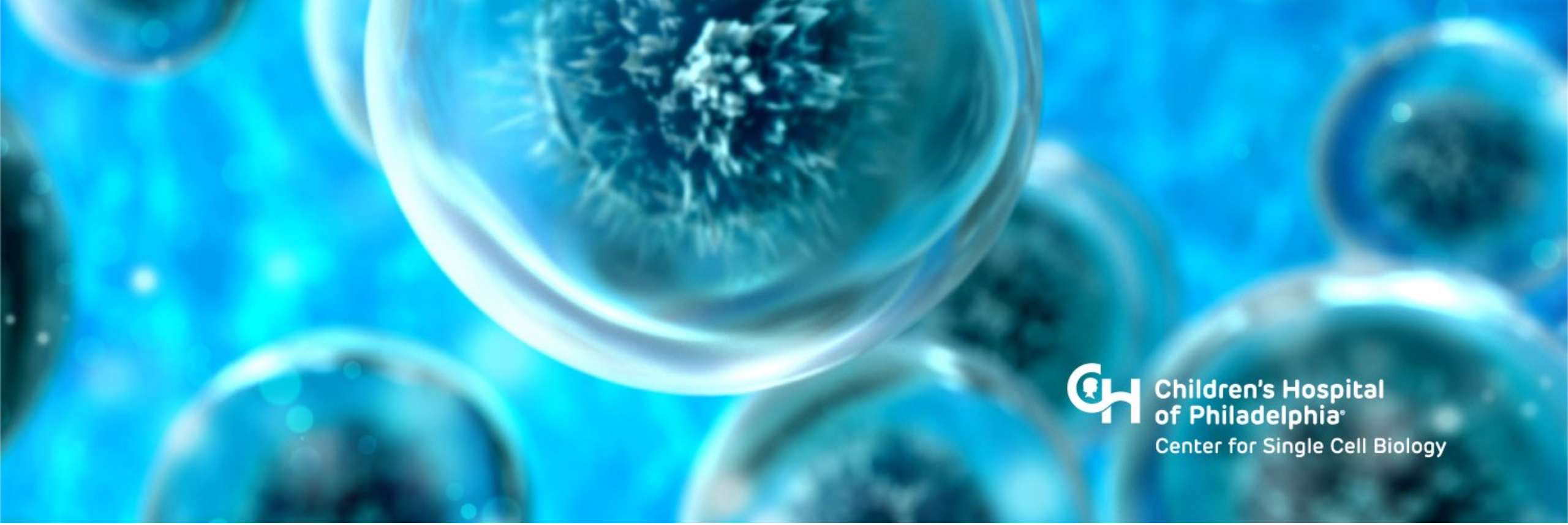
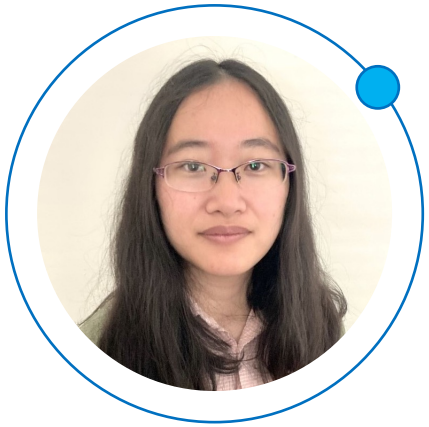


Advanced histology for spatial omics

Mei Zhang, PhD
Technical Director
Single Cell Technology Core
The Center for Single Cell Biology

Our Team

Children's Hospital of Philadelphia®
Center for Single Cell Biology



Xiaoxu Yang, MS

Research Assistant

Aliza Yousey, BS

Research Assistant

David Smith, PhD

Bioinformatics Scientist

Arta Seyedian, BS

Bioinformatics Scientist

Our Platforms

Children's Hospital of Philadelphia
Center for Single Cell Biology

10x Genomics Chromium Controller

10x Genomics Chromium X

10x Genomics Chromium Connect

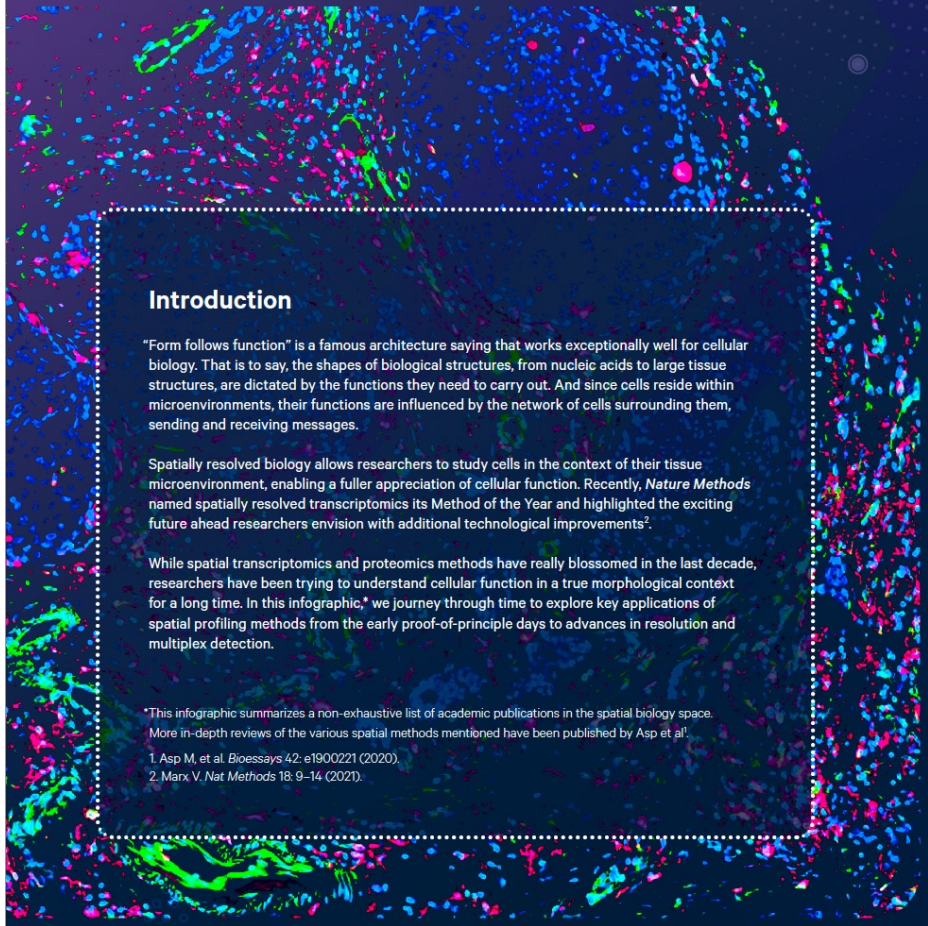
GeoMx Digital Spatial Profiler

Akoya PhenoCycler

Vizgen MERSCOPE

Milestones in spatial biology*

10x GENOMICS



Start

Introduction

"Form follows function" is a famous architecture saying that works exceptionally well for cellular biology. That is to say, the shapes of biological structures, from nucleic acids to large tissue structures, are dictated by the functions they need to carry out. And since cells reside within microenvironments, their functions are influenced by the network of cells surrounding them, sending and receiving messages.

Spatially resolved biology allows researchers to study cells in the context of their tissue microenvironment, enabling a fuller appreciation of cellular function. Recently, ***Nature Methods*** named spatially resolved transcriptomics its Method of the Year and highlighted the exciting future ahead researchers envision with additional technological improvements[2].

While spatial transcriptomics and proteomics methods have really blossomed in the last decade, researchers have been trying to understand cellular function in a true morphological context for a long time. In this infographic,* we journey through time to explore key applications of spatial profiling methods from the early proof-of-principle days to advances in resolution and multiplex detection.

*This infographic summarizes a non-exhaustive list of academic publications in the spatial biology space. More in-depth reviews of the various spatial methods mentioned have been published by Asp et al[1].

1. Asp M, et al. *Bioessays* 42: e1900221 (2020).
2. Marx V. *Nat Methods* 18: 9–14 (2021).

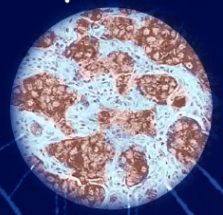
The Early Days

1941

Immunohistochemistry (IHC)

Coons et al. demonstrated the use of a fluorescently labeled antibody for visualizing protein targets in tissues[3]

3. Coons AH, et al. *Proc Soc Exp Biol Med* 47: 200–202 (1941).

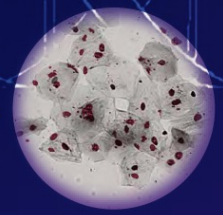


1969

DNA in situ hybridization (ISH)

Pardue et al. reported a DNA-DNA hybridization method using radioactive labeling for detecting the cellular localization of DNA sequences[4]

4. Pardue ML, et al. *PNAS* 64: 600–604 (1969).



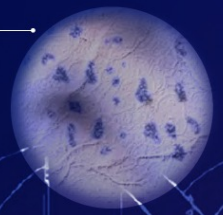
The Resolution Revolution

1990–2009

RNA ISH

Singer et al. described an in situ hybridization method for mapping mRNA species while maintaining the morphology of analyzed cells[5]

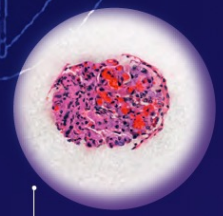
5. Singer RH, et al. *PNAS* 79: 7331–7335, (1992).



Laser capture microdissection

Emmert-Buck et al. published a method for visualizing and dissecting sections of cells microscopically from tissue samples for downstream profiling[6]

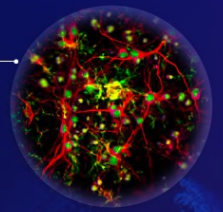
6. Emmert-Buck MR, et al. *Science* 274: 998–1001 (1996).



Tyramide signal amplification–based multiplexed IHC

Wang et al. showed the application of TSA to immunofluorescence confocal laser microscopy for visualization of protein localization[7]

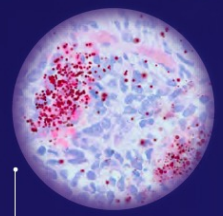
7. Wang G, et al. *Methods* 18: 459–64 (1999).



Branched DNA ISH

Player et al. presented the use of branched DNA signal amplification for detecting low-copy human papillomavirus DNA in subcellular compartments[8]

8. Player AN, et al. *J Histochem Cytochem* 49: 603–12 (2001).



Next

The Resolution Revolution

1990–2009

Hybridization chain reaction

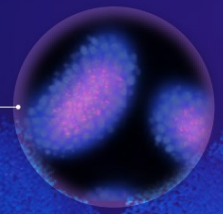
Dirks et al. described the use of nucleic acid probes as signal amplifiers for biosensing applications[9]

9. Dirks RM and Pierce NA. *PNAS* 101: 15275–15278 (2004).

Single molecule ISH (smISH)

Raj et al. demonstrated a method for multiplex gene expression profiling using singly labeled oligonucleotide probes[10]

10. Raj A, et al. *Nat Methods* 5: 877–879 (2008).



Fluorescence ISS (FISSEQ)

Lee et al. described the use of fluorescence in situ RNA sequencing for highly multiplexed subcellular RNA analysis[12]

12. Lee JH, et al. *Science* 343: 1360–1363 (2014).

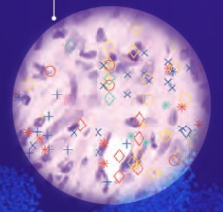
The Multiplex Expansion

2010–2021

In situ sequencing (ISS)

Ke et al. reported the application of sequencing for the multiplex detection of single mRNA molecules[11]

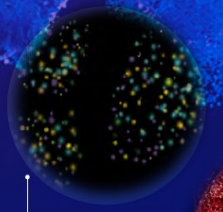
11. Ke R, et al. *Nat Methods* 10: 857–60 (2013).



seqFISH

Lubeck et al. showed a multiplexed mRNA detection method employing a sequencing barcoding strategy[14]

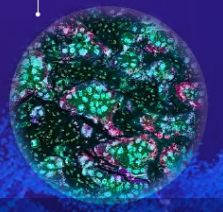
14. Lubeck E, et al. *Nat Methods* 11: 360–361 (2014).



Multiplexed Ion beam Imaging

Angelo et al. published a multiplexed IHC method to image proteins targets bound to metal-tagged antibodies via secondary ion mass spectrometry[13]

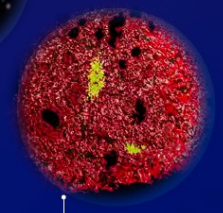
13. Angelo M, et al. *Nat Med* 20: 436–42 (2014).



Spatially encoded assays

Chee et al. demonstrated highly multiplexed spatial addressing of mRNA in fixed tissue samples[15]

15. Chee MS, et al. Poster #1682T. ASHG Annual Meeting (2014).

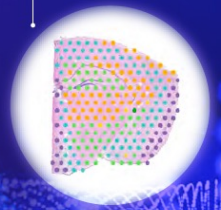


Next

Spatial transcriptomics (ST)

Stahl et al. demonstrated the visualization of tissue sections and quantification of their transcriptomes using unique positional barcodes[17]

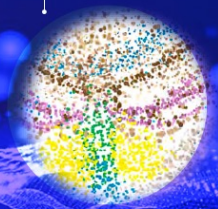
17. Stahl PL, et al. *Science* 353: 78–82 (2016).

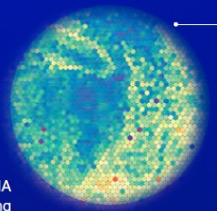


Slide-seq

Rodriques et al. reported a method for inferring RNA localization via sequencing of RNA transferred from tissue sections[19]

19. Rodriques SG, et al. *Science* 363: 1463–1467 (2019).





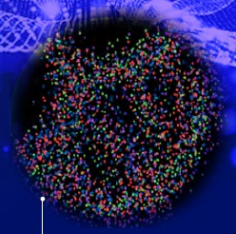
Visium Spatial Gene Expression

Ji et al. showed the application of Visium spatial whole transcriptome profiling to detail the spatial organization of tumor cell populations[21]

21. Ji AL, et al. *Cell* 182: 497–514.e22 (2020).

The Multiplex Expansion

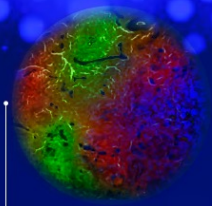
2010–2021



MERFISH

Chen et al. presented a specific error-tolerant approach to multi-plexed single-molecule counting and mapping of mRNAs in single cells[16]

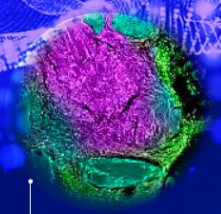
16. Chen HK, et al. *Science* 348: aaa6090 (2015).



Cyclic multiplex immunofluorescence

Goltsev et al. published an antigen staining method for cytometric multiplexed imaging of protein targets in single cells and tissue sections[18]

18. Goltsev Y, et al. *Cell* 174: 968–981 (2018).

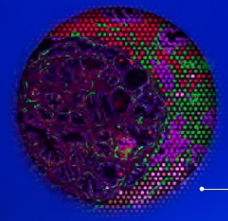


Digital spatial profiling

Amaria et al. published the use of digital spatial profiling for multiplexed IHC of immune markers[20]

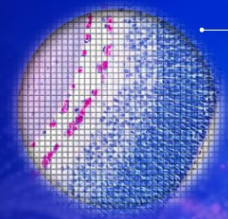
20. Amaria RN, et al. *Nat Med* 24: 1649–1654 (2018).
Erratum in: *Nat Med.* 2018 Oct 25.

Next



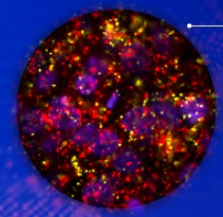
Visium Spatial Gene and Protein Expression

Will add highly multiplexed protein detection to Visium whole transcriptome profiling of tissue sections



Visium HD

Will bring single cell scale resolution to Visium spatial profiling



Xenium In Situ

Will allow precise spatial mapping of RNA and protein with a subcellular readout

The Next Frontier

2022 and beyond

Trailblazing the future of spatial biology

Spatially resolved biology, including whole transcriptomic and targeted in situ methods, allows scientists to build a more complete view of cellular function in the tissue context. Visium from 10x Genomics is a spatial discovery platform that allows whole transcriptome profiling of fresh and formalin-fixed paraffin-embedded (FFPE) tissues. And Xenium, our new in situ platform, provides the highest spatial resolution with targeted gene and protein detection, enabling translational and, ultimately, clinical applications. See biology in new ways with the most comprehensive spatial resolution and scale.

LIT000167 - Rev A - Poster - Visium | FFPE Infographic

10x GENOMICS

Spatial Genomics

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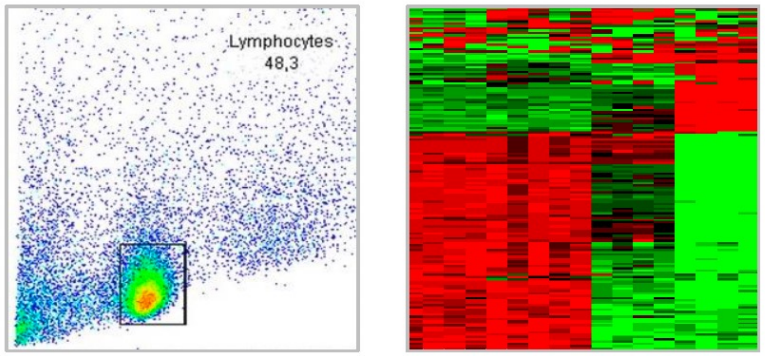
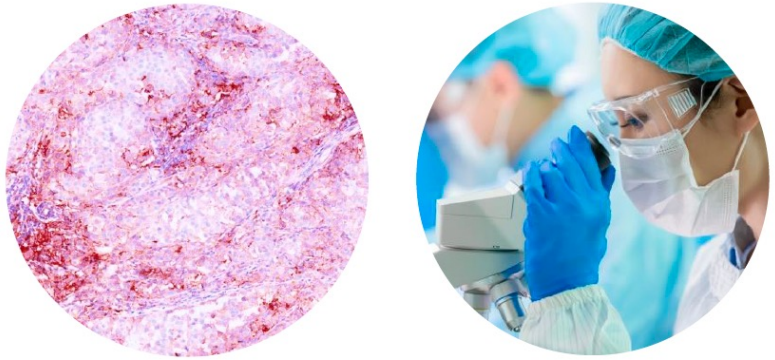
Genomics and Proteomics



- Quantitative, limited visualization
- Tissue is destroyed
- Loss of tissue architecture to analyze cellular distribution (loss of spatial context)

Pathology – Conventional IHC



- Visual assessment remains the gold standard for diagnosis
- Results are limited, qualitative and subjective
- Complexity of the TME: not able to reveal

Source: AkoyaBio

Spatial Genomics

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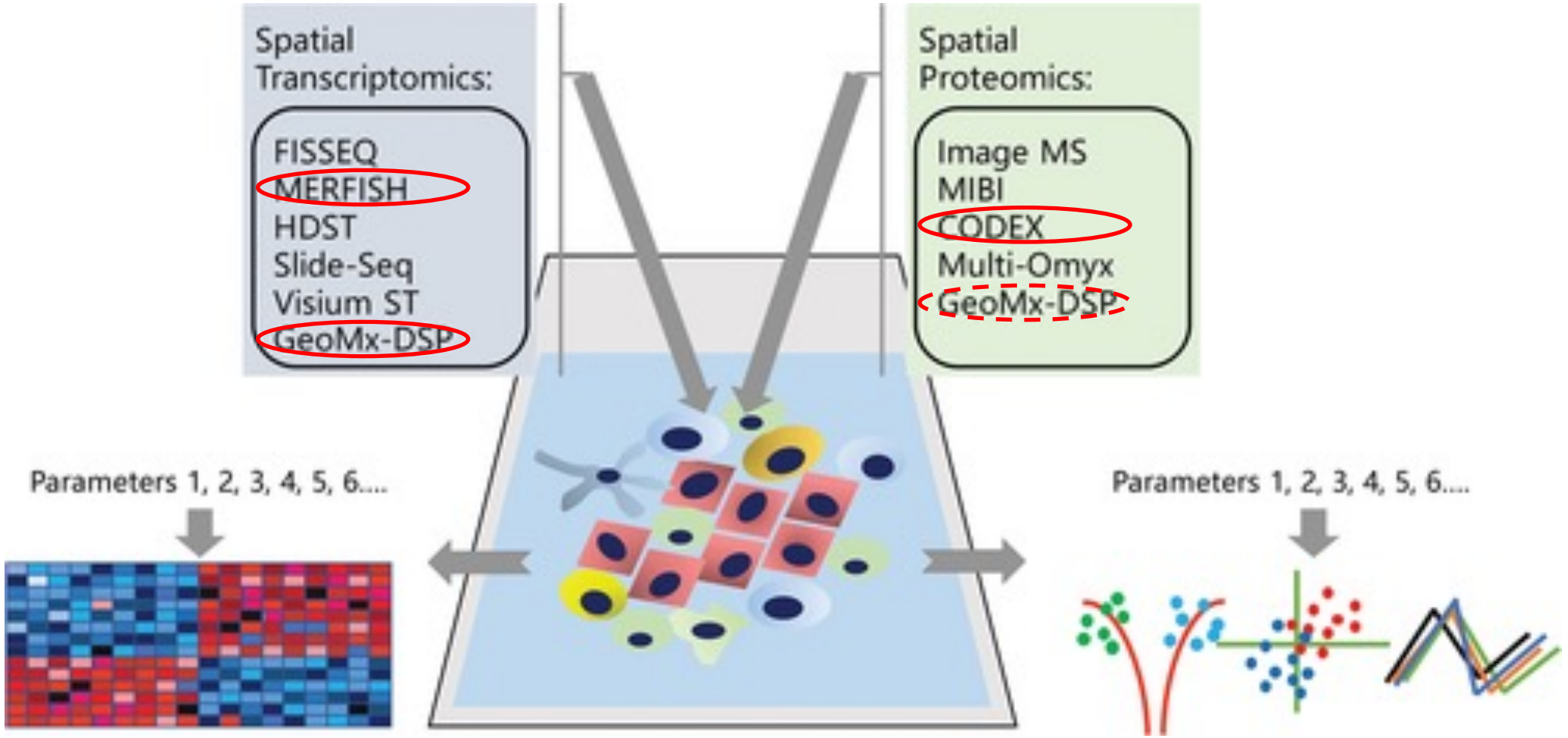

Wang, N. et al, Biotechnol J, 2021 Sept

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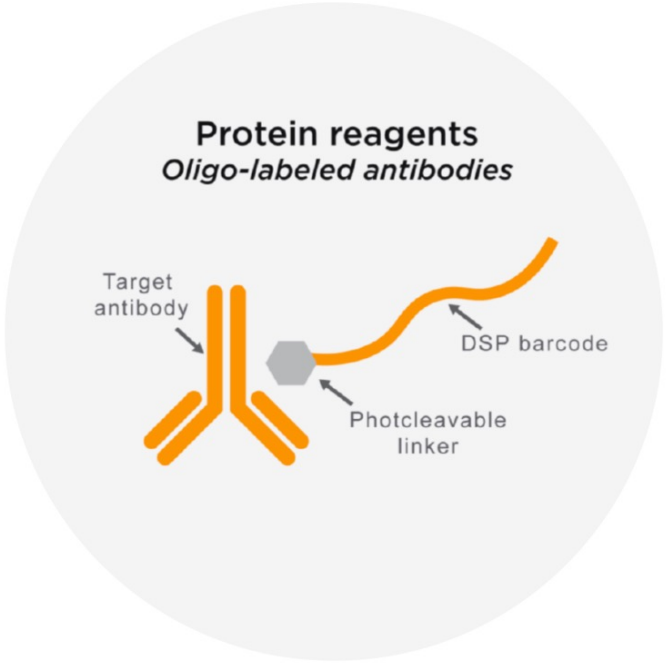
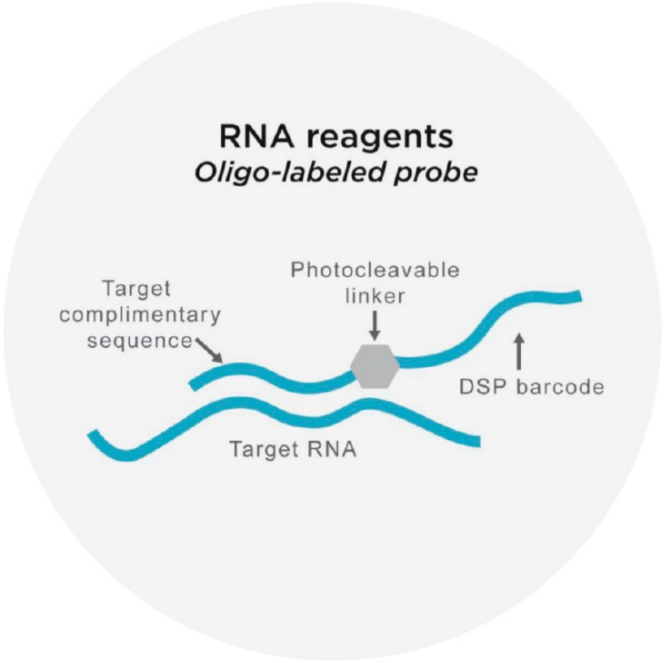
GeoMx Workflow

High-Plex Mixtures of Proprietary Reagents





GeoMx DSP technology

Source: nanoString

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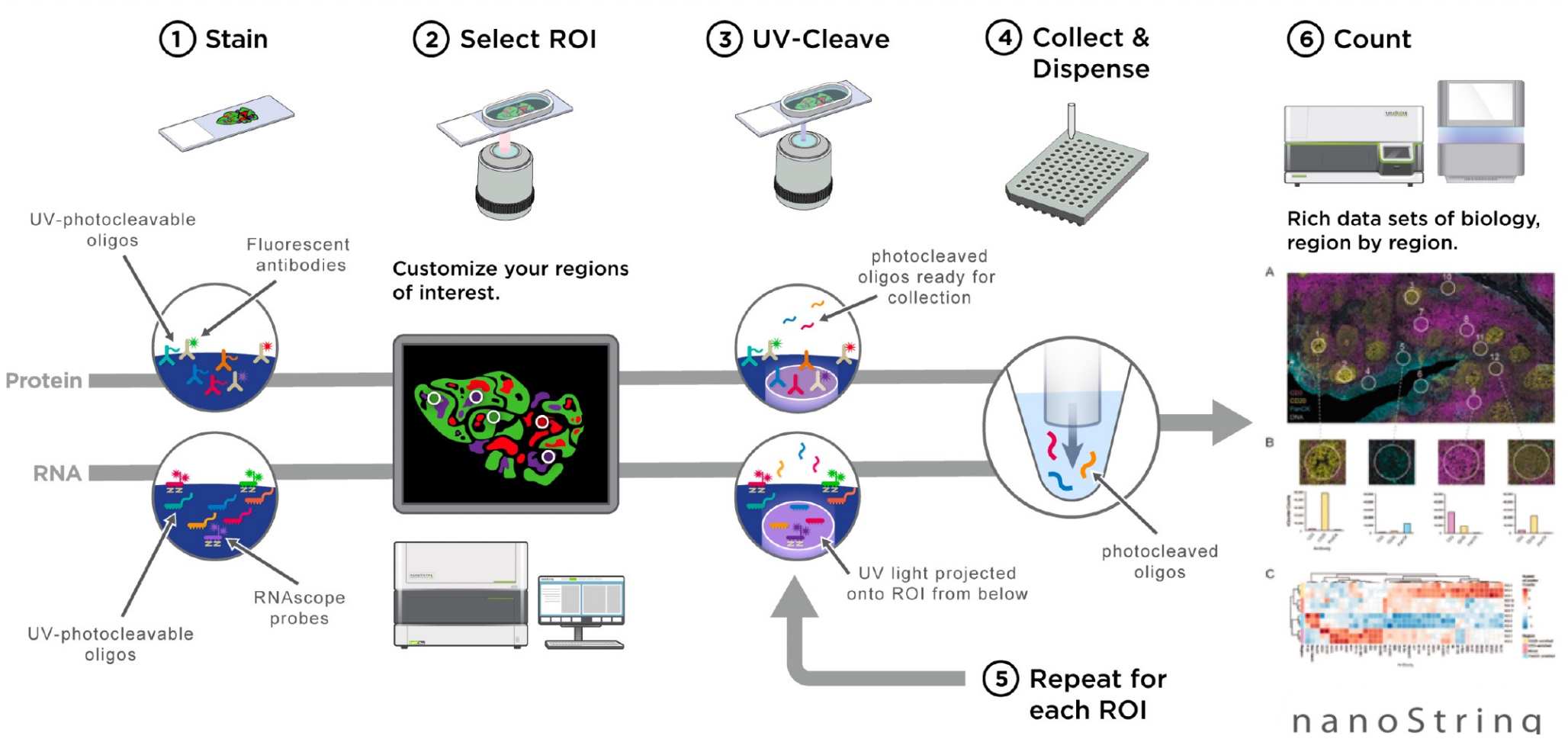
GeoMx Workflow



Source: nanoString

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Morphology Marker Guidelines

- up to 3 fluorescently labeled antibodies + 1 nuclear stain
- guide ROI selection and enable segmentation of specific compartments and cell types

ROI/AOI strategy

The biological questions ultimately help to define the right morphology markers to use and the best profiling selection strategy

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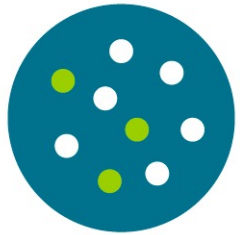
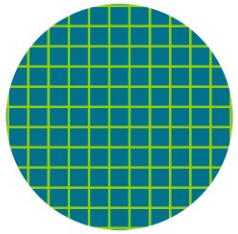
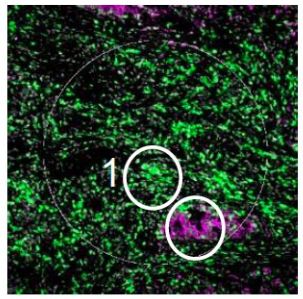
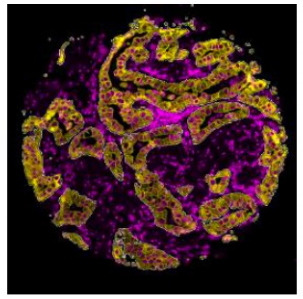
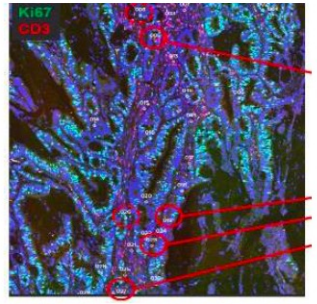
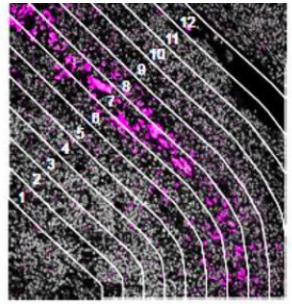
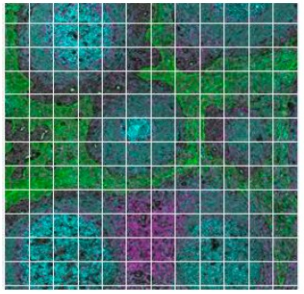
ROI/AOI selection strategies

Geometric	Segmentation	Rare cell	Contour	Gridded
				
				
What is the heterogeneity of expression in different regions of my tissue?	What is the expression profile of distinct biological compartments (e.g., Tumor-TME)?	What is the expression profile of a specific cell population in my tissue?	How does the immune environment change on either side of an infiltrate boundary?	What novel targets are uncovered with deep mapping of a specific tissue region?

50-100 cells are required for each collection to get a good signal over background noise

Source: nanoString

nanoString GeoMx Digital Spatial Profiler

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Supported Assays

Whole Transcriptome Atlas with NGS readout

- ~18,000 human genes
- ~20,000 mouse genes

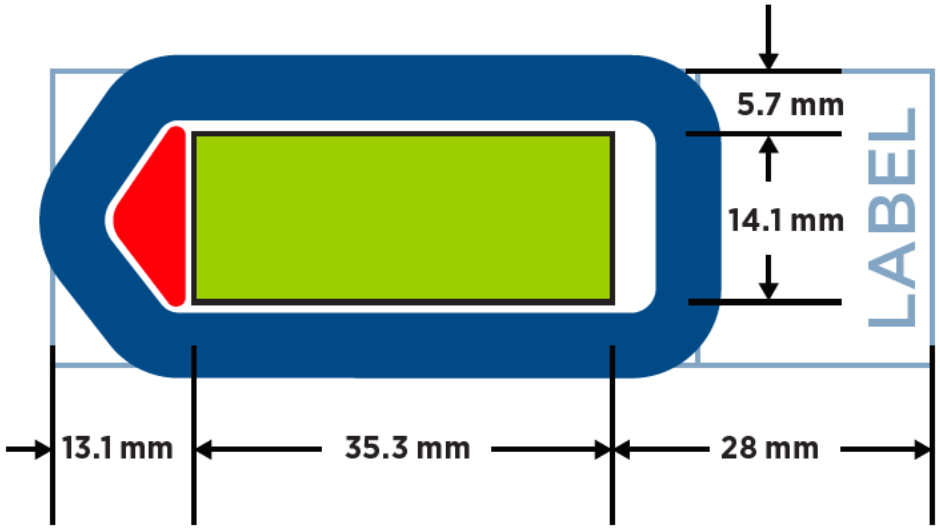
Sample Requirements/slide prep

FFPE, Fresh or Fixed Frozen tissue

Selecting and Sectioning FFPE Samples

- Immediately after excision (up to 1hr), tissues (<0.5 cm in thickness) should be fixed in 10% NBF for 18 to 24 hours at room temperature
- Avoid acidic decalcification; EDTA-based decalcification or special decalcification solutions can be used
- For best results, do not use FFPE blocks that are greater than 10 years old

- Fisherbrand SuperFrost™ Plus slides or Apex BOND® slides
- 5 µm thickness
- Scan area: 35.3mm x 14.1mm
- Multiple sections can be mounted on the same slide, at least 2-3 mm apart
- Slides stored in a desiccator at 4°C yield quality results for up to 2 weeks.



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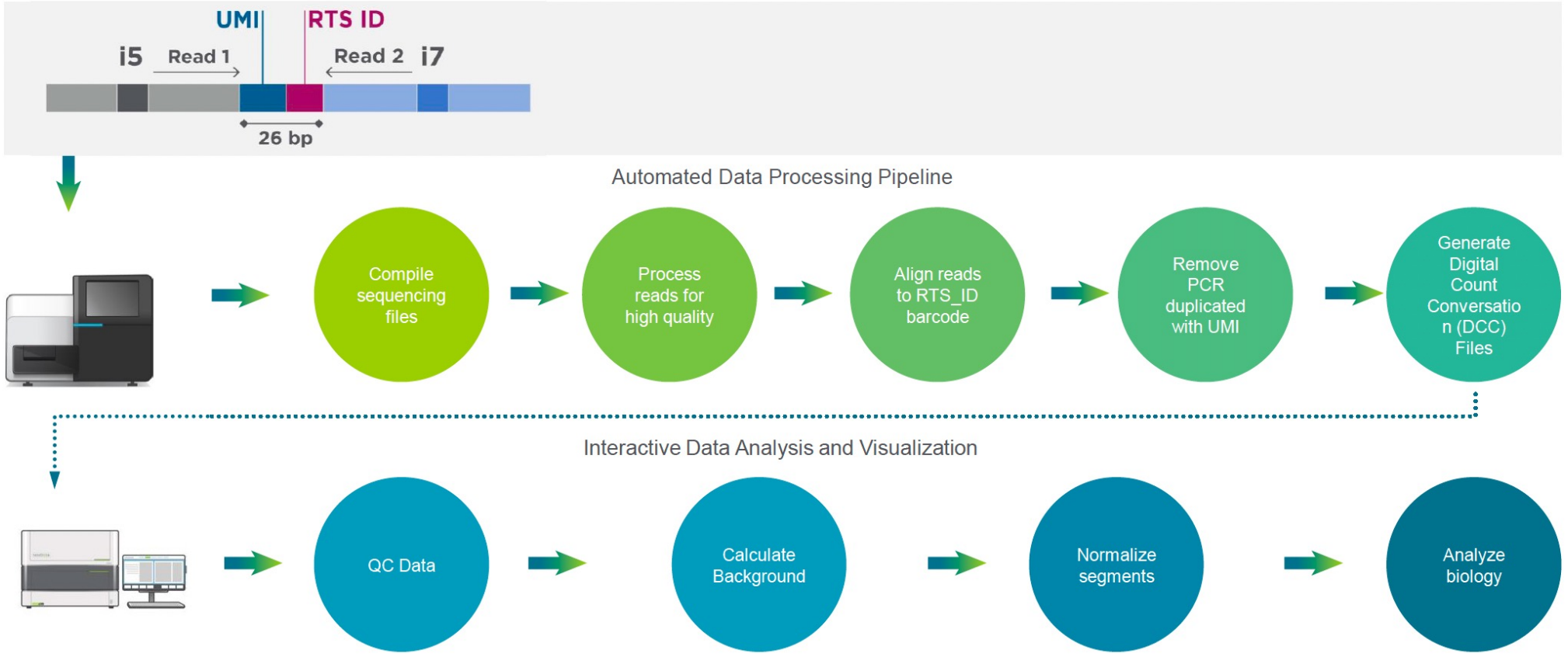
Complete NGS data processing and analysis capabilities



Source: nanoString

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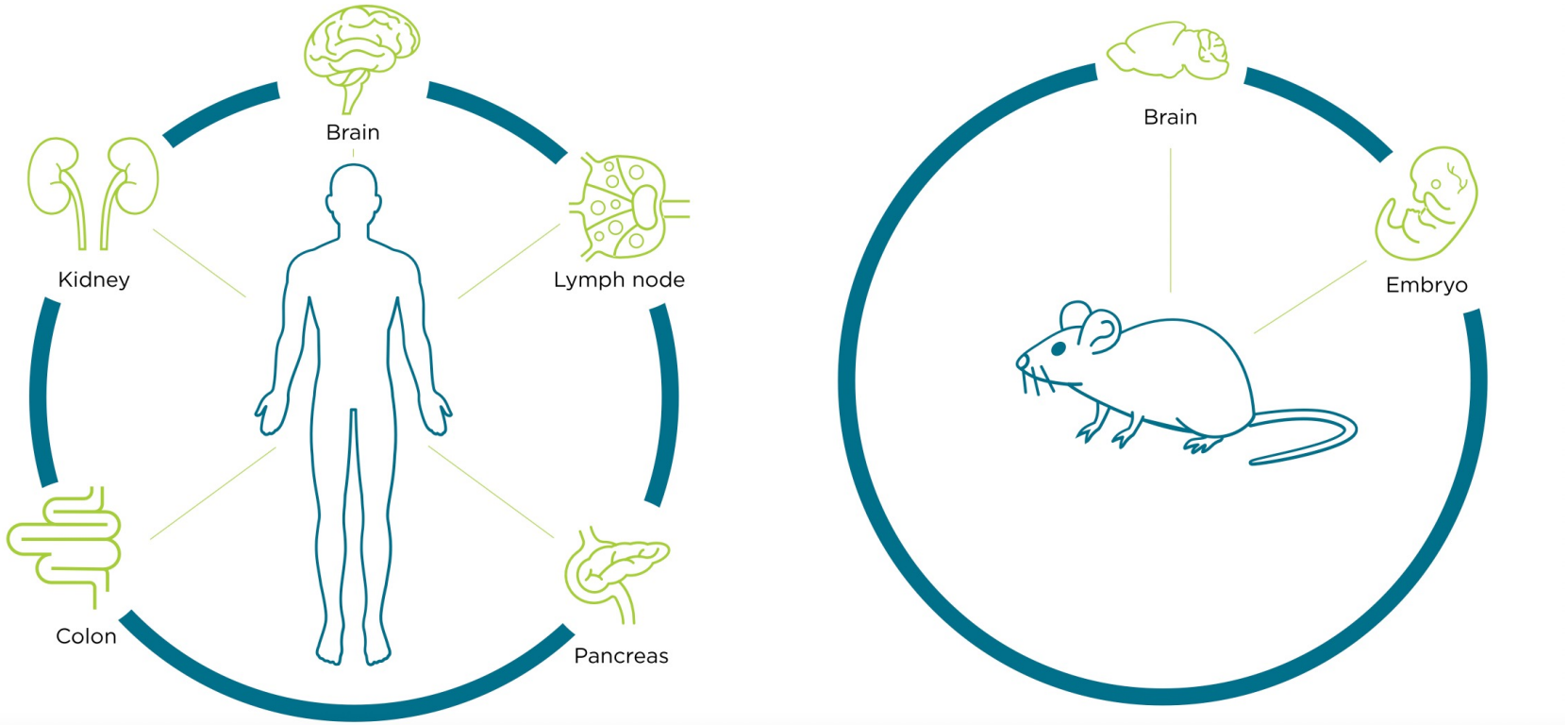
Spatial Organ Atlas



Source: nanoString

Akoya PhenoCycler (CODEX)

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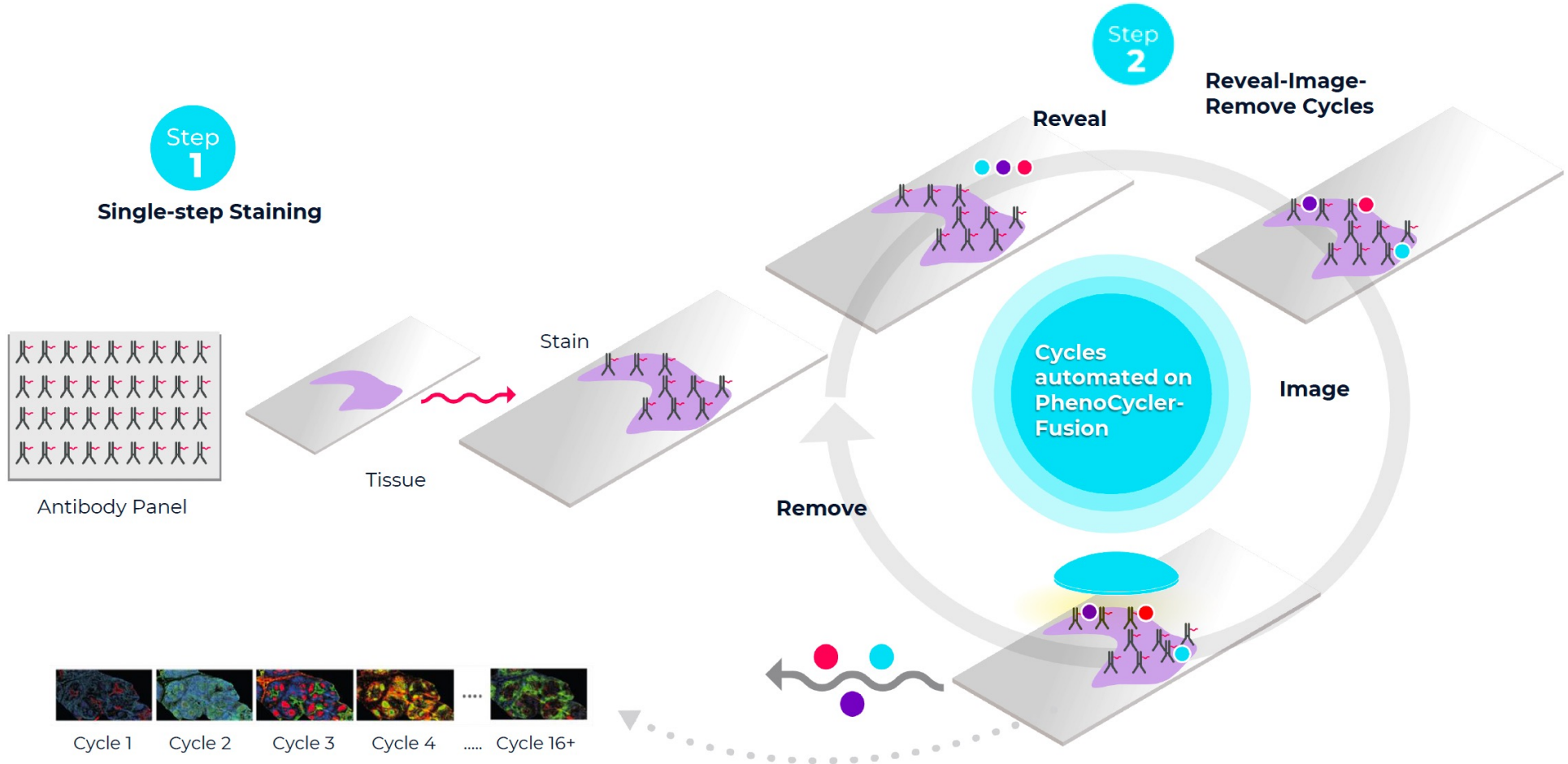
PhenoCycler Technology: Ultrahigh plex Imaging



Source: AkoyaBio

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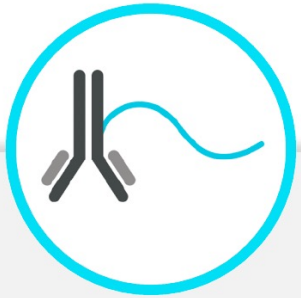
PhenoCycler Reagents and Consumables

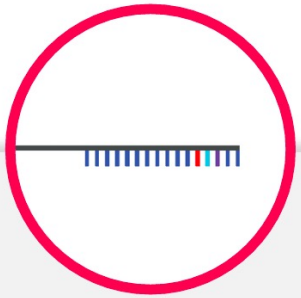
Staining Kit

PhenoCycler-Fusion Staining Kit for tissue staining and running iterative imaging (Processes 10 samples per kit)



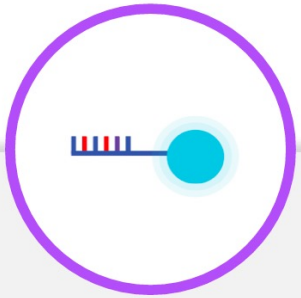
Antibodies

Conjugated antibodies for building antibody panels



Barcodes

Modified oligonucleotides for easy conjugation of custom antibodies



Reporters

Fluorophore conjugated oligonucleotides for visualization of custom antibodies

Source: AkoyaBio

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Oligo conjugated antibodies

- Akoya inventoried antibodies – ready to use
- Custom antibody conjugation and validation
- Akoya STEP panels – available for purchase as a core panel and additional modules

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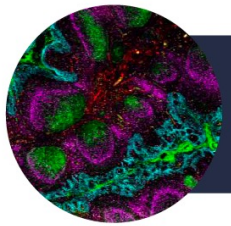
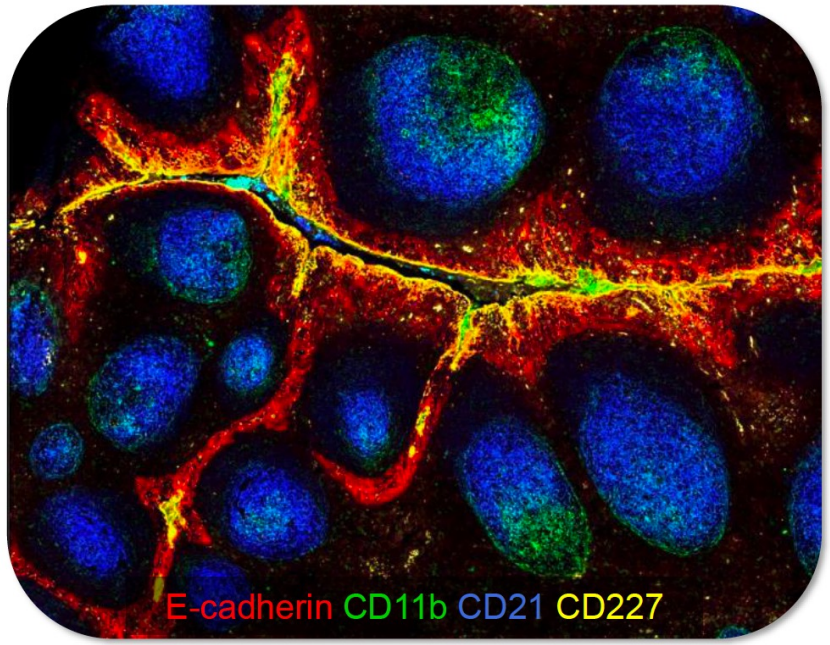


Immune Profiling Modules for Human FFPE

STEP Core Panel (15)	
CD4*	Helper T cells
CD68*	Macrophages
CD20*	B cells
CD11c*	Dendritic cells
CD8*	Cytotoxic T cells
HLA-DR*	APCs (MHC II)
CD3e*	T cells
CD44*	Activated T cells
CD45*	Immune cells
HLA-A	MHC I
CD14	Monocytes
Ki67*	Proliferating cells
Pan-CK*	Tumor cells
CD57	NK cells
CD45RO*	Memory T cells

Advanced Immune Module (6)	
CD163	M2 Macrophages
CD19	B-cells, FDCs
FoxP3	Regulatory T cells
Granzyme B	Activated T cells/NK cells
CD11b	Myeloid cells
CD21*	Dendritic cells, mature B cells, FDCs

Structural Module (7)	
E-cadherin*	Epithelial cells
SMA	Vascular, Fibroblasts, Smooth muscle
Vimentin	Cyto-structures
Collagen IV	Extracellular matrix
CD31*, CD34	Vascular structures
Podoplanin*	Lymphatics



COVID-19 Module (3)
SARS-CoV Spike, SARS-CoV Nucleocapsid, ACE2

Immune Activation Module (6)	
PD-1, LAG-3, TIM3, ICOS	Checkpoint receptor
PD-L1	Checkpoint ligand
IDO1	Multifunctional/Immune inhibitory

Breast Tissue Module (8)
Tp63, Keratin 5, Keratin 8, Keratin 14*, Keratin 19, ER, PR, HER2

Source: AkoyaBio

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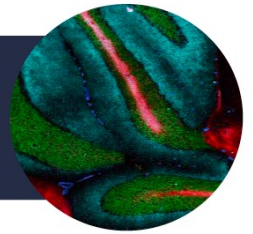
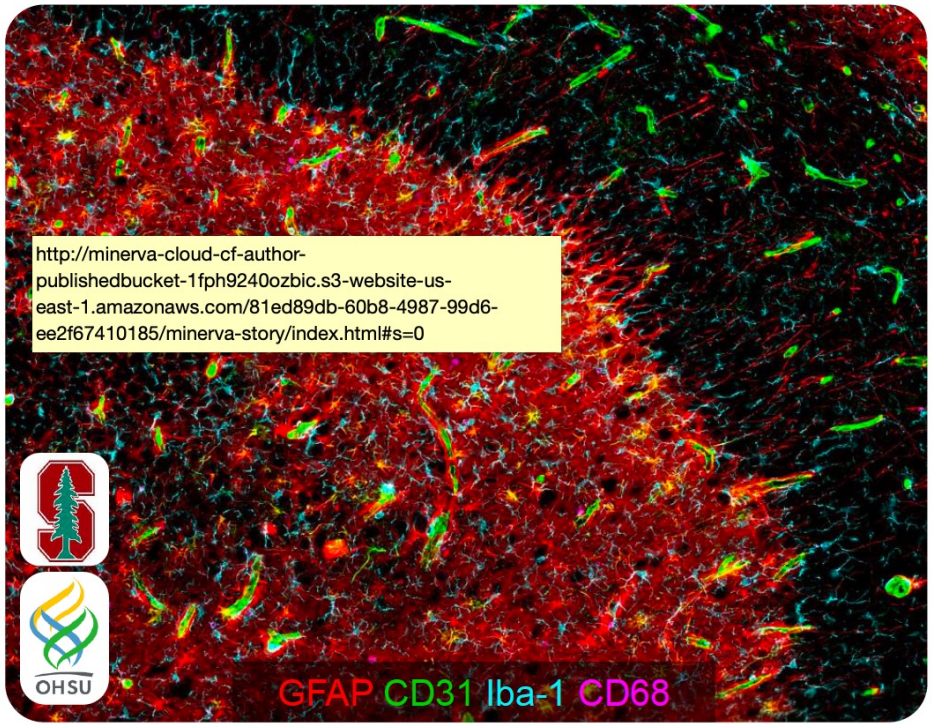
STEP for Neuro (Human FFPE)



Neuroinflammation Module (12)	
TMEM119	Microglia
AQP4	Blood-brain barrier
CD68*	Microglia, M1 Microglia
CD11c*	Microglia
CD163	M2 Microglia
Ki67*	Proliferating cells
PCNA*	Proliferating cells
Galectin-3*	Microglial activation
ApoE	Astrocytes/Microglia
β-Amyloid	β-amyloid peptide (Aβ)
Trem2	Microglia, Alzheimer's
iNOS	M1 microglia

Immune Module (9)	
CD8*	Cytotoxic T cells
HLA-DR*	APCs (MHC II)
CD3e*	T cells
CD44*	Activated T cells
CD45*	Immune cells
CD11b	Myeloid cells
CD4*	Helper T cells
CD14	Monocytes
HLA-A	MHC I

Neuroscience Core Panel (14)	
NeuN	Post-mitotic neurons
MAP-2	Neurons (dendrites)
H2A.X	Neurons
Neurofilament	Neurons
GFAP	Astrocytes
Iba-1	Microglia
Olig-2	Oligodendrocytes
Vimentin	Astrocytes, NSCs
CD31*	Vascular
Collagen IV	Vascular
Claudin-5	Vascular
CD34	Vascular
PSD-95	Synaptic
Synaptophysin	Synaptic



Source: AkoyaBio

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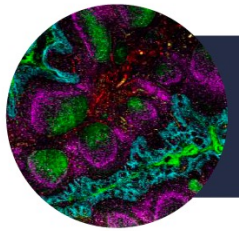
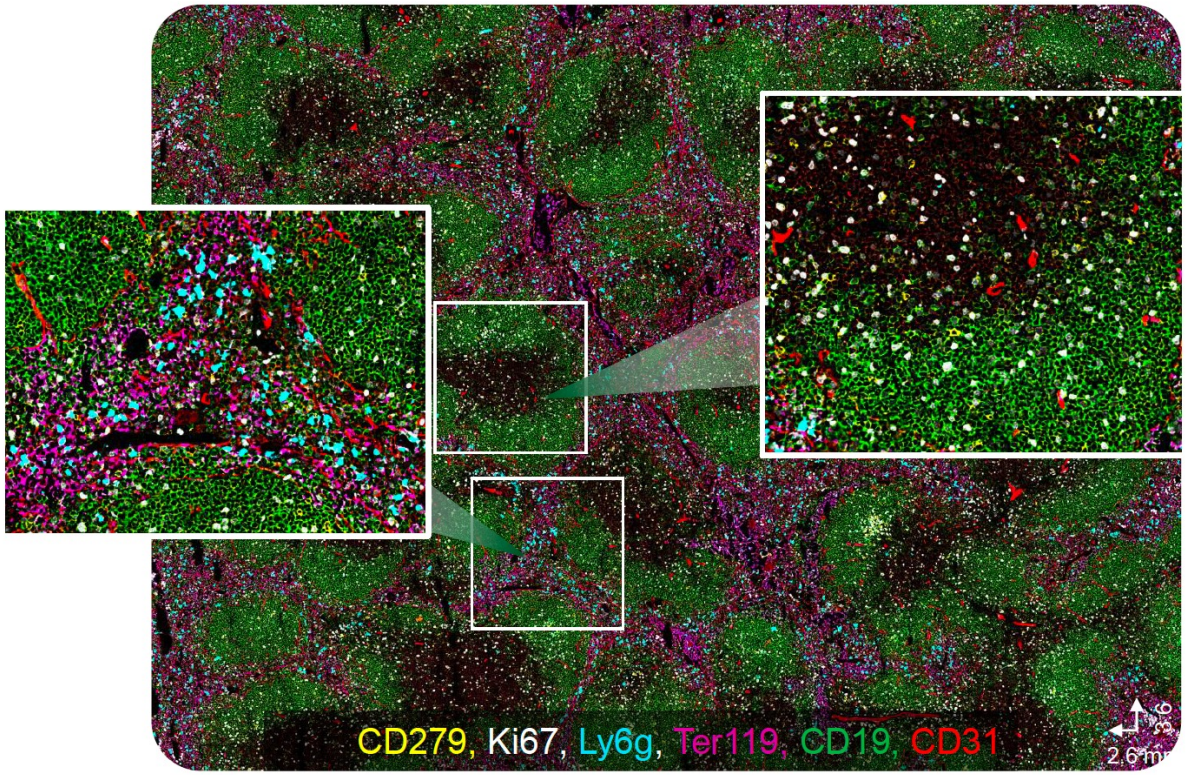


Immune Profiling for Mouse FF



STEP Core Mouse FF Panel (25)	
CD90*	HSCs, T cells, fibroblasts, vascular endothelial cells
CD31*	Vascular structures
TCR*	T cells
Ter119*	Erythrocytes
CD44*	Activated T cells
CD45*	Immune cells
CD19*	B-cells, FDCs
CD169*	Macrophages
CD45R/B220*	B cells
MHCII*	APCs
CD3*	T cells
IgM*	Immature B cells
CD5*	T cells
Ly6g*	Neutrophils
CD38*	NK cells, monocytes, activated B cells/T cells
CD21/35*	Mature B cells, FDCs
CD71*	Bone marrow blast cells
IgD*	Naïve B cells
CD4*	Helper T cells
CD11c*	Dendritic cells
CD24*	Dendritic cells
CD8a*	Cytotoxic T cells
CD49f*	Endothelial cells
CD11b*	Myeloid cells
Ki67*	Proliferating cells

Source: AkoyaBio

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Tissue samples requirement/coverslip preparation

- FFPE or Fresh Frozen
- Coverslips (22mm x 22 mm, Akoyabio) coated with poly-L-lysine
- Tissue sectioning: 5-10 µm (FF 8-10 µm, FFPE 4-5 µm); up to 15mm x 15mm
- Tissues sectioned onto poly-L-lysine-coated coverslips can be stored for up to six months

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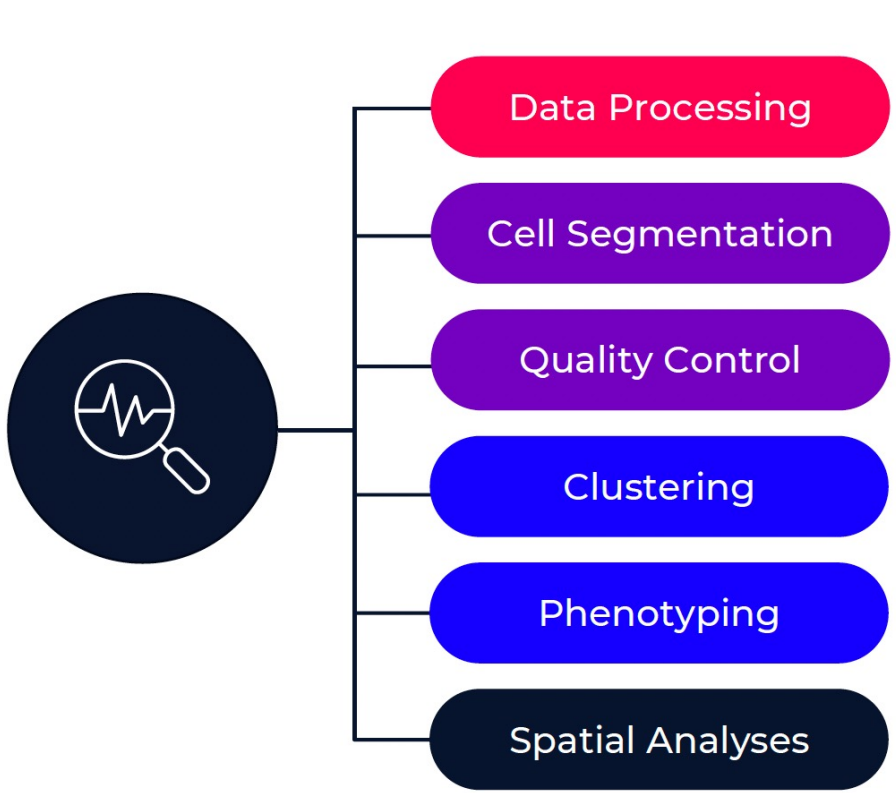
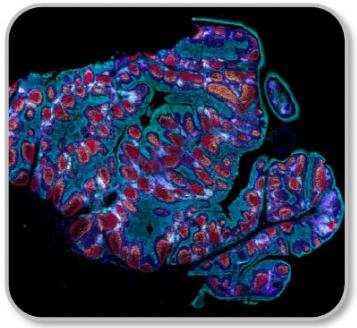
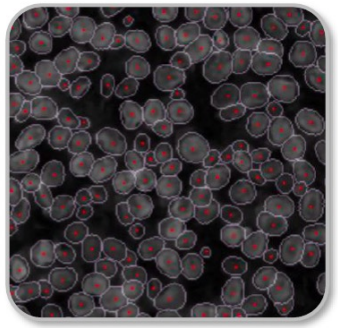
From Images to Phenotypes to Neighborhoods

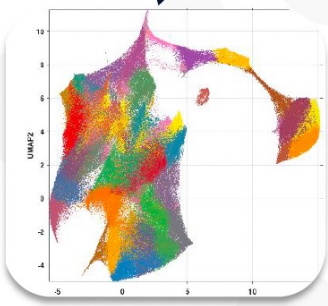




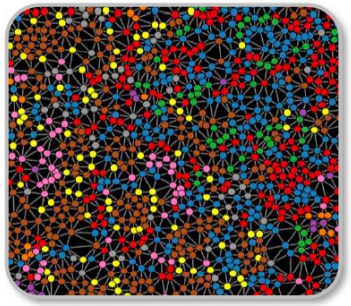
Top Quality Imaging Data



Segmentation & Quality Control



Clustering and Phenotyping



Cellular Neighborhoods

Source: AkoyaBio

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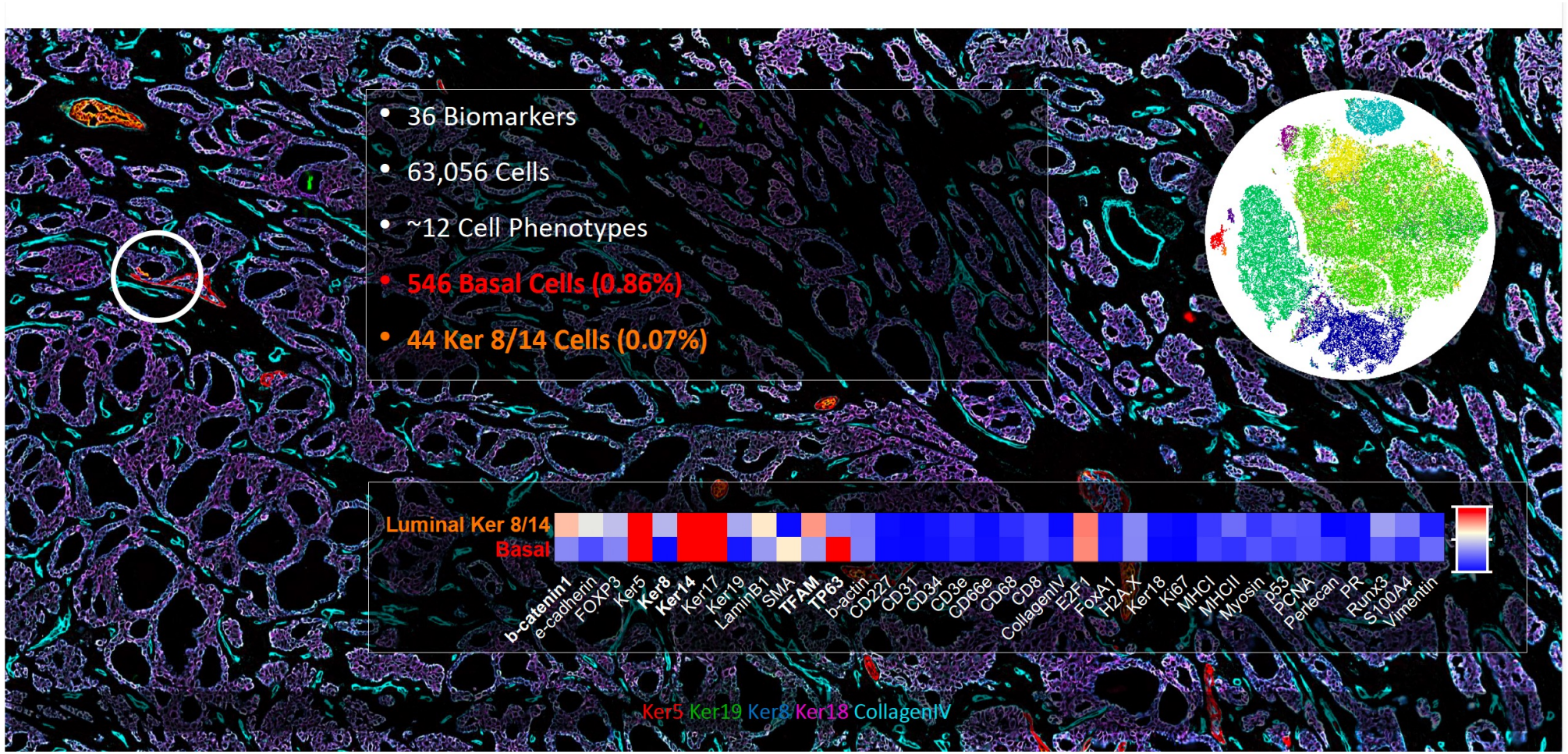
High-plex (up to 40+ biomarkers) whole-slide imaging at single-cell & sub-cellular resolution



Source: AkoyaBio

Vizgen MERSCOPE

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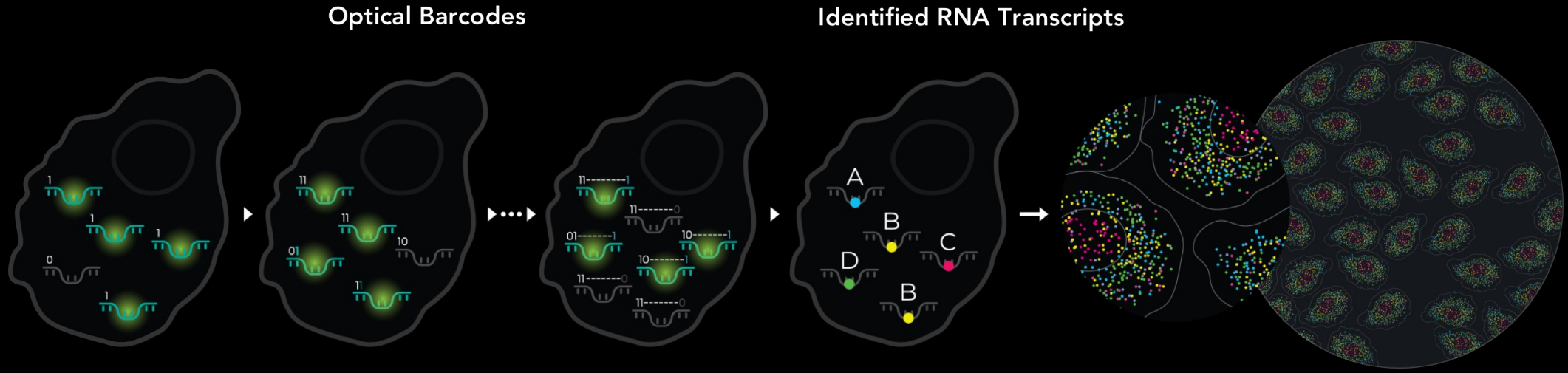
Spatial Transcriptomics with Single cell and Subcellular Resolution

MERFISH Technology



Source: Vizgen

Vizgen MERSCOPE

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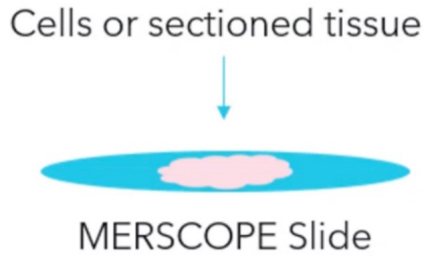
Sample preparation guidelines

- **Sample types:**
  - **Fresh Frozen**: harvest, snap-freeze, cut (Fix in 4% PFA only 15' prior to permeabilization)
  - **Fixed Frozen**: harvest, fix 4%PFA 6-12h, 30% sucrose, snap-freeze, cut
  - **FFPE** coming soon!
  - **Cell culture** (adherent and suspension)
  - **Maximum Size:** 1cm^3 for uniform freezing
  - **Check RNA quality**: RIN>7: Ideal; RIN 5-7: Detection Efficiency Diminishes; RIN<5: DO NOT Proceed
- **Cutting step:**
  - Fresh Frozen or Fixed Frozen tissue **embedded in OCT**
  - **RNAase Zap & 70% EtOH**: Clean glass slides, cryostat area & brushes
  - Equilibrate frozen tissue to -20 °C for at least 30 min
  - **Stage Angle**: 5 degrees; **Tissue Area**:1cm^2
  - Cut 10 µm thick. When tissue is on coverglass, wait 5 seconds at least & then allow to refreeze
  - You can warm coverglass (<70°C) to help tissue adhere better
  - Store coverglass at -20 °C for 5 – 45 min, then permeabilize in EtOH.



Source: Vizgen

Vizgen MERSCOPE

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Some Special Cases

- **Bone tissue:**
  - Requires decalcification after fixation. RNA may be degraded, and adhesion is more difficult.
    - Add RNAse inhibitors to decalcification solution
    - Reduce sucrose to 10% to help with adhesion
- **Fatty tissue**:
  - Harder to cut, reduce cryostat temp to -17 (-18) degrees to help
  - Extend thawing time of tissue on coverglass before refreezing to help with adhesion
  - More resistant to tissue clearing so use the "resistant" option from the user manual
- **Muscle tissue**: Closely packed cells challenge cell segmentation accuracy
  - Syncytium, cell boundary staining may be required

MERSCOPE Sample Verification Kit to verify and optimize sample preparation conditions

Source: Vizgen

Vizgen MERSCOPE

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Custom MERSCOPE Gene Panel Design Portal

A software tool for creating MERFISH gene panels to run on the MERSCOPE *in situ* spatial genomics platform. With the Portal you can customize your gene panels with real-time feedback about which genes are most suitable for a MERFISH measurement.

Source: Vizgen

Vizgen MERSCOPE

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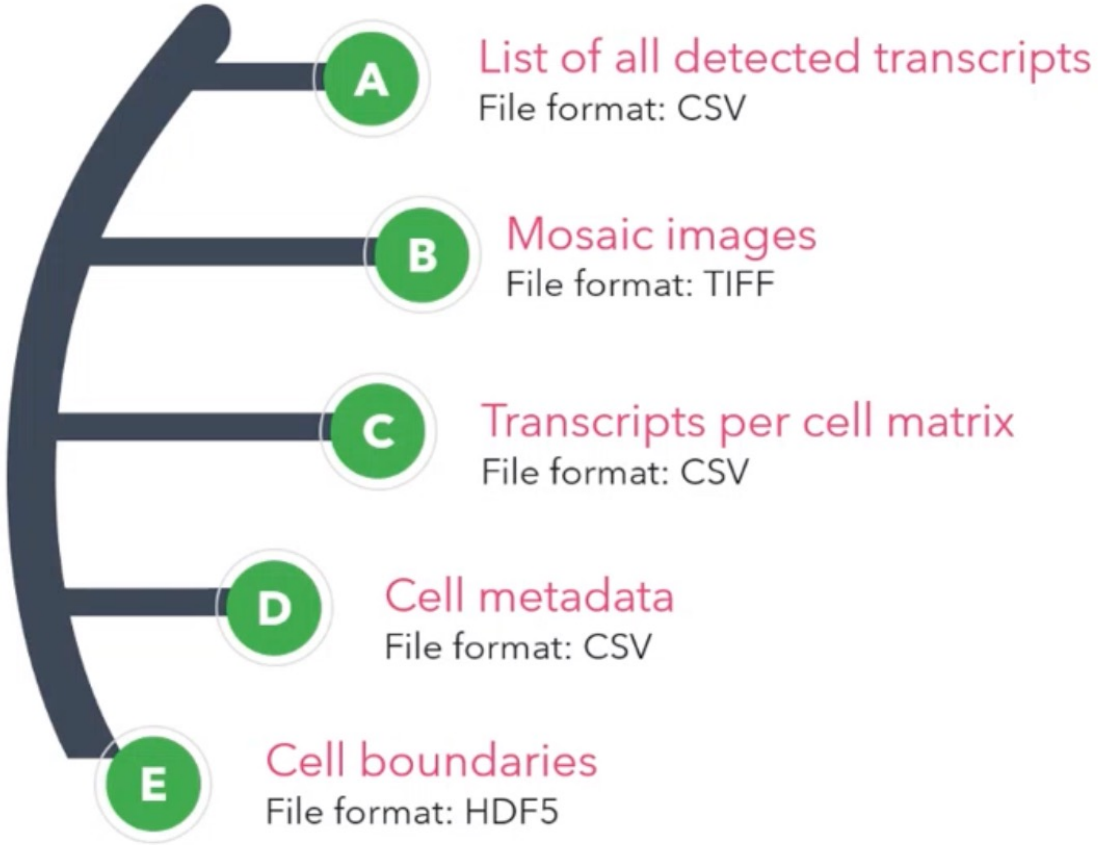
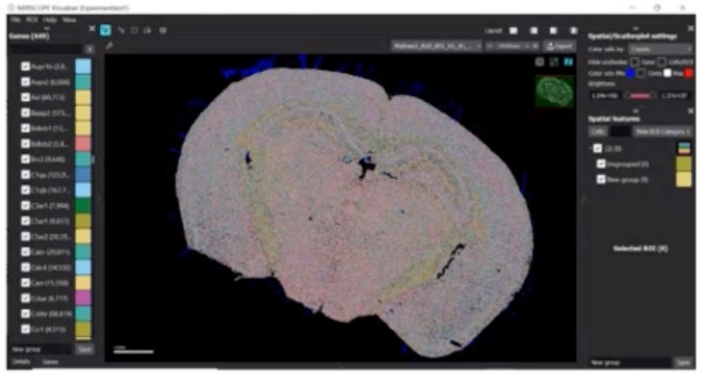
Vizgen Data output



MERSCOPE Vizualizer™



Data compatible with single-cell gene expression analysis software platforms

SEURAT scanpy

Source: Vizgen

Bioinformatics

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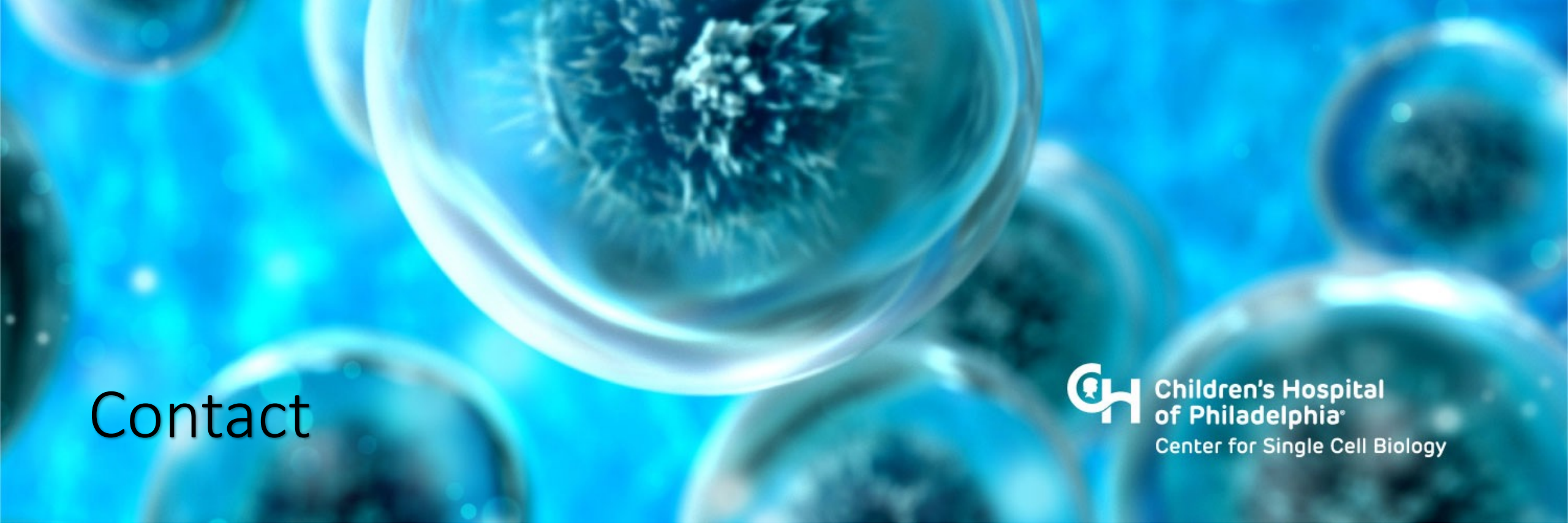
Our experienced bioinformaticians help with experimental design, developing reproducible workflows, analyzing high throughput next-generation sequencing data and spatial profiling data, and supporting manuscript development/publication. We generate visualizations of complex data and assist data uploads to public repositories.



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