Step 1 – Set up (6-colour protocols)*

Instrument set-up and optimisation for 6-colour analysis of paroxysmal nocturnal haemoglobinuria (PNH) granulocytes and monocytes by flow cytometry

For accurate and reliable PNH analysis it is recommended to perform the following:

1. Daily performance checks

Daily performance checks to measure the variation from the baseline from run to run.

Beckman Coulter platform

- Use Flow Check® fluorospheres, following the manufacturer's instructions.

Becton Dickinson platform

- Use Cytometer Set-up and Tracking (CS&T®) beads, following the manufacturer's instructions.

2. Colour compensation adjustment

Beckman Coulter platform

- Use the VersaComp Antibody Capture Bead kit or unstained whole blood cells (granulocytes and/or monocytes) to set up photomultiplier tube (PMT) voltages and compensation to correct for spectral overlap.
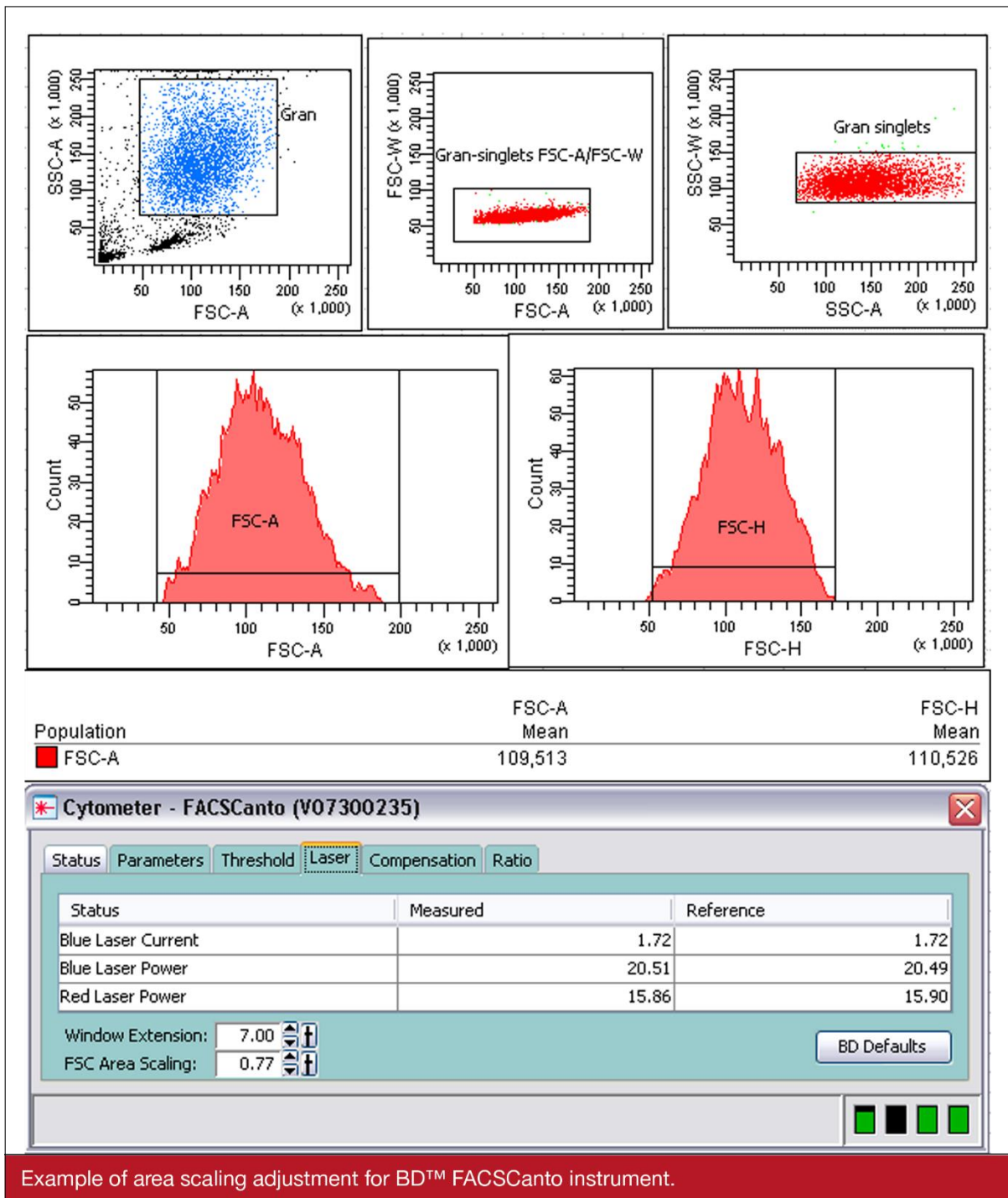
Becton Dickinson platform

- Use BD™ CompBeads or unstained whole blood cells (granulocytes and/or monocytes) and compensation to correct for spectral overlap.
- Use single colour stained microspheres/whole blood cells with each fluorochrome used in your application.

Verification of correct setting with an application control

- Update the control protocol with the settings derived from below. Run a biological control equivalent to the application or normal whole blood.
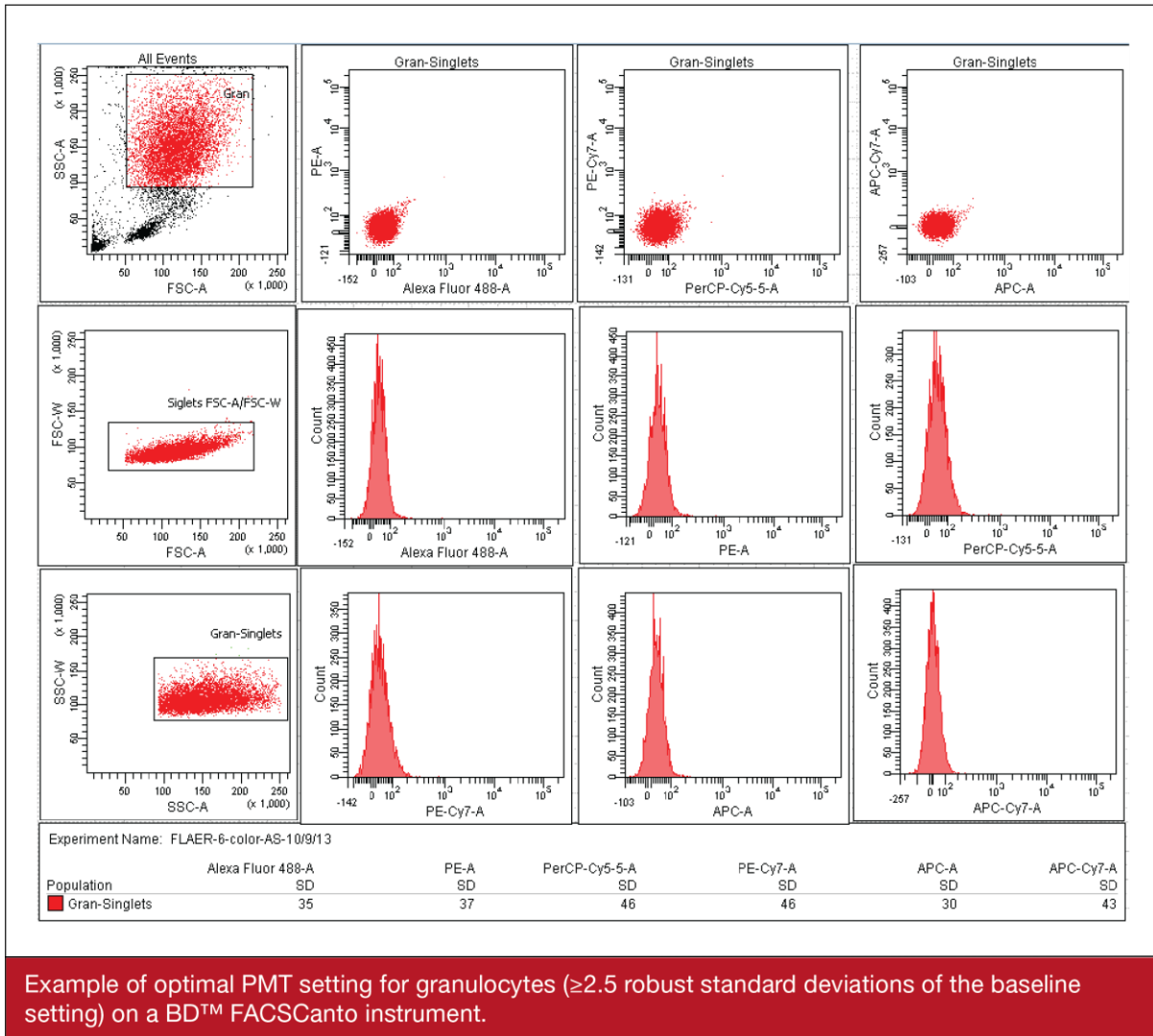
Area scaling

- Correction factor is set to match the area to the height measurement (shown below).
    - Use unstained whole blood cells (granulocytes, monocytes).
    - Use a forward scatter (FS) versus side scatter (SS) dot plot to gate granulocytes (monocytes).
    - Use FS-A versus FS-W and SS-A versus SS-W dot plots to define singlets.
    - Use FS-H and FS-A histograms to compare the mean channel values.
    - Set the FS area scaling factor to obtain identical values for FS-H and FS-A, respectively.

netflow PNH

Example of area scaling adjustment for BD™ FACSCanto instrument.

BD, Becton Dickinson; FS, forward scatter; SS, side scatter

PMT settings

- Use BD™ CompBeads or unstained whole blood cells (granulocytes and/or monocytes) for optimal baseline PMT voltage setting shown in the figure below.
  - Use FS versus SS dot plot to optimize FS/SS voltages (place the population of interest on scale) and gate granulocytes (monocytes).
  - Use FS-A versus FS-W and SS-A versus SS-W dot plots to define singlets.
  - Use single parameter histograms for all six fluorochromes used in the application to optimise the PMT voltages in order to obtain values ≥2.5 robust standard deviations of the baseline setting. This step should enable differentiation of dim populations from the negative population and place positive populations entirely on scale.

Experiment Name: FLAER-6-color-AS-10/9/13						
	Alexa Fluor 488-A	PE-A	PerCP-Cy5-5-A	PE-Cy7-A	APC-A	APC-Cy7-A
Population	SD	SD	SD	SD	SD	SD
■ Gran-Singlets	35	37	46	46	30	43

Example of optimal PMT setting for granulocytes (≥2.5 robust standard deviations of the baseline setting) on a BD™ FACSCanto instrument.

FLAER, fluorescent aerolysin; FS, forward scatter; PE, phycoerythrin; PMT, photomultiplier tube

Spectral overlap compensation

- For multicolour analysis performance of automatic spectral overlap compensation using single colour stained samples (compensation beads or normal whole blood cells) with each fluorochrome used in your application is recommended (as shown below).

Compensation Setup Details - PNH-6 color 05.03.2014

Parameters | Spectral Overlap | Cytometer

%	Alexa Fluor 488_FLAER	PE_CD24	PerCP-Cy5-5_C...	PE-Cy7_CD64	APC_CD15	APC-Cy7_CD45
Alexa Fluor 488	100.00	1.23	0.00	0.00	0.00	0.00
PE	15.07	100.00	0.00	0.10	0.00	0.00
PerCP-Cy5-5	1.97	20.25	100.00	0.40	2.34	0.00
PE-Cy7	0.33	5.43	63.94	100.00	1.33	4.88
APC	0.00	0.00	0.00	0.00	100.00	0.78
APC-Cy7	0.00	0.00	4.30	2.76	19.88	100.00

Spectral overlap compensation details for a 6-colour protocol for granulocyte and monocyte analysis on a BD™ FACSCanto instrument.

FLAER, fluorescent aerolysin; PE, phycoerythrin