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Flow Cytometry & Image Stream

Master Laboratory Techniques



Dr. Kristin Bieber and Simone Pöschel

29.01.2018





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Flow Cytometry Core Facility - FCF Berg



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University Hospital Tübingen
"Hill" (Berg) Hospitals



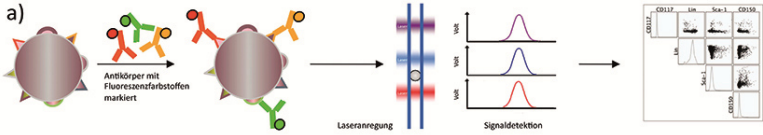
[https://www.medin.uni-tuebingen.de/Forschung/Forschungsinfrastruktur/
Core Facilities des IZKF/Durchflusszytometrie](https://www.medin.uni-tuebingen.de/Forschung/Forschungsinfrastruktur/Core%20Facilities%20des%20IZKF/Durchflusszytometrie)

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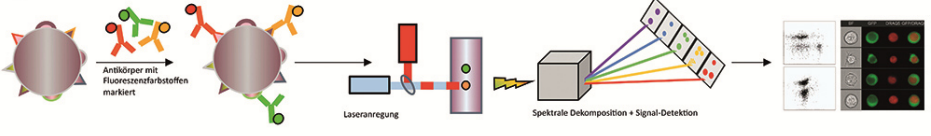
Today...




a)



b)




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Today...




Flow Cytometry

- Instrumentation: Into the black box
 - Fluidics
 - Optics
- Fluorochromes & Compensation
- Examples
- Service of FCF Berg

Image Stream

- Instrumentation
- Examples


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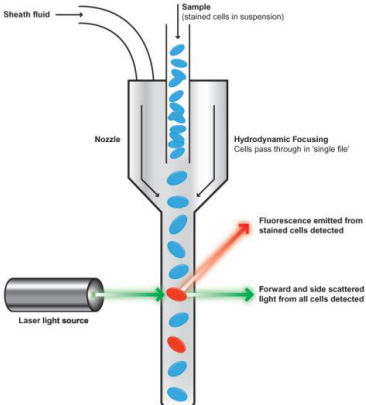
What means flow cytometry?



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- Flow ~ cells in motion
- Cyto ~ cell
- Metry ~ measure

> Measuring properties of cells while in a fluid stream



<https://www.withfriendship.com/user/mithunss/Flow-cytometry.php>



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What is flow cytometry?

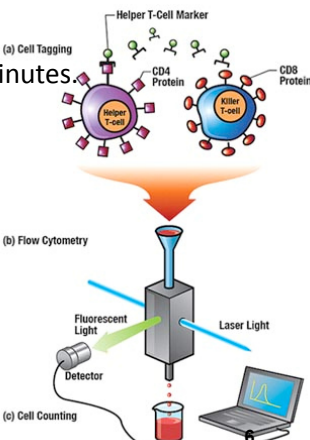


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- An instrument for making cell-based fluorescent measurements.
- Method for quantitating cellular or structural components of a cell using fluorescent antibodies or probes.

Allows analysis of tens of thousands of cells in minutes.

A method to sort and collect specific cell types.



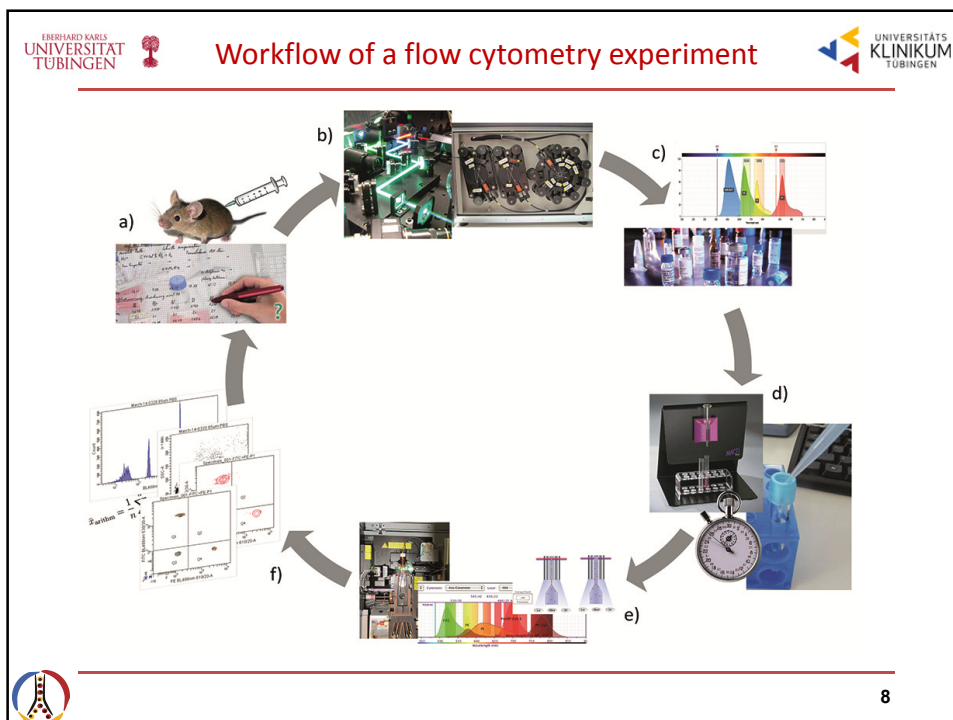
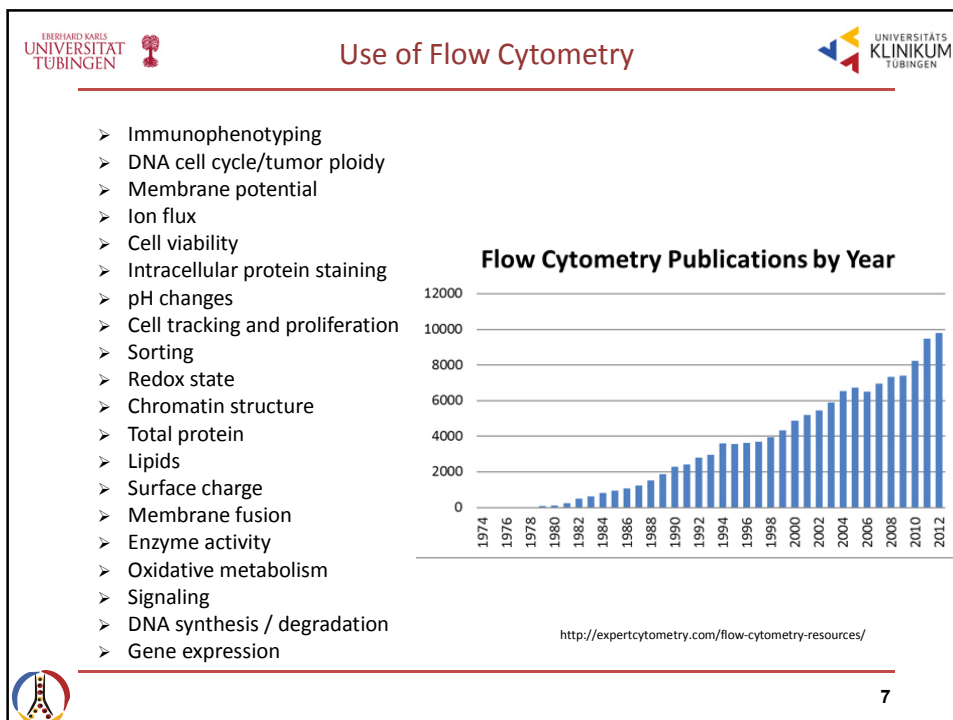
The BD FACS ARIA with sorter

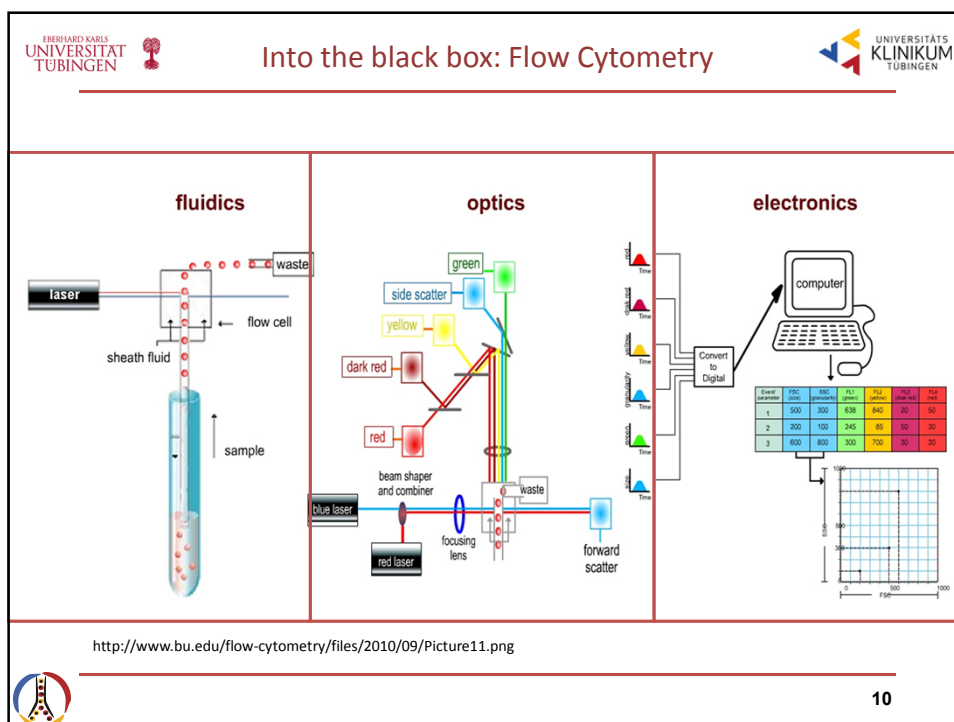
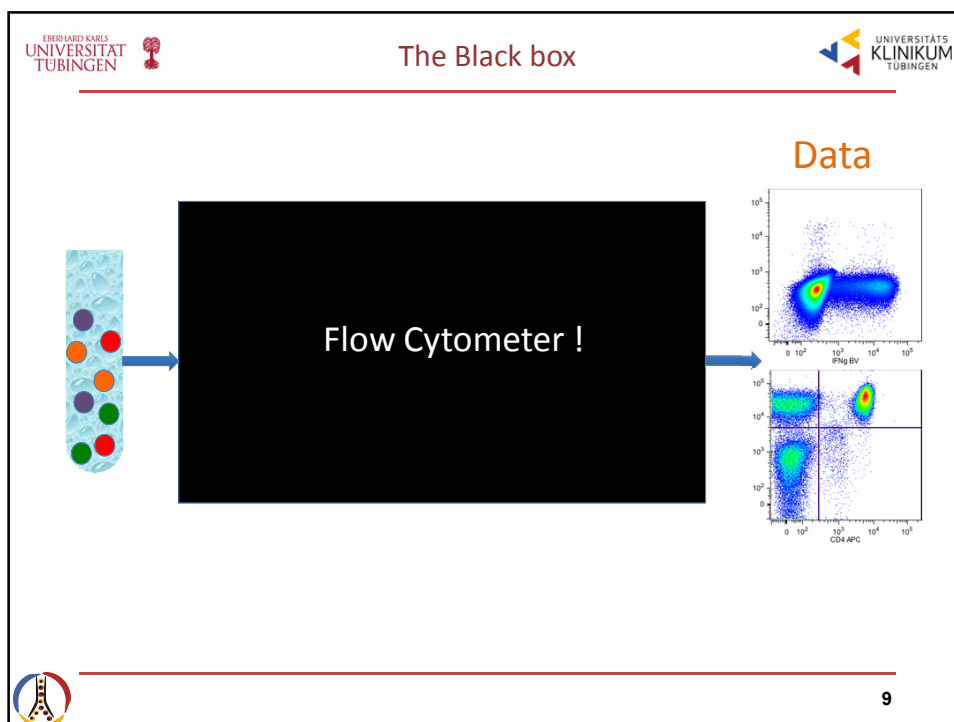


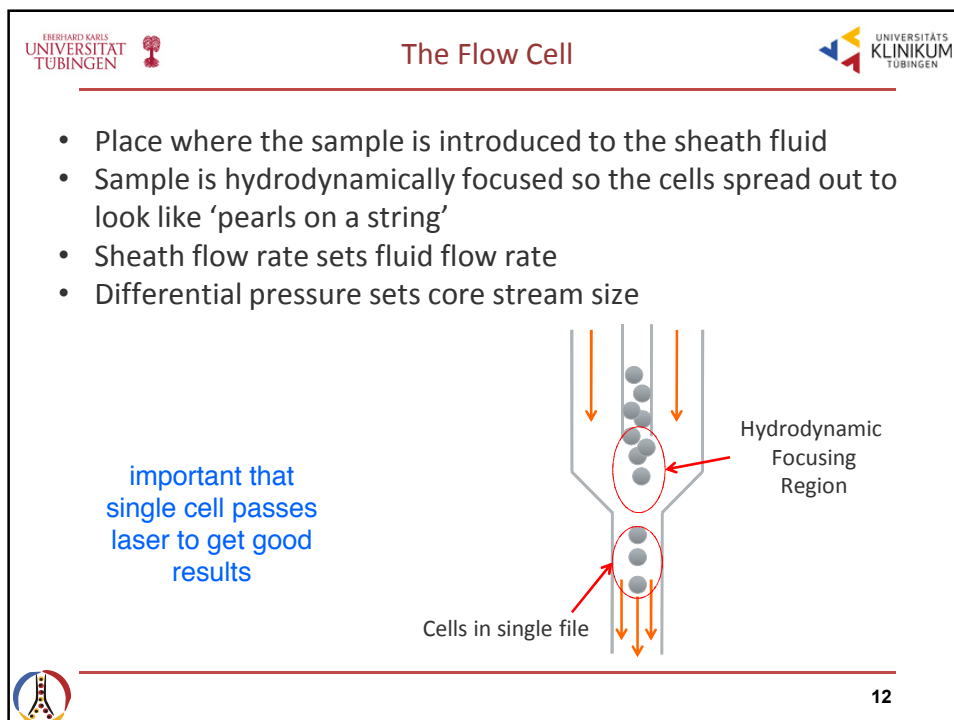
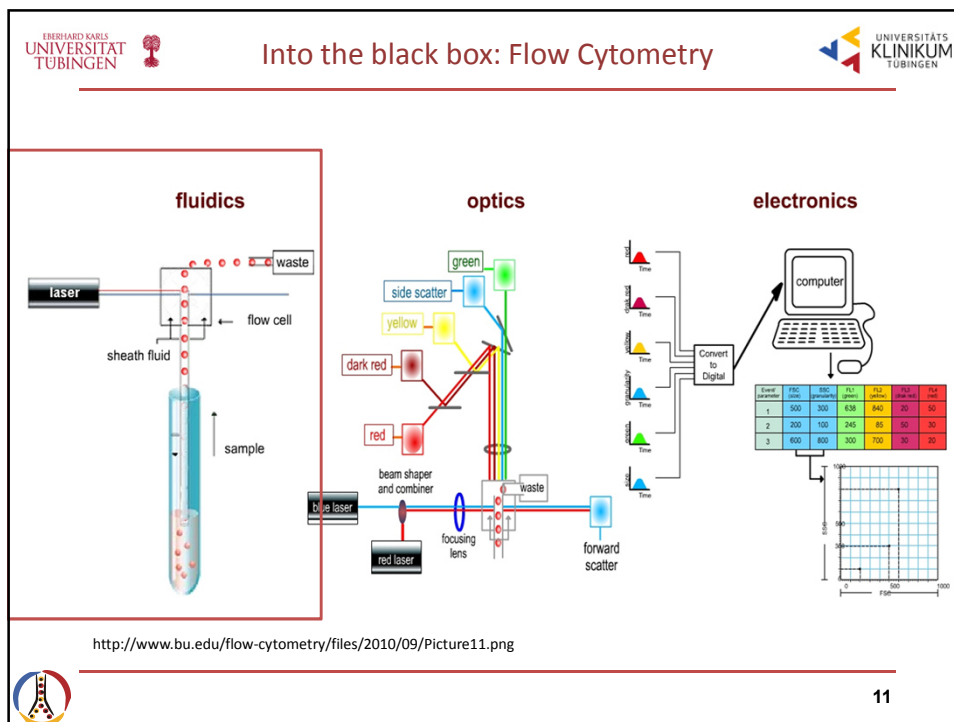


<https://www.withfriendship.com/user/mithunss/Flow-cytometry.php>

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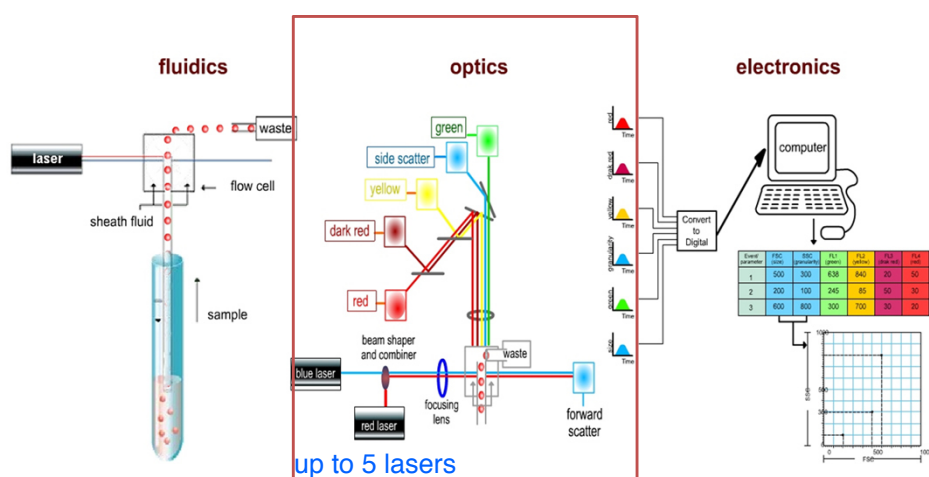


- Fluidics must be stable for cells to pass through the laser at the same point
 - Bubbles
 - Cellular disruption of the stream
- We want the cells to travel through the instrument in single file, where each cell will be exposed to the same intensity of illumination
passing laser beam one by one!!!!

Within the flow cytometer, the **flow cell** is the bridge between the fluidics and the optics



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<http://www.bu.edu/flow-cytometry/files/2010/09/Picture11.png>



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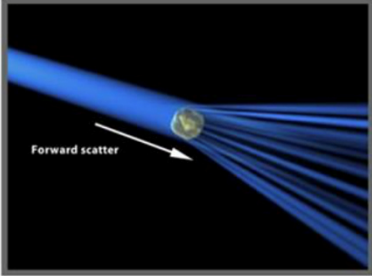


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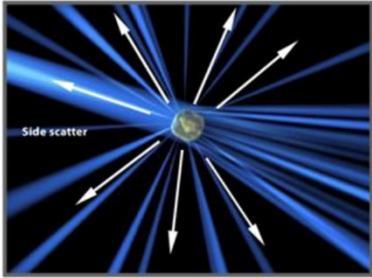
FSC & SSC



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cell surface



cellular components

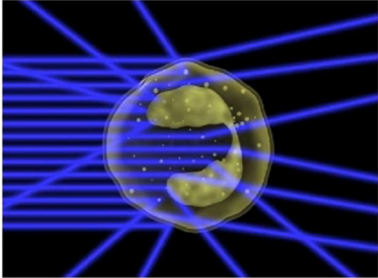


ABB.4 FORWARD SCATTER (FSC) UND SIDE SCATTER (SSC). FSC entsteht durch Beugung des Lichts an der Zelloberfläche, während SSC durch Beugung an Membranen und Vesikeln im Zellinneren entsteht. Quelle: <http://flow.csc.mrc.ac.uk>



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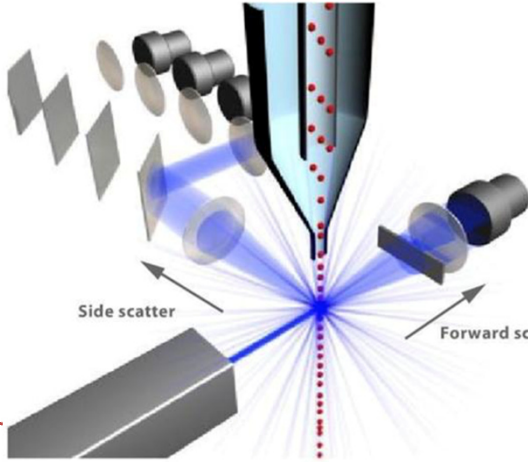
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Forward Scatter: FSC



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- Light that is scattered in the *forward* direction is detected in the Forward Scatter Channel.
- The intensity of this signal has been attributed to cell size



not how big the cell is but compared to other cell -> cell1 bigger than cell2 -> intensity is measured



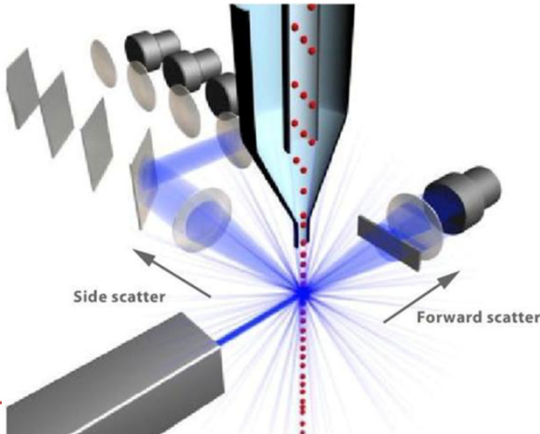
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Side Scatter: SSC

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- Laser light that is scattered at 90 degrees to the axis of the laser path is detected in the Side Scatter Channel
- The intensity of this signal is proportional to the amount of cytosolic structure in the cell (eg. granules, cell inclusions, etc.)



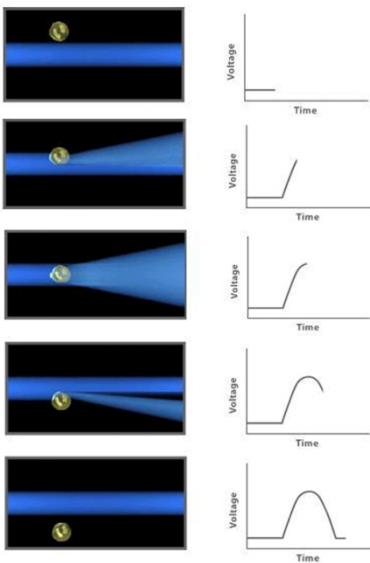


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Signalformation

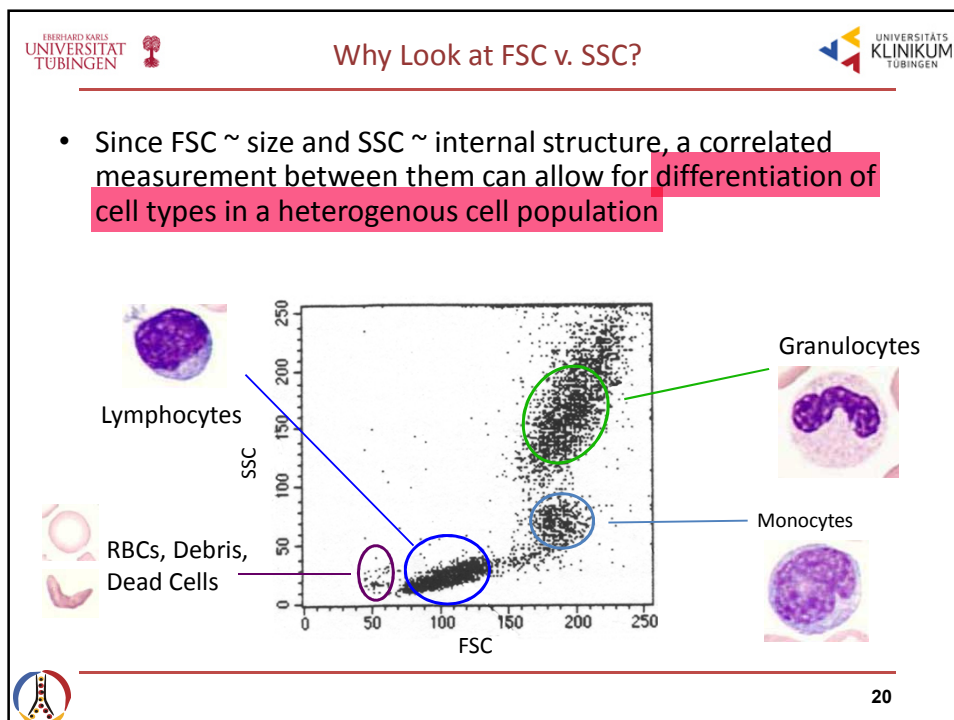
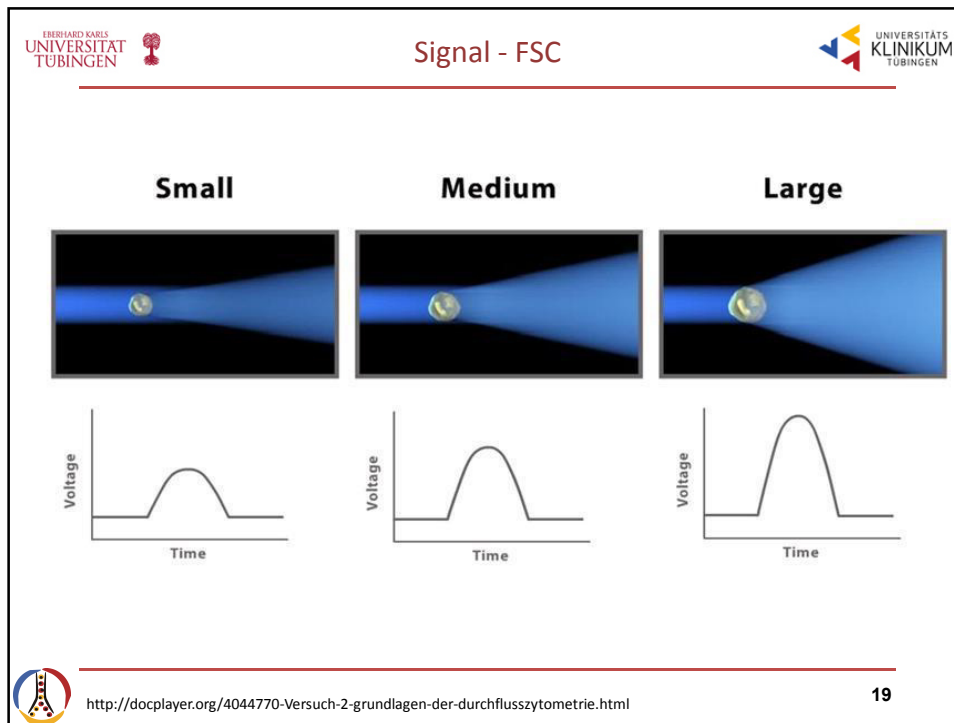
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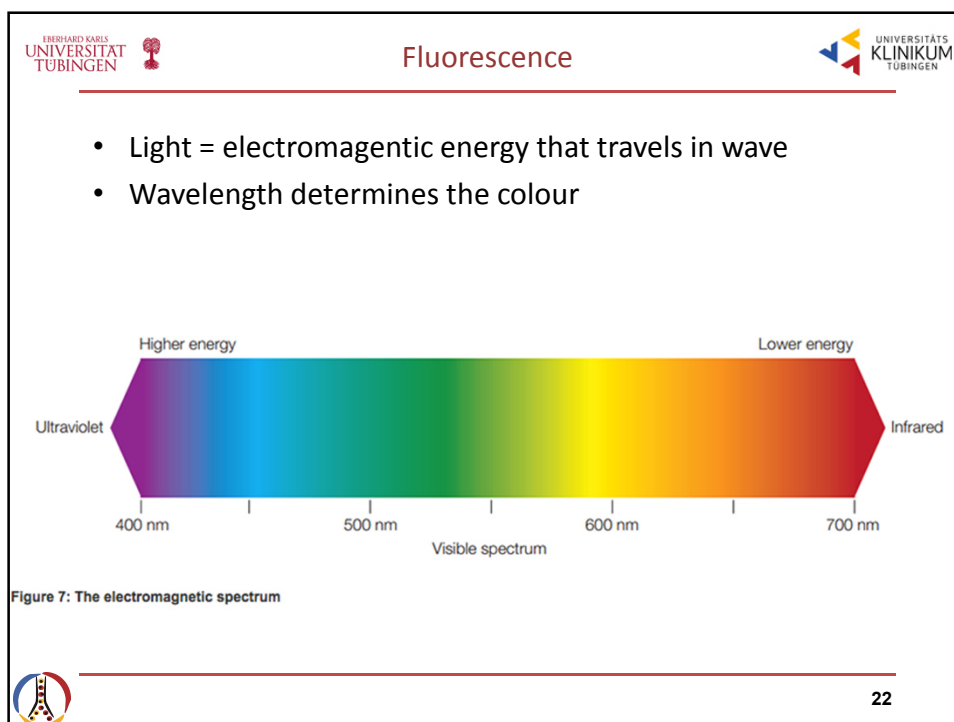
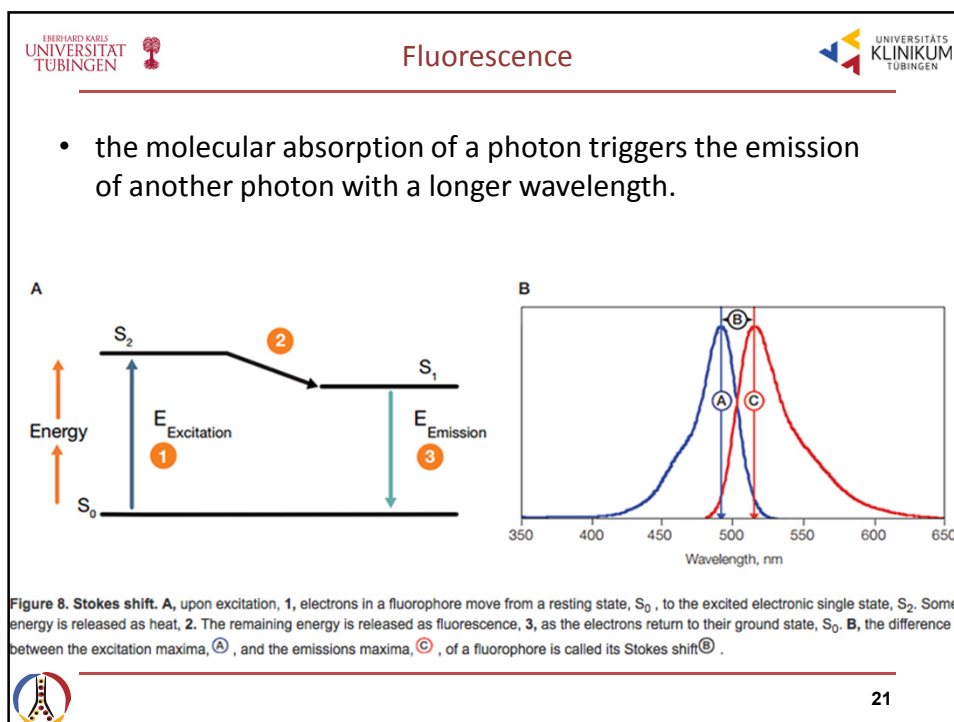




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<http://docplayer.org/4044770-Versuch-2-grundlagen-der-durchflusszytometrie.html>







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Filters



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- Many wavelengths of light will be scattered from a cell, we need a way to split the light into its specific wavelengths in order to detect them independently. This is done with filters
- Optical filters are designed such that they absorb or reflect some wavelengths of light, while transmitting other.
- 3 types of filters
 - **Long Pass filter**
 - **Short Pass filter**
 - **Band Pass filter**



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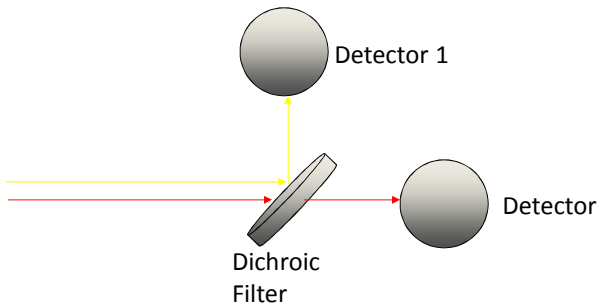
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Dichroic Filters



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- Can be a long pass or short pass filter
- Filter is placed at a 45° angle to the incident light
- Part of the light is reflected at 90° to the incident light, and part of the light is transmitted and continues on.



Dichroic
Filter



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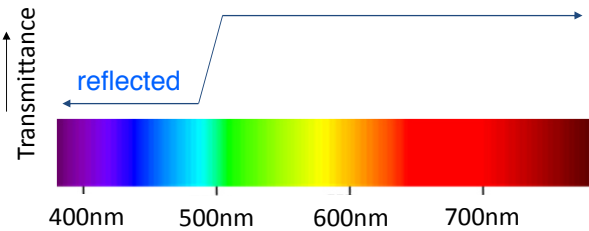
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Long Pass Filters



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- Transmit all wavelengths greater than specified wavelength
 - Example: 500LP will transmit all wavelengths greater than 500nm



short pass filter other way round



Original from Cytomation Training Manual, Modified by James Marvin

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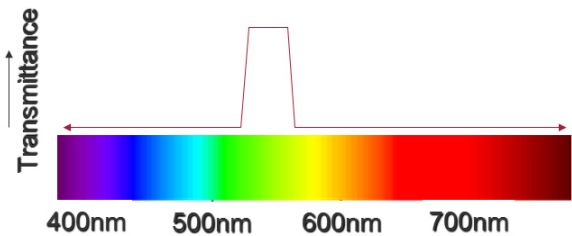
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Band Pass Filters



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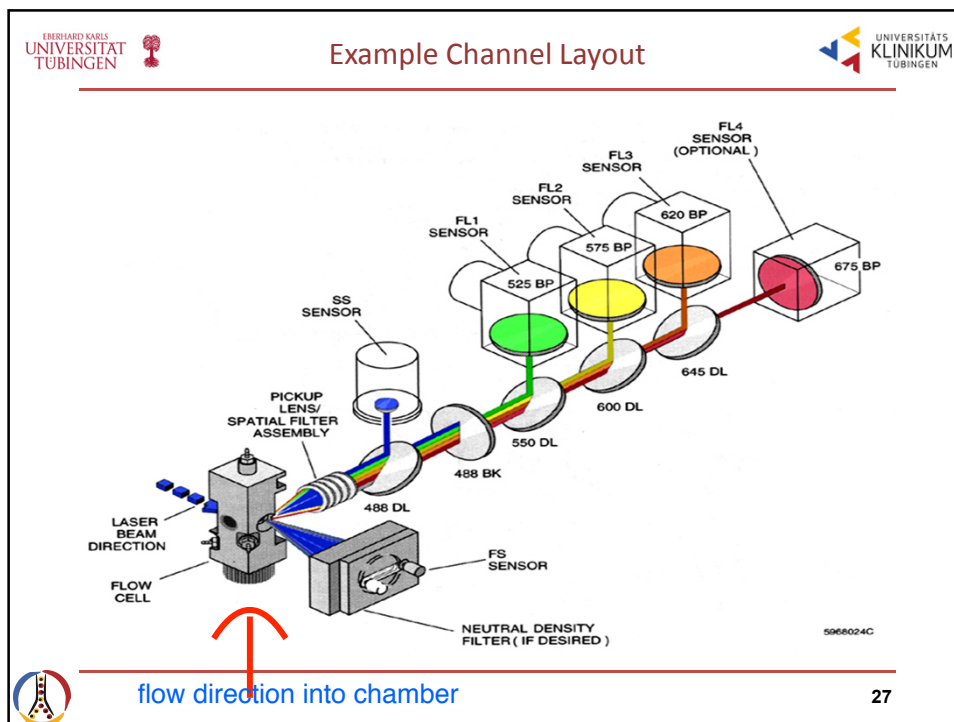
- Transmits a specific band of wavelengths
 - Example: 550/20BP Filter will transmit wavelengths of light between 540nm and 560nm ($550/20 = 550 \pm 10$, not 550 ± 20)
not 530 to 570 but 540 to 560



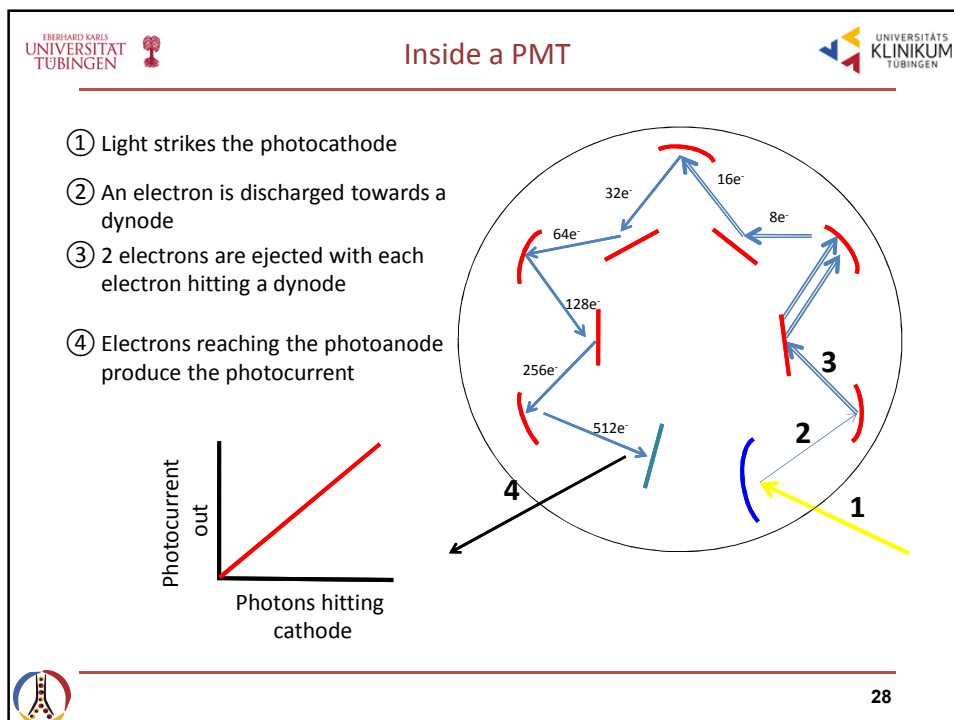


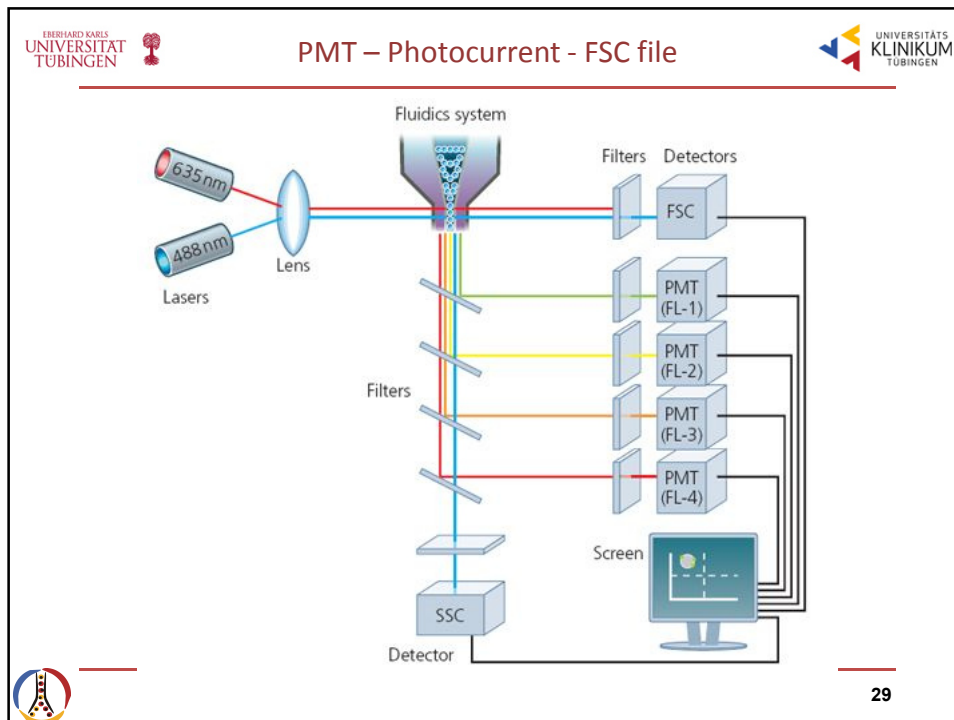
Original from Cytomation Training Manual, Modified by James Marvin

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photoMULTIPLIERtubes





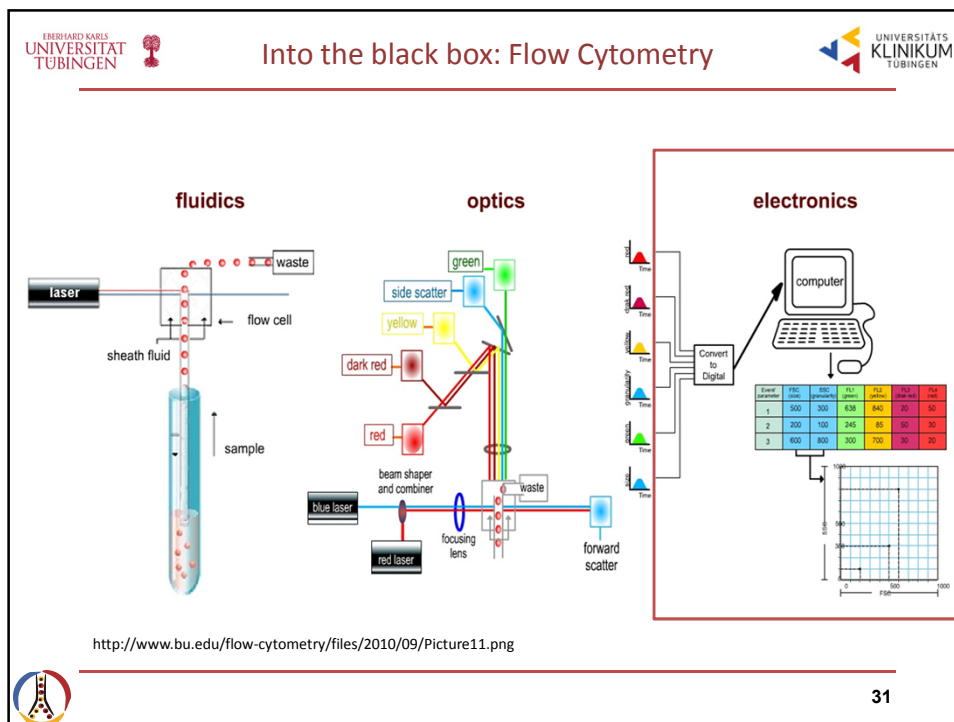



Optics Summary

- Move the laser light to the intercept point
 - Dichroic mirrors direct the lasers precisely to the flow cell
- Differentiate the emitted fluorescence and the scattered laser light into the detectors
 - Typically called the configuration or optical layout.
 - Uses a combination of filters to specify what wavelengths of light reach each PMT
- Convert the photons of light into a current so it can be turned into data
 - PMTs are the most used detectors in flow cytometers
 - The photocurrent emerging from a PMT is linearly proportional to the input.

Within the flow cytometer, the **PMT** is the bridge
between the optics and electronics.

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Electronics Summary

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- Converting the photocurrent from the PMT into discrete values for each cell
 - Continuous output from the PMT is turned into discrete values by the ADC
 - Most new systems are 18 or 24 bit
- **Timing Adjustment** **IMPORTANT!!!!**
 - Necessary for parallel-laser systems **to know that signal is from one cell**
- FCS files are generated from the collection of all of the values from a sample.
 - Data can be displayed in linear or log scale depending on dynamic range

The **FCS file** is the bridge between the flow cytometer and data analysis

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Types of Fluophores



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- **Fluorescent proteins: found in nature**
 - Phycoerythrin (PE), allophycocyanin (APC)
 - GFP, YFP, etc..
-
- **Synthetic Compounds**
 - Cyclic ring compounds
 - FITC, Texas Red, Alexa 488
 - Tandem dyes
 - BV570, Cy5-PE, Cy7-APC
 - Polymer and nanocrystal dyes
 - BV421, Quantum Dots



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DNA stains and cell viability



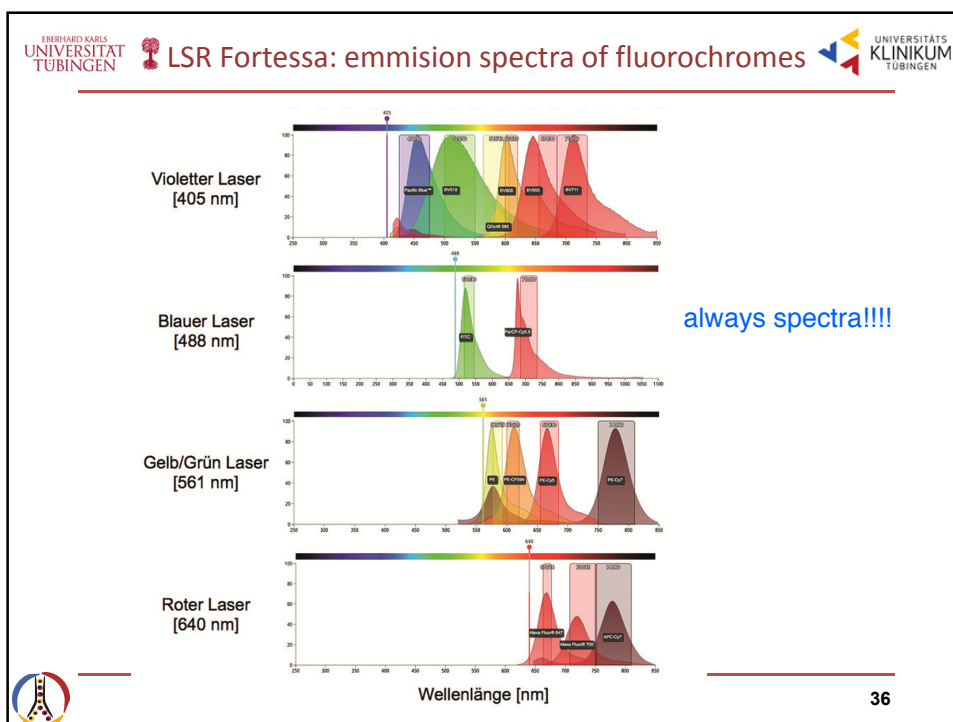
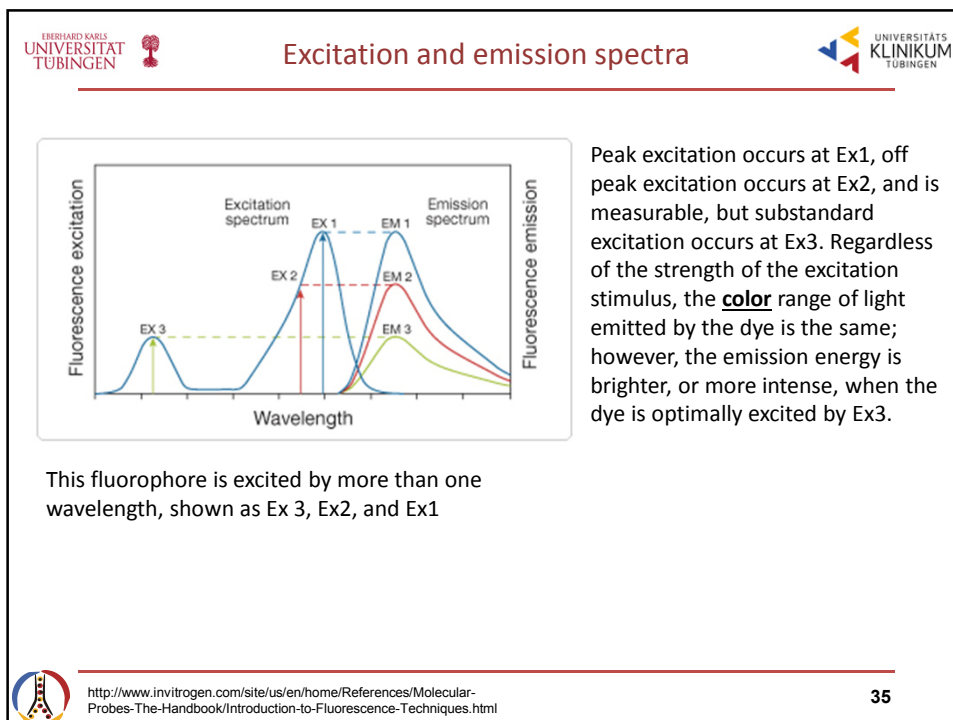
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- DNA dyes that are cell-impermanent can be used to identify dead cells
- Very critical for sorting applications
- Positive cells are 'Dead'
 - This removes from the analysis those cells that stain as false positives with antibodies that penetrated their permeable membranes

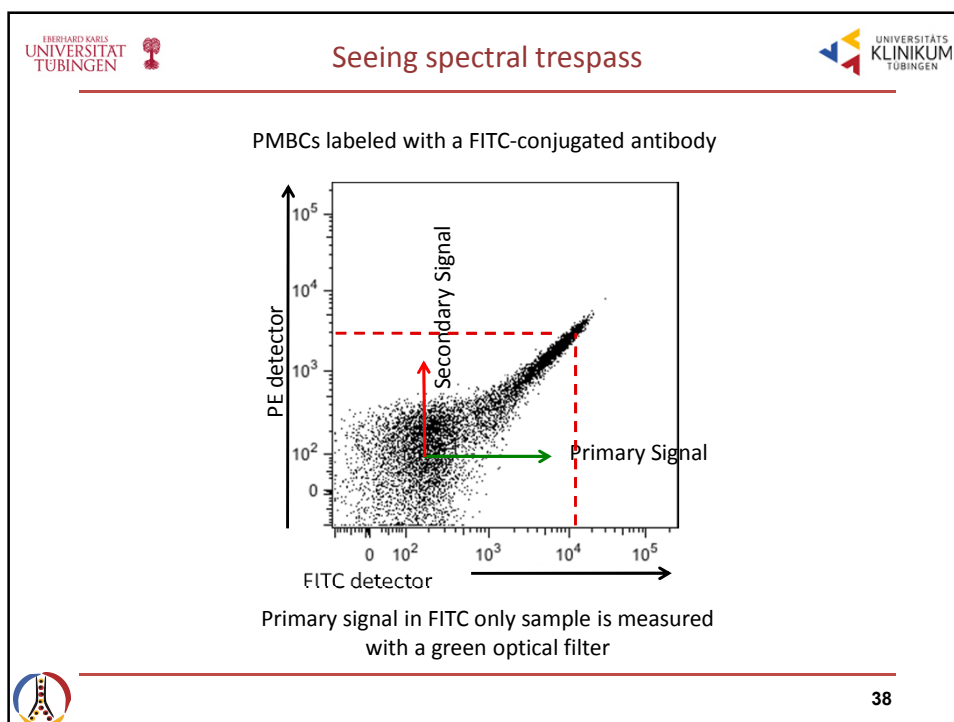
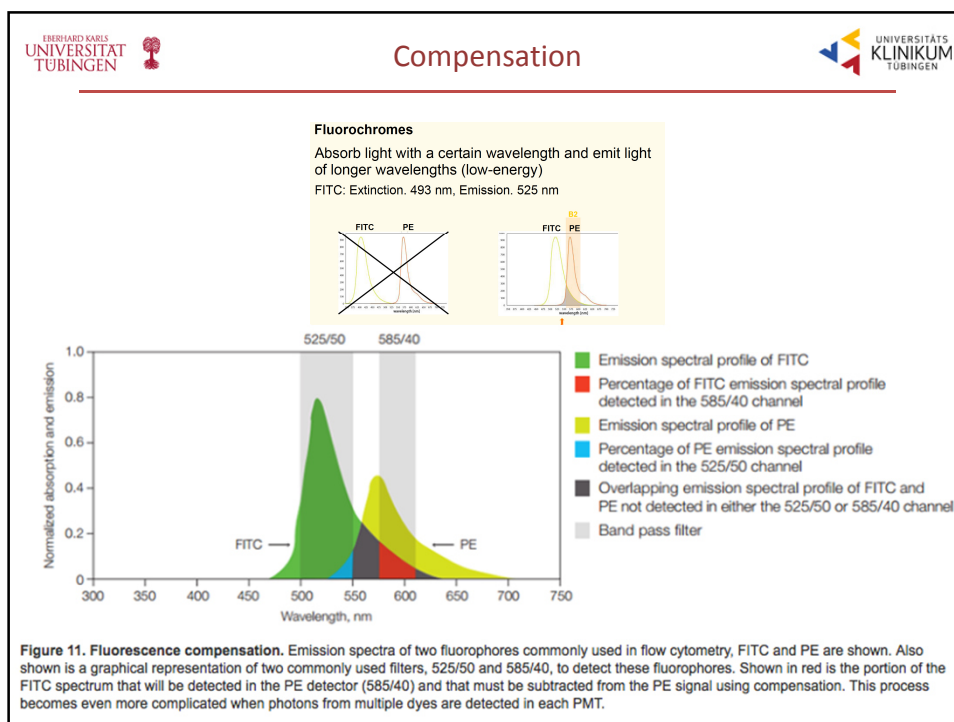
not useful for cell culture!

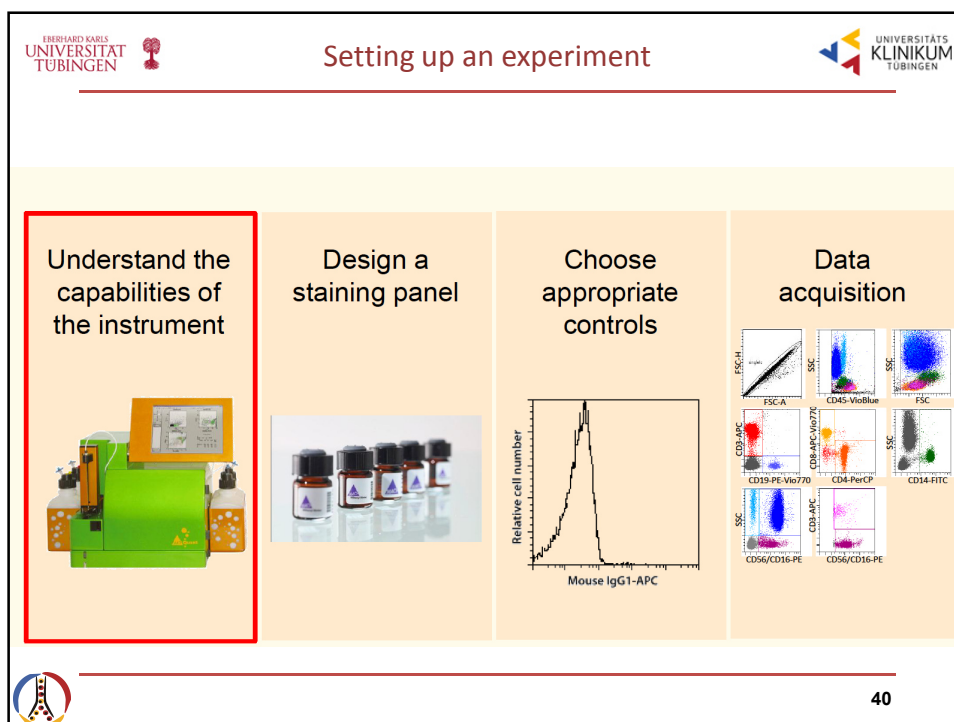
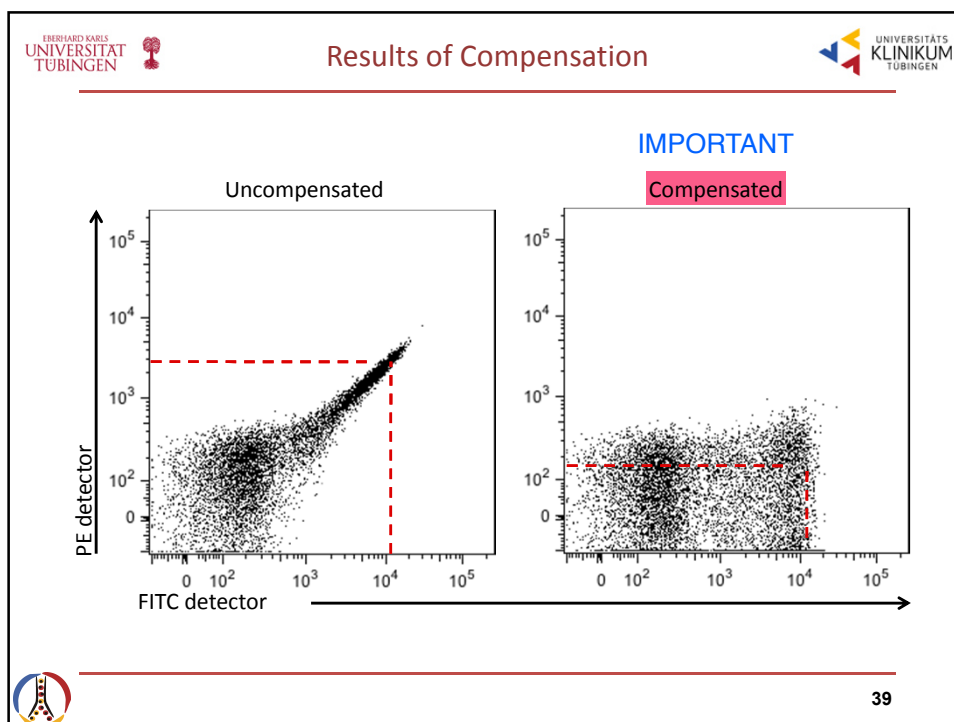


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get rid of spectra overlap





ERBERHARD KARLS UNIVERSITÄT TUBINGEN  **Understand the capabilities of the flow cytometer**  UNIVERSITÄTSKLINIKUM TUBINGEN

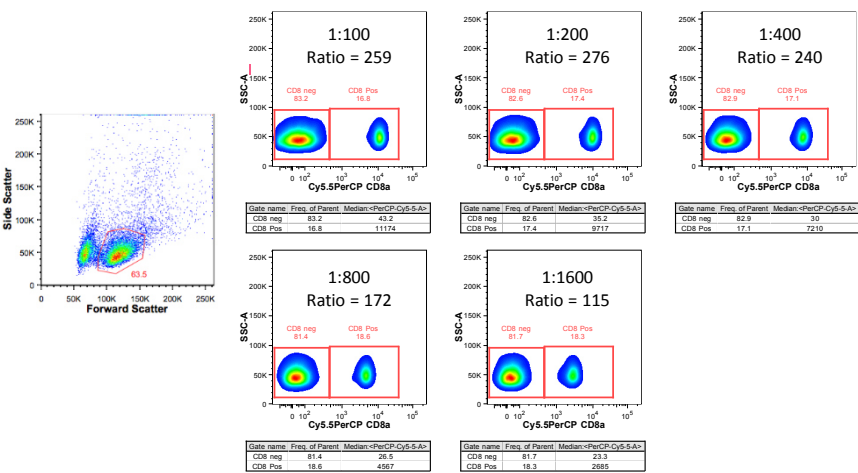
- How many lasers? What are the wavelengths?
- How many detector channels for each laser?
- Which fluorochromes can be detected?

Excitation	Channel	Filter (nm)	Sample Fluorochromes
405 nm	V1	450/50	VioBlue
	V2	525/50	VioGreen
488 nm	B1	525/50	FITC
	B2	585/40	PE
	B3	655-730	PI / 7-AAD / PerCP/PerCP-Vio700
	B4	750 LP	PE-Vio770
635 nm	R1	655-730	APC
	R2	750 LP	APC-Vio770

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ERBERHARD KARLS UNIVERSITÄT TUBINGEN  **Titration Example**  UNIVERSITÄTSKLINIKUM TUBINGEN

CD8a conjugated to Cy5.5PerCP
Tested as a surface stain in 100ul staining volume



1:100 Ratio = 259
1:200 Ratio = 276
1:400 Ratio = 240
1:800 Ratio = 172
1:1600 Ratio = 115

often to much AB for stain, not that much needed

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median of positive and negative and calculate the ration
less AB, less unspecific binding



Advantages of Titration



- Determines the appropriate staining concentration in order to saturate all antigens on cell
 - Improves accuracy of staining
 - Absolutely critical if measuring the amount of fluorescence
 - Saves money
 - Decreases non-specific binding caused by using high concentration of reagent


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Controls



Daily controls

- Quality control of flow cytometer (MACSQuant Calibration Beads)

Regular controls

- Compensation controls

Experiment controls - Controls to determine/prevent unspecific binding

- Isotype control
- FcR blocking reagent
- Isoclonic control
- Negative control
- FMO control quite important


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FMO: fluorescence minus one



- used to identify and gate cells in the context of data spread due to the multiple fluorochromes in a given panel
- **FMO control contains all the fluorchromes** in a panel, except for the one you want to set the cutoff

Antigen	FITC	PE	Cy5-PE	APC
CD3 FMO	---	CD4	CD8	CD19
CD4 FMO	CD3	---	CD8	CD19
CD8 FMO	CD3	CD4	---	CD19
CD19 FMO	CD3	CD4	CD8	---



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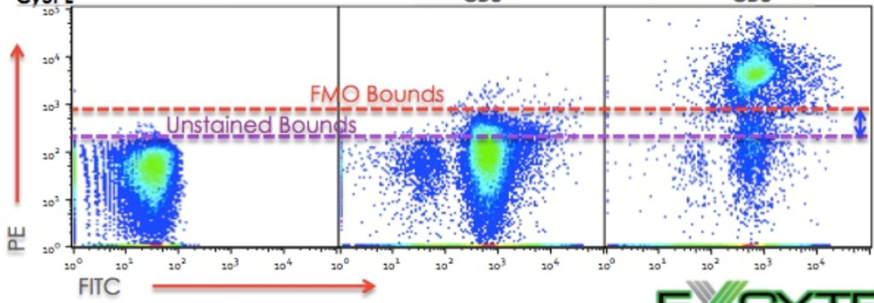


FMO: example



The FMO control ensures that any spread of the fluorochromes into the channel of interest is properly identified.

	Unstained Control	FMO Control	Fully Stained
FITC	---	CD3	CD3
PE	---	---	CD4
Cy5PE	---	CD8	CD8

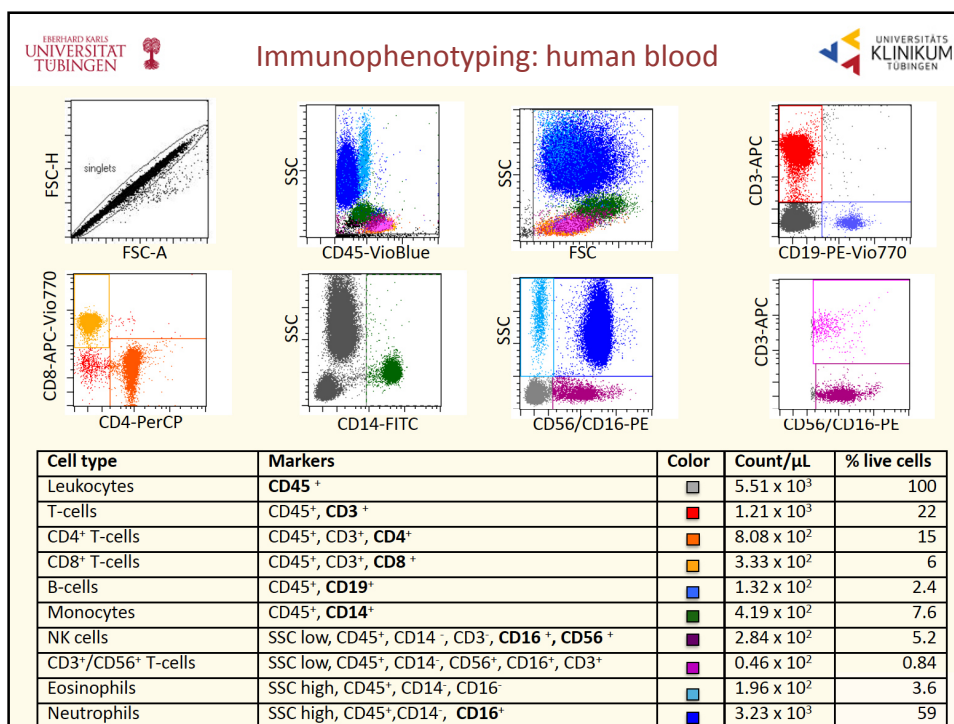
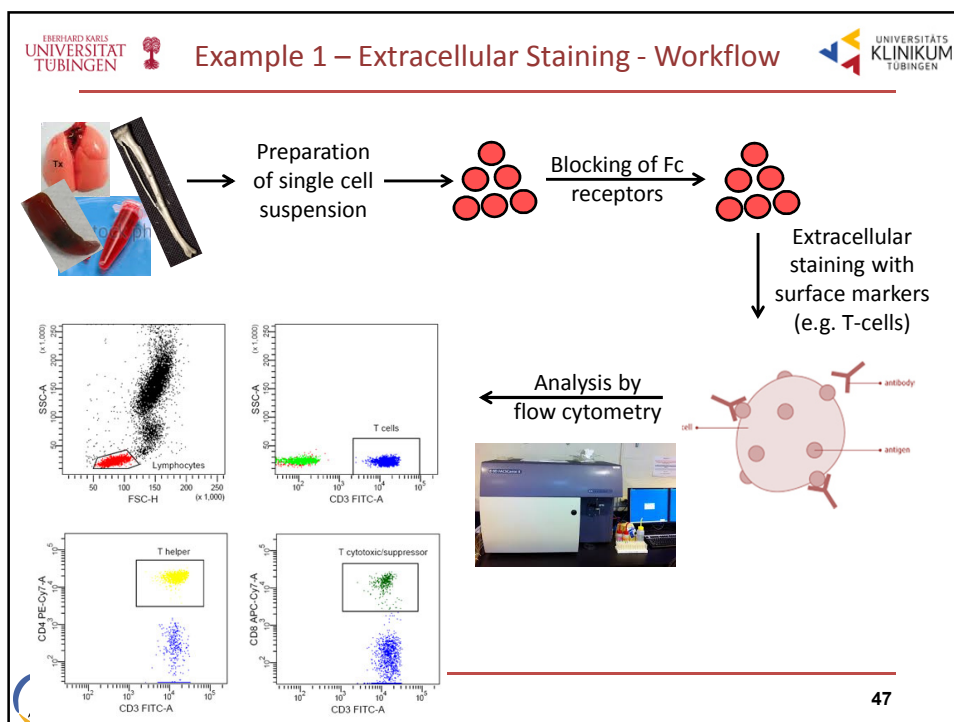


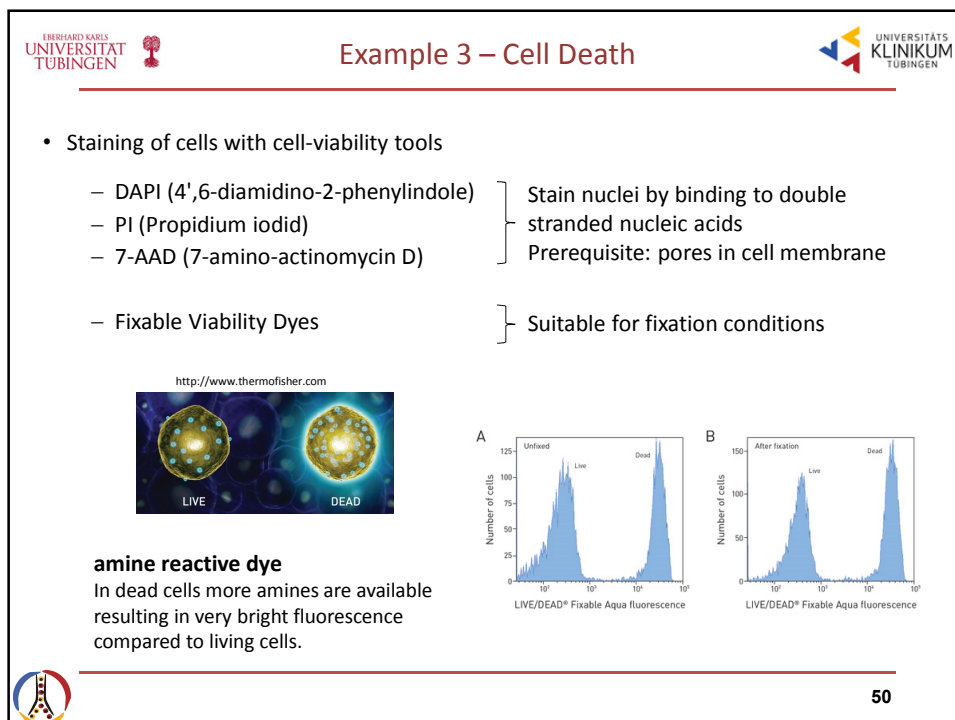
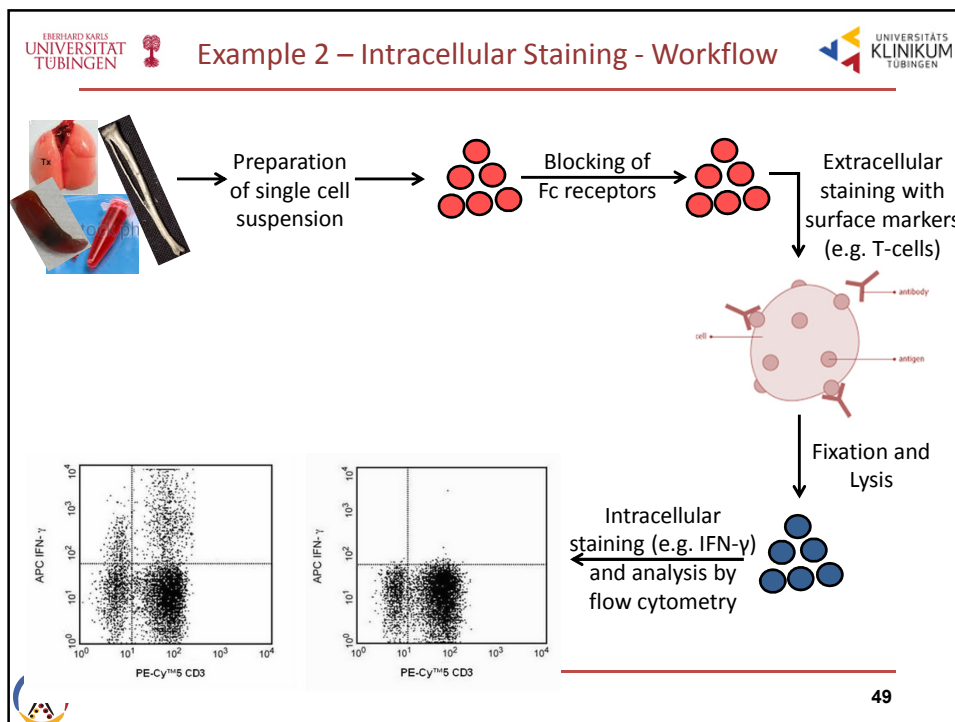
Slide Courtesy of Mario Roederer, Ph.D., NIH Vaccine Center

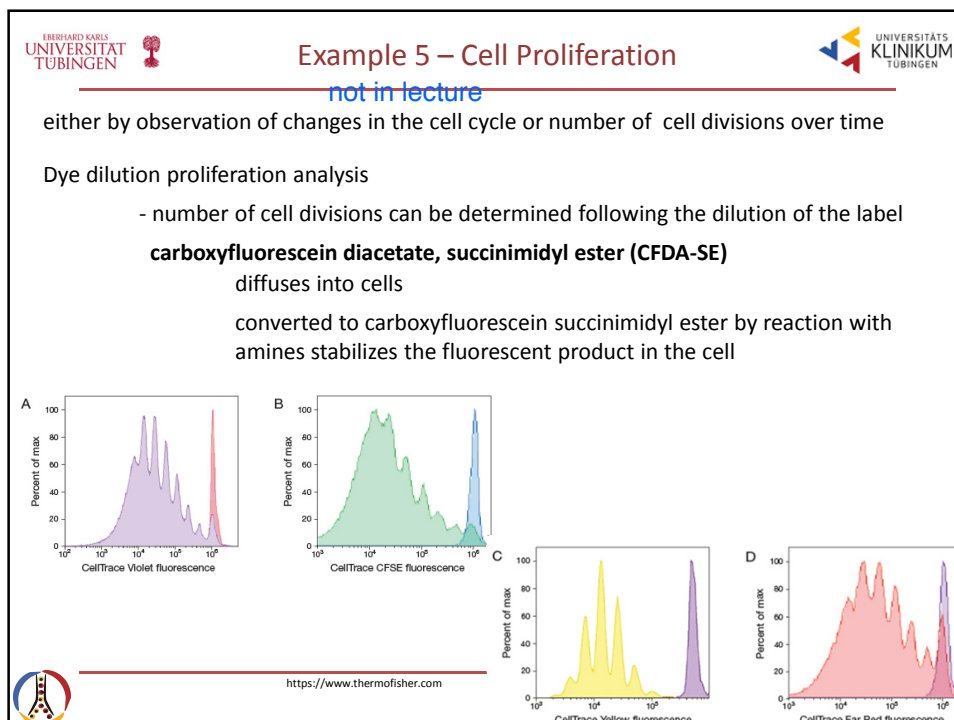
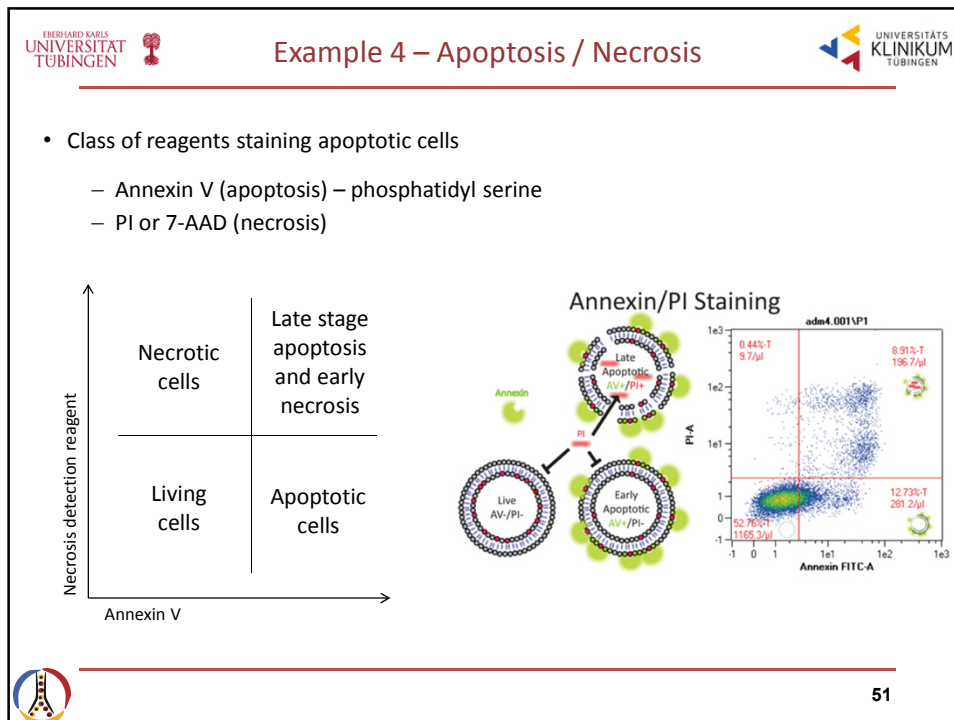




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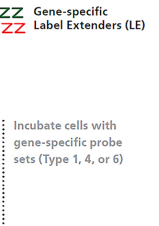
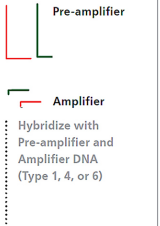
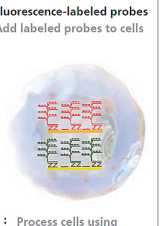
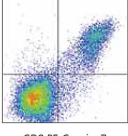
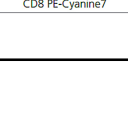
Example 6 – RNA Measurement

not in lecture



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- Analyze RNA and protein expression simultaneously
- fluorescent in situ hybridization (FISH) and branched DNA (bDNA) amplification technique in combination with immuno-phenotyping for cell surface and intracellular proteins

Sample Preparation	Target Hybridization	Signal Amplification	Detection
 Label proteins with antibody (optional) Fix and permeabilize cells in suspension Label intracellular proteins with antibodies (optional) Gene 1 Gene 2 Suspension cells with fixed RNA	 ZZ Gene-specific Label Extenders (LE) Incubate cells with gene-specific probe sets (Type 1, 4, or 6)	 Pre-amplifier Amplifier Hybridize with Pre-amplifier and Amplifier DNA (Type 1, 4, or 6)	 Fluorescence-labeled probes Add labeled probes to cells Process cells using a flow cytometer  CD8 mRNA Alexa Fluor® 647  CD8 PE-Cyanine7



<https://www.thermo-fisher.com>

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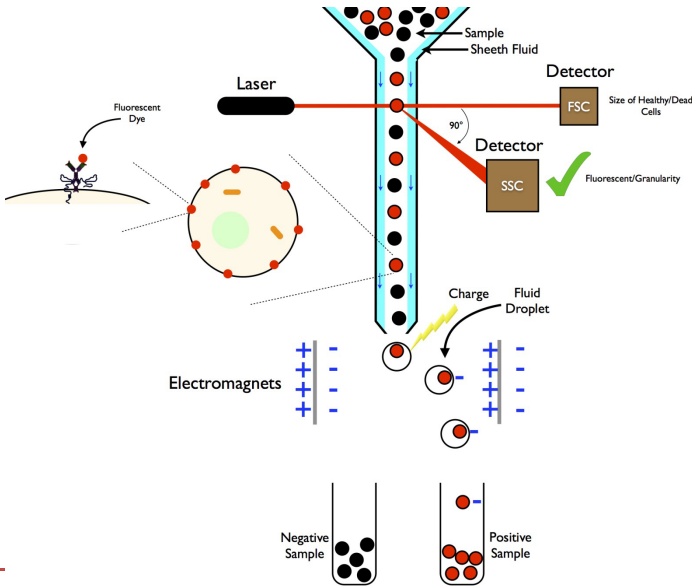


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Example 7 – Cell Sorting



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The diagram illustrates the process of cell sorting. A sample of cells is introduced into a stream of sheath fluid. A laser beam passes through the stream, and detectors (FSC and SSC) measure the cells. Electromagnets create a charge on the fluid droplet, which is then sorted into a negative or positive sample container.



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FACS Core Facility Berg - Equipment



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Cell Sorter

- **BD FACSARIA™ II**
3 lasers (375 / 405 nm, 488 nm, 640 nm)
10 parameters, up to 8 colors





Cell Analyzers

- **BD FACS LSRFortessa™**
4 lasers (402 nm, 488 nm, 561 nm, 640 nm)
18 parameters, up to 16 colors
- **BD FACSCanto™ II**
3 lasers (405 nm, 488 nm, 640 nm)
10 parameters, up to 8 colors
- **BD FACSLytic™**
3 lasers (375 / 405 nm, 488 nm, 640 nm)
12 parameters, up to 10 colors





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FACS Core Facility Berg - Service



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- Basic cell sorts (e.g. Lymphocytes, DCs, cells lines, transfected cells,...)
- Sorts into 96- or 384-well plates with defined cell numbers (even single cells)
- Complex sorts under sterile conditions
- Cell sorting of infectious samples (bio safety level 2)
- Flow cytometry analysis with up to 16 colors; 561nm-Laser
- Advice for panel design and analysis of multi-color experiments
- Seminars on flow cytometry
- Basic training for users on instruments, software and analysis



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Image Stream Core Facility (ISCF)

Prof. Dr. rer. nat. Katja Schenke-Layland
Simone Pöschel

*Department of Women's Health,
University Women's Hospital of the Eberhard-Karls-University Tübingen*

Tübingen, Januar 29th 2018



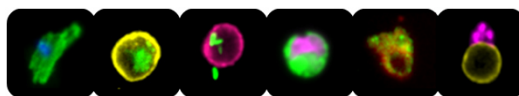
Introduction Imaging Flow Cytometry

STEP 1 Understanding the Image Stream

[What is the Image Stream? The ISx System.](#)
[How does it work?](#)
[Applications](#)

STEP 2 Analyzing the data with IDEAS®

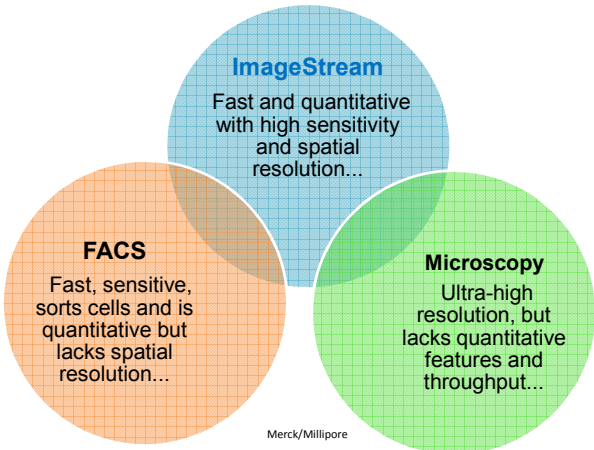
[How do I analyze the data?](#)
[Application examples](#)



Merck/Millipore




ImageStream^{MkII} combines FACS and Microscopy



Merck/Millipore

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ImageStream^{MkII} combines FACS and Microscopy

	Flow Cytometry	Microscopy	ImageStream
High speed	+	-	+
Statistical power	+	-	+
Imaging	-	+	+
Information content	-	+	+
Research benefit	Objective, statistical discrimination of cells based on intensity	Discrimination of cells based on appearance	Objective, statistical discrimination of cells based on appearance

ImageStream overcomes limitations of both techniques

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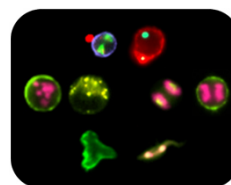
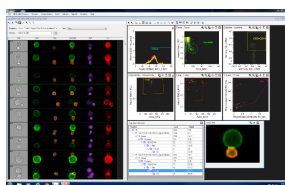
The ImageStream® System

High speed Imaging (ISx® MKII Imaging Flow Cytometer)
+
Quantitative image analysis (IDEAS® Image Analysis Software)
=
Advances your research

But WHY ???



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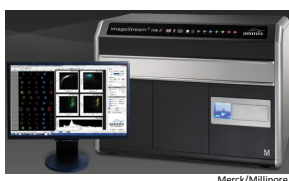


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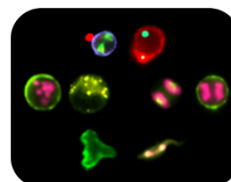
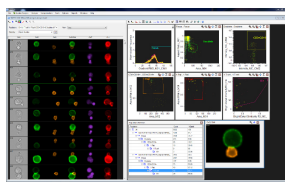
The ImageStream® System

High speed Imaging (ISx® MKII Imaging Flow Cytometer)
+
Quantitative image analysis (IDEAS® Image Analysis Software)
=
Advances your research

Because you can discriminate cells objectively and statistically based on their appearance



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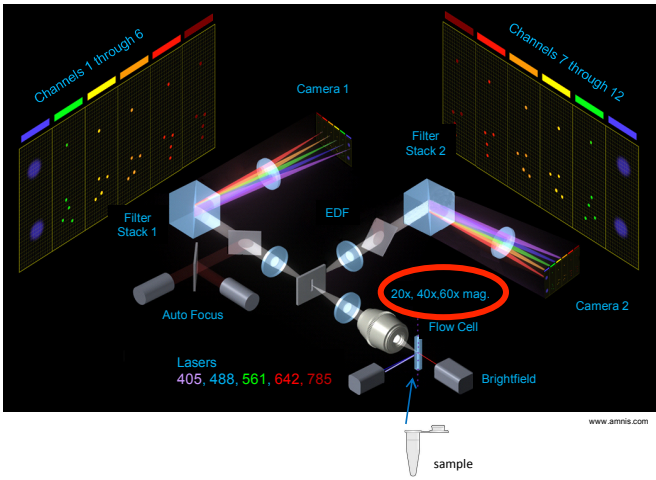
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How does the ImageStream work?

ImageStreamx Mark II Optical Layout



- 1.000 cells per second
- 4 lasers +SSC
- 2 CCD cameras
- 12 Image channels 6 channels per camera
- Multiple Magnifications
- EDF

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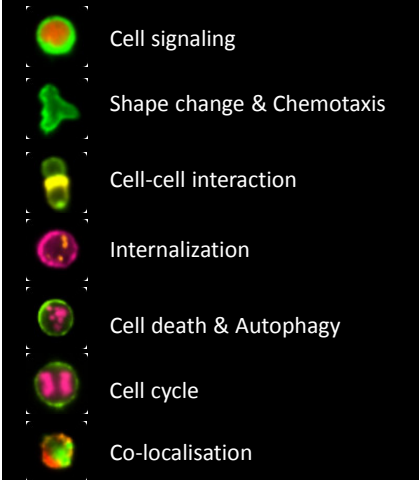
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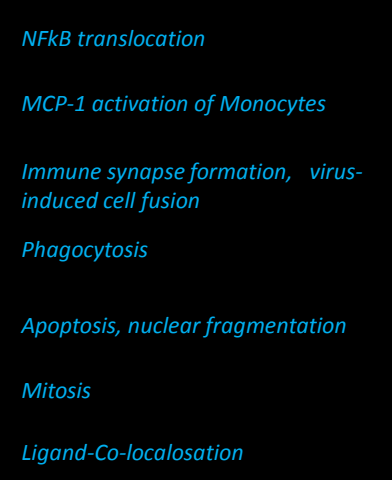
Primary applications

Field of study



- Cell signaling
- Shape change & Chemotaxis
- Cell-cell interaction
- Internalization
- Cell death & Autophagy
- Cell cycle
- Co-localisation

Example from ISx



- NFkB translocation*
- MCP-1 activation of Monocytes*
- Immune synapse formation, virus-induced cell fusion*
- Phagocytosis*
- Apoptosis, nuclear fragmentation*
- Mitosis*
- Ligand-Co-localisation*

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Analysing data with IDEAS Image analysis software

Combines image analysis, statistical rigor and visual confirmation



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Analysing data with IDEAS® : Features

Features Large selection of criteria to analyze images
85 base features per image

- Size: Area, Diameter,...
- Location: Angle, Spot distance,...
- Shape: aspect ratio, **Circularity**,...
- Texture Contrast, Bright detail intensity,...
- Comparison: **Similarity**, Internalization,...
- Signal strength: Intensity,...

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Analysing data with IDEAS® : Masks

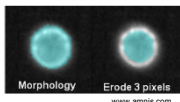
Masks



set of pixels in region of interest

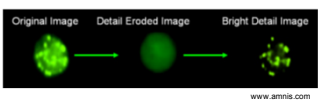
- 16 types of function masks
- combined masks

ERODE mask
removes pixels from edges



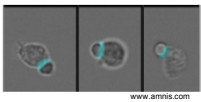
• cytoplasm
membrane removed

SPOT mask
extracts bright objects



• count spots
• quantify intensity in spots

INTERFACE mask
area where cell in contact with another object



• quantify immune synapses

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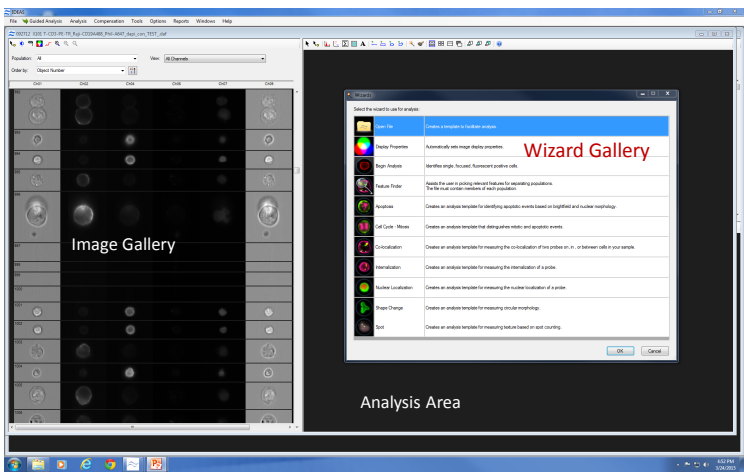


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Analysing data - Overview of IDEAS Application



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Analysing data - Interface of IDEAS Application

The screenshot displays the IDEAS Application interface with several key components labeled:

- Inspect your Population („virtual cell sort“)**: Points to the top-left panel showing a grid of colored dots representing different cell populations.
- Image Gallery**: Points to the bottom-left panel showing a grid of microscopy images corresponding to the cell populations.
- Images for every Dot**: Points to the bottom-right panel showing a large microscopy image of a single cell.
- Statistic Area**: Points to the bottom-center panel showing a table of statistical data for the selected population.
- Analysis Area**: Points to the top-right panel showing various flow cytometry plots and histograms.
- Images Display**: Points to the bottom-right panel showing a large microscopy image of a single cell.

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Analysing data – Feature Finder

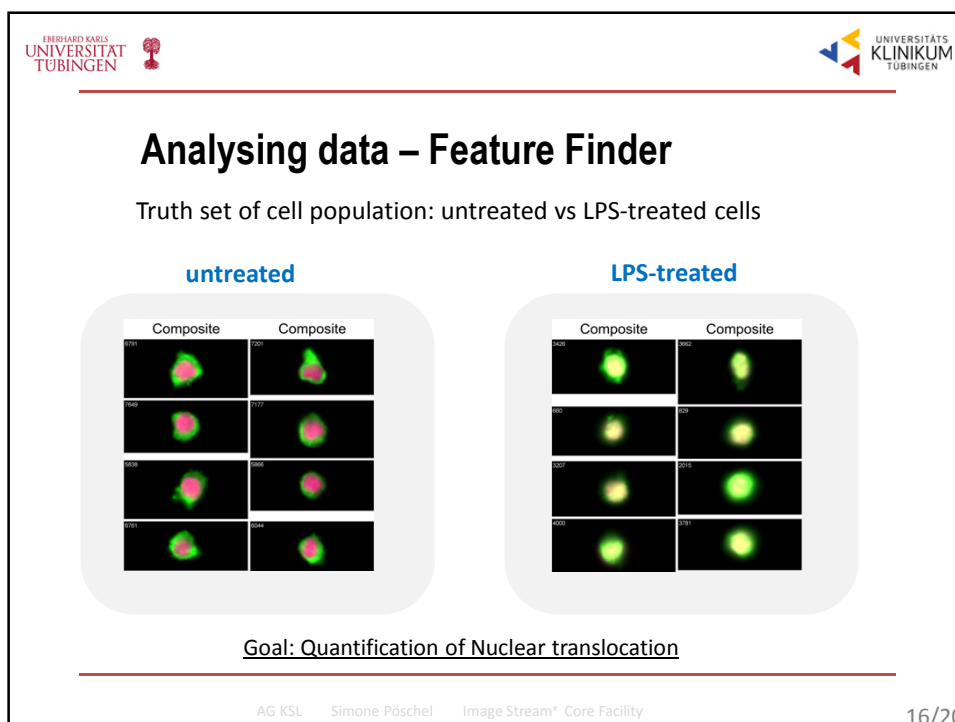
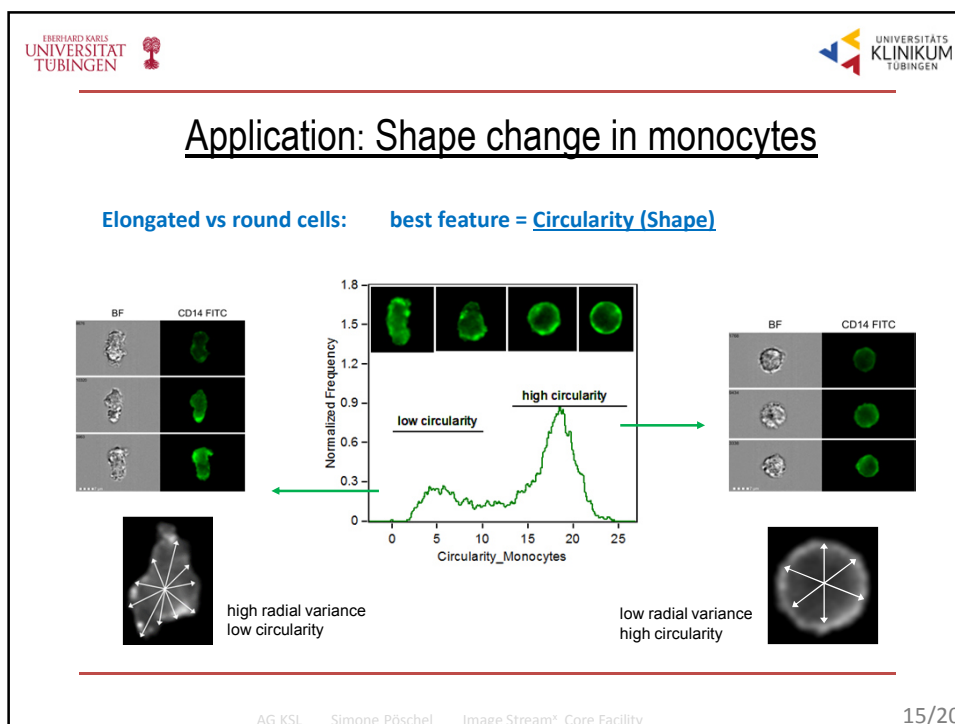
Truth set of cell population: elongated vs round cells

Round

Elongated

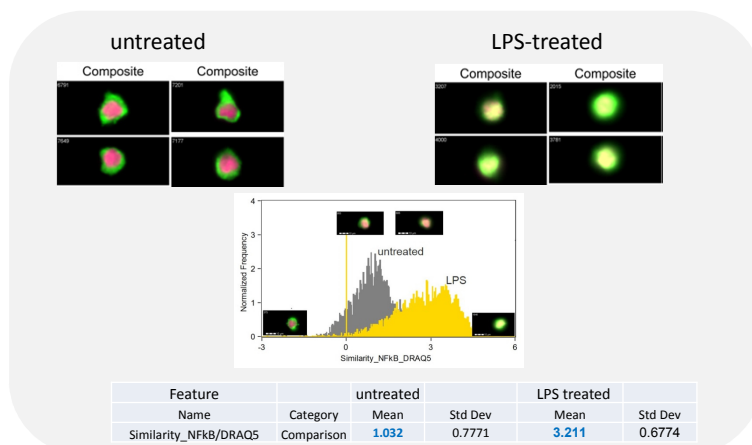
Goal: Quantification of cell activation

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Application: Nuclear Translocation

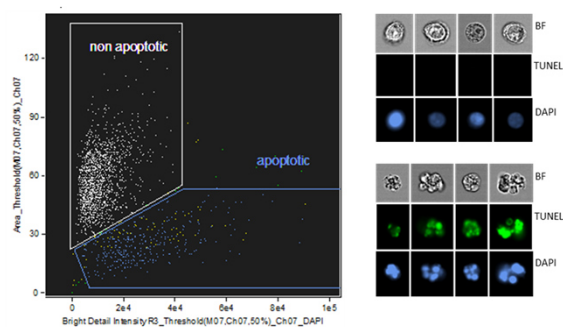
untreated vs LPS treated cells: best feature = Similarity



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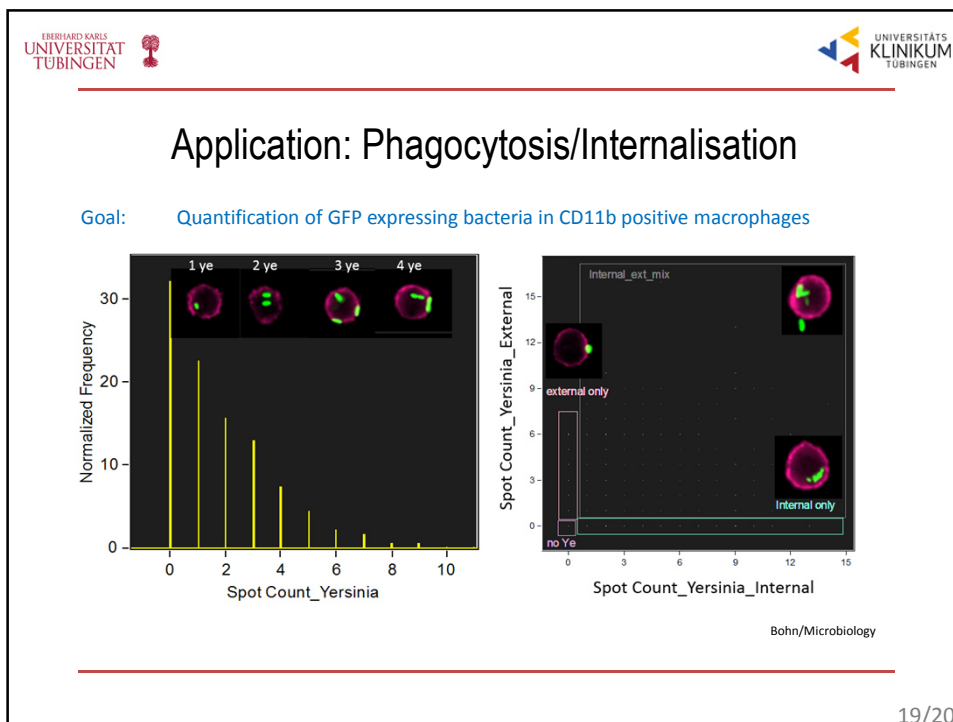
Application: Apoptosis

Measurement of TUNEL positives events and nuclear fragmentation in CPT* treated Jurkat cells



CPT*: Camptothecin

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Thank you for your attention!

For more information, go to:

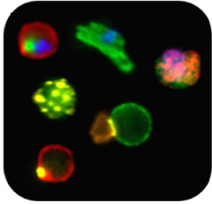
UKT website/Core Facilities/Image Stream
www.merckmillipore.com

Contact and Scheduling:



Department of Women's Health
AG Schenke-Layland

Operator: Simone Pöschel
 Phone: 07071-29-82745
 Mail: simone.poeschel@med.uni-tuebingen.de



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User Fees Core Facility Flow Cytometry (FCF) Berg Tübingen

Costs € / hour	Klinik	Uni Tübingen	MPG, industry *	Med II / SFB665	late cancellation	
					Policy	Charge [%]
Canto unassisted	10	20	30	5	<3h	50
Canto assisted	45	50	55	-	<24h	50
Fortessa unassisted	25	30	40	10	<3h	50
Fortessa assisted	55	60	65	-	<24h	50
Lyric unassisted	25	30	40	10	<3h	50
Lyric assisted	55	60	65	-	<24h	50
Sorting Arial assisted ¹	85	100	125	40	<48h	50
Panel design / Consultations / ask an "expert"	40	60	80	10	<24h	50
Data Analysis unassisted	5	5	5	0	<3h	50
Data Analysis assisted	45	60	80	20	<48h	50
Softwaretraining FlowJo 3 hours	100 / Person	150 / Person	200 / Person	80 / Person	<48h	50
Basic training 2 x 2 hours	150 / Training	200 / Training	250 / Training	100 / Training	<48h	50

¹ Prices are valid during working hours from 8 am to 6 pm. From 6.00 pm 20 € / h will be charged additionally.
 * plus Tax



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Fees and Charges

Costs in € / hour	MFT/UKT	Uni Tübingen	MPG/Industrie*
Imaging, ISCF staff	100	150	200
Analysis, ISCF staff	60	90	120
Imaging, unassisted	60	90	120
Analysis, unassisted	40	60	80
Consulting/Troubleshooting	50	75	100
Instrument Training ISxmkl, 2 x 1 hour	100/Person	150/Person	200/Person
Software Training IDEAS, 4 hours	200/Training	300/Person	400/Person

Free „proof-of concept run“ upon request

