APPLICATION NOTE

Directly Link T Cell Phenotype and Function to Genotype with the Opto™ Cell Therapy Development 1.0 Workflow

APPLICATION SPOTLIGHT

- Correlate cytokine secretion to target cell killing behavior in CAR-mediated antigen recognition
- Discriminate CAR-T cell subsets based on kinetics of target cell killing
- Link cytokine secretion and target cell killing behavior to TCR sequence in TCR-mediated antigen recognition

INTRODUCTION

T cell-based therapies have shown great promise for cancer treatment. However, developing these therapies is challenging because T cell-mediated tumor death relies on complicated cell-cell interactions and several complex mechanisms that depend on the perforin/granzyme pathway, cytokine-dependent killing and interactions between T cell-expressed Fas and tumor cell-expressed Fas Ligand (Fasl)[1]. Additionally, these therapies have been associated with significant side effects related to cytokine release syndrome (CRS) and neurotoxicity, some of which are associated with the intensity of T cell anti-tumor functions like cytokine release and killing kinetics[1,2]. Ideally the dose of T cell therapies would be tailored to mediate the rapid destruction of tumor cells while avoiding high levels of cytokine release, an effect that would be facilitated by relatively low numbers of T cells quickly killing multiple target cells.

Various techniques are currently used to assess T cell function in order to create more effective therapies, including bulk and single-cell RNA-Seq to measure gene expression; flow cytometry, live-cell imaging, and chromium release to assess target cell killing; and intracellular cytokine staining, ELISA, and ELISPOT to measure cytokine expression. However, these techniques only allow scientists to draw correlative rather than causative conclusions because it is impossible to collect all of the required data from the same individual cell. For example, it is not possible to determine whether a specific cytokine-secreting cell can kill tumor targets or link this behavior directly to gene expression or genotype. This is particularly relevant in TCR-mediated therapies since the TCR sequence and associated affinity for antigen is directly linked to the degree of cytokine secretion and kinetics of target killing[3]. Standard assays also make it challenging to discriminate between killing behaviors, such as multiple T cells killing multiple targets and single T cells capable of serial killing. They also make it difficult to assess key killing characteristics such as length of time in conjugation with tumor cells[1].

In this application note, we demonstrate how the Opto Cell Therapy Development 1.0 workflow on the Lightning™ system was used for CAR-T cell phenotypic and functional screening as well as the discovery of TCRs associated with specific T cell behaviors. The

ability to screen thousands of individual T cell target cell interactions in a single experiment, followed by live cell export for downstream analysis of T cells of interest (Figure 1) will enable scientists to accelerate the development of efficacious cancer therapies.

T Cell Analysis capabilities

The Opto Cell Therapy Development 1.0 workflow is a collection of software capabilities, reagents and protocols that allow scientists to define and test the function of individual T cells. Individual T cell-target cell interactions are precisely assembled in thousands of nanoliter-sized NanoPen™ chambers across an OptoSelect™ microfluidic chip using light in a process called optoelectropositioning (OEP™). T cells can be selectively penned based on fluorescent cell surface markers and co-cultured with target cells and IFNγ capture beads (Figure 2A-C). Timelapse brightfield and fluorescence imaging is then used to assess T cell function (IFNγ secretion) and killing activity (as assessed by caspase-3 activation in target cells) (Figure 2D-E). Importantly, individual T cells of interest are exported for downstream analysis such as sequencing (Figure 2F).

EXPERIMENTS AND DATA

Generating anti-CD19 CAR-T cells

We purchased healthy donor peripheral blood CD3+ T cells from StemCell Technologies and cultured these cells in T cell media containing Advanced RMPI 1640, 1X Glutamax, 10% human serum, and

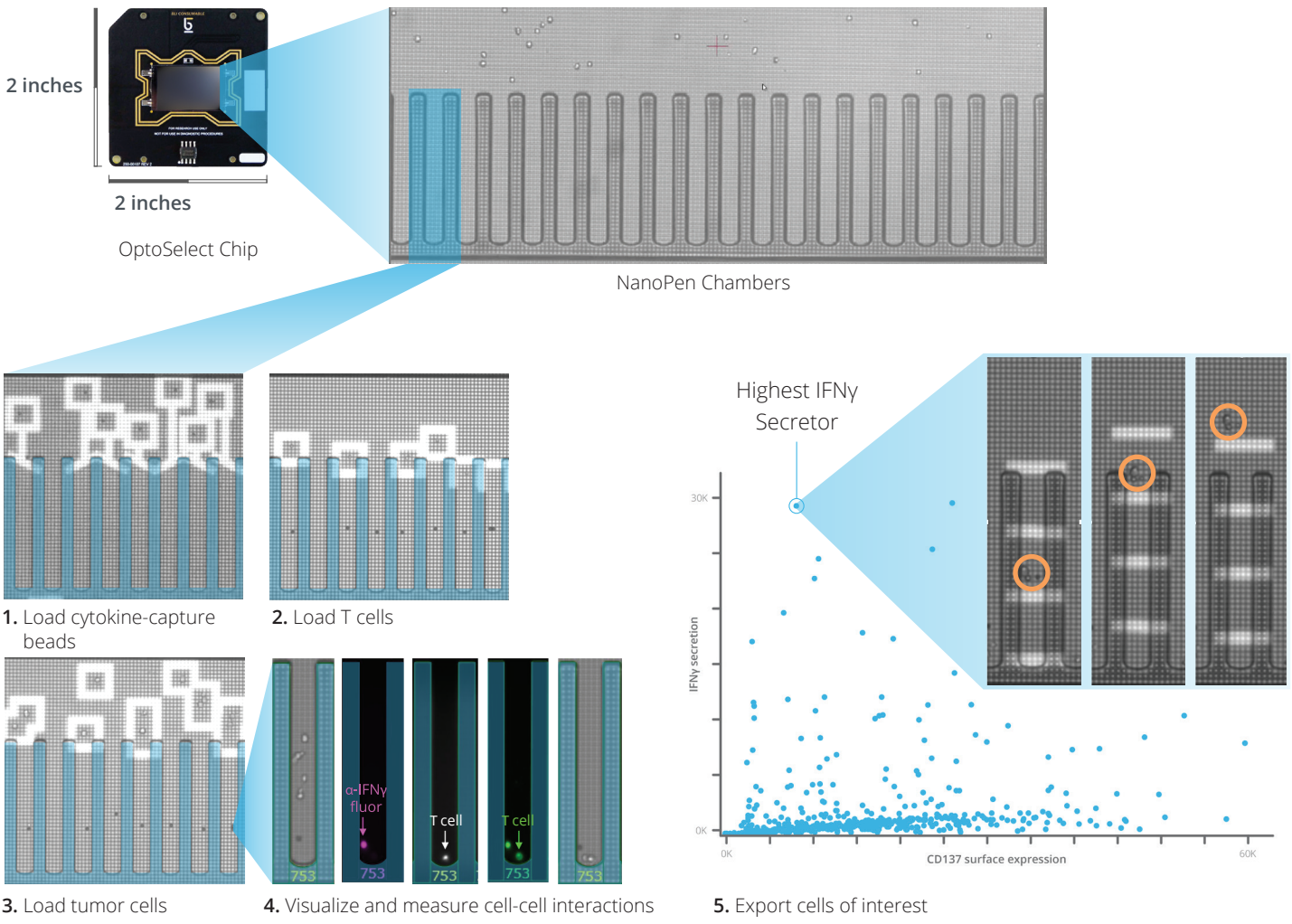
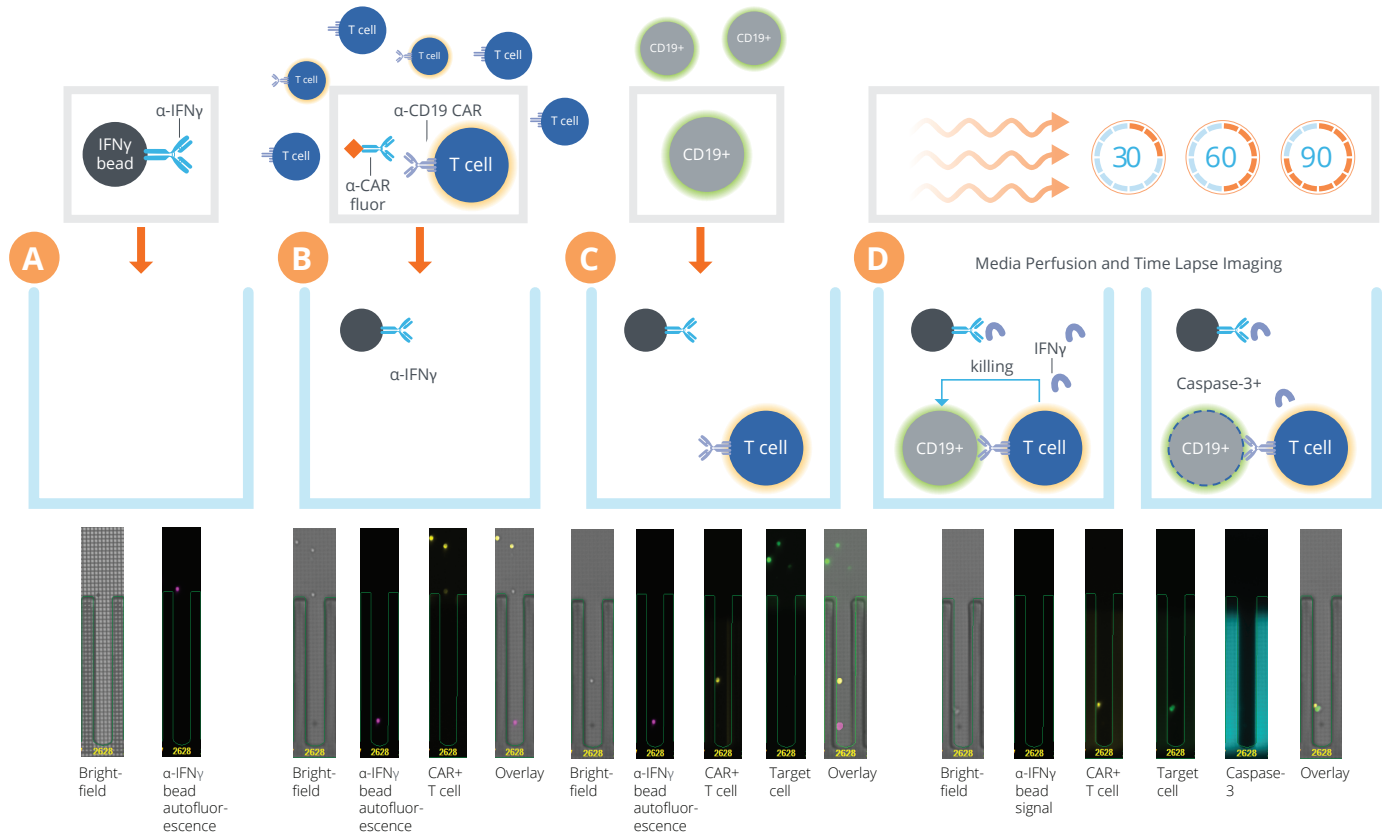


Figure 1. Assembly, visualization and characterization of individual cell-cell interactions and recovery of live cells of interest.



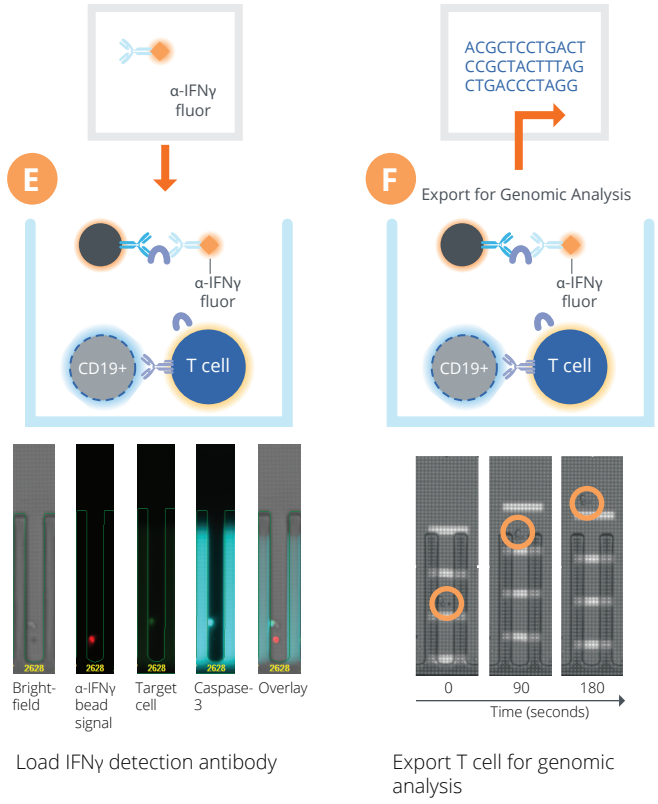


Figure 2. Assay set-up to link phenotype to function and genotype
A. Images from representative pen show loading of α-IFNγ capture beads, which are fluorescent in the Cy5 channel (magenta). **B.** Anti-CD19 CAR+ T cells are identified by fluorescence in the PE channel (yellow) and are selectively loaded into NanoPen chambers. **C.** CFSE+ Raji channels are identified by fluorescence in the FITC channel (green) and selectively loaded into NanoPen chambers with T cells and cytokine capture beads. **D.** T cells and target cells are co-cultured and perfused with cell culture media containing a fluorescent reporter of caspase-3 activity. Fluorescent images are captured every 30 minutes over 16 hours. **E.** Chip is incubated with PE-conjugated α-IFNγ antibody. Target cells which have activated caspase-3 are identified as DAPI+ (blue). **F.** Cells are exported for further analysis.

50 µM β-Mercaptoethanol (Thermo Fisher Scientific), supplemented with 25 ng/mL IL-7 and 10 ng/mL IL-15 (R&D Systems, Bio-Techne). Cells were activated for 3 days with anti-CD3/CD28 beads (Dynal beads, Thermo Fisher Scientific) at a ratio of 1 bead : 1 cell. To generate CAR cells, we then used an anti-CD19 chimeric antigen receptor (CAR) lentivirus expressing the scFv of anti-CD19 antibody linked to a CD28 transmembrane domain/endodomain, and CD137 (4-1BB) and CD3-zeta signaling domains (Creative Biolabs) for viral transduction. To allow us to easily identify and enrich for CAR-expressing cells the vector also contained a truncated version of the epidermal growth factor receptor (EGFRt) that is coexpressed with the CAR. Viral particles were mixed with cells with a multiplicity of infection (MOI) of 10 with 5 µg/mL polybrene (Sigma-Aldrich). Cells were centrifuged for 2 hours at 800g. After 7–10 hours, we diluted virus-containing media 6-fold with fresh media. After 4 days, we enriched for CAR expressing cells by sorting EGFRt+ cells on an Aria II flow cytometer (Becton Dickinson) after staining with a fluorescent antibody specific for EGFRt (R&D Systems, Bio-Techne). Sorted T cells were expanded for 13 days on irradiated feeder cells and cryopreserved. One day prior to the experiment, we thawed cells and incubated them in T cell media overnight. On the day of the experiment, we harvested and stained cells with fluorescent antibody specific for EGFRt (R&D Systems, Bio-Techne).

Generating FITC+ CD19+ and FITC+ CD19- target cells

To knockout CD19, we treated CD19+ Raji cells (ATCC) with CRISPR plasmids from Santa Cruz Biotechnology and expanded CD19- clones in Raji media containing Advanced RMPI 1640, 1X Glutamax, 10% Fetal Bovine Serum, and 50 µM β-Mercaptoethanol (Thermo Fisher Scientific). One day prior to the experiment, we labeled CD19+ and CD19- Raji cells with 1.25 µM CellTrace CFSE (Thermo Fisher Scientific) and incubated them in Raji media overnight.

Generating melanoma antigen-specific T cells

To generate melanoma antigen-specific T cells, we isolated naïve, CD8+ T cells from HLA-A2:01 donor peripheral blood mononuclear cells (STEMCELL Technologies) and stimulated them with autologous dendritic cells (DCs) that had been pulsed with an antigenic peptide from the melanoma-associated protein SLC45A2. T cells were cultured with DCs for two weeks. We then sorted and expanded tetramer+ cells as described above after harvesting and staining with a fluorescent tetramer targeting SLC45A2-specific T cells.

CAR-T chip experiments on the Lightning system

To assess CAR-T function, we loaded our OptoSelect chips onto the Lightning system and primed and loaded them with IFNγ capture beads using OEP. T cells and Raji cells were resuspended in T cell media containing our proprietary Loading Reagent. We assembled individual T cell-target cell interactions by selectively placing single EGFRt+ T cells and CFSE+ CD19+ or CD19- Raji cells into NanoPen chambers containing cytokine capture beads. 75% of NanoPen chambers were loaded with CD19+ Raji cells, while the remaining pens were loaded with CD19- Raji cells. We precisely tracked T cell and target cell phenotype and killing by co-culturing cells and beads with constant perfusion of T cell media containing 5 µM NucView 405 Caspase-3 Substrate (Biotium) and imaging pens every 30 minutes in all fluorescent channels. After overnight incubation, we directly measured secreted IFNγ captured by IFNγ capture beads by incubating chips with a PE-conjugated antibody against IFNγ (Berkeley Lights) and imaging in all fluorescent channels. Data were analyzed using Berkeley Lights software and Tableau.

TCR-T chip experiments on the Lightning system

To assess TCR function we loaded SLC45A2-specific T cells into NanoPen chambers containing IFNγ beads. CFSE-labeled T2 cells were pulsed with SLC45A2 peptide and loaded into 75% of the pens, while T2 cells pulsed with an irrelevant peptide were loaded into the remainder of the pens. Chips were incubated, labeled, imaged, and analyzed as above. We then used our TCRseq Well Plate Kit to selectively export single T cells of interest into a well plate and prepared them for sequencing of the variable domain on an Illumina MiSeq System with 50 X 250 paired end reads.

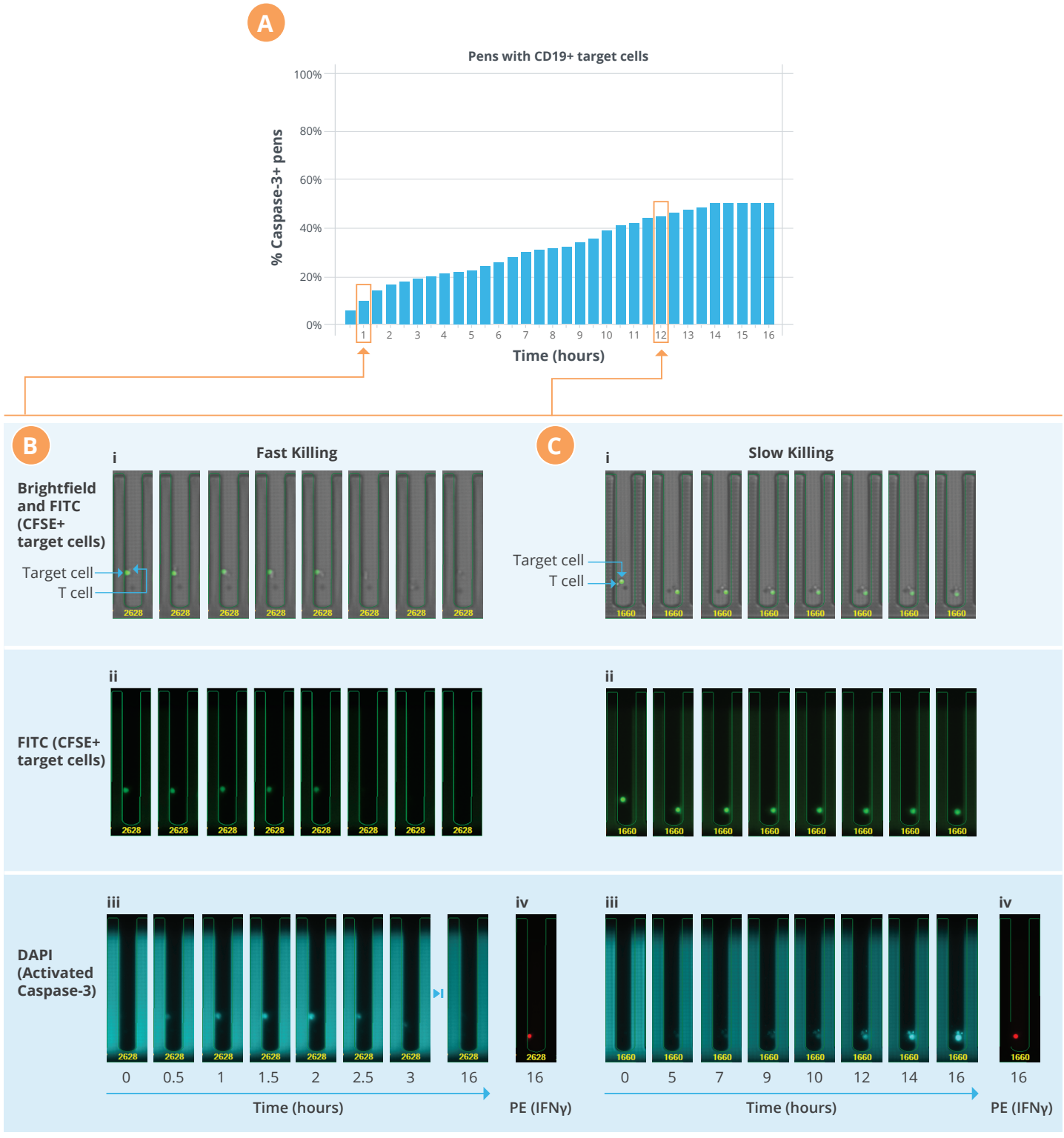


Figure 3. Direct assessment of CAR-T killing kinetics. Assay set-up to link phenotype to function and genotype A. Bar graph shows the cumulative percentage of caspase-3+ target cells over time in pens with CD19+ target cells. Measurements were made every 30 minutes for 16 hours. **B.** Images from a representative NanoPen chamber showing fast killing kinetics. *i.* Overlayed images in brightfield and FITC fluorescent channels over time course of culture. FITC+ cells are CFSE-labeled Raji cells and FITC- cells are T cells. *ii.* Images in FITC fluorescent channel over time. *iii.* Images in DAPI fluorescent channel over time. DAPI+ cells are those in which caspase-3 has been activated. *iv.* Image in PE channel after 16-hour culture. PE+ bead shows IFNγ captured on IFNγ capture bead over incubation time and stained with PE-conjugated anti-IFNγ antibody. **C.** Images from a representative NanoPen chamber showing slow killing kinetics. *i, ii, iii, and iv* have the same experimental setup as in B.

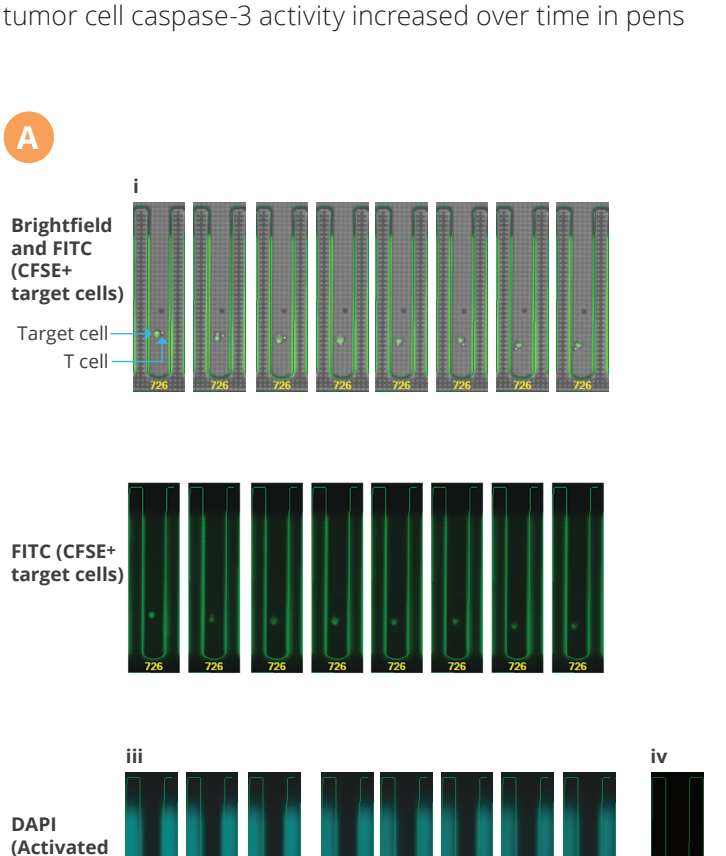
RESULTS AND DISCUSSION

Use case 1: CAR-T cell functional screening

We generated anti-CD19 CAR-T cells as described above and analyzed their phenotype and function using the Opto Cell Therapy Development 1.0 workflow on the Lightning system as outlined in Figure 2. We imaged cells at 30-minute intervals to assess tumor cell apoptosis as indicated by caspase-3 activation and directly measured IFNγ secretion using a PE-labeled anti-IFNγ antibody after overnight incubation.

As expected, the cumulative percentage of pens with tumor cell caspase-3 activity increased over time in pens loaded with CD19+ tumors, peaking at 50% tumor cell death after 16 hours of incubation (Figure 3A). This is in contrast to only 10% of pens displaying tumor cell death in control pens loaded with CD19- tumor cells; control pens also exhibited slower killing kinetics.

Importantly, the single-cell resolution of the OptoSelect microfluidic chip then enabled us to analyze each T cell-tumor cell interaction that contributed to these averaged killing data. We were able to directly compare differences in killing kinetics of individual T cells and link this tumor killing behavior to IFNγ secretion (Figure 3B-C). We identified fast-killing and slow-killing CAR-T cells in a single-day experiment, which could then be exported for



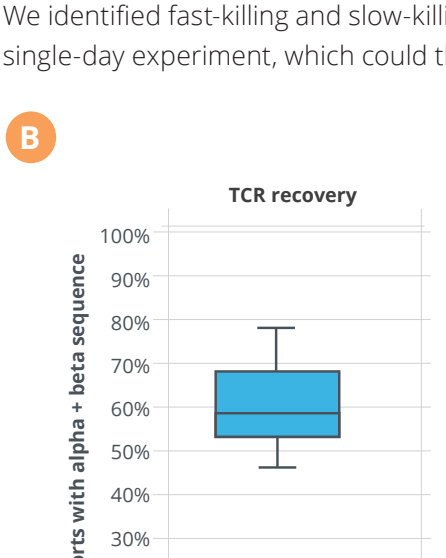


Figure 4. Directly linking T cell function to TCR sequence. A. Images from a representative NanoPen chamber showing TCR-dependent killing kinetics. *i.* Overlayed images in brightfield and FITC fluorescent channels over time course of culture. FITC+ cells are CFSE-labeled T2 cells and FITC- cells are T cells. *ii.* Images in FITC fluorescent channel over time. *iii.* Images in DAPI fluorescent channel over time. DAPI+ cells are those in which caspase-3 has been activated. *iv.* Image in PE channel after 16-hour culture. PE+ bead shows IFNγ captured on IFNγ capture bead over incubation time and stained with PE-conjugated anti-IFNγ antibody. **B.** Box and whisker shows percentage of exports in which alpha and beta TCR sequences were recovered. Upper extreme, upper quartile, mean, lower quartile and lower extreme are shown.

genomic analysis.

Use case 2: TCR recovery following functional assay

Using the same assay setup as above and the TCR-Seq capabilities of our Opto Cell Therapy Development 1.0 workflow, we analyzed killing kinetics of endogenous melanoma antigen-specific T cells co-cultured with tumor cells (**Figure 4A**) and exported cells of interest for downstream TCR sequencing. Using this approach and the Berkeley Lights TCRseq Well Plate Kit, we were able recover alpha and beta TCR sequences from an average of 60% of exported pens (**Figure 4B**). This capability enables direct linking of antigen-specific killing behavior to genotype in a single-day experiment. As a result, this assay facilitates the rapid identification of TCRs from antigen-specific T cells exhibiting varied functions and behaviors, as assessed by cytokine secretion and killing kinetics.

CONCLUSIONS

We demonstrated that the Opto Cell Therapy Development 1.0 workflow on the Lightning system can provide a direct link between T cell phenotype, function, and genotype in the context of different types of T cell based therapeutics. In particular, the NanoPen chambers and the workflow enabled us to segregate and track individual cells through time. This allowed us to assess and identify differences in engineered, CAR-mediated T cell killing kinetics as well as to recover TCR sequences that are directly associated with desired killing behavior of endogenous melanoma antigen-specific T cells. Importantly, these experiments were performed on more than a thousand of individual T cells in parallel in a single day and on a single system, enabling us to quickly draw actionable, causative conclusions about T cell function and biology. Such rapid and deep characterization has the potential to not only provide novel insights into T cell biology but also to accelerate the development of innovative, more efficacious cell therapies.

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