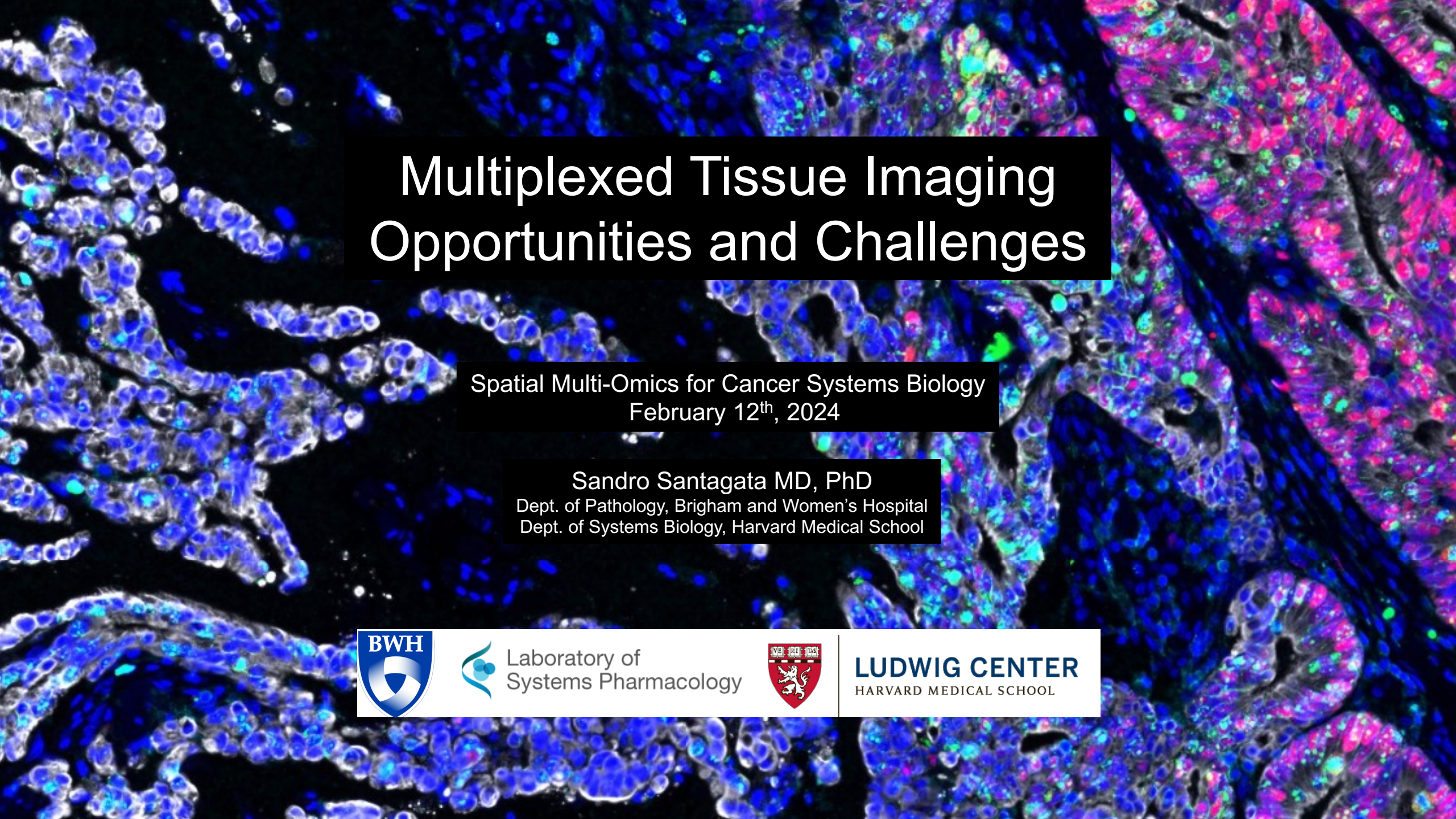


Multiplexed Tissue Imaging Opportunities and Challenges

Spatial Multi-Omics for Cancer Systems Biology
February 12th, 2024

Sandro Santagata MD, PhD
Dept. of Pathology, Brigham and Women's Hospital
Dept. of Systems Biology, Harvard Medical School

BWH

Laboratory of Systems Pharmacology

LUDWIG CENTER
HARVARD MEDICAL SCHOOL

No financial disclosures or COI to declare

Collaboration with RareCyte Inc. on the Orion method for tissue imaging

NCI SBIR (R44-CA224503)

Objectives

Our Approach for Antibody Multiplexed Tissue Imaging

Practical overview of:

Tools, Technologies, Software - Analysis, Visualization, Sharing

Include 3 approaches for multiplexed imaging

1. CyCIF (research) WSI
2. Orion WSI H&E + IF (research ←→ clinical) – clinical translation
3. 3D imaging (cell biology discovery → future)

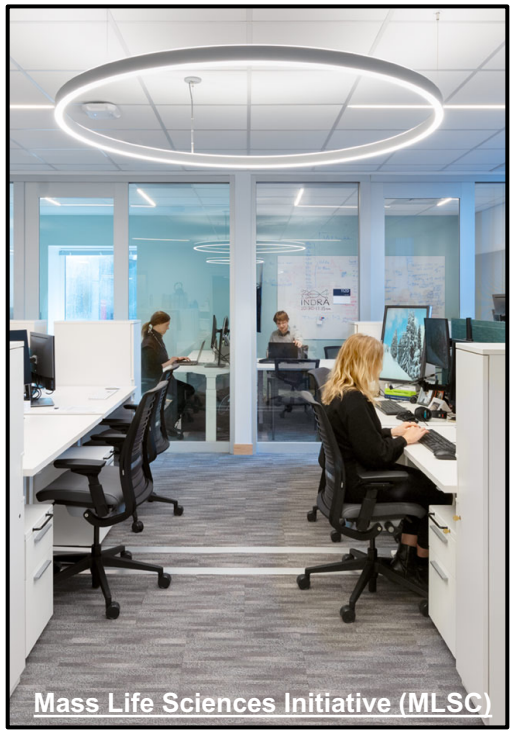
Applications are VAST - any normal or disease tissue (human or mouse)
FFPE (morphology) or Frozen (integrative)

- Basic Biology
- Tissue Biology
- Clinical and Clinical Trial Biomarkers
- Atlas Building
- etc.

Laboratory of Systems Pharmacology

"LSP"

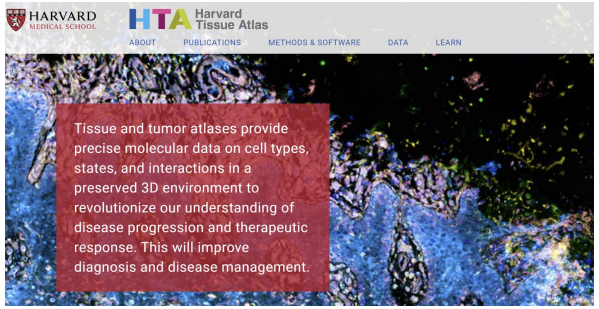
Peter Sorger
Director of LSP



Laura Maliszewski
Executive Director

Multi-institutional and multi-disciplinary team

tissue-atlas.org/



LUDWIG CANCER RESEARCH
FOUNDATION
gray.

NIH NATIONAL CANCER INSTITUTE

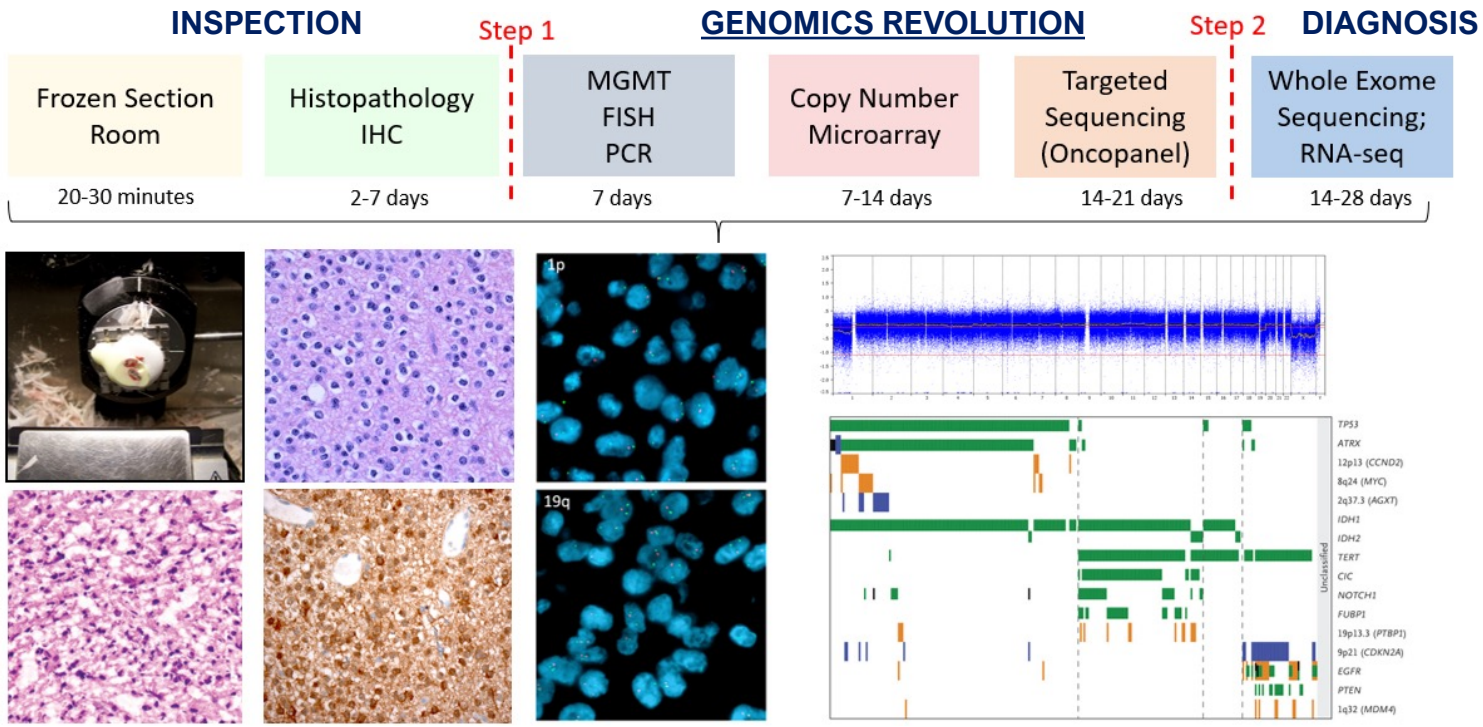
THE BRIDGE PROJECT

CANCER SYSTEMS BIOLOGY CONSORTIUM

HTAN
HUMAN TUMOR ATLAS NETWORK

R01-CA194005
U54-CA225088
U2C-CA233262
R44-CA224503
R41-CA224503
U01-CA284207
R01-CA279550

Expanding the Pathologist's Toolbox to Better Study Tissue



Improve classification
Improve risk assessment
Improve interception of early disease
Improve treatment and outcomes

IMPROVE DISCOVERY - GETTING BETTER RESULTS REQUIRES A MUCH BETTER UNDERSTANDING OF CANCER TISSUES

@NCIHTAN

Basic fundamental properties of cancer and immune cells WITHIN TISSUES and how do they change with progression and treatment

Cell
The Human Tumor Atlas Network: Charting Tumor Transitions across Space and Time at Single-Cell Resolution
Leading Edge
Perspective
236 Cell *181*, April 16, 2020 © 2020 Elsevier Inc.

Principal Research Method - Multiplexed Tissue Imaging of Whole Slides

- Simple
- Available reagents
- Flexible
- Reagent validation
- Relatively low cost

(~$150-200/slide)

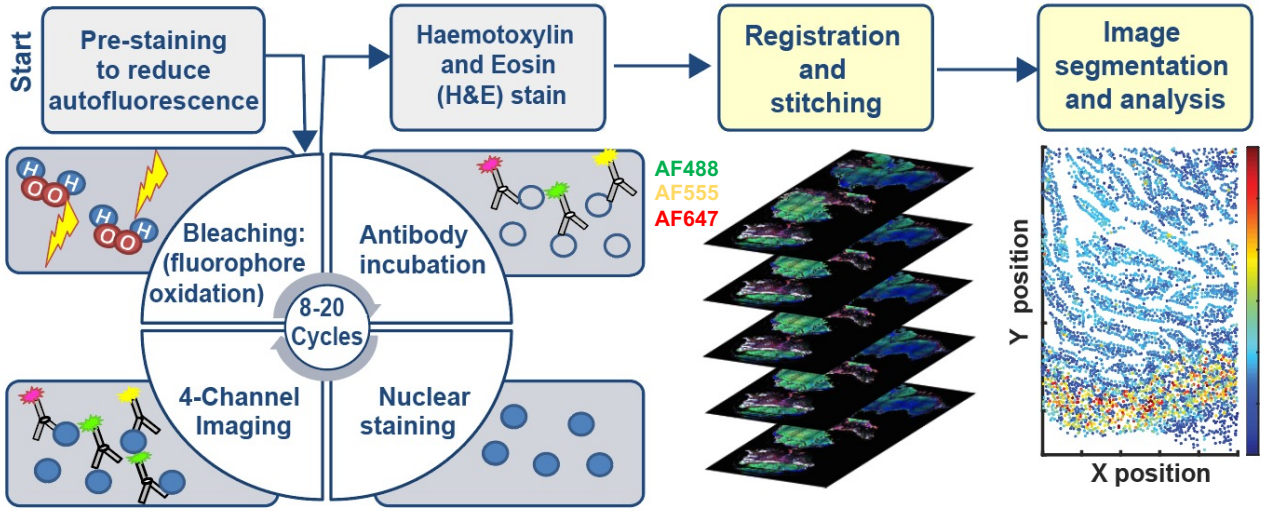
30-40 plex (>100-plex)

- Easy to perform
- Robust
- WSI

Jerry Lin

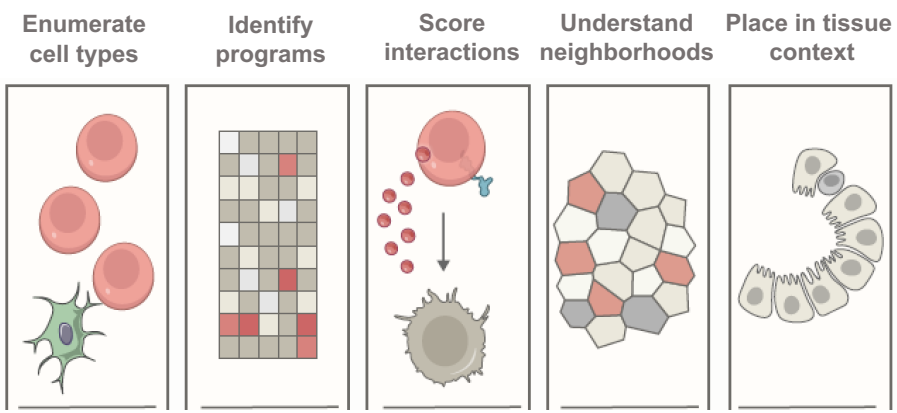
Peter Sorger

CyCIF (Cyclic Immunofluorescence) - generate multichannel images



Elife 2018 Jul 11;7:e31657 tissue-atlas.org/

Whole Slide Imaging - Tools for Data Processing & Sharing



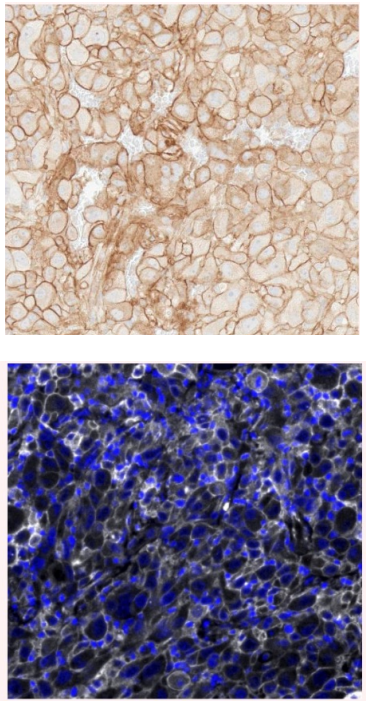
ASHLAR
CyLinter
IMAGE SEGMENTATION FILTER
MCMICRO
SCIMAP
SPATIAL SINGLE-CELL ANALYSIS TOOLKIT
miti
MINERVA

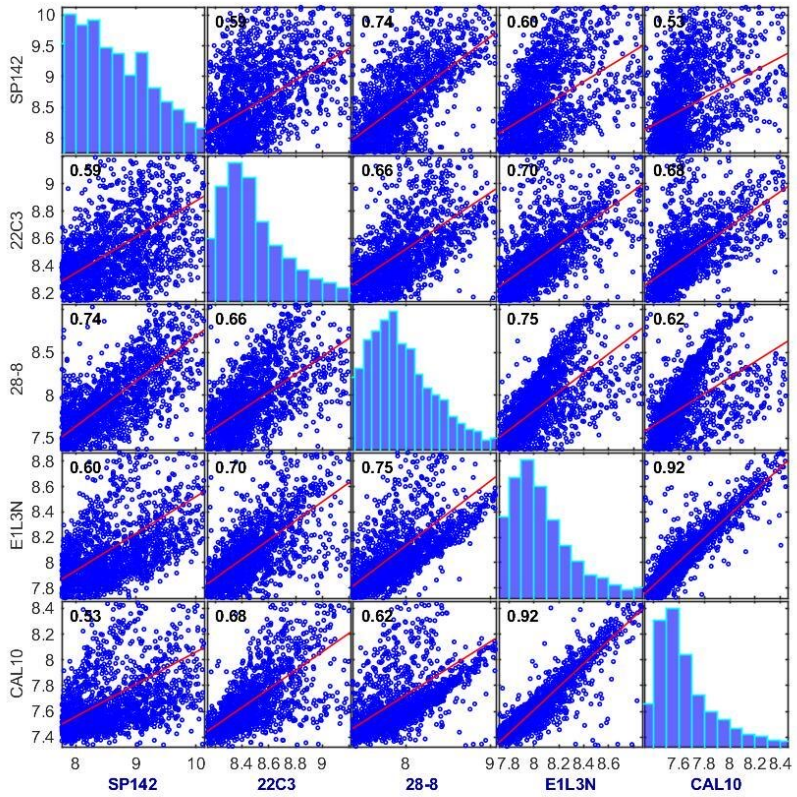
1. Elife. 2018 Jul 11;7:e31657
2. Nat Protocol. 2019 Oct;14(10):2900-2930
3. Nat Biomed Eng. 2022 May;6(5):515-526
4. Nat Methods. 2022 Mar;19(3):311-315
5. Nat Methods. 2022 Mar;19(3):262-267
6. Bioinformatics. 2022 Aug 16;38(19):4613-21
7. IEEE Trans Vis Comput Graph. 2022 Sep 28
8. Commun Biol. 2022 Nov 18;5(1):1263.
9. bioRxiv . 2023 Nov 1:2023.11.01.565120. doi: 10.1101/2023.11.01.565120.

Building Antibody Panel – Does an antibody 'work'?

Pixel-by-pixel quantitative comparison of multiple antibody clones directly on the same tissue section

PD-L1





Pixel-by-pixel analysis of
5 anti-PD-L1 clones on same section
(Gagne, Lin, unpublished)

- Signaling molecules
- Metabolic markers
- Post-translational modifications
- New targets
- Etc.

Approach:
Du Z, et al. Qualifying antibodies for image-based immune profiling and multiplexed tissue imaging. **Nature Protocols. 2019 Oct;14(10):2900-2930.** doi: 10.1038/s41596-019-0206-y. Epub 2019 Sep 18. PMID: 31534232; PMCID: PMC6959005.

Example in developing a breast cancer panel (HER2, ER, etc.):
Guerriero JL, et al. Qualification of a multiplexed tissue imaging assay and detection of novel patterns of HER2 heterogeneity in breast cancer. **NPJ Breast Cancer. 2024 Jan 2;10(1):2.** doi: 10.1038/s41523-023-00605-3. PMID: 38167908; PMCID: PMC10761880.

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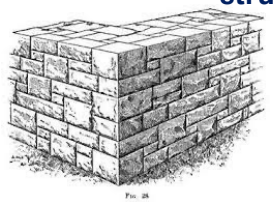
Whole slide tissue images are very large: Assembly is required

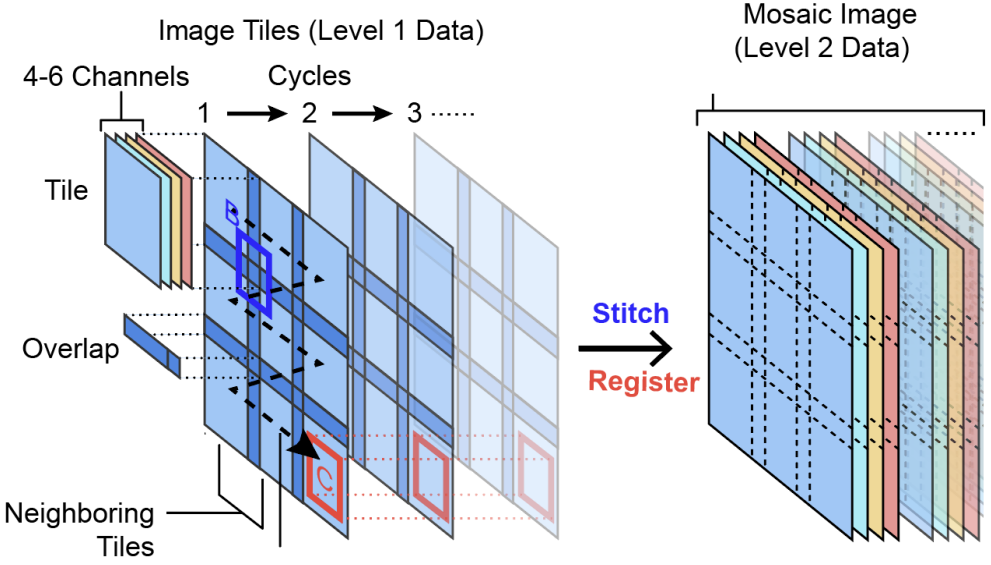
ASHLAR

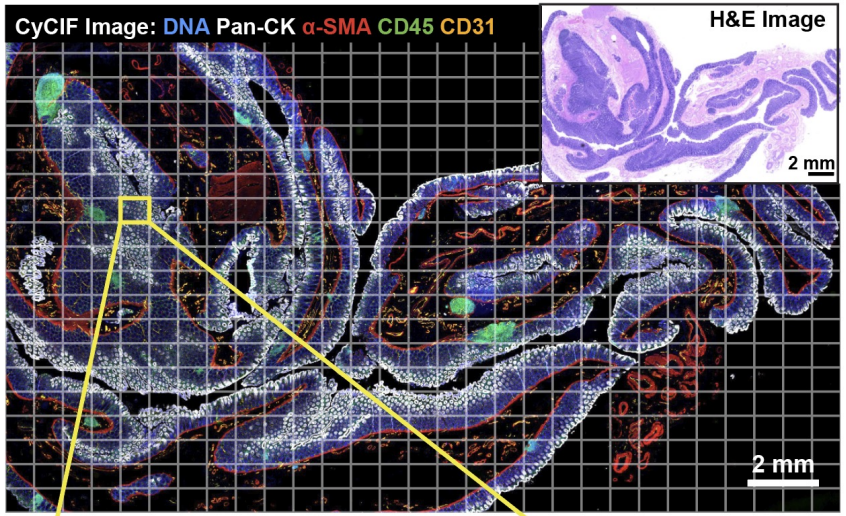
Ashlar masonry – building of stone structures from precisely cut stones

Jeremy Muhlich

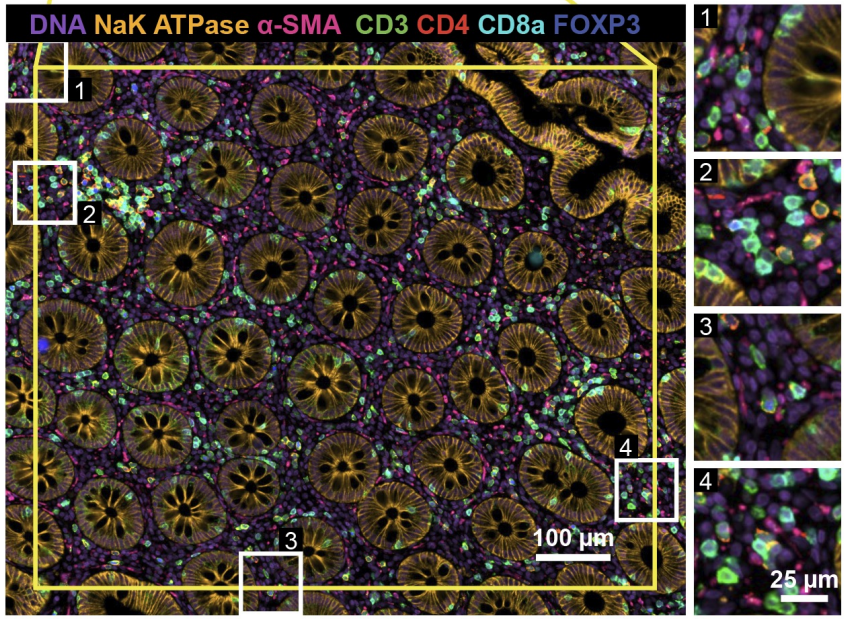


Mosaic





One tile



https://labsyspharm.github.io/ashlar/

Muhlich JL, et al. Stitching and registering highly multiplexed whole-slide images of tissues and tumors using ASHLAR. **Bioinformatics.** 2022 Sep 30;38(19):4613-4621. doi: 10.1093/bioinformatics/btac544. PMID: 35972352; PMCID: PMC9525007.

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Digging into datasets: Metadata and image processing

miti

- Link images to metadata standards; levels of data

NATURE METHODS | VOL 19 | MARCH 2022 | 262–267
MITI minimum information guidelines for highly multiplexed tissue images **HTAN consortium/DCC**

https://www.miti-consortium.org/

- Automate standard image processing tasks

MCMICRO

NATURE METHODS | VOL 19 | MARCH 2022 | 311–315
MCMICRO: a scalable, modular image-processing pipeline for multiplexed tissue imaging

https://mcmicro.org/

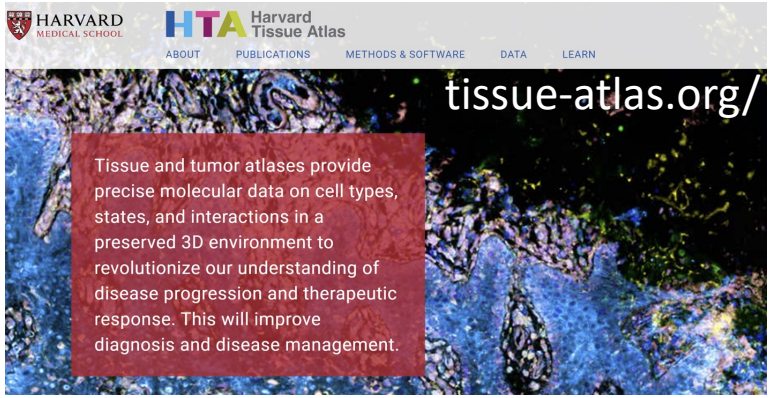
- Provide command line and graphical user interfaces
- Enable deployment across platforms and image scales
- Educate users through documentation, tutorials, and workshops (outreach)

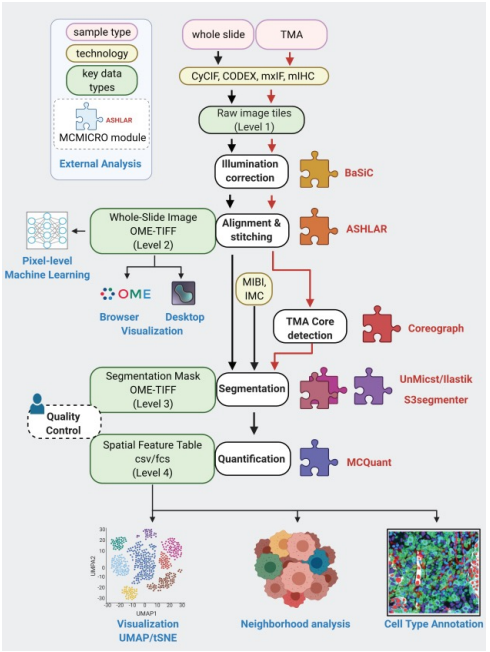

Sarah Arena


Allison Maier

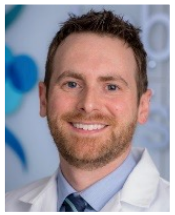

Juliann Tefft


Han Xu

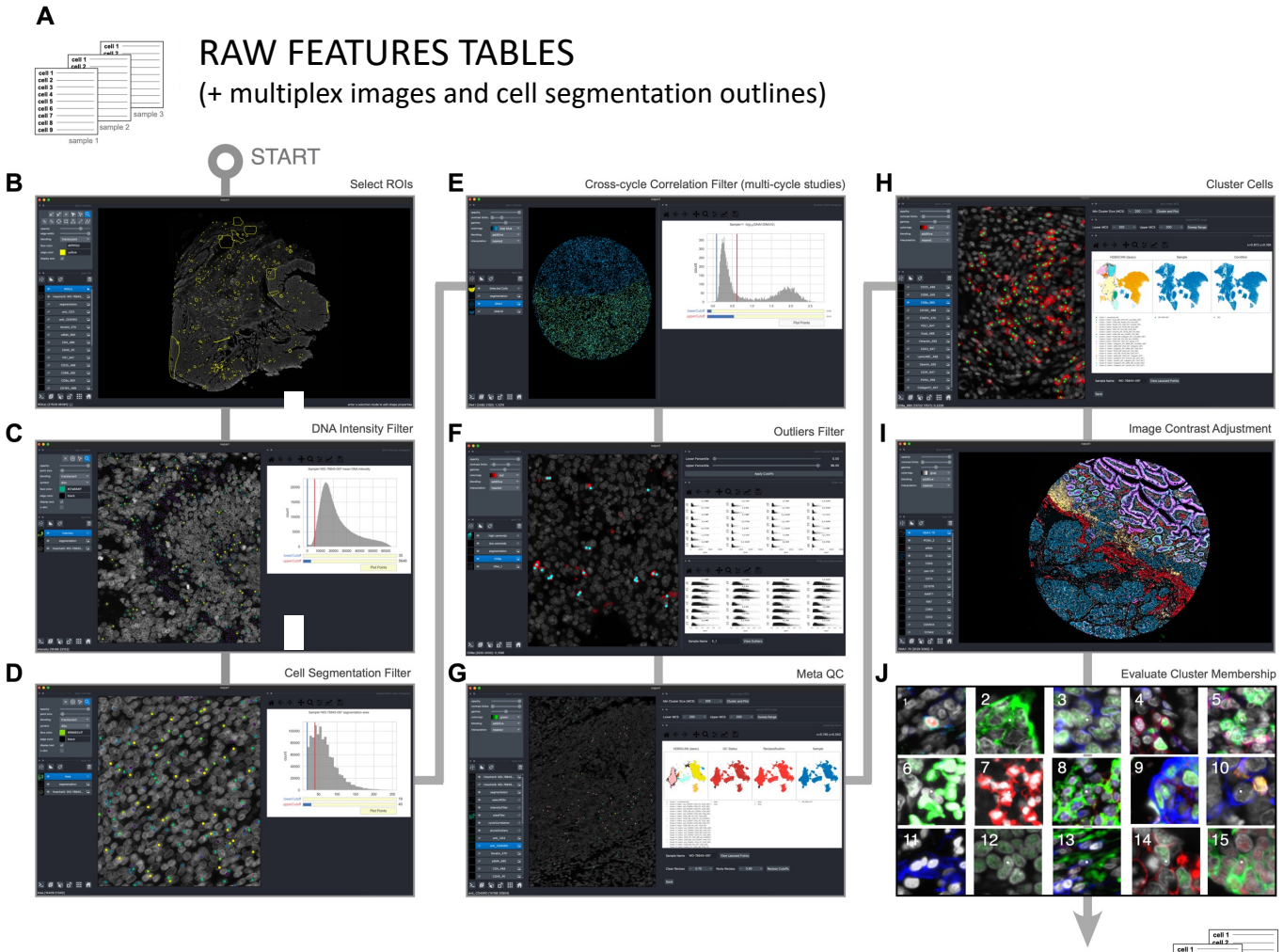




Interactive Quality Control for Highly Multiplex Microscopy



Greg Baker



https://labsyspharm.github.io/cylinter/

Baker GJ, et al. Quality Control for Single Cell Analysis of High-plex Tissue Profiles using CyLinter. **bioRxiv [Preprint].** 2023 Nov 1:2023.11.01.565120. doi: 10.1101/2023.11.01.565120. PMID: 37961235; PMCID: PMC10634977.

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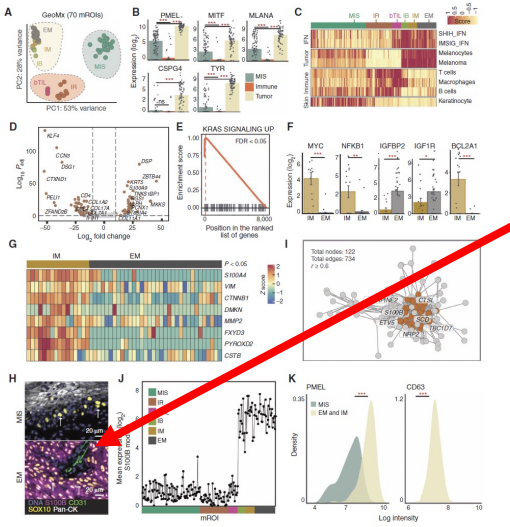
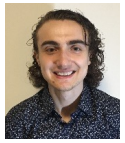
Challenge: How to interact with and share the resulting data?

Research



Postage stamp

Figure 6. Microregional transcript profiling. **A**, PCA plot of melanoma mrSEQ transcriptomes (GeoMx). Colors indicate regional histopathology: DTIL (pink), IR (brown), MIS (green), invasive front (IB; light green), EM (gray), and center of invasive melanoma tumor (IM; yellow). EM and IM are enriched for tumor cells in this analysis, and IB contains mostly tumor cells with marginal immune infiltration. **B**, Expression of selected melanoma-related marker genes in mrSEQ data (PickSeq) split into three broad groups based on the PCA of GeoMx data (**A**). Data are mean ± SEM. ****, $P < 0.001$; ns, not significant. **C**, ssGSEA on mrSEQ data (PickSeq). ssGSEA scores highlight enrichment of melanoma-related gene signatures in tumor mROIs (primarily IB, IM, and EM) and immune-related signatures in the immune-rich mROIs (IR, bTIL). **D**, Fold difference ($\log_2$) and significance ($\log_{10} P_{adj}$) for expression of 19,500 genes between EM (n = 34) and IM (n = 16) mROIs (PickSeq). Differentially expressed genes above (brown) and below (blue/gray) a significance threshold ($P_{adj} = 0.05$) and above a fold change threshold ($\log_2$ fold change = 1.0) are indicated. **E**, GSEA for upregulation of the KRAS pathway in IM (n = 16) compared with EM (n = 34) mROIs (PickSeq). FDR < 0.05. **F**, Expression ($\log_2$) of MYC, NFKB1, IGFBP2, IGF1R, and BCL2A1 in IM and EM mROIs (PickSeq). Data, mean ± SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. **G**, Heat map showing expression of genes (listed on the *y*-axis) known to play a role in EMT transition (PickSeq). All genes showed a significant difference between their mean expression in IM versus EM mROIs ($P < 0.05$). **H**, CyCIF image showing an FOV in MIS (top) and EM (bottom) regions. The tissue is stained for melanocytes (SOX10: yellow), endothelial cells (CD31: green), keratinocytes (pan-CK: white), and tumor cells (S100B: magenta). Arrows mark examples of melanocytes and tumor cells. **I**, Correlation network subgraph of genes associated with S100B expression in mrSEQ data (PickSeq). Nodes represent genes, and the edges correspond to the correlation between them. Brown nodes represent the genes that belong to the S100B module. Selected genes are annotated. **J**, Mean expression of 35 genes identified within the S100B module in mrSEQ data (PickSeq). The *x*-axis represents the mROIs grouped into the histopathologic annotation category from which they were isolated. **K**, Density plots illustrating the log-scaled protein expression of PMEL and CD63 in MIS and tumor (EM and IM) regions imaged with CyCIF. ****, $P < 0.001$.



John Hoffer

Rumana Rashid

Yu-An Chen

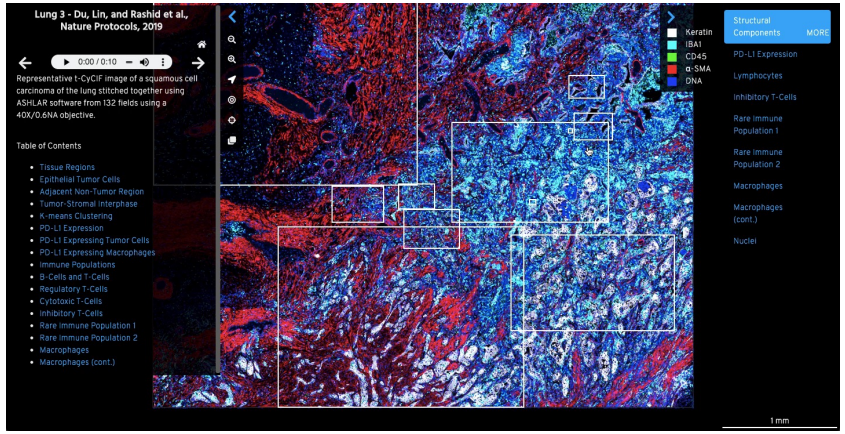
Robert Krueger

Jeremy Muhlich

Sarah Arena

Pathology Review

MINERVA Image Review & Interaction

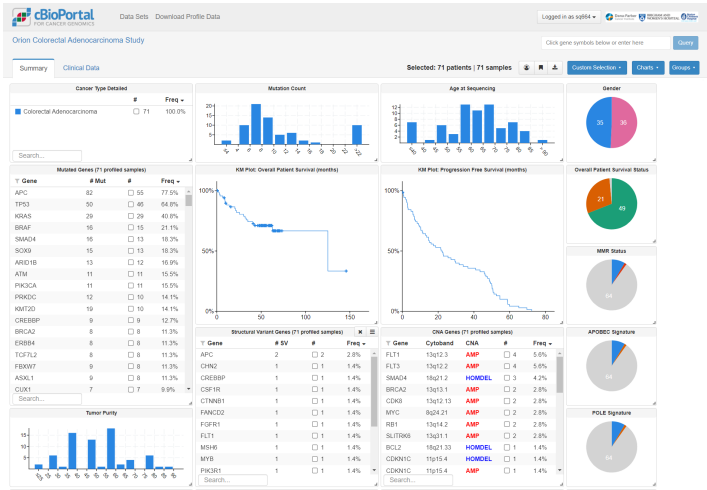


Minerva Story https://tinyurl.com/LungAdenoTour

HMS https://www.tissue-atlas.org/
https://www.minerva.im/

Additional Sharing

Integrating Minerva into cBioPortal



MINERVA v2.0 + cBioPortal FOR CANCER GENOMICS

Ritika Kundra

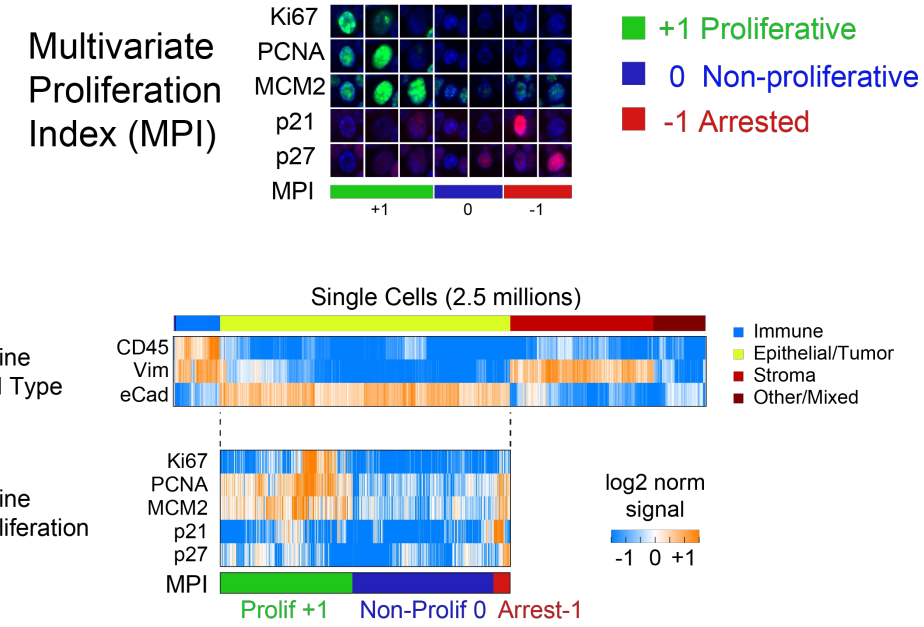
Niki Schultz

Memorial Sloan Kettering Cancer Center

Baby Anusha Satravada

Ino de Bruijn

Application: CyCIF for Measuring the Metrics of Cancer



Temporal and spatial topography of cell proliferation in cancer

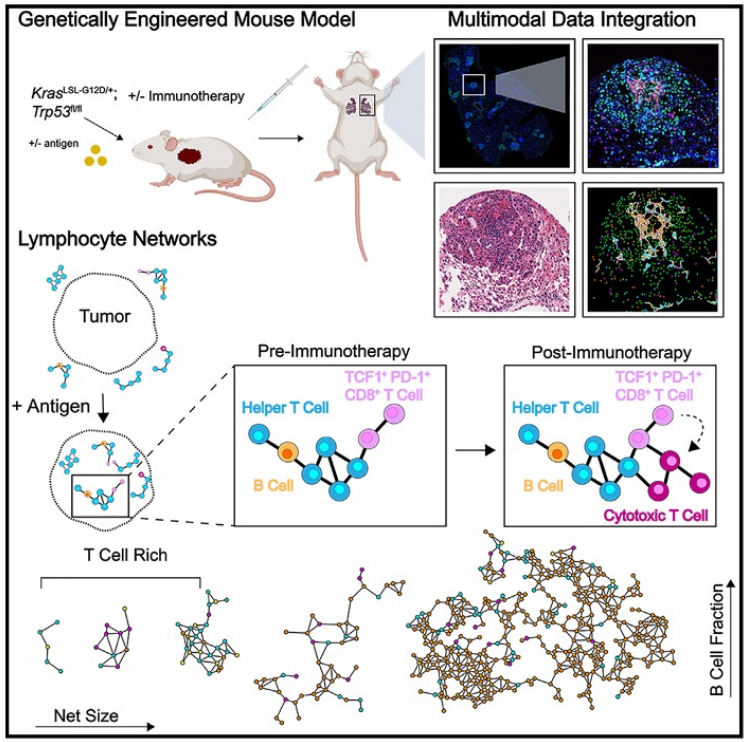
Giorgio Gaglia, Sheheryar Kabraji, Danae Rammos, Yang Dai, Ana Verma, Shu Wang, Caitlin E. Mills, Mirra Chung, Johann S. Bergholz, Shannon Coy, Jia-Ren Lin, Rinath Jeselsohn, Otto Metzger, Eric P. Winer, Deborah A. Dillon, Jean J. Zhao, Peter K. Sorger & Sandro Santagata

Nature Cell Biology **24**, 316–326 (2022) | Cite this article

Lymphocyte networks are dynamic cellular communities in the immunoregulatory landscape of lung adenocarcinoma

Giorgio Gaglia, Megan L. Burger, Cecily C. Ritch, Danae Rammos, Yang Dai, Grace E. Crossland, Sara Z. Tavana, Simon Warchol, Alex M. Jaeger, Santiago Naranjo, Shannon Coy, Ajit J. Nirmal, Robert Krueger, Jia-Ren Lin, Hanspeter Pfister, Peter K. Sorger, Tyler Jacks, Sandro Santagata

Cancer Cell
Volume 41, Issue 5, 8 May 2023, Pages 871-886.e10



Lymphocyte networks ('lymphonets') – smaller than TLS in mouse cancer models and in human cancer

Cancer Cell. 2023 May 8;41(5):871-886.e10.

HTAN
HUMAN TUMOR ATLAS NETWORK

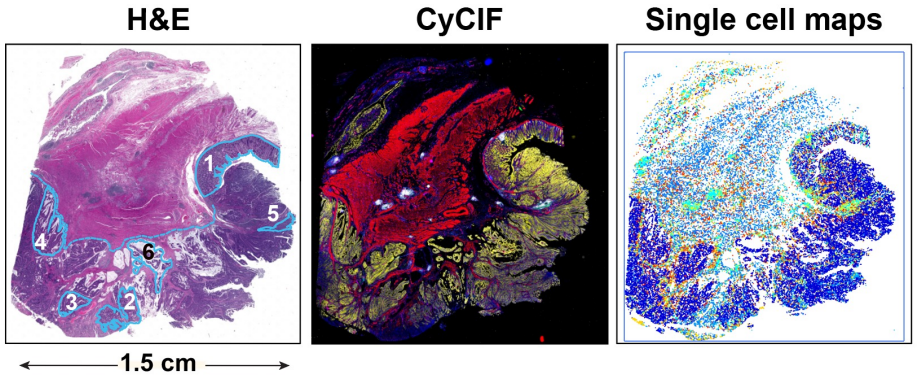
Laboratory of Systems Pharmacology

Application: First generation 3D imaging of colorectal cancer

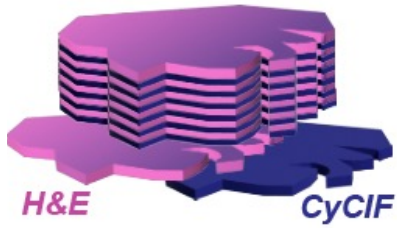
Multiplexed 3D atlas of state transitions and immune interaction in colorectal cancer

Jia-Ren Lin [1 7], Shu Wang [1 2 7], Shannon Coy [1 3 7], Yu-An Chen [1], Clarence Yapp [1], Madison Tyler [1], Maulik K. Nariya [1 4], Cody N. Heiser [5], Ken S. Lau [6], Sandro Santagata [1 3 4 8], Peter K. Sorger [1 4 8 9]

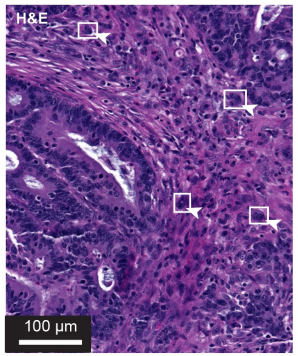
Cell Volume 186, Issue 2, 19 January 2023, Pages 363-381.e19

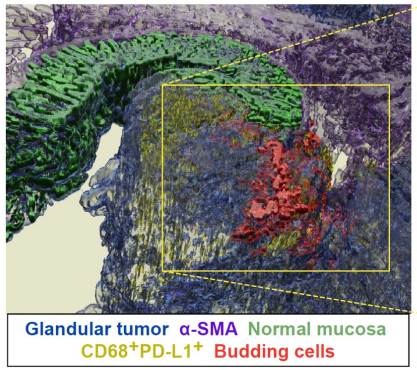


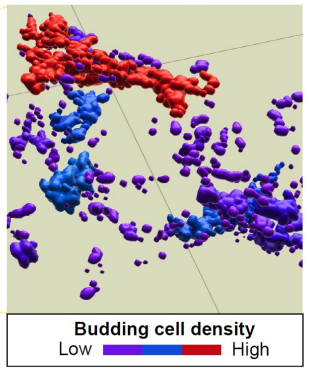
24-plex CyCIF – tumor intrinsic and immune markers (25 sections)



3D reconstruction of serial sections

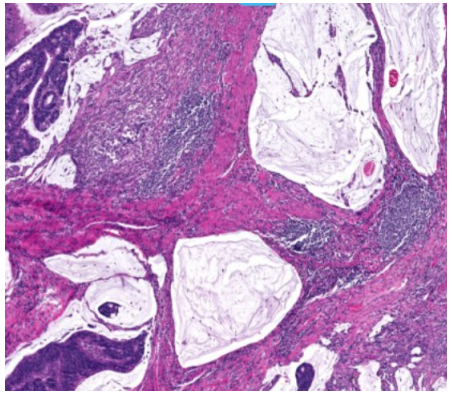


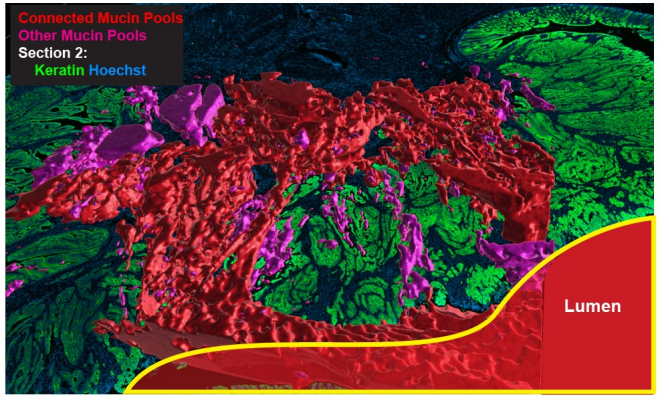




TUMOR BUDDING

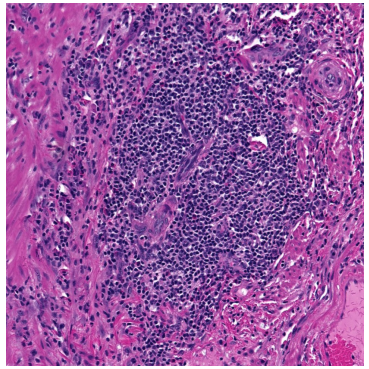
TUMOR TENDRILS



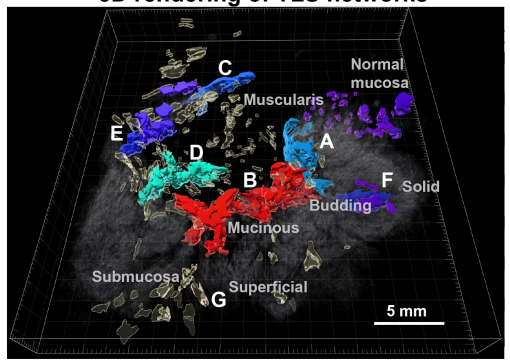


MUCIN POOLS

MUCIN CAVERNS



3D rendering of TLS networks

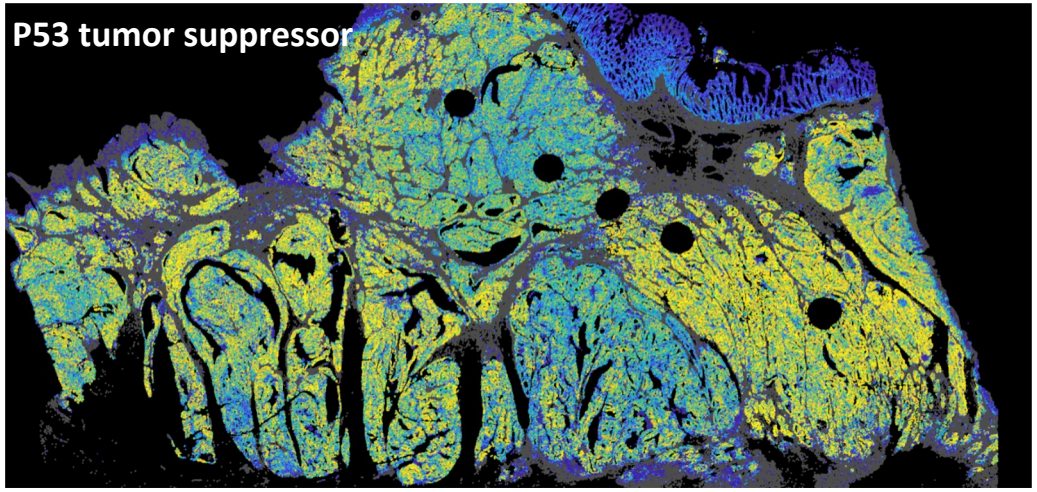


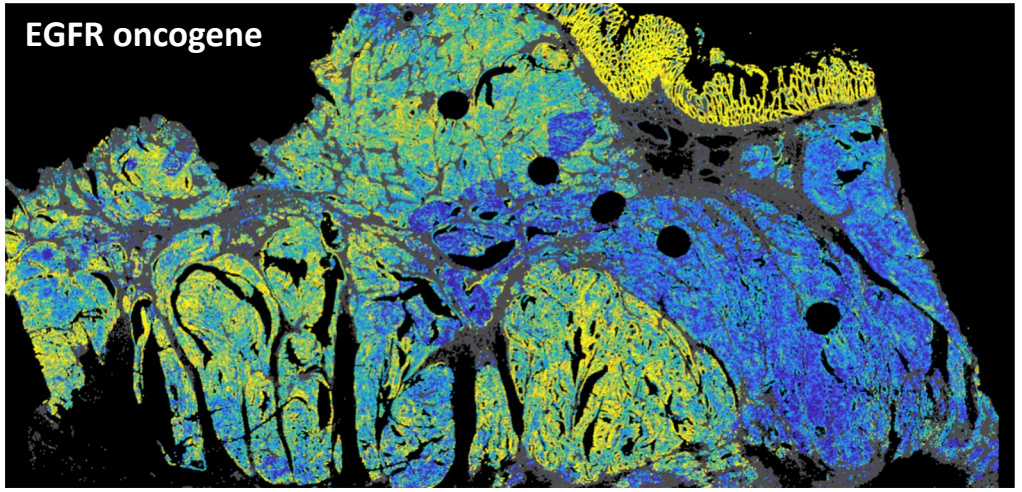
TLS

TERTIARY LYMPHOID NETWORKS

Challenges: Long and Short Molecular Gradients and Regional Diversity are Common in Tumors

Conceptual considerations: molecular gradients can be analogized to those in developing tissues

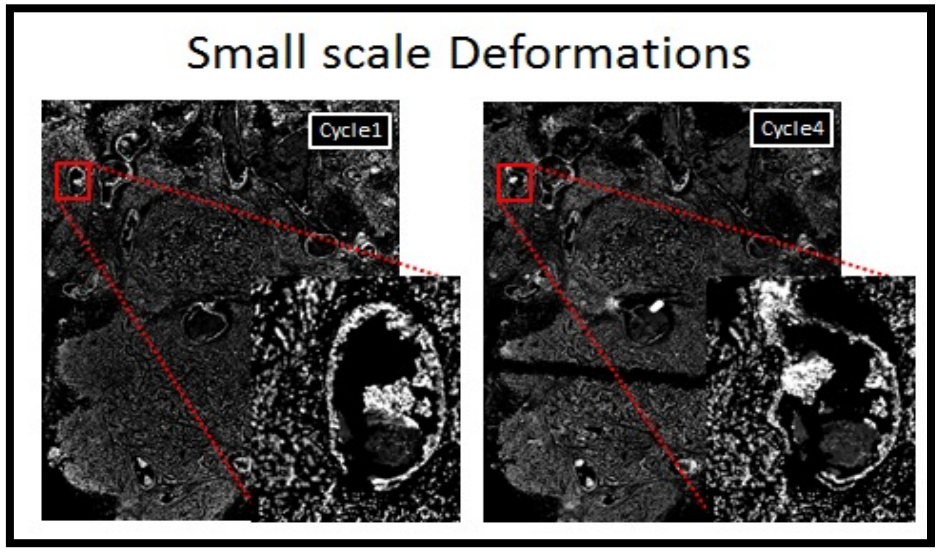


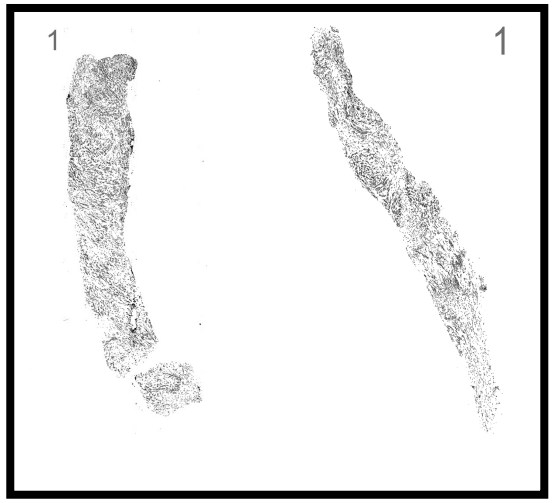


Practical considerations:

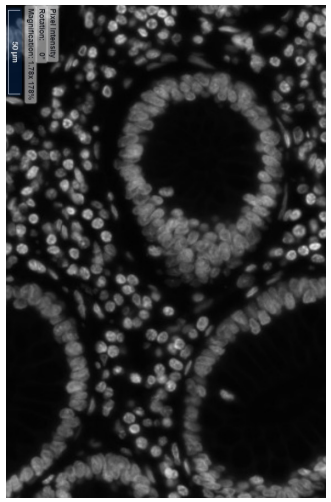
- Adequate tissue sampling is very important (motivates the need for WSI in many cases)
- Central limit theorem for correlated data - effective sample size (N_{eff}) for spatially correlated data is often 100- to 1,000-fold smaller than *N;* account for this in stats
- Subsampling and ROI selection can lead to spurious conclusions (particularly with patient outcomes)
- Applies to spatial transcriptomics data as well
- Considerations when analyzing core biopsies

Challenges of Cyclic Methods

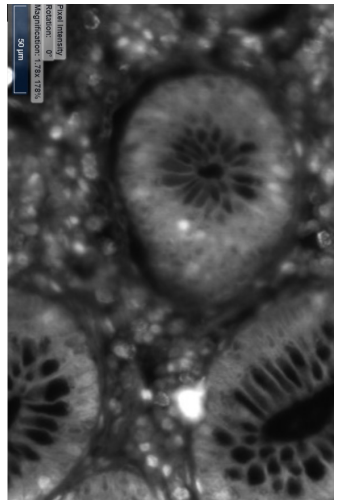




Cases with substantial tissue loss

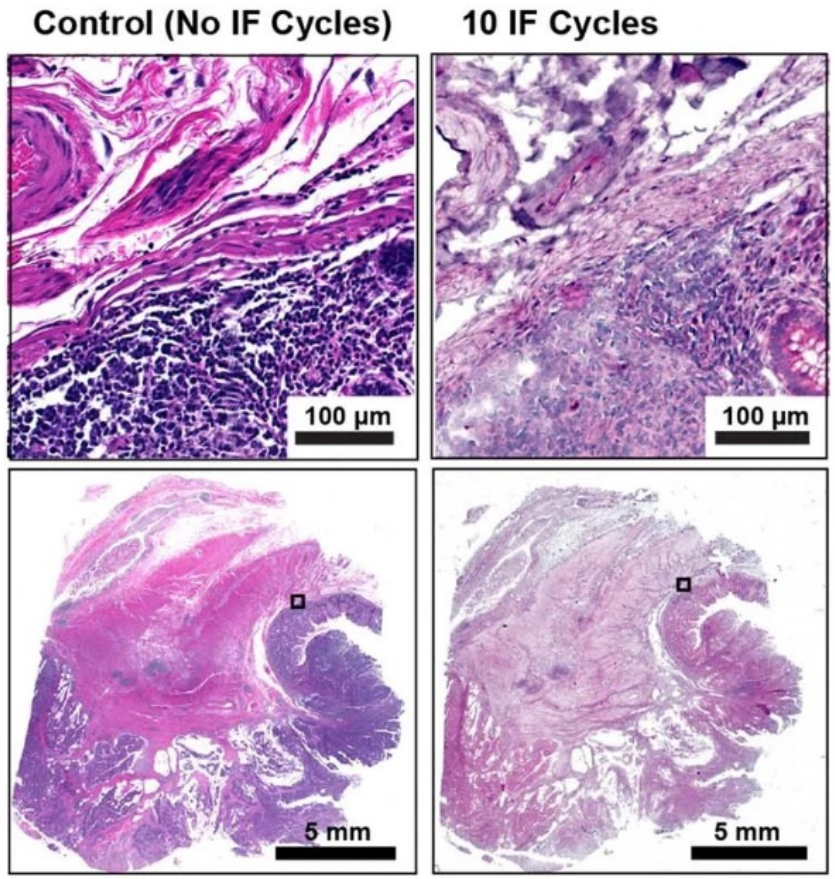


Early Cycle (5)
DNA Stain



Late Cycle (20)
DNA Stain

Many cycles of IF staining and bleaching reduce H&E image quality



First principles for high-plex imaging in diagnosis

Whole slide imaging: An FDA requirement for diagnosis and necessary to achieve statistical power

Sufficient plex for molecular analysis: Immunophenotyping, cancer cell identification, and pharmacodynamics requires ca. 16-18 markers

Same slide IF and H&E : Supports existing histopathology workflows and facilitates interpretation by pathologists

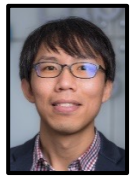
Sub-cellular resolution: to enable accurate cell segmentation and identification of organelles

Simplicity, reliability, sensitivity and speed: one-shot rather than sequential acquisition of IF data

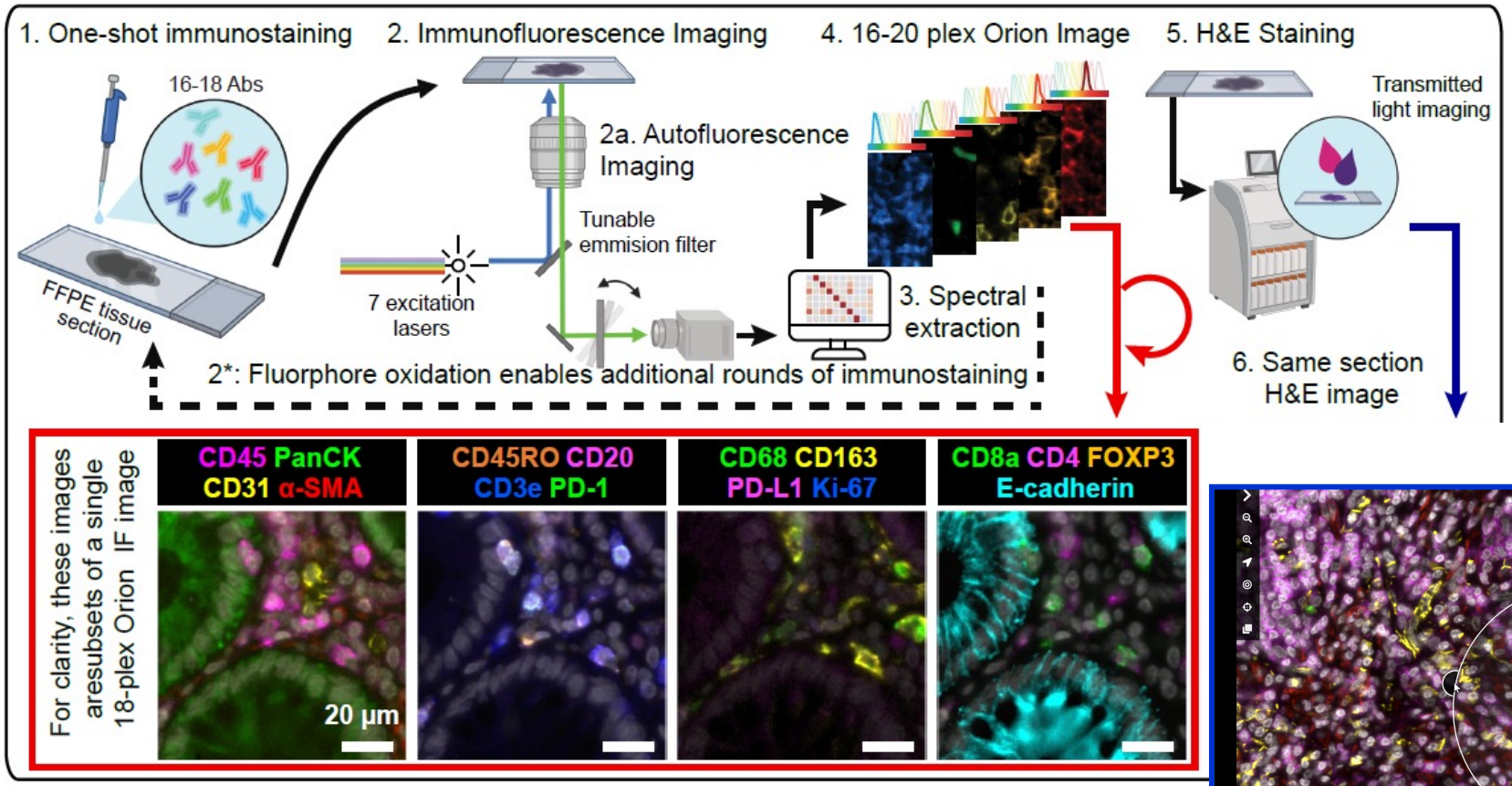
RareCyte Orion method:

One shot mIF (molecular features) and same slide integrated H&E (morphology)

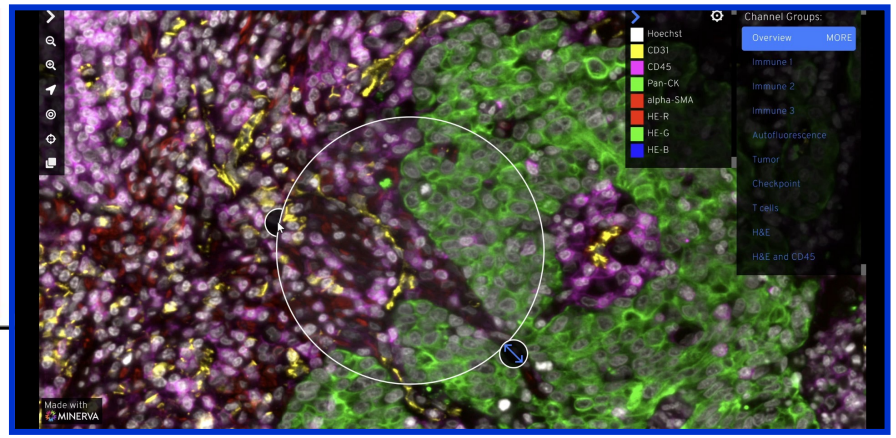
Jerry Lin



Yu-An Chen



up to 200+ whole slides



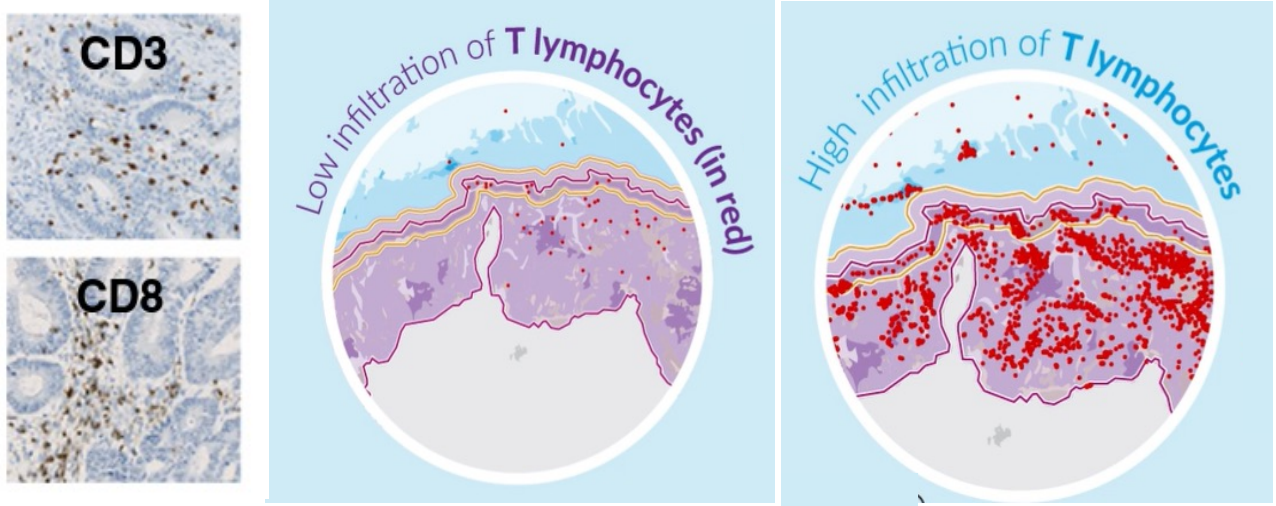
Bridge morphology (H&E) + molecular (IF)?

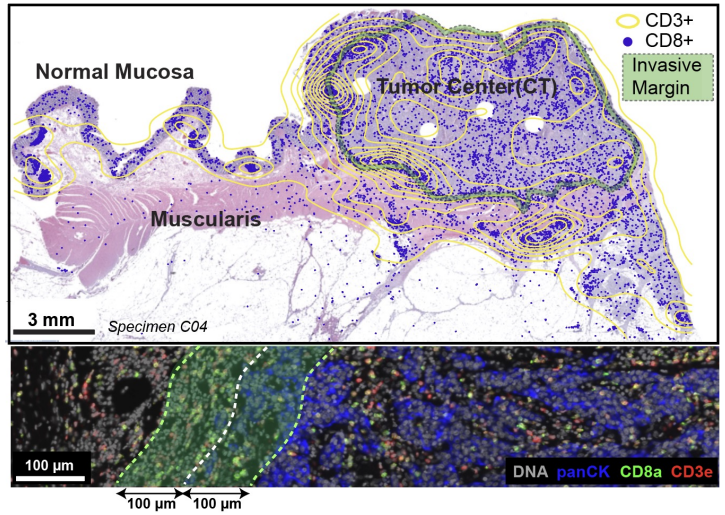
- Supports existing histopathology workflows and facilitates interpretation by pathologists.
- Enable one-to-one comparison of cell morphologies and molecular properties.
- Facilitate integration with ML/AI approaches being developed for H&E data.
- Two-way flow of information

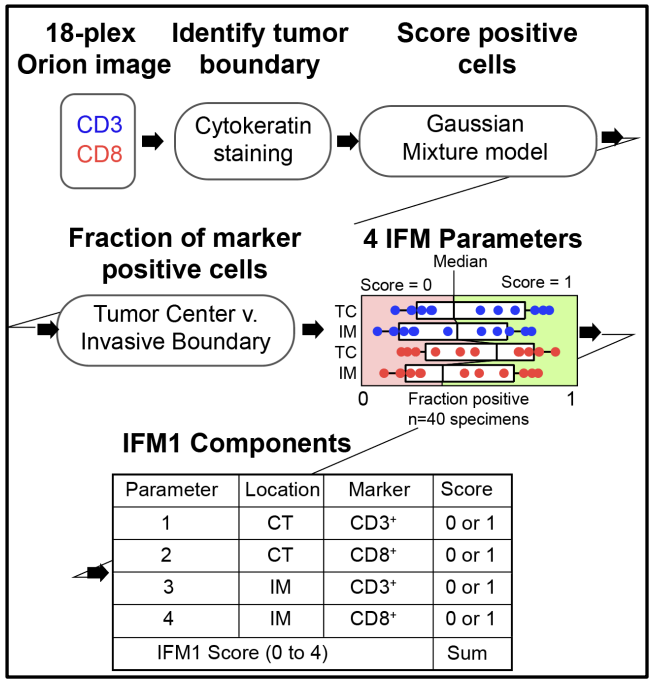
Data overviews of 40 CRC
https://labsyspharm.github.io/orion-crc/

Application: Recapitulating Immunoscore for CRC using Orion data

CD3 and CD8 at Tumor Center and Tumor Boundary

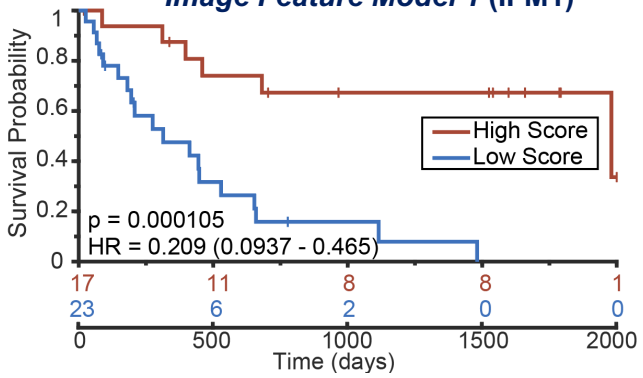






Parameter	Location	Marker	Score
1	CT	CD3⁺	0 or 1
2	CT	CD8⁺	0 or 1
3	IM	CD3⁺	0 or 1
4	IM	CD8⁺	0 or 1
IFM1 Score (0 to 4)			Sum

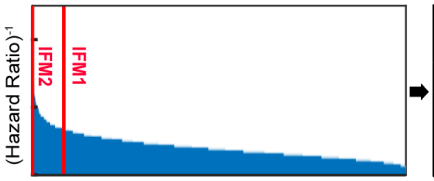
Image Feature Model 1 (IFM1)



Enhanced approach for biomarker development

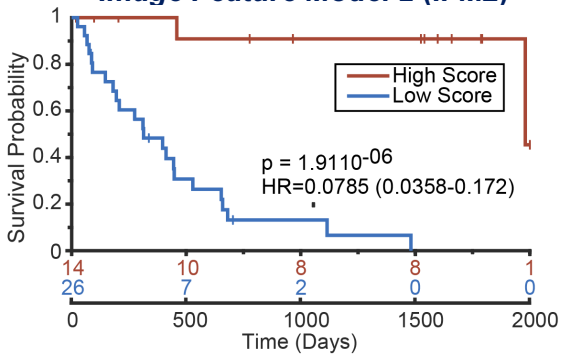
Select 13 Markers

CD3	CD163
CD4	FOXP3
CD8	PD1
CD20	PD-L1
CD45	CD31
CD45RO	α-SMA
CD68	



Parameter	Location	Marker	Score
1	CT	α-SMA⁺	0 or 1
2	IM	CD45⁺	0 or 1
3	IM	PD-L1⁺	0 or 1
4	IM	CD4⁺	0 or 1
IFM2 Score (0 to 4)			Sum

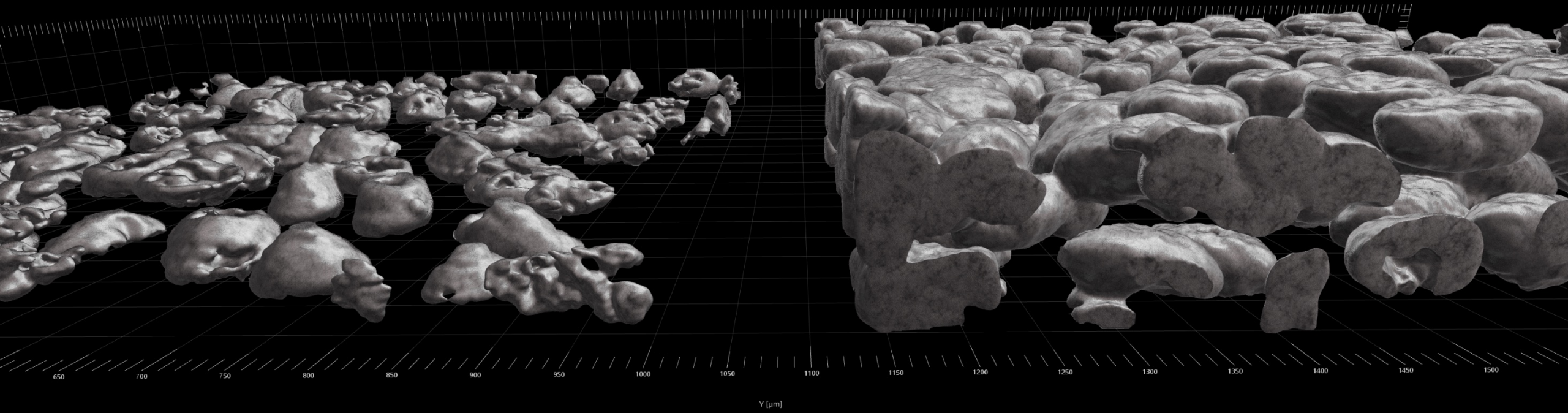
Image Feature Model 2 (IFM2)



3D multiplexed imaging of thick sections to better capture nuclear and cellular shapes and cell-cell interactions

5-micron thick tissue

20-micron thick tissue



2D and 3D multiplexed subcellular profiling of nuclear instability in human cancer

Shannon Coy, Brian Cheng, Jong Suk Lee, Rumana Rashid, Lindsay Browning, Yilin Xu, Sankha S. Chakrabarty, Clarence Yapp, Sabrina Chan, Juliann B. Tefft, Emily Scott, Alexander Spektor, Keith L. Ligon, Gregory J. Baker, David Pellman, Peter K. Sorger, Sandro Santagata

bioRxiv 2023.11.07.566063; doi: https://doi.org/10.1101/2023.11.07.566063

Multiplexed 3D Analysis of Cell Plasticity and Immune Niches in Melanoma

Clarence Yapp, Ajit J. Nirmal, Felix Zhou, Zoltan Maliga, Paula Montero Llopis, George F Murphy, Christine G Lian, Gaudenz Danuser, Sandro Santagata, Peter K. Sorger, Human Tumor Atlas Network

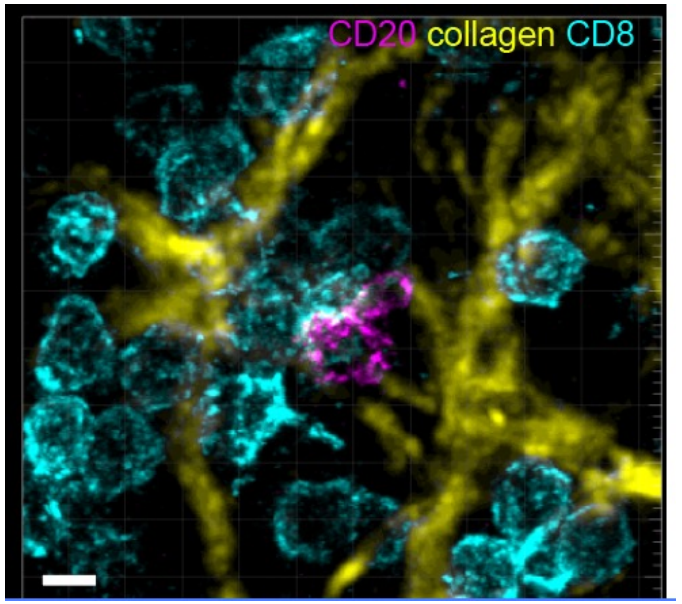
bioRxiv 2023.11.10.566670; doi: https://doi.org/10.1101/2023.11.10.566670

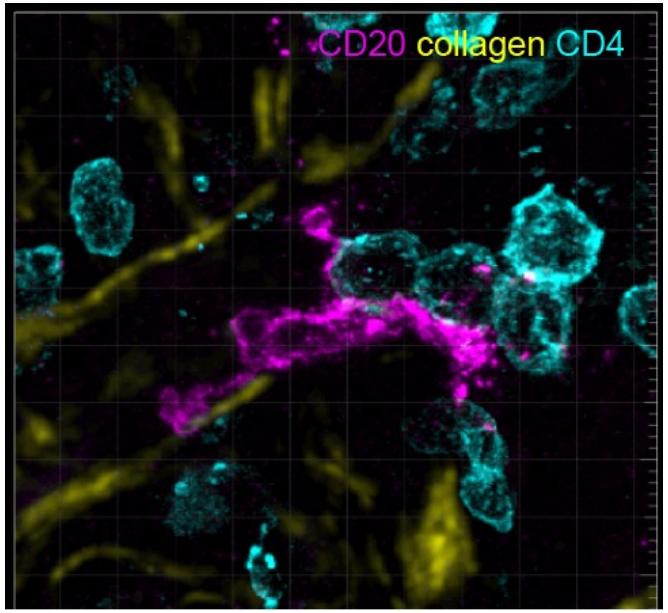
RESEARCH ARTICLES | JUNE 02 2022 CANCER DISCOVERY

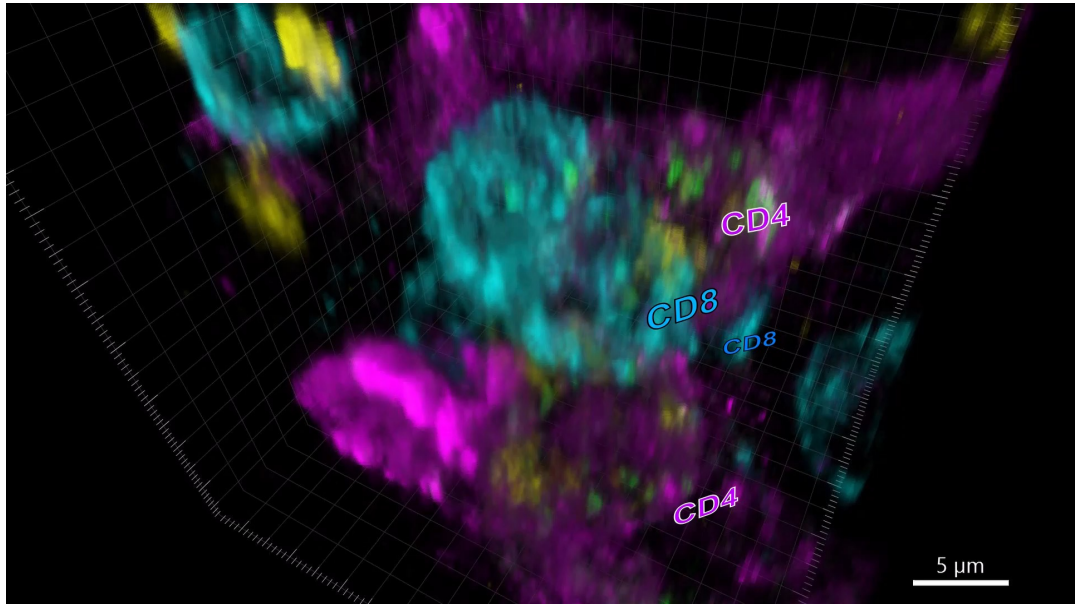
The Spatial Landscape of Progression and Immunoediting in Primary Melanoma at Single-Cell Resolution

Ajit J. Nirmal ; Zoltan Maliga ; Tuulia Vallius ; Brian Quattrochi; Alyce A. Chen ; Connor A. Jacobson ; Roxanne J. Pelletier ; Clarence Yapp ; Raquel Arias-Camison ; Yu-An Chen ; Christine G. Lian ; George F. Murphy; Sandro Santagata ; Peter K. Sorger

Application: Functional interactions between cells using high-resolution 3D imaging







High resolution 3D view of lymphocyte networks ('lymphonets')
Organization, cell-cell interactions, phenotyping

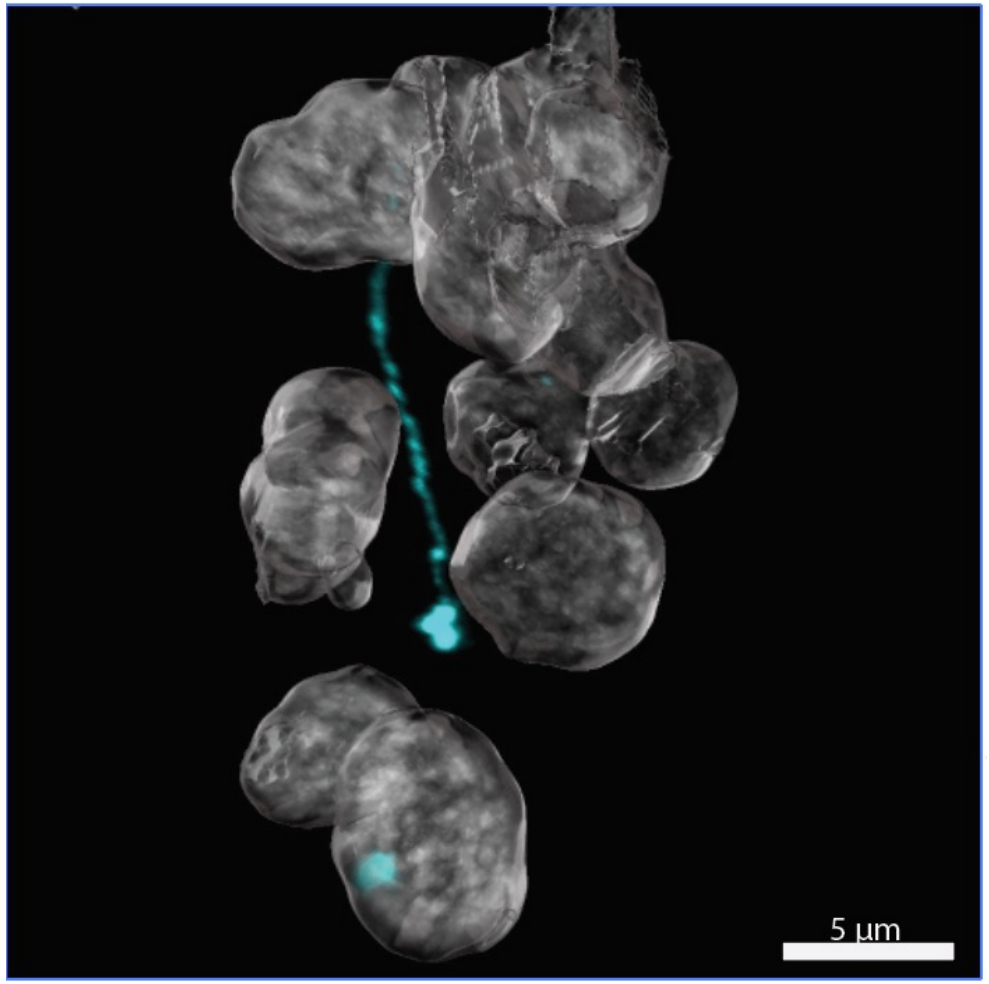
Multiplexed 3D Analysis of Cell Plasticity and Immune Niches in Melanoma

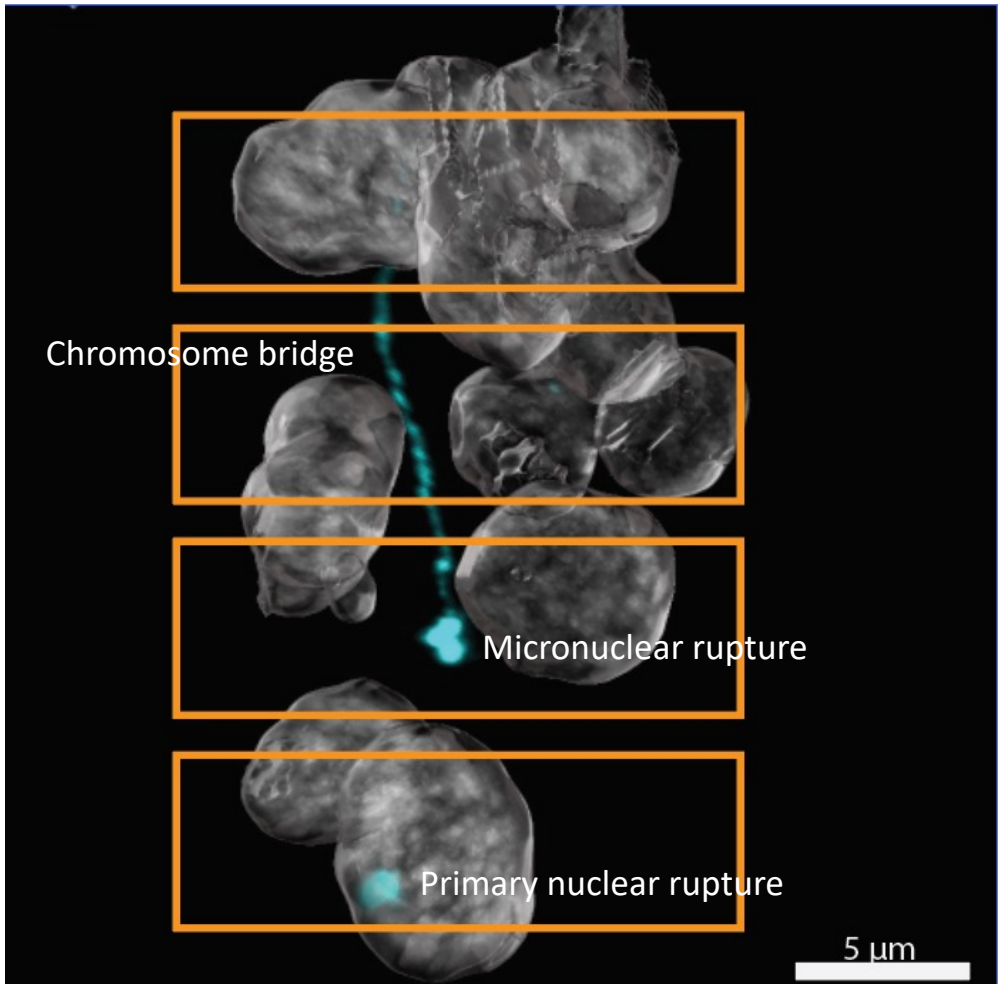
Clarence Yapp, Ajit J. Nirmal, Felix Zhou, Zoltan Maliga, Paula Montero Llopis, George F Murphy, Christine G Lian, Gaudenz Danuser, Sandro Santagata, Peter K. Sorger, Human Tumor Atlas Network

bioRxiv 2023.11.10.566670; doi: https://doi.org/10.1101/2023.11.10.566670

Application: Cell Biology Analysis in Tissues

Nuclear envelope ruptures detected using BAF (BANF1) and 3D high res multiplexed imaging

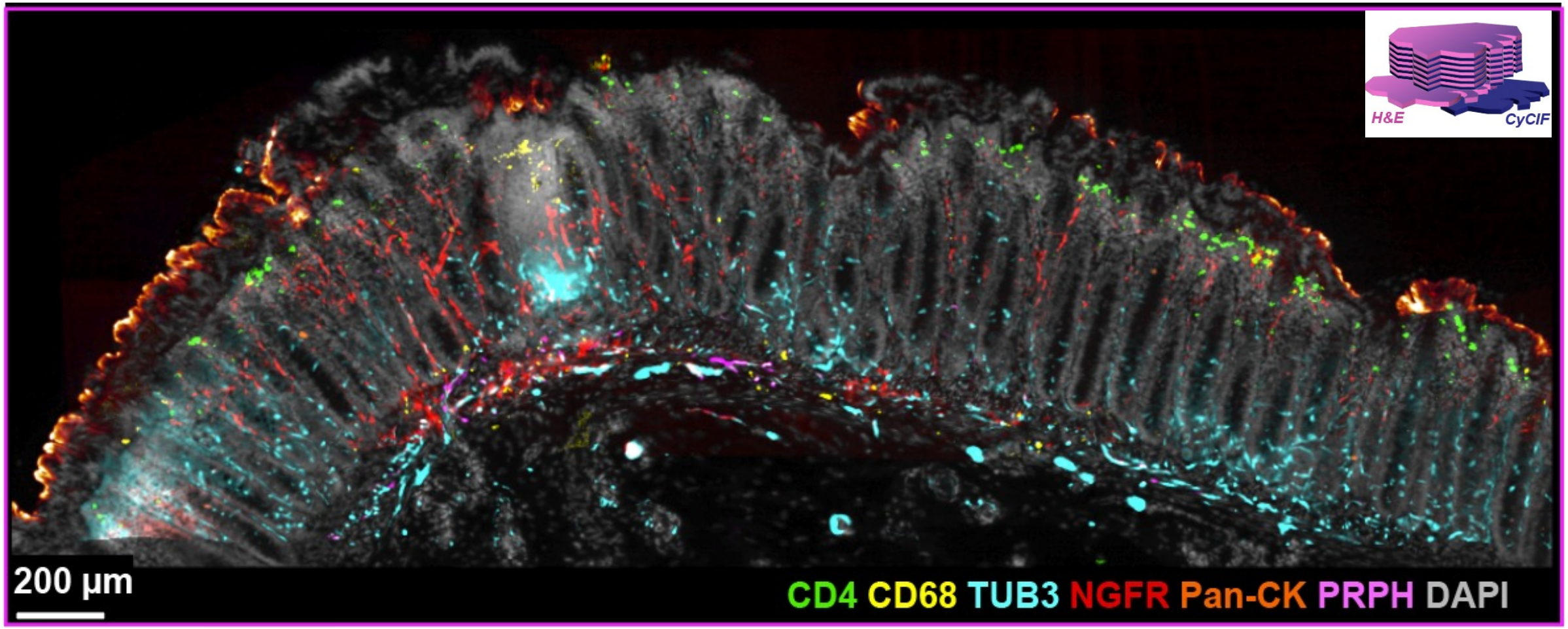





Clarence Yapp


Alex Wong

New Approach TME in context: Deep 3D CyCIF imaging



Laboratory of Systems Pharmacology

SUMMARY

POWERFUL NEW TOOLS

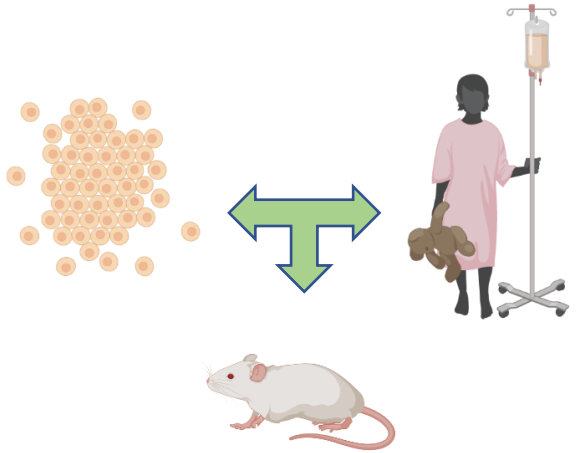
2D methods
CyCIF
Orion

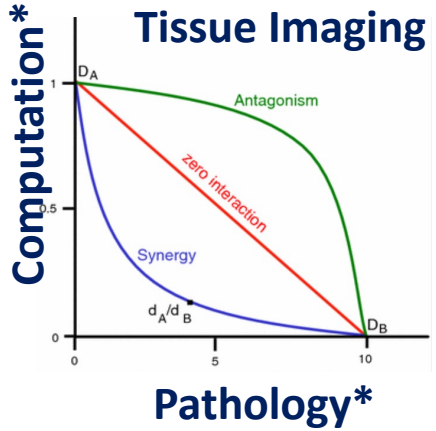
3D methods
Thick section (20-40 micron)
Deep 3D (1-3 mm)

SOFTWARE
(DEVELOPMENT!)

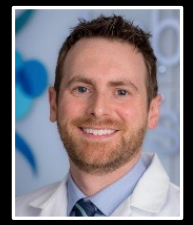
ASHLAR
CyLinter
MCMICRO
SCIMAP
miti
MINERVA

- **Selecting the right approach depends on your question**
- **Important phase of description of tissue features**



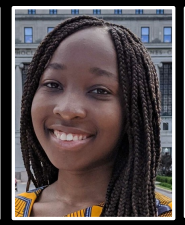


- **Morphology-molecular features and transitions**
- **Interconnected structures in 3D (buds, pools, TLS)**
- **METRICS!**
  - **Proliferative architecture of cancer cells**
- **Lymphonets**
- **Cell biology in cancer tissues w/ 3D high res multiplexed images**


Greg Baker

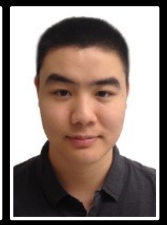

Roxy Pelletier


Zoltan Maliga


Yvonne Anang


Sabrina Chan


Rumana Rashid

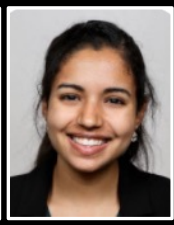

Shu Wang


Claire Ritch


Sarah Arena

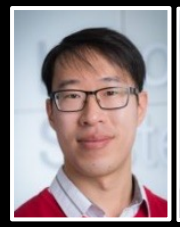

Allison Maier


Megan Burger


Ana Verma


John Hoffer


Scott Rodig


Clarence Yapp


Yu-An Chen


Alex Wong

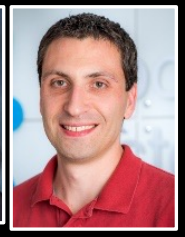

Jerry Lin


Mark Awad


Shannon Coy


Judith Agudo


Nathalie Agar


Jeremy Muhlich


Peter Sorger


Laura Maliszewski

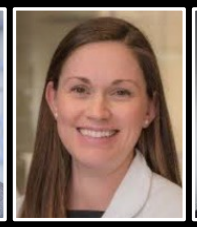

Alyce Chen


Cat Luria


Keith Ligon


Ken Lau


Ajit Johnson

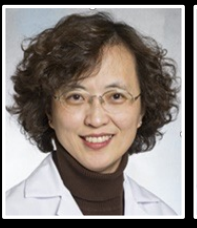

Jenn Guerriero


Sheheryar Kabraji


Jon Aster


George Murphy


Anniina Färkkilä


Christine Lian


Hanspeter Pfister


Ben Gaudio


Robert Krueger


Juliann Tefft

Giorgio Gaglia

John Lee

Brian Cheng

Lindsay Browning

Tuulia Vallius

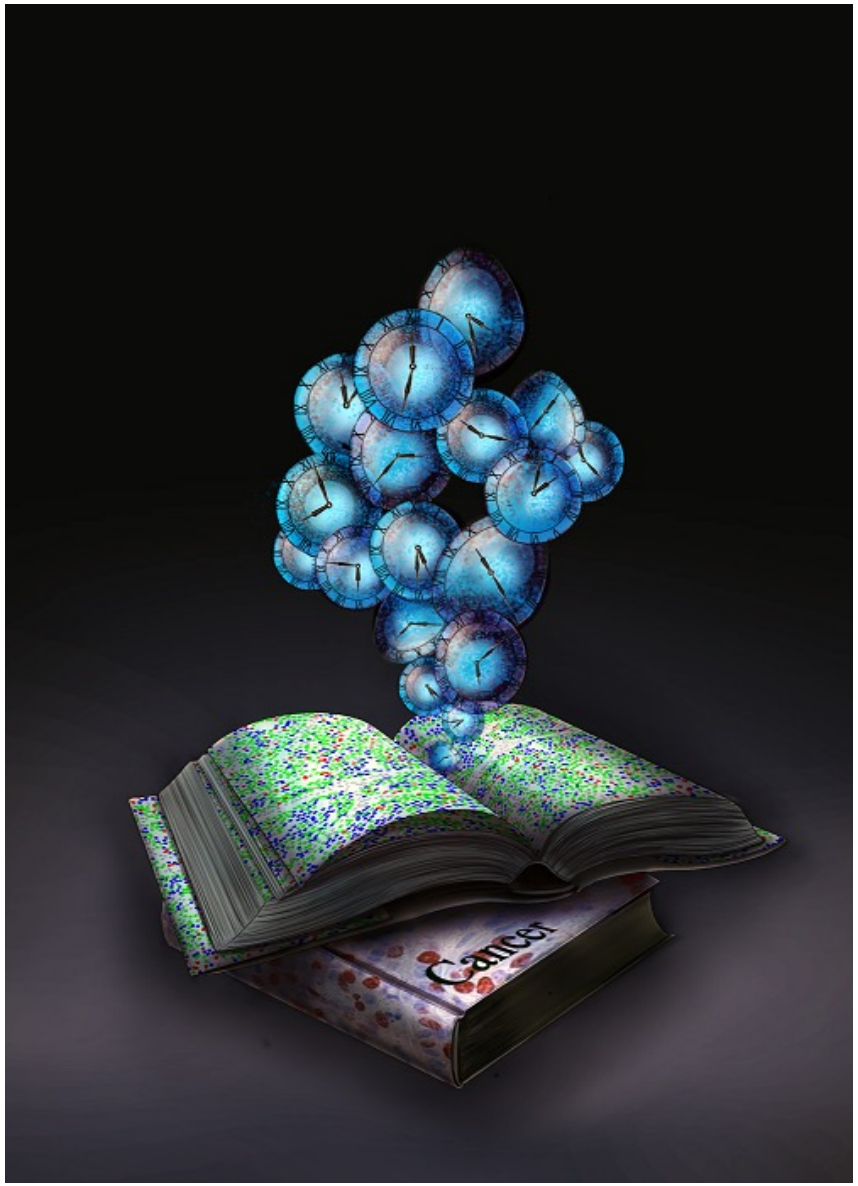
Han Xu

Madison Tyler

Artem Sokolov

Raquel Arias-Camison

Tanjina Kader



THANK YOU!