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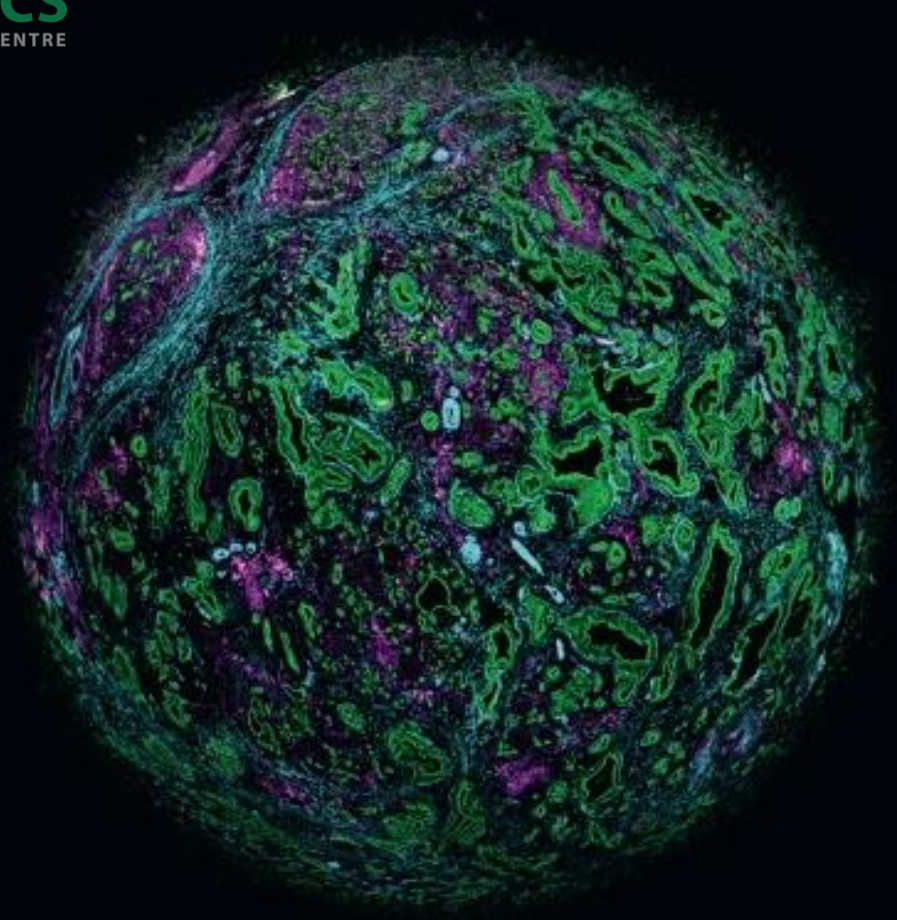


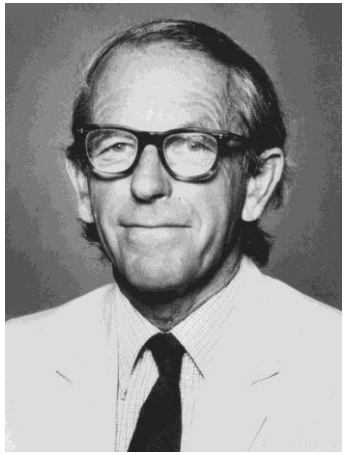
NGS technology & applications – the past, present & future

Dr. Martin Mau
Genomics Laboratory Manager
Omics Resource Centre | WCVM

VTMC 831 | May 12, 2025

What is sequencing, and why bothering?

Sequencing = A technique for determining the exact sequence of nucleotides, or bases, in a DNA or RNA molecule.



"... [A] knowledge of sequences could contribute much to our understanding of living matter."

[Frederick Sanger]

- 2 Noble prices
  - determining the amino acid sequence of insulin and other proteins
  - The Sanger (chain-termination) method for DNA sequencing
  - One of the first to sequence RNA (5S rRNA) in 1967 (Robert W. Holley and team sequenced first RNA (S. cerevisae Ala-tRNA in 1965)

Sadava, D. E. (2011). Life: the science of biology. 9. ed. Sinauer Associates.

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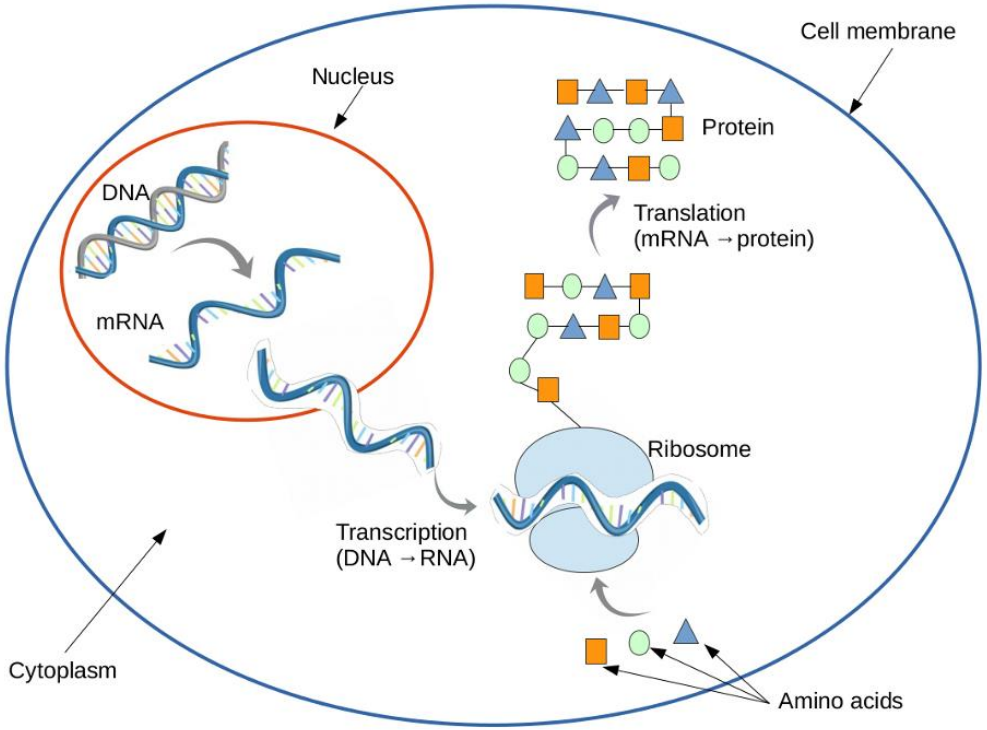
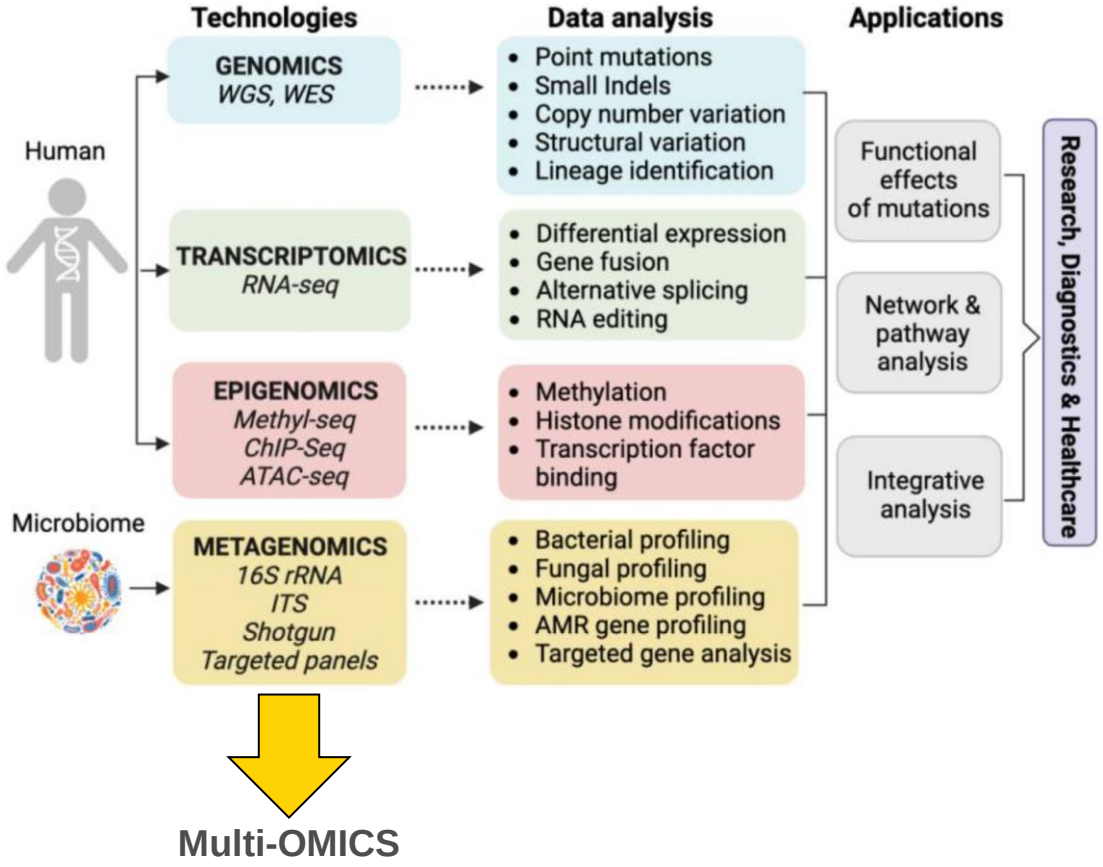
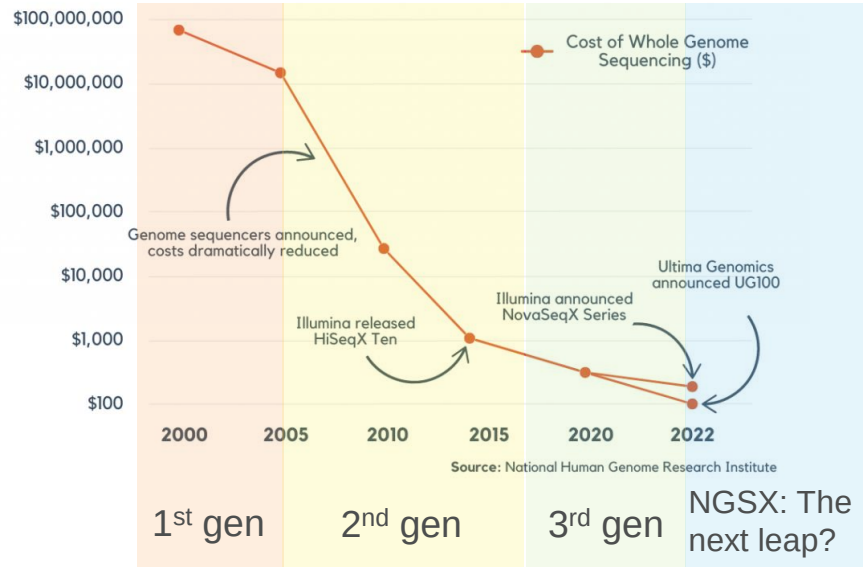




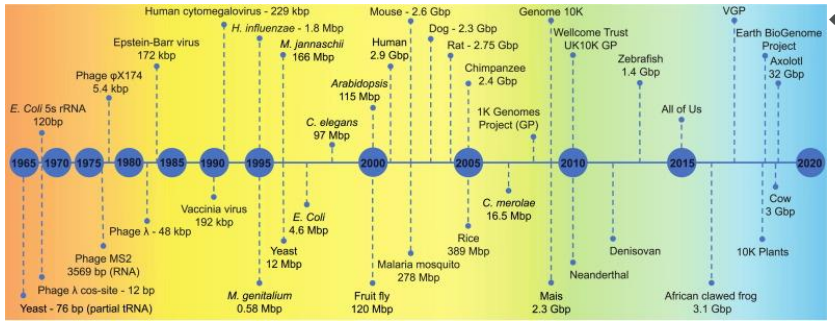
R. Hesketh, The War on Cancer, New York (2012) p.65; Satam H, et al. Biology 2023 12(7):997. doi: 10.3390/biology12070997

Sequencing on its way to be democratized

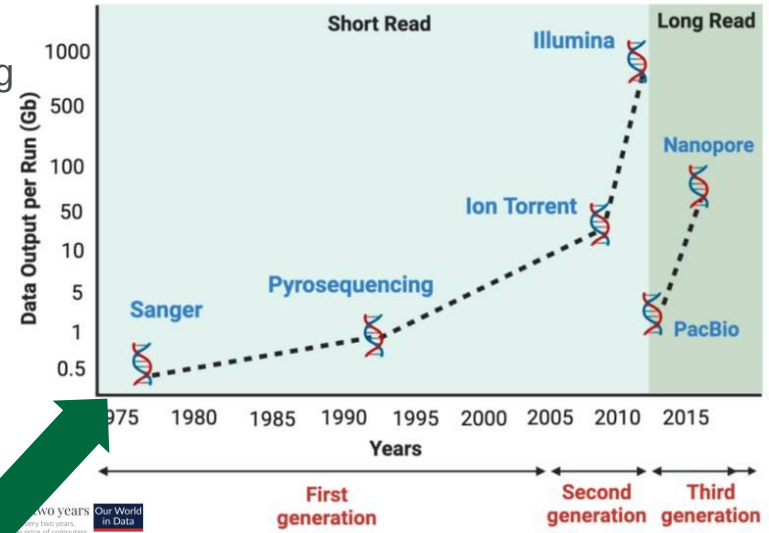
- Decreasing genome sequencing costs



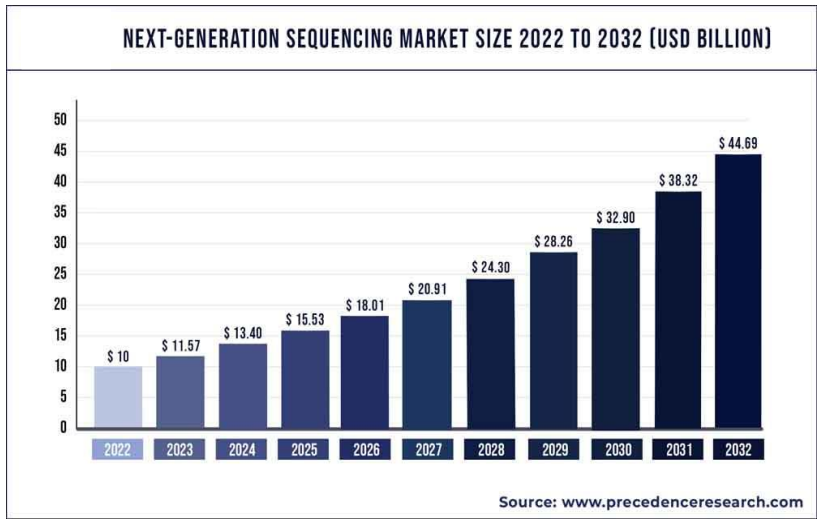
- Milestones

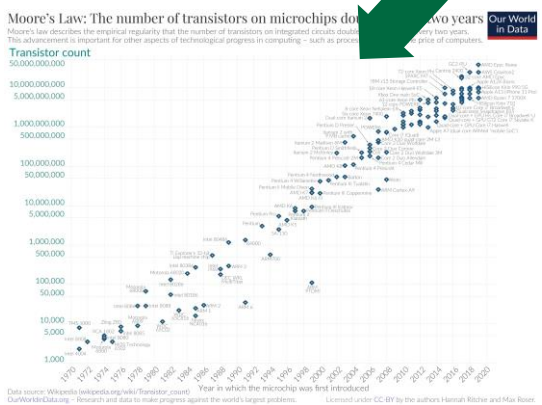


- Increasing sequencing output



- Increasing market size





https://genomize.com; https://www.precedenceresearch.com; wikipedia.org/wiki/transistor_count; Satam H, et al. Biology 2023 12(7):997, doi: 10.3390/biology12070997, Giani et al. Computational and Structural Biotechnology Journal,(18)2020,9-19, https://doi.org/10.1016/j.csbj.2019.11.002.

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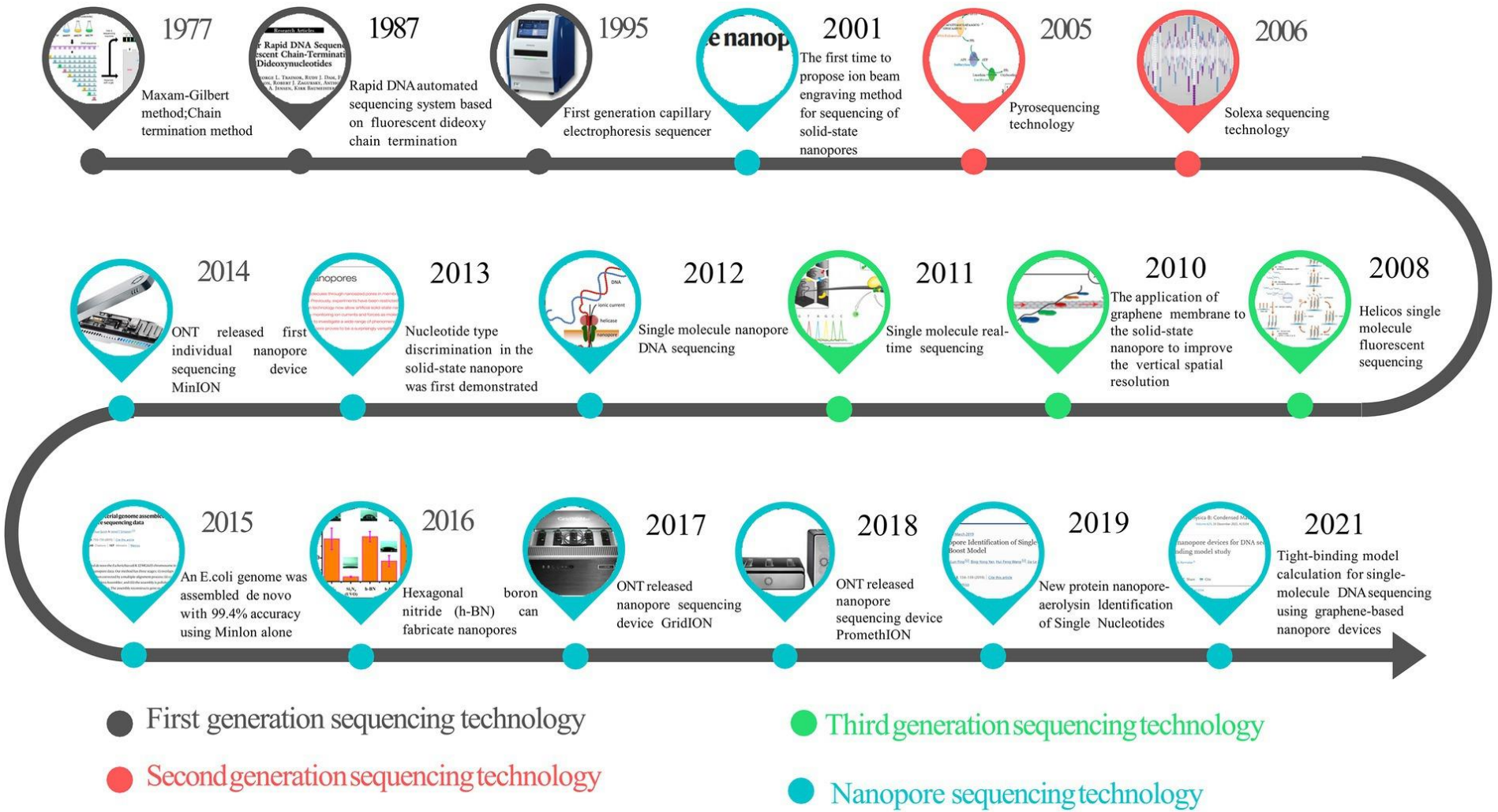
Evolution of sequencing technologies



James M. Heather, Benjamin Chain, Genomics, 2016, https://doi.org/10.1016/j.ygeno.2015.11.003

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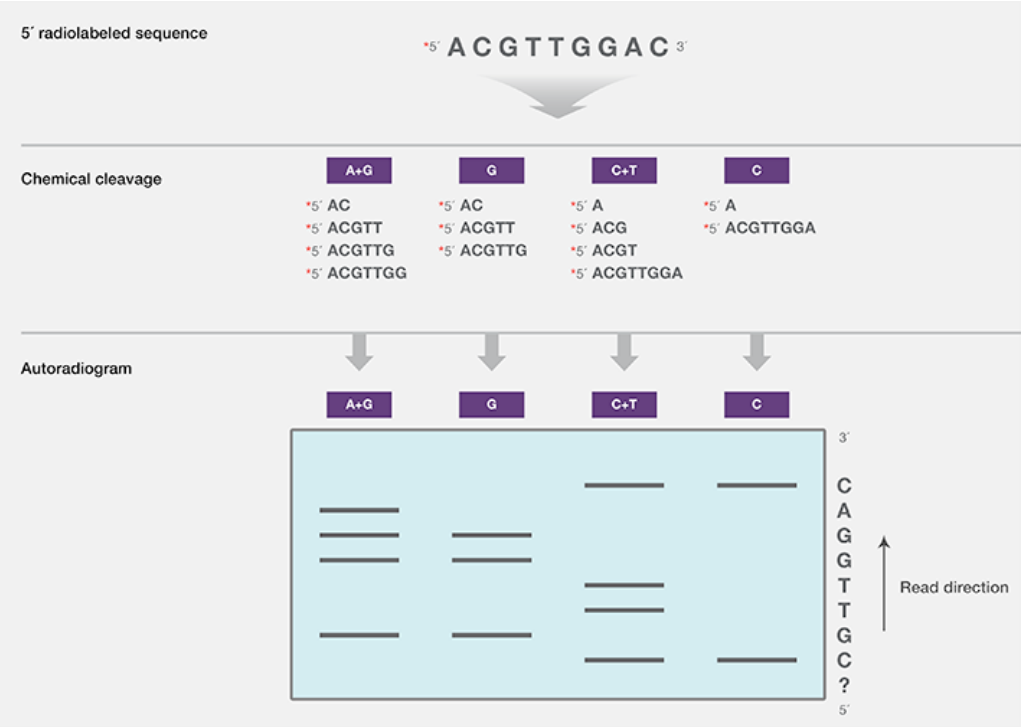
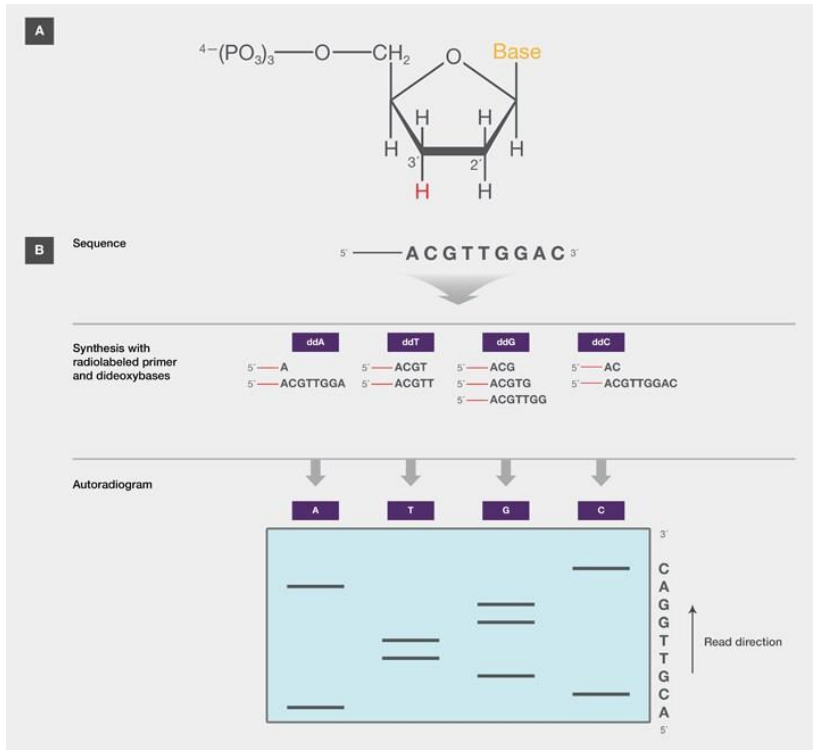
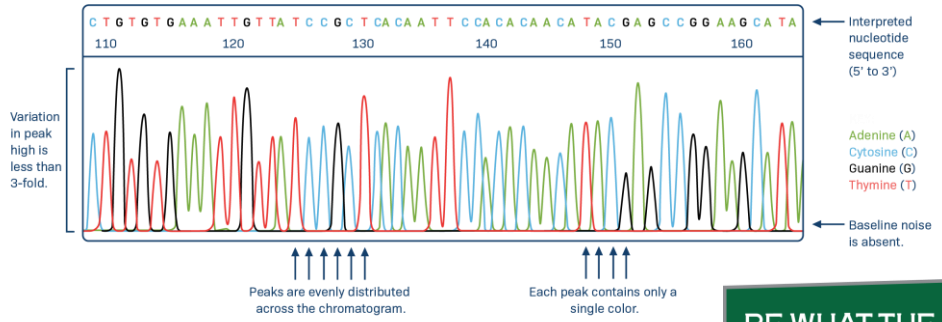
Classical sequencing – the gold standard

Maxam-Gilbert method



Sanger method





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https://www.thermofisher.com/ca/en/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/next-generation-sequencing/dna-sequencing-history.html

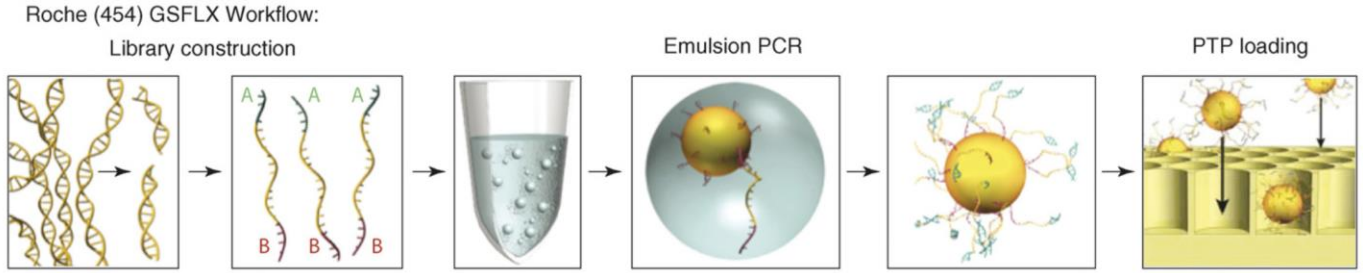
The beginnings of NGS technology

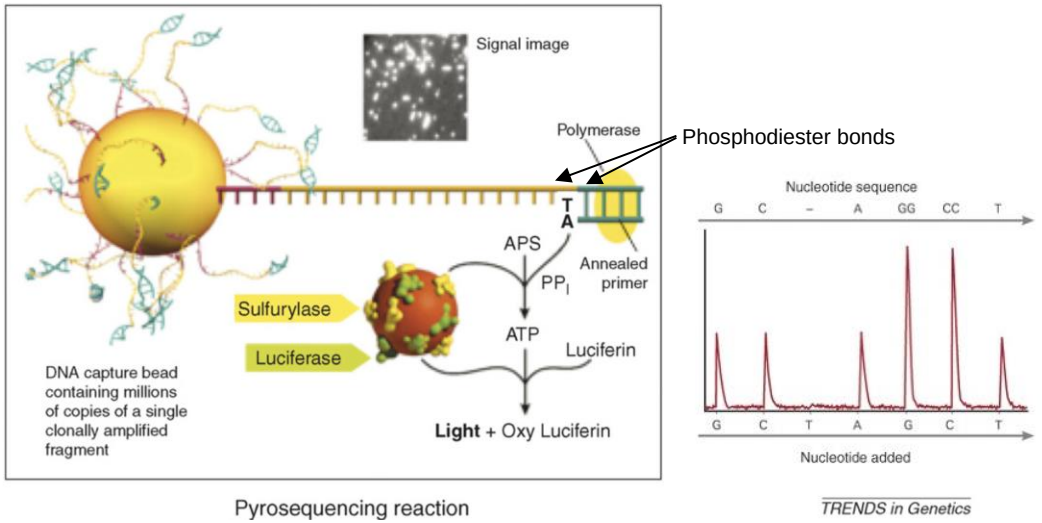
Pyrosequencing
- Principle in 1993 (Pal Nyren et al. U Stockholm), first commercial sequencer by 454 Life Sciences in 2004

Principle
- Sequencing by synthesis
- Utilizes pyrophosphate to detect nt incorporation
- Parallel sequencing
- First NGS technology

Advantages
- High throughput compared to Sanger (600Mbp/10hrs vs 2Mbp/day)
- Real-time monitoring
- No electrophoresis
- Suitable for SNP and indel detection

Disadvantages
- Short read length (100-300bp vs 500-1000bp)
- Poor sequencing capacity for homopolymer, repetitive and GC-rich regions

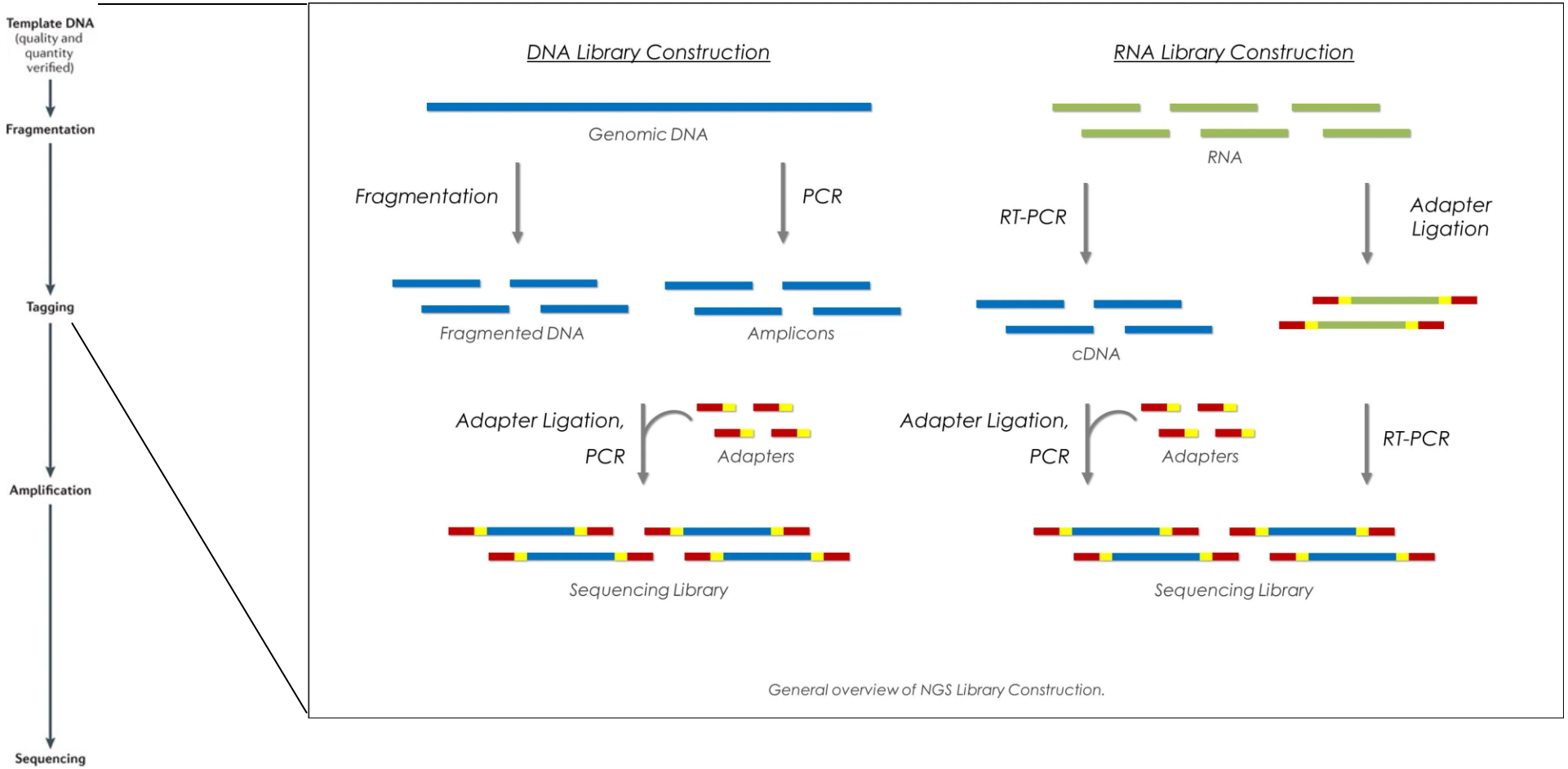




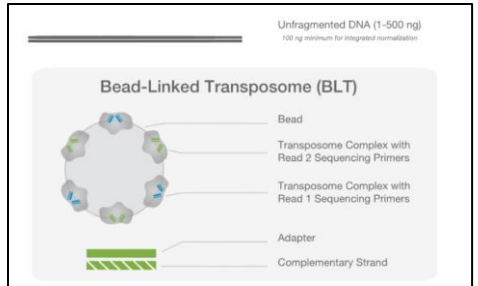
Mardis, Elaine R. Trends in Genetics Volume 24, Issue 3, 133 - 141 (2008)

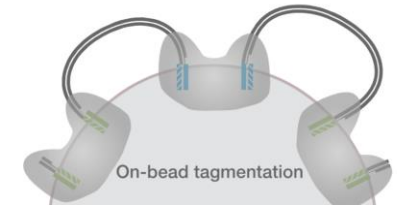
The 2nd generation: NGS/shotgun/massive parallel sequencing

Classic in solution fragmentation and adapter ligation

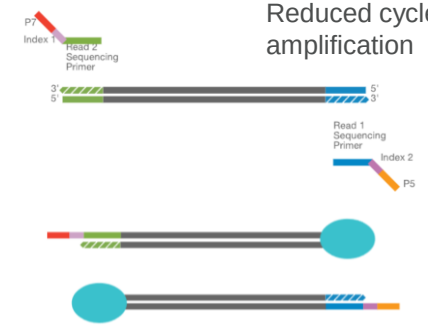


On bead Tagmentation

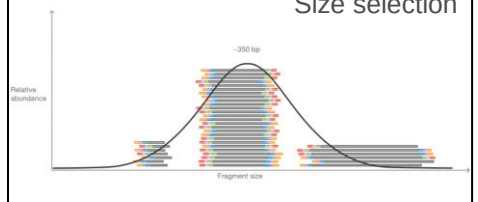




Reduced cycle amplification



Size selection



https://www.genome.gov/genetics-glossary/DNA-Sequencing;

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The 2nd generation: NGS/shotgun/massive parallel sequencing

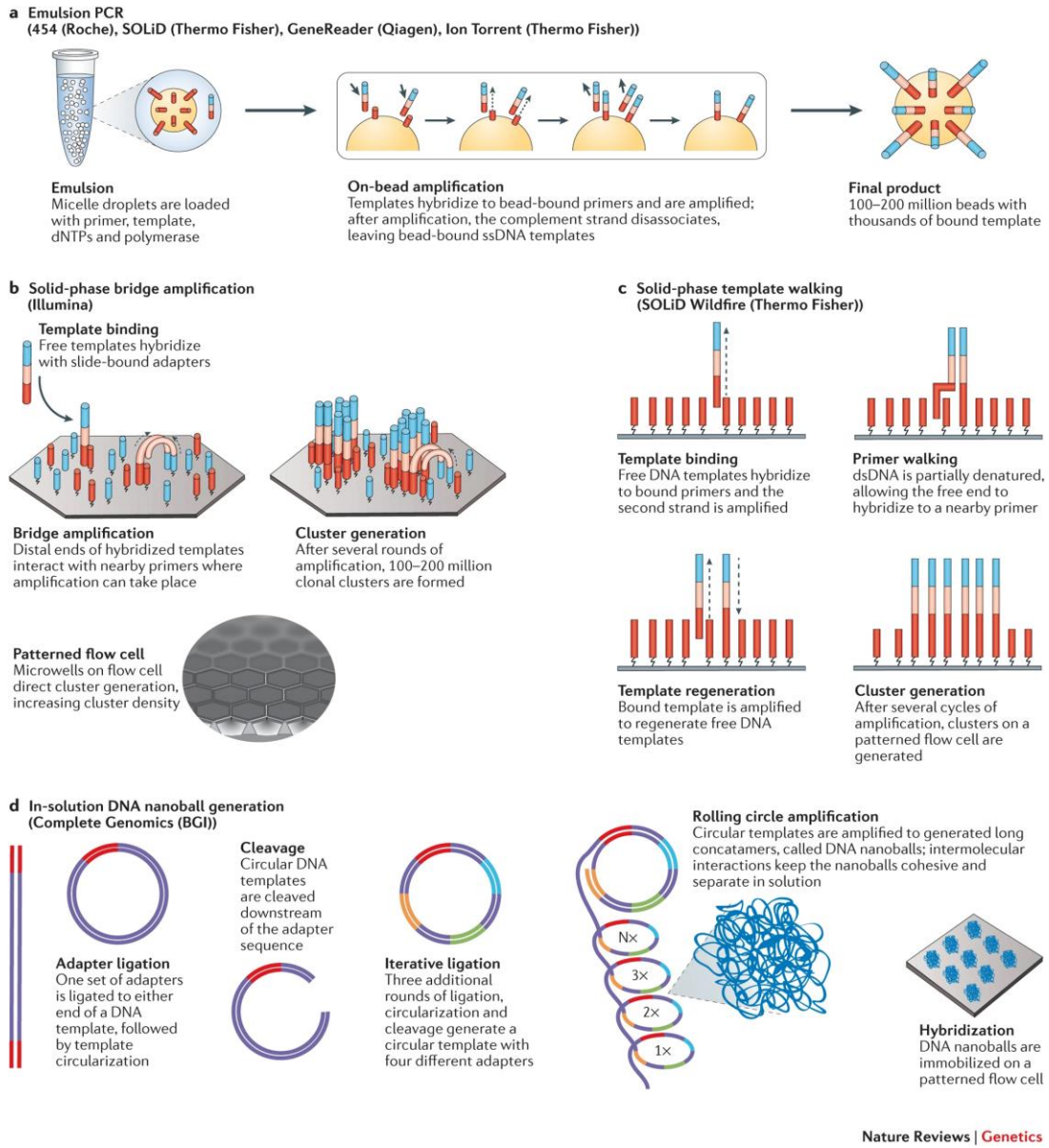
Template DNA
(quality and quantity verified)

Fragmentation

Tagging

Amplification

Sequencing



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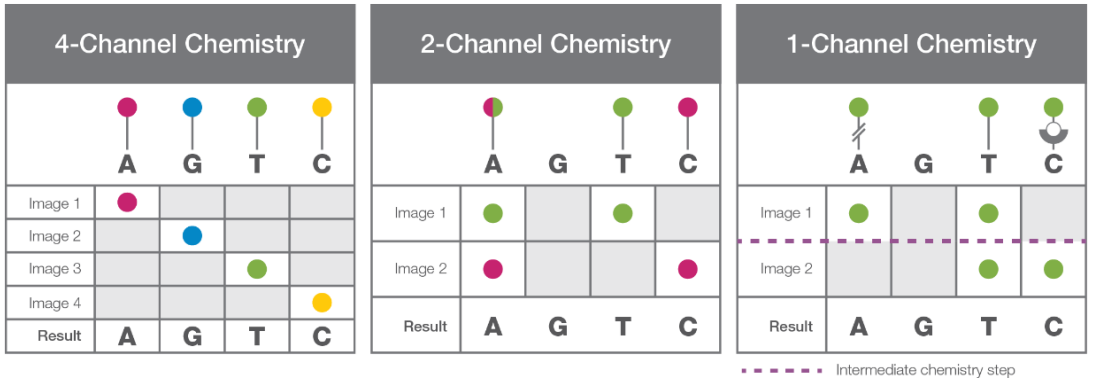
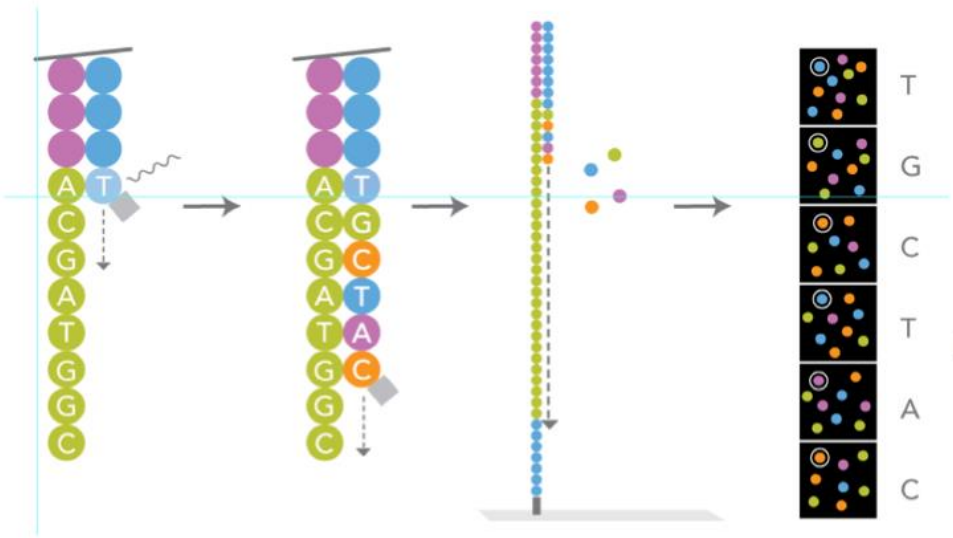
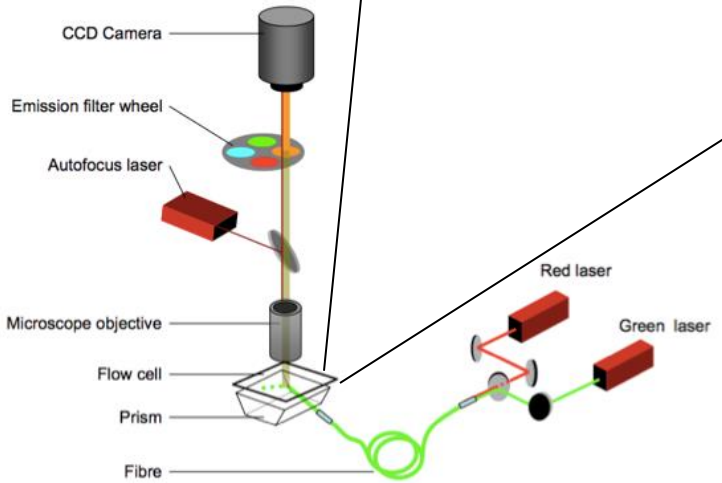
https://www.genome.gov/genetics-glossary/DNA-Sequencing

The 2nd generation: NGS/shotgun/massive parallel sequencing







Sequencing by synthesis (SBS)

https://www.illumina.com

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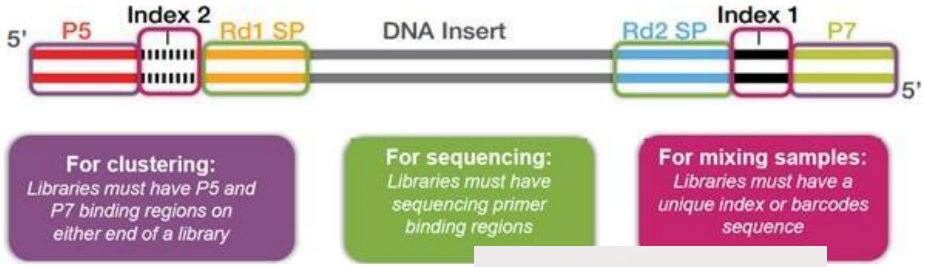
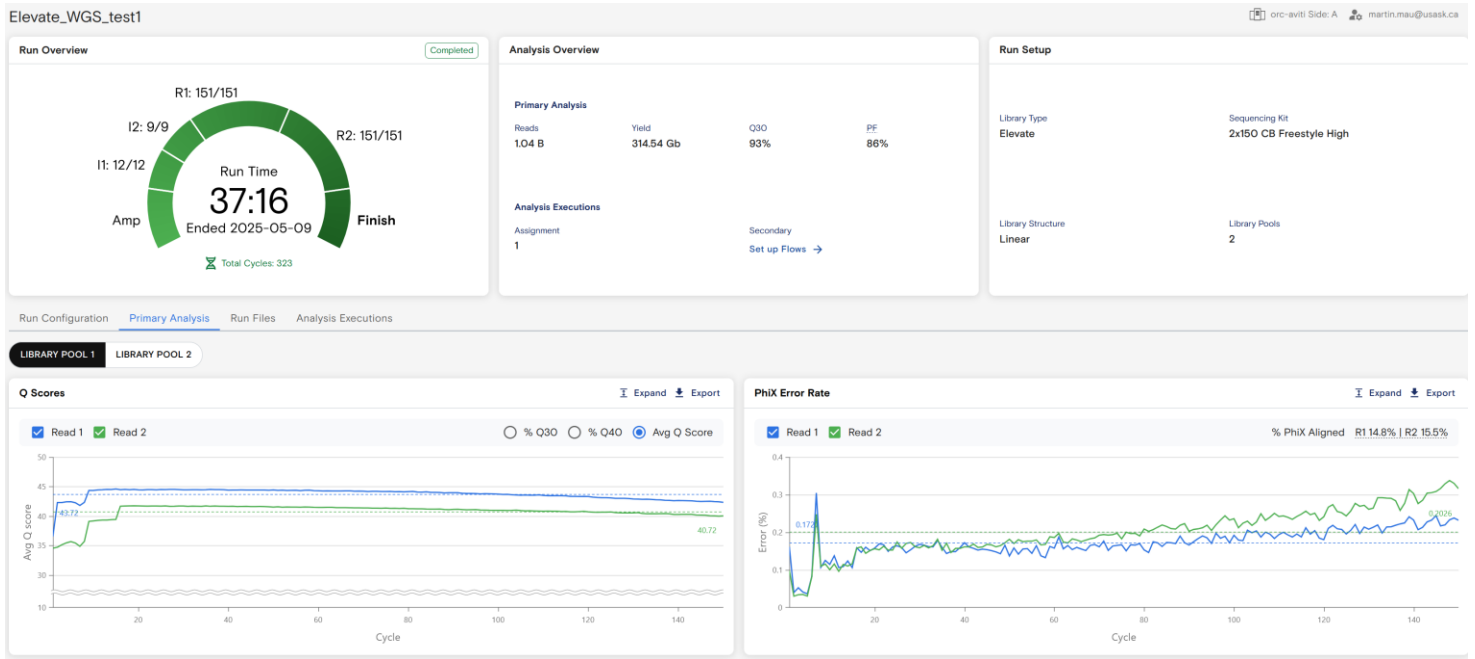
The 2nd generation: NGS/shotgun/massive parallel sequencing



For clustering: *Libraries must have P5 and P7 binding regions on either end of a library*

For sequencing: *Libraries must have sequencing primer binding regions*

For mixing samples: *Libraries must have a unique index or barcodes sequence*



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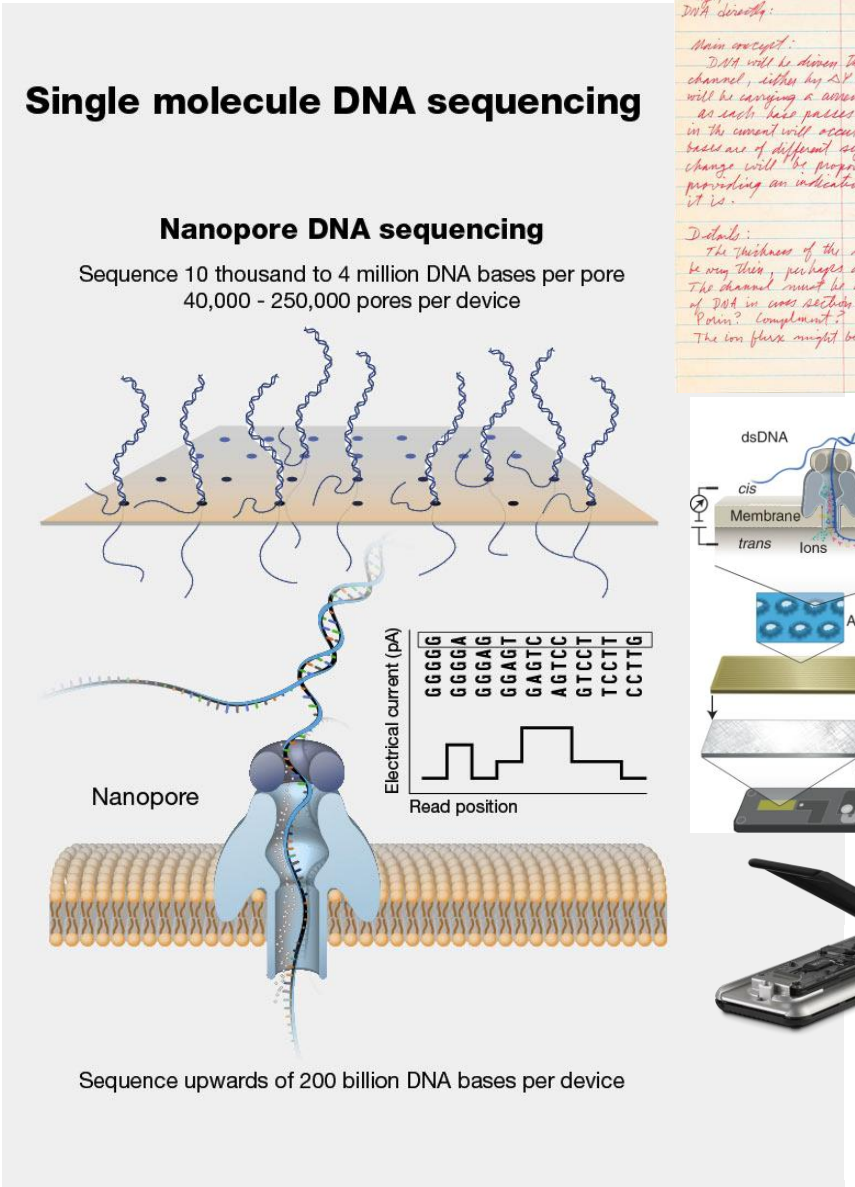
https://www.genome.gov/genetics-glossary/DNA-Sequencing: https://www.illumina.com/science/technology/next-generation-sequencing/sequencing-technology.html

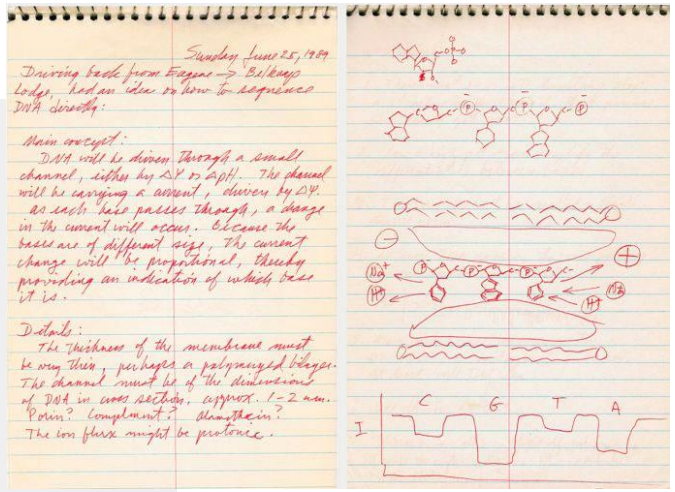
3rd Generation Sequencing - ONT

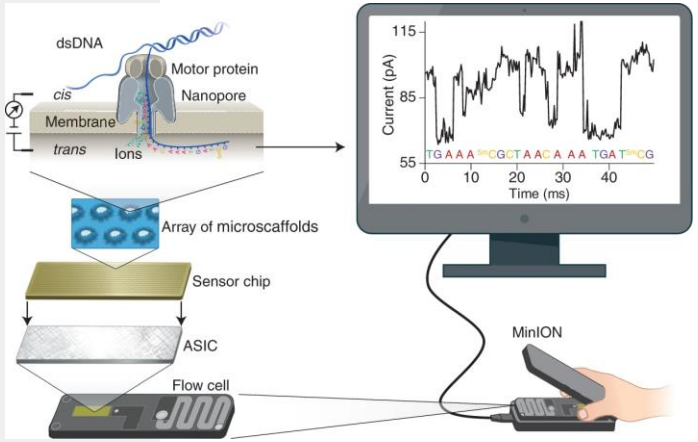
Technology
- Beginnings in 1989, seminal study in 2018
- Electrical current, drives an unwound oligomers (RNA/DNA) through an immobilized protein nanopore channel, disrupting the current as they passed through, in a manner characteristic of their base composition.
- Detection of electrical signals not fluorescence

Advantages
- Read limitations only by length of HMW DNA (Read length N50: 100-200kbp → 4Mbp)
- De novo genome assemblies
- Genomic + Epigenomic information
- Field sequencing

Disadvantages
- Lower accuracy (Phred Q score ~20)
- Lower output in gigabases (10s Gb/FC)







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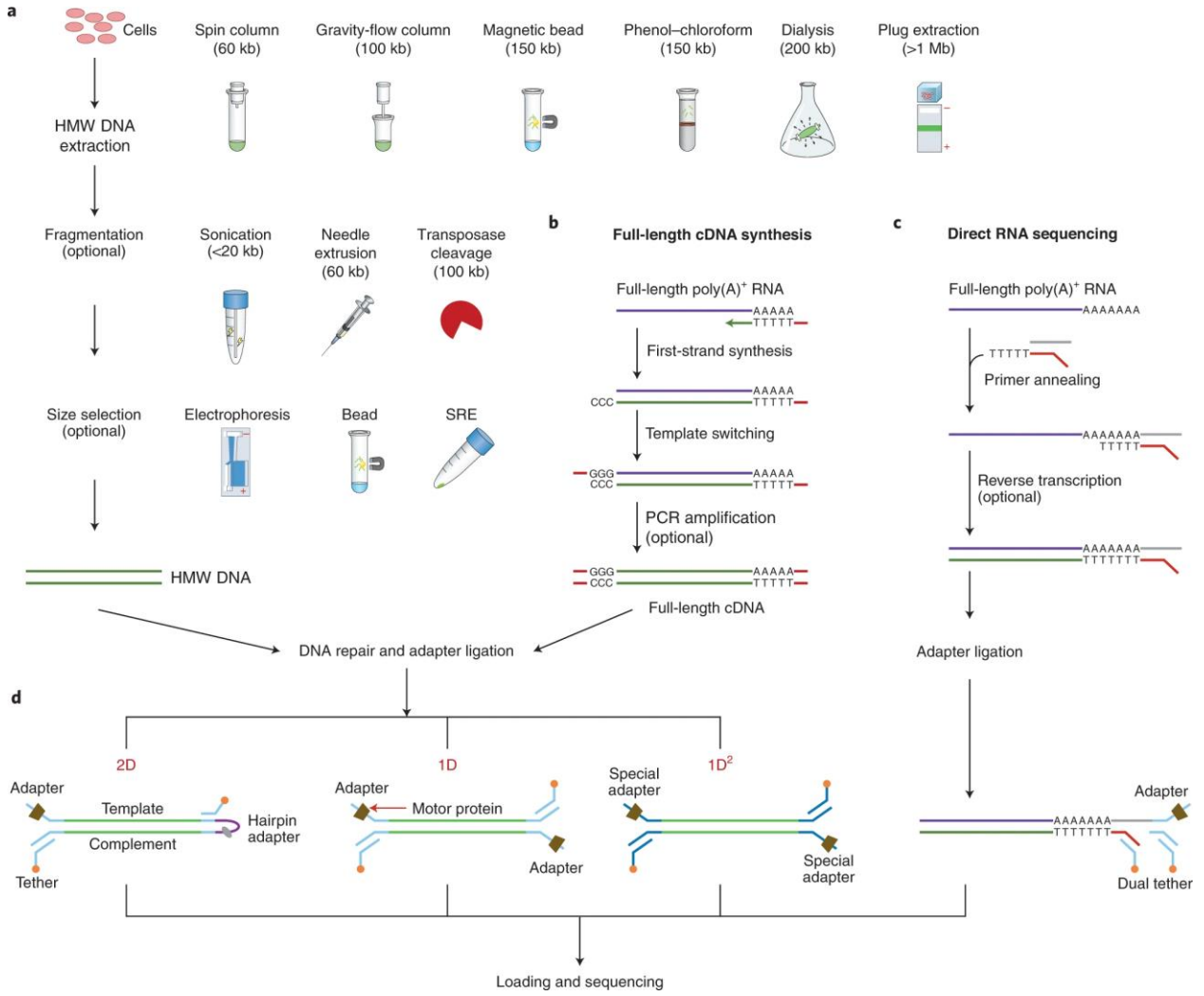
Wang et al. Nat Biotechnol 39, 1348–1365 (2021). https://doi.org/10.1038/s41587-021-01108-x

3rd Generation Sequencing - ONT

Sample preparation

- HMW DNA extraction is sensitive process
- QC requires specific equipment (Femtopulse)
- 2D (library for template + complement strand sequencing)
- 1D (each strand ligated and sequenced separately)
- 1D2 (sequential sequencing of both strands)



Wang et al. Nat Biotechnol 39, 1348–1365 (2021). https://doi.org/10.1038/s41587-021-01108-x

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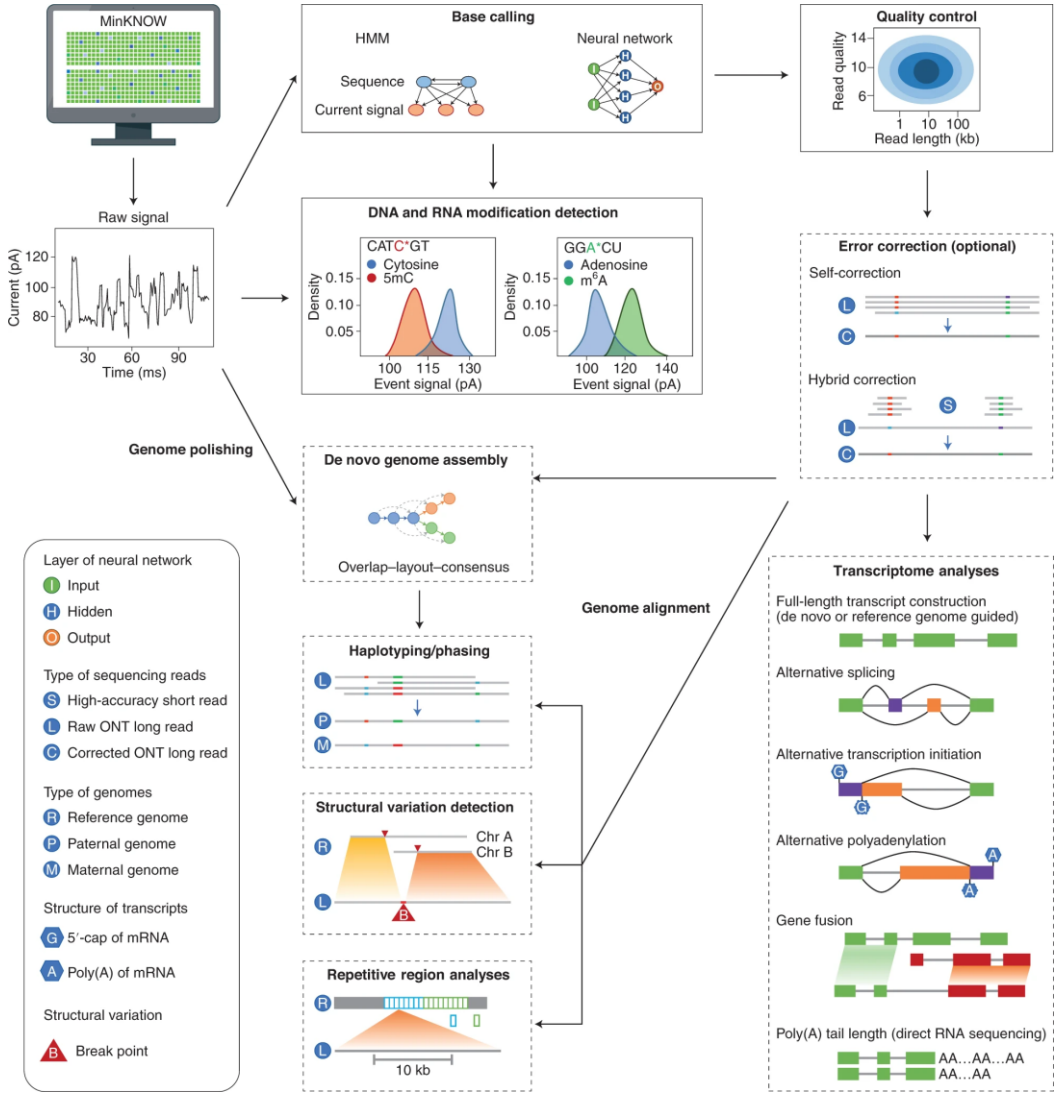
3rd Generation Sequencing - ONT

ONT data ideal for

- Multiploid organisms
- Complex genomes (e.g. recurrent hybrids, young and fast evolving genomes, genomes with high plasticity)
- Organisms without reference genomes or close relatives with reference genomes (de novo assembly)
- Detection of 5-Methylcytosin (5mC) modifications



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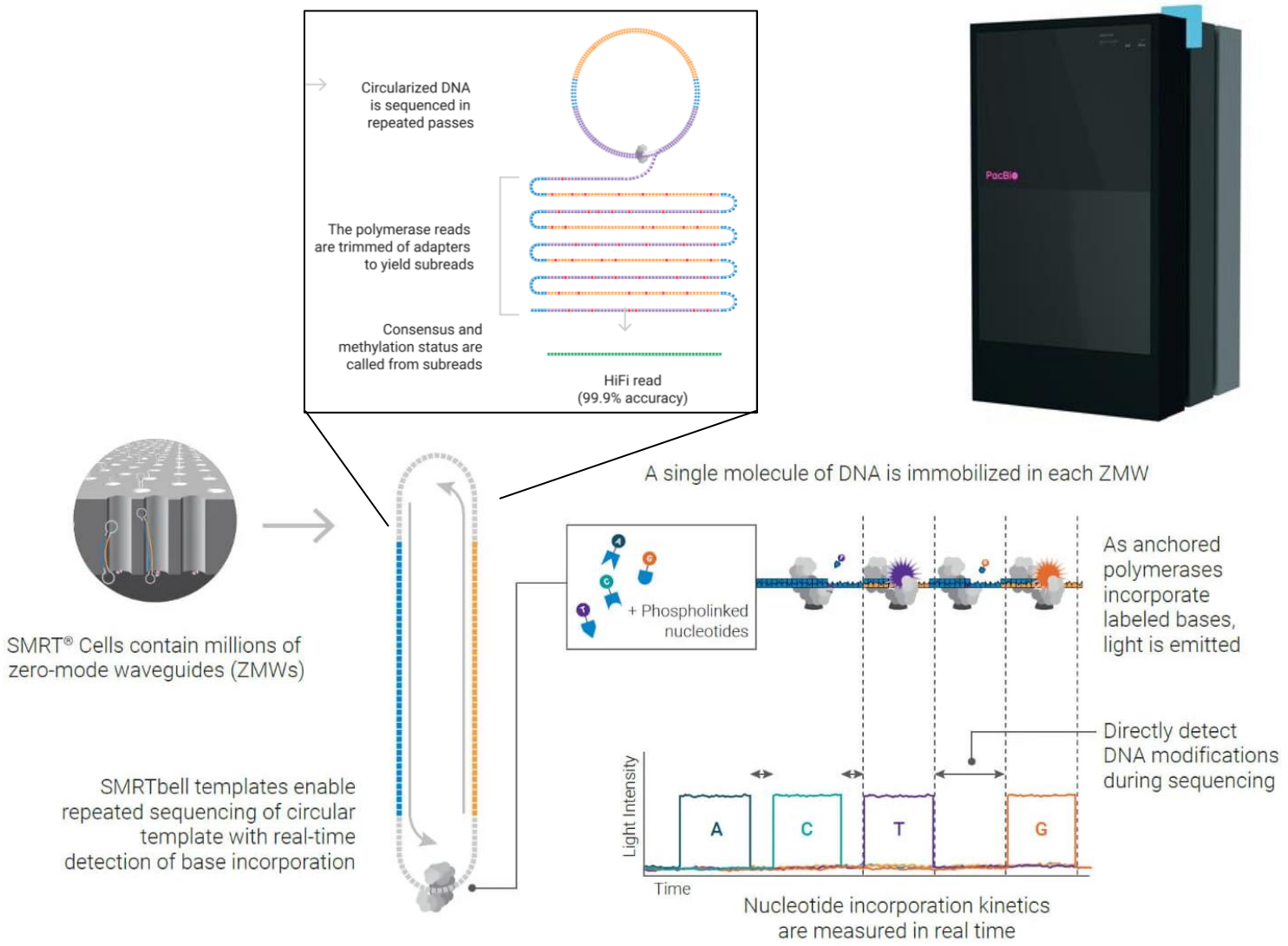
Wang et al. Nat Biotechnol 39, 1348–1365 (2021). https://doi.org/10.1038/s41587-021-01108-x

3rd Generation Sequencing – SMRT-Seq

Technology
- Single molecule sequencing in ZMWs
- SBS chemistry and photometrics

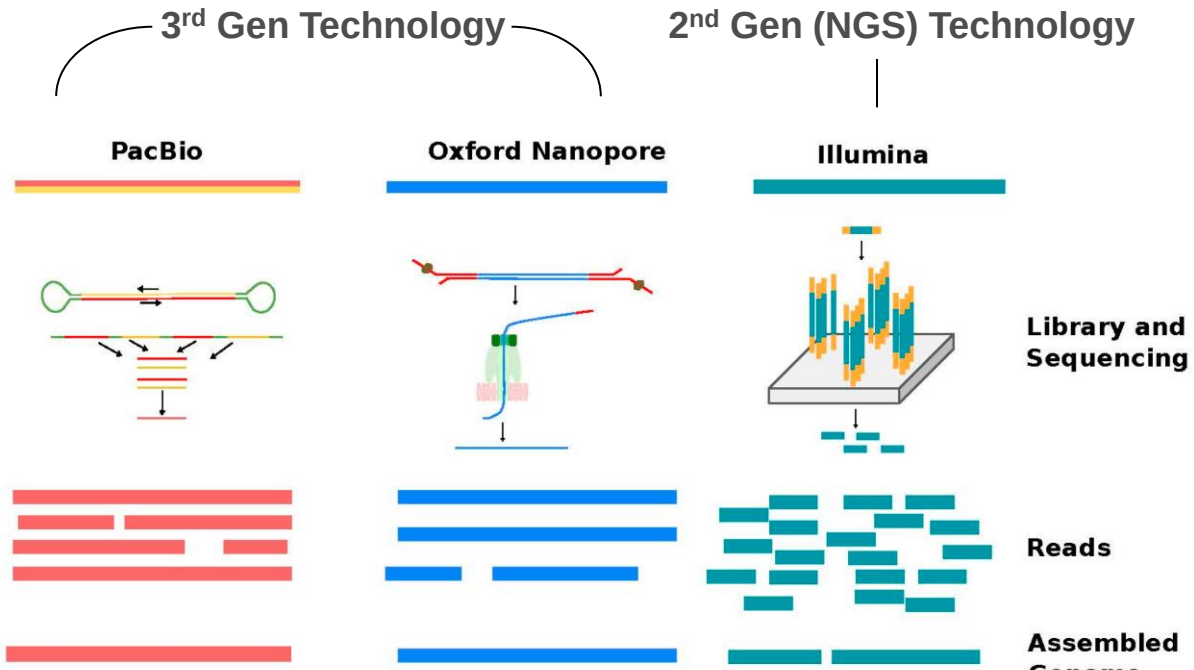
Advantages
- Merged reads from multiple passes enables higher accuracy compared to ONT (Q30+)
- Read length N50: 10-60kb
- Genomic + Epigenomic information (5mC in CHG context)

Disadvantages
- Higher costs compared to ONT sequencing ($43-86/Gb vs. $21-42/Gb)



https://www.pacb.com/technology/hifi-sequencing/how-it-works/

Sequencing technologies



Advantages

- **PacBio**
  - Long-read lengths
  - High consensus accuracy
  - Low degree of bias
  - Simultaneous epigenetic characterization
- **Oxford Nanopore**
  - Ultra-long read lengths
  - Real-time sequencing
  - Low degree of bias
  - Simultaneous epigenetic characterization and direct RNA sequencing
  - Cost-effectiveness
  - Scalability and portability
- **Illumina**
  - High throughput and speed
  - Accuracy and reliability
  - Scalable and versatile
  - User-friendly and well established technology
  - Low cost per gigabase

Disadvantages

- **3rd Gen Technology**
  - Higher costs per gigabase
  - Lower sequencing accuracy
  - Computationally demanding
  - Less suitable for targeted seq and high-indepth variant analysis
  - Requires larger input and high-quality samples
- **2nd Gen (NGS) Technology**
  - Read length limitation
  - Requires higher sequencing depth to cover genome
  - Not suitable for high-quality de novo assembly on itself

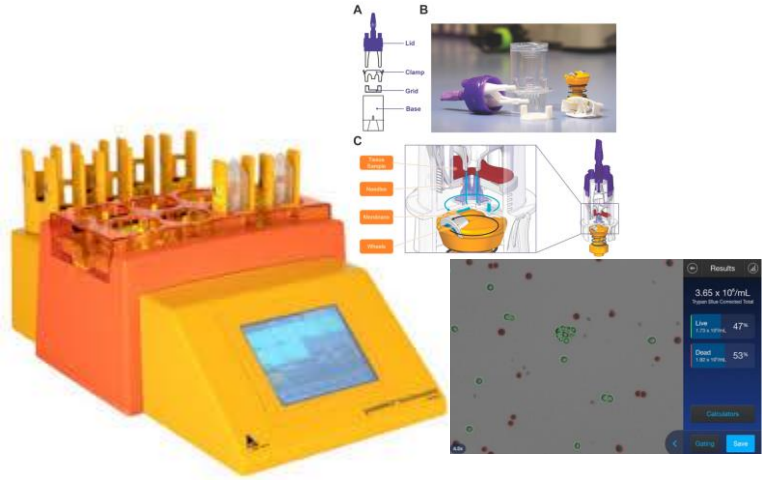
Ermini et al. Cancers 2024, 16, 1275. https://doi.org/10.3390/cancers16071275

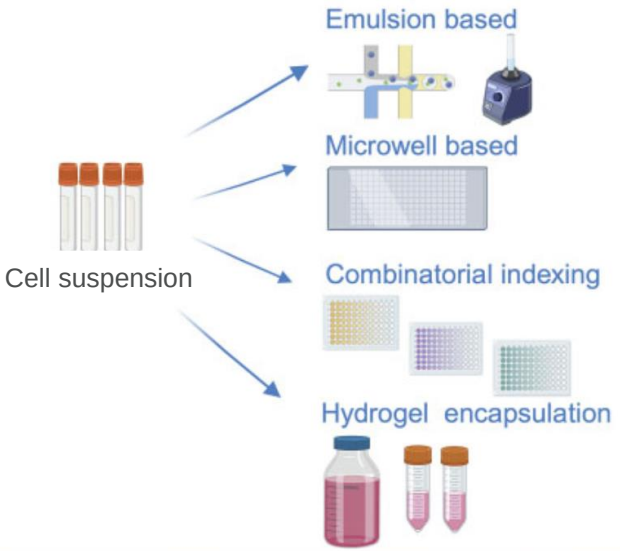
Changes to sample prep: scRNA-Seq

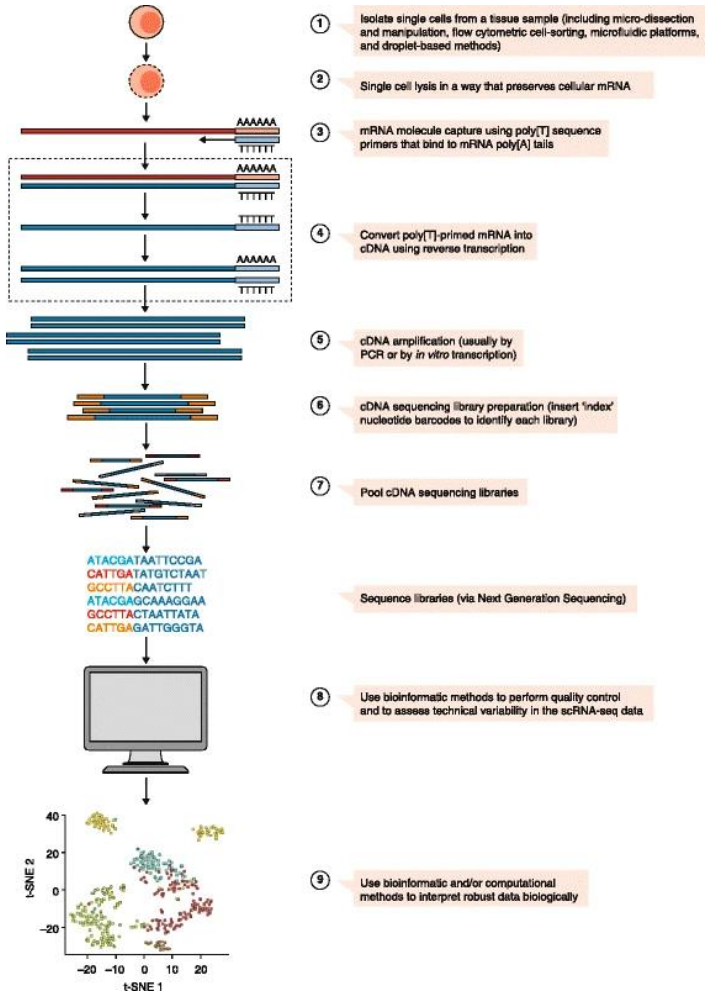
Technology
- various
- Utilizes any standard NGS technologies
- 10-50k reads per cell & 1k-mio cells

Advantages
- comparison of the transcriptomes of individual cells
- Addresses tissue heterogeneity (e.g. embryonic or immune cells)
- Identification of rare cell populations (e.g. normal-tumor interfaces)
- Characterization of unique cells (e.g. T-lymphocytes, neurons)
- Cell fate diversification

Disadvantages
- High costs
- Computation intense







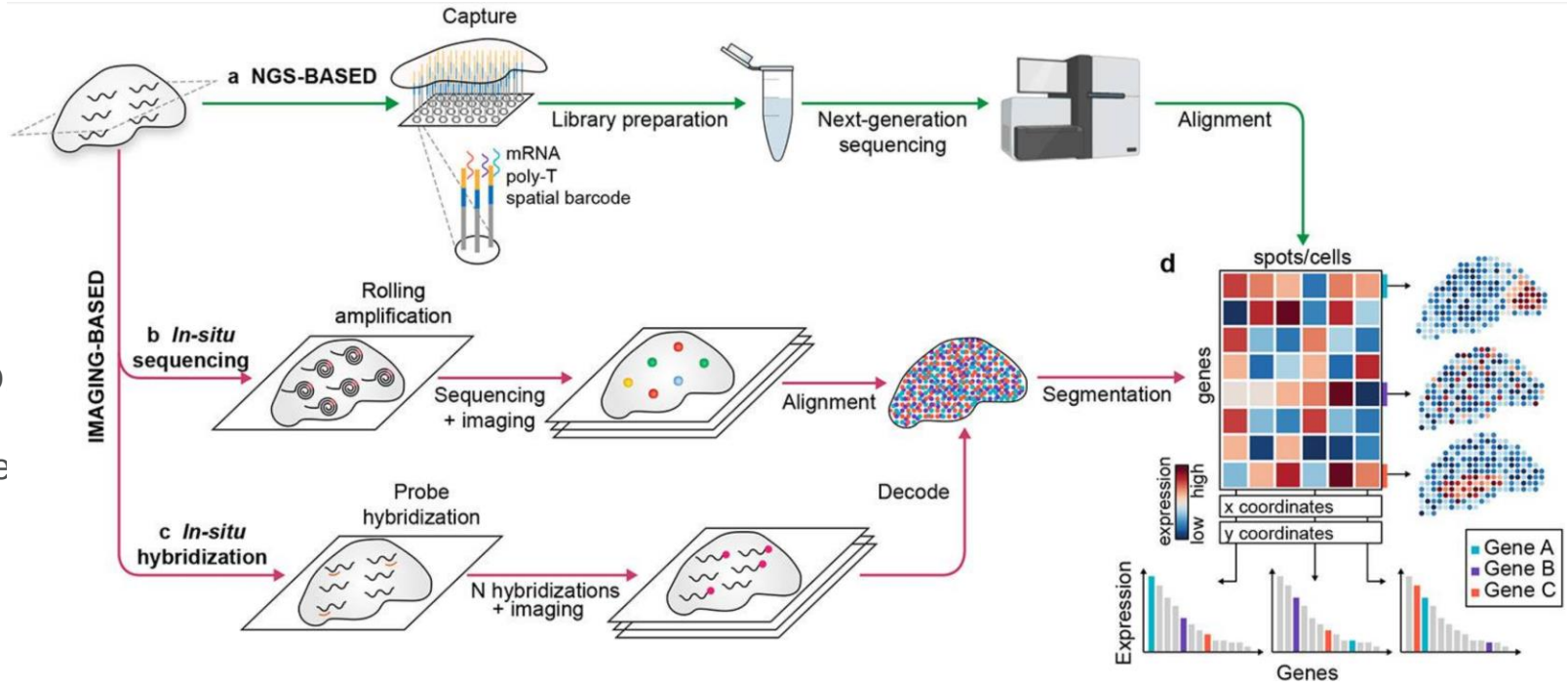
https://www.antteknik.com/en/products/?p=invitrogen-countess-3-automated-cell-counter; Simone, M., et al. (2024) Nucleic Acids Research 53(2); https://www.miltenyibiotec.com; Haque et al. Genome Med 9, 75 (2017). https://doi.org/10.1186/s13073-017-0467-4

Changes to sample prep : Spatial sequencing

Technology
- Combines FISH and standard NGS for spatial genomics (clusters - not single cell)
- Commercial ISS technologies:
  - 10X Genomics Xenium & Visium
  - Curio Bio SEEKER

Approaches
- NGS-based (Slide-Seq, DBiT-Seq)
- ISS (HybISS: Hybridization-based in situ sequencing (Nilsson lab, U Stockholm), FISSEQ fluorescent in situ RNA sequencing (Church lab, Wyss Institute), INSTA-Seq: in situ transcriptome accessibility sequencing (Lee lab, CSH)
- ISH (MERFISH, SeqFISH)

Applications
- Cell type identification
- Tumor-immune composition
- Cellular-molecular organization
- Tracking cell fate/development



Rao et al. Nature. 2021;596(7871):211-220. doi: 10.1038/s41586-021-03634-9

Changes to sample prep : In situ sequencing (ISS)

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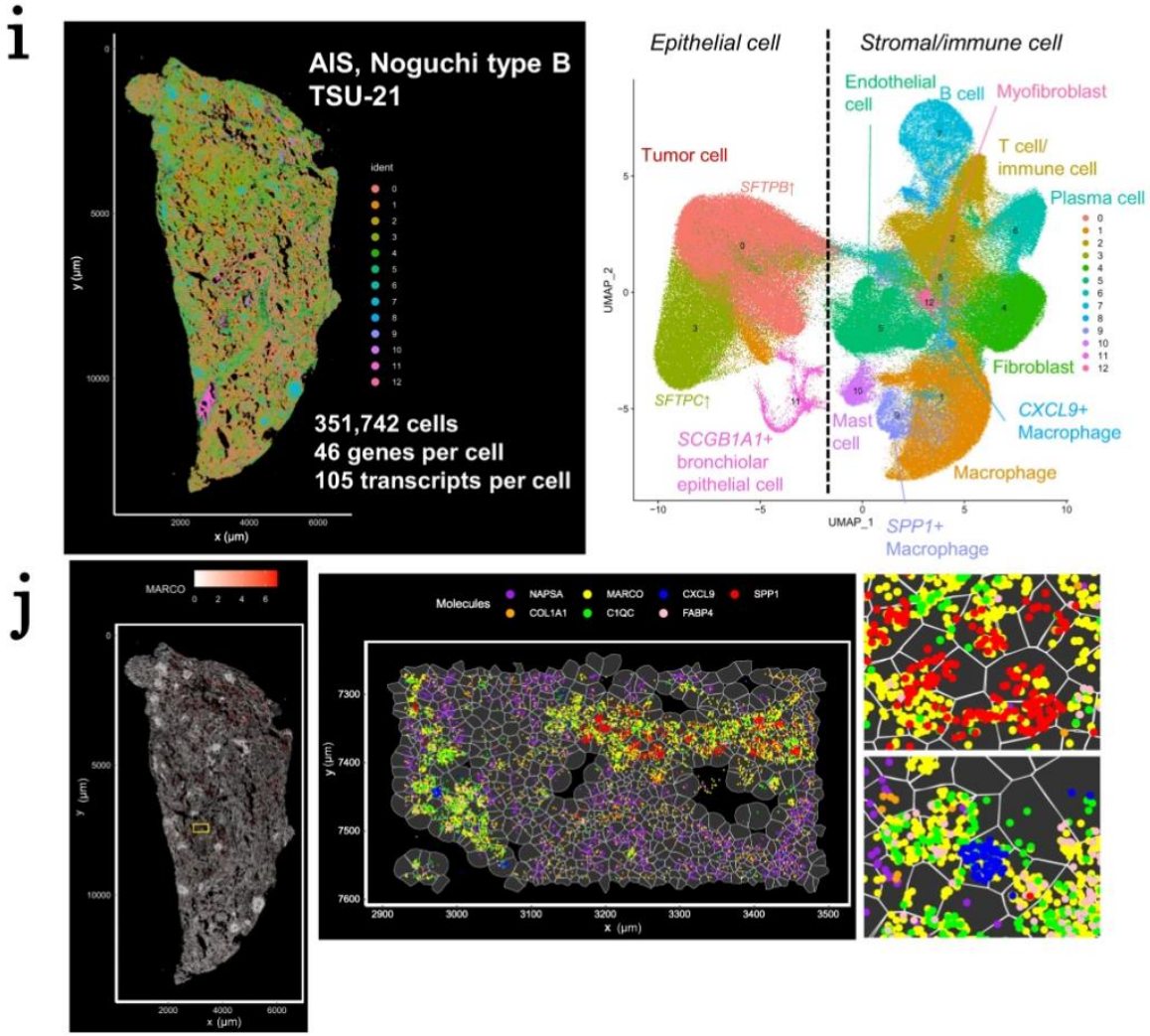

https://www.10xgenomics.com

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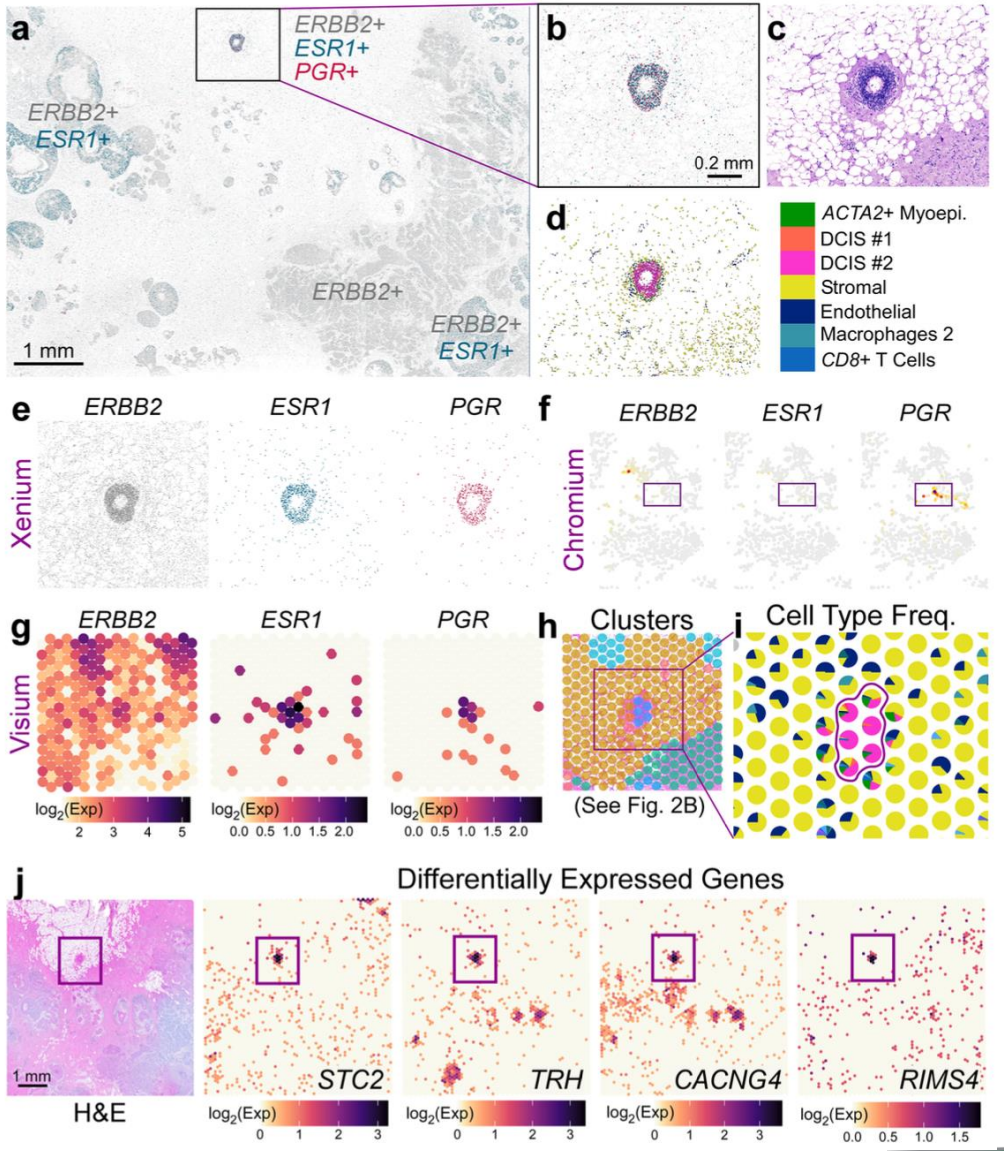

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  - Curio Bio SEEKER

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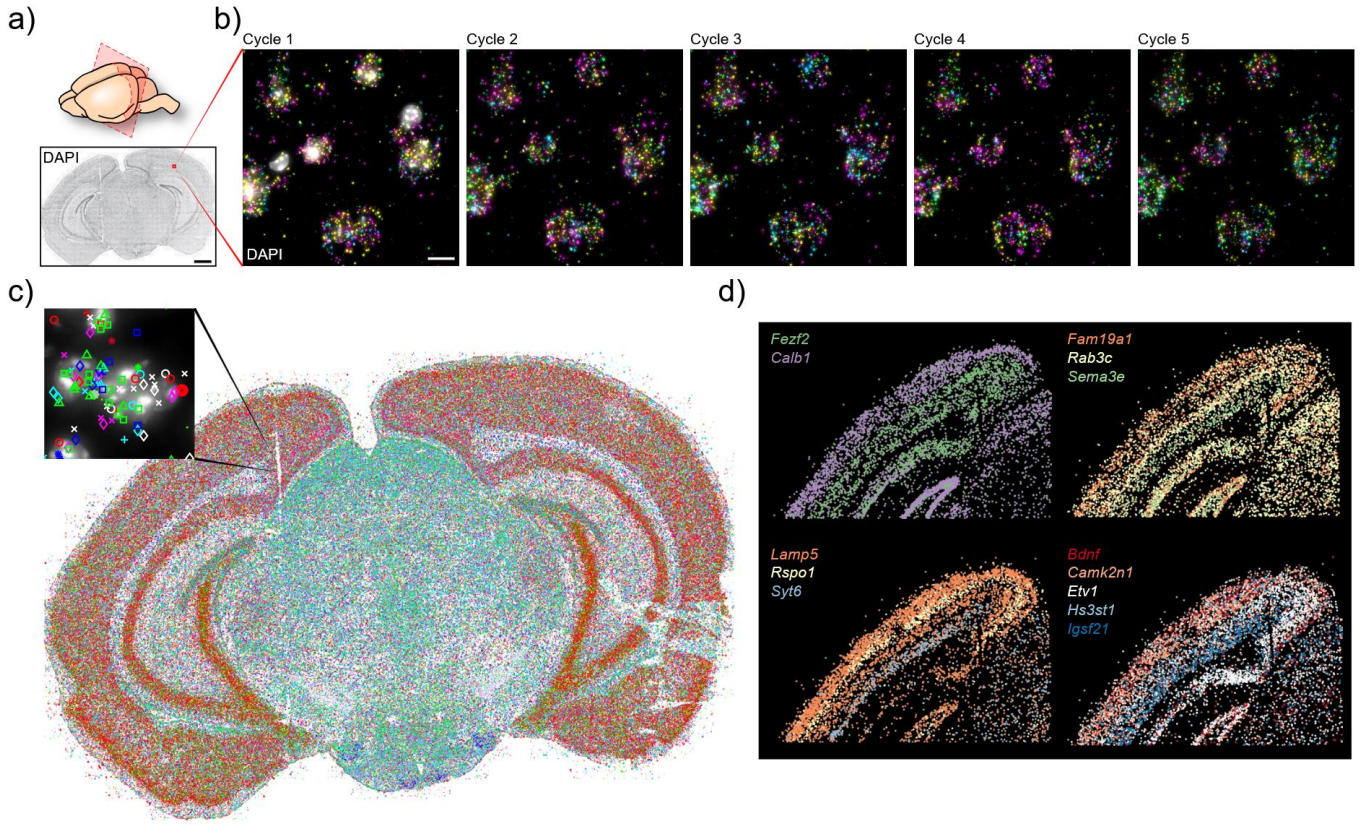
- NGS-based (Slide-Seq, DBiT-Seq)
- ISS (HybISS: Hybridization-based in situ sequencing (Nilsson lab, U Stockholm), FISSEQ: fluorescent in situ RNA sequencing (Church lab, Wyss Institute), INSTA-Seq: in situ transcriptome accessibility sequencing (Lee lab, CSH)
- ISH (MERFISH, SeqFISH)

Applications
- Cell type identification
- Tumor-immune composition
- Cellular-molecular organization
- Spatial mapping of transcript activity
- Tracking cell fate/development



Mouse coronal brain section

Gyllborg et al. (2020) bioRxiv preprint doi: https://doi.org/10.1101/2020.02.03.931618

Changes to sample prep : In situ sequencing (ISS)

Technology
- Combines FISH and standard NGS for spatial genomics (clusters - not single cell)
- Commercial ISS technologies:
  - 10X Genomics Xenium & Visium
  - Curio Bio SEEKER

Approaches
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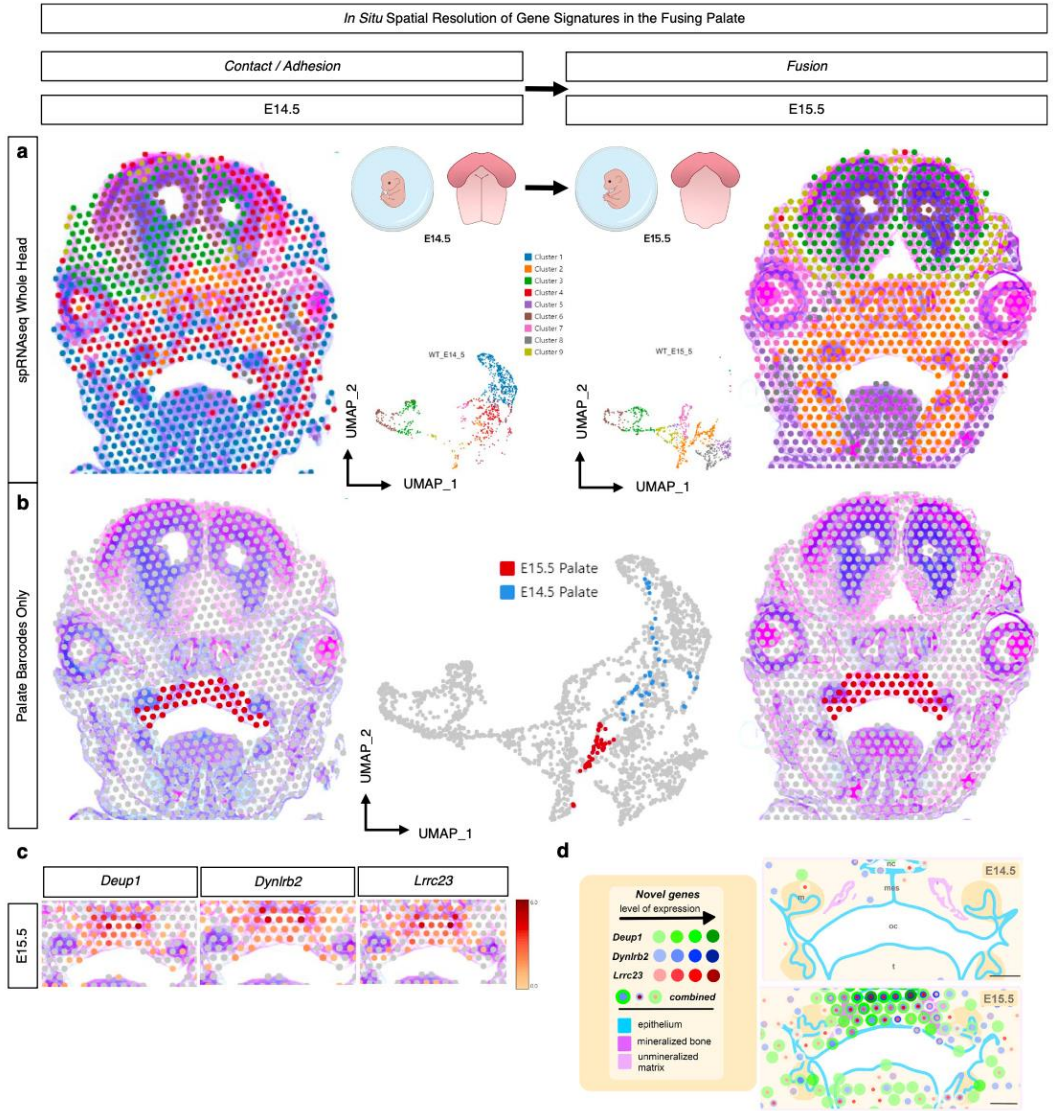
- ISH (MERFISH, SeqFISH)

Applications
- Cell type identification
- Tumor-immune composition
- Cellular-molecular organization
- Spatial mapping of transcript activity
- Tracking cell fate/development, *e.g.* to understand developmental disorders



Whole human embryo heads

https://www.10xgenomics.com

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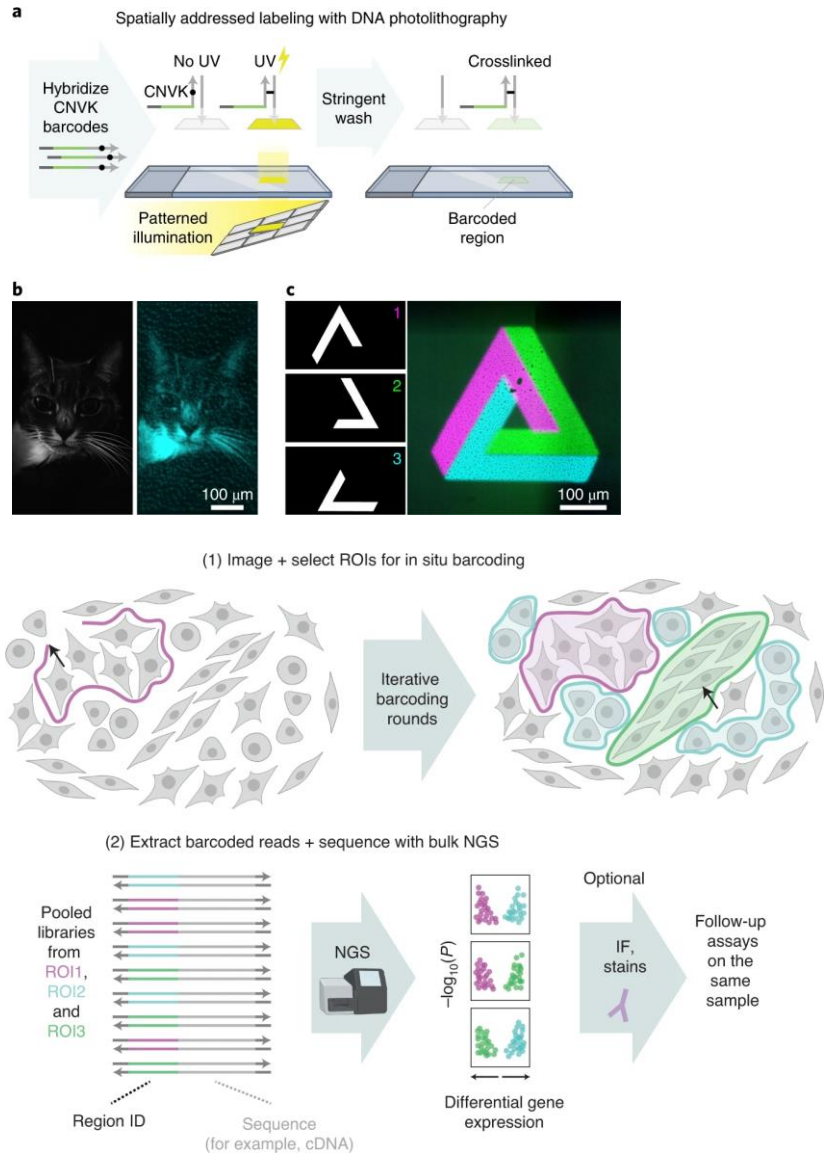
Changes to sample prep: Light-Seq

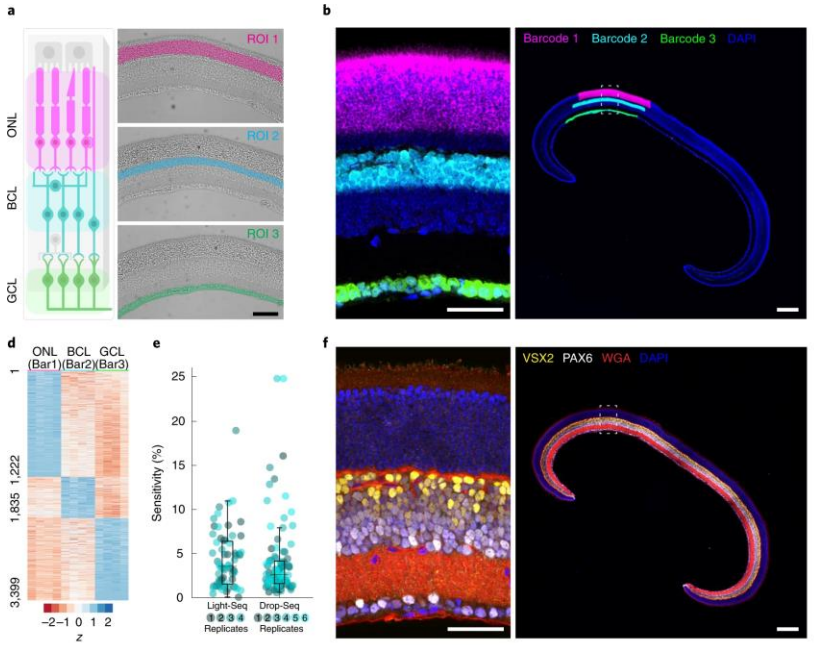
Technology

- photocrosslinkable nucleosides analog to ‘geotag’ the transcriptome of target cells in situ by light-controlled attachment of DNA barcodes
- high resolution imaging with standard NGS

Advantages

- Profiling of multiple different cell populations in fixed biological samples
- Spatial resolution of gene expression
- microscopically analyzed cells kept intact during RNA expression profiling which allows sequential measurements





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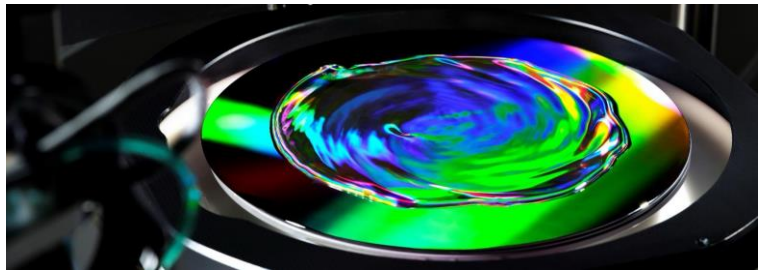
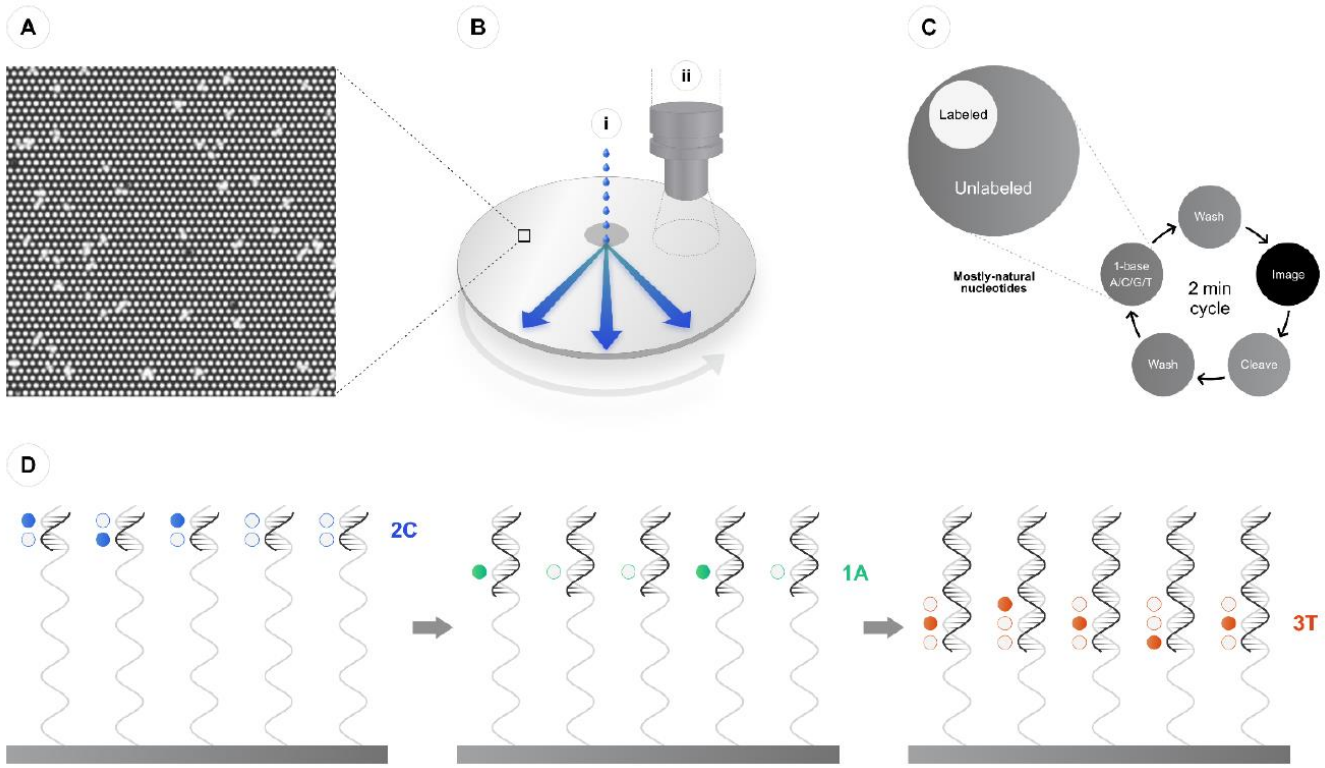
Kishi, J.Y et al. Nat Methods 19, 1393–1402 (2022). https://doi.org/10.1038/s41592-022-01604-1

Changes to the hardware: The spinning disc

- **Off-the-shelf semiconductor hardware**
  - spinning silicon wafer substitute flow cells
- **Traditional chemistry**
  - natural sequencing-by-synthesis (mnSBS)
- **Open-fluidics-system and fixed optics**
  - Sequencing reagents are added via spin-coating, and for readout of DNA sequence, the silicon wafer rotates while fixed dual high-speed cameras capture DNA clusters immobilized on the wafer.
- **Advantages:**

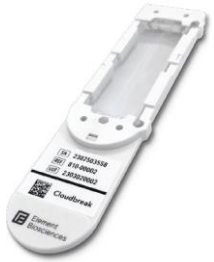
✓ *Ultra-high-throughput and ultra-low costs ($100 Genome)*
✓ *Short run-times, e.g. six human genomes in 20hrs*
✓ *Longer average read lengths (300-400+ cycles)*

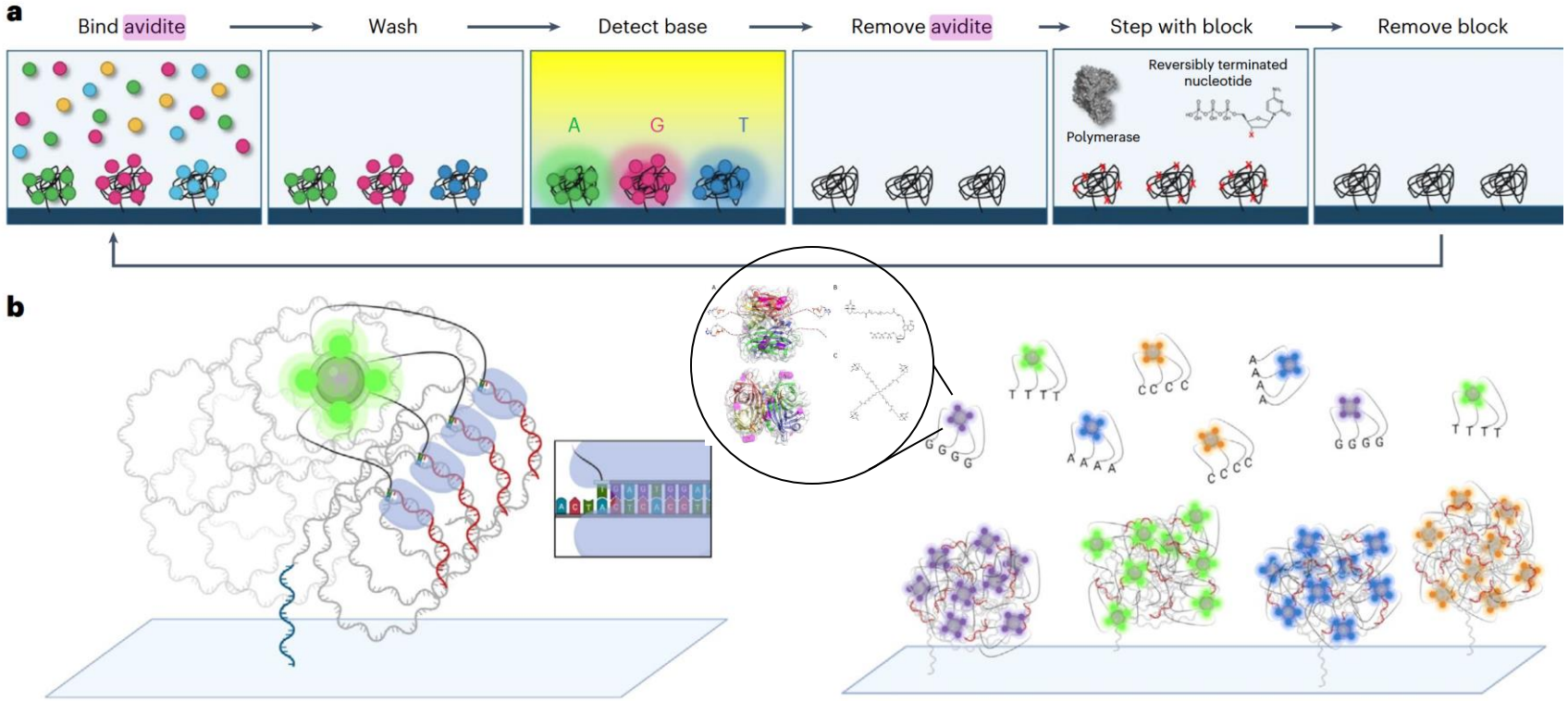




Almogy et al.bioRxiv 2022.05.29.493900; doi: https://doi.org/10.1101/2022.05.29.493900

Changes to the chemistry: ABC-sequencing





- **Changes to Illumina's SBS chemistry**
  - **Circularization** of the DNA library molecule on a capture primer
  - RC-**Amplification** copies ccDNA into a continues strand bound into a polony
  - **Sequencing** using Avidites
- **Advantages**

  - ✓ *No PCR artifacts*
  - ✓ *Reduced error rate*
  - ✓ *Reduced costs*
  - ✓ *No index hopping*

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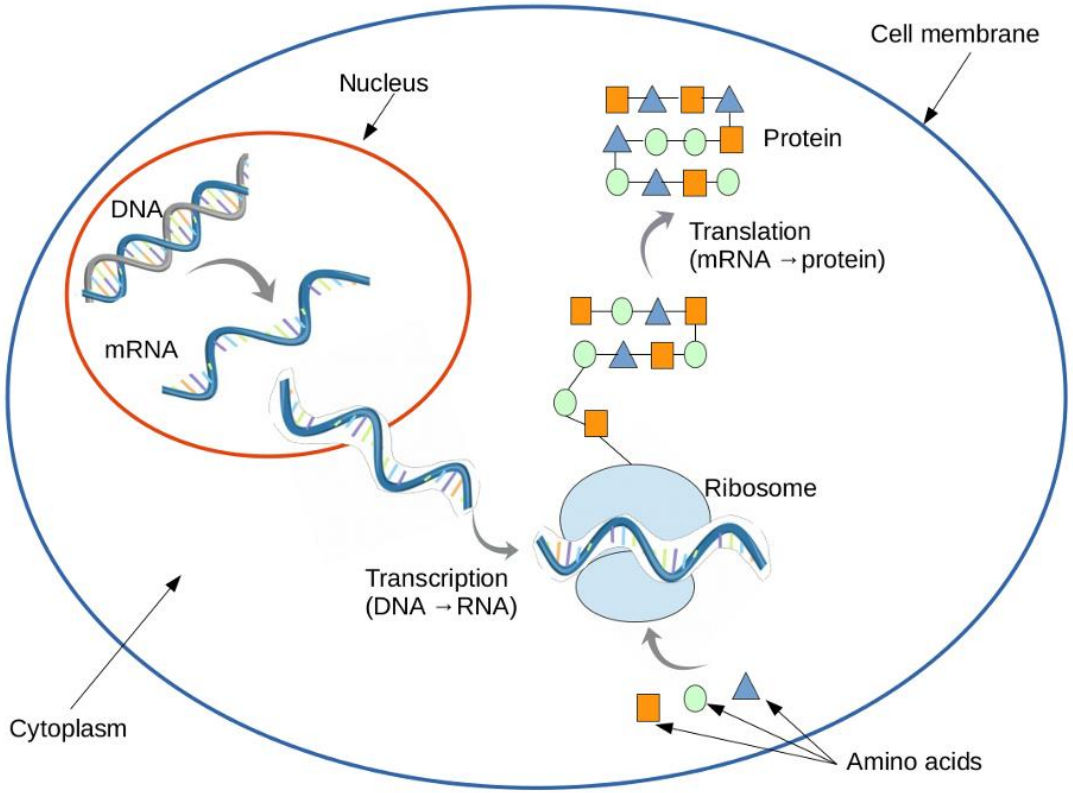
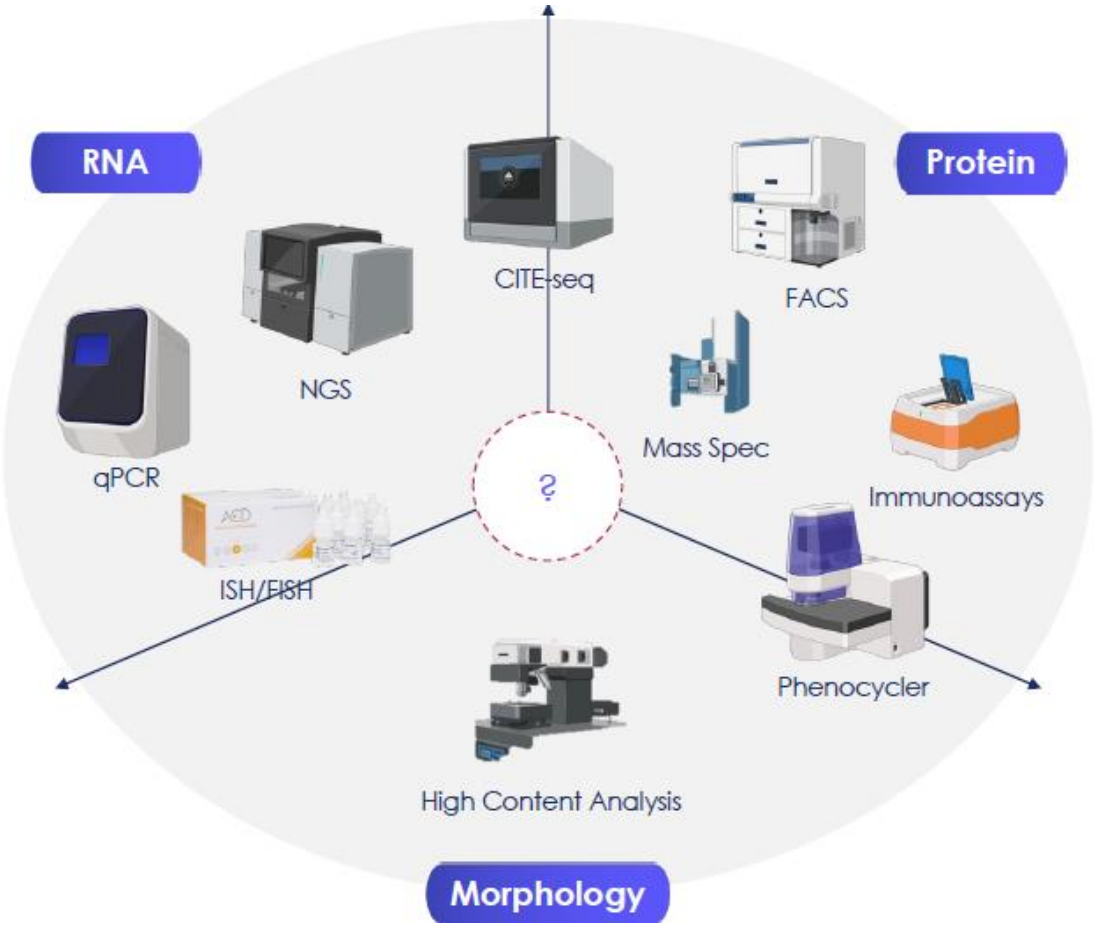
The "Future" of sequencing: NGSX



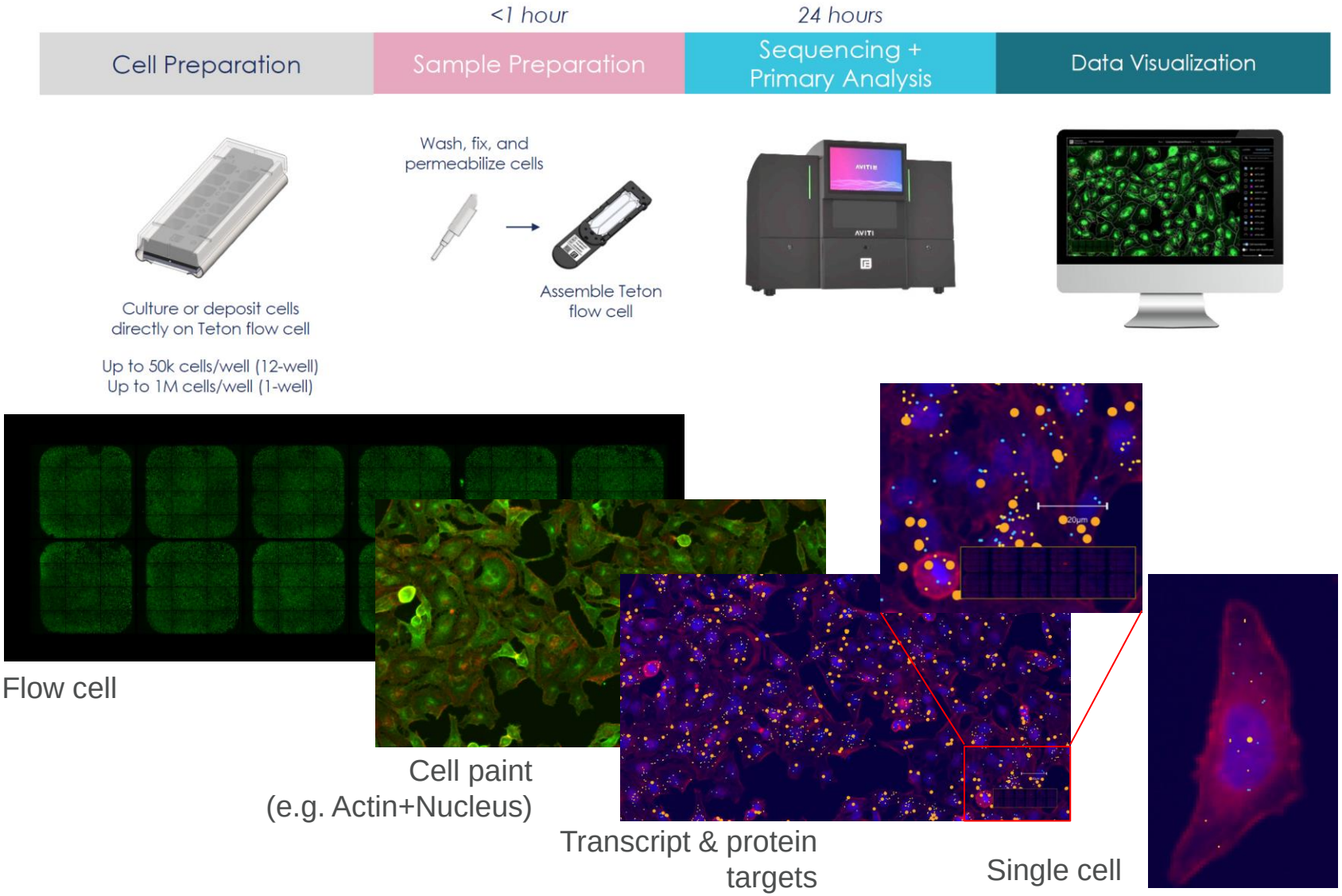


- Integration of true multiomics capabilities on a single instrument without the need of auxiliary equipment

https://www.elementbiosciences.com/; R. Hesketh, The War on Cancer, New York (2012) p.65; Satam H, et al. Biology 2023 12(7):997. doi: 10.3390/biology12070997

Direct in sample sequencing (DISS) for cells

- **Technology:**
  - Teton CytoProfiling + ABC sequencing from Element Biosciences
- **True multiomics on a single instrument**
  - Cell morphology
  - RNA detection (~350-plex)
  - Protein detection (~50-plex)
- **Use cases:**
  - Cellular profiling
  - drug response studies
  - *in situ* single cell RNA-Seq
  - cell synchronization

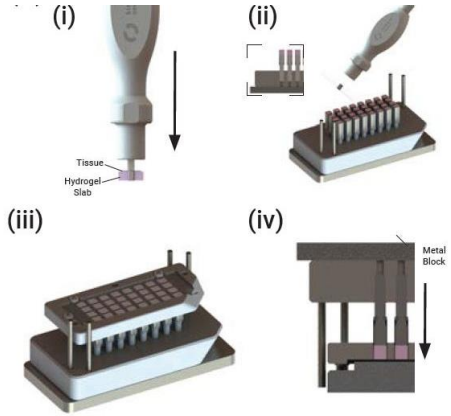


https://www.elementbiosciences.com/

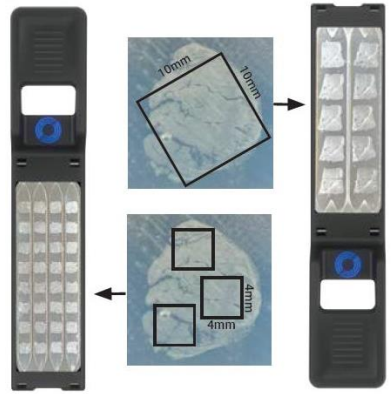
DISS for FFPE & fresh tissue

- **Technology:**
  - Tissue transfer + rapid 4-color SBS sequencing by Singular Genomics
- **True multiomics on a single instrument**
  - fH&E (Hematoxylin and eosin)
  - RNA detection (~153-plex)
  - Protein detection (~10-plex)
- **Use cases:**
  - late-stage translational research (T2-T4); e.g. tumor-immune interactions
  - Diagnostics; *e.g.* therapeutic biomarkers

Tissue cutting & transfer

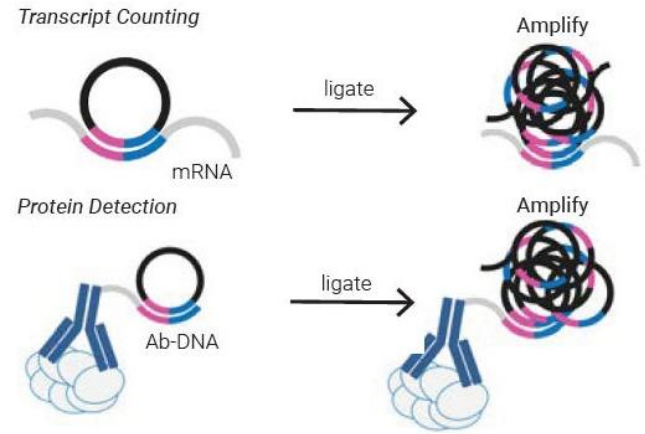


Flow cells with small/large samples

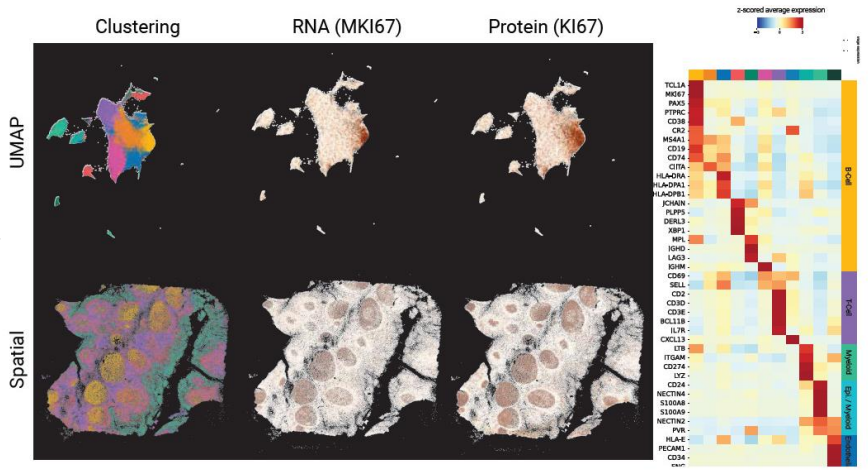


G4x sequencing

RNA/Protein detection by affinity or hybridization of antibodies/probes



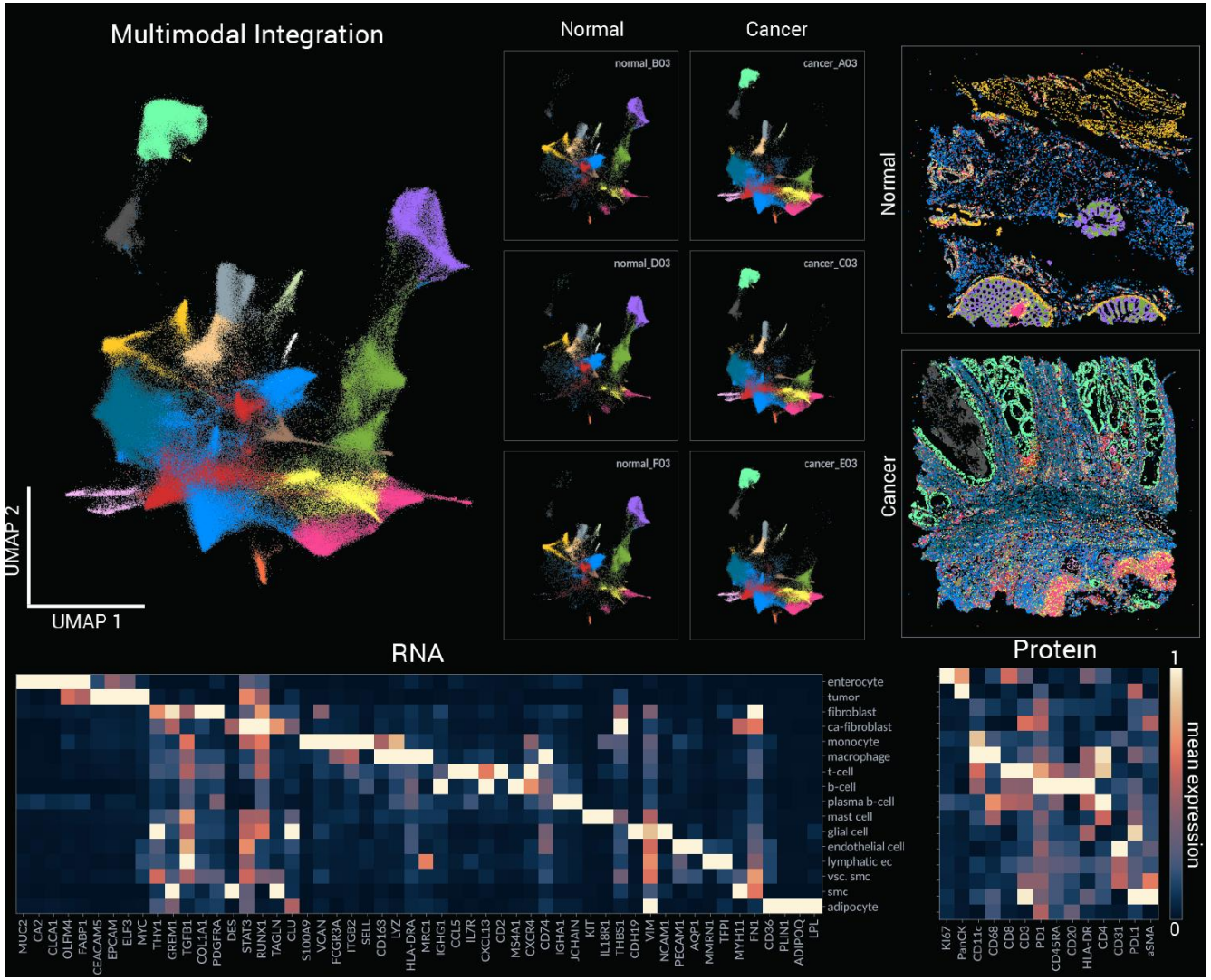
Quantification & analysis



https://www.singulargenomics.com/g4x

DISS use case #1: Visualize crosstalk of multiple cellular processes

- Tumor-Immune interactions (e.g. Colon normal vs cancer)
- Visualize & sequence tissue with (Sub-)Cellular resolution
- Targeting 100 millions of transcripts in millions of cells
- Integration of complex single cell RNA & protein expression profiles



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Kenneth *et al.* Spatial Multiomic Profiling of Tumor-Immune Interactions in 2D and 3D on the G4X Spatial Sequencer, AGBT (2025)

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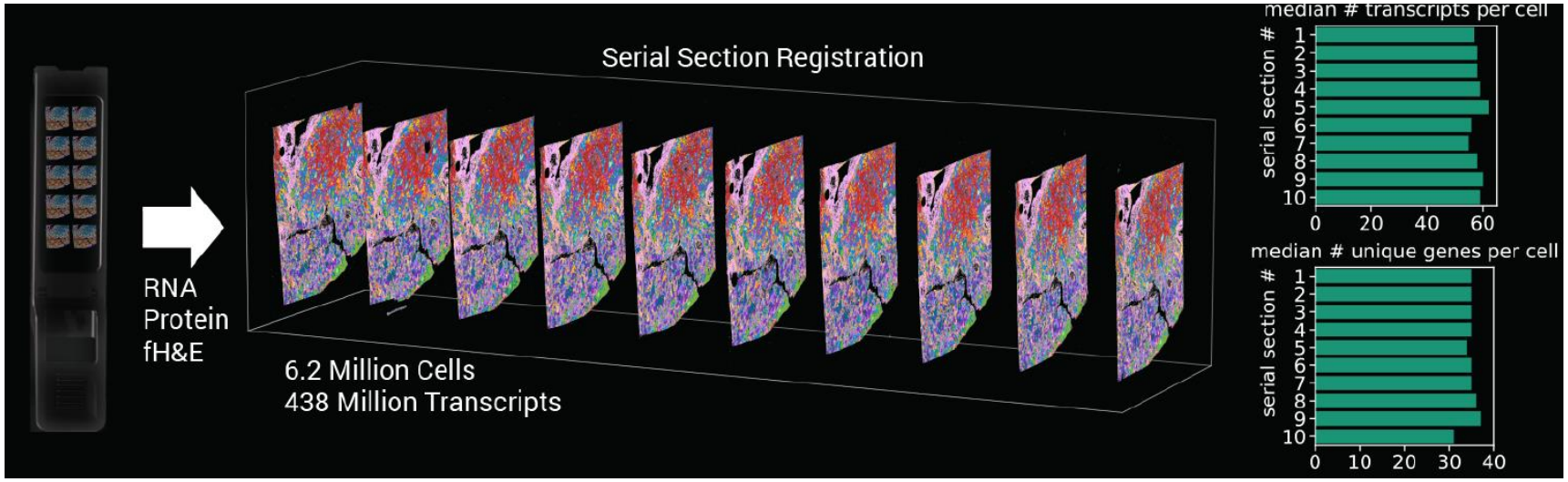
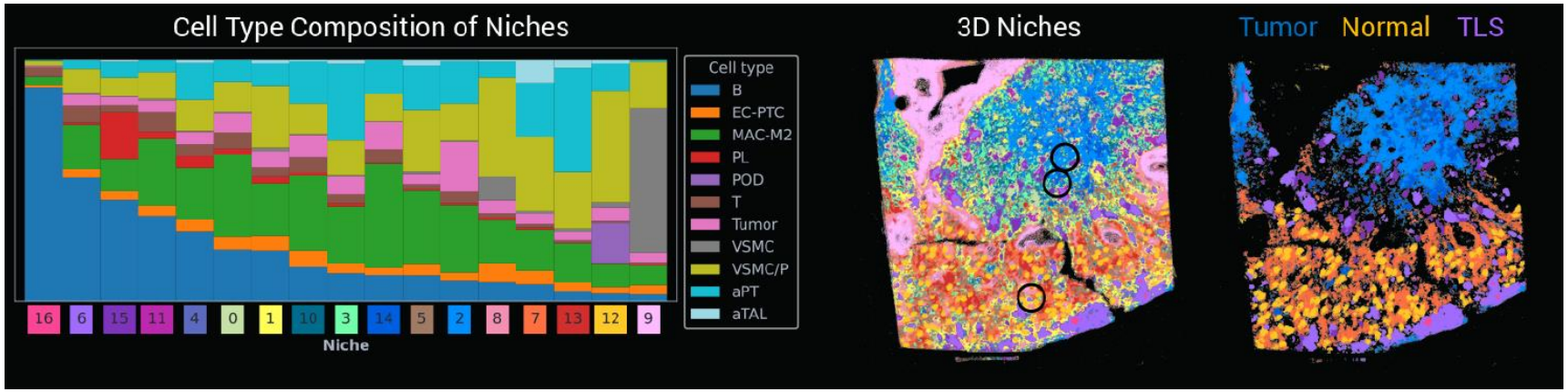
DISS use case #2: Enhance therapeutic success

- Reconstruction of Normal-Tumor Interfaces
- 3D spatial neighborhood analysis to identify niches of healthy vs cancerogenous tissue
- Monitoring effects of tumor-modulating drugs e.g. to enhance effectiveness of chemotherapies





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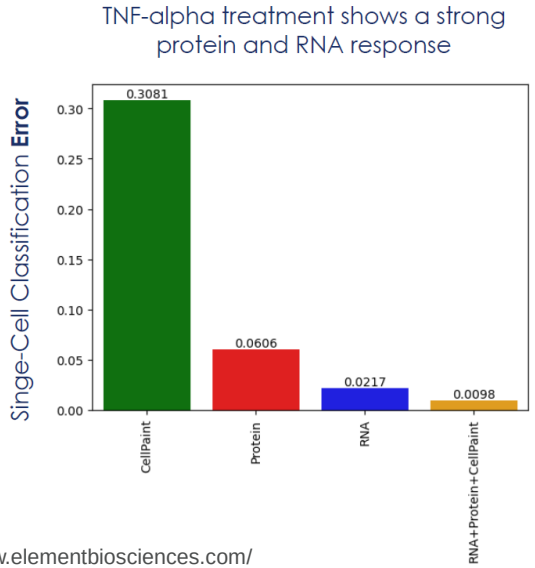
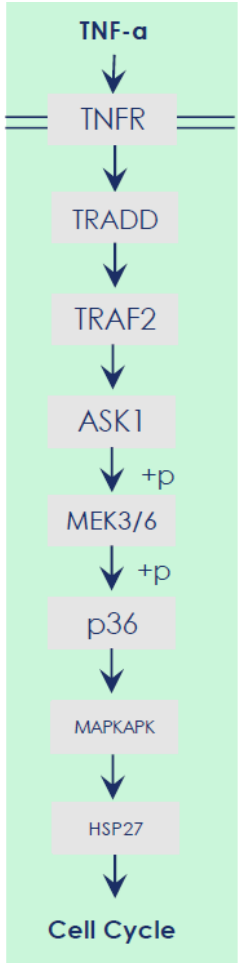
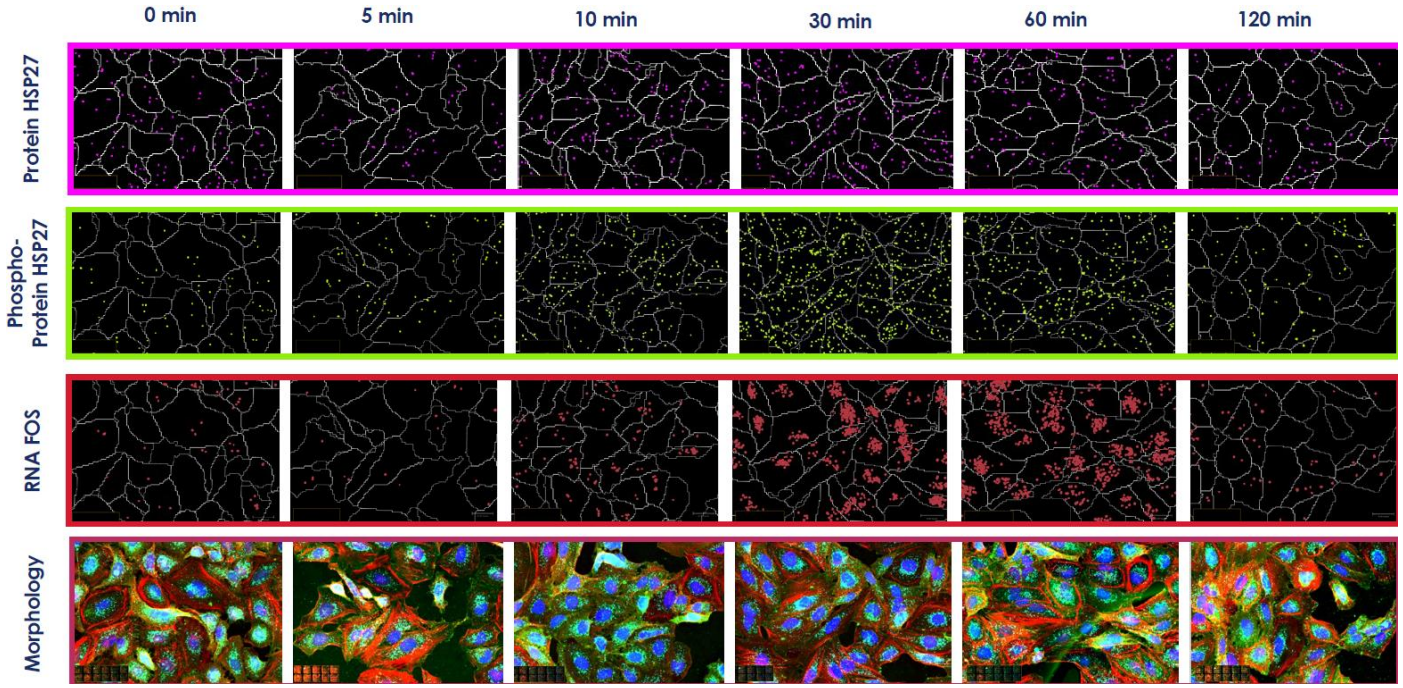
Kenneth *et al.* Spatial Multiomic Profiling of Tumor-Immune Interactions in 2D and 3D on the G4X Spatial Sequencer, AGBT (2025)

DISS use case #3: Personalized drug therapy

- Drug-induced changes to RNA, protein, phosphoprotein, and morphology
- Multiomic readouts identifies RNAs and Proteins driving the changes of morphological states & resolves complex mechanisms of drug response
- Multiomic readouts improve classification of cells based on their drug response







https://www.elementbiosciences.com/

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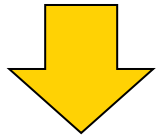
Summary & Trends

- ✓ Sequencing is a fast-evolving field of new emerging technologies
- ✓ New NGS methods are faster and more precise
- ✓ Necessitate smaller amounts of input DNA and reagents
- ✓ NGS technologies created new fields of research (e.g. pangenome analysis, multiomics, cell atlases)
- ✓ True multi-omics revolutionizes basic & translational research

How to handle the enormous amount of data (“big data”)?



Development of new bioinformatic tools, statistics, employ data sharing, unified file formats and AI to help integrate data from different “omes”

The Omics Resource Centre (ORC)

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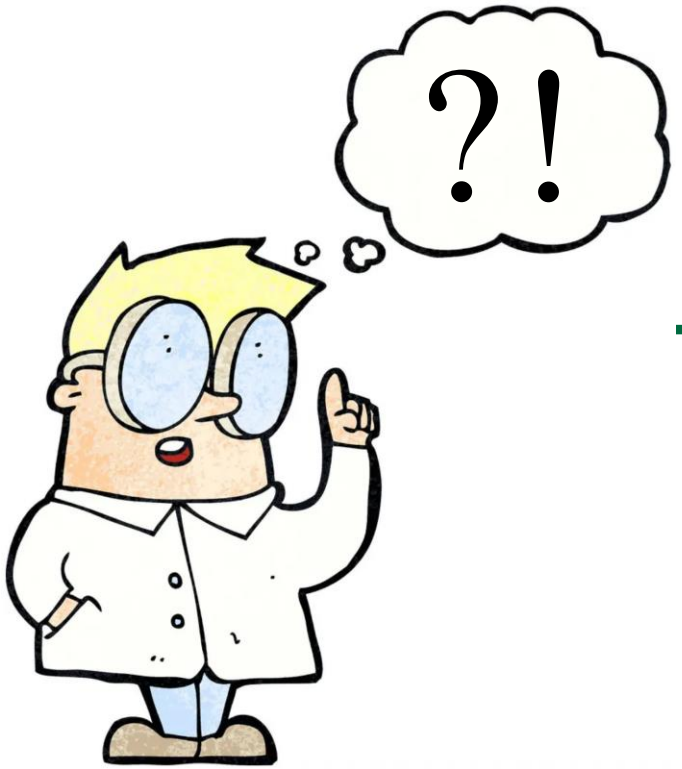

Thank you!

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https://www.vectorstock.com/royalty-free-vector/cartoon-scientist-with-thought-bubble-vector-5682036