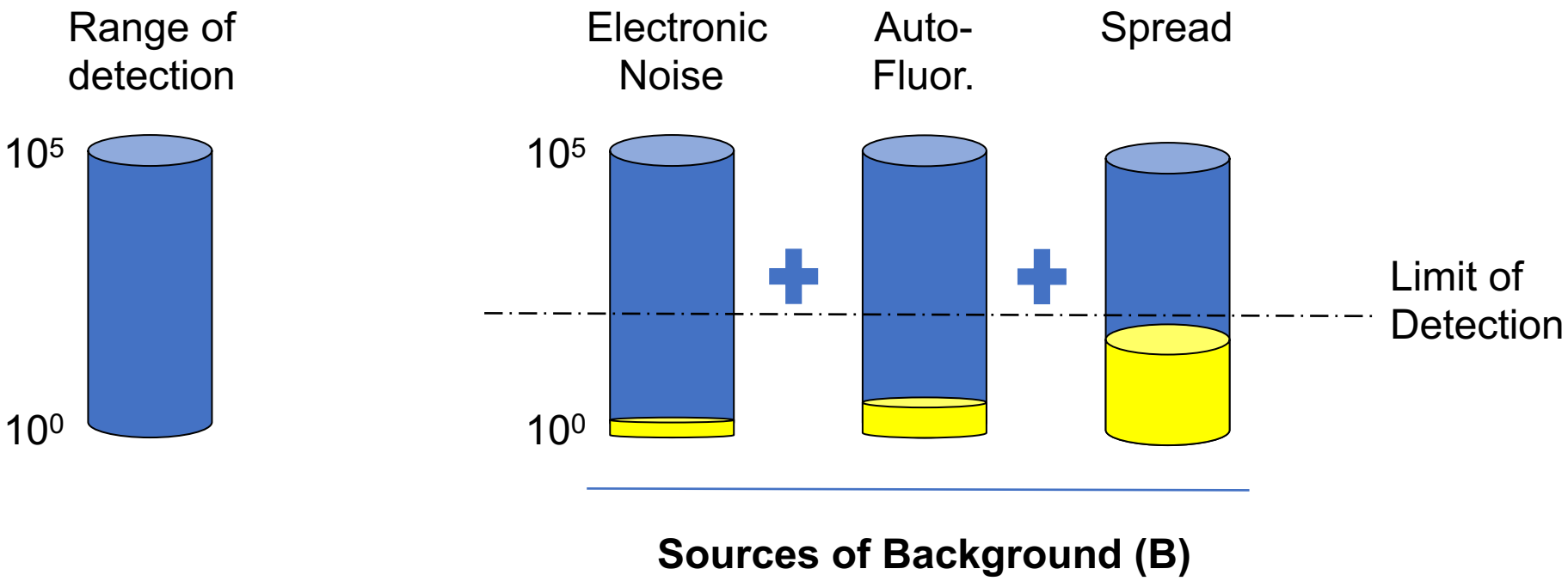
A LITTLE BIT OF BACKGROUND ABOUT BACKGROUND

- There are 3 main factors that contribute to the ability to clearly separate cells expressing a given marker
- The spread is the single most relevant factor!



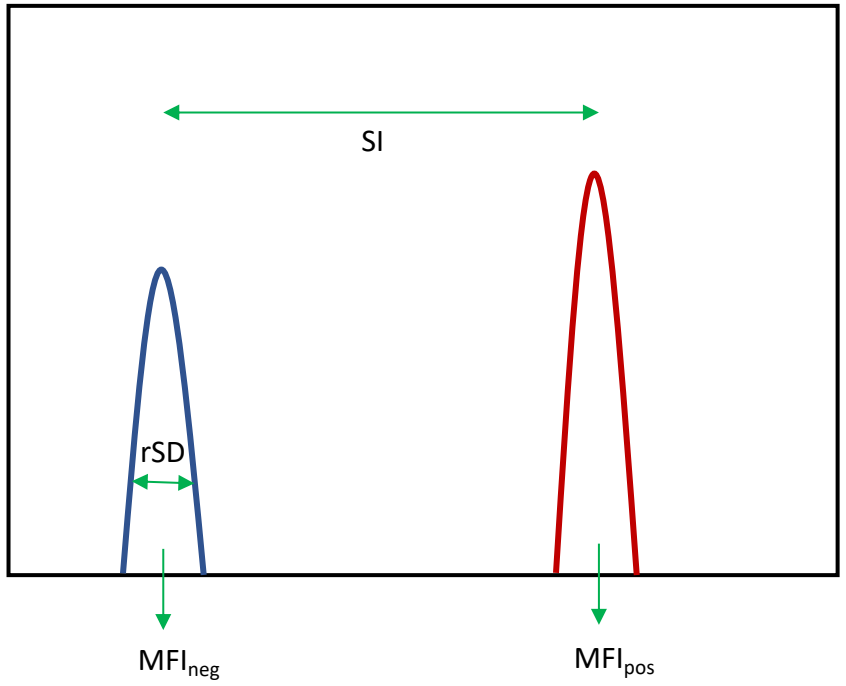
Adapted from Pratip Chattopadhyay

WHAT DOES THIS MEAN FOR FLUOROCHROME PERFORMANCE?

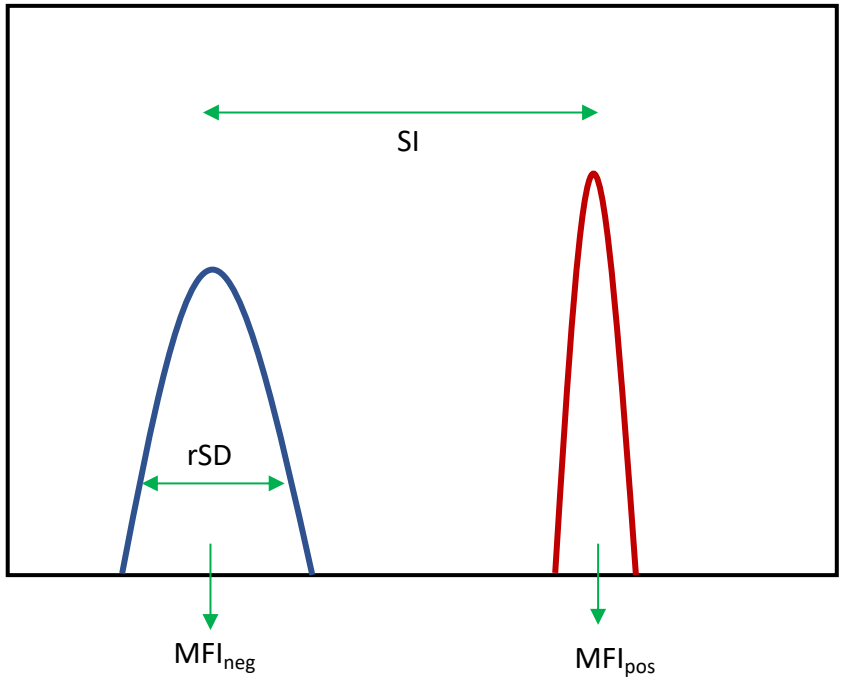
- The standard way to describe the performance of a given fluorochrome is to use the stain index

$$SI = \frac{(MFIpos - MFIneg)}{2 \, x \, rSDneg}$$

Single stain only



Increased background from spreading = reduced SI

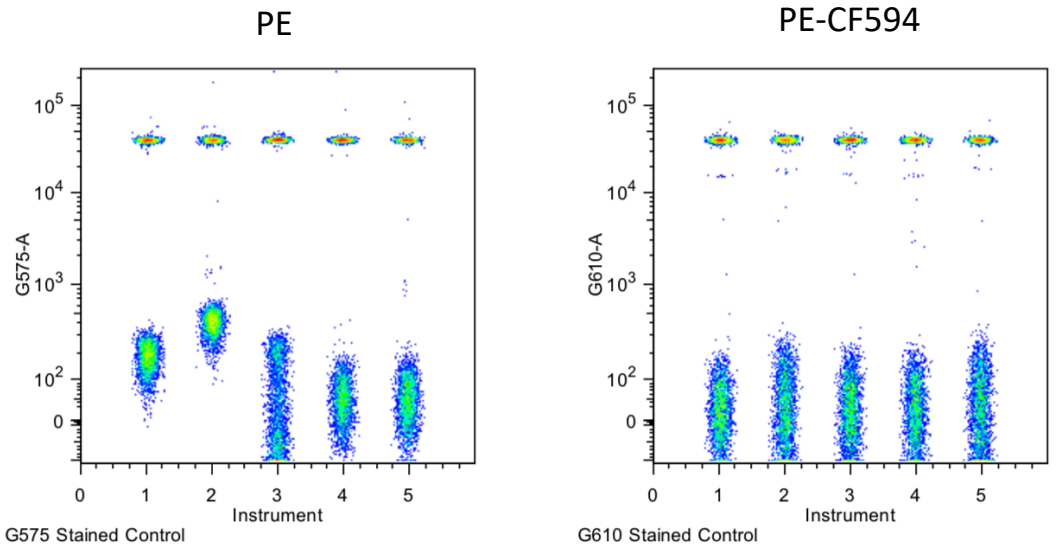


How to determine fluorochrome performance

- Fluorochrome properties can differ between instruments
  - Laser power, bandpass filters, instrument sensitivity…
- Optimal method: measure all fluorochromes conjugated to the same antibody and calculate SI
- For a rough estimate, use vendor information

Laser	Fluorochrome: Very Bright	Bright	Moderate	Dim
Ultraviolet (355 nm)		BD Horizon™ BUV661 BD Horizon™ BUV737	BD Horizon™ BUV395 BD Horizon™ BUV496	BD Horizon™ BUV805
Violet (405 nm)	BD Horizon™ BV421 BD Horizon™ BV650 BD Horizon™ BV711	BD Horizon™ BV605 BD Horizon™ BV786	BD Horizon™ BV510	BD Horizon™ V450 BD Horizon™ V500
Blue (488 nm)	BD Horizon™ BB515 BD Horizon™ PE-CF594 PE-Cy™5	PE PE-Cy™7	FITC Alexa Fluor® 488 PerCP-Cy™5.5	PerCP
Yellow/Green (561 nm)	PE BD Horizon PE-CF594 PE-Cy5 PE-Cy7			
Red (640 nm)		APC Alexa Fluor® 647 BD Horizon™ APC-R700		Alexa Fluor® 700 APC-H7 APC-Cy7

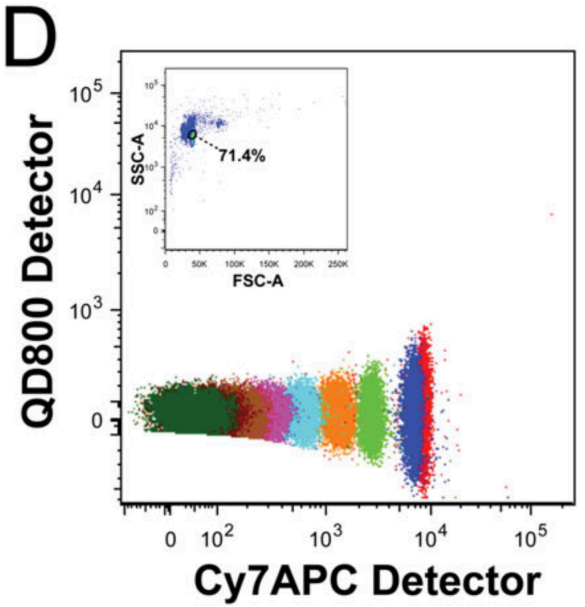
From BD Biosciences



HVTN Seattle Flow Cytometers

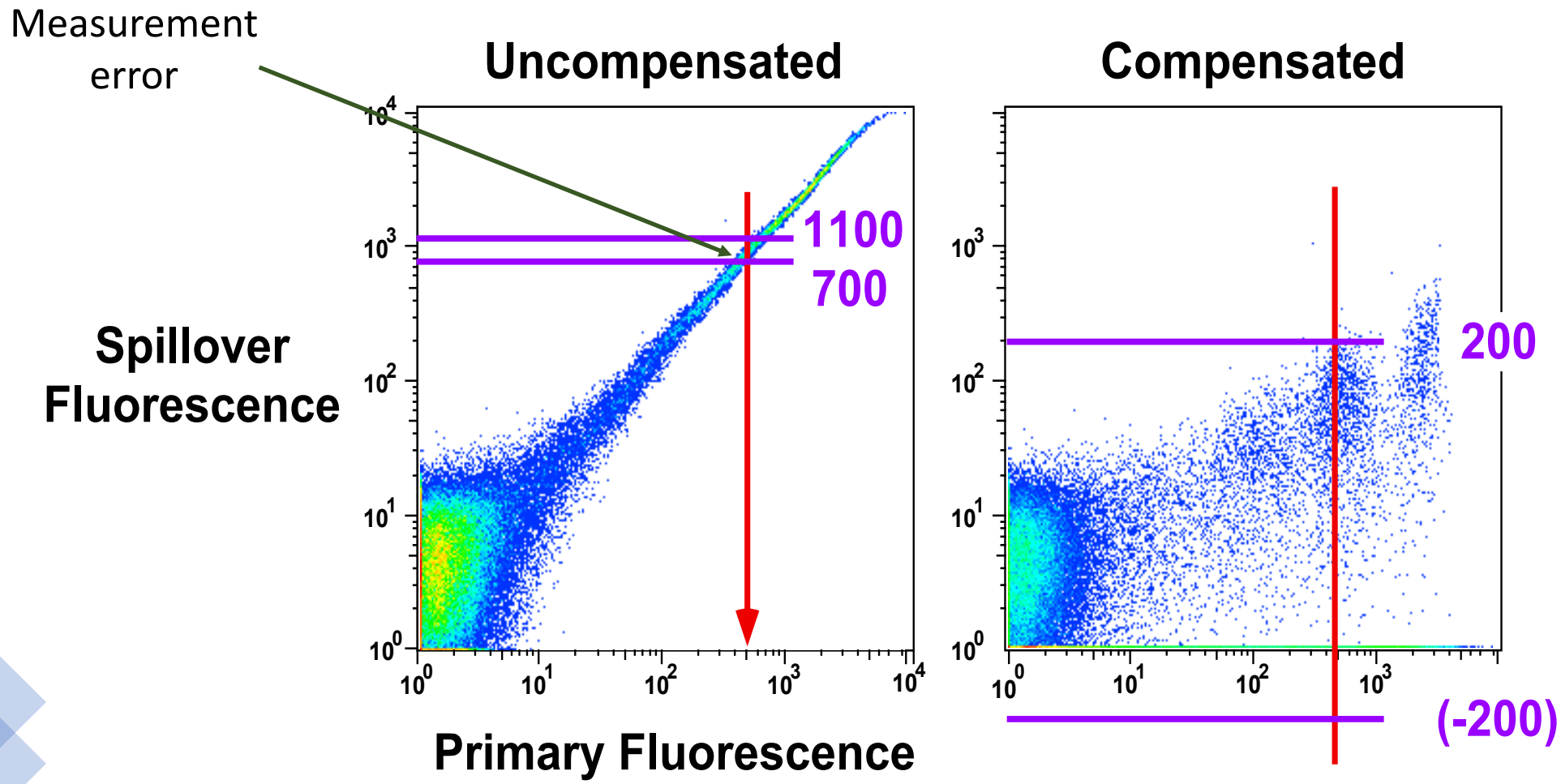
MEASUREMENT ERROR LEADS TO SPREADING

- The main source of the spreading error is the so-called, “Poisson Error” that occurs during photo counting in the detector (both PMT and APD)
- The error is proportional to the square root of the signal intensity
  - ➢ The stronger/more intense the signal, the greater the error (more photons!)
- Any error will be propagated throughout mathematical operations



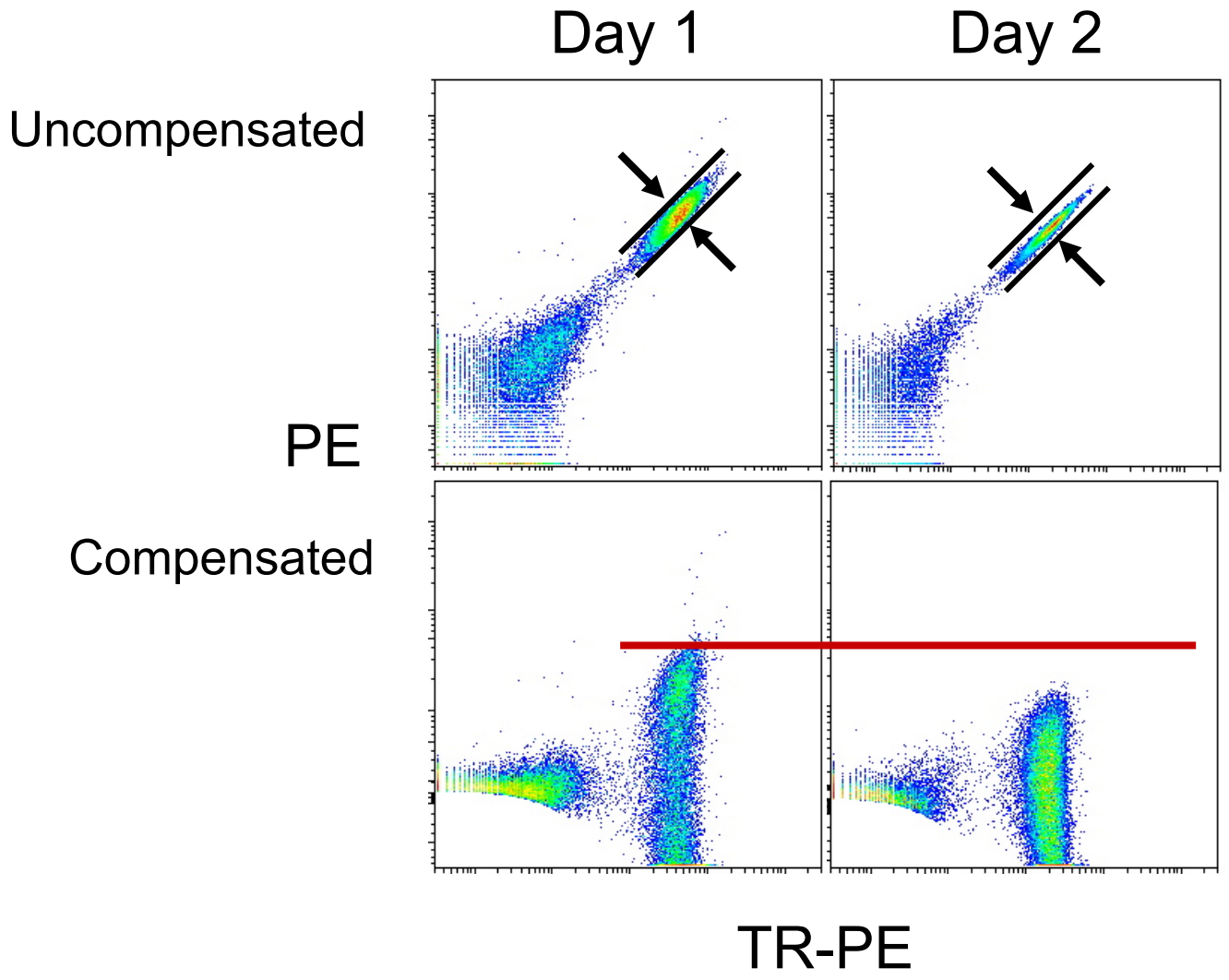
Ngyuen et al., Cytometry Part A 2013

EXAMPLE: SPREADING IN COMPENSATED DATA



Slide provided by M. Roederer, NIH

INSTRUMENT ALIGNMENT IS CRITICAL



- The compensation amount remains the same
- Improved instrument alignment means decreased measurement error
- Spreading decreases and limit of detection increases

Slide provided by S. Perfetto

COMPENSATION VALUES DO NOT PREDICT THE SPREAD

- The compensation value does not necessarily reflect the extent of the spreading error
  - Remember – compensation only subtracts the extra signal

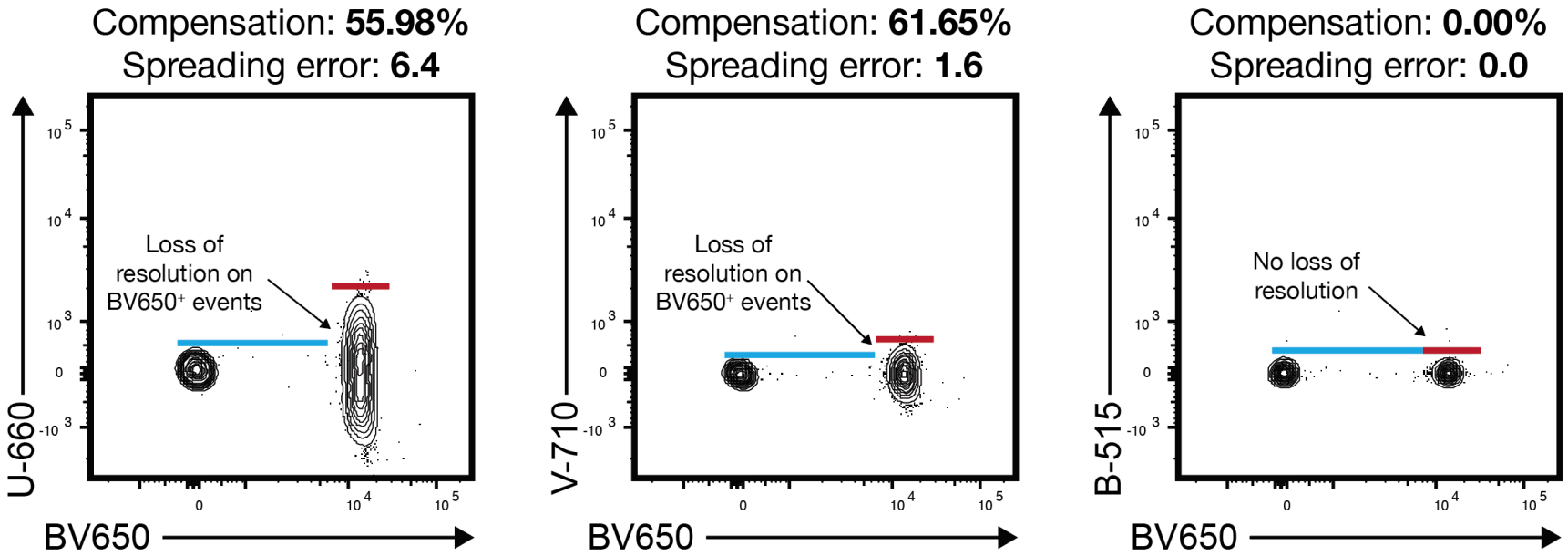
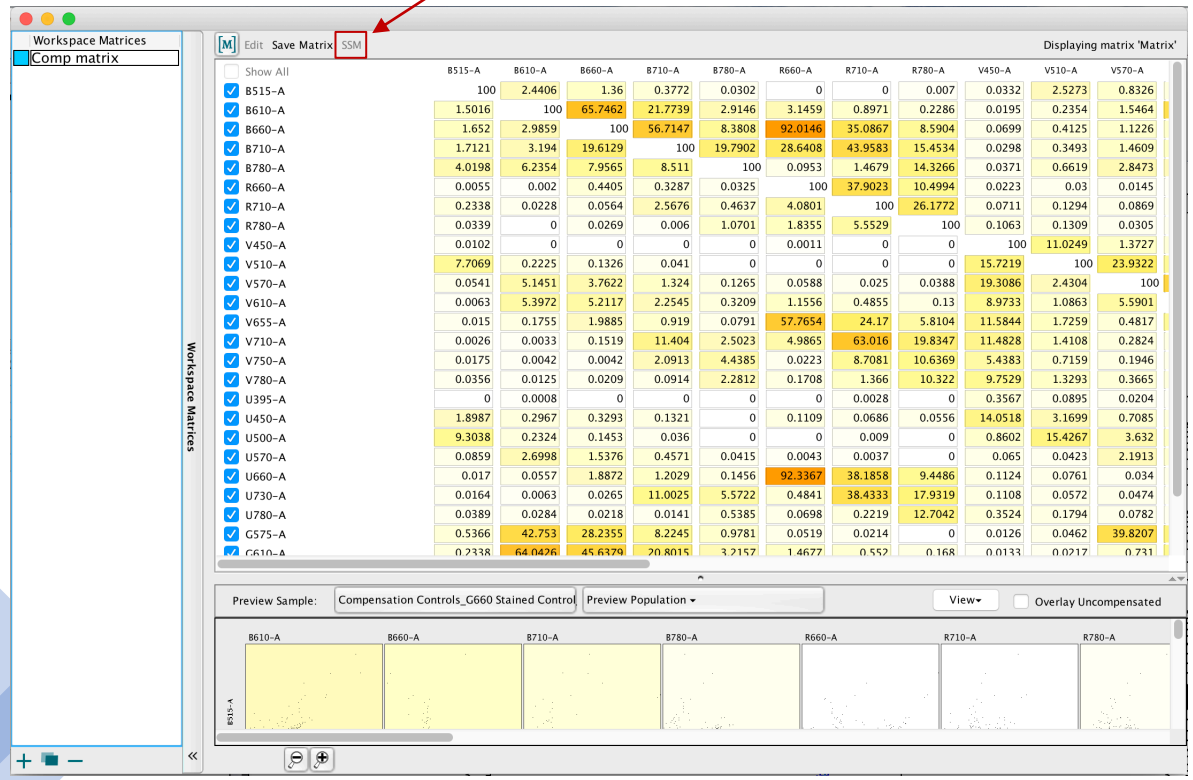


Image courtesy of Florian Mair

How to create an SSM in FlowJo

1. Gate compensation controls
2. Within the compensation module, there is a button for the SSM in both FlowJo 9.x and FlowJo 10.4

FlowJo 10.4:



FlowJo 9.x:

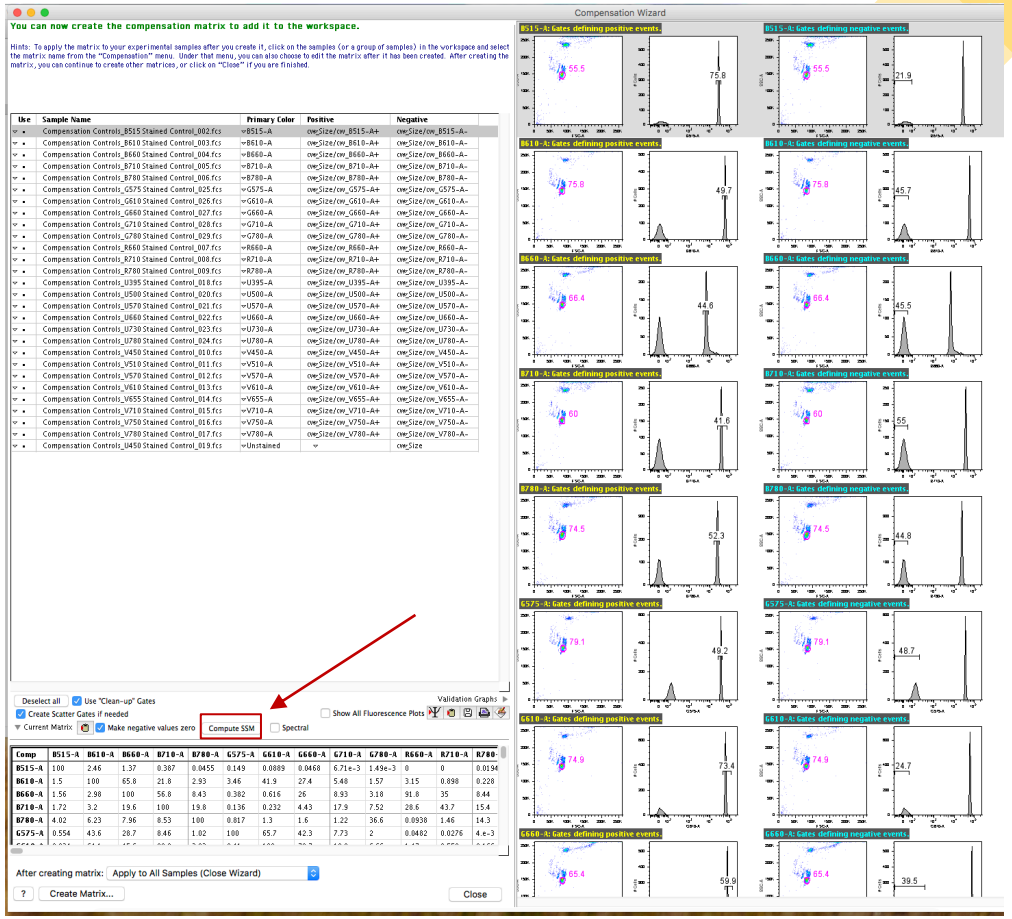


Image courtesy of Florian Mair

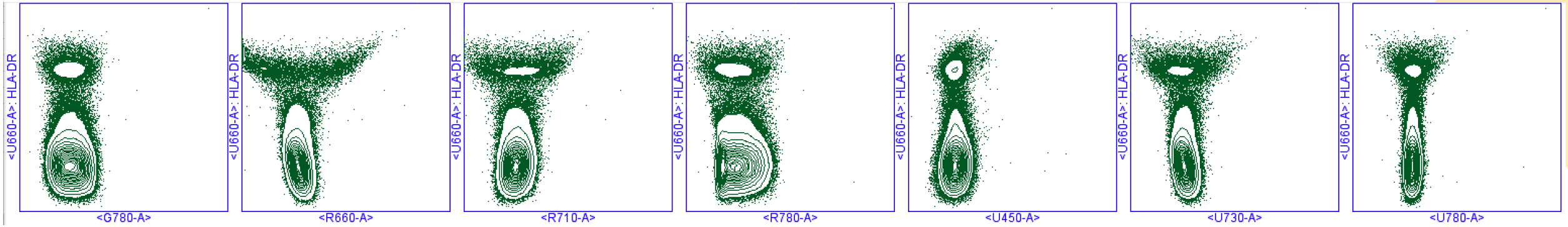
Understanding a Spillover/Spreading Matrix

Rows with high sums are fluorophores that donate a lot of spillover

		Detector																		
		B515	B710	G575	G610	G660	G780	R660	R710	R780	U395	U730	V450	V510	V570	V610	V655	V710	V780	
Fluorochrome	B515	0	0.165	0.0628	0.042	0.0697	0	0	0.0436	0.15	0.0537	0.161	0.102	0.0742	0	0	0	0	0	0.924
	B710	0.385	0	0.0966	0.067	0.508	1.15	0.324	2.26	0.977	0	1.54	0	0.197	0.183	0.166	0.343	1.41	1.38	10.9866
	G575	0.291	0.982	0	1.4	1.08	0.5	0.111	0.244	0	0	0.476	0.152	0.191	0.842	0.673	0.273	0.297	0.193	7.705
	G610	0.127	2.35	0.46	0	2.04	1.16	0.28	0.577	0.158	0.139	0.564	0	0	0.121	0.556	0.339	0.521	0.344	9.736
	G660	0.214	6.68	0.25	0.206	0	2.09	0.986	1.78	0.529	0	1.19	0	0.13	0	0.108	0.35	1.16	0.456	16.129
	G780	0.644	0.313	0.186	0.186	0.29	0	0	0.192	0.826	0	0	0	0	0.0205	5.06E-03	0.192	0.179	1.08	4.11356
	R660	0.211	0.903	0.112	0.12	1.75	0.692	0	2.22	1.15	0	1.35	0	0	0	0	0.463	0.451	0.479	9.901
	R710	1.09	0.639	0.289	0	0	0.877	0	0	1.84	0	1.67	0	0	0.882	0	0	0.608	0.741	8.636
	R780	0.419	0.17	0	0.121	0.504	1.69	0.728	0.63	0	0	0.679	0	0	0.332	0	0.131	0.256	1.42	7.08
	U395	0.875	0.463	0.27	0.259	0	0	0	0	0.479	0	0	0	0	0.37	0.367	0.28	0.202	0.245	3.81
	U730	0	1.12	0	0.113	0	0.493	0	2.01	1.52	0.468	0	0.333	0.513	0.0118	0	0.174	0.321	0.754	7.8308
	V450	0.363	0.205	0.141	0	0	0	0.0692	0	0	0	0	0	0.953	0.299	0.267	0.113	0.109	0.142	2.6612
	V510	0.256	0	0.412	0.324	0	0	0.12	0.194	0.304	0.16	1.23	0.831	0	1.38	1.45	0.707	0.563	0.669	8.6
	V570	0	0.61	1.1	1.07	0.915	0.582	0.149	0.161	0.46	0	0.927	0.817	0.253	0	1.77	0.879	0.827	0.518	11.038
	V610	0.257	0.969	0.358	1.23	1.45	0.783	0.345	0.486	0.333	0	2.19	0.329	0.031	0.369	0	1.41	1.22	1.18	12.94
	V655	0.992	0.573	0	0.218	0.861	0.457	1.12	1.25	0.797	0	2.15	0.747	0.503	0.493	0.593	0	1.77	1.63	14.154
	V710	0.648	1.52	0.237	0	0.385	0.353	0.219	1.87	0.866	0.292	4.71	0.395	0.393	0	0.313	0.246	0	3.3	15.747
	V780	0	0	0	0	0	0.894	0	0.25	0.839	0	0.939	0.461	0.386	0.268	0.187	0.207	0.25	0	4.681
		6.772	17.662	3.9744	5.356	9.8527	11.721	4.4512	14.1676	11.228	1.1127	19.776	4.167	3.6242	5.5713	6.45506	6.107	10.144	14.531	

Columns with high sums are detectors that collect a lot of spillover

WHAT DO THESE NUMBERS REPRESENT IN REALITY?

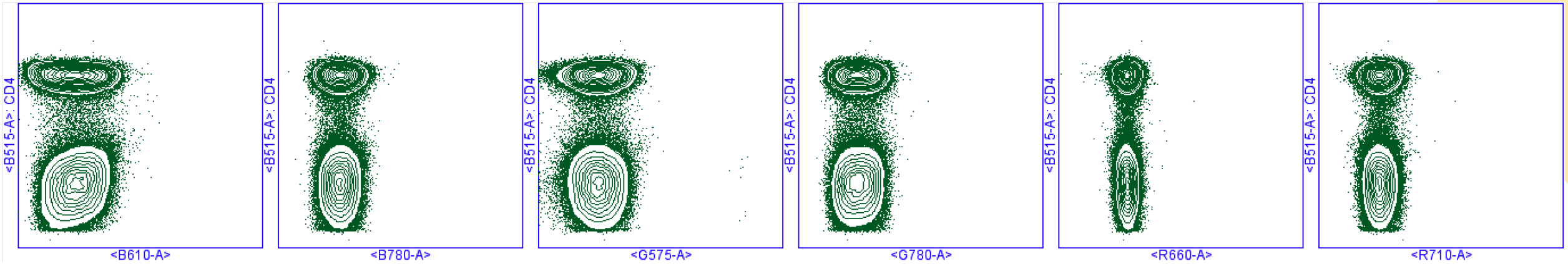


G780	R660	R710	R780	U450	U730	U780
0.873	10.3	3.58	1.41	0.287	3.41	2.48

Example of SSM values for U660 (HLA-DR BUV661) for several detectors

The larger the value, the more spread

WHAT DO THESE NUMBERS REPRESENT IN REALITY?



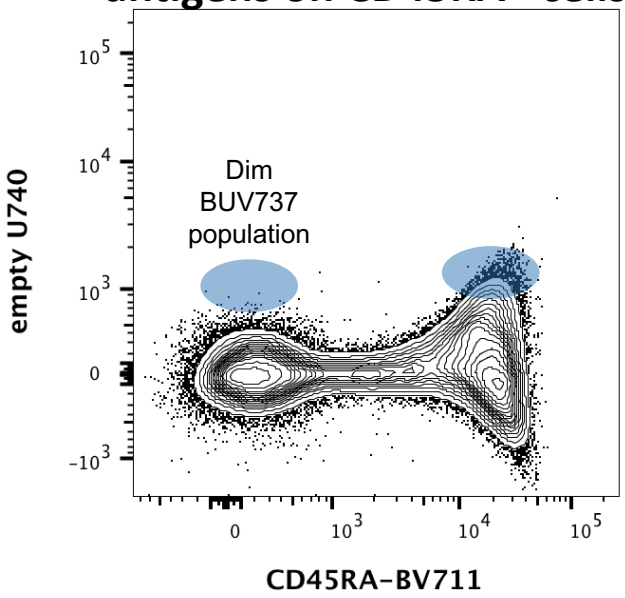
B610	B780	G575	G780	R660	R710
0.668	0.319	0.237	0.0928	0.315	0.179

Example of SSM values for B515 (CD4 Ax488) for several detectors

IMPLICATIONS OF SPILLOVER/SPREADING ERROR ON RESOLUTION

- Spreading error is the single most relevant contributor to loss of resolution
- KEY POINT: spreading error reduces the resolution in the detector that is collecting the spillover

BUV737 (U740): poor choice for dimly expressed antigens on CD45RA+ cells



PE-Cy7 (G780): good choice for dimly expressed antigens on CD45RA+ cells

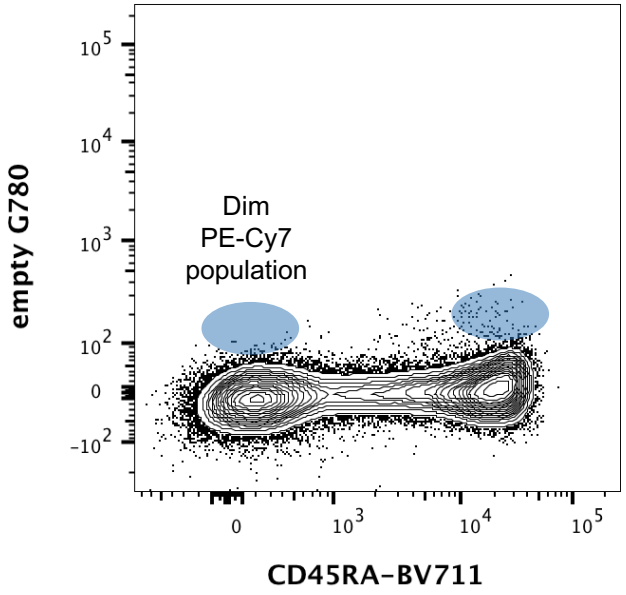
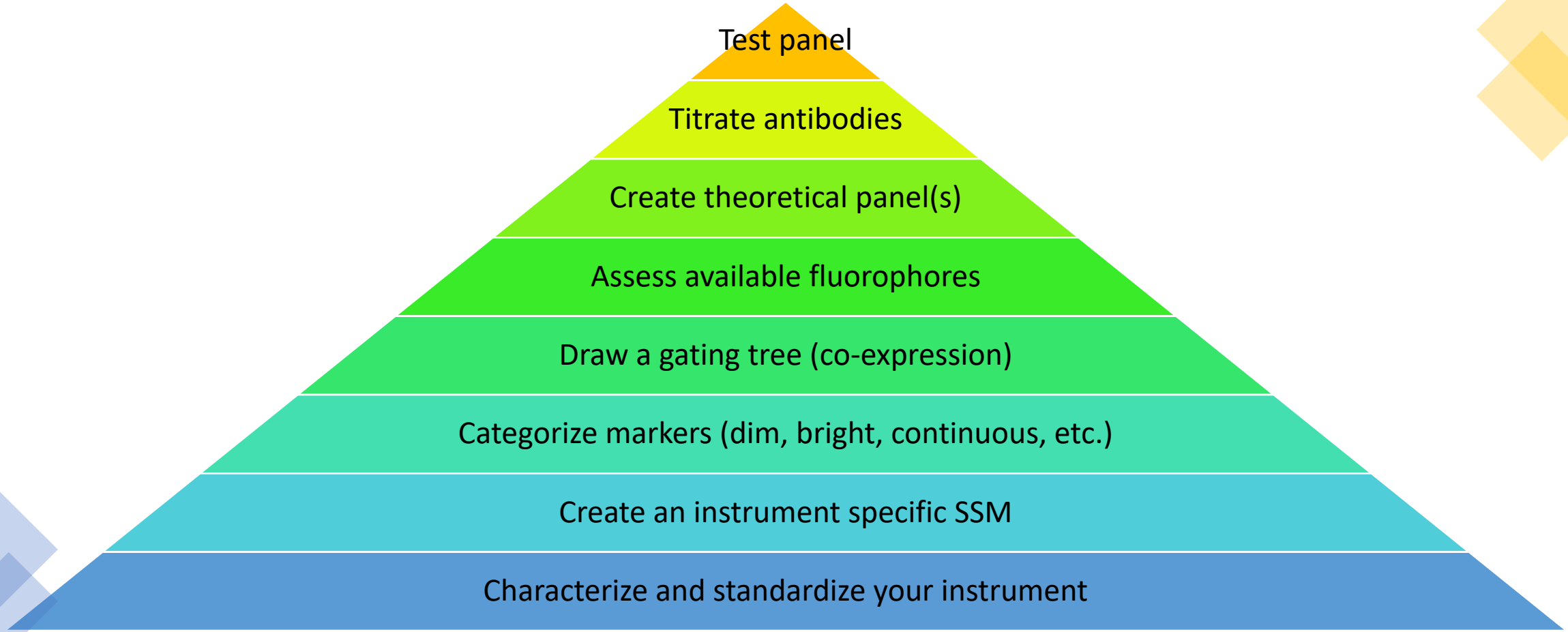


Image courtesy of Florian Mair

In Review

- Compensation
  - What is compensation and why it is necessary
  - Compensation controls
  - Transformation of data to confirm compensation
  - Diagnosis of compensation errors and how to fix them
- Spillover/Spreading
  - Background noise and how it impacts resolution
  - Stain index (fluorchrome performance) and how to calculate it
  - Measurement error (spreading)
  - How to generate and interpret a spillover spreading matrix
- Panel Design

Pyramid of panel design



Cytometer Standardization and Characterization

Note – this is for conventional flow cytometers

1. Perform voltage titration on each detector with CD4 stained cells (CD4 is available in every colour)
2. For each detector, calculate the stain index at each voltage reading and plot SI vs voltage to obtain a SI curve
3. Choose optimal voltage – minimum voltage where stain index is highest (optimize)

   NOTE: Can choose something slightly below optimal voltage to allow for populations that may be brighter than CD4
4. Run QC beads to determine target MFI for each detector (standardize)
5. Run QC beads and adjust voltages before every experiment so that bead MFI meets target MFI

Cytometer Specific (Representative) SSM

- For each detector…
  1. Stain cells with CD4 and acquire samples
  2. Create a compensation matrix
  3. Create SSM matrix

- Spillover/Spread matrix is representative (depends on markers in panel) but will always follow the same pattern **IF** the cytometer is kept at the same settings