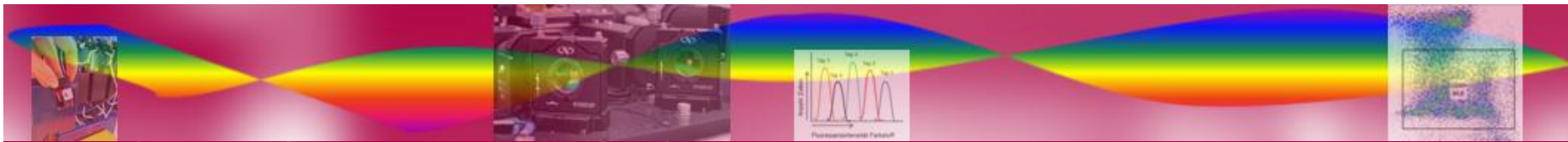


# What is wrong with my flow cytometry data?

## Hints, tricks and pitfalls



## ***Dublets – misinterpretation preassigned***

**Dublettenausschluss - Fehlinterpretation  
vorprogrammiert.**

# Doublets – What's the problem?

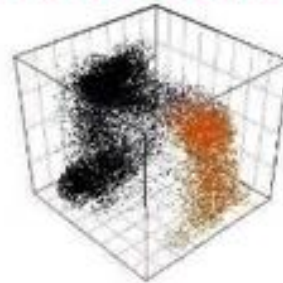
- flow cytometry → high-throughput analysis of single cell suspension
- doublets → 2 cells analysed at the same time
- signals can't be separated → loss of data accuracy
- false positive signal, higher MFI signals, more cells in G2/M phase in cell cycle

## Doublet discrimination — Youtube tutorial

### Flow Cytometry Basics: How to Gate-out Doublets and Clumps

This video is brought to you by:

**Flow Cytometry Network**  
**www.thefcn.org**



moovly

<https://www.youtube.com/watch?v=MjMvwSUucKI>

# Doublet discrimination — Youtube tutorial

## Objectives

By the end of this tutorial, you should be able to understand:

- What doublets and clumps are?
- Why we need to gate out doublets?
- How to identify doublets and clumps?
- Different ways to exclude doublets

moovly

  
www.thefcn.org

<https://www.youtube.com/watch?v=MjMvwSUucKI>

# Doublet discrimination - Next NEWLETTER

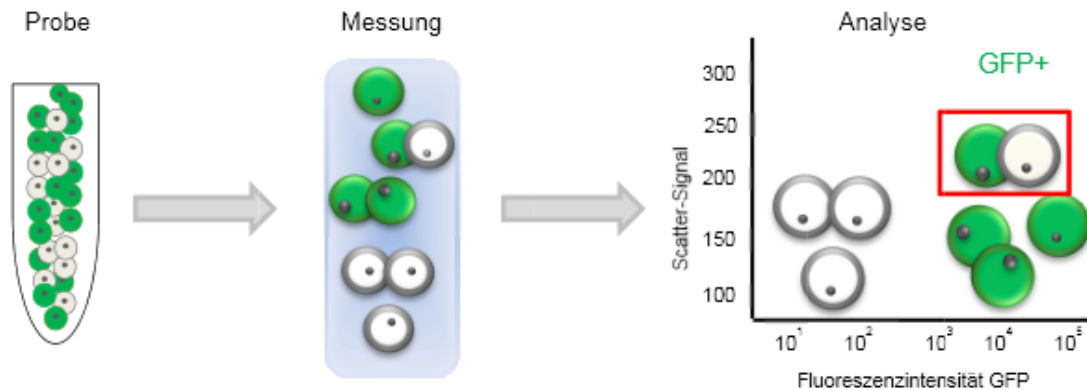
NEWS +++ NEWS +++ NEWS +++ NEWS +++ NEWS +++ NEWS +++ NEWS +++ NEWS

Aus der Core Facility für Zellsortierung und Zellanalyse

## DOUBLE TROUBLE - ÄRGER MIT DEN DUBLETTEN -

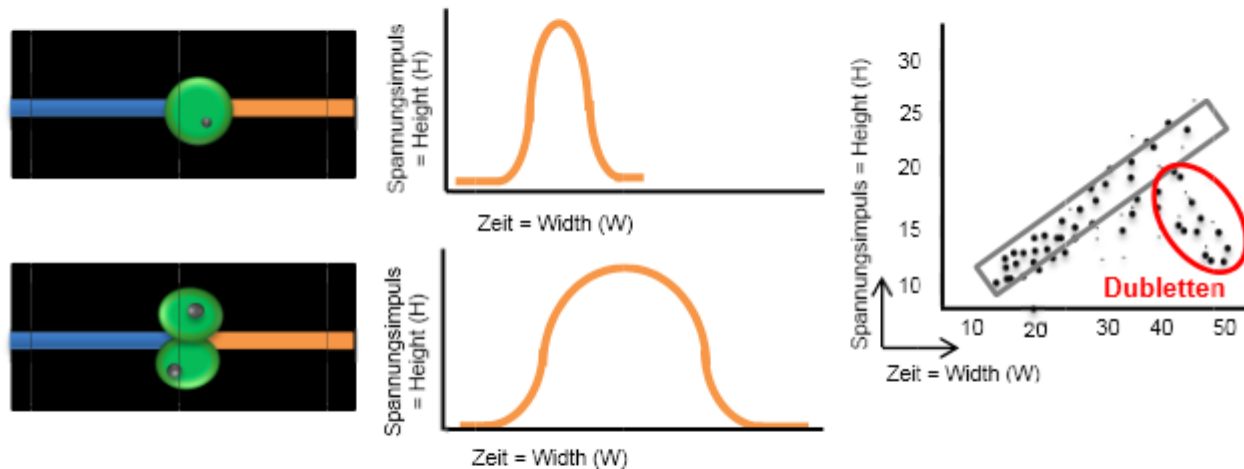
Kein Luxusproblem, sondern häufige Ursache von Fehlinterpretationen.

Trotz sorgfältiger Präparation kleben Zellen oft aneinander. Im Beispiel unten aggregiert eine GFP-negative mit einer GFP-positiven Zelle, so dass bei herkömmlichen Analysen beide als GFP-positiv wahrgenommen werden. Diese sogenannte Dublettenbildung führt bei 5- 20% aller Zellen – eher mehr bei Primärisolaten – zur Fehlinterpretation.



## Die Lösung: **Dublettendiskriminierung!**

Dubletten benötigen mehr Zeit, den Laserstrahl zu passieren – damit wird das Weite-Signal größer. Über dieses vergrößerte Weite-Signal können Dubletten aus der weiteren Analyse ausgeschlossen werden.



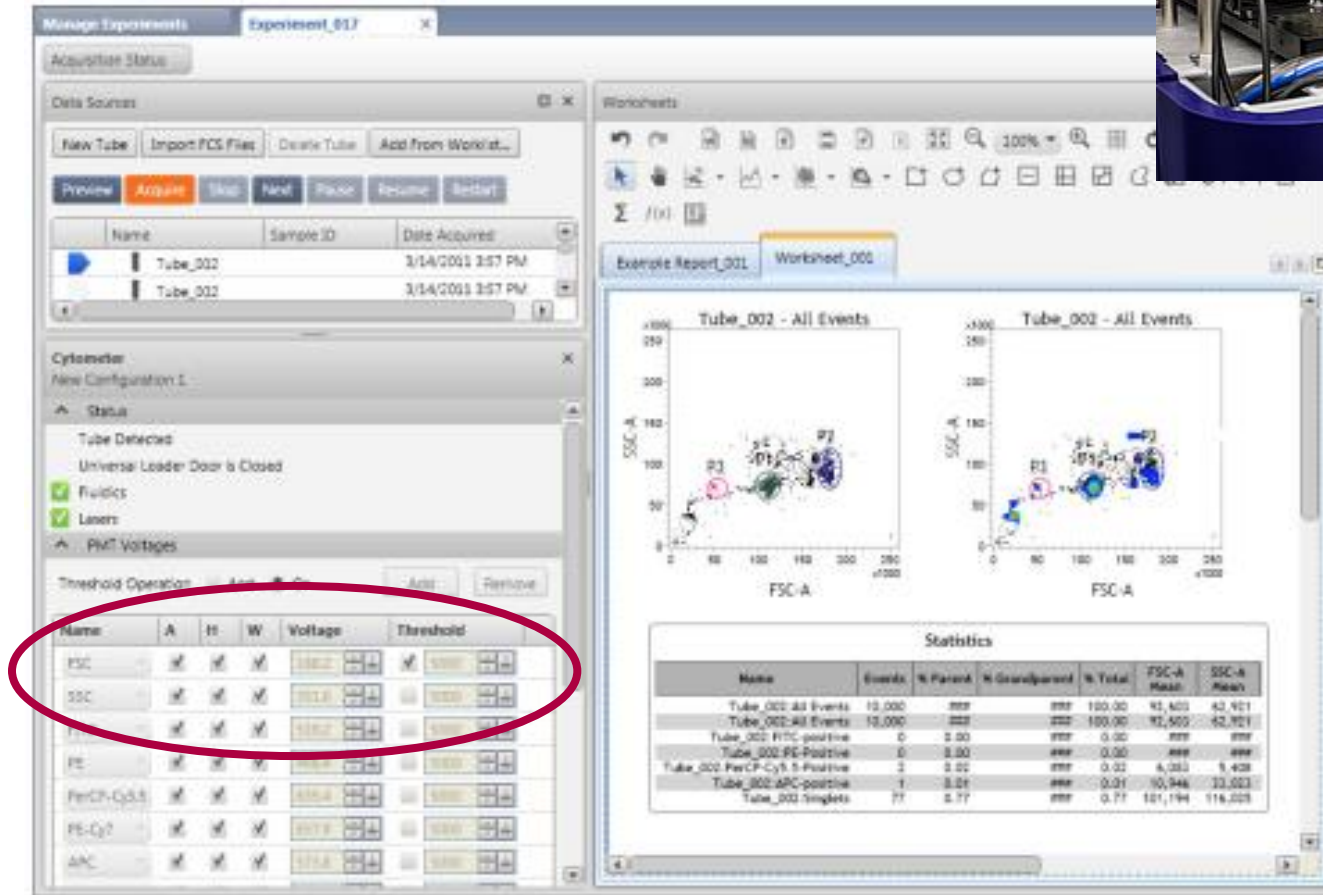
**Möchten Sie Ihre Analyse optimieren und haben Fragen dazu?**

Dann einfach anrufen oder Email schreiben!

Kontakt: [zsa@med.uni-rostock.de](mailto:zsa@med.uni-rostock.de) ☎0381-494 5876/5877/5883

# How to record FSC/SSC-Area Height Width

## FACS Verse – FACS Suite Software



The screenshot displays the FACS Suite software interface. On the left, the 'Data Sources' panel shows a table of acquisition data. The 'Threshold Operation' table is circled in red, showing parameters for FSC, SSC, PE, PerCP-Cy5.5, PS-Cy7, and APC. The main window shows two flow cytometry plots of SSC-A vs FSC-A for 'Tube\_002 - All Events'. Below the plots is a 'Statistics' table.

Name	A	H	W	Voltage	Threshold
FSC	✓	✓	✓	100.0	✓
SSC	✓	✓	✓	100.0	✓
PE	✓	✓	✓	100.0	✓
PerCP-Cy5.5	✓	✓	✓	100.0	✓
PS-Cy7	✓	✓	✓	100.0	✓
APC	✓	✓	✓	100.0	✓

Name	Events	% Parent	% Grandparent	% Total	FSC-A Mean	SSC-A Mean
Tube_002 All Events	12,000	###	###	100.00	95,403	62,921
Tube_002 All Events	12,000	###	###	100.00	95,500	62,921
Tube_002 FITC-positive	0	0.00	###	0.00	###	###
Tube_002 PE-Positive	0	0.00	###	0.00	###	###
Tube_002 PerCP-Cy5.5-Positive	2	0.02	###	0.02	6,385	5,408
Tube_002 APC-positive	1	0.01	###	0.01	10,946	22,023
Tube_002 Singlets	77	0.77	###	0.77	101,194	116,028



# How to record FSC/SSC-Area Height Width

## Cytek Aurora – SpectrFlo™ Software



The screenshot displays the SpectrFlo software interface. On the left, the 'Acquisition Control' panel shows 'Tube\_001' with a flow rate of 10.82  $\mu\text{L}/\text{Min}$ . The 'Instrument Control' panel shows 'User Settings: Default' and a table of channel parameters:

FSC	SSC	Violet	Blue	Red
V1: 357	V2: 426	V3: 335	V4: 243	V5: 225
V6: 254	V7: 283	V8: 224	V9: 259	V10: 561
V11: 548	V12: 735	V13: 491	V14: 544	V15: 044
V16: 322	All Channels %: 0			

The 'Instrument Control' panel also features a 'GAIN THRESHOLD SIGNAL LASERS' tab, which is highlighted with a red box. A red arrow points from this tab to the 'Signal' sub-tab, which is also highlighted with a red box. This sub-tab contains the following settings:

Channel	Area	Height	Width
FSC	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>
SSC	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>
SSC-B	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>

The 'Fluorescence Channels' section on the right shows settings for Violet, Blue, and Red lasers, with checkboxes for Area, Height, and Width, and dropdown menus for V9, B8, and R5.

# How to record FSC/SSC-Area Height Width

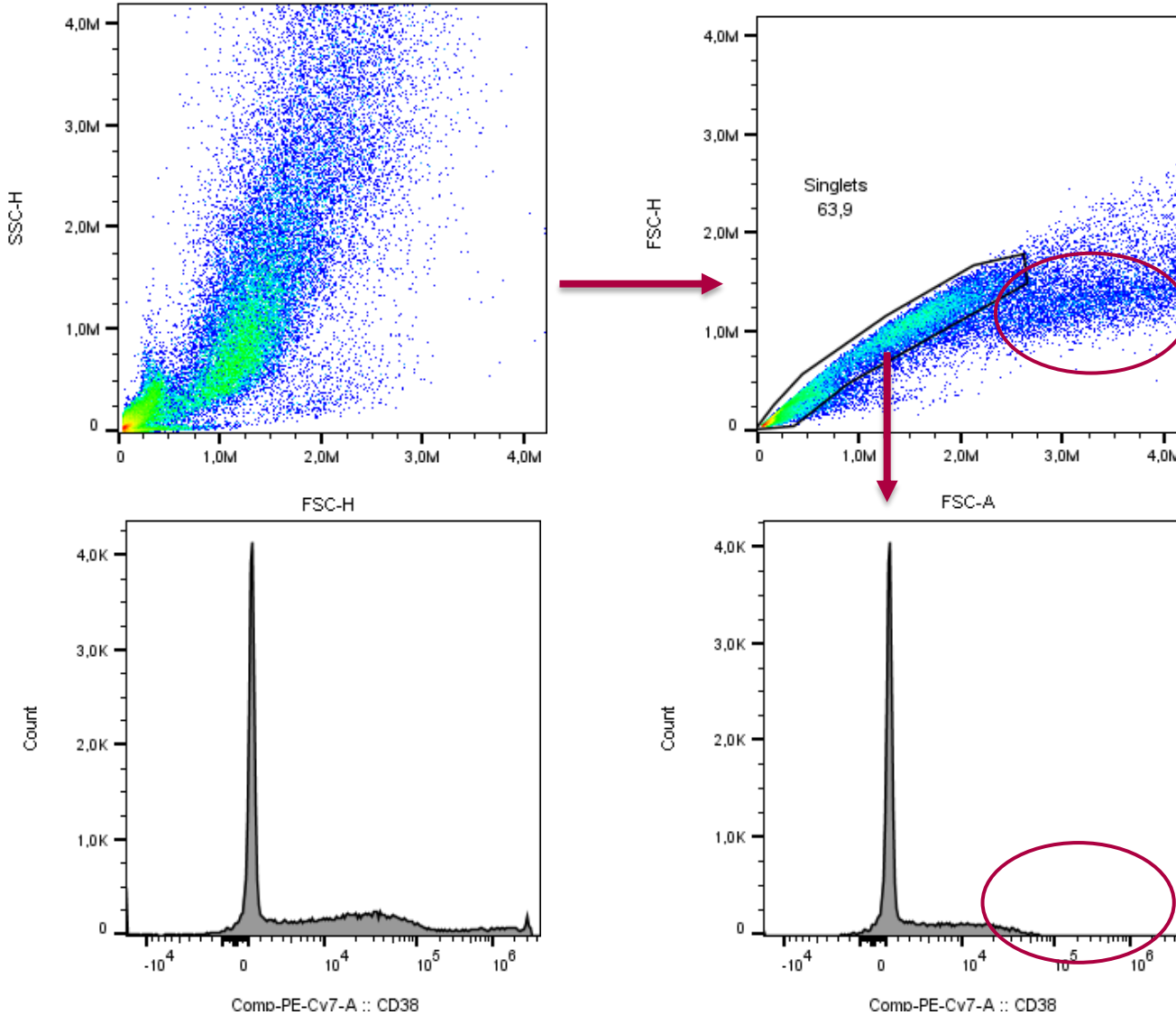
BD FACSCalibur™ – Cell Quest Pro

The screenshot displays the Cell Quest Pro software interface. At the top, two acquisition dot plots are shown: 'Acquisition Dot Plot' for FSC-H (X-axis) and SSC-H (Y-axis). A red circle highlights a context menu for the FSC-H plot, listing parameters: FSC-H, SSC-H, FL1-H, FL2-H, FL3-H, FL1-A, FL1-W, and Time (102.40 sec.). Below the plots, a 'Detectors/Amps' table is visible, with a red circle around it. The table lists parameters P1 through P7, their corresponding detectors (FSC, SSC, FL1, FL2, FL3, FL1-A, FL1-W, FL4), voltages, and gains. To the right, an 'Inspector: Dot Plot' panel shows settings for 'Basic Plot', 'Dot Plot', 'Geometry', 'Text Style', and 'FCS Keywords'. The 'Geometry' section shows dimensions: Location (Top: 0.31, Left: 0.11), Size (Width: 2.83, Height: 2.94).

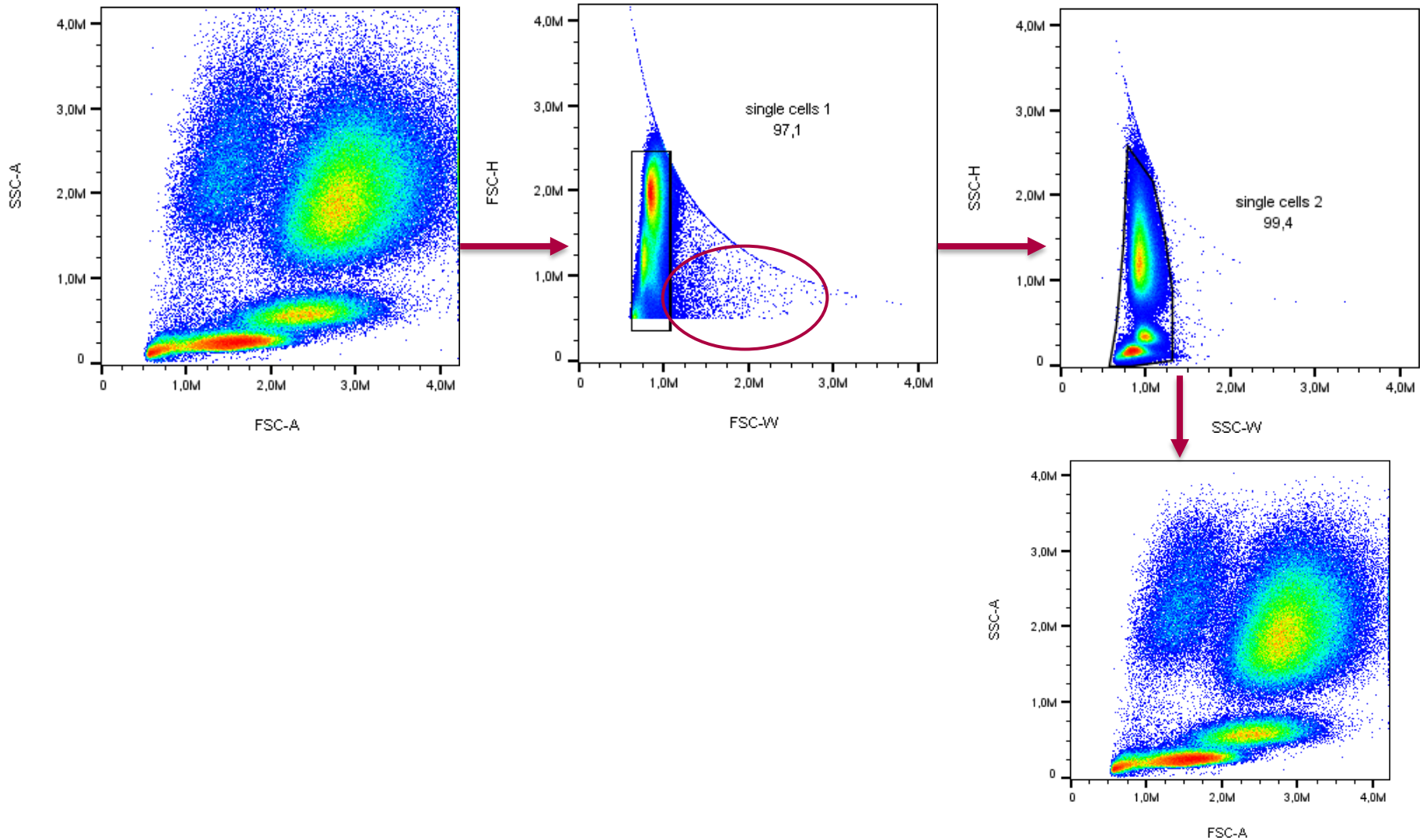
Param	Detector	Voltage	Amp Gain	Mode
P1	FSC	E00	2.00	Lin
P2	SSC	464	1.00	Lin
P3	FL1	541	1.00	Log
P4	FL2	472	1.00	Log
P5	FL3	602	1.00	Log
P6	FL1-A		1.00	Lin
P7	FL1-W		1.00	Lin
P7	FL4	600		Log



# example data and gating



# example data and gating



## Doublet discrimination - **TAKE HOME MESSAGE**

- Prevent doublet formation:
  - Filter cells
  - Resuspend cells
  - Vortex sample
  - Add EDTA
  - Reduce BSA/FCS concentration
  - Change to PP tubes instead of using PS tubes
- Select A, W and H parameter to be recorded
- Exclude doublet from analysis via A vs.W or H vs. W
- reduce acquisition speed / flow rate or dilute cells more

Thank you for your attention.

See you next month 02<sup>nd</sup> March.

Next topic:

The perfect control – what to take?

Die perfekte Kontrolle – aber wie und womit?