Protocol

CAR T Cytotoxicity Assay Against Suspension Tumor Cells with Whole-Well Imaging Analysis

Imaging-based cytotoxicity assay protocol using the Agilent xCELLigence RTCA eSight

Authors

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Abstract

The Agilent xCELLigence real-time cell analysis (RTCA) eSight gives its users the flexibility to use both label-free impedance and/or imaging in four channels for the real-time monitoring of cell proliferation and cytotoxicity. The xCELLigence RTCA eSight has three cradles that use proprietary 96-well plates (Agilent E-Plate VIEW 96 microplates) to collect impedance and imaging data. Additionally, it includes two cradles specifically designed for imaging-based studies. This protocol describes the use of regular 96-well tissue culture plates in the imaging-only cradles for determining the cytotoxicity of chimeric antigen receptor (CAR)-expressing T cells (CAR T cells).

Introduction

The process for the production of cellular immunotherapy products like CAR T cells is complex, and the cytolytic activity can be variable. This necessitates a thorough evaluation of potency in in vitro assays. The following instructions are for setting up a basic cytotoxicity assay for CAR T cells using the xCELLigence RTCA eSight to monitor the cytolysis of nonadherent tumor cells. The coculture assay has been optimized using the Raji B cell lymphoma cell line and CD-19 targeting CAR T effectors in regular tissue culture media. The assay conditions may require further optimization if other target cell lines or effectors are used.

Experimental

The reagents and materials used are displayed in Table 1, cell culture media and components are listed in Table 2, and equipment used is listed in Table 3.

Table 1. Reagents and materials.

Item	Manufacturer	Description	Part Number
Raji cells	ATCC	Burkitt's Lymphoma	CCL-2
RPMI	ATCC	RPMI 1640 medium	30-2001
FBS	VWR	Fetal bovine serum (Seradigm)	97068-085
Penicillin/ streptomycin (pen/strep)	HyClone	10,000 IU penicillin, 10,000 mg/mL streptomycin	30-002-CI
T cell media	StemCell Technologies	Immunocult-XF T cell expansion medium	10981
CD19 CAR antibody	Miltenyi Biotec	CD19 CAR FMC63 Idiotype antibody, REAfinity	130-127-342
CD19 antibody	Biolegend	APC anti-human CD19 antibody, clone SJ25C1	900005608
96-well plate	Falcon (Corning)	96-well, flat bottom	353072
PBS	HyClone	Phosphate-buffered saline without Ca^{2+} and Mg^{2+}	SH30256.01
eLenti Green	Agilent	Lentiviral vectors expressing GFP	8711010
Polybrene	Sigma-Aldrich	Transfection reagent	TR-1003-G
Human serum	Sigma-Aldrich	Human serum	H4522-100ML
Cell staining buffer	BioLegend	Cell staining buffer	420201

Table 2. Cell culture media.

Media	Description
Growth media for Raji-GFP cells	RPMI with 10% FBS, (100 IU/100 µg)/mL pen-strep, puromycin (0.25 µg/mL)
Assay media (c-RPMI)	RPMI with 10% FBS, (100 IU/100 µg)/mL pen-strep
T cell resting media	Immunocult-XF T cell expansion medium
Transduction media	c-RPMI with polybrene (8 µg/mL)
Selection media	c-RPMI with puromycin (0.4 µg/mL)

Table 3. Equipment

Item	Part Number
Agilent xCELLigence RTCA eSight	380601600
Agilent xCELLigence RTCA eSight software with Immunotherapy module, single license	S2807-90048
Agilent xCELLigence RTCA eSight Dilution Cloning module, single license	S2807-90042
5x objective	S2807-68000

Process overview

The coculture assay has been optimized using Raji cells expressing nuclear-localized green fluorescent protein (Raji-GFP cells) as targets (T) for the CD19 CAR T effectors (E) in regular tissue culture media and 96-well plates.

Workflow summary

Raji-GFP cells were seeded at a density of 15,000 cells/well and imaged overnight (18 to 24 hours). This seeding density was chosen to ensure reliable detection of target cell growth while minimizing the number of CAR T cells required for the assay. Meanwhile, CAR T cells were revived and rested. The next day, CAR T numbers needed to achieve effector:target (E:T) ratios of 0.67:1, 2:1, and 6:1. Untransduced T cells (UT) were added to Raji cells in similar numbers in control wells. Although cytolytic activity was monitored for 140 hours in this case, the typical duration of a cytolysis assay is between 24 and 72 hours following effector cell addition. Cytolysis was measured by the loss of green fluorescence signal from the targets.

Plate layout

In this example of process monitoring CAR T cell-mediated cytolysis, six replicate wells were used for each condition: targets only, targets with UT control T cells, and targets with CAR T cells at the respective (E:T ratios). Target layout is displayed in Figure 1, and effector layout is displayed in Figure 2.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)				
C		Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)				
D		Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)				
E		Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)				
F		Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)				
G		Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)	Raji-GFP (15,000)				
H												

Figure 1. Target layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		CAR T (10,000)	CAR T (30,000)	CAR T (90,000)	UT (10,000)	UT (30,000)	UT (90,000)					
C		CAR T (10,000)	CAR T (30,000)	CAR T (90,000)	UT (10,000)	UT (30,000)	UT (90,000)					
D		CAR T (10,000)	CAR T (30,000)	CAR T (90,000)	UT (10,000)	UT (30,000)	UT (90,000)					
E		CAR T (10,000)	CAR T (30,000)	CAR T (90,000)	UT (10,000)	UT (30,000)	UT (90,000)					
F		CAR T (10,000)	CAR T (30,000)	CAR T (90,000)	UT (10,000)	UT (30,000)	UT (90,000)					
G		CAR T (10,000)	CAR T (30,000)	CAR T (90,000)	UT (10,000)	UT (30,000)	UT (90,000)					
H												

Figure 2. Effector layout.

Detailed instructions

Generating fluorescently-labeled target cells:

1. Transduction of Raji cells with eLenti Green
   a. Prepare transduction medium by adding polybrene (8 µg/mL) to c-RPMI.
   b. Resuspend Raji cells in transduction media and add 50,000 cells (100 µL) per well to a 96-well plate.
2. Virus transduction and selection
   a. Add the virus aliquot at a MOI of 10 into the transduction medium.
   b. Adjust the final transduction volume to 200 µL per well.
   c. Five days post-transduction, replace transduction medium with a selection medium containing 0.4 µg/mL puromycin in c-RPMI.
   d. Maintain puromycin selection for 10 days to enrich successfully transduced cells.
3. Single-cell cloning and expansion
   a. After at least 10 days of puromycin selection, resuspend cells in c-RPMI (0.25 µg/mL puromycin). Dilute the suspension to a cell density of two cells per 100 µL. Add 100 µL of suspension to 96-well plates containing 100 µL selection medium.
   b. Perform single-cell cloning using the Agilent xCELLigence RTCA eSight Dilution Cloning module. The technical overview for this module can be found here.
   c. Monitor single-cell colonies and gradually expand them into 6- and 24-well plates for further analysis.

Day 1:

Preparation of target cells

4. Warm the assay media (c-RPMI) in a 37 °C water bath 30 minutes before the start of the experiment.
5. Use the (5x) objective for whole-well imaging.
6. Open the xCELLigence RTCA eSight software and enter the experiment and plate layout information as planned.
7. Cell preparation
   Critical: As with most cell-based assays, proper handling of cells is essential for the outcome of the assay.
   a. Culture Raji-GFP cells in growth media containing Puromycin selection (0.25 µg/mL) and passage every three days.
   b. Spin down the cells at 300 x *g* for five minutes and resuspend the cells in the assay media
   c. Count the cells and adjust the concentration of the cell suspension. In this example experiment, the concentration should be 15,000 cells/100 µL.
   Tip: Use reverse pipetting to avoid introducing bubbles.
8. Leave the plate undisturbed in the tissue culture hood at room temperature for about 30 minutes to allow the cells to settle evenly to the bottom of the well.
   Note: Immediately transferring the plate to 37 °C can result in a higher edge effect and an uneven distribution of cells.
9. Transfer the plate gently into cradle four (or five) of the xCELLigence RTCA eSight inside a 37 °C and 5% CO_2 incubator.
10. Click the **Schedule** tab of the xCELLigence RTCA eSight software and add an imaging step, as shown in Figure 3.

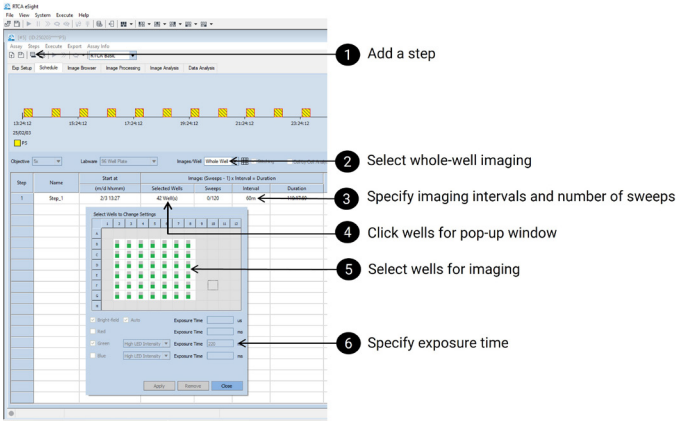

Figure 3. Steps in Agilent xCELLigence RTCA eSight software.

11. In this example, Raji-GFP cells were imaged every 60 minutes in brightfield at default exposure and in the green channel at 220 ms exposure for 180 hours.
    Note: Select whole-well imaging from the pull-down menu before starting, as shown in Figure 4.

Figure 4. How to select whole-well imaging in Agilent xCELLigence RTCA eSight software.

Reviving effector cells:

12. Warm the T cell resting medium in a 37 °C water bath for 30 minutes.
13. Revive the CAR T and UT cells frozen down in CryoStor CS10 (StemCell Technologies) by immersing and swirling the vial of frozen cells for approximately 1 minute in a 37 °C water bath.
    Add 1 mL of warm T cell resting media dropwise to the thawed contents and bring the total volume to 10 mL dropwise.
14. Spin down the cells at 300 x *g* for seven minutes. Resuspend the cells in fresh T cell resting media at 2 to 5 x 10^6 cells/mL and rest them in an incubator at 37 °C with 5% CO_2 overnight.

Day 2:

Addition of effector cells:

15. Transfer T cells from the resting media to the assay media. Spin down CAR T and UT cells at 300 x *g* for five minutes and resuspend the cells in assay media (c-RPMI).
16. Count the live cells and pipette the volume of CAR T and UT cell suspension needed for multiple replicates. Adjust the volume using assay media to achieve the final E:T ratio with the addition of 100 µL cell suspension. The total volume in every well will be 200 µL. The target-only control wells will receive 100 µL assay media.
    Tip: Abort the current step in RTCA eSight software and take the plate out of its cradle. Add T cell suspension gently and use reverse pipetting as before.
17. Add the CAR T and UT cells to the replicate wells as specified in the plate layout.
18. Leave the plate at room temperature for 5 minutes and then return it to the cradle.
19. In the RTCA eSight software, add a new step in the schedule. Select the wells and specify the exposure time. In this example, Raji-GFP cells were imaged every 60 minutes at an exposure time of 220 ms for 180 hours. The loss of green fluorescence from Raji-GFP cells was used as the marker for cytolysis by CD19 CAR T cells.
    Tip: Minimize the exposure times in blue and green channels to avoid phototoxicity. Recommended exposure times are around 80 ms on the blue channel and ≤ 200 ms on the green channel. Longer exposure time could also be avoided by using brighter cells for whole-well imaging.
    Note: While 190 ms was adequate for imaging the Raji-GFP cells using the 10x objectives on the xCELLigence RTCA eSight, a longer exposure time of 220 ms was needed with the 5x lens.

Data analysis:

Data analysis steps are shown in Figure 5.

20. The image processing method needs to be optimized for each target cell line and labeling approach. In this example, the loss of GFP signal (green channel) from the target cells was used to determine CAR T-mediated cytolysis.
21. Prepare a training set of at least 10 images spanning multiple wells/conditions at different time points. Include images taken right before and after the addition of T cells.

22. Optimize the segmentation in brightfield and/or fluorescence channels.

    a. Brightfield data was not used in this coculture assay.
    Tip: Setting the Segmentation Adjustment in this channel to 0.6 was found to be best suited for cytotoxicity assays with Raji cells.

    b. For this assay with Raji-GFP cells, the segmentation in the green channel was adjusted to 1.4.
    Critical: Segmentation can affect data analysis, and adjustments must be carefully balanced. When the fluorescent signal of labeled cells is weak, increasing the segmentation value can increase the sensitivity of the software to identify the cells. Larger segmentation could also lead to software artifacts. In this example, adjusting the slider to the default value of one or lower for the green channel resulted in a reduced background but fewer cells being detected, while increasing the value to 1.5 and higher resulted in a nonspecific background in several wells. Ensure that segmentation results in the correct application of fluorescence mask correctly across multiple wells representing different conditions.

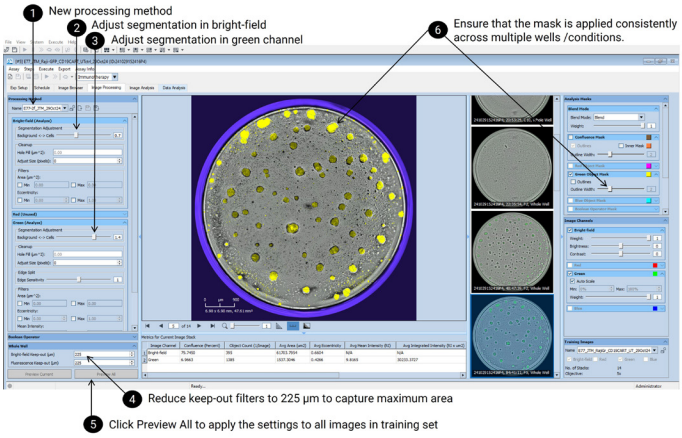

Figure 5. Data analysis steps.

24. The results of this experiment are presented in Figure 6. Cytolysis was determined using xCELLigence RTCA eSight software. The percentage of cytolysis for CAR T and UT treatments was calculated using untreated controls (target only) as a reference. Specific cytolysis in CAR T treated samples at each E:T ratio was calculated using the target cells treated with the corresponding UT numbers as reference. For instance, specific cytolysis for CAR T at an E:T ratio of 6:1 was calculated using UT at an E:T ratio of 6:1.

Note: Like all cell-based assays, this assay relies on the quality of cells used, the passage number, the killing kinetics, and the goal of the assay. This protocol is provided as an example and should be optimized for the actual conditions. The parameters that should be further considered and optimized are:

- Seeding density: The optimal seeding density range for target cell growth during the entirety of the assay must be identified. Avoid using low seeding densities, as these can disrupt normal growth kinetics, diminish cytotoxicity resolution, and reduce assay sensitivity
- Fluorescence labeling: Exposure time and imaging intervals. The fluorescence intensity of labeling in target cells can impact whole-well imaging data collected using the 5x objective. If the cells appear dim, exposure time can be increased in the red and green channels. At 220 ms, increased phototoxicity affected the controls and cytotoxicity calculations with the Raji cells when imaged at intervals of 45 minutes compared to 60 minutes. Extending imaging intervals when increasing exposure times can help mitigate phototoxicity, depending on the assay's purpose and duration.
- Imaging in the blue channel should be carefully optimized and used for imaging cells less frequently, with exposure times around 80 ms in assays shorter than 72 hours.
- E:T ratio: The ratio should be optimized for each target and CAR T effector pair.
- Untransduced T cell control: This control provides an estimate for the nonspecific killing of target cells in the presence of T cells and is used for calculating specific cytolysis. It is recommended to have a control with untransduced T cells for each corresponding E:T with CAR T cells. At optimal E:T ratios, cytolysis in this control would be minimal, indicating that cytolysis by CAR T is specific and due to the engineered CAR. Alternatively, targets that do not express the antigen for CAR could also be used as controls. The addition of T cells can affect the distribution of target cells in the wells and modulate the clustering of targets (Figure 7, representative figure).

Conclusion

The Agilent xCELLigence RTCA eSight platform enables real-time, whole-well imaging to assess CD19 CAR T cell cytotoxicity, measured by the reduction of green fluorescence in Raji-GFP target cells.

Fluorescence loss correlated with increasing effector-to-target (E:T) ratios, demonstrating a dose-dependent response.

This study highlights one of the many versatile applications of the RTCA eSight platform, which supports dynamic, label-free analysis across a broad range of cell therapy and immuno-oncology workflows.

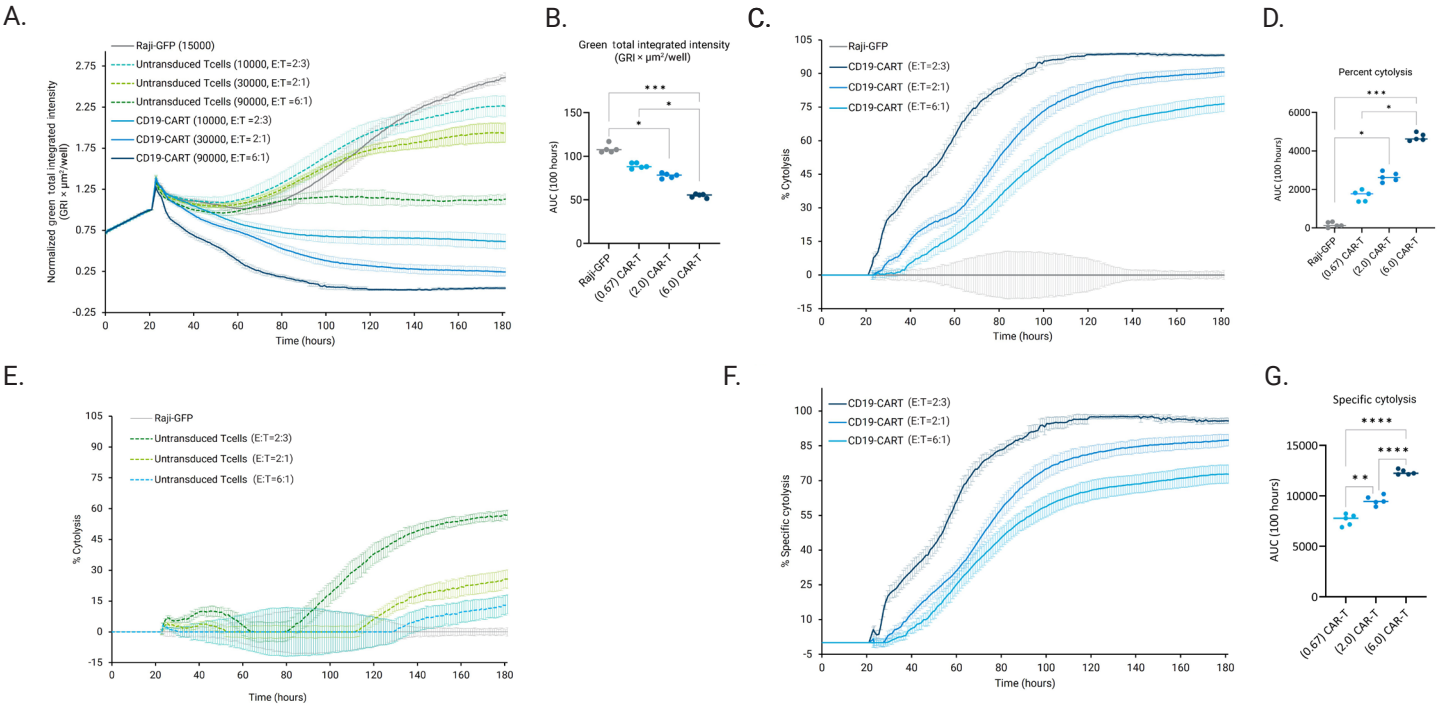

Figure 6. Whole-well imaging was performed on the Agilent xCELLigence RTCA eSight to monitor the loss of fluorescence signal from Raji-GFP cells for determining the cytotoxicity kinetics of CD19 CAR T cells. (A) Normalized data showing the growth of Raji-GFP cells (15,000/well, six replicates). The addition of CD19 CAR T cells at E:T ratios of 0.67:1, 2:1, and 6:1 results in the killing of target cells, evidenced by the loss of green fluorescence, which increases with CAR T cell numbers compared to untreated wells and wells with corresponding numbers of UT cells. (B) ANOVA analysis of the area under the curve (AUC) of normalized green signal detected up to 100 hours. (C) Percent cytolysis in CAR T-treated wells, calculated using untreated target wells as reference and (D) ANOVA test performed for the AUC (100 hours). (E) Percent cytolysis in wells with corresponding numbers of UT cells. (F) Specific cytolysis by CD19 CAR T cells (E:T ratios of 0.67:1, 2:1, and 6:1) calculated using Agilent xCELLigence RTCA eSight software, with corresponding UT cells (E:T ratios of 0.67:1, 2:1, and 6:1) as reference. (G) ANOVA analysis of the AUC for specific cytolysis during the first 100 hours.

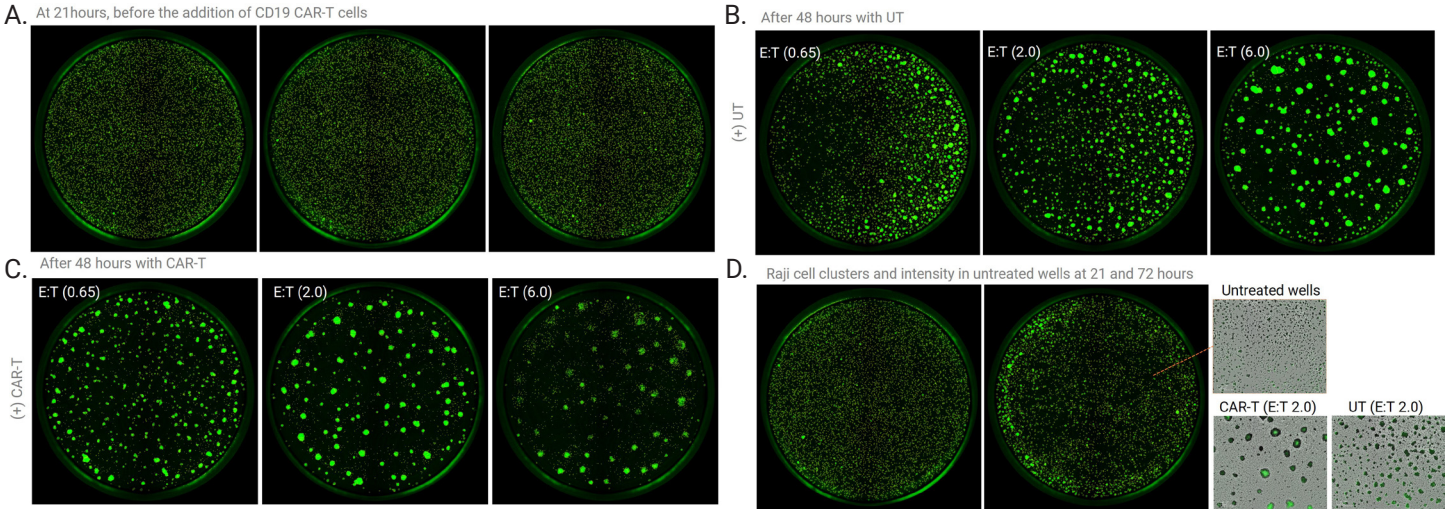

Figure 7. Representative whole-well images showing the changes in Raji-GFP cells during coculture with T cells. (A) Distribution of Raji cells at 21 hours before the addition of T cells, (B) after 48 hours of coculture with UT wells and (C) CD19 CAR T cells. The addition of CAR T cells results in the killing of target cells, as confirmed by fewer surviving Raji cell clusters and lower total green fluorescence intensity. Raji cells tend to form larger clusters in the presence of T cells (B and C) compared to untreated wells (D). Inset representative brightfield images show T cell distribution in the wells.

Products used in this protocol

Agilent xCELLigence RTCA eSight

www.agilent.com/lifesciences/esight

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