# Standard Protocols for Flow Cytometry for monocytes, macrophages, DC and T cells

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**Method Article**

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# Abstract

This document describes standard research protocols used for analysis of marker expression by flow cytometry in the Hutchinson work-group. In Riquelme et al., these protocols have been applied to the analysis of monocyte-derived macrophages, DC and cultured T cells.

# Introduction

This document describes standard research protocols used for analysis of marker expression by flow cytometry in the Hutchinson work-group. In Riquelme et al., these protocols were applied to the analysis of monocyte-derived macrophages and DC, and cultured T cells.

# Reagents

**General reagents** Biocoll Separating Solution \(L6115, Millipore-Biochrom) DPBS without $Ca^{2+}$ or $Mg^{2+}$ \(D8537, Sigma) BSA \(01400.1, Biomol) $NaN_3$ \(S8032, Sigma) EDTA 0.5M \(15575-038, Gibco Life) Human FcR blocking reagent \(130-059-901, Miltenyi) 7-AAD \(559925, BD) Intracellular fixation buffer \(00-8222, eBiosciences) Permeabilization Buffer \(00-8333, eBiosciences) FOXP3 Fixation/Permeabilization \(00-5521-00, eBiosciences) Fixable Live-Dead Viability DyeFluor660 \(65-0864, eBiosciences) Fixable Live-Dead Viability DyeFluor506 \(65-0866, eBiosciences) Faser Kit APC \(130-091-762, Miltenyi) **Antibodies** Please see Table 1

# Equipment

FACSCanto II cytometer: Standard configuration with 8 colour, 3 lasers \(BD Biosciences) DivaTM Software version v.6.1.3 \(BD Biosciences) Navios cytometer: Standard configuration with 10 colours, 3 lasers \(Beckman Coulter) Navios clinical software \(Beckman Coulter) FlowJoTM analysis software v.7.6.5 \(FlowJo, LLC.) Kaluza analysis software v.1.0 \(Beckman Coulter)

# Procedure

**1. REAGENT PREPARATION** **1.1 Preparation of FACS buffer** 1. Make stock solution of 10% $NaN_3$ in DPBS 2. Transfer 497ml DPBS to a Schott bottle 3. Add 1 ml 10% $NaN_3$ solution to DPBS for an end concentration of 0.02% $NaN_3$ 4. Add 2 ml 0.5 M EDTA to give an end concentration of 2 mM 5. Add 5 g BSA and allow to dissolve 6. Store solution at 4°C **2. PROTOCOLS FOR CELL-SURFACE STAINING OF MACROPHAGES, DC AND T CELLS** **2.1 Standard extracellular staining protocol** 1. Harvest cells into cold DBPS. $2 \times 10^5$ cells are required for each staining reaction. 2. Pellet cells by centrifugation at 300 g for 6 min 3. Aspirate supernatant using vacuum pump 4. Resuspend cells in 100 µl/reaction of FACS buffer + 10% FcR block 5. Incubate at 4°C for 30 min 6. Transfer blocked cell suspensions into FACS tubes at 100 µl/tube 7. Add primary antibodies as indicated 8. Incubate at 4°C in dark for 20 min 9. Add 5 µl 7-AAD per reaction. Incubate at 4°C in dark for 10 min 10. Add 2 ml DBPS per tube. Vortex 11. Pellet

cells by centrifugation at 300 g for 6 min 12. Aspirate supernatant using vacuum pump. 13. Resuspend cells in 200 µl DPBS for analysis by flow cytometry. **2.2 Amplification of PE or APC signals using FASER kit** 1. Harvest cells into cold DBPS. 2 × $10^5$ cells are required for each staining reaction. 2. Pellet cells by centrifugation at 300 g for 6 min 3. Aspirate supernatant using vacuum pump 4. Resuspend cells in 100 µl / reaction of FACS buffer + 10% FcR block 5. Incubate at 4°C for 30 min 6. Transfer blocked cell suspensions into FACS tubes at 100 µl / tube 7. Add primary PE or APC conjugated antibody as indicated 8. Incubate at 4°C in dark for 20 min 9. Add 5 µl 7-AAD per reaction. Incubate at 4°C in dark for 10 min 10. Add 2 ml DBPS per tube. Vortex 11. Pellet cells by centrifugation at 300 g for 6 min 12. Aspirate supernatant using vacuum pump. 13. Resuspend cells in 80 µl FACS buffer + 20 µl FcR block + 10 µl FASER reagent 1 14. Incubate at 4°C in dark for 10 min 15. Wash one time in 2 ml DPBS by centrifugation at 300 g for 6 min 16. Resuspend cells in 80 µl FACS buffer + 20 µl FcR block + 10 µl FASER reagent 2 17. Incubate at 4°C in dark for 10 min 18. Wash one time in 2 ml DPBS by centrifugation at 300 g for 6 min 19. Resuspend cells in 80 µl FACS buffer + 20 µl FcR block + 10 µl FASER reagent 1 20. Incubate at 4°C in dark for 10 min 21. Wash one time in 2 ml DPBS by centrifugation at 300 g for 6 min 22. Resuspend cells in 80 µl FACS buffer + 20 µl FcR block + 10 µl FASER reagent 2 23. Incubate at 4°C in dark for 10 min 24. Wash one time in 2 ml DPBS by centrifugation at 300 g for 6 min 25. Resuspend cells in 200 µl DPBS for analysis by flow cytometry. **3. INTRACELLULAR STAINING OF HUMAN MREGS FOR IDO** 1. Harvest cells into cold DBPS. 5 × $10^5$ Mregs are required for each staining reaction 2. Pellet cells by centrifugation at 300 g for 6 min 3. Aspirate supernatant using vacuum pump 4. Resuspend cells in 3 ml DBPS 5. Add 0.75 µl Fixable Live-Dead dye 6. Incubate at 4°C in dark for 30 min 7. Wash one time in 10 ml DPBS by centrifugation at 300 g for 6 min 8. Resuspend cells in 300 µl FACS buffer + 10% FcR block for 15 min 9. Transfer blocked cell suspensions into FACS tubes at 100 µl / tube 10. Add primary PE-conjugated CD33 or isotype antibody as indicated in Figure 1 \(3 reactions) 11. Incubate at 4°C in dark for 30 min 12. Wash samples one time in 2 ml DPBS by centrifugation at 300 g for 6 min 13. Add 100 µl IC Fixation Buffer for 30 min RT in the dark 14. Wash samples twice in 2 ml Perm Buffer by centrifugation at 300 g for 6 min 15. Resuspend samples in 100 µl of FACS buffer + 10% FcR block for 15 min at 4°C 16. Add intracellular staining antibodies as indicated for 30 min at RT in the dark 17. Wash samples once in 2 ml Perm Buffer by centrifugation at 300 g for 6 min 18. Resuspend cells in 200 µl DPBS for analysis by flow cytometry See figure in Figures section. **4. INTRACELLULAR STAINING OF HUMAN TREGS FOR FOXP3 AND HELIOS** **4.1 Intracellular staining of cultured T cells** 1. Harvest cells into cold DBPS. 5 × $10^5$ cells are required for each staining reaction 2. Pellet cells by centrifugation at 300 g for 6 min 3. Aspirate supernatant using vacuum pump 4. Resuspend cells in 1 ml DBPS 5. Add 1 µl Fixable Live-Dead dye 6. Incubate at 4°C in dark for 30 min 7. Wash one time in 10 ml DPBS by centrifugation at 300 g for 6 min 8. Resuspend cells in 100 µl FACS buffer + 10% FcR block for 15 min 9. Transfer blocked cell suspensions into FACS tubes at 100 µl / tube 10. Add cell surface antibodies as indicated in Figure 2 11. Incubate at 4°C in dark for 30 min 12. Wash samples one time in 2 ml DPBS by centrifugation at 300 g for 6 min 13. Aspirate supernatant using vacuum pump. Gentle vortex 14. Add 500 µl Fixation/Permeabilization 15. Incubate at 4°C in dark for 30 min 16. Wash samples in 2 ml Perm Buffer by centrifugation at 300 g for 6 min 17. Add intracellular staining antibodies \(FoxP3-PE, optional Helios-APC) as indicated 18. Incubate for 30 min at 4°C in the dark 19. Wash samples 3 times in 2 ml Perm Buffer by centrifugation at 300 g for

6 min 20. Resuspend cells in 200 µl Perm Buffer for analysis by flow cytometry See figure in Figures section. **4.2 Intracellular staining for FoxP3 in T cells from clinical blood samples** 1 1 ml blood is used for each staining reaction. Dilute blood 1:1 with DBPS 2 Layer blood carefully onto 6 ml Biocoll in a 15 ml tube 3 Centrifuge at 2000 rpm, 20°C, for 25 min with no brake 4 Recover cell layer in the interface 5 Wash with 14 ml DPSB by centrifugation at 1600 rpm for 10 min 6 Aspirate supernatant using vacuum pump 7 Resuspend cells in 100 µl / reaction of FACS buffer + 10% FcR block 8 Incubate at 4°C for 30 min 9 Transfer blocked cell suspensions into FACS tubes at 100 µl / tube 10 Add cell surface antibodies as indicated in Figure 3 11 Incubate at 4°C in dark for 30 min 12 Add 2 ml DBPS per tube. Vortex 13 Pellet cells by centrifugation at 300 g for 6 min 14 Aspirate supernatant using vacuum pump. Gentle vortex 15 Add 500 µl Fixation/Permeabilization 16 Incubate at 4°C in dark for 30 min 17 Wash samples in 2 ml Perm Buffer by centrifugation at 300 g for 6 min 18 Add intracellular staining antibodies as indicated 19 Incubate for 30 min at RT in the dark 20 Wash samples 3 times in 2 ml Perm Buffer by centrifugation at 300 g for 6 min 21 Resuspend cells in 200 µl Perm Buffer for analysis by flow cytometry See figure in Figures section. **5. Gating strategy for identifying TIGIT$^{+}$ Tregs among NSG mouse splenocytes** See figure in Figures section.

# References

1. Hutchinson J.A. et al. MITAP-compliant characterization of human regulatory macrophages. Transpl Int. 30\(8):765-775 \(2017). 2. Hutchinson J.A. et al. Cutting Edge: Immunological consequences and trafficking of human regulatory macrophages administered to renal transplant recipients. J Immunol. 1;187\(5):2072-8 \(2011) 3. Hutchinson J.A. et al. Human regulatory macrophages. Methods Mol Biol. 677:181-92 \(2011)

# Figures

| | | 488 Excitation | | 633 Excitation |
|---|---|---|---|---|
| | | FL2 (PE) | FL4 (PerCP-eFluor710) | FL6 (APC) |
| PO01 | ANTIGEN | CD33 | IDO | Live-Dead |
| | Clone name | P67.6 | eyedio | |
| | Isotype | mIgG1 | mIgG1 | |
| | Amount (µl) | 5,00 | 5,00 | 0,25 ul/ml |
| | Supplier | BD | eBiosciences | eBio |
| | Cat. # | 347787 | 46-9477-42 | 65-0864-14 |
| PO02 | ANTIGEN | CD33 | Isotype | Live-Dead |
| | Clone name | P67.6 | P3.6.2.8.1 | |
| | Isotype | mIgG1 | mIgG1 | |
| | Amount (µl) | 5,00 | 0,30 | 0,25 ul/ml |
| | Supplier | BD | eBiosciences | eBio |
| | Cat. # | 347787 | 9046-4714-120 | 65-0864-14 |
| PO03 | ANTIGEN | Isotype | Isotype | Live-Dead |
| | Clone name | MOPC-21 | P3.6.2.8.1 | |
| | Isotype | mIgG1 | mIgG1 | |
| | Amount (µl) | 10,00 | 0,30 | 0,25 ul/ml |
| | Supplier | BD | eBiosciences | eBio |
| | Cat. # | 550854 | 9046-4714-120 | 65-0864-14 |

## Figure 1

Staining panel IDO

| | | 488 Excitation | | | | 633 Excitation | | 405 Excitation | |
|---|---|---|---|---|---|---|---|---|---|
| | | FL1 (FITC) | FL2 (PE) | FL3 (PC5.5) | FL4 (PE-Cy7) | FL5 (APC) | FL6 (APC-H7) | FL7 (V450) | FL8 (Aqua) |
| TREG_PANEL | ANTIGEN | CD8 | FOXP3 | CD3 | TIGIT | Drop-in | CD25 | CD4 | Live-Dead |
| | Clone name | SK1 | PCH101 | SK7 | MBSA43 | | M-A251 | RPA-T4 | |
| | Isotype | mIgG1k | rat IgG2ak | mIgG1k | mIgG1 | | mIgG1k | mIgG1k | |
| | Amount (µl) | 20 ul | 10 ul | 20 ul | 10 ul | | 5 ul | 5 ul | + |
| | Supplier | BD | eBio | BD | eBio | | BD | BD | eBio |
| | Cat. # | 345772 | 12-4776-42 | 332771 | 25-9500-42 | | 560225 | 560345 | |
| | Status | CE/IVD | RUO | CE/IVD | RUO | | RUO | RUO | |

## Figure 2

Staining panel iTreg

| | | 488 Excitation | | | | 633 Excitation | | 405 Excitation | |
|---|---|---|---|---|---|---|---|---|---|
| | | FL1 (FITC) | FL2 (PE) | FL3 (PC5.5) | FL4 (PE-Cy7) | FL5 (APC) | FL6 (APC-H7) | FL7 (V450) | FL8 (Aqua) |
| TREG_01 | ANTIGEN | CD8 | FOXP3 | CD3 | TIGIT | CD226 | CD25 | CD4 | CD45 |
| | Clone name | SK1 | PCH101 | SK7 | MBSA43 | DNAX11 | M-A251 | RPA-T4 | 2D1 |
| | Isotype | mIgG1k | rat IgG2ak | mIgG1k | mIgG1 | mIgG1k | mIgG1k | mIgG1k | mIgG1k |
| | Amount (µl) | 20 ul | 10 ul | 20 ul | 10 ul | 10 ul | 5 ul | 5 ul | 5 ul |
| | Supplier | BD | eBio | BD | eBio | Miltenyi | BD | BD | BD |
| | Cat. # | 345772 | 12-4776-42 | 332771 | 25-9500-42 | 130-092-477 | 560225 | 560345 | 655873 |
| | Status | CE/IVD | RUO | CE/IVD | RUO | RUO | RUO | RUO | CE/IVD |
| TREG_02 | ANTIGEN | CD8 | FOXP3 | CD3 | TIGIT | Isotype | CD25 | CD4 | CD45 |
| | Clone name | SK1 | PCH101 | SK7 | MBSA43 | IS5-21F5 | M-A251 | RPA-T4 | 2D1 |
| | Isotype | mIgG1k | rat IgG2ak | mIgG1k | mIgG1 | mIgG1k | mIgG1k | mIgG1k | mIgG1k |
| | Amount (µl) | 20 ul | 10 ul | 20 ul | 10 ul | 10 ul | 5 ul | 5 ul | 5 ul |
| | Supplier | BD | eBio | BD | eBio | Miltenyi | BD | BD | BD |
| | Cat. # | 345772 | 12-4776-42 | 332771 | 25-9500-42 | 130-092-214 | 560225 | 560345 | 655873 |
| | Status | CE/IVD | RUO | CE/IVD | RUO | RUO | RUO | RUO | CE/IVD |
| TREG_03 | ANTIGEN | CD8 | FOXP3 | CD3 | TIGIT | CD278 | CD25 | CD4 | CD45 |
| | Clone name | SK1 | PCH101 | SK7 | MBSA43 | ISA-3 | M-A251 | RPA-T4 | 2D1 |
| | Isotype | mIgG1k | rat IgG2ak | mIgG1k | mIgG1 | mIgG1k | mIgG1k | mIgG1k | mIgG1k |
| | Amount (µl) | 20 ul | 10 ul | 20 ul | 10 ul | 5 ul | 5 ul | 5 ul | 5 ul |
| | Supplier | BD | eBio | BD | eBio | eBio | BD | BD | BD |
| | Cat. # | 345772 | 12-4776-42 | 332771 | 25-9500-42 | 17-9948-42 | 560225 | 560345 | 655873 |
| | Status | CE/IVD | RUO | CE/IVD | RUO | RUO | RUO | RUO | CE/IVD |

## Figure 3

Staining panel iTreg in peripheral blood

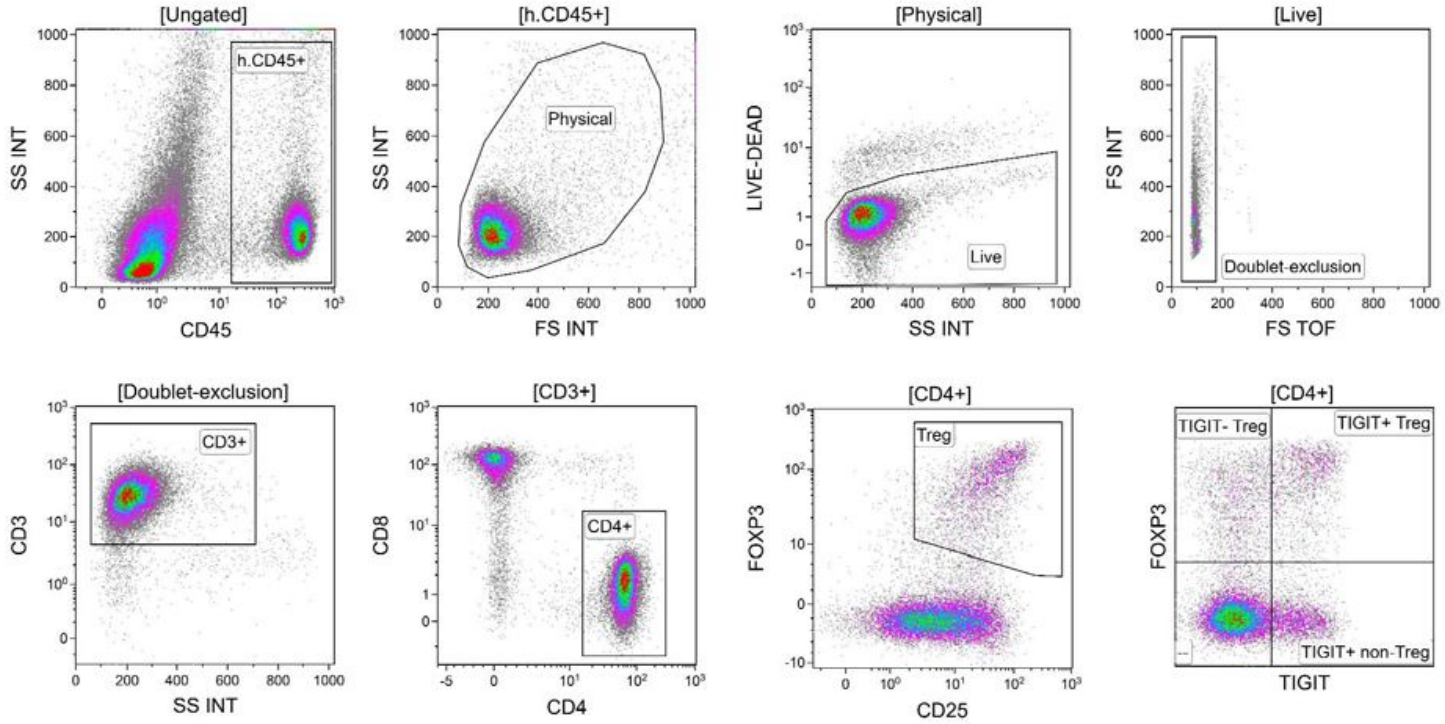


**Figure 4**

Gating strategy for identifying TIGIT^+^ Tregs among NSG mouse splenocytes

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement0.pdf