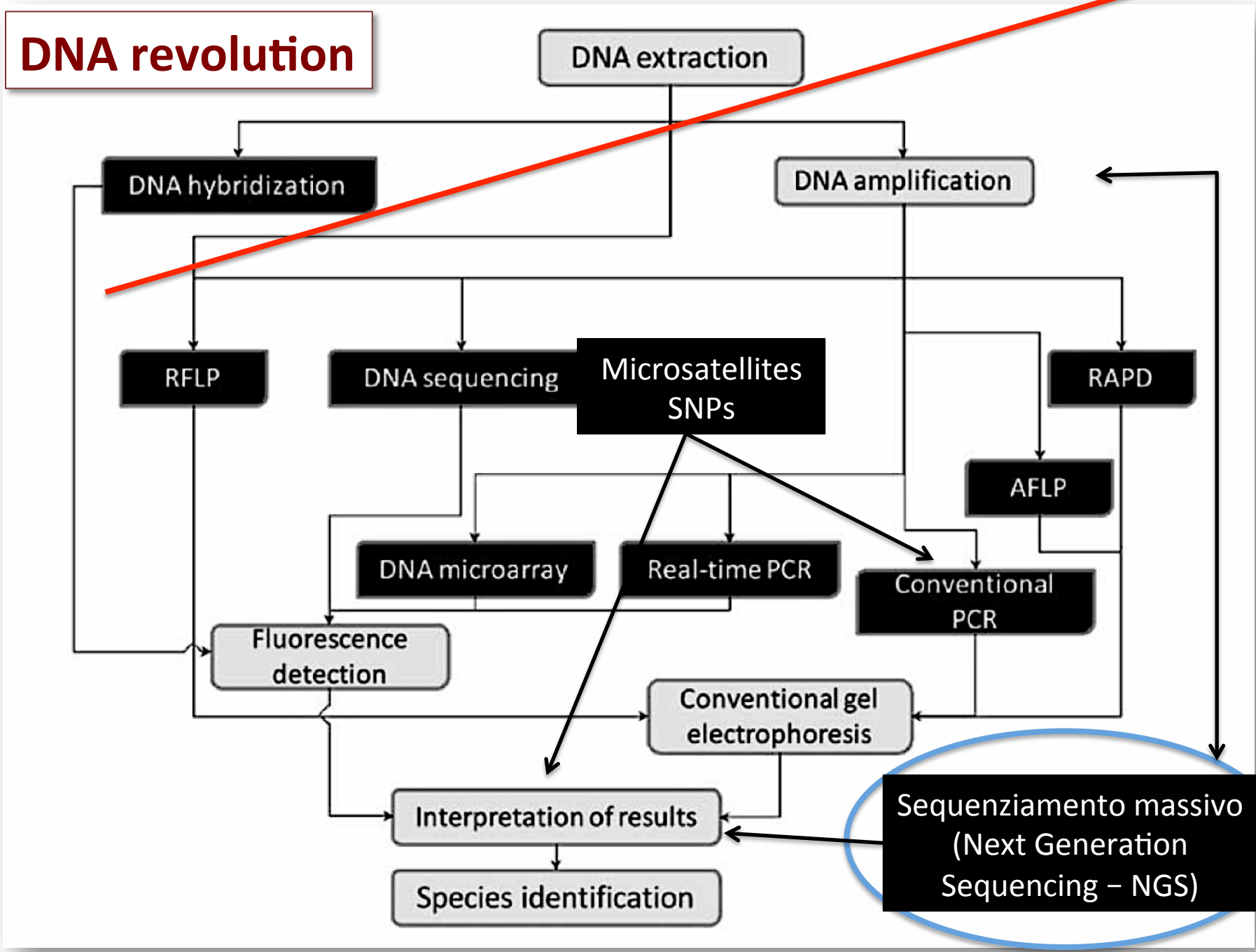
DNA revolution



NGS

2007

	454 GS FLX*	AB SOLiD	Illumina GAII
Chemistry	Pyrosequencing	Ligation based	Reversible terminators
	Standard	Fragment	Fragment
Run Time	7 hours	3-6.5 days	3 days
Read Lengths (bp)	250	25, 50	35, 50
Ave. Reads per Run	400K	$150x10^6$	$85x10^6$
Data per run	100MB	up to 7GB	up to 4.3GB
Throughput	100MB	1.1GB/day	1.4GB/day

Next-Generation Sequencing

Genome-wide genetic marker discovery and genotyping using next-generation sequencing

John W. Davey, Paul A. Hohenlohe‡, Paul D. Etter§, Jason Q. Boone‖, Julian M. Catchen‡ and Mark L. Blaxter*¶*

NATURE REVIEWS | GENETIC VOLUME 12 | JULY 2011 | 499

NGS

ics Proteomics Bioinformatics 12 (2014) 190–197

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www.elsevier.com/locate/gpb
www.sciencedirect.com

REVIEW

Application of Next-generation Sequencing Technology in Forensic Science

Yaran Yang [1], Bingbing Xie [1,2], Jiangwei Yan [1,*]

CrossMark

SLAF-seq: An Efficient Method of Large-Scale *De Novo* SNP Discovery and Genotyping Using High-Throughput Sequencing

Xiaowen Sun, Dongyuan Liu, Xiaofeng Zhang, Wenbin Li, Hui Liu, Weiguo Hong, Chuanbei Jiang, Ning Guan, Chouxian Ma, Huaping Zeng, Chunhua Xu, Jun Song, Long Huang, [...], Hongkun Zheng [view all]

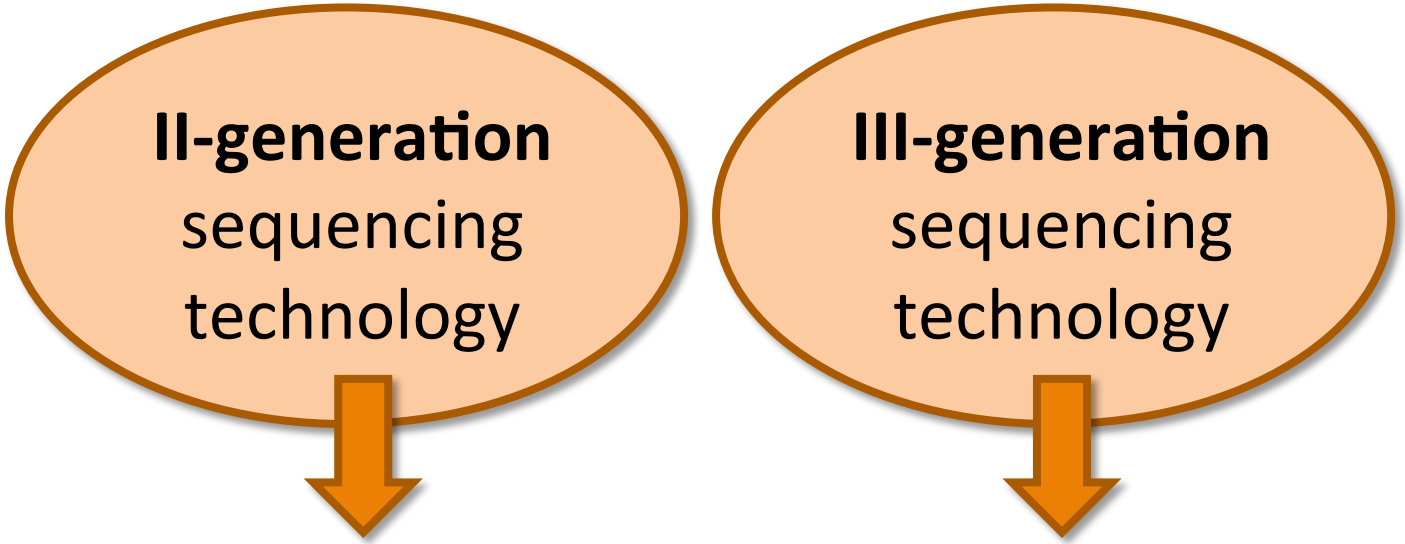
Published: March 19, 2013 • http://dx.doi.org/10.1371/journal.pone.0058700

PLOS ONE
TENTH ANNIVERSARY

NGS

Non-Sanger-based high-throughput DNA sequencing technology

- Increasing the throughput
- Minimizing the need for the fragment-cloning method often used in Sanger sequencing
- Enabling the sequencing of biological codes at a very rapid pace with low cost per operation

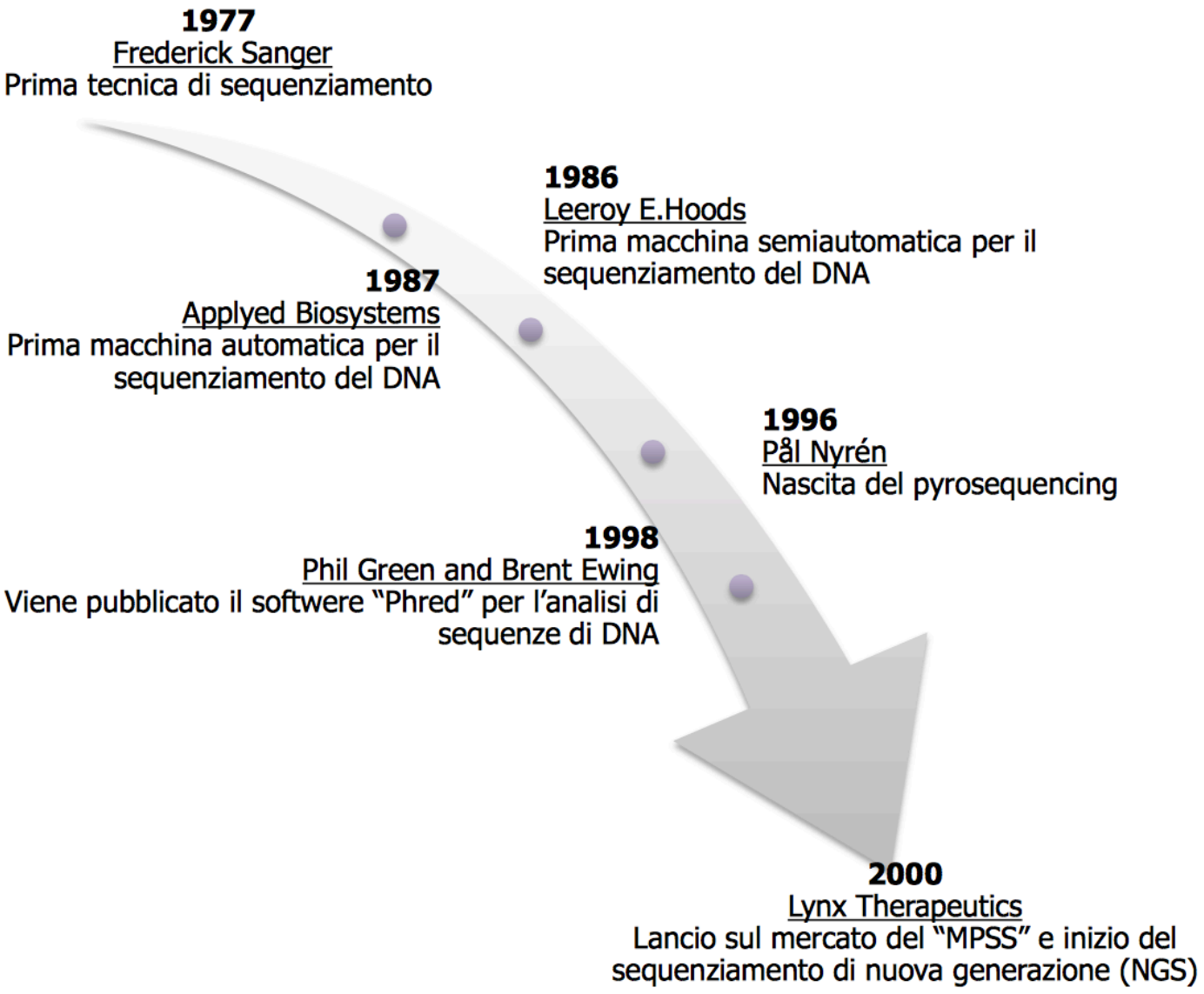


Le tecniche di sequenziamento

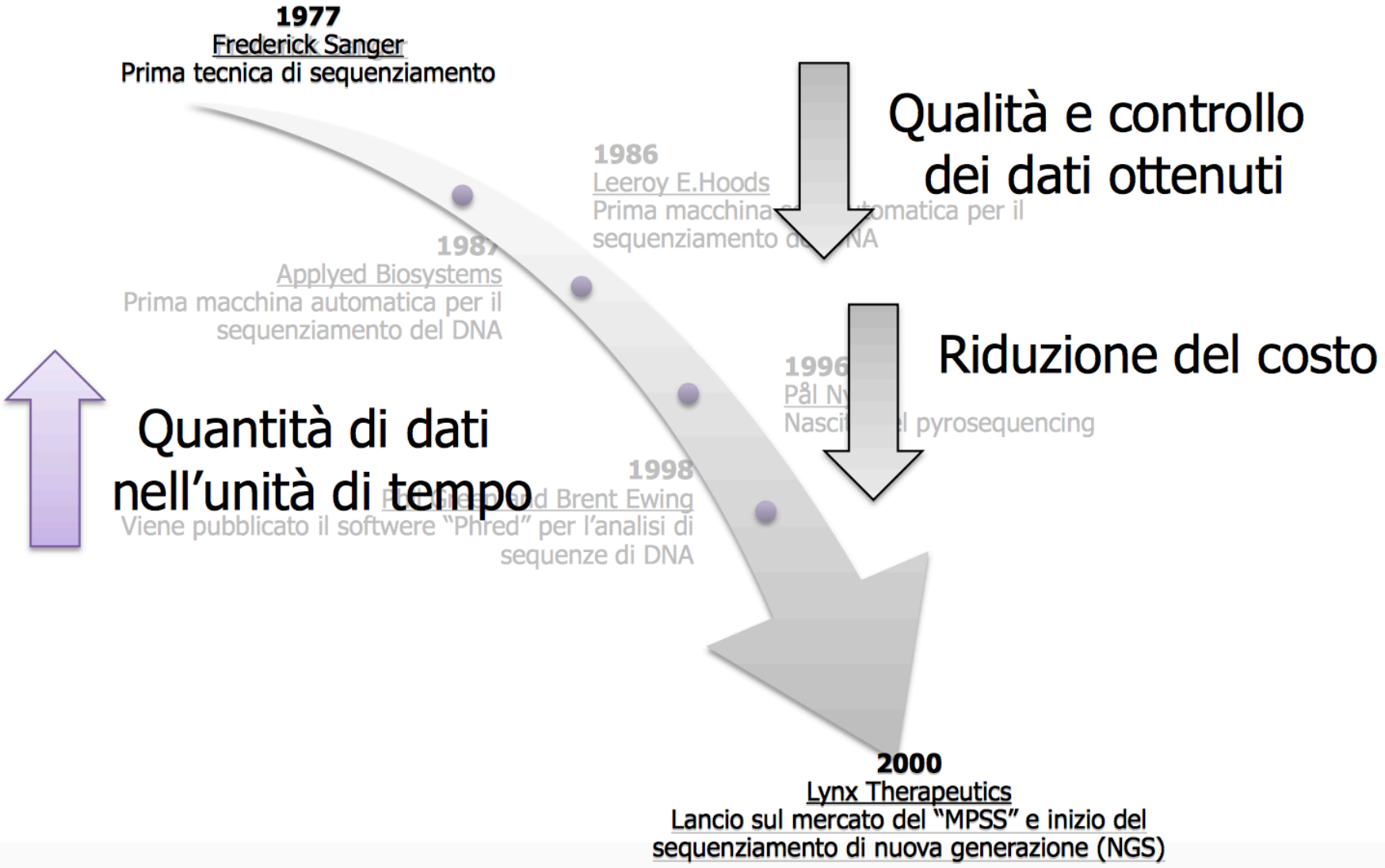
Generazione 0 - Maxam & Gilbert — Sequenziamento Chimico

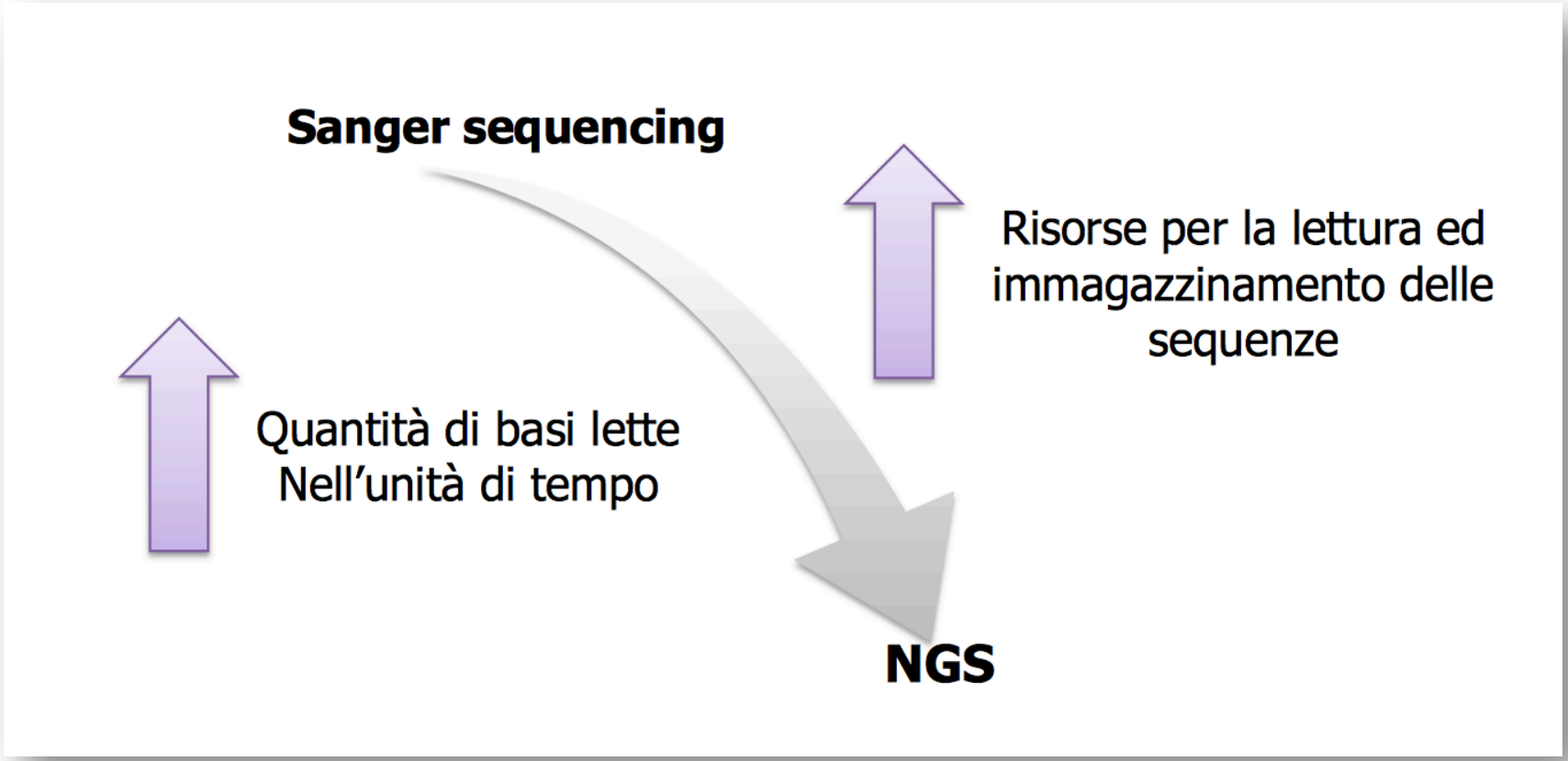
Generazione 1 - Sanger — Dye-Terminator

Evoluzione del sequenziamento



Evoluzione del sequenziamento




Sanger sequencing
Risorse per la lettura ed immagazzinamento delle sequenze
Quantità di basi lette Nell'unità di tempo
NGS

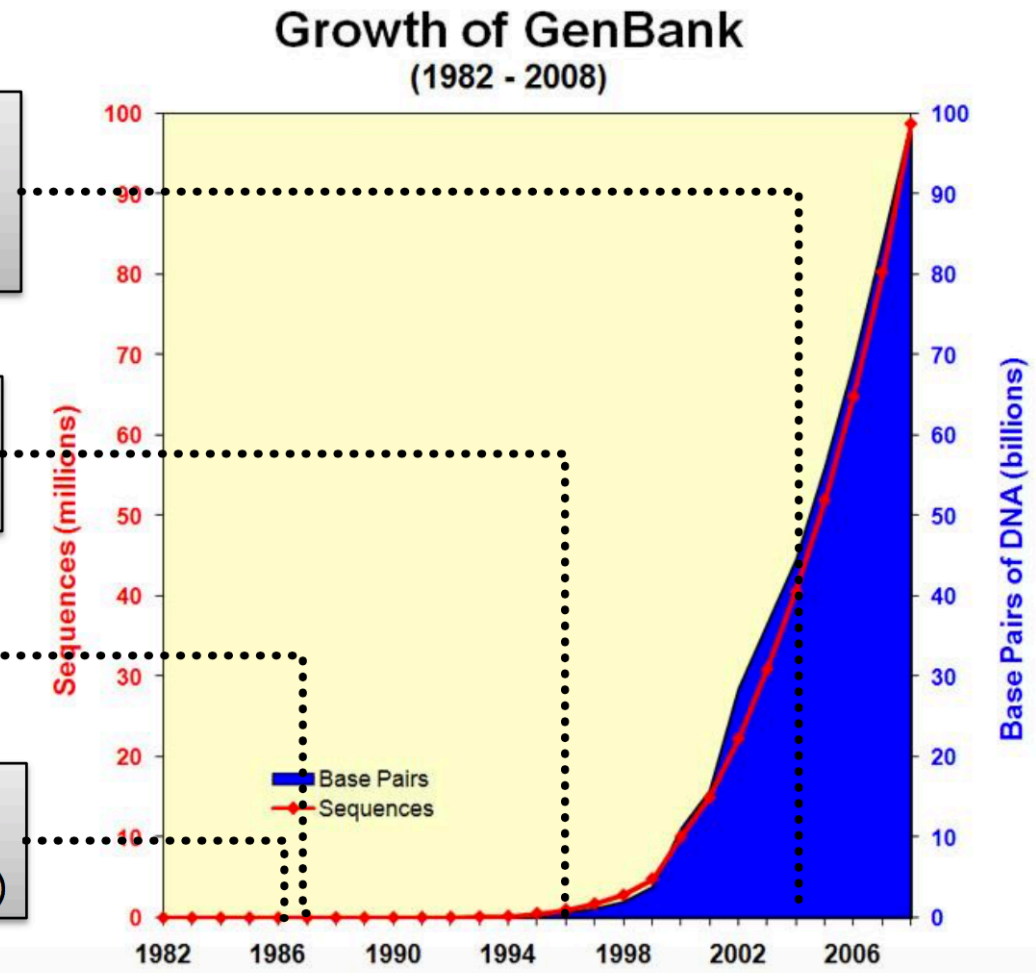
NGS, seconda generazione

Viene commercializzata la prima macchina automatica per il pirosequenziamento

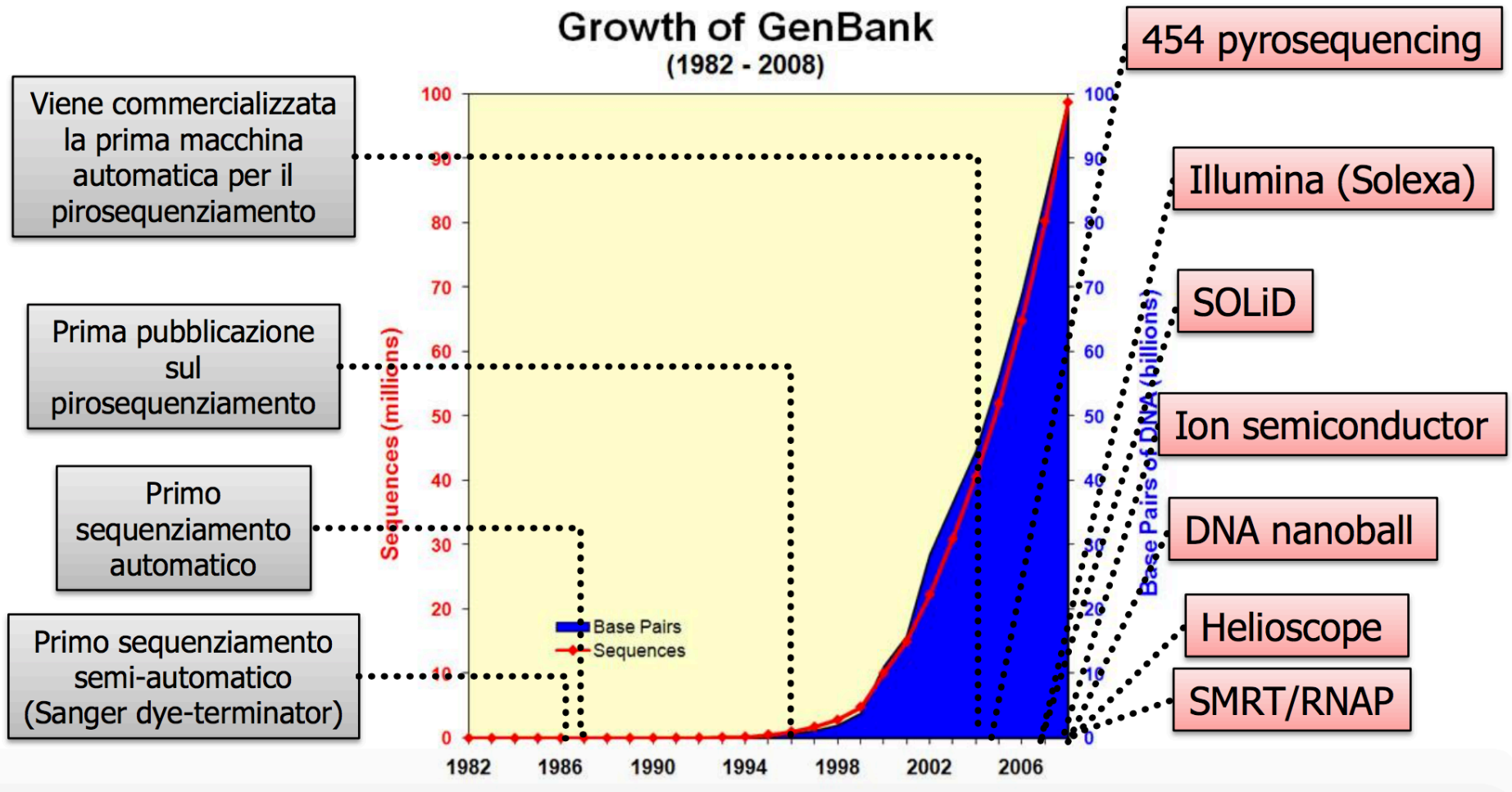
Prima pubblicazione sul pirosequenziamento

Primo sequenziamento automatico

Primo sequenziamento semi-automatico (Sanger dye-terminator)



NGS, seconda generazione



NGS

NGS technologies are a combination of strategies for:

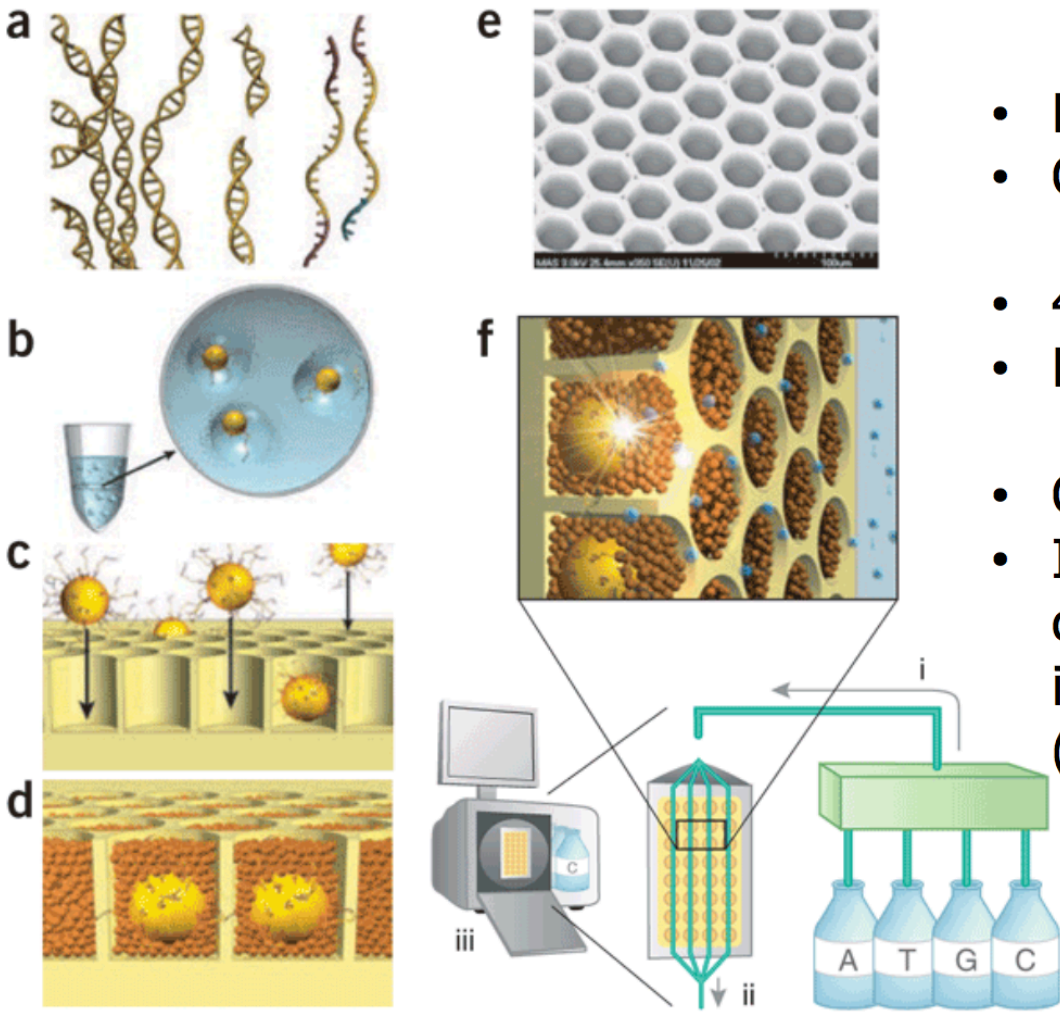
- Template preparation → **Library preparation**
- Sequencing and imaging
- Genome alignment
- Assembly methods

Major platforms for NGS:

1. **454** by Roche
2. **Solexa** by Illumina
3. **SOLiD** by Applied Biosystem

454 Genome Sequencing System (Roche)

- Pyrosequencing-based system **(2005)**



- Da 400 a 700 basi per *read*
- Circa la metà rispetto al Sanger

- 400-600 megabasi ogni 10 ore
- Più veloce del Sanger

- Costi medio alti
- Impossibilità di sequenziare correttamente più di 8 basi identiche consecutive (omonucleotidi)

- **200.000 reads**

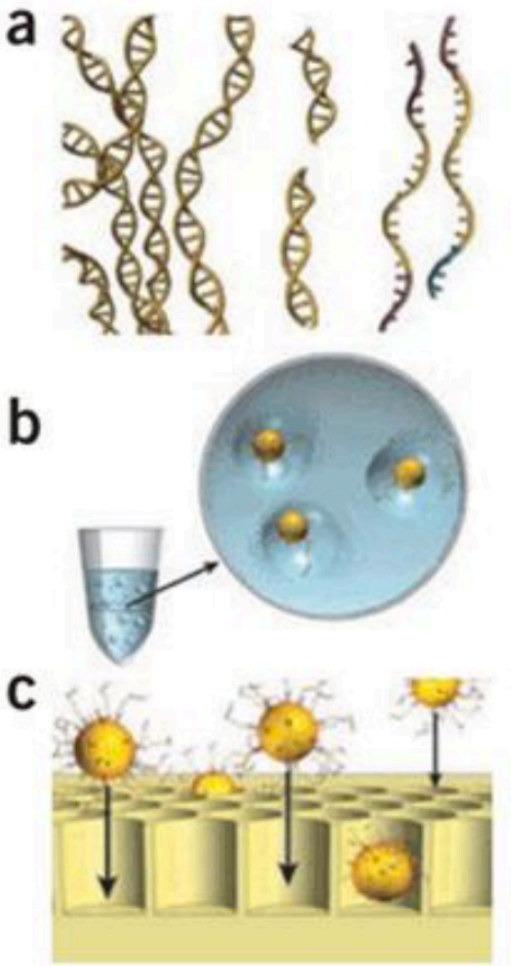
454 Genome Sequencing System (Roche)

In sintesi:

- Amplificazione tramite *emulsion PCR* (**emPCR**)
- Multiple reazioni di PCR nella stessa provetta
- Sospensioni acquose in "olio"

- Frantumazione DNA
- Primer di PCR legati a microsfere

- Intrappolamento microsfere in micropiastra
- Una microsfera per pozzetto



In pratica . . .

454 Genome Sequencing System (Roche)

Emulsion PCR (emPCR)

- Variazione della PCR usata nelle tecnologie NGS per replicare le sequenze di DNA

- Frammentazione della libreria di DNA:

1. **SONICAZIONE**
2. **NEBULIZZAZIONE**

NEBULIZATION	SONICATION
Compressed nitrogen is used to force DNA through a small hole, creating mechanically sheared fragments	Ultrasonic waves used to create gas bubbles in sample, and shear DNA by resonance vibration

454 Genome Sequencing System (Roche)

Emulsion PCR (emPCR)

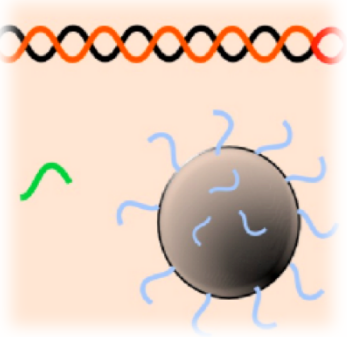
- Variazione della PCR usata nelle tecnologie NGS per replicare le sequenze di DNA

- Frammentazione della libreria di DNA:
  1. **SONICAZIONE**
  2. **NEBULIZZAZIONE**

  → **Produzione di frammenti di DNA di dimensioni tra 300 e 800 bp**

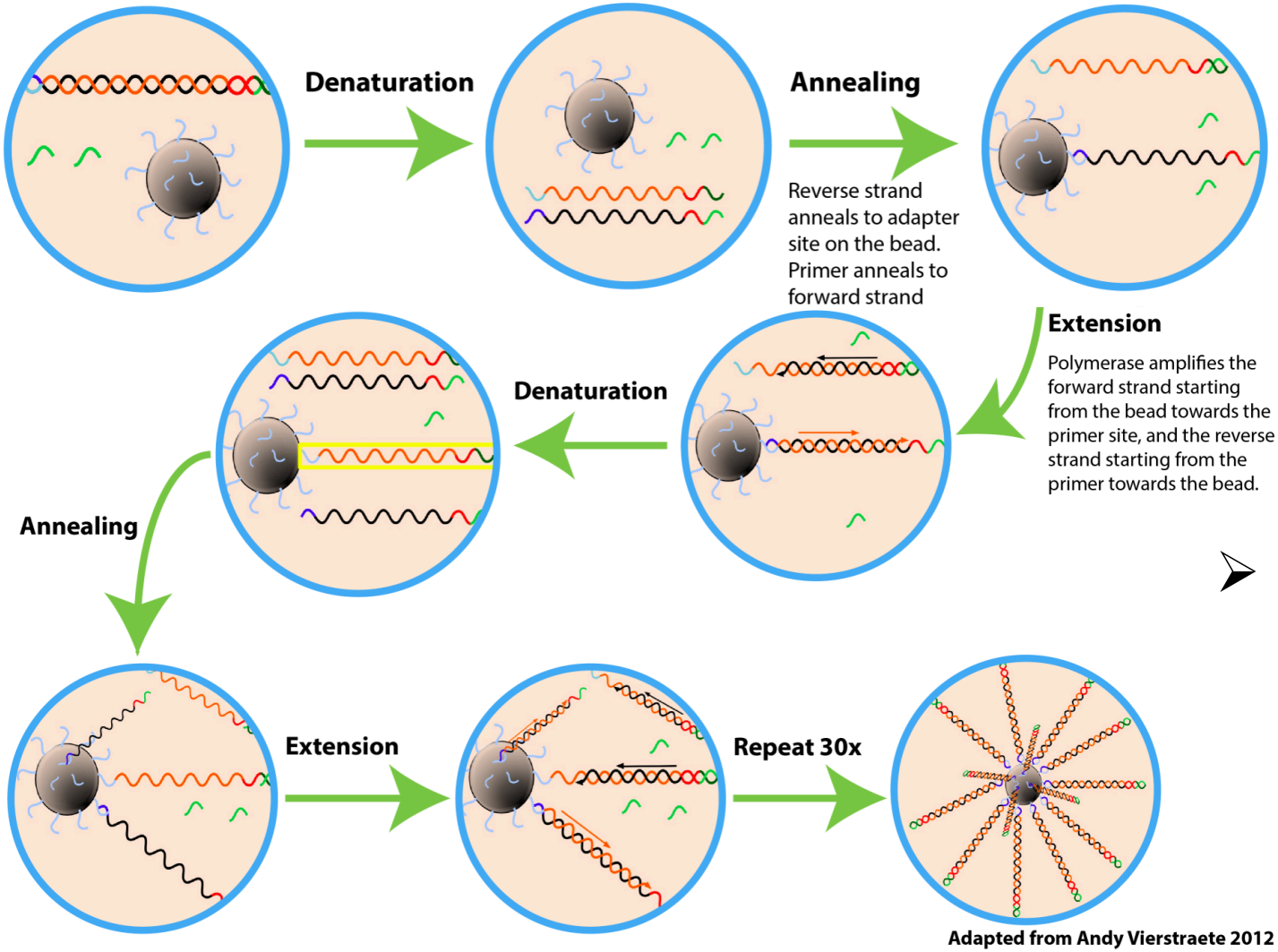
- Ligazione di **ADATTATORI** alle estremità dei frammenti di DNA. Questo permetterà a sua volta il legame dei frammenti con gli adattatori alle beads magnetiche.

- Legame dei frammenti con gli adattatori alle beads



454 Genome Sequencing System (Roche)

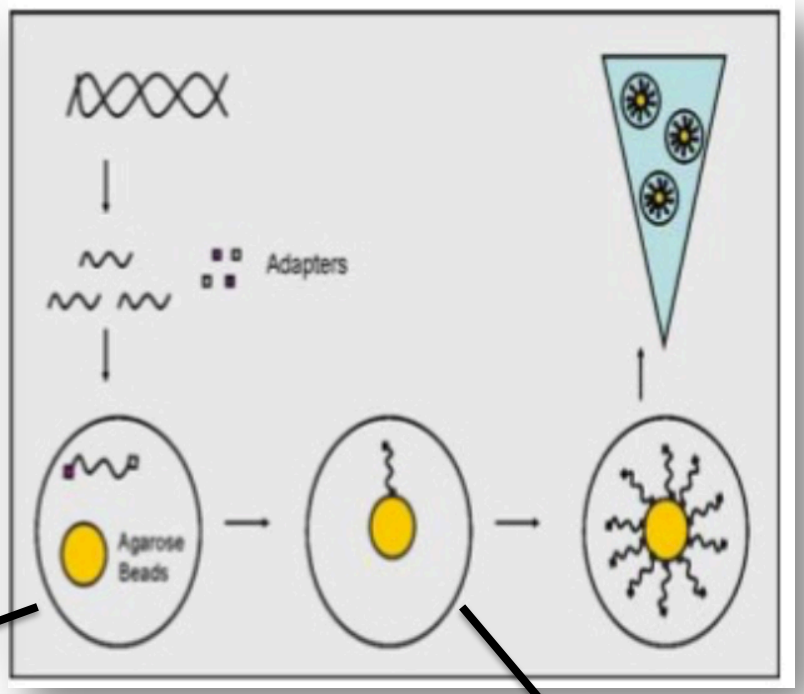
- Denaturazione della doppia elica, amplificazione



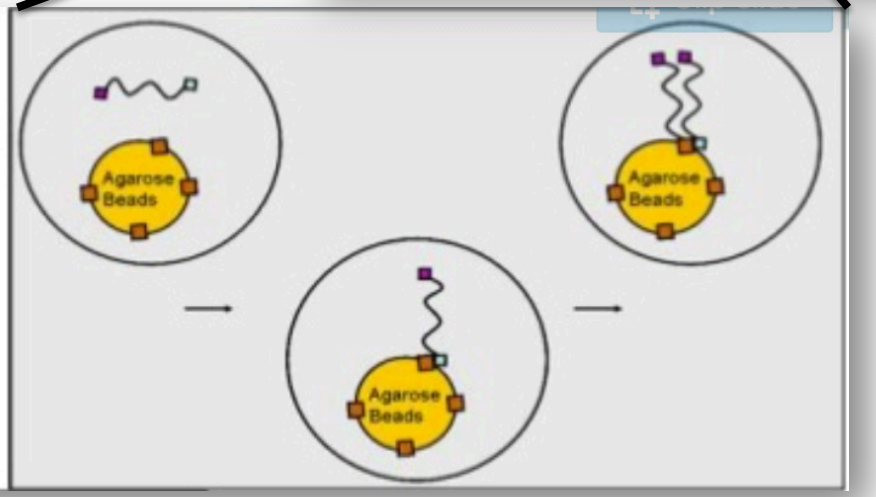
- Formazione di beads con **popolazioni di frammenti CLONALI**

454 Genome Sequencing System (Roche)

- DNA is fragmented, joined to adapters at either end of the fragmented DNA
- amplified in an emulsion PCR (includes agarose bead with complimentary adaptors to fragmented DNA)

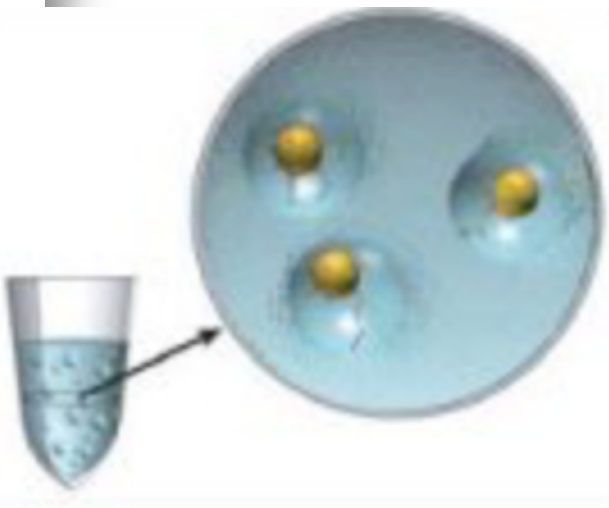


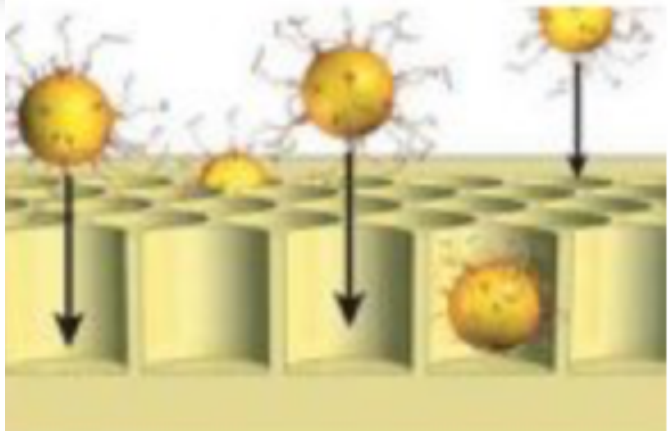
- Adapter containing the universal priming site are ligated to target ends
- Same primer can be used for amplification



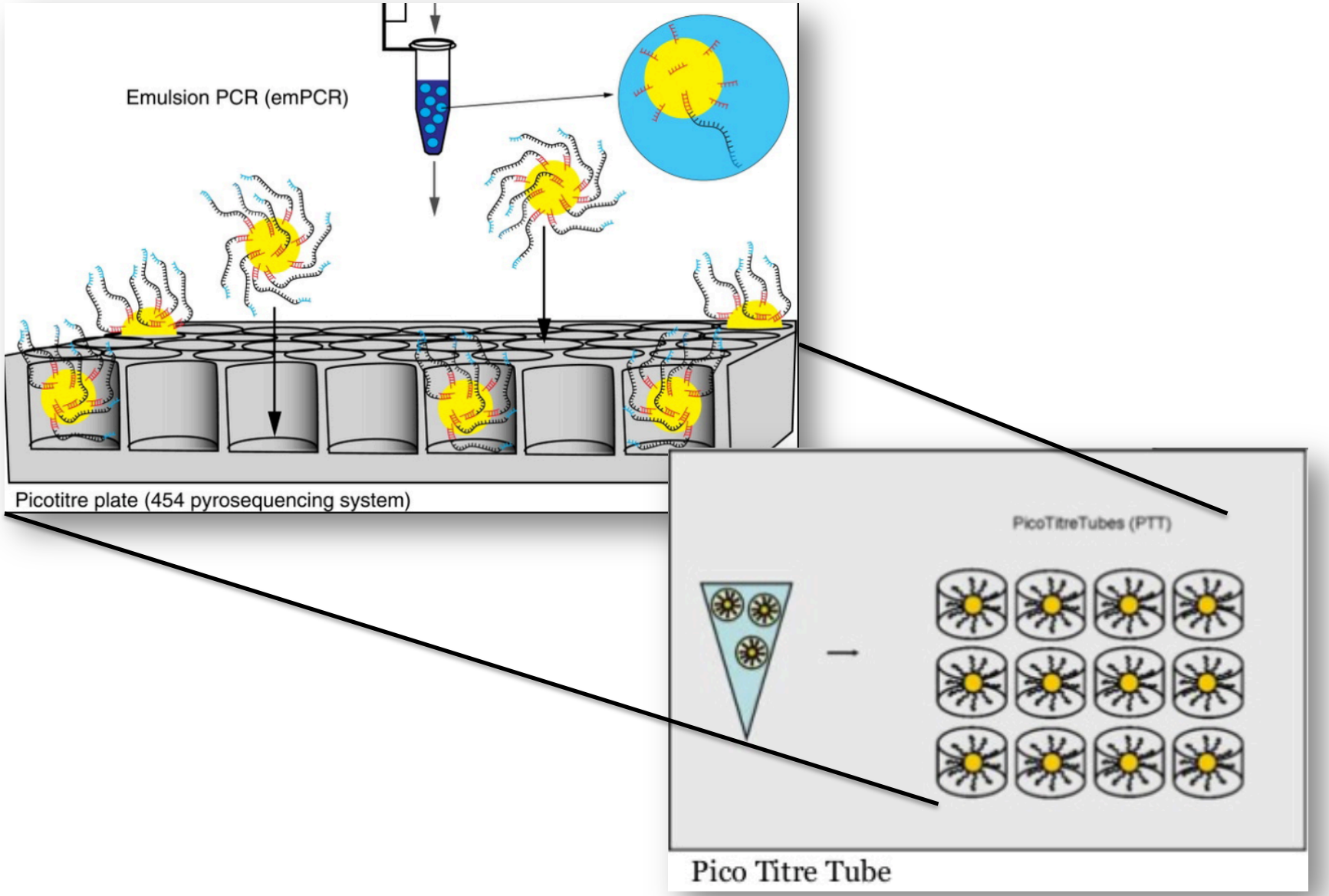
454 Genome Sequencing System (Roche)

- PCR amplified allowing up to 1 million identical fragments around one bead and finally dropped into a PicoTitreTube (PTT)





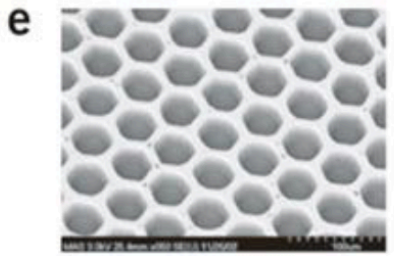
454 Genome Sequencing System (Roche)

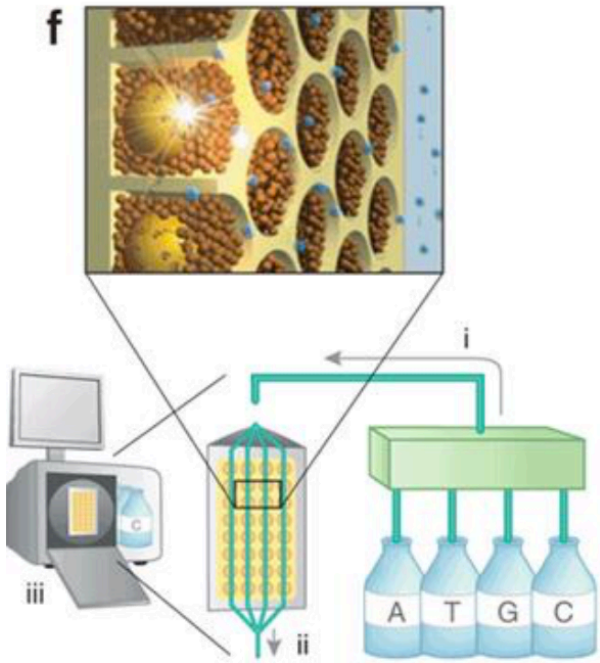


454 Genome Sequencing System (Roche)

Pirosequenziamento

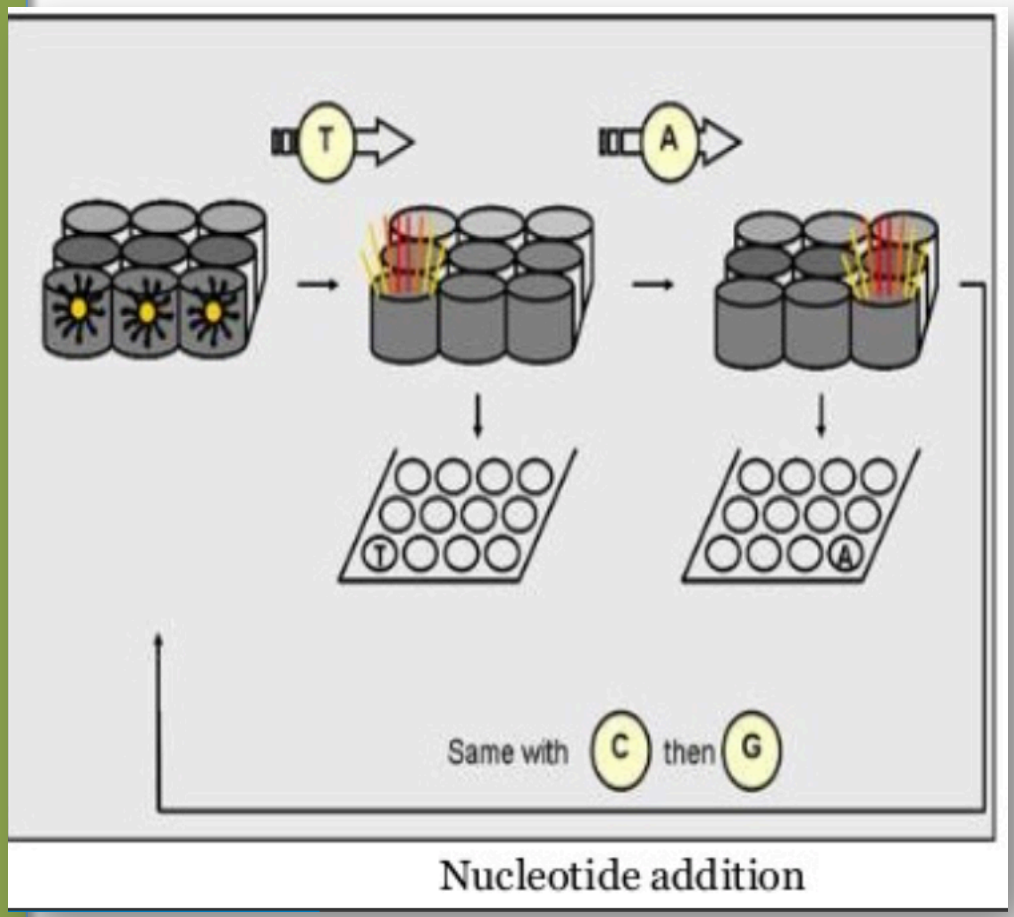
- Inserimento di un nucleotide per volta
- Incorporazione di un nucleotide da parte della DNA polimerasi comporta lo sviluppo di luce
- Rilevazione da parte di una videocamera

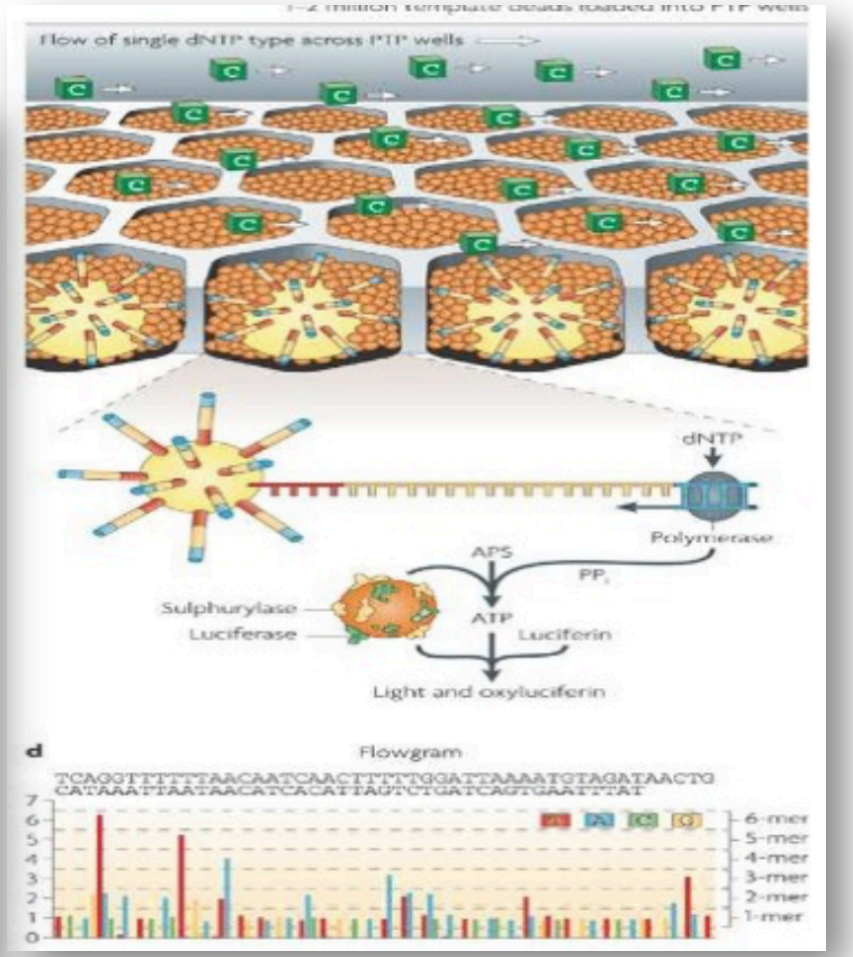




454 Genome Sequencing System (Roche)

In Pico trite tube reaction of fluorescence occurs with the addition of nucleotides

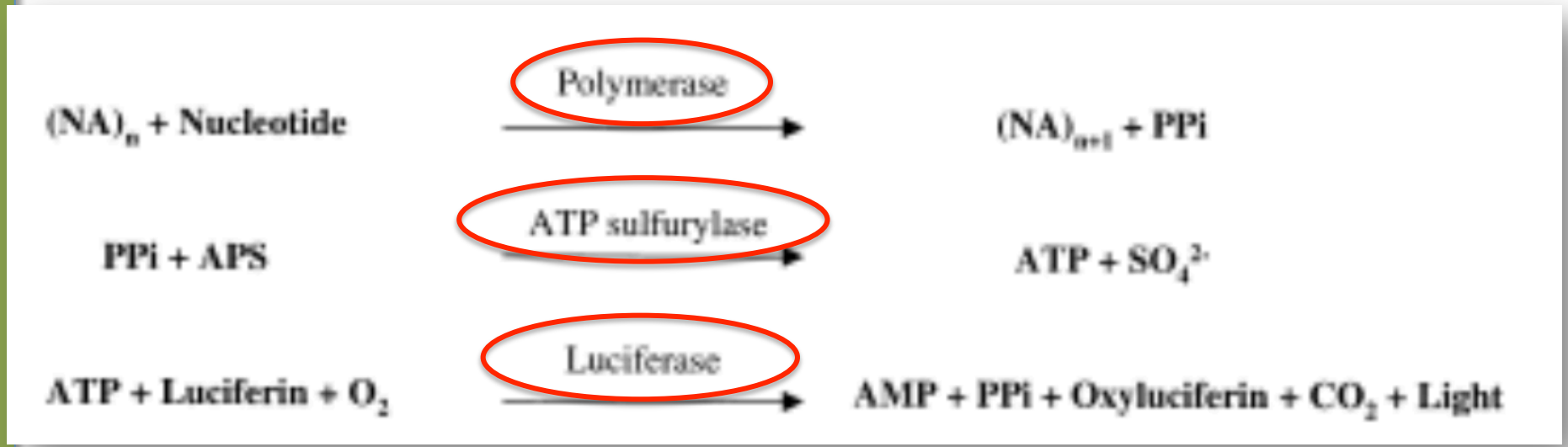




454 Genome Sequencing System (Roche)

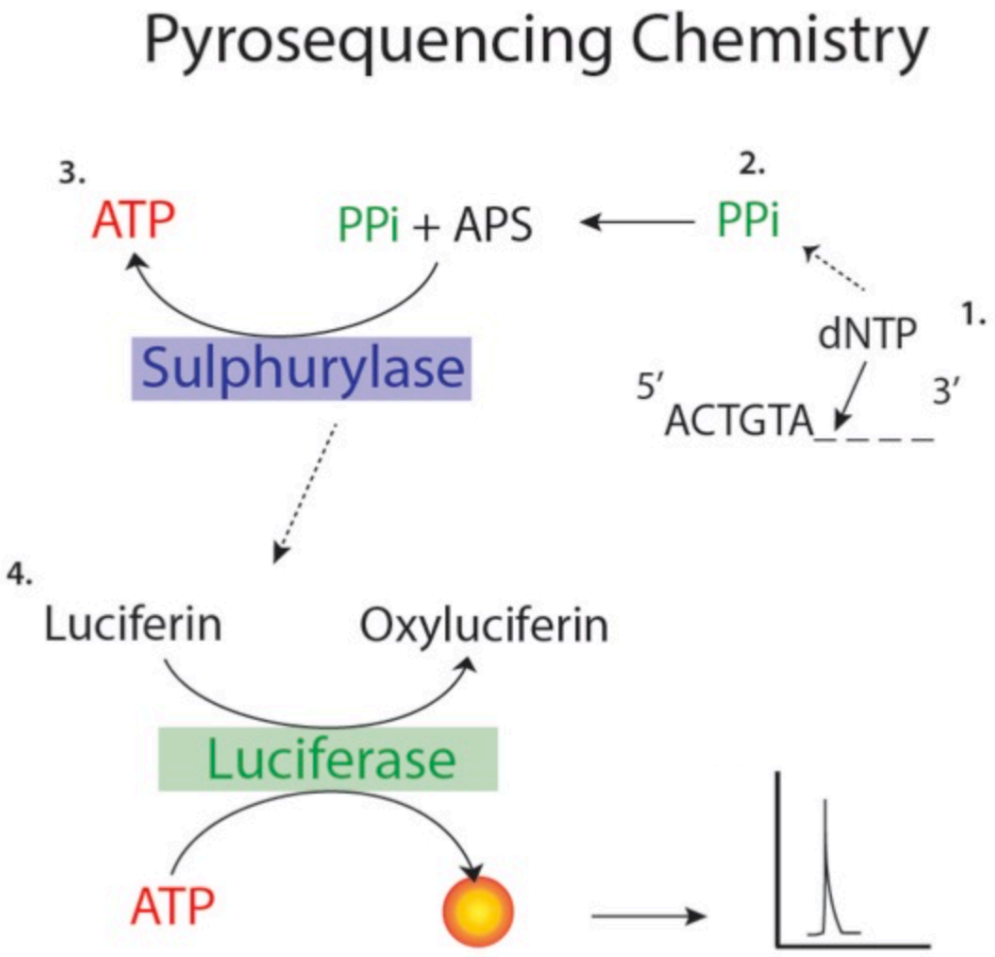
Principio generale alla base di tutti i sistemi di pirosequenziamento:

- Incorporazione dei nucleotidi grazie alla **DNA Polimerasi**
- Conversione del Pirofosfato (PPi) in ATP grazie alla **ATP Sulfurilasi**
- Produzione del segnale luminoso grazie all'ossidazione operata dalla **Luciferasi** di una molecola di luciferina



454 Genome Sequencing System (Roche)

Principio generale alla base di tutti i sistemi di pirosequenziamento:



454 Genome Sequencing System (Roche)

Possibile interferenza di segnale data dal residuo di dNTP's ad ogni ciclo??

The four different nucleotides are added STEPWISE and incorporation is followed using the enzyme ATP sulfurylase and luciferase,

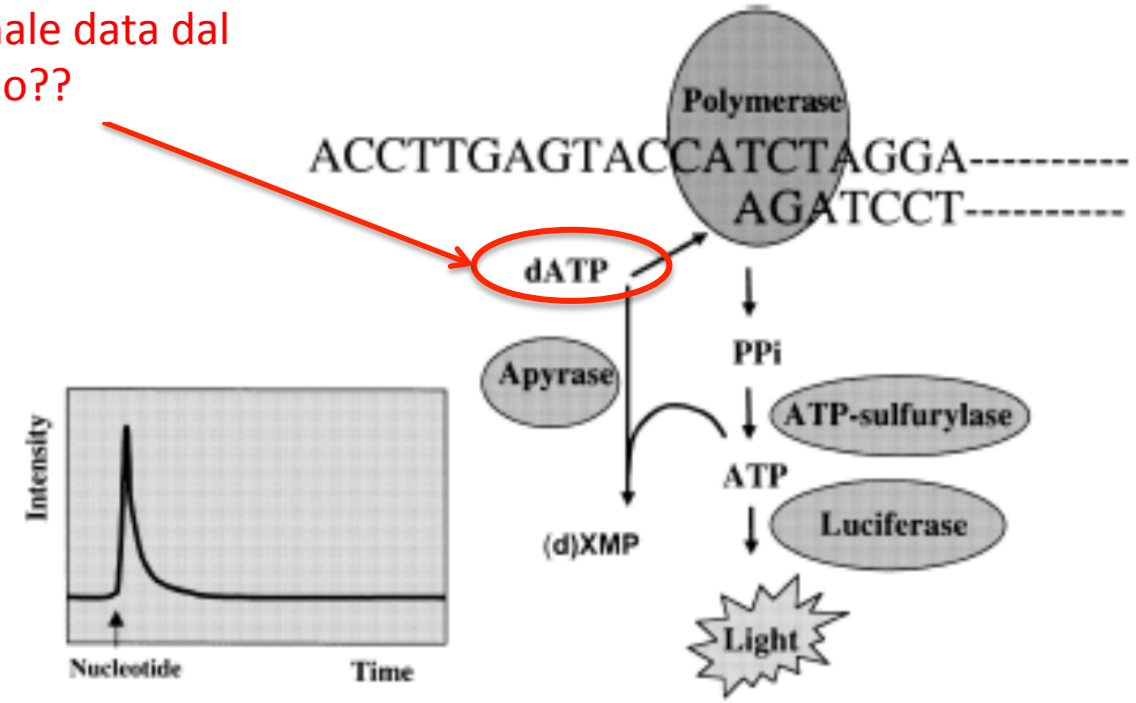
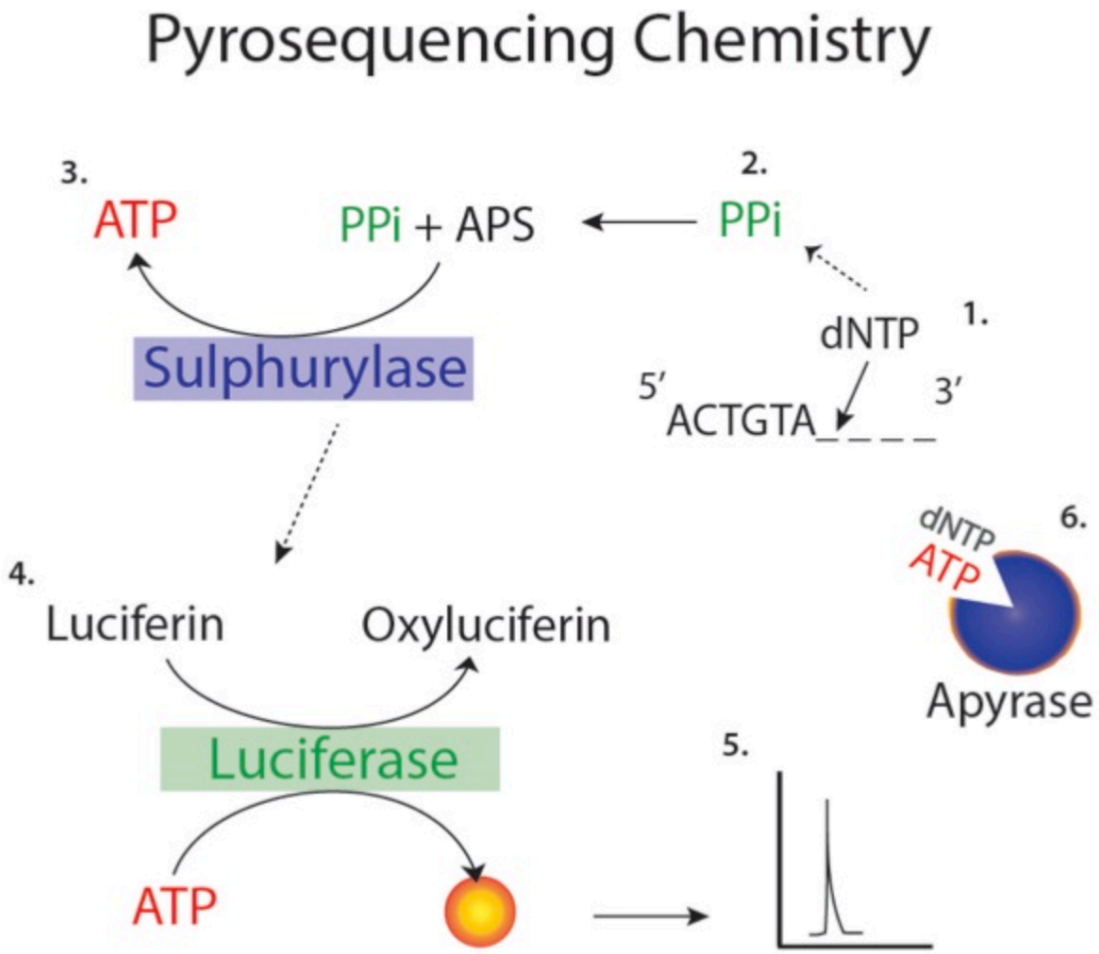


Figure 3 Schematic representation of the progress of the enzyme reaction in liquid-phase pyrosequencing. Primed DNA template and four enzymes involved in liquid-phase pyrosequencing are placed in a well of a microtiter plate.

The **nucleotides are continuously degraded** by nucleotide-degrading enzyme (**Apyrase**) allowing addition of subsequent nucleotide.

454 Genome Sequencing System (Roche)

Principio generale alla base di tutti i sistemi di pirosequenziamento:



454 Genome Sequencing System (Roche)

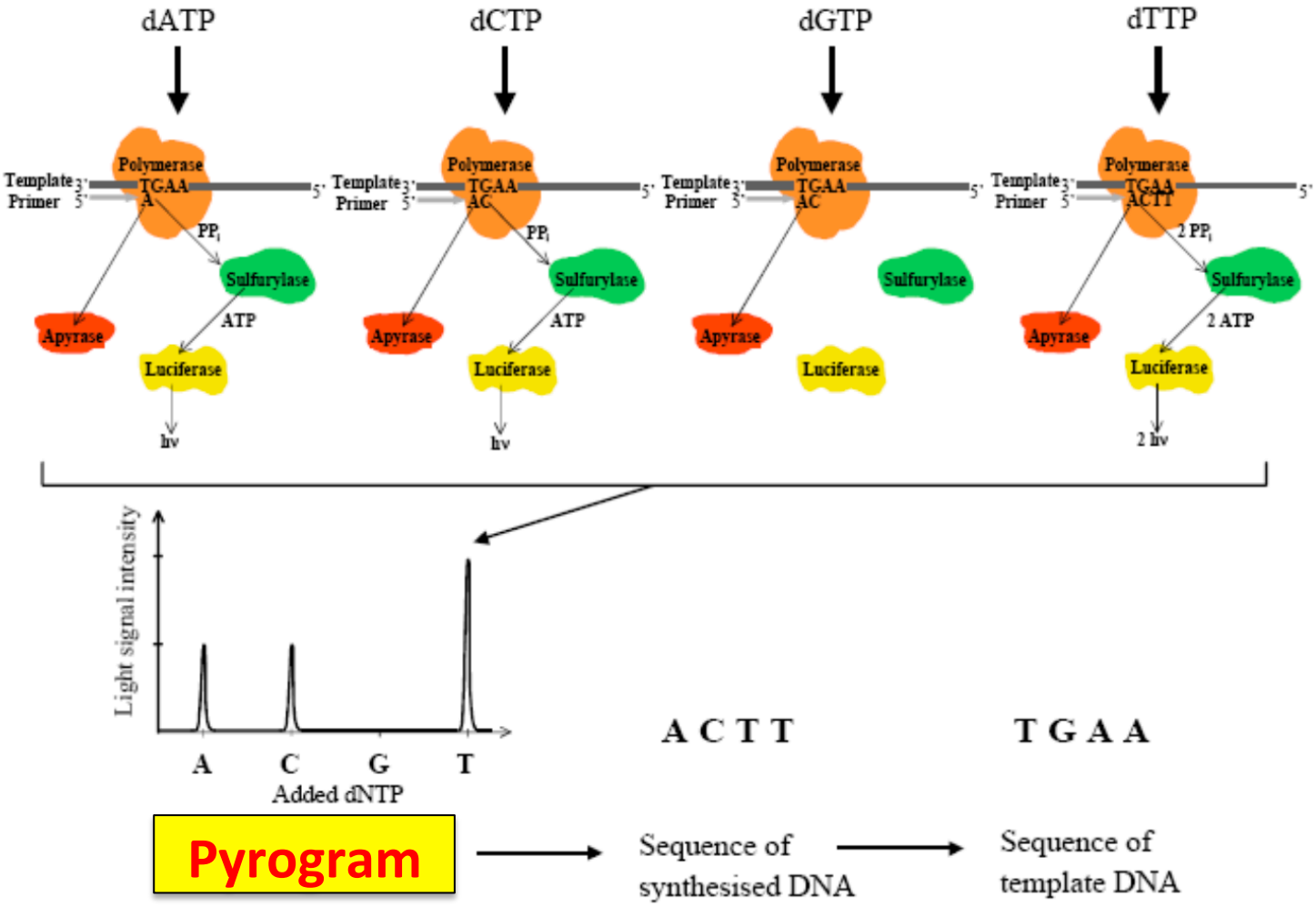
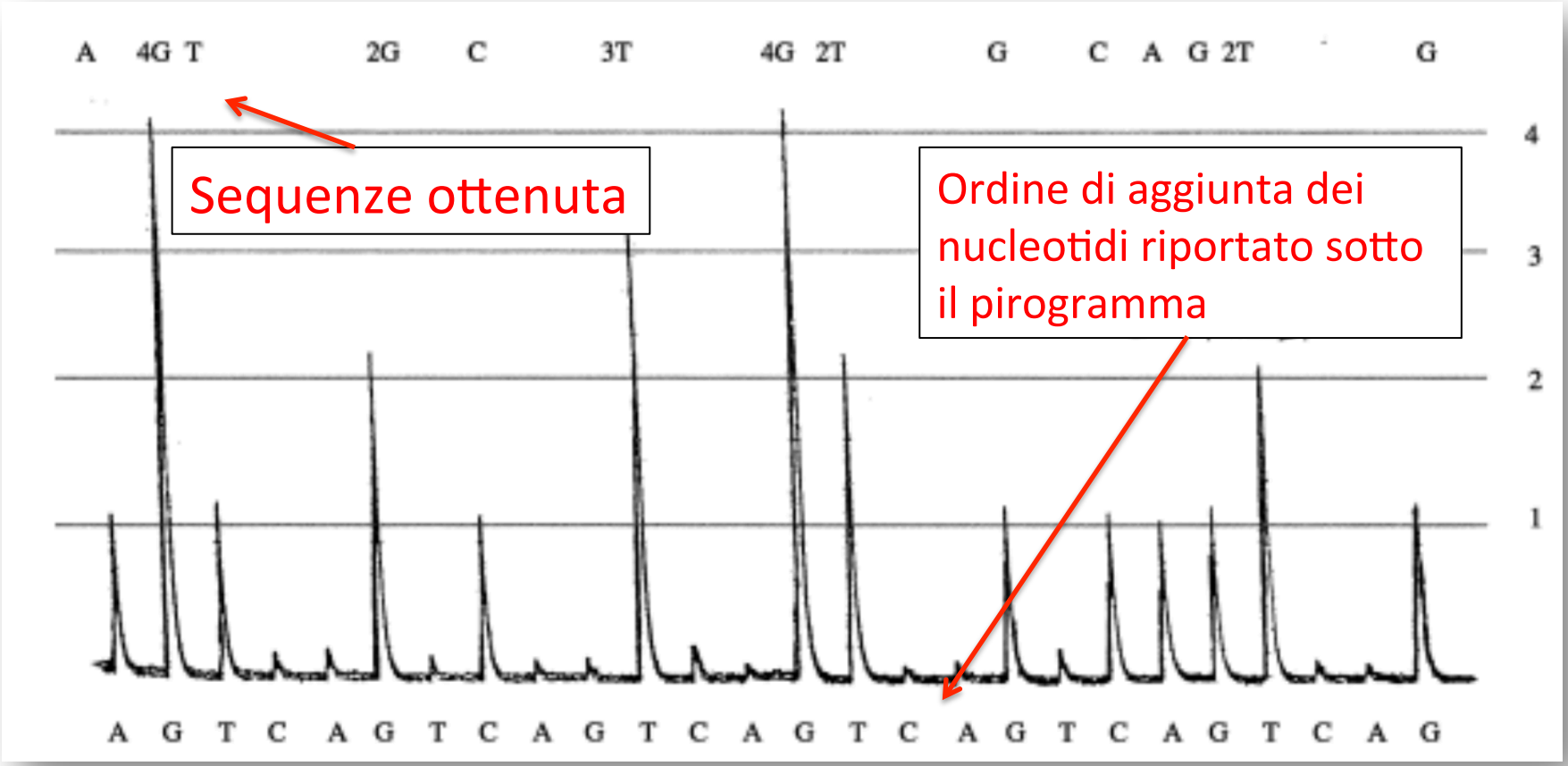


Fig. 1. Schematic representation of the pyrosequencing enzyme system. If the added dNTP forms a base pair with the template, Klenow Polymerase incorporates it into the growing DNA strand and pyrophospate (PPi) is released. ATP Sulfurylase converts the PPi into ATP which serves as substrate for the light producing enzyme Luciferase. The produced light is detected as evidence of that nucleotide incorporation has taken place.

454 Genome Sequencing System (Roche)

. . . AGGGGTGGCTTTGGGGTTGCAGTTG . . .

Pyrogram



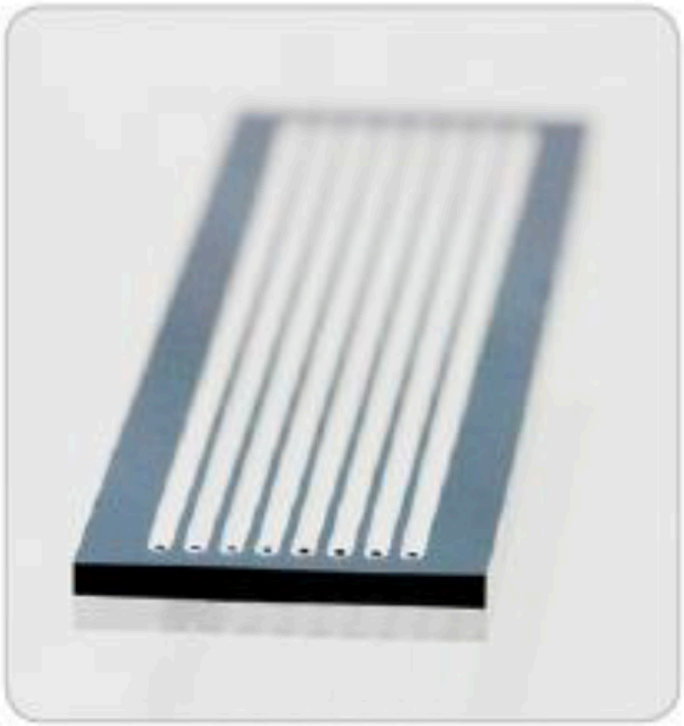
Proportional signals are obtained for one, two, three and four base incorporations.

454 Genome Sequencing System (Roche)

SEE VIDEO

https://www.youtube.com/watch?v=nFfgWGFe0aA

Solexa (2007)



Up to eight samples can be loaded onto the flow cell for simultaneous analysis on the Illumina Genome Analyzer.

- **Sequencing on-a-chip**
- Circa 100 basi per read
- 10 milioni di reads per spot
- Ridotti costi di sequenziamento
- Profondità di sequenziamento elevata

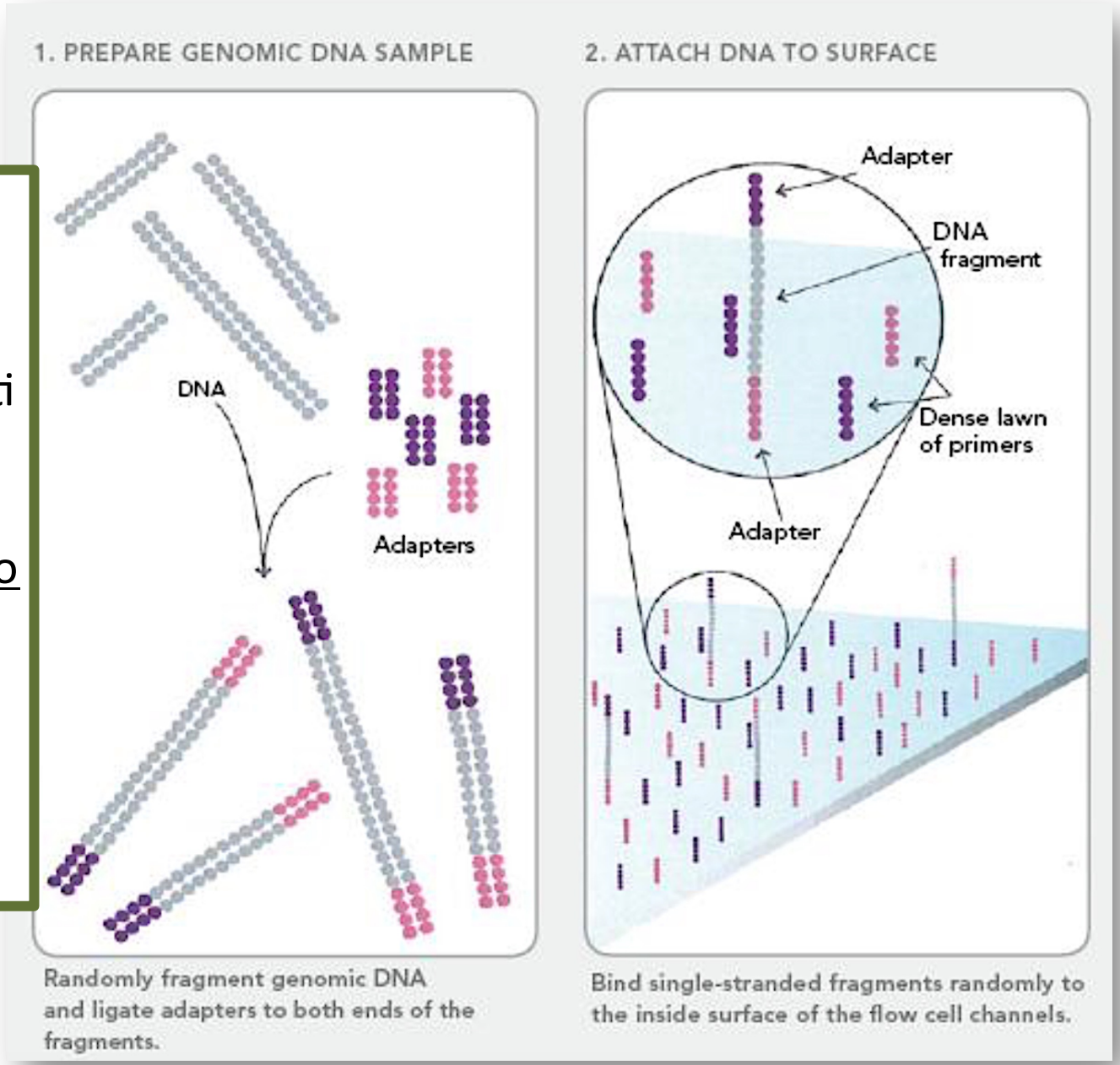
In sintesi:

- **Breaking up** DNA
- Adding **ADAPTORS**, but in this case attach not to a bead but **to a slide**
- **FOLD-BACK PCR** is then used to amplify the fragmented DNA into a cluster

Solexa

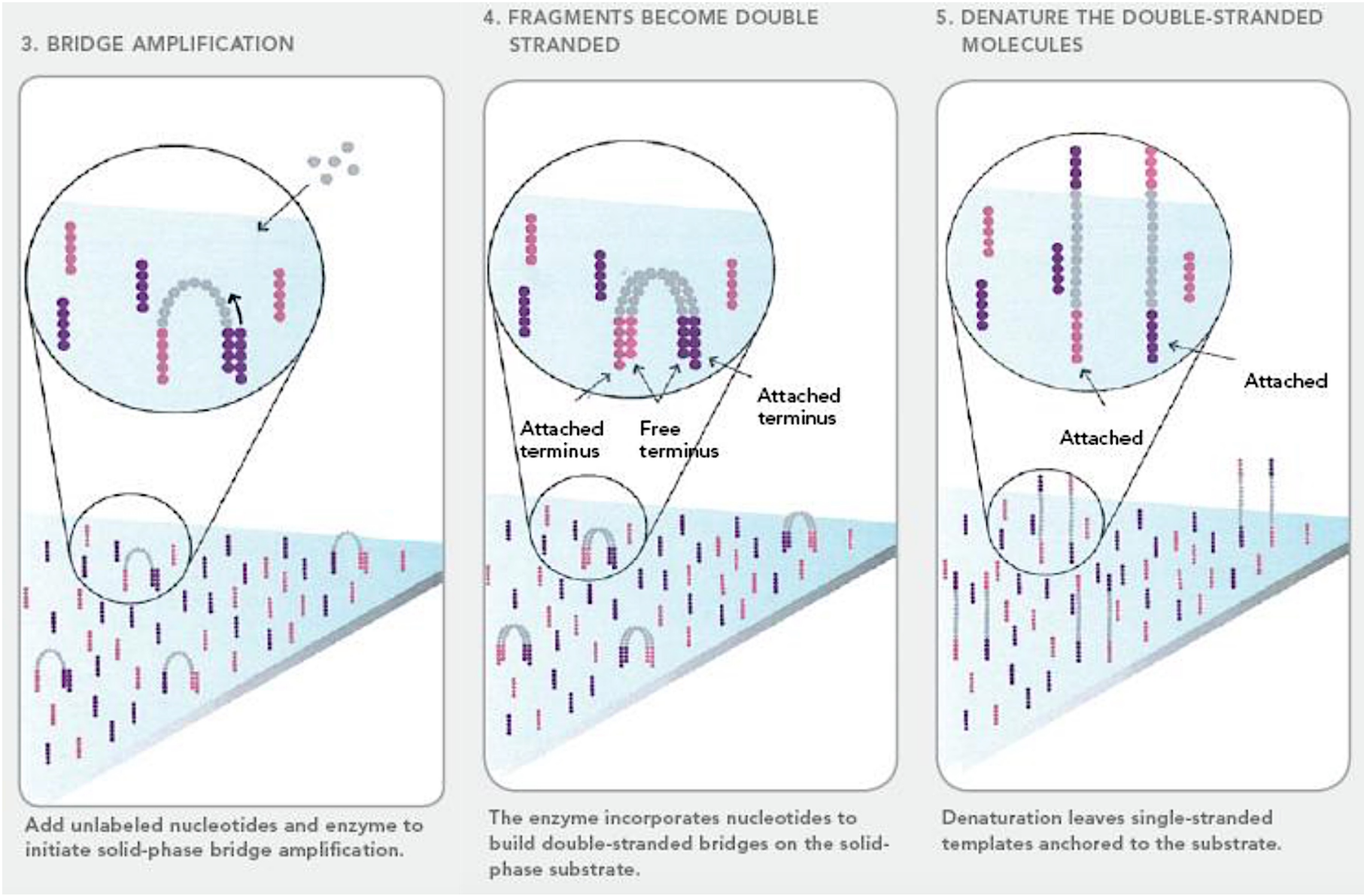
In pratica:

1) **Frammentazione** del DNA e **ligazione** dei frammenti ottenuti a degli adattatori
2) **ANNEALING** degli adattatori <u>a singolo filamento</u> alla superficie sulla quale avverrà la reazione di sequenziamento



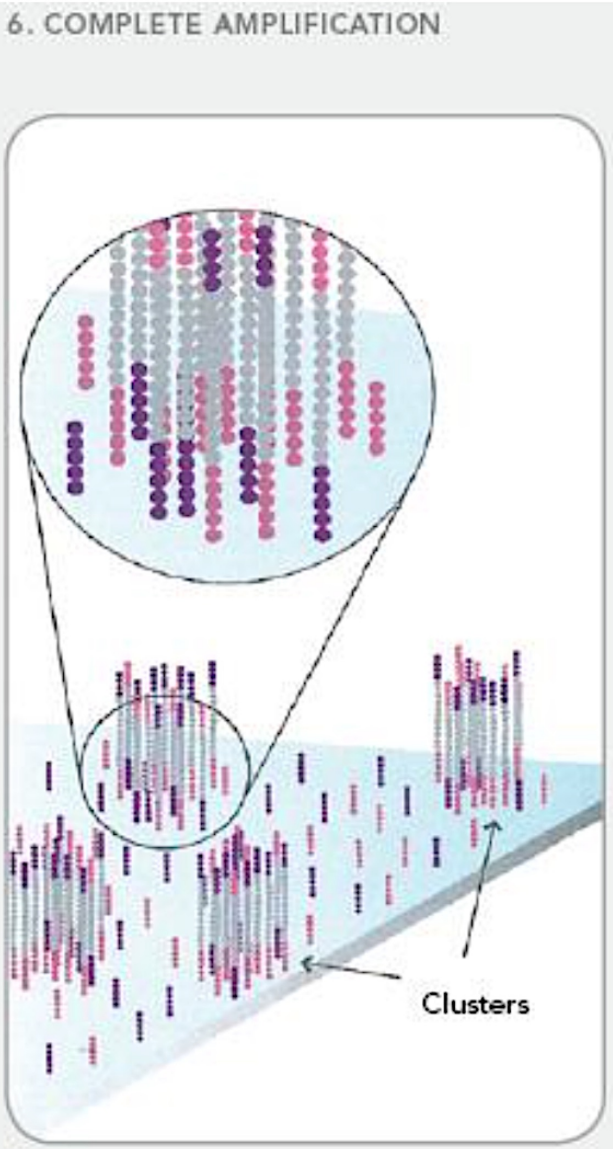
Solexa

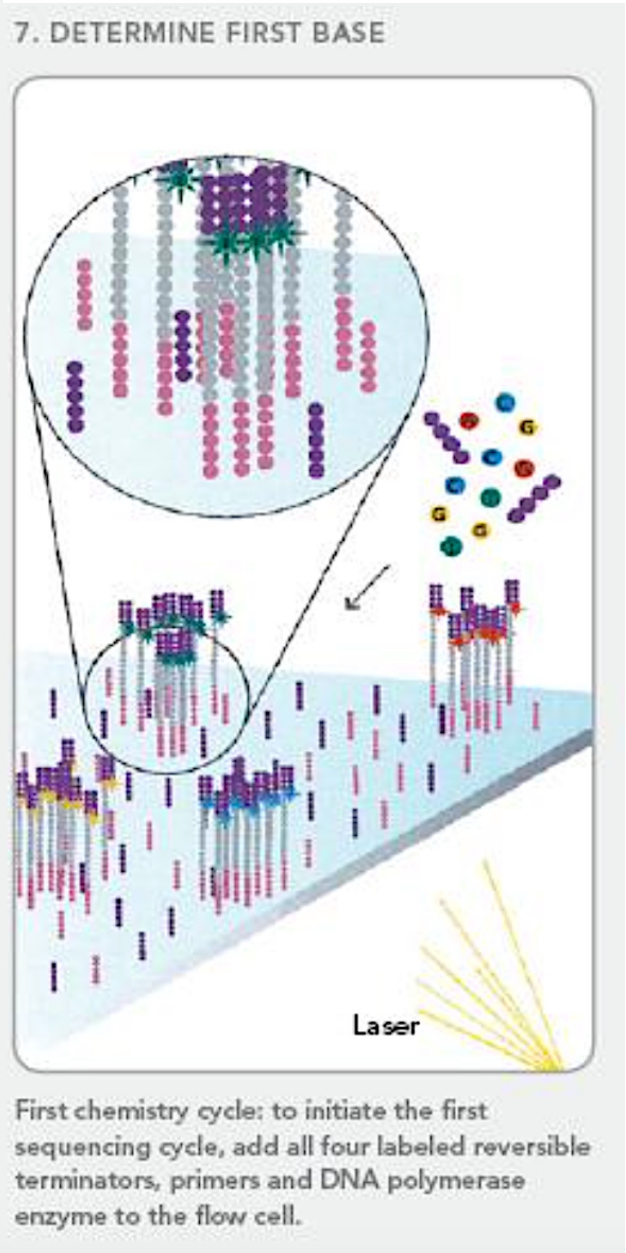
3-5) Amplificazione tramite BRIDGE-PCR



Solexa

6) **Formazione di cluster** densi e separati. Ogni cluster contiene una singlola sequenza
7) Aggiunta di **nucleotidi fluorescenti grazie ad una polimerasi**



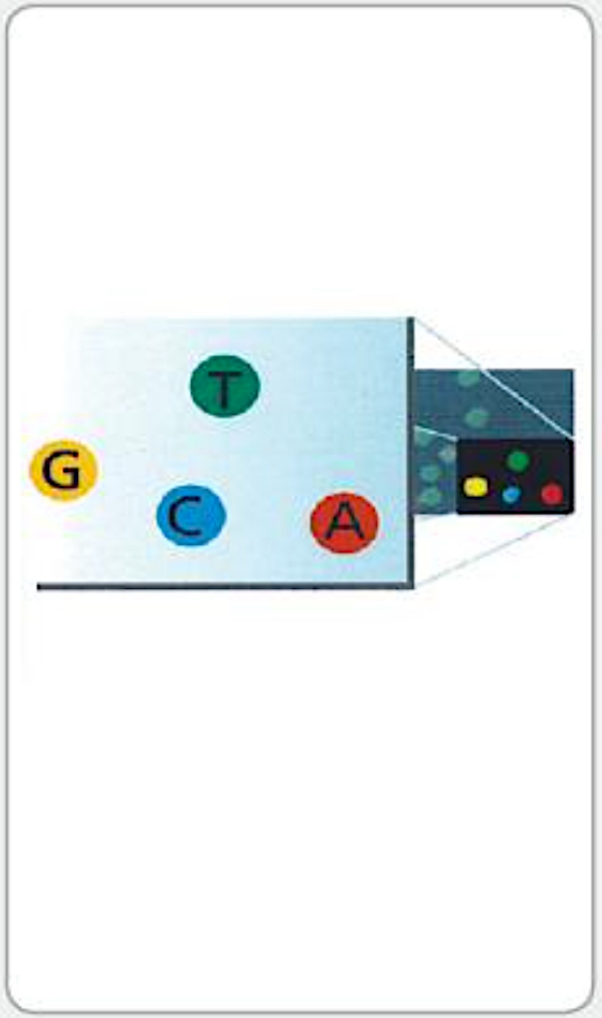


Solexa

8) Lettura della fluorescenza del primo nucleotide

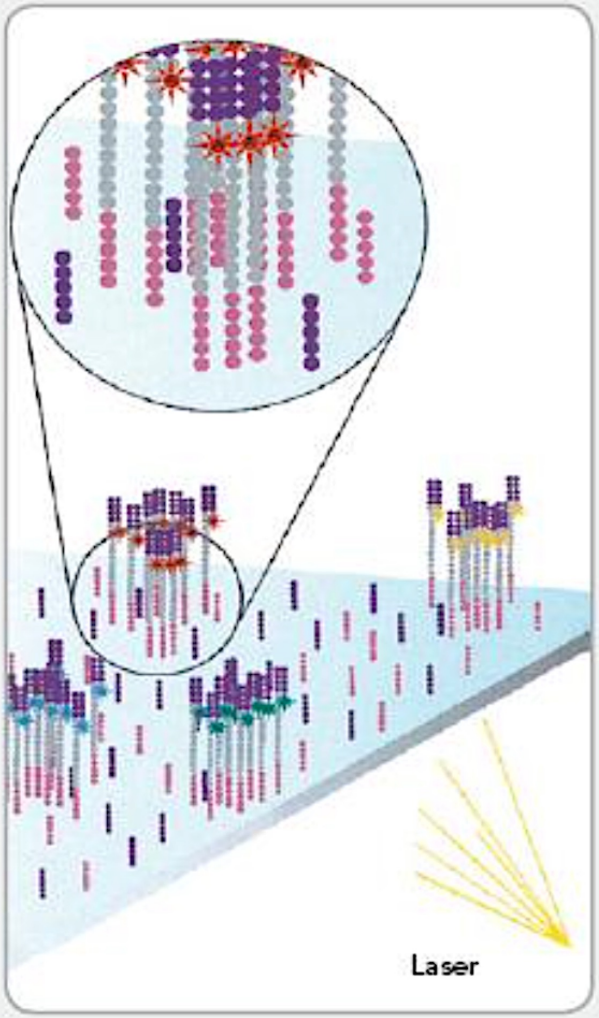
9) Utilizzo di terminatori reversibili (il fluoroforo può essere clivato)

8. IMAGE FIRST BASE



After laser excitation, capture the image of emitted fluorescence from each cluster on the flow cell. Record the identity of the first base for each cluster.

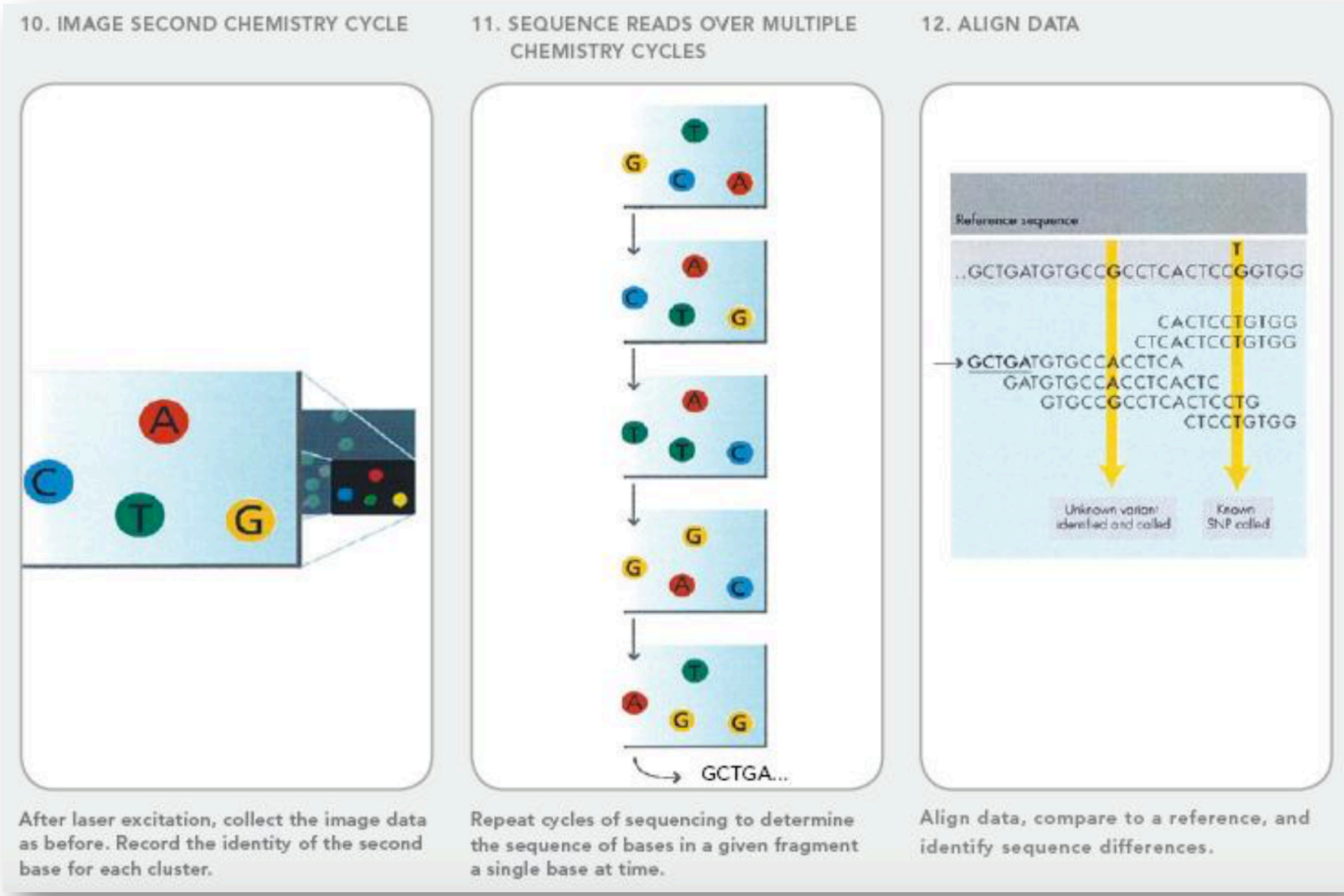
9. DETERMINE SECOND BASE



Second chemistry cycle: to initiate the next sequencing cycle, add all four labeled reversible terminators and enzyme to the flow cell.

Solexa

Lunghezza dei frammenti raggiunta: 100 bp (in pair-end)



Solexa

SEE VIDEO

https://www.youtube.com/watch?v=77r5p8IBwJk

https://www.youtube.com/watch?v=fCd6B5HRaZ8

SOLiD (2008)

- Developed by George M. Church at Harvard University
- Developed by Applied Biosystems, released in 2008
- **Very cost effective** ($0.13/million bases)
- Has trouble with palindromic sequencing

No DNA polimerasi

Utilizzo dell'enzima LIGASI

(***based on the oligonucleotide ligation tecnique***)

SOLiD (2008)

- **Reads di 150 bp massimo (generalmente 35 bp)**
- Massima accuratezza
- Vengono utilizzate sonde con due basi (***two-based encoding system***)
- Ogni base della sequenza viene letta 2 volte

SOLiD (2008)

In Sintesi:

1. LIBRARY PREPARATION:

Pre-amplificazione con emPCR su microsfere

Ligazione delle microsfere su un vetrino

2. LIGATION AND IMAGING

Ligation of the probes

Different cycles

SOLiD (2008)

1) LIBRARY PREPARATION

1a) Fragmentation of gDNA

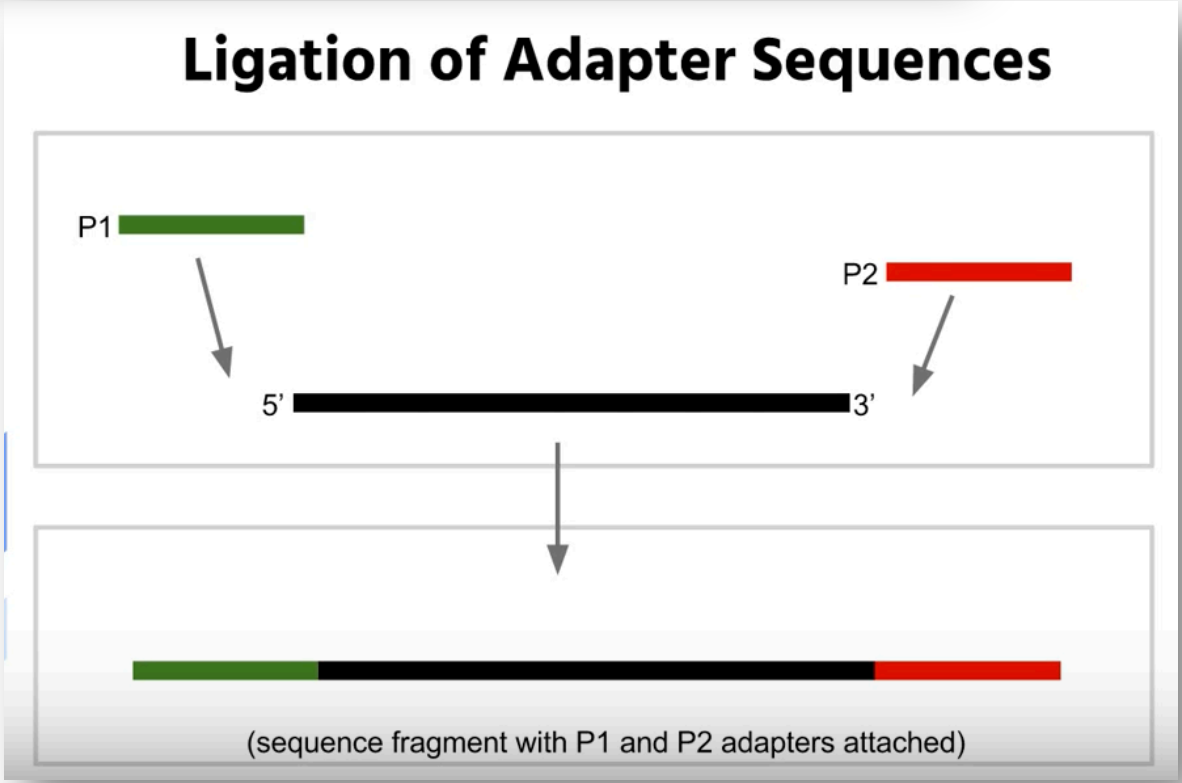
NEBULIZATION	SONICATION	DIGESTION
Compressed nitrogen is used to force DNA through a small hole, creating mechanically sheared fragments	Ultrasonic waves used to create gas bubbles in sample, and shear DNA by resonance vibration	Restriction enzymes used to cleave DNA, reaction kits with enzymes commercially available

SOLiD (2008)

1) LIBRARY PREPARATION

1b) Library Preparation

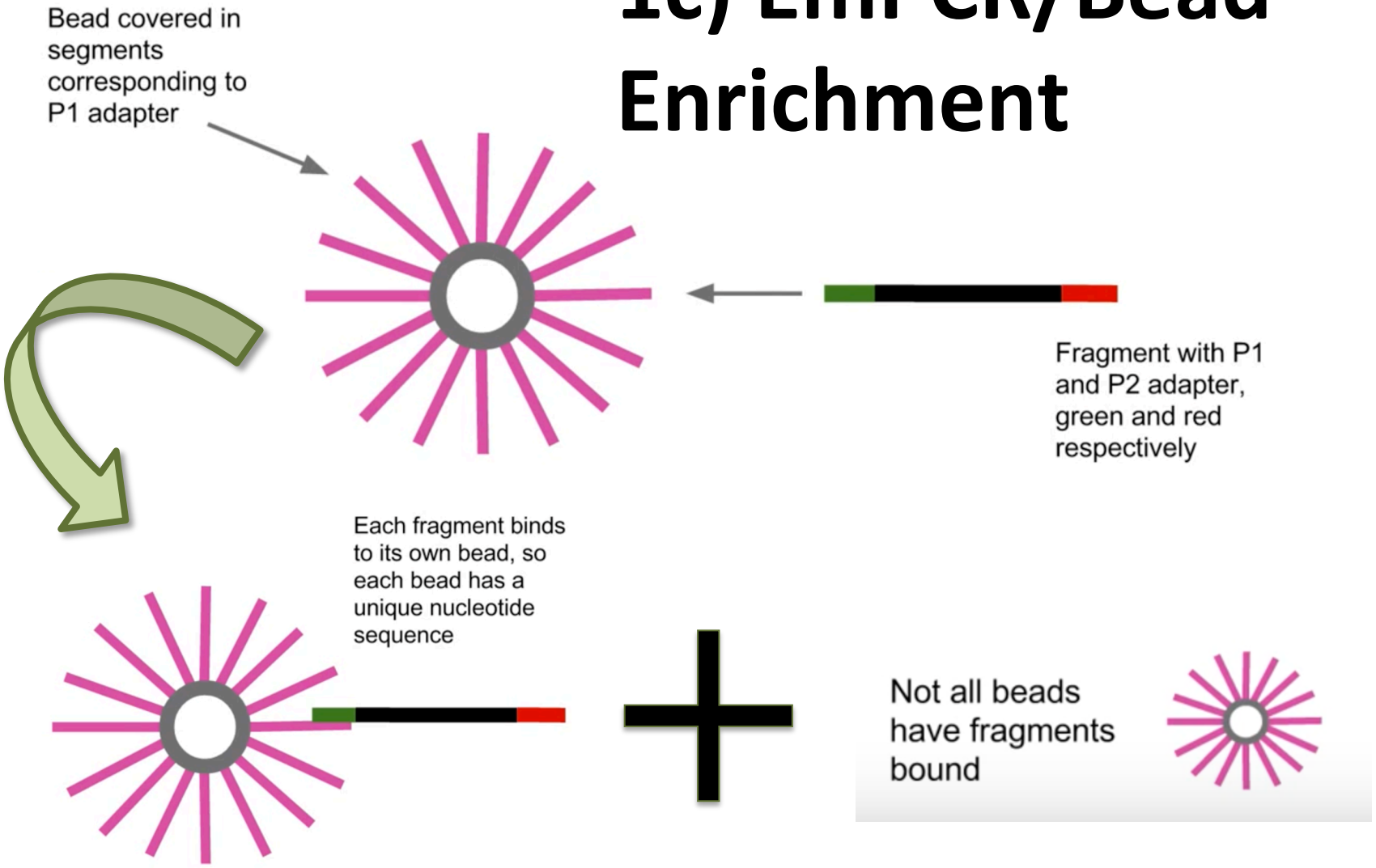
- two types of libraries sequencing-fragment or mate-paired are prepared.



SOLiD (2008)

1) LIBRARY PREPARATION

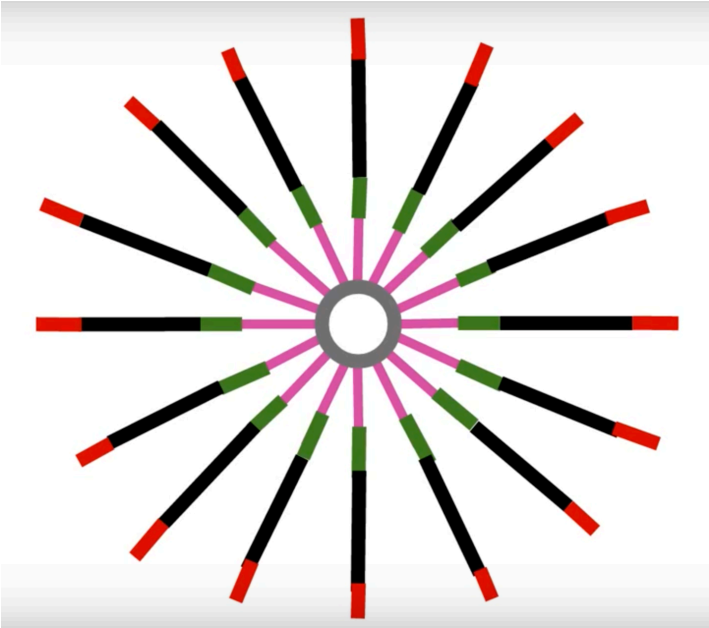
1c) EmPCR/Bead Enrichment



SOLiD (2008)

1) LIBRARY PREPARATION

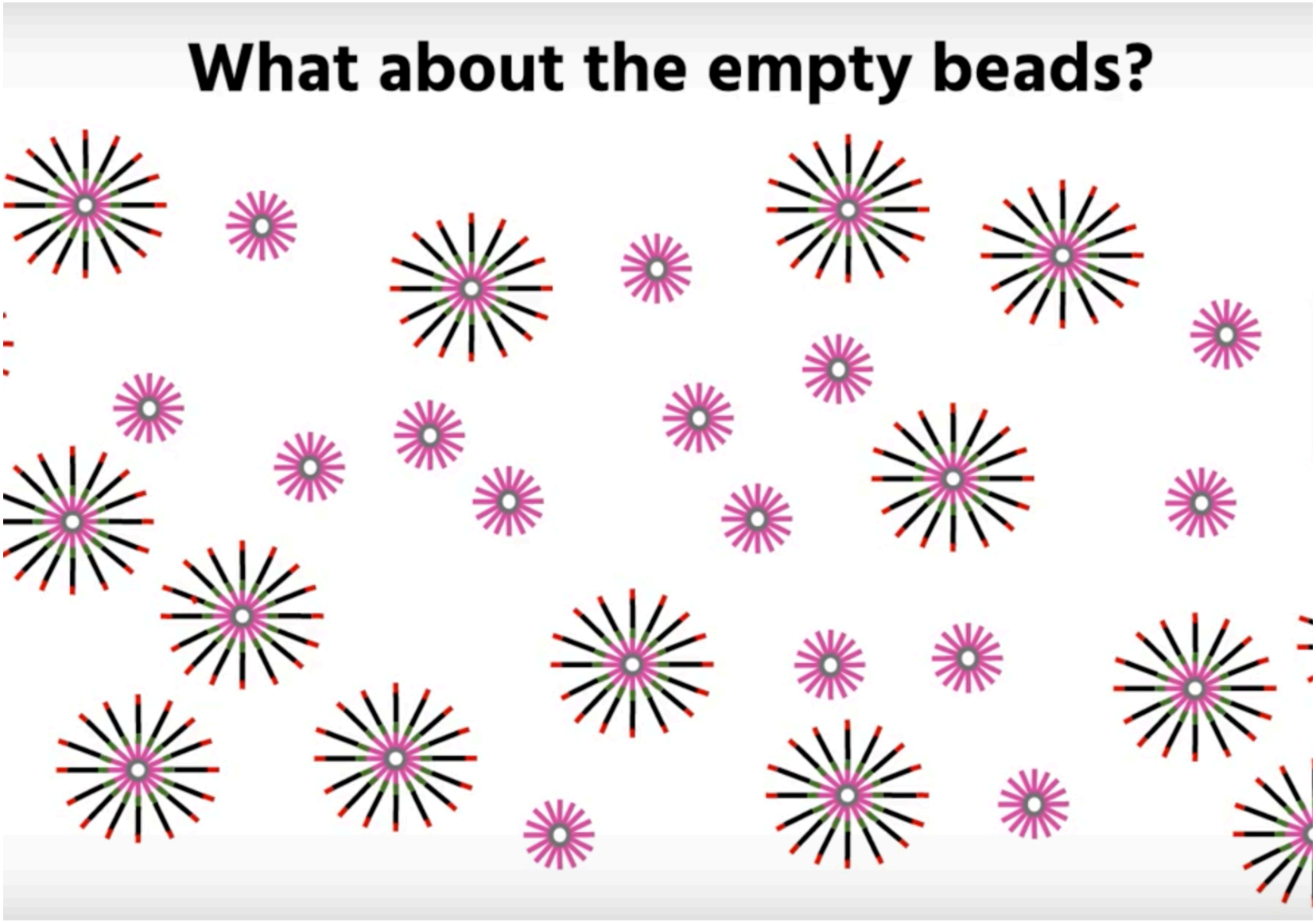
1c) EmPCR/Bead Enrichment



emPCR
Millions of copies of template strand on each bead

SOLiD (2008)

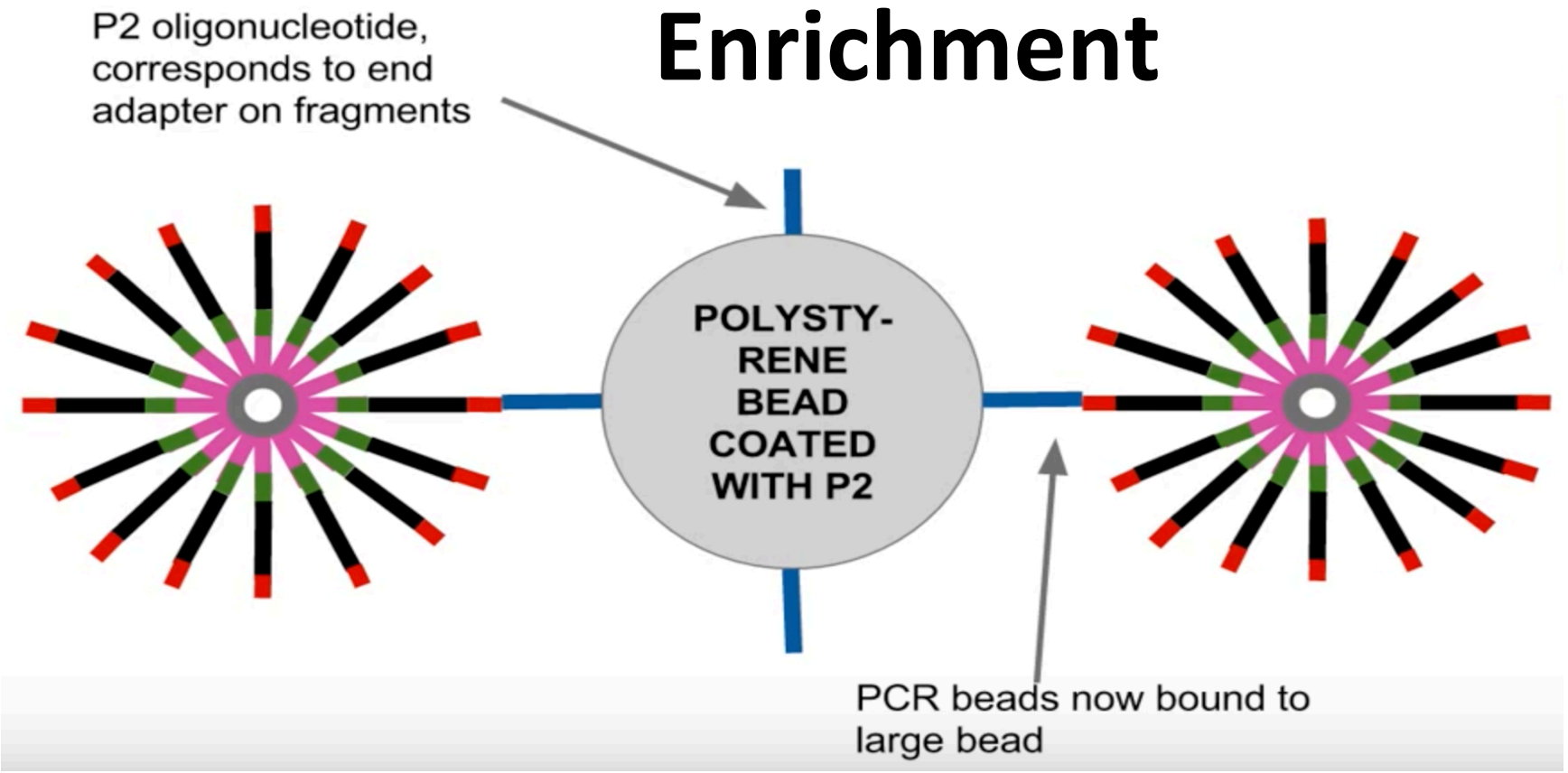
1) LIBRARY PREPARATION



SOLiD (2008)

1) LIBRARY PREPARATION

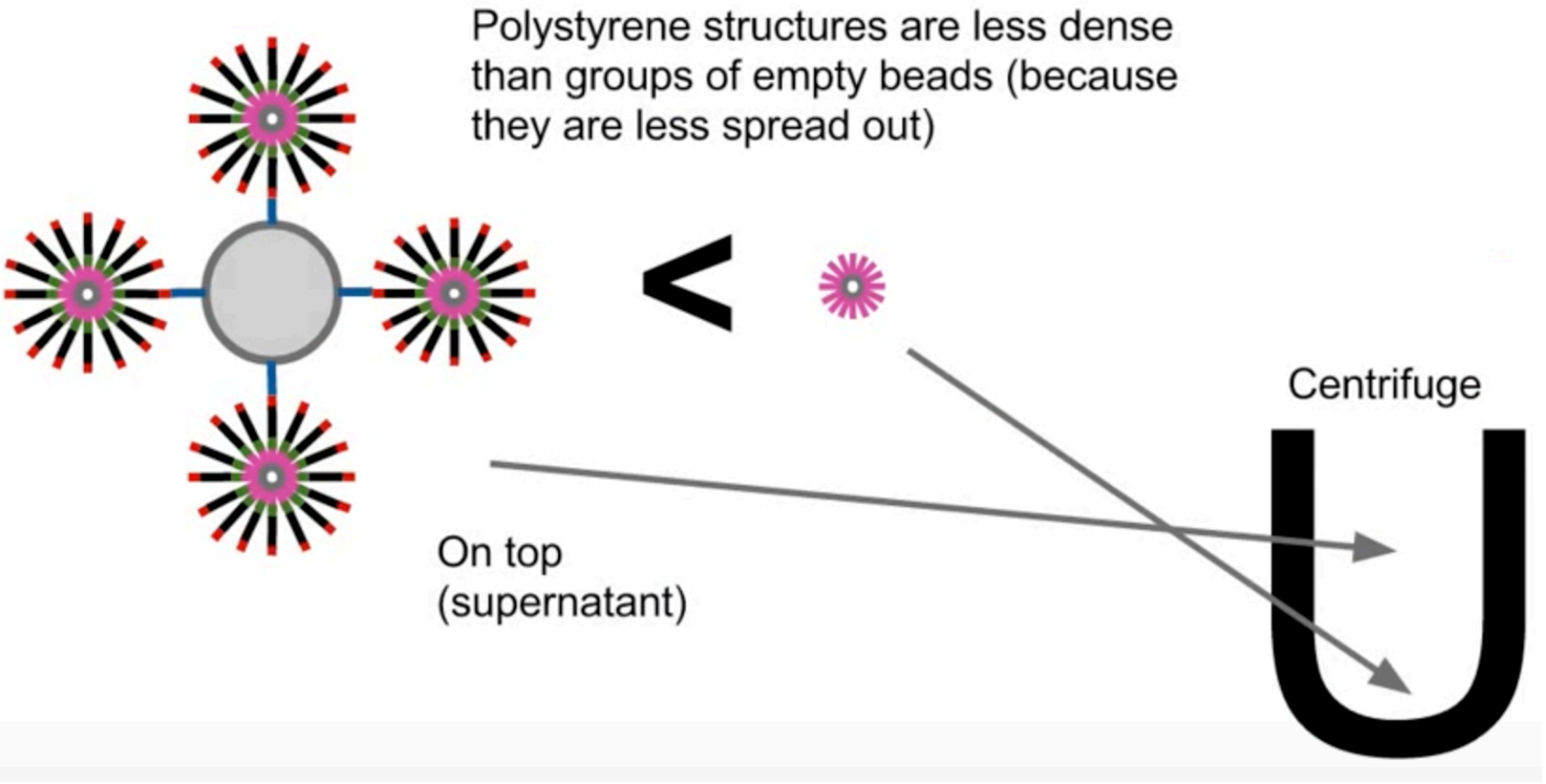
1c) EmPCR/Bead Enrichment



SOLiD (2008)

1) LIBRARY PREPARATION

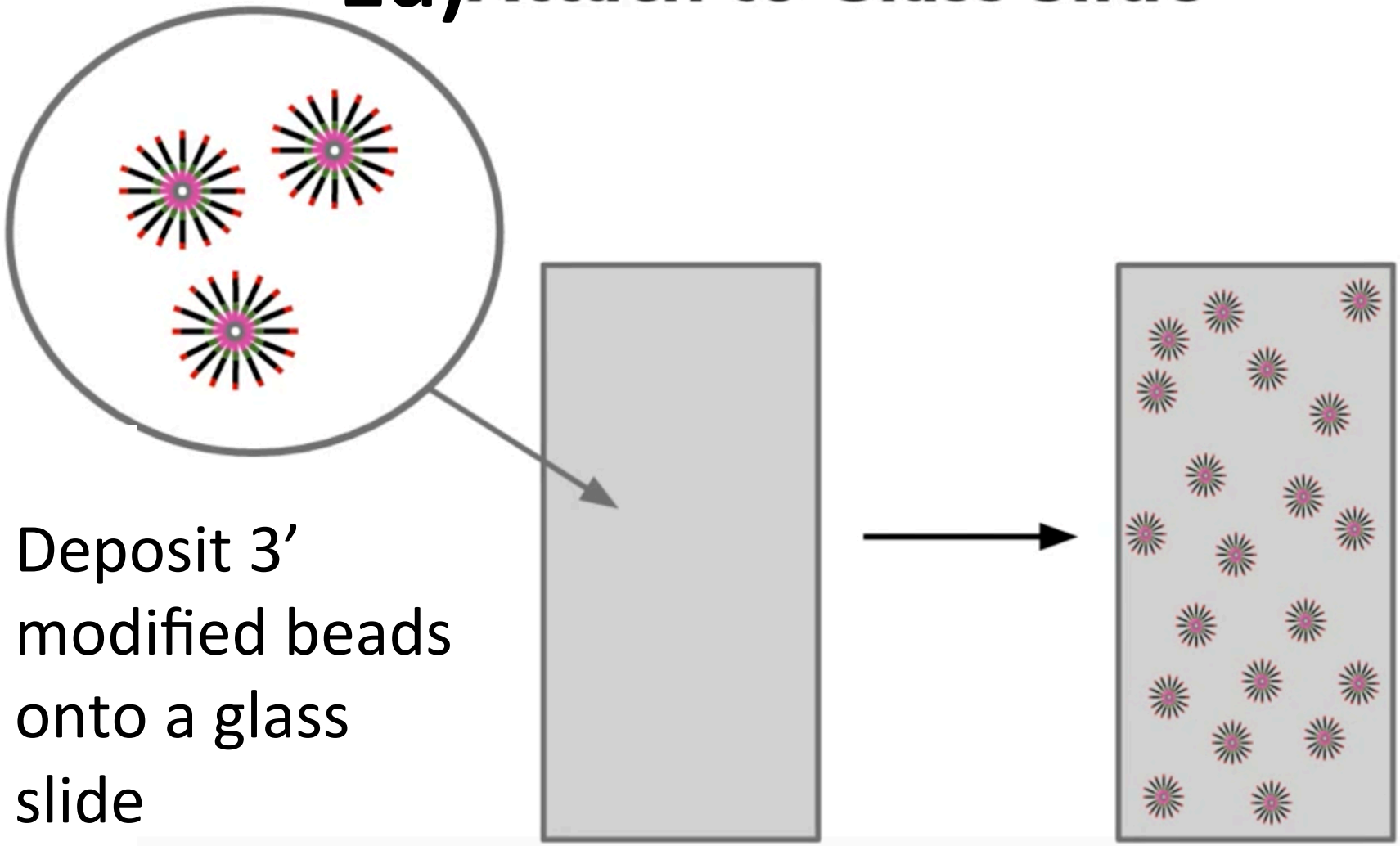
1c) EmPCR/Bead Enrichment



SOLiD (2008)

1) LIBRARY PREPARATION

1d)Attach to Glass Slide



Deposit 3’ modified beads onto a glass slide

SOLiD (2008)

2) LIGATION AND IMAGING

Reagents:

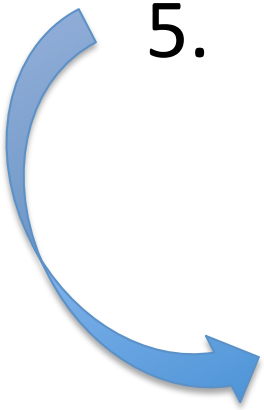
- Template Strands (from PCR)
- Primers (to start reaction)
- **8 nucleotide long probes with dye**
- Ligase to append probes to primers

SOLiD (2008)

2) LIGATION AND IMAGING

Ligation Chemistry Process (Overview):

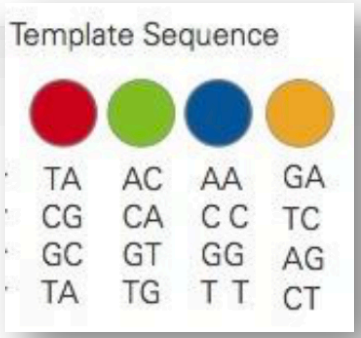
1. Primer binds to template strands
2. Probe hybridization and ligation
3. Fluorescent measured
4. Dye-end nucleotides cleaved
5. Steps 1-4 repeated 6+ times



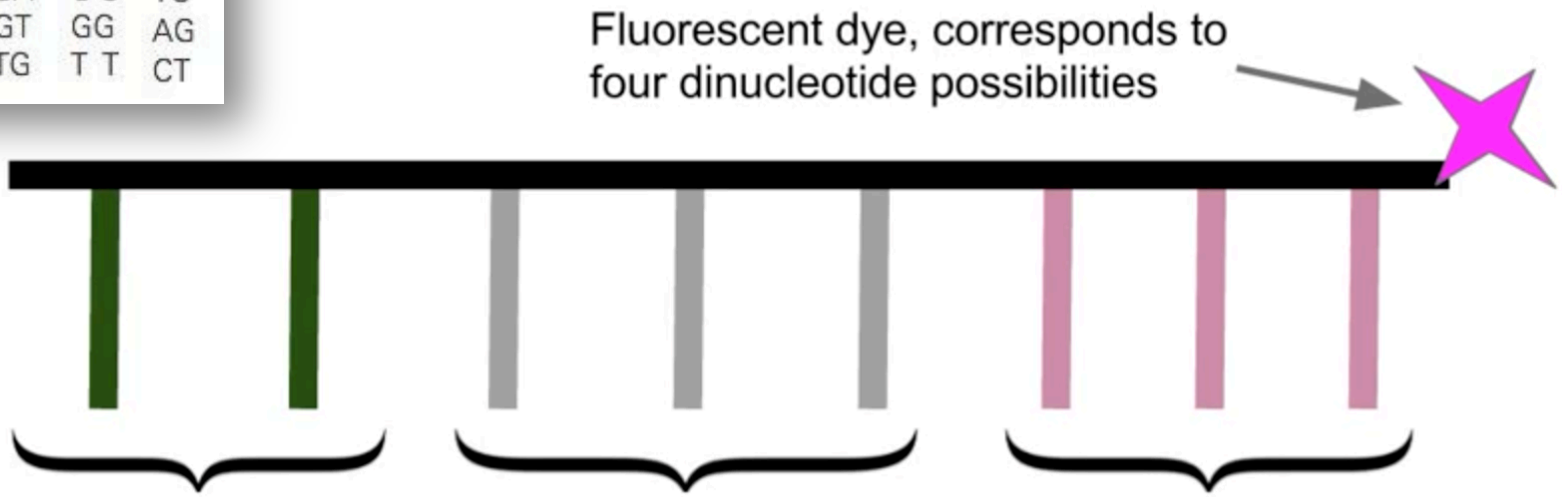
The entire process is completed 5 times.
Each time the primer is offset by 1 base

SOLiD (2008)

2) LIGATION AND IMAGING



Probe Anatomy



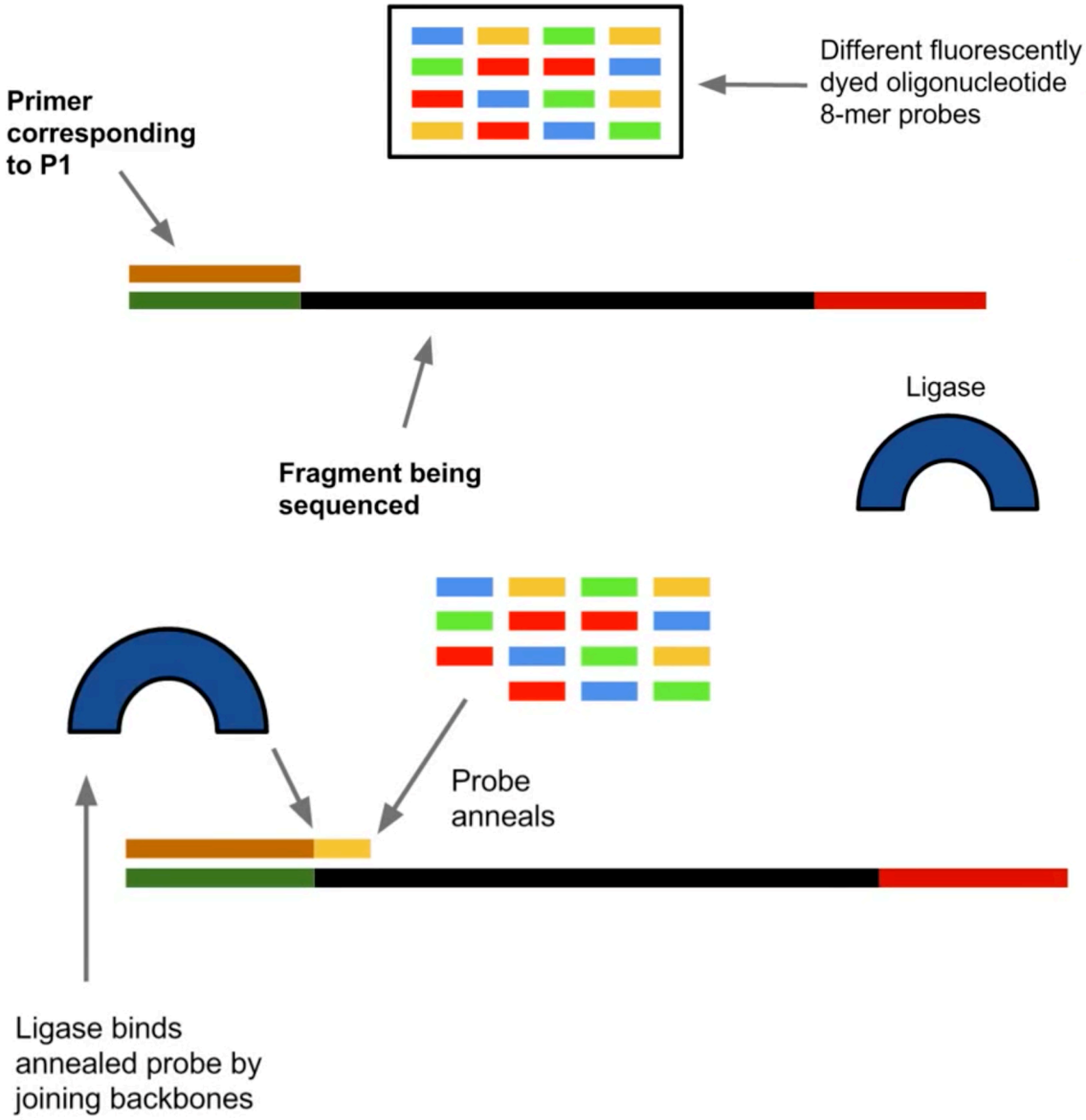
Two actual bases, each dinucleotide permutation corresponds to a dye color (red, green, blue, or yellow/orange) 16 possible dinucleotide permutations

Universal bases, bind to any of the 4 nucleotides

Universal bases with fluorescent dye, measure for fluorescence and cleaved in each cycle, so attached probe is only 5 nucleotides long

SOLiD (2008)

2) LIGATION AND IMAGING

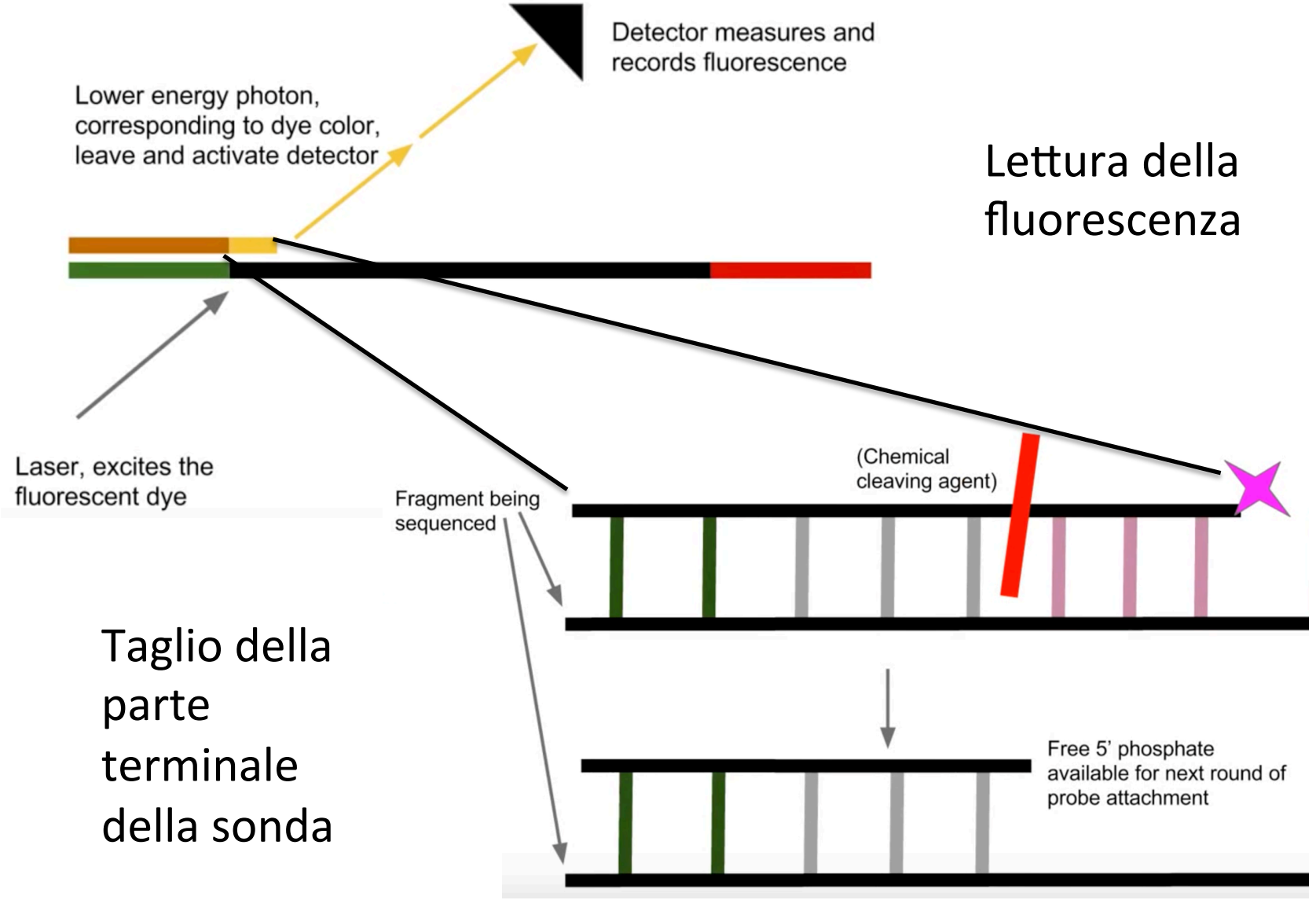


Ligazione del primer all'adattatore

Ligazione della prima sonda per compatibilità delle prime due basi

SOLiD (2008)

2) LIGATION AND IMAGING

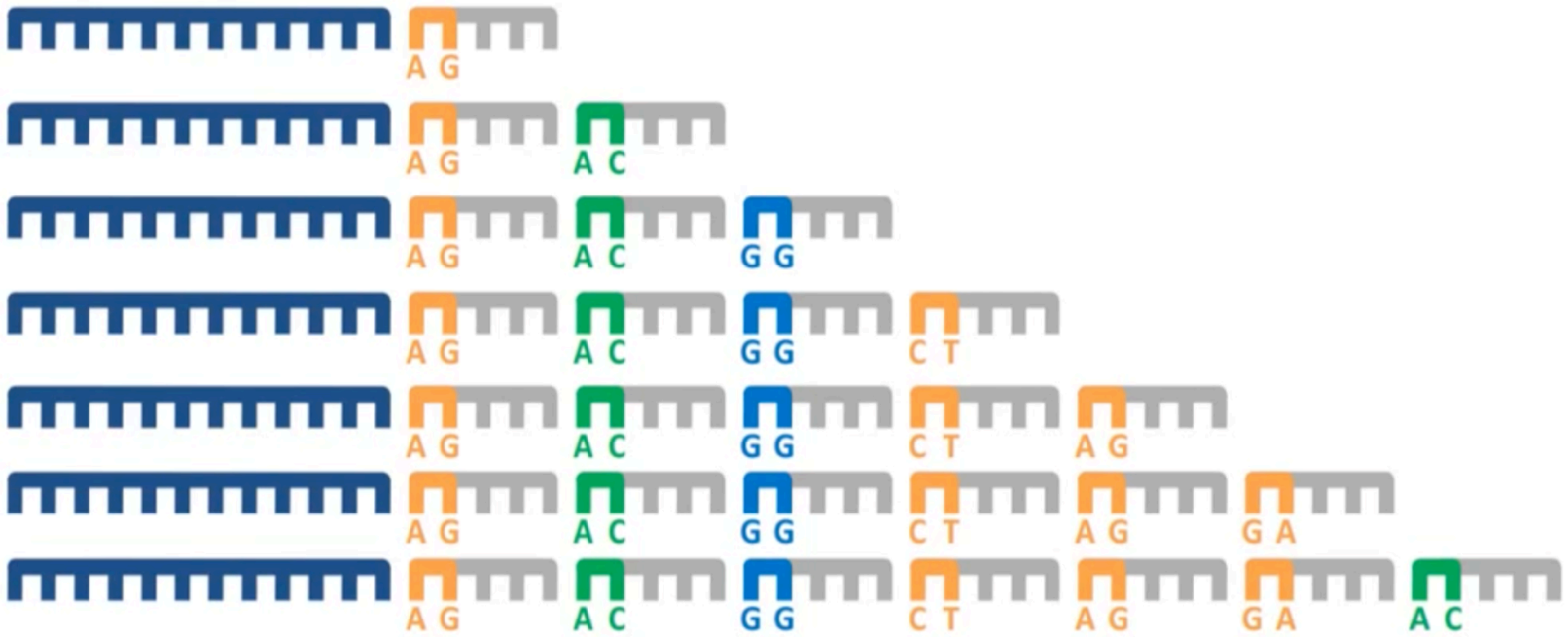


Lettura della fluorescenza

Taglio della parte terminale della sonda

SOLiD (2008)

2) LIGATION AND IMAGING

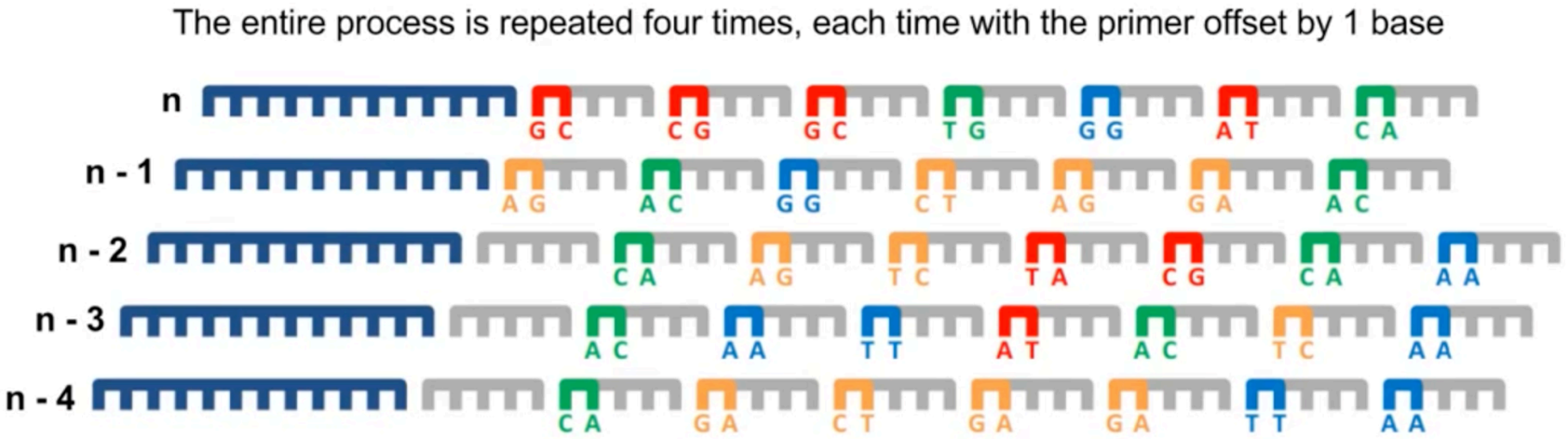


But we only have fluorescence measurements for every 5th base....

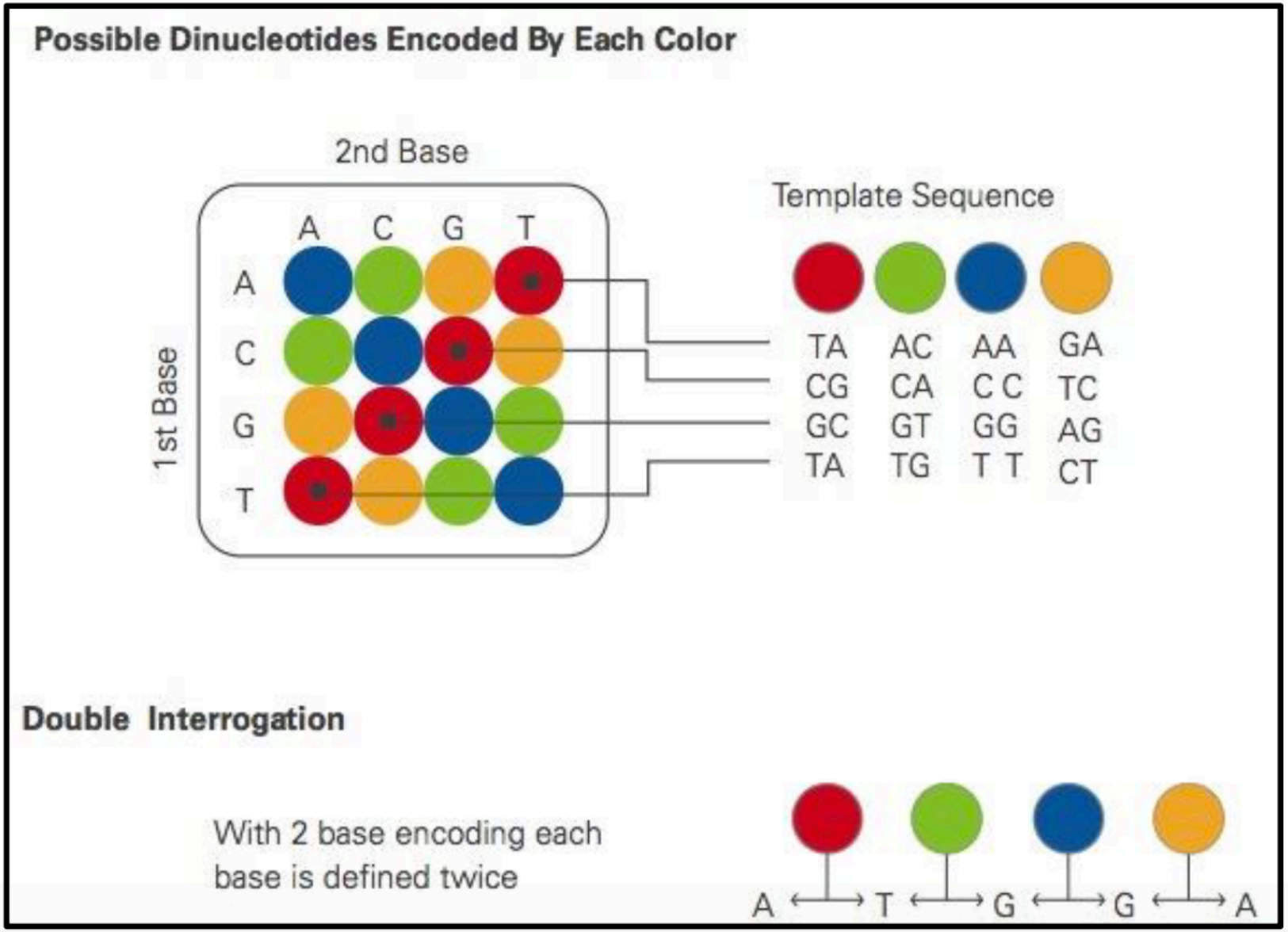
SOLiD (2008)

2) LIGATION AND IMAGING

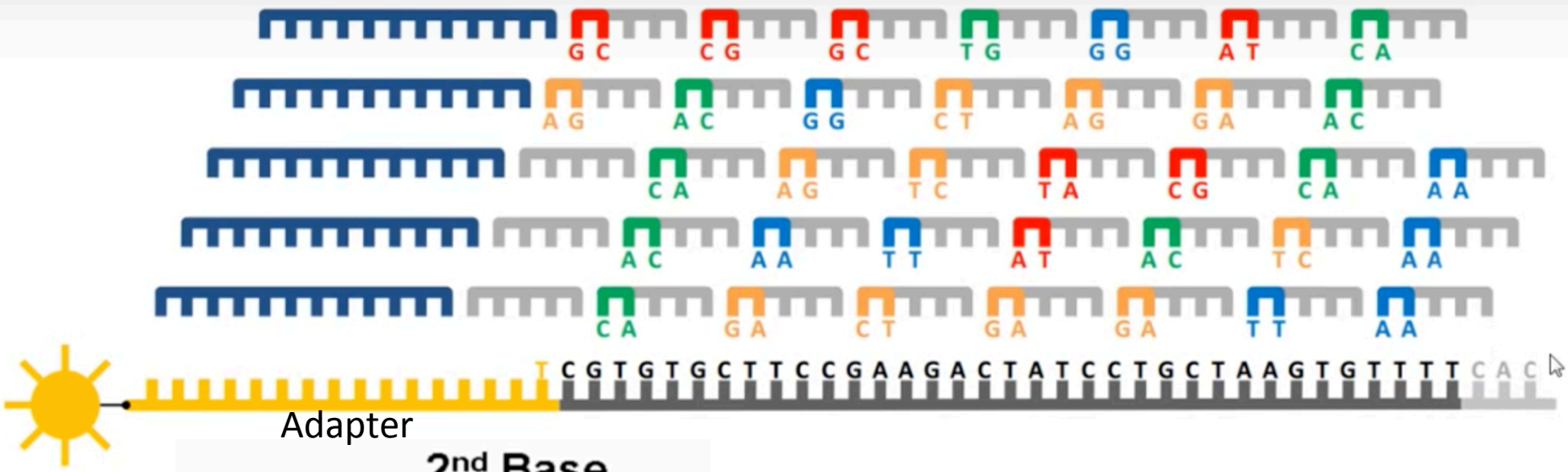
Offset by one base (and do the whole thing over again four times!)

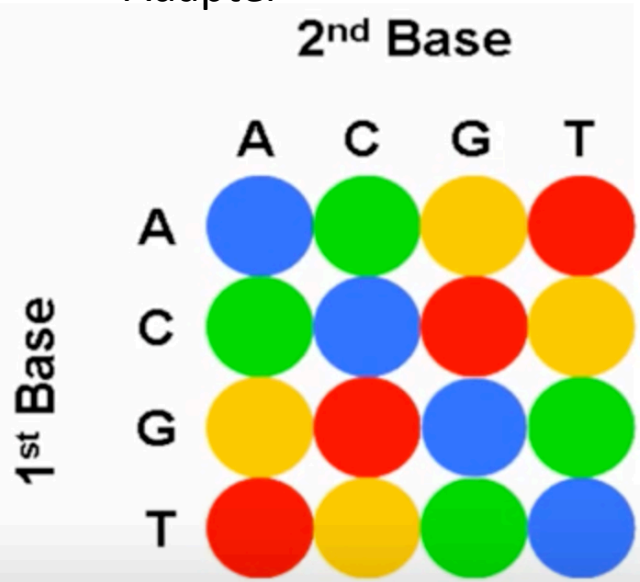


SOLiD (2008)


Possible Dinucleotides Encoded By Each Color
2nd Base
A C G T
1st Base
A
C
G
T
Template Sequence
TA AC AA GA
CG CA C C TC
GC GT GG AG
TA TG T T CT
Double Interrogation
With 2 base encoding each
base is defined twice
A T G G A

SOLiD (2008)





A **base** and a **color** define the next base in the sequence

SOLiD (2008)

SEE VIDEO

https://www.youtube.com/watch?v=nlvyF8bFDwM

https://www.youtube.com/watch?v=YLT-DUeaLms

SOLiD (2008)

Issues with palindromic sequences

What are palindromic sequences?

A sequence of DNA read in the 5' → 3' whose complement is the same when read in the 5' → 3' direction

5' - ATAT - 3'
3' - TATA - 5'

Palindromic

5' - ATTA - 3'
3' - TAAT - 5'

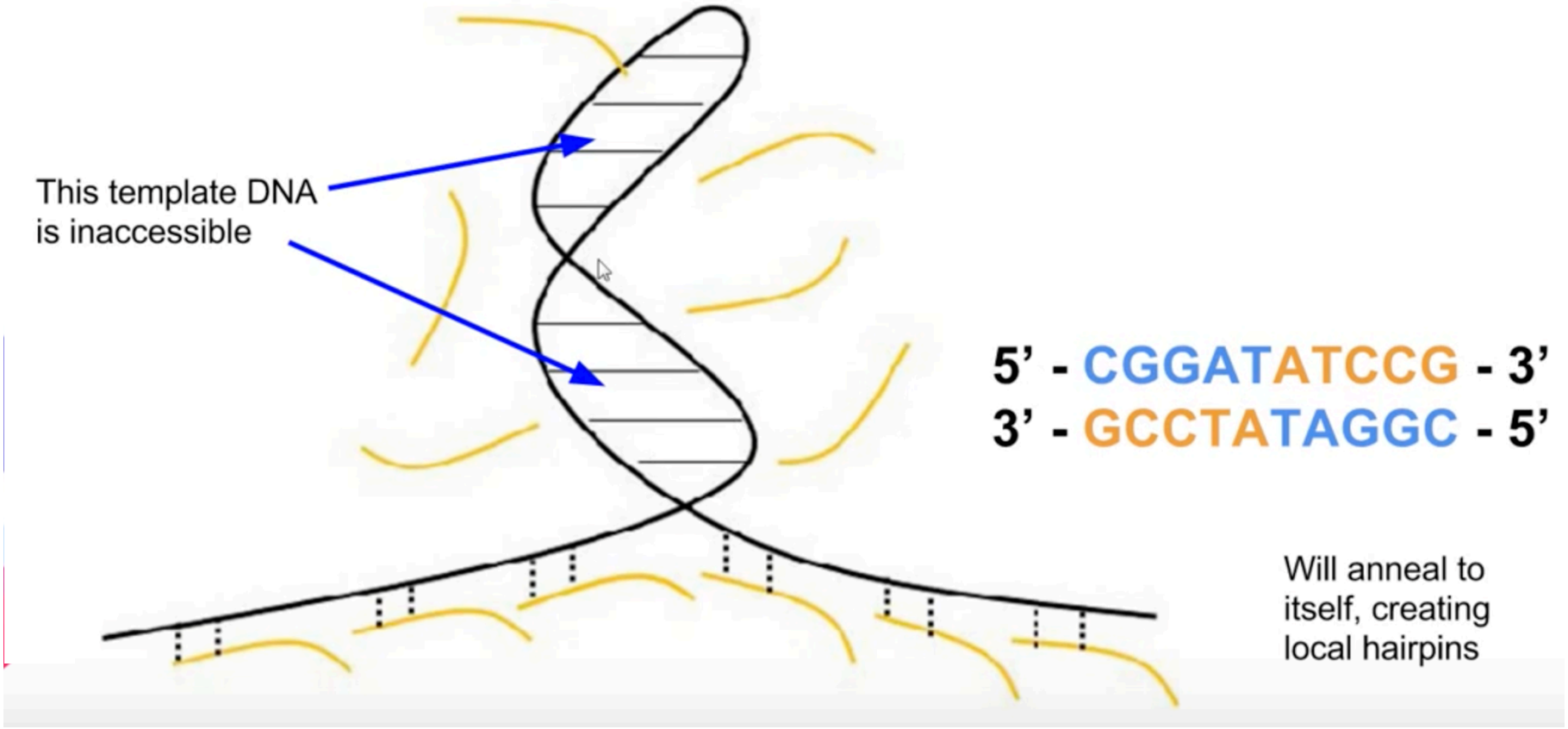
NOT Palindromic

5' - CGGATATCCG - 3'
3' - GCCTATAGGC - 5'

Palindromic

SOLiD (2008)

Issues with palindromic sequences



SOLiD and Solexa

- Generated much larger number of reads than the 454 system (**30 and 100 million reads, respectively**)
- But they were too short (**only 35 bp long in the SOLiD system)**

Ion Semiconductor – Ion Torrent (2011)

- Does not rely on fluorescence
- Maximum read length of up to 400 bp

ARTICLE

An integrated semiconductor device enabling non-optical genome sequencing

Jonathan M. Rothberg[1], Wolfgang Hinz[1], Todd M. Rearick[1], Jonathan Schultz[1], William Mileski[1], Mel Davey[1], John H. Leamon[1], Kim Johnson[1], Mark J. Milgrew[1], Matthew Edwards[1], Jeremy Hoon[1], Jan F. Simons[1], David Marran[1], Jason W. Myers[1], John F. Davidson[1], Annika Branting[1], John R. Nobile[1], Bernard P. Puc[1], David Light[1], Travis A. Clark[1], Martin Huber[1], Jeffrey T. Branciforte[1], Isaac B. Stoner[1], Simon E. Cawley[1], Michael Lyons[1], Yutao Fu[1], Nils Homer[1], Marina Sedova[1], Xin Miao[1], Brian Reed[1], Jeffrey Sabina[1], Erika Feierstein[1], Michelle Schorn[1], Mohammad Alanjary[1], Eileen Dimalanta[1], Devin Dressman[1], Rachel Kasinskas[1], Tanya Sokolsky[1], Jacqueline A. Fidanza[1], Eugeni Namsaraev[1], Kevin J. McKernan[1], Alan Williams[1], G. Thomas Roth[1] & James Bustillo[1]

The seminal importance of DNA sequencing to the life sciences, biotechnology and medicine has driven the search for more scalable and lower-cost solutions. Here we describe a DNA sequencing technology in which scalable, low-cost semiconductor manufacturing techniques are used to make an integrated circuit able to directly perform non-optical DNA sequencing of genomes. Sequence data are obtained by directly sensing the ions produced by template-directed DNA polymerase synthesis using all-natural nucleotides on this massively parallel semiconductor-sensing device or ion chip. The ion chip contains ion-sensitive, field-effect transistor-based sensors in perfect register with 1.2 million wells, which provide confinement and allow parallel, simultaneous detection of independent sequencing reactions. Use of the most widely used technology for constructing integrated circuits, the complementary metal-oxide semiconductor (CMOS) process, allows for low-cost, large-scale production and scaling of the device to higher densities and larger array sizes. We show the performance of the system by sequencing three bacterial genomes, its robustness and scalability by producing ion chips with up to 10 times as many sensors and sequencing a human genome.

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Ion Semiconductor – Ion Torrent

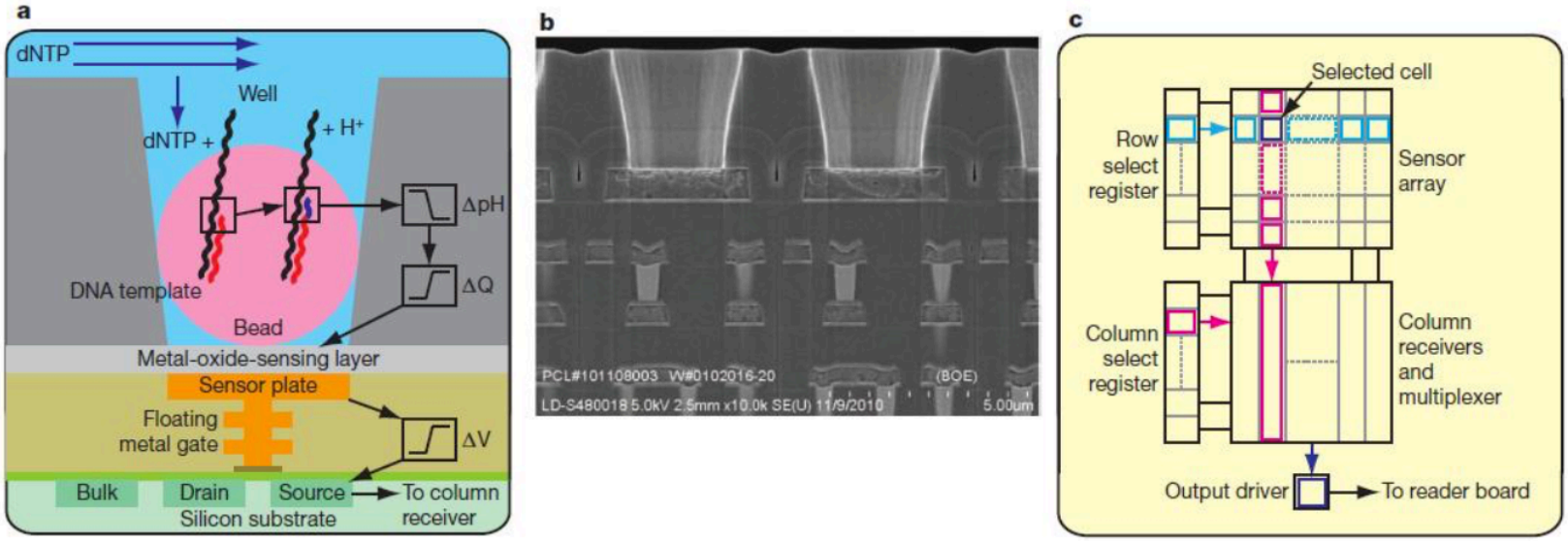


Figure 1 | Sensor, well and chip architecture. a, A simplified drawing of a well, a bead containing DNA template, and the underlying sensor and electronics. Protons (H^+) are released when nucleotides (dNTP) are incorporated on the growing DNA strands, changing the pH of the well (ΔpH). This induces a change in surface potential of the metal-oxide-sensing layer, and a change in potential (ΔV) of the source terminal of the underlying field-effect transistor. b, Electron micrograph showing alignment of the wells over the ISFET metal sensor plate and the underlying electronic layers. c, Sensors are arranged in a two-dimensional array. A row select register enables one row of sensors at a time, causing each sensor to drive its source voltage onto a column. A column select register selects one of the columns for output to external electronics.

- Amplificazione tramite *emPCR* su microsfere (intrappolate in micropozzetti)
- Utilizzo di normali dNTP
- L'incorporazione di un dNTP rilascia un protone (H+)
- Rilevazione della differenza di voltaggio (ΔV)
- ΔV proporzionale al numero di nucleotidi incorporati

Ion Semiconductor – Ion Torrent

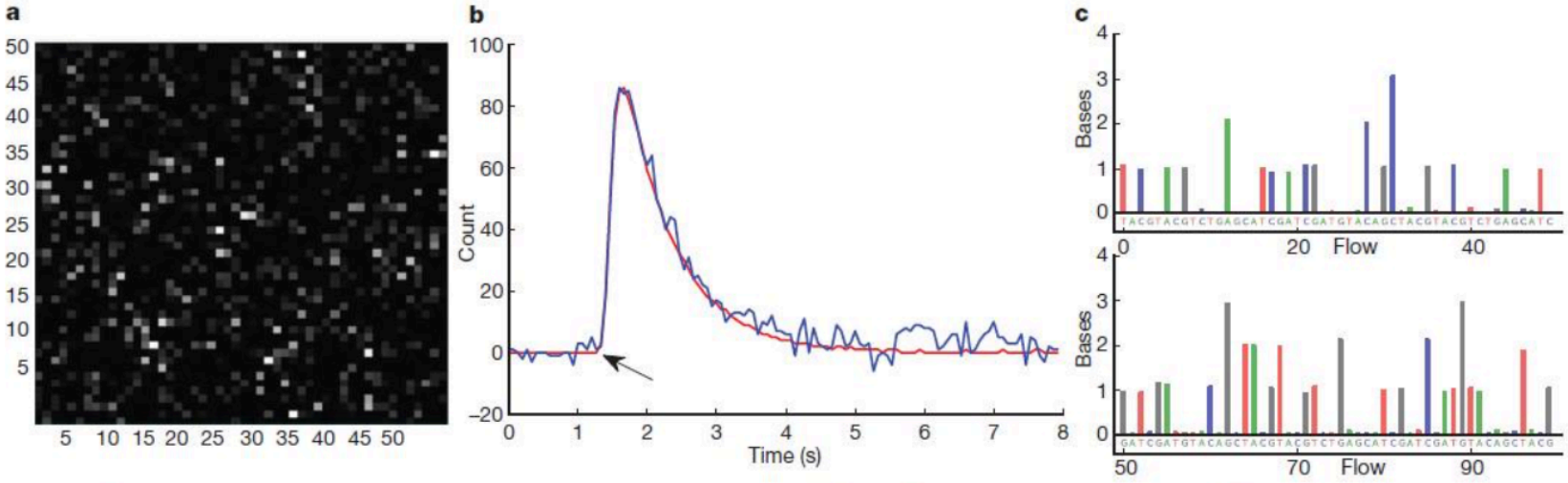


Figure 3 | Data collection and base calling. a, A 50 × 50 region of the ion chip. The brightness represents the intensity of the incorporation reaction in individual sensor wells. b, 1-nucleotide incorporation signal from an individual sensor well; the arrow indicates start of incorporation event, with the physical model (red line) and background corrected data (blue line) shown. c, The first 100 flows from one well. Each coloured bar indicates the corresponding number of bases incorporated during that nucleotide flow.

Lunghezza dei frammenti raggiunta: oltre 100 basi

Previsto nuovo CMOS chip da 400 basi entro dicembre 2011

Application of NGS

- Variants discovery in targeted region or whole genome by re-sequencing
- Reassembling genome of lower organism by *de novo* method.
- Cost-effective sequencing of complex samples at remarkable scale and speed.
- Sequencing entire transcriptome.
- In Meta genomics : Sequencing genome of entire biological communities
- Replacing ChIP-on-chip with ChIP-seq in case of multicellular eukaryotes.
- Personalized genome for personalized medicine

Advantages of NGS

- **Do not require bacterial cloning** of DNA fragments
- Rely on the preparation of NGS libraries in a **cell-free system**
- They can **parallelize** the thousands-to-many-millions of sequencing reactions
- The sequencing output is directly detected with **no need for electhroforesis**

Disvantages of NGS

- **Short read lengths** which has resulted in difficulties in subsequent sequence splicing, assembly, annotation and bioinformatic analysis
- **Efficiency of the starter PCR can be affected by complex structures** of the genome and the complete genomic sequencing cannot be represented in the library

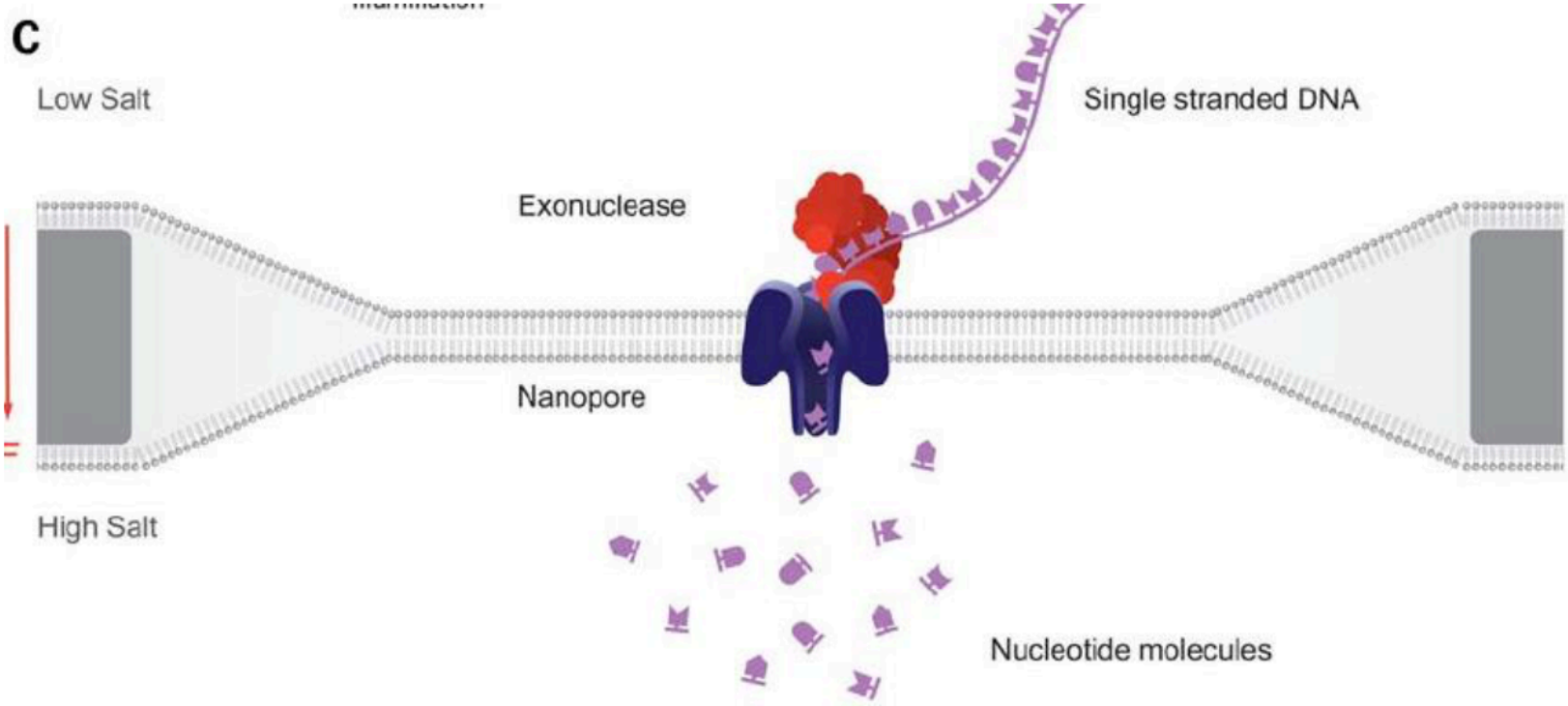
Generazione 3

Oxford Nanopore

PacBio – Single Molecule Real Time sequencing (SMRT)

- Sequenziamento a singola molecola di DNA
- Amplificazione iniziale tramite PCR non necessaria
- Riduzione di *bias* dovuti all'amplificazione
  (amplificazione differenziale di alcune parti della sequenza)

Oxford Nanopore



- Tecnologia ancora molto sperimentale
- Vari metodi per determinare i singoli nucleotidi

PacBio RS - SMRT Technology

Real-Time DNA Sequencing from Single Polymerase Molecules

John Eid,* Adrian Fehr,* Jeremy Gray,* Khai Luong,* John Lyle,* Geoff Otto,* Paul Peluso,* David Rank,* Primo Baybayan, Brad Bettman, Arkadiusz Bibillo, Keith Bjornson, Bidhan Chaudhuri, Frederic[illegible]ns, Ronald Cicero, Sonya Clark, Ravindra Dalal, Alex deWinter, John Dixon, [illegible] Foquet, Alfred Gaertner, Paul Hardenbol, Cheryl Heiner, Kevin Hester, David Holden, Gregory Kearns, Xiangxu Kong, Ronald Kuse, Yves Lacroix, Steven Lin, Paul Lundquist, Congcong Ma, Patrick Marks, Mark Maxham, Devon Murphy, Insil Park, Thang Pham, Michael Phillips, Joy Roy, Robert Sebra, Gene Shen, Jon Sorenson, Austin Tomaney, Kevin Travers, Mark Trulson, John Vieceli, Jeffrey Wegener, Dawn Wu, Alicia Yang, Denis Zaccarin, Peter Zhao, Frank Zhong, Jonas Korlach,† Stephen Turner†

Ruota

We present single-molecule, real-time sequencing data obtained from a DNA polymerase performing uninterrupted template-directed synthesis using four distinguishable fluorescently labeled deoxyribonucleoside triphosphates (dNTPs). We detected the temporal order of their enzymatic incorporation into a growing DNA strand with zero-mode waveguide nanostructure arrays, which provide optical observation volume confinement and enable parallel, simultaneous detection of thousands of single-molecule sequencing reactions. Conjugation of fluorophores to the terminal phosphate moiety of the dNTPs allows continuous observation of DNA synthesis over thousands of bases without steric hindrance. The data report directly on polymerase dynamics, revealing distinct polymerization states and pause sites corresponding to DNA secondary structure. Sequence data were aligned with the known reference sequence to assay biophysical parameters of polymerization for each template position. Consensus sequences were generated from the single-molecule reads at 15-fold coverage, showing a median accuracy of 99.3%, with no systematic error beyond fluorophore-dependent error rates.

www.sciencemag.org **SCIENCE** VOL 323 2 JANUARY 2009

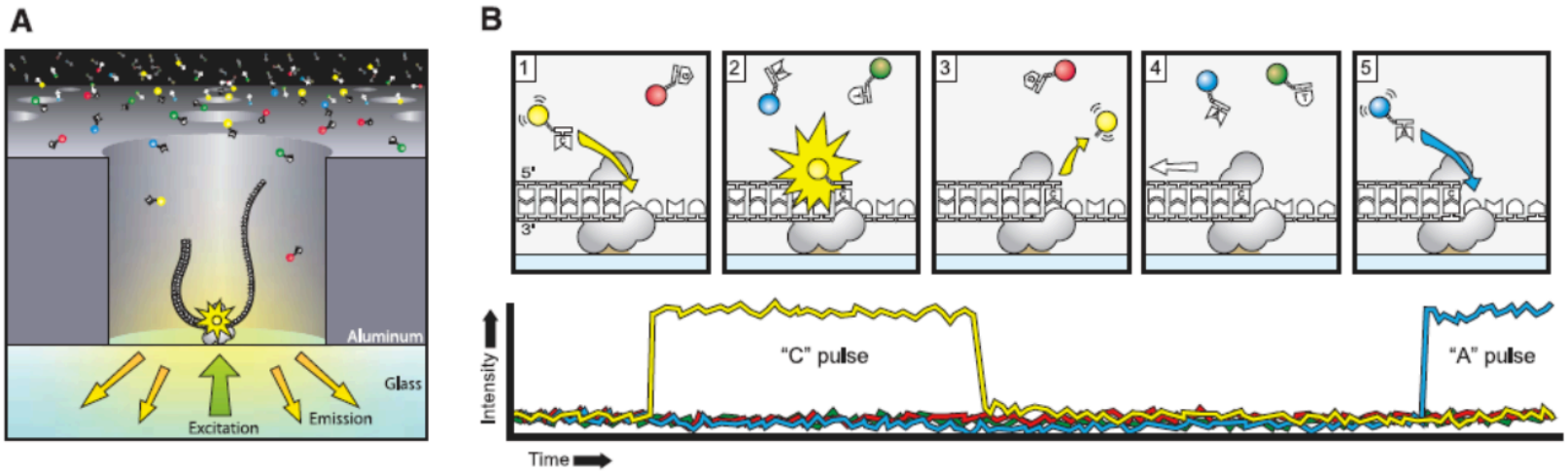


Fig. 1. Principle of single-molecule, real-time DNA sequencing. (**A**) Experimental geometry. A single molecule of DNA template-bound Φ29 DNA polymerase is immobilized at the bottom of a ZMW, which is illuminated from below by laser light. The ZMW nanostructure provides excitation confinement in the zeptoliter (10^{-21} liter) regime, enabling detection of individual phospholinked nucleotide substrates against the bulk solution background as they are incorporated into the DNA strand by the polymerase. (**B**) Schematic event sequence of the phospholinked dNTP incorporation cycle, with a corresponding expected time trace of detected fluorescence intensity from the ZMW. (1) A phospholinked nucleotide forms a cognate association with the template in the polymerase active site, (2) causing an elevation of the fluorescence output on the corresponding color channel. (3) Phosphodiester bond formation liberates the dye-linker-pyrophosphate product, which diffuses out of the ZMW, thus ending the fluorescence pulse. (4) The polymerase translocates to the next position, and (5) the next cognate nucleotide binds the active site beginning the subsequent pulse.

- Pozzetti con volume 10^{-21} litri (zeptolitri)
- Aggiunta sequenziale di dNTP marcati con fluorofori
- Determinazione incorporazione tramite ZMW (zero-mode waveguide)

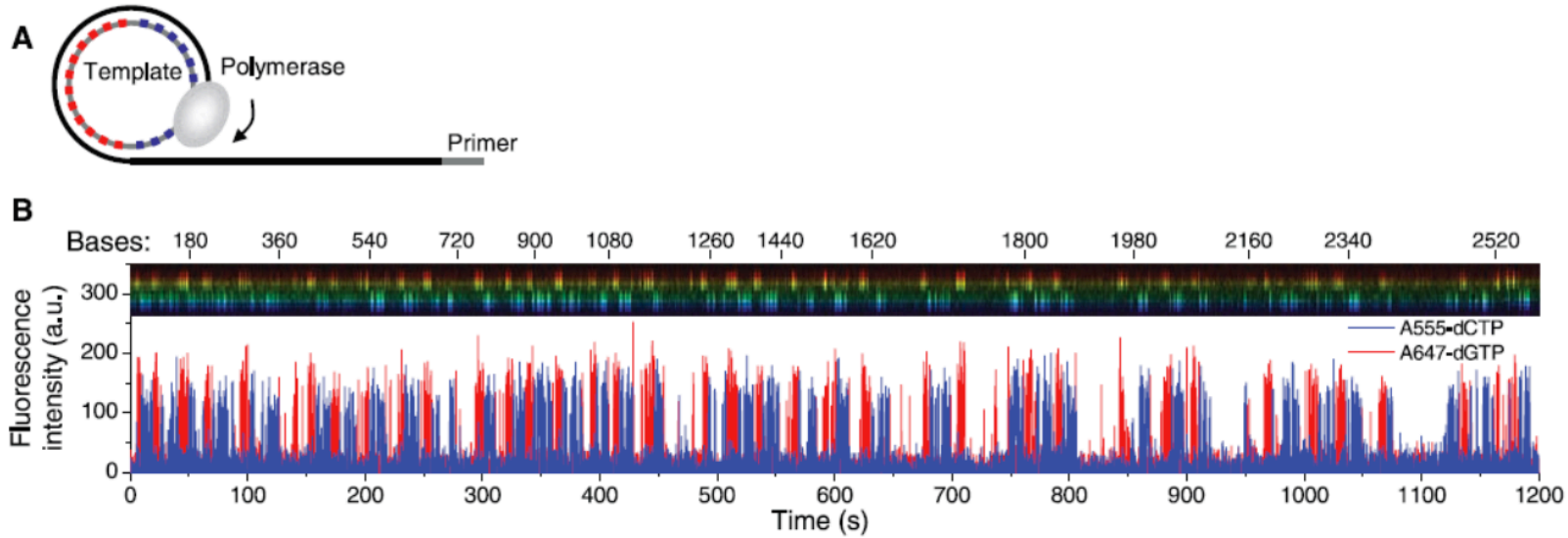
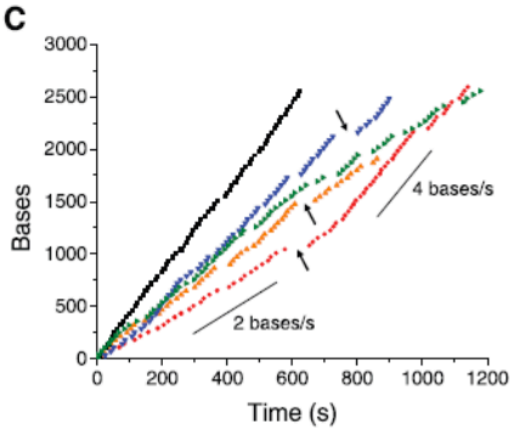
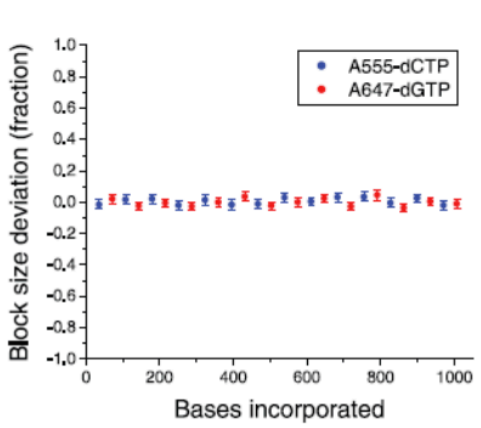


Fig. 3. Long read length activity of DNA polymerase. (**A**) DNA template design. The sequence of a circular, single-stranded template was designed to yield continuous incorporation via strand-displacement DNA synthesis of alternating blocks of two phospholinked nucleotides (A555-dCTP and A647-dGTP), interspersed with the other two unmodified dNTPs. (**B**) Time-resolved spectrum of fluorescence emission as in Fig. 2B with fluorescence time trace from a single ZMW. The corresponding total length of synthesized DNA is indicated by the top axis. (**C**) DNA polymerization rate profiles for several molecules. Examples of pause sites are indicated by arrows. The two lines indicate two persistent polymerization rates. (**D**) Error as a function of length of read for 14 rolling circle cycles (1008 total base incorporations; n = 186 reads). The fractional deviation from the average number of pulses per block (12 A555-dCTP and 12 A647-dGTP observed phospholinked dNTP pulses per cycle, respectively), mean ± SE, is plotted as a function of template position. The 95% confidence interval for the slope is −0.027 to +0.036 blocks per 1008 bases of incorporation.





Lunghezza dei frammenti raggiunta: 860-1100bp (esperimenti con circa 2.5 kb)

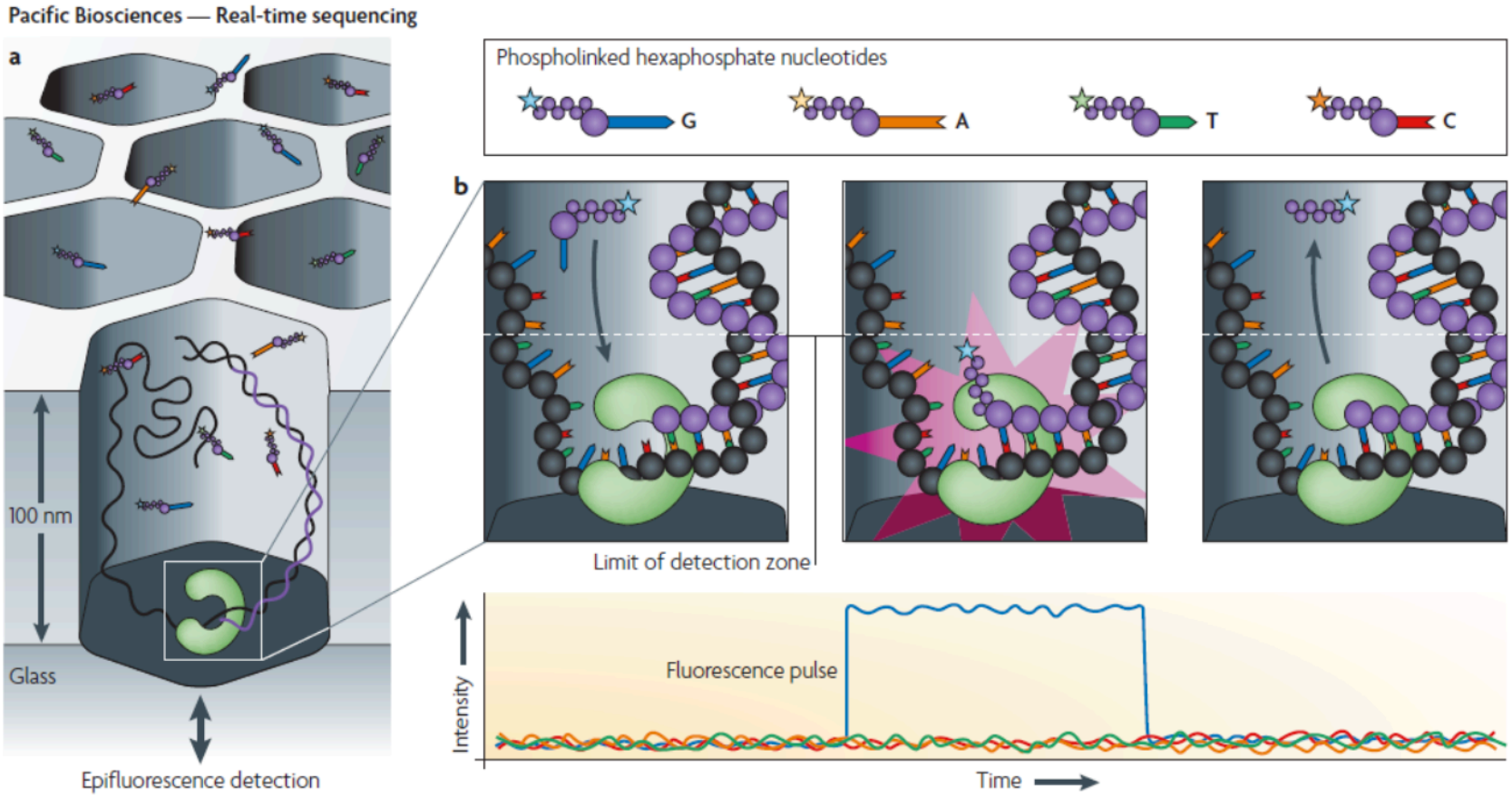


Figure 4 | **Real-time sequencing**. Pacific Biosciences' four-colour real-time sequencing method is shown. **a** | The zero-mode waveguide (ZMW) design reduces the observation volume, therefore reducing the number of stray fluorescently labelled molecules that enter the detection layer for a given period. These ZMW detectors address the dilemma that DNA polymerases perform optimally when fluorescently labelled nucleotides are present in the micromolar concentration range, whereas most single-molecule detection methods perform optimally when fluorescent species are in the pico- to nanomolar concentration range[42]. **b** | The residence time of phospholinked nucleotides in the active site is governed by the rate of catalysis and is usually on the millisecond scale. This corresponds to a recorded fluorescence pulse, because only the bound, dye-labelled nucleotide occupies the ZMW detection zone on this timescale. The released, dye-labelled pentaphosphate by-product quickly diffuses away, dropping the fluorescence signal to background levels. Translocation of the template marks the interphase period before binding and incorporation of the next incoming phospholinked nucleotide.

SEE VIDEO

https://www.youtube.com/watch?v=3UHw22hBpAk

NGS

NGS technologies are a combination of strategies for:

- Template preparation
- Sequencing and imaging
- Genome alignment
- Assembly methods

NGS

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- Template preparation
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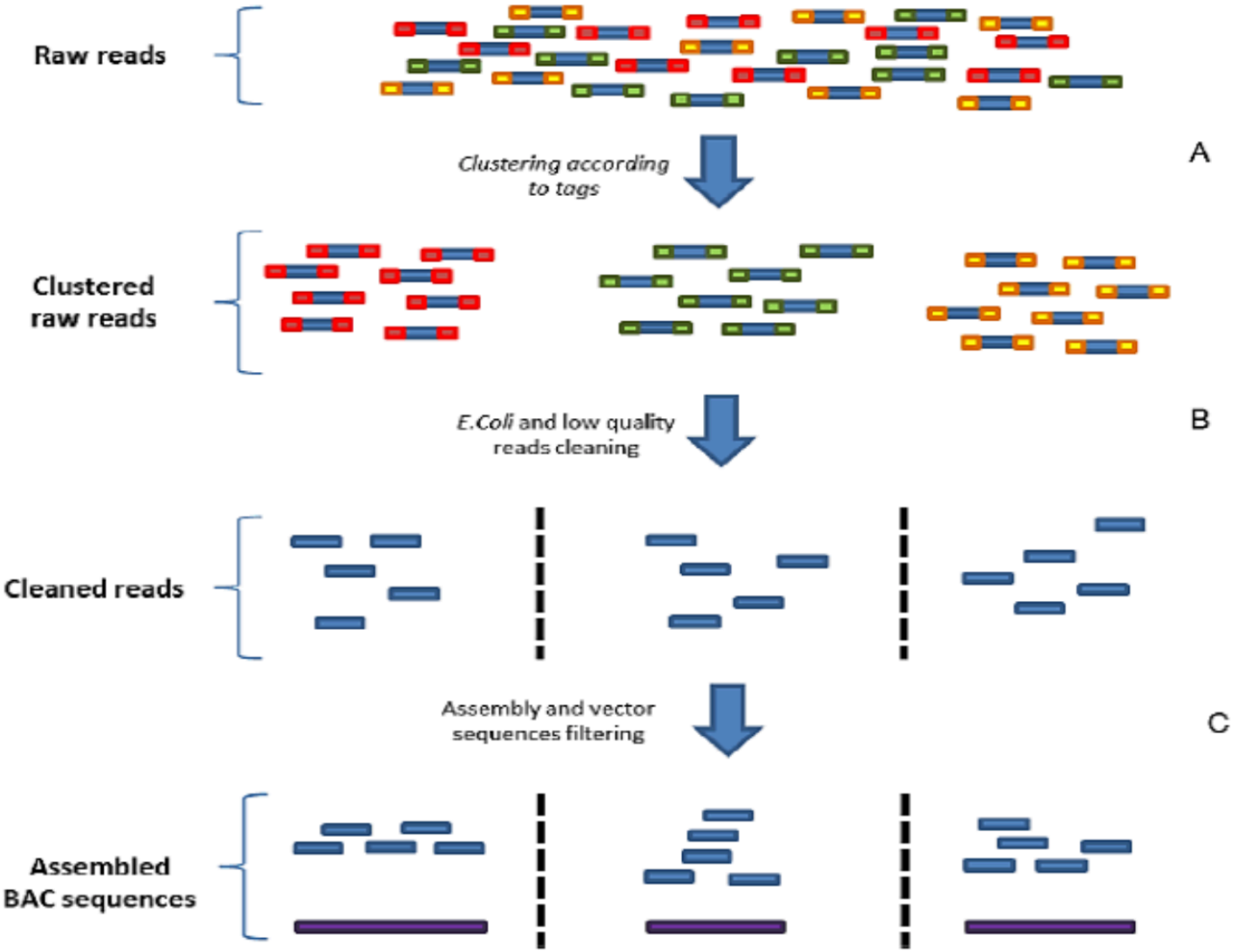
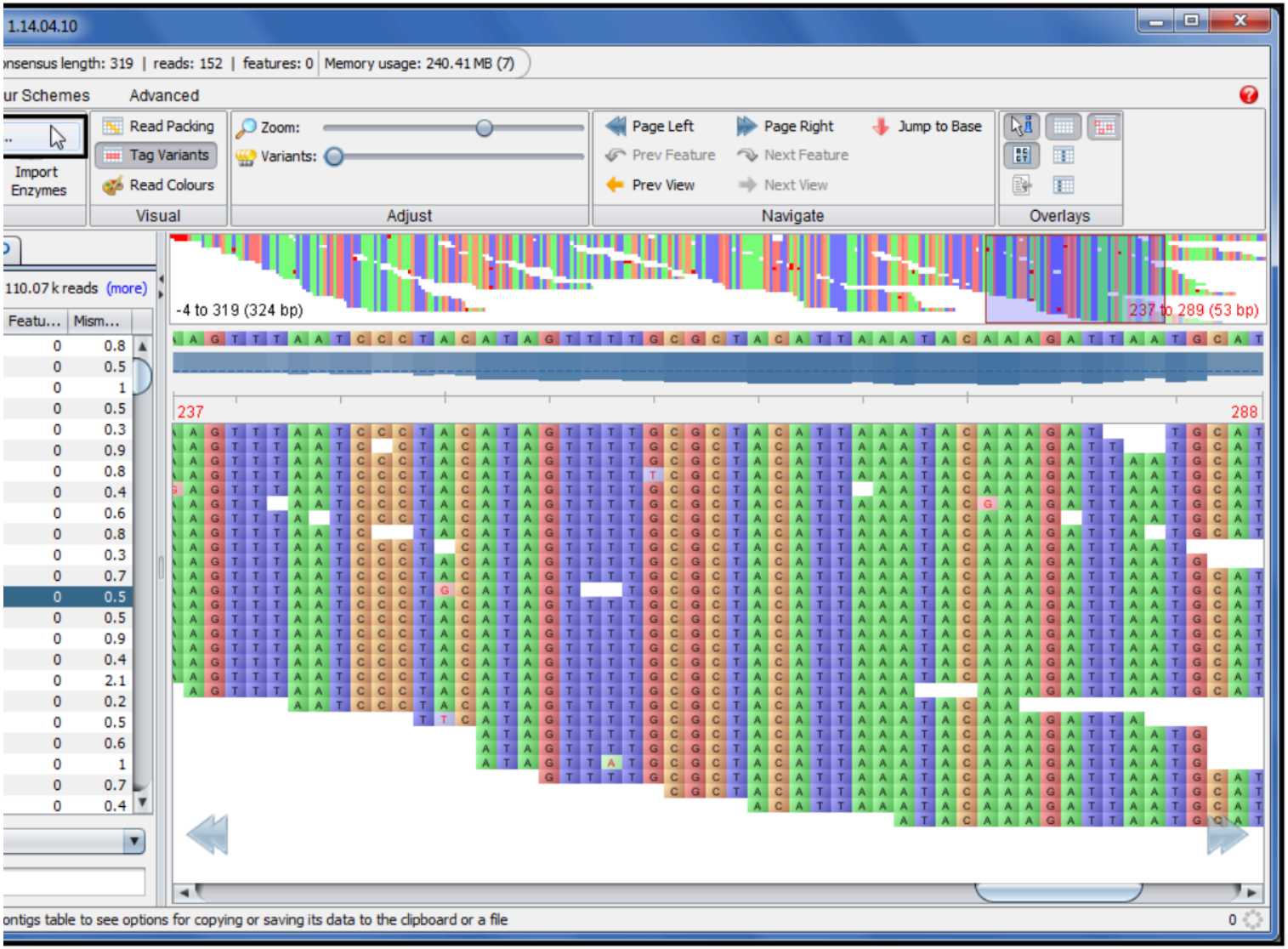
NGS


Raw reads
Clustering according to tags
A
Clustered raw reads
E.Coli and low quality reads cleaning
B
Cleaned reads
Assembly and vector sequences filtering
C
Assembled BAC sequences

NGS


1.14.04.10
nsensus length: 319 | reads: 152 | features: 0 | Memory usage: 240.41 MB (7)
ur Schemes
Advanced
Import Enzymes
Read Packing
Tag Variants
Read Colours
Visual
Zoom:
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Prev Feature
Next Feature
Prev View
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-4 to 319 (324 bp)
237 to 289 (53 bp)
237
288
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