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Combining Live-Cell Analysis and High Throughput Flow Cytometry to Gain Additional Insights into the Mechanisms of Immune Cell Killing of Tumor Cells

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Introduction

The ability to exploit the power of the immune system to destroy cancer cells provides huge hope for improved cancer therapies. For the body to defend and fight against cancer, immune cells must recognize, engage, kill and ultimately remove unwanted tumor cells. For example, the administration of antibodies targeting immune checkpoints (PD-1 | PD-L1 and CTLA-4 pathway inhibitors) and cytotoxicity inducers (CD3xCD19 and Herceptin) prevent cancer cells escaping the immune system, increasing engagement (for review see Wei *et al,* 2018). Introduction of CAR-T cells, engineered to recognize and kill tumor cells offer vast potential, with recent regulatory approvals (for review see Strati and Neelapu, 2019). Understanding the processes and interactions at the cellular level is central to identifying and validating new targets and cellular therapy approaches. Flow cytometry has been used extensively to study immune cells, yielding information regarding cellular subtypes, activation status and viability using expression profiles and cell health markers. In recent years live-cell analysis has become an established method by which temporal and spatial information can be gained from the interactions and resultant killing of tumor cells. Combining the benefits of these methodologies offers the potential to gain additional insights into the mechanisms of immune cell killing.

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In this application note we describe how the iQue® 3 high throughput flow cytometer and the Incucyte® Live-Cell Analysis System can be used within the same workflow to deliver complementary data from a single assay plate. Coupling these platforms with their reagents, namely T Cell Activation Cell and Cytokine Kit and the Immune Cell Killing application, allows us to gain deeper insight than previously reported. Morphological insights of immune cell activation, tumor cell death and quantification of cell numbers can be elucidated using Incucyte® time-lapse live-cell imaging and analysis, while the iQue® 3 yields cytokine release information and subset analysis. Importantly, the real-time data analysis on the Incucyte® platform enables educated selection of time points for flow cytometric determinations. Validation of the workflow utilized activation of immune cells by CD3 | CD28 Dynabeads® to engage and destroy adherent and non-adherent cancerous target cells. To exemplify how different mechanistic approaches may yield differential profiles we compared and contrasted CD3 | CD28 immune cell activation with the inclusion of a bispecific T cell engager antibody (BiTE) targeting CD3xCD19 epitopes.

Overall, using these techniques in conjunction enables more informed experimental design, mechanistic insights and rapid progress to actionable results.

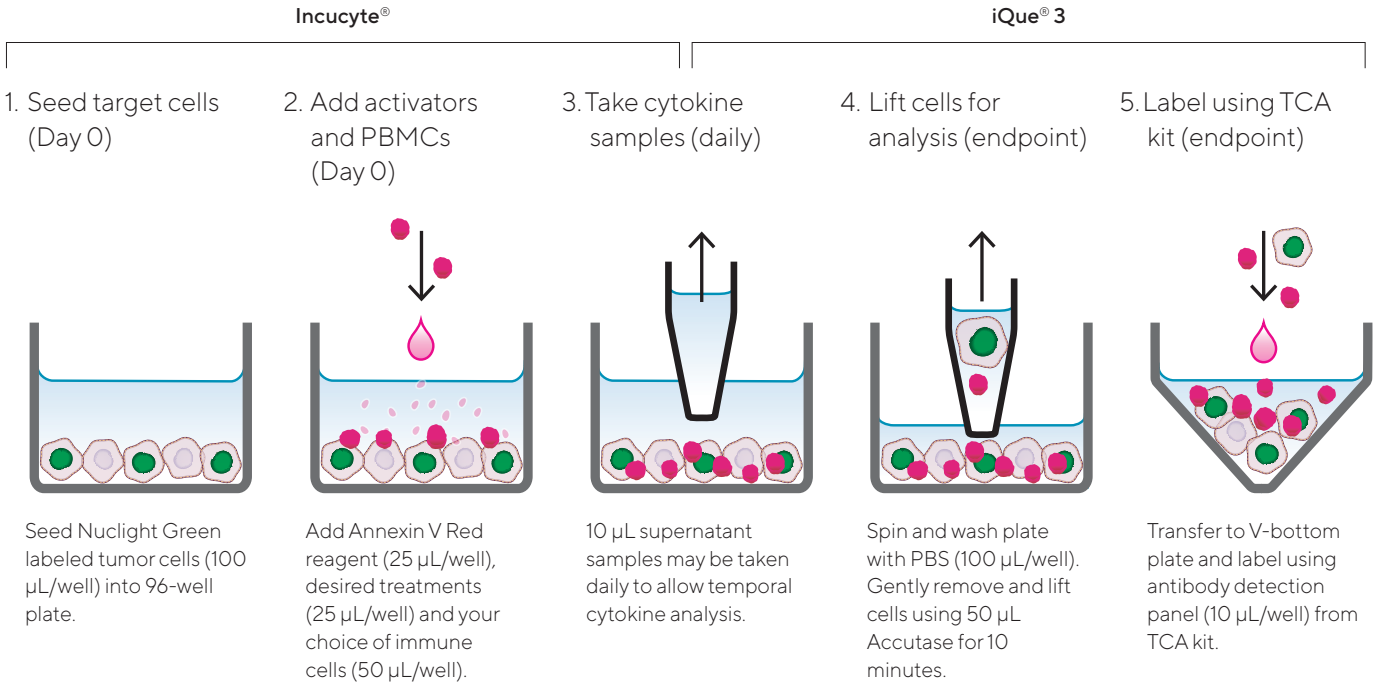

Figure 1: Schematic of the workflow enabling Incucyte® and iQue® 3 analysis from a single assay plate.

Materials and Methods

1. Seed Incucyte® Nuclight Green labeled target cells at an appropriate density into 96-well flat bottom plate. Note: Incucyte® Nuclight Red labeled cells are not compatible with the T Cell Activation Cell and Cytokine Profiling Kit (TCA kit #90560).
2. Add appropriate stimulants and activators (e.g., CD3 | CD28 Dynabeads®) to wells alongside an appropriate amount of effector cells (e.g., T cells, PBMCs). Different target-to-effector ratios should be tested (e.g., 1:5, 1:3). The cell death marker Incucyte® Annexin V Red can be used at this stage. Note: Incucyte® Annexin V Red will not interfere with TCA kit so can be used in conjunction.
3. Image cells in the Incucyte® at required time points. Cytokine samples can be collected (10 uL) each day or as necessary to run on iQue® 3 with the Qbeads® from the TCA kit (or Assay Builder: IFNγ option 1 and TNFα option 2).
4. Lift cells from flat bottom plate using Accutase, transfer to a V-bottom plate and label with antibodies from TCA Kit.
5. Once labeled, the plate can be run on the iQue® 3 with minimal alteration to the gating template provided with the TCA kit.

Validation of Combined Workflows

Compatibility of the iQue® 3 and Incucyte® workflows was demonstrated using a model of immune cell killing. Assay plates were prepared containing either SKOV-3 or Ramos target cells, stably expressing a nuclear targeted GFP (Incucyte® Nuclight Green), co-cultured with human PBMCs (1:5 effector ratio) to evaluate immune cell killing of tumor cells. Addition of CD3 | CD28 Dynabeads® at bead to effector cell ratios of 0.004:1 up to 1:1 induced immune cell activation. A single assay plate was monitored on the Incucyte® for 3–5 days. Every 24 h supernatant was removed for cytokine analysis using Qbeads® (Assay builder: IFNγ option 1 and TNFα option 2); this caused no perturbation or adverse effect on the cells. Once maximal killing had been observed cells were dissociated and evaluated using the iQue® 3 TCA kit antibody panel for identification of T cell subsets.

Morphological and Spatial Analysis

CD3 | CD28 Dynabeads® induced a marked morphological change in the PBMCs co-cultured with either Ramos or SKOV-3 target cells (Figure 2). At early time points the PBMCs were predominantly small and spherical in shape, however within 36 h a marked increase in size and elongation was observed. In contrast, under control conditions where CD3 | CD28 Dynabeads® were omitted, little or no morphological change was observed in the PBMCs.

Spatial observations also revealed marked clustering of the Ramos cells in the presence of activated PBMCs, alongside an increased association of target and effector cells. Similarly, inspection of the images taken from the plates containing activated PBMCs with the adherent SKOV-3 cells highlighted an increase in contact between the target and effector cells. These spatial effects were not observed when non-activated PBMCs were co-cultured with target cells.

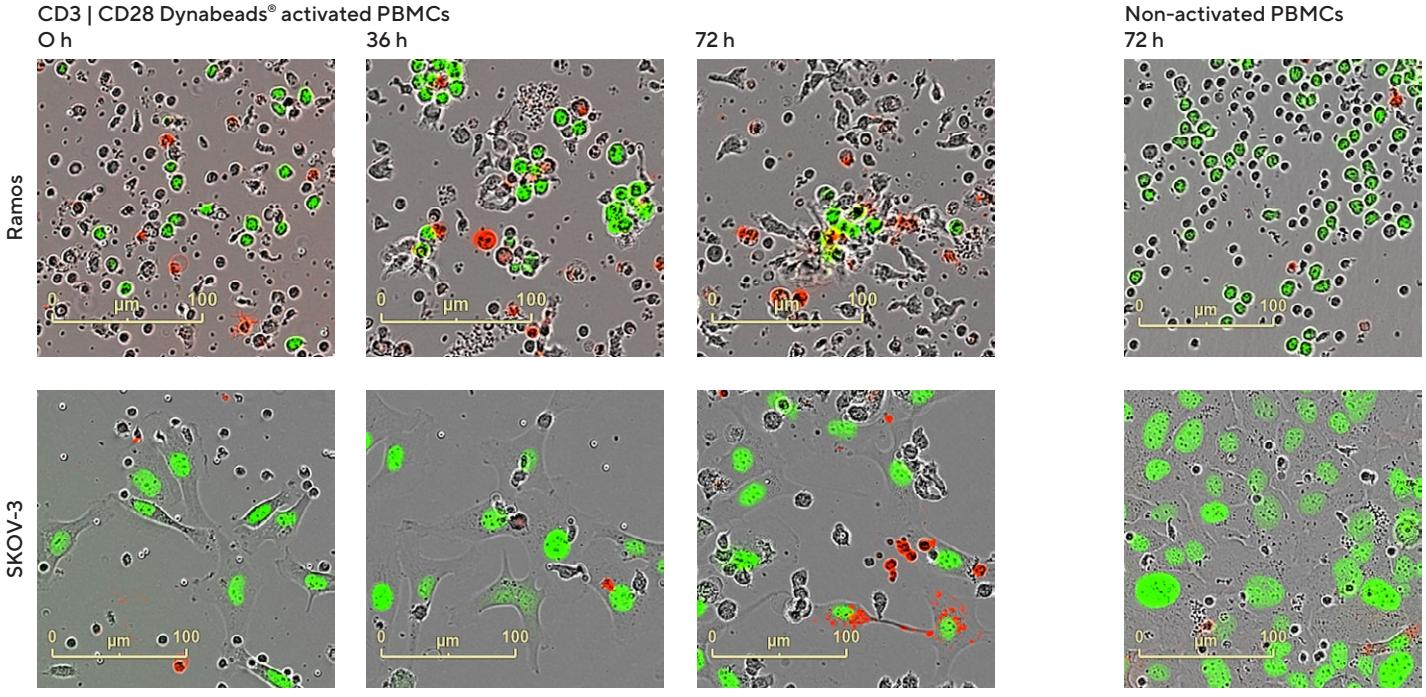

Figure 2: Representative images of immune cells (PBMCs) co-cultured with Ramos or SKOV-3 tumor cells. Ramos Nuclight Green cells (15K/well) or SKOV-3 Nuclight Green cells (4K/well) were seeded with PBMCs (1:5 Target to Effector) and activated with CD3 | CD28 Dynabeads® (1 bead to 1 cell). No beads were added to the non-activated controls. Incucyte® images were captured every 3 h using a 20X objective. Morphological changes of both immune cell and tumor cell can be visualized over time.

Quantification of Target and Effector Cell Numbers

Determination of the target cell number provides an indication of the degree of cytotoxicity elicited by the immune cells. Both the Incucyte® and iQue®3 systems are capable of enumerating target cells (Figure 3). Segmentation of the green nuclei using the Incucyte® integrated image analysis software enables the number of target cells per image to be quantified (Figure 3A and B). Dissociated cells passed through the iQue®3 platform enable gating based on size (forward scatter; FSC-A) and levels of green fluorescence (Figure 3C and D). As expected the largest target cell number was observed when cultured alone, with a small decrease in number when non-activated PBMCs were added. Inclusion of activated PBMCs induced high levels of cytotoxicity, markedly attenuating the target cell number (> 95%) compared to the SKOV-3 alone. Importantly, the trends of target cell enumeration were comparable across platforms, supporting the notion of compatibility of workflows.

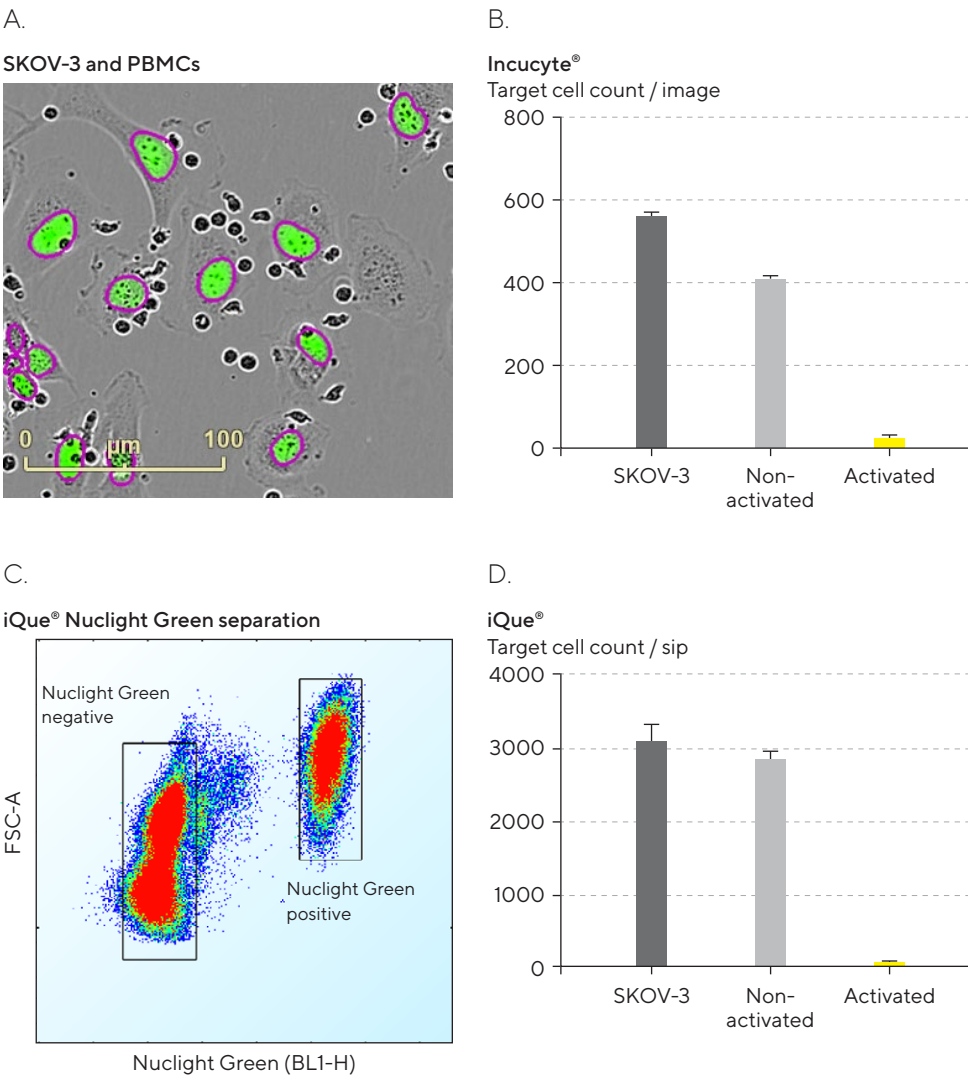

Figure 3: Target cell analysis yields comparable data across technologies. SKOV-3 Nuclight Green cells were seeded with PBMCs and activated with CD3 | CD28 Dynabeads® (1 bead to 1 cell). (A) Incucyte® basic analyzer was used to segment green nuclei of target cells (pink mask) from effector cells. (B) Incucyte® target cell quantification for SKOV-3 cells alone, in the presence of non-activated or activated PBMCs. (C) iQue®3 gating of target and effector cells using positive and negative green fluorescent populations. (D) iQue®3 target cell quantification for SKOV-3 cells alone, in the presence of non-activated or activated PBMCs.

Temporal Target Cell and Cytokine Analysis

Identifying temporal changes in the microenvironment using the iQue® 3 and relating these to the immune cell induced cytotoxicity visualized within the Incucyte® has the potential to offer deeper biological insight. Miniaturized volume requirements for the iQue® 3 enabled repeated non-perturbing supernatant sampling (10 µL, every 24 h) from the assay plate, thus allowing continued live-cell analysis. SKOV-3 Nuclight Green cells (4K/well) were co-cultured with PBMCs (10K/well) and activated with CD3 | CD28 Dynabeads® (41 to 10K beads/well) for 5 days, images were analyzed using Incucyte® software. Cytokine levels (IFNγ and TNFα) were quantified using the Qbeads® components from the T-Cell Activation Cell and Cytokine Profiling Kit (Reference related application for TCA application note.)

CD3 | CD28 activation elicited a marked concentration- and time-dependent increase in both cytokines (Figure 4A and B). IFNγ levels increased substantially after 24 h, reaching maximal levels after 96 h before plateauing, whereas the TNFα concentration increased more rapidly, reaching maximal levels after 72 h before declining. Cytokines above physiologically relevant levels (> 20 pg/mL) were observed at bead numbers greater than 1.1K/well.

From the same assay plate, proliferation of both effector and target cells were quantified using the Incucyte®. Under control conditions effector cell numbers decreased by 64 ± 0.1% after 120 h, as expected for non-activated PBMCs (Figure 4C). CD3 | CD28 activation induced a time- and concentration-dependent increase in effector cell numbers. Notably, conditions (1.1–10K beads/well) where marked effector cell proliferation was observed correlated to increased levels of cytokines. These increases were not observed until after 40–68 h. Under control conditions SKOV-3 target cells proliferated markedly, increasing from 45 to 550 cells/image as measured using the Incucyte® (Figure 4D). CD3 | CD28 activation yielded a time- and concentration-dependent reduction in SKOV-3 cell number; IC_{50} value of 1227 beads from AUC 0–120 h (data not shown). Little or no cytotoxic effects were observed until after 60 h, with the maximal amount of beads inducing near complete abolition (> 95% at 120 h) of the target cells compared to control.

Combining these datasets enabled mapping of an event timeline. As expected from previously performed studies utilizing independent methodologies, CD3 | CD28 activation yielded an initial rise in cytokine levels (within 2448 h), followed by proliferation of effector cells (post 40–68 h) and finally the death of target cells (post 60–72 h).

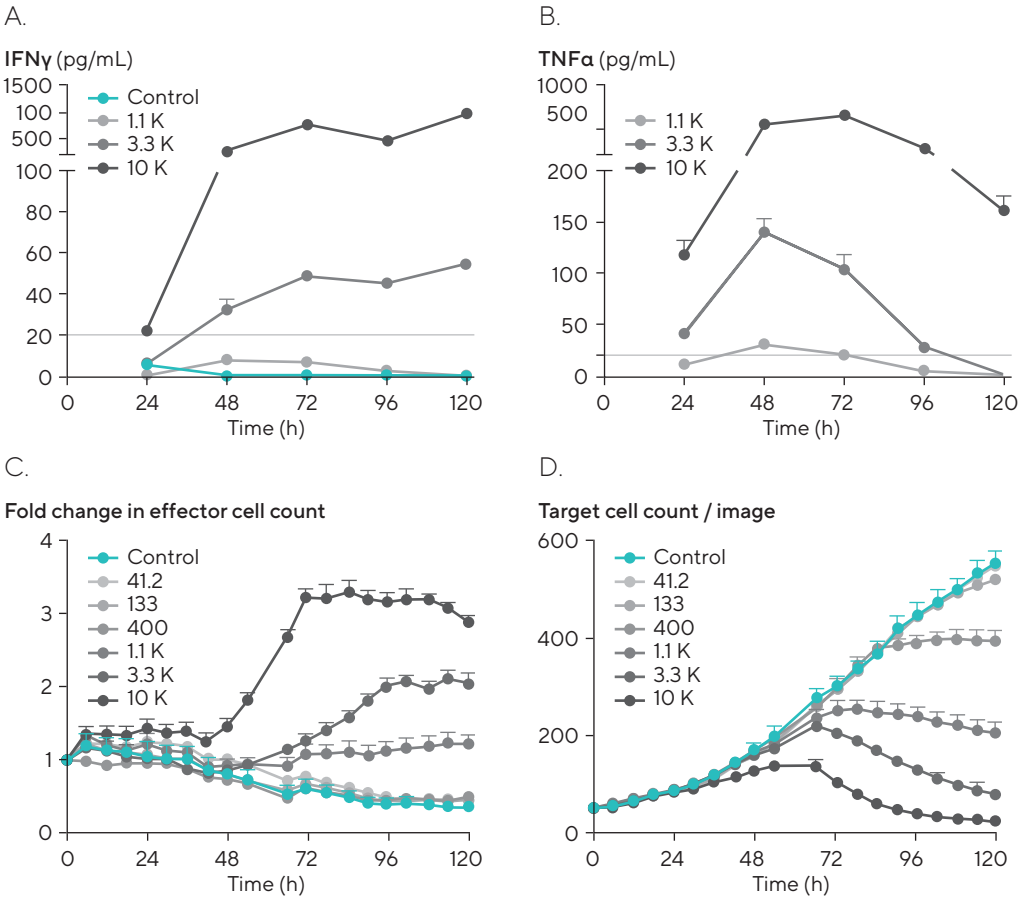

Figure 4: Temporal cytokine production is linked to target cell cytotoxicity. SKOV-3 Nuclight Green cells (4K/well) were seeded with PBMCs (10K/well) and activated with CD3 | CD28 Dynabeads® (41 to 10K beads/well). Temporal cytokine data for (A) IFNγ and (B) TNFα from 10 µL/day supernatant samples were quantified using Qbeads® components from the TCA kit (Qbeads® or Assay Builder: IFNγ option 1 and TNFα option 2). (C) Fold change in effector cell proliferation time-course analyzed using Incucyte®. (D) Target cell count time-course analyzed using Incucyte®.

Cellular Subset Analysis

Once immune cell killing has progressed to an appropriate point, as defined using the Incucyte® quantification of target cell number, the cellular contents of each well may be lifted and further analyzed using the iQue®3. Markers of early (CD69), mid (CD25) and late (HLA-DR) activation of the CD8+ and CD4+ population can be quantified using the TCA kit. CD69 and CD25 require T cell receptor (Tcr) stimulation alone, while HLA-DR needs additional co-stimulation of CD28 or antigen presenting cells. Data was compared for PBMCs obtained from two donors (Figure 5). When PBMCs from donor 1 were co-cultured with SKOV-3 cells, little or no cytotoxicity was observed under control conditions. However, the addition of CD3 | CD28 Dynabeads® induced a concentration-dependent killing of the target cells (Incucyte® data, not shown). For the non-activated controls, subset analysis revealed minimal levels of CD69, CD25 or HLA-DR positive cells in the CD8 positive population, consistent with no activation. CD3 | CD28 Dynabeads® evoked a concentration-dependent increase in all three markers yielding maximal population percentages of 13%, 94% and 26% for CD69, CD25 and HLA-DR, respectively. Determining the mid-point of activation yielded EC_{50} values of 916 beads for CD69, 1953 beads for CD25 and 1578 beads HLA-DR.

Studies for the second donor suggested pre-activation may have occurred. In the absence of CD3 | CD28 Dynabeads® notable levels of cytotoxicity of the target cells were observed (56% reduction in target cell number measured using the Incucyte®, data not shown). Similarly, the activation markers were elevated in the absence of exogenous stimulation; 13%, 12% and 10% for CD69, CD25 and HLA-DR in the CD8 positive population, respectively. Addition of Dynabeads® induced further increases in marker populations in a concentration-dependent manner, reaching levels notably higher than observed in donor 1 (maximal population percentages of 25%, 99% and 63% for CD69, CD25 and HLA-DR, respectively).

Evaluation of Target Cytotoxicity Induced by a CD3xCD19 Bispecific T Cell Engager (BiTE) Antibody

To exemplify the utility of combining both methodologies, we investigated target cytotoxicity induced by a CD3xCD19 BiTE antibody. BiTE antibodies, such as Blinatumomab, are being used clinically for treatment of B-lymphocytic leukemia alongside other late stage cancers (Ross *et al*, 2017). The CD3xCD19 construct, simultaneously engages the CD3 on T cells and the tumor associated antigen CD19 present on B cells. This interaction causes the clonal expansion and activation of T cells as well as direct contact between CD3+ T cells and CD19+ tumor cells, resulting in tumor specific cell lysis.

In order to model the therapeutic effects on B cell leukemia *in vitro*, an anti-hCD3xCD19 (Invivogen, cat #bimab-hCD19CD3) was evaluated alongside two control antibodies targeting CD3 alone (Ctrl Ab 1: anti-hCD3xβGAL) or CD19 alone (Ctrl Ab 2: anti-hCD19xβGAL). Data was generated from a single assay plate containing Ramos Nuclight Green cells co-cultured in the presence of PBMCs. Ramos cells were incubated briefly with the treatments before PBMCs were seeded. BiTE, or control antibodies at 10 ng/mL (5-fold serial dilution) or CD3 | CD28 Dynabeads® (75K/well, 3-fold serial dilution) were added. Images were captured every 3 h in the Incucyte®, supernatant samples were taken daily for cytokine analysis and endpoint subset analysis occurred at 72 h.

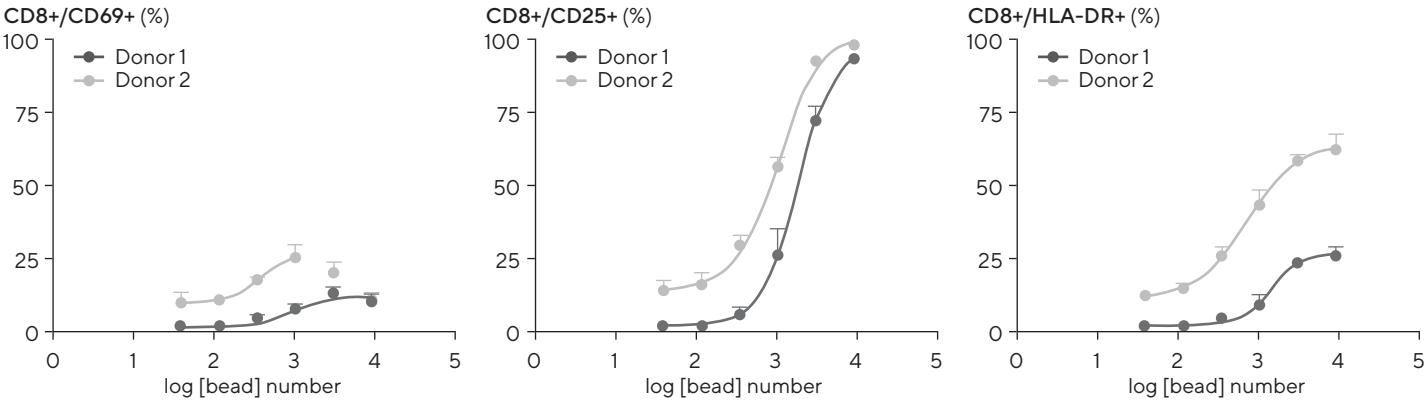

Figure 5: iQue®3 subset analysis highlights differences in activation of immune cells from different donors. SKOV-3 Nuclight Green cells (4K/well) were seeded with PBMCs (10K/well) from two donors and activated with CD3 | CD28 Dynabeads® (41 to 10K beads/well). Concentration-response curves show % expression of markers of early (CD69), mid (CD25) and late (HLA-DR) activation on CD8+ PBMCs. Quantification used the TCA kit components and was analyzed using the integrated Forecyt® software on the iQue®3.

CD3xCD19 BiTE Antibody Evokes Target Cytotoxicity with Temporal and Spatial Characteristics

Analysis of the green fluorescent integrated intensity using the Incucyte® platform enabled quantification of target cell death, while mitigating the impact of clustering of the Ramos B cells. In the absence of treatment (media alone) Ramos cells in the presence of PBMCs proliferated extensively (Figure 6A and C). Control Ab 1 (anti-hCD3xβGAL) induced little or no effect on the Ramos cell proliferation. Inclusion of the BiTE antibody elicited a concentration-dependent cytotoxicity of the Ramos cells (IC_{50} value of 0.09 ng/mL), with the highest concentration of BiTE Ab causing a 93% decrease in target cell viability compared to Control Ab 1 (Figure 6B, C and D).

Clustering and herding of non-adherent target cells by activated PBMCs is often observed (Figure 6A and B). In the presence of Control Ab 1 (Figure 6A, E and F) little or no clustering occurred. In contrast, the presence of the BiTE antibody caused the PBMCs to engage with target cells resulting in clear concentration-dependent clustering of cells, yielding an EC_{50} value of 0.12 ng/mL, similar in potency to the cytotoxicity effect (Figure 6B, E and F).

Note the PBMCs remain small and round in shape in the presence of the control antibody, whereas a marked increase in size and elongation is observed with the BiTE antibody. In addition, closer inspection of the images suggests additional interaction between BiTE-activated PBMCs with the target cells.

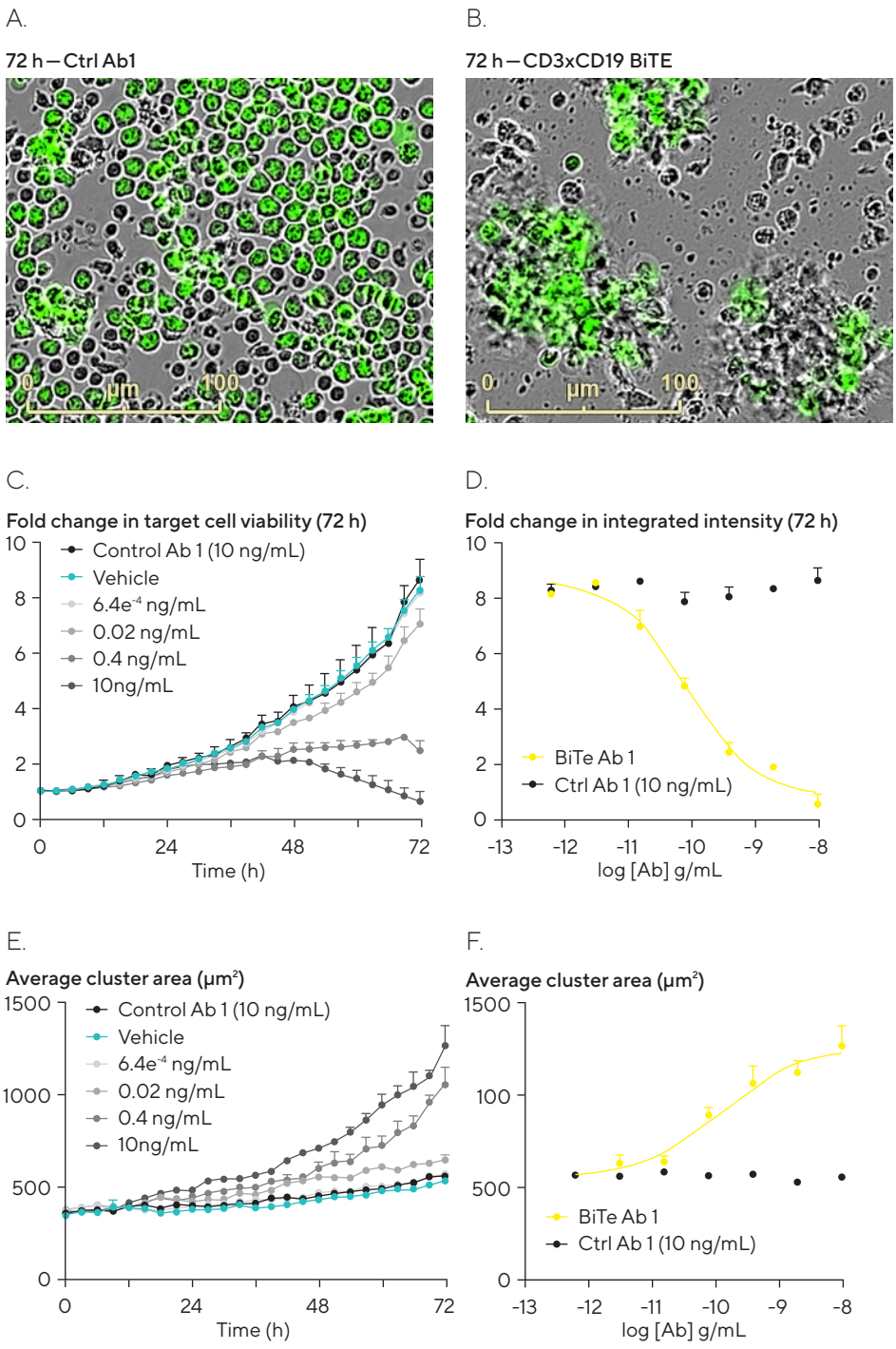

Figure 6: CD3xCD19 BiTE antibody induces cytotoxicity and target cell clustering. Ramos Nuclight Green cells (15K/well) were seeded with PBMCs (1:5 Target to Effector) and activated with BiTE antibody (anti-hCD3xCD19, 0.6 pg/mL to 10 ng/mL) or contrtol antibody (anti-hCD3xβGAL, 10 ng/mL). Representative images at 72 h in the presence of (A) control or (B) BiTE antibody. Incucyte® time-course data (images every 3 h) tracked the cell quantity (C) and clustering (E) of target cells. The fold change in green integrated intensity metric, which accounts for both brightness and size (GCU x µm²) was used to quantify target cells. Concentration-response curves at 72 h for target cell killing (D) and clustering (F) in the presence of the BiTE antibody compared with the control antibody.

CD3xCD19 BiTE Antibody Yields Reduced Levels of Cytokine Release

Cytokine production was evaluated from the supernatant samples taken daily, enabling comparison to target cell killing (Figure 6). The CD3xCD19 BiTE antibody induced production of IFNγ and TNFα in a concentration dependent manner, reaching maximal concentrations of 1.3±0.3 ng/mL and 1.5±0.3 ng/mL, respectively (Figure 7A and B). Interestingly, CD3 | CD28 Dynabeads® stimulation evoked vastly increased production of both cytokines, with IFNγ reaching 14.4±2.5 ng/mL and TNFα 3.8±0.2 ng/mL. Comparison to the levels of cytotoxicity (Figure 7C) highlight that, despite the high levels of target cell death, the BiTE antibody induces relatively low levels of cytokines compared to CD3 | CD28 stimulation.

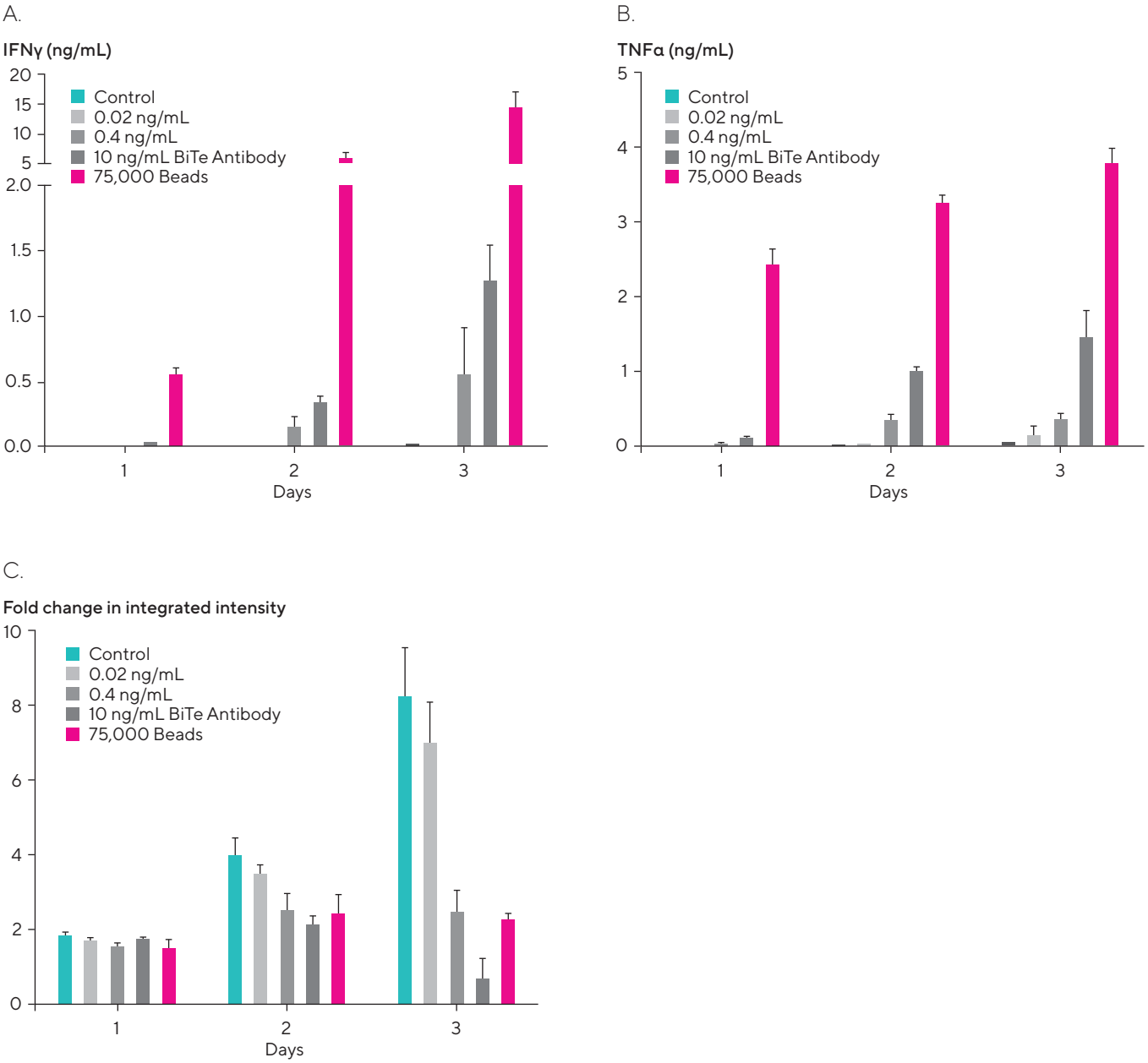

Figure 7: Temporal cytokine production and immune cell killing. Ramos Nuclight Green cells (15K/well) were seeded with PBMCs (1:5 Target to Effector) and either activated with increasing concentrations of BiTE antibody (anti-hCD3xCD19) or CD3 | CD28 Dynabeads® (75K/well). Images were captured by the Incucyte® for 72 h, with daily supernatant samples (10 µL) taken for analysis of cytokine (IFNγ and TNFα) concentrations by the iQue®3 ((A) and (B)). Temporal cytokine concentrations were compared to the level of immune cell killing as measured by fold change in green integrated intensity over time taken from Incucyte® data (C).

CD3xCD19 BiTE Antibody Preferentially Induces CD69+ | CD8+ Cell Subsets

Finally, after 3 days, cells were lifted from the assay plate and labeled with the T Cell Activation antibody panel. As expected the CD3 | CD28 Dynabeads® induced concentration-dependent increases in the proportions of CD69, CD25 and HLA-DR positive populations yielding comparable EC_{50} values of 4690, 16958 and 13865 beads/well, respectively (Figure 8A). The maximal population percentages were 29% for CD69, 69% for CD25 and 32% for HLA-DR.

In contrast, inclusion of the BiTE antibody displays a clear left shift in the CD69 expression pattern, with low concentrations (20 pg/mL) capable of inducing almost exclusive expression of this early activation marker (EC_{50} value of 5.5 pg/mL, Figure 8B). CD25 (mid activation) and HLA-DR (late activation) is induced but at much higher concentrations of BiTE, yielding EC_{50} values of 87 pg/mL and 72 pg/mL, respectively. Maximal subset percentages were 45% for CD69, 92% for CD25 and 46% for HLA-DR.

These data suggest different mechanisms of activation may yield altered proportions of CD8 positive subsets. The findings presented require further investigation to fully understand the implications of this observation.

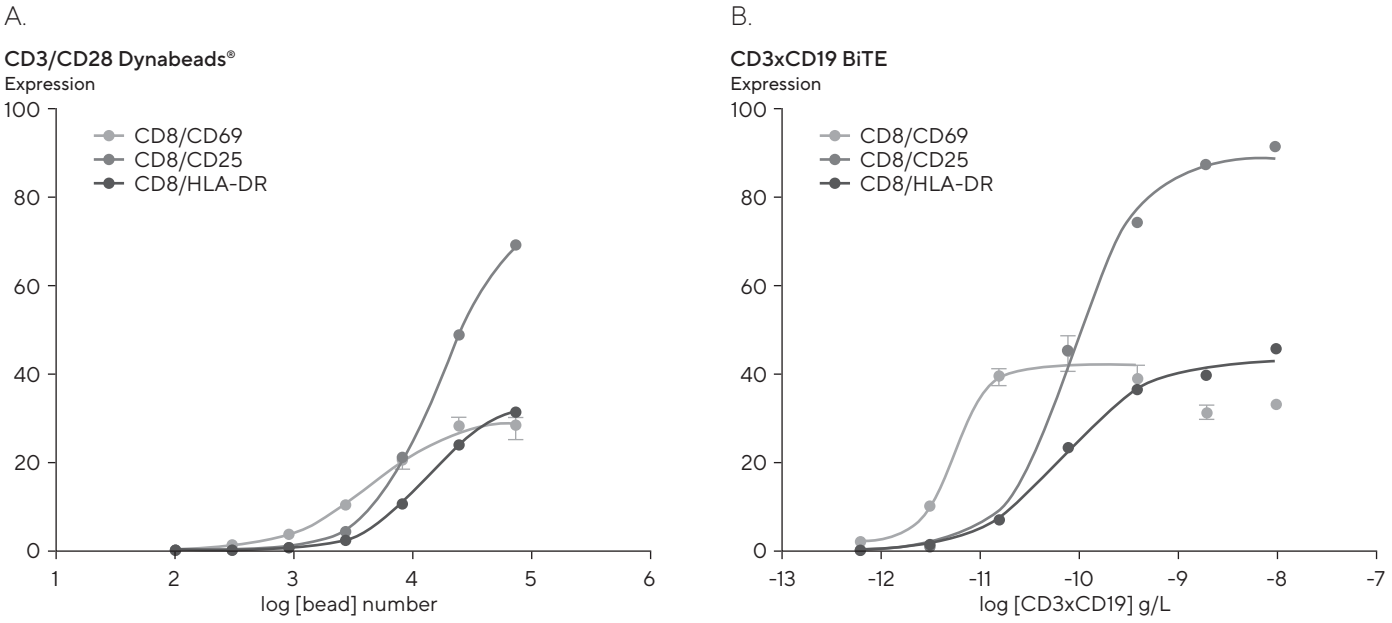

Figure 8: Subset analysis of activation markers in CD8+ immune cells. Concentration response curves to activation induced by (A) CD3 | CD28 Dynabeads® (102 to 75K beads/well) or (B) CD3xCD19 BiTE antibody (0.6 pg/mL to 10 ng/mL).

Conclusions

In this application note, we demonstrate the value of the combined Incucyte®–iQue®3 workflow to enhance insights into immune cell killing experiments. Utilizing the power of the Incucyte® automated temporal image acquisition and analysis for morphological information and target cell quantification, alongside the iQue®3 Human T Cell Activation Cell and Cytokine Profiling Kit and Qbeads® for cytokine profiling and activated subset analysis, we have shown:

- Simple transfer of assay plate from Incucyte® to iQue®3 maintains the trends in the data, supporting the use of a single assay plate for image-based temporal analysis (> 3 days), cytokine profiling over time and T cell activation subset analysis.
- Live-cell imaging in the Incucyte® can identify morphological changes and spatial interactions of tumor and effector cells.
- Measuring cytokine production of PBMCs over 5 days has no perturbing effect on the assay, enabling temporal cytokine data to be collected using the iQue®3.
- The Incucyte® was able to distinguish concentration-dependent morphological and cytotoxic changes in target cells dependent on PBMC activation.
- Combining cytotoxicity data with temporal cytokine production enabled the identification that T cell killing does not always correlate with cytokine production.
- Identifying subset populations on the iQue®3 allows further distinction between activation profiles of CD8+ T cells after stimulation with a therapeutically relevant antibody hCD3xCD19 BiTE.

Our findings within the present study support the notion that additional applications would benefit from the combination of Incucyte®–iQue®3 workflows enabling the benefits of both platforms to be maximized.

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Reagents

T Cell Activation Cell and Cytokine Profiling Kit (TCA)
Qbeads® Assay Builder: IFNγ option 1 and TNFα option 2
Incucyte® Nuclight Green Lentivirus
Incucyte® Annexin V Red

Related Applications

Incucyte® Immune Cell Killing application page
TCA application note—A Kinase Inhibitor Phenotypic Screen Using a Multiplex T Cell Activation Assay

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