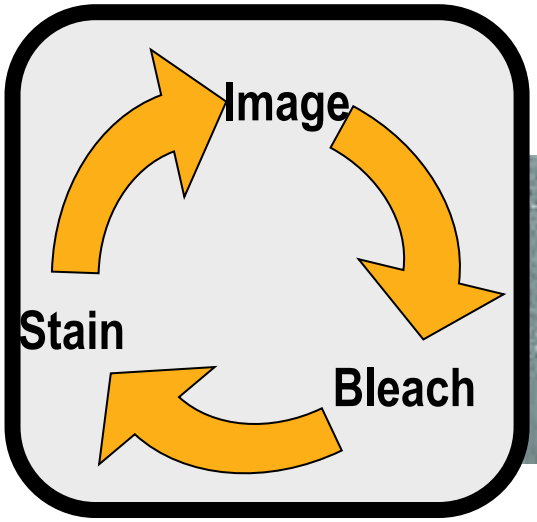

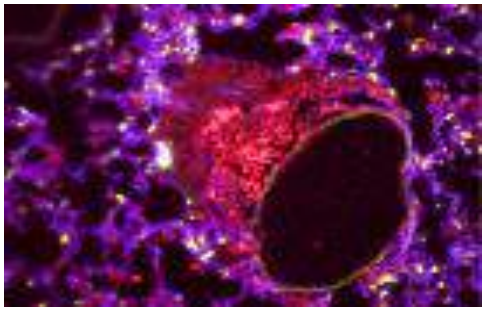

ChipCytometry identifies presence of uncommon B cell subset in inflamed tonsils associated to autoimmunity

Cyto2015 Glasgow 30.06.2015

Karl Welzenbach, PhD, ATI NIBR Basel, Collaboration with

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ZELLKRAFTWERK

Need for game-changing immunology therapeutics

Inappropriate immune responses in diverse diseases

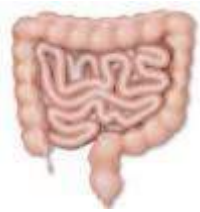
excessive *insufficient*

autoimmunity	autoinflammatory	alloimmunity	immune deficiency
misdirected; host's own cells	exaggerated; various sources	directed; beneficial / malignant tissues	insufficient protection; pathogen
Multiple Sclerosis, Rheumatoid Arthritis	Systemic JIA, Periodic Fever Syndromes	Graft rejection, Graft vs Host disease	Myeloablative chemotherapy, Immunodeficiency

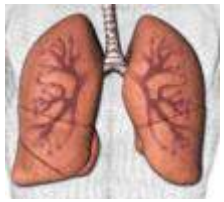
Immune mechanisms can mediate disease in every organ system











MULTIPLE ORGANS
Sys. Lupus Erythematosus
Sarcoidosis
Goodpasture's syndrome
...

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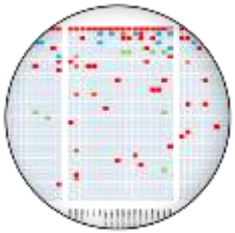
Resolving disease heterogeneity
enable delivery of optimal drug to stratified patient

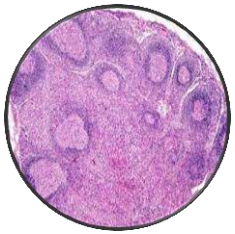
Pathways & Processes

Indications
- Alloimmunity
- Autoimmunity
- Autoinflammatory diseases

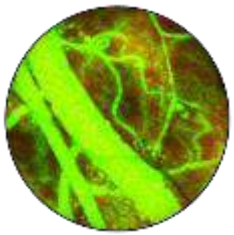
Human Research Studies
Targeted and unbiased analyses of
healthy and diseased tissue



X'omics



Staining



Imaging/Flow

Disease Pathophenotype

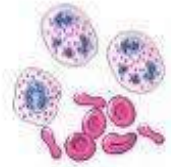
Therapeutic Approach

Deep Phenotyping of precious patient samples

NOVARTIS

Zellkraftwerk`s ChipCytometry: image-based multiplex-phenotyping of immobilized cells

Loading Sample on CellSafe Chip

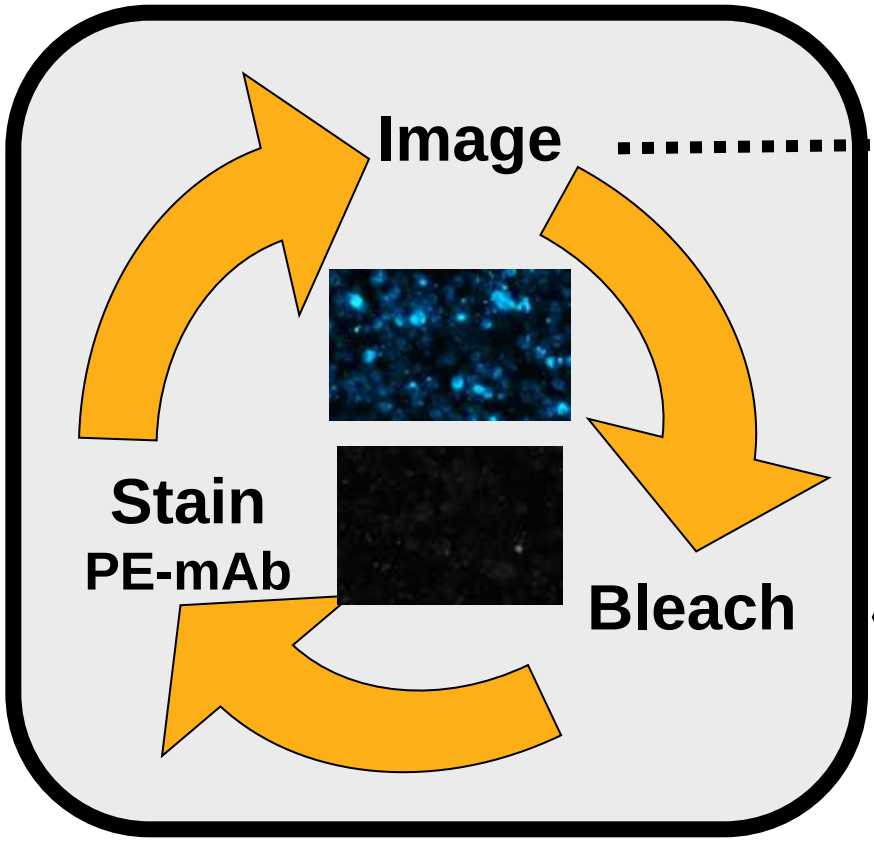


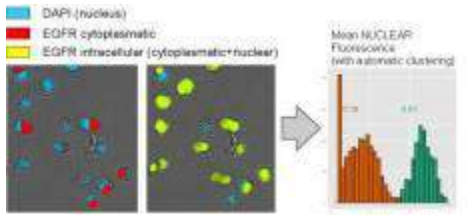
1

2

Loading Chip on ZellScanner

ZellScanner Instrument





Data processing

3

Storage for 20+ months

ZELLKRAFTWERK

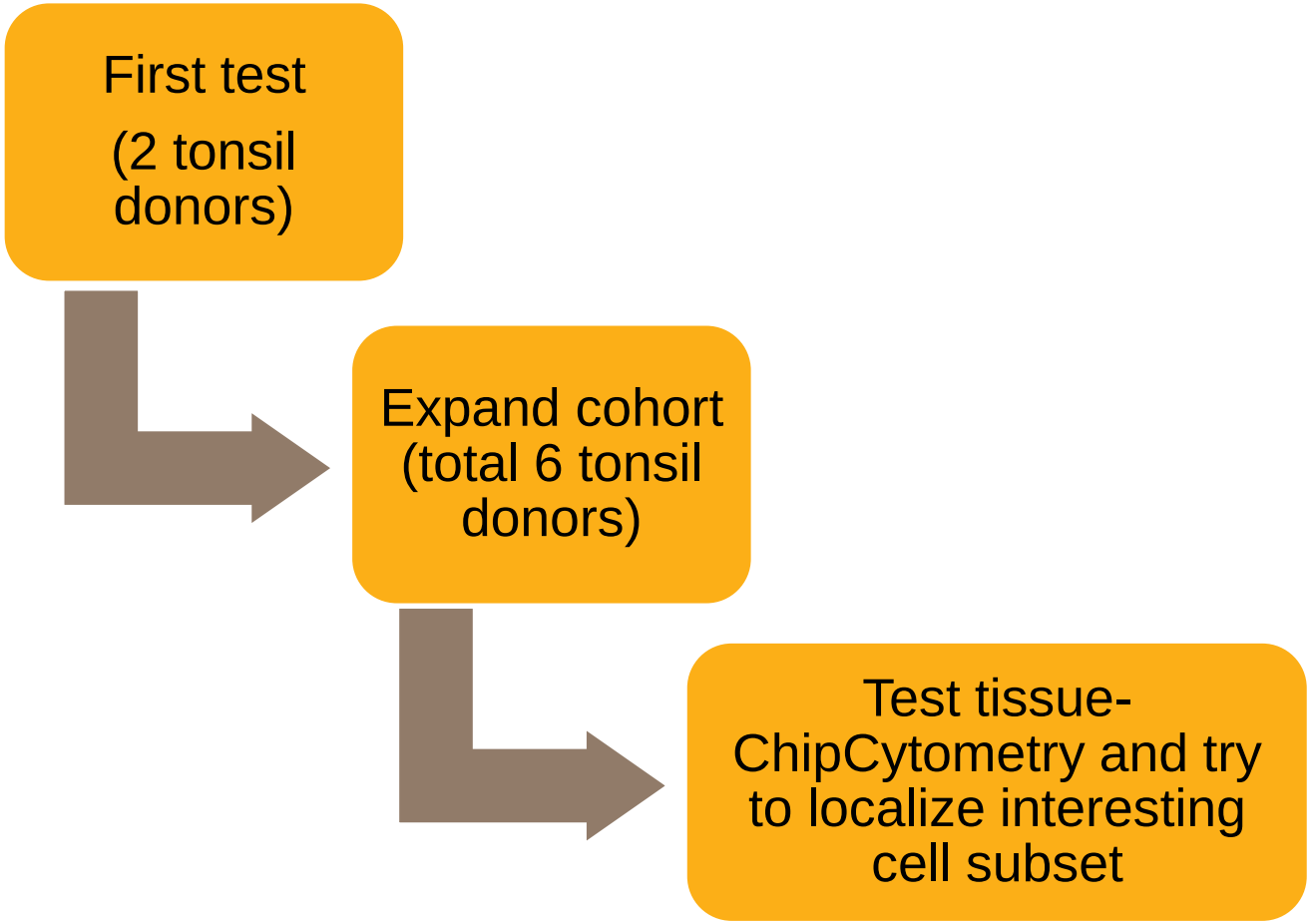
ChipCytometry addresses current limitations of classical flow cytometry

- **„EVERYCELL“-Cytometry**
  - Every cell is analyzed, <1% dropout.
  - Useful for **low-cell count samples** (**CSF, BAL, urine, synovial fluid**).
  - Go back to event and visualize
- **„BIOREPOSITORY“**
  - long-term biomarker stabilization on cells and tissues (>2 a) for sample storage, shipping, analysis and re-analysis in clinical studies.
- **„TISSUE-Cytometry“**
  - enables direct cellular biomarker quantitation in-situ without tissue disruption.

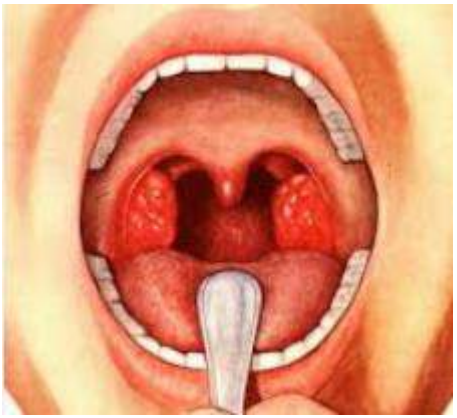
NOVARTIS

Our feasibility study on ChipCytometry

- Is ChipCytometry suitable for deep phenotyping of tonsil cells
- First glance on tissue cytometry

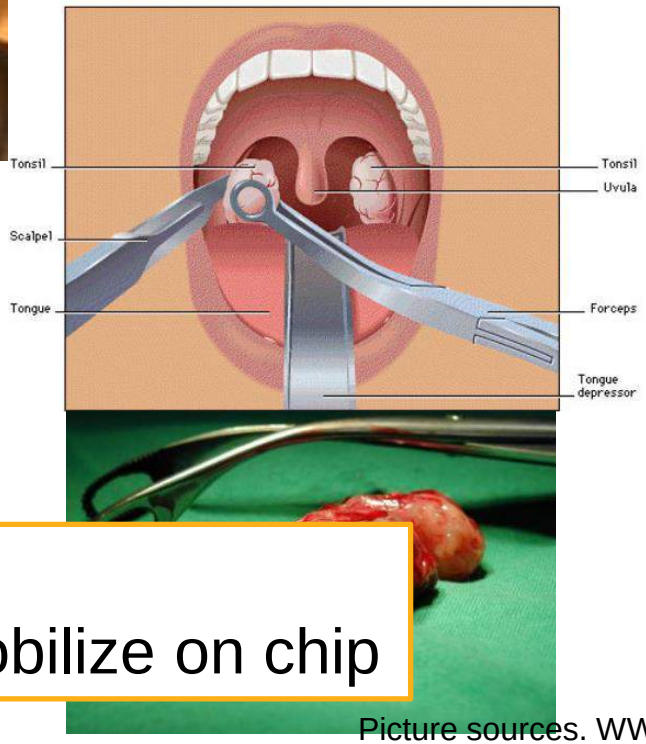

First test
(2 tonsil donors)
Expand cohort (total 6 tonsil donors)
Test tissue-ChipCytometry and try to localize interesting cell subset

Tonsils, good source for primary lymph node tissue



Inflamed palatine tonsils

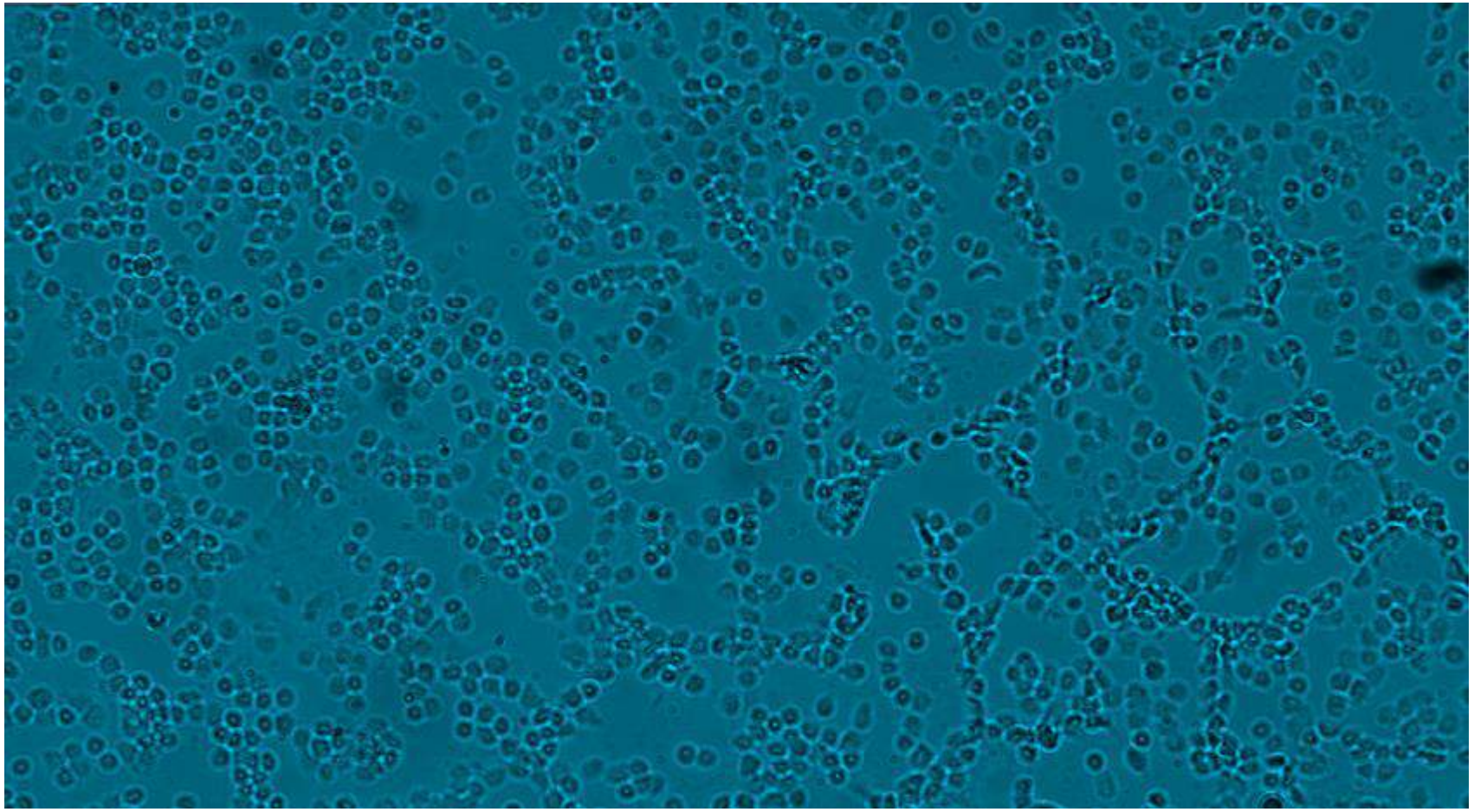
www.quickmeme.com



Make cell suspension
or cryosection and immobilize on chip

Picture sources. WWW.

Tonsillar leukocytes immobilized on a CellSafe chip

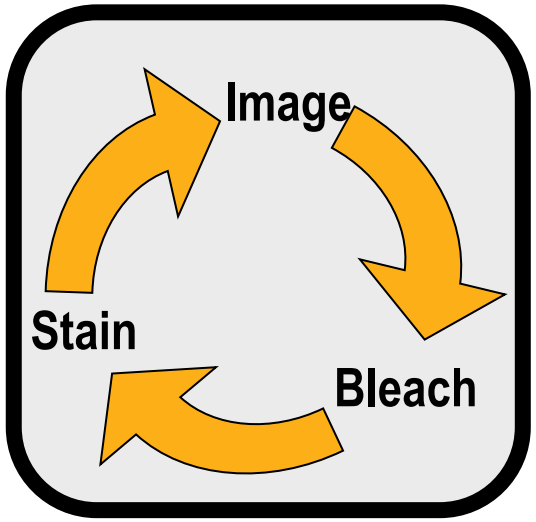


No significant loss of immobilized suspension cells after 80 washing cycles

Markers assessed

PE-labeled antibodies

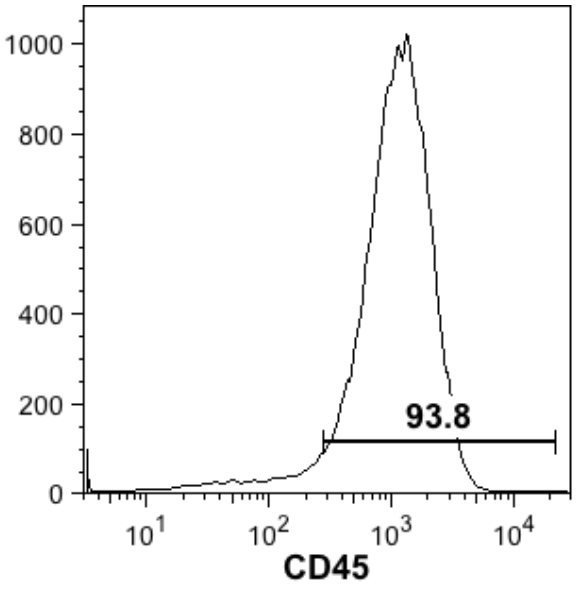
CD3	CD45
CD4	CD45RA
CD5	CD45R0
CD8	CD56
CD10	CD86
CD11b	HLA-DR
CD11c	IgD
CD14	T-bet
CD16	FoxP3
CD19	ROR gt
CD20	Helios
CD24	IL1b
CD25	IL8
CD27	IL10
CD31	IL12
CD38	IL17A



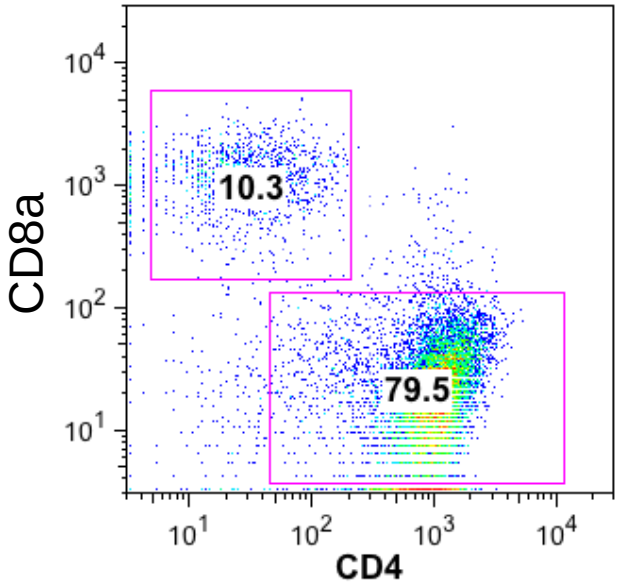
Done @

ZELLKRAFTWERK

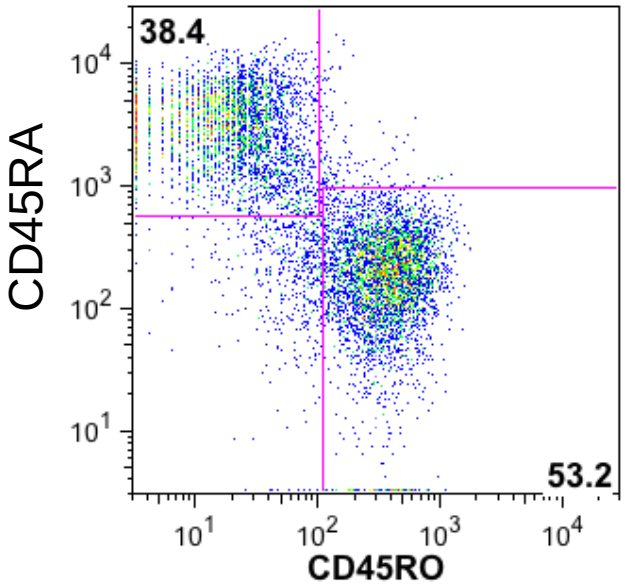
Conversion of fluorescent intensity of image into .FCS format



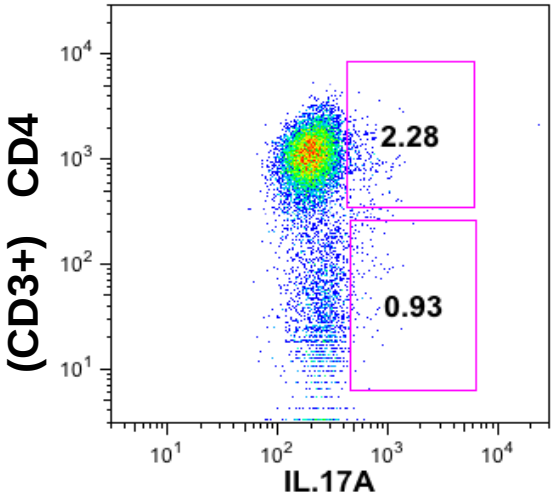
$CD45^+CD3^+$

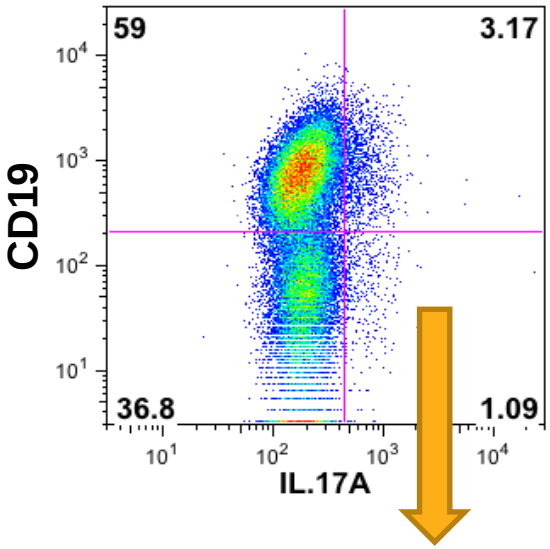


$CD45^+CD3^+CD4^+$



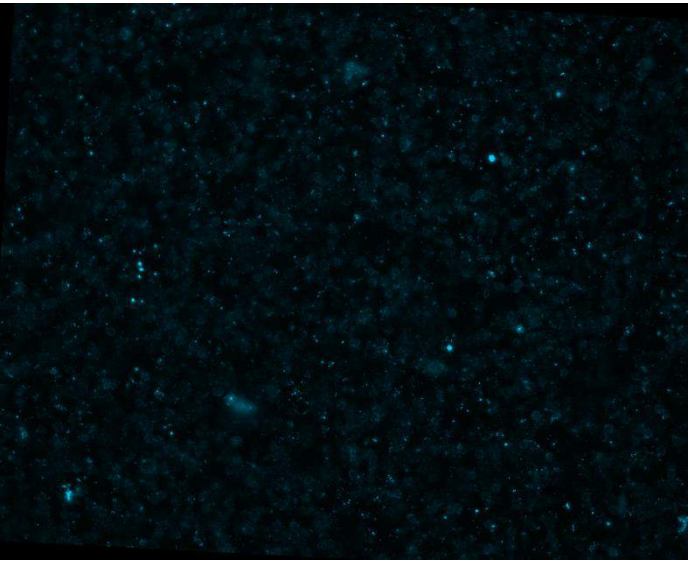
Active IL17A producers in inflamed tonsils



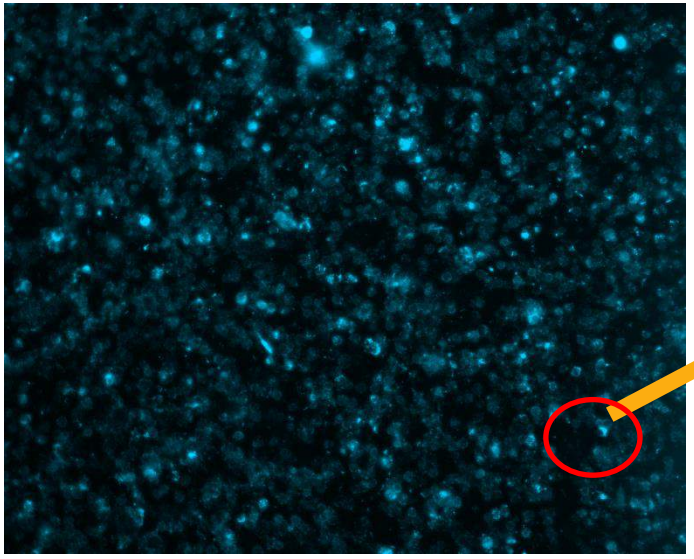


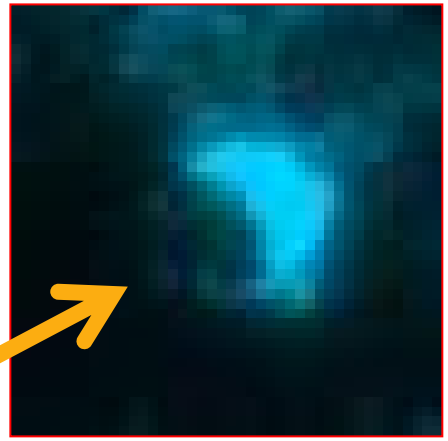
- Go back to picture and look at spacial information of individual cell

Isotype IgG1

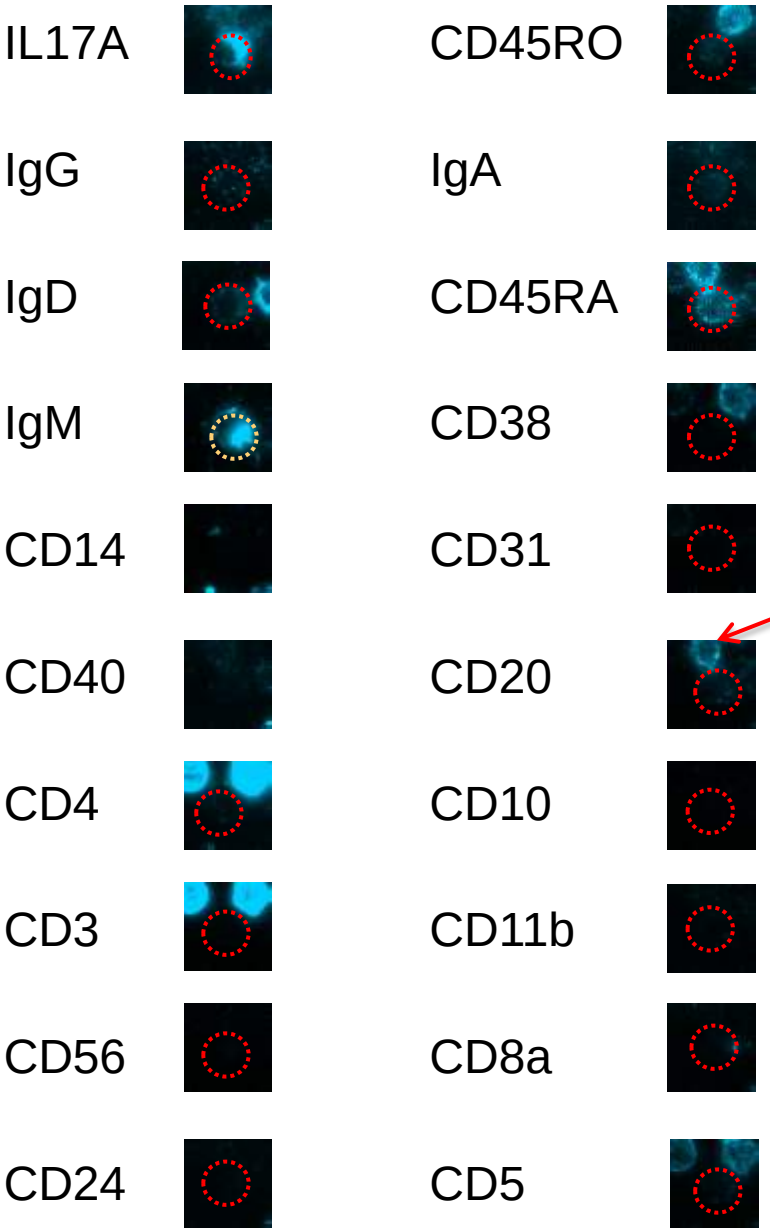


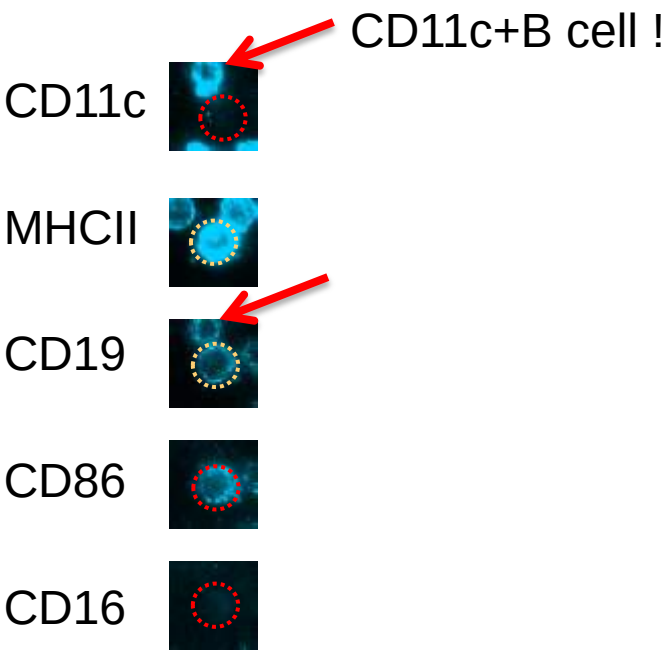
IL17A.intracellular





.... And its immuno-phenotype at single cell level

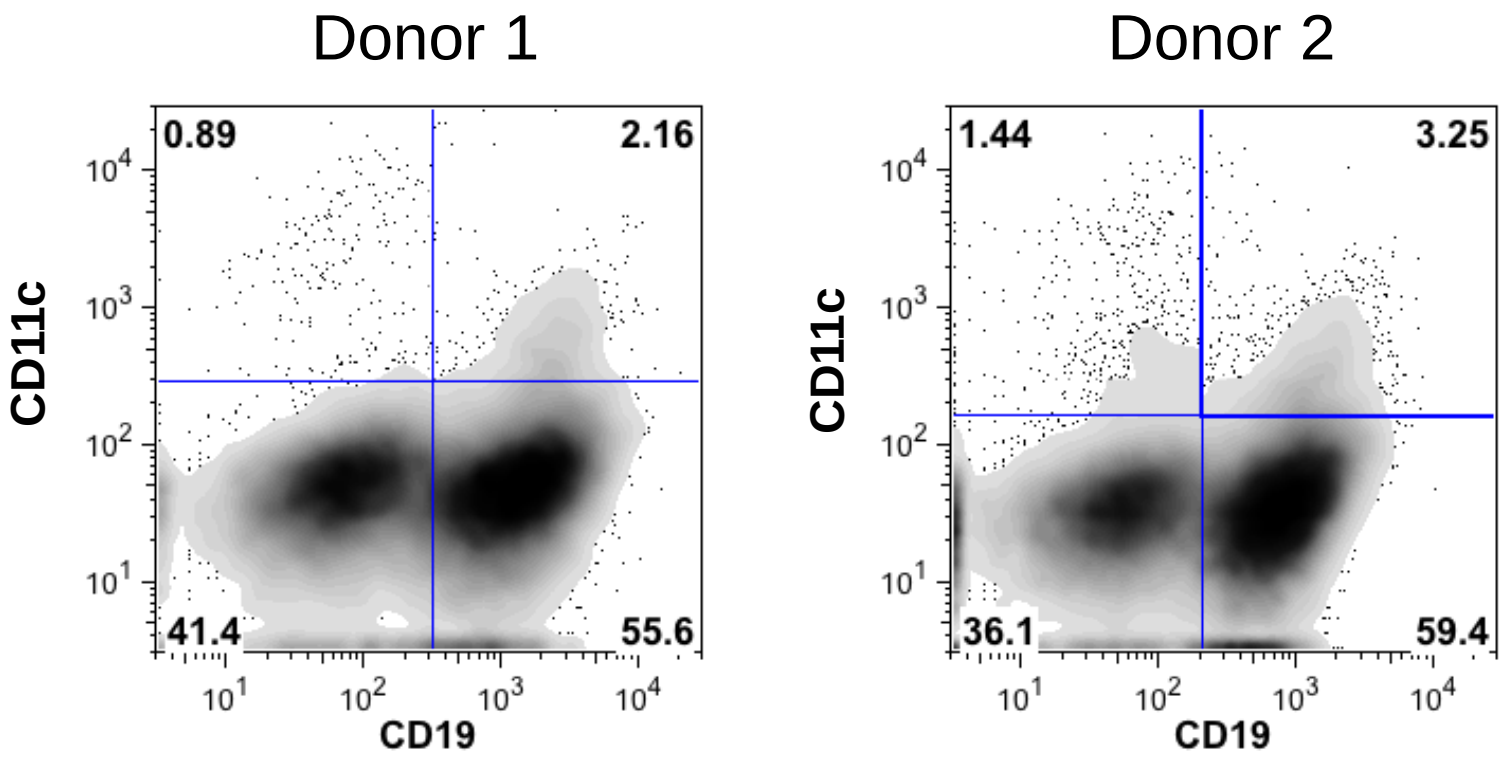




Negative:$CD11c^{-}CD16^{-}CD11b^{-}CD10^{-}$
$CD56^{-}CD24^{-}CD14^{-}CD3^{-}CD4^{-}CD38^{-}$
Positive: $CD19^{+}$ $CD20^{dim}$,$CD86^{+}CD45RA^{+}$
$MHCII^{+}CD86^{+}IgM^{+}IgA^{-}IgD^{-}IgG^{-}$

....IL17A producing B-cell

ChipCytometry identifies presence of uncommon $CD19^{+}CD11c^{+}$ B cells in inflamed tonsils



linked to autoimmunity.....

Rubtsov et al Blood 2011

CD11c^{+}B cells (ABC) in mice with SLE-like symptoms

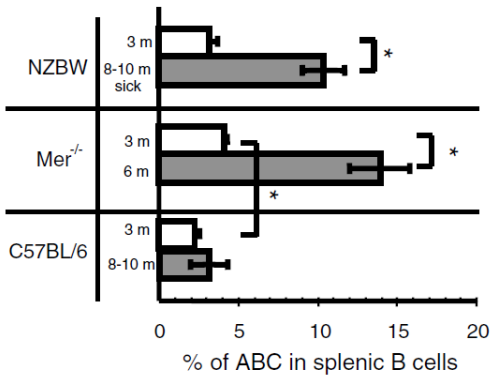
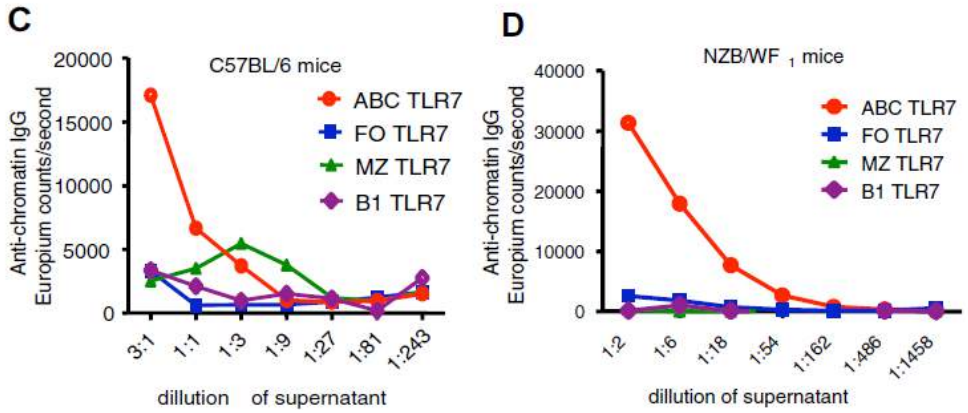


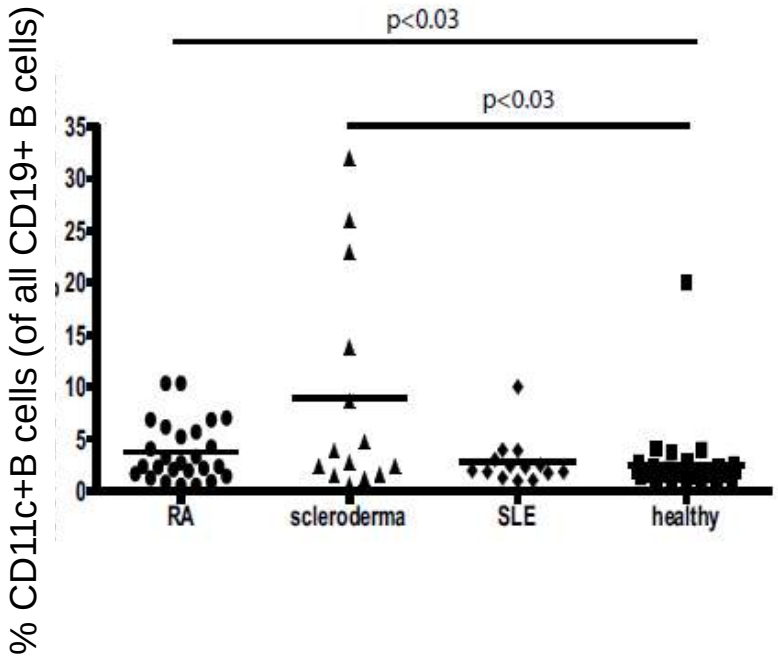
Figure 2. Increased number of ABCs in autoimmune prone mice at the time of onset of autoimmunity. The percentage of ABCs in splenic B cell populations was determined by flow cytometry in female mice of indicated strain and age. Bars represent mean (± SEM) of at least 5 mice per group. *$P < .01$ (Student 2-tailed t test).

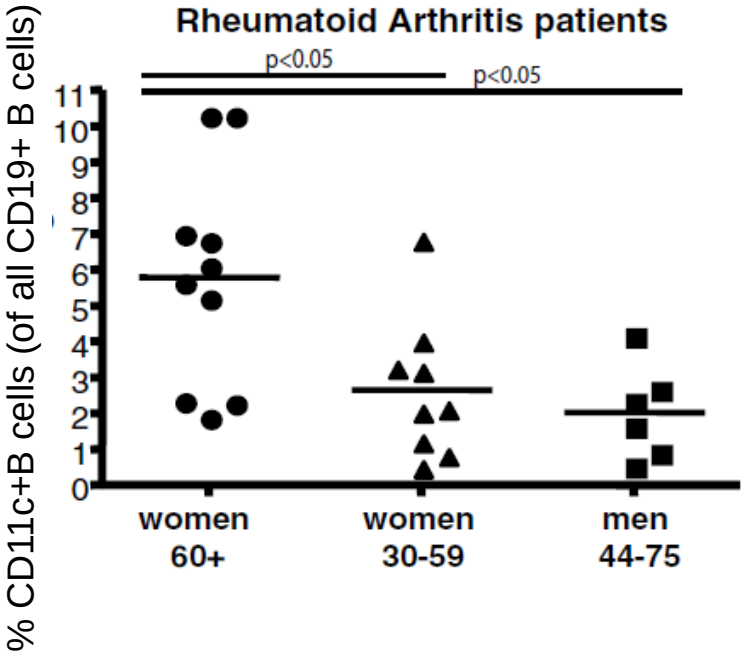
CD11c^{+} B cells (ABC) are TLR7-dependent and produce autoantibodies

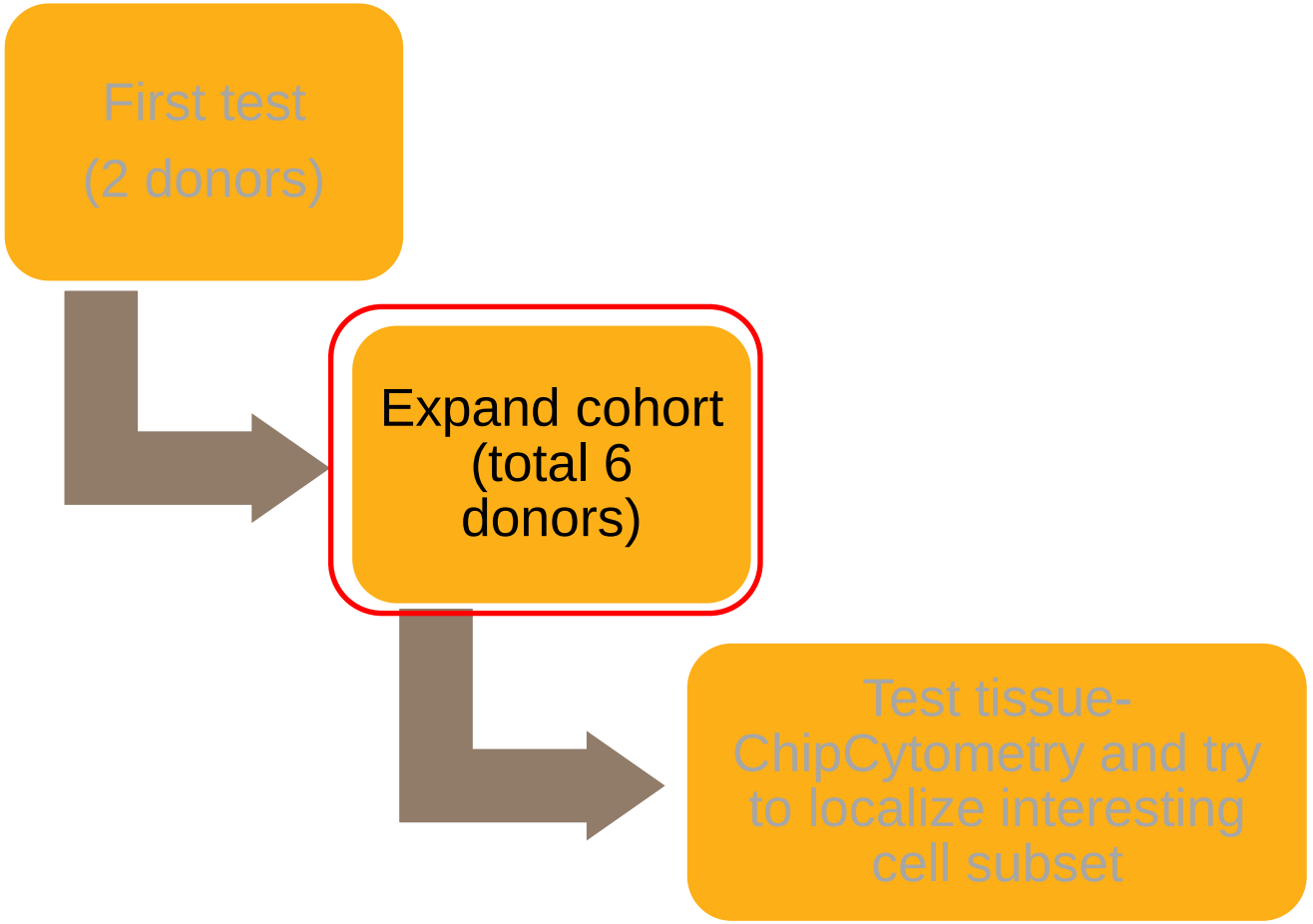


Increased number of CD11c^{+} B cells in circulation of aged women with autoimmune disorders

Rubtsov et al 2011






First test
(2 donors)
Expand cohort
(total 6
donors)
Test tissue-
ChipCytometry and try
to localize interesting
cell subset

2nd Phase: feasibility study with Zellkraftwerk`s ChipCytometry technology

- Increase cohort size, optimize staining conditions
- **Focused scientific question on the existence of CD11c+B cells in tonsils**

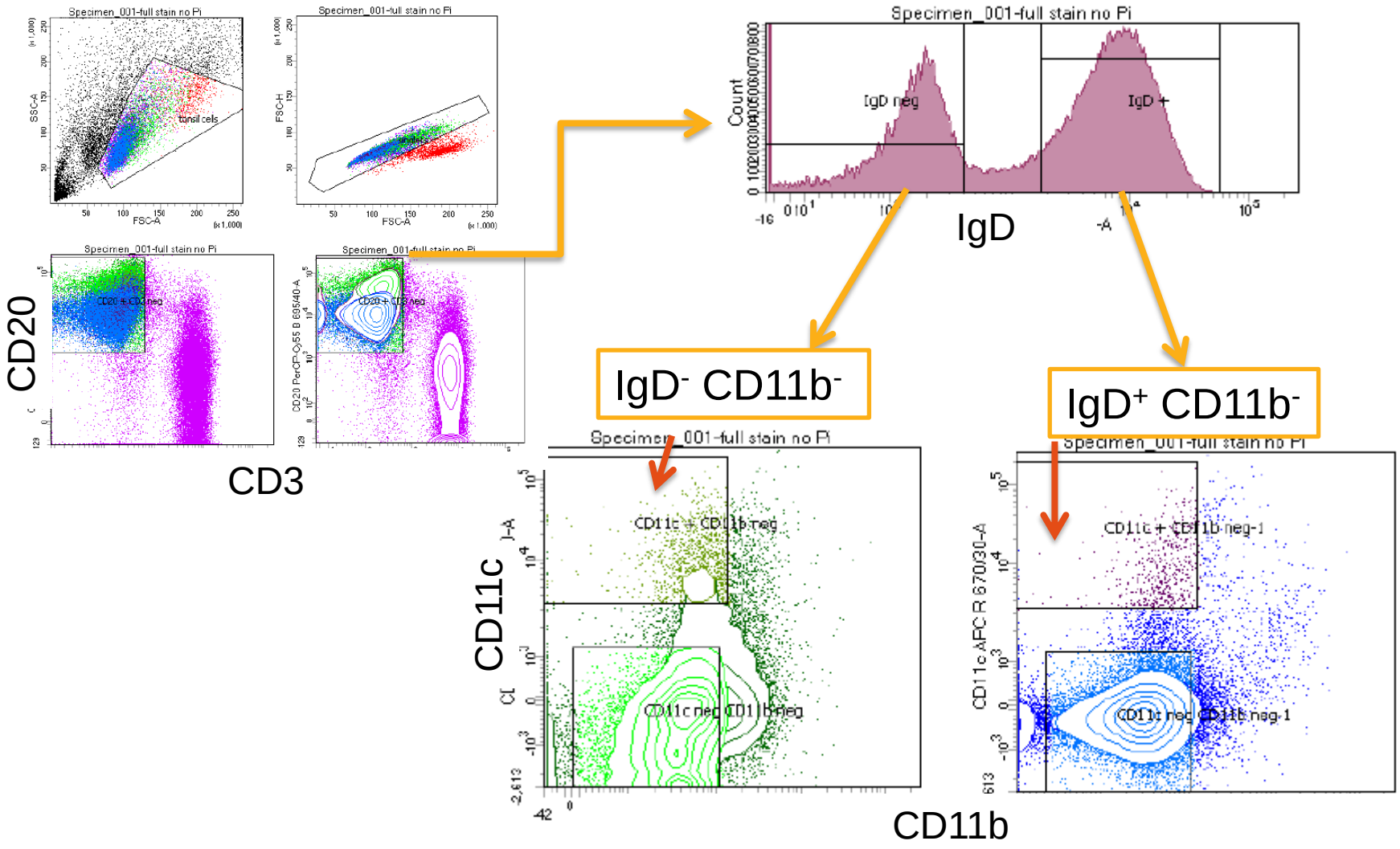
Markers

CD3	**IgG**
CD4	**CD45**
CD5	**IgD**
CD8a	**IgA**
CD10	**IgM**
CD11b	***ROR.GT***
CD14	**CD86**
CD16	**CD11c**
CD19	***IL17A***
CD25	**CD45RO**
CD27	**CD45.RA**
CD31	**HLA.DR..MHC.II**
CD38	

Confirmation of tonsillar CD11c^{+} B cells by Facs

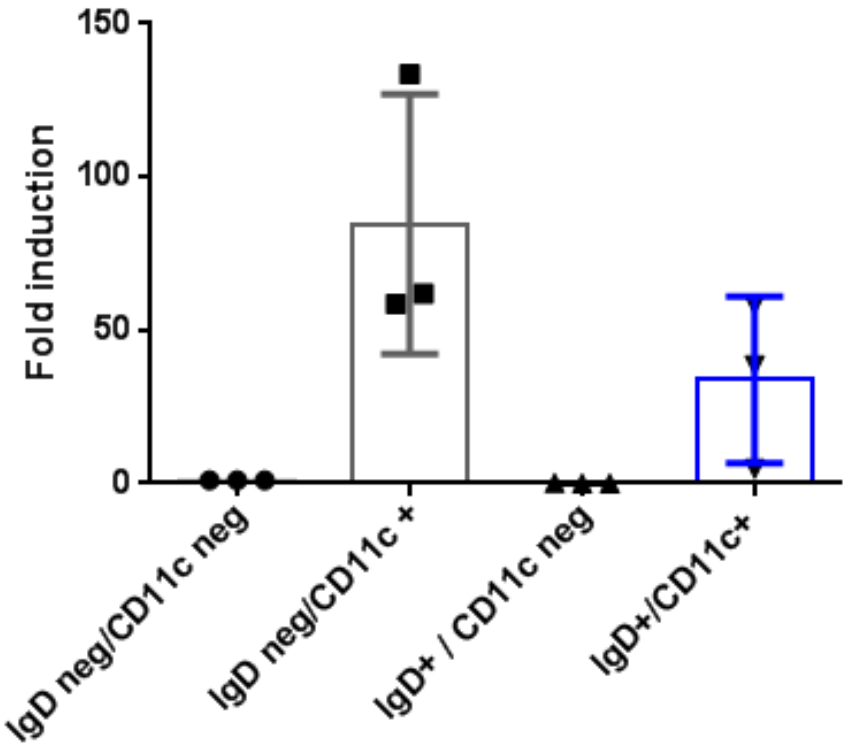
Gating: Single cells, PI- CD3- CD20+

Gating: IgD+/-



qPCR confirms CD11c expression in sorted B cells

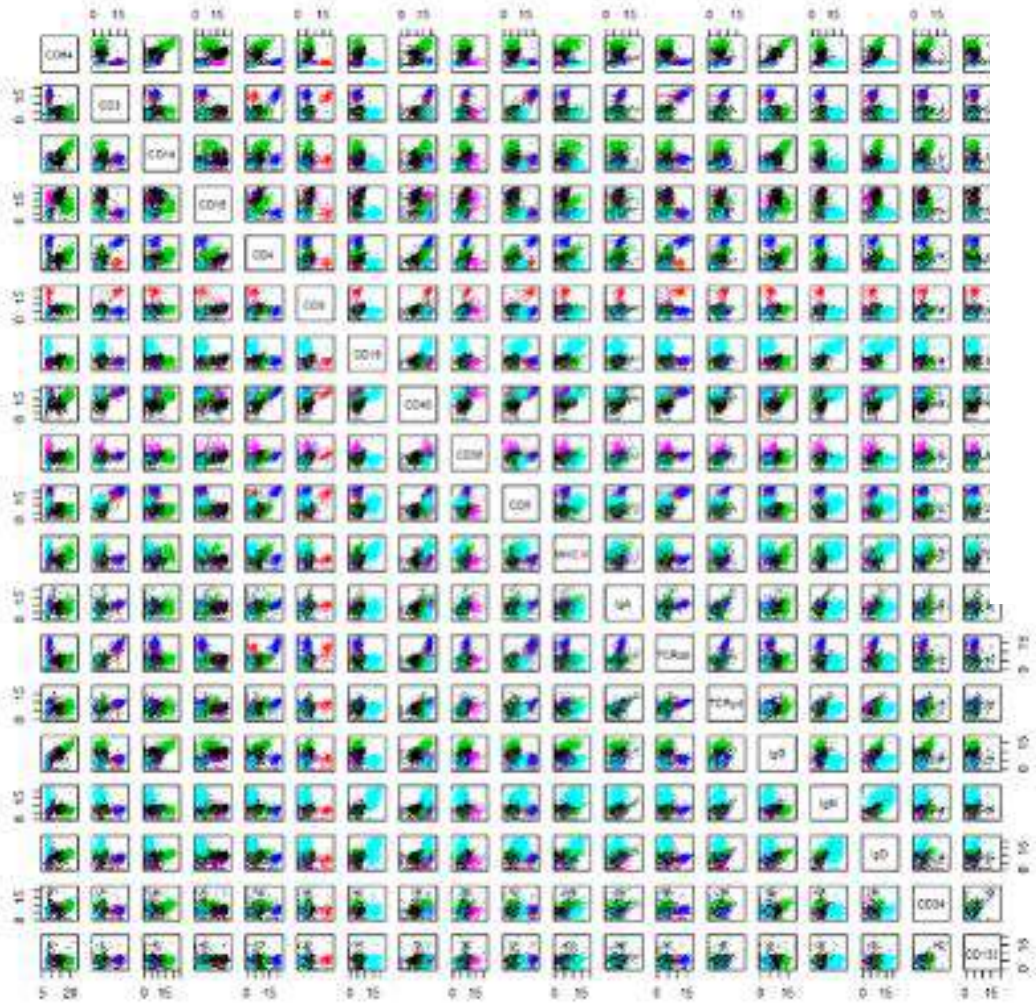
3 tonsil donors

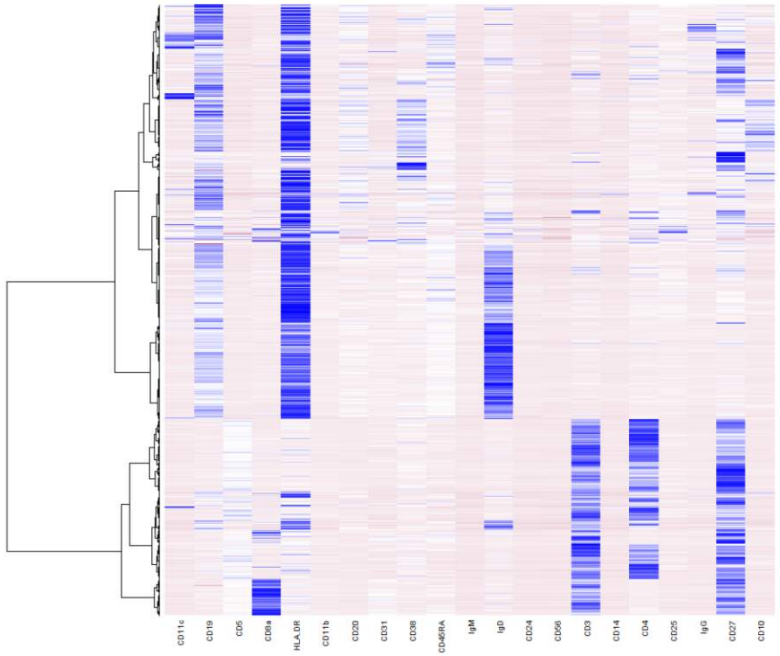


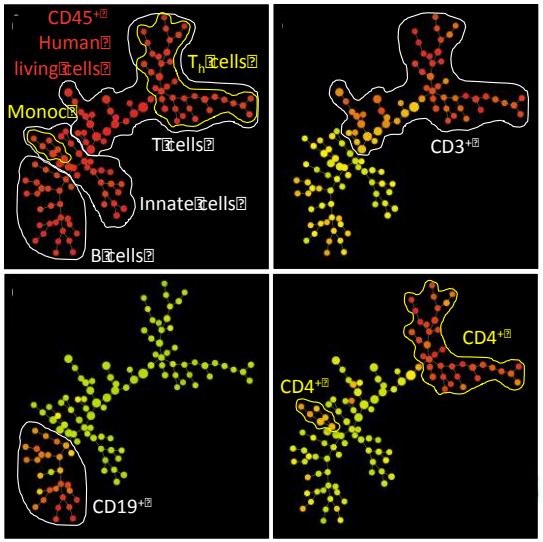
house keeping gene used H P R T

Understanding and evaluating high-dimensional data

.....one of the key challenges is an comprehensive visualization



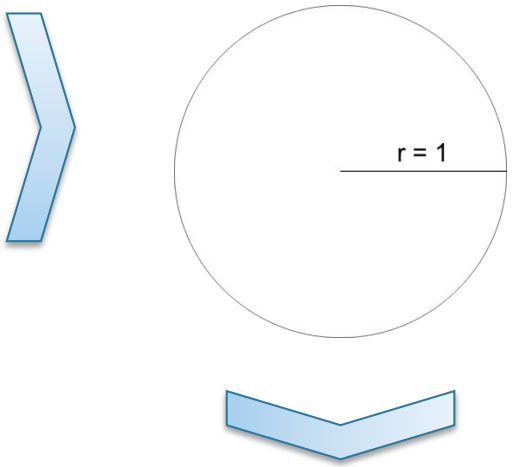




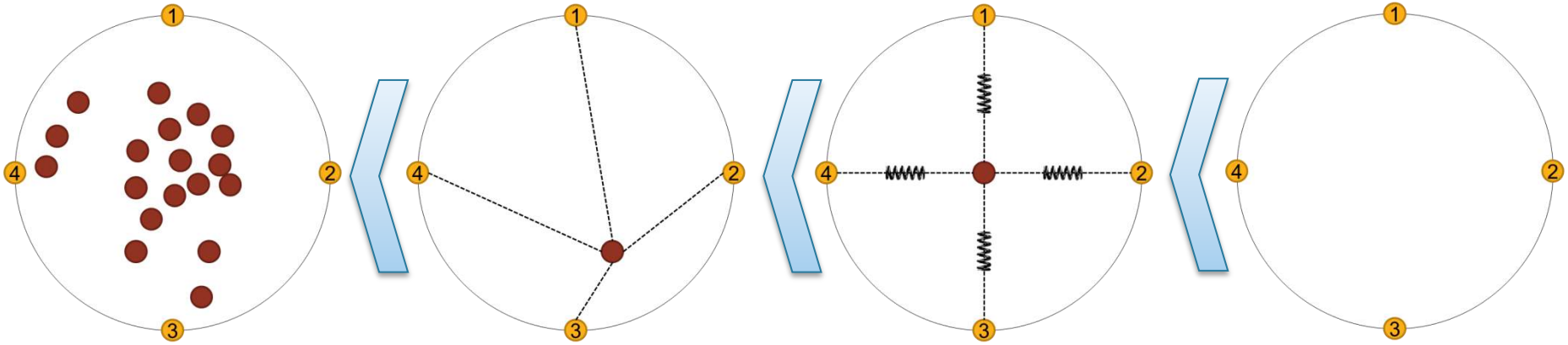
Building a Radviz: A N-Dimensional Scatter Plot

Channel 1	Channel 2	Channel 3	Channel 4
12	1254	37	44

Channel 1	Channel 2	Channel 3	Channel 4
0.1 [0,1]	0.5 [0,1]	0.7 [0,1]	0.05 [0,1]

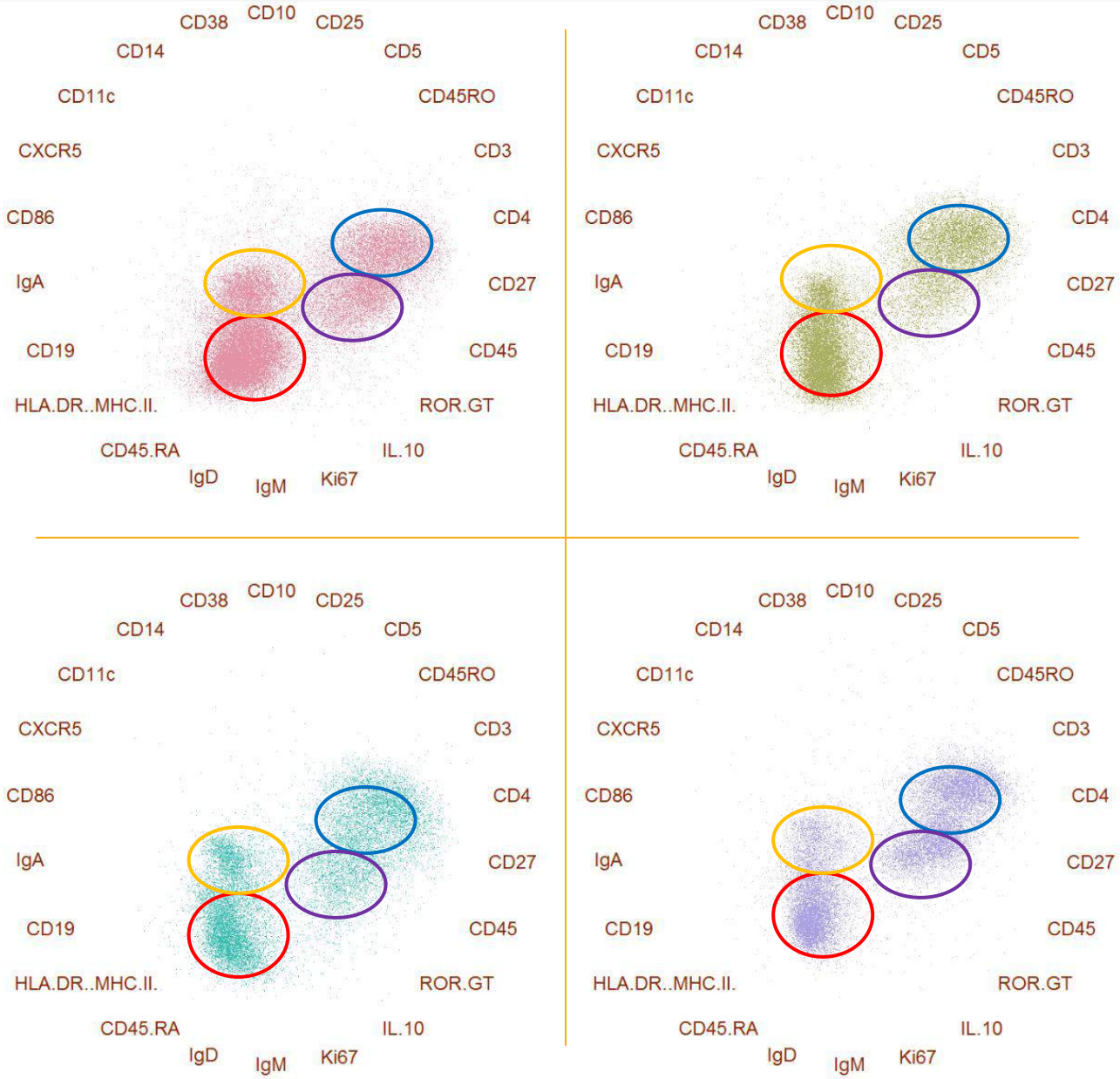


- *Measuring the activity of several pathways*
- *Identifying cell populations in complex samples*
- *Assessing selectivity in multiple assays*



NOVARTIS

RadViz: Unbiased view on the tonsil cytome of 4 donors

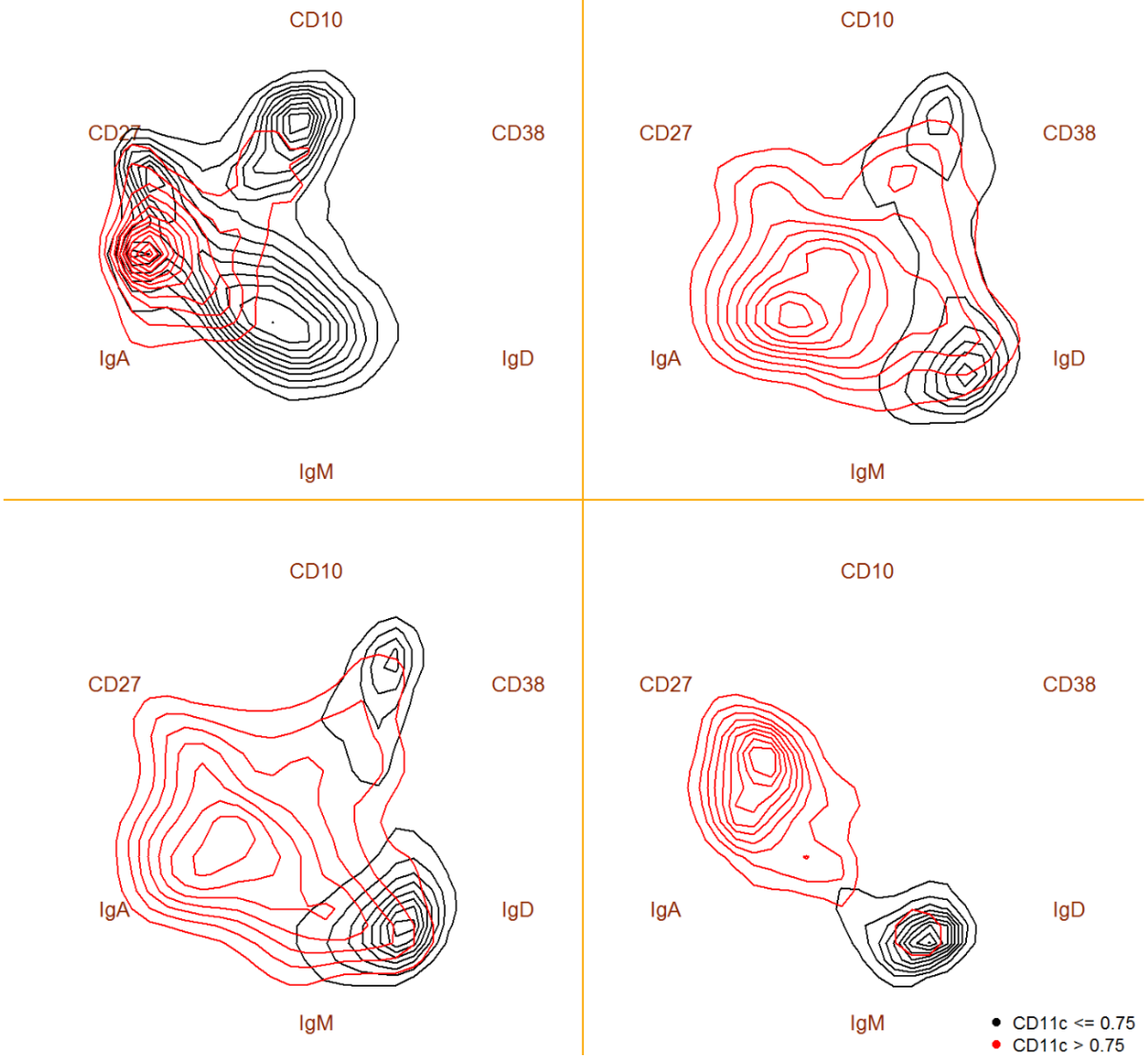


RadViz: Markers defining tonsillar B cell subsets

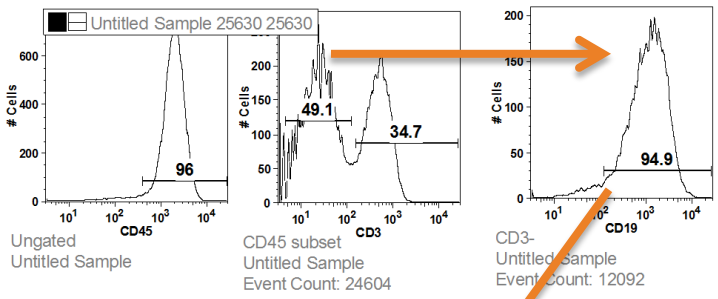
CD45^{+} CD19^{+} with or without CD11c expression

CD11c^{-} B cells

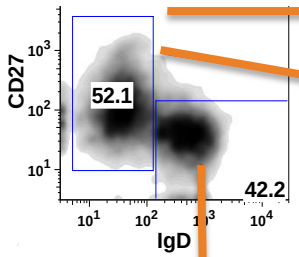
CD11c^{+}B cells



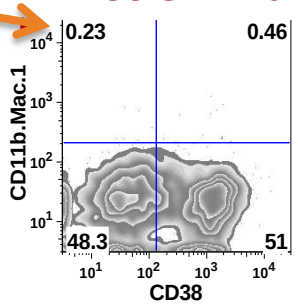
Radviz guided “classical gating” of CD11c+ B cells



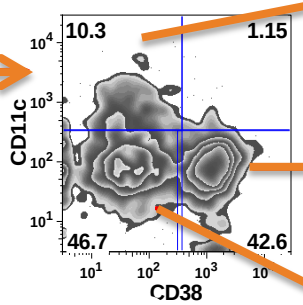
Memory B cells: CD27+ IgD-



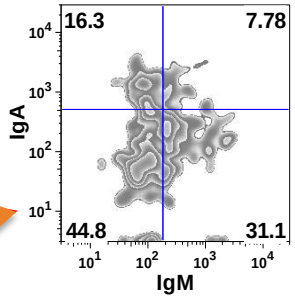
Not CD11b+

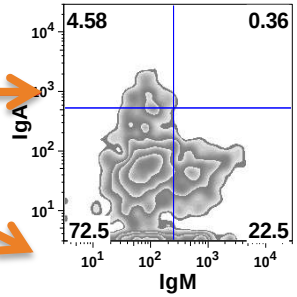


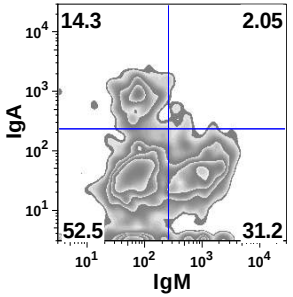
CD38- e.g.
no plasmablasts

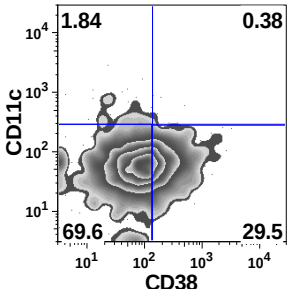


IgA+ vs IgM+







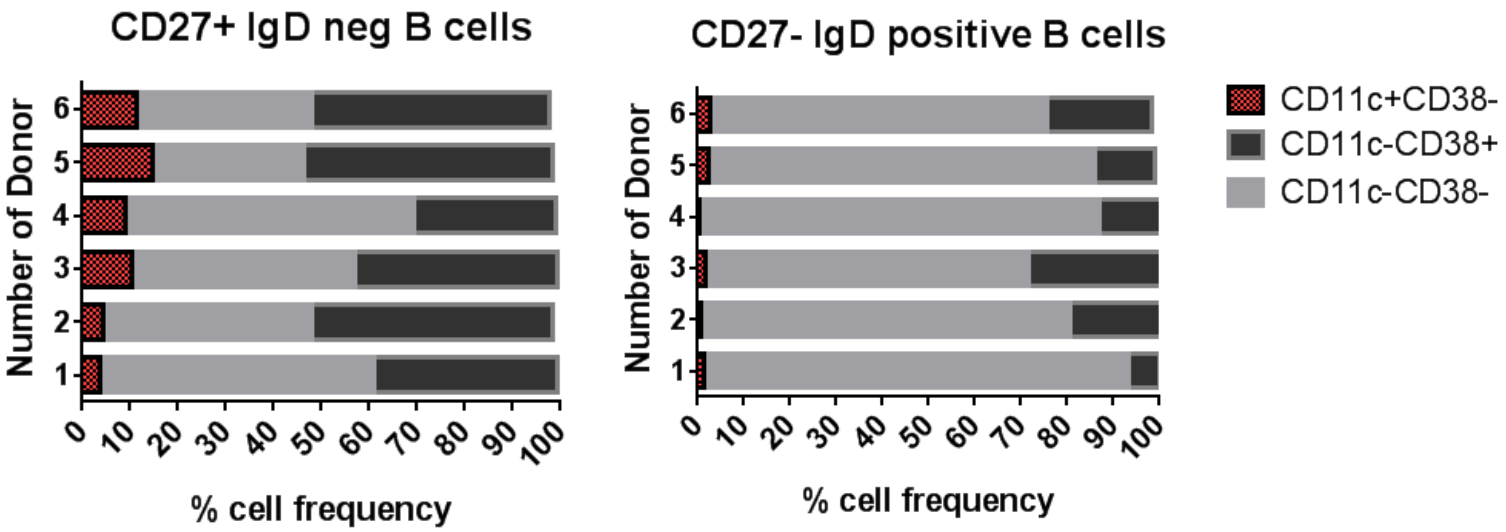


Only minority of IgD+, naive B cells are CD11c+

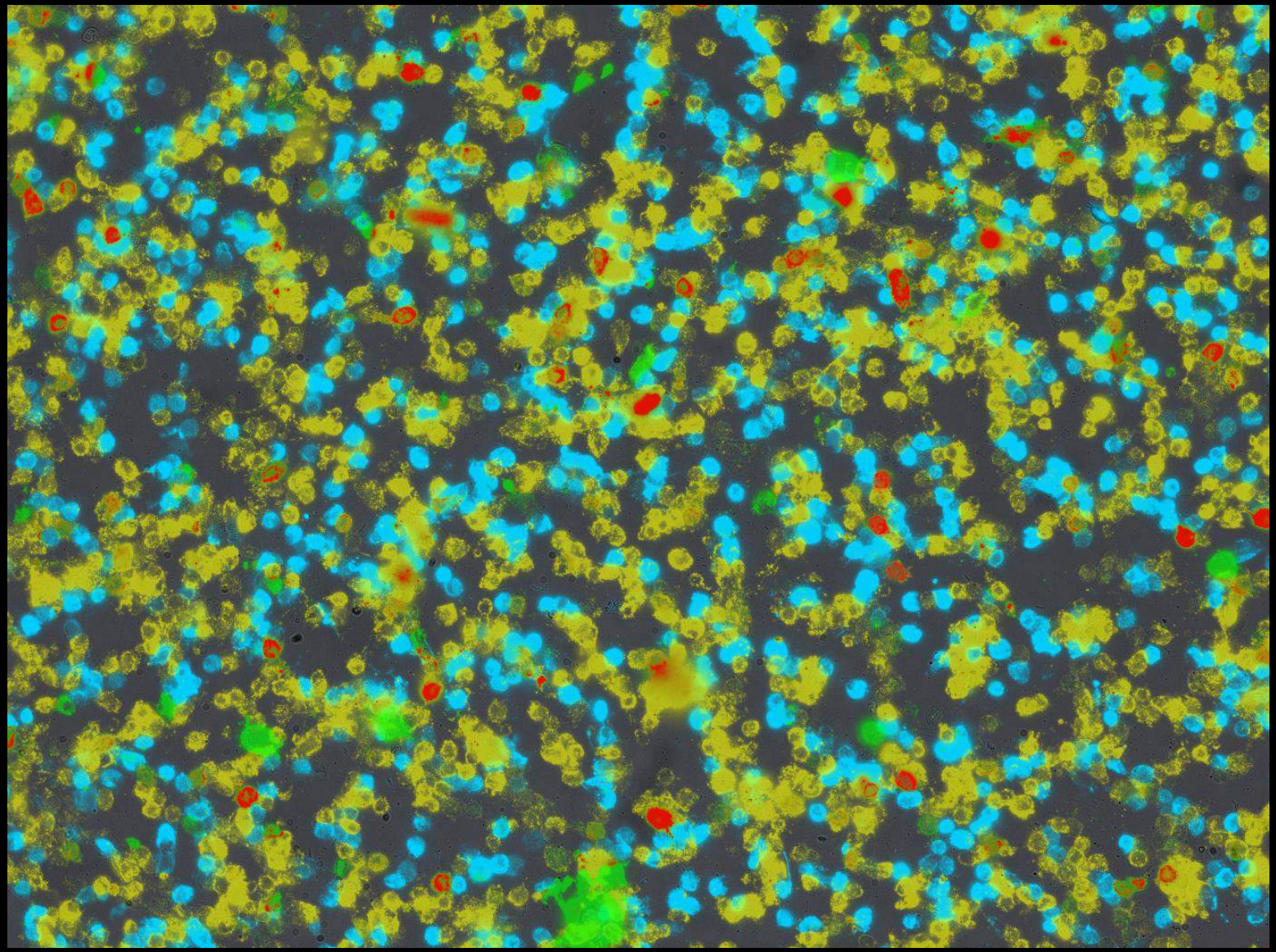
Composition of B cell subsets in inflamed tonsils of 6 donors

CD11c^{+} : 8.8 +/- 1.7 %

1.5 +/- 0.9 %



- Majority of CD11c^{+}B cells are CD27^{+} CD38^{neg} IgD^{neg}
- Tonsil CD11c^{+} B cells did not express CD5 which is different to published data on peripheral CD11c+B cells (Rubtsov et al 2011)
- Since CD5 staining is working, this may be a differentiator to those cells found in autoimmunity



CD45+

CD19+CD3-CD11c-

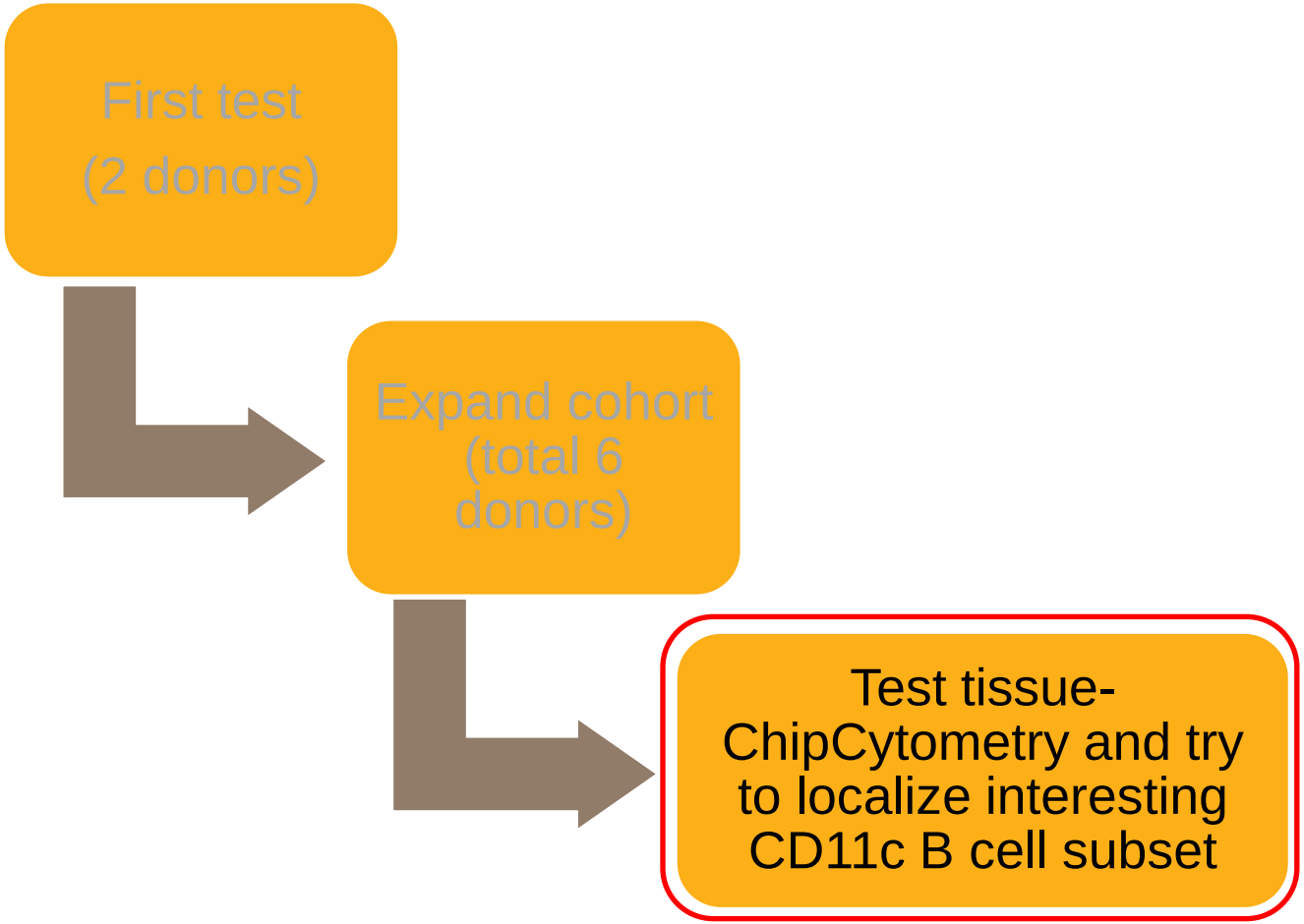
CD19-CD3+CD11c-

CD19+CD3-CD11c+

CD19-CD3-CD11c+

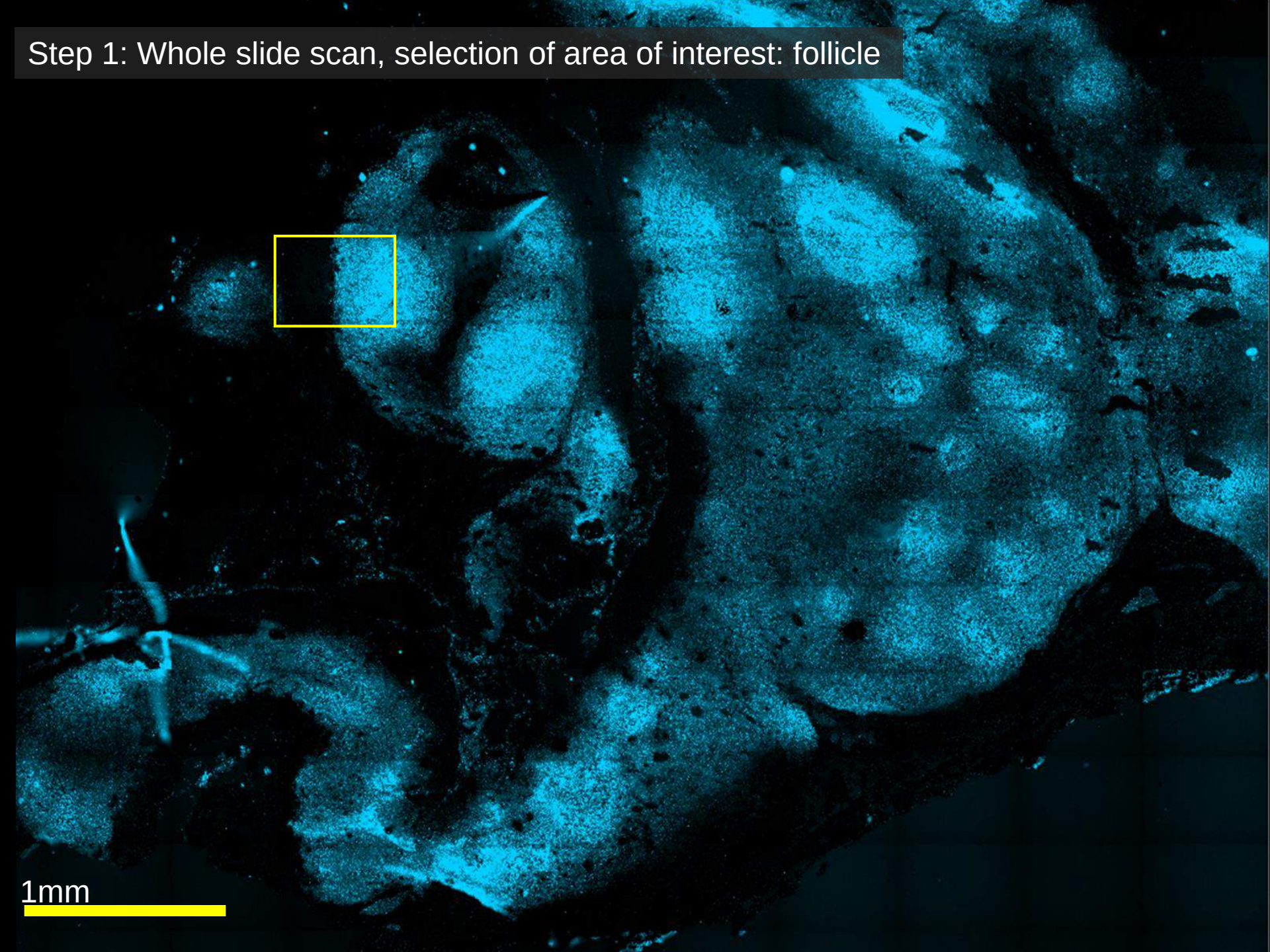
Sample ID: M29508
Single cells | human tonsil

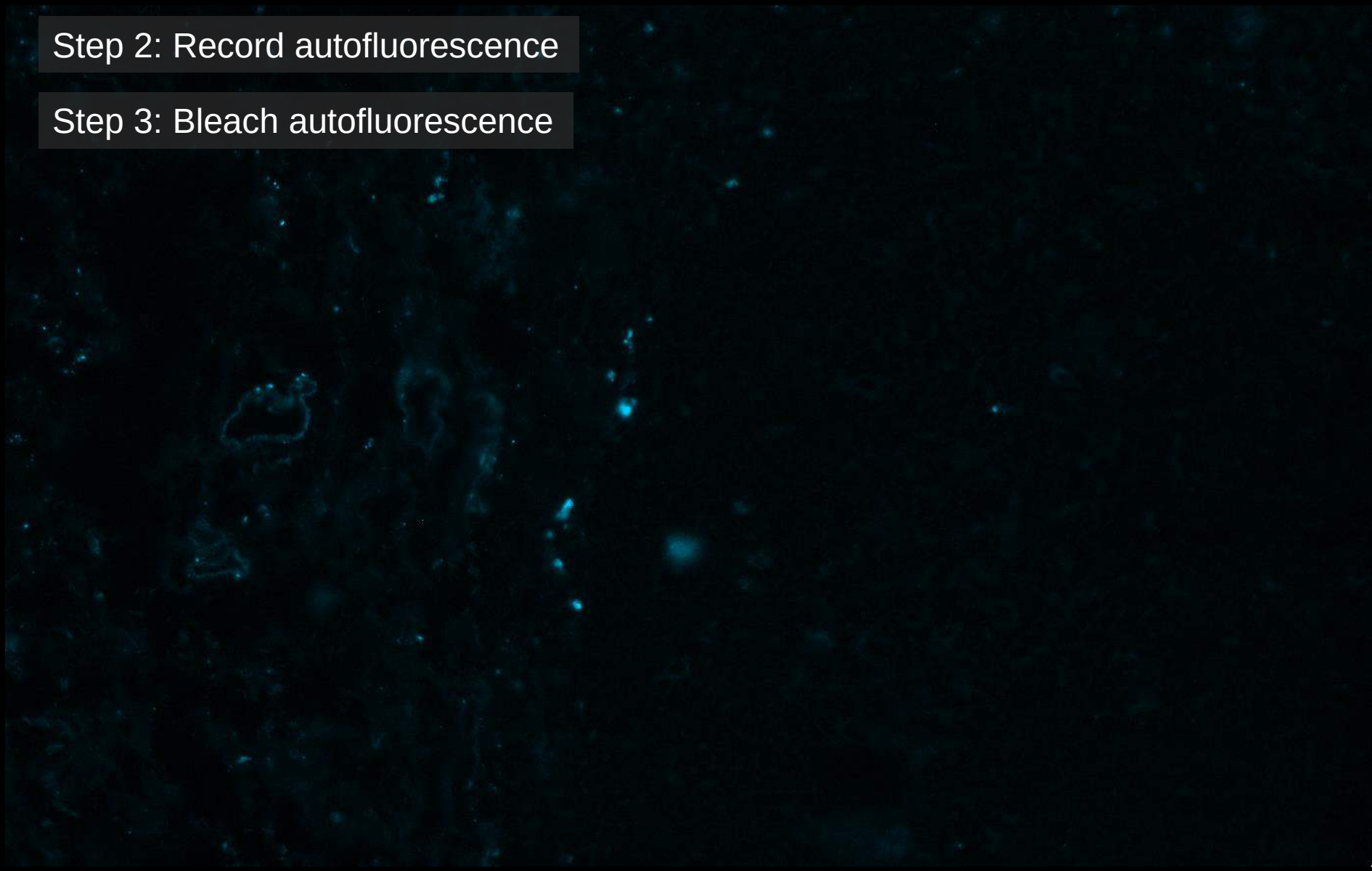
ZELLKRAFTWERK collaboration ATI Basel


First test
(2 donors)
Expand cohort
(total 6
donors)
Test tissue-
ChipCytometry and try
to localize interesting
CD11c B cell subset

Tissue cytometry

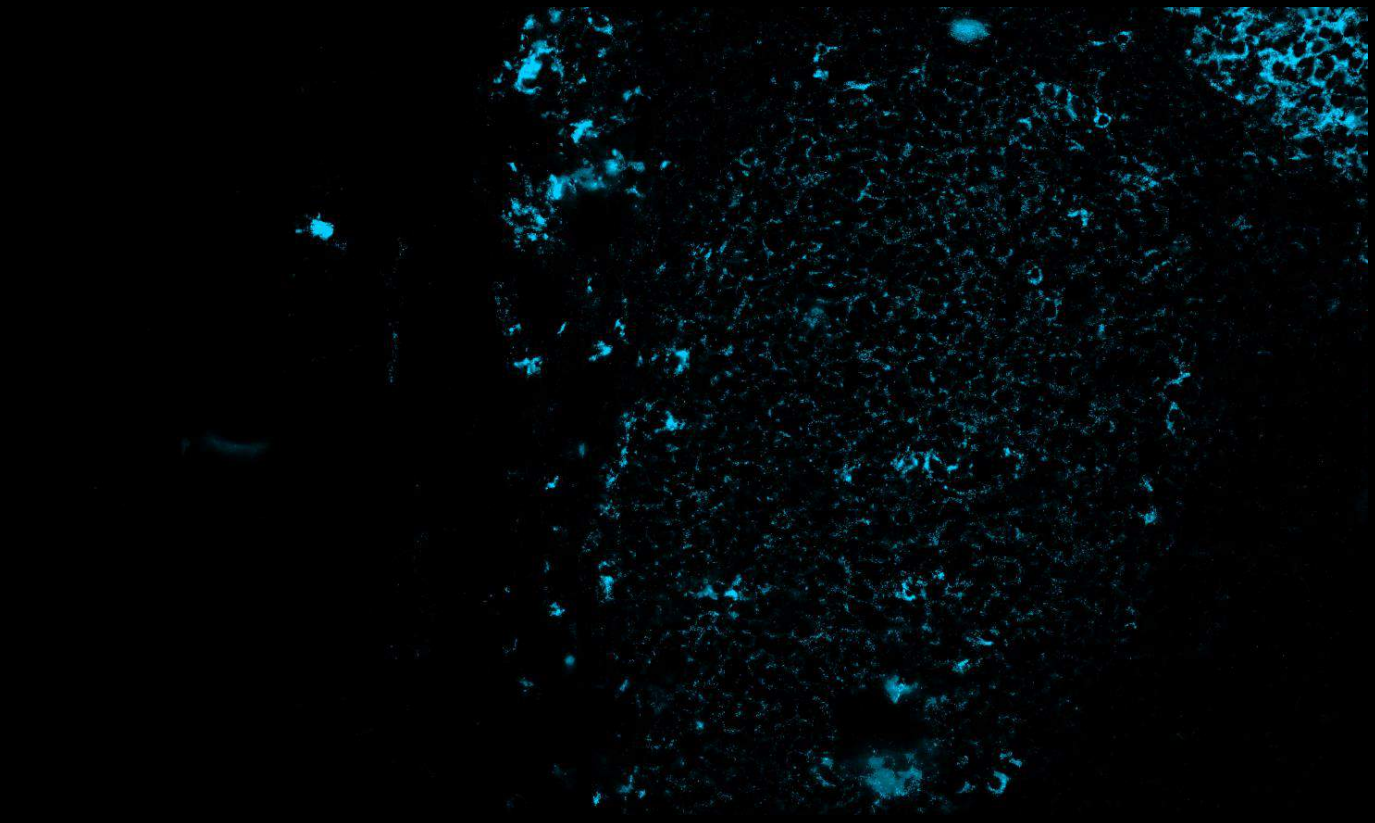
- Immobilize cryosections of tonsil on chip
- Perform iterative staining/imaging/bleaching cycles


Step 1: Whole slide scan, selection of area of interest: follicle
1mm

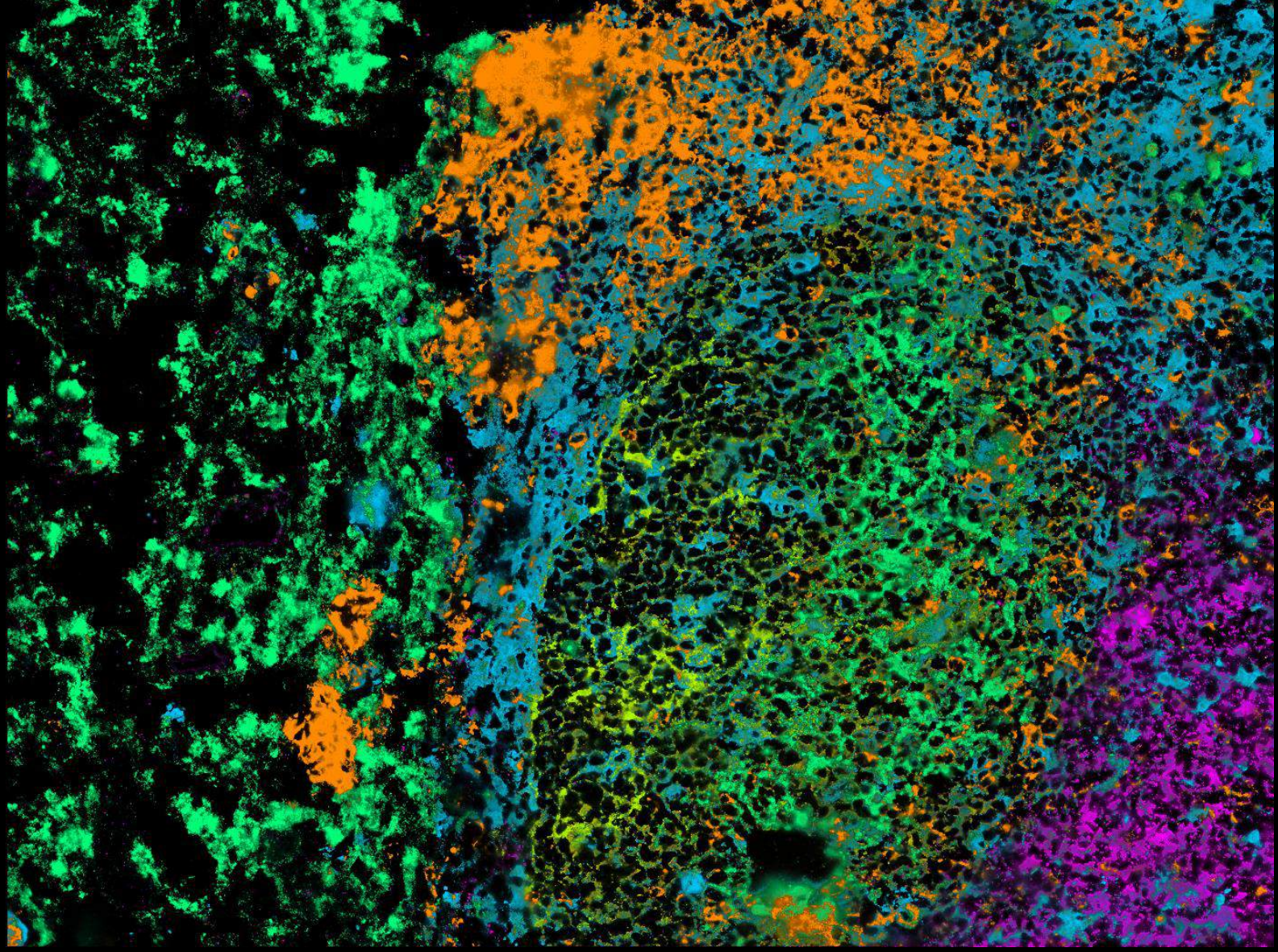

Step 2: Record autofluorescence
Step 3: Bleach autofluorescence

STEP 4

Measure Biomarkers
[autofluorescence substracted]



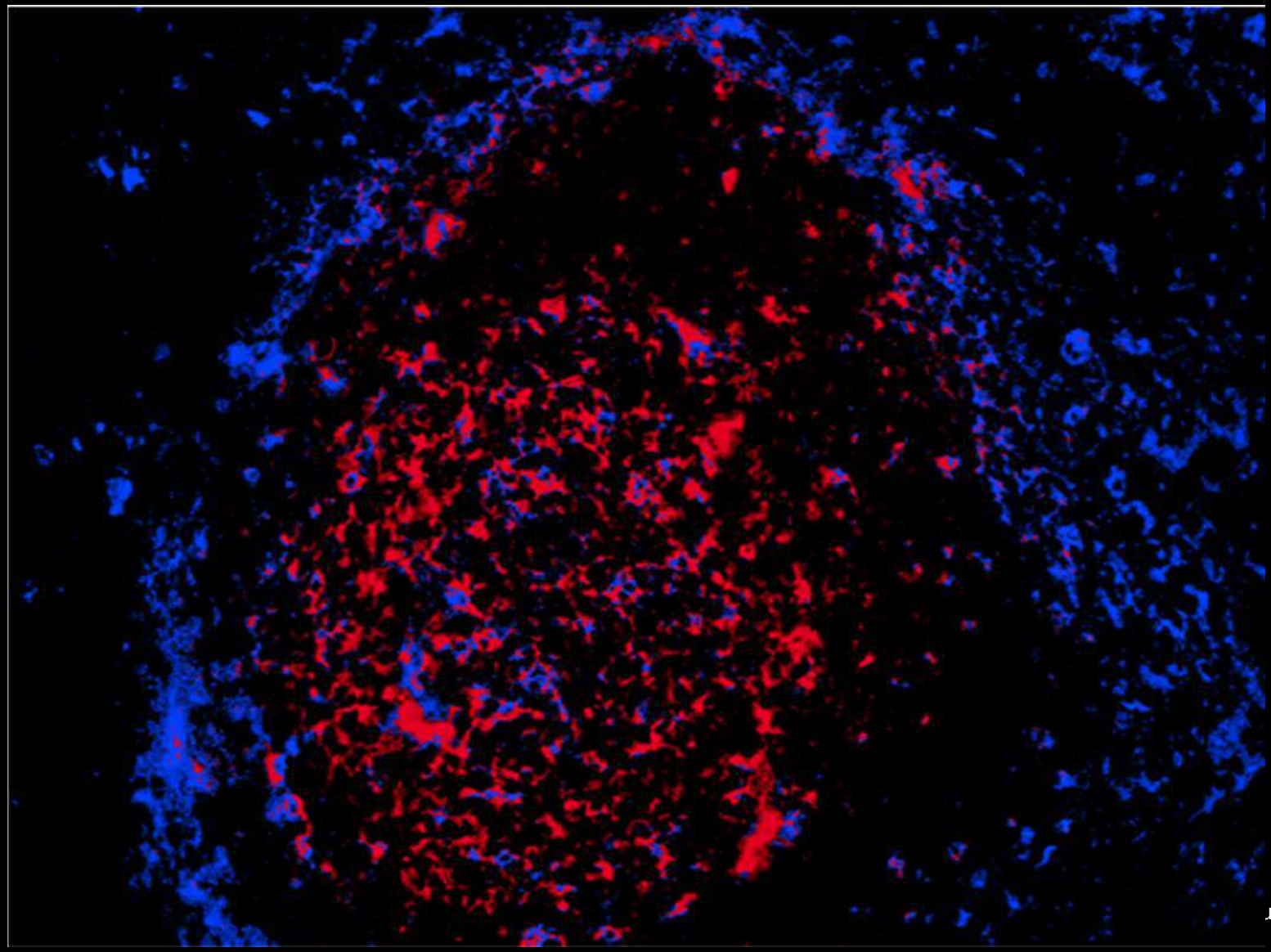
CD19 IgG CD11b IgD CD86 CD3 CD27 CD11c CD8a CD5 CD4 CD31…



IgG CD3 CD11c CD11b IgD

Sample ID: M82105

CD11c^{+}B cells reside in the germinal center



M28054
uman tonsil

Conclusion of feasibility study

Working

- for many readouts ....some parameters need further optimization

Enabling

- Assessment of immobilized cell and tissue with unprecedented possibility for multiplex readouts confirmed.
- Additional staining is possible after first round of phenotyping
- Biorepository and low cell number needed (ideal for TR studies)
- Localization of phenotyped cell in tissue of high scientific value

Overwhelming

- New technology with yet to be defined optimal way to display wealth of data, ideally have a specific question in mind and focus on this.....

Interesting

- Identification of interesting phenotypes (CD11c+ B-cells) has potential to serve as novel cellular biomarker candidate for several immune-cell based diseases

Evolving

- Validation and optimization of some readouts/stainings advisable
- Constant improvement/evolution of staining/measurement
- For clinical studies: sense of GLP, barcoding, databases, audit trails in place

Thanks!

- C Henning, J Detmers and J Schlegel
- NIBR Basel:
  - Grazyna W.+ lab
  - S Muller-Bentz, C Allard
  - Y Abraham
  - A Mir/J Arm
- Tonsil donors

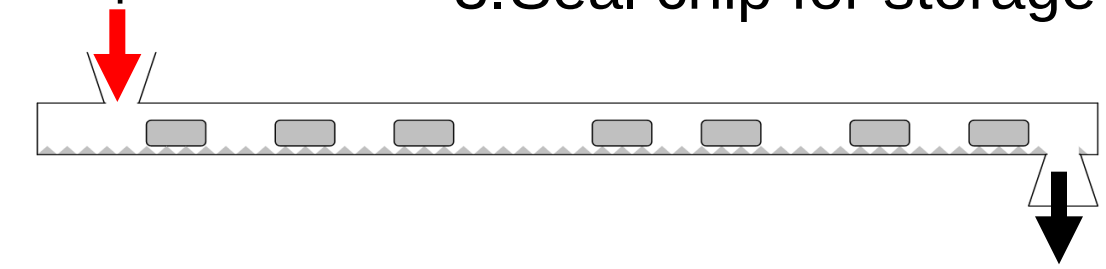
NOVARTIS

Overview: Next Generation Single-Cell Analysis

Manufacturer	MerckMillipore	Fluidigm/ DVS	Sony	Zellkraftwerk	Zellkraftwerk
Web	www.amnis.com	www.fluidigm.com	sonybiotechnology.com	www.zellkraftwerk.com	www.zellkraftwerk.com
Instrument	ImageStream-X MKII	CyTOF 2	SP6800 Spectral Analyzer	ZellScannerONE	Cytobot
Technology	imaging flow cytometry	mass cytometry	spectral cytometry	ChipCytometry	ChipCytometry
Technology Features					
Multiplexing: theoretical limit	max. 10 colors	100	25	unlimited	unlimited
Multiplexing: actual limit	12	40	19	65	65
subcellular localization	++	-	-	+	+
sample storage	1-5 days	1-5 days	1-5 days	at least 20 months	at least 20 months
cell-loss / drop out rate	5-50%	50%	?	<0.5%	<0.5%
tissue cytometry	-	Coming soon	-	+	Coming soon
Instrument Features					
cells/second	5,000	1,000	10,000	2,000	6,000
de-novo setup of a 15-marker panel	not possible	3 month	4 month	<1 day	<1 day
Total cost of ownership					
Basic instrument	≈400,000 USD	≈590,000 USD	≈400,000 USD	248,000 USD	1,000,000 USD
Energy supply	≈2,000 USD	≈8,000 USD	≈2,000 USD	2,000 USD	4,000 USD
Argon gas supply	not required	60,000 USD	not required	not required	not required
Maintenance Contract	≈40,000 USD	≈50,000 USD	≈35,000 USD	≈18,000 USD	≈50,000
Pros & Cons					
biggest pros	statistical microscopy with many morphological parameters	many publications by inventor available	discrimination of fluorescent proteins / fluorochromes	best instrument for low cell numbers and precious samples	precious samples: option for 20 months storage
biggest cons	limited to max. 10 colors / cumbersome panel development in case of more than 6 colors	proprietary labels required / dedicated user necessary / total costs of ownership	cumbersome panel development	bench-top instrument has medium-low sample throughput // fully automated system is expensive	price

ZellSafe: A Microfluidic BioBank

1. Load cells
2. Add fixation buffer
3. Seal chip for storage / shipment



			
Product	ZellSafe™ Cells	ZellSafe™ Rare	ZellSafe™ Tissue
Specimen	cell suspension	rare cells (<0.02%)	sections
Loading capacity	40-100µl	40-300µl	6 biopsies or 2x1cm section
Total cell number	approx. 250,000	approx. 1,000,000	tissue-dependent

ZellScannerONE specifications

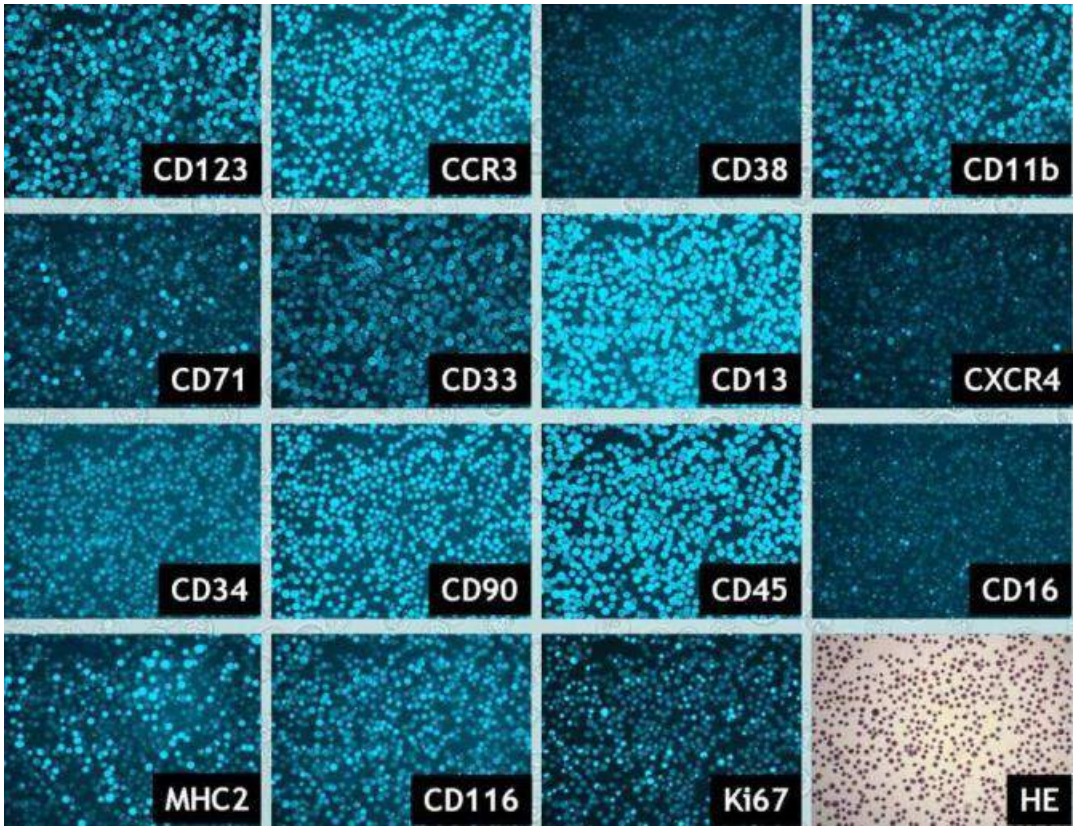
- autofluorescence detection and bleaching on single cell level
  - → high sensitivity+specificity
- max 5-colors at once (filterset with no/low spillover – no/low compensation)
- recovery of detection channels
  - → serial staining for unlimited number of markers
- works with all standard FACS antibodies
- 32bit HDR-imaging - ~200 pixel information/cell
  - → high sensitivity, high linear range
- Output: Raw images/FCS/CSV
- No spill-over effect => no color compensation required!

5	BUV395	BV421	FITC	PE	PerCP
10	BUV395	BV421	FITC	PE	PerCP
15	BUV395	BV421	FITC	PE	PerCP
20	BUV395	BV421	FITC	PE	PerCP
25	BUV395	BV421	FITC	PE	PerCP

…

unlimited number of markers

Stain same cell again and again ...



GATA1s induces hyperproliferation of eosinophil precursors in down syndrome transient leukemia.

Maroz A, Stachorski L, Emmrich S, Reinhardt K, Xu J, Shao Z, Käbler S, Dertmann T, Hitzler J, Roberts I, Vyas P, Juban G, **Hennig** C, Hansen G, Li Z, Orkin S, Reinhardt D, **Klusmann** JH.

Leukemia. 2013 Dec 13. doi: 10.1038/leu.2013.373. [Epub ahead of print]

PMID: 24336126 [PubMed - as supplied by publisher]