Application Note

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Enhanced Insights Into Immune Cell Killing in Single-Spheroid Tumor Models Using Confocal Live-Cell Imaging

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Abstract

Three-dimensional (3D) immune cell killing (ICK) assays, which incorporate tumor spheroids and immune cells, are increasingly being adopted to assess immune cell functions and evaluate potential immunotherapies for targeting solid tumors. However, these complex 3D models are technically challenging, and quantification methods struggle to reliably capture biological insights due to limited optical sectioning and depth resolution, or they are unable to temporally monitor the dynamic cellular processes.

In this application note, we demonstrate how confocal live-cell imaging and multiplexed, non-perturbing fluorescence readouts can be used to spatially and kinetically resolve *in vitro* ICK of tumor single-spheroids. We exemplify how it can flexibly be used across different immuno-oncology paradigms to evaluate mechanisms of immune cell engagement, naïve or engineered T-cell killing, and natural killer cell-mediated antibody-dependent cellular cytotoxicity (ADCC). By addressing challenges in 3D model evaluation, and bridging the gap between *in vitro* and *in vivo* studies, this approach facilitates the characterization of immunotherapeutic mechanisms and enhances the development of translational models.

Introduction

Immunotherapy leverages the body's innate immune system to combat cancer, with cytotoxic T-cells and natural killer (NK) cells playing crucial roles in inducing malignant cell death through immune cell killing (ICK).[1] This revolutionary approach to cancer treatment includes strategies like chimeric antigen receptor (CAR) T-cell therapy, immune checkpoint inhibitors (ICIs), monoclonal antibodies (mAbs), and adoptive cell transfer.[2,3] To develop these approaches, translational *in vitro* ICK assays are vital for understanding immune cell cytotoxic functions and evaluating immunotherapeutics.

Traditional techniques used to assess ICK *in vitro*, such as MTT, flow cytometry, or high-content imaging, often rely on single endpoint analyses which do not provide insights into dynamic cellular interactions.[4] Researchers are seeking new methods, such as live-cell analysis, to provide the temporal resolution necessary for comprehensively characterizing ICK. Furthermore, as *in vitro* cellular models become increasingly complex and move from two-dimensional (2D) monolayer cultures to three-dimensional (3D) ones, the challenges associated with evaluating ICK become significantly harder to surmount.[5,6] For example, tumor spheroid 3D models are increasingly being utilized to mimic *in vivo* conditions and examine how the tumor microenvironment (TME) affects tumor-immune interactions.[7,8] These models better replicate the complexity, architecture, and gradients of solid tumors and bridge the gap between *in vitro* and animal studies.[9] However, they often face challenges including lengthy or complex protocols, lack of reliable quantification of target cell killing or immune cell interactions, and limited availability of dyes that are non-perturbing and effective for staining target or effector cells over the duration of the assay.[6,10] Consequently, researchers require flexible ICK assays that can robustly be applied to 3D models and have the multiplexing capability to derive maximal insights from precious cultures.

The Incucyte® CX3 Live-Cell Analysis System (Incucyte® CX3) offers continuous imaging of 3D models within an incubator, combining spinning-disk confocal fluorescence with user-friendly features for meaningful and reproducible results. Confocal imaging improves the visualization of thick samples by capturing fluorescence from narrow focal planes, preserving biological detail across the Z-axis. This enables real-time monitoring of 3D ICK models and facilitates spatial differentiation of immune and tumor cell interactions.

This application note demonstrates how confocal live-cell imaging facilitates the assessment of immune-mediated killing of 3D tumor spheroids *in vitro* and, through non-perturbing, multiplexed fluorescence readouts, allows for the spatial and temporal resolution of killing responses. Furthermore, we show how this versatile approach can be adapted to examine naïve or engineered T-cells and be utilized for the assessment of antibody-dependent cellular cytotoxicity (ADCC) mediated immunotherapeutics.

Materials and Methods

Cell Culture

Media formulations and materials used for cell culture and assays are described below (Tables 1 and 2). Tumor cell lines, including A549 pulmonary adenocarcinoma, SKOV3 ovarian adenocarcinoma, and BT-474 breast ductal carcinoma, were transfected with Incucyte® Nuclight Orange 2.0 Lentivirus Reagent (Nuclight Orange) to induce expression of a nuclear restricted fluorescence protein. Nuclight cells were maintained in medium containing 0.5 – 1 µg/mL puromycin, however all assays were performed in the absence of puromycin.

Frozen peripheral blood mononuclear cells (PBMCs), NKs and CAR-Ts targeting HER2, were resuspended at 1E+06 cells/mL in RPMI medium supplemented with IL-2 in T25 or T175 flasks and rested overnight (37 °C, 5% CO_2).

Cell Culture	Base Medium	FBS	Pen/Strep	Supplements
A549 Nuclight Orange	F12K	10%	1%	1 µg/mL Puromycin
BT-474 Nuclight Orange	RPMI	10%	1%	0.5 µg/mL Puromycin
SKOV3 Nuclight Orange	F12K	10%	1%	0.5 µg/mL Puromycin
Human PBMCs	RPMI	10%	-	10 ng/mL IL-2
Human NKs	RPMI	10%	-	10 ng/mL IL-2
Anti-HER2 CAR-Ts	RPMI	10%	-	10 ng/mL IL-2

Table 1. Media formulations for cell culture.

Materials	Supplier	Cat. No.	Final Concentration
Human PBMCs	STEMCELL Technologies	70025.1	-
Human NKs	IQ Biosciences	Hu1-NK10	-
Anti-HER2 CAR-Ts	Creative BioLabs	-	-
Recombinant Human IL-2	Sartorius	CYK-0100-1001	10 ng/mL
Incucyte® Nuclight Orange Lentivirus 2.0 (EF1α, Puro)	Sartorius	BA-04891	MOI 3 - 6
F12K	Gibco	11580556	Base
RPMI 1640	Gibco	21875091	Base
Fetal Bovine Serum (FBS)	Cytiva HyClone™	SH30071.03	10%
Penicillin-Streptomycin	Gibco	15140122	1%
Puromycin	Gibco	12122530	0.5 – 1 µg/mL

Table 2. Materials used for cell culture.

Live-Cell Immunocytochemistry (ICC)

A simple mix-and-read, no wash, live-cell ICC protocol was used to label immune cells. Materials used are described in Table 3. Antibodies against CD45, an immune cell marker expressed on most nucleated hematopoietic cells, or an IgG1 isotype control were labeled with isotype matched Incucyte® IgG1 Fabfluor-488 Labeling Dye at a molar ratio of 1:3 in media for 15 minutes in the dark at 37 °C. The Fabfluor-antibody complexes (1 µg/mL FAC) in combination with Opti-Green background suppressor were then added to the ICK assay plate.

We performed image-based ICK assays of tumor single-spheroids following a previously described protocol outlined in Figure 1.[11] Materials used are described in Table 3. Tumor cells of interest stably expressing Nuclight orange were seeded at 1,000 – 3,000 cells/well in 100 µL for 96-well or 50 µL/well for 384-well ultra-low attachment (ULA) round-bottom plates and centrifuged (125 xg, 10 minutes) at room temperature (RT). For longer-term assays, PBS was placed in outer wells to mitigate evaporation. Spheroid formation was monitored using widefield imaging (4x or 10x) in an Incucyte® CX3 to ensure that by Day 3 spheroids have formed to the desired size (200 – 500 µM).

On Day 3 appropriate treatments, e.g., cytokines, antibodies, Fabfluor-antibody complexes and Opti-Green, or Annexin V NIR Dye for apoptosis, were added at 4 x FAC in 50 µL/well (96-well plate) or 25 µL/well (384-well plate). For pre-activation experiments, PBMCs were treated with media supplemented with anti-CD3/IL-2 to activate T-cell populations and NKs were activated using IL-12/IL-2 for 72 hours. Non-activated immune cells were supplemented with IL-2. Immune cells were incubated with DNase I (15 minutes, RT) if heavily clustered and then added to the assay plate at an optimized effector-to-target (E:T) cell ratio in 50 µL/well (96-well plate) or 25 µL/well (384-well plate).

Immune-mediated killing was monitored using the Incucyte® CX3 and single-spheroid confocal multiplane acquisition (4x or 10x magnification for 96-well, 10x for 384-well; default Z parameters) with phase + brightfield and fluorescence (green, orange, and/or near-infrared (NIR)) images acquired every 6 hours up to 13 days. The use of the 10x objective is highly dependent on the co-culture being in the field of view and may not be suitable for all spheroid morphologies and E:T ratios. Max projection images were analyzed using integrated software to quantify changes in fluorescence.

3D Immune Cell Killing (ICK) Assay

1. Seed target cells (Day 0)
2. Monitor spheroid formation (Day 0 - 3)
3. Add treatments and immune cells (Day 3)
4. Live-cell confocal imaging

Seed fluorescently labeled target cells into 96-well or 384-well ULA plate and centrifuge.

Place plate inside an Incucyte® CX3 Live-Cell Analysis System and scan every 6 hours using widefield imaging.

Add appropriate treatments and chosen immune cells at 4x final assay concentration.

Capture images using spheroid confocal acquisition every 6 hours to monitor spheroid proliferation and cytotoxicity.

Figure 1. 3D Tumor Spheroid Immune ICK Assay Workflow. Schematic shown of the workflow for 3D tumor single-spheroid ICK assay at 96- or 384-well throughput using the Incucyte® CX3 Live-Cell Analysis System.

Materials	Supplier	Cat. No.	Final Concentration
96-well round-bottom ULA plate	S-BIO	MS-9096UZ	-
384-well round-bottom ULA plate	S-BIO	MS-9384UZ	-
Recombinant Human IL-2	Sartorius	CYK-0100-1001	10 ng/mL
Recombinant Human IL-12	Sartorius	CYK-0100-1009	10 ng/mL
Incucyte® Mouse IgG1 Fabfluor-488 Antibody Labelling Reagent	Sartorius	4743	1 µg/mL
Opti-Green Background Suppressor	Sartorius	with above	1:200
Incucyte® Annexin V NIR Dye	Sartorius	4768	1:200
Anti-human CD3 Antibody	Biolegend	344802	10 ng/mL
Anti-human CD45 Mouse IgG1 Antibody	Biolegend	304002	1 µg/mL
Anti-human Mouse IgG1 Control Antibody	Biolegend	400165	1 µg/mL
Trastuzumab Human IgG1 (anti-HER2)	Absolute Antibody	Ab00103-10.0	10 – 0.08 µg/mL
Kadcyla® (Trastuzumab emtansine)	Midwinter Solutions	-	10 – 0.08 µg/mL
Anti-B-Gal Human IgG1 Control Antibody	Invivogen	Bgal-mab1	10 µg/mL
DNase I	STEMCELL Technologies	07469	100 µg/mL
Camptothecin	Sigma-Aldrich	C9911	3 µM

Table 3. Materials used for 3D Spheroid ICK Assay.

Results

Utilizing Confocal Live-Cell Imaging for the Assessment of 3D ICK Models

Immune-mediated tumor killing, either through T-cells or NK cell-driven ADCC, in 3D tumor spheroids requires precise visualization of effector-target interactions, such as immune-cell infiltration and localized tumor-cell killing. Widefield imaging often blurs these events, merging immune cells and tumor cells, and complicates the identification of cell interactions and initial cytotoxic responses. Spectral overlap in multiband emission systems further hinders multiplexed signal differentiation of individual cells, making single-channel fluorescent measurements advisable.[4,12] Therefore, whilst widefield imaging is powerful, the lack of optical sectioning and resolution can make it unsuitable for some 3D applications.

The Incucyte® CX3 utilizes spinning-disk confocal imaging to reject out-of-focus light and offers enhanced resolution of complex or dense 3D objects and high spatial fidelity, with a penetration depth of 50 – 100 µm (~5 cell layers), depending on the cell type and compactness of the 3D model. Additionally, by employing the integrated emission filter turret with single bandpass emission filters combined with laser-based excitation, the system minimizes the need for spectral unmixing of fluorescent signals. This improvement facilitates reliable fluorescence multiplexing in live-cell 3D co-cultures, which enables discrimination of labeled immune cells and allows for the assessment of their spatial and temporal interactions.

To exemplify this approach, we established a dual color single-spheroid ICK model using SKOV3 spheroids stably expressing an orange nuclear restricted fluorescent protein (Nuclight Orange) co-cultured with activated PBMCs (2.5:1 E:T), which were labeled using Fabfluor-488-CD45 in the presence of the background suppressor Opti-Green (Figure 2). Confocal multiplane acquisition was performed at 10x to capture fluorescence across two channels. A schematic is shown to illustrate confocal imaging of spheroids using the Incucyte® CX3 (Figure 2A). Briefly, autofocusing utilizes the brightfield image to determine the best focus position near the center of a spheroid (Figure 2B) and then acquires multiplane fluorescent images within a defined confocal Z-range from that point. Within the integrated software, users can view and analyze max projection images of the fluorescence channels (Figure 2C) alongside the brightfield image over time. Additionally, individual Z-plane images can be visualized as exemplified in Figure 2D where the top images are near to the midpoint of the spheroid (Z-plane 21) and images below are sweeping downwards through the spheroid co-culture until the bottom of the spheroid (Z-plane 17). The representative images show how confocal imaging enables fluorescence multiplexing in complex 3D models, demonstrating the ability to resolve target tumor cells (orange) and effector cells (green) at the spheroid interface and within the outer layers. This facilitates spatial differentiation of immune cell accumulation and early infiltration events that widefield fluorescence imaging cannot accurately distinguish.

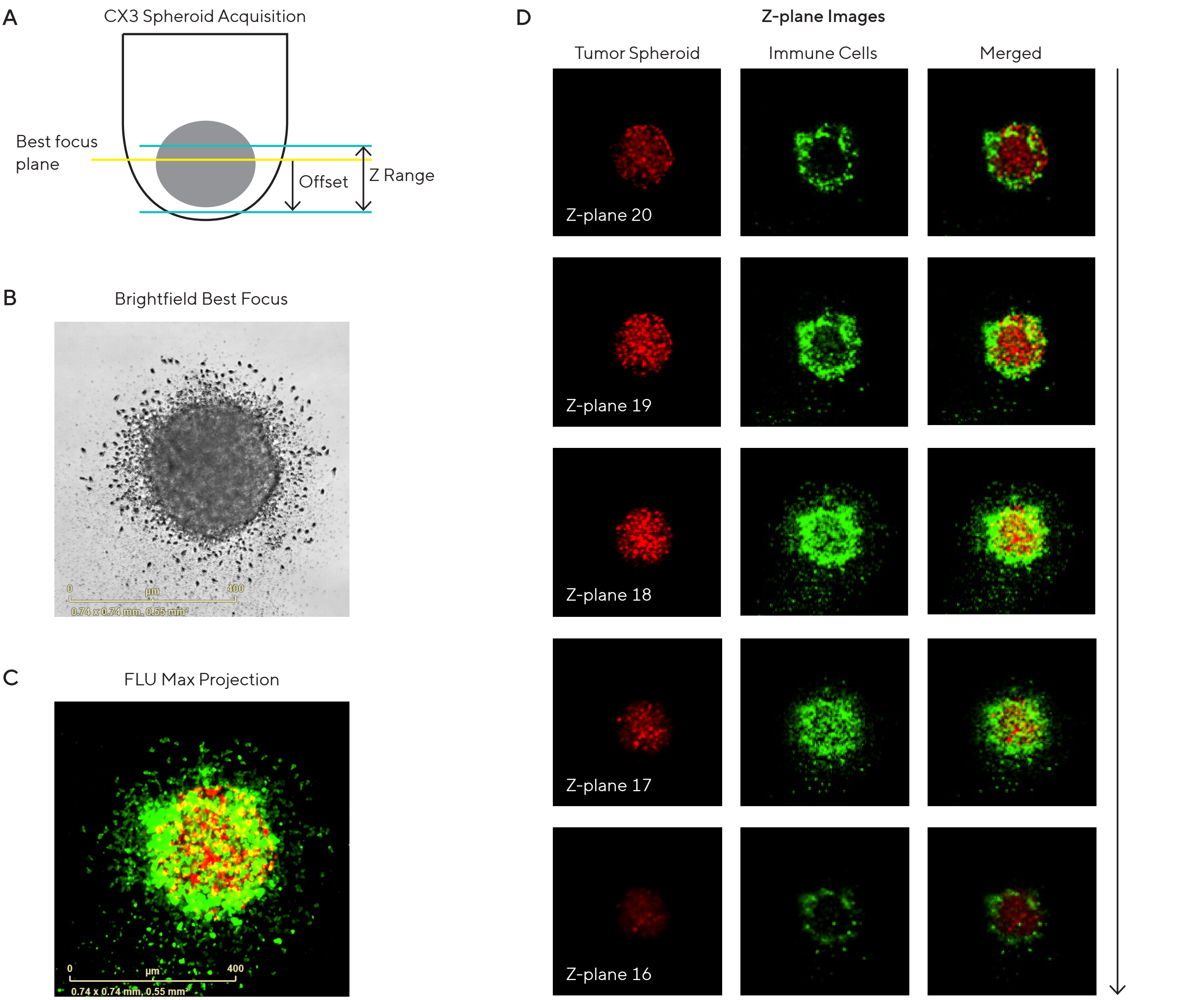

Figure 2. Confocal imaging enables multiplexed readouts to spatially resolve immune cells and tumor spheroids. SKOV3 Nuclight Orange spheroids were co-cultured with activated PBMCs which were labeled with Fabfluor-488-CD45 (green). Brightfield and dual color fluorescence images were acquired at 10x using confocal multiplane acquisition. A) Schematic of confocal spheroid acquisition using the Incucyte® CX3. B) Representative brightfield best focus image used for defining confocal fluorescence Z-range. C) Representative fluorescence (FLU) max projection image. D) Z-plane images for individual or merged fluorescence channels, sweeping down through the spheroid co-culture from Z-plane 20 to 17 (5 out of 21 Z-planes shown).

To demonstrate the ability to kinetically quantify ICK in 3D models and highlight assay flexibility, we further investigated SKOV3 and PBMC co-cultures using various configurations in 96-well plates (Figure 3). SKOV3 Nuclight Orange spheroids were co-cultured with PBMCs (5:1 E:T), which were either pre-activated, activated in-plate, or non-activated. Images were acquired every 6 hours using confocal spheroid acquisition at 4x or 10x magnification.

Representative brightfield and orange images at 10x, and fluorescence segmentation masks (blue overlay), are shown for the tumor spheroid alone, non-activated, and pre-activated PBMCs at 1.5 days (Figure 3A). The high-resolution images reveal rapid killing of the tumor spheroid in the presence of activated PBMCs as indicated by the loss of orange fluorescence, with little-to-no killing observed for non-activated PBMCs. Using integrated analysis, we can accurately segment and kinetically quantify the changes in tumor fluorescence over time.

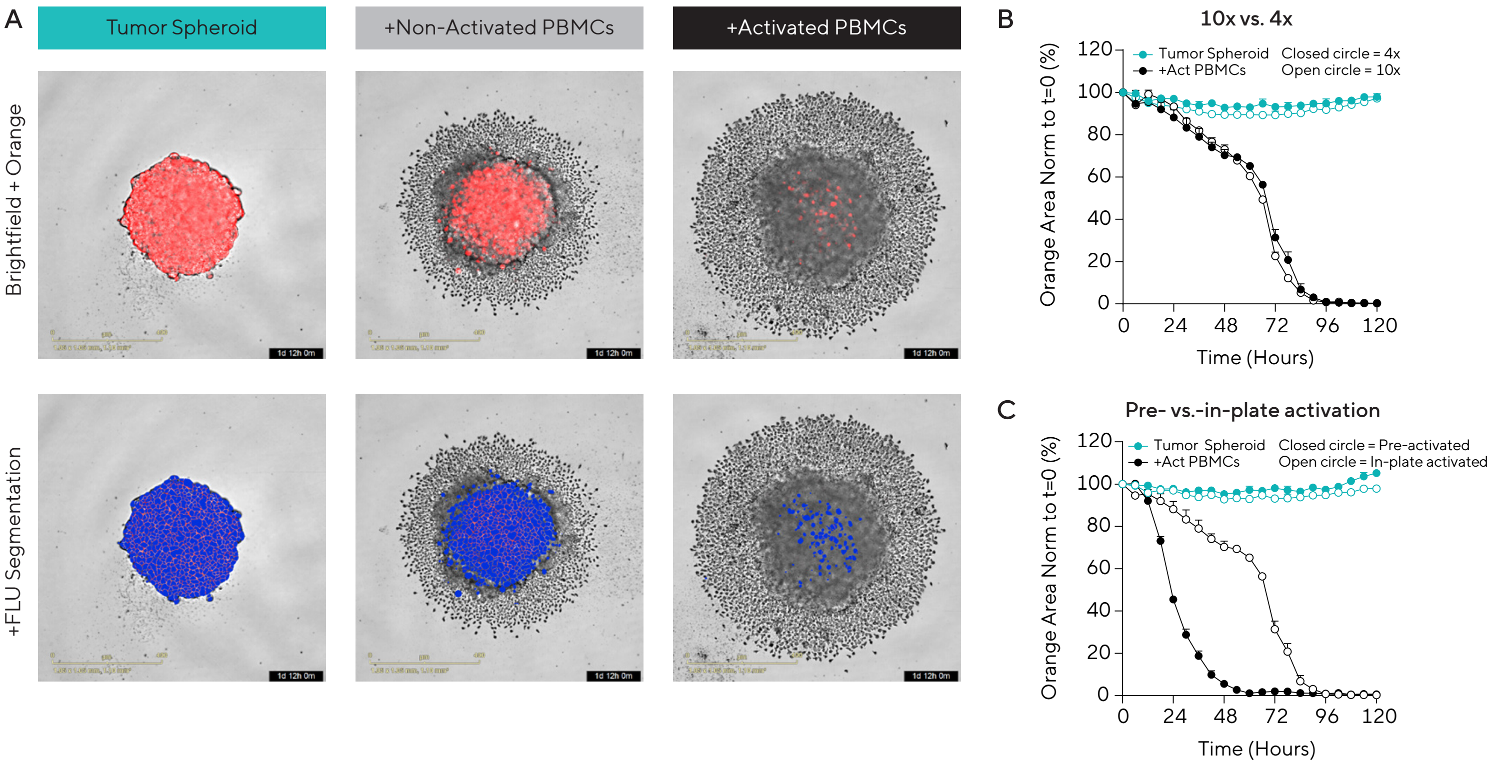

Figure 3. Flexible live-cell assays for assessment of immune-mediated tumor spheroid killing. SKOV3 Nuclight Orange tumor spheroids were co-cultured with PBMCs, which were pre-activated or activated in-plate. Immune-mediated killing was monitored using confocal fluorescence images acquired at 4x or 10x magnification. A) Representative brightfield and orange fluorescence images (10x) with fluorescence (FLU) segmentation shown (blue overlay mask). B) Time course of normalized orange area for in-plate activated PBMCs at 4x or 10x. C) Time course of normalized orange area for pre- or in-plate activated PBMCs at 4x. Data presented as mean + SEM, n = 3 replicates.

Figure 3B illustrates the observed loss in orange area for in-plate activated PBMCs compared to tumor spheroid alone with comparable kinetic data for images acquired at 10x and 4x magnification. Furthermore, flexibility can be introduced through various assay configurations, including using target or effector cells of choice, or comparing and optimizing methods of immune cell priming and activation.

For example, Figure 3C shows kinetic data for pre-activated or in-plate activated PBMCs, where we observe a delayed killing profile for in-plate activated PBMCs. Overall, this data shows the versatility and multiplexing capabilities of confocal live-cell analysis to spatially assess immune cell distribution near to and within outer layers of the spheroid and temporally resolve tumor cell death.

Enhanced ICK Insights using Multiplexed Fluorescence Confocal Imaging

By resolving spatial and temporal heterogeneity, confocal imaging supports the examination of detailed killing profiles, which more accurately reflect the complex biology inherent in 3D TMEs. Here, we further exemplify the insights that can be gained using multiplexed confocal imaging of ICK through the assessment of E:T ratios (Figure 4). SKOV3 Nuclight Orange spheroids were co-cultured with non-activated or pre-activated PBMCs at a range of E:T ratios (10:1 – 1.25:1). Immune cells were labeled using Fabfluor-488-CD45 or Fabfluor-488-IgG1 control and imaged using confocal acquisition at 4x every 6 hours over 5 days.

Representative images show immune-mediated killing in the presence of activated PBMCs at 2 days, as indicated by a visible loss in orange fluorescence, and show successful labeling of immune cells to aid with spatial resolution and effector cell proliferation (Figure 4A). To ensure the labeling of immune cells is not having an impact on the observed killing response, it is important to include appropriate controls (Figure 4B).

The results showed minimal difference in activated PBMC-mediated killing in the presence of Fabfluor-488 conjugated to CD45 or IgG1 control compared to media only (no Fab). We observed a temporal density-dependent decrease in orange area with increasing PBMC density for activated PBMCs compared to non-activated PBMCs, indicating target cell death (Figure 4C). There was some non-specific killing observed for higher densities of non-activated PBMCs for this donor. Furthermore, following the loss of orange fluorescence with activated PBMCs, we noticed a subsequent increase in green fluorescence.

This was density-dependent and indicates PBMC expansion following tumor destruction. For example, the highest activated PBMC density (20K) induced rapid killing of the tumor spheroid by 36 hours and 6 hours later the average total green area increased 4-fold from 2.73E+05 µm²/image at 42 hours to 1.11E+06 µm²/image at 120 hours. A subtle density-dependent increase in green fluorescence was observed for non-activated PBMCs, and minimal green was observed in Fabfluor-488-IgG1 conditions as expected.

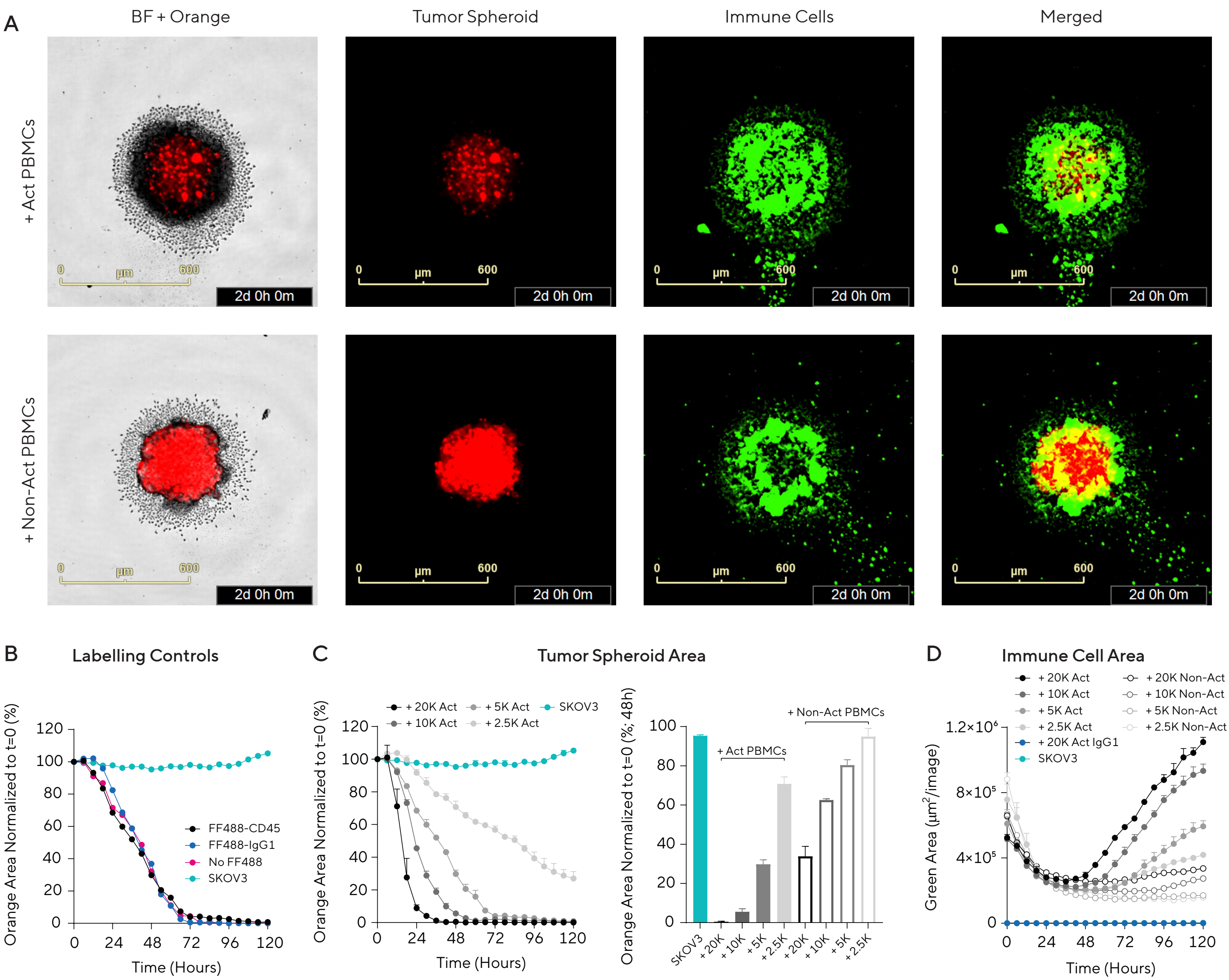

Figure 4. Measure Tumor Death and Effector Cell Proliferation using 2-color ICK Assay. SKOV3 Nuclight Orange spheroids were co-cultured with a density range of activated or non-activated PBMCs in the presence of Fabfluor-488-CD45 or Fabfluour-488-IgG1. Co-cultures were imaged using confocal multiplane acquisition at 4X. A) Max projection images shown at 2 days for E:T 2.5:1. B) Time course of normalized orange area for pre-activated PBMCs (2.5:1 E:T) in the presence or absence of Fabfluor-488 conjugated to CD45 or IgG1 control. C) Time course and bar graph (2 days) of normalized orange area for density range of pre-activated and non-activated PBMCs. D) Time course of green area. Data presented as mean + SEM, n = 3 replicates.

CAR-T Mediated Cytotoxicity of Tumor Spheroids

CAR-T cells are a form of personalized immunotherapy in which a patient's own T-cells are genetically engineered to recognize specific antigens expressed by tumor cells. This activates the patient's immune system to target and destroy cancerous cells.[13] To date, CAR-T cell therapies have shown remarkable results in treating hematological cancers, however developing therapies for targeting solid tumors has proven more challenging.[3,14]

To surmount these challenges and gain more translational insights, researchers have been integrating patient-derived material, such as CAR-Ts or biopsy tissue, into *in vitro* 3D tumor spheroid ICK models. This may improve the predictive value of potency assays in evaluating candidate CAR-T constructs, or other autologous therapies, and aid in the comparison of cell expansion and functional activity for QC purposes.

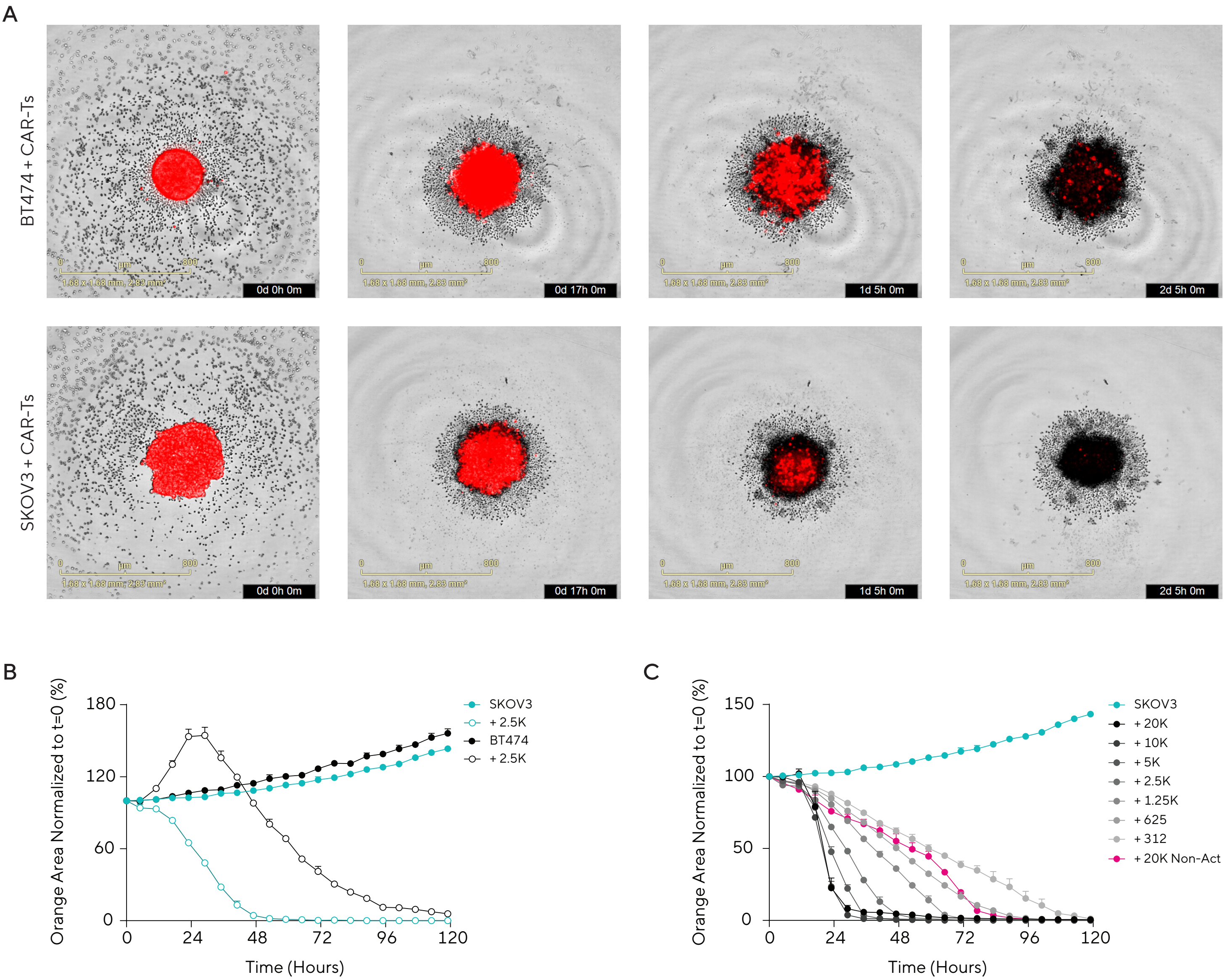

Figure 5. Visualization of kinetic changes accompanying CAR-T mediated tumor spheroid death. HER2-positive BT474 or SKOV3 Nuclight Orange spheroids were co-cultured with a density range of CAR-Ts (anti-HER2) and imaged using confocal acquisition at 10x. A) Representative brightfield and orange fluorescence timelapse images over 2 days. B) Time course of normalized orange area for SKOV3 and BT474 spheroids alone or in the presence of 2.5K CAR-Ts (1.25:1). C) Time course of normalized orange area shown for SKOV3 spheroids co-cultured with density range of CAR-Ts or 20K non-activated T-cells. Data presented as mean + SEM, n = 3 replicates.

To assess CAR-T cell-mediated killing using confocal imaging, we purchased CAR-T cells which were transduced with a second-generation CAR construct, specific for HER2, with reported transduction efficiencies of ~50%. Non-activated T cells were used as a control as matched mock transduced donors were unavailable. Tumor spheroids were formed using HER2-positive BT-474 or SKOV3 cells stably expressing Nuclight Orange. Spheroids were co-cultured with a density range of CAR-Ts (10:1 – 0.16:1 E:T) and confocal images were acquired at 10x every 6 hours over 5 days (Figure 5).

Cancer spheroid morphology is fundamental to their interaction with immune cells[8], and real-time imaging enables observation of the characteristics in solid tumors affecting immune responses. In Figure 5A, the representative images show CAR-Ts inducing target cell death in HER2-positive spheroids with differential killing profiles. In BT-474s, which typically form compact, rounded spheroids with well-defined borders, we see an initial increase in orange area as the spheroid is disrupted, followed by a decrease in area as killing occurs. Conversely, SKOV3 spheroids, which are less compact or uniform, show CAR-T engagement without notable changes in spheroid morphology. Quantification of orange area confirms this temporal response (Figure 5B). We observed an initial increase in area with CAR-T presence and a prolonged killing profile for BT-474s, whereas SKOV3 spheroids displayed a rapid loss in fluorescence. For both HER2-positive spheroids we observed CAR-T density-dependent killing, with data shown for SKOV3 co-cultured with CAR-Ts compared to non-activated T cells at the highest density or tumor spheroid alone (Figure 5C).

The results demonstrate how live-cell confocal imaging can be used for the functional assessment of CAR-Ts and the influence of solid tumor morphologies on cell interactions. Additionally, due to the non-perturbing nature of the assay, this can be coupled with downstream experiments to maximize the biological information obtained from precious cultures. For example, HTS by cytometry can be used for the assessment of cytokines and phenotypic profiling of CAR-Ts as we have shown previously.[15]

Anti-HER2-induced ADCC in 3D Tumor Spheroids

NK cells play a vital role in the immune system by recognizing and destroying abnormal cells, including pathogen-infected and cancerous cells.[16] They exert powerful cytotoxic capabilities, such as releasing cytotoxic granules, executing ADCC, and expressing apoptosis-inducing ligands.[17] Immunotherapies, such as mAbs or antibody-drug conjugates (ADCs), can enhance NK cell activity, for example by increasing ADCC, and can be used to treat challenging solid tumors.[18] ADCs combine both immunotherapeutic and chemotherapeutic interventions for an effective and targeted therapy through leveraging the specificity of therapeutic mAbs as a backbone for the delivery of potent cytotoxic drugs.[19] An increase in ADC development demands reliable techniques to screen and optimize ADCC-drug combinations incorporating more translational 3D ICK models.[20,21]

We assessed NK-mediated cytotoxicity in combination with two anti-HER2-hIgG1 antibodies, a trastuzumab biosimilar or Kadcyla® (trastuzumab emtansine), a therapeutic-grade ADC based on trastuzumab. Trastuzumab-based ADCs are anticipated to maintain the killing function of trastuzumab in addition to the payload-induced cytotoxic effects. An anti-β-Gal-IgG1 mAb was used as an isotype control. Firstly, we used a 3-color 3D ICK assay to assess trastuzumab-induced effects (Figure 6). HER2-positive SKOV3 Nuclight Orange spheroids were co-cultured with NK cells (2.5:1 E:T) in the presence of trastuzumab or hIgG1 control (10 µg/mL). IL-2/IL-12 treatment was used as a positive control for non-specific (natural) NK activation and camptothecin (CMP) was added to tumor spheroids as a positive control for cell death. NK cells were labeled with Fabfluor-488-CD45 or Fabfluor-488-IgG1 in combination with Opti-Green and Annexin V NIR, an indicator of apoptosis. Confocal images were acquired across three fluorescence channels at 10x every 6 hours over 7 days.

Trastuzumab induced NK-mediated tumor spheroid killing as evidenced by a temporal decrease in orange area and increase in Annexin V response compared to hIgG1 control (Figure 6A and 6B). We noted differences in the killing profile of trastuzumab compared to non-specifically activated NKs, which exhibited quicker killing and apoptotic responses. When we examined green fluorescence, we observed successful labeling of NKs using Fabfluor-488-CD45 and an increase in green area for trastuzumab and non-specifically activated NKs compared to hIgG1 control, indicating the beginning of NK expansion post killing at 7 days. Representative fluorescence images at 6 days are shown for the tumor spheroid alone or co-cultured with NKs in the presence of hIgG1 control or trastuzumab. These illustrate the decrease in nuclear fluorescence (orange) and increase in apoptosis (NIR) following treatment with trastuzumab compared to hIgG1 control and allow for spatial discrimination of NK cells (green) at or near the spheroid surface.

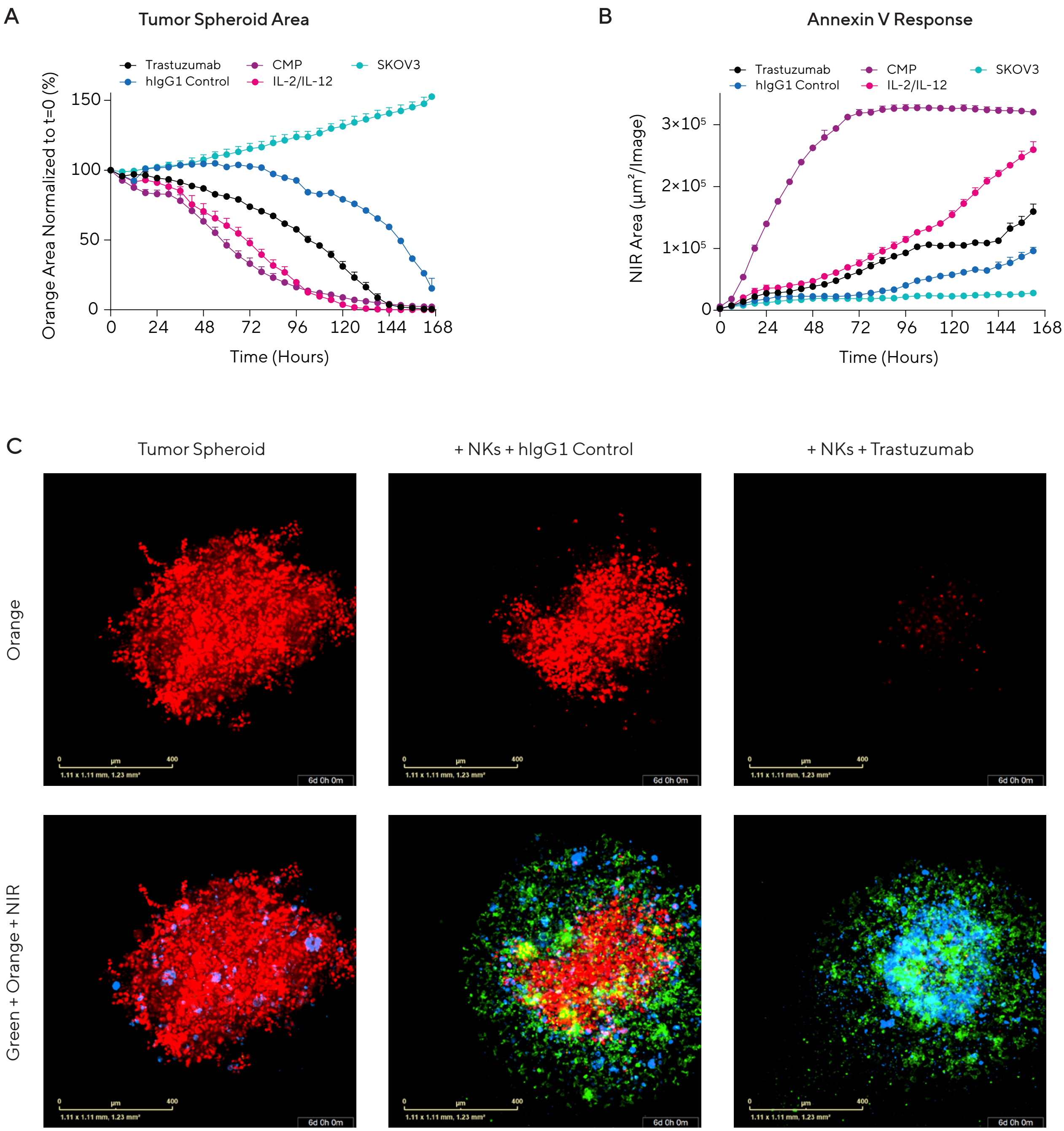

Figure 6. Examining trastuzumab-induced ADCC using a 3-color tumor spheroid ICK assay. SKOV3 Nuclight Orange HER2-expressing spheroids were co-cultured with NKs and treated with trastuzumab or hIgG1 control in the presence of Fabfluor-488-CD45, Opti-Green background suppressor, and Annexin V NIR. Confocal images were acquired at 10x every 6 hours. A) Time course of normalized orange area. B) Time course of total NIR area per image. C) Representative fluorescent images shown on Day 6 for tumor spheroid alone or in co-culture with NKs and hIgG1 control or trastuzumab. Data presented as mean + SEM, n = 3 replicates.

Next, we performed a single-color ADCC assay at 384-well throughput using HER2-positive (SKOV3) and HER2-negative (A549) tumor spheroids (Figure 7). Nuclight Orange spheroids were treated with 2-fold serial dilutions of trastuzumab and Kadcyla® or a single concentration of hIgG1 control in the presence or absence of NK cells (1.25:1 E:T). IL-2/IL-12 and CMP were used again as positive controls. Confocal images were acquired at 10x every 12 hours over 12 days. Intuitive software allows for an overview of the entire 384-well plate and facilitates quick assessments of compound or killing effects (Figure 7A); at 10 days we can see a concentration-dependent loss of fluorescence for HER2-positive, but not HER2-negative, co-cultures treated with Kadcyla® and a more subtle loss for trastuzumab as expected.

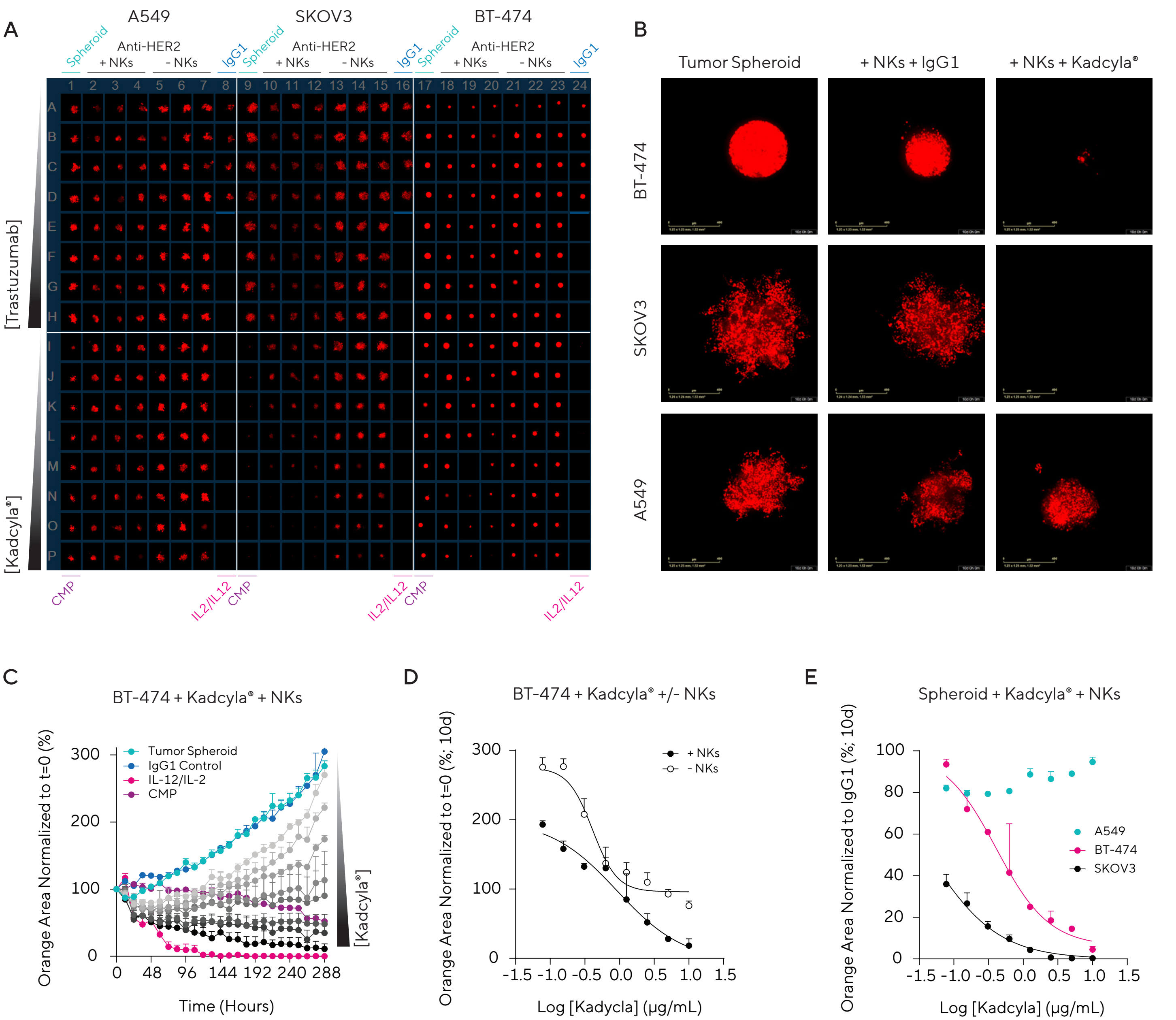

Figure 7. Assessment of ADCC in HER2-negative and -positive tumor spheroids at 384-well throughput. HER2-negative (A459) or HER2-positive (SKOV3, BT-474) spheroids were treated trastuzumab, Kadcyla®, or hIgG1 control in the presence or absence of NK cells. Confocal images were acquired at 10x every 12 hours. A) Vessel View of orange fluorescence across the 384-well plate at 10 days. B) Representative orange fluorescence images of tumor spheroids co-cultured with NKs in the presence of Kadcyla® or hIgG1 control at 10 days. C) Time course of normalized orange area for BT-474 spheroids co-cultured with NKs and Kadcyla® compared to control. D) Concentration-response curves at 10 days for BT-474s treated with Kadcyla® in the presence (closed circles) or absence (open circles) of NKs. E) Concentration-response curves at 10 days for HER2-negative or -positive spheroids in the presence of NKs and Kadcyla®. Data presented as mean ± SEM, n = 3 replicates.

On closer inspection, we can confirm tumor cell death for HER2-positive but not HER2-negative spheroid co-cultures, with representative images shown for Kadcyla® compared to IgG1 control or tumor spheroid alone (Figure 7B). When assessing HER2-positive BT-474 spheroid co-cultures, we can see temporal- and concentration-dependent killing for Kadcyla® over 12 days (Figure 7C).

To confirm that the observed target cell death results from ADCC rather than direct cytotoxicity, we compared BT-474 fluorescence area in co-cultures to conditions without NKs. This revealed a shift in potency for Kadcyla® (IC_{50} values of 0.76 µg/mL vs 3 µg/mL at 10 days, respectively) (Figure 7D). Additionally, we compared concentration-responses for the different HER2-expressing tumor spheroids in co-cultures with Kadcyla® (Figure 7E). Fluorescence area was normalized to hIgG1 control to account for differences in spheroid morphology. The results show concentration-dependent ADCC in HER2-expressing BT-474 and SKOV3 spheroids, with SKOV3s exhibiting increased sensitivity, as evidenced by reduced fluorescence even at the lowest Kadcyla® concentration compared to hIgG1 control. This may be reflective of differences in spheroid morphology and compactness impacting ADC penetration. We observed a slight loss of fluorescence for HER2-negative A549 spheroids towards later timepoints, indicating some non-specific killing.

Overall, these data highlight the capability of multiplexed confocal imaging for 3D ADCC assays at 96- or 384-well throughput, providing translational models for the assessment of immunotherapeutic effects on tumor killing, apoptotic pathways, and tumor-immune cell interactions.

Summary and Outlook

Emerging translational approaches, such as 3D cell models and advanced imaging techniques, are unlocking new potentials to address the challenges associated with treating solid tumors and develop effective immunotherapies. Utilizing live-cell confocal imaging, we investigated the dynamics of T-cell and NK cell-mediated tumor spheroid killing. In combination with multiplexed fluorescence readouts, we demonstrated the evaluation of apoptotic responses and spatial resolution of immune cells to enable the assessment of cell interactions and immunotherapeutic mechanisms of action. Collectively, the findings highlight the effectiveness and versatility of this approach in evaluating 3D spheroid co-culture models and advancing the development of immunotherapies.

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