WhitePaper

September 12, 2019

The Evolution of Immune Cell Killing Assays Using Live-Cell Analysis

How enhanced data analytics and translational models can provide new biological insights

Keywords: live cell analysis, cytotoxic T cells, natural killer cells, immune cell killing, 3D tumor immune cell killing, antibody dependent cytotoxicity

Introduction

In the ongoing battle to understand cancer and develop new therapeutic strategies, researchers are exploring the role of the patient's own immune system in defending the body against tumors. A critical component of this anti-cancer response is the ability of certain immune cells, such as cytotoxic T and natural killer cells, to induce malignant cell death through the process of immune cell killing (ICK). Modeling ICK *in vitro* is therefore of paramount importance.

There are multiple techniques traditionally used to assess ICK, such as flow cytometry and biochemical readouts. While these are valuable tools, they derive measurements from single time point analyses and do not characterize dynamic cellular interactions, limiting the biological insights that can be gained. Therefore, to develop a more comprehensive understanding of ICK, researchers are seeking new assays which can provide complementary information to supplement current workflows.

Scientists have a particular need for methods that can capture, visualize and quantify the dynamic changes associated with ICK. Additionally, as increasingly translational models become more widely used, researchers require flexible ICK assays that can be applied to 3D tumor spheroids as well as adherent and non-adherent 2D co-cultures. This whitepaper demonstrates how the technique of real-time live-cell analysis has evolved to meet these changing requirements of immuno-oncology research.

Assessing immune cell killing in cancer research: The key challenges

Given the complexity of ICK, researchers often face numerous challenges when modeling this process in vitro. One key difficulty is the inability to determine cell-specific cytotoxic signals in co-culture models due to global well measurements. For example, with techniques that measure enzyme release (such as the LDH and GAPDH release assays), it is not possible to determine whether signals derive from the death of tumor or immune cells within the co-culture model. To address this challenge, the ^{51}Cr release assay can be employed to isolate cell-specific signals. However, background signals can still limit the conclusions drawn from this assay as many cancer cells do not effectively take up or retain the chromium label[1,2].

Even with the most reliable analysis of tumor cell cytotoxicity, such aggregate measures of cell death cannot reflect the subtleties of the complex cellular interactions involved in ICK. Different T-cell subsets, for instance, can have distinct functional roles when inducing tumor cell death[3]. Researchers exploring these differences in behavior will need to study the morphological characteristics of the activated immune cells and their spatial relationship with cancer cells. However, methods traditionally used to assess ICK are non-image based and often require cell lifting. Therefore, scientists looking to gather phenotypic and spatial insight into this dynamic process will need to employ complementary techniques.

Alongside the difficulties in obtaining reliable and comprehensive data, traditional assays also lack the temporal resolution required to fully characterize the process of ICK. Since most methods assess parameters at a single pre-determined endpoint, it is not possible to gain insights into dynamic changes in biology[4]. An additional concern is the variability in cell maturity and health at the point of data collection, which can limit the quality and reliability of results. Moreover, when a problem is identified, troubleshooting to determine the cause can involve repeated experimental runs that consume valuable material and can take significant time.

The growing need for flexibility and multiplexing

As cell culture models become more complex, the challenges associated with measuring ICK become significantly harder to overcome. This is a pressing issue in immuno-oncology research, as scientists are often moving away from simple 2D culture systems to embrace advanced 3D models with greater physiological relevance. For example, tumor spheroid models are increasingly employed to reveal how the tumor microenvironment (TME) affects the interaction between cancer and the immune response[5,6]. As a further strategy to gain more translational insights, many researchers are now incorporating patient-derived material such as biopsy tissue or chimeric antigen receptor (CAR) T cells into ICK models, which could help unlock the full potential of personalized medicines. In this expanding landscape of translational cellular research, scientists are increasingly seeking flexible ICK assays which can be adapted to a range of different culture models.

The complex cultures involved in these translational models tend to be more delicate. As such, they are more susceptible to perturbations in environmental conditions, making it technically challenging to obtain reliable results. However, since these fragile cultures are much more precious than cell lines, it is vital to gain the most value from each assay. Consequently, researchers are recognizing the need for new ICK methods that enable better monitoring of cells and have the multiplexing capacity to extract the most information from the smallest possible amount of material. For scientists looking to meet these requirements, a particularly promising approach is live-cell analysis.

Live-cell analysis: A flexible solution to meet new requirements

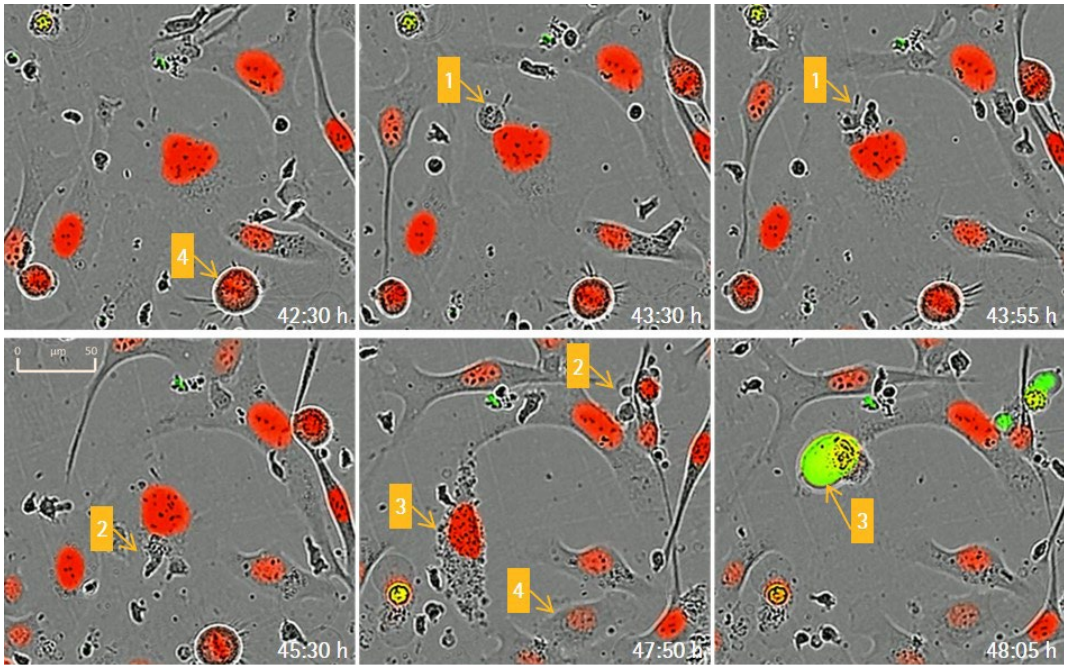
Live-cell analysis utilizes time-lapse imaging to capture the behavior of living cells in real-time. Cultures are maintained upon an imaging platform contained within the incubator, providing a dynamic view of biological events and behavioral changes throughout the experiment. Scientists can therefore continually assess the culture and schedule manipulations and measurements when most appropriate. Such flexibility is a key benefit for researchers contending with biological variability between cultures, particularly when there is the need to maximize the amount of data gained from precious material.

The technique of live-cell analysis can be applied to measure ICK across a variety of *in vitro* models, including both 2D and 3D cultures. In the simplest 2D application, tumor cells are maintained in a basic co-culture with selected immune cells such as T cells or human peripheral blood mononuclear cells (PBMCs) that are activated with soluble cytokines, antibodies or beads. Most typically, tumor cells will be labeled with a nuclear-restricted fluorescent protein which can be quantified as a marker of proliferation, while apoptosis will be measured in a second fluorescence channel using reagents such as Caspase 3/7 or Annexin V. To mitigate the contribution of immune cell apoptosis, size-gating filter settings are employed to exclude the smaller effector cells, thus reducing contaminating signals.

Crucially, since the mix-and-read reagents are added at the beginning of the experiment and the imaging platform is contained within the incubator, cells are undisturbed throughout the duration of the assay. This is a major advantage for delicate cultures which require a stable environment.

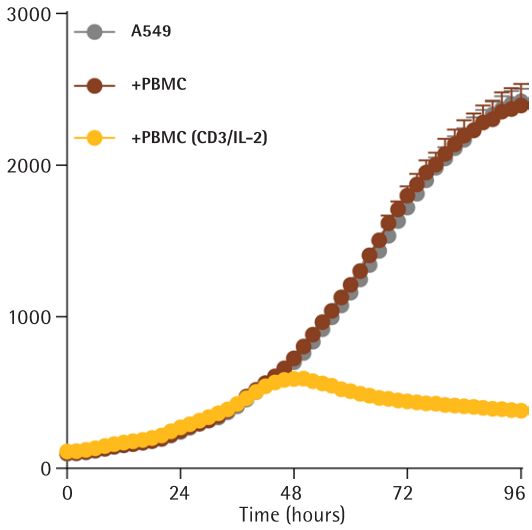
To illustrate the value of live-cell analysis for measuring ICK, A549 NucLight Red tumor cells (2,000 cells/well) were cultured in combination with PBMCs (20,000 cells/well, target: effector (T:E) ratio of 1:10). All wells contained Caspase 3/7 Green reagent to measure apoptosis. To activate the T cells in select wells, anti-CD3 in combination with IL-2 was added. Images were captured every two hours over a period of four days. These images were then analyzed to assess target cell proliferation using the count of red objects (nuclei), and to quantify target cell death using the count of green objects over time (Figure 1). Results revealed that wells containing activated T cells showed signs of target cell death and reduced proliferation.

A.

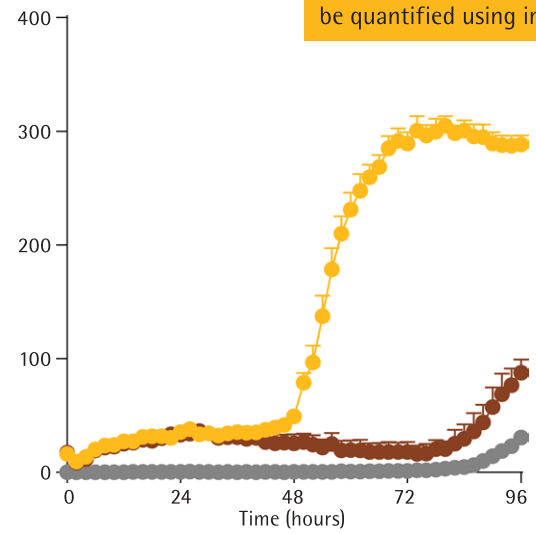


1. Physical contact between a small cytotoxic T cell and a larger labeled tumor cell (red).
2. Tumor cells under attack from a cytotoxic T lymphocyte: The "kiss of death".
3. Tumor cell cytoplasmic granulation immediately followed by Caspase 3/7 labeling (green), nuclear condensation and cell death.
4. Tumor cell mitosis: One cell becomes two.

B. Proliferation
Red Object count



Apoptosis
Green Object count



Real-time images capture morphological changes and cellular interactions, while tumor cell proliferation and apoptosis can be quantified using image analysis.

Figure 1: The use of live-cell analysis to assess immune cell killing in a simple 2D co-culture model. A. Visualization of the interplay between immune cells and tumor cells. B. Quantification of tumor cell proliferation and death in real-time. Images were analyzed for red object count to assess tumor cell proliferation and green object count to assess tumor cell death. Wells in which T cells were activated showed increased target cell death and reduced proliferation.

The images obtained from these wells clearly show the interactions between immune cells and T cells, illustrating the process of T-cell attack followed by cytoplasmic granulation and Caspase 3/7 labeling. In addition to static images, it is possible to create videos to fully capture the process of ICK. This wealth of visual data allows researchers to gather rich biological information and can also prove invaluable for troubleshooting and quality control (QC).

The study presented above demonstrates the basic use of live-cell analysis – to obtain aggregate measures of tumor cell proliferation and apoptosis, and to provide valuable qualitative data showing the interplay between cell types.

Gaining additional insights with enhanced data analytics

As the analytical capacity of live-cell analysis has evolved, scientists have gained new abilities to shed light on ICK. A key step forward has been the development of advanced image processing algorithms to define individual cells. Therefore, rather than simply using red object count to quantify tumor cells, scientists can now employ cell masking software (such as IncuCyte® Cell-by-Cell Analysis) to recognize all cells within the image. Since tumor cells will be labeled with RFP, the cells can then be classified into effector and target populations based on the presence or absence of red fluorescence.

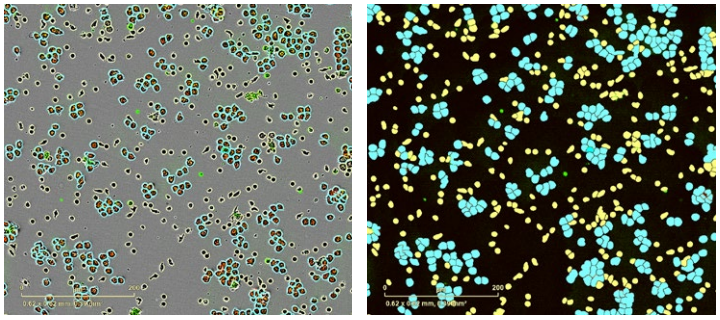
This ability to distinguish different cell types raises several advantages. Firstly, researchers can enumerate effector cells without the use of labels. Secondly, scientists can improve cell classification in studies involving non-adherent target cells. These tumor cells can look very similar to immune cells, making it challenging to isolate target apoptosis from contaminating effector cell signals using the size gating method employed in standard analysis.

To illustrate the use of cell masking software in non-adherent cultures, Ramos NucLight Red cells (10,000 cells/well) were mixed with an increasing ratio of pre-activated or non-activated PBMCs in the presence of Annexin V Green as a marker of apoptosis (Figure 2). Advanced image processing algorithms within the IncuCyte Cell-by-Cell Analysis Software were used to mask individual cells, which could then be classified into target and effector cell populations based on the presence or absence of red fluorescence. The target cell population (red) displayed a decrease in proliferation and an increase in apoptosis in the presence of increasing numbers of effector cells. In contrast, the effector cell population (non-red) displayed proliferation over time, but only for activated cells.

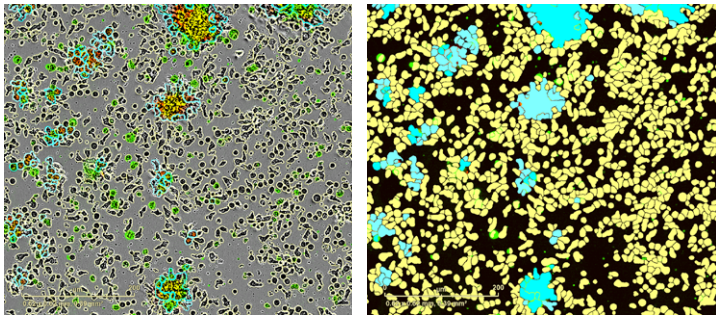
Advances in image analysis software also enable scientists to visualize and quantify interactions between immune and tumor cells. For instance, quantifying the coincidence or overlay of two cell masks enables the assessment of immune and target cell interaction. To demonstrate this, A549 CytoLight Red tumor cells (5,000 cells/well) were cultured with either pre-activated or non-activated PBMCs (25,000 cells/well, T:E ratio of 1:5) in the presence of IncuCyte® FabFluor-488-α-CD45 and IncuCyte® Opti-Green to label the total lymphocyte population (Figure 3). Two hours after PBMC addition, image processing software was used to mask the cells, enabling the spatial information of the target and effector cells to be quantified. Consistent with expectations, activated PBMCs showed a much greater interaction with target cells, aligning with increased ICK.

A. Effector cells (yellow mask)
Target cells (blue mask)

Ramos NucLight Red + act PBMC

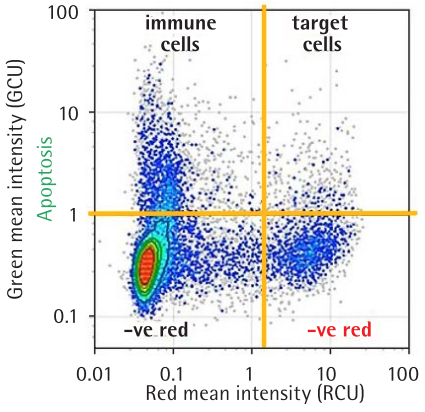


Ramos NucLight Red + non-act PBMC

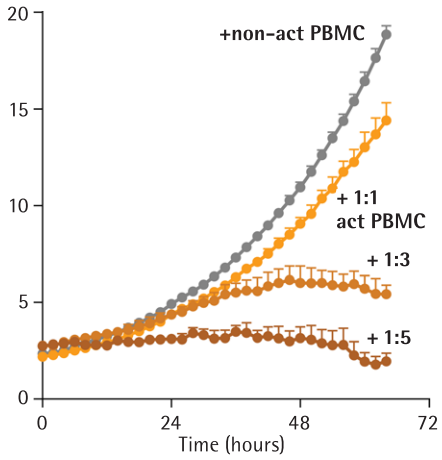


Non-adherent target cells and effector cells can easily be classified into distinct populations with the latest image analysis software.

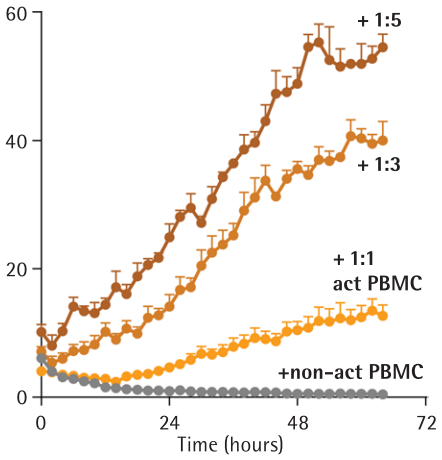
B. Classification of populations



C. Target cell proliferation
+ve Red Pop
Integrated Red intensity x 10^5



Target cell apoptosis
+ve Red Pop
Apoptotic index (%)



Effector cell proliferation
+ve Red Pop
Object count x 10^4

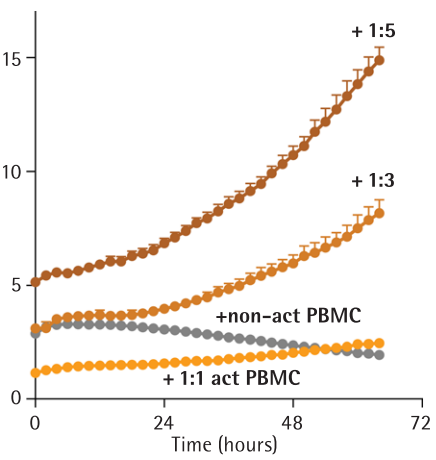


Figure 2: Enhanced image quantification using IncuCyte Cell-by-Cell Analysis Software. A. Images show individual cell masking of the total population with the Cell-by-Cell software. Target (blue) and effector (yellow) sub-populations were distinguished based on red fluorescence. B. Sub-populations were classified based on red and green fluorescence. C. Quantification of proliferation and apoptosis over time. The target cell population (positive red cells) show a decrease in proliferation and increase in apoptosis (% of red cells also green) in the presence of increasing numbers of effector cells. The effector cell population (non-red cells) show proliferation when activated. Data shown as mean +/- SEM, n=4 wells.

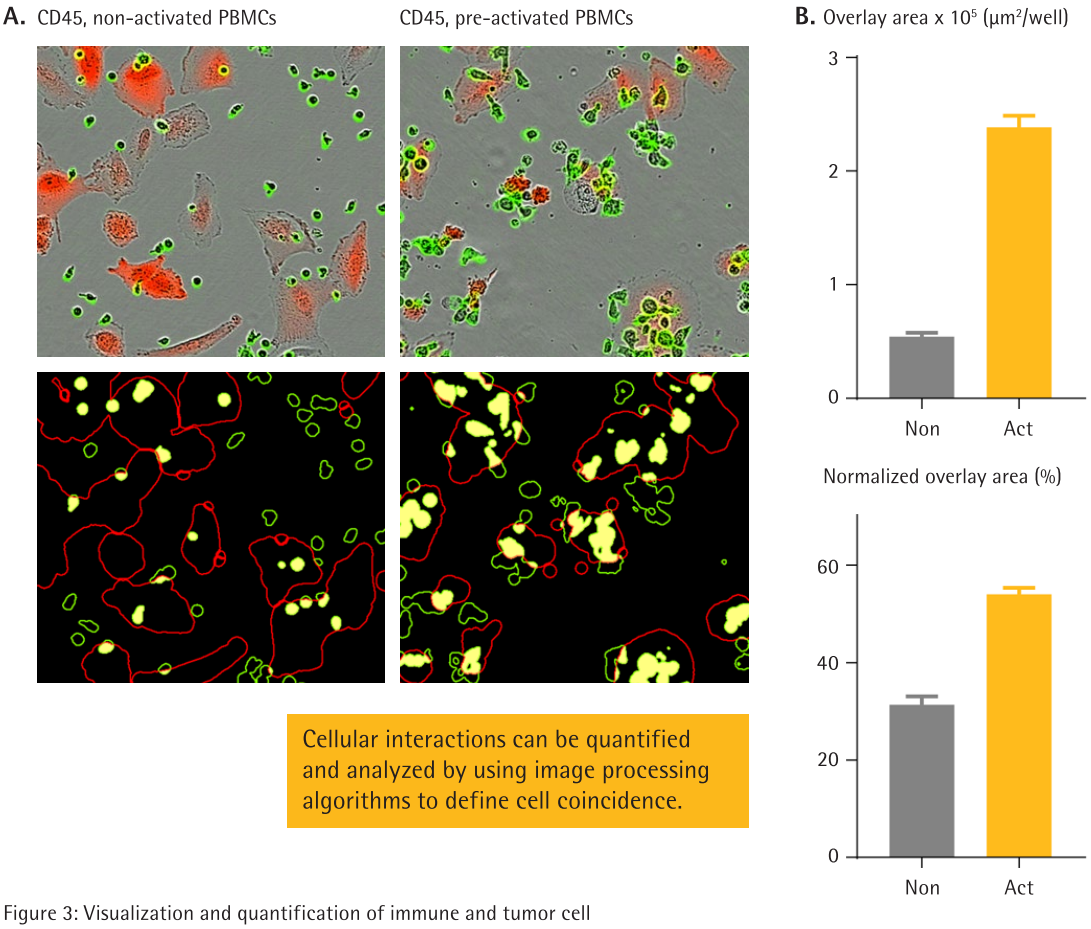


Figure 3: Visualization and quantification of immune and tumor cell interactions. A. Images at two hours after PBMC addition show interactions between CD45+ cells (green) and A549 cells (red). The overlay between the two cell types is shown with the yellow mask. B. Quantification of the overlay reveals a markedly higher interaction for activated compared to non-activated effector cells.

By measuring and quantifying cellular interactions, scientists can gain additional morphological and spatial insights to better characterize the process of ICK. Ultimately, this information could support the identification of novel therapeutic candidates. Assessing the duration of cellular interactions, for example, could prove very valuable for drug discovery programs, since a longer contact time between immune and cancer cells is potentially associated with increased ICK.

A further application of enhanced image processing is to use antibodies specific to surface markers to independently assess distinct effector cell subtypes. This type of subset analysis in conjunction with interaction assessment allows scientists to determine which immune cell populations are interacting with target cells. As a result, this approach could distinguish a greater therapeutic effect on a small subset of cells from a lesser therapeutic effect on all cells. Since heterogeneity between different effector cell types is a major complicating factor in immuno-oncology drug discovery, these new capabilities provide a significant advantage over assays that employ aggregate measures based on consolidating the information from all cells.

Applying live-cell analysis to 3D tumor spheroid models

A growing body of evidence highlights the importance of the tissue environment and architecture in modulating the complex relationship between immune and tumor cells. Consequently, many scientists are now employing 3D models to gain more physiologically relevant data when assessing cancer immunotherapy agents *in vitro*. Indeed, evidence shows that tumor cells cultured in 3D can exhibit heightened resistance to cytotoxicity, which is more reflective of the *in vivo* situation[6].

Tumor spheroid models, which incorporate 3D cellular aggregates formed of cancer cells, provide a useful way of reflecting the 3D TME as they model important features such as cell-to-cell contact and oxygen gradients[7,8]. One type of spheroid model commonly used in ICK research uses single spheroids, aggregates which can be formed by seeding tumor cells in ultra-low attachment plates. These provide a useful tool for studying ICK in solid tumors which may have hypoxic cores.

To demonstrate the use of single spheroid models to assess ICK, A549 NucLight Red tumor cells were seeded in a round-bottom 96-well plate (2,500 cells/well) and allowed to form spheroids over three days. Once formed, spheroids were co-cultured with PBMCs at a T:E ratio of 1:2.5 in the presence or absence of activating cytokines (anti-CD3 and IL-2). IncuCyte high definition phase and fluorescence images were then used to monitor the spheroids over several days. Results show a marked loss of fluorescence intensity for spheroids in the presence of activated PBMCs, reflecting an increased death of tumor cells.

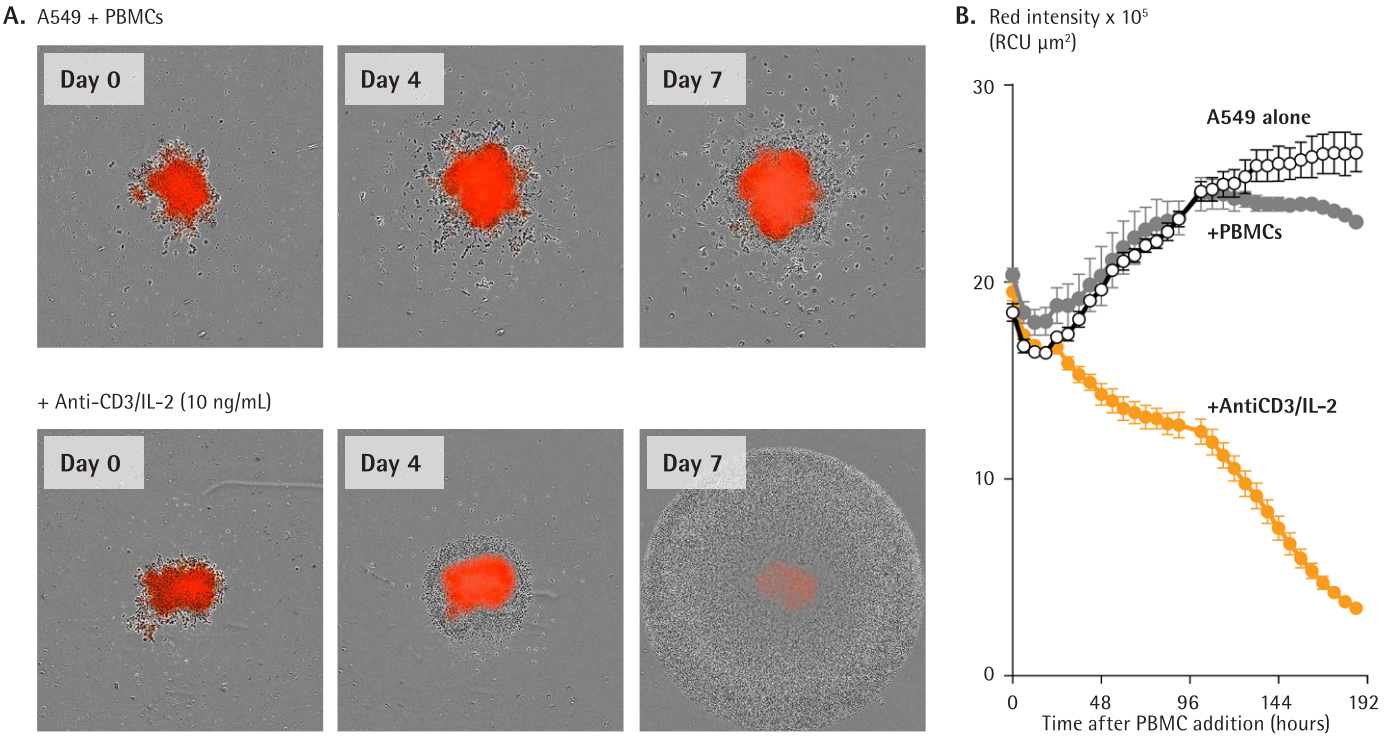


Figure 4: Impact of activated PBMCs on tumor spheroid proliferation. A. High definition phase and fluorescence images compare the effect of PBMCs on spheroid proliferation in the absence (top panel) and presence (bottom panel) of activating cytokines. A marked loss of fluorescence intensity can be seen in the presence of activated PBMCs. B. Time-course plot shows spheroid cytotoxicity quantified as a loss of fluorescence intensity over time. Data was collected over seven days at six-hour intervals. Data shown as mean +/- SEM, n=3 wells.

Multi-spheroid models

Multi-spheroid models, which incorporate components of the extracellular matrix (ECM), provide an alternative option for immuno-oncology researchers. The ECM plays an important role in ICK, as it can influence cellular interactions and affect the infiltration of immune cells into 3D structures. In multi-spheroid models, the biomatrix alters the formation process so that multiple heterogeneous spheroids are created in each well.

Live-cell analysis can easily be employed to measure ICK within these multi-spheroids. An example of one such study is shown in Figure 5. In this assay, the antibody dependent cytotoxicity (ADCC) of Herceptin on Her2 positive cells was assessed. Her2 negative MCF-7 cells expressing nuclear-restricted RFP (MCF7-NucLight Red) or Her 2 positive BT-474 cells expressing cytoplasmically-restricted green fluorescent protein (BT-474-CytoGreen) were seeded in flat-bottom 96-well plates (1,000 cells/well) on a bed of Matrigel®.

Multi-spheroids were allowed to form over three days before Herceptin was added in either the presence or absence of PBMCs (5,000 cells/well, T:E ratio of 1:5). Over the following seven days, brightfield and fluorescence images were used to monitor the spheroids. Results demonstrate a concentration-dependent loss of fluorescence in the presence of Herceptin in only the Her2 positive BT-474 cells and not the Her2 negative MCF-7 cells. A loss of fluorescence intensity was seen in both cell types with the addition of treatments activating the T-cell populations (anti-CD3 and IL-2).

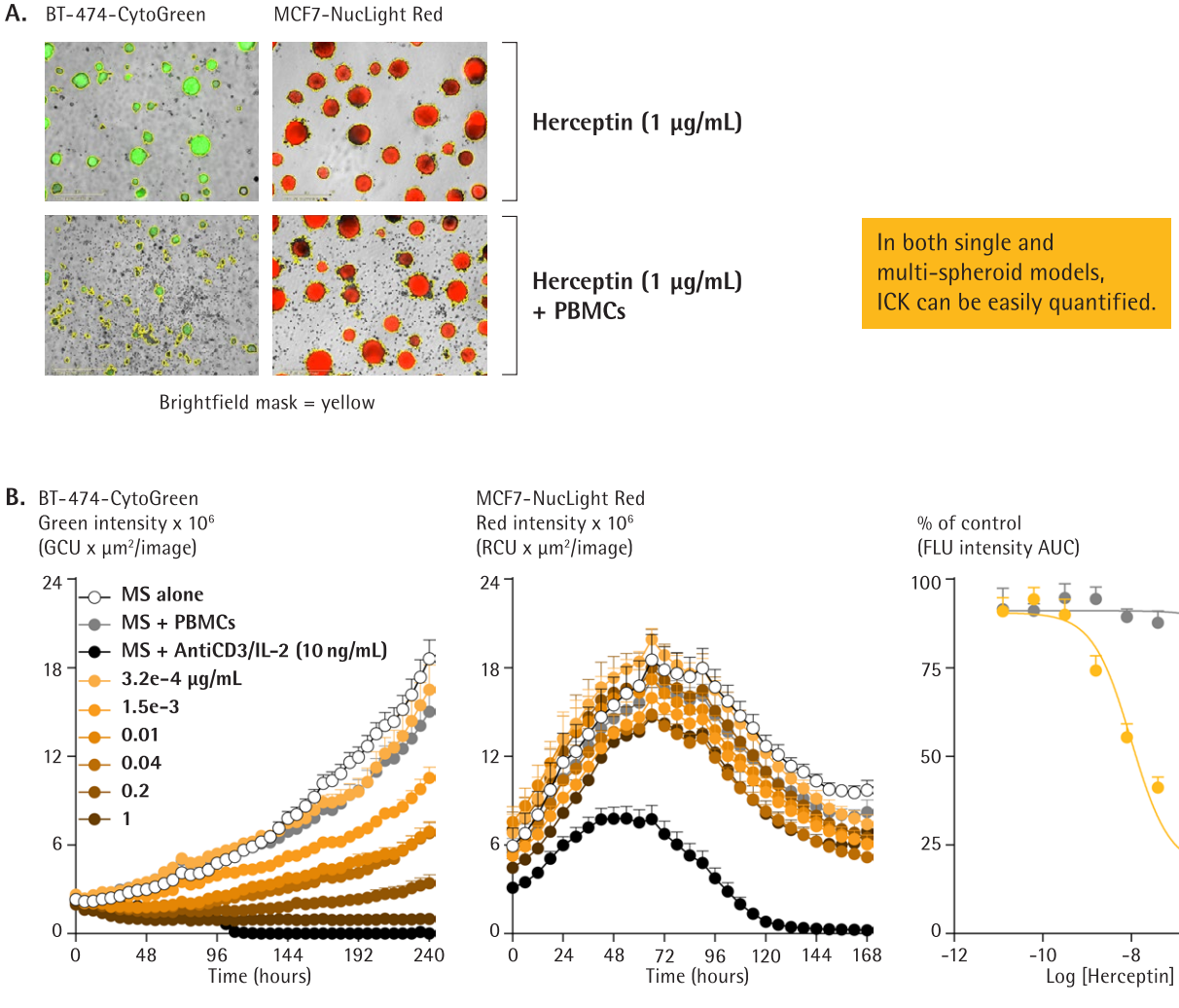


Figure 5: Impact of Herceptin-induced PBMCs on multi-spheroid proliferation. A. IncuCyte brightfield and fluorescence images taken at seven days (MCF7-NucLight Red) or ten days (BT-474) show the effect of Herceptin on spheroid proliferation in the absence (top panel) and presence (bottom panel) of PBMCs. The brightfield outline mask is shown in yellow. B. Time-courses show multi-spheroid death quantified as a loss of fluorescence intensity within the spheroid brightfield object. Increased cytotoxicity was seen with the addition of treatments activating T-cell populations (anti-CD3 and IL-2). Data was collected over ten days at six-hour intervals. Data shown as mean +/- SEM, n=4 wells.

Adapting to the evolving landscape of immunology research

In the quest to better understand the process of ICK, scientists are seeking deeper biological insights from more complex translational models. The evidence we have presented so far shows that live-cell analysis can easily be adapted to facilitate this research. It is also likely that this technique will be applied to newer models as the landscape of immunology research continues to evolve.

Multicultures: Live-cell analysis could be used in multicultures involving three or more cell types. Such models are likely to be increasingly used in immuno-oncology research, given the growing evidence for the important role of interactions with stromal cells in tumorigenesis[9]. For scientists assessing ICK in these complex models, live-cell analysis provides an ideal solution as the technique can flexibly accommodate complex culture models.

Another key translational approach is to incorporate patient-derived material, such as biopsy tissue or CAR T cells, into ICK assays. Again, live-cell analysis can easily be adapted to such studies. Indeed, this method has already been successfully used to develop and test new CAR T-cell constructs and assess their in vitro activity[10,11], and it could also prove useful in the comparison of expansion and activity between batches for QC purposes. So far, the use of live-cell analysis for CAR T-cell therapies is limited to the preclinical stage, but clinical applications are likely to develop as technology and instrumentation advances.

Immune cell infiltration: A further use for these assays could be in assessing the invasion of immune cells into 3D structures. This is a focus of interest in immuno-oncology, as the infiltration of T lymphocytes into solid tumors is correlated with clinical outcomes[12]. However, tumors employ many defense mechanisms which can limit the migration of immune cells. Therefore, the process of immune invasion is a developing area of research for scientists investigating new therapeutic approaches. In fact, this field is expanding fast, with the recent development of novel 3D high-throughput assays to investigate immune cell homing[6]. Given the flexibility of live-cell analysis in adapting to 3D models, it is likely that this method will play a key role in this exciting avenue of research.

Conclusion

In this whitepaper, we have illustrated how live-cell analysis has evolved to address the growing challenges of immuno-oncology research. The introduction of enhanced data analytics, coupled with the validation of this approach in more translational 3D culture models, has enabled live-cell analysis to become a flexible solution to meet the current and future requirements of this field.

Essentially, the unique value of this method stems from its capacity to maximize the amount of data gained from each culture. There are two main reasons for this: firstly, signals of interest will never be missed with real-time monitoring so cultures will not be wasted; and secondly, scientists can now increase the depth of information gathered from each sample by using new metrics and analyses. In this way, researchers can achieve a richer understanding of ICK without additional expense in terms of time or material. This resource-efficiency is crucial for translational models, which often incorporate more precious and delicate cultures.

To gain even greater value from each experiment, it is possible to couple live-cell analysis with other techniques. Since cells are unperturbed throughout the course of the experiment, researchers can remove supernatant samples, or alternatively lift cells after the assay and analyze them with methods such as flow cytometry. As such, scientists can maximize the biological information gained from precious cultures.

Overall, the flexibility and multiplexing capacity of live-cell analysis make this method a valuable solution to supplement traditional ICK workflows, offering great potential to advance immuno-oncology research and therapy discovery.

References

1. L. Zaritskaya, M. R. Shurin, T. J. Sayers, and A. M. Malyguine, **New flow cytometric assays for monitoring cell-mediated cytotoxicity.**, *Expert Rev. Vaccines*, vol. 9, no. 6, pp. 601–16, Jun. 2010.
2. D. L. Nelson, C. C. Kurman, and D. E. Serbousek, **^{51}Cr Release Assay of Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)**, in *Current Protocols in Immunology*, vol. 8, no. 1, Hoboken, NJ, USA: John Wiley & Sons, Inc., 2001, pp. 7.27.1-7.27.8.
3. V. Golubovskaya and L. Wu, **Different Subsets of T Cells, Memory, Effector Functions, and CAR-T Immunotherapy**, *Cancers (Basel).*, vol. 8, no. 3, Mar. 2016.
4. F. Cerignoli *et al.*, **In vitro immunotherapy potency assays using real-time cell analysis**, *PLoS One*, vol. 13, no. 3, p. e0193498, Mar. 2018.
5. E.-T. Verjans, J. Doijen, W. Luyten, B. Landuyt, and L. Schoofs, **Three-dimensional cell culture models for anticancer drug screening: Worth the effort?**, *J. Cell. Physiol.*, vol. 233, no. 4, pp. 2993–3003, Apr. 2018.
6. H. Sherman, H. J. Gitschier, and A. E. Rossi, **A Novel Three-Dimensional Immune Oncology Model for High-Throughput Testing of Tumoricidal Activity**, *Front. Immunol.*, vol. 9, p. 857, 2018.
7. S. Däster *et al.*, **Induction of hypoxia and necrosis in multicellular tumor spheroids is associated with resistance to chemotherapy treatment**, *Oncotarget*, vol. 8, no. 1, pp. 1725–1736, Jan. 2017.
8. S. Riffle and R. S. Hegde, **Modeling tumor cell adaptations to hypoxia in multicellular tumor spheroids**, *J. Exp. Clin. Cancer Res.*, vol. 36, no. 1, p. 102, Dec. 2017.
9. D. Radisky, C. Hagios, and M. J. Bissell, **Tumors are unique organs defined by abnormal signaling and context**, *Semin. Cancer Biol.*, vol. 11, no. 2, pp. 87–95, Apr. 2001.
10. A. E. Foster *et al.*, **Regulated Expansion and Survival of Chimeric Antigen Receptor-Modified T Cells Using Small Molecule-Dependent Inducible MyD88/CD40**, *Mol. Ther.*, vol. 25, no. 9, pp. 2176–2188, Sep. 2017.
11. H. Li *et al.*, **Antitumor activity of EGFR-specific CAR T cells against non-small-cell lung cancer cells in vitro and in mice**, *Cell Death Dis.*, vol. 9, no. 2, p. 177, Feb. 2018.
12. W. H. Fridman, F. Pagès, C. Sautès-Fridman, and J. Galon, **The immune contexture in human tumours: impact on clinical outcome**, *Nat. Rev. Cancer*, vol. 12, no. 4, pp. 298–306, Apr. 2012.

Essen Bioscience,
a Sartorius Company
300 West Morgan Road
Ann Arbor, Michigan, 48108
USA

www.sartorius.com/incucyte
Email: AskAScientist@sartorius.com

USA +1.734.769.1600
UK +44.1707.358688
Japan, Australia and other countries
around the world: +65.6872.3966