Preparation of tumoroids for flow cytometry

Purpose: Dissociate, stain, and analyze tumoroid cells by flow cytometry
Scope: Step-by-step protocol for tumoroid staining

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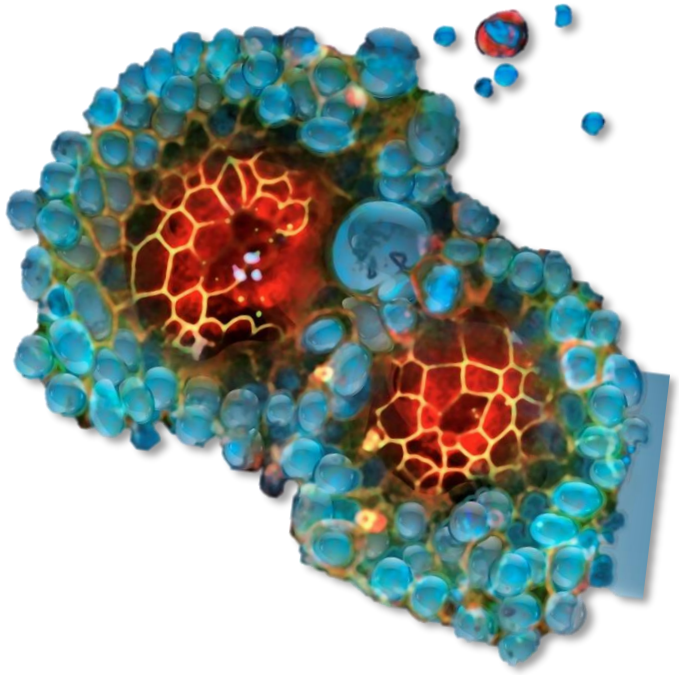
Introduction

Tumoroids, also known as tumoroid lines, cancer organoids, or patient-derived tumor organoids (PDTOs), are patient-derived cancer cells grown as 3D, self-organized multicellular structures.



Scientific literature suggests tumoroids are a promising tool for drug development studies and precision medicine applications because, compared to traditional 2D immortalized cancer cell lines, they:

- Better represent patient characteristics
- Support tumor heterogeneity
- Offer more clinically predictive results

Techniques such as immunofluorescence imaging, western blotting, and flow cytometry can be used to characterize tumoroids. However, the 3D structure of tumoroids and presence of basement membrane extract (BME), which is typically required for *in vitro* culture, can pose challenges when preparing these samples for downstream analysis. This protocol provides guidance on preparing tumoroids for analysis by flow cytometry.

Flow cytometry – general approach

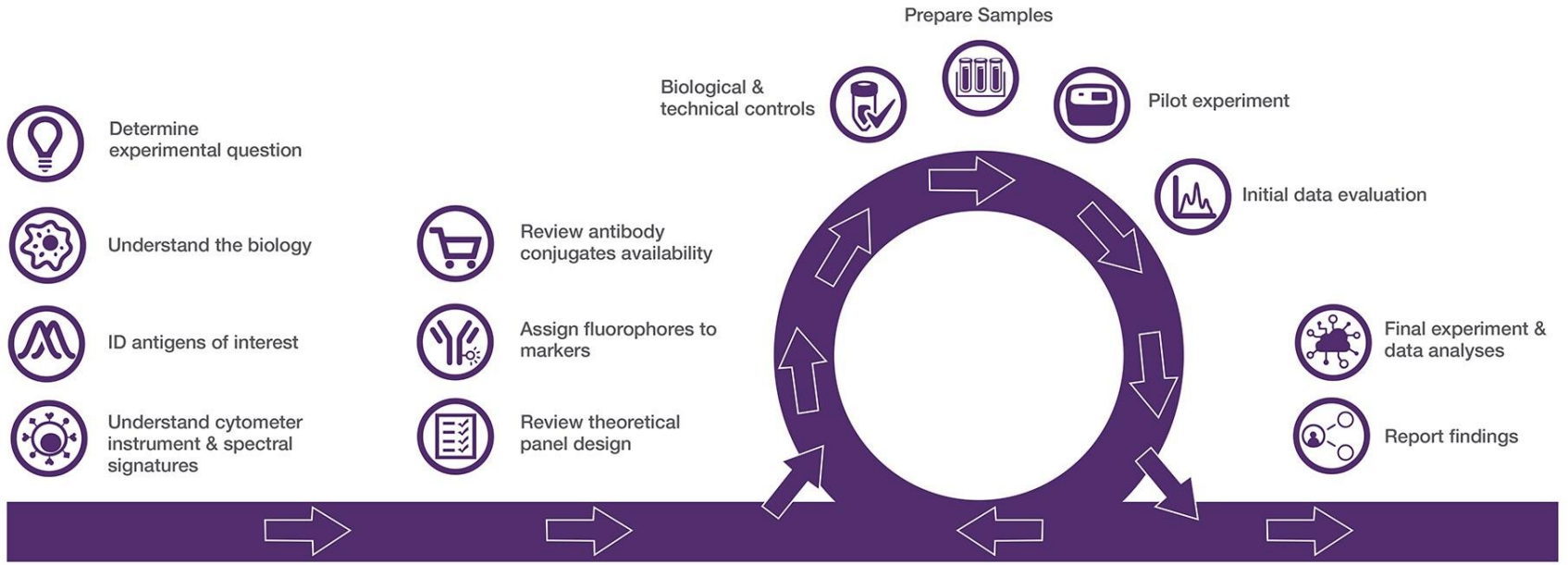


Image from Flow Cytometry Experimental Process guide

This guide provides a step-by-step protocol for preparing patient-derived tumoroid cells for analysis by flow cytometry. Resources on flow cytometry methods, instrumentation, antibody selection, antibody titration validation, and suggested controls can be found at thermofisher.com and are linked below.

Additional resources

- Flow cytometry methods
- General flow cytometry experimental process
- Flow cytometry panel builder
- Methods for antibody validation
- Antibody titration
- Controls for flow cytometry
- Panel development example

Required materials not supplied

Item	Cat. No.
Consumables	
Snap Cap Low Retention Microcentrifuge Tubes, 1.5 mL	3451PK
96 well plates or deep well plates	—
40 µm cell strainer	—
Nunc™ 15 mL Conical Sterile Polypropylene Centrifuge Tubes	339650
Nunc™ Serological Pipettes, 10 mL	170356N
Nunc™ Serological Pipettes, 5 mL	170366N
1000 µL Pipette Tips	—
200 µL Pipette Tips	—
20 µL Pipette Tips	—
Reagents and buffers	
Gibco™ DPBS, calcium, magnesium - DPBS (+/+)	14040133
Gibco™ DPBS, no calcium, no magnesium - DPBS (-/-)	14190144
Invitrogen™ eBioscience™ Flow Cytometry Staining Buffer	00-4222-57
Invitrogen™ Fc Receptor Binding Inhibitor Polyclonal Antibody, eBioscience™	14-9161-73
Invitrogen™ eBioscience™ Foxp3 / Transcription Factor Fixation/Permeabilization Concentrate and Diluent	00-5521-00
Gibco™ Goat Serum, New Zealand origin	16210064
Invitrogen™ ArC™ Amine Reactive Compensation Bead Kit	A10346
Invitrogen™ UltraComp eBeads™ Plus Compensation Beads	01-3333-42
Gibco™ OncoPro™ Tumoroid Medium Kit	A5701201
Gibco™ DMEM/F-12, GlutaMAX™ supplement	10565042
Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent	A1110501
Conjugated antibodies	Assay Dependent
Equipment	
Attune Flow Cytometer or other flow cytometer	Attune Models
Centrifuge	—
Microcentrifuge	—

Items with a Cat. No. can be purchased through **thermofisher.com**.

Items without a Cat. No. can be purchased from **fishersci.com** or other laboratory suppliers.

Warning: Read the safety data sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. SDSs are available from **thermofisher.com/support**.

LIVE/DEAD staining compensation setup

1. Vortex ArC™ Amine Reactive Compensation Bead Kit reactive beads (Component A) to mix.
2. Add 1 drop of ArC reactive beads (Component A) to a labeled microcentrifuge tube and vortex.
3. Incubate tube for 5 minutes at room temperature to warm.
4. Prepare fluorescent amine-reactive dye according to instructions included in the LIVE/DEAD™ Fixable Dead Cell Kit.
5. Add amount of reconstituted LIVE/DEAD fixable cell stain listed in table below to beads, pipetting directly into bead suspension and mixing well.

Amine-reactive dye for use with ArC™ reactive beads	Amount
LIVE/DEAD™ Fixable Blue stain	3 µL
LIVE/DEAD™ Fixable Violet stain	1 µL
LIVE/DEAD™ Fixable Aqua stain	3 µL
LIVE/DEAD™ Fixable Yellow stain	3 µL
LIVE/DEAD™ Fixable Green stain	3 µL
LIVE/DEAD™ Fixable Red stain	1 µL
LIVE/DEAD™ Fixable Far Red stain	3 µL
LIVE/DEAD™ Fixable Near-IR stain	1 µL

See MAN0002416 for additional details

6. Incubate 30 minutes at room temperature, protected from light.
7. Add 1 ml DPBS (+/+) to tube. Centrifuge for 5 min at 400 RCF.
8. Decant supernatant.
9. Resuspend bead pellet in 1 ml of Flow Cytometry Staining Buffer.
10. Vortex ArC™ Amine Reactive Compensation Bead Kit negative beads (Component B) to mix.
11. Add one drop of ArC negative beads (Component B) to sample tube, right before running on flow cytometer.
12. Vortex tube.
13. Analyze by flow cytometry, gating on the bead singlet population.

Antibody staining compensation setup

1. Label microcentrifuge tubes for compensation controls – one tube per antibody, plus one tube for unstained control beads.
2. Vortex UltraComp eBeads™ Plus Compensation Beads to mix.
3. Add 1 drop of compensation beads to each tube (without adding staining buffer).
4. Add 1 test or less of antibody conjugate to each tube – in general, this will be the amount of antibody used in staining a 100 µl final volume.

   Note: It is not necessary to use the antibody at its optimal conjugation, and most antibodies will use 0.03-0.125 µg. Use less antibody if high background is observed on negative bead population.

   Do not add any antibody to unstained control tube.

5. Vortex tube to mix.
6. Incubate at 4°C for 30 minutes, protected from light.
7. Add 1 ml of Flow Cytometry Staining Buffer to each tube. Centrifuge 400 RCF for 5 minutes.
8. Decant supernatant.
9. Resuspend bead pellet in 1 ml of Flow Cytometry Staining Buffer for each tube.
10. Vortex tube to mix.
11. Analyze by flow cytometry, gating on the bead singlet population.

Dissociate tumoroids and prep for flow cytometry

1. Transfer tumoroids and medium from the flask or well plate to a 15 mL conical tube.
2. Wash with cold DMEM/F12 + GlutaMAX™ and add wash to the 15 mL conical.

   Note: Adjust wash volumes based on culture vessel size, and use multiple 15 ml tubes if necessary.
3. Repeat the previous step with a second wash.
4. Centrifuge for 5 min at 400 RCF at 4°C.
5. Use a serological pipette to aspirate the supernatant without disturbing the cell pellet.
6. Add 12 mL of ice cold DPBS (-/-) to the 15 mL conical tube on top of the cell pellet and triturate ≥ 10 times to fully resuspend the cells.

   Note: Be careful not to overflow the 15 mL conical tube or draw solution into pipet filler.
7. Centrifuge for 5 min at 400 RCF at 4°C.
8. Prepare an appropriate volume of StemPro™ Accutase™ Cell Dissociation Reagent + 10 µM Y-27632 solution by supplementing StemPro™ Accutase™ Cell Dissociation Reagent 1:1000 with 10 µM Y-27632. 2 mL of StemPro Accutase + Y are needed to passage a T-25 flask.
9. Use a serological pipette to aspirate the supernatant, without disturbing the cell pellet.
10. Use a micropipette to aspirate the rest of the supernatant, removing as much of the DPBS (-/-) as possible without disturbing the cell pellet.
11. Add 2 mL of StemPro Accutase + Y solution to the conical tube on top of the cell pellet.
12. Use a micropipette set at 800 µL to triturate ≥ 10 times to fully resuspend the cells.
13. Place cells in water bath at 37°C for 10 minutes. Do not use a bead bath. Every ~2 minutes, swirl tube vigorously in order to resuspend cells in solution.

Dissociate tumoroids and prep for flow cytometry

14 After the 10 minute incubation, use a micropipette set at 800 µL to triturate the cells in the StemPro Accutase + Y.

15 Add 8 ml of OncoPro medium supplemented with 10 µM Y-27632 to tube containing cells.

16 Filter cell suspension using a 40 µm cell strainer. Discard retentate.

17 Centrifuge flow through at 400 RCF for 5 minutes at 4°C to pellet cells.

18 Proceed to count the number of viable cells according to your method of choice, centrifuging and resuspending if necessary.

19 Resuspend cells in DPBS(+/+) to desired concentration, centrifuging and resuspending post-counting as necessary. Target 1e6 cells/ml so that 50-100 µl = 50K-100K cells in a well.

20 Seed cells in 96-well plate for processing.
Recommend using ≥100,000 cells for panels of interest and ≥50,000 cells for controls.

21 Alternatively – divide cells into tubes, one tube per condition of interest.

Note

To remove non-dissociated tumoroid aggregates that may clog flow cytometry instrumentation, we recommend filtering dissociated cells through a 40 µm cell strainer as described in Step 16 prior to further processing.

LIVE/DEAD fixable dead cell staining

1. Bring vial of fluorescent reactive dye and vial of anhydrous DMSO to room temperature.
2. Add 50 µl DMSO to vial of reactive dye to make reconstituted LIVE/DEAD dye. Store unused portion at -20°C for up to 2 weeks.
3. Prepare working solution of LIVE/DEAD dye by diluting reactive dye solution in DMSO in DPBS (+/+) at 1:500 dilution.
4. Centrifuge plate containing cells at 400 RCF for 5 min at room temperature.
5. Decant supernatant.
6. Add 100 µl/well of LIVE/DEAD working solution to each well and mix by triturating 5 times.
7. Add 100 µl/well DPBS (+/+) to control and unstained wells.
8. Incubate at 4°C for 30 minutes, protected from light.
9. Centrifuge at 400 RCF for 5 min at room temperature.
10. Decant supernatant.
11. Wash cells with Flow Cytometry Staining Buffer by adding 200 µl/well and triturating 5 times.
12. Centrifuge 400 RCF 5 minutes at room temperature.
13. Decant supernatant.

Note

Dilution of LIVE/DEAD stain may depend on both fluorophore and cell type. Determine proper dilution by titration during initial panel design and validation experiments. Please see this resource for additional details.

Surface protein staining

1. Block non-specific Fc-mediated interactions by incubating cells with Fc Receptor Binding Inhibitor Polyclonal Antibody (FcBlock). Prepare Fc Blocking Buffer by mixing 10 µl of FcBlock with 40 µl of Flow Cytometry Staining Buffer per well.
2. Resuspend cells in 50 µl/well of Fc Blocking Buffer and mix by triturating 5 times when buffer is added.
3. Incubate at 4°C for 20 minutes.
4. Prepare the recommended quantity of each surface staining primary antibody at 2X in Flow Cytometry Staining Buffer so that the final staining volume is 100 µL (i.e. 50 µL of cell sample in Fc Blocking Buffer + 50 µL of antibody mix).
5. Add 50 µl/well of surface antibody cocktail to cells. Mix by triturating 5 times.
6. Stain 60 minutes at 4°C, protected from light.
7. Centrifuge 400 RCF for 5 minutes at room temperature. Decant supernatant.
8. Add 200 µl/well of Flow Cytometry Staining Buffer to wash. Mix by triturating 5 times.
9. Centrifuge 400 RCF for 5 minutes at room temperature. Decant supernatant.
10. Add 200 µl/well of Flow Cytometry Staining Buffer to wash. Mix by triturating 5 times.
11. Centrifuge 400 RCF for 5 minutes at room temperature. Decant supernatant.

Intracellular protein staining

1. Mix 1 part Foxp3 Fixation/Permeabilization Concentrate with 3 parts Foxp3 Fixation/Permeabilization Diluent.
   Note: IC Fixation Buffer was not successful for intracellular staining of these tumoroid models
2. Add 200 µl of Foxp3 Fixation/Permeabilization working solution to each well and fully resuspend cells in the solution by triturating.
3. Incubate overnight at 4°C. Protect from light.
4. Centrifuge samples at 400 RCF at room temperature for 5 minutes. Decant supernatant.
5. Prepare 1X Permeabilization Buffer by diluting 10X stock in distilled water.
6. Add 200 µl of 1X Permeabilization Buffer to each well. Mix by triturating 5 times.
7. Immediately centrifuge samples at 400 RCF at room temperature for 5 minutes. Decant supernatant.
8. Add 200 µl of 1X Permeabilization Buffer to each well. Mix by triturating 5 times.
9. Immediately centrifuge samples at 400 RCF at room temperature for 5 minutes. Decant supernatant.
10. Prepare Goat Blocking Buffer by adding 5 µl of goat serum to 45 µl of 1X Permeabilization Buffer per well.
11. Resuspend pellet in 50 µl of Goat Blocking Buffer for each well. Mix by triturating 5 times.

Intracellular protein staining

12. Block at room temperature for 15 minutes.

13. Prepare intracellular antibodies in 1X Permeabilization Buffer at 2X concentration to obtain correct final concentration with a staining volume of 100 µL.

    Note: If not adding intracellular antibody, directly add 50 µl of 1X Permeabilization Buffer per well.

14. Without decanting Goat Blocking Buffer, add the recommended amount of directly conjugated antibody for detection of intracellular antigens to each well. Mix by triturating 5 times.

15. Incubate for 60 minutes at room temperature. Protect from light.

16. Centrifuge samples 400 RCF for 5 minutes at room temperature. Decant supernatant.

17. Add 200 µl of 1X Permeabilization Buffer to each well to wash. Mix by triturating 5 times.

18. Centrifuge samples at 400 RCF at room temperature for 5 minutes. Decant supernatant.

19. Add 200 µl of 1X Permeabilization Buffer to each well to wash. Mix by triturating 5 times.

20. Centrifuge samples at 400 RCF at room temperature for 5 minutes. Decant supernatant.

21. Resuspend cells in appropriate volume of Flow Cytometry Staining Buffer (usually 200-300 µl per well).

22. Cells are ready to be analyzed by flow cytometry.

Flow cytometry run order

1. Unstained cells
   a. Determine appropriate FSC/SSC settings and PMT voltages for cells.
2. Compensation beads (single-stained samples)
   a. Adjust FSC/SSC to visualize beads.
   b. Apply a gate to singlet majority population for use in compensation setup.
   c. Decrease PMT voltage for any positive bead peak that is off-scale.
   d. Do not record data until all single-stained beads have been reviewed and all voltages are set.
   e. Run each single-stained bead sample and record files for compensation controls.
3. Readjust FSC/SSC setting for cell samples, but do not adjust PMT voltages for fluorescence detectors.
4. Collect and record FMO control samples.
5. Collect and record experimental samples.

Note

Suggested run order for an Attune™ NxT flow cytometer is shown. Adjust compensation and sample collection steps as needed for flow cytometer instrumentation.

Antibodies used for flow cytometry of tumoroids

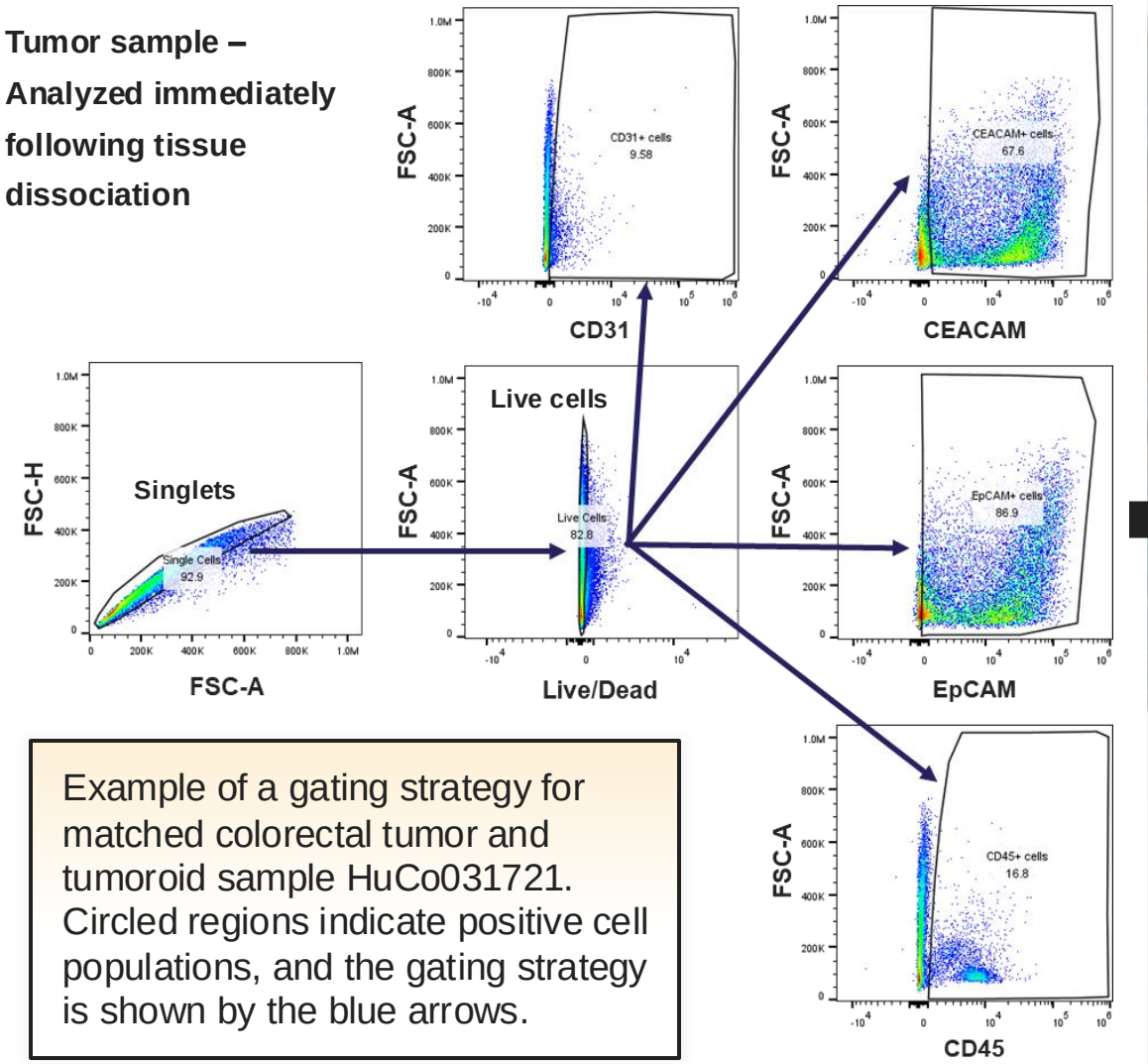
Target	Purpose	Location	Fluorochrome	Vender	Catalog Number	Host/IgG	Dilution
Cell viability marker	Identity live cells	Intracellular (stain prior to permeabilization)	Live/Dead Fixable Yellow	Thermo Fisher Scientific	L34959	N/A	1:500
PECAM-1 (CD31)	Endothelial cell marker	Cell surface	PerCP-eFluor 710	Thermo Fisher Scientific	46-0319-42	Mouse/IgG1, kappa	1:20
CD45	Hematopoietic cell marker	Cell surface	PE	Thermo Fisher Scientific	12-0459-42	Mouse/IgG1, kappa	1:20
EpCAM (CD326)	Epithelial cell marker	Cell surface	PE-cyanine7	Thermo Fisher Scientific	25-9326-42	Mouse/IgG1, kappa	1:20
CEACAM (CD66)	Colorectal cancer marker	Cell surface	Super Bright 436	Thermo Fisher Scientific	62-0668-42	Mouse/IgG2a, kappa	1:20
Cytokeratin 20	Colorectal cancer maker	Intracellular	PE	abcam	ab209953	Rabbit/IgG	1:750
Cytokeratin 7	Colorectal vs. lung cancer marker	Intracellular	Alexa Fluor 488	abcam	ab185048	Mouse/IgG2b	1:500

Note

Examples of antibodies that can be used for flow cytometry characterization of tumoroids are shown here. Specific panel design should be dictated by the biological question of interest. Do not combine antibodies with the same fluorochrome (e.g., multiple PE antibodies) in the same flow cytometry experiment.

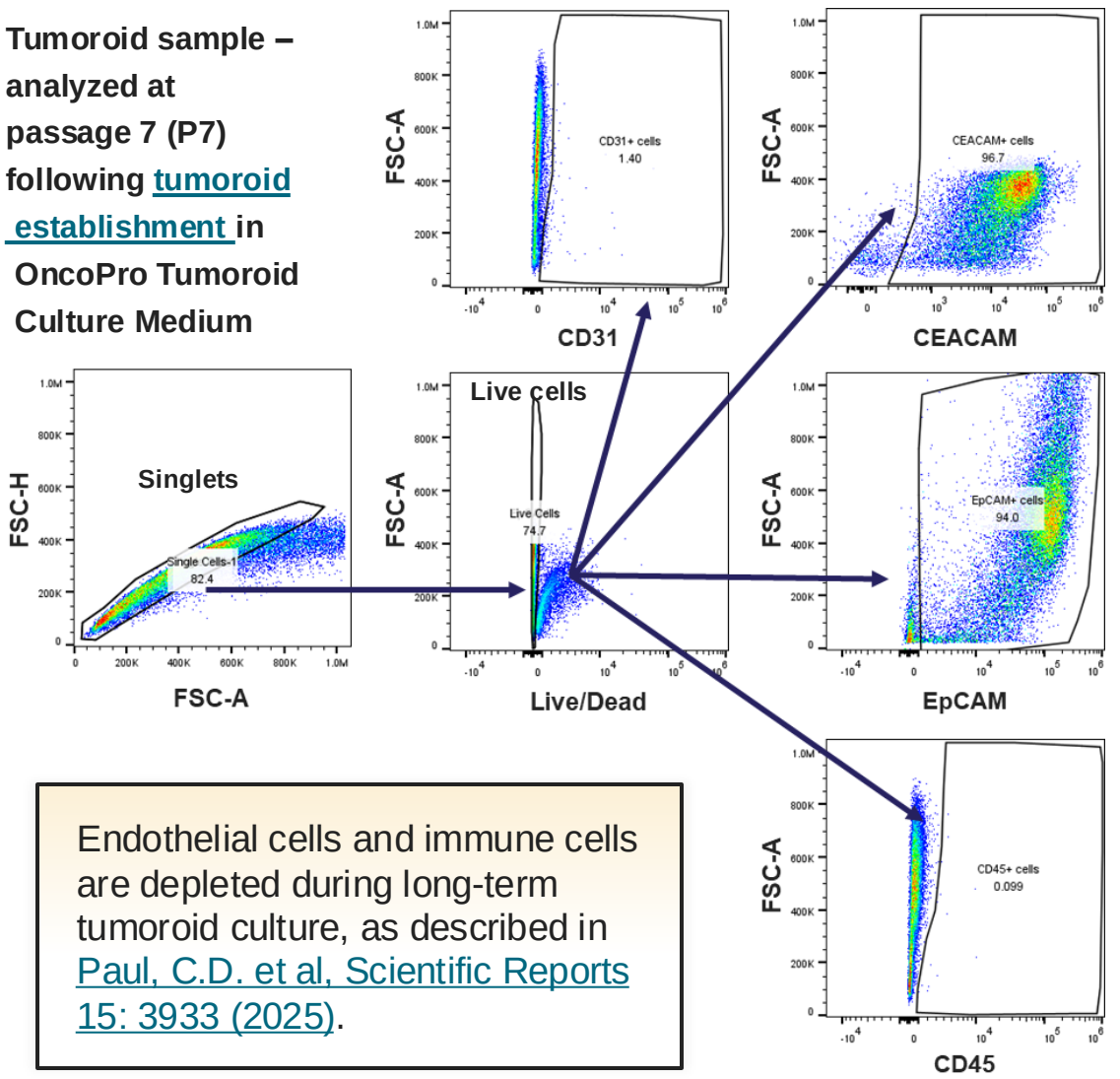
Example flow cytometry analysis of tumoroid samples

Tumor sample – Analyzed immediately following tissue dissociation



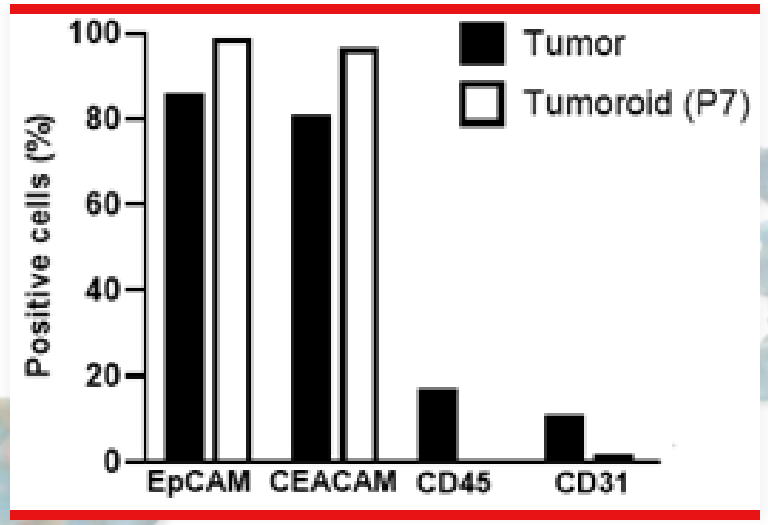
Example of a gating strategy for matched colorectal tumor and tumoroid sample HuCo031721. Circled regions indicate positive cell populations, and the gating strategy is shown by the blue arrows.

Tumoroid sample – analyzed at passage 7 (P7) following tumoroid establishment in OncoPro Tumoroid Culture Medium

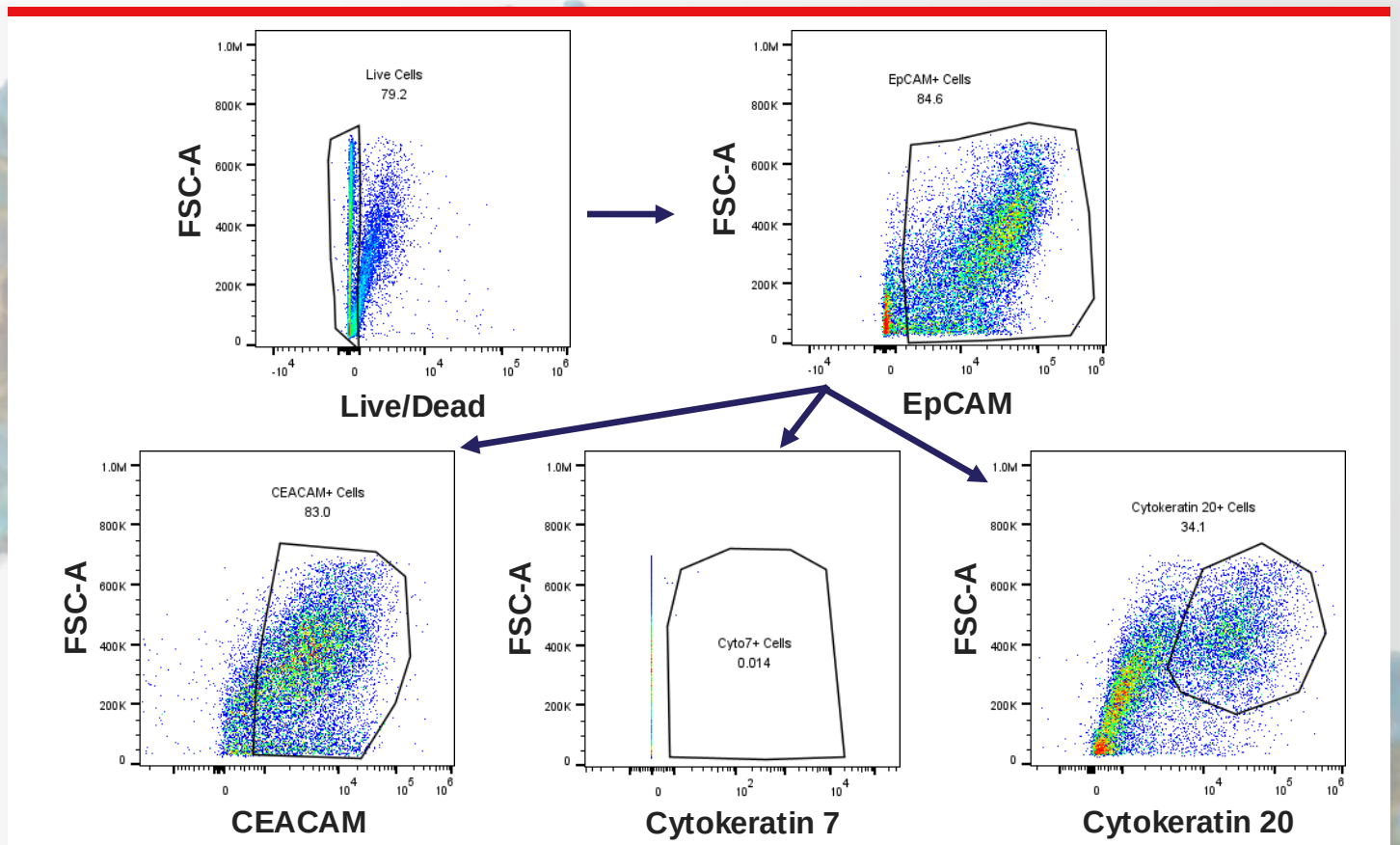


Endothelial cells and immune cells are depleted during long-term tumoroid culture, as described in Paul, C.D. et al, Scientific Reports 15: 3933 (2025).

Example flow cytometry analysis of tumoroid samples



Flow cytometry data for the expression of epithelial (EpCAM, CEACAM), immune (CD45), and endothelial (CD31) markers from matched colorectal tumor and tumoroid (P7) sample HuCo031721. Figure cropped from Supplementary Figure S3d of Paul, C.D. et al, Scientific Reports 15: 3933 (2025).



Flow cytometry profiling of the expression of extracellular (EpCAM, CEACAM) and intracellular (cytokeratin 7, cytokeratin 20) markers used for characterizing established colorectal tumoroid line HuCo021320. Circled regions indicate positive populations set from fluorescence minus one (FMO) controls. Inset shows percentage of positive cells compared to the parent gate.

Troubleshooting

Problem/question	Solution/answer
Tumoroids are too large after dissociation and are clogging the flow cytometer.	Dissociation in StemPro™ Accutase™ can be performed for up to 30 minutes in a water bath or on a shaker at 37ºC if tumoroids are not sufficiently singularized after the typical 10 minute dissociation period. Tumoroid lines that are difficult to dissociate can also be briefly vortexed during incubation in Accutase. We recommend passing the dissociated tumor cells through a 40 µm cell strainer prior to processing for flow cytometry. (Note: Count cells after straining for most accurate results.)
The tumoroids are sticking to the side of the pipette tip.	Add 0.1% BSA to wash buffers and DPBS to prevent tumoroids from sticking to the walls of pipette tips.
How do I design a panel for flow cytometry?	For help with designing panels for flow cytometry, see the Molecular Probes Flow Cytometry Panel Design Tool. The panel design tool can help researchers choose fluorescent antibody conjugates for flow cytometry panels in a few easy steps: pick the antibody species reactivity, select up to 14 targets of interest (choices include viability dyes), and choose appropriate lasers and fluorophores.
There is no signal for my antibody, or it is very dim.	Validate and titrate all antibodies using cells with known expression levels before testing samples with unknown expression. See this application note for an example of antibody titration.
Non-specific binding and/or high background observed.	Used verified antibodies and test with biological controls during initial experimental design.
What controls should I prepare for a flow cytometry experiment?	For compensation, prepare a singly stained sample (or compensation beads) for each fluorochrome used. Unstained controls are also helpful to quantify background autofluorescence. In addition, use fluorescence minus one (FMO) controls. These are controls in which cells are stained with nearly all dye or conjugated antibody in the panel, omitting one at a time. Make one FMO control for each color. These controls are important for properly setting gates on flow cytometry data and are discussed in detail here.

Thank you