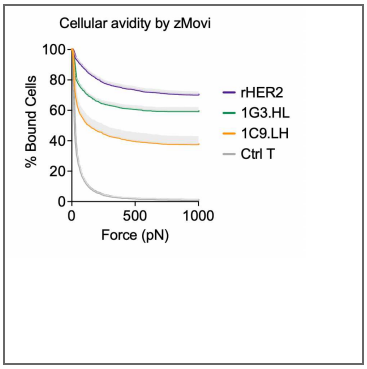

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Protocol status: Working
We use this protocol and it's working

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Cell Avidity analysis of murine CD8+ CAR T cells via z-Movi

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ABSTRACT

z-Movi is a cell based assay used to assess the avidity of specific immune cells against their targets. Perform this protocol at the end of generating CAR-T cells. For more information see "Retroviral transduction of primary murine CD8 T cells".

Target cell adhesion efficiency may vary between cell lines. Optimal chip-cell adhesion may be produced with adhesion media besides ConA, such as 0.01% Poly-L-Lysine. A cell-line specific protocol may be needed

This protocol was completed using a variant of B16-F10. "B16-F10 is a cell line exhibiting a morphology of spindle-shaped and epithelial-like cells that was isolated from skin tissue of a mouse with melanoma". We transduced B16-F10 with plasmid pTS194 (see attached ApE file) (*BF194*) to ectopically express murine HER2 (mHER2) with puromycin resistance driven by a strong lentiviral promoter. Successfully transduced cells were selected for via screening for drug resistance, and confirmed via flow cytometry.

For information on publications that used zMovi, please visit the link below:
https://lumicks.com/products/z-movi-cell-avidity-analyzer/

ATTACHMENTS

pTS194 pHIV7_SFFV_mHER2t_PuroR.ape

z-Movi - Chip cleaning V6.pdf

Cell Avidity analysis of murine CD8+ CAR T cells REAGENT AND EQUIPMENT LIST.xlsx

MATERIALS

See attached materials spreadsheet

Last Modified: Sep 21, 2023

PROTOCOL integer ID: 86833

Keywords: zMovi, Cellular avidity, CAR, murine CD8+, CAR T cell, Cell passage, Lumicks

BEFORE START INSTRUCTIONS

Prepare reagents listed below.

- 1 L **cDMEM** [1000 mL DMEM (1x), 100mL FBS (heat inactivated), 25 mL HEPES (1M), 10mL L-Glutamine (200 mM), 10 mL Pen/Strep. Filter the mix through 500 mL Bottle top Filter (0.2 µm aPES membrane)].

- 1 L **mTCM** [1000 mL RPMI1640 (w/ 20 mM HEPES) (1x), 100 mL FBS (heat inactivated), 10 mL Sodium Pyruvate (100 mM), 1 mL HEPES (1 M), 10 mL Pen/Strep, 100 uL 2-Mercaptoethanol (0.5 M). Filter the mix through 500 mL Bottle top Filter (0.2um aPES membrane)].

- 5 mL **Concanavalin A type IV-S (ConA)** [Reconstitute 5 mg vial of ConA lyophilized powder in 5 mL 1x D-PBS. Invert capped vial several times and incubate for several minutes at room temperature to ensure dissolution].

Day -2: Culture target cells (BF194)

1 Using Trypsin-EDTA (0.05%), passage B16-F10 transduced with pTS194 (BF194) cell culture at a ratio of 1:4 to prepare for experiment day.

Note

- We use a B16-F10 cell line that has been transduced to ectopically express murine HER2 with puromycin resistance driven by a strong lentiviral promoter. Successful transduction and selection is graded by flow cytometry. Please see description and attached files.

- A detailed protocol for dissociation of adherent cells can be found here. Dissociated cells can be enumerated using a hemocytometer (protocol link) or an automated cell-counter (e.g. Cellometer X2).

cDMEM recipe:
DMEM (gibco)
10% heat-inactivated FBS
100 U/mL penicillin/streptomycin
2mM L-Glutamine
25mM HEPES
Filter through 0.2um before use.

mTCM recipe:
RPMI 1640 (+HEPES)
10% heat-inactivated FBS
1 mM HEPES
100 U/mL penicillin/streptomycin
1 mM sodium pyruvate
50 µM b-mercaptoethanol
50 U/mL human IL-2 (Peprotech)
Filter through 0.2um before use.

1.1 Immediately following passaging, place target cell culture in T-150 back into incubator (37 °C, 5% CO2, 95% humidity).

Note

For optimal viability of target cells, passage adherent target cell culture to obtain 70-80% confluency on the day of in-chip seeding.

B16-F10 is an immortalized cell line that, if seeded at $3x10^6$ in a T-150 cell culture flask, will grow to be confluent in 5 days. If choosing to use a target cell line that exhibits different growth patterns, observe its rate of growth and plan accordingly to obtain 70-80% confluency on the day of experiment.

Day -1: Functionalization of clean, dry z-Movi chip

2 **In order to obtain quality data, it's a good idea to run 5 replicates of each effector construct. Plan chip use accordingly.**

3 Functionalize a clean, dry chip with 100 µL Concanavalin A (1 mg/mL) (ConA) for 16-18 hours.

3.1 Remove a clean, dry z-Movi chip from 37 °C dry incubator. Acquire z-Movi chip reservoir screw cap and set aside. Before screwing 5 mL luer-lock syringe on, pull back plunger to the 2.5 mL mark. Screw syringe onto z-Movi chip so that the measurement marks are facing upwards.

Note

It is critical that the z-Movi chip is kept at 37 °C before the experiment and all reagents added to it are pre-warmed before addition to the chip. **If bubbles nucleate in the chip observation chamber because of reagent warming, it will ruin your experiment.**

3.2 Using a P-200 micropipette, reverse-pipette 100 µL of pre-warmed 1x D-PBS (PBS) into the chip reservoir. Pipette up and down vigorously several times to dislodge any bubbles that have formed at the bottom of the reservoir during addition.

It is critical that no bubbles are present in the chip reservoir during the reagent addition steps.

Note

If bubbles get trapped in the flow cell during the addition of PBS or ConA, remove them by creating positive pressure: depress the syringe plunger until you notice that the bubble is dislodged and has floated to the top of the reservoir.

3.3 While chip valve is still closed, pull back chip plunger to 3 mL mark to create negative pressure. Fully pen the chip valve and allow PBS to flow into chip (1/2). When volume in the reservoir has reduced to approximately 10 µL (~3 mm in column height), quickly close chip valve.

Pipette an additional 100 µL pre-warmed PBS into the chip reservoir, and open the valve while maintaining negative pressure in chip flow cell.

3.4 Using a P-200 micropipette, add 100 µL ConA solution to the chip reservoir. Pull back syringe plunger back to the 3 mL mark, open valve, and draw liquid into the flow cell. Close the valve when there is a small layer of liquid left in the chip reservoir.

Ensure there are no bubbles present in the flow cell and immediately proceed to next sub-step.

3.5 While the flow cell valve is closed, rinse chip reservoir with 200 µL PBS (1/3)
Rinse chip reservoir with 200 µL PBS (2/3)
Rinse chip reservoir with 200 µL PBS (3/3) and remove liquid, leaving a small layer in the reservoir.
Pipette 200 µL PBS into the reservoir. Securely fasten z-Movi chip reservoir cap to z-Movi chip. Un-screw the luer-lock syringe from the system and place the ConA-functionalized chip into 37 °C dry incubator overnight (14-18 hours).

Day 0: Target cell seeding and assessing monolayer

4 Preparation of functionalized z-Movi chip for BF194 cell seeding

Note

Make sure you have obtained the following reagents before proceeding.
- PBS
- cDMEM
- Serum-free DMEM
- mTCM

Place reagents in the incubator for 00:15:00 **to ensure they have come to temperature.**

4.1 Following overnight (14-18 hour) incubation, remove the chip reservoir cap and screw the 5 mL luer lock syringe (plunger set to 2.5 mL mark) onto z-Movi chip. Use a P-200 micropipette to discard the PBS left in the reservoir, leaving a small volume.

Replace discarded volume with 400 µL fresh, pre-warmed PBS. Pipette up and down in reservoir to dislodge any bubbles that may have formed.

Pull the syringe plunger back to the 3 mL mark, and open the valve to create negative pressure. Gently pull through 400 µL warm PBS (1/2). Close the valve. Pull through an additional 400 µL warm PBS (2/2) and close the valve.

4.2 Creating negative pressure with the syringe plunger, open chip valve and pull 100 µL warm serum-free DMEM through the chip flow cell (1/2). close valve.
Pull an additional 100 µL warm serum free DMEM through the flow cell (2/2), leaving a small amount of liquid in the chip reservoir, and close the chip valve.

Screw on the reservoir cap and place the cell-ready functionalized z-Movi chip into 37 °C dry incubator.

Note

Following the second run of DMEM into the flow cell, the z-Movi chip is ready for target cell seeding. Step 4 may be performed before step 3, *if* step 4 elapsed time is kept to a minimum and cells are kept in an incubator in order to preserve adherent target cell viability.

5 Dissociate and prepare target cells for seeding.

5.1 Dissociate target cells cultured in T-150 with 5 mL Tyrpsin-EDTA (0.05%). Pipet dissociated cells into a sterile 15 mL conical. Quench Trypsin-EDTA reaction by pipetting 6 mL cDMEM into cell culture flask to rinse, then pipet into the 15 mL conical containing cell suspension. Homogenize cell suspension by pipetting up and down several times. 10m

Pipet the homogenous cell suspension through 40 µm cell strainer into a new sterile 15 mL conical.

Centrifuge cells 400 rcf, 00:05:00. With a pipet, resuspend cell pellet in 10 mL PBS.

Centrifuge cells 400 rcf, 00:05:00. With a pipet, resuspend cell pellet in 10 mL cDMEM.

Count cells. Take note of viability. Spin down, re-suspend BF194 cells at $1x10^8$ cells/ml in cDMEM.
Because of the very high concentration of cells in suspension, move quickly to ensure cellular viability is not compromised.

Note

An intermediate wash step applied to the dissociated target cells is vital to dilute out all left-over Trypsin-EDTA.

Cell population viability should be greater than 90% if the culture was passaged on Day -2. Passaging cells two days before experiment is integral to maintaining high viability and ideal cell cycle, which will translate to optimal target cell adherence.

e.g. $(5x10^6$ cells/mL)(10 mL) = $(1x10^8$ cells/mL)(x mL)
⇒ x=0.5 mL seeding volume

6 Seed target cells onto z-Movi chip flow cell

6.1 Retrieve cell-ready functionalized z-Movi chip from 37 °C dry incubator and place onto z-Movi chip pedestal. Using a small flathead screwdriver, tightly screw in the four corner screws equally to stabilize the chip during use. Remove the reservoir cap and set aside.

While attached to pedestal, screw on the 5 mL luer lock syringe to chip (set to 4.4 mL mark). Pull syringe plunger back to the 4.6 mL mark to create negative pressure.

Note

During and following the addition of adherent target cells to the z-Movi chip, it is very critical to keep the sheer forces applied in the flow cell kept to a minimum. Once syringe plunger is pulled back to the 4.6 mL mark, it should stay there for the remainder of the chips use without requiring being held back.

6.2 ***Before adding target cells, ensure cell suspension is homogeneous and there are no bubbles present in the suspension or chip reservoir.***

Add 20 µL target cell suspension to the bottom of the chip reservoir. With your hand on the chip valve, close the z-Movi pedestal lid mostly. Closing the lid to shade the pedestal gives a better visualization of the cells moving through. While watching the camera view, open the valve and count two seconds. Close the chip valve and immediately proceed with next step

Note

During the ~30 seconds taken to rinse the reservoir, target cells in the chip flow cell will begin to settle. Use this opportunity to determine if the monolayer will be sufficiently confluent.

6.3 Once valve is closed, Use a pipette to rinse chip reservoir out with 100 µL cDMEM (1/3).
Rinse chip reservoir out with 100 µL cDMEM (2/3).
Rinse chip reservoir out with 100 µL cDMEM (3/3).

Leave 100 µL of cDMEM in chip reservoir. Follow prompts on Lumicks Oceon software to center. Once the monolayer begins to form; replace reservoir cap, unscrew z-Movi chip from pedestal, and unscrew luer lock syringe. Place target-cell-seeded z-Movi chip in 37 °C dry incubator. Incubate for 2-4 hours.

Note

30 minutes before completing incubation, prepare the effector cells.

6.4 Following 2-4 hour incubation, inspect monolayer development on z-Movi pedestal again. Using a P200, pipette up and down several times to ensure there are no bubbles present in reservoir.

Replace the luer-lock syringe on z-Movi chip, and pull through fresh cDMEM in to the flow cell.

Day 0: Preparation of effector cells

7 Prepare 2x10^5 effector cells per run. Enumerate and collect effector cells.

Note

# of runs	# of cells
1	200,000
5	1,000,000
10	2,000,000
15	3,000,000

7.1 Pipette 20 µL of DMSO to a vial of CellTrace Far Red stain to prepare a 1000X solution.

In a sterile 15 mL conical, make a 1X dye solution by adding 1 µL reconstituted CellTrace Far Red to 1 mL of PBS.

Note

Prepare 1 mL of 1X dye solution *per sample condition*.

7.2 Pipet cells to a sterile 15 mL conical for staining. Centrifuge cells 400 rpm, 00:05:00 , discard supernatant. Using a P1000 micropipette, transfer 1 mL of 1X dye solution to the conical. Pipette up and down vigorously several times to dislodge and homogenize the cell pellet. 20m

Incubate cells in 1X dye for 00:15:00 @ Room temperature in the dark. .

7.3 Following 15 minute incubation, quench the dye reaction by adding 5 mL mTCM to the 15 mL conical. 5m

Centrifuge stained cells 400 rpm, 00:05:00. Re-suspend tracked cells at a concentration of 5x10^6 cells/mL.

Day 0: Avidity analysis

8 Follow prompts on Lumicks Oceon software to create an analysis protocol

If choosing to do avidity analysis replicate runs on several different chips, randomize the order of analysis to mitigate the influence of order and target antigen availability dependent results.

Note

It is vital that sheer force applied to adherent target cells be kept to a minimum. Be aware that once you start you will not need to change the plunger depth on the syringe at all.

8.1 Proceed by placing functionalized z-Movi chip with prepared monolayer on the z-Movi pedestal. Securely screw chip down.

Once chip is securely fastened and protocol is open, confirm and center microscope camera on the glass flow chamber. Test monolayer adherence by following the on screen prompts. Continue only if you notice that most of the monolayer does not become dislodged. Some cells may peel off, this is okay. Be sure to flow fresh complete media into the flow chamber to clear dislodged cells.

Note

Each functionalized z-Movi chip with a prepared monolayer can sufficiently accommodate up to 6 avidity analysis runs.

8.2 Once monolayer adherence is validated, continue to tracked effector cell addition.

Move quickly once cells are added to reservoir. As elapsed time increases before flowing cells into flow chamber, the more cells will sediment in the chip reservoir.

For each cell avidity analysis run, add 20 µL of tracked effector cells (5 x 10^6/mL) to the chip

reservoir. Open chip valve, wait 2 seconds, close valve, and check tracked cells on screen. Effector cells within the tracked area of the flow chamber should total between 200-500 for an efficient run.

8.3 Immediately after flowing cells into flow chamber and validating efficient numbers of cells, click "Start incubation" prompt.

Analysis will start automatically within the time specified on your protocol. Be sure to rinse reservoir 3 times with complete media before the incubation timer reaches the 1:30 mark. Once the timer reaches the 1:30 mark, make sure the z-Movi pedestal lid is closed.

8.4 Once acquisition is complete, flush fresh cDMEM into flow cell. It is likely not all cells will become detached and flow out. *Do not try to force them to detach as you will compromise the monolayer integrity.* Continue with up to 6 runs per chip.

Day 0: Cleaning z-Movi chips

2h

9 Immediately following completion of avidity analysis protocol, proceed to cleaning protocol which includes the steps outlined below. More information may be found in the Lumicks manual z-Movi chip cleaning protocol attached in description.

Insufficient cleaning may result in future adherence problems, and may impact the ability to run acoustic force ramps.

Note

Plan to clean chips immediately after protocol completion. **Cleaning may take up to 3 hours depending on number of utilized z-Movi chips.**

9.1 **During chip cleaning, place syringe on used z-Movi chip with plunger between 1-2 mL mark to maintain high negative pressure and increase sheer force acting on spent monolayer**.
Pull 400 µL DI water into the flow cell (1/2).
Pull 400 µL DI water into the flow cell (2/2).
Leave a small amount of water in the chip reservoir. Remove luer lock syringe, discard liquid volume in syringe into a sufficient biohazard container. Screw luer lock syringe back onto z-Movi chip.

9.2 Pull 400 µL 10% bleach solution into the flow cell (1/3). Pull completely through, then using the syringe plunger, push back and forth vigorously several times to create bubbles within the flow cell.

30m

Note

Bubbles within the flow cell mechanically scrub off the monolayer that was formed during adherence steps. Create as many bubbles as possible during scrubbing steps, as it will clean chips more efficiently.

Pull 400 µL 10% bleach solution into the flow cell (2/3) and 'scrub' vigorously.
Pull 400 µL 10% bleach solution into the flow cell (3/3), *making sure there are no bubbles present in the flow cell at this step*. Discard flow through held in syringe. With chip valve closed, rinse chip reservoir with 400 µL DI water twice. Incubate 00:30:00 at room temperature.

9.3 Immediately after 30 minutes has finished, completely pull through bleach solution. 30m
Pull 400 µL 10% bleach solution into the flow cell and scrub vigorously once.
Pull 400 µL 10% bleach solution into the flow cell, *making sure there are no bubbles present in the flow cell at this step*. with the chip valve closed, rinse chip reservoir twice with 400 µL DI water. Incubate 00:30:00 at room temperature.

Note

After second 30 minute incubation, you may check for cellular debris on z-Movi pedestal camera. If there is cell debris after completely pulling through, repeat more scrubbing steps and an additional 30 minute incubation before continuing.

9.4 1h
Pull through 400 µL DI water (1/5)
Pull through 400 µL DI water (2/5)
Pull through 400 µL DI water (3/5)
Pull through 400 µL DI water (4/5)
Pull through 400 µL DI water (5/5)
Pull through 400 µL 1M NaOH (1/2), and scrub back and forth several times before pulling through completely.
Pull through 400 µL 1M NaOH (2/2). Incubate 01:00:00 at room temperature. Rinse chip reservoir twice with 400 µL DI water, then screw cap over chip reservoir to prevent evaporation.

9.5 **Incubations longer than 1 hour may be detrimental to the chip, do not extend NaOH incubation time.**

Immediately following incubation, remove z-movi chip screw cap and pull through 400 µL DI water (1/3).
Pull through 400 µL DI water (2/3).

Pull through 400 µL DI water (3/3).
Pull through air multiple times *until flow cell is completely dry before long term storage*.

Store z-Movi chip in 37 °C dry incubator.