APPLICATION NOTE

# Identify and Select Optimal CAR T Cell Phenotypes with the Opto® Cell Therapy Development Workflow

## APPLICATION SPOTLIGHT

- Measure antigen-specific proliferation of a single CAR T cell using the T Cell Proliferation Assay
- Combine the T Cell Proliferation Assay with other functional assays to thoroughly characterize CAR T cells
- Correlate information generated using multiple assays to understand the mechanisms that drive optimal T cell behavior

## INTRODUCTION

Chimeric antigen receptor T cell (CAR T cell) therapy is an exciting form of immunotherapy due to its ability to use a patient's own genetically engineered T cells to recognize and eradicate cancer. Optimal CAR T cells should exhibit a less-differentiated memory phenotype, maintain proliferative capacity, exhibit polyfunctional cytokine secretion, and efficiently eliminate target cells. These phenotypes can be used to develop therapies where a few select T cells quickly destroy multiple target cells and persist in the patient to maintain long-term anti-tumor immunity.

To identify T cells exhibiting phenotypes associated with effective anti-tumor immunity, scientists must be able to assess T cell functions—such as antigen-specific proliferation—at the single-cell level to account for the inherent heterogeneity of T cell populations. However, many of the methods currently used to measure single-cell T cell proliferation are time-consuming and inefficient. More importantly, traditional methods cannot measure antigen-specific proliferation and then correlate that growth to other parameters such as cytokine secretion and cytotoxicity of the same cell. In addition, viability and expansion of single T cells isolated by FACS is often low to moderate, due to their sensitivity to hydrodynamic stress and/or resulting low-density culture conditions.

The Bruker Opto Cell Therapy Development workflow addresses these shortcomings. Run on the Lightning® or Beacon® optofluidic system, the workflow facilitates the thorough characterization required to develop effective T cell therapies and to understand the molecular mechanisms driving desired phenotypes. In a single experiment, multiple assays can be performed simultaneously or in series on more than one thousand individual T cells, enabling scientists to monitor cytotoxic activity and the secretion of multiple cytokines (polyfunctionality) for the same T cell. After characterization, single T cells can be recovered for genomic analysis or they can be expanded on the Bruker Platform to assess their proliferative potential.

In this application note, we demonstrate how the T Cell Proliferation Assay and the Multiplex Cytokine Assay in the Opto Cell Therapy Development workflow can be run in combination to identify single T cells that maintain

their proliferative ability while secreting one, two, or three cytokines simultaneously. We then demonstrate how tumor killing and proliferation can be correlated in the same cell and discuss how live cells of interest can be recovered for further genomic analysis.

## MATERIALS AND METHODS

### Generating and stimulating anti-CD19 CAR T cells

We cultured CD3+ T cells from healthy donor peripheral blood (STEMCELL Technologies) in T cell media containing Advanced RPMI 1640, 1X Glutamax, 10% human serum, and 50 µM β-mercaptoethanol (Thermo Fisher Scientific) that was supplemented with 10 ng/mL IL-7 and 10 ng/mL IL-15 (R&D Systems). We then activated cells with anti-CD3/CD28 beads (Dynabeads, Thermo Fisher Scientific) and performed viral transduction with anti-CD19 chimeric antigen receptor (CAR) lentivirus expressing the scFv of an anti-CD19 antibody linked to a CD28 transmembrane domain/endodomain and CD137 (4-1BB) and CD3-zeta signaling domains (Creative Biolabs). The vector also contained a truncated version of the epidermal growth factor receptor (EGFRt) that is coexpressed with the CAR and can be used to identify CAR-expressing cells. Four days after transduction, we harvested cells and stained them with a fluorescent antibody specific for EGFRt (R&D System). We then sorted EGFRt+ cells on an Aria II flow cytometer (Becton Dickinson) and expanded them for an additional 4 days before cryopreservation.

### Multiplex Cytokine Assay on the Lightning system

We loaded OptoSelect® 1500 chips with cytokine capture beads specific for IFNγ, TNF-α, and IL-2 (Biolegend) using Cell Analysis Suite (CAS®) 2.1 software and templates in the T Cell Multiplex Cytokine Assay Protocol (Bruker MAN-08058). We then loaded individual T cells into NanoPen® chambers followed by CD19-expressing Raji cells (CD19 WT). A subset of the chip was loaded with CD19-deficient Raji cells (CD19 KO) before the chip was perfused with T cell media overnight. We detected cytokines captured by the beads using a cocktail of biotinylated antibodies followed by PE-conjugated streptavidin (Biolegend). We then transferred chips to the Culture Station™ system and perfused T cell media for 5 days. We then detected expanded T cells using an APC-conjugated anti-CD3 antibody (Biolegend) and analyzed resulting data using Assay Analyzer 2.1 and Image Analyzer 2.1 software.

### Cytotoxicity Assay and Proliferation Assay on the Lightning system

We loaded single anti-CD19 CAR T cells and CFSE+ CD19 WT or CD19 KO Raji cells onto an OptoSelect 1500 chip using CAS 2.1 and templates in the T Cell Cytotoxicity Protocol (Bruker MAN-08103). We tracked target cell killing by co-culturing cells under constant perfusion of T cell media containing 5 µM NucView 530 Caspase-3 Substrate (Biotium) and imaged each NanoPen chamber every 30 minutes in FITC and PE. After overnight incubation, we transferred chips to the Culture Station system and perfused T cell media for 5 days. We detected expanded T cells using a PE-conjugated anti-CD8 antibody (Biolegend) and analyzed resulting data using Assay Analyzer 2.1, Image Analyzer 2.1 software, and Tableau.

## RESULTS AND DISCUSSION

### Observing antigen-specific CAR T proliferation

We loaded single anti-CD19 CAR T cells onto an OptoSelect chip, one T cell per NanoPen chamber. The cells were stimulated with either CD19 wild-type (WT) Raji cells or CD19 knockout (KO) Raji cells as a negative control (**Figure 1A**). After 5 days of culture on a Culture Station instrument, we identified expanded cells using brightfield imaging. We then stained the expanded cells with fluorescent anti-CD3 antibody to discriminate between the growth of CD3+ stained T cells and unstained Raji cells (**Figure 1B** and **C**).

When we quantified CD3+ T cell growth using Image Analyzer software, we found that 28% of the NanoPen chambers contained proliferating T cells after incubation with CD19-expressing tumor cells; only 5% of the NanoPen chambers contained proliferating T cells after incubation with CD19 KO cells (**Figure 1D**). This confirms that the expansion we observed is predominantly antigen-specific. A few NanoPen chambers contained growing cells that were CD3 negative colonies, indicating that tumor cells expanded.

### Correlating antigen-specific cytokine secretion with

**proliferation**

Understanding whether a T cell is monofunctional (i.e., secretes one cytokine in response to an antigen) or polyfunctional (i.e., secretes multiple cytokines) is key when developing therapeutics because polyfunctional cells are known to be more effective at clearing tumor cells[2]. An ideal T cell should also maintain its proliferative capacity, providing long-term anti-tumor protection. The Opto Cell Therapy Development workflow allows users to profile both antigen-specific cytokine secretion and proliferation on the same cell.

We loaded an OptoSelect chip with cytokine capture beads coated with antibodies against IFNγ, TNF-α, and IL-2. Next, single anti-CD19 CAR T cells were loaded and stimulated with either WT or CD19 KO Raji cells (Figure 2A). Cytokine secretion was evaluated to identify T cells producing one, two, or three cytokines in response to antigenic stimulation[3] (Figure 2B). The chips were subsequently transferred to the Culture Station system where cells were cultured on-chip for 5 additional days and then imaged to identify the NanoPen chambers that contained proliferating cells. CD3 staining was also performed to discriminate proliferating T cells from tumor cells. In the representative chambers shown in

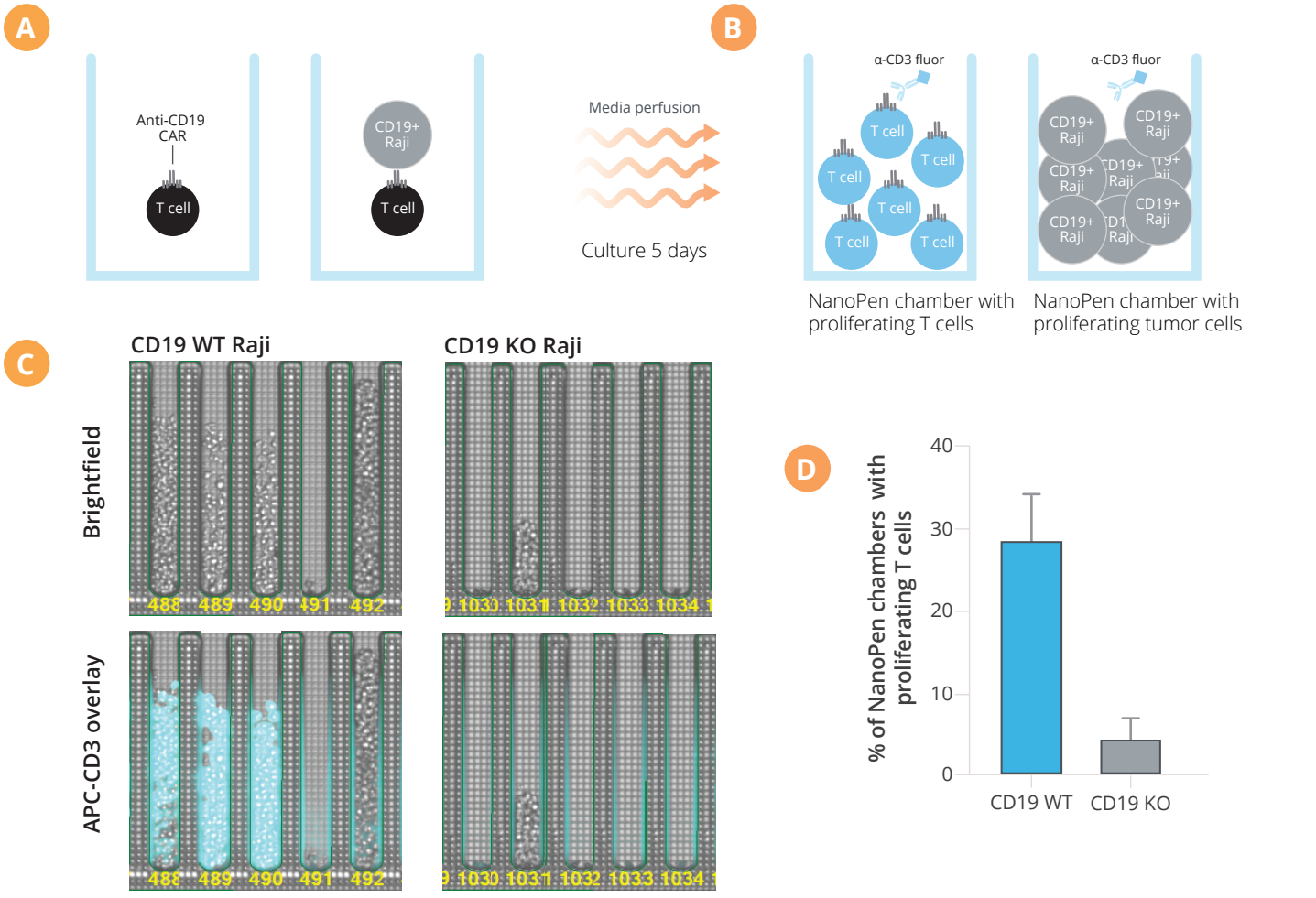


**Figure 1. The Opto Cell Therapy Development workflow enables antigen-specific proliferation of single T cells. A.** Schematic showing the stimulation of anti-CD19 CAR T cells with CD19-expressing Raji cells. **B.** Schematic showing how proliferating T cells are differentiated from tumor cells using anti-CD3 antibody staining (blue). **C.** Representative images of chambers containing T cells incubated with CD19 WT (left) or CD19 KO (right) Raji cells. Cell-surface staining of CD3 is used as a marker for T cells (blue). **D.** 29% of T cells loaded with CD19 WT Raji cells proliferated compared to less than 10% of those loaded with CD19 KO Raji cells after 5 days.

Figure 2B, we observed cytokine secretion in three out of four NanoPen chambers, indicated by yellow staining of cytokine beads, with NanoPen 348 exhibiting no cytokine secretion. Interestingly, T cell proliferation was observed in the NanoPen chambers where cytokine secretion was detected, while only tumor cells expanded in NanoPen 348 (Figure 2C).

This proliferation data was correlated with cytokine secretion data to identify which CAR T cells secrete one or more cytokines while maintaining antigen-specific proliferative capacity (Figure 2D). We found that cells secreting no cytokine and those secreting only TNF-α or IL-2 exhibited significantly less proliferation, as compared to those secreting IFNγ only or various combinations of the three cytokines. This type of nuanced analysis of T cell function may enable the development of cell therapies with cytokine secretion and proliferation profiles that are precisely tailored to the tumor microenvironment.

## Directly linking cytotoxicity to antigen-specific

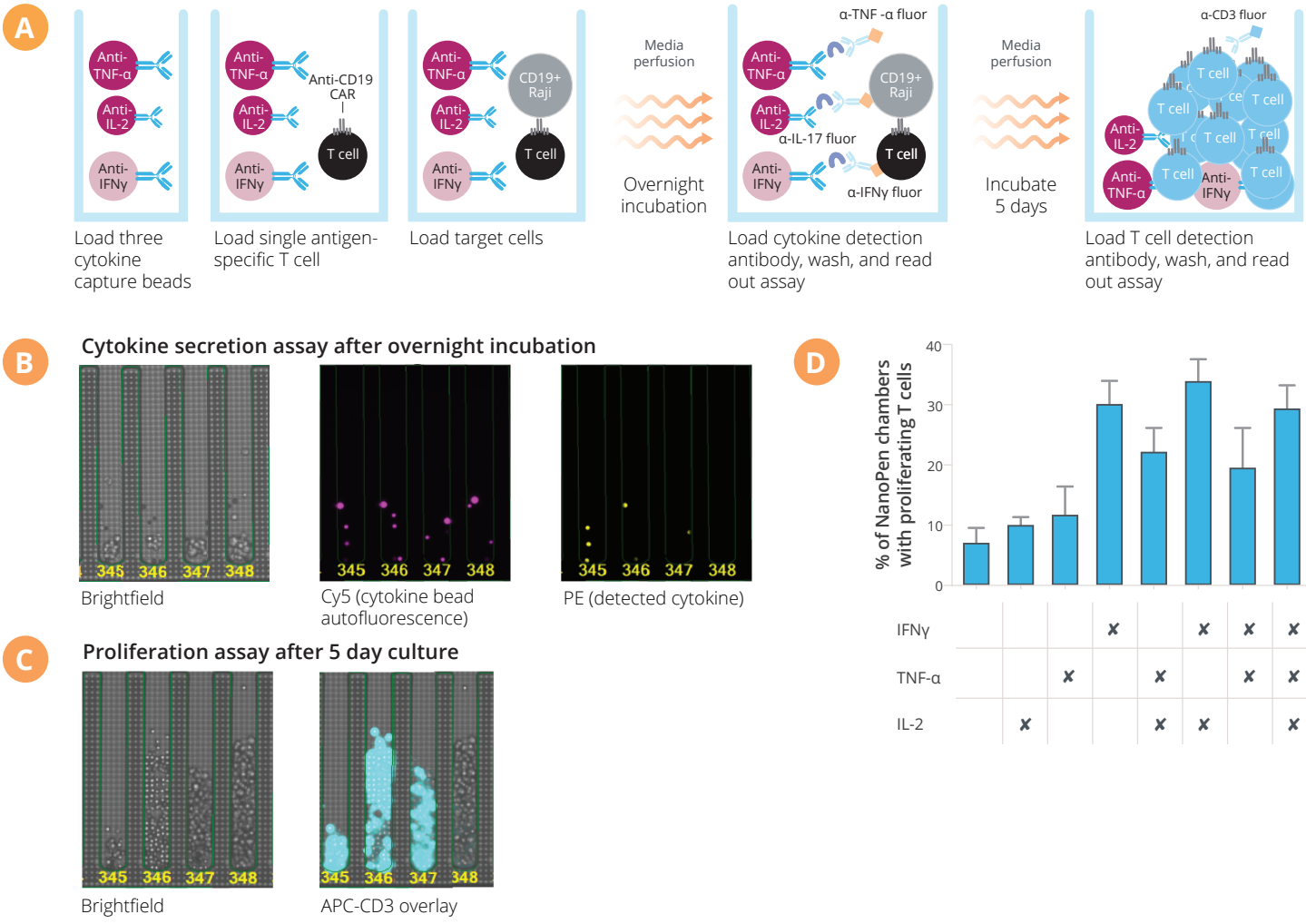


**Figure 2. The Multiplex Cytokine Assay and T Cell Proliferation Assay identify individual T cells that secrete IFNγ, TNF-α, and/or IL-2 and proliferate in response to antigenic stimulation. A.** Schematic showing three cytokine beads (magenta), single anti-CD19 CAR T cells, and Raji cells loaded into individual NanoPen chambers. Cytokine production is measured after overnight incubation by staining with fluorescent antibodies (yellow). After 5 additional days of incubation, proliferating T cells were identified by staining with a fluorescent anti-CD3 antibody (blue). **B.** Representative NanoPen chambers loaded with three cytokine beads (magenta) exhibiting cytokine production (yellow dots) in NanoPen chambers 345, 346, and 347. **C.** Same representative NanoPen chambers exhibiting T cell proliferation (blue cells). **D.** A greater percentage of T cells secreting TNF-α, IFNγ and IL-2, and all three cytokines proliferated than the other subpopulations.

**proliferation**

In our final experiment, we ran the Cytotoxicity Assay and T Cell Proliferation Assay on the same cells to identify CAR T cells with both tumor-killing and proliferative capacity (Figure 3A). Fluorescently labeled Raji target cells (green) were incubated with single anti-CD19 CAR T cells for approximately 16 hours. Fluorescent time-lapse imaging was performed during the culture and apoptosis was monitored using a caspase-3 substrate that fluoresces when caspase-3 is activated (red). After 5 additional days of culture on a Culture Station instrument, the cells were stained with a fluorescent anti-CD8 antibody to discriminate between CD8+ T cell growth (blue) and Raji cell growth (unstained). Images of a representative NanoPen chamber throughout this workflow are shown in Figure 3B. By quantifying and correlating data from both the Cytotoxicity and T Cell Proliferation Assays, we were able to identify subsets of T cells exhibiting different combinations of these functions (Figure 3C). We found that a very small subset of T cells exhibited both proliferation and target cell killing within the 16 hours that apoptosis was monitored, making these cells the most interesting subpopulation for further study.

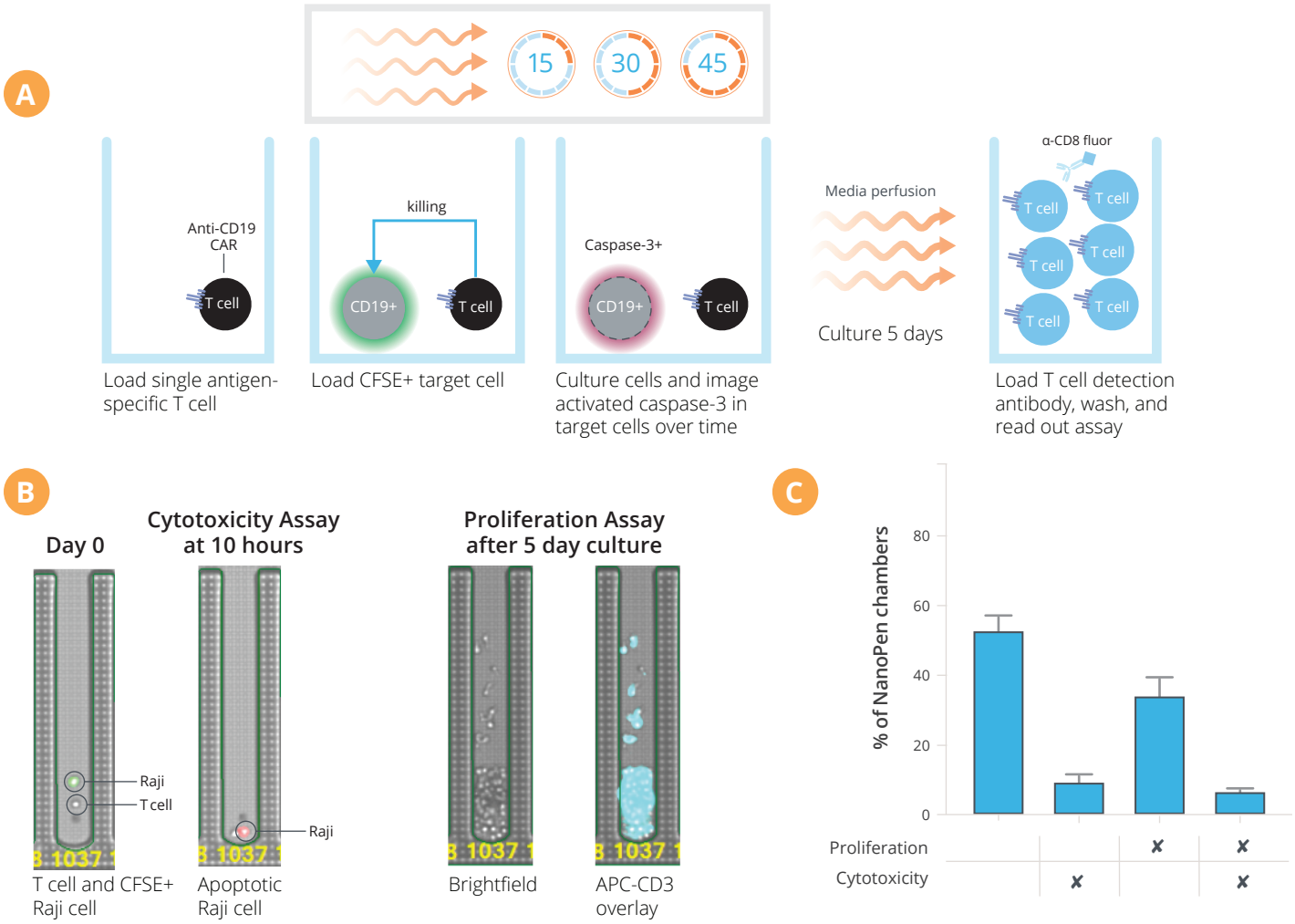


**Figure 3. The Cytotoxicity Assay and T Cell Proliferation Assay can be combined to identify individual T cells that effectively eliminate target cells and proliferate after antigenic stimulation. A.** Schematic showing single anti-CD19 CAR T cells and CFSE+ Raji (green) cells loaded into NanoPen chambers. Apoptosis of Raji cells is measured by perfusion with a fluorescent caspase-3 substrate. Cells with activated caspase-3 fluoresce in the PE channel (red). After 5 additional days of incubation, proliferating T cells were identified by staining with a fluorescent anti-CD8 antibody (blue). **B.** Representative NanoPen chamber showing T cell and Raji cell (green) at the start of culture. After 10 hours, activated caspase-3 is detected in Raji cell (red). After 5 days of culture, proliferating T cells are observed in brightfield and by staining with fluorescent anti-CD8 antibody (blue). **C.** A profile of our T cells shows that nearly 60% neither killed target cells nor proliferated; approximately 10% were able to do both.

### Correlating functional phenotype with gene expression

Because none of the assays described here are destructive, cells that have been identified as promising therapeutic candidates can be exported alive for additional downstream analysis. For example, cells of interest can be exported into a well plate and further processed for transcriptome sequencing, allowing scientists to link behaviors like antigen-specific proliferation, cytotoxicity, and cytokine secretion of an individual T cell to its gene expression.

## CONCLUSION

We have demonstrated how a T Cell Proliferation Assay can be run in combination with either the Multiplex Cytokine Assay or the Cytotoxicity Assay to identify cells that exhibit antigen-specific expansion as well as other desirable functions.

Adoptive T cell therapies have shown greatest clinical efficacy when the transferred T cells exhibit cytokine polyfunctionality, efficient target cell killing, and proliferation in response to antigens, leading to persistence of the transferred cell. However, many methods for defining T cell phenotypes fail to measure multiple parameters on individual T cells, forcing scientists to draw conclusions about samples that can be skewed by the extreme heterogeneity usually observed in bulk T cells measurements. The Bruker Opto Cell Therapy Development workflow is the only commercially available method that enables measurement of multiple functional parameters on a single cell and enables correlation of the functional phenotype of that same individual T cell with its gene expression. The flexibility of the workflow allows assay combinations that provide a comprehensive view of T cell behavior. The Opto Cell Therapy Development workflow has the ability to accelerate T cell-mediated therapeutic development by providing the most relevant information needed to identify and maintain optimal T cell phenotypes.

## REFERENCES


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3. Profile and Recover Polyfunctional T Cells Using Multiplexed Cytokine Secretion Assays. *Bruker Application Note*, 2020.


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**brukercellularanalysis.com**


BRUKER

5858 Horton Street | Suite 320 | Emeryville, CA 94608

**i+1-510-858-2855**
**brukercellularanalysis.com**