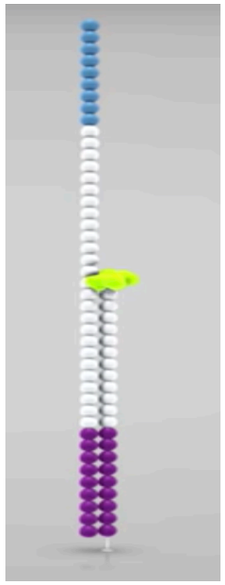

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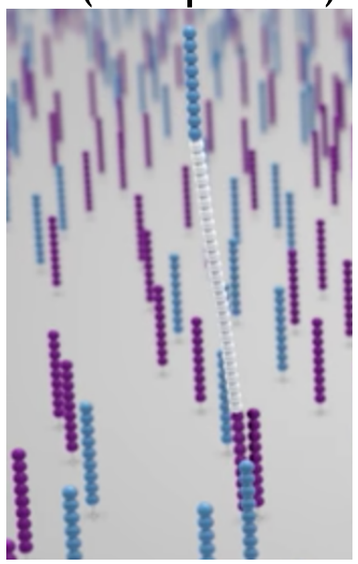

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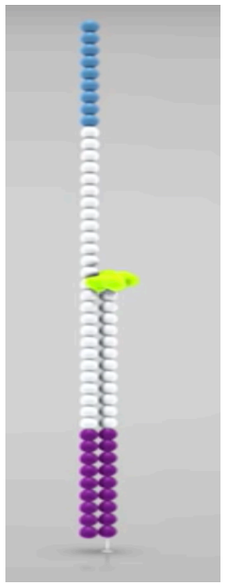

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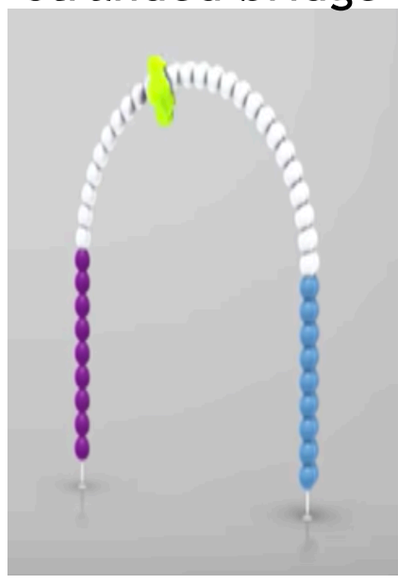

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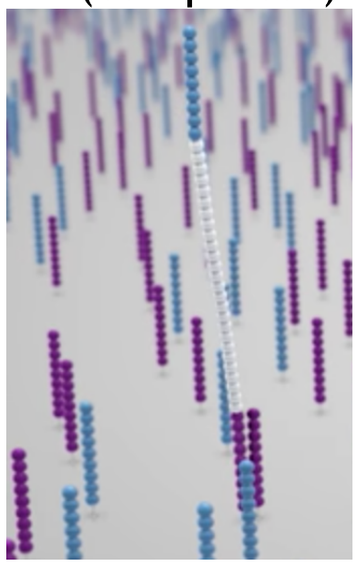

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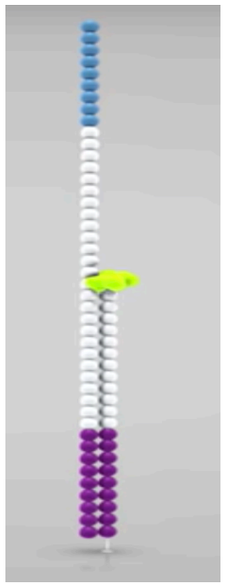

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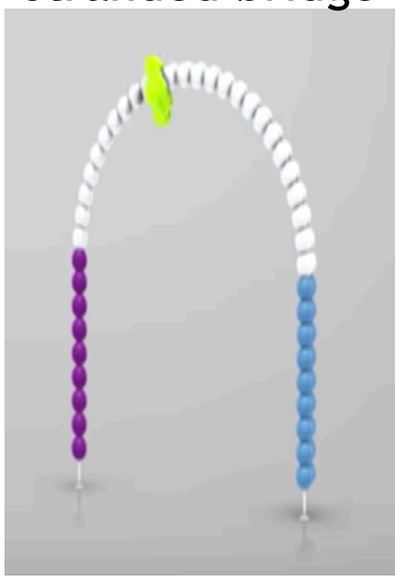
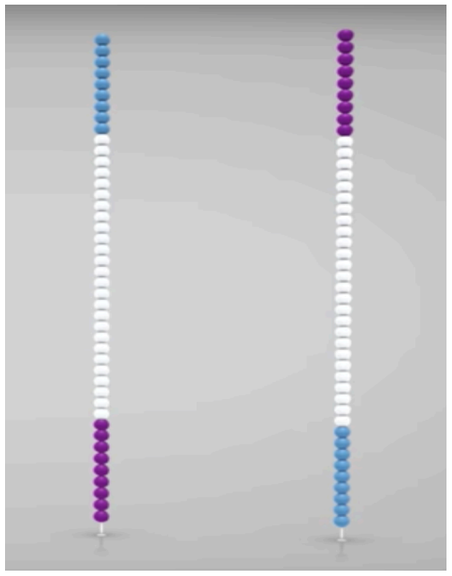


Single stranded DNA molecules tethered to the flow cell



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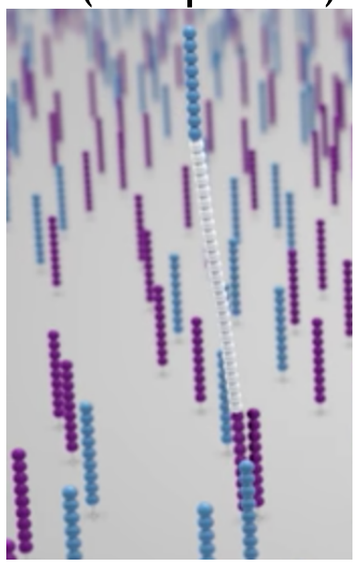

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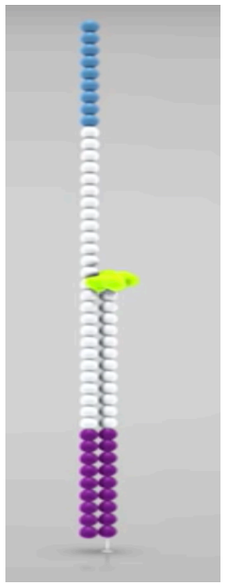

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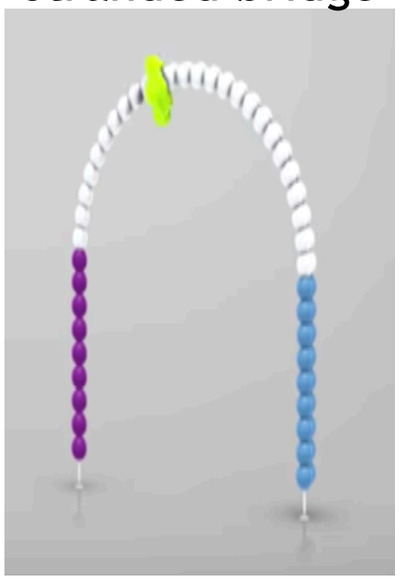

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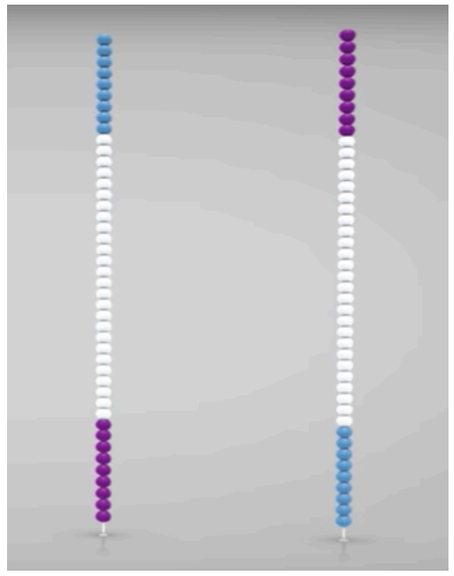
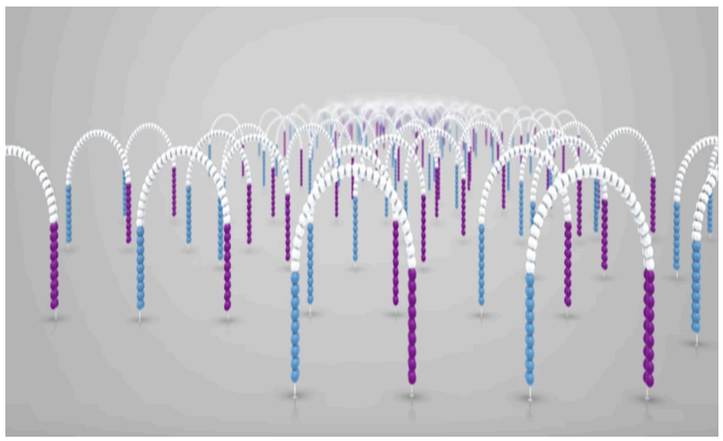


Repeat in parallel



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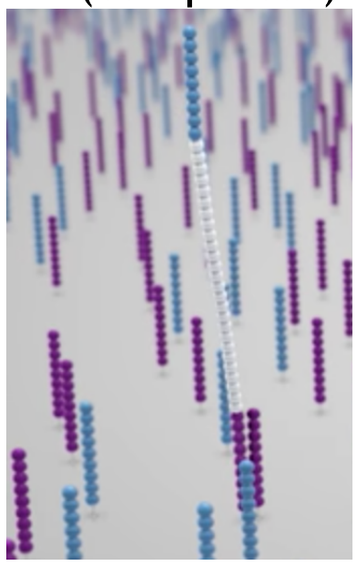

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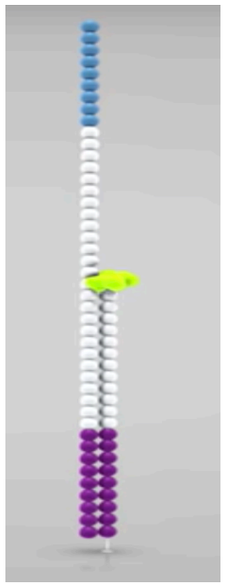

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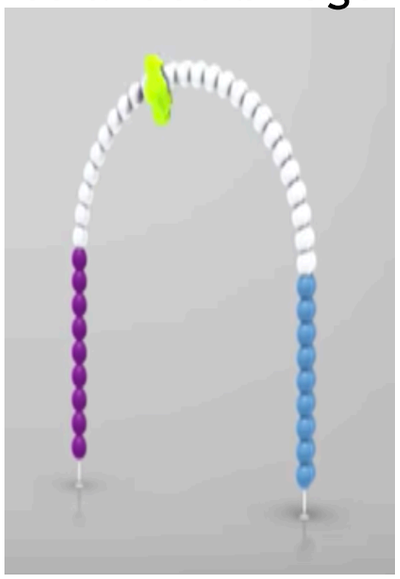

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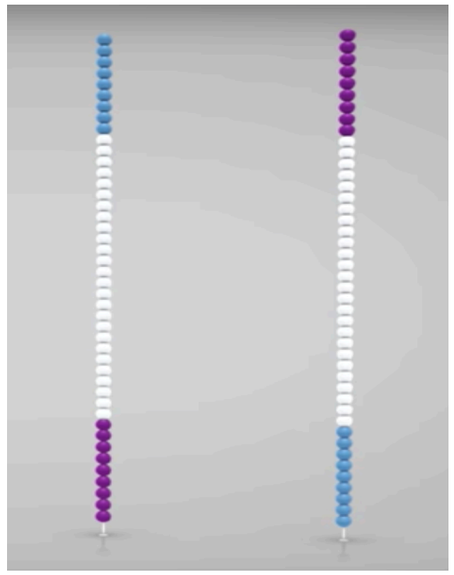

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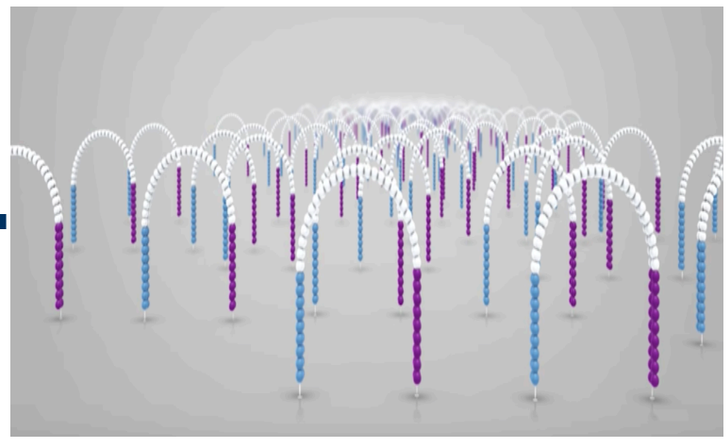
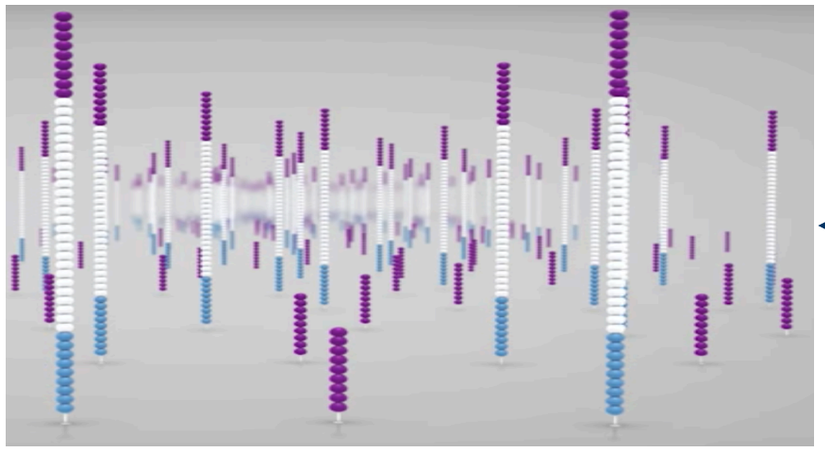


Millions of copies of forward Strand DNA tethered to the flow cell



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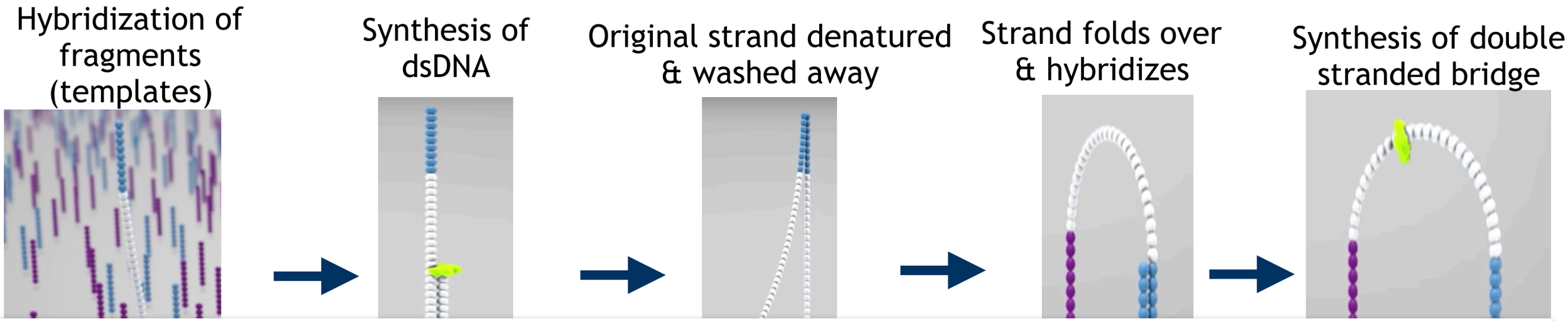
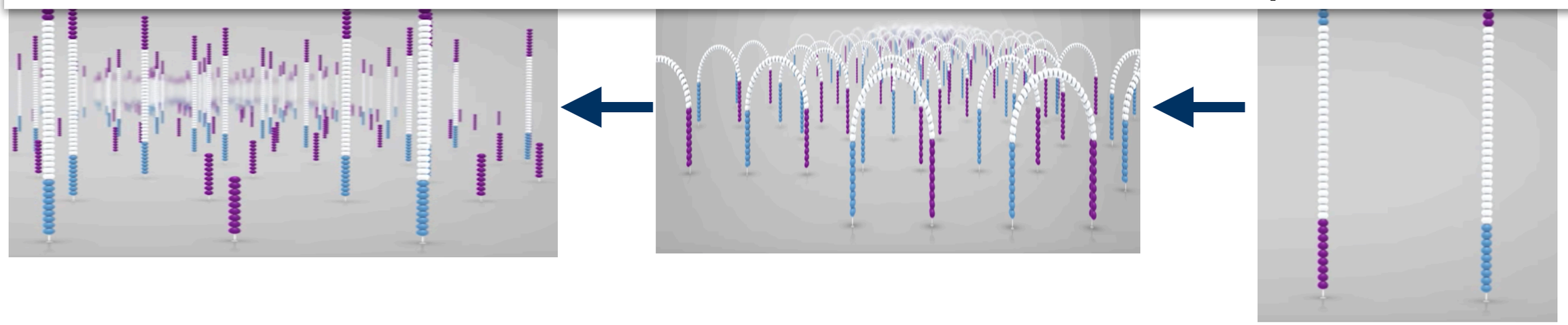
Clustering: Isothermal amplification of the DNA fragments on the solid surface



Cluster formation is a type of PCR ("bridge amplification")

PCR can introduce preferential amplification of some fragments

PCR can introduce artifacts, which will lead to false positive variants



Illumina Sequencing: Sequencing by synthesis

number of cycles => read length

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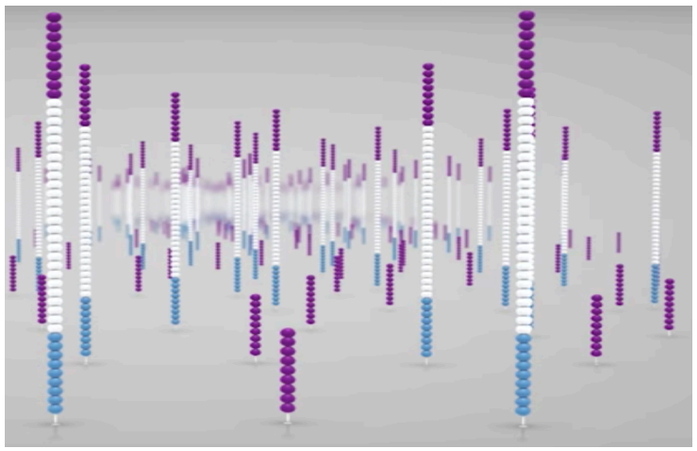

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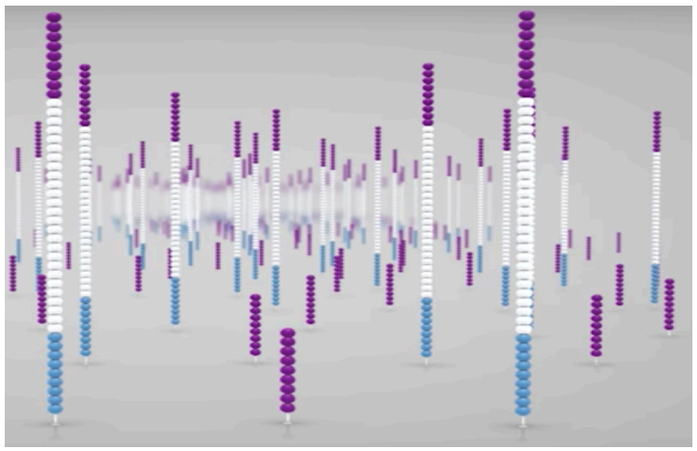
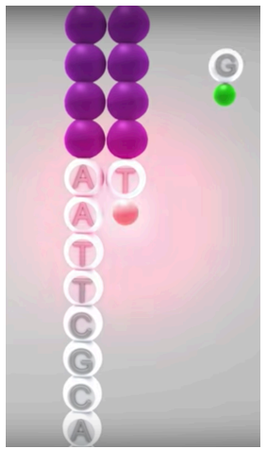


Sequencing primer extended, sequencing begins



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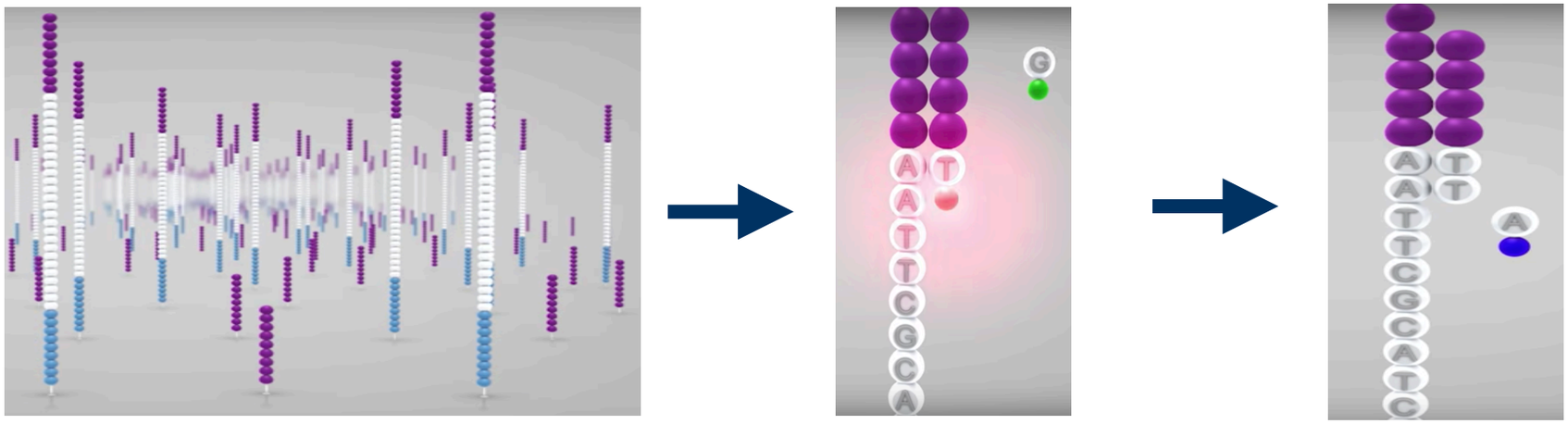
In each cycle, only one fluorescently tagged nucleotide is incorporated



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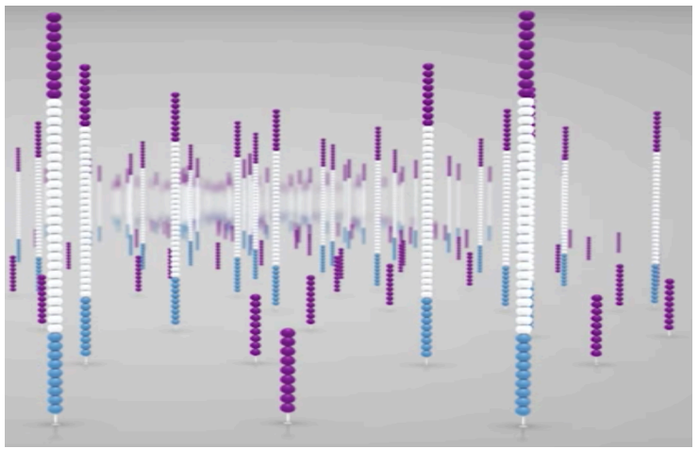

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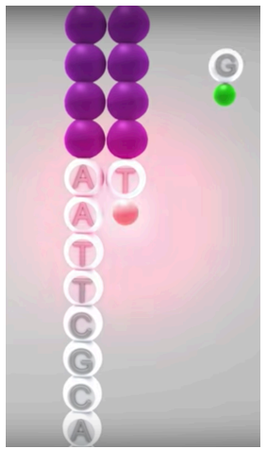

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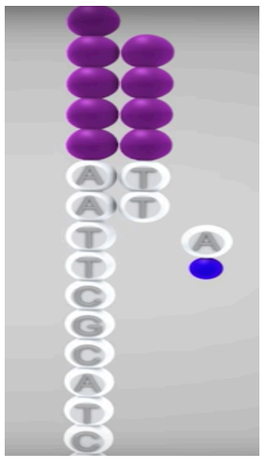
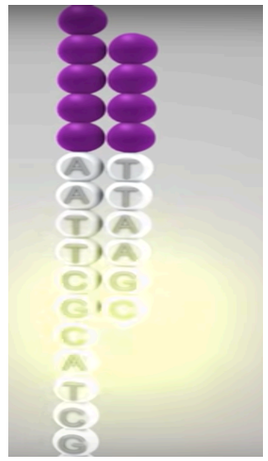


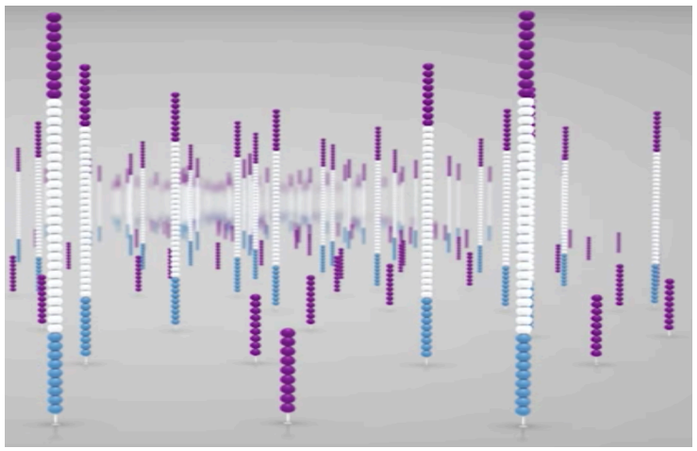
Excitation & Emission: After nucleotide addition, clusters are excited by a light source



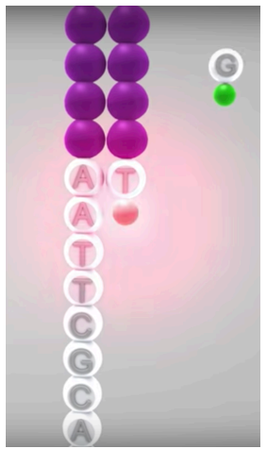
Illumina Sequencing: Sequencing by synthesis

number of cycles => read length

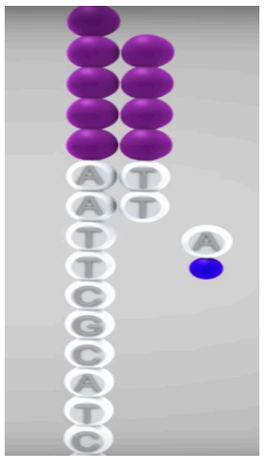
Millions of copies of forward Strand DNA tethered to the flow cell



Sequencing primer extended, sequencing begins



In each cycle, only one fluorescently tagged nucleotide is incorporated



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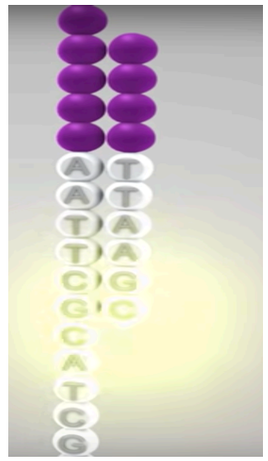
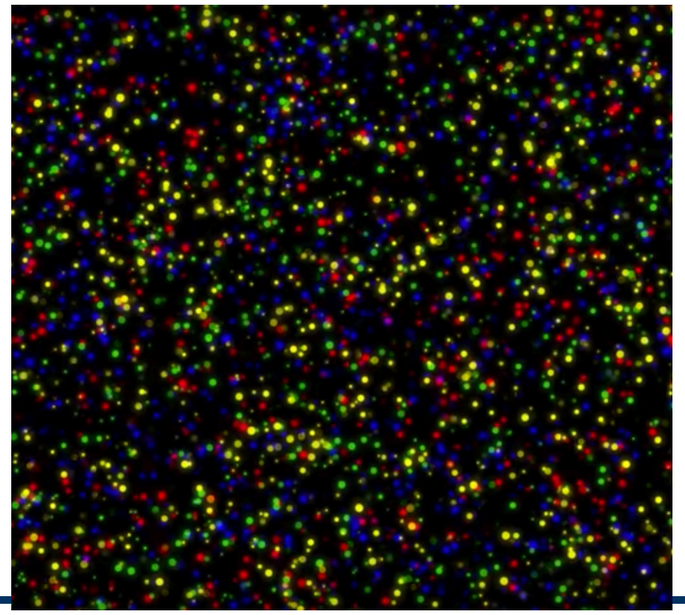


Four images taken per sequencing cycle



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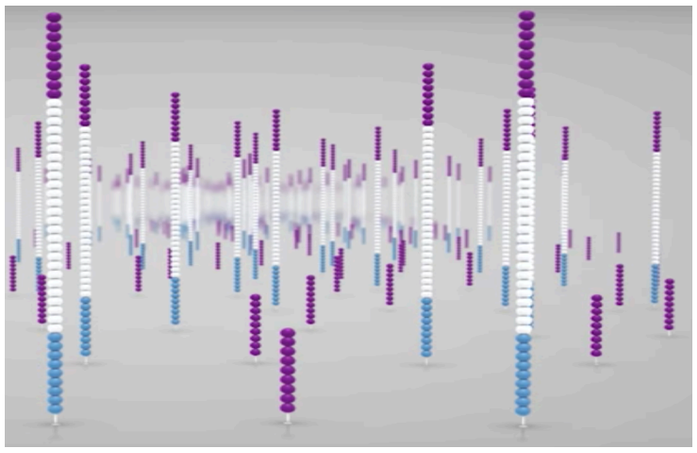

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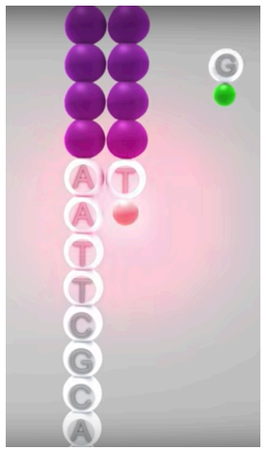

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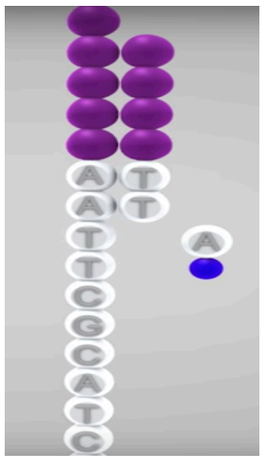

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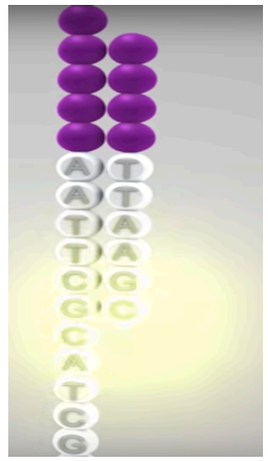

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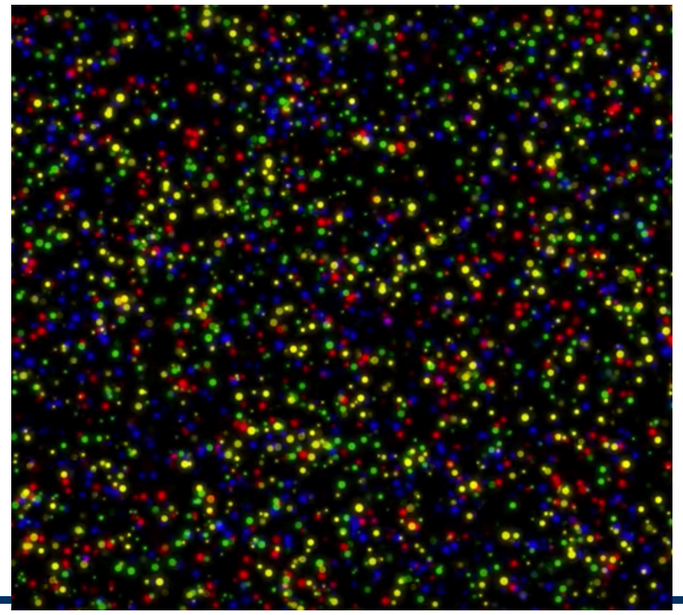
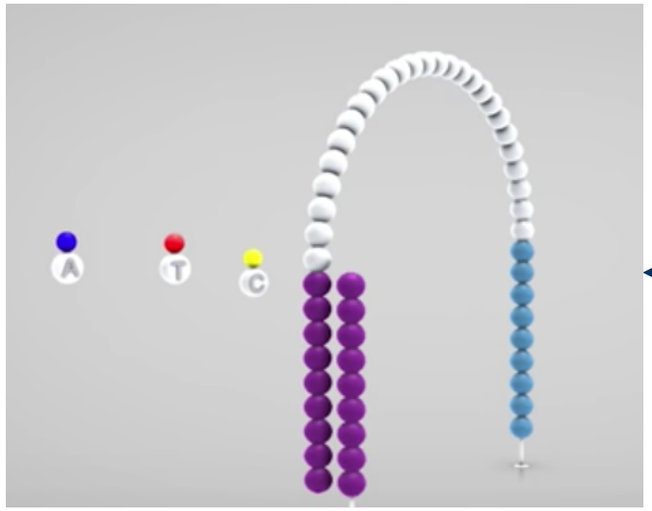


Generate Read 2, repeating for the reverse strand



...

Illumina Platforms

Miniseq

- small,benchtop
- 2x150 bp
- Two output modes:
  - high: ~6 Gb
  - mid: ~2 Gb

MiSeq

- benchtop
- v3 chemistry offers 2x300 bp reads
- Reverse read quality drops after ~200th cycle
- Throughput: 25 million reads/lane

<u>Illumina HiSeq</u>
~3 billion paired 100bp reads
~600Gb, $10K, 8 days
(or “rapid run” ~90Gb in 1-2 days)

<u>Illumina X Ten</u>
~6 billion paired 150bp reads
1.8Tb, <3 days, ~1000 / genome($$)
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<u>Illumina NextSeq</u>
One human genome in **<u><30 hours</u>**

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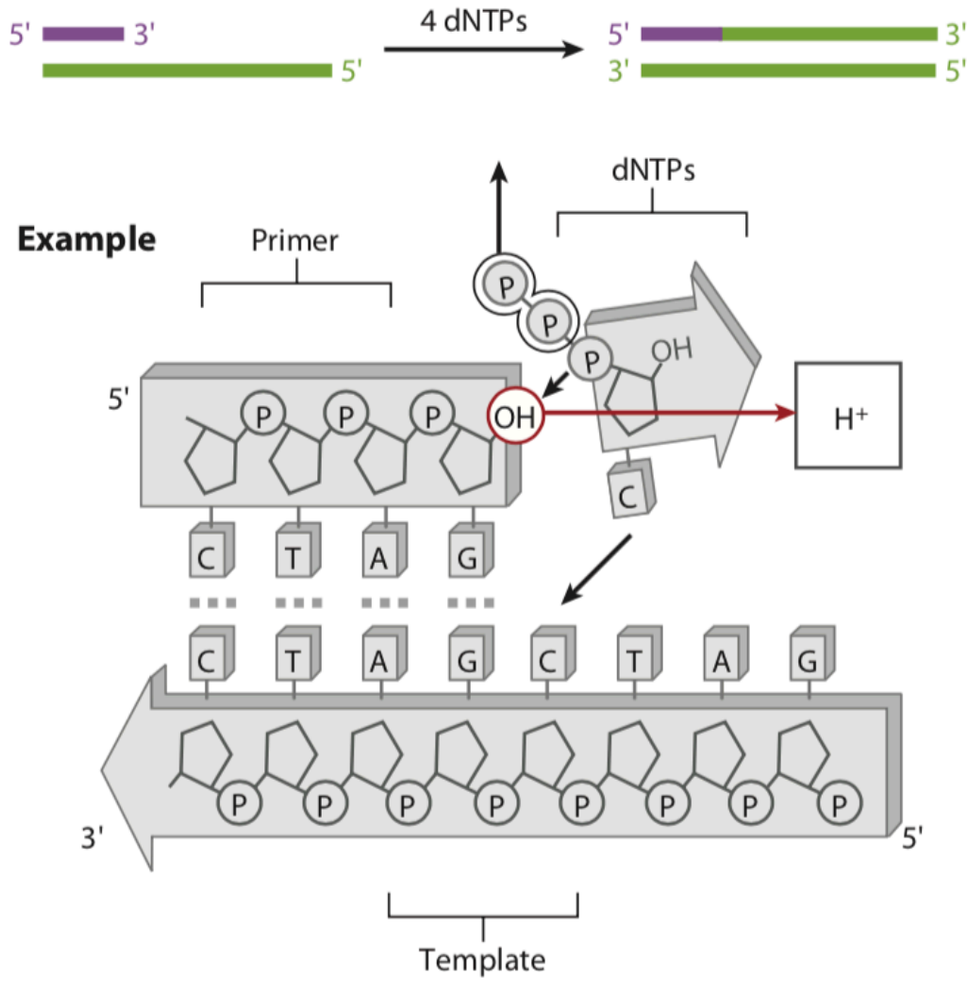
Ion Semiconductor Sequencing

Detection is electrochemical, no optics

What is detected?

Hydrogen ions (H^+) released during nucleotide incorporation



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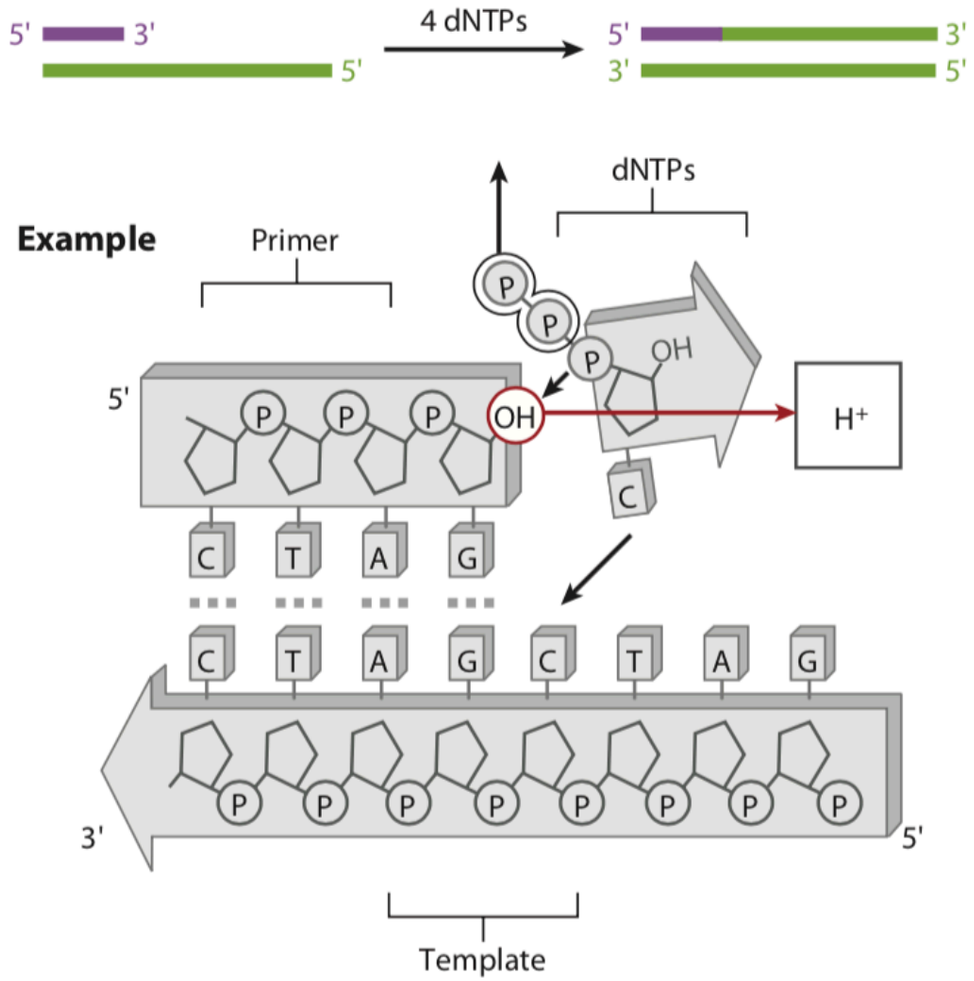
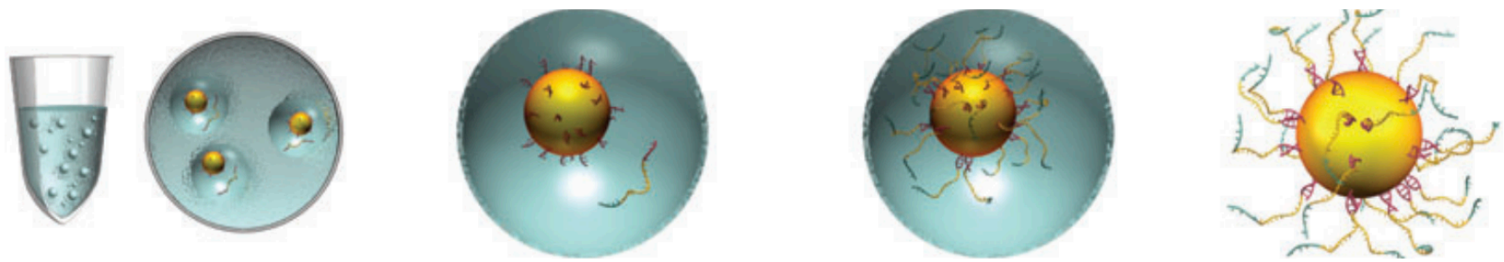


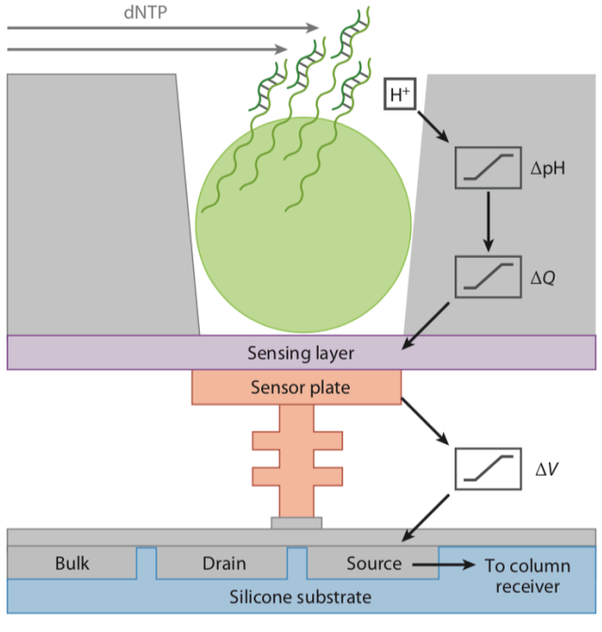
Amplification:

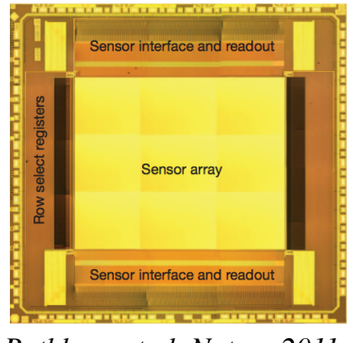
bead-based, emulsion-PCR

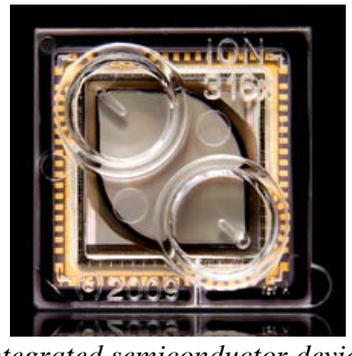


adapted from Mardis 2008. Annu. Rev. Genomics Hum. Genet.

Ion Semiconductor Sequencing







Rothberg et al. Nature 2011. An integrated semiconductor device enabling non-optical genome sequencing.

1-11 million wells

ion torrent
by Thermo Fisher Scientific

Ion Proton

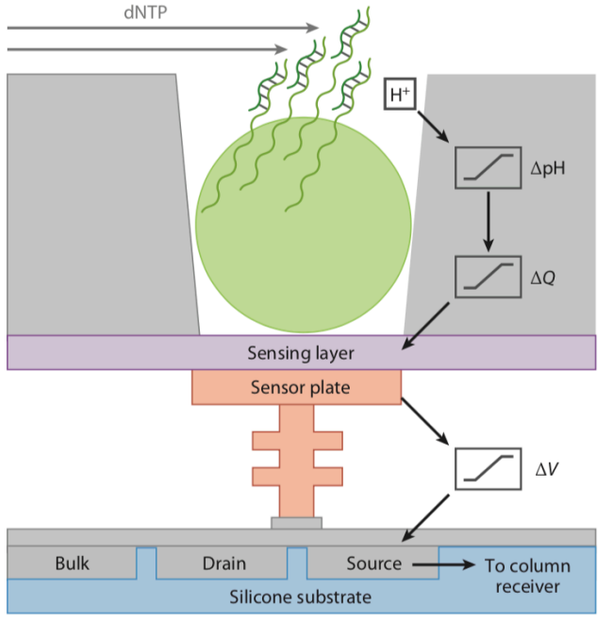
Ion PGM

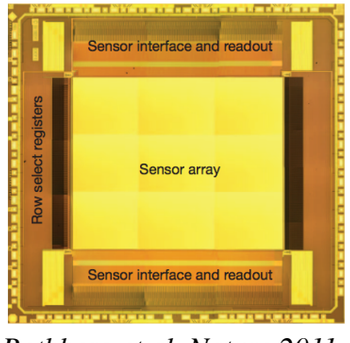
- linear dynamic range
- no substitution errors
- low startup cost
- short run time

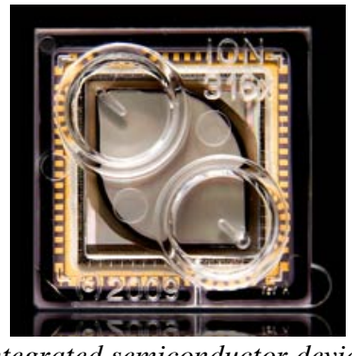
- paired-end reads not supported
- higher error rates
- cost/base high
- short read lengths (200 bp)

Ion Semiconductor Sequencing

Semiconductor based pH-meter







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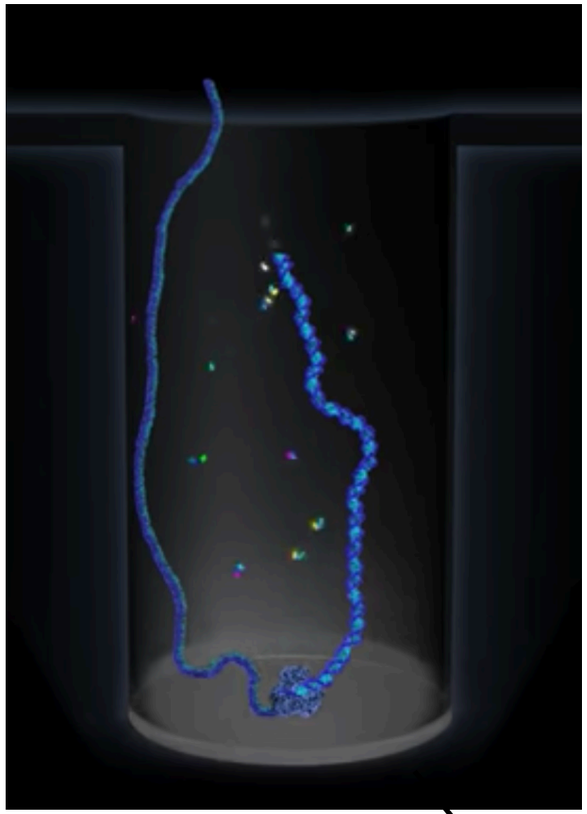
Third-generation Sequencing

Single Molecule Sequencing

"Real-time" Sequencing

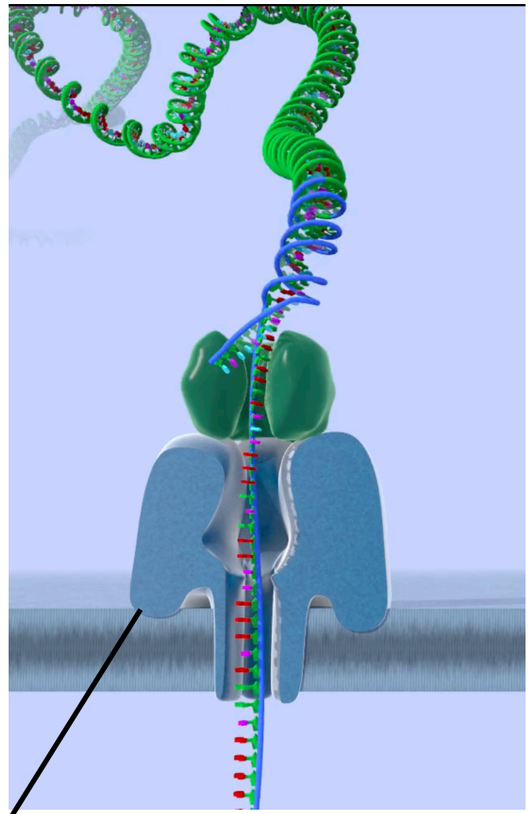
Third-generation Sequencing



nano-well

Single Molecule Sequencing

"Real-time" Sequencing



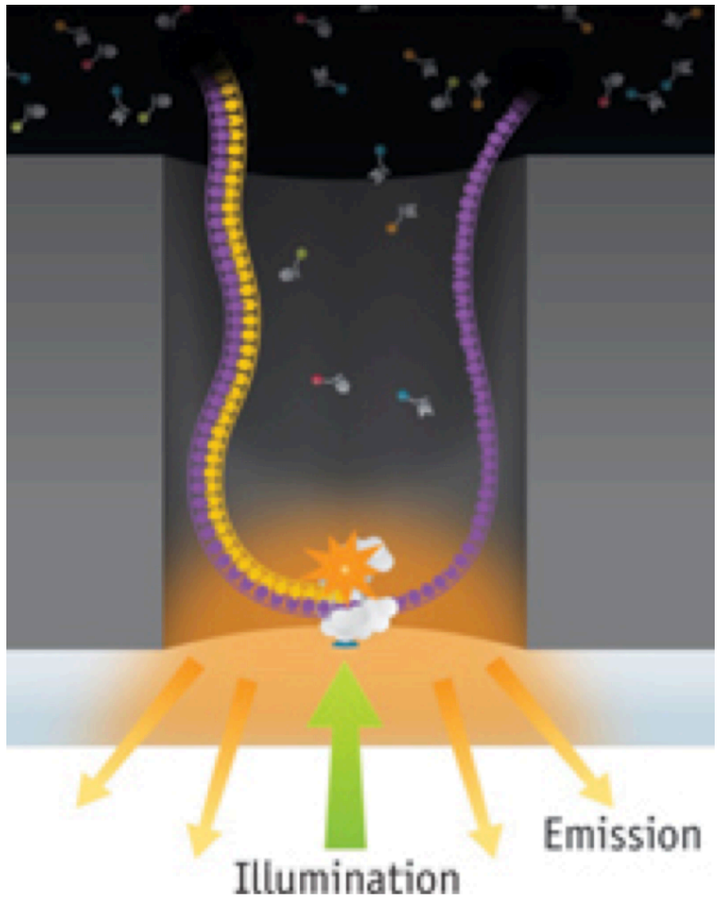
nanopore

PacBio Single Molecule Sequencing using *Zero-mode Waveguides*

Zero-mode Waveguide (ZMW):

provides *zeptoliter* (10^{-21}) scale detection volume

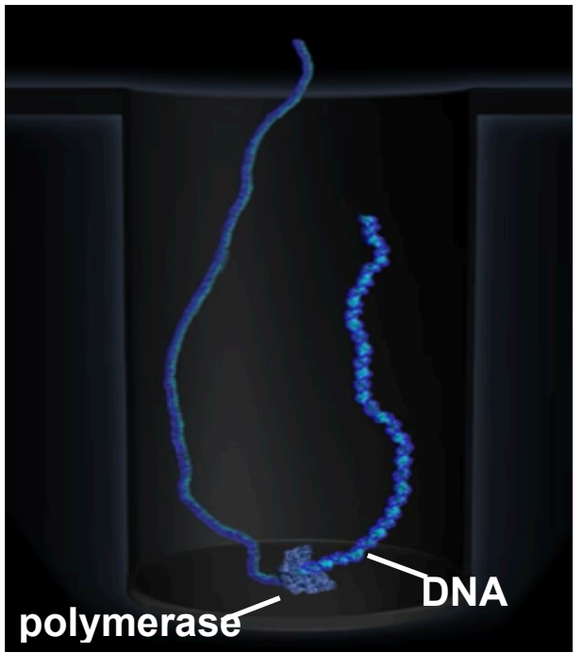
SMRT Cell:
nanofabricated array of ZMWs



Zero-mode Waveguide (ZMW)

PacBio Sequencing: Single-molecule, real time sequencing

I. DNA:polymerase complex, immobilized at the bottom of ZMW



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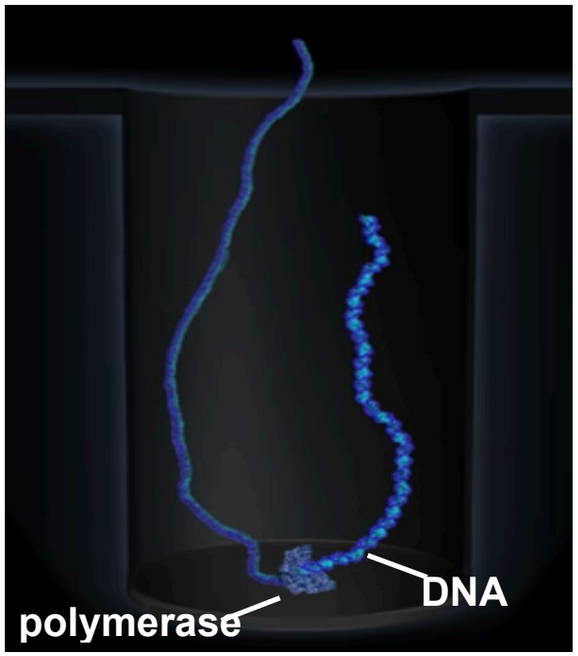
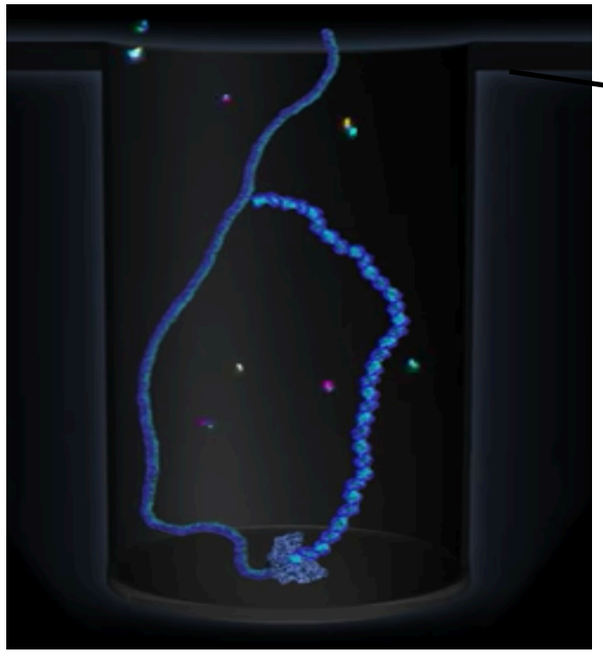
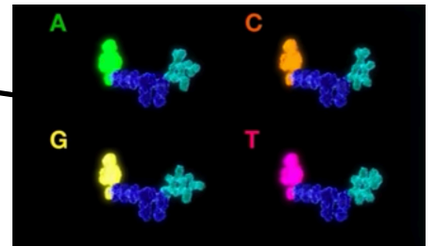


II. Flow in fluorescently labeled nucleotides





phospholinked nucleotides

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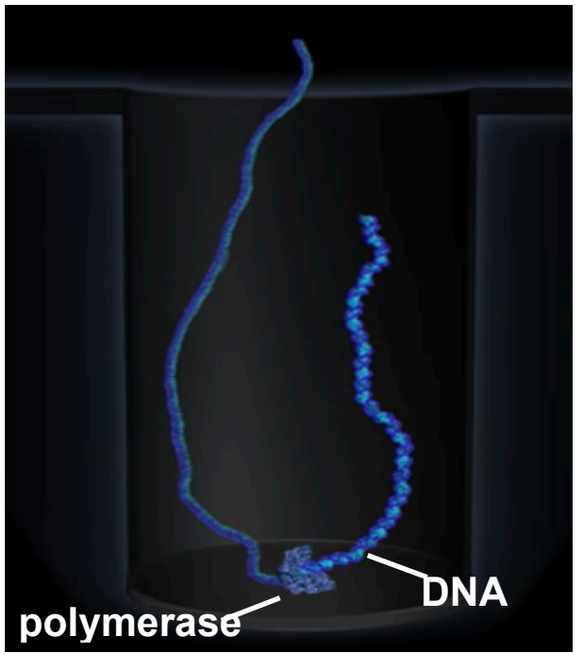

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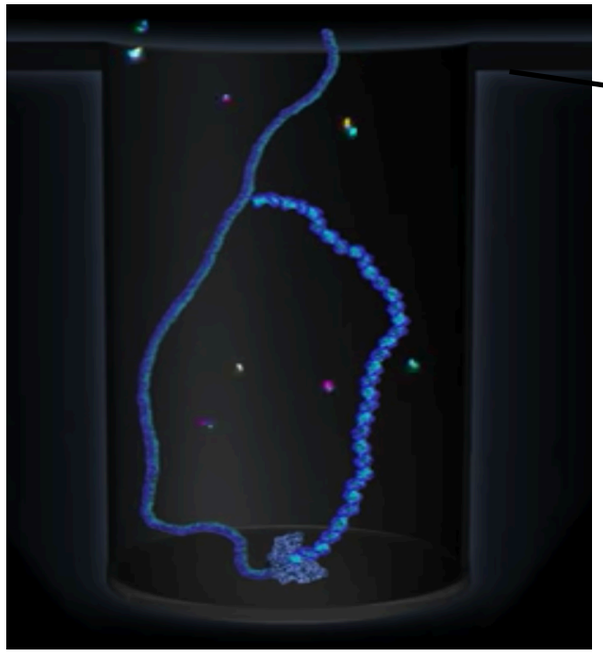

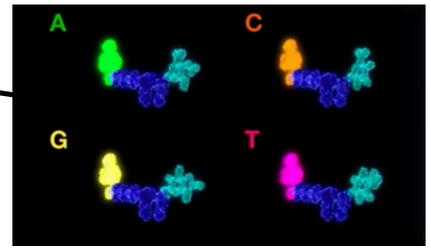

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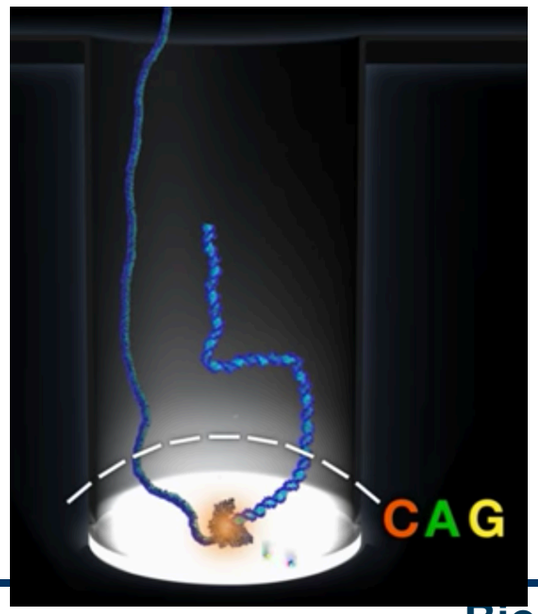
III. Fluorescent nucleotide in the active site, a light pulse is produced



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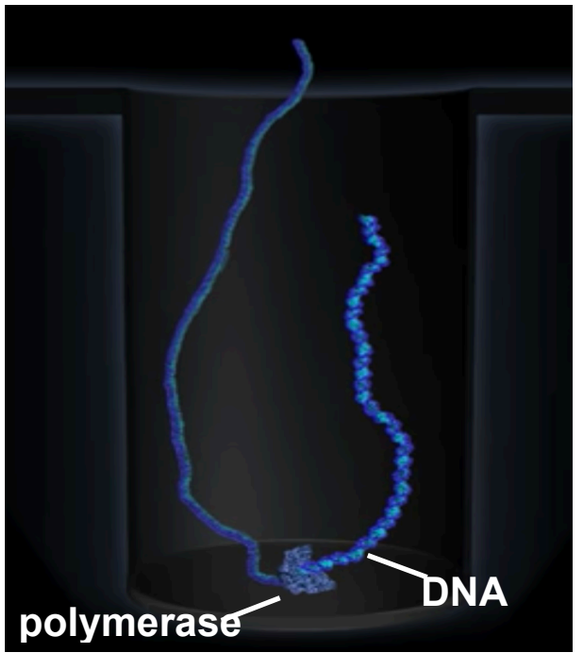

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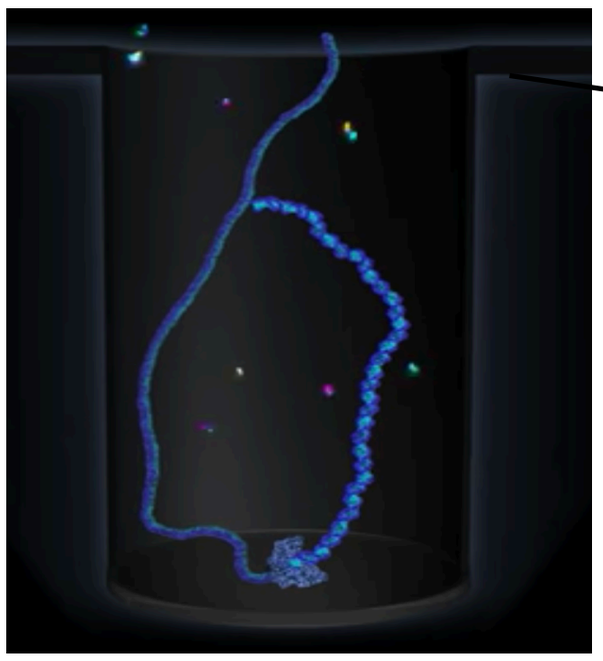

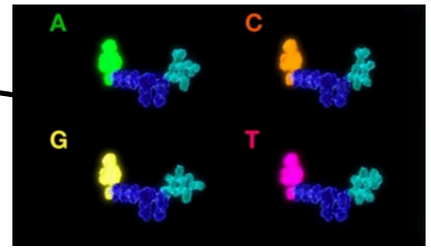

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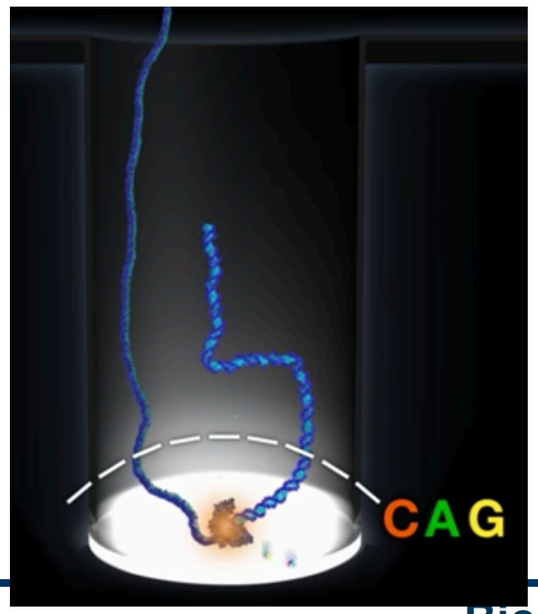
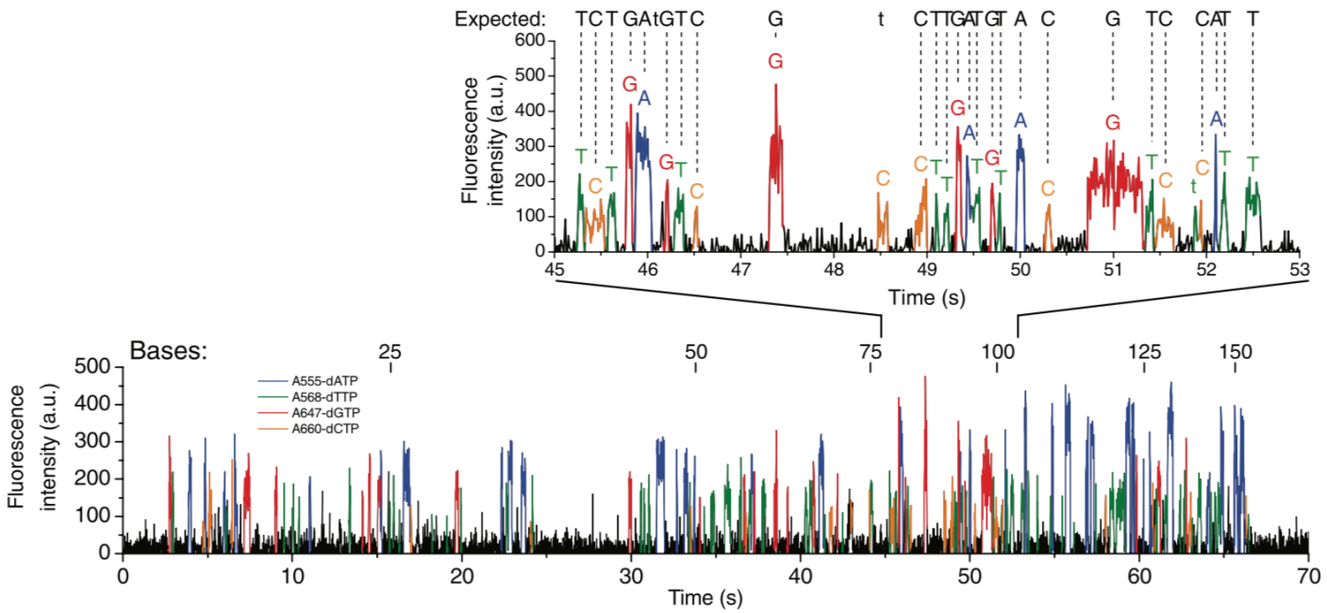
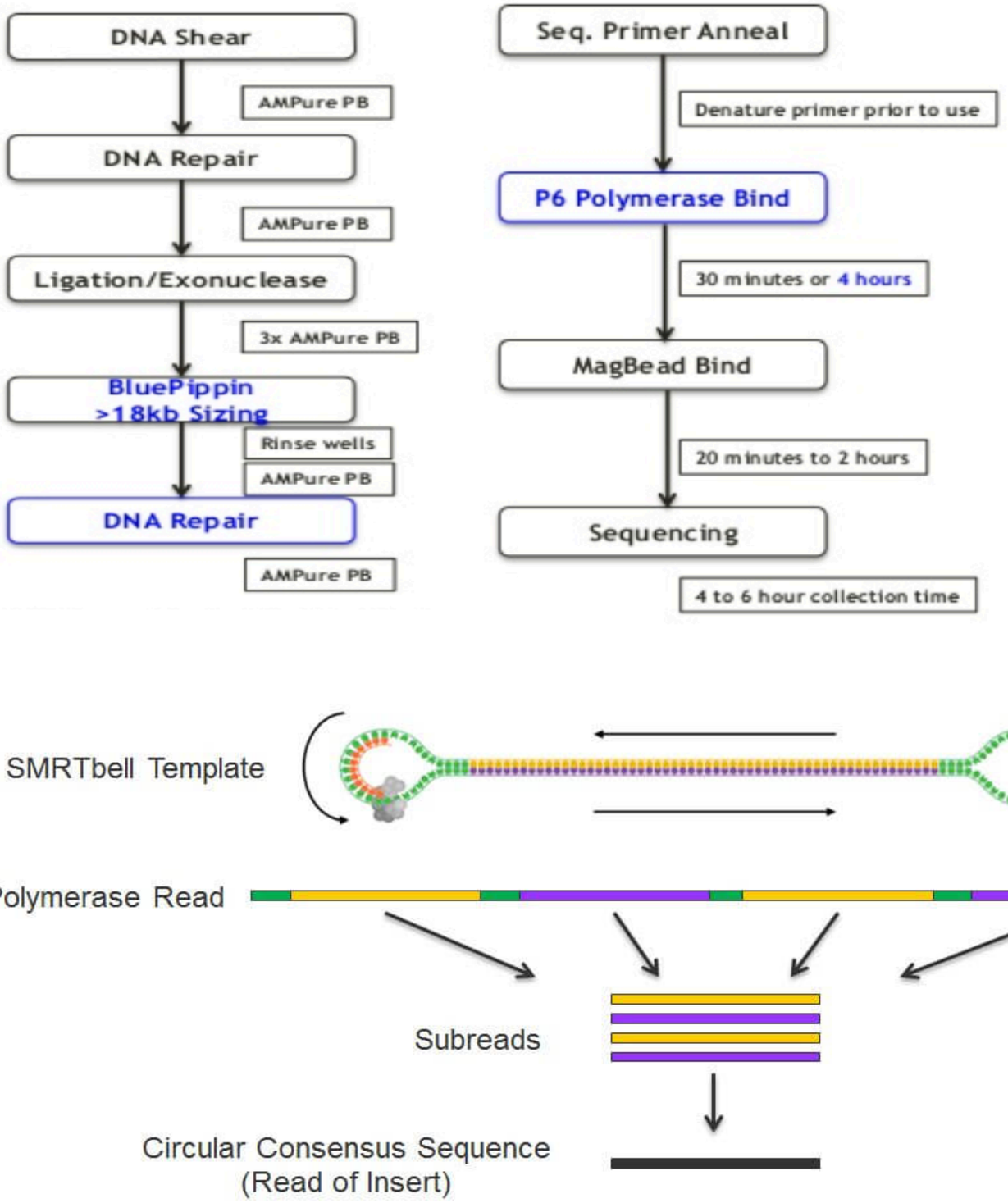




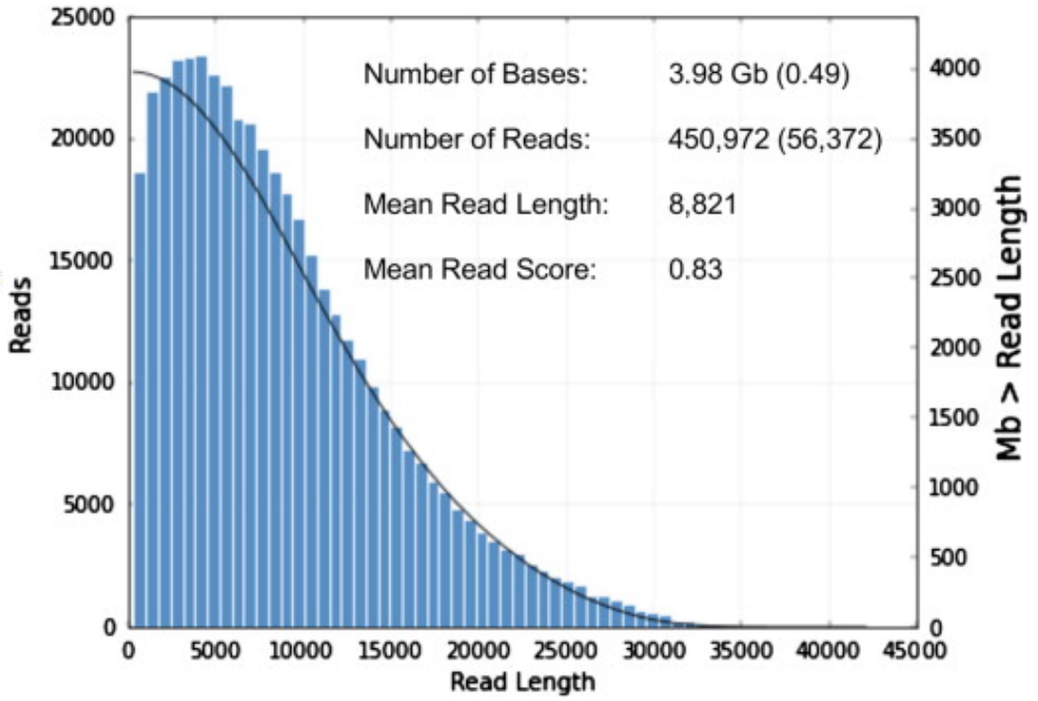
Eid et al. 2009. Science. Real-Time DNA Sequencing from Single Polymerase Molecules

PacBio Sequencing: Library Workflows

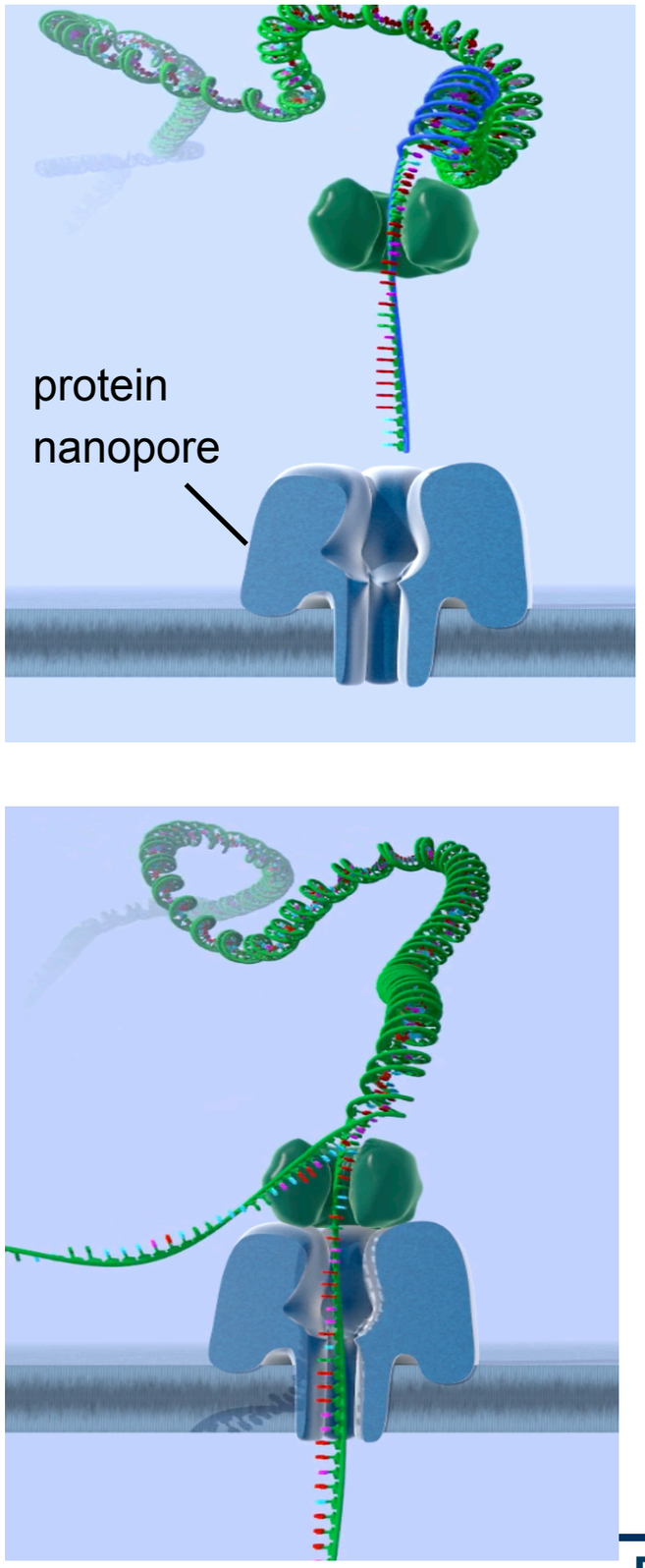


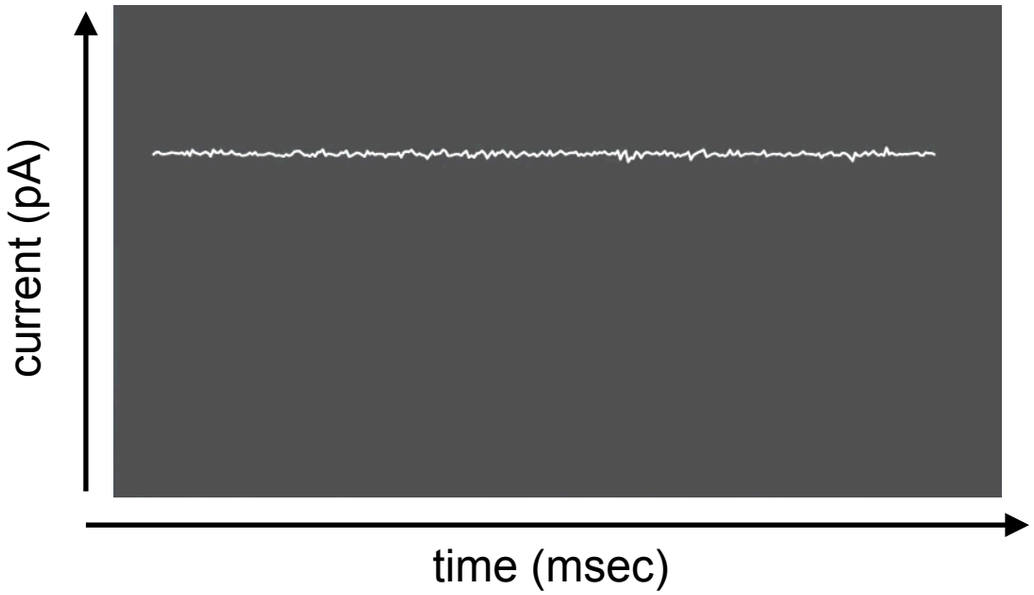
Considerations

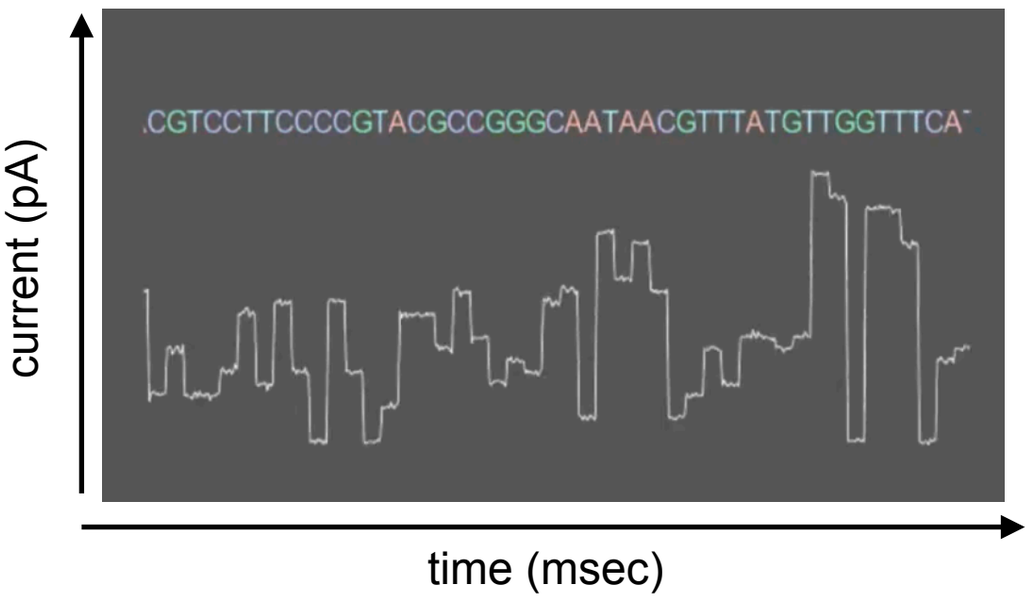
- High-quality, high molecular weight DNA
- Consistent shearing > 30 kb



Nanopore Sequencing

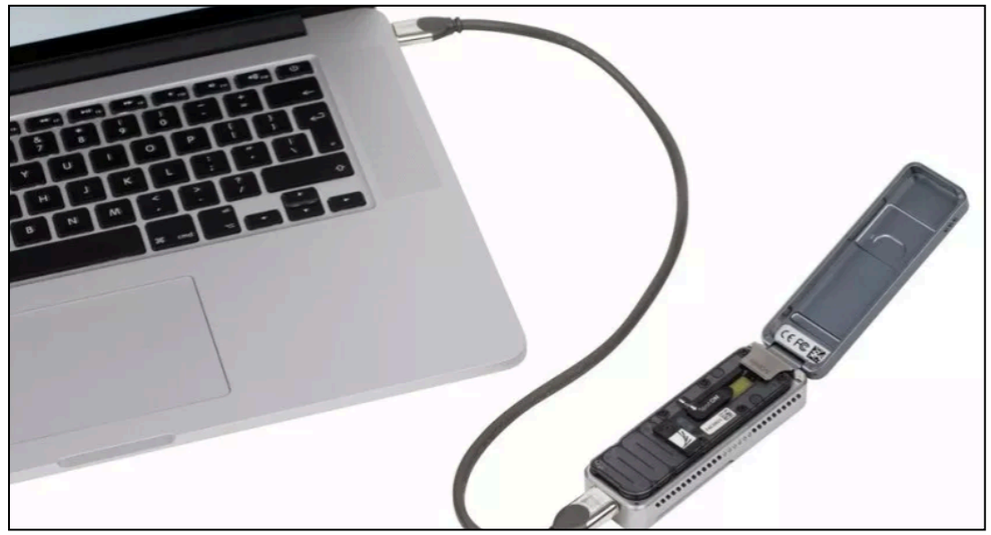






- Electrical current based detection of nucleotides in the pore
- Variable read lengths
- Error rate: 5-15%

Oxford Nanopore Sequencing Device



- Handheld
- Low power
- Low capital cost ($1000)

NGS vs. Single-Molecule Sequencing

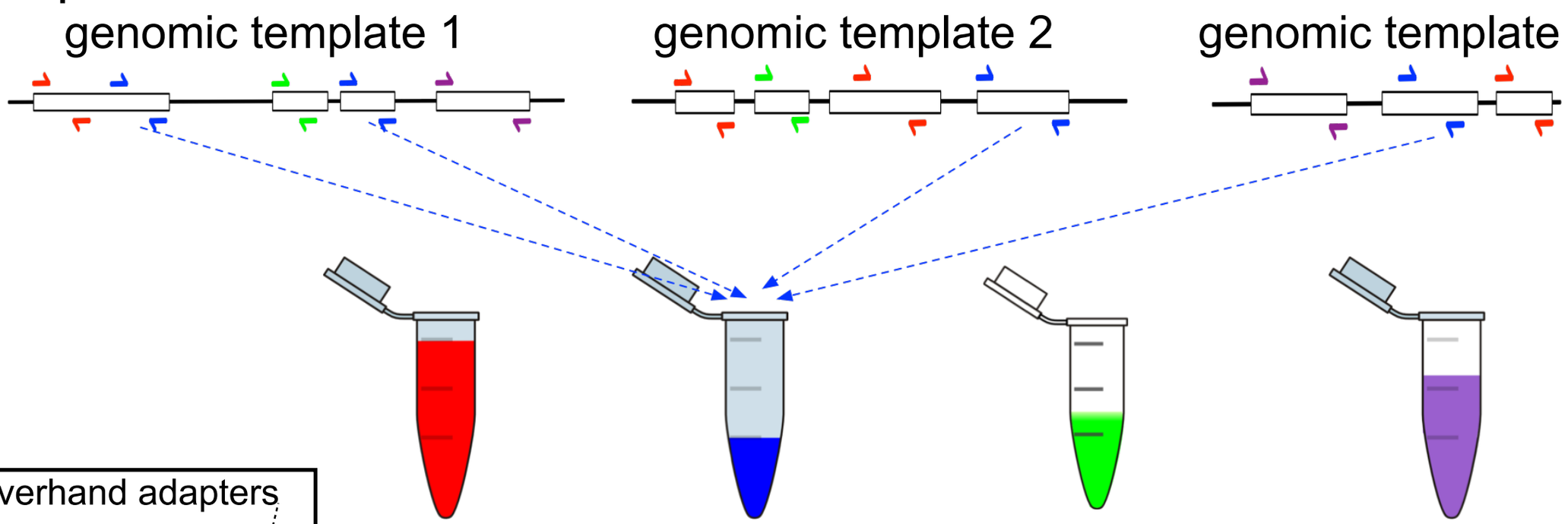
	Next-generation	Single Molecule
Amplification	needed	none
Cost (startup)	high	low
Cost (per bp)	low	high
Run Time	hours (IonTorrent)-days (Illumina)	hours
Read Length	short (<400bp)	long
Error Rate	low	high

Targeted Enrichment and Sequencing

- **Hybridization-based**
- **PCR-based**

Multiplex PCR Panels

12 primer pairs

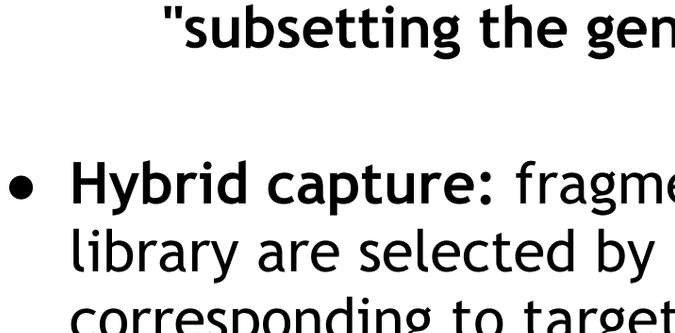


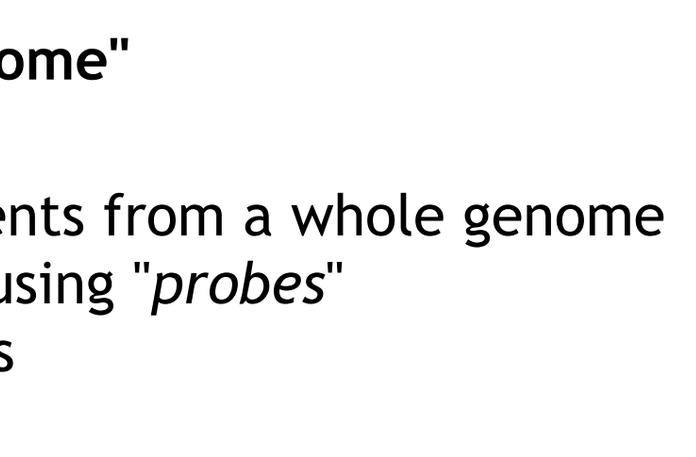
- Design primer pairs for targets, longer regions might need tiled primers
- Group primer pairs according to GC content, Tm and reaction condition specifics
- Amplify genomic DNA to generate multiple products from each primer set, pool products
- Create sequencing library by ligation or tail platform specific adaptors on the primer ends
- SEQUENCE

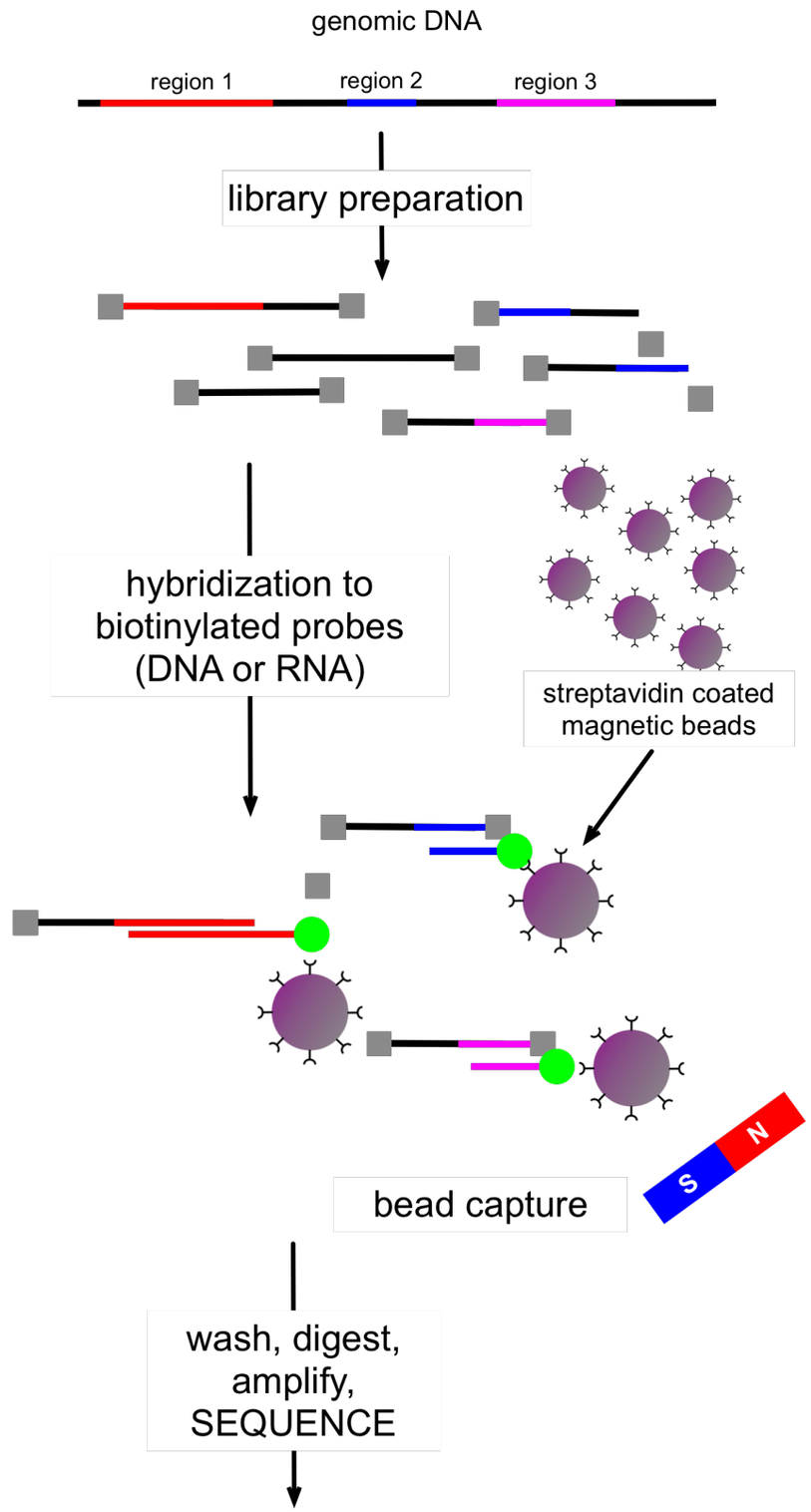
Hybrid Capture (since ~2010)

"subsetting the genome"

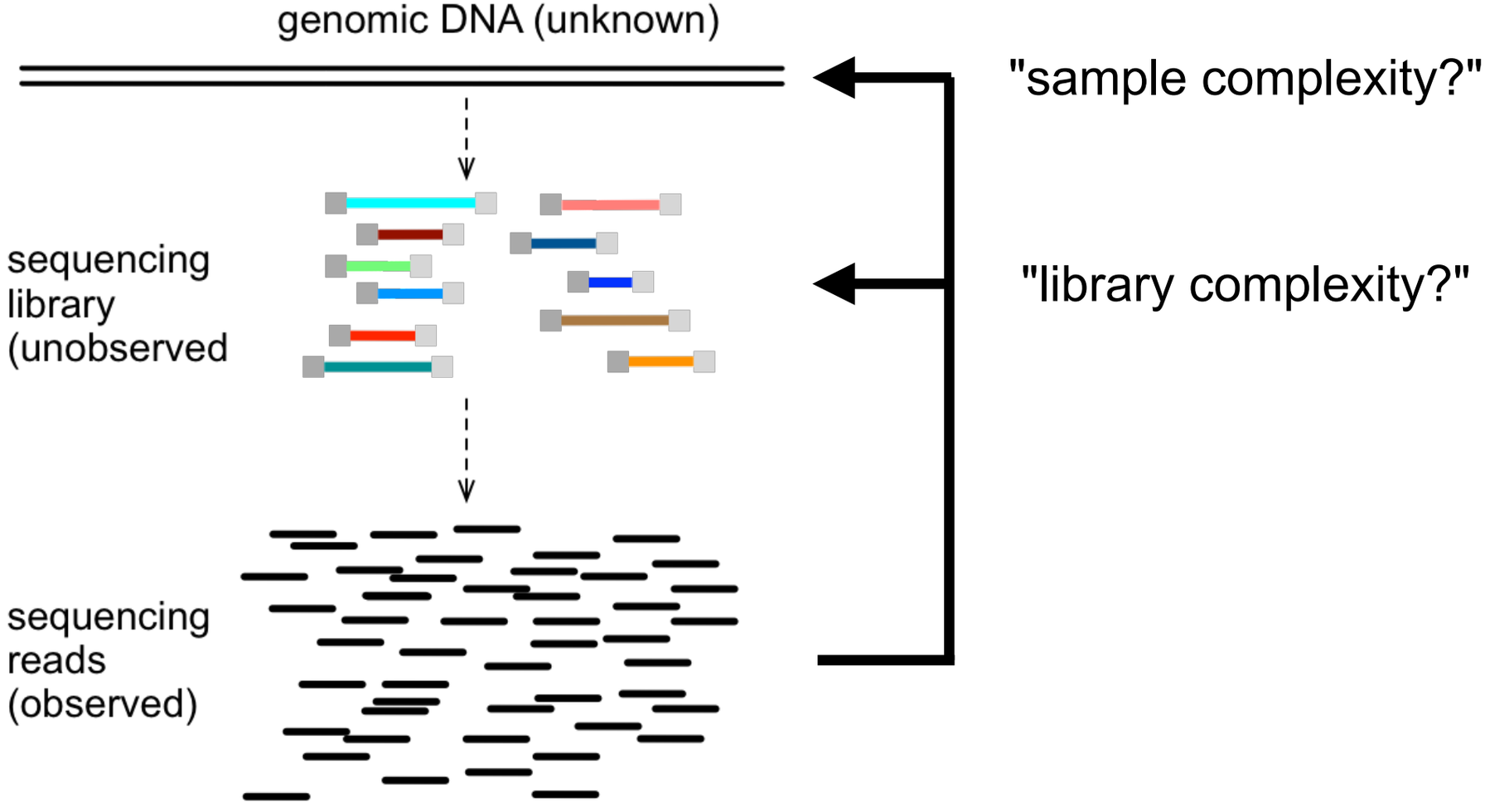
- **Hybrid capture:** fragments from a whole genome library are selected by using "*probes*" corresponding to targets
- DNA-DNA or DNA-RNA
- Probes are biotinylated, enabling selection from solution with streptavidin magnetic beads
- below 3-4 Mb of target sequence, target capture sequencing is not efficient, off-target effects etc.



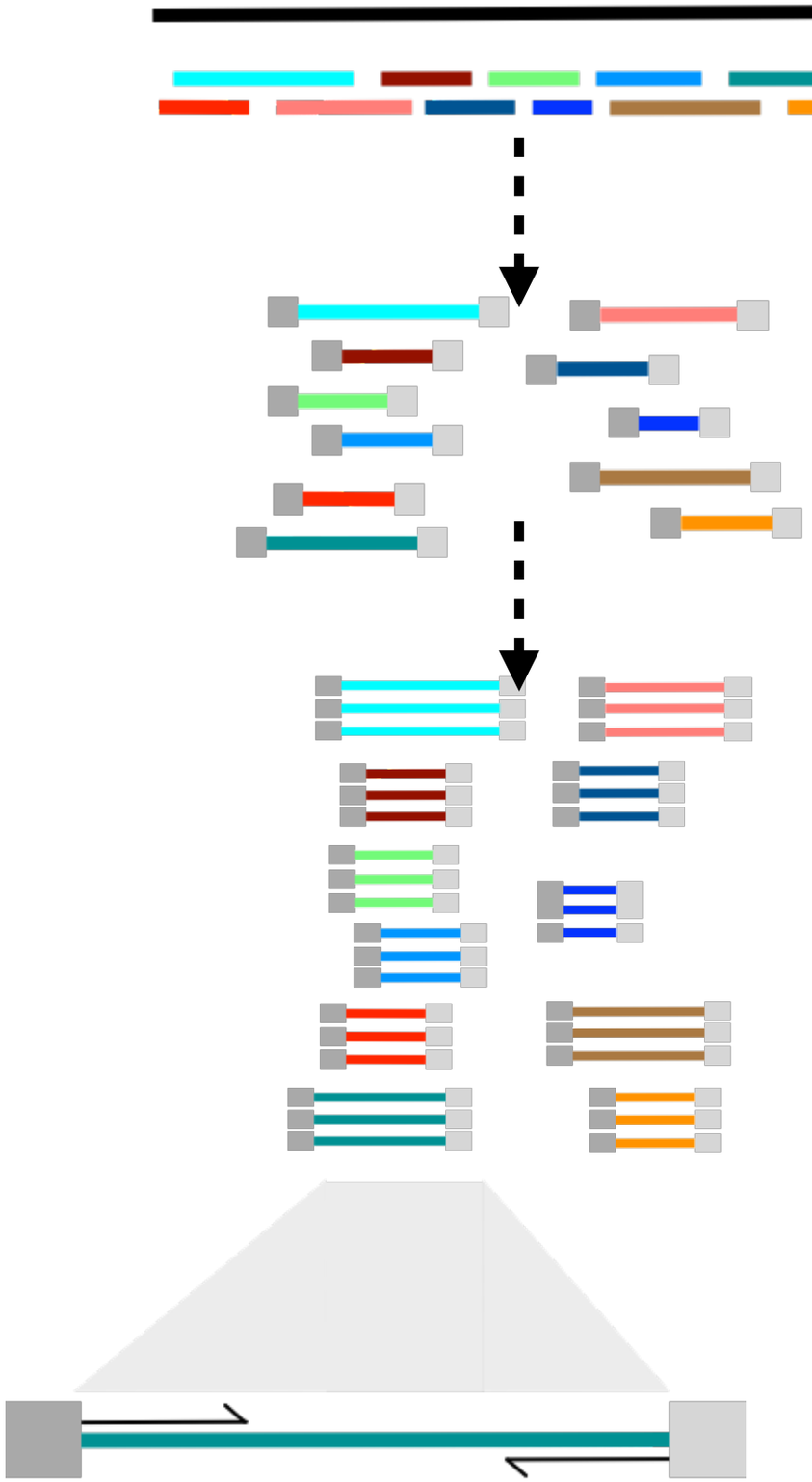




Library Complexity



Library Complexity



library complexity=11

Modeling Library Complexity: Poisson Model

Sequencing a library is *sampling* from it:estimate complexity from sequencing data

C: library complexity, sequence diversity of molecules, #types of molecules

N: number of reads we have

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- We don't see what we donot sequence: use a truncated Poisson-we only observe events that happened between a and b times

Truncated Poisson distribution:

$$Poisson(x_i \mid \lambda) = \frac{1}{K_{a,b}(\lambda)} \cdot \frac{e^{-\lambda} \cdot \lambda^{x_i}}{x_i!}; K_{a,b}(\lambda) = \sum_{x=a}^{b} P(x_i \mid \lambda)$$

Cohen et al, Estimating parameters in a conditional Poisson distribution. JASA 1960

Modeling Library Complexity: Poisson Model

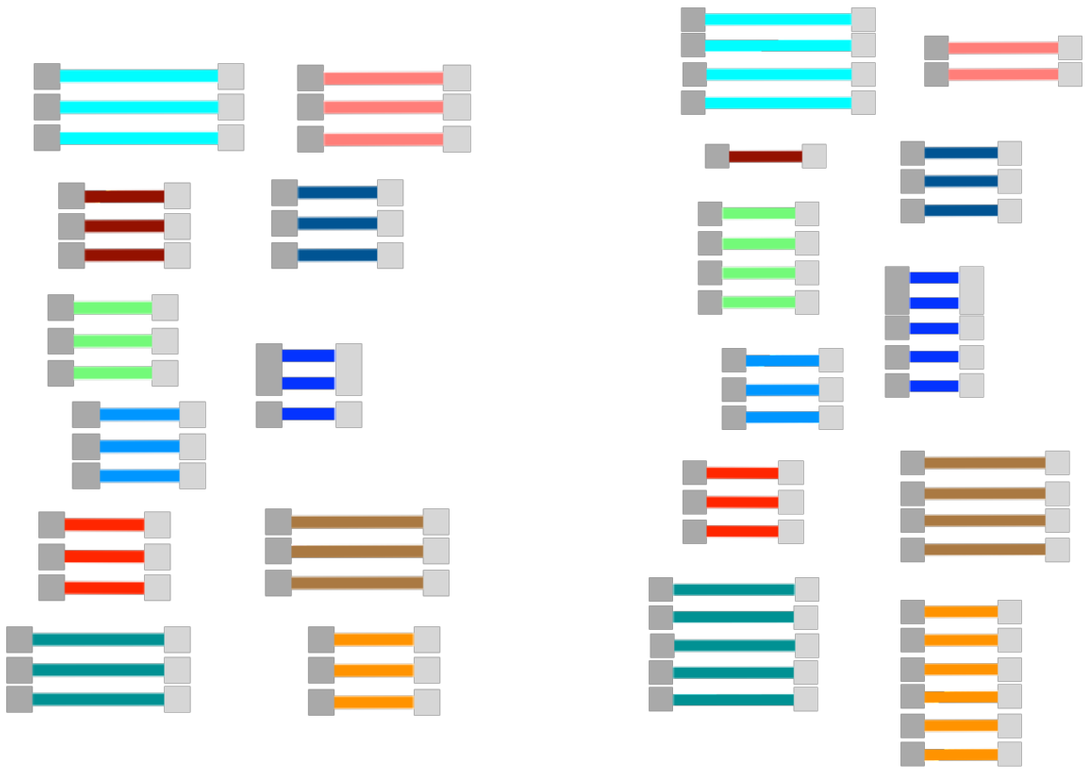
- Maximum Likelihood estimate of the library size:

$$\hat{C} = \frac{M}{K_{a,b}(\lambda)} \approx \frac{M}{1 - Poisson(0,\lambda)}$$

where M is the number of unique sequences

- Poisson model underestimates library complexity: non-uniformity in the original population
  - PCR bias
  - repeats...
  - Poisson:

$$mean = variance = \lambda$$



Modeling Library Complexity: Negative Binomial Model

$$Poisson(x;\lambda) = \frac{e^{-\lambda} \cdot \lambda^{x}}{x!}$$ ← Poisson sampling models sequencing

$$Gamma(x;\alpha,\beta) = \frac{\beta^{\alpha} x^{\alpha-1} e^{-\beta x}}{\Gamma(\alpha)}$$ ← models the entire population (library preparation)

Gamma distribution is a conjugate prior for Poisson:

$$NegBinomial(y;\alpha,\beta) = \int_{0}^{\infty} Poisson(y;x) Gamma(x;\alpha,\beta) \cdot dx$$

Complexity Estimate using Negative Binomial Model

$$P(x_i \mid \lambda, k) = NegBinomial(x_i \mid \lambda, k)$$

dispersion, sampling rate variance (latent variable)

$$= NegBinomial(x_i \mid n, p)$$

$$n = \frac{1}{k} \qquad p = \frac{\lambda}{(\lambda + 1/k)}$$

C: library complexity

N: number of reads we have

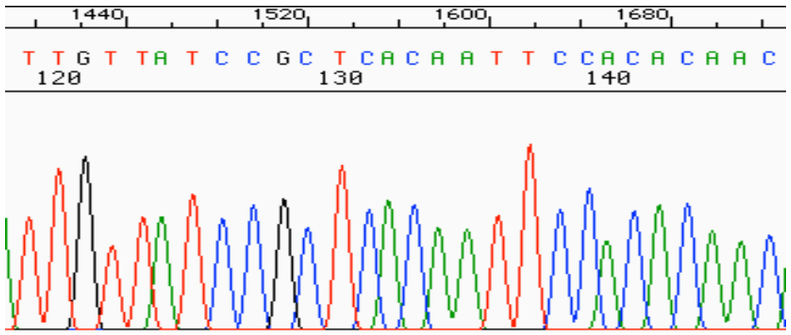
M: number of unique sequences

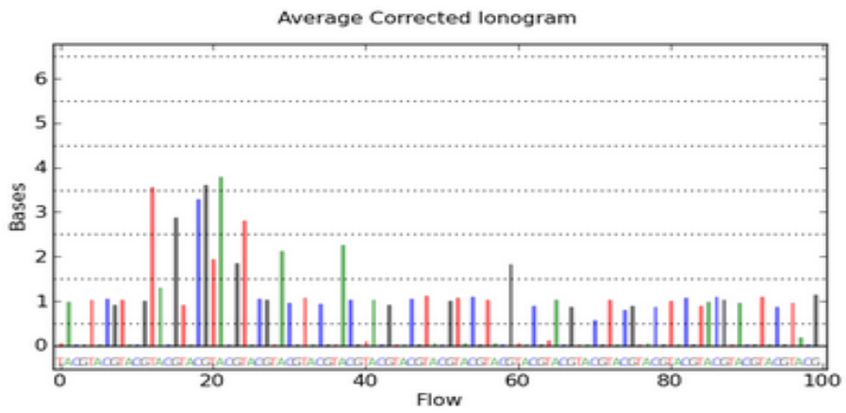
$$\text{M: } (1 - NegBinomial(0 \mid \lambda, k)) * C$$

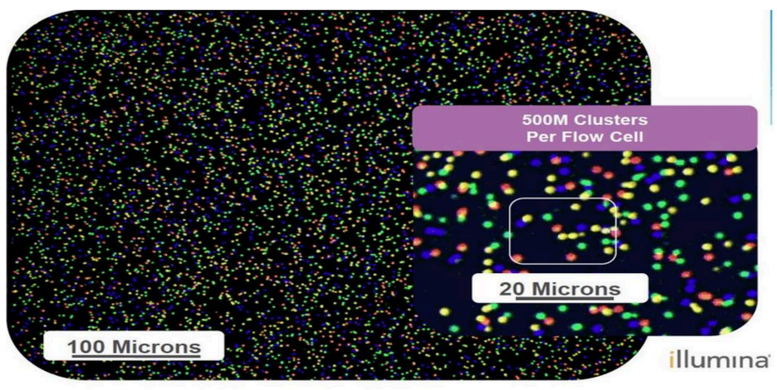
Sequencing Data Aspects

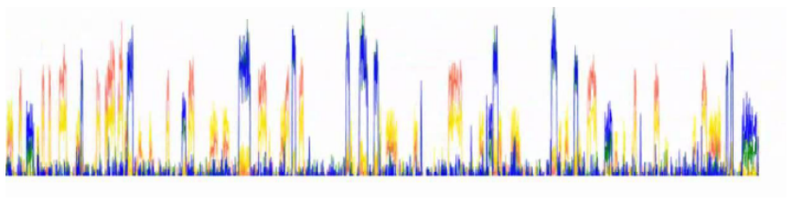
Basecalling

How do we translate the machine output to base calls?









...TGTAGCAGAGAAGACGCCTACTGAATT...

How do we quantify base quality?

FASTQ format & base qualities

```
@J00113:349:HMJHHBBXX:2:1101:12165:4110 1:N:0:TCACTCGA+TCGAGTGA
CTGTCGACCCGCGGAAAGCTTTGGAGCTACACCGAAAACCGGTACGCCCCGCCACCGCCGTAC
+
?AF<FJFJJJJFJJJJJJJJJJJJJAFJJJFJJJJFJJJJFJJJJJJJJAJJJJJJJFJJJJJJ
```

? = ASCII code 63

Base quality = ASCII code - 33 = 30

base quality	P_{error}
3	50%
5	32%
10	10%
20	1%
30	0.1%
40	0.01%

$$Q_{phred} = -10 \log_{10}(P_{error})$$

Error rates

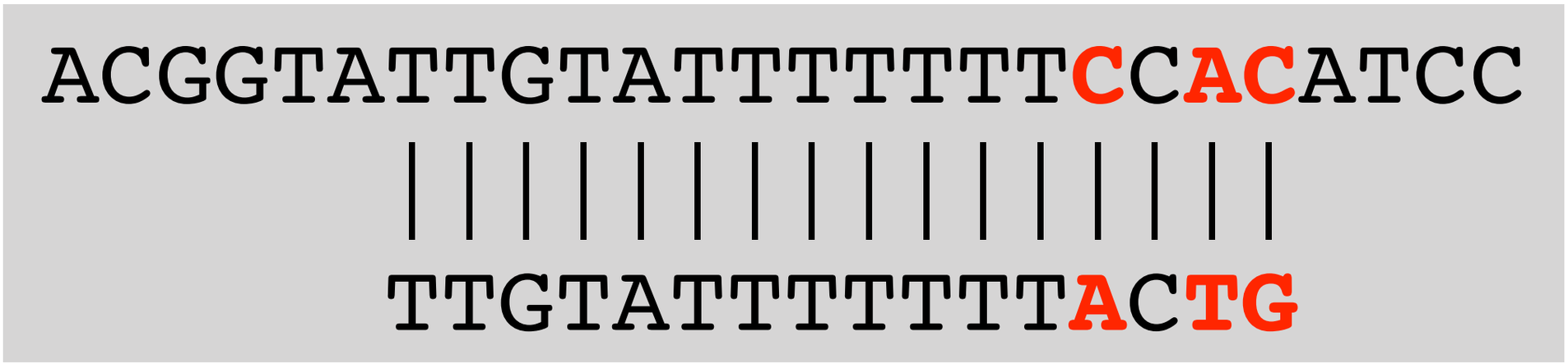
	Primary Errors	Single-pass Error Rate (%)	Final Error Rate (%)
3730xl (capillary)	substitution	0.1-1	0.1-1
Illumina	substitution	~0.1	~0.1
PacBio RS	indel	~13	<=1
Oxford Nanopore	deletions	>=4	4
Ion Torrent	indel	~1	~1

http://www.molecularecologist.com/next-gen-table-3c-2014/

Challenges: Sequencing by Synthesis

illumina®

Sequencing errors tend to be more prominent at the end of the reads



sequencing errors

Challenges: Single Molecule

PACBIO®

dominated by *indel* errors

reference

```
ACGGTATTGTATTTTTTTCCACATCC
|||||||||||||||||||||||||
TTGTAT-TT-TTCC-C
```

deletion errors

Challenges: Ion Semiconductor Sequencing

ion torrent
by Thermo Fisher Scientific

difficult to estimate the length of long homopolymers

homopolymer run

reference

```
ACGGTATTGTATTTTTTTTCCACATCC
||||||||||||||||||||||||||
TT--ATTTTT--CCAC
```

gap1 gap2

Take Home: Planning Experiments

Considerations

- **Number of biological replicates needed**
  - biological variability & technical "noise"
  - sequencing depth (effect size: will I see differences at this coverage?)
  - sample heterogeneity
- **Sequencing decisions (every company will tell you they have the greatest technology)**
  - coverage, coverage, coverage!: number of reads/sample (->sequencing depth)
  - read length
  - base-level quality: get your money's worth
  - paired end vs single end
  - be attentive about batch effects
  - consider library complexity

Sequencing experiment starts before sequencing:
PLAN, THINK, REVIEW and PLAN again