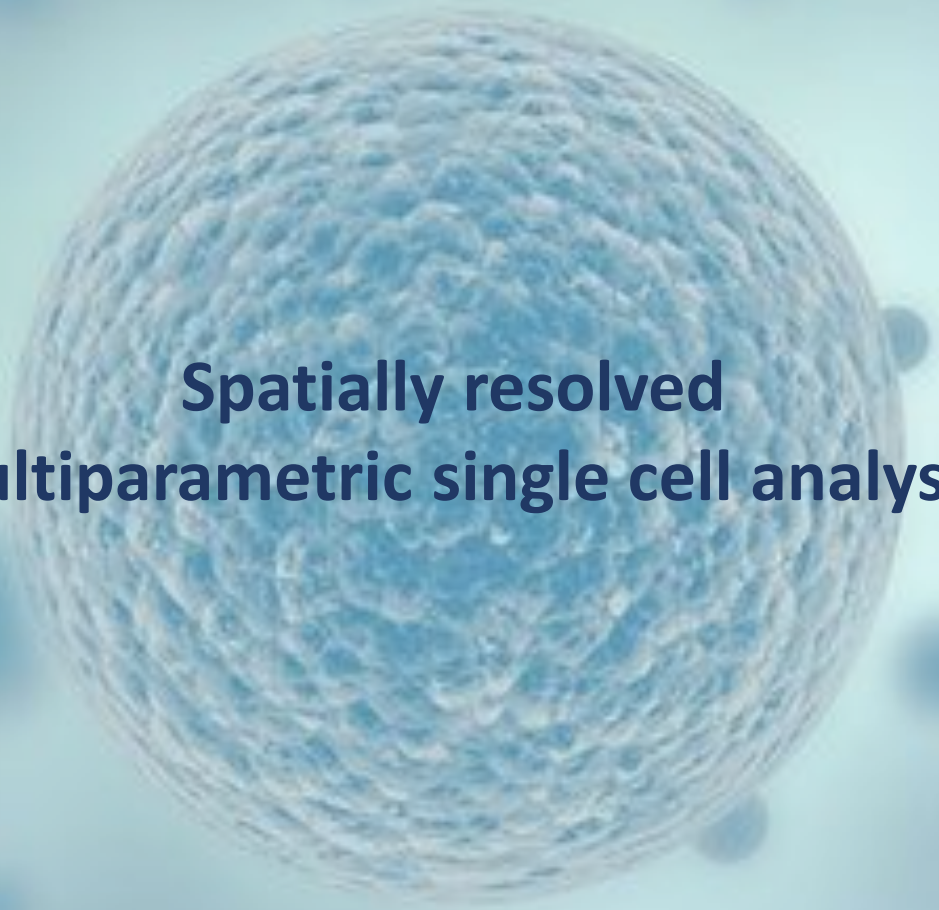


Spatially resolved multiparametric single cell analysis

Technical Journal Club

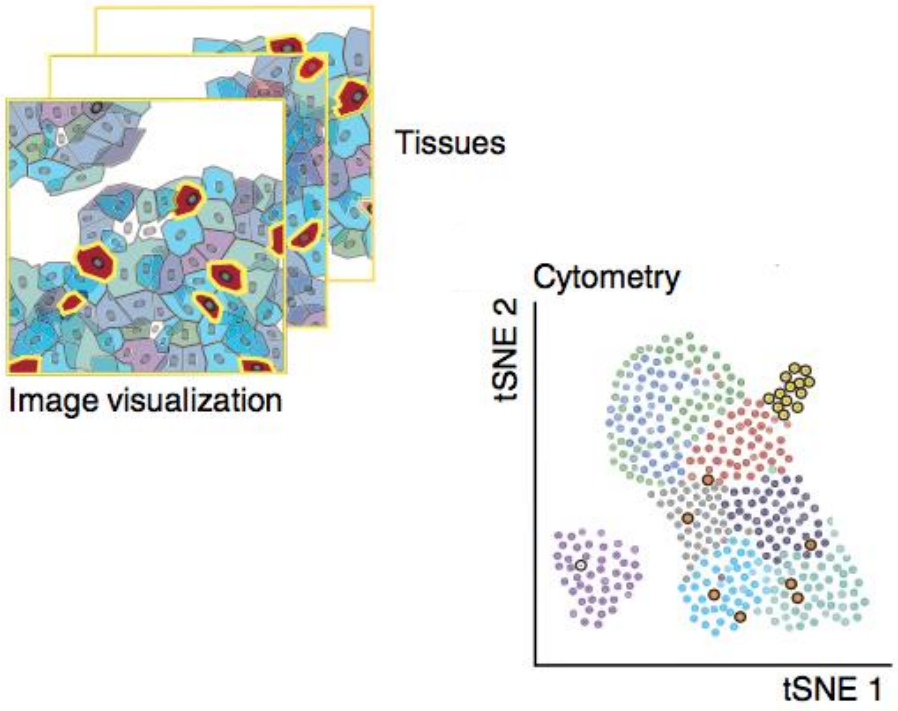
19th September 2017

Christina Müller (Group Speck)

Why spatially resolved multiparametric single cell analysis?

- Multiparametric single cell analysis uncovers the heterogeneity of cellular phenotypes and functional states within population based measurements
- Each cellular phenotype is defined by the interplay of its internal state as well as its environment

⇨ the ability to analyze single cell functional states with spatial resolution will improve the understanding of normal tissue function and disease biology



nature biotechnology

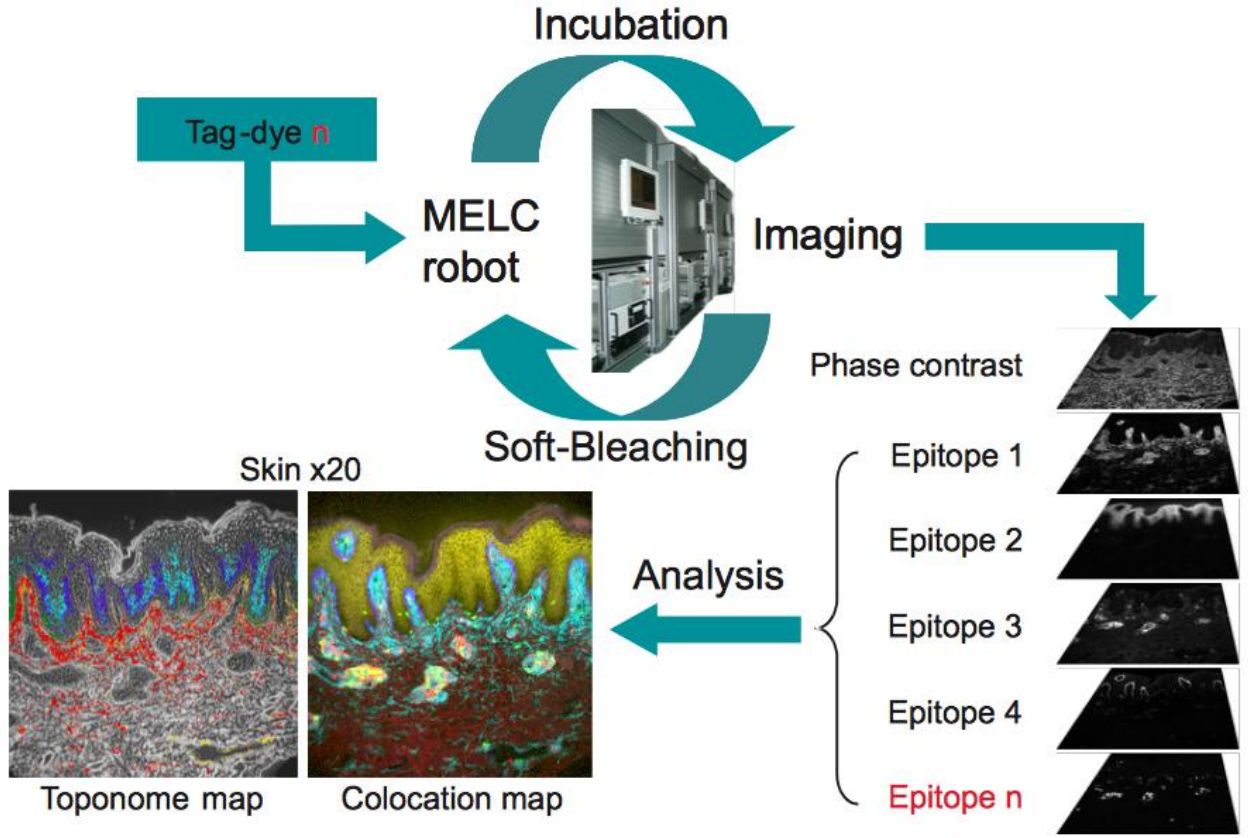
Analyzing proteome topology and function by automated multidimensional fluorescence microscopy

Walter Schubert[1,5], Bernd Bonnekoh[2], Ansgar J Pommer[5], Lars Philipsen[5], Raik Böckelmann[2], Yanina Malykh[5], Harald Gollnick[2], Manuela Friedenberger[1,6], Marcus Bode[1,5,6] & Andreas W M Dress[3,4,6]

Multi-epitope-ligand cartography (MELC) – Paper 1

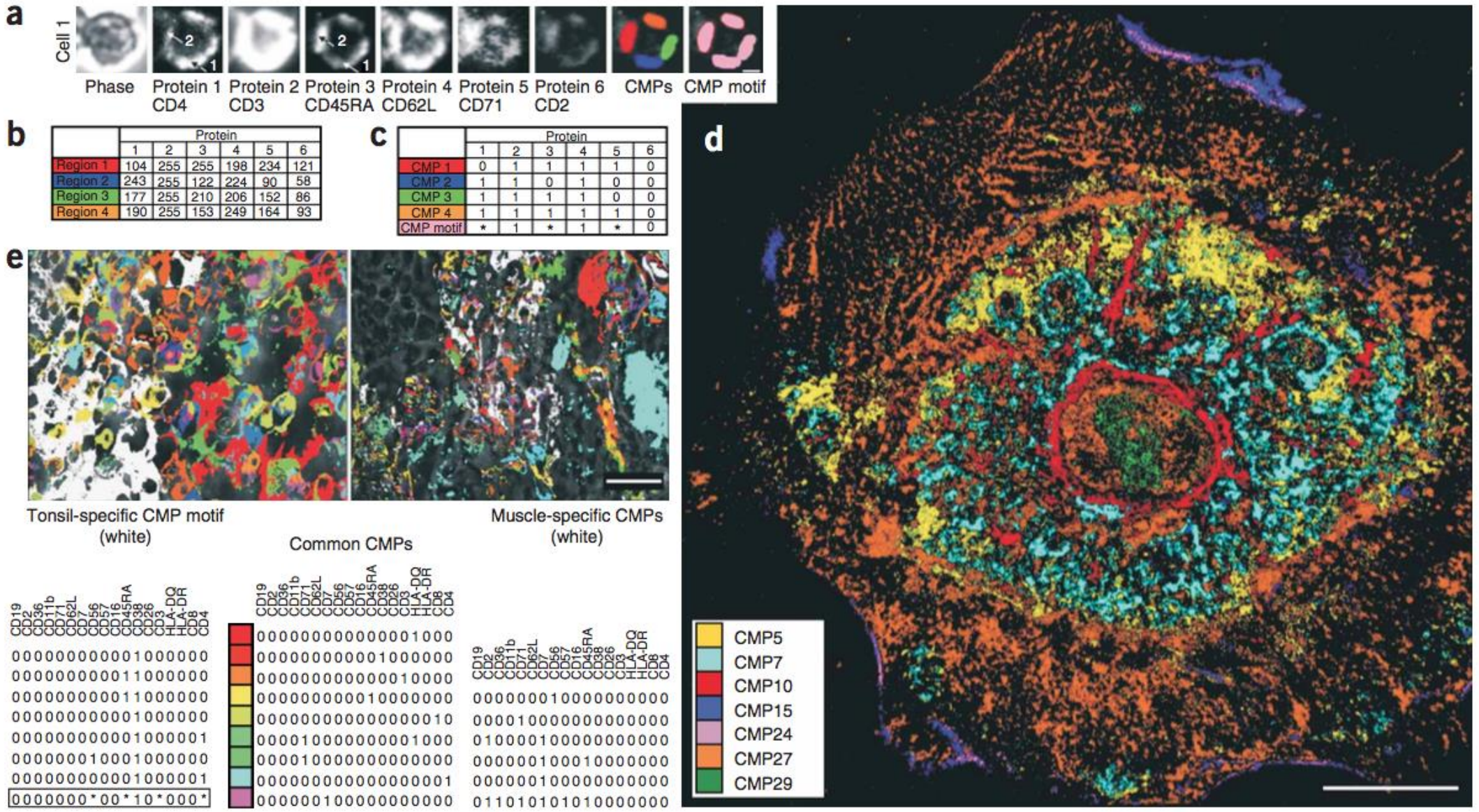
The principle of multi-dimensional MELC procedure:

- Multi-dimensional MELC is based on repeating staining, imaging and bleaching steps
- In each cycle the sample is incubated with one or more tags and imaged before bleaching by soft multi-wavelenght excitation (e.g. 485nm for FITC and 546nm for PE)
- Construction of co-localization maps and toponome maps



Multi-epitope-ligand cartography (MELC) – Paper 1

Construction of toponome maps:

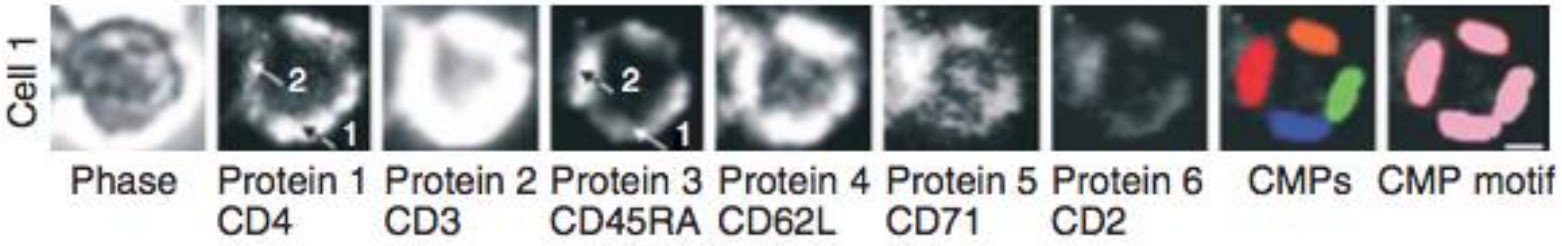


Multi-epitope-ligand cartography (MELC) – Paper 1

Construction of toponome maps:

- MELC data generates a list of the epitopes present at each pixel
- List can be based on fluorescence intensity (b) or on a binarized data set (c)
- Binarization requires the introduction of an appropriate threshold value for each epitope
- Binarized data set can be translated into combinatorial molecular phenotypes (CMPs)
- Some CMPs denote functional regions and were defined as CMP motifs

a



b

	Protein					
	1	2	3	4	5	6
Region 1	104	255	255	198	234	121
Region 2	243	255	122	224	90	58
Region 3	177	255	210	206	152	86
Region 4	190	255	153	249	164	93

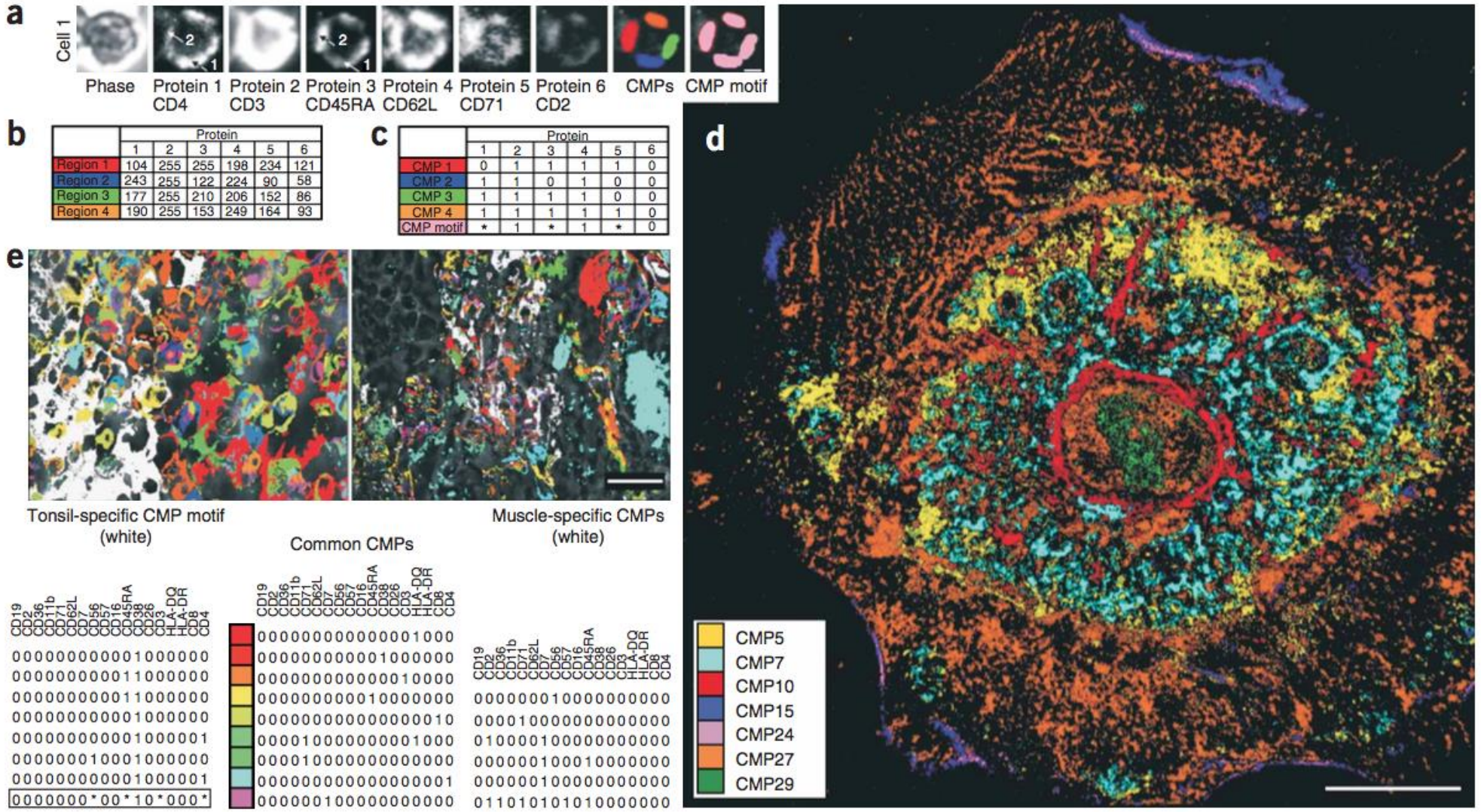
c

	Protein					
	1	2	3	4	5	6
CMP 1	0	1	1	1	1	0
CMP 2	1	1	0	1	0	0
CMP 3	1	1	1	1	0	0
CMP 4	1	1	1	1	1	0
CMP motif	*	1	*	1	*	0

Multi-epitope-ligand cartography (MELC) – Paper 1

Construction of toponome maps:

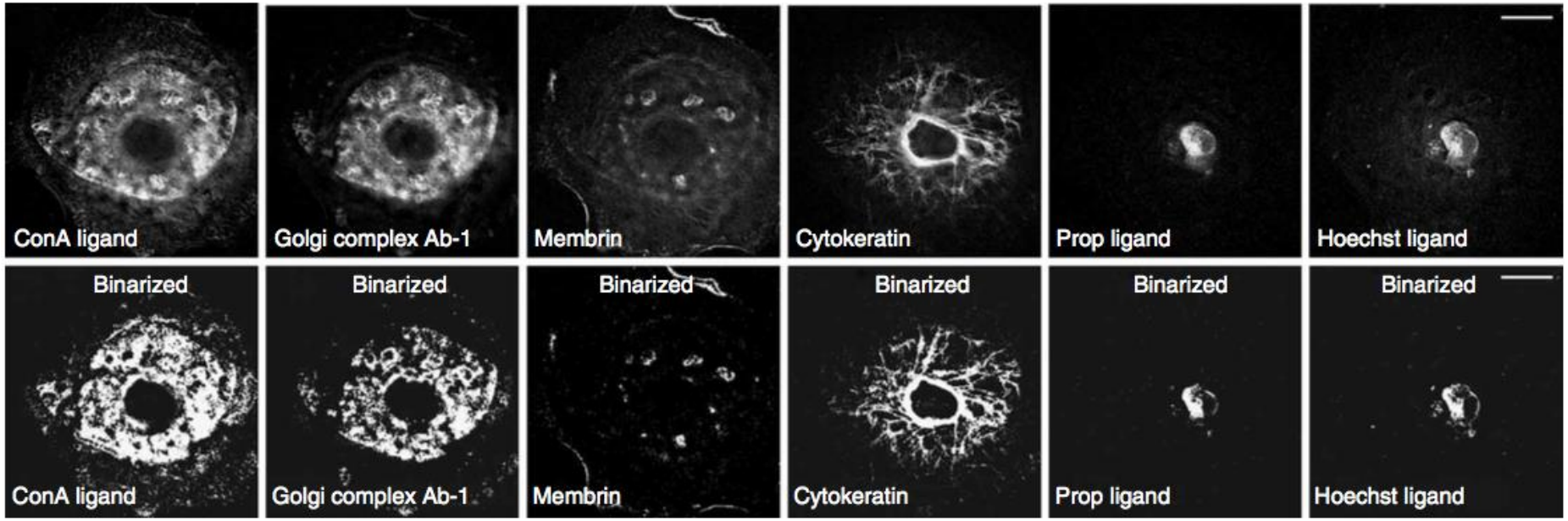
- Assignment of one color for each CMP motif can summarize protein organization in a 2D or 3D toponome map

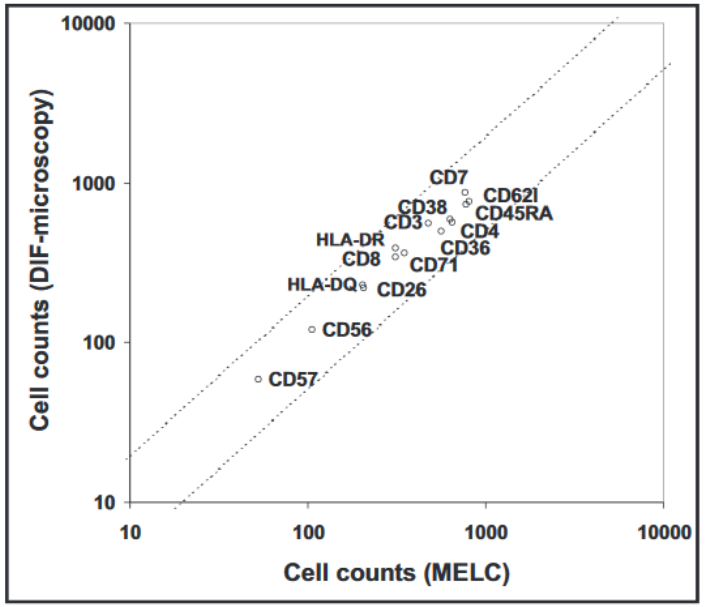


Multi-epitope-ligand cartography (MELC) – Paper 1

Test for signal separation and comparison to direct immunofluorescence microscopy:

- Staining with 6 molecular markers specific for cytoplasmic or nuclear structures
- Binarized data recapitulates established results

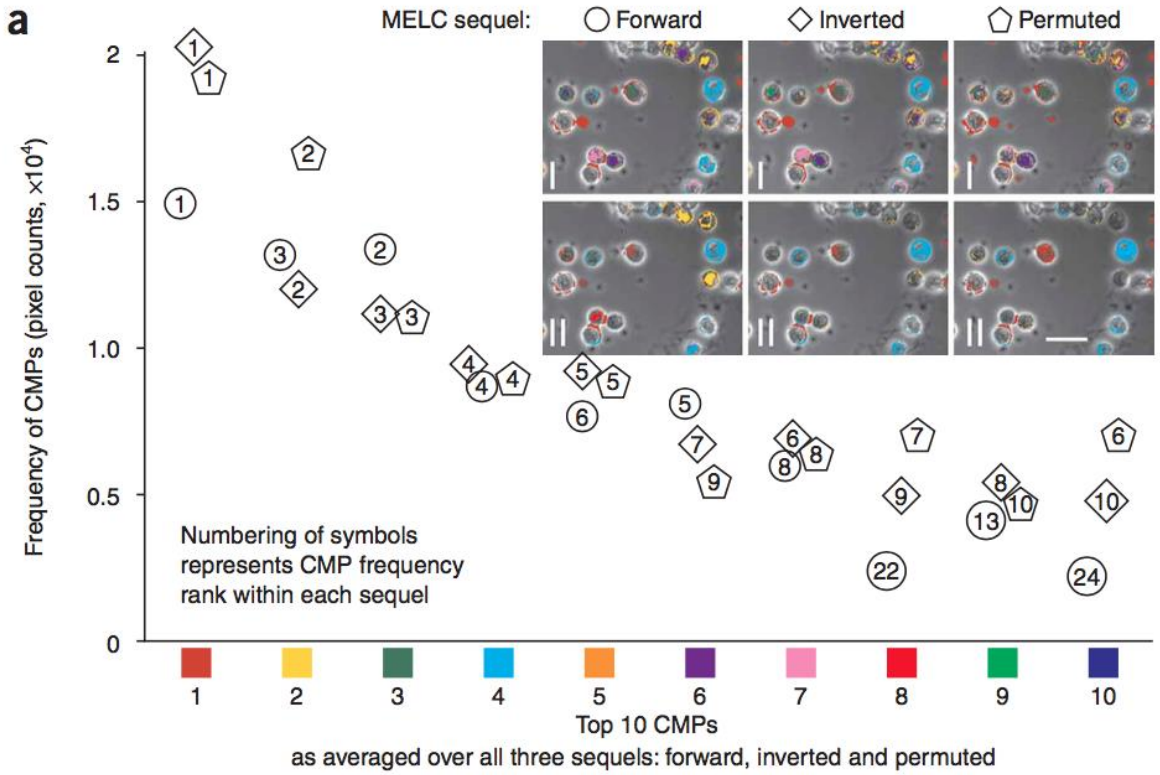




Multi-epitope-ligand cartography (MELC) – Paper 1

Does the staining order affect the result?

- Staining of PBMCs with 18 cell surface markers including a inverted labeling order of the antibody and a randomly permuted order
- CMPs are not significantly affected by the order

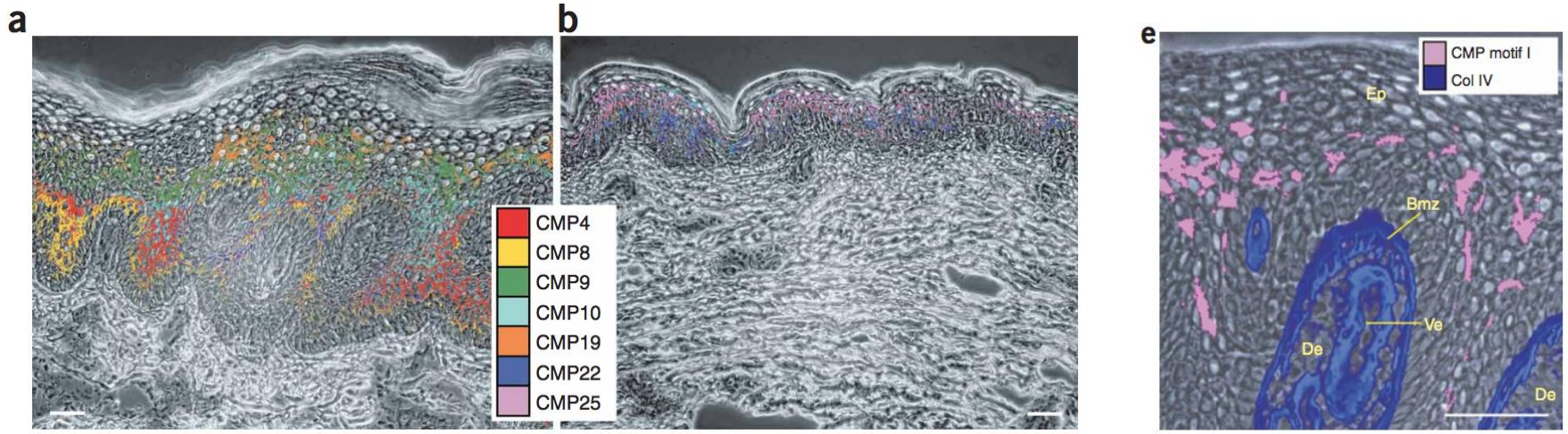


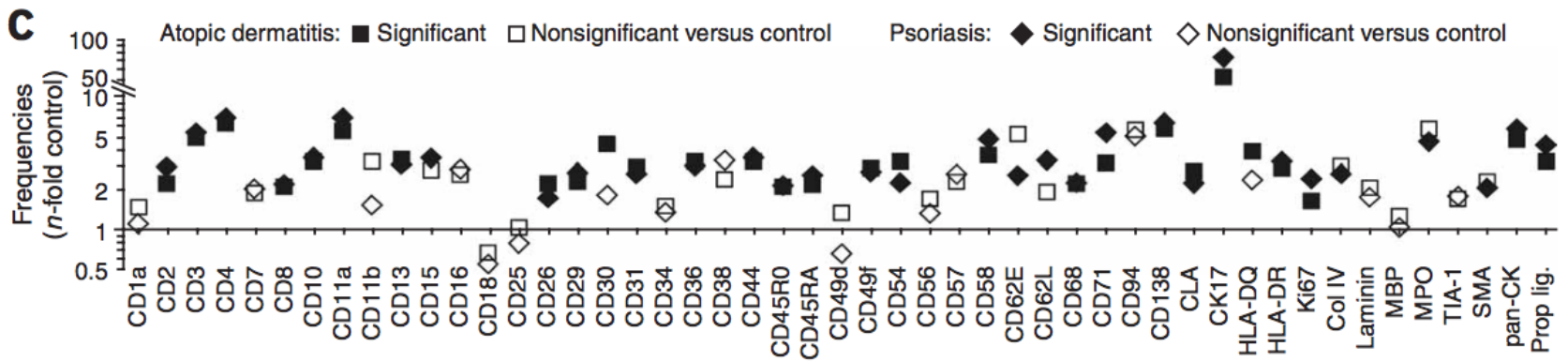
Cross-correlation of pixel fluorescence intensity			
Parameter	Forward/ Inverted	Forward/ Permuted	Inverted/ Permuted
CD4	0.95	0.93	0.96
CD8	0.97	0.95	0.98
HLA-DR	0.95	0.94	0.95
HLA-DQ	0.98	0.95	0.97
CD3	0.94	0.93	0.99
CD26	0.86	0.80	0.75
CD45RA	0.99	0.98	0.99
CD57	0.99	0.98	0.99
CD56	0.96	0.93	0.94
CD7	0.95	0.92	0.95
CD62L	0.91	0.77	0.80
CD71	0.81	0.73	0.67
CD11b	0.84	0.72	0.77
CD36	0.98	0.97	0.98
CD19	0.77	0.68	0.65

Multi-epitope-ligand cartography (MELC) – Paper 1

Protein organization in model diseases:

- Location of 48 proteins *in situ* in patients with psoriasis (b, e) , atopic dermatitis and healthy controls (a)
- Levels were general similar for psoriasis and atopic dermatitis compared to the control (c)





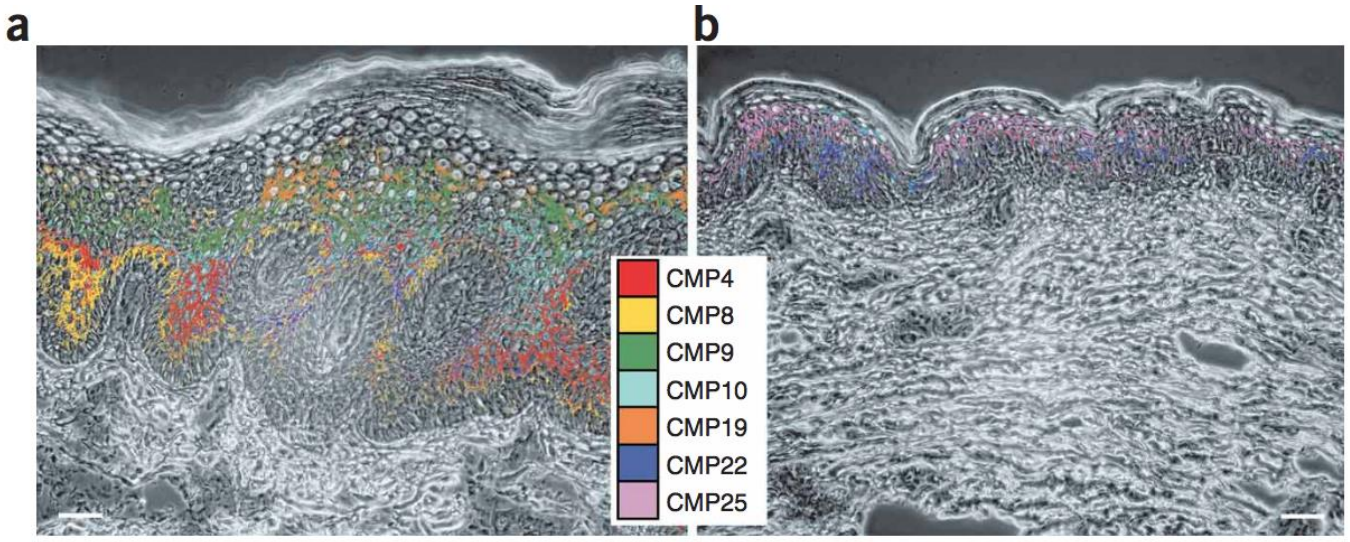
Multi-epitope-ligand cartography (MELC) – Paper 1

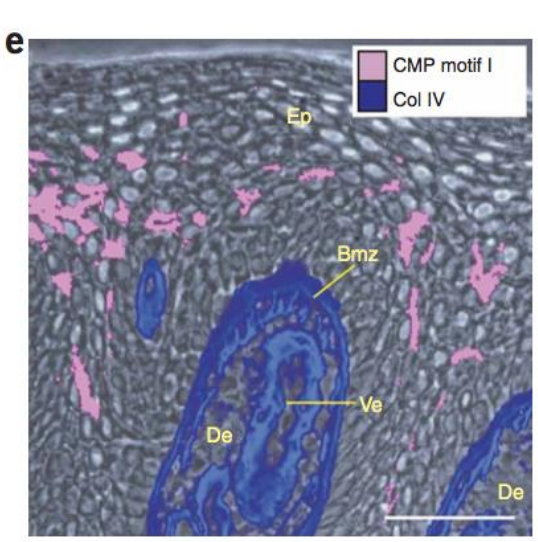
Protein organization in model diseases:

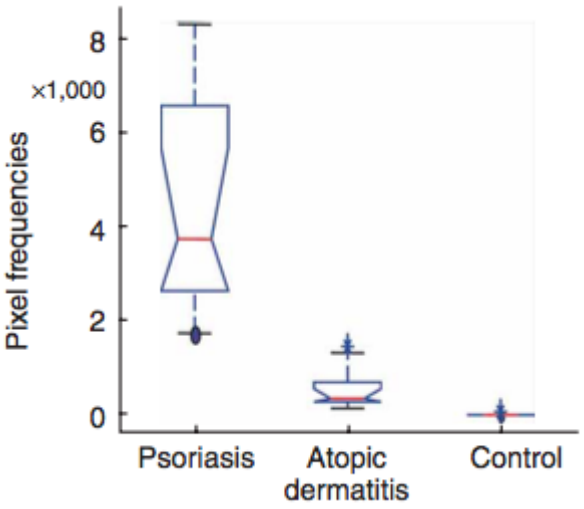
- Location of 48 proteins *in situ* in patients with psoriasis (b, e) , atopic dermatitis and healthy controls (a)
- Identification of disease-specific CMPs as e.g. CMP motif I:

  CD29-/CD36+/CD58+/CD138+/HLA-DR+/pan-CK

  → denotes keratinocytes from the upper epidermis and is a possible hallmark of the hyperactivated suprabasal keratinocyte islands



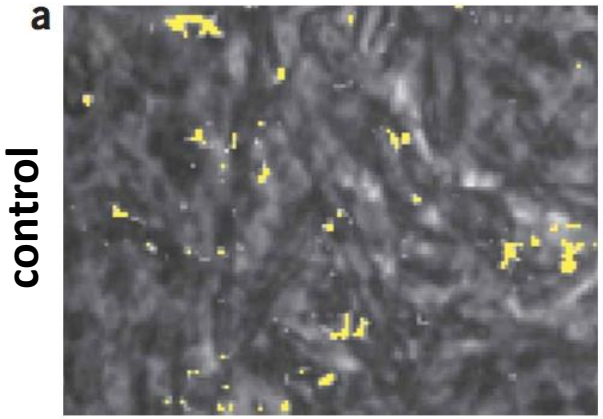




Multi-epitope-ligand cartography (MELC) – Paper 1

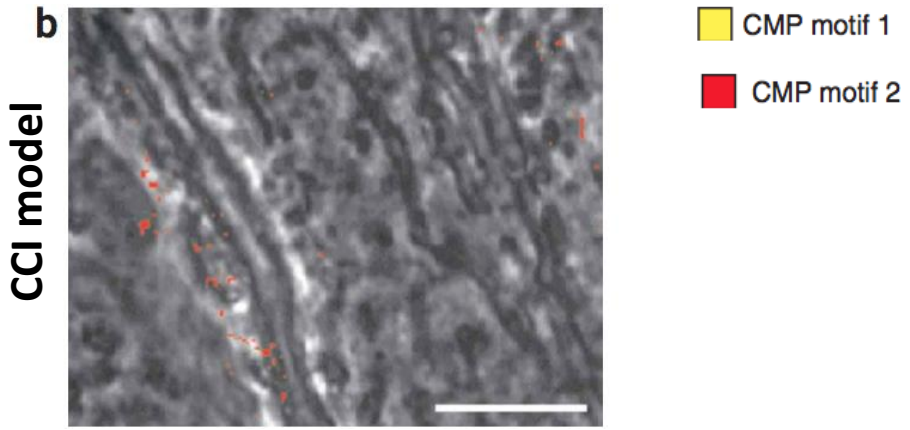
Protein organization in experimental pathology:

- Investigation of protein alternations using the CCI (chronic constriction injury) model in rats
- Procedure is thought to modulate interneuronal synapses
  - CMP motif 1 (yellow) characteristic for healthy control (a) is lost due to chronic neuropathic pain in the CCI model (b)
  - CMP motif 2 (red) only detected in the CCI model



CMP motif 1

- Lead proteins: GRIP-1, CT and GluR2/3



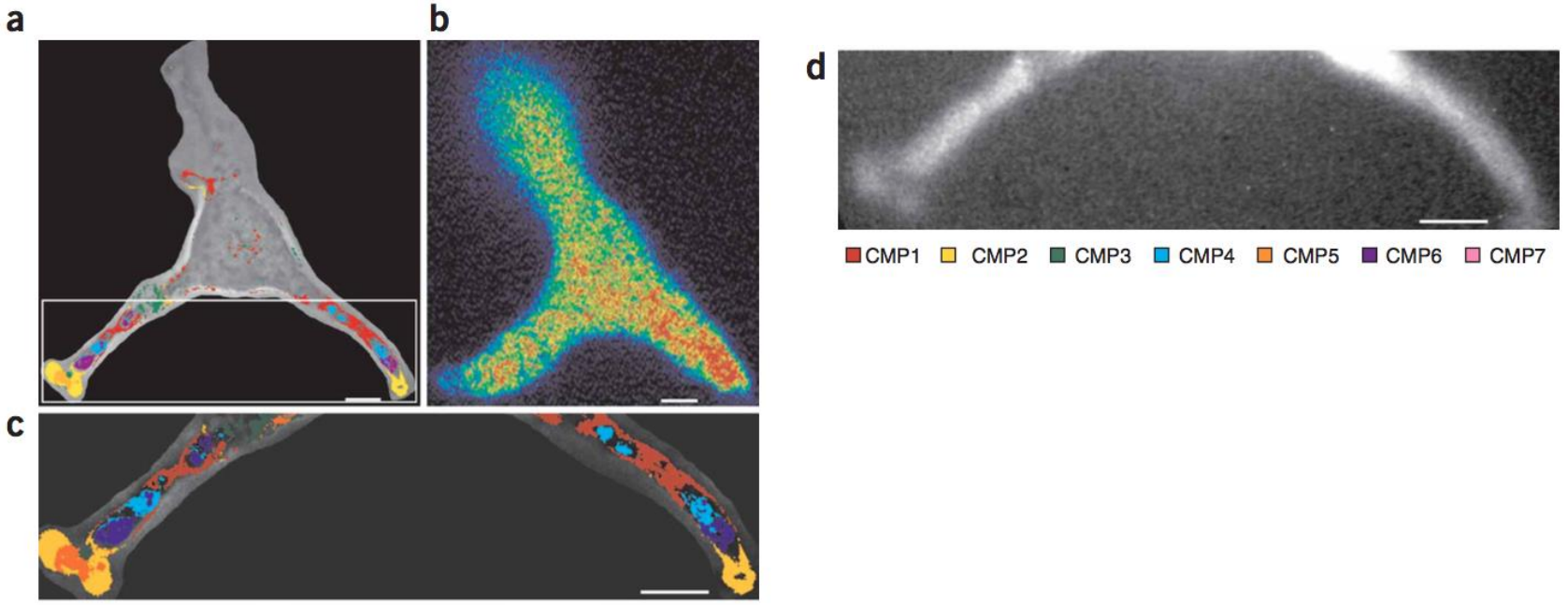
CMP motif 2

- Lead proteins: PSD-95, NR2A
- Restricted to single dendritic structures on the treated side

Multi-epitope-ligand cartography (MELC) – Paper 1

Identification of functional protein networks:

- Investigation of 23 cell surface proteins in a muscle tumor cell line TE671
- 4 CMPs were present while forming the exploratory state
- common lead protein of all 4 CMPs was the alanine-specific protease APN (CD13)
- Alanine-specific protease activity in the exploratory state as indicated by the breakdown of the fluorogenic substrate bis-ala-rhodamine 110 (b), correlates with the distribution of APN (d)



Multi-epitope-ligand cartography (MELC) – Paper 1

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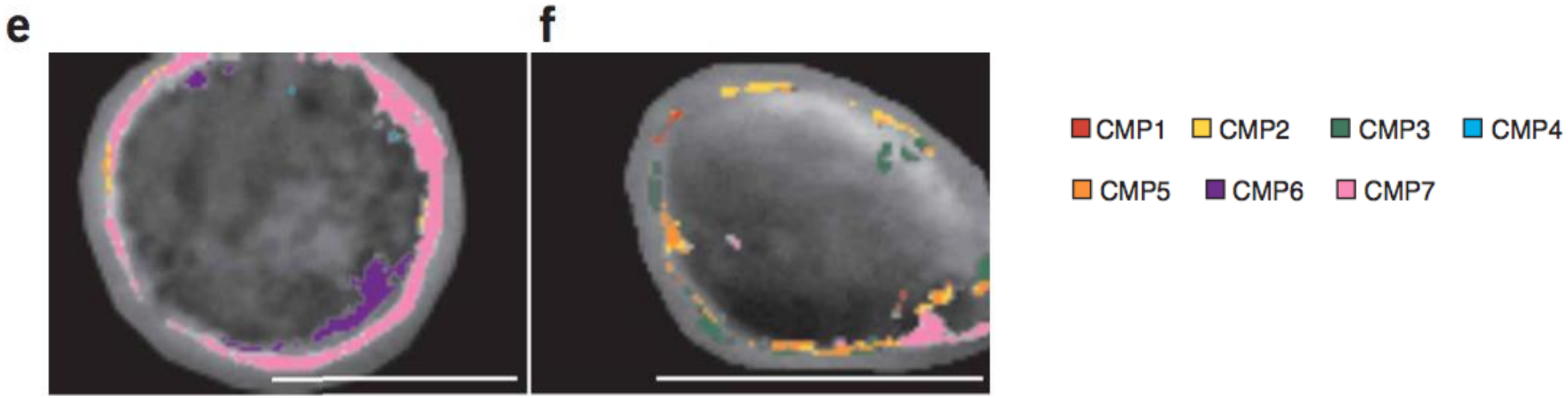
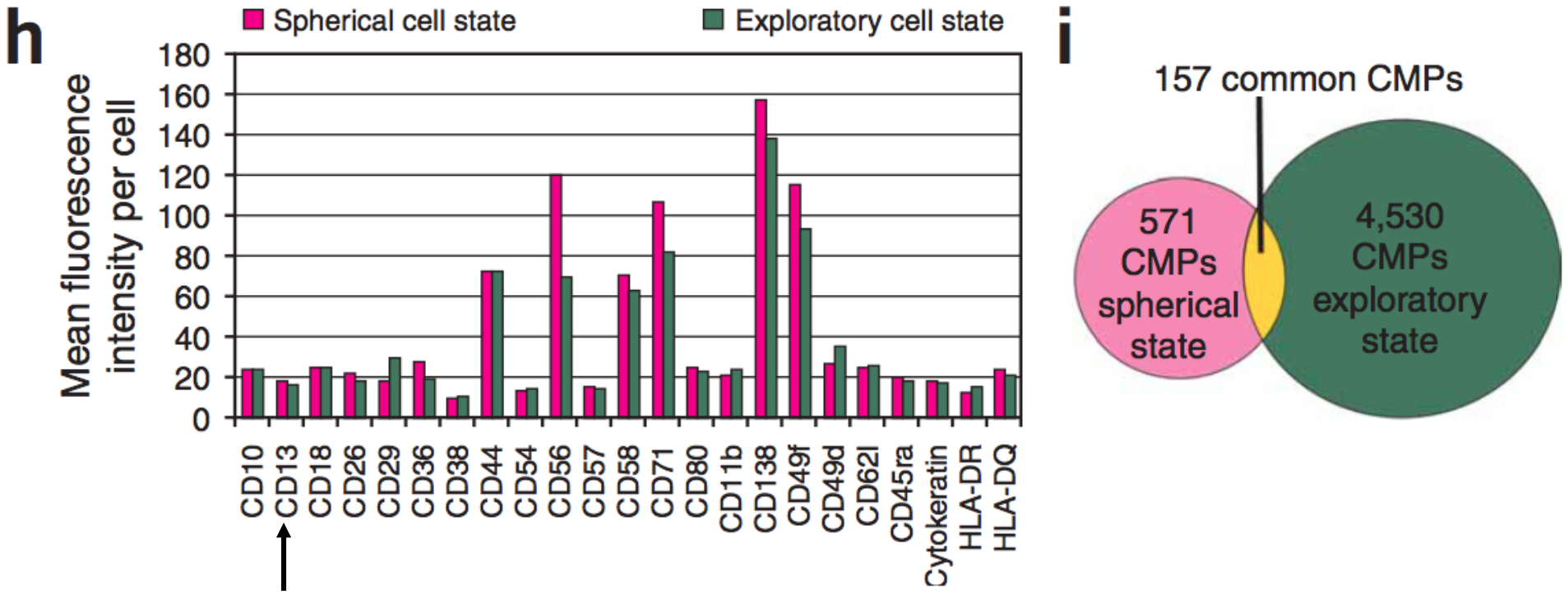
- Alanine-specific protease activity in the exploratory state as indicated by the breakdown of the fluorogenic substrate bis-ala-rhodamine 110 (b), correlates with the distribution of APN (d)
- Treatment with a APN specific inhibitor blocked the ability of the cells to transform into the exploratory state (e, f)



Multi-epitope-ligand cartography (MELC) – Paper 1

Identification of functional protein networks:

- Investigation of 23 cell surface proteins in the rhabdomyosarcoma cell line TE671
- 4 CMPs were present while forming the explorative state
- common lead protein of all 4 CMPs was the alanine-specific protease APN (CD13)
- Average fluorescence intensity of the 23 markers showed only little differences between the spherical and explorative state



Multi-epitope-ligand cartography (MELC) – Paper 1

Summary:

- multidimensional fluorescence imaging technology with functional resolution
- Allows the colocalization and detection of a large number of proteins
- Identifies transient or rare protein associations in 3D
- CMPs include weakly or transient interacting proteins
- Is able to reconstruct the dynamics of molecular networks
- Shows how protein associations are altered by pathology and disease

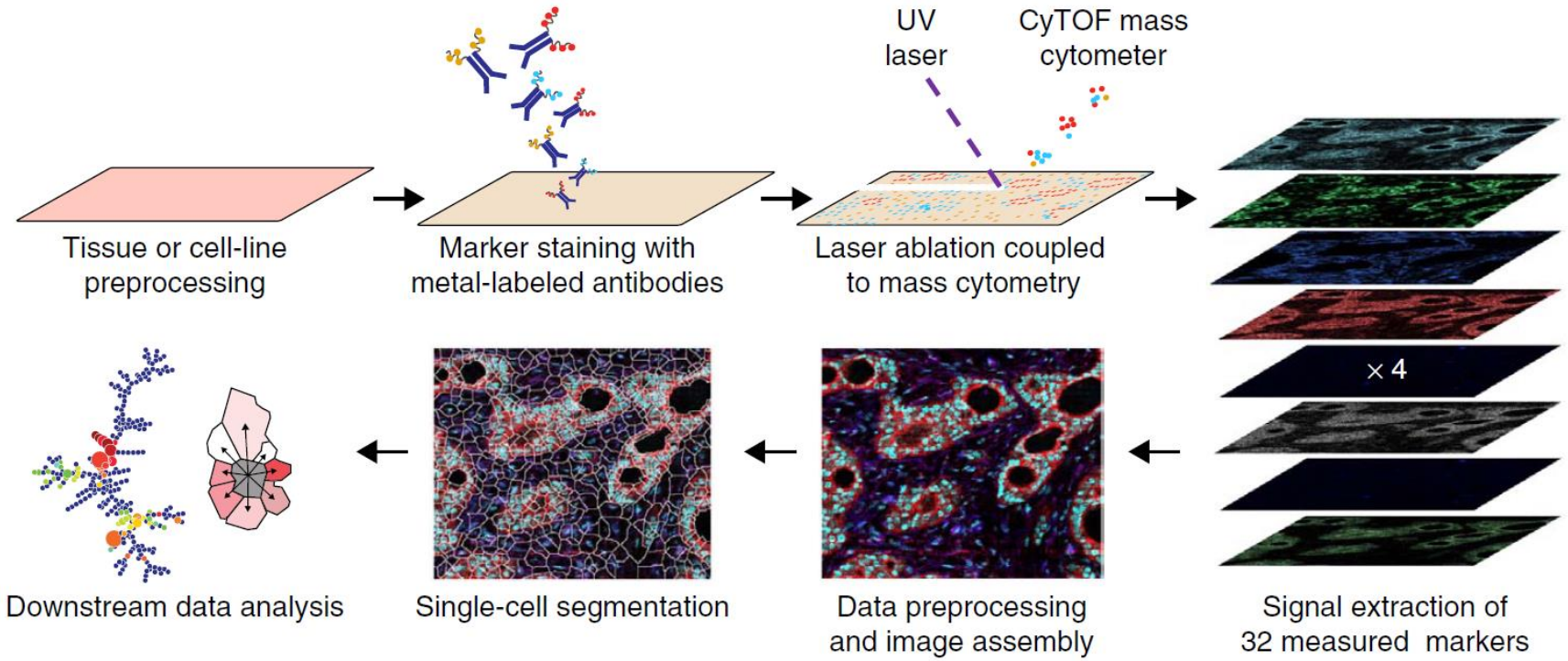
Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry

Charlotte Giesen[1,8], Hao A O Wang[2,3,8], Denis Schapiro[1,4], Nevena Zivanovic[1,5], Andrea Jacobs[1], Bodo Hattendorf[2], Peter J Schüffler[6], Daniel Grolimund[3], Joachim M Buhmann[6], Simone Brandt[7], Zsuzsanna Varga[7], Peter J Wild[7], Detlef Günther[2] & Bernd Bodenmiller[1]

Imaging mass cytometry (IMC) – Paper 2

The workflow of imaging mass cytometry:

- Preprocessing using routine immunohistochemistry protocols
- Laser beam of 1µm in diameter and 3.5J/cm^2 laser fluence used at a frequency of 20Hz
- Ablated sample aerosol gets directly transported to the CyTOF by argon and helium gas flow
- Single isotope signals are plotted using coordinates of each single laser shot
- Cell features are computationally segmented using the watershed algorithm



Imaging mass cytometry (IMC) – Paper 2

Validation of the approach using IHC:

- Comparison of metal-labeled and unlabeled antibodies in IFM using formalin-fixed paraffin-embedded breast cancer samples (a)

  nuclei: H3 and PR (not shown)

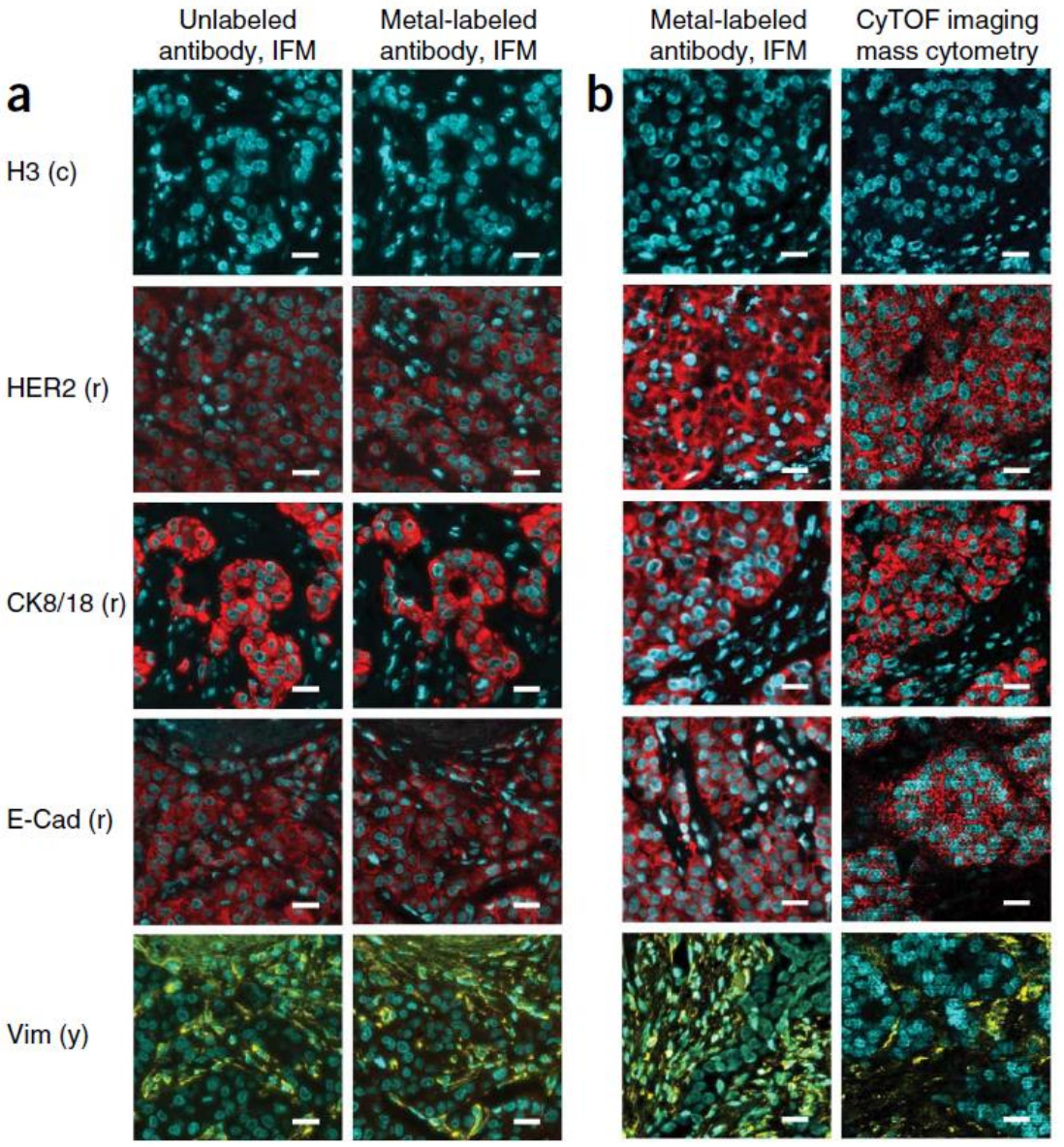
  plasma membrane: HER2, Cytokeratin 8/18, E-Cadherin

  stromal compartment: vitamin

- Investigation whether images generated by IMC are able to reproduce results obtained by IFM on luminal HER2+ breast cancer samples (b)

  → IMC is able to recapitulate IFM results with similar percentages of tumor cells expressing the analyzed markers

	IFM	IMC
H3	100%	100%
HER2	75%	79%
Cytokeratin 8/18	63%	66%

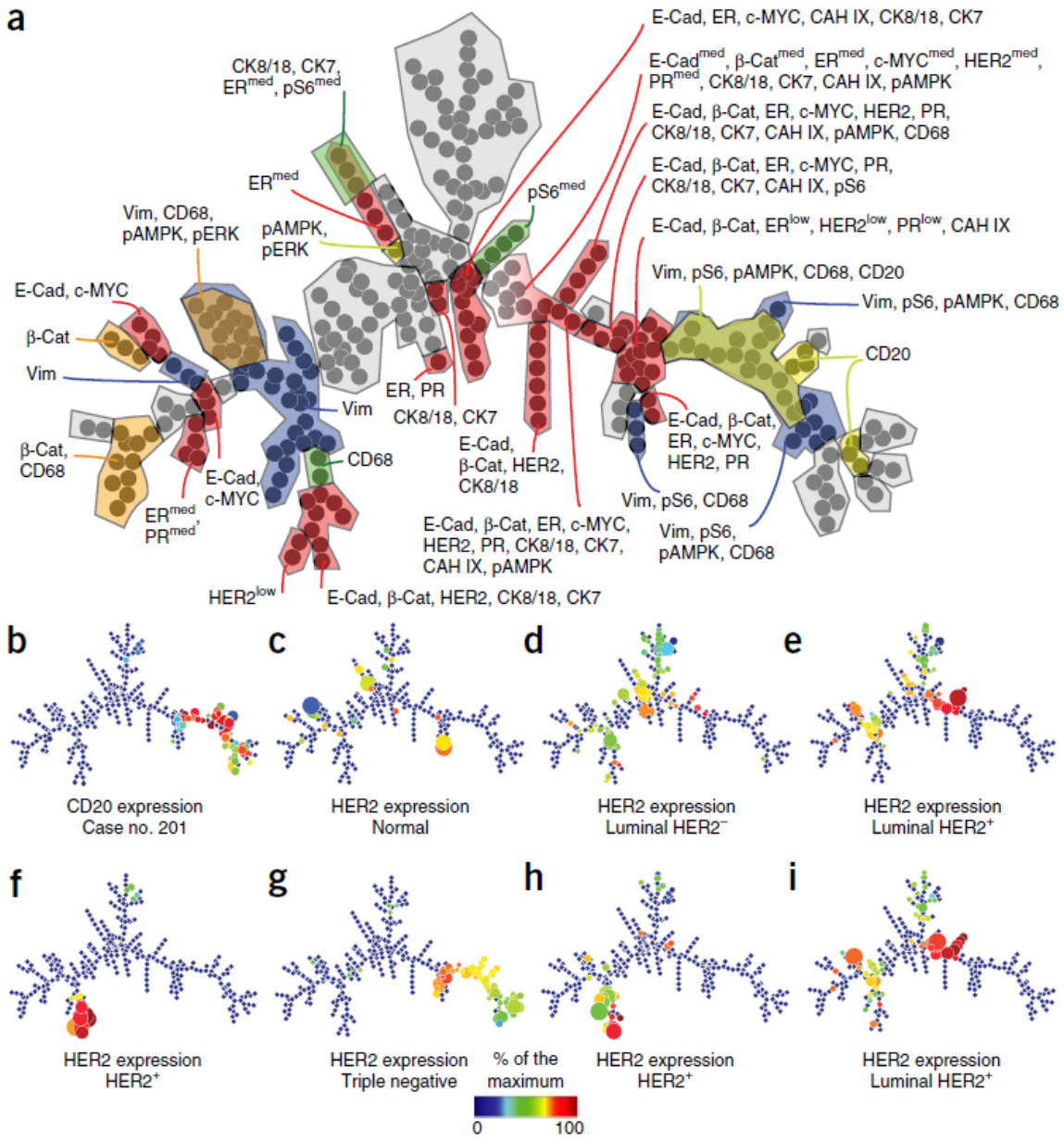


Imaging mass cytometry (IMC) – Paper 2

Analysis of tumor heterogeneity in breast cancer:

- in breast cancer, the expression of HER2, estrogen receptor (ER) and PR are used to define the main subtypes:
  - luminal HER2-
  - luminal HER2+
  - HER2+
  - Tripel negative
- Identification of cell subpopulation phenotypes using the IMC multiplexed measurements of 32 markers on 21 previously classified breast cancer samples by SPADE (spanning-tree progression analysis of density-normalized events) analysis (a)

→ Detection of breast cancer heterogeneity within and between the subtypes (c-i)



Imaging mass cytometry (IMC) – Paper 2

Summary:

- New method combining CyTOF with immunohistochemistry techniques resulting in spatially resolved multiplexed single cell analysis
- Advantages:

  no sample autofluorescence

  no matrix effects as seen for MALDI

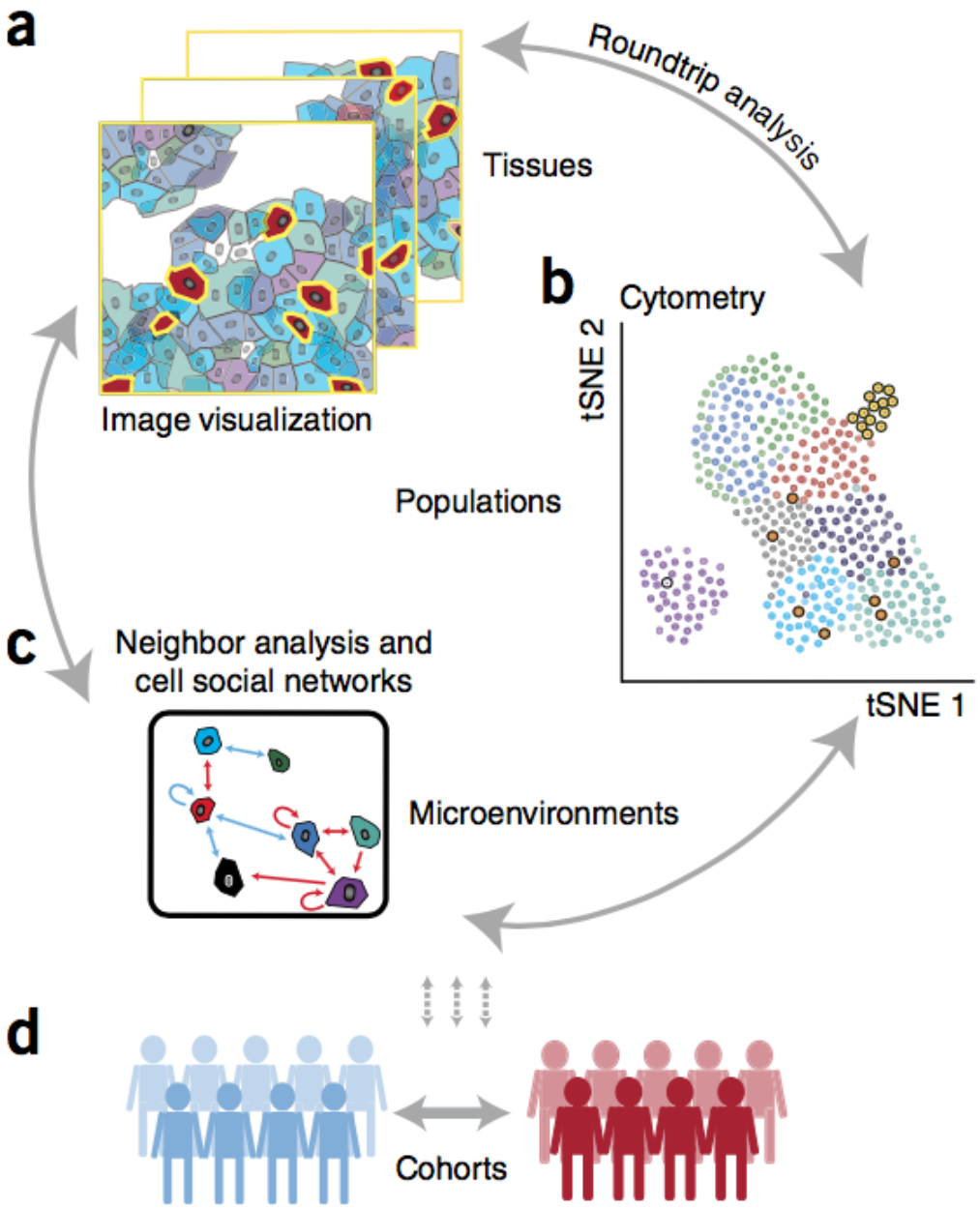
  no amplification step of the signal needed
- appropriate standards will enable the absolute quantification of cellular markers
- Current limitations are availability of antibodies, sampling time and resolution

histoCAT: analysis of cell phenotypes and interactions in multiplex image cytometry data

Denis Schapiro[1,2,5], Hartland W Jackson[1,5], Swetha Raghuraman[1,4], Jana R Fischer[1], Vito R T Zanotelli[1,2], Daniel Schulz[1], Charlotte Giesen[1,4], Raúl Catena[1], Zsuzsanna Varga[3] & Bernd Bodenmiller[1]

Histology topography cytometry analysis toolbox (histoCAT) – Paper 3

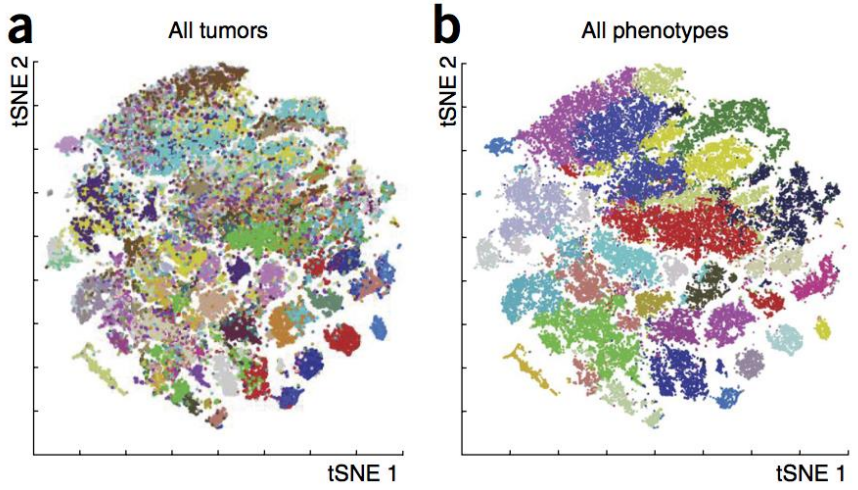
- need of new computational approaches to enable comprehensive, quantitative and interactive exploration of all levels of information
- histoCAT combines intuitive high dimensional image visualization and novel algorithms for the comprehensive study of cell-cell interactions and the social networks of cells
- use of “Round-Trip” analysis



Histology topography cytometry analysis toolbox (histoCAT) – Paper 3

Analysis example:

- Performance of IMC on 49 diverse breast cancer samples, 3 matched normal tissue samples and 6 additional healthy breast tissue samples
- Antibody panel contained markers for the identification of
  - cell lineages
  - signal pathway activation
  - proliferation
  - apoptosis
  - clinical markers
- To gain a tissue-wide overview of cell phenotypes present in a given image set, two approaches have been incorporated into histoCAT

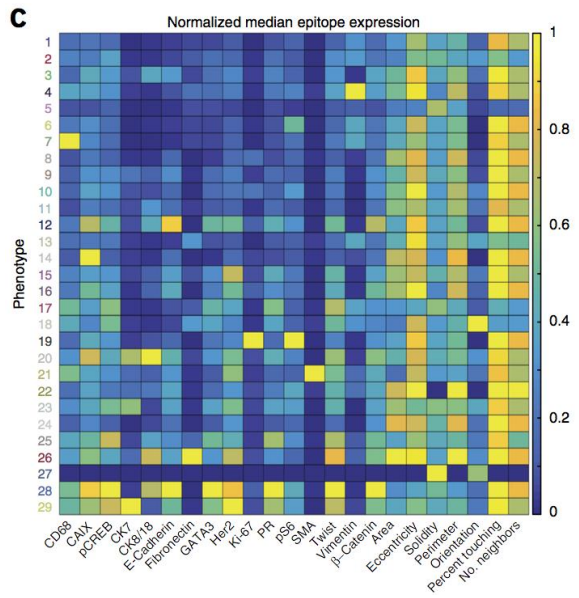


<u>First approach</u> is a supervised and based on tSNE

-> grouping similar cells, distinct colors distinguish cells from each source image

<u>Second approach</u> is based on the unsupervised clustering algorithm PhenoGraph

-> defines complex cell phenotypes based on marker expression and enables labeling of cell phenotype clusters on a tSNE plot or displays phenotypes as heatmaps taking into account the expression level

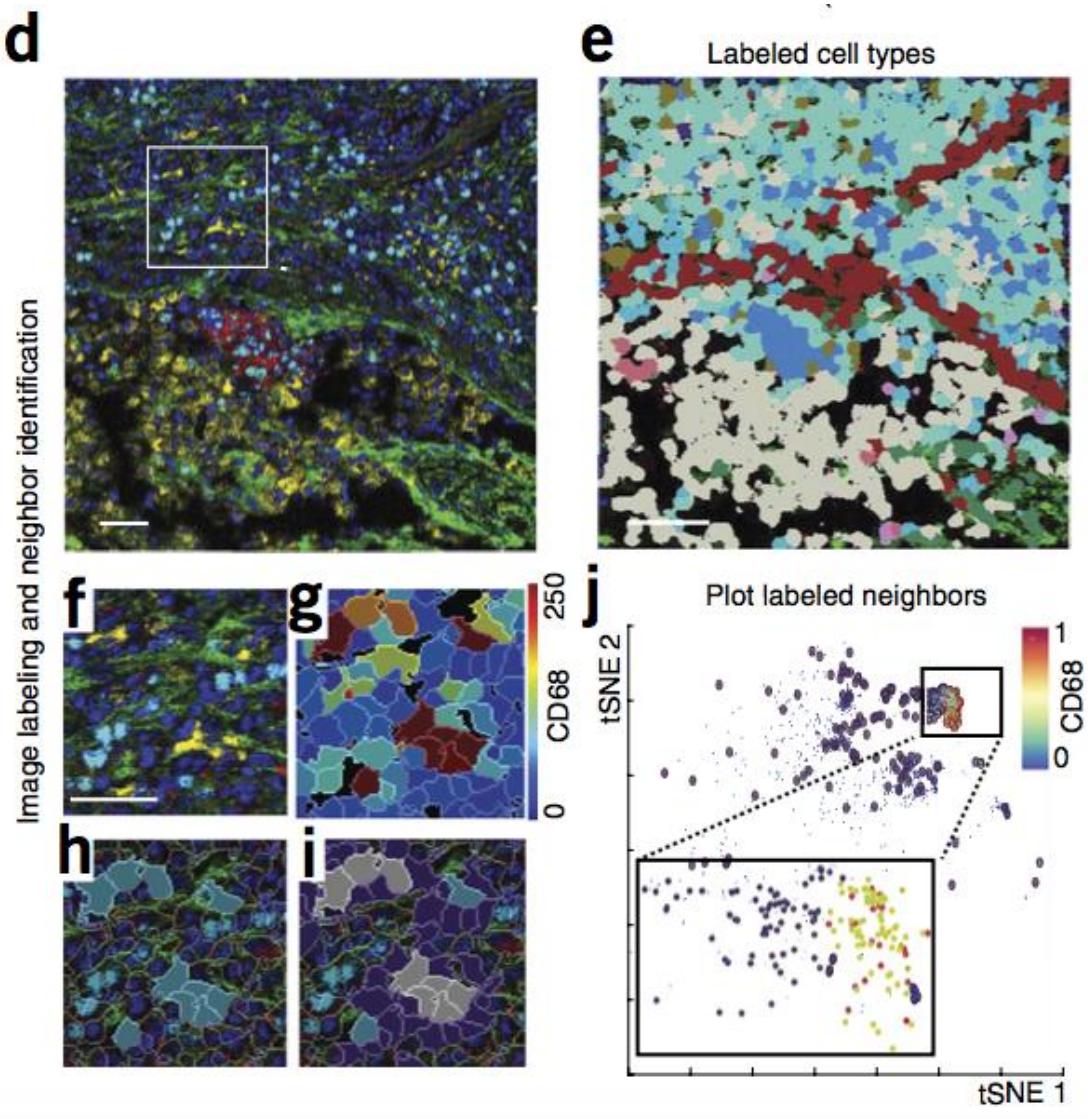


Histology topography cytometry analysis toolbox (histoCAT) – Paper 3

Analysis example:

- Marker intensities and cell populations can be linked back to their source images (d and e)
- Visualization by pseudocolors (f) or heatmap (g)
- Specific phenotype clusters can be selected for further analysis; e.g. PC7 with high CD68 expression for a deeper understanding of tumor-associated macrophages (TAMs) (g,h and j)
- histoCAT has two neighborhood functions that enable the investigation of the microenvironment as well
  - User guided, selection of cells touching or proximal to the cell population of interest (i and j)

    → downstream analysis revealed that distinct proliferative (Ki-67+, phospho-S6+) and hypoxic (carbonic anhydrase IX+) tumor cells neighbor CD68+ cells



Histology topography cytometry analysis toolbox (histoCAT) – Paper 3

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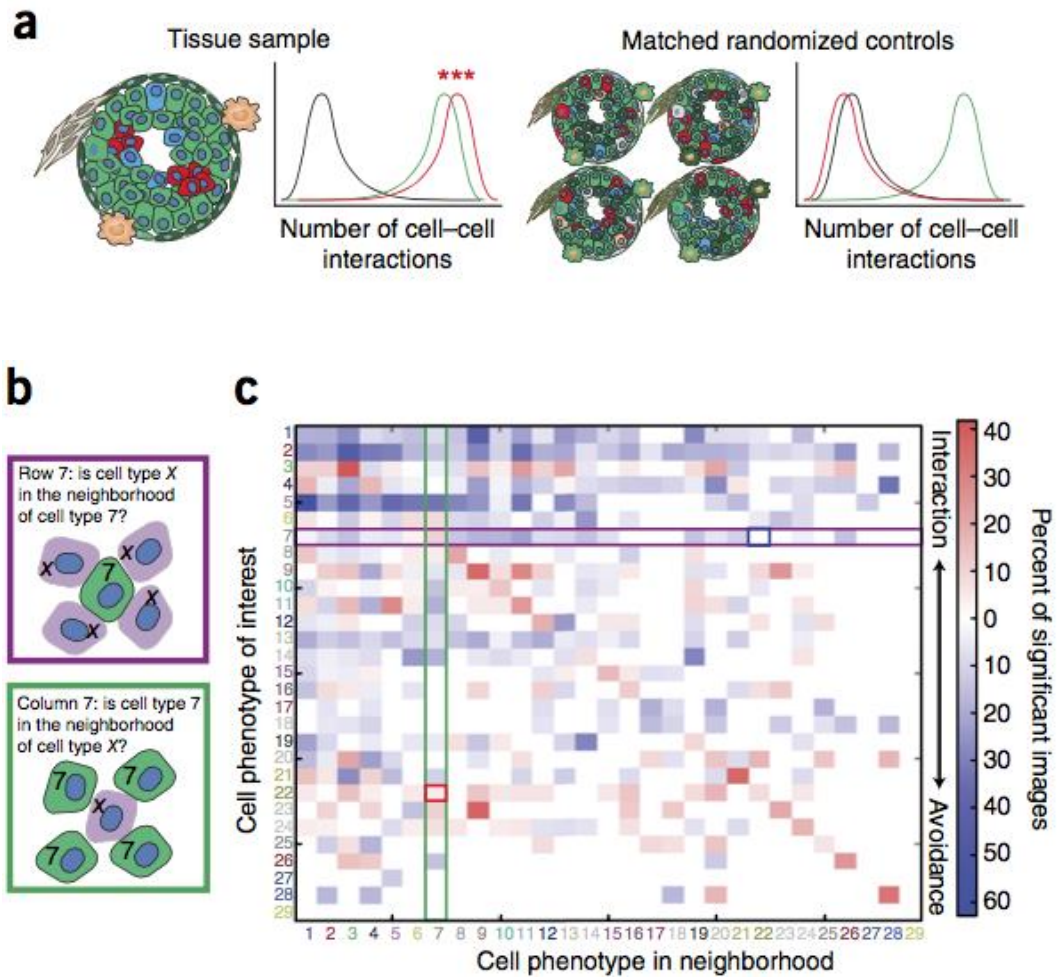
    → downstream analysis revealed that distinct proliferative (Ki-67+, phospho-S6+) and hypoxic (carbonic anhydrase IX+) tumor cells neighbor CD68+ cells
  - Unbiased and systematic study of cell-cell interactions using a permutation test to compare the number of interactions between all cell types in a given image to that of a matched control (a-c)



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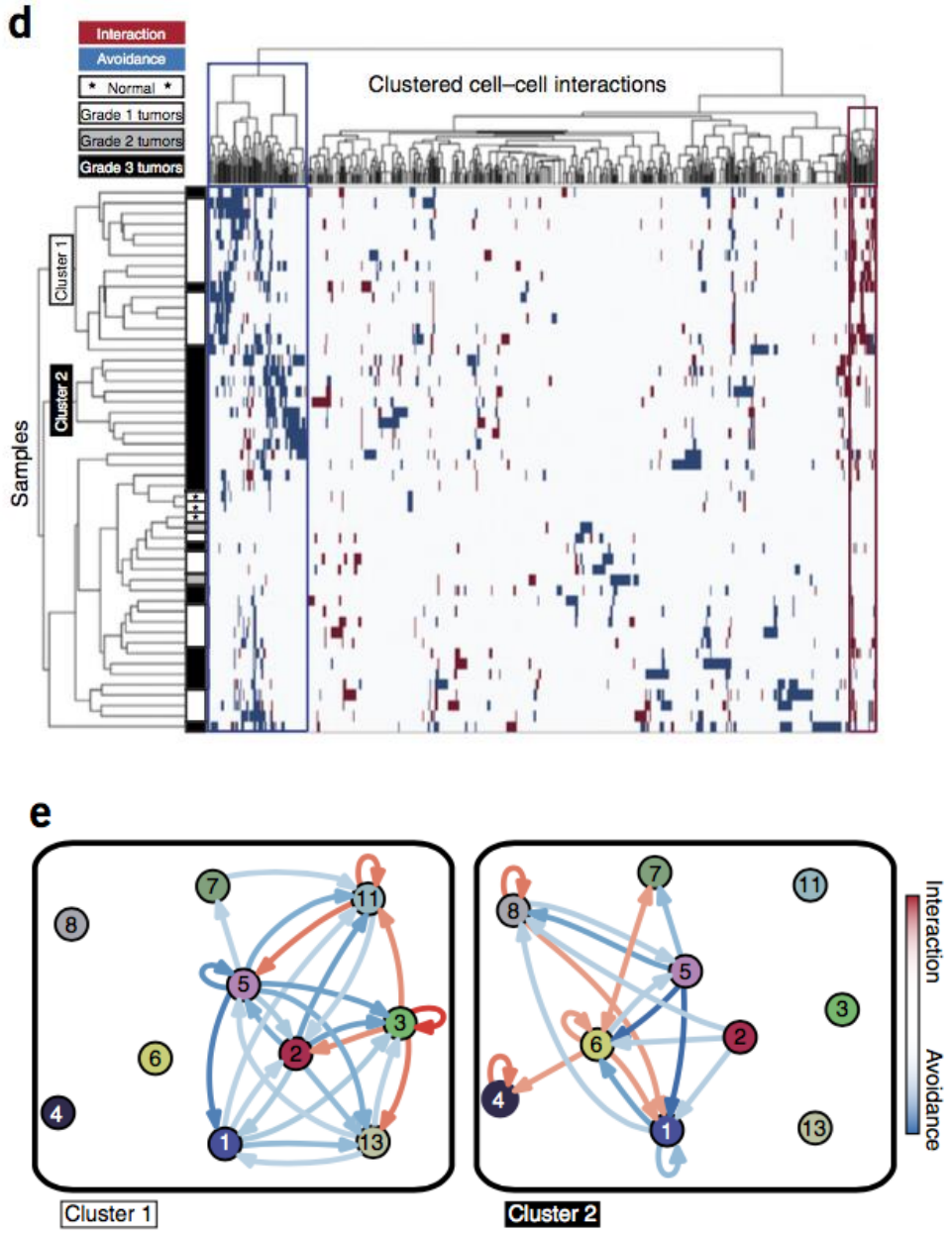
  - Unbiased and systematic study of cell-cell interactions using a permutation test to compare the number of interactions between all cell types in a given image to that of a matched control (a-c)

    → identification of cellular landscapes and social networks



Histology topography cytometry analysis toolbox (histoCAT) – Paper 3

Summary:

- histoCAT combines intuitive high dimensional image visualization and novel algorithms for the comprehensive study of cell-cell interactions and the social networks of cells
- quantitative and systematic analysis of cell-cell interactions

→ identification of defined groups with similar organization, revealing pathology-grade-associated cellular ecosystems that may distinguish unique disease states

- toolbox with a variety of different visualization options
- detailed user guide provided in the supplementary data

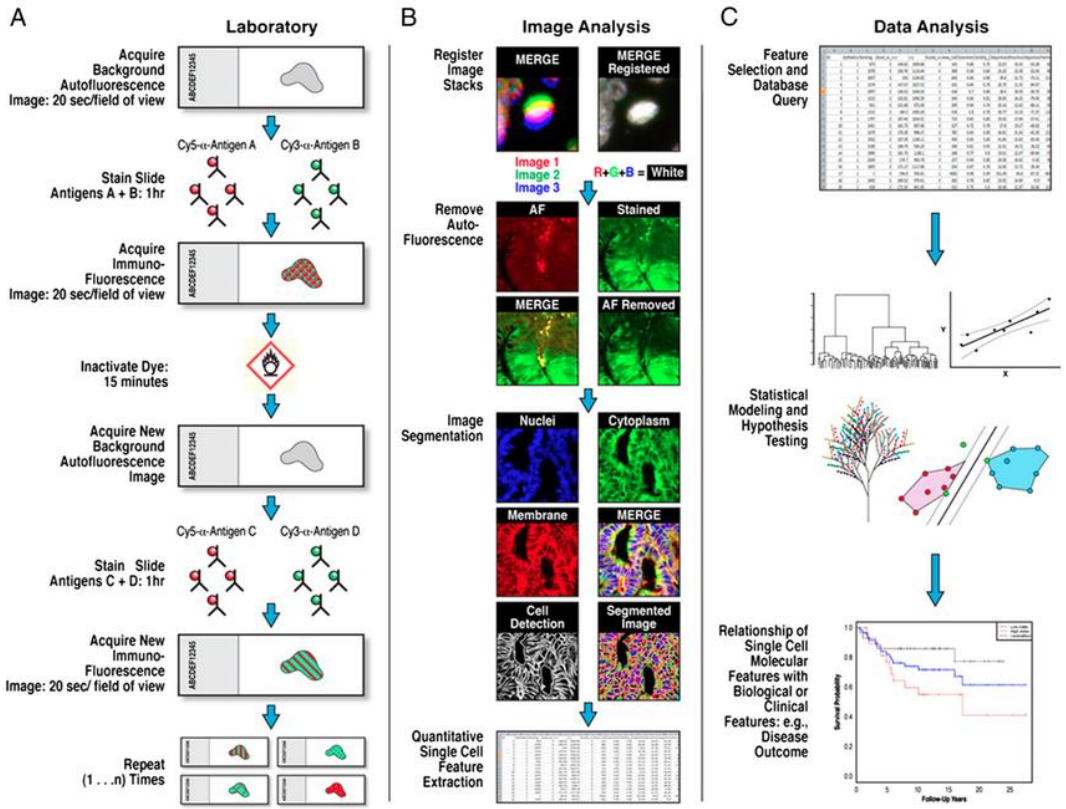
Thank you for your attention!

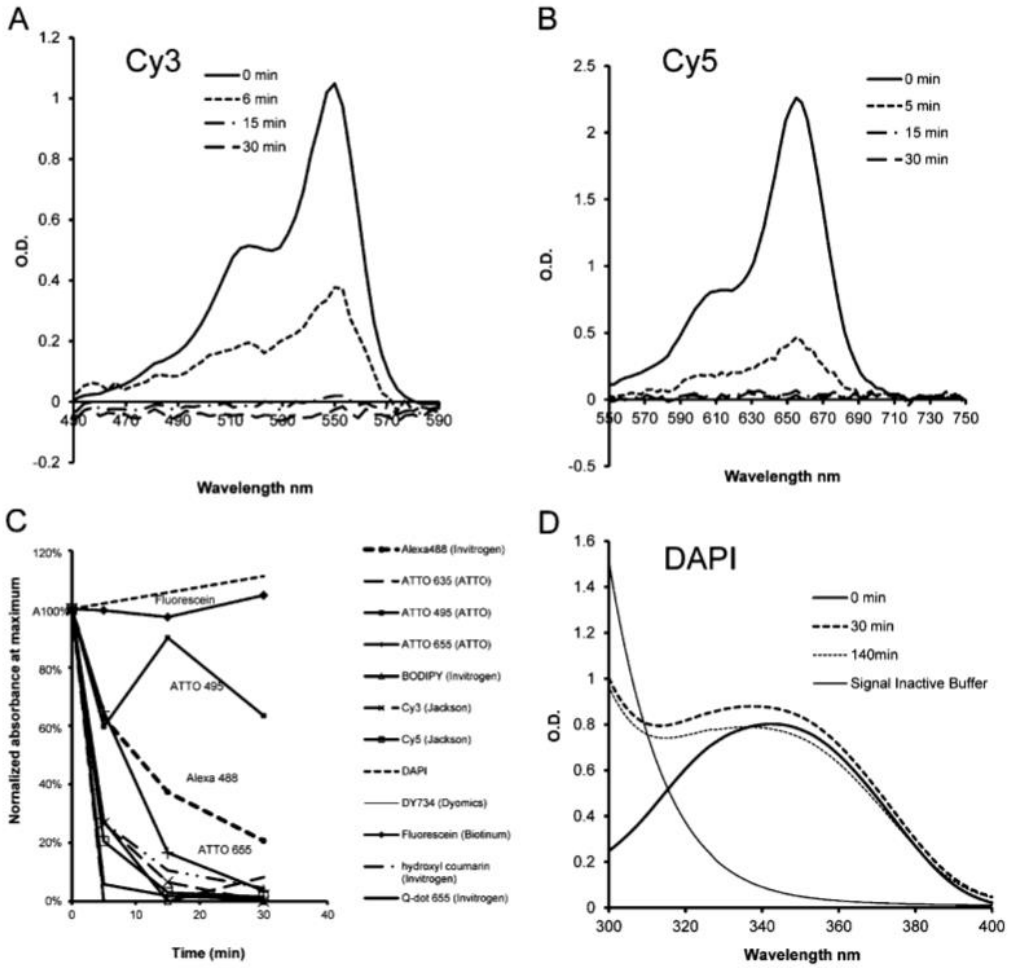
Questions?

Additional formats of cycling immunofluorescence methods

Highly multiplexed single-cell analysis of formalin-fixed, paraffin-embedded cancer tissue

Michael J. Gerdes[a,1], Christopher J. Sevinsky[b,1], Anup Sood[c,1], Sudeshna Adak[d], Musodiq O. Bello[e,2], Alexander Bordwell[c,3], Ali Can[e], Alex Corwin[f], Sean Dinn[c], Robert J. Filkins[g], Denise Hollman[b,3], Vidya Kamath[h], Sireesha Kaanumalle[c], Kevin Kenny[i], Melinda Larsen[a,4], Michael Lazare[j,3], Qing Li[j], Christina Lowes[j], Colin C. McCulloch[k], Elizabeth McDonough[j], Michael C. Montalto[l,5], Zhengyu Pang[b], Jens Rittscher[m], Alberto Santamaria-Pang[e], Brion D. Sarachan[n], Maximilian L. Seel[b], Antti Seppo[a], Kashan Shaikh[f], Yunxia Sui[k], Jingyu Zhang[b], and Fiona Ginty[o,6]





Additional formats of cycling immunofluorescence methods

nature COMMUNICATIONS

ARTICLE

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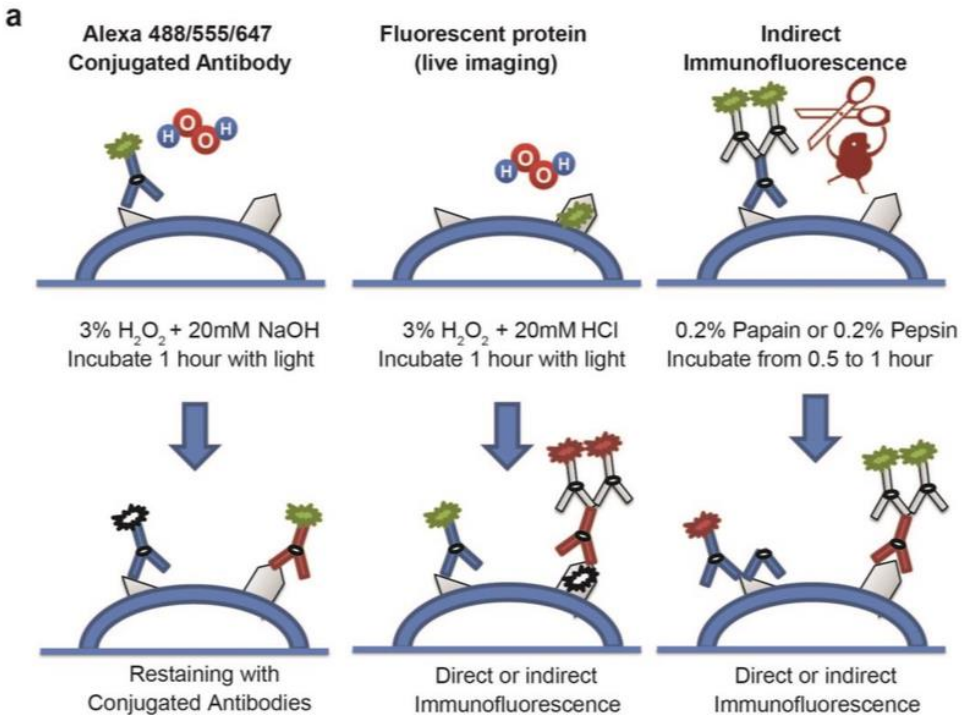
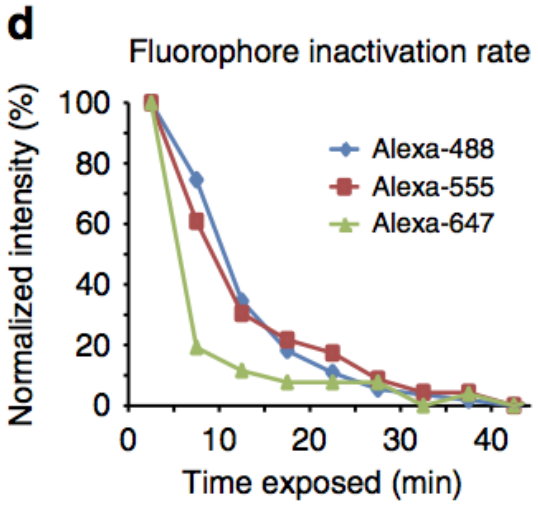
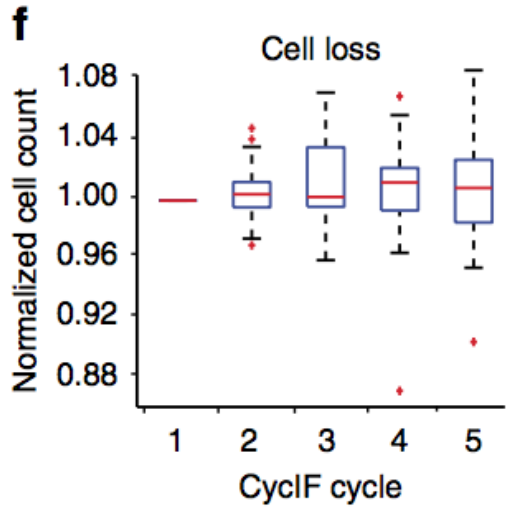
Highly multiplexed imaging of single cells using a high-throughput cyclic immunofluorescence method

Jia-Ren Lin[1], Mohammad Fallahi-Sichani[2] & Peter K. Sorger[1,2]







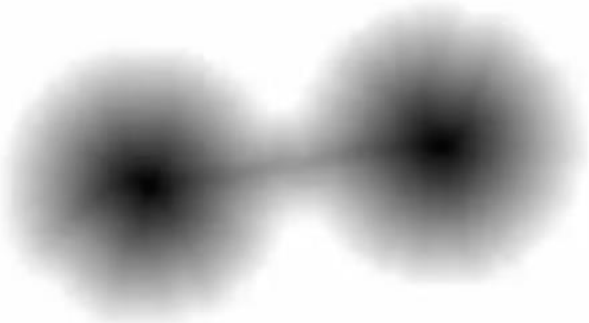
Imaging mass cytometry (IMC) – Paper 2

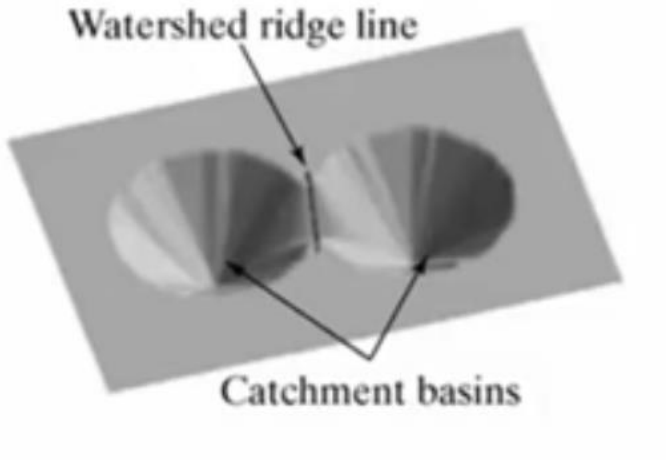
Watershed algorithm:

- Which pixel belongs to each object?
- Separating objects from the background or from each other
- grayscale image visualized as topological surface:

  bright areas = high (white corresponds to watershed lines)

  dark areas = low; catchment basins





1	1	0	0	0
1	1	0	0	0
0	0	0	0	0
0	0	0	0	0
0	1	1	1	0

0.00	0.00	1.00	2.00	3.00
0.00	0.00	1.00	2.00	3.00
1.00	1.00	1.41	2.00	2.24
1.41	1.00	1.00	1.00	1.41
1.00	0.00	0.00	0.00	1.00

Imaging mass cytometry (IMC) – Paper 2

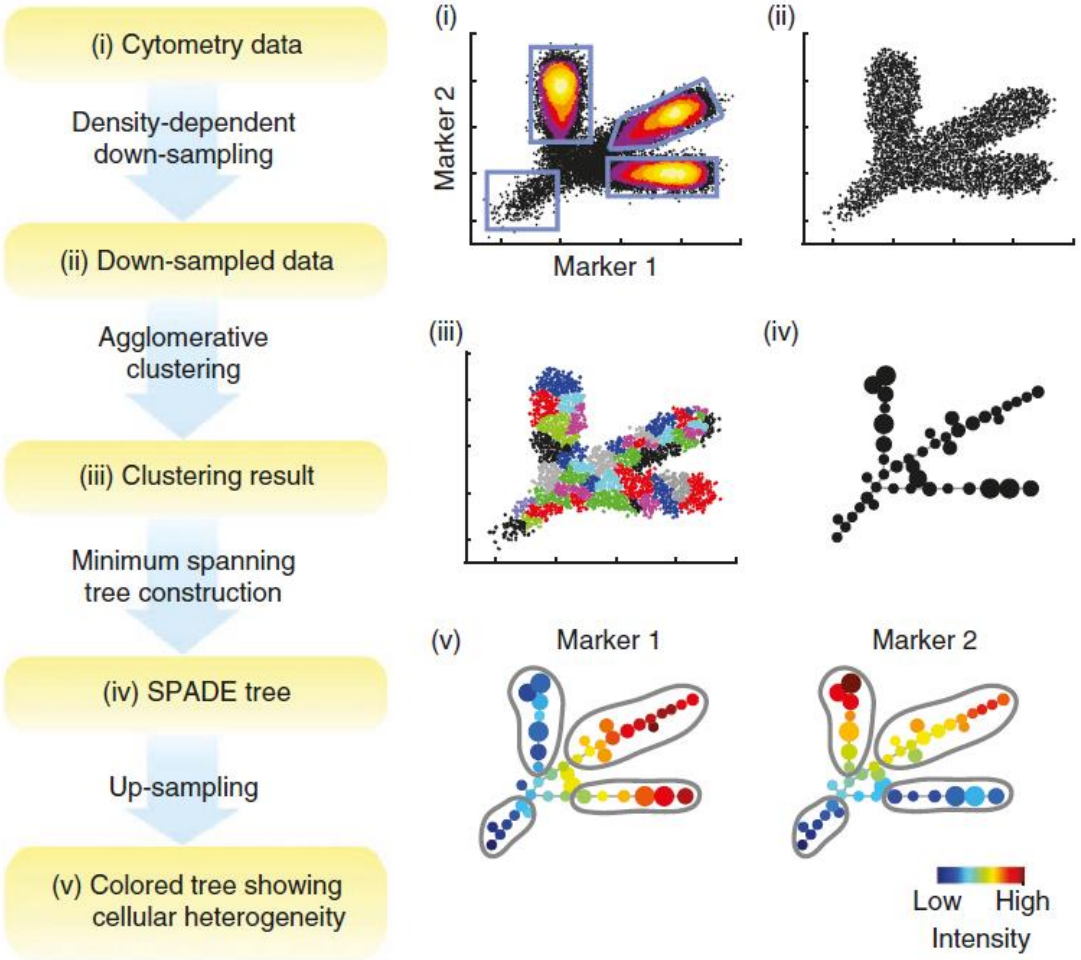
ANALYSIS

_computational BIOLOGY

Extracting a cellular hierarchy from high-dimensional cytometry data with SPADE

Peng Qiu[1,2], Erin F Simonds[3], Sean C Bendall[3], Kenneth D Gibbs Jr[3], Robert V Bruggner[3], Michael D Linderman[4], Karen Sachs[3], Garry P Nolan[3] & Sylvia K Plevritis[1]

SPADE = **s**panning-tree **p**rogression **a**nalysis of **d**ensity-normalized **e**vents



Histology topography cytometry analysis toolbox (histoCAT) – Paper 3

ARTICLES

nature biotechnology

viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia

El-ad David Amir[1], Kara L Davis[2,3], Michelle D Tadmor[1,3], Erin F Simonds[2,3], Jacob H Levine[1,3], Sean C Bendall[2,3], Daniel K Shenfeld[1,3], Smita Krishnaswamy[1], Garry P Nolan[2,4] & Dana Pe'er[1,4]

- viSNE plots individual cells in a visual similar to a scatter plot, while using all pairwise distances in high dimension to determine each cell's location in the plot
- viSNE finds the two dimensional representation of single-cell data that best preserves their local and global geometry
- The resulting viSNE map provides a visual representation of the single-cell data that is similar to a biaxial plot, but the positions of cells reflect their proximity in high-dimensional rather than two-dimensional space