White Paper

Suppressing single cell noise in T cell selection

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Introduction

Robust determining single cell kinetics including: killing rate, proliferation rate, engaging time… are the basis to compare and predict in-vivo performances among different cell clones, especially in CAR-T development or TCR-T identification. Conceptually, best performing clones as a single cell could possibly correlate to its superior behavior in bulk, given accurate monitoring and analysis can be achieved.

Highly accurate measurement can be achieved on single cell monitoring platform, which shows a greater spread among single cells carrying the same DNA materials than inter-genotype differences. Such spread can only be attributed to the noises such as, individual cell health, environmental turbulence and even the starting distances between T cells and tumor cells. Simply put, a modest clone in its good times might easily beat a good clone. Such measurement would cause ambiguity in identifying phenotypes and comparing different genotypes (DNA construct). In comparison, measurements in bulk are rather robust.

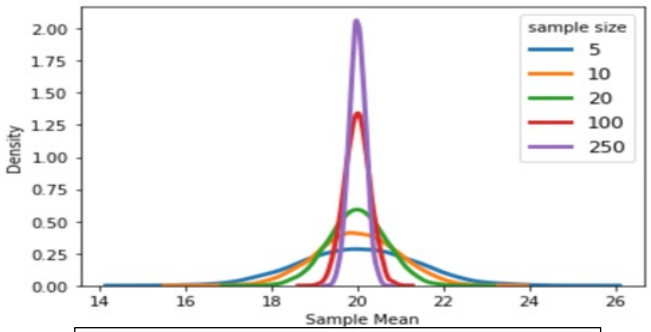

Figure 1. Parameter spread vs sample size.

Such discrepancies follow typical gaussian distribution. For any observables with gaussian randomness, the bigger the sampling size, the less spread (sharper peaks) the measurement will be. To make meaningful comparison between two distinct groups, adequate sample size is required to achieve a minimal peak spread.

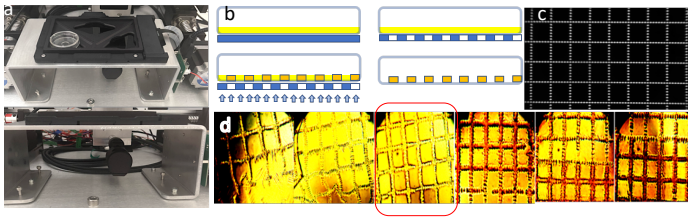

Figure 2. Generation of hydrogel wells.

Single cell measurements (killing time, doubling time) obtained from a single event by a short period of observation are usually too noisy to advise isolation of single clones from thousands of clones, instead, such platforms are better at assessing one construct with lots of wells.

To suppress single cell noise, different strategies to increase sample size are used on Enrich TroVo platform: time average and micro-group average. The former includes a long-term observation with several cycles (>3) of events over a single clone, and the calculated of the average kinetical properties of these events; the latter requires a cell proliferation step, so that multiple cells (>3) originated from one single clone can be obtained, which can be followed by monitoring and calculation the cumulative kinetical behavior by this micro-group.

We here use hydrogel based microwell system and CAR-T/Tumor cell coculture as an example.

Microcompartments generation (Fig.2):

The microwell printed followed the current optimal microwell print for coculturing cells. The gel mixture was then added to the 20mm glass bottom portion of the 35mm dish, and placed in the TROVO for printing.

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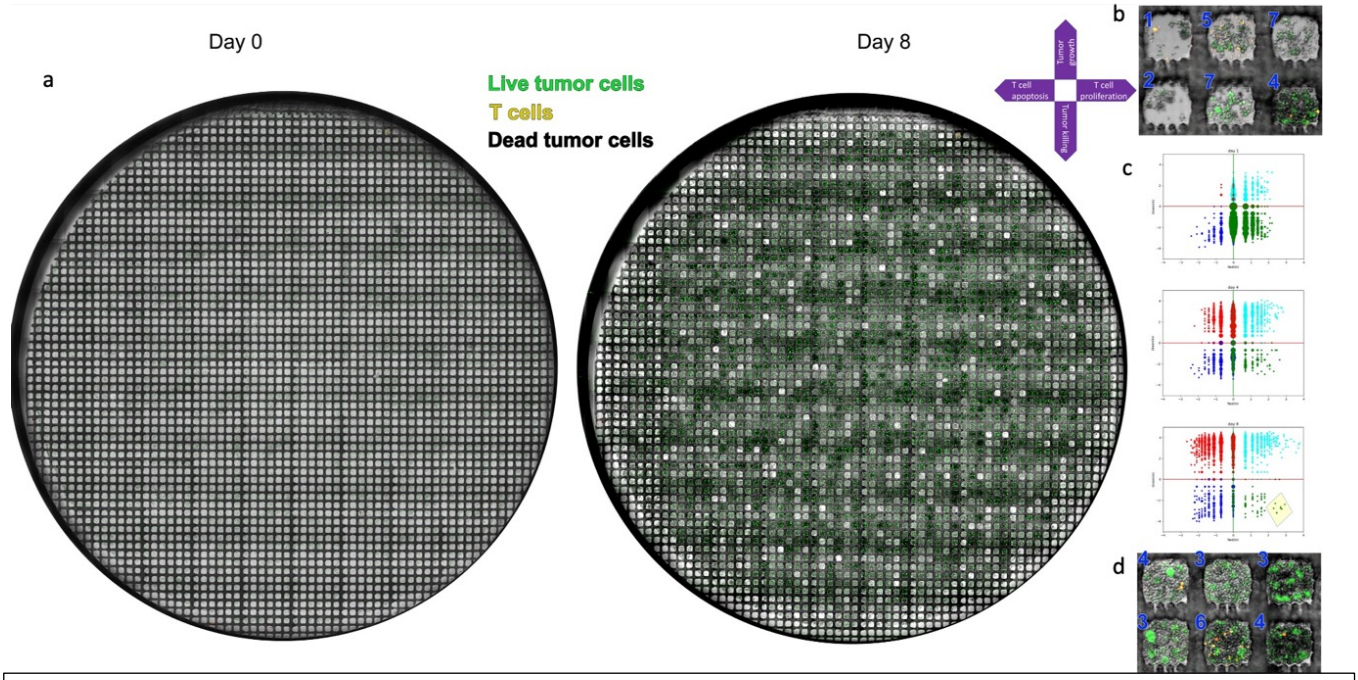

Figure 3. Long period evaluation of T cell/tumor cell coculture. (a) Day 0 and Day 8 Birdseye overview of microcompartment cocultures. (b,d) the close up view of typical Day 8 microwell cocultures (Yellow: T cells, Green: live tumor, Grey: dead tumor) . (c) Calculation of T cell proliferation and killing from enumeration of (T cells and tumor cells) in each microwell on day 0, day 4 and day 8

After printing, 2mL of pre-warmed 37°C PBS was added to the dish and incubated at 37°C for 5 minutes to wash. This washing step was repeated three times, and then complete medium was added for an overnight incubation at 37°C.

Cell Culture:
All work is done in a biosafety cabinet and sterile techniques are followed to maintain sterility throughout the experiment.

Car-T library cells (CAR-T) are thawed the day before setting up the microwell coculture. CAR-T containing 90% complete medium and 10% DMSO in 1mL was thawed from liquid nitrogen in a 37°C water bath and was then added dropwise to 9mL of pre-warmed 37°C complete medium.

The tube containing T cells was centrifuged at 400xg for five minutes to pellet the cells. Supernatant was removed and pelleted cells were resuspended in 2mL of complete medium to count. T cells is maintained at $1x10^6$ cells/mL in complete medium with 100U/mL of IL-2.

Tumor cell is thawed at least a week in advance to increase viability and cell number before setting up the microwell coculture. The thawing process for CAR-T above is followed for tumor. These cells are maintained at $3x10^5$ – $8x10^5$ cells/mL in complete medium.

Time averaged Tumor cell killing and T cell proliferation.
Microwell Coculture Setup:
The viability of both CAR-T and tumor cells-GFP should be at least >70% before setting up the microwell coculture. Having a higher cell viability will yield the more accurate results.

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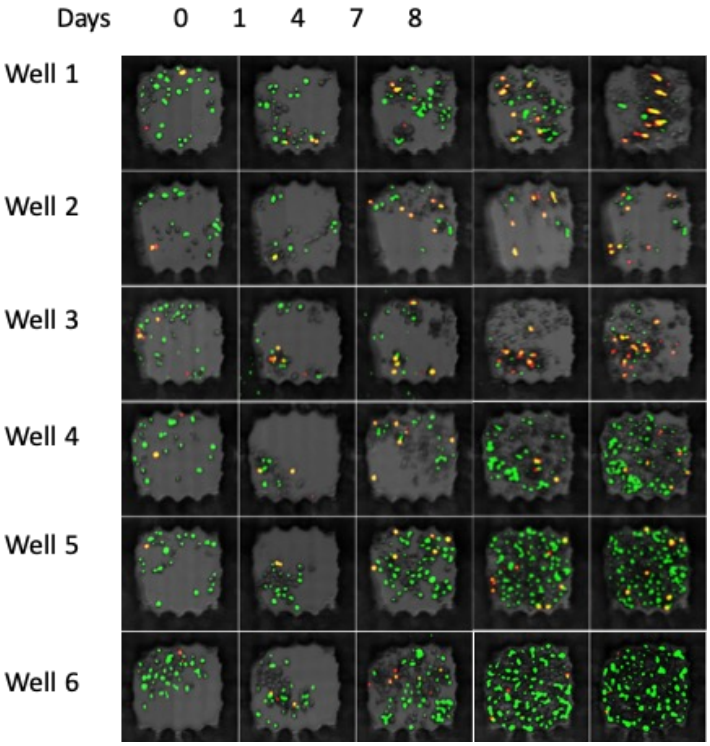

Figure 4. Less ambiguity using longer time culture, T cells (red and yellow), live tumor (green), dead tumor (grey), each represent one unique well. Each column represents the data collected on the same day.

CAR-T and tumor cells cells are combined at a 1:10 ratio in a microcentrifuge tube at a total volume of 200uL. The cell number plated follows the current most optimal for microwell coculturing, which is CAR-T and tumor cells at 10,000 and 100,000 cells respectively. Once the cell mixture is prepared, the 35mm dish containing the 20mm microwell is removed of all medium. The cell mixture is then added dropwise throughout the 20mm microwell and incubated for 15 minutes at 37°C to allow the cells time to settle into the microwells. After this incubation, 1.5mL of complete medium is added to the dish and it is incubated at 37°C. (Fig.2)

Cell Staining in Microwell:
Any stain used for a microwell is prepared in a total of 200uL. The stain used for this coculture is 1uL of both CD4 and CD8 monoclonal antibody (PE-Cyanine5.5) mixed with 198uL of complete medium. The CD4 and CD8 antibodies bind to the CAR-T cells so they fluoresce red when imaging. The 200uL prepared stain is added dropwise to the microwell and incubated at 37°C for 60 minutes. After this incubation, 2mL of complete medium is added and removed from the 35mm dish to wash the microwell.
Microwell Montage:

The microwell is montaged daily to over an eight-day period. To prepare the microwell for a montage, the microwell was first stained as described above. Then medium was removed from the sides of the 35mm dish without removing the media covering the microwell. The TROVO was then setup for a montage and the dish was placed into the machine.

When the montage is finished, the dish is removed from the TROVO and 1.5mL of complete medium is added. The dish is incubated until the following day for another montage. Long term time averaged coculture results were obtained and quantified to produce distinctive microwell phenotypes, where robust increase of T cells and reduction of tumors can be observed and quantified (Fig. 3). Typical microwell images were followed daily to remove ambiguity (Fig. 4), It is evident that up to day 4, the proliferation of T cells (red and yellow) and the growth of tumor cells (GFP green) is still ambiguous. Starting from day 7, the fate of each coculture became clear: In well 1,2,3, T cells kills almost all tumor cells and proliferated. In well 4,5,6, tumor cells take over and dominated, indicating a poor killing capacity of the enclosed T cells.

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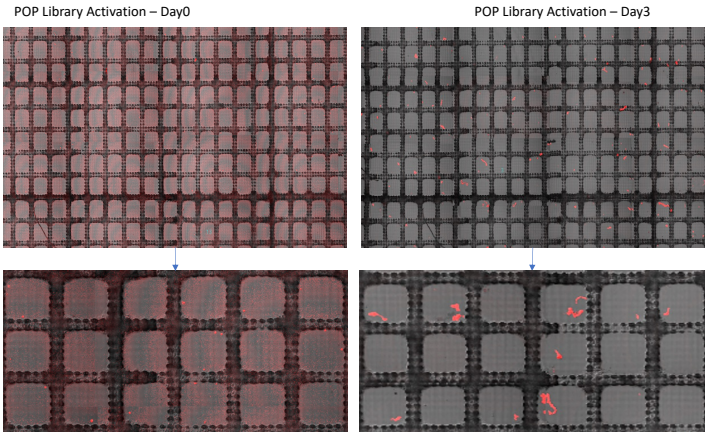

Figure 5. Generation of monoclonal T cell groups (mono-T) in each microwell, day0 and day 3 images were captured and T cells were stained with CD 4 and CD8 antibody-red.

Monoclonal T cell (monoT) group generation in microwell.

CAR-T and tumor cells cells are combined at a 1:10 ratio in a microcentrifuge tube at a total volume of 200uL. The cell number plated follows the current most optimal for microwell coculturing, which is CAR-T and tumor cells at 10,000 and 100,000 cells respectively. Once the cell mixture is prepared, the 35mm dish containing the 20mm microwell is removed of all medium. The cell mixture is then added dropwise throughout the 20mm microwell and incubated for 15 minutes at 37°C to allow the cells time to settle into the microwells.

After this incubation, 1.5mL of complete medium is added to the dish and it is incubated at 37°C. human CD3/CD28 T-cell activator (recommended 25uL/mL from protocol) and IL-2 are added to the culture. the coculture will be Incubated at 37°C for 3 days (recommended maximum incubation time for CD3/CD28 from protocol). Microwell was montages on Day0, Day2, and Day3. On day3, activation medium was removed and 100K tumor cells was added.

Dish is now montaged daily, and incubated in complete medium at 37°C. Monoclonal clusters of T cells were established from single T cell clones.(Fig.5), the killing and proliferation of the monoclonal T cell groups will be more robust by averaging out single cell noises.

Microwell Capture:

The capture gel used follows the current most optimal gel for capturing in microwells. The capture gel mixture is prepared and added to the 35mm dish containing the 20mm microwell. Then 100uL of capture gel is added dropwise to the microwell, and allowed to settle for one minute. After, the 100uL of capture gel is removed and the remaining 200uL of capture gel is added dropwise to the microwell. The purpose of adding and removing 100uL of capture gel is to ensure that the mixture is not diluted by any remaining medium.

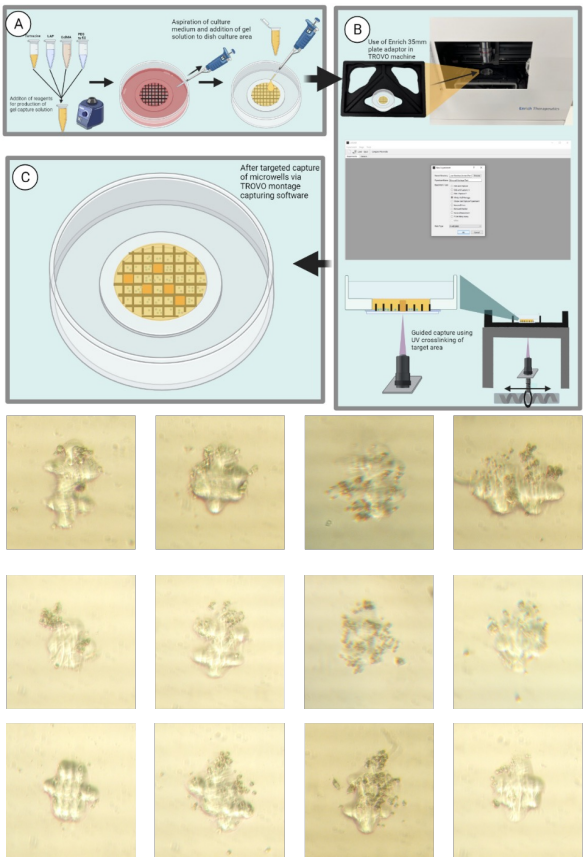

Figure 6. Cell retrieval process

The plate is ready for capturing and is placed into the TROVO to begin a montage with the same parameter as described in the microwell montage section. When complete, all montages are uploaded to the TROVO T-cell Killing Assay program, which processes the data to determine and plot the growth kinetics based on fluorescence for each microwell. For this experiment, the microwells of interest chosen from the growth kinetics plot were increasing T-cells (red) and decreasing cancer cells (green), which would be indicative of T-cell killing and proliferation. Once selected, a final check is done on the selected microwells before they are captured. The capture parameters used for this experiment are 10 seconds exposure, 4.0 step, and 3 pixels. When complete, the plate is removed from the TROVO to begin analysis.

Microwell Capture Analysis:
After capturing, PBS was added to the dish and incubated at 37°C for 5 minutes to wash. This PBS is then removed. Microwell will be peeled off from plate. The PBS is removed and replaced with 2mL of Hanks Enzyme Free Cell Dissociation Solution. After adding the Hanks solution, the plate is vigorously swirled for 30 seconds to remove any uncaptured cells. This is removed from the plate and the Hanks solution wash step is repeated two more times. After the final wash, 2mL of PBS is added for imaging.

At this point, the microwell and uncaptured cells have been removed from the dish and all that is remaining is the capture gels containing the cells of interest. Images of the capture gels are taken using a microscope camera before moving on to the recovery step.

Cell Recovery:
To begin recovery, 1uL of 10^4U/mL collagenase is added to a microcentrifuge tube containing 1mL of complete medium for a final concentration of 10U/mL collagenase. This is mixed and 200uL is removed and placed into a new microcentrifuge tube. All PBS is removed from the dish and capture gels should be visible on the plate when held to light. Using forceps, a tiny piece of alginate dressing is removed from its packaging, and is then dragged along the surface of the glass to lift the capture gels containing the cells into the alginate dressing. This is placed into the 200uL mixture of complete medium and collagenase. The microcentrifuge tube is incubated at 37°C for 20 minutes to dissolve the capture gel and free the cells. After this incubation, EDTA is added to the microcentrifuge tube at a final concentration of 30mM. This is incubated at 37°C for 30 minutes with vortexting every five minutes for a few seconds at low speed to dissolve the alginate dressing.

At this point, the capture gel and alginate dressing have been dissolved and all that remains is the cells of interest. PBS is added at 1mL and the tube is centrifuged at 1500RPM for five minutes to wash the cells. The supernatant is removed and the pellet is resuspended with 1mL of PBS. The centrifugation step is repeated one more time to wash the cells. After removing the supernatant for the second time, the pelleted cells are placed on ice for PCR, or further cell culture.

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