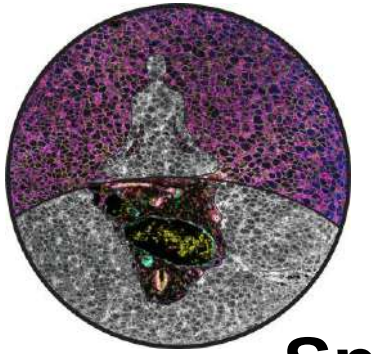

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Spatial mapping of the hepatocellular carcinoma landscape identifies unique intratumoural perivascular-immune neighbourhoods

Felix Marsh-Wakefield, Positha Santhakumar, Angela L Ferguson, Joo Shin, Ken Liu, Geoffrey McCaughan, Umaimainthan Palendira

Hepatocellular carcinoma (HCC)

- Most common primary liver cancer
- Sixth most common cancer worldwide, third most common cause of cancer death
  - Sung *et al.*, 2020 (*CA Cancer J Clin*)
- In 2021, mortality rates in Australia >80%, double that of the preceding two decades
  - Australian Institute of Health and Welfare, Cancer in Australia 2021

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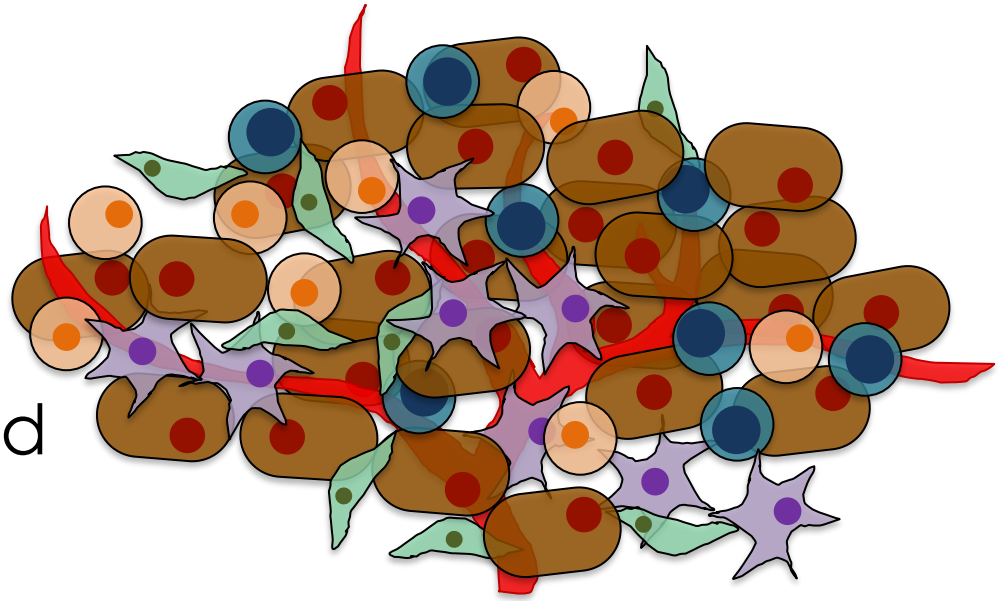
Tumour microenvironment

- Consists of cancer, immune, and stromal cells
- Associated with patient aetiologies and outcomes
- **More work needed to understand role in HCC**



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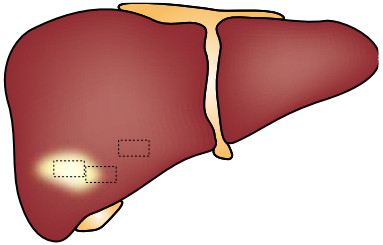
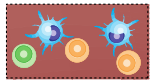
Sample collection

16 HCC patients
Liver resections

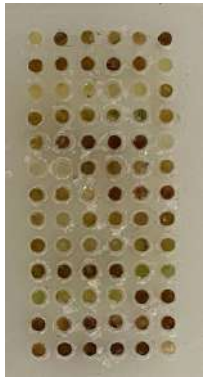


Non-tumour



Interface

Tumour



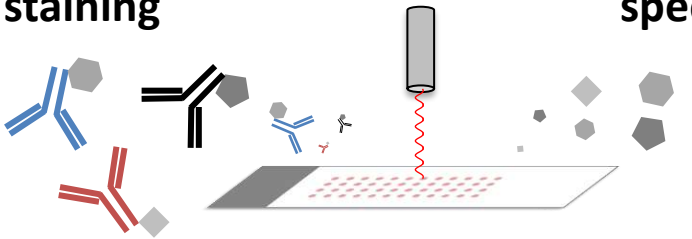
1mm circles

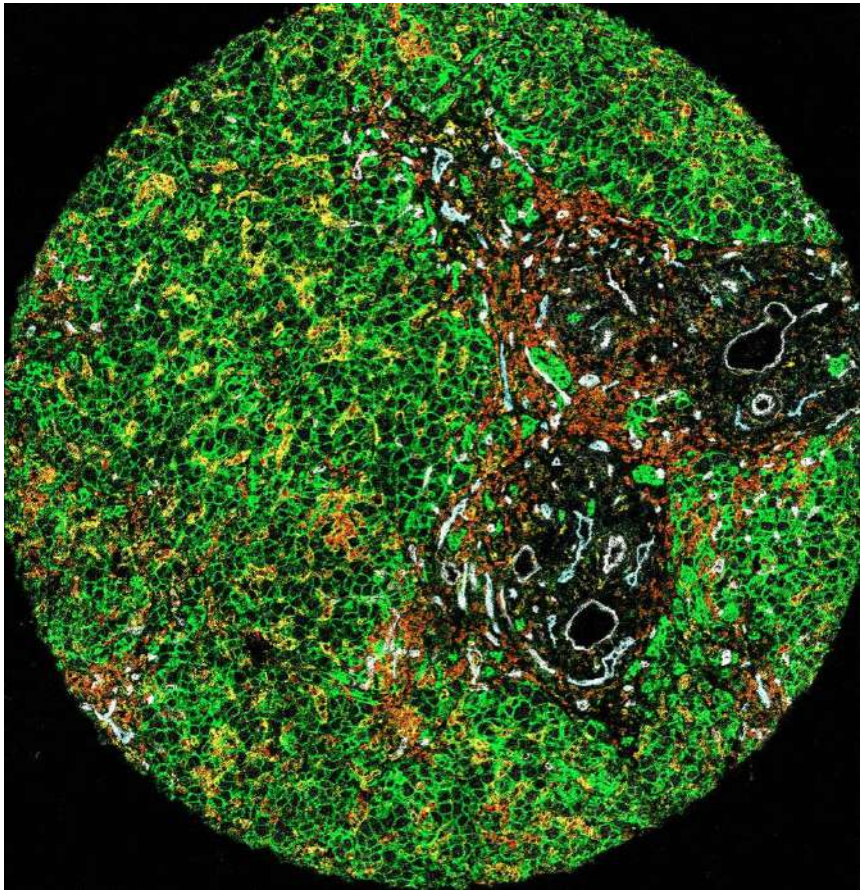
Imaging mass cytometry

Antibody staining

Tissue ablation

Mass spectrometry





Imaging mass cytometry panel

Hepatocytes

CK8/18
E-cadherin
HepPar1

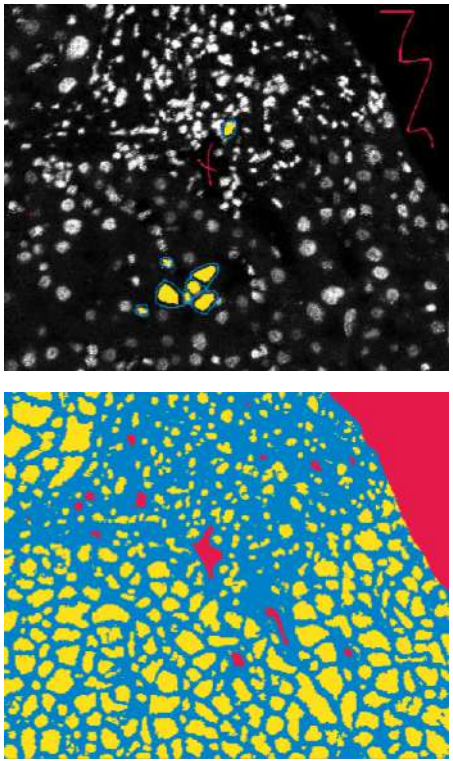
Structural markers

αSMA
CD34
podoplanin
vimentin

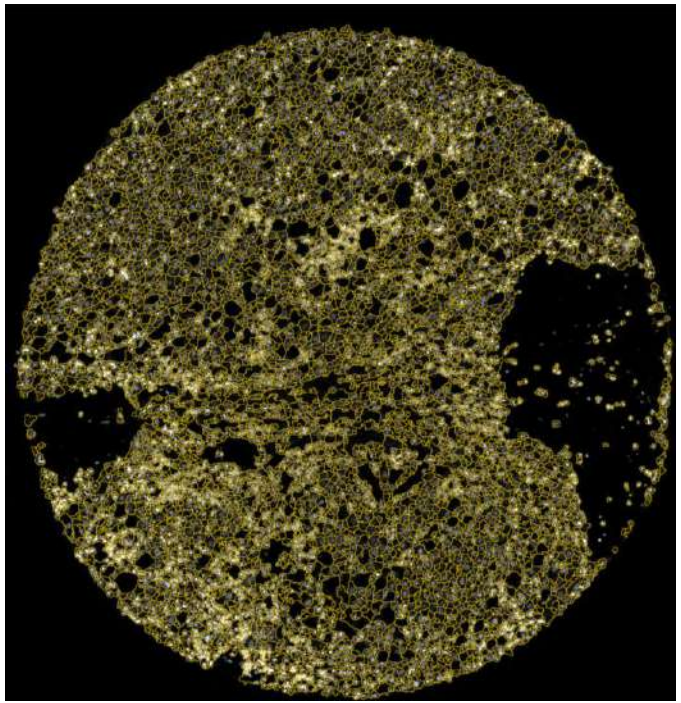
Immune cell markers

CD3	CD45RO	CD209	NaKATPase
CD4	CD56	CD303	PD-L1
CD8a	CD57	Foxp3	PD-L2
CD11b	CD66a	FXIIIa	SirPa
CD11c	CD68	Granzyme B	T-bet
CD14	CD69	HLA-DR	
CD16	CD86	IgA	
CD20	CD127	Ki67	
CD45	CD206	MPO	

Training

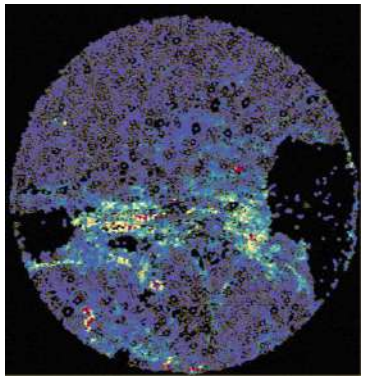


Cell segmentation

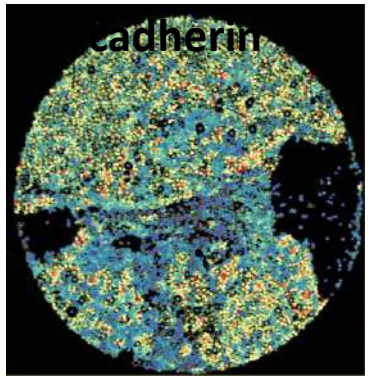


Marker quantification

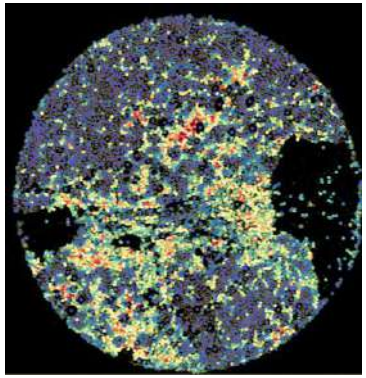
αSMA



E-cadherin

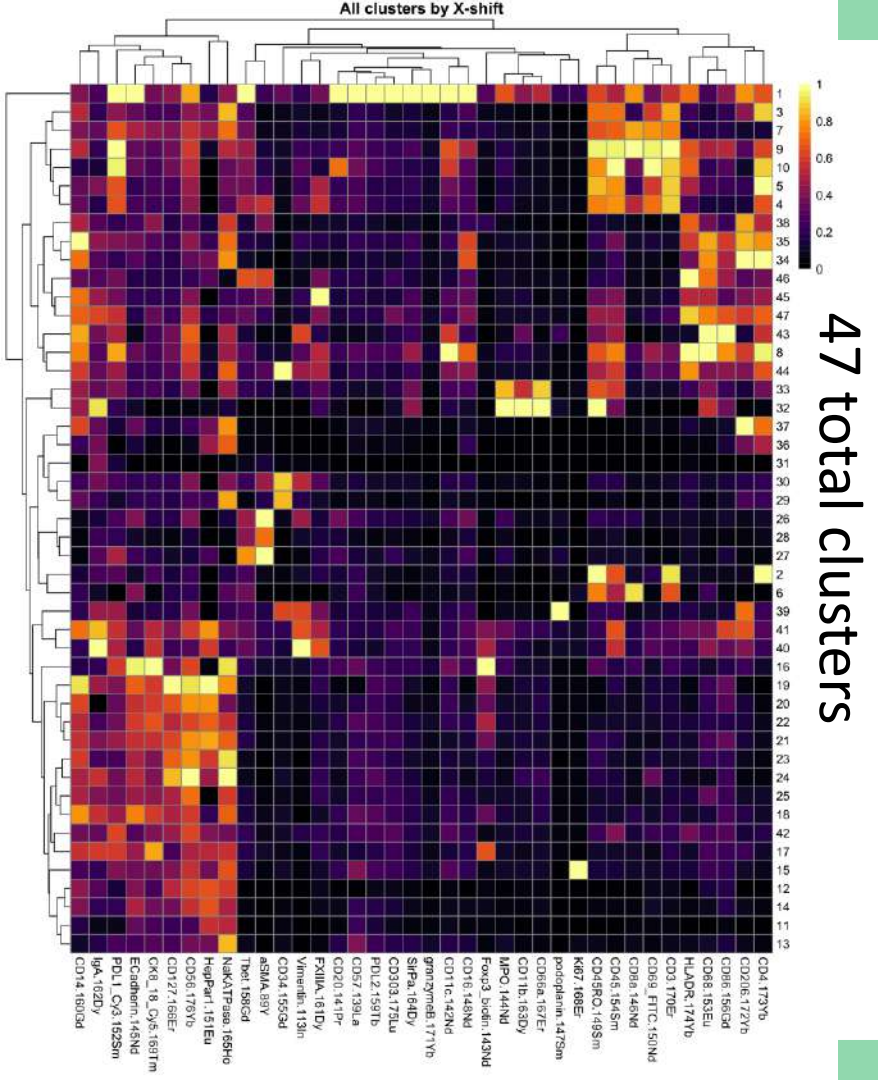


CD45



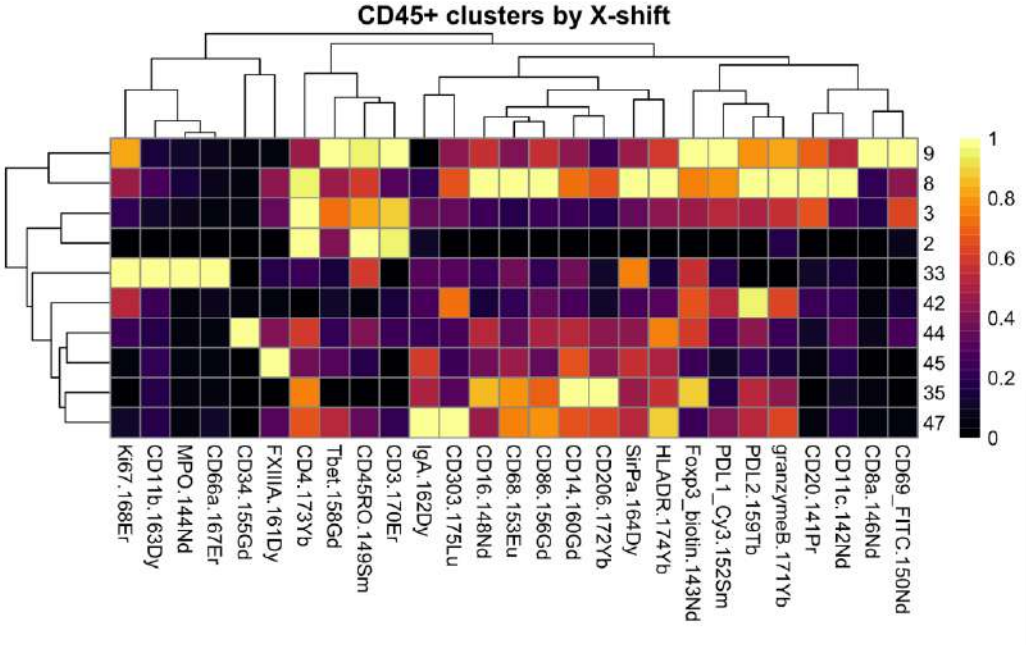
'ilastik' software, Berg *et al.*, 2019 (*Nat Methods*)

Clustering cells



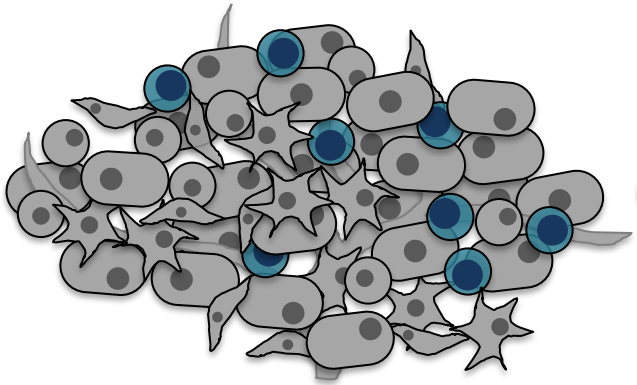
47 total clusters

10 CD45+ clusters



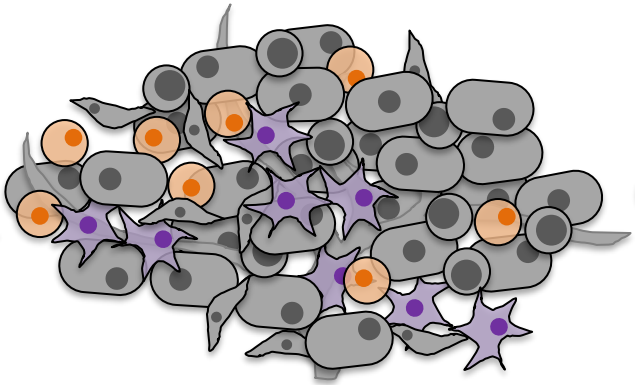
Quantify spatial data

Cell densities



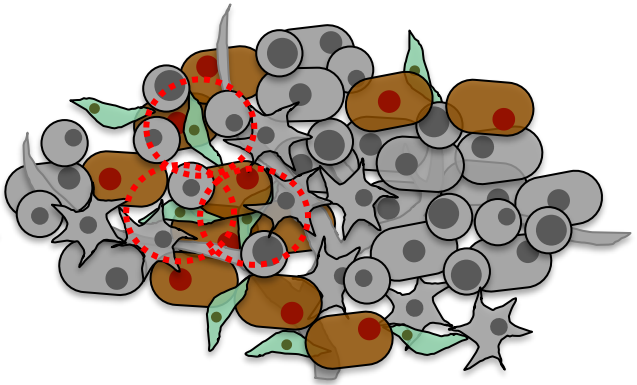
Cells per mm^2

Distance between cells



Distance between

Neighbouring cells



Interaction between

Done using 'Spectre' in R

Ashhurst, Marsh-Wakefield, Putri *et al.*, 2022 (*Cytometry A*)

Are there differences in neighbourhood interactions between liver regions?

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Linear dimensionality reduction

- Principal component analysis (**PCA**) reduces dimensions by summarising **overall variance**
  - All variability influence (human, experiment, etc.)
- Sparse partial least squares-discriminant analysis (**sPLS-DA**) reduces dimensions by summarising **differences between groups**
  - Need to know which groups to compare

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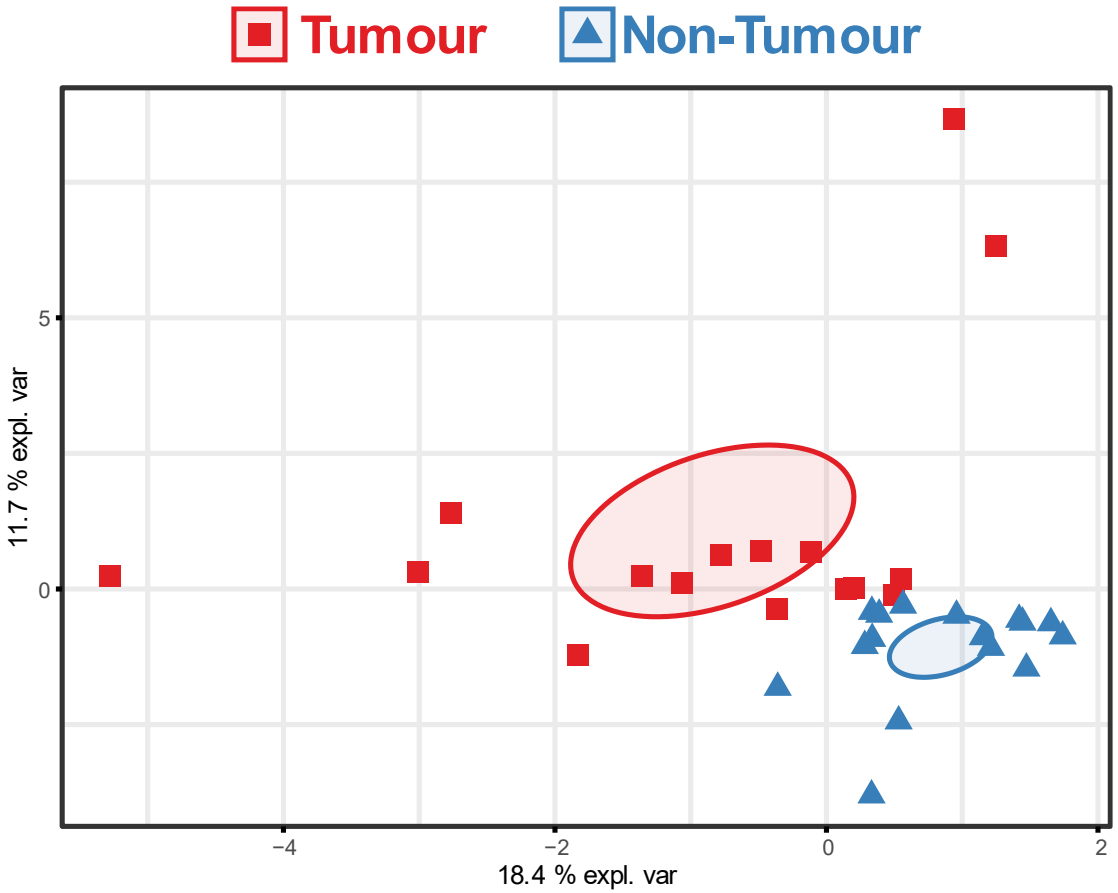
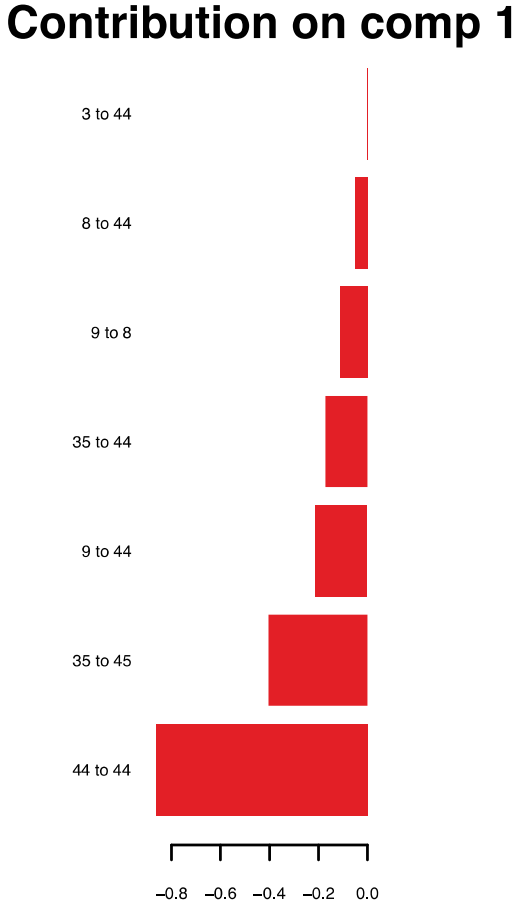
Regions differ in neighbourhood interactions





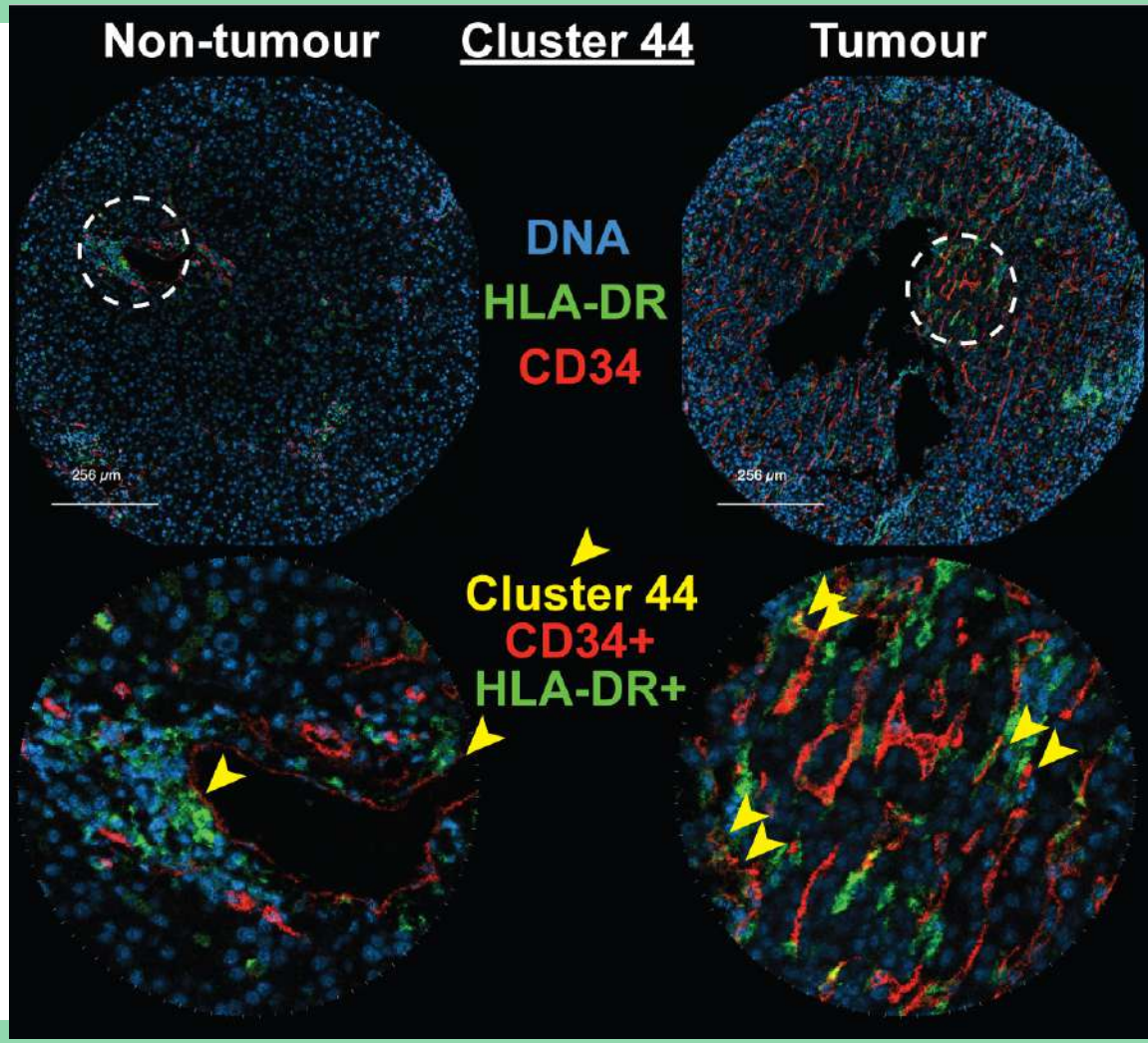
Sparse partial least squares-discriminant analysis (sPLS-DA)

Cluster 44
CD34+ macrophage?

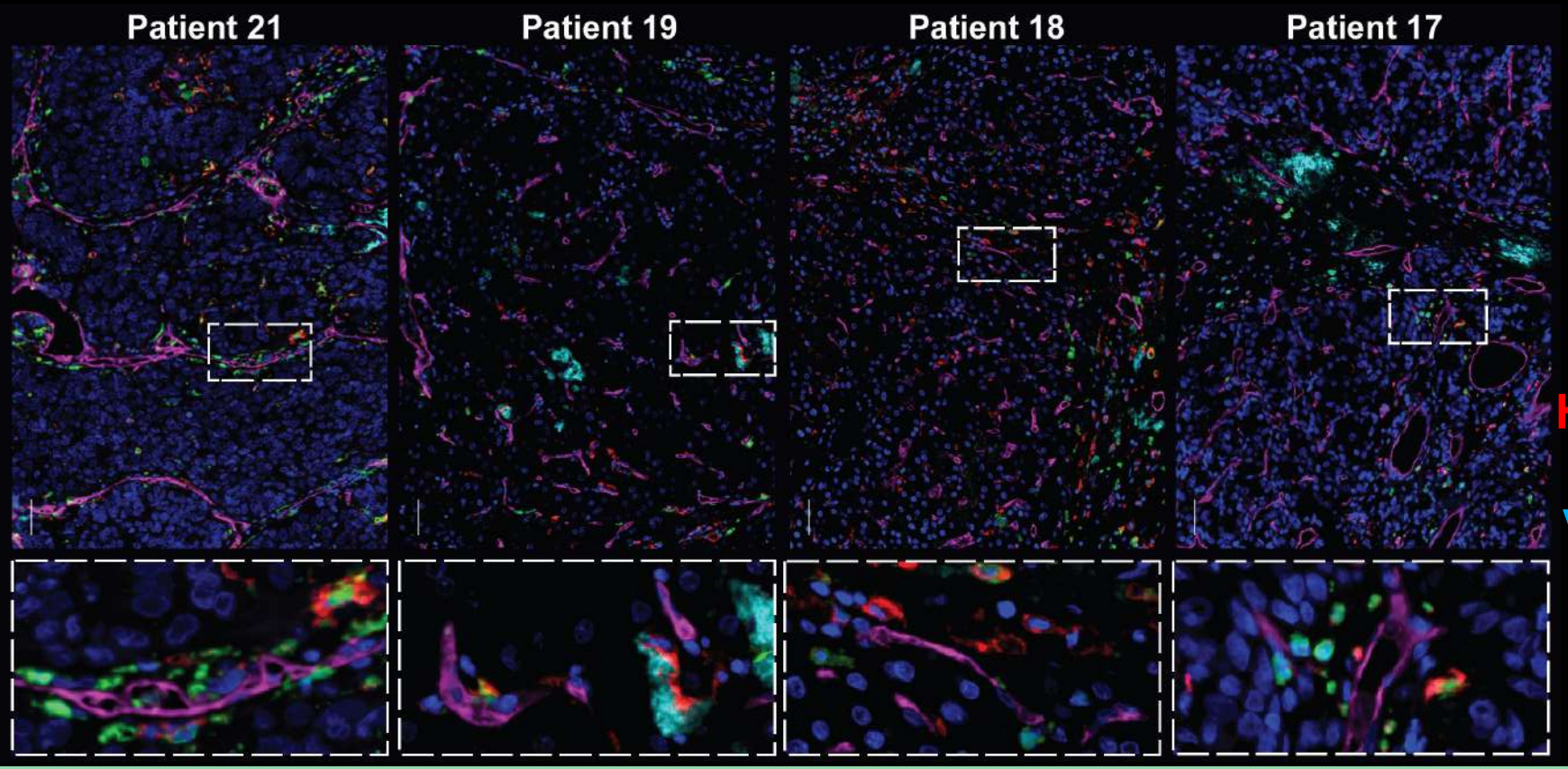
CD69_FITC.150Nd
CD8a.146Nd
CD11c.142Nd
CD20.141Pr
granzymeB.171Yb
PDL2.159Tb
PDL1_Cy3.152Sm
Foxp3_biotin.143Nd
HLADR.174Yb
SirPa.164Dy
CD206.172Yb
CD14.160Gd
CD86.156Gd
CD68.153Eu
CD16.148Nd
CD303.175Lu
IgA.162Dy
CD3.170Er
CD45RO.149Sm
Tbet.158Gd
CD4.173Yb
FXIIIA.161Dy
CD34.155Gd
CD66a.167Er
MPO.144Nd
CD11b.163Dy
Ki67.168Er

High

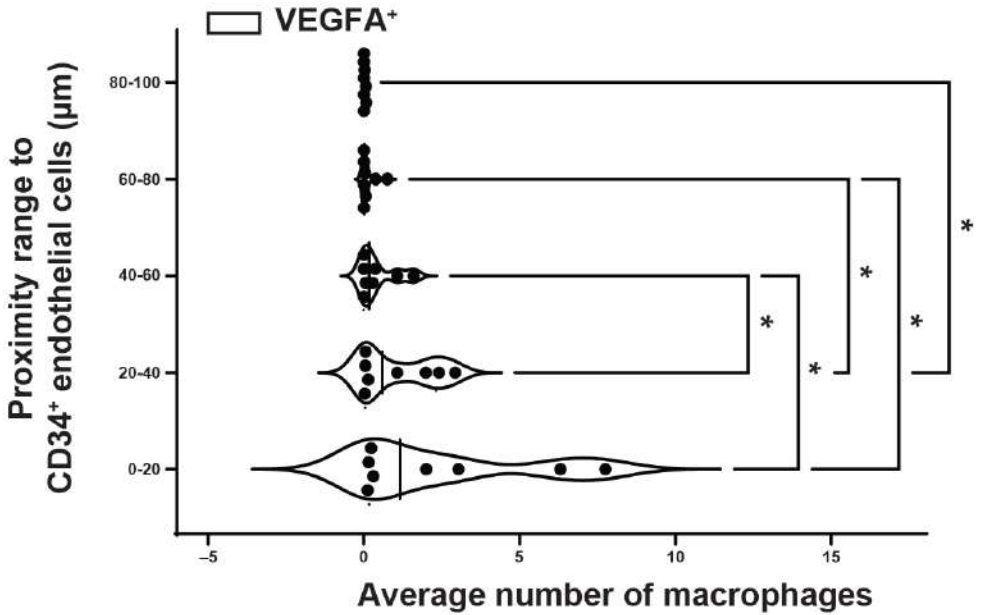
Low



VEGFA+ macrophages



Macrophages reside closely to CD34+ endothelial cells



Is there any communication between these cells?

Use scRNAseq to identify (potential) ligand-receptor communication

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Nat Commun. 2022; 13: 4594.
Published online 2022 Aug 6. doi: 10.1038/s41467-022-32283-3
PMCID: PMC9357016
PMID: 35933472

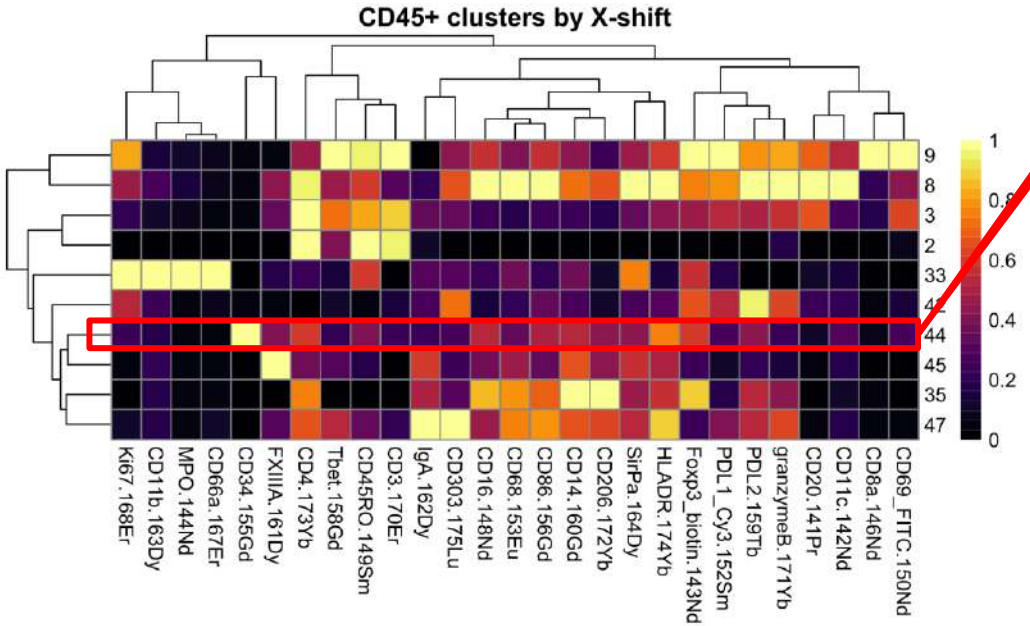
A single-cell atlas of the multicellular ecosystem of primary and metastatic hepatocellular carcinoma

Yiming Lu,[#1] Aiqing Yang,[#1] Cheng Quan,[#1] Yingwei Pan,[#2] Haoyun Zhang,[2] Yuanfeng Li,[1] Chengming Gao,[1] Hao Lu,[1] Xueting Wang,[1,3] Pengbo Cao,[1] Hongxia Chen,[1] Shichun Lu,[2] and Gangqiao Zhou[1,4]

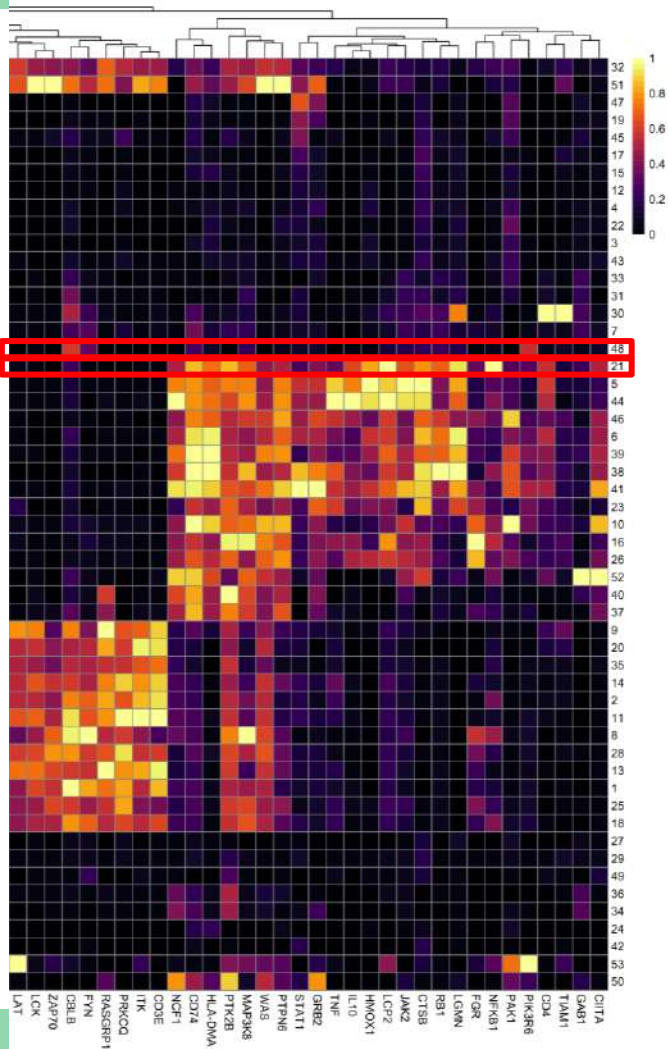
- >70,000 cells from 10 HCC patients
  - Sites include **non-tumour**, **tumour**, metastatic lymph node, and portal vein thrombus

Compare phenotypes between IMC and scRNAseq

IMC clusters

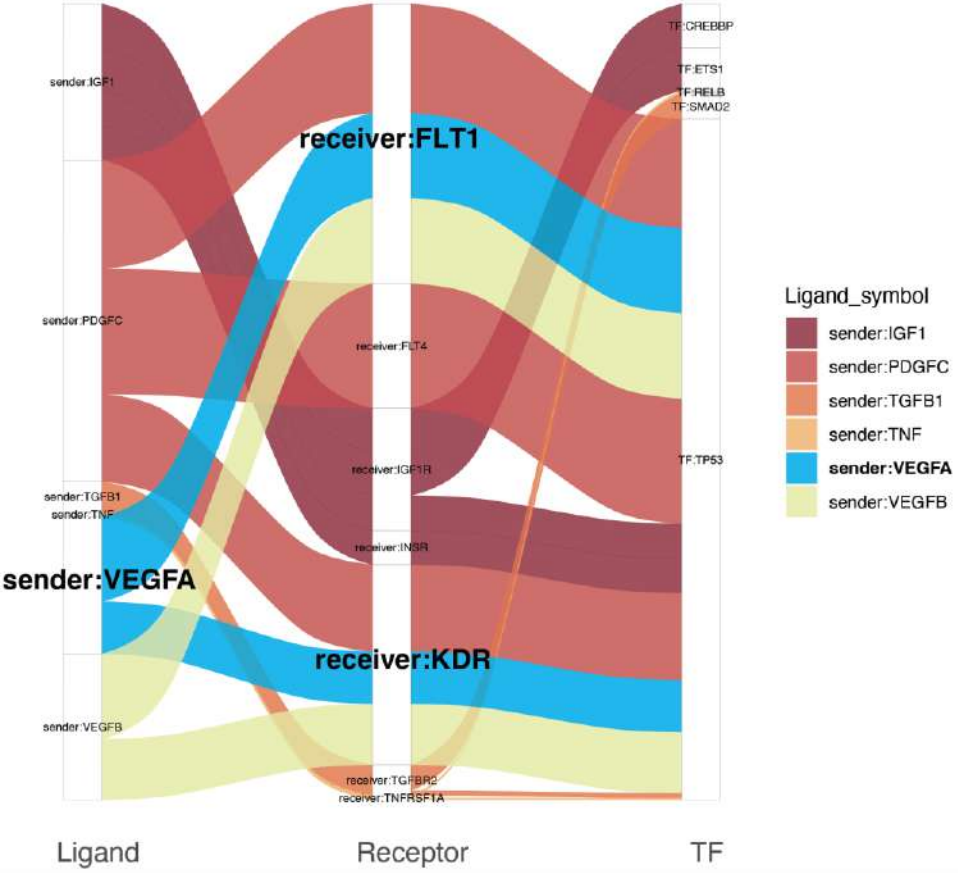


scRNAseq clusters



Macrophage-endothelial cell communication

Macrophage Endothelial



- Macrophages within tumour may communicate with endothelial cells

Lu *et al.*, 2022

Nature Communications

'CellCall' R package

Zhang *et al.*, 2021

Nucleic Acids Research

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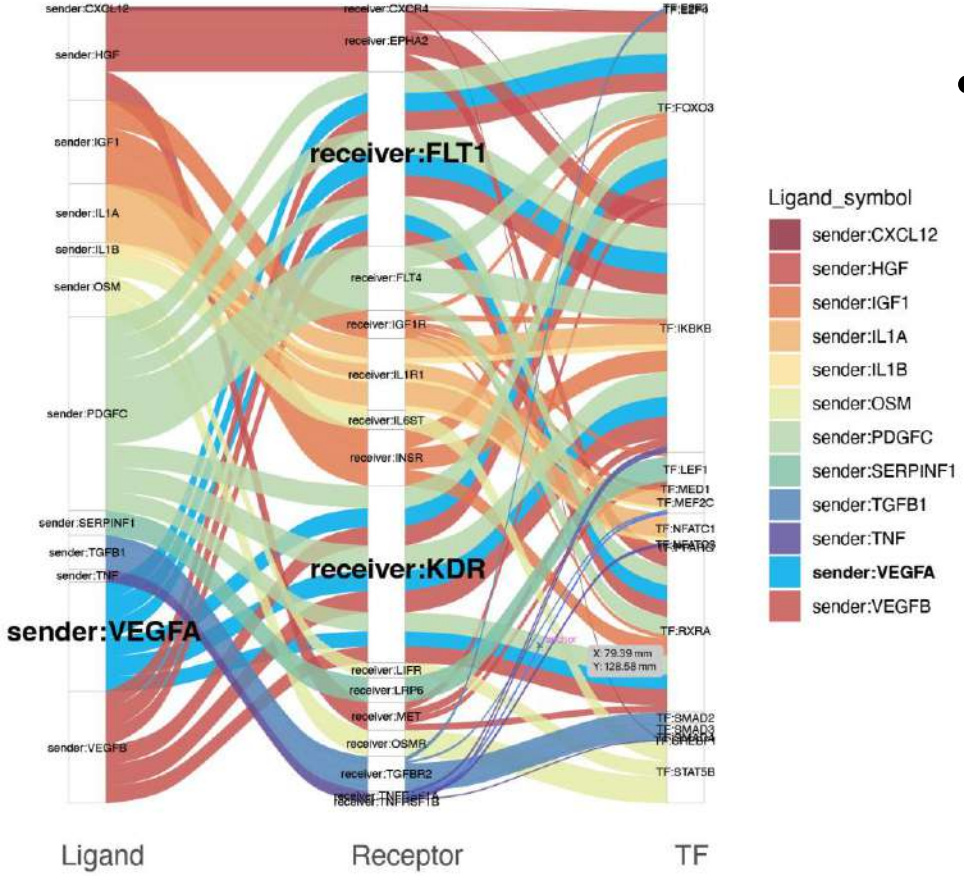
Macrophage-endothelial cell communication

Macrophage Endothelial



- Results confirmed in a second HCC cohort of 6 patients

Liu *et al.*, 2023

Journal of Hepatology

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Public spatial transcriptomic data

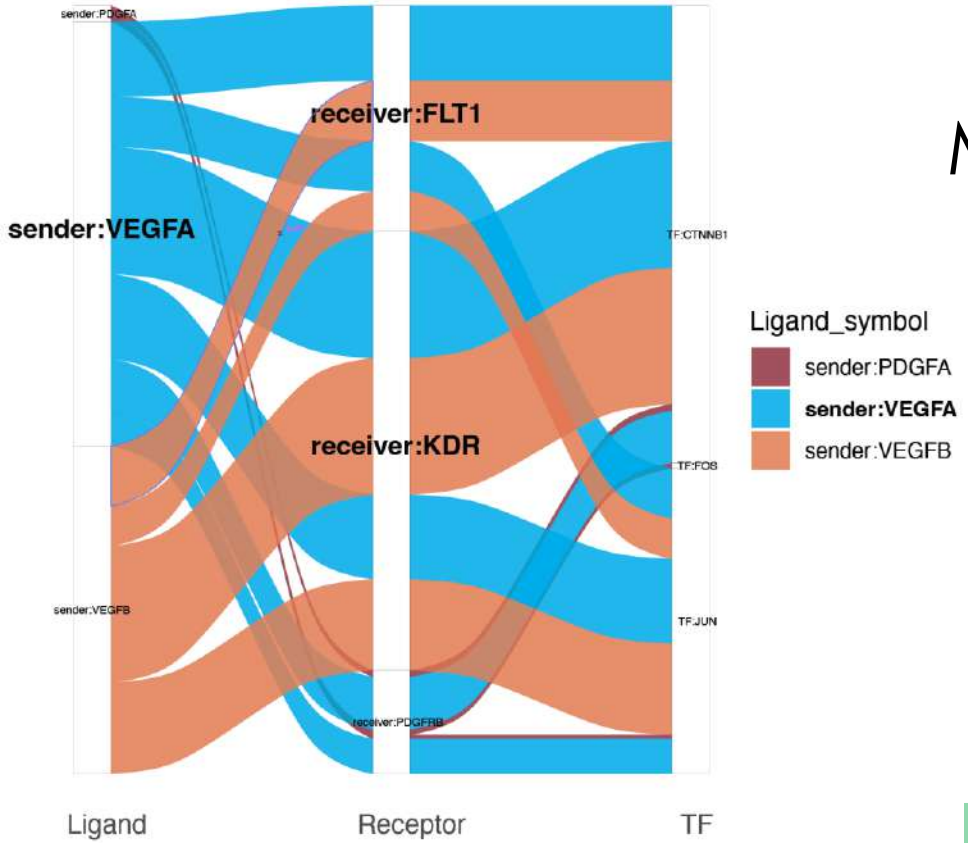
DATA SUMMARY

Tissue type	FFPE human normal liver	FFPE human hepatocellular carcinoma
Panel	Human Universal Cell Characterization Panel 1000 plex	Human Universal Cell Characterization Panel 1000 plex
Total scan area	76 mm^2	100 mm^2
Total cells	340,517	464,126
% Cells passed QC	97.8%	99.3%
Total transcripts detected	198,546,375	533,910,466
Mean total transcripts/cell	583	1150
Mean total transcripts/um^3	0.67	1.41
Mean negatives/target/cell	0.04	0.07
Mean FalseCode/target/cell	0.008	0.008

https://nanostring.com/products/cosmx-spatial-molecular-imager/human-liver-rna-ffpe-dataset/

Same communication occurs spatially

Macrophage Endothelial



Macrophage-endothelial cell communication by *VEGFA*

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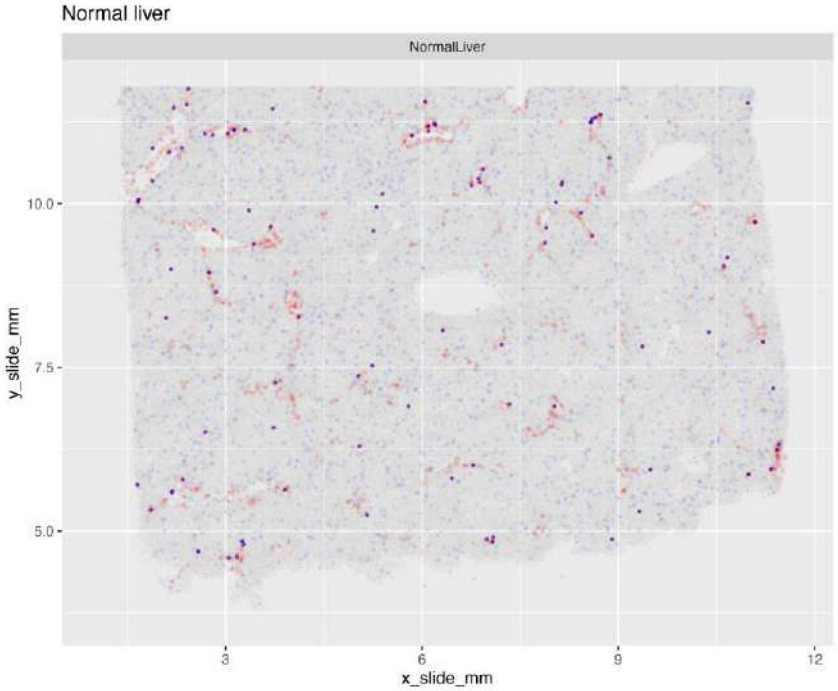
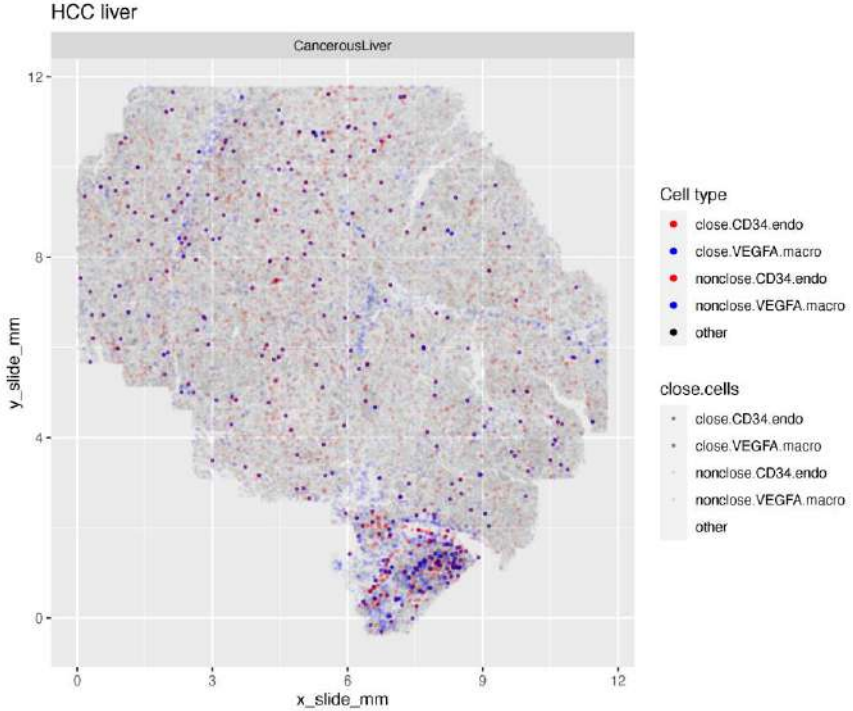
Close (≤20µm) cell interactions





Subset density (cells/mm²)	Normal	HCC
VEGFA⁺ macrophages	26.34	36.27
Close *VEGFA*⁺ macrophages	1.26	3.65
CD34⁺ endothelial cells	26.75	46.61
Close *CD34*⁺ endothelial cells	1.51	4.04

Subset proportions	Normal	HCC
Close *VEGFA*⁺ macrophages (%*VEGFA*⁺ macrophages)	4.80%	10.1%
Close *CD34*⁺ endothelial cells (%*CD34*⁺ endothelial cells)	5.66%	8.67%

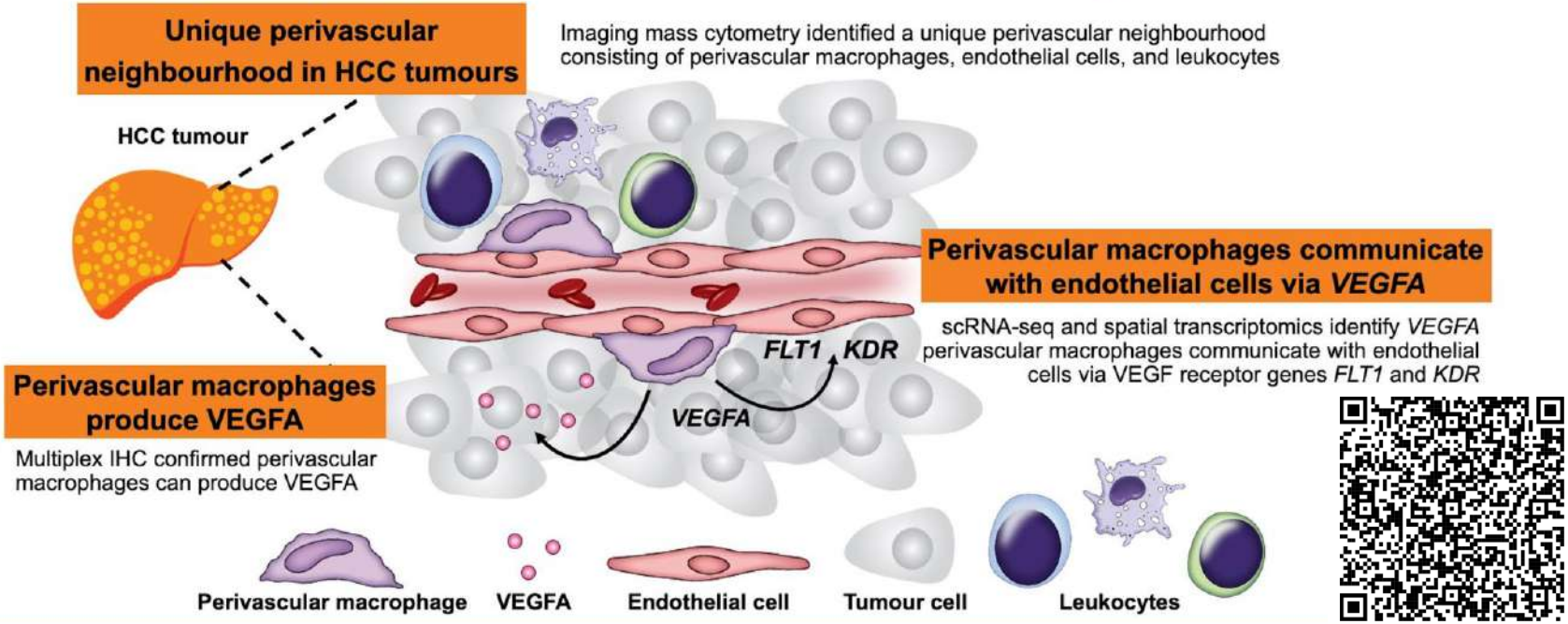
Average number within 20 µm	Normal	HCC
Macrophage to endothelial cell	0.060	0.132
Endothelial cell to macrophage	0.055	0.088

Anti-VEGFA treatment in HCC

- HCC patients treated with combination atezolizumab (anti-PD-L1) and bevacizumab (anti-VEGF-A) have better overall survival
  - Finn *et al.*, 2020, *New England Journal of Medicine*
- Depleting *Vegfa* in macrophages results in reduced vascular permeability in breast cancer mouse model
  - Harney *et al.*, 2015, *Cancer Discovery*

More information

Spatial Mapping of the Hepatocellular Carcinoma Landscape Identifies Unique Intratumoural Perivascular-immune Neighbourhoods



AASLD Marsh-Wakefield, et al | *Hepatology Communications*. 2024.
Hepatology Communications

Ongoing analyses

- Compare differences between aetiologies and tumour recurrence risk factors
- Non-immune cell interactions
  - Fibroblasts, hepatocytes…

αSMA	E-cadherin	Podoplanin
CD34	FXIIIa	T-bet
CK8/18	HepPar1	Vimentin
	NaKATPase	

More IMC!

- Additional 16 HCC patients (32 total)
  - 15 cirrhotic, 16 MVI
  - 8 HBV, 12 HCV, 6 MASH, 6 ALD, 3 unknown

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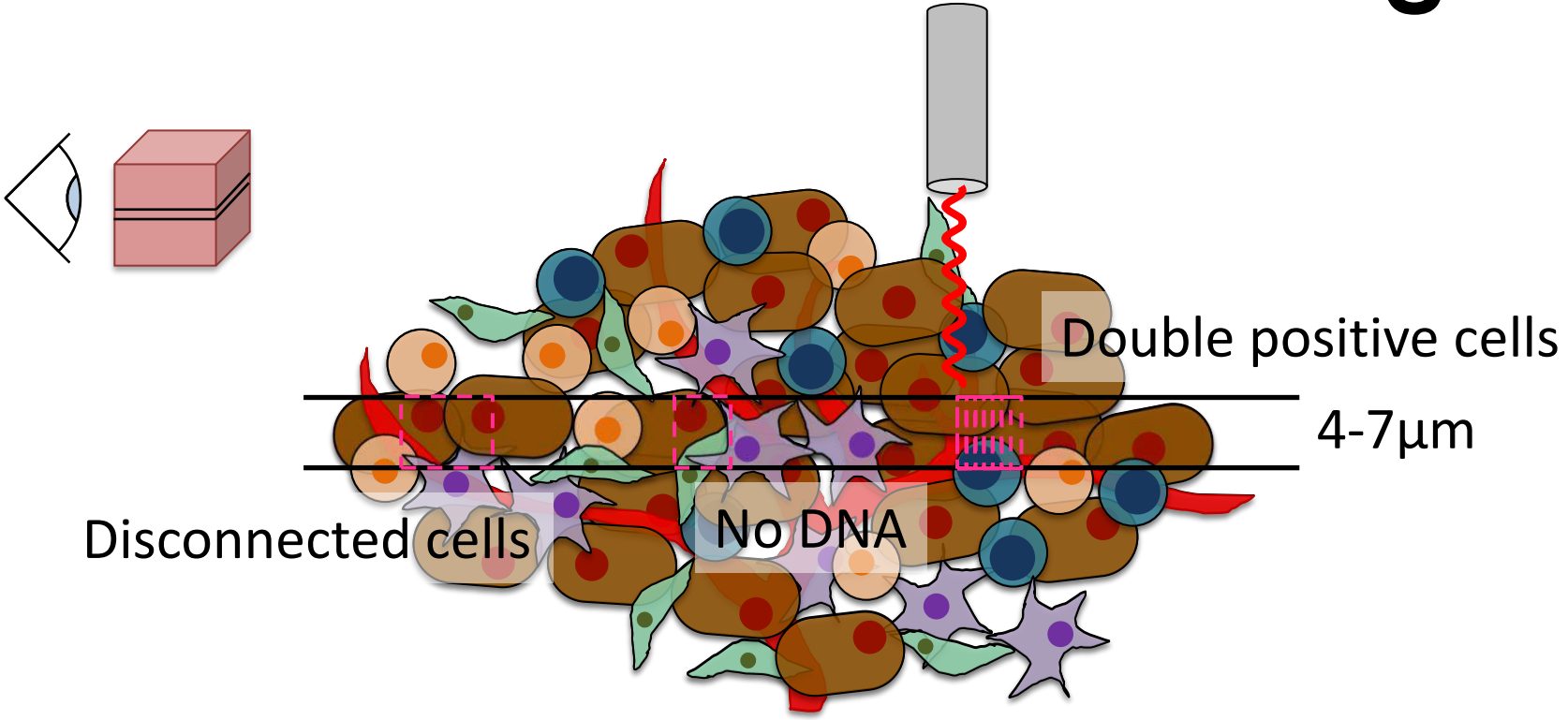
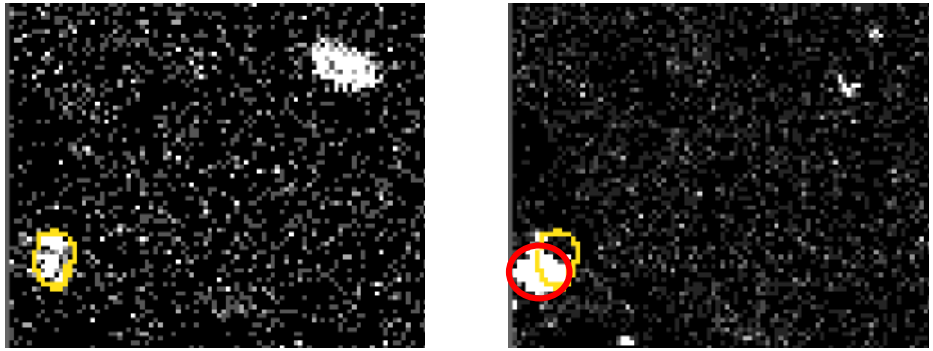
Limitations of tissue staining



Overlapping markers

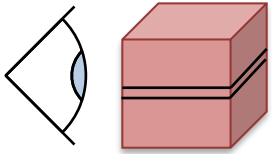
CD3 FXIIIA

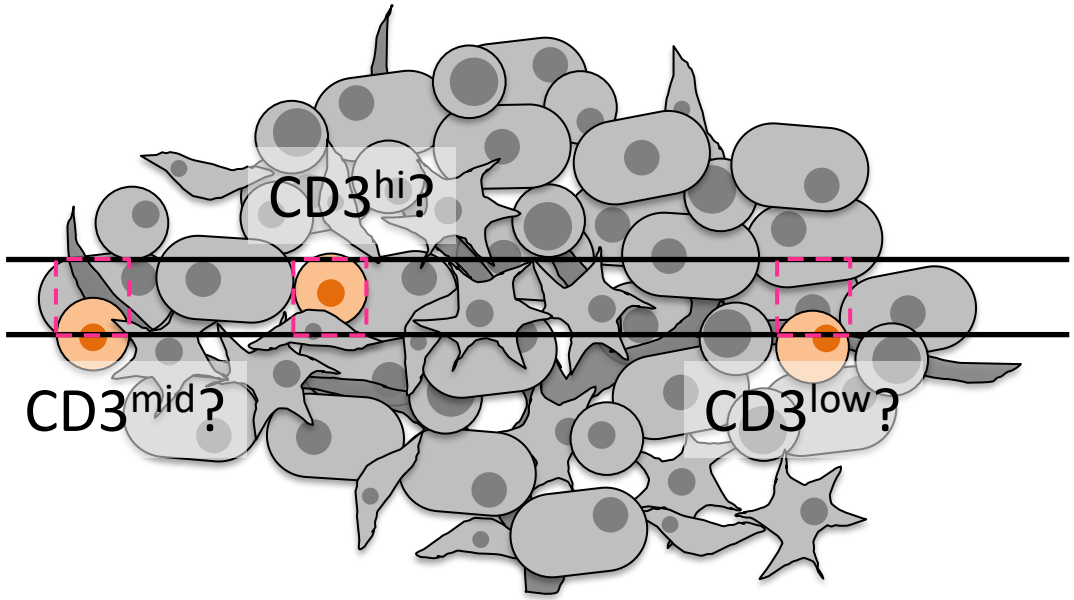


CD3+ macrophage?
FXIIIA+ T cell?
Double positive cell?

'ilastik' software, Berg *et al.*, 2019 (*Nat Methods*)

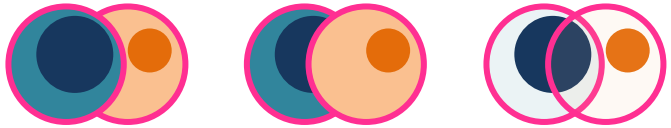
Sensitivity





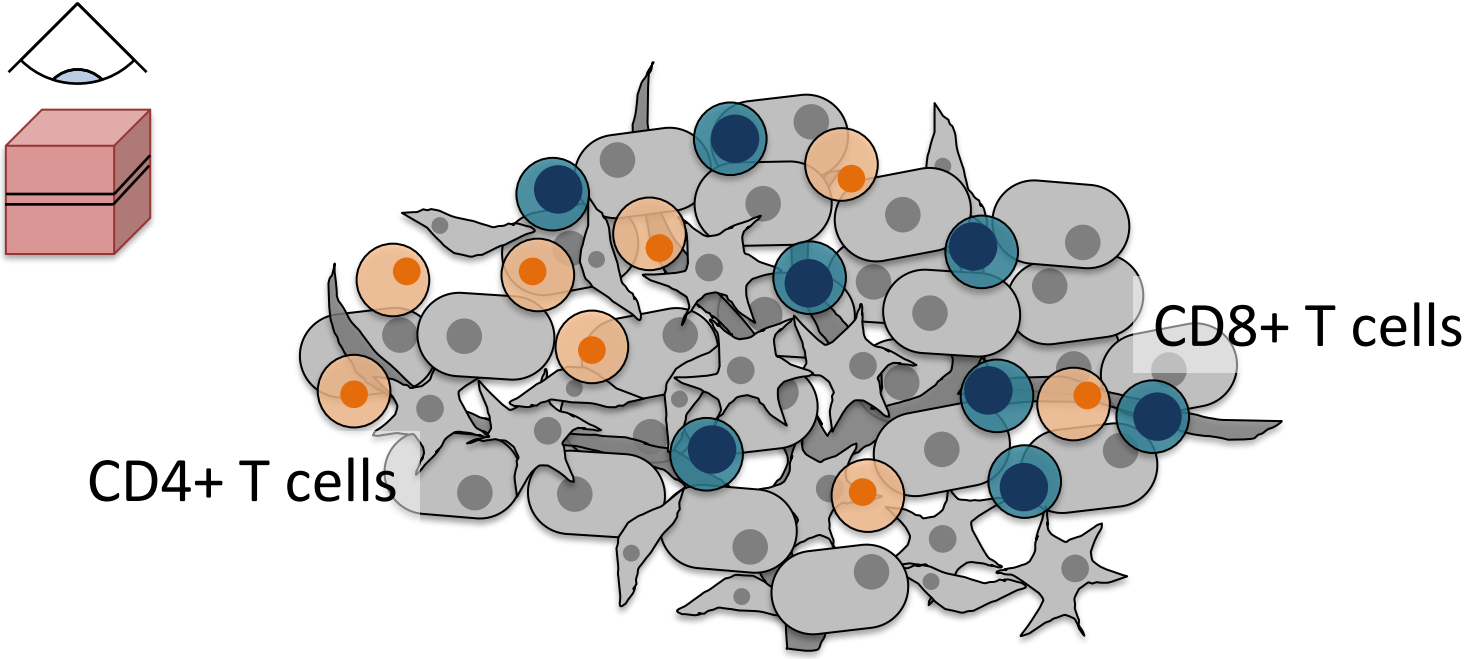
A solution?

- Cell segmentation doesn't (generally) allow border overlap
  - It's definitive

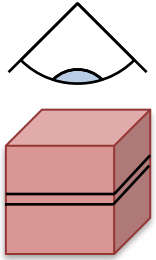


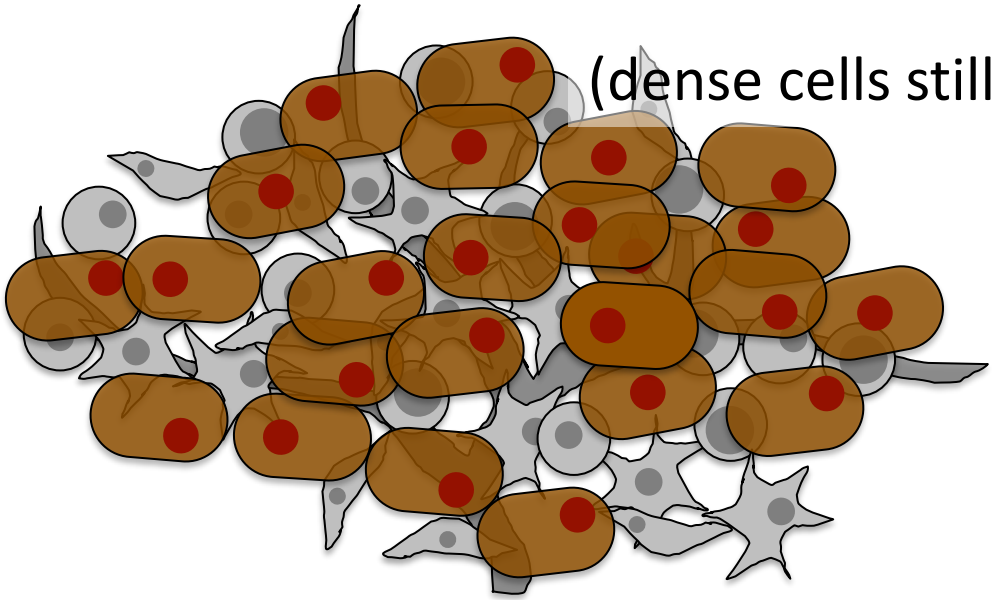
- Protein expression is quantified within border
- Cell coordinates are (generally) based on cell centres
  - Distances are not based on borders

Segment cell types separately



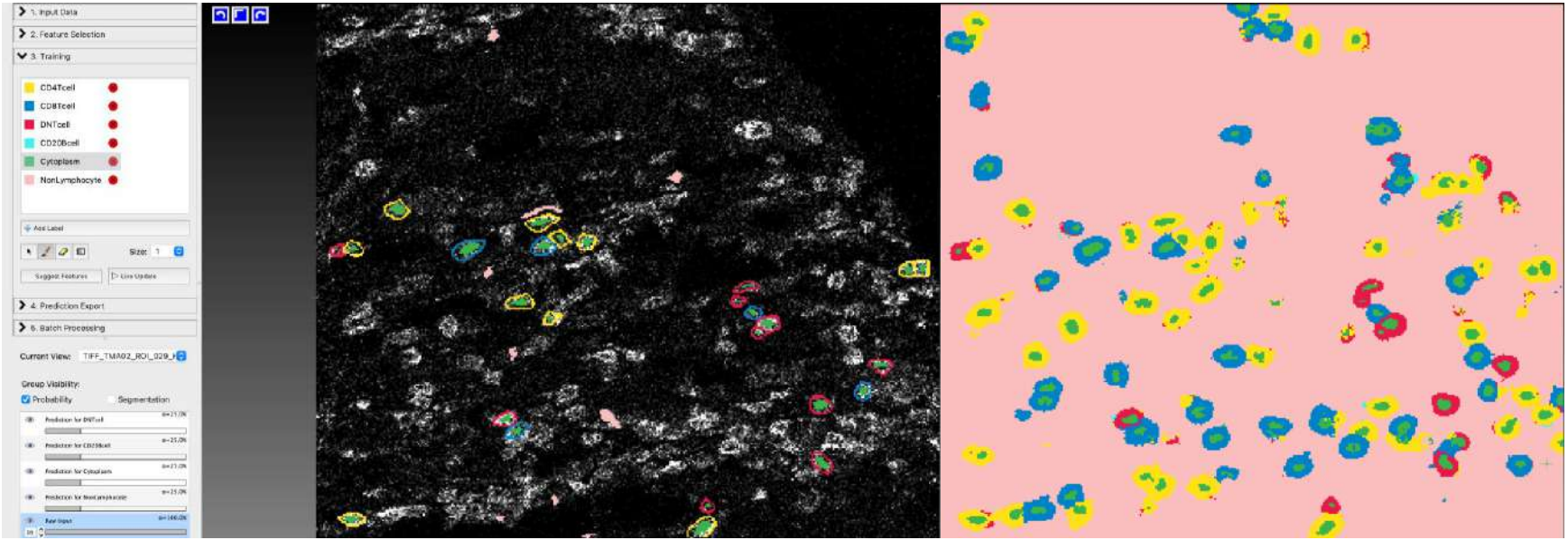
Segment cell types separately





(dense cells still an issue)

Lymphocyte segmentation



'ilastik' software, Berg *et al.*, 2019 (*Nat Methods*)

Limitations

- It is possible cells are being counted twice
  - May also miss “unknown” cells
- Based on your data and input
- **Huge time sink…**
  - Lymphocytes
  - CD45+ non-lymphocytes
  - Stromal cells
  - Hepatocytes

What's the point!?

- Only need to do this once…
- Do your findings match the biology?
- Have others found the same thing?
- What other experiments can be done?

Special thanks to…

- Cosi Santhakumar
- Angela Ferguson
- Geoff McCaughan
- Mainthan Palendira
- Ken Liu
- Joo Shin
- Thomas Ashhurst
- Givanna Putri

- Joan Krefft bequest
- Sydney Cytometry
- ACS

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INTERNATIONAL SOCIETY FOR ADVANCEMENT OF CYTOMETRY

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