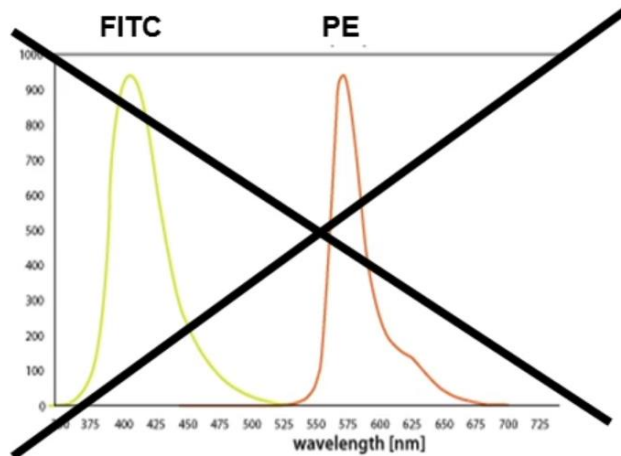


**4.) Aggravating compensation –
discover errors and correct them**

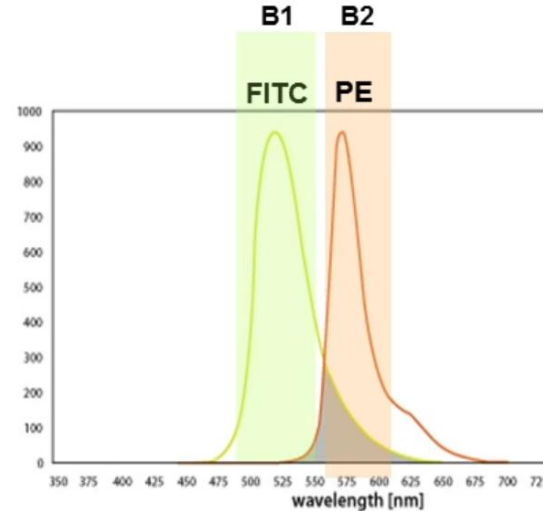
**4.) Die leidige Kompensation – Fehler
Erkennen und Nachbessern**

what is compensation?

- Compensation is the process to correct for spectral spillover of one dye on another detection channel
- empirically calculated from single stain controls in multicolour experiments



Wishful thinking

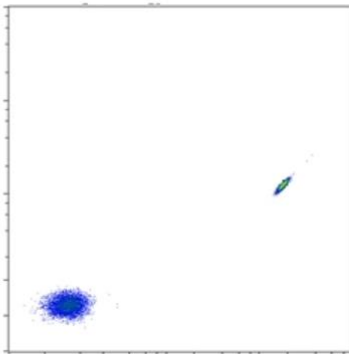


Spectral overlap

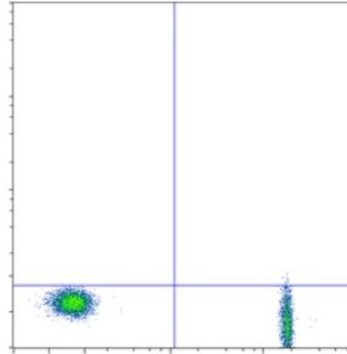
<https://www.miltenyibiotec.com/DE-en/resources/macs-academy/on-demand-webinars-and-tutorials.html#>

Which plot shows correct compensation?

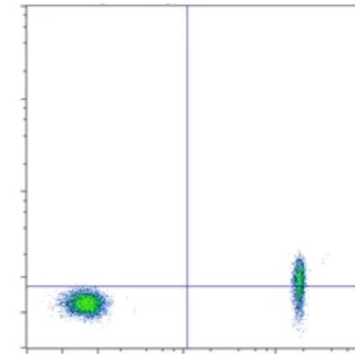
uncompensated



A

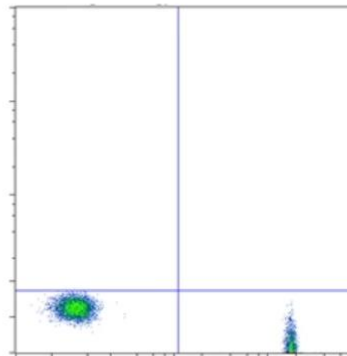


B

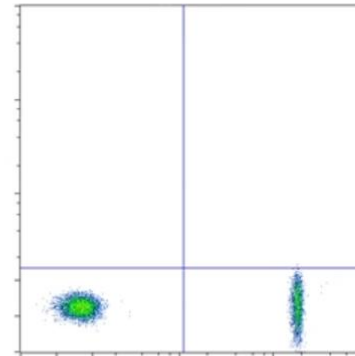


spillover
spread

C

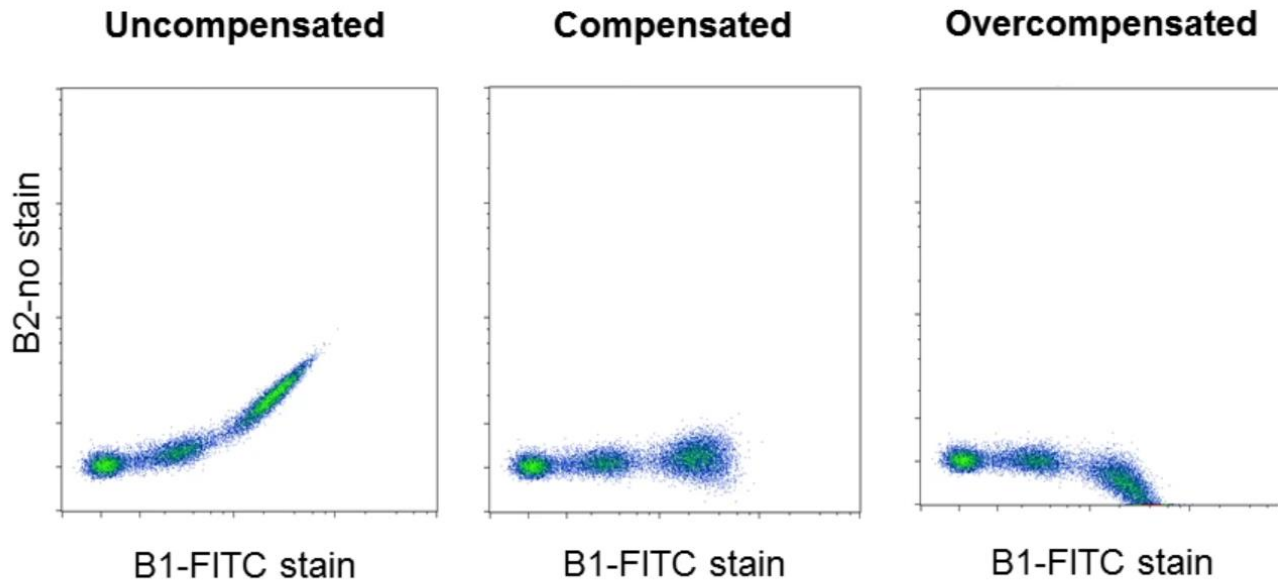


D



<https://www.miltenyibiotec.com/DE-en/resources/macs-academy/on-demand-webinars-and-tutorials.html#>

Compensation of spectral overlap

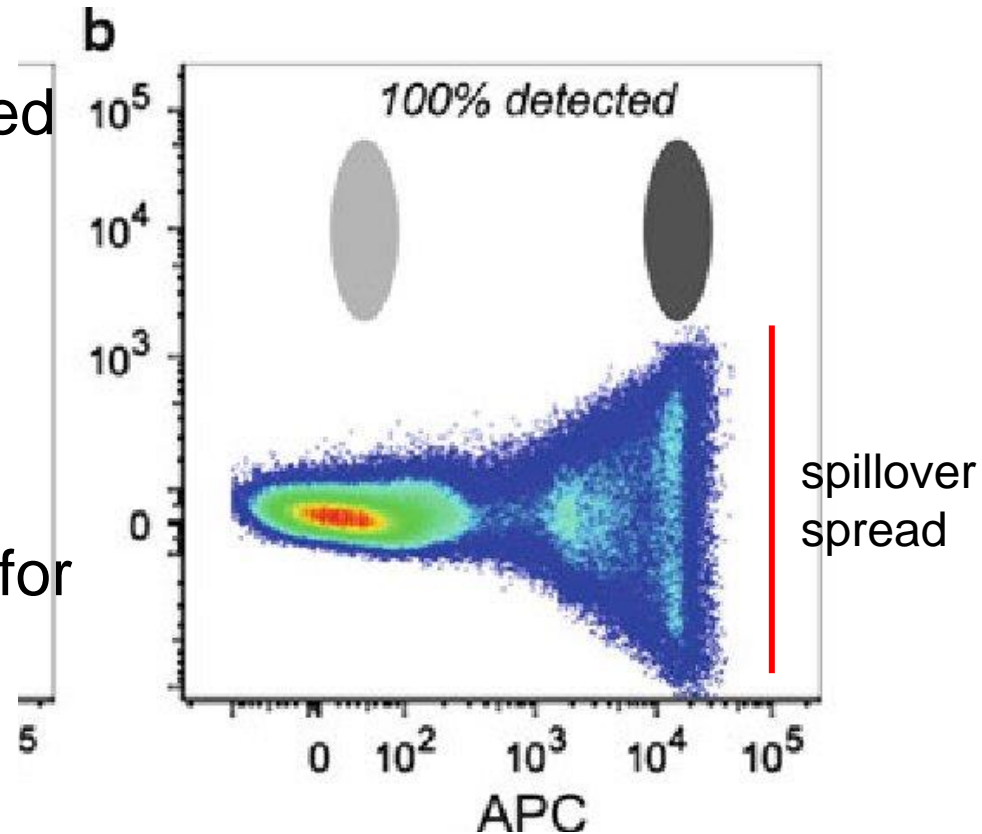


<https://www.miltenyibiotec.com/DE-en/resources/macs-academy/on-demand-webinars-and-tutorials.html#>

spillover spread

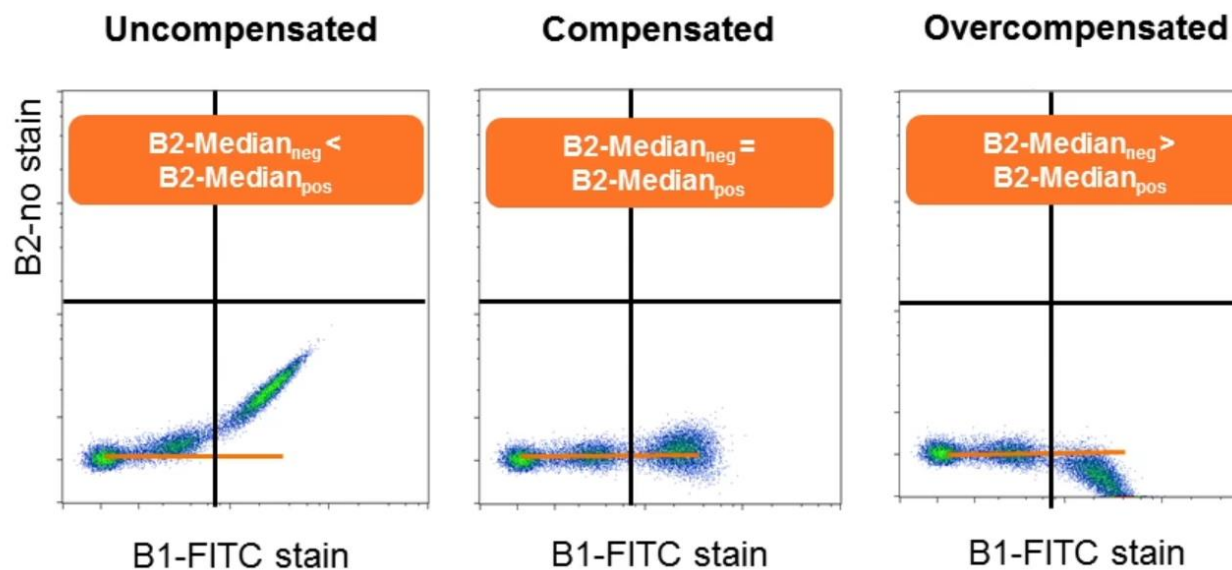
spread: mathematical introduced because of compensation

brighter dyes → bigger spread
depends on dyes → important for panel design



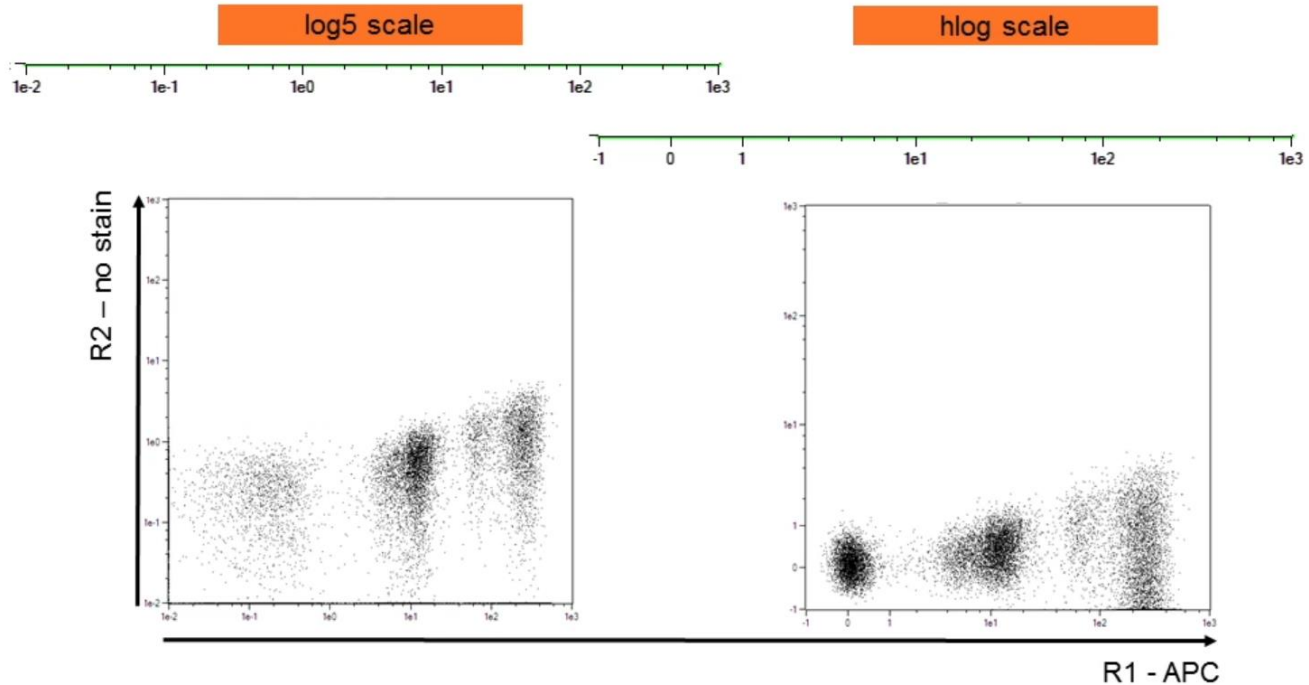
https://www.researchgate.net/figure/Spread-ing-error-and-loss-of-detection-sensitivity-a-APC-data-spread-into-the-Alexa_fig2_310494404

Use of MFI (median) for setting compensation



<https://www.miltenyibiotec.com/DE-en/resources/macs-academy/on-demand-webinars-and-tutorials.html#>

Avoid visual compensation



<https://www.miltenyibiotec.com/DE-en/resources/macs-academy/on-demand-webinars-and-tutorials.html#>

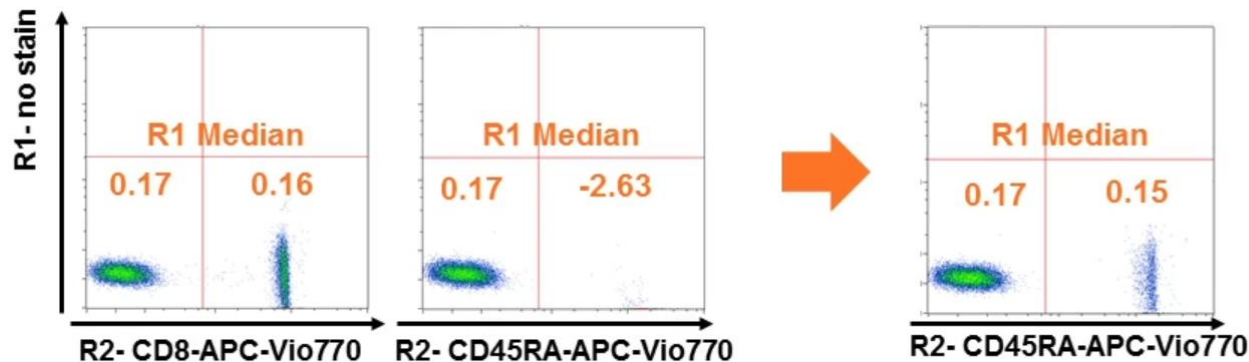
golden rules for compensation controls

- comp controls need to be as bright as the samples stained
- background (autofluorescence) of positive and negative population needs to be the same
- → compare cells with cells, beads with beads
- comp colour must fit experimental colour → FITC ≠ GFP ≠ AF488
- Beads good for rare cell markers
- Tandem-dyes differ from lot to lot
- buffers, fixation, ... may change the fluorophors → changes in compensation

Tandem fluorochrome conjugates need lot-specific compensation



- MACS Compensation Beads IgK, mouse were loaded with CD8-APC-Vio770™ or CD45RA-APC-Vio770™ antibodies
- Compensation done with CD8-APC-Vio770™ loaded beads vs APC-channel (R1)

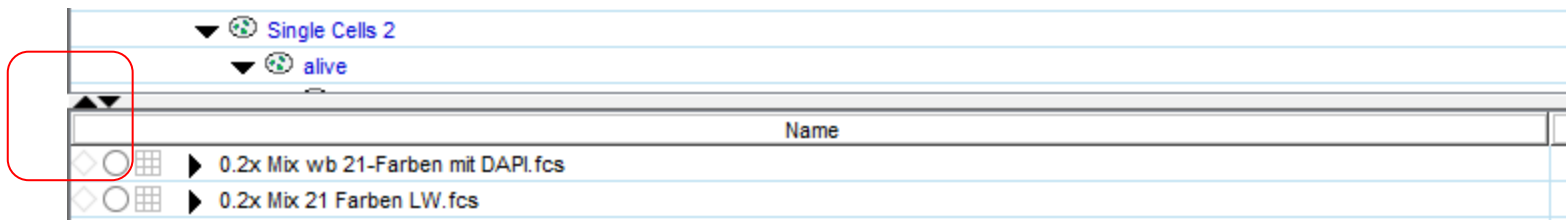


Compensation:
CD8-APC-Vio770 vs. R1 (APC): 8.2%

Compensation:
CD45RA-APC-Vio770 vs.
R1 (APC): 6.3%

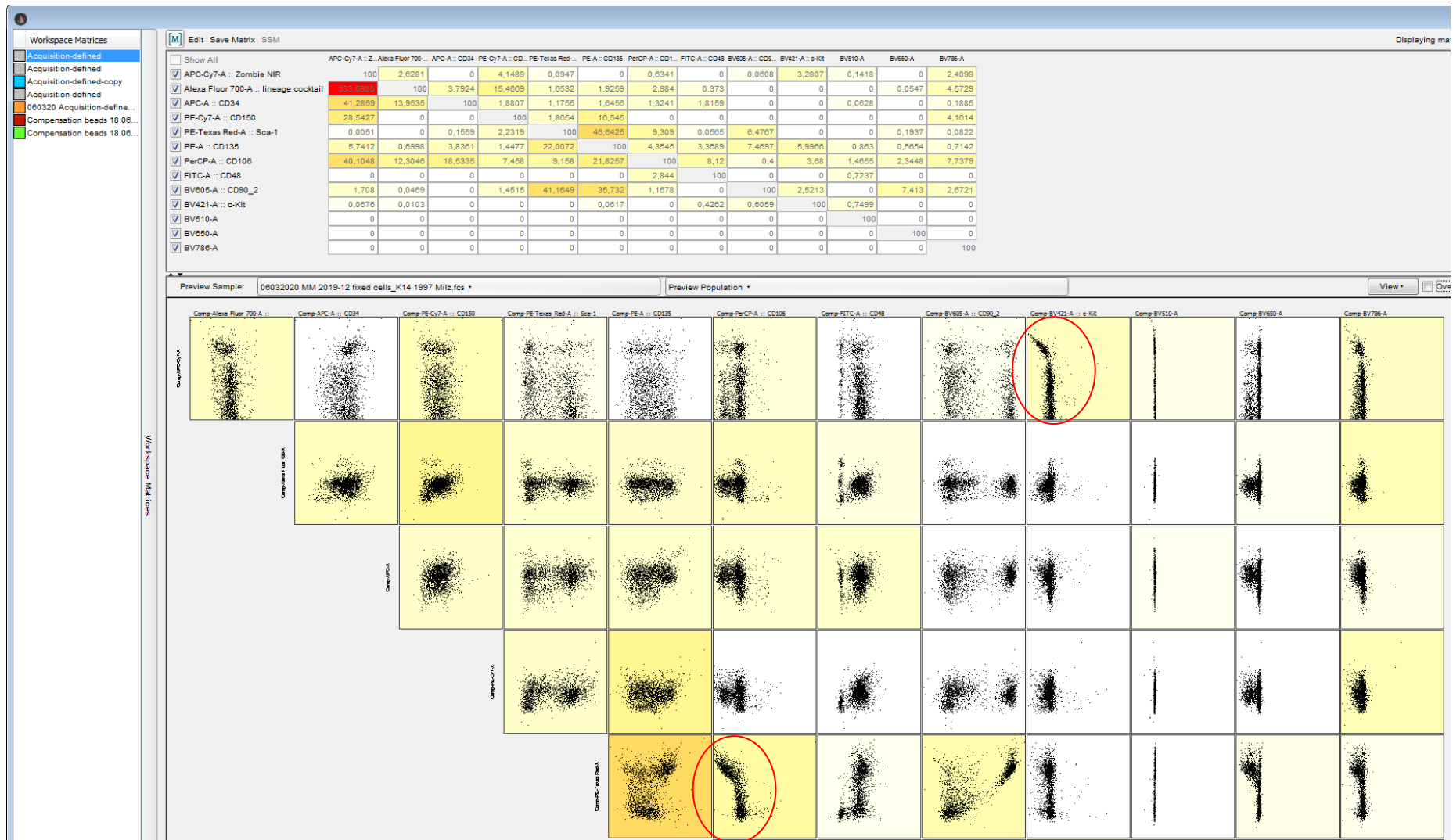
Compensation Matrix in FlowJo

- FlowJo allows different ways for compensation
- to start from scratch
- to use assisted calculation
- edit matrix from acquisition



- Special case – the BD FACS Calibur
- if compensated during acquisition the data will be saved changed
- → no way back to the uncompensated data

Compensation Matrix in FlowJo



TAKE Home Message

- compensation is calculated from single stain controls in multi- colour experiments
- autofluorescence of positive and negative fraction for each marker needs to be the same
- compare cells with cells, beads with beads
- spread is introduced and can't be avoided (only by panel design)
- FlowJo allows to view/edit or create new compensation matrices

Thank you for your attention.

Next topic (1st June): „The alpha and omega of preparing cells“

Nächstes Mal (am 1. Juni): Das A und O der Probenpräparation: Von Asservierung bis Zielzelle