Protocol

Tumor Infiltrating Immune Cell Assay

This protocol describes a solution for quantifying tumor infiltrating lymphocytes (TILs) from within a solid tumor model. The detailed method describes:

1) The formation of single spheroids
2) PBMC co-culture
3) Washing and dissociation of 3D spheroids

This allows phenotypic comparisons of non-infiltrating lymphocytes and TILs. Utilizing the iQue3 rapid sampling method allows increased replicates to be collected, this improves some of the issues associated with complex biological models without increasing acquisition time.

Required materials

- Target cells of interest
- Effector cells of interest: require labeling with CytoLight Rapid Green (Essen BioScience: 4705)
- Effector cell culture media
- Effector cell activator (e.g. CD3/CD28 Dynabeads)
- 96-well ULA microplate (e.g. Corning #7007)
- 2 x 96-well V-bottom microplate (e.g. Costar #3363)
- Plate stand (e.g. Diversified Biotech #WPST-1000)
- Accutase (e.g. Gibco #A1110501)
- T-Cell Activation Cell and Cytokine Profiling Kit (TCA Kit) (Intellicyt #90560)

Suggested materials

- Plate washer: for removal of antibodies after labeling, a wash step is required and while plates can be 'flicked' in order to remove unwanted supernatant, a plate washer is highly recommended.

NOTE: There is no recommended plate washer and instrument settings must be optimized before use.

General guidelines

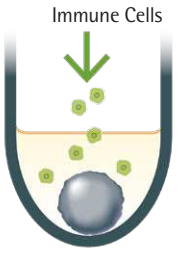
- It is strongly recommended that the following controls are included on the plate:
  1) Spheroids co-cultured with non-activated effector cells
  2) Spheroid in mono-culture
  3) Activated effector cells in mono-culture.
- It is strongly recommended to include 6-8 replicates per treatment.
- Once the assay plate is set up, it is recommended to allow at least 24 hours for pre-activated immune cells to infiltrate the spheroid and 36-48 hours for in well activation. This time point will need to be optimized depending on activator used.
- Remove bubbles from all wells by gently squeezing a wash bottle (containing 70-100% ethanol with the inner straw removed) to blow vapor over the surface of each well.
- The optional Cell Proliferation and Encoder Dye (B/Green) from the TCA kit cannot be included in this experiment due to the inclusion of CytoLight Rapid Green labeled effector cells.
- If care is not taken during washing steps it is possible for spheroids to be lost:
  A) If spheroid is inside tip with media: Hold pipette vertically and allow spheroid to sink to base of tip. Eject media back into wells until spheroid returns.
  B) If spheroid was transferred to a V-botton plate: Remove media from this well using a single channel P1000. Hold pipette vertically to allow spheroid to sink to base of tip. Eject media back into ULA well and observe under microscope for spheroid retrieval.
- The IncuCyte® can be used for QC purposes: After placing the plate in the IncuCyte live-cell analysis system, allow it to warm to 37°C for 30 minutes prior to scanning to avoid condensation.

Quick guide

1. Seed target cells (day 0)

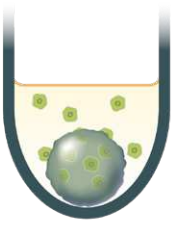
Seed tumor cells (100 µL/well) into 96-well Ultra Low Attachment Plate. Place in incubator and allow three days to form.

2. Add immune cells (day 3)



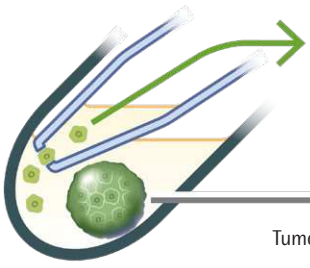
Add your choice of immune cells (50 µL/well). Add 50 µL/well of preferred activators (e.g. CD3/CD28 Dynabeads®).

3. Activation and infiltration (days 4–7)



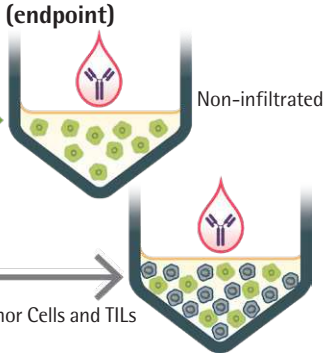
Monitor infiltration using IncuCyte or under microscope, assess for activation phenotypes of Immune cells (e.g. Clustering).

4. Wash off non-infiltrated cells (endpoint)



Wash off and collect Non-infiltrated cells for analysis. Dissociate spheroids using 100 µL/well Accutase.

5. Label using antibodies (endpoint)



Transfer dissociated spheroid to V-bottom plate and label using Antibody detection panel (10 µL/well) from TCA kit.

Protocol

1. Seed target cells

1.1 Seed target cells: Seed an appropriate density (100 µL/well) into a 96-well ULA plate such that by day 3, spheroids have formed with the desired size (e.g. 200–500 µm). Seeding density will need to be optimized for each cell line used, however 1,000 to 5,000 cells/well are reasonable starting points.

NOTE: Cell lines that require the addition of a basement membrane extract for tight spheroid formation (e.g. Matrigel) cannot be used in this assay.

1.2. Centrifuge the ULA plate (125 xg 10 minutes) at RT.

2. Monitor spheroid formation

2.1 Place the cell plate into the IncuCyte Live-Cell Analysis System. Schedule 24 hour repeat scans:

a. Scan Type: Spheroid
b. Channel Selection: Single spheroid, Phase + Brightfield
c. Objective: 10x or 4x
d. Scan Interval: 6 hours

NOTE: Using an IncuCyte for monitoring spheroid formation is optional and alternatively can be performed using a microscope.

3. Add immune cells and treatments

3.1. Once spheroids have reached desired size (e.g. 200 - 500 µm), remove the plate from the incubator and carefully add appropriate treatments if necessary (e.g. CD3/CD28 Dynabeads) at 50 µL/well.

3.2. Label immune cells with CytoLight Rapid Green (CLRG) labeling dye.

a. Add 21.5 µL of DMSO to CLRG vial (Stock 5 mM).
b. Dilute CLRG stock to 30 µM (100x FAC 1:166 dilution) with PBS.
c. Resuspend PBMCs at 5 x 10^5 cells/mL in pre-warmed PBS.

d. Add 1:100 dilution of CLRG to PBMCs and incubate at 37° C for 20 minutes, mixing by inversion after 10 minutes.
e. Add 6-fold volume of cell culture media to remove excess dye.
f. Centrifuge (1300 rpm 10 minutes).

NOTE: This protocol has been optimized for use with PBMCs on the iQue3. Other cell types may require further optimization.

3.3. Prepare the labeled immune cells at an appropriate density. It is recommended that different target-to-effector ratios are tested (e.g., 1:3, 1:5).

3.4. Seed 50 µL/well immune cells on top of spheroids to achieve a total assay volume of 200 µL.

3.5. Place the assay plate into the IncuCyte live-cell analysis system. If necessary schedule a new 24 hour repeat scanning. Or change scan interval to 3 hours.

NOTE: Using an IncuCyte for monitoring infiltration is optional.

4. Removal of Non-Infiltrated Cells

4.1. Once infiltration has occurred place assay plate on plate stand.

4.2. Gently triturate (5-10 times) 100 µL/well to suspend any non-infiltrated cells in the media. Allow spheroid to settle for 15 seconds.

4.3. Place tips at top edge of wells and gently remove 100 µL/well of media.

4.4. Transfer the non-infiltrated cells into a V-bottom plate (Plate 1). Centrifuge plate 1 (300 xg 5 minutes). Remove supernatant to allow further media additions.

4.5. Add 100 µL/well of media to assay plate and repeat 3x step 4.2-4.4.

NOTE: It is important to check spheroids have not been removed into tips after each wash step.

4.6. Centrifuge assay plate briefly (300 xg 1 minute) and assess washing of spheroids by scanning in IncuCyte or viewing under a microscope. If immune cells remain in wells repeat steps 4.2-4.5 until these are removed.

5. Spheroid Dissociation and Antibody Labeling

5.1. Add 100 µL/well PBS to assay plate (total volume 200µL), gently triturate, allow spheroid to settle for 15 seconds.

5.2. Gently remove 150 µL/well and discard.

NOTE: It is important to check spheroids have not been removed into tips after final wash step.

5.3. Add 100 µL/well of pre-warmed Accutase to assay plate and incubate at 37° C for 1 hour.

5.4. Shake plate at 1000 rpm (iQue3 manual mode) for 5 minutes. Gently triturate wells (5-10 times) to aid in spheroid dissociation.

5.5. Assess spheroid dissociation under a microscope, if cells are not in single cell suspension place back into incubator for a further 30 minutes and repeat step 5.4.

5.6. Once in single suspension transfer all well contents into a V-bottom plate (Plate 2).

5.7. Place 100 µL/well of media into assay plate and triturate to capture remaining cells. Transfer this to Plate 2.

5.8. Centrifuge V-bottom plates 1 and 2 (300 xg 5 minutes).

5.9. Remove supernatant and resuspend by shaking plate in residual liquid (3000 rpm 1 minute). Add 10 µL/well PBS + 2% FBS.

5.10. Add 10 µL/well Antibody Panel Detection Cocktail from TCA Kit to V-bottom plates 1 and 2, leave at RT in the dark for 1 hour.

5.11. Wash cells from both plates with 200 µL PBS + 2% FBS and centrifuge (300 xg 5 minutes). Aspirate supernatant and shake to resuspend cells in residual liquid (3,000 rpm 1 minute).

5.12. Add 20 µL/well PBS + 2% FBS and run on iQue3.

NOTE: Optimization may be necessary to achieve required results. A minimum sip time of 5 seconds is recommended.

6. Analysis using ForeCyt

6.1. TCA kit comes with a templated analysis. This includes automated compensation that should not require changes for this assay.

6.2. A change in the gating should be included in order to exclude tumor cells from the gates:
a. All events: select cell populations using SSC-H vs FSC-H.
b. Single cells: identify single cells using FSC-H vs FSC-A.
c. Effector cell identification: BL1-H vs FSC-H will identify two populations. High green are effector cells and low green target cells.
d. Live/Dead cells: RL1-H vs SSC-H can then be used to exclude any dead cells.
e. Continuation of TCA kit gates: copy gates onto new population to allow exclusion of target cells.

A complete suite of cell health applications is available to fit your experimental needs. Find more information at **www.sartorius.com/intellicyt**
For additional product or technical information, please email us at **AskAScientist@sartorius.com**

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