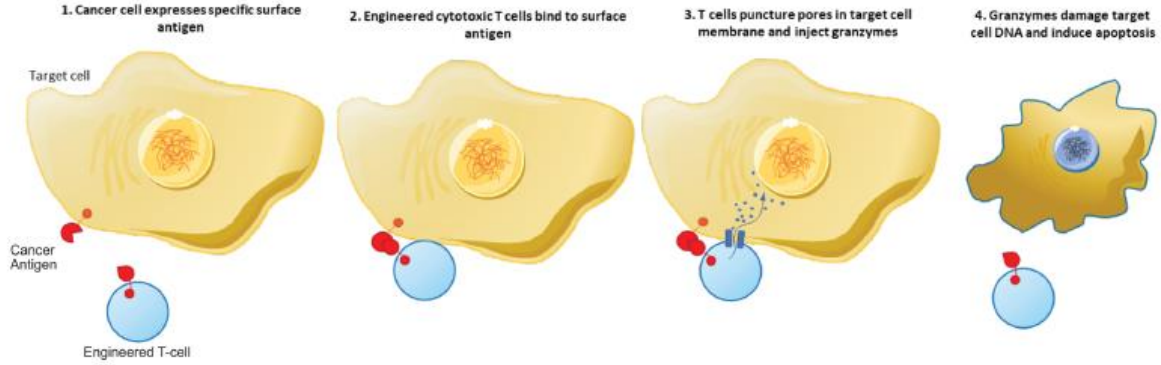
Investigate the efficacy and cytotoxicity of engineered T-cells in killing target cancers.

1. Introduction

T-cells are an integral part of the specialised adaptive immune response and act to eliminate targets and prevent infection. One of the major sub-types of T-cells are the CD8+ cytotoxic killer T-cells. These cells work to detect and kill virally infected and cancerous cells, and to secrete cytokines which recruit additional immune cells to the site of attack. Detection of target cells is performed with the aid of the Major Histocompatibility Complex (MHC). During killing of target cells, the T-cell cell binds to the MHC on the target cell via their T-cell receptor (TCR) and the CD8 glycoprotein co-receptor. The T-cell cell then uses the glycoprotein, perforin, to puncture pores into the membrane of the target cell enabling the subsequently secreted granzymes to enter the cell, leading to DNA damage resulting in target cell apoptosis.



A recent development in cellular immunotherapy manipulates this mechanism to treat certain types of cancer. These T-cells are taken from the patient and modified to have the desired receptors which enable the cells to recognise and bind to specific cancerous motifs, and then to deliver cytotoxic attacks to the cancer cells. The T-cells are then grown *in-vitro* and transfused back into the patient; these treatments have proven a valuable last resort treatment against various blood cancers.

Despite cellular immunotherapy's success in treating otherwise incurable forms of blood cancer the treatment has many limitations. For example, the high cost and length of time it takes to engineer and grow these modified cells *in-vitro*, the waning efficacy of the treatment over time as the infused T-cells die or lose functionality and the cancer cells adapt to evade detection by modifying their surface antigens, known as antigen escape. In addition, current treatments struggle to infiltrate and attack solid tumours, and there is a great difficulty in translating *in-vitro* observations to patients. For example, small changes in CAR-T receptors, often show little difference in conventional *in-vitro* assays but perform radically differently in clinical trials. Thus, additional detail is required when analysing these *in-vitro* experiments to determine the interaction dynamics between target and effector cells.

Livecyte T-cell killing assays observe and quantify T-cell killing

Using timelapse imaging, T-cell killing events can be seen in real time to quantify the efficiency of a treatment, indicating how long a treatment takes to become effective, and how persistent it is. Timelapse imaging, in conjunction with single cell analysis, can also help us to identify individual cells or outliers which interact with cells differently. This allows us to discern subtle changes in killing behaviour, enabling us to more closely anticipate how a treatment will work in the clinic. The current limitation of this timelapse imaging is that the analysis, performed by manually tracking cells and logging contacts between T-cell and targets, is extremely time and labour intensive. This leaves a gap in research techniques for a tool which can automatically monitor and quantify T-cell:Target contacts, giving a greater level of information compared to readings of whole wells.

All this is achieved with Livecyte's unique combination of Quantitative Phase Imaging, a specialised technique that generates fluorescence like images from unlabelled effector cells, and standard fluorescence labels to identify target cells and their killing. T-cell dynamics are then derived by backtracking from each cell death event. The fragile effector cells are not subject to the introduction of cellular labels, which can commonly perturb function. Livecyte's single cell segmentation and tracking algorithms can automatically quantify cell proliferation, cell death, T-cell kinetics such as average T-cell contact time, number of T-cell contacts with target cells, number of T-cells attached at death, and final T-cell contact time giving a more valuable, in-depth insight into T-cell killing.

Method

Cell Culture: CD8+ T-cell were extracted and isolated from OT1 murine spleens and cultured for 2 days in cRPMI containing ovalbumin and IL-2. mKate labelled Moc1 target cells were maintained in complete DMEM. Moc1 target cells were plated in an 8 well channel Ibidi µ-slide at a density of 5000 cells/ well. Each column contained

either OVA+ or OVA- target cells. OT1 cells were plated at T-cell: target ratios of 5:1 and 1:1. A Caspase-3 marker was utilised to indicate cell apoptosis.

Time-Lapse Imaging: High-contrast quantitative phase images were automatically captured using the Livecyte Kinetic Cytometer. Cells were imaged with an Olympus UPLXAPO 20X (NA 0.8) objective and a 500 x 500 µm field of view (FOV) per well for 48 hours at 5-minute intervals. Cells were maintained inside an environmental chamber at 37°C with 5% CO2 and 95% humidity. Fluorescent overlay images with red and green fluorescence channels were captured after each phase image.

Analysis & Results

Proliferation and cell death: Timelapse images captured every 5 min using fluorescence and QPI imaging showed significant levels of cell death in OVA+ target cells and T-cell. Livecyte's Cell Analysis Toolbox used gating, based on the apoptotic green fluorescence signal, to perform an automated Target cell death calculation. Figure 1 clearly indicates highest levels of T-cell killing in the T-cell:OVA+ 5:1 well. The well with a 1:1 ratio of T-cell to OVA+ showed moderate levels of T-cell killing. As expected, this is due to higher numbers of T-cell increasing the likelihood of T-cell - Target cell contacts occurring. There were low levels of cell death in the OVA- wells due to the T-cell being unable to recognise the target cells via the OVA protein.

In Figure 1 the OVA+ well with the highest count of T-cells showed the earliest signs of killing at roughly 2 hrs into the assay and showed the highest level of T-cell killing throughout, the 1:1 OVA+ treatment showed deaths a few hours later and at a lower rate. There were some cell death events in the OVA- treatments. This could be due to the high number of T-cell resulting in some target cell recognition.

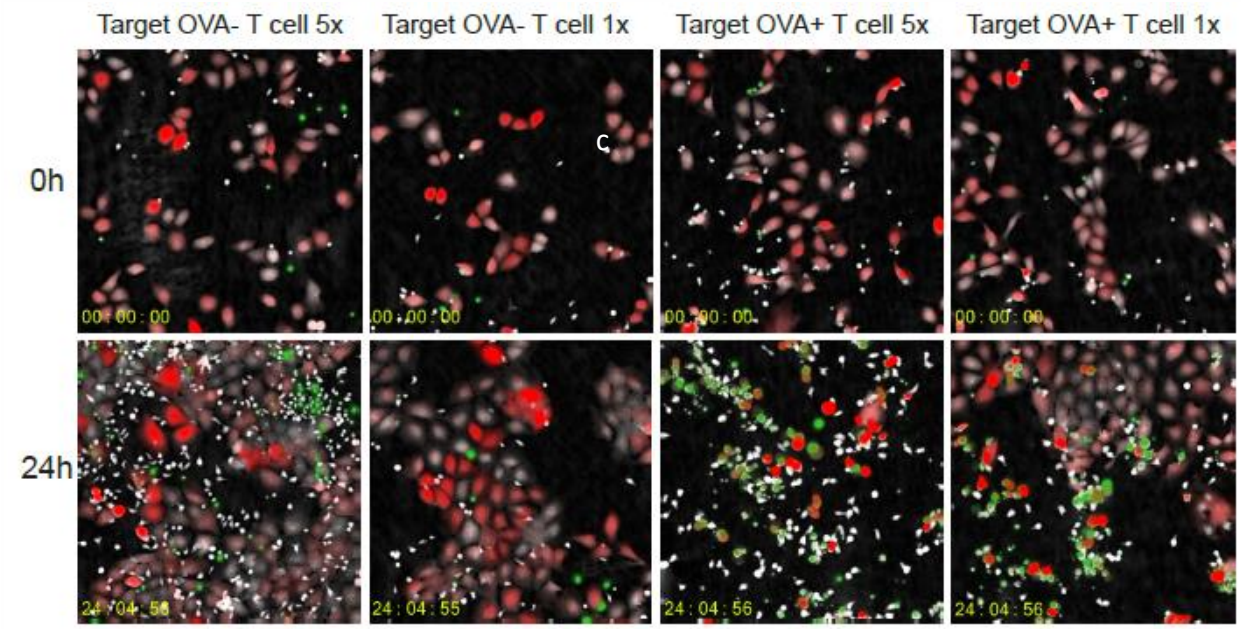


Figure 1: QPI and fluorescence images at 0 and 24 hrs

Gating was used to isolate live target cells. From this, cell count was quantified to give metrics on Target cell proliferation as seen in Figure 2.

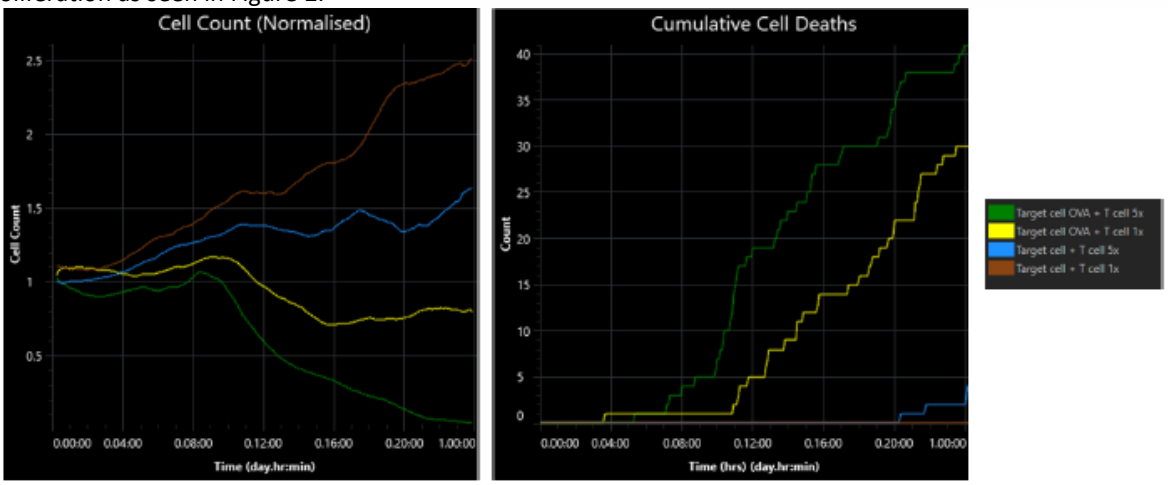


Figure 2: Target cell proliferation and Cumulative Target cell death over time.

From here we can see a clear reduction in cell count occurring roughly 8h into the assay for the OVA+ T-cell wells. The OVA+ T-cell 5x cells elicited a cell reduction response 2 hr earlier than the OVA+ T-cell 1x treatment. As these

metrics quantify T-cell effectiveness over time, a measure of T-cell persistence, or the level of how long a dose of T-cells can stay active and killing, can be ascertained.

T-cell – Target cell kinetics: Livecyte Analysis software tracked every Target cell and automatically quantified each contact between target cells and T-cell. From this, metrics on the number of T-cell contacts per cell, and total T-cell-Target contact time could be measured. The system was also able to quantify the individual times of each T-cell–Target contact and the time of the final T-cell contact prior to cell death. In addition to a higher number of death events in the 5:1 OVA+ well, when we compare this to the 1:1 OVA + ratio see there is a slight increase in the cumulative T-cell contact time (the total time over which a T-cell is in contact with that individual target cell prior to that target cell's death), number of T-cell contacts with target cells prior to death (incidence number of T-cell being in contact with a target cell), and overall average T-cell contact time (mean contact time of each T-cell visit for each target cell prior to death) being higher for OVA+ wells (Figure 3).

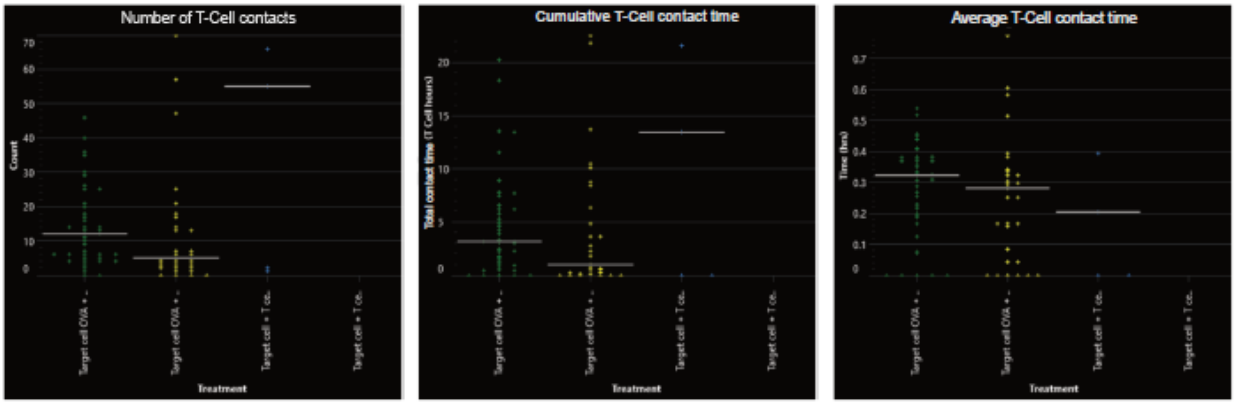


Figure 3: Number of T-cell contacts, Cumulative T-cell contact time, and Average T-cell contact time

Mechanistically, a higher E:T ratio will only increase the chances of contact, so it is unsurprising that statistically similar behaviour was observed in each population.

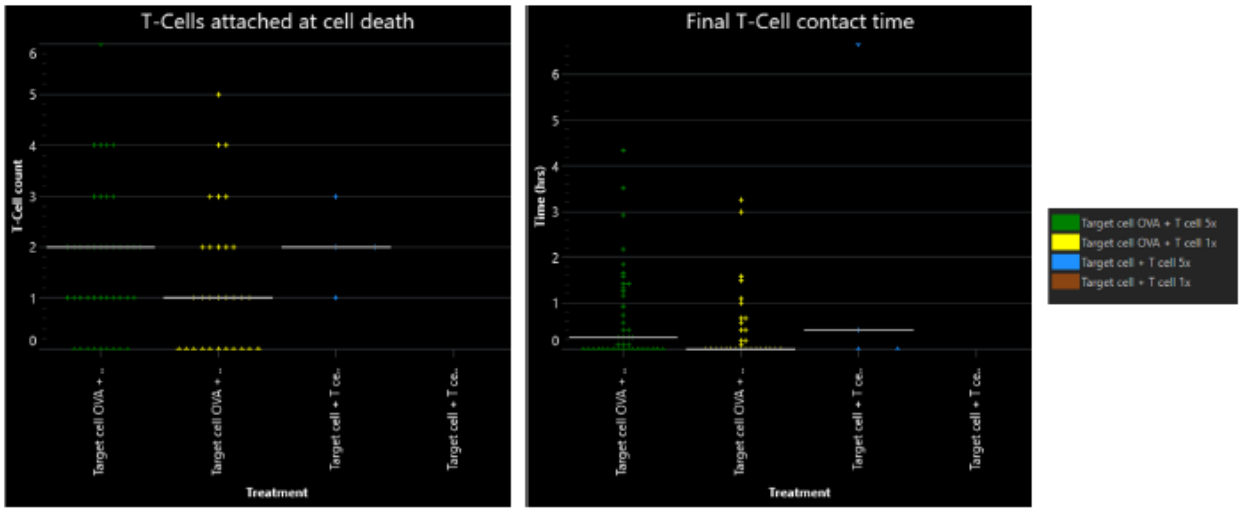


Figure 4: Number of T-cells attached at Target cell death and the Final T-cell contact time

Figure 4 shows the median Number of T-cell attached at cell death was constant (1 T-cell) for the wells showing incidences of cell death. The Final T-cell contact times ranged from almost instantaneous target death upon contact to over 34hrs and was marginally higher for wells with a higher T-cell to Target cell ratio. Overall statistical differences weren't observed between the different ratios of treatments, as the same OT1-specific cells were used, showing the same T-cell:Target cell kinetics. However, these metrics provide an opportunity to compare different T-cells which elicit different Target cell responses and observe the potency of T-cells. Research has shown that T-cell often kill target cells via an "additive cytotoxicity" mechanism, by which multiple T-cell contacts with the target result in an accumulation of sublethal DNA damage resulting in eventual apoptosis. Lower levels of T-cell contacts would indicate that each T-cell contact with a Target cell deals a greater level of DNA damage.

T-cell morphology: Livecyte was able to quantify cellular morphology of both cell types within the co-culture by gate formation. T-cell cells were selected for by a low dry mass and area. The median T-cell area and cell perimeter increased as target cell killing increased along with an increase in the median cell perimeter compared to the T-cell. This indicates spreading and flattening of the T-cell occurs when they are killing. T-cell morphology has been seen to change significantly during T-cell killing with some papers showing flattening and elongation of T-cells in-vivo when migrating and infiltrating tumours.

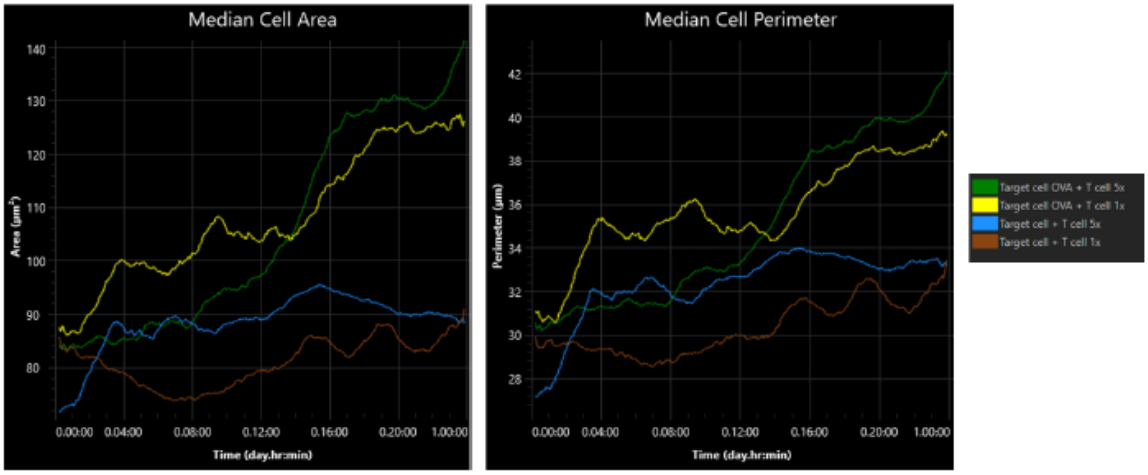


Figure 5: T-cell morphology: Median cell area and median cell perimeter over time (T-cells were gated based on low dry mass and cell area)

Target cell morphology: Target cell morphology was also quantified, giving information on target cell responses to T-cell attacks. Figure 7 clearly shows increases in median cell sphericity and area at the time points where cells begin undergoing apoptosis due to T-cell attacks. It is widely recorded that cells ball up and bleb when undergoing apoptosis, this can also be seen in the images in Figure 6 and is reflected in these results.

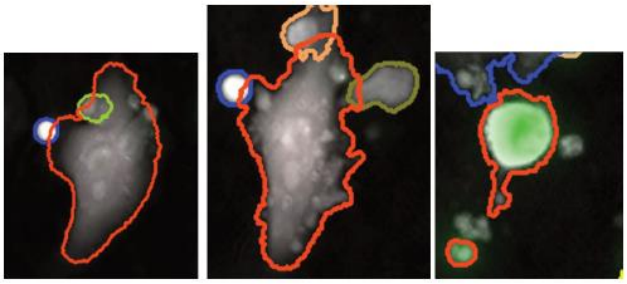


Figure 6: Target cell is attacked by multiple T cells, begins to bleb, and eventually balls up and dies several frames later

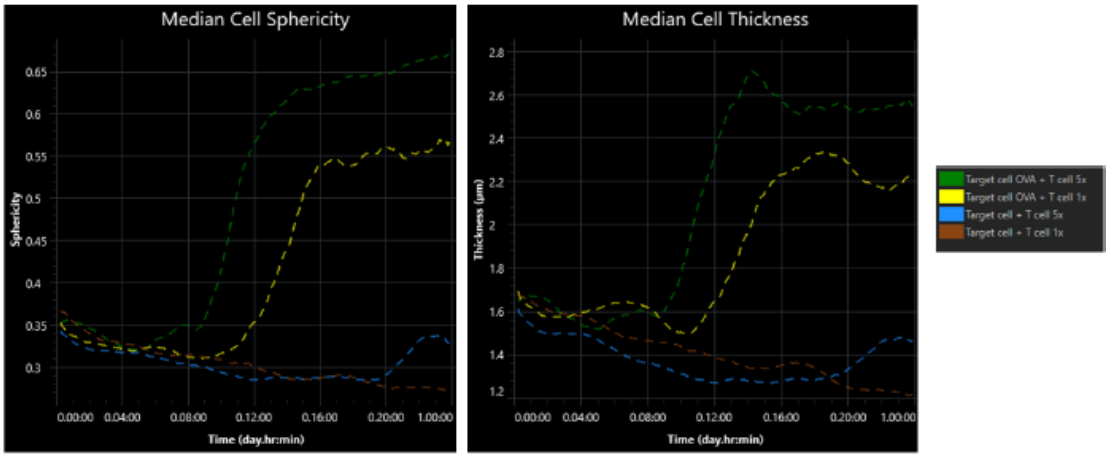


Figure 7: Target cell median sphericity and median cell area (target cells were gated for based on high red fluorescence)

Summary

Livecyte's combination of fluorescence capabilities with QPI imaging enabled automated analysis of T-cell killing, giving an enhanced level of detail not available with conventional imaging platforms. T-cell and Target Moc1 cells were automatically categorised in a co-culture and incidences of T-cell and target cell contact and when these led to cellular apoptosis were tracked. Anti-proliferative effects of the T-cell killing were quantified showing the point at which these effects began to reduce target cell count. More killing events and a quicker reduction in cell count was observed in wells with higher counts of T-cell and where OVA was expressed by target cells. T-cell and target cell morphologies were monitored showing changes between both target and T-cell wells when T-cell killing was underway. The level of understanding of T-cell function obtained far exceeds metrics obtained with conventional readers. Livecyte's cell death and T-cell:Target cell contact identification and quantification gives powerful, automated in-depth analysis, with the potential to quantify subtle differences in the efficacies of T-cells for the first time. This is achieved without fluorescent labelling of the fragile primary effector T-cells, or time intensive manual tracking. This leads to a higher level of insight about the T-cell target cell interaction and of the likelihood of cell death resulting from these behaviours.

Contact us to learn more about the Livecyte's capabilities or to request a guided demo.

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References

1. Phasefocus Livecyte Application Note AN021 - T-Cell Killing Analysis (Aug 2023). Available at: https://www.phasefocus.com/resources/app-notes/