

Video Article

Quantitative High-throughput Single-cell Cytotoxicity Assay For T Cells

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Abstract

Cancer immunotherapy can harness the specificity of immune response to target and eliminate tumors. Adoptive cell therapy (ACT) based on the adoptive transfer of T cells genetically modified to express a chimeric antigen receptor (CAR) has shown considerable promise in clinical trials¹⁻⁴. There are several advantages to using CAR⁺ T cells for the treatment of cancers including the ability to target non-MHC restricted antigens and to functionalize the T cells for optimal survival, homing and persistence within the host; and finally to induce apoptosis of CAR⁺ T cells in the event of host toxicity⁵.

Delineating the optimal functions of CAR⁺ T cells associated with clinical benefit is essential for designing the next generation of clinical trials. Recent advances in live animal imaging like multiphoton microscopy have revolutionized the study of immune cell function *in vivo*^{6,7}. While these studies have advanced our understanding of T-cell functions *in vivo*, T-cell based ACT in clinical trials requires the need to link molecular and functional features of T-cell preparations pre-infusion with clinical efficacy post-infusion, by utilizing *in vitro* assays monitoring T-cell functions like, cytotoxicity and cytokine secretion. Standard flow-cytometry based assays have been developed that determine the overall functioning of populations of T cells at the single-cell level but these are not suitable for monitoring conjugate formation and lifetimes or the ability of the same cell to kill multiple targets⁸.

Microfabricated arrays designed in biocompatible polymers like polydimethylsiloxane (PDMS) are a particularly attractive method to spatially confine effectors and targets in small volumes⁹. In combination with automated time-lapse fluorescence microscopy, thousands of effector-target interactions can be monitored simultaneously by imaging individual wells of a nanowell array. We present here a high-throughput methodology for monitoring T-cell mediated cytotoxicity at the single-cell level that can be broadly applied to studying the cytolytic functionality of T cells.

Video Link

The video component of this article can be found at <http://www.jove.com/video/50058/>

Protocol

1. Reagents Preparation

1. Prepare RPMI-PLGH by mixing 500 ml RPMI-1640 and 5 ml each of Penicillin-streptomycin, L-glutamine, and HEPES solution.
2. Prepare R10 solution by mixing RPMI-PLGH with 10% Fetal Bovine Serum (FBS). The FBS is heat-inactivated at 56 °C for 30 min prior to addition.
3. Pre-warm at 37 °C 50 ml of RPMI-PLGH, 15 ml of PBS, and 15 ml of R10 in sterile conical tubes.
4. Fabrication of arrays of nanowells in PDMS: The silicon master is fabricated using photolithography essentially as described previously¹⁰. Mix thoroughly the Sylgard 184 elastomer kit base and curing agent at 10:1 weight ratio in a disposable cup using a plastic knife. Degas the mixture in a vacuum chamber for 1 hr. Pour mixture onto the nanowell array, seal with a glass slide and let it sit for 30 min. Transfer the assembly into an oven set to 80 °C for 2 hr and let cool at room temperature for 1 hr. The elastomer will cure during this period and will bond to the glass slide. The glass slide containing the PDMS nanowell array is carefully lifted off the silicon master, covered with Scotch tape and stored until future use.

2. Target Cell (T) Labeling

1. Count 5 million target cells from cell culture using a hemocytometer and pellet the cells in a sterile 15 ml conical tube by centrifugation at 340 x g for 5 min. The cell culture is split the previous day in 5 ml total volume (density = 1 million/ml) and cultured in T-25 flask (VWR). Protocols for cell counting using hemocytometer have been described in detail previously¹¹
2. Aspirate excess media leaving behind approximately 50 μ l of media.
3. Prepare a working solution of Cell Tracker Red (CTR) by mixing 0.5 μ l CTR stock (1 mM) to 150 μ l RPMI-PLGH (final concentration 2.5 μ M). Add 150 μ l of the working solution to the target cells and mix thoroughly using a P200 pipette. Alternately, PKH 26 (final concentration of 2 μ M) can be used label target cells as per the manufacturer instructions.
4. Incubate at 37 °C/5% CO₂ for 20 min.

3. Effector (E) Cell Labeling

1. Count 5 million effector cells from cell culture using a hemocytometer and pellet the cells in a sterile 15 ml conical tube by centrifugation at 340 x g for 5 min.
2. Aspirate excess media
3. Prepare a working solution of Vybrant DyeCycleViolet Stain solution by adding 1 μ l of 5 mM Vybrant Violet stock to 1 ml of R10 media (final concentration of 5 μ M). Add the working solution to the cells and resuspend using P200 pipette. Alternately, PKH 67 (final concentration of 2 μ M) can be used label target cells as per the manufacturer instructions. If PKH 67 is used, then SYTOX Green should not be used since they are in the same fluorescence channel. The enumeration of apoptotic targets is done exclusively using AnnexinV staining in this case.

Note: If performing time-lapse and not endpoint measurements, UV dyes like Vybrant Violet should be avoided.

4. Incubate at 37 °C/5% CO₂ for 20 min.

4. Separation of Live and Dead Cells Using a Density Gradient

1. Add 6.8 ml and 6.0 ml of RPMI-PLGH to target and effector cells, respectively and mix by pipetting up and down gently.
2. Layer 3.5 ml of FICOLL-Paque Plus to bottom of each conical tube containing target and effector cells. Layer the FICOLL slowly to ensure good formation of layers between FICOLL and media.
3. Centrifuge both tubes at 340 x g for 30 min. with no brake and acceleration.
4. During centrifugation, transfer 7 ml of pre-warmed PBS to a 4-well plate. Prepare nanowell array (fabricated in PDMS): clean bottom of glass slide with ethanol and oxidize the PDMS at high RF setting using plasma oxidizer for 1 min. This step will sterilize the nanowell array while also rendering the PDMS hydrophilic. Immediately move the nanowell array into the plate containing PBS.
5. Transfer the plate to a biosafety cabinet and aspirate excess PBS. Make 10 ml 1% noble agar by adding 100 mg agar powder to 10 ml DI water and then microwave it for 30-45 sec. Apply warm 1% noble agar on top and bottom edge of glass slide that contains the nanowell array to immobilize the array and wait 10 min for agar to solidify. Add 3 ml PBS and aspirate excess agar.
6. Add 3 ml of R10 onto the top of nanowell array and let it equilibrate for ~10 min.
7. Subsequent to FICOLL centrifugation, aspirate 5 ml media from the top layer of each tube. Be careful not to aspirate the layer containing the cells.
8. Harvest the cells (white layer) by recovering 1-2 ml from the layer between FICOLL and media with P1,000 pipette and move to new sterile conical tubes. Repeat this for both sets of cells while making sure that you maximize the recovery cells.
9. Add 3 ml of pre-warmed RPMI-PLGH to each tube, mix by pipetting and pellet by centrifugation at 340 x g for 5 min on full acceleration and brake.
10. Aspirate excess media, add 5 ml of pre-warmed RPMI-PLGH to each tube and repeat centrifugation at 340 x g for 5 min.
11. Aspirate excess media and resuspend the cell pellet in 500 μ l of R10.
12. Count live cells using the Trypan blue exclusion method on a hemocytometer.

5. Cell Loading onto Nanowell Array

1. Aspirate excess media from nanowell array and add 2 ml of fresh R10 across the array. Aspirate again while making sure the top of nanowell array is not too wet/dry as it will affect distribution of cells.
2. Deposit 1×10^5 effector cells in 200 μ l of R10 onto nanowell array surface (density = 5×10^5 cells/ml).
3. Let cells settle for 5 min and using a standard tissue culture microscope check to ensure that the distribution of cells is desirable. Add more cells if necessary.
4. Deposit 1×10^5 target cells in 200 μ l R10 on nanowell array surface (density = 5×10^5 cells/ml).
5. Let cells settle for 5 min and using a standard tissue culture microscope check to ensure that the distribution of cells is desirable. Add more cells if necessary.
6. Rinse nanowell array carefully with 2 ml R10 and aspirate excess media.
7. Prepare 3 ml pre-warmed R10 in a 15 ml sterile conical tube. Add 3 μ l of 0.5mM SYTOX Green Nucleic Acid Stain (final concentration 0.5 μ M) and 60 μ l of Annexin V- Alexa Fluor 647 to prepare working solution. Add the working solution onto the 4-well plate carefully while making sure that cells are not displaced from the wells.
8. Incubate at 37 °C/5% CO₂ for 15 min.

6. Imaging 1

1. Acquire images of the nanowell array using a fluorescence microscope: In this example, we use a ZEISS Observer Z-1 microscope equipped with a motorized stage and Lambda-DG4.
2. Initialize the software and the microscope and check the signal intensity on the transmitted light and the other fluorescent channels. Overall, there will be five channels corresponding to: transmitted light, Annexin V-647 (Cy5 filter), CTR (DsRed filter), SYTOX Green (FITC filter), and Vybrant Violet (DAPI filter). Ensure that the microscope is setup to ensure maximal signal to noise while avoiding saturation.
3. Set X and Y position for the nanowell array. Begin process by setting the zero position and initializing focus on the center of 7 x 7 nanowell array block on top left corner of the nanowell array stamp. Set the x,y positions to coincide with the center of each block and the z-value to accurately reflect the focus on each block.
4. Once the position and focus are all set, make sure to set image acquisition in linear mode and acquire images. **Note:** If necessary turn on the incubator and CO₂ on the microscope at least 30 min before the experiment.

7. Post-imaging

Transfer the 4-well plate containing the nanowell array into an incubator (37 °C/5% CO₂) for 6 hr.

8. Imaging 2

Acquire the images at the second time-point as outlined in Step 6.

9. Image Analysis

1. The occupancy of cells within the nanowells is determined by the use of appropriate image segmentation programs (e.g. ImageJ, <http://rsbweb.nih.gov/ij/>) essentially as described previously^{9,12}.
2. Analysis of the initial set of microscope images ($t = 0$ hr; t_0) is used to generate a database of the individual nanowells containing exactly one live target cell (identified by CellTracker Red; red) and no dead cells (identified by Annexin V staining, green) [T1 t_0 nanowells].
3. Analysis of the second set of microscope images ($t = 6$ hr; t_6) is used to independently determine a second database of nanowells containing 1 target cell (red) [T1 t_6 nanowells]. An integrity check (database query) is implemented to ensure that all live targets at t_0 are matched to targets at t_6 to produce a final set of matched targets (implemented as nanowells that have T1 $t_0 = T1t_6$, designated as T1match)
4. The number of effector cells in nanowells is determined using Vybrant Violet staining at t_0 [E1 nanowells]
5. A database query is implemented to identify nanowells containing single effectors co-incubated with single targets (defined as nanowells that have E1 and T1_{match}, designated as E1T1)
6. Effector induced apoptosis is scored by identifying targets that are Annexin V negative at t_0 and Annexin V positive at t_6 (E1T1 with Annexin V target staining at t_6).

10. Optional Time-lapse Imaging of Killing Using Nikon's BioStation IM

1. Take a Petri dish with coverslip bottom and cut out a small piece of the nanowell array chip (prepared at Step 5) that will exactly fit on the coverslip. Make sure that the chip stays wet while cutting.
2. Mix 1×10^6 targets and 1×10^6 T cells in 100 μ l media, and pipette it onto the coverslip.
3. Allow about 15-30 min to let the cells settle.
4. Rapidly invert the small nanowell array chip and place it on the coverslip.
5. In order to ensure that the chip will not be able to move during the imaging, place one or two 20mm x 20mm coverslips onto the top of the chip. Apply pressure softly to push the chip to the bottom of the dish.

Note: The nanowell array chip's bottom is too thick for high-resolution imaging and therefore has to be turned upside down. During this, many of the cells are washed out. Since this cannot be easily controlled, cell numbers in Step 9.2 and incubation time in Step 9.3 have to be optimized.

Representative Results

An example of the application of the high-throughput cytolytic assay is demonstrated in **Figure 2**. Briefly, labeled CD19-specific CAR⁺ T cells were co-incubated with labeled mouse EL4 target cells in the individual wells of a nanowell array (Sections 1-5). An initial image was recorded on the automated fluorescent microscope to identify the occupancy (effectors and/or targets) of every single nanowell on the array (Section 6). Image processing was used to identify all nanowells containing exactly a single target and a single effector and to exclude nanowells containing dead targets (Section 9). The chip was transferred to an incubator for 6h and then a second image of the chip was recorded (Sections 7-8). The ability of effector to induce apoptosis in target cells was measured by staining with Annexin V, in nanowells containing exactly one effector and one target. Two parallel experiments were run with the same effectors and two sets of target cells (EL4-CD19⁺ and EL4-CD19⁻ (negative control)) to determine the frequency of antigen-specific lysis (**Figure 2**). It is desirable to achieve low frequencies of non-specific lysis (~2-4%). The methodology is dependent on the availability of the appropriate target cells and parallel experiments are run to determine apoptosis in targets independent of effectors. High frequencies of target apoptosis in the absence of effectors over the 6h period (> 8%) indicates a need to optimize culturing and assay conditions. The nanowell array can be adapted for time-lapse imaging when continuous monitoring is needed (Movie 1) and this allows for the observation of effector-target conjugation and subsequent cell-death.

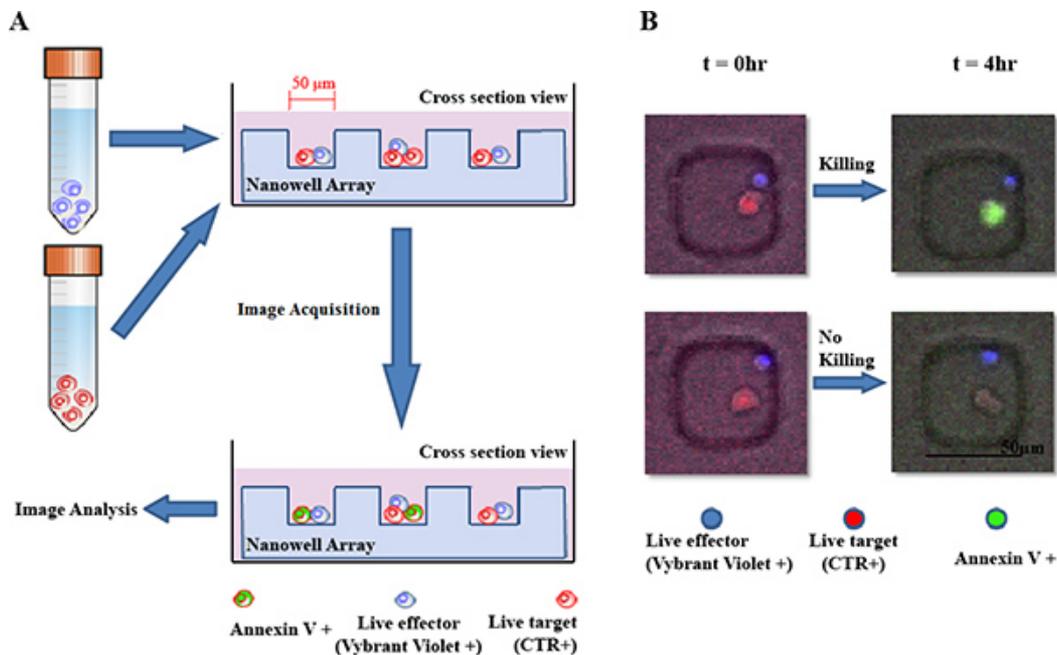


Figure 1. A. Schematic of nanowell array based cytolytic assay. Labeled CAR⁺ T cells (blue) and target cells (red) are incubated in an array of nanowells. Microscopy is used to monitor effector mediated target cytolysis. **B.** Representative composite micrographs of CD19-specific CAR⁺ T cells that induce apoptosis in EL4-CD19⁺ target cells and ones that do not.

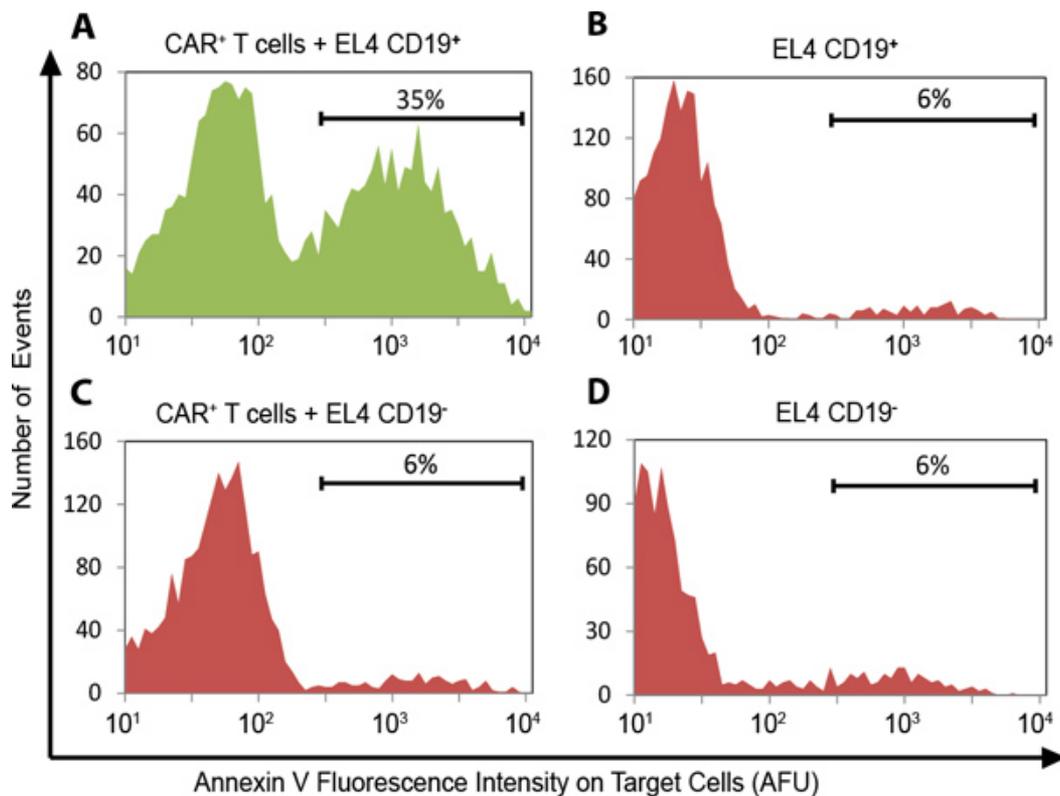


Figure 2. Antigen-specific cytolytic activity of CD19-specific CAR⁺ T cells. Target cell histogram plots of wells containing a single effector and a single target after 6 hr of co-incubation. Matched histograms of target cells without effectors are shown to report the frequency of background cell death.

Movie 1. Time-lapse microscopy of CAR⁺ T-cell mediated cytotoxicity. CAR⁺ T cells (unlabeled) were co-incubated with NALM-6-GFP⁺ target cells (green) in a nanowell and images were taken every 2 min for a total of 12 hr to enable dynamic monitoring. Annexin V was added to the culture media and apoptotic cells become labeled red.

Discussion

We have outlined the protocol for a high-throughput single-cell cytolytic assay enabled via co-incubation of effectors and targets in arrays of nanowells (**Figure 1**). In addition to throughput a major advantage of the technique is the ability to monitor effector-mediated cytotoxicity against desired target cells without the need for target cell engineering which in turn allows for the use of autologous or matched/primary tumor cells as target cells. The spatial confinement allows the retrieval and subsequent cloning of functional T cells at the end of the assay⁹. A limitation of the assay is the need for specialized microscopes with automated stages and fast-switching optics. This is not however a serious limitation given that these machines are now widely available as part of research core facilities.

There are a variety of flow-cytometry based assays that offer higher throughput while preserving single-cell resolution. These include assays that stain for the surrogate marker for degranulation (CD107a) or perforin content in effectors and caspase/granzymes activity in targets^{13,14}. These assays however do not truly monitor effector-target conjugation or the ability of the effector to engage and kill multiple targets. Compared to the gold standard assay such as a 4h 51Cr-release assay, run at E:T ratios of 1:1, our methodology typically reports the same overall number of cytolytic effectors, while still providing single-cell resolution. Although the protocol outlined here only details on cytotoxicity, as outlined previously this assay can be readily combined with the microengraving assay to determine cytokines secreted by single effector cells upon target ligation⁹. It is thus possible to generate composite functional profiles of CAR⁺ T cells that combine cytotoxicity and cytokine secretion at the single-cell level.

Disclosures

No conflicts of interest declared.

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