In recent years impressive progress has been made in our understanding of the basic mechanisms of immune and cancer cell biology. Indeed, this knowledge has translated to true benefits for cancer patients via novel immunotherapies such as checkpoint inhibitor drugs (e.g. ipilimumab (CTLA4), nivolumab (PD-1)) and adoptive T-cell transfer protocols. Despite this, the myriad complexity and dynamic nature of the interplay between the immune and cancer systems leaves many unanswered questions.

In vitro cell-based assays are critical for probing these mechanisms and evaluating new potential treatments. Cell activation, proliferation, and vitality (health, death, apoptosis, etc.) mean-
Continuous live-cell analysis (CLCA) has emerged as a powerful addition to the line-up. CLCA is a non-invasive cell monitoring and measurement method based on time-lapse, phase-contrast microscopy and fluorescence imaging (e.g. IncuCyte® ZOOM, Figure 1-3). In contrast to other methods, IncuCyte CLCA allows visualisation and direct quantification of the full time course of the biology of interest rather than relying on arbitrary end point measures. Moreover, the approach does not require cells to be removed from the incubator and lifting, washing, or labelling with perturbing (and expensive) antibodies is not

Figure 2: Continuous live-cell analysis workflow. In contrast to classic end point reads, images are taken repeatedly over time and analysed on the fly to facilitate real-time decision making.
necessary. Together, this eliminates a wide range of potential assay artefacts and affords significant additional biological insight. Interrogation of the time-lapse movies allows researchers to observe changes in morphology, movement, and spatial orientation of cancer and immune cells over days and weeks. Analysis is fully automated and assays are medium throughput (up to 6 x 384 well plates in parallel), thereby enhancing experimental productivity.

Here we highlight a series of short test cases to illustrate application, utility and value of CLCA to immuno-oncology research.

**Immune-Cell Killing Assays for Biosimilar Antibody Potency Determination**

More than 20 antibody-based drugs for cancer are now approved. These include checkpoint inhibitors and new immunotherapies...
Continuous Live-Cell Analysis of the Immune-Tumor Axis

for neuro-blastoma and multiple myeloma. A wide range of novel antibodies that target the tumor/immune axis are in clinical development. With further approvals, there is a growing opportunity to develop biosimilars, the antibody equivalents of generic copy-cat small molecule drugs. One challenge to achieve regulatory approval is to demonstrate true biosimilarity, particularly in relevant functional bio-assays.

Trastuzumab (Herceptin®) is a clinically used mAb treatment for hER-2 positive solid cancers. To compare the antibody-dependent (tumor) cell killing (ADCC) of trastuzumab and potential biosimilar mAbs, IncuCyte CLCA assays were assembled with co-cultures of hER-2 positive SKOV-3 ovarian cancer cells and peripheral blood mono-nuclear cells (Figure 4, 5). SKOV-3 cells were stably transduced with IncuCyte NucLight Red and plated at 5K cells per well on 96-well plates. PBMCs (25K cells per well) were added at t=0h. Apoptotic tumor cells were enumerated using IncuCyte Caspase 3/7 green

Figure 4: Additional biological insight with IncuCyte ZOOM continuous live-cell analysis. Sequential image montage of immune-cell killing (PBMCs) of SKOV-3 ovarian cancer cells. engagement of cancer cell by immune cells (1) and (2), tumor cell death, granulation & green caspase 3/7 signal (3) and tumor cell division(4).
substrate, a mix and read no-wash reagent suitable for live cell imaging.\textsuperscript{2} IncuCyte images were taken every 2h and automatically analyzed for red (tumor count) and green (apoptotic cell count) objects. In control (vehicle-treated) co-cultures, SKOV-3 cells proliferated over time (0-140h) and there was little or no observable apoptosis until post 100h. Activating PBMCs with anti-CD3/IL-2 caused cell killing as measured by a reduction in both tumor cell count and increase in apoptotic nuclei. Trastuzumab also produced a time-and concentration-dependent inhibition of proliferation, and a concomitant rise in apoptotic tumour cell death, with an IC\textsubscript{50} value of 8ng ml\textsuperscript{-1}. The three biosimilar mAbs each produced comparable killing effects to trastuzumab, with IC\textsubscript{50} values in the range 5-9ng ml\textsuperscript{-1}. Z' values ranged from 0.63-0.83 verifying assay robustness. Inspection of the image- and video-sets for each group verified the immune attack of tumor cells, the morphological hallmarks of apoptotic cell death (e.g. degranulation, nuclear condensation), and the fluorescent signal integrity (target vs. background). 

Figure 5: Quantification of trastuzumab biosimilar antibodies for antibody-dependent cell killing (ADCC) using IncuCyte continuous live cell analysis. Time-course and concentration-response curves for tumor cell number (A) and apoptotic target cell death measured using caspase 3/7 substrate (B). Concentration values in legend are ng ml\textsuperscript{-1}. Microplate views for 4 96-well plates with derived Z' parameters from the high and low control values (C).
Continuous Live-Cell Analysis of the Immune-Tumor Axis

CD47-Tumor Cell Engulfment Assay in Human Bone-Marrow Derived Macrophages

CD47 is a ubiquitously expressed immune-regulatory protein best known for its “don’t eat me” function that prevents phagocytic removal of healthy cells. Many cancerous cells express high levels of CD47, thereby circumventing anticancer immune responses. Based on this, CD47 has become a prominent target in the field of cancer immunotherapy. Indeed, preclinical studies indicate therapeutic benefit of anti-CD47 antibodies in both B-cell malignancies and solid cancers.3,4

CCRF-CEM, a CD47-expressing lymphoblastic leukaemia cell line, was prelabeled with a non-perturbing pH-sensitive dye, pHRodo (IncuCyte pHRodo Red Cell Labeling Kit for Phagocytosis, 250ng mL⁻¹). Following 1h pre-treatment with either anti-CD47 or IgG control, CCRF-CEM (15K cells per well) were added directly to pre-plated (4h) mouse bone-marrow derived macrophages (BMDMs, 10K). In-

Figure 6: IncuCyte continuous live cell assay for anti-CD47 Ab-mediated cellular engulfment of CCRF-CEM by human bone-marrow derived macrophages. Time course (A) and Area Under Curve (B) analysis. Fluorescent Area = total red object area (×10³ (µm²/Image) and Area Under Time Curve units are ×10³ (0-4h). Values are mean ± SD (n=4).
cuCyte images (20x) were taken every 15 min and analyzed for the appearance of red fluorescence objects over time (internalized CCRF-CEM cells in the acidic phagosome of the BMDM). Anti-CD47 caused marked and rapid (<1h), concentration-dependent cellular phagocytosis with a threshold concentration of 40ng mL-1 (Figure 6). BMDMs could be clearly seen to engulf CCRF-CEMs to trigger the appearance of the red signal. IgG had little or no effect and if BMDMs were omitted from the assay no signal was observed. Similar observations were made using antiCD-47 Ab in assays with J774.1 macrophages and pHrodo labelled CCRF-CEMs, albeit over longer time periods (i.e. 24h vs 4h). This assay is suitable for profiling novel CD47 modulators.

CXCR4-Receptor Mediated Chemotaxis of Human T-Cells

Tumor-associated chemokines, such as the CXCR-4 ligand CXCL-12, play a central role in cancer biology, promoting leukocyte infiltration, tumor growth, and immune evasion. CXCR4-mediated chemotaxis (gradient-dependent directional movement) is a key mechanism by which T-lymphocytes and other immune cells are drawn toward the tumor microenvironment. How
ever, tumor cells are able to hijack the chemokine receptor/chemokine system by switching infiltrating leukocytes from immuno-attack to immuno-tolerance. Accordingly, inhibitors of CXCR4 are of great interest as novel immuno-oncology therapeutics.

CLCA chemotaxis assays were conducted using IncuCyte ClearView™ 96-well plates, a novel transwell consumable that incorporates precision, laser-drilled pores in each well for cells to move through (Figure 7). Human T-lymphocytes (5K cells per well) were added to the upper wells of fibronectin-coated ClearView™ plates and monitored for migration via phase-contrast images (10x) collected automatically every 30 min for 24h using IncuCyte ZOOM. Addition of CXCL-12 (3-200nM) in the base-chamber caused clear concentration, and time-dependent chemotaxis, higher concentrations caused more rapid movement than lower concentrations and the measured EC$_{50}$ was 23nM. No directional migration was observed with vehicle control or the inactive chemokine, CXCL-11 (100nM). Co-addition of the CXCR-4 receptor antagonist AMD3100 abolished the response to CXCL-12. Similar observations were made with the
human T-cell line, Jurkat. In 4 assay plates run in parallel the Z' values for the high and low control groups ranged from 0.5-0.8 and CXCL-12 EC\textsubscript{50} values from 19-33nM indicating high assay precision and reproducibility. From the time-lapse videos, the movement of individual T-cells through the pores could be clearly observed.

**Summary**

For each of the immuno-oncology examples provided, IncuCyte\textsuperscript{®} ZOOM CLCA yielded robust, quantitative, and informative cellular assays based on direct phenotypic outcomes (killing, engulfment, directional movement). The key attributes of CLCA are fourfold: (1) the breadth and versatility of the types of assays and measures that can be made (2) the high relevance borne of measurements made non-invasively and from living cells (3) the additional biological insight gained from images and movies over time, and (4) the enhanced productivity provided by fully automated image capture and analysis. Moreover, unlike most microscope systems, IncuCyte is able to readily image and quantify non-adherent cells over long time periods thus allowing studies on hematologic cells.

Together these features enable researchers to probe and quantify the interplay between immune and cancer cells in ways not covered by other methodologies. Importantly, CLCA affords trustworthy and reliable analysis of the activities of new potential immune-therapies. CLCA and IncuCyte\textsuperscript{®} ZOOM is fast becoming an essential and obligate method in the immuno-oncology research field.

**Citations**

Measure dynamic interactions between immune cells and cancer cells

Immune cell activation, chemotaxis and transendothelial migration.

Tumor cell proliferation and metastatic potential.

Immune cell killing of tumor cells and clearance by phagocytosis.

Essenbio.com/immuno-oncology