Highly Multiplexed Imaging and Cell Phenotyping with ChipCytometry™

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Canopy Biosciences
22nd January 2024

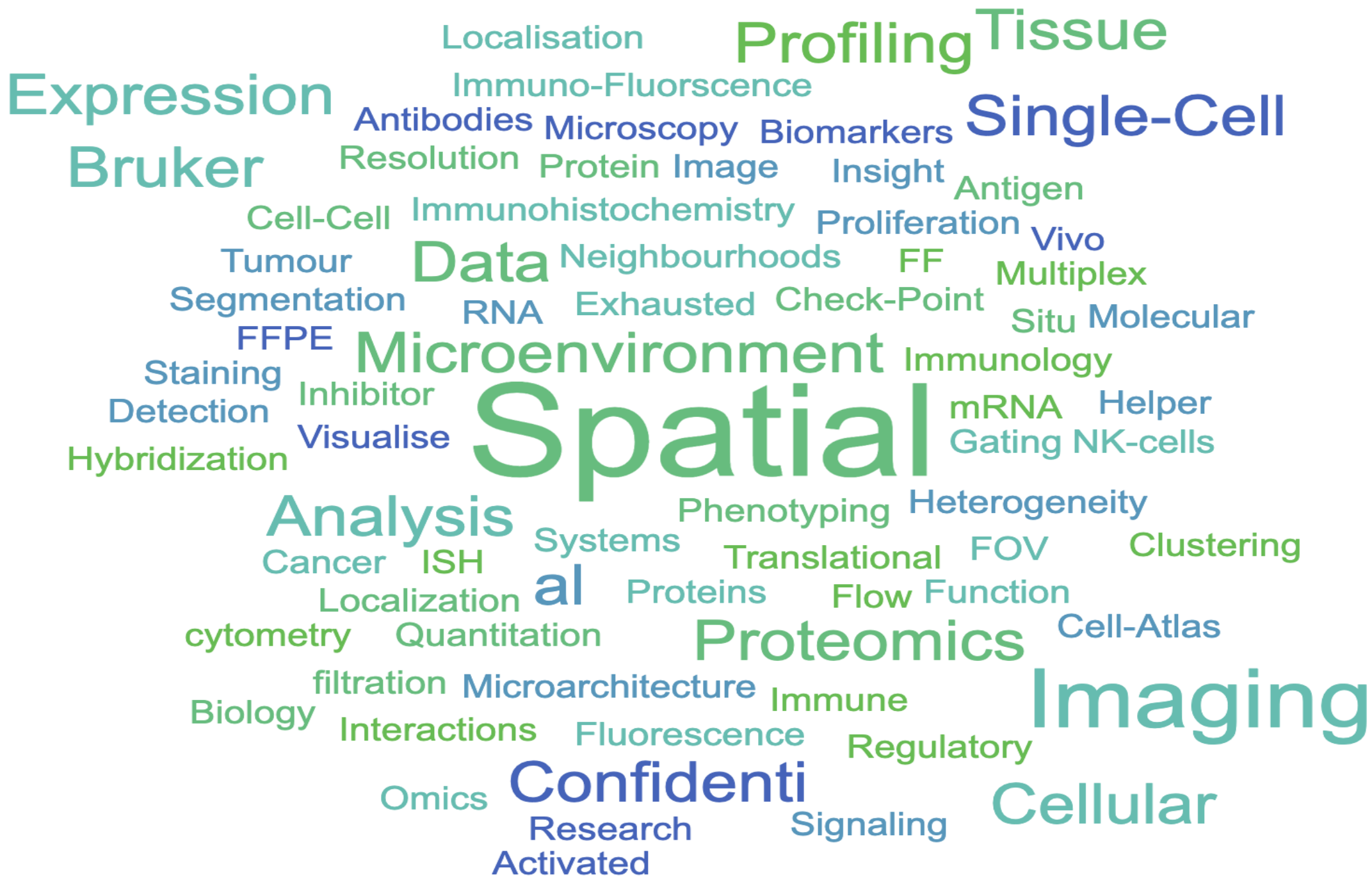
Agenda

What is Spatial Biology & Why is it important?

Case Studies and examples

ChipCytometry

Q&A


Localisation
Profiling
Tissue
Expression
Immuno-Fluorscence
Single-Cell
Antibodies
Microscopy
Biomarkers
Bruker
Resolution
Protein
Image
Insight
Antigen
Cell-Cell
Immunohistochemistry
Proliferation
Vivo
Tumour
Data
Neighbourhoods
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Multiplex
Segmentation
RNA
Exhausted
Check-Point
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Molecular
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Microenvironment
Immunology
Staining
Inhibitor
Detection
Spatial
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Helper
Visualise
Gating
NK-cells
Hybridization
Analysis
Phenotyping
Heterogeneity
Systems
Cancer
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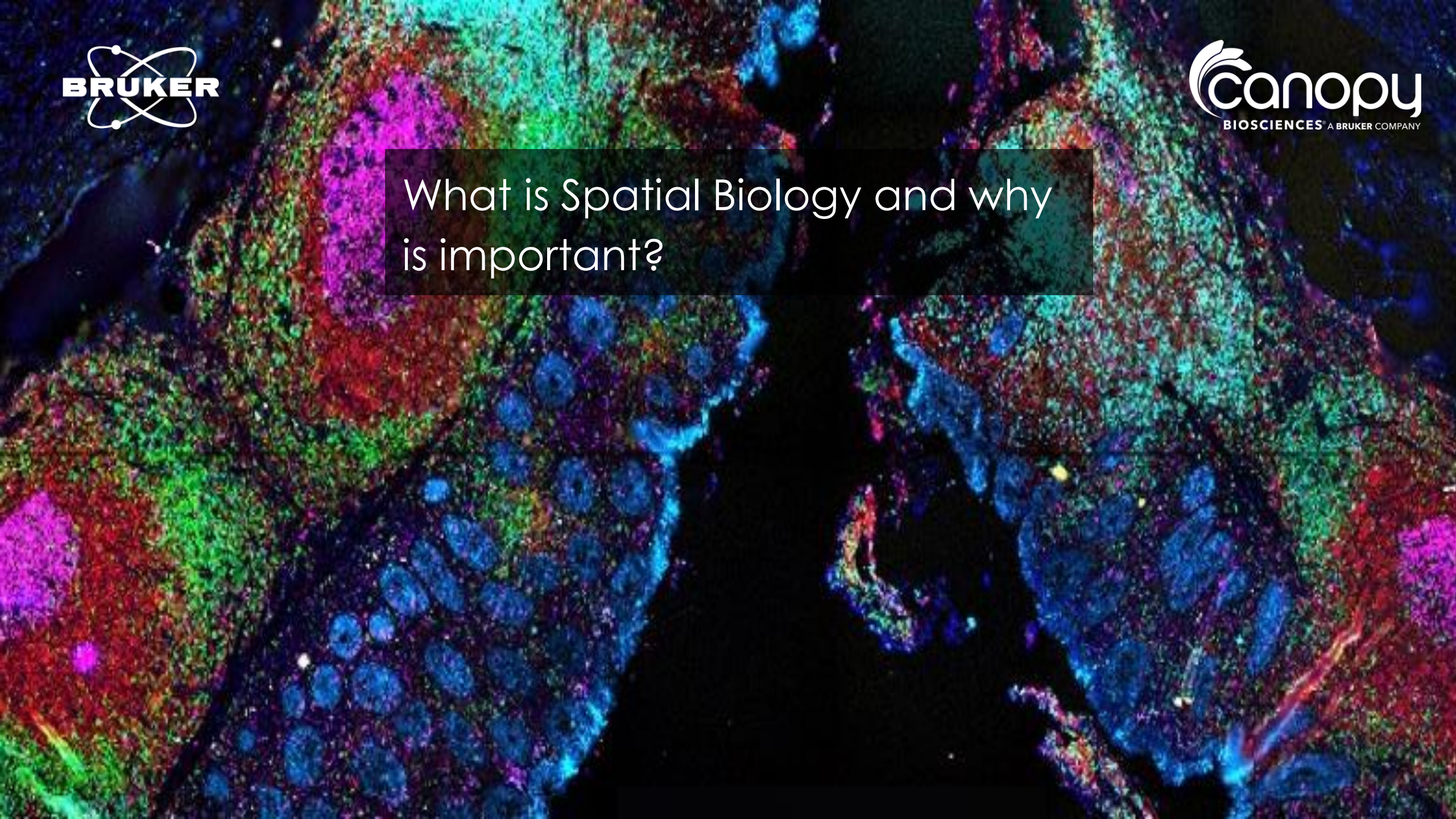
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What is Spatial Biology and why is important?

Quote from Emma Lundberg, Stanford University, September 2023

We are trying to understand the spatial distribution of all human proteins with the goal of building a spatiotemporal model of all human cells. This work can help us to address the question of how the cell functions as a system.

There are many, many interesting questions in spatial biology. A lot of diseases start with a protein being in the wrong place, or in the wrong place at the wrong time, which can have major functional consequences.

I think, in general, connecting location to function is broadly applicable to many questions in biology that can lead to thousands of interesting applications.

[The Spatial Perspective With Professor Emma Lundberg | Technology Networks]

Provides *in situ* Positional Context of Cells in Tissue

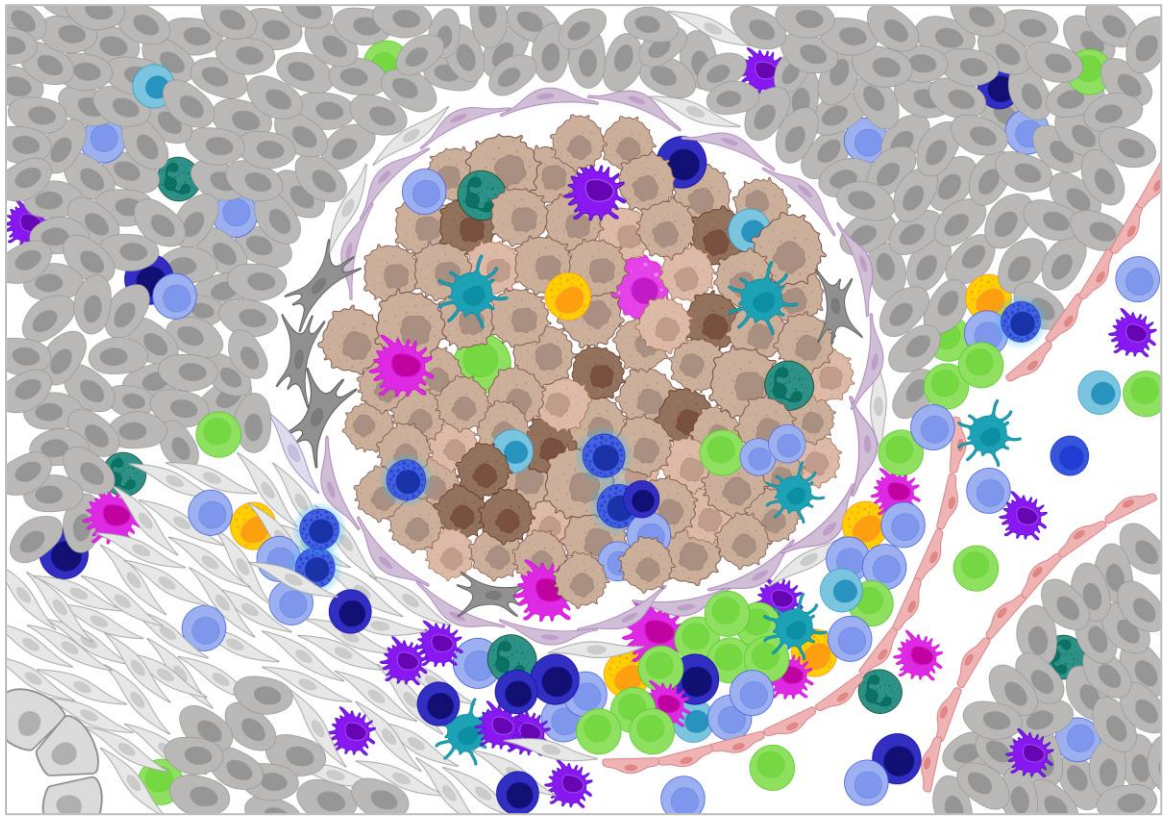
What are and where are the cell types in the tumor microenvironment (TME)?

What are the sub-types of these cell types?

What does the immune cell infiltration look like in the TME?

What is happening at the tumor-immune system interface?

The Tumor Microenvironment



What is the relationship between the cells? Is there inherent order in these neighborhoods?

Can we understand the cell-cell communication networks?

What are the key roles of these cells? What are they doing?

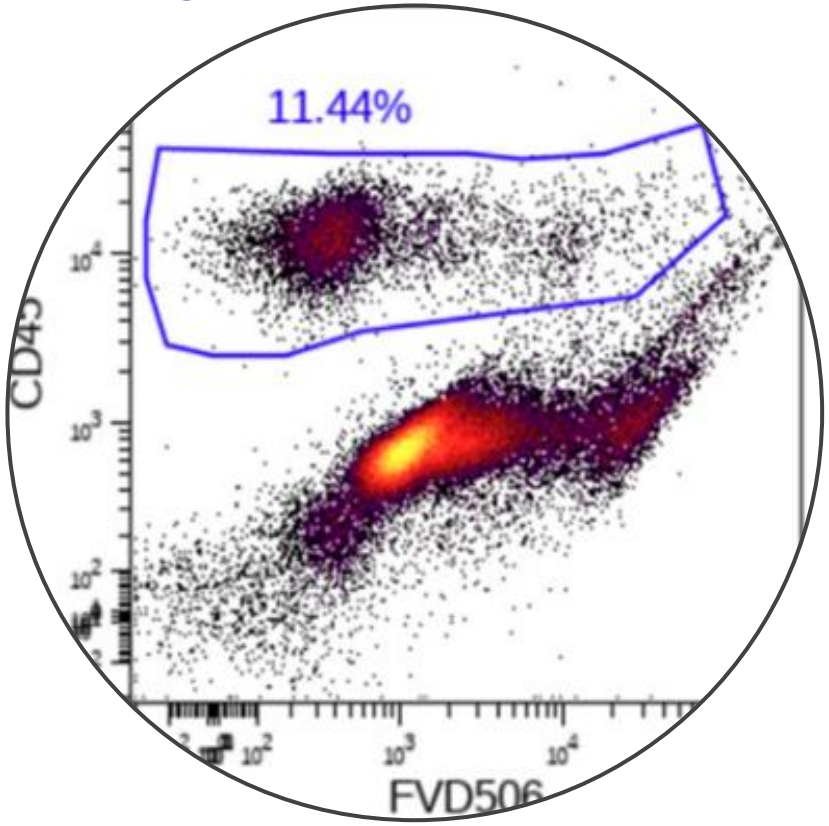
Can we develop better diagnostics? Can we design better therapies?

Spatial proteomics and single cell phenotyping

Flow cytometry & IHC have established uses, but at a tradeoff

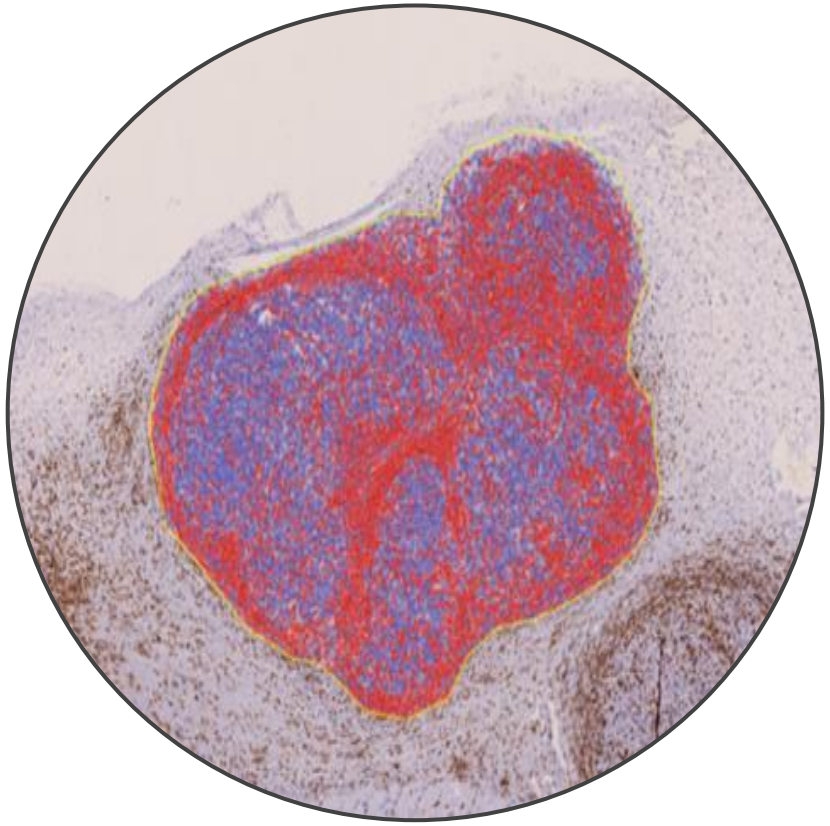
High multiplex

Can classify and quantify cell types but with no spatial context



Flow Cytometry

Spatial information



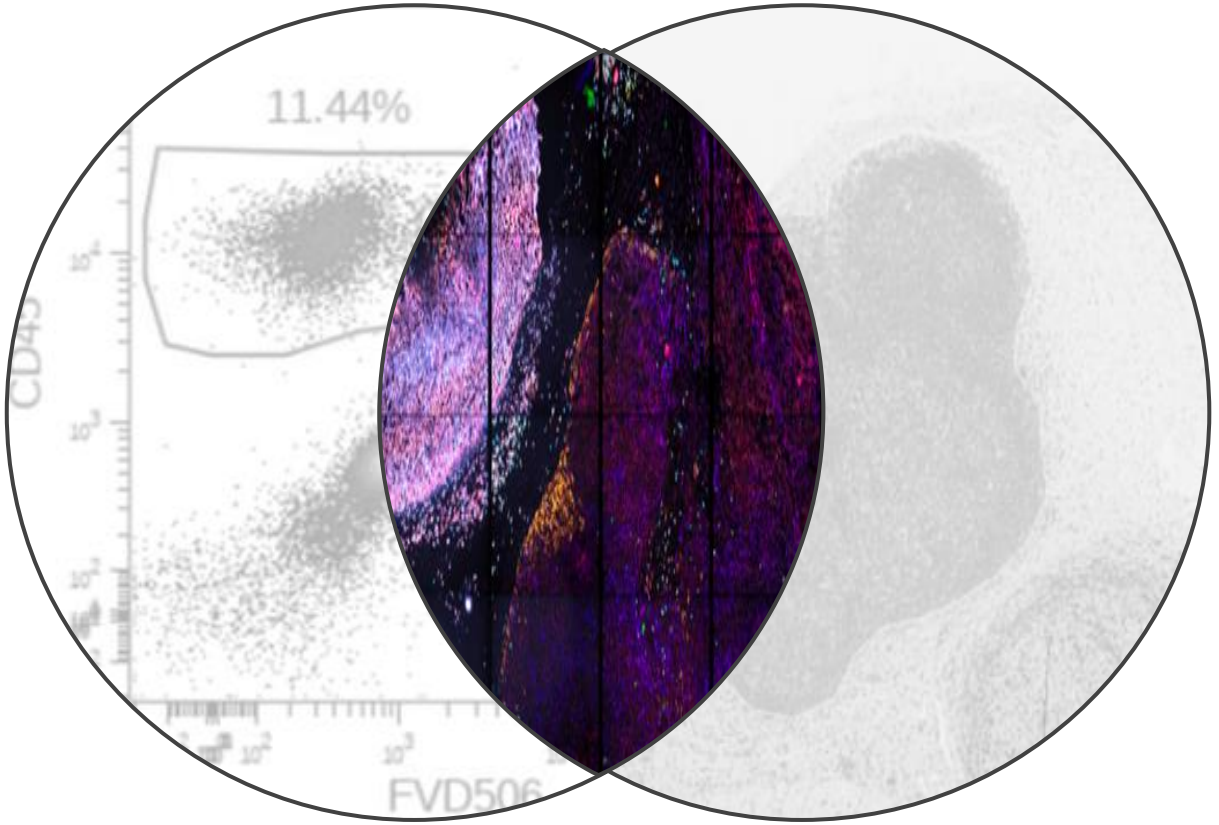
Spatial context but with limited markers

IHC

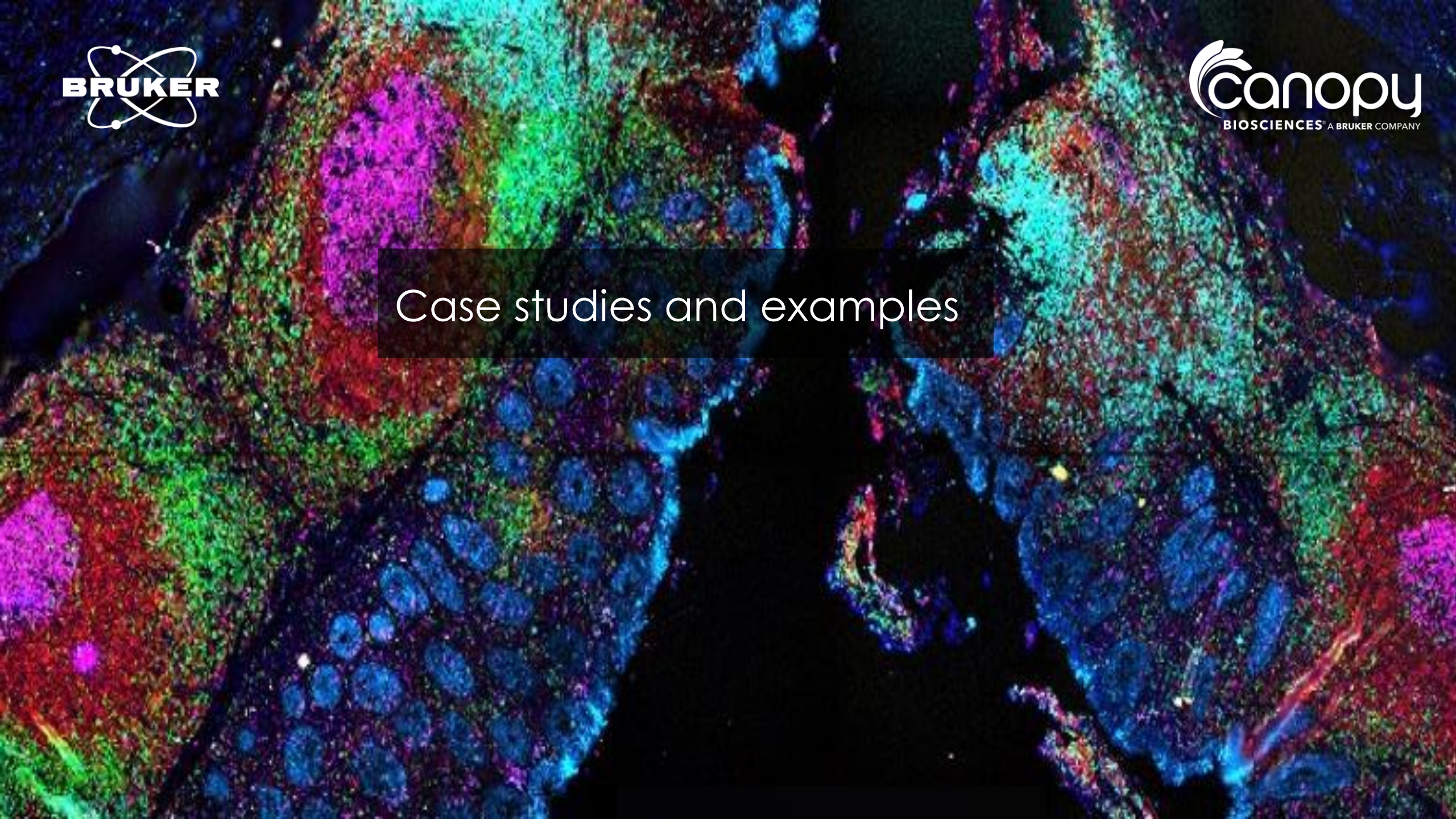
Why not both?

High multiplex potential **and** spatial information

Single Cell Quantification and sub-classification of immune response



Phenotyping within the tumour microenvironment with spatial conext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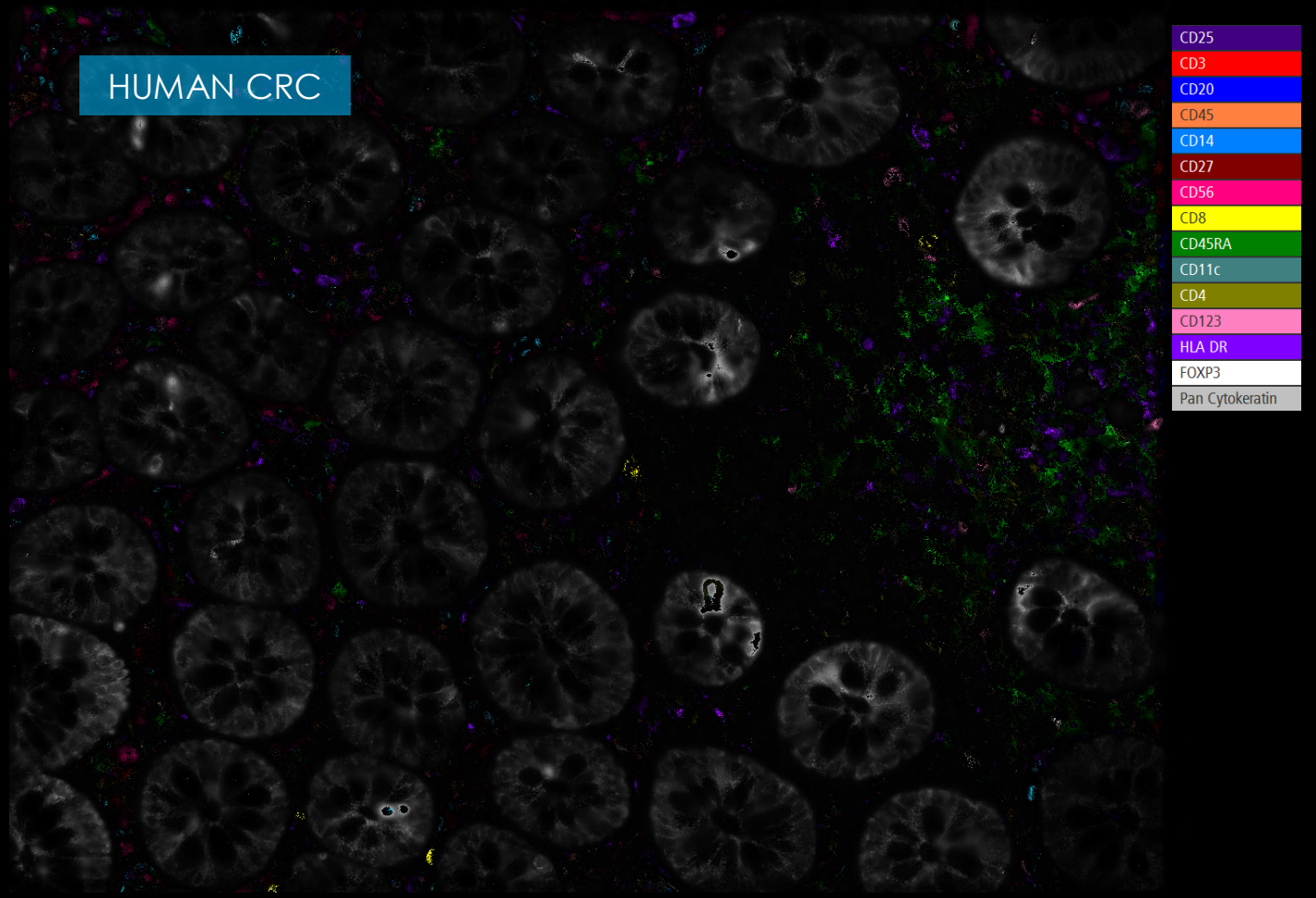
Case studies and examples

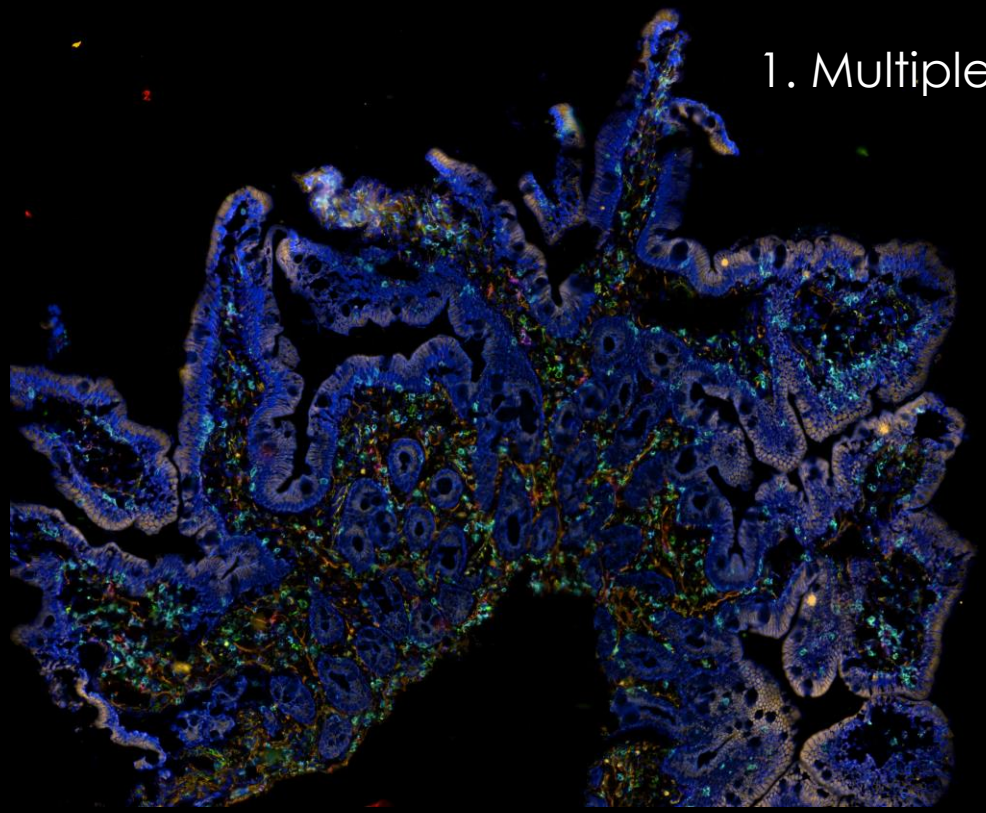
Iterative staining and imaging for detection of virtually unlimited protein biomarkers.

Individual cells in the tissue are segmented using a DNA stain.
Iterative staining with gives rise to high multiplex capacity.

5 different fluorescence filters available
--> 5 antibody stainings/ cycle



Generate Quantitative Single-Cell Data on intact Tissue Samples

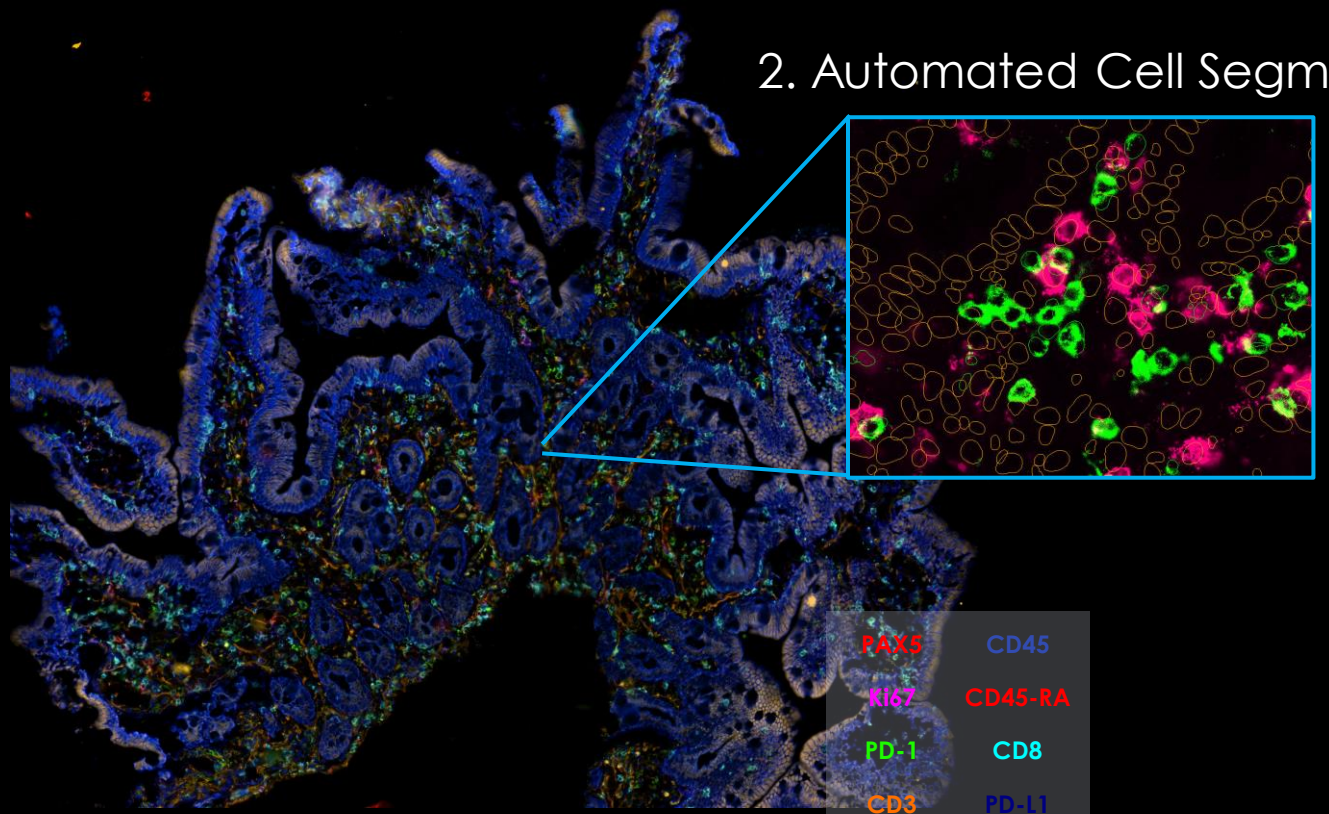


21-plex on
Colon FFPE

1. Multiplexed Imaging

CellScape Overview

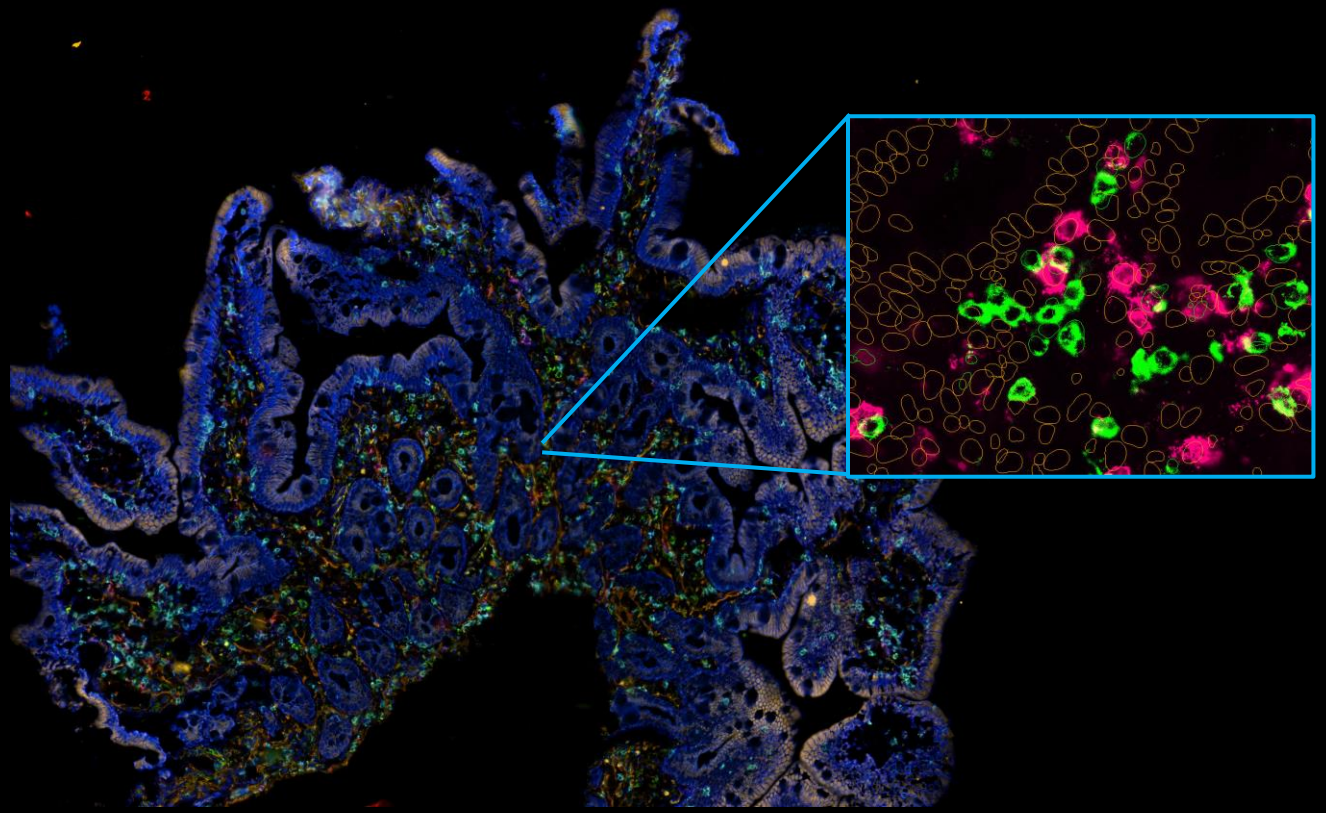
Generate Quantitative Single-Cell Data on In-Tact Tissue Samples



21-plex on
Colon FFPE

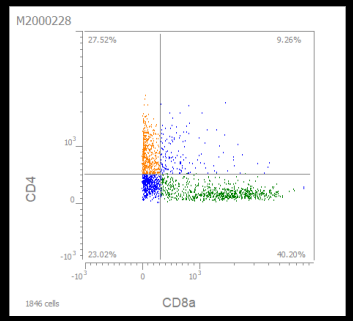
CellScape Overview

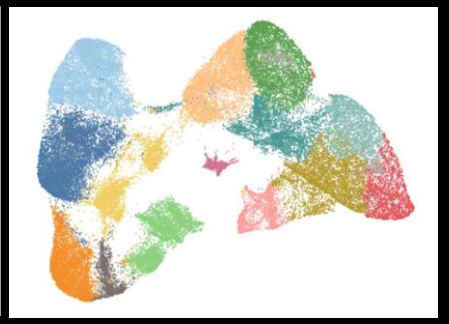
Generate Quantitative Single-Cell Data on In-Tact Tissue Samples



21-plex on
Colon FFPE

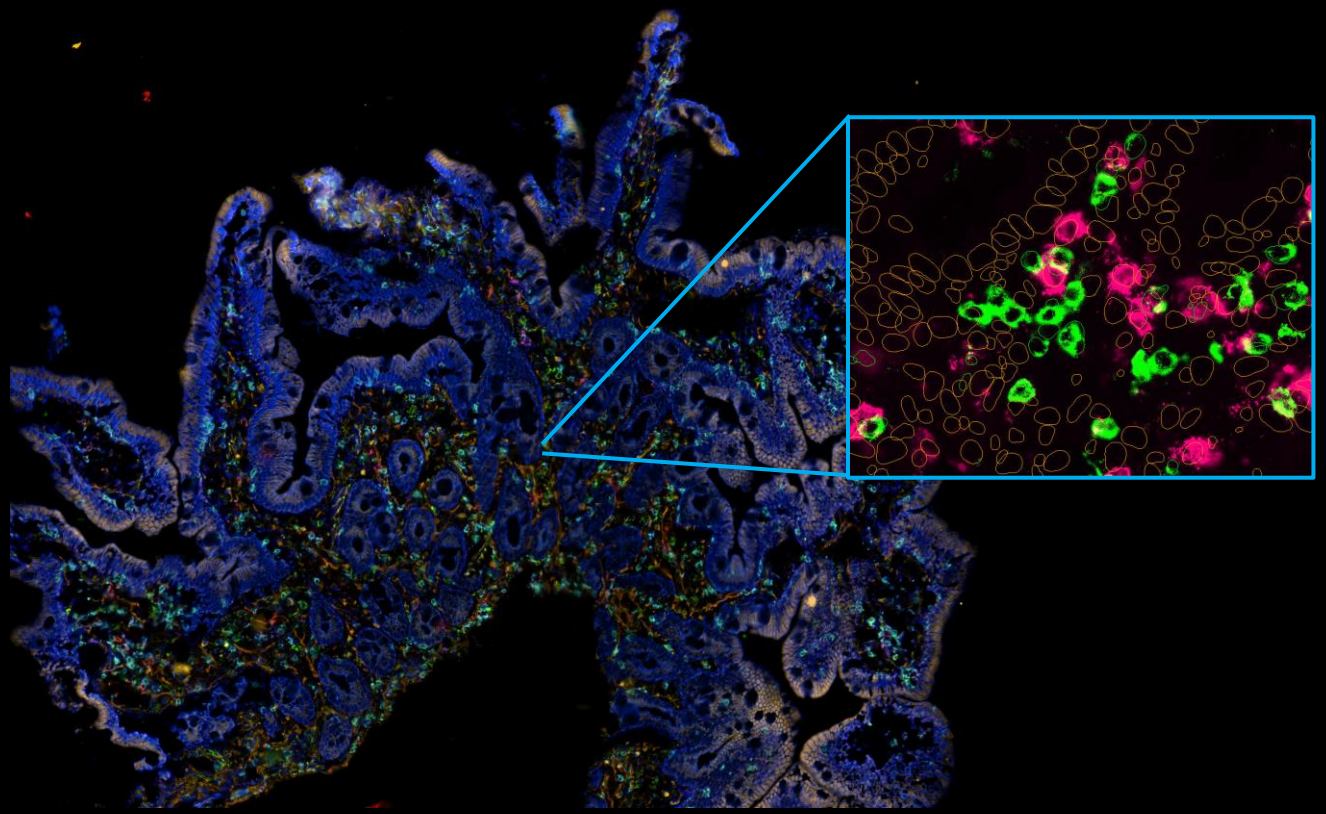
3. Phenotyping (Gating/Clustering)



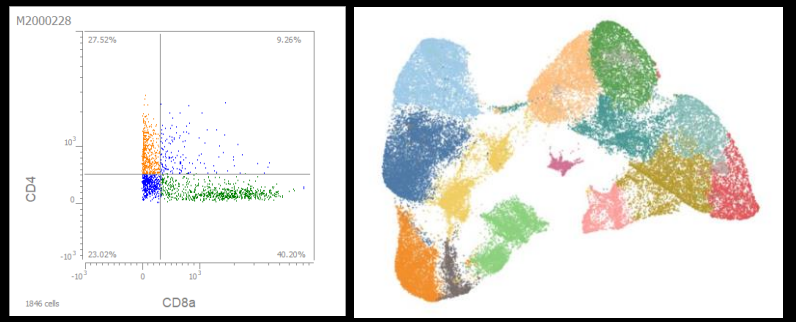


CellScape Overview

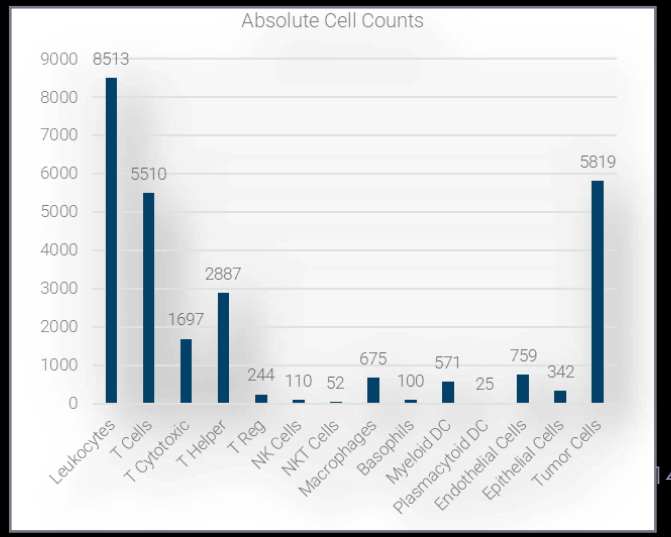
Generate Quantitative Single-Cell Data on In-Tact Tissue Samples



21-plex on
Colon FFPE



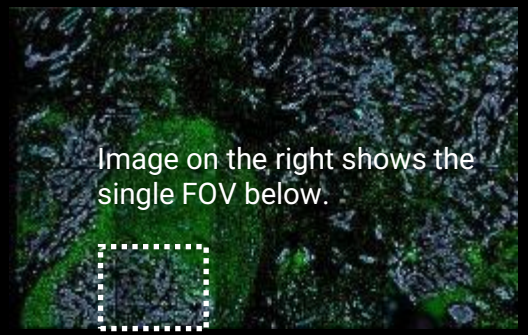
4. Population Analysis



Human breast carcinoma
(100-plex study)

SELECT CELL TYPES IDENTIFIED

T cells | CD8+ and CD4+
B cells | CD20+
NK cells | CD56+
Epithelial cells | pan-Cytokeratin+
Tumor cells | CD340+ (Her2)



Comprehensive study of breast tissue with **100 markers**

…able to perform **high-plex** with **quantitative phenotyping** across a **continuum** of cell types

CD16
CD8
CD335 (NKp46)
CD38
CD161
FoxP3
pan cytokeratin
CD31
Vimentin
CD340
CD4
CD56
Collagen IV
CD20
CD279 (PD
CD271

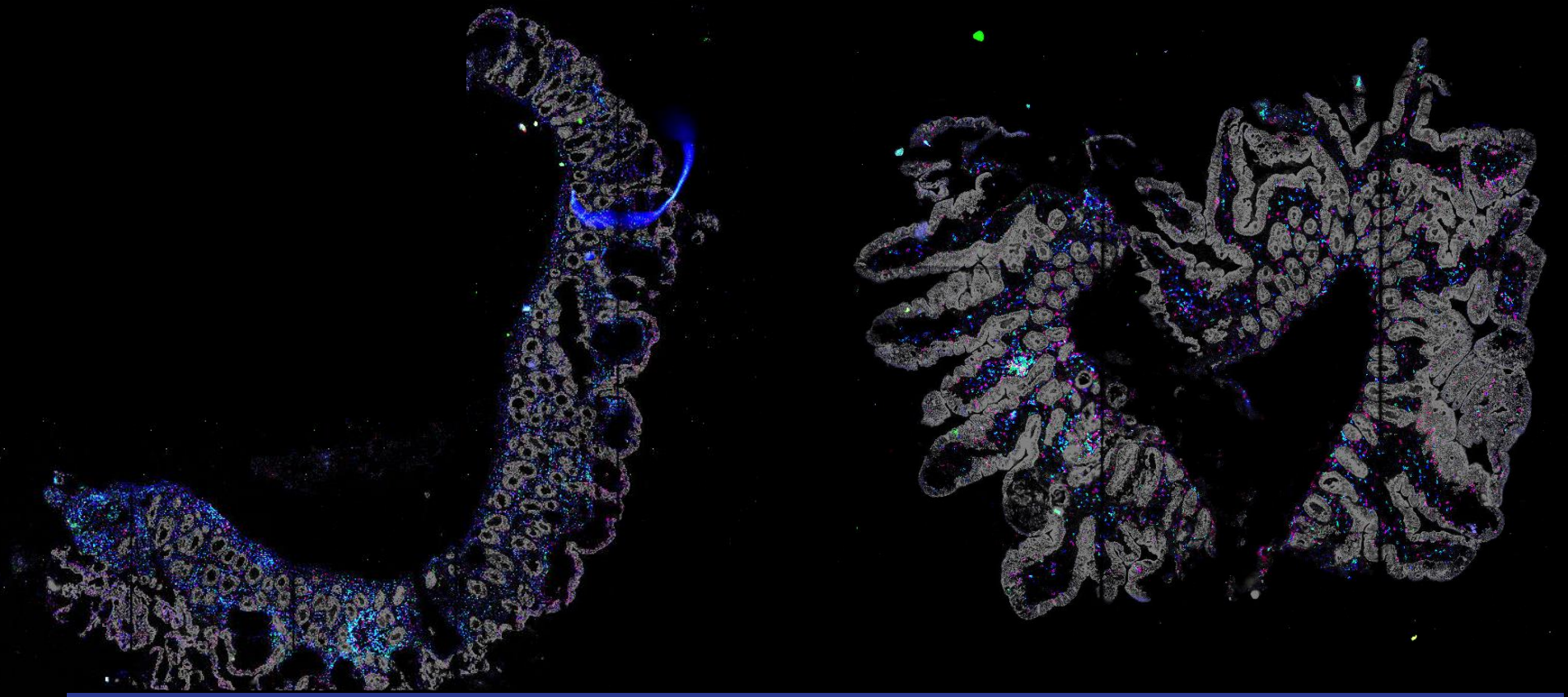
50µm 16 Aug 23

Application: Exploring the Immune Cell Landscape in Celiac Disease

21-plex Human FF Intestinal Tissue

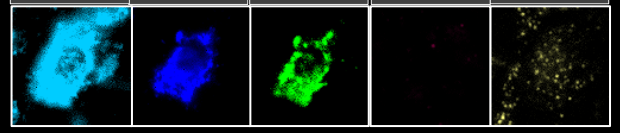
Active Celiac Disease

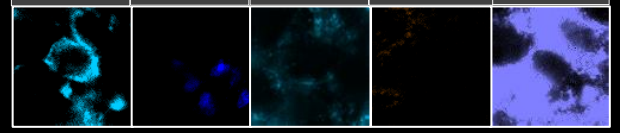
Healthy Control

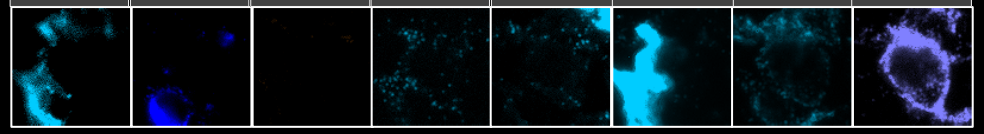


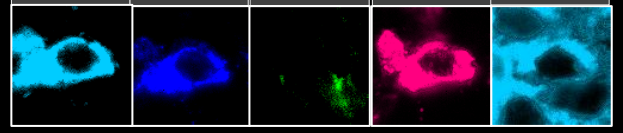
Observable differences in the immune composition of ACD vs HC samples

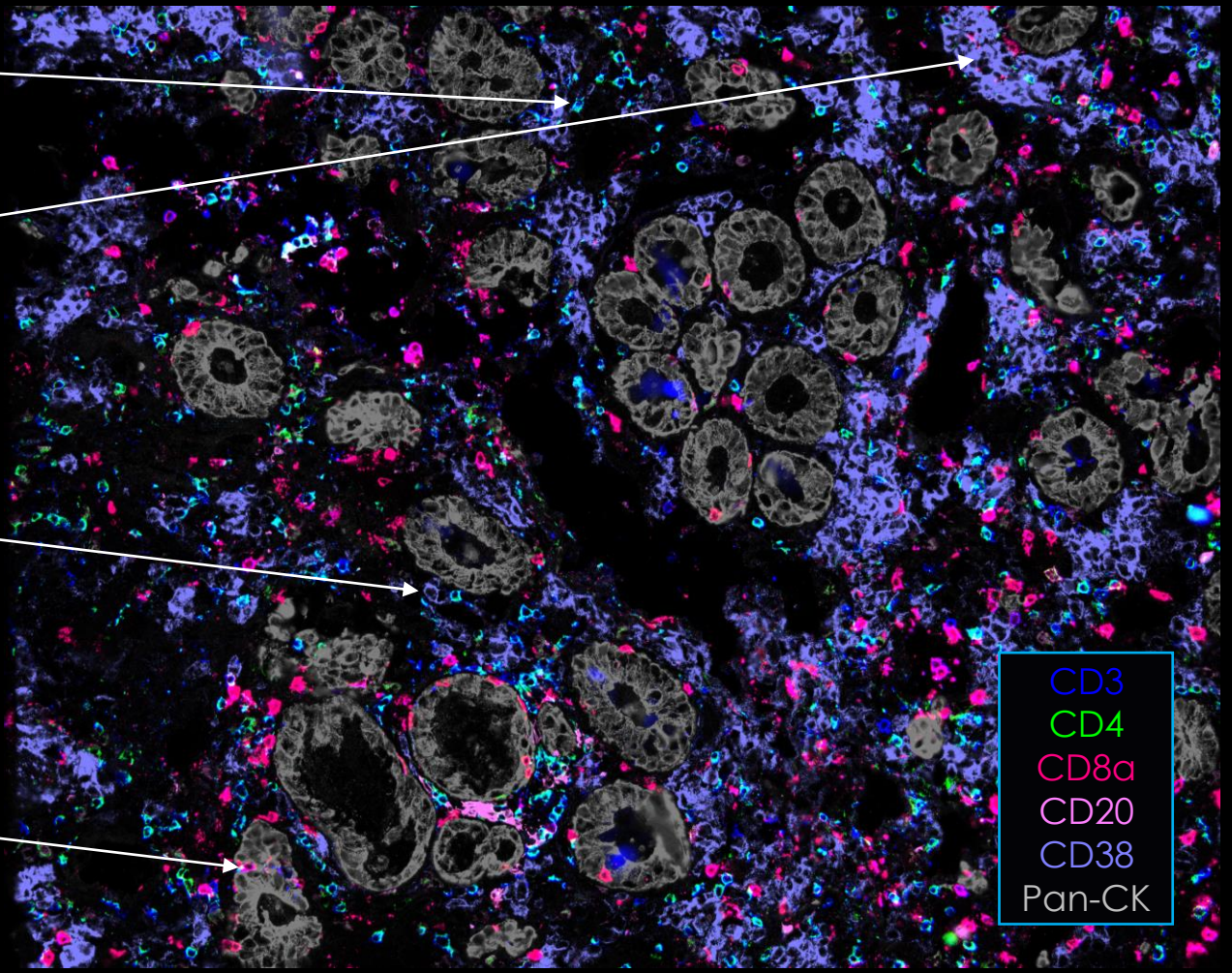
21-Plex Active Celiac Disease

Regulatory CD4+ T cells				
CD45+	CD3+	CD4+	CD8-	FoxP3+
				

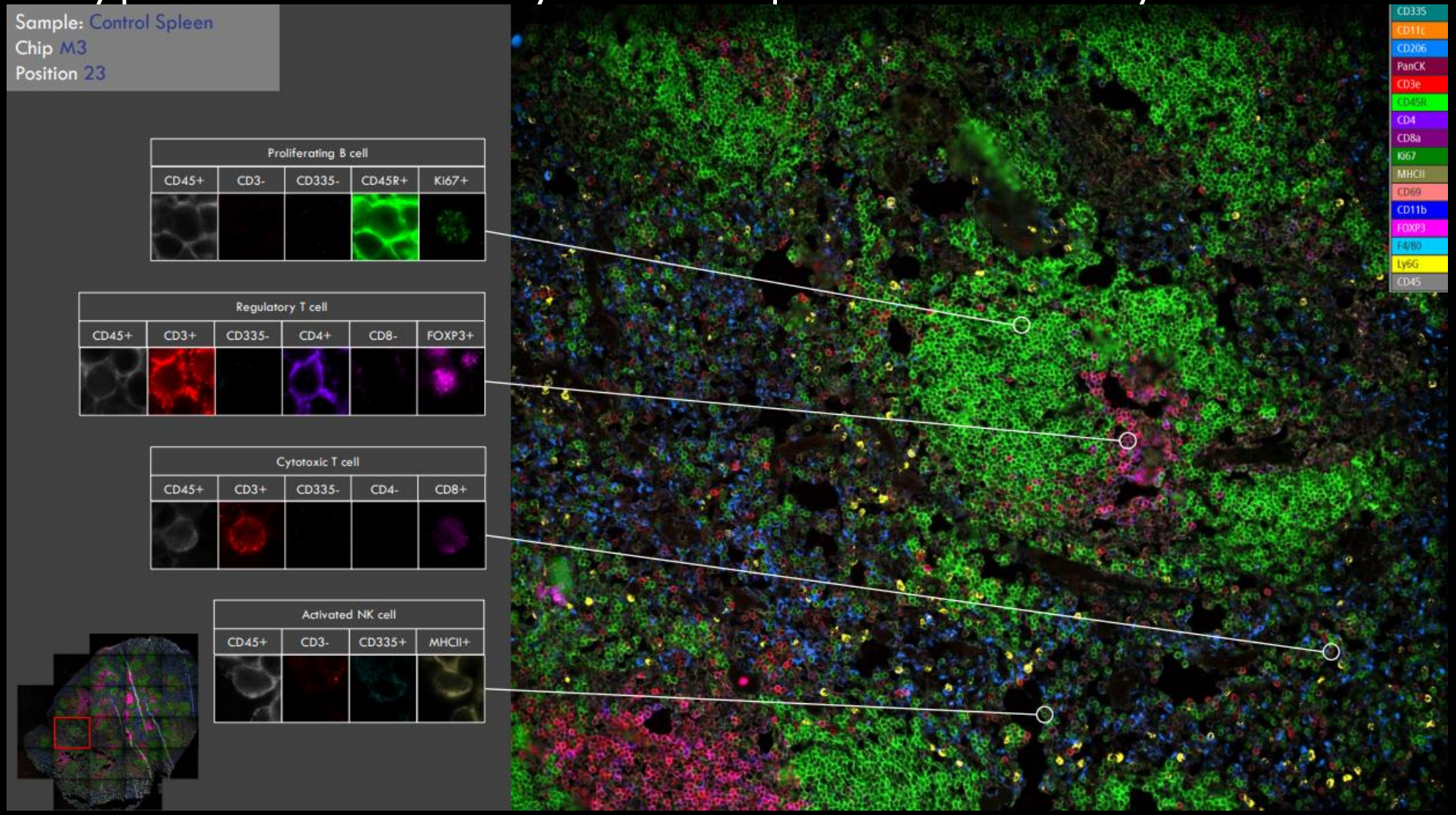
CD45+ Plasma cells				
CD45+	CD3-	CD56-	CD20-	CD38+
				

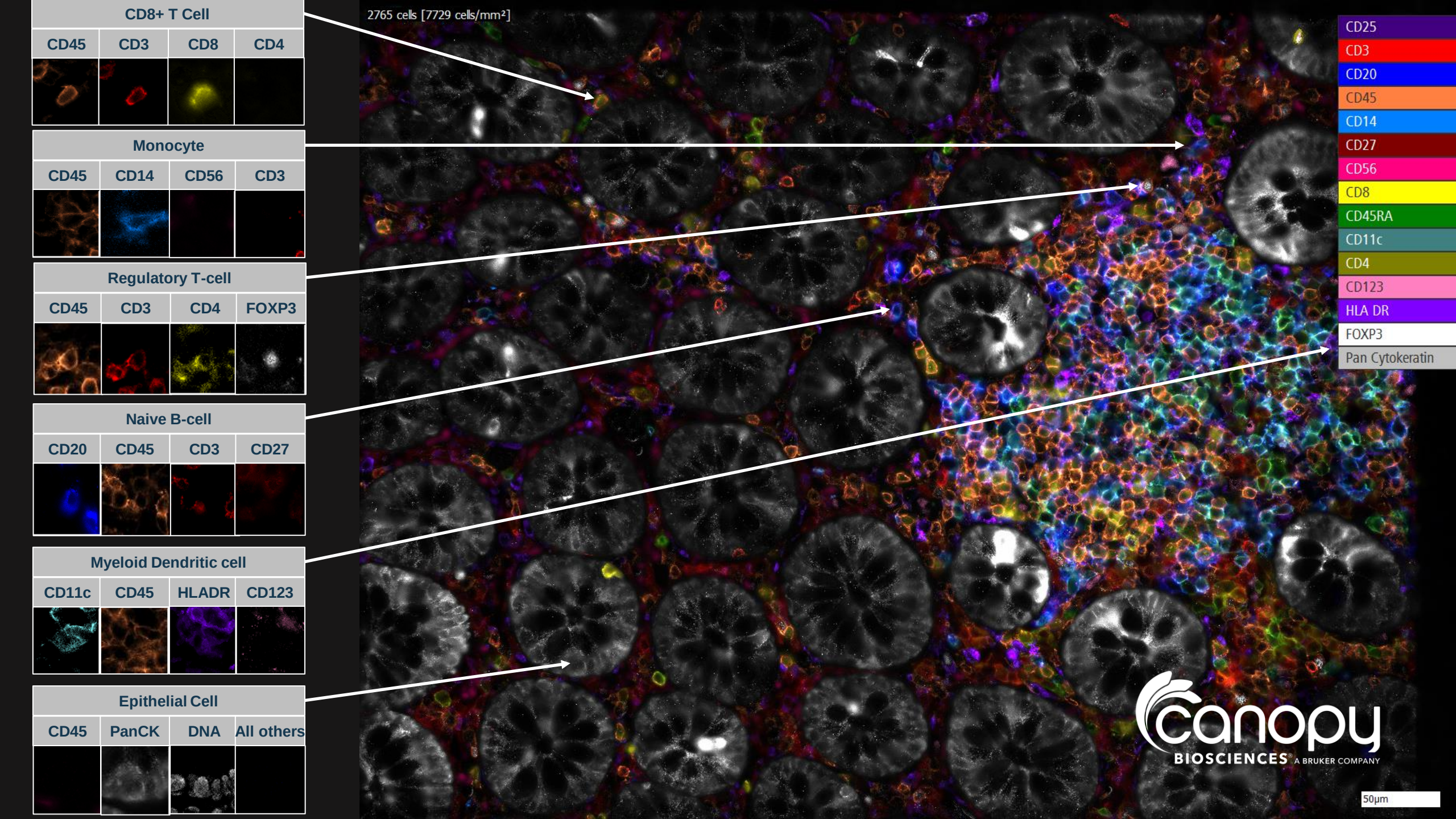
Lin-CD45-CD38+							
CD45-	CD3-	CD20-	CD123-	Epcam-	CD56-	CD11c-	CD38+
							

Intraepithelial HLADR+ Cytotoxic T cell				
CD45+	CD3+	CD4-	CD8+	HLADR+
				



Phenotype each cell in your sample individually.




CD8+ T Cell
CD45
CD3
CD8
CD4
Monocyte
CD45
CD14
CD56
CD3
Regulatory T-cell
CD45
CD3
CD4
FOXP3
Naive B-cell
CD20
CD45
CD3
CD27
Myeloid Dendritic cell
CD11c
CD45
HLADR
CD123
Epithelial Cell
CD45
PanCK
DNA
All others
2765 cells [7729 cells/mm²]
CD25
CD3
CD20
CD45
CD14
CD27
CD56
CD8
CD45RA
CD11c
CD4
CD123
HLA DR
FOXP3
Pan Cytokeratin
canopy
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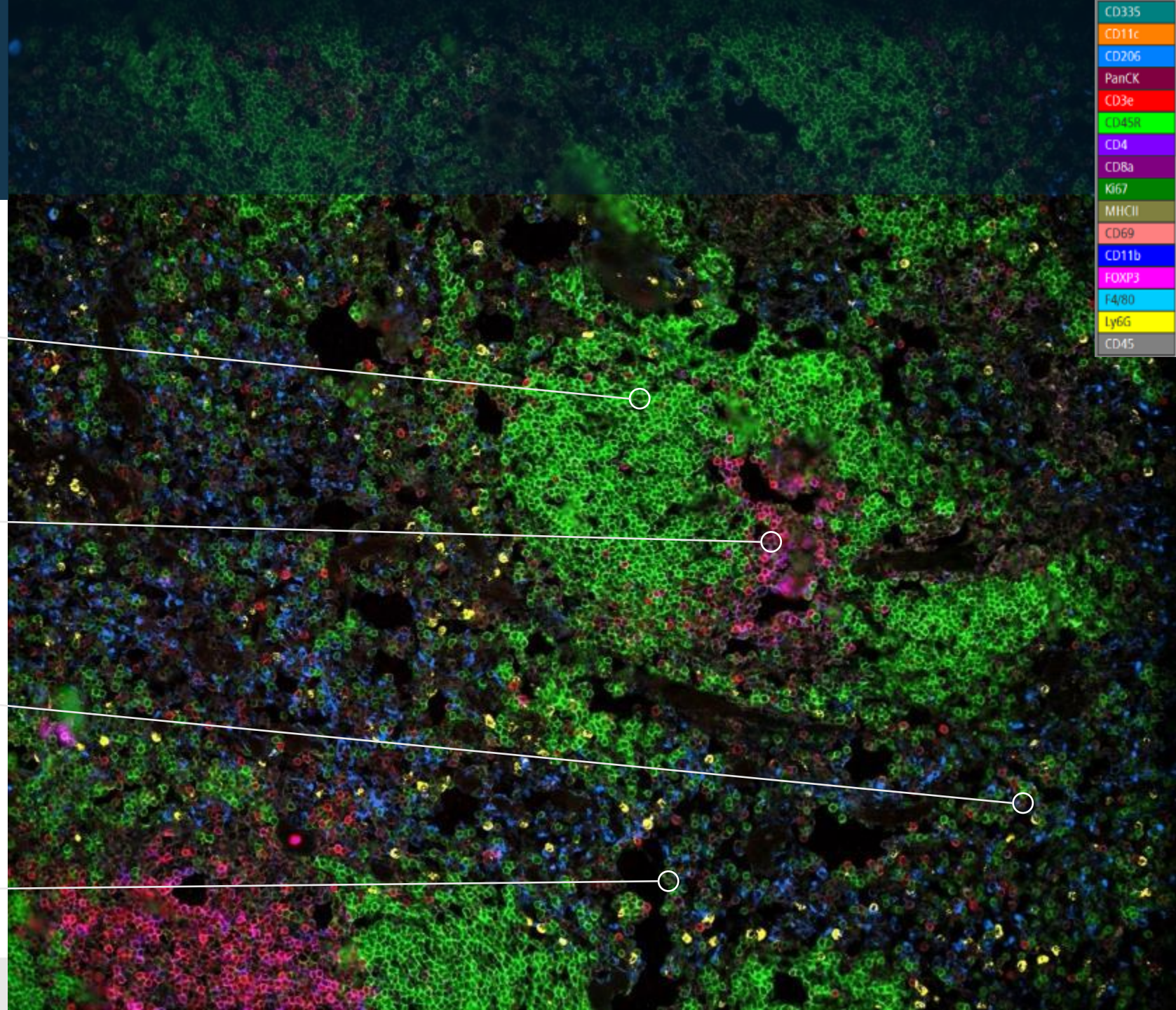
50µm

Human Spleen



Proliferating B cell

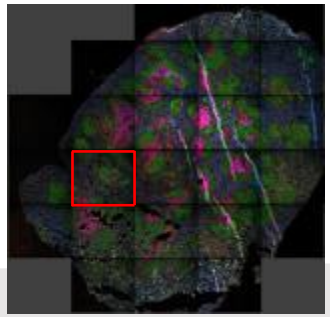
CD45+	CD3-	CD335-	CD45R+	Ki67+

Regulatory T cell

CD45+	CD3+	CD335-	CD4+	CD8-	FOXP3+

Cytotoxic T cell

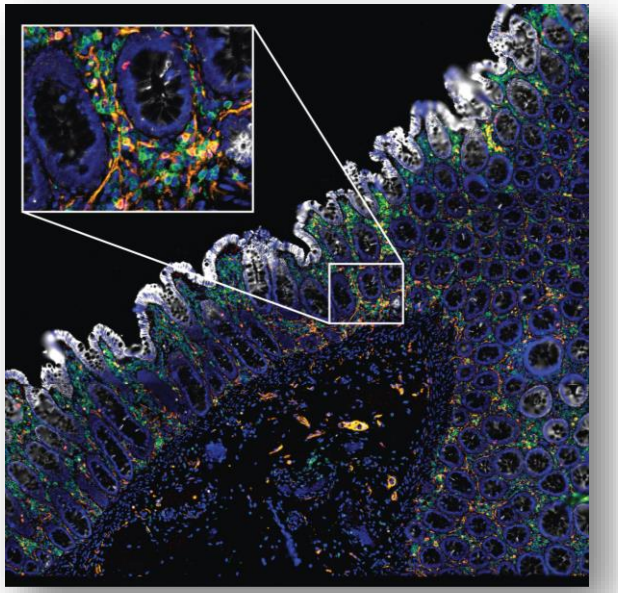
CD45+	CD3+	CD335-	CD4-	CD8+



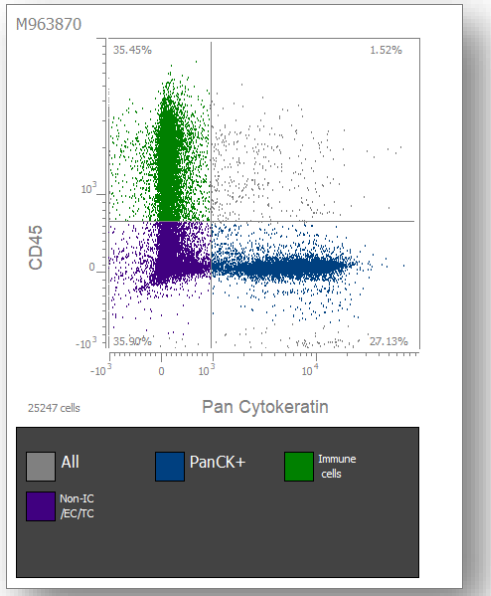
Activated NK cell

CD45+	CD3-	CD335+	MHCII+

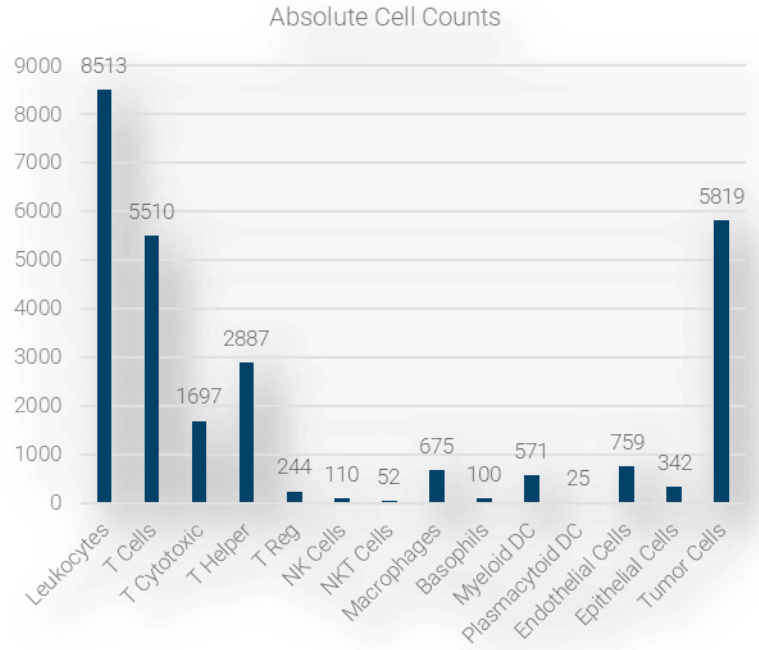
Convert images to quantitative flow-like data.



Image



Dot Plot

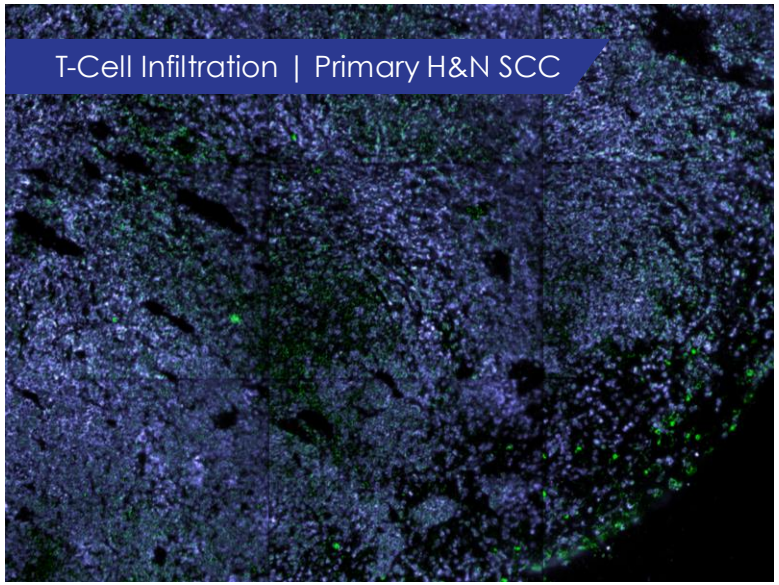


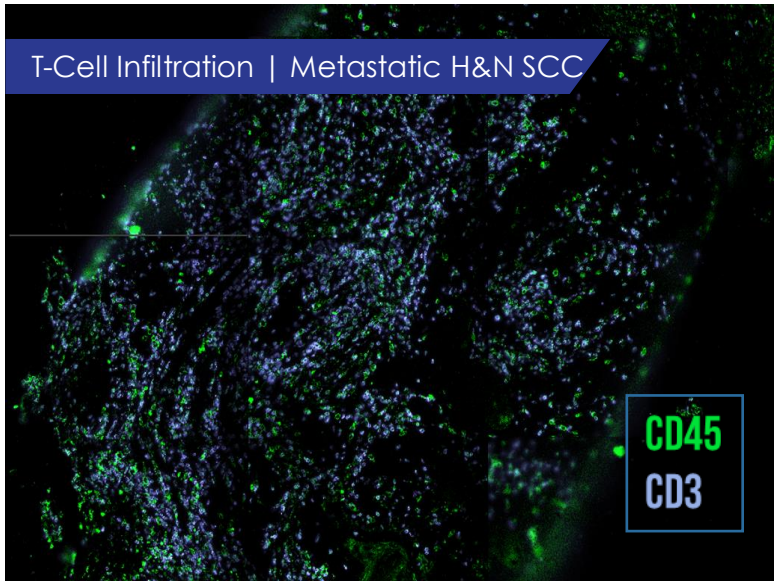
Quantify Populations

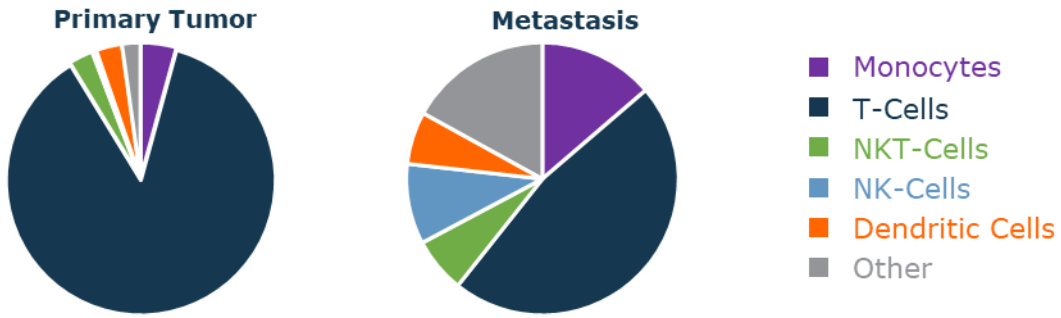
Overview

FCS files are generated from high-resolution images allowing for the phenotyping of each individual cell

Quantitative data. New discoveries.

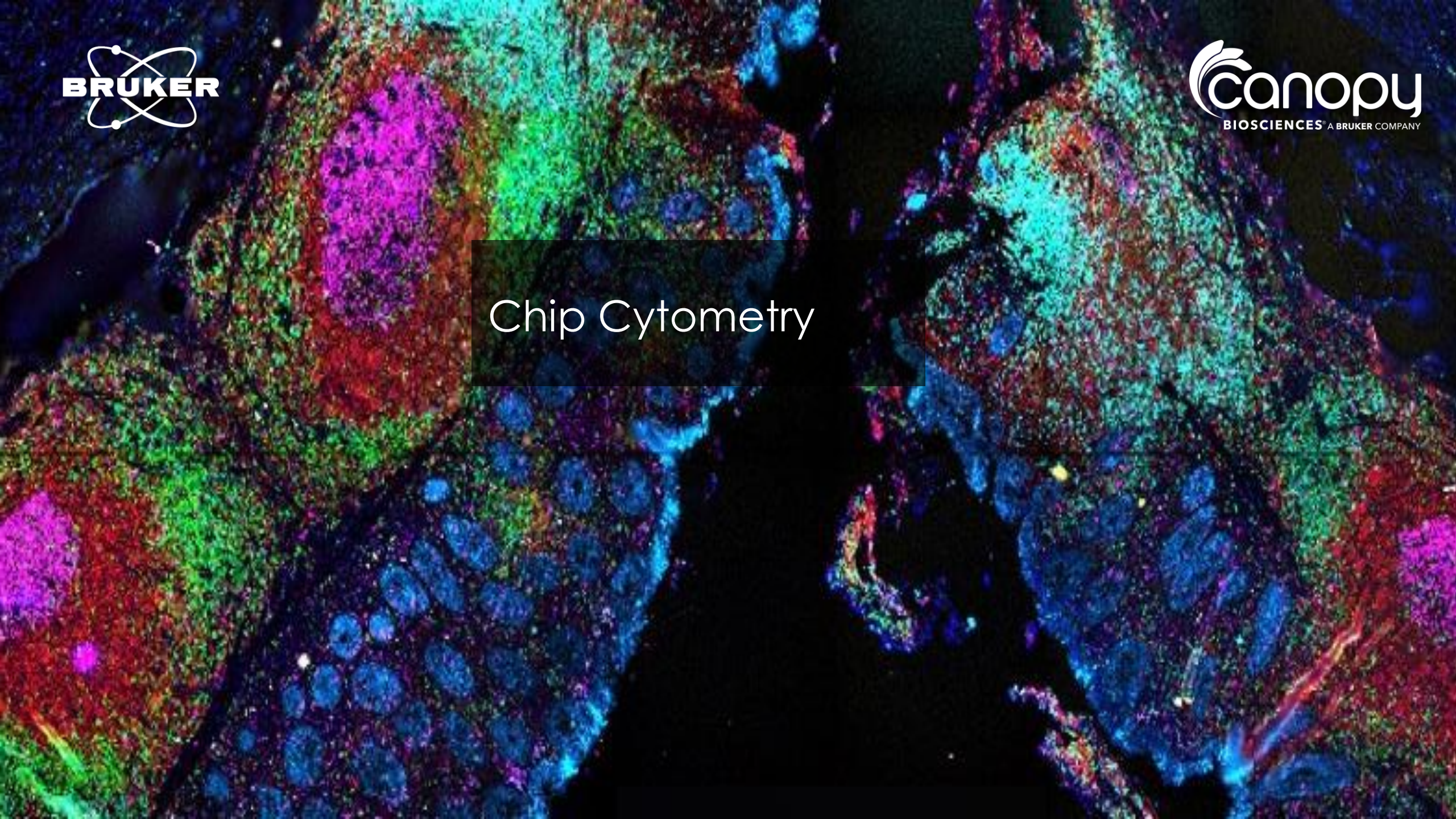






T Cell Infiltration.

Comparing the immune cell composition of a primary and metastatic tumor site from the same patient reveals significant differences between the T-cell infiltration at the two sites.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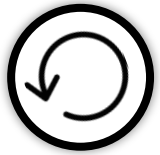
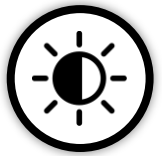
Chip Cytometry

Key Benefits of ChipCytometry

Virtually unlimited multiplexing: Successive rounds of immunostaining, imaging, and signal removal to detect virtually unlimited numbers of proteins on the same sample



Sample reinterrogation: Biobanking on proprietary chip technology preserves samples with the option to reinterrogate samples up to 2 years later



High dynamic range: Multiple exposure and fusion algorithms enable robust quantification of high and low expression markers

Open-source reagents: Fluorescently labeled antibodies from any commercial vendor are part of a flexible and practical solution for spatial immunophenotyping

Species and sample agnostic: High-quality staining results on any species or sample type, including dissociated cells, frozen tissues, and archival FFPE tissues

High resolution imaging: With the highest pixel density optical sensor available, visualize single cells as well as subcellular structures

Introducing CellScape™: The Next-Generation ChipCytometry™ Instrument

Seamless Fluidics Integration

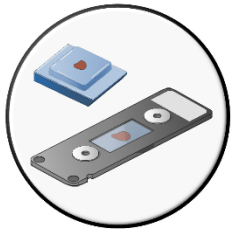
Enclosed imaging system

Purpose Built and Highly Optimized for High-throughput, High-plex Spatial Proteomics

4-gang sample holder

Stain. Image. Repeat.

Sample Prep



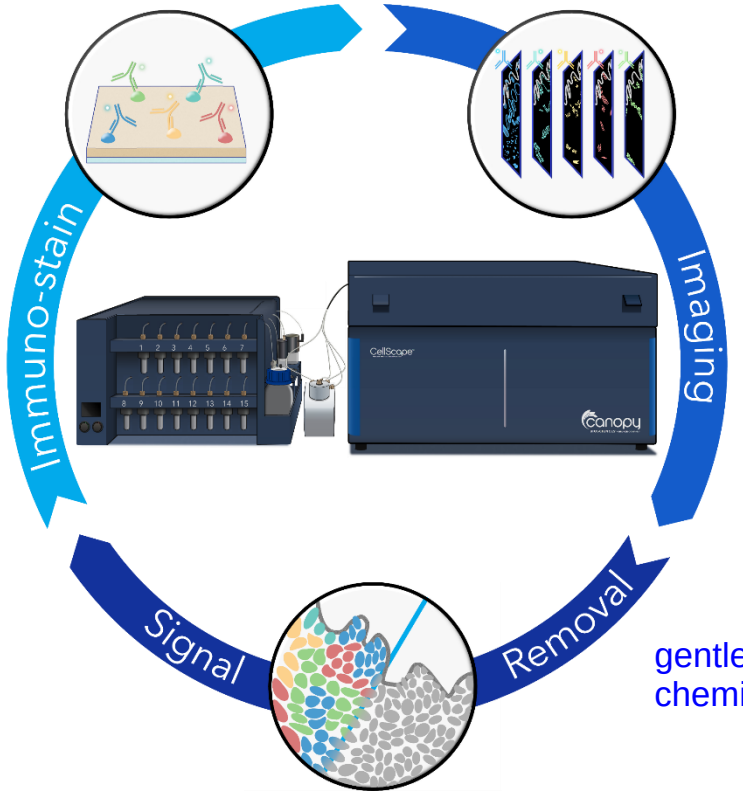
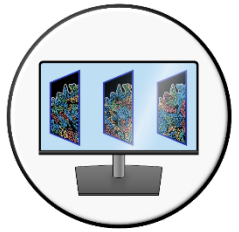


Image Overlay

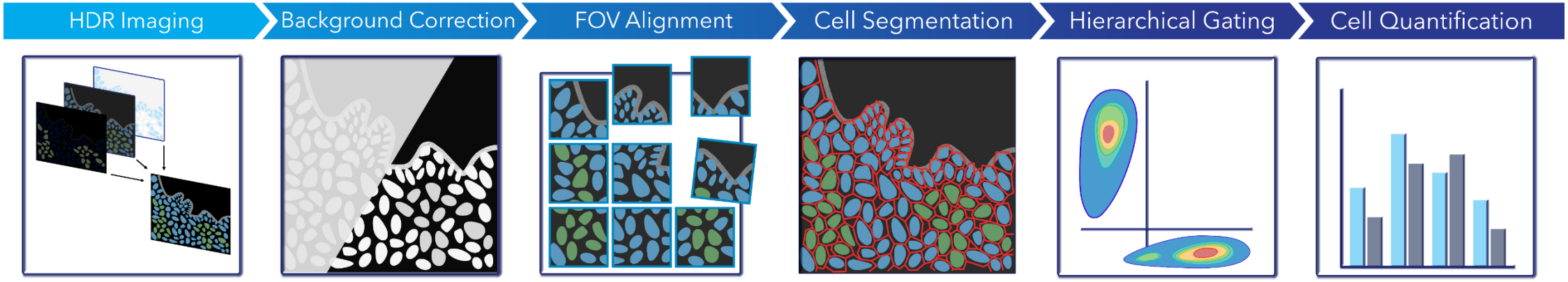


gentle photobleaching
chemical removal under development

Workflow

Samples are loaded onto CellScape™ chips and iteratively stained and imaged using fluorescently labelled antibodies. The serial images are digitally overlaid for virtually unlimited multiplexing of protein biomarkers.

Data Pipeline: Designed for Quantification

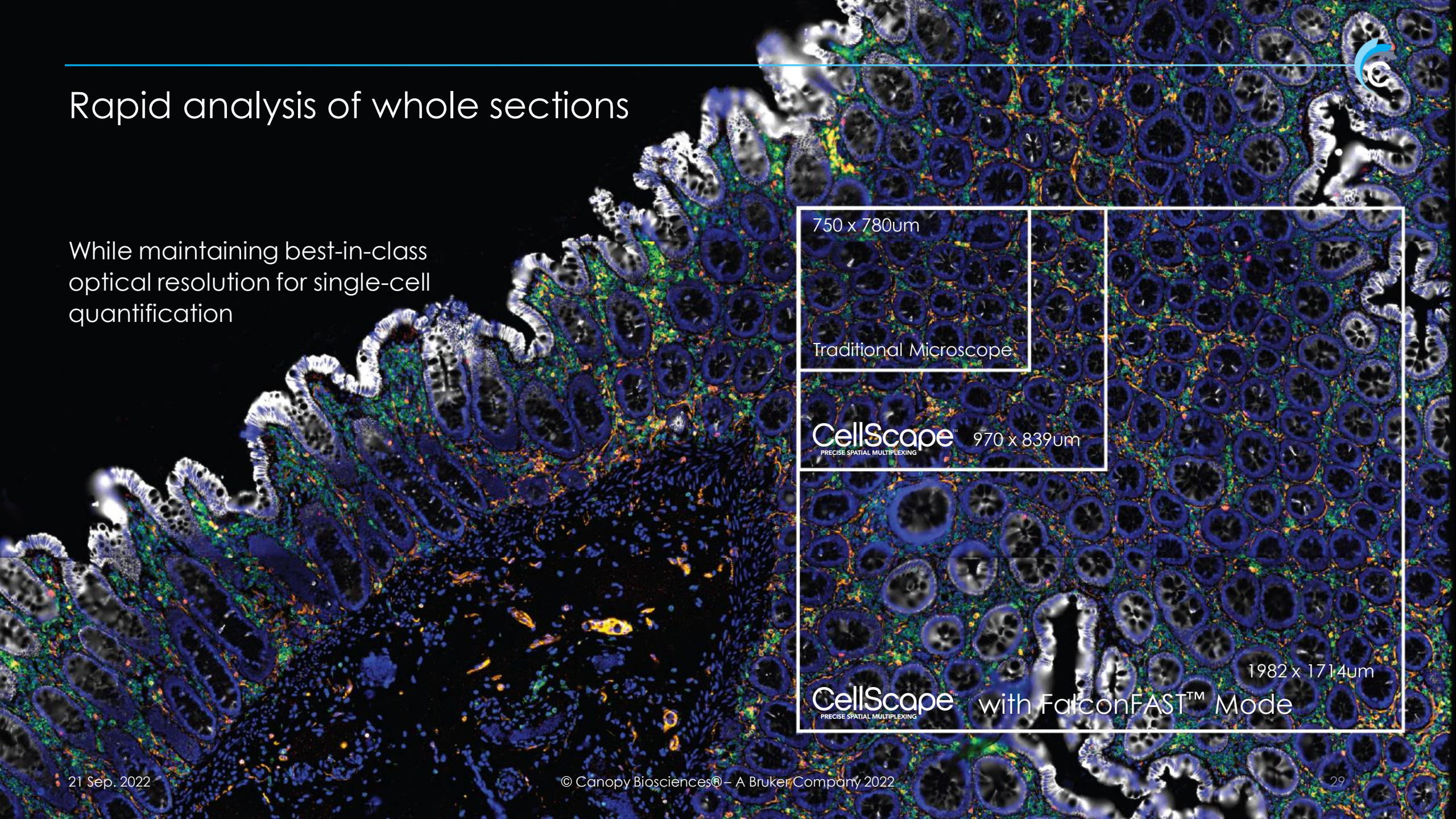


Workflow

HDR images are generated both pre-stain and post-stain. Background-corrected images are then aligned for before quantitative analysis is performed via cell segmentation and fluorescent value calculations.

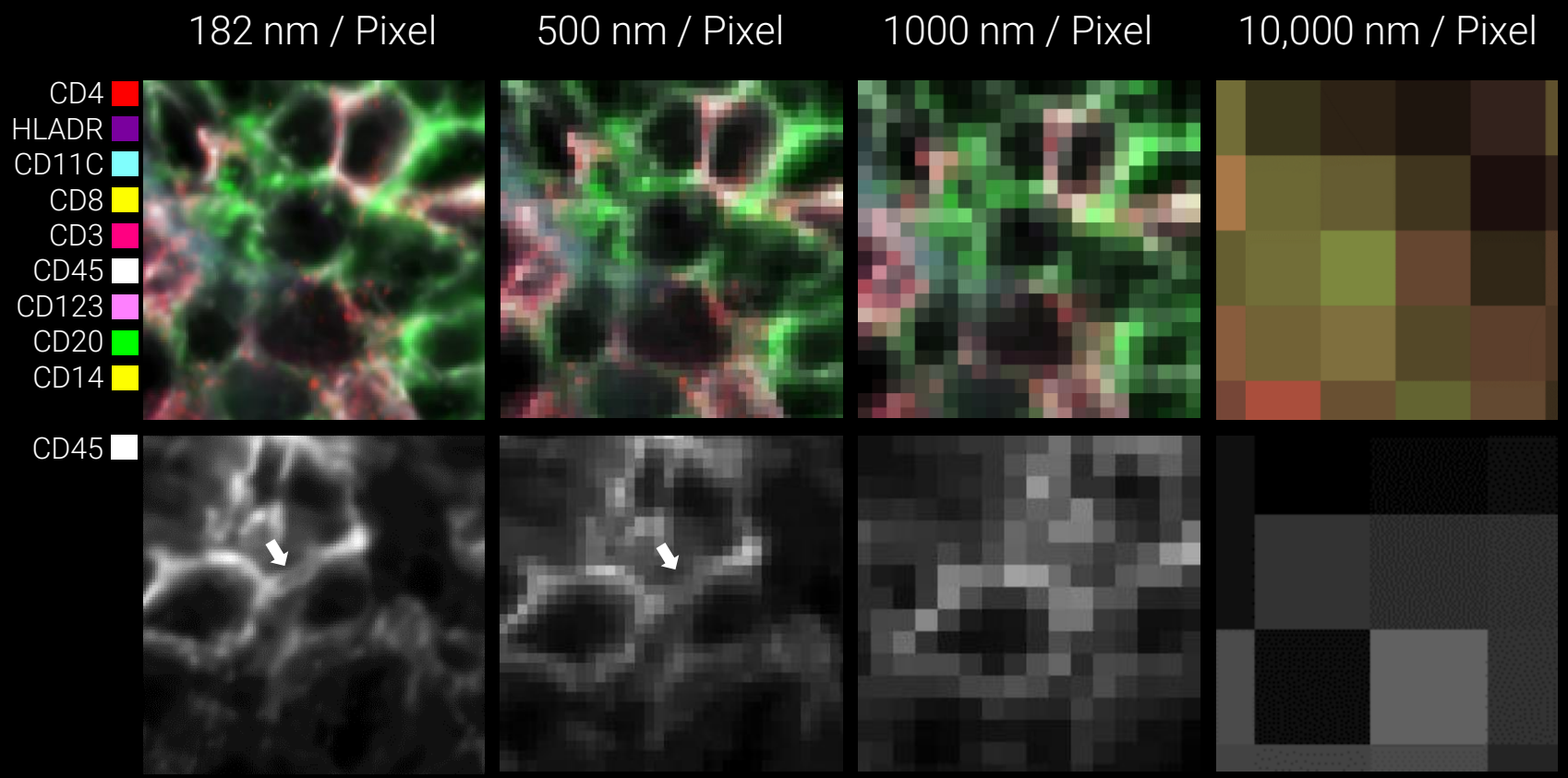
Rapid analysis of whole sections

While maintaining best-in-class optical resolution for single-cell quantification



Best-in-class optical resolution

Highest pixel density optical sensor available



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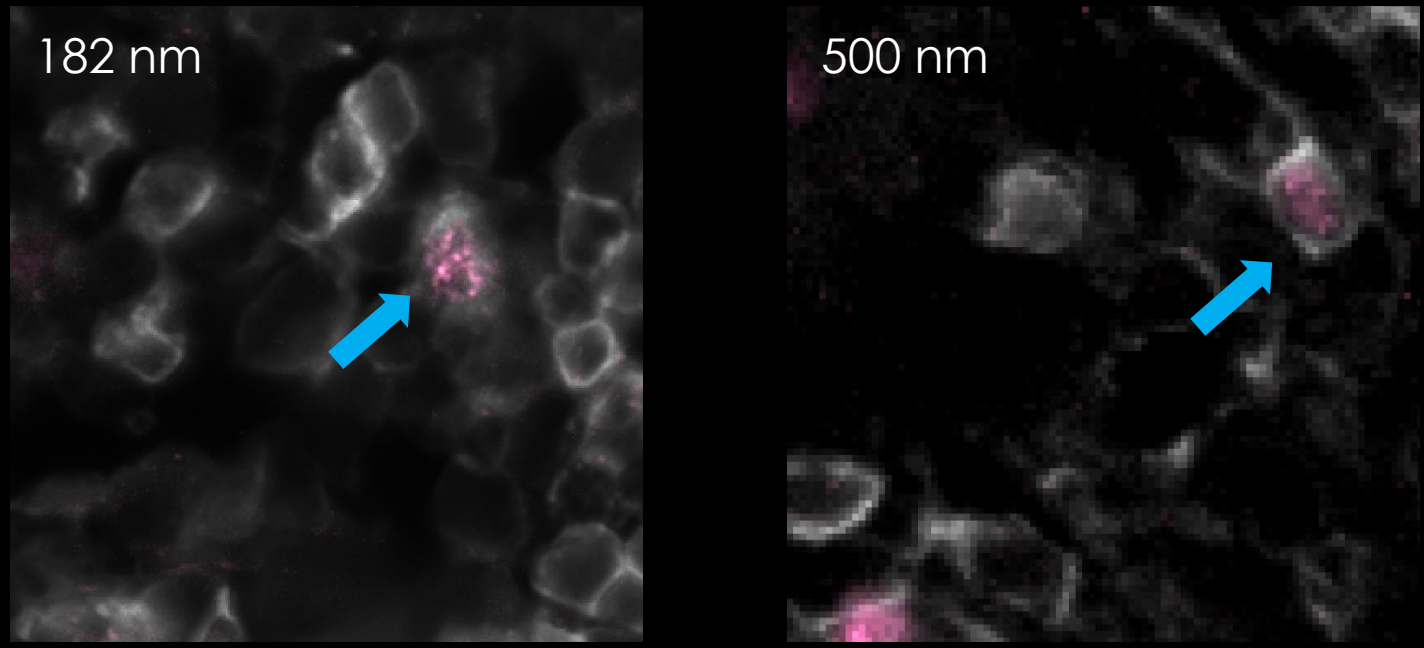
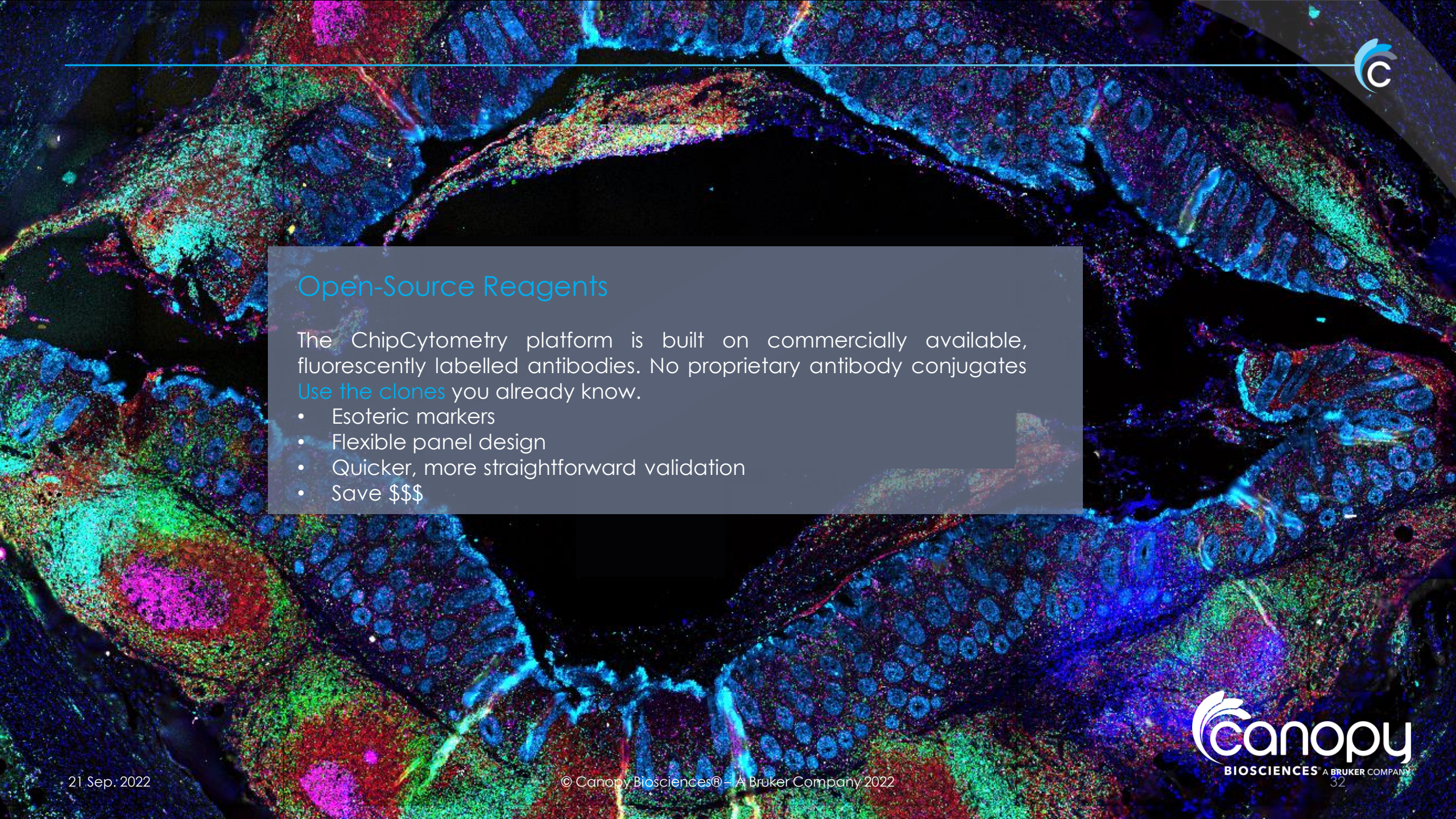


New biology to be discovered at this plex / resolution scale



Open-Source Reagents

The ChipCytometry platform is built on commercially available, fluorescently labelled antibodies. No proprietary antibody conjugates Use the clones you already know.

- Esoteric markers
- Flexible panel design
- Quicker, more straightforward validation
- Save $$$

Validated Antibodies

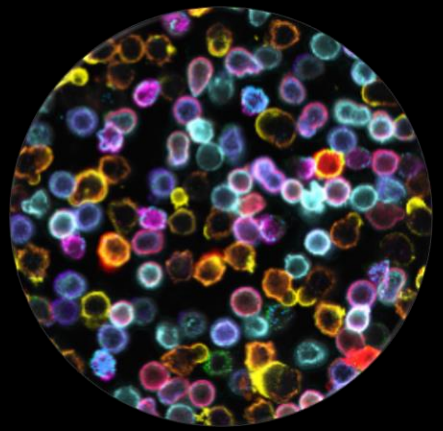
- Canopy Biosciences has validated more than >350 commercially available clones on the ChipCytometry platform
- Can customise marker panels
- All of the main immune cell markers
- Validated clones for Human Cell Suspensions (PBMCs), FF Tissue and FFPE Tissue (human, mouse, NHP)
- Open-source antibodies

Antibodies Validated for Human Cell Suspension

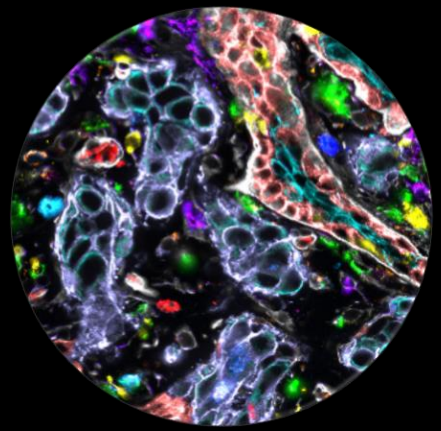
Active caspase-3	CD29	CD86	CD294	IL12
AhR	CD30	CD90	CD319	IL13
Aiolos (IKZF3)	CD31	CD95	CLA	IL17A
Bcl-2	CD33	CD102	Collagen IV alpha	IL17F
Beta-actin	CD34	CD115	CTLA-4	IL23R
CCR10*	CD36	CD117 (c-Kit)	CXCL10	Interferon gamma
CD1c	CD38	CD123	CXCL13	Ki-67
CD2	CD39	CD127	Endoglin	Lag-3*
CD3	CD40	CD134	EpCAM	Light chain kappa
CD4	CD45	CD137	FoxP3	Light chain lambda
CD5	CD45RA	CD138	Glycophorin A	MICA/B
CD8	CD45RO	CD141	Glycophorin A/B	p-Histone H3 (Ser10)
CD10	CD52	CD154 (CD40L)	GM-CSF	p-Stat1 (Tyr701)
CD11b	CD54	CD161	Granzyme B	p-Stat3 (Tyr705)
CD11c	CD56	CD172a/b	Helios	Pan-cytokeratin
CD14	CD57	CD183 (CXCR3)*	HLA-DR	PD-1
CD15	CD61	CD184 (CXCR4)*	IDO	PD-L1
CD16	CD62L	CD185 (CXCR5)*	IgA	Perforin
CD18	CD64	CD193 (CCR3)*	IgD	T-bet
CD19	CD66b	CD194 (CCR4)*	IgG	TCF1/TCF7
CD20	CD68*	CD195 (CCR5)*	IgM	TCR alpha/beta
CD21	CD69	CD196 (CCR6)*	IL1b	TIGIT*
CD22	CD71	CD197 (CCR7)*	IL2	TIM-3
CD24	CD73	CD206	IL4	TNF alpha
CD25	CD80	CD244	IL5	Vimentin
CD27	CD81	CD257 (BAFF)	IL8	Zap-70
CD28	CD83	CD278 (ICOS)	IL10	

Sample & Species Agnostic

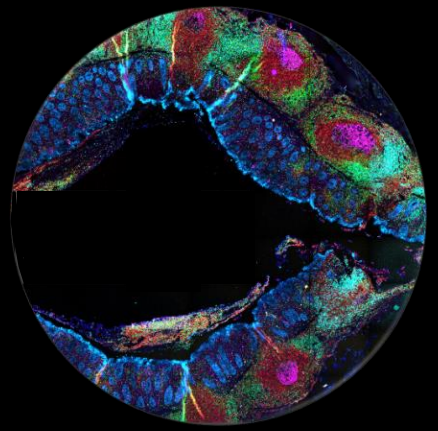
More than 350 validated antibodies



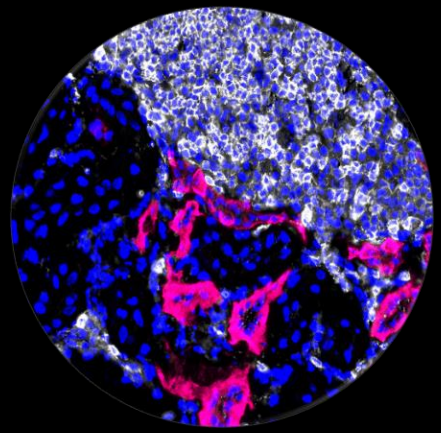
Dissociated cells
Human PBMCs



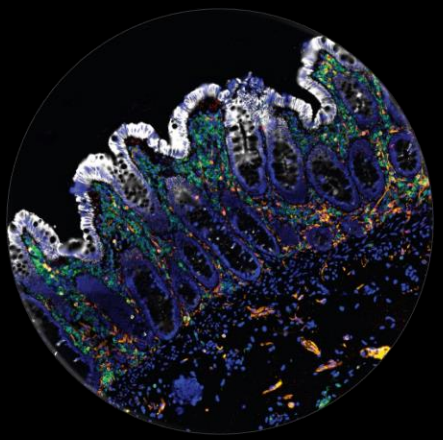
Fresh frozen tissue
Breast cancer



FFPE tissue
Appendix



Fresh frozen tissue
Mouse spleen



FFPE tissue
CRC

Human

Mouse

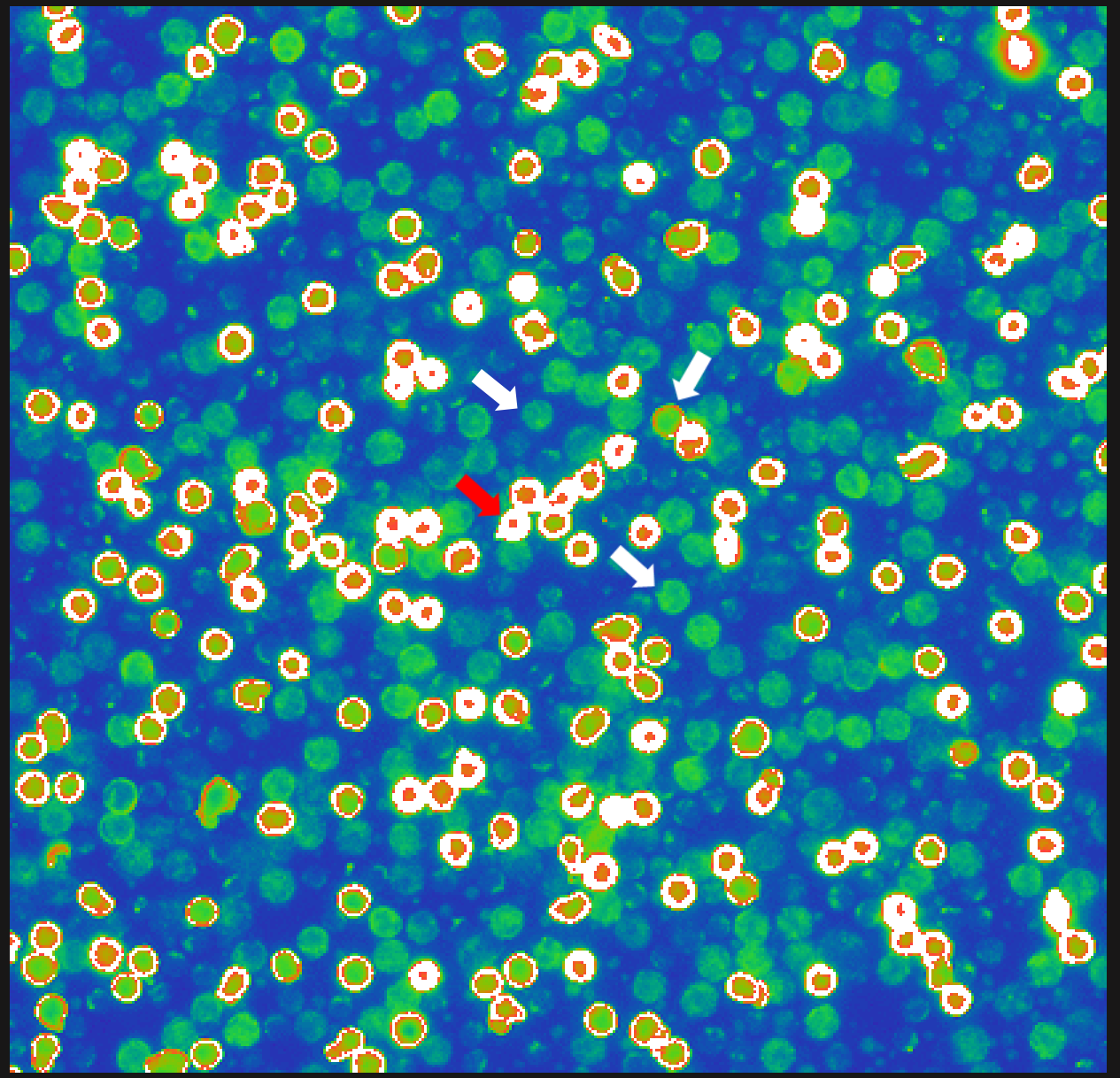
NHP

HDR imaging allows quantification over 8 logs of abundance

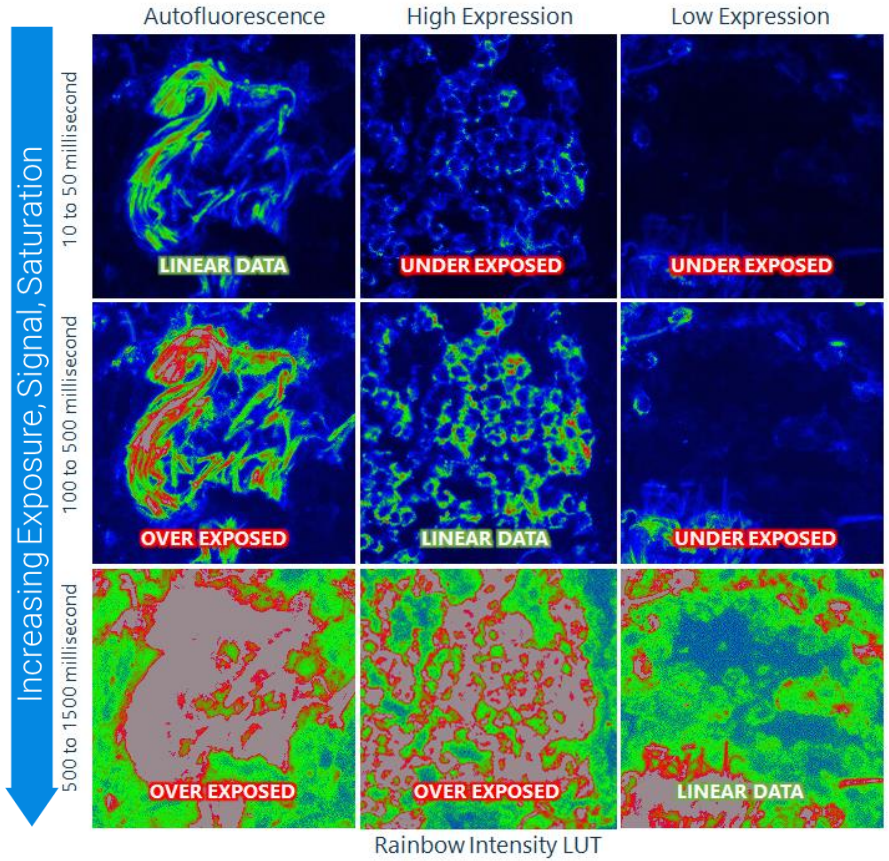
Both exquisite sensitivity and high-signal linearity are required for accurate sorting of all expression values.

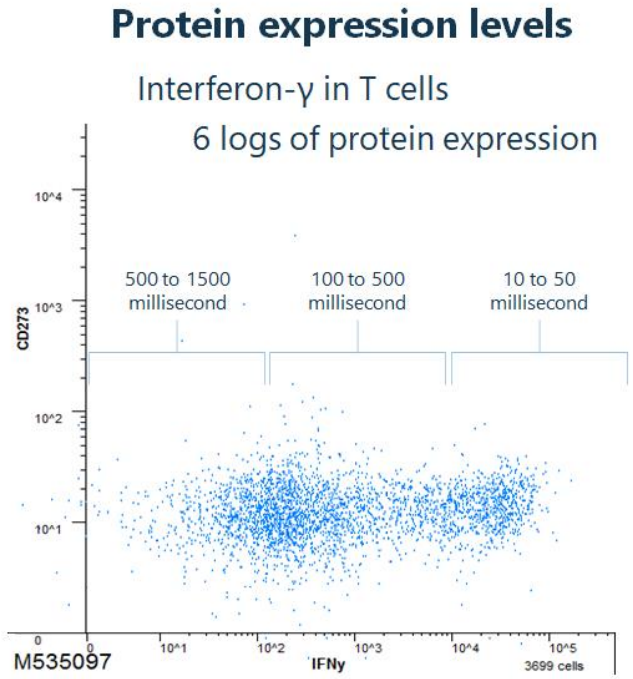
- Long Exposures capture quantitative information on dim cells, but over-saturate bright cells.
- Short Exposures capture quantitative expression among bright cells, but fail to provide any quantification on dim cells

Shown here: To accurately sort T cells, monocytes, and all other PBMC populations by CD4 expression, multiple exposure brackets are REQUIRED.



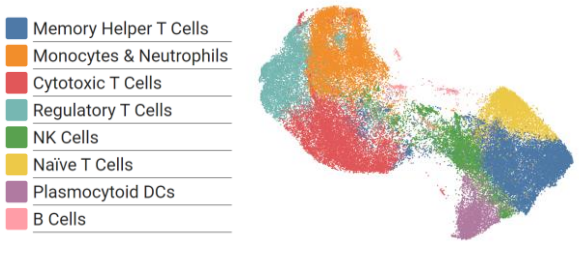
HDR image acquisition pipeline enables quantification of high- and low-expressing markers.



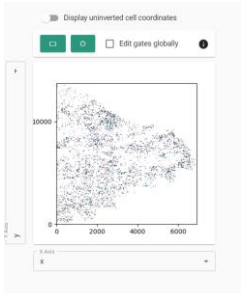


Multiple Exposure HDR is required to accurately capture biomarker expression

Open-source data analysis

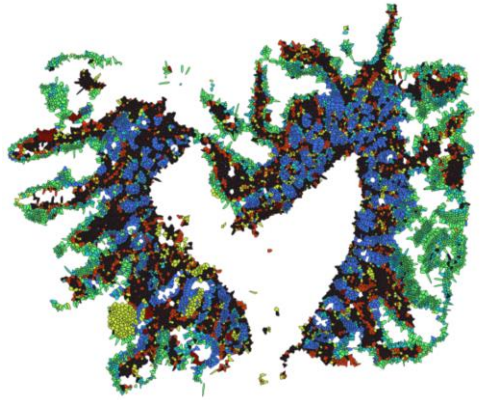


Unsupervised Clustering

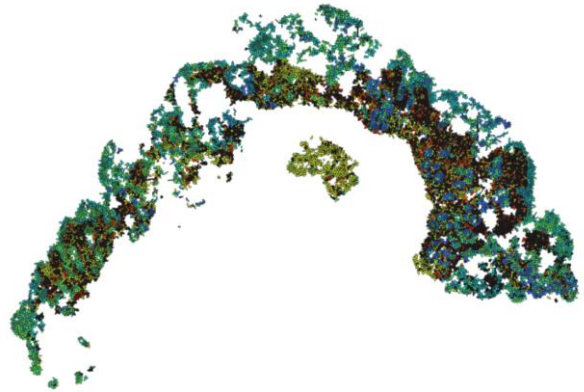


Automated Gating

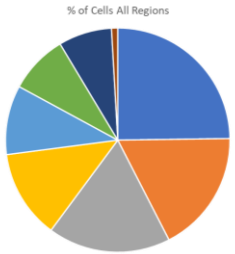
Active Celiac Disease



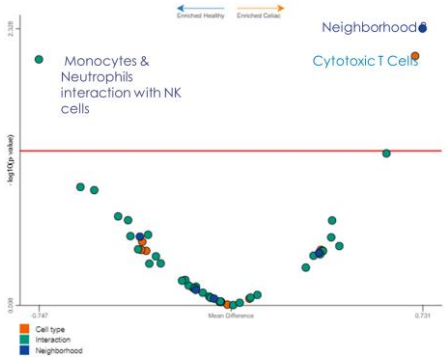
Healthy Control



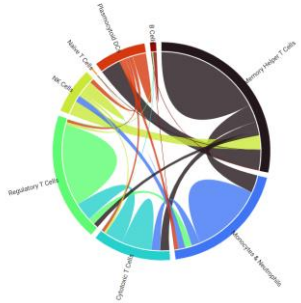
Interactive Voronoi Diagrams
Visual Map of Clusters in Space



Population Analysis

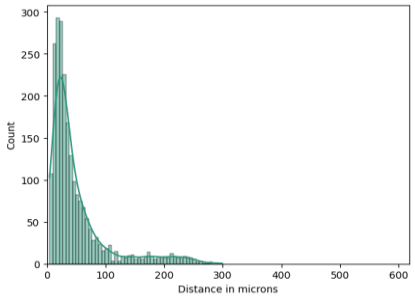


Volcano Plots
Two-cohort Analysis



Dendrograms
Similarity of Clusters

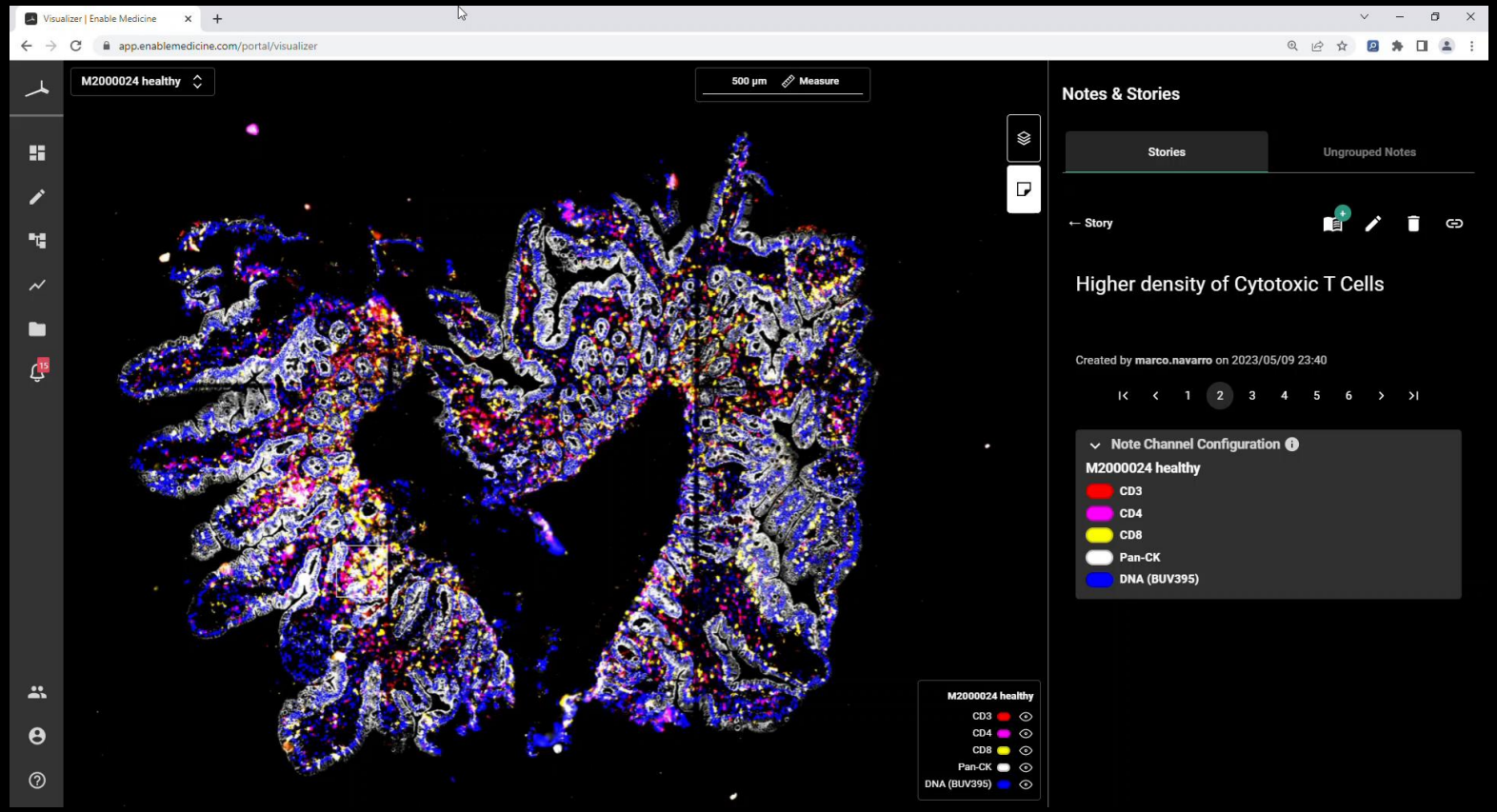
Celiac NK Cells to Monocytes and Neutrophils Clustered cells



Spatial Analysis
Nearest Neighbor & Distances

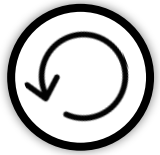
CellScape™ + ENABLE MEDICINE

PRECISE SPATIAL MULTIPLEXING

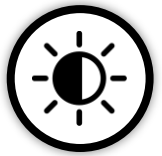


Key Benefits of ChipCytometry

Virtually unlimited multiplexing: Successive rounds of immunostaining, imaging, and signal removal to detect virtually unlimited numbers of proteins on the same sample



Sample reinterrogation: Biobanking on proprietary chip technology preserves samples with the option to reinterrogate samples up to 2 years later



High dynamic range: Multiple exposure and fusion algorithms enable robust quantification of high and low expression markers

Open-source reagents: Fluorescently labeled antibodies from any commercial vendor are part of a flexible and practical solution for spatial immunophenotyping

Species and sample agnostic: High-quality staining results on any species or sample type, including dissociated cells, frozen tissues, and archival FFPE tissues

High resolution imaging: With the highest pixel density optical sensor available, visualize single cells as well as subcellular structures

Questions and Answers

Steven.hawthorn@bruker.com

Spatial Biology Instruments & Services | Canopy Biosciences

Biobanking PBMCs on CellScape™ Chips using Density Gradient Separation Media

This protocol details the steps for separating peripheral blood mononuclear cells (PBMCs) from anticoagulated blood using density gradient separation media and loading onto cell chips for ChipCytometry experiments. The protocol uses CellScape™ cell chips and is compatible with the CellScape™ instrument. If you have additional questions, please contact us directly at samples.canopy@bruker.com.

MATERIALS & REAGENTS

- ☐ Swinging bucket centrifuge
- ☐ Tube adaptors for 12 × 75 mm tubes
- ☐ CellScape™ instrument or light microscope with 20X objective and phase contrast (optional)
- ☐ Falcon® Round Bottom Polystyrene Tubes (Corning, Cat. # 352058)
- ☐ Lymphosep™, Lymphocyte Separation Media (BioWest, Cat. # L0560-100)
- ☐ 2 pipettes (one *aspirating*, one *dispensing*)
- ☐ Pipette tips
- ☐ 1.5 mL Eppendorf Tubes® (Optional, for Step 3)
- ☐ CellScape™ Cell Chip Kit or CellScape™ Rare Cell Chip Kit
- ☐ Wash Station

Item	Size	Catalog #	Contents
CellScape™ Cell Chip Kit	10 chips	PRSM-CHP-CELL-010	10 CellScape™ Cell Chips 60 mL Wash Buffer 50 mL Storage Buffer 60 mL Fixation Buffer Chip Storage Box
CellScape™ Rare Cell Chip Kit	10 chips	PRSM-CHP-RARE-010	10 CellScape™ Rare Chips 60 mL Wash Buffer 50 mL Storage Buffer 60 mL Fixation Buffer Chip Storage Box
Wash Station	4 chips	PRSM-WASHSTAT-1	Wash Basin Frame

BEFORE YOU START

- Follow good laboratory practices and maintain a clean environment when working with samples. Note that the Storage Buffer is sterilized and should be replaced after 1 year to reduce risk of contamination.
- Blood should not be older than 2 hours and must be supplemented with anticoagulants (e.g., citrate, heparin, EDTA). Older samples may be compromised, and clinical data may not be readable.
- Use a centrifuge with a horizontal rotor (i.e., swinging bucket) and an adaptor for 12 × 75 mm tubes. Do not centrifuge at a higher speed or for longer time than noted, as this may lead to layer contamination.
- The protocol calls for careful aspiration of plasma or supernatant. Take care not to disturb the layers below with the pipette (i.e., gel barrier, PBMC layer or pellet).
- The protocol typically supports isolation of enough cells for a 1-chip experiment. However, this may vary for disease states where cell count is low. Verify cell density on the CellScape™ or with a light microscope.

STEP 1: BLOOD COLLECTION

a. Draw blood into tube
b. Mix blood and anticoagulant by gently inverting the tube 10 times.
c. Store at room temperature

Note: For optimum results, isolate PBMCs within 2 hours of blood collection.

STEP 2: PBMC ISOLATION

a. Add 1.5 mL Lymphosep to Falcon tube
b. In a separate Falcon tube, add 1 mL Wash Buffer to 1 mL of blood
c. Carefully layer the diluted blood on top of the Lymphosep phase. Do not mix.
d. Centrifuge for 10 minutes (465 g) at room temperature (Brake OFF, Acceleration 7, Deceleration 1)

Observation Point: The PBMCs are concentrated in a white layer just beneath the plasma layer (Figure 1). A reddish PBMC layer indicates**Error! Reference source not found.** contamination with red blood cells and poor sample quality.

e. Carefully collect the PBMC layer with a pipette
f. Transfer this fraction into a new Falcon tube

Note: For optimal results, collect PBMCs immediately after centrifugation.

g. Dilute cell suspension in 1 mL Wash Buffer. Place cap on tube.
h. Centrifuge for 5 minutes (100 g) at room temperature (Brake ON, Acceleration 9, Deceleration 9)
i. Carefully remove and discard the supernatant without touching the pellet.
j. Resuspend pellet in 1 mL CellScape™ Wash Buffer
k. Centrifuge for 5 minutes (100 g) at room temperature (Brake ON, Acceleration 9, Deceleration 9)
l. Carefully remove and discard the supernatant without touching the pellet.

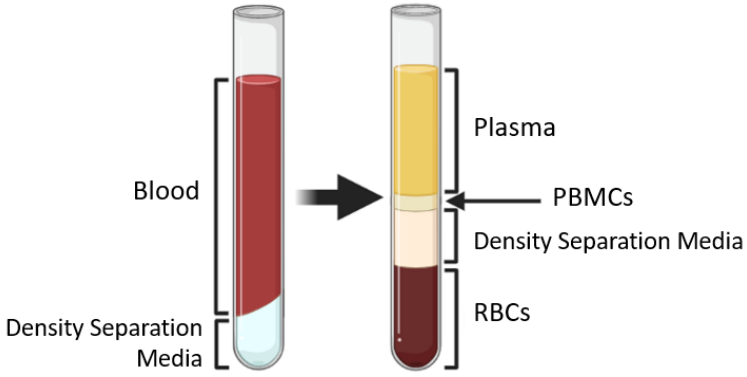


Fig. 1 | Density gradient layers pre- and post-centrifugation

OPTIONAL STEP 3: PRESTAIN CELLS

This step is optional. Skip this step if your experiment does not require pre-staining with select antibodies.

a. Prepare 300 µL antibody solution diluted in Storage Buffer in a 1.5mL Eppendorf Tube
b. Vortex gently to mix
c. Resuspend pellet in 300 µL antibody solution
d. Incubate for 5 minutes at room temperature
e. Centrifuge for 5 minutes (300 g) at room temperature (Brake ON, Acceleration 9, Deceleration 9)
f. Carefully pipette off the supernatant
g. Resuspend pellet in 300µL Wash Buffer
h. Centrifuge for 5 minutes at (300 g) at room temperature (Brake ON, Acceleration 9, Deceleration 9)
i. Carefully pipette off the supernatant

STEP 4: PREPARE CHIPS

a. Resuspend cell pellet in 200µl Wash Buffer.
b. Apply the patient identification label on the CellScape™ chip at the position indicated in Figure 2 (optional; label not included in the kit).
    - Label size must not exceed 1.3cm × 2.5cm.
    - Do not place label on the bottom of the chip.
    - Do not bend label over chip edges.
    - Do not write on the QR-code label.

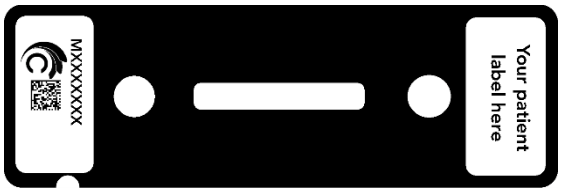


Fig. 2 | Space for patient identification label on the CellScape™ chip (label not included)

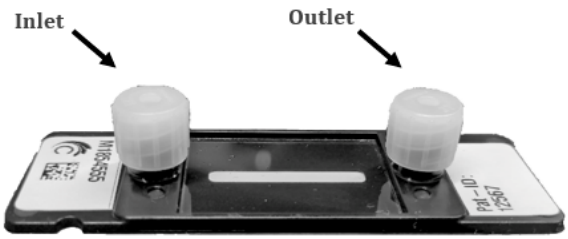


Fig. 3 | CellScape™ chip with sealing plugs blocking the inlet and outlet

c. Place the chip with label side up in the washing station.
d. Remove the sealing plug from the inlet of the chip (Figure 3) while leaving the outlet plug sealed.

Note: Do not discard sealing plugs as they are reusable.

e. Pipette a few drops of Wash Buffer into the inlet with *dispensing* pipette to prevent contracting air into the channel when removing the outlet plug.
f. Remove the sealing plug from the chip outlet.
g. Wash 3 times with 200 µL Wash Buffer (10 times for CellScape™ Rare chips) to remove sodium azide solution in chamber
    - Without touching the bottom of the well, aspirate buffer from the outlet with *aspirating* pipette.
    - Pipette 200µl Wash Buffer into the inlet with *dispensing* pipette. Observe liquid level rising in the outlet.
h. Aspirate buffer from the outlet with *aspirating* pipette.

STEP 5: LOAD CELLS

Before this step ensure that all air bubbles are removed from the inlet and outlet as well as the channel. Pipetting of all solutions (buffers and cell suspension) should be done drop-by-drop. **The chip should NEVER run dry!**

a. Pipette 100 µl cell solution into the inlet.
b. Tilt the chip with the inlet high to move the cell solution to the channel (Figure 4).

Fig. 4 | Tilting the chip ensures cell solution settles in the visible channel

c. Allow the cells to settle (5 min; RT).
d. Level the chip before rinsing.
e. Wash the chip with 5 × 200µl Wash Buffer.
f. Verify cell density with the CellScape™ or a standard light microscope (Figure 5).

Fig. 5 | Examples of cell density measurements

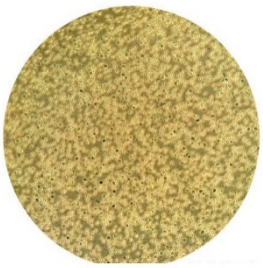


Fig. 5a | Acceptable cell density (200X)

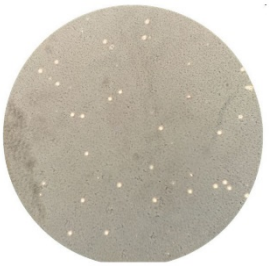


Fig. 5b | Low cell density (200X)

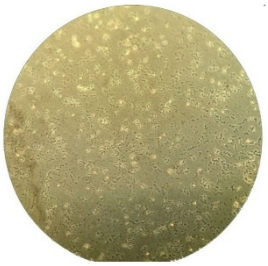


Fig. 5c | Dirt, no cells (200X)

STEP 6: FIX CELLS

a. Wash the chip with 5 × 200µl Fixation Buffer.
b. Incubate for 45 min at 4°C.
c. Following incubation, rinse the chip with 5 × 200µl Wash Buffer.

STEP 7: STORE CHIPS

a. Wash chip with 5 × sterile 200µl Storage Buffer.
b. Replace outlet sealing plug first, then inlet sealing plug on CellScape™ chip
c. Store at 4°C

Note: Exchange with fresh, sterile CellScape™ Storage Buffer after approx. one year to prevent contamination.

Note: CellScape™ chips that are to be shipped should be stored in a CellScape™ Box. The shipping conditions are 4°C with temperature tracking (RFID). **DO NOT FREEZE!**

TROUBLESHOOTING

See table below for troubleshooting.

Problem	Possible cause	Solution
No defined PBMC layer	Incorrect adapter size	Use centrifuge tube adapter
	Centrifuge not calibrated	Refer to centrifuge manual to recalibrate
	Centrifugation speed too low	Increase speed to 465 g
	Centrifugation time too short	Increase centrifugation time (up to 30 min)
Reddish PBMC layer	Blood sample too old	Process blood within 2 hours after collection
	Blood volume too high	Decrease sample volume to 1 mL
Air bubbles in the chip	Air infiltration	Move air bubbles towards the channel end by tilting the barcode side of the chip up. Carefully insert the pipette tip onto the inlet bottom, pointing toward the channel, then rapidly push ca. 500µl Wash Buffer into the channel. The bubble will exit the channel through the outlet. CAUTION: THE OUTLET WILL OVERFLOW. USE GOGGLES AND GLOVES. DO NOT CLEAR CHANNEL BUBBLES IN THIS MANNER IF UNFIXED CELLS ARE LOADED
Low cell count on chip	Low cell count	If possible, reload the chip with 100 µL of sample BEFORE fixation