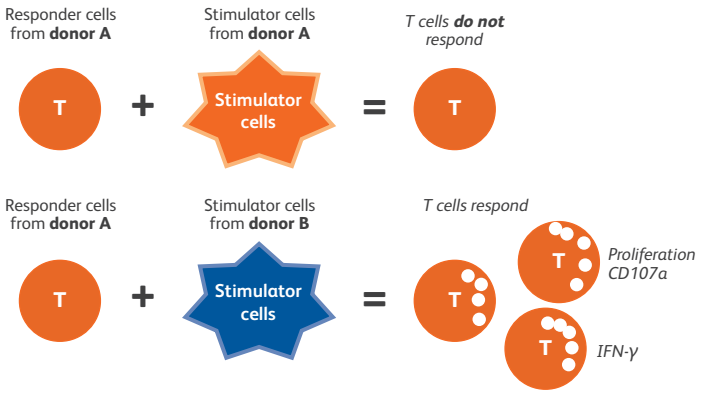
# Flow cytometric assessment of CD8+ cytotoxic T-cell activation in a mixed lymphocyte reaction

## Immuno-oncology applications for the BD FACSCelesta™ flow cytometer

### Features

- Assess multiple indicators of cytotoxic activity at the single-cell level
- Assess T-cell proliferation, cytokine production and degranulation simultaneously
- Multiplex both cell surface and intracellular activation indicators in the same panel
- Identify specific T-cell subsets with potential cytotoxic activity

Figure 1




Immuno-oncology has become the subject of intense scientific and therapeutic interest, as researchers probe the mechanisms by which cancer cells circumvent the effector functions of cytotoxic T and NK cells. Because these research questions are inherently multivariate, involving the complementary push-and-pull signaling of activation and inhibition, they are ideal for multicolor flow cytometry, which can assess multiple parameters in heterogeneous cell populations at the single-cell level. With up to 3 lasers and 12 fluorescence parameters, the BD FACSCelesta™ flow cytometer can unravel effector functions among specific subsets of cytotoxic cells in a single-tube assay.

**Figure 1. One-way MLR assay to evaluate CD8+ T-cell response**
Responder T cells from donor A are co-cultured with inactivated stimulator cells from either the same donor A (autologous) or a different donor B (allogeneic). T-cell responses include proliferation (measured using CFSE dilution), degranulation (exposed CD107a) and cytokine production (intracellular IFN-γ).

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To assess the cytotoxicity of CD8⁺ T cells, we employed a one-way mixed lymphocyte reaction (MLR) assay (illustrated in Figure 1). This assay can provide a multifaceted window into T-cell function, including proliferation (measured by CFSE dilution), degranulation (exposed CD107a) and cytokine production (intracellular IFN-γ). Responder (effector) T cells from one donor were magnetically enriched from peripheral blood mononuclear cells (PBMCs) and co-cultured with the remaining T-cell-depleted (stimulator) fraction, either from the same donor (autologous) or a different donor (allogeneic). To discriminate effector vs stimulator cells–and simultaneously measure T-cell proliferation–we labeled the responder T cells with CFSE. The stimulator cells were treated with mitomycin C to inhibit their proliferation.

The cells were analyzed using an 8-color panel on the BD FACSCelesta Blue/Violet/Red (BVR) configuration. Figure 2 shows overall proliferation of live CFSE-labeled T-cell responders ($CD3^{+}FVS780^{-}CFSE^{+/low}$). Analysis of CFSE dilution showed substantial T-cell proliferation ($CFSE^{low}$, boxed) in response to allogeneic stimulation (top plots), as opposed to minimal proliferation in response to autologous stimulation (bottom plots). As expected, the frequency of proliferating T cells was proportional to the ratio of T cells to stimulator cells in the co-cultures. As negative and positive experimental controls, responder T cells were cultured in media alone (showing no proliferation) or with Staphylococcal enteroxin B (SEB, showing high proliferation).

Figure 2

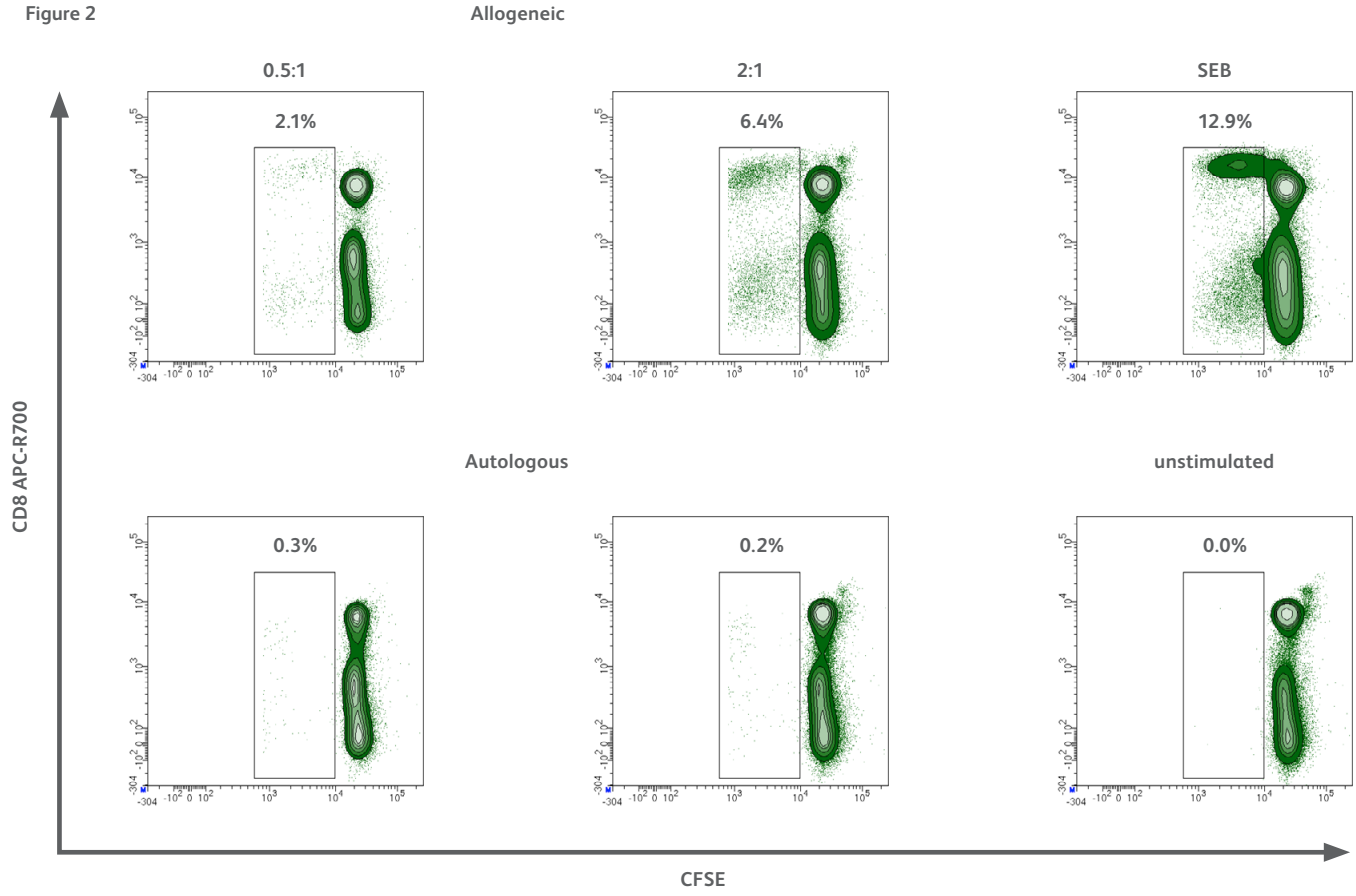


**Figure 2. T-cell proliferation in mixed lymphocyte reaction**

T cells were isolated from the peripheral blood of healthy donors using BD IMag™ Human T Lymphocyte Enrichment Set – DM and then labeled with BD Horizon™ CFSE for analysis of cell proliferation. The T-cell depleted counterparts (stimulator cells) were treated with mitomycin C to cease proliferation of any remaining T cells in this cell fraction. CFSE-labeled T cells were co-cultured with mitomycin-C treated allogeneic or autologous stimulator cells for 4 days. Then, fresh culture medium containing BD GolgiPlug™ Protein Transport Inhibitor (Containing Brefeldin A), BD GolgiStop™ Protein Transport Inhibitor (Containing Monensin) and BD Horizon™ BV421 Mouse Anti-Human CD107A was added to the cells. After 5 hours of incubation, the cells were harvested and stained with BD Horizon™ BV510 Mouse Anti-Human CD3, BD Horizon™ APC-R700 Mouse Anti-Human CD8, BD Horizon™ BV786 Mouse Anti-Human CD45RO and BD Pharmingen™ Alexa Fluor® 647 Mouse Anti-Human CD197 (CCR7). Surface-stained cells were further labeled with BD Horizon™ Fixable Viability Stain 780 for exclusion of dead cells from analysis and fixed and permeabilized with BD Cytofix™ Fixation Buffer and BD Perm/Wash™ Buffer, respectively. Permeabilized cells were stained with BD Pharmingen™ PE Mouse Anti-Human IFN-γ. The cells were analyzed on a BD FACSCelesta BVR flow cytometry system. Stimulation with Staphylococcal enterotoxin B (SEB) was used as a positive control. Cells were gated on live responder T cells ($CD3^{+}FVS780^{-}CFSE^{+/low}$, gating not shown). **Results:** T-cell proliferation was determined by analyzing CFSE dilution vs CD8 counterstain. A higher proportion of T cells proliferated ($CFSE^{low}$, boxed) in response to either SEB or allogeneic stimulation (top plots) compared to unstimulated T cells or those co-cultured with autologous cells (bottom plots). Optimal proliferative response was observed when the ratio of T cells to stimulator cells in the co-cultures was 2:1 (middle plots), while specific allogeneic response was still detectable at cell ratios as low as 0.5:1 (left plots).

Figure 3 shows the analysis of cytotoxic CD8⁺ T cells' effector functions in response to allogeneic stimulation. The cells were gated as in Figure 2 and further gated on CD8⁺ cells (Figure 3A). Figure 3B shows the cell surface analysis of externalized CD107a (top plots) and intracellular levels of IFN-γ (bottom plots) in CD8⁺ cells. As observed, allogeneic stimulation induced cellular degranulation (CD107a externalization) and IFN-γ production in CD8⁺ T cells undergoing proliferation.

The abundance of detectors in the BD FACSCelesta allowed us to include immunophenotypic markers and extend the analysis to T-cell subsets. In Figure 4A, the CD8⁺ T cells were categorized as naïve, central memory, or effector/effector memory cells based on differential expression of CD197 (CCR7) and CD45RO. Figure 4B shows that cell proliferation ($CFSE^{low}$), CD107a externalization (top plots) and IFN-γ production (bottom plots) were restricted to the central memory and effector/effector memory CD8⁺ T cell subsets, as very few naïve CD8⁺ T cells proliferated in response to allogeneic stimulation.

The use of flow cytometry to assess cytotoxicity allows you to answer important immuno-oncological questions. The BD Accuri™ C6 Plus personal flow cytometer, as explained in our companion product information sheet, can assess immune cell proliferation and activation simultaneously in two panels on a benchtop. By adding a third laser and additional fluorescence channels, the BD FACSCelesta can further identify, in a single panel, which cell subsets contributed to the immune response.

Combining innovations in instrumentation with optimization for bright new reagents and BD's unparalleled service and support, the BD FACSCelesta flow cytometer is designed to help you extract a deeper level of biological information from your cell types of interest. By enabling simultaneous analysis of multiple parameters, and by supporting both cell surface and intracellular investigations, the BD FACSCelesta helps you gain new understanding and insights into immuno-oncological processes.

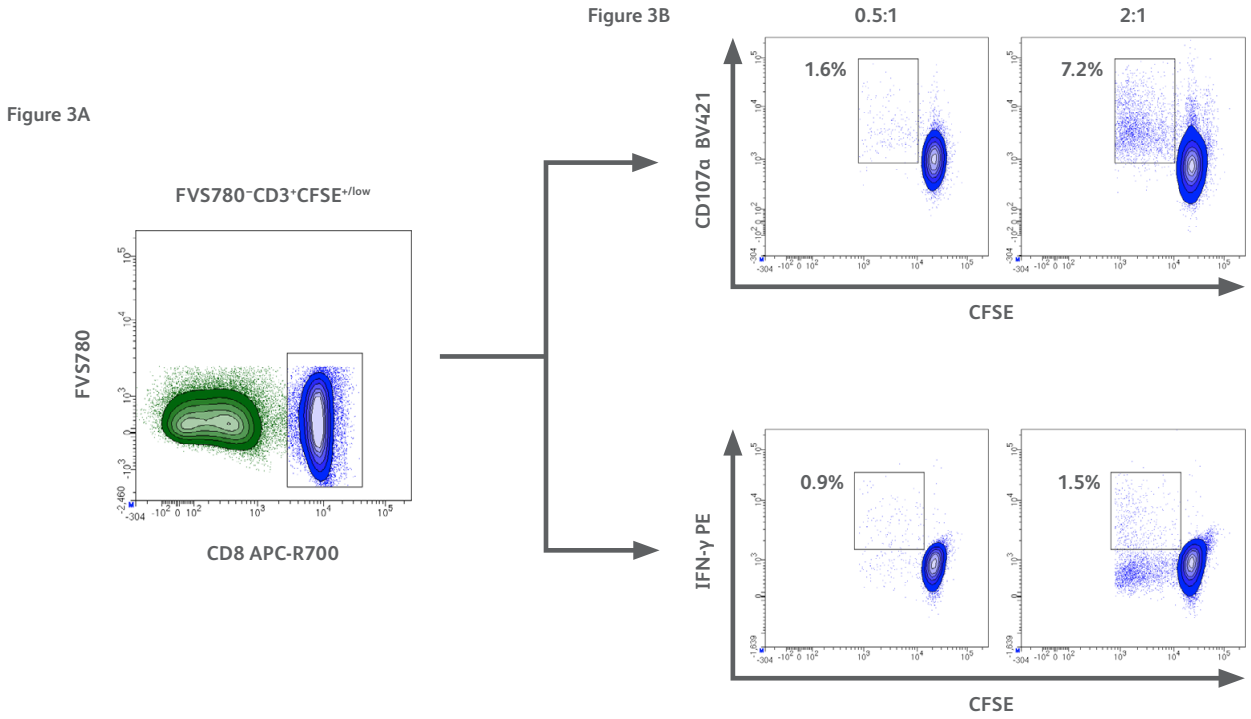


**Figure 3. Activation of CD8⁺ T cells in mixed lymphocyte reaction**
CFSE-labeled T cells were cultured with allogeneic mitomycin C-treated stimulator cells and stained as described in Figure 2. Cells were gated on live responder T cells ($CD3^{+}FVS780^{-}CFSE^{+/low}$, gating not shown). **Results: A.** Cells were further gated on CD8⁺ T cells for analysis. **B.** Externalization of CD107a on the cell surface (top plots) and expression of IFN-γ (bottom plots), were primarily observed among the $CFSE^{low}CD8^{+}$ proliferating T cells, suggesting that these cells were fully activated and functional upon stimulation with allogeneic cells.

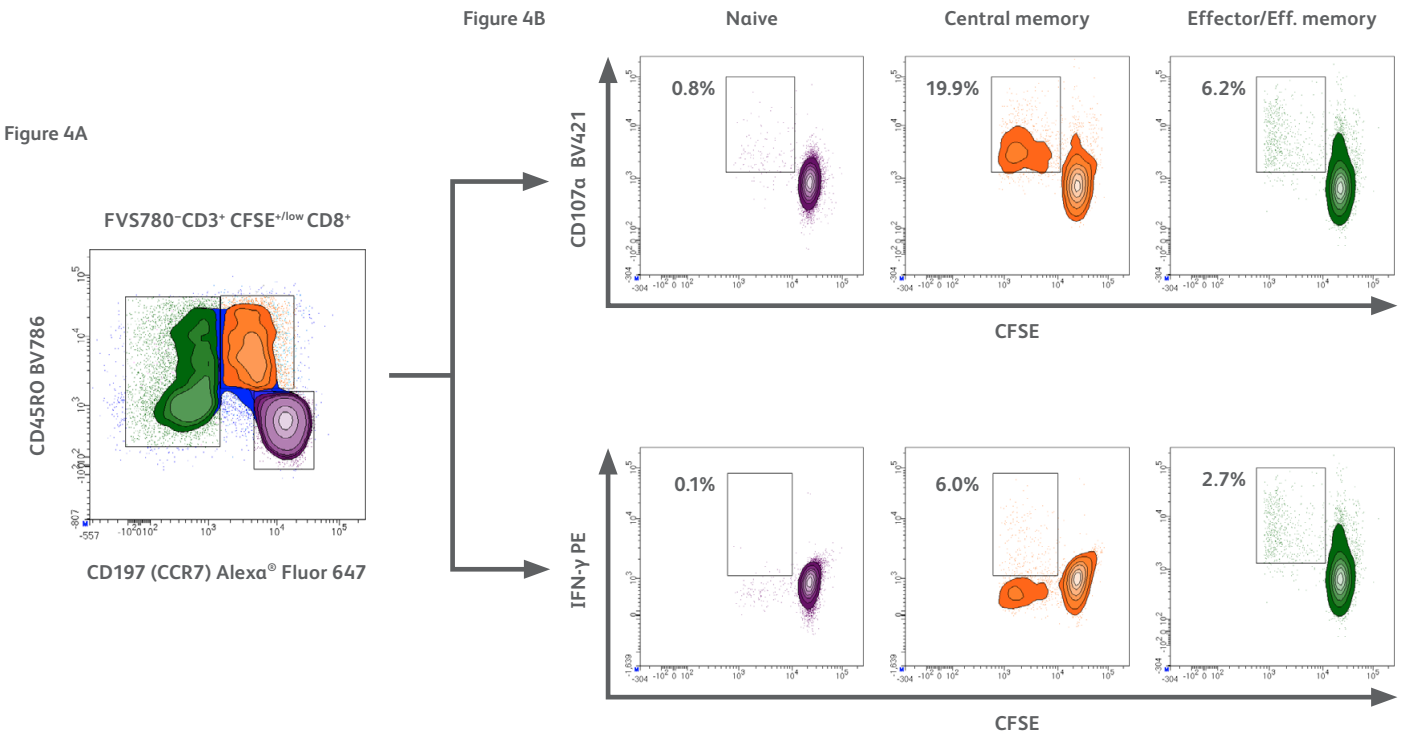


**Figure 4. Analysis of alloresponsive CD8⁺ T-cell subsets**

CFSE-labeled T cells were cultured with allogeneic mitomycin C-treated stimulator cells and stained as described in Figure 2. Cells were gated on live responder T cells ($CD3^+FVS780^-CFSE^{+/low}$) and then on $CD8^+$ for further analysis (gating not shown). **Results: A.** $CD8^+$ T cells were grouped into three main subsets–naïve (purple), central memory (orange) and effector/effector memory cells (green)–based on CD197 and CD45RO staining profiles. **B.** In contrast to naïve $CD8^+$ T cells, more central memory and effector/effector memory $CD8^+$ T cells proliferated in response to allogeneic stimulation. These proliferating cell subsets also externalized CD107a on the cell surface (top plots) and expressed IFN-γ (bottom plots).

## Ordering information: Systems and software

| Description | Cat. No. |
|---|---|
| BD FACSCelesta™ Flow Cytometer, BVR Configuration | 660344 |
| BD FACSCelesta™ Flow Cytometer, BVYG Configuration | 660345 |
| BD FACSCelesta™ Flow Cytometer, BVUV Configuration | 660346 |
| BD FACSCelesta™ Flow Cytometer, BV Configuration | 660343 |

## Ordering information: Reagents

| Description | Cat. No. |
|---|---|
| BD IMag™ Human T Lymphocyte Enrichment Set – DM | 557874 |
| BD Horizon™ CFSE | 565082 |
| BD GolgiPlug™ Protein Transport Inhibitor (Containing Brefeldin A) | 555029 |
| BD GolgiStop™ Protein Transport Inhibitor (Containing Monensin) | 554724 |
| BD Horizon™ BV421 Mouse Anti-Human CD107A | 562623 |
| BD Horizon™ APC-R700 Mouse Anti-Human CD8 | 565166 |
| BD Horizon™ BV510 Mouse Anti-Human CD3 | 563109 |
| BD Horizon™ BV786 Mouse Anti-Human CD45RO | 564290 |
| BD Pharmingen™ Alexa Fluor® 647 Mouse Anti-Human CD197 (CCR7) | 560816 |
| BD Horizon™ Fixable Viability Stain 780 | 565388 |
| BD Cytofix™ Fixation Buffer | 554655 |
| BD Perm/Wash™ Buffer | 554723 |
| BD Pharmingen™ PE Mouse Anti-Human IFN-γ | 559327 |

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