# Using spectral flow cytometry and BD Horizon RealYellow™ and BD Horizon RealBlue™ Reagents to dissect the expression of metabolic proteins in resting and activated T cells

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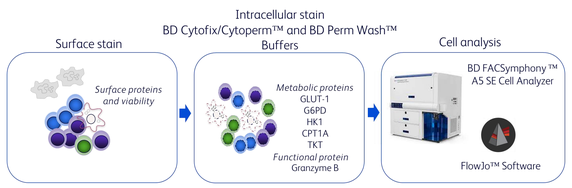
## Abstract

In response to activation, T cells undergo metabolic adaptations to meet the energetic and biosynthetic demands required for their rapid cell growth and proliferation. T cell receptor (TCR) engagement and CD28 costimulation trigger the initial metabolic changes that support T cell activation including increase in glucose uptake. PI3K signaling is critical for upregulation of GLUT-1, the main glucose receptor in T cells. In this study, we describe an assay for detection of GLUT-1 and key metabolic enzymes using fluorochrome-conjugated antibodies that have been validated for flow cytometry. Our analyses showed a broad expression of GLUT-1 and other metabolic markers across human immune cell populations. In T cells, GLUT-1 was expressed in resting T cells and upregulated in response to anti-CD3/CD28 stimulation. GLUT-1$^{high}$ cells were found among CD69$^{+}$ activated T cells, which also showed higher expression levels of glucose-6-phosphate dehydrogenase (G6PD) compared to the CD69$^{-}$ cells. Notably, the PI3K inhibitor Ly294002 abrogated the upregulation of GLUT-1 and G6PD expression in activated CD69$^{+}$ T cells. Ly294002 cell treatment did not affect the expression of hexokinase 1 (HK1), CD69, PD-1 or CD25, suggesting that PI3K inhibition impacted the expression of select metabolic proteins and not overall T cell activation. These results support literature evidence showing the importance of the glycolytic pathway on T cell activation while demonstrating optimized experimental conditions for resolution of multiple metabolic proteins.

## Methods

**Cell preparation and staining**

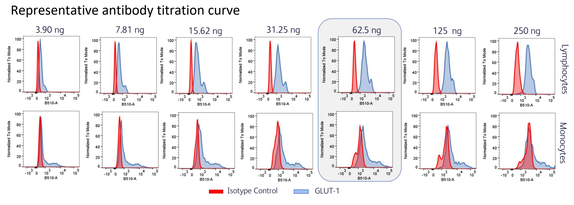
1. Freshly isolated peripheral blood mononuclear cells (PBMC) were stained with a cocktail of antibodies against surface markers and BD Horizon™ Fixable Viability Stain UV440. The cells were fixed with BD Cytofix/Cytoperm™ Fixation Buffer for 20 min at 4 °C prior to 1 h-intracellular staining in BD Perm/Wash™ Buffer with antibodies against five metabolic proteins and granzyme B.
2. PBMCs were cultured overnight in four different conditions: a) media alone (resting condition); b) stimulated with Dynabeads™ anti-CD3/CD28 in a 0.5:1 ratio; c) Dynabeads™ anti-CD3/CD28 plus 50 μM Ly294002; d) Dynabeads™ anti-CD3/CD28 plus 6 mM 2-Deoxy-D-glucose (2-DG).




**Assessment of metabolic proteins**

Prior to multicolor staining, antibody titrations were performed as demonstrated below (optimal concentration indicated by the gray box). Matching isotype controls were utilized to determine the contribution of nonspecific background. Stain index values were calculated using median of fluorescence of the positive population (metabolic protein stain) and median of fluorescence and standard robust deviation of the negative population (isotype control).

Representative antibody titration curve




30-color immunophenotyping panel

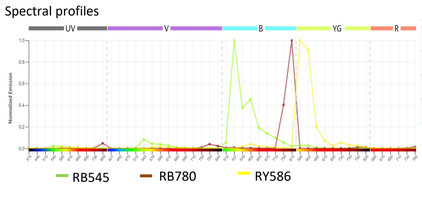
| A5 SE | Fluor | Broad panel | Clone | Purpose |
|---|---|---|---|---|
| | **UV379** | BUV395 | G6PD | EPR6292 | Oxidative pentose phosphate pathway |
| | **UV446** | | FVS440UV | | Viability |
| | **UV515** | BUV496 | HLA-DR | G46-6 | Dendritic cells and activated T cells subsetting |
| | **UV540** | | AutoF | | Autofluorescence measurement |
| | **UV585** | BUV563 | CD45 | HI30 | Leucocyte identification |
| | **UV610** | BUV615 | CD45RA | HI100 | Distinction between naive and memory T cell subsets |
| | **UV660** | BUV661 | HK1 | EPR10134(B) | Glycolysis |
| | **UV736** | BUV737 | CD27 | M-T271 | T and B cells subsetting |
| | **UV809** | BUV805 | CD19 | HIB19 | B cells identification |
| | **V427** | BV421 | CD197 | 2-L1-A | Distinction between naive and memory T cell subsets |
| | **V450** | V450 | CPT1A | 8F6AE9 | Fatty-acid synthesis |
| | **V470** | V480 | CD38 | HB7 | T and B cells subsetting |
| | **V510** | BV510 | TCR Cb1 | JOVI.1 | Distinction between TCR heavy chain b1 and b2 |
| | **V576** | BV570 | CD3 | UCHT1 | T cells identification |
| | **V615** | BV605 | CD56 | R19-760 | NK cells identification |
| | **V680** | BV650 | CD127 | HIL-7R-M21 | Regulatory T cells identification |
| | **V710** | BV711 | IgM | G20-127 | B cells subsetting |
| | **V750** | BV750 | CD16 | 3G8 | NK cells and monocytes subsetting |
| | **V785** | BV786 | CD279 | NAT105 | Analysis of immunocheckpoint regulator |
| | **B510** | AF488 | GLUT-1 | EPR3915 | Glucose uptake |
| | **B537** | RB545 | GzmB | GB11 | NK and T cell activation |
| | **B602** | BB630 | CD4 | SK3 | CD4 T helper cells identification |
| | **B660** | BB660 | IgD | IA6-2 | B cells subsetting |
| | **B675** | PerCP | CD8 | SK1 | Cytotoxic T cells identification |
| | **B710** | BB700 | CD123 | 7G3 | Plasmacytoid dendritic cells and basophils identification |
| | **B810** | RB780 | TKT | 7H1AA1 | Non-oxidative pentose phosphate pathway |
| | **YG585** | PE | CD11c | B-ly6 | Dendritic cells subsetting |
| | **YG602** | RY586 | CD25 | 2A3 | Regulatory T cells identification |
| | **R660** | APC | CD14 | M5E2 | Monocytes subsetting |
| | **R730** | R718 | CD28 | L293 | T cells subsetting |
| | **R780** | APC-H7 | CD20 | 2H7 | B cells subsetting |

24-color T-cell activation panel

| A5 SE | Fluor | T cell panel |
|---|---|---|
| | **UV379** | BUV395 | G6PD |
| | **UV446** | | FVS440UV |
| | **UV515** | BUV496 | HLA-DR |
| | **UV540** | | AutoF |
| | **UV610** | BUV615 | CD45RA |
| | **UV660** | BUV661 | HK1 |
| | **UV736** | BUV737 | CD27 |
| | **UV809** | BUV805 | CD69 |
| | **V427** | BV421 | CD197 |
| | **V450** | V450 | CPT1A |
| | **V470** | V480 | CD38 |
| | **V510** | BV510 | TCR Cb1 |
| | **V576** | BV570 | CD3 |
| | **V615** | BV605 | CD56 |
| | **V680** | BV650 | CD127 |
| | **V750** | BV750 | CD16 |
| | **V785** | BV786 | CD279 |
| | **B510** | AF488 | GLUT1 |
| | **B537** | RB545 | GrzmB |
| | **B602** | BB630 | CD4 |
| | **B675** | PerCP | CD8 |
| | **B810** | RB780 | TKT |
| | **YG602** | RY586 | CD25 |
| | **R660** | APC | CD14/CD19 |
| | **R730** | R718 | CD28 |

A5 SE, BD FACSymphony™ A5 SE Cell Analyzer; BUV, BD Horizon Brilliant™ Ultraviolet; BV, BD Horizon Brilliant™ Violet; V, BD Horizon™ Violet; BB, BD Horizon Brilliant™ Blue; RB, BD Horizon RealBlue™; RY, BD Horizon RealYellow™; R, BD Horizon™ Red; AF, Alexa Fluor™

Spectral profiles

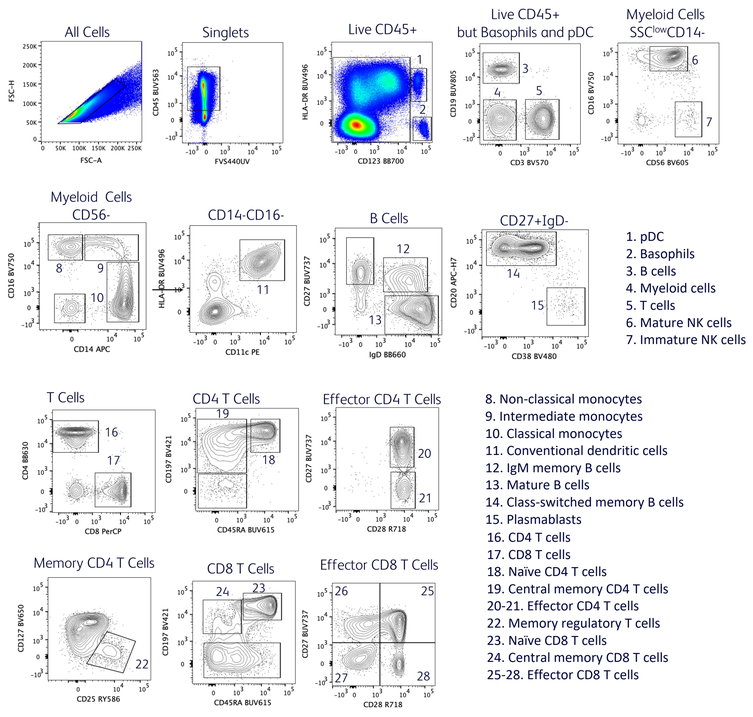



Similarity scores

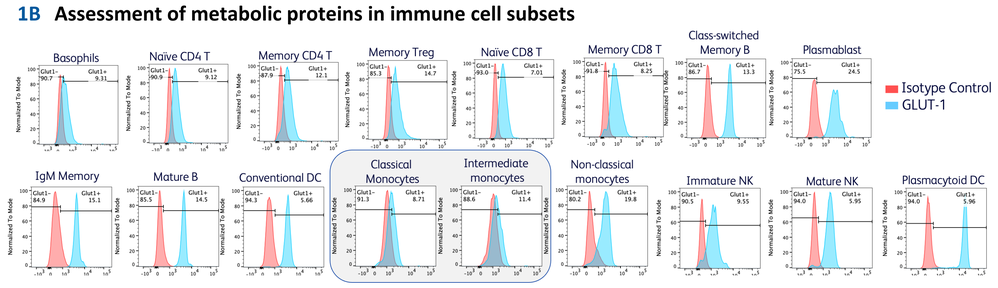

The curves show normalized spectral emission of three new BD Horizon™ Fluorochromes. Scores calculated on BD® Spectrum Viewer indicate that BD Horizon™ RB545 and Alexa Fluor™ 488 Dyes have distinct spectral profiles (low score of 0.63). In contrast, BD Horizon™ RY586 and PE Dyes have higher spectral overlap (high score of 0.9) and were carefully used in the 30-color panel to minimize impact from spillover spreading.

## Results (1)

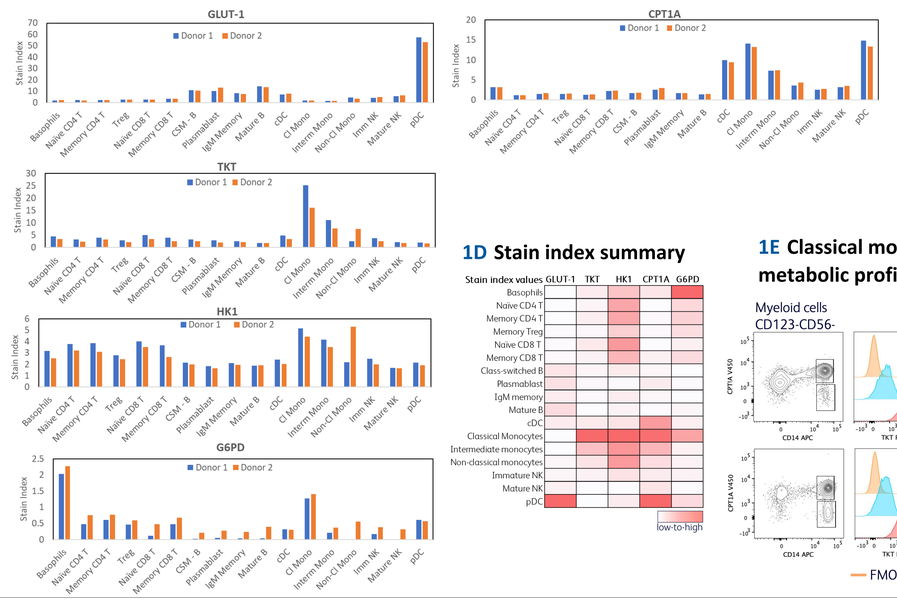
### 1A Identification of immune cell subsets with a 30-color panel




### 1B Assessment of metabolic proteins in immune cell subsets



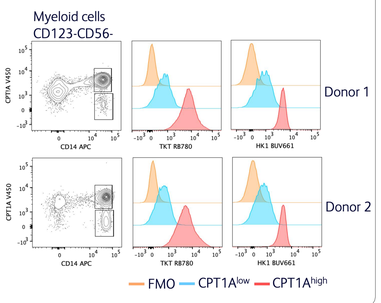

### 1C Relative expression of metabolic proteins across immune cell subsets




### 1D Stain index summary

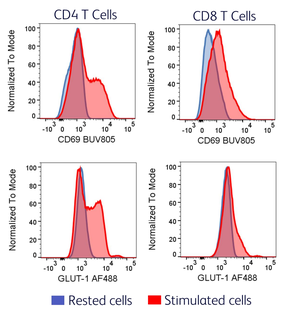



### 1E Classical monocytes metabolic profile




## Results (2)

### 2A GLUT-1 upregulation in activated T cells




### 2B Effect of PI3K and glycolysis inhibition on T cell activation

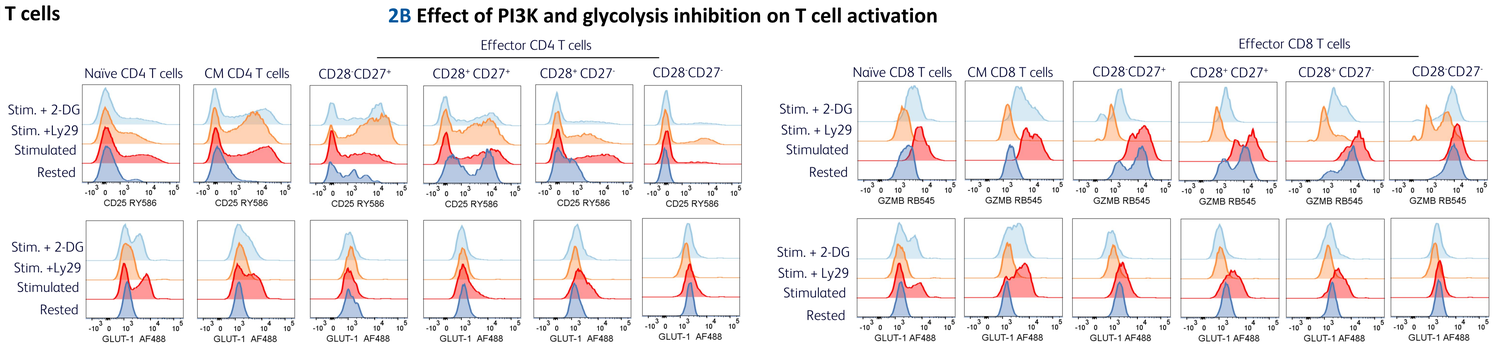
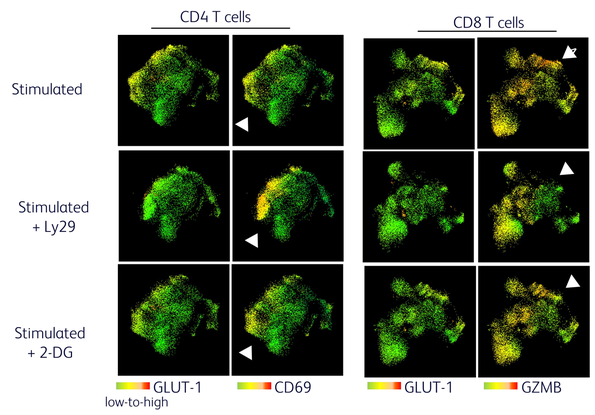


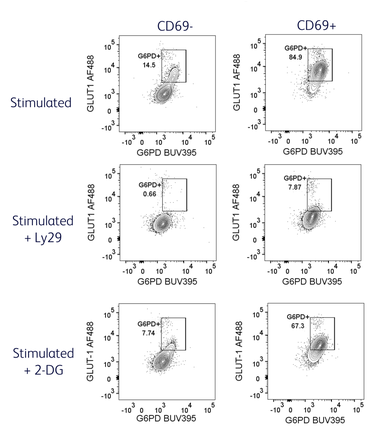

Figure 2: **Analysis of T cell activation and metabolic status upon TCR stimulation with anti-CD3/CD28 beads. A)** Histogram overlays depict the expression of the activation marker CD69 and the glucose transporter (GLUT-1) after overnight cell stimulation. **B)** Expression of the activation marker CD25, GLUT-1 and GZMB on different T cell subsets following cell stimulation in the presence of either a PI3K inhibitor (Ly294002) or glycolysis inhibitor (2-DG). GLUT-1 upregulation occurs predominantly on naïve and central memory cells (newly activated cells) and Ly294002 disrupted GLUT-1 upregulation in CD4 and CD8 T cell subsets while glycolysis inhibition only partially impacted GLUT-1 upregulation. PI3K inhibition also affected GZMB expression in CD8 T cell subsets.

### 3A UMAP demonstrates co-expression of GLUT-1 and activation markers in a PI3K-dependent manner

Figure 3: **Co-expression of upregulated GLUT-1 and activation markers. A)** Uniform Manifold Approximation and Projection (UMAP) shows co-expression of GLUT-1 and CD69 on CD4 T cells. It also depicts an increase in the proportions of CD69$^{+}$ cells in response to Ly294002 treatment. This is consistent to an increase in CD25$^{+}$ cells proportions (Fig.2B). In CD8 T cells, Ly294002 led to the loss of GLUT-1$^{+}$GZMB$^{+}$ double positive cells. **B)** G6PD expression is upregulated in activated CD69$^{+}$ T cells concurrently with GLUT-1. Glycolysis inhibition with 2-DG partially perturbed the formation of GLUT-1$^{+}$G6PD$^{+}$ cells.




### 3B GLUT-1 and G6PD co-expression




## Conclusions

- Cellular metabolism is crucial for the activation and specialized functions of immune cells. Here we offer tools and strategies for a comprehensive analysis of metabolic proteins in a variety of cell subsets:
  1. Five clones for flow cytometry applications and specific for catabolic or anabolic pathways (GLUT-1 - EPR3915; G6PD - EPR6292; HK1 - EPR10134(B); CPT1A - 8F6AE9 and TKT - 7H1AA1).
  2. Buffer system and protocol that allow concomitant measurement of these five metabolic proteins. Also, we show an approach for reagents' titrations and application of isotype controls for accurate measurements in diverse populations.
  3. New instrumentation (BD FACSymphony™ A5 SE Cell Analyzer) and fluorochromes (BD Horizon™ RB545, RB780 and RY586) for panel expansion from 24 to 30 colors.
- T cell activation leads to dramatic shifts in cell metabolism from oxidative phosphorylation to aerobic glycolysis. To measure such changes in vitro, we evaluated specially the expression of the glucose transporter GLUT-1. We detected GLUT-1 upregulation in activated cells. This process was disrupted by PI3K inhibition demonstrating the relevance of this signaling pathway for the uptake of nutrients (glucose) during cell activation. Importantly, PI3K inhibition led to the loss of a GZMB$^{+}$ functional CD8 T cell population. Feeding the cells with 6 mM of a glucose analog (2-DG) that cannot be metabolized only partially affected T cell populations.