CH-413 Nanobiotechnology

Next-Generation Sequencing (NGS)

Angela Steinauer

April 3, 2025

The Human Genome Project

Venter Collins

"Bill Clinton wanted biotech entrepreneur Craig Venter (left) and Francis Collins (centre) of the US National Institutes of Health to patch up their differences." Credit: Ron Sachs/Shutterstock

https://www.nature.com/articles/d41586-020-01849-w

Start: 1990

Completed: April 2003

Goal:

Sequencing of all 3 billion (3×10^9) base pairs of human genome

Competition between private (Celera) and public projects

Recommended resources:

https://www.genome.gov/human-genome-project

https://www.nature.com/articles/464649a

The Human Genome Project

What about the naysayers who asked, "Where are the cures for diseases that we were promised?"

I became director of this institute three and a half years ago, and I remember when I first started going around and giving talks. Routinely I would hear: "You are seven years into this. Where are the wins? Where are the successes?"

I don't hear that as much anymore. I think what's happening, and it has happened in the last three years in particular, is just the sheer aggregate number of the success stories. The drumbeat of these successes is finally winning people over.

We are understanding cancer and rare genetic diseases. There are incredible stories now where we are able to draw blood from a pregnant woman and analyze the DNA of her unborn child.

Increasingly, we have more informed ways of prescribing medicine because we first do a genetic test. We can use microbial DNA to trace disease outbreaks in a matter of hours.

These are just game changers. It's a wide field of accomplishment, and there is a logical story to be told.

A version of this article appears in print on April 16, 2013, Section D, Page 3 of the New York edition with the headline: Human Genome, Then and Now. Order Reprints | Today's Paper | Subscribe

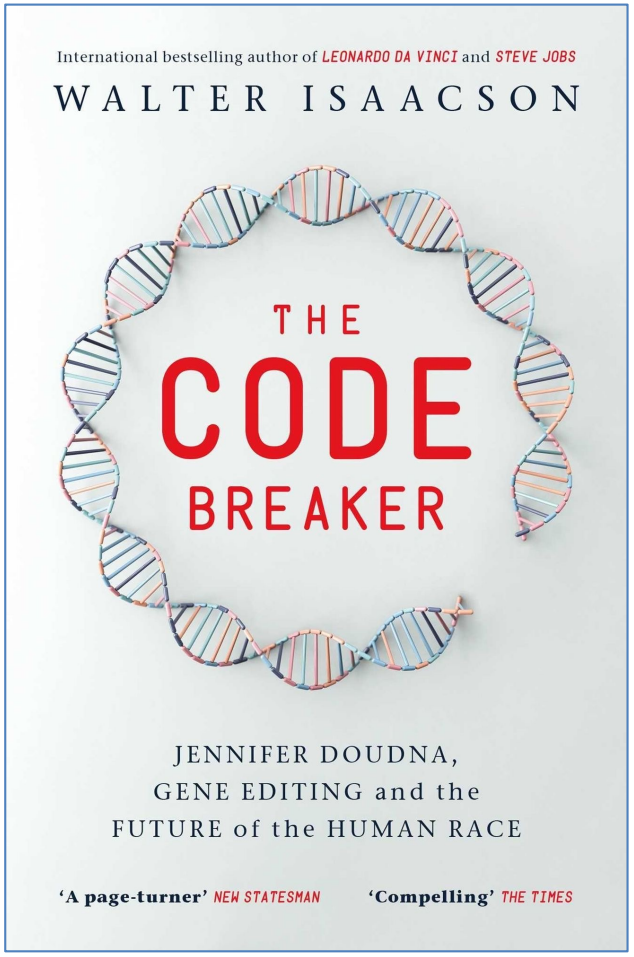
Excerpt from an interview in the *New York Times* with **Eric Green**, then director of the National Human Genome Research Institute at the National Institutes of Health

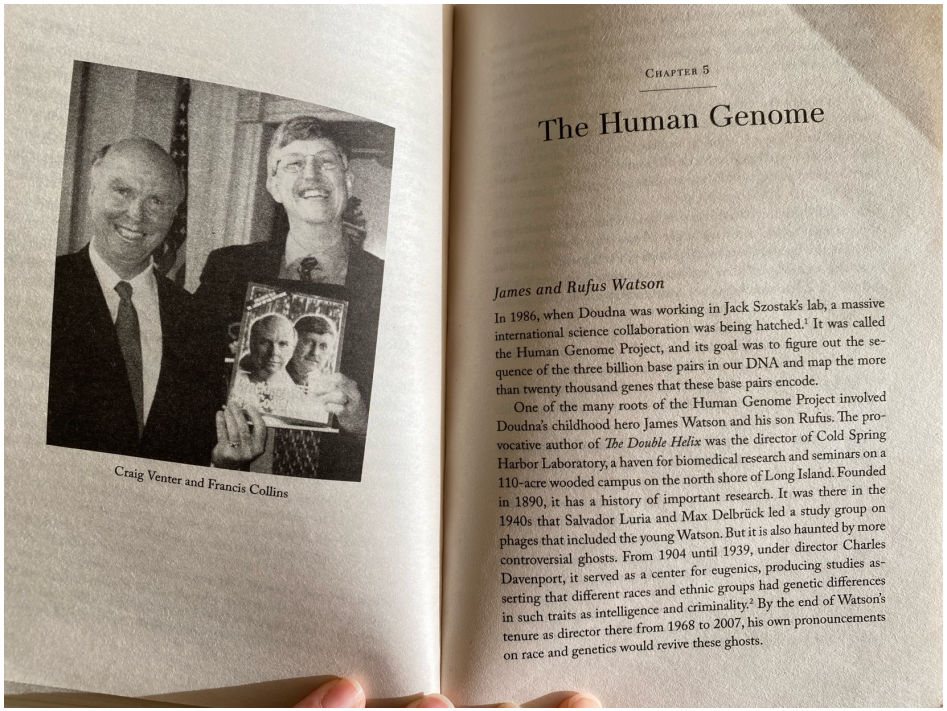
https://www.nytimes.com/2013/04/16/science/the-human-genome-project-then-and-now.html

Impact of the Human Genome Project

Area	Impact
Genomics	Reference genome, gene catalog, understanding of variation
Technology	Rapid drop in sequencing cost, rise of NGS and bioinformatics
Medicine	Precision therapies, diagnostic tests, carrier screening
Science policy	Data-sharing norms, ELSI frameworks, global collaboration
Functional genomics	Mapping of non-coding regions, transcriptomics, epigenetics

Book recommendation





Craig Venter and Francis Collins

CHAPTER 5

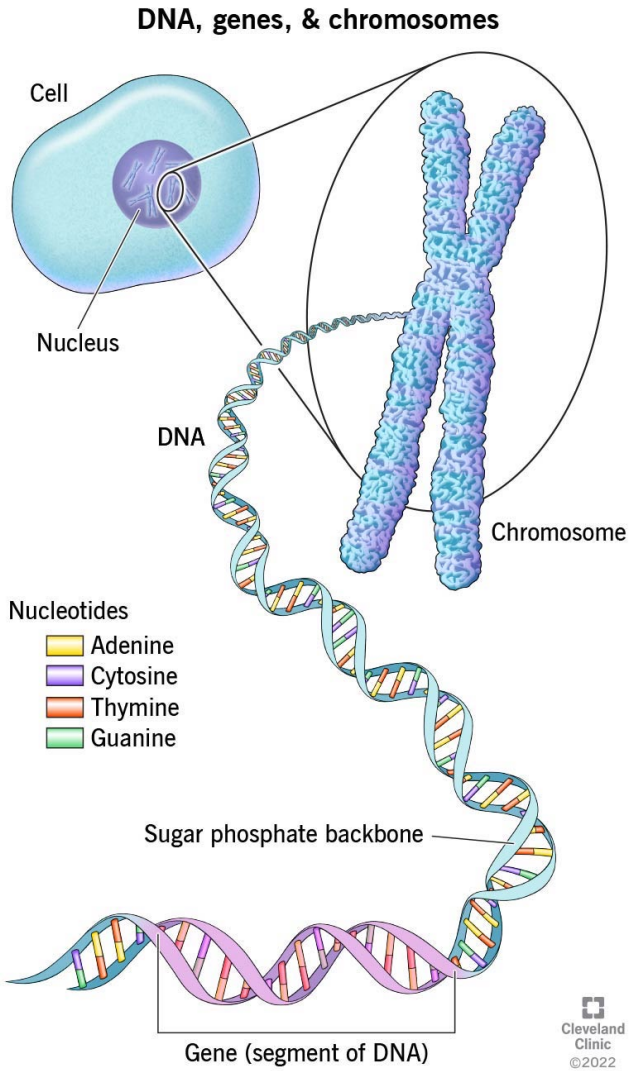
The Human Genome

James and Rufus Watson

In 1986, when Doudna was working in Jack Szostak's lab, a massive international science collaboration was being hatched.[1] It was called the Human Genome Project, and its goal was to figure out the sequence of the three billion base pairs in our DNA and map the more than twenty thousand genes that these base pairs encode.

One of the many roots of the Human Genome Project involved Doudna's childhood hero James Watson and his son Rufus. The provocative author of *The Double Helix* was the director of Cold Spring Harbor Laboratory, a haven for biomedical research and seminars on a 110-acre wooded campus on the north shore of Long Island. Founded in 1890, it has a history of important research. It was there in the 1940s that Salvador Luria and Max Delbrück led a study group on phages that included the young Watson. But it is also haunted by more controversial ghosts. From 1904 until 1939, under director Charles Davenport, it served as a center for eugenics, producing studies asserting that different races and ethnic groups had genetic differences in such traits as intelligence and criminality.[2] By the end of Watson's tenure as director there from 1968 to 2007, his own pronouncements on race and genetics would revive these ghosts.

Motivation: Read the genetic code to understand life itself



DNA:

carrier of genetic information
→ information storage

easily synthesized

can be amplified

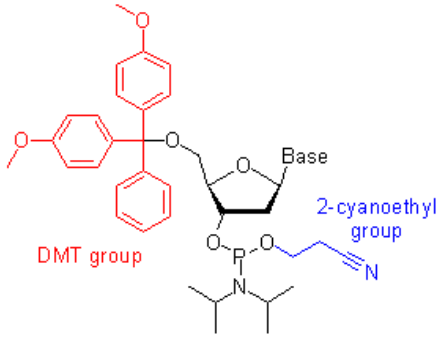
Ideal information medium

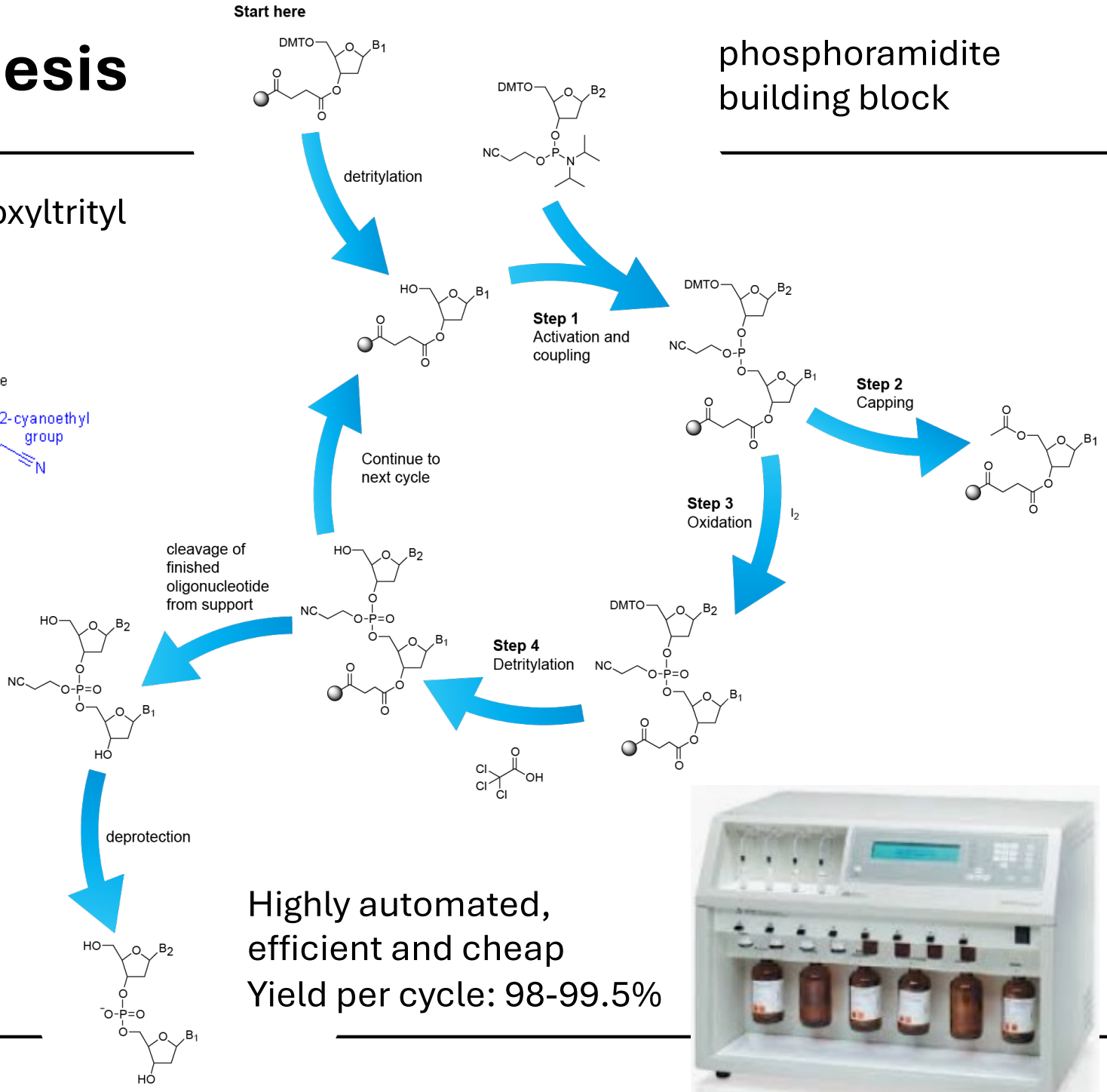
→ However, we need efficient methods to read the molecular information

DNA synthesis

phosphoramidite building block

(DMT) dimethoxyltrityl

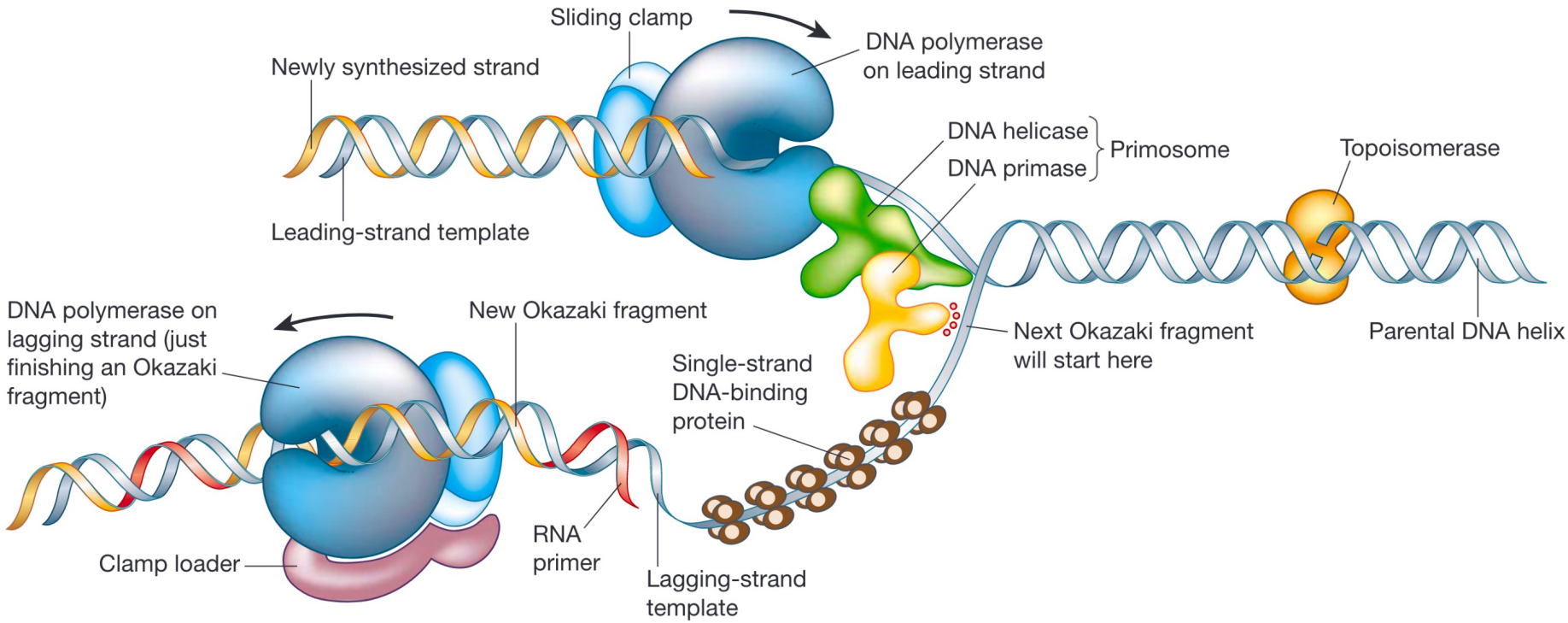




Source: atdbio.com

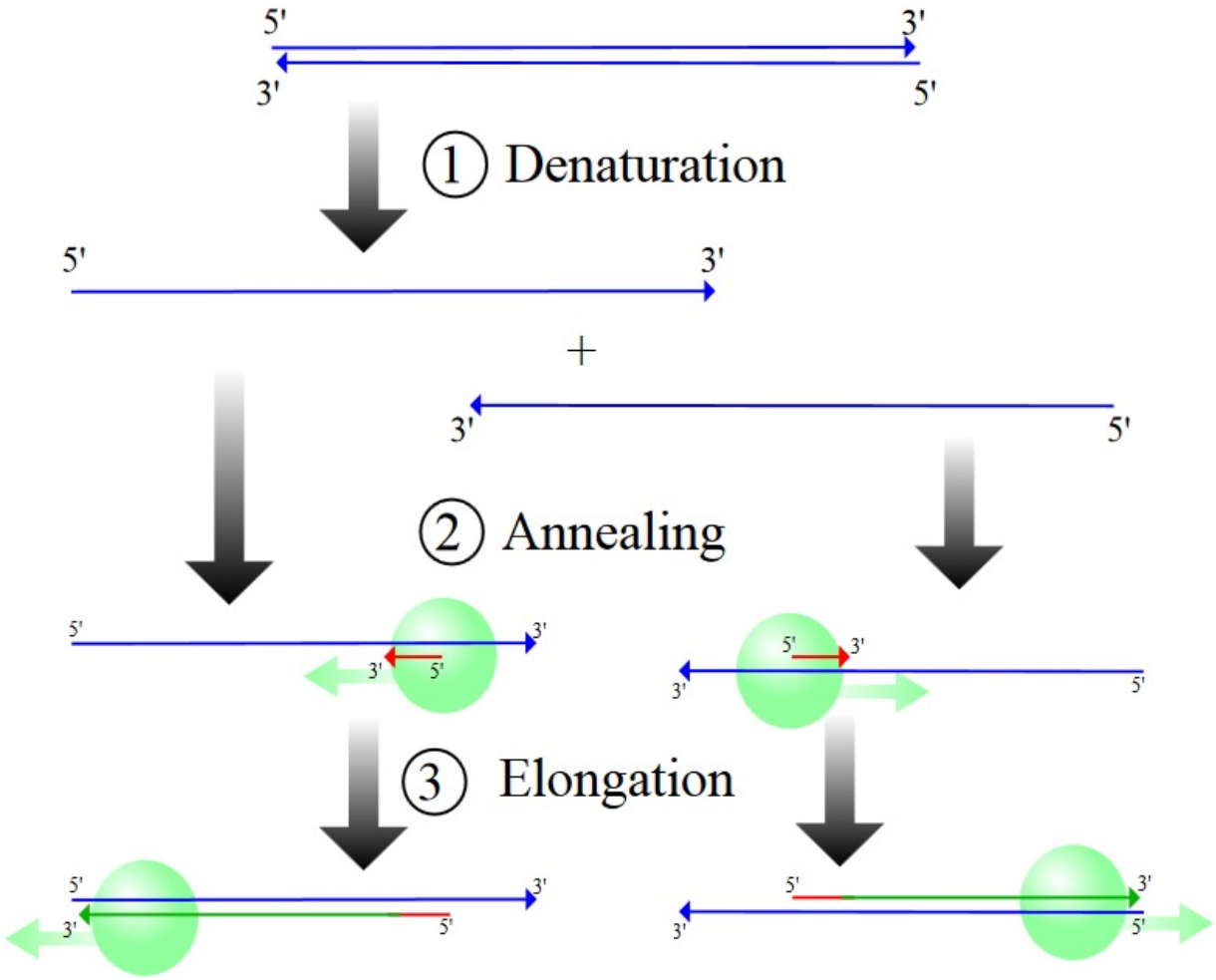
Highly automated, efficient and cheap
Yield per cycle: 98-99.5%

DNA synthesis in vivo: replication



Alberts, Nature 421, 431-453 (2003).

PCR - Polymerase chain reaction



1. dsDNA is denatured by heating to 95 °C
2. Temperature is lowered to 50-65 °C: synthetic primers are annealed to the single-stranded DNA
3. Temperature is increased to 72 °C: DNA is elongated by heat-resistant polymerase
4. Cycle is repeated 20-35 times
5. **Exponential** increase in sequence of choice

First-generation method: Automated Sanger sequencing

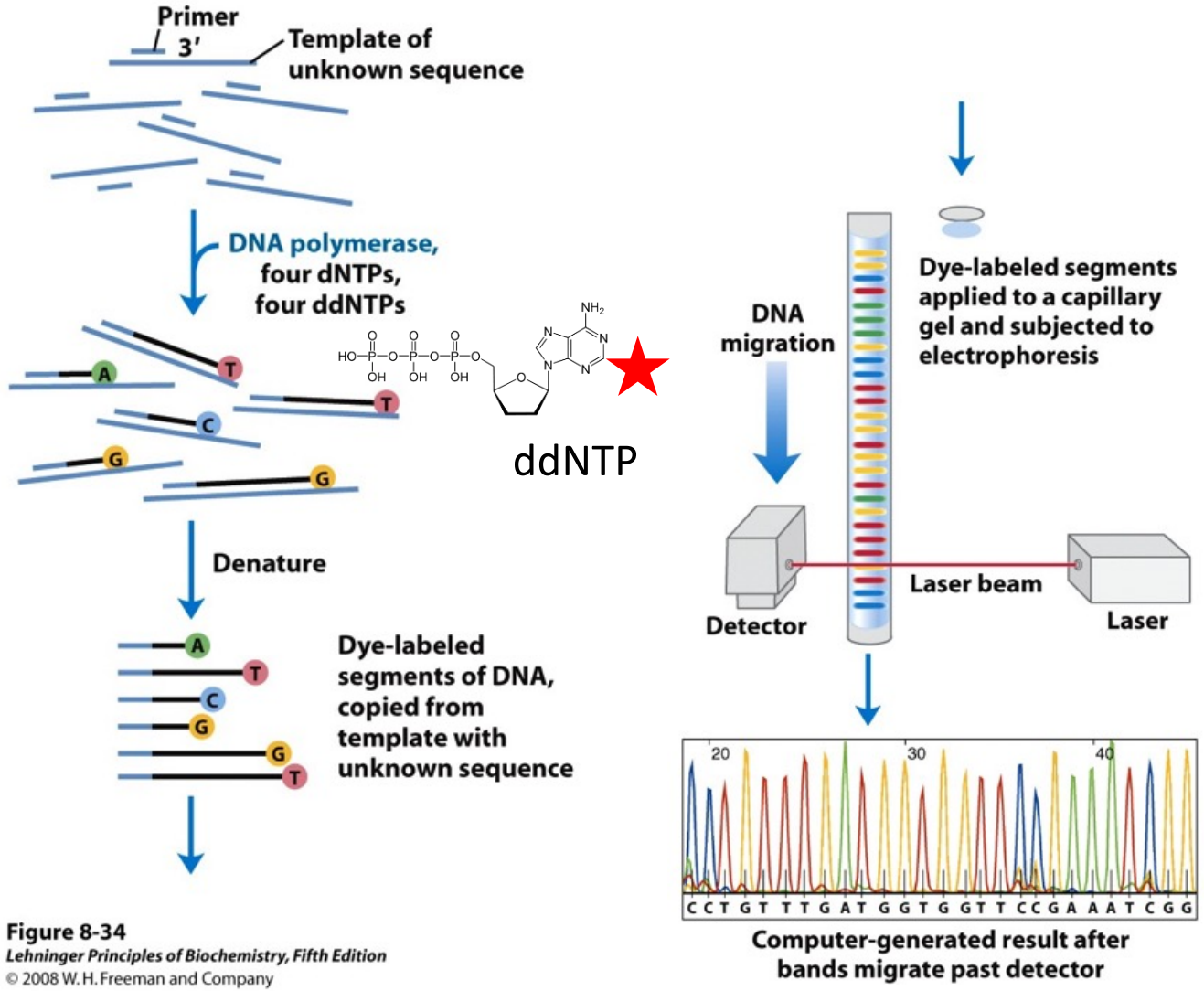


Figure 8-34
Lehninger Principles of Biochemistry, Fifth Edition

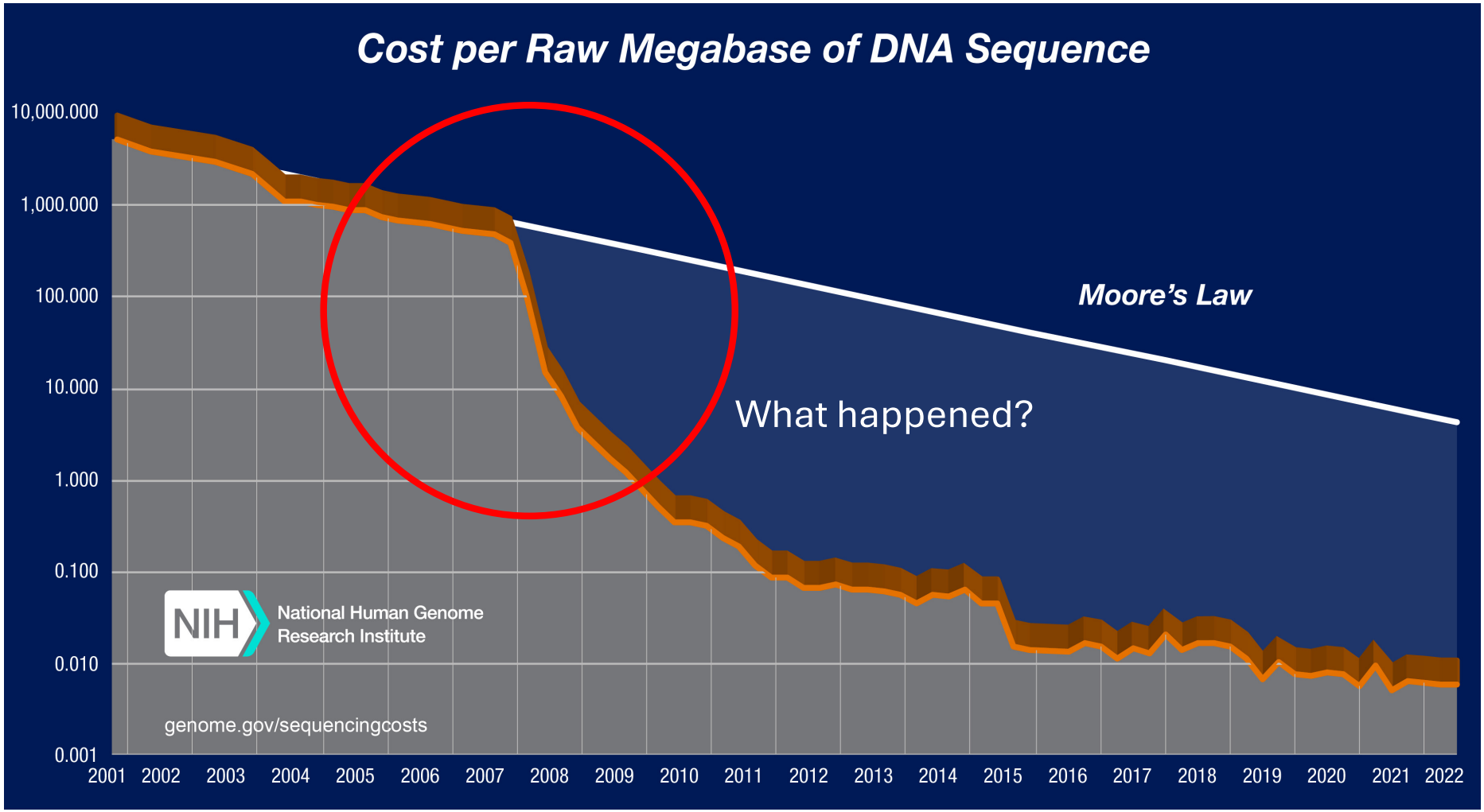
Sequencing is based on PCR reaction

- PCR with mixture dNTPs and **fluorescent dideoxynucleotides (ddNTPs)**
- DNA synthesis stops at ddNTP
- Sequences are separated by capillary electrophoresis (shortest fragments elute first)
- Last nucleotide added is identified by its fluorescence signal
- Sequence (up to a 1000 bp) is reconstructed

Disadvantage

- Slow
- Max 1000 bp read length
- **One DNA fragment per sequencing run**
- Material intensive

Sequencing costs



Next-generation sequencing (NGS) methods

A revolution of DNA analysis:

- Cheap generation of large amounts of data
  - one billion of short reads per instrument run
- Low amount of material required (single-molecule sequencing possible)
- Evolution of bioinformatics methods to assemble genome
- Methods very versatile, not limited to genome sequencing
  - Replacing microarray methods
  - Readout of experiments
  - barcoding

NGS workflow

Library preparation

Amplification

Sequencing

Data analysis

Next generation sequencing

Two fundamentally different approaches:

1. **Clonally amplified templates** originating from a single DNA molecule
2. Directly from a **single DNA molecule**

Range of templates:

- Genomic DNA (randomly broken into fragments of suitable size)
- DNA/RNA fragments from biological assays (ChIP, RNA from cells, etc.)
- DNA from sensor application

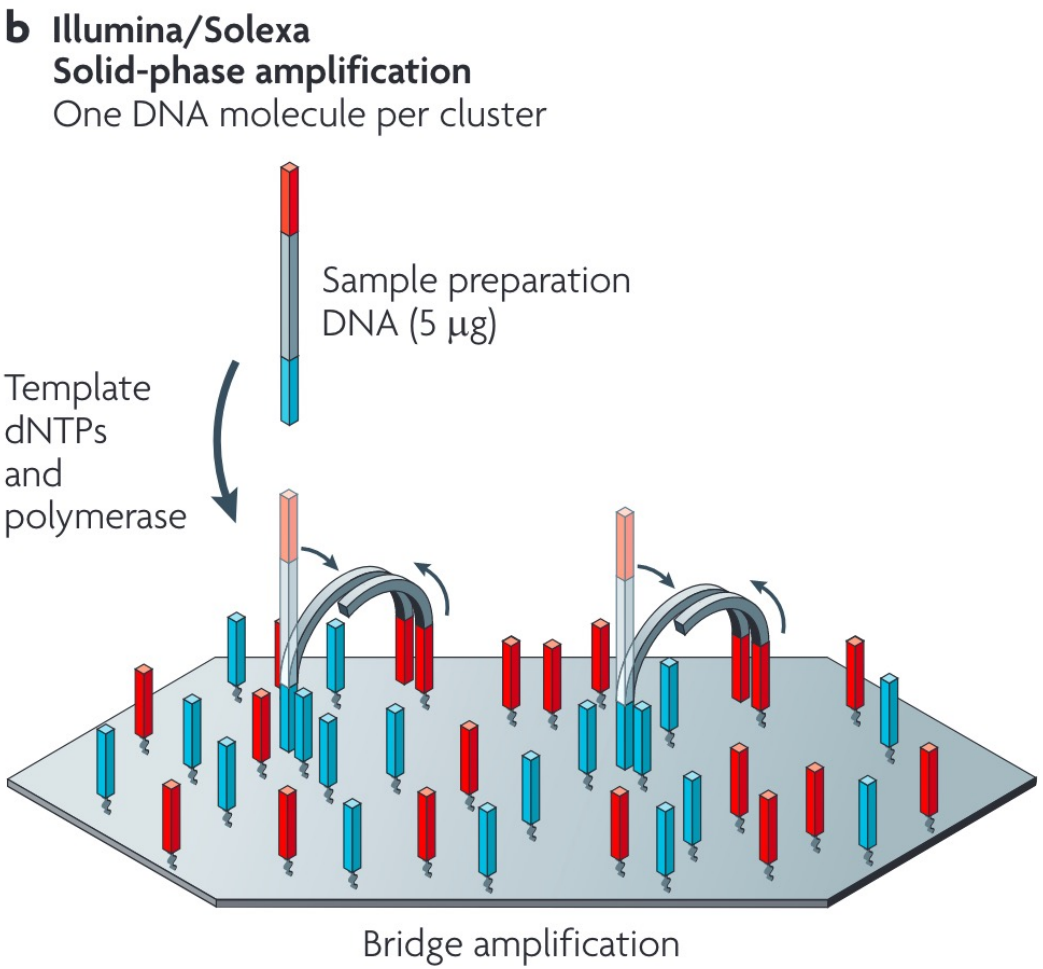
Next-generation sequencing methods

- Roche/454
- Illumina/Solexa
- Life/APG
- Helicos BioSciences
- Polonator
- Pacific BioSciences
- Ion torrent sequencing
- Qiagen GeneReader
- Oxford Nanopore sequencing
- 10X Genomics
- …

Next-generation DNA sequencing

- Introduction
- **Illumina: short-read, sequencing-by-synthesis**
- Helicos: short-read, single-molecule, sequencing-by-synthesis
- PacBio: long-read, single-molecule, real-time sequencing
- Nanopore: long-read, nanopore-based electrical sensing (see last week!)

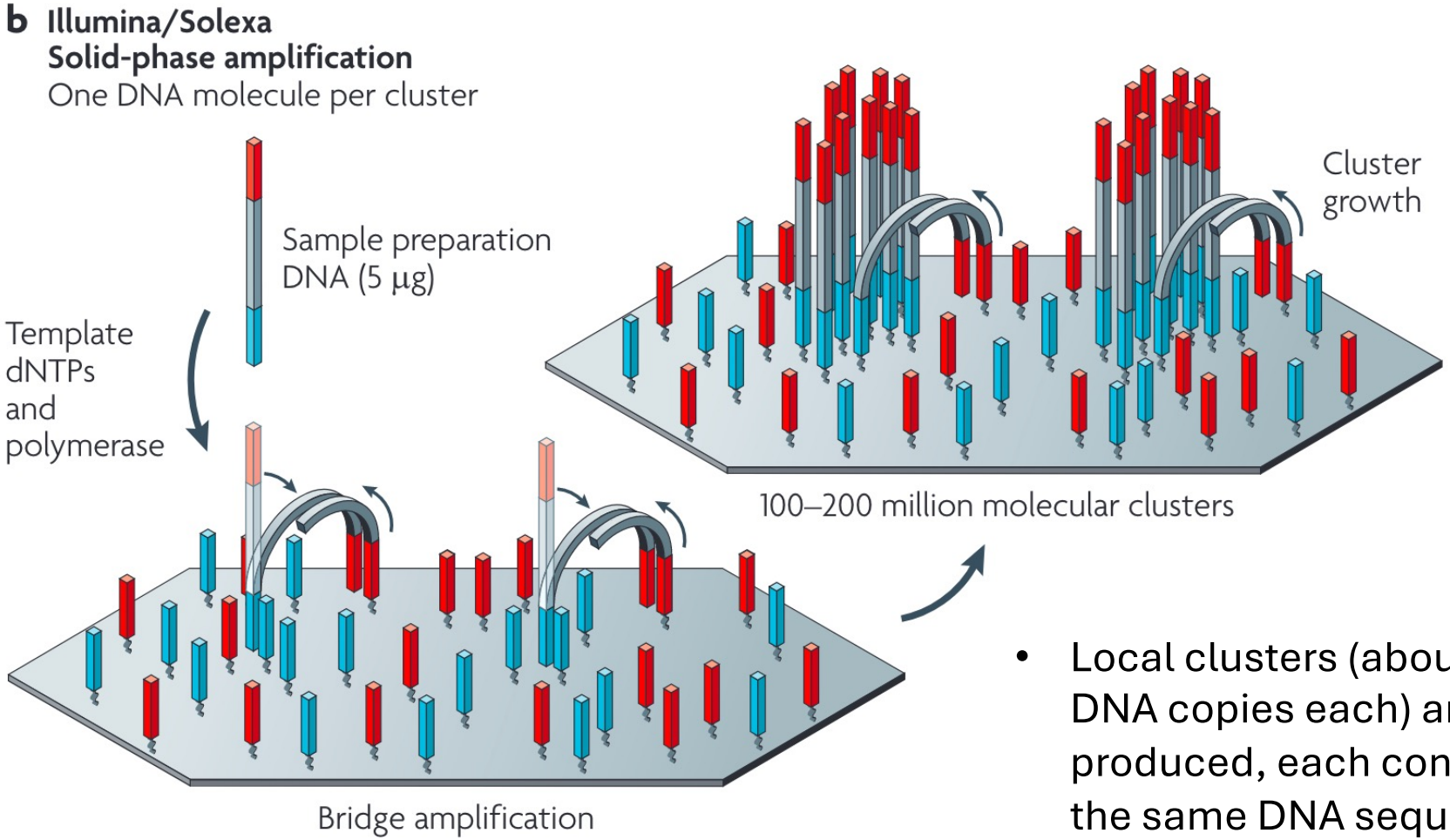
Illumina: Solid-phase amplification



- Short DNA fragments are produced by enzymatic digestion (about 300 bp max) and standardized adapters are ligated
- Adapters contain primer binding sites, flow cell attachment sequence and indices/barcodes
- DNA strands are annealed to solid support
- High-density forward and reverse primers covalently attached (=attachment sequences)
- Ratio of the primers to template defines surface density
- Bridge amplification is employed to produce local enrichment of the same sequence

Metzker et al. Nature Rev Genetics 2010

Illumina/Solexa: Solid-phase amplification

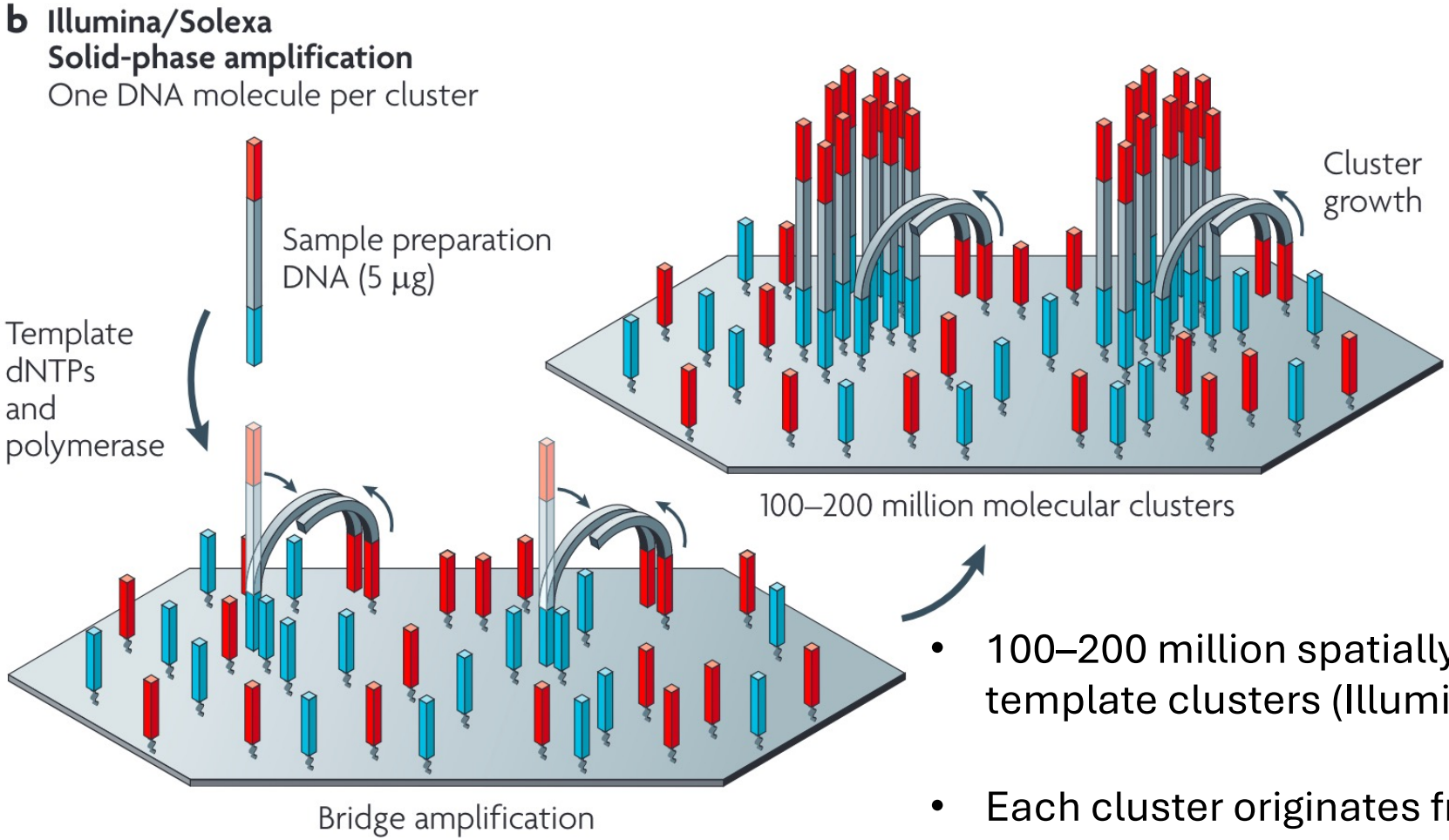


- Local clusters (about 1000 DNA copies each) are produced, each containing the same DNA sequence

→ **Clonal amplification**

Metzker et al. Nature Rev Genetics 2010

Illumina/Solexa: Solid-phase amplification



- 100–200 million spatially separated template clusters (Illumina/Solexa),
- Each cluster originates from a single molecule, and will produce a single, strong signal during sequencing.

Metzker et al. Nature Rev Genetics 2010

Illumina sequencing

- https://www.youtube.com/watch?v=fCd6B5HRaZ8&ab_channel=Illumina

What are index reads? (see video above)

- *Short sequences used as **unique barcodes***
- *Added to each DNA sample before sequencing*
- *Not part of the DNA samples studied, synthetic sequences*

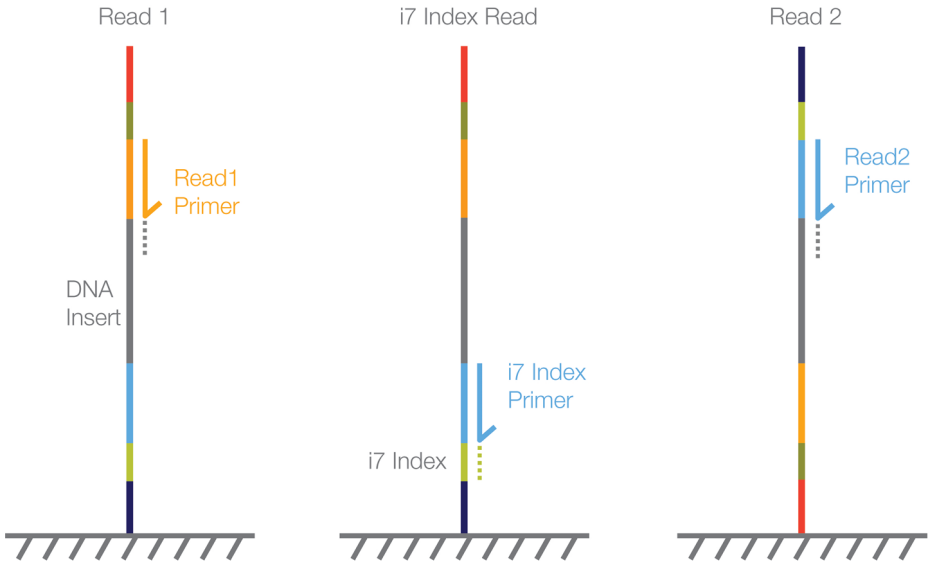
Index 1:

- First barcode sequence added to samples during the library preparation phase
- Read after the first sequencing read (Read 1) for paired-end sequencing

Index 2:

- Second barcode, also added during library preparation
- Used in a different configuration:
- For paired-end sequencing, this index is read after Read 2.

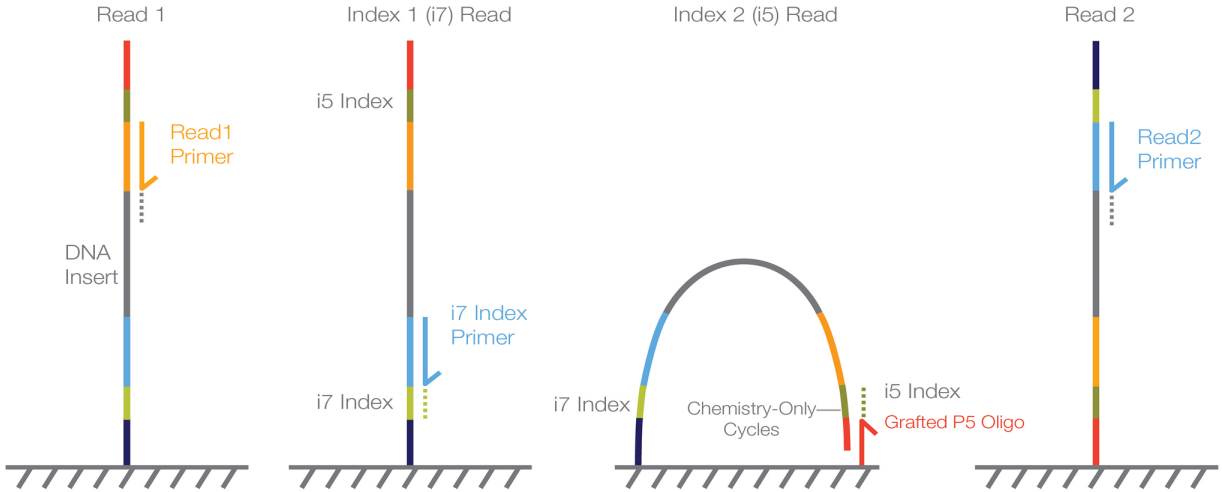
Figure 1 Single-Indexed Sequencing



Source: Illumina “Indexed Sequencing Overview Guide”

Dual-indexed sequencing on a paired-end flow cell

Figure 2 Dual-Indexed Sequencing on a Paired-End Flow Cell (Forward Strand)



1. **Read 1**—Read1 follows the standard Read 1 sequencing protocol using SBS reagents. The Read 1 sequencing primer is annealed to the template strand during the cluster generation step.
2. **Read preparation**—The Read 1 product is removed and the Index 1(i7) sequencing primer is annealed to the same template strand
3. **Index 1 (i7) Read**—Following Index Read preparation, the Index 1(i7) Read performs up to 20 cycles of sequencing. The maximum number of cycles in each Index Read depends on the system and run parameters.
4. **Index 2 (i5) Read**—The Index1(i7) Read product is removed and the template anneals to the grafted P5 primer on the surface of the flow cell. The run proceeds through an additional seven chemistry-only cycles (no imaging occurs), followed by up to 20 cycles of sequencing.
5. **Read 2 resynthesis**—The Index Read product is removed and the original template strand is used to regenerate the complementary strand. The original template strand is then removed to allow hybridization of the Read 2 sequencing primer.
6. **Read 2**—Read 2 follows the standard paired-end sequencing protocol using SBS reagents.

Source: Illumina “Indexed Sequencing Overview Guide”

Activity: Index reads

- Take a look at the figure in the previous slide
- Go through the process by yourself, do you have questions about the index read workflow? (3 min)
- Find someone in the room you haven’t talked to yet: Help each other answer your remaining questions.
- Discuss: what are the benefits of using 1 vs. 2 index reads?

Benefits of using index 1 and 2

- **Increased Multiplexing:** Using two indices allows for a higher degree of sample multiplexing within a single sequencing run, reducing costs and increasing throughput.
- **Error Reduction:** Dual indexing reduces the risk of sample misassignment and cross-contamination, providing more accurate sequencing results.
- **Flexibility:** Researchers can customize the combination of Index 1 and Index 2 to fit the scale and needs of their project.

Overall, Index 1 and Index 2 reads are fundamental for efficient, accurate, and high-throughput sequencing in the Illumina platform, enabling the simultaneous processing of multiple samples and ensuring data integrity.

Clonal amplification sequencing: Advantages and disadvantages

Advantages

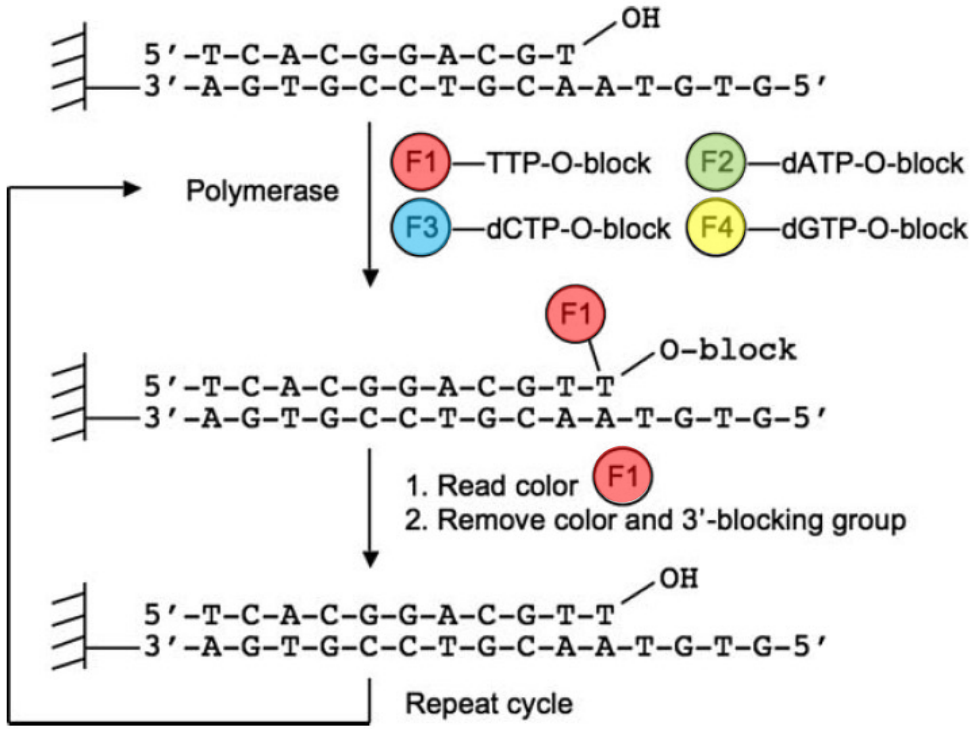
- Improved signal strengths (exponential signal increase)
- Higher accuracy and base-calling confidence
- Error correction via redundancy
- Multiplexing and high throughput
- No lost sequences

Disadvantages

- PCR can introduce mutations, multiple coverage required
- Amplification bias due to sequence (AT and GC rich)
- Could lead to dropout of rare species
- Not compatible with long-read sequencing

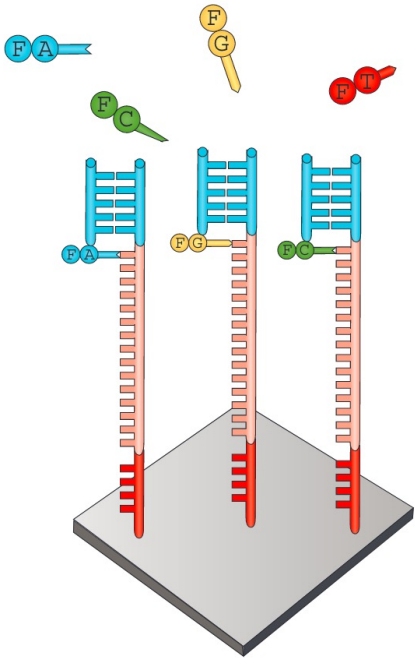
Sequencing chemistry (Illumina/Solexa): Cyclic reversible termination (CRT)

- Addition of a nucleotide
- Wash
- Readout
- Deprotection / cleavage

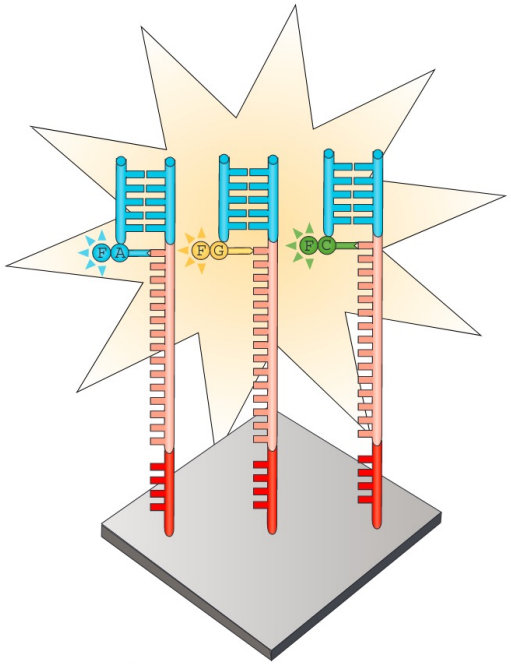


Hutter et al., Nucleosides, Nucleotides and Nucleic Acids, 29:879–895, 2010

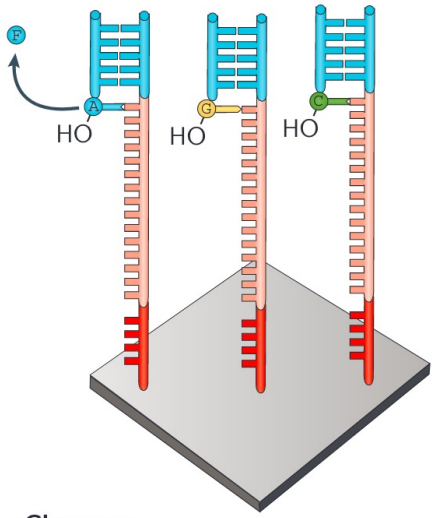
Illumina/Solexa: Sequencing by synthesis



Nucleotide addition
Fluorophore-labelled, terminally blocked nucleotides hybridize to complementary base. Each cluster on a slide can incorporate a different base.



Imaging
Slides are imaged with either two or four laser channels. Each cluster emits a colour corresponding to the base incorporated during this cycle.

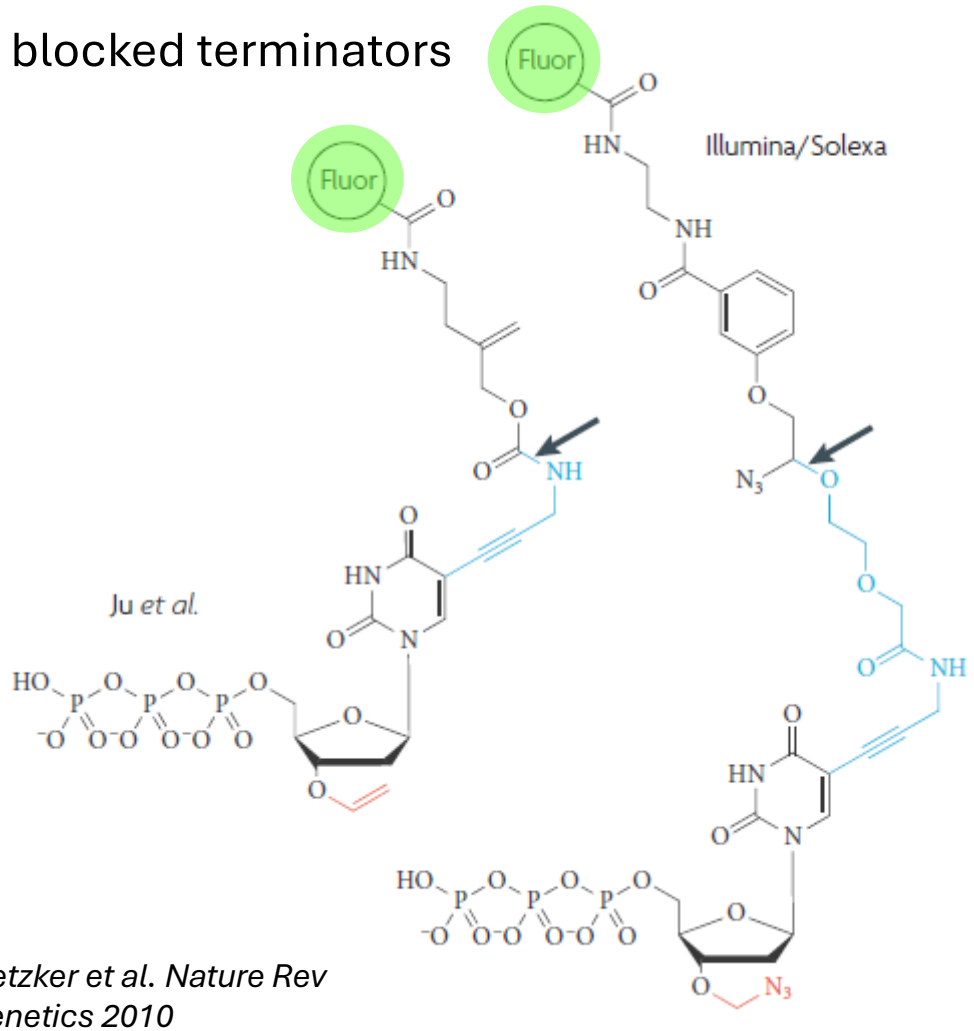


Cleavage
Fluorophores are cleaved and washed from flow cells and the 3′-OH group is regenerated. A new cycle begins with the addition of new nucleotides.

Goodwin et al. Nature Rev Genetics 2016

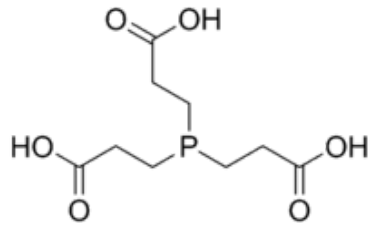
Nucleotides used in CRT

3' blocked terminators



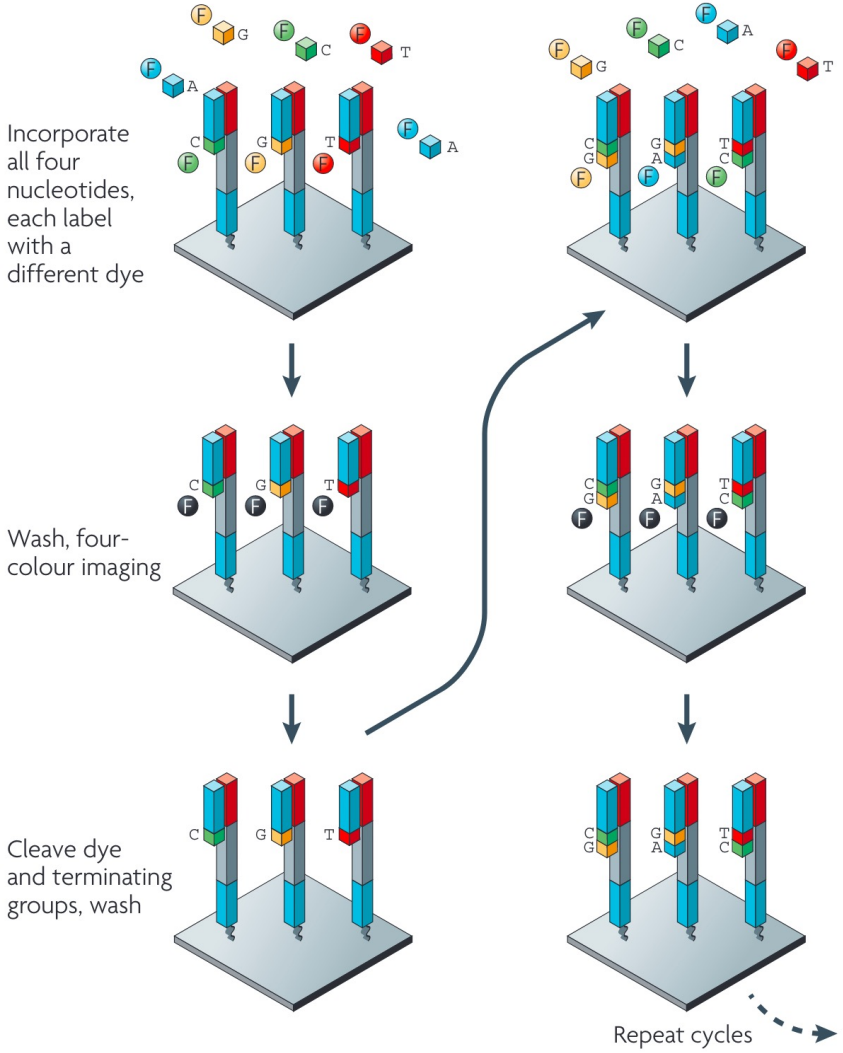
Metzker et al. Nature Rev Genetics 2010

- All protecting groups can be simultaneously cleaved using a reducing agent. Usually, tris(2-carboxyethyl) phosphine **TCEP**, is used

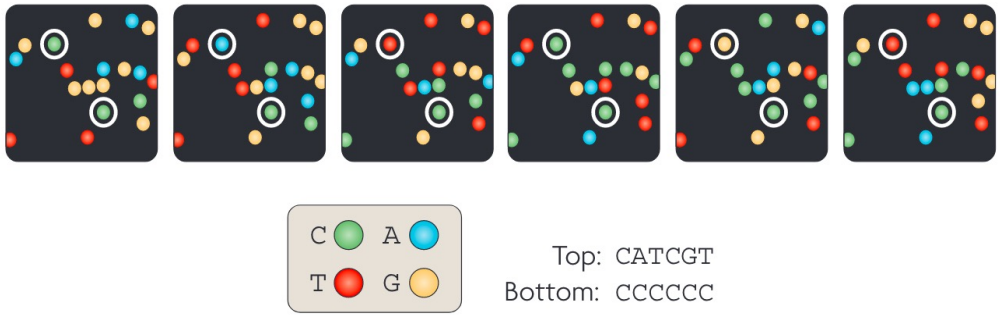


- Cleavable **fluorophore** is used for nucleotide identification
- **Blue**: residual linker/molecular scar
- **Red**: cleavable terminating functional groups
  - *O*-allyl group
  - 3'-azidomethyl group

Illumina/Solexa:
Cyclic reversible termination (CRT)

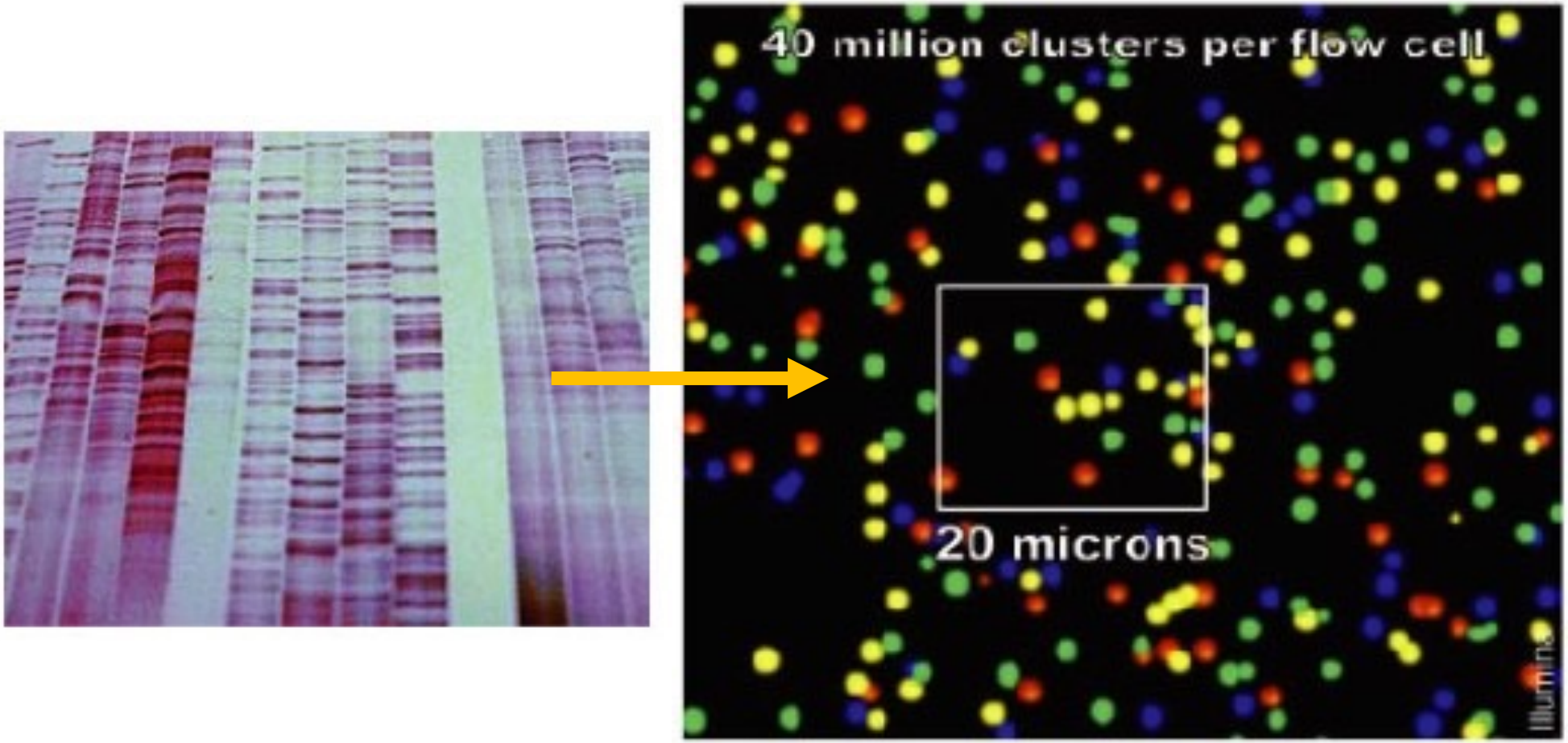


Metzker et al. Nature Rev Genetics 2010



- **Elongation**: Requires modified polymerase (tolerant to modified bases)
- **Deprotection**: Reducing agent, TCEP
- **Readout / imaging**: two-laser **TIRF** imaging of fluorescence emission
- Cycle is repeated until the sequences are determined (max length: 300 bp)

From bands (Sanger) to colorful dots (Illumina)

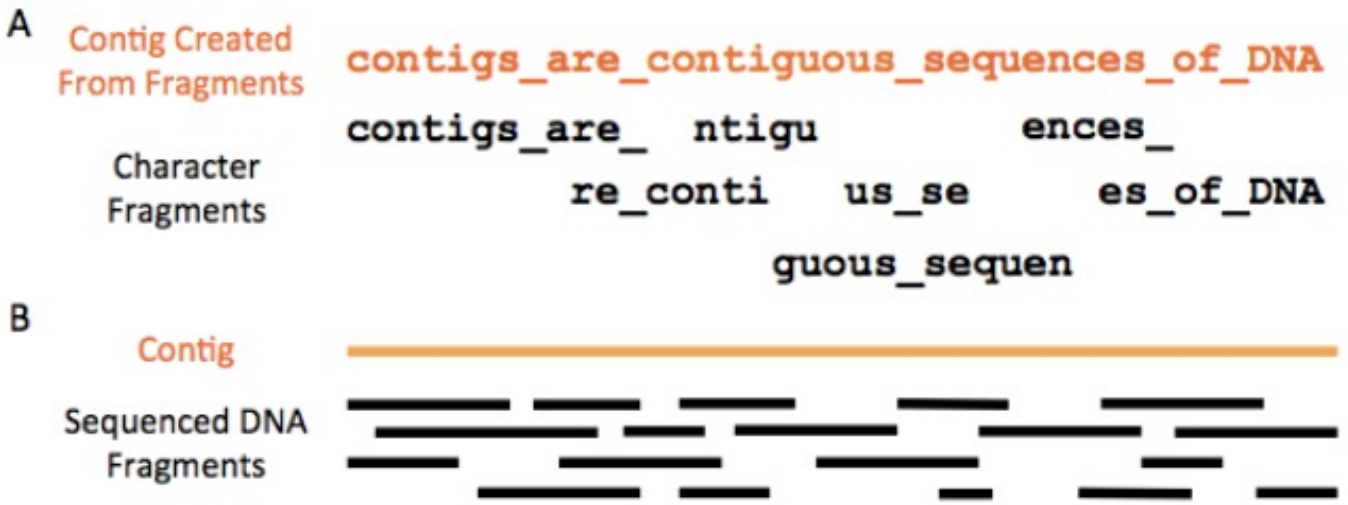


Color dots replace bands as a sequence is read with reversible terminators on a Solexa (Illumina) sequencer.

Image source: https://www.nature.com/articles/nmeth1154

Data analysis for NGS

- Alignment to reference genome
- De-novo assembly



http://gcat.davidson.edu/phast/

Computational challenge

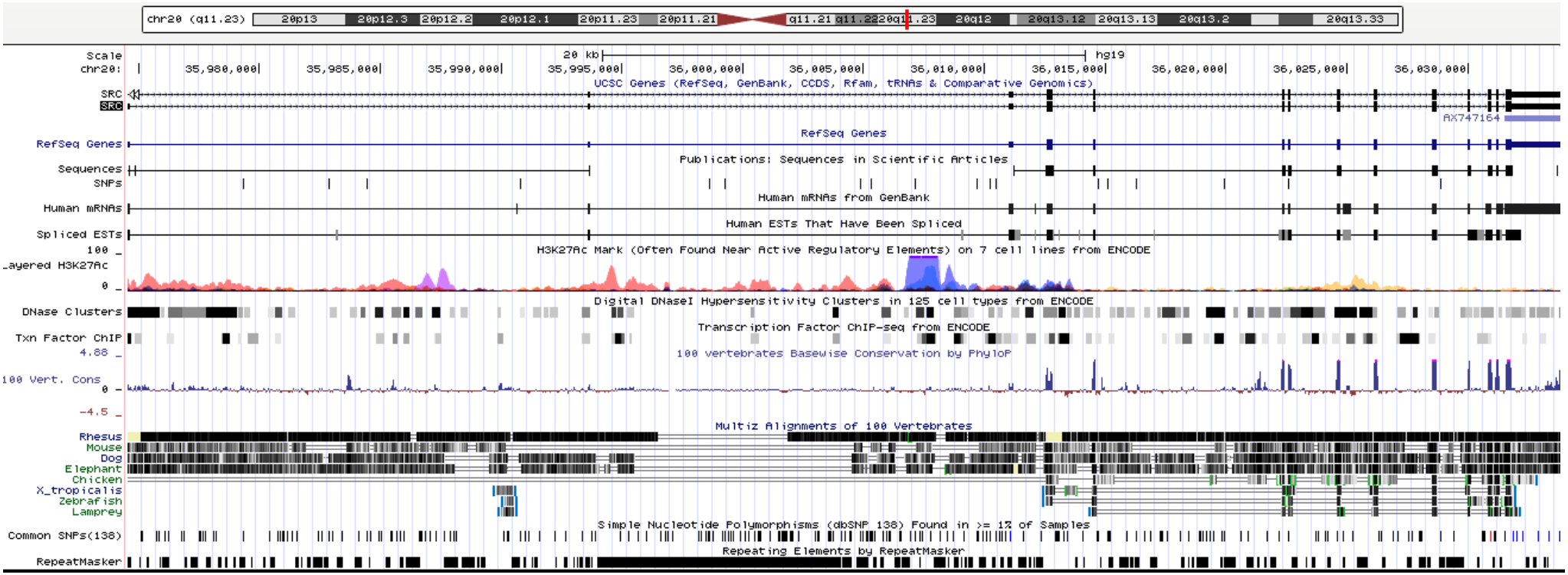
- Huge amounts of complex data
- SNPs and structural variants
- Sequencing errors
- Complex search algorithms required

→ Bioinformatic challenge

- From NGS reads (short and high volume) high oversampling is required to construct a genome:
- 30-40x oversampling is required

Applications of next gen DNA sequencing

- Whole genome sequencing
- Sequence variants
- Single cell sequencing
- cDNA, RNA, micro-RNA sequencing



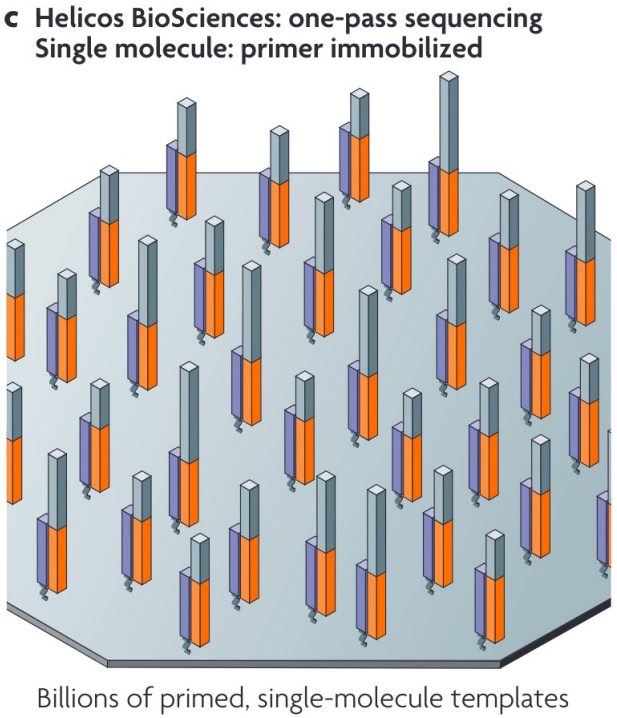
Next-generation DNA sequencing

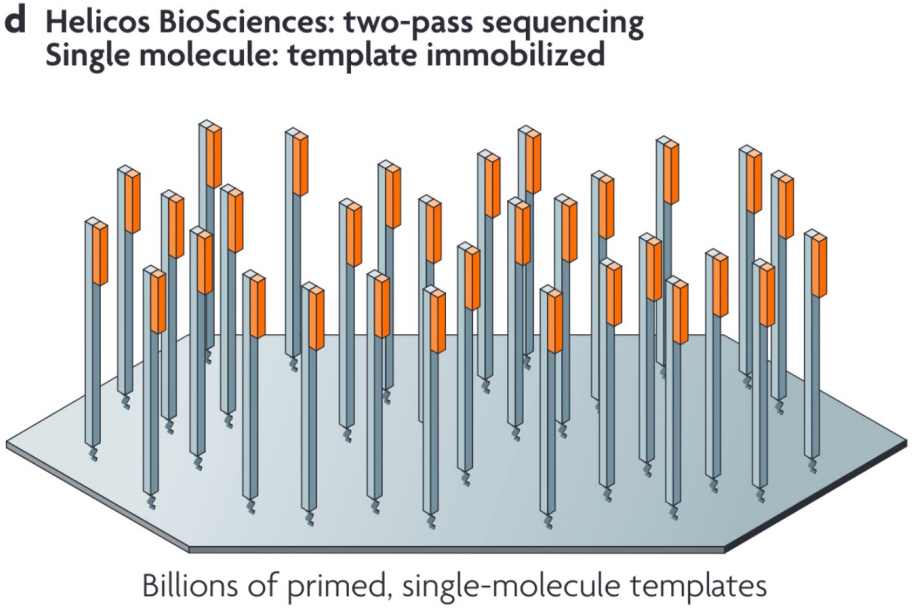
- Introduction
- Illumina: short-read, sequencing-by-synthesis
- Helicos: short-read, single-molecule, sequencing-by-synthesis
- PacBio: long-read, single-molecule, real-time sequencing
- Nanopore: long-read, nanopore-based electrical sensing (see last week!)

Next-generation DNA sequencing

- Introduction
- Illumina: short-read, sequencing-by-synthesis
- **Helicos: short-read, single-molecule, sequencing-by-synthesis**
- PacBio: long-read, single-molecule, real-time sequencing
- Nanopore: long-read, nanopore-based electrical sensing (see last week!)

Single-molecule templates: Helicos (defunct)





Why important?

First example of:

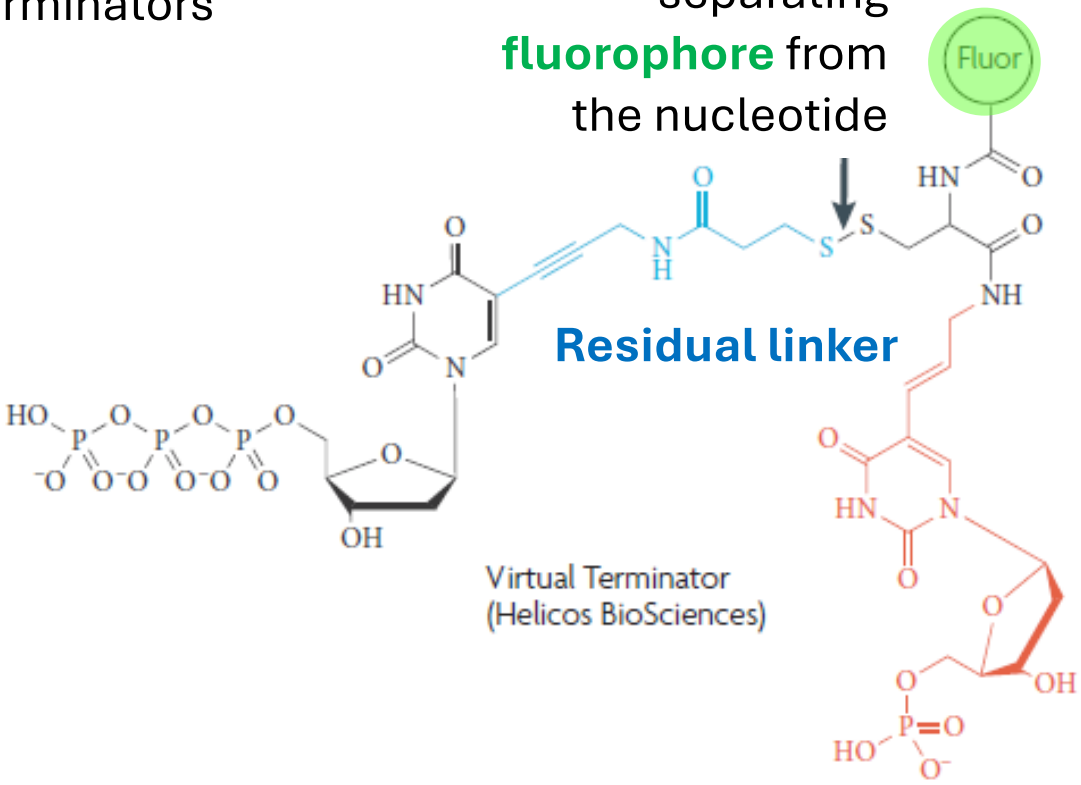
- Amplification-free sequencing
- Direct sequencing of RNA molecules
- Single-molecule sensitivity

Metzker et al. Nature Rev Genetics 2010

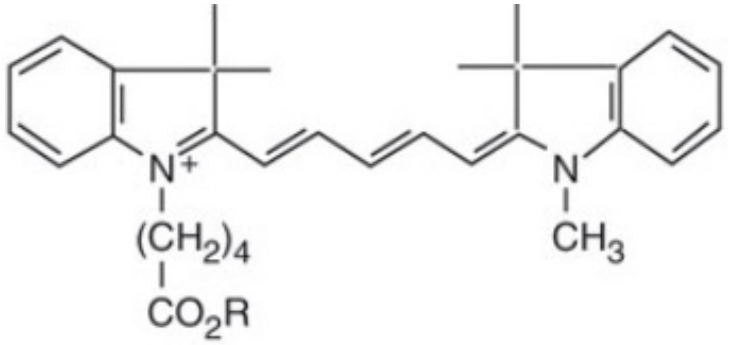
Single-molecule CRT

3' **unblocked** terminators

Site of cleavage: separating **fluorophore** from the nucleotide



fluorophore



Deprotection by reduction

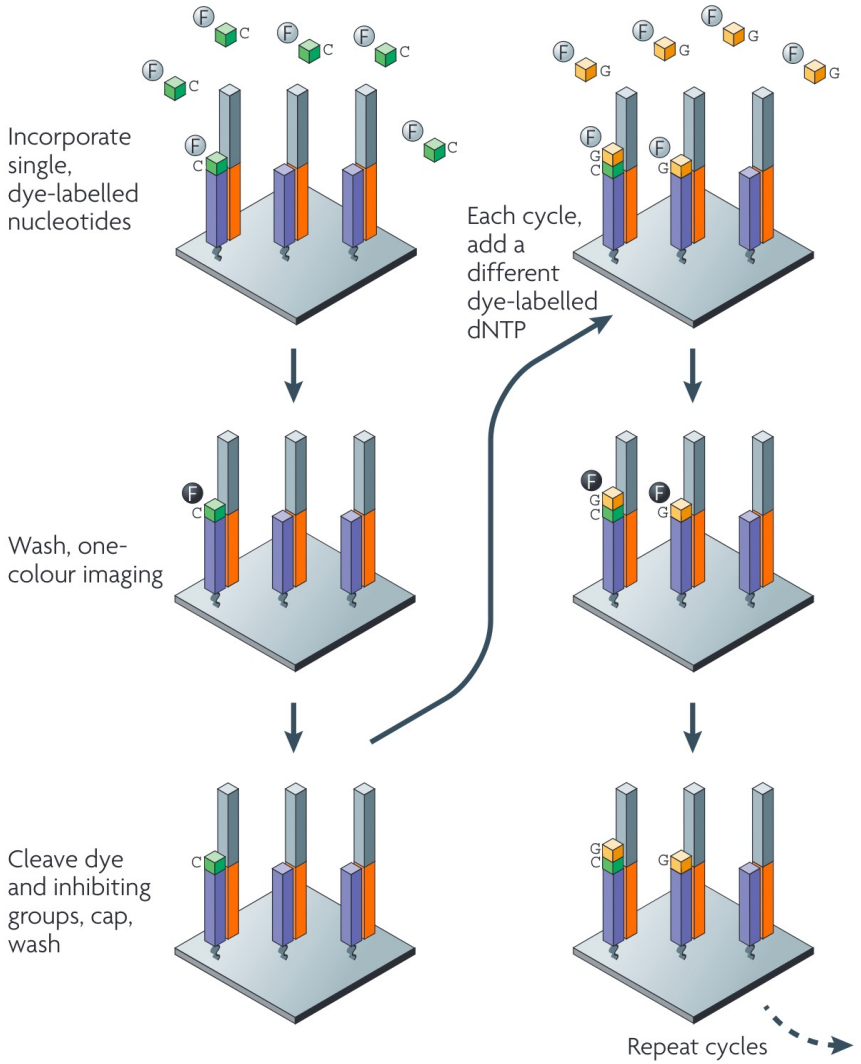
Steric hindrance: inhibitory function

Metzker et al. Nature Rev Genetics 2010

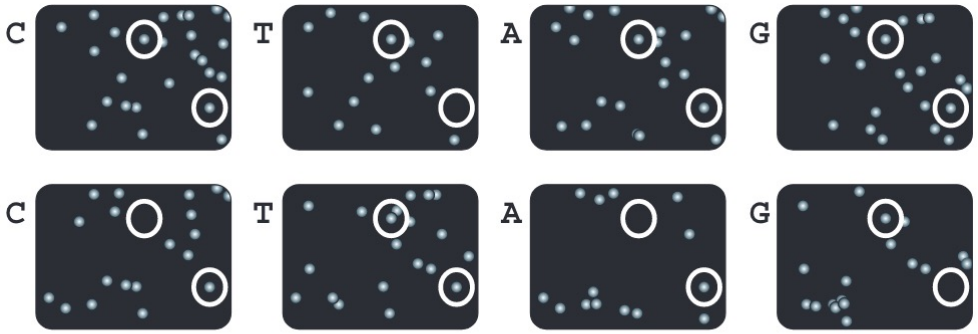
Activity: Comparing Illumina and Helicos sequencing methods

- Take a look at the figure on the next slide (1-color CRT, Helicos)
- What is the same, what is different compared to Illumina/Solexa CRT sequencing (see previous slides)?
- Why is only one dye needed for Helicos sequencing?
- What are the implications of using only one color vs. multiple colors?
- Given what you've learned, why might Helicos sequencing have struggled commercially, despite its innovative design?

1-color CRT, Helicos



Metzker et al. Nature Rev Genetics 2010



Top: CTAGTG
Bottom: CAGCTA

- **Elongation:** Less stringent for the polymerase
- **Deprotection:** Only one step
- **Readout / imaging:** single molecule Cy5 TIRF imaging

Solution 2025

🔍 1. What is the same, what is different compared to Illumina CRT sequencing?

✅ Similarities

- Both are **sequencing-by-synthesis (SBS)** methods.
- Use **cyclic reversible terminators (CRT)**: nucleotides with blocking groups to ensure **single-base addition per cycle**.
- Require **cleavage of fluorescent labels and blocking groups** before the next cycle.
- Fluorescent signals are used to identify the incorporated base.

❌ Differences

Feature	Helicos (1-color CRT)	Illumina (4-color CRT)
Amplification	No (single-molecule)	Yes (bridge amplification into clusters)
Fluorophores used	One dye (same for all bases)	Four distinct dyes (one per base)
Nucleotide addition	One type of base per cycle	All four bases added simultaneously
Signal decoding	Base identity inferred from **cycle position**	Base identity from **fluorescence color per cluster**
Throughput and density	Lower (due to weaker signal, no amplification)	Higher (stronger signal from clusters)

Solution 2025

🎨 2. Why is only one dye needed for Helicos sequencing?

- Helicos uses **only one type of nucleotide per cycle** (e.g., only A, then only T, etc.).
- Since **only one base is available** for incorporation at any given step, the **presence or absence of fluorescence** reveals whether that base was incorporated.
- Therefore, **color is not used to encode identity—cycle order encodes identity**.

⚖️ 3. What are the implications of using only one color vs. multiple colors?

✅ Advantages of One-Color (Helicos)

- **Simpler optics**: no need to separate fluorescent spectra.
- **No spectral crosstalk** or dye-specific calibration.
- Easier base discrimination in theory (only “on” or “off” per cycle).

❌ Disadvantages

- Requires **four full synthesis/imaging cycles to determine one base position**, reducing **speed and throughput**.
- More cycles → **more opportunities for phasing errors** and reagent degradation.
- Lower efficiency for large-scale applications compared to multi-color methods.

Solution 2025

🧠 4. Why might Helicos sequencing have struggled commercially, despite its innovative design?

Several factors contributed:

Factor	Issue
Short read lengths	Typically ~35–55 bp—insufficient for many genomics applications
Lower accuracy	Single-molecule signal prone to noise; no redundancy from clonal amplification
Low throughput	Imaging single molecules without clusters limits yield
High cost and complexity	Long sequencing time due to one-base-per-cycle, high imaging demand
Market competition	Illumina emerged with superior throughput, scalability, and support
Limited ecosystem	Fewer software tools, protocols, and user community support

Despite its clever approach, Helicos couldn't keep pace with rapidly improving competitors like **Illumina**, **Ion Torrent**, and later **single-molecule platforms (PacBio, Nanopore)** that offered either better throughput, longer reads, or lower cost.

Why are Helicos reads limited to 55 bp?

Limitation	Explanation
Signal decay	Fluorophore bleaching and background noise accumulate with each cycle.
Polymerase limitations	Reduced efficiency with modified nucleotides leads to poor incorporation and error accumulation.
No amplification	Single-molecule detection makes error correction and signal averaging impossible.
Imaging noise	CCD-based imaging systems add background that limits cycle length.

Advantages/limitations of single-molecule sequencing

Advantages

- No amplification bias
- Simpler sample preparation
- Relaxed efficiency for extension
- Sequencing of low abundance samples
- **Longer** read lengths
- Greater sensitvity to DNA modifications

Limitations

- Single-molecule detection can be challenging
- Multiple nucleotide or probe additions: higher error rates
- Complex data analysis
- Quenching/blinking/bleaching of fluorophores: deletion errors
- Often: a sequence coverage >1 for verification required

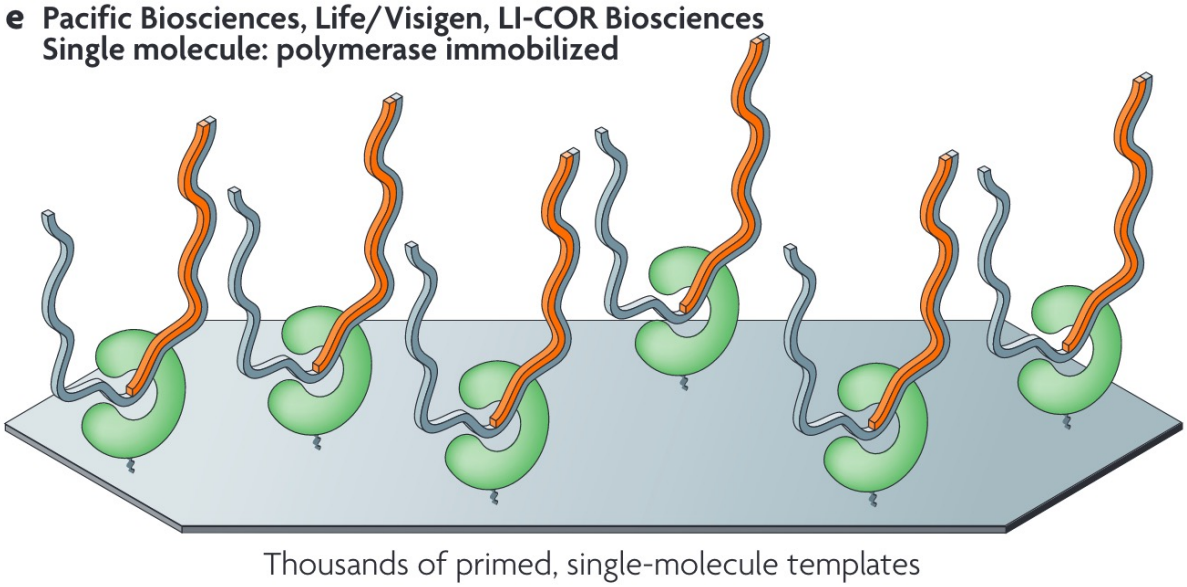
Next-generation DNA sequencing

- Introduction
- Illumina: short-read, sequencing-by-synthesis
- Helicos: short-read, single-molecule, sequencing-by-synthesis
- **PacBio: long-read, single-molecule, real-time sequencing**
- Nanopore: long-read, nanopore-based electrical sensing (see last week!)

PacBio sequencing

- https://www.youtube.com/watch?v=_lD8JyAbwEo&ab_channel=PacBio

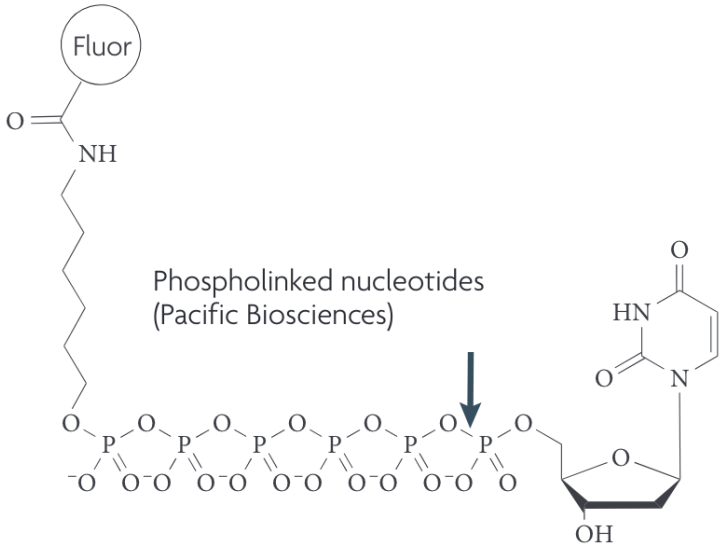
Single-molecule real-time sequencing: PacBio



- Immobilization of DNA polymerase molecules
- Observation of the **incorporation of single, fluorescently labeled nucleotides** (no reversible terminators)

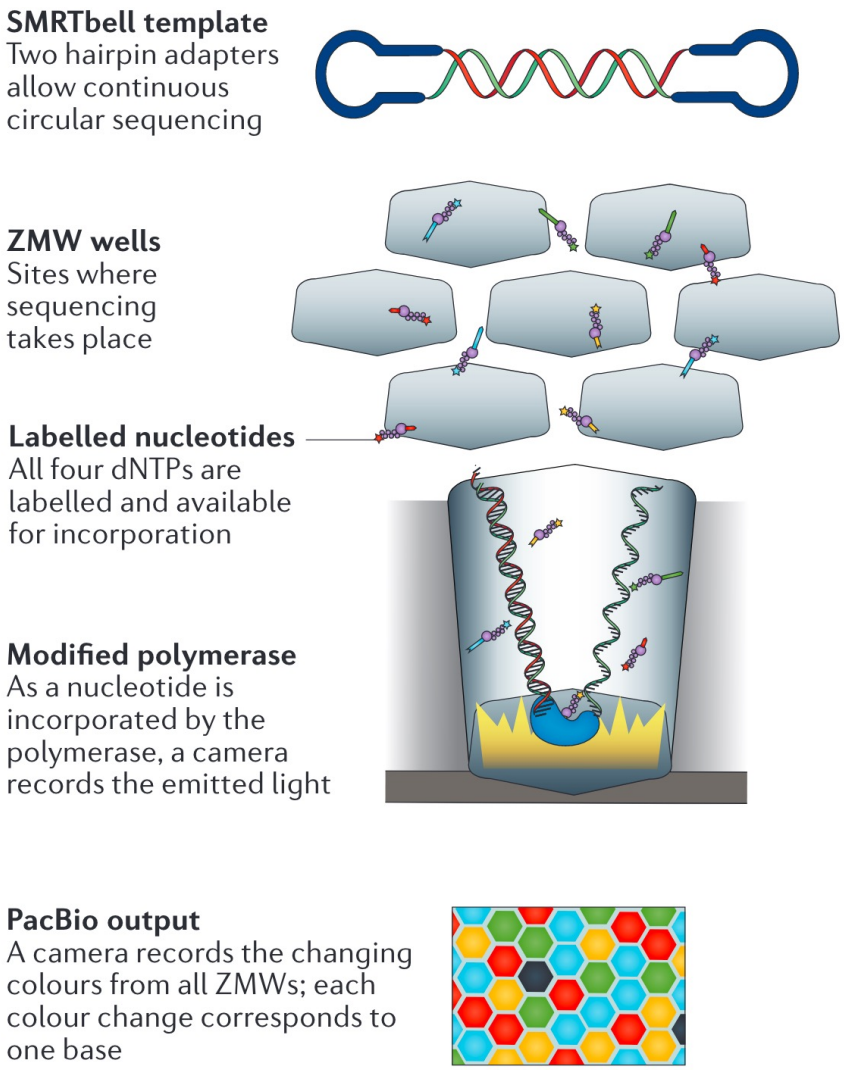
Phospholinked nucleotide (hexaphosphate linker)

- Hexaphosphate slows down incorporation process slighly
- PacBio DNA pol was engineered to recognize and incorporate these modified nucleotides
- Hexaphosphate group serves as a cleavable linker



Metzker et al. Nature Rev Genetics 2010

Single-molecule real-time sequencing: PacBio



Single-stranded circular DNA:

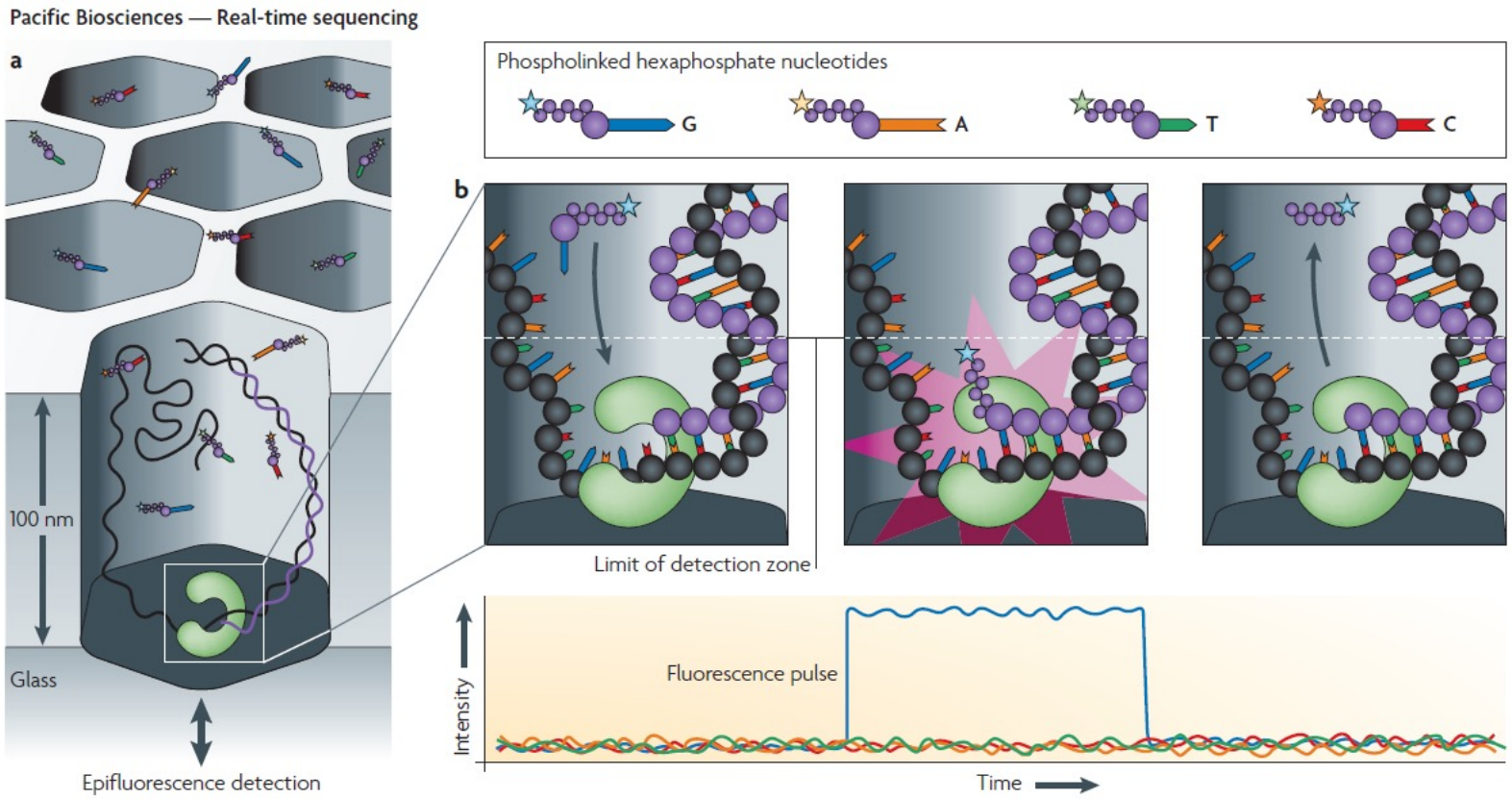
- Template fragments processed and ligated to hairpin adapters at each end
- Primers and ϕ29 DNA polymerase are attached to ssDNA regions on SMRTbell template

Zero mode waveguides (ZMW):

- Cavity in thin metal film with
- Reduction of illumination volume to zeptoliter size
- Allows to observe molecules at high concentration, **one at a time**

Goodwin et al. Nature Rev Genetics 2016

Real-time sequencing



- Residence time of phospholinked nucleotides in the active site: millisecond scale
- Long enough to record a **fluorescent pulse**
- Released, dye-labeled pentaphosphate by-product quickly diffuses away

Metzker et al. Nature Rev Genetics 2010

Performance of real-time sequencing

- Average read-length: several thousands of nucleotides
- Error sources:
  - Deletions
  - Insertions
  - Mismatches
- Mostly due to very fast reaction times

- **Accuracy** per run: 83%
  - Requires muliple sequencing runs for the template with multiple runs: 99.9%

Next-generation DNA sequencing

- Introduction
- Illumina: short-read, sequencing-by-synthesis
- Helicos: short-read, single-molecule, sequencing-by-synthesis
- PacBio: long-read, single-molecule, real-time sequencing
- **Nanopore: long-read, nanopore-based electrical sensing (see last week!)**

Summary of NGS methods

Feature	Illumina	Helicos	Ion Torrent	PacBio (HiFi)	Oxford Nanopore
Detection method	Fluorescence (SBS)	Fluorescence (single-molecule SBS)	pH sensing (semiconductor)	Fluorescence (real-time SMRT)	Ionic current through nanopore
Read length	75–300 bp	25–55 bp	100–400 bp	10–25 kb (HiFi reads)	10–100+ kb
Accuracy (raw)	>99.9%	~98%	~98–99%	>99.9% (HiFi)	~95–98% (raw); improving
Throughput	Very high (up to terabases/run)	Moderate (low density vs. modern SBS)	Moderate	Moderate	Variable (scalable)
Run time	Hours to days	~1-2 days	Hours	~24 hrs	Real-time (minutes–hours)
Library amplification	Yes	No (single molecule)	Yes	No (SMRTbell prep)	No
Molecule type	Amplified DNA	Native DNA	Amplified DNA	Native DNA or RNA	Native DNA or RNA
Strengths	Accuracy, throughput, cost per base	Amplification-free, early SMS platform	Fast, inexpensive instruments	Long reads + high accuracy	Ultra-long reads, portability
Limitations	Short reads, PCR bias	Short reads, discontinued platform	Lower accuracy, pH drift	Higher cost per base, lower throughput	Higher error rate, context bias
Best for	WGS, RNA-Seq, panels, clinical use	Single-molecule transcription profiling	Targeted panels, quick runs	Isoform sequencing, genome assembly	Structural variation, field use
Manufacturer	Illumina	Helicos BioSciences (defunct)	Thermo Fisher	PacBio	Oxford Nanopore Technologies

Connections

- https://connections.swellgarfo.com/game/-NvBOf2_nOaAEYOnwTU5