Modern immuno-oncology assays enabled by high-throughput image cytometry.

revvity

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Introduction

Immunotherapy utilizes a patient's immune system to interrupt and inhibit cancer progression from both hematologic to solid tumor malignancies. CAR T cell-based therapies, checkpoint inhibitor-based therapies, bispecific antibody therapies, and cancer vaccines are all immunotherapy tools in the ongoing fight against cancer.

As research and development evolves, scientists face additional cell analysis challenges as developing cell therapy products targeting solid tumors requires physiologically relevant *in vitro* models that provide cell-matrix and cell-cell interactions.

High-throughput assays are needed to screen many therapeutic candidates and investigate possible combinatorial treatment strategies. Researchers are also looking into other cell fitness assays beyond viability to better characterize cell therapy products. Revvity is proud to be at the leading edge, offering novel technologies, assays, and research expertise that contribute to the success of immunotherapy development.

Revvity offers a comprehensive array of image-based cell analysis instrumentation offering many unique advantages to the immunotherapy field. This handbook offers real accounts of experiments performed with access to procedures, materials, and methodologies for optimizing cell-based research and development. This content acts as a catalyst for easily obtaining critical insight, removing analytic barriers, and streamlining workflow for advanced scientific development in less time with less headache.

Introduction to image cytometry

An image cytometer captures cellular images in brightfield and fluorescence for quantification in terms of number, morphology, and fluorescent intensity. The image provides visual context to understand the quantitative results and enable concrete comparison between experimental conditions.

Revvity's Celigo® high-throughput plate-based imaging system is a robust platform that can be utilized for monitoring immune cell expansion, product health, viability, potency assays, and *in vitro* characterization of immunotherapy products.

The Celigo system can be utilized to monitor the efficacy of targeted cancer cell killing in a variety of complex assay formats. This highly versatile system can be implemented to characterize and screen a vast array of immunotherapy targets with ready- to-use signal-to-noise assays that take biologists from imaging to quantitative results in less than ten minutes.

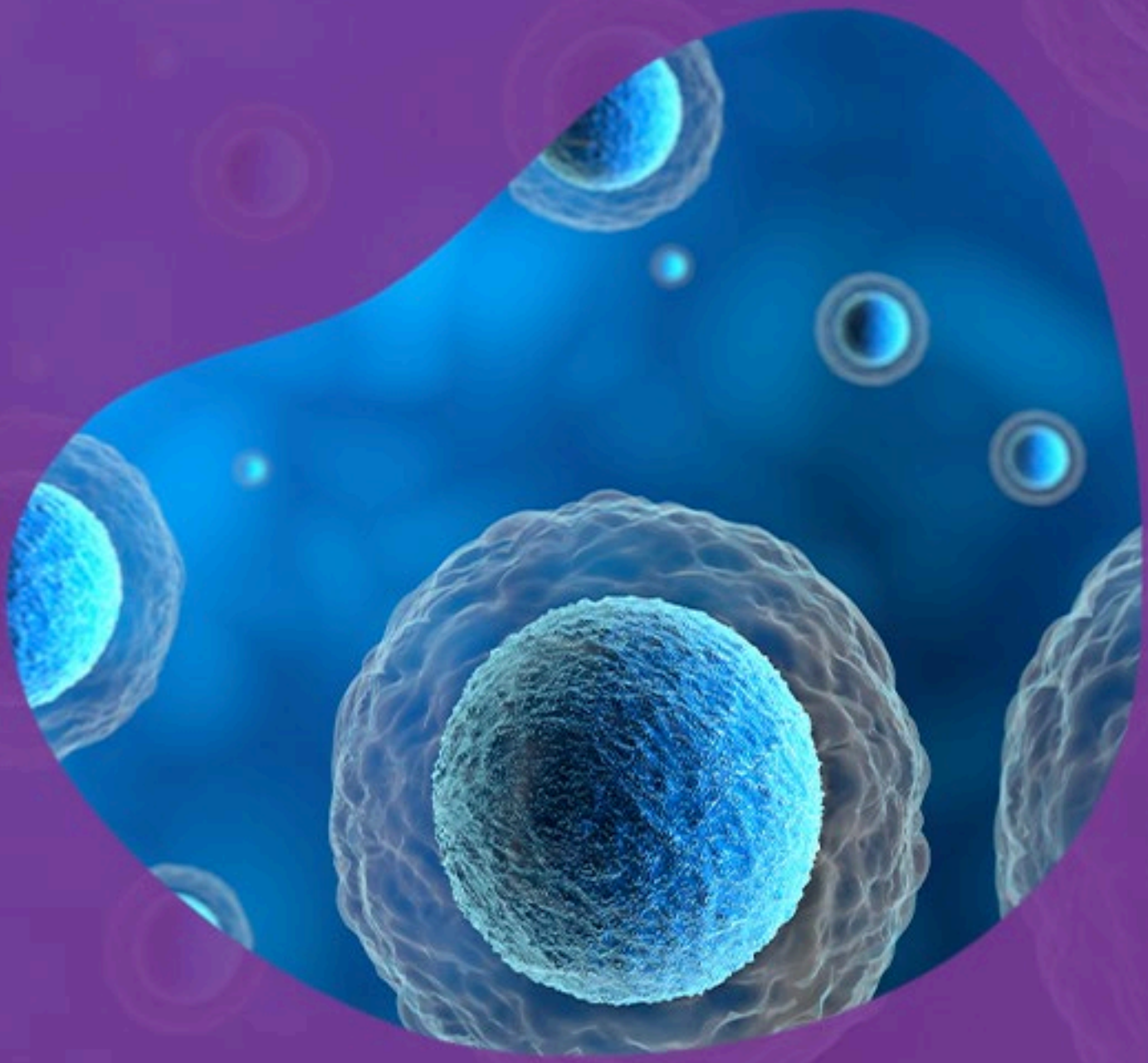
Sensitive whole-well cell level analysis in brightfield and up to four color fluorescence for both adherent and suspension cells.

Built-in gating function allows flow like population analysis directly in plates without the need to terminate cell culture.

From assay development to screening therapeutic target quality and efficiency, Celigo software is designed for biologists, with streamlined workflow and real-time graphic feedback for intuitive analysis throughout the immuno-oncology research and development pipeline.

Chapter 1

Immune cell phenotyping



Introduction

Immunophenotyping refers to the enumeration and classification of a heterogeneous population of cells via surface antigens. It can also provide information on the developmental status and physiological function of an immune cell.[1] Identifying the pathways associated with therapeutic response or resistance is critical to understanding a patient's response to treatment. Obtaining a high resolution of T cell phenotype coupled with genomic and functional information can provide insight into mechanisms of treatment response.[1] The development of novel and efficacious therapies relies on the successful sorting and classification of the correct immune cells for treatment and is also beneficial to understand the cellular composition of the tumor microenvironment.

Immunophenotyping is a valuable tool utilized extensively in biomedical research, clinical diagnosis, and the development of cancer therapies. Conventional fluorescence-based methods routinely used in this type of assay include flow cytometry, fluorescence microscopy, and image cytometry. The goal is to identify various populations including T cells, B cells, natural killer cells, dendritic cells, monocytes, neutrophils, eosinophils, and basophils, as well as circulating stem cells, progenitor cells, and endothelial cells. Additional analysis can be performed to determine activation markers and further sort cell types into their subsets.[4]

Unfortunately, many of the available tools are expensive and the instrumentation is complex and difficult to maintain. Image cytometers offer a cost-effective, quick, and simple option. By combining brightfield and fluorescence microscopy, cell populations can quickly be analyzed for population quantification and percent of differentially labeled cells.

Assay principles

Multi-color flow cytometry-based assays assemble a variety of panels for sorting the major immune cell populations. Current methods remove red blood cells and platelet contaminants and then isolate the lymphocytes. Once the lymphocytes are isolated, enrichment can be done on certain subsets of populations for selection.[2]

There is a wide range of antibodies and fluorochromes used to stain cells in preparation for immunophenotyping.[3] These antibodies target typical clusters of differentiation (CD) markers since they are found on the surface of the cell making immunodetection straightforward. Other cell-surface and intracellular markers for chemokine receptors, cytokines, adhesion molecules, transcription factors, signaling molecules, and glycoproteins are also included in immunophenotyping to further identify specific subsets of immune cells.

Adoptive cell therapies like Chimeric Antigen Receptor (CAR) T cell therapies, involve obtaining immune cells from patients/donors which often require *ex vivo* manipulation and/or expansion.[5] Immunophenotyping is critical to immunotherapy development and implementation to ensure that the correct immune cells are chosen and expanded for treatment. Since high cost and difficulty of use can be prohibitive in traditional fluorescence detection methods for smaller institutions that need quick on-site diagnostics, the Celigo cell imaging system is a sensible option. The cytometer takes both brightfield and fluorescence images. The target cells are then rapidly counted and quantified using the Celigo software. The cell count threshold and fluorescence intensity can be easily adjusted, and data quickly obtained. Cell population analysis is performed with scatter plots and gating, just as with flow cytometry. Additionally, the images can be directly exported into Bitmap files and the fluorescence measurements can be directly exported. The Celigo cell imaging system provides the ability to effectively perform immunophenotyping at a lower cost and potentially boost productivity for clinical diagnosis and biomedical research.

Models/probes and dyes

Antibodies used:

- CD3-AF488 (Clone SK7) (Biolegend)
- CD19-PE (Clone HIB19) (Biolegend)
- CD14-AF647 (Clone HCD14) (Biolegend)
- CD45-V450 (Clone HI30) (BD Biosciences)
- Hoechst 33342
- Human IgG

** Any optimized primary and or secondary antibody can be used on the Celigo for detection as long as the fluorophore used is compatible with the Celigo's four fluorescence channels.*

Methods and Celigo set-up

These assays require cell samples, antibodies, FACS buffer (PBS, 2mM EDTA, 3%FCS), FACS tubes, imaging microtiter plates (Corning 3603 used here), a swing bucket centrifuge, Cellometer automated cell counter, and the Celigo imaging cytometer.

Apheresis and PBMC samples:

Using a Cellometer obtain a cell count for each sample.

Using $1x10^6$-$4x10^6$ total cells:

- Wash cells with cold PBS (~3mL) in FACS tubes. Centrifuge 400g for 5min, discard the supernatant.
- Resuspend pellet in FACS Buffer containing 10µg/mL human IgG to block Fc receptors (5 min).
- Wash with a FACS buffer (~3mL).
- Centrifuge 400g for 5min, discard the supernatant.
- Resuspend cells in 100µL of antibody master mix (FACS Buffer with antibodies at a 1:100 dilution). Incubate at 4oC for 1hr.
- Wash with FACS Buffer (~3mL).
- Centrifuge 400g for 5min, discard the supernatant.
- Resuspend in ~1mL FACS Buffer for imaging.
- In imaging microtiter plates (96-well) add 20µL of stained cell sample followed by 180µL of PBS.
- This should result in 20,000-40,000 cells.
- Centrifuge 100g for 1min, acceleration, and brake set 5 on a scale of 0-9.
- Quantify cells on Celigo.

Expression analysis assay: Target 1 +2 +3 +4 +Mask where the Mask channel is Blue fluorescence. Channels Target 1-4 are set according to the channel compatible with the fluorophore (here Brightfield, Red, Far-Red, and Green).

The Celigo expression analysis assay Target 1+2+3+4+Mask was used. Exposure times are optimized for each fluorescence channel (~ Approximate exposure 50 and 400ms for blue, red, far-red, and green). The Mask channel was set to Blue for either v450-CD45 or Hoechst 33342. The analysis was defined to identify all cells of interest (total population) in the Mask channel. Background correction was applied to each fluorescence channel and object-level fluorescence data was exported directly to a predefined analysis template using FCS Express 6 for scatterplot gating.

Challenges

The major challenge for Celigo in these assays is poor image contrast. This is due to either poor staining (low brightness of dye or poorly binding antibody) and/or high fluorescence background. Antibodies other than those listed here should be optimized for use on Celigo both for dilution and incubation time. High fluorescence background can be significantly mitigated by adhering to the post staining washing steps as described in the methods.

TIPS & TRICKS

Use Celigo optimized fluorophores: V450, BV421, Pacific Blue, AlexaFluor488, FITC, PE, AlexaFluor 568, AlexaFluor 647, APC.

Ideally use antibodies validated for fluorescence microscopy.

Exposure time ranges for typical staining protocols will range from 50ms – 400ms depending on the antibody, clone, binding affinity, and fluorophore.

Centrifuge plates before imaging to ensure all cells are imaged.

Results

In this example, crude human apheresis products and human PBMCs were evaluated and results were compared to flow cytometry analysis performed on the BD™ LSR II (Beckman Dickinson). As shown in Figure 1, immunostaining for each CD marker can be clearly seen in their respective Celigo fluorescence images with a high signal to noise ratio and no crosstalk (contribution of a fluorophore to multiple channels). The high signal to noise ratio for each fluorophore provides a quick visual quality control step for the accurate classification of these cell types. Additionally, a comparison of the brightfield and merged Celigo images illustrates that not all cells in the sample are CD45 +, likely due to incomplete RBC removal. Indeed, nuclear staining with Hoechst 33342 (image not shown) reveals that most CD45 - cells in these samples are anucleated (RBC or debris).

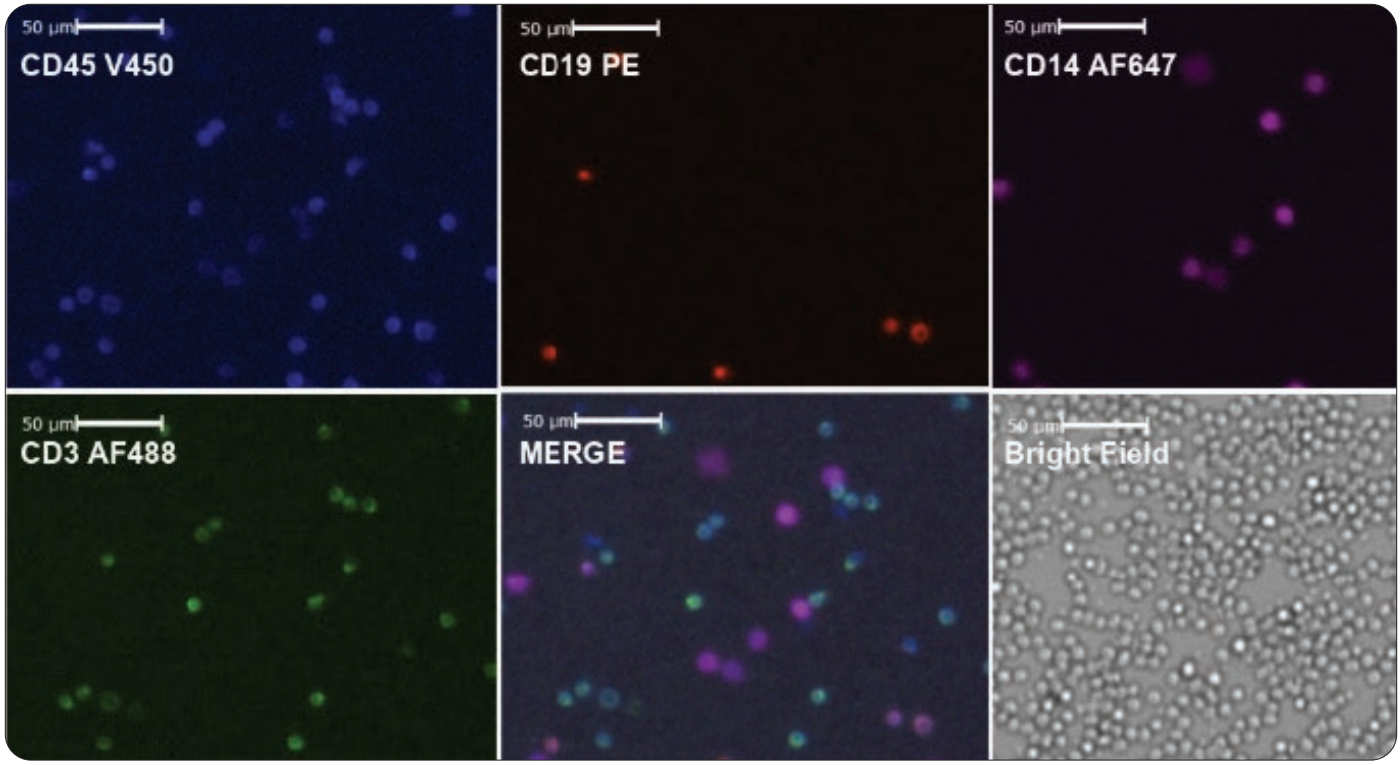
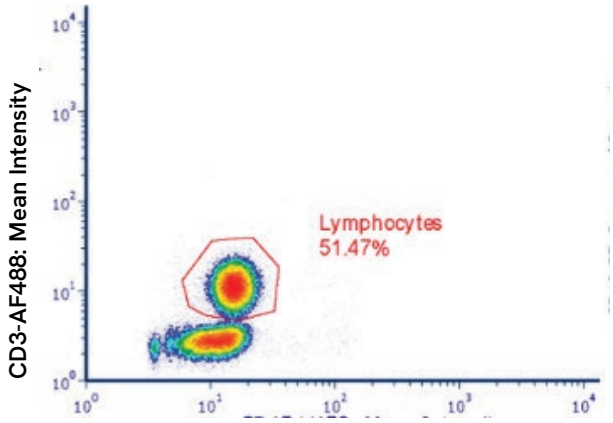
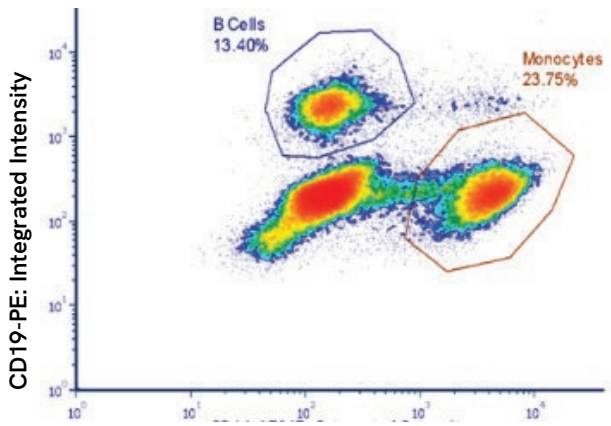


Figure 1. Representative images acquired from Celigo.

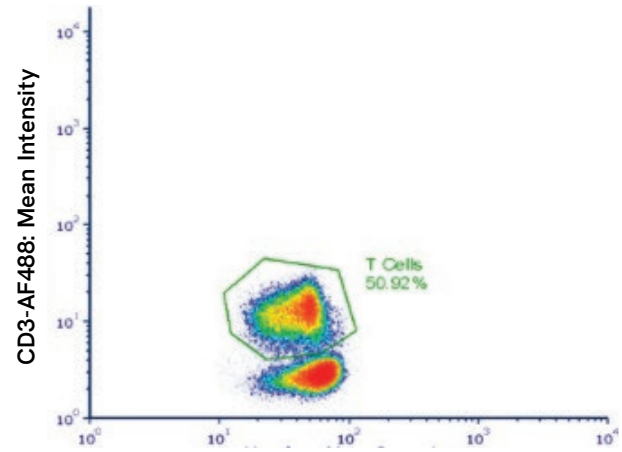


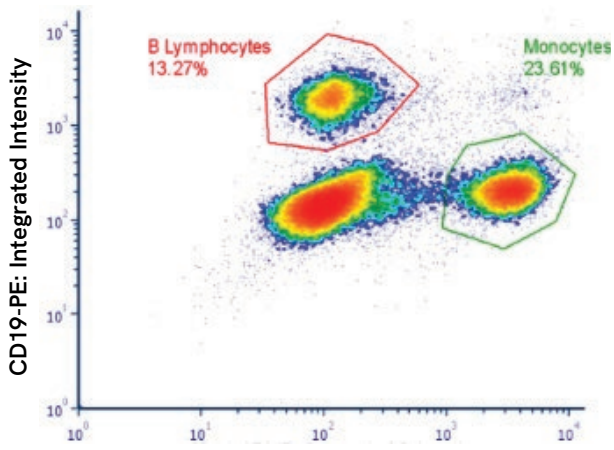


Subsets	% of Total Celigo	% of Total LSRII	# of Events	[cell/mL] x 10^6
Total Leukocytes (CD45+)	N/A 88.62%*	93.3%	68,482	102.72
T Cells (CD3+CD45+)	51.47%	45.8%	32,250	52.87
Monocytes (CD14+CD45+)	23.75%	23.8%	16,263	24.39
B Lymphocytes (CD19+CD45+)	13.40%	19.2%	9171	13.76

*Percent based on sum of subsets - 100%

Figure 2. Human apheresis immunophenotyping using CD45-V450.

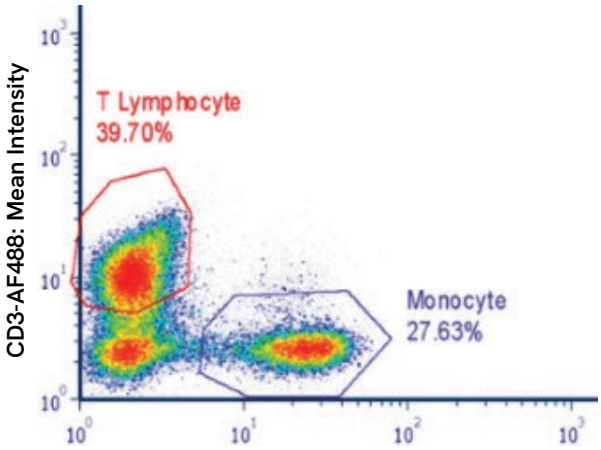


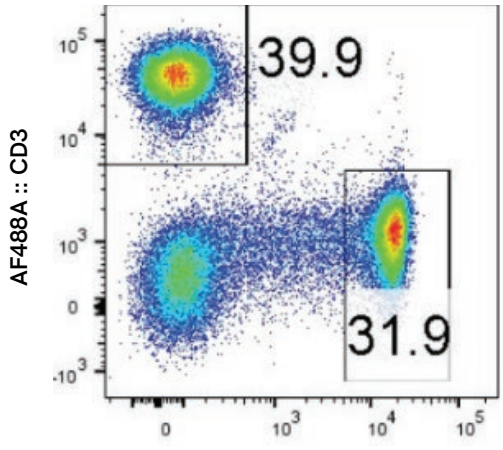


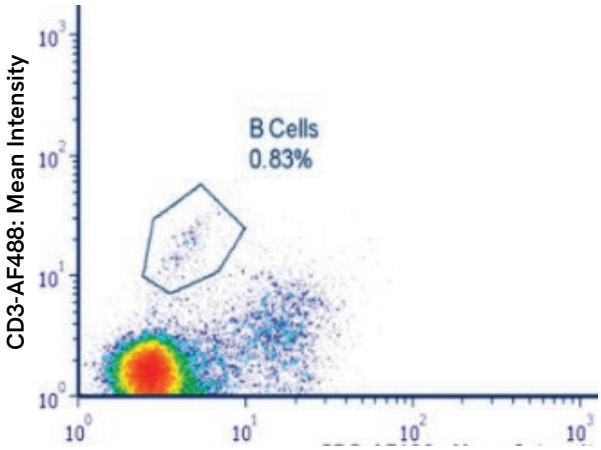
Subsets	% of Total Celigo	% of Total LSRII	# of Events	[cell/mL] x 106
Total Leukocytes (CD45+)	N/A 87.8%*	93.3%	66,877	100.32
T Cells (CD3+CD45+)	50.92%	45.8%	34,057	51.09
Monocytes (CD14+CD45+)	23.61%	23.8%	15,792	23.69
B Lymphocytes (CD19+CD45+)	13.27%	19.2%	8873	13.31

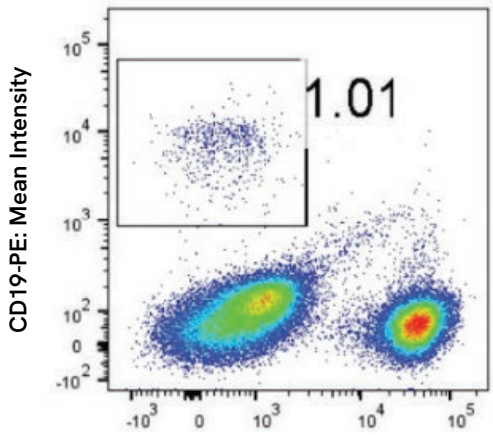
*Percent based on sum of subsets - 100%

Figure 3. Human apheresis immunophenotyping using Hoechst 33342 as a mask.









Subsets	% of Total Celigo	% of Total LSRII	# of Events	[cell/mL] x 106
Total Leukocytes (CD45+)	N/A 87.8%*	93.3%	66,877	100.32
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B Lymphocytes (CD19+CD45+)	**13.27%**	**19.2%**	**8873**	**13.31**

*Percent based on sum of subsets - 100%

Figure 4. Human apheresis immunophenotyping using Hoechst 33342 as a mask.

Conclusions

The Celigo image cytometer allows for rapid assessment of T cell numbers and health using small sample volumes in multiwell plates. This enables ongoing monitoring of patient sample expansion and post-transformation proliferation as well as assessment of cell handling conditions such as cryopreservation and recovery protocols.

Including these methods into the T cell product research workflow will provide increased throughput allowing for more efficient product development. This will enable teams to use more patient samples, and assess multiple transduction and product handling protocols.

Collaborators

Data collected at Dr. James L. Riley's Lab, University of Pennsylvania

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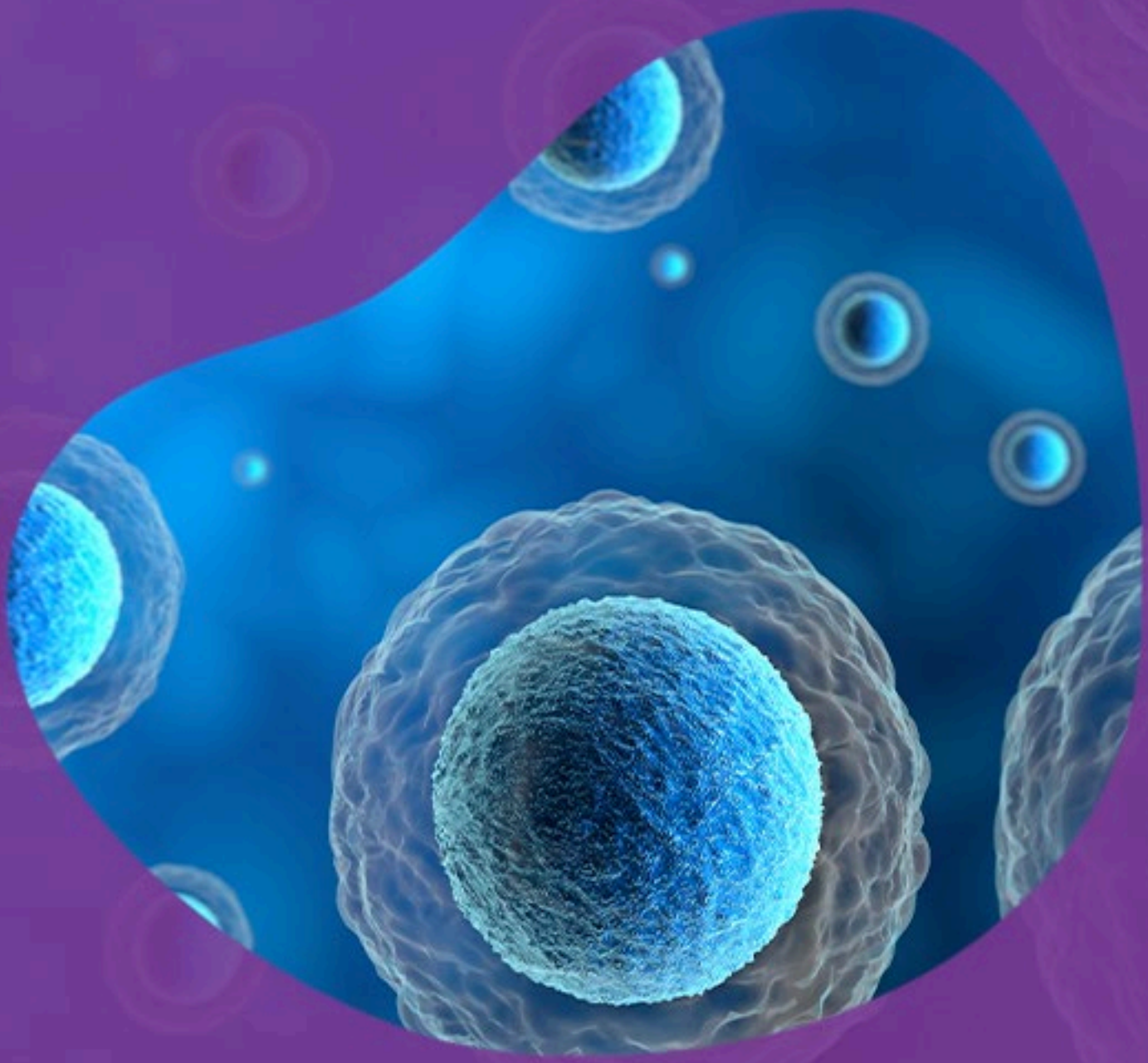
Chapter 2

T cell activation and proliferation



Introduction

T-cell therapies have become one of the major treatment methods against hematological cancers and solid tumors. One critical aspect of T-cell therapy is to characterize the proliferative and activation potential before infusing back to the patients. To determine the T cell proliferation and activation, the cells are commonly measured by Coulter counting devices, which can provide cell concentrations and cell size information. However, this method can be time-consuming and is difficult to produce kinetic data.

The Celigo Image Cytometer was used to perform rapid, high-throughput, and label-free T cell proliferation and activation measurements (Figure 1). First, the Celigo was able to directly count T cells in 96-well plates in brightfield imaging at different concentrations. Next, the Celigo was able to perform kinetic measurement of T cell proliferation and activation for 48 hours to demonstrate the increase in cell number as well as cell size, which are important parameters for the characterization of therapeutic T cells.

The initiation and regulation of the immune response are dependent on the activation and proliferation of T cells. Cancer immune evasion mechanisms prevent the immune system from exerting a powerful response. New and improved cancer immunotherapies using T cells are being developed to target cancer by eliciting a specific and potent immune response to a tumor. The activation of a T cell is initiated by an interaction between an antigen-presenting cell (APC) and the T cell receptor (TCR), and the subsequent engagement of co-stimulatory molecules, such as CD28 receptor-associated co-receptors. Once activated, T cells undergo a phenomenon known as clustering. Clustering is when T cell receptors aggregate at the surface of the cell causing morphological changes. The next phase is activated T cell proliferation, which aids in obtaining high cell densities required for T cell expansions and clinical therapeutic use.

T-cell immunotherapies are a growing area of biomedical research that not only strives to improve current T-cell therapies but also to explore novel designs to use in other disease indications. Many groups are working on genetic modification or selection of T cells to create certain phenotypes to persist *in vivo*, improve potency, and potentially reduce toxicity.

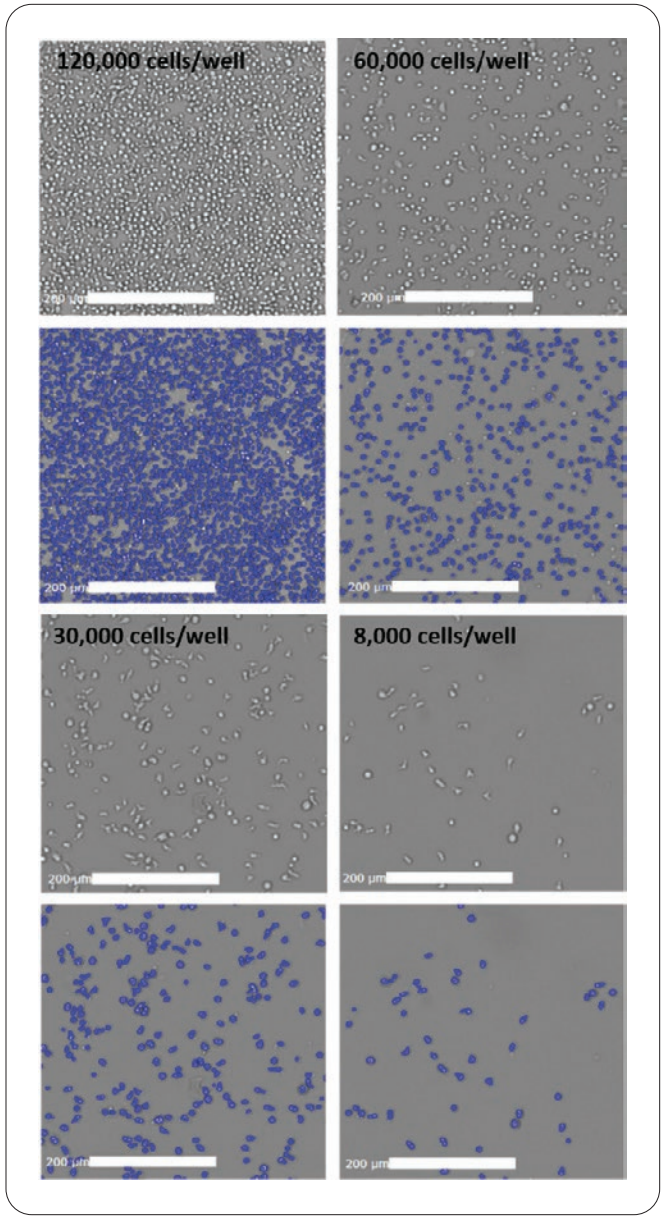


Figure 1. Example brightfield and counted images of T cells in 96-well plate, demonstrating accurate counting using the Celigo Image Cytometer.

Assay principles

Recapitulating the activation and proliferation processes of T cells *in vitro* can be accomplished through the isolation of splenocytes. Primary splenocytes can be stimulated with a variety of compounds depending on the experimental goals. For example, anti-CD3 is commonly used to stimulate T cells to drive proliferation and expands only antigen-specific T cell populations. Pigeon cytochrome C (PCC) peptide is used to stimulate CD4+ T cell proliferative responses derived from PCC-specific TCR transgenic mice. Following stimulation and expansion, cells can be tagged and analyzed by flow cytometry using

antibodies such as anti-Bcl-2, anti-CD4, and anti-Foxp3, which are indicative of apoptosis suppression (cancer marker), helper T cells, and regulatory T cells, respectively. Flow cytometry can identify, sort, and quantify T cells with a desired phenotype or expression of a specific TCR. After sorting, the cells can be reseeded for the proliferation assay and then analyzed using stain or dye like Trace CFSE or eFluor670 to assay for proliferation. Unfortunately, flow cytometry is a terminal assay with a single endpoint that does not recapitulate the dynamic data cellular behavior that T cell activation and proliferation encompass.

Given the limitations of flow cytometry, and the short observation window of classical imaging modalities, it can be difficult to capture the relevant biological processes and mechanisms of T cell activation and proliferation that may dictate drug efficacy. With the ability to take multiple measurements over longer periods, imaging cytometry can capture the dynamic changes, cell-to-cell interactions, and clustering that occurs during T cell activation and provide insight into biologically relevant mechanisms. For example, clustering kinetics can be calculated over time without the need for cell labels, while also eliminating debris for accuracy.

The growth curves and cluster formation can be compared across experimental conditions informing the expansion efficacy of desired T cell phenotypes as well as cell viability, proliferation rate, and doubling times. The Celigo cell imaging system makes it possible to observe these changes and the success of activation in the targeted population in real-time. Decisions can be made immediately to optimize conditions ensuring success. The data generated via growth curves, clustering formation, viability, and proliferation rate can inform T cell selection for novel immune-oncology applications and therapeutics.

Models/probes and dyes

CD4 and CD8 T cells can be isolated from patient blood samples through different separation kits. After collecting the T cells, they can be cultured in 6 to 96-well plates, depending on the number of T cells required for the downstream assays.

The T cells can be stimulated with anti-CD3 and anti-CD28 bound beads for activation. Other stimulation cocktails can be used to induce T cell proliferation, which enables the screening of various media for optimal conditions.

Methods and Celigo set-up

The Celigo Application "Target 1" was used to measure the T cells with a specific diameter in the brightfield channel at an exposure time of ~2,800 µs. The Celigo software was used to directly count the number of T cells on the bottom of the 96-well plates at different concentrations and time points. The ANALYZE parameters for the brightfield channel were set to:

1. "Algorithm = Fluorescence"
2. "Intensity Threshold = 8"
3. "Precision = High"
4. "Cell Diameter = 10"
5. "Dilation Radius = 1"
6. "Background Correction = Check"
7. "Separate Touching Objects = Check"
8. "Minimum Cell Area = 20"

TIPS & TRICKS

To accurately determine the T cell number, the plate must be centrifuged to allow T cells to settle to the bottom for imaging and counting.

The T cells over time may clump and cluster together as they become activated. To accurately count the cells, they can be separated by simply pipetting each well and allowing the cells to settle through a centrifuge for imaging.

Challenges

T cell proliferation and activation assays require the use of 96-or 384-well plates for high-throughput, and high-speed measurement.

Materials and methods

T cell proliferation and activation experiment

The isolated T cells were seeded into each well at 10,000 cells/well with 100 µL of media in a 96-well plate. Next, two formulations of T cell stimulation cocktails were added to separate wells at 100 µL, bringing the total volume to 200 µL. Cocktail 1 has a stronger stimulation potential than the Cocktail 2 formulation. A negative control was also set up without cocktail or growth factors.

On day 0, immediately after adding the T cells and stimulation cocktails, the plate is centrifuged at 1500 RPM for 2 min to allow the cells to settle to the bottom of the well. Subsequently, the plate is imaged and analyzed using the Celigo Image Cytometer to directly count the T cells in each well. The T cells were allowed to incubate with the cocktails for up to 5 days, where the plate is imaged and analyzed on days 1, 2, and 3

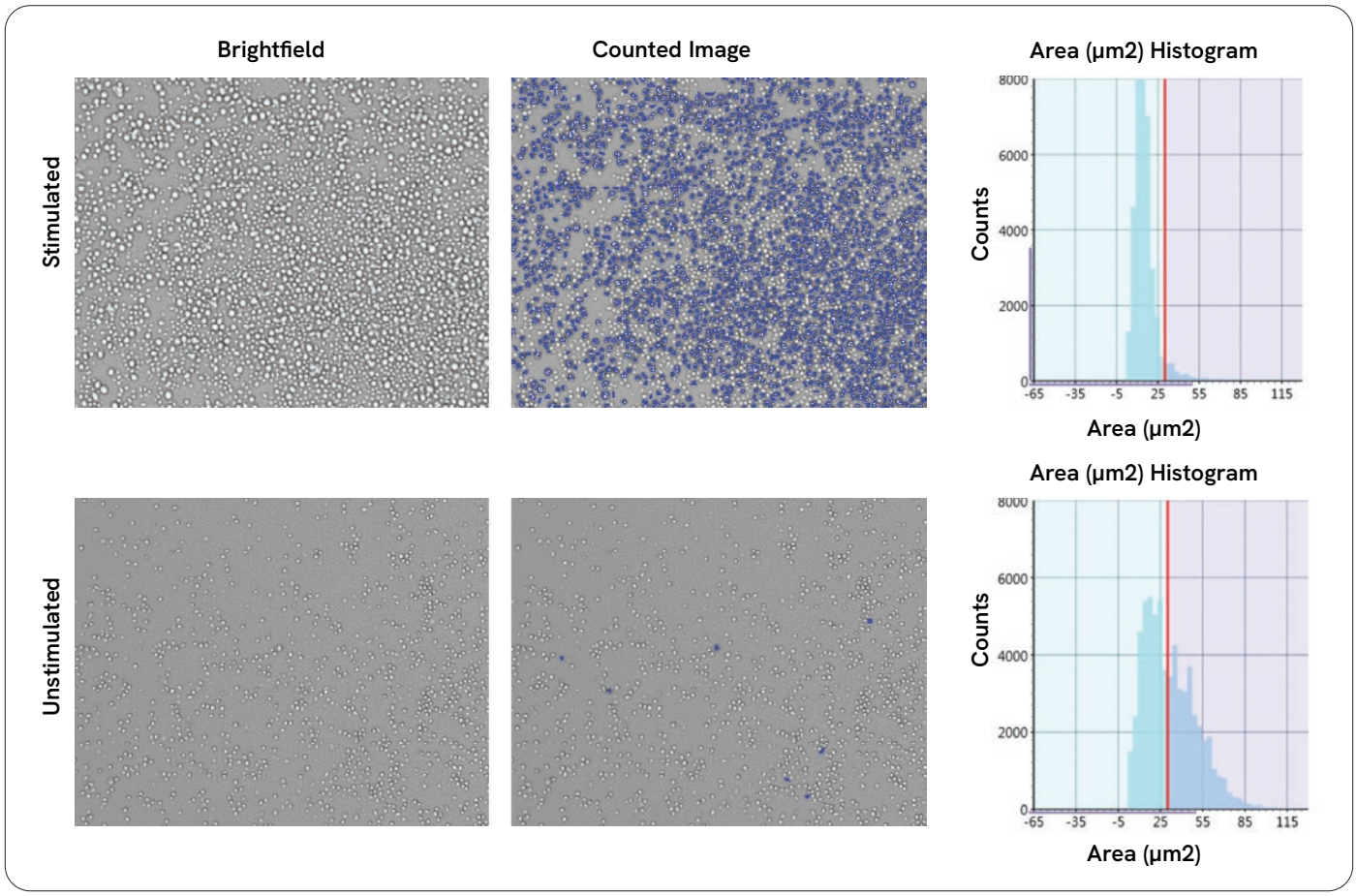


Figure 2. Brightfield and counted images of stimulated and unstimulated T cells, which were directly gated in the Celigo software for counting large, activated T cells.

Results

T cell proliferation and activation results

The Celigo Image Cytometer was used to track the number of activated T cells over time. By using the gating function on the Celigo software, the larger activated T cells can be directly gated at each time point showing the differences in the counted images for stimulated and unstimulated T cells (Figure 2).

The Celigo software can automatically generate growth curves for each well on the 96-well plate (Figure 3). The results again showed that T cells treated with Cocktail 1 showed significant proliferation and activated populations, while Cocktail 2 showed a minimum increase in T cell counting overtime. Finally, the negative control or unstimulated T cells did not proliferate as expected.

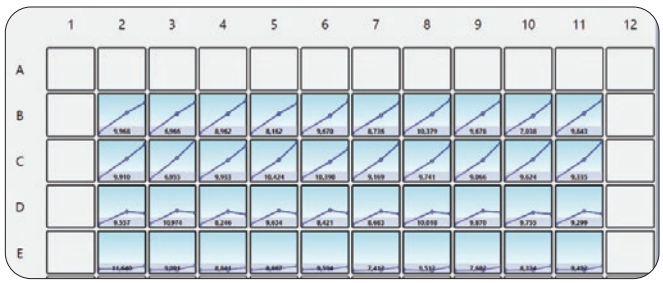


Figure 3. Celigo software-generated T cell proliferation curves for each well.

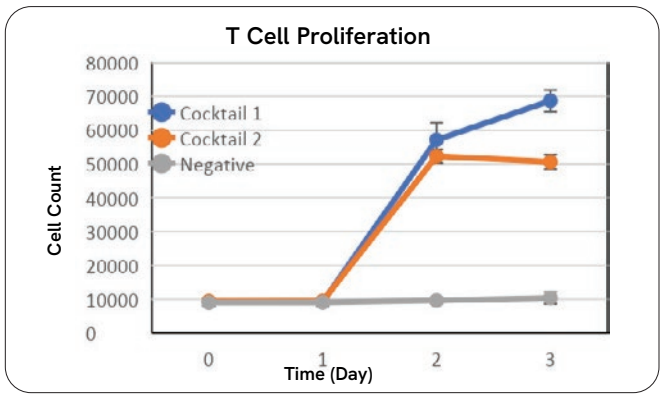


Figure 4. The average cell counts for Cocktail 1, 2, and the negative control, which showed enhanced T cell stimulation for Cocktail 1 compared to 2.

The initial T cell seeding generated cell counting results (Figure 4) ranging from 7,000 to 11,000 cells/well. After 3 days of stimulation, the T cells proliferate to approximately 70,000 cells/well for Cocktail 1 and 50,000 cells/well for Cocktail 2. The negative control remained consistently around 10,000 cells/well.

Additionally, after the stimulation has been induced, the T cells can be fluorescently labeled with specific activation biomarkers to determine the activated populations. The T cells are labeled with CD4-AF488, CD8-PE, CD69-AF647, and Hoechst. The Celigo was used to identify all the Hoechst positive cells, and then analyze specific CD69 positive cell populations (Figure 5).

The results showed the CD69 percentages for Molecule 1 were higher than 2, as expected, where both molecules demonstrated dose-response behavior with respect to the activated T cell populations (Figure 6).

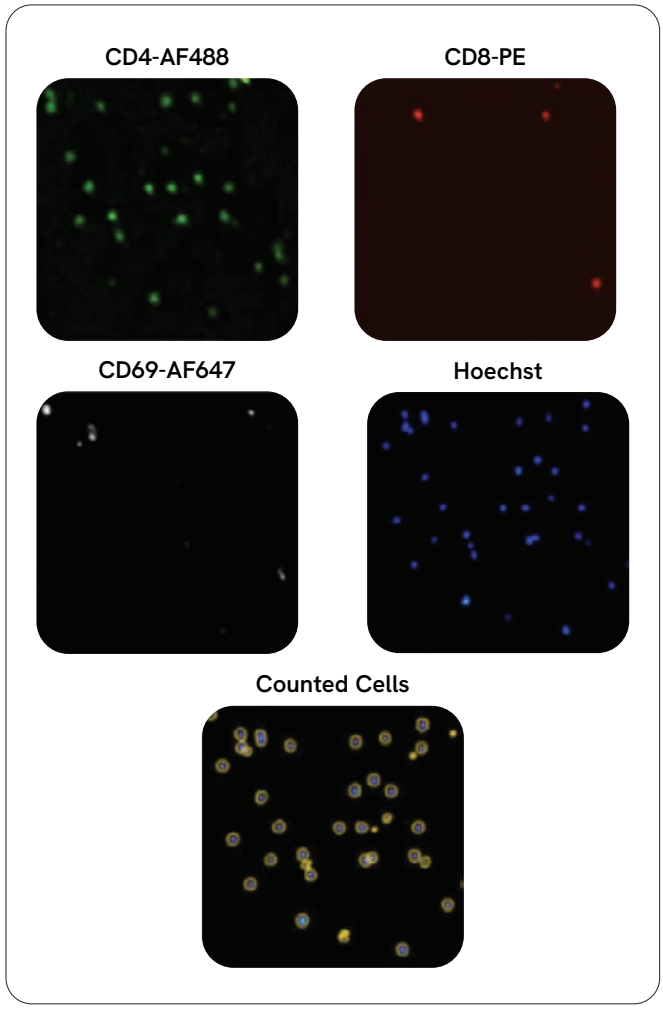


Figure 5. Fluorescent images of T cells labeled with CD4-AF488 (Green), CD8-PE (Red), CD69-AF647 (White), and Hoechst (Blue). The counted cells are circled in yellow outlines.

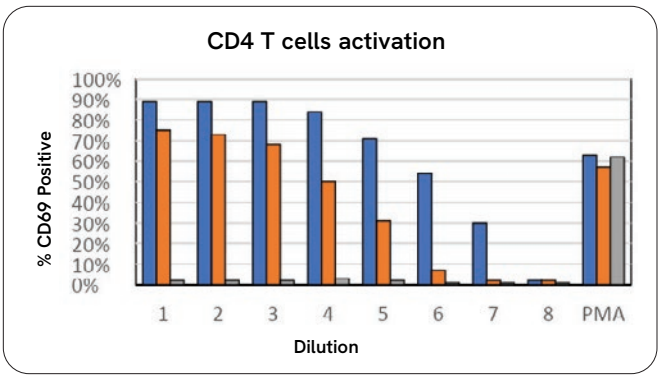


Figure 6. Dose-dependent response of Percent CD69 positive or activated T cell populations for Molecule 1, 2, and IgG (Negative control).

Conclusions

The proposed image cytometry method can scan and analyze the entire well area of the entire 96-well plate in less than six minutes for directly counting T cells. The method is highly efficient and rapid for determining T cell proliferation and activation for downstream assays such as cytotoxicity or ELISA.

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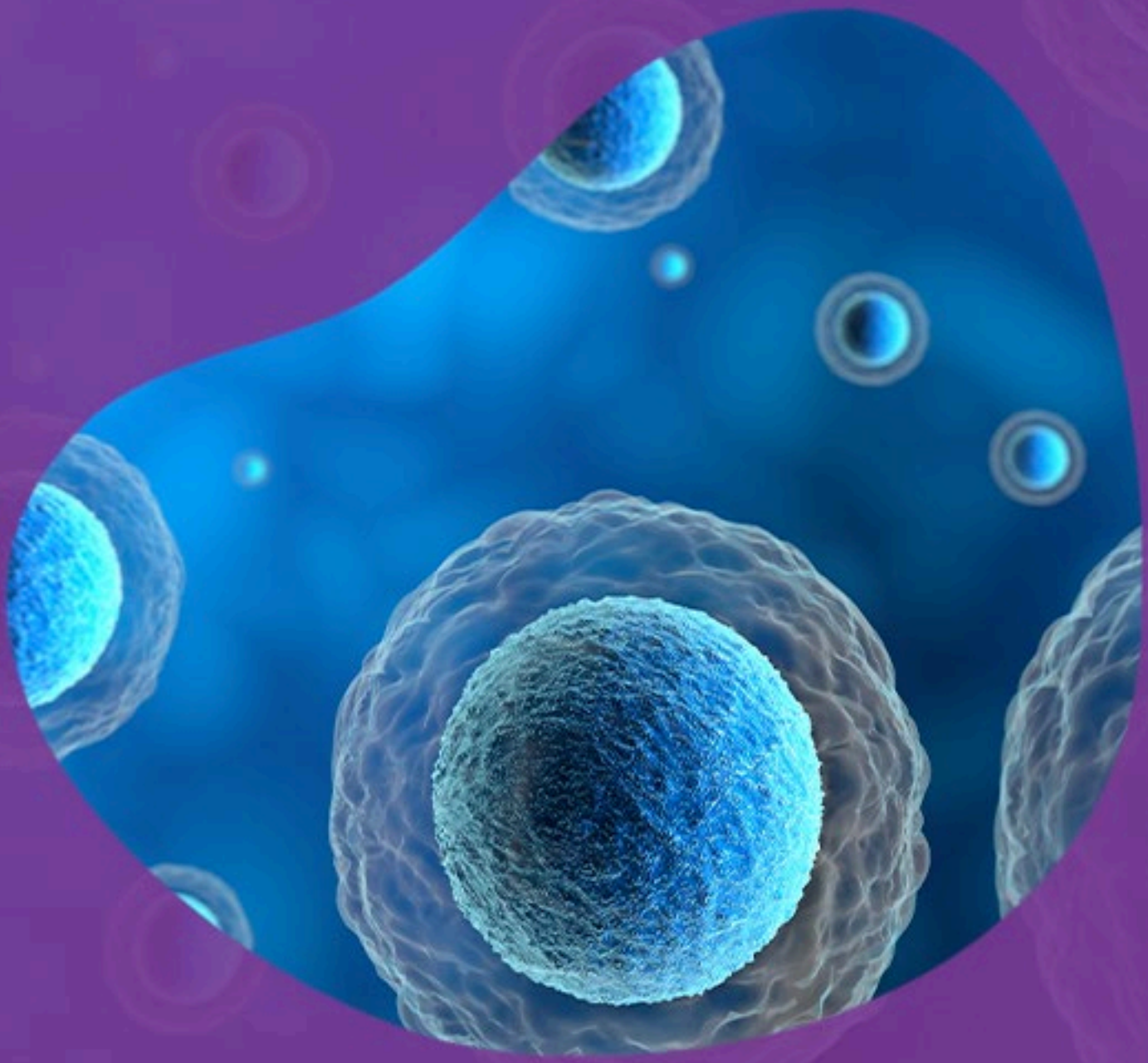
Chapter 3

Assessing T cell viability and fitness



Introduction

T cells play a central role in the body's adaptive immune response, continually demonstrating therapeutic potential in mitigating tumor development and encouraging tumor destruction.1 Chimeric Antigen Receptor (CAR) T cells have demonstrated clinical efficacy in hematological malignancies targeting CD19 in lymphomas and are currently under investigation as a novel treatment for solid tumors.1 Since the T cells themselves are the treatment, it is critical to measure cell number, health, and viability to optimize cell behavior, growth conditions, and treatment efficacy.

CAR-T cell therapies are prepared by T cell selection and/or activation, and genetic modification followed by large-scale expansion and end-of-process formulation.2 In the development of CAR T therapies, a major limitation is the low survival rate of the T cells sorted from a patient's blood. It is critical that the correct subset of T cells is selected for the expansion and that the viable cells are enumerated accurately. T cell viability is a requirement when sorting cells for therapeutic use and experimental studies because their functionality is dependent on producing viable treatments.

When filing an application for an Investigational New Drug, part of the process is to establish the stability of a drug product. In the case of T-cell immunotherapies, stability is dependent on cell health over time. It is beneficial to understand the timeline of maintaining the T cell therapies' stability to translate it to shelf life for clinical use.

Assay principles

Typical cell viability assays fall into the following four categories:

1. Dye exclusion assays (e.g. trypan blue)
2. Colorimetric assays (e.g. MTT(3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide))
3. Fluorometric assays (e.g. protease viability marker assay, glycyl phenylalanyl-amino fluoro coumarin (GF-AFC)
4. Luminometric assays (e.g. ATP assay)[3]

Dye exclusion assays work by staining only dead cells and excluding viable cells. Trypan blue is a common dye exclusion assay that is used at the end of an experiment. While the trypan blue assay is a direct assay that can identify membrane-compromised dead cells, it cannot measure changes in cell viability over time without repeated sampling and also struggles in complex or low viability samples.[3]

Colorimetric assays are indirect, terminal assays that generally measure a biochemical marker that corresponds to a relative value of metabolic activity in cells. Metabolic activity induces a color change which is measured using a spectrophotometer. In the commonly used MTT assay, cell viability is determined by measuring the activity of mitochondrial enzymes via the reduction of MTT to formazan which results in a purple color.[3] Luminometric assays also provide indirect, relative measurements of metabolic activity. Luciferase requires cellular ATP to be active and will cleave luciferin, resulting in a bioluminescence output that is proportional to the amount of ATP present.

Fluorometric assays have a wide range of methodologies and reagents, typically using fluorescence plate readers, flow cytometers, or fluorescence imaging to measure readouts. One example is the GF-AFC assay, which measures protease activity in cells and results in decreased fluorescent signal as viability decreases. The substrates are relatively non-toxic and the cells can be measured and then used for subsequent assays.3

These typical viability assays generally indirectly assess a homogenous cell population. For T cell therapy, upstream processes include selecting T cells from patient blood samples. Samples are therefore heterogeneous and complex, and usually require pre-processing red blood cell (RBC) lysis. 2

Instead, rapid assessment of cell viability can be accomplished using Acridine Orange and Propidium Iodide AO/PI. The AO/PI method is appropriate for primary samples such as whole blood or PBMC samples that have been enriched via leukapheresis or density gradients as well as cultured cells.

AO is cell-permeant, will enter all cells, and fluoresce green in the presence of Genomic DNA (nucleated cells). PI is cell impermeant and will only be able to enter cells with a compromised membrane. Upon entering a compromised cell, PI will suppress AO fluorescence (through FRET), bind the nucleus, and fluoresce red. This will result in green intact nucleated cells and red membrane-compromised nucleated cells (dead). Debris and red blood cells will not be fluorescent.

The Celigo cell imaging system can measure AO/PI viability in a heterogeneous cell population like peripheral blood samples, reduce perturbation of the T cells by negating the need for RBC lysis, and be used as a quality assurance check on the AO manufacturing process of T cell expansion and end-of-process formulation. Taken together, the ability to quickly, reliably, and efficiently secure a sufficient number of viable T cells increases the chances for success in the development of immunotherapies for clinical use.

Models/probes and dyes

- PBMC, Expanded T cells, or CAR T cells
- ViaStain™ AO/PI Staining Solution (CS2-0106-5mL, Revvity)
- ViaStain™ Live Caspase 3/7 Detection for 2D/3D Culture (CS1-V0002-1, Revvity)
- ViaStain™ PI Staining Solution for Apoptosis (CS1-0116, Revvity)

Methods and Celigo set-up

These assays require cell samples, imaging microtiter plates (recommended: Greiner 675090), viability reagents, a swing bucket centrifuge, and the Celigo imaging cytometer.

AO/PI cell count and viability

First, 75 µL of a 10% AO/PI solution in Phosphate buffered saline (PBS) is pipetted into each well of the plate followed by the addition of 5-25 µL of cell sample. This will result in accurate cell counts up to a stock sample concentration of 3.6 x10^6 cells / mL (25 µL cell sample) to 1.8 x10^7 cells / mL (5 µL cell sample).

The microtiter plates are then centrifuged in a swing bucket centrifuge at 400xg for 3 minutes with the acceleration and brake set to 5 (on a scale from 0-9).

Cells can be quantified on the Celigo image cytometer immediately after centrifugation using Green and Red fluorescence channels. Live cells are AO (green) positive and cells with a compromised membrane are PI (red) positive. If the user specifies the cell volume added to each well under the Analyze tab, the Celigo software will report the concentration of both the Live and Dead cell populations.

Caspase 3/7 with PI

75 µL of Caspase 3/7 (2µM) and PI (2µg/mL) in culture media are placed into each of the wells of the culture plate followed by 5-25 µL of cell sample. This will result in accurate cell counts up to a stock sample concentration of 3.6 x10^6 cells / mL (25 µL cell sample) to 1.8 x10^7 cells / mL (5 µL cell sample).

The plates are then replaced in the incubator for 40-60 minutes.

If needed, T cell clumps can be disrupted by gentle resuspension with a multichannel pipette followed by centrifugation using a swing bucket rotor centrifuge for five minutes at 400xg for 3 minutes with the acceleration and brake set to 5 (on a scale from 0-9).

Cells can be quantified on the Celigo image cytometer immediately after centrifugation using Brightfield, Green, and Red fluorescence channels. Live cells are unstained, Caspase 3/7 positive cells are green fluorescent, and cells with a compromised membrane are PI red positive.

Challenges

The Celigo can accurately count a monolayer of T cells (up to 180,000 cells in a full-sized 96-well). However, active T cell clumps and piles of cells pose challenges for accurate counting. To overcome these challenges, T cell clumps can be dissipated by pipetting followed by centrifugation, or fewer cells can be placed in the dish: try 10 µL instead of 25 µL. If cells are piling up at the edges of the well during centrifugation either the swing buckets are not clean/lubricated, or the acceleration and break need to be reduced.

With the Caspase 3/7 reagent, culture media that has significant green autofluorescence can be a problem. Instead, use Hanks Balanced Salt Solution (HBSS) or a culture medium designed for live-cell imaging like Fluorobrite (Thermo).

TIPS & TRICKS

Disrupt cell clumps by pipetting followed by centrifugation.

Set centrifuge acceleration and break to half power to avoid cell clustering at the edges of the well.

Results

The process of producing CAR T cells starts with the expansion of patient leukocytes. As shown in Figure 1A, initial PBMC samples contain platelets and RBC that can confound both manual and automated cell counting methods that use brightfield alone (such as Trypan blue). However, AO/PI staining clearly identifies the nucleated cell population of interest, while also measuring its viability. At the early stages of expansion (day 3) of the patient samples, there is still debris and overall viability can decrease somewhat (73%) as non-stimulated cells begin to die and cells form clumps (Figure 1B). At later stages of initial and post-CAR transformation expansion, actively proliferating cells are dominant, with little to no debris and high cell viability (Figure 1C).

The use of the Celigo to quantify T cell health allows researchers to rapidly screen large numbers of PBMC donors and culture conditions without sacrificing a large portion of the cell sample. Typically, imaging and quantification of cell density and viability for 96 samples is accomplished in 9 - 15 minutes.

Post cryopreservation of T cell products, a more sensitive viability assay is called for. While AO/PI is an excellent viability reflection of fresh or growing cultures, it is ultimately a measure of cellular membrane integrity. In Figure 2, assessing viability in this way would overestimate the truly viable portion of cells in media conditions 1-3. By assessing caspase 3/7 activity, those cells with intact membranes that are undergoing apoptosis can be eliminated from the true viable cell count. With the combination of caspase 3/7 dye and PI only, those non-fluorescent cells can be considered truly viable. It should be noted that the caspase 3/7 and PI method is unsuited for PBMC samples because RBC and other debris could be miscounted as viable (unstained) cells, thus overestimating the viable cell count. Therefore, it is critical to choose the appropriate combination of reagents to measure T cell health at different stages of development.

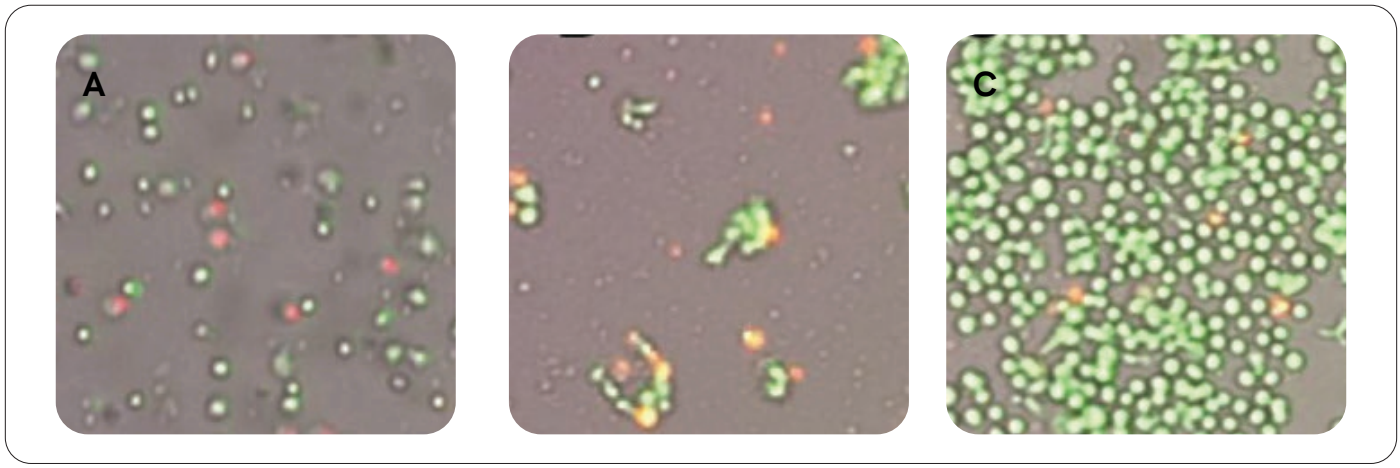


Figure 1. AO/PI viability of PBMC and CAR-T cell culture. 5-25µL of cells are sampled during PBMC culture to expand patient T cells and after CAR transduction. The green AO signal highlights all live nucleated cells while red PI fluorescence highlights nucleated cells with a compromised membrane. Red blood cells, platelets, and other debris are not fluorescent as can be seen on day 0 of PBMC culture (A). In panel (B) clumps of activated T cells can be observed. Post CAR transduction, proliferating T cell cultures are denser and have high viability (C).

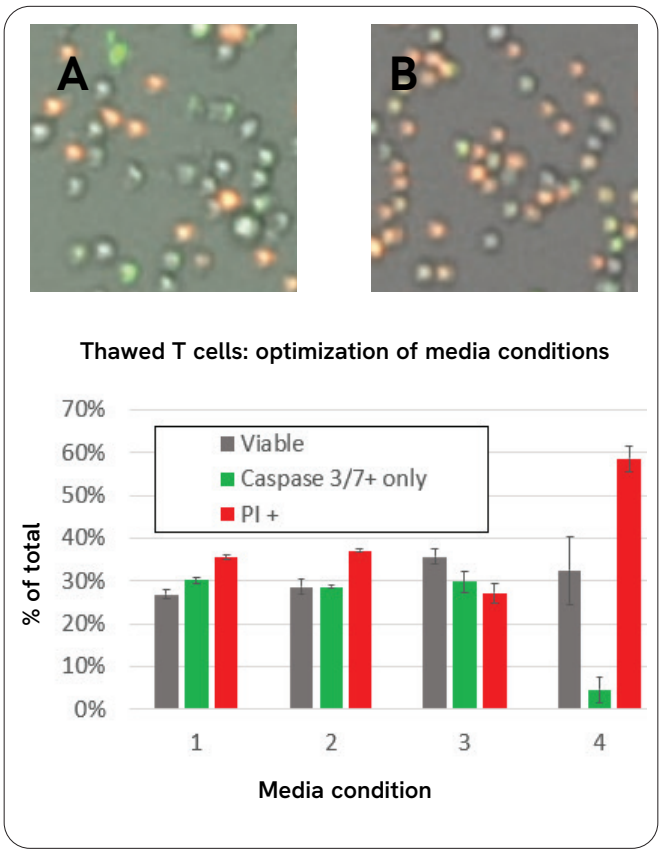


Figure 2. Caspase 3/7 and PI viability of cryopreserved CAR-T cell product. Incubation of T cell cultures with Caspase 3/7 dye and Propidium Iodide is used to segment early apoptotic (green Caspase 3/7 positive) and cells with a compromised membrane (red PI-positive) from intact, viable cells. After thawing cryopreserved CAR-T cells, four recovery media conditions are evaluated. In panel (A) media condition 3 results in similar levels of viable, caspase 3/7 + and PI + populations. However, media condition 4, (panel B) results in mostly PI + cells and a similar percentage of viable cells to condition 3.

Conclusions

The Celigo image cytometer allows for rapid assessment of T cell numbers and health using small sample volumes in multiwell plates. This enables ongoing monitoring of patient sample expansion and post-transformation proliferation as well as an assessment of cell handling conditions such as cryopreservation and recovery protocols.

Incorporating these methods into the T cell product research workflow will provide increased throughput allowing for more efficient product development. This will enable teams to study more patient samples and assess multiple transductions and product handling protocols.

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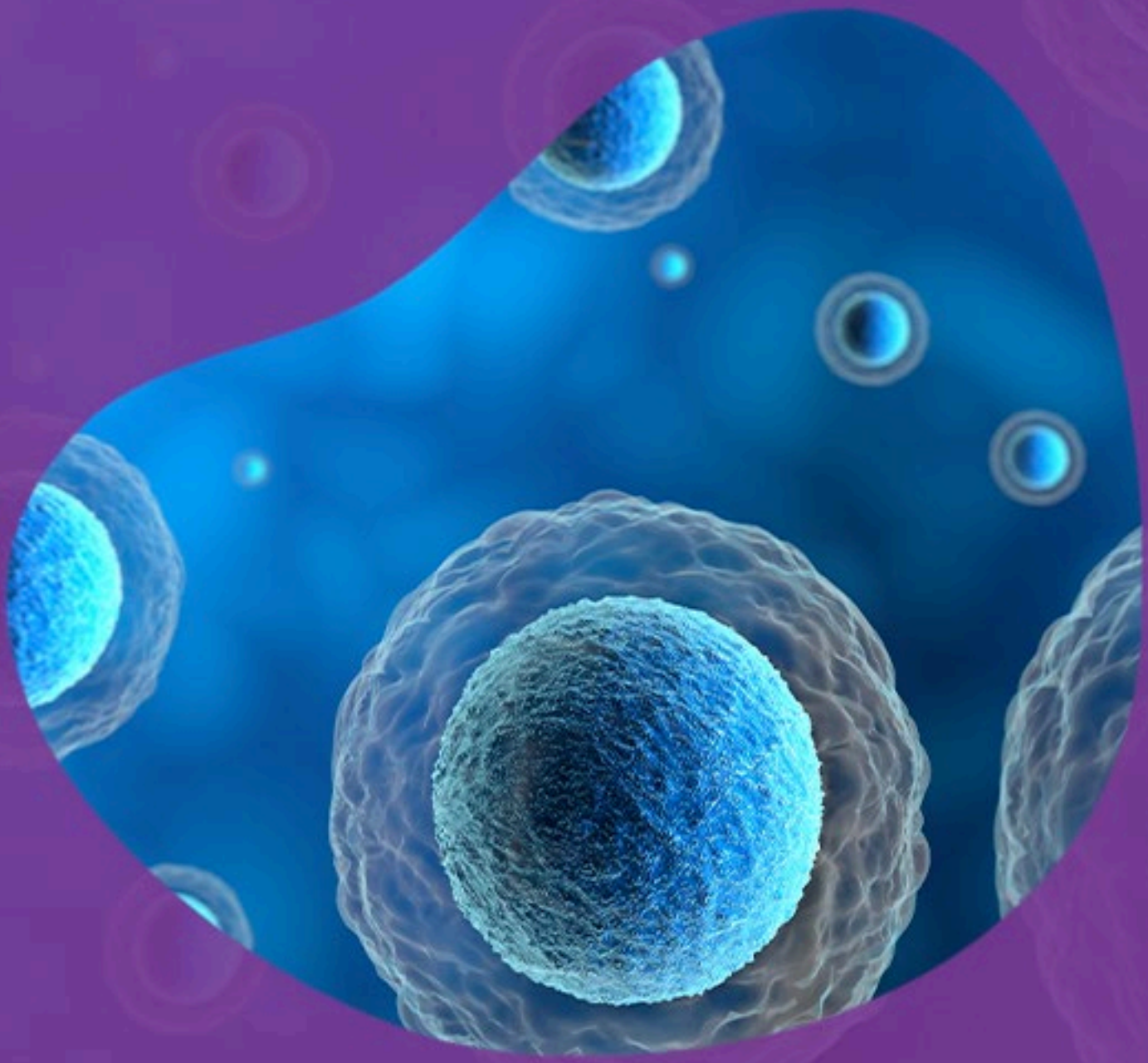
Chapter 4

Transwell T cell migration assay



Introduction

T cells are a vital part of generating an effective immune response to infection and cancer. Part of this process includes T cell migration. T cells are recruited via chemotaxis, and functional subsets are localized to the site of disease for immune modulation.[1,2] It has been shown that certain T cell populations can enhance anti-tumor response during immunotherapy while other types can suppress anti-tumor activity. Therefore, the ability to regulate chemotaxis of specific T cell populations can aid in increasing the efficacy of immunotherapeutics. One such population of interest is regulatory T cells (Tregs).

Tregs are a subpopulation of $CD4^+$ T cells that phenotypically express CD4, $CD25^{high}$, and forkhead box P3 (FOXP3). Tregs suppress the immune cell response by producing immunosuppressive molecules such as transforming growth factor-β (TGF-β) and interleukin-10 (IL-10).[3] An increase in Treg recruitment creates a favorable environment for the dysregulation of antitumor immunity and tumor growth. Clinically, the increase of Tregs to the tumor has been correlated with lower survival rates.[3,4] Targeting and restricting the recruitment of Treg cells to the tumor microenvironment could increase immunotherapeutic efficacy.

Researchers are attempting to understand and quantify this critical, complex, and dynamic process to improve outcomes in patients treated with immuno-oncology therapies. Rapid screening assays can be performed *in vitro* to investigate, characterize, and modulate migration patterns of different populations of T cells. This knowledge could help scientists improve outcomes of immunotherapeutics by restricting Treg recruitment into the tumor microenvironment.

Assay principles

T cell migration is a longitudinal, kinetic biological process that is important in immune regulation. Conventional assay approaches include the classic Boyden Chamber assay, microfluidics assays, flow cytometry, and time-lapse microscopy.[2,5] Most commonly, cell migration is measured in a transwell assay. The set up consists of a hollow plastic chamber that is sealed at one end with a porous membrane which is then suspended over a larger well containing medium or chemoattractants. For example, to measure Treg inhibition, the well may contain chemokine ligands (CCL)17 and CCL22. Cells are then placed inside the chamber and allowed to migrate through the membrane for a defined time-point. At the terminal endpoint, the cells that have migrated through the membrane are stained and then counted.[2] This assay can be done in a wide range of plate formats, from 12- well, 24-well, 96-well, or 384-well.

While traditionally this type of assay would be assessed via microscopy and manual counting, image cytometry has emerged as a more streamlined approach to study the complex T cell migration response. By combining imaging, increasing time-points, and streamlining a quantitative analysis that is produced in real-time and requires no post-processing of data, image cytometry can overcome some of the laborious methods used for measuring T cell migration.

The Celigo plate-based imager can measure direct *in situ* T cell migration of label-free or fluorescently labeled cells in a high-throughput manner, rapidly interrogating a large field of view across multiple time points. Potential immunotherapeutics that regulate T cell migration can be identified from high-throughput screening using the Celigo cell imaging cytometer, ultimately facilitating better clinical outcomes.

Models/probes and dyes

- Hoechst 33342 (CS1-0128-5mL, Revvity)
- $CCR4^+$ T cells

Methods and Celigo set up

There are several key parameters to consider for performing the transwell chemotaxis assays. First, the transwells can be selected with different pore sizes that are appropriate for certain target cells.

If the target cells are in suspension, the cells can migrate through the pores and fall to the bottom of the plate for image and quantification either in brightfield or fluorescence.

The Celigo has the focusing range to image cells above the transwell. Therefore, researchers can determine the ratio of migrated cells.

If the target cells are adherent, the cells can migrate through the pores and adhere to the underside of the transwell. FluoroBlok™ transwell can be used, which is a black transwell that enables fluorescence-based counting of the migrated cells without nonspecific counting of the cells above the transwell.

CCL-mediated CCR4⁺ T cell migration chemotaxis assay

Preparation of chemokine ligands and Hoechst staining in the transwell plates

1. Chemokine ligands CCL17 and CCL22 (100 µL) were pipetted into the lower reservoirs of two separate 96-well Transwell plates (CLS3388-2EA, Sigma-Aldrich).
2. Next, the Hoechst stain (100 µL) was added to the lower reservoirs, making a final staining concentration of 8 µM.
3. The final CCL17 concentrations were 12.500, 4.167, 1.389, 0.463, 0.154, 0.051, 0.017, 0.006, and 0.002 nM.
4. The final CCL22 concentrations were 50.000, 16.667, 5.556, 1.852, 0.617, 0.206, 0.069, 0.023, and 0.008 nM.
5. Additionally, wells without CCL17 and CCL22 were set up as the negative controls (spontaneous migration). Each concentration was performed in duplicates.

Preparation of CCR4⁺ T cells in the transwell inserts

1. The transwell inserts were placed into each well, and ~300,000 CCR4⁺ T cells in 80 µL of T cell media were pipetted into the top reservoirs.
2. The transwell plates were covered and incubated for 3 hours before the initial image cytometry analysis.

Image cytometric analysis of the migrated T cells

1. After 3 hours of incubation, the Transwell inserts were removed from each well, and the plate was centrifuged at 1200 RPM at 5 min to settle the T cells still in suspension.
2. The plate was imaged and analyzed to generate CCL17 and CCL22 dose-dependent migrated CCR4⁺ T cell counts.
3. The results were exported into EXCEL and plotted using GraphPad Prism to determine the EC50 values.

CCL-mediated CCR4⁺ T cell migration inhibition assay

1. The CCL17 and CCL22 were prepared to working concentrations of 0.6 and 1.0 nM for the chemotaxis inhibition assay, respectively.
2. CCL17 and CCL22 solutions (100 µL) were pipetted into the lower reservoirs of two separate 96-well Transwell plates.
3. Next, the prepared Hoechst stain (100 µL) was added to the lower reservoirs.
4. The final CCL17 and CCL22 concentrations were 0.3 nM and 0.5 nM, respectively.
5. Similarly, wells without CCL17 and CCL22 (media only) were set up as the negative controls.

Preparation of CCR4+ T cells in the transwell inserts

1. The transwell inserts were placed into the plates, then ~300,000 CCR4+ T cells in 40 µL of T cell media were pipetted into the top reservoirs.
2. Next, the titrations of anti-CCL17 and anti-CCL22 were added separately into the top reservoirs of the two plates, resulting in the final concentrations at 20, 4, 0.8, 0.16, and 0.032 µg/mL.
3. Additionally, anti-HA F10 control antibodies were added serving as the negative control.
4. Finally, the inserts were moved into the plates with CCL17 and CCL22 then covered and incubated for 3 hours before the initial image cytometry analysis.

Image cytometric analysis of inhibition of T cell migration

1. After 3 hours of incubation, the Transwell inserts were removed from each well, and the plate was centrifuged at 1200 RPM at 5 min to settle the T cells still in suspension.
2. The plate was imaged again and analyzed to generate migrated CCR4⁺ T cell counts in the presence or absence of antibodies.
3. The counting results were exported into EXCEL, where spontaneous migrated cell counts were subtracted from each sample and plotted using GraphPad Prism to determine the IC50 values.

The Celigo software application "Target 1 + 2" was utilized to autofocus and acquire brightfield and blue fluorescent images at 1 µm2/pixel with exposure times set to auto-exposure and 50,000 µs with a gain of 150, respectively.

The Celigo software was used to directly count the number of Hoechst positive T cells on the bottom of the 96-well plates at different chemokine and antibody concentrations. The ANALYZE parameters for the Blue channel were set to:

1. "Algorithm = Fluorescence"
2. "Intensity Threshold = 4"
3. "Precision = High"
4. "Cell Diameter = 10"
5. "Dilation Radius = 0"
6. "Background Correction = Check"
7. "Separate Touching Objects = Check"
8. "Minimum Cell Area = 20"
9. The BF images were not utilized by setting the "Intensity Threshold" to 255

TIPS & TRICKS

The transwell chemotaxis assay can be performed kinetically by imaging and analyzing the plate at different time points without removing the transwells.

However, during the migration time frame, there will still be cells floating in the medium that have not settled down to the bottom.

To properly count the migrated cells, the plate will need to be centrifuged with the transwells removed.

If the transwells are not removed during centrifugation, the cells may be forced through the pores of the membrane.

Challenges

Performing the T cell migration assay requires the centrifugation of the transwell plate to allow the T cells in suspension to settle to the bottom for imaging. In addition, the transwell inserts must be removed, otherwise, the T cells in the insert can be forced through the micro-pores and increase nonspecific migration cell counts. Therefore, the final method required the removal of inserts and centrifugation at the endpoint of the migration assay.

Results

The Celigo was used to image and count every Hoechst+ T cell that migrated into the bottom of the well (Figure 1).

Measurement of CCL17-and CCL22-induced CCR4+ T cell migration.

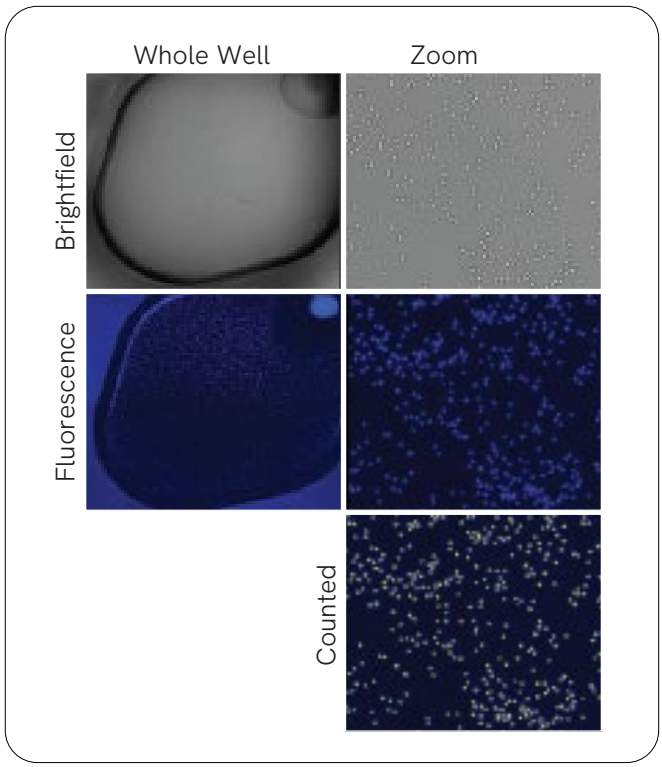


Figure 1. Whole-well and zoomed images were taken using Celigo. Hoechst+ T cells are identified and counted in the Celigo software.

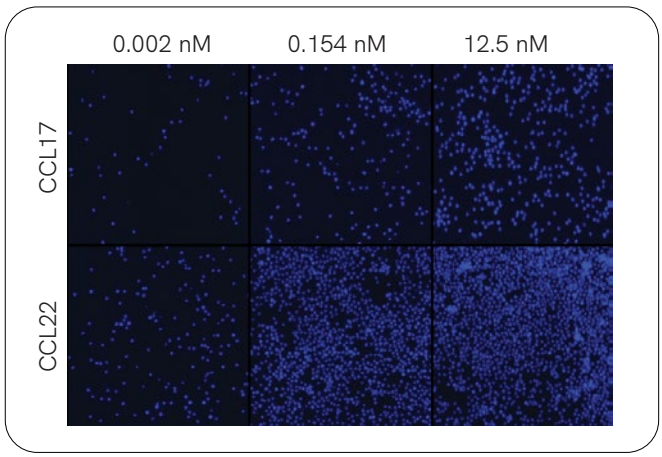


Figure 2. Chemokine ligands CCL17 and CCL22 increase T cell migration in a dose-dependent manner. Hoechst-stained T cells are imaged and quantified with Celigo.

The dose-dependent fluorescent images are shown in Figure 2, where the number of T cells migrated to the bottom reservoir increased with the increase of CCL17 or CCL22 concentrations.

The dose-response curves are shown in Figure 3, where the measured EC_{50} values are 0.163 and 0.339 nM for CCL17 and CCL22, respectively, which indicated that CCL17 induced a circa 2-fold stronger chemotactic response for CCR4+ T cells. Fewer T cells migrated to the bottom reservoirs at higher chemokine ligand concentrations.

The chemotaxis inhibition assays were performed to demonstrate the capability of image cytometry to measure inhibitory dose-response with commercially available antibodies. As expected, the tested antibodies were able to inhibit the migration of CCR4+ T cells through the Transwell membrane. Examples of dose-dependent fluorescent images are shown in Figures 4a and 4b, where the migrated number of T cells decreased with respect to the increase of anti-CCL17 or anti-CCL22 antibody concentrations.

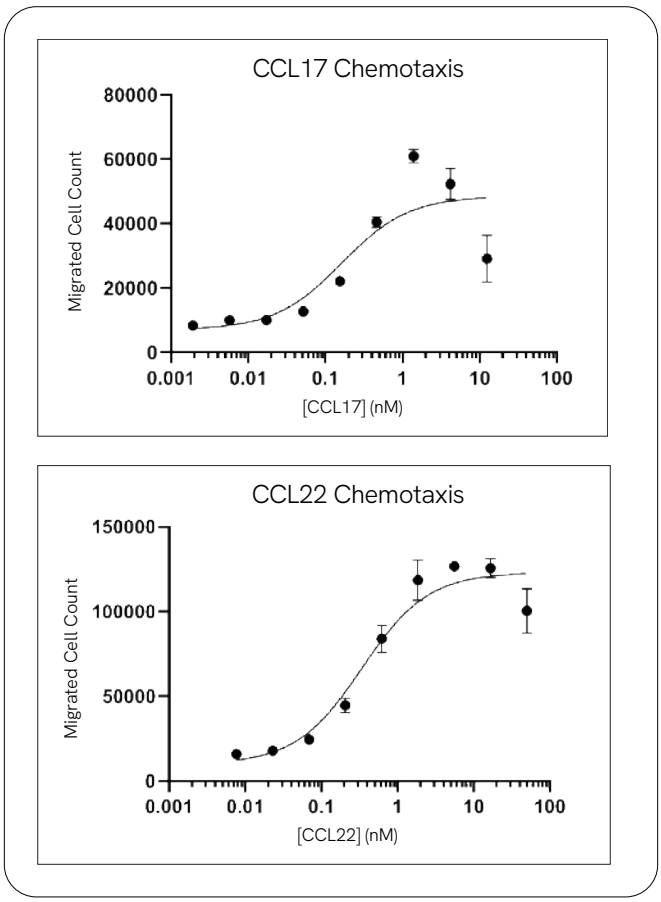


Figure 3. Dose-response curves of Treg chemotaxis. Measurement of anti-CCL17 and anti-CCL22- inhibited T cell migration.

The dose-response curves are shown in Figures 5a and 5b, where the IC50 values are 0.234 and 2.304 µg/mL or 1.56 nM and 15.36 nM for anti-CCL17 and anti-CCL22 antibodies, respectively. In contrast, the anti-HA F10 antibodies did not inhibit cell migration, as expected.

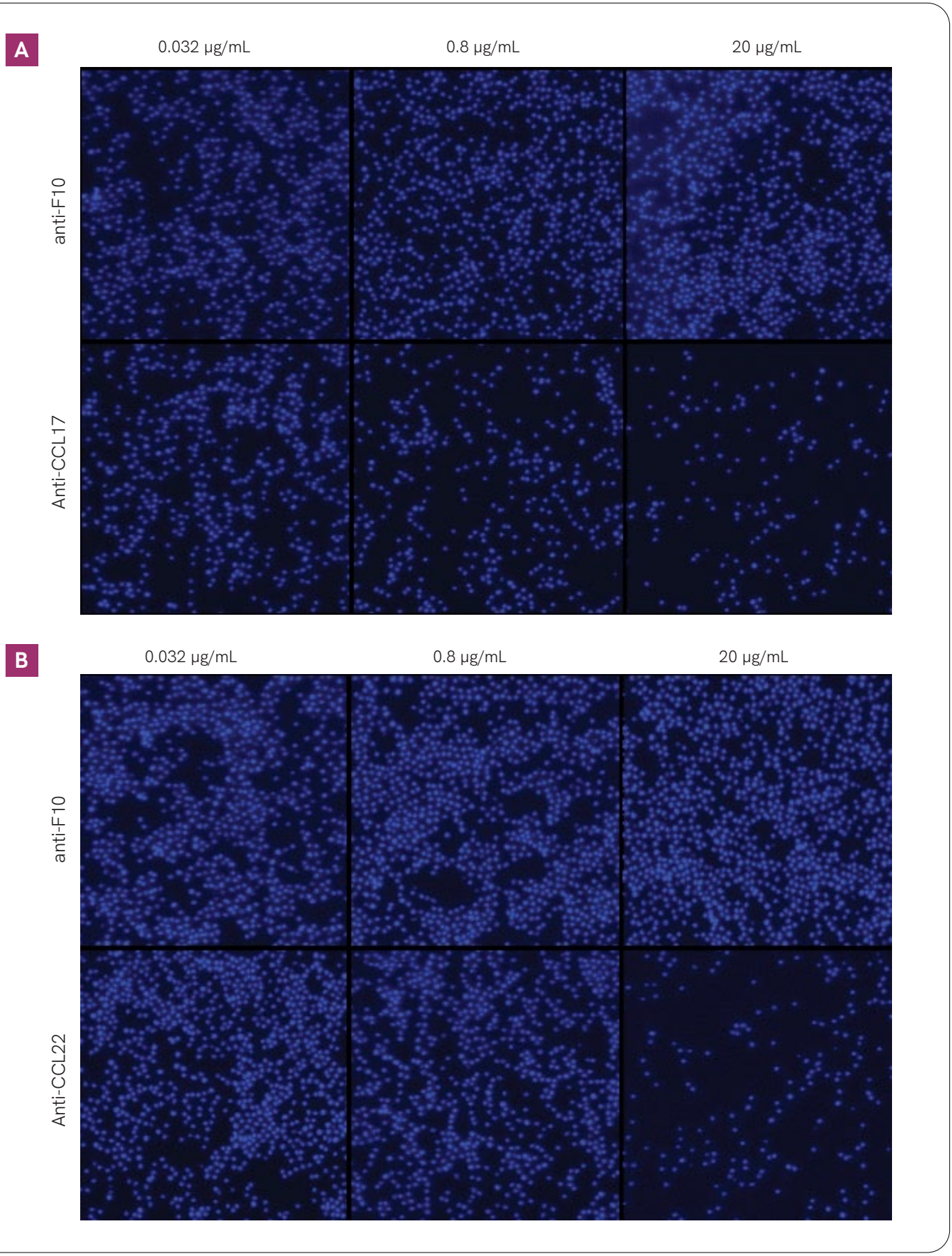


Figure 4. Control antibody anti-F10 does not affect Treg migration, but Anti-CCL17 (a) and Anti-CCL22 (b) does decrease migration in a dose-dependent manner.

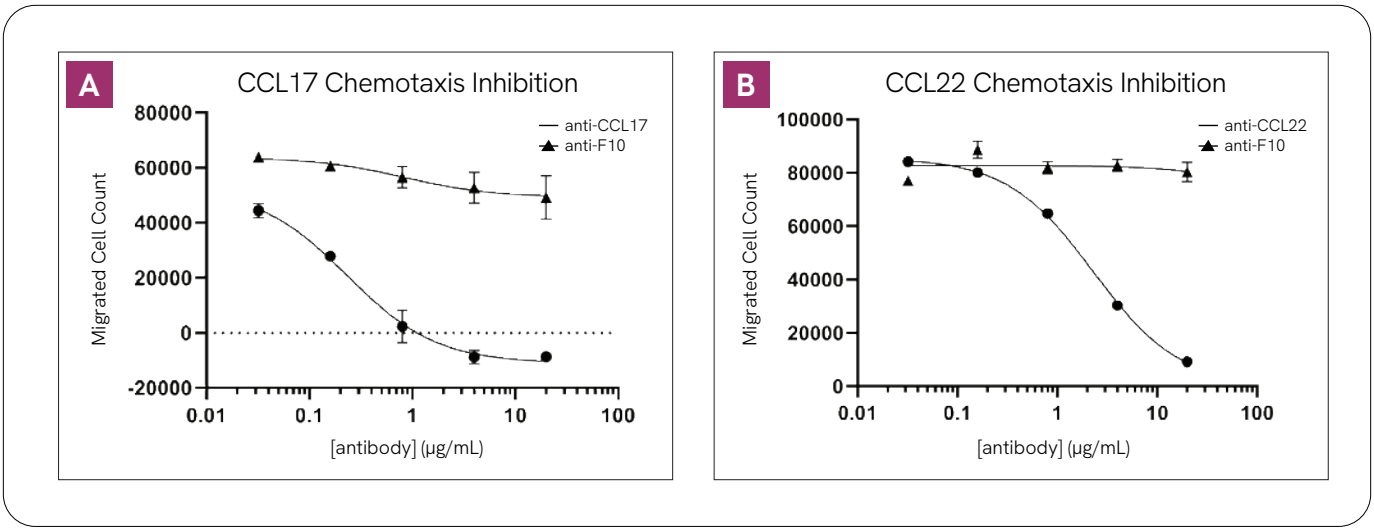


Figure 5. Dose-response curves showing chemotaxis inhibition of Tregs with antibodies anti-CCL17 (a) and anti-CCL22 (b) as compared to controls.

Conclusions

The Celigo Image Cytometer was able to perform high-throughput counting of migrated Hoechststained CCR4+ T cells using the Transwell microplates. The method can perform rapid imaging and analysis of the entire 96-well plate in less than 10 min for one brightfield and one fluorescent channel. The Celigo allows the screening of multiple antibodies at various concentrations, which can improve the efficiency of discovering highly qualified antibody candidates for the stimulation or inhibition of immune cell recruitment to the tumor microenvironment.

The work presented here was a collaboration with Dana Farber Cancer Institute, Department of Cancer Immunology and Virology, and has been published in the Journal of Immunological Methods (ref 4).

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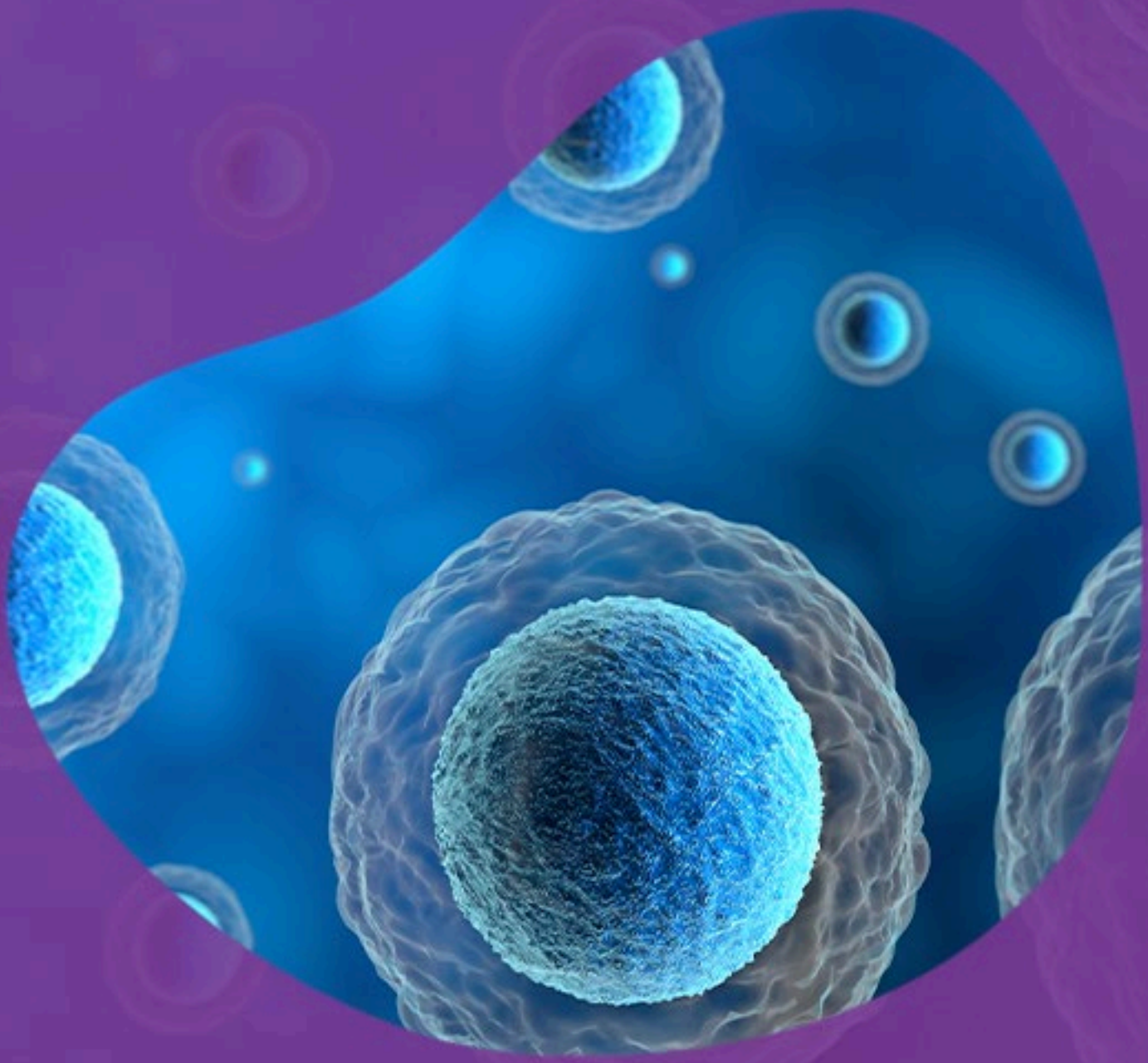
Chapter 5

CAR T cell cytotoxicity assay



Introduction

Chimeric Antigen Receptor (CAR) T-cell therapies are growing in popularity due to their success in treating patients with hematologic malignancies. CAR T cells expressing CD19 and CD20 have been used as targeted antitumor immunotherapeutics in hematologic malignancies, successfully leading to sustained tumor regression.[1]

CAR designs are synthetic constructs that - in cancer therapies - link an extracellular ligand- binding domain specific for a tumor cell surface antigen to an intracellular signaling molecule that activates T cells during antigen binding. When the CAR is expressed in the selected T cells, it mimics the T cell receptor activation, which then redirects its specificity and effector function towards a specific antigen found on a tumor cell. Another strategy to target CAR T therapies is engineering the T cells with CAR expression that is regulated by input signals primarily found in the tumor microenvironment. Designing the CAR with specificity for tumor cells can help minimize off-target toxicity, which allows for the use of more potent therapies. CAR-T cell therapies can be selected based on *in vitro* affinity for, and potency against, the target tumor cells.[2] Currently, the development of CAR-T cell therapies is swiftly expanding to improve CAR designs for solid tumor indications, gene-engineering approaches, and to optimize manufacturing.[3]

Assay principles

The ability to test CAR T cell efficacy and functional variations across different CAR designs *in vitro* is critical in the development process. Co-culture cytotoxicity assays are regularly performed to inform the selection of CAR T designs for immunotherapies.3 T cells are co-cultured with target tumor cells at a defined effector (T cell)-to-target ratio (E:T). The target tumor cells are fluorescently labeled and monitored at multiple timepoints with microscopy or flow cytometry. The efficacy of the CAR-T induced cytotoxicity is determined based on the evaluation of target cell number and viability at each timepoint. A reduction in live target tumor cells could indicate a viable CAR design for immunotherapy.

The analysis of adherent tumor cells and the suspension T cells can be limited when investigating CAR T cytotoxicity in co-cultures. Classical imaging techniques such as brightfield microscopy and fluorescence microscopy can only analyze adherent cell populations reliably. To quantify the viability of both tumor cells and CAR T cells flow cytometry is employed. Multiparametric flow cytometry is a common method that employs the use of panels of a variety of cellular markers to evaluate and separate a heterogeneous cell population, including adherent and suspension cell lines. However, co-culture cytotoxic assays utilizing flow cytometry are not high-throughput and tend to be time, resource, and labor-intensive. Additionally, a kinetic profile of T cell killing by FACS requires harvesting samples at multiple time points.

The Celigo cell imaging system presents a new method of analyzing CAR T cell cytotoxicity in a high-throughput manner. Utilizing both brightfield and fluorescence microscopy, the Celigo cell imaging system can image whole- microwells at a time enabling the capture of data from each cell including suspension cells. The Celigo cell imaging system can aid in overcoming the need to create a terminal sample per time point, minimizing the use of precious primary cell samples, supplies, labor, and time. Multi-time point analysis can easily be done within one well in real-time informing of the change in E:T ratios over long periods.

CAR T therapies are dependent on their selective toxicity for tumor cells, and therefore, the efficacy of CAR T therapies is dependent on kinetic profiling of E:T ratios. The Celigo cell imaging system can use brightfield and fluorescence imaging to determine E:T ratios to evaluate immunotherapeutics, providing comprehensive information on cell health, potency, and efficacy. By limiting the use of primary cell and drug supply and measuring multiple time points without needing a terminal sample, real-time kinetic analysis, and characterization of the cells beyond the E:T ratio can be obtained. Taken together, the use of image cytometry to assess both adherent and suspension cell cultures is a powerful tool for drug development that can quickly provide kinetic efficacy and potency data *in vitro* on newly engineered CAR-T cell therapies.

Models/probes and dyes

Total Cell labels:

- Calcein AM (ViaStain™ Calcein AM - CS1- 0119)
- CMFDA (ViaStain™ CMFDA - CS1-P0001-1)
- Fluorescent Proteins (nuclear or cytosolic)

Viability stains:

- DAPI - CS1-0127-2mL

Methods and Celigo set-up

These assays require cell samples, total cell, and viability stains, imaging microtiter plates (Corning 3603 used here), a cell culture incubator, a Cellometer automated cell counter, and the Celigo cell imaging system.

Suspension cells, short term assay.

1. Stain target (DAOY) cells with Calcein AM:
    1. Incubate with 5 µM Calcein AM in PBS 30 min at 37oC.
    2. Wash three times in PBS (pellet cells by centrifugation, discard supernatant, resuspend pellet in PBS).
    3. Resuspend cells in culture medium
2. Count cells and seed 3500 cells per well into a 96-well plate.
3. Add effector cells at various E:T ratio of 1.25:1, to 40:1
4. Bring the total volume of each well to 150 µL with culture medium
5. Centrifuge plates at 400xg for 3 min, acceleration, and brake set to 5 on a scale of 0-9.
6. Image on Celigo at 0 hr, 4 hr.

Celigo set-up:

The Celigo application Target 1+ Mask was used. Target 1 was set to Brightfield and the Mask channel was set to Green. The analysis settings were adjusted to only count the live, bright green fluorescent cells (Fluorescence algorithm, Intensity ratio of 8).

To calculate the CAR T cell-mediated cytotoxicity the number of live target cells at t=4hrs can be compared to the number of initial target cells at t=0hrs.

$$\text{Cytotoxicity} = \frac{(\#\text{Target cells t0} - \#\text{Target cells t4hrs}) \cdot 100\%}{\#\text{Target cells t0}}$$

Adherent cells, longer-term assay recommendations:

1. HPAC cells expressing a nuclear-targeted RFP were used. Alternatively, target cells can be labeled with a cell trace dye such as CMFDA.
2. Count stock of HPAC cells with a Cellometer, and seed 5000 cells into each well of a 96-well plate.
3. Allow cells to adhere for 24 hours in the cell culture incubator.
4. CAR T cells added to each well at E:T ratios of 1:1, 2:1, 5:1 (T=0 hrs.)
5. Centrifuge plates at 400xg for 3 min, acceleration, and brake set to 5 on a scale of 0-9.
6. Image on Celigo.
7. Incubate for 24 hours.
8. Add DAPI to each well (1 µM final concentration)
9. Image on Celigo. (T=24 hrs.)

The Celigo application Target 1+ 2 + Mask was used. Target 1 was set to Brightfield, Target 2 Blue, and the Mask channel was set to Red. The Mask channel analysis settings were adjusted to detect the RFP-positive nuclei. Identification algorithm: Fluorescence, Intensity Threshold:

4. Under the Gating Tab, a histogram of blue fluorescence mean intensity was used to separate DAPI positive from DAPI negative (live) tumor cells. DAPI negative cells were counted as the total number of Target cells (#Target cells).

To calculate the CAR-T mediated cytotoxicity the number of live target cells at t=24hrs can be compared to the number of initial target cells at t=0hrs.

$$\text{Cytotoxicity} = \frac{(\#\text{Target cells t0} - \#\text{Target cells t24hrs}) \cdot 100\%}{\#\text{Target cells t0}}$$

Challenges

Calcein AM is an excellent intact cell label for assays up to 24 hours duration. However, some cell types will spontaneously pump the green fluorescent Calcein out, limiting the assay time window. As an alternative, cells expressing a cytoplasmic soluble (non-fusion protein) GFP or a whole-cell label like CMFDA can be used in parallel with a viability stain such as PI or as demonstrated here, with DAPI.

In assays using a viability stain, CAR T cell clumps can make an accurate determination of target cell viability difficult as dead CAR T cells on top of target cells can be mistaken for dead target cells. To improve detection, CAR T cell clumps can be dispersed by pipetting to resuspend the culture followed by centrifugation and imaging. Active CAR T cells will re-clump around target cells within 30 minutes with limited impact on assay kinetics.

TIPS & TRICKS

Target cell lines expressing either nuclear or cytoplasmic fluorescent proteins are best suited for killing assays.

For long-term assays choose a target cell density such that at the endpoint, the cells in the no treatment control are not over confluent.

Results

The Celigo cell imaging system is a high-throughput imaging platform capable of performing CAR T cell based cytotoxicity assays of both suspension and adherent growing tumor cells.

In these assays, CAR T cell-induced cytotoxicity is mediated by a loss of target cell membrane integrity. This disruption of membrane integrity can be detected as either the loss of a target cells's fluorescent contents or the gain of fluorescence from a cell impermeant dye. In the former case, as shown in Figure 1, intact DAOY cells contain cytoplasmic Calcein, which upon membrane disruption is released into the surrounding medium rendering the dead DAOY cells dark or dimly fluorescent. In the latter case, the use of a nuclear stain that is normally excluded from intact cells can be used to highlight target cells with disrupted membranes as in Figure 2.

The Calcein AM method is well suited for cytotoxicity assays that; evolve over 0-24 hours, use high E:T ratios and can be used with primary cells. In Figure 1, it is clear from the brightfield image that segmenting individual cells in wells with high E:T (40:1) ratios may prove difficult based on brightfield contrast alone. However, in the green fluorescence image, only live, intact, tumor cells are detectable, greatly simplifying the analysis. Comparison of the green fluorescence images of the same location at 4 hours reveals fewer live green cells.

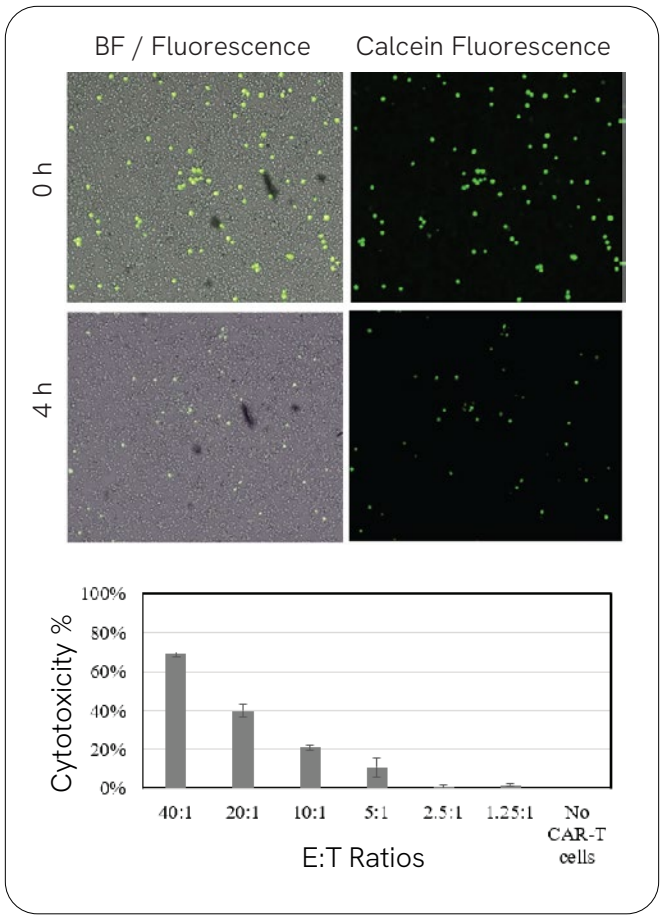


Figure 1. Merged brightfield and green fluorescence images of Calcein labeled DAOY cells (green) and effector cell (visible in brightfield) at an E:T ratio of 40:1. Bar chart: CAR T cell-mediated cytotoxicity measured at 4 hours.

Four hours post-incubation, effector cells are measured, and the target DAOY cells provide a histogram displaying higher cytotoxicity at higher E:T ratios. By having the t=0hrs DAOY cell count, we can account for any well-to-well variation in target cell seeding as each cytotoxicity value is normalized to time 0. Alternatively, for longer assays where cell growth is expected to be a significant factor, cytotoxicity values should be calculated by comparison to the no effector cell control group as in Figure 2. This will help account for the dual kinetics of cell growth and CAR T cell-mediated cytotoxicity.

For cytotoxicity assays using adherent target cells, an alternative approach can be used. In Figure 2 HPAC target cells expressing a nuclear targeted red fluorescent protein (RFP) are used. Labeling of the nucleus with RFP improves the counting accuracy of adherent cells growing in clusters as compared to whole-cell labels, or cytoplasmic fluorescence (as with Calcein or cytoplasmic GFP). Here, both the RFP-tagged target cells and the much smaller CAR T cells can be seen in the combined brightfield and fluorescence image. Following 24 hours of coculture, the addition of DAPI highlights both the dead T cells as well as the dead target cells. The use of DAPI as a viability stain is needed because the nuclear RFP does not immediately disperse from target cells with a compromised membrane. In the cytotoxicity analysis for this assay, only live (DAPI negative) RFP-positive cells are counted. As in Figure 1, clusters of effector cells visible in brightfield do not impede the detection of RFP expressing target cells in fluorescent images.

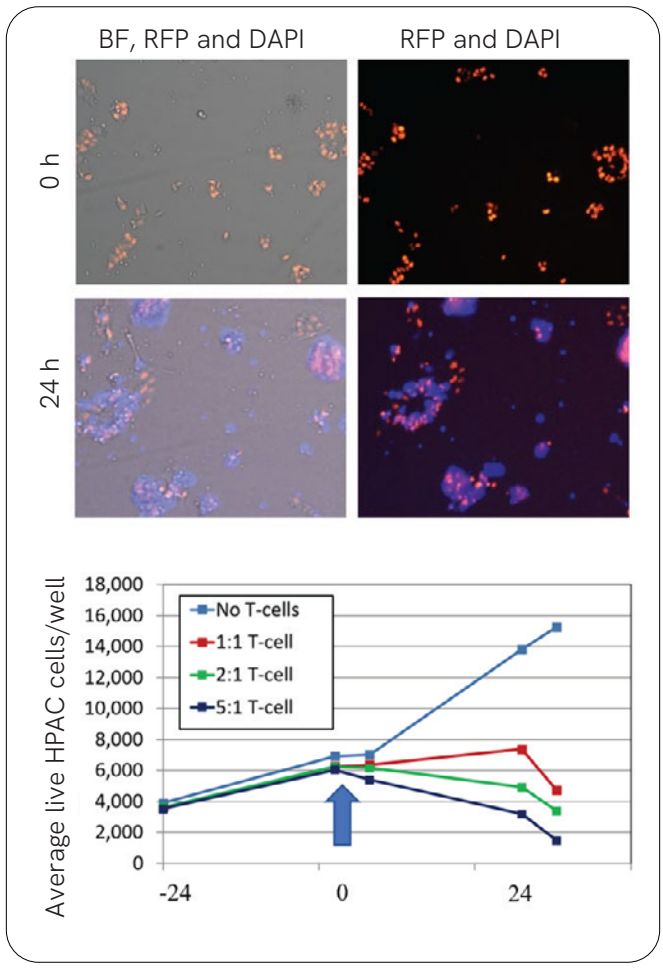


Figure 2. Merged Brightfield, RFP, and DAPI fluorescence images of the same field of view at 0 and 24 hours post addition of effector cells. HPAC cells are labeled with a nuclear RFP. DAPI highlights dead cells. Line graph: HPAC cell number is quantified at multiple time points before and after the addition of effector cells (indicated by the blue arrow).

For longer duration cytotoxicity assays, proliferation of the target cells must be taken into account when calculating cytotoxicity.

In Figure 2, the line graph shows the proliferation of target cells from seeding to the addition of T cells (arrow) and in the no effector cell condition. By normalizing to the no T cell control group it is possible to account for the dual-rate kinetics of target cell proliferation and T cell-induced cytotoxicity. The same can be done for the data in Figure 1, however, target cell proliferation over 4 hours is negligible.

Overall, the Celigo cell imaging system is a fast platform for perform CAR T cell cytotoxicity experiments which provides the following benefits:

1. Direct in situ quantification of cytotoxicity without disrupting the assay.
2. Easy-to-use interface to image and identify fluorescently labeled target cells.
3. Applicable to both adherent and suspension target cell types
4. Accommodates a multitude of microwells and T-flasks, allowing for increased flexibility for users
5. Provides rapid real-time readouts: a 96-well plate can be analyzed in under 20 minutes

Conclusions

Performing CAR T cell-mediated cytotoxicity assays on the Celigo image cytometer allows for in situ non-destructive quantification of target cell viability within the same well at different time points under standard cell culture conditions. This provides a flexible, robust, and easy-to-use method for evaluating different CAR designs in a streamlined and rapid workflow.

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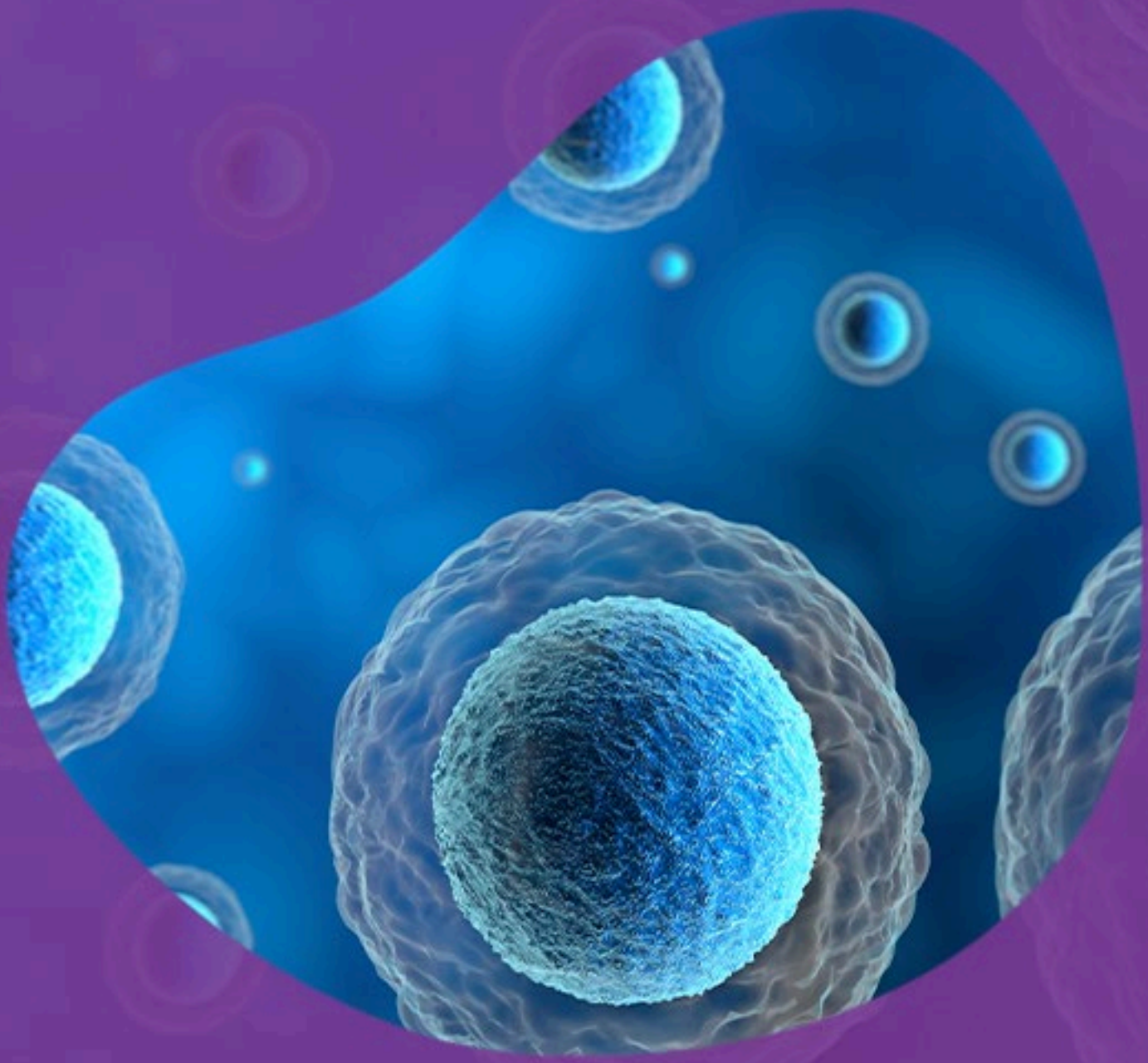
Chapter 6

Measuring target specificity of CAR T cell killing in a mixed co-culture



Introduction

Chimeric antigen receptor (CAR) therapies are immunotherapies that target cancer by eliciting a specific and potent immune response to a tumor. Typical CAR designs involve three components: a monoclonal antibody, a linker, and an antigen.[1] Of the CAR T cells that have been developed, those targeting CD19 have been particularly successful in treating hematological malignancies and producing tumor regression in many patients.[2,3] CD19 is an antigen that is expressed universally on B-cells and is also expressed in B cell malignancies. Antigenic targets using CAR therapies can range from a number of different proteins, carbohydrates, and glycolipids, thus making them an extremely versatile construct for targeting.[4] The utilization of CAR therapies is currently under investigation towards hematological as well as solid tumor types. This targeted approach to anti-cancer therapies provides a platform in which potent, cell-mediated cytotoxicity can be utilized and tailored to mitigate off-target effects on normal tissues.[3]

The first steps for evaluating the efficacy of CAR therapies are *in vitro* assays that assess stability, cytotoxicity, specificity, and target recognition. Target selection is important for the success and safety of CAR therapies. Ideally, the target should be abundantly overexpressed exclusively on tumor cells and not expressed on normal cells. Identification of these targets is difficult due to the inconsistent and incomplete expression of unique tumor epitopes throughout tumor tissue.[5] Efforts are underway to engineer CAR constructs that recognize multiple antigens to increase target specificity. Researchers are also working towards developing new CAR constructs that reduce off-target toxicity and increase target specificity.

To facilitate the optimization of CAR therapies, it is imperative to understand the toxicity to bystander cells. The ability to longitudinally understand viability and growth kinetics in the same well without system perturbation is important but a limitation of traditional techniques like flow cytometry or dual color luminescence, in which samples must be sacrificed at each measured time point. The Celigo cell imaging system is advantageous over these and traditional microscopy methods because it can image and quantify the cells at multiple time point measurements without needing a terminal sample and can provide realtime kinetic analysis and characterization of cell interactions.

Assay principles

When assessing a CAR therapy, building a safety profile is critical to understanding the off-target effects and toxicity of the immunotherapy. Bystander-killing assays are *in vitro* assays that can show the specificity of a drug on tumor cells.

To examine off-target effects on non-cancerous cells, bystander killing assays are performed by incubating the CAR therapy cells (effector cells) with normal cells (bystander cells) and target tumor cells, which are fluorescently labeled. Ideally, the fluorescent label persists in live cells for the duration of the assay until the membrane is compromised by the effector cells and the fluorescent contents spill into the surrounding media rendering the cell non-fluorescent. Cell labeling can be accomplished either through the expression of a cytosolic fluorescent protein (GFP and mCherry) or with labels like Calcein AM (Blue and Green variants).

The labeled cells are typically monitored and enumerated at specific timepoints via the Celigo live-cell imager or flow cytometry. Cytotoxic efficacy is measured as a reduction in the number of remaining intact fluorescent cells. A viable CAR design for immunotherapy would result in a significant reduction in target tumor cells without a corresponding drop in bystander cell number.

Models/probes and dyes

Total Cell labels:

- Cytosolic Fluorescent Proteins (GFP, mCherry)

Alternatives:

- Calcein AM (ViaStain™ Calcein AM - CS1- 0119)
- CMFDA (ViaStain™ CMFDA - CS1-P0001-1)
- CellTrace Violet

Viability stains:

- DAPI - CS1-0127-2mL
- PI
- DRAQ7™

Methods and Celigo set-up

These assays require cell samples, total cell and viability stains (as appropriate), imaging microtiter plates (Corning 3603 used here), a cell culture incubator, a Cellometer automated cell counter, and the Celigo cell imaging system.

Target cells

Two Hek293 cell lines were generated: GFPHek293 expressing the CAR target epitope ("on target") and RFP-Hek293 not expressing the CAR epitope ("bystander").

1. Count target and bystander cells and seed 25,000 cells of each type per well into a 96-well plate.
2. Add effector cells at various E:T ratio of 1:1, 1:5, 1:25
3. Bring the total volume of each well to 150 µL with the culture medium
4. Centrifuge plates at 400xg for 3 min, acceleration, and brake set to 5 on a scale of 0-9
5. Image on Celigo at 0hr, 18hr, 22hr and 42hr.

Celigo settings:

The Celigo application Target 1+ 2 was used. Target 1 was set to Green and Target 2 to Red The analysis settings were adjusted to count the bright green and red fluorescent cells (Fluorescence algorithm, Intensity ratio of 8).

Due to the length of this assay, the calculation of the CAR T cell-mediated cytotoxicity must take into account the kinetics of cell proliferation. This is accomplished by normalizing the number of live target cells in a treatment group at each timepoint to the number of live target cells in the no effector cell treatment group. For each time point:

$$\text{Cytotoxicity \%} = \frac{(\text{Target cells}_{\text{No effector cells}} - \text{Target cells}_{\text{Txgroup}})}{\text{Target cells}_{\text{No effector cells}}} * 100\%$$

Challenges

Cytosolic fluorescent proteins. are an excellent intact cell label for both short and long-term assays. However, it can be impractical to express fluorescent proteins in some cell types (primary cells for example). As an alternative, Calcein AM can be used for assays up to 24 hours. There are multiple color variants of Calcein AM and the assay principle is the same where only live, intact cells remain brightly fluorescent. For longer-term assays, up to 72 hours in duration whole-cell labels like CMFDA can be used in parallel with a viability stain such as PI, DAPI, or Draq 7.

In assays using a viability stain, CAR T cell clumps can make an accurate determination of target cell viability difficult as dead CAR T cells on top of target cells can be mistaken for dead target cells. To improve detection, CAR T cell clumps can be dispersed by pipetting to resuspend the culture followed by centrifugation and imaging. Active CAR T cells will re-clump around target cells within 30 minutes with limited impact on assay kinetics.

TIPS & TRICKS

Target cell lines expressing cytoplasmic fluorescent proteins are best suited for killing assays.

For long-term assays choose a target cell density such that at the endpoint, the cells in the no treatment control are not over confluent.

Results

The Celigo cell imaging system is a high-throughput imaging platform that is capable of monitoring fluorescently labeled adherent and suspension growing cells in multiple channels.

Whole-well imaging at each timepoint enables the accurate enumeration of all fluorescent target and bystander cells. In both figures 1 and 2, the top portion of the wells has large cell-free areas, due to

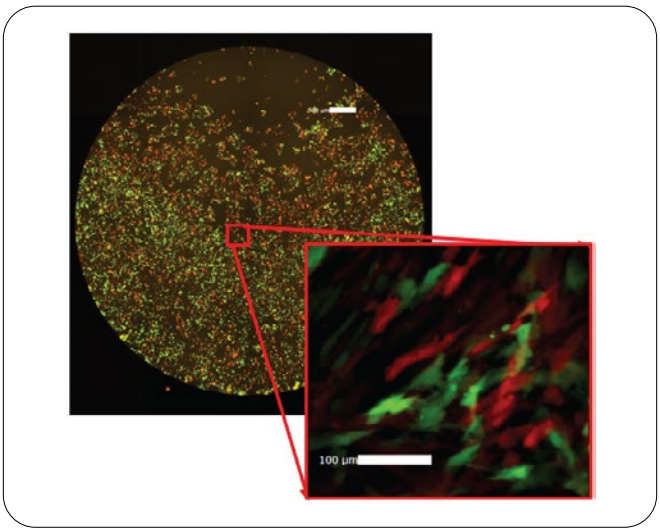


Figure 1. Merged, whole-well Red and Green fluorescence images of GFP-Hek293 and mCherry-Hek293 cells. Celigo enables quantification of fluorescent cells.

pipetting pressure during the seeding of effector cells. However, because all cells are imaged and counted in whole-well images, displaced cells are still included in the total cell count.

Repeated imaging at 18.5 22- and 42-hours post seeding enables the direct monitoring of both CAR T cell cytotoxicity efficacy and specificity for the GFP-Hek293 cells (Figure 2). In wells without effector cells, both target and bystander Hek293 cells continue to proliferate over 42 hours of culture (not shown). In wells with E:T ratios of 1:01 and 1:05 specific cytotoxicity for GFP-Hek293 cells can be observed after initial seeding. This is illustrated qualitatively

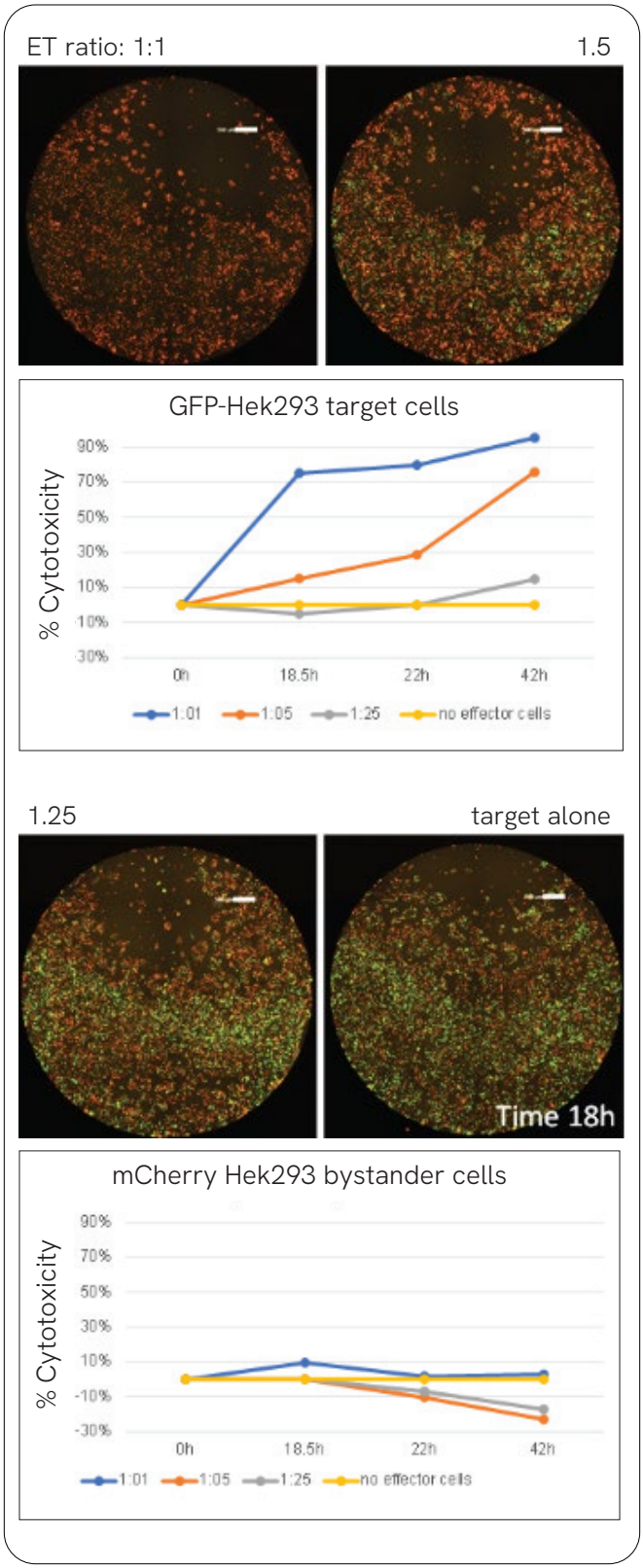


Figure 2. Merged, whole-well Red and Green fluorescence images of GFP-Hek293 and mCherry- Hek293 cells. Line graphs represent repeated Celigo whole-well imaging of GFP-Hek293 and mCherry- Hek293 cells at 18.5, 22, and 42 hours.

by the whole-well images in Figure 2. Here, few GFP-Hek293 cells are visible at an E:T ratio of 1:01 18.5 hours post-seeding while there is no discernable difference in the images of wells with no effector cells and those with a ratio of 1:25.

Quantitatively, an E:T ratio of 1:01 results in ~70% cytotoxicity of GFP-Hek293 cells by 18.5 hrs. while the 1:05 treatment group does not reach this level of cytotoxicity until 42 hours post-seeding. Only minor cytotoxicity of GFP-Hek293 cells is observed in the 1:25 E:T ratio treatment group. Interestingly, some cytotoxicity (~10%) of mCherry-Hek293 cells is observed at 18.5 hours in the 1:01 E:T ratio treatment group. However, this initial cytotoxicity is not observed at subsequent time points or with other treatment groups. Indeed, at 42 hours the bystander mCherry-Hek293 in the 1:05 and 1:25 E:T ratio groups exhibit more proliferation than the no effector cell control group.

Overall, the Celigo cell imaging system is a fast platform for perform CAR T cell cytotoxicity experiments which provides the following benefits:

1. Direct in situ quantification of cytotoxicity in multiple cell types simultaneously without disrupting the assay.
2. Easy-to-use interface to image and identify fluorescently labeled target cells.
3. Applicable to both adherent and suspension target cell types
4. Accommodates a multitude of microwells and T-flasks, allowing for increased flexibility for users
5. Provides rapid real-time readouts: a 96-well plate can be analyzed in under 20 minutes

Conclusions

The cytotoxic specificity of a CAR T cell construct can be assessed on the Celigo image cytometer by in situ non-destructive quantifications of target and bystander cell populations within the same well at different time points under standard cell culture conditions. This provides a flexible, robust, and easy-to-use method for evaluating different CAR designs in a streamlined and rapid workflow.

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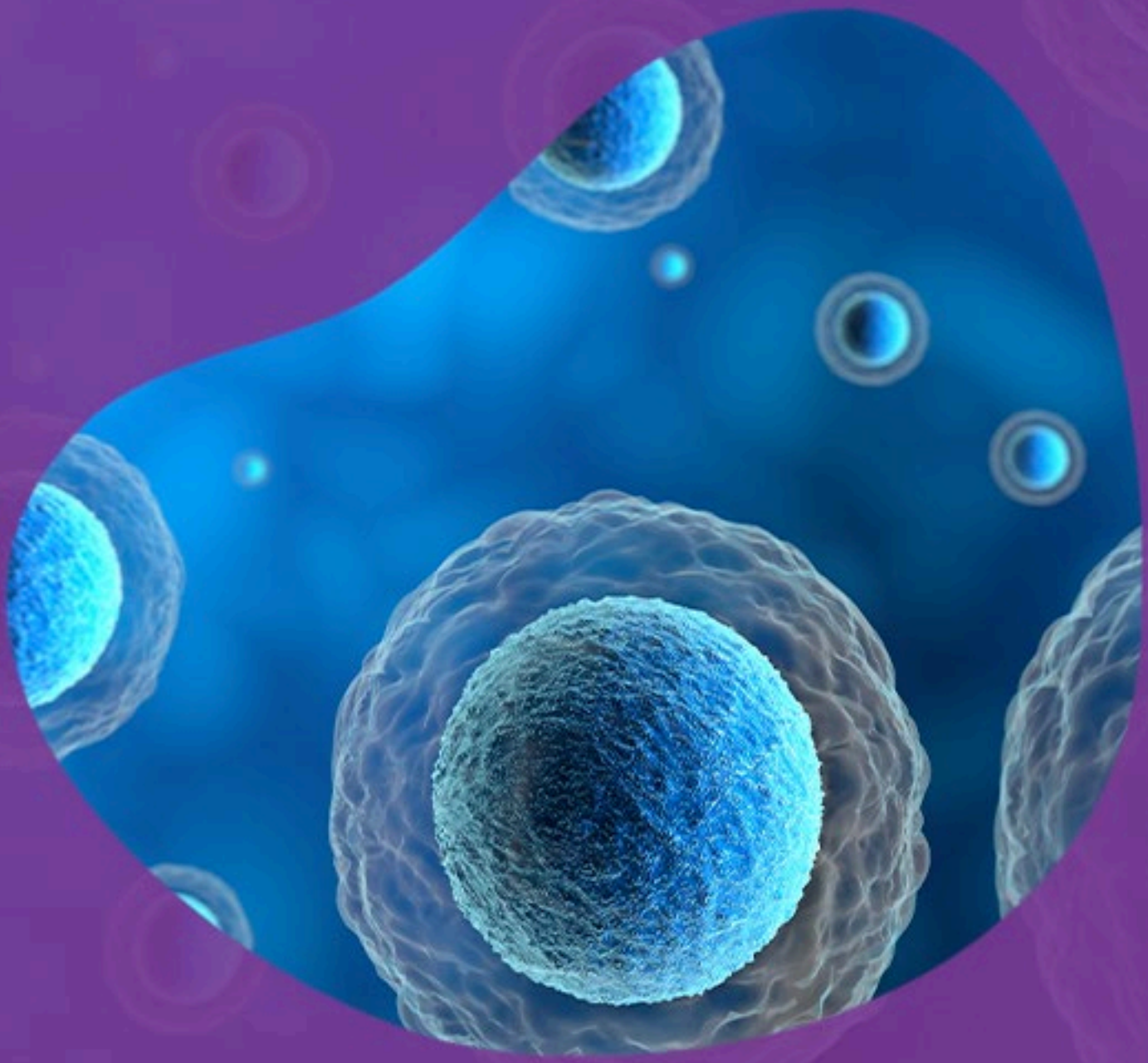
Chapter 7

3D spheroid T cell killing assay



Introduction

T cell dependent cellular cytotoxicity (TDCC) is a mechanism in which T cell receptors (TCR) expressed on the surface of T cells can target a specific antigen on a different cell and induce apoptosis.[1] To increase the efficacy and targeting of TDCC, T cells are being genetically engineered with chimeric antigen receptors (CARs). CAR therapies have been successful in treating hematological malignancies however, treatment for solid tumors has been modest.[2] Currently, researchers are investigating optimization strategies for CAR therapies to target and destroy tumor cells.

Several factors could hinder the efficacy of immunotherapeutics for solid tumors. Solid tumors are complex and dense structures that can limit access for immune cells and antibodies to target tumor cells. In addition to the extracellular matrix and signaling molecules, the tumor microenvironment (TME) hosts rich and variable interactions between tumor cells, immune cells, and stromal cells. All of which support an immunosuppressive environment through the proliferation, survival, and metastasis of tumor cells.[3]

The gold standard of *in vitro* testing is using 2D cell culture. However, this poorly recapitulates the complex 3D nature of the TIME including cell-to-cell interactions, *in vivo*-like phenotypes, and metabolic gradients. [4] Researchers have begun to use 3D cell culture systems such as organoids and spheroids, to better retain tumor architecture and recapitulate *in vivo*-like efficacy that can translate *in vitro* efficacy into clinical outcomes.

Assay principles

Part of evaluating novel T cell therapies is testing the functionality and variations *in vitro* via T cell killing assays. T cell killing assays assess the efficacy of T cell therapies to target and destroy target tumor cells. Multicellular tumor spheroids (MCTS) are currently utilized to test T cell killing in 3D cell culture. Typical 3D T cell killing assays involve the manual monitoring of MCTS using brightfield or confocal microscopy. Generally, 3D T cell killing assays follow a similar paradigm: MCTS are formed using the target tumor cell type of interest, treated with the T cell therapeutic at varying dosages to create a titration curve, imaged at predetermined time points, and then harvested at a terminal endpoint for further analysis.

For better visualization and insight, MCTS can be created using target tumor cells with intrinsically tagged fluorescent protein such that a reduction in fluorescent intensity signifies the T cell killing of target cells. Other methods of analysis use a variety of fluorescent dyes and stains to determine T cell killing such as Calcein AM stain for live-cell nuclei and Propidium Iodide (PI) for dead cells. Viability ratios and dose-response curves can be generated from the respective signal intensities between the live and dead cells.

Cell tracking dyes can be used to visualize a therapeutic T cell invasion of the MCTS. These dyes are nontoxic, cell membrane permeable protein dyes, which use non-fluorescent precursor compounds that diffuse across the cell membrane into the cytoplasm creating a cell-impermeant reaction once inside. The acetate substituents of the dye are cleaved by non-specific esterases in the cell which traps the fluorescent reaction product and then labels arbitrary proteins via covalent bonding between the free amino substituents on the protein and the succinimidyl esters of the dye.[5]

Tracking dyes have a multi-generational and long-term fluorescent signal, however only denote live cells. For T cell tracking, a popular tracking dye is a violet cell tracker. The emission spectrum is separated from commonly used green and red fluorescent signals used in many biological assays and allows for multiplexing.5 For example, using violet cell tracker with the live/dead fluorescent staining Calcein-AM (green) and ethidium homodimer-1 (red), a more comprehensive evaluation can be made that includes T cell therapeutic invasion and survival as well as viability and apoptosis of target cells in MCTS. A comprehensive profile of cellular fate in response to treatment could facilitate investigation into resistance mechanisms.

In addition to being resource and labor intensive, typical imaging modalities and analysis are not high-throughput. The Celigo cell imaging system utilizes both brightfield and fluorescence imaging to quantify cells and evaluate changes in size and morphology of cells and MCTS in a high-throughput manner providing indicators of T cell therapy efficacy and potency. T cell tumor infiltration using fluorescent dyes can also be monitored in realtime to determine the mechanism of action of T cell therapies and screening compounds enabling rapid identification of clinically translatable immunotherapeutics.

Models/probes and dyes

MDA-MB-453 GFP reporter cell line was constructed.

Methods and Celigo set-up

1000 MDA-MB-453 GFP cells were plated per well in 96-well ultra-low attachment U bottom Revvity plates.

Cells were cultured in media containing a 2x spheroid formation buffer containing Matrigel and allowed to form spheroids for three days.

Primary T cells at 1:50 E:T ratio were added in combination with ImmTAC at 10, 1, 0.1, 0.01, and 0.001nM to the spheroids and imaged on the Celigo.

Celigo Set-up:

The plate is scanned using the Tumorsphere 1 + Mask application in green fluorescent and brightfield illumination. The application segments the tumorsphere in brightfield for size measurement, and quantifies GFP signal in green fluorescence for viability measurement.

Challenges

Traditional methods for tumor spheroids size measurements include manual monitoring using a light microscope or confocal microscopy. Both methods are time-consuming and can be subjective to operator interpretation.

Results

The Celigo image cytometer allows one to image and monitor the size and viability of spheroids under cytotoxic conditions. Figure 1 shows representative images of MDA-MB-453 GFP spheroids with T cells at 1:50 E:T ratios in the presence or absence of ImmTAC at 10, 1, 0.1, 0.01, and 0.001nM. After 72hrs of exposure, a concentration-dependent effect of the ImmTAC activated T cells was observed. Concurrently, pockets of dead cells shown by loss of GFP signal were observed starting at 0.1nM ImmTAC, and destruction of the spheroid was seen under 1 and 10nM ImmTAC treatments. Spheroid size and fluorescent intensity amongst other parameters can be reported in the Celigo software and a plot depicting the average mean GFP intensity of the spheroids in the presence or absence of the ImmTAC-activated T cells was generated (Figure 2). The increases in killing are represented by the reduction in the average mean intensity of the GFP spheroids. This cytotoxicity of the spheroids is due to the concentration-dependent activation of the T cells.

TIPS & TRICKS

To set up a tumor spheroids culture, seeding density and growth condition need to be optimized for individual cancer cell lines. It is ideal to use a cell line that forms compact spheroids without the need for Matrigel.

If Matrigel is added to aid tumor spheroids formation, to ensure proper interaction between effector cells and target spheroid. The viscosity of the Matrigel can be reduced by incubating the plate at 4oC and spinning in a precooled centrifuge.

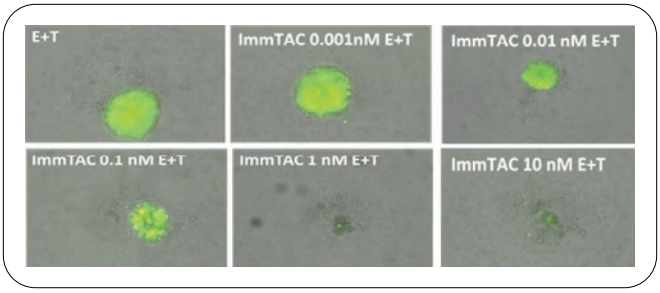


Figure 1. Representative images of MDAMB- 453 GFP spheroids at 1:50 E:T ratio at 72h.

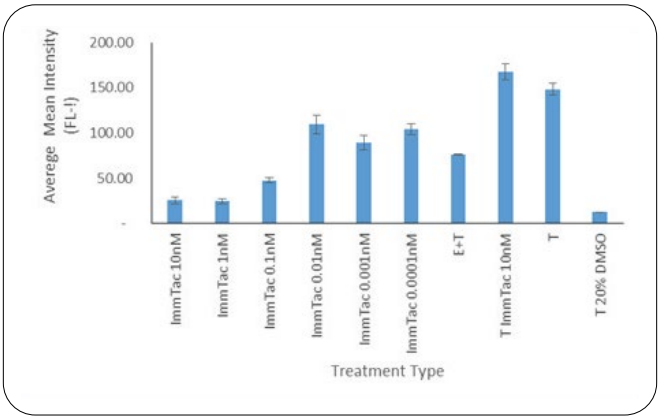


Figure 2: A kill curve of MDA-MB-453 GFP spheroids plus 1:50 ratio of ImmTAC activated T cells.

Conclusions

3D spheroid killing application on the Celigo image cytometer allows for in situ spheroid growth and viability analysis at different time points under cytotoxic conditions. This provides a rapid and easy-to-use method to quantify cytotoxicity of 3D spheroids without the need for subjective manual analysis. The primary T cells have a minimal observable killing effect on their own. The ImmTAC molecule is designed to bring T cells into proximity of the antigen-expressing target cells. This proximity and receptor antigen interaction is key to the activation of the T cells and T cell-dependent cellular cytotoxicity. This is observed by the ImmTAC dose-dependent killing of the GFP target cells in the spheroids.

Collaborators

Immunocore, 101 Park Dr, Milton, Abingdon OX14 4RY

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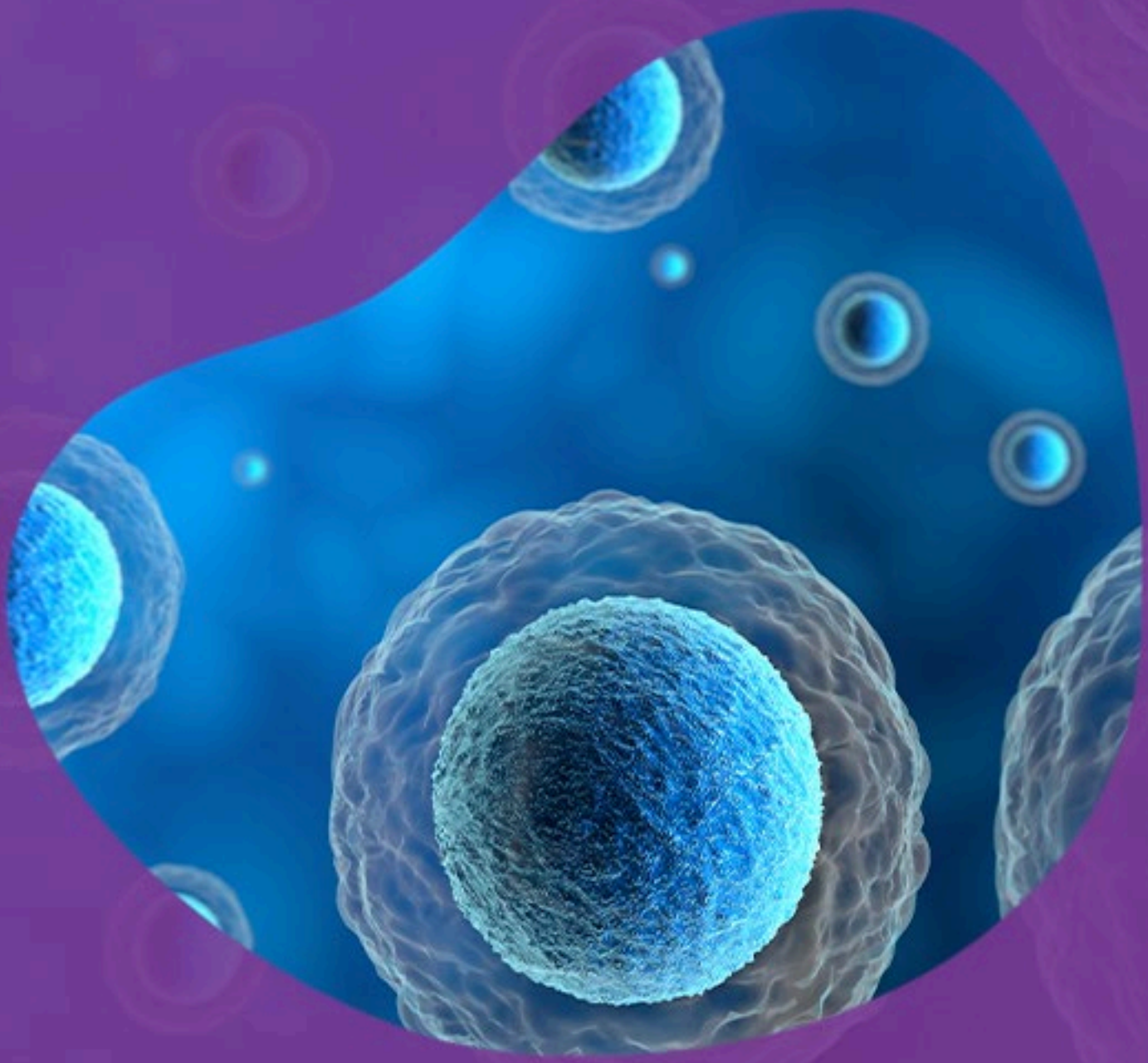
Chapter 8

Cytotoxicity of Bispecific Antibody Therapeutics



Introduction

Bispecific antibodies (bsAb) combine the binding sites of two monoclonal antibodies (mAbs), which enables the recognition of two different epitopes either on the same or different antigens.[1] Particular application to immunooncology includes the ability of bsAbs to redirect specific immune effector cells - like T cells - to tumor cells and thereby enhance cytotoxicity.[2] The ability to target two disease mediators for immuno-therapeutic applications could provide greater target specificity, modulate off-target effects, increase drug safety, overcome immune evasion by tumor cells, and improve clinical outcomes.[3]

Currently, most of the available bsAbs are designed with specificity to CD3, to recruit T cells to tumor cells, which in turn are targeted by a variety of tumor antigen-specific antibodies such as CD19, CD20, HER2, etc.[4] The recruited T cells then exert potent cytotoxicity toward the tumor cells. In addition to T cells, natural killer (NK) cells and dendritic cells (DCs) have also been investigated as potential targets of bispecific antibodies.[5]

Immunotherapeutic bsAbs can be separated into three main categories: (1) Cytotoxic effector cell redirectors, including T cell redirectors and NK cell redirectors; (2) tumortargeted immunomodulators; and (3) dual immunomodulators.[6] Of these three, the most advanced in development are the T cell redirectors.[6]

As an example, bispecific T cell engager antibodies (BiTEs) contain one antigen-binding site directed against the CD3 receptor that activates cytotoxic T cells while the second antigen-binding site is directed against tumor antigens such as CD19, CD20, HER2, etc.[4] Another bsAbs design is called BiKEs (bispecific killer engagers), which induce tumor cytotoxicity through binding CD16a (FcRγIIIa) on NK cells through CD3.[7]

To date, bsAbs have been successful in clinical development for hematological malignancies but have limited efficacy in solid tumors. Part of the challenge is the physical density and complexity of tumors themselves, as well as the scarcity of known tumor antigen targets.[8] The modification of bsAbs can customize functionality, enhance efficacy, modulate immunogenicity, and change effector functions, which is a valuable characteristic in developing immunotherapeutics.[9]

Assay principles

Before bsAbs can be evaluated for *in vivo* efficacy, characterization should be performed *in vitro*. Cytotoxicity is an important parameter to measure in immunotherapeutic development. Traditional cell killing assays used to measure bsAbs cytotoxicity are Calcein-acetoxymethyl (Calcein-AM), ^{51}CR release assay, lactate dehydrogenase release assays, 3-(4,5-dimethylthiazol- 2-yl)-2,5 diphenyltetrazolium bromide (MTT), (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethyl phenyl)-2-(4-sulfophenyl)-2Htetrazolium) (MTS), lactate dehydrogenase (LDH) assay, or various flow cytometric assays.[10]

Calcein AM cytotoxicity assays are commonly used. Calcein AM stains live cells for intracellular esterase activity, then the fluorescent signal dissipates once the cells die. Generally, the target tumor cells are incubated with Calcein AM, then undergo a wash step and are plated. The bsAbs-containing cells are spiked in at varying doses over the time course. Once the endpoint is reached, the cells are harvested and analyzed using a fluorometer. Cytotoxicity analysis is based on the intensity of the fluorescent Calcein signal. Unfortunately, Calcein-AM has a high spontaneous release rate thus increasing variability in measurements.[11]

Flow cytometry allows for quantification of both live and dead cell populations to measure viability and cytotoxicity. Typically, a coculture of differentially labeled effector cells (engineered bsAbs containing cells e.g., T cells) and target tumor cells with varying ratios are incubated over a time course. Target cells and effector cells can be labeled with a non-toxic cell tracking dye like violet cell tracker. Cell tracking dyes are multigenerational and permeable to cell membranes. Once the dye enters the cell, the dye binds nonspecifically to proteins and is retained in the cell for several days. The cell tracking dyes have a variety of colors with different emission spectra to enable several cell populations to be stained and tracked at the same time.[12] The fluorescent signal from

both populations is calculated as a ratio to indicate efficacy. When the time-course is completed, the cells are harvested and stained with near infrared Live/Dead, a far-red tracker to evaluate cell killing.[12,13] Unfortunately, flow cytometry is not a high-throughput method, is laborious, and costly.

Image cytometry provides several advantages to evaluating cytotoxicity due to real-time live cell imaging, analysis over a longer time-course, and high-throughput abilities. The Celigo cell imaging system offers high-content imaging by utilizing brightfield and fluorescence imaging to determine the selective toxicity of bsAbs therapeutics, while also tracking the size and morphology of cells in the evaluation of novel bsAbs immunotherapeutics.

Models/probes and dyes

CellTracker™ Green CMFDA (Thermo Fisher Scientific)

Violet BMQC (Thermo Fisher Scientific)

Methods and Celigo set-up

To evaluate selective cytotoxicity of dual-antigen targeting bsAb, parental NCI-H358 (expressing two disease mediators, EGFR and HER2) and HER2 KO cells were stained with CellTracker™ Green CMFDA and Violet BMQC (Thermo Fisher Scientific), respectively, according to the instructions from the manufacturer.

Cells were then combined at a 1:1 ratio in RPMI-1640 with GlutaMAX supplemented with 10% HI FBS and seeded in 96-well plates at a density of 1×10^4 cells/well.

Two antibody constructs, either the parental EGFR/HER2 targeting parental DuetMab or an EGFR affinity-reduced variant (VκF94A+VHP97A), were added at various concentrations to triplicate samples.

The cells were incubated for 72 hours at 37 °C and 5% CO2 in a humidified incubator.

To enumerate each cell population after 72 hours, the wells were scanned on the Celigo imaging cytometer in the brightfield, green fluorescence, and blue fluorescence channels.

Celigo Set-up:

The plate was scanned on Celigo using Target 1+Target 2+Target 3 Analysis.

Viable parental NCI-H358 cells were quantified as CMFDA positive cells in green fluorescence, and viable HER2 KO cells were enumerated via BMQC signal in blue fluorescence, using the fluorescence algorithm.

Percent cell viability was calculated based on the change in cell number relative to a no antibody treatment control.

Challenges

Selective cytotoxicity assay poses a challenge for traditional cell killing methods, which only provide bulk measurements and lack cell composition analysis. Imaging cytometry allows researchers to use fluorescence cell tracing dyes to label and track different cell types in mixed cocultures, making it possible to provide sensitive and specific measurements to assess the selective cytotoxicity of bsAb.

TIPS & TRICKS

To minimize background signal and autofluorescence around the well edges, it is best to use a black-walled plate.

When setting up the co-culture assay, seeding density, and ratio need to be optimized.

To use the fluorescence cell labeling dyes in the co-culture assay, leakage of the fluorescence dye into neighboring cells needs to be tested and ruled out.

Impermeable membrane dyes such as Propidium iodide can be used to monitor dead cell populations in conjunction with tracking dyes.

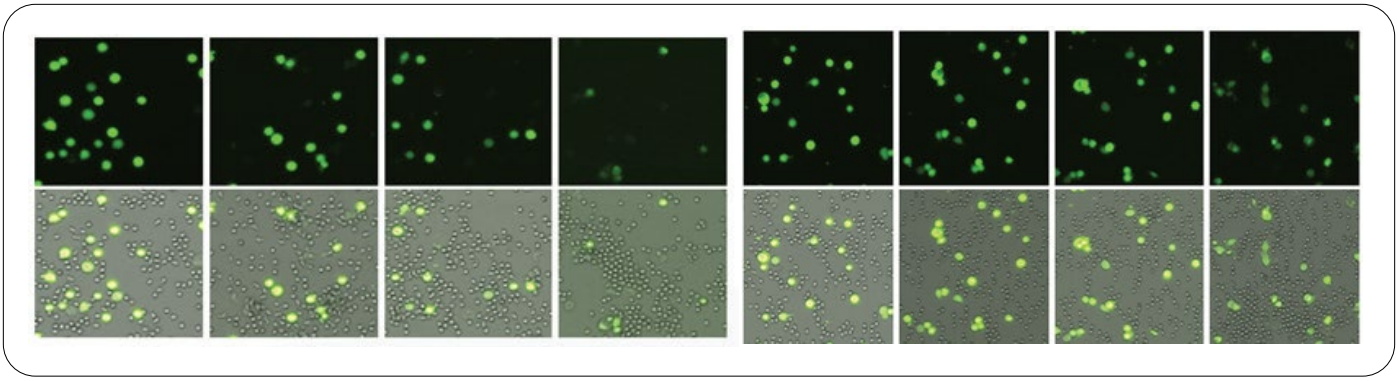


Figure 1: Measuring cytotoxicity of dual-antigen targeting bsAb with image cytometry

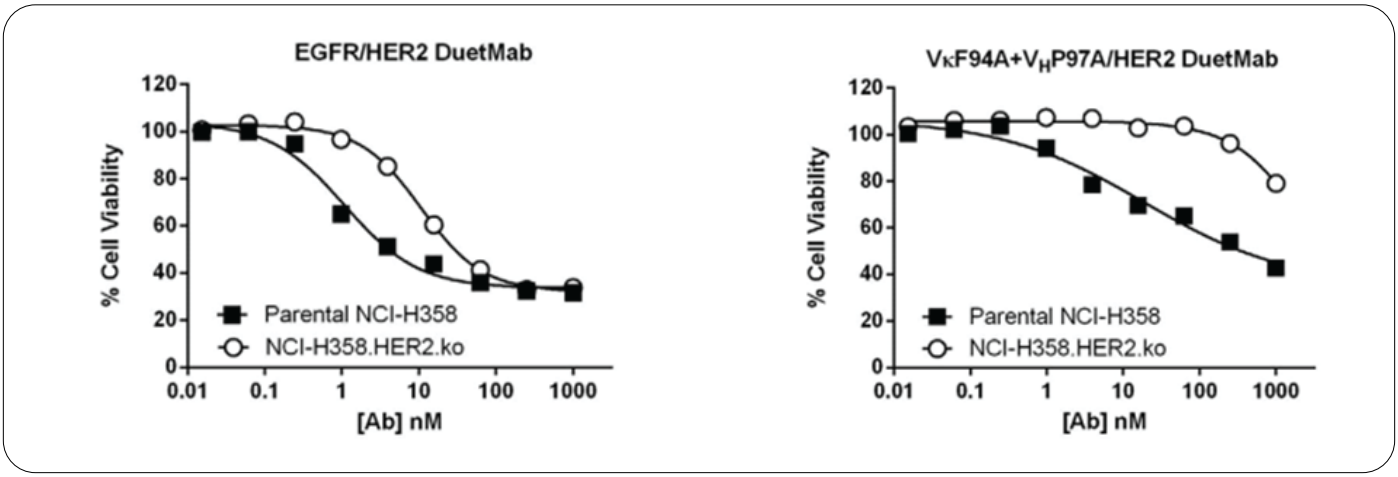


Figure 2: Reduced affinity bsAb variant leads to selective tumor cell cytotoxicity.

Results

The Celigo can be used to determine the efficacy of bsAb candidates targeting dual disease mediators on target cancer cells by labeling the target cells with a tracking dye (such as Calcein AM) and monitoring the change in viable cell numbers relative to no antibody treatment controls (Figure 1). Positive control antibodies (top panel) and negative control antibodies (bottom panel) were incubated with target tumor cells (labeled green) over time. Cells are quantified in the green channel. A clear decrease is observed over time in target cells treated with a positive control antibody.

Conclusions

BsAbs remain an extremely promising area of anti-cancer therapeutic development. The therapeutic advantage of a dual-antigen targeting bsAb is considerable, as modulating the affinity of each arm to their targets (thus the overall avidity of the bsAb) can affect the selective cytotoxicity of the antibody and ensure the targeted killing of only those tumor cells expressing both antigens.

Collaborators

Customer Publication: Mazor, Y., Sachsenmeier, K., Yang, C. *et al*. Enhanced tumor-targeting selectivity by modulating bispecific antibody binding affinity and format valence. Sci Rep 7, 40098 (2017).

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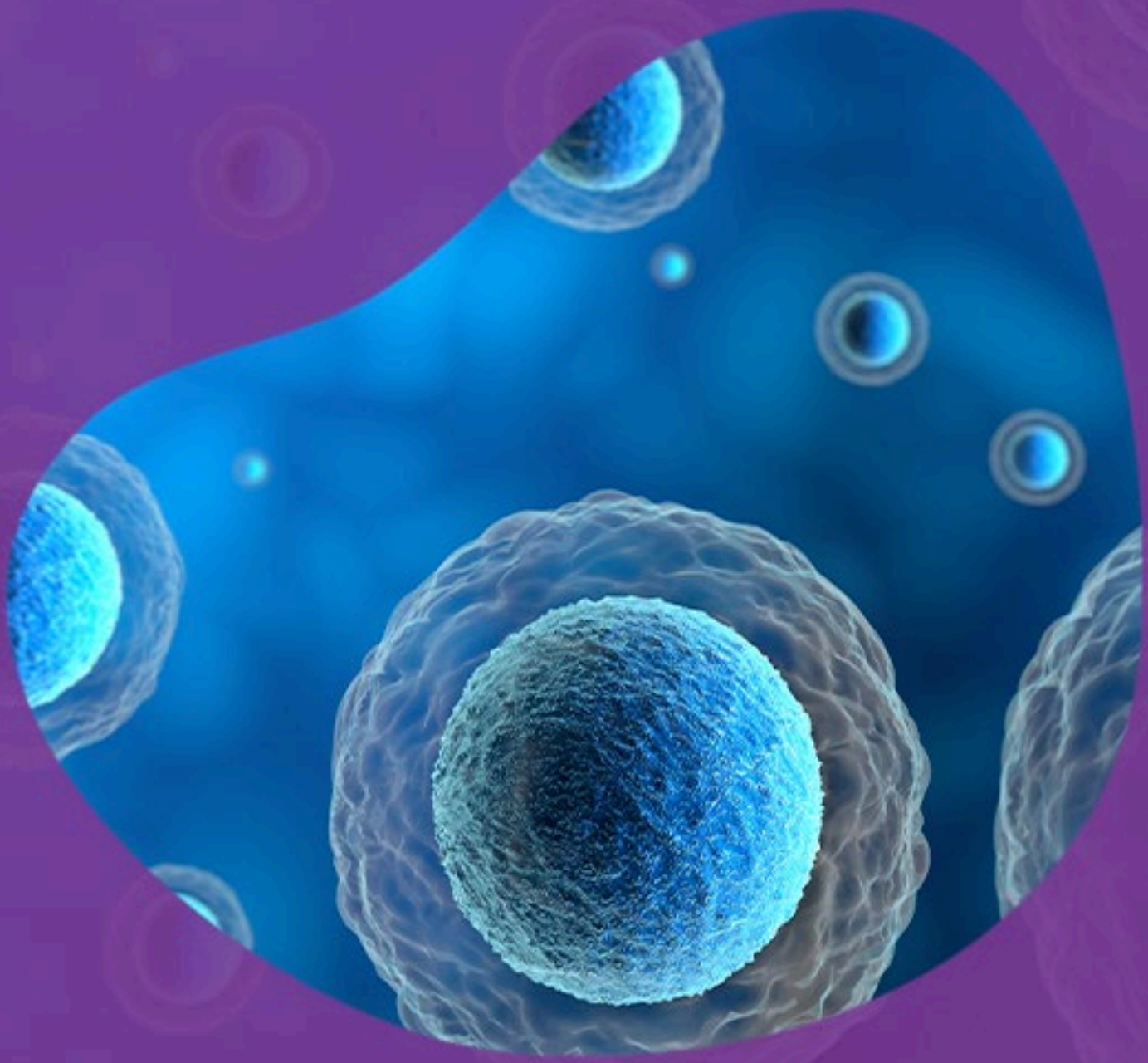
Chapter 9

Quantifying direct cell cytotoxicity by NK cells



Introduction

Natural killer (NK) cells are a type of immune cell that participates in early immune defense responses against pathogens and tumors, contributing as an immune effector and regulatory cell. NK cells secrete cytokines such as interferon-γ and TNF-α and fragment crystallizable (Fc) proteins such as CD16 (FcγRIII) and CD56bright/dim.[1]

The interface between an NK cell and its target tumor cell is called an immunological synapse. In this synapse, cytolytic proteins such as perforin and granzymes are released and result in direct target tumor cell lysis or apoptosis.[2] Due to their ability to target and kill tumor cells, NK cells have been investigated for cell therapy. However, since NK cells are functionally impaired in many cancer types, researchers are attempting to develop NK cell-based immunotherapies to increase sensitivity and specificity to tumor cells. Since NK cell activity and etiology differ between patients, it is key to establish and utilize a variety of methods to evaluate NK cell function.[3]

Assay principles

There are two types of assays for measuring NK cell function: degranulation and cytotoxic (direct cell killing) assays. Degranulation assays are indicative of NK cell activation and the percent of NK cells that respond to a stimulus such as a target tumor cell. Cytotoxic assays yield more information by assessing the functional killing efficacy of NK cells that are co-cultured with tumor target cells at varying ratios. Peripheral blood mononuclear cells (PBMCs) are frequently used as a source of NK cells for these direct cell killing assays. These tests can be used to evaluate potential treatments, measure the innate immune responses of cancer patients, and screen for features like monogenic defects affecting the NK cell compartment.[2,3]

Cytotoxicity assays have traditionally been performed using the ^{51}Chromium (^{51}Cr) release assay, which involves labeling the tumor cells (target) with radioisotopes. When the target cells are lysed by the immune cells (effector), they release the entrapped radioactive ^{51}Chromium, which is measured to determine the level of cytotoxicity induced by the effector cells. The ^{51}Chromium release assay is highly hazardous, time-consuming, and can only acquire endpoint readout. Nowadays, cytotoxic assays are more commonly analyzed via a plate reader or flow cytometer utilizing fluorescence-based assays, bioluminescence-based assays, and lactate dehydrogenase (LDH)- release assays. [4] Table 1 shows a summary of different assay used for cytotoxicity measurements, including assay principles and limitations of each method.

Several flow cytometry-based assays have been developed for evaluating NK cell cytotoxicity. For example, target tumor cells are pre-labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and co-cultured with NK cells at varying ratios in the presence of PBMCs. After a predetermined incubation time, the cells are stained with a nucleic acid dead stain and enumerated via flow. The cytotoxicity of the NK cells is analyzed across varying concentrations by a reduction in tumor cell fluorescent signal and an increase in dead cell stain signal.[2]

Table 1. Summary of traditional assays to measure NK cell cytotoxicity

Detection Method	Description	Existing Issues
Radioactivity Release	Measure release or radiolabels, 51Cr, 101In in the supernatant	Handling hazardous material and indirect measurement of cell death
Fluorescence Release	Measure release of Calcein AM fluorescent molecules in the supernatant	Indirect measurement of cell death, endpoint assay only
LDH Release	Measure release of a cytosolic enzyme in the supernatant	Indirect measurement of cell death, endpoint assay only
Luciferase Reporter Assay	Measure luciferase as the cells die	Indirect measurement of cell death
Flow Cytometry	Measure the number of viable cells and viability in the sample	Cannot perform in plates, must trypsinize for adherent cells

Unfortunately, assays run on a flow cytometer are terminal, which only allows one time point to be measured thereby limiting the ability to derive kinetic information. The time in which NK cells activate and respond to therapies can vary depending on donor and target cells.

Celigo image cytometry can overcome this obstacle and provide kinetic information by capturing multiple measurements over time within every well in a cell culture plate. Target cells are typically stained with a fluorescent dye which will be released from the cells when they are lysed by the NK cells. Or, a counterstain to identify dead cells can be added. The Celigo imaging system enables direct images as well as quantitative measurements of NK cell killing of tumor cells and thereby provides multiparametric and longitudinal analysis rather than a snapshot. Additionally, data can be normalized to the amount of target tumor cells present in the well at time zero, allowing for more robust assays even when seeding density is not perfectly uniform. Finally, because Celigo captures whole-well images, every cell in each condition is included in the analysis, meaning fewer cells must be used for each condition and more conditions can be studied from a single donor (especially important when using primary cells).[1] Taken together, imaging cytometry with Celigo provides the ability to effectively generate real-time data and analysis of NK cell cytotoxicity that can inform therapy efficacy.

Models/probes and dyes

ViaStain Calcein AM (CS1-0119, Revvity)

Human PBMCs (effector cells)

K562 cells (target tumor cells)

This data set specifically shows NK cell killing using whole PBMCs as the effector cell population and K562 cells as the target tumor cell population. However, this general assay set up could work with a wide range of target tumor cells. Calcein AM typically works well to stain target cells for a 4-6-hour assay, but other dye combinations or fluorescent reporters could be used in longer-term assays

Methods

The Celigo Image Cytometer was used to perform a high-throughput cytotoxicity screening assay to detect direct killing of Calcein-stained K562 target cells co-cultured with fresh PBMCs from two healthy donors in a flat-bottom 96-well plate in the presence or absence of IL-2. The effector-to-target ratios (E:T) were set up at 50:1, 25:1, 12.5:1, 6.25:1, and 3:1, and direct cell counting of Calcein positive cells was performed at times 0, 1, 2, 3, and 4 hours. As the target cells die, the Calcein fluorescence is diminished and only the live Calcein positive target cells remain. Therefore, the percent lysis or cytotoxicity can be calculated from cell count at time 0 and 4 hours.

Cell-mediated cytotoxicity assay

Calcein AM staining

1. Prior to staining, dilute the Calcein-AM stock solution (1 mM) in PBS to 5 µM staining concentration
2. Collect the Target cells from the culture flask, spin down, and remove cell media
3. Resuspend the cells in 5 µM Calcein AM and incubate for 30 min in 5% CO_2, 37 °C
4. After incubation, wash the Target cells three times with PBS and resuspend in cell culture media

PBMC and K562 co-culture cytotoxicity assay

1. Seed approximately 10,000 Calcein AM stained target cells/well in a 96-well plate in a final volume of 100 µl/well.
    1. The seeding density range will vary depending on Target cell type, Target cell size, as well as the availability of Effector cells
2. Centrifuge the 96-well plate containing the Target cells at 1,000 RPM for 5 min in a swing bucket rotor centrifuge
3. Scan the 96-well plate on Celigo at t = 0 h- (prior to the addition of control reagent and Effector cells)

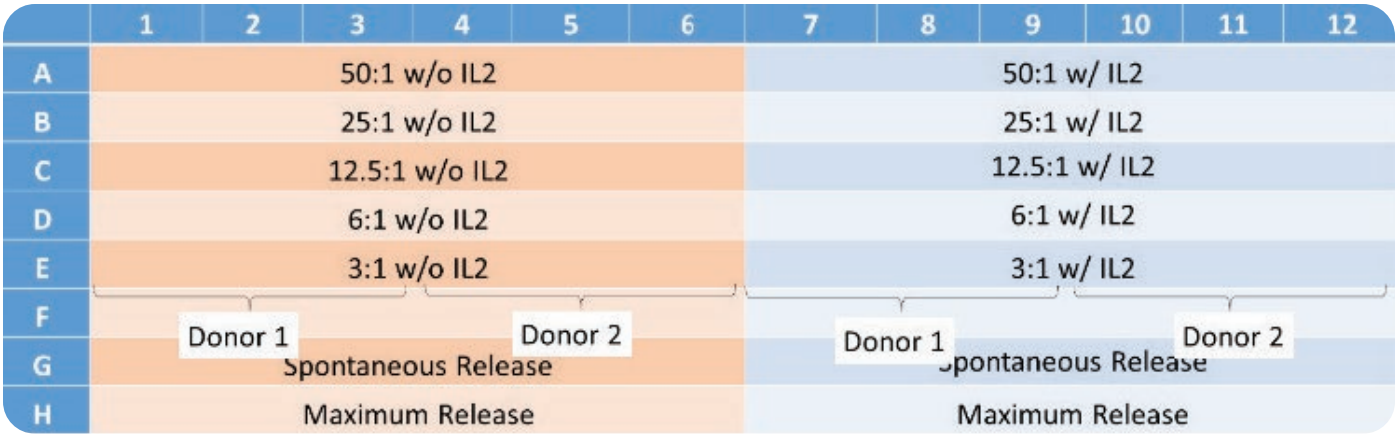


Figure 1. Example plate map with appropriate E:T ratios, cytokines, and controls

4. Add in 50 µl of IL-2 to activate the PBMC, and 50 µl media to the other half of the wells, so the total in the wells is 150 µl
5. Add in different E:T ratios of Effector cells in the volume of 50 µl, bringing the volume to 200 µl
    1. 1 x 107 cells/ml (50:1), 5 x 106 cells/ ml (25:1), 2.5 x 106 (12.5:1), 1.25 x 106 cells/ml (6:1), and 0.63 x 106 cells/ml (03:1)
6. Add Triton X-100 control treatment to maximum release control wells
    1. Prepare Triton X-100 at 20% in a volume of 50 µl cell culture media
    2. Pipette the 50 µl of Triton X-100 into each treated well, so the volume in the well is 200 µl
7. Prepare spontaneous release control wells
    1. Prepare some wells without any Effector cells, with a final volume of cell culture media at 200 µl
8. See the example plate map above in Figure 1

Image cytometric analysis of PBMC-mediated cytotoxicity

1. Centrifuge the plate after all the reagents and Effector cells have been added
2. Scan the 96-well plate on Celigo at t = 0 h+ (after adding control reagent and Effector cells)
3. Incubate cells at 5% CO_2, 37 °C for 1 hour before the next scan
4. Scan the 96-well plate on Celigo at future time points depending on the assay
    1. Commonly performed at 4 – 20 hours duration
    2. For 4 – 6 hours incubation, the Celigo is typically used to scan on an hourly basis
    3. For 20 hours of incubation, the Celigo is typically used to scan on an hourly basis up to ~6 hours, and then incubate overnight to scan an endpoint between 18 – 20 hours

Celigo set-up

The Celigo software application "Target 1 + 2" was utilized to autofocus and acquire brightfield and green fluorescent images at 1 µm2/pixel with exposure times set to auto-exposure and 10,000 – 30,000 µs, respectively.

The Celigo software was used to directly count the number of Calcein-positive K562 cells on the bottom of the 96-well plates at different chemokine and antibody concentrations. The ANALYZE parameters for the green channel were set to:

1. "Algorithm = Fluorescence"
2. "Intensity Threshold = 8"
3. "Precision = High"
4. "Cell Diameter = 10"
5. "Dilation Radius = 0"
6. "Background Correction = Check"

7. "Separate Touching Objects = Check"
8. "Minimum Cell Area = 20"
9. The BF images were not utilized by setting the "Intensity Threshold" to 255

Challenges

It is important to check the target cell viability before the beginning of the assay. Additionally, the dye you use to label the target cells should persist for the duration of your experiment. Calcein AM is a great dye for many target cells, but some target cell types will pump out the dye. Other dyes such as CFSE or Cell Tracker dyes can be good alternatives. Fluorescent reporters such as GFP or RFP are also good options if you encounter poor staining with Calcein-AM.

Results

Endpoint PBMC-mediated cytotoxicity results

The Celigo was used to count every Calcein-positive K562 target cell in the well. The example Calcein AM fluorescent images of K562 Target cells at t = 4 hours are shown in Figure

2. You can visually see the reduction in green fluorescent target cells in conditions with high E:T ratios, especially in the presence of IL-2. The resulting cell counting quantification (reported as % Lysis in

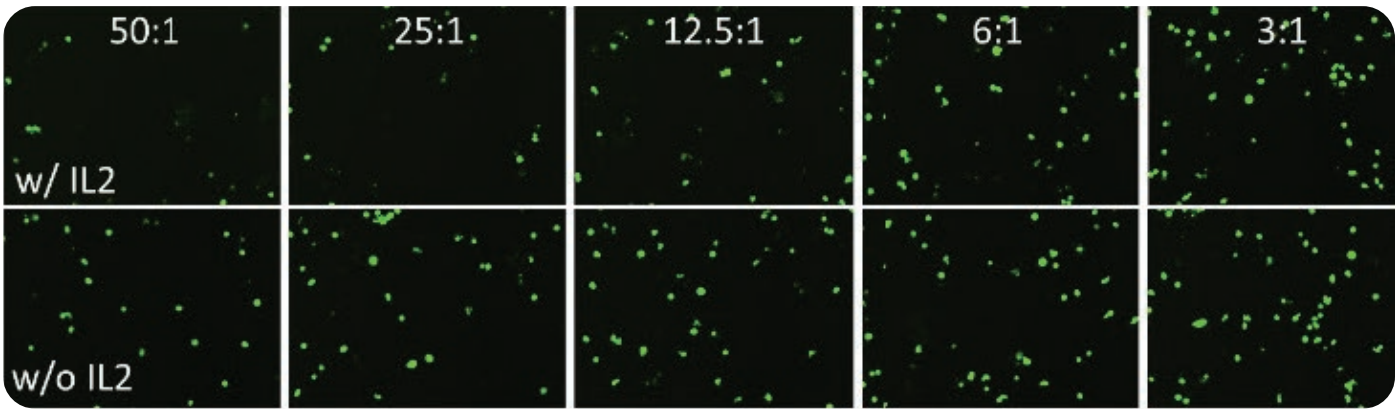


Figure 2. Example images from Celigo showing lower Calcein AM stained K562 target cells in wells with high E:T ratios in the presence of IL-2.

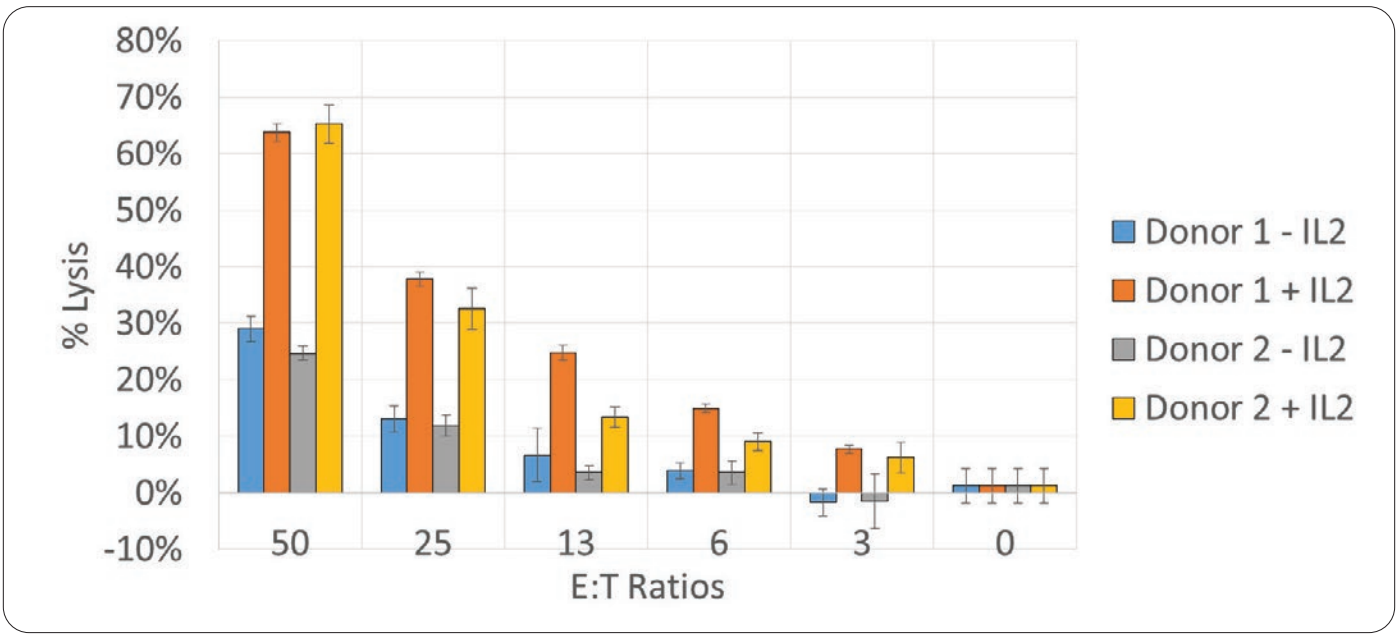


Figure 3. Quantification of % Lysis based on Donor and E:T ratio.

Figure 3) showed an increase in specific lysis as E:T ratio increased. The addition of IL-2 also showed a significant increase in the killing of target cells. The results indicate that Donor 1 has a slightly higher cytolytic potential compared to Donor 2

Time-dependent PBMC-mediated cytotoxicity results

The Celigo was used to track the number of live Calcein-positive target cells over time. Figure 4 shows the time-dependent fluorescent images, where you can visually see the decrease in fluorescent target cells over time.

Time-course tracking of % lysis can eliminate the need for multiple controls and the effect of non-uniform cell seeding in the final cytotoxicity calculation. Figure 5 shows the quantified % lysis over time for the 50:1 E:T ratio. In the presence of IL-2, NK direct cell killing is twice as high for both donors.

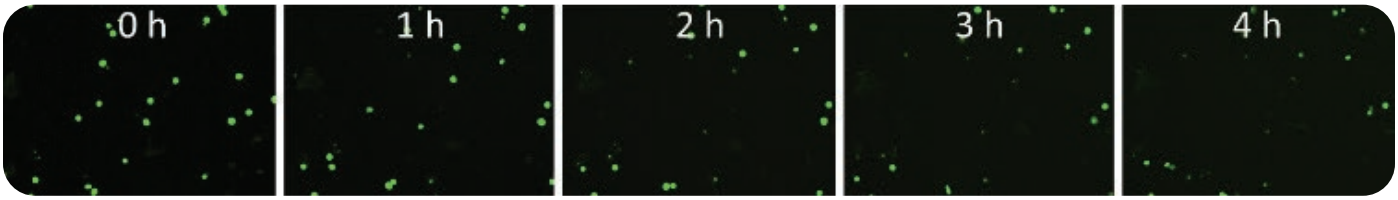


Figure 4. Celigo images showing a decrease in K562 target cells over time.

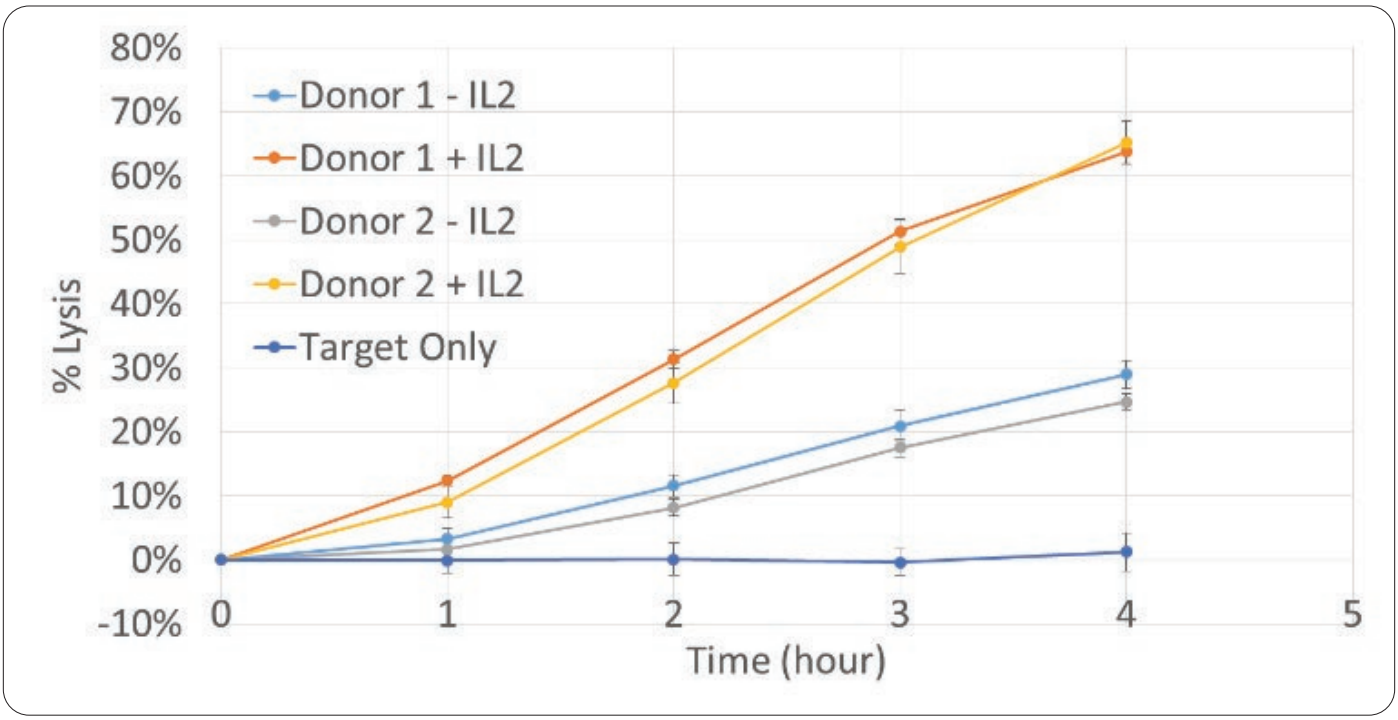


Figure 5. Time course direct NK cell killing of K562 cells.

TIPS & TRICKS

The cell-mediated cytotoxicity percentages can be determined using the "self-referencing" calculation. The percent killing is generated by referencing the number of live target cells to time 0, to obtain a more accurate representation of target cell status in the wells.

Be sure to spin down the plate to get an accurate starting target cell count at time zero.

Purified cultured NK cells can also be used in this experiment instead of NK cells in whole PBMC. Cultured NK cells typically have very high cytotoxicity effects on target cells and therefore would be tested with much lower E:T ratios.

Conclusions

The Celigo image cytometry method can scan and analyze the entire well area of an entire 96-well plate for this assay in 7 minutes, making it ideal for high-throughput kinetic screening of direct NK cell killing of target tumor cells. Adherent cells can be measured and analyzed directly in the plate without trypsinization. The number of cells used is significantly less than the cells needed for Release assays and Flow Cytometry. Overall, the Celigo offers an efficient method for academic, industry, and clinical research that overcomes the limitations of previous methods.

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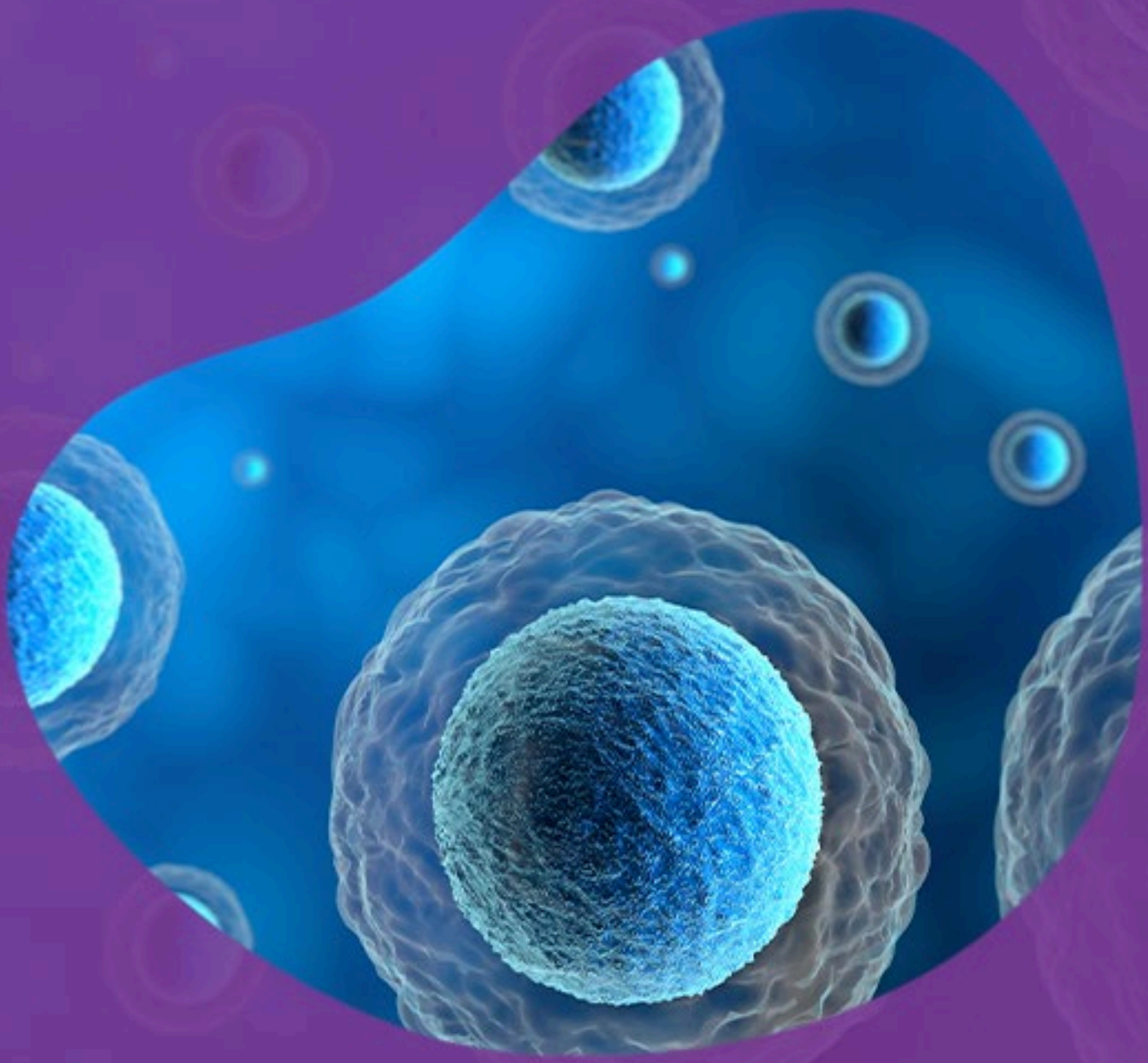
Chapter 10

Long-term antibody-dependent cell-mediated cytotoxicity



Introduction

Antibody-dependent cell-mediated cytotoxicity (ADCC) is an important mechanism used by the immune system to target and destroy diseased cells. This mechanism is exploited in immuno-oncology to develop powerful and effective cancer treatments. The ADCC mechanism is triggered when tumor-specific monoclonal antibodies (mAb) of the effector cell recognize tumor-selective antigens on the surface of the target/tumor cell. Effector cells include natural killer (NK) cells, monocytes, macrophages, neutrophils, eosinophils, and dendritic cells.1 Inside the tumor microenvironment there are rich and variable interactions among immune cells, tumor cells, and stromal cells along with the extracellular matrix and signaling molecules like cytokines and chemokines. The tumor microenvironment supports proliferation, survival, and metastasis of tumor cells by creating an immunosuppressive environment.2 ADCC is an important mechanism to modify so that immune response can be rescued in the tumor microenvironment. In drug development, ADCC assays that target the ability of NK cells to bind mAb for target tumor cell killing are important in measuring the efficacy of potential cancer therapeutics.

Traditional ADCC assays can be difficult to standardize since primary target cells like PBMCs or effector cells like NK cells are typically from different donors, contributing to variability. This vial-to-vial variability decreases reproducibility and increases inconsistency in the performance of the assays.

An imaging cytometer can overcome the need to use a large number of cells and acquire multiple time points for real-time assessment of experimental parameters. Reducing the need for multiple wells could enable researchers to use one cell lot for analysis and reduce the variability of data.

Assay principles

Typical ADCC assays used to assess efficacy are: Calcein-acetoxymethyl (Calcein-AM), ^{51}CR release assay, or lactate dehydrogenase-release assays, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), a luciferase reporter assay, or various flow cytometric assays using Annexin V, propidium iodide, or 7-amino-actinomycin D.[3,4]

The MTT assay is a colorimetric assay that measures metabolic activity in cells. Active metabolism of cells reduces MTT to a purple formazan product. MTT cannot be reduced if cells die. The color change is measured via a spectrophotometer and generates absorbance values which are indirect measurements of cell viability.[5]

Calcein AM is a non-fluorescent dye that becomes fluorescent when live cells transport the dye inside and the intracellular esterases remove the acetomethoxy group. Viable cells are enumerated and quantified. Unfortunately, Calcein-AM has a high spontaneous release rate thus increasing variability in measurements.[6]

Brightfield imaging can capture cell behavior such as size and morphology changes as well as activation and proliferation. In this case, the cytotoxicity of a target tumor cell can be indicated by the appearance of clusters of immune cells like NK cells surrounding the target tumor cells. With imaging, the response to increasing amounts of immunotherapeutic can be assessed and optimized over time.

Unfortunately, many of these assays have poor reproducibility and high variability. As previously mentioned, the performance of effector and target cells in a frozen thaw-and-use format can exhibit vial-to-vial and lot-to-lot variation. These assays also do not provide longitudinal data and are limited by having one terminal endpoint, thereby causing the need to run many plates to optimize culture conditions.

The Celigo cell imaging system decreases the need for large cell numbers by virtue of its ability to monitor and measure longitudinally, enabling multiple experiments using the same vial of cells for ADCC assays. Another benefit of imaging the same well over and over is that the dynamic killing process of target tumor cells can be analyzed

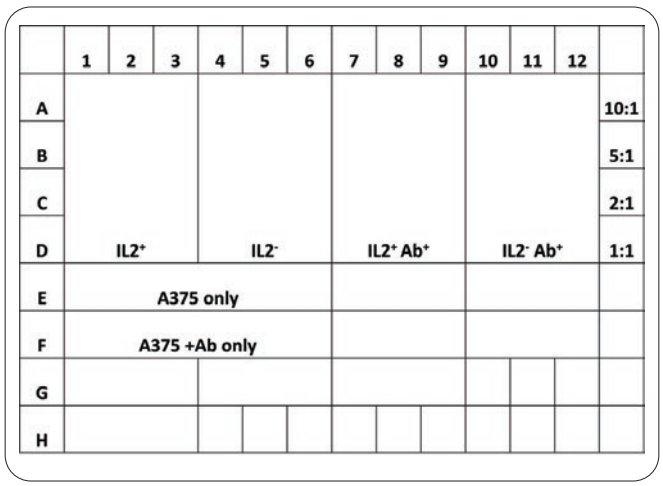
visually and quantitatively. The Celigo cell imaging system can enhance data sets that support novel immunotherapeutics that target the ADCC mechanisms by taking multiple longitudinal measurements, reducing variability, increasing assay reproducibility, and real-time assessment of efficacy and immune cell response.

Models/probes and dyes

ZsGreen A375 cells were constructed for the long-term NK cell-mediated ADCC assay.

Methods and Celigo set-up

1. The ZsGreen A375 cells are collected from cell culture in the presence or absence of MICA/B antibodies after 24-hour incubation
2. Seed approximately 5,000 cells/well in 96-well plate in a final volume of 100 µl/well.
    1. The seeding density range will vary depending on Target cell type, Target cell size, as well as the availability of Effector cells
3. Centrifuge the Target cells at 1,000 RPM for 5 min in a swing bucket rotor centrifuge
    1. Follow standard settings for other cell types
4. Scan the 96-well plate on Celigo at t = 0 h-(prior to addition of reagent and Effector cells)
5. Add in 100 µl of IL-2 pretreated NK cells at E:T ratios 1:1, 2:1, 5:1, and 10:1
6. Prepare control wells



    1. Prepare some wells without Effector cells, with a final volume of cell culture media at 200 µl
    2. See plate map (below)
7. Centrifuge the plate after all the reagents and Effector cells have been added
8. Scan the 96-well plate on Celigo at t = 0 h+ (after adding control reagent and Effector cells)
9. Incubate cells at 5% CO_2, 37 °C for 3 hours before the next scan
10. Scan the 96-well plate on Celigo at t = 3, 4, 6, 24, 27, 30, 51, 74, and 76 hours.

$$Specific\ Killing\ \% = \left(\frac{\Delta Count}{Count_{t=0}}\right) \times 100\,,$$
$$\Delta Count = Count_{t=0} - Count_{t=4}\,,$$

11. The number of ZsGreen positive cells is counted for each well at each time point
12. The specific killing is calculated by comparing the number of cells present at t = 0 and a future time point of t = x

Celigo set-up:

The Celigo software application "Target 1 + 2" was utilized to autofocus and acquire brightfield and green fluorescent images at 1 μm^2/pixel with exposure times set to auto-exposure and 10,000 µs, respectively.

The Celigo software was used to directly count the number of ZsGreen positive A375 cells on the bottom of the 96-well plates at different antibody concentrations. The ANALYZE parameters for the green channel were set to:

1. "Algorithm = Fluorescence"
2. "Intensity Threshold = 4"
3. "Precision = High"
4. "Cell Diameter = 20"
5. "Dilation Radius = 0"
6. "Background Correction = Check"

7. "Separate Touching Objects = Check"

8. "Minimum Cell Area = 20"

9. The BF images were not utilized by setting the "Intensity Threshold" to 255

Challenges

When designing a co-culture cytotoxicity assay on an imaging cytometer, a bright fluorescent label is required for accurate quantification of the target cells specifically. There are several models and fluorescent labels that can be considered depending on the desired assays.

For short-term cytotoxicity assays (shorter than 24 hours), the simplest method is to stain the target cells with Calcein AM and then count the live cells over time.

For long-term cytotoxicity assays (longer than 24 hours), the best method is to utilize target cells expressing fluorescent proteins such as BFP, GFP, ZsGreen, RFP, mCardinal, etc., and then count the fluorescent positive target cells over time.

Results

The Celigo was used to image and count ZsGreen-positive A375 cells over time (Figure 1).

The E:T ratio-dependent fluorescent images showing NK cell-mediated killing at the 74-hour time point are shown in Figure 2. A subset of co-cultures was supplemented with IL-2 to support NK cell survival and compared against co-cultures without IL-2. The images showed that NK cells induced high cytotoxicity on A375 with the addition of MICA/B antibodies. Additionally, A375 co-cultured with IL-2 pretreated NK cells showed higher cytotoxicity.

TIPS & TRICKS

When adding the target and effector cells into the wells, pipette the liquid in the wells up and down at least five times to ensure uniform distribution so that the cells are not highly dense in a certain area, which makes accurate cell counting difficult.

The target cell numbers can vary based on their growth rate and well size, thus an initial target cell proliferation assay should be considered for the duration of the killing assay.

If a tracer dye or metabolic dye is used, the spontaneous release rate should be considered to ensure the target cells do not release too quickly during the killing assay, which may cause inaccurate signal generation.

E:T ratios should be considered depending on the physiological relevance of the assay, lower E:T ratios can be used for prolonged killing

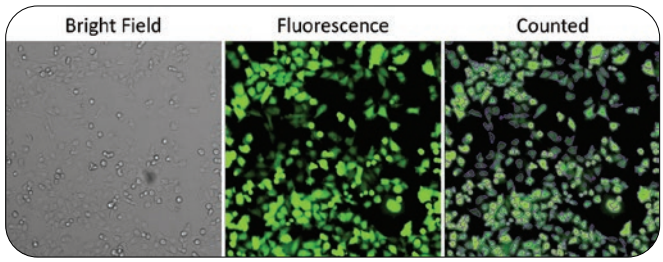


Figure 1. Representative images of ZsGreen A375 cells from Celigo.

A kinetic profile of NK cell-mediated cytotoxicity is illustrated in Figure 3 which shows BF/FL overlay images for wells representing control, IL-2 as well as IL-2 plus MICA/B antibody conditions.

The quantitative kinetic analysis of the long-term NK cell-mediated ADCC is shown in Figure 4. In the absence of NK cells, the A375 tumor cells grew quickly over time. In the absence of IL-2 or MICA/B antibodies, the NK cells were able to inhibit tumor cell outgrowth, and the effect is E:T ratio-dependent. The NK cell-mediated killing kinetics may be difficult to observe in a standard 4-hour assay (Figure 4), while longer co-culturing time can reveal additional killing characteristics such as the ability of NK cells to eliminate target cancer cells and prevent the regrowth of residual cancer cells.

The addition of the MICA/B antibodies induced a strong NK cell response towards A375 melanoma cells, resulting in the eradication of most tumor cells at a high effector to target ratios (5:1 and 10:1). The addition of IL-2 plus MICA/B antibody was synergistic and resulted in the elimination of most live tumor cells.

Conclusions

The Celigo Image Cytometer was used to measure ADCC effects of NK cells co-cultured with A375 for an extended period. The Celigo was used to image the killing of human ZsGreen+ melanoma cells by primary human NK cells in the presence of an antibody targeting MICA and MICB on the tumor cell surface. The number of live ZsGreen+ A375 cells was counted in 96-well plates over 72 hours, and the results were used to calculate % specific killing. Image cytometry demonstrated that the combination of the MICA/B antibody and IL-2 induced near-complete eradication of A375 melanoma cells by NK cells at later time points. This novel image cytometry-based approach will be suitable for the discovery of combination therapies that enhance the cytotoxic function of NK cells against tumor cells.

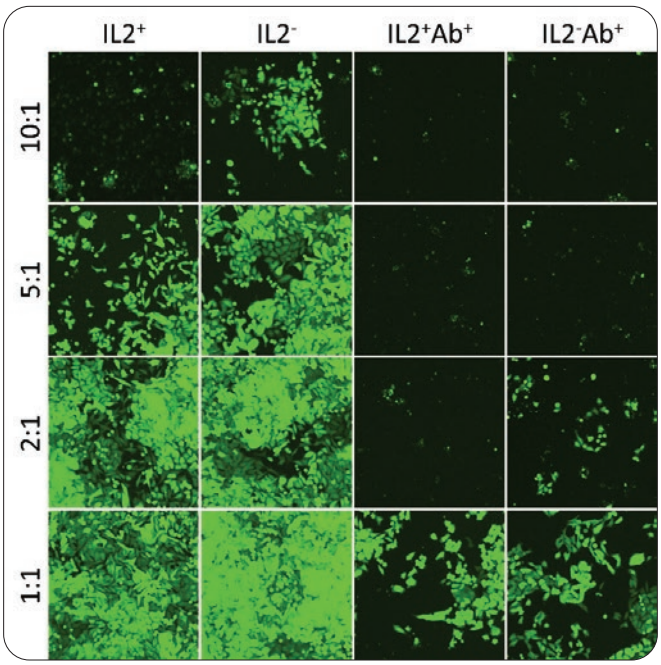


Figure 2. NK cell-mediated ADCC results at 74h.

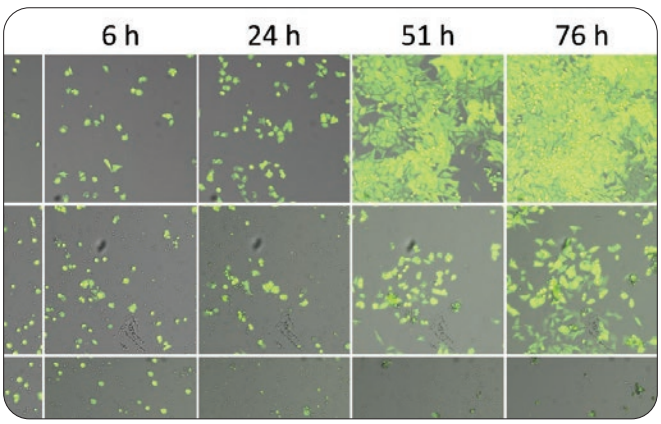


Figure 3. Representative images of co-culture at 0, 6, 24, 51, and 76h.

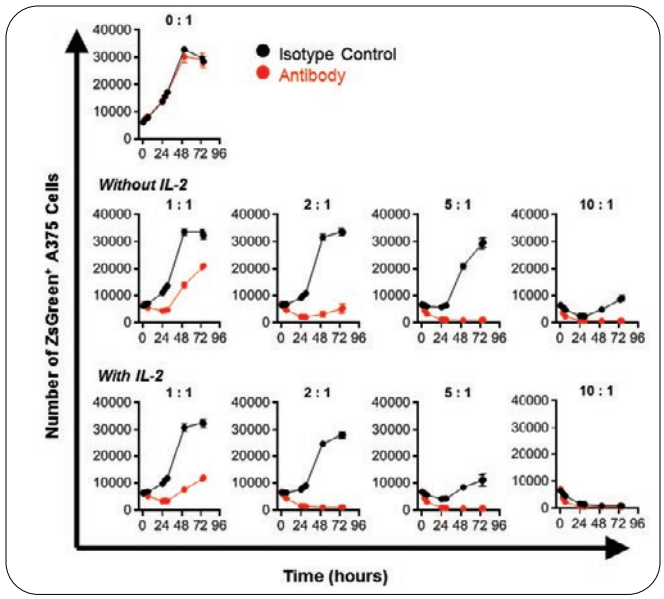


Figure 4. Quantitative kinetic analysis of the long-term NK cell-mediated ADCC.

Collaborators

The work presented here was a collaboration with Dana Farber Cancer Institute, Department of Cancer Immunology and Virology, and has been published in the Journal of Immunological Methods (ref 3).

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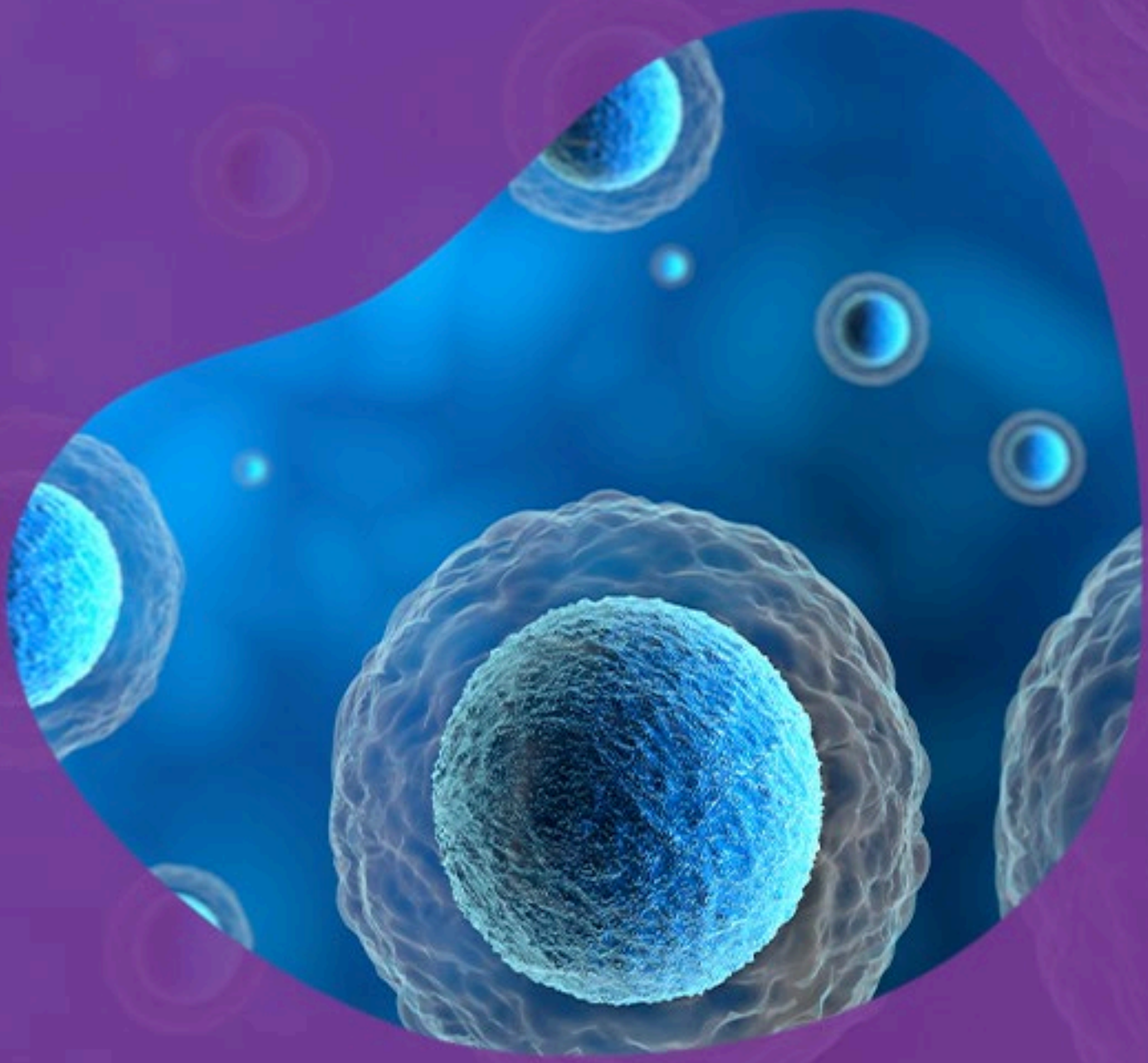
Chapter 11

3D spheroid antibody-dependent cell-mediated cytotoxicity



Introduction

Antibody-dependent cell-mediated cytotoxicity (ADCC) is a principal effector mechanism of natural killer (NK) cells. NK cells express fragment crystallizable (Fc) gamma receptors (FcγR), predominantly CD16a (FcγRIIIA), which recognize the Fc domain of an antibody-like IgG. Binding to the target cell via the Fc domain causes cytokine and cytotoxic granule release from the NK cell, which induces target cell lysis/apoptosis.[1,2] The interaction of F cγRs and mAbs creates an abundance of functional diversity in immune responses. The ADCC mechanism can be mediated therapeutically by antigen directed monoclonal antibodies (mAbs) that are overexpressed in tumor cells. Immunotherapies are utilizing the overexpression of unique antigens and engineering mAbs to create targeted and more potent treatments.

Targeting ADCC has been successful in the treatment of hematological malignancies however, treatment for solid tumors has been limited. Factors hindering the efficacy in immunotherapeutics for solid tumors utilizing ADCC include difficulty with access to the tumor and the complex nature of the tumor and its microenvironment.

To gain access to the solid tumor, immune cells and antibodies must extravasate from the blood vessels, then diffuse and migrate to the tumor. Solid tumors are dense structures that impede drug penetration and migration of cells and have the potential to interfere with the ability of NK cells and antibodies to reach the target cells in the hypoxic tumor center. The tortuous path leading to and inside the tumor must therefore be considered when developing immunotherapeutics for solid tumor indications.

Furthermore, the tumor microenvironment and tumor structure are complex, difficult to recapitulate *in vitro*, and poorly correlated in a standard 2D cell culture. Typically consisting of a monolayer of cells on a plastic surface, a 2D cell culture lacks the complexity of a 3D structure set to mimic the tumor microenvironment and allow for cell-to-cell interactions, *in vivo*-like phenotypes, and metabolic gradients. Researchers have begun to use 3D cell cultures of organoids and spheroids to better recapitulate *in vivo* efficacy and better predict clinical outcomes *in vitro*.[3]

Assay principles

A multicellular tumor spheroid (MCTS) is a translational tool that can improve predictions of the clinical efficacy of immunotherapeutics. MCTS can be created by different methods, the most common are the hanging-drop method, the suspension method, and the use of microfluidic devices. In the hanging drop method, cells are suspended inside a droplet of culture media and aggregate based on gravitational forces. The suspension method utilizes ultra-low attachment (ULA) plates that inhibit cells from attaching to the plate and forces them to adhere to each other, thereby creating MCTS. The formation of MCTS using microfluidic devices utilizes hydrodynamic trapping of cells in micro-chambers with controlled geometries. The device will perfuse fresh media and control the size of the MCTS via perfusion flow rate.[4]

Typical 3D ADCC assays measure the viability of spheroids post immunotherapy treatment. An MCTS is created and then treated with an immunotherapeutic and harvested at a terminal endpoint and stained with various stains, dyes, probes indicating cell health and viability.

A common stain used for viability analysis is live/dead staining which consists of Calcein-AM (green stain) and ethidium homodimer-1(red stain). The intensity of red and green are measured via imaging and can be calculated as a ratio to signify efficacy. Calcein AM stains live cells for intracellular esterase activity. Ethidium homodimer-1 stains for loss of plasma membrane integrity that indicates an apoptotic cell.[5,6]

Another common set of stains used together is Hoechst staining, SYTOX red dead cell staining, or propidium iodide (PI). Hoechst stains the nuclei of cells and SYTOX and PI stain dead cells. SYTOX dye only permeates the membrane of dead cells and binds to DNA and emits a fluorescent signal. PI binds to DNA by intercalating between the bases with little or no sequence preference. The viability can be quantified via the signal intensity of the SYTOX dye or PI.[7] Viability can be quantified from the signal intensity and a dose-response curve can be generated.

Microfluidic systems can give real-time longitudinal data via automated widefield fluorescence microscopy for quantification. However, microfluidic systems are multicomponent, not high-throughput, and expensive and laborious to maintain. For the purposes of drug development and discovery, the use of microfluidics is not ideal.

Part of the difficulty in having concise measurements of viability in MCTS is the 3D nature of the system. The Celigo cell imaging system enables analysis of MCTS using both brightfield and fluorescence imaging in a high-throughput manner. MCTS can be imaged over longer periods of time and the changes in morphology indicating tumor stability can be monitored, suggesting drug potency and efficacy. The ability to image an MCTS in one well over time would better recapitulate the ADCC process and minimize the noise of the data. The ability to measure MCTS size and morphology using the Celigo cell imaging system gives an abundance of qualitative and quantitative data. This enables researchers to determine a more comprehensive and reproducible ADCC profile and identify highly effective immunotherapeutics via multiparametric analysis.

Models/probes and dyes

Calcein AM (Revvity)

Methods and Celigo set-up

K562 tumor spheroids are cultured in the appropriate media for a predetermined time for proper growth and formation.

NK cells are also grown and stimulated as needed.

K562 tumor spheroids are stained with Calcein AM and subjected to NK cells under various E:T ratios.

The plate is scanned on Celigo at time 0 and 4h in brightfield and green fluorescence to measure the size and viability of tumor spheroids.

Celigo Set-up:

The plate is scanned using the Tumorsphere 1 + Mask application in fluorescent and brightfield illumination. The application segments the tumorsphere in brightfield for size measurement and quantifies Calcein signal in green fluorescence for viability measurement.

Challenges

To set up a tumor spheroids culture, seeding density and growth condition need to be optimized for individual cancer cell lines.

Traditional methods for tumor spheroids size measurements include manual monitoring using a light microscope or confocal microscopy. Both methods are time-consuming and can be subjective to operator interpretation.

TIPS & TRICKS

NK or effector cells can be stained with fluorescent live tracker dyes (Cell Trace Violet) to identify and quantify effector cell penetration and killing capacity

Calcein AM staining conditions need to be optimized to ensure proper staining of tumor spheroids. Residual Calcein AM can cause high levels of background and may require a washing step.

Results

The Celigo image cytometer images and monitors the size and viability of spheroids under cytotoxic conditions. Figure 1 shows representative images of K562 spheroids stained with Calcein-AM viability dye at various E:T ratios. After 4hrs of exposure to NK cells, pockets of dead cells appear shown by loss of Calcein-AM staining. Higher E:T ratios have increased pockets of dead cells compared to T=0.

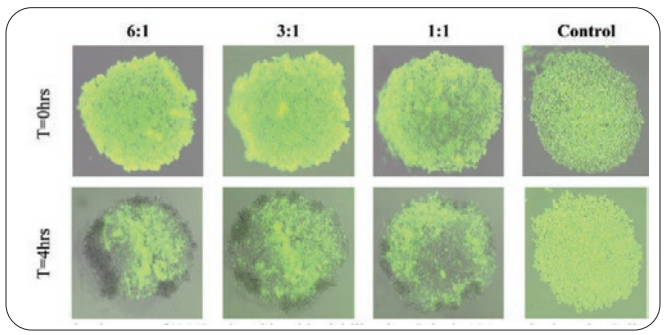


Figure 1. Representative images of K562 spheroids with Calcein AM on Celigo image cytometer at 0 and 4h.

Presented in Figure 2 is a killing curve depicting the percentage of dead cells through the loss of Calcein-AM. Total mean intensity was exported for each of the E:T ratios and normalized to T=0 hrs. Higher killing is observed with increasing E:T ratios, with the highest level of cytotoxicity seen in the E:T 6:1 ratio.

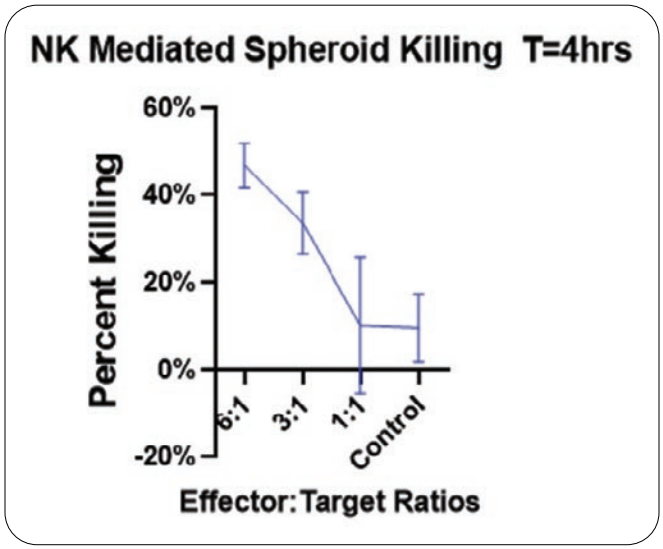


Figure 2. NK cell-mediated spheroid killing at 4h.

Celigo image cytometer was utilized to perform high-throughput 3D killing experiments. Ready to use spheroids application automatically reports spheroid size, volume, fluorescent intensity, and morphological characteristics.

Conclusions

3D spheroid killing application on the Celigo image cytometer allows for in situ spheroid growth and viability analysis at different time points under cytotoxic conditions. This provides a rapid and easy-to-use method to quantify cytotoxicity of 3D spheroids without the need for subjective manual analysis. Celigo accommodates a multitude of microwells and embedding methods, allowing for increased flexibility for users.

Data contributed by Celigo customers at an academic research university.

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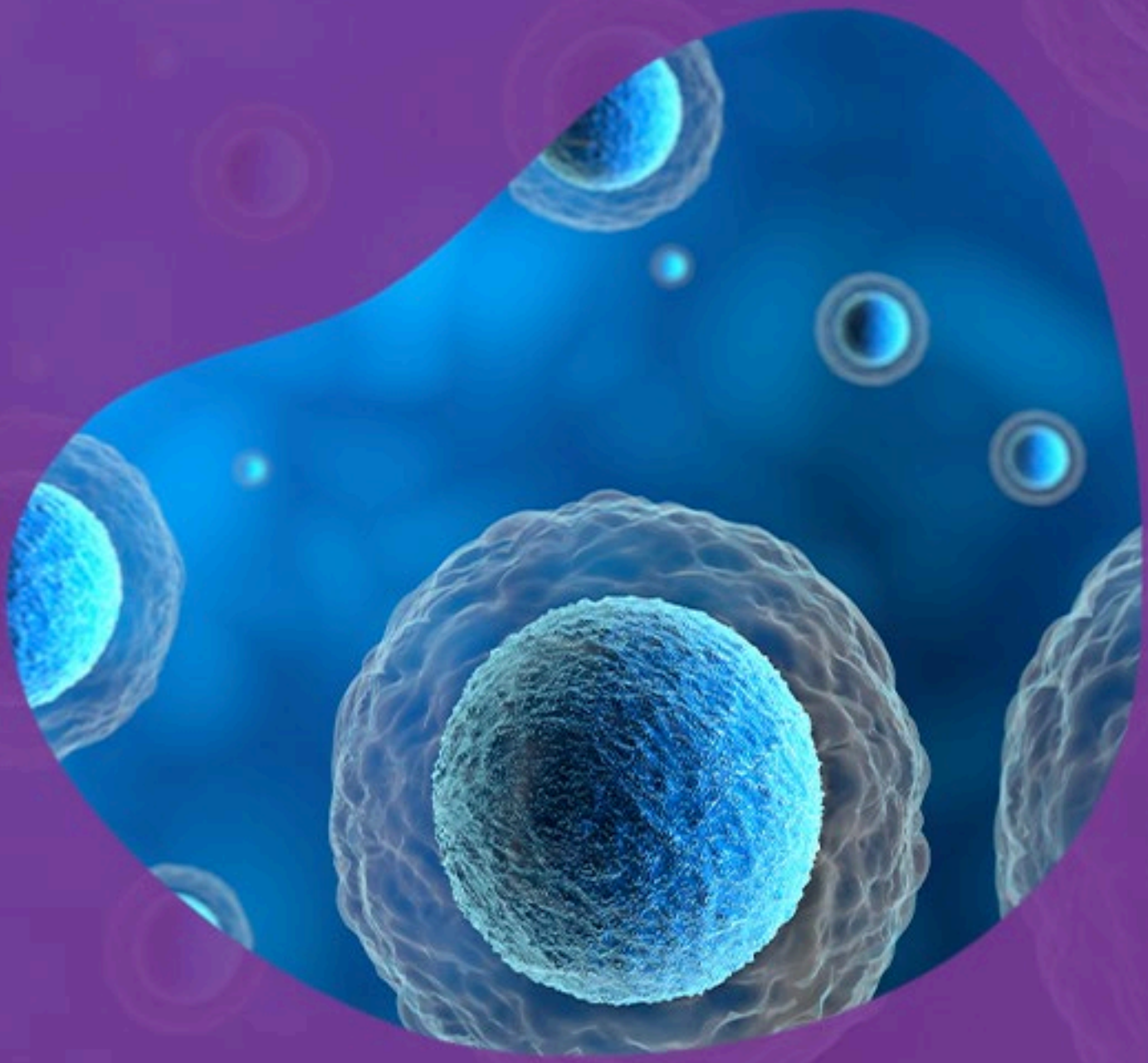
Chapter 12

Efficacy screening of antibody-drug conjugates



Introduction

An increasingly investigated type of immuno-oncology therapeutics are antibody-drug conjugates (ADCs). ADCs are complex molecules constructed from recombinant monoclonal antibodies (mAb) that are covalently attached with a linker molecule to a cytotoxic drug. ADCs combine the specificity of antibodies with the potency of a drug to then selectively destroy tumor cells while minimizing off-target effects to normal cells.[1–3] The FDA has stressed the imperative on the analytical characterization of all monoclonal antibody-based therapeutics creating the need for rigorous and reliable analytical techniques.

The three components of ADCs - antibody, linker, and drug - result in heterogeneity of ADCs when engineering immunotherapeutics. Modifying the chemistry of any one of these components results in changes in their stability, conjugation, concentration, and distribution. Current techniques assess the drug-to-antibody ratio (DAR), ADC stability, aggregation degree assessment, bioanalysis, quantification of unconjugated drug molecules, and post-translational modifications (PTMs).[3]

Efficacy of cytotoxicity is a key bioanalytical measurement made to verify the quality of the engineered ADC. Typical cytotoxic assays to measure ADC efficacy are cell viability and proliferation using flow cytometry, colorimetric assays, and luminescent assays. Efficacy is directly related to cell viability analysis in which the total number of viable cells are assessed post-exposure to ADCs. There are challenges in using both of these assays. Typically, these assays are terminal and have multi-point titration curves with a specific end time-point. Measurements cannot be made over longer time periods, which could cause sub-optimal measurements and neglect biological mechanisms that occur over longer time periods. An imaging cytometer overcomes many of these challenges by allowing for longer measurement times and real-time decision making for faster optimization of the best endpoint and cell culture system parameters.

Assay principles

In the drug discovery pipeline, regulatory safety assessment includes *in vitro* toxicity and efficacy assessment using ADCs before moving to *in vivo* testing. Typical cytotoxic assays for assessing the efficacy of ADCs are *in vitro* viability assays that measure the total number of cells left viable after exposure to the ADC therapeutic. Viability is generally measured by fluorescence, luminescence, or colorimetric outputs. The most common method for high-throughput applications in drug development is the measurement of Adenosine Triphosphate (ATP) using firefly luciferase. During apoptosis, cells not only lose membrane integrity and the ability to synthesize ATP but also endogenous ATP is expelled from the cytoplasm. Therefore, ATP is used as a marker of viability as non-viable cells will not have ATP. For this assay, cells are seeded in standard multi-well plates (e.g., 96-well or 384-well plate) and ADCs are spiked in using a titration curve. After some time, the ATP detection reagent is added to the wells. The regent generally consists of a detergent to lyse the cells, an ATPase inhibitor to stabilize the ATP released, and luciferin as a substrate. Luciferase is used to catalyze the reaction, which generates a photon of light that is then quantified using a spectrophotometer. The advantage of this method is that it can be done all in one plate, cells do not need an additional staining step, and the measurements can be made within ten minutes of adding the ATP reagent.[4] However, this indirect method is an endpoint measurement, and the drug tested as part of the ADC may affect the ATP metabolic pathway and impact the readout. Furthermore, as these assays do not require imaging one cannot examine the effect of uneven cell seeding or pipetting error which can dramatically affect results.

A major hurdle in cytotoxic assays is the optimization of the time course of cell growth for the best endpoint for viability and proliferation. Ideally, researchers could use a non-invasive, non-destructive, direct measurement of cell number, and growth over time. The use of the Celigo cell imaging system can address this need by its capacity to use brightfield imaging and cellular quantification to make multiple measurements

and analysis in real-time, facilitating immediate decisions and adjustments with minimal perturbation to the cell culture system. Proliferation kinetics can easily be calculated based on confluence which reflects the effect of the ADC on the target cells. A time-dependent growth curve can be generated with multiple data points that inform efficacy (in a dose-dependent manner), viability, and proliferation of the ADC treated cells. Cells are monitored over time and multiple images are taken of the cells, including time zero to ensure consistent starting cell density. During the time-course, the Celigo cell imaging system enumerates cells as they proliferate and measures changes in morphology as cells die. A growth curve and viability curve can easily be generated. Compared to classical endpoint methods like luminescence or flow cytometry, efficacy measurements can be taken over longer time periods, leading to additional data to drive experimental decisions. The ability to characterize ADC viability and proliferation using non-terminal multiple time-points and imaging enables mechanistic insight allowing for the engineering of efficacious immunotherapeutics that are more predictive of clinical outcomes.

Models/probes and dyes

Any mammalian cell type, adherent or suspension, works well for the ADC screening assay. No dyes are needed, as the Celigo can image and quantify cell number or confluence label-free.

Methods

The antibody-drug conjugate killing assay requires the following materials: Adherent and or suspension target cells, growth media for the target cells, the antibody-drug conjugate prepared at different concentrations, the antibody isotype control, and another positive control if deemed necessary (e.g., 20% DMSO), and a tissue culture 96-well plate. 5000 cells were seeded in 150 ul media within each well and then spun down at 1000 rpm for 1 minute and left to adhere for 4-6 hours and imaged at time 0. 50ul of the positive control, isotype control, and the ADC antibody were added to the wells and the plate was imaged. The plate was then incubated for the desired time and imaged at several time points throughout the experiment. Row A-C contains the isotype control for the ADC candidate and Row D-F contains the titration of the targeted ADC candidate. Row G and H contain no treatment and a positive control (20% DMSO) in alternate quadrants.

Celigo Set-up:

The Celigo's user-interface is designed to sequentially set up an experiment by clicking through the tabs in the software. First, the cell plate is imaged by setting up a new scan using brightfield illumination and the machine is set to analyze the area covered in each well, confluence, using the CONFLUENCE 1 application. When all imaging parameters are properly set, the plate is scanned. Following the algorithm selection, the user can set simple parameters to properly identify cells/ confluent areas in each well and ensure proper segmentation. Typically, the plate scanning and image analysis are performed simultaneously. Data can be exported as raw data and imported into third-party software. This workflow can be used as an end-point readout or at different time points while live cells are maintained in culture. An entire 96-well plate can be captured and quantified in about 5 minutes, so the cells can be scanned periodically and then returned to the incubator until the next time point. This label-free method is direct, kinetic, and non-destructive.

TIPS & TRICKS

Choose a low starting cell seeding density so that your untreated control wells are not overly confluent by the end of your experiment.

Spin down the plate before capturing time zero confluence measurements.

Appropriate controls are essential: use an isotype control as a negative control and a positive control of 20% DMSO to show death.

Use black-walled plates with few imperfections and scratches, filled with 200 uL liquid for ideal imaging conditions.

Challenges

For the first time performing this assay with a new cell type or ADC molecule, choosing the appropriate cell seeding density and time points for measurements may be unknown, but Celigo can provide label-free insight into kinetic cell growth over time. To see dose-dependent results from your ADC, cells should be in the exponential phase of cell growth. Cells should be seeded at low enough density so that there is room to grow over the entire course of your experiment, and ADC molecules should be titrated out in multiple doses. Cells in high-treatment wells may not result in a reduction of confluence, but can still be compared to wells in which the cells are growing and the confluence is increasing over time. To truly measure cell death, using a fluorescent dye like propidium iodide may be another approach to consider.

Suspension cells may present a challenge for most imaging methods, as cells will move about in the plate. The Celigo overcomes this challenge through its ability to capture whole-well images and measure cell coverage of the plate, even if the cells have moved around.

Results

The Celigo image cytometer rapidly captures whole-well images to monitor ADC-based cell killing using cell confluence by measuring the area of the well covered by cells. Figure 1 is a representative image of a 96-well plate depicting cell confluence with a green fill for ease of visualization. This fill view can be seen in the Results tab of the Celigo software. The ADC candidate in Rows DEF reduces the area of confluence in a concentration-dependent manner, as shown by the reduction in green area from right to left in the plate view at 24hrs.

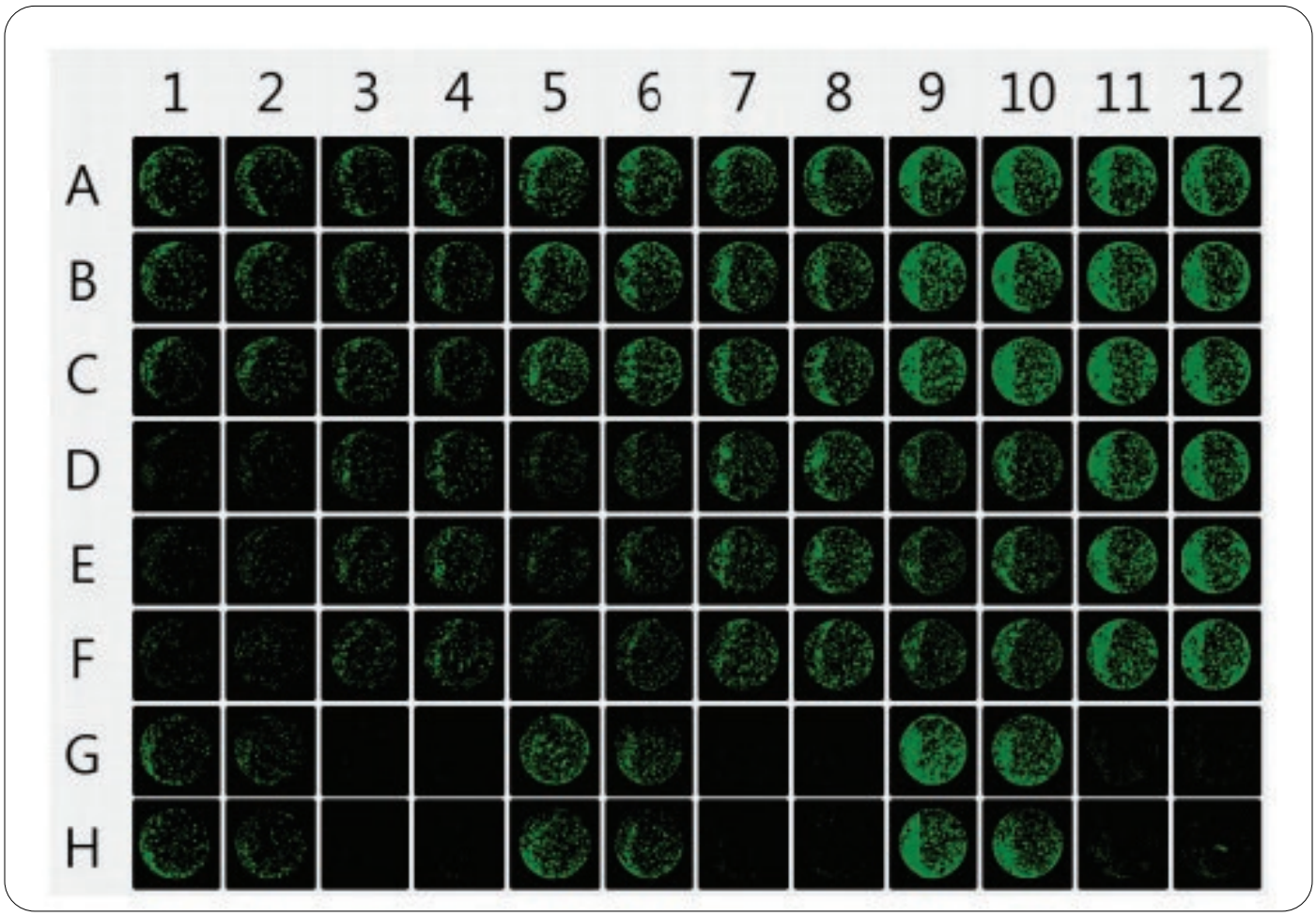


Figure 1: Representative image of a 96-well plate depicting whole-well confluence with a green overlay to ease visualization at 24hrs. Note the increasing green overlay and thus cell confluence as one moves from well A1 to well A12 in row A.

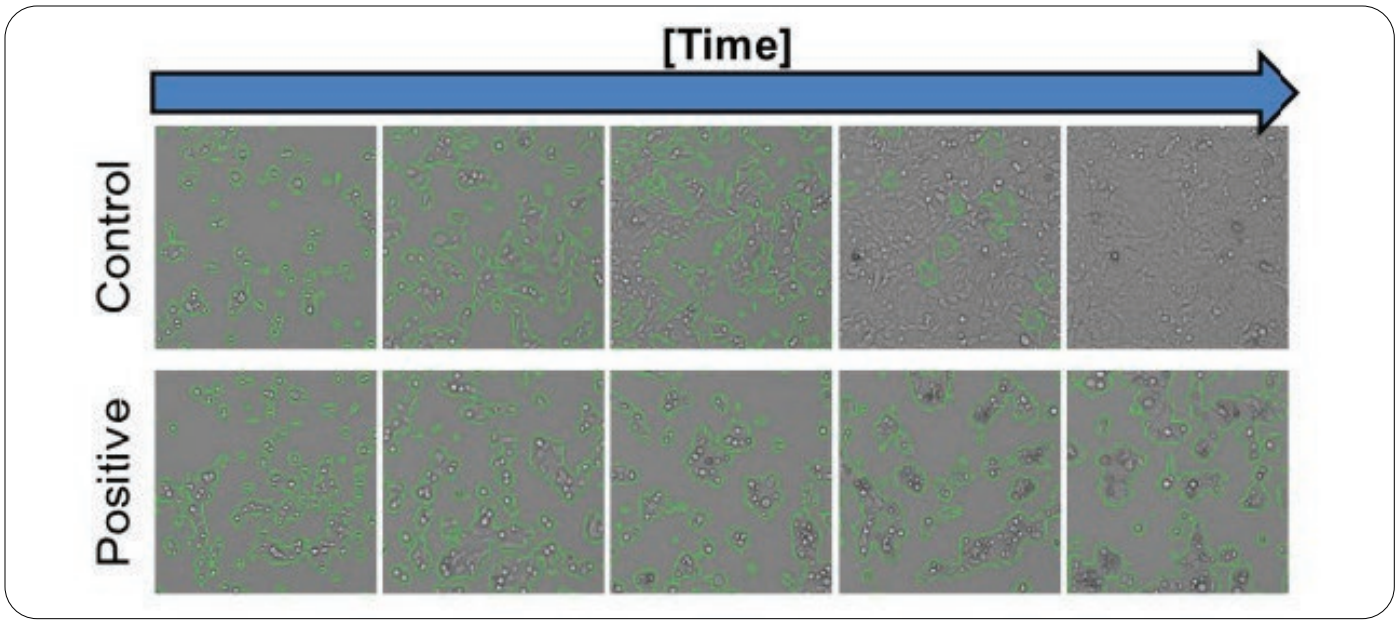


Figure 2: Representative zoomed-in images of Wells B5 (control) and E5 (positive) over the time course of the assay. Note the area of the cells is identified by a green outline that reduces as the image area is increasingly covered by cells in the control image and stays the same or reduces slightly with the positive ADC.

This cytotoxic effect can also be observed kinetically as shown in Figure 2. A selected area from well B5 (control) and E5 (positive) was chosen and a zoomed-in image was created where one can observe that wells containing the control sample have an increase in confluence over time whereas the positive ADC cell area stays the same if not reduces a little.
One can also see the morphology of the cells alter as a function of time in the control sample, the cells adhere and spread out, whereas upon cytotoxic treatment the cells dye and thus round up and cannot adhere to the well.

$$\%\ Cytotoxicity\ (control) = 1 - \frac{\%\ Confluence_{treated}}{\%\ Confluence_{control}}$$

The % cytotoxicity was calculated using the equation below:

The graph in Figure 3 uses this equation and plots the % cytotoxicity for different antibody concentrations.

This rapid, simple, label-free method to monitor ADC cytotoxicity overcomes the limitations of non-image-based traditional methods.

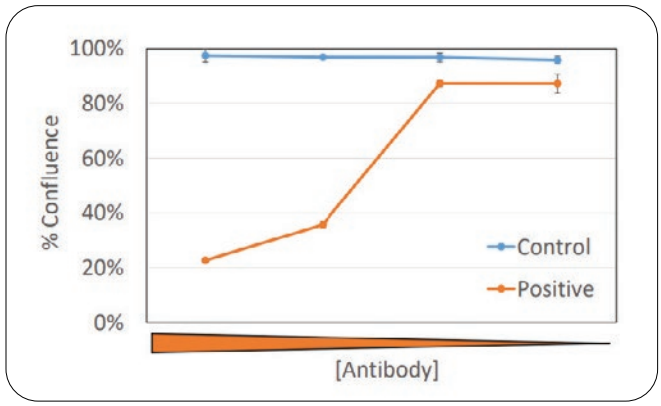


Figure 3: Cytotoxicity curves examining the dose-dependent effect of an ADC compared to control cytotoxic conditions.

Overall, the Celigo image cytometer allows for rapid whole-well plate imaging to perform high-throughput cytotoxicity killing experiments with the following benefits:

1. Easy-to-use interface to image and identify a wide variety of cells and cell well area coverage (confluence) in brightfield and fluorescent channels

2. Output includes quantitative data such as area and fluorescent intensity, along with images enabling observation of morphological characteristics

3. Accommodates a multitude of microwells and embedding methods, allowing for increased flexibility for users

4. Provides rapid real-time readouts: a 96-well plate can be analyzed in under five minutes

5. Using brightfield imaging gives one the flexibility to combine fluorescent probes to gather extra information in confirming cytotoxic readouts. This can be done as an endpoint readout using viability and total stains like Propidium Iodide and Acridine orange and Hoechst. Furthermore, some of these dyes can also be used in a kinetic manner, e.g., Propidium Iodide, DRAQ 7 for viability, and Caspase 3/7 for apoptosis.

Conclusions

The brightfield confluence application on the Celigo image cytometer allows for in situ kinetic ADC cytotoxicity detection and analysis without the need for invasive fluorescent dyes or stains. Celigo enables flexibility to multiplex with kinetic or endpoint stains and dyes to confirm experimental data and gather extra information from orthogonal assays that the traditional plate reader methods cannot. This provides a rapid and easy-to-use method to quantify cytotoxicity effects of antibody-drug conjugates without the need for subjective manual analysis.

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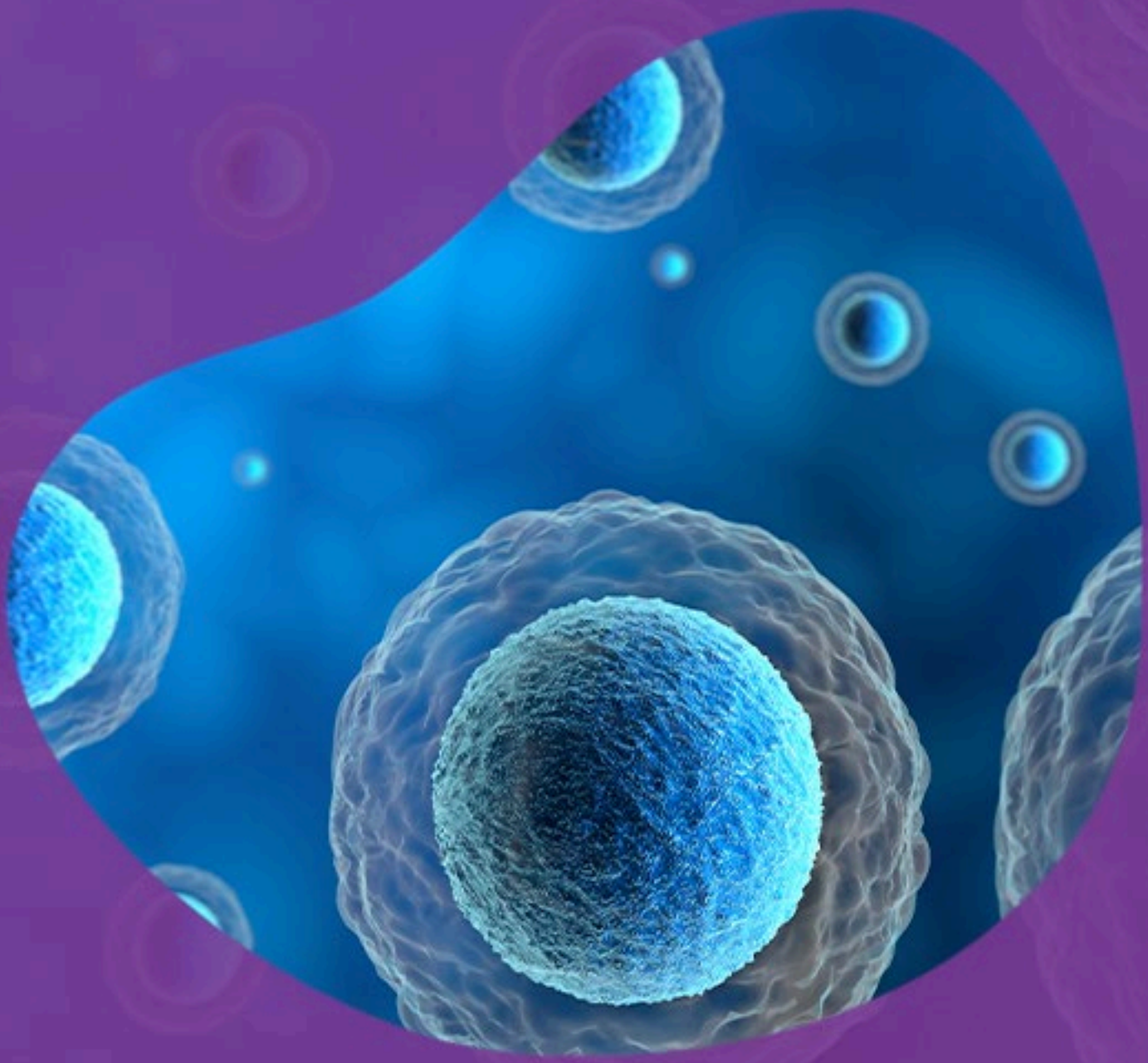
Chapter 13

Complement-dependent cytotoxicity assays



Introduction

Monoclonal antibodies (mAbs) are one of the fastest growing therapeutics.[1,2] Complement dependent cytotoxicity (CDC) is a well-known mechanism that utilizes mAb immunotherapy designs to target and kill tumor cells. CDC occurs when antibodies lyse the unwanted target by activating a cascade of complement-related reactions. The classical complement pathway is activated by antibodies - typically IgG1 and IgG3. These antibodies bind C1q, the first component of the C1 complement complex, to the fragment crystallizable (Fc) region of the cell-bound antibodies. Several other complements (i.e., C3, C4, C5) are recruited from serum, and a series of enzyme activations and cleavage events take place. This results in the eventual formation of a membrane attack complex (MAC) that permeabilizes the membrane and ultimately leads to cell lysis of the target tumor cells.[3] A real-world example of an approved CD20 mAb developed to utilize CDC in the control and eradication of tumor cells is rituximab, which is now well established in treating hematological malignancies such as B-cell lymphoma and leukemias.[1]

CDC is a complex and dynamic process with many variables requiring optimization to create a successful mAb for immunotherapy. The ability to monitor the kinetics of CDC using mAb over long periods without perturbing the system and taking multiple measurements would enable the development of more efficacious and clinically relevant immunotherapies while also considering mechanisms of resistance. Imaging cytometry overcomes some of these issues by enabling label-free methods of tracking cells that are high-throughput, non-radioactive, and can take measurements at multiple time points. The ability to take longitudinal measurements becomes especially important in the investigation of resistance mechanisms. Not all cells are destroyed via targeted mAb immunotherapies and those cells can have intrinsic resistance to CDC. This could be due to low mAb target expression, complement exhaustion, and increased activity or expression of complement regulatory proteins, which would result in decreased generation of membrane attack complexes.[7]

Assay principles

Optimization and characterization of *in vitro* potency and efficacy of mAb designs is critical to the success of immunotherapies moving from bench to bedside. Typical CDC assays utilize lactate dehydrogenase (LDH) activity, resazurin, and chromium-51 (^{51}Cr) tagging.

LDH is a soluble and stable ubiquitous cytoplasmic enzyme that is released by cells undergoing apoptosis, necrosis, or other cellular damage. LDH assays measure the amount of LDH in the cell culture medium using a colorimetric or fluorometric method to quantitate the relative amounts of dead cells.[4]

Resazurin is a cell membrane permeable water soluble, stable, non-fluorescent blue dye that becomes a highly fluorescent pink when it undergoes a redox reaction. Resazurin becomes reduced when it interacts with mitochondrial enzyme activity from an electron donor like NADPH (nicotinamide adenine dinucleotide phosphate) or cytochromes like cytochrome c. The number of viable cells can be correlated with the signal intensity of the dye reduction. Resazurin can therefore be used as an indicator of metabolic activity and cellular health.[5]

The chromium-51 release assay was developed in 1968 and measures cytotoxicity of T cells and NK cells. The target cells are tagged with chromium (^{51}Cr). When the cells lyse or undergo apoptosis, the chromium is released. The released chromium can then be measured in the supernatant via a liquid scintillation counter or gamma counter, which can then be correlated to the number of dead cells. Chromium-51 assays use radioactive isotopes which pose safety threats and waste disposal concerns.[6]

All of these assays require a stain or probe and are usually terminal. CDC is a dynamic process and the ability to monitor the cell culture system and build a kinetic profile allows researchers to better inform mAb efficacy and mechanism.

The Celigo imaging cytometer can enable the recapitulation of CDC *in vitro* using Daudi cells stained with Calcein-AM. Daudi cells are a CD20+ B cell Burkitt lymphoma cell line, which can be targeted by CD20 mAbs. The changes of Calcein-AM expression can be captured in live cells as cells begin to die. A time

course of the Calcein-AM expression changes and cell morphology can easily be plotted into cytotoxicity and growth curves and compared across experimental conditions. Taken together, the Celigo cell imaging cytometer can provide comprehensive and informative analysis for therapy design, potency, and efficacy.

Models/probes and dyes

Calcein AM (Revvity)

SYBR Green (Thermo Fisher)

TO-PRO-3 (Thermo Fisher)

Methods

Kinetic CDC experiment

Target Daudi cells were stained with 5 µM Calcein-AM for 30 min and washed.

Labeled Daudi cells were seeded 10,000 cells/ well in a 96-well plate and treated with titrations of target antibodies and 2% human sera (complement)

Plates were scanned at 0, 30, 60, 90, and 120 min to count the number of live target cells (Calcein+) and calculate cytotoxicity percentages over time

An equation was used to calculate cytotoxicity percentages using the number of Calcein+ cells at each time point, where it represented the number of live target cells remaining in the well, thus referencing or

$$Cytotoxicity\ \% = \frac{(\#Calcein_{t=0} - \#Calcein_{t=X})}{\#Calcein_{t=0}} \times 100$$

normalizing on a per well basis.

The negative control cytotoxicity percentage can be subtracted from the experimental sample to normalize against baseline cell death to improve the accuracy of the results.

Endpoint CDC experiment

Target Daudi cells were stained with SYBR Green and seeded in 384-well plates at 500 cells/well.

After incubation with a titration of target antibodies and serum for 15 min, TO-PRO-3 was added for 1 h.

Green and red FL images were captured to calculate viabilities at different target antibody concentrations.

The viabilities are calculated with the equation

$$Viability\ \% = \frac{(\#SYBR\ Green_{t=end\ point})}{\#SYBR\ Green + TOPRO3_{t=0}} \times 10$$

Celigo Set-up:

The Celigo software was used to directly count the number of Calcein positive Daudi cells or Sybr Green and TOPRO3 positive Daudi cells on the bottom of the plates at different antibody concentrations. The ANALYZE parameters for the green and red channels were set to:

1. "Algorithm = Fluorescence"
2. "Intensity Threshold = 8"
3. "Precision = High"
4. "Cell Diameter = 10"
5. "Dilation Radius = 0"
6. "Background Correction = Check"
7. "Separate Touching Objects = Check"
8. "Minimum Cell Area = 20"
9. The BF images were not utilized by setting the "Intensity Threshold" to 255

Challenges

Several parameters need to be optimized to set up a successful CDC assay, including choosing the right target cell lines that express sufficient target antigens and optimizing the complement source. When designing a CDC assay using imaging cytometry readout, careful consideration needs to be given to the choice of cell

TIPS & TRICKS

Optimize the labeling dye's concentration and incubation time before setting up the CDC assay. Considerations include ensuring low spontaneous release to minimize background signals and the longevity of the staining signals.

labeling dyes. For kinetic readout, a live cell-based tracing dye that can be retained by target cells for the duration of the experiment is needed.

Results

Time-course CDC experiment results

Brightfield and Calcein green fluorescent images for high and low antibody concentrations at 90 min time point are shown in Figure 1. The low antibody concentration

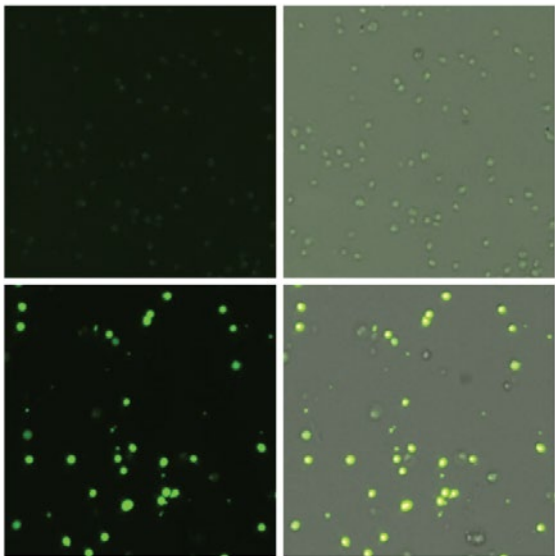


Figure 1. Kinetic CDC brightfield and Calcein images at 90 min.

showed more Calcein+ cells than the high concentration antibody treatment, where all of the cells have been killed evidenced by a loss of Calcein signal.

The cytotoxicity results, presented as a time dependent

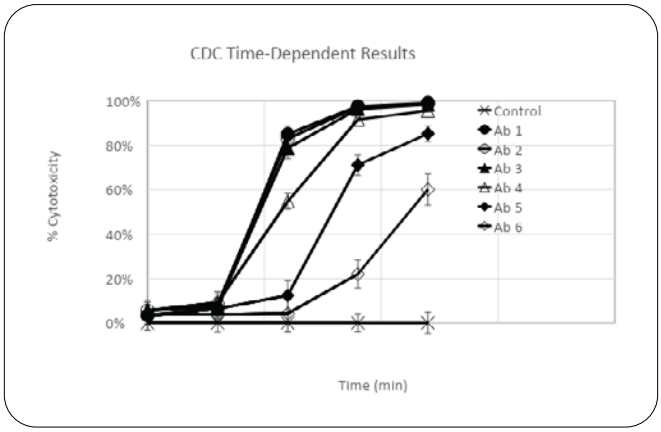


Figure 2. Kinetic CDC time-dependent cytotoxicity results.

plot, are shown in Figure 2, which showed an increase in cytotoxicity % over 120 min. Over time, target cell cytotoxicity increased for the positive antibodies, while the control condition did not show significant changes.

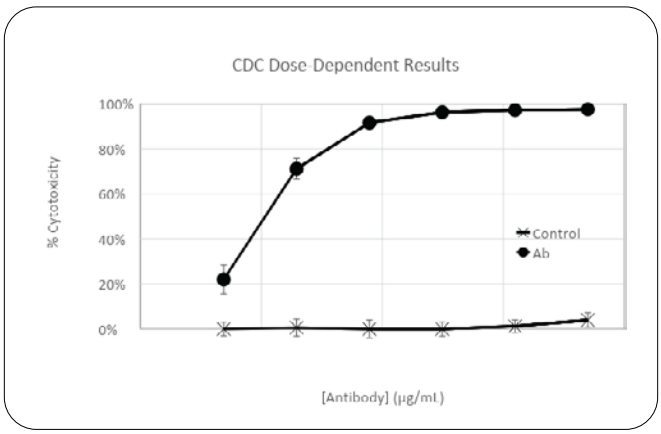


Figure 3. Dose-dependent cytotoxicity results at 120 min.

The dose-dependent plot is shown in Figure 3. The dose response at 120 min showed that a positive antibody induced a dose-dependent CDC effect in target cells, where the negative control showed no cytotoxic effects.

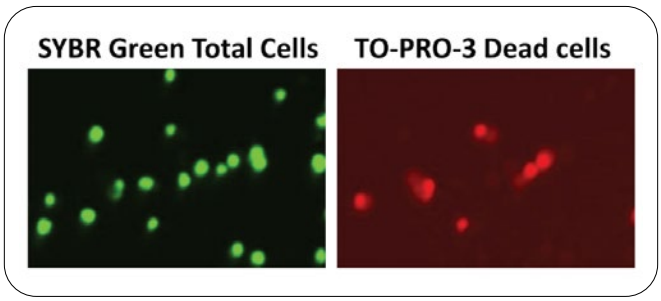


Figure 4. Representative images of SYBR Green stained total cells and TO-PRO-3-stained dead cells.

Endpoint CDC viability results

The live Daudi cells are stained with SYBR Green dye and the membrane-compromised dead cells are

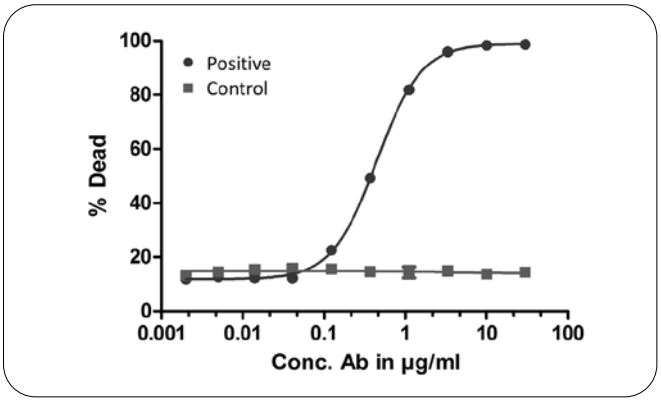


Figure 5. Dose-dependent cytotoxicity results from endpoint CDC assay.

stained with TO-PRO-3 dye. The fluorescent images are shown in Figure 4.

In Figure 5, the positive antibody showed a clear dose-response for the CDC effect, showing an increase in the percentage of dead cells, while there was no change in the percent of dead cells for the negative control.

Conclusions

The proposed image cytometry method provides a non-radioactive, high-throughput method to determine complement-dependent cytotoxicity based on direct cell counting. A typical 96-well plate can be scanned and analyzed in less than 10 minutes. By choosing a live cell-based cell tracing dye, CDC effects can also be tracked over time.

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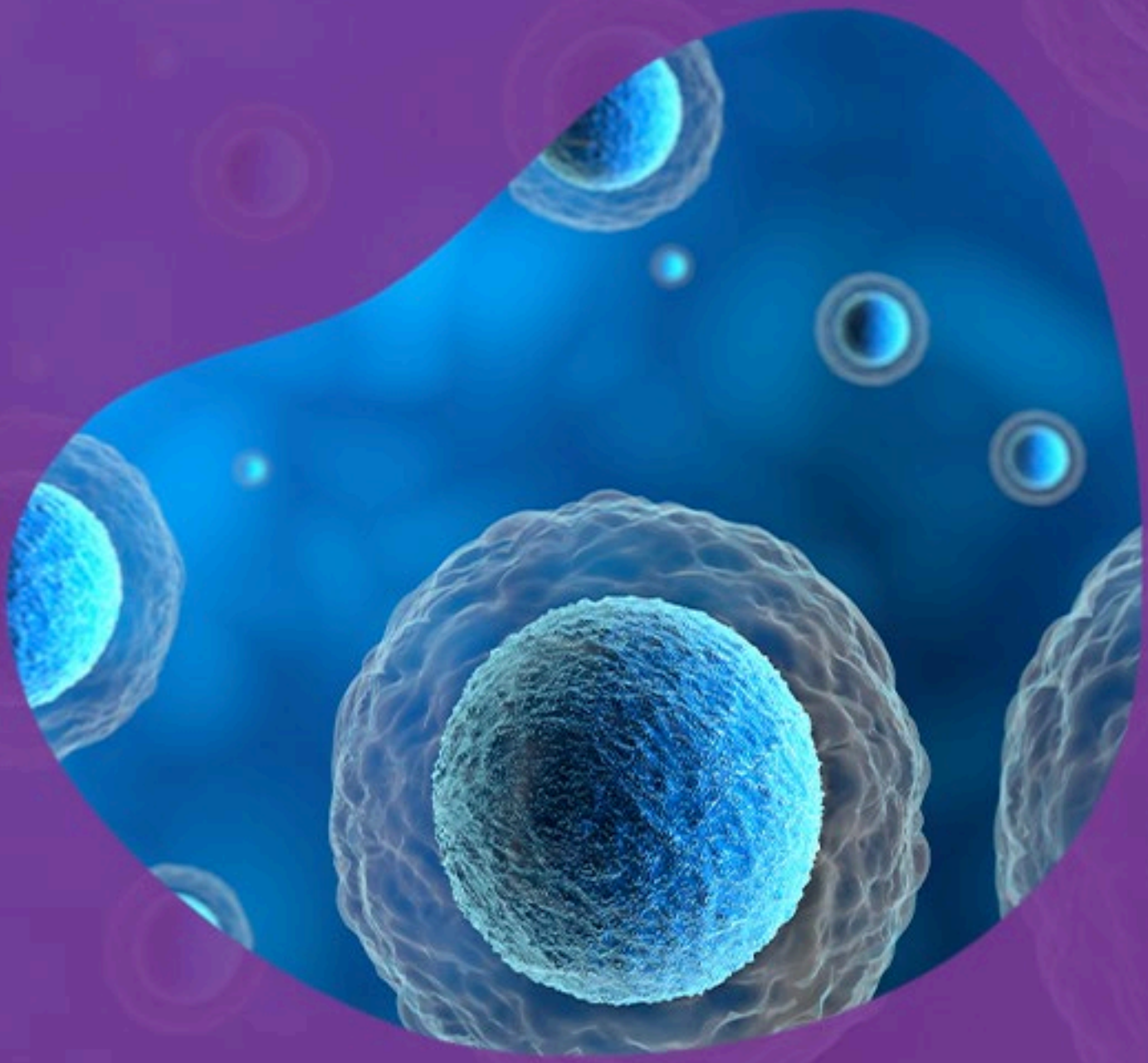
Chapter 14

High-throughput patient-derived organoid screening assays



Introduction

Traditional drug development processes use *in vitro* assays before moving drug candidates to preclinical and clinical studies. However, *in vitro* assays can be poor predictors of *in vivo* reactivity and clinical outcomes. Efforts have been made to develop better assays and testing that predict clinical efficacy. The development of patient-derived organoids (PDOs) has recently emerged as an *in vitro* preclinical model that could potentially better recapitulate the tumor microenvironment.[1–3]

PDOs are 3D multicellular clusters derived from patient tumor tissue or normal tissue from either pluripotent stem cells or isolated organ progenitors. PDOs can differentiate to form tissue that contains multiple cell types[4] and can be expanded indefinitely.[5] Phenotypic and genotypic profiling of PDOs from tumors has shown that PDOs retain the histological complexity as well as genomic, transcriptomic, and morphological features of the original tumor. Functional analysis of individual tumors can be done on PDOs because, even after several months of subsequent culturing, tumor PDOs preserve the mutational status, copy number alteration, and identification of fusion genes of the original tumor.[4] The characteristics of PDOs can be exploited as clinically relevant models for immuno-oncology drug development, single and combination agent testing, investigation of predictive biomarkers, mechanisms of resistance, and the exploration of patient specific pathology for developing individualized treatment regimens.[6,7] A challenge in developing immunotherapies is that the mechanism of tumor immune evasion is highly variable, which creates difficulty in predicting not only patient-to-patient sensitivity to treatment but also to resistance.[6] Co-culturing immune cells with PDOs can better recapitulate the *in vivo* tumor microenvironment and provide a better model for the prediction of therapeutic response and mechanism.

Assay principles

While there is much interest in using PDOs for drug screening, implementing high-throughput methods is difficult due to the complexity and heterogeneity of the model.[8] Typical methods of characterizing PDO viability and toxicity in response to immunotherapeutics are image based assays utilizing brightfield, confocal, and fluorescence microscopy. Flow cytometry can be utilized; however, PDOs must be mechanically and enzymatically dissociated to the single-cell level at the assay endpoint for analysis.

For imaging analysis in drug screening assays, PDOs are typically assessed for viability and apoptosis markers. PDOs can be stained with a cell tracker dye and an apoptosis probe detecting caspase-3/7 cleavage to visualize apoptotic cascade activation. As the tumor cells die, the apoptosis probe will fluoresce green and the chosen cell tracker dye fluorescent signal will decrease. The PDO will also change in size and morphology as it dies or continues to proliferate. The different parameters measured can provide a cytotoxic profile of the immunotherapy treatment.[6] Cell tracking dyes can also be used to visualize immune cell infiltration.[3,9]

Organoid cytotoxicity can also be quantified after fixation and staining with apoptotic or proliferative markers such as histone-2AX (γH2AX) to mark DNA double-strand breaks and Ki-67 to identify tumor cell proliferation, with DAPI as a nuclear stain.[3] Many staining techniques are terminal endpoints for the PDOs, therefore if multiple time points are needed to create a dose-response curve, several PDO replicates are required. Traditional imaging techniques and flow cytometry are cost and labor-intensive processes and are not high throughput. The PDOs must undergo a great deal of manipulation which also introduces variability into the data. These techniques also lack the ability to capture the dynamic processes and underutilize the PDO property of long-term cell culture and self-renewal.

The Celigo cell imaging system can visualize PDOs over long time courses, with brightfield and fluorescence imaging allowing for rapid scanning ability to

measure larger data sets. PDO characterization in Celigo includes quantitative measurements of size, morphology, and fluorescence intensity in up to 4 different channels. Using Celigo image cytometry, PDOs can be assessed in real-time, enabling kinetic growth tracking and viability analysis. Since the PDOs are not destroyed by analysis, further genomic evaluation can be performed at the end of the drug treatment. The Celigo platform captures the dynamic and complex processes of PDO response to treatment in a standardized, high-throughput assay, thus enabling scientists to robustly measure kinetic cytotoxic profiles for novel immunotherapeutics.

Models/probes and dyes

ViaStain PI Staining Solution (CS1-0109-5mL, Revvity)
Esophageal patient-derived organoids

This data set specifically shows esophageal organoids from three different patients under six targeted cancer drugs, however, this methodology could be applied to PDOs from any organ or tumor site. Propidium Iodide was used for PDO viability measurement in these experiments, but other fluorescent viability, proliferation, or apoptotic dyes could be used.

Methods

PDO growth and viability assay requires the following materials: patient-derived cells, microplates, an incubator, appropriate cell culture media with embedding matrix, a drug of interest, and the Celigo imaging cytometer.

Cells of choice are embedded in a microwell plate at a predetermined assay-appropriate density. It is then suggested to centrifuge the microplate at 1000 rpm for 5 minutes to allow for cells to move towards one focal plane at the bottom of the well. This "Celigo Spin Method'' brings PDOs to a single focal plane without requiring more matrix and without negatively impacting organoid growth. However, it is also possible to image PDOs without spinning the plate in what is called the "Dome method". This requires multiple focal planes to be captured and data analysis is merged afterwards.

In this experiment, esophageal organoids from three different patients were treated with six targeted cancer drugs. Six concentration dilutions were used per drug as well as a comparison between control fibroblast media and conditioned media from cancer-associated fibroblasts. Organoids were embedded in Matrigel on a 96-well plate and spun down to achieve a single focal plane. Propidium iodide was used to kinetically measure PDO viability over the course of drug treatment.

Celigo set-up:

The Celigo image cytometer rapidly captures whole-well images for PDO count and analysis. The Celigo's user interface is designed to sequentially set up an experiment by clicking through the tabs in the software. First, the cell plate is imaged by setting up a new scan using brightfield and/or fluorescent illumination and the machine is set to analyze colony growth and viability intensities. In this experiment, the Celigo software applications "Colony 1" and "Colony 1 + 2" were used to acquire brightfield only or brightfield and red fluorescent images at 1 μm^2/pixel resolution.

Using simple parameters that can be defined or edited by the user, the Celigo software directly identified and measured each organoid for each treatment condition in the 96-well plate. Plate scanning and image analysis were performed simultaneously, and the data was exported. The plates were scanned at time zero and then every 24 hours for three days.

Challenges

PDOs must be grown to an adequate size before imaging. We recommend at least a 3-day growth period after embedding in Matrigel. The Celigo spin method is advised as a traditional dome method can be imaged but analysis becomes difficult as various focal positions must be merged to get proper counts. Fluorescent dyes should also be tested for proper penetration before imaging.

TIPS & TRICKS

Use smaller well formats with fewer cells. 6-wells are no longer required as is typical in manual counting.

Celigo spin method is beneficial for easy imaging.

Dyes and antibody stains should be tested for ease of penetration into Matrigel.

Results

The Celigo image cytometer quickly imaged and analyzed PDO count and growth over time for the different treatment conditions. Figure 1 shows an example of one well in a 96-well plate with hundreds of PDOs embedded within the matrix. On the left is a whole-well brightfield image that captures every PDO in that well while on the right is a segmented image showing all the PDOs properly identified. Figure 1 depicts the versatility of the Celigo to capture and analyze numerous PDOs of various sizes and shapes.

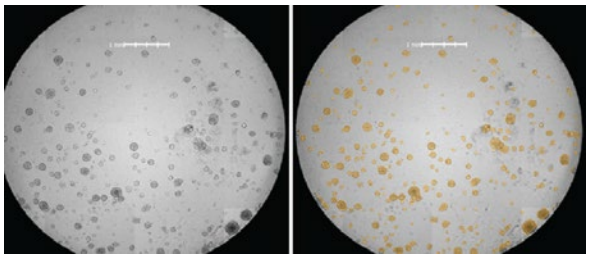


Figure 1: Individual PDOs can be detected with brightfield whole-well imaging and segmented properly regardless of quantity.

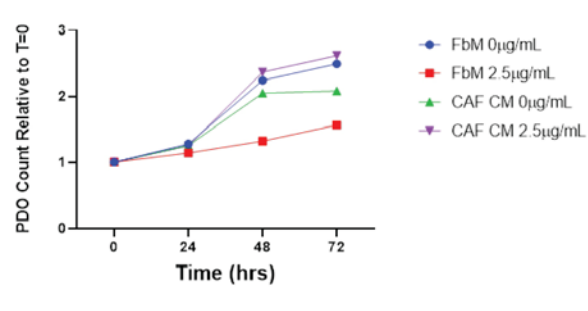


Figure 2: Overall PDO counts can also be monitored kinetically to see growth inhibition under cancer drug treatment. Here we see that drug treatment is very effective in normal fibroblast media (FbM) but not as effective with CAF CM.

Total PDO counts can be exported and plotted to compare different drug conditions. Figure 2 shows an example of a time-course experiment comparing two different media conditions in the background of a cancer drug dosage response. As you can see, the concentration of the drug has more of an effect on total PDO growth as compared to the conditional media. Example images can also be exported and showcased as seen in Figure 3.

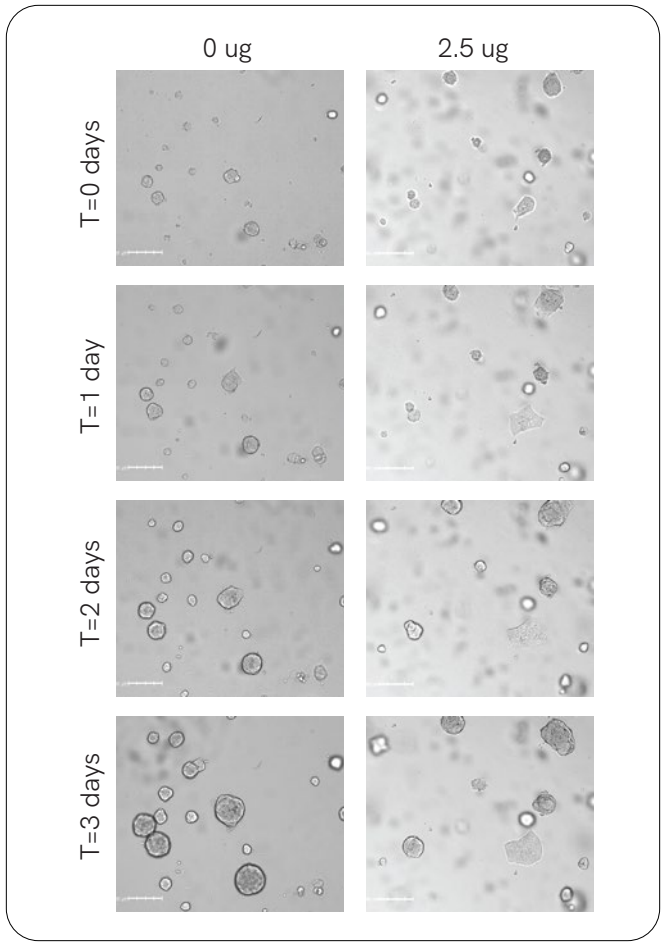


Figure 3: Example images of PDO growth over time. Cancer drug treatment slows the growth of PDOs as seen with 2.5ug of the drug as compared to no drug control.

PDOs under drug treatment had minimal growth as compared to untreated controls. The Celigo image cytometer allows for capturing snapshots of key areas in a well over the designated time course. This analysis is routinely used for spheroid growth and drug targeting and can be easily accomplished with high-throughput imaging on the Celigo.

PDO viability can also be monitored kinetically or as an end-point assay using fluorescent viability dyes. In Figure 4 we show a comparison of PDO viability using Propidium Iodide (PI) staining for cell death. Here we see the conditioned media does affect the level of cell death after three days of treatment as compared to Day 0. This data was captured similarly as PDO count analysis and example images showing PDO death over time are depicted in Figure 5. Here we can see the untreated well shows clear signs of PDO growth with minimal PI staining while in comparison the treated well PDOs are beginning to degrade and have a high level of PI.

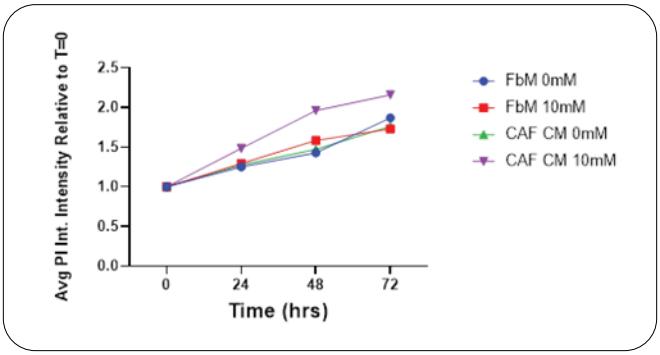


Figure 4: Celigo image cytometers can be used to monitor PDO viability kinetically and show differences in cell death under various conditions. Here we show an increase in PI staining when cancer-associated fibroblasts conditioned media (CAF CM) is added to the highest dosage of cancer drug.

Conclusions

Overall, the Celigo image cytometer is a fast and reliable platform to perform PDO growth and viability measurements suitable for high throughput drug screening which provides the following benefits:

1. No label needed to measure PDO count and size.
2. Easy-to-use interface to image and identify a wide variety of PDO shapes and sizes.
3. Easily compare many drug targets with concentration ranges within a single plate with minimal patient tissue.
4. Accommodates a multitude of plate formats, allowing for increased flexibility for users.

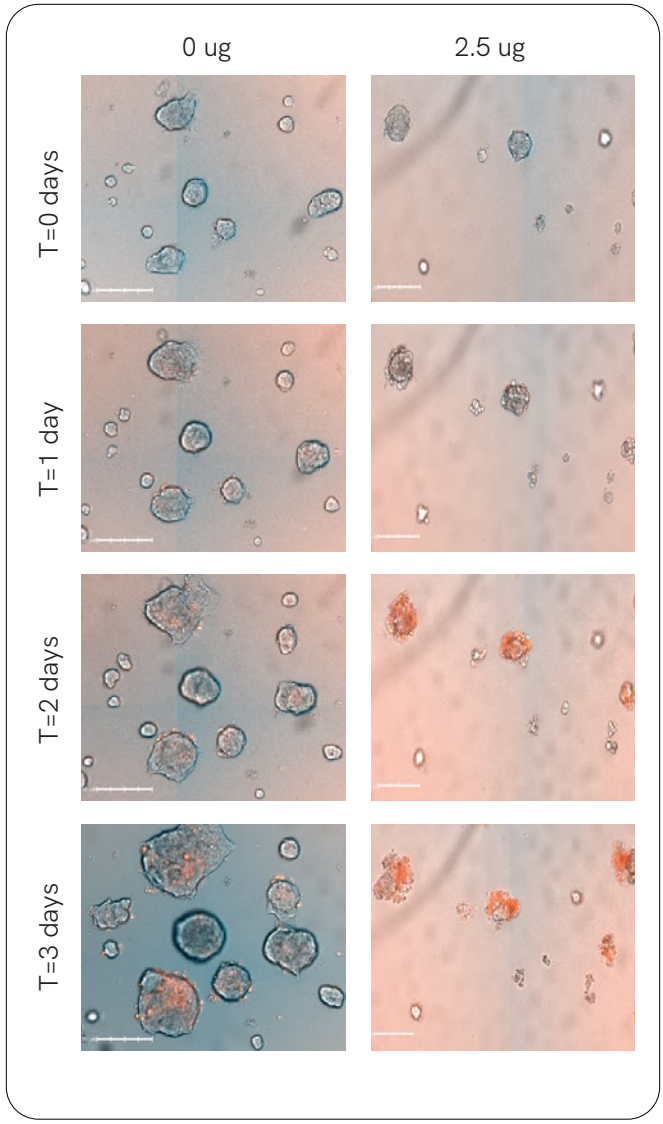


Figure 5: Representative images of kinetic cell death monitoring. The addition of CAFs to 2.5ug cancer drug increases PI staining and drastically inhibits the growth of PDOs.

5. Provides rapid real-time readouts: a 96-well plate can be analyzed in under five minutes and a 6-well plate can be analyzed in under 30 minutes.

PDO growth tracking and viability on the Celigo image cytometer allows for fast high-throughput screening of drug targets and components in a single plate. Imaging can be done in a label-free manner for counts and size measurements as well as with fluorescent dyes for cell health analysis. This provides a flexible, robust, and easy-to-use quantitation of PDO growth and/ or viability in live conditions over time in a streamlined and rapid workflow.

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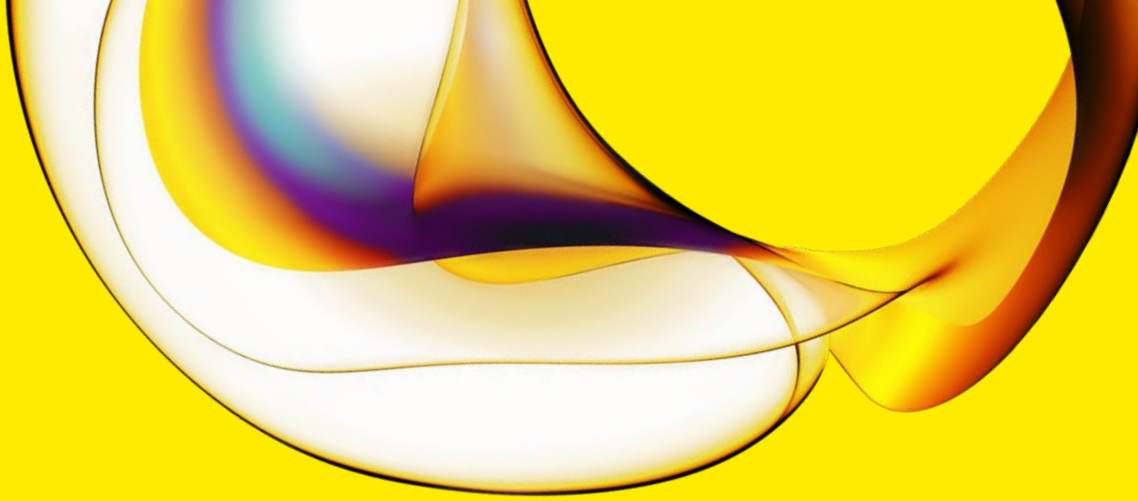

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