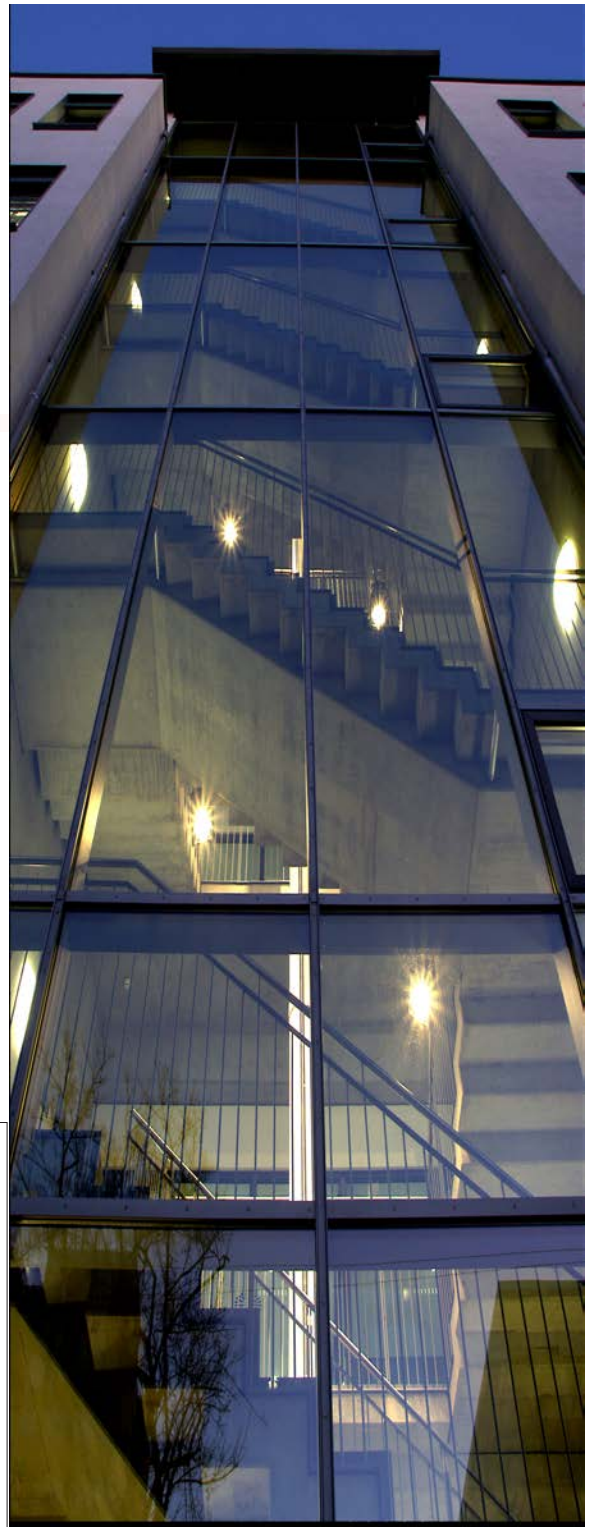
Astana – September 21th, 2018

Kazakhstan

Flow Cytometry Workshop



Customized cellular products

Institut für Medizinische Mikrobiologie Immunologie und Hygiene

Matthias Schiemann

Technische Universität München, Germany

ifcc International Federation of Clinical Chemistry and Laboratory Medicine

Flow Cytometry units of the Technischen Universität München (CyTUM)

CyTUM

Steering Committee	
Chair:	M. Schiemann
Members:	D. Busch, D. Zehn, C. Traidl-Hoffmann

Core MIH

Institut für Medizinische Mikrobiologie Immunologie und Hygiene

Core UNIKA-T

UNIKA-T

Core LTI

Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

Lehrstuhl für Tierphysiologie und Immunologie

CyTUM

Flow Cytometry FACS Unit`s – located at MIH, LTI and UNIKA-T

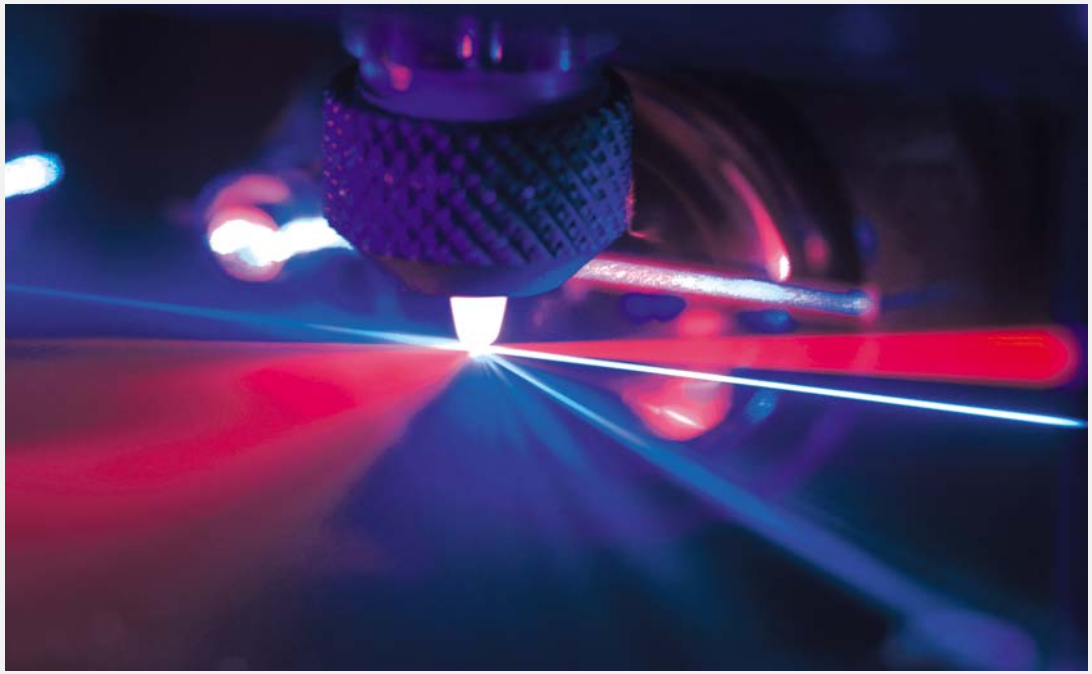
→ First University overarching facility in Germany

680 user
- 300 (Analysis)
- 380 (Cell Sorting)

Instruments:

- 28 Analyzer
- 7 Cell Sorter

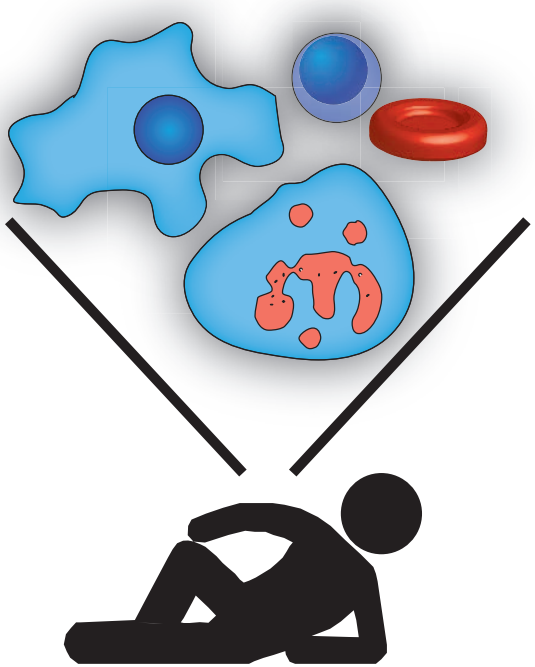
- 10 employees

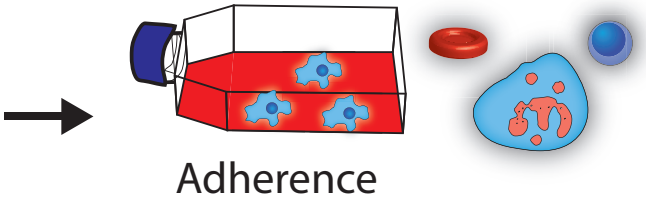


Instrumentation of the CyTUM MIH

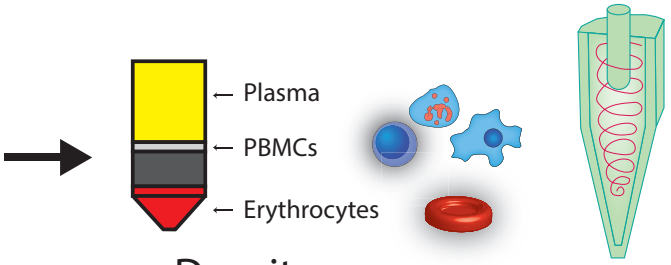
Cell separation – an overview

>200 different cell types

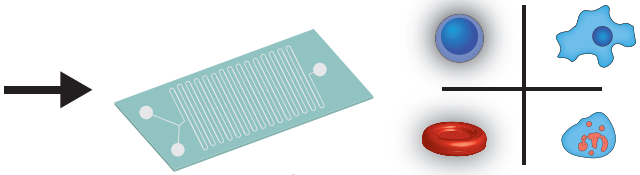




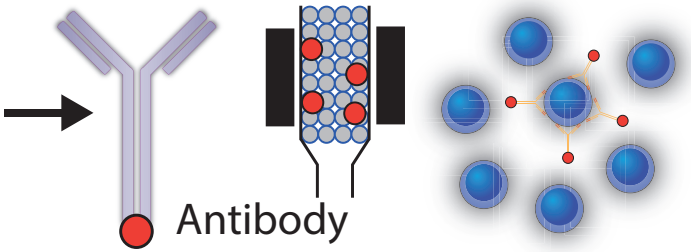
Adherence



Density



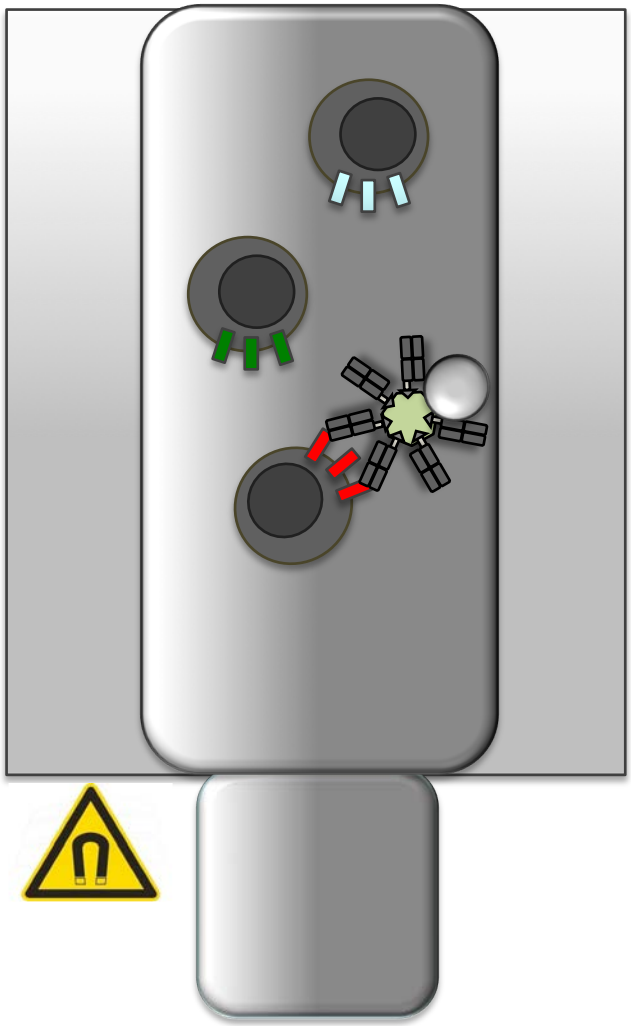
Microfluidics



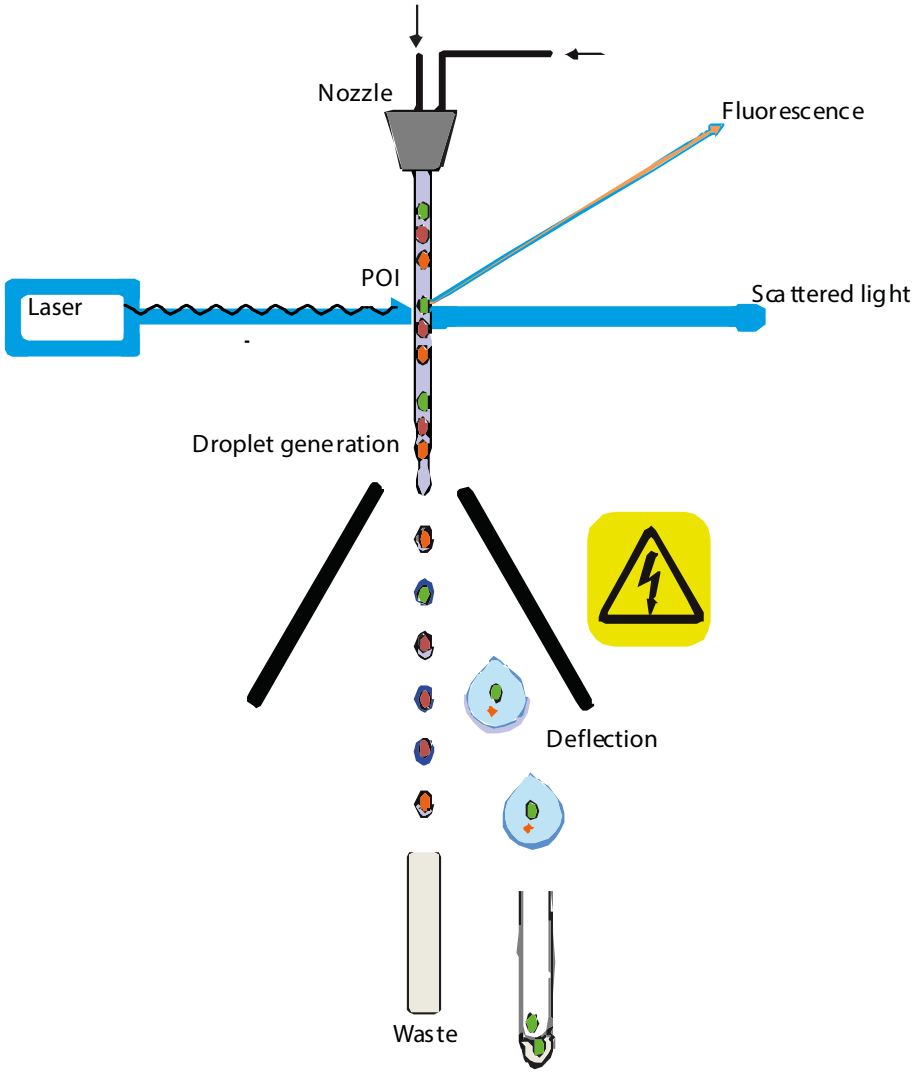
Antibody

Antibody based cell separation methods

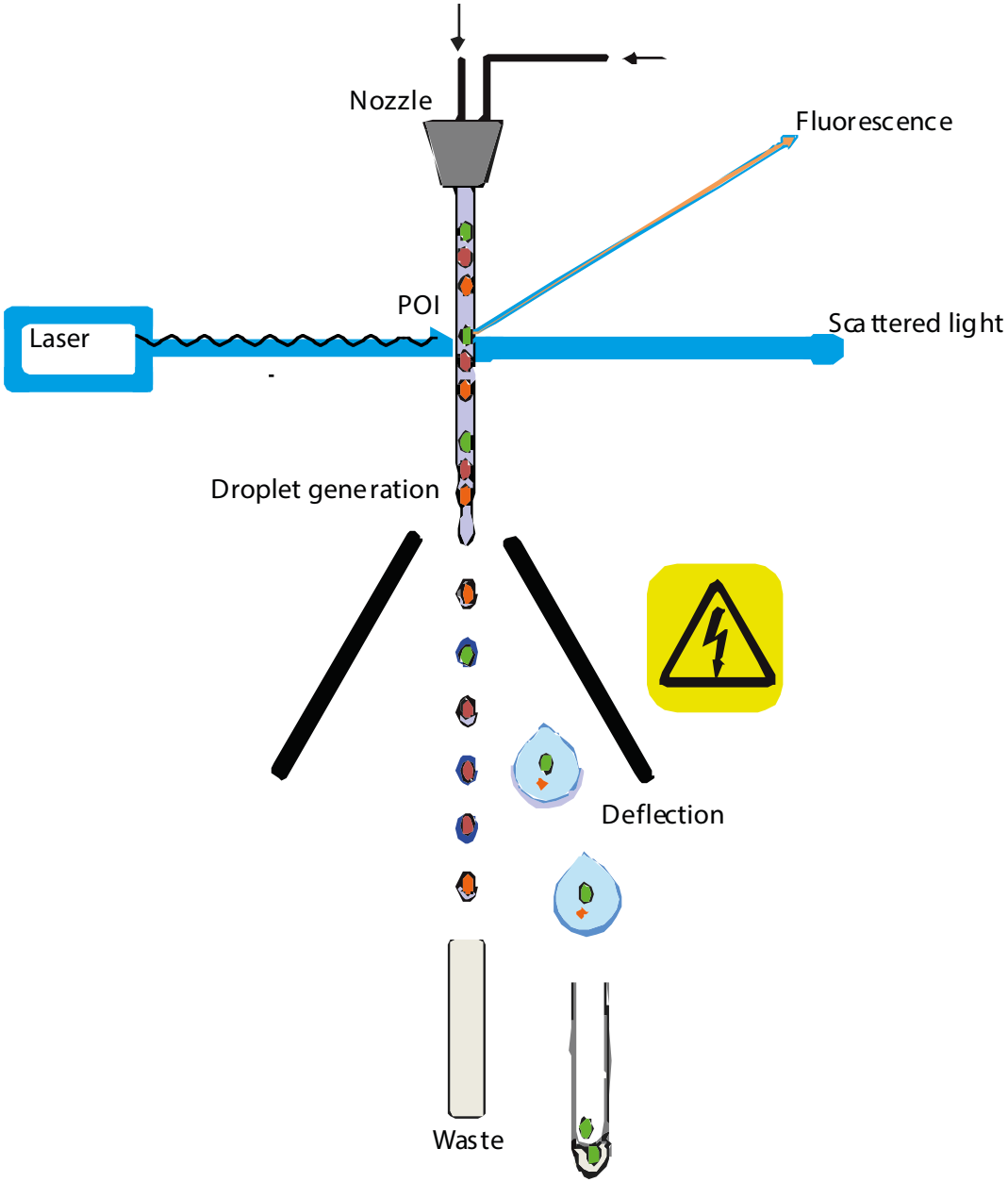
MACS

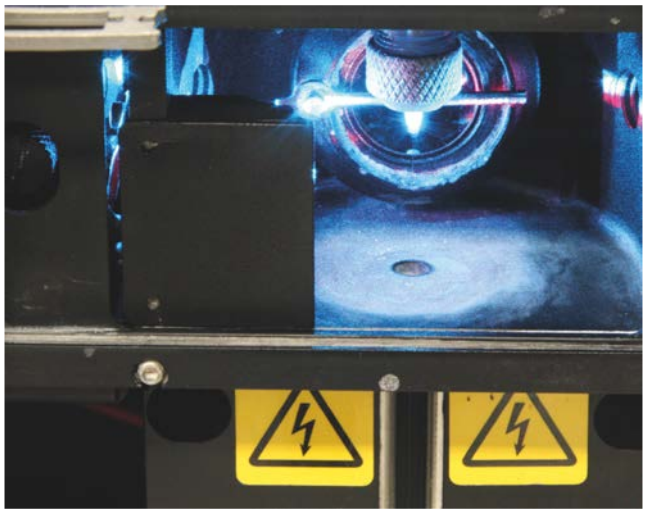


FACS



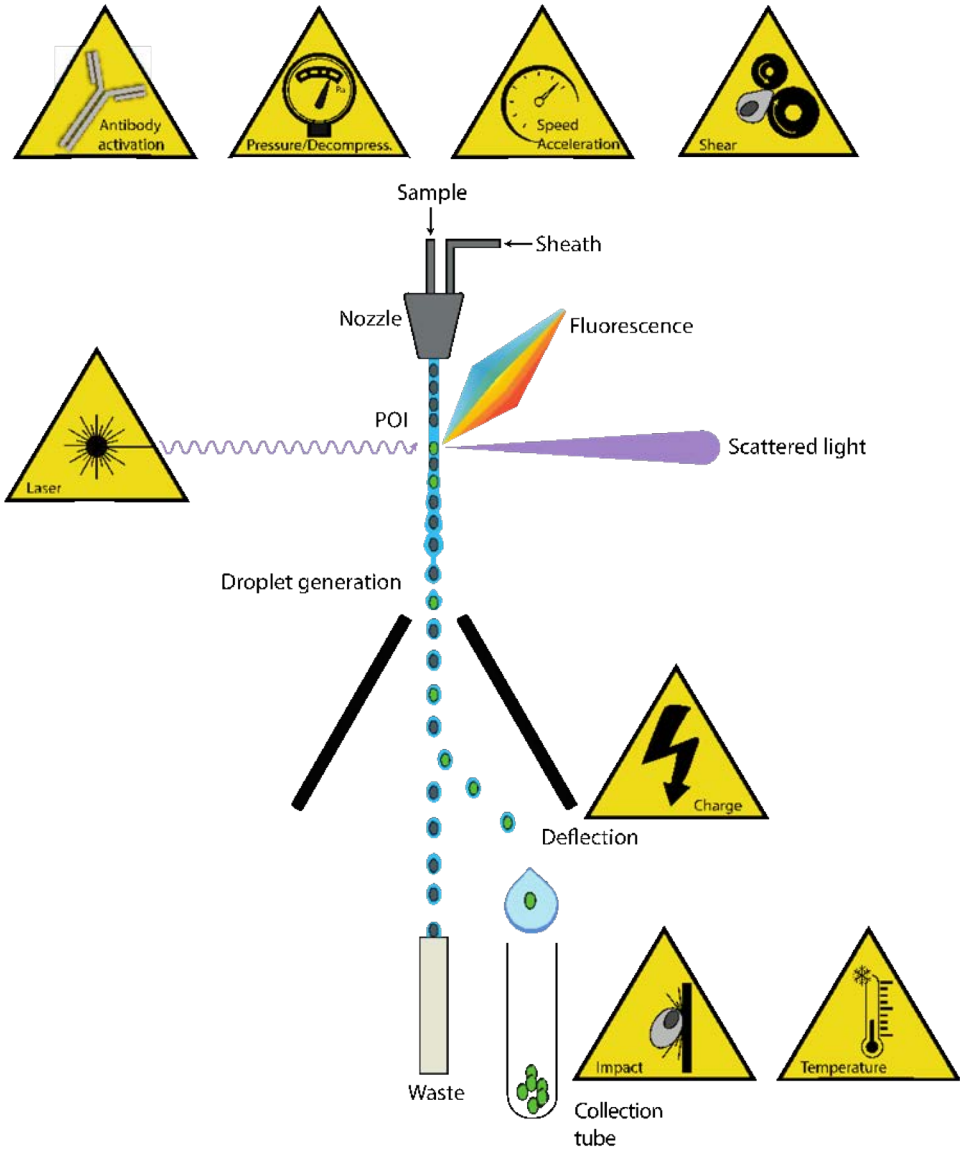
Introduction FACS





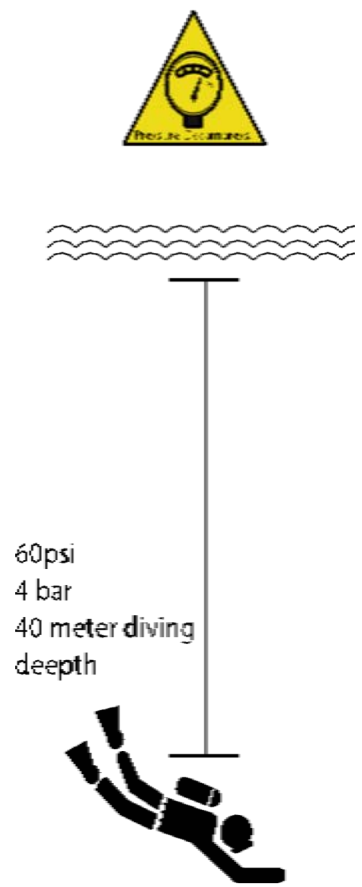
Scientific Question and considerations

- **Sample processing**
  - **Cell isolation/preparation**
  - **Staining/biological durability/activation**
  - **Temperature**
- **Sorting parameters**
  - **Pressure**
  - **Shearing effects/Acceleration**
  - **Decompression/expansion**
  - **Laser light**
  - **Electrostatic charge**
  - **Impact**
  - **Energy Dissipation Rate**

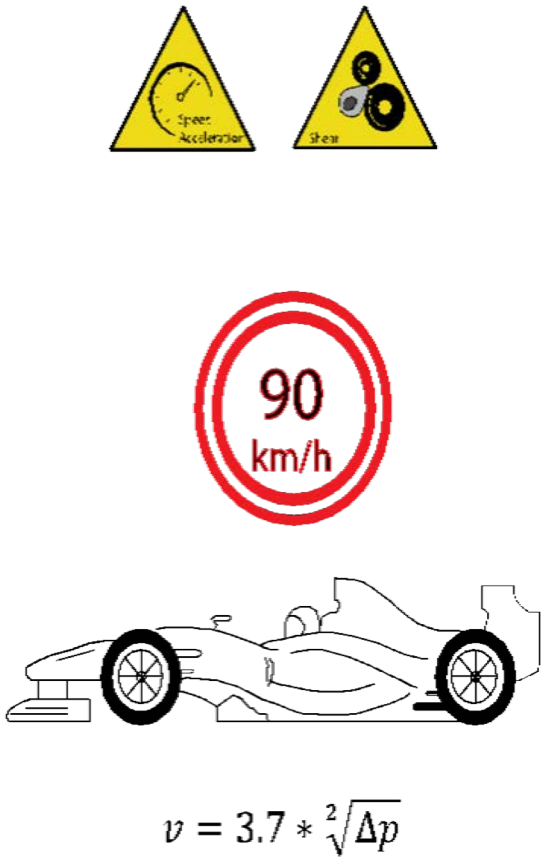


Varma, S. *et al*., Analytical chemistry (2017)
Richardson, G. M. *et al.*, Cytometry A (2015)
Mollet, M. et al., Biotechnology and Bioengineering (2007)

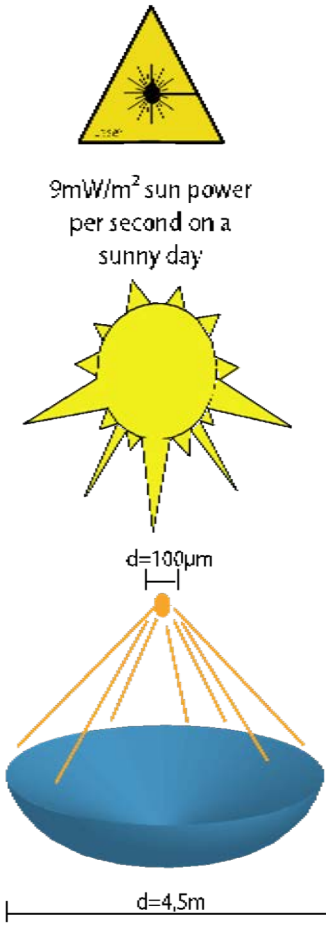
A closer look at some FACS forces



Eisinger, J. *et al.*, Biophysical chemistry (1987)



Olesen, S. P. *et al.*, Nature (1988)
Seidl, J. *et al.*, Cytometry (1999).



Ge, J. *et al.*, Cytometry A (2013)
Catt, S. L. *et al.*, Molecular human reproduction (1997)

Characterization of cell alterations caused by FACS

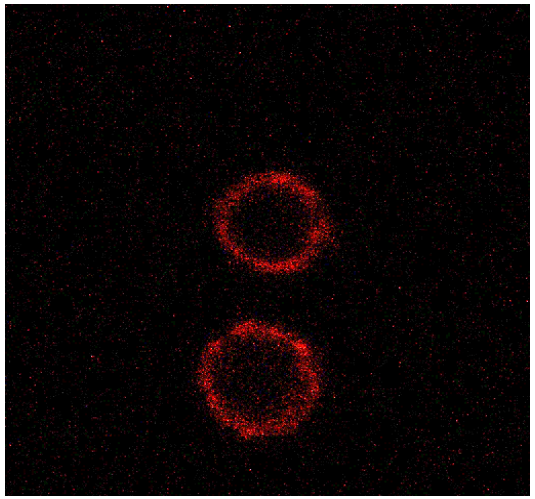
In versatile applications FACS is unrivaled to other purification methods, thus limited to applications for research use only.

In the work of our group we evaluate assays to determine cell fitness, in order to validate current research data. These results might also be useful for other clinical cell processing's.

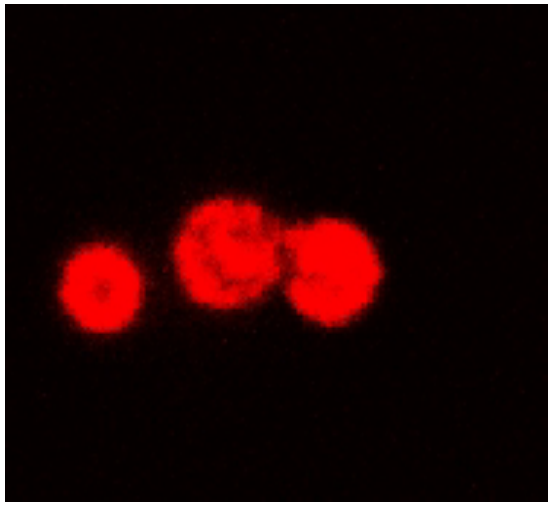
Method – “optimized” cell sorter

We built a modified MoFlo XDP™ cell sorter, for controlling temperature sort conditions of the whole system, enabling us to evaluate the influence of temperature. In this approach we used different technologies to evaluate every single parameter during sorting, like in an equation, with only one variable.

4° C



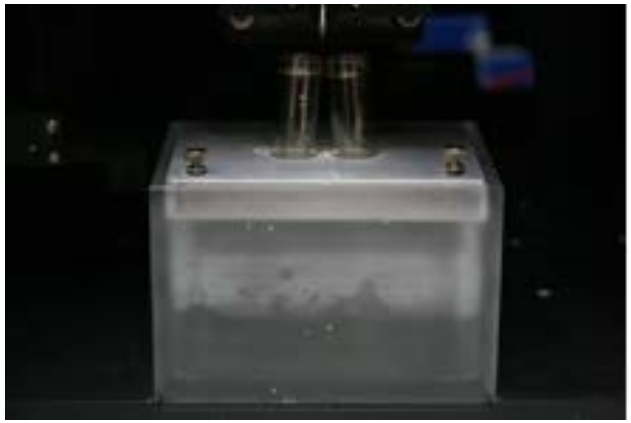
37° C

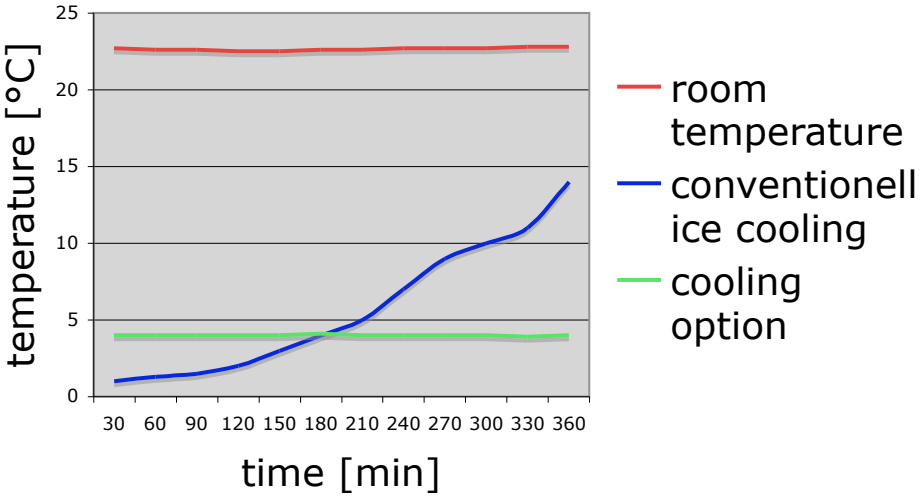


LLO_{91-99}–specific T cell line
H2-K^d/LLO_{91-99}/mb_2m SA-Alexa546
45 min staining

FACS Optimizing - Temperature

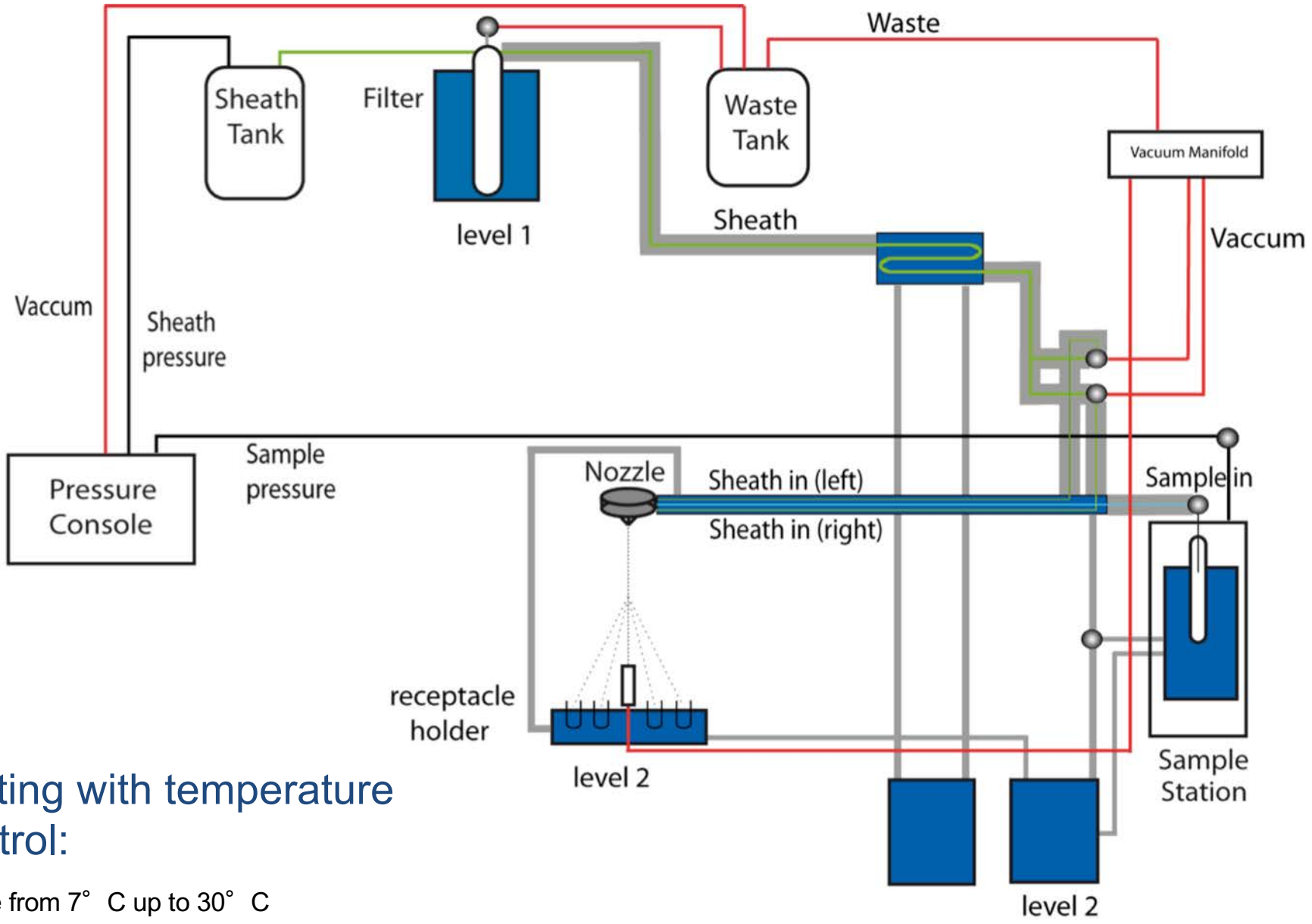
Ice cooling





Centralized cooling solution for all conventional instruments

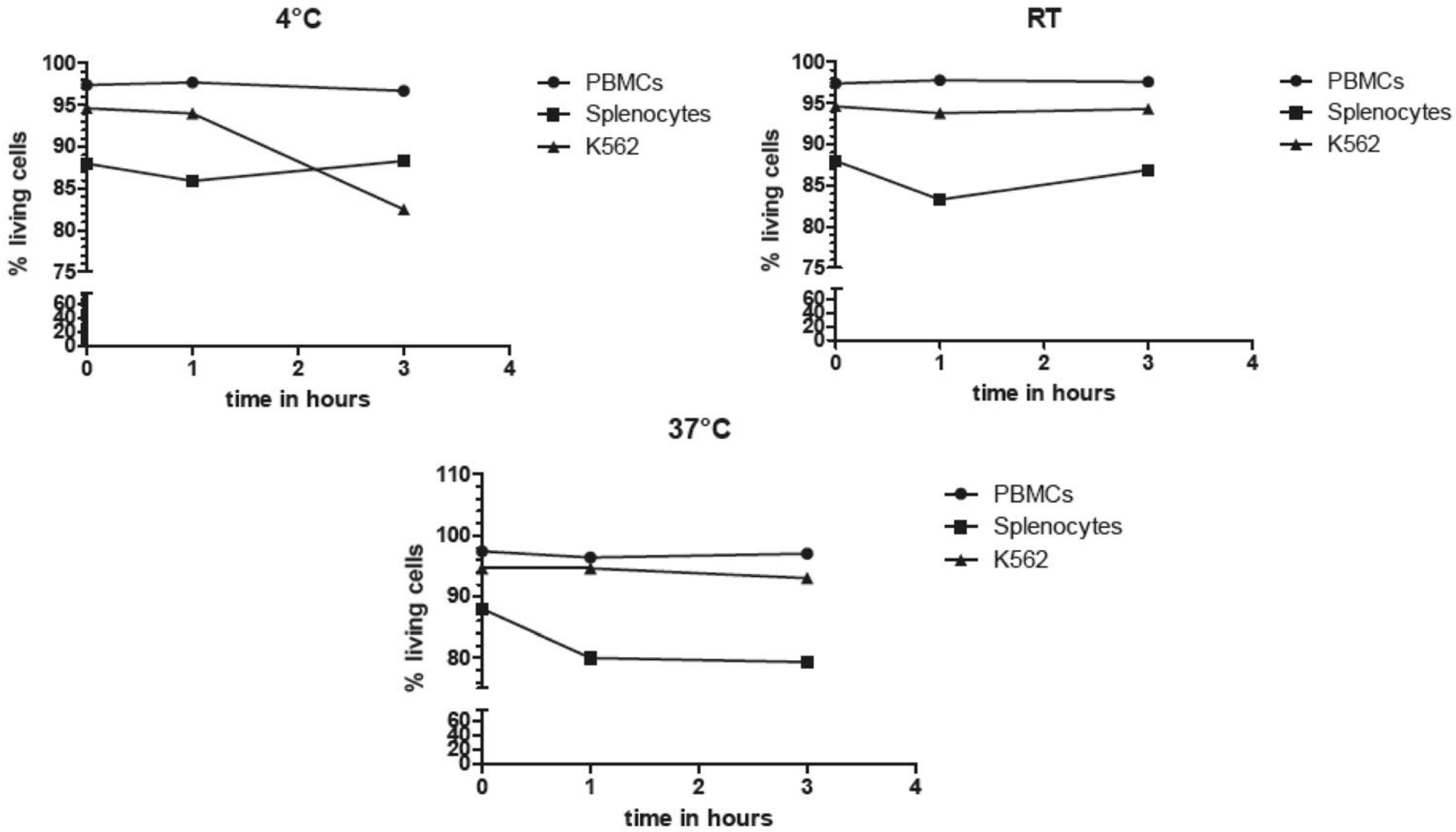
Temperature control on the MoFlo XDP™ fluidic system



Sorting with temperature control:

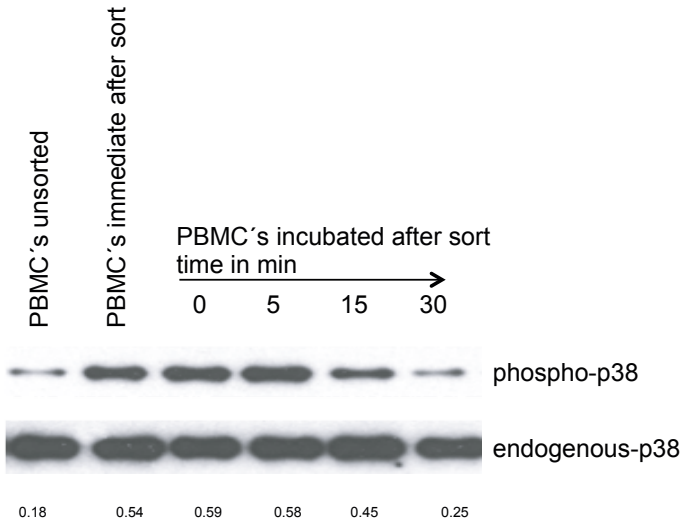
Stable from 7° C up to 30° C

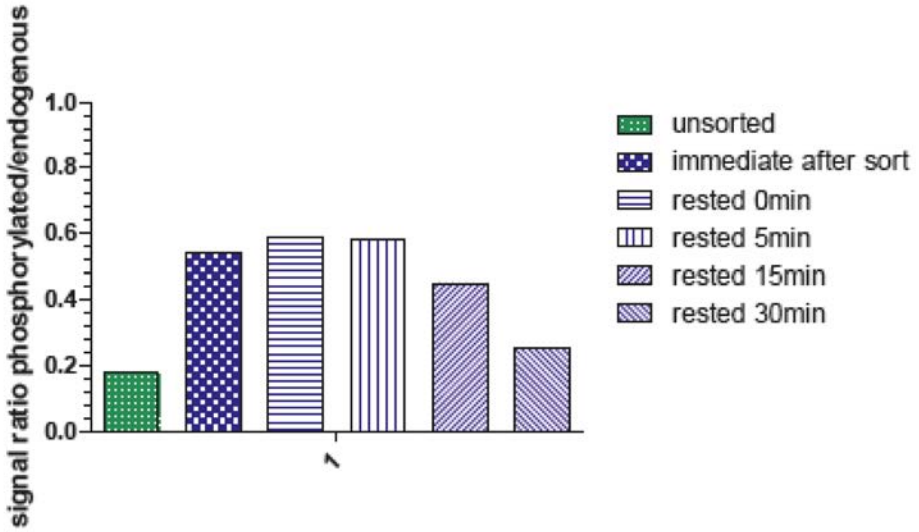
Cell death after temperature storage by propidium iodide (PI) staining

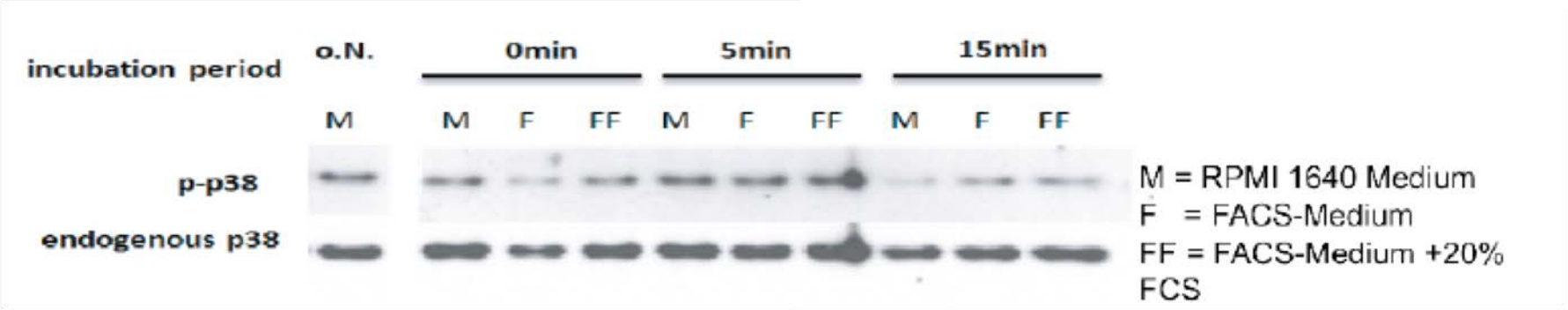


Flow cytometric analysis of cell death after temperature storage by propidium iodide (PI) staining. Graphs show percentages of living cells in a time course for human PBMCs, murine splenocytes and K562 cells.

Phosphorylation of the MAPK p38 signaling pathway

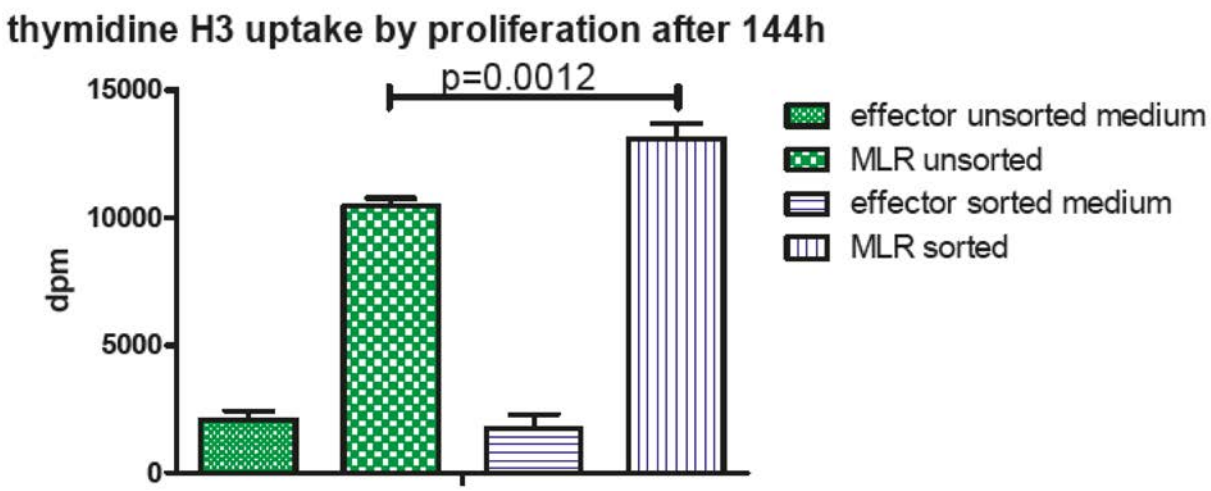


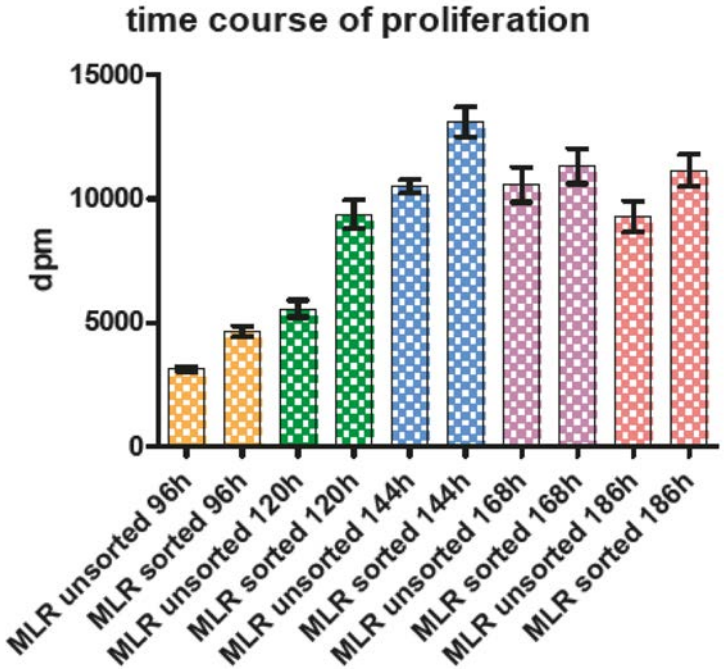




Immunoblot (top left graph) for phosphorylated p38 in ficoled PBMCs from BuffyCoat Donors, with exclusion of Buffer related phosphorylation effects (lower graph). p38 signal strength was determined via ImageJ Software shown as a bar chart (top right graph).

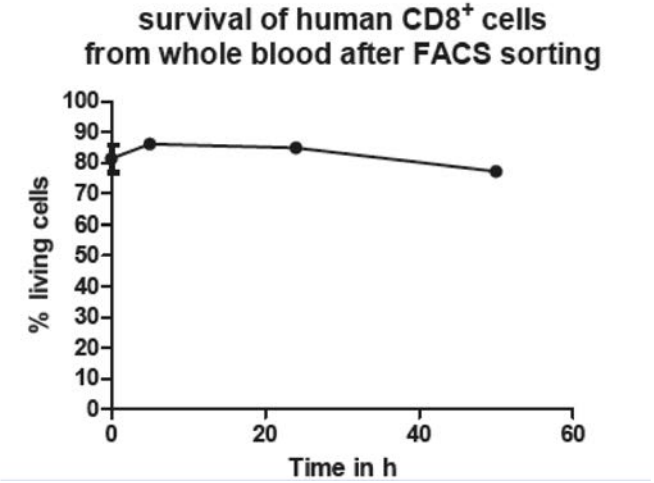
Functional Analysis of sorted cells



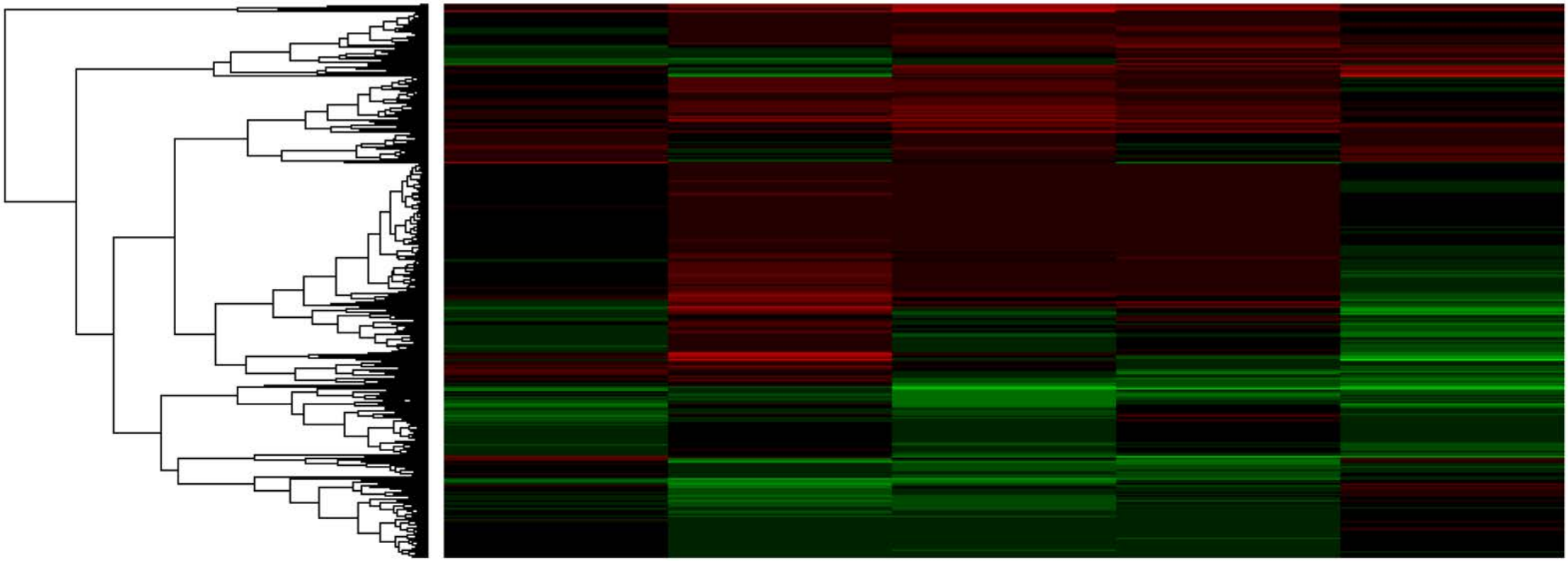


Two graphs (both left) show the proliferation based thymidine uptake of sorted CD8 positive cells in a mixed lymphocyte reaction (MLR).

Lower graph displays the survival of sorted CD8 cells from whole blood in a time course over two days.



mRNA levels via MicroArray



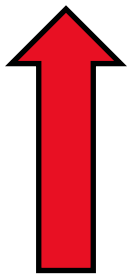
sorted – 0h
unsorted - 0h

unsorted
4h
resting

unsorted - 0h
sorted – 4h

over time
sorted – 4h

sorted – 4h
unsorted - 4h



Conclusion and Discussion

- We were able to observe sorting induced cell alterations in different cell lines, as well as altered cell physiology.

- Primary cells, specially PBMCs and T-cells seemed very robust und unaffected by the use of FACS.

- The parameters evaluated in this project could also be applied to other clinical cell processing methods.

- mRNA levels via MicroArray
  - no activation – more down regulation

General problem: remaining sort-marker

- changes of the cell population

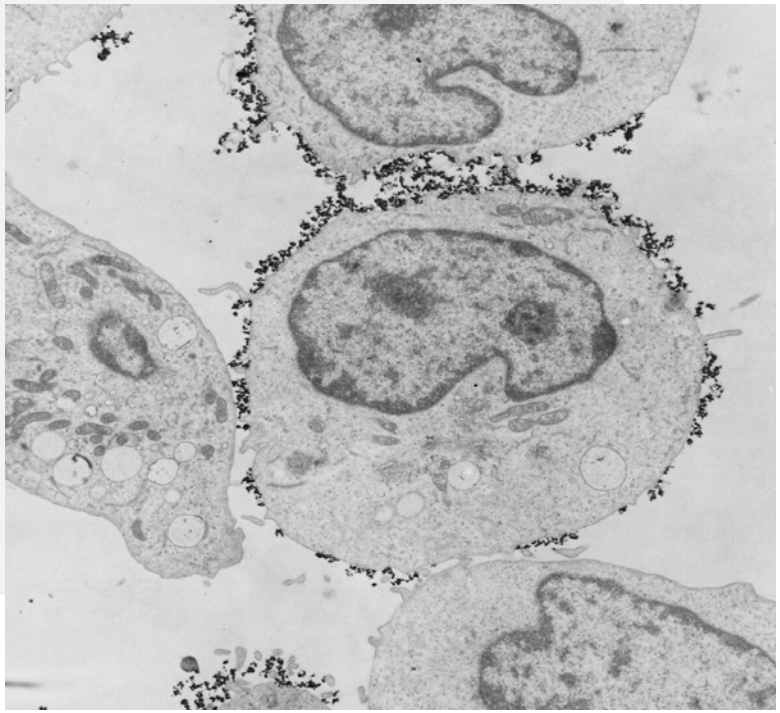
cross linking, activation, cell death, receptor blockade

- clinical issues

toxicity, immunogenicity, allergic reactions

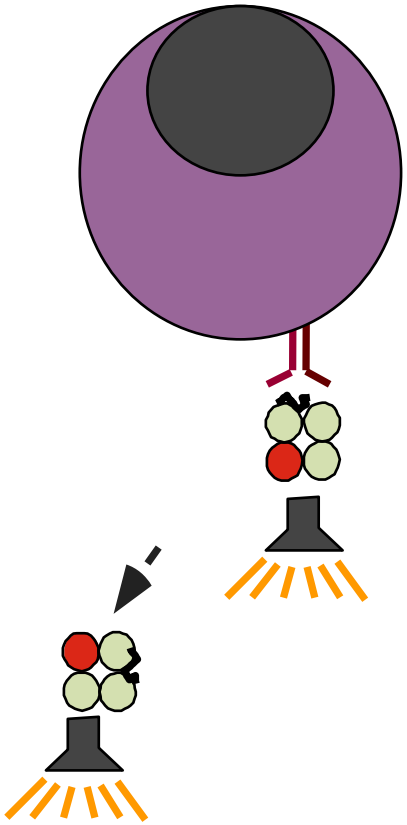
- regulatory/economical aspects

difficult process for reagent approval



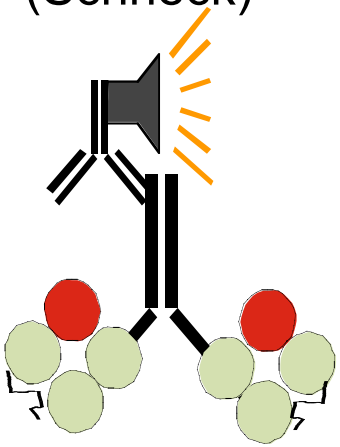
MHC Multimers

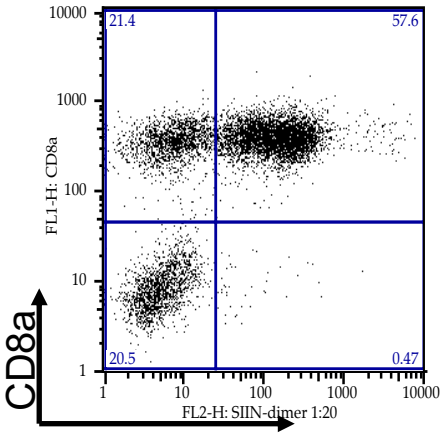
MHC monomer



MHC dimers

(Schneck)

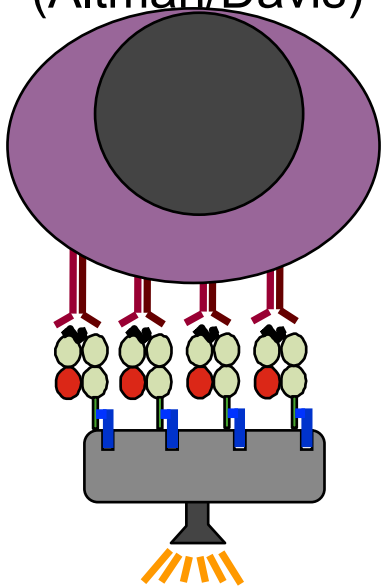


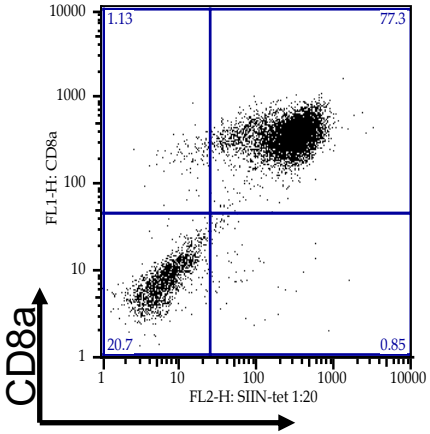


H2-Kb/SIINFEKL dimer

MHC tetramers

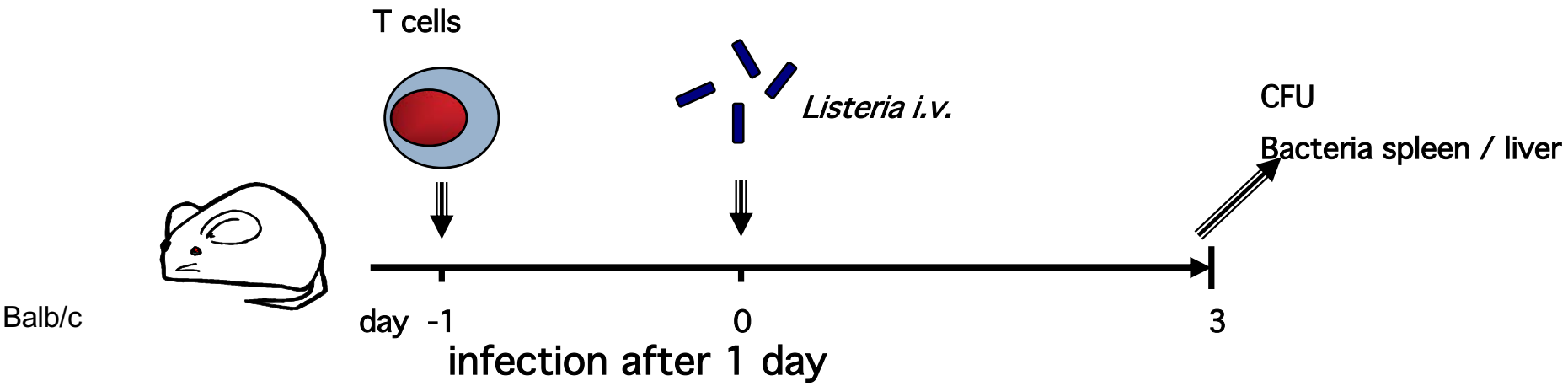
(Altman/Davis)

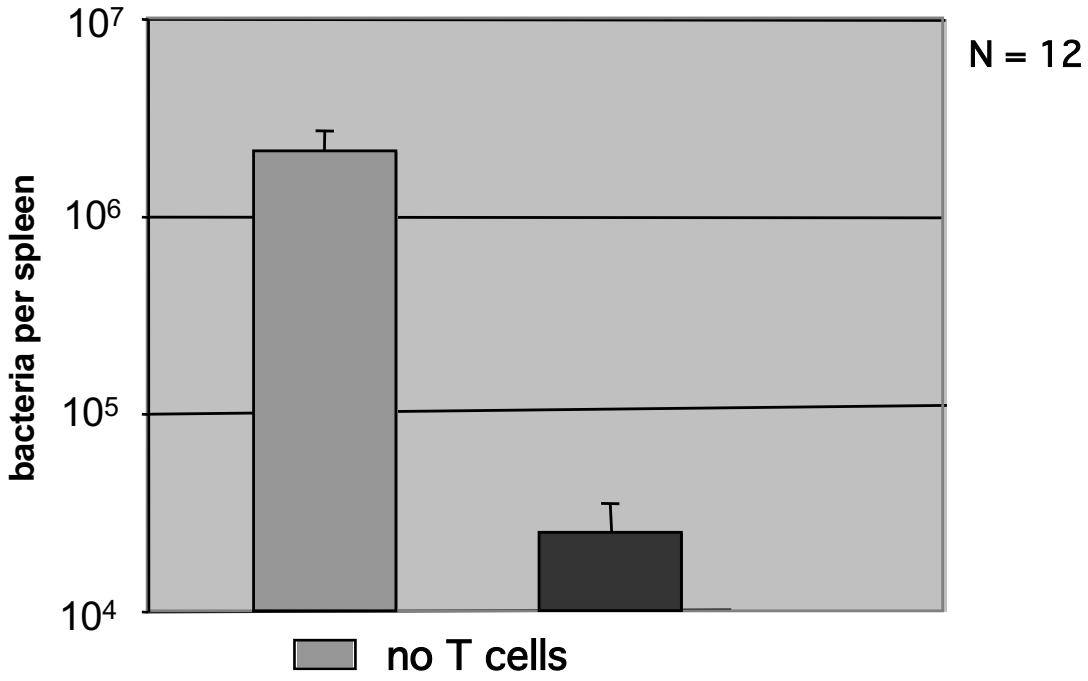




H2-Kb/SIINFEKL tetramer

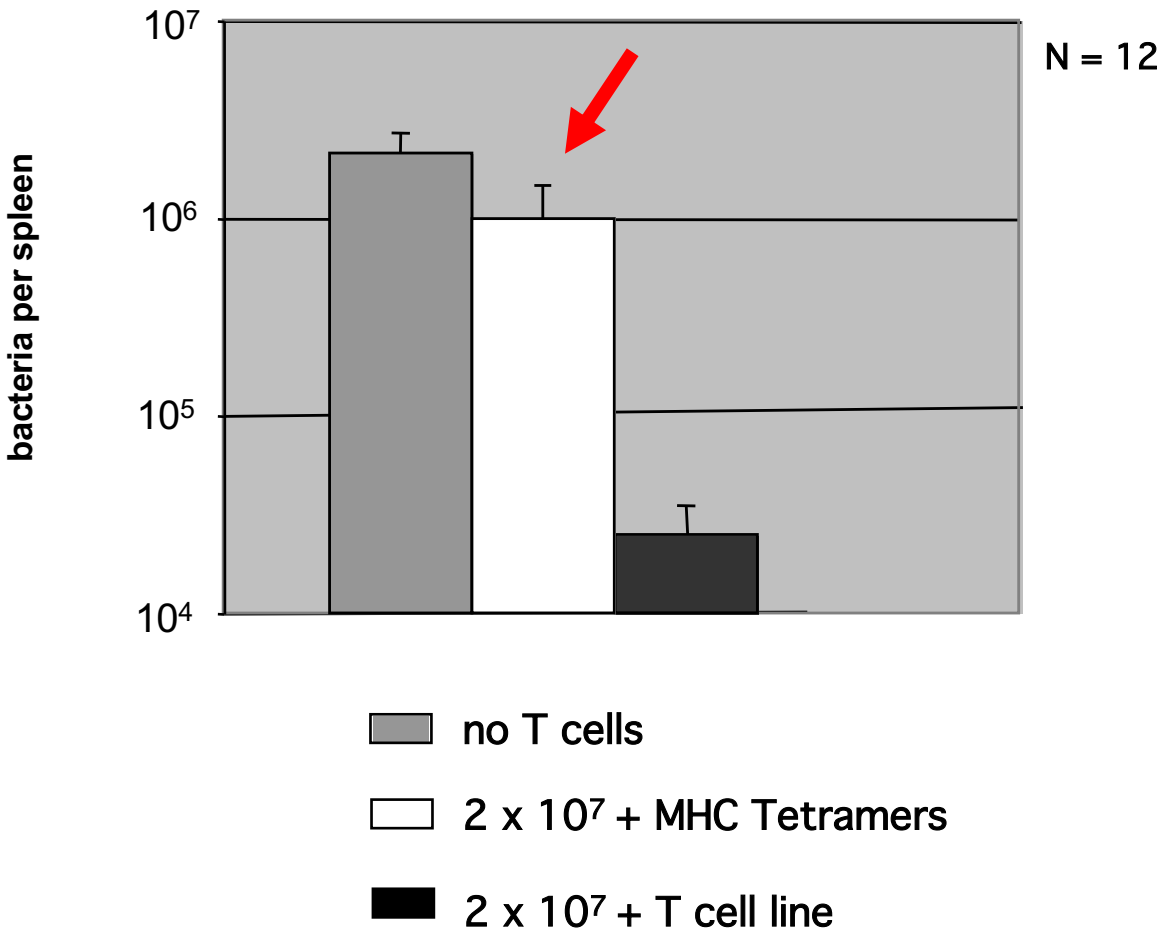
MHC Multimers - adoptive Transfer





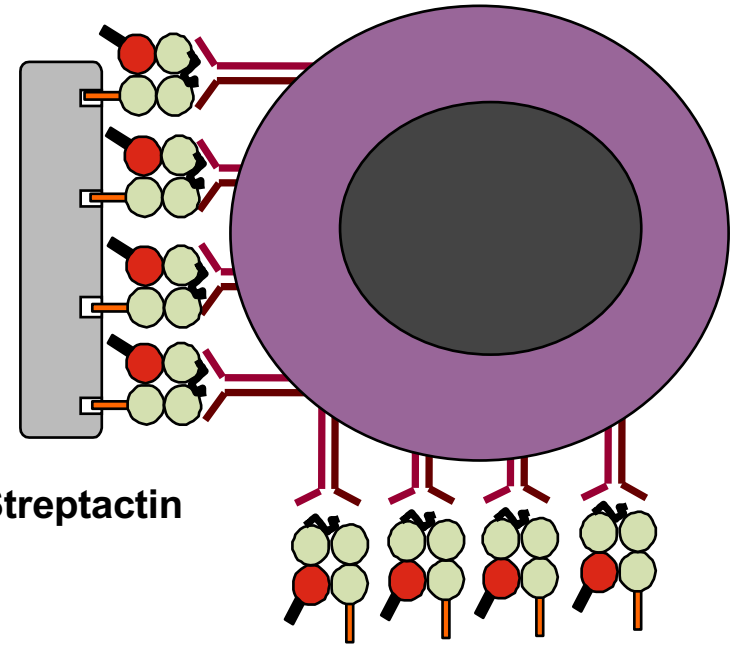
LLO_{91-99}-specific T cells

MHC Multimers - adoptive Transfer



LLO_{91-99}-specific T cells

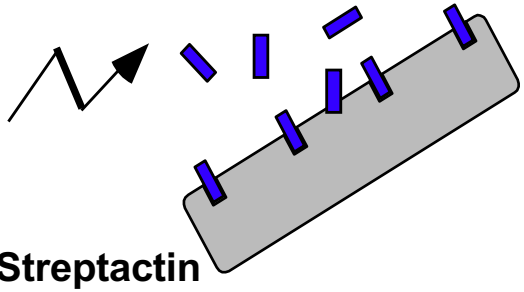
Reversible MHC Multimers - Streptamers



StreptagIII/Streptactin

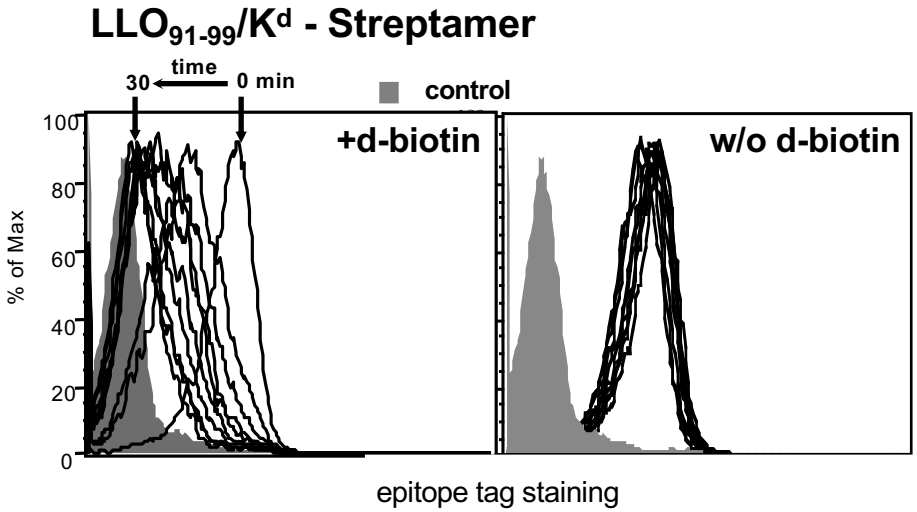
K_D 10^{-6} M

d-Biotin



d-Biotin/Streptactin

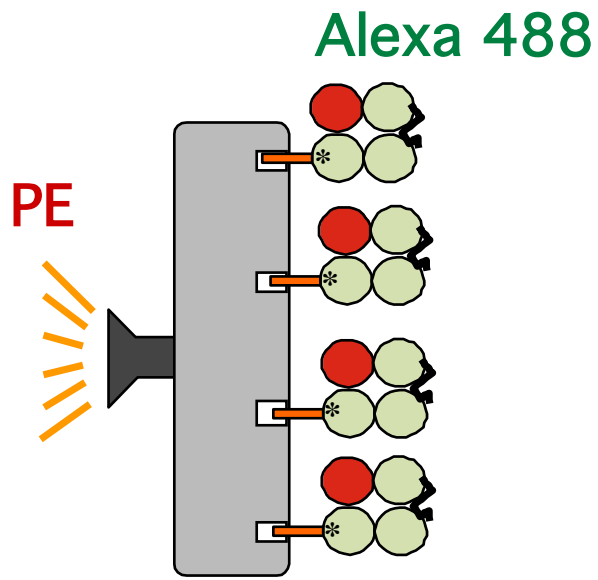
K_D 10^{-12} M

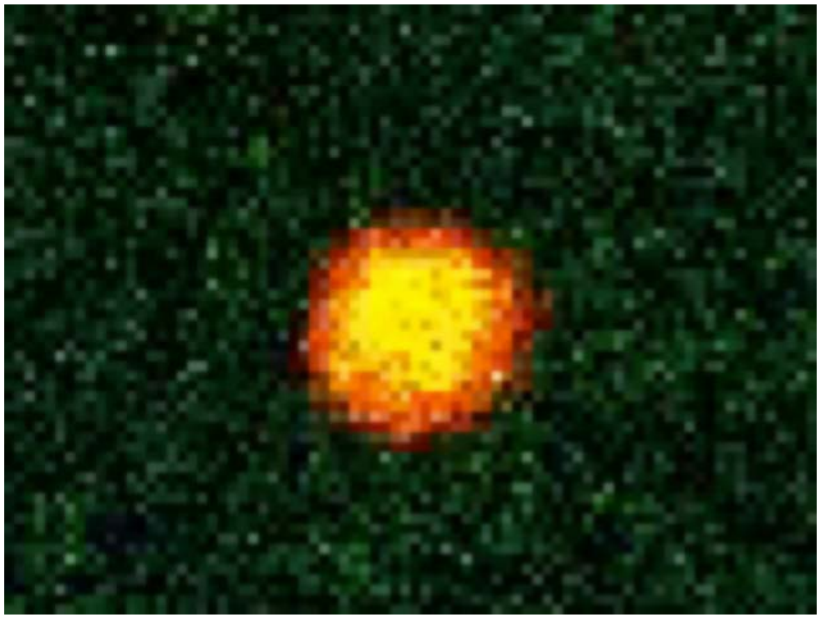


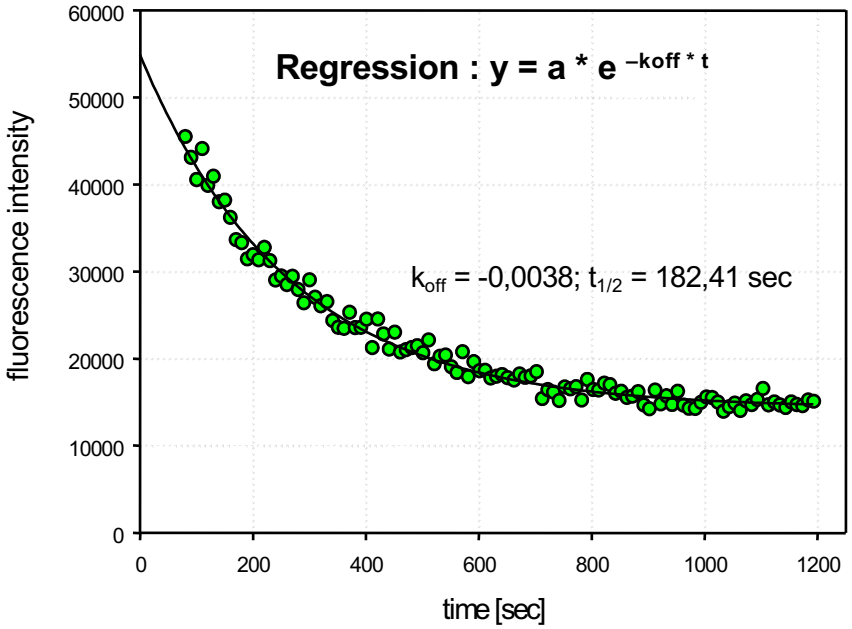
Knabel, Franz, Schiemann et al.

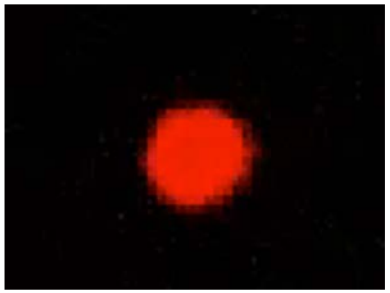
Nature Medicine 2002

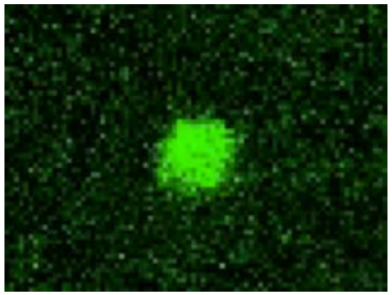
MHC Streptamers



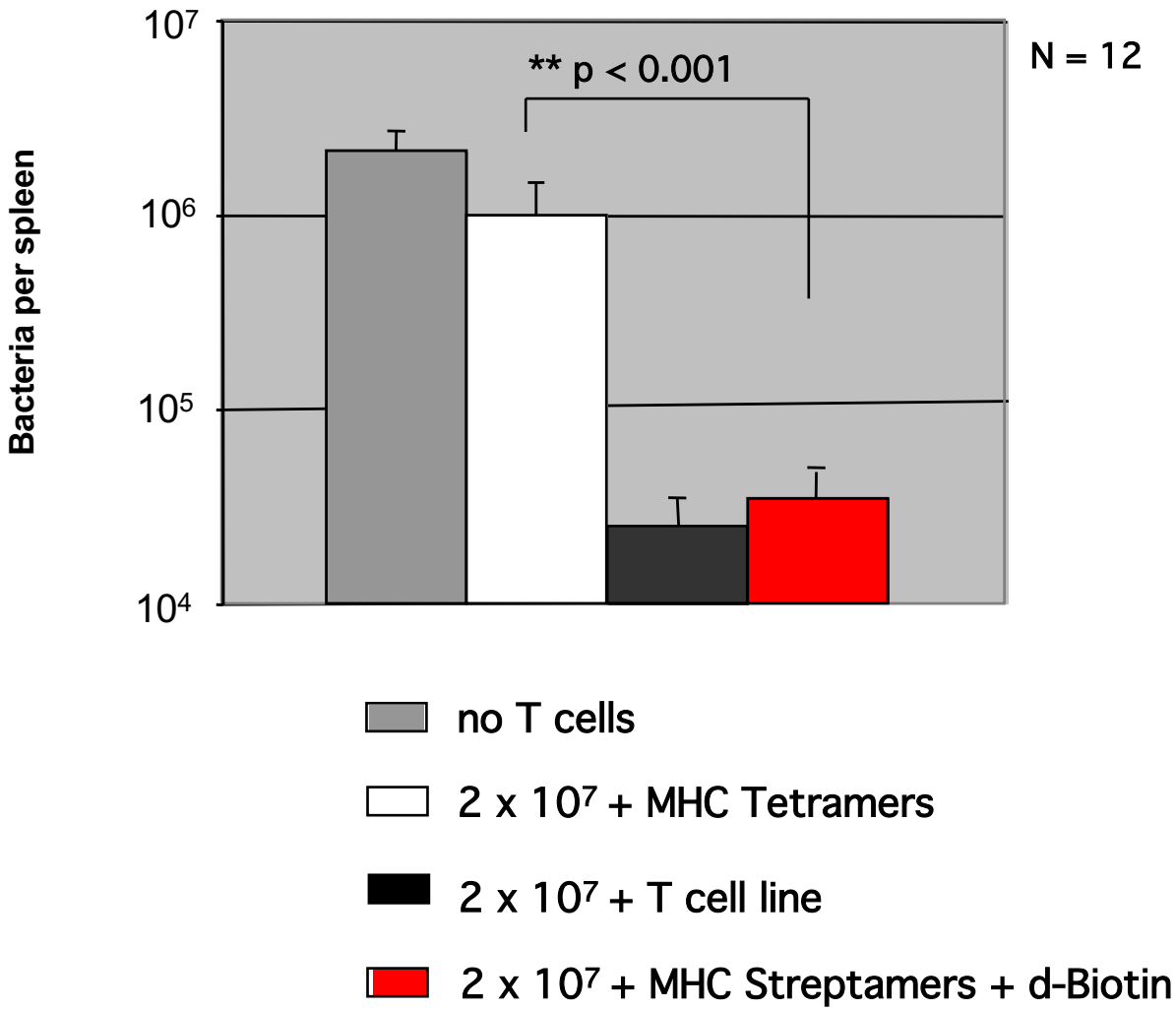






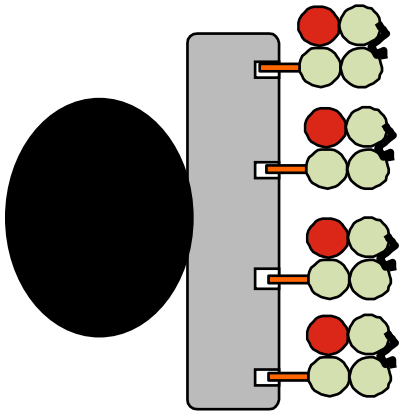


MHC Streptamers and effective adoptive Immunotherapy



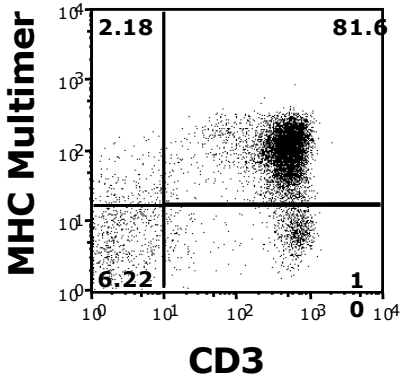
LLO_{91-99}-specific T cells

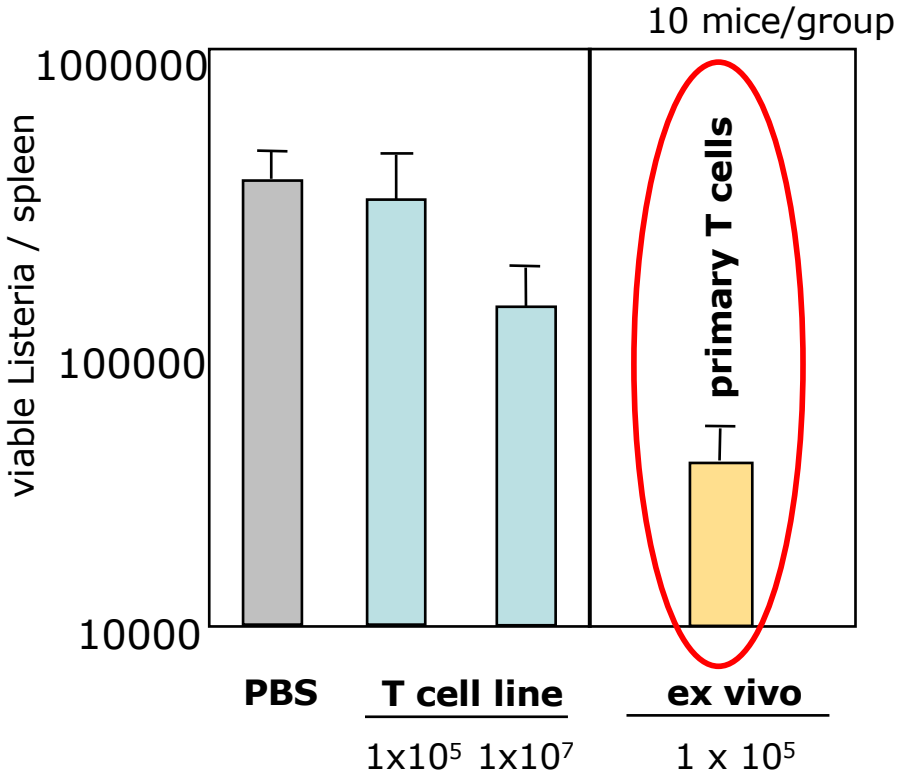
Directly *ex vivo* sorted T cells



magnetic beads / reversible Multimers

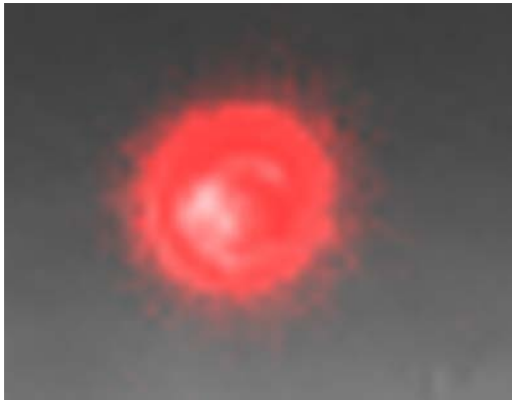
positive fraction





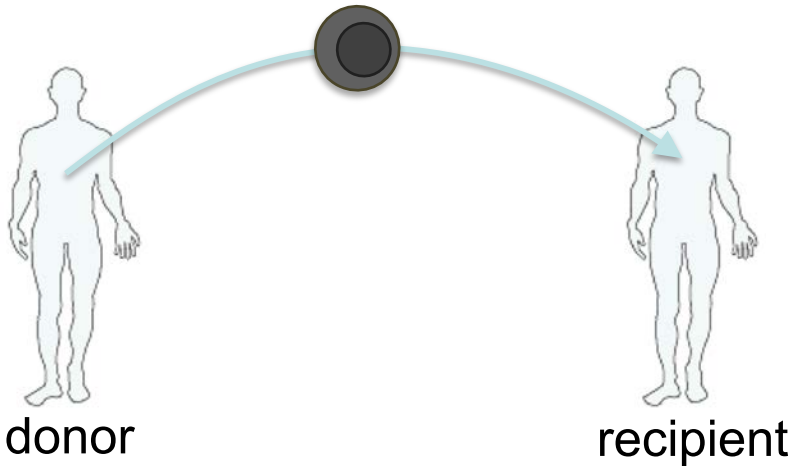
Preclinical research summary

- remaining marker (MHC multimers) can alter stained cells
- fully reversible staining with MHC Streptamers
- low numbers of primary T cells for effective adoptive transfer
- identification of highly effective memory subsets for adoptive transfer

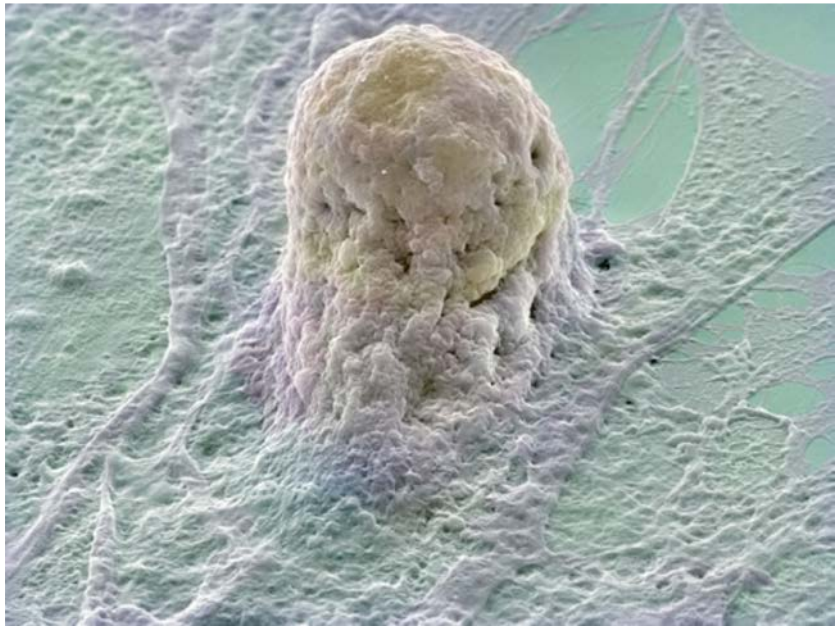


Cell therapy

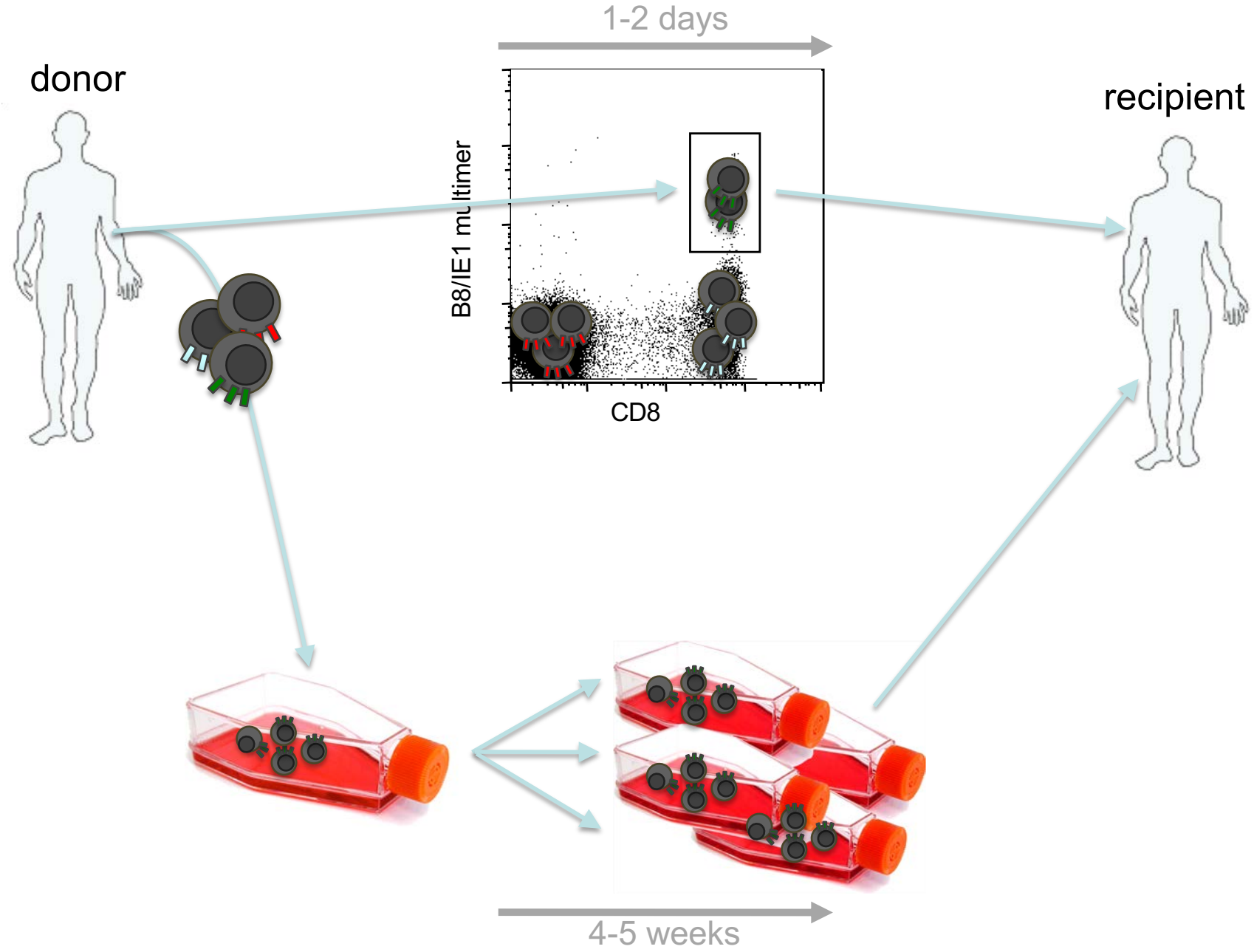
Increasing targets and applications



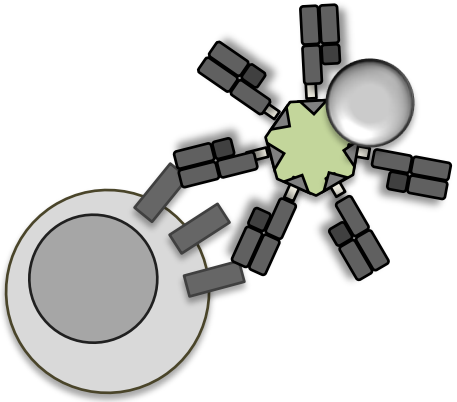
- hematopoetic / mesenchymal stem cells
- T cells (antigen-specific T cells, regulatory T cells)
- endothelia, chondrocytes, NK cells …….



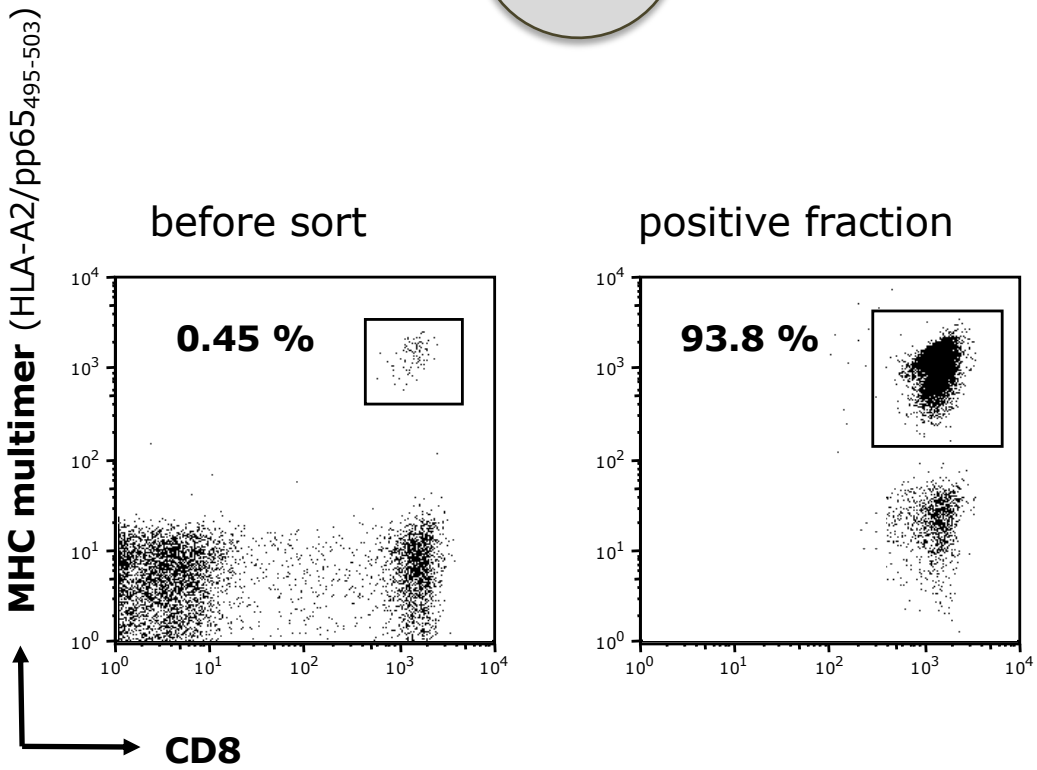
T cell therapy



Clinical cell sorting



magnetic particle



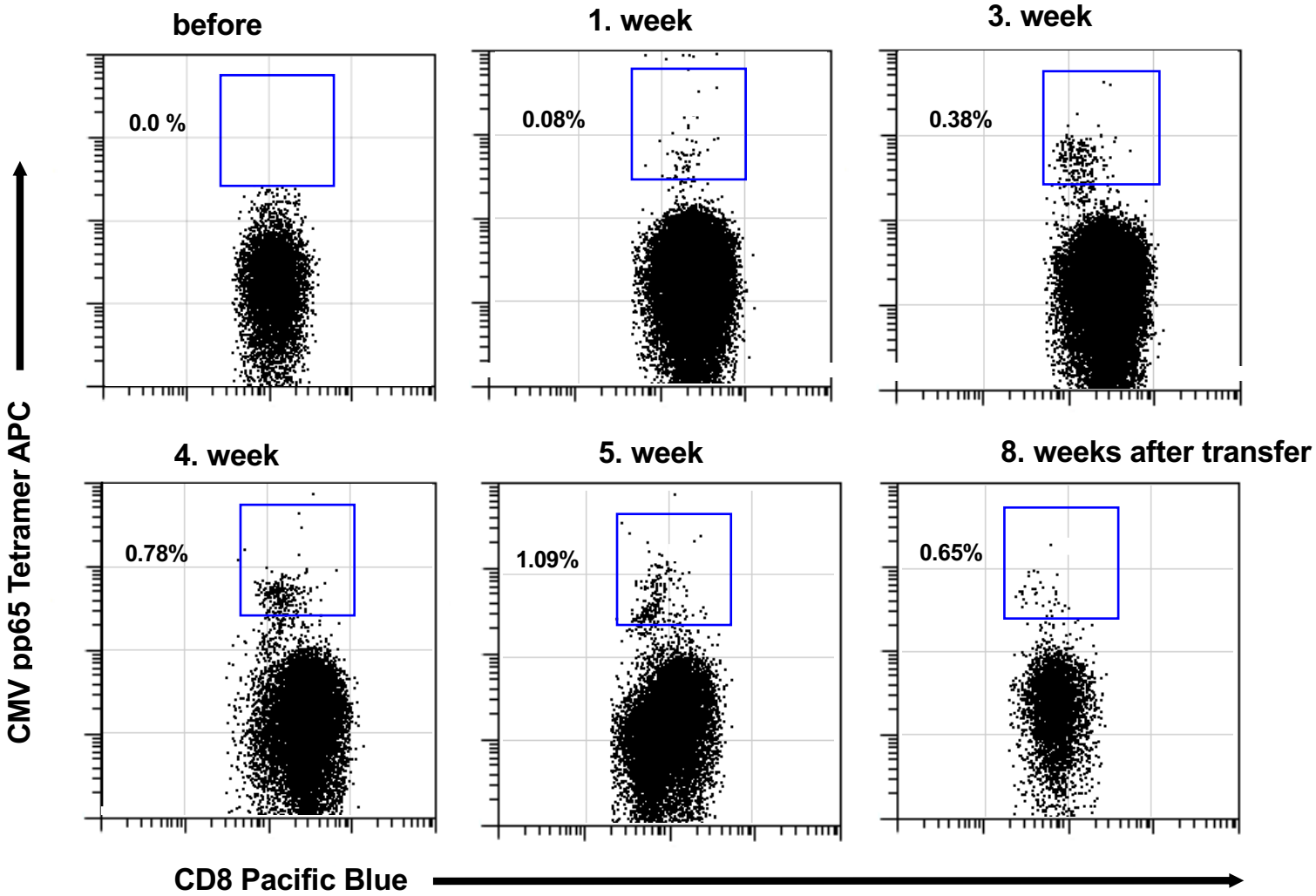
A2/pp65 -Streptamer-Beads

CliniMACS, Miltenyi

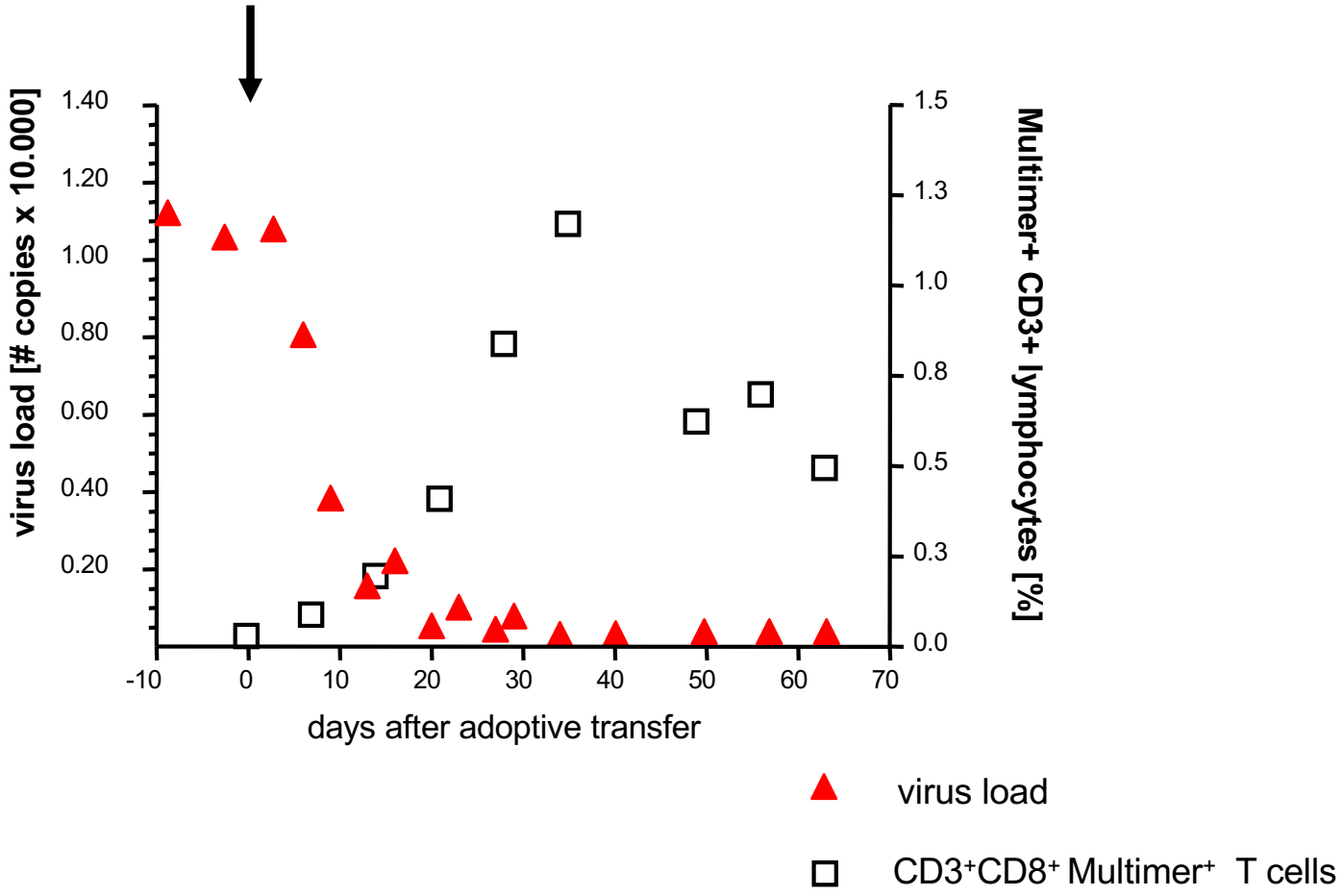
Example: Patient (Utrecht)

- 14 years, ALL, CMV positive
- stem cell transplantation (father)
- after 2 weeks, CMV reactivation, Ganciclovir resistant $\Rightarrow$ Foscarnet
- MHC Multimer-purification: $pp65_{495\text{-}503}$-specific T cells
- adoptive transfer of 100.000 specific cells

Immune-Monitoring upon T cell transfer

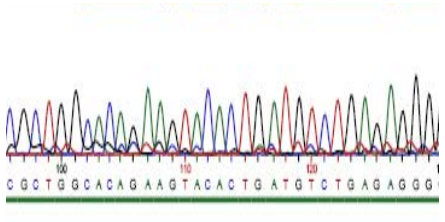


T cells, virus load

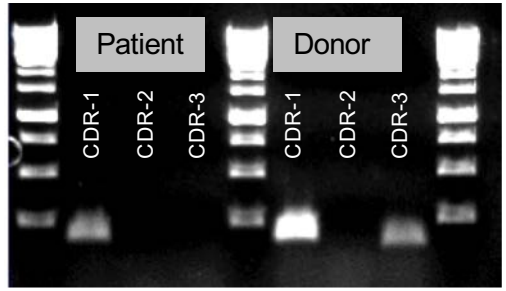


Single cell sort on 48 spot slides for CDR3-T cell Tracking in CMV patients

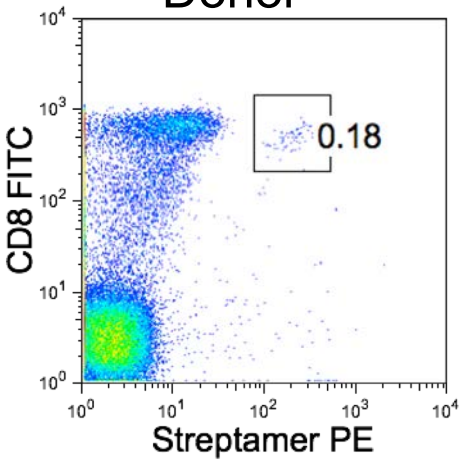
Bulk sorting of at least $5/10^5$ Donor T cell for PCR screening with V-beta specific primers



Identification of donor derived T cells by CDR3 specific primers



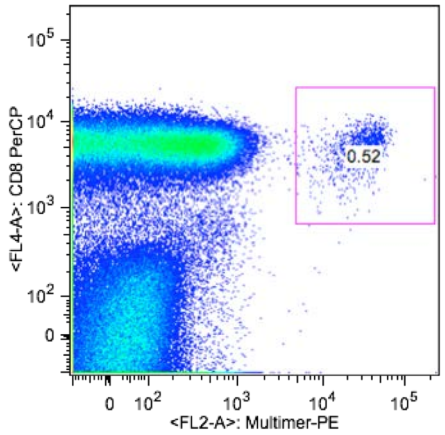
Donor

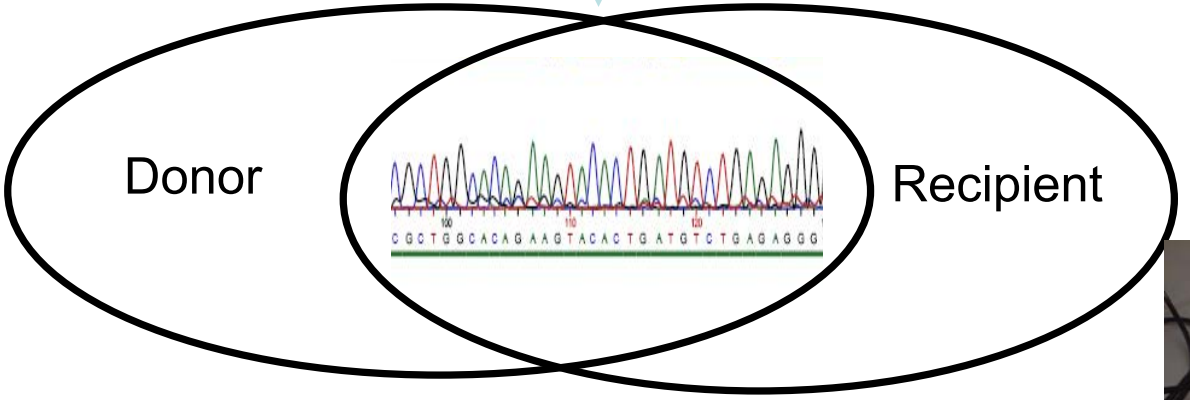


Sorting of 1 antigen specific donor T cells per PCR sample

Sorting of 1 antigen specific patient T cells per PCR sample

Patient



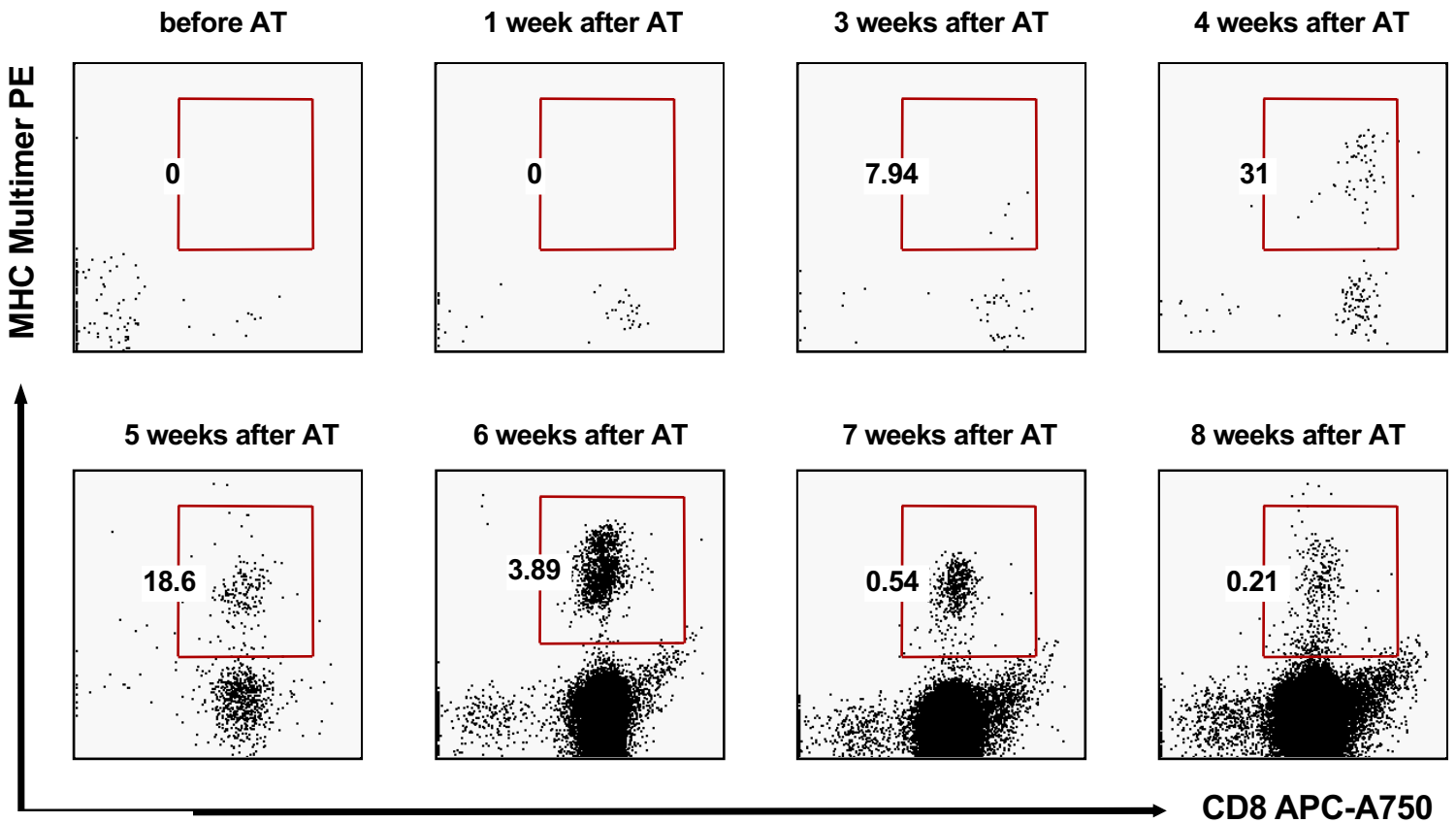


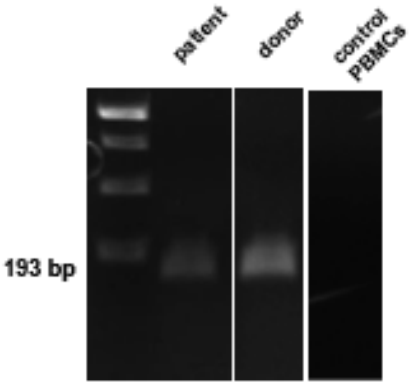
Patient (SCID, Tunesia)

A. Borkhardt (Düsseldorf)

- 8 Mo, severe combined immunodeficiency
- stem cell transplantation (father)
- CMV in urine, blood, ascites, trachea, liquor, skin biopsy
- Ganciclovir resistant
- Streptamer-purification: $pp65_{495\text{-}503}$ specific T cells
- adoptive transfer of 30.000 specific cells

Immunmonitoring upon T cell transfer





Sequence (Patient) V□13N …TGTGCCAGCTTTTTTGGGGGTGGGGATCAACC…

Sequence (Donor) V□13N …TGTGCCAGCTTTTTTGGGGGTGGGGATCAACC…

TCRVb13.6 BD2.1 BJ1.2

TUMCells

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Michael Neuenhahn **Nicole Frisch**

Julia Albrecht **Tanja Rossmann-Bloeck**

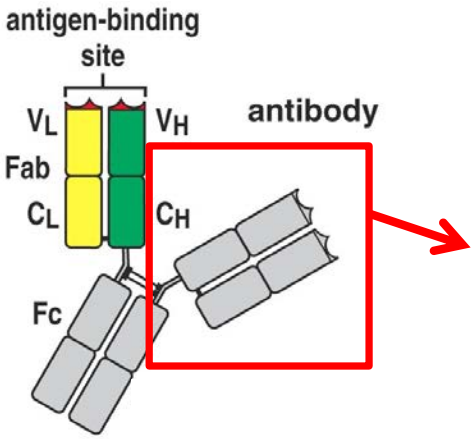
TUMCells
Technische Universität München
Zentrum für zelluläre Therapien

Products - designed for the production of:

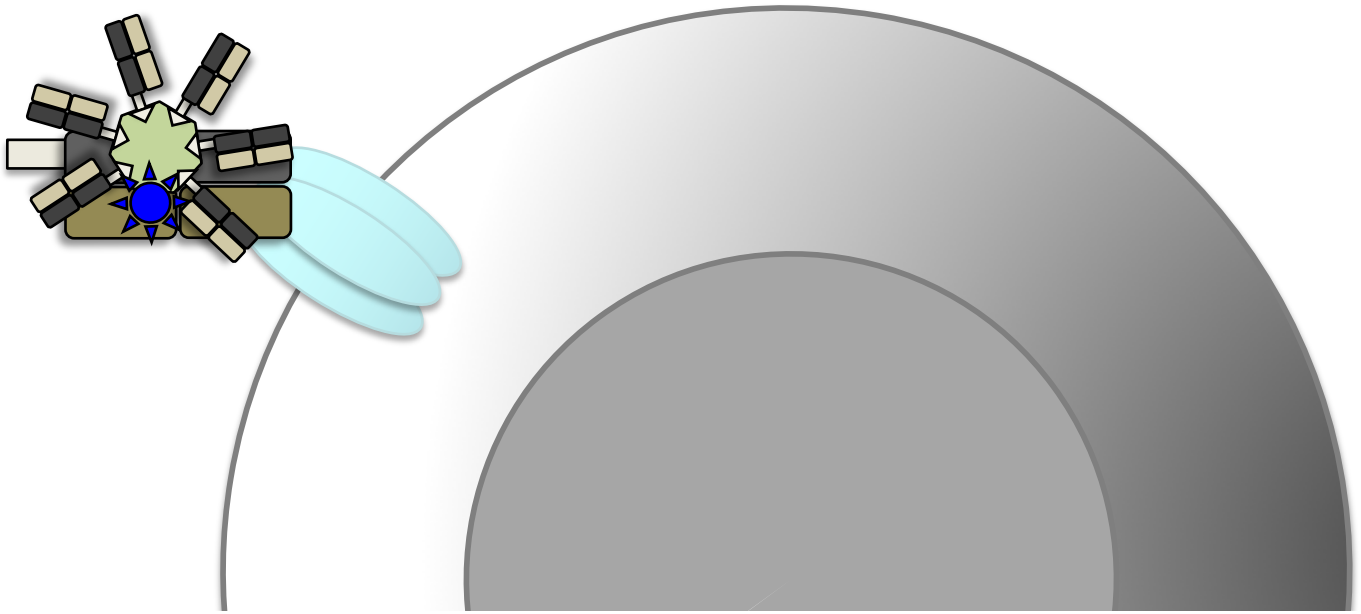
- **Blood cell products**
- **Investigational drugs**
- **Advanced Therapy Medicinal Products (ATMPs), which include:**
  - **Somatic cell therapy; Gene transfer drugs; Tissue-engineered products (combination products).**

Clinical cell processing

Interaction of TCR – MHC Streptamers ➔ only T cells are accessible

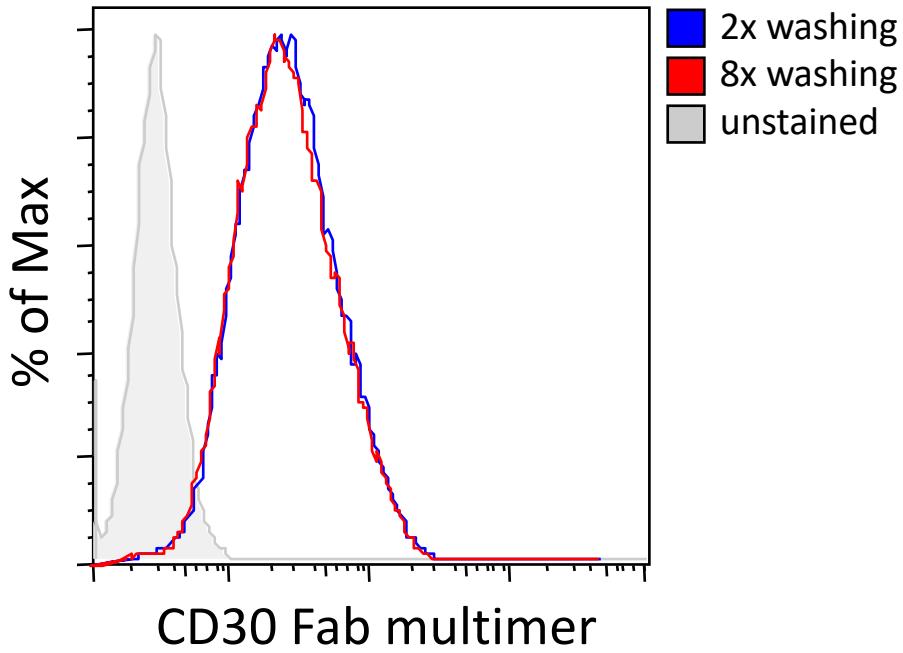


- recombinant Fab-streptag monomers
- if necessary, affinity modification



Fab Streptamers

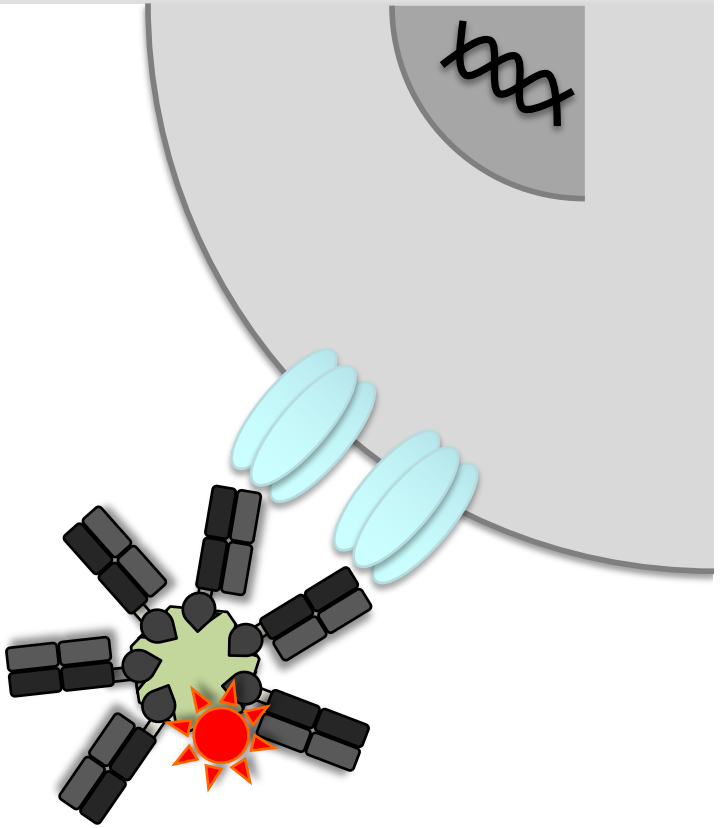
Stable binding of αCD30 multimers even after excessive washing

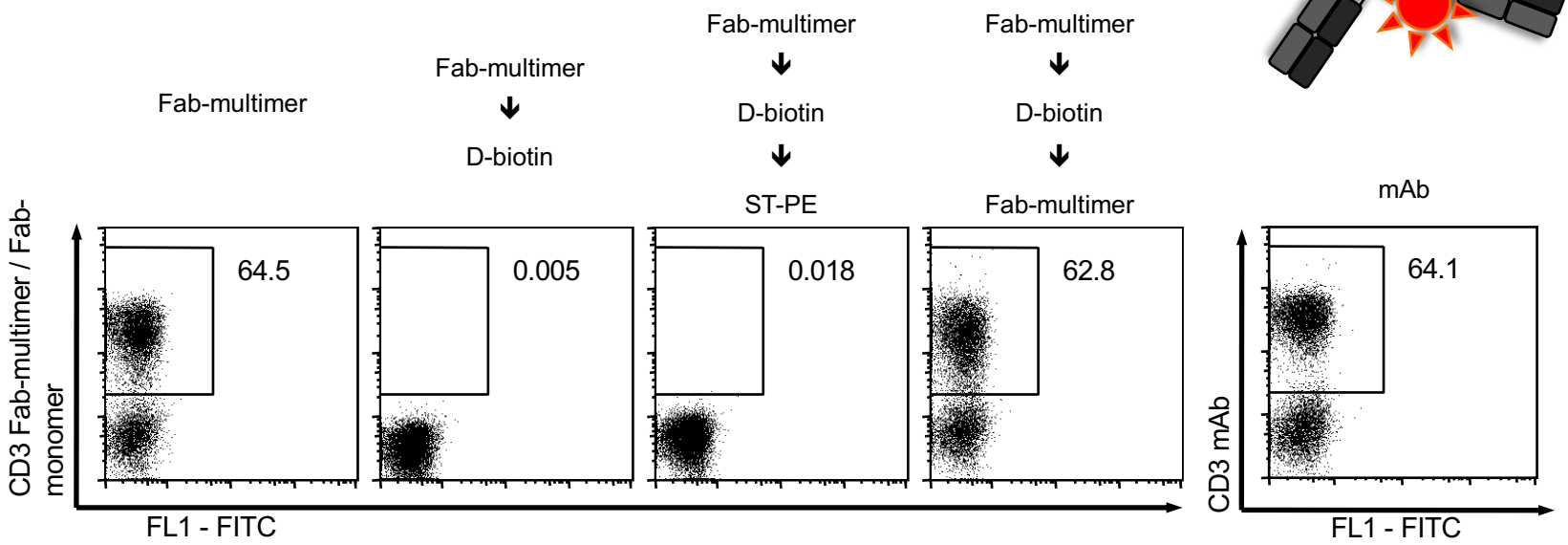


Fully reversible staining with Fab-*Strep*tamers

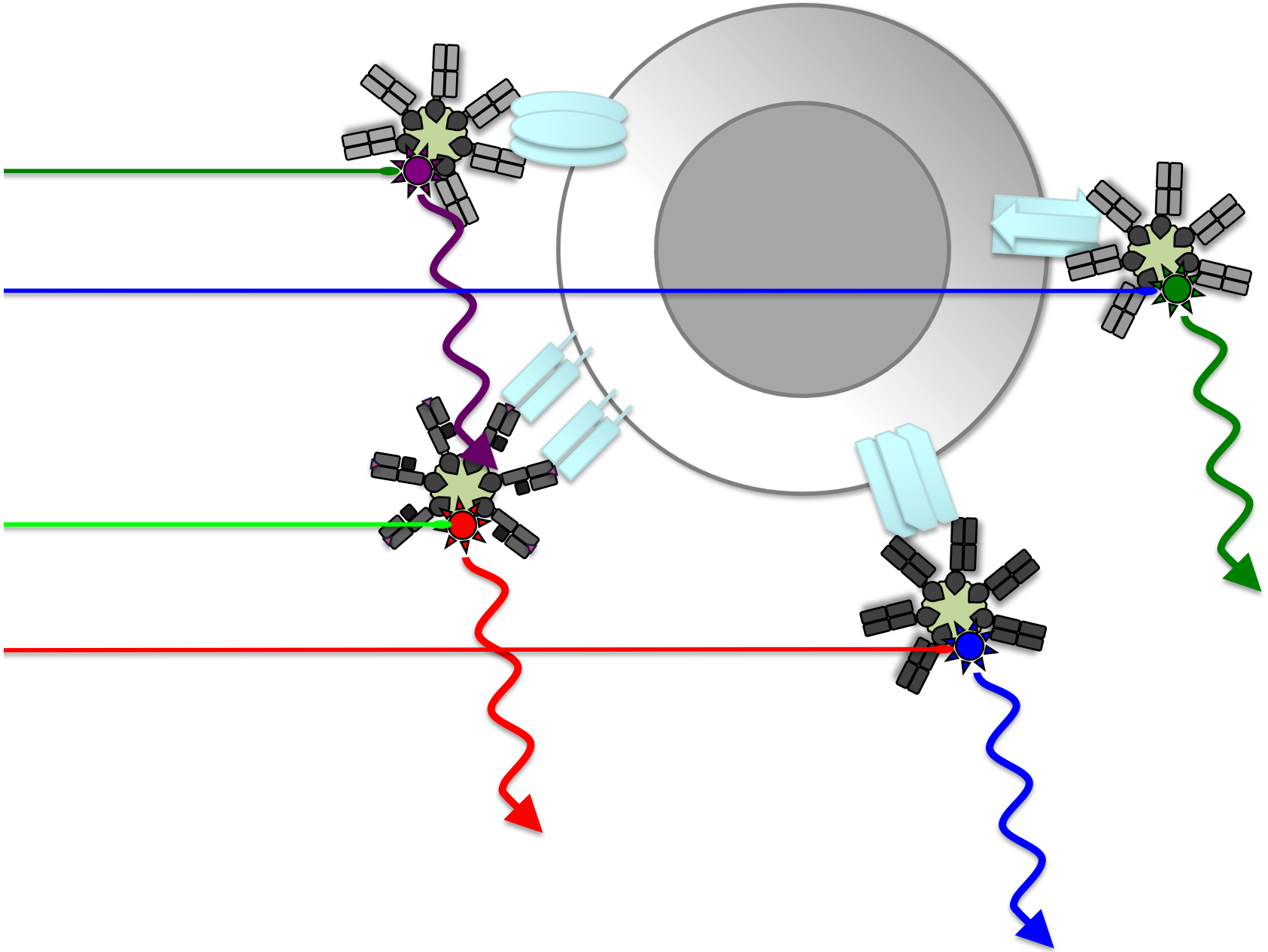
Stemberger, Dreher, Tschulik, Schiemann et al.

PloS One 2012

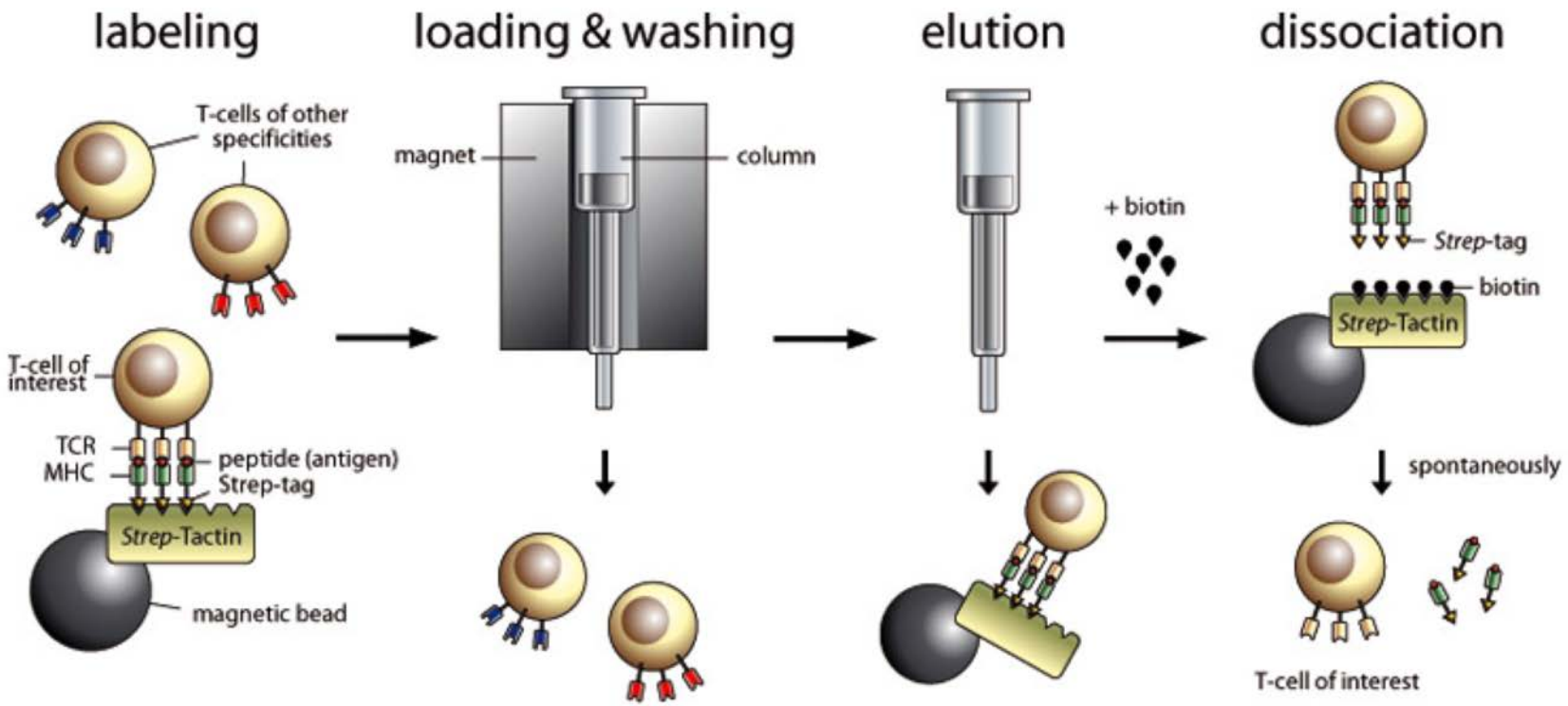




Multiplex Streptamer staning and sorting



Method – “untouched” cells



www.iba-lifesciences.com

Fully reversible staining with Fab-Streptamere

www.iba-lifesciences.com

www.fabian-online.com

FAB.ion®
CELL SELECTION
REINVENTED

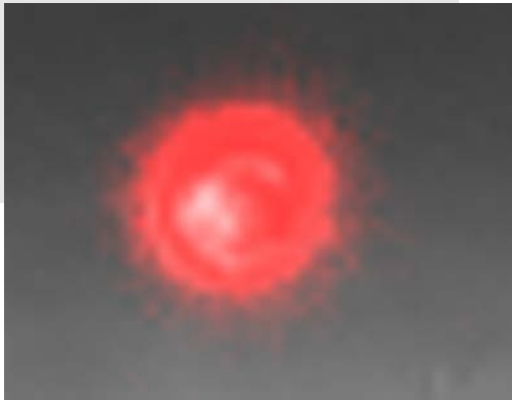
>90% purity

Cell selection and expansion:

- **FABian® for automated cell selection - with reversible reagents**
- **PBMCs, T and B cell isolation directly from whole blood without magnetic beads and density gradient centrifugation.**

Summary

- MHC Streptamers
- Tool for basic research and clinical applications
- Fab Streptamers – transfer of Streptamers to virtually any cell surface marker
- Reversibility as a standard for clinical cell purification



From bench to beside - collaboration groups

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BECKMAN COULTER