Impact of Fixation/Permeabilization Buffers on Fluorescence Background and Marker Selection for Panel Design

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Abstract

The selection of permeabilization buffers is a critical factor in the design and optimization of flow cytometry assays. In this study, we assessed the impact of different fixation/permeabilization buffers on background fluorescence and marker detection. We tested the eBioscience™ FoxP3/transcription factor staining buffer set (Thermo Fisher Scientific), BD FACS™ permeabilizing solution 2 (BD Biosciences) and True-Nuclear™ transcription factor buffer set (Biolegend) on whole blood. Samples were either left unfixed or processed with these buffers and stained accordingly.

Our results demonstrated that the eBioscience™ FoxP3/transcription factor staining buffer set caused higher background fluorescence (or higher autofluorescence) in several channels in violet laser (including 448/45, 528/45, and 606/36), and blue laser (including 527/32, and 586/42), with noticeable shifts in negative populations. In contrast, BioLegend® True-Nuclear™ transcription factor buffer set and BD FACS permeabilization solution 2 resulted in lower or no background across most channels. However, when evaluating marker expression, the eBioscience™ FoxP3/transcription factor staining buffer set consistently yielded better resolution for both FOXP3 and Ki-67 expression. Although BD FACS permeabilizing solution 2 and BioLegend® True-Nuclear™ transcription factor buffer set displayed reduced background signal, we were unable to detect the expression of FOXP3 and Ki-67. These findings underscore the importance of buffer selection based on both background signal and staining efficiency. Although the eBioscience™ FoxP3/transcription factor staining buffer set shows high background, its superior performance in detecting intracellular markers makes it a preferred choice for FOXP3 and Ki-67 detection. This study reinforces the need for careful buffer selection and optimization to ensure accurate and reproducible flow cytometry results.

Method

Sample Preparation

1. Lysed 2 mL WB with 40 mL 1X BD Pharm Lyse (BD Biosciences, 10 min at room temperature, continuously mixing in a rotator).
2. Resuspended cells at 20x10^6 cells/mL in BSA stain buffer (BD Biosciences).
3. 100 µL of cell suspension is transferred to 5 mL tube (12 x 75mm).
4. Surface staining: cells were either unstained or stained with CD56-V450, CD19-eF506, CD25-BV605, TCRγδ-BV711, TCRVδ2-FITC, FOXP3-PE, NKG2D-RB780, and CD3-APC-Cy7, by incubating for 30 min in dark at ambient temperature. Cells were washed twice with BSA stain buffer and continued for the following procedures:

1. No fixation	2. BD Cytofix™ fixation buffer (BD biosciences)	3. BD FACS permeabilization solution 2	4. eBioscience™ FoxP3/transcription factor staining buffer set	5. BioLegend® True-Nuclear™ transcription factor buffer set
↓	fixation (15 min, 4°C)	fixation (10 min, RT)	fixation (30 min, RT)	fixation (30 min, RT)
	↓	↓	↓	↓
	wash twice with BSA (2 mL)	wash twice with BSA (2 mL)	wash twice with permeabilization buffer (2 mL)	wash twice with permeabilization buffer (2 mL)
	↓	↓	↓	↓
		BSA/antibody (30 min, dark)	perm buffer/antibody (30 min, dark)	perm buffer/antibody (30 min, dark)
		↓	↓	↓
		wash twice with BSA (2 mL)	washed twice with permeabilization buffer (2 mL)	wash twice with permeabilization buffer (2 mL)
		↓	↓	↓
		resuspend in BSA	resuspend in BSA	resuspend in BSA
↓	↓	↓	↓	↓
resuspend in BSA	resuspend in BSA	ready for acquisition on BD FACS Lyric	ready for acquisition on BD FACS Lyric	ready for acquisition on BD FACS Lyric
↓	↓			
ready for acquisition on BD FACS Lyric	ready for acquisition on BD FACS Lyric			

Results

Impact of Fixation/Permeabilization Buffers on Channel-Specific Autofluorescence

Unstained samples were processed as outlined in the sample preparation method. Data show the following:

Violet Laser (448/45, 528/36, 606/36, 715/50, 780/60)

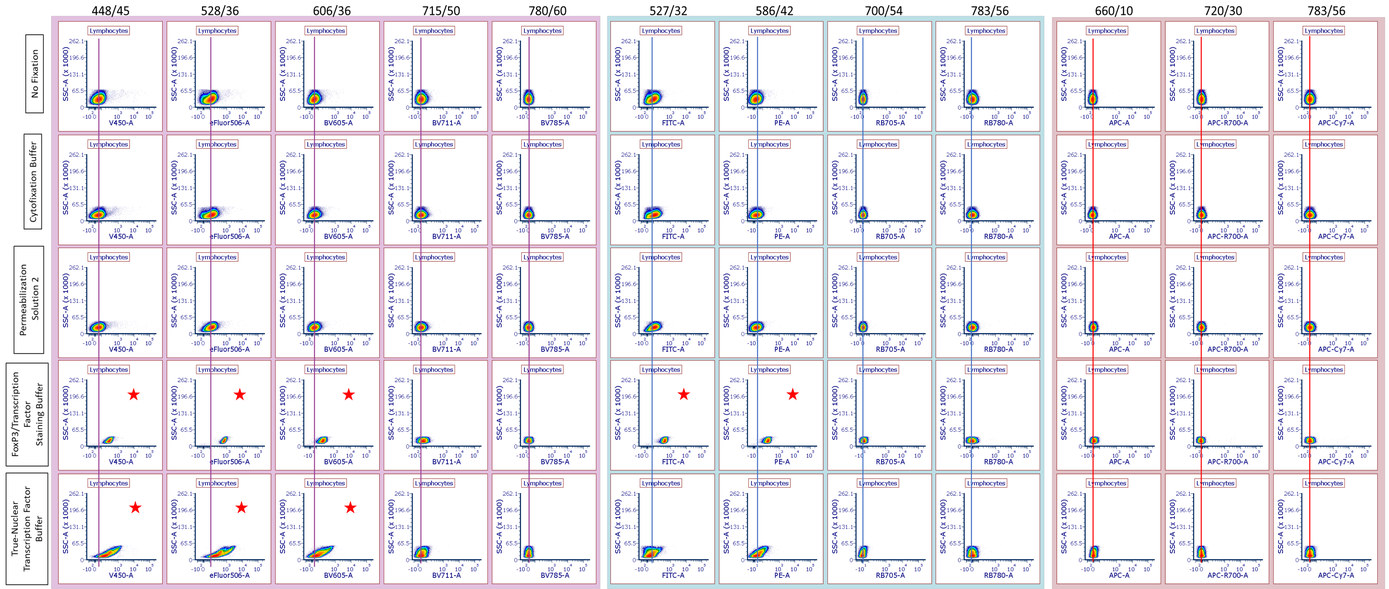
Autofluorescence was observed in **448/45**, **528/45** and **606/36**, with the **eBioscience™ FoxP3/transcription factor staining buffer set** and the **BioLegend® True-Nuclear™ transcription factor buffer set**.
No autofluorescence was observed in **715/50** and **780/60** under any condition.
No autofluorescence was observed with **BD Cytofix™ fixation buffer** and **BD FACS permeabilization solution 2** in any channels.

Blue Laser (527/32, 586/42, 700/54, 783/56)

Autofluorescence was observed in **527/32** and **586/42** with the **FoxP3/transcription factor buffer set**.
No autofluorescence was observed with **BioLegend® True-Nuclear™ transcription factor buffer set**, **BD Cytofix™ fixation buffer** and **BD FACS permeabilization solution 2**.
No autofluorescence was observed in **channels 700/54** and **783/56** with all conditions.

Red Laser (660/10, 720/30, 783/56)

No autofluorescence was observed in any red laser channels.



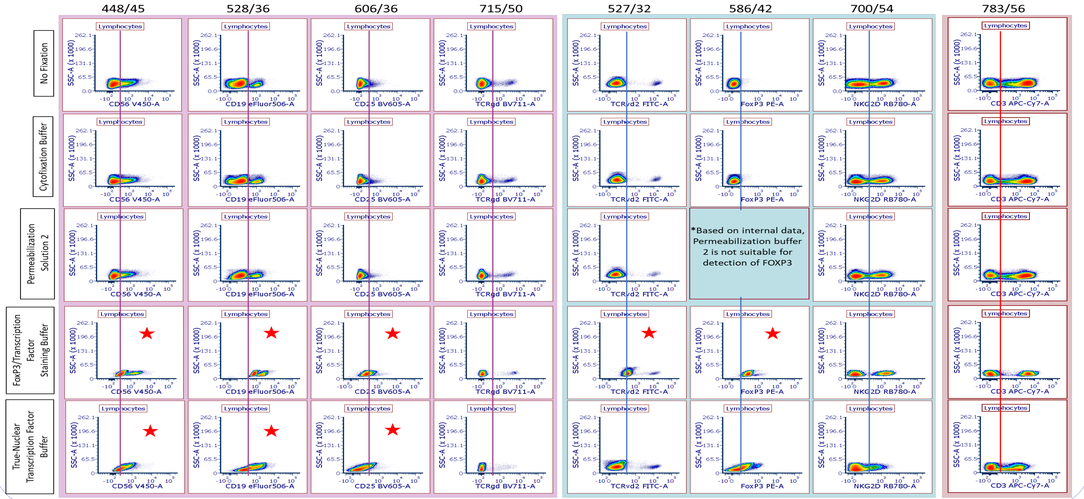
NOTE: Results shown are based on lymphocyte gating, but similar trend was observed in monocytes (data not shown). Granulocytes were not detected after processing with all the permeabilization buffers.

Detection of Markers with Various Buffers

To assess the detection of specific markers, cells were stained with the following markers under different conditions as shown in the figure below: **CD56-V450**, **CD19-eF506**, **CD25-BV605**, **TCRγδ-BV711**, **TCRVδ2-FITC**, **FOXP3-PE**, **NKG2D-RB780**, and **CD3-APC-Cy7**.

- Autofluorescence was observed in **CD56-V450 (448/45)**, **CD19-eF506 (528/36)**, and **CD25-BV605 (606/36)** with either the **eBioscience™ FoxP3/transcription factor buffer set** or the **BioLegend® True-Nuclear buffer set** (excited by violet laser channels).
- Autofluorescence was observed in **TCRVδ2-FITC (527/32)** and **FOXP3-PE (586/42)** with **eBioscience™ FoxP3/transcription factor buffer** Set only (excited by the blue laser).
- No autofluorescence was observed for **TCRγδ-BV711 (715/50, violet laser)**, **NKG2D-RB780 (700/54, blue laser)**, or **CD3-APC-Cy7 (783/56, red laser).**

NOTE: With higher autofluorescence, resolution for certain markers was poor, specifically for markers on rare populations (e.g. CD56 V450).



Intracellular Marker Comparison with different Fixation Buffers

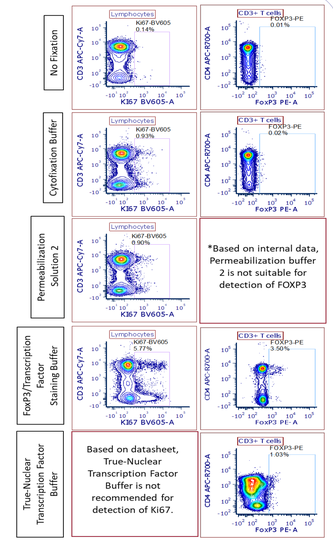
BD Cytofix™ fixation buffer
Not suitable for intracellular staining and was used as control for FOXP3 and Ki-67 gating.

FOXP3 Expression

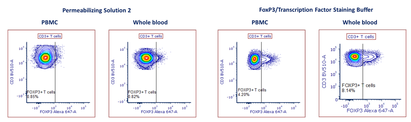
- **eBioscience™ FoxP3/transcription factor staining buffer set**
  showed the highest FOXP3 expression despite some autofluorescence and is recommended for FOXP3 detection.
- **BioLegend® True-Nuclear™ transcription factor buffer set**
  showed a significant reduction in FOXP3 expression compared to FoxP3/transcription factor staining buffer set.
- **BD FACS permeabilizing solution 2***
  is **not** suitable for FOXP3 detection. Cerba research internal data showed significantly reduced detection compared to Foxp3/transcription factor staining buffer set.

Ki-67 Expression

- **eBioscience™ FoxP3/transcription factor staining buffer set**
  showed the highest Ki-67 expression despite some autofluorescence and is recommended for Ki-67 detection.
- **BioLegend® True-Nuclear™ transcription factor buffer set**
  is **not** suitable for Ki-67 staining according to the manufacturer's datasheet.
- **BD FACS permeabilizing solution 2**
  showed reduced Ki-67 expression compared to FoxP3/transcription factor buffer set



*Based on our internal data, permeabilization solution 2 resulted in lower FOXP3 expression in both PBMC and whole blood samples compared to the FoxP3/transcription factor staining buffer set. As a result, permeabilization solution 2 was excluded from subsequent experiments for comparing different permeabilization buffers for FOXP3 staining.



Conclusion

The **eBioscience™ FoxP3/transcription factor staining buffer set** showed higher autofluorescence in **violet and blue laser channels**, while **red laser channels remain unaffected**. Despite this, it remains the **best option** for intracellular staining of **FOXP3 and Ki-67**, as it provides **higher** resolution compared to **BD FACS™ permeabilizing solution 2** and **BioLegend® True-Nuclear™ transcription factor buffer set**.

Consideration for panel design:

Assign **higher level expressing markers with clear separation** of positive and negative populations to affected channels and use low expression or markers on rare population to channels not impacted by the higher autofluorescence when intracellular staining is required.

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