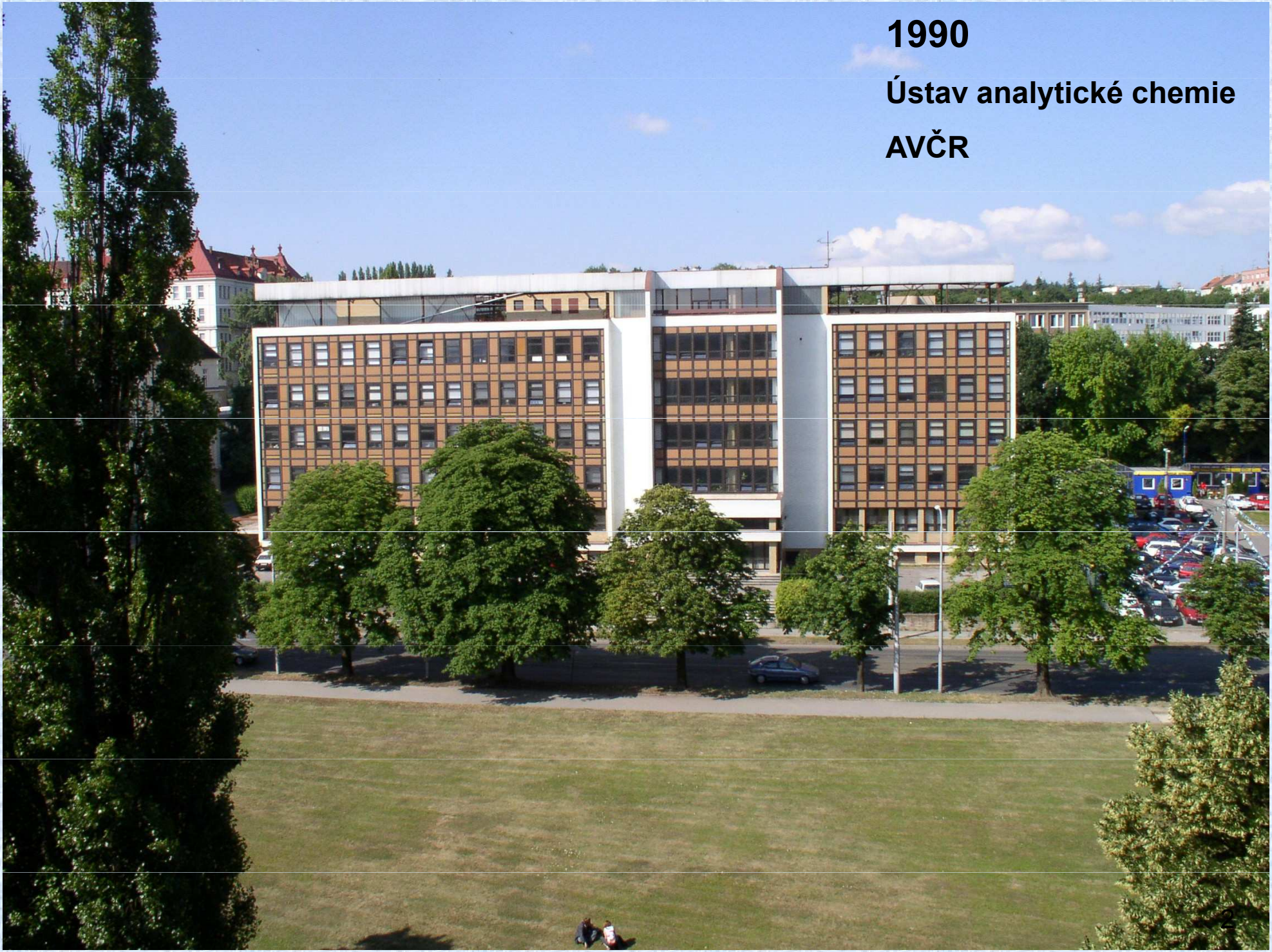
Moderní analytická instrumentace pro genetický výzkum, lékařskou diagnostiku a molekulární identifikaci organismů

Karel Klepárník
(klep@iach.cz)

Department of Bioanalytical Instrumentation
Institute of Analytical chemistry
Czech Academy of Sciences
Brno
(www.iach.cz)



1990

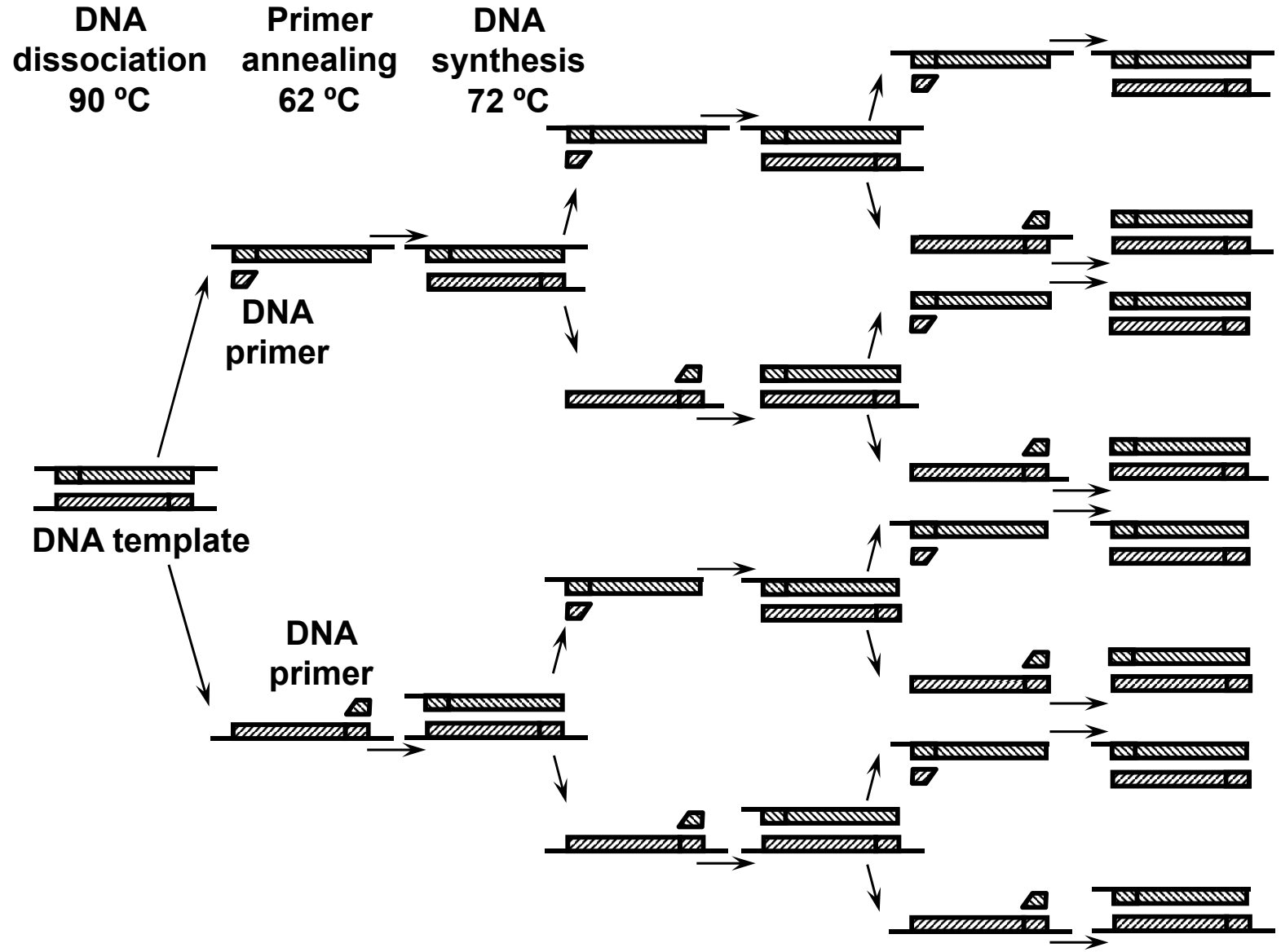
Ústav analytické chemie

AVČR

Polymerase chain reaction

PCR amplification

PCR amplification scheme



Correct copies	1st cycle: n=1	2nd cycle: n=2	3rd cycle: n=3
$N=2^{n+1}-2(n+1)$	$2^2 - 2\cdot 2 = 0$	$2^3 - 2\cdot 3 = 2$	$2^4 - 2\cdot 4 = 8$

Kary B. Mullis

born 1944

La Jolla, CA, USA

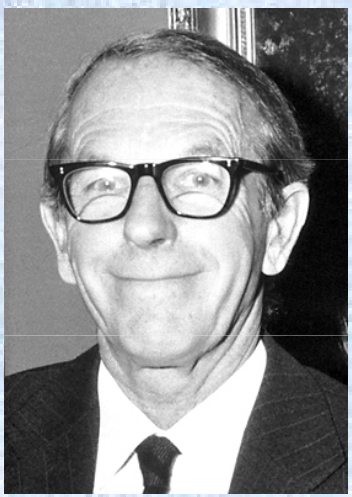
University of British Columbia

The Nobel Prize in Chemistry 1993

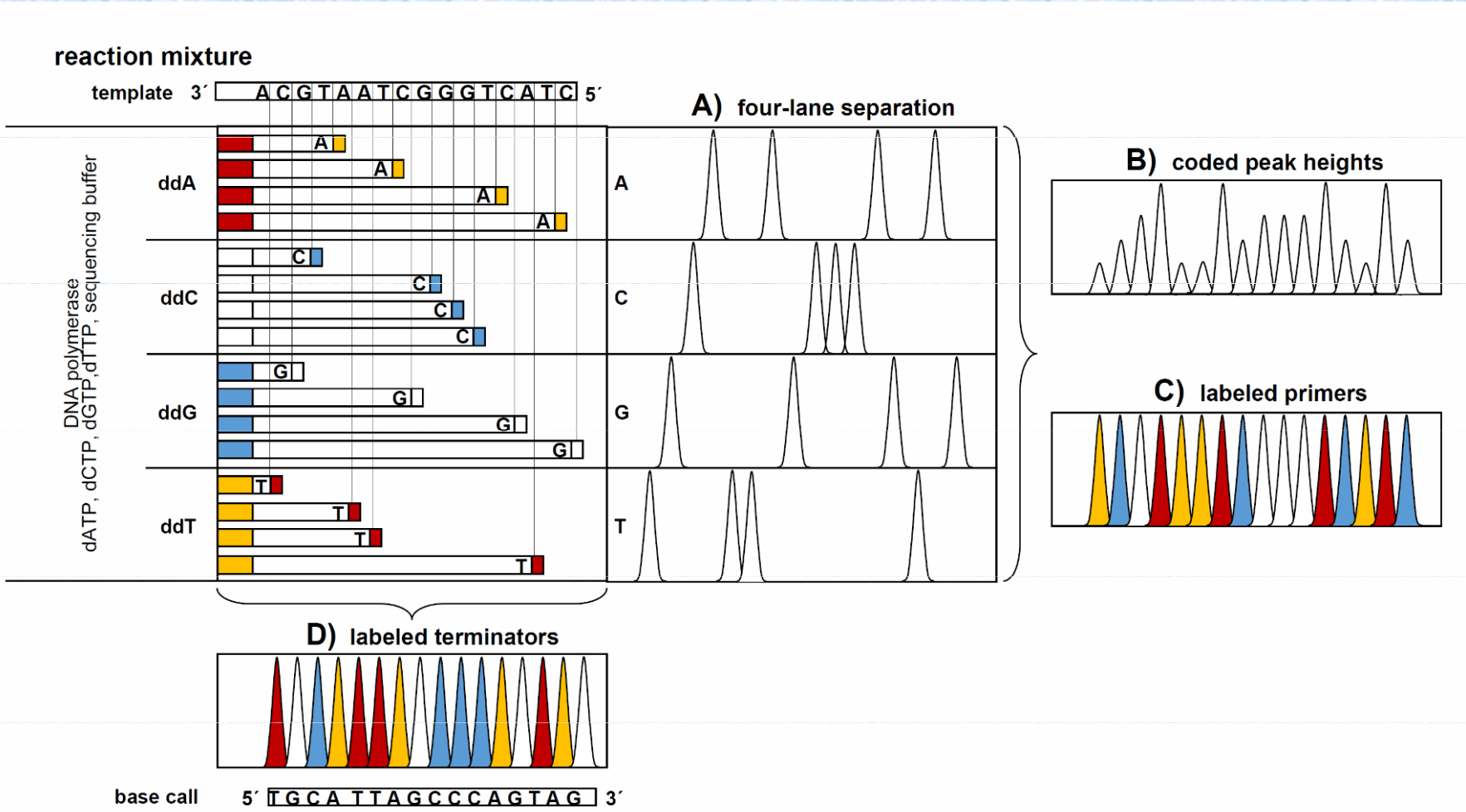
For his invention of the polymerase chain reaction (PCR) method

DNA sequencing

Synthesis of Sanger sequencing fragments

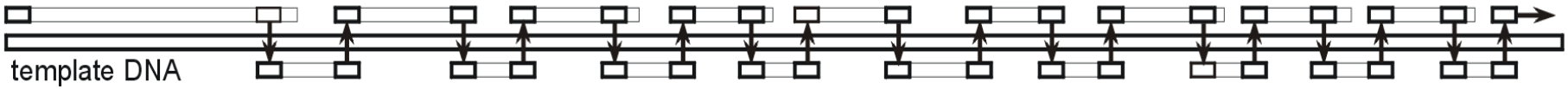


Frederick Sanger
MRC Laboratory of Mol. Biol.
Cambridge, UK
1918 – 2013
Nobel Price in Chemistry 1980



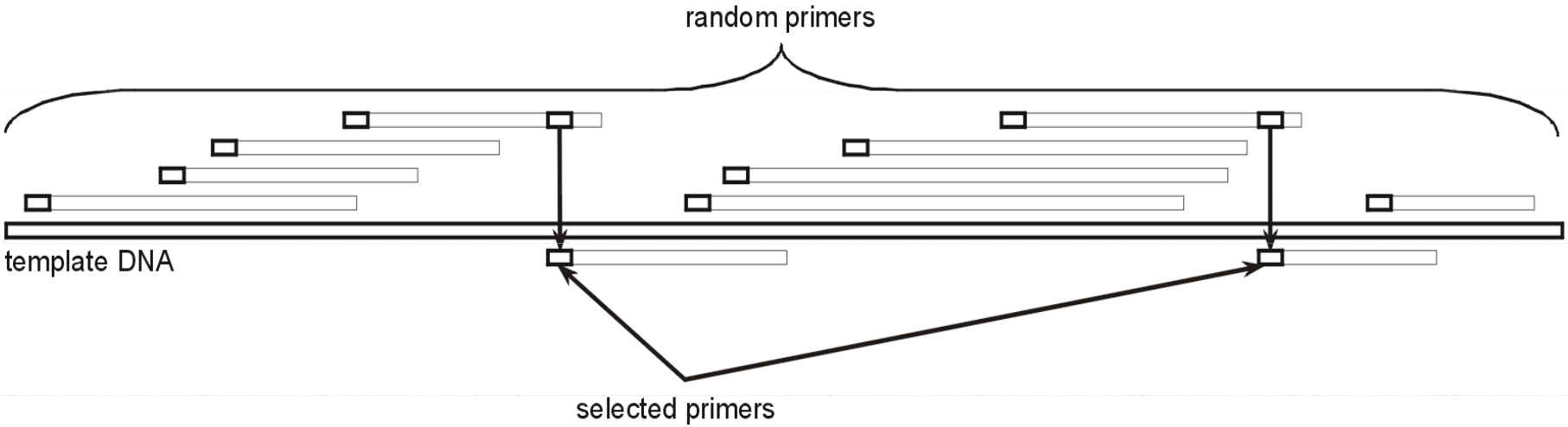
DNA sequencing strategy

Primer walking



consecutive primer selection

Shotgun

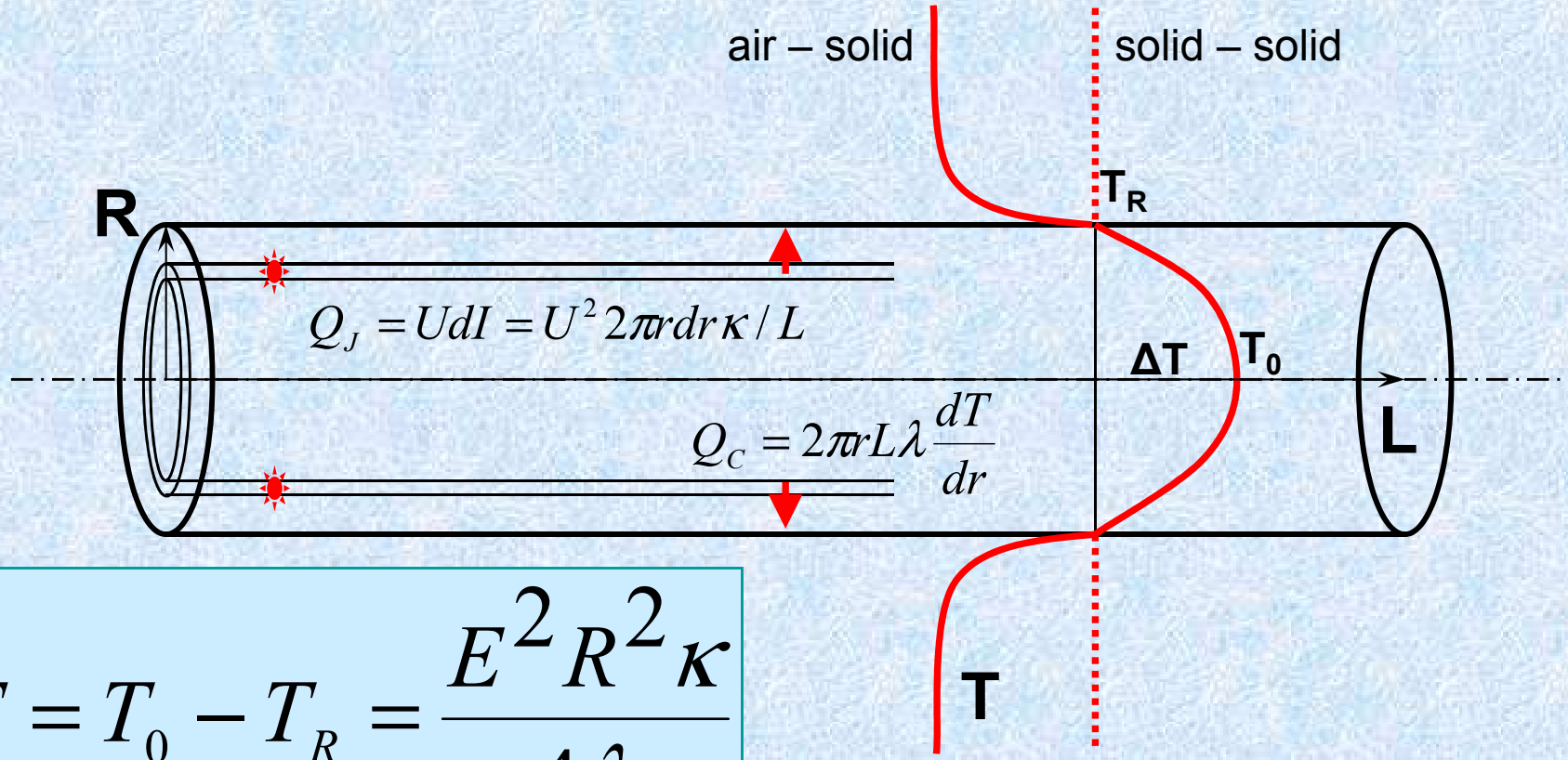


Separation methods

Capillary electrophoresis

CE

Why capillary electrophoresis?



$$\Delta T = T_0 - T_R = \frac{E^2 R^2 \kappa}{4\lambda}$$

Miniature capillary: low R => fast separation

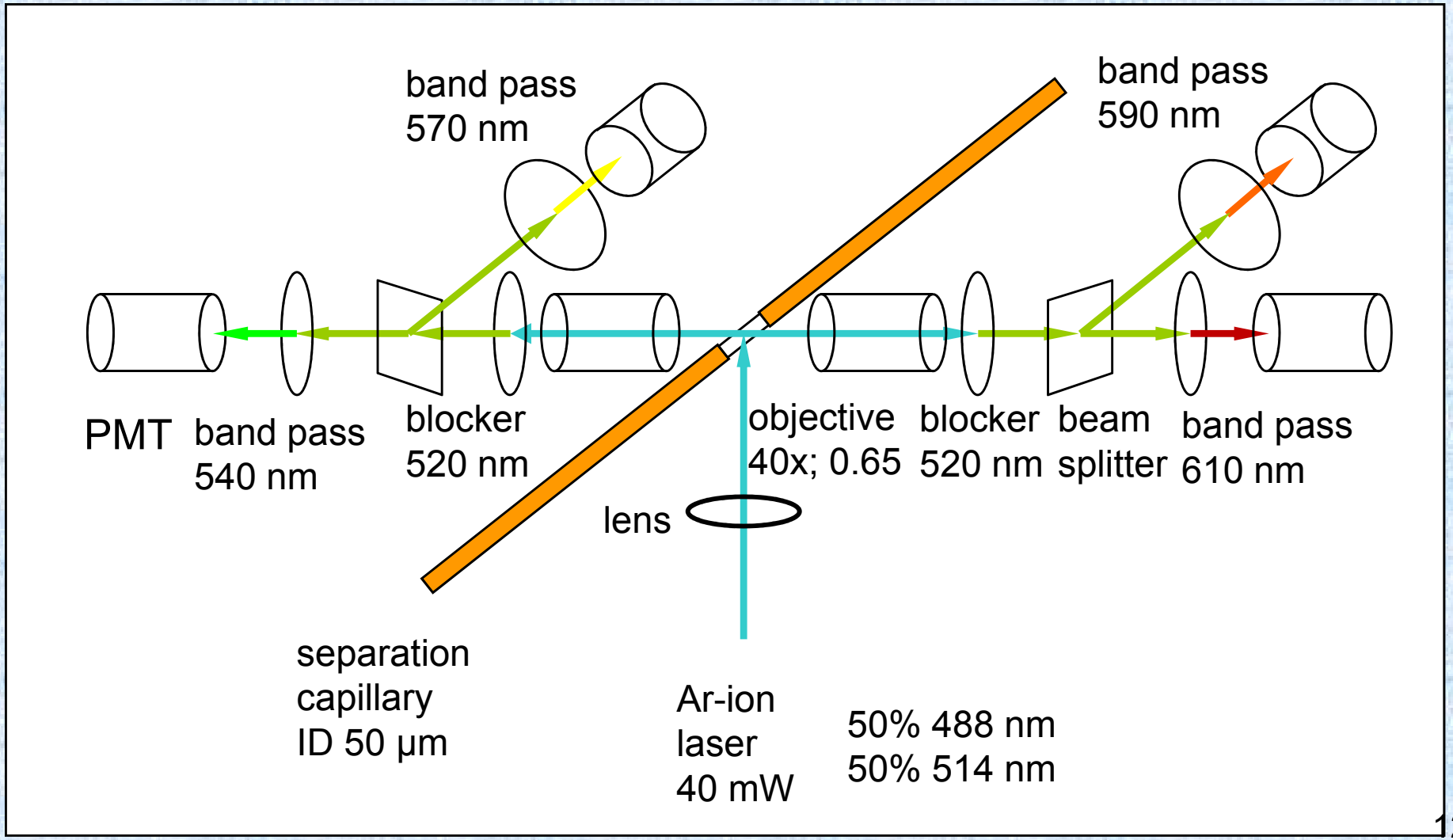
1) high resistivity ➧ low current at high voltage ➧ low heat production

2) efficient heat transport ➧ low temperature difference inside the capillary

LIF detection

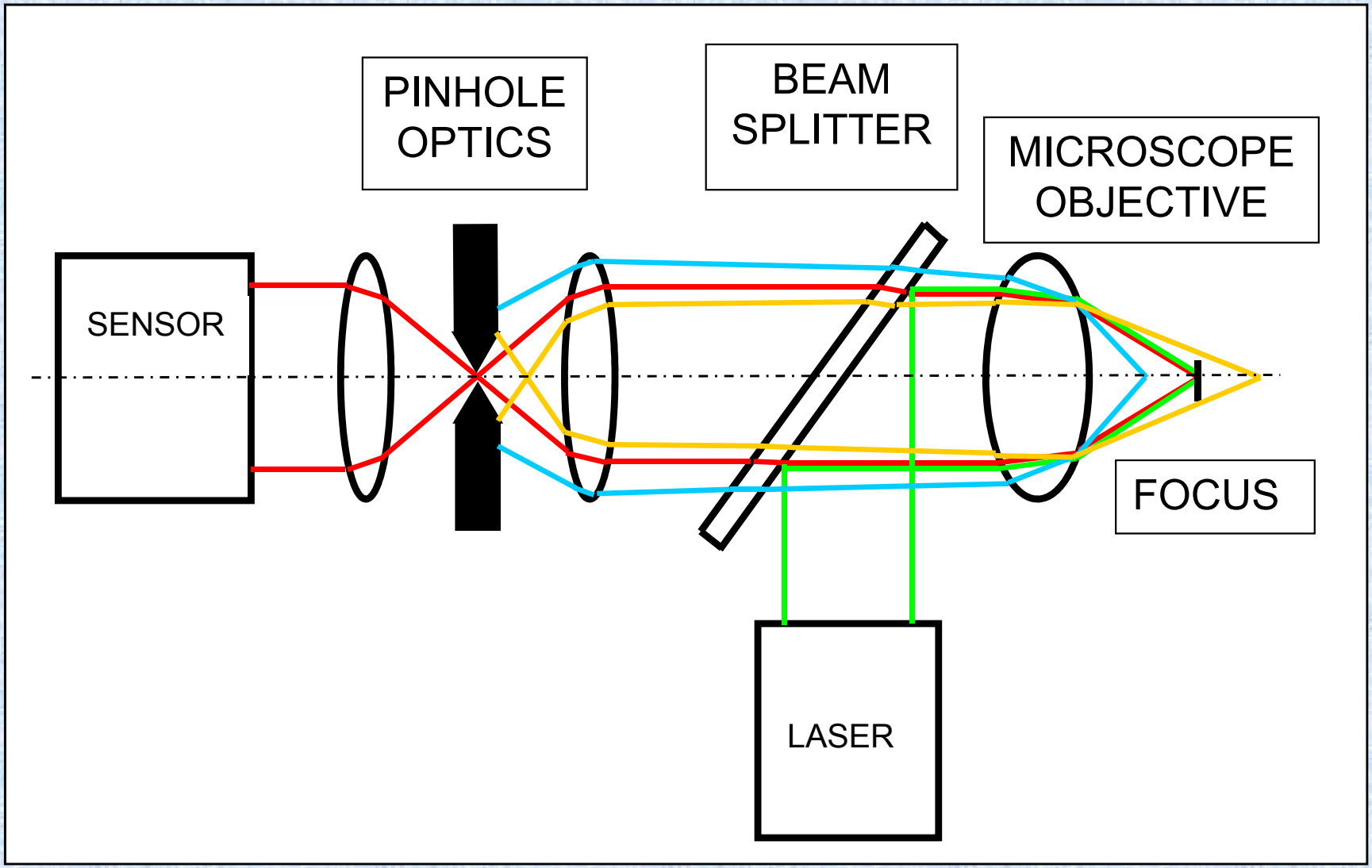
Spectral filtering

Four channel LIF detection arrangement



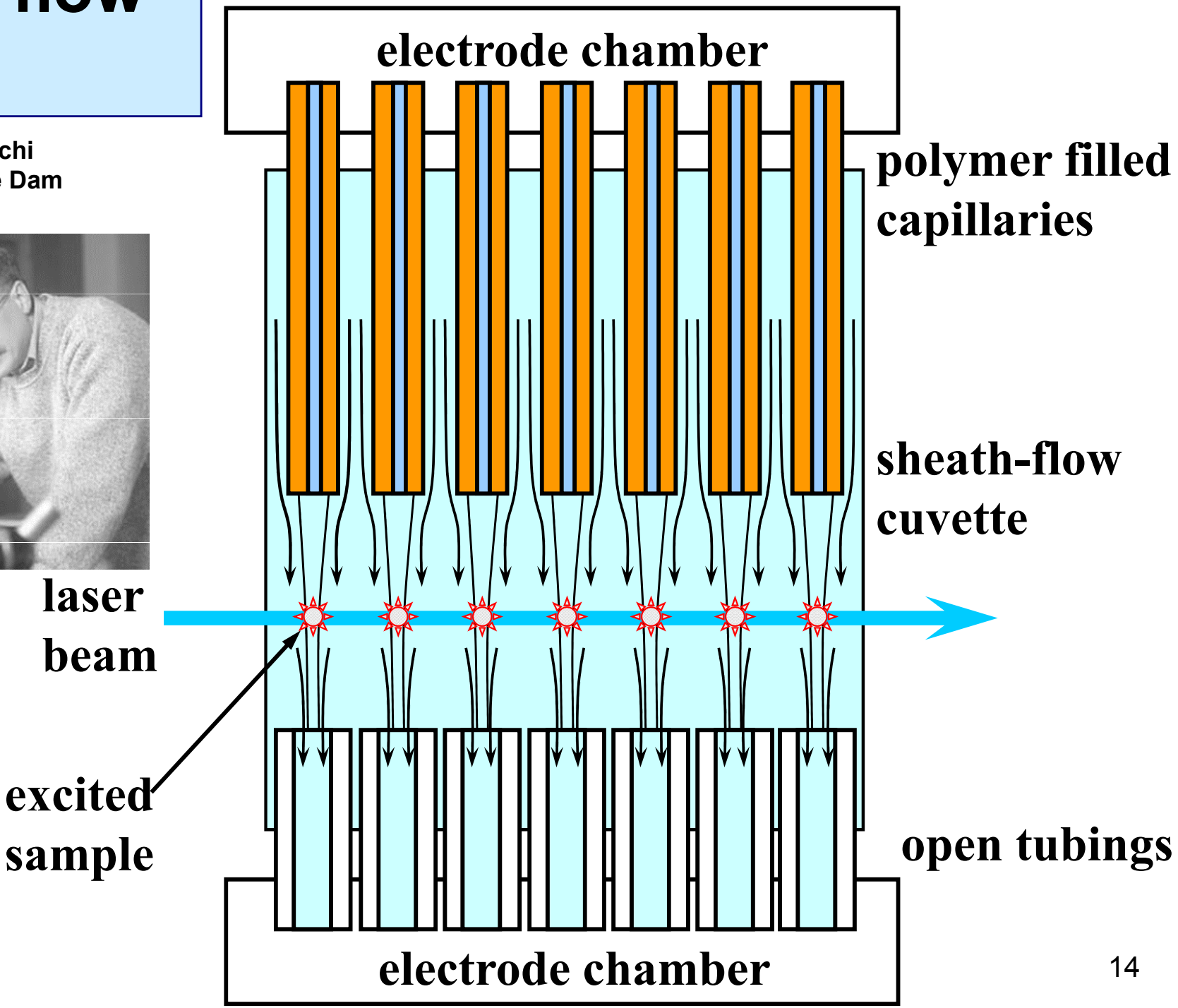
Space filtering

SCHEME OF CONFOCAL DETECTOR



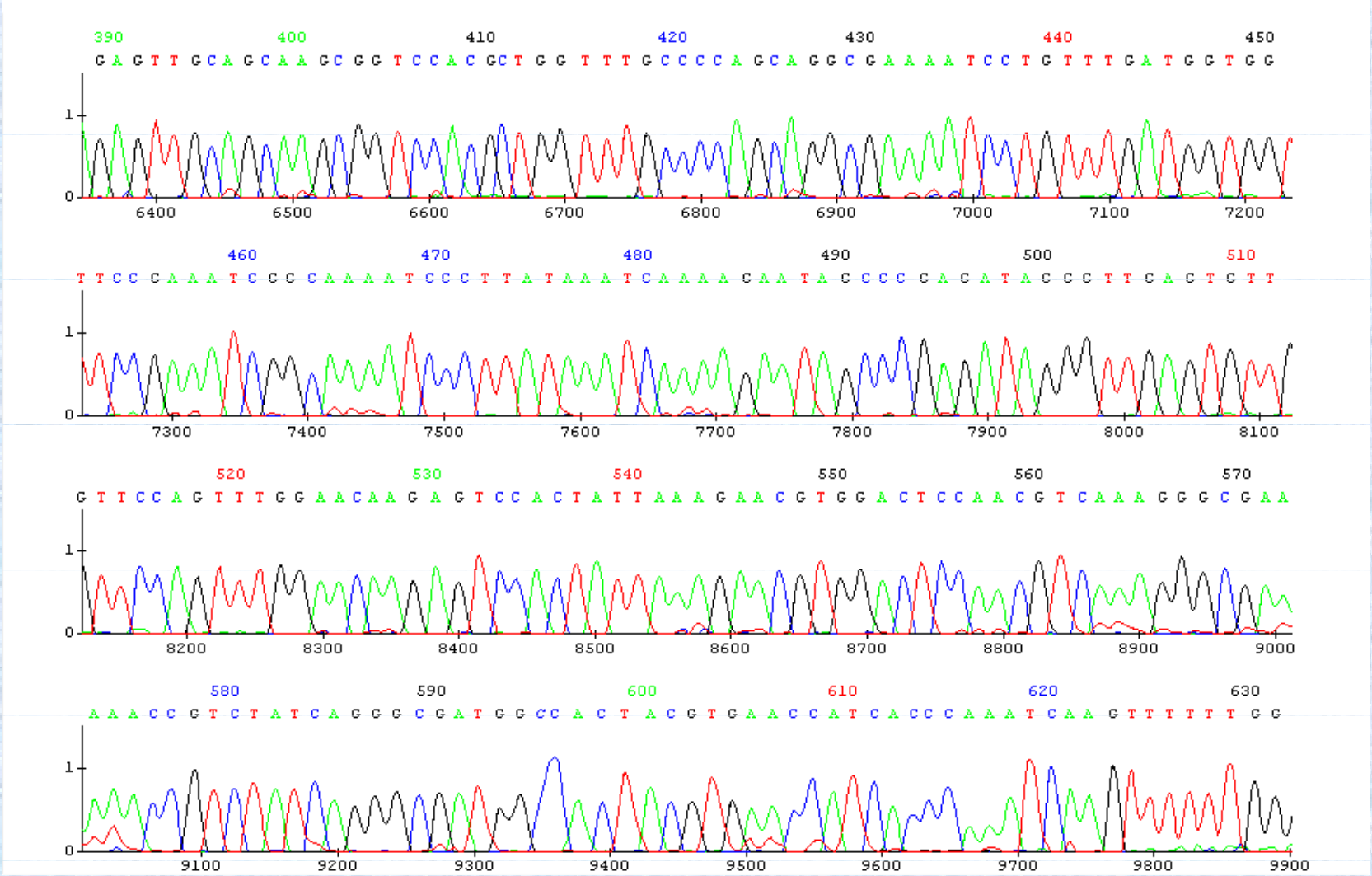
Sheath-flow cuvette

Prof. Norman Dovichi
University of Notre Dam
Indiana, USA



Prof. Hideki Kambara
Hitachi Central Research Laboratory
Tokyo, Japan

DNA sequencing record

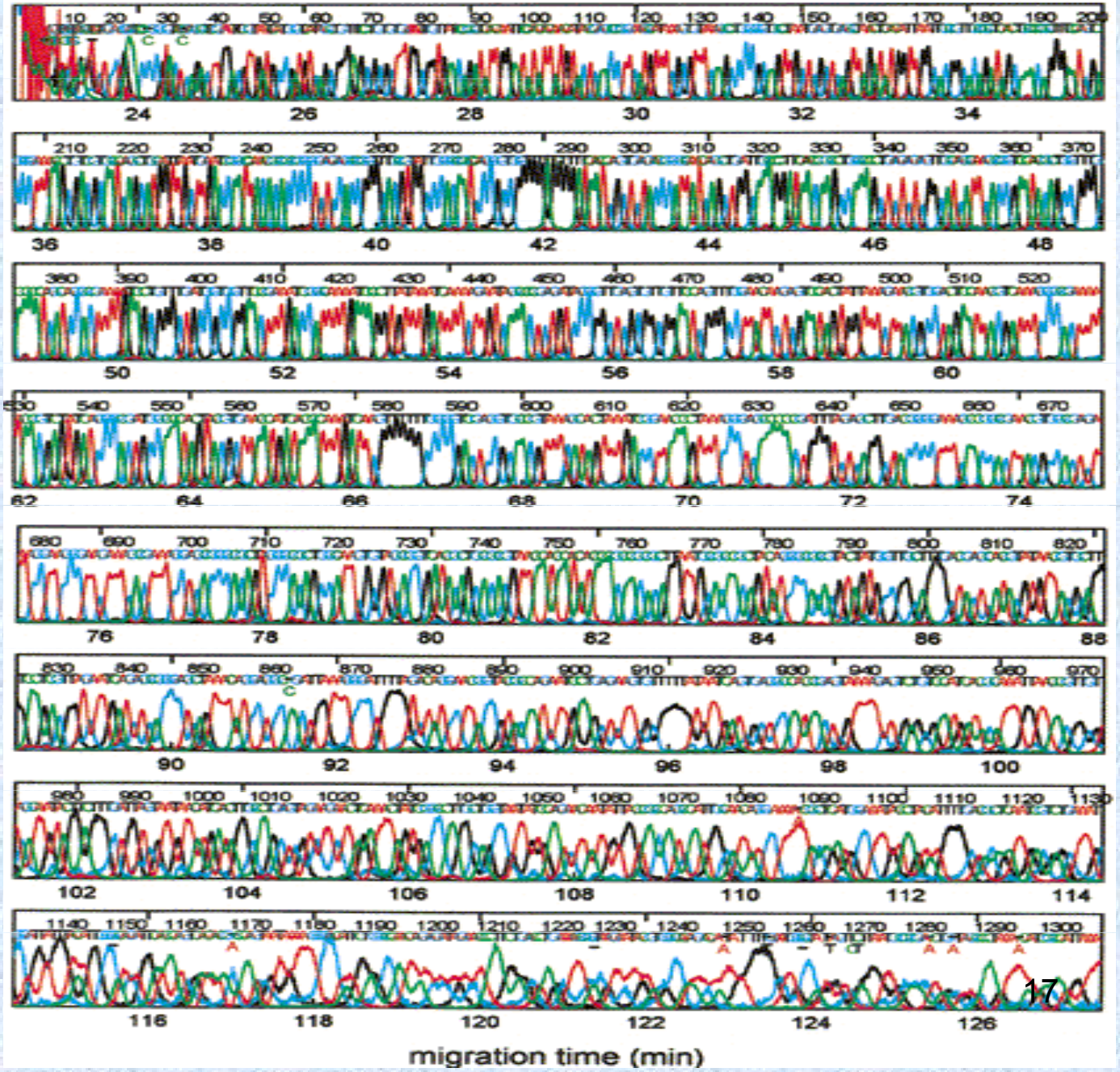


DNA sequencing up to 1300 bases in 2 hours

Separation matrix: LPA 2.0% (w/w) 17 MDa, 0.5% (w/w) 270 kDa

E: 125 V/cm, T: 70 °C

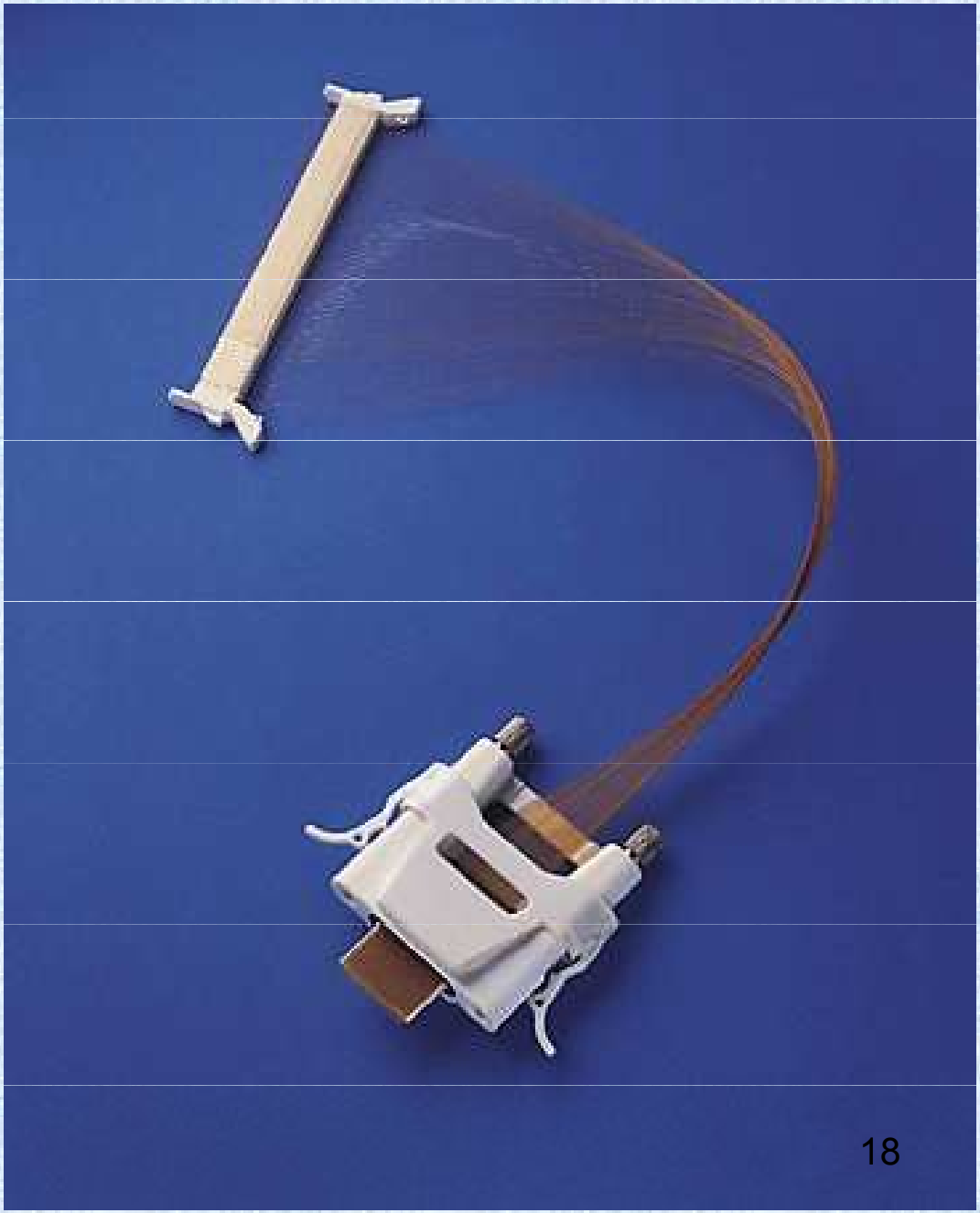
Barry L. Karger
The Barnett Institute
Northeastern University
Boston MA



ABI PRISM® 3700 DNA Analyzer

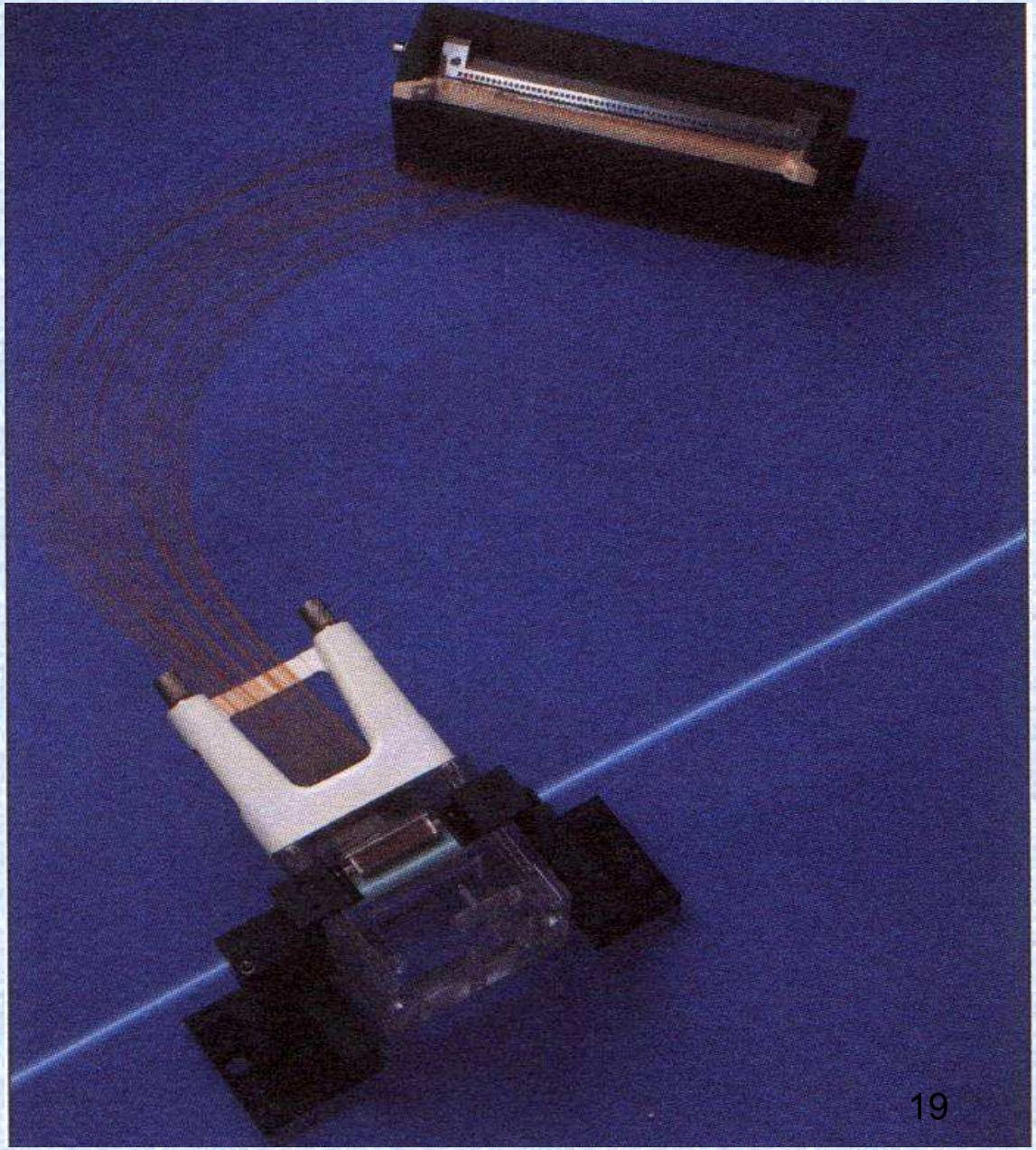
96 active

eight reserve capillaries



ABI PRISM® 3700 DNA Analyzer

Sheath flow cuvette



J. Craig Venter

The Institute for Genomic Research (**TIGR**)

The first president of **Celera Genomics**

The completed sequence of the human genome was published in February 2001 in Science.

Venter, C. J. et al. *Science* **2001**, *291*, 1304-1351.

J. CRAIG VENTER, Ph.D., PRESIDENT, CELERA GENOMICS
REMARKS AT THE HUMAN GENOME ANNOUNCEMENT
THE WHITE HOUSE
MONDAY, JUNE 26, 2000

Mr. President, Honorable members of the Cabinet, Honorable members of Congress, distinguished guests.
Today, June 26, 2000 marks an historic point in the 100,000-year record of humanity. We are announcing today that for the first time our species can read the chemical letters of its genetic code. At 12:30 p.m. today, in a joint press conference with the public genome effort, Celera Genomics will describe the first assembly of the human genetic code from the whole genome shotgun sequencing method. Starting only nine months ago on September 8, 1999, eighteen miles from the White House, a small team of scientists headed by myself, Hamilton O. Smith, Mark Adams, Gene Myers and Granger Sutton began sequencing the DNA of the human genome using a novel method pioneered by essentially the same team five years earlier at The Institute for Genomic Research in Rockville, Maryland. The method used by Celera has determined the genetic code of five individuals....

…There would be no announcement today, if it were not for the more than **$1 billion that PE Biosystems invested in Celera and in the development of the automated DNA sequencer** that both Celera and the public effort used to sequence the genome…

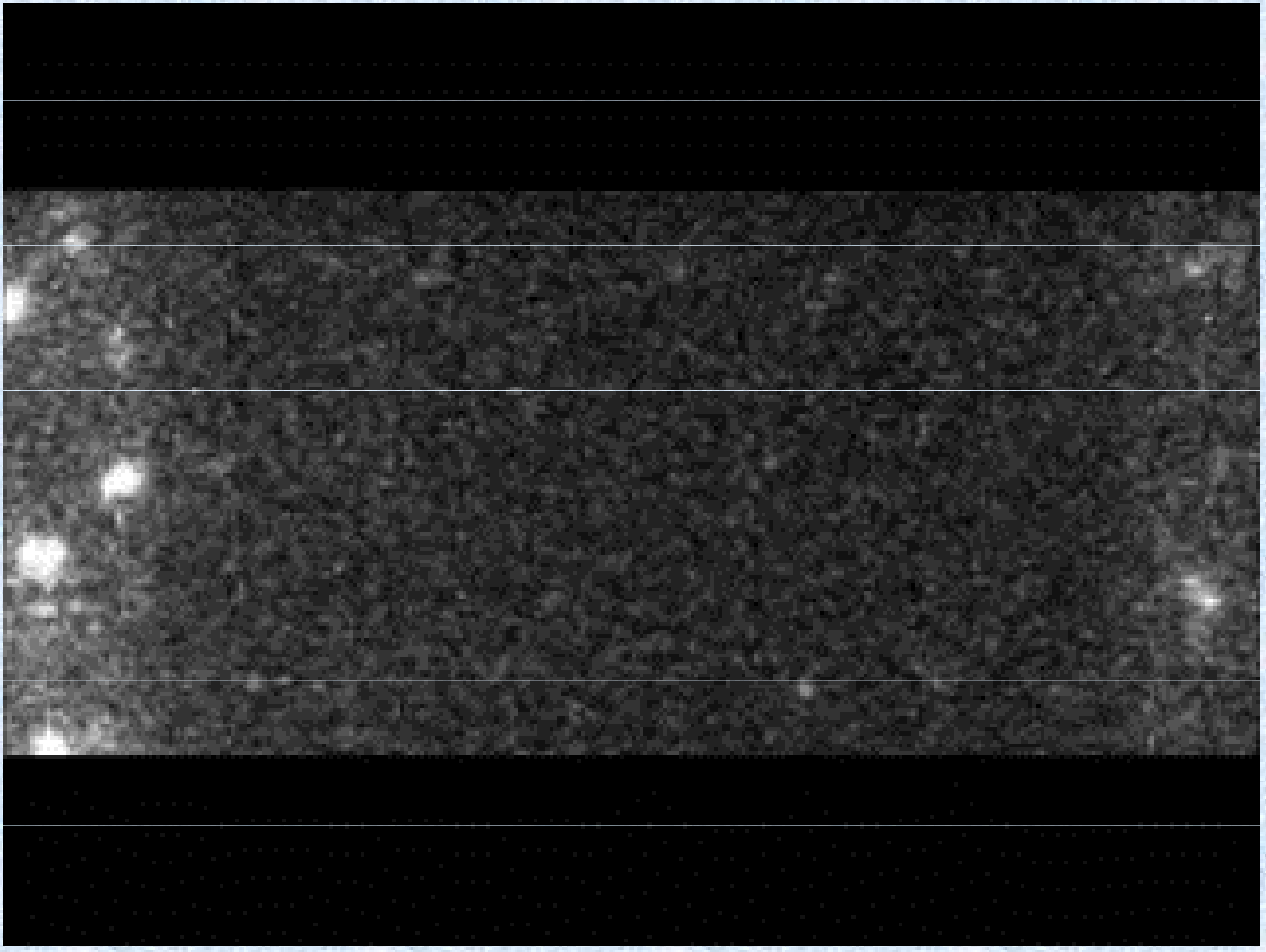
DNA mutation analysis

Next generation sequencing

Single molecule detection

Stretching of dsDNA in Nanochannels

- evaluation of size
- chromatography or electrophoresis
- detection of nucleotides consecutively cleaved by exonuclease



Single molecule reaction monitoring

Parallel single molecule sequencing by synthesis

Helicos

The HeliScope™ Sequencer

2 . 10^9 b/day

10^9 reads/run
25 – 55 bp read lengths

454 LIFE SCIENCES

Genome Sequencer FLX System

3 . 10^8 b/day

100 Mb/7.5 hour run
400 000 reads/7.5 hour
200 – 300 bp read lengths

Solexa

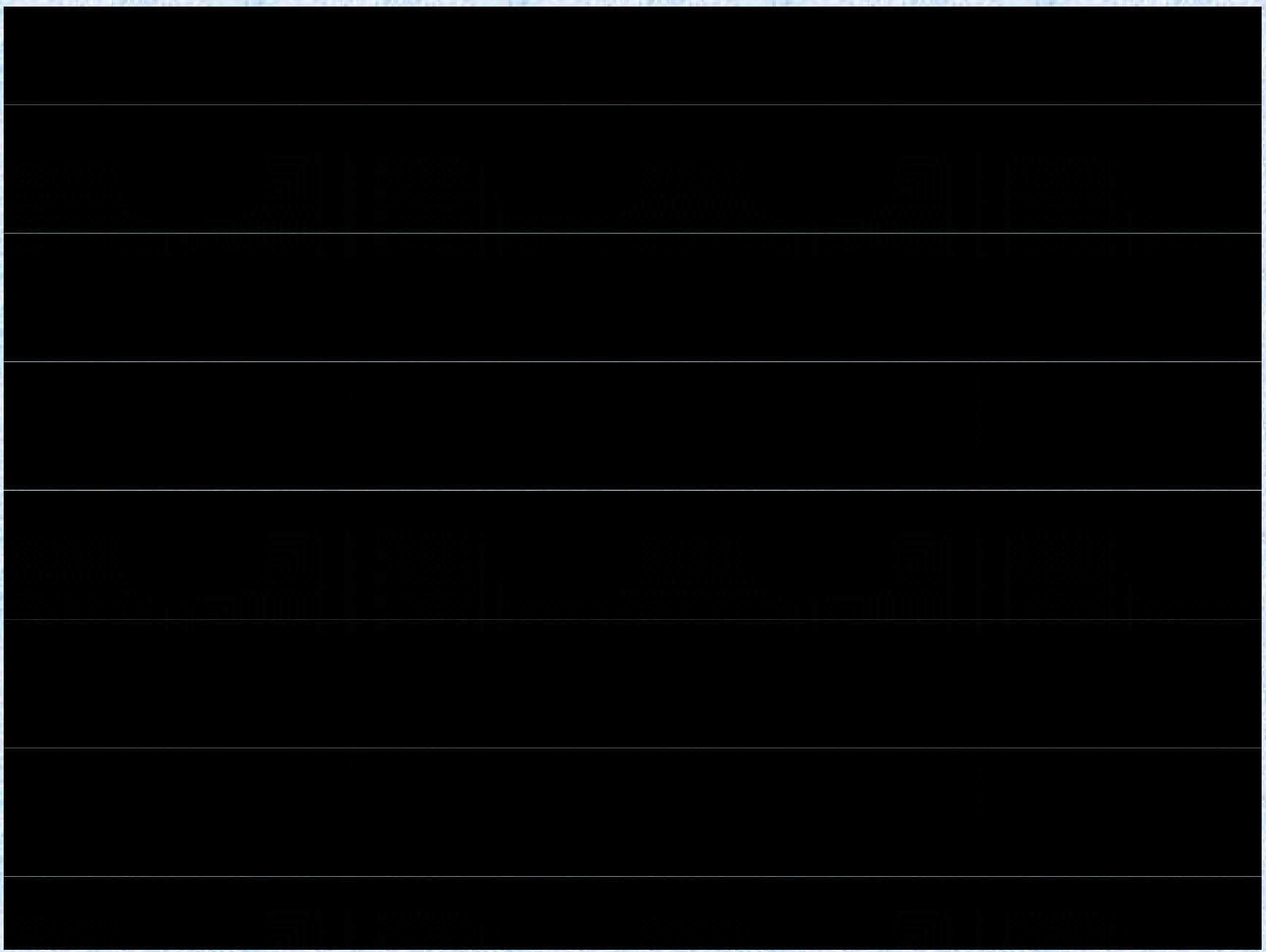
Illumina Genome Analyzer

6 . 10^8 b / day

3 . 10^9 b / 5 days run
50 . 10^6 oligo clusters
36 – 50 bp read lengths

The HeliScope™ Sequencer

http://helicosbio.com/



Photocleavable dideoxy nucleotides

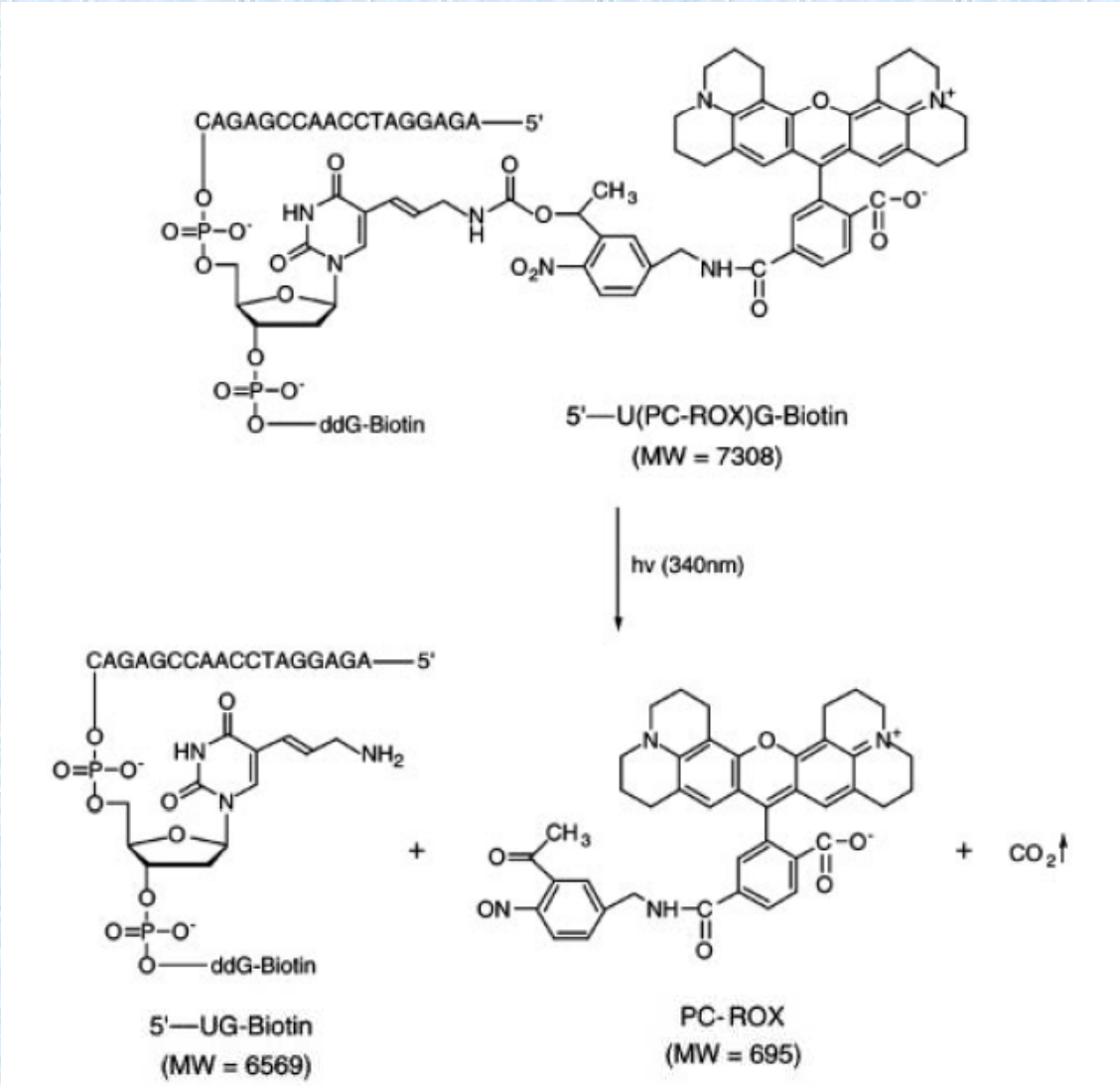


Fig. 2. DNA product 5′-U(PC-ROX)G-biotin, formed by incorporating a dUTP-PC-ROX into a primer in a polymerase reaction and its photocleavage, producing DNA fragment 5′-UG-biotin and PC-ROX. MW, molecular weight.

Next generation DNA sequencing

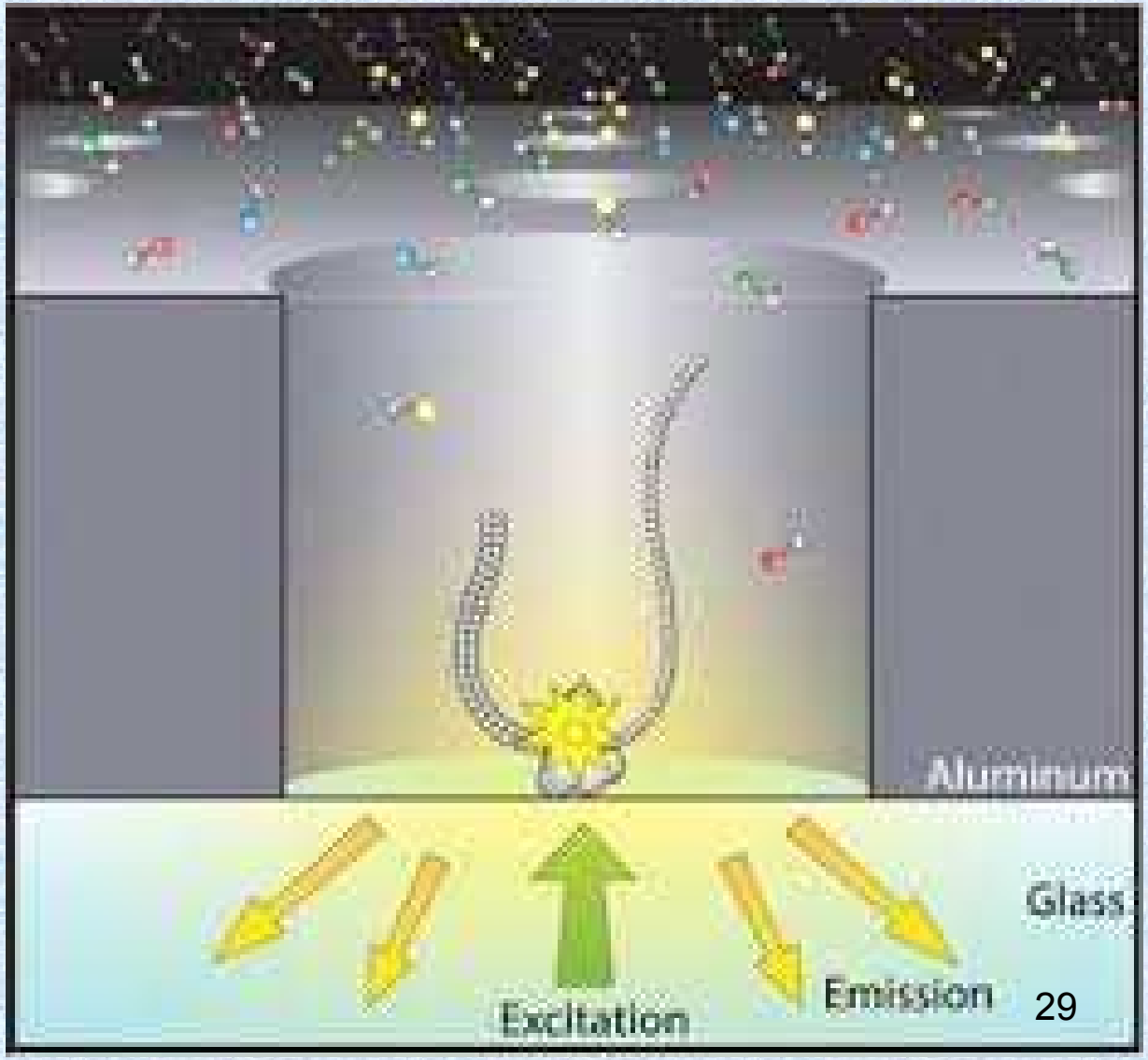
Single molecule real time sequencing (SMRT™)

Pacific Biosciences

www.pacificbiosciences.com

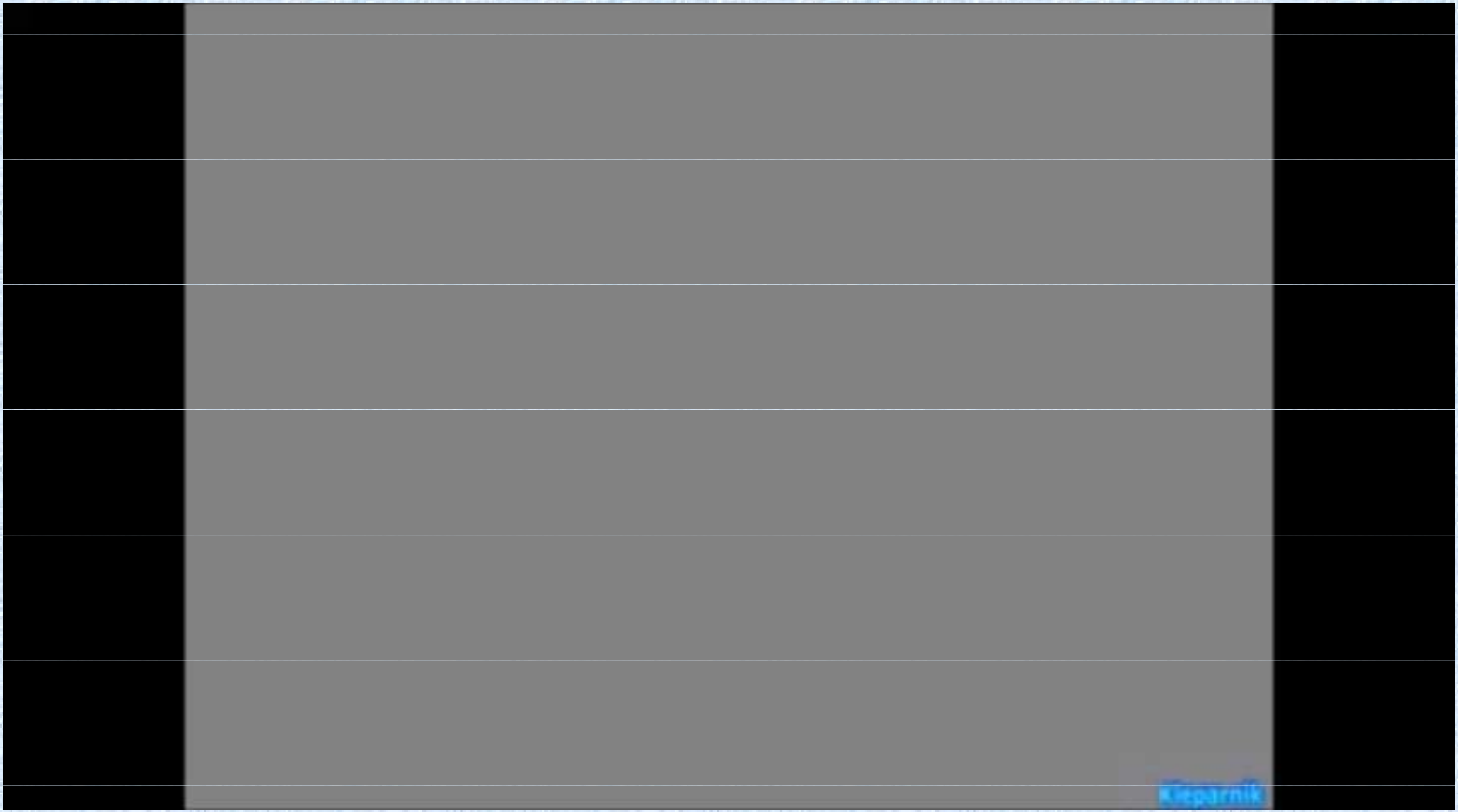
DNA sequencing – DNA polymerase
RNA sequencing – reverse transcriptase
Codone-resolved translation elongation by single ribosomes

Tens of nucleotide peaks in 1 sec
Read length 1 – 15 kb
80 000 detection points
15 min/genome: 50 n/s * 80 000 points * 15 min * 60 s = 3.6 Gb
DNA polymerase 529 processivity 20 kB – 400 b/s
Some enzymes are not processive
$ 100/genome



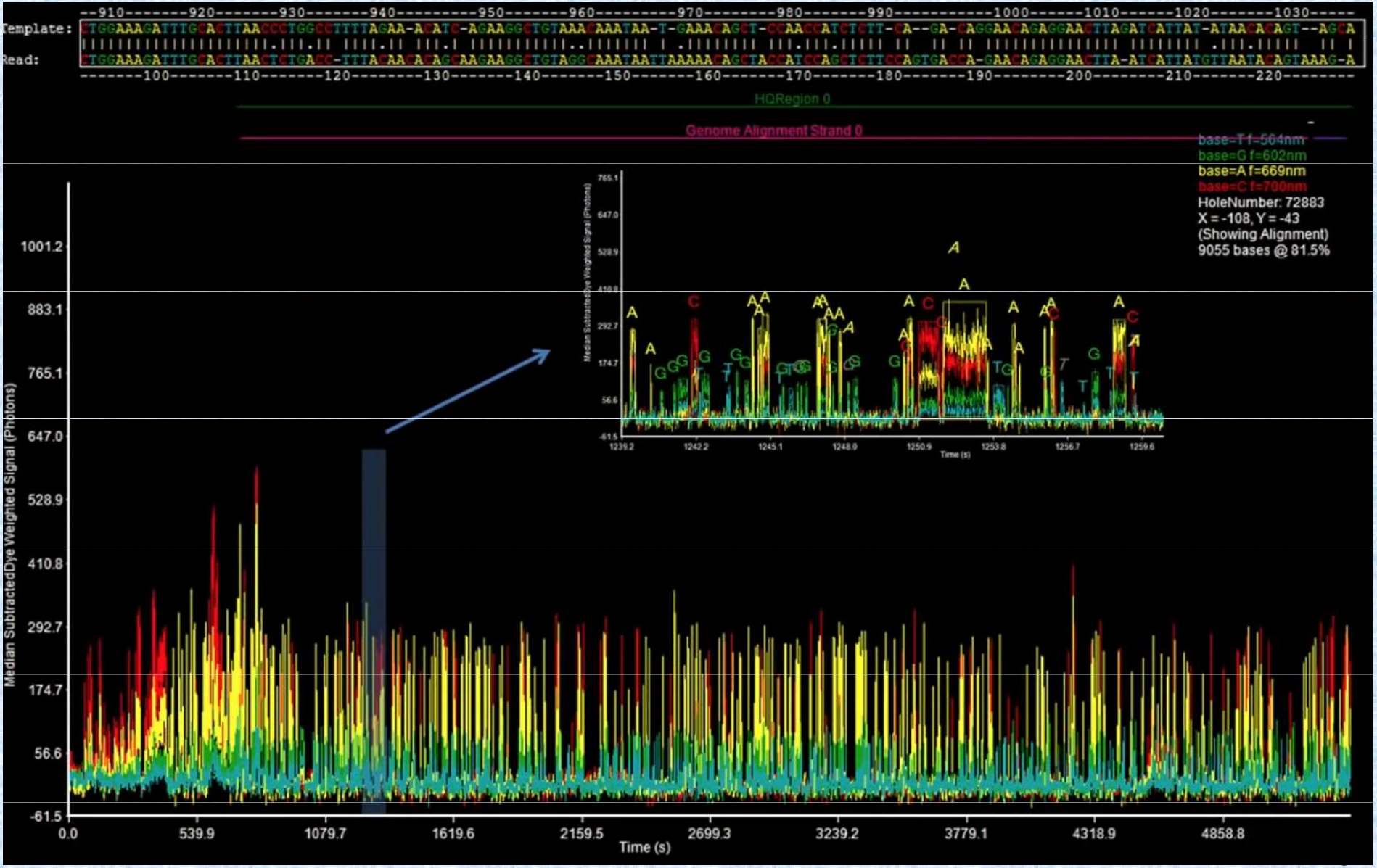
Pacific Biosciences

Single Molecule Real Time (SMRT™) DNA sequencing

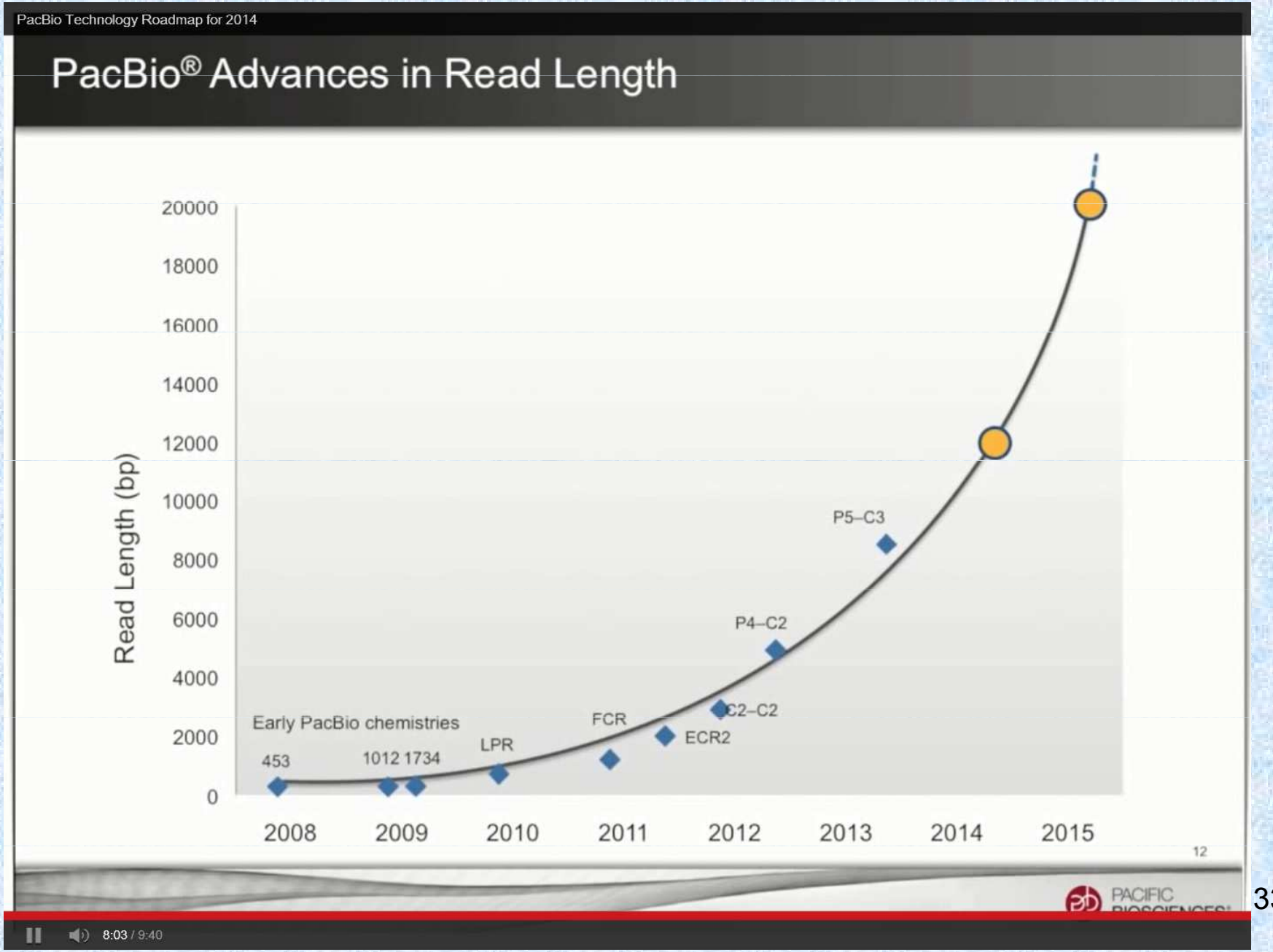


PacBio RS *instrument*

Single molecule real time sequencing



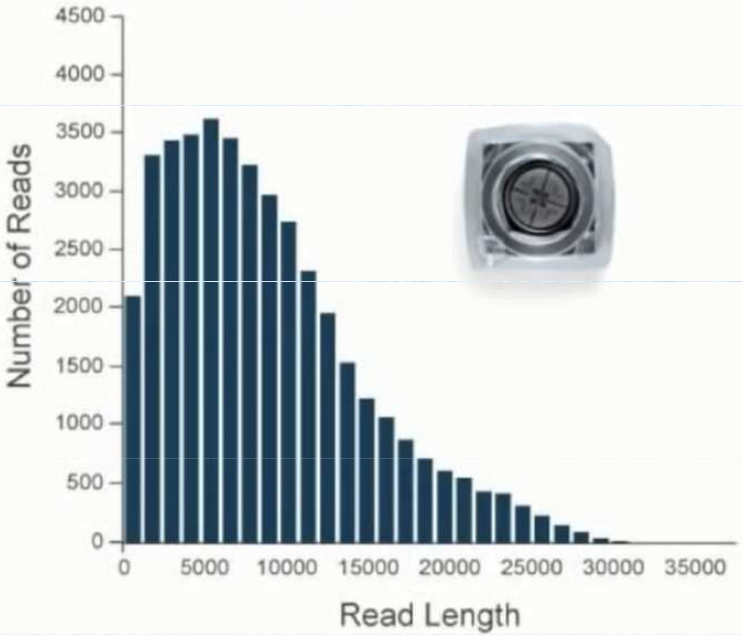
Pacific Biosciences Read Length



Pacific Biosciences Read Length

New P5-C3 Sequencing Chemistry

Read Length Distribution



Typical Results

Read Length:

Average:	~ 8.5 kb
Maximum:	> 30 kb
Top 5% of reads:	> 18 kb
Half of data in reads:	> 10 kb
Data per SMRT® Cell:	~ 375 Mb

Based on data from a 20 kb size-selected E. coli library using a 180-minute movie.
Each SMRT Cell yields ~ 50,000 reads.

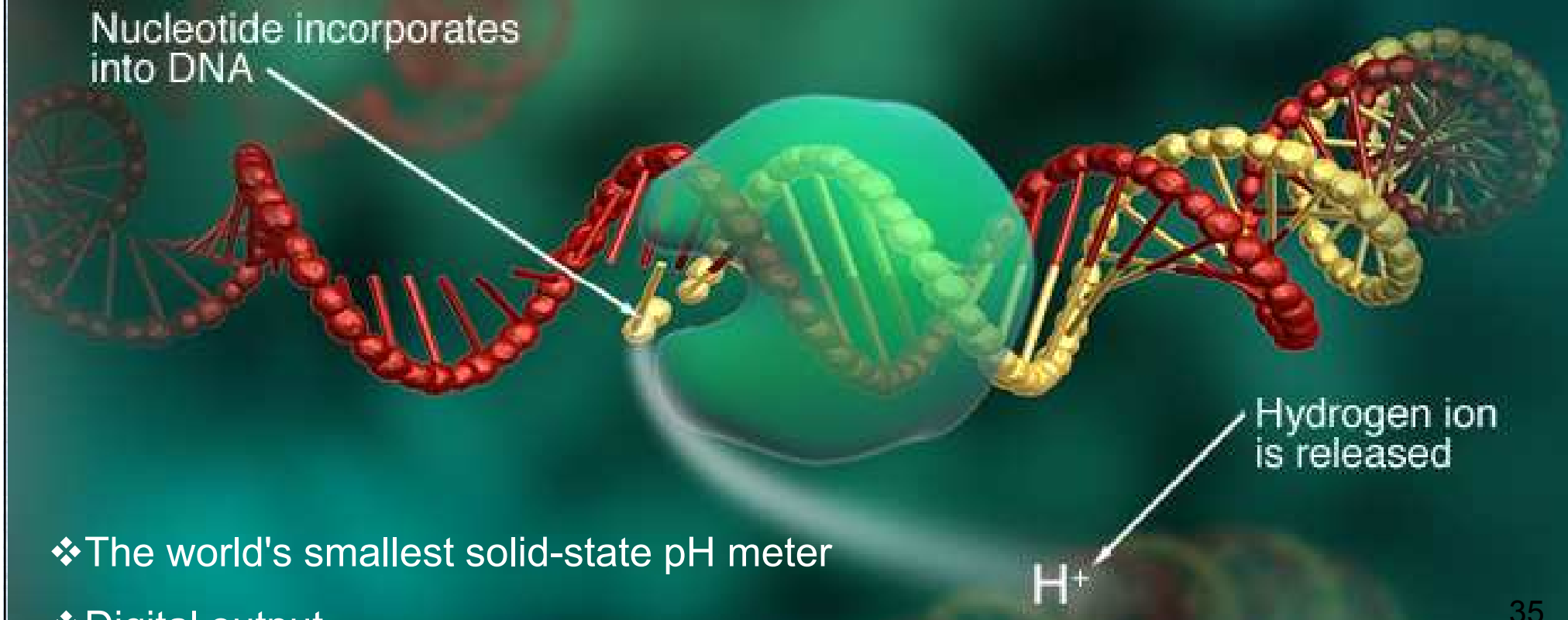
PACIFIC BIOSCIENCES®

Ion Torrent

The Ion Personal Genome Machine (PGM™) sequencer

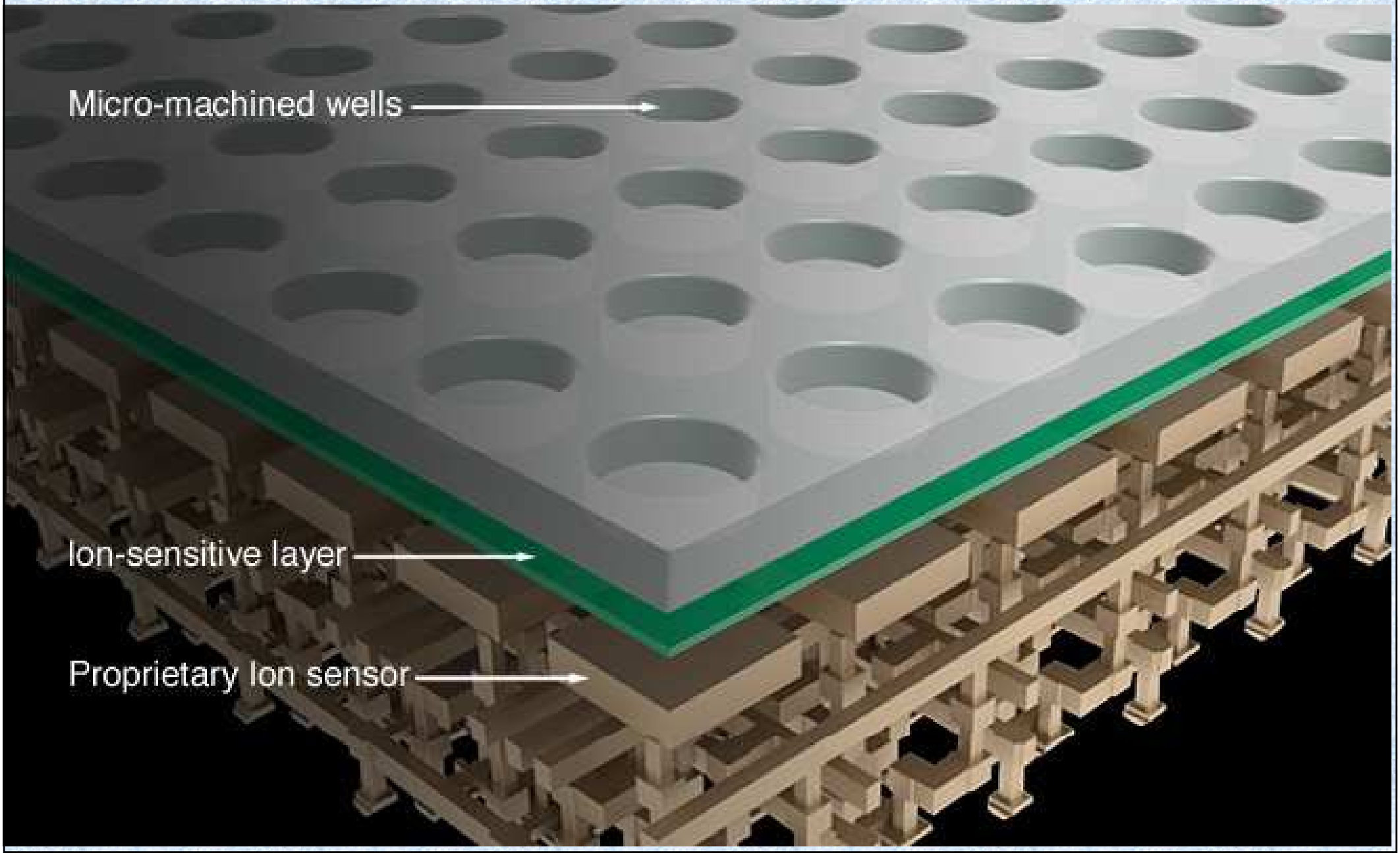
http://www.iontorrent.com/

Hydrogen ion is released as a byproduct when a nucleotide is incorporated into a strand of DNA by a polymerase

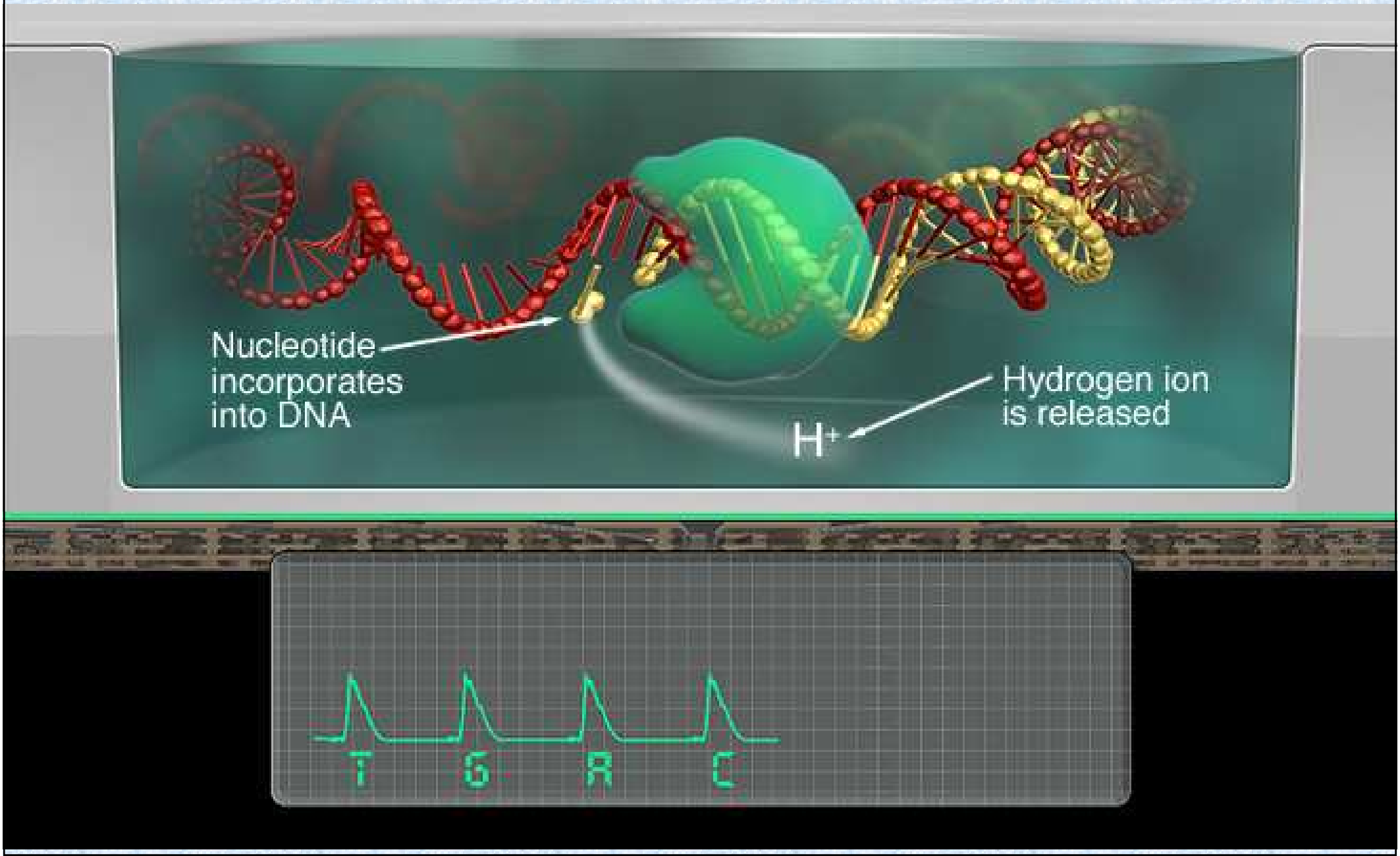


- The world's smallest solid-state pH meter
- Digital output

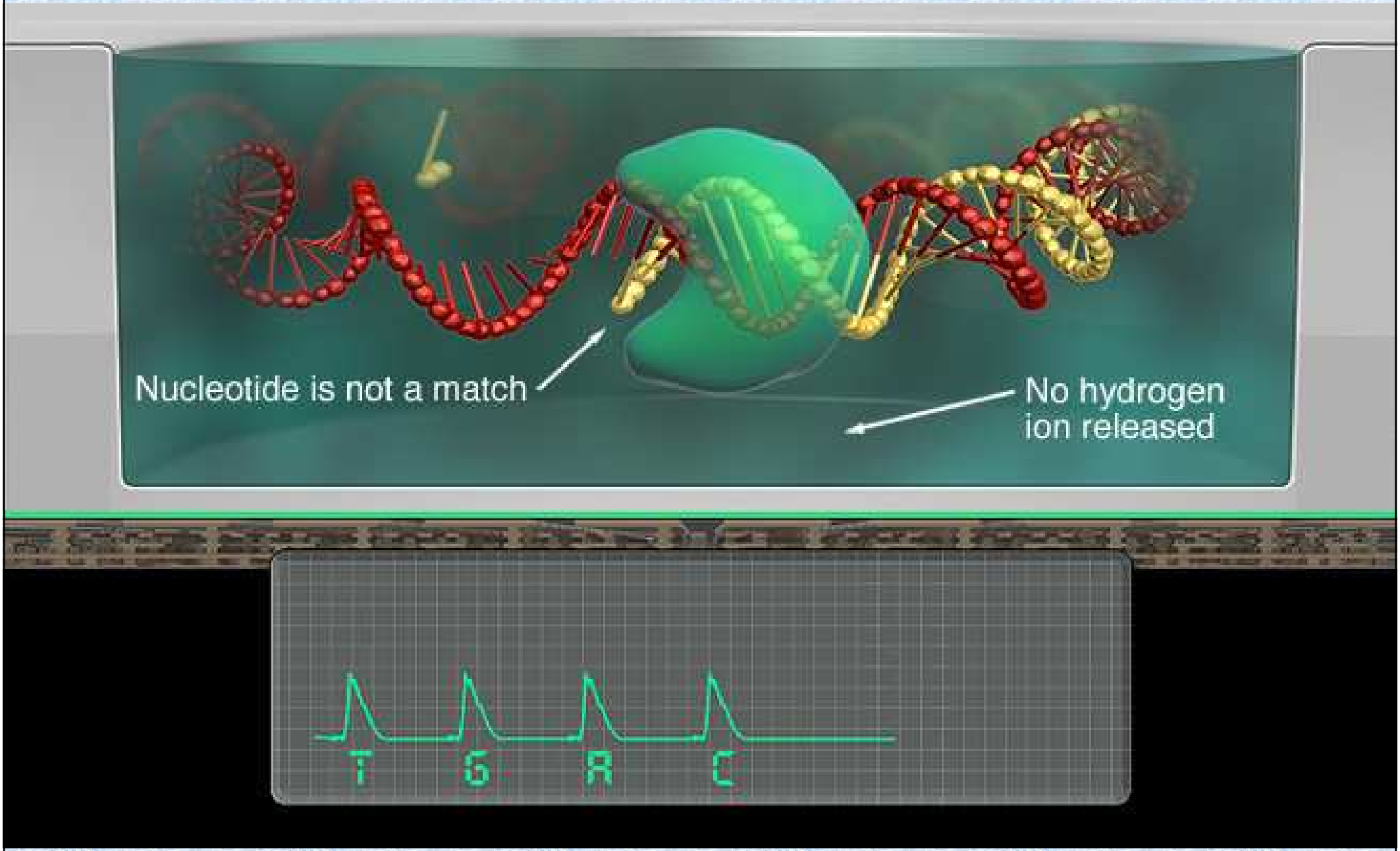
High-density array of micro-machined wells. Each well holds a different DNA template. Beneath the wells is an ion-sensitive layer and a proprietary ion sensor.



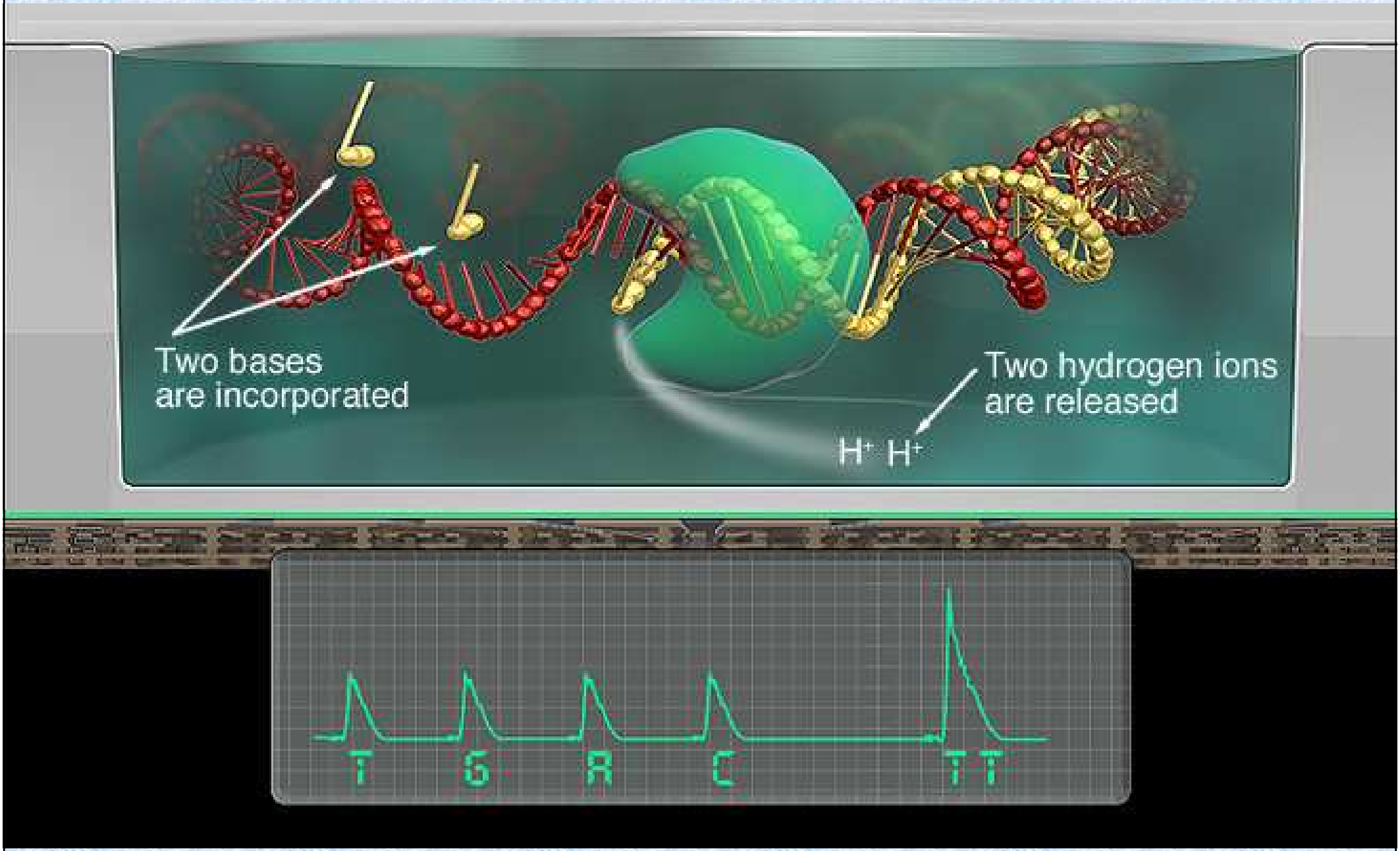
If a nucleotide is added to a DNA template and is then incorporated into a strand of DNA, a hydrogen ion is released. The charge from that ion will change the pH of the solution. The world's smallest solid-state pH meter—will call the base.



The sequencer sequentially floods the chip with one nucleotide after another. If the next nucleotide that floods the chip is not a match, no voltage change will be recorded.



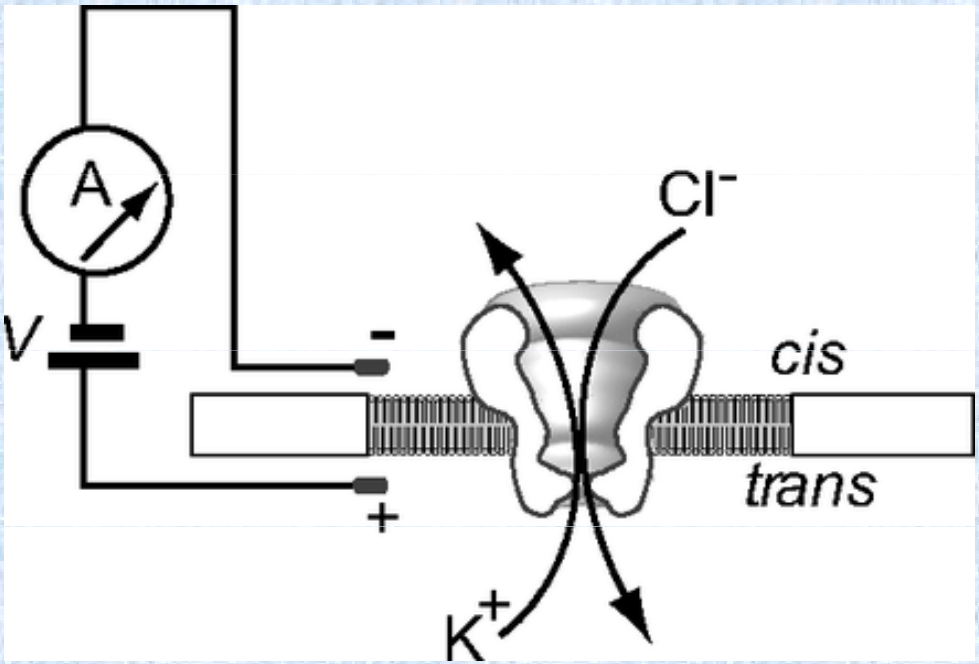
If there are two identical bases on the DNA strand, the voltage is double, and the chip records two identical bases.



Single molecule passage through a pore

Oxford Nanopore Technologies

Schematic of the nanopore device.



Oxford Nanopore Technologies

Principle and Instrumentation

DNA sequencing development

2001: Genome draft of 5 individuals in 9 months
– more than billion $

2015: Complete human genome in an hour – ~100 $

Sample preparation
for next gen. DNA/RNA sequencing

single cell profilling

Single-Cell RNA-Seq

- transkriptomy tisíců jednotlivých buněk různého typu a stavu

Tradiční techniky:

- Analýza několika genů v souborech tisíců buněk (např. *in situ* hybridizace)
- Profil exprese tisíců genů v homogenátu tkání.

Příklady „*Single-Cell RNA-Seq*“ aplikací:

- Pochopení heterogenity: nádorů, evoluce klonů, metastatických klonů, rezistence k léčivům atd.
- Pochopení komplexních tkání, např. neuronové tkáně. (Úplný transkripční profil jednotlivých neuronů aktivovaných externími stimuly představuje zásadní krok pro odhalení principu zachycení a uložení paměťové stopy.)
- Spolehlivá identifikace typů buněk a markerů, pochopení diferenciačních drah ve vývojové biologii a biologii systémů

Experimentální podmínky pro „single-cell sequencing“ tisíců buněk

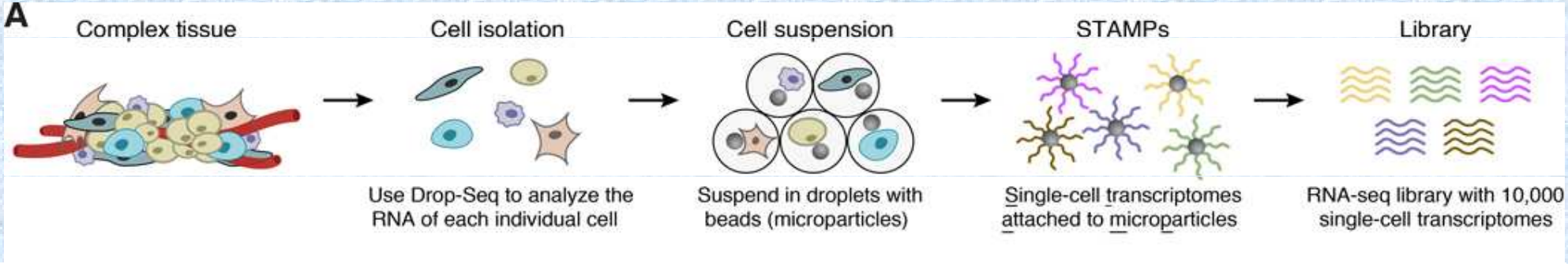
Manipulace s tisíci buňkami tkání	- mikro kontejnery (10^5 kapek/min)
Lýza buněk	- uvnitř kontejneru
Sekvenování oblasti genů	- RNA
RNA jedné buňky v kontejneru	- specificky značená částice pro hybridizaci
Kompletní transkriptome	- hybridizace RNA uvnitř kontejneru - nadbytek oligo primerů na jedné částici
Identifikace buněk	- buněčný barcode pro každý RNA fragment
Identifikace sekvence	- molekulární barcode - tatáž sekvence jednoho fragmentu může být analyzována vícekrát
RNA konstrukty vhodné pro	- reverzní transkripci - PCR - vysoce výkonný „next gen. sequencing“

Drop-RNA seq

enables highly parallel analysis of thousands of individual cells by RNA-seq

(Macosko et al., Cell, 2015, 161,1202-14)

- Analysis of RNA or transcriptome variation in identified cells



separation of thousands of cells in suspension

cell – RNA assignment – barcoding

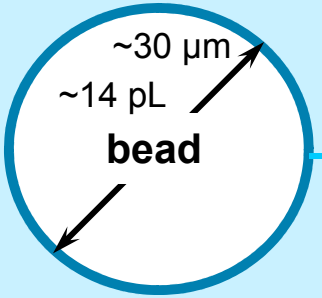
analyses of cellular transcriptomes

47

Molecular barcoded cellular transcriptomes

inside 0.5 nL droplets

10^8 reads on a single bead



PCR handle	cell barcode	mol. identifier	poly dT_{30}
	12 nts (4^{12} = 1.7*10^7)	8 nts (4^8 = 65536)	cellular mRNA hybridized
identical for all beads	identical for all primers on a bead, i.e. for the cell in the drop	different on each primer (reveals PCR duplicates)	captures polyA on mRNA and primes reverse transcription

1000 beads in µL

outside droplets

reverse transcription - cDNA

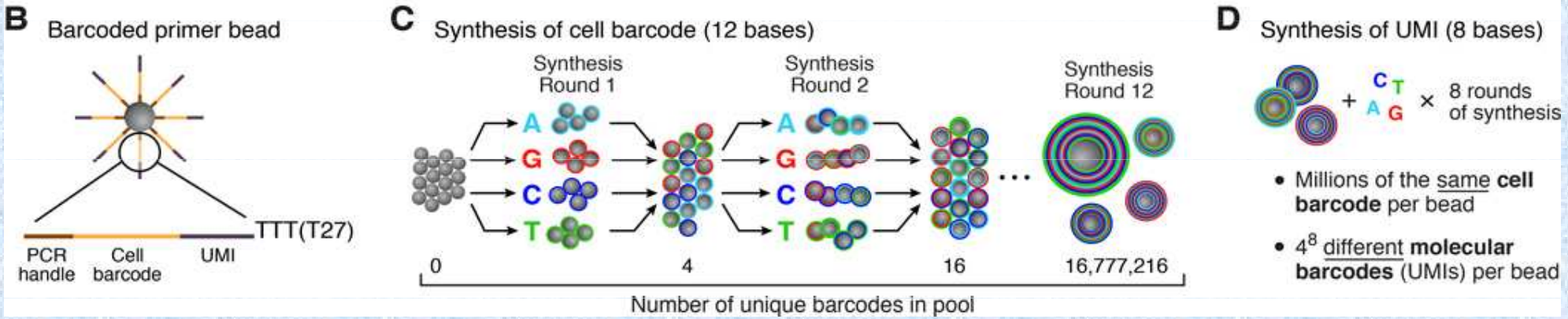
PCR amplified cDNA

high throughput sequencing

Synthesis of cellular barcodes and molecular identifiers on microparticles

Millions of primers on a microparticle

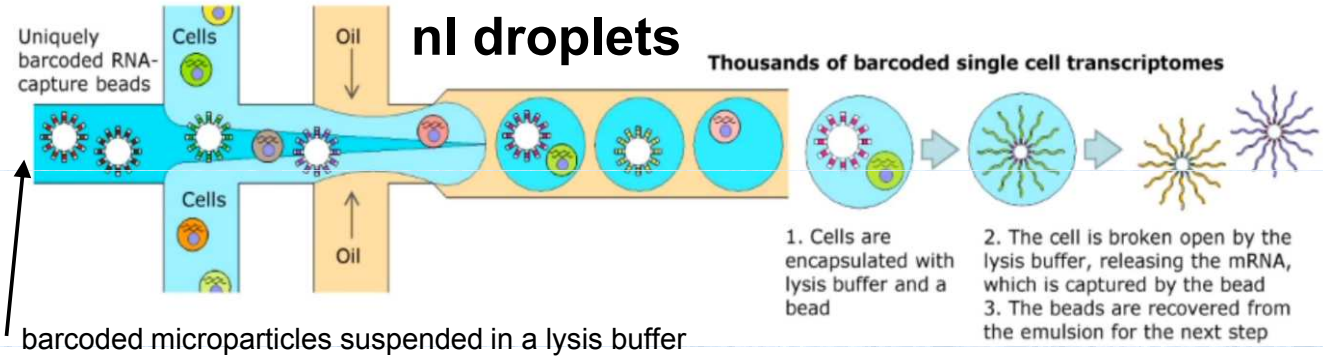
"split-and-pool" strategy	- the same sequence of all primers on a single bead
„bar codes"	- 4^{12} (16,777,216) possible barcodes after 12 rounds
	- different microparticles have different sequences
degenerative synthesis	- 8 synthesis rounds with 4 DNA bases
„univ. mol. identifier" (UMI)	- 4^8 (65,536) possible sequences on each particle
	- specific sequences for each primer
30 dT sequence	- complementary for polyA RNA



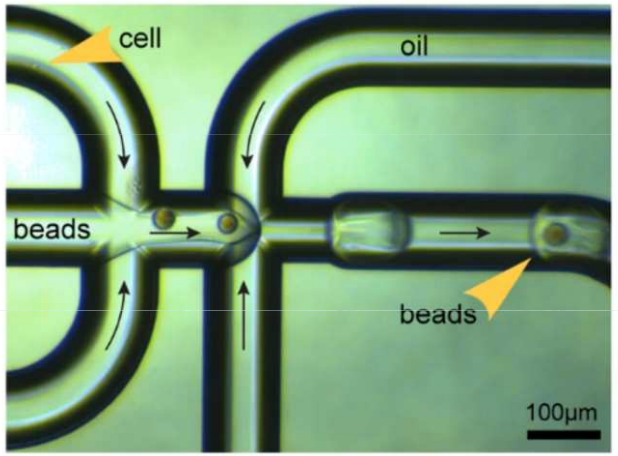
dolomite bio

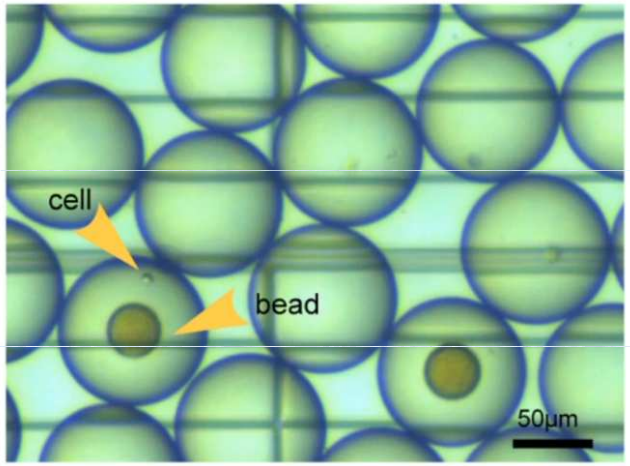
Single cell RNA-Seq

- Individual cells are captured in droplets with mRNA-capture beads
  - Each bead has a unique DNA sequence 'barcode'
  - There are 16 million unique barcodes in the bead library
  - Beads capture ~11% of mRNAs/cell (i.e., ~20,000 transcripts)

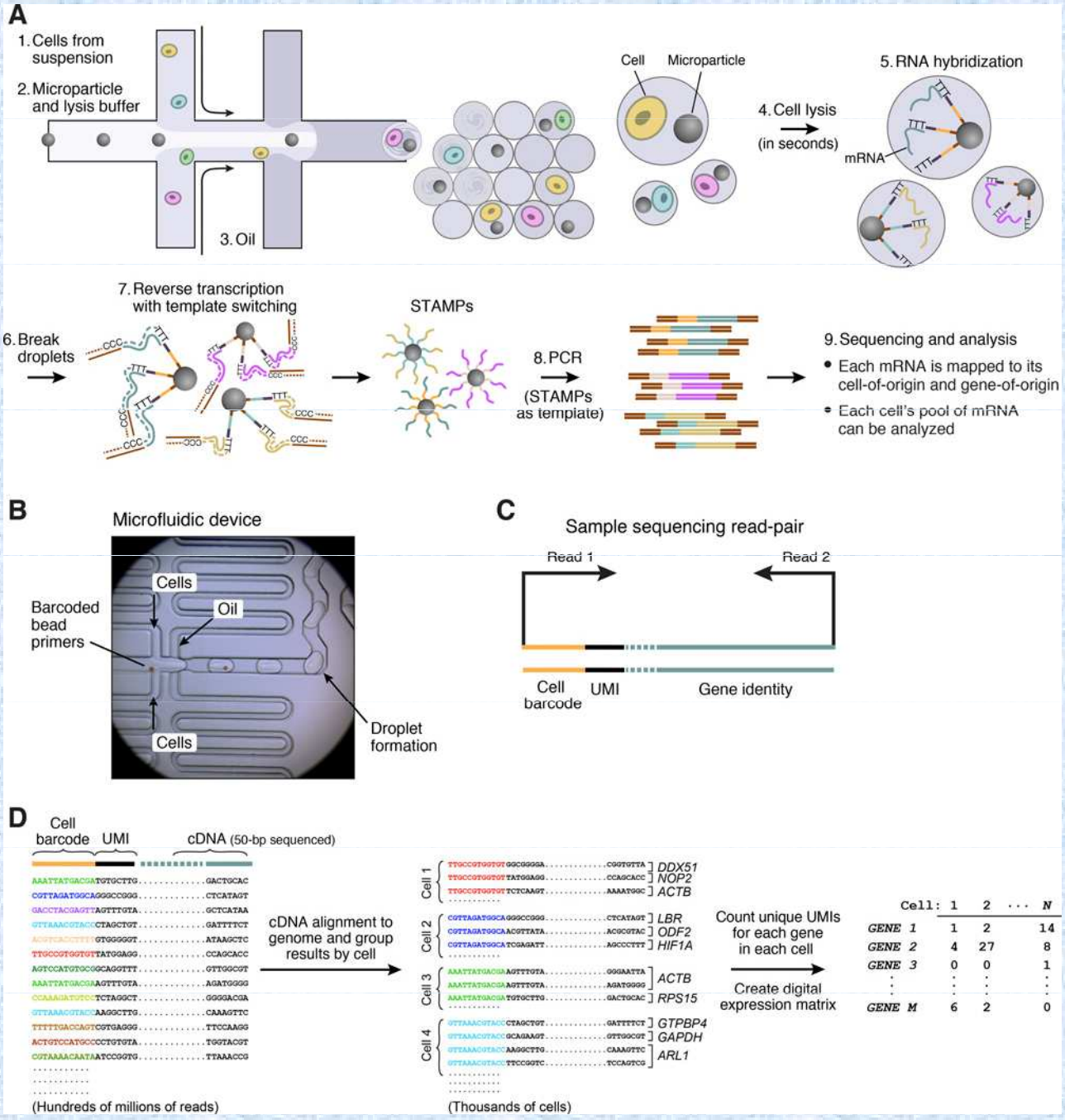


100,000 nl-sized droplets/min





Single Cell RNA-Seq



Complex neural mouse retina tissue

- transcriptomes from 44,808 mouse retinal cells analyzed
- 39 transcriptionally distinct cell populations identified

1) V čem spočívá princip polymerázové řetězové reakce (*PCR amplification*)?

Enzymatická reakce (DNA polymeráza) na templátu genomové DNA za přítomnosti dvou specifických primérů (krátkých oligonukleotidových řetězců vymezujících počátek a konec amplifikační syntézy) a deoxy nukleotidů (dATP, dTTP dCTP, dGTP) jako základních stavebních jednotek vede k cílené syntéze zvolených fragmentů. Cyklováním mezi teplotami 92, 62, 72 ˚C dochází postupně k disociaci dvouřetězcové DNA, asociaci primérů a syntéze fragmentů na obou komplementárních řetězcích. Takto se produkty předchozího cyklu stávají templáty cyklu následujícího a počet zvolených fragmentů tak narůstá exponenciálně (2^{n+1}, kde n je pořadí cyklu).

2) Princip Sangerovy sekvenační reakce?

Enzymatická syntéza (DNA polymeráza) komplementárního řetězce DNA k templátu (genomová DNA) za přítomnosti specifických primérů (krátkých oligonukleotidových řetězců vymezujících počáteční místo syntézy) dideoxy terminátorů (ddATP, ddTTP ddCTP, ddGTP) a deoxy nukleotidů (dATP, dTTP dCTP, dGTP) jako základních stavebních jednotek vede ke směsi různě dlouhých fragmentů. Poloha každého koncového nukleotidu je zde zakódována jako délka příslušného Sangerova sekvenačního fragmentu. Separací těchto fragmentů (specificky fluorescenčně značených na primérech, nebo dideoxy terminátorech), tedy dostáváme sekvenci nukleotidů v genomu.

3) Jaký je princip nejmodernějších metod sekvenování DNA?

a) Multiparalelní monitorování inkorporace jednotlivých nukleotidů do jedné molekuly dsDNA v reálném čase polymerázové syntézy.

b) Multiparalelní monitorování proudu, při průchodu molekul DNA přes póry umělé membrány.

Single Cell RNA-Seq

- transcriptomes of thousands of single cells varying in type and state

Traditional Techniques:

- analysis of a few genes in thousands of individual cells (e.g., in situ hybridization)
- expression profile of thousands of genes only on a tissue homogenate.

Examples of *Single Cell RNA-Seq* applications:

- Understanding tumor heterogeneity and clonal evolution – lineage analysis, cancer stem cells, and drug resistant and metastatic clones.

- Understanding complex tissues (e.g. neural tissues - the first look at the entire transcriptional profile in individual neurons activated by external stimuli - a critical step in ultimately discovering how a memory is captured and stored).

- High resolution identification of cells types and markers, and understanding differentiation pathways in developmental and systems biology.

Experimental conditions for single-cell sequencing

Thousands of cells from a tissue	– capturing containers (10^5 droplets/min)
Gene coding regions	– RNA
Complete transcriptome	– excess of capturing oligo primers
Cell identification	– cell barcode for each RNA fragment
Sequence identification	- one sequence could be analyzed many times
RNA constructs amenable to	- reverse transcription
	- PCR
	- high throughput next gen. sequencing