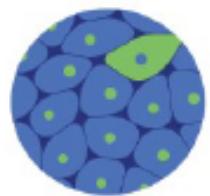
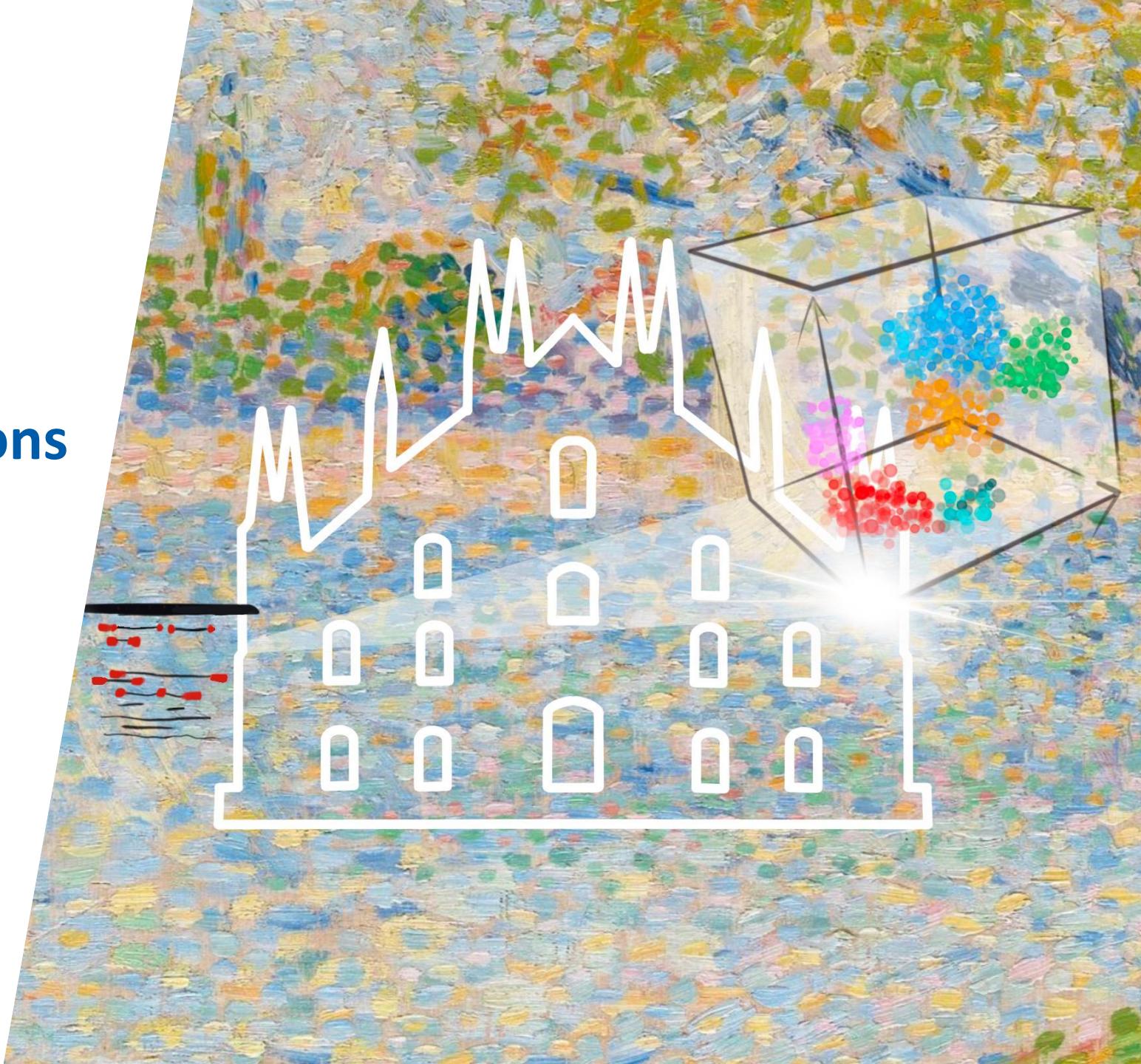


Single-cell sequencing Technologies and Applications



Clelia Peano

University of Milan, 14 July 2025



IMPORTANCE OF SINGLE-CELL AND SPATIAL MULTIMODAL OMICS

Single-cell sequencing
selected as 2013
Method of the Year



Single-cell multimodal omics
selected as 2019
Method of the Year



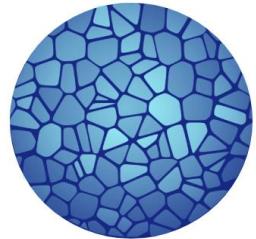
Spatially resolved
transcriptomics
selected as 2020
Method of the Year



Spatial Proteomics
selected as 2024
Method of the Year



Single-cell sequencing and Spatial Transcriptomics Technologies allowed large international initiatives :

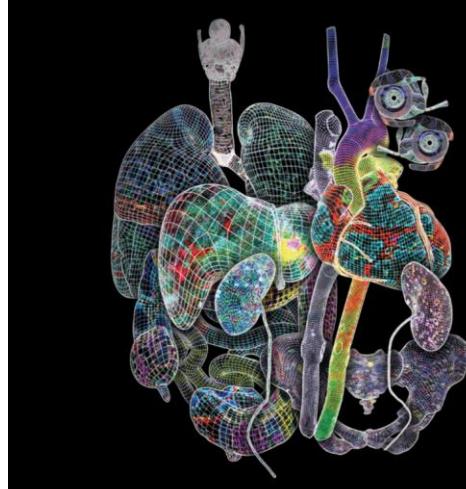


HUMAN
CELL
ATLAS

MISSION

To create comprehensive reference maps of all human cells—the fundamental units of life—as a basis for both understanding human health and diagnosing, monitoring, and treating disease.

nature



Human BioMolecular Atlas Program

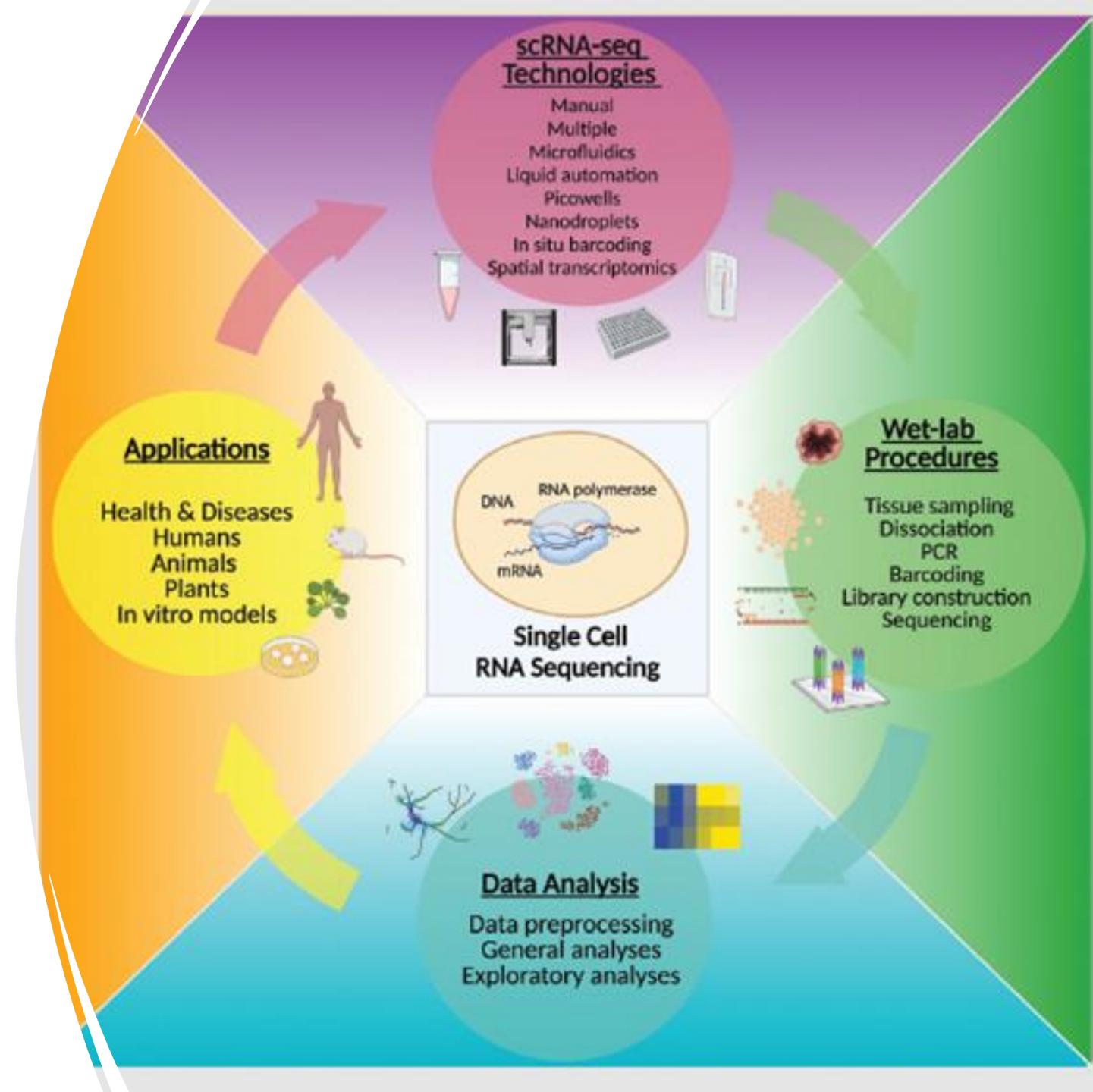
A collection of research articles and related content from the Human BioMolecular Atlas Program describing the distribution of biomolecules across single cells, tissues and organs in the human body.

- An international consortium that charts the cell types in the healthy body, across time from development to adulthood, and to old age.
- **HCA was founded in 2016** and includes more than 2,900 members, from over 1,500 institutes and 94 countries.
- HCA data is being made available at the [Data Coordination Platform for researchers around the globe](#)
- **HuBMAP was launched in 2018** to assemble spatial maps of biomolecules, including RNA, proteins and metabolites, in human organs at single-cell resolution.
- The ultimate goal is to enhance our understanding of how cells work and how they interact with each other in the human body.
- All the data collected by the HuBMAP consortium are freely available on [the HuBMAP Data Portal](#)

OUTLINE

FIRST PART:

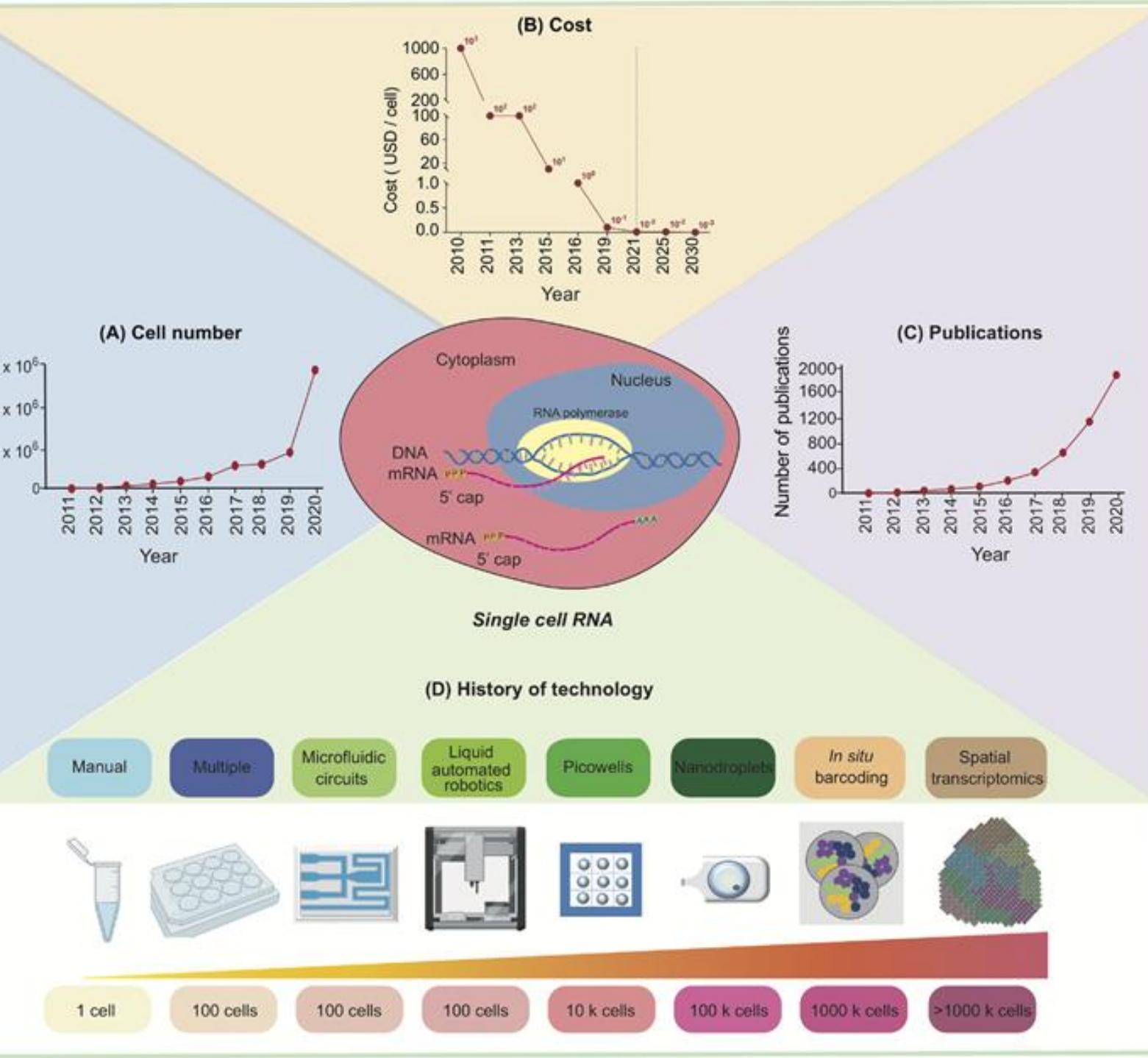
1. Evolution of Single-cell RNA and multi-omics technologies
2. Applications of single-cell sequencing technologies
3. Overview of single-cell RNA seq analysis:
 - 10x Genomics technology
 - BD Rhapsody technology
4. Overview of single-cell RNA seq data analysis



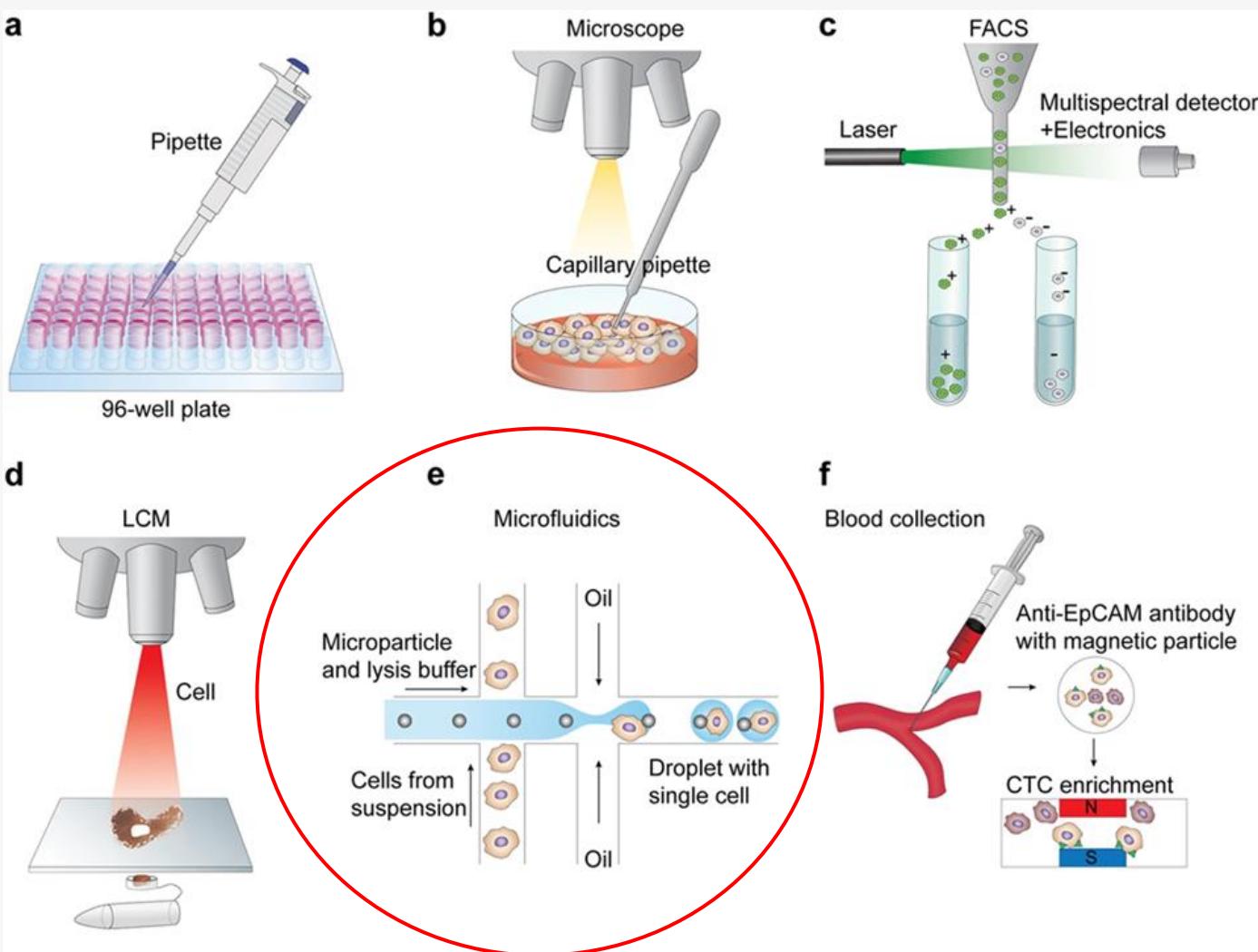
SECOND PART:

1. Spatial Transcriptomics technologies and evolution
 - 10x Genomics Visium
 - StereoSeq
 - Nanostring GeoMX DSP
 - Spatial analysis at high resolution

Evolution of single-cell sequencing technologies



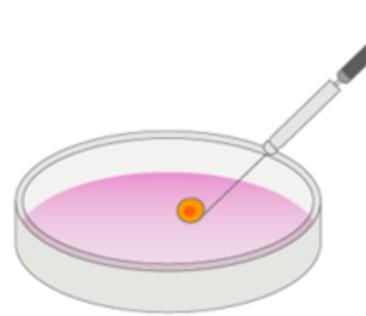
Evolution of single-cell sequencing technologies



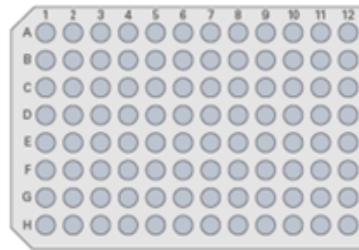
- The limiting dilution method** isolates individual cells, leveraging the statistical distribution of diluted cells.
- Micromanipulation** involves collecting single cells using microscope-guided capillary pipettes.
- FACS** isolates highly purified single cells by tagging cells with fluorescent marker proteins.
- Laser capture microdissection (LCM)** utilizes a laser system to isolate cells from solid samples.
- Microfluidics for single-cell isolation** is based on cells partitioning in droplets.
- The CellSearch system** isolates CTCs from patient blood samples by using a magnet conjugated with CTC binding antibodies.

Evolution of single-cell isolation strategies to improve sequencing throughput

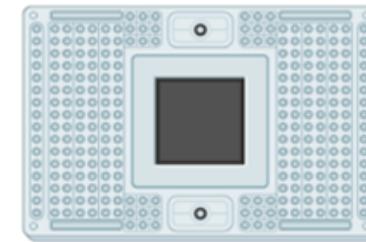
The advancement of scRNA-seq technology coupled with decreasing sequencing expenses offers an exceptional opportunity to explore the transcriptomes of millions of individual cells.



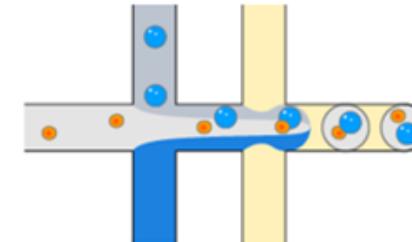
Micro-manipulation



Plate



Microwell



Droplet



Combinatorial indexing

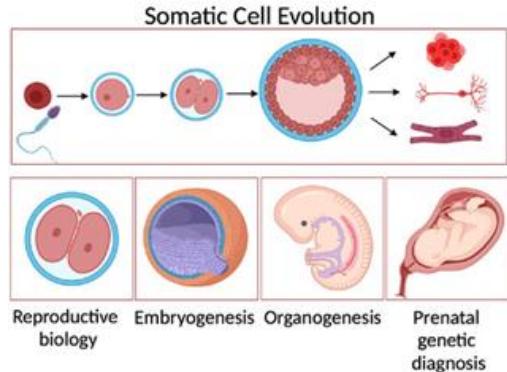
1–1,000s scale

Full coverage, high sensitivity, low throughput

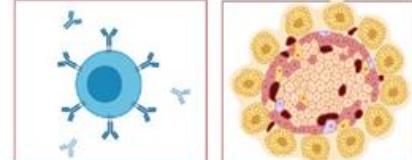
10,000s–1,000,000s scale

End counting, low sensitivity, mRNA only

(A) Development



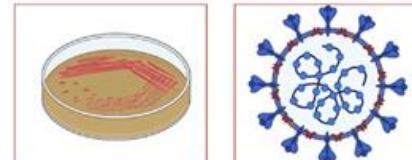
(B) Immunology



(C) Diabetes

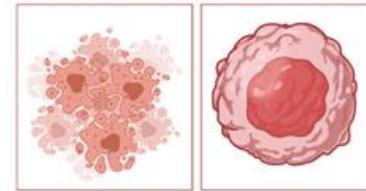


(D) Microbiology

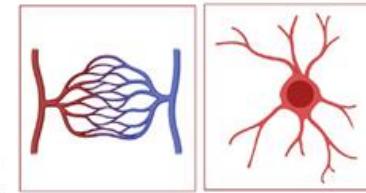


(E) COVID-19

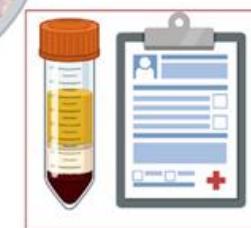
(F) Cancer Biology



(G) Vascular Biology



(I) Clinical Diagnostic



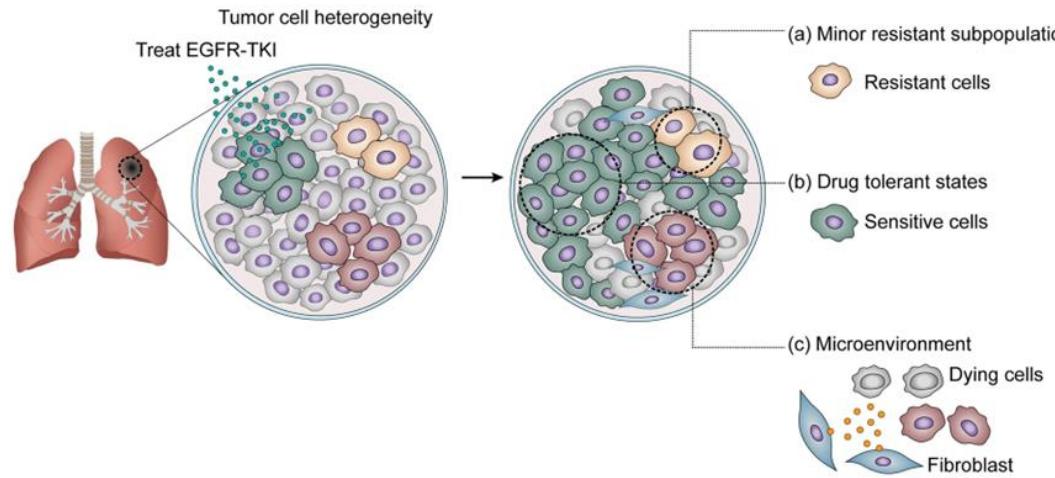
Applications of single-cell sequencing technologies

Single-cell RNA sequencing has been applied in different species to improve understanding of normal and disease models.

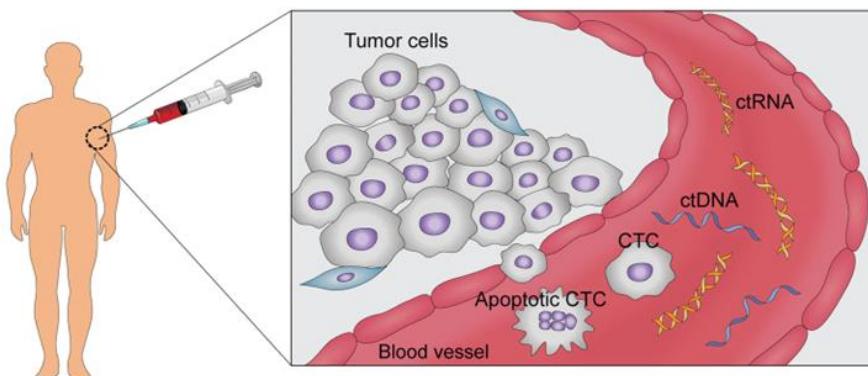
Many scRNA-seq methods are focused on understanding:

- Development,
- Immunology,
- Metabolic diseases,
- Infections,
- Cancer biology,
- Vascular biology
- Neurobiology
- Clinical diagnostics.

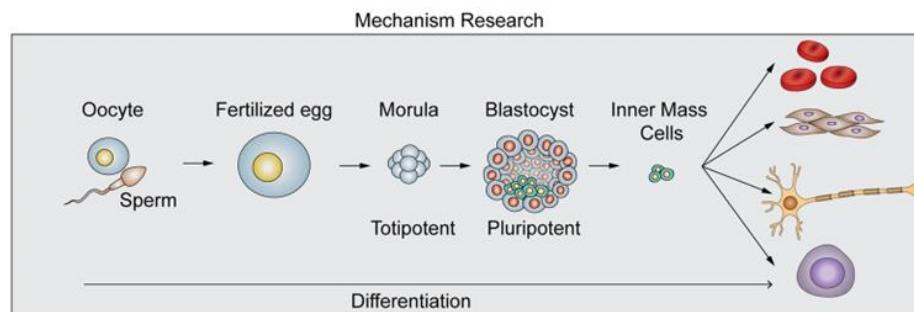
a. Drug resistance clone identification



b. Non-invasive biopsy diagnosis



c. Single-cell lineage and stem cell regulatory network



Applications of single-cell sequencing technologies

a. Intratumor heterogeneity

scRNA-seq can tackle this problem by effectively identifying subgroups of tumoral cells within a tumor.

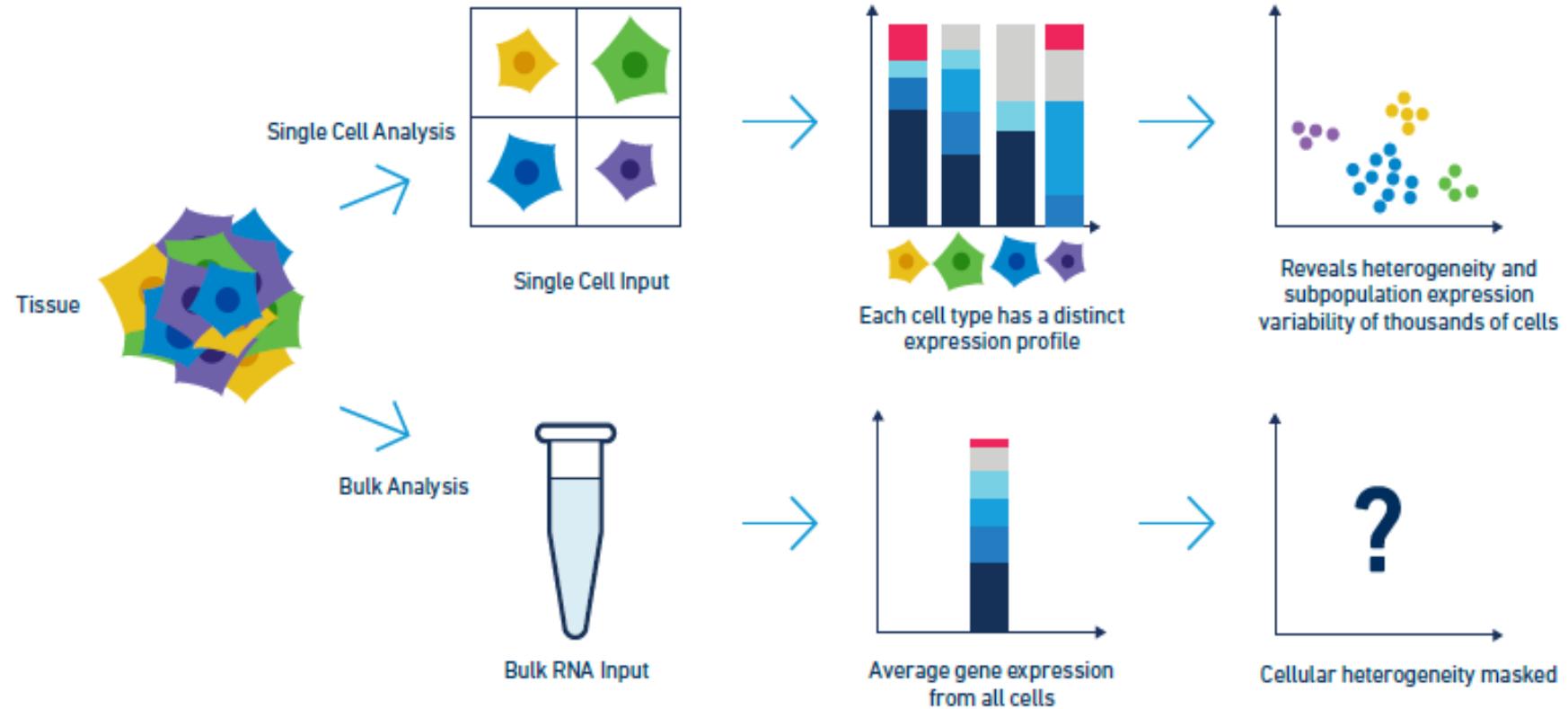
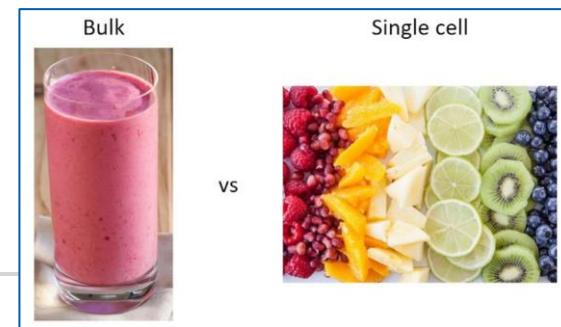
b. Liquid biopsy

scRNA-seq of CTCs could provide novel insights into biomarker characterization useful for diagnosis or prognosis.

c. Stem cells differentiation studies

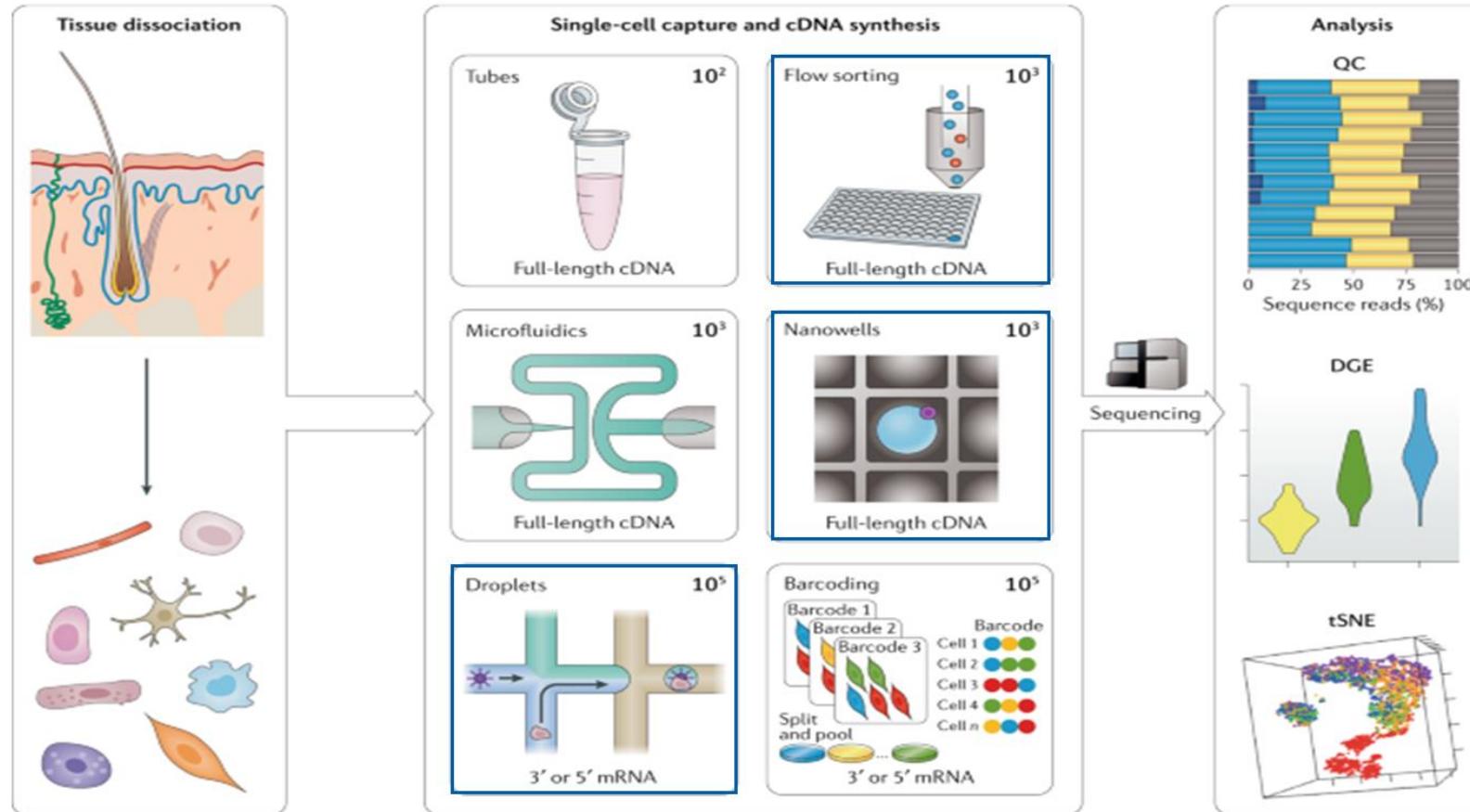
scRNA-seq can infer lineage information from the early developmental stage and can identify novel differential markers.

IMPORTANCE OF SINGLE-CELL LEVEL ANALYSIS



- Bulk methods measure the average gene expression from all cells.
- Single-cell technologies measure the gene expression profile of each individual cell.

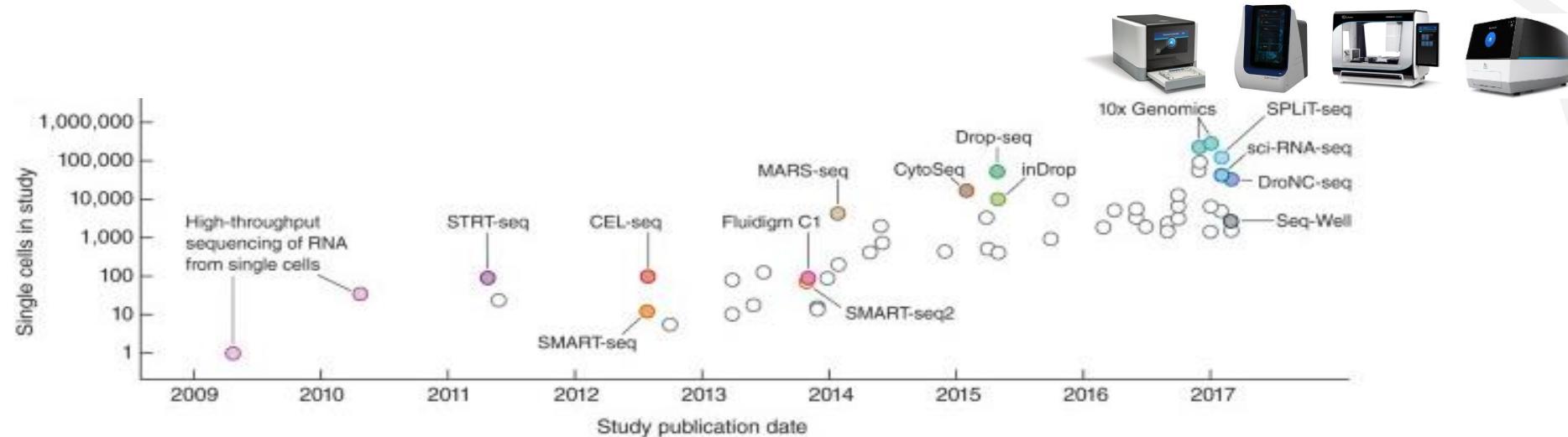
OVERVIEW OF SINGLE-CELL RNASeq ANALYSIS



Main steps of Single-cell RNA seq:

- Tissues/samples dissociation
- Partitioning of single cells
- Single-cells' RNA capture
- cDNA synthesis
- Library preparation
- Sequencing
- Data analysis

SINGLE-CELL sequencing PLATFORMS



Droplet-Based Platforms

Chromium Controller



Chromium Connect



Chromium X



Microwell-based Platform

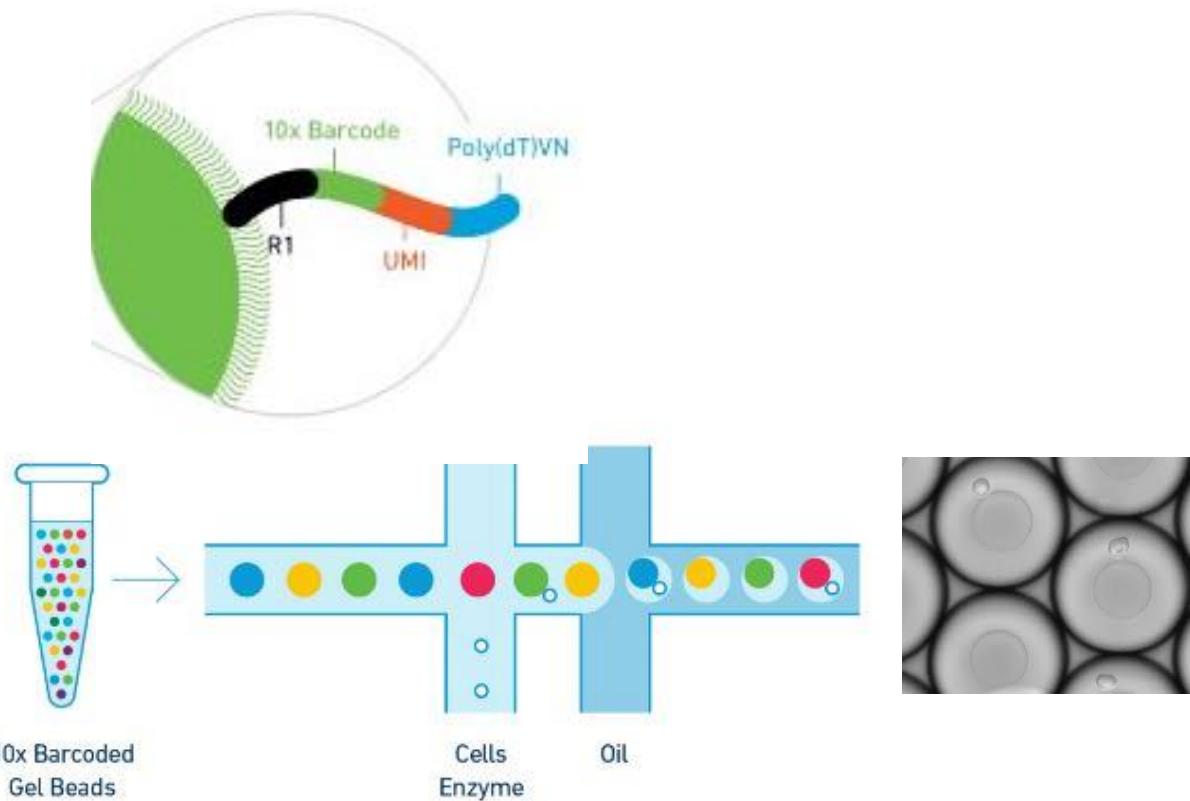
BD Rhapsody



ISOLATION OF SINGLE CELLS IN DROPLETS OR WELLS WITH FUNCTIONALIZED BEADS

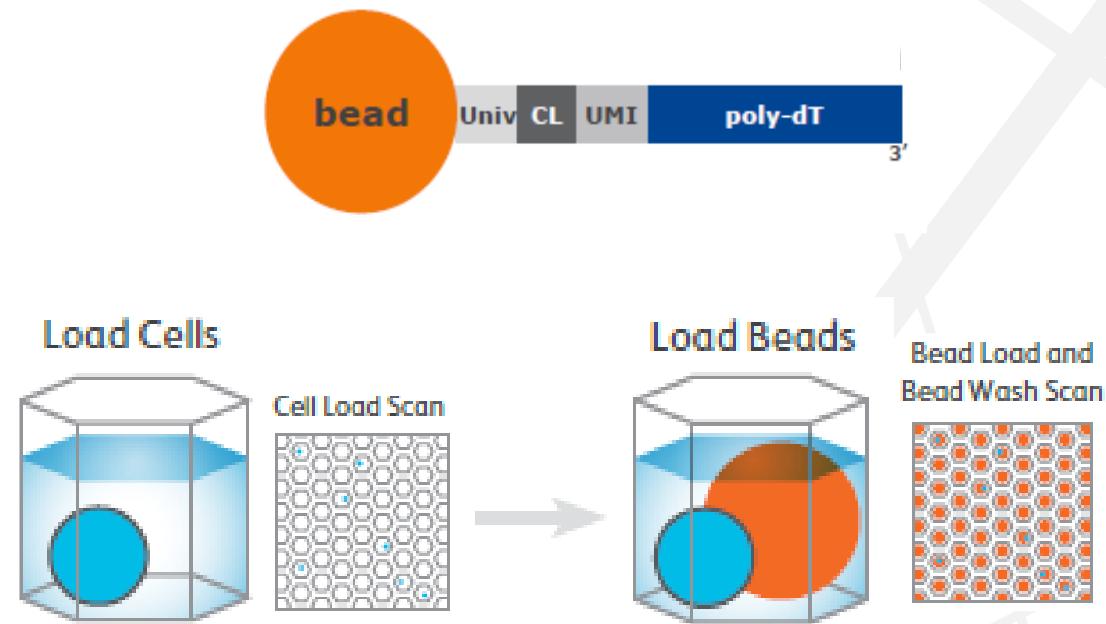


Droplet-Based Platforms



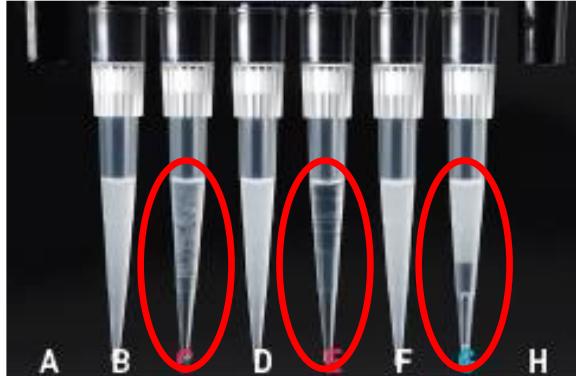
Barcoded Gel Beads mixed with cells, enzymes, oil to form single-cell droplets

Microwell-based Platform



Single cells and single barcoded beads deposited into microwells

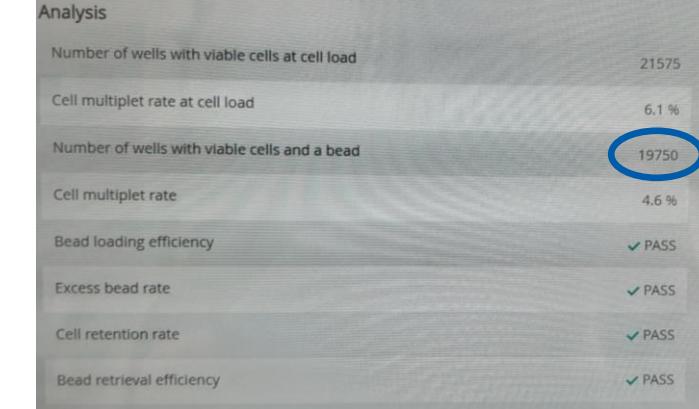
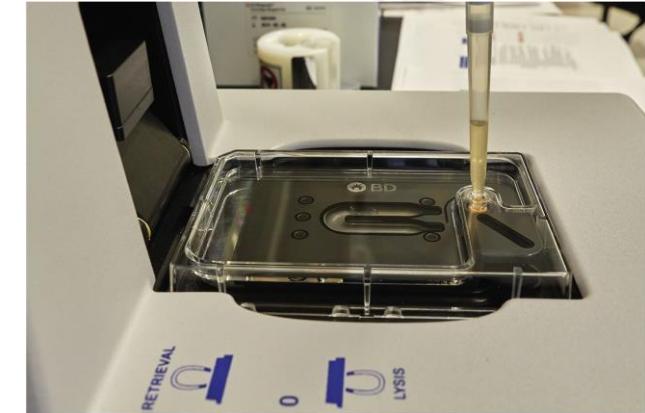
CAPTURE OF SINGLE CELLS WITH FUNCTIONALIZED BEADS



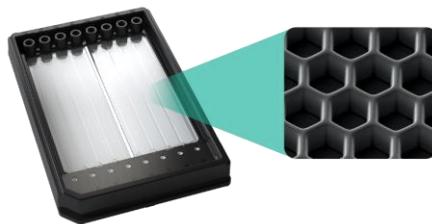
Visual inspection of emulsion



Indication on the number of captured cells



Components of the BD Rhapsody™ HT Single-Cell Analysis System



BD Rhapsody™ 8-lane Cartridge:

- 8 independent lanes for processing of 100 – 100.000 cells per lane, up to 80% capture rate (up to 800.000 cells/cartridge)
- Partial loading enabled: Use the number of lanes needed and use the rest on another day
- One cartridge for ANY assay / assay combinations
- Highly efficient , even for fragile cells like Neutrophils



BD Rhapsody™ HT Xpress

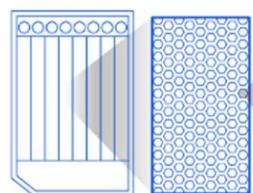
- Small and purely mechanical device
- Needed for the BD Rhapsody Cartridge loading
- Robust and easy to use



BD Rhapsody™ Scanner (Optional)

- Imaging device for both: Sample quality control AND cartridge workflow quality control
- Get sample quality information BEFORE the cartridge workflow
- Counts the numbers of single cells and multiplets captured:
→ You can precisely allocate the sequencing depth

Load cells and beads

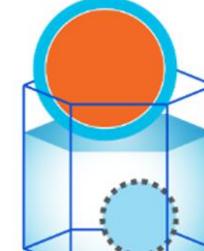


Lyse cells, barcoding



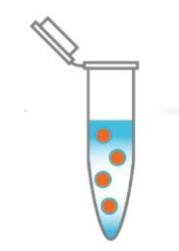
Microwell
Barcoded bead
Cell

Retrieve beads (magnet)

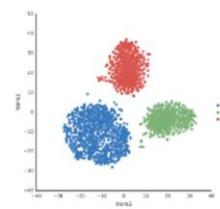


mRNA
Barcoded bead
Lysed cell

Synthesize cDNA

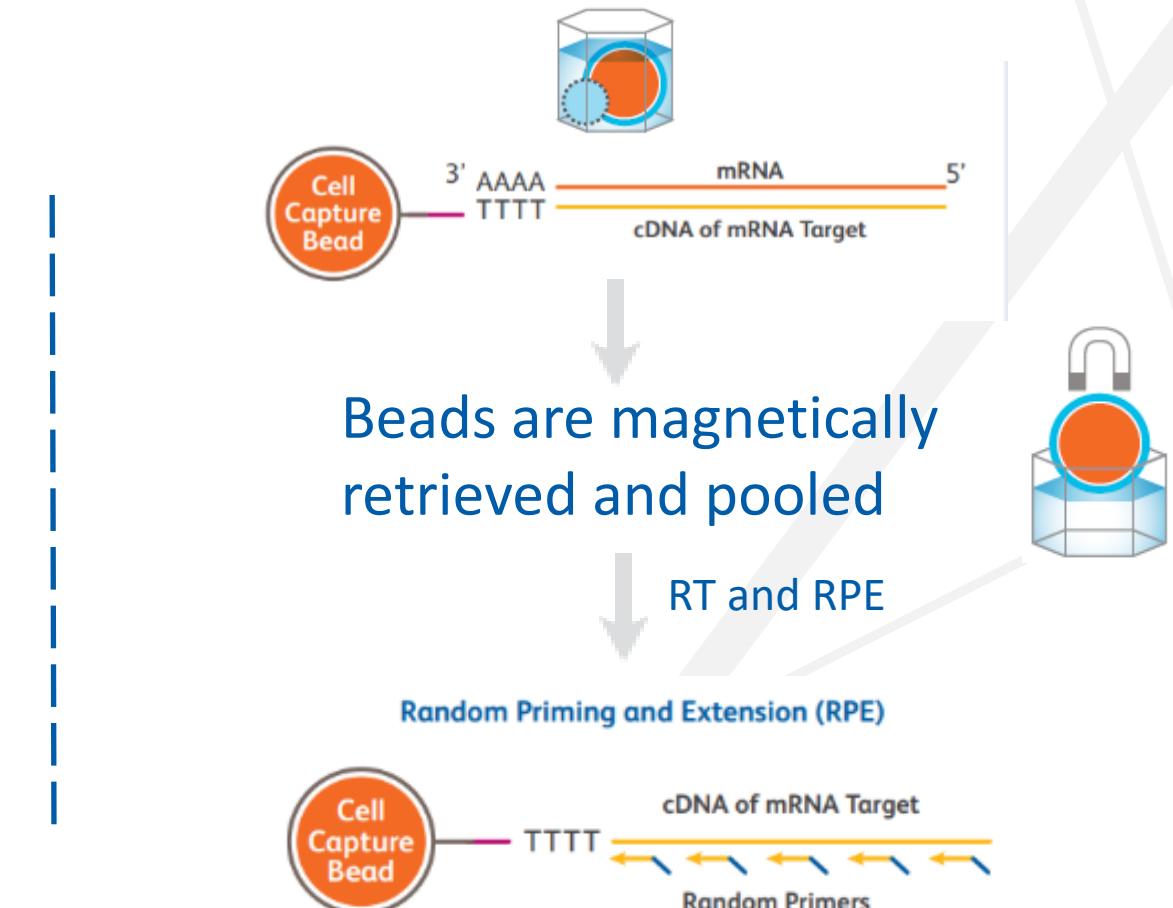
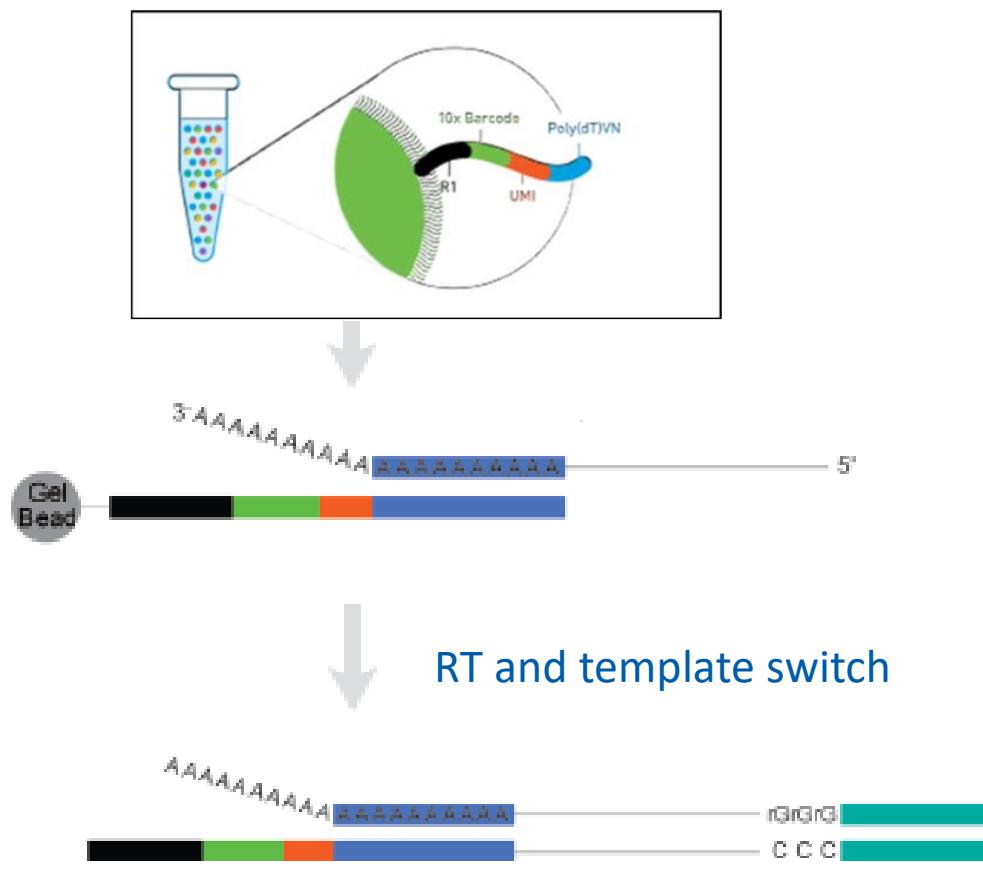


Analyze data



Into a droplet and into a microwell:

1. cells are lysed and release mRNA
2. mRNA is captured by the oligodT of the functionalized beads
3. Retro-Transcription is performed to obtain single-cells cDNAs



NGS LIBRARY PREPARATION



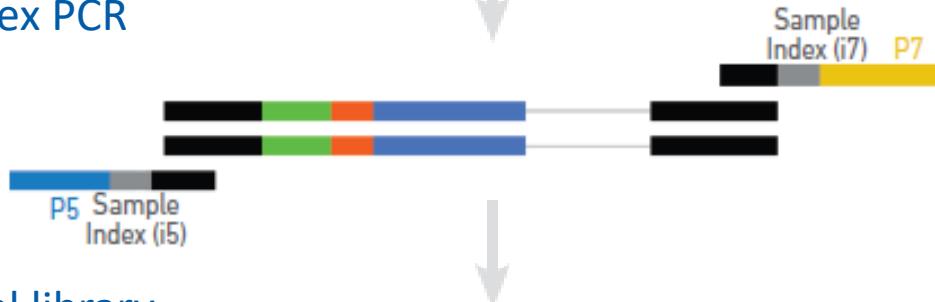
cDNA fragmentation



end repair, A tailing, ligation



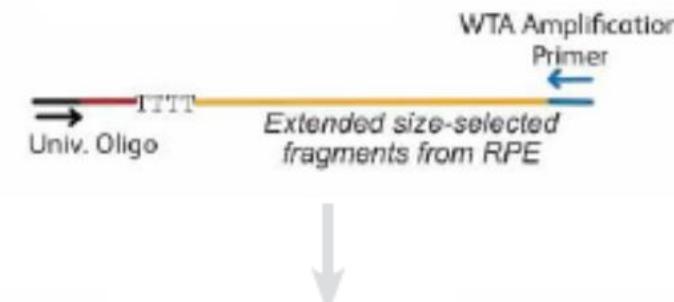
index PCR



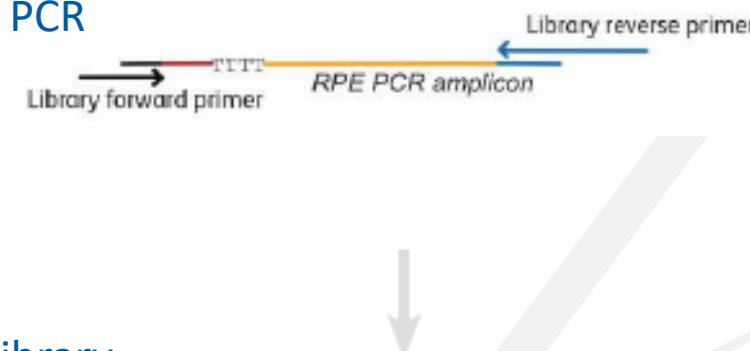
final library



Random Priming and Extension PCR



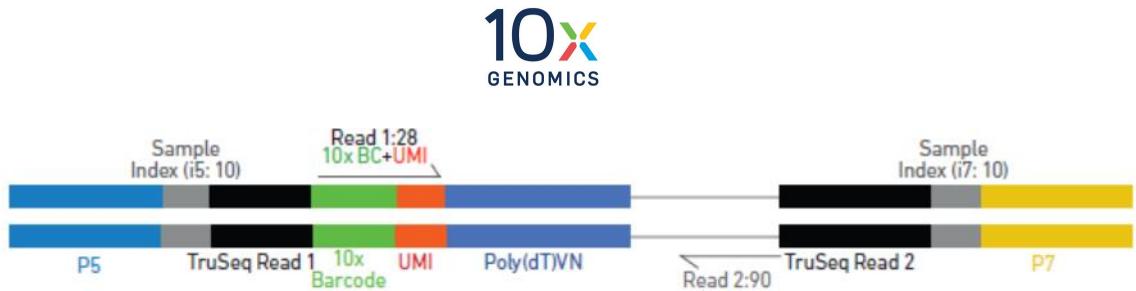
index PCR



final library



SEQUENCING



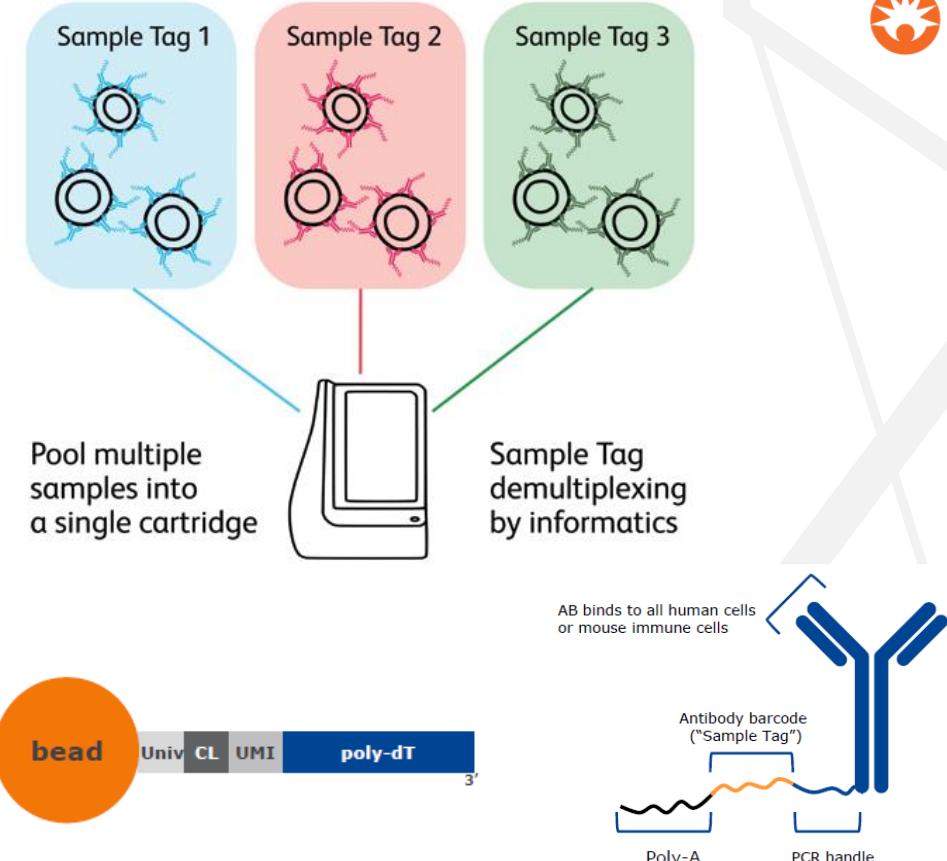
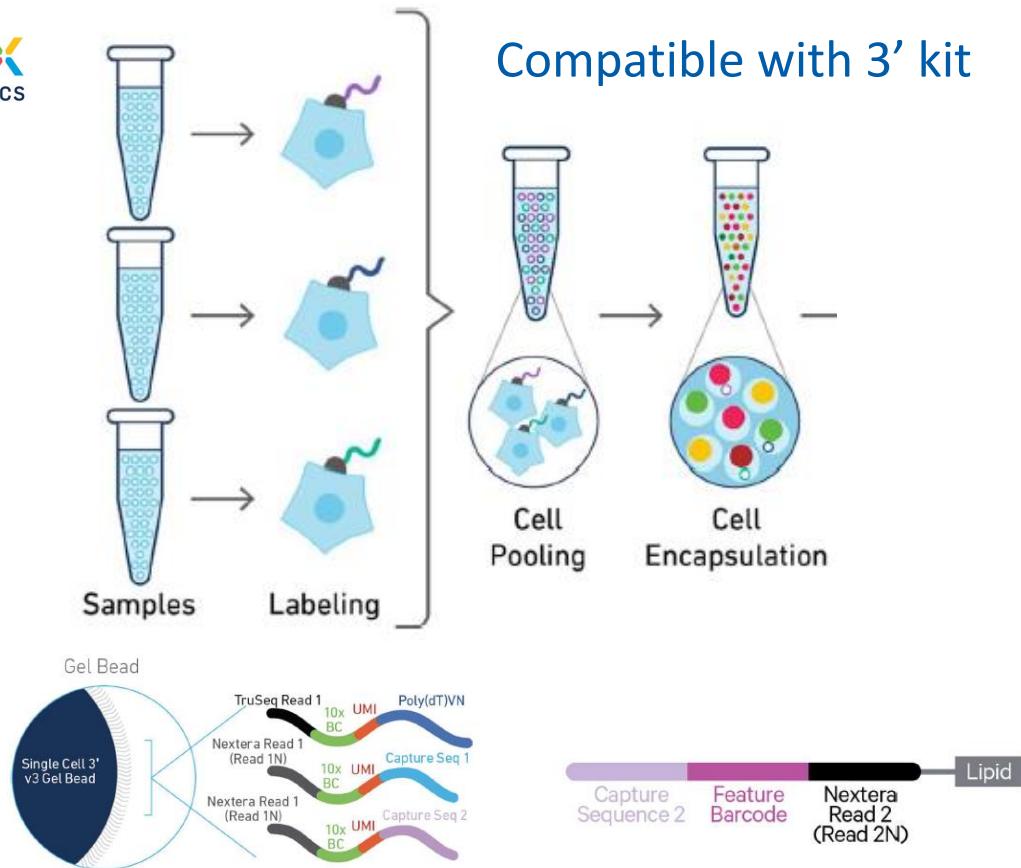
- Read 1 ➔
- **Cell Barcode** -> identification of the Cell
 - **UMI** -> transcripts count and normalization
- Read 2 ➔
- **RNA insert** -> identification of transcript



10x and BD libraries are sequenced paired-ends on high-throughput Illumina sequencers

The sequencing depth should be at least 50'000 reads per cell

SAMPLES MULTIPLEXING

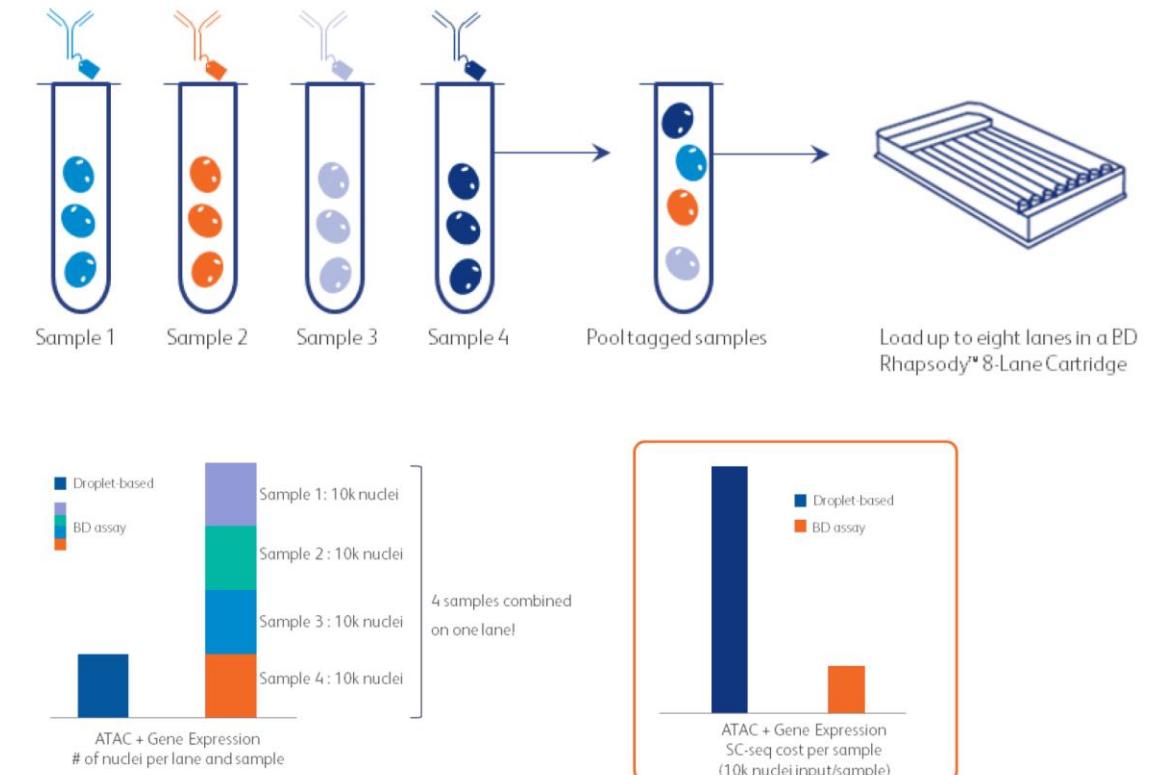
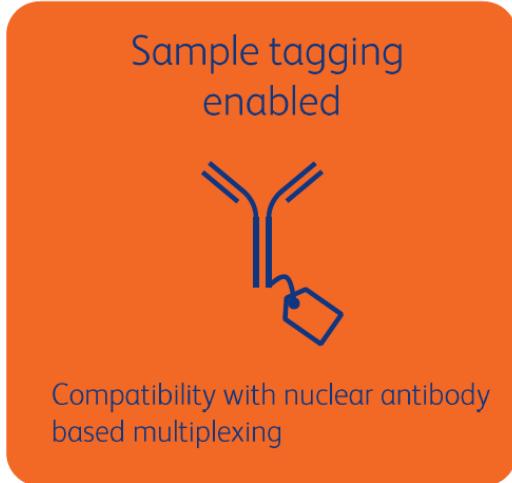


Multiplex up to 12 samples (10x or BD) to optimize cost and time:

- 10x uses a lipid-conjugated barcoded oligo
- BD uses an antibody-conjugated barcoded oligo

New BD Rhapsody workflows

- Multiplexing nuclei for Multiomics analyses ATACseq + Nuclear Gene Expression



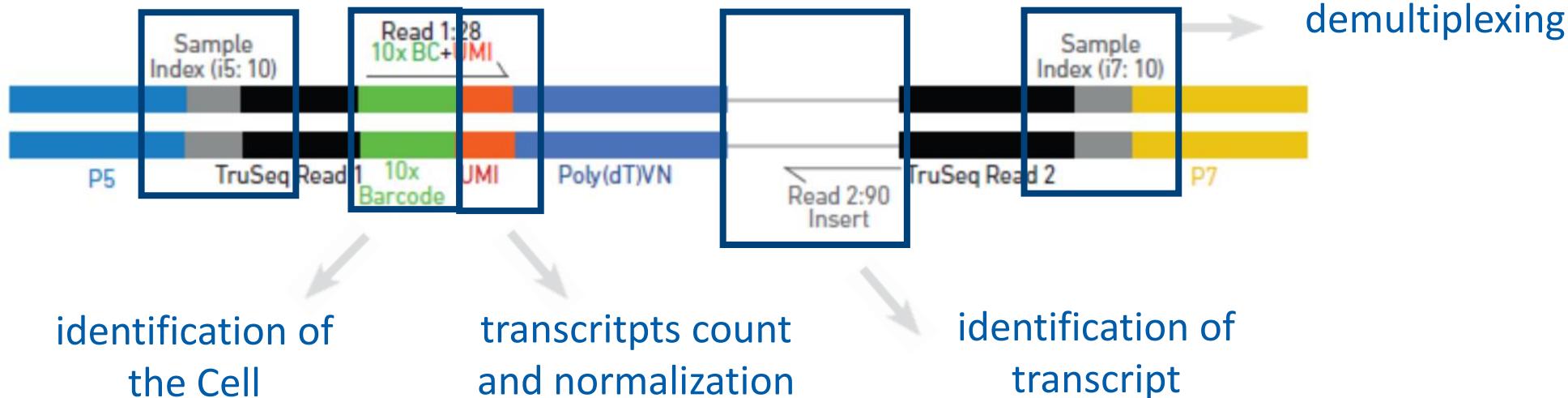
COMPARISON OF 10X AND BD PLATFORMS



	10X APPLICATIONS	BD Rhapsody
➤ Work well with <i>Fragile cells</i>	Problematic with some fragile cells as neutrophils	Work well with fragile cells
➤ Capture efficiency of loaded cells	~50%	~80%
➤ Information on truly captured cells	no	Yes
➤ SC ATAC and Multiome available	yes	Yes (multiome multiplexed)
➤ Work with FFPE tissues	Yes, kit with probes	No (can work with cell in preservation buffer)
➤ Maximal number of targeted cells per run	Almost 1 million (full chip of multiplexed samples on Chromium X)	800'000 cells

BD Rhapsody should be considered when processing fragile cells, as deposition in microwells is gentler than droplet generation.
 10x is ideal when processing a high number of cells or to analyse fixed cells.

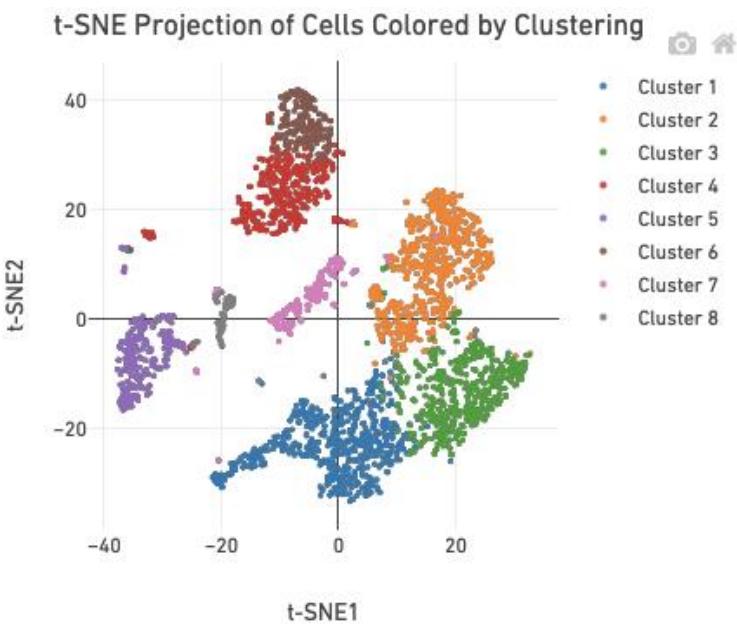
PRELIMINARY DATA ANALYSIS



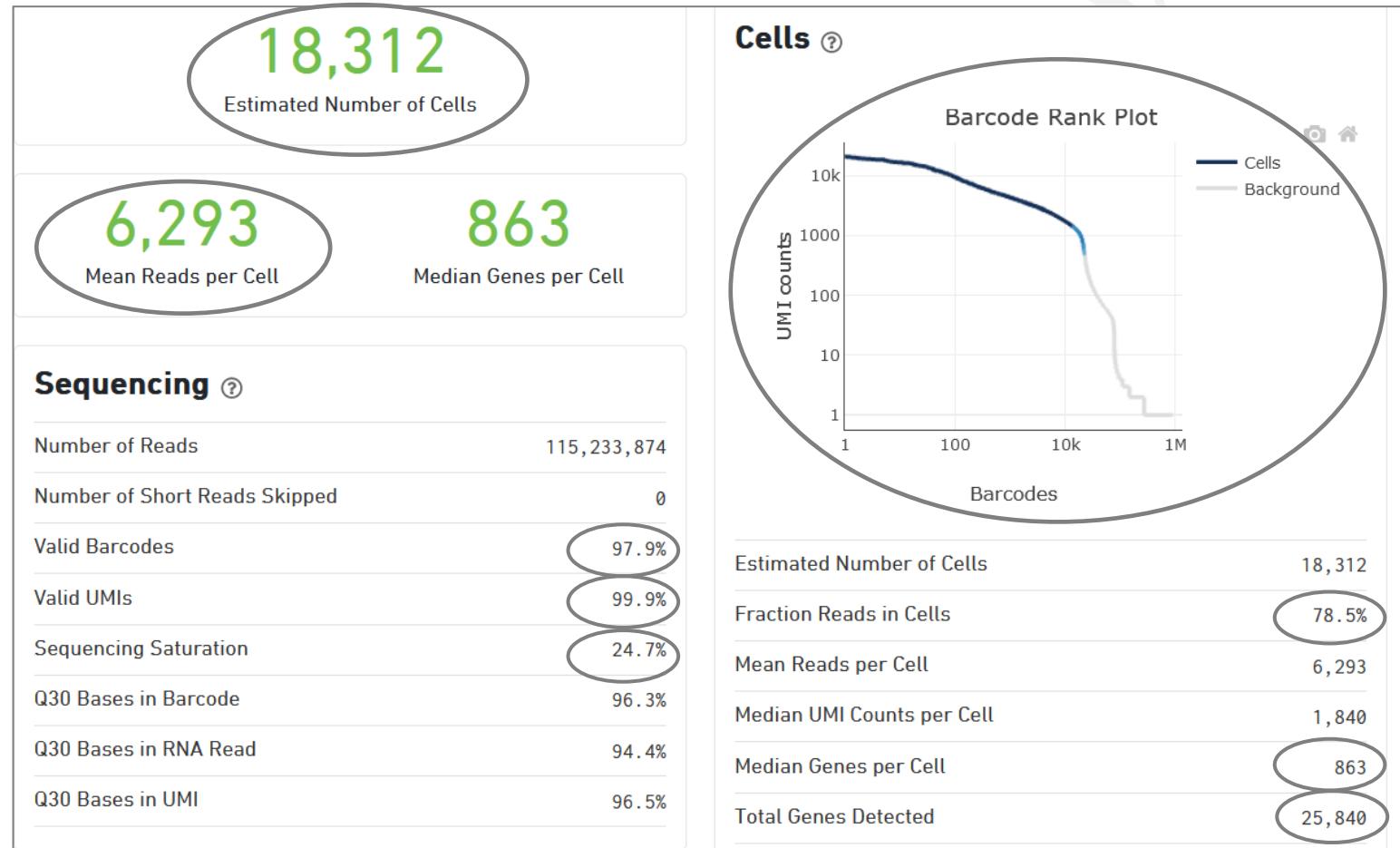
- Pipelines available: Cell Ranger (10x) and Seven Bridges (BD)
- Demultiplexing-alignment-raw count matrix

	Cell1	Cell2	...	CellN
Gene1	3	2	.	13
Gene2	2	3	.	1
Gene3	1	14	.	18
...
...
...
GeneM	25	0	.	0

PRELIMINARY DATA ANALYSIS



Sequencing Depth	Minimum 20,000 read pairs per cell
Sequencing Type	Paired-end, single indexing
Sequencing Read	Recommended Number of Cycles
Read 1	28 cycles
i7 Index	8 cycles
i5 Index	0 cycles
Read 2	91 cycles

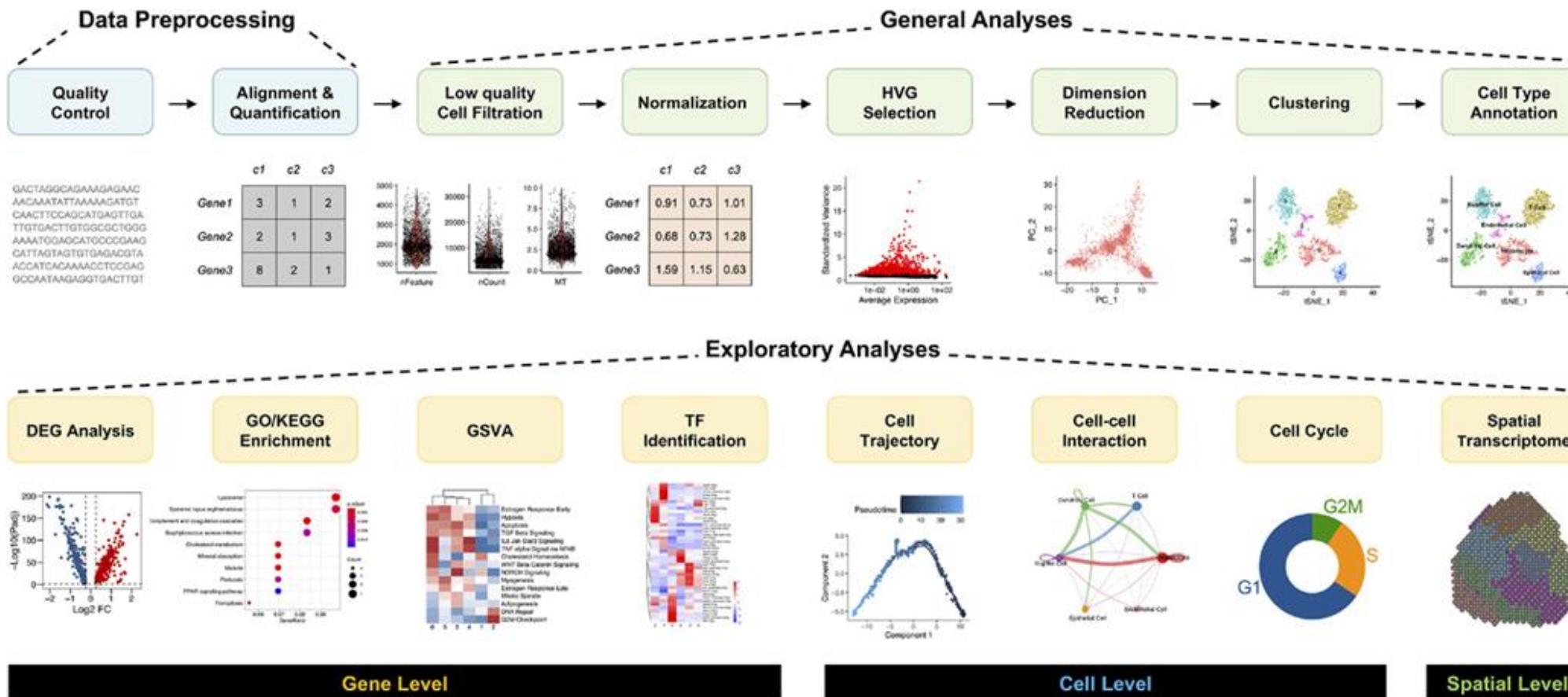


Web Summary obtained with Cell Ranger

- Check that targeted cells near the estimated number of cells
- Check that the barcode rank plot has a steep drop-off
- Check the fraction reads in cells
- Check the sequencing saturation and median genes per cell

Roadmap for typical single-cell RNA seq data analysis

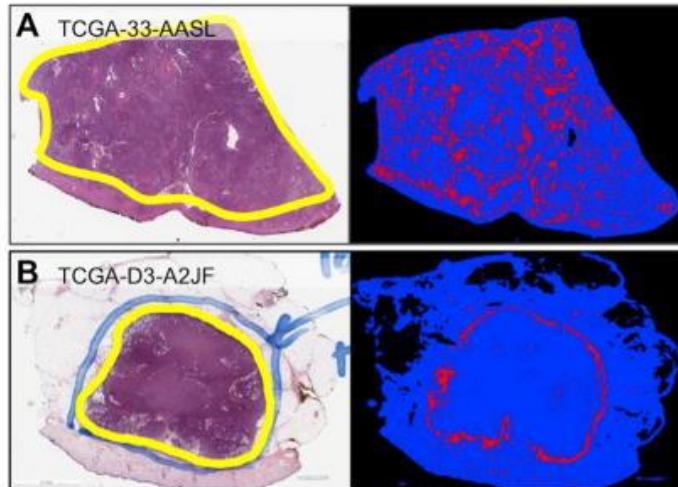
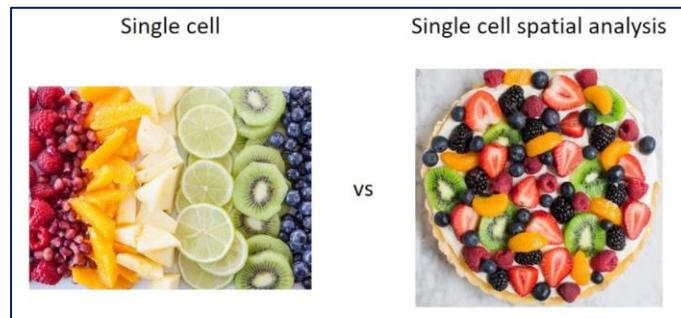
1. **Data pre-processing** includes quality control, alignment and quantification;
2. **General analyses** include low-quality cell filtering, normalization, HVG selection, dimension reduction, clustering and annotation of cell types;
3. **Exploratory analyses** include: DEG analysis, function enrichment, GSVA, TF prediction, cell trajectory, cell-cell interaction, cell cycle and spatial transcriptome analysis.



SPATIAL TRANSCRIPTOMICS

Method of the Year 2020: Spatially resolved transcriptomics

The analysis of spatial organization is fundamental for understanding biological processes



Lymphocytes infiltrating tumors: **good prognosis**

Lymphocytes stopped at tumor boundary: **poor prognosis**

The need for single cell transcriptomics with spatial analysis

Bulk



VS

Single cell



Single cell spatial analysis

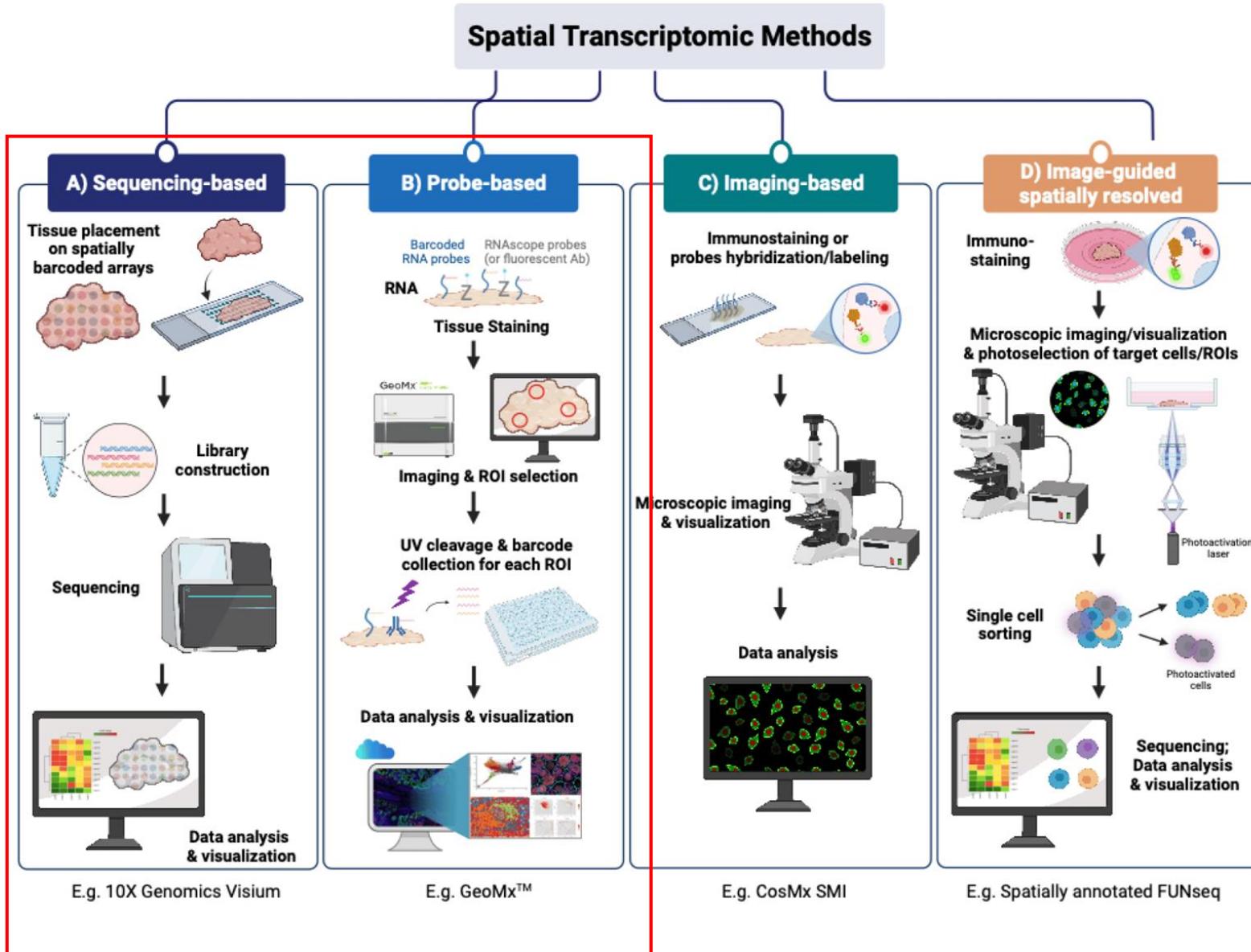


Bulk transcriptomic analyses lose spatial and single cell information

Single cell transcriptomic analyses retain single cell information but lose spatial organization

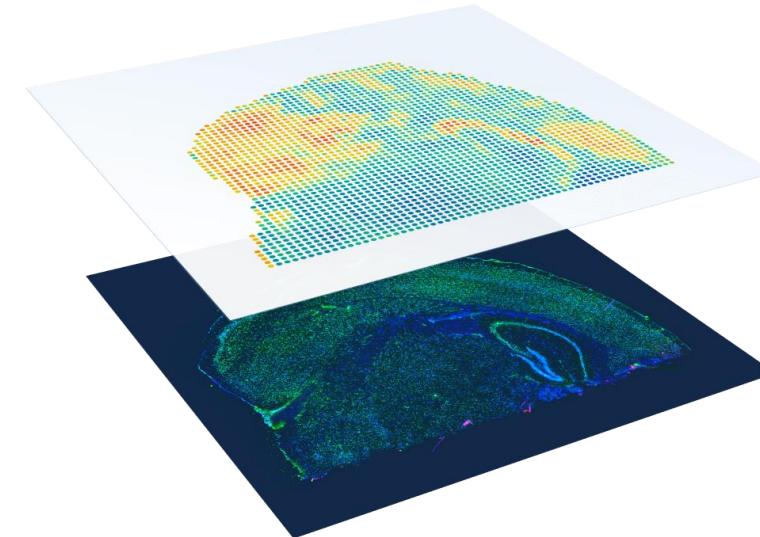
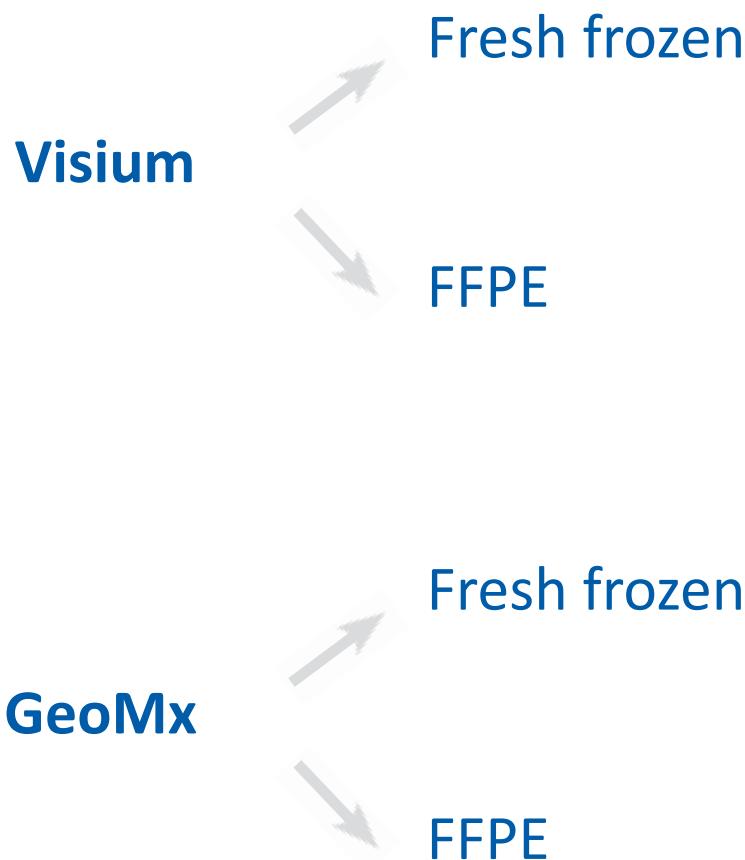
Single cell, multiplexing spatial analyses provide transcriptomic information with spatial organization while retaining single cell resolution

SPATIAL TRANSCRIPTOMICS



- **Sequencing-based methods** use barcoded DNA arrays to capture polyadenylated RNA transcripts from tissues followed by NGS.
- **Probe-based methods** capture panels of transcripts in manually selected regions of interest (ROIs) or in the full tissue.
- **Imaging-based methods**, rely on *in situ* hybridization of fluorescent probes panels to detect the targeted transcripts.
- **Image-guided spatially resolved scRNASeq methods** select spatially different single cells in ROIs followed by fluorescence-activated cell sorting and scRNASeq.

SPATIAL TRANSCRIPTOMICS NGS BASED



10X
GENOMICS

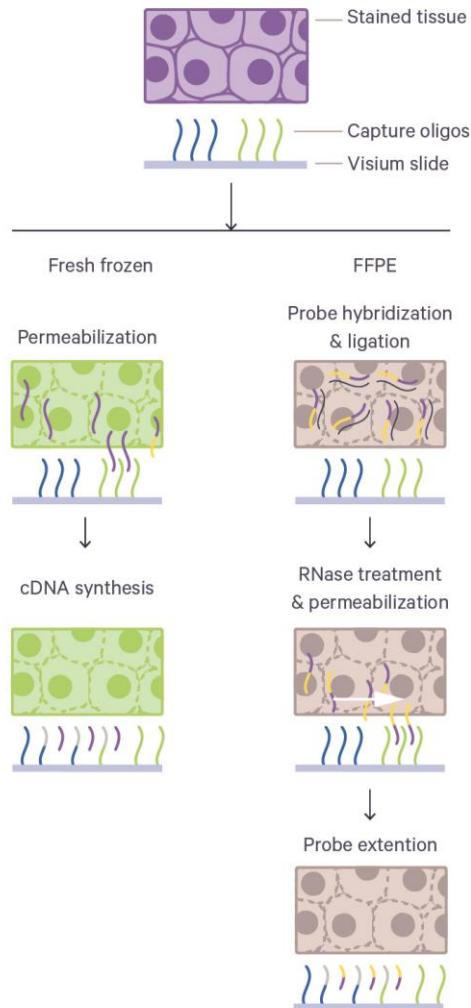


nanostring®

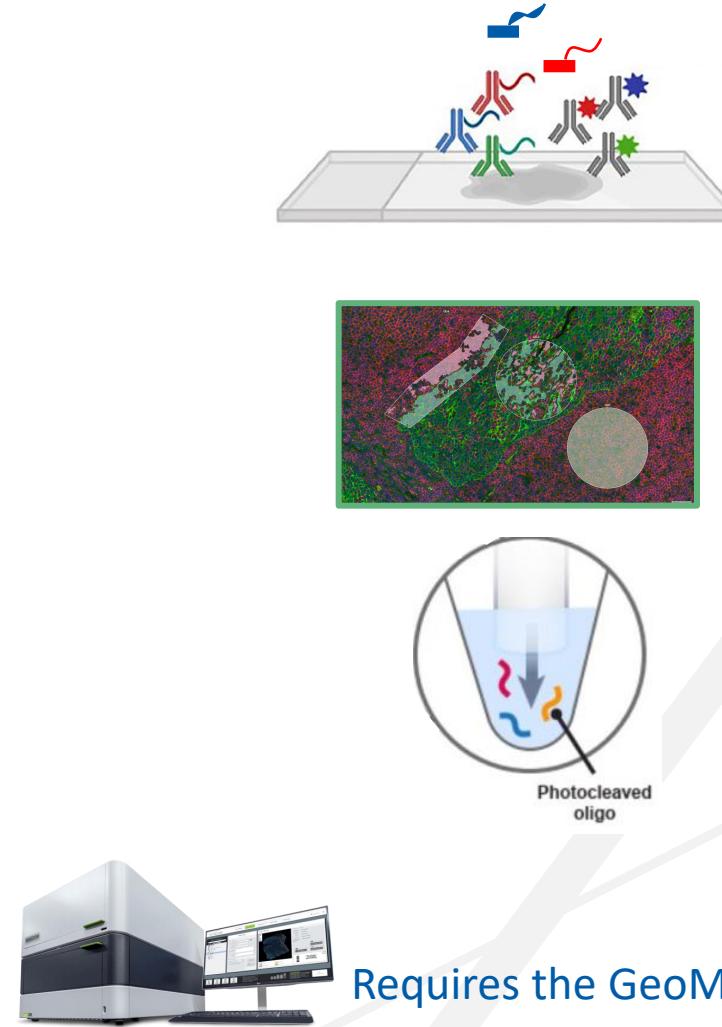
OVERVIEW OF VISIUM AND GEOMX



mRNA is captured by oligos on the slide surface



mRNA is captured by probes panels linked to oligos



FRESH FROZEN VISIUM WORKFLOW

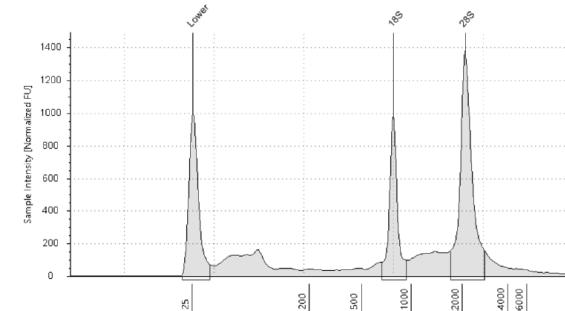
Embed tissue in OCT
and freeze it



Cut 10 μ m sections extract totRNAs
and place on Visium slides



QC of bulk RNA (RIN)
extracted from tissue

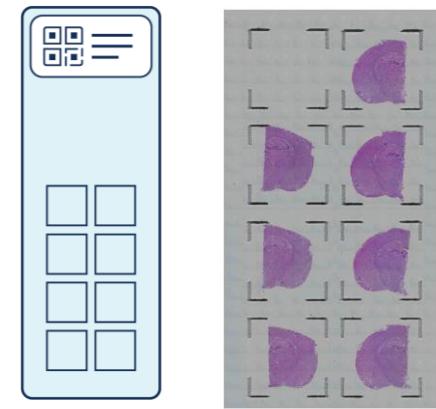


OK if RIN > 7

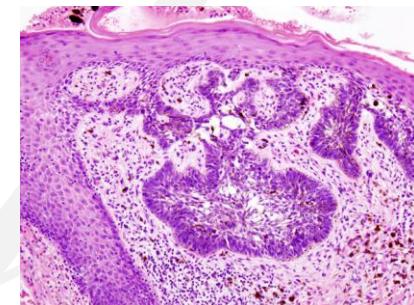
Gene expression assay



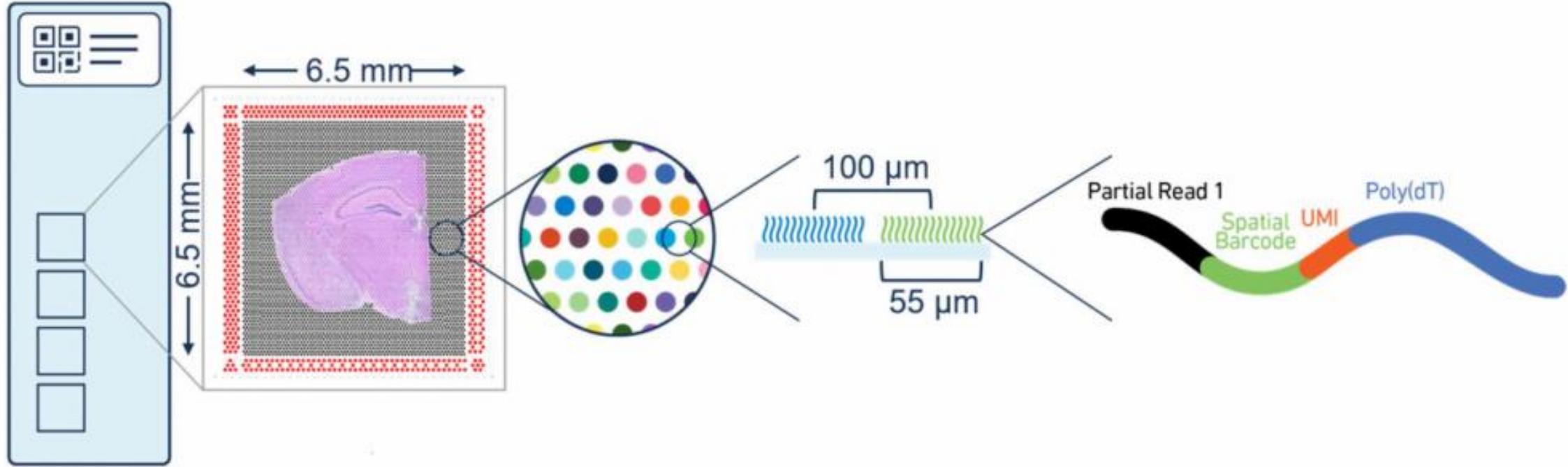
Tissue optimization



Methanol fixation, H&E staining
and imaging



FRESH FROZEN GENE EXPRESSION ASSAY



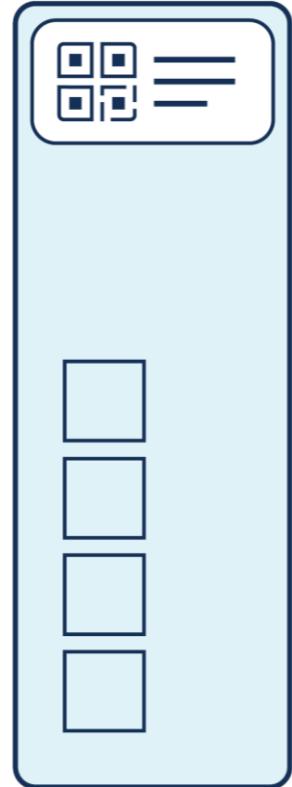
Four capture areas per slide

Each capture area has 5'000 spots of 55 μm diameter

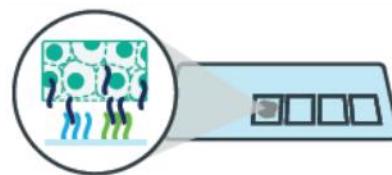
Spot-specific barcoded Poly (dT) oligos

FRESH FROZEN GENE EXPRESSION ASSAY

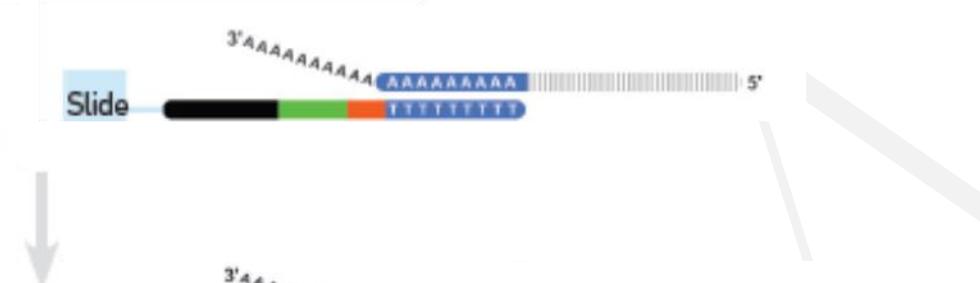
On the slide



Permeabilization



Reverse transcription



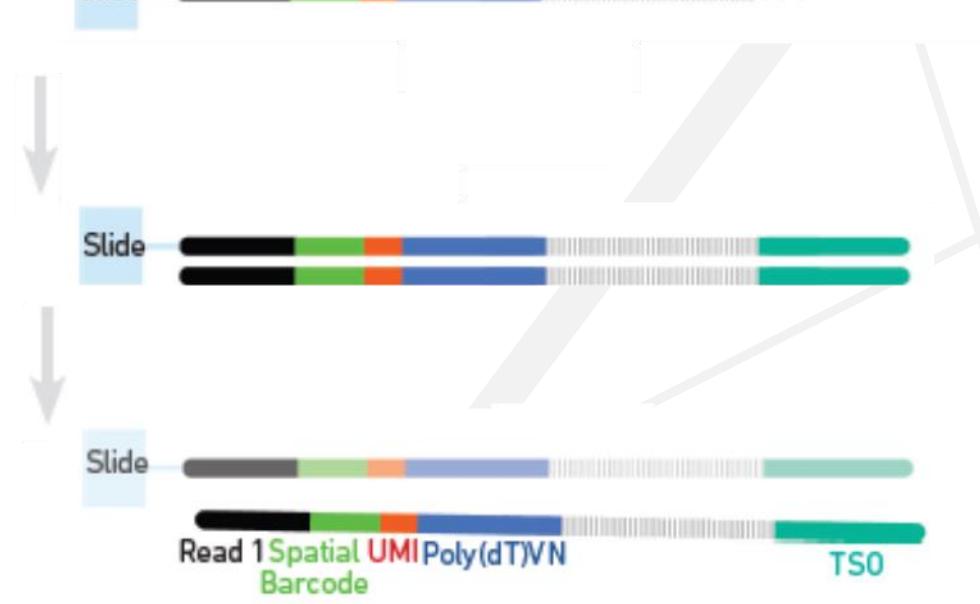
Template switching



Second Strand Synthesis



Denaturation



FRESH FROZEN GENE EXPRESSION ASSAY

In the tubes

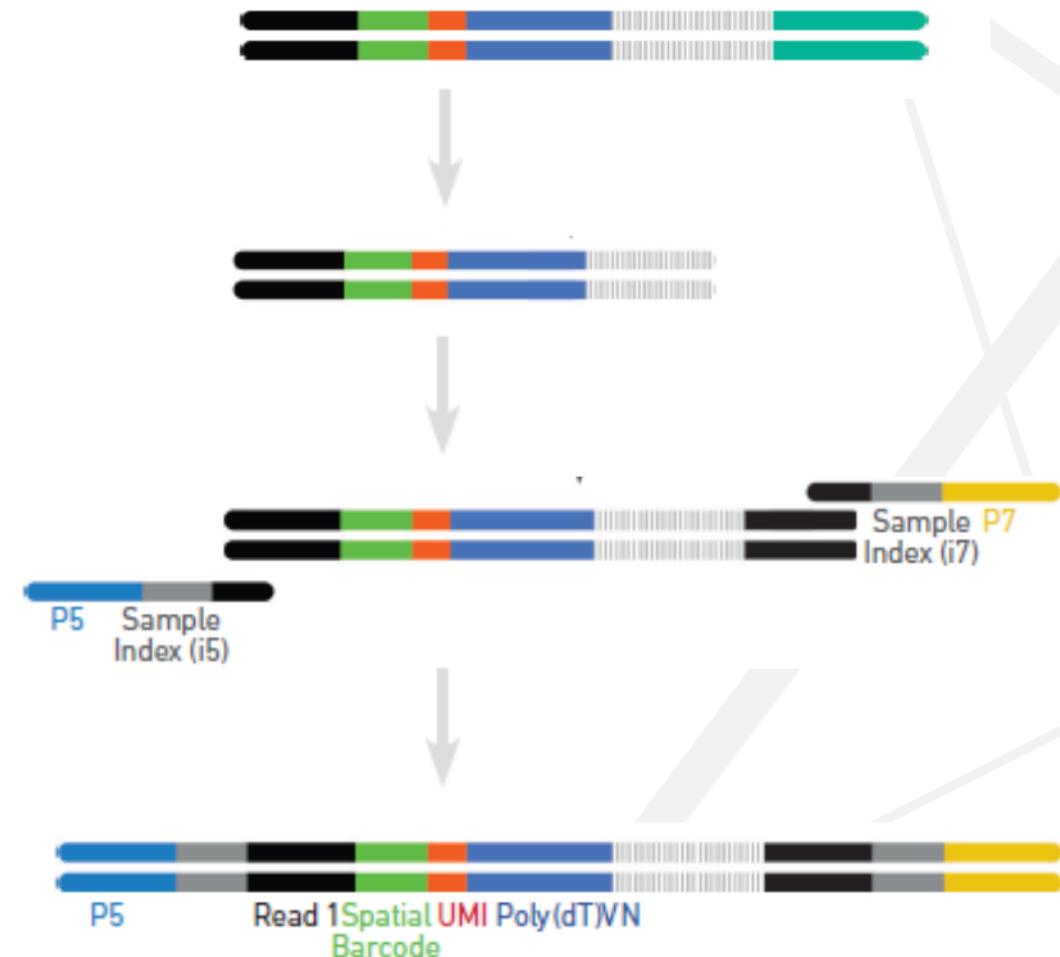


cDNA amplification

Enzymatic fragmentation

End repair, A-tailing, ligation and index PCR

Final Library



FFPE VISIUM and VISIUM HD WORKFLOW

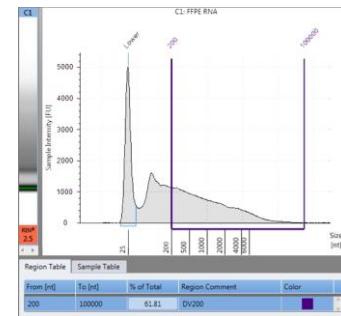
FFPE embedded sample



Cut 5 µm sections and extract totRNA



QC of bulk RNA (DV200) extracted from tissue

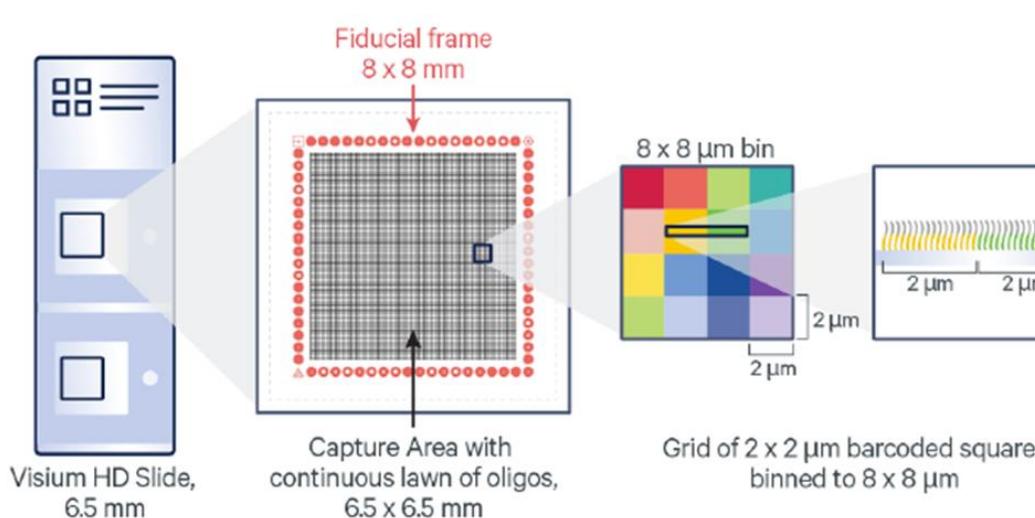


OK if DV200 > 30%

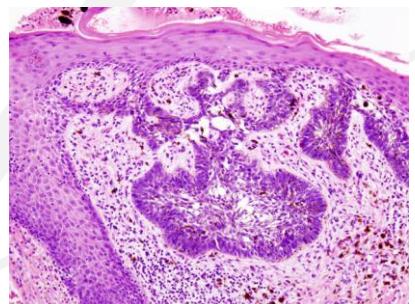
Transfer FFPE tissues ROI on Visium slide with Cytassist



Probe hybridization and FFPE gene expression assay



Deparaffinization, H&E staining and imaging



FFPE GENE EXPRESSION ASSAY

Probe hybridization
and ligation



Permeabilization
and extension



Denaturation,
probe elution and
index PCR

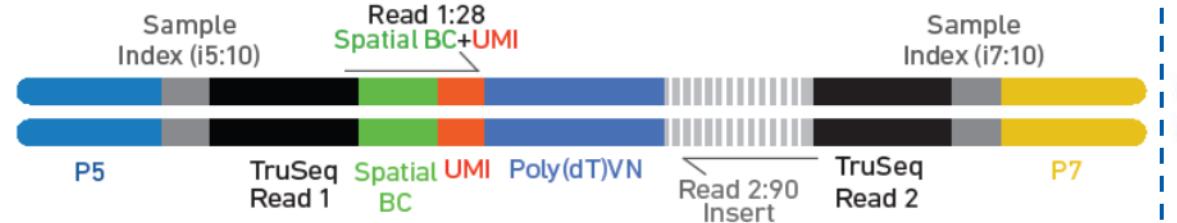


Final library

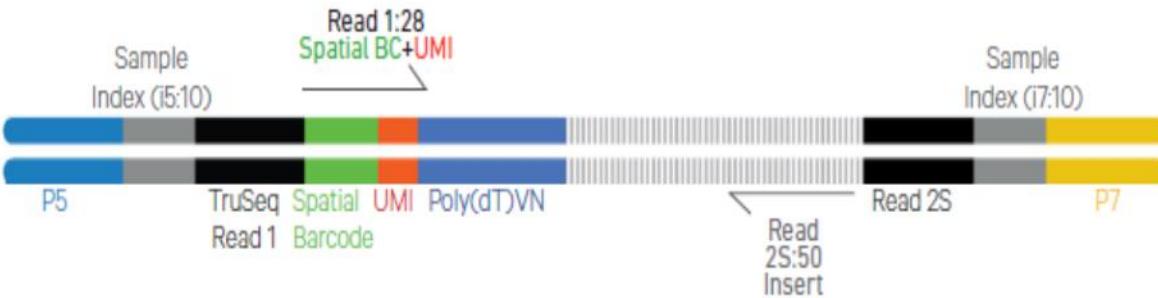


VISIUM LIBRARIES SEQUENCING

Fresh Frozen Visium



FFPE Visium



Read 1 →

- **Spatial Barcode** -> identification of the spot
- **UMI** -> transcripts count and normalization

Read 2 →

- **RNA insert or probe** -> identification of transcript

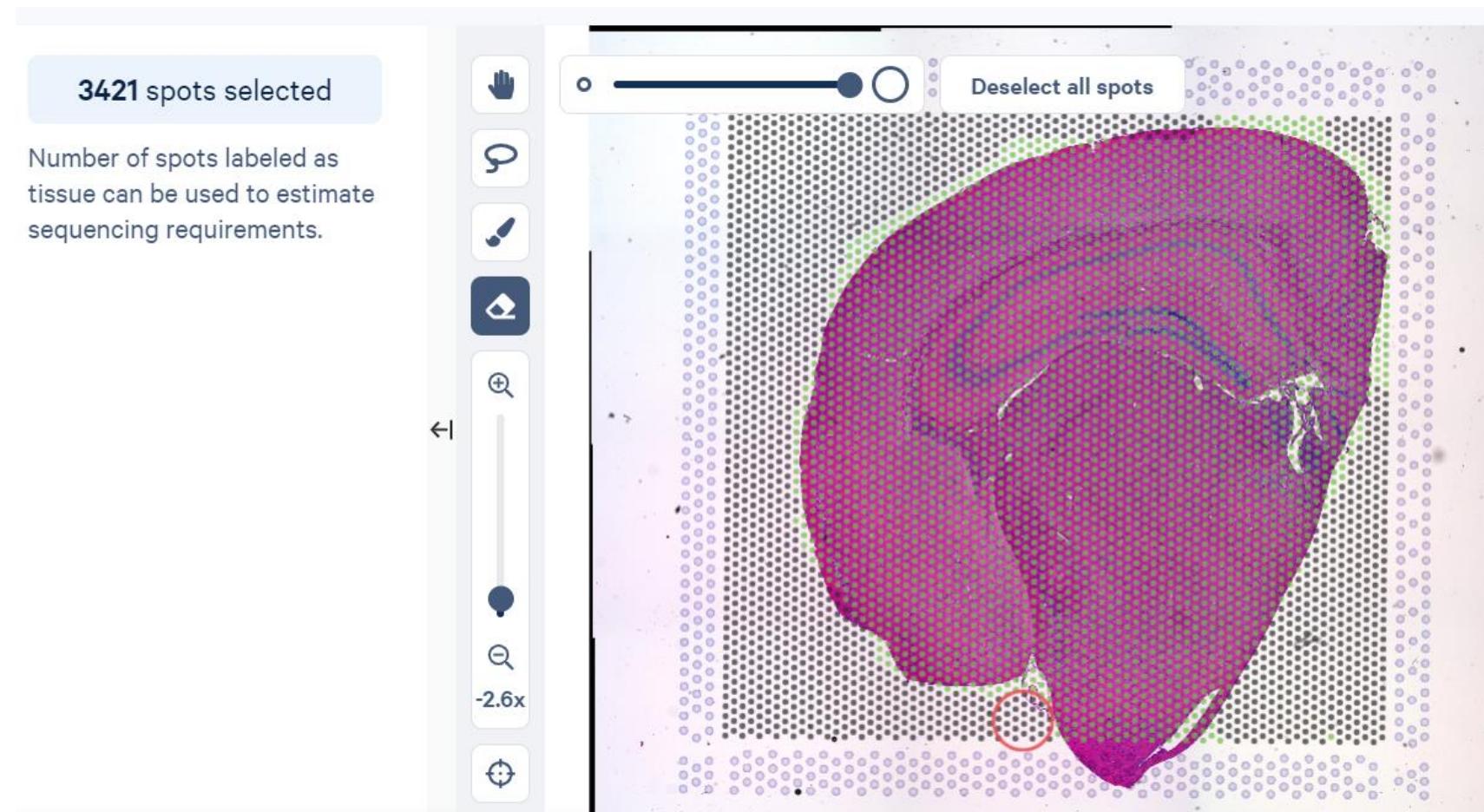


FF and FFPE Visium libraries are sequenced paired-ends on high-throughput Illumina sequencers

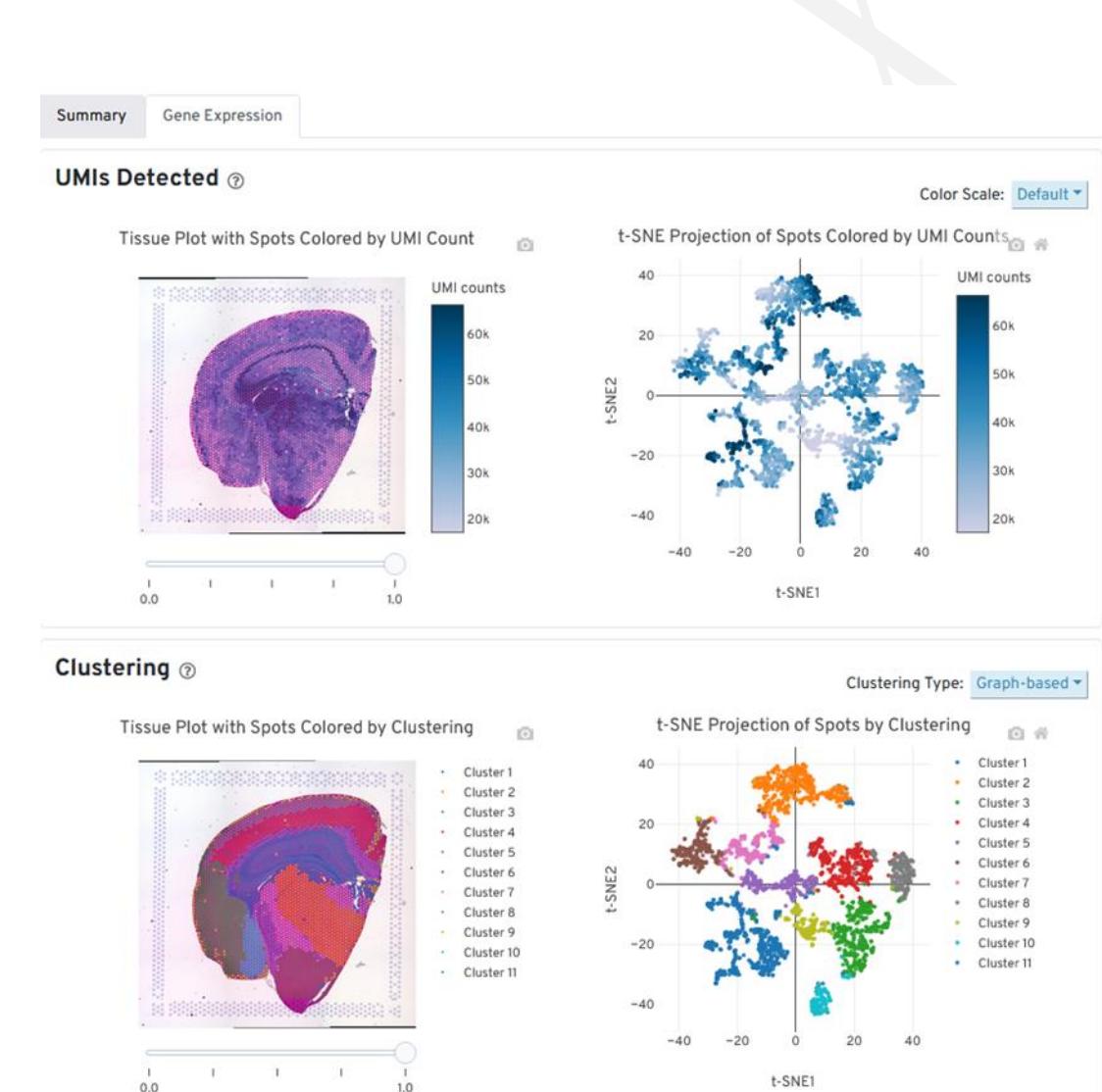
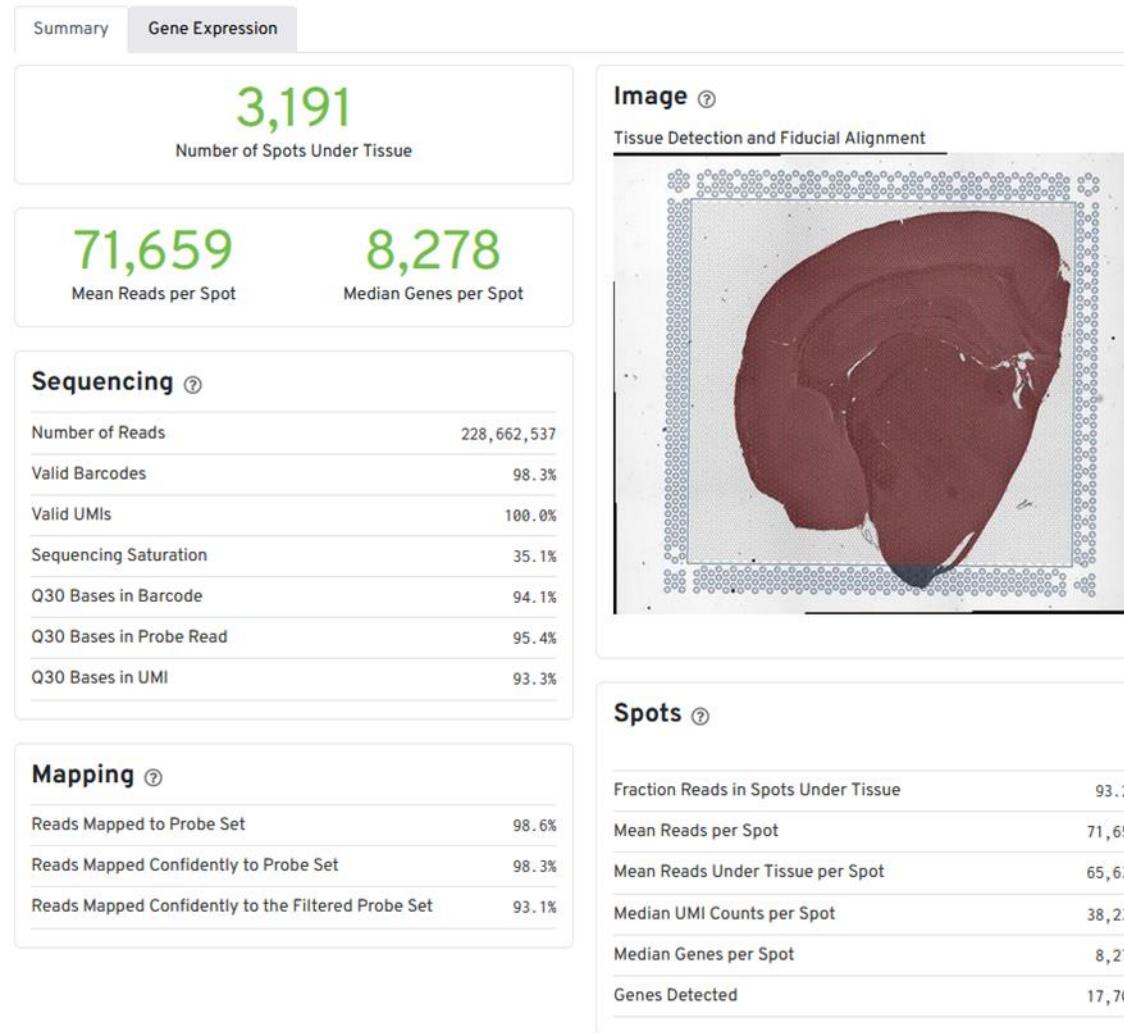
The recommended sequencing depth is 25'000/50'000 reads per spot

VISIUM LIBRARIES SEQUENCING

Number of spots covered by tissue is determined with Loupe Browser by overlapping the image of the tissue slice to the Visium matrix



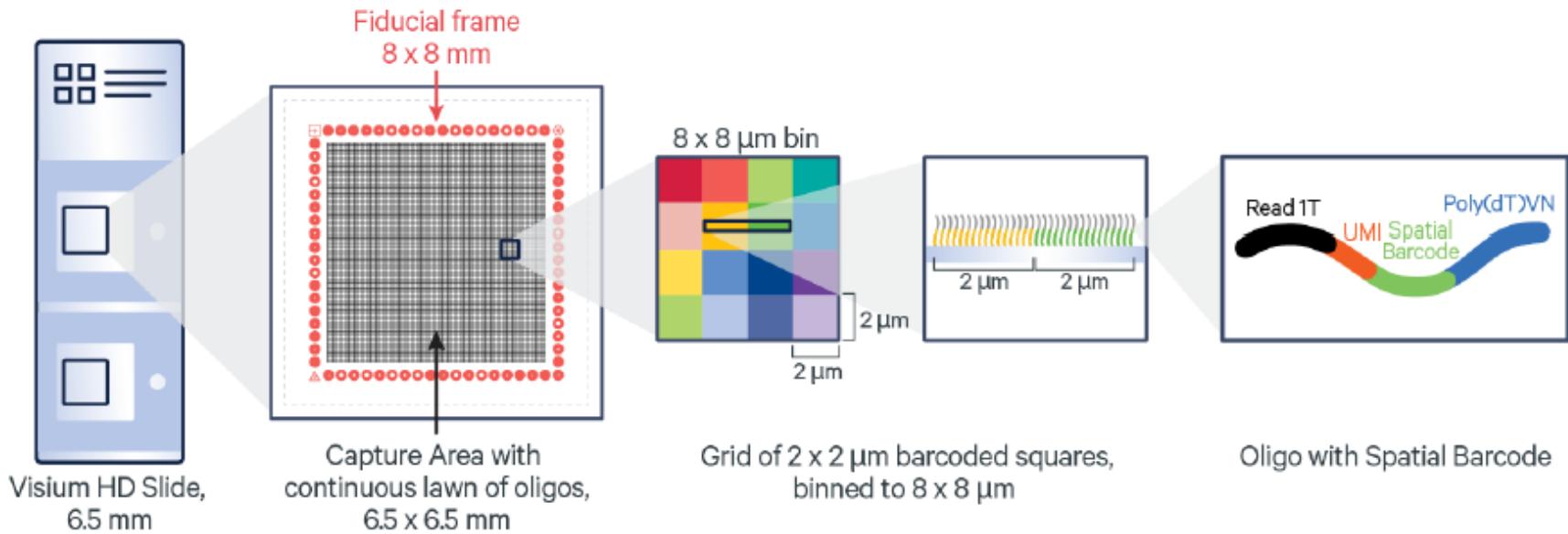
PRELIMINARY DATA ANALYSIS WITH SPACE RANGER



Visium HD 3'



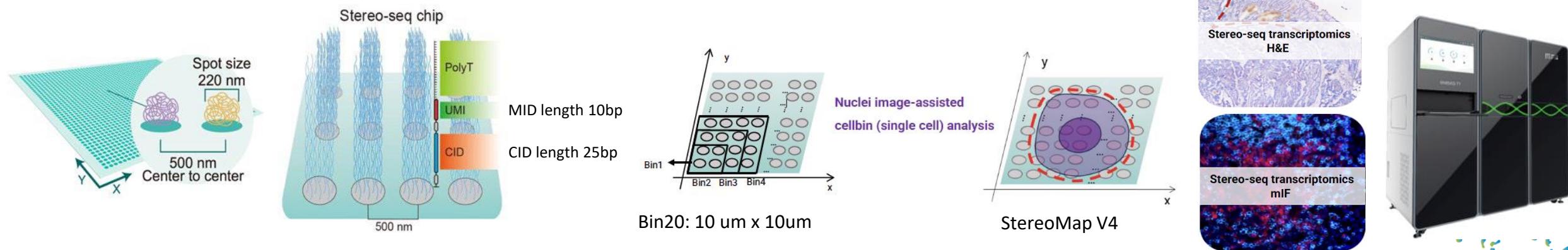
- Compatible across species
- 3' poly(A) capture-based chemistry enables *de novo* discovery applications –feasibility for isoforms, TCRs/BCRs, SNVs, etc.
- Single cell-scale resolution with gapless tissue coverage
- CytAssist-enabled workflow streamlines slide handling and provides high spatial fidelity
- Enhanced histology with same section H&E-staining



StereoSeq 3' polyA (STOmics-MGI)

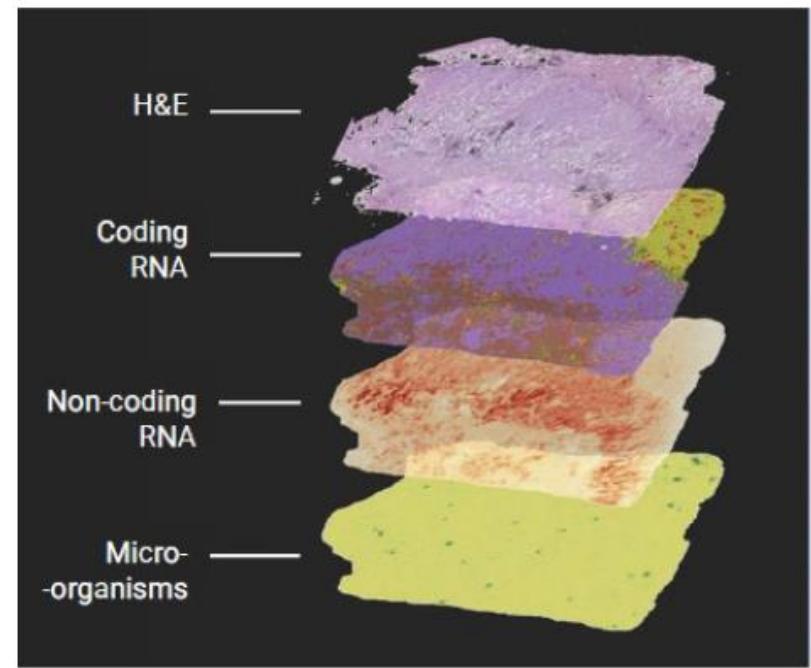
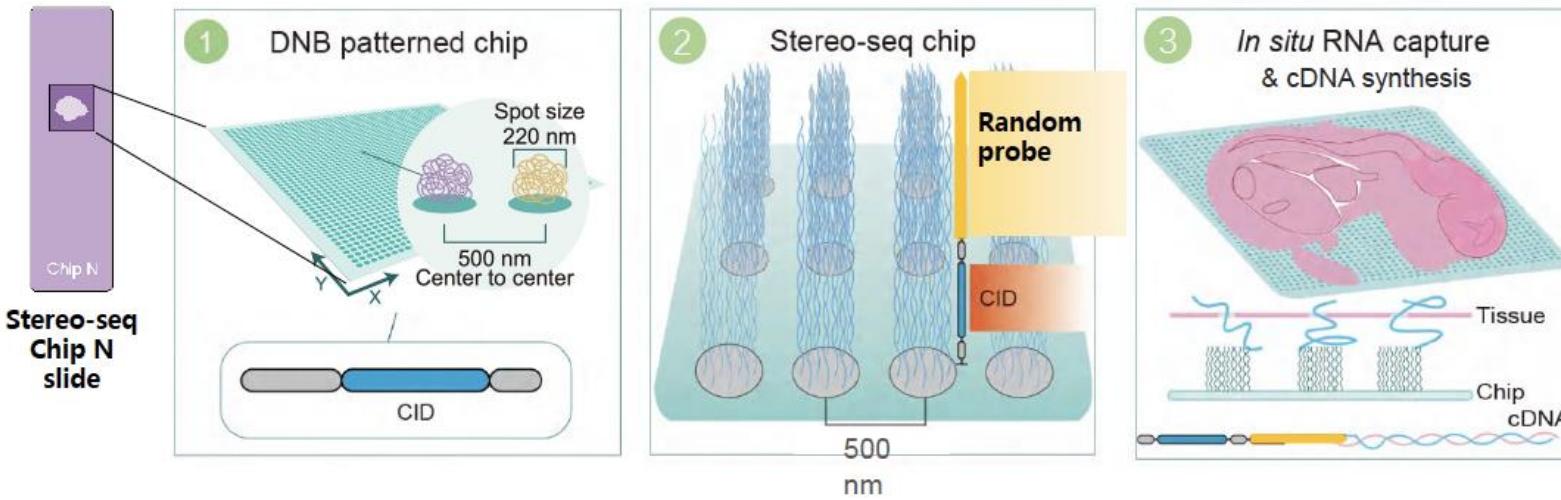
Stereo-seq technology is only spatial-omics technology that could achieve both centimeter level panoramic field of view and subcellular resolution

- Nanoscale subcellular resolution (200-500 nm)
- Up to 13cm*13cm capturing area
- Unbiased whole transcriptomics & Proteomics (100+)



Stereo-seq OMNI: Unbiased Total RNA for FFPE samples

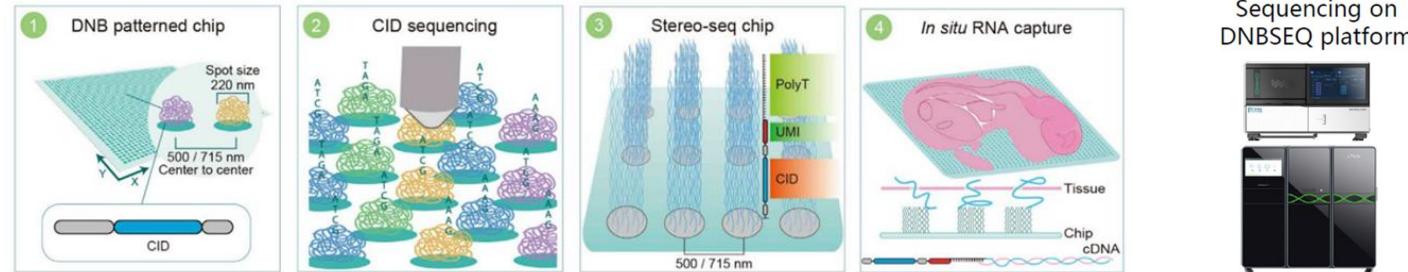
- Random probe design for total RNA capturing
- Compatible with low-quality samples (DV200>30)
- Species agnostic, enabling the study of host-microorganism interaction



Comparison StereoSeq vs Visium HD:

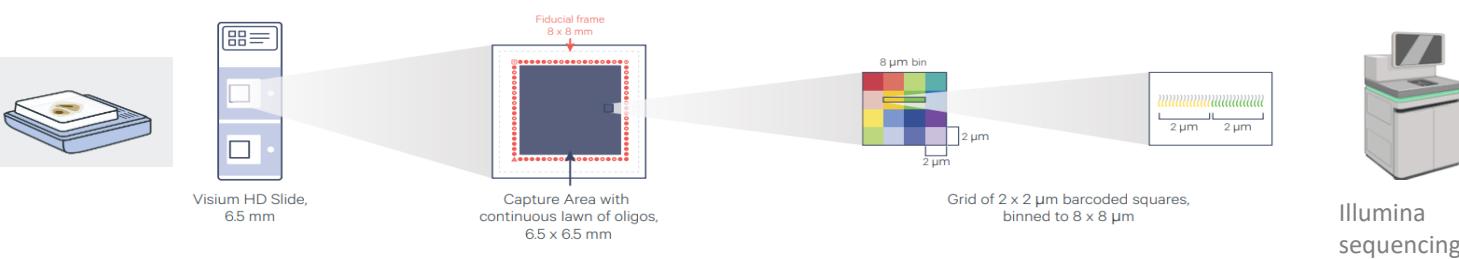
➤ **StereoSeq:** cellular/subcellular level (nanoscale resolution) – high flexibility – **NO instrument**

Features	StereoSeq
Spot size (μm)	0,22
Center to center (μm)	0,5
Field of view (mm)	upto 132X132
Sample type	Fresh Frozen FFPE

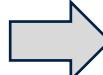
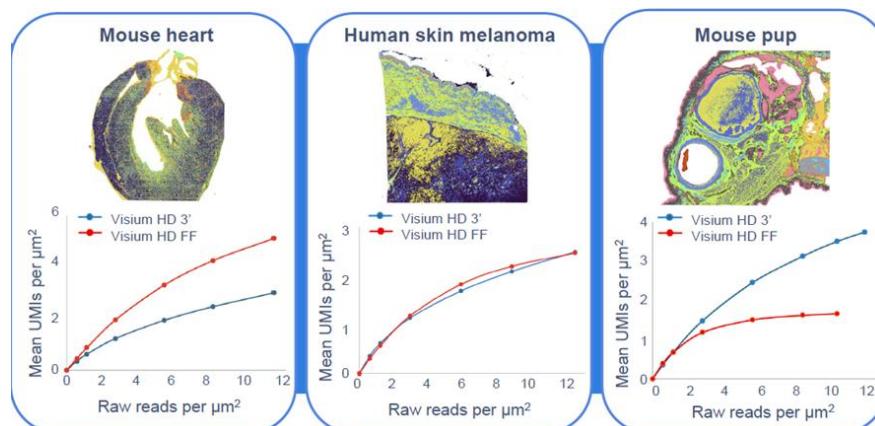


➤ **Visium HD:** cellular level –high throughput–**NO instrument**

Features	Visium HD
Spot size (μm)	8(2x2)
Center to center (μm)	na
Field of view (mm)	6,5x6,5
Sample type	FFPE Fresh Frozen

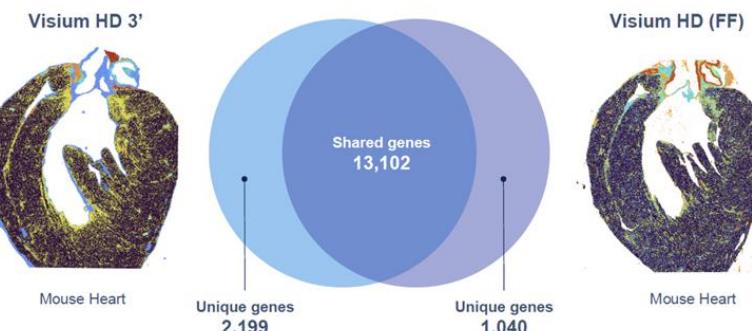


Data Comparison: Visium HD 3' vs. Visium HD (FF): Sensitivity results depend upon tissue type and sample quality



Uncover more unique genes with Visium HD 3'

Expanded discovery beyond the protein coding transcriptome



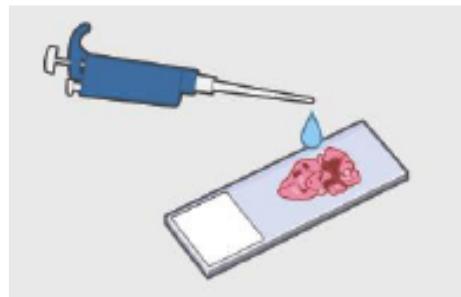


GEOMX Digital Spatial Profiler

<https://nanostring.com/products/geomx-digital-spatial-profiler/>

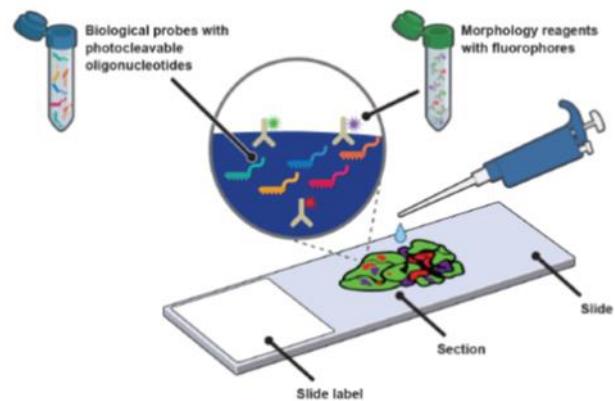
GEOMX WORKFLOW

Slide preparation with 5µm of FFPE or FF tissue



Standard histological slides,
large tissue area

Incubation with imaging and
probes panels



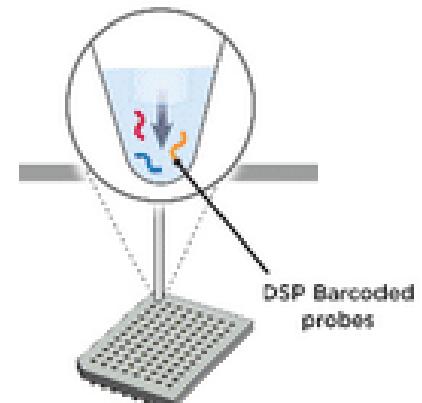
Select regions of interest



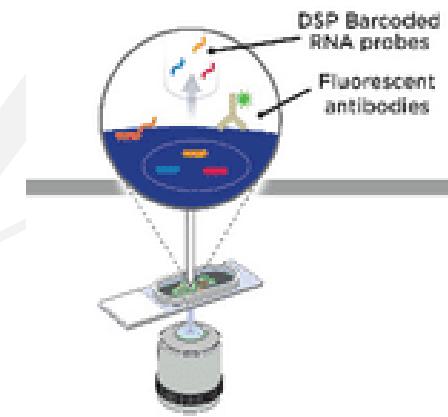
Prepare library and
sequence



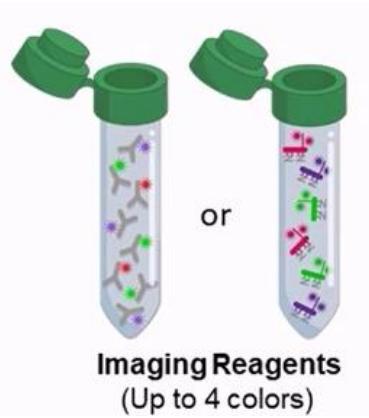
Dispense barcodes in
plate



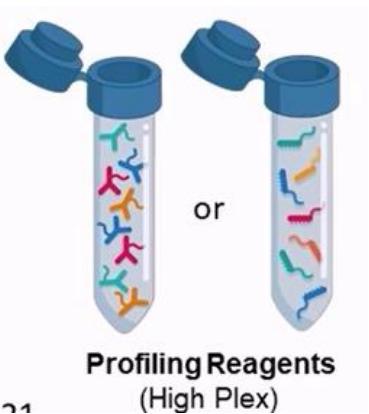
UV cleave of barcodes



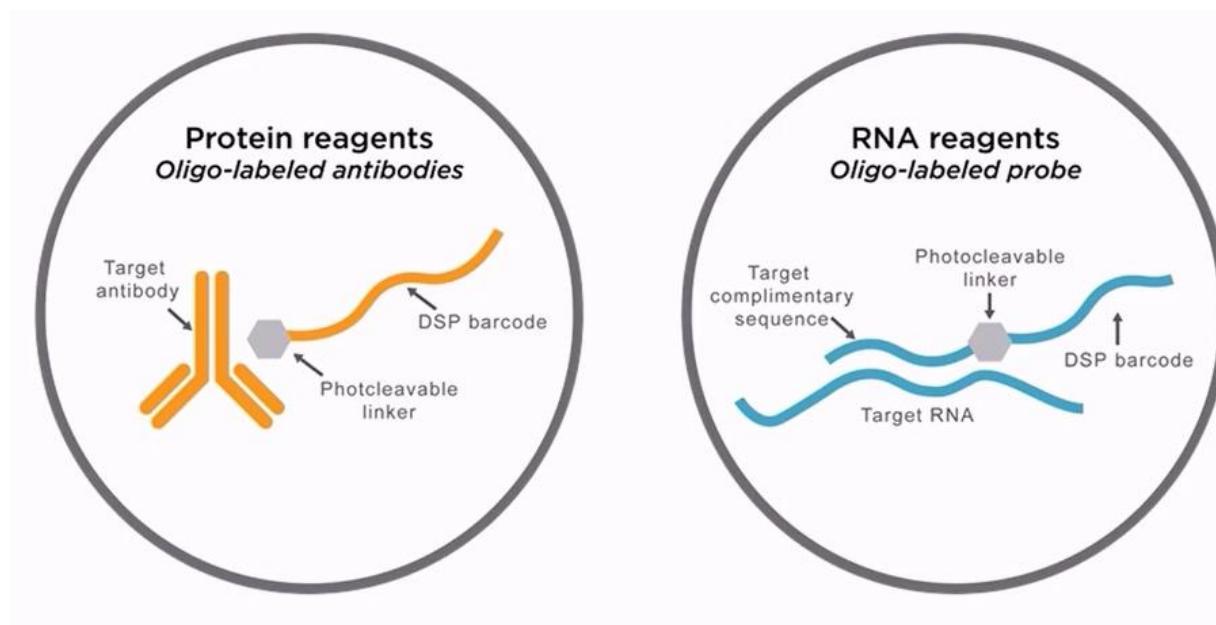
INCUBATION WITH IMAGING AND PROFILING REAGENTS



Imaging reagents to get morphological info and select ROI
(four fluorescent antibodies can be used at the same time
DAPI for nuclei always included to count cells in ROIs)

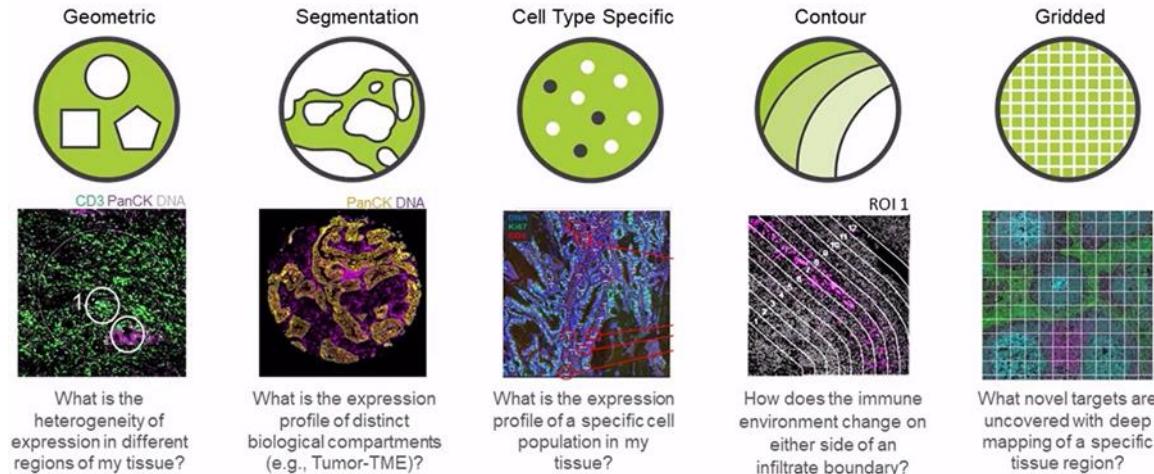


Protein and RNA profiling probes are linked to UV
photocleavable DSB (digital spatial barcodes)

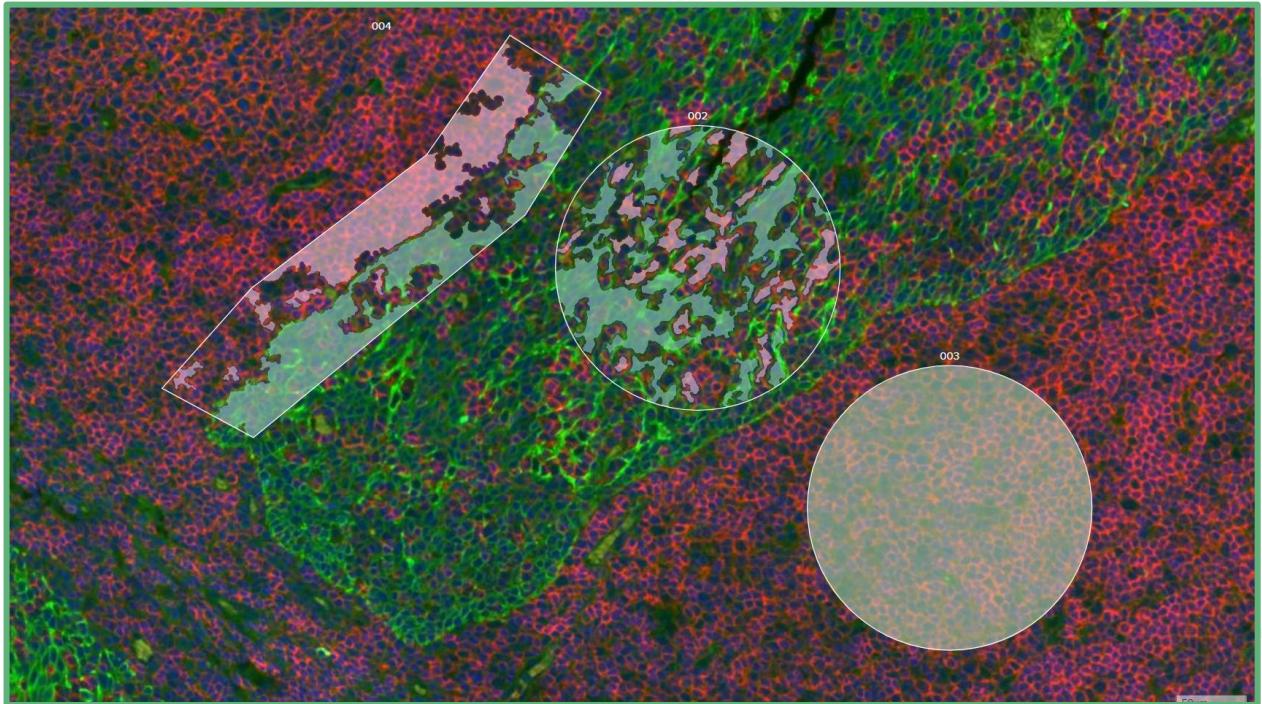




SELECTION OF REGION OF INTEREST



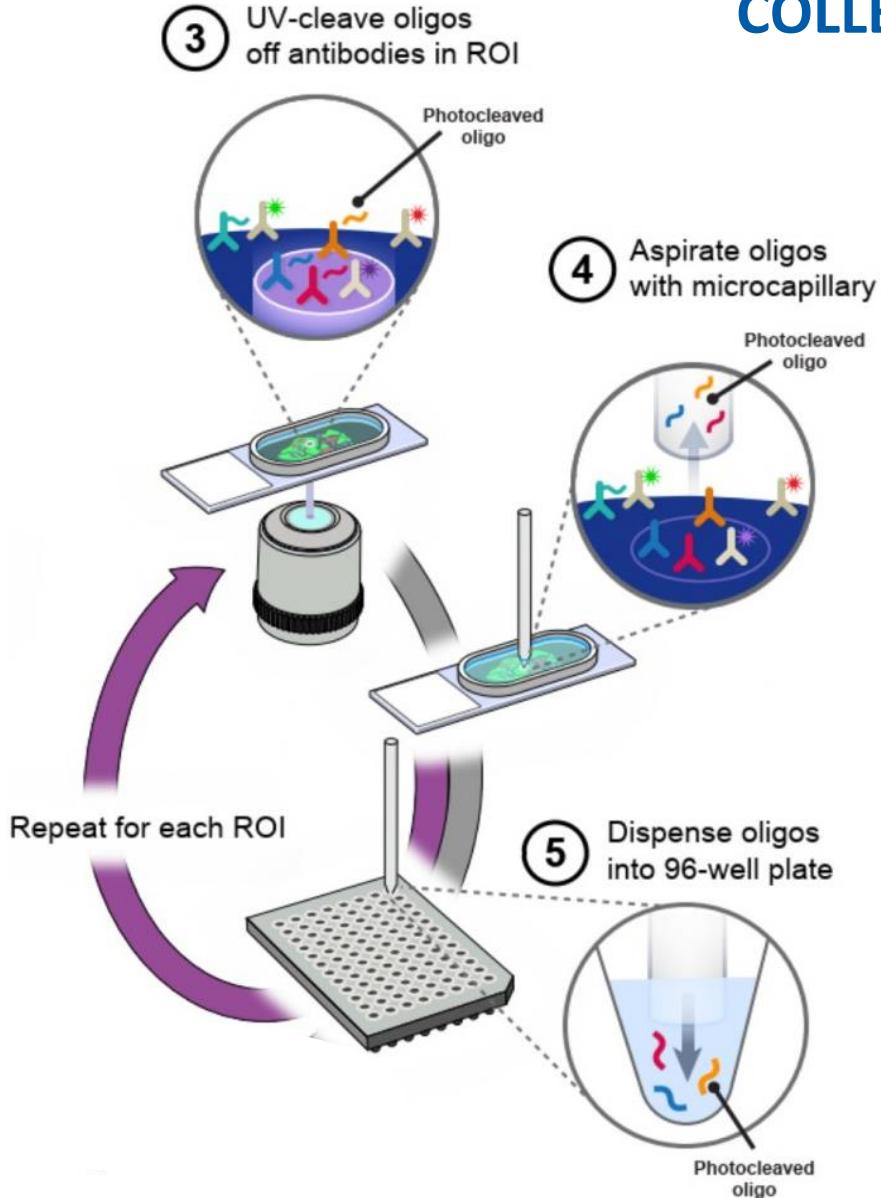
Tonsil tumor sample



Scan slides and draw ROI based on morphology markers

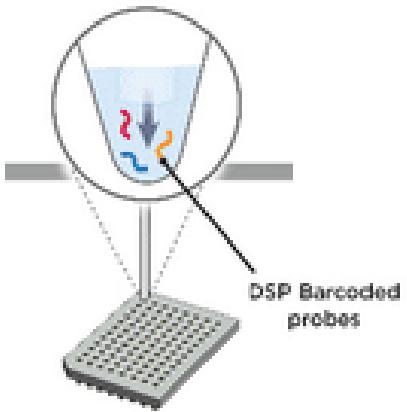
Nuclei
Pan Cytocheratin
CD45

COLLECTION OF DSB (digital spatial barcodes) FROM ROI



1. UV light to release spatial barcoded oligos from RNA probes and/or antibodies
2. Oligos are aspirated with microcapillary and dispensed into 96 well plate
3. Each well of the plate contains oligos from the same ROI

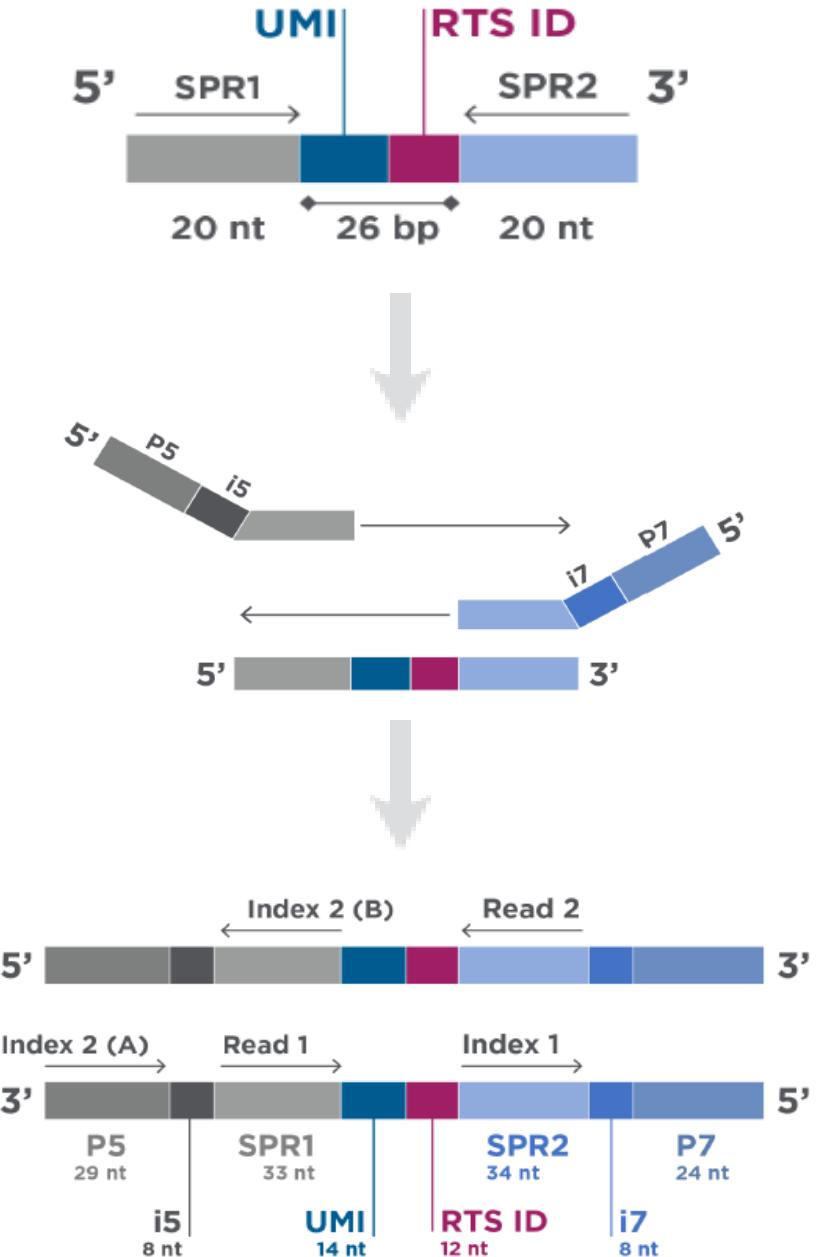
NGS LIBRARY PREPARATION



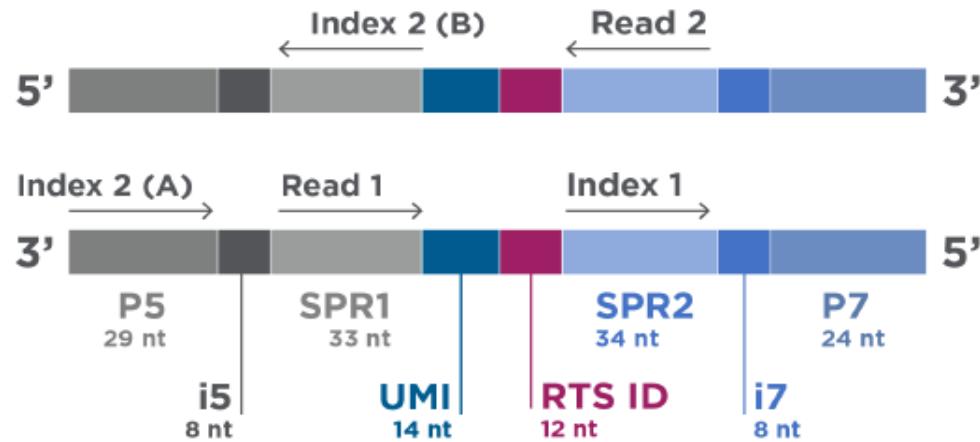
Photocleaved DNA oligos
collected into a plate

Index PCR on DNA oligos

PCR products purification
to get final libraries



GEOMX LIBRARY SEQUENCING



Nextseq 2000



Novaseq 6000



GEOMX libraries are sequenced paired-ends on high-throughput Illumina sequencers

Sequencing depth depends on assay type and size of ROI

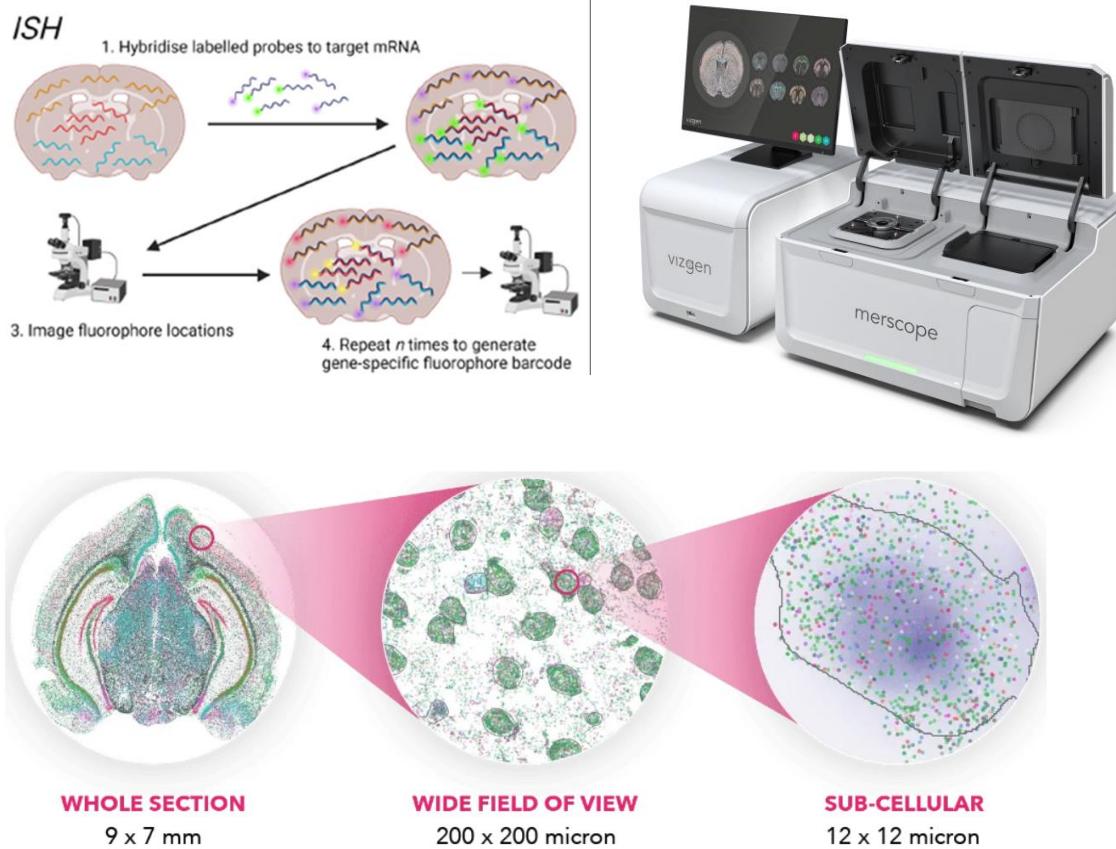
In WTA assay, it is recommended 100 reads per μm^2 of collection area

COMPARISON OF METHODS

	Visium FF	Visium HD-FFPE	GeoMx
Resolution	55µm (1-10 cells per spot)	8 µm (1 cell per spot)	50-100 cells (WTA)
Detection method	Capture poly(A) transcripts	Probe-based (18k protein-coding genes) also polyA	Probe-based (18k protein-coding genes + 570 proteins)
Sample per slide	4	2	Up to more than 4
Possible to add custom targets	no	no	Up to 50/60 custom probes
Possible to select ROI	No	No	yes
Sequencing depth	50k reads/spot	50k reads/spot	100 reads/µm ²

Technologies for spatial multi-omics analysis based on Imaging:

➤ MERSCOPE Platform (Vizgen)

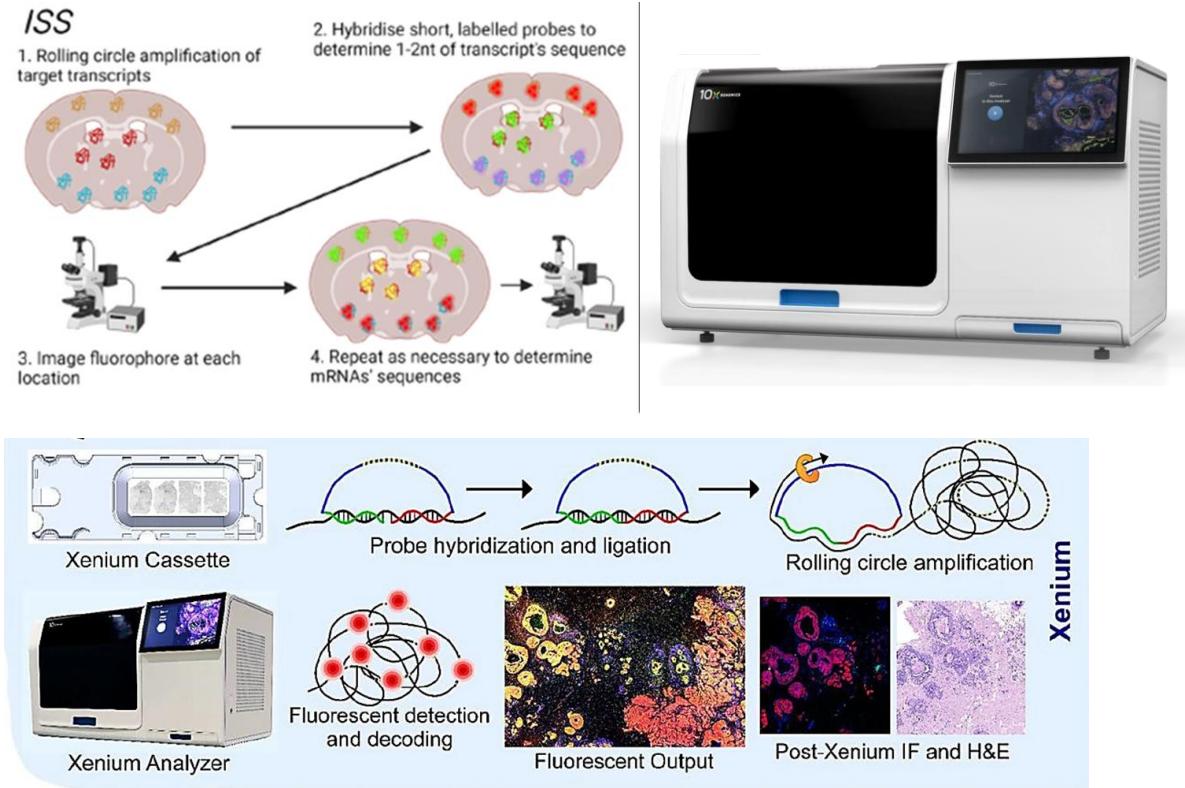


Resolution: < 100 nm

Gene panel : up to 500 -> 1000

Tissue types: Fresh Frozen and FFPE

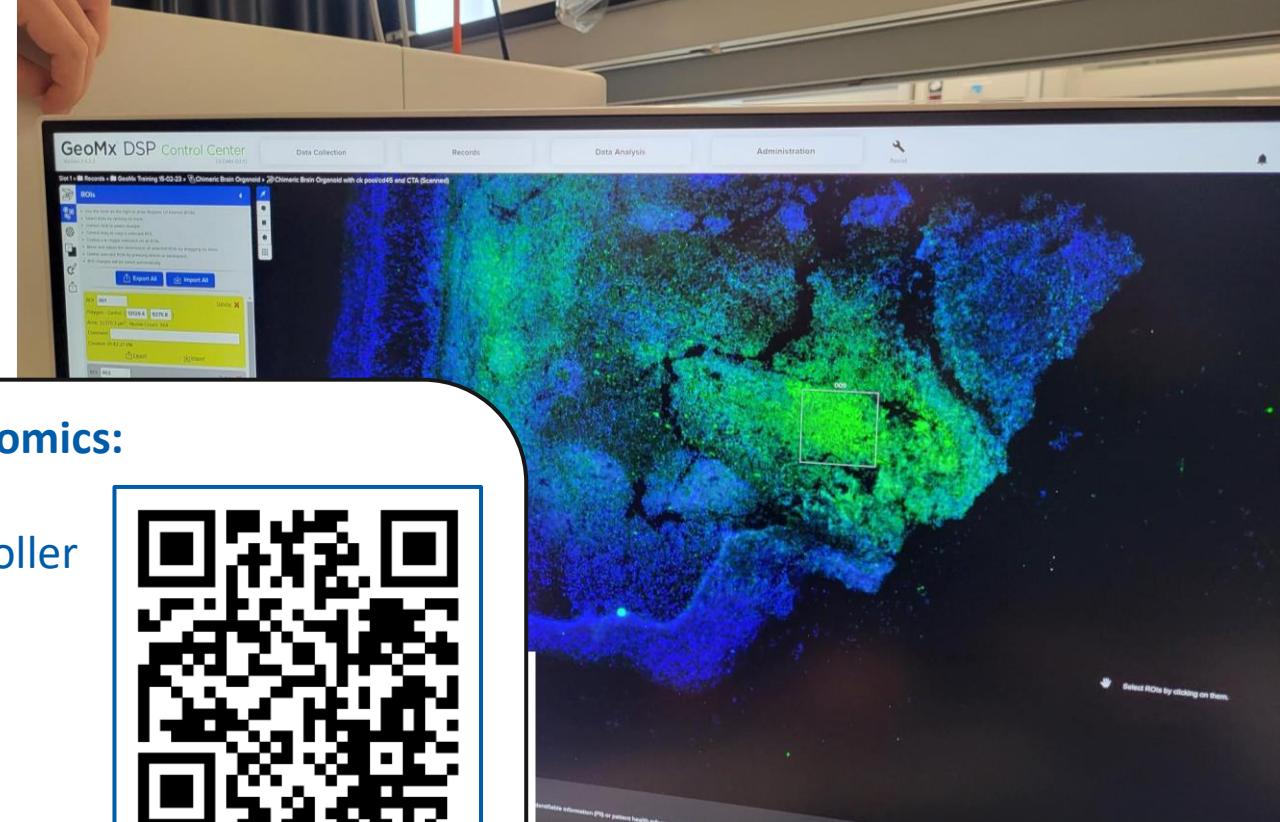
➤ Xenium Analyzer (10x Genomics)



Resolution: XY-localization precision < 30 nm
Z-localization precision < 100 nm

Gene panel : up to 5000 (predesigned +custom)

Tissue types: Fresh Frozen and FFPE



National Facility for Genomics:

10x Genomics:

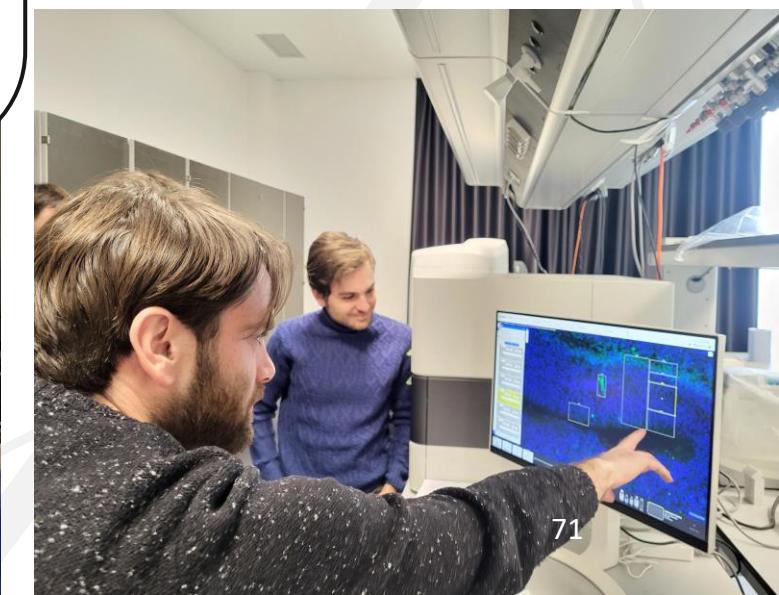
- Chromium controller
- Chromium X
- Visium Cytassist

BD:

BD Rhapsody

Nanostring:

- GeoMX Digital Spatial Profiler



Thank you!



We are Hiring!



Human Technopole

Bioinformatic Technician

Milan, Lombardy, Italy · 4 days ago · Over 100 applicants
Promoted by hirer · Actively reviewing applicants

 Hybrid · Full-time · Entry level

 Skills: Computational Biology, Genomics, +7 more

**Deadline
24 August**

Questions?