Pitfalls of Multicolor Flow Cytometry Staining

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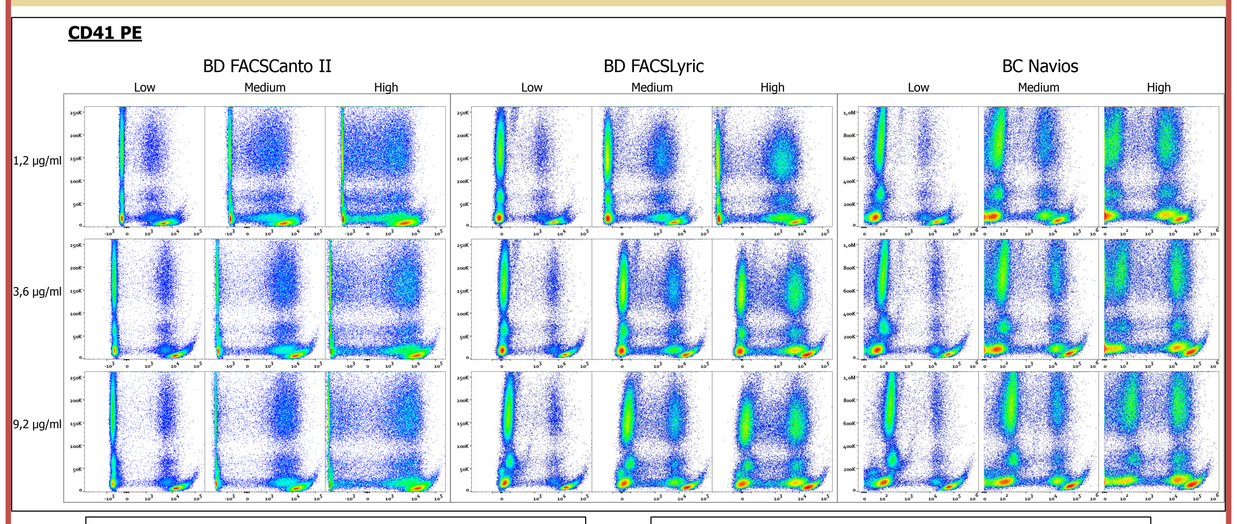
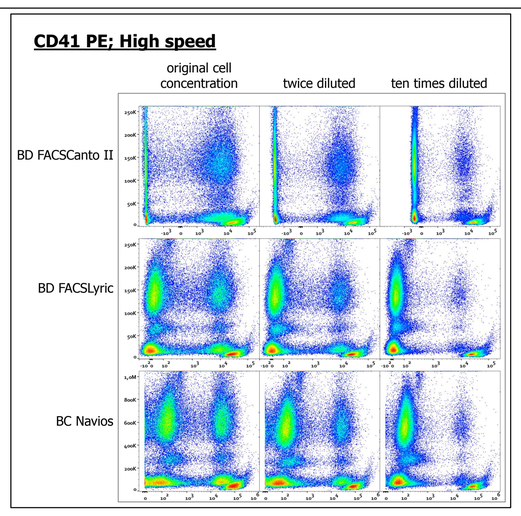
Introduction

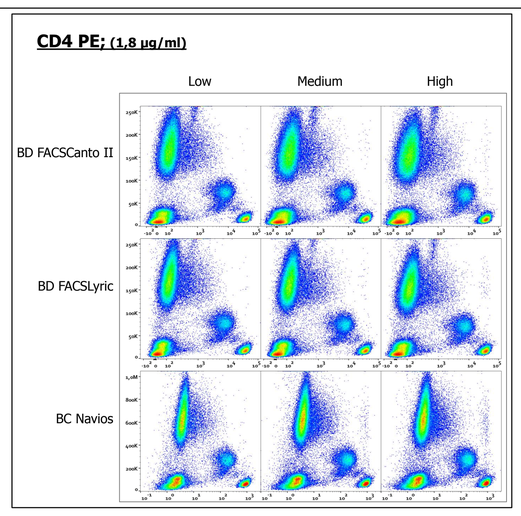
With years of daily use as a core method in hematology-oncology and immunology laboratories, flow cytometry has become a very powerful tool aiding in diagnostics. Latest advancements in clinical flow cytometry allow for staining of many cell populations or target cell molecules simultaneously. Hence a demand for easy, fast and efficient flow cytometry staining protocols goes hand-in-hand with the advancements in diagnostics.

Here we address the most frequent concerns and questions that we are receiving from our customers, like stability of monoclonal antibody reagents, selection of optimal flow cytometry platform or effects of different staining protocols and daily monoclonal antibody cocktailing on quality of the measurement. As a manufacturer of fluorescence- labeled monoclonal antibody reagents we attempt to provide helpful hints to flow cytometry users in their long path to optimum design of multicolor flow cytometry panels and protocols.

Which flow cytometry platform is suitable for my flow cytometry assay?







Conclusion:

On a specific case of anti-human CD41 labeled monoclonal antibody (clone MEM-06), we demonstrate the effect of negative fluorescence value of events not expressing the CD41 antigen, based on the platform used.

Very bright antigens like CD41 in PE require lower PMT voltages causing negative events to have fluorescence lower than actual baseline. Further subtraction of the baseline in-between acquired events may then cause negative fluorescence value of negative events. High cell concentration in flow cuvette increases fluorescence background to be subtracted.

Results show strong dependence of negative events fluorescence on acquisition event rate and antibody concentration.

While not completely eliminating negative fluorescence value, dilution of cells affects acquisition rate and background fluorescence in a way that negative fluorescence values of CD41 negative events may become less negative and get closer to zero. Same effect is achieved by dilution of antibody.

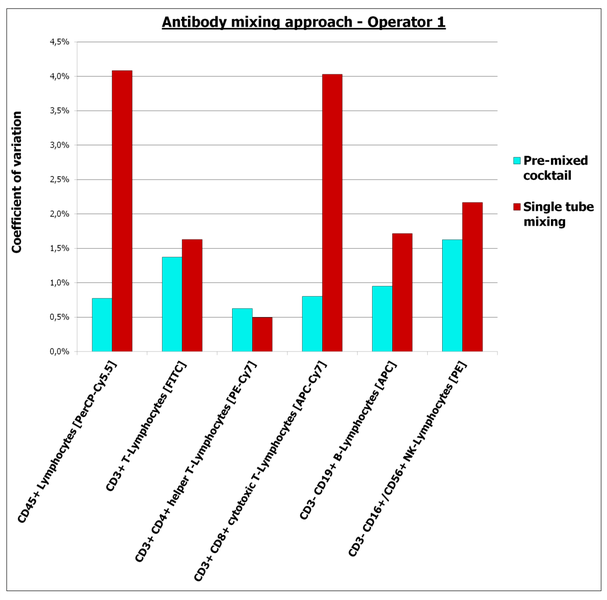
No similar effect was observed when using leukocyte marker antibody. For comparison combination of a bright antigen and a bright fluorochrome CD4 PE was used.

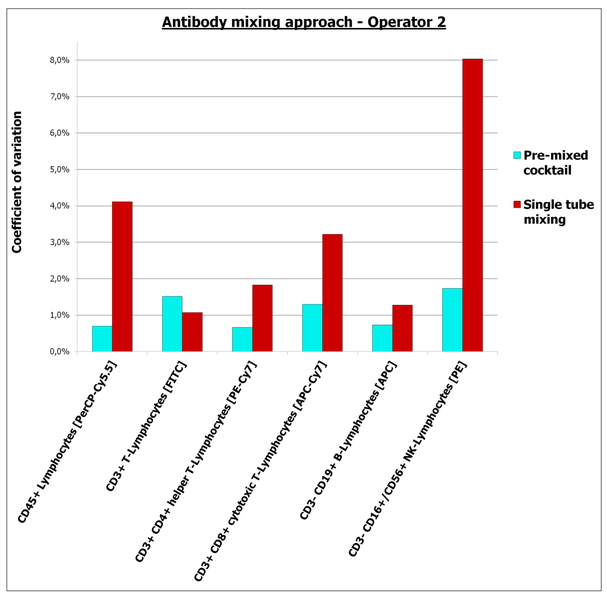
We strongly advise the user to be aware of cytometer platform used and the limitation of a combination of bright antigen with bright fluorochrome.

How does daily monoclonal antibody mixing affect the precision of measurement?

Six color cocktail TBNK:

CD45 PerCP-Cy™5.5 / CD3 FITC / CD4 PE-Cy™7 / CD8 APC-Cy™7 / CD19 APC / CD16+CD56 PE





Conclusion:

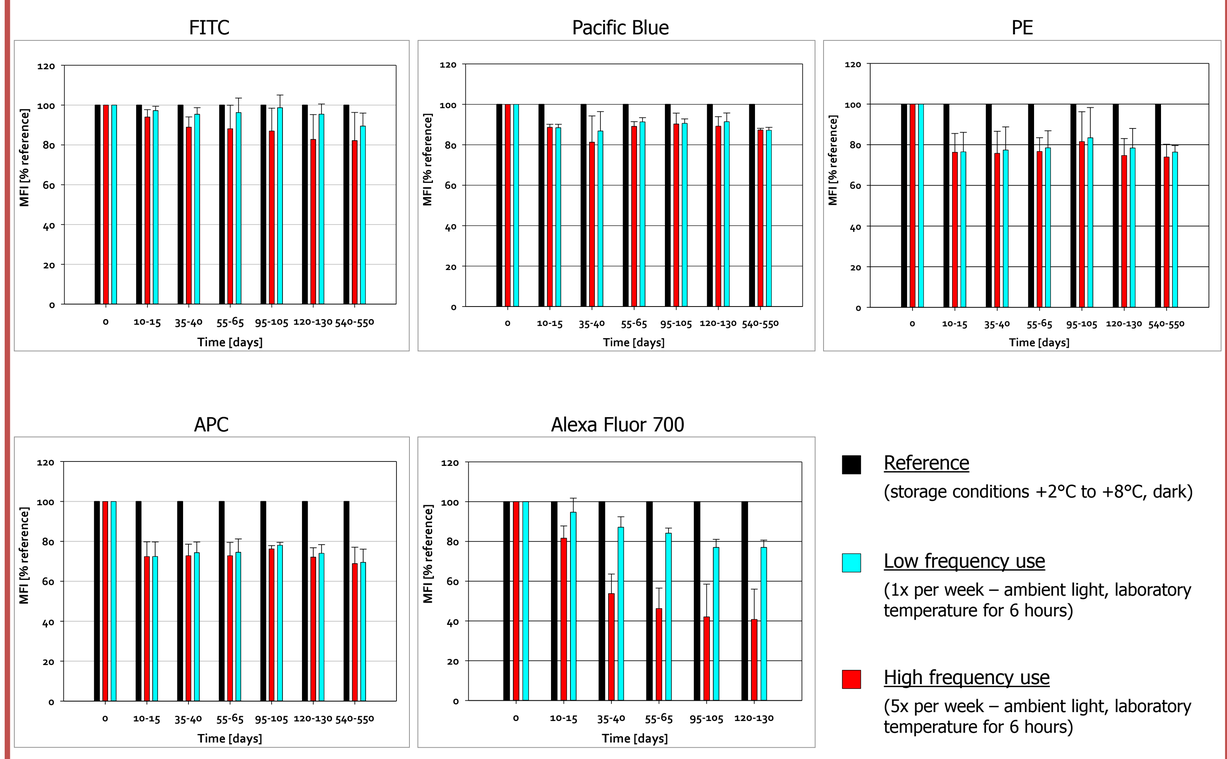
Here we compare 2 different antibody mixing approaches typical to a clinical laboratory daily routine where staining by a pre-mixed antibody cocktail or a single tube antibody mixing may be required.

Based on 2 separate measurements by 2 different operators we show observations on positive cell populations fluorescence variability (% CV) between a multicolor TBNK pre-mixed cocktail prepared by mixing antibodies individually prior to staining and a single tube antibody mixing approach in six different tubes for both approaches.

Measured fluorescence variability show data spread to be higher for single tube mixing approach than in a pre-mixed cocktail staining approach.

In order to minimize intra-laboratory variability of measurement we recommend the use of pre-mixed antibody cocktails rather than mixing selected antibodies in a single tube where possible.

How does daily use affect stability of monoclonal antibody reagents?



Conclusion:

By exposing several labeled monoclonal antibodies to laboratory temperature and ambient daylight on regular daily basis, we show changes in performance for all tested fluorochromes.

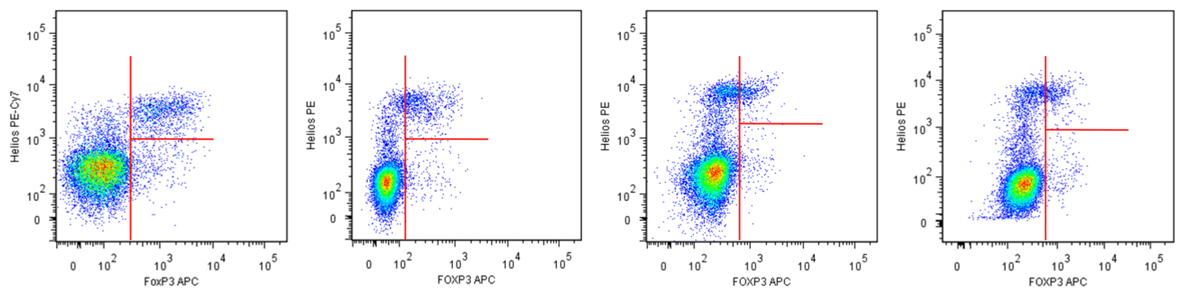
For each fluorochrome 4 different antibody clones labeled by the fluorochrome were used. To accelerate the stability study each antibody-fluorochrome conjugate was subjected to 2 freeze/thaw cycles and exposed to temperatures in the range of +25°C to +45°C to simulate worst case scenario during product transport to a customer.

Changes of antibody conjugate performance occurred with varying intensity for different fluorochromes. While prolonged exposure of most antibody-fluorochrome conjugates to ambient light and temperature had little to no effect on conjugate performance, antibodies labeled with Alexa Fluor® 700 were strongly affected by high frequency of use and exposure to ambient light and temperature.

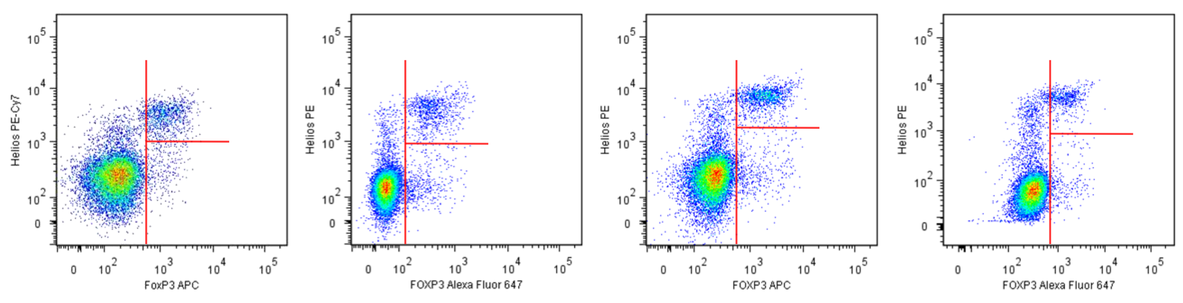
Our recommendation is to minimize the required exposure time of antibody conjugates to ambient light and temperature as much as possible when used.

How different staining protocol effects detection of dimly expressed molecules such as FoxP3?

Clone 3G3



Clone 259D



Permeabilizing buffer A (Whole peripheral blood)

Permeabilizing buffer B (Whole peripheral blood)

Permeabilizing buffer C (Isolated PBMCs)

Permeabilizing buffer D (Isolated PBMCs)

Conclusion:

In this very specific case we tried to show the importance of staining protocol and its effect on antibody performance.

2 different anti Fox-P3 antibodies (clone 3G3 and 259D) were used for intra-nuclear staining in combination with 4 different commercially available permeabilizing buffers and anti-Helios, anti-CD4 and anti-CD25 antibodies as control reagents.

While 3G3 may seem to be a dimmer anti-FoxP3 clone than 259D using most permeabilizing buffers, permeabilizing buffer A allows for separation of CD4+CD25+FoxP3+ T-regulatory cells similar to that of 259D clone.

Different antibody clones reactive against the same antigen may require different staining protocols, hence the selection of staining protocol directly affects the performance of selected antibody panel.