SARDRICS

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.

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

Non-infiltrated	Infiltrated (TILs)	BT474 (breast cancer) spheroids

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



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

µL/well)

10 mins.

	mmune Cell Killing (I	CK)		
Tumour Infiltrating Lymphocytes (TILs)		TILs	ICK	
1. Seed target cells	2. Add effector cells	3. Monitor infiltration	4. Wash off non-infiltrated cells, dissociate spheroids	4. Dissociate spheroids & label



- D1474 (DIEast Calicer) splicion 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 $67.1 \pm$ 0.6%) regardless of the number of Dynabeads present.
- Non-infiltrated cells required 40fold 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.

T cell activation and exhaustion increase during spheroid killing





Cytokine samples can be

taken and stored at -20

°C for later analysis.



Select appropriate end point (once killing has occurred). a) Dissociate spheroid and label with antibodies. b) Assess T cell phenotypes and target cell count on the iQue®

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.



6×10

 Quantification of spheroid green intensity after the final wash indicated increased numbers of green labeled TILs with increasing concentrations of Dynabeads.

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:

- 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[®]. IFNy and Granzyme B (not shown) both increased over time and with Dynabead density.

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.

- 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.

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

