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]

Nat Met, April 2014

Multiplexed ion beam imaging of human breast tumors

Michael Angelo[1,2], Sean C Bendall[1], Rachel Finck[1], Matthew B Hale[1], Chuck Hitzman[3], Alexander D Borowsky[4], Richard M Levenson[4], John B Lowe[5], Scot D Liu[5], Shuchun Zhao[6], Yasodha Natkunam[6] & Garry P Nolan[1]

Nat Med, April 2014

Next-Generation Immunohistochemistry: **Multiplex tissue imaging with mass cytometry**

Journal Club – Timo Böge

Overview

Introduction

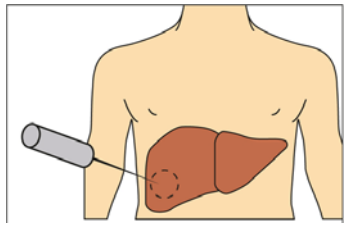
- Conventional Immunohistochemistry
- Mass Spectrometry
- Mass Cytometry (Cytof)
- Multiplexed Mass Cytometry

- Paper: Giessen et al, Nat Met 2014 & Angelo et al, Nat Med 2014

- Discussion:
- Advantages
- Limitations

Immunohistochemistry (IHC)

Samples preparation:



- Patients undergo surgery/biopsy
- Formalin Fixation and Paraffin Embedding (FFPE) of tissue
- fixation time depends on the thickness of the tissue, time: 1 mm/h

Staining preparation

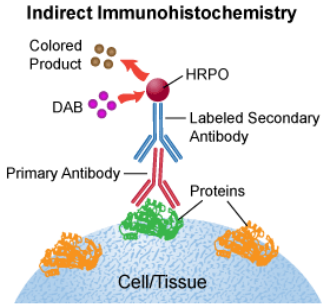
- Cutting (5um) and deparaffinization/rehydration

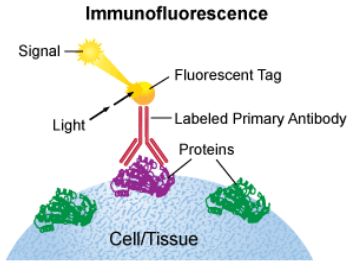
Block slide

- Heat or enzymatic digestion for antigen retrieval to unmask the antigenic epitope

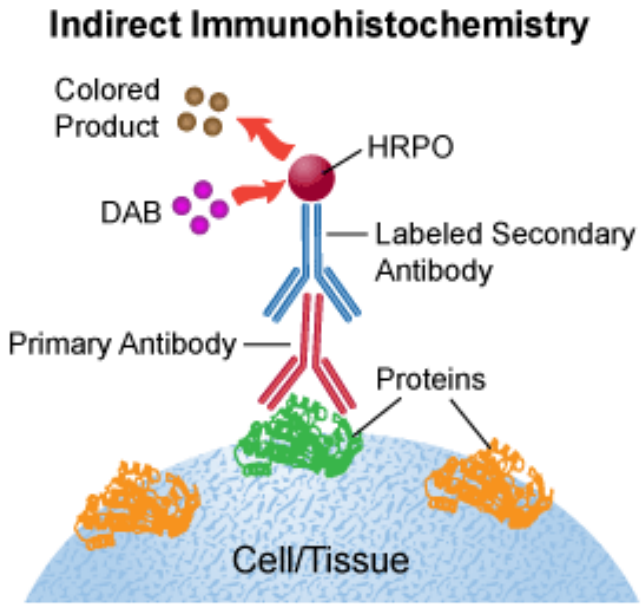
Immunohistochemical Staining

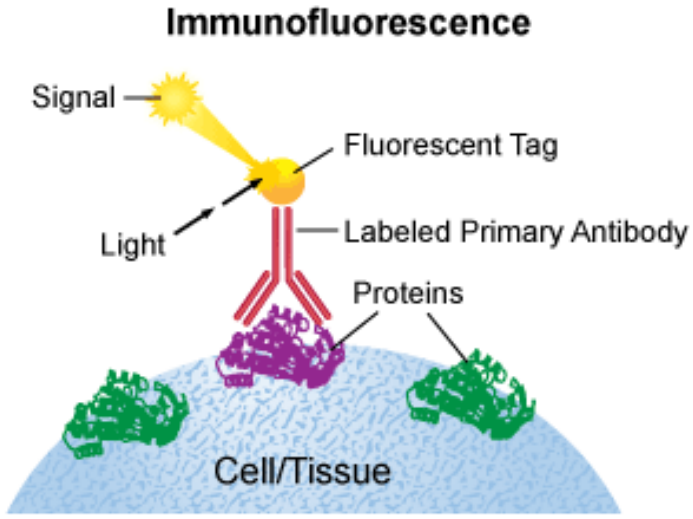
- Blocking, Incubation with 1° ab - directly labeled or
- washing incubation 2° ab
  - HRP (plus DAB substrate & Hematoxylin counterstaining)
  - or flourophore coupled





Immunohistochemistry (IHC)





- Colometric detection:

max. 4 different colours

- IF:

max. 11 different colours (Alexa, 2001)

max. 18 diffenerent colours (quantum dots, 2011)

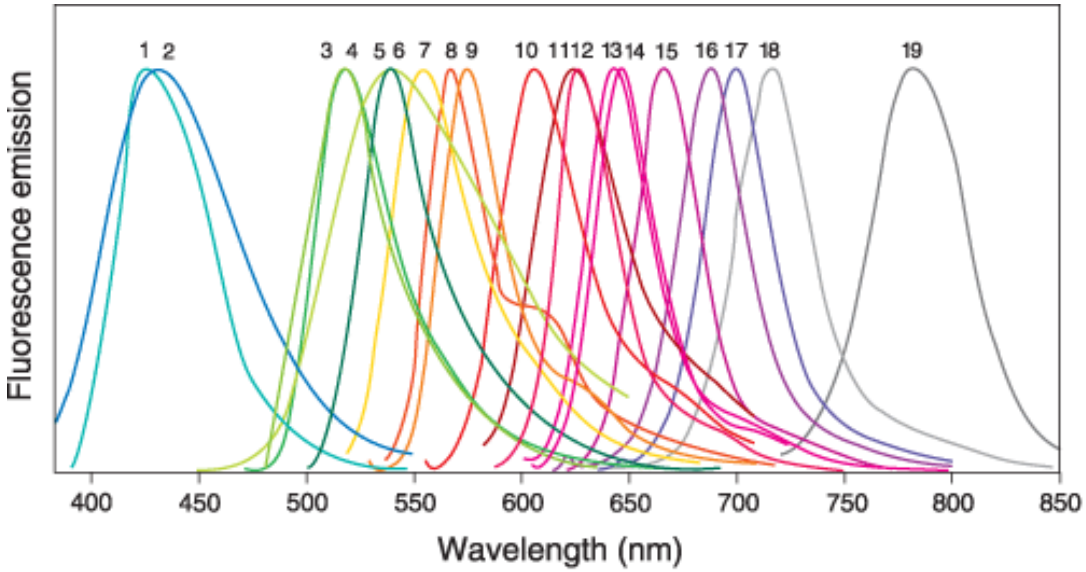
Limitations:

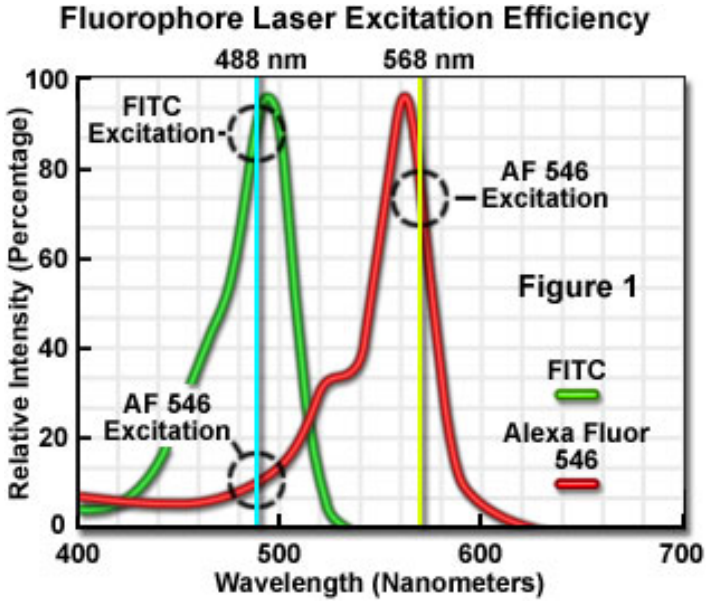
→ Need for 1°ab from **different host species**

→ non-overlapping **reporter emission spectra**

Bendall et al, 2012

Immunohistochemistry (IHC)





Limitations:

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Bendall et al, 2012

Mass Spectometry (MS)

Mass spectrometry measures molecules/fragments based on their mass-to-charge ratio (m/z)

1. Ionization

- Molecules bombarded with a stream of electrons
- Produces positively charged ions/fragements
- Protein have «pre-determined breaking points»

2. Acceleration

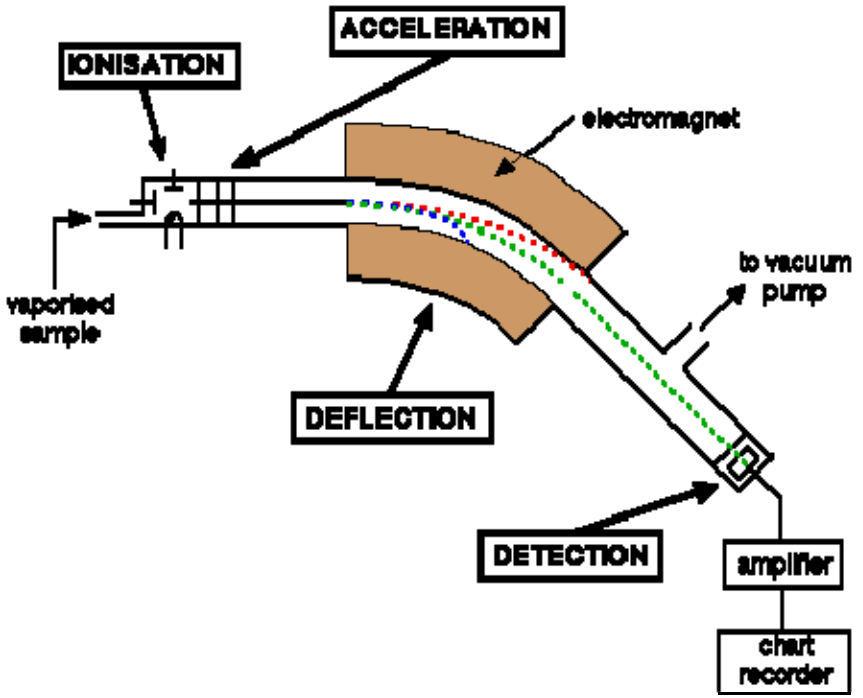
- same kinetic energy

3. Deflection

- by a magnetic field,
- according to their masses & charges

4. Detection

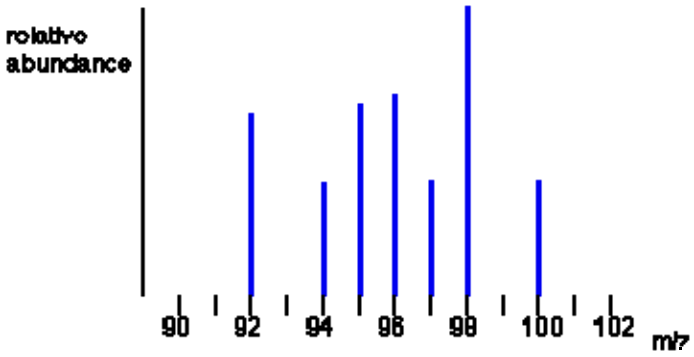
- mass to charge ratio: m/z



Mass Spectometry (MS)

5. Output & Data analysis:

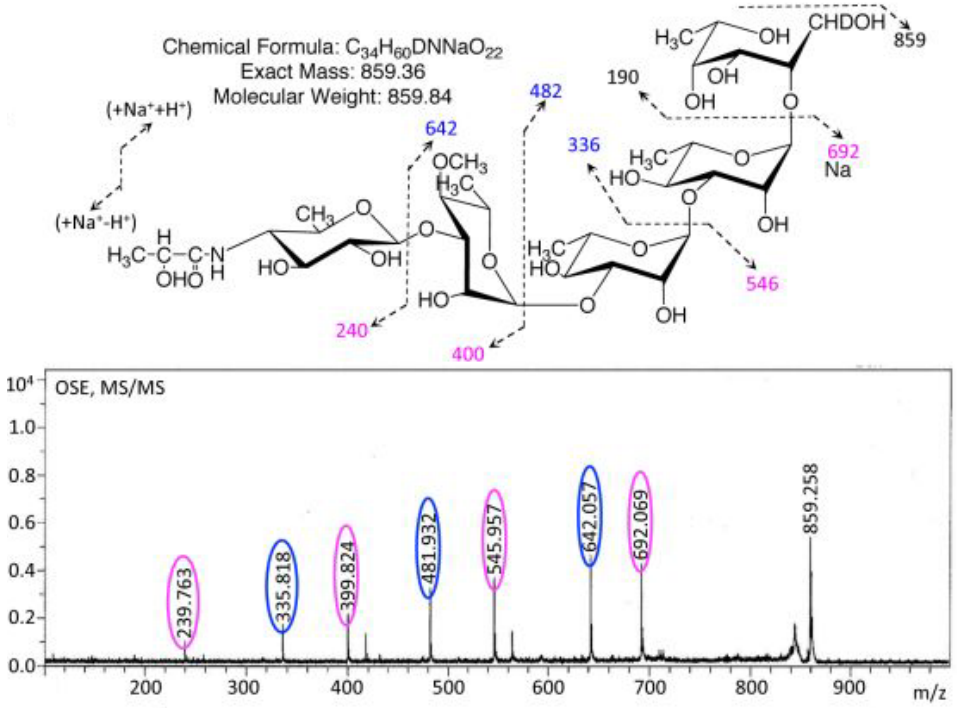
atoms → positively charged ions, **stick diagram**



m=100 & z= +1 → m/z = 100

m=200 & z=+2 → m/z = 100 !

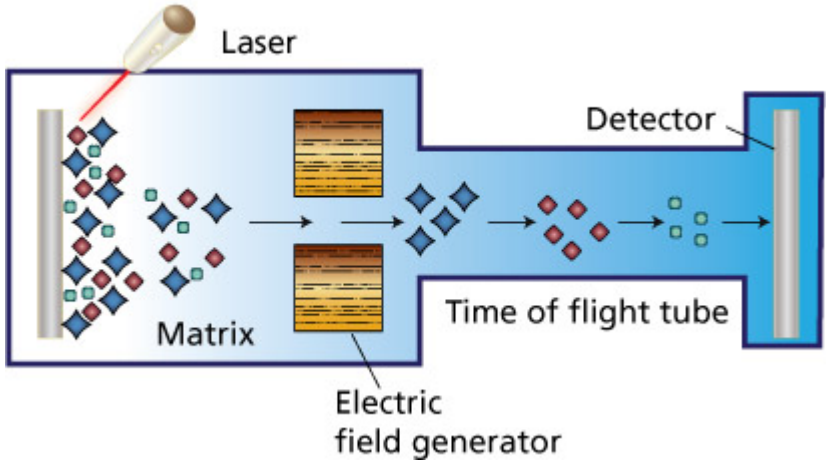
Proteins → «**fingerprint**» of fragments



Mass Spectometry –MADLI-TOF

1. Matrix-assisted laser desorption/ionization (MALDI)
   - Improves the fragmentation of large molecules

2. Time of Flight (TOF)
   - Improves the detection of large molecules/fragments



Mass Cytometry (CYTOF)

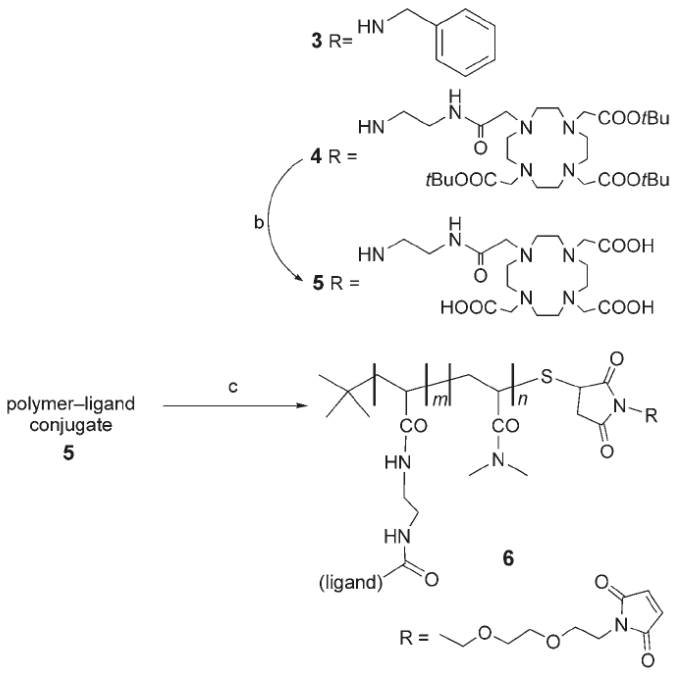
- Single cell **Flow cytometry** combined with **Mass Spectometry**

- Antibodies are labeled with **rare** transition element isotypes
- no fluorochromes → no spectral overlapp
- Read-out: time-of-flight mass spectrometry

Workflow:

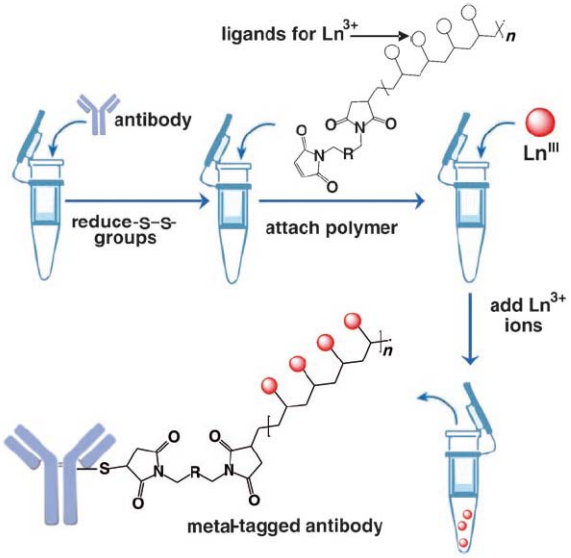
1. Cells are labeled with metal tagged antibodies
2. Cells are vaporized
3. Analysis by time-of-flight mass spectrometry
4. Read-out: fcs files («FACS plots», SPADE trees)

Antibody tagging with a unique isotope



ligand preparation

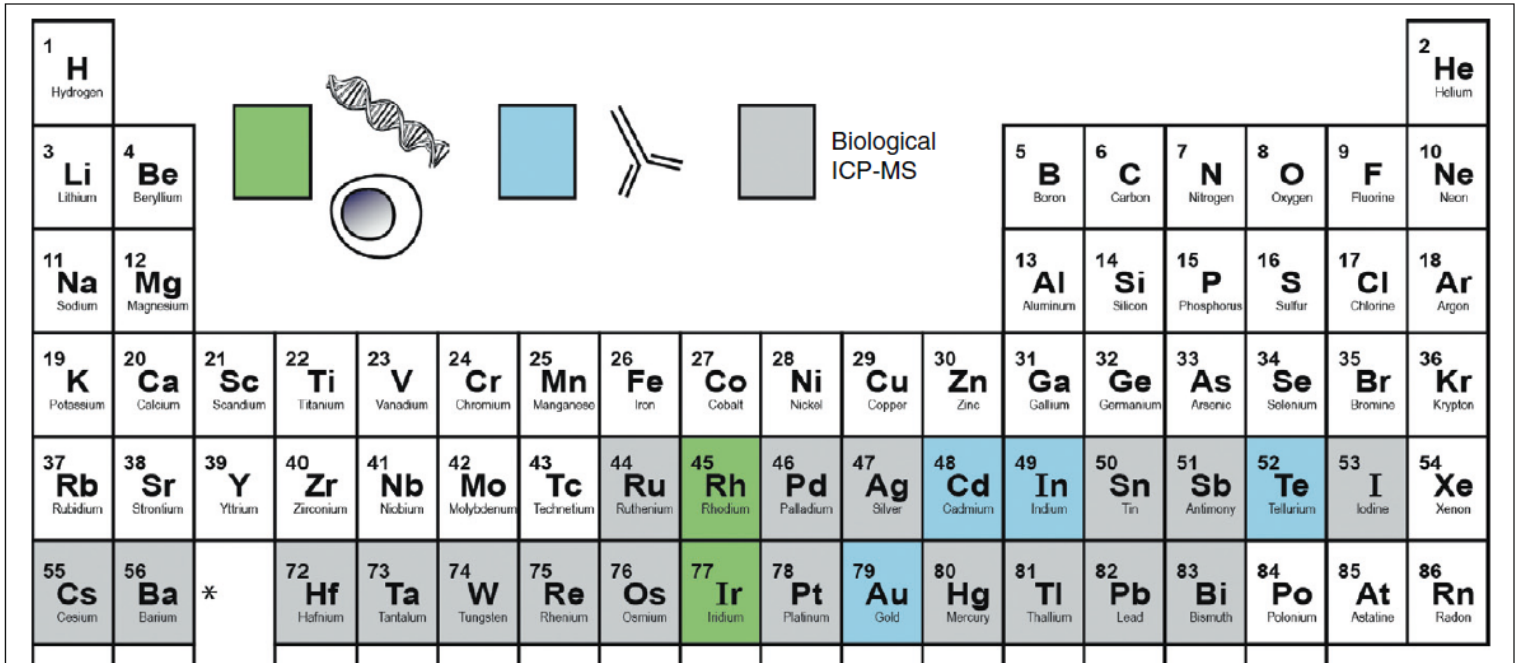
Polymer-ligand combination

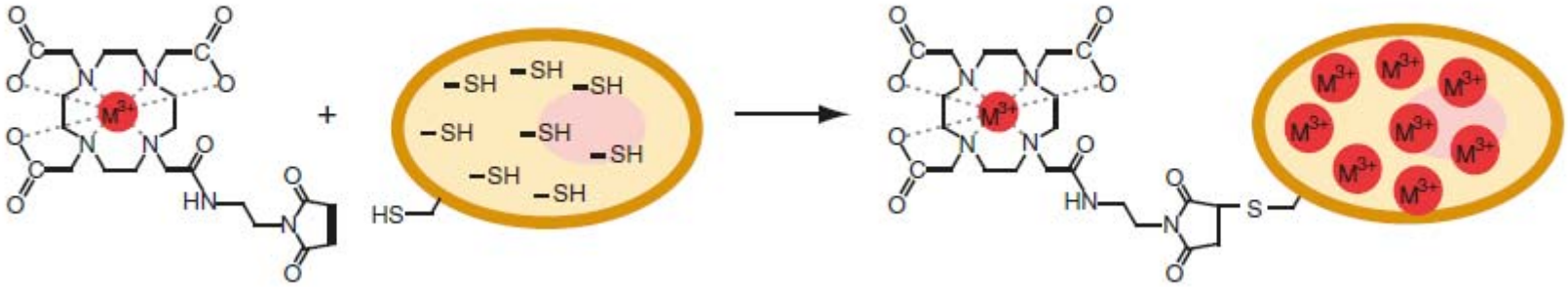


1. Metal chelating ligand show high affinity to Lanthanids
2. Ligand and polymer are combinde
3. Metal-chelating polymer with reactive –SH residues is added to the ab

Lou et al, 2007

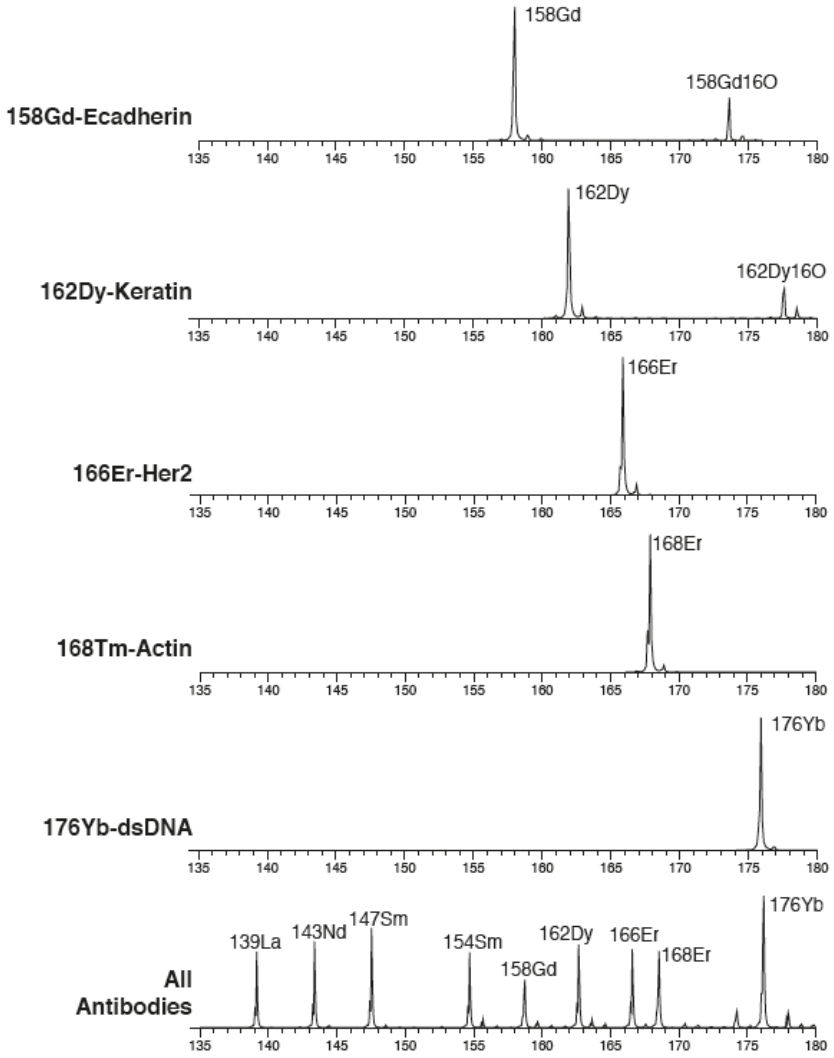
Isotopes for antibody tagging

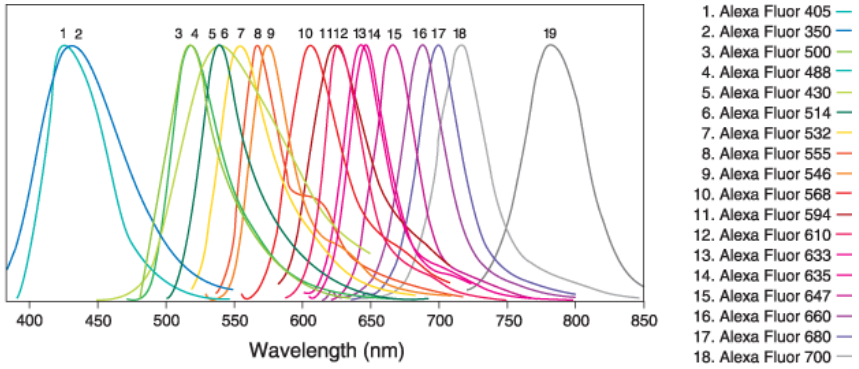




→ Combination of 7 different isotypes leads to 128 different ab-tags

CYTOF: rare isotopes diagrams

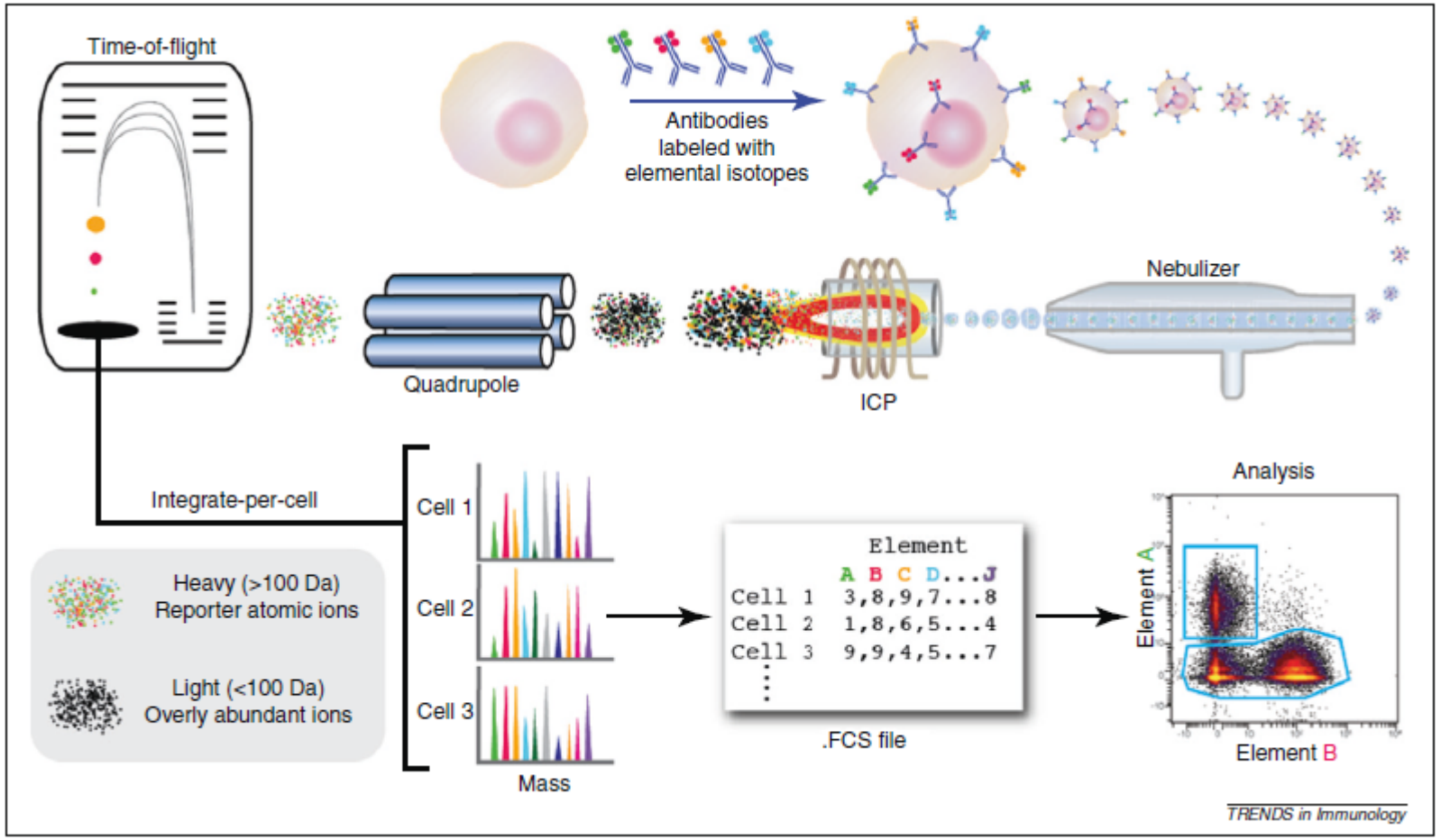




1. Alexa Fluor 405
2. Alexa Fluor 350
3. Alexa Fluor 500
4. Alexa Fluor 488
5. Alexa Fluor 430
6. Alexa Fluor 514
7. Alexa Fluor 532
8. Alexa Fluor 555
9. Alexa Fluor 546
10. Alexa Fluor 568
11. Alexa Fluor 594
12. Alexa Fluor 610
13. Alexa Fluor 633
14. Alexa Fluor 635
15. Alexa Fluor 647
16. Alexa Fluor 660
17. Alexa Fluor 680
18. Alexa Fluor 700
19. Alexa Fluor 750

→ isotope peaks are **non-overlapping !**

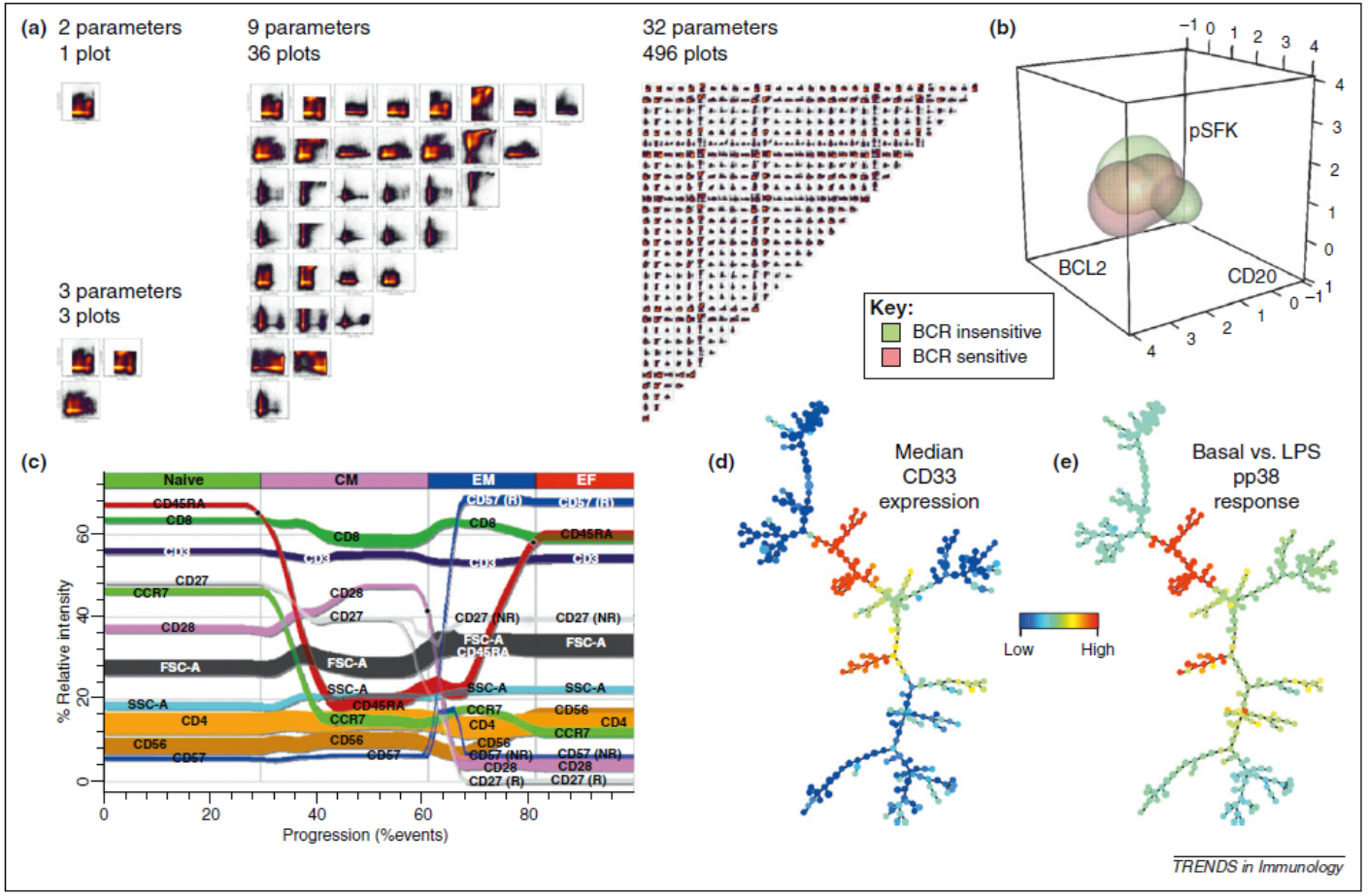
CYTOF: Work-flow



CYTOF: Read-out

a) «FACS plots»

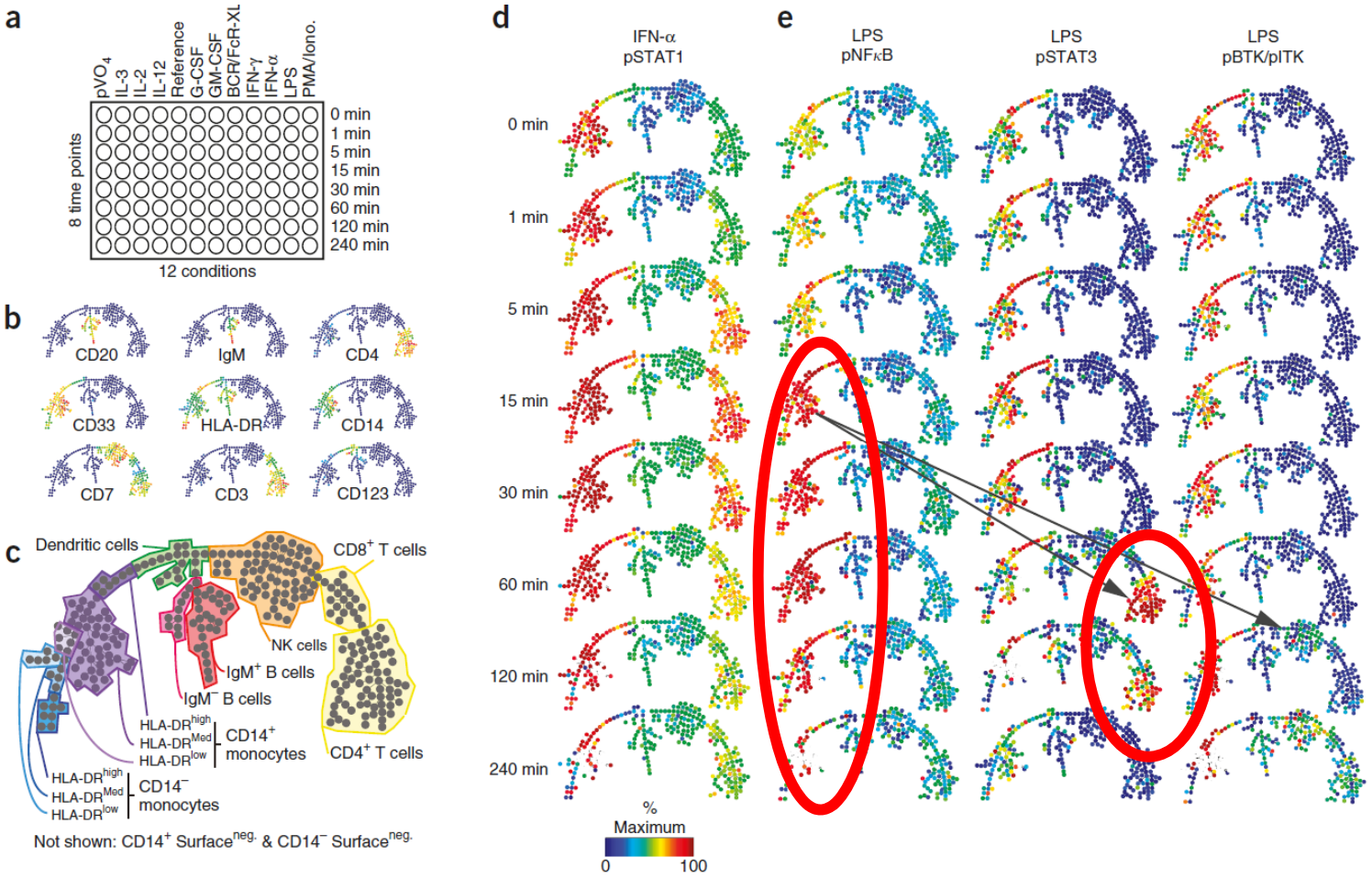
b) Clustering: «FLAME»



C) GEMSTONE Analysis

D) SPADE Tree

CYTOF: SPADE Trees



Stimulation of PBMCs with LPS activates e.g.:

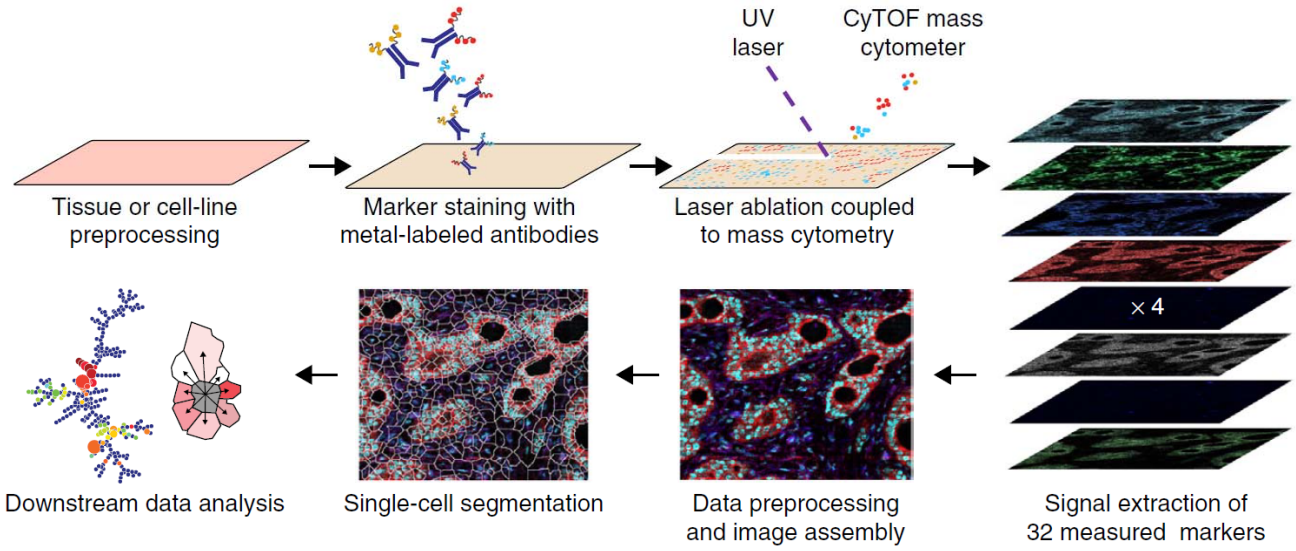
- pSTAT3 in CD4+ T cells after 60min
- NFKB acitvation of CD14» monocytes

Bodenmiller et al, 2012

Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry (Giessen et al, 2014)

Aim:

- Combine conventional IHC with Mass Spec (CyTOF) and high resolution laser ablation for multiplex imaging at subcellular resolution in human breast cancer samples



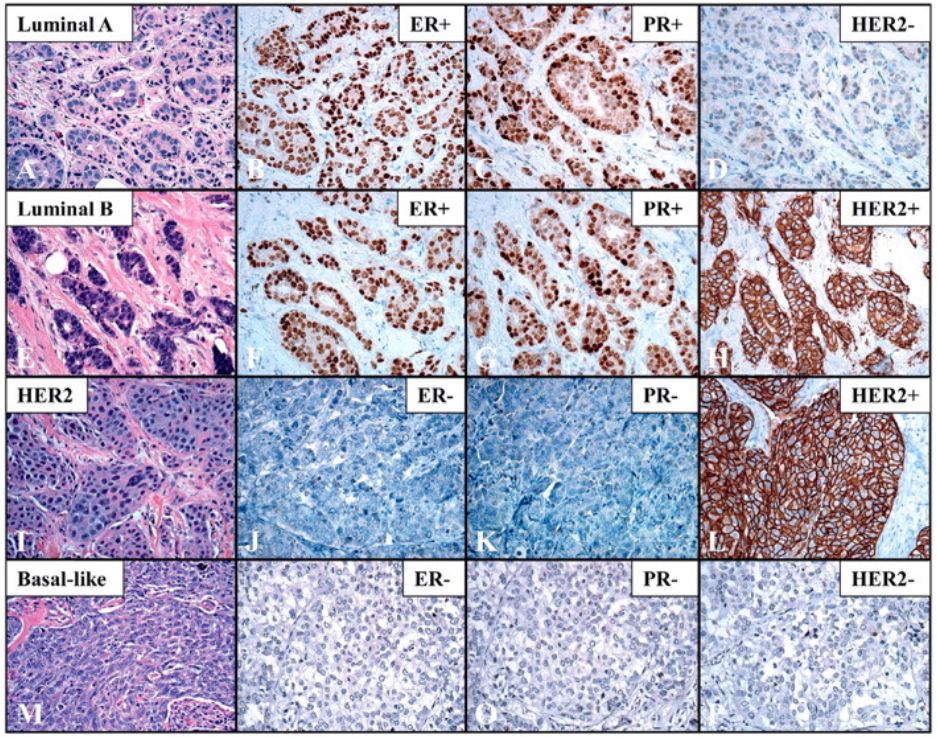
Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry (Giessen et al, 2014)

I. Tissue: Human Breast Cancer

- Human formalin-fixed, paraffin embedded (FFPE) tissue, (routine protocols)
- TMA with tumor and non tumor tissue
- Subtypes of breast cancer according to histopathology and rumor marker expression

Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry (Giessen et al, 2014)

I. Tissue: Human Breast Cancer Subtypes



Ductal carcinoma
invasive/metastatic - 55%

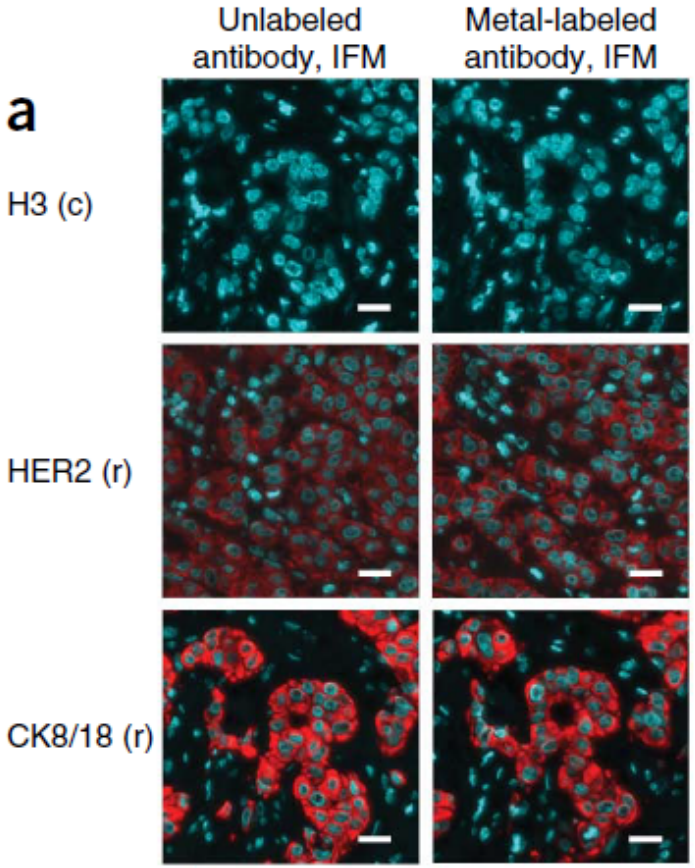
Ductal carcinoma
in situ/ non-invasive - 13%

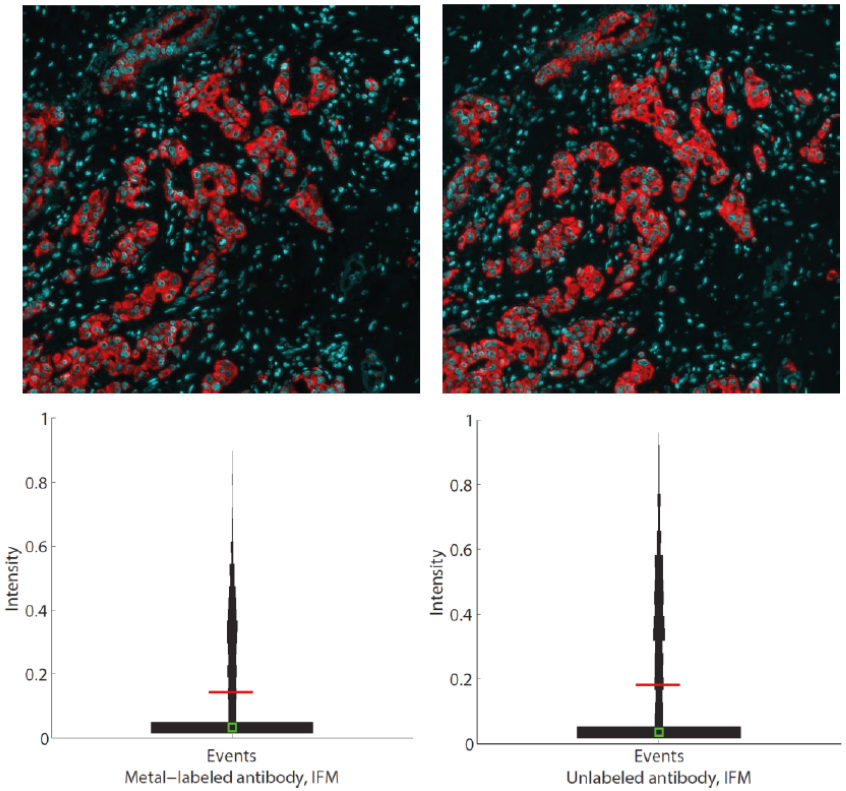
Invasive lobular carcinoma
(metastatic) - 5%

ER = estrogen receptor
PR = progesterone receptor
HER2 = human epidermal growth factor receptor 2

Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry (Giessen et al, 2014)

11. Antibody validation on serial sections by IF and intensity quantification



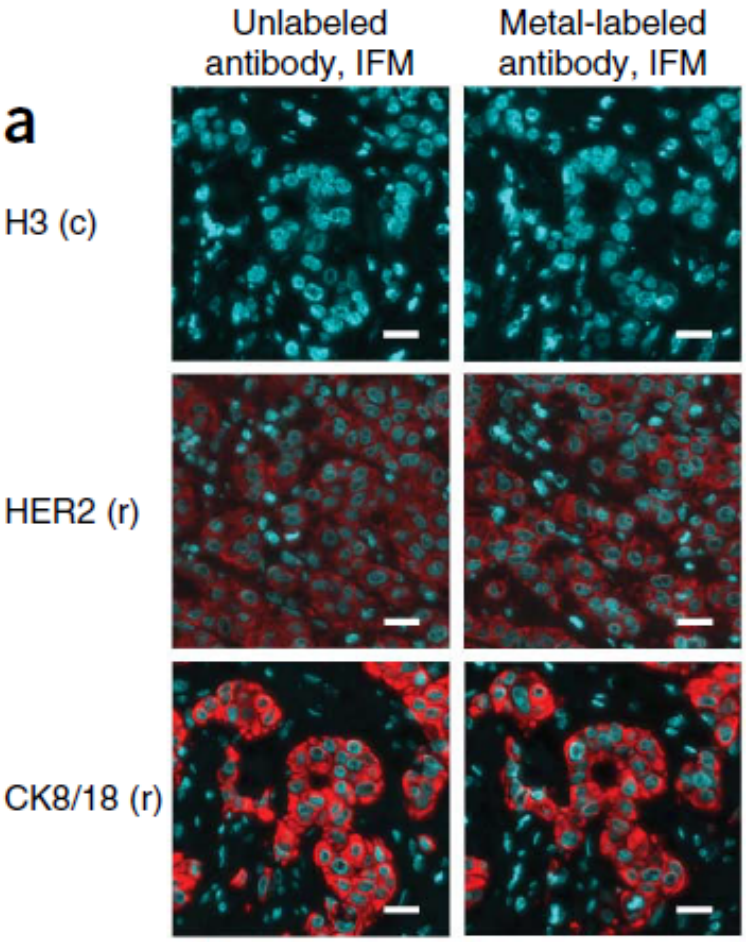


→ metal labeling of ab`s does not interfere with target specificity

→ metal labled ab`s show th same intensity (2-7% difference)

Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry (Giessen et al, 2014)

III. Validation of mass cytomtery imaging vs IF for **single stainings**

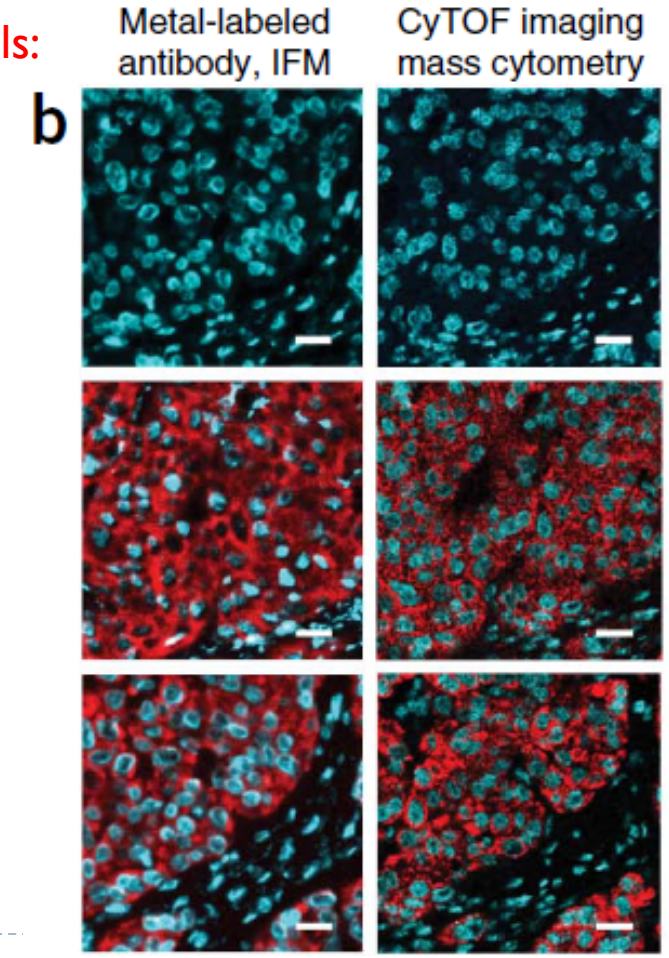


Positive tumor cells:

100% - 100%

75% - 79%

63% - 68%

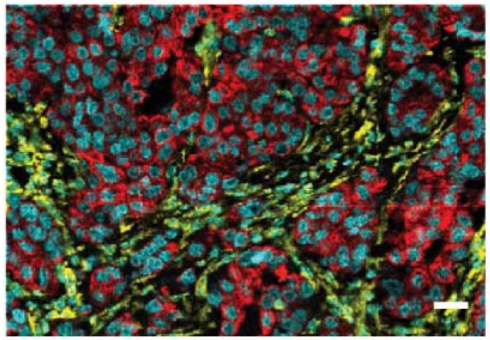


Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry (Giessen et al, 2014)

III. Validation of mass cytomtery imaging vs IF for **multiplex stainings**

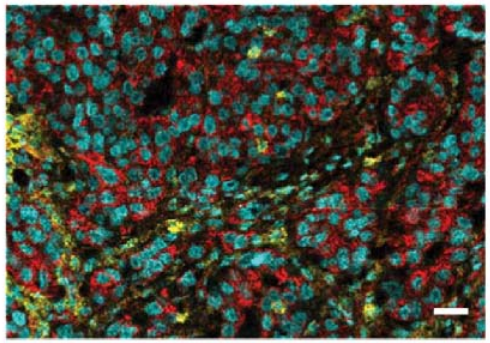
→ **One section stained for 32 different antibodies**

a



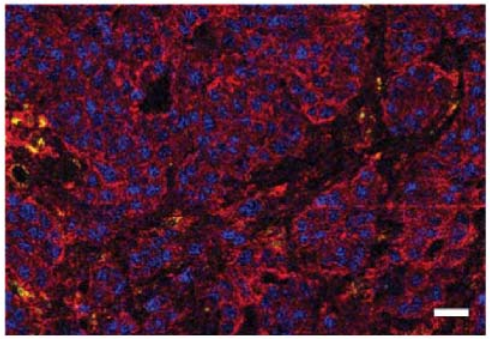
Mass Cytometry imaging:
Overlay of cytokeratin 8/18 (red), H3 (cyan) and vimentin (yellow).

b



Overlay of cytokeratin 7 (red), H3 (cyan) and CD44 (yellow).

c

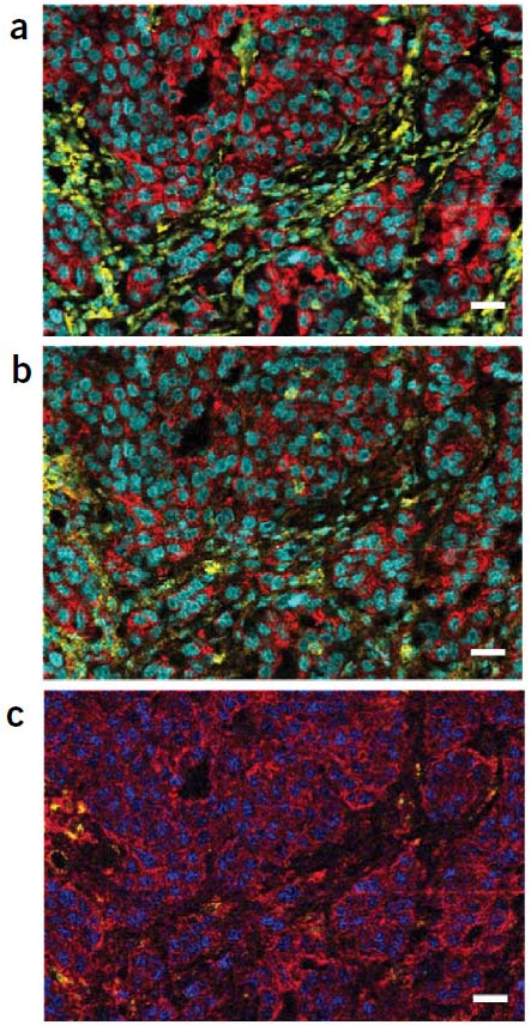


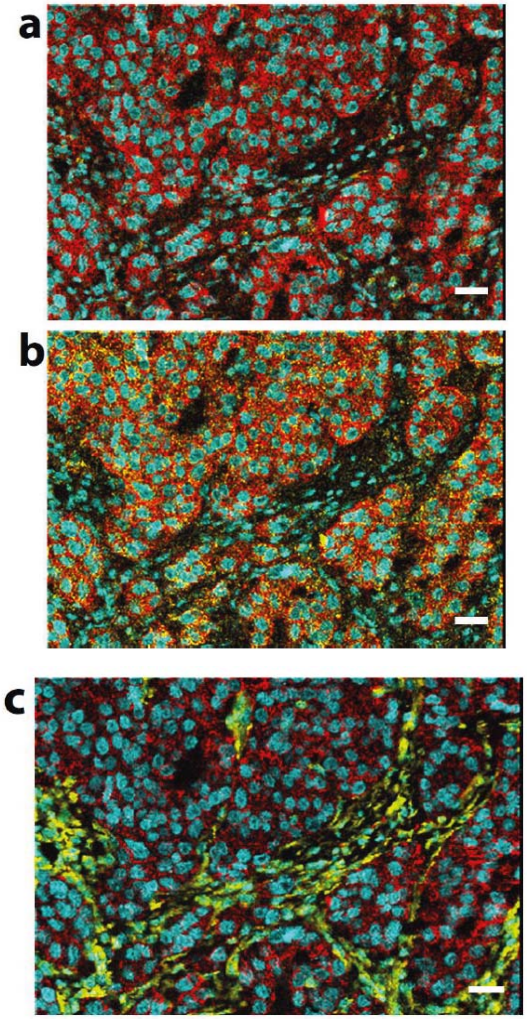
Overlay of pan-actin (red), progesterone receptor (blue), CD68 (yellow).

Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry (Giessen et al, 2014)

III. Validation of mass cytomtery imaging vs IF for **multiplex stainings** (FFPE)

→ **One section stained for 32 different antibodies**





Mass Cytometry imaging:

bcatenin (red)
H3 (cyan)
pS6 (yellow)

HER2 (red)
H3 (cyan)
CAH IX (yellow)

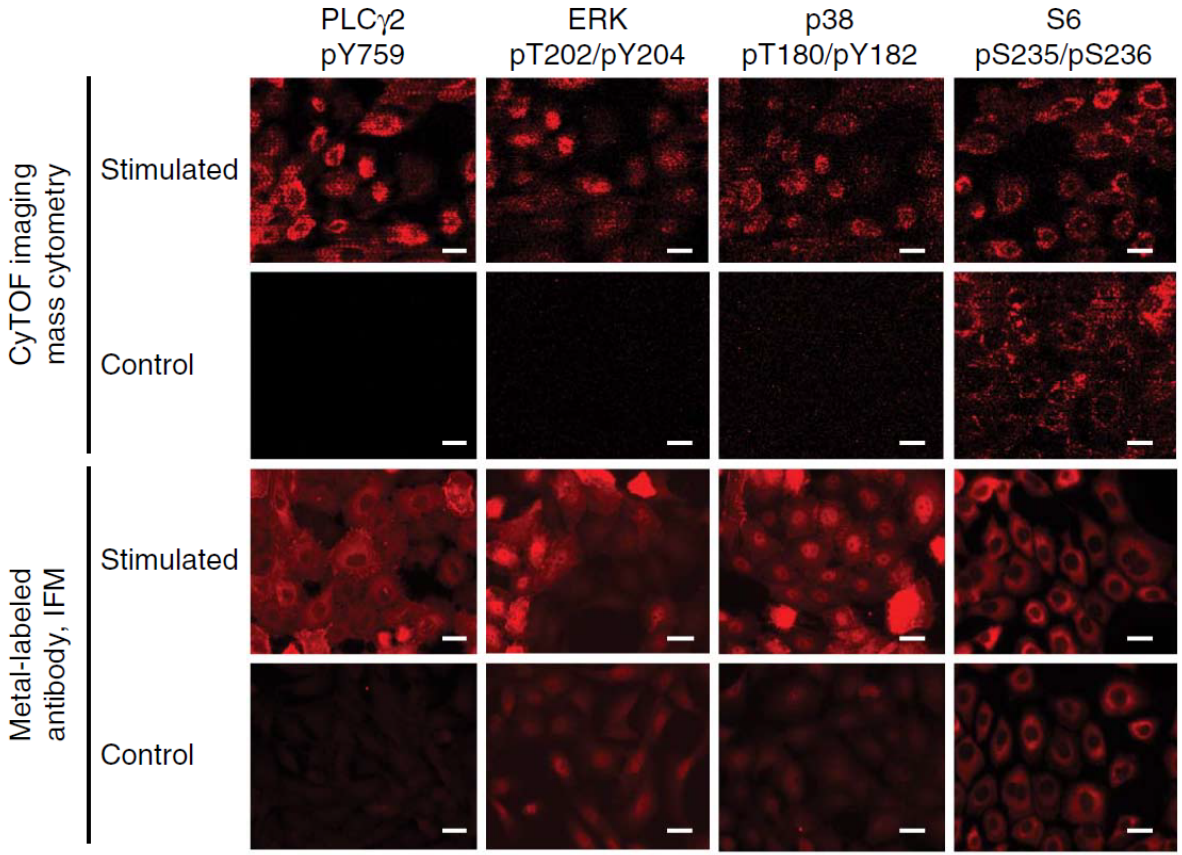
E-cadherin (red)
H3 (cyan)
vimentin (yellow).

Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry (Giessen et al, 2014)

III. Validation of mass cytomtery imaging for non FFPE tissue

→ Human breast **cancer cell line** grown cells were grown (~80% confluency)on glass coverslips

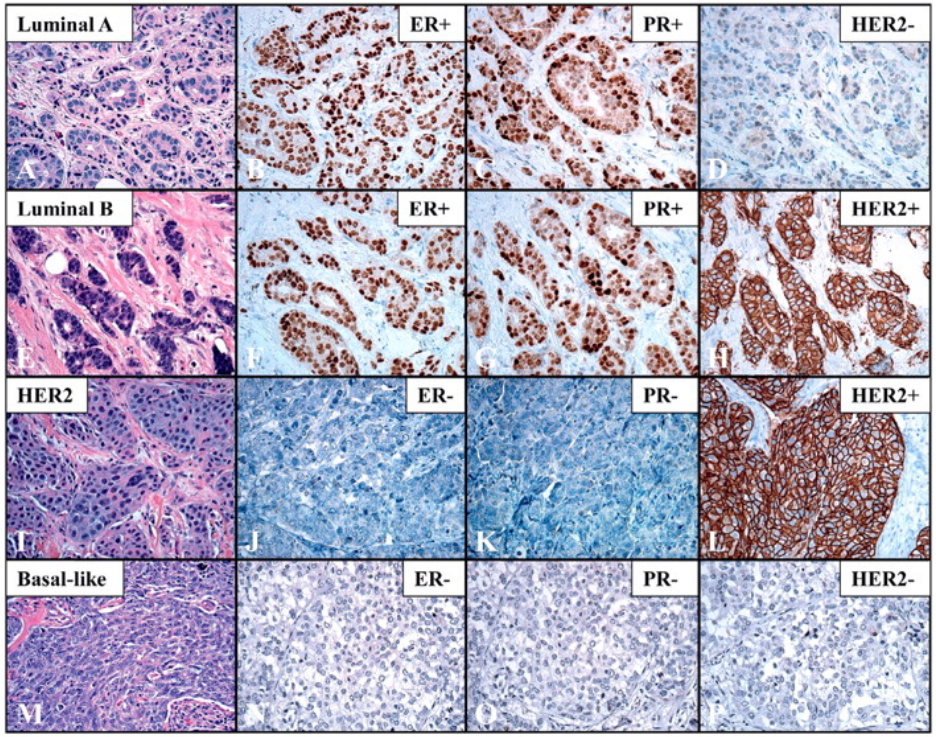
→ and treated with tyrosine phosphate inhibitor vandate for 30min



Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry (Giessen et al, 2014)

IV. Analysis of tumor heterogeneity

→ Her2, PR & ER expression define breast cancer subtypes



Ductal carcinoma
invasive/metastatic - 55%

Ductal carcinoma
in situ/ non-invasive - 13%

Invasive lobular carcinoma
(metastatic) - 5%

→ Analysis of 21 FFPE samples (TMA) by 32-plex imaging

Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry (Giessen et al, 2014)

IV. Analysis of tumor heterogeneity

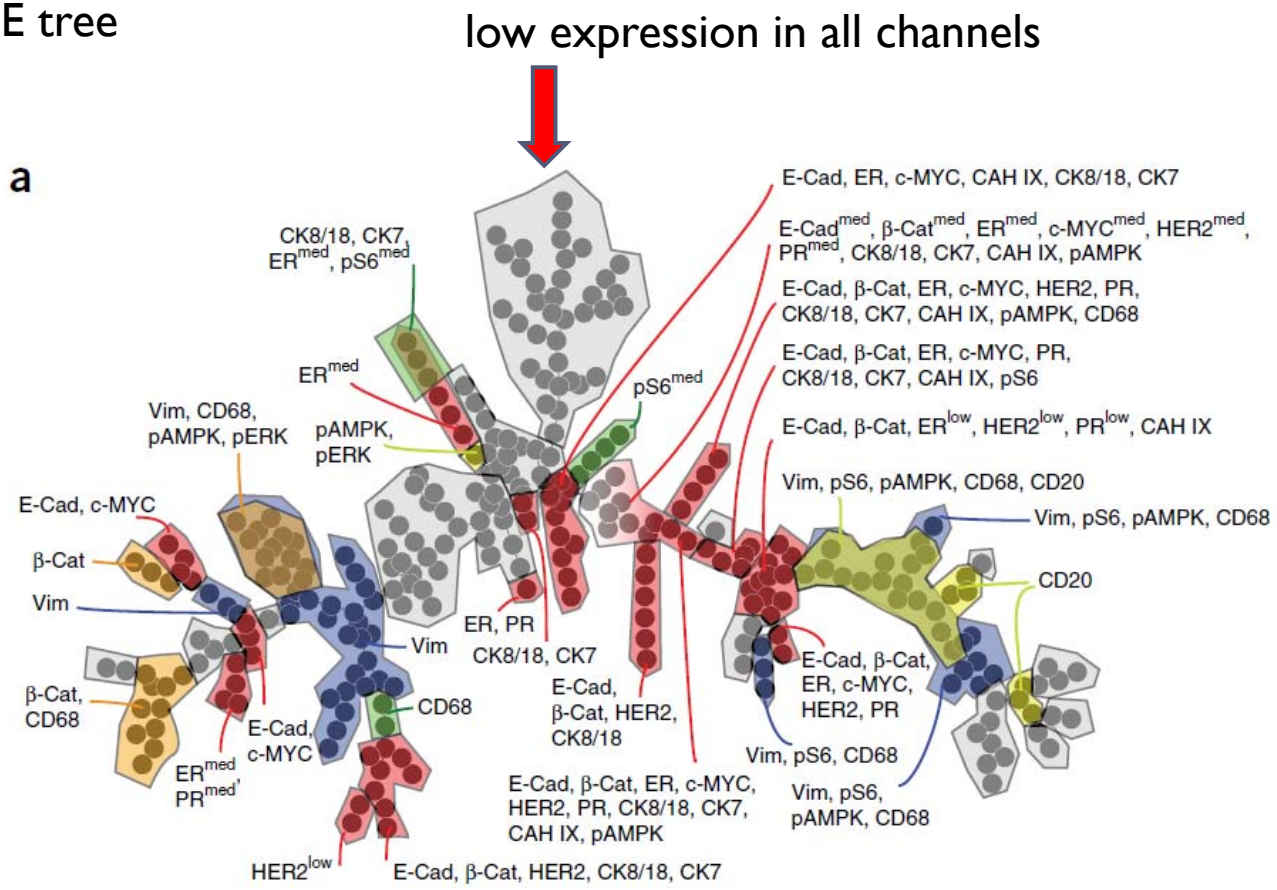
→ Analysis of 21 FFPE samples (TMA) by 32-plex imaging (previously defined by pathologists)

HER2 FISH	Mol. Signature (IHC)	Tissue type	ID	Resolution	Tumor subtype	pT	pN	M	Grade	Multifocal_centric
Normal	Luminal (HER2 neg)	Primary tumor	79	1 µm	Invasive ductal	pT2	pN0 (sn)	0	G3	Unifocal
Normal	Luminal (HER2 neg)	Primary tumor	95	1 µm	Invasive ductal	pT2	pN1	0	G3	Multicentric/focal
Amplification	HER2 (non luminal)	Primary tumor	96*	1 µm	Invasive ductal	pT3	pN1	0	G3	Unifocal
Normal	Triple negativ	Primary tumor	162	1 µm	Invasive ductal	pT2	pN1	0	G3	Multicentric/focal
Amplification	HER2 (non luminal)	Primary tumor	199*	1 µm	Invasive ductal	pT2	pN1	1	G2	Unifocal
Normal	Triple negativ	Primary tumor	201	1 µm	Invasive ductal	pT1c		0	G2	Unifocal
Normal	Triple negativ	Primary tumor	201*	1 µm	Invasive ductal	pT1c		0	G2	Unifocal
Amplification	Luminal (HER2 pos)	Primary tumor	210	1 µm	Invasive ductal	pT1c	pN0	0	G2	Unifocal
Normal	Luminal (HER2 neg)	Primary tumor	254*	1 µm	Invasive cribriform	pT1c	pN0	0	G1	Unifocal
Amplification	Luminal (HER2 pos)	Primary tumor	257	1 µm	Invasive ductal	pT2	pN3	0	G3	Unifocal
Normal	Luminal (HER2 neg)	Tumor recurrence	260	2 µm	Invasive ductal	pT1c				
Normal	Luminal (HER2 neg)	Primary tumor	261	2 µm	Invasive ductal	pT1c	pN0 (sn)	0	G2	Unifocal
Amplification	HER2 (non luminal)	Primary tumor	273	1 µm	Invasive ductal	pT4	pN1	0	G3	Unifocal
Amplification	Luminal (HER2 pos)	Primary tumor	276	1 µm	Invasive ductal	pT2	pN3	0	G3	Unifocal
Normal	Luminal (HER2 neg)	Primary tumor	283	1 µm	Invasive ductal	pT1c	pN0	0	G2	Multicentric/focal
Normal	Luminal (HER2 neg)	Lymph node metastasis	290	2 µm	Invasive ductal					
Amplification	HER2 (non luminal)	Primary tumor	294*	1 µm	Invasive ductal	pT1c	pN0 (sn)	0	G3	Unifocal
Normal	Triple negativ	Primary tumor	304	1 µm	Invasive ductal	pT2	pN1	0	G3	Unifocal
Normal	Luminal (HER2 neg)	Tumor recurrence	321	1 µm	Invasive lobular					
Normal	Normal breast	Normal breast tissue	343*	1 µm						
Normal	Normal breast	Normal breast tissue	359*	1 µm						

Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry (Giessen et al, 2014)

IV. Analysis of tumor heterogeneity

→ SPADE tree



Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry (Giessen et al, 2014)

IV. Analysis of tumor heterogeneity

→ SPADE tree)

low expression in all channels

Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry (Giessen et al, 2014)

Summary & Conclusion:

- ➢ Validation of imaging mass cytometry
- ✓ Metal labeling of ab`s did not influence antigen binding
- ✓ Multiplex imaging mass cytometry reproduced IF staining pattern
- ✓ No background autofluorescence
- ✓ High resolution 1um
- ✓ Samples preparation is identical to conventional IHC
- ✓ No amplification step needed

- ✓ SPADE analysis confirmed intra- and interpatient heterogeneity

Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry (Giessen et al, 2014)

Disadvantages:

- Technique depends on antigen-antibody interaction
- antibodies against protein of interest are needed
- Samples preparation is not normalized

- Time consuming protocol (outlook: 100marker in 1h)
- Complex analysis for daily practice in diagnostics
- Accumulation of data, need/usage?
- Imaging represents only a snapshot of tumor development

Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry (Giessen et al, 2014)

Outlook:

- Analysis of 100 markers in 1h
- Combination of multiple monoclonal ab`s against the same protein (different epitopes)
- Housekeeping-protein for normalization and tissue quality index

- In situ detection of RNA oder DNA ?