

Making sense of cells in space

Challenges in interpreting spatial transcriptomics data

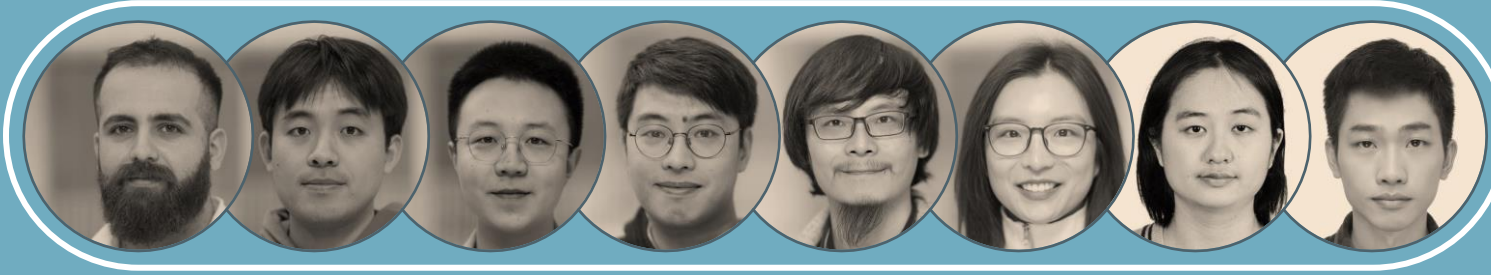
Naveed Ishaque

Berlin Institute of Health at Charité University Hospital, Germany
Imperial College London: Better Bioinformatics Seminar Series

Ishaque lab: bioinformatics to understand biological heterogeneity



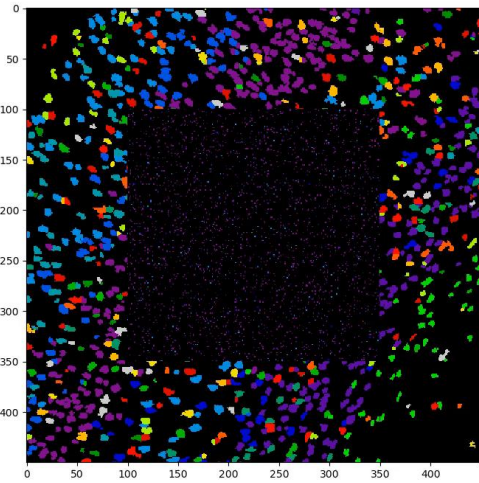
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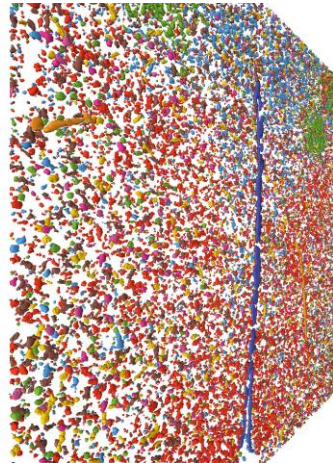
PhD



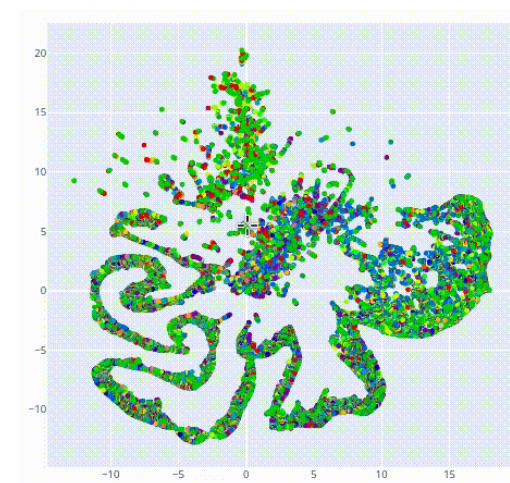
Clinician Scientist



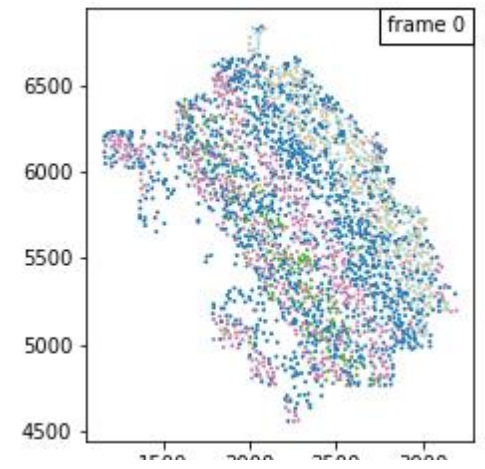
In-silico tissue generation



3D reconstruction



Interactive analysis tools



Domain identification

Major themes in my group

Using single-cell and spatial omics to better understand development and disease

Accurate cell segmentation remains a problem

- **Cell-segmentation-free** analysis as an alternative

Tissue sections are not 2D

- Identifying **cell overlaps** and **preparation artifacts** within tissue sections

Making sense of cells in space (e.g. niches, domains, tissue units)

- Advanced **spatial statistics**
- Identifications of **tissue regions** (domains/niches/spatial clusters)

Resolution revolution – transcriptomics

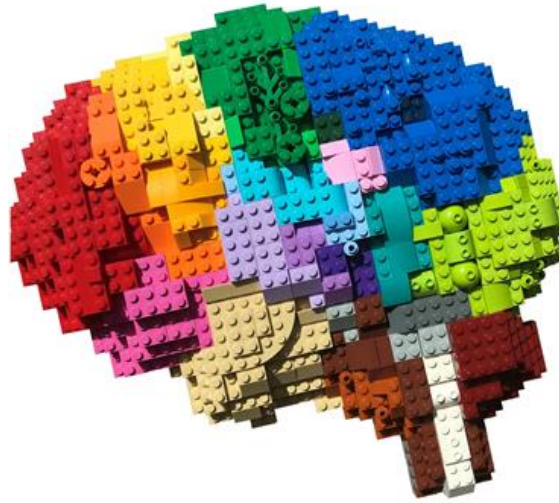
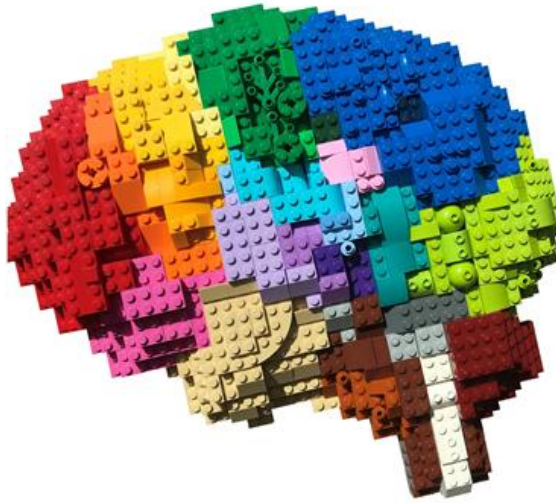


Image credit: Bo Xia (@BoXia7)

Resolution revolution – transcriptomics: bulk



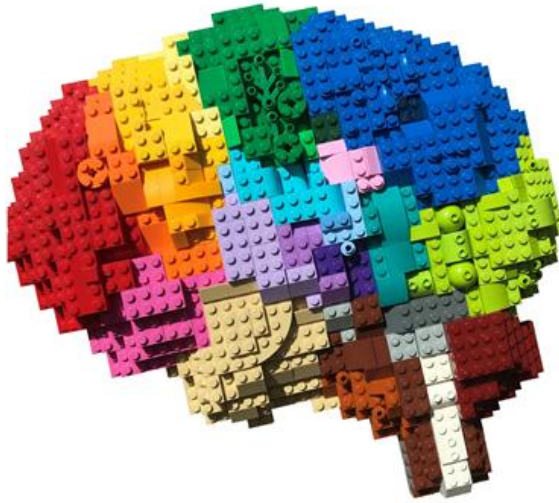
Bulk

Image credit: Bo Xia (@BoXia7)

Resolution revolution – transcriptomics: single cell

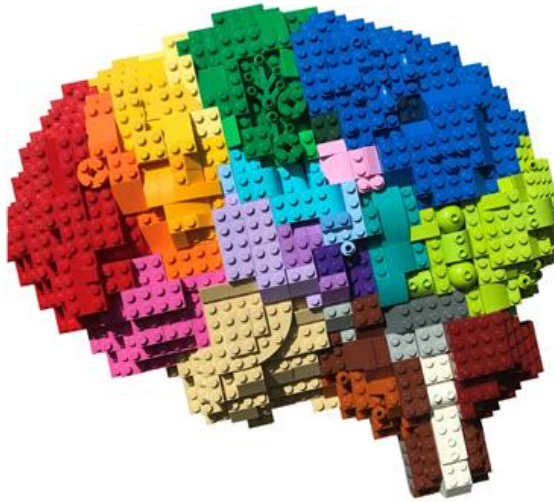


Bulk



Single-cell

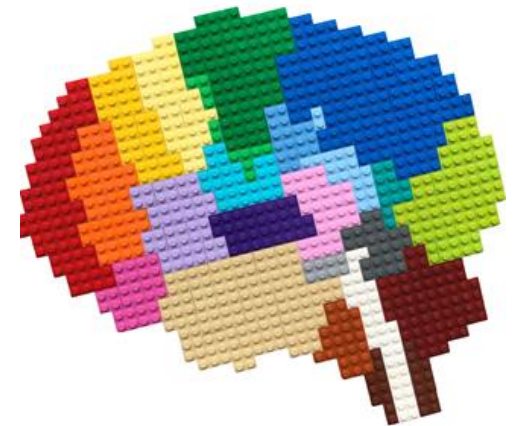
Resolution revolution – transcriptomics: spatial



Bulk

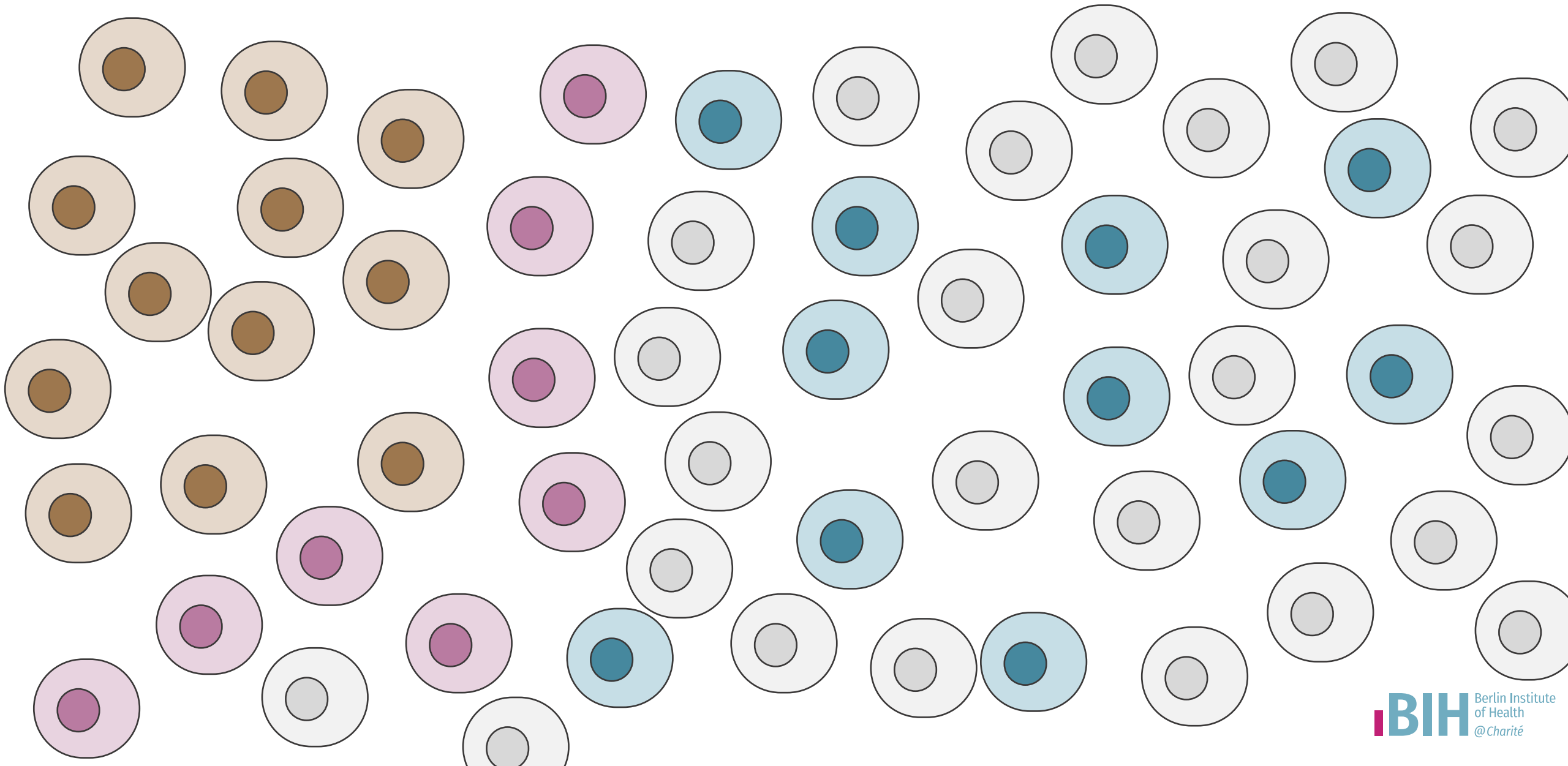


Single-cell

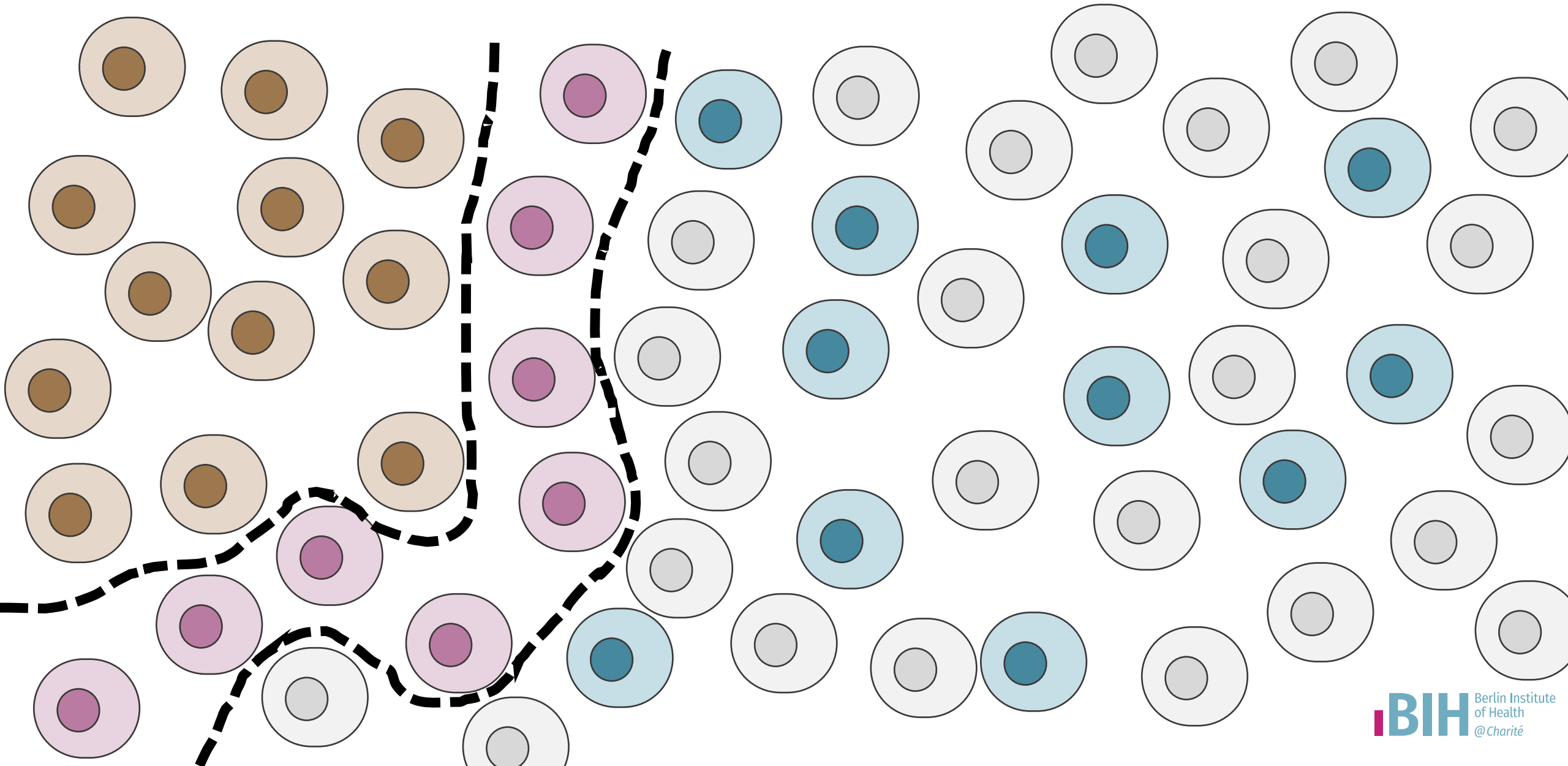


Spatial

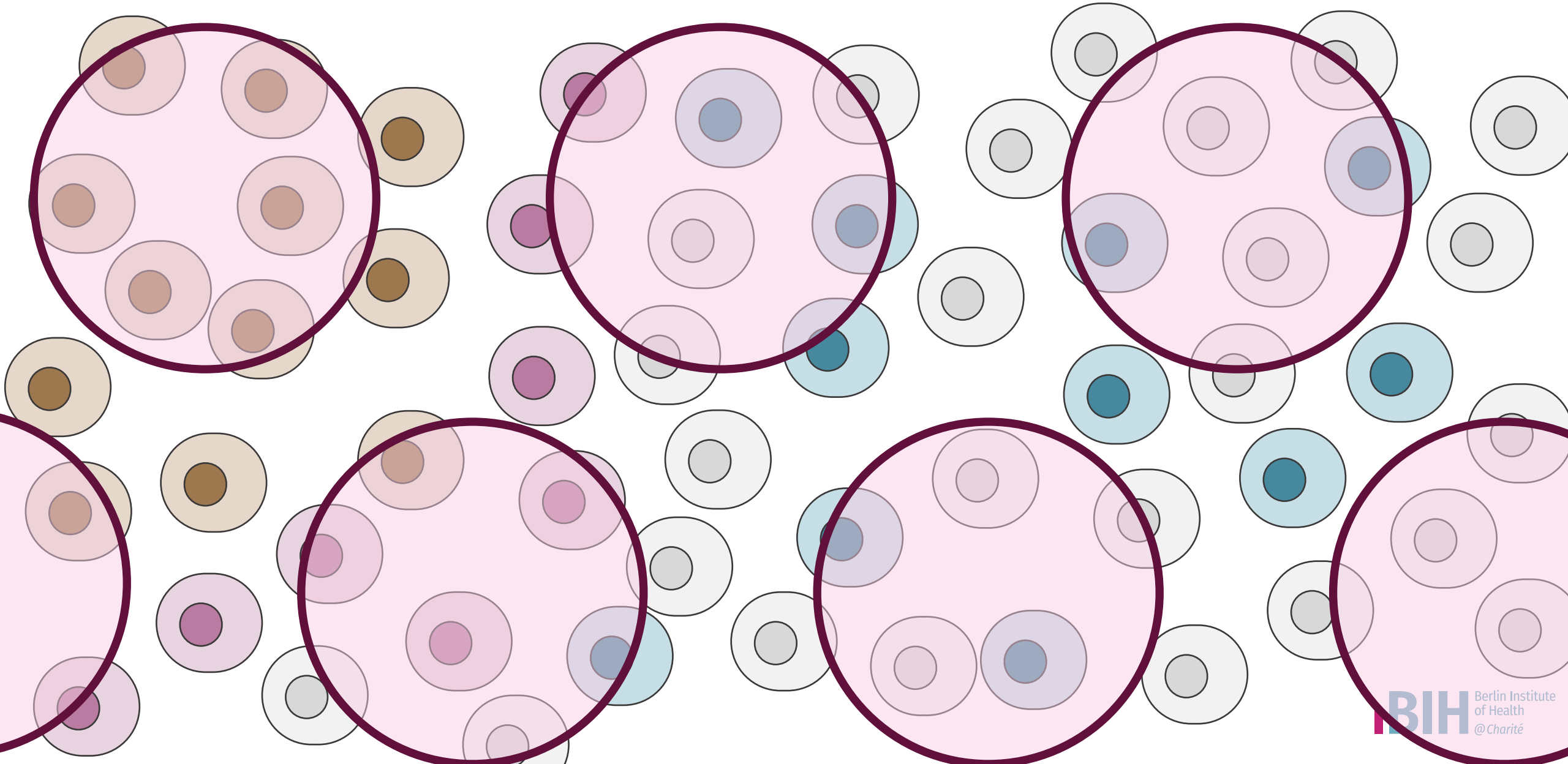
Spatial resolution



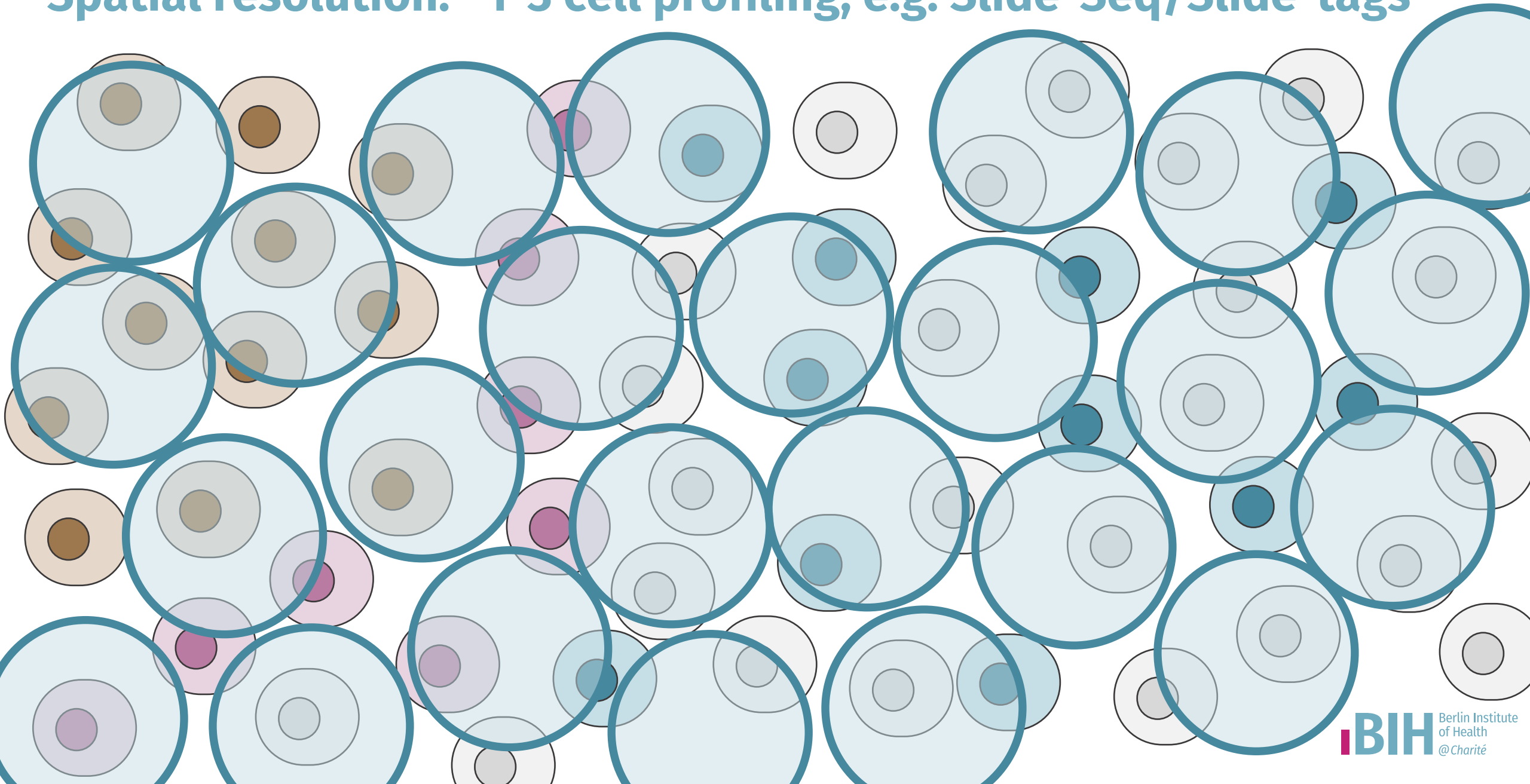
Spatial resolution: microdissection, e.g. TIVA, Geo-seq, etc



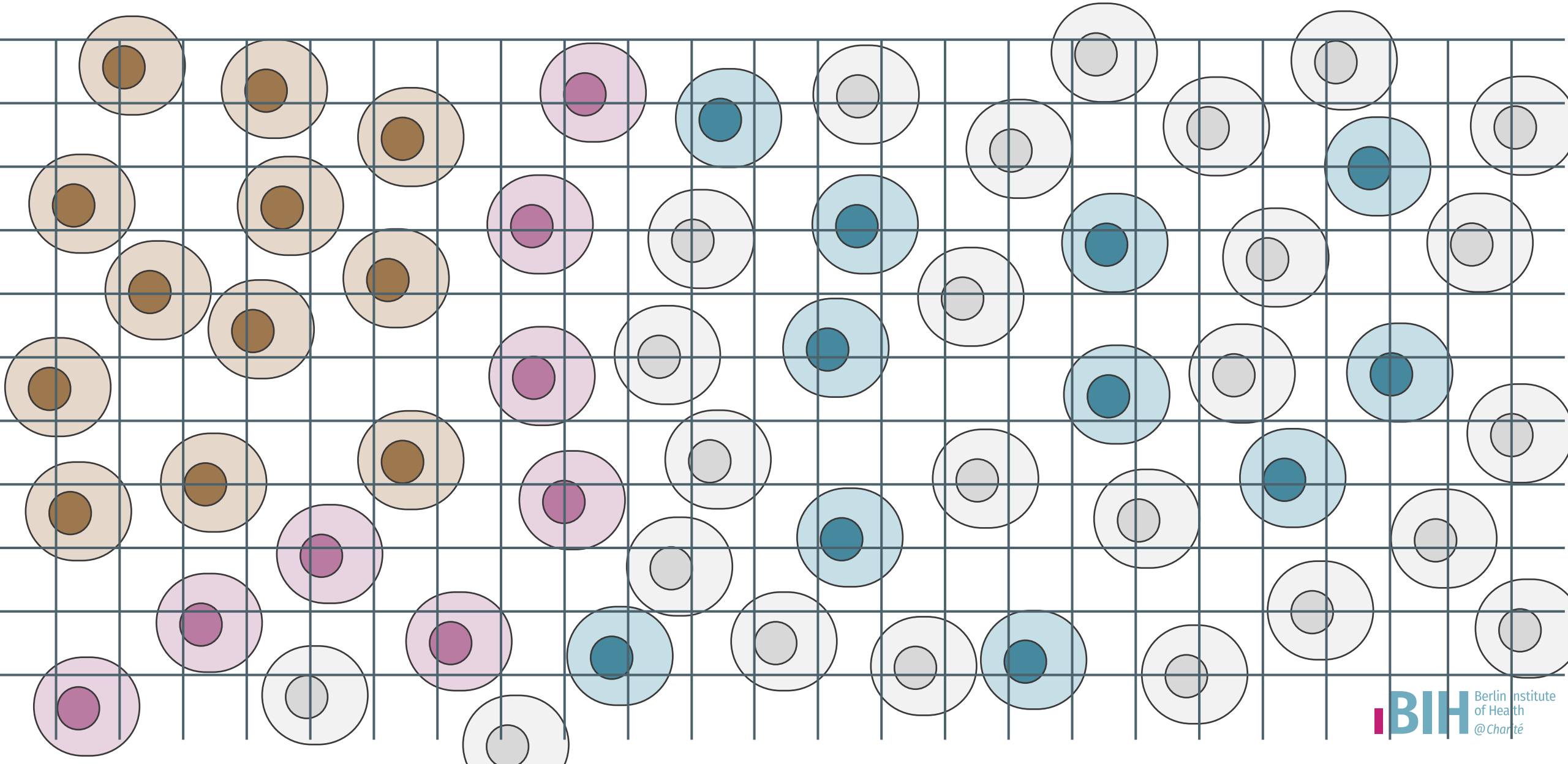
Spatial resolution: supracellular grid, e.g. Visium



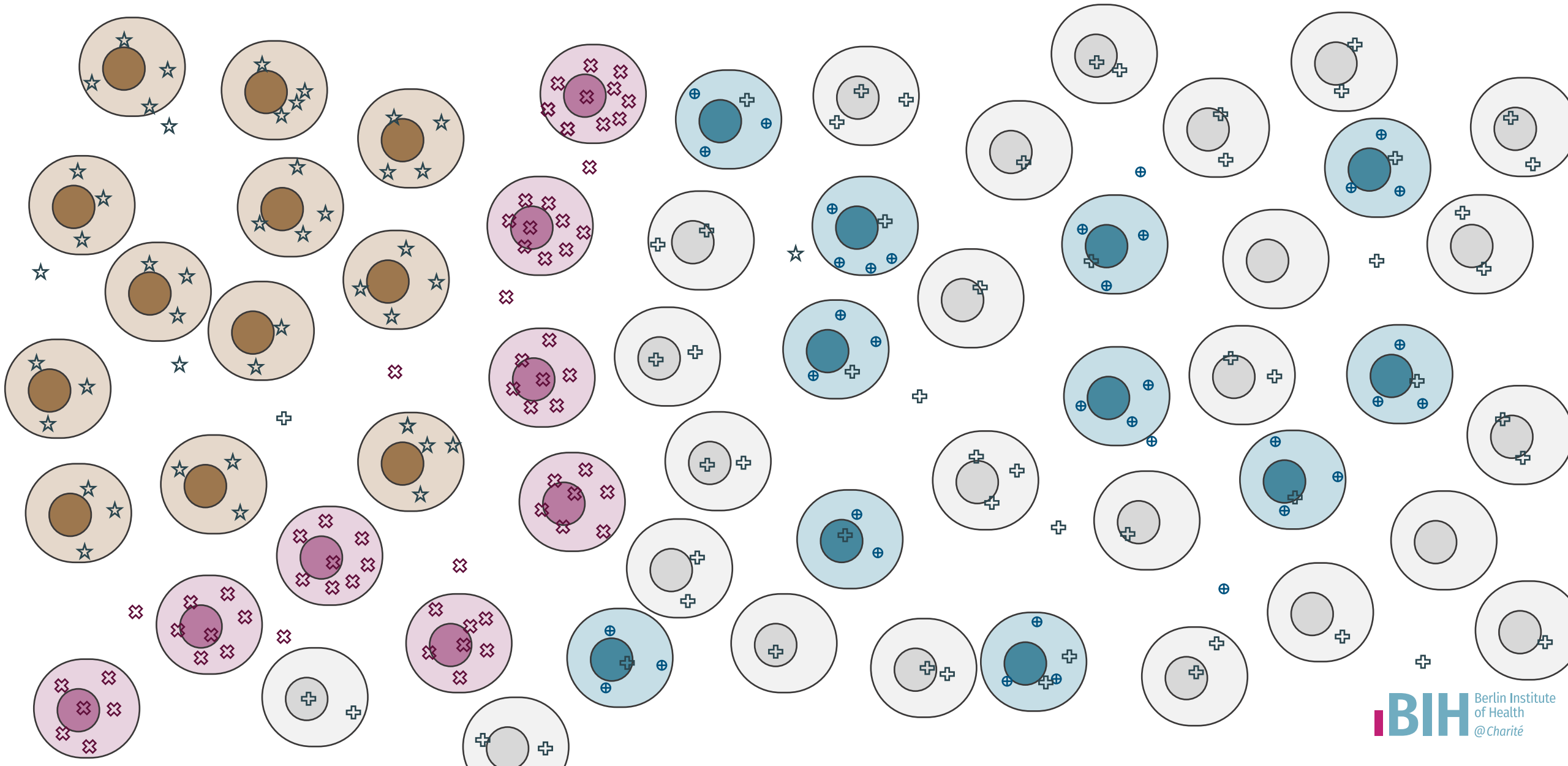
Spatial resolution: ~1-3 cell profiling, e.g. Slide-Seq/Slide-tags



Spatial resolution: sub-cellular, e.g. VisiumHD

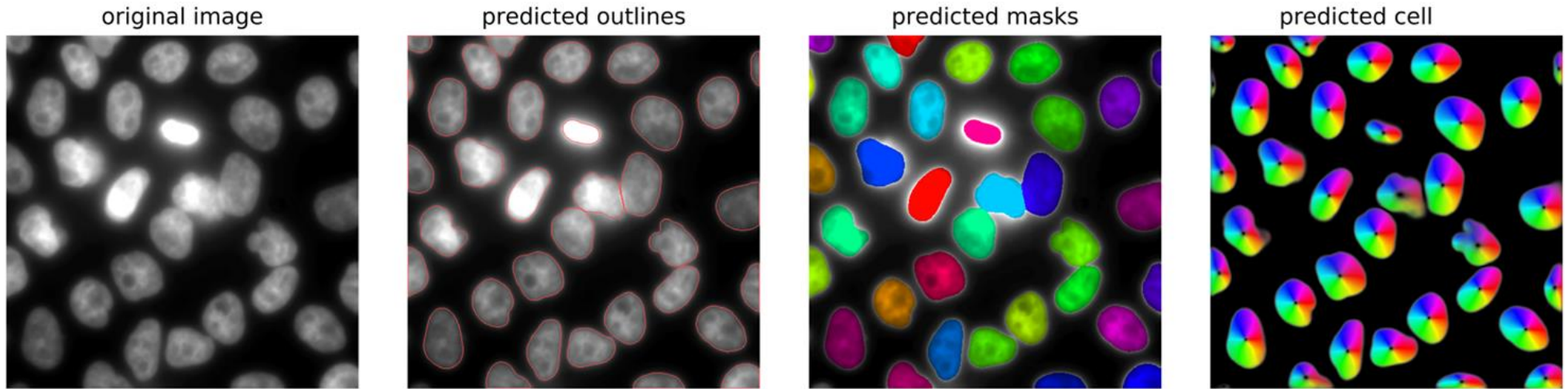


Spatial resolution: single molecule, e.g. MERSCOPE, cosMX, Xenium

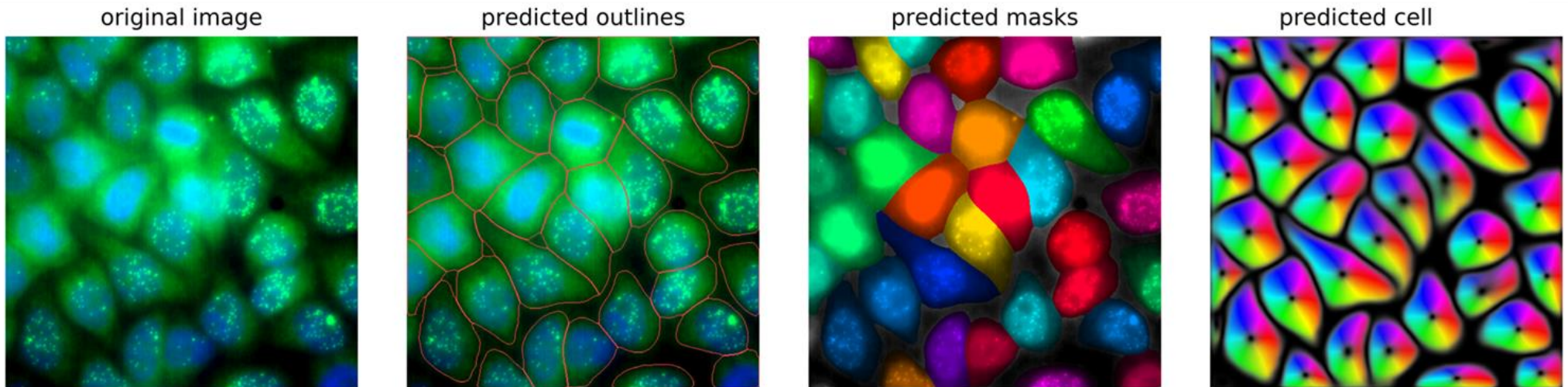


Cell segmentation by staining cell landmarks

**Nucleus
Segmentation**

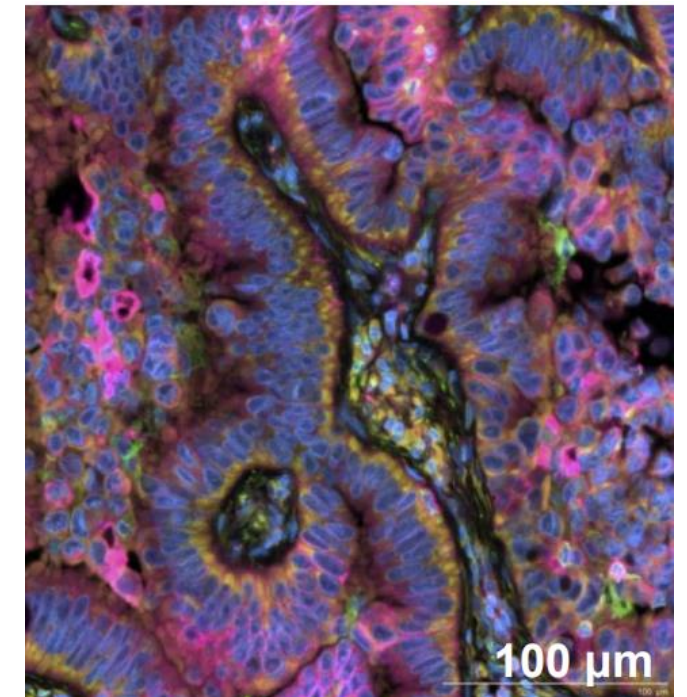
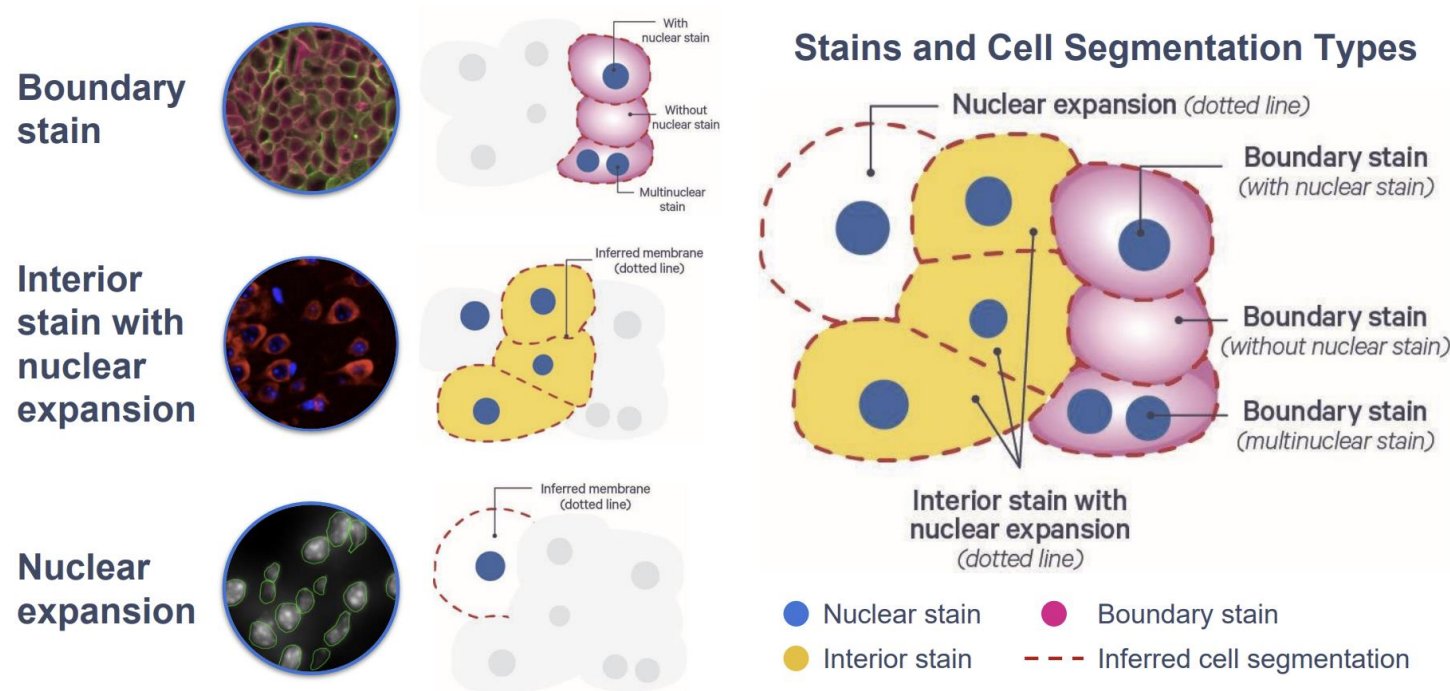


**Cytoplasmic
Segmentation**



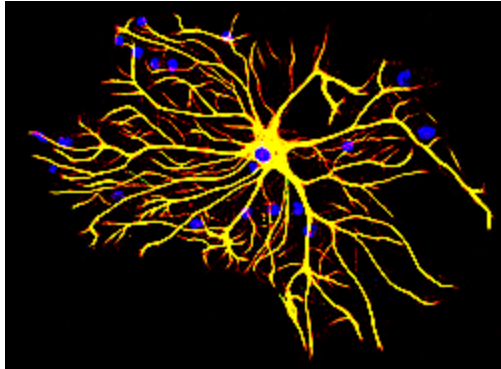
Improving cell segmentation - staining multiple cell landmarks

Vendors offer staining of multiple cell landmarks for “multi-modal” cell segmentation, e.g. 10x:

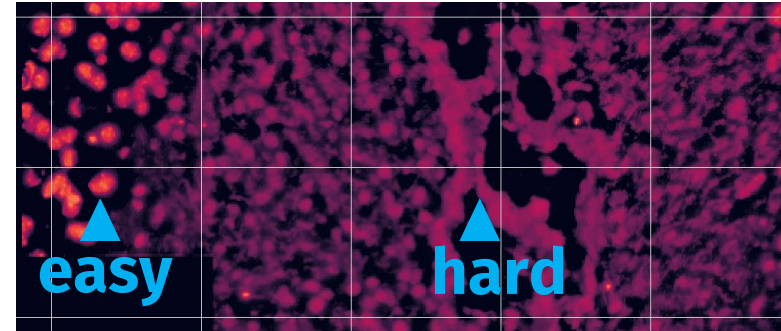


[1] https://pages.10xgenomics.com/rs/446-PBO-704/images/AGBT_2024_Cell_Segmentation_Poster.pdf

Identifying borders of cells (cell segmentation) is challenging



Cell shapes can be very challenging: eg. Astrocytes



Data of DAPI stained nuclei cannot always be distinguished

We are involved in many efforts to evaluate cell segmentation

I will talk more about in cell-segmentation-free analysis....

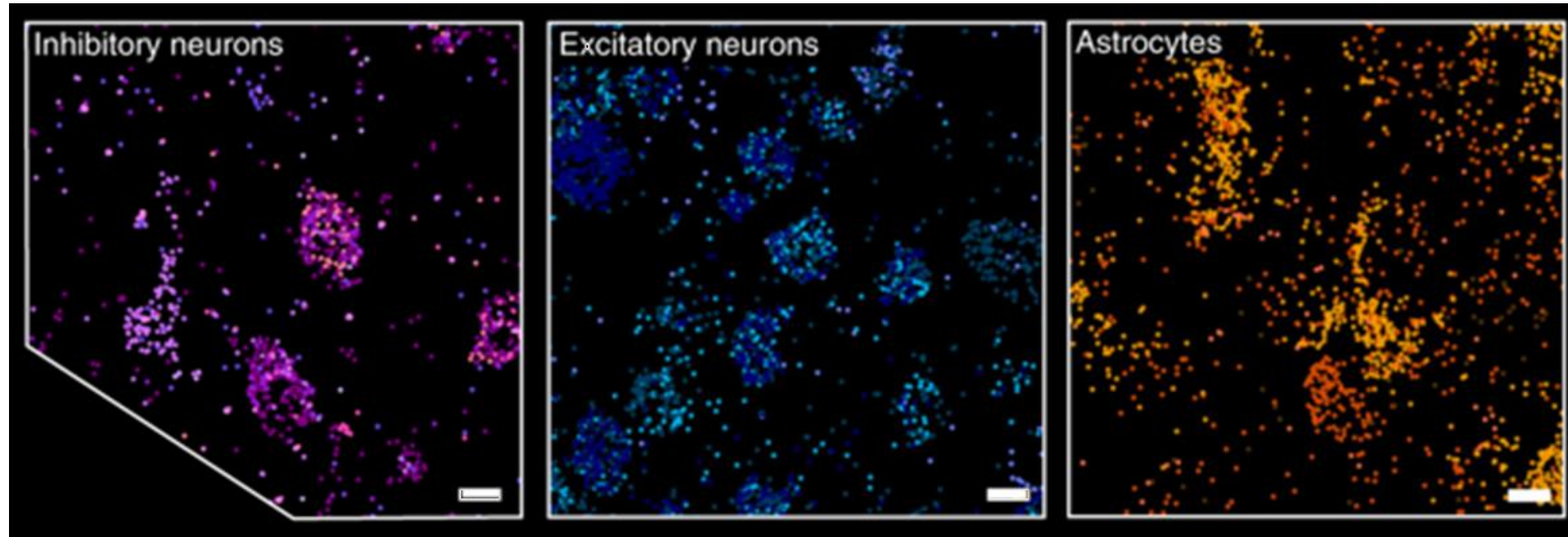
Cell Segmentation Free Analysis

Park et al 2021 “Cell segmentation-free inference of cell types from in situ transcriptomics data”. [Nature Communications](#)

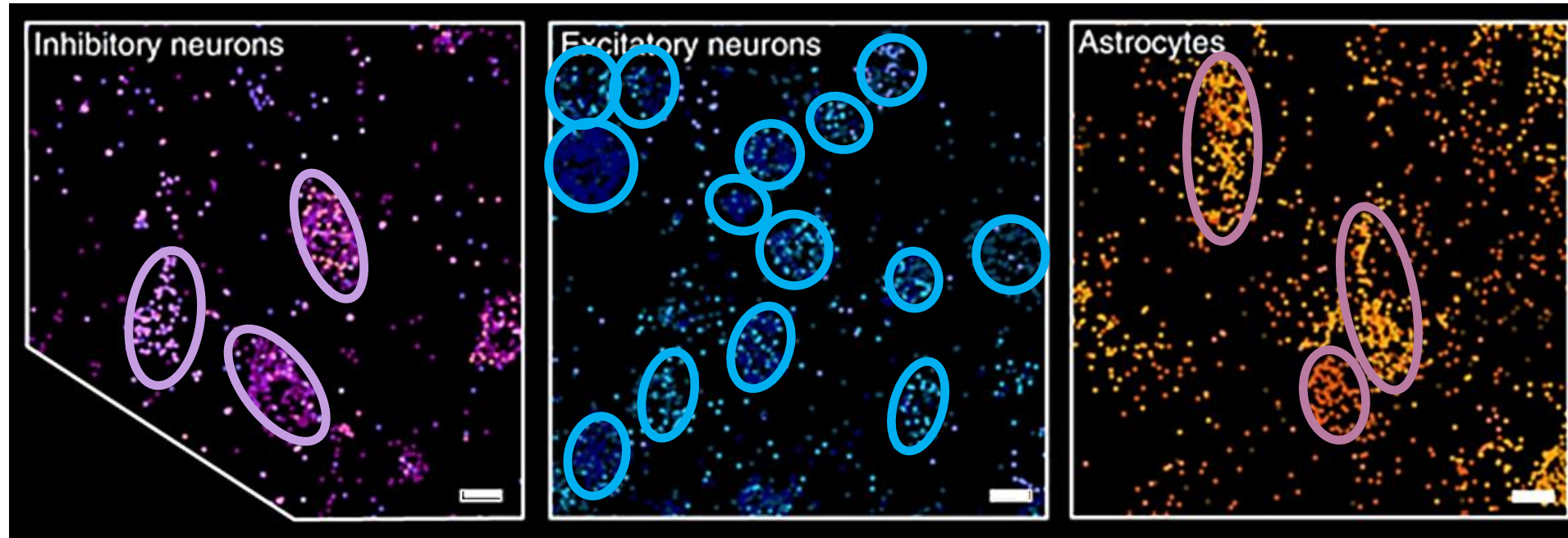
Tiesmeyer et al 2025 “SSAM-lite: A Light-Weight Web App for Rapid Analysis of Spatially Resolved Transcriptomics Data”. [Frontiers in Genetics](#)

Mueller-Boettcher et al 2025 “Sainsc: A Computational Tool for Segmentation-Free Analysis of In Situ Capture Data”. [Small Methods](#)

Transcript organisation is not random

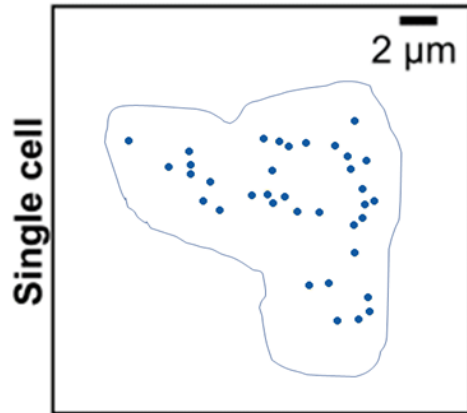


Transcript organisation is not random



Our approach – gene expression density is a proxy for cells

Analyse spatial gene expression density, not cells



Our approach – gene expression density is a proxy for cells

Analyse spatial gene expression density, not cells



Our approach – gene expression density is a proxy for cells

Analyse spatial gene expression density, not cells



Sainsc: optimised for millions of cells & organism-scale

Segmentation-free Analysis of IN Situ Capture data

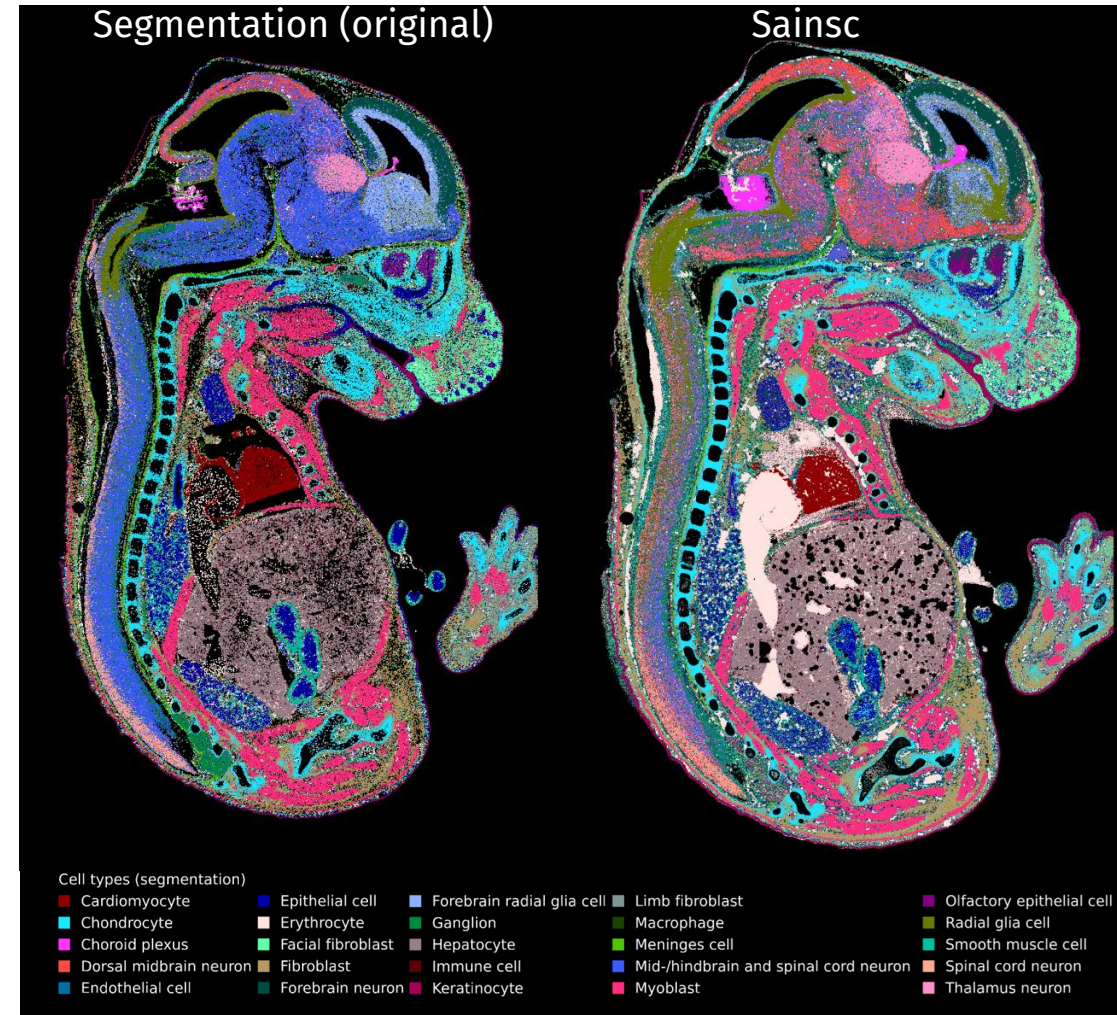
- Segmentation-free identifies red blood cells in the spleen and umbilical cord

Optimised for organism scale analysis

- Can analyse most samples in <1 hour
- Suitable for use on a (high-end) laptop

Suitable for imaging and sequencing-based spatial transcriptomics

- E.g. Stereo-seq, Open-ST, Nova-ST, VisiumHD



Sainsc: optimised for millions of cells & organism-scale

Segmentation-free Analysis of IN Situ Capture data

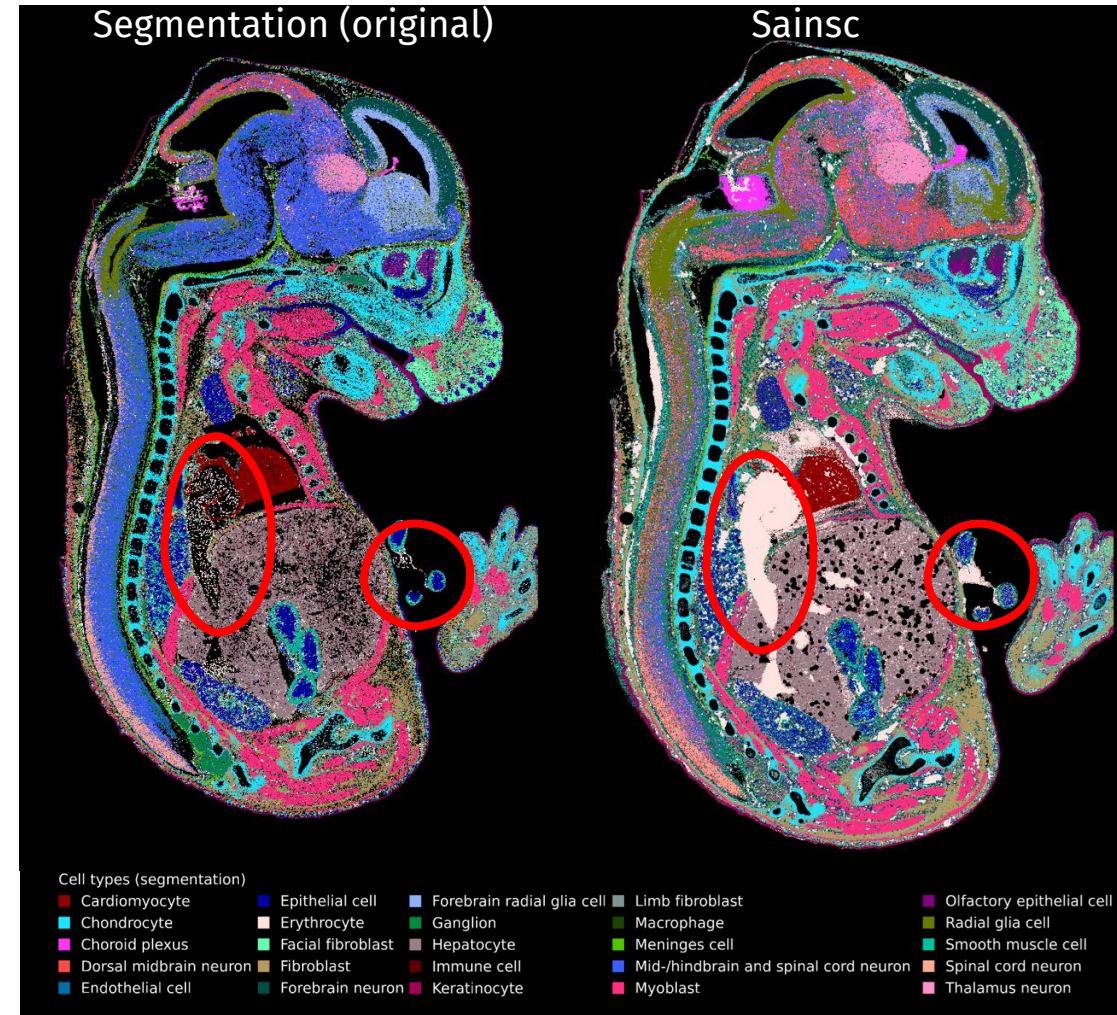
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Suitable for imaging and sequencing-based spatial transcriptomics

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Summary 1: cell-segmentation free analysis

Why do cell-segmentation free at all?

- Cell segmentation is **not trivial**
- Segmentation-free is not limited by cell-segmentation
- Segmentation-free allows let's you interrogate your data rapidly

How?

- Using tools like **Sainsc** and **FICTURE**
- Generate **cell type maps**
- Use as a **ground truth** for evaluating cell segmentation results

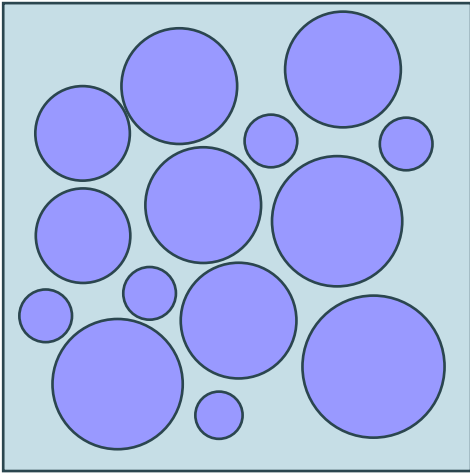
2D or not 2D? That is the question!

(... investigating cell overlaps in spatial transcriptomics data)

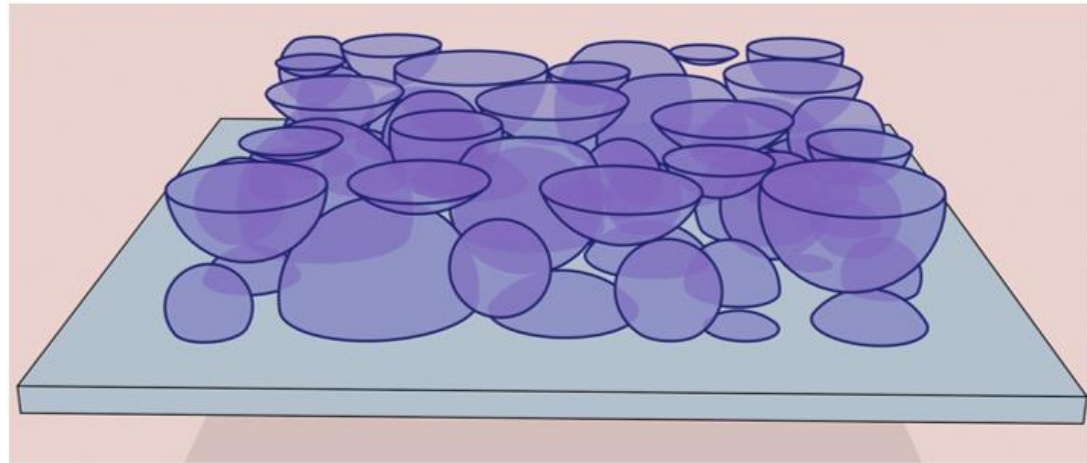


Salas et al 2025 “Optimizing Xenium In Situ data utility by quality assessment and best-practice analysis workflows” Nature Methods
Tiesmeyer et al 2025 “2D, or not 2D? Investigating vertical signal integrity of tissue slices”. bioRxiv
Tiesmeyer et al 2026 “Identifying 3D signal overlaps in spatial transcriptomics data with ovrlpy”. Nature Biotechnology
Ishaque et al 2026 “The challenges of cell segmentation in spatially resolved transcriptomics” arXiv

Our restricted view of spatial transcriptomics data

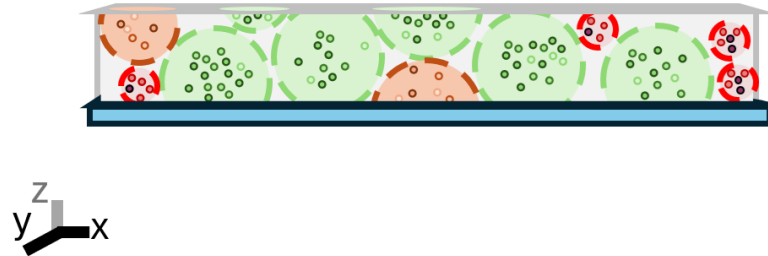


or

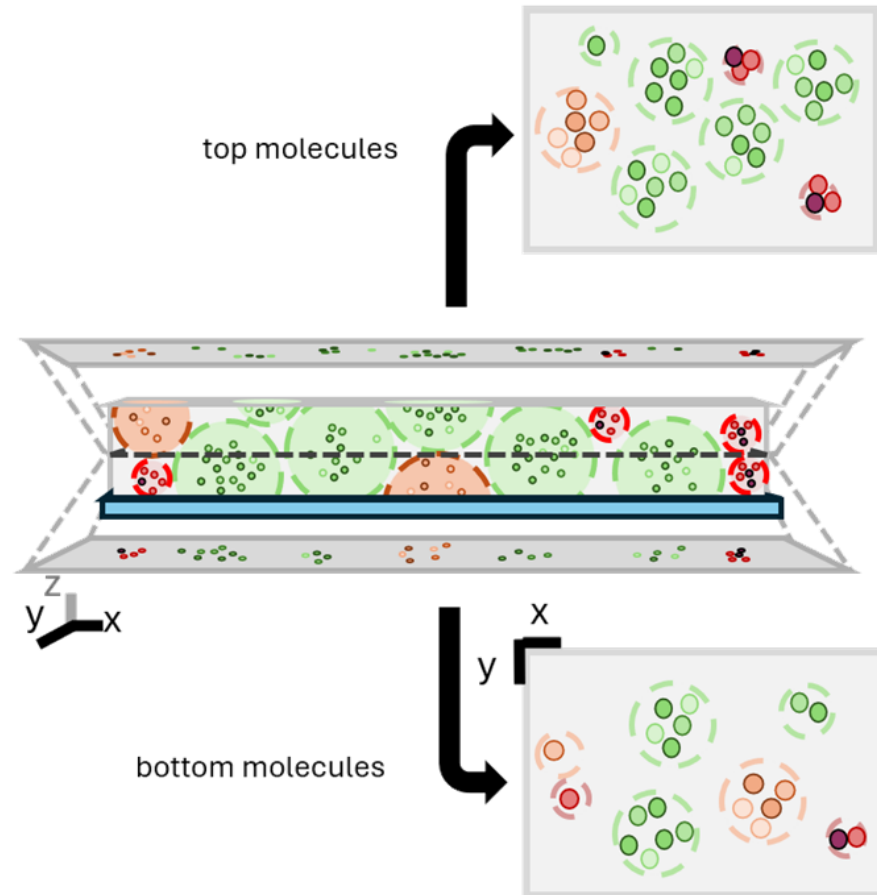


?

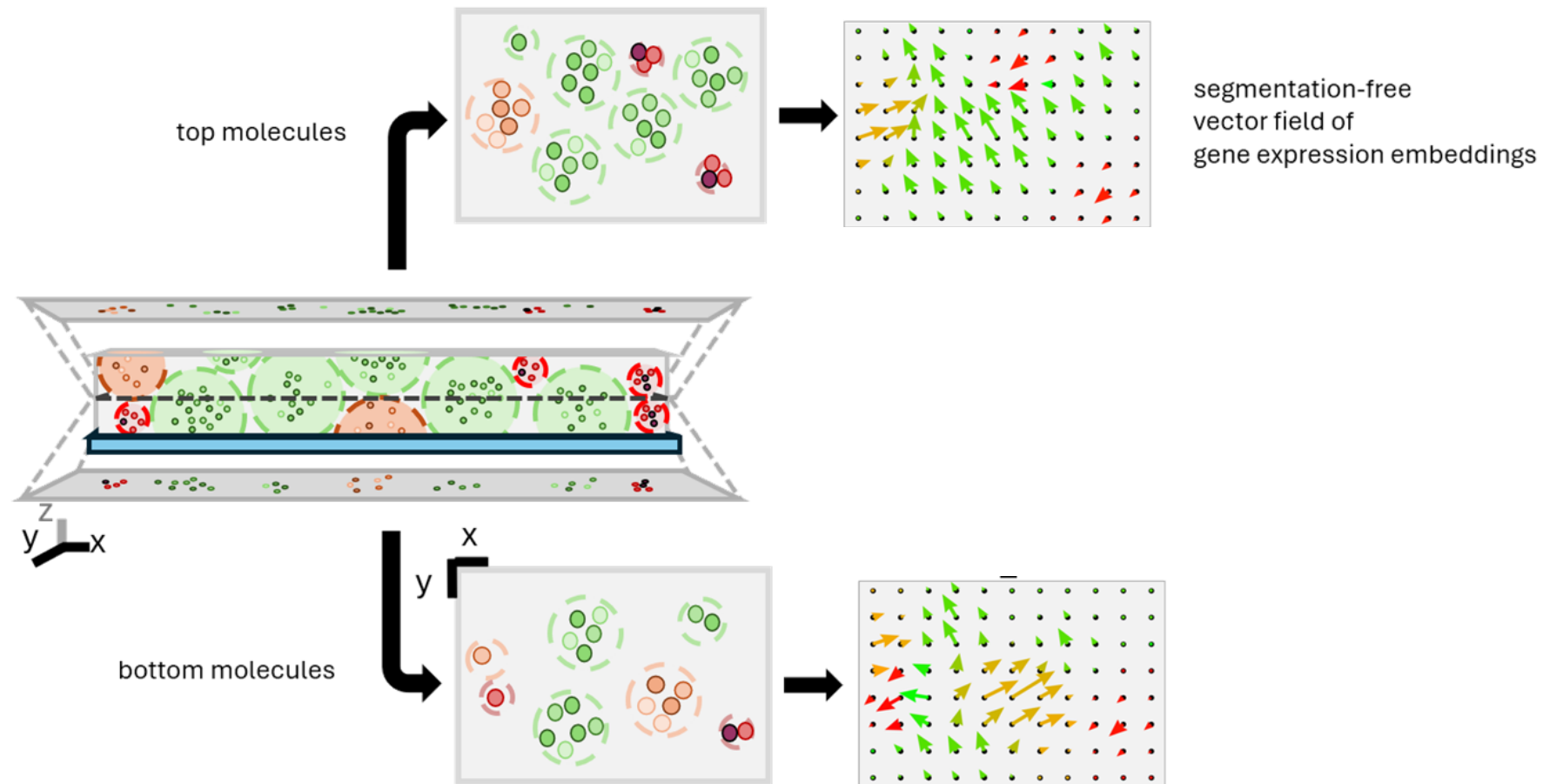
ovr1.py algorithm – let's start with a side view of our data



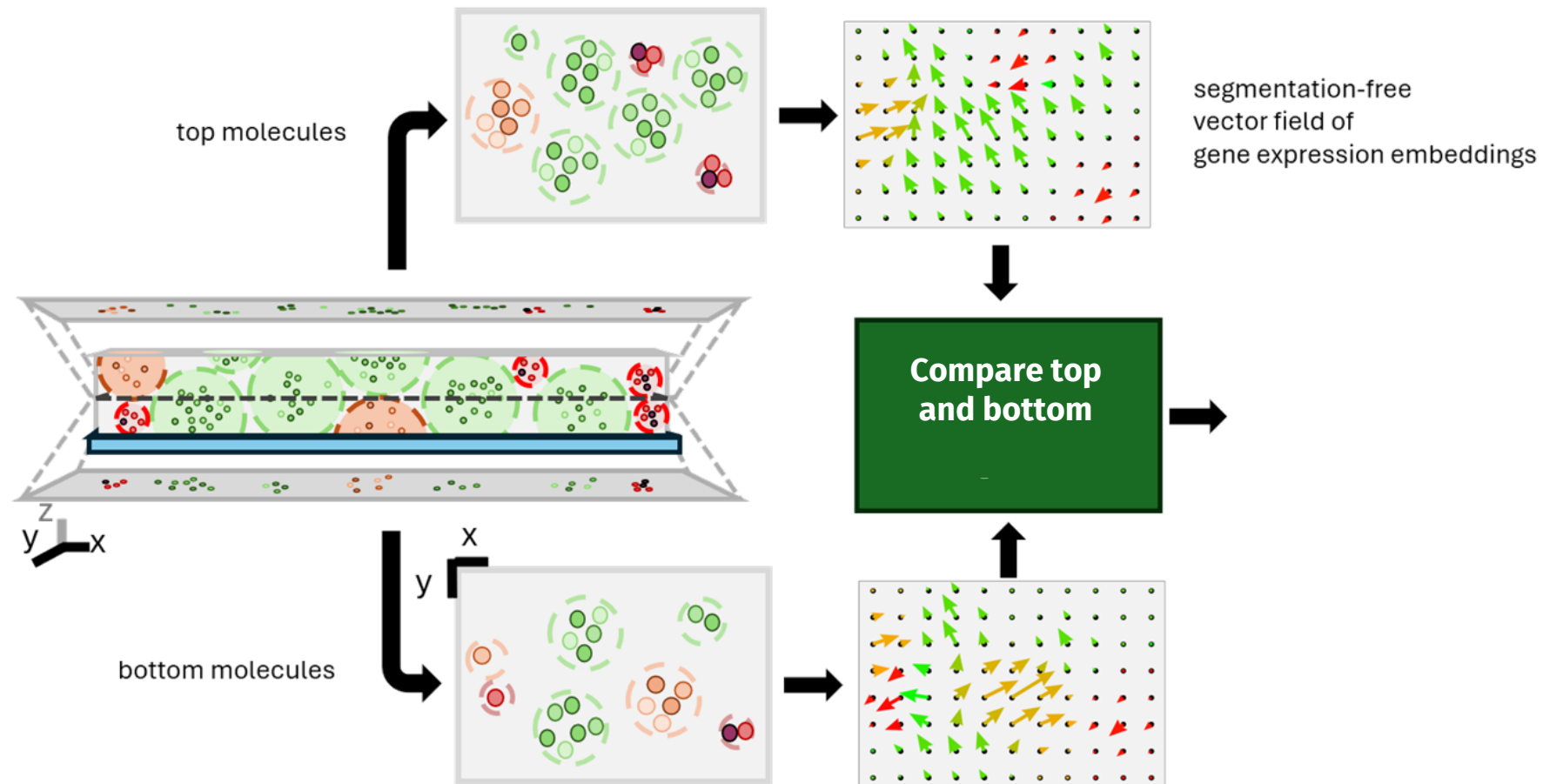
Step 1: virtual vertical sub-sectioning



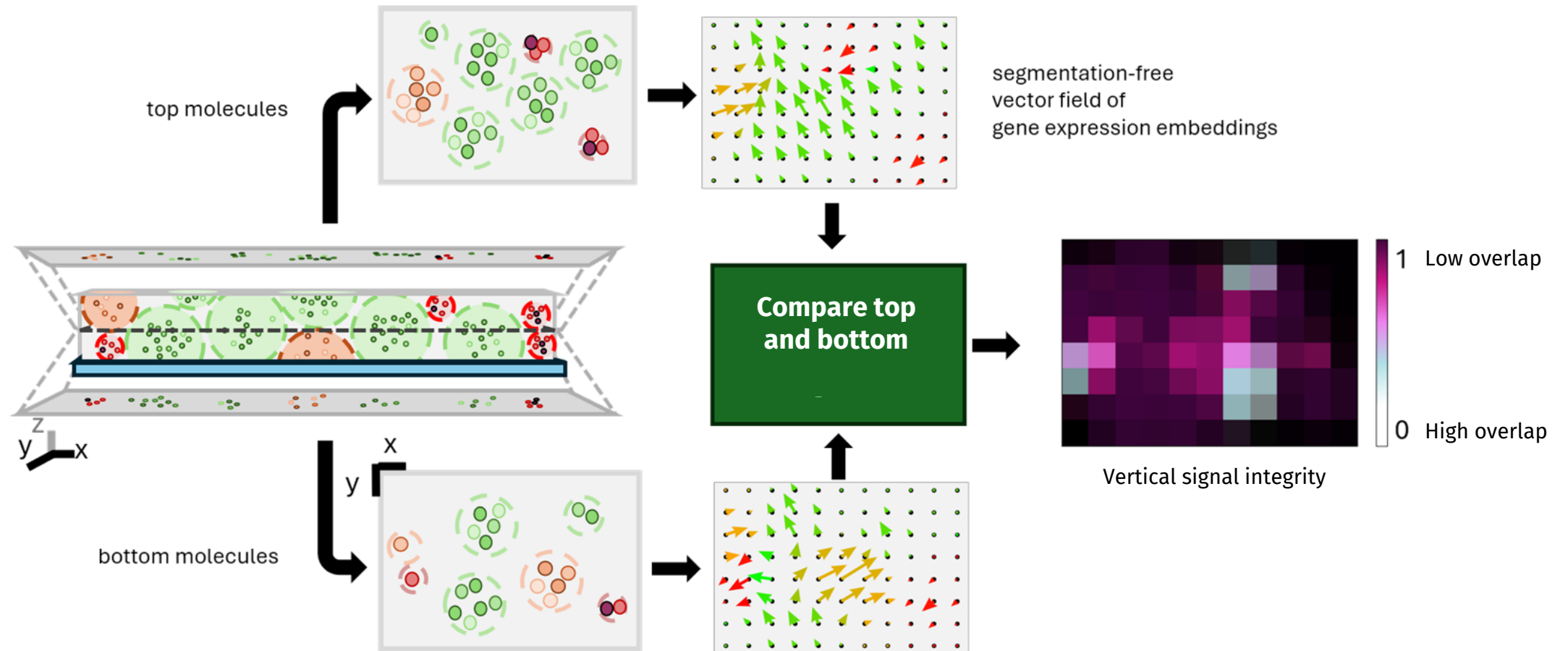
Step 2: create a gene expression embedding



Step 3: compare the top and the bottom

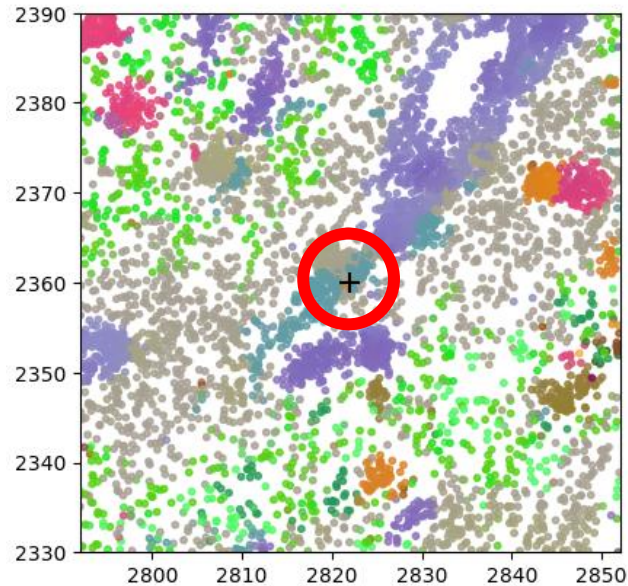


Step 4: identify vertical signal integrity ($\approx$ cell overlaps)

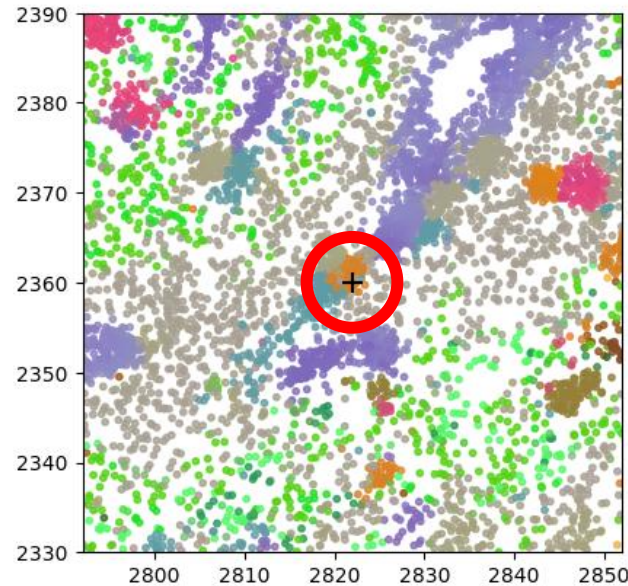


ovr1.py example 1: 3-way cell overlap!

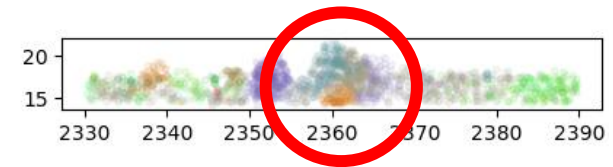
Microglial cell (blue) and astrocytes (khaki) cell on top of an inhibitory neuron (orange)



ROI: top view



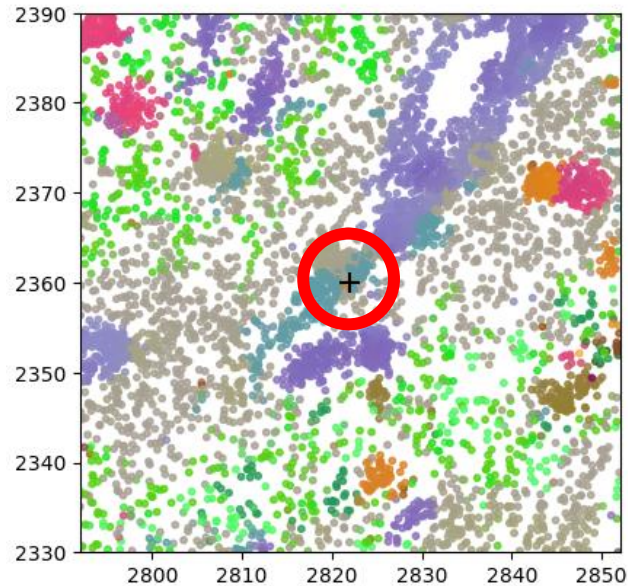
ROI: bottom view



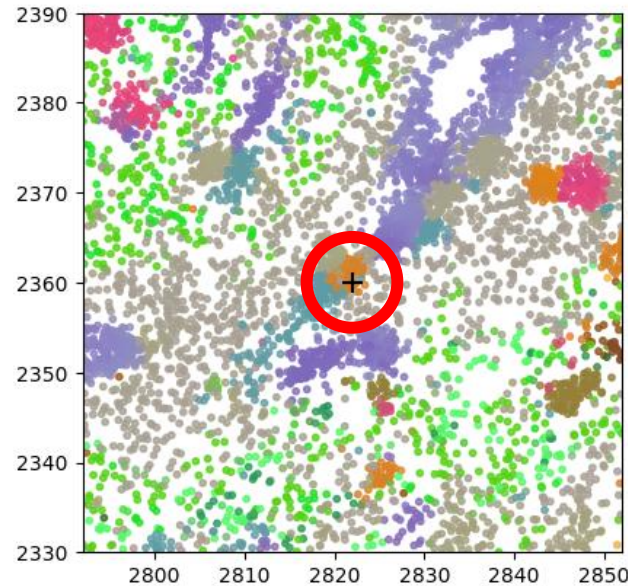
ROI: side view, y

ovr1.py example 1: 3-way cell overlap!

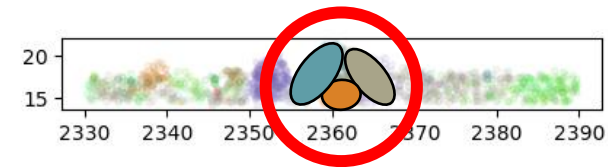
Microglial cell (blue) and astrocytes (khaki) cell on top of an inhibitory neuron (orange)



ROI: top view

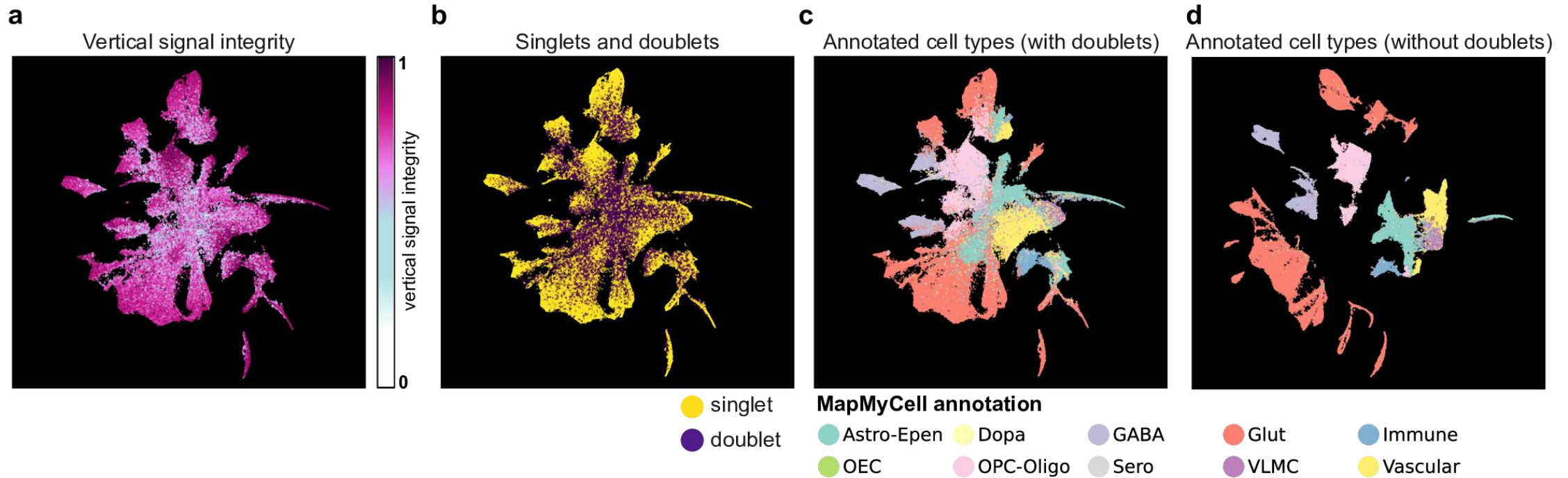


ROI: bottom view

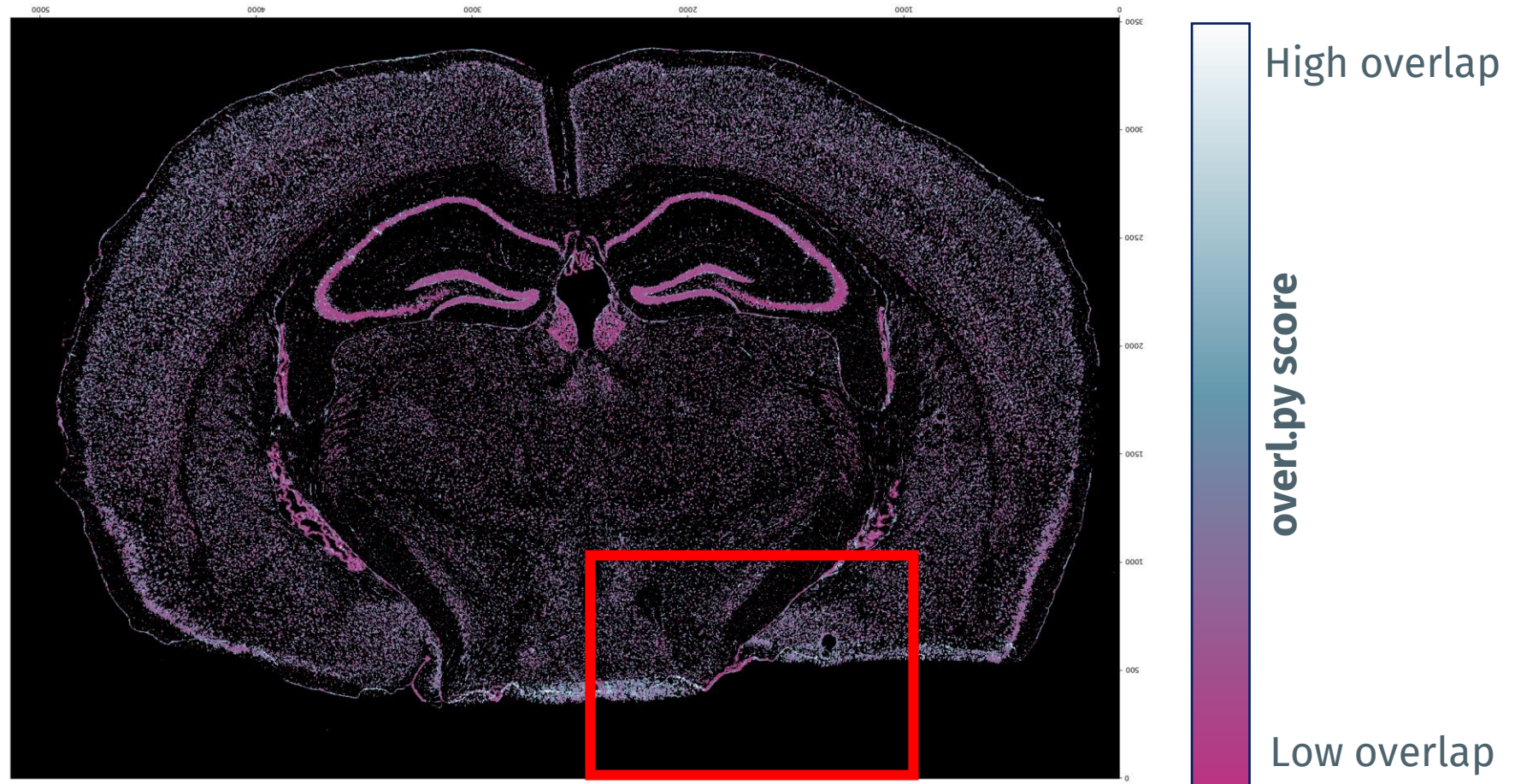


ROI: side view, y

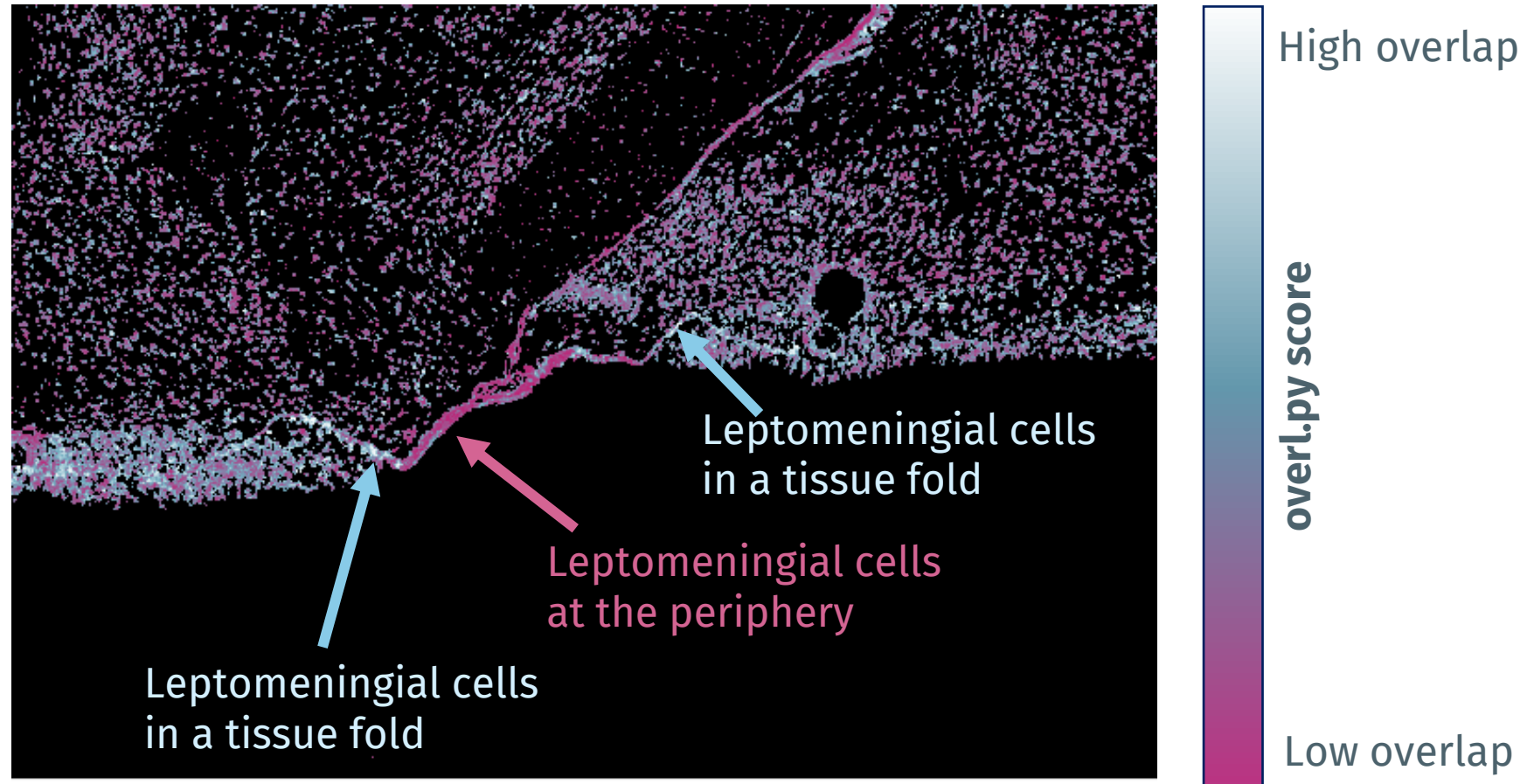
Removing overlapping cells improves cell-type clustering



ovr1.py example 2: tissue fold



ovr1.py example 2: tissue fold



Summary 2: segmentation is hard (... recurrent theme)

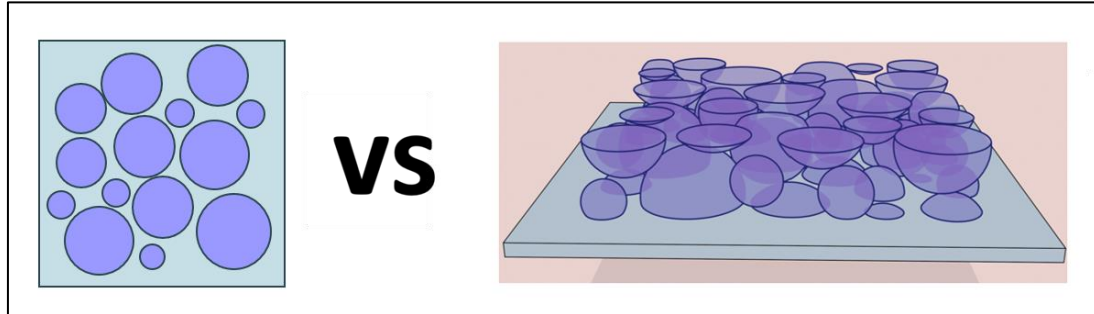
Why do overlap analysis at all?

- Most cell segmentation methods are restricted to 2D, but cells overlap in 3D
- Gene expression from overlapping cells mix, and cause “vertical doublets” = contamination

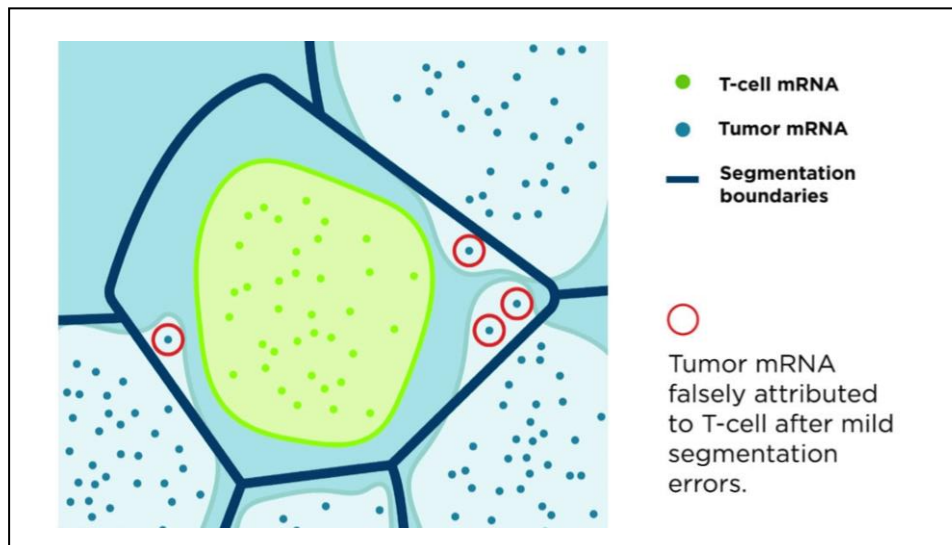
How?

- There is no tool like it, so you can only use **ovrl.py** or tools that implement it (e.g. **SegTraQ**)
- Check your tissue for **tissue folds**
- **Identify and filter cells** with high overlap likelihood

Summary 2.1: Perspectives on cell segmentation



Tissue sections are 3D and not 2D. Cell overlaps are a problem!



Transcripts from adjacent cells can be miss-assigned!

arXiv > q-bio > arXiv:2606.09675

Quantitative Biology > Other Quantitative Biology

[Submitted on 8 Jun 2026]

The Challenge of Cell Segmentation in Spatially Resolved Transcriptomics

Naveed Ishaque, Peter Kharchenko, Daria Lazic, Jieran Sun, Jean Yee Hwa Yang, Martin Emons, Florian Heyl, Wolfgang Huber, Daniel Jones, Louis B. Kuemmerle, Alex R. Lederer, Malte D. Luecken, Vinicius Maracaja-Coutinho, Matthias Meyer-Bender, Andrew Moorman, Evan W. Newell, Quan Nguyen, Shyam Prabhakar, John Randell, Daria Romanovskaia, Oliver Stegle, Gary D. Bader, Raphael Gottardo

Spatially resolved transcriptomics (SRT) is transforming how we study tissues by measuring gene expression in cells in their spatial context. However, the field lacks robust methodological guidance on one of its most fundamental analytical steps: how to accurately segment cells and assign spatially localized transcripts to them. Major technical challenges include sparse molecular signals, transcript displacement, complex cellular morphologies, and the projection of three-dimensional tissue architecture onto two-dimensional imaging planes. These challenges make segmentation a major source of uncertainty, with errors that can propagate through downstream analyses and ultimately lead to misleading biological interpretations. Here, we argue that segmentation should be treated as a central unresolved problem in spatial omics rather than a routine preprocessing step. We review current approaches, highlight key methodological limitations, including the lack of appropriate metrics and gold-standard benchmarks, and propose a community-driven path forward. Establishing shared evaluation frameworks, scalable benchmark datasets, and transparent reporting standards will be essential for transforming SRT into a robust and reproducible foundation for biological discovery and clinical translation.

Perspectives on where things stand, the challenges, and what's next

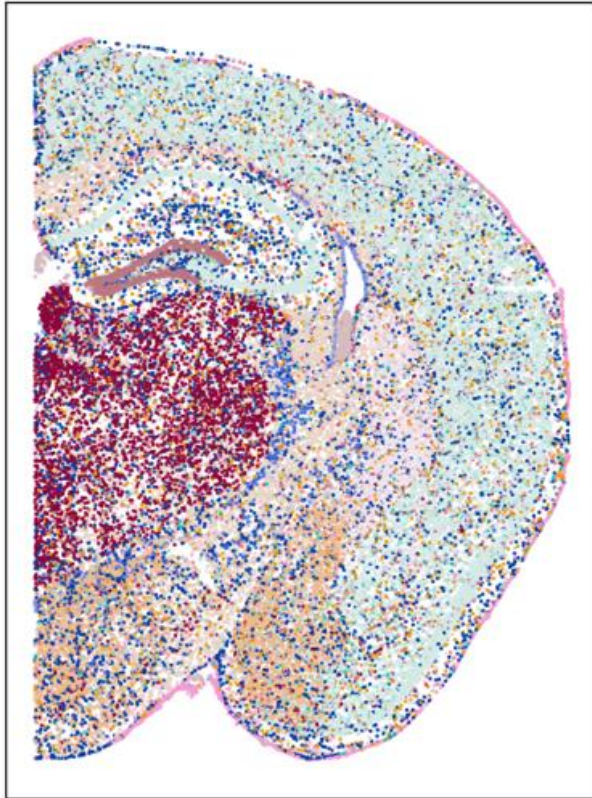
Making sense of cells in space

Mueller-Boetticher et al 2025 “SpatialLeiden: spatially aware Leiden clustering”. [Genome Biology](#)

Sun et al 2025 “Beyond benchmarking: an expert-guided consensus approach to spatial clustering”. **bioRxiv** (provisionally accepted)

From cells in space

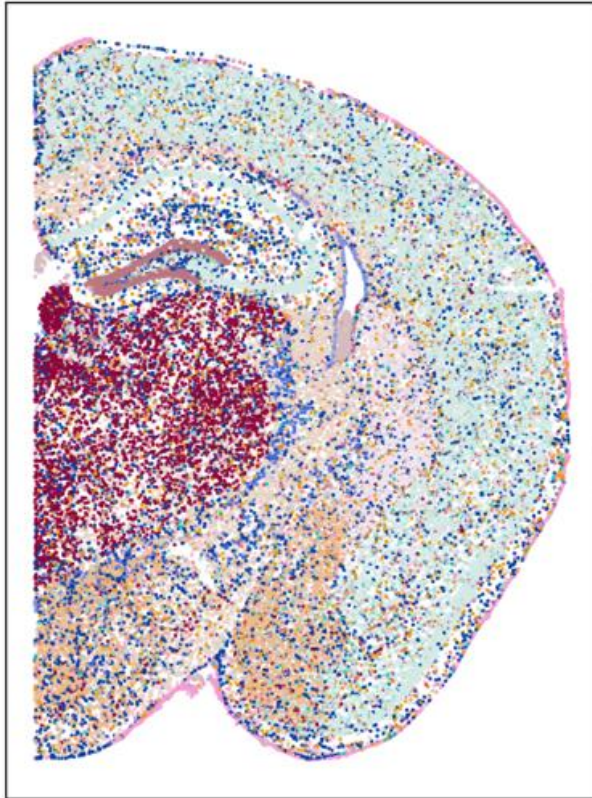
Cell map



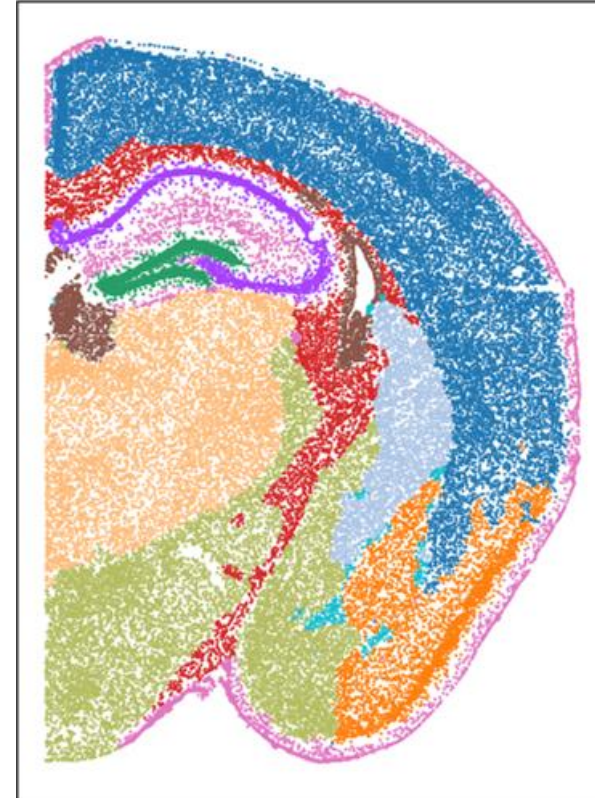
Adapted from Shang, Lulu, and Xiang Zhou. "Spatially aware dimension reduction for spatial transcriptomics." *Nature communications* 13.1 (2022): 7203.

From cells in space to tissue regions – spatial clustering

Cell map



Tissue regions



Adapted from Shang, Lulu, and Xiang Zhou. "Spatially aware dimension reduction for spatial transcriptomics." *Nature communications* 13.1 (2022): 7203.

SpatialLeiden – a spatially aware Leiden clustering algorithm

The Leiden algorithm is the clustering method of choice for single-cell data

We discover that Leiden has native support for spatial clustering by adding a spatial graph



Genome Biology

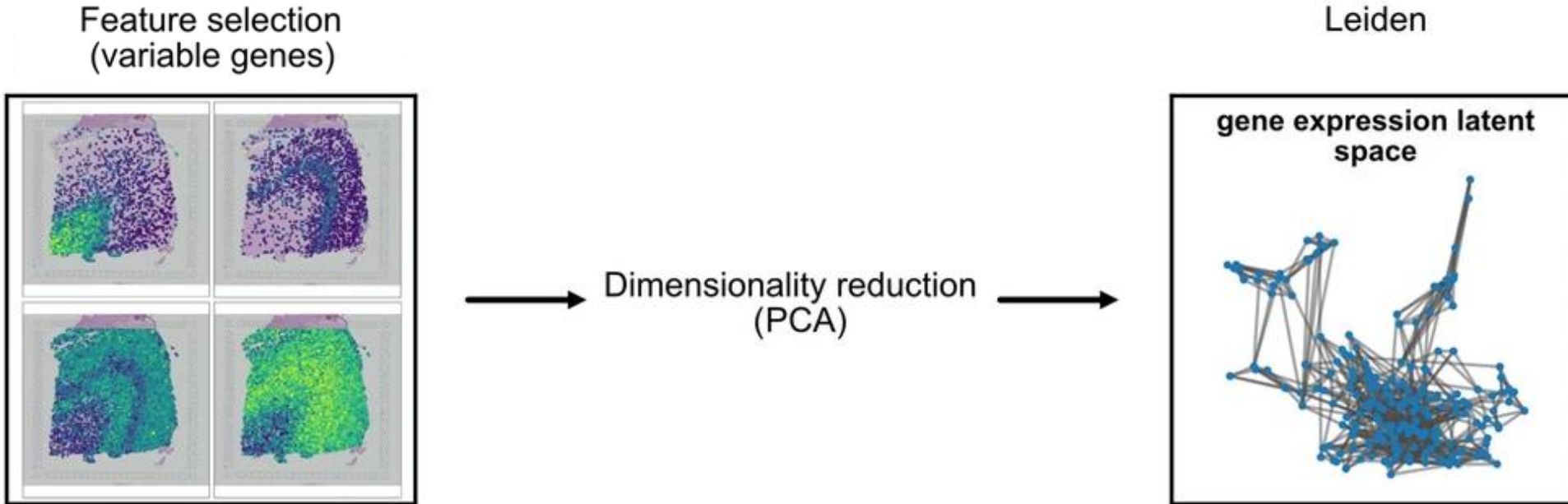
Brief Report | [Open access](#) | Published: 07 February 2025

SpatialLeiden: spatially aware Leiden clustering

[Niklas Müller-Böttcher](#), [Shashwat Sahay](#), [Roland Eils](#) & [Naveed Ishaque](#) 

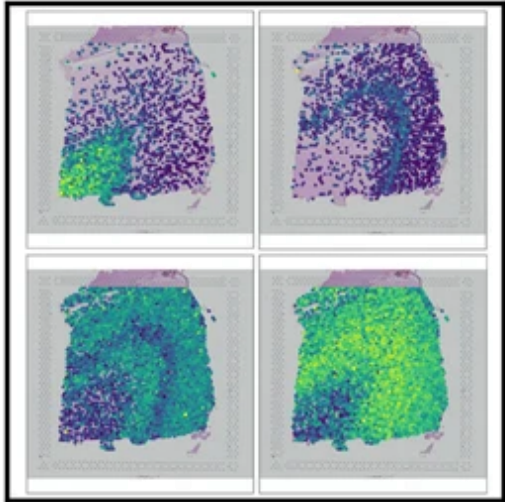
[Genome Biology](#) **26**, Article number: 24 (2025) | [Cite this article](#)

Leiden clustering: from single-cell



Leiden clustering: from single-cell to spatial

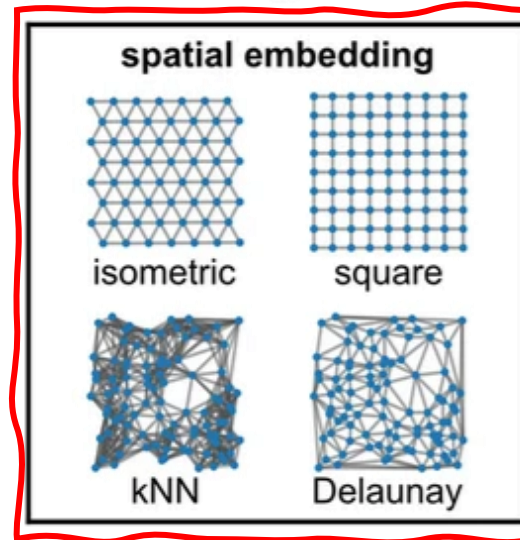
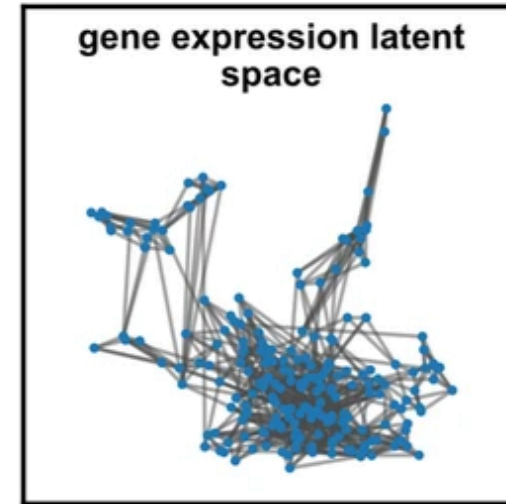
Feature selection
(spatially variable genes)



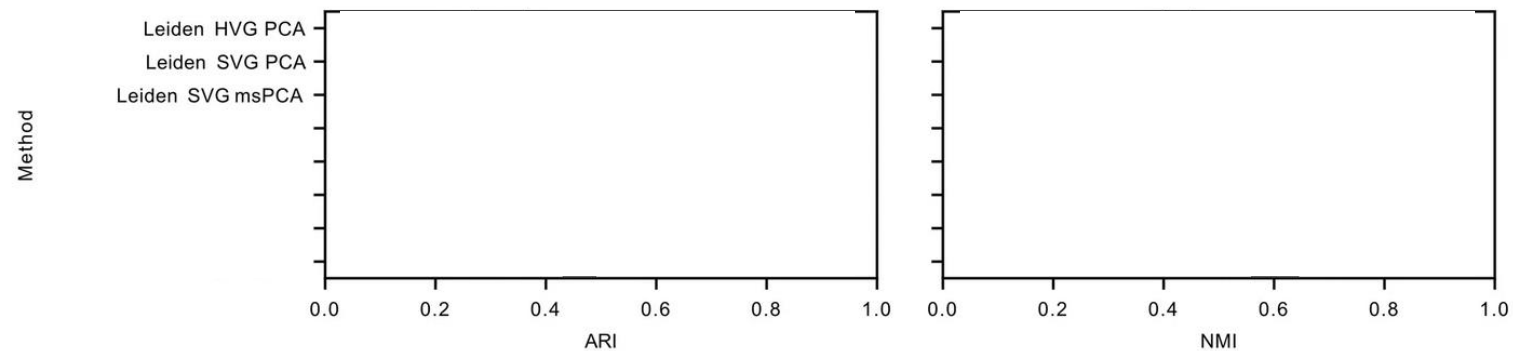
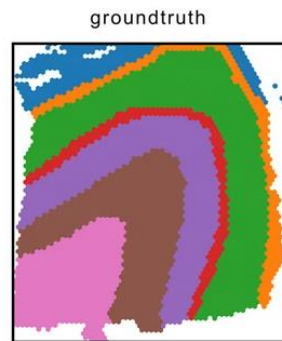
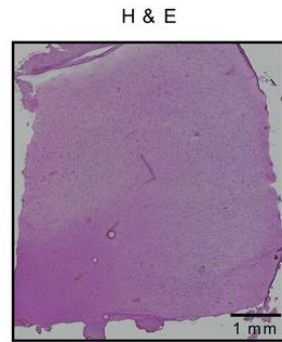
Dimensionality reduction
(MULTISPATI-PCA)



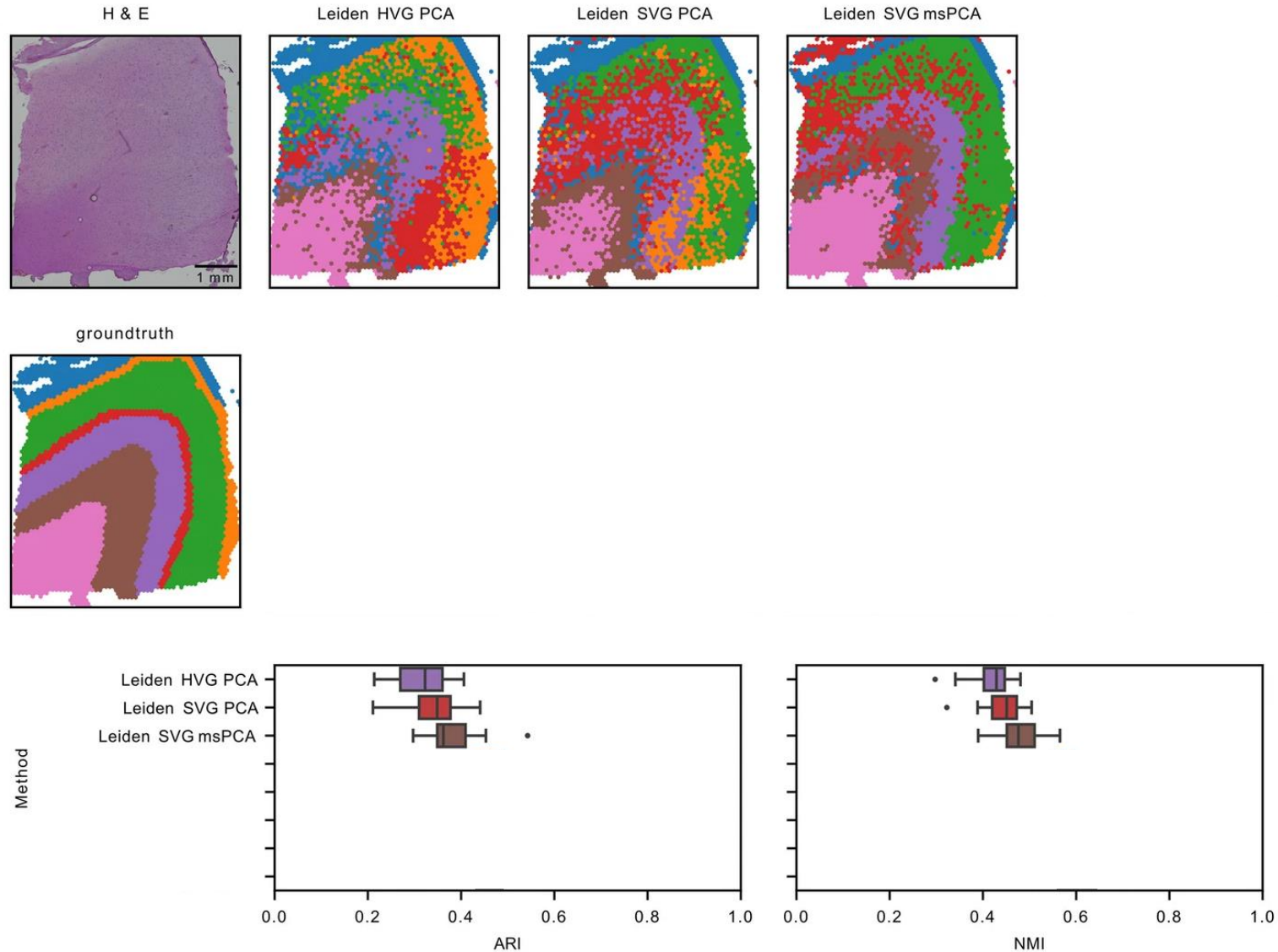
SpatialLeiden
(Leiden layer multiplex clustering)



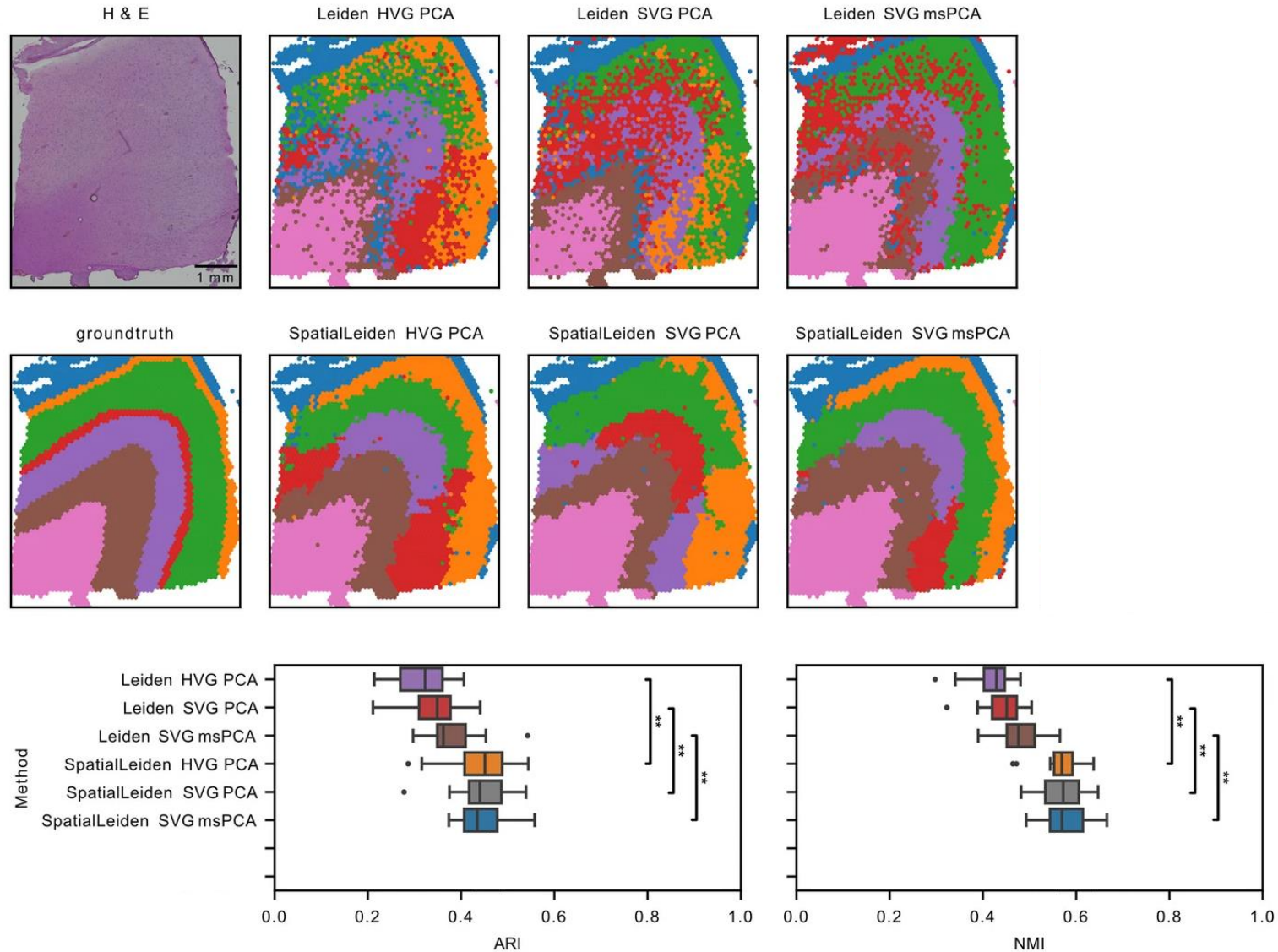
SpatialLeiden is significantly better than standard Leiden



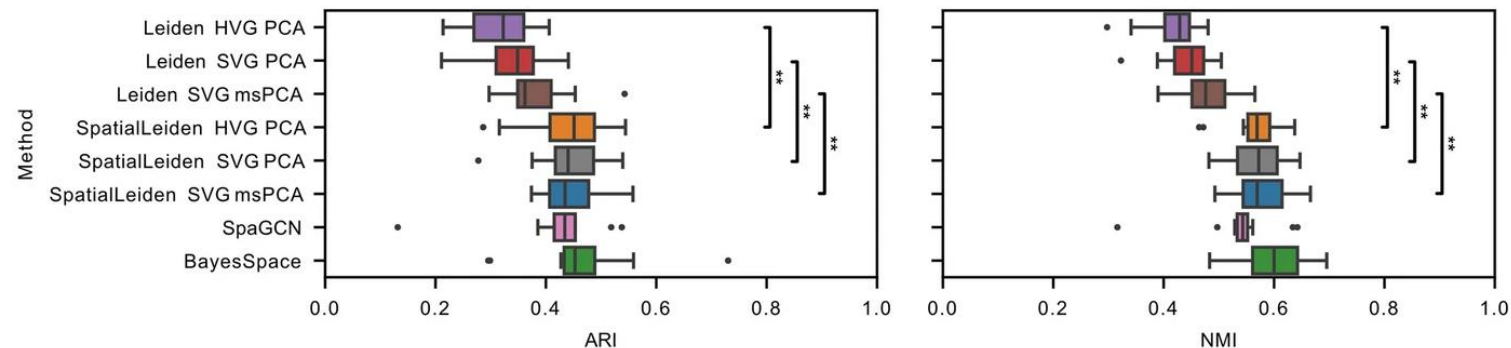
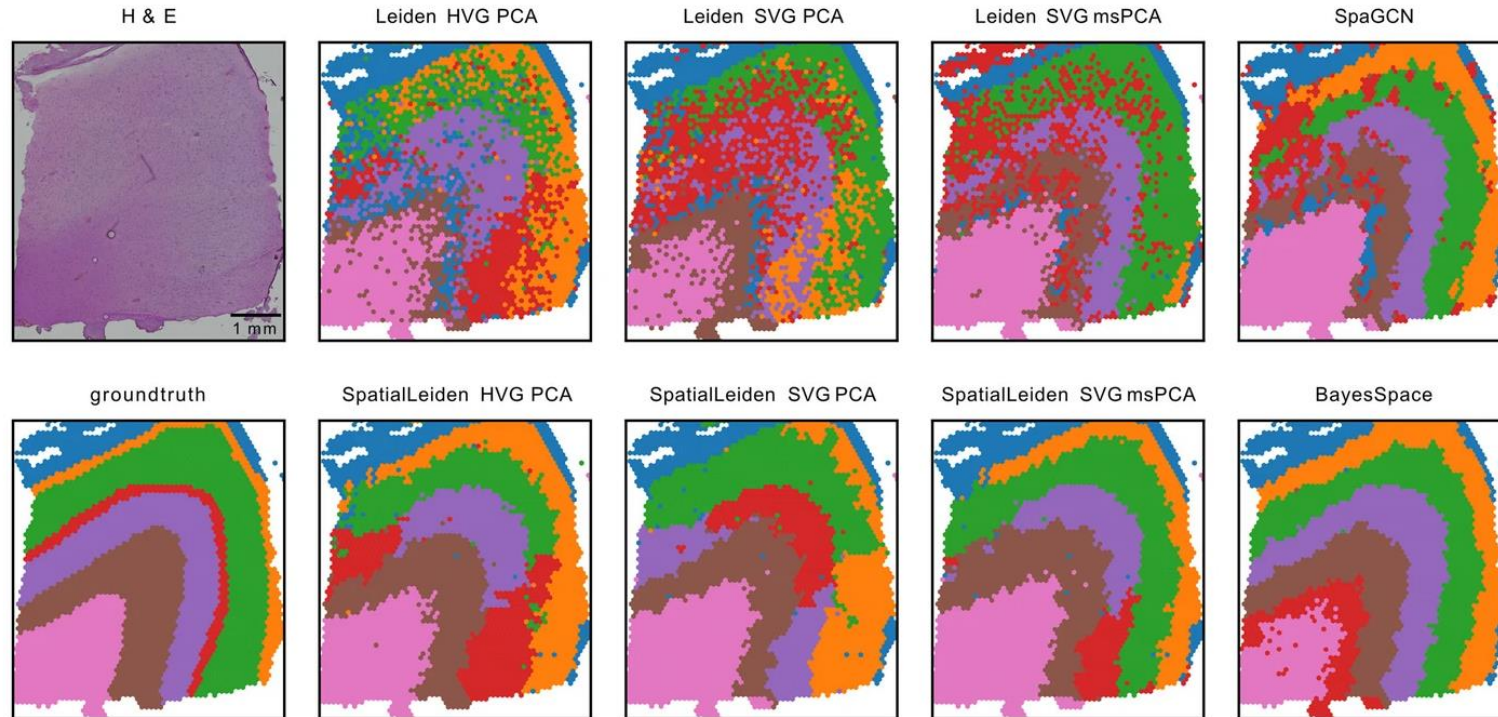
SpatialLeiden is significantly better than standard Leiden



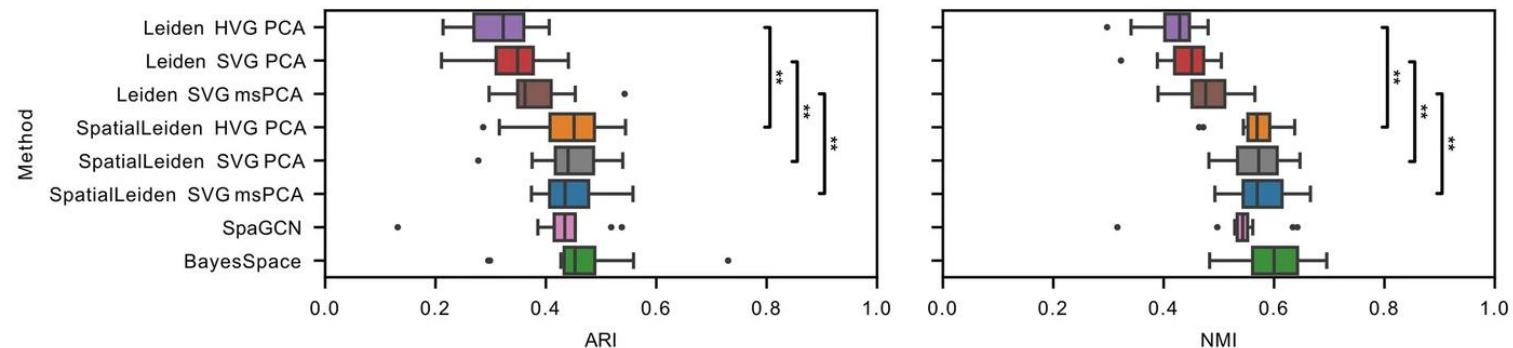
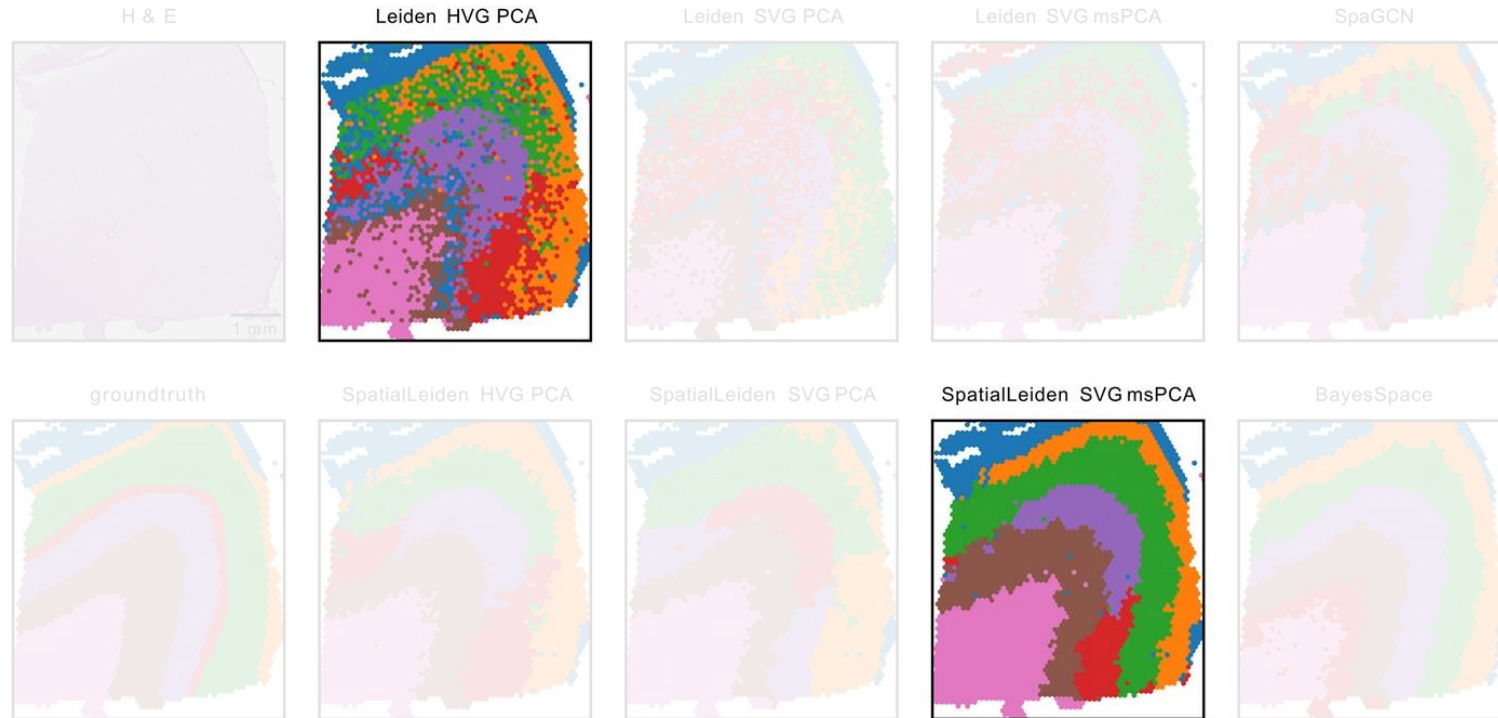
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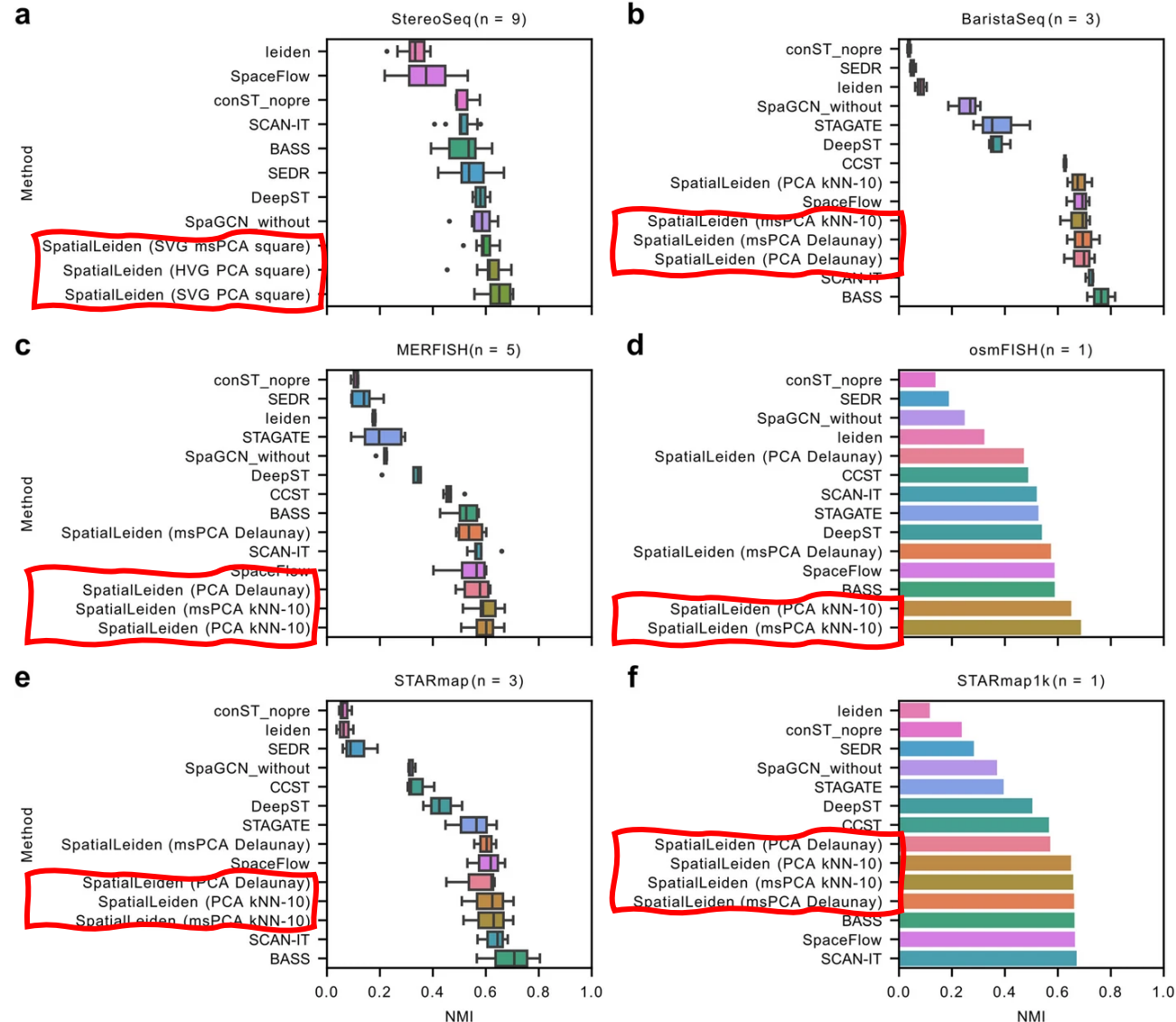
SpatialLeiden is significantly better than standard Leiden



SpatialLeiden is significantly better than standard Leiden



SpatialLeiden achieves top tier performance across datasets



Summary 3: SpatialLeiden... a success?

Why does SpatialLeiden matter when there are >50 other competing tools?

- Single-cell data analysts understand how to use Leiden, so SpatialLeiden is intuitive.
- SpatialLeiden is fast, so exploring parameters (e.g. resolution) is feasible

How can I identify structures in my tissue?

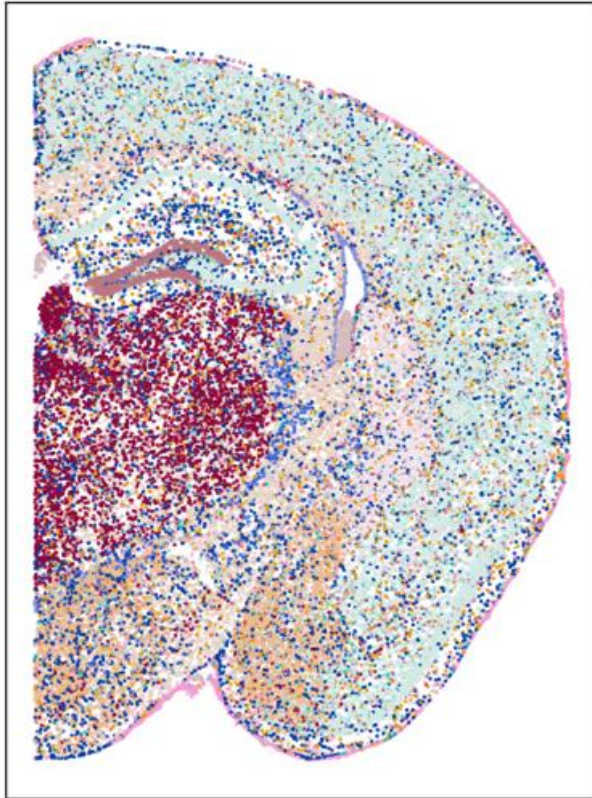
- Use performant tools like **SpatialLeiden**, **CellCharter**
- Run different set of parameters
- ... more coming up in the next section

Beyond Benchmarking

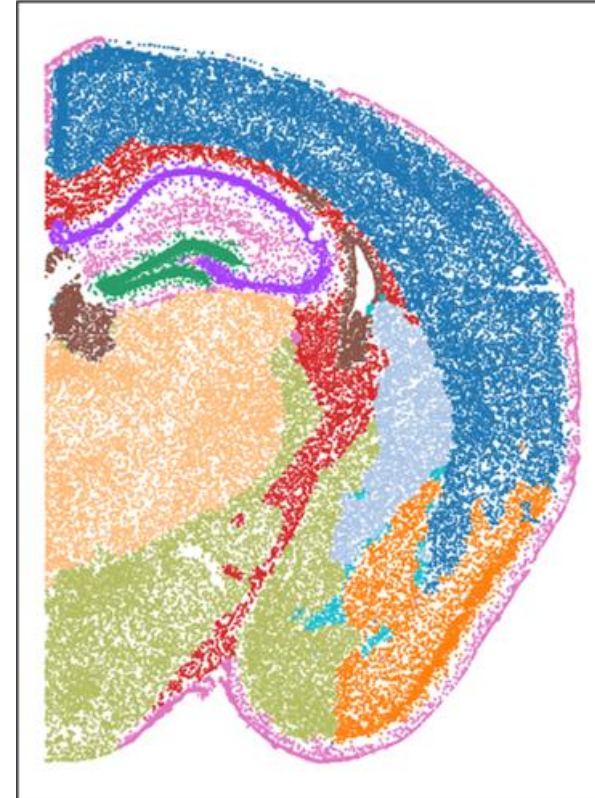
An Expert Guided Consensus Approach To Spatially Aware Clustering

From cells in space to tissue regions – spatial clustering

Cell map



Tissue regions



Adapted from Shang, Lulu, and Xiang Zhou. "Spatially aware dimension reduction for spatial transcriptomics." *Nature communications* 13.1 (2022): 7203.

Benchmarking spatially aware clustering

What do I expect?
(Ground truth)



Benchmarking spatially aware clustering

What do I expect?

(Ground truth)

What does the data look like? (data)



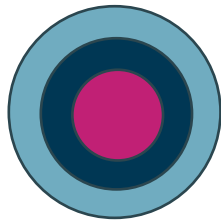
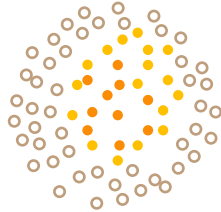
Benchmarking spatially aware clustering

What do I expect?

(Ground truth)



What does the data look like? (data)



What does my spatial clustering method give me?

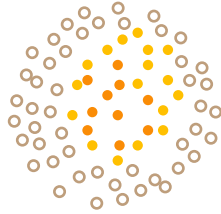
Benchmarking spatially aware clustering

What do I expect?

(Ground truth)



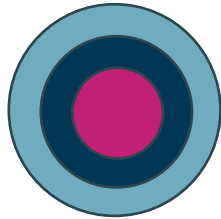
What does the data look like? (**data**)



Compare using a **metric**



What does my spatial clustering **method** give me?



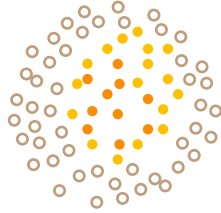
Benchmarking spatially aware clustering

What do I expect?

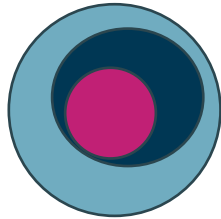
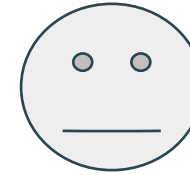
(Ground truth)



What does the data look like? (**data**)



Compare using a **metric**



What does my spatial clustering **method** give me?

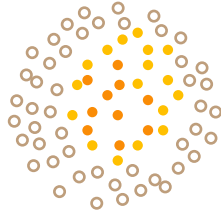
Benchmarking spatially aware clustering

What do I expect?

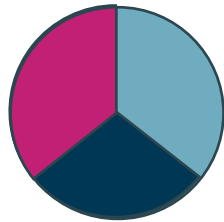
(Ground truth)



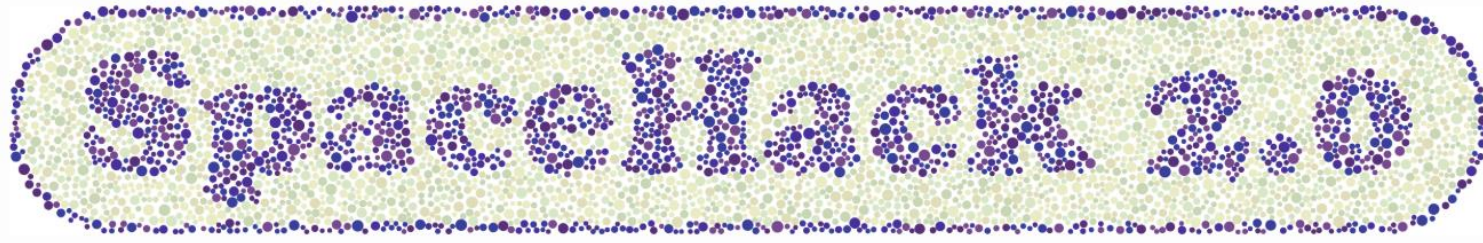
What does the data look like? (data)



Compare using a metric



What does my spatial clustering method give me?

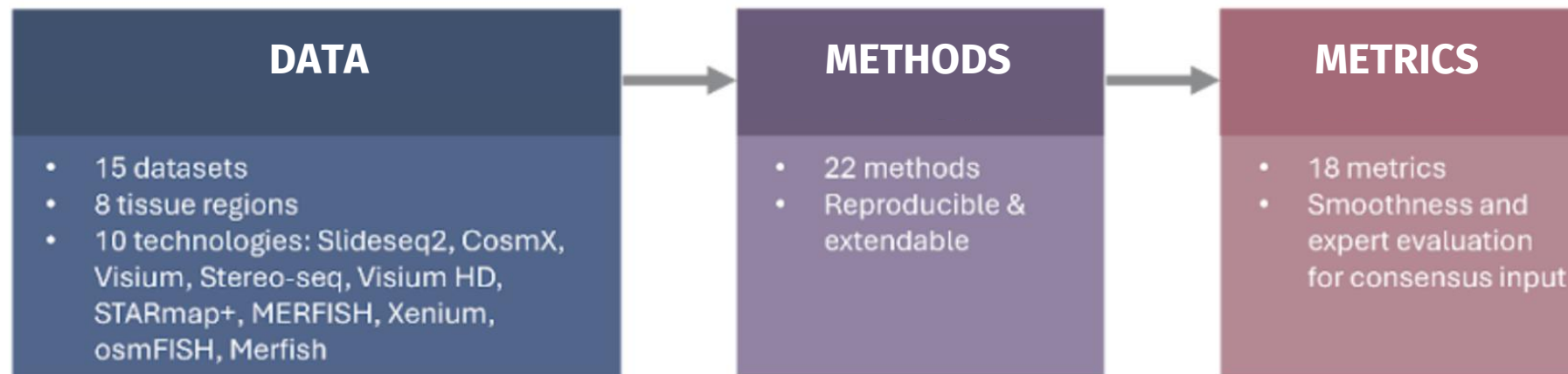


● ELIXIR-Germany 2nd BioHackathon ● 11-15 December 2023 ● Bielefeld, Germany ● Hybrid event ●



SpaceHack 2.0: a hackathon to establish best practices for spatial clustering/domain identification

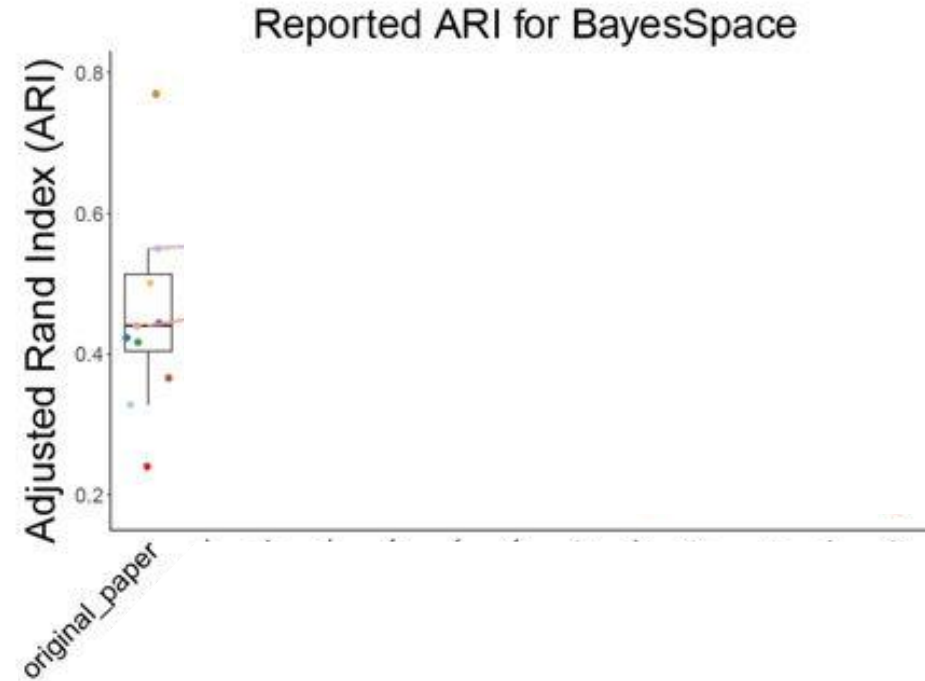
- 1 week, 28/62 people, 263 GIT commits, 400 slack messages
- 8 dedicated students, 4 PIs and 2 years later.... >20,000 results to evaluate...



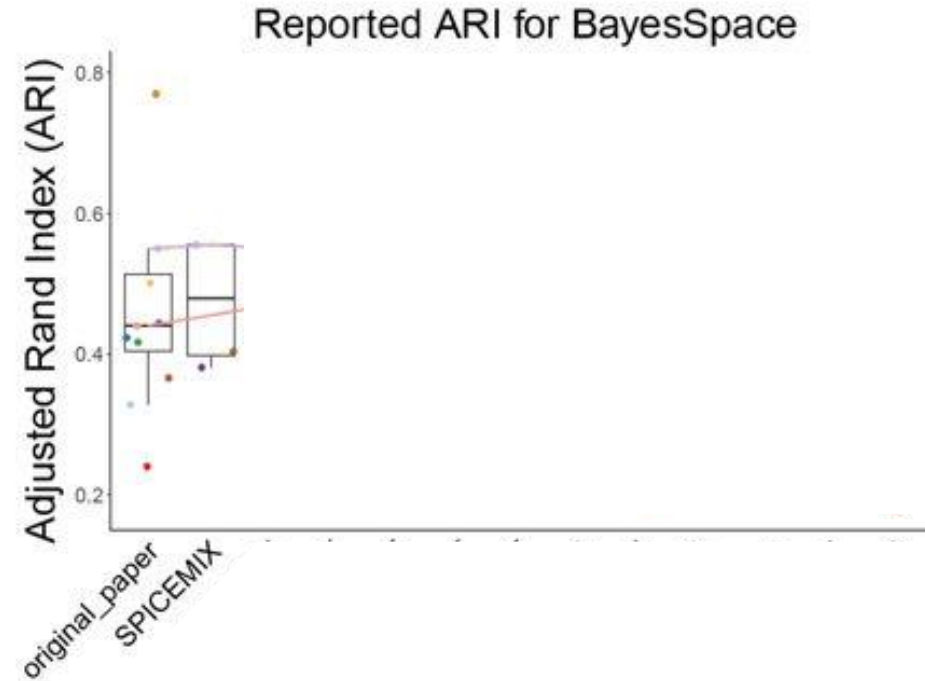
Where to start with so many results?

... sanity check – do our results align with previously reported performance?

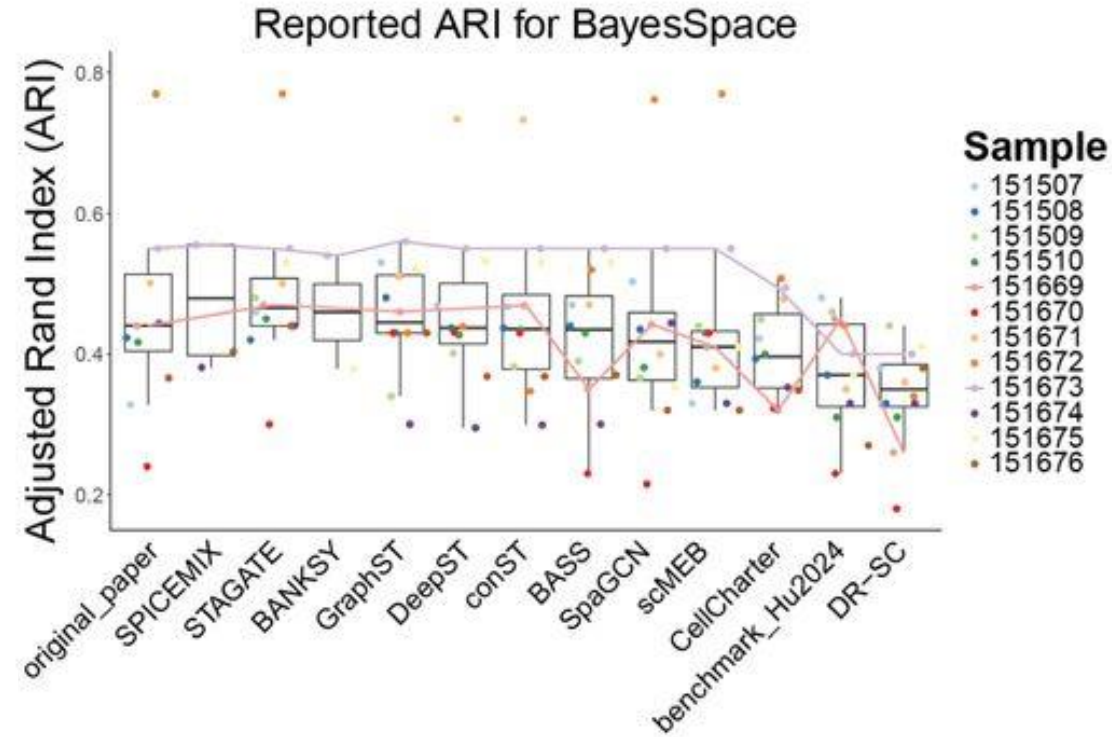
Performance reports are inconsistent between studies



Performance reports are inconsistent between studies

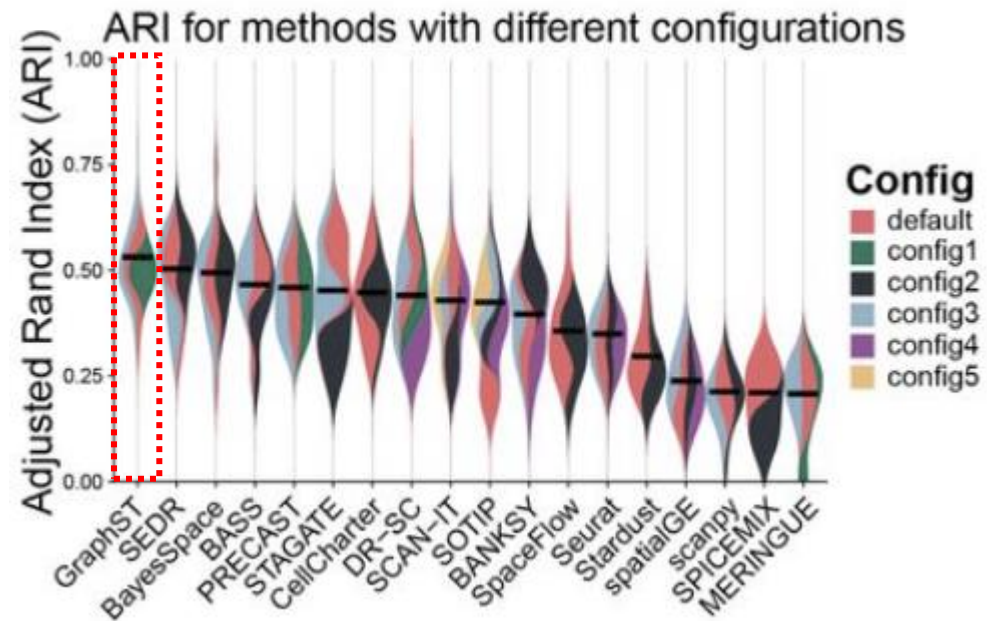


Performance reports are inconsistent between studies



Method performance can be sensitive to parameters

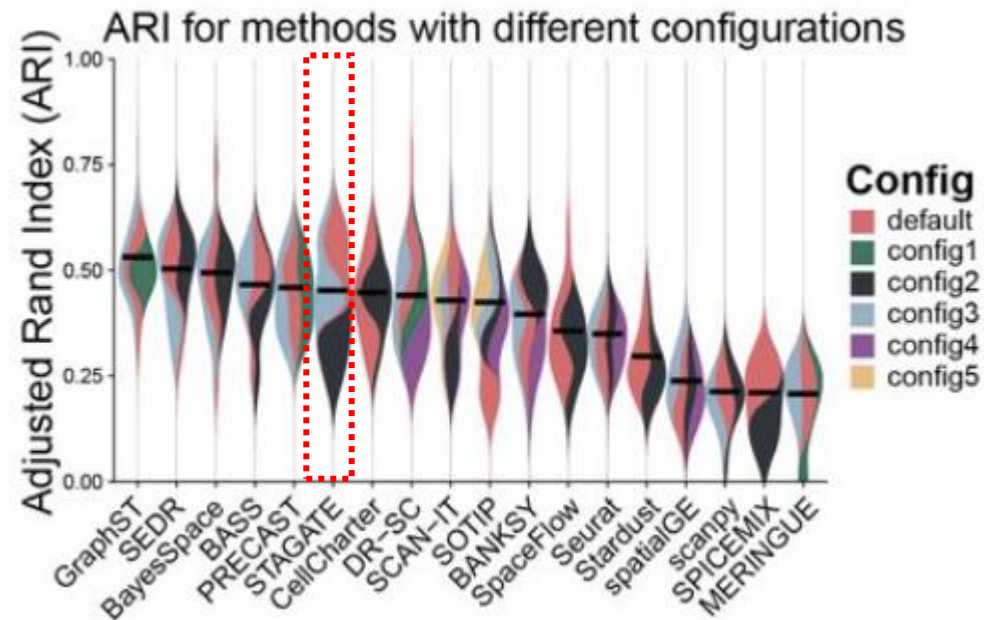
Some method seem robust to configuration parameters...



Method performance can be sensitive to parameters

Some method seem robust to configuration parameters...

... while others are highly sensitive



Conclusion 1: method implementations are not reproducible/robust

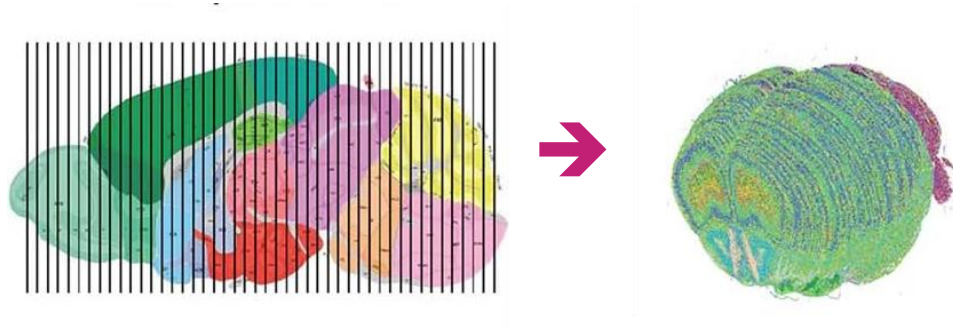
... great start! Maybe it gets better?

Spatial transcriptomics data keeps getting bigger...

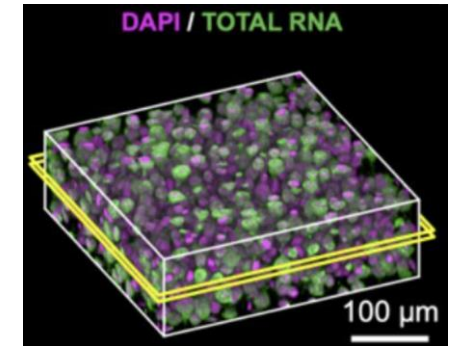
Size and resolution



3D reconstruction

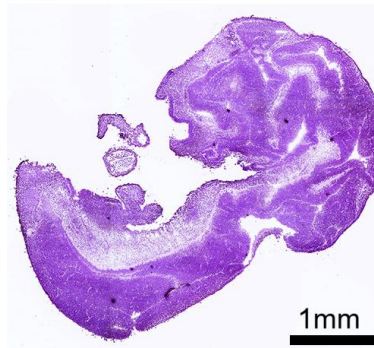


Real 3D

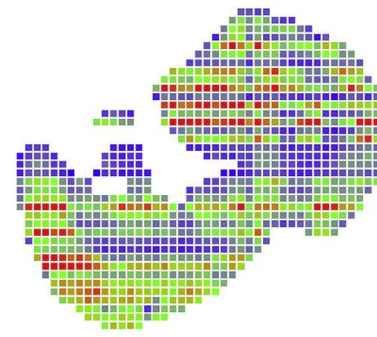


Multi-omics

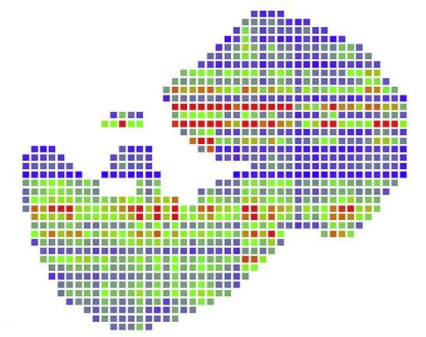
H&E (E.10 Embryo)



Pan-mRNA map



Pan-protein map

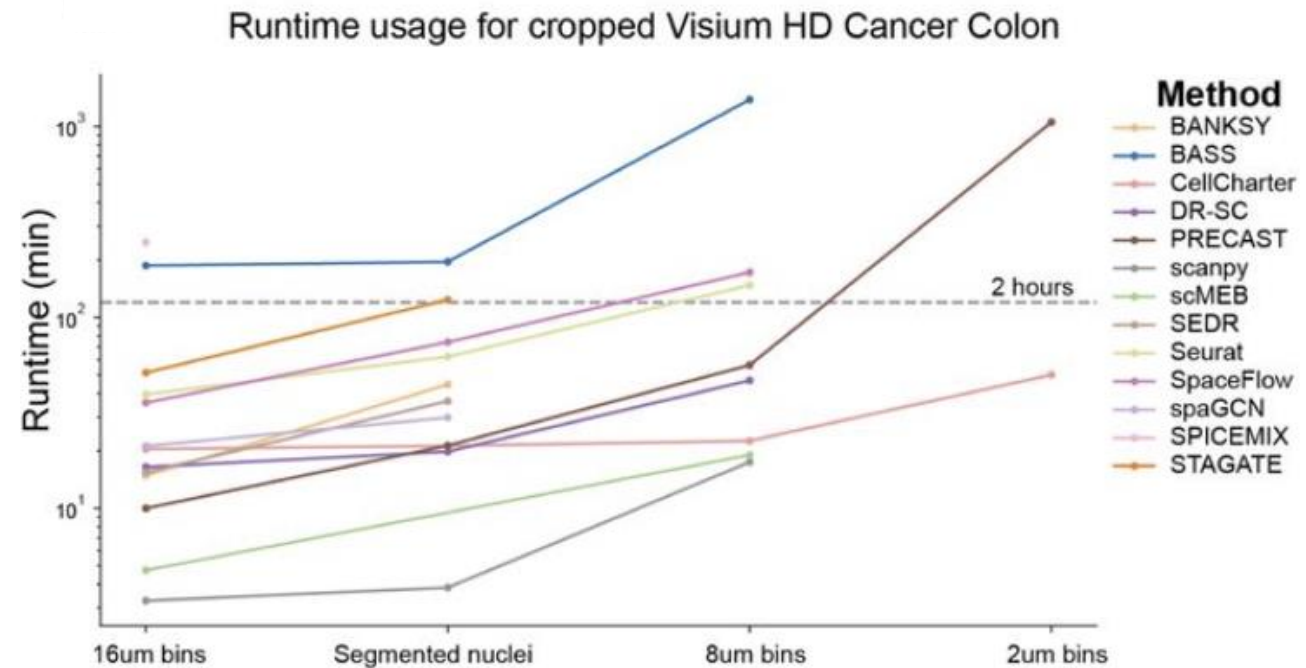
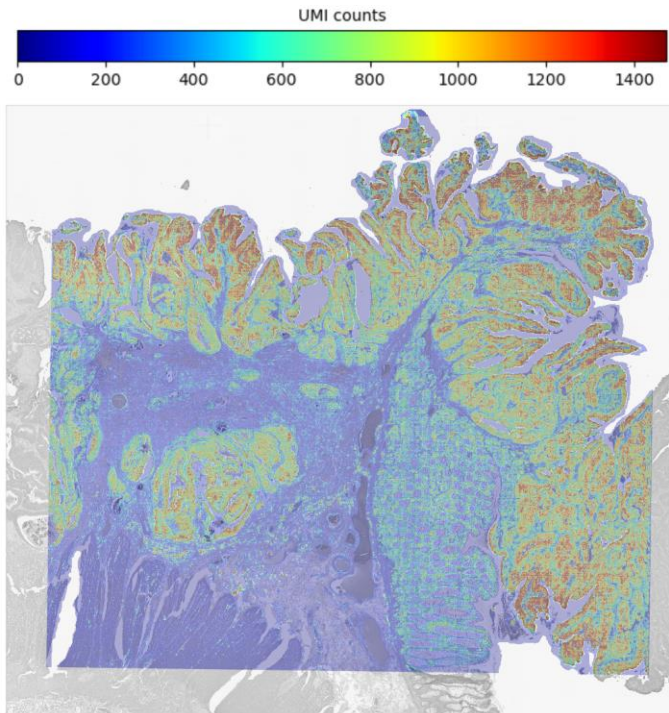


Most methods do not run on large single samples

Dataset: 1/4 10x Genomics VisiumHD (full transcriptome) colon cancer sample

Native resolution: 2 μm

Typical bin size for analysis: 8 μm

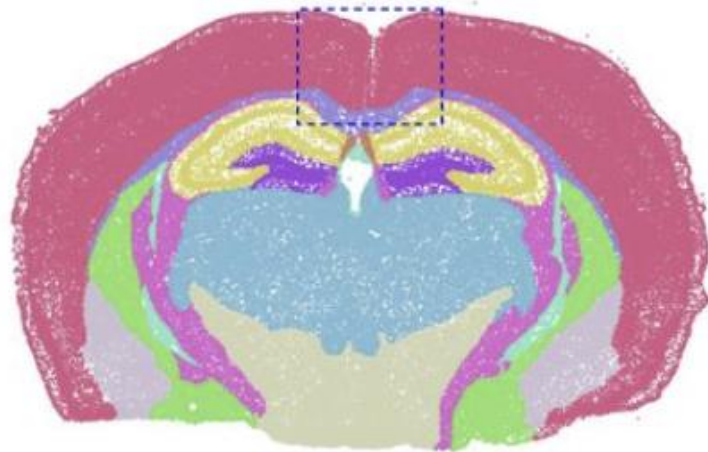


Conclusion 2: method do not scale

... I hope the 8 students working on this aren't getting too annoyed...

Problems with ground truths: they do not consider granularity

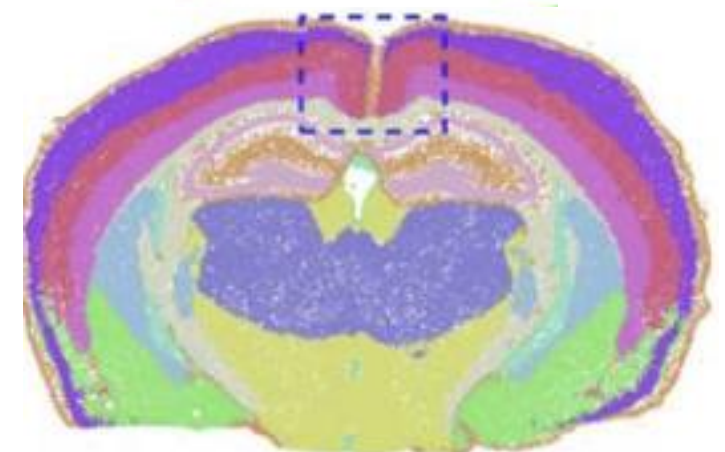
Xenium dataset GT



Label

● CTXpl	● FC	● MFBs	● VS
● DG	● LFBs	● TH	● CTXsp
● HY	● CA	● STR	

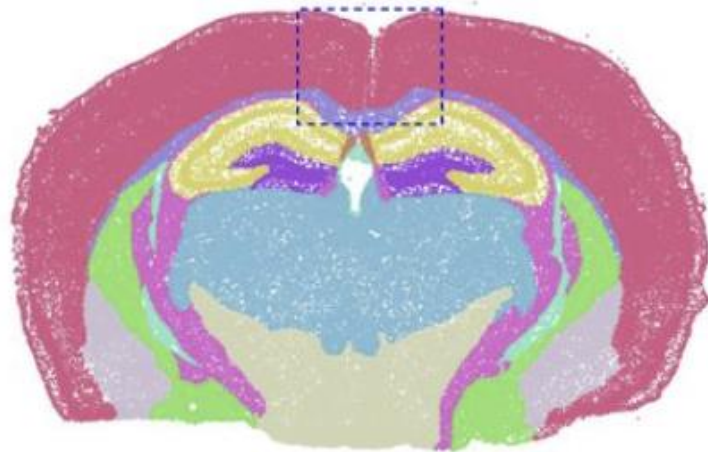
CellCharter results



11 clusters

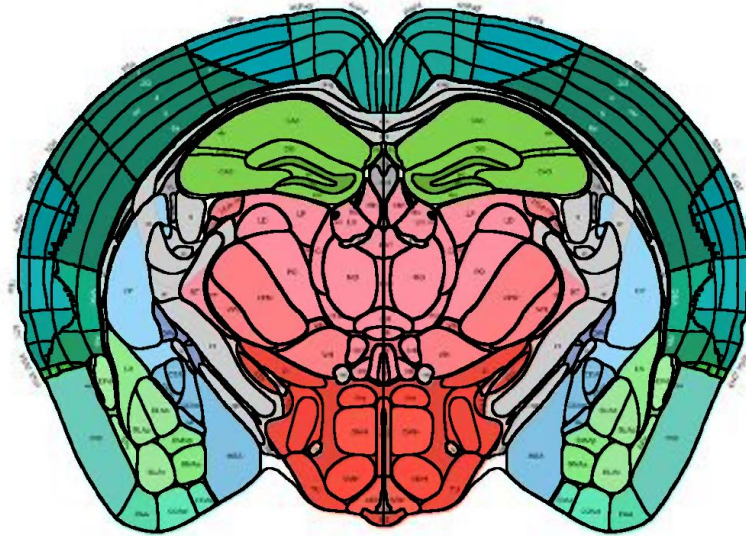
Problems with ground truths: they do not consider granularity

Xenium dataset GT

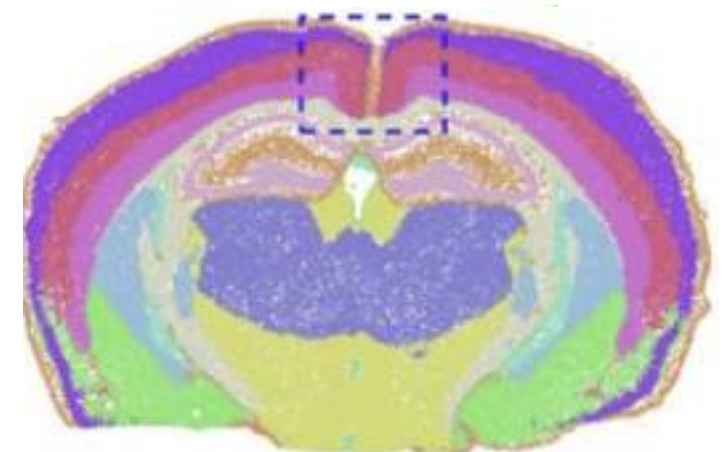


Label

CTXpl	FC	MFBs	VS
DG	LFBs	TH	CTXsp
HY	CA	STR	



CellCharter results



11 clusters

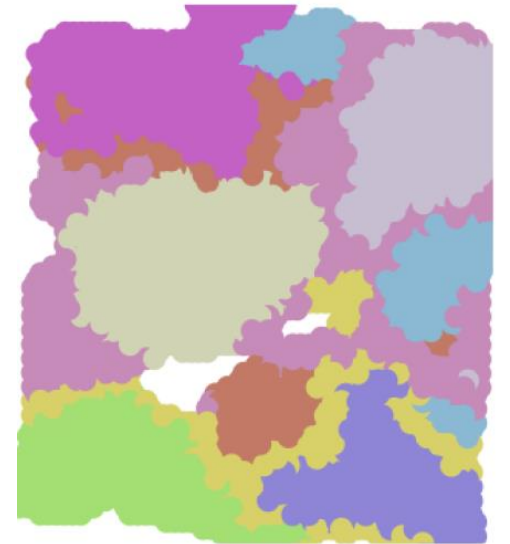
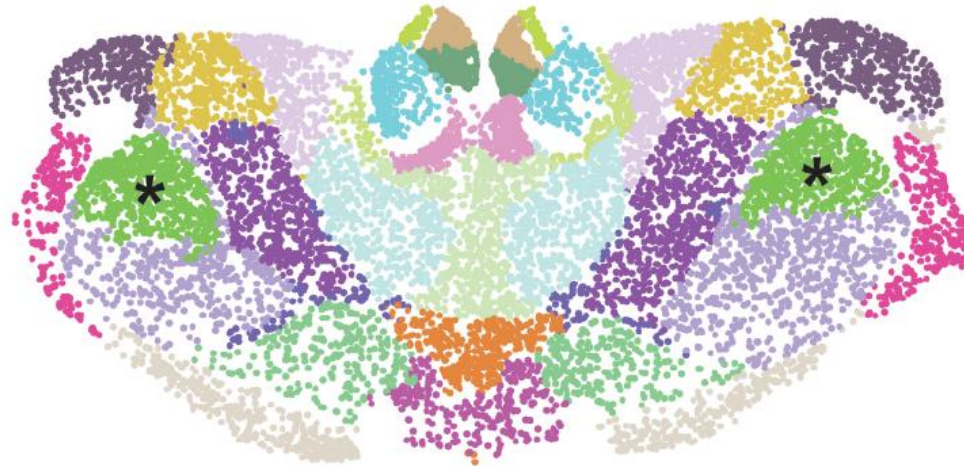
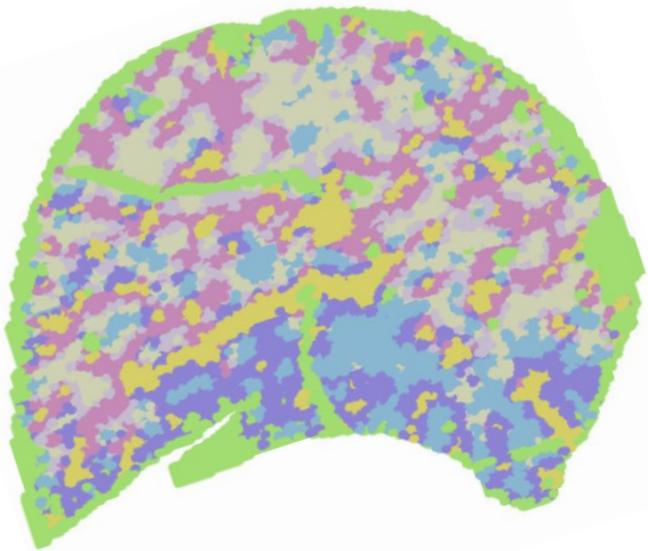
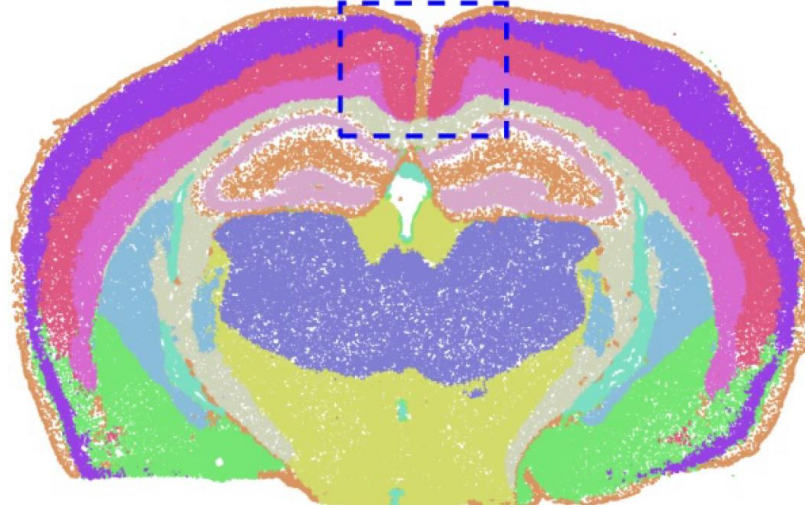
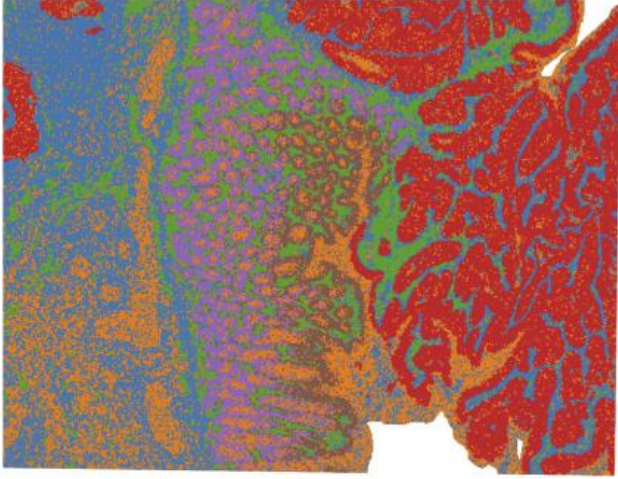
Problems with ground truths: they can be computer generated

- **What do we want to replicate: human behaviour or other computational tools?**
- **Should ground truths be defined by humans or computational tools?**
- **Many GTs are computationally generated:**
 - Stereo-seq mouse embryo ground truth labels → SCC, which used Leiden
 - **(No) Surprise:** Leiden implementations in Seurat and Scanpy had best performance!
 - STARmap mouse cortex ground truth labels → BASS
 - **(No) Surprise:** BASS was a top tier performer
 - **Surprise:** ... it was not the best!?!?

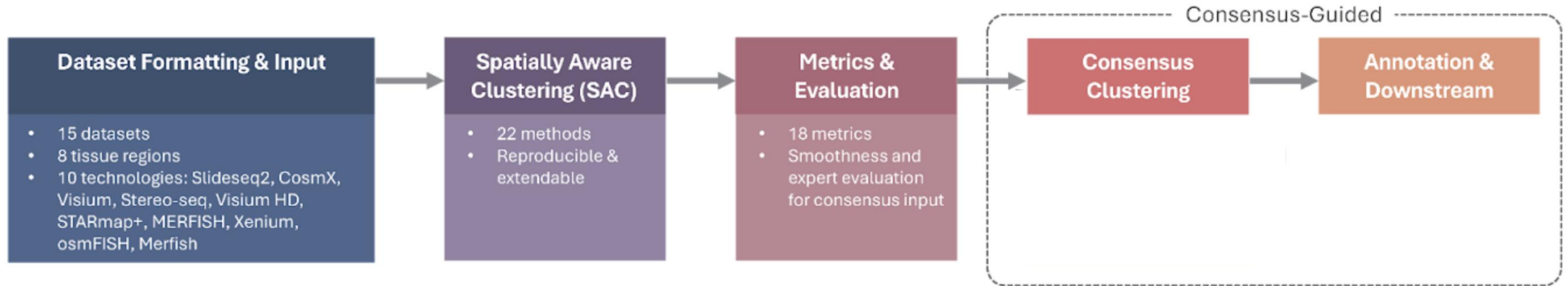
Conclusion 3: ground truth annotations are suboptimal

... time to run and hide from those 8 students that have been working on this for 2 years?

Question: can we trust the output of methods?



SACCELERATOR: a framework to evaluate spatial clustering



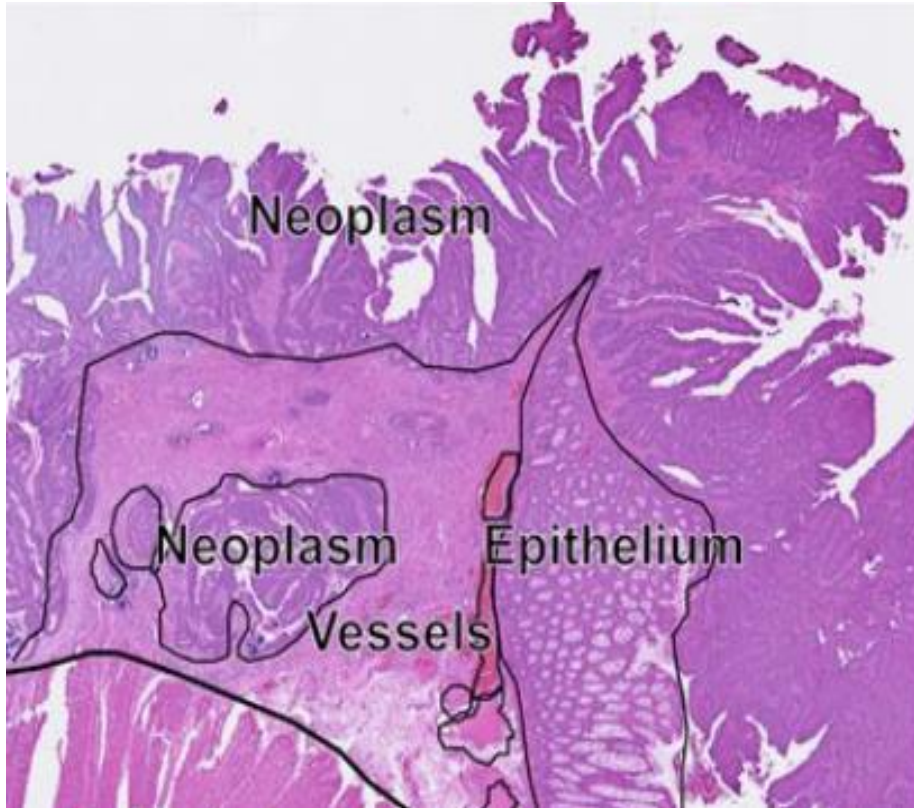
Challenging case study: colon cancer

VisiumHD

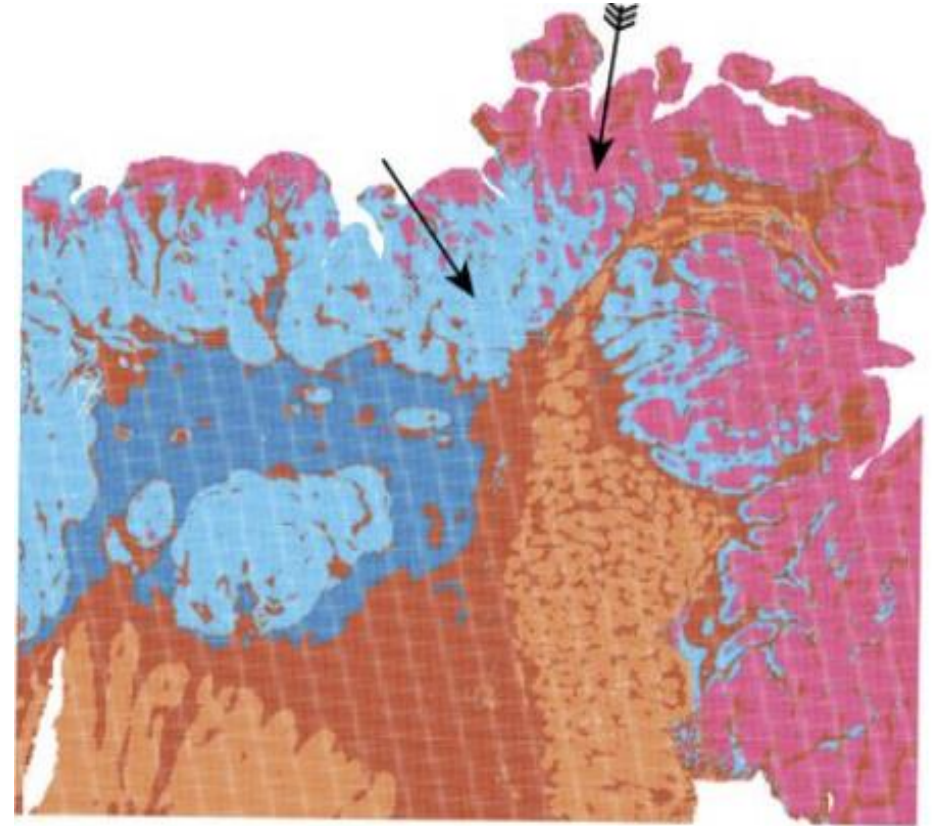
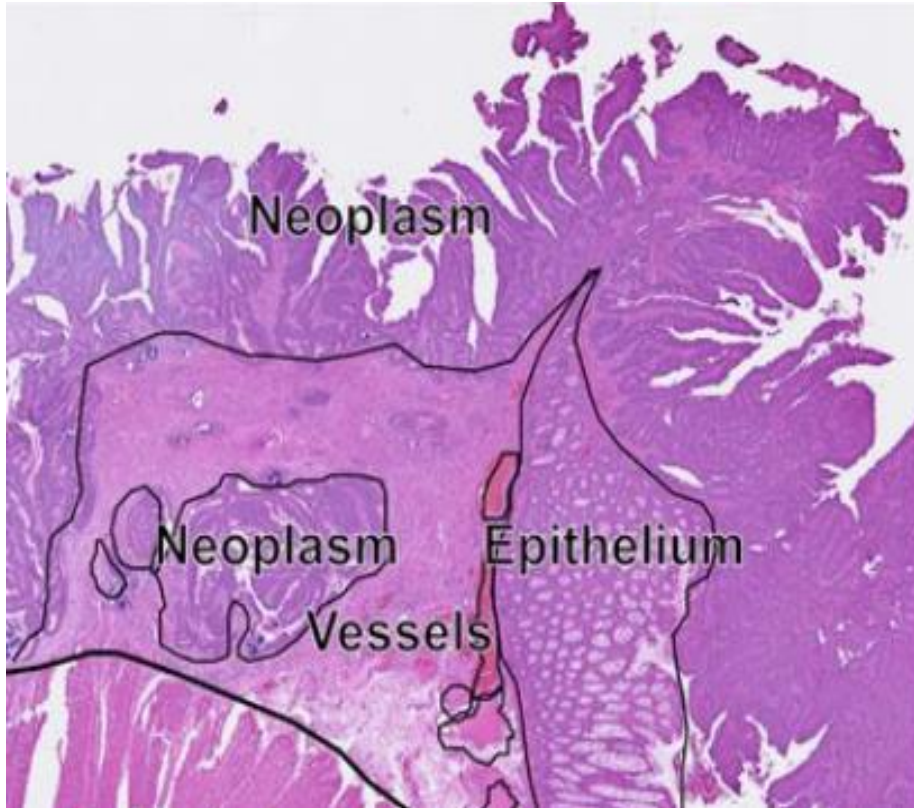
No ground truth...

Work done by Paul Kiessling (AG-Kuppe)

Step 1: define some sort of ground truth for optimizing methods



Step 2: run consensus spatial clustering



Step 3: evaluate results

Expert Pathology Ranking

Can identify neoplastic and non-neoplastic tissue	+	+	+	+	+	+	+	+	+	+	+	+	~
Can identify all major tissue compartments (muscle, mucosa, etc)	+	+	+	+	+	+	+	+	+	+	+	+	~
Can phenotype the connective tissue	+	+	+	+	+	+	~	+	+	+	+	+	-
Can identify small groups of tumor cells	~	+	~	~	~	~	~	~	~	~	~	~	~
Can identify different differentiation states (grades) of epithelial cells	~	~	~	~	~	~	~	~	~	~	~	~	~
Can detect lymphovascular and perineural structures	+	~	~	~	~	~	+	-	-	-	-	-	-
Can identify immune populations	-	-	-	-	-	-	-	-	-	-	-	-	-
	STAGATE_slide_stereo	DR-SC_FindSVGs	CellCharter_default	DR-SC_default	Seurat_leiden_VisiumHD	CellCharter_dlpfc	spaGCN_default	SCAN-IT_default	SpaceFlow_seqfish	SpaceFlow_default	SEDR_dlpfc	STAGATE_default	scanpy_leiden_tutorial

Good +

Average ~

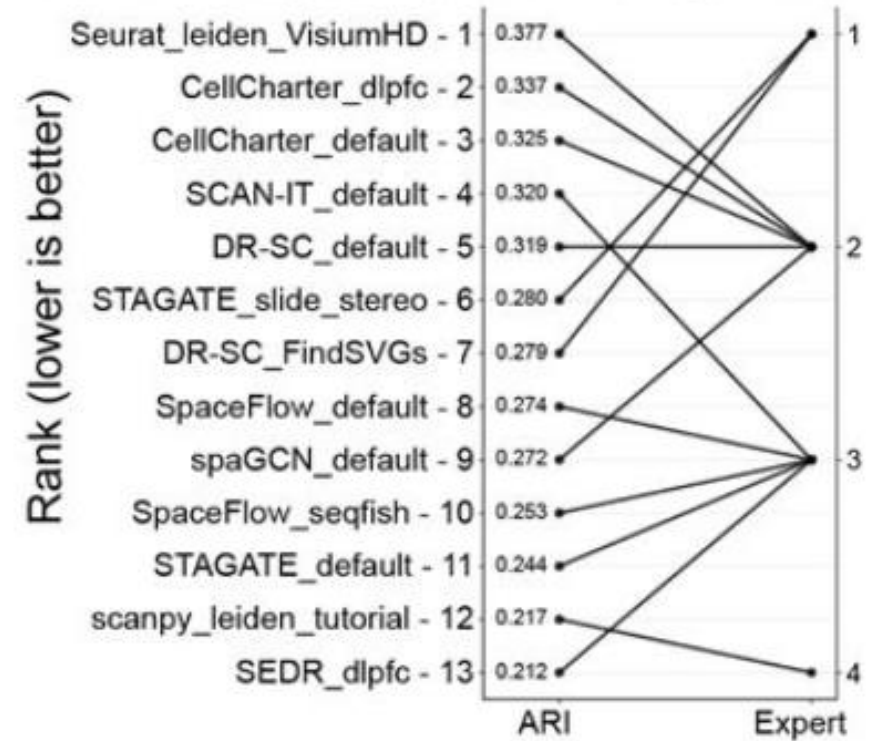
Poor -

Step 3: evaluate results

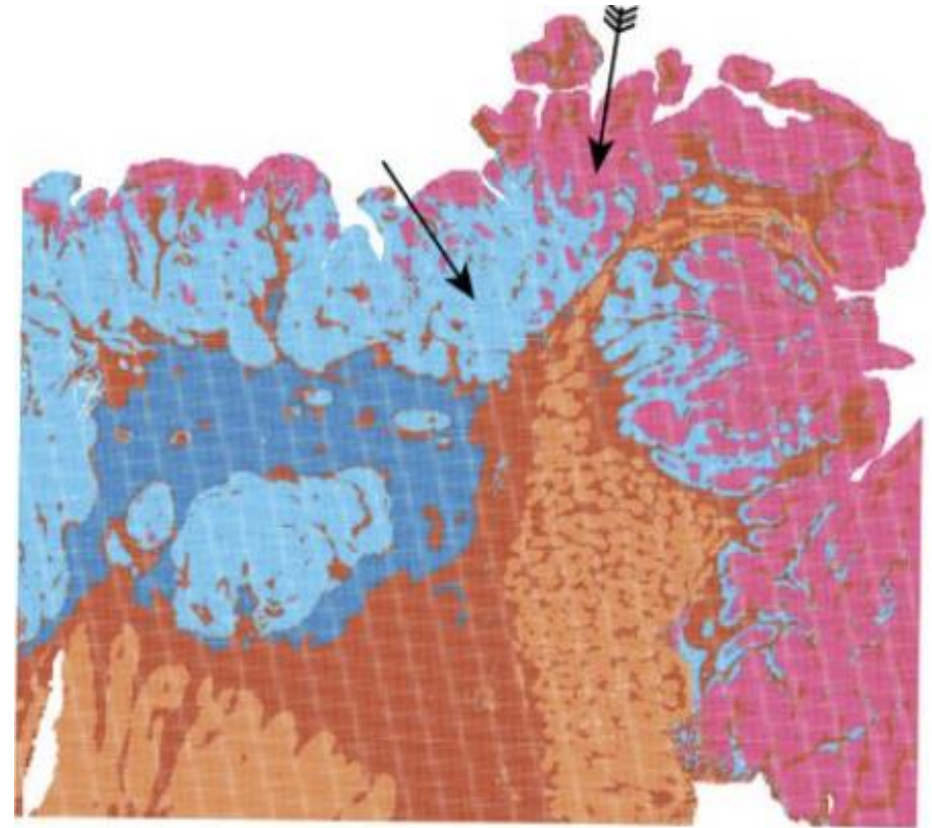
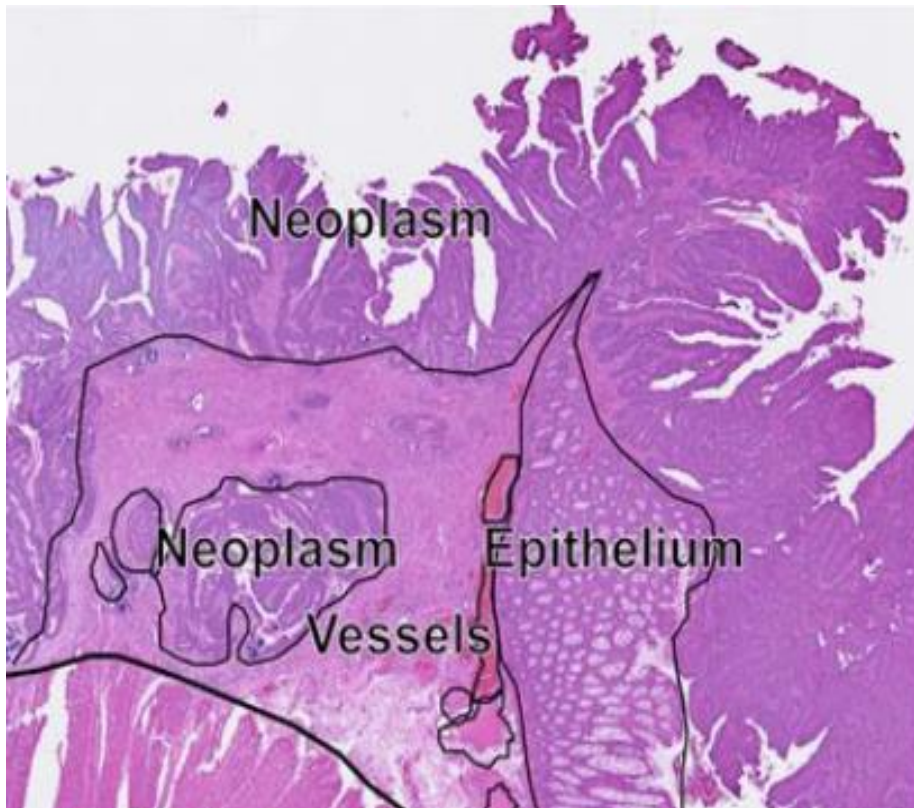
Expert Pathology Ranking

Can identify neoplastic and non-neoplastic tissue	+	+	+	+	+	+	+	+	+	+	+	+	~
Can identify all major tissue compartments (muscle, mucosa, etc)	+	+	+	+	+	+	+	+	+	+	+	+	~
Can phenotype the connective tissue	+	+	+	+	+	+	~	+	+	+	+	+	-
Can identify small groups of tumor cells	~	+	~	~	~	~	~	~	~	~	~	~	~
Can identify different differentiation states (grades) of epithelial cells	~	~	~	~	~	~	~	~	~	~	~	~	~
Can detect lymphovascular and perineural structures	+	~	~	~	~	~	+	-	-	-	-	-	-
Can identify immune populations	-	-	-	-	-	-	-	-	-	-	-	-	-
	STAGATE_slide_stereo	DR-SC_FindSVGs	CellCharter_default	DR-SC_default	Seurat_leiden_VisiumHD	CellCharter_dlpfc	spaGCN_default	SCAN-IT_default	SpaceFlow_seqfish	SpaceFlow_default	SEDR_dlpfc	STAGATE_default	scanpy_leiden_tutorial
Good	+												
Average	~												
Poor	-												

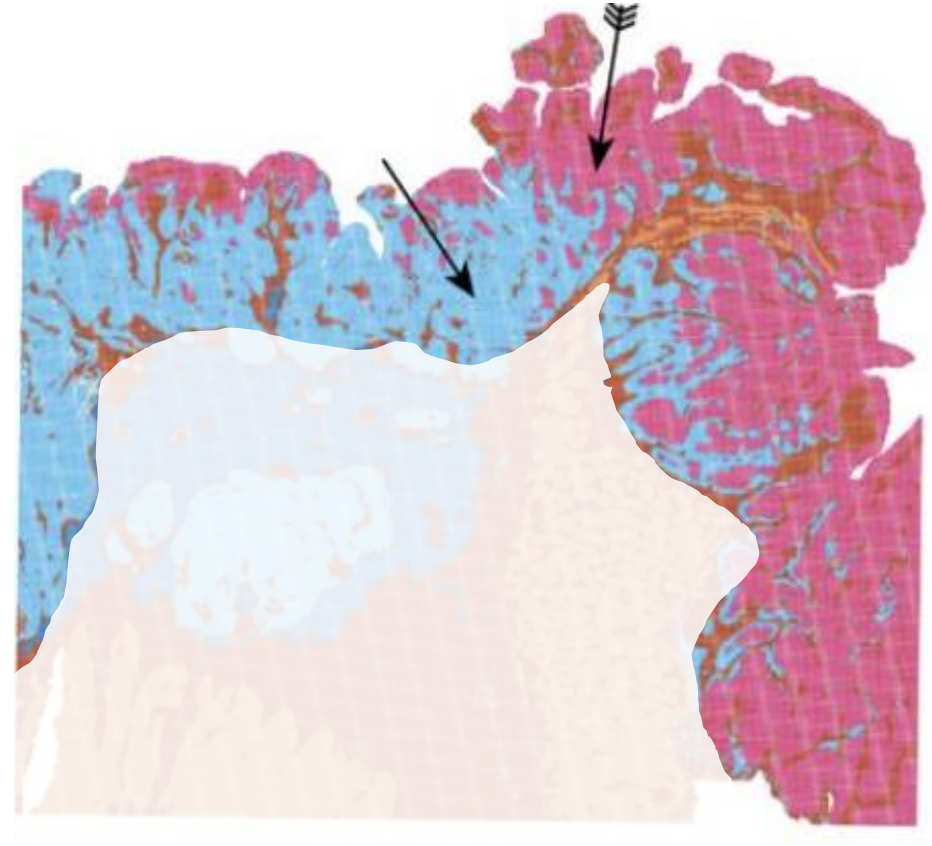
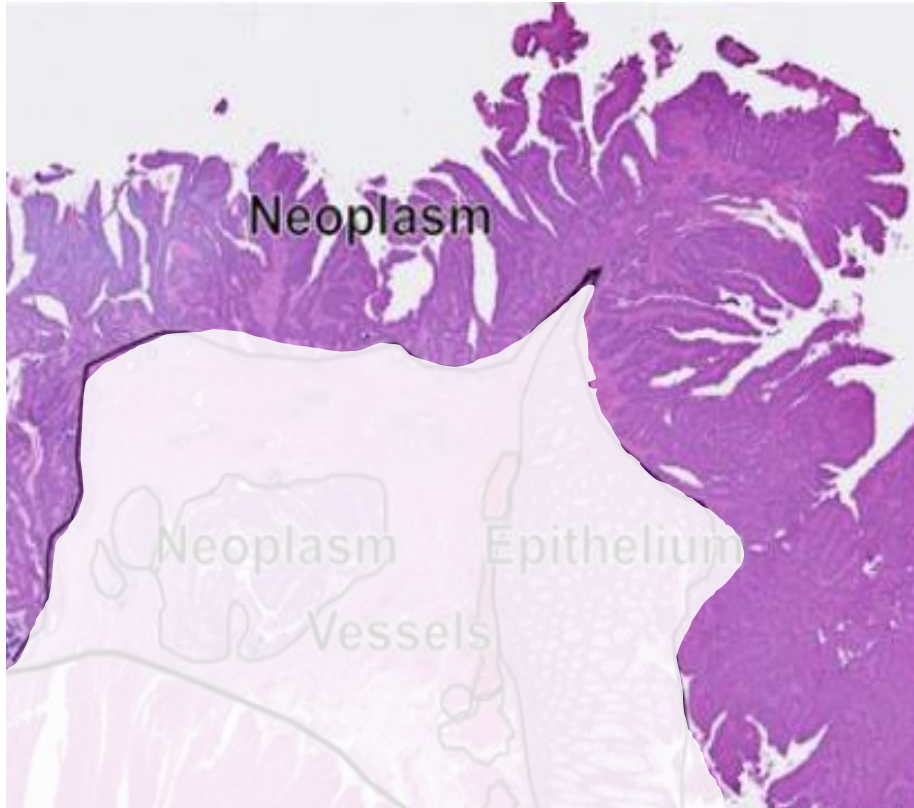
ARI Ranking vs Expert Pathology Ranking



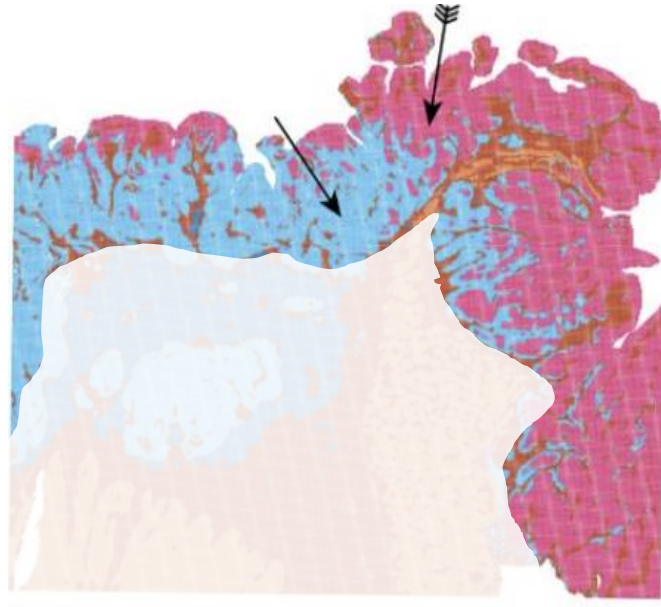
Step 4: discover something new



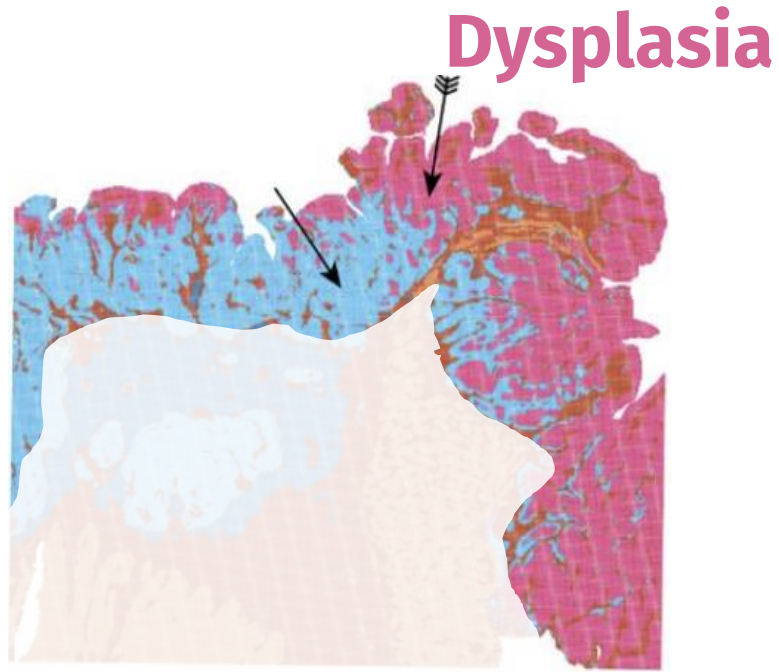
Step 4: discover something new



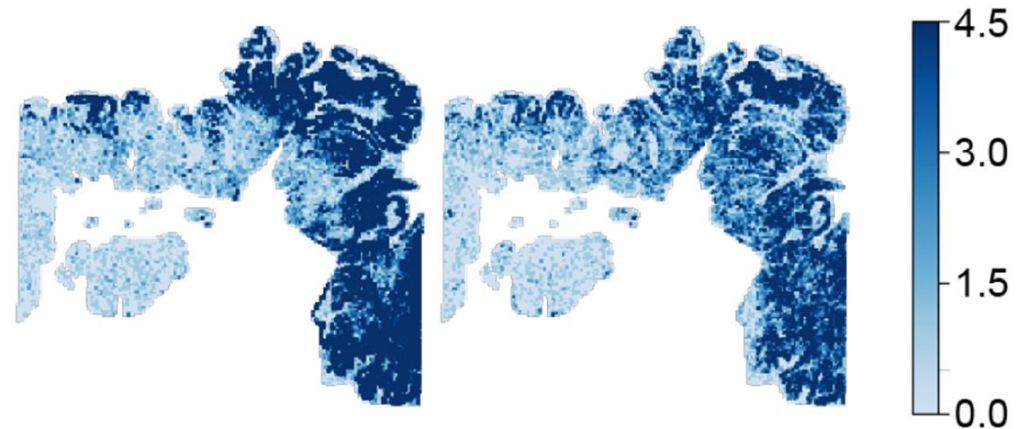
Step 4: discover something new



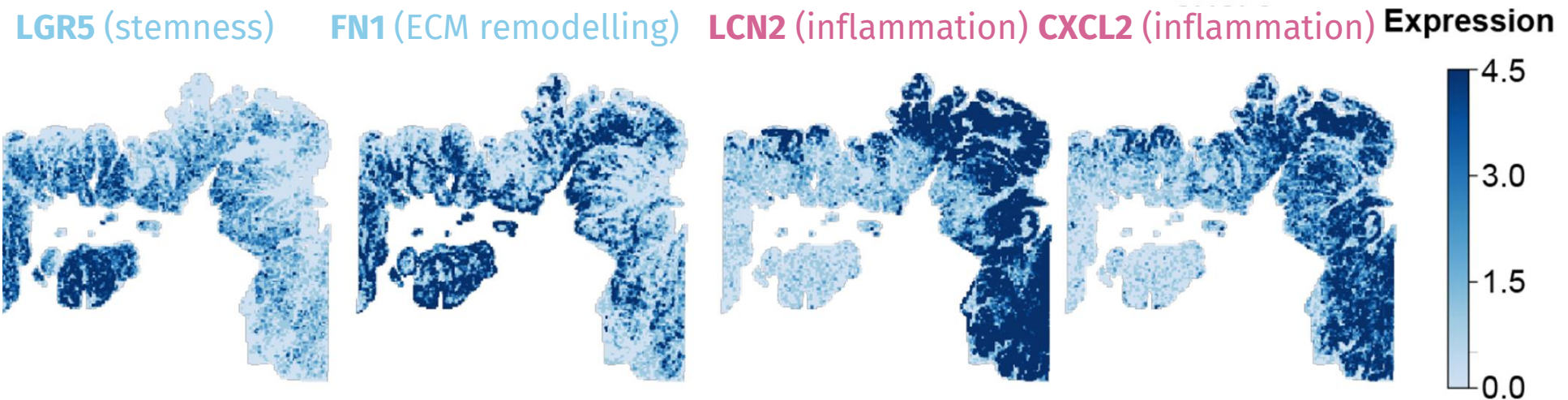
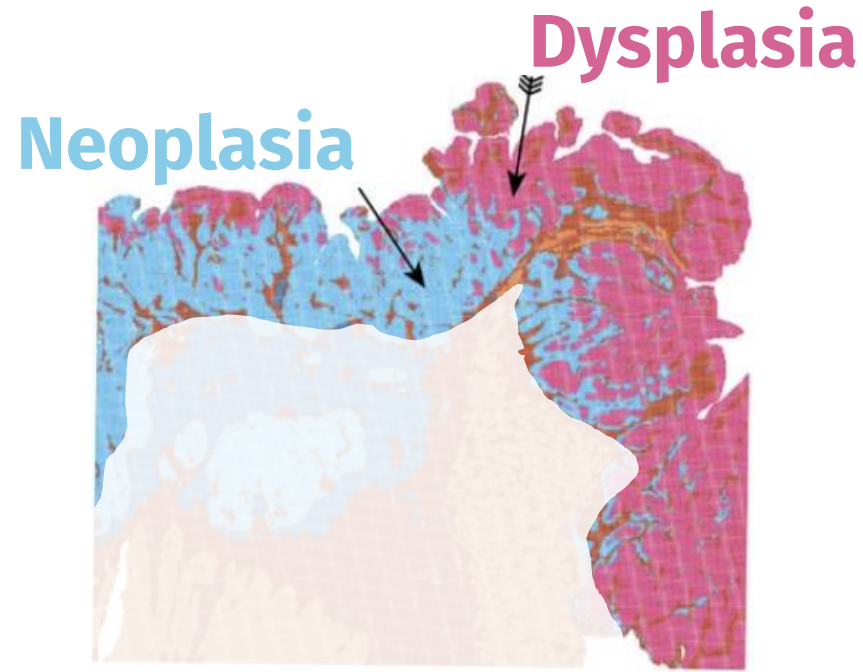
Step 4: discover something new



LCN2 (inflammation) CXCL2 (inflammation) Expression



Step 4: discover something new



Summary 4: benchmarking is hard

- **Why is benchmarking hard?**
 - Previous benchmarks do not seem to be **reproducible**
 - Evaluation metrics do not consider **granularity** of spatial biology
 - You can only trust results as much as you can trust the **ground truth**
 - **Some bioinformatics benchmarking efforts are working well – e.g. protein structures**
- **How can I proceed to do spatial clustering if you have no tool recommendations?:**
 - **Use tools that your colleagues have used** and have had good experiences with
 - Use a tool that is **fast and efficient** e.g. CellCharter (or SpatialLeiden)
 - There is no substitute for a **team of experts**

Acknowledgements

All donors and patients

AG-Ishaque

Sebastian Tiesmeyer
Niklas Mueller-Boetticher

Alexander Malt
Yiheng Jiang
Kaixin Huang
Yizhen Xiang
Fei Wang
Peiru Du
Haoxin Wang
Jianpeng (Raphael) Zhang
George Gavrilidis
Timon Last

BIH Center of Digital Health

Project management team
Compute infra. Teams
Roland Eils

SpaceHack Participants

HCA Cell Segmentation Team

BIH Digital Health Accelerator

David Aagten-Murphy
Dorothee Marie-Louise Döpfer
Tim Huse

CCCC, Charite

Lukas Roelke
Anastasia Dielmann
Anna Kotarac
Loredana Vecchione
Ulrich Keilholz

ECRC, Charite

Olivia Nonn
Kerim Secener
Florian Herse
Sunhild Hartmann
Dominik Mueller & Ralph Dechend

Dept. of Hematology, Oncology and Tumorimmunology, Charite

Shashwat Sahay
Sophy Denker
Bjoern Chapuy

Dept of Gastroentroylogy, Charite

Felix Bolduan
Bertram Wiedenmann
Frank Tacke

University of Zurich

Peiying Cai
Martin Emons
Samuel Gunz
Mark Robinson

Dept. of Pathology, Charite

Franz Dressler
Markus Morkel
David Horst

University of Lausanne/CHUV

Jieran Sun
Raphael Gottardo

Dept. of Gynecology, Charite

Andreas Lackner
Ioana Braicu

Pusan National University

Jeongbin Park

Med Uni Graz

Katja Sallinger
Amin El-Heliebi

RWTH Aachen

Paul Kiessling
Christoph Kuppe

Allen Brain Institute

Meghan Turner
Brian Long

Technical University Delft

Kirtie Biharie
Marcel Reinders
Ahmed Mahfouz

Science for Life Laboratory

Sergio Marco Salas
Mats Nilsson

INAB/CERTH, Greece

Fotis Psomopoulos

Technical University of Denmark

Søren H. Dam

Hannover Medical School

Christian Hinze

DKFZ Heidelberg/GHGA

Florian Heyl
Oliver Stegle

Helholtz Munich

Francesca Drummer
Sara Jimenez
Licas Arnoldt
Malte Luecken
Fabian Theis

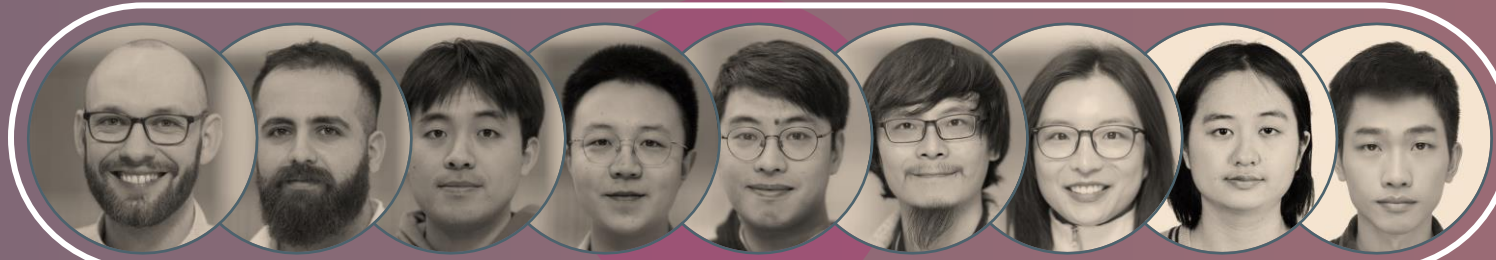
ELIXIR Single-Cell Omics

Paulo Czarnewski
Andrew Stubbs
Eija Korpelainen

AG-Ishaque



Postdoc



PhD



Clinician Scientist



Swiss Institute of Bioinformatics



Swiss National Science Foundation



Leiden University Medical Center



Centre hospitalier universitaire vaudois



Delft University of Technology



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