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Simplifying Progress

Characterisation of Bi-specific T-cell Engager (BiTE) Antibody-mediated Cell Killing Using a Combined Live-cell and Flow Cytometry Workflow

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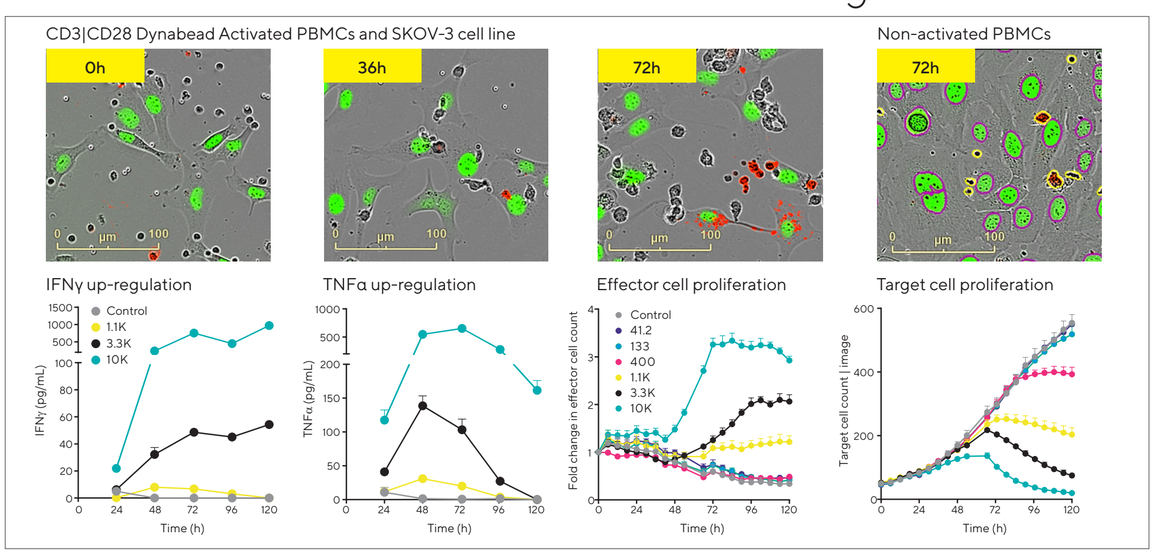
Summary & Impact

- The development of novel immunotherapies depends on the ability to evaluate immune cell effects on tumor cells.
- Here we describe a workflow combining live-cell analysis and flow cytometry enabling complementary parameters to be quantified.
- To demonstrate the potential insight gained by combining platforms, we have characterised immune cell killing induced by a α-hCD3 × CD19 BiTE antibody.
- Phase and fluorescent images were captured with Incucyte® and segmented objects were quantified.
- Cytokine secretion and T-cell activation markers were evaluated using the iQue® platform.
- This combined approached has the potential to facilitate a greater understanding of the complicated biology involved in immune cell activation and killing of tumor cells.

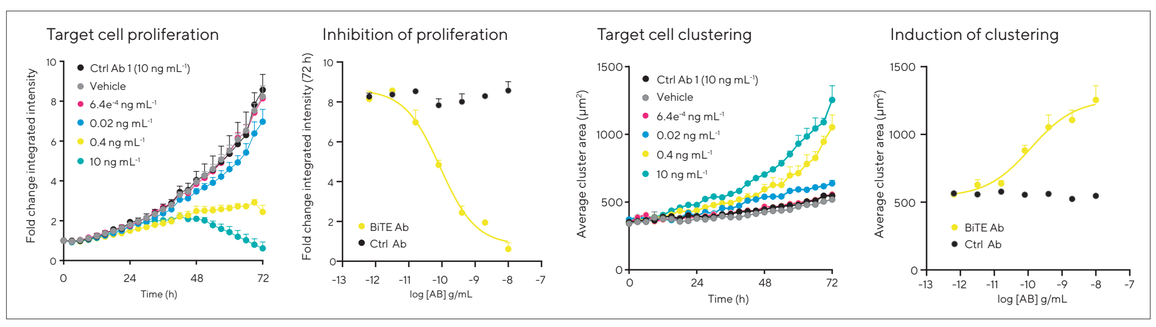
Timeline of Immune Cell Activation and Killing



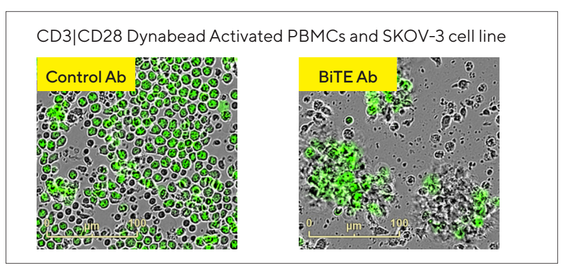
- Adherent SKOV-3 NucLight Green target cells (nuclear segmentation, pink mask) were seeded (4K | well) with non-labelled PBMCs (Cell-by-Cell segmentation, yellow mask) at a 1:5 target to effector cell ratio.
- Cell health was monitored using Annexin V Red reagent and PBMCs were activated with CD3 | CD28 Dynabeads® (41-10,000 beads) for 5 days, images were captured and analysed using Incucyte®.
- Supernatant samples were taken daily (10 µL) and secreted cytokines IFNγ and TNFα were quantified using the QBeads component from the T-Cell Activation Cell and Cytokine Profiling Kit.
- Cytokine levels were shown to increase in both a time- and concentration-dependent manner.
- Effector cell population increased over time with the top three bead densities eliciting the greatest response 48h post bead addition.
- CD3 | CD28 activation yielded a time- and concentration-dependent decrease in SKOV-3 cell numbers with the maximal amount of beads inducing near complete abolition (>95% at 120h).
- This timeline of events details how activation of the T-cell subpopulation ultimately leads to immune cell-mediated cytotoxicity.

BiTE Induced Spatial and Proliferative Changes in Target Cells

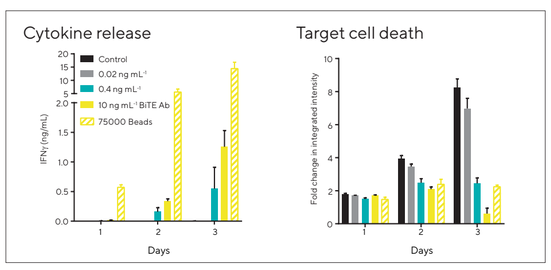
N2A cells were pre-treated with staurosporine (24 hrs), labeled with the Incucyte® pHrodo® Orange Cell Labeling Kit, and added to pre-plated primary rat microglia (Brain Bits, 20,000 K/well). N2A cells alone have minimal fluorescence (left image). Engulfment of labeled apoptotic N2A cells by microglia causes an increase in orange fluorescence (florescent image) that is segmented using automated Incucyte® Live-Cell Analysis Software (fluorescent image with mask). A kinetic N2A density-dependent response is quantified over time (right).



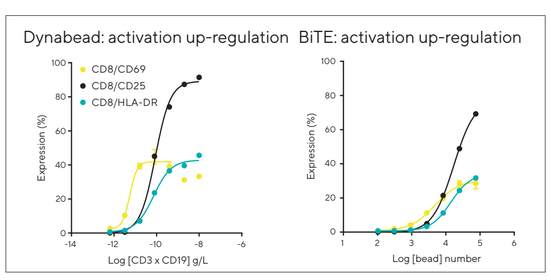
- Ramos NucLight Green target cells were seeded (15K | well) with non-labelled PBMCs (1:5 target to effector cell ratio) and activated with BiTE antibody (α-hCD3 × CD19, 0.6 pg-10 ng mL^{-1}) or control antibody (α-hCD3 × βGal, 0.6 pg - 10 ng mL^{-1}).
- Representative images at 72h in the presence of control or BiTE Ab.
- Phase and fluorescence images acquired using Incucyte® were quantified, taking into account both the brightness and size of green objects (GCU × µm^2) and expressed as fold change in integrated intensity.
- Inclusion of BiTE antibody induced a concentration-dependent increase in target cell cytotoxicity as well as target cell clustering between 24 and 72h.



Differential Profiles Between BiTE- and CD3 | CD28-induced Activation



- Cytokine samples taken daily (10 µL) from the BiTE (α-hCD3 × CD19) co-culture were compared with CD3 | CD28 Dynabead activation.
- BiTE Ab elicited a time- and concentration-dependent increase in IFNγ levels, reaching a maximal concentration of 1.3 ± 0.3 ng mL^{-1}.
- Although CD3 | CD28 activation yielded a significantly higher IFNγ level (14.4 ± 2.5 ng mL^{-1}), BiTE stimulation resulted in greater target cell death (93% compared to 72% following Dynabeads).



- After 3 days cells were lifted and labelled using the TCA-kit antibody cocktail and membrane integrity dye prior to analysis using the iQue® platform.
- Inclusion of BiTE antibody induced a clear left shift in CD69 expression pattern when compared to bead activation, with low concentrations of BiTE (20 pg mL^{-1}) capable of producing almost exclusive expression of the CD69 early activation marker (EC_{50} value 5.5 pg mL^{-1}).

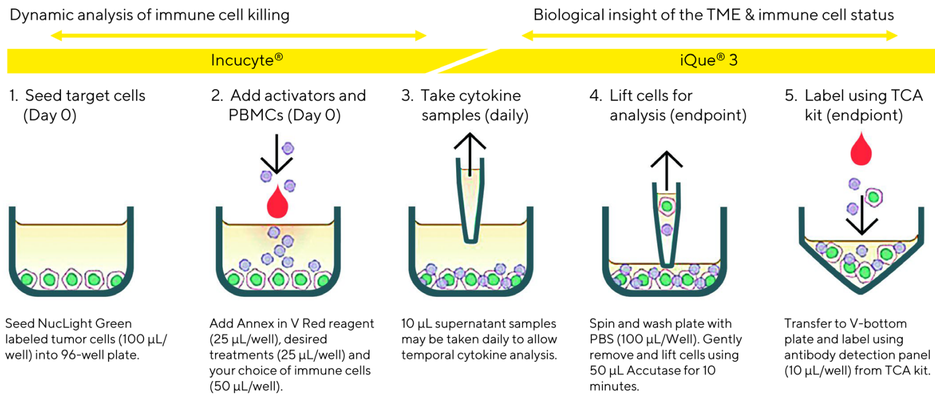
Incucyte® System and iQue®3 Combined

Incucyte® S3 Live-Cell Analysis System
A fully automated phase contrast and two-color fluorescence system that resides within a standard cell incubator for optimal cell viability. Designed to scan plates and flasks repeatedly over time.

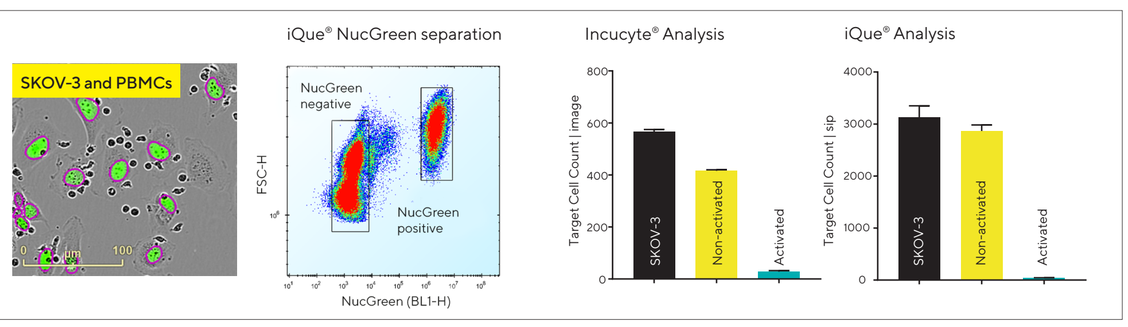
iQue®3 Flow Cytometer
An advanced flow cytometry platform with a patented sampling method allowing for rapid sample acquisition which delivers fast actionable results.. Capable of handling 96 and 384 well plates.

Sartorius Reagents
A suite of reagents, kits and protocols for cell health and function screening.

Live-cell Analysis and Flow Cytometry Workflow: Methodology

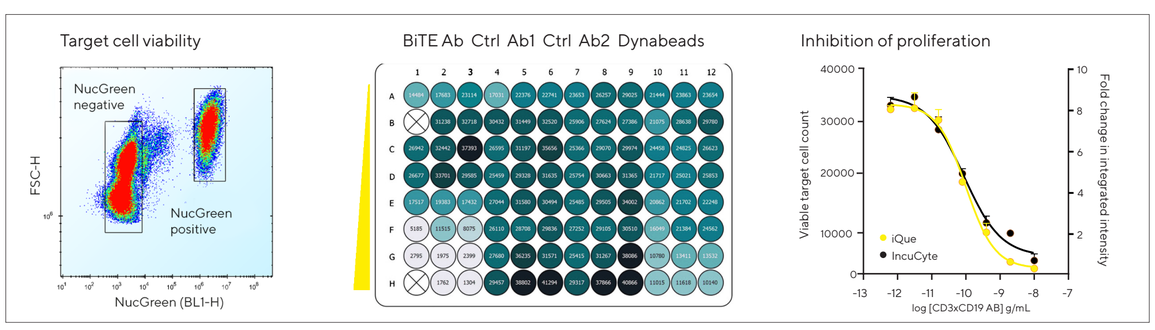


Reagents and Kits are Compatible Across Both Platforms



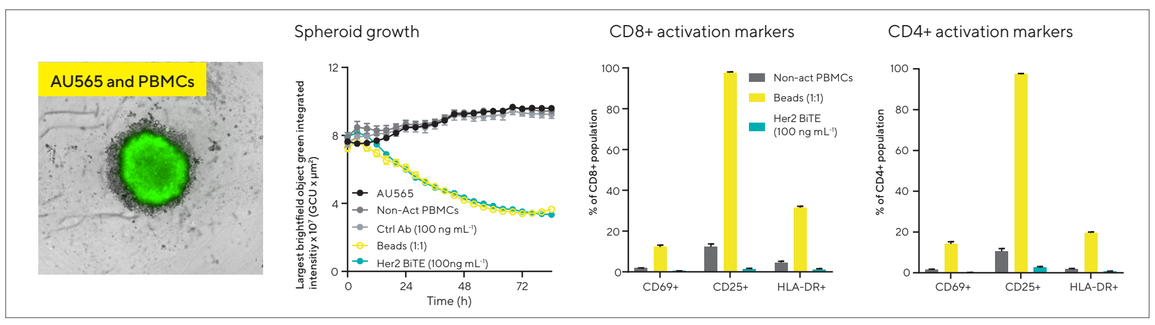
- NucLight Green cell lines were seeded with PBMCs (label free) in the presence of an activator e.g. CD3 | CD28 Dynabeads, BiTE Ab.
- Incucyte® target cell quantification of adherent SKOV-3 cells (nuclear segmentation, pink mask) describes the effect of activated PBMCs, where target cell numbers were significantly reduced in comparison to the target cells alone.
- Cells were then lifted from the assay plate and analysed using the iQue®. Gating using Forecyt software enabled separation of the target and effector cell types based on positive or negative green fluorescent populations.
- Cell count analysis from iQue® analysis showed activated PBMCs induced high levels of cytotoxicity, this trend was highly comparable across both platforms.

Quantitative Pharmacology Demonstrates Comparable Results



- Ramos NucLight Green target cells were seeded (15K | well) with non-labelled PBMCs (1:5 target to effector cell ratio) and activated with BiTE (α-hCD3 × CD19, 0.6 pg-10 ng mL^{-1}) or control Abs (α-hCD3 × βGal and α-hCD19 × βGal, 0.6 pg-10 ng mL^{-1}).
- Viable and non-viable target cells were identified using a cell membrane integrity dye (Sartorius) and gated populations were quantified.
- Live events were displayed using a heat map where increasing shades of purple equated to increased number of target cells.
- Activation with BiTE and CD3 | CD28 Ab induced concentration-dependent decrease in target cell numbers.
- Importantly proliferation potency concentrations were comparable between the iQue® and Incucyte® (IC50 values 99 pg ml-1; 88 pg ml-1 respectively).

Immune Cell Killing of 3D Spheroids



- AU565 NucGreen labelled breast cancer target cells were seeded in a round bottom ULA plate (4K | well) and allowed to form spheroids over 3 days.
- Spheroids were subsequently treated with PBMCs (1:5 target to effector cell ratio) which were either non-activated or in well-activated with an α-hCD3 × Her-2 BiTE Ab or CD3 | CD28 Dynabeads.
- Spheroid growth in the presence of BiTE-activated (100 ng ml^{-1}) PBMCs was inhibited by 64 ± 0.02% (comparable to Dynabead activation).
- Interestingly, the BiTE Ab did not elicit a classic T-cell activation response suggesting an alternative mechanism of action.