Simplifying Progress

Quantify T Cell Response in 3D Tumor Spheroids Using Advanced Flow Cytometry and Live-Cell Analysis

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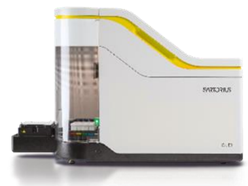
Summary & Impact

- Antigen recognition by T cells induces a cascade of downstream events which are critical in the immune system's fight against cancer.
- Robust *in vitro* assays are required to evaluate the potential for novel therapeutics, for example CAR-T cells and bispecific antibodies, to enhance T cell response against cancer.
- Conventional assays rely on the use of suspension cells or 2D cell monolayers, but these lack many of the complex cell-cell and cell-ECM interactions found *in vivo*.
- Here we provide two 3D tumor spheroid-based solutions to study immune cell-tumor interactions: immune cell killing (ICK) and tumor infiltrating lymphocytes (TILs).
- Therapeutics can improve T cell function in ICK by several mechanisms, including: enhancing activation and killing, reducing exhaustion and promoting memory T cell formation.
- High numbers of TILs are linked to increased response to neoadjuvant chemotherapy and improved pathological complete response rates.
- These data demonstrate the ability to study complex 3D tumor models using advanced flow cytometry and live-cell analysis-based workflows as a translational approach to *in vitro* characterization of immunotherapeutics.

Incucyte® & iQue® 3 Systems

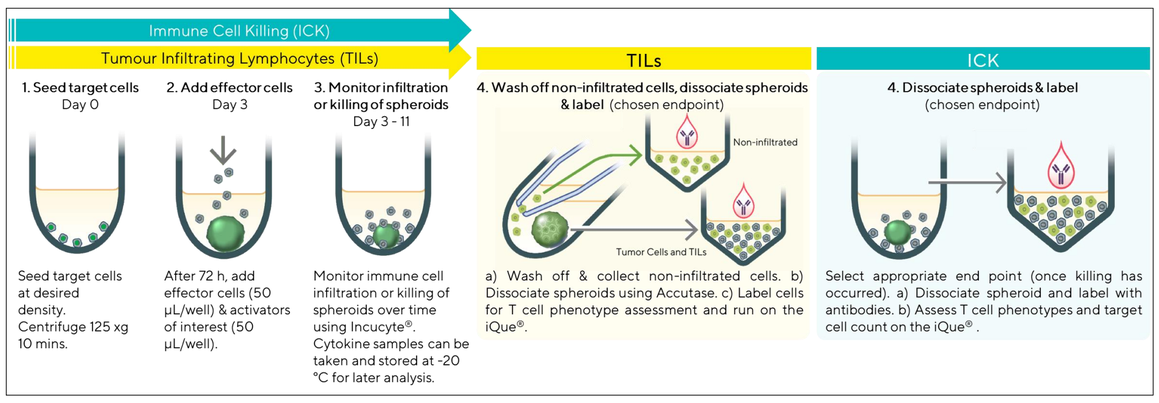
Incucyte® Live-Cell Analysis System
A fully automated phase contrast and multi-color fluorescence system that resides within a standard cell incubator for optimal cell viability. Designed to scan plates and flasks repeatedly over time.



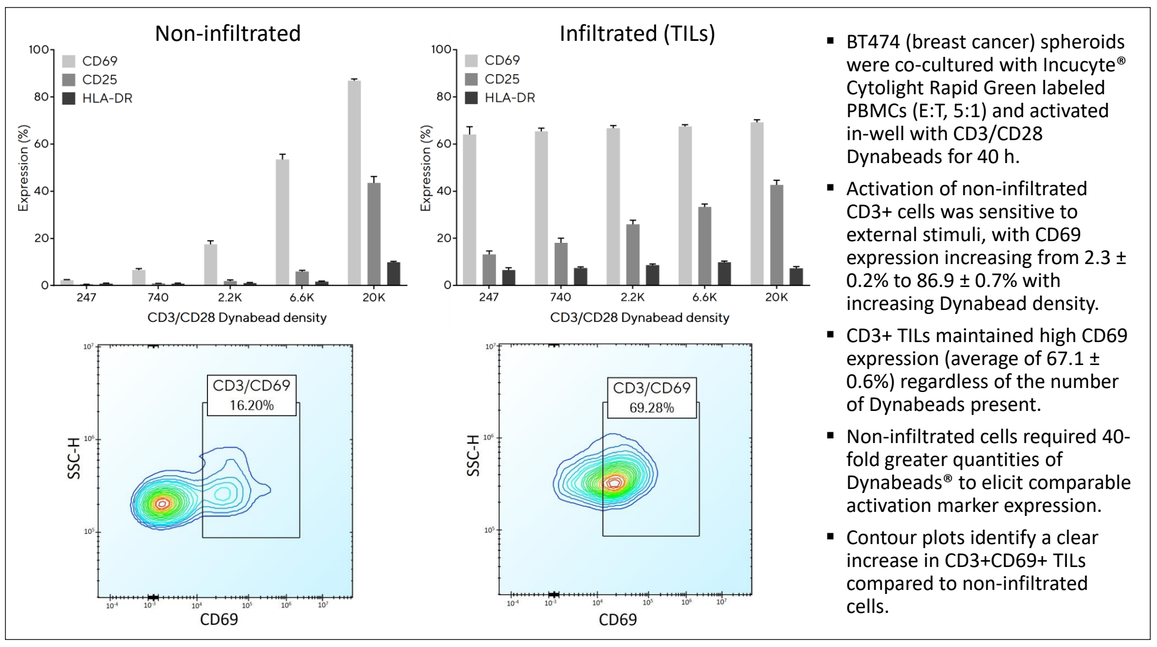
iQue® 3 Advanced Flow Cytometer
An advanced flow cytometry platform with a patented sampling method allowing for rapid sample acquisition to deliver fast actionable results. Capable of handling 96 and 384 well plates.

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

Assay Workflow

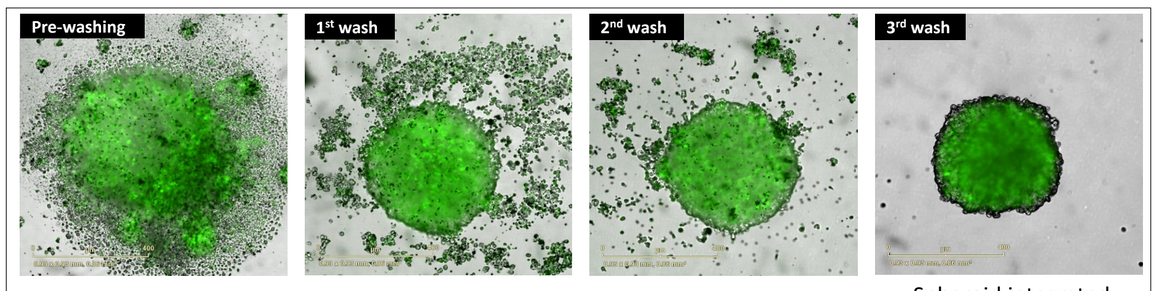


Activation status of TILs is higher than non-infiltrated T cells

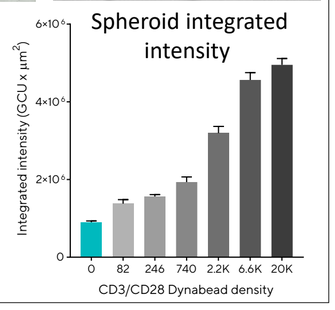


- BT474 (breast cancer) spheroids were co-cultured with Incucyte® Cytolight Rapid Green labeled PBMCs (E:T, 5:1) and activated in-well with CD3/CD28 Dynabeads for 40 h.
- Activation of non-infiltrated CD3+ cells was sensitive to external stimuli, with CD69 expression increasing from 2.3 ± 0.2% to 86.9 ± 0.7% with increasing Dynabead density.
- CD3+ TILs maintained high CD69 expression (average of 67.1 ± 0.6%) regardless of the number of Dynabeads present.
- Non-infiltrated cells required 40-fold greater quantities of Dynabeads® to elicit comparable activation marker expression.
- Contour plots identify a clear increase in CD3+CD69+ TILs compared to non-infiltrated cells.

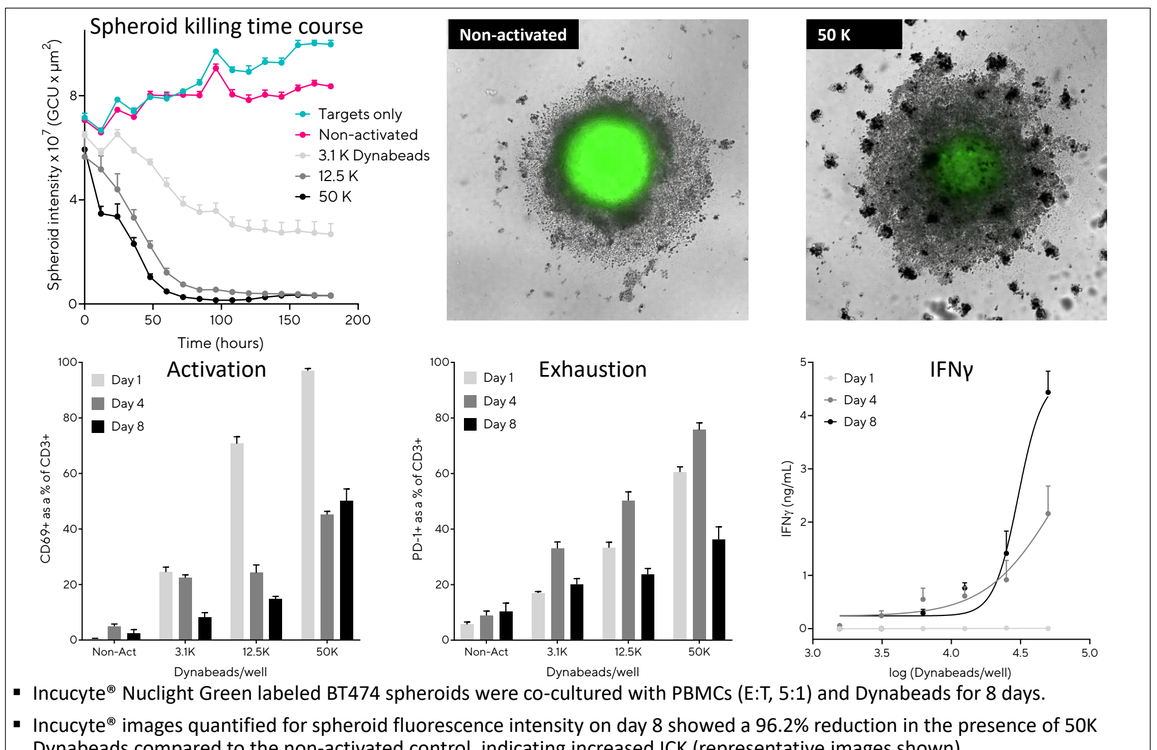
Validation of the TILs washing protocol using the Incucyte®



- BT474 (breast cancer) spheroids were formed for 3 days before pre-activated Incucyte® Cytolight Rapid Green labeled PBMCs were added for 24 h.
- Spheroids were washed three times with PBS as per the iQue® TILs assay protocol.
- Images taken using the Incucyte® after each wash step were used to validate the washing protocol.
- These images showed a clear reduction in the number of non-infiltrated immune cells surrounding the spheroid with each successive wash step, with negligible numbers remaining after the 3rd wash.
- Quantification of spheroid green intensity after the final wash indicated increased numbers of green labeled TILs with increasing concentrations of Dynabeads.

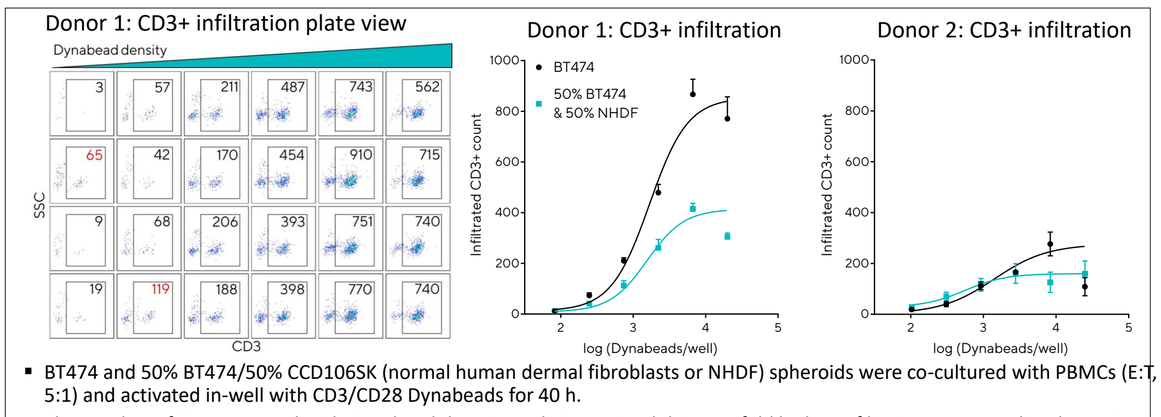


T cell activation and exhaustion increase during spheroid killing



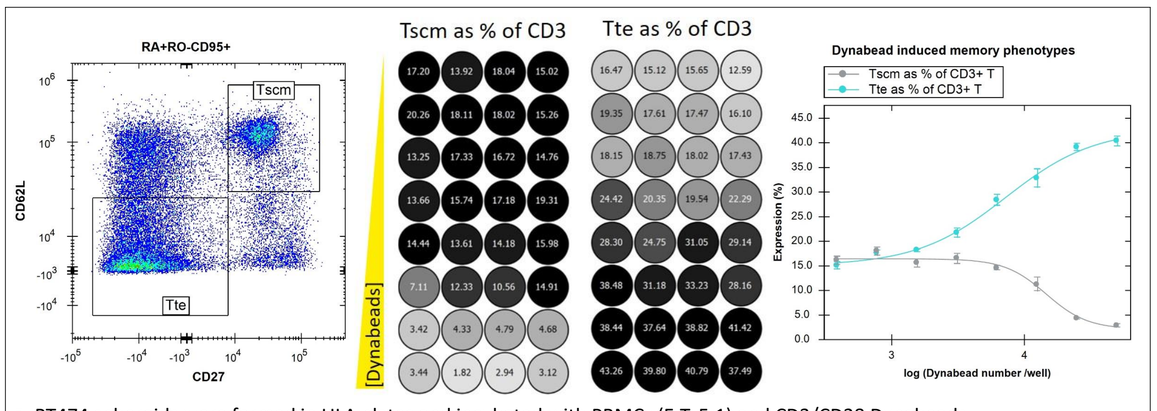
- Incucyte® Nuclight Green labeled BT474 spheroids were co-cultured with PBMCs (E:T, 5:1) and Dynabeads for 8 days.
- Incucyte® images quantified for spheroid fluorescence intensity on day 8 showed a 96.2% reduction in the presence of 50K Dynabeads compared to the non-activated control, indicating increased ICK (representative images shown).
- Subset analysis using iQue® T cell characterization kits showed peak CD69 (early activation marker) expression on day 1 (98 ± 0.5 %, 50K Dynabeads). PD-1 peaked on day 4 then declined by day 8, suggesting recovery from exhaustion after antigen clearance.
- Supernatant samples were analyzed for cytokine concentrations using iQue Qbeads®. IFNγ and Granzyme B (not shown) both increased over time and with Dynabead density.

Spheroids containing fibroblasts have reduced numbers of TILs



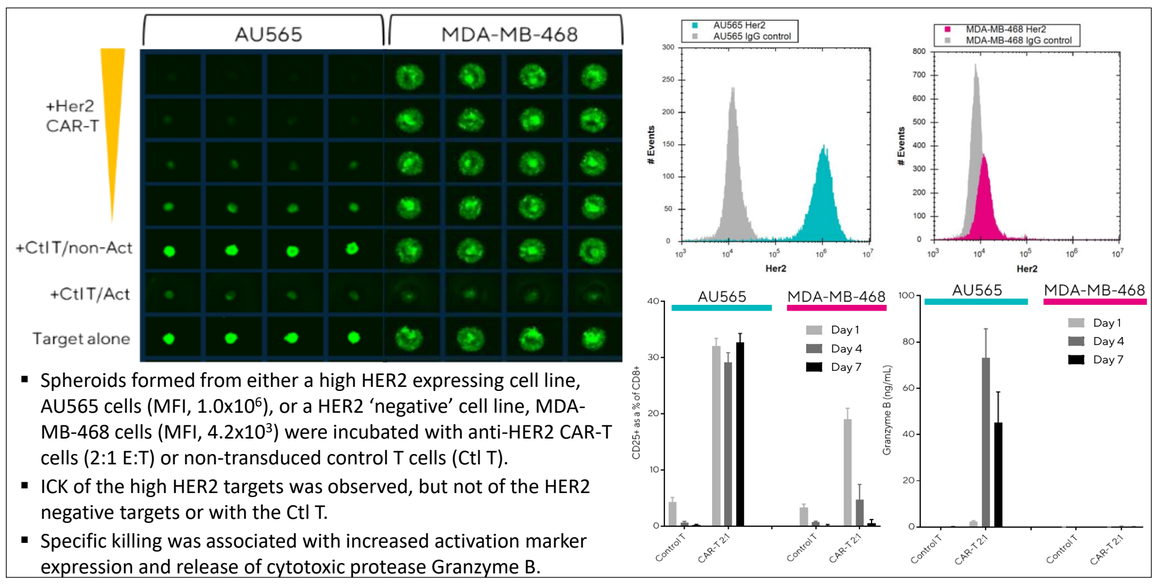
- BT474 and 50% BT474/50% CCD106SK (normal human dermal fibroblasts or NHDF) spheroids were co-cultured with PBMCs (E:T, 5:1) and activated in-well with CD3/CD28 Dynabeads for 40 h.
- The number of TILs increased with Dynabead density, with Donor 1 exhibiting 3-fold higher infiltration compared to donor 2.
- Fibroblast inclusion caused the maximal CD3+ infiltration to significantly reduce by >50% in both donors.
- At high ratios of Dynabeads (1:1) the immune cells begin to attack the tumor spheroid, causing the model to break down.

CD3/CD28 promotes transition to T memory cells during ICK



- BT474 spheroids were formed in ULA plates and incubated with PBMCs (E:T, 5:1) and CD3/CD28 Dynabeads.
- After 24 h, spheroids were dissociated and cell subsets analyzed using the iQue® Human T Cell Memory Kit.
- Dot plot shows gating of Tscm (stem cell memory) and Tte (terminal effector) populations using the template provided in the kit.
- Heat maps and concentration response curves from iQue Forecyt® show the shift from the early stage Tscm phenotype towards the later stage Tte phenotype with increasing Dynabead concentration. This is accompanied by a loss in self renewal potency.

Targeted activation and killing by anti-HER2 CAR-T cells



- Spheroids formed from either a high HER2 expressing cell line, AU565 cells (MFI, $1.0x10^6$), or a HER2 'negative' cell line, MDA-MB-468 cells (MFI, $4.2x10^3$) were incubated with anti-HER2 CAR-T cells (2:1 E:T) or non-transduced control T cells (Ctl T).
- ICK of the high HER2 targets was observed, but not of the HER2 negative targets or with the Ctl T.
- Specific killing was associated with increased activation marker expression and release of cytotoxic protease Granzyme B.