12 – 13 December 2024
Berlin, Germany

Programme & Abstract book

Inaugural conference of the

European Society for Spatial Biology (ESSB e.V.)

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Want to become a member of the ESSB?

Join us at the Members´Assembly
on Thursday, 12 December 2024,
at 6:00 pm, Ballroom 3

Inaugural conference of the European Society for Spatial Biology (ESSB e.V.)

12 – 13 December 2024, Berlin, Germany

Venue

Hotel & Conference Center
Titanic Chaussee Berlin
Chausseestr. 30
10115 Berlin, Germany

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Congress President

Anja Hauser
Charité – Universitätsmedizin Berlin
&
German Rheumatology Research Center Berlin (DRFZ),
a Leibniz Institute; Germany

TITANIC Convention
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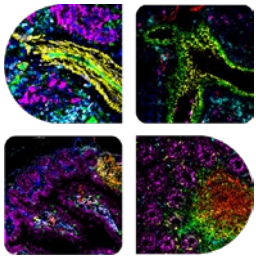
Anja Hauser

Dear Friends of Spatial Biology,



It is an immense pleasure for me to welcome you to the inaugural conference of the European Society for Spatial Biology here in the center of Berlin.

The ESSB is still a young society, it was only founded this year. Therefore, we were all the more surprised and, in a positive sense, overwhelmed by the enthusiasm we received during the preparation for this conference. The number of registrations far exceeded our expectations. In addition to the great interest of participants from academia, we are very grateful for the support from industry partners who make this event possible.

Together, this highlights the relevance and timeliness of the interdisciplinary, rapidly developing field of spatial biology, as well as the need for scientific exchange and for meeting other scientists working in this area.

I hope that with this conference we can create a forum for exactly those interactions and lay the foundation for many more ESSB events that will advance this interesting and important field.

Now I wish all participants a successful conference - enjoy the science, immerse yourself in discussions and meet old and new friends!

Anja Hauser

Congress President and ESSB Vice President

Program DAY 1: Thursday, December 12, 2024

Titanic Hotel Chaussee, Ballroom 3

8:15 am Welcome

8:30 am Session 1 New Developments in Spatial Biology

- *Keynote speaker Joakim Lundeberg, Sweden: Resolving the tissue ecosystem into its components
- **Nicole Strittmatter, Germany: Studying disease states in cancer and infection using spatial metabolomics
- Short talk by Xavier Rovira Clavé, USA: In Situ Tracking of Clonal Evolution and Phenotypic Heterogeneity in Tumors by Spatial Epitope Barcoding
- Short talk by Carsten Hopf, Germany: Multimodal MALDI Lipid-Protein Imaging of Amyloid-β Plaques and Machine Learning reveal Alzheimer's Disease Signatures

10:15 am Coffee break, posters & industry exhibition

11:00 am Session 2 - Image Analysis for Spatial Biology

- **Carolina Wählby, Sweden: Spatial (transcript)omics – a joint mapping of tissue function and architecture
- Short talk by Jan Schröder, Germany: Deep Spatial Phenotyping of the Bone Marrow Microenvironment in NPM1-Mutant Acute Myeloid Leukemia
- Short talk by Malte Kuehl, Germany & Denmark: PathoPlex – Next-generation multiplexed image analysis

12:00 pm Lunch Symposium Akoya Biosciences *ESSB Lunchtime Raffle!*

- Alessio Colombo, Akoya Biosciences: Ultrahigh-Plex and High-Throughput Spatial Biology Solutions: From Discovery to the Clinic

12:30 pm Lunch, posters & industry exhibition

1:30 pm Lunch Symposium Miltenyi Biotec *ESSB Lunchtime Raffle!*

- Katrin Schönborn & David Agorku, Miltenyi Biotec: Unlocking new insights in colorectal cancer with spatial multiomic on the MACSima™ Platform

2:00 pm Session 3 - Machine Learning and Data Integration in Spatial Biology

- **Leeat Keren, Israel: Escalating high-dimensional imaging for cancer research
  Talk sponsored by Ionpath
- Short talk by Josef Lorenz Rumberger, Germany: Automated classification of cellular expression in multiplexed imaging data with Nimbus
- Short talk by Iva Buljan, Austria: Tissue-specific prediction of biological age from histopathological images

3:00 pm Coffee break, posters & industry exhibition

4:15 pm Session 4 - New Spatial Biology insights for disease and pathology

- *Keynote speaker Bernd Bodenmiller, Switzerland: Highly multiplexed imaging of tissues with subcellular resolution by imaging mass cytometry
- Short talk by Maria Puschhof, Germany: Molecular drivers of low- to high-grade transition in IDH-mutant glioma
- Short talk by Gesa Krueger, Germany: Modeling immune responses of cattle to mycobacteria using magnetic bioprinted granulomas

5:30 pm Dinner Symposium - 10xGenomics *ESSB Dinner Raffle!*

- Sarah Taylor, 10X Genomics: Mapping Immune Cell Populations Using High-Definition Spatial Technologies

6:00 pm Members´ assembly

7:00 pm Poster session & get-together at the industry exhibition

Food and drinks will be provided.

***Keynote: 45 min (40+5), **Invited speaker: 30 min (25+5),**
*****Short talks selected from abstracts: 15 min (12+3)**

DAY 2: Friday December 13, 2024

Titanic Hotel Chaussee, Ballroom 3

8:15 am Short Breakfast Talk by Vizgen & Ultivue

- *Dries Van Hemelen und Friedrich Preusser: Merging Expertise: Vizgen and Ultivue Join Forces to Innovate in Spatial Multi-Omics"*

Breakfast Buffet will be provided!

08:30 am Session 5 Spatial Immunology

- **Camilla Engblom, Sweden: Spatially resolving B cell clonal dynamics
- Short talk by Raluca Niesner, Germany: MetaFLIMB – longitudinal *in vivo* NAD(P)H fluorescence lifetime imaging of the femoral marrow
- Short talk by Katharina Imkeller, Germany: Connecting the Spatial Architecture and Immune Function of Tertiary Lymphoid Structures in Glial Tumors
- Short talk by Vladimir Sukhov, USA: Advanced Spatial Analysis Highlights Immune Cell Organization in Successful Tumor Rejection After Immune Checkpoint Therapy
- Short talk by Francesca Bosisio, Belgium: Omics Integration Reveals Spatial and Molecular Predictors of Response to Anti-PD-1 Immunotherapy in Metastatic Melanoma
- Short talk by Aysegül Adam, Germany: Spatial characterization of Innate Lymphoid Cells in oral inflammatory diseases

10:15 am Coffee break, posters & industry exhibition

11:00 am Session 6 New Dimensions in Spatial Biology

- **Harsharan Singh Bhatia, Germany: Clear-Omics: Spatial molecular maps in optically cleared 3D intact specimens
- Short talk by Peter Androvic, Germany: Beyond Genes: Integrating Transcriptomics with Structure and Metabolism to Map Neurodegeneration
- Short talk by Jorge Trojanowski, Spain: Single-round Profiling by Amplification and Color Encoding (SPACE)-FISH in 3D microvasculature on-chip

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***Keynote: 45 min (40+5), **Invited speaker: 30 min (25+5),**
Short talks selected from abstracts: 15 min (12+3)

12:00 Lunch Symposium - Lunaphore *ESSB Lunchtime Raffle!*

- Daniel Azorin, ETH Zurich & Müge Akpinar, Lunaphore: Spatial multiomics for precision target identification in the tumor microenvironment

12:30 pm Lunch, posters, industry exhibition

1:30 pm Lunch Symposium Standard BioTools *ESSB Lunchtime Raffle!*

- Melissa Klug, Standard BioTools™: Whole Slide Imaging Modes for Imaging Mass Cytometry Reveal Cellular Diversity of the Tumor Immune Microenvironment in Mouse Glioblastoma

02:00 pm Session 7 Tumor Microenvironment

- **Raza Ali, UK: Charting the intact breast tumour microenvironment
- **Oliver Stegle, Germany, Data infrastructures for spatial omics and advances in cell segmentation
- Short talk by Spencer Watson, Switzerland: Investigating Glioblastoma Recurrence with Spatial Multi-Omics
- Short talk by Ann-Marie Baker, UK: Spatial analysis reveals reshaping of tumour-immune dynamics at the transition to invasive colorectal cancer
- Short talk by Christina Schniederjohann, Germany: Microenvironmental Factors Impacting Chemotherapy Response in B-NHL

3:45 pm Coffee break, posters & industry exhibition

04:15 pm Session 8 Visions for Spatial Biology

- *Keynote speaker: Peter Sorger, USA: Spatial profiling of human tissues for discovery and diagnosis

Talk sponsored by Ionpath

5:15 pm Poster Award Ceremony, Concluding remarks and Farewell

The conference would not be possible without sponsorship.

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Special thanks go to:

The abstract booklet is sponsored by Lunaphore

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Travel Grant Awardees

We are pleased to announce the following EFIS Travel Scholarship Awardees:

Erison Santos, Brazil

Noel Thombiano, Burkina Faso

Sarthak Sahoo, India

Sefiyat Odunola Adebiyi, South Africa

Sogunmez Erdogan Nuray, Turkey

Anna Pascual-Reguant, Spain

Ada Junquera, Finland

Welcome to Berlin, Spatialists! Here are some fun facts for you:

▸ There are over 90 Christmas markets in Berlin - - We will be visiting one of them on Friday evening. Who's coming along?

▸ The Berliner Currywurst turned 75 years in summer 2024. According to statistics, 70 million Currysausages are consumed in Berlin every year.

▸ Traffic lights lovingly called the crow's nest at the crossing Kurfürstendamm/ Joachimsthaler Str. were controlled by a traffic policeman pressing a button until 1962.

▸ The memorial church nicknamed the hollow tooth and next to it the two church buildings, the chapel – "powder puff" and the church – "lipstick"

▸ With over 1600 kebab stands, Berlin is the 'capital of the kebab' Around 950 kebab skewers are eaten every day in Berlin

 All pictures: Adobe Express or Adobe Stock

- Berlin's oldest tree is the "Dicke Marie" in Tegeler Forst. It is said that the Humboldt brothers (Wilhelm 1767-1835, Alexander 1769-1859) baptised the English oak because of its impressive size, thinking of their cook "Marie". Today, the tree is 26 metres tall and is estimated to be 600 to 700 years old. Nobody knows for sure.

- The oldest bridge is the Jungfern-brücke in Mitte, the only remaining bascule bridge.

- Berlin's oldest animals probably live in the Zoo. The current leader is "Ingo the flamingo", who lives in the Zoo and was caught in Cairo on 23 June 1948. He came to the Zoo in 1955. The pink pensioner does not need any special treatment. He gets the same food as everyone else, is totally fit and a completely normal member of the flamingo group.

- Berlin has the longest open-air gallery in the world - the East Side Gallery. It is 1,316 metres long and displays over 100 paintings by artists from all over the world shown on the remains of the Berlin Wall.

- Berlin's public transport system is so long that it circles the globe 8.7 times a day - if it travels...

- Berlin has over 1000 "Spätis" - where you can buy drinks or anything else around the corner at any time of day or night. The concept originates from the GDR.

- Adlergestell is with 11.9 km the longest street in Berlin. A total of 9473 streets run through the city, most of them - namely 1303 - in Treptow Köpenick.

- Berlin has more museums than rainy days - with 180 museums, Berlin surpasses the average of 106 rainy days a year.

DAY 1: Thursday, December 12, 2024

8:30 am Session 1 New Developments in Spatial Biology

Chairs: Denis Schapiro, Christian Schürch

Keynote Speaker: Joakim Lundeberg

SciLifeLab, KTH Royal Institute of Technology, Sweden

Resolving the tissue ecosystem into its components

Tissue represents an ecosystem of different cells carrying out various tasks. Specific types of cells exist in every organ and serve specialized functions defined by the specific genes and proteins active in each cell type. Comprehensive maps of molecularly defined human cell types are underway through the Human Cell Atlas effort using primarily single-cell RNA sequencing. The technologies to assemble spatial maps that describe and explain the cellular basis of health and disease are still being discussed. We have developed and established the Spatial Transcriptomics technology, in which tissue imaging is merged with spatial RNA sequencing and resolved by computational means. Spatial Transcriptomics technology was the first method to provide unbiased whole transcriptome analysis with spatial information from tissue using barcoded array surfaces and has, since its initial publication, been used in multiple biological systems in health and disease. This presentation will cover the technology's novel methodological and analytical aspects in the context of biological applications from cell atlas, neurology, and cancer.

Nicole Strittmatter

Department of Bioscience, Technical University of Munich, Germany

Studying disease states in cancer and infection using spatial metabolomics

Mass Spectrometry Imaging (MSI) is a powerful technique to characterise complex biological samples such as tissues or plant material, enabling the mapping of hundreds of chemical constituents simultaneously. In the Strittmater lab, we are using MSI predominantly to map changes in the metabolome (spatial metabolomics) of biomedical specimen ranging from organoids to preclinical and clinical tissue specimen using Desorption Electrospray Ionisation (DESI) MSI, an ambient technique operating under atmospheric conditions and enabling analysis without prior sample preparation. This makes DESI-MSI particularly suited for deployment in multimodal imaging studies, such as in combination with imaging mass cytometry, which allows the targeted, multiplexed detection of proteins. In this talk, I will highlight some recent applications that cover some of our dominant research interests such as host-microbe

interactions, tumour biology and anti-tumour drug disposition and metabolism.

Short talk by Xavier Rovira Clavé

Department of Pediatrics, Genetics, Stanford University, USA

In Situ Tracking of Clonal Evolution and Phenotypic Heterogeneity in Tumors by Spatial Epitope Barcoding

Understanding the dynamics of clonal evolution and phenotypic shifts within the tumor microenvironment is crucial for developing effective therapeutic strategies. Previously, we introduced a combinatorial tagging system coupled with multiplexed ion beam imaging for in situ tracking of barcodes within tissues. This approach enabled us to dissect the spatial component of cell lineages and phenotypes in small cell lung cancer xenograft models, revealing emergent properties from mixed clones and the preferential expansion of clonal patches. We are now extending this approach by integrating it with genome editing technologies, allowing the investigation of the impact of genetic perturbations on clonal dynamics and phenotypic heterogeneity within the tumor microenvironment. We applied this integrated approach to study the effects of knocking out a set of key genes for small cell lung cancer development in a xenograft model. Our analysis revealed distinct patterns of clonal expansion and phenotypic shifts associated with specific gene knockouts and identified unique subclonal interactions within the tumor. This work highlights the complex interplay between genetic alterations, clonal dynamics, and phenotypic heterogeneity in the tumor microenvironment. Our findings demonstrate the potential of this approach to uncover novel insights into the mechanisms driving tumor progression and treatment resistance, paving the way for the development of more effective personalized therapies.

Short talk by Carsten Hopf

Center for Mass Spectrometry and Optical Spectroscopy (CeMOS), Mannheim University of Applied Sciences, Mannheim, Germany. Faculty of Medicine, Heidelberg University, Germany Mannheim Center for Translational Neuroscience (MCTN), Medical Faculty Mannheim, Germany

Multimodal MALDI Lipid-Protein Imaging of Amyloid-β Plaques and Machine Learning reveal Alzheimer's Disease Signatures

Alzheimer's disease (AD), the leading cause of dementia, is characterized by deposition of amyloid-β peptides and various lipids in plaques. This study investigates the lipid and protein heterogeneity of amyloid-β (Aβ) plaques in the APP PS1 mouse model and in post-mortem brain samples from AD patients as well as in plaque-bearing but cognitively unimpaired individuals. We combined multimodal MALDI imaging of lipids and proteins at a single-plaque level on various mass spectrometry platforms with computational assessment of lipid and protein composition in large plaque populations. We identified distinct populations of amy-

loid plaques characterized by differential Aβ and lipid composition. These findings suggest that the heterogeneity in Aβ metabolism and lipid homeostasis is a key factor in the pathogenesis of AD and imply that total amyloid burden alone is an insufficient marker for the disease.
Brain sections of APP PS1 mice were analyzed by MALDI imaging on a neoflex benchtop MALDI-TOF mass spectrometer (Bruker). Lipid and HiPlex-MALDI-IHC information were obtained from the same tissue section. Multimodal MALDI lipid-protein imaging of fresh-frozen human brain sections was performed on a timsTOFflex mass spectrometer (Bruker). Computational evaluation of amyloid plaque-like objects was performed using the PlaquePicker R tool (Enzlein et al. 2020). Different machine learning approaches were utilized, in order to identify a mixed lipid-protein classifier characteristic for AD (Enzlein et al. 2024).
Amyloid beta plaques were segmented using PlaquePicker and differentiated based on their lipid and peptide composition. Combination of MALDI lipid imaging and HiPLEX-IHC demonstrated extensive co-localization of gangliosides as well as with microglia and activated astrocytes with Aβ plaques. However, we identified only one class of plaques in the APP PS1 mouse model. In contrast, multimodal MALDI lipid-protein imaging of human brain samples followed by statistical single-plaque analysis and machine learning, revealed marked differences between plaques in AD tissue and in brains from plaque-bearing but cognitively unimpaired (AP-CU) individuals. Surprisingly, three different machine learning models suggested that Aβ1-38 and distinct gangliosides rather than Aβ1-42 which is believed to drive amyloid plaque formation, most strongly distinguishes between AD- and APCU amyloid plaques. The combination of these molecular markers in multimodal MS imaging could be used to better understand AD pathology.

11:00 Session 2 Image Analysis for Spatial Biology

Chairs: Denis Schapiro, Carolina Wählby

Carolina Wählby

Uppsala Universitet and SciLifeLab, Uppsala, Sweden

Spatial (transcript)omics – a joint mapping of tissue function and architecture

Optical microscopy combined with specific staining techniques has been crucial for much of the understanding we have today of both developmental processes, and disease, such as cancer. Via single cell transcriptomics, we have learnt that mRNA sequencing is an efficient way to identify different cell types and their function. By doing the sequencing directly in the tissue, via in situ sequencing, we can now map function to subcellular tissue architecture, and not only know which cells and functions are present, but also where, and in what con-

text, they are present. This information can further be combined with detection of specific interactions between e.g., a drug and drug target. The techniques rely on computational methods, specifically addressing transcript detection, gene decoding, visualization, and finally quantification and interpretation. Visual assessment of microscopy image data becomes limiting as dataset size and complexity grows. It can also be difficult to draw confident conclusions if the observed processes are subtle and the samples are heterogenous. In the past ten years, AI, and particularly learning-based approaches relying on deep convolutional neural networks, have gained enormous popularity in all fields of image-based science. The methods have great potential, but they must also be used with care, not to fool us in our findings. We apply AI to map tissue morphology in relation to spatially resolved gene expression in tissue, with applications in cancer and development.

Short talk by Jan Schröder

Department of Hematology, Oncology, Clinical Immunology and Rheumatology, University Hospital and Comprehensive Cancer Center Tübingen, Germany.
Department of Pathology and Neuropathology, University Hospital and Comprehensive Cancer Center Tübingen, Germany.

Deep Spatial Phenotyping of the Bone Marrow Microenvironment in NPM1-Mutant Acute Myeloid Leukemia

Despite generally favorable outcomes in NPM1-mutant acute myeloid leukemia (AML), 30% of patients have refractory/relapsing (R/R) disease and exhibit dismal outcomes. Long-term survival rates remain below 20% and R/R patients may only be cured with allogeneic hematopoietic cell transplantation. It is therefore critical to identify 1) biomarkers to predict therapy response and 2) novel disease mechanisms and therapeutic targets to improve the outcome of R/R patients with NPM1-mutant AML. The bone marrow microenvironment (BMME) has been implicated in leukemogenesis and drug resistance and therefore may account for prognostic disparities within genetically defined AML subsets and offer novel targeting opportunities.
We established a 60-plex co-detection by indexing (CODEX) antibody panel and spatially phenotyped the BMME in BM trephine biopsies from 68 NPM1-mutant AML patients at first diagnosis and 58 matched controls with normal BM (NBM). We imaged a total of 3.901.827 cells and used unsupervised clustering and manual annotation to identify 30 cell types, including leukemic cell subpopulations, myeloid, lymphoid, erythroid, vascular and stromal cells, megakaryocytes, and hematopoietic stem/progenitor cells. Spatial analysis of NBM marrows revealed 12 cellular neighborhoods (CNs) corresponding to known hematopoietic niches. The leukemic BMME showed a severe loss of structural organization with fragmentation of NBM CNs and expansion of leukemia-specific CNs. Furthermore, there was a shift towards immunosuppressive and regulatory lymphoid and myeloid cell types such as PD-1+ CD8+ T cells, regulatory T cells and myeloid-derived suppressor cells. Enrichment of suppressor cells and leukemia-specific CNs was associated with treatment response and

clinical outcomes independently of clinical risk stratification. In conclusion, we captured the spatial landscape of the BMME in NPM1-mutant AML and for the first time identified leukemia-specific and prognostically relevant CNs in a real-world cohort of AML patients.

Short talk by Malte Kuehl

Institute of Medical Systems Biology, Center for Biomedical AI (bAIome), Center for Molecular Neurobiology Hamburg (ZMNH), University Medical Center Hamburg-Eppendorf, Hamburg, Germany
Department of Clinical Medicine, Aarhus University, Denmark
Department of Pathology, Aarhus University Hospital, Denmark

PathoPlex – Next-generation multiplexed image analysis

Protein expression and localization are critical to understanding health and disease. While transcriptomic methods have advanced, they miss important post-translational modifications and structural details critical to disease. However, current spatial proteomics methods are limited by cellular resolution (>200nm/pixel), number of targets (approx. 40-50), and focus on cell segmentation, often overlooking sub- and extracellular signals.
Here, we present Pathology-oriented multiPlexing (PathoPlex), a scalable, quality-controlled and interpretable framework that combines deep multiplexed imaging capable of mapping >120 proteins at 80 nm per pixel using commercial antibodies and light microscopy with a novel software package (spatiomic) that integrates pixel-level signals to identify altered protein co-expression.
To validate our approach, we first applied PathoPlex to a model of immune-mediated kidney disease and identified c-Jun as a key mediator. Next, we used PathoPlex to dissect diabetic nephropathy, revealing integrative pathological features such as Ca2+-mediated stress and enabling druggability profiling. In a final example, PathoPlex uncovered features of renal stress in patients with type 2 diabetes who lacked pathological evidence of kidney disease, which were then used to assess the impact of sodium-glucose cotransporter 2 inhibitors.
In summary, PathoPlex identifies integrative features of tissue biology and supports the development of next-generation pathology atlases.

12:00 Lunch Symposium Akoya Biosciences *ESSB Lunchtime Raffle!*

Alessio Colombo

Akoya

Ultrahigh-Plex and High-Throughput Spatial Biology Solutions: From Discovery to the Clinic

Spatial phenotyping, which enables whole-slide imaging at single-cell resolution, is transforming our understanding of cellular organization and interactions. This technology is uncovering new insights into complex biological processes, such as tumor progression, inflammation, and immune responses, while also revealing novel therapeutic targets. In this presentation, our speaker will:

- Showcase how to scale spatial discovery efforts, turning insights into actionable spatial phenotypic signatures for therapeutic development.
- Highlight Akoya's Spatial Biology 2.0 Solutions, which offer unparalleled speed and scalability for advancing spatial biology studies.
- Explore the new PhenoCode Discovery Panels, designed to accelerate breakthroughs in immuno-oncology (IO) and drive advancements in preclinical research.

Lunch, Posters, Industry Exhibition

01:30 pm Lunch Symposium Miltenyi Biotec *ESSB Lunchtime Raffle!*

Katrin Schönborn & David Agorku

Miltenyi Biotec

Unlocking new insights in colorectal cancer with spatial multiomic on the MACSima™ Platform

Using the MACSima™ Platform with its advanced RNAsky® assay for same-section spatial multiomics, we comprehensively mapped cancer-associated fibroblast (CAF) heterogeneity in colorectal cancer (CRC) through integrated transcriptomic and proteomic analysis. While CAF subpopulations, such as inflammatory, antigen-presenting, and myofibroblastic CAFs, have been well-documented in cancers like pancreatic ductal adenocarcinoma and breast cancer, our study reveals their presence and diversity in CRC as well. Additionally, we identified a novel CAF subtype, termed TinCAFs (T cell-inhibiting CAFs), marked by NECTIN2 expression that mediates T cell suppression and exhaustion in vitro. Our findings emphasize the complexity of CAF heterogeneity in CRC and demonstrate the power of the MACSima™ Platform in unveiling new insights into immune evasion and tumor progression, advancing immuno-oncology research through spatial biology.

2:00 pm Session 3 - Machine Learning and Data Integration in Spatial Biology

Chairs: Julio Saez-Rodriguez, Leeat Keren

Talk sponsored by Ionpath

Leeat Keren

Molecular Cell Biology, Weizmann Institute of science, Rehovot, Israel

Escalating high-dimensional imaging for cancer research

Tumors are spatially organized ecosystems that are comprised of distinct cell types, each of which can assume a variety of phenotypes defined by coexpression of multiple proteins. To underscore this complexity it is essential to interrogate cellular expression patterns within their native context in the tissue. I will describe technological and computational advancements in multiplexed imaging, and demonstrate its application to study the tumor microenvironment.

Short talk by Lorenz Rumberger

Max-Delbruck-Center for Molecular Medicine in the Helmholtz Association, Berlin, Germany Humboldt-Universität zu Berlin, Faculty of Mathematics and Natural Sciences, Berlin, Germany, Helmholtz Imaging

Automated classification of cellular expression in multiplexed imaging data with Nimbus

Multiplexed imaging offers a powerful approach to characterize the spatial topography of tissues in both health and disease. To analyze such data, the specific combination of markers that are present in each cell must be enumerated in a process known as cell clustering. For clustering, the image data is transformed to tabular data, by integrating the marker expressions of the individual cell segments. This step introduces errors due to multiple confounders: signal can spill over from adjacent cells, uneven illumination and background staining can introduce false positives. To tackle this, we constructed the Pan-Multiplex (Pan-M) dataset containing 197 million distinct annotations of marker expression across 15 different cell types. We used Pan-M to create Nimbus, a deep learning model to predict marker positivity. Nimbus is a pre-trained model that can predict marker expression across distinct cell types, from different tissues, acquired using different microscope platforms, without requiring any retraining. We demonstrate that Nimbus predictions capture the underlying staining patterns of the full diversity of markers present in Pan-M. We then show how Nimbus predictions can be integrated with downstream clustering algorithms to robustly identify cell subtypes in image data. All underlying data, models, and code have been released as a community resource.

Short talk by Iva Buljan

CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria,
Ludwig Boltzmann Institute for Network Medicine at the University of Vienna, Vienna, Austria

Tissue-specific prediction of biological age from histopathological images

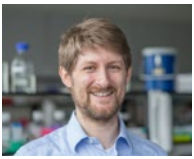
Aging, the leading risk factor for many diseases, manifests in diverse forms across human tissue architecture, providing an opportunity to quantify tissue-specific aging. To comprehensively understand the relationship between tissue structure, aging and pathology in humans, we present a comprehensive analysis of changes during aging using whole slide histopathological images from the Genotype-Tissue Expression Project (GTEx). Analyzing 25,712 images from 40 tissue types across 983 individuals, we apply deep learning to quantify age-related morphological changes.
We develop ‚tissue-clocks'—predictors of biological age from tissue images—achieving a mean prediction error of 4.9 years. These clocks correlate with telomere attrition, subclinical pathologies, and comorbidities. Assessing biological aging rates across organs reveals non-uniform aging patterns, with some organs showing early changes (20-40 years) and others displaying bimodal age-related patterns. We also identify associations between demographic, lifestyle, and medical history factors and tissue-specific acceleration or deceleration of biological age, highlighting modifiable risk factors.
This work bridges molecular and cellular changes to tissue-level contexts and demonstrates the value of histopathological imaging for understanding age-associated pathology in humans.

4:15 pm Session 4 New Spatial Biology insights for disease and pathology

Chairs: Anja Hauser, Bernd Bodenmiller

Keynote: Bernd Bodenmiller

University of Zurich /ETH Zurich, Zurich, Switzerland



Highly multiplexed imaging of tissues with subcellular resolution by imaging mass cytometry

Cancer is a tissue disease where heterogeneous tumor cells, stromal cells and immune cells form a dynamic ecosystem that evolves to support tumor expansion and ultimately tumor spread. The complexity of this system is the main obstacle to treat cancer. The study of the tumor ecosystem and its cell-to-cell communications is thus essential to enable an understanding of tumor biology, to define new biomarkers, and to identify new therapeutic routs and targets. To understand the workings of the tumor ecosystem, highly multiplexed image information of tumor tissues is essential. Such multiplexed images will reveal which cell types

are present in a tumor, their functional states, and how they interact together. Our analysis reveals surprising level of inter and intra-tumor heterogeneity.

Short talk by Maria Puschhof

Heidelberg University, Faculty of Medicine, and Heidelberg University Hospital, Institute for Computational Biomedicine, Bioquant, Heidelberg, Germany
European Molecular Biology Laboratory, Genome Biology Unit, Heidelberg, Germany

Molecular drivers of low- to high-grade transition in IDH-mutant glioma

Gliomas are the most common type of brain tumors in adults and the prognosis for patients strongly varies with the subtype and clinical grade. While 5-year survival can be as high as 80% for patients with low-grade tumors, it drops to under 5% for high-grade gliomas. While some gliomas are always highly aggressive (e.g. glioblastoma), IDH-mutant astrocytoma tumors are able to transition from low- to high-grade state. Thus, there is an urgent need to better understand the molecular drivers of this transformation to ultimately develop counteracting therapeutic approaches.
To this end, we have performed in-depth multi-modal profiling of whole astrocytoma tumors from 6 patients combining single nuclei multi-omics and spatial transcriptomics. Applying the Visium CytAssist platform to tissue sections from 69 tissue blocks, we were able to obtain both detailed pathology annotations as well as spatial transcriptomic data for each tissue slice, enabling the identification of transforming (high-grade) regions next to low-grade tumor tissue at the resolution of sequencing spots. Differential gene expression and functional analysis of biological activities was performed using decoupler, a framework to infer biological activities with the aid of prior knowledge database Omnipath. Across donors, low grade regions were enriched for axon ensheathment and the related processes of myelin assembly and oligodendrocyte development. Conversely, tumor cells residing in high grade regions exhibit enhanced cross-talk towards the microenvironment, both through cytokines and voltage-gated channels. Furthermore, high grade lesions were enriched for various biological processes related to neural development, suggesting distinct developmental programs associated with these areas. These observations were consistent with cell type deconvolution of spatial data via cell2location that further characterized cellular niches associated with low versus high grades. Taken together, we present the first spatial characterization of glioma grade transformation and identify candidate cellular and molecular targets for therapeutic intervention.

Short talk by Gesa Krueger

Institute of Immunology, Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Greifswald-Isle Riems, Germany

Modeling immune responses of cattle to mycobacteria using magnetic bioprinted granulomas

Tuberculosis (TB) remains a threat for human and livestock health. Mycobacteria causing TB are host-adapted pathogens which occasionally spillover to other species. Mycobacterium bovis causes bovine TB, a well-known zoonosis. A hallmark of TB in all hosts are multicellular tissue lesions termed granulomas. Using bovine leukocytes and nanotechnologies we developed a three-dimensional, multicellular granuloma model which we designated in vitro granuloma-like structure (IVGLS). We generated stable IVGLS resembling TB granulomas at innate, made of macrophages, or adaptive stages, containing also lymphocytes. Evaluation of IVGLS by confocal high-content imaging, accessing single-cell data on both image and statistical level, revealed progression of macrophages towards foamy phenotypes and increased apoptotic cell death over time. IVGLS, unlike conventional macrophage monolayers, were permissive for mycobacterial replication and released abundant phagocyte chemoattractants and Th1 cytokines. Magnetic bioprinted bovine granulomas facilitate studying immune responses to mycobacteria in a three-dimensional manner, including spatial mapping. Deciphering protective immune responses within IVGLS could contribute to vaccine development for cattle, whereas unveiling resistance mechanisms may help devise novel interventions for human TB.

5:30 Dinner Symposium – 10xGenomics *ESSB Dinner Raffle!*

Sarah Taylor

10XGenomics

Mapping Immune Cell Populations Using High-Definition Spatial Technologies

6:00 Members assembly

Ballroom 3

7:00 pm Poster Session & get together

Food and Drinks will be provided.
Later in the evening, a DJ will play for all those who like to dance.

DAY 2: Friday, December 13, 2024

8:15 am Short Breakfast Talk by Vizgen + Ultivue

Breakfast Buffet will be provided!

Dries Van Hemelen & Friedrich Preusser

Vizgen & Ultivue

Merging Expertise: Vizgen and Ultivue Join Forces to Innovate in Spatial Multi-Omics

08:30 am Session 5 Spatial Immunology

Chairs: Anja Hauser, Christian Schürch

Camilla Engblom

SciLifeLab, Division of Immunology and Allergy, Department of Medicine Solna, Center of Molecular Medicine, Karolinska Institutet, Stockholm and Department of Clinical Immunology and Transfusion Medicine, Karolinska University Hospital, Stockholm, Sweden

Spatially resolving B cell clonal dynamics

B cells perform functions critical to human health, including antibody production and antigen presentation. B cells develop, differentiate, and expand in spatially distinct sites across the body. B cells express clonal heritable B cell receptors (BCR), either as membrane-bound or secreted antibodies, that confer exquisite molecular (i.e., antigen) specificity. B cell receptors can be defined by sequencing, but these methods require tissue dissociation, which loses the anatomical location, and the surrounding functionally relevant environmental cues. Linking specific BCR sequences to their molecular and cellular surroundings, i.e., 'clonal niche', could help us understand and harness B cell activity. A technological bottleneck has been to capture the location of BCR sequences, and by extension B cell clonal responses, directly within tissues. We recently developed a spatial transcriptomics-based approach (Spatial VDJ) and associated computational pipelines to reconstruct B cell clonality in human tissues. Here, we present adaptation of Spatial VDJ to murine tissue to enable preclinical studies and B cell receptor dynamics under inflammatory conditions, including cancer.

Short talk by Raluca Niesner

Freie Universität Berlin; Germany Rheumatology Research Center, Berlin (DRFZ) – A Leibniz Institute

MetaFLIMB - longitudinal in vivo NAD(P)H fluorescence lifetime imaging in the femoral marrow at single-cell level

Alexander Fiedler[1,2], Ruth Leben[1,2], Anja E. Hauser[2,3], Raluca Niesner[1,2]
[1] Freie Universität Berlin; [2] DRFZ, Berlin – A Leibniz Institute; [3] Charité, Berlin

Phenotypes and functions of immune cells are tightly linked to their metabolic profile, while all being affected by the continuously changing tissue microenvironment. Focusing on bone regeneration, we aim to understand changes in single myeloid cell metabolism in bone marrow, over time. However, technologies providing such information are missing.
We developed a microendoscopic implant for the mouse femur to enable longitudinal NAD(P)H fluorescence lifetime imaging (FLIM) and used it for metabolic profiling at single-cell level in marrow tissue (MetaFLIMB). Based on reference enzyme-dependent NAD(P)H fluorescence lifetimes, we identified in these data preferential enzymatic activities and dominant metabolic pathways voxel-wise, i.e. (1) fatty acid β-oxidation (FAO), (2) anaerobic glycolysis, (3) carbohydrate metabolism pathways, (4) oxidative mechanisms, including oxidative phosphorylation. Additionally, (5) oxidative burst induced by NADPH oxidases activation and (6) dormancy or death, i.e. unbound NAD(P)H, were detected.
Using MetaFLIMB in osteotomized femurs of mice with red fluorescent myeloid cells (LysMCre/+R26LSL-tdRFP), we found that these cells display highly heterogeneous metabolic profiles both spatially and temporally during bone regeneration. Our results go beyond the binary paradigm of myeloid cells using either glycolytic or oxidative signaling pathways (linked to pro- or anti-inflammatory functions) predicted in vitro, presumably due to a dynamic marrow microenvironment. In vivo, myeloid cells with various metabolic profiles, i.e. using other pathways for energy production than the pro-inflammatory anaerobic pathway, performed oxidative burst needed for phagocytosis, at different time-points after injury. These data suggest that a high metabolic flexibility of myeloid cells in vivo is related to high functional flexibility.
As a label-free method, MetaFLIMB retains the power to bring spatio-temporal insight into the metabolism of diverse marrow cells, when using adequate fluorescent reporter mice.

Short talk by Katharina Imkeller

Institute of Neurology (Edinger Institute), University Hospital, Goethe University, Frankfurt
Cancer Institute (FCI), University Cancer Centre (UCT), Frankfurt, Germany;
MSNZ Group of Computational Immunology, University Hospital, Frankfurt, Germany

Connecting the Spatial Architecture and Immune Function of Tertiary Lymphoid Structures in Glial Tumors

Adult-type diffuse gliomas, the most common primary brain tumors, pose significant clinical challenges due to limited treatment options, restricted anti-tumor immune response and dismal patient prognosis. In this study, we elucidate the immunological function and clinical relevance of intra-tumoral tertiary lymphoid structures (TLS) in adaptive anti-glioma immunity. We conducted a comprehensive, unbiased analysis of lymphoid aggregation in 642 human gliomas using a multi-modal approach that combines RNA sequencing with spatial transcriptome and proteome profiling. Our findings reveal that TLS are present in

15% of tumors and correlate with improved overall survival. Gliomas with TLS exhibit a remodeled perivascular space, marked by transcriptional upregulation and spatial redistribution of collagens associated with barrier functions. Furthermore, we demonstrate that TLS maturation into sites of dynamic adaptive immune responses, characterized by clonal T and B cell expansion and IgA+ and IgG+ plasma-cell formation, is driven by efficient early T cell recruitment to the perivascular space.

Short talk by Vladimir Sukhov

Washington University School of Medicine, St. Louis MO, USA

Advanced Spatial Analysis Highlights Immune Cell Organization in Successful Tumor Rejection After Immune Checkpoint Therapy

A deeper understanding of how immune cells organize within tumors is crucial for developing more effective cancer therapies, as cell organization affects their function and treatment outcomes. By leveraging a reproducible tumor model with well-defined rejection kinetics, here we used advanced analytical methods to interrogate a longitudinal CODEX data set and identify spatiotemporal changes that drive successful tumor rejection upon treatment with immune checkpoint immunotherapy (ICT). Mice with T3 MCA sarcomas were treated with Control Ab or α-PD-1 and α-CTLA-4 antibodies (ICT), and tumors were analyzed on days 7, 9, 10, 11, and 13 with a 32 antibody panel providing detailed images of 42 tumors. Using unsupervised clustering and classifiers, we analyzed over 13 million cells and identified 13 distinct cell types. Tumor cells decreased by day 10 and were completely eliminated by day 13. This was driven by increased CD4+ T cells clustering with CD8+ T cells and type 1 conventional dendritic cells, forming high-contact clusters. Cellular neighborhood (CN) analysis revealed a lymphoid-rich CN, containing most CD4+ and CD8+ T cells that progressively increases in ICT-treated tumors and is enriched for granzyme B-positive and proliferating T cells. Interestingly, after day 10, these effector T cell populations display distinct kinetics as cytotoxic T cells moved toward remaining tumor cells while proliferating T cells concentrated in the tumor tissue periphery. We next examined the communication rules between CNs by identifying cells at the CN interface and constructing a network of interactions. This technique showed different architectural patterns between control and ICT conditions, indicating that effective immunotherapy alters spatial organization by promoting expansion of the lymphoid-rich CN over the tumor boundary and nearby myeloid CNs. Overall, this study allowed us to develop bioinformatical methods to comprehensively study how T cells coordinate the antitumor response during successful immunotherapy with α -CTLA-4 and α-PD-1.

Short talk by Francesca Bosisio

Translational Cell and Tissue Research Unit, Department of Imaging and Pathology, KU Leuven, Leuven, Belgium
The Leuven institute for single-cell omics (LISCO), KU Leuven, Leuven, Belgium
Department of Imaging and Pathology, University Hospitals Leuven, Leuven, Belgium

Omics Integration Reveals Spatial and Molecular Predictors of Response to Anti-PD-1 Immunotherapy in Metastatic Melanoma

While immune checkpoint-based immunotherapy (ICI) yields promising results in cancer treatment, only a subset of patients responds favorably. The efficacy of ICI is tied to complex interactions within the tumor microenvironment, involving malignant and non-malignant cells. Despite insights into the antitumor role of cytotoxic T cells (Tcy), traditional single-cell analyses miss critical spatial information necessary for understanding cellular interactions and their therapeutic impacts.
Previously, our team used high-dimensional multiplexed immunohistochemistry (mIHC) to study the immune landscape in metastatic melanoma, distinguishing between ICI responders and non-responders. We found that interactions between Tcy and PD-L1+ M1-like macrophages at the tumor-stroma interface predict responses to anti-PD-1 therapy.
Expanding on this, we integrated mIHC with single-cell RNA sequencing and spatial transcriptomics (ST, Nanostring CosMx), delving into the dynamics of these key immune populations. Our findings not only reinforce the pivotal role of these cellular networks but also reveal significant expression gradients of functional markers based on the proximity of macrophages to Tcys. This deeper understanding of spatial and molecular profiles further elucidates the mechanisms driving the response to anti-PD-1 immunotherapy, suggesting pathways for more precise and effective treatments.

Short talk by Aysegül Adam

Charité – Universitätsmedizin Berlin, Department of Periodontology, Oral Medicine and Oral Surgery, 14197 Berlin, Germany
Immune Dynamics, Deutsches Rheuma-Forschungszentrum (DRFZ), a Leibniz Institute, Charitéplatz 1, 10117 Berlin, Germany

Spatial characterization of Innate Lymphoid Cells in oral inflammatory diseases

The oral mucosal barrier is under constant exposure to a variety of antigens and commensals via the upper respiratory tract and the oral cavity which is home to one of the most prevalent chronic-inflammatory diseases, periodontitis. Periodontitis affects up to 47% of the adult population worldwide (~11% in its severe forms). Besides its local effects on tooth supporting structures– destruction of the alveolar bone and the gingiva, periodontitis is coupled with an increased risk of chronic and potentially grievous diseases, incl. cardiovascular diseases, diabetes, and rheumatoid arthritis. However, the immunological me-

chanism and cellular players regulating the switch from immunity to immunopathology, as well as its possible role in promoting systemic inflammation remain ill-defined. In the last few years innate lymphoid cells (ILCs) emerged as sentinels of mucosal barriers, maintaining barrier integrity, tissue healing and remodeling, features that are disturbed in patients suffering from periodontitis. Since barrier disintegration is one of the main characteristics of periodontitis we set out to investigate ILC localization, and tissue niches in the inflamed gingival tissues. Fluorescent immunophenotyping indicates an expansion of ILCs, and a shift in subpopulations to TNF-α and IL-17A producing subtypes in periodontitis compared to periodontally healthy individuals. MELC (multi-epitope-ligand-cartography), a multiplex immunofluorescence imaging technique allowed for an analysis of distribution patterns of ILCs and characterization of their putative hematopoietic and non-hematopoietic interactions partners within the tissue. Features of innate immune activation such as neutrophil extracellular traps (NETs), as well as chronic stimulation of the adaptive immune system resulting in tertiary lymphoid structure formation were identified for the first time at those sites. Combining our proteomic approach with spatial transcriptomics, we aim to distinctly define immune dynamics within the gingiva in the context of periodontitis.

11:00 am Session 6 New Dimensions in Spatial Biology

Chairs: Sinem Saka, Harsharan Singh Bhatia

Harsharan Singh Bhatia

Helmholtz Munich and Ludwig Maximilian University (LMU), Munich, Germany

Clear-Omics: Spatial molecular maps in optically cleared 3D intact specimens

Spatial molecular profiling of complex tissues is crucial for understanding cellular function in both physiological and pathological contexts. To achieve this, optical tissue clearing and 3D imaging provide an unbiased view of deep tissues within intact organs and organisms. However, while these techniques offer detailed morphological insights, they fall short in addressing deeper mechanistic questions. To bridge this gap, our lab has been developing novel tools that integrate whole-organ/organism optical clearing and imaging, deep learning-based image analysis, robotic tissue extraction, and ultrahigh-sensitive mass spectrometry-based proteomics and transcriptomics. This presentation will delve into the methodology and the recent biological applications of these groundbreaking technologies, exploring their potential to investigate a range of pathologies such as acute brain injuries, infections, cancer, and metabolic dysfunction.

Short talk by Peter Androvic

Department of Old Age Psychiatry and Cognitive Disorders, University Hospital Bonn, Bonn, Germany
Institute for stroke and dementia research, LMU University Hospital, Munich, Germany

Beyond Genes: Integrating Transcriptomics with Structure and Metabolism to Map Neurodegeneration

Deciphering the functional implications of cell state change in complex diseases requires integrating multiple views of cellular phenotype within native tissue context. We present spatially-resolved, multi-modal approaches to characterize cellular responses to brain aging and neurodegeneration, combining spatial transcriptomics, structural analysis, and spatial lipidomics.
Our Spatial Transcriptomics-correlated Electron Microscopy (STcEM) method links spatial gene expression of single cells with their ultrastructural morphology by integrating MERFISH and large area electron microscopy on adjacent tissue sections. Applying STcEM to a mouse model of demyelinating brain injury, we characterized and linked transcriptional and structural states of microglia and infiltrating T-cells in situ. By further integrating these datasets with single-cell RNA-Seq we uncovered correlations between genome-wide gene expression and ultrastructural features of microglia. We identified distinct microglia state within remyelinating lesions, characterized by aberrant inflammatory signature and accumulation of lipid droplets. To further probe functional implications, we have expanded our approach to include spatial lipidomics via mass spectrometry imaging (MSI). Our preliminary data reveal distinct lipid distributions associated with vulnerable brain regions and pathological microenvironments. By integrating MSI with MERFISH, we are mapping gene-metabolic networks and tracking their alterations in the aging brain and during amyloidosis progression.
Our framework provides an integrated, multi-scale view of the spatial, structural, transcriptional, and metabolic reorganization of the brain in response to pathology. By applying machine learning to these rich datasets, we next aim to develop predictive models of cellular behavior and disease progression, providing critical new insights into the mechanisms of brain aging and neurodegeneration.

Short talk by Jorge Trojanowski

European Molecular Biology Laboratory (EMBL), Tissue biology and disease modeling Unit, Barcelona, Spain
European Molecular Biology Laboratory (EMBL), Genome biology Unit, Heidelberg, Germany

Single-round Profiling by Amplification and Color Encoding (SPACE)-FISH in 3D microvasculature on-chip

Self-assembly of endothelial and supporting cells into a perfusable vessel network establishes the conduit for blood supply of tissues during development, and restores it during wound healing. Vessel formation involves multiple cell types and cell states that need to

be coordinated spatially. So far, this process could only be studied in dissociated cells or for a small number of selected target genes in situ, but a direct mapping of transcriptional states to the tissue positions is missing. To understand the spatio-temporal regulation of the cellular states during vessel formation, we employ multiplexed fluorescence in situ hybridization (FISH) to detect gene transcription in a complex 3D in vitro vascularized tissue. Performing FISH in 3D tissues with cycling-based multiplexing approaches is challenging due to long staining times, which limits efficient multiplexing. To resolve these challenges, we developed Single-round Profiling by Amplification and Color Encoding (SPACE)-FISH which is based on multi-color labeling of the nascent RNA at 18 target gene loci with a single round of staining and imaging. The combinatorial color barcodes extend the multiplexing capacity compared to traditional FISH methods, hence SPACE-FISH circumvents prohibitively long staining and imaging times making it a viable approach for scalable high-plex 3D tissue imaging. Applying this method to microvasculature from human-derived primary cells grown on-chip over different time points, we are able to visualize a spatiotemporal expression map for relevant genes involved in the self-assembly process. In particular, we investigated the expression profiles of genes related to angiogenesis, cell type and cell cycle. This approach will be valuable for applications that require the scalable and inexpensive detection of tens of nascent transcripts in 3D tissues ranging from cell type detection to studies of transcriptional mechanisms such as mono- vs bi-allelic expression.

12:00 pm Lunch Symposium - Lunaphore

Daniel Azorin & Müge Akpinar

ETH Zurich & Lunaphore

Spatial multiomics for precision target identification in the tumor microenvironment This work introduces an innovative spatial multiomics methodology for the simultaneous analysis of RNA and protein within tumor tissue. The approach offers in-depth characterization of the tumor microenvironment, facilitating the discovery of critical biomarkers and therapeutic targets.

Lunch, Posters, Industry Exhibition

1:30 pm Lunch Symposium Standard BioTools

Melissa Klug

Standard BioTools™

Whole Slide Imaging Modes for Imaging Mass Cytometry Reveal Cellular Diversity of the Tumor Immune Microenvironment in Mouse Glioblastoma

Mouse models are used for translational research and permit detection of therapeutic-induced immune-related modification in the tumor immune microenvironment (TIME). Imaging Mass Cytometry™ (IMC™) is a spatial biology technique capable of quantitative evaluation of multiparametric tumor and immune cell composition in brain tissue. Here we demonstrate the application of a 40-marker panel composed of tumor and immune markers on mouse normal and glioblastoma (GBM) tissue using two new whole slide imaging (WSI) methods for IMC. Tissue Mode rapidly scans the entire tissue followed by pixel-clustering analysis to uncover spatial distribution of relevant markers. Preview Mode samples the entire tissue at predefined spacing to rapidly capture a low-resolution image of all expressed markers, improving informed identification of areas for high resolution Cell Mode imaging on the same slide. Tissue Mode imaging with pixel clustering analysis provided quantitative spatial expression patterns of tumor and immune markers across the GBM tissue. Necrotic cores, areas with high immune infiltration, extracellular matrix deposits and activated tumor cells were quantified. Preview Mode in combination with Cell Mode imaging and single-cell analysis revealed extensive tissue vascularization, replicating tumor cells and a variety of infiltrating immune cells. Overall, we highlight the capability of IMC to provide quantitative insights into the spatial biology of the TIME.

02:00 pm Session 7 Tumor Microenvironment

Chairs: Raza Ali, Julio Saez-Rodriguez

Raza Ali

CRUK Cambridge Institute, University of Cambridge, Addenbrooke's Cambridge University Hospital ,NHS Foundation Trust

Charting the intact breast tumour microenvironment

The diagnosis and treatment of breast cancer continues to rely on decades-old techniques in traditional histopathology. Immunotherapy has proved effective among some patients but not others, and this variation is poorly explained by traditional assays. Using imaging mass cytometry – a technique that couples antibodies conjugated to rare earth metal reporters and time-of-flight mass spectrometry to infer epitope abundance at subcellular resolution – my group has shown that the complexity of the TME can be reliably enumerated in situ and used to predict response in a large randomized trial of neoadjuvant immunotherapy in triple-negative breast cancer. Moreover, we show how immunotherapy remodels the TME, and how resistant cancer cells endure treatment by analyzing serial samples collected over the treatment course. I will share our results and offer some insights on the wider implications for spatial cancer biology.

Oliver Stegle

Computational Genomics & Systems Genetics, German Cancer Research Center (DKFZ), Heidelberg Germany

Data infrastructures for spatial omics and advances in cell segmentation

Oliver Stegle is the Head of the Computational Genomics and Systems Genetics Division at the German Cancer Research Center (DKFZ) and group leader at EMBL in Heidelberg, Germany. His laboratory is developing and applying statistical and machine learning methods for deciphering molecular variation across individuals, space and time. He coordinates the German Human-Genome-Phenome Archive, the health program of the European Laboratory for Learning and Intelligent Systems and is an ERC investigator.

Short talk by Spencer Watson

Department of Oncology, University of Lausanne, Ludwig Institute for Cancer Research, University of Lausanne, Switzerland; Agora Cancer Research Center Lausanne
Agora Cancer Centre, University Hospital Lausanne
Lundin Brain Tumour Centre, University Hospital Lausanne

Investigating Glioblastoma Recurrence with Spatial Multi-Omics

Glioblastoma recurrence is currently inevitable despite advances in standard-of-care treatment. An alternative approach of targeting the tumor microenvironment, specifically tumor-associated macrophages via CSF-1R inhibition, was found to dramatically regress established tumors in preclinical trials. While tumor regression was sustained in ~50% of individuals, recurrent tumors emerged over time in the remaining subset. These recurrences were ubiquitously associated with fibrotic scars that had formed during treatment. This same fibrotic response to treatment was also observed following surgical resection, radiotherapy, and in patient samples of recurrent glioblastoma. Investigating the complex evolving post-treatment tumor landscape required integrating multiple -omics approaches, including mass-spec proteomics, single-cell RNAseq, high-dimensional digital pathology, and spatial transcriptomics. Spatial multi-omics analyses of the post-treatment tumor microenvironment identified specific fibrotic domains as pro-tumor survival niches that encapsulated surviving glioma cells, and inhibited immune surveillance while maintaining the tumor cells in a dormant state. Integrated analyses revealed that targeting neuroinflammatory signaling and the TGF-beta pathway in a unique population of fibroblast-like perivascular cells could abrogate the fibrotic response to anti-CSF-1R therapy. Combinatorial therapies timed to the activation window of fibroblast-like cells both inhibited treatment-associated fibrosis and significantly improved survival in long-term preclinical trials of anti-CSF-1R therapy.

Short talk by Ann-Marie Baker

Centre for Evolution and Cancer, Institute of Cancer Research, London, United Kingdom

Spatial analysis reveals reshaping of tumour-immune dynamics at the transition to invasive colorectal cancer

The introduction of bowel cancer screening has led to an increase in the detection and removal of pre-malignant adenomas, however many of these would never progress to colorectal cancer (CRC) within a patient's lifetime. This implies a key evolutionary bottleneck at the transition from adenoma to carcinoma, with immune surveillance likely playing a central role in suppressing the outgrowth of invasive cells. Here we analysed a cohort of "ca-in-ads" (adenomas with a small focus of cancer) to provide a unique snapshot of the invasive transition. We combined genomics, spatial transcriptomics, digital pathology and multiplex imaging, concurrently deriving the distribution of tumour clones, their gene expression and their interplay with the immune microenvironment.

We performed multi-region low-coverage whole genome sequencing and T-cell receptor sequencing on formalin-fixed paraffin-embedded ca-in-ads from 40 patients. For a subset of cases we performed spatial transcriptomics using the 10x Genomics Visium platform, including custom probes to detect T-cell clones of interest. We applied a deep learning cell classifier to haematoxylin and eosin (H&E) stained sections to determine the abundance and spatial distribution of eight cell types, with detailed immunophenotyping on selected cases (using a 50-marker panel on Akoya's PhenoCycler-Fusion).

We found a significant increase in copy number alterations in carcinoma regions relative to adenomas, and this was accompanied by a large shift in the composition of the T-cell repertoire. Examples of T-cell clonotypes that appeared unique to either the adenoma or the invasive cancer were validated using Visium. Digital pathology and cyclic immunofluorescence revealed significantly increased infiltration of macrophages and neutrophils in regions of invasive cancer. Taken together our analysis suggests a striking reshaping of tumour-immune dynamics at the transition to an invasive phenotype in CRC. Understanding what governs this invasive shift could highlight new avenues for cancer prevention or identify individuals at risk of CRC progression.

Short talk by Christina Schniederjohann

Department of Hematology, Oncology and Clinical Immunology, Medical Faculty and University Hospital Düsseldorf, Heinrich Heine University Düsseldorf, Düsseldorf, Germany
Center for Integrated Oncology Aachen-Bonn-Cologne-Düsseldorf (CIO ABCD), Düsseldorf, Germany
Molecular Medicine Partnership Unit (MMPU), Heidelberg, Germany
Department of Medicine V, Heidelberg University Hospital, Heidelberg, Germany.

Microenvironmental Factors Impacting Chemotherapy Response in B-NHL

The tumor microenvironment (TME) and its spatial architecture are known to influence therapy effectiveness. However, the spatial organization of diffuse large B-cell lymphoma (DLBCL) remains largely unexplored. Here, we examine the differences in cellular composition and spatial architecture in DLBCL and correlate these findings with clinical outcomes following chemoimmunotherapy.
Using CO-Detection by indEXing (CODEX) we analyzed the cellular composition of aggressive B-cell non-Hodgkin lymphoma samples from 193 patients at initial diagnosis, who received chemoimmunotherapy. Samples were assembled in tissue microarrays and stained with a 54-plex antibody panel targeting microenvironmental cells along with key functional markers of malignant B cells.
The cellular composition of the DLBCL microenvironment varied significantly among samples but remained consistent across technical replicates. Among others, lymphoma-infiltrating T-cells ranged from 0.7% to 85% of all cells, with a mean frequency of 29%. We could show that a higher infiltration of cytotoxic T-cell is linked to favorable outcomes, while exhausted cytotoxic T-cells are correlated with poor outcomes.
We examined the spatial interactions between different cell types and identified seven distinct cellular neighborhoods across patients, based on the 30 nearest neighboring cells. The composition of these neighborhoods varied between patients, likely reflecting different interaction patterns between the tumor cells and their microenvironment. Patient samples could be divided into three distinct groups based on these spatial patterns including one immune-cell deficient group and two distinct immune-cell enriched groups.
Our results underline the importance of microenvironmental factors such as T-cell infiltration for clinical outcome after chemoimmunotherapy in DLBCL. Further, the neighborhood analysis revealed not only a strong heterogeneity between patients but also recurrent patterns that are similar across patients. Linking these spatial subgroups to clinical and genetic annotations might allow us to better understand the spatial architecture of DLBCL and reveal insights into spatial patterns that mediate therapy response.

04:15 pm Session 8 Visions for Spatial Biology

Chair: Denis Schapiro

Talk sponsored by Ionpath

Keynote Speaker: Peter Sorger

Program in Therapeutic Science, Harvard Medical School, Boston, USA

Spatial profiling of human tissues for discovery and diagnosis

The effective treatment of cancer and many other diseases is increasingly dependent on a precision approach in which the quantification of molecular features at the level of individual patients is used to guide treatment plans. Currently, cancer diagnosis and staging are performed primarily via direct examination of biopsy and resection specimens by histopathologists. However, these classical methods provide insufficient molecular insight to guide the use of targeted and immunotherapies even when supplemented by knowledge of tumour genotypes. I will describe the recent development of several high-plex 3D tissue imaging methods that provide new insight into cell states and interactions in the preserved environment of human specimens. I will also discuss how sophisticated data of this type can be used to advance simpler methods that represent a practical approache to the development of a new generation of multiplexed histopathological test for use in clinical trials and patient care. Such tests promise to improve outcomes and reduce the burden of therapy. Realizing these research and diagnostic possibilities requires the development of new instruments, reagents, and machine-learning algorithms. I will describe progress in these areas with reference to the characterization of immune landscapes in primary melanoma and predicting tumour progression in colorectal cancer.

5:00 pm Poster Award Ceremony, concluding remarks and farewell

Poster Abstracts

Posters are numbered alphabetically according to the last name of the presenting author/ first author.

001 Spatial Transcriptomics of Human TB Lymph Nodes

Odunola Adebiyi [1], Tracey Jooste, Gerhard Walzl[1], Nelita du-Plessis [1],

[1]*Immunology Research group, Stellenbosch University, Cape Town, South Africa*

Tuberculosis (TB) granulomas exist along a spectrum of phenotypes, ranging from protective, sterile granulomas that contain the infection, to permissive granulomas that allow uncontrolled growth and dissemination of Mycobacterium tuberculosis (M.tb). These heterogeneous granulomas can coexist in an individual host. TB clinical and treatment outcome in a host is determined locally by the overall cumulative effect of this granuloma heterogeneity. However, the factors driving the formation and persistence of distinct granuloma phenotypes remain largely unclear. To address this, in this study, we employed the 10x Genomics Visium spatial transcriptomics platform, combined with routine histological staining and immunofluorescence, to characterize the immune landscape of four granulomas (necrotic and non-necrotic) across eight tissue sections from archived lymph nodes of children who underwent excision due to TB lymphadenitis. Our findings reveal that non-necrotic granulomas harbor a higher accumulation of CD8+ T cells within the granuloma center, in contrast to the more dispersed distribution in necrotic granulomas. Non-necrotic granulomas were enriched for both interferon-alpha and interferon-gamma responses, with widespread expression of interferon-stimulated genes (IFI44L, IFI27, IFITM3, IFI6, IFIT3, IFI44, and IFIH1). Conversely, the low IFNG expression was restricted primarily to the myeloid-rich core of the necrotic granulomas, accompanied by widespread expression of TGFB1 across both granuloma types. In addition, both granuloma phenotypes exhibited immunosuppressive features, marked by widespread expression of IDO1. PD-1 and IL10 expression were notably low across all granulomas, whereas PD-L1 was more prominently expressed in non-necrotic granulomas and localized to myeloid regions within necrotic granulomas. These ongoing findings suggest that distinct, spatially coordinated compensating immunoregulatory programs are present within different TB granuloma types during active disease, highlighting the complex local immune responses that may influence TB progression and treatment outcomes.

*Kindly Note: Bioinformatic Analysis is still ongoing.

Multiomic characterization of colorectal cancer using MICS technology reveals close interaction of cancer-associated fibroblasts and T cells 002

David Agorku[1], Emily Neil[2], Rebecca C. Hennessey[2], Dongju Park[2], Julia Femel[1], Michael DiBuono[2], Hanna Lafayette[2], Erica Lloyd[2], Hsinyi Lo[2], Alex Makrigiorgos[2], Shaina Lu[2], John Lee[2], Sameh Soliman[2], Dominic Mangiardi[2], Paurush Praveen[1], Philipp Ströbel[3], Silvia Rüberg[1], Fabian Staubach[1], Ryan Hindman[2], Thomas Rothmann[1], Olaf Hardt[1], Hansueli Meyer[2], Tanya Wantenaar[1], Jinling Wang[2], Werner Müller[1], Robert Pinard[2], and Andreas Bosio[1]
1Miltenyi Biotec, Bergisch Gladbach, Germany;
[2]Miltenyi Biotec, Waltham, MA;
[3]University Medical Center Göttingen, Göttingen, Germany

In solid tumors, cancer-associated fibroblasts (CAFs) have been described as key players in the tumor microenvironment (TME). They have been shown to affect cancer progression and response to treatment via interaction with diverse cell types in the TME including cancer and immune cells. To better resolve potential subtypes, spatial relationships, and signaling occurring between CAFs and other cell types within the TME, we have performed multiomic profiling of CAFs by single-cell RNA sequencing and same-section multiomic profiling using the MACSima™ Imaging Cyclic Staining (MICS) technology.

Our analysis revealed different subtypes of fibroblasts in the TME of CRC, which were characterized by differential gene expression and were spatially separated. Characterization of cellular neighborhoods and potential cell-to-cell interactions occurring within the TME showed that one of the CAF populations we named TinCAFs (T cell-inhibiting CAFs), closely interacted with T cells to potentially suppress them. By using RNAsky™ technology, which allows for detection of RNAs in combination with proteomic profiling, we demonstrated that TinCAFs and activated lymphocytes did not overlap in the TME.

Our findings provide an atlas of fibroblast heterogeneity in colorectal cancer and highlight a T cell-suppressive CAF subtype. Together, the data deepen our understanding of crosstalk within the TME and how it contributes to tumor progression.

NiCo Identifies Extrinsic Drivers of Cell State Modulation by Niche Covariation Analysis 003

Ankit Agrawal[1*], Stefan Thomann[1], Sukanya Basu[1], Dominic Grün[1]

[1]Würzburg Institute of Systems Immunology, Max Planck Research Group at the Julius-Maximilians- Universität Würzburg, Würzburg, Germany

Cell states are modulated by intrinsic driving forces such as gene expression noise and extrinsic signals from the tissue microenvironment. The distinction between intrinsic and extrinsic cell state determinants is essential for understanding the regulation of cell fate in tissues during development, homeostasis and disease. The rapidly growing availability of single-cell resolution spatial transcriptomics makes it possible to meet this challenge. However, available computational methods to infer topological tissue domains, spatially variable gene expression, or ligand-receptor interactions are limited in capturing cell state changes driven by crosstalk between individual cell types within the same niche. We present

NiCo, a computational framework for integrating single-cell resolution spatial transcriptomics with matched single- cell RNA-sequencing reference data to infer the influence of the spatial niche on the cell state. By applying NiCo to mouse embryogenesis, adult small intestine and liver data, we demonstrate the capacity to predict novel niche interactions that govern cell state variation underlying tissue development and homeostasis. In particular, NiCo predicts a feedback mechanism between Kupffer cells and neighboring stellate cells limiting stellate cell activation in the normal liver. NiCo provides a powerful tool to elucidate tissue architecture and to identify drivers of cellular states in local niches.

004 Landscape of spatial B cell organisation and immunotherapy response in TNBC

Lubna Ahmad[1], Ellen Schrader[1], Xiao Qian Wang[1], Neus Masque-Soler[1], Maurizio Callari[2], Matteo Dugo[3], Pinuccia Valagussa[2], Giuseppe Viale[4,5], Luca Gianni[2], Giampaolo Bianchini[2,3,6*], H. Raza Ali[1,7*]

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Although tertiary lymphoid structures (TLS), have emerged as indicators of immunotherapy efficacy, the broader spectrum of B cell spatial organisation within the tumour microenvironment (TME) remains largely unexplored. B cells are fundamental to adaptive immunity, and their spatial structures provide key insights into their functional roles. Yet, the broader diversity of their organisational patterns within the TME has not been well-understood. This spatial heterogeneity, influenced by inflammatory cues and a myriad of factors within the TME, underscores the need for a comprehensive and systematic investigation. Such research is essential to uncover the diverse functional roles of B cells in cancer and to identify novel biomarkers that could predict treatment response.
To characterise the spatial landscape of B cells, we have implemented a novel approach of simultaneous detection of RNA and protein in situ by imaging mass cytometry. We applied this assay to a large-scale randomised neoadjuvant immunotherapy clinical trial, NeoTRIPaPDL1, where patients with high-risk triple-negative breast cancer (TNBC) were treated with atezolizumab. Samples from 268 patients were collected at three timepoints: baseline, on-treatment and at surgery. All tissues were stained with RNA probes for chemokines and antibodies targeting B cells and TME phenotypes. Regions of interest of immune cell infiltration and aggregation in tumour biopsies were targeted and acquired using IMC. This yielded a dataset of over 2000 images capturing snapshots of B cell organisation and immune signalling molecules in the TNBC TME. To date, this study provides the largest B cell and immune signalling single-cell dataset of TNBC, allowing us to characterise the landscape of

B cell organisation in cancer. Overall, this retrospective study will create a valuable resource and framework to understand the roles of B cells in cancer and will inform us of their value as potential biomarkers for immunotherapy response.

Spatial reconstruction of single-cell enhancer activity in a developing embryo 005

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Understanding the spatial and temporal regulation of gene expression is fundamental to unraveling the mechanisms underlying development. Enhancers play a crucial role in orchestrating these processes, yet identifying their spatio-temporal activity remains a significant challenge in developmental biology.
In this study, we present spatial-scERA, a novel in vivo/in silico single-cell enhancer reporter assay designed to predict the spatial activity of multiple enhancers in a multicellular organism. Our method combines scRNA-seq with spatial reconstruction using novoSpaRc. By using prior knowledge of gene expression patterns within a virtual tissue, called an atlas, novoSpaRc can map cells from their transcriptomic profiles back onto a virtual embryo. This approach, with custom improvements, enables the prediction of candidate enhancers' spatial activity with cellular resolution.
We tested our protocol on 25 developmental enhancers in stage 6 Drosophila embryos, comparing the predictions to HCR imaging. Remarkably, spatial-scERA faithfully predicts their spatial activity, even when the expression of the enhancer-reporter construct was detected in as few as 10 cells.
Moving forward, the next step involve generating our own atlas using MERFISH, This will lead to the creation of 3D atlases for any developmental stage. We believe that, combined with our approach, this could significantly advance the study of gene regulation in complex multicellular organism.

Cell segmentation for immunofluorescence multiplexed images using a DeepLabV3+ deep neural network 006

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Highly multiplexed imaging technologies tackle how the phenotype and the function of cells are influenced by their tissue niche.
Cell segmentation is a crucial part of imaging-based single-cell analysis, but manual labeling is time-consuming. This has led to the rise of automated segmentation solutions, particularly using deep learning algorithms, which have shown strong performance. However, challenges remain in cytometry due to irregular cell shapes, cell overlap, and varying intensities. Inconsistencies in imaging conditions, such as staining intensity and microscope settings, also complicate accurate segmentation.
Traditional deep learning models may struggle with these challenges, particularly when cells overlap or cluster densely in tissues. Moreover, tissues and immune cells are highly heterogeneous, making it difficult to distinguish between different cell types, especially in complex environments like tumors or inflamed tissues. Many cell types share markers, complicating the task of differentiating them.
Using a 26+-plex MELC dataset from murine lung and SI tissue that was previously analyzed semi-automatically, we propose a pipeline for automated cell segmentation using the DeepLabv3+ architecture, enhanced with Atrous convolutions. DeepLabv3+ is well-suited for this task due to its ability to adjust the field of view in convolutional layers without increasing computational complexity or the number of parameters. Atrous convolutions allow the model to effectively segment cells at multiple scales, preserving spatial resolution, which is crucial for capturing the complex structures in diverse tissue environments.
In our study, DeepLabv3+ achieved impressive performance metrics on the dataset: a mean Intersection over Union (mIoU) of 85.29%, a mean Pixel Accuracy (mPA) of 93.06%, a mean Precision of 91.22%, and a mean Recall of 93.06%. These results highlight the effectiveness of the model in addressing the challenges of cell segmentation, such as irregular cell shapes, overlapping cells, and variations in staining intensity.

007 Deep Learning approach for Detecting and Segmenting Perineural Invasion in Colon, Prostate, and Pancreatic Cancers

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Perineural invasion (PNI) represents a significant pathological feature where cancer cells surround or infiltrate the nerves within the tumour microenvironment, indicating an aggres-

sive tumour behaviour and a potential mechanism for tumour spread. This phenomenon is observed across various types of cancers, including squamous cell carcinoma, pancreatic cancer, prostate cancer, and colon cancers. PNI is associated with a worse prognosis, including increased risk of local recurrence, distant metastasis, and reduced overall survival.
We present a deep learning framework for the automated detection and segmentation of PNI within whole slide images (WSIs) of colon, pancreas, and prostate tumour tissue. Leveraging a dataset of 150 pathologist-annotated slides, our model's architecture uses a pre-trained EfficientNet-B3 for organ-specific classification and a pre-trained UNet for segmentation. For preprocessing, we have extracted 224 x 224 patches for WSI and masks using sliding window with 50% overlapping. Patches containing about 90% white background were removed. Preliminary models show accuracies of 0.74 for colon, 0.73 for prostate, and 0.72 for pancreas. These will be used to build a framework to model the tumour neuro-immune environment, the association between PNI, tumour-infiltrating lymphocytes, tumour mutational burden and their collective impact on response to immune oncology treatment strategies, potentially enhancing patient stratification based on PNI status, and development of personalized cancer treatment approaches.

Spatial and Multiomics Characterization of Solid Tumor: Ovarian Cancer MICS Panel **008**

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Solid tumor immunotherapies, including CAR-T cell treatments, face significant challenges due to the immunosuppressive tumor microenvironment (TME). Our investigation centers on ovarian cancer due to its high mortality rate, aggressive nature, and the critical need for innovative therapeutic strategies, particularly as conventional treatments often show limited efficacy. We have developed a comprehensive multiplex immunofluorescence panel using MACSima™ Imaging Cyclic Staining (MICS) technology to characterize major cell types within primary ovarian tumor samples. This panel enables deeper phenotyping of cells within the TME through an unbiased clustering workflow, providing valuable insights into immune-tumor dynamics.
Combination of the developed panel with RNAsky™ technology allows to complement the analysis with transcriptomic characterization, offering a more comprehensive understanding of protein and RNA dynamics. The flexibility of this integrated panel allows incorporation of further research-dependent markers, identified through other omics methods such as single- cell RNA sequencing. This multiomics approach has the potential to identify immune- suppressive pathways and guide the development of enhanced CAR constructs to overcome therapeutic resistance. Our platform demonstrates broad applicability in solid cancer

009 DISSCOvery: A Unified Computational Pipeline for Enhanced Analysis of Multiplexed Immunohistochemistry Data Across Various Platforms

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High-dimensional multiplexed immunohistochemistry (mIHC) is at the forefront of spatial single-cell analysis, providing detailed insights into cellular organization within native tissue environments. However, relying solely on raw data without thorough inspection risks introducing errors that can compromise downstream measurements. Unlike conventional (dissociated) single-cell methods, mIHC data require intricate processing steps such as cycle alignment, background subtraction, and cell segmentation.To address these challenges, we present **DISSCOvery**, a comprehensive computational pipeline designed for the full-scale analysis of mIHC images. From image acquisition and quality control to the characterization of cell populations and spatial relationships, DISSCOvery streamlines the process. Its integration into a user-friendly web interface allows researchers without computational expertise to perform complex analyses.
In a benchmarking exercise using consecutive sections from multiple tissue types, we demonstrate the adaptability of DISSCOvery across multiple mIHC platforms, including MILAN (LISCO), CODEX (Akoya), COMET (Lunaphore), and MACSima (Milteny). Our study shows that depending on specific study requirements—such as tissue type, panel size, or scanning area—different technologies offer distinct advantages and challenges. Regardless of the platform, our results emphasize the necessity of a robust, integrated image analysis pipeline to ensure high-quality, reliable data.

010 Cancer-Associated Fibroblast Subsets Shape the Immune CellComposition of Non-Melanoma Skin Cancer

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Cancer-associated fibroblasts (CAFs) are key drivers of cancer progression and therapy response. We explored the diversity of CAFs in Basal Cell Carcinoma and Squamous Cell Carcinoma at molecular and spatial single-cell resolution.
Our analysis identified three distinct CAF subtypes: myofibroblast-like RGS5^{+} CAFs, matrix

CAFs (mCAFs), and immunomodulatory CAFs (iCAFs). Large-cohort tissue analysis revealed significant shifts in CAF subtype patterns with increasing malignancy. Two of these subtypes exhibited immunomodulatory properties through different mechanisms: mCAFs synthesized extracellular matrix, potentially restricting T cell invasion in lower-grade tumors by encapsulating tumor nests, while iCAFs were enriched in late-stage tumors and expressed high levels of cytokines and chemokines, facilitating immune cell recruitment and activation. Building on these findings, we are currently investigating the immune microenvironment and the expression of key immunomodulatory factors, such as exhaustion markers and proliferation/activation markers, through Imaging Mass Cytometry. By correlating these immune parameters with CAF composition in each sample, we aim to elucidate the spatial interactions between CAF subtypes and immune cells within the tumor microenvironment. Our study underscores the complexity of CAF-driven immunomodulation in non-melanoma skin cancers and suggests that targeting specific CAF subtypes may enhance immunotherapeutic efficacy by reshaping the immune landscape within tumors. These findings hold promise for improving immunotherapy outcomes, not only in skin cancers but potentially across a broad range of solid tumors.

sPCA: A simple, linear and interpretable approach for spatially aware clustering of spatial transcriptomics data 011

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Spatial Transcriptomics (ST) uncovers gene expression patterns within the elaborate spatial layout of tissues, a level of detail absent in single-cell transcriptomics analysis, which enhances our comprehension of cell-environment interactions. Accurate spatial information is critical for clustering cell domains and for a better understanding of their functional connections in intricate biological tissues. In this study, we adapt spatial principal component analysis (sPCA)[1] with ST data to efficiently reveal complex gene expression profiles while preserving the spatial context of tissues. The main idea behind sPCA consists in identifying the principal components (PCs) that best maximize the product of spatial autocorrelation (Moran's Index) and transcriptomic covariance, reflecting both the structure of genetic expression and its spatial distribution. By combining dimensionality reduction and emphasis on spatial correlations, sPCA refines the ability to detect spatial gene expression patterns and variations, thereby improving the outcome of domain clustering. Based on sparse matrices, this approach reduces computational complexity, improves scalability, which makes it ideally adapted to the analysis of large-scale ST datasets. The interpretability of this approach is reinforced by its linear structure, which provides a clear understanding of the impact of each variable on the clustering results, in contrast to current more complex approaches based on

Graph Neural Networks. In order to explore the effectiveness of sPCA in detecting spatial information and clustering cell types, we integrated it with several popular clustering techniques such as K-means, Gaussian mixture and Leiden. We evaluated our approach using several widely recognized and open-access ST datasets, featured in numerous benchmarks and obtained by different technologies. Our approach demonstrated robust performance, comparable to various state-of-the-art methods.

[1] Jombart, T., et al. "Revealing cryptic spatial patterns in genetic variability by a new multivariate method." Heredity 101.1 (2008): 92-103.

012 Multiomic Mapping of the Brain: same-section, fully-automated spatial RNA and protein detection on mouse frozen tissues

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Multiplexed immunofluorescence (mIF) and RNA in situ hybridization (ISH) allow simultaneous detection of multiple protein and RNA biomarkers. Combining spatial assays on the same tissue section is essential to increasing our knowledge of complex tissues, such as tumor microenvironments or neural tissues. Extracting precise information on cellular interconnections, neuronal connectivity, and signaling activity is key to understanding the biological processes involved in development and disease.

Here, we present a novel, fully automated approach that integrates the RNAscope™ HiPlex-Pro assay [1] and sequential immunofluorescence (seqIF™) [2] protocols for the co-detection of RNA and protein targets on the same tissue section on the COMET™ platform. The multiomics protocol was applied to mouse tissues and frozen sections for the first time, demonstrating the versatility and robustness of the approach.

We automated RNAscope™ and seqIF™ protocols on COMET™ for the simultaneous detection of RNAs and proteins. The system ensures precise temperature control and reagent distribution, critical for maintaining the integrity of frozen sections, and allows for a multiplexing capability of up to 12-plex RNA and 24-plex protein targets.

We demonstrated that the combination of RNAscope™ and seqIF™ protocols on COMET™ enables the simultaneous detection of RNA and protein biomarkers on sensitive frozen tissues while ensuring high reproducibility and minimal user intervention.

RNAscope™ probes targeting biomarkers relevant to neuronal function, including neurotransmitters and receptors, and other glial cells, were combined to protein markers profiling multiple cell types in their microenvironment, including several types of infiltrating immune cells (such as $CD3^+$, $F4/80^+$, $CD11c^+$, or $CD56^+$ cells).

Our findings demonstrate the successful application of the combined RNAscope™ and seqIF™ protocols on the COMET™ platform to analyze delicate, high-autofluorescence tissues

and non-human tissues. These results demonstrate the approach's versatility and robustness an open the door to potential new applications in the immuno-oncology field, including biomarker and drug development.

A spatial atlas of human gastro-intestinal acute GVHD reveals epithelial and immune dynamics underlying disease pathophysiology 013

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Acute graft-versus-host-disease (aGVHD) is a significant complication of allogeneic hematopoietic stem cell transplantation (aHSCT). Alloreactive donor T cells are known as a driving factor in GVHD development in the gut. However, the roles of additional donor and host cells in this process are not fully understood. Here, we performed multiplexed imaging to spatially characterize epithelial, stromal and immune cells from diagnostic biopsies of 57 patients with gastrointestinal GVHD and 10 healthy controls. Normal duodenum was stereotypical across individuals, showing organization and zonation of epithelial, stromal and immune cells. GVHD manifested in increased fibrosis, alterations in crypt morphology, loss of Paneth cells, and accumulation of endocrine cells in the crypts. Homeostatic immune organization was broken, with a prominent reduction in IgA-secreting plasma cells. $CD8^+$ T cells were enriched in only a subset of patients, whereas others were characterized by non-canonical enrichments of other immune cell types, including macrophages and neutrophils. Time after transplantation was a major determinant of immune composition, with stereotypical dynamics across individuals. We used *in situ* hybridization (ISH) to distinguish between donor and host immune cells in sex-mismatched samples. Host cells dominated the plasma and T cell compartments in the gut for extended periods of time following transplantation, suggesting that additional players may drive GVHD across individuals in addition to donor-derived T cells. Overall, this spatial atlas of healthy duodenum and GVHD uncovers non-canonical immune dynamics, offering insights into disease pathophysiology and potential clinical applications in GVHD and other inflammatory bowel diseases.

S^3-CIMA: Supervised spatial single-cell image analysis for the identification of disease-associated cell type compositions in tissue 014

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The spatial organization of various cell types within the tissue microenvironment is a key element for the formation of physiological and pathological processes, including cancer and autoimmune diseases. Here, we present S^3-CIMA, a weakly supervised convolutional neural network model that enables the detection of disease-specific microenvironment compositions from high-dimensional proteomic imaging data. We demonstrate the utility of this approach by determining cancer outcome- and cellular signaling-specific spatial cell state compositions in highly multiplexed fluorescence microscopy data of the tumor microenvironment in colorectal cancer. Moreover, we use S^3-CIMA to identify disease onset-specific changes of the pancreatic tissue microenvironment in type 1 diabetes using imaging mass cytometry data. We evaluated S^3-CIMA as a powerful tool to discover novel disease-associated spatial cellular interactions from currently available and future spatial biology datasets.

015 The Spatial Proteomics Unit at SciLifeLab

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SciLifeLab in Stockholm, Sweden.

Track: Spatial multiomics techniques and approaches

Spatially resolved omics technologies have emerged in recent years and have undoubtedly changed the way we understand the spatial organization of complex multicellular biological systems. The aim of the Spatial Proteomics unit is to do full-service multiplexed immunofluorescence projects covering discovery, translational and diagnostic research questions.
In the unit we offer two main technologies: the Phenocycler-Fusion from Akoya Biosciences uses conjugated antibodies detected in cycles by addition of fluorescent reporters; and the COMET from Lunaphore uses off-the-shelf antibodies in sequential rounds of immunofluorescence. Both these methodologies allow us to run targeted spatial proteomics analysis of up to 40 markers at single cell level in tissue sections.
Furthermore, the unit also works on method development and implementation of new services such as isPLA and multiomics analysis.

016 Revealing the Spatial Distribution of the Microenvironment Across Distinct Tumor Sites in Immunotherapy-Treated Urothelial Carcinoma

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Immunotherapy, particularly the use of PD-1/PD-L1 inhibitors, has become a cornerstone

in the treatment of advanced urothelial carcinoma (UC). However, the response rate remains relatively low, with only about 25% of UC patients responding to treatment. This underscores the challenges in predicting treatment outcomes based on PD-L1 assessments. Additionally, it highlights the dynamic nature of UC biology, where therapeutic responses are heavily influenced by the tumor microenvironments (TME) of primary tumors and metastatic niches. To avoid unnecessary treatments in non-responders, and to enhance patient selection for immunotherapy, we aim to deepen our understanding of the spatio-dynamic changes in UC and their TME in relation to immunotherapy.
We identified a cohort of 43 patients treated with immunotherapy at the University Hospital Tübingen, including 17 patients (39.5%) with lymph node metastasis and 15 patients (34.9%) with distant metastasis. Available tumor material from primary tumor, lymph node and distant metastasis has been collected, pathologically reviewed, and incorporated into tissue microarrays (TMAs). Six TMAs were constructed to include all included histological subtypes, tumor centre and invasion front, and tumor-associated tertiary lymphoid structures. We created a CODEX (CO-Detection by indEXing) panel, a highly multiplexed microscopy method with 72 markers—including markers related to basal and luminal differentiation of UC — to image these samples. We performed comprehensive image processing, including cell segmentation, cell type annotation, and clustering.
By imaging the TMA cores, we achieved spatial resolution of single cells, allowing for detailed clustering to investigate interactions among cancer, stromal, and immune cells. Finally, we compare the TME of responders versus non-responders, as well as across different tumor sites. These methods enable us to comprehensively profile UC cancer cells at distinct sites and their adjacent TME in relation to immunotherapy. The ultimate goal is to better predict treatment outcomes and develop personalized therapies for UC patients.

When QuPath meets Python for cell phenotyping

017

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The field of spatial biology has rapidly expanded, offering a wide range of wet lab and analytical pipelines solutions. Users can choose between an overwhelming amount of commercial and open-source solutions to analyze complex, high-dimensional data.
A crucial first step in a successful analysis is framing questions that have biological meaning and can be quickly validated and visualized. In our facility, we use QuPath - an open source software for visualization, quality control, and cell phenotyping. QuPath offers a variety of cell segmentation and classification tools. What has been missing is using gold-standard, hierarchical phenotyping algorithms within QuPath without the need of exporting data from and to different softwares.
Here, I am presenting a use-case of a Python-to-QuPath bridge: paquo.
Paquo can be run from a jupyter notebook, to read cell measurements for any clustering methods, it can display dimensionality reduction plots, and send cell clustering data back

to QuPath to 'color-code' cells belonging to different clusters.
I will present an example workflow and give an outlook for further developments of using *paquo* and QuPath.

018 In-Depth Characterization of Lung Organoids: An Imaging-Multi-Omics Approach

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Understanding the characteristics of human lung organoids is essential for their use in mimicking the human lung and facilitating the investigation of its (patho-)physiology and host-pathogen interactions. However, their constant development and increasingly complex cellular and structural composition intensify existing challenges, such as donor and batch-to-batch variability. To address these challenges, it is necessary to quantify organoid complexity and heterogeneity at the molecular, cellular, and morphological levels in a standardized manner.
Here, we present an integrative approach to characterize adult stem cell (ASC)-derived human lung organoids from ten donors, over thirteen passages and nine months of culture, using imaging, image segmentation, single-cell, and spatial transcriptomics methods. We employ multiplex imaging, high-content scanning, and semi-automated image segmentation to analyze the cellular composition by characteristic cell markers established in reference lung samples. This reveals not only the cellular composition but also morphological characteristics such as organoid size, number of cells, and number of lumens per organoid. These 2D parameters from histological sections are compared to both 3D fixed and live organoid samples imaged using light sheet microscopy. Furthermore, we analyze molecular and cellular composition via RNA single-cell sequencing and spatial transcriptomics. For the latter, we present cell type annotation in lung and organoid samples, enabling spatial cell-specific analysis at the transcriptome level.
With the analysis pipelines presented here, organoid researchers across all organ fields can achieve comprehensive characterization of their organoids at the protein, transcriptome, and structural levels. This approach not only enhances our understanding of the organoid model in use but also paves the way for the development of more advanced organoids and their reliable and reproducible applications in research and medicine.

Escalating High-dimensional Imaging using Combinatorial Multiplexing and Deep Learning 019

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Understanding tissue structure and function requires tools that quantify the expression of multiple proteins at single-cell resolution while preserving spatial information. However, current imaging technologies are inherently limited in throughput by their design: using a separate measurement channel for each individual protein. As such, increasing the number of proteins measured in an experiment is linear, drastically limiting scalability. In contrast, combinatorial staining approaches, whereby each target is encoded by several channels have revolutionized measurements of mRNAs, enabling measurements of thousands of mRNAs *in situ*. Current combinatorial approaches require spatial separation between the measured entities, which holds for mRNAs, but not for proteins, which are ~ 10,000-fold more abundant. As a result, to date it was considered mathematically impossible to apply combinatorial staining methods to proteins, generating a significant gap in the field.
CombPlex (COMBinatorial multiPLEXing) is a combinatorial staining platform coupled with an algorithmic framework to exponentially increase the number of proteins that can be measured using channels from up to . In CombPlex, each protein is imaged in several channels, and every channel contains agglomerated images of several proteins, resulting in compressed images. These combinatorically-compressed images are then decompressed to individual protein-images using deep learning. To demonstrate CombPlex's capabilities, I measured 22 proteins using 5 channels, multiplexing 9-10 proteins per channel and achieving a 4.5-fold compression. I showed successful compression and accurate reconstruction, with a median F1 score of 0.95 ±0.13 and median Pearson correlation of 0.98±0.08. Overall, combinatorial staining coupled with deep-learning decompression can escalate the number of proteins measured using any imaging modality, without the need for specialized instrumentation. Coupling CombPlex with instruments for high-dimensional imaging could pave the way to image hundreds of proteins at single-cell resolution in intact tissue sections (Ben Uri*, Ben Shabat* et al, *bioRxiv* 2023, under revision in *Nature Biotechnology*).

Smoothing of GSEA-Scores from Spatial Omics Data 020

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In my thesis, I explored the application of network propagation techniques to spatial omics data from breast cancer samples, aiming to enhance the identification of tumor versus normal tissue regions. Gene Set Enrichment Analysis (GSEA) is a commonly used method for assessing differential expression of gene sets, which can be integrated with spatial omics data to account for the tissue's spatial architecture. In my thesis I applied network-smoothing, specifically using the netSmooth package, to improve GSEA predictions by propagating

scores through networks based on either spatial proximity or gene expression similarity of the sample spots. I computed GSEA scores for individual spots and applied various network-smoothing methods, followed by thresholding to evaluate the predictions against known ground truth data. Results indicated that many smoothed scores significantly outperformed unsmoothed ones in terms of prediction accuracy. The most effective approach was to first smooth based on expression similarity, and then apply spatial neighborhood smoothing. The extent of improvement was closely related to the tissue's spatial organization; larger distinct tumor and normal areas benefitted more than tissues with smaller, intermixed regions. For datasets where the original GSEA scores were poorly predictive, smoothing provided only limited enhancement.

021 Spatial multi-omics of acute myocardial infarction reveals a novel mode of immune cell infiltration

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Myocardial infarction (MI) continues to be a leading cause of death worldwide. Although it is well-known that the complex interplay between different cell types determines the overall healing response after MI, the precise changes in the tissue landscape are still poorly understood. We generated an integrative cellular map of the acute phase after murine MI using a combination of imaging-based transcriptomics (Molecular Cartography) and antibody-based highly multiplexed imaging (Sequential Immunofluorescence), allowing us to assess cell-type compositions and changes at subcellular resolution over time. Unexpectedly, we identified a novel route of leukocyte infiltration to the infarcted heart via the endocardium. To investigate the underlying mechanisms driving this previously unknown route, we performed unbiased spatial proteomic analysis using Deep Visual Proteomics (DVP). When comparing endocardial cells of homeostatic hearts and infarcted hearts, DVP identified von

Willebrand Factor (vWF) as an upregulated mediator of inflammation 24 hours post-MI. To further explore vWF's immune role and impact on tissue repair, we performed functional blocking of vWF during acute murine MI which resulted in a reduced infiltration by $CCR2^+$ monocytes and worse cardiac function post-MI. Our study provides the first subcellular spatial map of acute murine MI, revealing a novel immune infiltration route and identifying vWF as a key mediator of endocardial immune cell infiltration.

SpaceTrooper, an R package for the preprocessing and quality control of imaging-based spatial transcriptomics data 022

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Emerging technologies in spatially resolved single-cell omics enable high-throughput analysis of the molecular characteristics of cells within their tissue environment. The rapid advancement of these techniques requires the development of novel computational methods to analyze the massive amounts of data they generate.
Several computational pipelines have been proposed for preprocessing and quality control of spatial omics data. However, these pipelines primarily rely on methods adapted from single-cell RNA-seq data analysis, with geospatial features only marginally considered during exploratory steps. While this approach is reasonable for sequencing-based platforms, the different nature of imaging-based spatial transcriptomics requires the development of specialized methods to identify cell segmentation errors and compute simple metrics for assessing the quality of cell boundaries. Moreover, although some quality control metrics can be derived from probe counts and morphological characteristics of the inferred cells, it remains unclear how to effectively combine these metrics for efficient flagging or removal of low-quality cells.
To address these challenges, we introduce *SpaceTrooper*, an R package specifically designed for the preprocessing and quality control of spatial transcriptomic data obtained from imaging-based technologies. The core framework of *SpaceTrooper* is built on existing Bioconductor data structures for spatial transcriptomics and leverages the extensive ecosystem of geospatial R packages to generate cell geometries directly from image and shape files in various formats.
SpaceTrooper employs a statistical test to detect outliers and performs an accurate cleansing based on a score that combines several metrics (cell-level probe expression and spatial features derived from cell geometries). We tested *SpaceTrooper* on various public datasets, covering four distinct tissues (breast cancer, lung tumor, healthy brain, and colon) across

human and mouse samples. Unlike other computational pipelines, *SpaceTrooper* effectively integrates probe expression with cell morphological characteristics to identify low-quality data, boundary effects and unrealistic cell polygons that would impact negatively on downstream analysis.

023 The tumor microenvironment architecture correlates with early recurrence of Head and Neck Squamous Cell Carcinoma

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Emerging evidence suggest that not only the frequency and composition of tumor-infiltrating leukocytes but also their spatial organization might be a major determinant of tumor progression and response to therapy.
Here we performed an explorative, prospective clinical study to assess whether structures within the tumor microenvironment can predict recurrence after salvage surgery in Head and Neck Squamous Cell Carcinoma (HNSCC).
We employed co-detection by indexing (*CODEX*) multiparametric imaging to measure the major leukocyte subsets and an *ad-hoc* computational framework to identify and analyze discrete cell types and cellular neighborhoods (CN) and correlate the presence of CT and or CN with clinical parameters and recurrence.
Unsupervised clustering identified the expected leukocyte subsets but also additional populations such, for example, “capsule cells” that, when present, contour the neoplastic nests. Cell neighborhood and cell-to-cell spatial analysis reveal the presence of 11 distinct cellular neighborhoods. Five CNs were characterized by homotypic cell-to-cell interactions (cold tumor, neutrophil rich, MDSC rich, NK CN, M1 rich), while in six CNs cells interacted with cells of a different type (Tertiary lymphoid structure (TLS) Type 1, TLS type 2, Hot tumor CN, vasculature CN, peritumoral CN, and Stroma CN).
Finally, we employed tensor deconvolution and uni- and multivariate analysis to evaluate whether the tumor composition in terms of cell type and cellular neighborhood discriminates the tumors of patients that recur from those of patients that remain tumor-free. We found that the frequency of type 1 tertiary lymphoid structure composed of $CD31^{high}CD38^{high}$plasma cells is associated with the lack of recurrence after surgery in HNSCC.
Our data support the notion that the structural architecture of the tumor microenvironment plays an essential role in tumor progression and indicate that type 1 tertiary lymphoid

structures and long-lived CD31^{high}CD38^{high}plasma cells are associated with a good prognosis in HNSCC.

Whole transcriptome spatial profiling at single cell scale resolution. 024

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Recent developments in the resolution and sensitivity of spatial profiling technologies have enabled researchers to assess previously unseen aspects of biology. The new Visium HD Spatial Gene Expression assay enables whole transcriptome profiling at single cell scale resolution. Visium HD slides have a ~25 fold reduction in feature size compared to standard Visium Capture Areas, with two 6.5 mm x 6.5 mm Capture Areas composed of ~11 million 2 x 2 µm barcoded squares. As there are no gaps in between the squares, the assay enables uninterrupted profiling of finer anatomical features. Achieving single cell scale resolution requires precise localization of transcripts relative to their point of origin in the tissue. The assay workflow, Visium HD slides, and CytAssist instrument have all been optimized to control the probe transfer process and ensure precise transcript localization. Using 15 different human and mouse tissues, we performed H&E staining of tissues on standard glass slides followed by the Visium HD workflow allowing for morphological assessment and single cell scale whole transcriptomic profiling from the *same* tissue section. We demonstrate robustness of the assay and high confidence transcript localization. We show that Visium HD offers profound insights into the heterogeneity and organization of tissues at single cell scale resolution, empowering researchers to uncover novel discoveries in normal development, disease pathology, and clinical translational research.

Integrated spatial analysis defines the pathological niches of diverse autoimmune diseases 025

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The last decade has been the witness of a meteoric development of multiplexed imaging (MI) technologies, allowing in theory the in-depth spatial profiling of biological tissues. Together with single-cell genomic assays, MI methods have thus the potential to shed light on poorly understood biological systems, ranging from developmental pathways to diseases pathogenesis. However, unlike single-cell genomic, the potential of MI methods is still constrained as it has not resulted in significant biological discoveries.

Here we show that the potential of MI can be unlocked by using tailored experimental sampling and data analysis approaches that embody the intrinsically spatial nature of MI. First, by coupling in-depth sampling simulation with a large scale spatial transcriptomic atlas, the

rules governing MI experimental design were elucidated [1], and more efficient sampling strategies were established. Second, inspired by spatial ecology, we developed statistical models to analyze cell count derived from MI data, resulting in a drastic increase of statistical power for differential abundance analysis, as illustrated by the reanalysis of MI COVID-19 data [2]. Finally, we developed a new approach to model the distribution of cells through space in a quantitative manner, thus providing an interpretable feature extraction method to describe the shape of a group of cells. Combining it with the automated analysis and annotation of several MI RNA datasets, we identify a unique and recurring spatial pattern of immune infiltrating cells in auto-immune diseases, consisting in the aggregation of naive CD4-T cells into dense patches near TLS while activated CD8-T cells are homogeneously spread through the tissue.
Altogether, we provide a theoretical and practical basis for the experimental design and analysis of MI experiments, unleashing the potential of MI as a powerful technology for patient diagnosis and biological discovery, complementary to single-cell genomic.

026 Spatially resolved analysis of TGF-β / BMP signaling in pancreatic ductal adenocarcinoma by digital pathology

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Transforming growth factor (TGF)-β and bone morphogenetic protein (BMP) signaling has a dichotomous (tumor-promoting and -suppressive) role in pancreatic ductal adenocarcinoma (PDAC). Smad proteins, intracellular signal transducers for the TGF-β family, and BMPs are drivers of aggressive biological behavior in cancer, known to promote epithelial-mesenchymal transition (EMT); Gremlin1 (GREM1) is an important BMP antagonist. Here, we quantify key signaling molecules of both pathways in PDAC.
We analyzed a cohort of 117 curatively resected PDAC cases using a multi-core tissue microarray (cores from tumor front/center/stroma each). We assessed the spatially resolved mRNA expression levels of TGF-α, TGF-β1, TGF-β2, GREM1, and BMP4 through in-situ-hybridization and performed immunohistochemical staining for Inhibitor-of-DNA-binding-1 (ID1) and phospho-smad2 (psmad2) for comprehensive characterization of TGF-β/BMP signaling in PDAC and associated tumor stroma by digital pathology.

In PDAC glands, immunohistochemical ID1 expression was mostly high and correlated inversely with GREM1 mRNA expression. While TGF-β1 transcript numbers were higher in tumor parenchyma ($p<0.01$) than in stroma, the opposite was true for TGF-α ($p=0.02$), TGF-β2 ($p<0.01$) and GREM1 ($p<0.01$). The expression of TGF signaling molecules was positively correlated in tumor parenchyma and stroma. TGF-α^{high} ($p=0.05$) and GREM1^{high} ($p=0.01$) PDAC correlated with higher T-stage. Higher tumoral TGF-β2 ($p=0.01$) correlated with the presence of perineural invasion. Significantly higher tumor budding counts per core were observed for high epithelial ($p=0.02$) but low stromal TGF-β1 expression ($p=0.01$). Lower stromal TGF-β2 transcript numbers significantly correlated with worse survival ($p<0.01$). No significant differences were found between tumor center/front, tumor size or lymph node-status.
Here, advanced T-stages were observed in TGF-α^{high} PDAC. While increased tumoral TGF-β1/2 signaling was associated with tumor-suppressive features, reduced stromal TGF-β2 signaling correlated with worse survival in PDAC.

Communication – A case study on melanoma 027

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Cell-cell communication (CCC) plays a pivotal role in organismal cell biology across a lifetime, from development, aging, to disease etiopathogenesis. CCC occurs through different cell extrinsic mechanisms, broadly categorized as direct contact-based (juxtacrine) and secreted factor-based (paracrine) communication, resulting in modifications of the intrinsic properties of the interacting cells, like gene expression, lipid and protein alterations. In vitro co-cultures with model cell lines are highly relevant to study CCC, however, conventional workflows do not allow to precisely position different cell types and thereby study the influence of the spatial context. Multiplex cell patterning may help overcome this major limitation. However, the available approaches are not compatible with multi-omics readouts, which drastically limits our abilities to monitor rewiring of CCC-mediated intrinsic properties of the target cells.
We therefore developed a microfluidics-based workflow for multiplex cell micropatterning, which enables full control over the spatial configurations and distances between interacting cell populations. The patterned cells are integrated into a microfluidic chip in which

they can grow and communicate, enabling real-time study of dynamic cell behavior. After removal of the chip, the cell patterns are accessible for downstream state-of-the-art spatial omics methodologies, for which we are investigating antibody staining, spatial lipidomics and spatial transcriptomics. We are applying this workflow to study the interaction between melanoma cells and cells from their tumor microenvironment to enhance our understanding of the impact of CCC on tumor progression and metastasis.
In conclusion, our innovative workflow for multiplex cell micropatterning provides precise spatial control of interacting cell populations and compatibility with spatial biology methods, resulting in a highly relevant tool to aid researchers in gaining new fundamental insights into the importance of CCC in their favorite biological system.

028 Programming-free single cell analysis of spatial proteomics whole-slide-images

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The analysis of spatial proteomics scans is predominantly conducted using software packages that require coding skills and custom development. This limits usability by experts with medical or biological rather than computer science knowledge. We propose an analysis workflow that can be generically tailored to meet the heterogenous requirements in life science research and is usable without programming skills. The workflow is realized in the MIKAIA software.
Multiple image analysis blocks are involved: (1) detection of tissue, (2) dearraying of tissue micro arrays (TMAs), (3) creation of ROIs either manually or by thresholding, (4) cell segmentation, (5) cell qualification, (6) assignment of cells into ROIs, (7) creation of annotations per individual marker or by co-expression, (8) subsequent spatial analysis and (9) batch analysis and export of quantitative measurements. Cell segmentation is available via a computer-vision algorithm (faster) or CellPose AI models (more robust). Cell qualification is done based on user-provided cut-offs or in an unsupervised fashion using clustering, incl. UMAP visualization. Cells can be grouped by two levels of ROIs, for instance tumor-vs.-stroma and additionally per TMA core. Spatial postprocessing includes cell-cell connections analysis based on Delauney triangulation, identification of cellular neighborhoods (CN), or proximity analysis.

The workflow is compatible with various instruments, incl. CODEX or Comet, and formats, incl. OME-TIF.

Microanatomical principles of organ organization in the human body and its change with age 029

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Aging is a complex process that affects human physiology at various scales, from cellular alterations to tissue and organ level integrity. Microanatomical domains – cell arrangements that contribute to organ-specific function, provide a highly physiologically relevant perspective on tissue homeostasis yet are severely understudied in the context of aging.
To unravel how microanatomy changes with age, we utilized large-scale whole slide imaging datasets of human tissue together with deep learning techniques to detect and quantify the abundance and morphology across 40 tissues of 983 donors along the axis of adult human lifespan. We developed an unsupervised algorithm to detect microanatomical domains, which enabled us to systematically characterize principles of organ architecture across the human body. We found 218 domains which we categorized into 74 classes across the 40 tissues. Domains varied greatly in tissue specificity, with 11% shared between 3 or more organs, while 74% were tissue-specific. Interestingly, we found that age was the dominant factor in the variance of domain abundance, with 27% changing significantly with age. Finally, by incorporating information on pathologies, we identified domains linked to age-related pathologies such as fibrosis and atrophy across organs.
This multi-organ analysis provides insights into fundamental principles of tissue organization, unveiling the dynamics of microanatomical structures of the human body across the human lifespan.

CellTune: An integrated software for multiplexed image visualization, annotation, and human-in-the-loop active learning for cell classification 030

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Multiplexed imaging enables measurement of multiple proteins *in situ*, offering an unprecedented opportunity to capture the molecular and spatial complexity of tissues. Accurate cell classification is essential for understanding tissue organization. However, current methods

struggle, primarily due to signal overlap of densely packed cells, and often fail to utilize spatial expression information or learn from human annotations efficiently. Supervised deep learning approaches, which require large amounts of labeled data, are constrained by the scarcity of high-quality, manually labeled datasets, exacerbated by the lack of efficient tools for interactive cell labeling. Here, we present **CellTune**, an integrated software for visualization, annotation and classification of multiplexed images that overcomes these challenges using a human-in-the-loop active learning framework. CellTune computes and incorporates spatial features, including subcellular expression, protein colocalization, and neighborhood expression as input for the classification task. It trains gradient-boosted tree-based models with iterative rounds of model refinement, guided by human input. It employs query-by-committee sampling to maximize the information gained from manual labels. The graphical user interface is tailored for large multiplexed imaging datasets, easily streamlining tasks such as cell classification by automatically displaying the relevant channels for determining a cell's lineage and quickly moving between cells across images. To test CellTune, we generated *CellTuneDepot*, the largest available manually-annotated dataset, comprised of 40k cells from 30 types. We tested CellTune against 10 methods and showed that it achieved approximately 10-15% higher accuracy than state-of-the-art methods, on par with human performance. CellTune doubled the number of distinguishable cell types, enabling finer-grained classification. CellTune also facilitates the discovery of new cell types by interactively engaging users with cells confused between existing classifications. Together, CellTune and CellTuneDepot make an invaluable tool for researchers, offering speed, accuracy and precision in multiplexed imaging analysis while promoting the discovery of novel cell types.

031 Spatial Transcriptomics Reveals Localized Responses and Interactions in the Small Intestine During *Heligmosomoides polygyrus* Infection

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Parasitic helminths like *Heligmosomoides polygyrus* can cause chronic infections in hosts despite immune defences. In mice, this intestinal parasite establishes infection by entering the small intestine, breaching the epithelial layer, and residing in the submucosa for the first week. The immune response during these early stages is not well understood, though it involves granuloma formation and the recruitment of macrophages, eosinophils, and neutrophils in an IL-4R-dependent manner. Using spatial transcriptomics, we investigated localised transcriptional changes in the intestinal epithelium and lamina propria during early infection. Our analysis revealed distinct cellular compositions in *H. polygyrus* granulomas, with macrophages clustering near larvae and mast cells migrating to more distant sites as infection progresses. Deconvolution techniques identified common and infection-specific cell type interactions and potential ligand-receptor pairs facilitating communication between granulomas and epithelial crypt cells. We also observed upregulation of genes like

Ccl9, *Fcer1g*, and *Tmsb4x*, indicating roles in type 2 inflammation, along with genes such as *Reg3b* and *Mxra7* linked to wound healing. These findings deepen our understanding of the transcriptional changes in the murine small intestine during *H. polygyrus* infection and provide a platform for exploring host-pathogen interactions.

Spatial analysis of the Myeloma Tumor Microenvironment in Murine Bone Marrow 032

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Multiple myeloma (MM) is a severe and often incurable cancer of plasma cells. Despite advances in treatment, relapses are common following therapy. Plasma cells, or myeloma cells, are primarily located in the bone marrow (BM), a complex and spatially organized microenvironment that includes various cells, vascular structures, and extracellular matrix (ECM). This microenvironment plays a critical role in the invasion, progression, and relapse of MM. Myeloma cells interact with multiple BM components, including stromal cells, myeloid cells, immune cells, and the ECM, all of which can have pro-tumor or anti-tumor effects at different stages of tumor development. This study aims to analyze the tumor bone marrow microenvironment in multiple myeloma using a mouse syngeneic model (5TGM1) and multiplex imaging techniques. These methods enable us to investigate MM cell dynamics from early invasion to later expansion within the BM, and describe the spatial organization and cell composition of the MM BM microenvironment.

MM cells migrate in the bone marrow (BM) preferentially start seeding in proximal, metaphyseal BM regions, where they proliferate and cluster near blood vessels. $CXCR4^{high}$ myeloma cells were observed to cluster closely around vessels, suggesting that chemotactic signals in these vascular niches are crucial for their localization and retention. Angiogenesis was evident within large tumor clusters, where stromal cells and fibroblasts were closely associated with endothelial cells. Strong Vimentin signals were detected in both stromal and endothelial cells, indicating a key role of Vimentin in angiogenesis and in mediating interactions between myeloma cells and stromal components. $CD11b^{+}$ myeloid cells frequently aggregated around tumor clusters and within tumor areas. Many of these cells expressed CD206 and CD68, identifying them as tumor-associated macrophages (TAMs). These TAMs often co-localized with T cells and were implicated in promoting immune suppression and tumor progression.

033 Biological information derived from clinical samples is hindered by upstream processing steps in Image- based Spatial Transcriptomics data

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Image-based Spatial Transcriptomics (iST) methods allow single-molecule RNA readout at subcellular resolution. Given the similarities between the data generated by iST and single-cell RNA-sequencing (scRNA-seq) technologies, current processing strategies rely on the most common steps used for the latter. However, the ability of these pipelines to retrieve unbiased biological information have just started to be investigated.

Here, we investigated the effects of upstream processing methods of scRNA-seq data, when applied to iST, with a focus on data normalization. Addressing a clinically relevant question, we evaluated how each step impacted the ability to characterize cell populations and gene expression heterogeneity in a cohort of primary colorectal cancer (CRC) patients with matched liver metastasis (LM).

A tissue microarray (TMA) comprising cores from 13 patients with matched primary CRC and LM was used. CosMx Spatial Molecular Imaging (SMI, Nanostring) was used to generate a transcriptomic readout of 1000 genes for all samples. AtoMx (Nanostring) was used for cell segmentation. Quality control was performed to discard cells with low expression, cells with area below the 5^{th} percentile, low-quality cores, and genes with low signal-to-noise ratio.

Seven normalization workflows that account for the cell area, varying number of RNA molecules detected per cell, and gene variance across cells were performed. The resulting data were then used to identify highly variable genes (HVGs), followed by automatic cell annotation using singleR.

Our results show that HVG identification is impacted by the upstream analysis, with only 50% HVGs being consistently detected. The disparity in HVG identification results in inconsistent cell type annotation. This creates a bottleneck for the identification of sample differences in the downstream analysis that might be clinically relevant.

In conclusions, iST-specific methods are needed to account for the methodological differences between iST and scRNA-seq for the application of iST for clinically relevant research questions.

034 Quantifying spatial metabolic remodeling upon mitochondrial inhibitor treatment in HER2-negative early breast cancer patients

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Breast cancer remains a leading cause of cancer-related mortality among women, often diagnosed at advanced stages when metastasis has occurred, leading to poor prognosis.

The tumor microenvironment plays a pivotal role in disease progression, shaped by intricate metabolic and immune dynamics. Recent therapeutic strategies target these dynamics through the use of anti-angiogenic agents like bevacizumab to normalize tumor vasculature and stroma. This vascular normalization triggers a significant metabolic shift in cancer cells from glycolytic suppression to enhanced mitochondrial respiration, termed 'metabolic synthetic lethality'.
Building on this understanding, a phase 0/I clinical study combined bevacizumab with ME-344, a mitochondrial complex I inhibitor, and demonstrated promising results. Post-treatment patient biopsies revealed a reduction in Ki67 expression, suggesting reduced tumor proliferation compared to pre-treatment samples. These findings suggest that vascular normalization enhances nutrient and oxygen delivery, facilitating a shift toward oxidative phosphorylation in tumor cells. However, a gap remains in understanding how the tumor microenvironment influences these metabolic changes, particularly how cancer and immune cells alter their metabolism in response to therapy. The metabolic niches of these cells may differ before and after treatment, but it is unclear how these shifts impact therapeutic efficacy. A deeper understanding of these metabolic alterations within the tumor microenvironment, and their relationship to treatment outcomes, is critical for improving predictive models and optimizing future therapeutic strategies.
To investigate these metabolic alterations within the tumor microenvironment, we are utilizing clinical trial biopsies from the same study to compare pre- and post-treatment samples. We aim to explore the role of metabolic dysregulation in early breast cancer through an innovative combination of single-cell metabolic profiling (scMEP) and multiplexed ion beam imaging (MIBI). MIBI enables 40-dimensional proteomic imaging, allowing us to visualize multiple proteins simultaneously, including tumor and immune antigens.
We have developed a robust antibody panel consisting of 40 markers that cover cell lineage markers, metabolic markers, transcription factors, immune state markers, and additional features for cell segmentation. We meticulously validated each antibody through immunohistochemistry (IHC) to confirm accurate binding patterns using both healthy and diseased tissues before conjugating them to heavy metals for use in MIBI. To comprehensively explore the metabolic and immune spatial niches, we have successfully tested our antibody panel on multiple healthy and cancerous tissue samples using MIBI.
So far, we have obtained samples and proceeded with initial data analysis, including cell segmentation with Cellpose and clustering via FlowSOM. We are currently exploring the cell type expressions and spatial niches. Looking ahead, the insights gained from this work have the potential to identify novel biomarkers, enable better predictions of therapeutic efficacy, and ultimately guide the development of more personalized and effective treatment strategies for early breast cancer. Additionally, the integration of high-dimensional proteomic imaging will offer a more nuanced understanding of immune-tumor interactions, opening new avenues for targeted interventions within the tumor microenvironment.

035 avenues for targeted interventions within the tumor microenvironment.Highly multiplexed image analysis of murine ORal-Esophageal-Stomach (ORES) rolls

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Distal regions of the GastroIntestinal tract (GI) are immunologically highly connected, which is critical in intestinal inflammation and other diseases. However, it has not been possible to image and investigate the GI tract of one single specimen in a single experiment. Monasterio et al. recently developed a new technique enabling researchers to roll the murine upper GI tract - ORal-Esophageal-Stomach (ORES) - and image these ORES rolls at once. In this project, we analyzed highly multiplexed immunofluorescent images of four such novel rolls. They were imaged by Canopy Biosciences on the CellScapeTM platform with an 18-plex antibody panel optimized to detect immune cells and major epithelial cell populations. We aimed to investigate variability and the role of immune cell populations between regions of the GI tract. Additionally, we studied the impact of inflammation of the gut onto the upper GI tract. Leveraging these unique samples and advanced imaging techniques, we show that the amount of immune cells drastically reduces upon gut inflammation.

036 Unveiling Spatial Dynamics of Cancer-Immune Crosstalk in Diffuse Midline Glioma

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Diffuse midline glioma (DMG) is an aggressive pediatric brain tumor with no curative treatment, prompting exploration of T cell immunotherapy. However, the unique tumor microenvironment (TME) of DMG, influenced by its anatomical location and developmental stage, presents therapeutic challenges. To dissect this complex niche, we developed an immunocompetent mouse model via in-utero electroporation (IUE) to induce DMG formation during embryonic development. Employing snRNAseq, Spatial Transcriptomics, and advanced multispectral imaging techniques on matched human and mouse samples, we characterized the spatial and phenotypic landscape of DMG.
Our analysis unveiled significant intratumoral heterogeneity, revealing two distinct spatial

patterns of cancer-immune interdependence. The mesenchymal-like niche facilitated recruitment of blood-derived immune cells, while another pattern displayed an immunologically cold phenotype, akin to a normal immune-privileged brain. Further exploration of spatially-resolved cell-cell communications in the TME identified a novel immune checkpoint pathway implicated in these patterns.
In vivo validation underscored the therapeutic potential of targeting this pathway, demonstrating notable survival benefits in our syngeneic mouse model. Thus, our comprehensive findings shed light on the intricate interactions within the DMG microenvironment, offering a promising therapeutic target and laying the foundation for novel immunotherapeutic strategies against this devastating pediatric brain tumor.

circVDJseq for streamlined TCR sequencing from 3'-directed single cell and spatial transcriptomics workflows **037**

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Antigen-specific T cells are key mediators of the adaptive immune response and play a central role in autoimmune disorders and cancer. Sequencing based TCR profiling methods can determine individual T cell repertoires at single cell resolution and provide insights into clonotype dynamics in various disease settings. Particularly, paired analysis of TCR sequences and mRNA expression within the same cell has established a direct correlation between TCR repertoire and cellular phenotypes, illuminating T cell development and function.
However, most sequencing-based spatial transcriptomics workflows are based on 3'-barcoding of transcripts coupled with short read sequencing, and therefore fail to recover the VDJ sequence information located in the 5' end of TCR transcripts. Since monitoring of lymphocyte clonality within human tissues can provide important insights into adaptive immune response mechanisms, several NGS-library construction workflows have been developed to

overcome this limitation and retrieve spatial VDJ sequence information from 3'-barcoded cDNA libraries. However, these methods are resource intensive as they rely on the amplification of TCRA and TCRB sequences via multiplexed PCR with hundreds of primers targeting all V genes, sometimes combined with hybridization capture and long-read sequencing.
Here, we present circVDJseq, a simplified workflow for paired full-length VDJ sequencing from 3'-barcoded cDNA libraries that greatly reduces the number of required gene-specific primers and obviates the need of specialized sequencing equipment. circVDJseq efficiently recovers T cell clonotype information from 3'-barcoded cDNA libraries, while the identified VDJ sequences and clonotype frequencies are highly concordant with data obtained by the 5'-directed Immuneprofiling v2 workflow (10X Genomics). We furthermore show that circVDJseq is compatible with single nucleus RNA sequencing, as well as the RNA+ATAC Multiome and Visium workflows (10X Genomics) and can retrieve spatial TCR clonotype information from freshly resected as well as autopsy tissue.

038 Imaging functional perturbations in lung tissue slice cultures - understanding chronic lung disease one cell at a time

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Chronic lung diseases (CLD), including chronic obstructive pulmonary disease (COPD) and pulmonary fibrosis (PF), are a leading cause of death worldwide. Causes of failed regenerative responses in CLD remain unknown and no curative therapy is available. Recent studies defined *Regenerative Cell States* (RCS) that transiently appear upon injury and resolve upon successful regeneration. We hypothesise that defects in generating RCS cause organ degeneration, while persistent RCS cause fibrosis. This is likely driven by dysregulated pathway signalling involved in tissue remodelling. We have associated RCS with distinct cell niches already found in early stages of IPF as assessed by applying multiplexed fluorescence imaging (4i) to micro-CT staged patient tissue cores. Pathway activity of major morphogenic pathways, visualised by nuclear shuttling of downstream targets, differs in these niches compared to healthy controls. We are now functionally investigating the role of morphogenic pathways on fibrosis development and treatment by perturbing human precision cut lung slices (hPCLS) pharmacologically. Perturbations will be analysed using multiplexed IF imaging and single nuclei RNA-seq to link pathway signalling activity with cell state changes. Investigating the regulation of injury related cell state trajectories, this project will enhance our understanding of IPF pathophysiology, paving the way for novel treatment strategies.

DIY Spatial Proteomics: Affordable Solutions for a Generalizable Pipeline 039

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Spatial proteomics, a rapidly expanding field within spatial biology, provides critical insights into protein distribution within cells and tissues. However, challenges such as limited sample quantities, contaminants, and sample loss hinder protein quantification. Overcoming these challenges requires innovative and cost-effective strategies. Recent advancements including artificial intelligence-driven image analysis, single-cell proteomics, and high-throughput techniques, have opened up new possibilities in spatial proteomics (1, 2),. Moreover, the possibility to use formalin-fixed paraffin-embedded samples, widely available in biobank archives, presents a promising opportunity for studying various pathologies.
We introduce a generalizable spatial proteomics pipeline, developed at EPFL's Research Core Facilities (Proteomics, Histology, Imaging, and Flow Cytometry). Our approach combines microdissection with bottom-up mass spectrometry, allowing the study of proteome variability across samples. Leveraging innovative, do-it-yourself techniques, we developed a streamlined workflow that can be implemented on most proteomic platforms with minimal investment. Combining laser-capture microdissection and data-independent acquisition (DIA) on an Orbitrap Exploris 480, we can quantify up to 7,000 proteins from regions containing fewer than 100 mouse hepatocytes. Our method provides rapid results within 24 hours and can be adapted to flow cytometry.
Collaborations with EPFL research groups have validated this pipeline across diverse biological models, including tissues and organoids, addressing key questions in development

and pathology.

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040 Structured Illumination Microscopy Improves Spot Detection Performance in Spatial Transcriptomics

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Spatial biology is a rapidly growing research field that focuses on the transcriptomic or proteomic profiling of single cells within tissues with preserved spatial information. Imaging-based spatial transcriptomics uses epifluorescence microscopy, which has shown remarkable results for the identification of multiple targets in situ. Nonetheless, the number of genes that can be reliably visualized is limited by the diffraction of light. Here, we investigate the effect of structured illumination microscopy (SIM), a super-resolution microscopy approach, on the performance of single-gene transcript detection in spatial transcriptomics experiments. We performed direct mRNA-targeted hybridization in situ sequencing for multiple genes in mouse coronal brain tissue sections. We evaluated spot detection performance in widefield and confocal images versus those with SIM in combination with 20×, 25× and 60× objectives. In general, SIM increases the detection efficiency of gene transcript spots compared to widefield and confocal modes. For each case, the specific fold increase in localizations is dependent on gene transcript density and the numerical aperture of the objective used, which has been shown to play an important role, especially for densely clustered spots. Taken together, our results suggest that SIM has the capacity to improve spot detection and overall data quality in spatial transcriptomics.

041 Colorectal Cancer Through the Lense of Whole Transcriptome Imaging

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Multi-cellular systems orchestrate function through an interplay of their molecular compo-

nents and structural organization (e.g., tissue composition and architecture). Recent spatial molecular imaging (SMI) technologies can profile tissues at molecular resolution under retention of physical coordinates — albeit limited in the number of (transcript) targets measurable thus far.
In an immune-oncology context, these data have the potential to help i) understand the compositional and organizational principles that govern tumor heterogeneity; ii) categorize tumors and patients under consideration of clinically relevant metadata; and, iii) make personalized medical decisions that are guided by prognostic predictions regarding, e.g., efficacy of treatments.
Here, we acquired whole-transcriptome SMI data on 1.5M+ cells from colon tissue sections that cover the full range of tumor evolution, including normal epithelia as well as progression to colorectal cancer (CRC) via premalignant Tubullovillous adenoma (TVA) lesions that can give rise to different CRC subtypes. In addition, we leveraged single-cell data (from snPATHO-seq) on adjacent slices for semi-supervised annotation of cell subpopulations.
We first set out to characterize immune, stromal and epithelial cell compartments in terms of their molecular and structural makeup. Next, histology-based annotation of normal, lesion and tumor domains allowed us to perform comparative analyses between key regions of interest. Lastly, we studied what drives TVA emergence and progression by linking the transcriptional (pseudotime) ordering of cells with tissue-wide compositional, microenvironmental, and morphological changes.
In this talk, I will present a multi-dimensional, holistic view of our results — encompassing whole-transcriptome single-cell and SMI data, pathology-guided and unsupervised, as well as established and problem-tailored analysis approaches.

An overview of the past 4 years analyzing spatial omics data

042

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Over the past four years, I have delved into the fascinating world of single-cell spatially resolved data, encompassing both proteomics and transcriptomics, and integrating this with data from various other omics using sophisticated machine learning algorithms. This multidisciplinary approach has allowed us to uncover a richer and more detailed understanding of biological systems, pinpoint crucial biomarkers, and unravel pathological mechanisms across diverse infectious diseases. Our ultimate goal is to harness these cutting-edge, high-dimensional techniques to pioneer more precise, targeted, and personalized treatment strategies. To showcase the transformative potential of these methods, I will share some of our groundbreaking findings. These include the application of spatial proteomics and spatial transcriptomics, along with omics integration, in COVID-19 autopsy studies, particu-

larly focusing on under-represented populations (in the Amazon and Sub-Saharan Africa) in published major study cohorts. Additionally, I will discuss our research on malaria infection and various oncology projects that I have been working on over the last four years.

043 A spatially-resolved single cell lung atlas integrated with clinical and blood signatures distinguishes COVID-19 disease trajectories

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COVID-19 is characterized by a broad range of symptoms and disease trajectories. Understanding the correlation between clinical biomarkers and lung pathology over the course of acute COVID-19 is necessary to understand its diverse pathogenesis and inform more precise and effective treatments. Here, we present an integrated analysis of longitudinal clinical parameters, peripheral blood biomarkers, and lung pathology in COVID-19 patients from the

Brazilian Amazon. We identified core clinical and peripheral blood signatures differentiating disease progression between recovered patients from severe disease and fatal cases. Signatures were heterogenous among fatal cases yet clustered into two patient groups: “early death” (< 15 days of disease until death) and “late death” (> 15 days). Progression to early death was characterized systemically and in lung single-cell spatial proteomics by rapid, intense endothelial and myeloid activation/chemoattraction and presence of thrombi, associated with SARS-CoV-2^{+} macrophages. In contrast, progression to late death was associated with fibrosis, apoptosis and abundant SARS-CoV-2^{+} epithelial cells in post-mortem lung, with cytotoxicity, interferon and Th17 signatures only detectable in the peripheral blood 2 weeks into hospitalization. Progression to recovery was associated with higher lymphocyte counts, Th2 and anti-inflammatory-mediated responses. By integrating ante-mortem longitudinal systemic and spatial single-cell lung signatures, we defined an enhanced set of prognostic clinical parameters predicting disease outcome for guiding more precise and optimal treatments. Finally, this study represents a major advance in the investigation of acute respiratory infections by integrating serial clinical data and peripheral blood samples with histopathological and spatially-resolved single-cell analyses of post-mortem lung samples.

Longitudinal Spatial Multi-Omics of 3D organoids and patient biopsies for Predicting Radiation Response in Breast Cancer 044

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Radiation (Rx) is a primary treatment for breast cancer (BC). Currently, all patients receive the same standard course of Rx, regardless of the molecular profile of their cancer. This standardized approach assumes uniform radiosensitivity across all BC, despite multiple studies proving this assumption to be incorrect. Cancer cells evolve in variable tumor microenvironment (TME) and adapt to harsh microenvironmental conditions such as acidosis, starvation, and hypoxia. Adaptation to these conditions can set cancer cell states to be unresponsive to radiation therapy due to adaptation to reactive oxygen species (ROS) in both conditions. Hypoxia and acidosis followed by increased ROS that will kill the cells if they don’t evolve resistance to it. One main mechanism of killing cancer cells by Rx is also Rx. Therefore, preexisting adaptation of cancer cells to ROS can change their response to treatment in different patients. Here we use a single cell, spatially resolved multi-omics (MALDI mass spectrometry

for lipidomics and metabolomics and multiplex IF) approach on 3D spheroid and patient derived organoid models to demonstrate that the pre-existing cell states can influence the evolutionary trajectories of cancer cells responding to Rx. Integration of spatial data at multiple biological level produced a detailed tumor landscape profile confirming detection of unique cell states in our patients' pre-treatment biopsies, which can indicate a more precise, targeted mode of therapy for the patient.

045 PROXIMAP: A robust and platform independent tool to uncover spatial topology of tissue by neighborhood co-localization analysis

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Introduction: Understanding tissue architecture and niche-specific microenvironments through spatial transcriptomics (stRNA-seq) necessitates the precise annotation of individual spots or cells to accurately decipher gene expression patterns in their spatial contexts. Despite recent advances, a platform-independent tool to quantitatively measure the degree of colocalization of diverse gene signatures or cell types in spatial contexts is still missing. To address this gap, we present PROXIMAP, a robust tool to analyze spatial neighborhood co- localization in tissue sections across different platforms.

Method: PROXIMAP represents a standalone Python and R package that quantitatively and qualitatively decodes the co-occurrence of gene signatures within the spatial context of tissue. It features two core methods: *Enclave*, which computes a density-based likelihood score to minimize false positives and assigns certainty scores based on median ranked-sum scores of gene-set enrichment algorithms to enhance co-localization reliability; and *Area*, which estimates spatial density from per-spot data for the identification of spatial compartments and disjoint regions, providing insights into the underlying tissue topology.

Results: We applied PROXIMAP across multiple platforms (e.g., 10X Visium, Xenium, Vizgen Merfish), organs (e.g., brain, tonsils), and organisms (human and mouse). The tool demonstrated that co-localization informs downstream spatial transcriptomics analysis, uncovering deeper insights than single-cell transcriptomics alone. In metastatic melanoma patient samples undergoing immunotherapy, PROXIMAP identified distinct spatial architectures: non-responders exhibited overlapping lipid-associated macrophages and exhausted T cells, while responders showed colocalized interferon-producing macrophages and activated T cells, indicating differential spatial architectures influencing therapy outcomes.

Discussion: PROXIMAP provides a novel approach to quantifying the co-localization of diverse cell states, enhancing our understanding of tissue organization and supporting clinical decision-making. Its platform-independent design, compatibility with Python and R, is adaptable to future spatial high-throughput data, providing a versatile tool for advancing spati-

al transcriptomics research.

Characterization of therapeutic effects in Glioblastoma through integrated spatial single-cell multiome profiling of longitudinal samples: unveiling clinically relevant subtypes of pathological changes 046

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Glioblastoma remains a highly malignant, inevitably recurring brain tumor with dismal prognosis. Tumor progression is influenced by cellular interactions between the tumor and its microenvironment, yet quantitative data on these interactions, particularly under standard of care therapy, remains sparse. We utilized spatial, single-cell, multiome profiling to map therapy-induced changes in the architecture of 357 paired glioblastoma samples from 52 patients at diagnosis and recurrence. This approach categorized patients into groups reflecting specific evolutionary responses to therapy, with implications for prognosis and treatment. Favourable outcomes were linked to shifts towards enriched perivascular, oligodendrocyte-progenitor-like niches with deep tissue infiltration of activated immune cells, whereas poor outcomes correlated with shifts towards an immunosuppressive mesenchymal environment with poor immune infiltration. Finally, while mutations in EGFR/PTEN, or MGMT-promoter methylation influenced tumor evolution, we also identified a subset of patients that were more responsive to lomustine after early recurrence. Our findings offer a framework to stratify patient treatment based on tumor and microenvironmental evolution.

047 An optimized computational pipeline for single-cell phenotyping from multiplex immunofluorescence imaging data

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Single-cell phenotyping is pivotal to identify the best treatment strategy, and to improve patient outcomes.
Multiplex immunofluorescence imaging (mIF) is a powerful state-of-the-art approach for single-cell phenotyping with high spatial resolution. However, mIF often struggles with ambiguous cell type assignments and low accuracy when identifying low abundance cell types. And while great advances have been made in quantifying highly multiplexed images, imaging noise continues to affect cell segmentation and marker thresholding. This ultimately results in inaccurate phenotyping.
Here, we developed a highly parallelized image processing pipeline that provides: (1) cytosolic background removal with doubled SNR improvements, (2) an advanced combinatorial approach for cell and nuclei segmentation that captures more diverse phenotypes, (3) hundreds of fluorescent intensity and morphology measurements across different cellular regions to aid in cell phenotyping, (4) machine-learning guided auto-thresholding to reduce laborious manual annotations, (5) combining hundreds of parameters into a single composite index for thresholding and assigning marker status, and (6) a distance-based cellular phenotyping approach. For broader interoperability, this pipeline can either be used as a stand-alone tool or as a post-processing pipeline together with MCMICRO.
We demonstrate the power of our scalable pipeline on an immuno-oncology head and neck

cancer cohort, where we characterized the relationship between treatment response and the colocalization of tumor cells with immune and stromal cells in different outcome groups.

Challenges of multiplexed imaging on a core facility 048

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Tissue multiplexing imaging is a powerful tool to answer at specific scientific questions by tracking the location and abundance of specific proteins of interest. Over the past few years, there has been a surge in highly multiplexed tissue imaging technologies [1, 2], surpassing the limitations of conventional immunophenotyping by allowing simultaneous staining of the same sample with several dozen markers. While multiplexed imaging technologies may vary in terms of imaging methods or biomarkers detection, challenges are shared for successful experiments. The quality of the final image analysis step depends heavily on the overall workflow, including tissue preparation and biomarkers panel design.

Given that approaches based on these technologies can be expensive, time-consuming, and require specific expertise, we offer this service at our facility, MicroPICell, based on the PhenoCycler System (Akoya). MicroPicell is both part of the national infrastructure France BioImaging and the european infrastructure EuroBioImaging, and then open to all interested users. The type of sample (tissue, organoids), the preparation of tissue (fresh frozen or formalin-fixed paraffin embedded), biomarkers of interest or scientific questions are elements which may vary between each project and require to solve several challenges for each new project requested by a user of our facility.

The first challenge lies in achieving high-quality multiplexing staining, which requires attention of various points from tissue preparation to data acquisition. Proper preparation of sample sections is critical to ensure tissue integrity : quality controls can be added during the experimental workflow to validate this point. The quantity of biological material must be optimized for each run. Autofluorescence can be problematic in fluorescence imaging of tissue sections, requiring the implementation of a quenching protocol. Designing a multiplex panel with available antibodies or performing antibody conjugation for biomarkers of interest while getting the best signal to noise ratio is the key to multiplexed analysis.

The tissue image analysis is the second challenge and recent developments in the bioinformatics methods offers exciting perspectives for exploring the generated data [3], requiring a strong user involvement to guide the analysis. But even before obtaining this data and analysing the cell populations, the final image for analysis from the raw acquisitions has to undergo several preprocessing steps [4] . In particular cell segmentation is essential to identify precisely every cell or other compartments in an image and may vary depending on the project. Finally, another challenge is the data management, including file organisation, description and sharing capabilities.

In conclusion, the biggest challenge of multiplexed imaging on a core facility is to propose

a standardized workflow from tissue preparation to data acquisition, while offering a diverse panel of analysis tools to address each scientific question. We present the solutions implemented on our facility, exemplified on different projects, and discuss the encountered difficulties.

049 Integrating whole-organ light-sheet and highly multiplexed microscopy to investigate adoptive T cell responses in metastatic breast cancer lesions

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Immunotherapy has the potential to achieve long-lasting cancer remission, but therapy efficacy is often limited by heterogenous responses of metastatic lesions. To systemically characterize tissue niches that mediate immune evasion, we developed a workflow for image-guided tissue extraction to correlate whole-organ light-sheet microscopy (LSM) with highly multiplexed microscopy using "CO-Detection by indexing" (CODEX).
Systemic metastases of ovalbumin (OVA)-expressing breast cancer cells were generated by tail artery injection and targeted by adoptive transfer of *in vitro* activated OVA-specific (OT1) $CD8^+$ T cells. Using LSM, we created a spatio-temporal atlas of immunotargeting efficacy in lung and liver, along with immune activation in corresponding lymph nodes by mapping OVA-specific and endogenous $CD8^+$ T cell infiltration and tumor cell viability in relation to anatomical organ niches.
While OVA-expressing tumor cells were near-completely eradicated *in vitro*, adoptive transfer of OT1 $CD8^+$ T cells reduced the number and volume of metastases but failed to achieve complete eradication. Different immune evasion mechanisms occurred in parallel within the same animal, with immune exclusion frequently observed in liver metastases, whereas lung metastases were highly infiltrated by $CD8^+$ T cells that failed to suppress tumor growth.
To further characterize the cellular composition of identified regions in LSM with CODEX, tissue clearing was reversed and contrast-agent based micro-computer tomography (CT) imaging was used to define the regions in the frozen tissue block for precise extraction using a milling robot. The resulting volumes were arranged in a tissue array and stained using a validated, clearing-compatible CODEX antibody panel of >30 markers.
In summary, we established a workflow to integrate the detection of immunosuppressive anatomical niches in 3D with high-dimensional cell type characterization and cellular neighborhood analysis in 2D. This method will help identify novel therapeutic targets and guide the design of combination therapies to address diverse immunosuppressive mechanisms in metastatic cancer.

Novel workflow for matrix-free generation and fast-track, high-throughput characterization of human brain organoids by lightsheet microscopy 050

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The processes of human brain development and its function are incompletely understood. Development of new *in vitro* models as well as implementation of state-of-the-art, high-throughput analytical workflows are urgently required to enable a better understanding of neurological diseases and to advance therapeutic developments.
Therefore, we developed a novel, standardized workflow including matrix-free generation of human pluripotent stem cell (hPSC)-derived brain organoids followed by detailed characterization of the cellular composition using 3D lightsheet microscopy.
First, we established a xeno- and Matrigel-free differentiation protocol for highly reproducible generation of hPSC-derived cerebral organoids, successfully recapitulating early brain development in 3D. Next, we developed an efficient and fast, non-toxic clearing protocol for organoids, allowing as well for preservation of endogenous fluorescence markers, like GFP. Multiplex labeling of organoids with REAfinity™ conjugates was optimized for lightsheet microscopy.
In order to facilitate high-throughput imaging of up to 48 organoids in a single run we developed a dedicated multi-sample holder, the MACS® UltraMount 48. This imaging holder for the UltraMicroscope Blaze™ is also suited for parallelized sample preparation, thereby minimizing sample handling times.
Our data indicate development of a straight forward differentiation protocol allowing for standardized generation of hPSC-derived cerebral organoids with high efficiency.
Whole mount staining using REAfinity™ antibody conjugates was optimized for brain organoids at different developmental stages and combined with a non-toxic tissue clearing procedure. Parallelized organoid sample processing followed by light sheet imaging revealed the formation of multiple ventricular zones and cortical plate-like regions, indicative of early cortical development.
Next, this workflow will be used for characterization of patient-derived organoid models and in vitro evaluation of cellular therapeutics.

Spatially resolved single-cell proteomics for treatment response prediction in metastatic colorectal cancer 051

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Colorectal cancer (CRC) is the fourth leading cause of cancer death worldwide. Around 50% of patients develop metastatic CRC (mCRC), with a 5-year survival of only ~14%. Choosing the optimal first-line therapy among several possible combinations of chemotherapies and targeted drugs has been shown to be essential to achieve higher objective response rate and overall survival. However, in clinical practice no biomarkers that predict a patient's response to the different therapy options exist. Thus, there is an urgent clinical need for new diagnostic strategies aimed at capturing mCRC heterogeneity and predicting individual response to treatment.
To address this challenge, we established a cohort of mCRC patients (n=400) with tissue samples and matched treatment and response data available and employed targeted spatial single-cell proteomics to identify predictive biomarkers. We developed two 40-plex antibody panels for imaging mass cytometry (IMC) with markers capturing tissue architecture, the tumor and immune compartments, and potential drug targets. Tissue samples were organized in tissue microarrays, consecutive sections were stained with the two antibody panels, and multiplexed images were acquired using IMC. Data were analyzed using a computational pipeline to identify stratifying signatures that distinguish between responders and non-responders.
Our preliminary results demonstrate that our approach can generate a deep understanding of mCRC tumor ecosystems and identify features with potential predictive value for treatment-specific response. We found that single-cell marker expression and spatial features based on the interaction of the identified cell types were associated with response to therapy. These initial findings will be extended and consolidated using larger mCRC cohorts, with the eventual aim to validate our approach in the context of a prospective study, allowing clinicians to more accurately identify the most effective therapy for individual mCRC patients.

052 Cellular influence maps pinpoint antitumor immunity hotspots at the oral squamous cell carcinoma invasive front

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Introduction: Oral tongue squamous cell carcinoma (OTSCC) is the most common cancer of the oral cavity and is associated with a poor prognosis (five-year mortality rate of 30-40%). Predictive biomarkers are critically needed for identifying patients at risk of OTSCC recurrence, enabling targeted development of novel treatment strategies. The tumor microenvironment (TME) plays a crucial role in OTSCC progression, contributing to the formation of immunosuppressive hotspots which promote tumor proliferation, dedifferentiation, and immune evasion.

Methods: We are conducting a retrospective, two-center cohort study utilizing 49-plex imaging mass cytometry (IMC) to understand the spatial organization of tissue functions in primary OTSCC resection samples (579 1.5mm cores) from 198 treatment-naïve patients, 27.3% of whom developed recurrence within three years of primary resection with clear margins. The panel includes eight structural, 18 immune lineage, three immune checkpoint and three tumor markers. Additionally, 17 functional markers of cellular signaling, metabolism, and enzymatic activity are quantified across all cell types.

Results: In a pilot study (24 patients), we used standardized tissue zones to identify TME patterns associated with loss-of-differentiation. We achieved accurate histological grade classification (AUC = 0.88) using Stabl, a machine-learning algorithm that combines data-driven feature selection and multivariable modeling. Now, we generated a large dataset comprising >7M single cells of the heterogeneous tumor-immune interface of OTSCC. Single cell functional influence (Sc-fi) mapping captures each individual cell's ability to modify neighboring cells' intracellular signaling, metabolism, or enzymatic activity and allows identifying functional tissue regions that differentiate patient outcomes. We will next apply Stabl to train a multivariable model to predict 3 year-recurrence at the time of primary resection.

Conclusion: This study offers a comprehensive analysis of the OTSCC TME, highlighting its role in tumor progression and recurrence. Potential spatial biomarkers will be confirmed using IHC and could inform future studies to improve clinical management.

Spatial insights into tumor dissemination of colorectal cancer 053

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Background: Understanding the release mechanisms of circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) is crucial for understanding metastatic disease, yet the underlying biology remains controversial. We hypothesize that specific biological processes and cell types are facilitating tumor dissemination. We utilize spatial transcriptomics to analyze tumor tissues, exploring the spatial distribution of point mutations and specific transcripts in the tumor microenvironment of colon cancer patients.
Methods: We employ Xenium and in situ sequencing to analyze primary tumor tissues from colorectal cancer (CRC) patients, focusing on mutations detected in the primary tumors. Gene panels ranging from 220 to 322 genes are evaluated, emphasizing biological pathways potentially contributing to tumor dissemination, including angiogenesis, proliferation, hypoxia, inflammation, and invasion. Additionally, immune cell phenotyping is conducted through in situ sequencing. Data analysis is performed using CellProfiler, MATLAB, TissUMaps and Xenium Explorer.
Results: In a proof-of-concept study, patient-specific mutations were identified by sequencing CRC tissue. Mutation-specific spatial analysis by in situ sequencing revealed distinct spatial distribution patterns in primary tumor tissue sections. By spatial gene expression analysis of neoplastic versus non-neoplastic tissue multiple biological processes contributing to tumor dissemination were identified, including invasion, tumor-associated stromal cells, and immune cell populations.
Conclusion: Spatial analysis allows the identification of distinct cell types and biological processes associated with tumor dissemination and disease progression.

054 Recording the high-resolution spatial transcriptome and electrophysiology of cellular networks using CMOS MEA chips

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Microelectrodes Arrays (MEA) are great tools for investigating electrophysiology in Micro Physiological Systems (MPS). However, studying both electrophysiology and transcriptomics concurrently has not yet been done. Current methods for *in vitro* spatial transcripto-

mics (like FISH) on cell culture are incompatible with performing live-cell high-resolution spatiotemporal electrophysiology, as they require cell tissues to be fixed and permeabilized on a dedicated functionalized glass slide. Therefore, we propose to combine electrophysiology recordings and spatial cell barcoding on the same MEA platform.
We have developed High Density Multielectrode Arrays (HDMEA) that can take simultaneous multi-channel voltage recordings with single-cell spatial resolution (electrode pitch of 15um) and temporal resolution of 30kHz. All >16k electrodes on the chip can be addressed, and 1024 electrodes can be used simultaneously. This chip was previously employed in neural network monitoring, using the chip's integrated amplifiers to record *in situ* from cultured hippocampal neurons at day 15 onwards for more than an hour [1], showcasing its capability for long-term electrophysiology monitoring.
Here, we present our HDMEA chip as an *in vitro* spatial cellular transcriptomics platform. We performed spatially targeted single-cell electroporation on Human Dermal Fibroblasts using the chip's integrated stimulator to introduce spatially tagged oligonucleotide barcodes with multiplexing. We report the successful introduction of the fluorophore-tagged ssDNA barcodes into single cells on pre-defined locations on the chip, with 78% of cells successfully delivered with barcodes. We also designed a microfluidic setup together with an image-based electrode selection program to aid the targeted delivery of different barcodes to single cells. We are in the process of performing de novo scRNA-seq on these location-tagged cells to confirm the unique introduction of spatial barcodes.
Combining both sources of information, this chip will enable spatial transcriptomics and electrophysiology measurements to be done on the same cell.
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Miccoli Beatrice et al. Frontiers in Neuroscience (2019). 10.3389/fnins.2019.0064

An adaptable 20-plex immuno-oncology antibody core panel for human FFPE cancer samples: REAplex IO Core for MACSima **055**

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Spatial biology is becoming essential for understanding immune cell dynamics, the tumor microenvironment (TME), and therapeutic responses in cancer research. Ultra-high-plex immunofluorescence has revolutionized deep phenotyping and cell neighborhood analysis, offering unprecedented insights into complex tissue samples. While many platforms automate image acquisition, selecting appropriate markers, finding compatible antibody clones, and optimizing dilutions remain time-consuming tasks. To streamline the panel-building process, we developed pre-defined antibody panels for the MACSima™ Imaging Cyclic Staining (MICS) technology, ranging from screening panels (205-plex) to application-specific panels, such as the REAscreen™ Immuno Oncology panel, with 61 cancer and immune-related antibodies.

With REAplex IO Core, we introduce a new 20-plex, pre-defined and flexible antibody panel, designed to identify major tumor-infiltrating leukocyte populations, focusing on T-cells, tumor vascularization, and cellular activity in human FFPE tumor samples. REAplex IO Core can serve as a stand-alone panel for basic sample analysis or be expanded with additional antibodies to create custom panels tailored to specific research questions. Here, we demonstrate its performance and broad applicability across several cancer types, and present adaptable templates for data analysis with the MACS iQ View analysis software, highlighting the simplicity and potential of this integrated end-to-end workflow.

056 A high-resolution and high-speed confocal imaging pipeline for simultaneous assessment of tissue organization and subcellular protein quantification in large patient cohorts

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The slow acquisition speed of point scanning confocals and the inconsistent autofocus performance across imaging cycles have so far limited the use of high resolution tissue multiplex immunofluorescence to selected smaller tissue areas. Larger studies rely on lower-resolution (usually 20x widefield) images, which have two main disadvantages: first, due to overlapping cells, segmentation in dense areas is often unsatisfactory. Second, (sub)cellular morphology and protein localization are largely ignored in routine analysis pipelines.
To address these problems, we developed a high throughput and high resolution cyclic immunofluorescence imaging pipeline where we combine high-speed spinning disk confocal image acquisition with very bright 40x water immersion objectives. The key innovation is a highly optimized autofocus strategy that allows the precise imaging of the same z-plane over up to 10 staining cycles in hundreds of tissue microarray cores, enabling consistently correct automated stitching and registration.
The resulting multiplex images show consistently even illumination and an excellent resolution in x, y and z even in datasets from large patient cohorts with notoriously difficult samples like biopsies. This excellent image quality allows the routine use of parameters like cell shape, subcellular protein distribution etc during analysis of datasets and solves many problems usually encountered during automated image analysis. Importantly, increased resolution of the images also results in up to 3x improved segmentation accuracy in dense tumor areas compared with 20x widefield fluorescence.
To illustrate the potential of this pipeline, we will present preliminary results from a high-throughput project designed to reveal new therapeutic biomarkers in triple negative breast cancer (TNBC) using this pipeline to image tumor samples obtained from 110 patients treated with neoadjuvant therapy containing microtubule binding cancer drugs.

Innovations in mass spectrometry imaging open-source software and application for spatial phenotyping of solid tumors 057

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Mass spectrometry imaging (MSI) is a powerful method for oncological research, offering detailed spatial maps of hundreds of molecules such as metabolites, peptides and n-glycans. In translational research, MSI enhances our understanding of pathophysiology, reveals candidate biomarkers and provides insights into tumor heterogeneity. However, analysis of the large and complex data often represents a major hurdle in MSI projects. We introduce the open-source software i2nca that streamlines MSI data handling and preprocessing. It reduces raw file sizes by about 99% while maintaining precise mass values and molecular information. I2nca can read and write the standard file format imzML allowing to embed its functions into modular interoperable workflows. We have integrated i2nca and additional open-source software such as Cardinal into the Galaxy plattform. Galaxy has a graphical user interface and allows for reproducible and transparent data analysis in public clouds and via standardized workflows. We have applied the developed software to analyze MSI data of pancreatic cancer and intrahepatic cholangiocarcinoma (ICC), two highly aggressive tumors characterized by very complex spatial biology. Pixel-wise classification distinguished with high accuracy between pancreatic ductal adenocarcinoma and neuroendocrine tumors of the pancreas. In ICC, supervised classification distinguished tumor-positive regions from non-malignant tissue with over 90% accuracy. Unsupervised segmentation revealed novel insights into molecular inter- and intratumor heterogeneity. In summary, MSI in combination with powerful and easy to use analysis software paves the way towards highly multiplexed spatial phenotyping of solid tumors.

058 Spatial transcriptomics validate the impact of JAK-STAT signaling on homeostasis

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JAK-STAT signaling is well known to mediate responses to external signals, but it also regulates expression profiles of cells in homeostasis. Yet, the role of individual JAK-STAT proteins, the source of signals, and the extent of regulation in homeostasis are unclear.

Using transgenic mice, we performed large-scale analyses of *in vivo* transcriptomic and epigenomic effects of JAK-STAT perturbations on immune cells from spleens in homeostasis. We found broad and unexpected effects of JAK-STAT perturbations, some of which led to a rewiring of JAK-STAT pathways. To validate these intriguing findings, we performed spatial transcriptomics (Visium assay) and RNA *in situ* hybridization analyses of STAT1 knockout compared to wildtype murine spleens. While the overall tissue architecture was unaffected by the STAT1 knockout, spatial expression patterns validated a clear down-regulation of JAK-STAT target genes in knockout compared to wildtype samples. These effects were focused on white pulp, an area that contains T cells and macrophages (the immune cell populations we focused on). Further analyses of tissue-context-deprived cells and re-analyses of public expression data from the spleen predicted interactions of cells with their microenvironment as a source of low-level JAK-STAT stimulation.

Taken together, our multi-level analyses demonstrate a strong role of the tissue environment, resulting in non-canonical JAK-STAT activation that critically shapes gene expression in homeostasis.

059 Tumor-immune spatial and molecular signatures predictive of ovarian cancer histotypes

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Immunotherapy has shown potential for a long-term response and a possible cure across a multitude of cancer subtypes but has so far shown a limited effect in ovarian cancers (OC), hence why no immunotherapy treatment against OC has been approved by the FDA. Despite the underwhelming results studies have shown ovarian tumors to be immunogenic containing anti-tumor responses and the immune infiltration to be prognostic for patient survival. There is thus a need for comprehensive characterization of tumor immune microenvironments (TIME) to predict optimal treatment regimens for individual patients, as well as to identify novel targets. OC is traditionally grouped into five primary histotypes high-grade serous carcinoma (HGSC), low-grade serous carcinoma (LGSC), mucinous carcinoma, clear cell carcinoma, and endometroid carcinoma. Here we used the GeoMx Digital Spatial Profiling (DSP) to characterize spatially distinct TIME niches in OC and identify TIME signatures associated to OC histotypes and prognosis. FFPE tissue microarrays from population-based, mixed-histology OC cohort, with 3x1mm cores per tumor, were analysed. Selection of regions of interest (ROIs, n=1-8 per tumor) were guided by 4-color immunofluorescence. We identified distinct differences in the spatial TIME and the molecular signatures in OC histotypes. The histotypes LGSC, mucinous, clear cell and endometroid carcinoma has an overall suppressed TIME whereas HGSC is characterised by infiltrating T- and NK cells. STING was observed to be upregulated in LGSC tumor regions and therefore identified as a potential immune marker of interest for further studies. These results indicate that the OC histotypes have a vastly heterogenous TIME, proving that the potential of immunotherapy in OC rests on a deeper dive into the TIME of OCs.

Cell type resolved tissue proteomics to identify drivers of cancer therapy resistance in head and neck cancer **060**

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Head and neck squamous cell carcinoma (HNSCC) incidence rates are rising globally, mainly due to the consumption of carcinogen-containing products and HPV infections. However, treatment success especially in HPV-negative patients is limited [1]. Patients with recurrent and metastatic disease are often treated with anti-cancer immunotherapy targeting the PD-1 pathway. However, most patients do not respond to the treatment with checkpoint inhibitors despite being selected based on PD-L1 positivity in immunohistochemistry. This suggests that patient selection cannot rely entirely on PD-L1 alone due to alternative checkpoints or pathways that affect the immune response. To systematically profile the tumor-immune microenvironments of responders and non-responders with single-cell and spatial resolution, we selected a patient cohort of 90 individuals and applied multiplex immunofluorescence imaging of 25 markers to capture cellular phenotypes and their functional states. We employed machine learning based image analysis, which identified more than 1 million cells, covering various tumor and immune cell phenotypes, including T cells, B cells, macrophages as well as cancer cells and their states (epithelial, mesenchymal, proliferative). We identified distinct patterns of cellular organizations and interactions that

significantly varied between responders and non-responders. Our data revealed strong differences in immune cell abundances and their spatial distributions especially at the tumor invasive front. In the future, we will combine these promising data with the deep visual proteomics workflow employing single-cell laser microdissection followed by deep mass-spectrometry based low-input tissue proteomics. By pooling phenotypically similar cells, we will link cellular neighborhood information to functional proteome sates. With this integrated, multimodal approach, we can combine the advantages of targeted and untargeted spatial proteomics, enabling us to identify potential new drug targets, prognostic biomarkers of treatment response and infer mechanisms of treatment resistance.

061 Identification of functional tumor-immune interactions with a multiplexed in situ proximity ligation approach

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Cancer cells can exploit immune inhibitory pathways as mechanisms for immune evasion. Blocking these pathways has become a prominent immunotherapeutic strategy, but with limited success even among patients positive for relevant biomarkers. This highlights the necessity of a comprehensive understanding of the tumor microenvironment (TME), focusing on functional interactions rather than mere expression profiles. We hereby introduce a novel immuno-oncology three-plex panel (Naveni®Plex) which leverages the *in situ* proximity ligation assay (*is*PLA) to detect PD1/PD-L1 and CD8/MHC-I interactions, alongside with CD3 expression in human cancer tissues.

Multiplex *is*PLA successfully identified its targets in healthy tonsil tissues, while the negative control (muscle tissue) showed no signals. Samples from head-and-neck cancer, lung cancer, Hodgkin lymphoma, and melanoma were also examined. Lung cancer tissues displayed varying levels of PD1/PD-L1 activation in CD3+ cells, while Hodgkin lymphoma showed a high number of activated T cells (CD8/MHC-I+) and abundant PD1/PD-L1 interactions. In melanoma, one sample was immune-cold with no detectable signals, while another exhibited limited immune checkpoint activation and active immune cells.

A deeper understanding of immune checkpoint dynamics within the TME can aid in developing strategies to counteract cancer cells' immune evasion. Functional assessments of pathways like PD1/PD-L1 and CD8/MHC-I offer a more biologically relevant perspective than traditional IHC, enabling more meaningful spatial profiling. Further research is needed to explore the therapeutic potential of protein interactions as superior biomarkers and the possibility to improve patient outcomes.

062 Comprehensive Evaluation of Automated Tissue Pre-Staining and Staining Conditions for Reliable Multiplexed Imaging

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The emergence of spatial proteomics has enabled the simultaneous detection of multiple tissue biomarkers. These methods, now widely available commercially, have revolutionized the study of biological and pathological processes. With the increased adoption of multiplexed protein imaging, particularly in pre-clinical studies in pharmaceutical and biotechnology companies, there is an urgent need for a comprehensive evaluation of various tissue preparation steps, including antigen retrieval and antibody staining conditions on automated commercial immunostainers. The automation of the staining process not only enhances the consistency and reliability of results but also significantly reduces the hands-on time required for manual protocols. This not only increases efficiency but also allows for the simultaneous processing of multiple samples, further optimizing laboratory productivity. In this work, we aim to compare several pre-staining conditions on a small scale using formalin-fixed paraffin-embedded mouse and cynomolgus monkey tissue microarray sections. We will utilize the Leica Bond RX and Ventana Discovery Ultra systems for downstream staining with a neuro/immune-focused 15-plex conjugated antibody panel on the Akoya Phenocycler. We will perform quantitative image analysis to assess the impact of these conditions, such as signal intensity, background, specificity and morphology preservation.
The insights derived from this study will be incredibly helpful in guiding those who are new to multiplexed imaging, providing support in experiment design, reagent selection, experimental conditions, and the quantitative evaluation of results.

The Spatial-Cell-ID spatial transcriptomics facility **063**

Yad GHAVI-HELM

Functional Genomics Institute of Lyon, France

I will present Spatial-Cell-ID, a spatial transcriptomics facility located in Lyon, France. At Spatial-Cell-ID, we provide various services going from single-cell transcriptomics to sequencing- and imaging-based spatial transcriptomics. In particular, I will showcase our latest advances in the development of a 3D MERFISH setup that uses super-resolution microscopy to gain deeper insights into the spatial location of hundreds of transcripts within a whole-mount embryo.

Simultaneous and Integrated Spatial Gene and Protein Expression Analysis on Xenium *In Situ* platform **064**

Gabriele Girelli, Thanutra Zhang, Smritee Dadhwal, Anatalia Robles, Cheyenne Christopherson, Joshua Thao, Minkyung Park, Hsin Wen Chan, Janine Hensel, Cedric Espenel, Hayato Ikoma, Paul Ryvkin, Veronica Gonzalez Muñoz

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In recent years, high-resolution spatial gene expression analysis in intact tissue sections has established itself as a powerful tool to uncover the molecular characteristics of human diseases. However, integrating it with protein expression is instrumental for a comprehensive understanding of the underlying molecular mechanisms. Recent developments in the Xenium platform have enabled high-resolution spatial capture of gene expression in conjunction with cell morphology and protein expression in the same instrument run, without any post-run hands-on time. These novel protein detection capabilities seamlessly integrate with other tools of the Xenium ecosystem, facilitating the exploration of automatically registered cell segmentation, RNA detection, and protein expression images via the Xenium Explorer tool. Furthermore, the non-destructive nature of the Xenium protocol allows for subsequent hematoxylin and eosin (H&E) staining on the same sample post-run, which can be automatically aligned to the workflow's nuclei staining in Xenium Explorer. Our studies demonstrate that Xenium is a well-rounded platform which can integrate additional post-run steps to complement the initial results. We tested this across a wide variety of human and mouse tissue types and our data showcase the ability of Xenium to simultaneously capture and integrate spatially-resolved *in situ* gene and protein expression making it a powerful tool for advancing our understanding of disease mechanisms.

065 Integrated Multiomics Uncovers Distinct Macrophage Alterations in Human Metabolic dysfunction-Associated Steatohepatitis Progression

Markus Boesch[1,2,3,4], Tessa Ostyn[2], Asier Antoranz[2,3], Trieu My Van[5], Daniel Newhouse[5], Caitlyn Myers[6], Lukas Van Melkebeke[1,7], Najmeh Saffarzadeh[6], Rita Feio-Azevedo[1], Lena Smets[1], Gautam Shankar[2,3], Paula Longás Calvo[3,4], Thierry Voet[3,4], James Clark[8], Simon Cockell[8], Matthias Lannoo[9], Ellen Deleus[9], Joris Jaekers[9], Halit Topal[9], Baki Topal[9], Kai Markus Schneider[10], Michele Vacca[11,12], Michael Allison[13], Mattias Ekstedt[14], Jerome Boursier[15], Elisabetta Bugianesi[16], Vlad Ratziu[17] , Frederik De Smet[2,3], Bram Boeckx[18,19], Ann K. Daly[8], Jörn M. Schattenberg[20], Jef Verbeek[1,7], Francesca Bosisio[2,3], Diether Lambrechts[18,19], Tania Roskams[2], Carolin V. Schneider[10], Sven Francque[21,22], Hannelie Korf[1], Anetta Härtlova[6,23], Dina Tiniakos[8,24], Quentin M. Anstee[8,25]*,Schalk van der Merwe[1,7]*, Olivier Govaere[2,3,8]*

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Single-Cell Spatial Atlas of the Ageing Human Breast

066

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Breast cancer can develop across a wide age range, with tumours in younger women differing significantly from those in older women. Ageing alters the spatial context of early tumour development and may explain these differences, but tissue-level changes in the ageing breast remain poorly characterised.
Using imaging mass cytometry to profile the in-situ expression of 40 proteins at subcellular resolution, we explored age-related remodelling of normal breast tissues in over three million cells from 527 reduction mammoplasties. Aged breast tissue was less cellular and less proliferative for all cell types (epithelial, stromal and immune). Tissue architecture was restructured with fewer heterotypic epithelial cell-cell interactions, a marked reduction in lobules, and increased fat. Older tissues had a more inflammatory microenvironment with increased M2 macrophages and granzyme B^+ T cells, contrasted by younger tissues in which B cells were most enriched.
While the age-incidence of cancer is largely attributed to the accumulation of somatic mutations, our data suggest that the ageing tissue ecosystem becomes more permissive to carcinogenesis. In conclusion, this extensive multiscale atlas of healthy breast tissue reveals the changing spatial context in which tumours develop, providing a critical reference for understanding mammary biology, breast cancer, and ageing.

067 Spatially resolving B cell clonal responses in breast cancer patients

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Breast cancer remains the most prevalent cancer among women, with certain subtypes lacking targeted therapeutic options. Recent evidence shows that tumor-infiltrating B lymphocytes may play a critical role in anti-tumor immunity, particularly in association with tertiary lymphoid structures. However, the clonal dynamics of B lineage cells within the tumor stroma remain poorly understood. Unraveling the association between tumor niches and B cell clones can provide insights into clonal specificities, factors triggering clonal expansion, and class switching, together with cell dynamics at the site.

To address this knowledge gap, we employed our recently developed spatial transcriptomics-based method – Spatial VDJ - to map B and T cell receptors in breast cancer tumors from untreated triple-negative and HER2+ subtype patients. This approach enables the elucidation of B and T cell lineages within tumor microenvironment structures. In total, we analyzed 22 tumor sections using 10x Visium and Spatial VDJ, processed with a tailored Ig-Discover clonotype calling module, to survey inter- and intra-tumoral heterogeneity.

Our analysis revealed a remarkable diversity of immune repertoires, identifying over 14,000 IGH, IGK, and IGL clones across all samples. Several clones were shared between two or more patients, with higher prevalence in light chain clones. In parallel, we captured ~2,000 TRB and 400 TRA clones, all of which were found solely within one tumor. This, together with in-depth analysis of one tumor sample with 12 sections from 4 tumor regions, highlights high clonal diversity between and within patients. Linking the clones to the microenvironment, we identified clones specific to tertiary lymphoid structure (TLS)-like structures, clones enriched in tumor areas, and clones, including class-switched ones, that were shared between multiple microenvironmental niches. In sum, these data start to uncover B cell lineage dynamics in breast cancer and identify putative antibody sequences for functional studies.

Segmentation-Free Approach for Interpretable Single-Cell Analysis in Imaging Mass Cytometry 068

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Imaging Mass Cytometry (IMC) enables the multiplexed visualization of over 40 proteins with subcellular resolution, offering insight into cell-type heterogeneity and spatial organization within tissues. This technique provides spatial context, which is absent in single-cell sequencing approaches. However, current computational pipelines for IMC rely heavily on segmentation-based algorithms, where cell representations are derived from the mean expression across segmentation masks. Errors in segmentation propagate into the analysis, leading to artifacts such as the coexpression of mutually exclusive markers (e.g. CD20 and CD3), compromising downstream cell-type identification and tissue interpretation.

In this study, we propose a novel deep learning-based method that eliminates the need for segmentation by utilizing cell detection. This approach leverages grouped convolutions, allowing the model to independently learn meaningful features for each channel. The architecture enables direct interpretability by mapping channel contributions to the embedding space, providing insights into which proteins drive the formation of the representation space. This interpretability is crucial for analyzing high-dimensional IMC data, where manual inspection and ground truth annotations are not feasible due to the data's complexity and scale.

We validated our approach using an IMC dataset comprising 1.8 million cells from the bone marrow of 84 neuroblastoma patients. Our method yielded an interpretable single-cell representation that allowed the accurate identification of known bone marrow and tumor cell types, providing results in line with traditional methods, without the need for segmentation or manual feature selection. The learned feature space is biologically interpretable, enabling experts to assess cell types using clustering algorithms and domain knowledge. This provides insights into the phenotypes and spatial relationships within the microenvironment.

Our framework is applicable to other high-dimensional single-cell imaging modalities, such as multiplex immunofluorescence and multiplexed ion beam imaging. It provides a scalable, interpretable, and segmentation-free approach to analyze highly multiplexed imaging data.

069 Benchmarking cell-phenotyping methods for antibody-based multiplexed imaging

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Accurate cell phenotyping in multiplexed images is crucial for characterizing cellular composition within complex tissues and for subsequent downstream analysis. Current techniques, such as manual gating and clustering, rely heavily on expert input, making them time-consuming or highly subjective. Recently, automated algorithms including Astir, CELESTA, STELLAR, MAPs, etc have been proposed each with their own strengths and weaknesses involving their robustness, accuracy, phenotype definitions, and dataset availability. Furthermore, these methods face significant challenges specifically due to imaging noise, segmentation artifacts, and the presence of rare cell types.
In this study, we present a comprehensive benchmarking analysis of key cell type classification methods using a collection of novel, publicly available dataset specifically designed for this evaluation. Our dataset includes multiplexed imaging data with diverse staining qualities and cell type distributions for multiple tissues obtained from multiple platforms, providing a rigorous framework for assessing the different approaches. We evaluate cell gating as Gold-Ground Truth comparing alongside advanced algorithms, focusing on performance metrics such as accuracy, F1-score, and computational efficiency.
Our results highlight trade-offs between automation, interpretability, and the ability to detect rare populations. This study provides practical insights for selecting the most suitable phenotyping methods and introduces a standardized dataset to drive future improvements in multiplexed imaging analysis.

070 Spatial mechano-transcriptomics: mapping at single-cell resolution mechanical forces and gene expression in tissues

Adrien Hallou, Ruiyang He, Benjamin D. Simons and Bianca Dumitrascu

Advances in spatial profiling technologies are providing insights into how molecular programs are influenced by local signaling and environmental cues. However, cell fate specification and tissue patterning involve the interplay of biochemical and mechanical feedback. Here, we propose a new computational framework that enables the joint statistical analysis of transcriptional and mechanical signals in the context of spatial transcriptomics. To illustrate the application and utility of the approach, we use spatial transcriptomics data from the developing mouse embryo to infer the forces acting on individual cells, and use

these results to identify mechanical, morphometric, and gene expression signatures that are predictive of tissue compartment boundaries. In addition, we use geoadditive structural equation modeling to identify gene modules that predict the mechanical behavior of cells in an unbiased manner. This computational framework is easily generalized to other spatial profiling contexts, providing a generic scheme for exploring the interplay of biomolecular and mechanical cues in tissues.

Spatial biology to understand host-parasite interactions – schistosomes in the lung 071

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Schistosomiasis is a major Neglected Tropical Disease (NTD) caused by infection with parasitic flatworms in the genus *Schistosoma*, affecting >200 million people world-wide. The life cycle involves two hosts where the parasite is exposed to multiple environments and tissue micro-niches promoting multi-organ morbidities. Thus, the worm expresses life-stage specific transcriptomic and morphologic profiles to survive in diverse landscapes. The parasite lung stage remains understudied, with a paucity of knowledge about its interaction with the lung tissue. In hosts such as rats, parasites are cleared in the lung, however in humans and mice these mechanisms are absent allowing the worm to traverse the lung without causing evident pathology. To date there is limited characterisation of the spatio-transcriptomic landscape of both host and parasite cells interacting with each other.

Here we present single cell transcriptomics of lung-migrating juveniles of *S. mansoni* in a murine infection model (n=4) identifying cell populations underlying body organisation and developmental axis of the worm. We also explore tentative transcriptome perturbation and host-parasite interactions that may point to mechanisms of parasite migration in the absence of inflammation.

To further support our findings, we decided to profile the lung-stage of the worm using histology and microscopy. We reconstructed putative morphological compartments observed throughout the life cycle and identified juvenile-specific morphological features. Additionally, to interrogate the host-parasite interface we are currently generating a single cell spatial atlas of pulmonary *S. mansoni* infection utilising the new 5K Xenium Prime platform with a bespoke *S. mansoni* gene panel.

Our data shine light not only on the biology of the poorly understood lung-stage of S. mansoni, but also the host-parasite interface within the lung. Most importantly, we establish the feasibility of using high-dimensional spatial approaches to provide insight into the patho-

logy of a major NTD.

072 Spatial metabolic profiling of bone marrow niches in Multiple Myeloma patients with bone disease using Imaging Mass Cytometry

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Multiple Myeloma (MM) is the second most common hematological malignancy characterized by aberrant proliferation of monoclonal plasma cells (PCs) in the bone marrow. Approximately 80% of MM patients present with radiologically detectable bone lesions at the time of diagnosis. Despite extensive research on the tumor microenvironment (TME) associated with MM-induced bone disease, the underlying mechanisms responsible for the absence of bone lesions in the remaining patients remain elusive, highlighting the need for a more comprehensive understanding of the MM-TME interactions.
We employed imaging mass cytometry (IMC) to analyze bone marrow samples from a cohort of 72 patients, consisting of 58 MM patients with bone disease (MM_BD) and 14 MM patients without bone disease (MM_nBD).
Preliminary results show that MM_BD patients have a more compact TME and a significantly higher number of Osteoclasts (OCs) relative to MM_nBD. Spatial analysis reveals enriched proximity between OCs, PCs, and other key immune cell populations in MM_BD patients. While myeloma cells have been previously described to highly depend on glycolysis, our metabolic expression profile suggests that malignant PCs may rely more on oxidative phosphorylation than previously thought.
In summary, we provide a comprehensive spatial metabolic perspective on MM and associated bone disease.

073 Fast and accurate cell segmentation of highly multiplexed spatial omics using graph neural networks with segger

Elyas Heidari, Andrew Moorman, Gleb Rukhovich, Dana Pe'er, Moritz Gerstung, Oliver Stegle

Imaging-based spatial omics datasets present challenges in reliably segmenting single cells. Achieving accurate segmentation at single-cell resolution is crucial to unravelling multicel-

lular mechanisms and understanding cell-cell communications in spatial omics studies. Despite the considerable progress and the variety of methods available for cell segmentation, challenges persist, including issues of over-segmentation, under-segmentation, and contamination from neighbouring cells. While combining multiple segmentation methods with distinct advantages has been proposed, it does not completely resolve these issues. Additionally, scalability remains an obstacle, particularly when applying these methods to larger tissues and gene panels in targeted studies.

Here we introduce Segger, a cell segmentation model designed for single-molecule resolved datasets, leveraging the co-occurrence of nucleic and cytoplasmic molecules (e.g., transcripts). It employs a heterogeneous graph structure on molecules and nuclei, integrating fixed-radius nearest neighbor graphs for nuclei and molecules, along with edges connecting transcripts to nuclei based on spatial proximity. A heterogeneous graph neural network (GNN) is then used to propagate information across these edges to learn the association of molecules with nuclei. Post-training, the model refines cell borders by regrouping transcripts based on confidence levels, overcoming issues like nucleus-less cells or overlapping cells. Benchmarks on 10X Xenium and MERSCOPE technologies reveal Segger's superiority in accuracy and efficiency over other segmentation methods, such as Baysor, Cellpose, BIDcell, and simple nuclei-expansion. Segger can be pre-trained on one or more datasets and fine-tuned with new data, even acquired via different technologies. Its highly parallelizable nature allows for efficient training across multiple GPU machines, facilitated by recent graph learning techniques. Compared to other model-based methods like Baysor and BIDCell Segger's training is orders of magnitude faster, and more accurate making it ideal for integration into preprocessing pipelines for comprehensive spatial omics atlases. Segger is available as an open-source python package: https://elihei2.github.io/segger_dev/

Single cell expression predicted from low-plex immunofluorescence images and GeoMx bulk ROI protein counts in ovarian cancer **074**

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Spatial omics enables detailed profiling of individual tumors. Most patients are however still left with unpredictable treatment options due to lack of implementation of high-plex and costly technologies and solutions for harnessing key patterns in clinical and molecular data. Recent efforts have demonstrated the potential for image deep learning to predict outcome and/or molecular profiles from just a limited set of markers.

GeoMx protein data is comprised of digital counts collected in spatially defined regions of interest, each region typically encompassing hundreds of cells. To increase resolution and extend the potential applicability of spatial proteomics, we developed a deep learning mo-

del for deconvolution of single cell expression of >80 protein markers of tumor, immune and stromal cells from 4-plex (PanCk, CD45, CD8 and nuclear stain) images. Explicitly, our model learns visual representations of cells in a contrastive manner, utilizing Graph Neural Networks to predict expressions from a cell graph of learned cell representations. The model was benchmarked using an ovarian cancer GeoMx dataset, and its performance evaluated using a CycIF dataset from single cell resolution, high-plex staining of colorectal cancer tissue. While the model struggles with accurate single cell expressions of particularly low abundant markers, accuracy is dramatically improved when considering groupings of up to ~200 cells, enabling future stratification by tumor immune microenvironment based on low-plex immunofluorescence images, while allowing for standard single-cell analysis.

075 iprm-PASEF: accurate and confident molecular identification in MALDI Imaging

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Spatial multiomics strategies are increasingly used to explore molecular mechanisms in disease and treatments. MALDI Imaging is a crucial technique, revealing spatial patterns of multiple omics layers in tissue sections. Its label-free nature demands orthogonal analyses for accurate molecule identification. While MS/MS-based methods are ideal for this, obtaining spatial MS/MS data at a reasonable throughput is difficult. We introduce iprm-PASEF, an integrated software workflow for targeted, multiplexed MALDI-MS/MS Imaging, enabling acquisition, analysis, and annotation of fragment ion images for up to 25 molecules in one run.

CCS-enabled MALDI Imaging of lipids was performed on rat kidney cryosections with a timsTOF fleX to select fragmentation precursor ions. After iprm-PASEF acquisition, data were imported into SCiLS™ Lab for precursor identification and fragment annotation using the MetaboScape Lipid Species annotation tool, integrated in SCiLS Lab. Chimeric MS/MS spectra, containing fragment ions from multiple isomeric molecular species were observed. For instance, m/z 766.54 was identified as PE(38:4) via headgroup fragment, with side chain fragments revealing the presence of PE(18:0_20:4), PE(16:0_22:4), PE(18:1_20:3), and PE(18:2_20:2).

This workflow allows for a complete and comprehensive analysis of spatial MS/MS data in an intuitive and integrated manner and enables confident molecular identification for meaningful interpretation of multiomics MALDI Imaging data.

Spatial Mapping of IL4/IL13 Signaling Dynamics in Tuberculous Granuloma Formation 076

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Tuberculosis (TB), an infectious disease that has coexisted with humans for millennia, remains a significant global health challenge, causing approximately 1.5 million fatalities annually. The persistence of TB is partly attributed to the complex interactions between the human immune system and *Mycobacterium tuberculosis* (Mtb), the causative agent of TB. A critical aspect of this interaction and defining pathological hallmark of this disease is the formation of tuberculous granulomas.

Tuberculous granulomas are highly organized structures that form upon infection with a member of the *Mycobacterium tuberculosis* complex (MTBC) or close relatives like *M. marinum*. While crucial for infection containment, these structures can paradoxically promote disease progression, impede antibiotic treatment, and foster antibiotic resistance. Understanding the spatial organization and cellular interactions within granulomas is essential for developing effective therapies.

Using the zebrafish-*M. marinum* (Mmar) infection model, we are investigating the molecular determinants that underlie the formation of these spatially highly organized structures. Through a combination of genetic perturbations and advanced spatial biology imaging techniques, we are mapping the distribution and dynamics of IL4/IL13 signaling in granuloma formation.

Our analysis revealed distinct spatial compartmentalization of IL4/IL13 signaling within granulomas. In IL4/13 deficient animals, we observe a disruption of granuloma organization patterns and these altered spatial organization correlates with increased bacterial burden. Furthermore, we can map the dynamic changes in signaling patterns over the course of infection.

By mapping the spatial dynamics of IL4/IL13 signaling, we provide new insights into granuloma formation and maintenance. Our findings may inform novel host-directed adjunct therapeutic strategies for antibiotic treatment in TB by disrupting the spatial organization of tuberculous granulomas. Understanding the spatial aspects of immune signaling in granulomas could lead to more targeted and effective treatments for tuberculosis.

Automated pipeline for spatial phenotyping of fibroblasts in thoracic malignancies 077

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Cancer associated fibroblasts (CAFs) are associated with poor survival in multiple disease types. Our group's application of self- learning artificial intelligence methods (AI) to H&E images of resected lung adenocarcinomas and mesotheliomas find
unexpected high prognostic significance of tumour regions made up entirely of fibroblasts.

These regions are both morphologically diverse and strongly predictive of patient outcome, highlighting the importance of understanding how CAF subtypes interact with both tumour cells, and microenvironment.
Fibroblasts are difficult to phenotype: myofibroblastic CAFs (mCAFs) express either SMA or FAP, inflammatory CAFs (iCAFs) express IL-6, antigen-presenting CAFs (aCAFs) express HLA-DR, and podoplanin-positive CAFS are strongly related to poor outcome. We have used all these biomarkers along with cytokeratin (to assist with tissue segmentation) to develop an automated pipeline to spatially phenotype CAFs in thoracic malignancies.
We have developed and optimised a multiplex immunofluorescent panel for all these markers using the Ventana Discovery Ultra platform (Roche Tissue Diagnostics). The panel is being applied to a large series of lung adenocarcinoma tissue microarrays (1025 cases) with linked RNASeq and genomic data and is to be applied to a further set of mesothelioma tissues (n=450). Digital images are being passed through our Visiopharm quantitative pipeline to derive cellular lineages of CAFs in their full spatial context. We are currently in the process of analysing the association between patient outcome and lung adenocarcinoma fibroblast phenotypes.
This data will be combined with clinicopathological data to associate CAFs with patient outcome, to discover the relationship between CAF subtypes and key clinicopathological data including grade, subtype, AI-derived metrics and molecular data. We will discover the key molecular and prognostic associations of detailed fibroblast subtypes in a large set of human tumours.

Strategies for biomarker identification from mass spectrometry imaging data

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A common task in biology is to identify relevant features in a data vector of mostly irrelevant data. In spatial biology, the local composition of features and structures adds an additional layer of complexity but also provides valuable information. Our question is how spatial information can help identify spatial biomarkers for macroscopic phenotypes, such as drug side effects, from mass spectrometry imaging data. We evaluate and adapt machine learning approaches to this end. We consider a case study based on tissue slides from lung, liver and kidney of rats that are affected by drug-induced phospholipidosis. Specifically, we applied two embedding techniques, Topic Modeling and Autoencoders, and compared them to simple feature selection tools. Embedded and selected feature images subsequent-

ly were used to associate with the target side effects by S3-CIMA, CNN and VQ-VAE. We find that our embeddings work well for image classification and we identified features with tissue- and treatment-specific spatial pattens and compare these to the performance achieved on spot-level inputs without including spatial information. Our approach is applicable to any kind of comparative analysis on the basis of imaging mass spectrometry readout. We therefore expect the results of this survey to enhance future studies that aim at identifying spatial biomarkers from this type of data.

Vascular patterns of clear cell renal cell carcinoma are linked to immune cell infiltration and immunotherapy responsiveness 079

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Clear cell renal cell carcinoma (ccRCC) is a highly vascularized tumor with histological heterogenous appearance. Here, we defined vascular patterns of ccRCC into three categories: high-branching (HB), low-branching (LB) and sinusoid. We developed a learning-based algorithm for the classification of vascular patterns (VP) based on CD31 immunohistochemistry, an endothelial cell marker. VP-based gene signatures were generated by integrating the transcriptomes of matched ccRCC samples. We identified a trajectory from a HB to LB vascular phenotype that was paralleled by a decline in the expression of proximal tubule cell lineage traits, the presumed cell of origin of ccRCC. Applying the VP gene signatures to transcriptome datasets from two phase III clinical trials that compared the inhibition of angiogenesis with or without immunotherapy, we found that benefit from additional immunotherapy was limited to low-branching ccRCC and linked to an immune cell infiltrated tumor microenvironment. Using ccRCC patient-derived organoid modelling grown as air-liquid interface (ALI) cultures, we confirmed the association between LB vascular pattern and higher T cell infiltration that resulted in reduced viability of LB ccRCC organoids under immune-stimulating conditions. Multiplex immunofluorescence (CODEX) and analysis using the SPACEc pipeline revealed profound differences in cellular neighborhoods and characterized the dense immune cell infiltration of ccRCC with LB vascular patterns.

Multiplexed SARS-CoV-2 detection in brain tissue – methodological challenges and possibilities 080

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Following initial claims for virus detection in the CNS during COVID-19, it was observed that several of the antibodies lack specificity and many of the electron microscopy studies misinterpreted cellular structures as virus. Robust and specific detection methods for the presence of viral products in the brain are therefore of utmost importance.
In a multicentre approach we collected brain tissue from deceased COVID-19 patients and prepandemic negative controls, and generated tissue microarrays with in total 280 different samples. SARS-CoV-2 abundance was analyzed with Xenium probe-based spatial transcriptomics platform and the VoltRon spatial omics software package. Digital PCR and immunohistochemistry were as used as validation method.
In the lung (SARS-CoV-2 positive control), virus containing cells can contain up to 400,000 viral RNA molecules. In contrast the granular cell layer of the cerebellar cortex showed in 12 cases including prepandemic negative controls a substantial signal of the Nucleocapsid but no other viral probe, and no detection of SARS-CoV-2 in the digital PCR. Inflammatory activation of the cells was pointing to a bystander activation of the CNS during systemic infection.
Our multiplexed spatial transcriptomics assay using tissue microarrays allowed efficient screening of hundreds of samples in parallel. We found probably unspecific binding of SARS-CoV-2 nucleocapsid probes to (yet unknown) genuine cerebellar structures, which could explain published data on supposed virus abundance in the cerebellum with similar patterns, highlighting the importance of appropriate controls and usage of orthogonal detection methods.

An optimized analysis pipeline to study human Peyer's Patches in Crohn's disease using Imaging Mass Cytometry 081

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Crohn's disease (CD), is a common form of inflammatory bowel disease (IBD). The disease is described as a chronic inflammation of the gastrointestinal tract in a discontinuous pattern. Since CD is marked by organ- and compartment-specific changes, we aim to study the differences in T-cell populations in specialized lymphoid follicles, so called Peyer's Patches (PPs). To study the effect of inflammation on the immune cell composition in CD, we designed a 33-plex imaging mass cytometry panel (IMC). Biopsies of CD patients and healthy individuals were taken during endoscopy and prepared for staining. An optimized analysis pipeline was established where images were first subjected to background removal in order to integrate the different patients' samples. Next, single cell segmentation was performed using the deep-learning based framework Mesmer after validating different segmentation strategies. To further identify differences in the distribution of cell populations, images were manually annotated into different tissue segments like follicles or lamina propria. Lastly, a cell type prediction step was included to separate individual cell populations like T and B cells, which were not separable solely based on the mean expression due to spatial spillover. By implementing these improvements into the final preprocessing pipeline, we were able to show the presence of a proinflammatory environment within PPs of CD patients, even in the absence of inflammation in the terminal ileum.

Voyager - bringing geospatial data analysis methods to spatial omics in R 082

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Recent advances in spatial omics technologies posed a challenge on exploratory data analysis of such datasets. Usage of spatial information however, is not yet part of standard bioinformatic analysis workflows. A number of software packages (both in R and python) have been developed to tackle this problem. There is tendency to analyze spatial omics datasets

as single-cell data which would not be entirely correct. The utilization of spatial information is the key for any analysis of spatially-resolved datasets.
Here we introduce two Bioconductor packages, SpatialFeatureExperiment (SFE) S4 class to arrange spatial data and Voyager that takes the SFE object as an input for exploratory spatial data analysis using geospatial statistics from various R packages. Voyager implements plotting functions for gene expression, cell attributes, and spatial analysis results. Spatial technology specific image data, such as H&E, DAPI, Cadherin staining (including OME-TIFF format) can also be loaded and used for visualization purposes. Functionality to convert from Seurat object to SFE is supported and would allow users to convert already existing and processed spatial technology-specific objects for downstream spatial analysis using Voyager. This functionality is currently supported for Visium, Visium HD, Vizgen/MERSCOPE and Xenium technologies.

083 micronuclAI: Automated quantification of micronuclei for assessment of chromosomal instability.

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Chromosomal instability (CIN) is a hallmark of cancer that drives metastasis, immune evasion and treatment resistance. CIN results from chromosome mis-segregation events during anaphase, as excessive chromatin is packaged in micronuclei (MN). CIN can therefore be effectively quantified by enumerating micronuclei frequency using high-content microscopy. Despite recent advancements in automation through computer vision and machine learning, the assessment of CIN remains a predominantly manual, time-consuming, and imprecise labor, which limits throughput and may result in interobserver variability. Here, we present micronuclAI, a novel pipeline for automated and reliable quantification of micronuclei of varying size, morphology and location from nuclei-stained images. The pipeline

was evaluated against manual single-cell level counts by experts as well as against routinely used micronuclei ratio within the complete image. The classifier was able to achieve a weighted F1 score of 0.937 on the test dataset and the complete pipeline can achieve close to human-level performance on various datasets derived from multiple human and murine cancer cell lines. The pipeline achieved an R^2 of 0.87 and a Pearson's correlation of 0.938 on images obtained at 10X magnification. We also tested the approach on a publicly available image data set (obtained at 100X) and achieved an R^2 of 0.90, and a Pearson's correlation of 0.951. By identifying single cell associated biomarkers, our approach enhances accuracy while reducing the labor-intensive nature of current methodologies. Additionally, we provide a GUI-implementation for easy access and utilization of the pipeline.

Spatial transcriptomic analysis elucidates tissue patterns associated with PARP inhibitor resistance in high grade serous ovarian cancer patients **084**

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High grade serous ovarian cancer (HGSOC) is an aggressive disease, characterized by mutations imposing DNA repair deficiency, delayed diagnosis, and poor prognosis. In addition to being the most lethal subtype, HGSOC is also the most common subtype of ovarian cancer. Despite significant advancements in clinical targeting of HGSOC, particularly with PARP inhibitors (PARPi), therapy resistance in patients remains a major challenge. Tumor microenvironment (TME) profiles have been associated with patient prognosis, but tissue organizational patterns underlying disease resistance are not yet fully understood. In this study, we propose the use of spatial transcriptomics (ST) to delineate simultaneous spatial and transcriptional features of treatment-resistant HSGOC tumors. Utilizing the recently released Xenium 5K Plex platform from 10X Genomics, we quantified the expression of 5000 genes in over 2.5 million cells from matched longitudinal samples taken at the time of diagnosis and following PARPi for three HGSOC patients. Overall tissue characterization between timepoints showed that post-PARPi samples harbored fewer cancer cells but more exhausted immune cells when compared to diagnosis tumor samples. Evaluation of the transcriptomic profiles within cancer cells proximal or distal to immune cell populations highlighted genes involved in immune-cancer interfaces, which also varied according to treatment status. Harmonizing both the clinical and spatial aspects of our dataset permitted unique insights regarding tumor ecosystem evolution under therapeutic selective pressure. Our results emphasize the utility of ST to characterize features of resistant disease in clinical samples. Expanding this approach to a larger cohort could further uncover general mechanisms of disease resistance, enhancing our understanding and guiding future therapeutic strategies.

085 TCMR-induced epithelial injury patterns in kidney transplants

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Background: Acute T-cell mediated rejection (TCMR) is a major challenge after kidney transplantation, posing risks to long-term allograft outcomes. Previous research highlighted the critical role of TCMR-induced renal epithelial injury, but the cellular origins and associated gene expression profiles remain poorly understood.
Methods: To study the molecular changes, we used C57BL/6 and BALB/c mouse models for allogeneic kidney transplantation and syngeneic controls. We analyzed renal gene expression during TCMR using single nucleus RNA sequencing (snRNA-seq) and spatial transcriptomics on allogeneic and syngeneic kidneys 7 days post-transplant. Differentially expressed genes and a gene set predictive of allograft outcomes were investigated per cell type, and results were compared to snRNA-seq data from human TCMR kidney biopsies and stable allografts without rejection.
Results: Mouse kidneys from allogeneic transplantation showed all histological hallmarks of TCMR. SnRNA-seq revealed a strong gene expression response, especially in C57BL/6 kidneys transplanted into BALB/c mice. These responses were most pronounced in kidney epithelial cells, particularly in the proximal tubules (PT) and thick ascending limbs (TAL), inducing distinct injury-associated cell states. Spatial transcriptomics identified a heterogeneous spatial distribution of these cell states between cortex and medulla. Published genes indicative of allograft outcome were mostly expressed in injured PT and TAL but showed heterogeneous differential expression in the different injured PT and TAL cell states. Cross-species analysis revealed a substantial overlap of differential gene expression and injured epithelial cell states between mouse and human TCMR.
Conclusion: Our study offers a detailed exploration of cell type-specific gene expression changes during TCMR in human and mice. The analysis of allograft outcome-associated ge-

nes revealed their origin from various injured epithelial cell states. This insight may help in identifying injured cell states most responsible for reduced graft function, potentially enabling targeted therapeutic interventions.

InterScale: Towards understanding long-range interactions in spatial transcriptomics 086

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Spatial transcriptomics has revolutionized our understanding of the complex spatial gene expression patterns within tissues. Leveraging information about local spatial neighborhoods, current cell-cell communication methods have gone beyond ligand-receptor analysis, which was the standard interaction analysis for dissociated datasets. However, these models fail to consider long-range interactions in heterogeneous niches because extending interactions beyond a local neighborhood often leads to over-smoothing. Here, we present InterScale, a cell communication model that considers local neighborhood graphs and global attention. The InterScale framework consists of a graph neural network followed by a transformer. The first module learns cell interactions in a short range, while the second module goes beyond and learns long-range relationships. We tested the model using high-resolution spatial molecular profiling data from the human pancreas, brain, and lung. We recovered cell-cell interaction signatures at local and long scales known to underlie critical cellular processes across conditions, tissues, and technologies.

087 Chemotherapy induces myeloid-driven spatial T-cell exhaustion in ovarian cancer

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To uncover the intricate, chemotherapy-induced spatiotemporal remodeling of the tumor microenvironment, we conducted integrative spatial and molecular characterization of 117 high-grade serous ovarian cancer (HGSC) samples collected before and after chemotherapy. Using single-cell and spatial analyses, we identify increasingly versatile immune cell states, which form spatiotemporally dynamic microcommunities. We describe Myelonets—networks of interconnected myeloid cells—as a previously unrecognized functional compartment within the spatial tumor ecosystem. We demonstrate that chemotherapy triggers spatial redistribution and exhaustion of CD8+ T cells due to prolonged antigen presentation by macrophages, which associates with poor responses to chemotherapy. Single-cell and spatial transcriptomics identifies prominent myeloid-T-cell interaction programs induced by chemotherapy. Using a functional patient-derived immuno-oncology platform, we show that CD8+T-cell activity can be boosted by immune checkpoint blockade therapies administered after chemotherapy. Our discovery of the clinically relevant myeloid-driven spatial T-cell exhaustion paves the way for novel immunotherapeutic strategies to unleash CD8+ T-cell-mediated anti-tumor immunity in HGSC.

Spatial cell cycle phenotypic variations among clinical subgroups post-neoadjuvant chemotherapy in ovarian cancer

088

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Introduction

High-grade serous carcinoma (HGSC) is the most common and lethal subtype of ovarian cancer., exhibiting a 70% relapse rate. Neoadjuvant chemotherapy (NACT) is a well-established treatment modality for advanced-stage patients with HGSC. However, eventual relapse and platinum resistance remain major clinical challenges. The complex interplay of multiple biological processes, like cell cycle progression, DNA repair, and immune surveillance is known to have an impact on disease progression. Therefore, exploring the spatial roles of cell cycle regulation in patients undergoing neoadjuvant NACT and its interactions with the tumor microenvironment is crucial to improve current clinical standards.

Materials and methods

We prospectively recruited 24 newly diagnosed patients to the Oncosys-OVA clinical trial (NCT06117384). We performed BRCA1/2 mutational profiling and a genomic scarring (ovaHRDscar) to annotate the HGSCs to distinct genomic groups. Detailed surgical and clinical data were collected to assess chemotherapy responses, while histological examination using H&E staining annotated the morphological response patterns of HGSC. We employed a single-cell spatial profiling technology, tissue cyclic immunofluorescence (tCycIF), on FFPE samples collected from the same cohort. t- CycIF is a multiplex imaging method, detecting up to 80 antigens from one sample, while preserving the tissue architecture. We built and validated a tCycIF panel with antibodies targeting cell cycle-regulators and checkpoint inhibitors (Cyclin A, B, D, E, pRb, p21, p27), proliferation (Ki-67, PCNA) and DNA damage markers (γH2AX), alongside cell-type markers for distinct cell populations. We utilized GeoMx spatial transcriptomics to capture spatially resolved and cell-population deconvoluted gene expression profiles within regions of interest from tCycIF spatial analyses.

Results and discussion

We employed tCycIF utilizing a validated custom-made antibody panel targeting up to 50 markers to characterize the cell populations in the TME, their functional states, cell cycle profiles and DNA damage patterns. Via advanced image analysis, we revealed the spatial topographies of cellular communities, and cell-cell interactions within the TME. Through computational methods, we reconstructed the cell cycle dynamics and assessed the expression of markers in cells in pseudotime. Furthermore, by conducting a multivariate assessment

of the proliferation markers, we were able to assess spatial distribution and neighborhoods of cells with distinct proliferative characteristics. To explore the transcriptome of these regions, we developed a pipeline that integrates tCycIF and GeoMx imaging data, enabling the precise targeting of cell populations in adjacent slides. These analyses were integrated with the matching clinical, pathological, and genomic profiling information, leading to the identification of recurring cellular neighborhoods with potential therapeutic implications.

Conclusion

By integrating multi-omics and spatio-temporal analyses with clinicopathological features, we identified response-related patterns that could inform patient stratification and treatment selection. This study has the potential to drive advancements in immuno-oncology and personalized therapeutic approaches for patients with HGSC.

089 Deep learning-based mapping of intact murine kidneys: clearing the way for virtual organs

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Glomeruli are the primary structures responsible for kidney filtration and thereby organ function. Understanding the patterns of glomerular loss at the organ level, which ultimately poses the risk for kidney failure, remains a challenge. A major reason for this is the lack of an automated method that provides accurate and reliable glomerular segmentation on large-scale 3D datasets.

We have access to multiple datasets of optically cleared kidneys, which were imaged using light sheet microscopy. The renal vasculature was fluorescently labelled. To obtain an organ-wide glomerular map, we introduced GlomPose, a Cellpose-based 3D segmentation tool for glomeruli with domain-specific post-processing, yielding an average dice score of 95% on manually annotated patches. This allows us to quantify the size and shape of glomeruli, as well as to analyze their distribution throughout the kidney and the glomerular composition of specific anatomical regions. Furthermore, we have shown that optical clearing is reversible and compatible with subsequent complementary analyses, including electron microscopy and indirect immunofluorescence. Thus, we propose that our 3D kidney atlases can be utilized as a guide for region-of-interest selection that links organism-level models of kidney function to molecular and ultrastructural data, paving the way to the construction of

multidimensional virtual organs.

Influence of the Lymphoma Immune Microenvironment on Therapy Resistance vs. Cure in Diffuse Large B-Cell Lymphoma 090

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BACKGROUND: Biological heterogeneity in diffuse large B cell lymphoma (DLBCL) arises not only from cell-of-origin subtypes and related genomic alterations but also from the variety of cell types and their functional states within the tumor microenvironment (TME). Despite the use of poly-immunochemotherapy, patient outcomes vary and the reasons of treatment failure often remain unclear. The impact of different cell types and their spatial organization on therapy response remain poorly understood, with significant knowledge gaps in niche-specific drivers of lymphomagenesis.
OBJECTIVES: To create a single-cell multiomics tissue atlas of the DLBCL TME in cured and relapsed/refractory (R/R) patients at initial diagnosis. Characterization of stromal and immune cells, their functional states, cell-cell interactions, cellular neighborhoods, gene expression profiles, and mutational signatures. Integrative analysis to uncover complex relationships within the TME, identify predictive biomarkers and potential new therapeutic targets.
METHODS: Tissues from 80 cured and 80 R/R DLBCL cases are analyzed by CODEX highly multiplexed microscopy, RNAseq and NGS. The CODEX antibody panel includes markers for immune, tumor, stromal and vascular cell types and their functional states. TME characteristics will be compared between therapy responders and non-responders and correlated to clinical data and molecular DLBCL subtypes.
RESULTS: R/R patients have higher clinical risk scores (p=0.0027) and age (p=0.021) and the non-GCB subtype is more frequent in this group, whereas sex distribution is similar between both groups. Median follow-up is 78 months for cured patients and time to relapse is 12 months. We imaged ~4,33 million cells by CODEX and identified various cell types, T cell subsets, dendritic cells, macrophages, myeloid cells, NK cells, tumor cells, stromal cells and vasculature. Cells were grouped into distinct cellular neighborhoods: Tumor-cell enriched with few immune cells, tumor-cell enriched with a significant presence of macrophages and dendritic cells, tumor-cell poor, and extracellular matrix-rich (preliminary data).

091 SPACEc: A Streamlined, Interactive Python Workflow for Multiplexed Image Processing and Analysis

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Multiplexed imaging technologies offer profound insights into complex tissue architectures. However, analyzing the generated data presents challenges, including software fragmentation, cumbersome data handoffs, inefficiencies in processing large images, and limited spatial analysis capabilities. To address these issues, we developed SPACEc, a scalable end-to-end Python solution. SPACEc handles image extraction, cell segmentation, and data preprocessing and incorporates machine-learning-enabled, multi-scaled, spatial analysis. We designed the workflow to be user-friendly and interactive, enabling experimental and computational scientists to collaborate effectively during the multistep analysis. We envision that this enhances transparency and accessibility of data analysis, promoting reproducibility. SPACEc efficiently manages the analysis of extensive imaging data (~8,000,000 cells) and provides a comprehensive workflow for cross-condition comparisons. It is scalable with GPU acceleration and meets the growing demand for efficient tools usable by non-experts, as the commercialization of multiplexed imaging technology attracts new entrants into the spatial-omics realm. SPACEc offers interactive annotation and inspection features and is compatible with scVerse, interfacing with various pre-existing libraries. This ensures that SPACEc remains a valuable asset for researchers navigating the increasing complexities of spatially resolved multiplexed imaging data analysis.

Mapping Molecular Subtypes in HNSCC: From Bulk RNA to Spatial Data

092

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Head and Neck Squamous Cell Carcinomas (HNSCC) arise from different anatomical locations in the upper aerodigestive tract and exhibit significant heterogeneity. To elucidate the complexity of this heterogeneous malignancy Walter et al. introduced a microarray- based molecular subtyping method that classified samples into four subtypes, with each subtype having its own distinct molecular profile. This subtyping has been effectively applied in several clinical cohorts to predict prognosis and stratify the patients into uniform groups. However, conflicting results from different studies suggest that a single subtype may not be indicative of the whole tumor, but rather a tumor contains hybrid subtypes and cannot be clearly categorized.

The aim of this study is to analyze these subtypes in spatial transcriptomics data and determine how much it would resemble the bulk RNA results. We hypothesize that HNSCC may constitute different subtypes rather than a single one and the ratio of the different subtypes may be helpful for better molecular understanding of the tumor behavior. Using bulk transcriptome data from 13 different HNSCC untreated xenograft models, we observed that only two models were consistently aligned to a specific subtype, whereas the other models across samples showed diversity. Currently, we are investigating spatial transcriptomic data from 7 of these HNSCC xenograft models to generate a spatial subtype pattern to compare with the bulk transcriptome data. This comparison presents a novel opportunity to refine the original Walter et al. algorithm by connecting it to tumor microenvironment for a more precise classification of tumors into subtypes.

After finalizing the spatial data pipeline, we plan to integrate these xenograft data with ra-

diomic signatures and histopathology microscopic data of these tumors to gain a better molecular understanding of the intertumoral complexity and ultimately be able to generate a translatable, applicable subtype-specific molecular profile to test in clinical HNSCC samples.

093 Standard processing and analysis of spatial Xenium in situ sequencing data with the nf-core workflow spatialxe

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Spatial omics technologies represent a transformative approach in biological research, enabling the comprehensive analysis of molecular profiles within their native spatial context. By preserving the spatial relationships between cells, spatial omics technologies, such as 10x Xenium, offer critical insights into tissue architecture, cellular heterogeneity, and the microenvironment's role in a healthy and disease context. As there is an increase in demand for understanding spatial patterns to study diseases, there is a need for standardized and reproducible workflows. The nf-core community thus presents spatialxe, a blueprint for the analysis of Xenium data. Spatialxe supports featured benchmarked tools, such as Xenium Ranger. It generates a spatial object data that includes the cell feature matrix that can be used for further downstream analysis. We would also implement a number of segmentation algorithms like Cellpose, Baysor and QuPath for image annotation. Spatialxe will be an extensive pipeline to cover not only standard processing but also single cell and spatial omics quality control, conversion of the data to be SpatialData ready, and automated image annotation. The workflow will be deployed within the German Human Genome-Phenome Archive (GHGA - www.ghga.de) as the default analysis workflow for incoming Xenium data. The standardization as well as the reproducibility of the Nextflow/nf-core pipelines combined with the infrastructure for FAIR omics data usage and ethico-legal framework offered by GHGA will enable cross project analysis and hence promote new collaborations and research projects.

094 Spatial transcriptomics reveals mechanisms of degeneration and regeneration in multiple sclerosis

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Background/Objectives: In multiple sclerosis (MS), various lesion types are found across the central nervous system, with varying potential for demyelination, remyelination and scar formation. Mixed active/inactive (mA/I) lesions with foamy microglia correlate with neuro-axonal damage while those with ramified microglia do not, suggesting biological differences. Investigating these differences may elucidate critical mechanisms driving MS lesion progression and repair.
Methods: Postmortem tissue of n=8 MS donors and n=3 control donors was provided by the Netherlands Brain Bank. We performed high-resolution spatial transcriptomics and immunohistochemistry analyses, focusing on the demyelinated center, demyelinating border and the peri-lesion border of mA/I lesions with ramified microglia to those with foamy microglia within the same donors.
Results: Stereo-seq and IHC results at the single-cell and bin50 level showed that lesion border and demyelinated center had significantly different cellular composition as compared to the peri- lesion region. Unsupervised clustering and cell-bin pseudo bulk analysis identified distinct gene expression patterns. Gene expression of mA/I lesions with foamy microglia indicated higher levels of ongoing tissue damage, demyelination, complement activation, iron metabolism, reactive astrocytes, and immunoglobulin production in the centre, border, and peri-lesion region. In contrast, mA/I with ramified microglia showed higher levels of neurite outgrowth, stability of myelin, and lipid export in the border and peri-lesion.
Conclusion:
mA/I lesions with ramified microglia may be more prone to remyelination, while those with foamy microglia may be more prone to lesion expansion. This study sheds light on the molecular mechanisms driving MS lesion dynamics and identifies potential targets for therapeutic interventions.

Quantitative analysis of CODEX multiplexed histological imaging data in the tumor microenvironment **095**

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The tumor microenvironment (TME) is a complex biological niche populated by tumor cells, stromal cells and immune cells. A better quantitative understanding and characterization of this environment and its heterogeneity between patients is of vital importance in developing effective treatment strategies. The CODEX multiplexed histology platform allows for the simultaneous measurement of more than 40 fluorescent markers on a single tissue section by fully automated staining and imaging cycles, but systematic analysis of the resulting

large data sets is still challenging. Here, we describe a quantitative, modular open-source work-flow for processing data from the CODEX platform. Our work-flow addresses several challenges in currently available data analysis protocols such as background subtraction, detection of staining artifacts and treatment of structured noise, overall leading to more robust single-cell features. Downstream custom semi-automated unsupervised classification yields reproducible cell-type annotations. We expect that the resulting spatial information will contribute to the quantitative characterization of spatial tissue architectures and to the design of data-driven mathematical models of immune-cell dynamics in the TME.

096 Multiomic profiling of healthy and diseased brains with high-plex single-cell spatial molecular imaging

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Single-cell transcriptomics and proteomics can provide complementary information about the form and function of neurons and glia throughout the brain. However, most high-plex spatial analyses to date have primarily utilized one of these two modalities to interrogate cell activity and cell-to-cell communication. Here, we simultaneously leveraged the detection of 68 proteins and over 6,000 RNA targets on the same formalin-fixed paraffin-embedded (FFPE) human brain sections to perform extended segmentation of neural processes and integrated analyses of protein and RNA expression.

Using a multiomic approach with the CosMx™ Spatial Molecular Imager (SMI), first high-plex protein panel targets were imaged via cyclic in situ hybridization chemistry. Next RNA targets on the same tissue section were exposed then hybridized, and finally RNAs are imaged using the same chemistry. The human neuro protein panel targets are particularly well-suited for dissecting neurodegenerative disease pathology, including various phospho-tau species and amyloid beta variants. Moreover, the protein panel includes markers for diverse neural cell types and enables robust cell typing, especially alongside the >4,900 neuroscience-related genes covered by the Human 6K Discovery Panel. RNA targets focus on >80 pathways, cell typing, and key ligand-receptor interactions. To demonstrate the capability of the single-cell high-plex multiomic technique, we collected data from sections of FFPE male human brains, with samples derived from frontal, parietal, and occipital lobes, as well as the precentral/ postcentral gyri and cerebellum, of healthy individuals and Alzheimer's Disease patients.

Drawing on both the protein and RNA data, we achieved unparalleled segmentation of neurons and glia and increased transcript counts per cell. We also annotated cells with neuronal, glial, and vascular subtypes. By comparing RNA and protein expression, we identified genes and proteins with correlated and divergent patterns across our tissue space, highlighting the advantage of including the functional readout, protein, in understanding cell activity. Using open-source tools, we assigned cells into niches based on protein patterns

and then applied differential expression models to identify genes and gene sets which varied based on niche for individual cell types. Overall, by applying a multiomic approach to human brain samples, we were able to simultaneously probe cell shapes, cell types, cell neighborhoods, and cell activity in one experiment on a single slide.

Chemokine receptor expression in health and disease 097

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Background: Chemokines orchestrate multiple aspects across the lifespan of human B lymphocytes, including migration, development, and activation. The expression of chemokine receptors (CKRs) is tightly regulated throughout B cell development, modulating the cellular response to chemokine signals. In autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), the migration of B cells into affected tissues represents a critical step in disease progression. While the expression of key CKRs (CXCR4, CXCR5, CCR6) has been documented, a comprehensive characterization of CKR expression across B cell subpopulations remains elusive. Moreover, CKR expression patterns are known to vary with age and tissue type, adding another layer of complexity to their study. Elucidating these intricate expression patterns is crucial for understanding B cell ontogeny and homing mechanisms, ultimately providing insights into both normal immune function and pathological conditions.
Objectives: Study CKR expression patterns focussing on B cell (sub)populations across tissues, age-groups in healthy and pathological conditions.
Methods: Spectral flow cytometry analysis (30/31-color panels, 12 CKR targets) of peripheral blood mononuclear cells (PBMCs) of a healthy donor (HD) cohort across age and sex, and PBMCs of diseased/vaccinated patients. CKR expression analysis of HD tissues (cord blood, bone marrow, tonsil, spleen).
Results: The established 30-color panel allows the identification of 12 CKRs on the surface of up to 25 early and mature B cell subpopulations. Moreover, the 31-color panel uncovers CKR expression patterns in major monocyte, T-, natural killer- and dendritic cell subpopulations. The developed (un)supervised analysis pipeline reveals age-, sex- and tissue-dependent differences in CKR expression patterns on immune cell subpopulations.
Conclusions: Our study provides a comprehensive map of CKR expression on major immune cell subpopulations across ages, tissues, and pathological conditions. These results underscore the complexity of immune cell trafficking and form a foundation for future studies investigating functional consequences and potential therapeutic applications.

098 CO-Detection by IndEXing in bone marrow biopsies

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Investigating the microenvironmental architecture of hematological malignancies is critical to identify mechanisms contributing to disease progression, therapy resistance and patient outcome.
This study focuses on immunophenotyping of formalin-fixed paraffin-embedded (FFPE) bone marrow biopsies. In hematological malignancies, patients can suffer from a progressive perturbation of the bone marrow microenvironment, however, there is only little information on a multiparametric analysis of the cellular composition, neighbourhoods and tumor cell heterogeneity present in this particular tissue. We aim to identify the malignant cells and display their survival niches and the immune cell neighbourhood without destruction of their spatial relationship.
The generation of high-quality bone marrow FFPE sections requires decalcification, which influences the tissue durability, causes cavitations and decreases antigenicity. We therefore optimized our CO-Detection by IndEXing (CODEX) pipeline according to these particular challenges. Adjustments regarding antigen retrieval, tissue bleaching as well as antibody selection and titration allowed the establishment of a custom 50-plex antibody panel.
We acquired multicentric sample cohorts to cover the clinical heterogeneity in myelodysplastic syndromes and splenic B-cell lymphomas. We currently investigate the spatial architecture by established bioinformatic pipelines to define cell-cell interactions, distribution patterns and cellular neighborhoods. Additionally, the cellular morphology will be compared to cytological staining in bone marrow smears to account for the distinct dysplastic features.
Correlating our findings to the corresponding clinical data, we assume that the defined immunophenotypes will provide deeper insight into disease pathology, and may uncover cell-interactions or tumor survival niches that impact on disease progression or therapy resistance. Moreover, with the determination of novel biomarker signatures, we aim to improve diagnosis, treatment stratification and potentially identify novel therapeutic approaches.

Mapping Organ-wide Immune Interfaces at Near Single-cell Resolution Using Spatial Transcriptomic Approaches **099**

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Advances in RNA sequencing technologies, such as single-cell RNA sequencing (scRNAseq) and single-nucleus RNA sequencing (snRNAseq), have revolutionized our understanding of cellular diversity in complex tissues. However, these approaches often lack spatial context, making it difficult to map immune responses within tissue structures.
We are developing a mouse atlas that maps organ-wide immune interfaces at near single-cell spatial resolution, with a particular focus on tissue healing processes and infection responses, such as cardio-immune interfaces after myocardial infarction and immune responses during acute brain infection by Toxoplasma gondii. Utilizing spatial transcriptomic technologies like Visium HD and Curio Bioscience Seeker, we explore immune cell interactions within the brain and heart.
In the heart, we are investigating the interaction between immune cells and cardiovascular tissues after myocardial infarction, with a particular focus on tissue repair processes. In the brain, we are examining how acute Toxoplasma infection impacts immune cells, such as T-cells, astroglia, and oligodendrocytes, altering their roles in infection response.
By integrating spatial transcriptomics with single-cell approaches, we aim to gain a more comprehensive organ-wide and spatial understanding of these processes at both the single-cell and transcriptional levels.

GLI-YAP interactions in basal cell carcinoma promote immune evasion by regulating the spatial distribution of immune responses. **100**

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Basal cell carcinoma (BCC) is the most common form of skin cancer. The disease is driven by mutational activation of the Hedgehog (HH)/GLI signaling pathway, leading to uncontrolled cell proliferation and tumor formation in the absence of an effective anti-tumor response despite a remarkably high mutational burden. Therefore, we aimed to elucidate the underlying reasons for HH/GLI-driven immune evasion to improve current treatments and identify novel therapeutic approaches beyond the use of HH/GLI pathway inhibitors. Using GeoMx-based spatial protein profiling of human BCC tissues, we discovered an abundance of Ki-67+ CD68+ macrophages within the intratumoral infiltrates and a striking lack of activated CD8+ T cells. Interestingly, CD8+ T cells were preferentially located in regions distant from the tumor, revealing a characteristic spatial distribution of immune cells in BCC. In silico analysis of single-cell RNA-sequencing data from primary human BCC revealed an inverse correlation between HH/GLI and Hippo/YAP activity in tumor cells with chemokine expression and relative CD8+ T-cell numbers. To mechanistically analyze these findings, we generated 3D organotypic skin models harboring GLI-transformed keratinocytes with or without constitutively active YAP signaling and analyzed their transcriptome and pathway activation profile. In combination with humanized mice harboring skin grafts simulating oncogenic GLI and YAP mutations, we provide evidence for a role of GLI-YAP signaling in regulating the intricate spatial distribution of immune responses within BCC, highlighting the role of these oncogenic signals in shaping the tumor immune microenvironment and shedding light on potential new targets for rational combination immunotherapy in BCC.

101 Deciphering tissue niches of ILCs in murine lung and small intestine using multiplexed microscopy in health and inflammation

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ILCs are rare immune cells owning various roles in processes controlling tissue homeostasis, barrier integrity, autoimmunity, and pathogen defense. Although being scarce in numbers, they are potent tissue sensors and cytokine secretors. The subgroups ILC1s, ILC2s, and ILC3s are the innate counterpart of T helper (Th) 1, Th2, and Th17 cells, respectively, but unlike T cells, ILC do not express a T cell receptor (TCR). The similarities to T cells and different subtypes with additional markers represent some of the challenges faced when studying ILCs in tissue context. Here, we aim to identify ILC subtypes in murine lung and SI tissue as well as changes in their microenvironment during inflammatory processes.
We combined multi epitope ligand cartography (MELC) and a Type 2 systemic inflammation model based on consecutive IL-33 injections. A semi-automated image analysis workflow was used to segment nuclei and cells, and extract single cell information with spatial information from 30-plex MELC data in murine lung and SI. Dimensionality reduction, cluster analysis and spatial co-enrichment analysis was adapted and optimized for the needs of MELC data.
This approach enabled to identify ILC subtypes, namely ILC2s, ILC1s/NK cells, and ILC3s, as well as major immune, epithelial and endothelial cell types in both organs. Lung ILC2s clustered in tissue niches shared with LYVE-1$^+$ CD90.2$^+$ lymphatic endothelial cells and myeloid cells, while NK cells/ILC1s rather localized in parenchymal tissue areas associated with EMCN$^+$ CD31$^+$ blood endothelial cells in the lungs. Interestingly, we observed intestinal NK cells/ILC1s/ILC3s that were predominantly present in basal regions of the villi and crypts under healthy conditions shifting towards the luminal site of the villi after induction of inflammation by IL-33.
In this study, ILC subtypes were resolved for the first time in MELC data from murine lung and SI tissue and we determined different predominant tissue niches of ILC subtypes in both organs which represents the basis for further investigations linking localization and functional behavior.

Visual Analysis of Cell-Cell Interactions in 2D & 3D Cancer Imaging Data **102**

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Studying cell-cell interaction patterns in human tissue has garnered significant interest in cancer research due to its potential to advance our understanding of immune responses and to develop new therapeutics. Novel imaging technologies have enabled computer-aided analysis of biological tissue at scale. However, as such spatial analysis is initially exploratory in nature, human intervention and interpretation is essential. We designed and evaluated two visual analytics tools that combine automated methods with visual inter-

faces, integrating biomedical experts in the analysis loop. With Visinity (1) we developed an approach that infers interaction based on the proximity of cells in large 2D multi-channel immunofluorescence images. We compute range-query based neighborhood vectors and allow users to interactively detect and search patterns of similar cellular neighborhoods in and across specimens with millions of cells. By contrast, our newest approach, Cell2Cell (2), analyzes cell-cell interactions on a smaller scale but in high-resolution 3D data. We trace and compare protein intensity distributions between cells and integrate these profiles into a multi-volume viewer. Evaluation showed that our approaches are capable of confirming known biological patterns and assisting in cutting-edge cancer research.

103 Mapping Neurotoxicity in the Brain Using Spatial Transcriptomics

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The intricate nature of the brain and the difficulties in understanding the fundamental processes make neurotoxicity a major obstacle in the development of central nervous system (CNS) -targeting drugs. Spatial transcriptomics (ST) presents new opportunities for elucidating these complex molecular pathways. In this study, we investigated changes in gene expression in the brain of mice exposed to CNS-targeting drugs known for their neurotoxic properties, compared to control groups. Different dosages of these compounds were given to mice, and neurotoxicity was assessed by histological analysis, neurofilament light chain (NfL) measurements, and ST. Neurotoxicity was histologically identified as Purkinje cell necrosis, confirmed by Calbindin immunohistochemistry (IHC). NfL levels were shown to be highly linked with the degree of neuronal necrosis detected. The ST analysis detected significant modifications in pathways associated with inflammation, brain function, and other important biological processes. These results highlight the capacity of ST to detect important indicators of neurotoxicity, therefore enhancing our understanding of how the CNS reacts to therapeutic treatments. High-resolution gene expression mapping provided by ST allows for the identification of region-specific alterations that may be overlooked using traditional approaches. This study highlights the significance of ST in neurotoxicological research and its potential as a powerful tool for advancing CNS drug safety, with future work needed to fully elucidate the broader implications.

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Immunoglobulin A (IgA) plays a crucial role in maintaining the gut barrier. By binding to gut microbiota, IgA helps regulate microbial diversity and influences host-microbe interactions. A deficiency in IgA has been linked to changes in the gut epithelium transcriptome and microbial composition, which, in turn, impacts plasma cell dynamics in the gut. We hypothesized that plasma cells adapt to environmental signals and exhibit distinct positional and phenotypic features along the villus-crypt axis, shaped by IgA-epithelium-microbiota interactions. To investigate this, we developed a deep learning model (nnUNet) capable of accurately segmenting key anatomical regions of the intestine, such as the lamina propria, epithelium, and muscularis. Additionally, we implemented multiplex immunostaining targeting specific markers of interest and used a pre-trained Cellpose model to accurately segment IgA-producing plasma cells. By integrating predictions from the nnUNet model, particularly of the lamina propria, with cell segmentation tools, we were able to perform detailed analyses of plasma cell characteristics within this region.

Our findings revealed that plasma cells display considerable variability in IgA content, suggesting varying rates of production and secretion. These variations were confirmed using additional techniques such as ELISpot and metabolic labeling. Furthermore, gnotobiotic mice exhibited significant differences in plasma cell behavior compared to SPF mice, with lower plasma cell frequencies and a preference for localization within crypt areas. These differences are most likely driven by epithelial cell signals, such as CCL28 and pIgR expression, which are reduced in germ-free mice.

Our image analysis pipeline provides a robust framework for integrating data from H&E staining with multiplex immunohistochemistry. This approach has enabled us to develop a more comprehensive understanding of the IgA system and its function, linking IgA to both intestinal epithelial cells and the immune compartment, and advancing our knowledge of the complex interplay between these components.

Spatial transcriptomics applied to the human choriodecidua 105

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The molecular mechanisms that trigger parturition in humans remain poorly understood, but gene expression is altered during labor primarily in the choriodecidua, a tissue composed of the maternal decidua (uterine lining) and the fetal chorion (outermost layer of membranes). This tissue is a mixture of maternal stromal and immune cells and fetal cells (primarily trophoblasts). We applied 10X Genomics Visium technology to human choriode-

ciduas collected between 37 and 42 weeks of gestation in two groups, one with labor and vaginal deliveries and the other with planned prelabor cesarean deliveries –two samples per group– to obtain a spatial overview of genome-wide gene expression with and without labor.

We applied a workflow composed of raw data normalization, dimensionality reduction, integration of data from the four samples and clustering steps. We applied this workflow recursively as many times as the size of each of the obtained clusters enable the integration step. At each round of sub-clustering, we evaluated multiple parameter values for all the steps and several clustering algorithms. Among the hundreds of evaluated parameter values combinations, we selected the best clustering based on the mean silhouette score and the size of the smallest cluster.

Despite the challenge of the tissue thinness and its multilayered organization with respect to the Visium spatial resolution, we were able to finely define spatial clusters whose transcriptomic profiles reflected the diversity of expected cell types. For example, we characterized four clusters of trophoblasts based on the regional expression of specific genes. This detailed characterization of tissue substructure will allow better exploration of spatially differentially expressed genes with and without labor. Furthermore, using a single nucleus RNA-seq dataset of this tissue, we will further characterize the cell types driving gene expression differences between clusters using reference-based spatial transcriptomics deconvolution methods.

106 NAD(P)H-FLIM and PHIP-based NMR identify metabolic alterations in human lymphoma cells after inhibition of NAD+ restoration

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The co-enzymes NAD(P)+/NAD(P)H are crucial for cellular metabolism and pathogen defense. Since the oxidized forms are autofluorescent and their fluorescence lifetime change with the enzyme to which they bind to, they can serve as markers to monitor these life-sustaining mechanisms, in a label-free manner and in vivo by fluorescence lifetime microscopy (FLIM). However, interpreting NAD(P)H-FLIM data is challenging due to the low two-photon action cross-sections and debated enzyme-bound fluorescence lifetimes (Sharick 2018, Blacker 2019, Chacko 2019, Leben 2019, Ranjit 2019).

Here we performed NAD(P)H-FLIM on human lymphoma cells using two-photon microscopy and analyzed the data with our vector algebra algorithm (Leben 2019). In brief, the measured fluorescence lifetimes of key NAD(P)H-dependent enzymes in solution were used to benchmark the universal circle in the phasor plot. Our algorithm assigned each pixel in the FLIM image to specific enzymes, creating maps of enzyme distribution and metabolic activity. Due to similar lifetimes, we categorized enzyme-bound NAD(P)H into four groups: unbound NAD(P)H, LDH-like, PDH-like, and NOX-bound, helping differentiate between metabolic inactivity, anaerobic glycolysis, oxidative phosphorylation (OxPhos), and oxidative burst/stress.
We challenged lymphoma cells by inhibiting the NAD+ salvage pathway, essential for regenerating NAD+. Our NAD(P)H-FLIM data showed a significant increase in NAD(P)H activity, indirectly indicating a reduced pool of freely available NAD(P)H. Enzyme maps revealed a drastic reduction in NADH bound to the LDH group, suggesting decreased anaerobic glycolysis with constant OxPhos, supported by the unchanged PDH-group binding. Additionally, NOX activation significantly increased post-intervention.
Our findings, confirmed by PHIP-based NMR (Hövener 2018), underscore the efficacy of our NAD(P)H-FLIM evaluation framework in capturing metabolic complexity in living cells and tissues.

Developing novel spatial technologies for the detection of breast cancer metastases in the murine lung **107**

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Breast cancer affects 1 in 7 women, and the risk of death from metastatic (stage 4) disease remains high. In recent years, chemotherapy and mastectomy have improved the overall survival of breast cancer patients and reduced the incidence of breast cancer in at-risk individuals. However, these treatments are non-specific, and do not eliminate the risk of cancer development, patient relapse, or progression to advanced metastatic disease. Understanding the interactions between heterogeneous lesions and the blood vessels that facilitate their spread, will enable better characterisation of these metastasis-initiating cells. New methodologies and technologies are required to facilitate such discoveries and are rapidly developing in the fields of microscopy, spatial omics and cancer. Here, we have develo-

ped two novel protocols for the detection of cancer clones in a murine model of metastatic breast cancer. First, we use light-sheet microscopy and optical barcoding to locate individual clones and blood vessels within whole mount organs. Next, we use MERSCOPE technology (Vizgen) to detect clones that are labelled with genetic barcodes, in their original spatial context. Here, we reveal the value of spatial information for insight into the behaviour of aggressive breast cancer clones.

108 Three-dimensional spatial analysis throughout the complete bone marrow at subcellular resolution

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A heterogeneous architecture with distinct cellular niches is characteristic of the bone marrow, which serves both as site of hematopoiesis, but also maintains immunological memory in specialized niches. After bone injury, immune cells infiltrate into the fracture gap to fulfill their role within the healing cascade. Myeloid cells are of particular interest, as they have been shown to promote the growth of osteogenic type H vessels.

Here, we aim to investigate the organization of myeloid cell types within the vascular network in whole mouse femurs both in steady state and after injury. It is a challenge to image whole bones due to their physical properties. We developed a tissue clearing, staining and 3D-imaging pipeline called MarShie, optimized to image the entire intact femur at subcellular resolution down to the deepest bone marrow regions. To analyze the three-dimensional dataset, we apply a machine learning approach (LABKIT) enabling us to segment thousands of cells. We show that in aged mice the draining sinus massively decreases in volume while transcortical vessels also decline. We find that during homeostasis CX_3CR1^+ myeloid cells localize in perivascular niches, whereas $CD169^+$ myeloid cells are dispersed in the parenchyma. After injury, CX_3CR1^+ myeloid cells relocate and sequester the injury site prior to vascularization. Analysis of the femur after full osteotomy reveals that vessel sprouting is initiated at periosteal regions. Combining 3D imaging with machine learning will help us in future to characterize tissue niches and patterns of cellular interactions, which are hard to study on small tissue sections in 2D.

109 Dual Spatial Host-Bacterial Gene Expression: Unveiling Bacterial Pathogenesis and Regulatory Mechanisms of Virulence Factors in Tissues

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Co-localization of spatial transcriptome information of host and pathogen can revolutionize our understanding of microbial pathogenesis, such as mycobacterial infection. Here, we aimed to demonstrate that customized bacterial probes can be used to simultaneously identify host-pathogen interactions in formalin-fixed-paraffin-embedded (FFPE) tissues by probe-based spatial transcriptomics technology (10x Visum).
We performed the spatial gene expression of selected bacterial transcripts (2 targets) with the whole host transcriptomic profile (18k mouse gene) in murine lung tissue chronically infected with Mycobacterium abscessus embedded in agar beads and uninfected mice.
Histological characterization showed that infected/inflamed tissues formed by granuloma-like structures were confirmed by the analysis of spatial transcriptional profiles, distinct from host profiles in surrounding areas. The customized probes allowed for mapping of the spatial distribution of bacteria, revealing a complex landscape within the lungs. Customized mycobacterial probes were designed for the constitutively expressed rpoB gene (an RNA polymerase β subunit) and the virulence factor precursor lsr2, modulated by oxidative stress.
Customized mycobacterial probes were designed for the constitutively expressed rpoB gene (an RNA polymerase β subunit) and the virulence factor precursor lsr2, modulated by oxidative stress. We found a correlation between the rpoB expression, bacterial abundance in the airways, and an increased expression of lsr2 virulence factor in lung tissue with high oxidative stress.
Additionally, the application of a deconvolution step reinforced these observations, revealing how host transcriptomics clusters in bacteria-positive and -negative regions were correlated with distinct cellular compositions. Based on these results, we are now developing a new bacterial panel of over 180 probes and targeting more than 50 mycobacterial transcripts.
Overall, we demonstrate the potential of dual bacterial and host gene expression assay in FFPE tissues, paving the way for the simultaneous detection of host and bacterial transcriptomes in pathological tissues.

Integrating spatial transcriptomics and immune repertoires based on multi-graph clustering to elucidate intra- and intercellular signalling **110**

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Understanding the role of adaptive immune cells, particularly T and B lymphocytes, is cru-

cial for advancing treatments for diseases such as cancer, autoimmune disorders, and for improving vaccination strategies. Recent biotechnological advancements enable the spatial mapping of T and/or B cell receptor sequences (VDJ sequences) within tissues, offering insights into spatially resolving clonal diversity with respect to tissue morphology and gene expression. Existing computational tools, primarily designed for single-cell or bulk VDJ sequencing, are inadequate for spatial immune profiling. This highlights the need for new methodologies to comprehensively explore the relationship between antigen receptors, cellular patterns, and signalling pathways.

We propose a strategy to elucidate immune-related intra- and intercellular signalling relationships at spatial resolution, leveraging both spatial and sequence-based similarities inherent in the B and T cell repertoires. We first perform cell deconvolution to obtain the proportion of cell types at each spot, followed by estimating the conditional expected gene expression. We construct a gene co-expression graph from the gene expression profiles of specific cell types, and generate T-cell and B-cell clonal graphs using the similarities in their respective latent space. The latent spaces are learned by Variational Graph Auto-Encoders, from an adjacency (based on spatial similarity) and a feature matrix (based on VDJ sequences). We next identify clone/gene clusters on individual graphs, leveraging within- and cross-graph dependencies, thereby unravelling both intracellular signalling (within-graph relationships) and intercellular communications (cross-graph relationships).

We showcase our approach on spatial B and T cell VDJ sequencing and transcriptomics data from tonsil sections of a healthy subject and breast cancer sections from 2 patients. Compared to general multi-omics integration methods, our approach not only validates known immune interactions but also uncovers potential cellular interactions, promising insights into immune dysregulation and facilitating the exploitation of antigen-specific clones for personalised therapies.

111 Spatial Transcriptomics analysis of Ewing Sarcoma Cell Plasticity and Tumor Microenvironment

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Ewing sarcoma is a highly aggressive fusion-driven cancer of childhood and adolescence that can arise in both bone and soft tissue.

The sarcoma tumor microenvironment (TME) is composed of diverse cell types from the mesenchymal and hematopoietic lineages. Complex interactions between tumor cells and their TME are believed to shape the TME structure and the tumor cell plasticity. However, the TME of Ewing sarcoma remains unexplored due to limited accessibility to patient samples

and the constraints of contemporary staining methods.
In this study, we chart the TME and study the tumor cell plasticity in Ewing sarcoma at single-cell and spatial resolutions.
We first generated a single-cell dataset from four archived patient samples using scFFPEseq for initial exploration and observed heterogeneity in tumor cells.
With the single-cell dataset, we designed a panel targeting 480 genes to directly profile the gene expression of Ewing sarcoma TME in situ with 10X Xenium. In the Xenium dataset (n=30 patients), we found three tumor cell substates shared by scFFPEseq and Xenium. The first shared state is the cycling tumor cell. These cells are characterized by high expression of canonical proliferation markers (eg. MKI67) and cycling markers (eg. CENPA). The second shared state is the conserved tumor cell, which shows higher expression of CCND1 and other genes potentially driven by fusion activity. The third shared state is the tumor cells with higher expression of hypoxia markers. The hypoxic tumor substates are highly enriched in the area far away from the tumor-normal interface and show a co-localization pattern with SPP1+ macrophages.
Furthermore, we profiled ˜30 patient samples with Xenium to find more TME structures and to find the connection between the TME structures and the clinical data.
We believe that the spatial resolution will provide us insights on the tumorigenesis and progression of Ewing sarcoma.

Weave: a software package for integrated spatial multi omics visualization and data analysis **112**

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Spatial omics technologies enable detailed molecular analyses directly from tissue. In parallel, multi-omics approaches that combine different omics levels to obtain a holistic view are increasingly in demand. However, performing spatial multi-omics data analysis presents several challenges. Data is typically acquired on serial tissue sections, at different spatial resolutions, in a variety of data formats, requiring expertise across multiple domains to obtain the best results from each individual technology. We present software that addresses the need for spatial multi-omics bioinformatics solutions, enabling efficient integration and joint visualization of different assays.
Weave is a cloud-based spatial omics data management and data analysis software platform. We demonstrate the software via two spatial multi-omics use cases:
Combining lipid mass spectrometry imaging (timsTOF fleX, Bruker) with multiplexed sequential immunofluorescence (COMET, Lunaphore) and histology images of fresh-frozen mouse brain,
The comparison of two antibody-based spatial proteomic assays; MALDI-IHC (Ambergen)

and IMC (Standard Biotools) measured on mouse lymph node.
Image registration was performed using a non-rigid algorithm, allowing accurate translation of the data acquired from different modalities. Data integration was conducted to obtain a homogeneous data structure across assays of different spatial resolutions, providing a foundation for joint downstream data analysis and visualization. Joint visualization and data analysis reveals spatial correlations between different lipids and proteins in the mouse brain. Similarities and differences in IMC and MALDI-IHC readouts were confirmed by overlaying both datasets onto H&E-stained images of the samples.

113 Synovial-lining macrophage defined by high VSIG4 and TREM2 expression, driven by PRG4, protect joint homeostasis whilst Rheumatoid Arthritis results from their SPP1-producing inflammatory counterpart

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Distinct synovial tissue macrophage (STM) phenotypes are responsible for disease prevention, initiation and resolution of Rheumatoid Arthritis (RA)1. We previously demonstrated that MerTKposTREM2pos STMs dominate the healthy synovium whereas RA inflammation is driven by SPP1-producing MerTKneg STMs, resulting in local fibroblast activation and enhanced neutrophil and monocyte infiltration. However, the mechanisms that underpin this transition remain unclear.
We performed imaging-based single-cell spatial transcriptomics of healthy, active RA and RA in remission synovium using Nanostring's CosMx 6000 gene panel. As in MacDonald et al., single cells were segmented with Mesmer and Baysor and celltypes annotated by integration with our scRNAseq reference. Cell neighbourhoods were identified; tissue regions segmented and, spatially clustered to identify niches. RNA velocity analysis of cellular trajectories was performed, and ligand-receptor interactions of spatially co-localized cell-types investigated.
Localization of STM clusters and dissection of interacting cells found that healthy TREM-

2high exist in synovial lining-layer (LL) in close contact with lubricin-producing (PRG4pos) LL fibroblasts in the joint-space interface and AXLpos DC2 in the superficial sublining. These STMs have high expression of immunoregulatory VSIG4, restricting local T cell activation and in-vitro experiments confirmed PRG4 drives this phenotype. Active RA results from loss of homeostatic VSIG4posTREM2high macrophages. Their replacement by infiltrating SPP-1posFCN1pos cluster leads to population of the LL by SPP1posVSIG4negTREM2low macrophages, which colocalize with MMP3pos diseased LL fibroblasts and drive joint destruction. Together, these cells create inflammation-permissive myeloid-rich LL niche, which enables inflammatory DC3 activation of CCL5posCD4 T cells, driving chronicity of arthritis.
Single-cell spatial transcriptomics characterised STM and their neighbours between healthy tissue and RA disease states, highlighting the transition of healthy VSIG4posTREM2high to SPP1posVSIG4negTREM2low macrophages, creating a niche for inflammatory DC3 activation of CCL5posCD4 T cells driving disease chronicity. PRG4 is protective of this phenotype and can resolve VSIG4 expression in vitro.
MacDonald, L., Elmesmari, A., Somma, D., Frew, J. et al. (2024) Distinct tissue-niche localization and function of synovial tissue myeloid DC subsets in health, and in active and remission Rheumatoid Arthritis. bioRxiv 2024.07.17.600758

VoltRon: A Spatial Omics Analysis Platform for Multi-Resolution and Multi-omics Integration using Image Registration **114**

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The growing number of spatial omics technologies have created a demand for computational tools capable of storing, analyzing, and integrating spatial datasets with multiple modalities and diverse spatial resolutions. Meanwhile, image processing is becoming an integral part of analyzing spatial data readouts where image registration and alignment of tissue sections is essential for accurate spatially aware data integration. Hence, there is a need for computational platforms that process and analyze images of microanatomical tissue structures as well as those that integrate datasets across distinct spatial resolutions and pathological images. To this end, we have developed VoltRon, an R package for spatial omics analysis with comprehensive image processing capabilities and a novel spatial data framework that supports a large selection of distinct spatial resolutions. Computer vision toolboxes are fully embedded in VoltRon that allow users to automatically synchronize spatial coordinates across adjacent/serial tissue sections for data transfer. To connect and integrate spatially diverse omics profiles, VoltRon accounts for spatial organization of tissue blocks, layers and assays given a multi-resolution and agnostic collection of spatial data readouts. Our framework offers a unique data structure that accommodates data readouts with many levels of spatial resolutions (i.e., multi-resolution). These include single cells, spots, and molecules as well as regions of interest (ROIs) and image tiles/pixels that are often ignored in currently available spatial data analysis platforms. More information on the framework can be found on https://bioinformatics.mdc-berlin.de/VoltRon.

115 Characterisation of the immune infiltrate of bladder cancer

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Urothelial bladder cancer is the 10th most common tumor worldwide with a high incidence in the Western world. It is characterised by a morphological heterogeneity with coexisting prognostic favourable or adverse features. Current treatment of bladder cancer includes radical cystectomy accompanied by adjuvant chemotherapy but recently also different immunotherapies are being studied in this disease. In order to improve their implementation against this form of cancer, a deeper understanding of its immune microenvironment is needed.

In this project tissue microarray (TMA) including cores from 273 patients, taken both from the tumor center and its invasive margin were evaluated for the presence of the major immune subsets, namely CD4$^+$, CD8$^+$ and regulatory T cells, B cells and CD163$^+$ macrophages.

The overall level of immune infiltrate was highly heterogeneous among the patients as well as between the tumor center and its invasive margin. With respect to the immune subsets, CD163$^+$ macrophages were the most frequent infiltrating cell type, both in the center and invasive margin, followed by the CD8$^+$ T cells. When the TMA cores were divided into stroma

and tumor portions, a skewed distribution of the immune cells was found, with the Treg being highly enriched within the tumor cells, in particular in the center but not in the invasive margin.
Preliminary association with clinical data from 234 patients highlighted a significant positive correlation between higher tumor stage and increased frequency of macrophages, particularly in the tumor invasive margin.

Uncovering the multicellular structure of ovarian cancer and its impact on patient survival 116

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Ovarian cancer is one of the deadliest gynecological malignancies, characterized by a high mortality rate due to late diagnosis and poor response to treatment. It has been shown through single-cell RNA sequencing approaches that ovarian cancer is highly diverse due to significant clonal heterogeneity, but the overall structure remains poorly studied and understood. Current treatment strategies are often ineffective, underscoring the urgent need for a deeper understanding of the multicellular structure of this cancer.
Our study aims to comprehensively characterize the multicellular architecture of ovarian tumors. We have assembled a large cohort of 980 ovarian cancer patient samples, which have been stained for 43 markers, enabling detailed analysis of the tumor and the tumor microenvironment. By leveraging this high-dimensional data alongside extensive clinical information, we are examining the correlation between tumor architecture and patient survival outcomes.
This study provides profound insights into the structural and cellular composition of ovarian cancer, potentially leading to the development of improved prognostic tools and targeted therapeutic interventions.

Pegivirus encephalitis, a distinctive viral CNS infection in immunosuppressed patients 117

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Introduction: *Pegivirus hominis* (HPgV-1), a single-stranded RNA flavivirus, has been considered non-pathogenic in humans. Here, we report a series of four chronically immunosuppressed patients with encephalomyelitis associated with HPgV-1 infection. We focus on a tissue-based characterization of the virus-host response using spatial transcriptomics.
Methods: Evaluation of clinical and radiological data. Quantification of HPgV-1 RNA in the CSF, serum and autopsy samples using RT-qPCR. We performed spatial transcriptomics on brain autopsy samples using Xenium In Situ Platform (10x Genomics) targeting 300 genes. To investigate the frequency of HPgV-1 in brain tissue we performed RT-qPCR on 60 fresh-frozen brain tissue samples from tissue donors of the Brainbank (Charité).
Results: MRI revealed hyperintensities in T2/DWO-weighted images along the whole pyramidal tract. Post-mortem tissue analysis showed higher HPgV-1 RNA viral loads in the CNS samples, compared to non-CNS tissue. The genetically distinct HPgV-1 population in CNS compared to non-CNS compartments is indicative of independent HPgV-1 replication within the CNS. All tested brain autopsy tissue samples from the Brainbank (n = 60) were negative for HPgV-1 RNA. Histologically the lesions showed infiltration of CD68+ macrophages and CD8+ T cells, upregulation of HLA-DR, bizarre astrocytes, destruction of myelin and axons. Using Xenium, we examined the spatial heterogeneity of the viral host response and found a spatially heterogenous upregulation of interferon related genes. We correlated this with the histopathology using image alignment tools available within the VoltRon software.
Conclusion: Symmetrical bilateral affection of the pyramidal tracts should elicit testing for HPgV-1 RNA. The genetically distinct HPgV-1 population in CNS as compared to non-CNS compartments and the spatially emphasized interferon response are indicative of independent HPgV-1 replication within the CNS. It is still not clear which host or viral factors determine a possible neurotropism leading to encephalitis as HPgV-1 RNA is found in ~ 5% of the

population.

Spatial Mapping of serotonin metabolism-related genes in human brainstem 118

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Introduction: Pathomechanisms of cognitive impairment and/or fatigue occurring after an infection with SARS-CoV-2 are poorly understood. Recently, it has been shown that systemic viral infections including infections with SARS-CoV-2 can result in a reduction of circulating serotonin levels via various mechanisms such as reduced intestinal tryptophan absorption, impaired serotonin storage, and altered activation of the vagus nerve and autonomic nervous system.
Methods: Here, we performed single-nucleus RNA sequencing and Spatial Transcriptomics (StereoSeq) of fresh-frozen human autopsy brainstem samples from deceased individuals who have passed away months after the acute infection.
Results: We detected a heterogeneous expression of serotonin receptors across different cell types. HTR1A, HTR1E, HTR2C and HTR5A were expressed mainly on neurons and HTR2B in glial and endothelial cells. HTR4 and HTR7 were expressed mainly in macrophages and microglia. By additionally integrating the spatially resolved sequencing data from StereoSeq (STOmics) with subcellular resolution, we can assign the detected transcripts to individual nuclei of the respective cells. Serotonin receptors were abundantly expressed in the brainstem tissue, whereas genes involved in serotonin synthesis were only scarcely detected.
Conclusion: Serotonin receptor genes are abundantly expressed throughout the human brainstem. Alterations of serotonin metabolism could potentially cause functional disturbances that contribute to neurological complications of post viral conditions.

Empowering Spatial Biology through Strategic Grants: Meet Miltenyi´s Grant Management Team 119

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The Miltenyi Biotec Grant Team is dedicated to supporting and facilitating cutting-edge research through pioneering grant projects. We initiate, coordinate, and actively participate in projects that drive transformative technologies and advance clinical translation.
Through collaborations, we empower scientists to fully explore spatial biology's potential

and promote multidisciplinary partnerships that spark innovation in research and therapeutic applications. We offer strategic grant support to connect Miltenyi Biotec with visionary researchers, collectively pushing the boundaries of science and medicine.
We are always on the lookout for innovative partners who share our vision for transforming biomedical research, aiming to unlock new therapeutic possibilities and advance our understanding of complex biological systems.

120 Vitessce Link: A Hybrid Approach Combining Mixed Reality and 2D Displays for Visualization of 3D Tissue Maps

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Figure 1 Mixed Reality and 2D display hybrid approach for the analysis of 3D tissue maps. Interactions in both the 2D display space as well as the MR space are synced with each other. For each output space, we use the appropriate input space.

We introduce a novel hybrid system, Vitessce Link, that combines a Mixed Reality (MR) stereoscopic view of 3D spatial data with linked views on a conventional 2D display (see Figure 1). Our system addresses key visualization challenges in spatial biology, a field dedicated to examining molecular components within their native spatial context, resulting in complex, high-resolution 3D tissue maps. With the diversity of techniques generating these maps, practical visualization tools are essential. To tackle this, we collaborated closely with spatial biology experts throughout the design process (see Figure 2), conducting iterative development and testing through three case studies. These case studies include: 1) single-cell cyclic immunofluorescence (CyCIF) imaging to investigate early melanoma development (see Figure 3 C1); 2) lightsheet microscopy of kidney tissue to understand the function of glomeruli, showcasing insights into spatial relationships within kidney structures (see Figure 3 C2); and 3) multiplexed immunofluorescence imaging (MxIF) to study various kidney structures, highlighting the benefits of our hybrid approach in controlling channel information and conducting distance measurements (see Figure 3 C3). By extending the web-based Vitessce (http://vitessce.io) framework for single-cell analysis, a tool already familiar to domain experts, with a WebXR spatial view, we ensured usability and integration with existing workflows (see system overview in Figure 4). A qualitative evaluation of our prototype revealed widespread recognition of the hybrid system's value, even among those initially skeptical of MR technology.User feedback strongly supports combining direct hand interaction in MR with traditional mouse input on a 2D display, underscoring the effectiveness of this approach in enhancing interaction with complex spatial data.

CellsFromSpace: new functionalities for the fast and accurate reference-free spatial transcriptomics workflow 121

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Sequencing-based Spatial Transcriptomics (ST) allow the study of cell dynamics in healthy and diseased organs by capturing transcriptomic profiles histological samples. However, the mini-bulk nature of ST data poses computational challenges for identifying cell populations, analyzing their distribution, and studying their interactions. To address this, we developed the CellsFromSpace framework, utilizing independent component analysis (ICA) to decompose and analyze ST data without relying on single-cell references. ICA helps identify distinct cell types or activities, reduce noise, and enable focused analysis of specific populations. CellsFromSpace has demonstrated its flexibility and accuracy in identifying both common and rare cell populations across multiple technologies, including Visium, Slide-seq, MERSCOPE, CosMX, Visium HD, and Stereoseq. It has also proven faster, and more scalable and accurate than other reference-free methods, and supports the joint analysis of multiple samples.
Since its initial release, several features were added to CellsFromSpace, including spatial correlation analysis to examine the spatial relationships between features and create network representations of samples. Users can now identify shared niches between samples, explore component signatures more deeply, and conduct ligand-receptor analyses. These updates and more are integrated in CellsFromSpace's graphical interface, empowering non-bioinformaticians to fully analyse their ST data.

Developing a coculture model to unravel respiratory virus-induced fibrosis in the spatial context 122

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<Aim> Respiratory viruses cause life-threatening exacerbations in patients with chronic obstructive pulmonary disease, a chronic and progressive lung disease driven by small airway fibrosis. However, it is not fully known how the infected airway epithelial cells and fibroblasts communicate with each other in the spatial context. As the availability of human lung samples during exacerbations are limited, a model that mimics the airway in the acute phase of infection is needed. **<Methods>** Primary human bronchial epithelial cells were seeded on a membrane and differentiated at the air-liquid interface with a method validated for influenza A virus infection (Nakayama et al. Front Microbiol 2023). Primary human lung

fibroblasts were added to the other side of the membrane 4 weeks after airlift, and coculture was continued for 2 weeks. Cells were observed under the inverted microscope and at the end, fixed with 4% paraformaldehyde. Cross-sections were made from formalin-fixed paraffin embedded blocks and Heamatoxylin and Eosin stain was performed. **<Results>** The presence of both cell-types was confirmed. More than 20 cross-sections could be placed within a compact capture area suitable for spatial transcriptomics. **<Conclusion>** An airway epithelium-fibroblast coculture model for respiratory viral infection and spatial transcriptomics has been established. Plans are to do infections and spatial transcriptomics as well in dissected human airways as an in situ version of this model.

123 CONCLAVE: CONsensus CLustering with Annotation-Validation Extrapolation

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Background and Aim: Spatial single-cell omics is crucial for understanding tissue architecture and cell phenotyping. Cell identification involves multiple steps (normalization, sampling, dimensionality reduction, clustering), each affecting outcomes. While individual clustering algorithms have been explored, comprehensive evaluation of method combinations for spatial multiplexed immunohistochemistry (mIHC) is lacking. We present CONCLAVE, a consensus-based method improving phenotyping accuracy and stability in mIHC datasets, especially under noise.
Materials & Methods: We analyzed spatial mIHC data using real and in-silico datasets. Methods tested included four normalization strategies (z-score, MinMax, log-normalization, none), different sampling techniques (random, stratified), and DR methods (PCA, t-SNE, UMAP, PaCMAP). Clustering was performed using unsupervised (Phenograph, FlowSOM, k-means, Meanshift) and supervised methods (Random Forest, SVM, LDA, UMAP-projection). CONCLAVE integrated these clustering results, and its performance was assessed, focusing on accuracy and stability in noisy conditions.
Results: CONCLAVE consistently outperformed individual clustering algorithms, providing higher phenotyping accuracy and more stable results. It remained robust under various noise levels and datasets with high marker overlap.
Conclusion: CONCLAVE improves phenotyping accuracy and stability compared to individual methods, demonstrating resilience to noise. This adaptability makes it a strong tool for single-cell data analysis. Integrated into our web tool “DISSCOvery”, CONCLAVE enables accessible cell phenotyping for researchers without bioinformatics expertise.

Spatial changes in structure and activity of lymphoid organs during rodent malaria infection 124

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Malaria remains a significant global health threat, causing ca. 620,000 deaths annually. Most fatalities occur in sub-Saharan Africa among young children and pregnant women, making it a critical focus for prevention and treatment efforts.
We aim to understand how the immune system responds to parasite infection across different tissues. Specifically, we tracked host response to Plasmodium chabaudi infection, the rodent malaria model, using flow cytometry and suspension single cell transcriptomics (10X) in blood and tissues (bone marrow, lymph node and spleen). Cell-cell interaction (CCI) analysis of spleen showed a response changing from macrophage-dominated at peak infection to more T-cell-dominated at recrudescence. We then performed and compared spatial transcriptomics tools, incl. 10X Visium and Nanostring CosMX (1000 mouse panel). We were able to identify the major sub-structures of the spleen and expected cell types, in both Visium and CosMx data. Visium data reveal a marked increase of macrophages in the spleen red pulp in response to infection while CosMx data enabled us to dissect CCI spatially. Notably, technical limitations resulted in underrepresentation of specific cell types, such as B- and T-cells in the white pulp of the spleen.
Ongoing work aims to develop a Xenium 5000 probe panel for mouse combined with 50 parasite markers, for simultaneous multi-species analysis. Overall, spatial transcriptomics is a unique opportunity to explore host-parasite interaction, with challenges specific to organs studied and methods chosen.

Multiomics and Multimodal investigation of different kidney disease states 125

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Introduction: Spatial localization of analytes on tissue has expanded to target proteins using the MALDI HiPLEX-IHC workflow. The addition of this workflow to clinical research allows for high multiplexing of antibody targets, combining targeted proteins with multiomics, and whole slide profiling in under an hour. Here, a comparative spatial biology analysis of healthy and diseased kidney will be demonstrated using traditional staining, immunofluorescence, MALDI Imaging and IR Imaging. The characterization of cellular mechanisms behind solid organ transplant rejection will help with understanding the localiza-

tion of immune response mechanisms within the transplanted tissue.
Methods: FFPE human kidney tissues were first investigated with IR Imaging in a very short timeframe. Afterwards the tissue got heated up to 80°C for 15 min and washed two times with Xylene. Lipid Imaging was performed using DHAP matrix. After MALDI Imaging matrix was removed and standard MALDI HiPLEX-IHC workflow was performed. Serial sections were prepared for chromogenic IHC comparison. All visualization and data processing were done using SCiLS™ Lab software.
Results: The multiplexing capabilities allow for visualization of spatial interactions between cell types, such as macrophages (CD68), fibrillar collagen (COLA1A), tubules (ATPase-1A1) and glomeruli (VIM). Additionally, IR Imaging and lipid Imaging results can be correlated and give some insights in lipid profile changes within transplant rejected and inflammatory tissue.
Small molecule targets for lipid regulation are well researched and present additional multiomics options. Recent method development for MALDI Imaging from FFPE show that lipids and metabolites are accessible from fixed tissue and will be demonstrated.

126 Spatially Tracking Copy Number Alterations in High-grade Serous Ovarian Cancer

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Being the most prevalent subtype of ovarian cancer, High-Grade Serous Ovarian Carcinoma (HGSOC) is known for its aggressive nature and significant intra- and intertumoral heterogeneity. Computational tools have been developed to track subclones within tumor tissues from spatial transcriptomics (ST) data (A. Erickson et al., Nature, 2022), to better resolve such heterogeneity in a histological context. In this study, we are spatially tracking copy number variations in ST data acquired from 35 tissue samples across 16 HGSOC patients. The samples were taken from patients belonging to groups with either long or short progression-free intervals. In a previous study, an artificial intelligence model had been trained to classify whole-slide images from H&E-stained tissue samples of HGSOC patients, associating them with either of these outcomes (A.R. Laury et al., Sci Rep, 2021). Here, we compare spatially inferred copy number variations between these groups, providing both biological underpinnings for the model's predictions, as well as general insights into the copy number landscape of patients with long versus short progression-free intervals.

Whole slide imaging guides deep spatial proteomics of chemoresistant triple negative breast cancer 127

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Triple negative breast cancer (TNBC) is an aggressive disease whose standard treatment is neoadjuvant platinum-based chemotherapy.
However, chemoresistance and relapse are common for this cancer subtype. Currently the field aims to increase treatment efficacy by better understanding the tumor microenvironment (TME) to reveal therapeutic targets. However, systematic analyses with high phenotypic and omic-level resolution remain limited.
To tackle this, here for the first time, we combined multiplex immunofluorescence imaging with spatial neighborhood analysis, and untargeted mass spectrometry-based proteomics. As proof of concept, we analyzed a set of 6 patient biopsies, 3 non-relapsing and 3 relapsing tumors (initial and remission), to characterize key biomarkers predictive of the variable treatment outcomes. Whole slide images were stitched, registered, segmented, and cells classified with a combination of MCMICRO and Snakemake pipelines --- taking advantage of its high transparency and replicability. Whole slide imaging allowed us to phenotype more than 5 million cells across 10 sections, providing high statistical power for identifying recurrent neighborhoods across treatment outcomes.
Importantly, our workflow was optimized for the applicability to laser microdissection, requiring specialized membrane slides. Additionally, this allowed us to deeply profile (6000+ proteins) unique cellular neighborhoods linked to treatment outcome and connect single-cell phenotypes to functional proteome states. These multimodal data provide us with unique insights induced by chemotherapy of TNBC, potentially identifying novel response markers that could improve clinical decision making in the long term.

The super-resolved structure of the *Salmonella* 3D genome 128

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Recent single cell studies of bacteria reveal a phenotypic heterogeneity of response compared to population averages. The ability of single cells to respond uniquely to stress allows subsets of cells to adapt to changing environmental conditions, which benefits the popu-

lation. To date, no single prokaryote genome has been described with high spatial and genomic resolution. Here, we used sequential OligoSTORM, Hi-C, and modeling, to determine genome folding at high genomic and spatial resolution of *Salmonella enterica* serovar Typhimurium. The foundation of this chromosome tracing technology is the sequential labelling and detection of Oligopaint oligos (fluorescence in situ hybridization probes). In each round of imaging, different genomic loci are targeted and visualized. Image reconstruction features the entire chromosome walk and represents the first high-resolution view of an entire genome. Our results demonstrate substantial single cell heterogeneity with respect to macrodomains, and other features that were based on ensembles.

129 Spatial registration of thyroid hormone-responsive genes in cerebral organoid models of human cortical neurogenesis

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Thyroid hormone (TH) function is essential for mammalian nervous system development. In pediatric patients, this role of TH is evident from a spectrum of neurological phenotypes resulting from conditions of maternal hypothyroidism or resistance to TH syndromes during fetal development. We used human cerebral organoids (hCOs) as a powerful 3D model system to delineate local TH action during early human cortex development. To generate a first atlas of TH-responsive genes for human cortical neurogenesis, we differentiated hCOs in media containing different concentrations of T3 (biologically active TH) and performed longitudinal transcriptome analyses by single cell RNA-seq. Along a cortical excitatory neuron differentiation trajectory, we identified genes responding to T3 treatment in radial glia cells, intermediate progenitors and excitatory neurons, revealing a pleiotropic nature of TH action during neurogenesis. To validate our single cell analyses, we used a panel of T3-responsive genes in multiplexed RNAscope assays for spatial registration of TH responses. This approach provided the flexibility needed for spatial transcript mapping across many timepoints and treatment conditions and faithfully resolves even subtle differences in cell state-dependent T3 responses in a spatial dimension. Correlating spatial mRNA expression patterns along laminar cell type distributions revealed an excellent agreement with gene expression response profiles derived from lineage trajectory analysis of single-cell data. Our results demonstrate the value of complementary spatial transcript mapping to enhance the biological significance of single cell transcriptome data towards a comprehensive understanding of developmental processes governing early neurogenesis.

A spatial multiomics workflow for the combined analysis of lipids and expressed proteins in tissues 130

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Introduction: MALDI (matrix-assisted laser desorption/ionization) mass spectrometry imaging (MALDI Imaging) is a powerful technique for the spatial analysis of biomolecules in tissue. Multiomic spatial localization of lipids in combination with expressed protein distributions can be archived from a single tissue section, thus increasing our understanding of localized cellular processes. Here, we introduce a workflow on a new benchtop mass spectrometer that starts with human colorectal cancer FFPE tissue sections and ends with simultaneous visualization of lipids and expressed proteins in a clinically relevant sample.
Methods: FFPE sections of a human colorectal cancer sample were prepared for lipid imaging. Spatially resolved mass spectra were acquired on the new neofleX instrument (Bruker) at 20 µm pixel size. To assess the spatial protein landscape, the same slide was processed by MALDI HiPLEX-IHC using 14 Miralys™ antibodies (AmberGen) and measured on the same instrument. Visualization of the data was done in SCiLS™ Lab 2024b and SCiLS Scope 1.0.
Results: The lipid imaging experiment revealed fifty-eight annotated lipid species, mainly phosphatidylcholines and sphingomyelins from the FFPE sample.
Protein expression and lipid imaging data were combined and mapped for examination of feature colocalization. Lipids colocalizing with individual tumor or immune markers from the MALDI HiPLEX-IHC experiment were thereby obtained using the mapped images. For example, PC_32:0 was detected with higher abundance in the same region, where also cytokeratin marker PanCK was binding. An accumulation of phosphatidylcholines in cancer is well known and occurs to fuel the higher energy demands of cancer cells.
Our workflow provides a simple method to visualize the targeted spatial protein expression profiles from FFPE tissue. As an add on, our method can be combined with lipid imaging from the same tissue section to receive deeper insights into the cellular mechanisms of disease.

Making spatial spatial again: High dimensional segmentation of morphological features provides transcriptomically unbiased clustering of cellular identities 131

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Background: Spatial transcriptomics frequently rely on clustering of dimensionality reduced gene expression patterns to digitally dissect the tissue. Our approach aims to improve transcriptomic data digital dissection by integrating standard histology. By using HE-images from a mouse model of cholestatic liver disease we aimed to determine if it is possible to identify areas with differential cell distributions and integrate transcriptomic data after defining regions of interest, without the need for reference datasets.
Materials and Method: Mice underwent bile duct ligation and were sacrificed at 6 different timepoints to capture key molecular and immunological events during the acute phase of cholestasis (4h, 8h, 24h), from the non-acute phase (3d, 5d, 7d) as well as sham surgeries. Liver samples were snap frozen and processed for spatial transcriptomics using 10X Visium. Standard HE-images were used to extract spatial features (HE intensity and morphological distribution of staining) per nuclei and cytoplasm as detected by watershed segmentation. After alignment and clustering, the abundances of unlabelled cell classes were correlated with liver specific cell lineage markers.
Results: Cell class abundances were correlated with known markers for cholangiocytes, hepatocytes, stellate cells, immune cells, as well as inflammatory, apoptotic and cell migration signalling molecules on a group level. We can trace the co-occurrence of signals of neighbouring cells, such as overlap of cholangiocyte and stellate markers which being highly separated from hepatocytes. Along the time axis we observe significant correlations of specific cell classes and hepatocytes, stellate cells, monocyte-like cells and chemotaxis receptors.
Conclusion: We have demonstrated the potential of integrating quantitative image analysis with spatial transcriptomic data and suggest this as an alternative approach to digital dissection of spatial transcriptomic datasets. In conclusion, this additional information extracted from the HE image data can improve spatial transcriptomics results and make them more robust and reproducible.

132 The case when the spatial microtubules' arrangement determines the ability of red algae spores to swim

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As it is known, red algae spores are devoid of such transport means as flagella, cilia or the ability of non-reversible cyclic cell shape changes. But most of them are reported to swim with the velocity within the framework from to (Pickett-Heaps et al., 2001). Newly it was proposed a model considering a released spore in unbound water as a spherical particle en-

closing a liquid incompressible cytosol, in which oscillates a solid spherical organelle (Pundyak, 2023). It is shown that in the case when the cytosol has Newtonian or Maxwell properties, the spore may swim only when the forward and backward trajectories of the organelle are different. In the case of the shear thinning cytosol properties the spore may swim also when the organelle trajectories are the same, but the velocities of forward and backward movements of the organelle should differ. The swimming of the model spores completely satisfies experimental data. As it is shown, in some polarized cells (including monospores of red alga *Porphyra yezoensis*) microtubules are mostly arranged symmetrically to the cell axis, while their minus and plus-ends are clearly orientated relatively to the cell poles. Intracellular organelles are able to be actively translocated on long-distances by means of motor proteins, such as dynein and kinesin, which move towards microtubule minus- and plus-ends, respectively, with different velocities [7, 11, 12]. Ca^{2+}-ions may decrease the relation of active kinesin and dynein concentrations determining the direction of a cargo movement in polarized cells. Not only in animal, but also in plant cells including algae there may take place oscillations of Ca^{2+} level and bound with it oscillations of Ca^{2+} gradient [14, 15]. Thus the conditions are created, under which a cell organelle may be translocated in turn towards different cell poles with different velocities and by different ways.

These data may inspire one to investigate detail intracellular spatial and temporal organization of red algae spores.

Plastids' movement and their clear spatial arrangement as also complicated cytoskeleton organization of protonemal outgrowth were shown in germinating moss spores (Pundiak at al., 2002; Pundiak et al., 2003; Pundiak, Demkiv, 2008; Pundiak, 2011). In some cases such a movement was fast. Moss spores do not have a significant supply of nutrients, so for survival their protonema should as quick as possible get to the soil surface for sun light. But the growth of the protonema is slow. Thus it is natural to assume that analogically with red algae spores moss spores may also swim within the soil capillaries to the soil surface. So contemporal investigation of spatial intracellular structure and dynamics of germinating moss spores is also very desirable.

Spatial characterization of follicular lymphoma transformation 133

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Follicular lymphoma (FL) is the most common indolent non-Hodgkin lymphoma. FL is capable of transforming into the much more aggressive diffuse large B cell lymphoma (DLBCL), which is characterized by poor prognosis and treatment outcomes (5-year survival rate 90% vs 65% respectively). This complex process is associated with the gain of additional genetic mutations and dramatic stromal niche remodeling. However, cell signaling-dependent mechanisms underlying both tumor and tumor microenvironment (TME) remodeling during FL transformation remain unclear.

In order to understand how changes in cell composition and signaling activity within the TME are linked to FL transformation, we performed complementary spatial (10X Visium and CODEX) and single cell profiling (10X Multiome) methods on composite tumor samples containing both follicular and diffuse entities. We used patient-matched single cell references to deconvolve the Visium data, and identified preferential enrichment of myeloid and cytotoxic T cells in the diffuse tumor compartment compared to the follicular zones. Furthermore, we used collective optimal transport theory (COMMOT) to infer cell-cell communication and downstream signaling events which correlate with the preservation or loss of follicular structure. We observed pro-inflammatory (IL-1, TNF-alpha) cross-talk exclusively restricted to the follicular zones, while the diffuse zones show increased protease-activated receptor (PAR) signaling. Through our characterization of the structurally distinct niches within the composite tumors, we aim to uncover clinically insightful cell-types and signaling pathways promoting FL transformation.

134 Investigating tertiary lymphoid structures in paediatrics patients of rhabdomyosarcoma for promoting immunotherapy responses.

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Embryonal-rhabdomyosarcoma(ERMS) is the most common soft tissue sarcoma in children, posing significant clinical challenges due to limited effectiveness of conventional treatments namely surgery, radiation, and chemotherapy. While cancer immunotherapy called immune check point blockade (ICB) shows promise in solid tumors including non-small cell lung cancer, ERMS remains largely unresponsive. Despite the presence of tertiary lymphoid

structures (TLS) in the tumor microenvironment, which often predicts responses to ICB in other solid tumors, patients with ERMS do not respond to ICB. This suggests intrinsic differences in the tumor microenvironment including TLS in these patients. Our preliminary data provide critical insights into the immune composition of ERMS. Using Haematoxylin&Eosin and immunohistochemistry staining, we identified that most ERMS cases contain immature lymphoid aggregates, or early forms of TLS, which lack germinal center or follicular dendritic cells. Initial findings from ultra-high multiplexing tissue imaging platform (PhenoCycler-Fusion) reveal a predominance of naïve CD8+ T cells in TLS of ERMS, suggesting inadequate priming likely due to reduced dendritic cells. Next, we observed significantly higher densities of ICB-sensitive CD8+TCF1+PD1+ stem-like T cells in TLS with primary follicle or germinal center as compared to the early forms of TLS. We hypothesize that the absence of mature TLS with germinal centers leads to insufficient priming and activation of CD8+ T cells, resulting in ICB unresponsiveness in ERMS. To investigate this, we will analyze the spatial interactions of functional subsets of CD8+ T cell with other immune cells across different TLS maturation stages in ERMS and compare these findings with TLS in non-small cell lung cancer patients who respond to ICB. By exploring how mature TLS influence antigen presentation and T cell activation in ICB responders, our study aims to uncover mechanisms behind ICB resistance in ERMS and develop strategies to enhance tumor-specific T cell responses, potentially improving immunotherapy outcomes.

A transcriptional map of human tonsil architecture 135

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Recent advancements in spatial molecular imaging (SMI) technologies have enabled the profiling of tissues at molecular resolution while preserving their intact architecture. However, analyzing SMI data remains challenging due to its sparsity, low dynamic range, and inaccurate segmentation. Despite these obstacles, SMI data hold significant potential for expanding our biological understanding by viewing tissues as hierarchical systems with various functional units- molecules, cells, niches, and entire organs. Therefore, developing computational tools specifically designed for SMI data is crucial to fully leverage its potential and gain deeper insights into the complex organization of tissues.
In this study, we developed a specialized analysis pipeline for CosMx SMI data and applied it to three palatine tonsil samples, using the Universal Cell Characterization Panel, to ge-

nerate a comprehensive spatial resource. We identified 1.75 million cells that underwent quality control and were further analyzed using both cell-type-dependent (e.g., clustering) and cell-type-agnostic tools (e.g., gradients of gene expression, pathway scoring). Even with a limited panel of 980 target genes, we robustly identified 51 distinct cell populations from hematopoietic, epithelial, and mesenchymal compartments, each with unique transcriptional signatures and spatial distributions consistent with existing literature.
When comparing these results to the Tonsil Cell Atlas, including matched samples, we observed a complete loss of mesenchymal cells and a reduction in tissue-resident immune cells in the scRNA-seq reference, which generally captures less compartmental heterogeneity. Importantly, we identified various immune cell types that were overlooked in the Tonsil Cell Atlas and characterized, at the molecular, cellular and spatial level, previously unrecognized cell interactions involved in maintaining tonsil homeostasis. We also unbiasedly identified 10 distinct spatial contexts representing homogeneous, functional tissue units and charted their cellular and transcriptional composition, including that of the mantle zone, a poorly characterized region, where distinct lymphomas originate.

136 Combining spatial transcriptomics and ECM imaging in 3D for mapping cellular interactions in the tumor microenvironment

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Tumor are complex ecosystems composed by malignant and non-malignant cells supported by the extracellular matrix (ECM). Cell-cell/ECM interactions occur in 3D cellular neighborhoods (CN) and control molecular phenotypes in the tumor microenvironment. Despite their inhibition can stop tumor progression, routine molecular tumor profiling cannot capture cellular interactions in CN. Conversely, single cell-resolved spatial transcriptomic methods (ST) hold great promise to profile receptor-ligand interactions but are limited to 2D tissue sections and lack ECM readouts. Here, we profile one clinical lung carcinoma combining 3D ST and ECM imaging in serial sections to systematically investigate molecular states, cell-cell interactions, and ECM remodeling in CN. Our integrative analysis pinpointed known immune escape and tumor invasion mechanisms, revealing several druggable drivers of tumor progression in the patient under study. In this proof-of-principle study, we showcase the utility of in-depth CN profiling in a routine clinical sample to inform microenvironment directed therapies.

Multiplexed imaging: having your raw data, overkill or a need ? 137

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A variety of devices to produce multiplexed images are currently available. The hardware usually comes with an accompanying software tailored to process and analyze the images acquired with the specific device. While some companies offer access to the raw data, i.e. tiles, others only provide the already registered and stitched image. When it comes to processing your data the latter case allows you to jump directly into downstream analysis, yet if the stitched image is sub-optimal, further correction on it will be hindered since no raw data is available. In the first case, where access to the raw tiles is possible, the user has the flexibility to try out and find the methods that are more suitable for correcting artifacts and/or suppressed unwanted signals in the data, this custom processing yields images that are more adequate for downstream analysis than the accompanying software. *In this project we present a couple of cases from different multiplexed technologies where processing the raw data "in-house "allows for better vignetting correction, stitching and preservation of pixel intensities.*

138 Mapping B and T cell clones in murine tissue using spatial transcriptomics

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B and T cells are essential for detecting and responding to infections and cancer. Their unique antigen receptors critically regulate their development and differentiation and can be used for lineage tracing during clonal expansion, which is tightly regulated based on their tissue environment.
Capturing the spatial distribution of B cell receptors (BCR) and T cell receptors (TCR) could help understand the dynamics and functionality of clones in their native tissues.
Spatial VDJ is a spatial transcriptomics-based method that maps B and T cell receptor sequences in human tissues. However, pre-clinical models are needed for mechanistic studies. Here, we adapt Spatial VDJ to mouse tissues. We first tested the BCR and TCR-targeted capture probes using Chromium single-cell 3' libraries prepared from mouse colon. In wild-type mice, we detected prominent levels of BCR and TCR. Further, IgG2A and IgG2C isotype expression were restricted to BALBc and B6 genetic backgrounds, respectively. In immunodeficient mice that received adoptive T cell transfers, TCR clonal counts were high, while B cell counts were negligible, indicating high probe specificity.
As a proof-of-concept, we applied murine Spatial VDJ to capture B and T cell clones from spatial transcriptomics libraries of tumor-affected murine colon. IgA was the prominent clonal isotype concordant with high Igha expression in the colon. Most of the IgA B cell clones were positioned within tumor-adjacent or normal tissue, rather than the tumor area. Nonetheless, as tumors progressed, B cell clones were expanded and more broadly distributed in the tissue, suggesting that tumor-associated inflammation may induce B cell clonal expansion throughout the colon.
Taken together, we faithfully capture antigen receptors in mouse single-cell and spatial transcriptomics libraries. This opens up the possibility to use Spatial VDJ to mechanistically and comprehensively investigate B and T cell clonal dynamics within their native tissue microenvironment in pre-clinical studies.

139 From Biomarker Measurement to Clinical Insights: An AI-Enabled Workflow for Spatial Image Analysis of Multiplexed Immunofluorescence Images at Scale.

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Measuring reliable biomarkers at scale across patient cohorts facilitates patient selection and the formulation of exact combination therapies. Specifically, in the case of precision

cancer therapy, profiling tumor microenvironment with high accuracy increases the precision of state-of-the-art treatments that depend on immune system activation. While traditional immunohistochemistry (IHC) approaches measure individual biomarkers per section and with good precision, they lack the possibility to extend the number of biomarkers and their automated quantitative assessment can be challenging. Ultivue's InSituPlex assays are designed to fill this gap and provide users with an end-to-end platform for performing multiplex immunofluorescence (mIF) at scale, compatible with standard fluorescence scanners. In the work presented here, we will focus on the analysis of cancer biopsies and reveal how Ultivue's proprietary STARVUE software platform can extract important parameters for patient selection from mIF images created with InSituPlex assays. We will present a cloud-based AI-enabled workflow leveraging STARVUE, comprising deep learning cell segmentation and phenotyping that scales to whole cohorts and millions of cells overcoming the challenges of data size and data complexity. Additionally, we will present how data extracted with STARVUE can be used to perform more detailed spatial analysis and thereby go beyond traditional analysis of cell type abundance. Taken together, we will show an end-to-end analysis workflow, from scalable mIF staining to complex data analysis and clinical insight generation.

Enhanced analysis of tumor microenvironment and immune regulation via an automated adjustable signal amplification technique for multiplex immunofluorescence 140

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Multiplex immunofluorescence (mIF) has become fundamental for tumor microenvironment (TME) [1]. Markers with different expression levels can provide crucial insights [2], but detecting low-expressed markers often benefits from signal amplification [3]. Here, we demonstrate a new automated method enabling the amplification of individual markers within multiplex panels.

COMET™ is an automated platform that performs sequential immunofluorescence (seqIF™) assays [4]. This amplification method increases the number of detection antibodies per primary antibody in a cyclic manner, leading to a stronger signal. The amplification complex can be eluted, enabling subsequent staining cycles while preserving tissue integrity. Human colorectal and breast carcinoma samples were stained using a 26-plex panel including 20 markers in standard seqIF™ and 6 amplified markers, together covering most basic immune cells, functions, and stroma. The performance was assessed by comparing marker expression with and without amplification and amplified staining to single-plex chromogenic immunohistochemistry (IHC) for each marker [5].

Six amplified markers were integrated within a 20-plex seqIF™ panel. Amplified low-expressed markers were reliably detected with a controllable signal intensity increase between low and high-expressing cell populations, providing a higher dynamic range than unamplified seqIF™. Amplification enabled the detection of the full spectrum of PD-1 and PD-L1 expression intensity and the identification of several low-expressing subtypes of regulatory immune cells, such as Treg and macrophages, within the TME. The staining patterns of amplified markers showed a good correlation with chromogenic IHC, and elution of the amplification complex did not damage the tissue.
Hyperplex seqIF™ panels integrating this novel amplification technique will enable the detection of low-expressed markers, such as the full expression range of critical immune checkpoint markers that could not robustly capture with non-amplified methods. Analyzing a high number of markers simultaneously while allowing selective amplification will improve the profiling of immune and tumoral cells within their environment.

141 Novel insights of stroma-immune interaction during immunization revealed by CODEX multiplexed imaging

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142 In situ detection and subcellular localization of 5,000 genes using Xenium Analyzer in ovarian cancer tissue samples

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The Xenium Analyzer (10X Genomics) enables researchers to perform spatial analysis with highly sensitive and specific detection of RNA within intact tissue.
FFPE tissue sections of human ovarian cancer were prepared and placed onto the Xenium slide. The slides were assayed with the Xenium Prime 5K Human Pan Tissue and Pathways panel and a custom panel of 100 additional differentially expressed genes identified from 10x Genomics single cell Flex data (scFFPE-seq) on matched serial sections. The same sections were stained for automated morphology-based cell segmentation as per the Xenium Cell Segmentation workflow. The slides were then loaded onto the Xenium Analyzer instrument for automated in situ analysis. Transcript assignments, segmented cell boundaries, and clustering outputs were visualized using the Xenium Explorer software. H&E staining was performed after instrument analysis to overlay histological imaging with in situ visualizations. We used sketch-based subsampling in Seurat to define custom clusters. Cell annotations were determined based on the literature, gene ontology, cell atlases, and the matched scFFPE-seq data.
Ultra high-plex gene detection and multi-modal cell segmentation successfully resolved bio-

logically distinct subgroups of immune, stromal, endothelial, epithelial, and tumor cells. For example, Xenium segregated ciliated and nonciliated epithelial cells of the fallopian tube, as well as ovarian granulosa cells. Tumor and stromal endothelial cells were also distinguished by their spatial localization as well as differential gene expression. Xenium identified four distinct tumor subtypes, including proliferative, VEGFA+, and hypoxic/karyorrhectic tumor cells. Spatial zonation of these malignant cells suggests a transcriptional trajectory, whereby cells transition from a neoplastic state towards one characterized by abnormal vasculature (implied by the expression of VEGFA, contributing to leaky and disorganized vessels), ultimately culminating in hypoxia.
The Xenium platform facilitates targeted and discovery research with ultra high-plex gene detection and multi-modal cell segmentation, delivering unprecedented high-resolution spatial gene expression data to enhance understanding of tissue biology.

Mathematical modelling of multi-axis plasticity in ER+ breast cancer 143

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Resistance of anti-estrogen therapy is a major clinical challenge in treating estrogen receptor positive (ER+) breast cancer. Recent studies highlight the role of non-genetic adaptations in drug tolerance, yet the mechanisms remain unclear. One of the key processes that underlies enhanced drug tolerance as well as metastatic potential is the process of epithelial-mesenchymal plasticity (EMP). Furthermore, lineage switching, and acquisition of immunosuppressive traits are also commonly observed. We investigate the role of genes involved in EMP to elucidate origins of such multi-axis cellular plasticity by mathematical modelling of underlying gene regulatory networks. Specifically, we show that six co-existing phenotypes are enabled by underlying gene regulatory network, with epithelial-sensitive and mesenchymal-resistant being dominant. Population dynamics analysis demonstrates how phenotypic plasticity promotes survival among sensitive and resistant cells, suggesting that mesenchymal-epithelial transition inducers could enhance anti-estrogen therapy effectiveness. Additionally, hybrid epithelial/mesenchymal (E/M) phenotypes, which exhibit high PD-L1 levels, contribute to immune evasion and enhanced metastatic fitness, obviating the need for a full EMT. Finally, multi-modal transcriptomic data analysis shows associations between EMT and luminal-basal plasticity, linking luminal breast cancer with epithelial states and basal breast cancer with hybrid E/M phenotypes and higher heterogeneity. Our mechanistic modelling of ER+ breast cancer recapitulates observed clinical and pre-clinical findings in spatial transcriptomic datasets, thus offering a predictive framework for characterizing intra-tumor heterogeneity and potential therapeutic interventions given a spatially heterogenous tissue sample. These insights underscore the importance of understanding phenotypic plasticity and non-genetic heterogeneity and its integration with spatial transcriptomics to improve treatment strategies for ER+ breast cancer.

144 Aggregating nuclei segmentation methods to quantify cell counts in spatial transcriptomics data

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Spatial transcriptomics allows for the characterization of spatial variations in gene expression within a tissue. Sequencing-based approaches, such as 10x Genomics Visium, implement micro-bulk RNAseq-like measurements in thousands of spatially located spots across a histological tissue section. These spots contain only a few cells, providing insight into the spatial organization within a tissue and the variability between cells at a refined scale. However, Visium data are complex to normalize and analyze because the number of cells in each spot is unknown and varies across the tissue. In this context, estimating the number of cells per spot could help refine data preprocessing, such as normalization, and improve the accuracy of downstream analyses, including cell type composition deconvolution. To address this challenge, we propose nucount, an approach based on state-of-the-art machine learning methods for cellular segmentation, namely Stardist and Cellpose, to automatically count cells in each spot using microscopy section images provided with Visium datasets. In order to avoid the need for tuning segmentation model hyperparameters and to improve prediction performance, we employ an ensemble learning approach to aggregate cell count predictions from the segmentation methods across a grid of hyperparameter values. We have used various datasets with annotated images and ground truth segmentation to assess the performance of our approach. Our findings show that the aggregated predictions outperform the results from the individual segmentation methods when used separately. Additionally, we have analyzed pituitary adenoma samples from unrelated patients (without ground truth), and our results are consistent with human interpretation of the images and with results from independently performed deconvolution analyses. Our method is implemented using the Squidpy framework for spatial transcriptomics data analysis and is available as a Python package.

145 Characterizing germinal centers in murine and human specimens using automated ultra-plex imaging

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Germinal centers (GCs) are found in secondary lymphoid tissues like lymph nodes, spleen,

and tonsils. Its primary role in mammals is to generate plasma cells and memory B cells that can protect them against future infection. Within GCs, other cell types such as T follicular helper cells and follicular dendritic cells (FDCs) interact with these cells to support B cell diversification. Immunohistochemistry (IHC) and immunofluorescence (IF) are standard techniques to study the tissue microenvironment, but they have limitations. Advanced methodologies like customized multiplex imaging have emerged to overcome these limitations. We optimized and validated up to 50 markers to analyze the tissue microenvironment in lymph nodes, spleen, and tonsils in both mouse and human samples using the MACSima automated imaging platform. Additionally, we developed an image analysis workflow combining CellProfiler and MACSima software to identify different cell types. This approach provides a more comprehensive assessment of the tissue microenvironment during immune responses.

Combining discovery-based and spatial proteomics to interrogate molecular signatures of malignant transformation of oral leukoplakia into oral squamous cell carcinoma **146**

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Discovery of spatial programs from cross-condition and spatio-temporal data **147**

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We present OFFSET (Optimization of diFFerential Spatial Expression paTterns), a framework to infer differentially spatially patterned molecular programs from cross-condition and

temporal spatial omics data. Combining matrix factorization, measures for spatial autocorrelation, and a newly developed multimodal optimization algorithm, OFFSET efficiently identifies a set of programs that exhibit distinct spatial patterning across conditions or time points. For example, a response-specific molecular program can exhibit high spatial autocorrelation after acute tissue damage, while not showing any patterning in normal tissues. We first validated OFFSET on simulated data. We next showcase our approach on a spatial transcriptomics dataset from spinal cord tissue of human amyotrophic lateral sclerosis patients, where we identify not only previously described processes but also identified potential new molecular programs. To explain the emergence of these programs we use prior knowledge to identify transcription factors and cellular interactions that may underlie the context-specific coordination. Finally, in a spatio-temporal context, we also present how OFFSET can be used to analyze changes in spatial patterns of molecular programs in a single-cell resolution spatial transcriptomics dataset of murine experimental autoimmune encephalomyelitis which includes four time points after disease induction.

148 Distinct cellular distribution gradients at the tumor interface zone of clear cell renal cell carcinoma

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Clear cell renal cell carcinoma (ccRCC) is the most prevalent kidney malignancy characterized by an expansive growth pattern and formation of a pseudocapsule at the tumor boundary. Here, we focus on a detailed analysis of this interface zone (TI) of ccRCC employing a non-proprietary cyclic immunohistochemistry method (cycIHC). 3D reconstruction of whole-slide-images (WSI) of the TI revealed only modest differences, whereas granular analysis in a set of more than 30 patients using tissue-microarrays (TMA) showed distinct distribution gradients for major immune cell populations. Correlation of cellular distribution patterns with defined extracellular-matrix (ECM) components further indicated ECM-instructive features defining cellular micro-niches within the pseudocapsule zone. Side-by-side comparison towards other available low-plex multiepitope staining technologies further demonstrated equal performance levels of cycIHC. Altogether, our data demonstrate that the pseudocapsule zone of ccRCC exhibits a complex microenvironment defined by distinct ECM patterns.

149 Comparison of computational methods analyzing cellular colocalization in tissues

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Studying the spatial characteristics of tissues is essential for understanding their function in health and disease. Spatial omics technologies have enabled the study of single cells in their native spatial context, providing valuable information about cellular architecture and interactions. A variety of methods for spatial cellular neighborhood analysis have been developed to retrieve these spatial patterns from biological data. However, these methods differ in their biological and algorithmic approaches, and have not been systematically compared. In this project, we identified a common algorithmic framework of neighborhood analysis tools that allows for a modular understanding of the methods. Within this framework, we conducted a systematic comparison of cellular colocalization methods by assessing the ability to differentiate tissue cohorts based on their analysis results. To generate cohorts with different tissue architecture, we utilized the recently released framework for in silico tissue simulation. This allowed us to also evaluate the tool's ability to recover ground-truth cellular colocalizations, which is not possible with biological tissue data. After identifying advantageous algorithmic features across cell type co-localization tools, we combined them to create an optimal co-localization method. The method can recover directionality of co-localizing cell types and is sensitive to variations in co-localization strength among different cohorts. Our study serves as a first comprehensive guide for users and method developers in the field of neighborhood analysis, while offering a novel approach to evaluate spatial omics tools.

Building a classifier to predict occurrence of postoperative pancreatic fistula with MALDI Imaging of tryptic peptides **150**

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Postoperative pancreatic fistula (POPF) is defined as leakage of the pancreatic exocrine secret after pancreatic surgeries. Even though several risk factors such as body mass index, mean pancreatic duct diameter and soft pancreatic texture have been identified, POPF still remains a major postoperative complication with a mortality rate of approximately 1% (1990-2015). A defined routine diagnostic evaluation to estimate the risk of POPF occurrence would provide a standardized method for better prognosis. In this study, mass spectrometry imaging (MSI) of tryptic peptides was applied to build a classifier for prediction of POPF development after pancreatoduodenectomy.

This cohort consists of formalin-fixed, paraffin-embedded pancreatic resection margins of 24 patients of whom 12 developed POPF. Measurements were performed with a spatial resolution of 100 µm on a 4800 MALDI-TOF/TOF Analyzer resulting in 247250 spectra. Data analysis was performed with the reproducible "Cardinal" tools on the European Galaxy Server. Supervised classification and segmentation were performed on the preprocessed dataset to build a c and to determine regions of interest that might be relevant for distinguishing POPF samples from control. The classified regions of interest show a correlation with m/z-features associated with collagens, in accordance of the suggested impact of fibrosis on POPF development. For identification of the detected m/z features, an optimized workflow was applied to match LC-MS/MS with peptide imaging data by using trapped ion mobility.
Altogether, this study aims to find molecular and spatial features by MSI to predict the occurrence of POPF after pancreatoduodenectomy and investigate its correlation with ECM structure.

151 Implementing imaging based spatial omics at EPFL Core facilities

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Spatially resolved omics have become key technologies in the research community to investigate specific gene transcripts and proteins in a spatial context. However, due to their high cost and required expertise in molecular biology, high-throughput sequencing or imaging, and advanced data analysis, only few specialized labs have access to them.
Our goal as core facilities is to democratize these tools, and help users select the best option for their research questions. For this, collaborations between facilities are paramount. We offer a wide variety of complementary and combinable solutions, described below and on a dedicated website.
For spatial transcriptomics, the Genomics Facility offers sequencing-based solution like Visium HD (10X Genomics). Imaging-based solutions are available at the Histology and Microscopy Facilities, with either RNAscope (ACDbio), Xenium (10X Genomics), or a version of hybridization-based in situ sequencing (HybISS)[1] implemented in an automated way in-house. This allows a high level of flexibility in species and protocol, and is open-source at every step.
For proteomics, Laser Capture Microdissection followed by mass-spectrometry is offered at the Proteomics Facility. At the Histology Facility, the Comet (Lunaphore) multiplexing platform integrates staining and imaging of up to 40 spatial markers using off-the-shelf antibodies.
By offering a wide range of targeted and untargeted approaches, including commercial and in-house solutions, we can cater to the precise needs of researchers. Our integrated workflows enable analysis of both transcriptomic and proteomic landscapes in situ, providing access to advanced spatial tools to meet the diverse needs of the research community.

A glance at the single-cell data from the first 1200 cancer patients collected within the IMMUcan consortium

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Many cancer patients benefit from immune checkpoint inhibitor therapy. However, response rates vary across cancer types and patients and biomarkers to predict treatment response are scarce. To investigate the tumor microenvironment (TME) across cancers and identify novel biomarkers, large-scale, standardized single-cell and multi-omic studies are required. The EU-funded IMMUcan consortium is a partnership of industrial and academic organizations with the goal to profile the tumor microenvironment from up to 2700 patients suffering from either breast, colorectal, renal, head and neck or lung cancer. Each tumor sample is analyzed using Imaging Mass Cytometry (IMC), multispectral imaging, bulk RNAseq and whole exome sequencing. Here, we highlight our IMC workflow generated within IMMUcan. We provide a first comparison of the TME of 1'200 cancer patients across three cancer indications. Further, using a cohort of non-small cell lung cancer we developed a simple prognostic score to quantify the heterogeneity of T cell infiltration across whole slide images. Using IMC and bulk RNAseq we show that uniform T cell infiltration was accompanied by signs of inflammation and tumor reactivity while non-uniformly infiltrated tumors contained less dendritic cells, more fibroblasts and showed increased gene expression of collagen related genes. In summary, the IMMUcan consortium will generate one of the richest, standardized multiplexed imaging and multi-omic dataset to date, thereby enabling a detailed characterization of the TME of different tumor types for computational tool development, early biomarker discovery and hypothesis testing.

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153 Identification and functional phenotyping of lymphoid aggregates in brain metastases

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Brain metastases (BrM) are the most common malignant tumors affecting the central nervous system (CNS). Due to the unique brain microenvironment and the blood-brain barrier, treatment of BrM has remained mostly ineffective and challenging and is still prognosis limiting for the patients. Recent studies have shown the presence of lymphoid aggregates, such as tertiary lymphoid structure (TLS), can be used to predict overall survival and response to immune checkpoint therapy in nonCNS cancers. A retrospective cohort of 461 FFPEBrM samples, derived from the biobank of the University Cancer Center (UCT) Frankfurt (Germany), was screened in a 4step approach to identify and characterize TLS, as well as investigating their clinical relevance. 1. Immunohistochemistry staining against CD20 was used to screen for lymphoid aggregates. 2. Tumors with lymphoid aggregates were further characterized by highpleximmunofluorescence (IF) imaging with 30 antibodies at the protein level. 3. Region-based and single-cell resolution spatial transcriptomics were used to investigate the whole transcriptome and expression of 468 genes, respectively. This deep phenotyping on protein and geneexpression level allows for identification of immune cell subtypes and their spatial context inside the TLS and their surrounding tumor tissue. 4. TLS expression data was correlated with clinical data to further understand the effects of different TLS subtypes on a clinical level.
TLS were observed in 50,1% of BrM and were more common in lung and melanoma, compared to breast BrM. Our preliminary survival data analysis suggests a prolonged overall survival in patients with TLS+ BrM. Due to their high prevalence, we focused our TLS phenotyping analysis on lung, breast and melanoma BrM. Different TLS subtypes were identified based on their cellular composition.

154 High throughput spatial immune mapping reveals an innate immune scar in post-COVID-19 brains

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The underlying pathogenesis of neurological sequelae in post COVID-19 patients remains unclear. Here, we used multidimensional spatial immune phenotyping methods on brains from initial COVID-19 survivors to identify the biological correlate associated with previous SARS-CoV-2 challenge. Compared to healthy controls, individuals with post COVID-19 revealed a high percentage of TMEM119$^+$P2RY12$^+$CD68$^+$Iba1$^+$HLA-DR$^+$CD11c$^+$SCAMP2$^+$ microglia assembled in prototypical cellular nodules. In contrast to acute SARS-CoV-2 cases, the frequency of CD8$^+$ parenchymal T cells was reduced, suggesting an immune shift towards innate immune activation that may contribute to neurological alterations in post COVID-19 patients.

Machine learning on single-cell spatial data reveals that MHCII expression by cancer cells shapes ovarian cancer microenvironment **155**

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Objective: To unravel the association between the spatial tumor microenvironment of High-grade serous cancer's with molecular profiles and clinical outcomes.

Materials and methods: We employed highplex tissue imaging (tCycIF) of 250 HGSC tumors using 1000 TMA-cores and 34 different protein markers. We categorized single cells to distinct subpopulations using the TRIBUS and SCIMAP software. The immune cell infiltration was also assessed by pathologists using IHC for CD8, CD20, CD68, and CD103. Using DNA sequencing and methylation assay we categorized the tumor samples in four molecular profiles: BRCAloss, homologous recombination deficient (HRD) or proficient (HRP), and CCNE1-amplification. We developed a machine-learning random forest pipeline that uses single-cell marker intensity, morphology, and neighborhoods, to classify cells into molecular profiles, and also into long or short survival groups. For validation sets, we used 1) deconvoluted cancer-cell specifc RNA-expression from The Cancer Genome Atlas (TCGA), 2) Whole-slide highplex tCyCIF imaging from 15 samples from an independent cohort and 3) Publicly available Visium data of 8 samples.

Results and discussion: Our machine learning pipeline achieved an accuracy (F1 score) of 0.77 in classification of individual BRCAloss cancer cells from HRP cancer cells in chemotherapy-naive samples. We detected that BRCAloss and HRD cancer cells exhibited enrichment in immunogenic signals (MHC-I and MHC-II) compared to HRP and CCNE1-amplified cancer cells. Furthermore, when training our model to predict high or low survival within HRD or HRP profiles, MHC-II and MHC-I were identified among the most important features for the prediction and also in our validation sets. We observed that MHCII modulates the spatial association with immune cells creating immune 'hot' niches and increasing INF-γ; this was corroborated in our independent validation sets.

Conclusion: Our study revealed distinct phenotypic cell subpopulations linked to antigen presentation which were associated with molecular profiles and clinical outcomes.

156 Unsupervised detection and characterization of immune cell clustering in tissues

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Analyzing immune cell clustering in tissues is crucial to understanding inflammatory responses, particularly in the bladder, where chronic inflammation is associated with neutrophils and diseases such as urinary tract infections. By using the Markov Cluster Algorithm (MCL), we have developed an unsupervised cluster analysis workflow to study the spatial distribution of immune cells. We calculated the pairwise shortest paths between neutro-

phils using a breadth-first search approach that takes into account the complex, curved structure of the urothelium. To further emphasize spatial proximity, we performed a sigmoid transformation before applying the MCL algorithm, strengthening the influence of neighboring cells. To ensure the statistical significance of the observed clusters, we compared the results with randomly distributed cell patterns using permutation. In addition, we used alpha shape analysis to assess cluster density and the spatial relationship of clusters to tissue boundaries providing insights into neutrophil localization within the tissue architecture. Our findings show that the application of the MCL algorithm on the sigmoid-transformed cell-cell distances combined with the subsequent alpha-shape analysis effectively identifies and characterizes neutrophil clusters in the infected bladder tissue. This study highlights the power of unsupervised graph clustering techniques in spatial biology and provides new insights into the organization of immune cells in tissues.

Tertiary lymphoid structures contain stem-like T cell reservoirs and act as amplifiers of immunotherapy response **157**

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Despite the success of PD1 blockade in cancer, the mechanisms of T cell reinvigoration are not fully understood. Recent research emphasizes the role of tertiary lymphoid structures (TLS) as crucial immune niches that influence therapy response. TLS are spatially organized immune hubs linked to better clinical outcomes, yet their role in T cell-driven antitumor immunity is unknown.

We used spatial omics and ex vivo technologies with transcriptional and functional profiling of lung cancer tissue to study how TLS affect T cell specificity, state and capacity for reinvigoration. RNA sequencing revealed enriched T cell activation pathways in TLS, indicating they are hotspots for T cell activity. TCRβ sequencing showed clonal overlap between TLS and tumor bed, suggesting that T cell activity in TLS is not driven by a distinct repertoire. Shared clones displayed dysfunctional states, indicative of tumor reactivity. Analysis of spatial transcriptomics datasets revealed that T cells in TLS enriched for early dysfunctional

states, while the tumor bed contained more effector-like cells. Using patient-derived tumor fragment (PDTF) ex vivo technology, we found that T cells in both TLS and tumor bed were reactivated upon PD1 blockade, with TLS showing greater intensity and diversity in cytokine secretion.
These findings suggest TLS enhance therapy response by acting as reservoirs for precursor-like cells that replenish the antitumor T cell pool and amplify T cell responses upon PD1 blockade.

158 Resolving Cell-Type Spatial Profiles with Weight-Induced Sparse Regression

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Technological advances have led to the collection of large, complex datasets, requiring sophisticated processing techniques. A major challenge is whether existing deconvolution tools can accurately predict cell-type localizations in spatial transcriptomics (ST) data. While single-cell RNA sequencing (scRNA-Seq) provides high-resolution gene expression data and captures cell heterogeneity, it lacks spatial information. Conversely, spatially resolved gene expression profiles obtained from spatial transcriptomics (ST) help us to understand tissue organization and function, but ST lacks single cell resolution with whole-genome coverage. Existing tools try to bridge this gap through various deconvolution models. Yet, it turns out that many of these models fail to account for the sparsity characteristic of biological systems and use brute-force algorithms to predict even spatially non-existing cell-types. Here, we developed a machine learning model called **weight-induced sparse regression (WISpR)**. WISpR integrates high-resolution cell-types from scRNA-Seq to deconvolute their spatial profiles from ST, by preserving their biological relevance. The performance superiority of WISpR was benchmarked against 5 recently published models using 4 matched and 6 blended simulated datasets and revealed how brute-force approaches fail when the cell-types in scRNA-Seq data do not always match with ST content. Moreover, WISpR was applied on 2 healthy datasets as the developing embryonic human heart and mouse brain and correctly mapped the cell profiles and defined overall tissue architecture, successfully. Finally, WISpR revealed the zone-specific cellular cancer heterogeneity in human breast cancer. Overall, the principle of WISpR was driven by the sparsity of nature and it will provide promising contributions to personalized medicine as it enables the high-resolution molecular profiling of biological tissues.

Properties of Image Transformers in Spatial Transcriptomic data 159

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In order to digitally study Spatial Transcriptomics (ST) and their accompanying histological images, many mathematical and statistical models have been developed, most recently in the artificial intelligence (AI) field. After a long reign of convolutional neural networks (CNN) as an ubiquitous tool for analyzing images and spatial data, a more recent technology has taken the digital pathology (DP) world by storm.
In recent years, large DP groups have already created and published several "Foundation models," based on Transformer architectures from the field of large language modeling. These models are trained on millions of whole slide images (WSI) and a wide variety of cancer types, other diseases, different organs, scanners and laboratories. These Transformer models make it possible to efficiently combine both gene expression data and the morphology of histological sections underlying the ST data and gain insight on virtually any tissue and disease to a molecular level.
The properties of the resulting features (or descriptions) of Transformers are however not yet studied, they are mostly evaluated in terms of their usefulness in downstream tasks but not their properties as features and their effect in ST data. It is also important to understand how they differ from their CNN counterpart before using them as freely as other AI methodologies.
We present an exploration of data in both lumbar spinal cord tissue sections for the study of amyotrophic lateral sclerosis (ALS) and Consensus Molecular Subtypes (CMS) in Metastatic Colorectal Cancer. In this explorative study we discovered the good, the bad and the ugly of features extracted with image transformers specifically trained for DP. For example, some advantages include the use of attention maps, to obtain deeper insight for the tissue characterization, while some disadvantages include artifacts that appear in some dimensions of the features that behave like scanlines even when the image does not contain this issue. We propose strategies for making use of these advantages and also dealing with potential problems Transformer features can pose when working with ST data.

Spatial mapping of the human bone marrow in health and disease 160

Thomas, Emily, Anna Sozanska

The bone marrow is a complex and highly organised tissue that is primarily responsible for blood production (haematopoiesis). It contains a diverse population of haematopoietic, stromal, endothelial and immune cells. The myeloproliferative neoplasms (MPN) are a group of blood cancers arising in the bone marrow that result in the over-production of certain blood lineages that may result in scarring of the marrow (myelofibrosis) and bone

marrow failure. They are characterised by a disruption of the normal bone marrow architecture that is incompletely understood and only partially described.
We have developed a workflow for performing high quality spatial transcriptomic (ST) profiling of archival diagnostic formalin-fixed paraffin embedded (FFPE) human bone marrow trephine (BMT) material. Using the 10x Xenium platform, we profiled 30 human BMT samples, including 5 normal BMTs and 25 from MPN patients. Within the MPN cohort, we profiled patients with non-fibrotic (n=11), pre-fibrotic (n=3) and fibrotic (n=11) disease subtypes.
We present the first whole-section spatially resolved description of the bone marrow in biopsy samples from healthy individuals and patients diagnosed with blood cancer (MPN) using the Xenium platform. At single cell resolution, we identify all the main haematopoietic cell populations and describe their spatial distribution across normal, non-fibrotic and fibrotic bone marrow trephines. By overlaying ST data onto an H&E stained image of the same section, we integrate expert pathologist annotations alongside outputs from algorithms designed for H&E morphological profiling into the ST spatial landscape. Incorporating knowledge from H&E has allowed us to improve segmentation and interpret morphological profiles in the context of gene expression. In addition, using existing continuous index of fibrosis ('CIF') AI-algorithms on the H&E sections, we integrate quantitative descriptions of fibrosis with spatial profiling to explore cellular spatial features associated with fibrotic foci.

161 Whole Slide Imaging Modes for Imaging Mass Cytometry Reveal Cellular Diversity of the Tumor Immune Microenvironment in Mouse Glioblastoma

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Mouse models are used for translational research and permit detection of therapeutic-induced immune-related modification in the tumor immune microenvironment (TIME). Imaging Mass Cytometry (IMC) is a spatial biology technique capable of quantitative evaluation of multiparametric tumor and immune cell composition in brain tissue. Here we demonstrate the application of a 40-marker panel composed of tumor and immune markers on mouse normal and glioblastoma (GBM) tissue using two new whole slide imaging (WSI) methods for IMC. Tissue Mode rapidly scans the entire tissue followed by pixel-clustering analysis to uncover spatial distribution of relevant markers. Preview Mode samples the entire tissue at predefined spacing to rapidly capture a low-resolution image of all expressed markers, improving informed identification of areas for highresolution Cell Mode imaging on the same slide. Tissue Mode imaging with pixelclustering analysis provided quantitative spatial expression patterns of tumor and immune markers across the GBM tissue. Necrotic cores, areas with high immune infiltration, extracellular matrix deposits and activated tumor cells were quantified. Preview Mode in combination with Cell Mode imaging and single-cell analysis revealed extensive tissue vascularization, replicating tumor cells and a variety of infiltrating immune cells. Overall, we highlight the capability of IMC to provide quantitative

insights into the spatial biology of the TIME.

msiFlow: Automated Workflows for Reproducible and Scalable Multimodal Mass Spectrometry Imaging and Immunofluorescence Microscopy Data Processing and Analysis 162

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Multimodal imaging by matrix-assisted laser desorption ionisation mass spectrometry imaging (MALDI MSI) and immunofluorescence microscopy (IFM) holds great potential for understanding pathological mechanisms by mapping molecular signatures from the tissue microenvironment to specific cell populations. However, existing open-source software solutions for MALDI MSI data analysis are incomplete, require programming skills and contain laborious manual steps, hindering broadly applicable, reproducible, and high-throughput analysis to generate impactful biological discoveries across interdisciplinary research fields. Here we present msiFlow, an accessible open-source, platform-independent, and vendor-neutral software for end-to-end, high-throughput, transparent, and reproducible analysis of multimodal imaging data. msiFlow integrates all necessary steps, from import and preprocessing of raw MALDI MSI data to visual analysis output, as well as registration, along with state-of-the-art and newly developed algorithms, into automated workflows. Using msiFlow, we unravel the molecular heterogeneity of leukocytes in infected tissues by spatial regulation of ether-linked phospholipids containing arachidonic acid. We anticipate that msiFlow will facilitate the broad applicability of MSI in the emerging field of multimodal imaging to uncover context-dependent cellular regulations in disease states.

163 Cost-Effective Bleach & Stain Multiplex Immunostaining Approach for Enhanced Tumor Characterization in Breast Cancer

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Multiplex immune-staining technology is a rapidly evolving technique that offers high-resolution spatial and quantitative data for the comprehensive analysis of complex biological systems. However, it remains expensive, highlighting the need for more cost-effective methods and innovations.

To address this need, we have developed a bleach & stain multiplex approach for FFPE breast cancer tissues, utilizing Akoya PhenoImager technology to extend the standard 6-plex to an 11-plex format. This enhancement improves the characterization of luminal-basal differentiation in breast cancer. The key challenges of this approach is the development of an efficient bleaching method to accommodate additional markers meanwhile preserving tissue integrity.

Two 11-marker panels were developed through an iterative process that included DAB IHC validation, positioning experiments, panel composition refinement, and Opal single-plex and multiplex optimization. The bleaching process was optimized using LED full-spectrum lamps in a hydrogen peroxide plus sodium hydroxide solution, ensuring efficient bleaching while preserving tissue architecture.

We achieved high-quality staining and tissue preservation by optimizing cooking steps, minimizing exposure to pH9 treatments, and applying mechanical protection using PAP-Pen barriers and electrostatically and biochemically adhesive-coated slides specifically for breast cancer tumor tissue. These refinements enabled efficient antibody removal and facilitated 11 staining cycles per section, successfully finalizing the Bleach & Stain Multiplex approach. Additionally, small tissue microarrays (TMA) consisting of cells from 7 cell lines were created to provide marker-specific positive controls, enhancing the reliability and consistency of staining across all 11 markers and enabling quantification of marker expression levels based on signal intensity.

Our bleach & stain multiplex approach enables two 11-plex panels with optimized staining protocols, offering a significant advancement in breast cancer subtype characterization while maintaining tissue integrity and cost-efficiency.

Mapping the human pancreas by imaging mass cytometry reveals early dysregulation in the endocrine and immune compartment in type 1 diabetes progression 164

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Background: The pathogenesis of type 1 diabetes (T1D), particularly during the auto-antibody positive (AAb+) stages preceding clinical onset, is incompletely understood.
Aims: We performed a novel, single-cell analysis of the human pancreas over T1D progression, using highly multiplexed imaging.
Methods: We analyzed pancreas sections from 88 organ donors from the Network for Pancreatic Organ donors with Diabetes (nPOD) using imaging mass cytometry. The study included single AAb+ (N=28), multiple AAb+ (N=10), recent-onset T1D (N=21), long-standing T1D donors (N=14), and non-diabetic controls (N=15). We profiled over 16 million cells, creating, to our knowledge, the largest human multiplexed T1D dataset to date.
Results: We identified gradual infiltration of myeloid, B, CD4+ T helper (T-CD4) and CD8+ cytotoxic T cells (T-CD8) into islets. T-CD4 and T-CD8 cells co-infiltrated islets and all four cell types were associated with β-cell MHC-I, and insulitic β-cell MHC-II expression.
We further showed that, along disease progression, activated macrophages and conventional dendritic cells (DC) acquired M1-polarized and mature DC phenotypes respectively and localized near islet-enriched PD1+ T-CD8 cells. These cells, just as PD1+ T-CD4 cells, were highly reflective of T1D progression.
Conclusion: We identified β-cell state, PD1+ T cells, and pro-inflammatory myeloid phenotypes as critical indicators of early disease progression, suggesting that these are potential therapeutic targets.

Spatial Mapping of Innate Immune System Activation in the Central Nervous System Following Systemic Infection 165

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Post COVID-19 condition (PCC) can be similar to chronic fatigue syndrome. The underlying pathomechanisms of cognitive impairments and/or fatigue after an infection with SARS-CoV-2 are poorly understood, and therapeutic options and management of persisting symptoms are very limited. Therefore, we analyzed cerebrospinal fluid (CSF) from patients with PCC (n=20) suffering from neurological impairment one year after infection. We detected an activation of the innate immune system response similar to marked microglial activation in the CSF of these patients compared to non-infected controls. This activation was also still observable in the tissue of patients who had passed away months after contracting COVID-19, as revealed by snRNA sequencing. To validate these findings, we applied spatial transcriptomics (StereoSeq) to fresh-frozen brainstem samples from the same donors. With this method, we can map the detected transcripts to individual nuclei to distinguish cell types and employ lower-resolution binning to delineate tissue niches. To achieve this, we performed spatial neighborhood-aware clustering on an integrated dataset of patients and controls (n=8). This allowed niche analysis of the innate immune system in the human post-mortem brain tissue and shows the spatial interaction of microglial cells with their microenvironment after systemic viral infection.

166 Benchmarking Spatial Domain Identification Methods on Spatially-Resolved (Transcript)Omics Data

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Spatially-resolved omics technologies present molecular information within its spatial context, enabling an unprecedented understanding of tissue architecture and cellular heterogeneity. Taking spatially-resolved transcriptomics (SRT) as an archetype, since 2020, over 50 methods have been developed to identify spatial domains, also referred to as clusters or niches, in SRT datasets. Existing benchmarking efforts[1,2] are limited by imbalances in dataset and technology inclusion, hindering a comprehensive overview of current methods. In this project, we introduce SpaceHack2023 (https://github.com/SpatialHackathon/SpaceHack2023), a community-driven benchmarking effort initiated by over 40 researchers worldwide. Using the SpaceHack implementation, we evaluate over 20 methods on 13 datasets across 7 SRT technologies. Method performance is assessed in terms of accuracy, spatial continuity, scalability, and usability.

Our overall assessment reveals that no single method excels in all metrics or in all datasets, rather that method performance is closely linked to the SRT technology and tissue type of the dataset. We also found that certain domains are more accurately identified across methods. For instance, in the brain dataset, the hypothalamus domain consistently achieves the highest F1 score for most methods and samples. Additionally, we incorporate continuity to showcase methods' ability to define smooth domain boundaries.

Given the overall similarity in accuracy performance, scalability and usability become critical factors. For example, we observe that Bayesian inference-based methods and Graph Neural Network-based methods tend to be runtime and memory-intensive. Usability-wise, we underscore the challenges in reproducing functional software environments and achieving reported performances from existing studies. We further question the ground truth annotations, which may suffer from technical errors and subjective biases, and propose a flexible alternative via ensemble clustering.

Through our comprehensive benchmarking efforts we share critical insights into the domain calling processes and devise practical guidelines to assist life scientists in data analysis and inspire methodologists for future development.

Untangling the Complexity of Lewy Body Disorders Using Spatial Proteomics 167

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Neurodegenerative diseases (NDDs) are a heterogeneous group of disorders affecting loco-

motor, cognitive and behavioural processes. Parkinson's disease (PD), Parkinson's disease dementia (PDD) and Lewy Body dementia (LBD), belong to the group of Lewy Body disorders and are characterized by the accumulation of alpha-synuclein aggregates (Lewy Bodies) and loss of dopaminergic neurons in the substantia nigra. To improve the prognostics, treatment and management of Lewy Body disorders, it is first necessary to improve the diagnostics. However, PDD shares many clinical and pathological features with LBD, making proper diagnosis challenging. In fact, it has been estimated that around 20% of LBD patients are incorrectly diagnosed. Furthermore, the cellular and molecular factors that govern these diseases (PD, PDD, and LBD) are poorly understood.
By applying high-parametric spatial protein mapping on post-mortem human brain (including hippocampus, amygdala, prefrontal cortex, parietal cortex, cingulate cortex, substantia nigra, and caudate) in a cohort of 68 patients (including 15 PD, 5 PDD, 23 LBD and 25 controls), our study aims to 1) shed light on the cellular features involved in PD, PDD and LBD, and 2) discover potential clinical biomarkers.
Additionally, by integrating our spatial protein maps with spatial transcriptomics, metabolomics and lipidomics data, we aim to unravel the pathophysiological mechanisms, and better understand and differentiate these Parkinsonian conditions.

168 Advancing personalized medicine by spatial biology - evaluating individual drug responses in the tumor microenvironment in advanced human *ex vivo* tumor models

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In addition to cancer cells, solid tumors consist of a tumor microenvironment (TME) containing fibroblasts, immune cells, blood and lymphatic vessels, and the extracellular matrix. Each tumor has its individual complex and dynamic microenvironment which influences its development, progression, and response to therapy. However, suitable model systems are needed to spatially track the tumor microenvironment's response to chemotherapy or immunotherapy. Here, we combined advanced human *ex vivo* tumor models with multiplex immunofluorescence stainings to trace the treatment response of single cells while preser-

ving their location.
Heterotypic spheroids consisting of tumor cells and fibroblasts or patient-derived precision-cut tumor slices (PCTS) were treated with chemotherapeutic agents or immune therapies. To spatially analyze therapeutic effects, 6-plex multiplex immunofluorescence stainings for cell types and functional biomarkers were performed. Images were analyzed using a machine-learning based workflow, which enables the comparison of biomarker expression and immune cell spatial distribution in the stromal and tumor areas before and after treatment. By analyzing co-localizations, the treatment response can be related to the different cell types present in the TME. In response to cisplatin, patient-derived PCTS showed an individual induction of apoptosis (cleaved caspase-3), DNA damage (γH2AX) and immune check point inhibitor (PD-L1). To better study responses to immune therapy, we introduced PBMCs to our model systems. Heterotypic spheroids were thus co-coltured with allogeneic PBMCs. Patient- derived PCTS were co-cultured with autologous PBMCs. These systems enable us to track T cell infiltration, T cell activation (granzyme B) and cell death induction (cleaved caspase-3). Individual patient responses to the immunotherapy were observed.
By integrating spatial biology with our advanced ex vivo tumor models, we can map the relationship between the tumor, stromal, and immune cell components within individual TMEs, visualize inter- patient differences, and predict *in vivo* therapy response. This makes our platform a valuable tool for personalized cancer medicine.

Identification and antimicrobial resistance of Bats 1 Enterobacteria in Burkina Faso (West Africa) 169

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The world is in turmoil with the re-emergence of various diseases in which bats are playing a key part. Indeed, bats are known to host antibiotic-resistant bacteria that can create major public health problems. This first survey in Burkina aimed to evaluate the antibiotics resistance profile of enterobacteria isolated from bats to 03 families of antibiotics (Betalactams, 3rd generation Cephalosporins and Carbapenems). Mist nets were employed to catch bats in six locations in Burkina Faso from December 2020 to September 2021. Swabs were collected from each bat and processed for bacterial isolation, identification, characterisation, and antimicrobial resistance. Total of 204 bats captured, 84 bats were positive for the presence of at least one enterobacteria with a prevalence of 41.17%. The infected bats were composed of nine species. 29 species of enterobacteria were isolated and tested with 03 families

of antibiotics. 35% of the bacterial species are resistant to 3rd generation cephalosporins (CRO), 46% are resistant to Betalactam (AMC) and 2.4% are resistant to Carbapenems (IMI). Resistance to CRO was observed in 11 species of bacteria and was 100% in Salmonella Typhi and Salmonella paratyphi A. Variability within bat species was observed when analyzing the bacterial resistance profile of bat species to CRO. However, there was no difference in the resistance of their bacteria to AMC. The analyses showed that bacterial resistance to the three families of antibiotics is not related to the sex of the bats. However, the percentage of metallo-betalactamase (NDM) and Imipenemase (IMP) resistance genes in the population of bacterial strains is abundant, with a rate of 96.7% for NDM gene, and 3.3% of the IMP gene. The findings of this study confirm Antibiotic resistance in strains of enterobacteria isolated from bats calls for particular attention to the implementation of global strategies in the fight against antibiotic resistance.

170 Profiling gene expression in primary cholangiocarcinoma and paired lymph nodes through Xenium in situ technology

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Background and Aims: Cholangiocarcinoma is an aggressive cancer type with very limited therapeutic options. Lymph node metastases are present in about 50% of patients at the time of diagnosis and are correlated with higher recurrence rates. The highly complex and heterogenous tumor microenvironment (TME) of cholangiocarcinoma consists of multiple cell types and is known to contribute to tumor progression, therapy resistance, and immune evasion. Specifically, cancer-associated fibroblasts (CAFs) create an immunosuppressive TME via cell-to-cell interactions and the secretion of immunosuppressive cytokines. This study aimed to profile the gene expression of the TME in cholangiocarcinoma and to reveal cell-to-cell interactions in primary tumors and paired lymph node metastases through single-cell spatial transcriptomics.
Method: A formalin-fixed paraffin-embedded (FFPE) tissue microarray composed of primary tumor and paired lymph node metastases from 8 cholangiocarcinoma patients was constructed. Spatial single-cell gene expression data was generated through the Xenium in situ hybridization technology (10X Genomics) using the pre-designed “Human Multi-Tissue and Cancer” panel for 377 genes. First, we performed a differential gene expression analysis

focused on T-cells comparing between the primary tumour and lymph node samples. We further statistically analyzed the cell neighborhood composition using a k-nearest neighbors approach based on spatial coordinates to identify populations of cells that co-localize, revealing biologically relevant interactions.

Results: As expected, the amount of immune cell infiltration was higher and the fibroblasts infiltration was lower in lymph node metastases than in primary tumors. Small duct carcinomas had less fibrosis and T-cell infiltration compared to large duct types. The detailed analysis identified differentially expressed genes in T lymphocytes in primary tumors and lymph node metastases. Regarding the spatial distribution, T cells were associated with intratumoral vessels, while the myeloid cells were localized close to the fibroblasts.

Conclusion: Spatial biology offers valuable insights into the cellular distribution and interactions within malignant tumors by providing high-resolution information on the complex and heterogeneous tumor microenvironment. This deeper understanding is essential for identifying mechanisms of tumor resistance and for guiding the development of novel therapeutic strategies.

Unraveling the phenotypic heterogeneity in melanoma through multiplex immunofluorescence **171**

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Melanoma originates from the uncontrolled proliferation of melanocytes and is the deadliest form of skin cancer. Early-stage melanoma can be successfully treated with surgical resection, however, this approach is not suitable for late-stage tumors, which are also resistant to classical anticancer therapies. Immune checkpoint inhibitors and targeted therapy have significantly improved the survival rate of patients with advanced melanoma. However, the effectiveness of both therapeutic options is often impaired by innate or acquired resistance.

Melanoma cells are characterized by great heterogeneity and possess the capacity to rearrange the transcriptomic programs, in response to the environmental conditions.

Therapy-driven inflammation has been proposed as a condition that promotes the switching of melanoma cells to a dedifferentiated phenotype, characterized by the loss of melanoma differentiation antigens. This switch allows melanoma cells to evade the anti-tumor immune response and to invade tissues.

Our study aims to characterize the complexity of the melanoma tumor microenvironment, considering both spatial context and immune infiltration. Specifically, we investigated how tissue pigmentation correlates with melanocytic differentiation marker expression, at single cell level, and whether immune infiltration depends on the spatial organization of differentiated or dedifferentiated melanoma cells. To achieve this, we performed multiplex

immunofluorescence experiments (PhenoCycler®) on FFPE samples from 38 patients with advanced melanoma. Samples included primary tumors and metastases, 17 of which were annotated by pathologists as pigmented, and 21 as unpigmented.
The macroscopic pigmentation status was compared to the protein expression of differentiation markers and immune infiltration. Cellular neighborhood analysis was also perfomed. Analyses were performed using a newly developed Python workflow (SPACEc).
Here, we present a novel and detailed characterization of the melanoma microenvironment, contributing to a deep understanding of the phenotypic heterogeneity. This provides insights into the mechanisms of therapy resistance and immune evasion, ultimately guiding the development of more effective, personalized treatment strategies.

172 Unveiling tissue compartments through multimodal feature extraction and archetypal analysis with Chrysalis

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Spatial transcriptomics (ST) has revolutionized tissue biology by enabling the profiling of gene expression in cells while preserving their native tissue context. However, existing computational methods for ST data analysis, such as clustering and deconvolution, face challenges due to resolution limits and reliance on biased reference data from single-cell or single-nucleus RNA sequencing.
To overcome these challenges, we introduce Chrysalis, a novel computational method that integrates spatially variable gene detection, morphological feature extraction from histology, and archetypal analysis. Chrysalis identifies tissue compartments (i.e., cellular niches) by modeling their emergence as convex combinations of transcriptional features in the latent gene expression space, with the option to incorporate morphological features. Additionally, it offers a unique visualization approach that facilitates rapid tissue characterization and provides information about the contribution of underlying genes, enabling the identification of spatially and functionally distinct cellular niches.
The performance of Chrysalis was evaluated through various benchmarks and validated against cell type deconvolution data, demonstrating superior performance compared to competing methods in both in silico and real-world test examples. We further demonstrated its capabilities through the analysis of a diverse set of tissue samples from multiple

ST platforms, including Stereo-seq and Visium HD. As a case study, we applied Chrysalis to breast cancer to decipher tissue heterogeneity and explore the mechanisms behind minimal residual disease, a key driver of cancer recurrence. In both mouse models and human clinical samples, Chrysalis enabled the identification of a unique drug-tolerant cancer cell phenotype with epithelial-mesenchymal transition characteristics that persists in the tumor microenvironment despite chemotherapy, offering new insights into tumor recurrence mechanisms and potential therapeutic targets in breast cancer.

Integrating in situ Proximity Ligation Assay for PD1-PDL1 interaction with multiplexed immunofluorescence imaging 173

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Background: Programmed cell death protein 1 (PD1) and its ligand PD-L1 are pivotal components of the immune checkpoint machinery, playing a crucial role in the regulation of immune responses in cancer. The interaction between PD1 and PD-L1 is a significant therapeutic target in oncology, as its inhibitors have shown improved outcomes across a variety of malignancies. However, the treatment response to immunotherapies such as pembrolizumab has shown a modest response (~30%) in previous studies. This could be explained by the lack of molecular insight pertaining to the interaction of PD1 on T-cells and PD-L1 in the tumor cells in malignant tissues at the time of clinical diagnosis. The recent development of *in situ* Proximity Ligation Assay (*is*PLA), marks a significant advancement in the field. This method allows for the visualization of direct protein interactions within tissues, for example between PD1 and PD-L1. This adds an important layer of information compared to multiplexed immunofluorescence, which is useful for deep phenotyping of cell types in a tissue but lacks the ability to detect direct interactions between specific cells.

Methods: PD-L1-PD1 interactions in human FFPE tonsil and bladder cancer tissue were identified using the NaveniFlex Tissue Atto647N kit, following the guidelines provided by the manufacturer. This method is capable of detecting proteins within a proximity of less than 40 nm, utilizing oligonucleotide-antibody conjugates that generate an enhanced fluorescent signal. After completing the Naveni® detection process, we performed multiplexed immunofluorescence (IF) using the Phenocycler platform and a panel of 14 barcoded antibodies, to generate *is*PLA and multiplexed IF data on the same tissue section.

Results & Discussion: We have successfully established an automated workflow for the *is*PLA protocol. In this work, we aim to continue this effort and combine the *is*PLA, with downstream highly multiplexed immunofluorescence using the Phenocycler platform. We have generated proof of concept data for the successful integration of the two methods, using tonsil and bladder cancer tissue to detect protein interactions between PD1 and PD-L1, as well as phenotypic profiling of the tumor microenvironment with a 14 plex panel. This

panel allows us to phenotype the relevant cell types (B cells, dendritic cells, T cells, macrophages, tumor cells and their proliferation status) and subsets of T-cells like cytotoxic CD8+ cells. The integration of the *is*PLA and multiplexed IF is poised to significantly advance our understanding of the TME and hopefully aid in patient stratification for immune checkpoint inhibition therapy. The clinical significance of this method integration will later be explored in a retrospective cohort of muscle invasive bladder cancer, receiving immune checkpoint inhibition therapy with pembrolizumab as a second line treatment.

174 In situ characterization of a clonally hyperexpanded T-cell clone in a severe case of SARS-CoV-2 vaccine-associated liver injury

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Background: SARS-CoV-2 vaccine-associated liver injury (SVALI) was first described after the implementation of mass vaccination programs against COVID-19. Recent studies have shown that spike antigen-specific T-cells can be detected in the liver of patients with SVALI and the disease is associated with an oligoclonal T-cell repertoire. Therefore, we aimed to evaluate the T-cell repertoire and immune microenvironment of a SVALI case with a severe clinical course.
Methods: Liver biopsy and liver explant formalin-fixed paraffin-embedded (FFPE) tissue sample materials were obtained from a patient with severe SVALI. T-cell receptor sequencing was performed from isolated total FFPE RNA. An RNA in situ hybridization (RISH) probe was designed against the complementarity determining region 3 (CDR3) sequence of the hyperexpanded T-cell clone. Combined multiplex immunofluorescence (mIF) and RISH staining was performed by sequentially applying the Phenocycler-Fusion System (formerly CODEX) and BaseScope RISH assay to the same liver explant section. The acquired mIF and RISH images were further co-registered to localize and phenotype the hyperexpanded T-cell clone in situ.
Results: T-cell repertoire analysis identified a hyperexpanded T-cell clone with a frequency of more than 14% in both the patient's liver biopsy and explant. This clone was shown to have a similar CDR3 sequence to a previously identified spike-specific T-cell clone. The presence of this clone was validated in situ by combined mIF/RISH assay. RISH+ clones were characterized as CD8+ Granzyme B+ effector T-cells and a small proportion of which expressed memory (CD45RO) and tissue-resident memory (CXCR6) cell markers.
Conclusions: In the liver of a severe SVALI patient, we have identified a clonally expanded

persistent effector CD8+ T-cell clone that shows sequence similarity to a T-cell clone with a known spike antigen specificity. Therefore, this study provides further evidence for the potential role of oligoclonal T-cell response and spike-like effector CD8+ T-cells in this rare entity.

Single-cell and spatial multi-omics to understand human cell biology from embryogenesis to aging and disease 175

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Innovative single-cell and spatial multi-omics technologies are revolutionizing the study of complex biological systems by enabling the analysis and integration of data from the genome, epigenome, transcriptome, proteome and/or metabolome of single-cells at unprecedented resolution. Over the past decade, the field has experienced remarkable technological advances that enable crucial new insights into cellular heterogeneity, spatial organization, and the molecular mechanisms that govern development, aging and disease. At the lab of Prof. Thierry Voet and the KU Leuven Institute for Single Cell Omics (LISCO), our research is dedicated to developping, benchmarking an applying both experimental and computational single-cell and spatial (multi-)omics technologies, with a strong focus on human embryogenesis, cancer biology and neuropathology. By highlighting the latest advances in these rapidly evolving technologies and showcasing our recent applications in biomedical research, we demonstrate their transformative potential and substantial value for advancing fundamental cell biology and clinical translational research.

ActiveVisium: An AI-Based Assistant for Rapid Visium Spot Annotation 176

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Introduction: Spatial transcriptomics technologies such as 10x Visium enable studying gene expressions within tissue context. A crucial step in the validation of experimental and computational analysis methods includes cross-referencing the findings with spot-level pathologist annotations. With typical experiments comprising numerous tissue samples containing 5,000 to 11,000 spots each, manual annotation quickly becomes impractical. To address this challenge, we introduce ActiveVisium - an AI-based assistant for rapid Visium

spot annotation.

Material and methods: ActiveVisium leverages foundation models in digital pathology combined with active learning methods to facilitate spot annotations. Starting from a small fraction of annotated spots, ActiveVisium predicts annotations for remaining unannotated spots. The model is iteratively refined by the pathologist's feedback and annotations that are provided for the most informative unannotated spots identified via active learning strategies.

Results: ActiveVisium is validated on FFPE breast and FF colorectal cancer samples, demonstrating its effectiveness in complex and heterogeneous tumour environments. By annotating around 10% of all spots in the breast cancer sample, the model achieves high predictive performance, with an average f-score exceeding 0.8. In the case of colorectal cancer samples, the model demonstrates strong annotation transfer capabilities across replicates and donors: an f-score over 0.7 is achieved for tumour spots without any prior annotations.

Conclusion: As an AI assistant, ActiveVisium facilitates the annotation process**,** enabling pathologists for the first time to annotate previously prohibitively large ST datasets and to focus on refining annotations. This greatly facilitates ST data analysis and development and validation of novel computational approaches, e.g. prediction of transcriptomic signatures from HE images.

Keywords: Spatial transcriptomics, active learning, digital pathology

177 sPCA applied to single-cell morphometrics data reveals T-box dependent spatial patterns of epithelial tension in the Second Heart field

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Spatially structured cell heterogeneity within tissues is essential for healthy organ function. This heterogeneity arises from a tightly regulated interplay of cell proliferation, cell differentiation and spatial organization during development. Here, we present a quantitative single-cell morphometric approach, including force inference analysis, to characterize the interacting dynamics of epithelial mechanics and genetic mechanisms [1]. We introduce spatial Principal Component Analysis (sPCA) as a relevant method to analyze multivariate spatial patterns in epithelial tissue and compare multiple conditions, at different time points and with different genetic perturbations. sPCA uncovers multivariate spatial patterns by maximizing the product of spatial autocorrelation (Moran's index) and feature covariance

[2]. We use our approach to study the development of the vertebrate heart tube, which extends by progressive addition of epithelial second heart field (SHF) progenitor cells from the dorsal pericardial wall. Our approach reveals that cell orientation and stress direction are the main parameters defining apical cell morphology and distinguishes cells adjacent to the arterial and venous poles in the SHF. At the genetic level, we show that while the T-box transcription factor Tbx1 is necessary for cell orientation towards the arterial pole, activation of Tbx5 in the posterior SHF correlates with the establishment of epithelial stress and SHF deletion of Tbx5 relaxes the progenitor epithelium. Collectively, our results show that integrating findings from cell-scale feature patterning and mechanical stress provides new insights into cardiac morphogenesis.

[1] "Single-cell morphometrics reveals T-box dependent patterns of epithelial tension in the Second Heart field", Clara Guijarro , Solène Song*, Benoit Aigouy, Raphael Clement, Paul Villoutreix#, Robert Kelly#, Nature Communications, in press*
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Spatial Immune Profiling of a Brain Border Region with Multiplex Imaging Proteomics **178**

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Recent advancements in spatial biology have positioned it as a rapidly emerging field with significant potential. Imaging proteomics has also seen substantial upgrades, notably in utilizing extended antibody panels exceeding 100 markers. With this innovation, researchers can capture an unprecedented level of biologically relevant data, facilitating the identification, quantification, and profiling of diverse cell populations and tissue structures, as well as mapping their spatial interactions. In this study, we present a comprehensive profiling of a brain border region, the dura mater, using the Akoya PhenoCycler-Fusion platform with a curated 32-antibody panel. Given the complex nature of the data, we specifically developed or refined various computational methodologies to enable a comprehensive analysis. Our approach includes accurate semantic segmentation of diverse immunological cell populations within the dura, utilizing extensively fine-tuned models that sequentially segment multiple input channels. The downstream analysis features (1) morphological profiling of macrophage populations across different conditions, (2) cell-to-cell interaction analysis, (3) vasculature network registration coupled with identification of various types of vessels, and (4) neighborhood analysis. Taken together, these methods provide a holistic understanding of the immune landscape within the brain border region and the relationships between different cellular and structural elements. Lastly, we highlight the challenges in the field that continue to hinder imaging data analysis.

179 Mass spectrometry based spatial analysis of postnatal human lung development

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Postnatal lung development is crucial for a healthy respiratory system. Although numerous studies have been conducted in animal models, many key mechanisms during postnatal development are not well understood in humans.

In this study, we aim to characterize the dynamic changes of postnatal lung development by implementing state-of-the-art laser-capture microdissection mass spectrometry (LCM-MS) based spatial proteomics on FFPE samples. In our pilot study, 4813 proteins were profiled from 5 donors.

The preliminary proteomic data indicates spatiotemporal changes in protein composition of different tissue niches across donors spanning 0-11 years of age. We observed enrichment of specific cellular marker proteins in subtissular niches, such as the proximal and distal airways, secretory glands, bronchioles and alveolar septae as well as arteries and veins; this cell marker enrichment also shifts with age, suggesting a development-driven cell population shift. Moreover, certain proteins exhibited expression gradients along the proximal-distal axis of the airway. After narrowing down to only proteins with high correlation with age of each tissue niche, approximate linear trends in abundance with age are observed particularly in extracellular matrix (ECM) associated protein families (e.g., laminin, collagen).

In conclusion, our findings provided a draft of protein in postnatal lung development. Meanwhile, experiments and analysis of the full tissue cohort of the study are underway.

180 Multimodal spatial atlas of non-small cell lung cancer decodes niche-specific tumor-immune regulation along the axis of tertiary lymphoid structure maturation

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Tertiary lymphoid structures (TLSs) are highly organized aggregates of immune cells that form ectopically in non-lymphoid tissues. TLSs have been observed in many cancer types and are often associated with good prognosis and improved response to immunotherapy. However, the mechanisms of cancer-associated TLS formation are still poorly understood. We therefore constructed a multimodal spatial atlas of TLSs in non-small cell lung cancer (NSCLC) in order to better characterize its cellular organization and maturation process.
Our atlas consists of three synergistic data modalities: single-nucleus RNA-Seq from FFPE tissue alongside single-cell spatial transcriptomic and proteomic profiling by Nanostring CosMx SMI and MIBIscope, respectively. With the guidance of established histopathological classification, the atlas spans more than 200 regions-of-interest across 10 unique patients. Capturing the full spectrum of TLS maturity, from early lymphoid aggregates to mature TLSs, our atlas provides a unique spatial perspective on TLS development. Furthermore, we developed a new method inspired by recent advances in geometric deep learning to derive a more holistic characterization of these structures. Specifically, we use a graph variational autoencoder (GVAE) to learn embeddings that encode both spatial and phenotypic properties of our data, allowing us to identify niches with different compositional and transcriptional patterns correlating with substructures like germinal centers, mantle-like zones, and stromal regions.
The pseudo-temporal alignment of TLS progression-specific spatial niches, identified using these embeddings, suggests that localized communities of antigen-presenting cells, their interacting partners, and tumor infiltrating lymphocytes may play key roles in the regulation of tumor microenvironment immunogenicity. Here, we observe a series of coordinated, multi-cellular state changes that could potentially be involved in the remodeling of the tumor-microenvironment along the axis of initial lymphoid aggregation to mature TLS formation. By identifying key transitional states across spatial niches and cellular communities, this atlas can be used to accelerate the identification of TLS initiation and progression-targeted therapeutics which modulate multi-cellular interactions.

Analysis of kidney biopsies from patients with glomerulonephritis using Imaging Mass Cytometry reveals an increase in immune cells with associated dedifferentiation and injury of tubular cells **181**

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Background: This study was conducted to develop mechanistic understanding of the tubulointerstitial impact of glomerulonephritis. For both ANCA-associated vasculitis (AAV) and

Lupus nephritis (LN), there is a lack of patient-targeted therapies.
Methods: 30 FFPE cortex samples from 15 patients (5 with AAV, 5 with proliferating LN, and 5 tumor-remote nephrectomy samples (reference)) were analyzed in a Tissue Microarray with imaging mass cytometry after staining with 37 markers. Cells were segmented with Mesmer. Clustering was performed using the Phenograph algorithm while interactions were analyzed with the kNN method.
Results: Analysis resulted in identification and segmentation of 106,455 cells (37.2% from reference, 31.2% from AAV, 31.6% from LN) with total analyzed tissue areas of 9.7, 5.1 and 5.8 mm^2, respectively. Reference kidneys had 119 +/- 67.57 immune cells per mm^2 tissue area whereas AAV kidneys contained 1560 +/-1100/mm^2 and LN kidneys 1372 +/- 1363/mm^2, including T cells, B cells and mononuclear phagocytes.
Cluster annotation resulted in 6 different proximal tubule (PT) clusters. H-PT was most predominant containing 47.4% of all PT cells with resident tubular markers Aquaporin 1 and Megalin being highly expressed. The second most prevalent PT cluster was FR-PT, making up 24% of all PT cells and showing predominance in injured conditions. It showed high expression of vimentin and pRIPK3 (necroptosis). 1% of all PT cells expressed Ki-67.
Interaction analysis revealed that in injured samples, FR-PT had the highest number of interactions with immune cells. They made up 15.8 % of all interaction in AAV and 22.5 % in LN, respectively.
Conclusion: AAV and LN kidney samples reveal a marked increase in interstitial immune cells with associated injury and dedifferentiation of resident proximal tubule cells. Failed repair is highlighted by increased necroptosis marker and vimentin expression with concurrent downregulation of resident tubular markers and increased interactions with immune cells.

182 MALDI Imaging applications on a new benchtop MALDI axial TOF instrument

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Expanding analytical utility from discovery-based research to translational utility requires tools that can meet the challenge. Most research grade instrumentation has not been designed with the intention of use in translational spaces, which have different requirements. Here, we characterized a new benchtop MALDI axial TOF mass spectrometer for broad application use cases and showcase the analytical applicability to perform MALDI Imaging measurements in clinically relevant research areas. A new enhanced imaging detector was installed which has stable gain even for many hours long imaging runs with high ion currents. MALDI Imaging acquisitions were conducted at an imaging speed of more than 20 pixels per

second at 20-30 µm pixel size.
Targeted expressed protein data were acquired on the system using the MALDI HiPLEX-IHC workflow on a human tonsil tissue. Additionally, a 116-plex MALDI HiPLEX-IHC experiment of lung cancer was performed. A pLSA analysis distinguished cancerous from healthy lung tissue and discriminated adenocarcinoma from squamous cell carcinoma in this pilot experiment. Individual antibody profiles were characteristic for the adenocarcinoma tumor region or the squamous cell carcinoma tumor region. The microscopy image of the same section was co-registered with the MALDI HiPLEX-IHC data. A human carcinoma section was prepared for N-glycan analysis and resulted in complex spectral profiles. It is known that changes in N-linked glycosylation occur with the development of many cancers. Specific glycans were present in the tumor vs non-tumor regions or co-localized with specific cell types as demonstrated by MALDI HiPLEX-IHC.

In situ **multiplexed imaging of single-cell drug distribution uncovers spatial features underlying chemoresistance in epithelial ovarian cancer** **183**

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TGFβ-1-Thrombospondin-1 axis regulates neutrophil infiltration into the urothelium during urinary tract infection **184**

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Background: Neutrophil migration to infection sites is crucial for clearing pathogens, but this process alters tissue microenvironments through antibacterial mechanisms. However, the cellular and molecular adaptations of neutrophils in situ are not fully understood.
Aim: This study investigates the cellular interactions between neutrophils and the microenvironment at the molecular level, focusing on intrinsic neutrophil changes to gain insights into their molecular adaptations in infected tissue niches.
Method: We used multimodal imaging (multiplex-microscopy and mass spectrometry ima-

ging) and deep learning algorithms to analyze cell- and tissue-specific signatures during *E. coli*-induced urinary tract infection in mice.

Result: Thrombospondin-1 was upregulated and secreted by urothelial cells upon UPEC infection. Inhibiting Thrombospondin-1-mediated TGFβ-1 maturation blocked neutrophil infiltration into infected urothelium. Mass spectrometry imaging showed significant lipidome changes in neutrophils during migration. Inhibiting Thrombospondin-1-dependent TGFβ-1 maturation led to LTB4 precursor accumulation in neutrophils, indicating that the Thrombospondin-1/TGFβ-1 axis plays a role in leukotriene-mediated neutrophil migration in bacterial infections.

Conclusion: Multimodal imaging identified a molecular signature and adaptation strategies of neutrophils in infected tissue, enhancing our understanding of neutrophil migration, potentially offering targets to modulate this process.

185 Enhancing Multiplex Immunofluorescence Workflows: An Automated Imaging Solution for Improved Efficiency and Reproducibility

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Background:

Spatial biology is evolving rapidly from primary research to routine application, with an increasing focus on workflow efficiency, result accuracy, and reproducibility. Multiplex immunofluorescence (mIF) stands out as a powerful tool to characterize multi-parametric disease processes within the tumor microenvironment (TME). Despite its high potential, implementing mIF protocols for routine research is challenging: mIF staining protocols are more complex than traditional chromogenic staining technology and can reveal high intensity variability intrinsic to tissue samples. The latter is usually countered by manual image acquisition processes which are time-consuming and introduce additional sources of sample independent variability preventing comparability of results.

To address these challenges, we present an innovative approach to combine the high reproducibility of Ultivue's multiplex immunofluorescent technology with a co-optimized and user-friendly automated image acquisition process. This approach significantly reduces image acquisition time and enhances reproducibility of results.

Methods:

A 4-plex OmniVUE assay was utilized to stain formalin-fixed paraffin-embedded (FFPE) tissue samples over three consecutive days. The stained slides were initially imaged on a ZEISS Axioscan 7 by three unique operators following a typical pre-defined manual routine.

Subsequently, the operators reimaged the slides on the ZEISS Axioscan 7 featuring our novel software solution. The duration of imaging process and the reproducibility of scans between the two approaches, as well as the inter-day replicates, were compared. The key metrics evaluated included the total hands-on time, image quality, and consistency of biomarker signal detec-

tion across different operators and imaging sessions.
Results:
The results demonstrated that combining highly reproducible ISP staining technology with our novel scan automation suite lead to highly consistent results, with minimal variability introduced by the imaging process. Moreover, we confirmed a reduction in hands-on time required for obtaining multiplex immunofluorescence images.
Conclusion:
The innovative automated imaging solution featuring Ultivue's mISP technology and ZEISS' optimized Axioscan 7 automation software presents a significant advancement in mIF workflows. By reducing the hands-on time required and enhancing the reproducibility and consistency of image acquisition, this system effectively addresses the challenges associated with traditional mIF imaging methods. The improved efficiency and reliability of this automated solution has the potential to accelerate research in spatial biology, enhancing speed and depth of insights into complex biological processes and more accurate profiling of the tumor microenvironment.

Enhancing Spatial Biology with Kromnigon's Streptaclick Technology and ZEISS Axioscan 7 186

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Background: Spatial biology is at the forefront of scientific research, focusing on achieving high accuracy and deeper insights into complex biological processes. Multiplex immunofluorescence (mIF) is a crucial tool in this field, allowing for detailed analysis of biomarker signatures in tumor microenvironments (TME). However, developing novel mIF assays supporting more than five biomarkers is complex and time-consuming, often requiring cyclic staining and imaging, which despite being powerful, can limit throughput and extend time to results. Imaging multiple biomarkers on the same section in a single step increases workflow efficiency to approach the throughput of standard histopathology.
Kromnigon's StreptaClick® technology for tyramide signal amplification addresses these challenges by a streamlined combination staining reagents: (I) Antibody conjugation and purification processes are streamlined by monovalent streptavidin-HRP conjugates forming direct labeled complexes with biotinylated antibodies without antibody aggregation and washing steps; (II) Mild chemical HRP quenching replaces tissue-damaging heat treatment between staining cycles, and (III) a pre-optimized selection of fluorophores enables staining of up to eight colors.
A co-optimized ZEISS Axioscan 7 microscope complements the Streptaclick® technology by providing optimized, high throughput imaging for mIF with excellent separation of all 8 fluorescence channels without the need for spectral unmixing and robust imaging protocols.

Methods: An 8-plex StreptaClick® assay was used to stain tissue samples across a typical range of tissue types. The resulting slides were imaged on several ZEISS Axioscan 7 microscopes using the same scan settings. Key metrics evaluated included image quality and precision of biomarker signal detection across different devices and imaging trials.
Results: The results demonstrated that the combination of Streptaclick® technology and the ZEISS Axioscan 7 significantly enhanced the efficiency and precision of mIF imaging. The optimized Axioscan 7 provided exceptional detection and separation of all 8 channels, ensuring high- quality images and consistent biomarker signal detection across different devices and tissue types.
Conclusion: The integration of Kromnigon's Streptaclick® technology with the ZEISS Axioscan 7 microscope presents a significant advancement in mIF workflows. By leveraging the high multiplexing capability of Streptaclick® and the precise imaging performance of the Axioscan 7, this system addresses the challenges associated with traditional mIF methods. This powerful combination enhances the speed and depth of insights into complex biological processes and provides more accurate profiling of the TME. These advancements empower researchers to push the boundaries of cellular and molecular biology, enabling more comprehensive analysis in spatial biology.

187 Deciphering Intra-Tumoral Heterogeneity and Metastatic Processes in Pancreatic Ductal Adenocarcinoma Using In Situ Sequencing

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Despite progress in clinical research leading to improved survival rates for many cancers, pancreatic ductal adenocarcinoma (PDAC) remains a challenging exception. PDAC is marked by intra-tumoral heterogeneity (ITH), which is one of the main causes of poor patient survival rates. To increase the efficacy of treatments and improve the overall prognosis of PDAC patients, advancing the personalized molecular stratification for the late stage and metastatic disease is imperative.
Spatially resolved single-cell transcriptomics (scST) methodologies such as Xenium In Situ (XIS) map individual RNA molecules at subcellular resolution and thus determine the transcriptional state of single cells in a tissue section. Their application to clinical samples promises to improve the understanding of ITH, metastasis, and treatment responses.
In collaboration with an expert team, we created a panel of 477 genes to study central pathogenic processes of PDAC. The panel includes marker genes for all major immune cells, cancer-associated fibroblasts (CAFs), "classical" and "basal-like" tumor cells as well as other pathologically relevant processes like epithelial-to-mesenchymal transition, metastasis,

and hypoxia. We used this panel to acquire scST datasets of three tissue microarrays (TMAs) comprising a total of about 100 samples from both primary tumors and metastases of 51 PDAC patients. After XIS analysis, TMA sections were stained histologically and thoroughly annotated by pathologists. To analyze the data, we established a novel framework to process and visualize XIS data of TMAs, facilitating the efficient integration of pathological annotations with computational analysis.
We identified differentially expressed genes between cells of the primary tumor site and the metastasis side, focusing especially on the cancer cells and cells of the tumor microenvironment, including CAFs and different immune cells. Further, we will use spatially aware analysis algorithms to investigate cellular interaction networks, identify markers for patient stratification and validate them on the protein level.

Adaptation of automated cell segmentation approaches for highly multiplexed tissue images. 188

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Spatial analysis of malignant tissue is imperative for accurate cancer diagnosis and treatment evaluation. Leveraging methods such as Immunofluorescence, which enable the simultaneous recording of multiple markers on a single tissue slice, known as multiplex images, holds promise in understanding differences in immune cell subsets. However, effective cell segmentation, a pivotal step for subsequent data analysis, remains a challenge. Existing approaches vary from labor-intensive manual annotation to fully automatic deep neural networks, yet few methods harness the information from multiple channels.
In this study, we propose a novel segmentation approach capitalizing on the sequence properties of transformer models, allowing the input of arbitrary sized images. Our model adopts a transformer U-net architecture comprising sliding window transformer encoder, bottleneck and decoder blocks. The encoder downsamples the data using patch merging layers until it reaches its most compact form in the bottleneck layer. Subsequently, the decoder upsamples the data back to its original size using patch expansion layers. Additionally, the decoder layers compute cross attention using the corresponding encoder blocks' key and

value vectors.
In the output function, the data is pooled to predict a binary mask and flows, resulting in a topology map. Using gradient tracking on this map generates the instance segmentation masks.
Notably, our model can be seamlessly integrated into the Cellpose model zoo, offering accessibility through a user-friendly graphical user interface. Thus, our approach holds promise in enhancing the efficiency and accuracy of spatial analysis in cancer research.

189 Unraveling Subcellular Organization in the Tumor Microenvironment Using Multiplexed Imaging of Human Tissue

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Subcellular organization reflects cellular states and functions. Advances in imaging enabled the generation of high-dimensional data with ~200 nanometer spatial resolution, allowing the study of subcellular organization in human cell lines. However, the clinical relevance of such organization remains underexplored, due to technical incompatibility with clinical samples and limited analysis tools.
We present a novel framework for subcellular quantification in cancer formalin-fixed paraffin-embedded (FFPE) samples, the most common format in pathology. Our approach leverages multiplexed ion beam imaging (MIBI), capable of visualizing over 40 proteins simultaneously in FFPE samples at confocal-level resolution. The framework covers the entire workflow—from data acquisition to interpretation—using a tailored antibody panel.
Our high-resolution images capture over ten key structures, including mitochondria and the Golgi body. Our custom analysis tools quantify subcellular features, distinguishing between cell and tissue types and potentially indicating disease progression. Ongoing work aims to link these subcellular descriptors to metabolism and clinical outcomes, offering deeper insights into disease biology.
This framework could expand quantifiable signatures in FFPE samples, aiding in patient stratification for improved prognosis and personalized treatments. It will be available to the spatial biology community, lowering barriers to subcellular studies in clinical settings.

190 Neuromelanin granules as a use case for spatial proteomics

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Neuromelanin granules (NMGs) are organelle-like structures present in the human *substantia nigra* (SN). NMGs are of high clinical relevance, since neurons with a high NMG content are the most vulnerable ones in the context of Parkinson's disease (PD) and dementia with Lewy bodies (DLB). Nevertheless, the role of NMGs in health and disease remains incompletely understood. Since NMGs are absent in the brain of common laboratory animals without genetic modifications, researchers are dependent on human *post-mortem* brain tissue, which is highly requested and therefore limited.
To account for this, we established a protocol for the proteomic characterization of NMGs in comparison to the surrounding SN ($SN_{Surr.}$) tissue, based on the use of laser microdissection and bottom-up proteomics (Wulf et al., 2021) requiring limited sample amounts. Two follow-up studies have been conducted using this workflow, with the proteome of NMGs being characterized under physiological conditions (CTRL, Wulf et al., 2022a) and neurodegenerative conditions using tissue of DLB cases (Wulf et al., 2022b). A comparison of the proteomic profiles of NMGs from CTRL and DLB cases revealed that alpha-synuclein and protein S100A9 were significantly higher abundant in NMGs of DLB cases, indicating the presence of a DLB-specific proteomic profile in NMGs.
In the current study, we analyzed *post-mortem* brain tissue from eight PD and ten CTRL cases. In concordance to our study on DLB-specific proteomic changes, alpha-synuclein was again significantly higher abundant in PD NMGs compared to CTRL NMGs. Despite this concordance, other proteins of higher abundance differed. For example, S100A9 was not changed in abundance between PD and CTRL NMGs, although it has been found to be changed in abundance in our DLB study. This and other findings indicate that proteins potentially coaggregating with alpha-synuclein in NMGs under neurodegenerative conditions differ between PD and DLB, pointing towards diseaGermanyse specific pathomechanisms.

Investigating paracrine effects in skin organoids infected with herpes simplex virus 1 191

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Organoids originating from human iPSCs are increasingly being used to investigate the molecular and cellular processes in viral infections. However, comprehensive mechanistic studies in such complex infection models are still scarce.
In this study, we generated highly complex skin organoids from human-induced pluripotent stem cells encompassing a stratified epidermis, fat-rich dermis, hair follicles, sebaceous glands, Merkel cells, neurons, and other cell types. Following infection with herpes simplex virus (HSV-1), we measured organoid slices on the Xenium probe-based spatial transcriptomics platform and analyzed using the VoltRon spatial omics software package.
Within the spatial analysis, we focused on paracrine effects of cytokines secreted following infection. For example, we detected a cell type-specific inflammatory response triggering expression of *TNF* (coding for TNFα) and *TNFSF9* (4-1BBL) following HSV-1 infection. Closely adjacent cells then underwent activation of genes that may be a consequence of paracrine signaling by the TNF cytokines. In turn, this activation of innate immunity pathways could initiate a local antiviral environment.
In conclusion, we show that combining skin organoid model with spatial transcriptomics can deepen our understanding of various virus infections within human tissue.

192 Pathologist-level cell type annotation of the mononuclear phagocyte system (MPS) in precursor B-cell lymphoma versus leukemia

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Background: MPS cells possess complex morphologies, characterized by large cell bodies, long protrusions and heterogeneous protein expression, making their accurate quantifi-

cation in tissue challenging. Our model are B-cell precursor (BCP) neoplasms which either present as leukemia (BCP-ALL) disseminated in blood, bone marrow and limited tissue manifestation or lymphomas (BCP-LBL) exclusively residing within tissues. Despite these differences, both share molecular features and are considered part of spectrum of the same disease.

Methods: QuPath-based workflows to detect MPS cell were tested and controlled by visual inspection through experienced pathologists. Two 5-marker multiplex immunofluorescence panels: CD68, CD14, CD11c, HLA-DR, CD163; Lymphoid: CD3, CD8, PD1, LAG3, FoxP3) were applied to FFPE-tissue of BCP-LBL, tissue manifestation of BCP-ALL, and Burkitt lymphoma (BL) control group.

Results: Among the methods tested, a supervised, nuclei-independent machine-learning algorithm-based pixel classifier matched the expectation by pathologists. BCP-LBL showed higher infiltration of M0 and M2 macrophages, as well as CD3, CD4, and CD8 T cells, compared to BCP-ALL. T cells in both showed low or no PD1 and LAG3 expression.

Conclusion: Detection of MPS cells required segmentation workflows different to other inflammatory cells. Our findings indicate that the immune-infiltrate differs between lymphomas and leukemias suggesting differences in the pathomechanisms of tissue invasion.

The single-cell spatial immune architecture of hepatocellular carcinoma defines patient outcomes to immunotherapy: integrative multi-omic investigation of tumor stroma between different immunotypes **193**

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The determinants of the response to checkpoint immunotherapy in Hepatocellular Carcinoma (HCC) remain poorly understood. The organization of the immune response in the tumor microenvironment (TME) is expected to govern immunotherapy outcomes but spatial

immunotypes remain poorly defined. We used the deconvolution of spatial immune network architecture to identify clinically relevant immunotypes in HCC by conductig highly multiplexed imaging mass cytometry on HCC tissues from two independent patient cohorts. We performed in-depth spatial single-cell analysis in a discovery cohort to deconvolute the determinants of the heterogeneity of the HCC immune architecture and develop a spatial immune classification that was predictve in a immune checkpoint inhibitor (ICI) therapy cohort. Three major spatial HCC immunotypes that reflect a higher level of intratumor immune cell organization were identified based on single-cell immune networks with differing involvement of immune cells and immune checkpoints dominated by either CD8 T-cells, myeloid immune cells or B- and CD4 T-cells: depleted, compartmentalized and enriched. Progression-free survival under ICI therapy differed significantly between the spatial immune types with improved survival of enriched patients. These data point to a pivotal role of the tumor stroma in regulating immune responses in the tumor microenvironment. We thus integrate spatial transcriptomic and metabolomic data with the IMC data to identify molecular and metabolic programs involved in immune evasion. These analyses indicate that pathways regulating ECM remodeling, cell adhesion, immune modulation, and particularly metabolic reprogramming differentiate the stromal features in the different immunotypes.

Conclusion

Spatial single-cell profiling and dissection of the immune architecture in the HCC TME identified major spatial immunotypes and is predictive of immunotherapy. Different immunosuppressive mechanisms in different immunotypes point to potentially alternative targets for therapy. A better understanding of the stromal immunoregulation may allow targeted conversion of compartmentalized into enriched immunotypes.

194 Uncovering the spatial pattern in tumor heterogeneity for PDAC using spatial omics

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Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy with an overall 5-year survival of less than 7%, predicted to be the biggest cancer killer in the near future. PDAC is highly heterogeneous, promoting a range of phenomena including immune escape, tumor progression, and treatment resistance, as well as different molecular subtypes dynamics, posing significant challenges to the advancement of precision oncology in PDAC. Our team has investigated the heterogeneity of PDAC using spatial regional strategies (GeoMx DSP), exploring the tumour microenvironment (CD45+), tumor epithelial (PanCK+), fibroblast-rich (αSMA+) regions. However, the lack of single-cell resolution limits the ability to investigate

the spatial dynamics and heterogeneity at a cellular level. In this study, we aim to integrate single-cell spatial transcriptomics analysis with pathological insights, generating a novel pipeline to investigate the spatial patterns influencing tumor heterogeneity in PDAC. Single-cell spatial omics is an emerging, cutting-edge technique that combines high-resolution spatial location with single-cell transcriptomics and proteomic data. Nanostring's CosMx Spatial Molecular Imager (SMI) is a spatial-omics platform enabling high plex in situ hybridization using a 6000 panel). This Pilot study analyzed tissue microarray (TMA) samples from treatment-naïve PDAC patients (n=23). Our analysis uncovered significant heterogeneity in the PDAC tumor microenvironment across patients. We identified three malignant subtypes - classical malignant cells, basal-like malignant cells, and FN1+ MT2A+ malignant cells, which demonstrated distinct spatial patterns within tumor microenvironments and epithelial structures. Furthermore, we examined the spatial correlation of stromal cells and immune cells within the context of the different malignant cells, exploring the molecular subtypes in terms of spatial context. Single-cell spatial transcriptomics provides the exact spatial locations for each cell, allowing in-depth exploration of the tumor microenvironment. By defining accurately annotating and defining cellular phenotypes, we can begin to explore cell-cell interactions related to spatial distances and correlations, further advancing our understanding of tumor heterogeneity and the immune landscape. In the future, integrating multi-scale spatial-omics techniques will allow for comprehensive cancer exploration, and has the potential to develop precise strategies for clinical diagnosis and treatment. The development of 3D spatial-omics represents the next frontier for understanding pancreatic cancer at an unprecedented depth.

Spatially resolving B cell receptor dynamics in tissues 195

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B cells are essential components of the immune system and the spatial distribution of B cells plays a crucial role in their development and functionality. B cell receptors (BCR) have two forms: membrane-bound BCRs, which mediate B cell signaling, activation, and differentiation, and secreted BCRs, i.e. antibodies, which perform important effector functions. However, where and when membrane versus secreted BCRs are expressed within tissues, for example in germinal centers, is not fully resolved, but is relevant to understand B cell clonal dynamics in health and disease.
Here, we developed a bioinformatical method based on in-house technology Spatial VDJ to map the membrane versus secreted BCR *in situ*. Membrane and secreted BCR expression were consistently identified in human tonsil tissues, and quantitative analysis showed that

their spatial distribution matched with their known functions in the tissue, gene expression clustering, and single-cell deconvolution. Integrating our receptor mapping method with single-cell gene expression data, also showed that the distribution of membrane and secreted BCRs matched with cell types. Different BCR isotypes exhibited distinct spatial patterns, likely reflecting their different functional roles. We utilized membrane versus secreted BCR information to infer B cell differentiation through spatial lineage analysis, which showed transition from membrane BCR to secreted BCR within clones.
Taken together, we can spatially resolve membrane versus secreted BCRs within different tissues. Validation using single-cell data showed that the mapping method is consistent and accurate. Spatially resolving membrane and secreted BCR information adds another dimension to the events regulating B cell clonal expansion and selection, which could help dissect BCR receptor dynamics in healthy and diseased conditions directly in their native tissues.

196 Spatiotemporal transcriptomic map of glial cell response in mouse model of acute brain ischemia

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The role of non-neuronal cells in the resolution of cerebral ischemia remains to be fully understood. To decode key molecular and cellular processes that occur after ischemia, we performed spatial and single-cell transcriptomic profiling of male mouse brain during the first week of injury. Cortical gene expression was severely disrupted, defined by inflammation and cell death in the lesion core, and glial scar formation orchestrated by multiple cell types on the periphery. The glial scar was identified as a zone with intense cell-cell communication, with prominent ApoE-Trem2 signaling pathway modulating microglial activation. For each of the three major glial populations, an inflammatory-responsive state, resembling the reactive states observed in neurodegenerative contexts, was observed. The recovered spectrum of ischemia-induced oligodendrocyte states supports the emerging hypothesis that oligodendrocytes actively respond to and modulate the neuroinflammatory stimulus. The findings are further supported by analysis of other spatial transcriptomic datasets from different mouse models of ischemic brain injury. Collectively, we present a landmark transcriptomic dataset accompanied by interactive visualization that provides a comprehensive view of spatiotemporal organization of processes in the post-ischemic mouse brain.

Automating a spatial profiling workflow to explore the effects of hypoxia in the tumor microenvironment in head and neck cancer

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Background: Head and neck cancer (HNC) is a heterogeneous group of malignancies that arise from the mucosal surfaces of the upper aerodigestive tract. The tumor microenvironment (TME) of HNC is characterized by the presence of immune cells, stromal cells, and extracellular matrix components. A key feature of the TME is hypoxia, which promotes tumor growth, invasion, and metastasis by altering the expression of genes involved in angiogenesis, cell survival, and metabolism. Understanding the complex interplay between hypoxia and immune infiltrates in the TME of HNC is crucial for the development of novel therapeutic strategies for the treatment of this disease. Whole transcriptome analysis by digital spatial profiling is an excellent method of probing the TME, but assessing large cohorts can be time consuming. Automating a profiling workflow to reduce hands-on time and region of interest (ROI) selection bias will enable exploration of large cohorts to identify mechanisms of action, potential drug targets, and biomarkers.

Methods: We developed an optimized spatial multi-omic workflow to enable high-throughput spatial analysis on GeoMx® Digital Spatial Profiler (DSP) using the Whole Transcriptome Atlas (WTA) and immuno-fluorescent morphology markers: SYTO82 (nuclei), CAIX (hypoxia), pan-cytokeratin (epithelium), CD3 (T-cells). A.I.-based analysis (Oncotopix® Discovery) of serial section H&E images and GeoMx IF images was developed to identify ROIs for GeoMx collection. Immune hot and cold selection used leukocyte density; tumor/ tromal interface selection used epithelial areas. Areas of illumination (AOI) were chosen using concentric CAIX expression gradients. Integrated analysis of digital images using Oncotopix Discovery and the whole transcriptome was done to assess the above TME compartments.

Results: Automated ROI placement based on tumor/stroma, hypoxia and immune infiltration and AI /Deep Learning based AOI segmentation reduced AOI selection time and improved accuracy of tissue compartment enrichment, especially between samples and tissue types. Automated development of hypoxia gradient-based AOI enabled a selection strategy not possible in the standar DSP software. Cell phenotyping using IF morphology scan was used to supervise cell deconvolution.

Conclusions: This work shows that ROI-based spatial analyses can be used to explore the effects of hypoxia levels on immune infiltration in HNC. Automated AI-based ROI selection provides a means of sampling relevant tumor subtypes based on hypoxia and immune infiltrate criteria in an unbiased, reproducible manner, and can provide a standardized, automated method for selecting ROIs and segmenting AOIs across a cohort of mixed tissue types and pathological subtype, improving throughput.

198 A Deep-Learning Approach to Guide Acquisition Region Selection for Imaging Mass Cytometry

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Background: Imaging Mass Cytometry™ (IMC™) is the method of choice for single-step staining and high-plex imaging of tissues while avoiding the complications of autofluorescence and cyclic imaging. IMC has three new imaging modes: Preview Mode (PM), Cell Mode (CM) and Tissue Mode (TM). PM rapidly scans a stained tissue to provide a comprehensive overview, mapping out the distribution of over 40 markers and revealing tissue heterogeneity. This enables researchers to make informed decisions about which areas warrant closer examination. Following PM, regions of interest (ROIs) are selected for high-resolution imaging. This a critical step that is informed by biomarker expression using automated AI algorithms. CM offers high-resolution imaging for detailed analysis of the ROIs identified during PM, all using the same slide. TM provides fast acquisition of the entire tissue at 5-micron resolution, optimal for quantitative pixel-based analysis. These modes support automated, continuous imaging of more than 40 large tissue samples (400 mm2) weekly.

Methods: Tissue sections of colon adenocarcinoma were stained with a 30-marker IMC panel of structural, tumor, stromal, immune cell and immune activation markers. Images were acquired on the Hyperion XTi™ Imaging System (Standard BioTools™), first in PM and then in CM with automatic selection of ROIs using Phenoplex™ software (Visiopharm®). ROIs were automatically selected based on two criteria: 1) actively proliferating and non-proliferating tumor regions; 2) cold and hot tumor regions as identified by immune hotspots within stromal or epithelial tumor regions. An adjacent serial section was acquired in TM.

Single-cell analysis of the images obtained in CM was performed using Phenoplex. Tissue segmentation divided the tissue into tumor epithelial and stromal regions; cell segmentation was based on iridium DNA channels; and phenotyping was performed using the guided workflow. This data was used to compare the immune contexture and spatial distributions via interactive t-SNE plots partitioned by spatial region and clinical variables.

Results

A high degree of immune infiltration was observed in the tumor, with significant levels of infiltrating myeloid cells. Hotspots of tumor-associated neutrophils expressing granzyme B were found, implicating their role in the recruitment and activation of intratumor CD4+ and cytotoxic CD8+ T cells.

Conclusion: This work demonstrates that Hyperion™ XTi can greatly accelerate the ability of IMC users to gain useful insights from complex biological samples. Phenoplex enables a comprehensive workflow for the analysis of this data, providing automated ROI selection, phenotyping, and spatial analyses of high-resolution IMC images for biological assessment.

Spatial Transcriptomics Reveals Systemic Functional Changes in Mouse Brain 5 Hours After Sleep Deprivation

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Introduction: Sleep is crucial for homeostasis, emotion regulation, memory consolidation, and spatial orientation. Chronic insomnia can lead to severe physiological changes and is often an early sign of neurodegenerative diseases. Previous studies have shown that sleep deprivation (SD) in mice results in memory deficits and increased foraging behavior. However, molecular-level changes have been limited due to technical constraints.
Objective: To investigate changes in gene expression and cellular interactions in various mouse brain regions following acute sleep deprivation.
Methods: Six 9-week-old male C57BL/6J mice were used, with three undergoing 5 hours of sleep deprivation and three as controls. Brains were cryo-embedded and analyzed using updated Spatially enhanced-resolution omics-sequencing V1.3 (Stereo-seq V1.3).
Results and Conclusion: Using Stereo-seq V1.3, we obtained 827,653 cells (137,942 cells per sample), successfully annotating over 84 subtypes. Significant gene expression changes were observed in the neocortex, hypothalamus, and hippocampus. In the neocortex, changes were noted in circadian regulation, synaptic signaling, and postsynaptic regulation. In the hippocampus, immediate early genes (Rps6, Elk1, Nr4a1, Egr1, and Homer1) and pathways related to neuron projection development and synapse organization were significantly altered. Using spatially informed cell bins (averaging 903 genes per cell), we analyzed paracrine/autocrine interactions and cell-cell contact interactions. We found reduced interactions between microglia and surrounding neurons in the neocortex after SD. These findings indicate that sleep deprivation affects synaptic organization, circadian rhythm, and neural plasticity, providing insights into the molecular mechanisms underlying the effects of sleep deprivation and offering a valuable transcriptomic profiling database for future research on related diseases.

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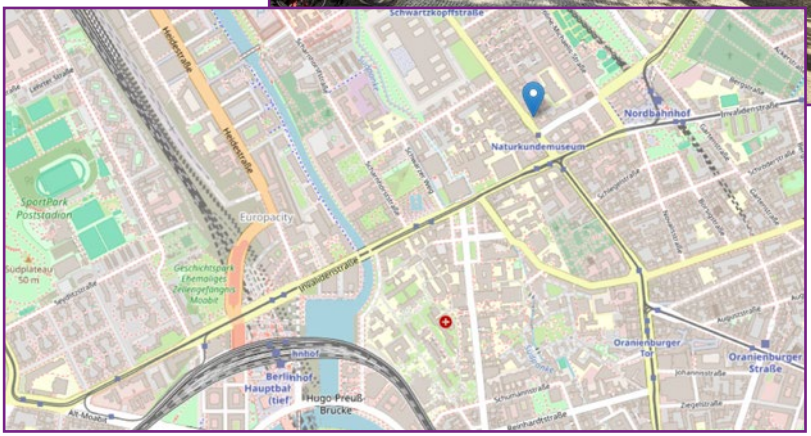

From the main train station Hauptbahnhof Berlin

From Hauptbahnhof you are just 10 minutes away by using the public transport. With the tram-line M5 oder M10 from Europaplatz direction Hohenschönhausen you will reach the stop Naturkundemuseum. Please get off at this station and turn on your left on the Chausseestraße. Just a 3-minute walk and you will see our Titanic Chaussee Berlin to your right hand side.

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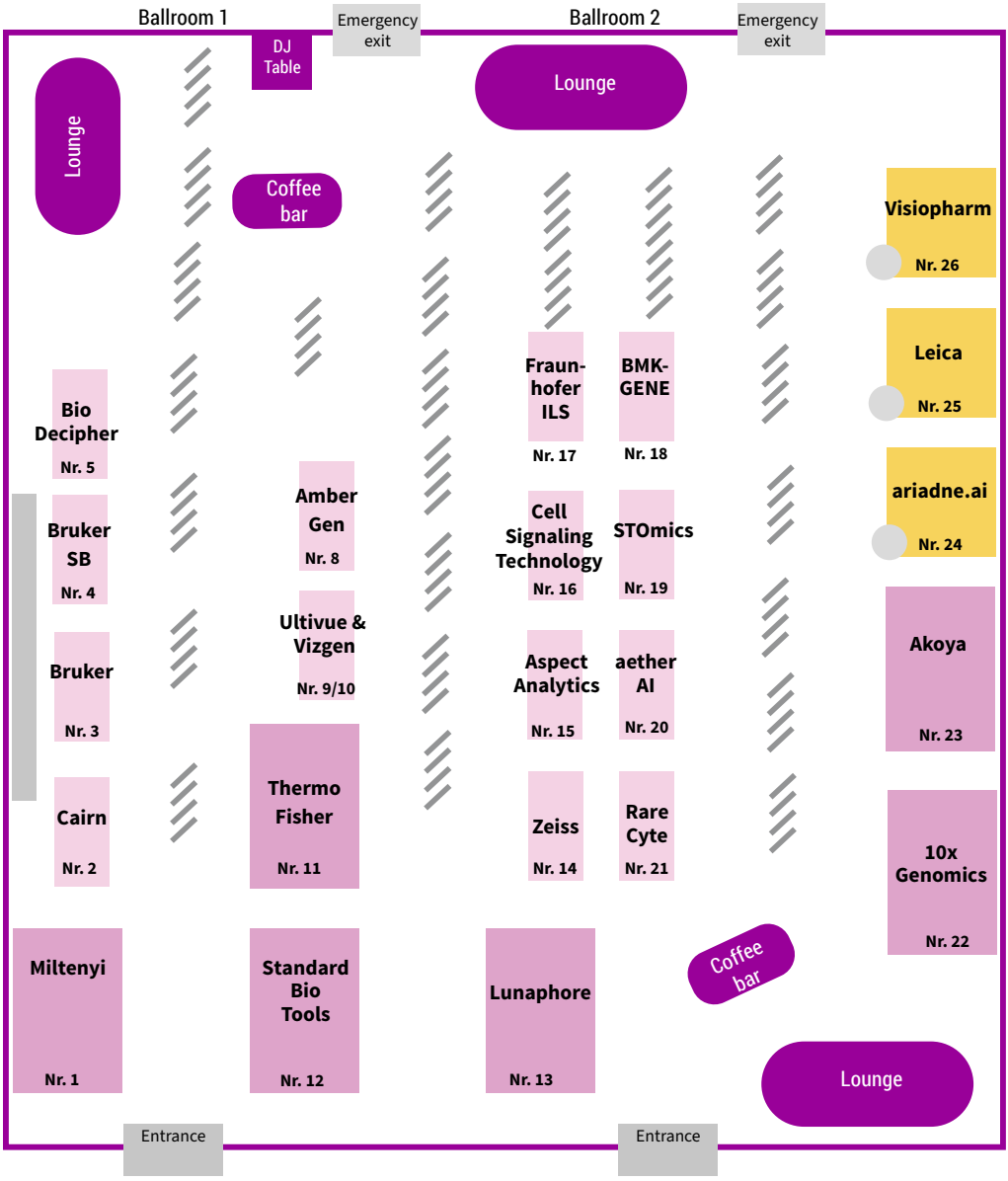
Exhibition area and Poster session



Exhbition area in Ballroom 1+2, Size of room: 580 m²

Poster wall

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Conference Organizer

Leeat Keren, PhD
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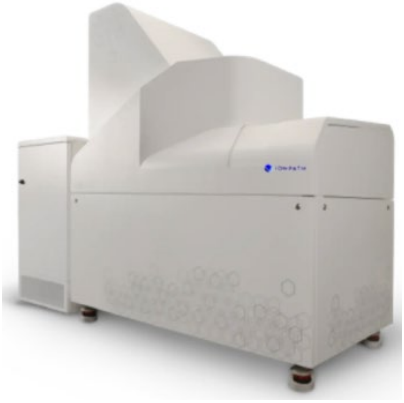

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