Quality Control and Standardization of Flow Cytometry

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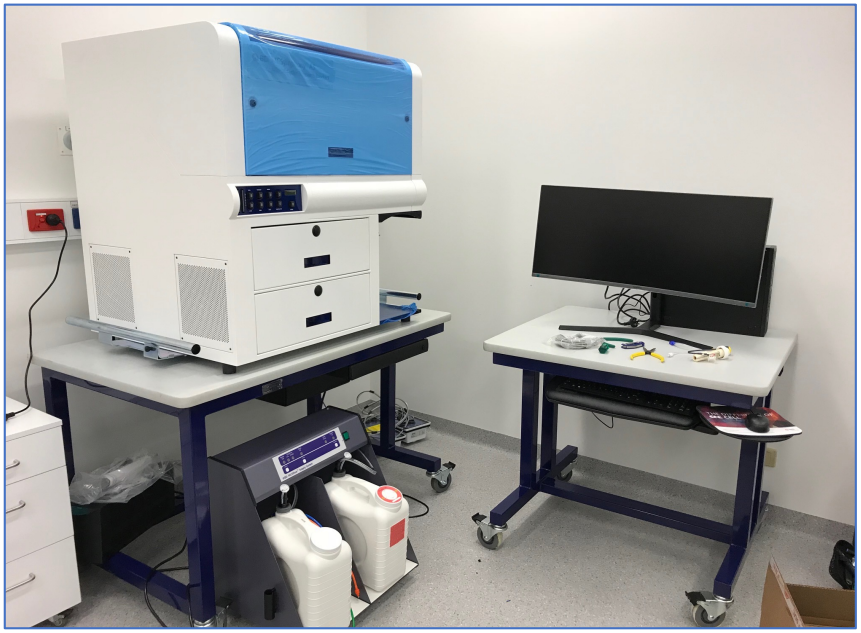
Westmead Cytometry Facility

Instrument	Lasers	Detectors	Complexity
CytoFlex	2	4	Simple
CantoA	2	8	Simple
CantoII	3	10	Intermediate
CantoII	3	10	Intermediate
LSRII	5	20	Advanced
Fortessa	5	20	Advanced
Symphony V 5.2	8	50	High Parameter
AriaIII	3	16	Intermediate
Influx	5	24	Advanced



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What is the Quality Control?

- A set of procedures performed by the laboratory staff for the continuous and immediate monitoring of laboratory work in order to decide whether the results are reliable enough to be released

Why is Quality Control Important in Flow Cytometry?

- Flow cytometer is highly sensitive. The success of flow cytometry experiments depends on a number of key factors which QC will be able to verify.
- A routine sequence of QC is essential to ensure
  - Accuracy
  - Resolution (dim subpopulation): sensitivity
  - Reproducibility
  - Standardisation
- A well designed monitoring procedure should identify both immediate and potential problems
- Allow data comparison within an instrument on different dates or across different instruments and facilities
- It will also help to
  - Establish credibility in the field
  - Reduce waste and rework

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Flow Cytometry QC

- Instrument QC
- Procedure/Method QC: SOP
- Other QCs in the lab: specimen integrity verifications, reagent QC, internal QC

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Instrument QC Key Factors

Hardware Variables

- Lasers
- PMTs
- Optical filters
- Flow cell condition
- Electronics

Dependent Variables

- Laser alignment
- Detector PMT voltages
- Software - Laser delay, area scaling factors, and window extension
- Linear range of each parameter
- Target MFI for each parameter
- Detector efficiency
- Optical background
- Electronic noise

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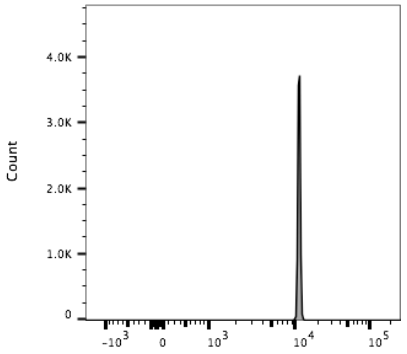
What is the ideal sample for Instrument QC

Bead particles are ideal for this purpose

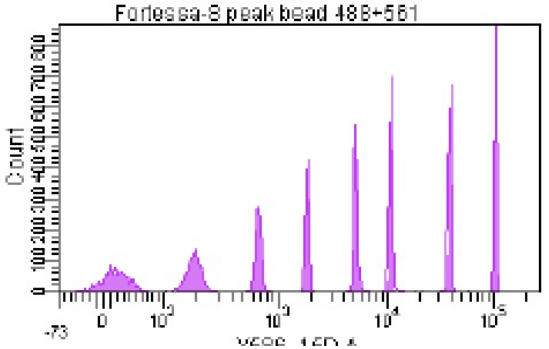
- Measurement values can be reproduced over long period of time
- Contains broad-spectrum dyes: can be excited by most currently supplied lasers and emit in the respective detectors for virtually any fluorescent dye

Beads

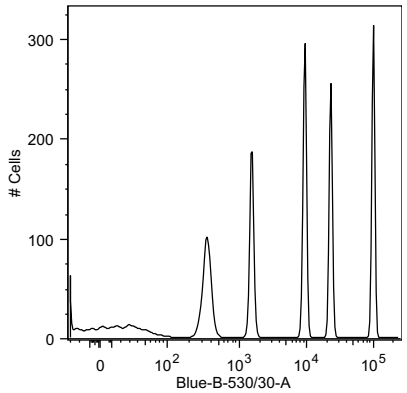
- Single peak Rainbow beads:
  - Standardisation
  - Calibration
- Multi-peak beads:
  - Show the dynamic range
  - Offer quick visual check of relative PMT response of a detector



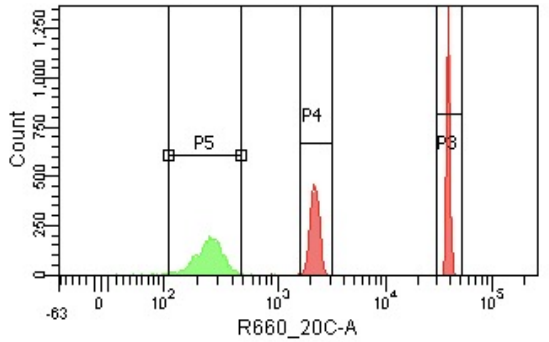
Single peak



8 peak rainbow beads



Cytocal



CS&T beads

Baseline Performance

When to do it?

- Upon instrument installation
- Major part replacement e.g. laser, PMT
- Following PM

Why to do it?

- Check the critical components to ensure optimal performance

It is essential for all flow cytometers.

Cytometer Baseline Report

Cytometer:	LSRFortessa	User:	BDService
Cytometer Name:	YellGrn Fortessa	Institution:	N/A
Serial Number:	H647800L006	Software:	BD FACSDiva 6.2
Input Device:	Manual	Date:	09/11/2015 03:31 PM
Cytometer Configuration:	WMI_2015_3B_6V_3U_3R_4Y		

Setup Beads

Bead Product: CST Setup Beads, Part #: 910723
Lot ID: 49706, Expiration Date: 12/31/2016
Bead Lot Information: Available

Detector Settings

Laser	Detector	Parameter	PMTV	New Target Value	Old Target Value	Bright Bead %Robust CV	Mid Bead Median Channel	Mid Bead % Robust CV	Dim Bead Median Channel	Dim Bead % Robust CV
Blue	FSC	FSC	427	125000	N/A	1.58	129114	1.55	22103	2.96
Blue	C	SSC	254	125000	N/A	2.44	131859	2.23	70549	1.4
Blue	B	B530_30B	444	15743	N/A	1.61	615	6.29	82	28.09
Blue	A	B710_50A	645	43327	N/A	2.5	1305	10.08	196	29.52
Red	C	R670_14C	541	34452	N/A	1.78	1616	6.92	135	26.79
Red	B	R730_45B	498	39700	N/A	1.87	1018	5.94	114	22.24
Red	A	R780_60A	527	42716	N/A	2.05	934	6.34	107	24.71
Violet	F	V440_40F	401	17876	N/A	2.63	1083	6.73	207	20.38
Violet	E	V525_50E	367	60953	N/A	2.08	2759	3.91	198	15.41
Violet	D	V610_20D	578	37639	N/A	3.08	1336	12.98	172	45.75
Violet	C	V660_20C	587	41219	N/A	3.08	2116	8.13	181	32.86
Violet	B	V710_50B	519	56839	N/A	3.53	2031	7.4	190	25.98
Violet	A	V780_60A	655	126431	N/A	4.55	1806	12.68	178	37.98
UV	C	U379_28C	434	29425	N/A	2.8	917	5.09	156	15.34
UV	B	U515_30B	477	79449	N/A	2.41	1215	9.98	151	41.48
UV	A	U740_35A	860	82473	N/A	6.04	1801	21.84	165	80.58
Yellgrn	D	Y586_15D	397	15471	N/A	3.57	560	7.69	150	18.33
Yellgrn	C	Y610_20C	383	13545	N/A	3.67	481	8.46	125	18.06
Yellgrn	B	Y710_50B	450	44410	N/A	3.68	1173	7.75	152	32.16
Yellgrn	A	Y780_60A	471	62607	N/A	3.94	1257	8.09	149	22.44

Detector Settings (Continued)

Laser	Detector	Parameter	Linearity Min Channel	Linearity Max Channel	Slope	Intercept	Electronic Noise Robust SD	Qr	Br
Blue	FSC	FSC	N/A	N/A	0.0038	3.5	N/A	N/A	N/A
Blue	C	SSC	N/A	N/A	6.3914	-10.3	N/A	N/A	N/A
Blue	B	B530_30B	45	245862	7.5104	-15.7	15.5	0.0839	765
Blue	A	B710_50A	513	230327	7.3266	-16.0	20.7	0.03	264
Red	C	R670_14C	23	239658	7.4909	-15.9	13.3	0.1356	18
Red	B	R730_45B	93	237973	7.3948	-15.3	13.9	0.0189	3251
Red	A	R780_60A	77	235042	7.3418	-15.3	14.5	0.0158	2033
Violet	F	V440_40F	19	248075	7.7003	-15.8	17.5	0.0747	2414
Violet	E	V525_50E	30	240765	7.9870	-15.7	16.1	0.0383	511
Violet	D	V610_20D	70	235960	7.5012	-16.1	17.5	0.0503	123
Violet	C	V660_20C	54	234976	7.4116	-15.9	18.2	0.0837	87
Violet	B	V710_50B	80	234632	7.4160	-15.4	19	0.1178	51

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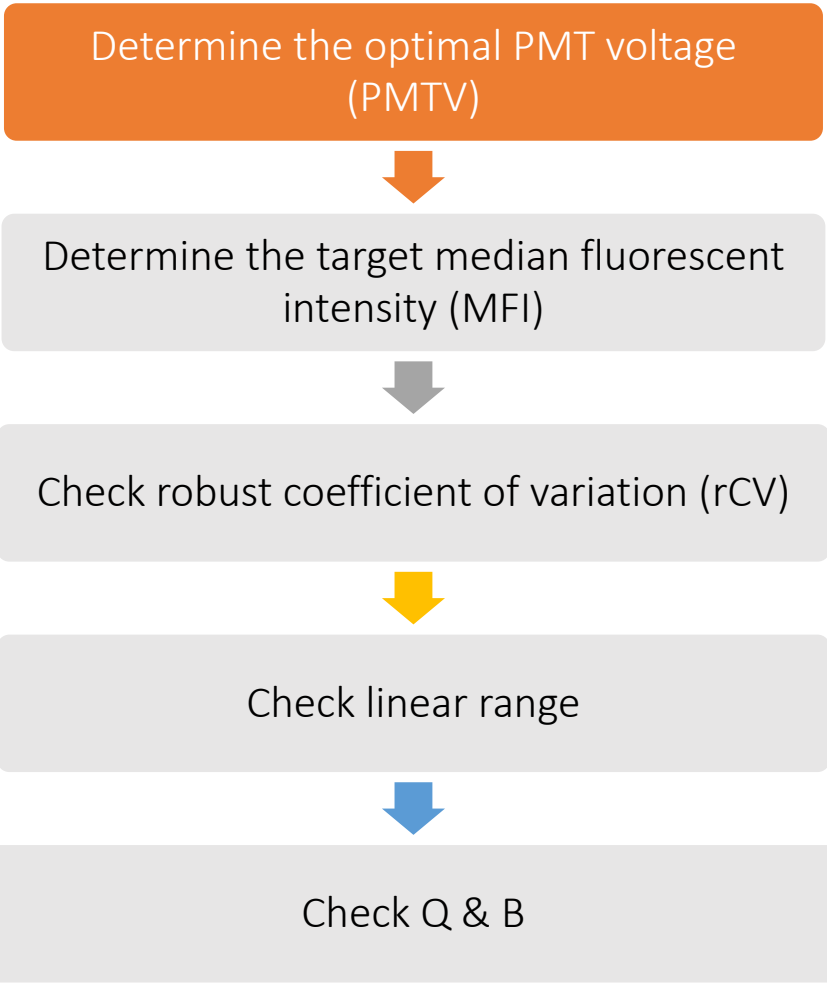
QC Key Factors

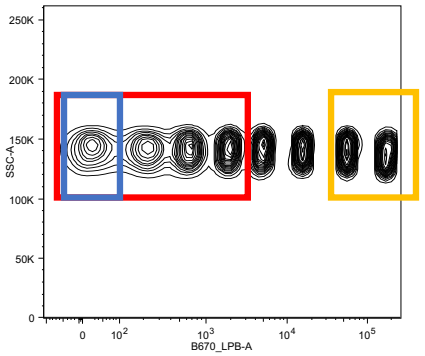


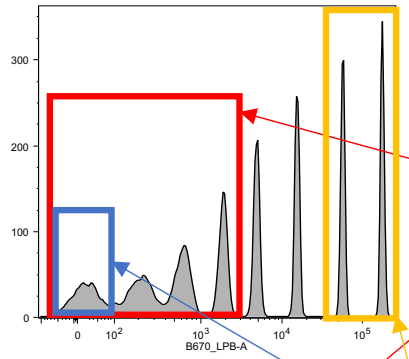
What Happens if PMTV is not Optimal?

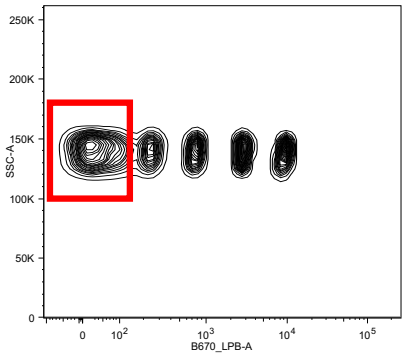
Optimal
(PMTV: 523)

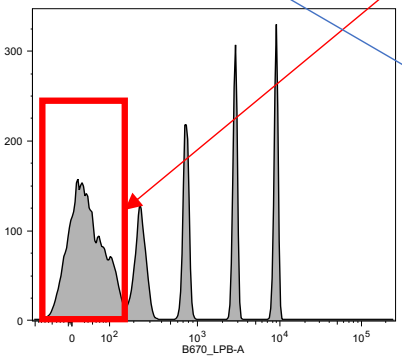
Too low
(PMTV: 350)

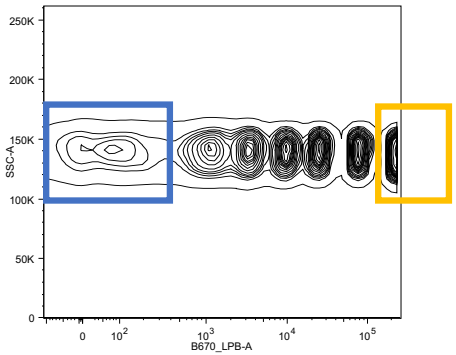
Too high
(PMTV: 650)

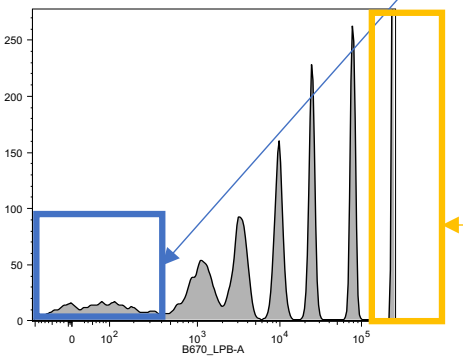












Loosing resolution in dim population

Increasing spreading error

Signal off the scale

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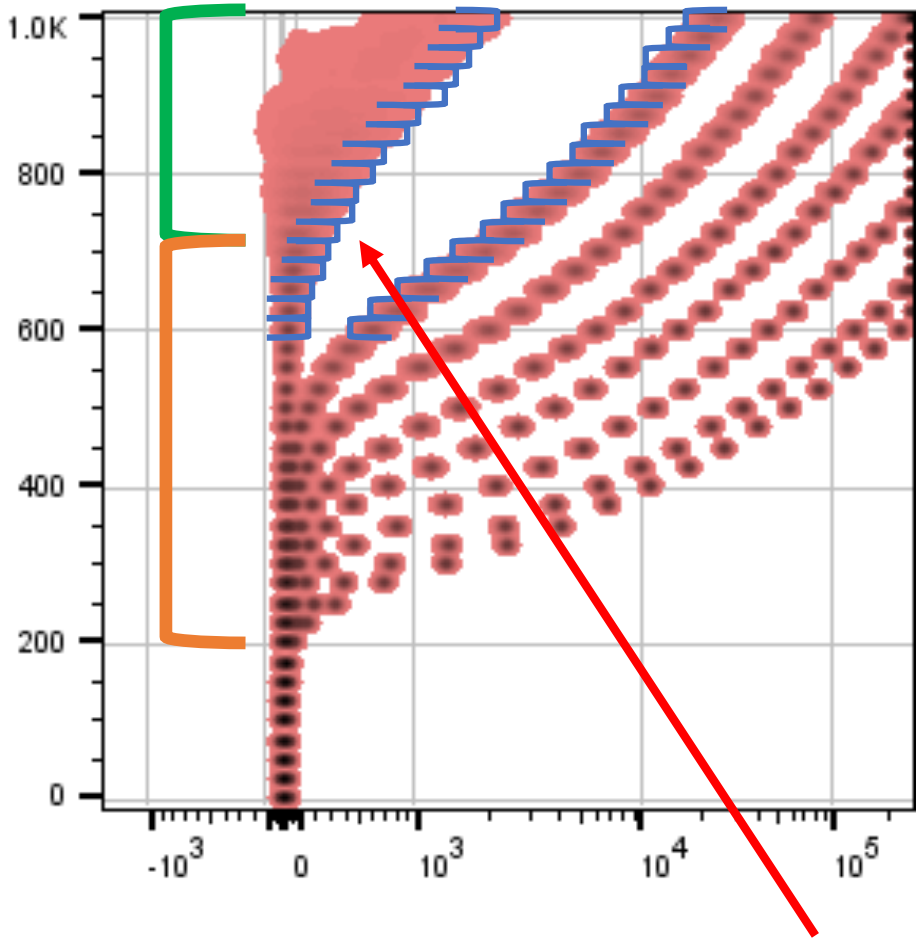
How to Determine the Optimal PMT Voltage

Similar distance

Increasing detector sensitivity



Optimal voltage is 670

- 8 peak beads (other beads can be used)
- PMTs varied from 0-1000V with 25V increments
- Increasing voltages maximises detector sensitivity to a maximum
- Past this maximal sensitivity there is no increase in resolution for dim populations, but only increase the background

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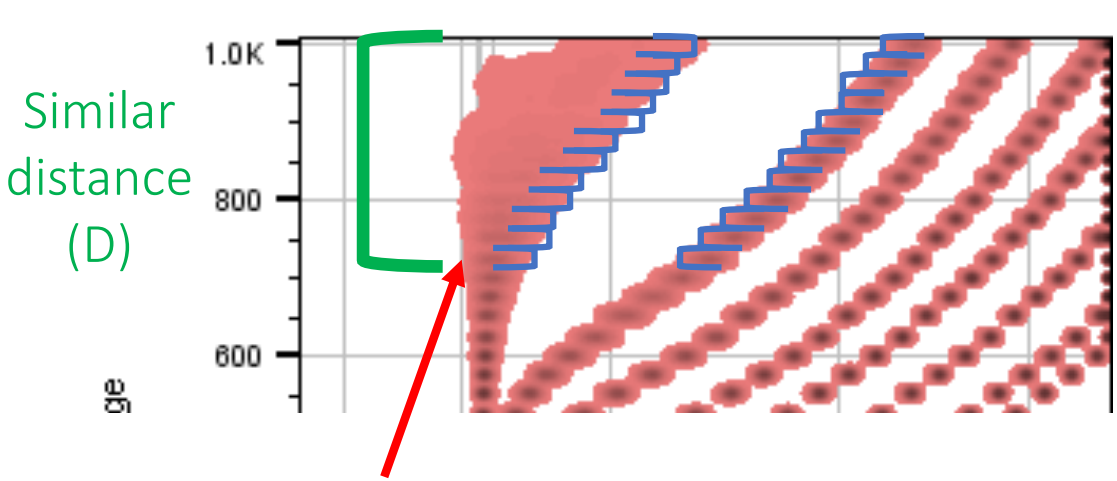
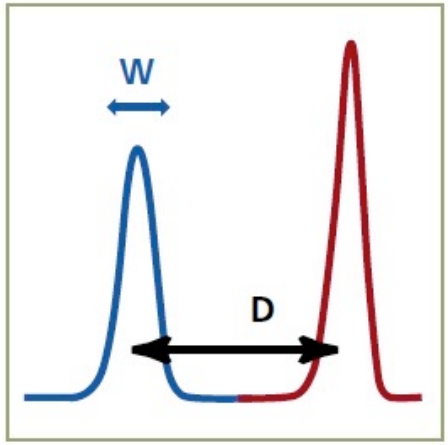
Why Use the lowest PMTV in the Optimal Range?



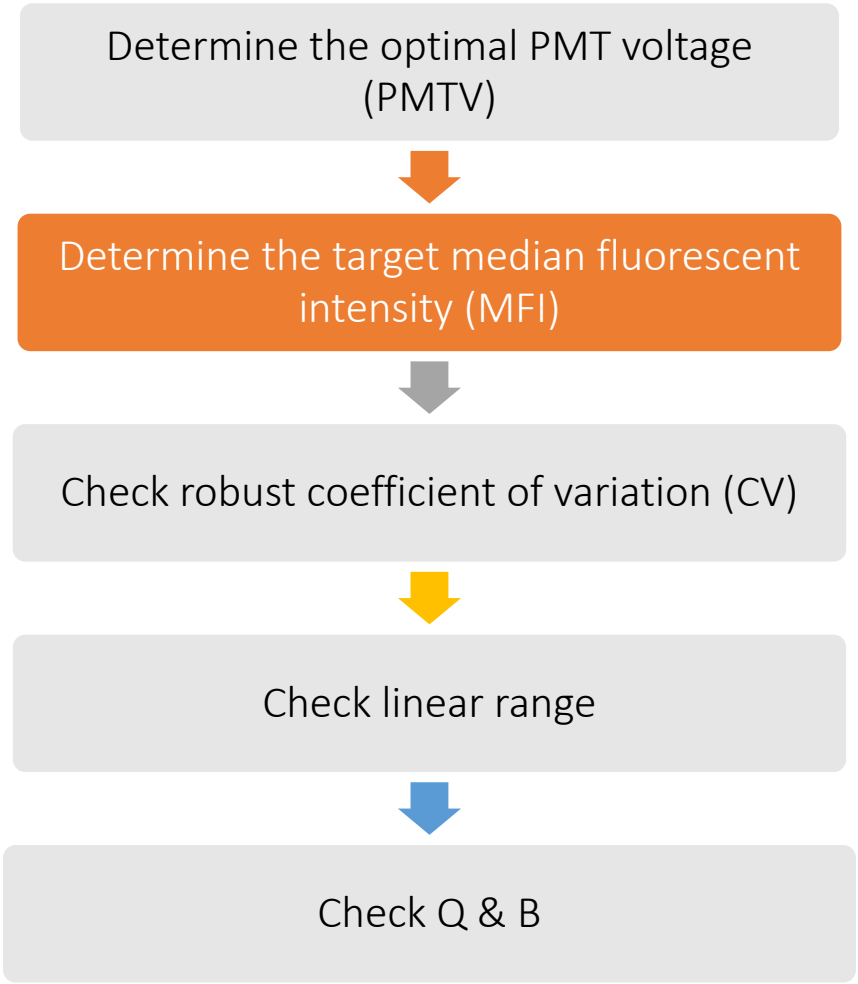
Optimal voltage is 670



Stain Index = D/W

- D remains the same; ↑ PMTV; ↑ spreading of negative population
- Therefore, ↑ W, stain index ↓

QC Key Factors



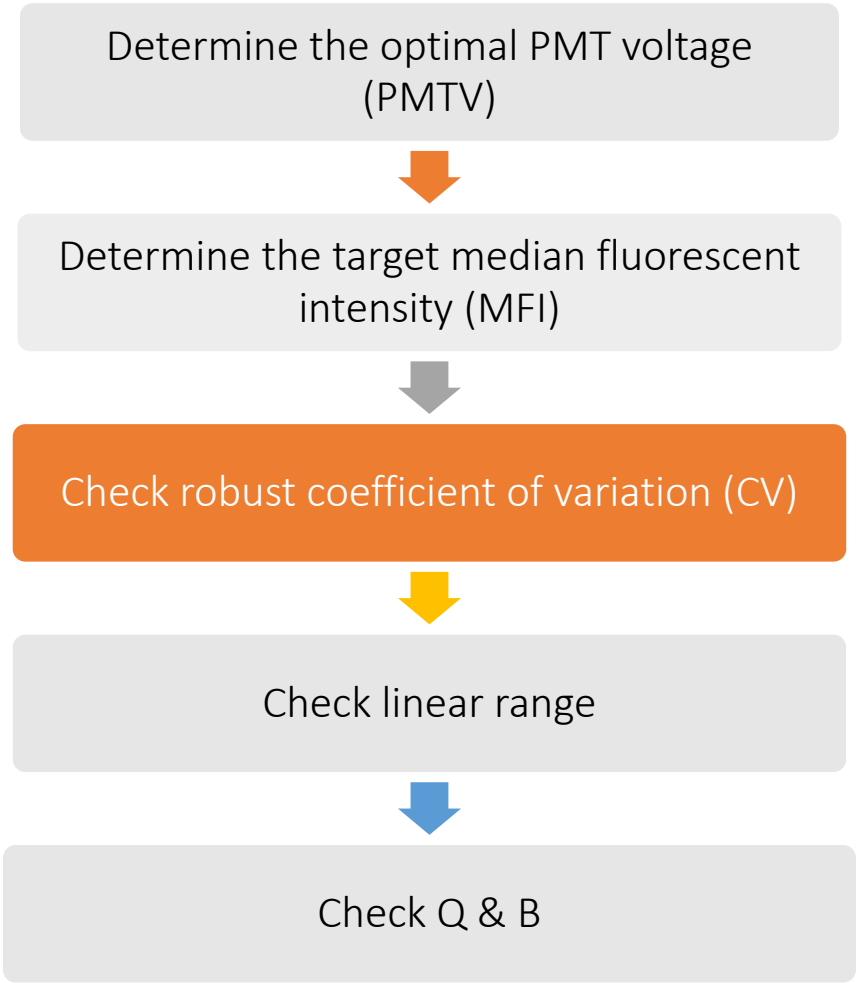
Target Medium Fluorescent Intensity (MFI)

- MFI of the bright bead at the Optimal PMTV

Population	#Events	%Parent	B530_30E-A Median	B585_42D-A Median	B670_LPB-A Median	B780_60A-A Median	R660_20C-A Median	R780_60A-A Median	V450_50B-A Median	V510_50A-A Median
P1	8,442	84.4	10,437	10,691	10,960	11,575	10,292	11,190	11,393	11,380

- Ensure the data reproducibility
- Allow the data comparison for a longitudinal study

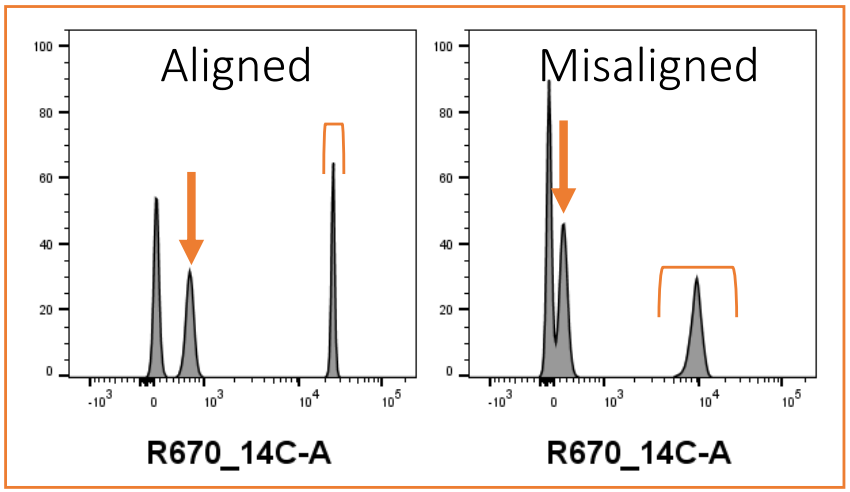
QC Key Factors



Robust Coefficient of Variation - % rCV

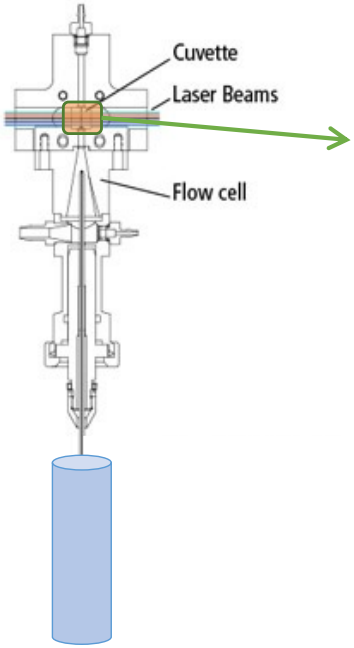
- Indicate Laser alignment

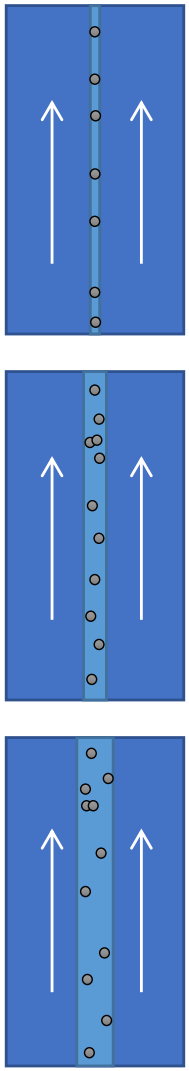
Population	#Events	%Parent	B530_30... %rCV	B585_42... %rCV	B670_LP... %rCV	B780_60... %rCV	R660_20... %rCV	R780_60... %rCV	V450_50... Mean	V450_50... %rCV	V510_50A... %rCV
P1	8,442	84.4	1.7	1.7	2.3	5.5	3.4	5.0	11,389	1.8	1.7



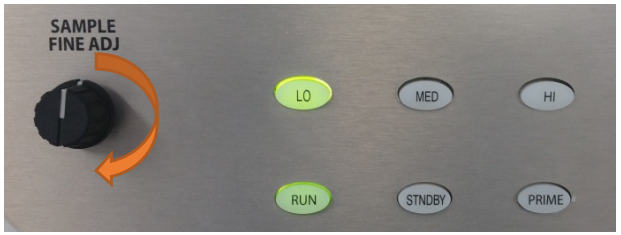
- Important for resolving populations

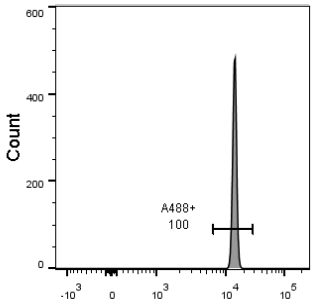
Flow rate can affect CV



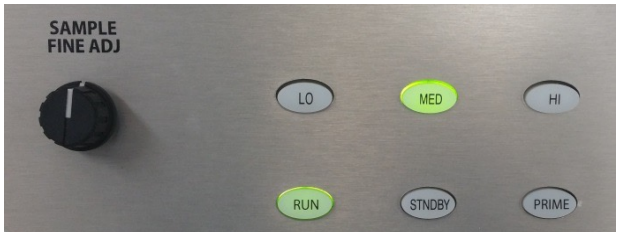


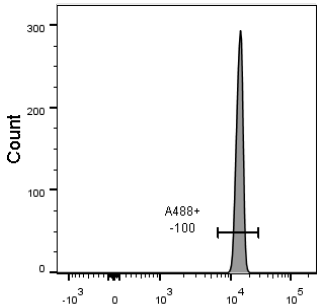
Fine control



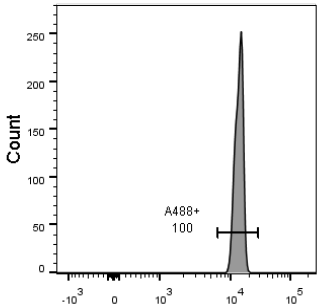


CV : 5.68





CV : 9.90



CV : 12.5

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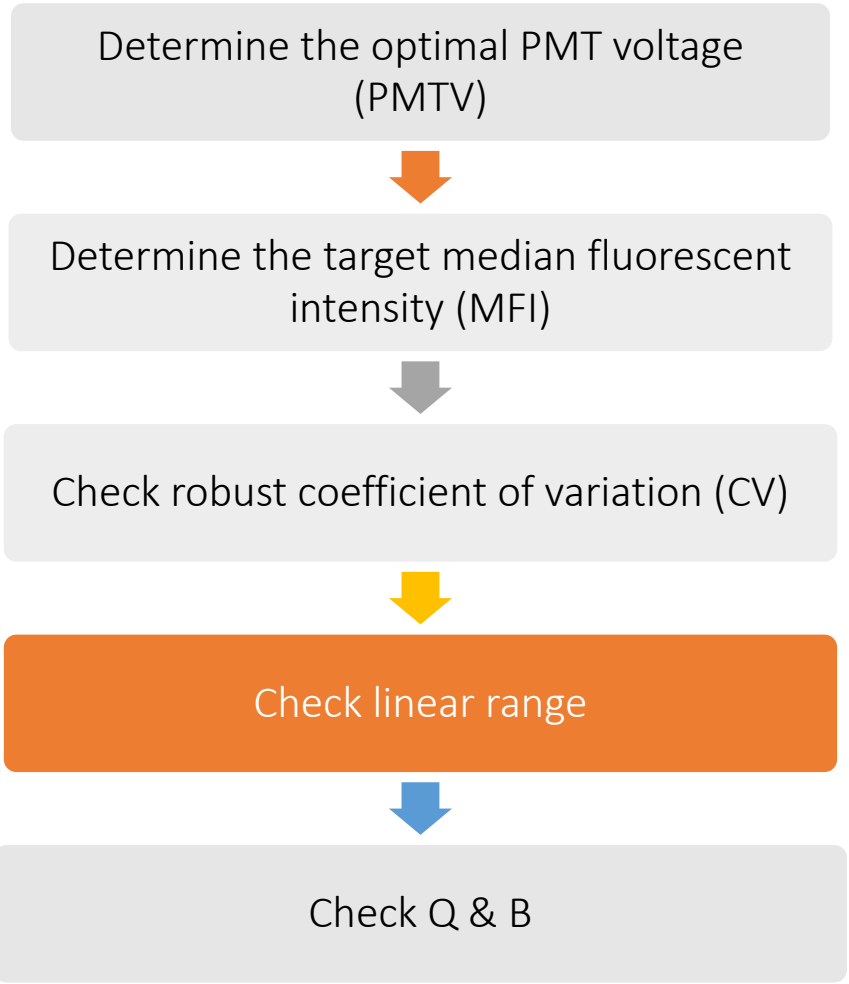
QC Key Factors

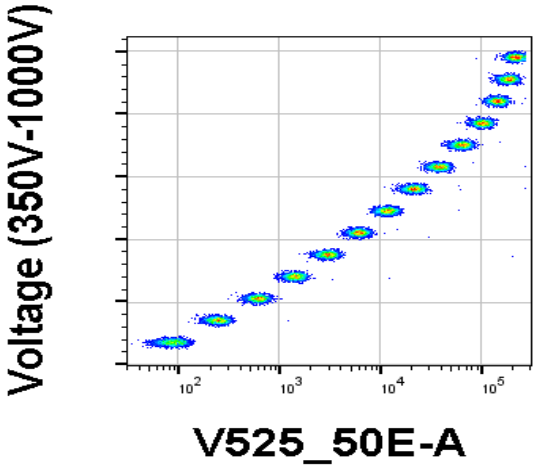


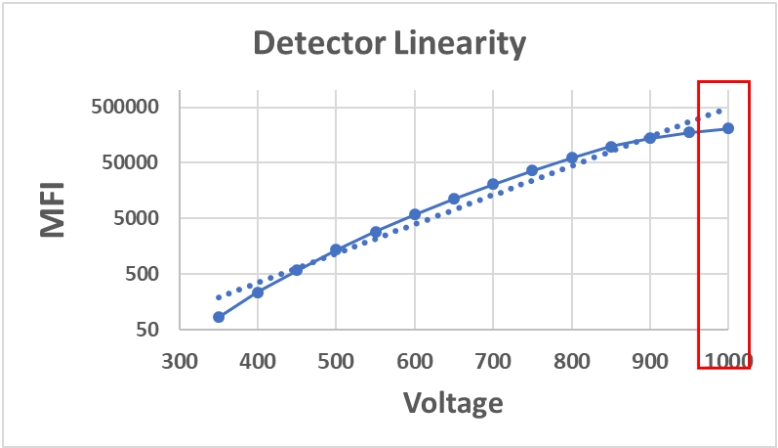
Linear Range

- Linearity: defined as proportionality of output (MFI) to input (Fluorescence signal/ # of photons)

- Acquire data with increasing PMT voltages
- Plot Voltage vs. MFI
- Observe that signals at very high or very low levels deviate from detector linearity

- Important for compensation: compensation won't be accurate if out of linear range





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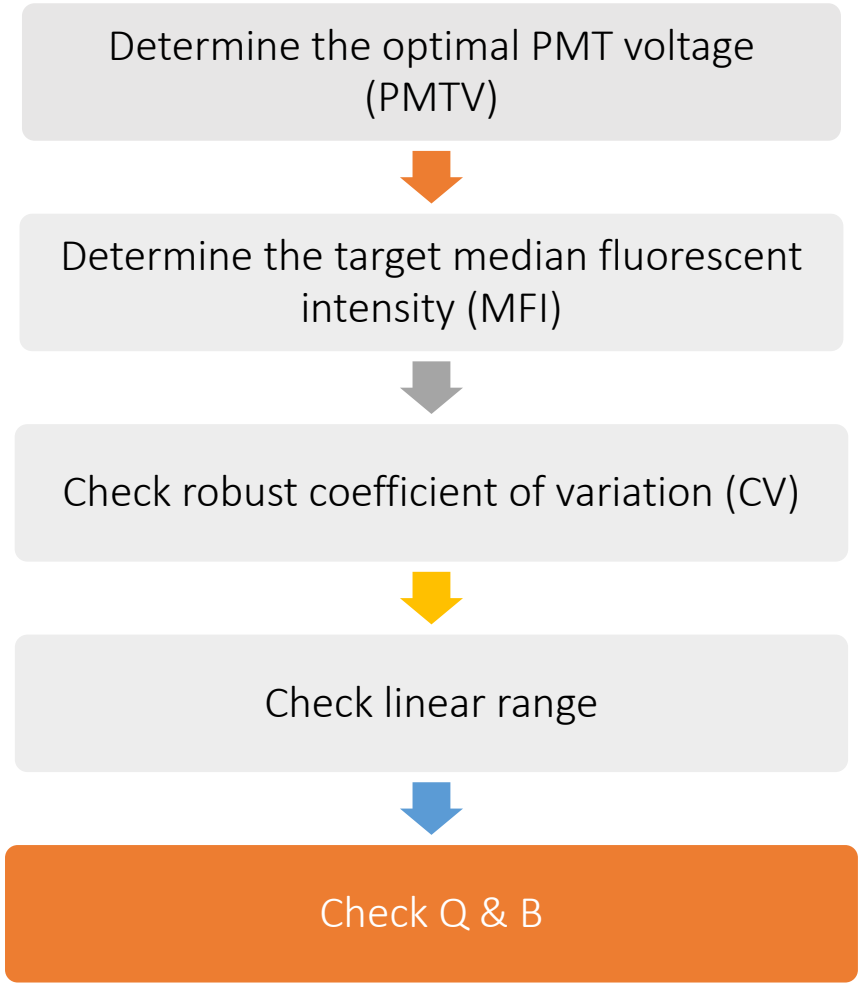
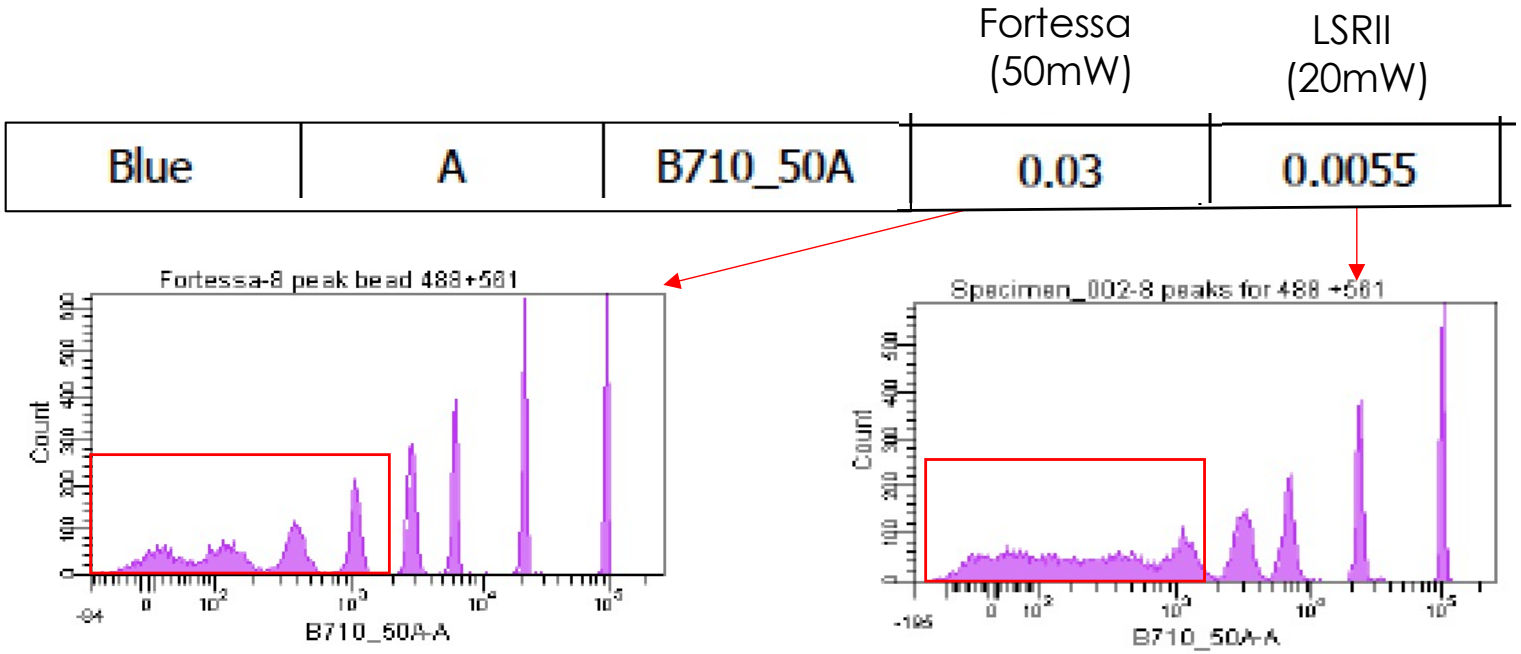
QC Key Factors



Q - Fluorescence Detector Efficiency

Q is *photoelectrons per fluorescence unit* and indicates how bright a reagent will appear on the sample when measured in a specific detector.

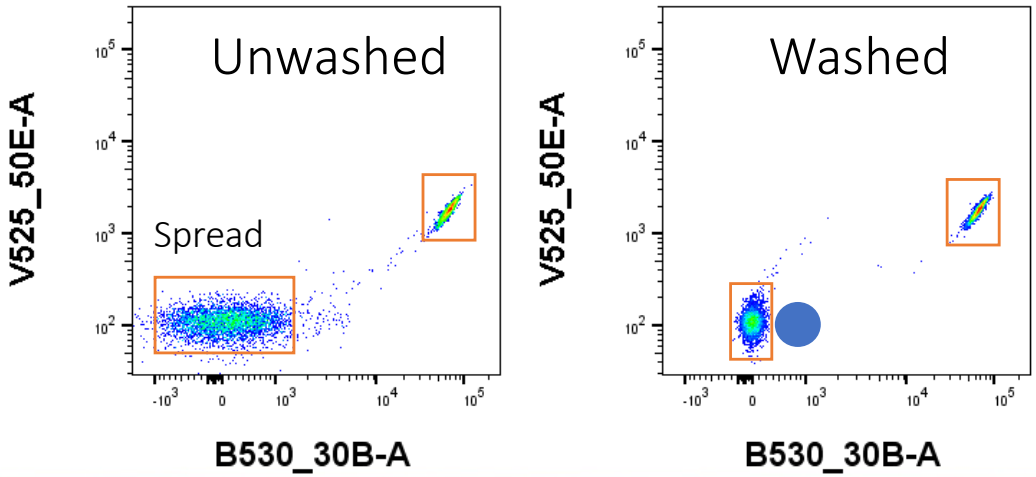
- Higher Q value means higher resolution
- Higher laser power, higher Q
- Optical design: dichroic mirrors, collection lens….



B - Background “noise”

B: optical background - how easily dim signal may be resolved from the background

- Higher B widens negative and dim populations
- Factors increases B:
  - Samples: non-specific binding, auto-fluorescence, unbound dye/fluorochrome
  - Instruments: Raman Scattering, light, optics (e.g. incorrect filter orientation), electronic noise, dirty flow cells



Sensitivity

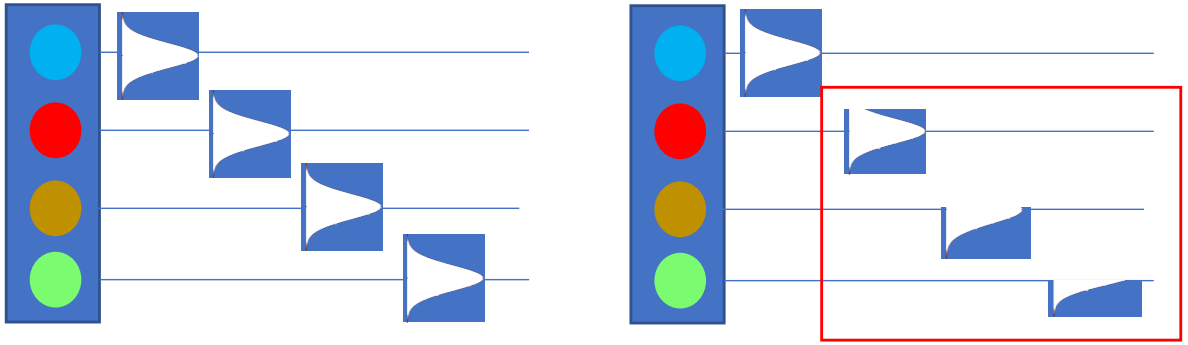
- Q and B are independent variables, but both affect sensitivity.
- Increases in B or decreases in Q can reduce sensitivity and the ability to resolve dim populations.

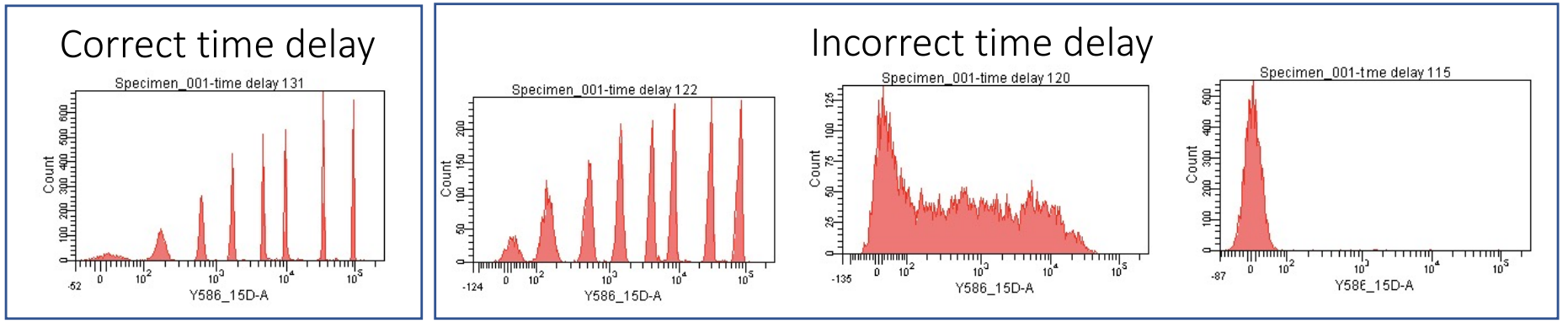
➢Dim fluorescent markers: high Q and low B

Time Delays

- Laser movement & incorrect time delays can cause signal variations.

Time delays must be set to accommodate laser movement to avoid loss of signal.





When to Perform an Instrument QC?

- Daily, normally in the morning
- When the data doesn't look right
- An experiment without internal controls, especially for the long term clinical projects

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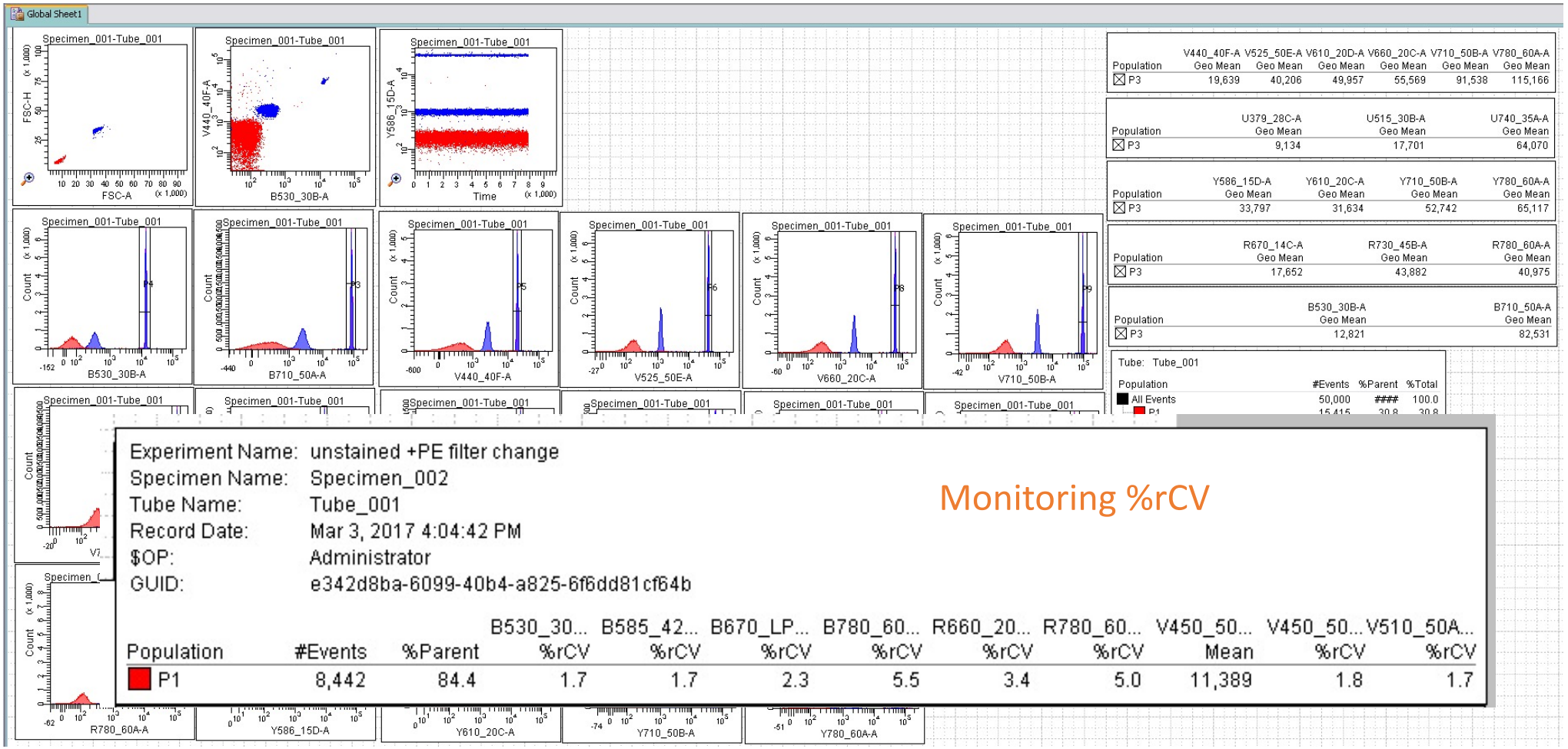
How to Setup a QC Template & Perform a QC?

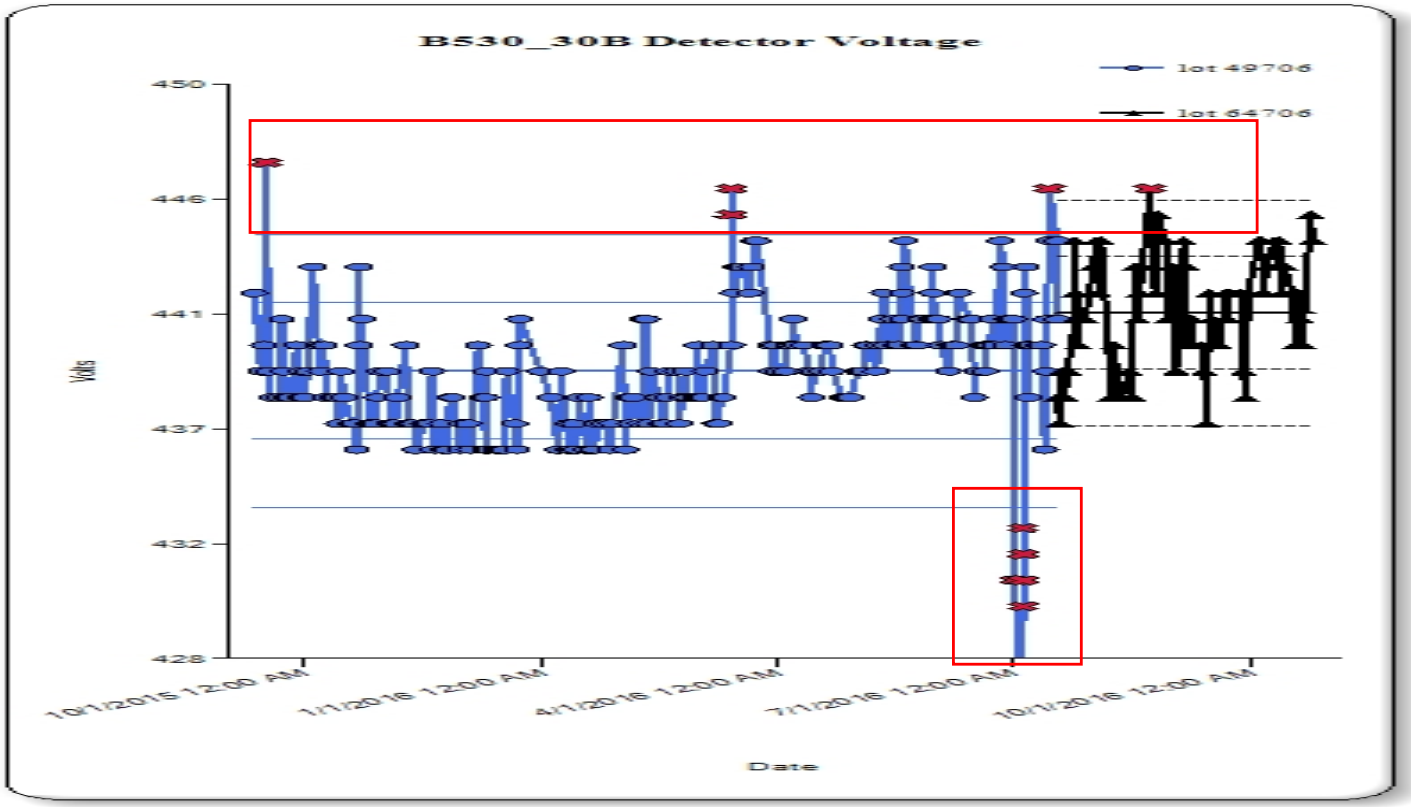
- CS&T, single peak rainbow, 8 peak beads
- to the target gate for all detectors
- for all parameters
- for daily QC
- use the same lot# beads

QC Reference Template



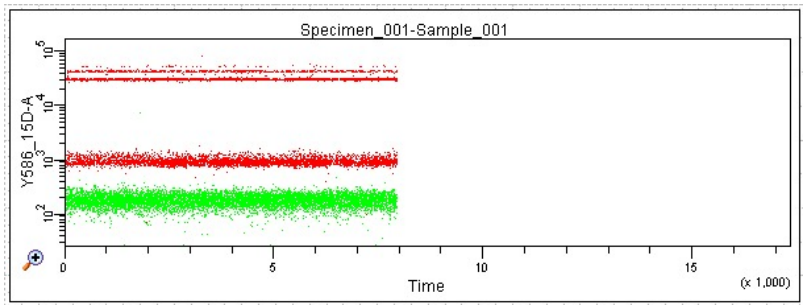
Instrument Monitoring

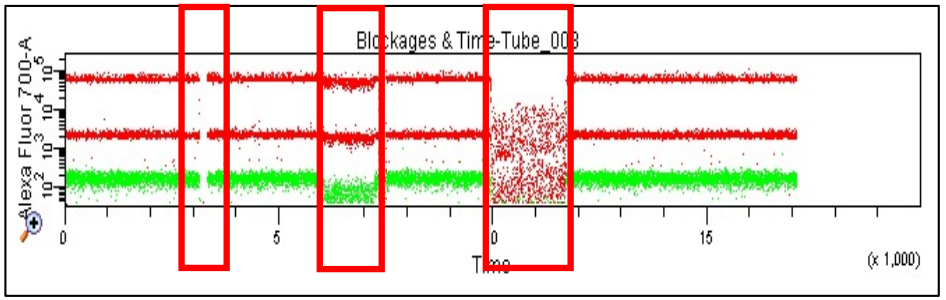
Levey-Jennings type plots: visually inspect longitudinal data for monitoring instrument performance and changes in precision, sensitivity and accuracy



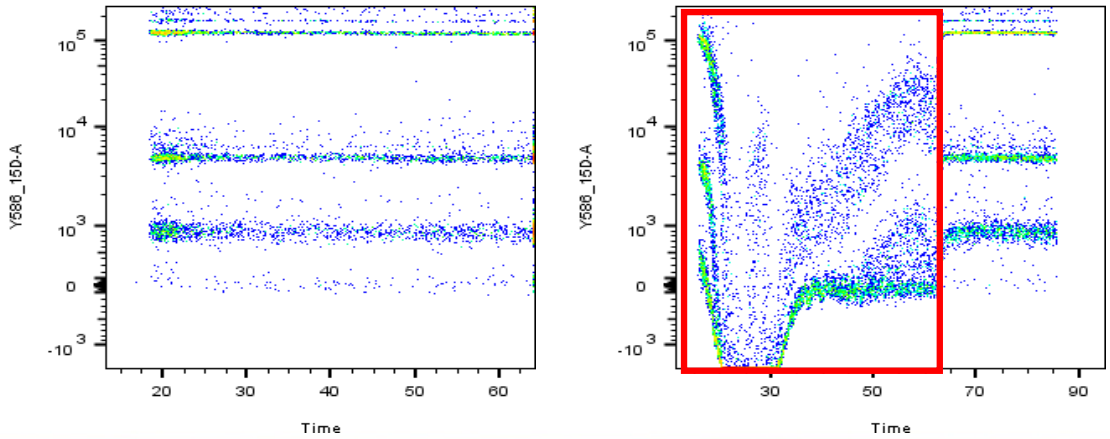
Instrument Monitoring - TIME

- Viewing time on X axis and letting samples stabilize.





- How often should we change the filter on the analyser?



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How to Interpret the QC data

Scenarios


THE WORST-CASE SCENARIO IS NEAR.
CARTOONSTOCK .com
Search ID: hscn160

Case #1

Issue

- Many things went wrong (%rCV increased, Q negative value and B not calculated in multiple channels with multiple lasers)

Possible cause

- Machine has not warmed up yet

Solution

- Leave the machine on for 20 minutes and rerun QC

Detector Settings

Laser	Detector	Parameter	Target Value	Actual Target Value	% Difference Target Value	Bright Bead % Robust CV	Mid Bead Median Channel	Mid Bead % Robust CV
Blue	FSC	FSC	125000	123934	-1	4.71	123870	4.73
Blue	C	SSC	125000	124679	-1	3.19	125198	3.03
Blue	B	B530_30B	15743	15349	-3	2.82	603	5.99
Blue	A	B710_50A	43327	42717	-2	3.11	1205	9.1
Red	C	R670_14C	34452	33383	-4	5.6	1535	8.83
Red	B	R730_45B	39700	38370	-4	5.59	918	7.99
Red	A	R780_60A	42716	41632	-3	5	828	8.11
Violet	F	V440_40F	17876	17586	-2	4.19	1040	6.9
Violet	E	V525_50E	60953	61720	1	3.92	2796	5.23
Violet	D	V610_20D	37639	36963	-2	4.42	1325	12.74
Violet	C	V660_20C	41219	40859	-1	3.63	2041	8.34
Violet	B	V710_50B	56839	55976	-2	4.48	1866	7.54
Violet	A	V780_60A	126431	125284	-1	5.56	1675	12.94
UV	C	U379_28C	29425	28711	-3	3.24	894	5.37
UV	B	U515_30B	79449	78990	-1	3.49	1238	11.13
UV	A	U740_35A	82473	82189	-1	7.31	1720	24.91
Yellgrn	D	Y586_15D	15471	15754	1	2.61	574	7
Yellgrn	C	Y610_20C	13545	14017	3	2.69	498	7.38
Yellgrn	B	Y710_50B	44410	43949	-2	2.97	1099	6.8
Yellgrn	A	Y780_60A	62607	61878	-2	3.08	1150	7.2

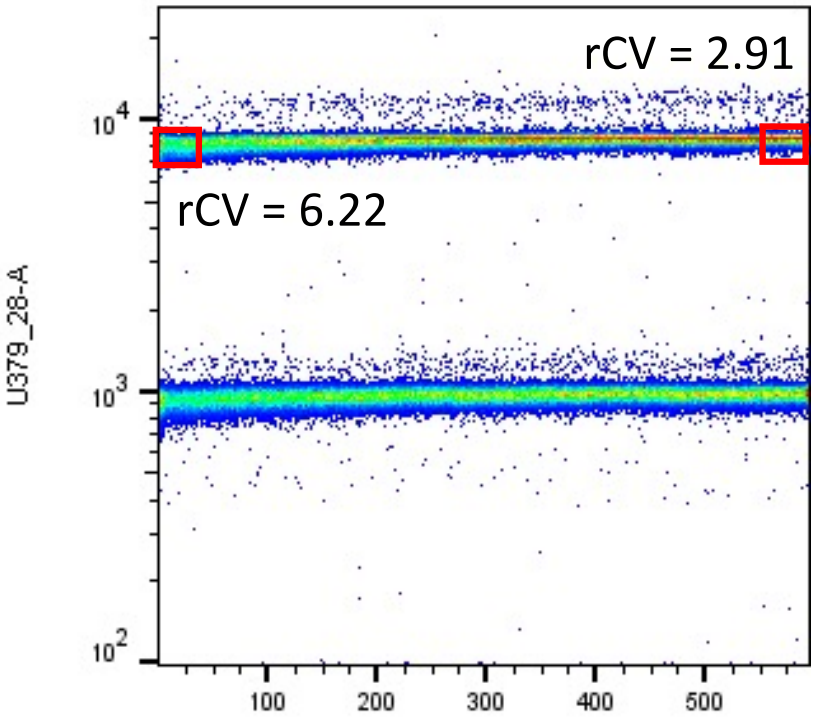
Detector Settings (Continued)

Laser	Detector	Parameter	Dim Bead Median Channel	Dim Bead % Robust CV	PMTV	Δ PMTV	Qr	Br	P/F
Blue	FSC	FSC	20480	11.85	400	-27	N/A	N/A	Pass
Blue	C	SSC	68187	2.33	246	-8	N/A	N/A	Pass
Blue	B	B530_30B	82	26.16	433	-11	0.1284	1045	Pass
Blue	A	B710_50A	186	25.78	622	-23	0.0444	260	Pass
Red	C	R670_14C	127	29.65	543	2	0.1392	40	Pass
Red	B	R730_45B	101	25.31	497	-1	0.021	4467	Pass
Red	A	R780_60A	93	28.61	526	-1	0.0157	2503	Pass
Violet	F	V440_40F	194	18.8	388	-13	0.1137	2634	Pass
Violet	E	V525_50E	203	15.24	362	-5	0.0339	73	Pass
Violet	D	V610_20D	181	41.92	573	-5	0.0567	125	Pass
Violet	C	V660_20C	177	32.51	588	1	0.0872	80	Pass
Violet	B	V710_50B	180	25.75	516	-3	0.1519	82	Pass

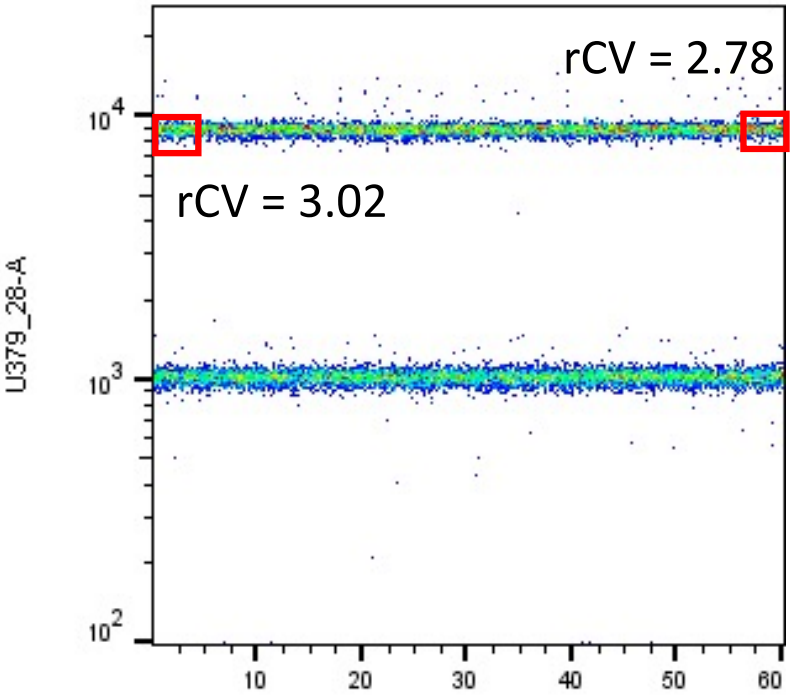
Research Use Only Page 1 of 2

Case #1

- Signal CVs are affected without warmup



10min run without warmup



After 20min warmup

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Case #2

Issue

- MFI decreased for one detector
- both ΔPMTV and %rCV increased for one detector

Possible cause

- Faulty PMT
- Dirty optics
- Electronics

Possible Solution

- Replace PMT
- Clean filter
- Fix the electronics

						% rCV	ΔPMTV
UV	C	U379_28C	29425	29062	-2	3.43	19
UV	B	U515_30B	79449	78571	-2	2.97	9
UV	A	U740_35A	82473	83442	1	5.92	3

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Case #3

Issue

- MFI signals appear dim in the far-red detectors
- Q is low in the far-red detectors

Possible cause

- Low-sensitive PMTs were used in these detectors

Possible Solution

- Replace PMT with far-red sensitive detectors

Q

Original PMT

Red	B	R730_45B	107	24.3	501	3	0.0094	417	Pass
Red	A	R780_60A	99	30.45	529	2	0.0087	1191	Pass

High sensitive PMT

Red	B	R730_45B	101	25.31	497	-1	0.021	4467	Pass
Red	A	R780_60A	93	28.61	526	-1	0.0157	2503	Pass

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Case #4

Issue

- MFI signals reduced in all detectors of one laser
- ΔPMTV and % rCV increased in all detectors of one laser

Possible cause

- Laser delay setting is incorrect
- Blockage, especially for latter lasers
- Laser power

Possible Solution

- Check and adjust timing delay
- Clean the sample probe & flow cell
- Replace the laser

				% rCV	ΔPMTV
Yellgrn	D		-2	2.15	-7
Yellgrn	C		0	2.18	-9
Yellgrn	B		-1	2.35	-3
Yellgrn	A		-1	2.9	1

Dirty flow cell

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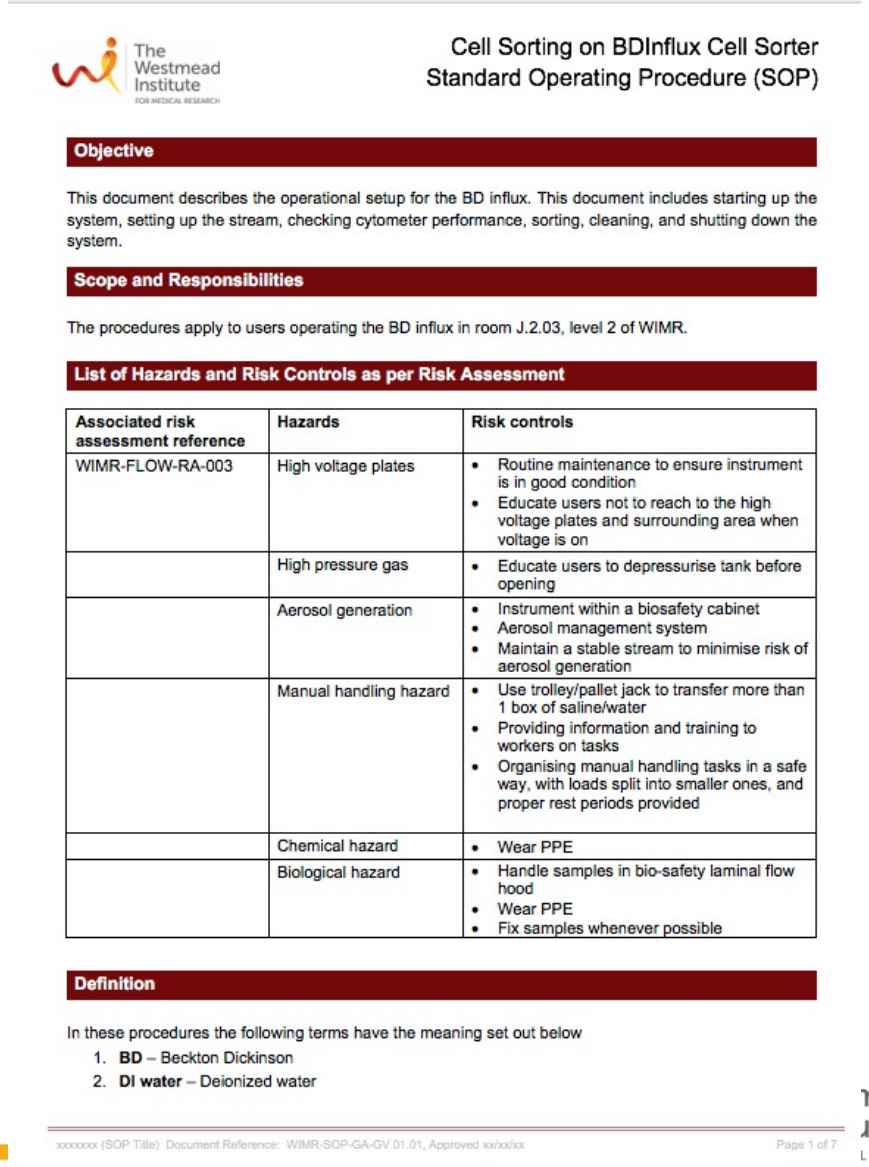
QC - SOPs

Generating valid SOPs is critical for flow cytometry lab

- Contents
  - Objectives
  - Hazards and risk control and assessment
  - Resources required: instrument, AMO, PPE
  - Step by step procedure
  - Troubleshooting
  - Emergency procedures: e.g. spill
  - Waste disposal



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Cell Sorting on BDInflux Cell Sorter
Standard Operating Procedure (SOP)

Objective

This document describes the operational setup for the BD influx. This document includes starting up the system, setting up the stream, checking cytometer performance, sorting, cleaning, and shutting down the system.

Scope and Responsibilities

The procedures apply to users operating the BD influx in room J.2.03, level 2 of WIMR.

List of Hazards and Risk Controls as per Risk Assessment

Associated risk assessment reference	Hazards	Risk controls
WIMR-FLOW-RA-003	High voltage plates	• Routine maintenance to ensure instrument is in good condition • Educate users not to reach to the high voltage plates and surrounding area when voltage is on
	High pressure gas	• Educate users to depressurise tank before opening
	Aerosol generation	• Instrument within a biosafety cabinet • Aerosol management system • Maintain a stable stream to minimise risk of aerosol generation
	Manual handling hazard	• Use trolley/pallet jack to transfer more than 1 box of saline/water • Providing information and training to workers on tasks • Organising manual handling tasks in a safe way, with loads split into smaller ones, and proper rest periods provided
	Chemical hazard	• Wear PPE
	Biological hazard	• Handle samples in bio-safety laminal flow hood • Wear PPE • Fix samples whenever possible

Definition

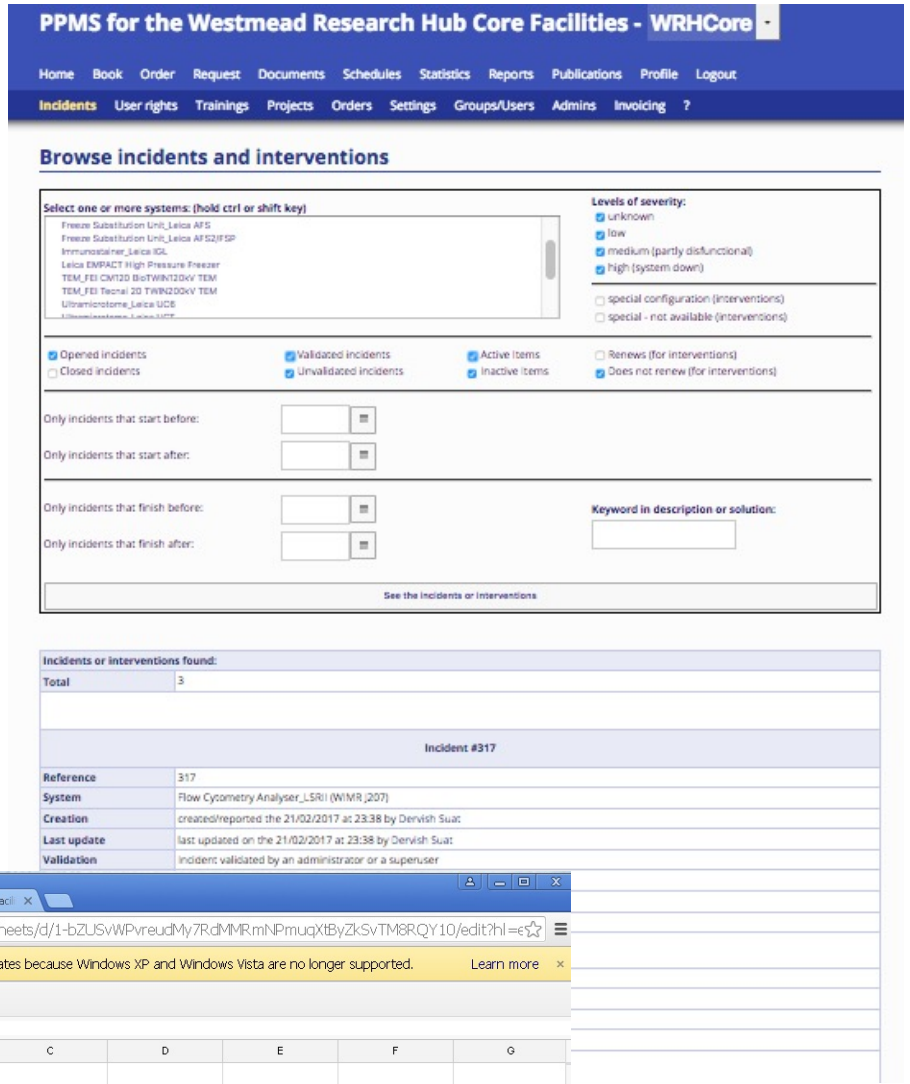
In these procedures the following terms have the meaning set out below

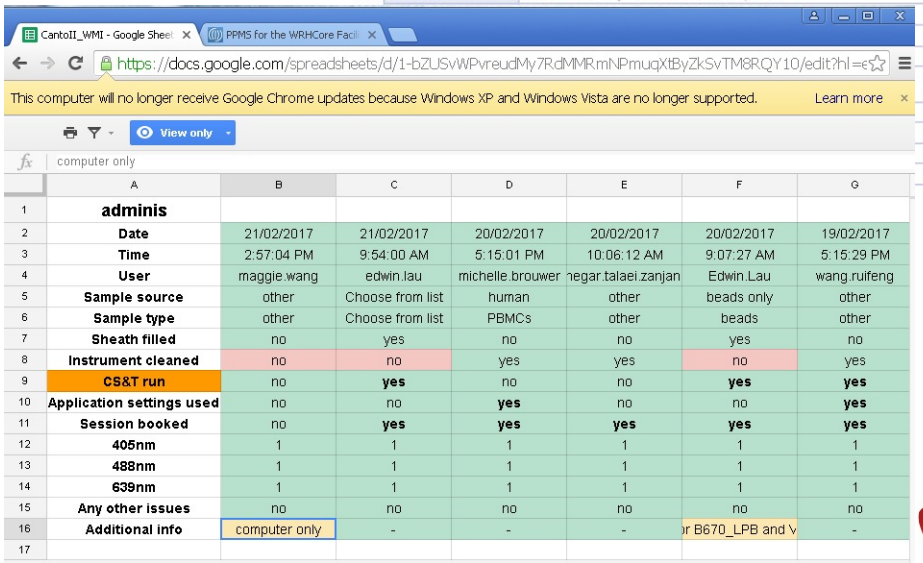
1. **BD** – Beckton Dickinson
2. **DI water** – Deionized water

xxxxxxx (SOP Title) Document Reference: WIMR-SOP-GA-GV.01.01, Approved xx/xx/xx Page 1 of 7

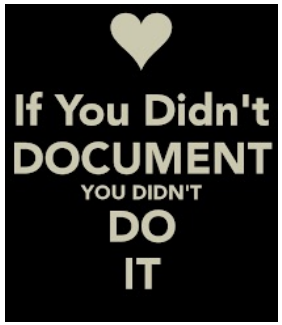
Record Keeping

- Installation/baseline report
- Routine maintenance
- Service and repair records – online system
- Troubleshooting





	A	B	C	D	E	F	G
1	**adminis**						
2	**Date**	21/02/2017	21/02/2017	20/02/2017	20/02/2017	20/02/2017	19/02/2017
3	**Time**	2:57:04 PM	9:54:00 AM	5:15:01 PM	10:06:12 AM	9:07:27 AM	5:15:29 PM
4	**User**	maggie.wang	edwin.lau	michelle.brouwer	negar.talaei.zanjan	Edwin.Lau	wang.ruifeng
5	**Sample source**	other	Choose from list	human	other	beads only	other
6	**Sample type**	other	Choose from list	PBMCs	other	beads	other
7	**Sheath filled**	no	yes	no	no	yes	no
8	**Instrument cleaned**	no	no	yes	yes	no	yes
9	**CS&T run**	no	**yes**	no	no	**yes**	**yes**
10	**Application settings used**	no	no	**yes**	no	no	**yes**
11	**Session booked**	no	**yes**	**yes**	**yes**	**yes**	**yes**
12	**405nm**	1	1	1	1	1	1
13	**488nm**	1	1	1	1	1	1
14	**639nm**	1	1	1	1	1	1
15	**Any other issues**	no	no	no	no	no	no
16	**Additional info**	computer only	-	-	-	r B670_LPB and V	-
17							



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QC is a MUST-DO step

- For ALL flow cytometry experiments
- Ensure instrument is optimal & result is consistent and reproducible
- At least DAILY, not weekly or monthly
- It will spend your 10-20 minutes everyday for $, but to SAVE your troubleshooting time (hours, days, months), cost $$$$ (antibodies, labor, other reagents), stress, but most importantly, precious SAMPLES and DATA

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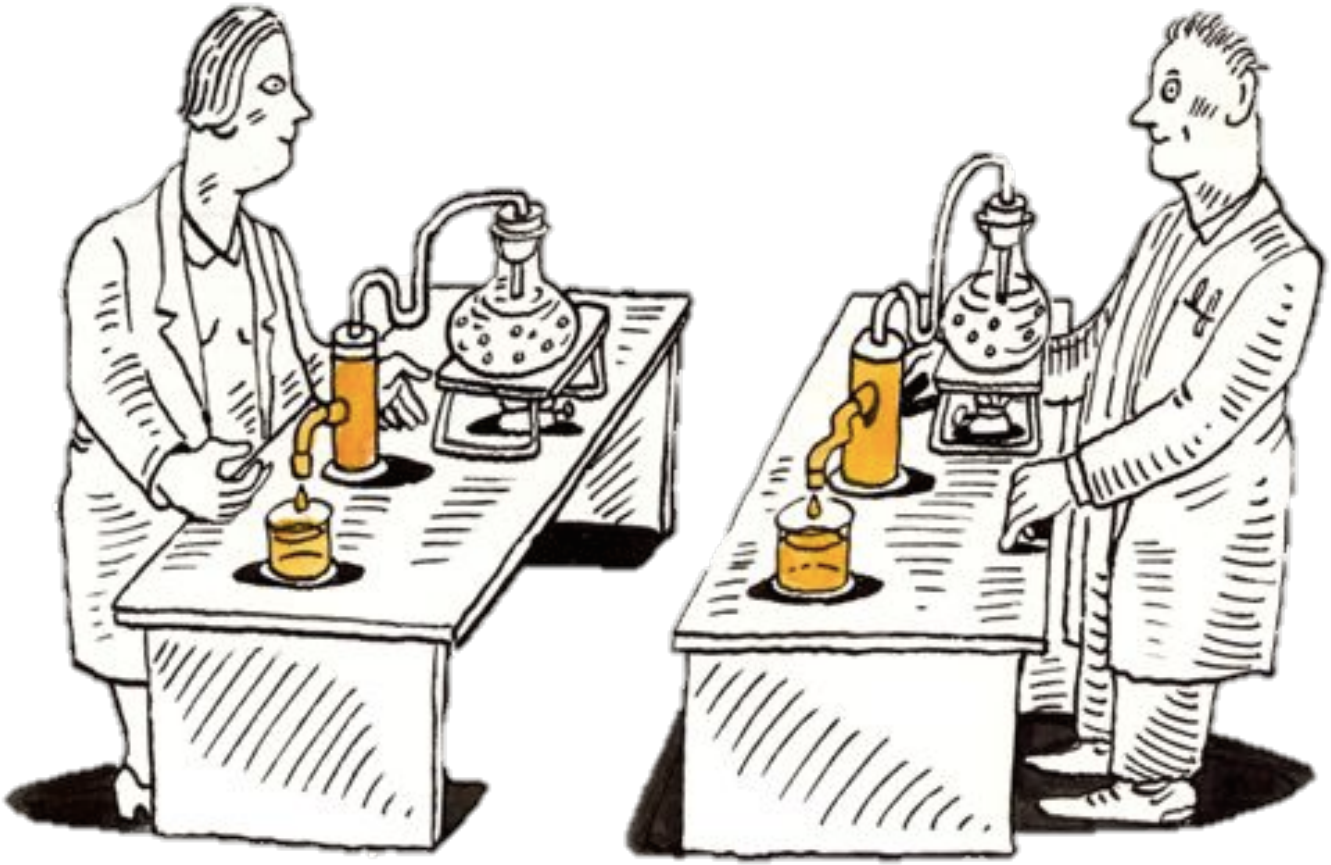
How to compare data cross multiple Instruments?



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Principles

To ensure the assay will perform acceptably on the poorest performing instrument

- Use same reagent, QC particles and QC method for a common assay
- Establish minimum instrument performance for assay quality assurance
- Ensure the MFIs are consistent and reproducible between the instruments.

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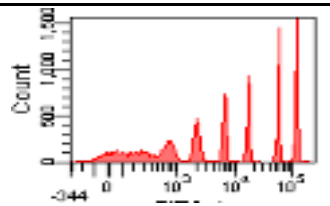
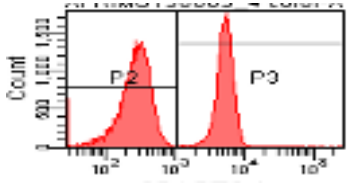
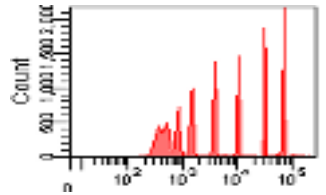
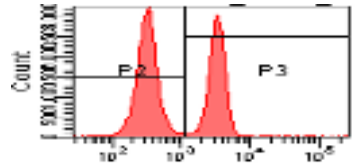
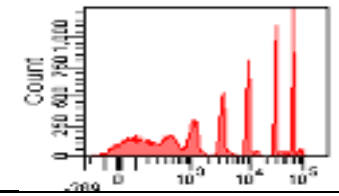
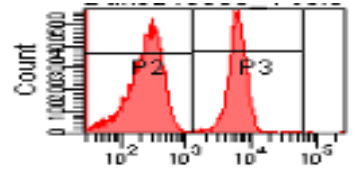
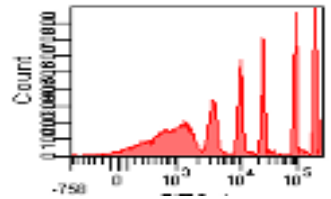
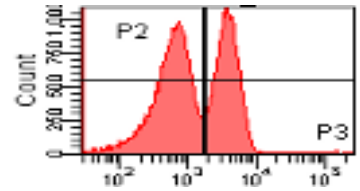
Variation in instrument performance

Instrument	SDen	Qr	Br	SI Comp beads	8 peak beads	Lymphocytes (CD4 FITC)
1	26	0.015	976	55.6		
2	17	0.042	92	88.2		
3	22	0.010	613	66.0		
4	9	0.007	2322	13.7		

(Adapted from BD)

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Setting voltage on primary instrument

- PMTV needs to be set according to the limitations of the poorest performing instrument.

Inst. No.	SDen	Qr	Br	Sens	Upper End of Linearity
1	26	0.015	976	3.9	230,000
2	17	0.042	92	21.4	200,000
3	23	0.01	298	5.8	180,000
4	22	0.01	613	4.0	200,000
5	9	0.007	2322	1.7	230,000
6	20	0.018	2768	2.6	190,000

- Instrument 3 has the lowest upper end of linearity: 180,000.
  - PMTV should be low enough so the brightest population in the assays is lower than 180,000 on any instrument
- Instrument 1 has the highest electronic noise: 26.
  - PMTV should be high enough if you are measuring dim events
- If both conditions can't be met, then you must choose which is more important for this channel: identification of bright populations or resolution of dim populations.

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Using CS&T Application Settings to standardize assay fluorescence cross instruments

- The CS&T system is designed to set PMTV to optimize low-end sensitivity for each instrument.
- Using CST setting, Users can create their own application settings for each assay type.
- MFIs can be set by the user, saved, and reproducibly reused.
- It is possible to standardize multiple instruments to give equivalent MFI

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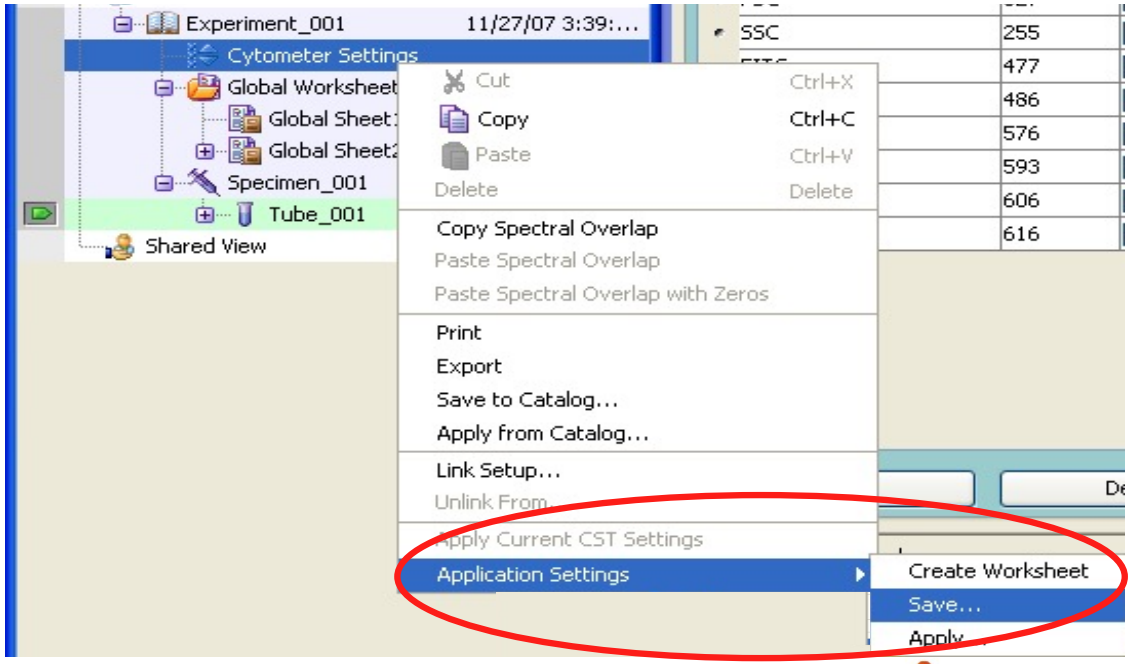
CS&T saves your assay specific MFI targets

- Run a CS&T Performance Check
- Adjust PMTV to have MFI that are appropriate for your assay.
- Select “Application Settings-Save”: Software remembers the target MFI values.

- These settings can then be applied to future experiments.
- Gives reproducible data experiment to experiment or; instrument to instrument



Transferring assays cross instruments

- Between instruments with equivalent laser & filter combinations

On the primary instrument, adjust PMTV for all your channels as required for the assay, then save Application Settings/template

→

On the primary instrument, use beads as a sample (e.g. CS&T, 8 peak beads) & determine the MFI target values for all required channels

→

Using the same lot # beads on all other instruments, adjust PMTV to meet MFI targets as the primary instrument and then save Application Settings/template

- Between instruments with different laser and/or filter combinations (e.g. PE off a 488nm vs 561nm laser)

On the primary instrument, adjust PMTV for all your channels as required for the assay, then save Application Settings/template

→

Using fluorochrome-matched controls (e.g. CompBeads) as samples, determine the MFI target values for all required channels

→

Using the same lot # controls on all other instruments, adjust PMTV to meet MFI targets as the primary instrument and then save the template

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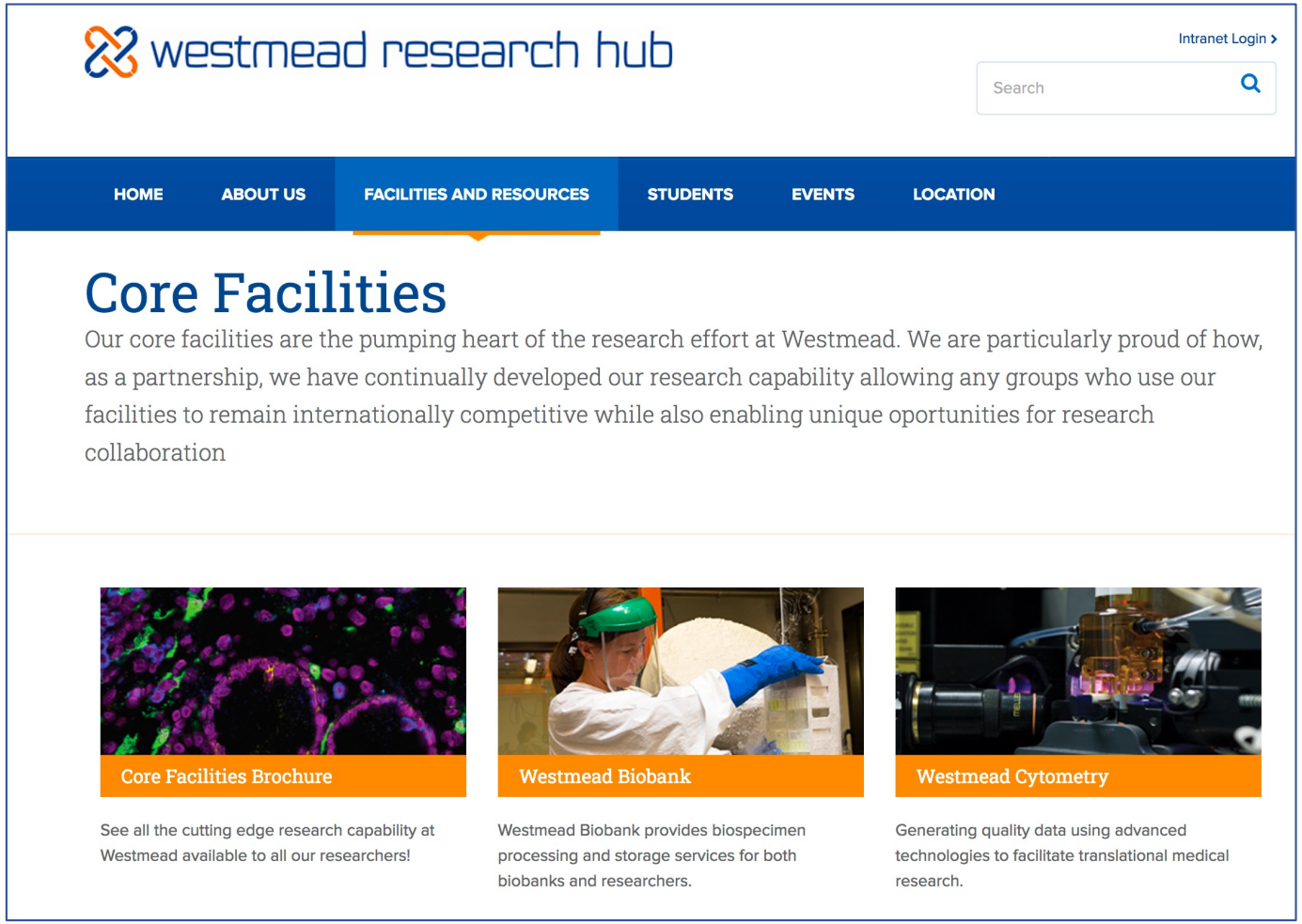

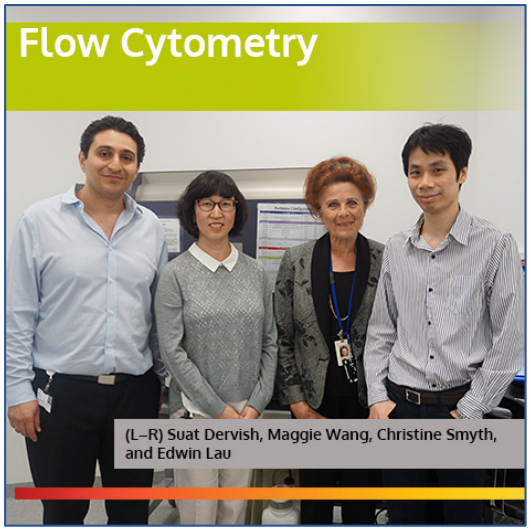

- Tips & tricks
- SOPs
- Q&A
- Data analysis codes
- Lots of more

http://www.westmead.org.au/

Email: westmead.cytometry@sydeney.edu.au

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