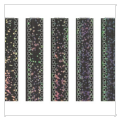

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Image-based 3D cell culture cytotoxicity assay

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ABSTRACT

This protocol describes an imaged-based 3-dimensional (3D) cell culture cytotoxicity assay using multicellular tumor spheroids (in this case MC38 cells) sensitized with hgp100 peptide and killing them with pmel-1 T cells or the apoptosis-inducing antibiotic, Staurosporine. Our goal was to explore an *in vitro* 3D cell culture system to study T cell-mediated cytotoxicity as a potential way to more rapidly and relevantly test the effect of T cell manipulation on T cell cytotoxicity before moving to the mouse model.

In this setup, the T cells are required to migrate towards the tumor spheroids that are already suspended in a gel matrix, there is minimal manipulation of the samples post-coculture because the samples are imaged directly in the chips to measure cell death, and there is somewhat higher throughput than confocal imaging provides (more spheroids being imaged per sample via widefield microscopy).

We used 3D cell culture chips from AIM Biotech (Singapore) and adapted protocols published by:

- AIM Biotech: https://www.aimbiotech.com/general-protocols.html
- Pavesi, Andrea, Anthony T. Tan, Sarene Koh, Adeline Chia, Marta Colombo, Emanuele Antonecchia, Carlo Miccolis, et al. 2017. “A 3D Microfluidic Model for Preclinical Evaluation of TCR-Engineered T Cells against Solid Tumors.” *JCI Insight* 2 (12). https://doi.org/10.1172/jci.insight.89762.
- Jenkins, Russell W., Amir R. Aref, Patrick H. Lizotte, Elena Ivanova, Susanna Stinson, Chensheng W. Zhou, Michaela Bowden, et al. 2018. “Ex Vivo Profiling of PD-1 Blockade Using Organotypic Tumor Spheroids.” *Cancer Discovery* 8 (2): 196–215.

For image analysis, refer to:

- Czech, Eric, Bulent Arman Aksoy, Pinar Aksoy, and Jeff Hammerbacher. 2019. “Cytokit: A Single-Cell Analysis Toolkit for High Dimensional Fluorescent Microscopy Imaging.” BMC Bioinformatics 20 (1): 448.

GUIDELINES

We worked from protocols described by others and we highly recommend reading the following:

- AIM Biotech protocols on gel preparation and working with their 3D cell culture chips: https://www.aimbiotech.com/general-protocols.html
- Pavesi, Andrea, Anthony T. Tan, Sarene Koh, Adeline Chia, Marta Colombo, Emanuele Antonecchia, Carlo Miccolis, et al. 2017. “A 3D Microfluidic Model for Preclinical Evaluation of TCR-Engineered T Cells against Solid Tumors.” *JCI Insight* 2 (12). https://doi.org/10.1172/jci.insight.89762.
- Jenkins, Russell W., Amir R. Aref, Patrick H. Lizotte, Elena Ivanova, Susanna Stinson, Chensheng W. Zhou, Michaela Bowden, et al. 2018. “Ex Vivo Profiling of PD-1 Blockade Using Organotypic Tumor Spheroids.” *Cancer Discovery* 8 (2): 196–215.

A fluorescence microscope with automation for multipoint imaging is required for this assay. We used the Keyence BZ-X710 system with a 20X objective, DAPI and Cy5 filters and brightfield, and a 20 µm step-size.

MATERIALS

NAME	CATALOG #	VENDOR
Staurosporine	S5921	Sigma Aldrich
Sterile water		
Ice & ice bucket		
HyClone™ Dulbeccos High Glucose Modified Eagles Medium	SH30022FS	Fisher Scientific
Trypsin 0.05% 1X Solution	16777-202	VWR Scientific
Conical Tubes (50 mL) (racked)	AM12501	Thermo Fisher
Collagen I Rat Tail	354236	Corning
CytoOne 10 cm TC dish	CC7682-3394	USA Scientific
100 µm cell strainer	352360	Fisher Scientific
40 µm cell strainer	22-363-547	Fisher Scientific
3D Cell Culture Chip	DAX-1	AIM Biotech
10X PBS pH 7.4	70011044	Thermo Fisher Scientific
Phenol Red Sodium Salt	P4758	Sigma Aldrich
0.5 M NaOH	View	Sigma Aldrich
NucBlue™ Live ReadyProbes™ Reagent	R37605	Thermo Fisher Scientific
SYTOX™ Red Dead Cell Stain for 633 or 635 nm excitation	S34859	Thermo Fisher Scientific
Recombinant Murine I-TAC (CXCL11)	250-29	peprotech
Interleukin-2 human	I2644	Sigma Aldrich

BEFORE STARTING

1. If conducting a T cell-mediated cytotoxicity assay, decide which day post-activation the T cells will be co-cultured with the tumor spheroids and determine how many T cells will be needed. Accordingly, activate an appropriate number of T cells however many days ahead of time is needed.
2. Expand MC38 cells in culture. The number of 3D cell culture chips that will be used for the experiment will determine how many MC38 cells will be needed when plating the cells for spheroid formation. Per 10 cm dish, 2 million cells are needed. For a good density of spheroids in the collagen gel you will need 50 ul of gel per 10 cm dish and 30 ul gel per 3D culture chip. Because the gel is viscous and some gets lost when mixing, make twice as much as you think you need.
3. Reconstitute the CXCL11 as per the manufacturer's directions.
4. On the day of co-culture:

- Collect reagents in an ice bucket with ice that fits into the hood for preparing collagen mixture (Collagen type I, 10X PBS with phenol red, 0.5 M NaOH, sterile water).
- Calculate how much collagen gel will be needed. The amounts listed in the protocol are for 1 ml collagen gel mixture. We were usually harvesting 10 x 10 cm tissue cultures dishes of spheroids and resuspending the spheroid pellet in 500 ul of gel to get a good density of spheroids.

1 If using T cells to kill the tumor spheroids, start culturing them and activate them.
Maintain the cells, supplementing the culture media and splitting when necessary, until the desired day of co-culture.

2 The day before co-culture.

Prepare MC38 cells to form spheroids:

- Make a single cell suspension of MC38 cells at 2.5 x 10^6 cells per ml (in DMEM, 10% FBS, and 1% pen/strep)
- With a multichannel pipettor, pipet 5 rows of 20 ul droplets of cell suspension on the inner surface of a 10 cm tissue culture dish lid (40 droplets per dish)
- Pour 5-10 ml of sterile water in the bottom half of the dish (to humidify the chamber and stop the droplets from drying out)
- Carefully invert the lid with the droplets over the bottom half of the dish (try not to bump the lid while picking it up and turning it over or the droplets with flatten out and the spheroids will not grow uniformly)
- Incubate the droplets overnight **37 °C** **5% CO2** .

3 Day of co-culture.

Harvest the droplets containing MC38 spheroids.

- Working in the hood, flip the lid upside down to expose the droplets
- (at this point, an empty dish bottom can be placed on top and the droplets can be checked for spheroid formation on an inverted microscope
- Harvest the spheroids using a p1000 and 2 ml DMEM per dish lid and transfer into a 50 ml tube
- Pass the spheroid suspension through a 40 µm nylon mesh filter
- Invert the 40 µm filter over a new 50 ml tube and wash the retained spheroids into the tube
- Pass the spheroid suspension through a 100 µm filter over a new 50 ml tube (collect the flow through)
- Centrifuge the spheroid suspension (now containing spheroids between 40 and 100 µm) at **200 x g** **5 minutes** .

Make the collagen gel while spheroid suspension is centrifuging:

- Perform all steps on ice
- Recipe for 1 ml collagen gel:

10x PBS + phenol red	95 ul
Collagen (3.9 mg/ml)	641 ul
0.5M NaOH	26 ul (this may change)
dH2O	157 ul
CXCL11 (stock at 0.1 mg/ml)	27 ul
IL-2	4 ul
Spheroid pellet	about 50 ul

- Mix the above components in a sterile eppendorf tube on ice in the hood. Mix by pipetting up and down and avoid adding air bubbles to the mixture. Mix until the color from the phenol red is uniform. To check the pH, make sure the color is red (not yellowish or purplish). If the mix is slightly yellow, add 0.5 M NaOH in 1 ul increments, mixing well in between, until the color is red. If the mixture goes too far into the purple, it's easier to start over.

Seed the 3D cell culture chips.

- Each 3D cell culture chip has 3 channels. We used 1 chip per condition, which resulted in 3 replicates per condition.
- Carefully aspirate the DMEM from the spheroid pellet. Try to aspirate as much liquid as possible.
- Resuspend the spheroid pellet with collagen mix. Test the density of spheroids by pipetting a 10 ul droplet onto a tissue culture dish and checking on the microscope. Add more gel mix if the spheroids look too dense. We have found that 50 ul gel mixture per 10 cm dish worth of spheroids works well.
- Pipette 10 ul into the central channels in the 3D cell culture chips. Pipette very slowly so that the gel doesn't escape into the side channels. Pipet into the top port until the gel reaches half way and then pipette into the bottom port until the gel fills the central channel.
- If using the chip holders from AIM Biotech, add sterile water to the chambers to humidify the chips while in the holder.
- Incubate the seeded chips at **37 °C** **5% CO2** for **00:30:00** to allow the gel to polymerize.

4 Prepare the T cells or drug treatment dilutions while the gel is polymerizing.

For the T cells:
- Count and harvest the desired number of T cells.
- The T cells can be stained with a marker at this point, if desired. We have used IncuCyte Rapid live cell labeling with success.
- Centrifuge the T cells **350 x g 5 mins**
- Resuspend the T cells in T cell media supplemented with IL-2 (200 IU/ml) to obtain the desired concentration (we were using T cells at various concentrations so serially diluted them from 4 million as our highest concentration).

For the Staurosporine treatment:
- Dilute Staurosporine stock to desired concentrations in DMEM (supplemented with 10% FBS and 1% pen/strep).

5 Add the T cells or drug treatment to the 3D cell culture chips.

- Remove the chips from the incubator and place in the tissue culture hood

For the co-culture with T cells:
- Carefully and slowly pipette 20 ul of T cell media supplemented with IL-2 (200 IU/ml) and with or without hgp100 peptide (2 µM) into the top port of the right side channel for each channel in the chip (we had conditions without the peptide as a negative control for our assays). Add 50 ul of the same media to the top and bottom ports of the same side channel.
- To the left side channel, carefully and slowly pipet 20 ul of the T cell suspension in. Make sure to mix the T cell suspension before each addition (the T cells tend to settle quite quickly). Do not top off the ports on this side channel because it can displace the T cells.

For the Staurosporine-treated samples:
- Carefully and slowly pipet 20 ul of the Staurosporine diluted in DMEM into the left and right side channels in each chip. Top off the 4 side channel ports with 50 ul of the same media.

Replace all chips in the incubator in their humidified chip holders for overnight incubation.

6 Day of imaging.

Make staining solution:
- 5 ml PBS
- 20 drops of NucBlue
- 18 ul of SYTOX red dead cell stain

Stain one chip at a time:
- Remove any media in troughs around the side channel ports (don't try and suck media directly out of the port or you run the risk of pulling the gel out)
- Gently pipette 20 ul of staining buffer directly into the top port of each of the side channels and watch it push the T cells or media out the bottom port of the channel
- Removed any media from bottom port troughs
- Gently pipette 50 ul of stain into the top port of each side channel until it comes out the bottom port
- Incubate the chip for 30 mins at room temperature, protected from light

Image:
- Set exposure times for each channel based on the positive control chip (in our case this was the one containing the Staurosporine-treated spheroids) and use the same settings for all other samples:
- CH1=Cy5 (SYTOX red for any dead cells). We found that the SYTOX dye tended to photobleach if it was imaged after the DAPI channel so we imaged it first.
- CH2=DAPI
- CH3=brightfield
- Set a 3x11 grid for each channel (and set multipoint imaging for the 3 channels on each chip)
- We used the high resolution setting (but this may not be necessary)
- With these settings, it took about 30 mins to image each chip

Once the imaging has started for one chip, starting staining the next chip to keep the staining time consistent across conditions.

7 For image analysis, refer to the Cytokit repository (https://github.com/hammerlab/cytokit). Methods detailed in:

Czech, Eric, Bulent Arman Aksoy, Pinar Aksoy, and Jeff Hammerbacher. 2019. “Cytokit: A Single-Cell Analysis Toolkit for High Dimensional Fluorescent Microscopy Imaging.” *BMC Bioinformatics* 20 (1): 448.