Application Note

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Real-Time Potency Assay for CAR T Cell Killing of Adherent Cancer Cells

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Introduction

Harnessing the exquisite potency and molecular specificity of the immune system, and directing these towards cancer cell destruction, immunotherapy in its various forms has permanently changed the landscape of clinical oncology. Among the frontrunners that have helped to usher in this new paradigm are adoptive cell therapies such as chimeric antigen receptor (CAR) T cells. CARs are engineered proteins that consist of an extracellular antigen-binding domain that is linked, via a hinge region and transmembrane domain, to intracellular activation domains that drive T cell activation, proliferation, and target cell killing.[1] A few decades of CAR engineering efforts have made it clear that merely linking these protein domains together, like "beads on a string", is insufficient for generating an effective CAR; subtle differences in how the domains are linked to each other can greatly impact functionality.[1,2] While today's third-generation CARs are improved relative to their predecessors, a universally efficacious CAR architecture has not been identified and efforts to design CARs with novel or refined functionality continue to require empirical optimization on a case-by-case basis.[2]

During CAR optimization, biochemical/biophysical assays can be used to study critical quality attributes such as the antigen affinity of the extracellular domain[3] or the cytokine profile of CAR T cells stimulated by soluble ligands.[4] Although these reductionistic approaches are useful, their results don't necessarily correlate with cancer-killing efficacy within a more physiologically relevant context.[3] To rigorously compare different CAR constructs and identify unwanted behavior such as tonic (antigen-independent) signaling, it is widely acknowledged that CAR function should be evaluated within the complex milieu of the immune synapse, using bona fide cancer cell killing as the readout. For this purpose, release assays (^{51}Cr, LDH) have historically been the gold standard, but suffer from the fact that they require significant "hands-on" time and only provide end-point data. Requiring just target cancer cell seeding and a subsequent CAR T-cell addition step, herein we use the Agilent xCELLigence RTCA eSight to continuously monitor CAR T cell-mediated killing of cancer cells over the course of multiple days. Providing a direct and objective assessment of target cell number, cell size, and cell-substrate

attachment strength, impedance biosensors embedded within the base of eSight microplates quantitatively track the continuum of target cell killing, spanning from early (reduced cell-substrate adhesion strength) to late (lysis) events. Concurrently, eSight captures live cell images in brightfield and three fluorescence channels (red, green, and blue), providing an orthogonal readout of the killing process. By combining the strengths of real-time impedance monitoring (simplicity, analytical sensitivity, and objectivity) with that of live cell imaging (specificity of the readout), eSight increases the information richness of the CAR T cell-killing assay without increasing the workload.

Assay principle

At the core of the eSight assay is a specialized electronic microplate. Incorporated within the glass bottom of all 96 wells, a gold biosensor array continuously and noninvasively monitors cellular impedance. As shown at the bottom of Figure 1, the adhesion of target cells to these biosensors impedes the flow of a microampere electric current—providing an exquisitely sensitive readout of cell number, cell size, and cell substrate attachment strength. This cellular impedance signal is recorded at a user-defined temporal frequency (every minute, once per hour, etc.), and is reported using a unitless parameter called Cell Index. Importantly, the CAR T cells that are subsequently added to the wells are nonadherent and therefore do not contribute to the impedance signal. Consequently, within this heterogeneous assay, the impedance signal exclusively reflects target cell health and behavior. The CAR T cell-induced biochemical and cellular changes (cell rounding, detachment, lysis) that occur in the target cells are detected as a progressive drop in the impedance signal. At the same time that impedance is being monitored, a viewing window in the center of the biosensor array of each well enables eSight to also track CAR T cell-killing efficacy via live cell imaging in brightfield and three (red, green, blue) fluorescence channels (Figure 1; only the brightfield and red channels were used in this study).

In order for target cells to generate an impedance signal, and in order for them to be positioned within the focal plane of the eSight microscope/camera, they must be in intimate contact with the well bottom. While this occurs naturally for adherent solid tumor cells, two different strategies can be employed for studying nonadherent liquid cancers. First, liquid cancer cells can be tethered to the well bottom using antibodies that are cell-type specific. For this purpose, Agilent has developed kits that are

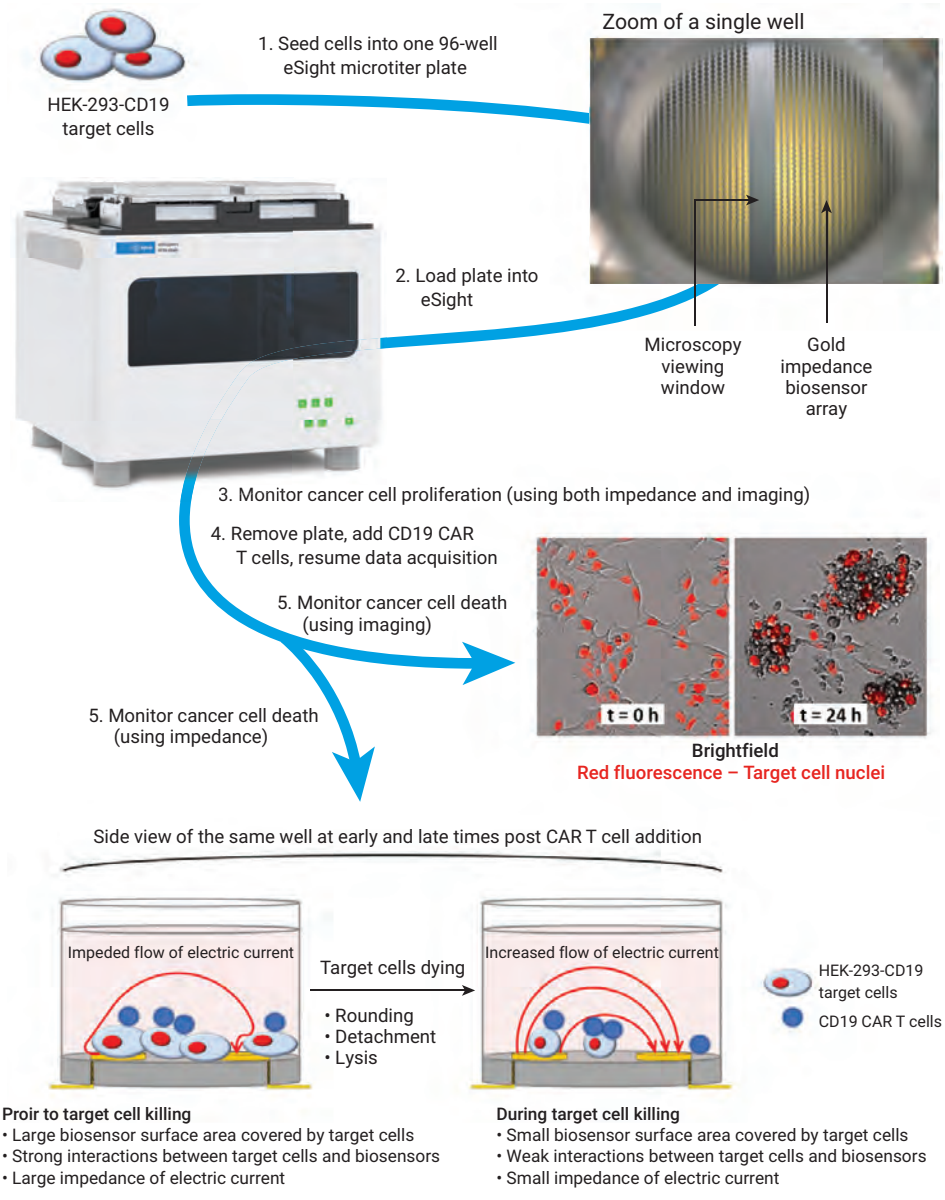

Figure 1. Agilent xCELLigence RTCA eSight workflow for CAR T cell-mediated killing assay.

specific for CD9, CD19, CD29, CD40, and CD71, all of which are preferentially expressed on the surface of normal B cells and tumors of B-cell lineage. Alternatively, the liquid cancer antigen of interest can be exogenously expressed in an adherent cell line, as was done in this study where the B lymphocyte protein CD19 was expressed in HEK-293 cells.

Materials and methods

Target cells

HEK-293 cells were maintained at 37 °C/5% CO_2 in MEM/EBSS (HyClone, catalog number SH30024.01) supplemented with 10% FBS (Gibco, catalog number 16050-122). These cells were engineered to express CD19 using lentiviral transduction. The resulting HEK-293-CD19 cells, as well as the negative control parental cell line (HEK-293), were subsequently engineered to express nuclear-localized red fluorescent protein (RFP) by transduction with eLenti Red (Agilent Technologies, catalog number 8711011) at a multiplicity of infection of 3. From day 3 to day 14 postinfection, 2 µg/mL puromycin was included in the growth medium to select for transductants.

Effector cells

Growth medium and conditions were the same as those described above for the target cells. CD19-specific CAR T cells were constructed using peripheral blood mononuclear cells from a healthy donor. For 9 days posttransduction, cells were stimulated with anti-CD3/anti-CD28 beads in the presence of 200 IU/mL of IL-2. Mock CAR T cells were prepared in an identical manner, minus the transduction step. Because this project was conducted in collaboration with a company developing a proprietary CD19 CAR T for future clinical use, specifics of the CAR gene construction and subsequent cell manufacturing protocol aren't being disclosed here.

Characterizing target and effector cells by flow cytometry

All flow cytometry analyses were performed on an Agilent Quanteon flow cytometer. Expression of CD19 on the surface of HEK-293-CD19 cells was verified using a CD19-specific antibody (clone HIB19, Agilent Biosciences Hangzhou, catalog number 8920007). T-cell lineage was evaluated using a CD3-specific antibody (clone UCHT1, BioLegend, catalog number 300436), a CD4-specific antibody (clone OKT4, BioLegend, catalog number 317441), and a CD8-specific antibody (clone SK1, Agilent Biosciences Hangzhou, catalog number 8921023). Expression of the CD19-specific CAR on T cells was verified using a CD19 antigen fused to an Fc tag, followed by an anti-Fc secondary antibody.

Killing assay

All steps of the killing assay were conducted in an E-Plate VIEW microplate (Agilent Technologies, catalog number 00300601030) using the same growth medium and conditions described above for the target cells. After measuring background impedance using 50 µL of media/well, 10,000 target cells (in 100 µL of media) were added to each well. After monitoring proliferation for 23 hours, 50 µL of media was aspirated followed by the addition of 50 µL of media containing either mock or CD19 CAR T cells. T-cell numbers were varied to achieve E:T ratios of 0.06, 0.12, 0.25, 0.5, 1, 2, or 4. While impedance was measured every 15 minutes, images were acquired every 90 minutes. In each well, four fields of view were captured for each channel (brightfield and red fluorescence). Exposure times were as follows: red (300 ms), brightfield (automatically optimized by the eSight software). When using the impedance data, % cytolysis = [1 – Normalized $CI_{treatment}$ /Normalized $CI_{target\ only}$] × 100. When using target cell counts from the imaging data, % cytolysis = [1 – Normalized $Count_{treatment}$ /Normalized $Count_{target\ only}$] × 100.

Results and discussion

Characterizing engineered effector and target cells

After peripheral blood mononuclear cells were transduced with the CD19 CAR, T-cell proliferation was stimulated using anti-CD3/anti-CD28 beads for 9 days, and the end product was characterized on an Agilent Quanteon flow cytometer. Consistent with the expected T-cell expansion, 91.9% of the live cells were CD3+ (Figure 2A). Among these, 19% were CD8+ and 77% were CD4+. Using a CD19 peptide-based detection strategy (Figure 2B), roughly 50% of the CD4+ and CD8+ cells were found to express a CAR capable of binding the CD19 target antigen (Figure 2C). Finally, ~99% of the HEK-293-CD19 target cells expressed the intended CD19 target (Figure 2D).

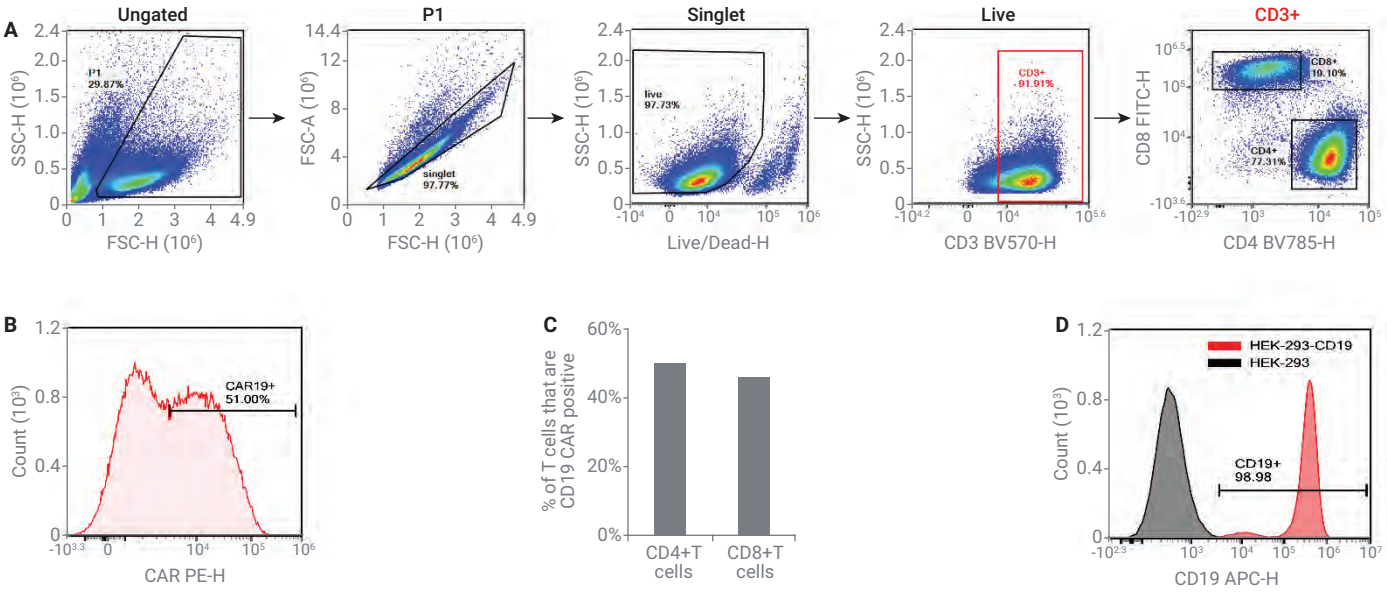

Figure 2. Verifying CAR and CD19 expression using an Agilent Quanteon flow cytometer. (A) After transduction and expansion, the final cell product was comprised of 91.9% CD3+ T cells, of which 19% were CD8+ and 77% were CD4+. (B) A CD19 peptide linked to an Fc tag was used to probe cells for CD19 binding activity. (C) Roughly 50% of all CD4+ and CD8+ cells expressed the CD19-specific CAR. (D) The CD19 protein is present on the surface of ~99% of the engineered HEK-293-CD19 target cells.

Monitoring CAR T-cell killing activity by live cell imaging

When left untreated for 48 hours, the RFP-expressing HEK-293-CD19 cells proliferate to the point of confluence (Figure 3A). However, after 48 hours of exposure to CAR T cells there is a very clear reduction in the number of target cells present. As expected, this killing response is dose dependent, with the highest E:T ratios causing the most pronounced killing. As the E:T ratio is increased, the unlabeled/grey CAR T cells become more prominent in the field of view, and clustering of these T cells (which is a characteristic of activation) becomes more robust. Note that at late time points these T-cell clusters contain a large number of red target cells which, because they display rounding/detachment and cytoplasmic shrinkage, appear to be progressing through apoptosis. Finally, when the assay is repeated using a fixed E:T of 4:1, the time dependence of the killing response is clearly evident (Figure 3B).

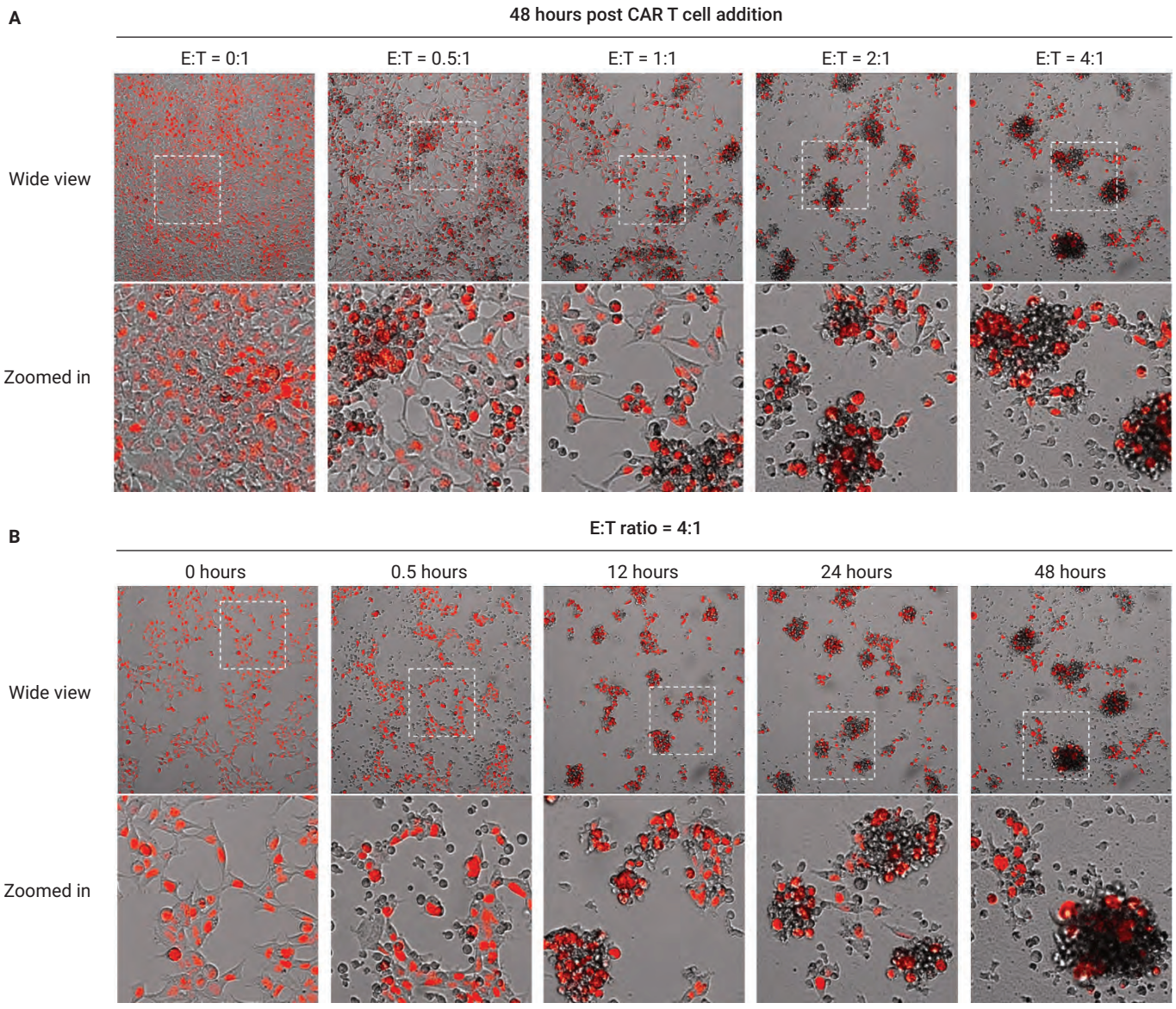

Figure 3. Killing of RFP-expressing HEK-293-CD19 cells by CD19 CAR T cells. (A) Comparison of different E:T ratios 48 hours post CAR T-cell addition. The white squares in the upper panels denote the regions that are blown up in the lower panels. The unlabeled CAR T cells are grey. (B) Comparison of different time points for a constant E:T ratio of 4:1.

Quantifying CAR T-cell killing efficacy

While the above photos markedly demonstrate CAR T cell-mediated killing, they are inherently qualitative. To extract quantitative information, the eSight software was used to count the number of red target cell nuclei present as a function of time (Figure 4). The masking algorithm accurately demarcates the red target cell nuclei (yellow outlines); neither false positives nor false negatives are present. Importantly, when the unlabeled CAR T cells are added (some of which are denoted by white arrows), they are appropriately excluded from the recognition mask (Figure 4, right side).

The number of red target cells is plotted as a function of time in Figure 5A. Note that this plot excludes the first 18 hours of target cell proliferation; the zero-hour time point corresponds to the moment that CAR T cells were added to the well. At this time point, each well contains ~15,000 target cells, which is consistent with the published HEK-293 doubling time of 24 to 34 hours and the fact that 10,000 target cells were initially seeded. In the absence of CAR T cells, the target cells continue proliferating until the 50-hour time point (Figure 5A, black data trace). When CAR T cells are added at the very low E:T of 0.06:1, a killing response isn't observed until the 30-hour time point (Figure 5A, orange data trace). Progressively increasing the E:T ratio causes the killing response to manifest at earlier time points and ultimately results in a greater number of target cells being destroyed.

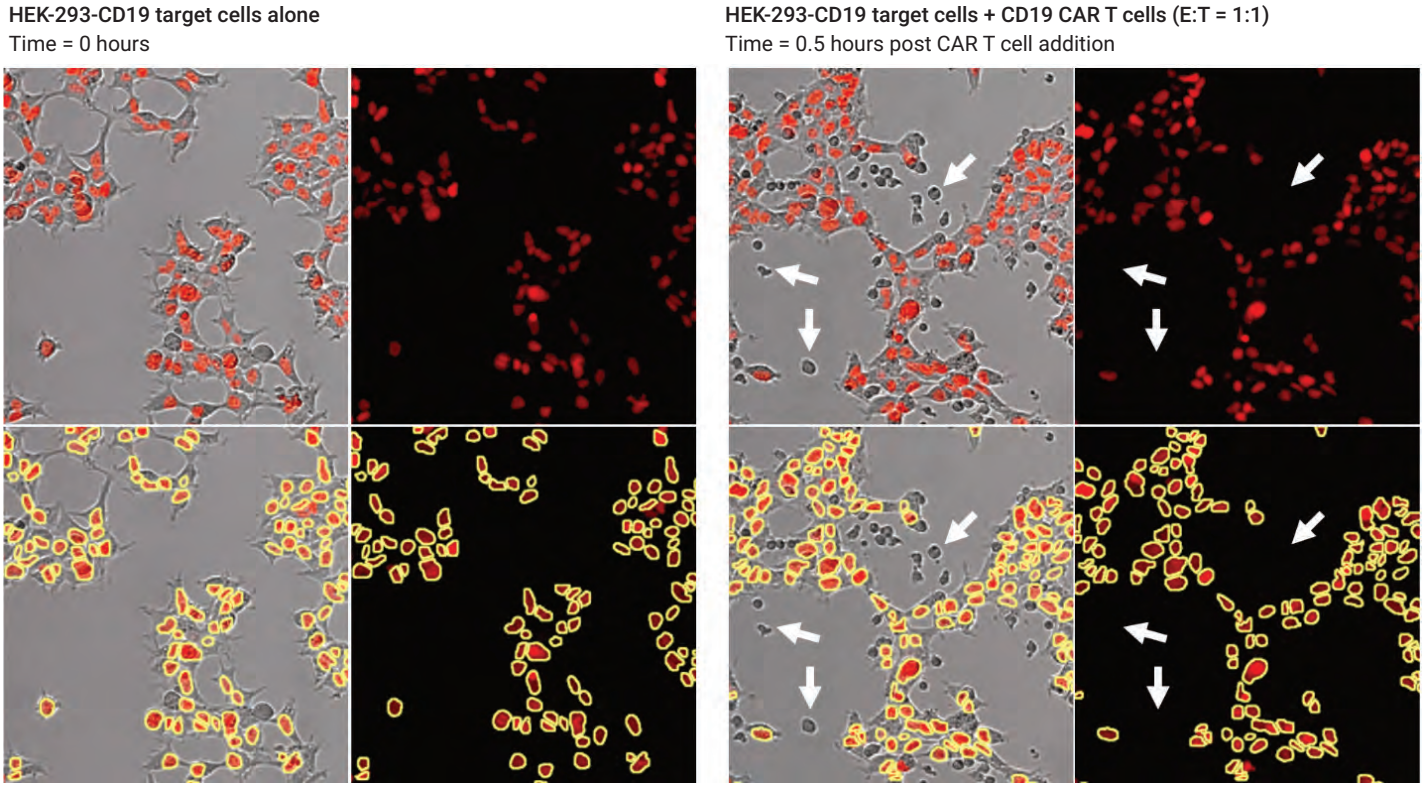

Figure 4. Examples of using the Agilent eSight's segmentation mask to identify red target cell nuclei. The set of four panels on the left side show target cells alone. To highlight the accuracy of the masking algorithm, four different formats are used to show the same well. The set of four panels on the right depict the same well as the set of panels on the left, but 30 minutes later (after CAR T cells have been added at an E:T of 1:1). Numerous unlabeled CAR T cells are visible in the brightfield images; three of these cells are highlighted by white arrows. Because the CAR T cells aren't fluorescent, they are excluded from the mask and do not get counted.

Tracking the killing response using impedance (Figure 5C) produces cytolysis curves that are largely similar to the image-based curves in terms of their time- and dose-dependency. One salient difference between the two readouts is the fact that at E:T = 4:1, the impedance response drops to zero, whereas the number of target cells never drops below ~5,000. This persistence of target cells even at late time points is consistent with the photos shown in Figure 3. That the impedance signal concurrently falls to zero suggests that these lingering target cells are no longer strongly adhered to the well bottom. Consistent with this, nearly all of the red cells that are visible after 48 hours of exposure to CAR T cells at E:T = 4:1 are rounded and appear to be loosely resting on the well bottom (Figure 3).

Using the simple equations shown in the materials and methods section, both the impedance data and the image-based data were converted into % cytolysis plots (Figures 5B and D). When plotted this way, the kinetics of the killing responses are strikingly different between the two data sets. Even though both the impedance and imaging data were collected from the exact same population of cells (i.e., cells in the same well), for a given time point, the impedance signal universally gives a higher % cytolysis than the image-based readout. This contrast is highlighted by

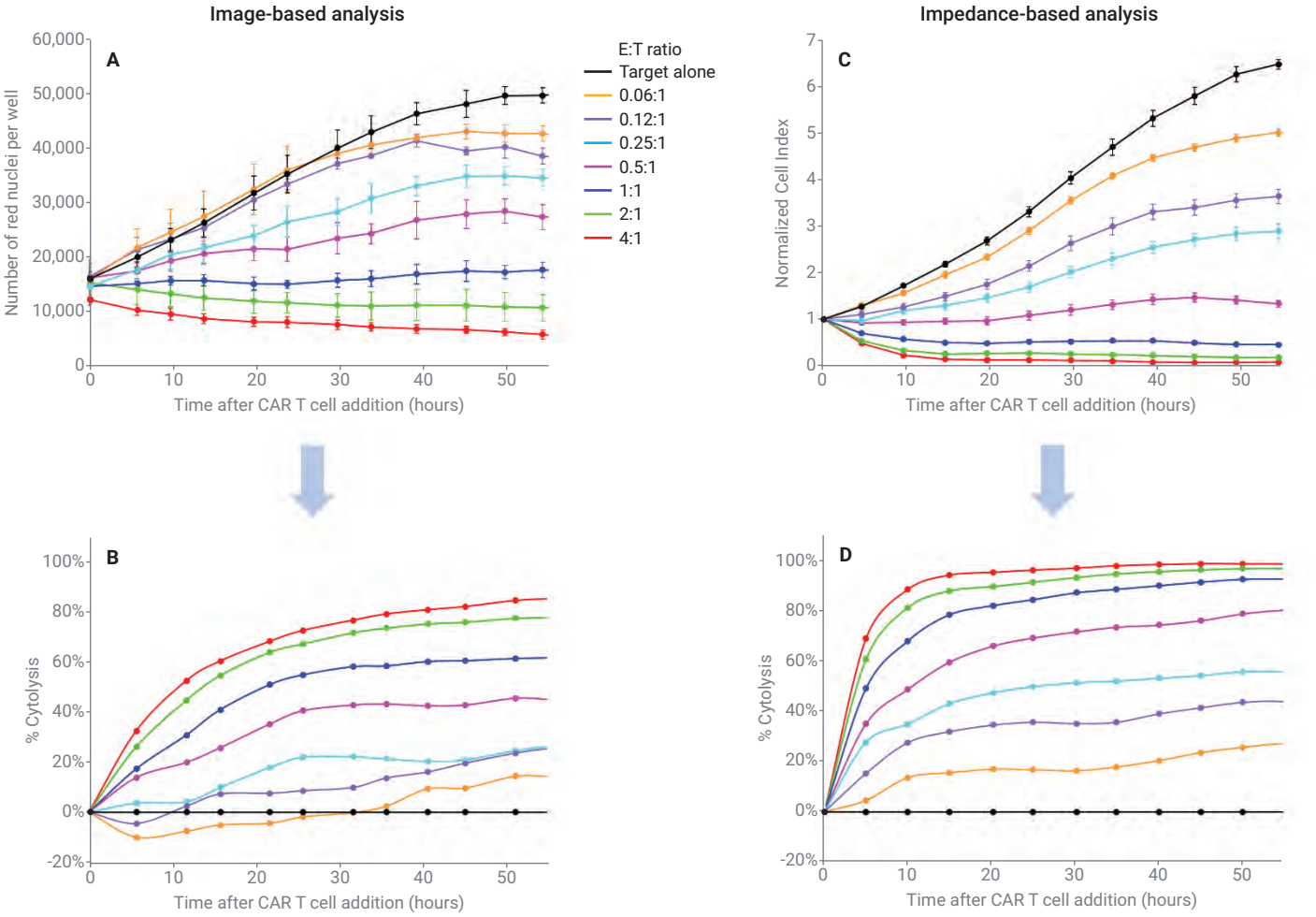

Figure 5. Time courses for HEK-293-CD19 killing by CD19 CAR T cells as measured by imaging (A-B) and impedance (C-D). While upper panels display the primary data, lower panels display % cytolysis, calculated as described in the materials and methods section. Assays were run in duplicate; error bars represent standard deviation.

juxtaposing the two different readouts within the same plot (Figure 6A–B). The reason that these two detection methods display different kinetics is that they are tracking CAR T cell-mediated killing from different perspectives (Figure 7). In the very earliest phases of CAR T cell-induced cytotoxicity, the HEK-293-CD19 targets begin to round and adhere to the well bottom less tightly, both of which are detectable by impedance. In contrast, the image-based tracking used in this assay doesn't register a change until the target cells actually lyse, resulting in a reduction in the number of countable red foci.

One of the most striking features of the eSight assay described here is the amount of information that it provides despite requiring minimal hands-on time to conduct. Since each well of the E-Plate provides a continuous readout over the entire length of the assay, the collection of endpoints is unnecessary. Moreover, every well simultaneously provides the two totally independent data sets of impedance and live cell images. By conservative estimate, attempting to match eSight's dual readout using the traditional endpoint methods of a ^{51}Cr

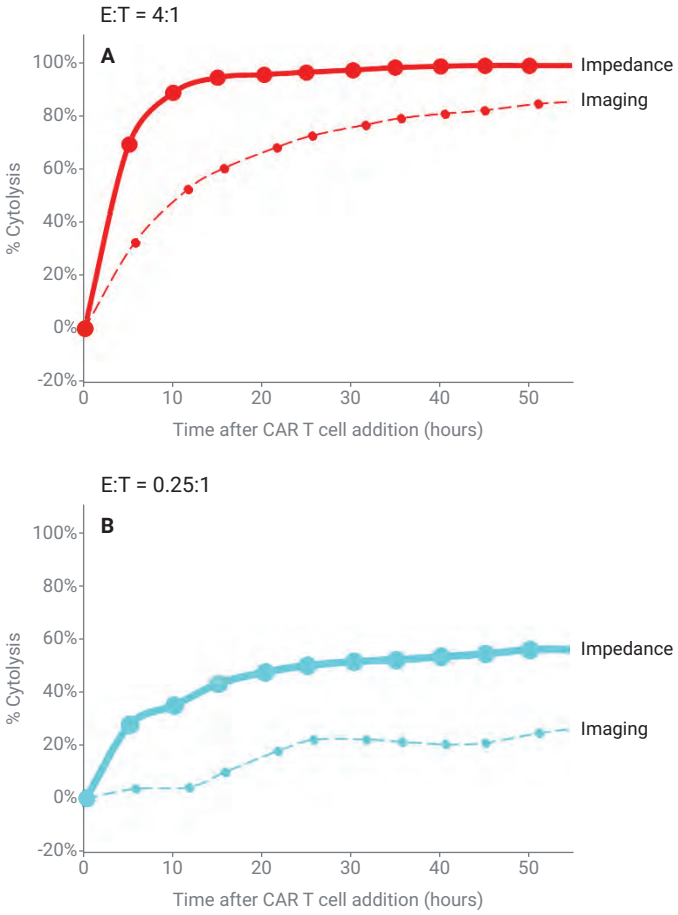

Figure 6. Comparison of percent cytolysis when determined using impedance or imaging. (A) E:T = 4:1. (B) E:T = 0.25:1.

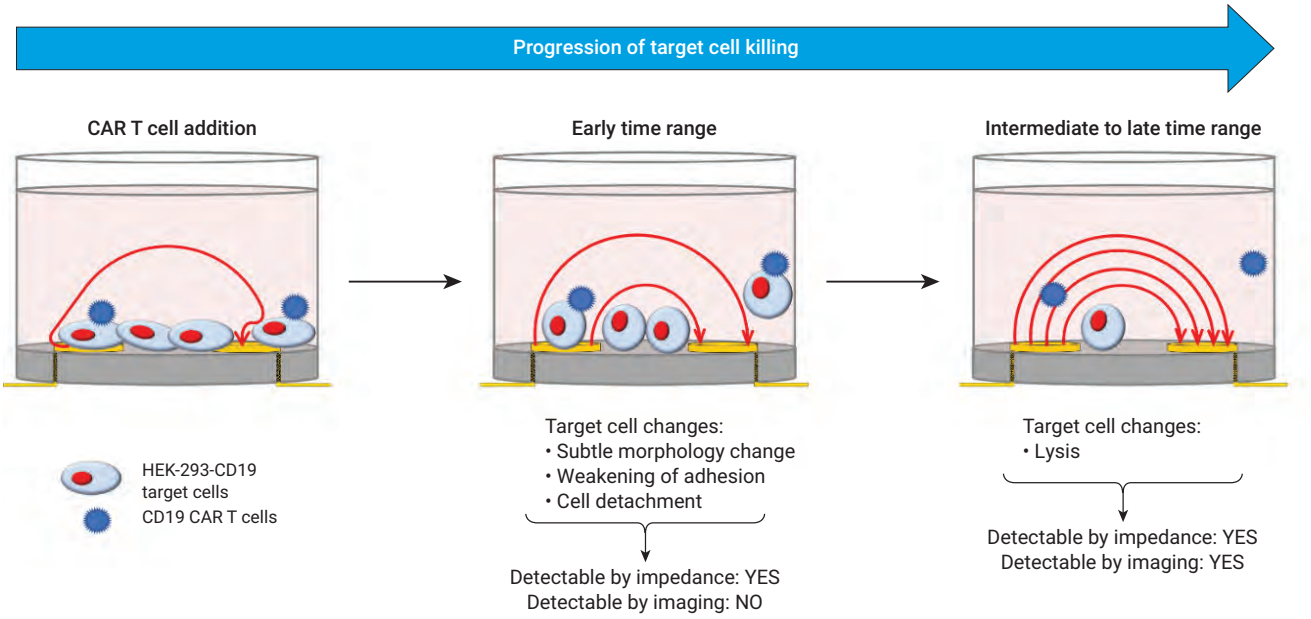

Figure 7. Explanation for why CAR T cell-mediated killing is detected earlier by impedance than it is by imaging.

release assay plus manual microscopy would require 12 times the amount of hands-on work (Table 1; note that this estimate assumes a crude temporal resolution of just four time points). The value of simply adding effector cells and walking away while data is recorded continuously and automatically cannot be overstated.

Another distinguishing feature of the eSight CAR T assay is its sensitivity. Because ^{51}Cr spontaneously leaches out of target cells, resulting in a progressive increase in background signal, ^{51}Cr release assays must be conducted over relatively short periods of time. This necessitates the use of high E:T ratios that are physiologically irrelevant. In contrast, there are no inherent temporal limits for eSight assays. Given a longer assay window, CAR T cell-killing activity can be interrogated at much lower E:T ratios that mimic *in vivo* scenarios (see E:T = 0.06:1 in Figure 5).

Because impedance is so sensitive to changes in cell size and cell-substrate adhesion strength, it is able to detect the very earliest stages of the target cell death process. In contrast, the image-based readout used here tracks target cell lysis—which occurs much later. Despite this difference, impedance and imaging ultimately yielded similar quantitative assessments of the CD19 CAR T cells' killing efficacy. Using the data shown in Figures 5A and C, the area under the curve was plotted as a function of E:T to yield dose response curves (Figure 8). The calculated EC_{50}, which is the E:T ratio required to generate a 50% killing response, was 0.56 (based on imaging) and 0.30 (based on impedance). Contributing to this consistency between the two data sets is the fact that both types of measurements are made on the exact same population of cells (that is, cells that are present in the same well).

Such tight correlation of data from measurement techniques that are physically very different provides a great deal of confidence in the conclusions being drawn, and suggests that eSight can serve as both the primary and secondary readout for cell-mediated killing assays.

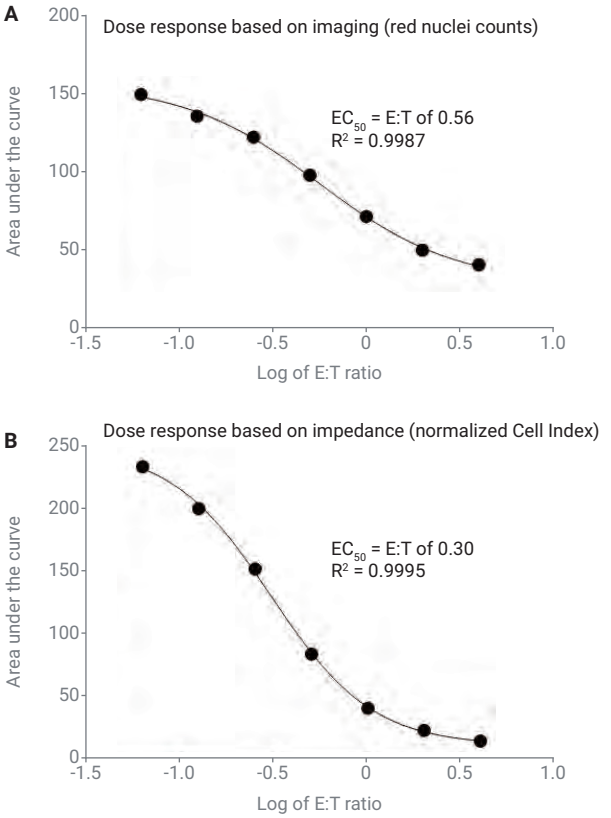

Figure 8. Dose-response curves based on the imaging versus impedance readouts.

Table 1. Comparing the hands-on work load of the continuous, dual-readout Agilent eSight assay vs. combining a traditional ^{51}Cr release assay with manual imaging. Only a single assay condition is considered, where E:T = 4:1.

Method	No. of Wells Needed to Monitor a Single Condition Where E:T = 4:1	No. of Cell Handling Steps	Time Points to Collect	Total No. of Manipulations
eSight	1 (impedance and images collected from the same well)	2 Target seeding Effector addition	0 (monitoring is automatic and continuous)	2
^{51}Cr Release Assay	2 One well for the release assay One well for image collection	3 Target seeding Effector addition Endpoint collection	4 (12, 24, 36, and 48 hours)	24

Beyond the benefit of having two independent measurement techniques, it is important to note the objectivity of the impedance readout, which is reported directly, without any processing or input from the user. Conversely, for eSight and all other imaging-based instruments, the raw image files get converted to outputs (such as number of red target cell nuclei) by user-informed algorithms where the expected size range, eccentricity, and brightness of cells must be defined. Although potential problems associated with this approach, such as inter-user variability, can be minimized through proper training and consistent usage of the same segmentation parameters, having impedance as an objective comparator helps build confidence in the assay's results.

Although not utilized in this study, eSight can image cells in three different fluorescent channels. In addition to labeling target and effector cells with different colors, the third channel can be used to track cell death in general or apoptosis specifically through the use of annexin V- or caspase 3-specific reagents.

Conclusion

The xCELLigence RTCA eSight couples the simplicity, analytical sensitivity, and objectivity of real-time impedance monitoring with the highly specific readout of live cell imaging to characterize CAR T cell-killing efficacy with unparalleled ease and information richness.

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Printed in the USA, November 9, 2020
5994-1712EN

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