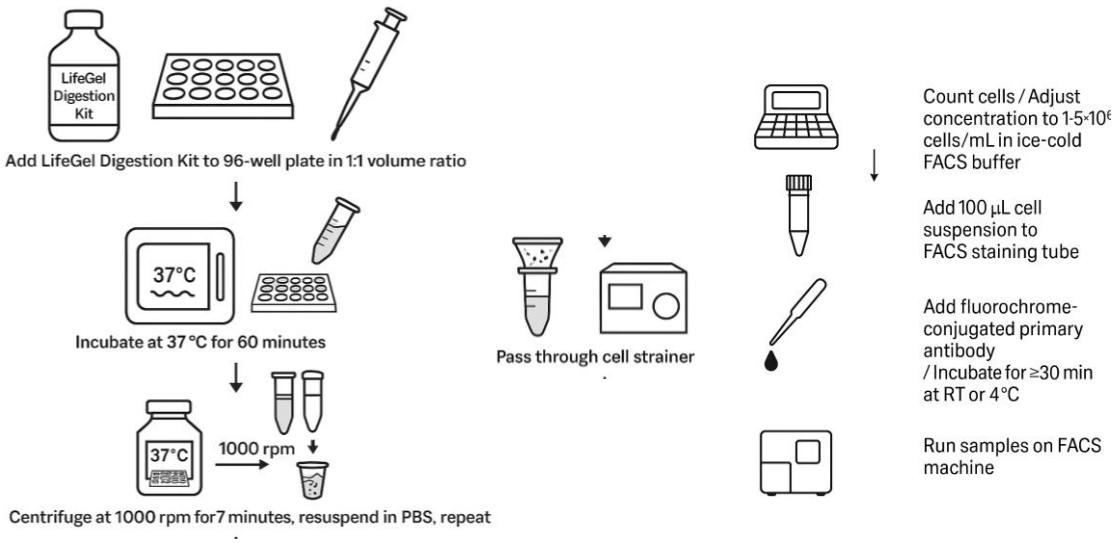
In Vitro Application

Flow Cytometry Staining Protocol for Cells Isolated from LifeGel-Grown Spheroid Cultures.

Three-dimensional (3D) spheroid cultures more accurately mimic the in vivo tumor microenvironment compared to traditional two-dimensional (2D) monolayer cultures. LifeGel-based spheroid models support complex cell-cell and cell-matrix interactions, making them ideal for studying cell behavior, drug responses, and phenotypic heterogeneity in a more physiologically relevant context.

Flow cytometry (FACS) is a widely-used technique for immunophenotyping cells by detecting surface and intracellular markers at the single-cell level. However, successful FACS analysis of cells from LifeGel-grown spheroids requires efficient dissociation into a viable single-cell suspension without compromising antigen integrity.

This protocol outlines the steps required to prepare, stain, and analyze cells derived from LifeGel-based spheroids for flow cytometric evaluation. It includes recommendations for cell dissociation, antibody staining, and optional intracellular marker detection, enabling accurate assessment of cellular phenotypes and subpopulations.



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PROTOCOL:
Cell preparation using LifeGel Digestion Kit [RRLDK4] for Flow Cytometry

Materials required:

- 15 mL and 50 mL Conical Tubes
- Cell Strainer (35–70 µm)
- LifeGel Digestion Kit (cat. No RRLDK4)
- TrypLE or Accutase if needed
- Phosphate-Buffered Saline (PBS) (without Ca^{2+}/Mg^{2+})
- FACS buffer (PBS containing 0.5–2% BSA or 5–10% FBS, and 0.1% sodium azide)
- Serological pipettes
- Orbital shaker
- Centrifuge

1. Add LifeGel Digestion Kit to LifeGel in a 1:1 volume ratio. (For 48-well 300 µL, for 96-well 150 µl).
2. To aid digestion, pipette well contents up and down a few times.
3. If most or all of the plate is to be digested, incubate it at 37°C on an orbital shaker (e.g. 30-60 rotations per minute) for 60 minutes. If only a few wells are to be digested, transfer well contents to a vial and vortex every 15 minutes for 60 minutes (time depends on cell line and size of spheroids).
4. Centrifuge the cell suspension at 1000 rpm for 7 minutes. Carefully discard the supernatant, then resuspend the cell pellet in PBS or if additional digestion of spheroids is needed use TrypLE or Accutase depending on further stainig.
5. Repeat the centrifugation step once more under the same conditions. After the second spin, resuspend the cells again in fresh PBS.
6. Pass the cell suspension through a cell strainer to eliminate debris and cell clumps.
7. Count the single-cell suspension using a hemocytometer or an automated cell counter.
8. Adjust the concentration to 1–5 × 10^6 cells/mL in ice-cold FACS buffer (PBS containing 0.5–2% BSA or 5–10% FBS, and 0.1% sodium azide*).

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PROTOCOL:

FACS Staining Protocols - Direct immunostaining of surface antigens

Materials required:

- FACS Tubes (5 mL polystyrene round-bottom with caps)
- Phosphate-Buffered Saline (PBS) (without Ca^{2+}/Mg^{2+})
- FACS Buffer – PBS + 0.5–2% BSA or 5–10% FBS, and 0.1% sodium azide*
- Fc Receptor Blocking Reagent *(optional)*
- Fluorophore-Conjugated Antibodies
- Viability Dye *(optional but recommended)*.
- Fixation Buffer – e.g., 2–4% Paraformaldehyde (PFA) in PBS.

*Note: Do **not** include sodium azide if downstream functional assays are planned, as it inhibits metabolic activity.

1. Add 100 µL of the cell suspension to each staining tube.
2. Fc Receptor Blocking (Optional) - If cells express high levels of Fc receptors, add 100 µL of Fc block (1:50 dilution in FACS buffer) to each sample. Incubate on ice for 20 minutes.
3. Centrifuge at 1500 rpm for 5 minutes at 4°C and discard the supernatant.
4. Add 0.1–10 µg/mL of fluorochrome-conjugated primary antibody. Dilute as needed in FACS buffer. Incubate for at least 30 minutes at room temperature or 4°C in the dark.
   Note: This step may require optimization.
5. Wash cells with ice-cold FACS buffer by centrifugation (1500 rpm, 5 minutes) and resuspend cells in 200 µL to 1 mL of ice-cold FACS buffer. Keep samples on ice or at 4°C in the dark until analysis.
6. Fixation (Optional) - To preserve cells for several days.
7. After step 7, instead of resuspending in FACS buffer, add 100 µL of 1–4% paraformaldehyde.
8. Incubate for 10–15 minutes at room temperature.
9. Centrifuge at 1500 rpm for 5 minutes, discard the fixative, and resuspend cells in 200 µL to 1 mL of ice-cold PBS.
   Note: Fixation conditions may require optimization depending on the sample type.
10. Analyze cells as soon as possible, preferably on the same day. For short-term storage (up to 16 hours), fixed samples can be stored at 4°C in the dark in 1–4% paraformaldehyde to maintain sample integrity.

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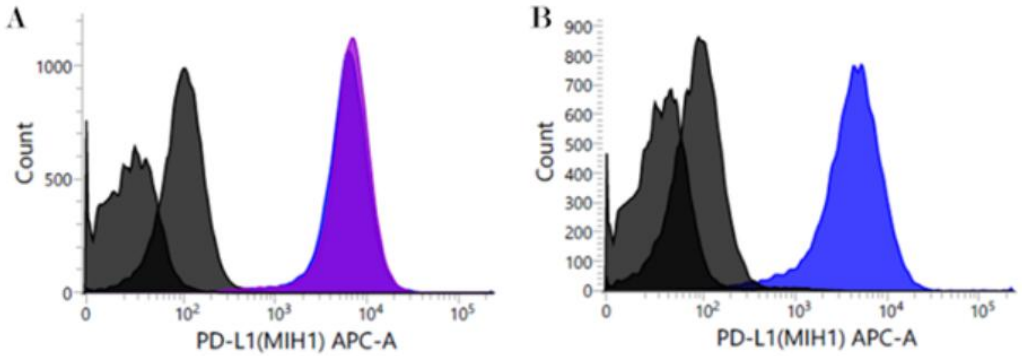
Flow cytometry results with detected human PD-L1 on the surface of thehPD-L1 RKO cells.

(A) Results from 2D cell culture, blue - RKO 2D MIH1 (hPD-L1),purple - RKO 2D after LDK treatment MIH1 (hPD-L1), grey - isotype control, grey (inthe range of 0-102) - unstained cells, alive.

(B) Results from 3D cell culture, blue - RKO3D MIH1 (hPD-L1), grey - isotype control,grey (in the range of 0-102) - unstained cells,alive. All of the probes were stained with DAPI (alive cells), instead of unstained samples.



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PROTOCOL:

FACS Staining Protocols - intracellular antigens staining

1. Prepare a single-cell suspension following the Cell preparation using LifeGel Digestion Kit for Flow Cytometry protocol in staining buffer.
2. Dispense 100 µL of the cell suspension into individual staining tubes for each condition, including appropriate controls.
3. To fix cells choose one of the following methods:

   Formaldehyde Fixation:
   - Resuspend cells in 1–4% formaldehyde in PBS.
   - Incubate for 10–15 minutes at room temperature.
   - Wash cells with PBS + 1% BSA or 2% FBS.

   Methanol/Acetone Fixation (alternative):
   - Add ice-cold methanol (or acetone) dropwise to cell pellet while gently vortexing.
   - Incubate at –20°C for 10 minutes.
   - Centrifuge and wash with PBS + 1% BSA.
4. Incubate cells in permeabilization buffer. For nuclear staining use Triton-X100 or NP-40 (0.1-1%) in PBS + 1% BSA; for antigens in the cytoplasm or the cytoplasmic face of the plasma membrane and soluble nuclear antigens use Tween 20 or Saponin for 15 minutes at room temperature.
5. Incubate cells in blocking buffer (e.g., PBS + 1–2% BSA or FBS) for 10–15 minutes at room temperature.
6. Add fluorochrome-conjugated antibody or primary antibody (if using indirect staining).
7. Incubate for 30 minutes at 4°C or room temperature, protected from light.
8. If using an unconjugated primary antibody, wash and follow with a fluorochrome-conjugated secondary antibody under the same conditions.
9. Wash cells 2–3 times with permeabilization buffer or PBS (depending on fixation method).
10. Resuspend in 100–200 µL FACS buffer for analysis.
11. Keep samples on ice and analyze on a flow cytometer within 24 hours.
12. For fixed samples, store at 4°C in the dark if acquisition is delayed.

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TIPS:

- Pre-formulated fixation, fixation/permeabilization, and permeabilization/wash buffers—as well as complete intracellular staining kits—are available from major flow cytometry suppliers such as BD Biosciences, Abcam, R&D Systems, and others.
- For antigens located near the plasma membrane or soluble cytoplasmic proteins, use mild permeabilization without fixation to preserve antigen integrity.
- Cytoskeletal proteins, viral antigens, and certain enzymes typically require fixation with high concentrations of acetone, alcohol, or alternatively formaldehyde for optimal detection.
- Detecting antigens within cytoplasmic organelles or granules often requires customized fixation and permeabilization protocols, tailored to maintain epitope accessibility while preserving cellular architecture.