Optimizing Advanced Cell Models: Protocols and Recommendations for the iQue® HTS Platform

Benefits of characterization of marker expression in advanced cell models (ACM)

Technical Note

This technical note provides an overview of the methods for analysis of complex 3D cellular structures by cellular marker characterization using the iQue® High Throughput Screening (HTS) Cytometry Platform. It outlines methods for single cell dissociation of ACM such as spheroids and organoids for surface marker analysis using flow cytometry.

Required Materials

- 3D cell models of interest (organoids/spheroids)
- 96-well Ultra Low Attachment (ULA) microplate (e.g. Corning 4515)
- 96-well V-bottomed microplate (e.g. Corning 3363)
- Accutase (Sigma-Aldrich; A6964-100ML)
- Cell culture media/culture conditions
- Cell Recovery Solution (Corning; 354253)
- Centrifuge with plate adaptors
- Conjugated antibodies with appropriate controls
- Gentle Cell Dissociation Reagent (GCDR) (STEMCELL Technologies; 100-0485)
- iQue® Cell Membrane Integrity Kit (90342, 90346, 90350, 97057)
- Phosphate buffered saline (PBS)

This technical note is a collection of methods for dissociation of various 3D cell models in addition to marker staining and analysis on the iQue® Platform. For more information on the application of some of these methods, please see:

T-Cell Killing in Spheroids

Quantifying T-Cell Response in 3D Tumor Spheroids Using Advanced Flow Cytometry Workflows

Quick Guide

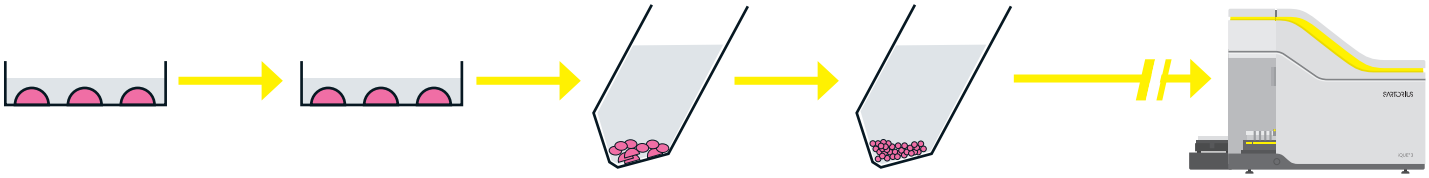
Organoids

1. Aspirate media and add GCDR

2. Pipette domes ~20 times and collect in tube

3. Centrifuge at 4 °C, resuspend in Accutase, incubate for 1 hour at 37 °C

4. Single cell suspensions are stained and analyzed on iQue®

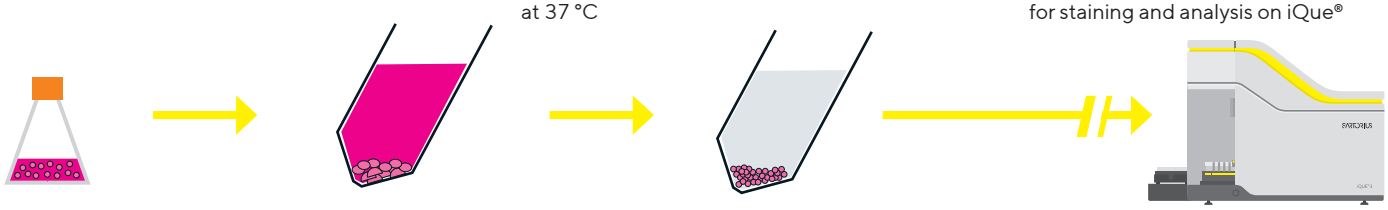


iPSC Spheroids

1. Collect spheroids from culture suspension into tube

2. Centrifuge tube, resuspend in Accutase, incubate for 12 minutes at 37 °C

3. Resuspend cell suspension until single cell suspension, cells are ready for staining and analysis on iQue®



Single spheroids ULA plates

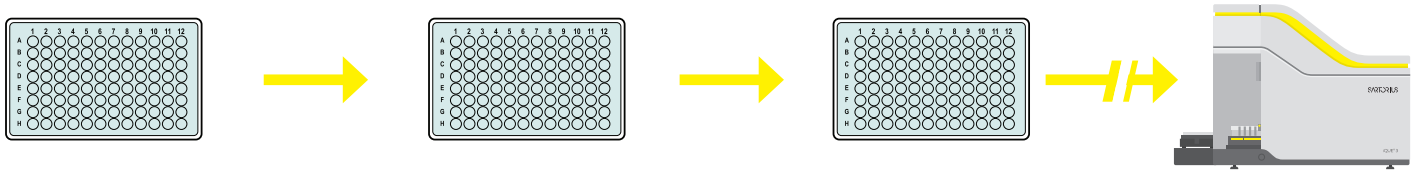
1. Wash plate with PBS, centrifuge and collect **supernatant in V-bottom plate**

2. Add Accutase, incubate for 1 hour at 37 °C

3. Triturate plate, check for single cell suspension

4. Transfer cell suspension to **V-bottom plate**

5. Single cell suspensions are stained and analyzed on iQue®



Single spheroids ULA plates (Matrigel®)

1. Wash plate with PBS, centrifuge and collect **supernatant in V-bottom plate**

2. Add Cell Recovery Solution, shake for 30 minutes at 4 °C

3. Collect supernatant in **V-bottom plate**

4. Add Accutase, shake for 1 hour at 4 °C, centrifuge, resuspend, incubate for 1 hour at 4 °C

5. Triturate plate, check for single cell suspension

6. Transfer cell suspension to **V-bottom plate**

7. Single cell suspensions are stained and analyzed on iQue®

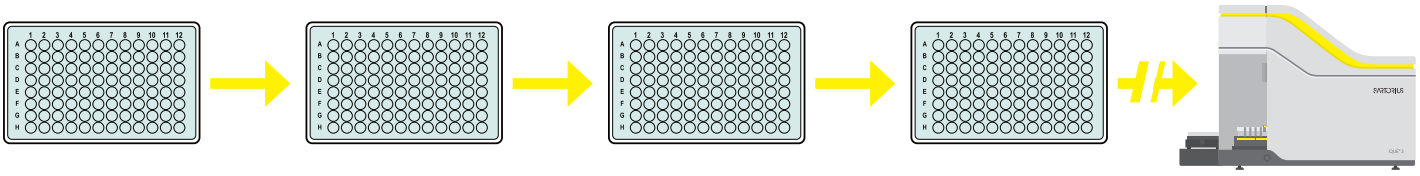


Figure 1: Workflows for ACM dissociation.

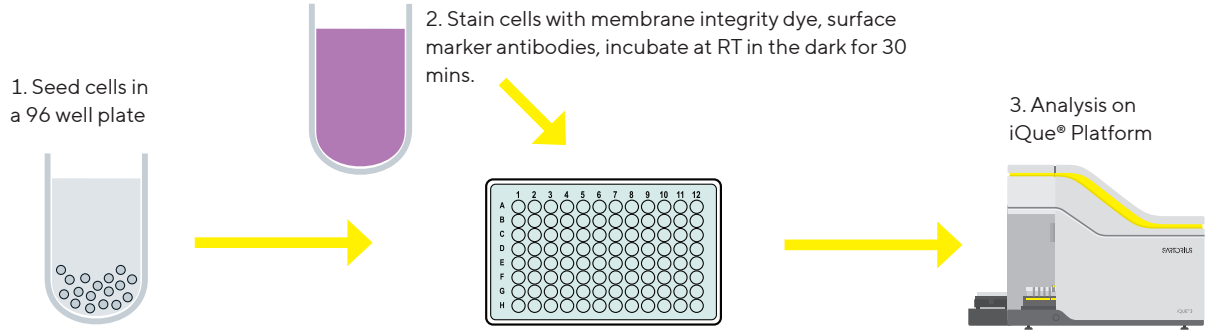


Figure 2: Workflow for surface marker staining. Surface marker staining can be performed during this incubation step concurrently with fixable viability dye staining.

Protocol

Dissociation of 3D models to single cells

Organoids cultured in Matrigel® domes

1. Aspirate media and add 1 mL of Gentle Cell Dissociation Reagent (GCDR) per well of a 6-well plate.
2. Using a P1000, pipette ~20 x to disintegrate the Matrigel®.
3. Pool the supernatant in a 15 mL falcon tube and wash each well with a further 1 mL of GCDR and add to the tube.
4. Centrifuge tube at 300 x g at 4 °C for 5 minutes.
5. Aspirate supernatant and resuspend the pellet in 1 mL Accutase and incubate at 37 °C for 1 hour.
6. Check suspension during incubation for single cell dispersion.
7. After incubation, resuspend the cell suspension 3-4 times with a P1000. If cell clusters remain, repeat the incubation for a further 10 minutes and resuspend 3-4 times. Repeat until a single cell suspension.
8. Once cells are in a single cell suspension, they are ready for staining and analysis using the iQue® HTS Platform.

Single Spheroids in ULA 96-well plates (Liquid media)

1. Gently remove 150 µL/well of supernatant and transfer to a V-bottom plate.
2. Add 150 µL/well PBS to the ULA plate.
3. Centrifuge ULA plate at 125 x g for 1 minute.
4. Remove 150 µL/well and add to the V-bottom plate.
5. Add 150 µL/well Accutase to ULA plate and incubate at 37 °C for 1 hour.
6. During incubation, check wells for single cell dispersion, if spheroids are dissociated end incubation.
7. Centrifuge V-bottom plate at 300 x g for 5 minutes. Flick ULA plate to remove supernatant and resuspend cells in residual media by shaking 2400 rpm for 1 minute on iQue® HTS Platform. Place plate at 37 °C.
8. After 1 hour triturate ULA plate. Use a light microscope to examine spheroid dissociation. If cell clusters remain, repeat incubation for a further 15 minutes and triturate. Repeat until a single cell suspension.
9. Transfer entire contents of wells to V-bottom plate. Add 100 µL/well PBS into the ULA plate to wash wells and transfer to the V-bottom plate. Centrifuge V-bottom plate at 300 x g for 5 minutes.
10. Flick V-bottom plate to remove supernatant, resuspend cells in residual media by shaking 2400 rpm for 1 minute.

iPSC spheroids

1. Collect spheroids from culture suspension media into a tube.
2. Centrifuge at 200 x g for 4 minutes.
3. Aspirate supernatant and resuspend the pellet in Accutase.
4. Incubate at 37 °C for 10-12 minutes.
5. During incubation, check the spheroids every 2-3 minutes for signs of dissociation.
6. After incubation, resuspend the cell suspension 3-4 times with a P1000.
7. If cell clusters remain, repeat the incubation for a further 10 minutes and resuspend 3-4 times. Repeat until a single cell suspension.
8. Once single cell, resuspend cells in media or PBS + 0.5% FBS to quench Accutase.

Surface marker staining

1. Plate 2 x 10^4 cells in 10 µL of medium per well in a V-bottom 96-well plate.
2. Wash cells once by adding 90 µL PBS and centrifuging at 400 x g for 5 minutes, aspirate supernatant.
3. Shake plate at 3000 rpm for 60 seconds.
4. Add 10 µL of antibody and membrane integrity dye cocktail to each well.
5. Briefly centrifuge the plate at 400 x g for 5 seconds and then shake at 2000 rpm for 20 seconds.
6. Incubate the samples at RT in the dark for 30 minutes.
7. Add 90 µL PBS per well.
8. Centrifuge at 400 x g for 5 mins, aspirate supernatant and shake at 3000 RPM for 60 seconds.
9. Add 20 µL PBS + 0.5% BSA to each well and run on the iQue® HTS Platform.

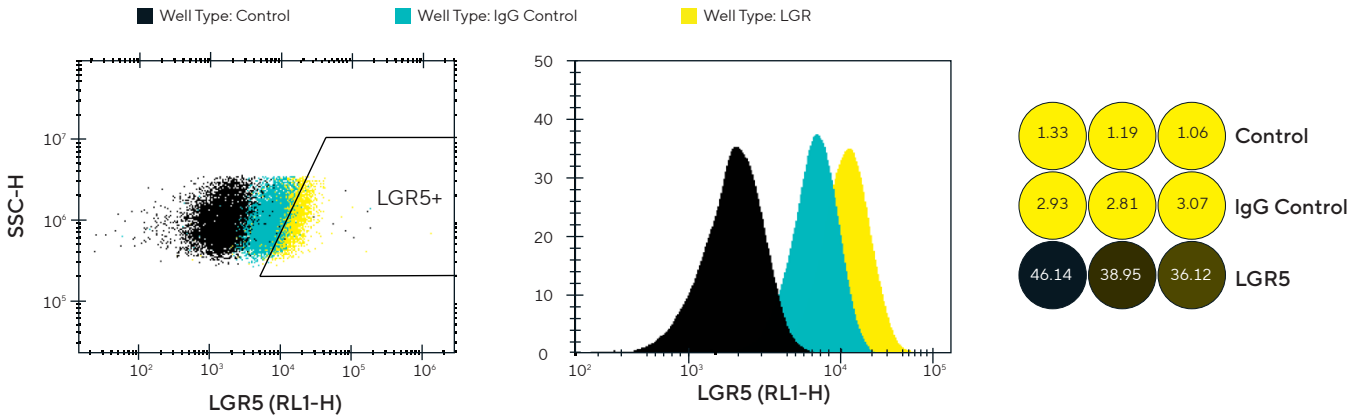


Figure 3: iPSC-derived hepatic organoid LGR5 expression characterized using the iQue® Platform. Analysis of LGR5+ stem cell populations in iPSC-derived organoids can be achieved using the organoid dissociation and surface marker staining protocols described. Organoids were dissociated to single cells and stained with membrane integrity dye to assess cell health and viability and stained with LGR5 antibody to characterize the stem cell niche within the organoids. LGR5 is a marker found in mature stem cells in a variety of organoid and tissue models, its presence is indicative of proliferative organoids capable of further differentiation. Hepatic organoids were derived from iPSCs and their levels of LGR5 were measured on the iQue® HTS Cytometer. Dot plot and gating strategy is shown in addition to a histogram of the same data taken from iQue Forecyt® software. A heatmap provides another method for displaying the data, showing the percentage of the iPSC-derived hepatic organoids positive for LGR5 expression.

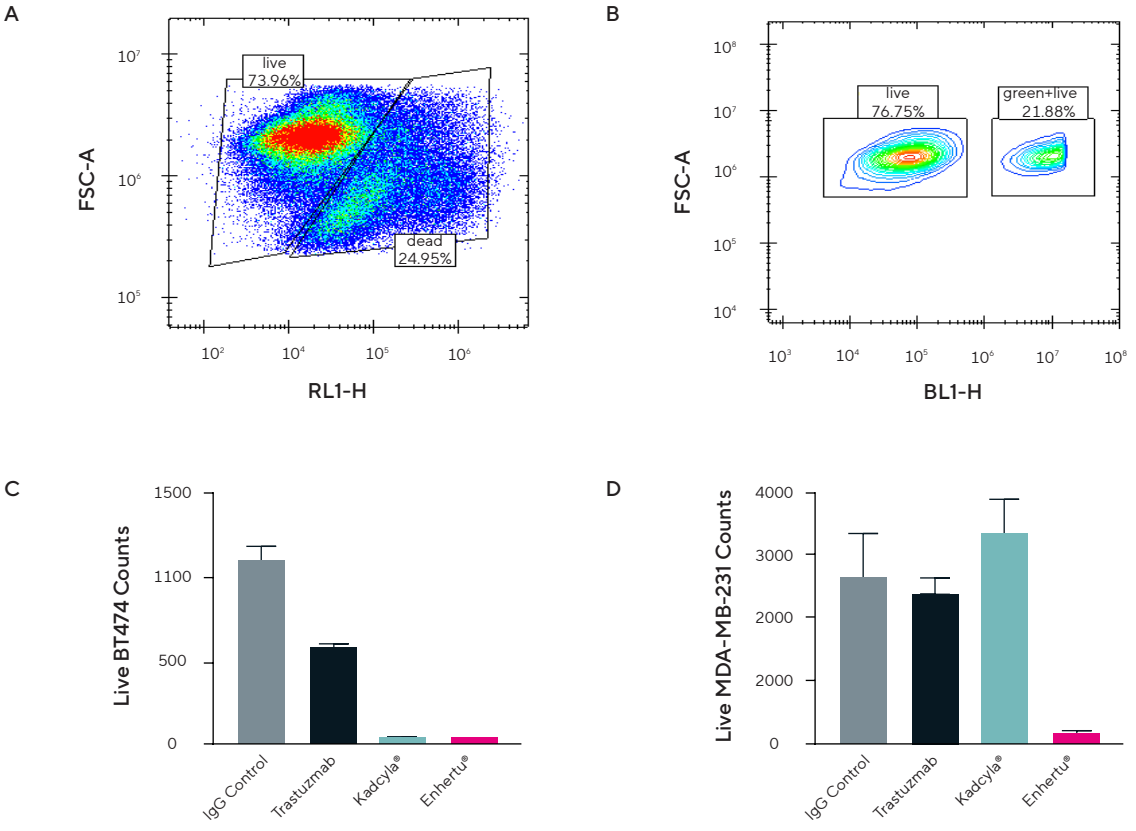


Figure 4. Investigation of antibody drug conjugate (ADC) bystander killing in a single spheroid advanced cell model. Single spheroids were formed from a 2:3 ratio of high HER2 expressing BT474 cells (labeled with Incucyte® Cytolight Green Lentivirus) to low HER2 expressing, unlabeled MDA-MB-231 cells. Anti-HER2 antibodies were added after 72 hours. On day 8, spheroids were dissociated, cells labeled using the iQue® Cell Membrane Integrity (R/Red) Dye. Live cell counts were quantified using the iQue® HTS Platform. A) iQue Forecyt® dot plot showing gating strategy for selection of live and dead cells. B) Contour plot outlining the gating strategy for separation of green positive (BT474) and green negative (MDA-MB-231) cells (C and D Bar graphs show viable cell counts from the iQue® HTS Platform).

Data Acquisition and Analysis

Acquisition

1. Using the integrated software, iQue Forecyt®, import the iQue® Fixable Viability Dye Kit template for the viability dye colour of choice and open a new experiment.
2. Place your plate on the plate stage and select run on the software.
3. The iQue® HTS Platform will acquire the data and populate the plots and gates in real-time.

Analysis

1. Once your plate has been acquired, you can select different methods for displaying your data, and manipulate gates and create new plots and histograms.
2. Heatmaps are also an option for a visually clear method of displaying data.

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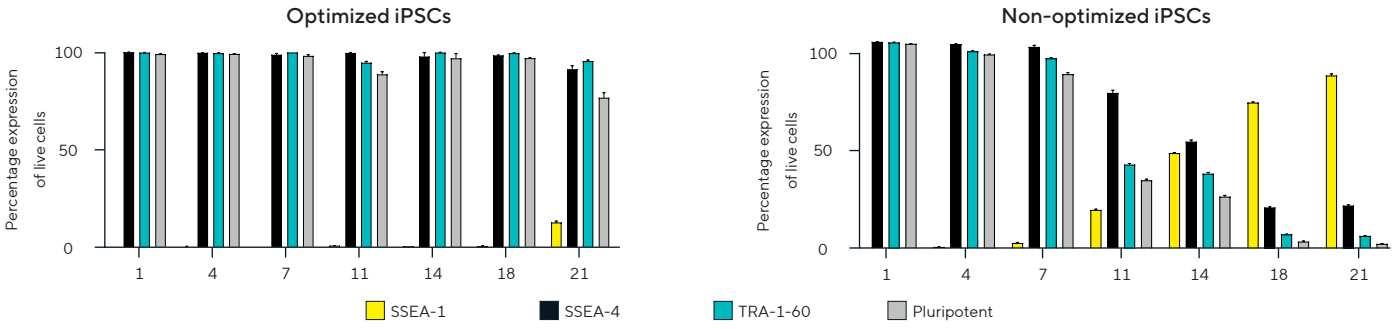

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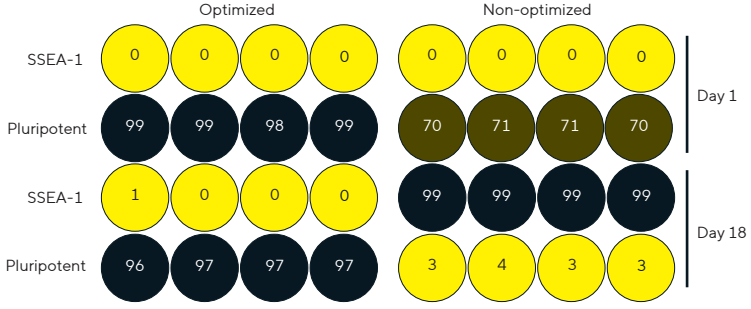

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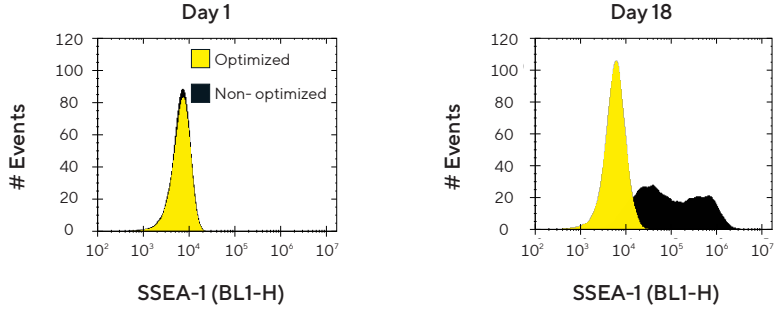

Figure 6. Characterization of marker expression in iPSC spheroids using the iQue® HTS Platform.
Characterization of pluripotency in iPSC spheroid advanced cell models can be determined using the iPSC spheroid dissociation and surface marker staining protocols outlined here. SSEA-1 was used as a marker of non-pluripotent cells, while SSEA-4 and TRA-1-60 were used to characterize pluripotent cells. Pluripotent is the SSEA-1- cell population that is both SSEA-4+ and TRA-1-60+. A) iPSC spheroids were grown in optimized (passaged) and non-optimized (non-passaged) conditions to induce 'differentiation' for up to 21 days and marker expression was measured on the iQue® HTS Platform (± SEM, n=4). The spheroids were grown until their first passage, at which point they were subjected to the described growth conditions. B) Heatmaps produced in the iQue Forecyt® software showing the percentage of cells positive for marker expression at days 1 and 18 of Optimized and Non-optimized growth conditions (n=4). C) iQue Forecyt® histograms highlighting the shift in SSEA-1 marker expression of Non-optimized iPSC spheroids.

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