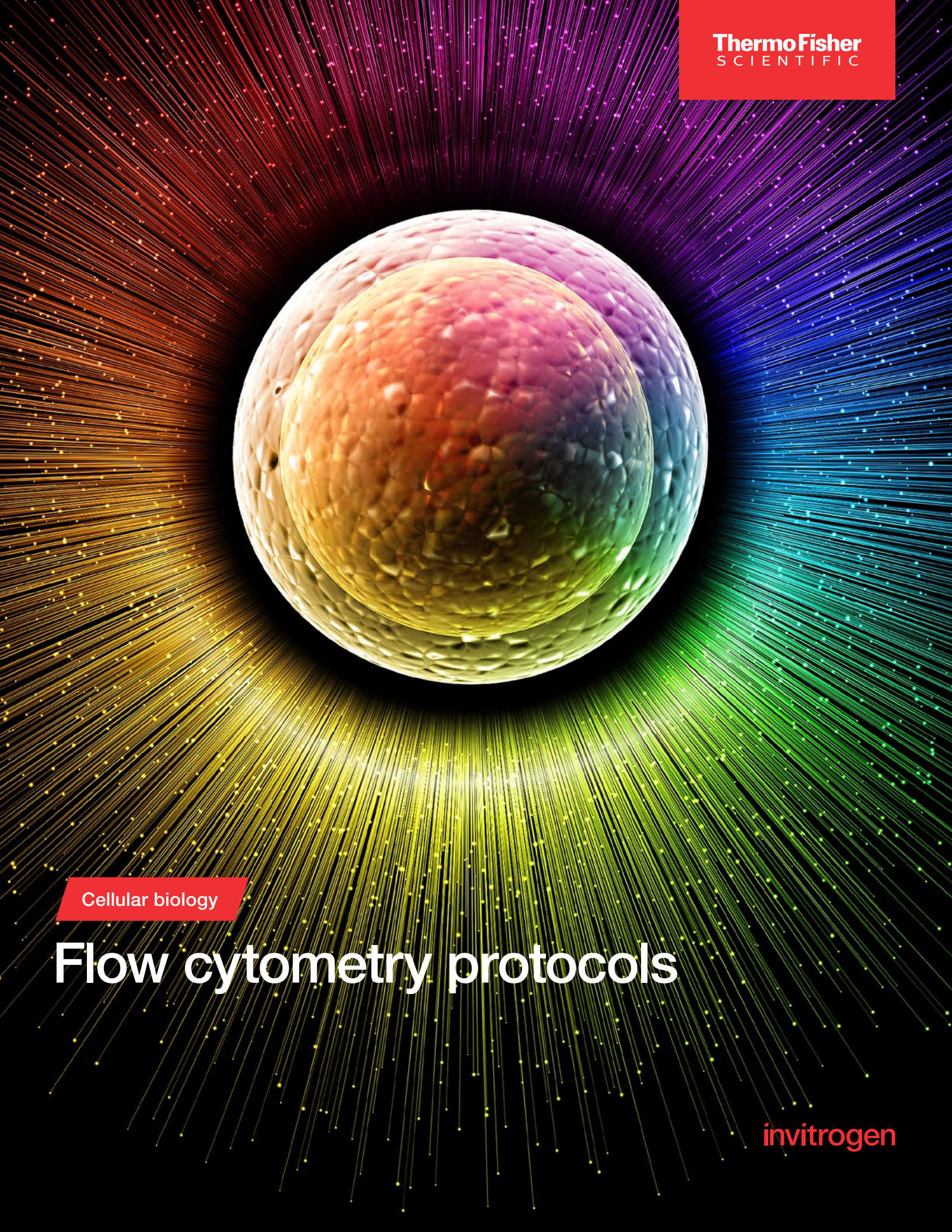
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Flow cytometry protocols
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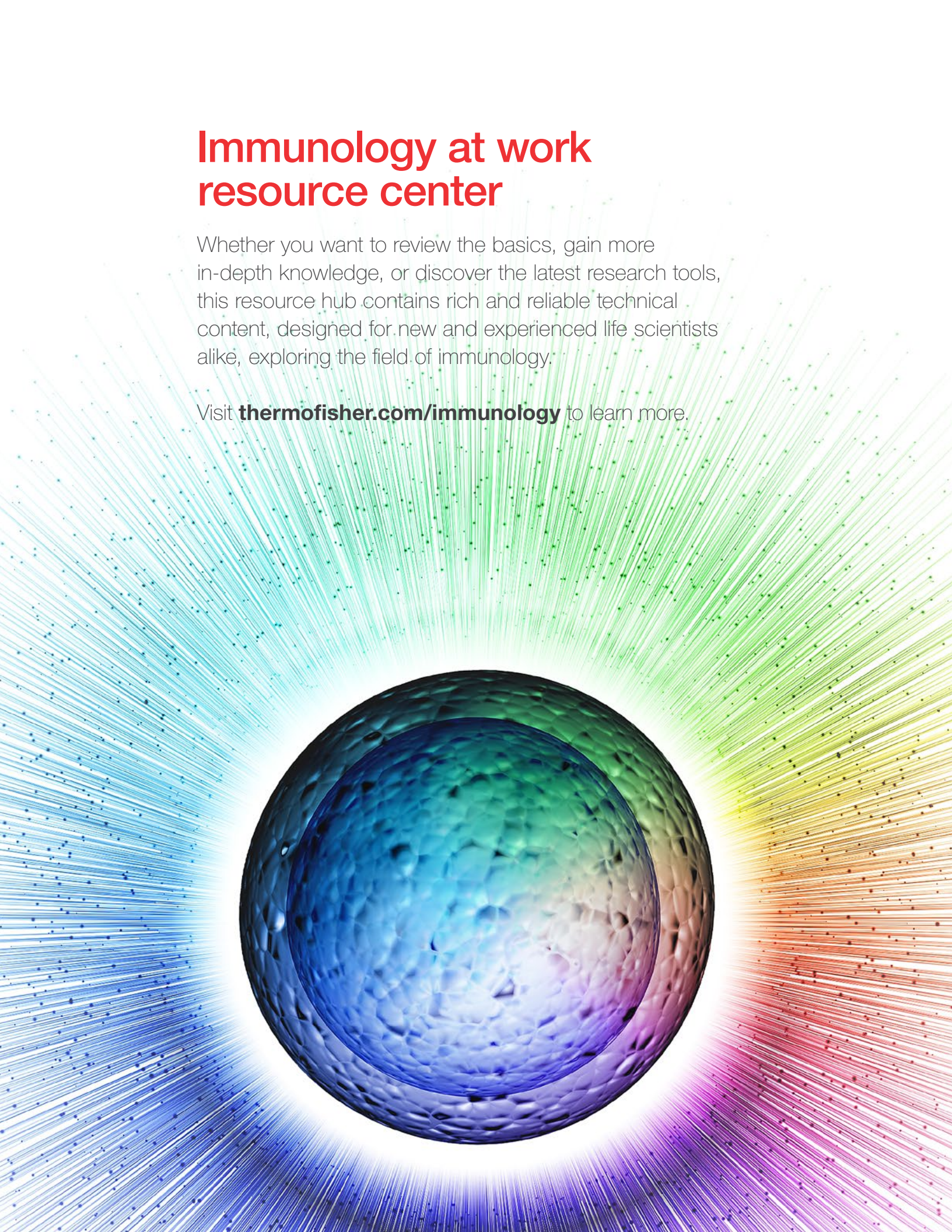

Contents

Flow cytometry

Sample preparation

Isolation of human peripheral blood mononuclear cells from whole blood

Introduction

Single-cell suspensions are required for all flow cytometry assays. Thus, peripheral blood cells or cells that grow in suspension are well suited for analysis by flow cytometry. Adherent cell lines, solid tissue samples, and tumors require processing into single-cell suspensions before they can be analyzed. Numerous protocols are available and may involve enzymatic digestion or mechanical dissociation of the tissue. Care should be used when chelation or enzymatic digestion are used, as these may result in the destruction of the antibody epitope. In all situations, removing cell clumps, dead cells, and debris is essential to eliminate false positives and obtain results of the highest quality.

Note

This protocol is intended for use with the specific products mentioned within it. Substituting different products is not recommended.

Materials

- Gibco™ PBS (Phosphate-buffered saline), pH 7.4, (Cat. No. 10010023)
- Ficoll-Paque™ PLUS medium (GE Healthcare, Cat. No. 17-1440-02) or other density separation medium
- Flow Cytometry Staining Buffer (Cat. No. 00-4222) or other buffer of choice
- 15- or 50-mL conical centrifuge tubes

Note

If cells are to be cultured, perform all steps using aseptic technique and buffers that do not contain azide.

Experimental procedure

1. Dilute blood sample at least 1:1 with PBS in a conical tube.
2. Underlay the diluted sample with a volume of Ficoll that is equal to the original sample volume.
3. Centrifuge at 400 x *g* for 20 minutes at room temperature with the brake OFF.
4. Harvest PBMC located at the interface of the PBS and Ficoll layers into a fresh tube.
5. Fill the tube with PBS to wash the cells.
6. Centrifuge the cells at 300–400 x *g* for 4–5 minutes at 2–8°C. Discard supernatant.
7. Resuspend the cell pellet in an appropriate volume of Flow Cytometry Staining Buffer or buffer of choice and perform a cell count and viability analysis.
8. Centrifuge cells as in Step 4 and resuspend in appropriate volume of Flow Cytometry Staining Buffer or buffer of choice so that the final cell concentration is 1 x 10^7 cells/mL (other cell concentrations may be appropriate for different experiments).

Mouse bone marrow cell isolation

Introduction

This protocol describes the isolation of cells from the marrow of bone [1,2]. As compared with whole blood, bone marrow yields a more complex set of cells, including hematopoietic stem cells–responsible for the production of leukocytes, erythrocytes, and thrombocytes–and stromal cells, such as endothelial cells, fibroblasts, macrophages, osteoblasts, osteoclasts, and adipocytes.

Materials

- Cell culture dishes (e.g., Thermo Scientific Nunc EasYDish Dishes, 100 mm, Cat. No. 150466)
- Lab wipes (e.g., Fisher Scientific Kimberly-Clark Professional Kimtech Science Kimwipes Delicate Task Wipers, 1-PlyFisher)
- Ethanol (e.g., Absolute Ethanol, 200 Proof, Molecular Biology Grade)
- RPMI Complete Media
  - RPMI 1640 medium (e.g., Gibco BenchStable RPMI 1640, Cat. No. A4192301)
  - 2 mM L-glutamine (e.g., Gibco L-Glutamine, 200 mM, Cat. No. 25030149)
  - Fetal bovine serum (e.g., Gibco Fetal Bovine Serum, Cat. No. 26140087)
  - **Optional** Penicillin-streptomycin (e.g., Gibco Penicillin-Streptomycin (5,000 U/mL), Cat. No. 15070063, or Gibco Penicillin-Streptomycin (10,000 U/mL), Cat. No. 15140122)
- Scalpel and blades
- 5–10 mL syringe (e.g., Fisherbrand Sterile Syringes for Single Use, 10 mL)
- 25-gauge needle (e.g., Fisher Scientific BD General Use and PrecisionGlide Hypodermic Needles)
- 70 µm cell strainers (e.g., Fisherbrand Sterile Cell Strainers)
- 50 mL conical tubes (e.g., Thermo Scientific Nunc 50 mL Conical Sterile Polypropylene Centrifuge Tubes, Cat. No. 339652)
- Red blood cell (RBC) lysis buffer (e.g., Invitrogen eBioscience 1X RBC Lysis Buffer, Cat No. 00-4333-57 or Invitrogen eBioscience 10X RBC Lysis Buffer (Multi-species), Cat. No. 00-4300-54)
- Cell counter (e.g., Invitrogen Countess 3 Automated Cell Counter, Cat. No. AMQAX2000)
- Gibco™ PBS (Phosphate-buffered saline), pH 7.4, (Cat. No. 10010023)

This protocol is continued on page 3

Procedure

Cell isolation

1. Under sterile conditions, isolate bone marrow femurs from mice, and place into a sterile cell culture dish.

2. Use forceps and small scissors to cut away the muscle and fibrous tissues from the bone. If muscles are still intact, wipe the bones with a Kimwipe saturated with 70% ethanol to remove any excess muscle fibers.

3. Add 5–7 mL RPMI complete media to a new sterile cell culture dish. Prepare complete RPMI 1640 medium by supplementing RPMI 1640 medium with fetal bovine serum to a final concentration of 10%, 2 mM L-glutamine (if using medium not currently supplemented with GlutaMAX).

4. Cut both femur ends with a scalpel or sharp scissors so that both ends are clean.

5. Attach a 25-gauge needle to a 10 mL syringe. Draw up 5 mL RPMI complete media into the syringe.

6. Using forceps, hold the bone above the cell culture dish containing 5–7 mL RPMI complete media, and carefully flush out the marrow into the cell culture dish using the syringe containing 5 mL RPMI complete. Repeat flushing as necessary until the bone is white, which indicates that all the marrow has been removed. Repeat this step for all bones.

7. Place a 70 µm cell strainer on top of a new 50 mL conical tube, held upright in a tube rack.

8. Using a sterile serological pipet, transfer all marrow from cell culture dish into the strainer. Add more RPMI complete media to the cell culture dish and continue transferring any leftover marrow to the strainer.

9. Using just the plunger from a 10 mL syringe (DO NOT TOUCH the black rubber end of the plunger; keep it sterile), mash the marrow contained in the 70 µm cell strainer in a downward circular motion. Verify there are no large pieces of bone marrow left.

Protocol tip

After flushing the femur bones, they may be crushed by mortar and pestle for additional cells. Bone marrow can also be isolated from crushed pelvic bones and tibias.

Optional

Supplement media with 1% penicillin-streptomycin (5,000 units/mL).

This protocol is continued on page 4

Mouse bone marrow cell isolation, cont.

10. Rinse any leftover marrow from the plunger end with RPMI complete media into the strainer to collect as many cells as possible.

11. Rinse strainer with 5–7 mL RPMI complete media. Remove the strainer and cap the conical tube containing the sample.

12. Spin down cells at 600 x *g* for 4 minutes at 4°C. Discard the supernatant.

13. Add about 5 mL RPMI complete media to the cell pellet, and pipet up and down to resuspend.

14. Place a new 70 µm cell strainer on top of a new 50 mL conical tube in a rack.

15. Strain the resuspended marrow sample through the cell strainer and into the conical tube. Count viable cells if desired.

16. Resuspend cell sample to desired cell concentration with appropriate media.

Cell counting

1. Lyse a small sample of the red blood cells with an RBC lysis buffer to easily count immune cells. Add 1 mL 1X RBC lysis buffer to 100 µL sample.

2. Incubate at room temperature for 4–5 minutes with occasional shaking or rotating.

3. Add 2–3 mL 1X PBS to stop the reaction.

4. Spin down the cells at 600 x *g* for 4 minutes at 4°C. Discard the supernatant.

5. Add about 500 µL complete RPMI to pellet, and pipet up and down to break the pellet.

6. Count viable cells with a hemocytometer or the Invitrogen Countess 3 Automated Cell Counter.

Optional

Proceed to immune cell isolation protocols using Invitrogen MagniSort Kits or Invitrogen Dynabead Kits or to cell stimulation protocols.

References

1. *J Biol Methods* (2014) 1(1):e1.
2. *Bio Protoc* (2015) 5(20):e1631. PMID 27441207.

Mouse spleen cell isolation protocol

Introduction

The spleen is the site for hematopoiesis, red blood cell clearance, and immunologic functions, and therefore, a good source of cells. It filters cell debris, pathogens, and irregular cells. It is a source for both red blood cells and leukocytes and for several immune cell subtypes including granulocytes, monocytes, macrophages, dendritic cells (DCs), NK cells, T cells, and B cells. Leukocytes can be found in the crude spleen preparation. DCs and macrophages can be isolated by enzymatic release of the cells from the crude cell preparation.

Materials

- Red blood cell (RBC) lysis buffer (e.g., eBioscience 10X RBC Lysis Buffer (Multi-species), Cat. No. 00-4300-54)
- Gibco™ PBS (Phosphate-buffered saline), pH 7.4, (Cat. No. 10010023)
- Scalpel and blades
- Cell culture dishes (e.g., Thermo Scientific Nunc EasYDish Dishes, 100 mm, Cat. No. 150466)
- Disposable transfer pipette (e.g., FisherBrand Disposable Graduated Transfer Pipettes)
- 50 mL conical tubes (e.g., Thermo Scientific Nunc 50 mL Conical Sterile Polypropylene Centrifuge Tubes, Cat. No. 339652)
- 70 µm cell strainers (e.g., Fisherbrand Sterile Cell Strainers)
- 5–10 mL syringe (e.g., Fisherbrand Sterile Syringes for Single Use, 10 mL)
- Hank's balanced salt solution (e.g., Gibco Hank's Balanced Salt Solution, HBSS 10X, Cat. No. 14060040)

Preparation of myeloid cells

- Collagenase (e.g., Gibco Collagenase, Type IV, Cat. No. 17104019)
- DNase (e.g., Thermo Scientific DNase I solution, Cat. No. 90083)
- Fetal Bovine Serum (e.g., Gibco Fetal Bovine Serum, Cat. No. 26140087, or Fetal Bovine Serum One Shot format, Cat No. A3160401)
- EDTA (e.g., UltraPure 0.5M EDTA, pH 8.0, Cat. No. 15575020)

This protocol is continued on page 6

Mouse spleen cell isolation protocol, cont.

Procedure

1. Obtain fresh whole mouse spleen.
2. Place mouse spleen into petri dish with 5 mL HBSS buffer.
3. Carefully mince the spleen into small pieces (~0.2 cm^2) with a razor or scalpel blade.
4. For preparation of myeloid cells (continue to step 5 for crude preparation): Incubate the excised spleen pieces for 20–30 min at 37°C with 5 mL of HBSS containing Collagenase IV (100 U/mL) and DNase (20 µg/mL) solution with 1% FBS.
5. Add EDTA 1 mM/mL for 5 minutes at room temperature to stop the enzymatic reaction.
6. Place cell strainer over a 50 mL conical tube.
7. With a disposable transfer pipette, transfer the excised spleen into the cell strainer.
8. With the plunger end of a syringe, mash or press the spleen through the strainer. Add 5–10 mL PBS if necessary.
9. Wash the cells through the strainer with excess PBS. Repeat step 6 and 7, if needed.
10. Centrifuge the cells at 400–600 x *g* for 5 minutes at 4°C; discard the supernatant.
11. Resuspend the cell pellet in 2–5 mL of cold 1x RBC Lysis buffer.
12. Incubate the suspension for 5 minutes on ice.
13. Wash the cell suspension with 10–20 mL cold PBS.
14. Centrifuge the cells at 400–600 x *g* for 5 minutes at 4°C; discard the supernatant.
15. Resuspend the cell pellet in PBS at 2–3 x 10^6 cells/mL.

Critical note

Perform steps 1–8 at room temperature and steps 9–13 on ice with cold buffers.

Whole blood staining protocol for flow cytometry analysis

Introduction

This protocol describes the immunophenotyping of cells in a whole blood sample by flow cytometry with minimal sample manipulation, thereby preserving cell structure and function while also reducing cell loss [1,2]. Whole blood samples stained with antibodies for cell-surface markers can be analyzed directly on a flow cytometer. To facilitate this analysis, red blood cells (RBCs) in the whole blood sample can be lysed after antibody staining. The ratio of RBCs to mononuclear cells in whole blood is approximately 600:1 (depending on sample and species); thus, when RBCs are present, the acquisition of many more events is required to observe rare cell events [1,3–4]. After RBC lysis, cells in the whole blood sample can be permeabilized and stained with antibodies for intracellular markers for subsequent analysis by flow cytometry.

Materials

- Blood collection tubes with anticoagulant, either heparin, K3EDTA, or K2EDTA tubes
- 12 x 75 mm round-bottom polystyrene tubes (e.g., Thermo Scientific Samco 12 x 75 mm Disposable Culture Tubes, Cat. No. 12-007S)
- Flow cytometry staining buffer (e.g., Invitrogen eBioscience Flow Cytometry Staining Buffer, Cat. No. 00-4222-26)
- Fluorophore-labeled primary antibodies (e.g., validated Invitrogen Flow Cytometry Antibodies)
- Gibco™ PBS (Phosphate-buffered saline), pH 7.4, (Cat. No. 10010023)
- Red blood cell (RBC) lysis buffer (e.g., Invitrogen eBioscience 1X RBC Lysis Buffer, Cat No. 00-4333-57 or Invitrogen eBioscience 10X RBC Lysis Buffer (Multi-species), Cat. No. 00-4300-54)
- Permeabilization buffer (e.g., Invitrogen eBioscience Permeabilization Buffer (10X), Cat. No. 00-8333-56)
- **Optional** 0.45 µM cell strainer (e.g., Falcon Cell Strainer, Cat. No. 08-771-1)
- **Optional** Non-fixable cell viability dye (impermeant nucleic acid dye) (e.g., Invitrogen SYTOX Dead Cell Stain Sampler Kit, for flow cytometry, Cat. No. S34862)
- **Optional** Fix/Lyse solution (e.g., Invitrogen eBioscience 1-Step Fix/Lyse Solution (10X), Cat. No. 00-5333-54)

This protocol is continued on page 8

Whole blood staining protocol for flow cytometry analysis, cont.

Procedures

Whole blood preparation

1. Collect whole blood into heparinized tubes or into tubes containing 1 µL 10% potassium EDTA per 100 µL of whole blood.

2. For each antibody staining experiment, aliquot 100 µL unlysed whole blood into a 12 x 75 mm tube. For each single-color control (used to set compensation), aliquot 100 µL unlysed whole blood into a 12 x 75 mm tube. Reserve additional unlysed whole blood for an unstained control sample.

Cell surface staining

1. Prepare desired antibody cocktail—containing fluorophore-labeled primary antibodies for cell-surface markers—in Flow Cytometry Staining Buffer. We recommend testing antibody dilutions from 1:50 to 1:100 initially. **Protect from light**.

2. Add the antibody cocktail to a 100 µL aliquot of whole blood.

3. Incubate for 30 minutes to 1-hour at 2–8°C with rotation. **Protect from light**.

4. Wash samples with 2 mL Flow Cytometry Staining Buffer.
   This wash step can be repeated.

5. Centrifuge at 500 x *g* for 5 minutes at 4°C. Discard the supernatant and resuspend in 500 µL Flow Cytometry Staining Buffer.

   Optional (Do not include this step when using fixation or lysis reagents.) An impermeant nucleic acid dye such as a SYTOX Dead Cell Stain can be added to gate viable cells. Do not add a wash step after adding this impermeant nucleic acid dye. Add 0.5 µL of SYTOX Dead Cell Stain solution per 500 µL sample, and mix well. Incubate for 20 minutes at room temperature, protected from light.

6. **Optional** Filter cells at room temperature through 0.45 µm cell strainer to remove large debris if running directly on a flow cytometer.

Protocol tip

As compared with the antibody concentrations required in staining protocols for lysed blood samples or serum, the antibody concentrations used in whole blood staining protocols should be higher.

Protocol tip

The No-Wash, No-Lyse Detection of Leukocytes In Human Whole Blood on the Attune NxT Flow Cytometer application note provides an example of gated, stained leukocytes. To access the application note, visit **thermofisher.com/nowashnolysewholeblood**

This protocol is continued on page 9

Optional: Red blood cell lysis

1. For 100 µL of whole blood, add 2 mL of room temperature 1X 1-Step Fix/Lyse Solution, and invert gently. Lysing red blood cells creates easier gating conditions during flow cytometry analysis, though it can potentially interfere with immune cell function. The 1-Step Fix/Lyse Solution is formulated to lyse non-nucleated erythrocytes while maintaining a fixed and labeled leukocyte population.
2. Incubate for 15 to 60 minutes at room temperature, protected from light.
3. Store samples in buffer for up to 48 hours at 2–8°C, protected from light. Single color-stained samples, which are used to set compensation, and an unstained sample should be stored under the same conditions.

Flow cytometry analysis

1. Analyze samples on a flow cytometer equipped with appropriate filters, corresponding to the selected fluorophore labels.
2. To achieve a CV of 1–10%, a minimum of 10^7–10^5 cell events must be acquired to find an event that occurs at a frequency of 0.1% in the cell population. The acquisition rate should be set to slowly collect events (e.g., 200–500 µL/min collection rate on the Invitrogen Attune Flow Cytometers).

Protocol tip

The concentration of white blood cells varies from 4,000 to 10,000 cells per microliter [3]. We recommend calculating the number of events required for a statistically significant result to estimate the amount of blood needed for an experiment [5].

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Whole blood staining protocol for flow cytometry analysis, cont.

Intracellular staining

1. Before staining with antibodies that recognize intracellular markers, we recommend lysing the red blood cells and fixing the leukocyte population as described above using 2 mL 1X 1-Step Fix/Lyse Solution per 100 µL of whole blood. Invert gently, and incubate for 15 to 60 minutes at room temperature, protected from light.
2. Dilute 10X Permeabilization Buffer to 1X by mixing 1-part 10X concentrate with nine parts distilled water.
3. Centrifuge sample at 500 x *g* for 5 minutes at room temperature. Discard the supernatant.
4. Resuspend the pellet with 2 mL 1X Permeabilization Buffer, and centrifuge at 500 x *g* for 5 minutes at room temperature. Discard the supernatant. Repeat once. **Protect from light**.
5. Resuspend the cell pellet with 100 µL of Flow Cytometry Staining Buffer.
6. Prepare desired antibody cocktail—containing labeled primary antibodies for intracellular markers—in Flow Cytometry Staining Buffer. **Protect from light**. We recommend trying antibody dilutions from 1:50 to 1:100 initially.
7. Add the antibody cocktail to the cell suspension.
8. Incubate for 20–60 minutes at room temperature with rotation. **Protect from light**.
9. Wash samples with 2 mL Flow Cytometry Staining Buffer. This wash step can be repeated.
10. Centrifuge at 500 x *g* for 5 minutes at 4°C. Resuspend with 500 µL Flow Cytometry Staining Buffer.
11. Analyze samples by flow cytometry.

Protocol tip

Fixation and permeabilization may affect antibody specificity. Refer to the Intracellular Staining Buffer Selection Guide at **thermofisher.com/intracellularstainingbuffer** and specifically the "Fixation & Permeabilization" column for a non-exclusive list of antibodies that will work under fixation conditions.

References

1. *Methods* 134–135:149 (2018). PMID 29269150.
2. *Oncotarget* 10(65):6969 (2019). PMID 31857851.
3. *Clinical Methods: The History, Physical, and Laboratory Examinations* (1990) 3rd edition. Boston: Butterworths; 1990. Chapter 153.
4. *Clin Diagn Lab Immunol* 9(3):708 (2002). PMID 11986282.
5. *J Oncol* 2010:426218 (2010). PMID 20049168.

Red blood cell lysis

Introduction

Before using peripheral blood or certain lymphoid tissue suspensions for flow cytometric analysis or for *in vitro* assays, red blood cells (RBC) should be removed. The 1X RBC Lysis Buffer (Cat. No. 00-4333) and 10X RBC Lysis Buffer (Multi-species) (Cat. No. 00-4300) are formulated for optimal lysis of erythrocytes in single-cell suspensions of human peripheral blood and mouse tissue (such as spleen). The buffers contain ammonium chloride, which lyses RBC with minimal effect on leukocytes. When using human peripheral blood for flow cytometric analysis, the RBC lysing step can be incorporated into the staining protocol. The 1-step Fix/Lyse Solution (10X) (Cat. No. 00-5333) is formulated for the combined lysis of RBC and fixation of peripheral blood leukocytes after staining with fluorochrome-conjugated antibodies. All of the RBC lysis reagents are compatible with fluorochrome-conjugated antibodies.

Red Blood Cell (RBC) lysis protocols

- Protocol A: Using 1X or 10X RBC Lysis Buffers
  - A1 Antibody Staining Followed by Lysis of Whole Peripheral Blood
  - A2 Bulk Lysis of Human Whole Blood
  - A3 Lysis of Mouse/Rat Spleen or Bone Marrow Cells
- Protocol B: Using 1-step Fix/Lyse Solution

Protocol A: Using 1X or 10X RBC Lysis buffers

Both the 1X and 10X RBC Buffers are designed to lyse RBC in whole blood (using heparin or EDTA as the anti-coagulant) or tissue preparations using ammonium chloride-based osmotic shock. The 10X RBC Lysis Buffer (Multi-species) is specially formulated for optimal lysis of RBC in peripheral blood. It has been validated to work on whole blood from human, mouse, rat, canine and non-human primate sources. The 1X RBC Lysis Buffer is optimized for lysis of RBC in human peripheral blood or single-cell suspensions of mouse hematopoietic tissues such as spleen or bone marrow.

Note

This protocol is intended for use with the specific products mentioned within it. Substituting different products is not recommended.

General notes

- Before use, the 10X RBC Lysis Buffer (Multi-species) must be diluted 1:10 with room temperature, reagent-grade water.
- The 10X RBC Lysis Buffer (Multi-species) has been shown to work equivalently in blood collected using either heparin or EDTA as the anti-coagulant.
- In general, a small number of residual RBC does not interfere with subsequent use of cells and can be gated out during flow cytometric analysis; however, a second round of lysis can be performed, if desired.

This protocol is continued on page 12

Red blood cell lysis, cont.

Materials

- 1X Gibco™ PBS (Phosphate-buffered saline), pH 7.4, (Cat. No. 10010023)
- 10X RBC Lysis Buffer (Multi-species) (Cat. No. 00-4300) or 1X RBC Lysis Buffer (Cat. No. 00-4333)
- 50 mL conical tubes
- Flow Cytometry Staining Buffer (Cat. No. 00-4222) or other buffer of choice
- 12 x 75 mm round-bottom test tubes
- Primary antibodies (directly conjugated)

Note

Refer to bulk lysis protocol (Protocol A2) for RBC lysis before antibody staining (page 13).

Experimental procedure

A1. Antibody staining followed by Lysis of whole peripheral blood

1. Aliquot a sample of whole blood into a tube.

Protocol tip

- For human, use 100 µL of blood.
- For mouse, use 50-100 µL of blood.
- For rat, use 50-100 µL of blood.
- For canine, use 100 µL of blood.
- For non-human primate, use 100 µL of blood.

2. Add the antibody(s) needed for staining (in a volume no greater than 50 µL) and mix thoroughly. Refer to Staining Cell Surface Targets for Flow Cytometry Protocols as found in our Best Protocols at **thermofisher.com/cellsurfacetargets**.

3. Incubate for 30 minutes in the dark at room temperature.

4. Add 2 mL of room temperature 1X RBC Lysis Buffer, and then pulse vortex or invert to mix.

5. Incubate at room temperature in the dark.

Protocol tip

- For human, incubate for 10–15 minutes.
- For mouse, incubate for 4–10 minutes.
- For rat, incubate for 4–10 minutes.
- For canine, incubate for 10–15 minutes.
- For non-human primate, incubate for 10–15 minutes.

6. After lysis, centrifuge immediately at 500 x *g* for 5 minutes at room temperature. Decant the supernatant.

Note

Observe turbidity to evaluate red blood cell lysis. Once the sample becomes clear, lysis is complete.

7. **Optional** Repeat Steps 4–6.

Note

Step 7 is not typically necessary, as small numbers of residual red blood cells do not interfere with subsequent assays and can be gated out during flow cytometric analysis.

8. Resuspend cells in 2 mL of Flow Cytometry Staining Buffer and centrifuge as in Step 6.

This protocol is continued on page 13

9. Decant the supernatant and resuspend the cell pellet in an appropriate volume of Flow Cytometry Staining Buffer.

10. Analyze the samples by flow cytometry.

A2. Bulk Lysis of human whole blood

1. Add 10 mL of 1X RBC Lysis Buffer per 1 mL of human blood.

2. Incubate for 10–15 minutes at room temperature (no more than 15 minutes).

3. Centrifuge at 500 x *g* for 5 minutes at room temperature. Decant supernatant.

4. Resuspend the pellet in the appropriate volume of Flow Cytometry Staining Buffer or buffer of choice.

5. Perform a cell count and viability analysis.

6. Proceed with cell staining or culture, as desired.

Note

If cells are to be put in culture, perform all steps using aseptic techniques.

Note

Observe turbidity to evaluate red blood cell lysis. Once the sample becomes clear, lysis is complete.

This protocol is continued on page 14

Red blood cell lysis, cont.

A3. Lysis of mouse/rat spleen or bone marrow cells

1. Harvest tissue and prepare a single-cell suspension. Refer to Cell Preparation Protocols for Flow Cytometry found in our Best Protocols at **thermofisher.com/cellpreparation**.
2. Pellet the cells by centrifugation at 500 x *g* for 5 minutes at room temperature and decant the supernatant.
3. Resuspend the pellet in 3–10 mL of 1X RBC Lysis Buffer.
4. Incubate for 4–5 minutes at room temperature.
5. Stop the lysis reaction by adding 20–30 mL of 1X PBS.
6. Centrifuge immediately at 500 x *g* for 5 minutes at room temperature. Decant the supernatant.
7. Resuspend cells in 2 mL of Flow Cytometry Staining Buffer or buffer of choice and centrifuge as in Step 6. Decant supernatant.
8. Resuspend cells in an appropriate volume of Flow Cytometry Staining Buffer or buffer of choice.
9. Perform a cell count and viability analysis.
10. Proceed with cell staining or cell culture, as desired.

Note

The use of 1X RBC Lysis Buffer (Cat. No. 00-4333) is recommended for use with mouse and rat tissues.

Note

If cells are to be put in culture, perform all steps using aseptic techniques.

This protocol is continued on page 15

Protocol B: Using 1-step fix/lyse solution

The 1-step Fix/Lyse Solution both lyses the RBC and fixes the remaining leukocytes. It is ideal for use when antibody-stained blood samples are to be lysed and fixed before analysis. It may also be used to lyse RBC and fix cells before staining with antibodies; however, it is important to confirm that the antibodies to be used will recognize fixed epitopes on the antigens of interest. Mechanical disruption of lymphoid tissue is generally sufficient to release cells to a single cell suspension.

Materials

- 1X Gibco™ PBS (Phosphate-buffered saline), pH 7.4, (Cat. No. 10010023)
- 1-step Fix/Lyse Solution (10X) (Cat. No. 00-5333)
- 50 mL conical tubes
- Flow Cytometry Staining Buffer (Cat. No. 00-4222)
- 12 x 75 mm round-bottom test tubes
- Primary antibodies (directly conjugated)

Experimental procedure

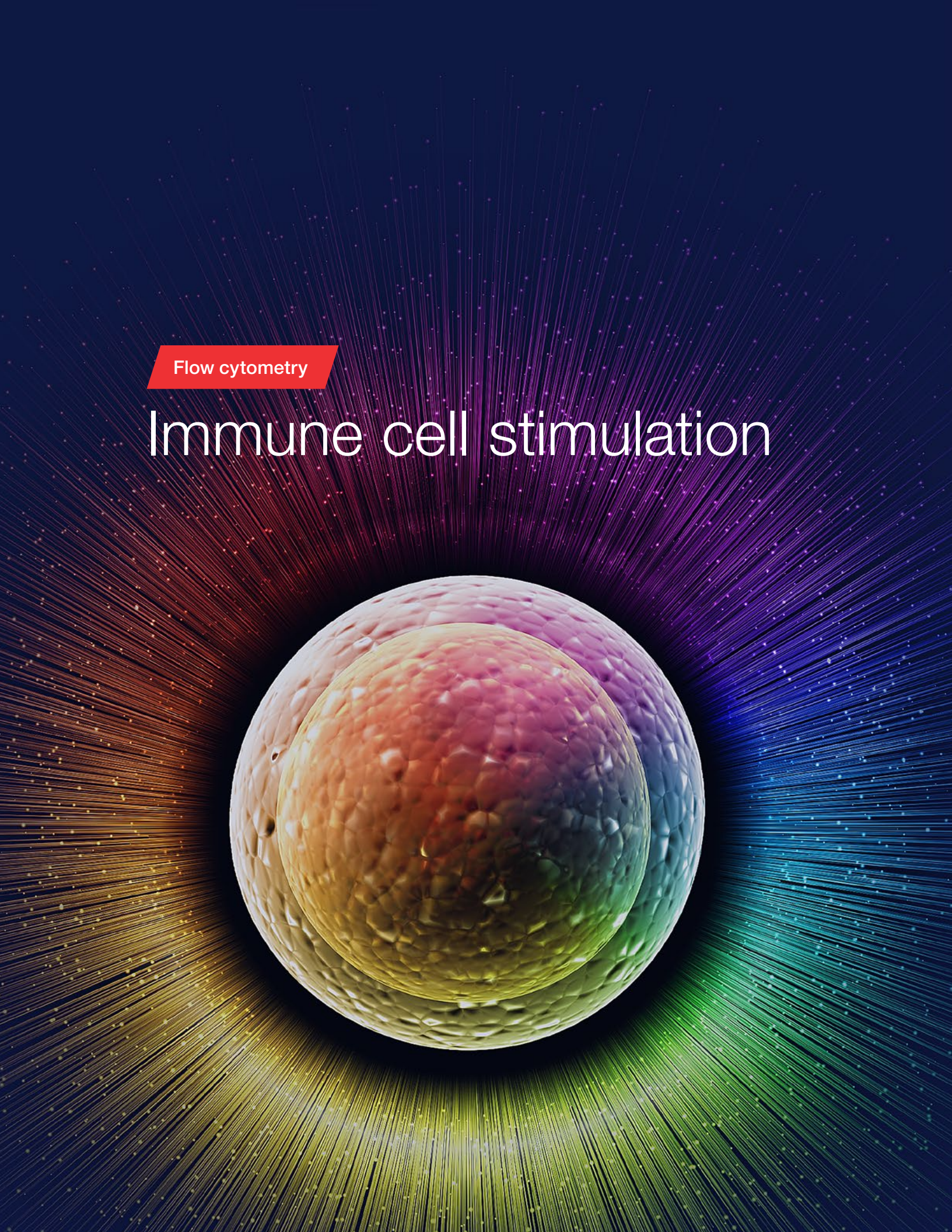
1. To 100 µL of whole blood, add the appropriate antibodies needed for surface staining and mix thoroughly.
2. Refer to Staining Cell Surface Targets for Flow Cytometry Protocols as found in our Best Protocols at **thermofisher.com/cellsurfacetargets**.
3. Incubate for 30 minutes in the dark at room temperature.
4. Add 2 mL of room temperature 1X 1-step Fix/Lyse Solution, then invert gently.
5. Incubate for 15–60 minutes at room temperature in the dark.
6. **Optional** Samples can be stored in 1X 1-step Fix/Lyse Solution for up to 3 days at 2–8ºC in the dark with minimal effect on brightness.

General notes

- Before use, the 1-step Fix/Lyse Solution must be diluted 1:10 with room temperature, reagent-grade water.
- The 1-step Fix/Lyse Solution has been shown to work equivalently in blood collected using either heparin or EDTA as the anticoagulant.

Flow cytometry

Immune cell stimulation



In vitro differentiation of macrophages from monocytes

Introduction

After isolation from whole blood and culture in media with serum, adherent monocytes will differentiate into macrophages. For a pure macrophage culture, we recommend that you add factors such as M-CSF. Adding other factors including IL-4, IL-10, or TGF-β can help improve viability. The protocol below is for a T25 or T75 flask but can be scaled down for 100 mm culture dishes or plates.

Materials

- T25 or T75 sterile flasks with vented caps (e.g., Nunc EasYFlask Cell Culture Flasks, T25, filter, Cat. No. 156367)
- RPMI 1640 medium (e.g., BenchStable RPMI 1640 Medium, Cat. No. A4192301)
- Fetal Bovine Serum (e.g., Gibco Fetal Bovine Serum, Cat. No. 26140087, or Fetal Bovine Serum One Shot format, Cat. No. A3160401)
- L-glutamine (e.g., 200 mM L-Glutamine, Cat. No. 25030149)
- **Optional** penicillin-streptomycin (e.g., Gibco Penicillin-Streptomycin, Cat. No. 15140148)
- M-CSF purified protein (e.g., Gibco M-CSF Recombinant Human Protein, Cat. No. PHC9504)
- **Optional** IL-4 purified protein (e.g., IL-4 Recombinant Human Protein, Cat. No. A42602)
- Gibco™ PBS (Phosphate-buffered saline), pH 7.4, (Cat. No. 10010023)
- EDTA (e.g., UltraPure 0.5M EDTA, pH 8.0, Cat. No. 15575020)
- 50 mL conical tube (e.g., Nunc 50 mL conical sterile centrifuge tubes, Cat. No. 339652)
- Flow cytometry staining buffer (e.g., eBioscience Flow Cytometry Staining Buffer, Cat. No. 00-4222-26)

Protocol tip

Culturing monocytes on Nunc UpCell Dishes can help preserve cell viability and surface proteins during cell harvest. Nunc dishes with UpCell Surface enables harvesting of cells by temperature reduction without the need for dissociation enzymes (e.g., trypsin, EDTA), thereby maintaining cellular membrane and surface receptors.

This protocol is continued on page 18

In vitro differentiation of macrophages from monocytes, cont.

Procedure

1. Using aseptic techniques under sterile conditions, isolate PBMC from whole blood (See supplemental protocol A on page 20).

2. Prepare complete RPMI 1640 medium by supplementing RPMI 1640 medium with fetal bovine serum to a final concentration of 10%, 2 mM L-glutamine (if using medium not currently supplemented with GlutaMAX). Bring medium to 37°C.

Optional

Supplement media with 1% penicillin-streptomycin (5,000 units/mL).

3. Resuspend cell concentration to 2 x 10^6 cells/mL in complete RPMI 1640 medium.

4. Transfer resuspended cell solution to a cell culture dish.

5. Incubate the culture dish for 24 hours in 5% CO_2 incubator at 37°C to allow the monocytes to adhere to dish.

6. In a sterile conical tube, prepare complete RPMI 1640 medium with M-CSF Recombinant Human Protein at a final concentration of 40–50 ng/mL.

Optional

Add 20 ng/mL IL-4 Recombinant Human Protein.

7. Replace media in culture dish with the prepared media containing M-CSF and IL-4 (if using).

8. Incubate cells for 6 days in 5% CO_2 incubator at 37°C. Within the 6 days, replenish media with new complete RPMI 1640 medium supplemented with 40–50 ng/mL of M-CSF Recombinant Human Protein every 3–4 days. Check under microscope for cell health and confluence.

Optional

You may also add 20 ng/mL IL-4 Recombinant Human Protein.

9. Cells are ready to harvest when cells exhibit more granules in the cytoplasm and are a bit elongated. In addition, cells should be more adherent to the culture plate. When cells are ready to harvest, discard old media, and rinse dish twice with 1X PBS, discarding PBS after each rinse.

10. Add 10 mL 10 mM EDTA to each culture dish, and let sit for 10 minutes, or until cells dissociate from dish at room temperature.

11. Collect cells in a 50 mL conical tube and centrifuge at 300–400 x *g* for 4–5 minutes at room temperature.

This protocol is continued on page 19

12. Discard the supernatant and rinse cells with 1X PBS.

13. Centrifuge cells at 300–400 x *g* for 4–5 minutes.

14. Discard the supernatant, and resuspend cells in flow cytometry staining buffer or desired medium.

This protocol is continued on page 20

In vitro differentiation of macrophages from monocytes, cont.

Supplemental Protocol A: Isolation of PBMC from whole blood

Materials

- Gibco™ PBS (Phosphate-buffered saline), pH 7.4, (Cat. No. 10010023)
- 15 mL or 50 mL conical tube (e.g., Nunc 15 mL conical sterile centrifuge tubes, Cat. No. 339650)
- Ficoll-Paque™ density separation medium
- Flow cytometry staining buffer (e.g., eBioscience Flow Cytometry Staining Buffer, Cat. No. 00-4222-26)

Procedure

1. Dilute blood sample at least 1:1 with PBS in a conical tube.
2. Underlay the diluted sample with a volume of Ficoll-Paque™ medium that is equal to the original sample volume.
3. Centrifuge at 400 x *g* for 20 minutes at room temperature with the brake OFF.
4. Harvest PBMC located at the interface of the PBS and Ficoll-Paque™ medium layers into a fresh tube.
5. Fill the tube with PBS to wash the cells.
6. Centrifuge the cells at 300–400 x *g* for 4–5 minutes at 2–8°C. Discard supernatant.
7. Resuspend the cell pellet in an appropriate volume of flow cytometry staining buffer or buffer of choice, and perform a cell count and viability analysis.
8. Centrifuge the cells as in step 6, and resuspend in appropriate volume of complete RPMI 1640 so that the final cell concentration is 2 x 10^6 cells/mL.

Note

As cells will be cultured, perform all steps using aseptic techniques, and use buffers that do not contain azide.

Protocol tip

Significantly improve the accuracy of assessing cell health and concentration from freshly harvested peripheral blood mononuclear cells with automated counters. See detailed protocol on how to count PBMC using the Countess 3 FL Automated Cell Counter at **thermofisher.com/countess3**.

Stimulation of cytokine production in immune cells

Introduction

Stimulation reagents, phorbol 12-myristate 13-acetate (PMA), ionomycin, Brefeldin A, and monensin are useful to activate transcription factors for intracellular signaling and production of cytokines of many different immune cell types. Brefeldin A solution is required for intracellular retention of signaling proteins and cytokines. Brefeldin A is an inhibitor of intracellular protein transport. Incubation of cells in culture with brefeldin A leads to blockade of protein transport to the Golgi complex (GC) and accumulation of proteins in the endoplasmic reticulum (ER). Addition of Brefeldin A during the last hours of *in vitro* activation of cells results in enhanced detection of intracellular cytokines. If performing an immunoassay on secreted protein (e.g., ELISA, western or multiplex protein detection), cells do not require treatment with Brefeldin A.

Materials

- T25 or T75 sterile flasks with vented caps (e.g., Nunc EasYFlask Cell Culture Flasks, T25, filter, Cat. No. 156367)
- RPMI 1640 medium (e.g., BenchStable RPMI 1640 Medium, Cat. No. A4192301)
- Fetal Bovine Serum (e.g., Gibco Fetal Bovine Serum, Cat. No. 26140079)
- Cell Stimulation Cocktail: phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A, and monensin (e.g., Invitrogen Cell Stimulation Cocktail with or without protein transport inhibitors, Cat. No. 00-4975-93 or 00-4970-93)
- Brefeldin A solution (e.g., eBioscience Brefeldin A solution, Cat. No. 00-4506-51)
- Cell scraper (e.g., Nunc Cell Scrapers, Cat. No. 179693)

This protocol is continued on page 22

Stimulation of cytokine production in immune cells, cont.

Procedure

1. Using aseptic techniques under sterile conditions, isolate PBMC from whole blood (supplemental protocol A, page 23) or prepared lymphoid tissue (supplemental protocol B, page 24).

2. Prepare complete RPMI 1640 medium by supplementing RPMI 1640 Medium with fetal bovine serum to a final concentration of 10%. Bring medium to 37°C.

3. Resuspend cells to a concentration of 3 x 10^6 cells/mL in complete RPMI 1640 medium. Prepare the volume needed based on the flask size you will be using (T25 or T75).

4. Prepare 2 culture flasks: one labeled ‘stimulated’ (activated) and the other as ‘non-stimulated’ (non-activated).

5. Divide the cell solution (prepared in step 3) evenly into the prepared flasks.

6. Add Cell Stimulation Cocktail at a concentration of 1X, 2 µL/mL into the stimulated (activated) flask.

7. Add Brefeldin A solution at a concentration of 1X, 3 µL/mL to each of the stimulated and non-stimulated flasks.

8. Cover both flasks with vented filtered caps, and incubate for 5 hours in 5% CO_2 incubator at 37°C.

9. Harvest cells.

Protocol tip

Use of ready-made fixation buffers can help improve detection of cytokines and transcription factors in flow cytometry applications.

This protocol is continued on page 23

Supplemental Protocol A: Isolation of PBMC from whole blood

Materials

- Gibco™ PBS (Phosphate-buffered saline), pH 7.4, (Cat. No. 10010023)
- 15 mL or 50 mL conical tube (e.g., Nunc 15 mL conical sterile centrifuge tubes, Cat. No. 339650)
- Ficoll-Paque™ density separation medium
- Flow cytometry staining buffer (e.g., eBioscience Flow Cytometry Staining Buffer, Cat. No. 00-4222-26)

Procedure

1. Dilute blood sample at least 1:1 with PBS in a conical tube.
2. Underlay the diluted sample with a volume of Ficoll-Paque™ medium that is equal to the original sample volume.
3. Centrifuge at 400 x *g* for 20 minutes at room temperature with the brake OFF.
4. Harvest PBMC located at the interface of the PBS and Ficoll-Paque™ medium layers into a fresh tube.
5. Fill the tube with PBS to wash the cells.
6. Centrifuge the cells at 300–400 x *g* for 4–5 minutes at 2–8°C. Discard supernatant.
7. Resuspend the cell pellet in an appropriate volume of flow cytometry staining buffer or buffer of choice, and perform a cell count and viability analysis.
8. Centrifuge cells as in step 6, and resuspend in appropriate volume of complete RPMI 1640 so that the final cell concentration is 3 x 10^6 cells/mL.

Note

As cells will be cultured, perform all steps using aseptic techniques, and use buffers that do not contain azide.

Protocol tip

Significantly improve the accuracy of assessing cell health and concentration from freshly harvested peripheral blood mononuclear cells with automated counters. See detailed protocol on how to count PBMC using the Countess 3 FL Automated Cell Counter at **thermofisher.com/countess3**.

This protocol is continued on page 24

Stimulation of cytokine production in immune cells, cont.

Supplemental Protocol B: Isolation of immune cells from lymphoid tissue

Materials

- 60 x 15 mm cell culture dish (e.g., Nunc Cell Culture Petri Dishes)
- Plastic 3 mL syringe or two frosted glass microscope slides
- Cell strainer (nylon mesh)
- 15 mL or 50 mL conical tube (e.g., Nunc 15 mL conical sterile centrifuge tubes, Cat. No. 339650)
- Flow cytometry staining buffer (e.g., eBioscience Flow Cytometry Staining Buffer, Cat. No. 00-4222-26)

Procedure

1. Harvest tissue (spleen; thymus; lymph nodes) into a cell culture dish containing 10 mL of Flow Cytometry Staining Buffer or buffer of choice. Tease apart into a single-cell suspension by pressing with the plunger of a 3 mL syringe. Alternatively, mash tissue between the frosted ends of two microscope slides using 10 mL of Flow Cytometry Staining Buffer.

2. Place a cell strainer on top of a 15 mL conical tube. Pass cells from the cell culture dish through the cell strainer to eliminate clumps and debris.

3. Centrifuge cell suspension at 300–400 x *g* for 4–5 minutes at 2–8°C. Discard the supernatant.

4. Resuspend the cell pellet in an appropriate volume of Flow Cytometry Staining Buffer or buffer of choice and perform a cell count and viability analysis.

5. Centrifuge cells as in step 3, and resuspend in appropriate volume of complete RPMI 1640 medium to the final cell concentration is 3 x 10^6 cells/mL.

Note

Mechanical disruption of lymphoid tissue is generally sufficient to release cells into a single-cell suspension.

Note

As cells are to be cultured, perform all steps using aseptic technique, and use buffers that do not contain azide.

T cell activation

Introduction

Product Contents

- Gibco Dynabeads Human T-Activator CD3/CD28
  - 1 x 0.4 mL (Cat. No. 111.61D)
  - 1 x 2 mL (Cat. No. 111.31D)
  - 5 x 2 mL (Cat. No. 111.32D)

Product Description

This product is intended for physiological activation of human T cells, e.g. $CD4^+$ T cells, $CD8^+$ T cells, polyclonal cells, or antigen specific T cells.

Downstream Applications

The activated T cells can be analyzed shortly after activation (for transfection/transduction or to study e.g. TCR signaling, proteomics or gene expression). T cells can be left in culture to differentiate into T helper cell subsets [1–3], T cell proliferation or expansion of polyclonal/Ag-specific T cells [4–5].

Additional materials required

- Buffer: Gibco™ PBS (Phosphate-buffered saline), pH 7.4, (Cat. No. 10010023) with 0.1% bovine serum albumin and 2 mM EDTA, pH 7.4 (PBS w/0.1% BSA)
- Magnet (DynaMag): See **thermofisher.com/magnets** for magnet recommendations
- Culture medium: Advanced RPMI Medium 1640 (Cat. No. 12633-012) with 2 mM L-Glutamine, 10% FCS/FBS and 100 U/mL penicillin/streptomycin can be used. Alternatively Gibco CTS OpTmizer T Cell Expansion SFM (Cat. No. 0080022SA) with 100 U/mL penicillin/streptomycin, or an equivalent culture medium
- Heat inactivated Fetal Calf Serum (FCS)
- Recombinant human IL-2
- Flat bottom tissue culture plates or tissue culture flasks of suitable size
- Humidified CO_2 incubator

Note

Cat. No. 111.31D and 111.32D are formerly know as Dynabeads CD3/CD28 T Cell Expander. Each product contains 4 x 10^7 beads/mL in phosphate buffered saline (PBS), pH 7.4, with 0.1% human serum albumin (HSA).

Critical notes

- Resuspend the Dynabeads in the vial carefully before use, i.e. vortex for >30 sec., or tilt and rotate for 5 minutes.
- This product should not be used with Dynal MPC-1.
- Never use less than the recommended volume of Dynabeads.
- Carefully follow the recommended pipetting volumes.
- Avoid air bubbles during pipetting.
- Prior to flow cytometric analysis, Dynabeads and bead-bound cells should be removed. Upon activation and for 2–3 days thereafter, some cells will bind strongly to the beads. Resuspend the bead/cell suspension thoroughly by pipetting to increase cell recovery, separate on a magnet (after transfer to a suitable tube) and collect supernatant containing the T cells. The bead-bound cell fraction can be cultured overnight and the above process repeated to further increase T cell recovery. When using cells for proteomics or gene expression studies, lyse the cells prior to bead removal.

This protocol is continued on page 26

T cell activation, cont.

Protocols

This product allows for easy physiological activation of human T cells, without the need for preparing antigen-presenting cells (APCs) or antigen.

Preparations

- See **thermofisher.com/cellisolation** for recommended Dynabeads products for positive or negative isolation of all human T cells, or specific T cell subsets.
- Prepare cell culture medium

Dynabeads washing procedure

Note

Dynabeads should be washed before use.

1. Resuspend the Dynabeads Human T-Activator CD3/CD28 in the vial.
2. Transfer the desired volume of Dynabeads to a tube.
3. Add an equal volume of Buffer, or at least 1 mL, and mix (vortex for 5 seconds, or keep on a roller for at least 5 min).
4. Place the tube on a magnet for 1 min and discard the supernatant.
5. Remove the tube from the magnet and resuspend the washed Dynabeads in the same volume of Culture Medium as the initial volume of Dynabeads taken from the vial (step 2).

This protocol is continued on page 27

Activation of human T cells

1. Start with 8 x 10^4 purified T cells in 100–200 µL medium in a 96-well tissue culture plate.
2. Add 2 µL Dynabeads Human T-Activator CD3/CD28 to obtain a bead-to-cell ratio of 1:1.
3. Incubate in a humidified CO_2 incubator at 37°C, according to your specific experimental requirements.
4. Harvest the activated T cells and use directly for further analysis.
5. For flow cytometry applications, remove the beads prior to staining. Place the tube on a magnet for 1–2 minutes to separate the beads from the solution. Transfer the supernatant containing the cells to a new tube.

Expansion of human T cells

1. Start with 1–1.5 x 10^6 purified T cells/mL in culture medium in a suitable tissue culture plate or tissue culture flask.
2. Add Dynabeads Human T-Activator CD3/CD28 at a bead-to-cell ratio of 1:1.
3. Add 30 U/mL rIL-2.
4. Incubate in a humidified CO_2 incubator at 37°C, according to your specific experimental requirements.
5. Examine cultures daily, noting cell size and shape. Cell shrinking and reduced proliferation rate is typically observed in exhausted cell cultures.
6. Count the cells at least twice weekly after thorough re-suspension.
7. When the cell density exceeds 2.5 x 10^6 cells/mL or when the medium turns yellow, split cultures back to a density of 0.5–1 x 10^6 cells/mL in culture medium containing 30 U/mL rIL-2.

This protocol is continued on page 28

T cell activation, cont.

Re-stimulation

Cell cultures showing signs of exhaustion (typically at day 7–10 of expansion) can be re-stimulated several times by adding fresh Dynabeads Human T-Activator CD3/CD28 and rIL-2. The $CD8^+$ T cells remain cytotoxic after repeated re-stimulations. Re-stimulation is typically necessary when cell shrinking and a reduced rate of proliferation is observed. Do not use an excess volume of Dynabeads Human T-Activator CD3/CD28, as excess Dynabeads per cell may inhibit expansion. Prior to re-stimulation, remove the used Dynabeads by transferring the cells to a suitable tube. Place the tube on a magnet for 1–2 minutes until the Dynabeads have moved to the side of the tube. Transfer the supernatant containing the cells to a new tube. Continue as described.

1. Count the cells and resuspend to a density of 1 x 10^6 cells/mL in culture medium with 30 U/mL rIL-2 in a suitable culture plate or tissue culture flask.
2. Add Dynabeads Human T-Activator CD3/CD28 at a bead-to-cell ratio of 1:1.
3. Add 30 U/mL rIL-2.
4. Incubate in a humidified CO_2 incubator at 37°C for the length of your specific experiment.
5. Examine cultures daily, noting cell size and shape. Cell shrinking and reduced proliferation rate is typically observed in exhausted cell cultures.
6. Count the cells at least twice weekly after thorough re-suspension.
7. When the cell density exceeds 2.5 x 10^6 cells/mL or when the medium turns yellow, split cultures back to a density of 0.5–1 x 10^6 cells/mL in culture medium with 30 U/mL rIL-2

This protocol is continued on page 29

Table 1. Volume recommendations for bead-to-cell ratio = 1:1.

	8 x 10^4 T cells	1 x 10^6 T cells	50 x 10^6 T cells
Type of culture plate/flask	**Per well in 96-well plate**	**Per well in 24-well plate**	**175 cm^2 tissue culture flask**
Dynabeads Human T-Activator CD3/CD28	2 µL	25 µL	1,250 µL
rIL-2	30 U/mL	30 U/mL	30 U/mL
Seeding volume (medium)	100–200 µL	1–2 mL	50–100 mL

Table 2. Re-stimulation guidelines for anti-CD3/CD28-expanded cultures.

Cell type	First re-stimulation*	Subsequent re-stimulations*
$CD4^+$ (polyclonal)	8–10 days	8–11 day intervals
$CD8^+$ (polyclonal)	7–9 days	7–10 day intervals
T cells	7–9 days	10–12 day intervals

* Establish optimal times for your particular cells. Please note that these are only generic guidelines.

References

1. *Nat Immunol* 9(6):650 (2008).
2. *Blood* 109(10):4343 (2007).
3. *Blood* 111(3):1366 (2008).
4. *J Imm Methods* 275:251 (2003).
5. *Blood* 111(2):680 (2008).

General Information

Certification

Invitrogen Dynal AS conforms to the Quality Systems Standard ISO 9001:2000 and ISO 13485:2003 with the following scope: "Development, manufacturing, marketing and sales of Dynospheres, Dynabeads and associated products to customers that work within immunology, biological and clinical research, cell based therapy and *in vitro* diagnostics." In the United States, Dynabeads ClinExVivo CD3/CD28 is available for use in clinical trials under an approved IND or IDE.

USA (Device Master File)

A Device Master File is held with the United States Food & Drug Administration (FDA), which will assist users with their application for FDA approvals on their clinical trials. If cross-referencing the Device Master File is of interest to an Investigational New Drug (IND) Application or other applications, please contact Invitrogen Dynal with the sponsor's and/or investigator's full name and address, along with project name and aim. This information is required by Invitrogen Dynal to issue a Letter of Authorisation, informing the FDA who has been authorized to cross-reference the Master File for their IND application.

Technical Service

Please contact Invitrogen Dynal AS for further technical information at **www.thermofisher.com** Certificate of Analysis (CoA) is available upon request.

Precautions

Material Safety Data Sheet (MSDS) is available at **www.thermofisher.com**.

Flow cytometry

Phenotypic analysis

Staining cell surface targets for flow cytometry

Introduction

Cell surface markers can be used to define cell subsets based on lineage and developmental stage, as well as function when they are labeled with fluorochrome-conjugated antibodies and analyzed by flow cytometry. These surface markers have different forms and functions, including receptors for both soluble and cell-bound ligands, ion channels, glycoproteins, phospholipids, and more. For example, CD4 is a surface marker for T helper cells that can be further differentiated into unique sub populations based on expression of other chemokine receptors and cluster of differentiation (CD) markers. Live cells stained with antibodies can be sorted based on unique staining patterns and used for additional experiments.

Staining cell surface targets protocols

- Protocol A: Cell Suspensions
- Protocol B: Human Lysed Whole Blood

Protocol A: Cell suspensions

Materials

- 12 x 75 mm round-bottom test tubes or 96-well U- or V-bottom microplates
- Primary antibodies (directly conjugated or purified)
- Secondary reagents, if necessary (for indirect staining)
- Anti-Mouse CD16/32 Purified (Cat. No. 14-0161) or Human FC Receptor Binding Inhibitor Purified (Cat. No. 14-9161)
- Flow Cytometry Staining Buffer (Cat. No. 00-4222)
- **Optional** Viability solutions:
  - 7-AAD Viability Staining Solution (Cat. No. 00-6993)
  - Propidium Iodide Staining Solution (Cat. No. 00-6990)
  - Invitrogen™ product line such as Fixable Viability Dyes eFluor™ 455UV (Cat. No. 65-0868), eFluor™ 450 (Cat. No. 65-0863), eFluor™ 506 (Cat. No. 65-0866), eFluor™ 660 (Cat. No. 65-0864) and eFluor™ 780 (Cat. No. 65-0865)

This protocol is continued on page 32

Note

This protocol is intended for use with the specific products mentioned within it. Substituting different products is not recommended.

General notes

- For optimal performance of fluorochrome conjugated antibodies, store vials at 2–8°C in the dark. Do not freeze.
- Prior to use, quick spin the antibody vial to recover the maximum volume. Vortexing the antibody vial is not recommended.
- Except where noted in the protocol, all staining should be done on ice or at 2–8°C with minimal exposure to light.
- If storage of samples is necessary after staining with fluorochrome-conjugated antibodies, store the samples in IC Fixation Buffer (Cat. No. 00-8222) by combining 100 µL of sample with 100 µL of IC Fixation Buffer, or add 2 mL of 1-step Fix/Lyse Solution (Cat. No. 00-5333). Samples can be stored in these buffers for up to 3 days in the dark at 2–8°C.
  - Notes: There appears to be minimal impact on brightness of staining or FRET efficiency/compensation of tandem dyes, such as Invitrogen™ APC-eFluor™ 780 or PE-Cyanine7, when using the IC Fixation Buffer (Cat. No. 00-8222) or 1-step Fix/Lyse Solution (Cat. No. 00-5333) for storage of samples. Differences in fixation buffer quality can affect fluorochrome brightness or FRET efficiency. Some generalizations regarding fluorophore performance after fixation can be made, but clone-specific performance should be determined empirically.
  - Notes: Staining with Fixable Viability Dyes (FVD) is recommended before fixing samples to allow for gating on live cells during analysis by flow cytometry.

Staining cell surface targets for flow cytometry, cont.

Experimental procedure

1. Prepare cells as described in Cell Preparation Protocols for Flow Cytometry found in our Best Protocols section at **thermofisher.com/cellpreparationflow**.

2. **Optional** Block non-specific Fc-mediated interactions:
   For mouse cells: Pre-incubate the cells with 0.5–1 µg of Anti-Mouse CD16/CD32 Purified per 100 µL for 10–20 minutes at 2–25°C before staining.
   For human cells: Pre-incubate the cells with 20 µL of Human Fc Receptor Binding Inhibitor Purified per 100 µL for 10–20 minutes at 2–25°C before staining.

3. Aliquot 50 µL of cell suspension (from 10^5–10^8) to each tube or well.

4. Combine the recommended quantity of each primary antibody in an appropriate volume of Flow Cytometry Staining Buffer so that the final staining volume is 100 µL (i.e. 50 µL of cell sample + 50 µL of antibody mix) and add to cells. Pulse vortex gently to mix.

5. Incubate for at least 30 minutes at 2–8°C or on ice. **Protect from light**.

6. Wash the cells by adding Flow Cytometry Staining Buffer. Use 2 mL/tube or 200 µL/well for microplates. Centrifuge at 400–600 x *g* for 5 minutes at room temperature. Discard supernatant.

7. Repeat Step 6.

8. Incubate for 60 minutes at 2–8°C or on ice.

9. Wash the cells by adding Flow Cytometry Staining Buffer. Use 2 mL/tubes or 200 µL/well for microplate. Centrifuge at 400–600 x *g* for 5 minutes at room temperature. Discard supernatant.

10. Repeat Step 9.

Note

Antibody-binding kinetics are temperature-dependent. Staining on ice may require longer incubation times. Furthermore, some antibodies may require non-standard incubation conditions that will be noted on the technical data sheet provided with the antibody.

Note

Proceed to Step 8 for purified or biotinylated primary antibodies.

Note

Proceed to Step 14 if all primary antibodies were directly conjugated to fluorochromes.

This protocol is continued on page 33

11. Dilute the appropriate fluorochrome-labeled secondary reagent in 100 µL of Flow Cytometry Staining Buffer and add to the cells. Incubate for at least 30 minutes at 2–8°C or on ice. **Protect from light**.

12. Wash the cells by adding Flow Cytometry Staining Buffer. Use 2 mL/tubes or 200 µL/well for microplates. Centrifuge at 400–600 x *g* for 5 minutes at room temperature. Discard supernatant.

13. Repeat Step 12.

14. **Optional** Stain samples with a viability dye according to the appropriate Viability Dye Staining Protocols found at **thermofisher.com/viabilitystaining**.

15. **Optional** For storage of samples before analysis, resuspend cells in 100 µL of Flow Cytometry Staining Buffer and add 100 µL of IC Fixation Buffer or 2 mL of 1-step Fix/Lyse Solution.

16. Resuspend cells in an appropriate volume of Flow Cytometry Staining Buffer.

17. Analyze samples by flow cytometry.

This protocol is continued on page 34

Staining cell surface targets for flow cytometry, cont.

Protocol B: Lysed whole blood (human)

Either the 10X RBC Lysis Buffer (Multi-species) or 1-step Fix/Lyse Solution (10X) may be used to lyse RBC after whole blood has been stained with fluorochrome-conjugated antibodies. Additionally, the 1-step Fix/Lyse Solution (10X) lyses RBC and fixes leukocytes in a single step. If lysed whole blood cells will be prepared using the 1-step Fix/Lyse Solution before staining, confirm that the antibodies in the staining panel recognize fixed epitopes on the antigens of interest. Please refer to the "Antibody Clone Performance Following Fixation" table for antibody clone performance following fixation/permeabilization at **thermofisher.com/antibodyfixation**.

Materials

- 10X RBC Lysis Buffer (Multi-species) (Cat. No. 00-4300-54) or 1-step Fix/Lyse Solution (10X) (Cat. No. 00-5333)
- 12 x 75 mm round-bottom test tubes
- Primary antibodies (directly conjugated or purified)
- Secondary reagents, if necessary (for indirect staining)
- Human FC Receptor Binding Inhibitor Purified (Cat. No. 14-9161)
- Flow Cytometry Staining Buffer (Cat. No. 00-4222)
- **Optional** Viability solutions:
  - 7-AAD Viability Staining Solution (Cat. No. 00-6993)
  - Propidium Iodide Staining Solution (Cat. No. 00-6990)
  - Invitrogen™ product line such as Fixable Viability Dyes eFluor™ 455UV (Cat. No. 65-0868), eFluor™ 450 (Cat. No. 65-0863), eFluor™ 506 (Cat. No. 65-0866), eFluor™ 660 (Cat. No. 65-0864) and eFluor™ 780 (Cat. No. 65-0865)

Note

Before using, the 10X RBC Lysis Buffer (Multi-species) and 1-step Fix/Lyse Solution (10X) must be diluted to 1X by adding 1 part buffer with nine parts room temperature, reagent-grade water.

Experimental procedure

1. Aliquot 100 µL of whole blood to each tube.

2. **Optional** Block non-specific Fc receptor-mediated interactions with 20 µL of Human Fc Receptor Binding Inhibitor Purified per 100 µL of blood. Incubate for 10–20 minutes at 2–8°C or room temperature.

Note

Antibody-binding kinetics are temperature-dependent. Staining on ice may require longer incubation times. Furthermore, some antibodies may require non-standard incubation conditions that will be noted on the technical data sheet provided with the antibody.

This protocol is continued on page 35

3. Combine the recommended quantity of each primary antibody in an appropriate volume of Flow Cytometry Staining Buffer so that the final volume of antibody mix is 50 µL. Add to cells. Pulse vortex gently to mix.

4. Incubate for 20–30 minutes at room temperature. **Protect from light**.

Note

Proceed to Step 7 if all primary antibodies were directly conjugated to fluorochromes.

For purified or biotin conjugated antibodies

5. Wash the cells by adding 2 mL of Flow Cytometry Staining Buffer. Centrifuge at 400–600 x *g* for 5 minutes at room temperature. Carefully discard supernatant.

6. Dilute the appropriate fluorochrome-labeled secondary reagent in 100 µL of Flow Cytometry Staining Buffer and add to the cells. Incubate for 15–30 minutes at 2–8°C or on ice. **Protect from light**.

7. **Without washing** cells, add 2 mL of freshly prepared 1X RBC lysing solution and pulse vortex briefly. Incubate for 10–20 minutes at room temperature. **Protect from light**.

Note

Do not incubate longer than 20 minutes if using the 10X RBC Lysis Buffer (Multispecies) (Cat. No. 00-4300)

8. Centrifuge samples at 400–600 x *g* for 5 minutes at room temperature. Discard supernatant.

9. Add 2 mL of Flow Cytometry Staining Buffer and centrifuge at 400–600 x *g* for 5 minutes at room temperature. Discard supernatant.

10. Repeat Step 9.

11. **Optional** For cells lysed using the 10X RBC Lysis Buffer, stain samples with a viability dye according to the appropriate Viability Dye Staining Protocols at **thermofisher.com/viabilitystaining**.

Note

Viability dyes, such as propidium iodide or 7-AAD, should not be used on cells lysed using the 1-step Fix/Lyse Solution as fixation can cause permeabilization of the cells. Fixable Viability Dyes (FVD) may be used to stain whole blood before using the 1-step Fix/Lyse Solution; refer to Viability Dye Staining Protocols found at **thermofisher.com/viabilitystaining**.

12. Resuspend cells in an appropriate volume of Flow Cytometry Staining Buffer.

13. Analyze samples by flow cytometry.

Staining intracellular antigens for flow cytometry

Introduction

A modification of the basic immunofluorescent staining and flow cytometric analysis protocol can be used for the simultaneous analysis of surface molecules and intracellular antigens at the single-cell level by flow cytometry. Typically, cells are fixed with formaldehyde to stabilize the cell membrane, and then permeabilized with detergent or alcohol to allow antibodies against intracellular antigens access to stain intracellularly.

When staining proteins inside the cell, it is important to consider their location as this may dictate the protocol and buffer system that will perform optimally. For example, nuclear proteins and many secreted proteins work well with the Foxp3/Transcription Factor Staining Buffer Set (Cat. No. 00-5523), while secreted proteins such as cytokines and chemokines work well with the Intracellular Fixation & Permeabilization Buffer Set (Cat. No. 88-8824). Lastly, there are several phosphorylated signaling proteins that may not work in the two previously-mentioned buffer systems but will work with the Fixation/Methanol Protocol. Antibody performance in different buffer systems and protocols should be determined empirically. Please contact Technical Support (**techsupport@thermofisher.com**) for more information.

Note

This protocol is intended for use with the specific products mentioned within it. Substituting different products is not recommended.

Staining intracellular antigens protocols

- **Protocol A:** Two-step protocol: intracellular (cytoplasmic) proteins
- **Protocol B**: One-step protocol: intracellular (nuclear) proteins
- **Protocol C:** Two-step protocol for Fixation/Methanol

Protocol A: Two-step protocol: intracellular (cytoplasmic) proteins

The following protocol allows the simultaneous analysis of cell surface molecules and intracellular antigens at the single-cell level. In this protocol, fixation is followed by permeabilization resulting in the creation of pores in the cell membrane that require the continuous presence of the permeabilization buffer during all subsequent steps. This allows antibodies to have access to the cytoplasm of the cell. Thus, all intracellular staining must be done in the presence of the permeabilization buffer.

This protocol is recommended for the detection of cytoplasmic proteins, cytokines, or other secreted proteins in individual cells following activation *in vitro* or *in vivo*. For cytokine detection, the appropriate stimulation conditions and kinetics of cytokine production will vary depending on the cell type and the particular cytokine being

General notes

- For optimal performance of fluorochrome-conjugated antibodies, store vials at 4°C. **Protect from light**. Do not freeze.
- Prior to use, quickly spin the antibody vial to recover the maximum volume. Vortexing the antibody vial is not recommended.
- Except where noted in the protocol, all staining should be done on ice or at 4°C with minimal exposure to light.
- The fixation and permeabilization steps that are required for the detection of intracellular antigens may alter the light scatter properties of cells and may increase non-specific background staining. Including extra protein such as BSA or fetal calf serum (FCS) in the staining buffer may help reduce non-specific background. The use of Fixable Viability Dyes (FVD) is recommended to help eliminate dead cells during the analysis.

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assayed. For example, to stimulate T cells to produce IFN-γ, TNF-α, IL-2, and IL-4, a combination of PMA (a phorbol ester, a protein kinase C activator) and Ionomycin (a calcium ionophore) or anti-CD3 antibodies can be used. To induce IL-6, IL-10, or TNF-α production by monocytes, stimulation with lipopolysaccharide (LPS) can be used. For *in vitro* stimulation of cells, it is necessary to block secretion of cytokines with protein transport inhibitors, such as Monensin or Brefeldin A Solution, during the final hours of the stimulation protocol. It is advised that investigators evaluate the use and efficacy of different protein transport inhibitors in their specific assay system.

For the detection of nuclear proteins such as transcription factors, please see Protocol B: One-step protocol: intracellular (nuclear) proteins on page 40. For detection of some phosphorylated signaling molecules such as MAPK and STAT proteins, it may be preferential to use Protocol C: Two-step protocol: Fixation/Methanol (page 44).

Materials

- 12 x 75 mm round bottom test tubes
- **Optional** Invitrogen™ product line such as Fixable Viability Dyes (FVD) eFluor™ 455UV (Cat. No. 65-0868), eFluor™ 450 (Cat. No. 65-0863), eFluor™ 506 (Cat. No. 65-0866), eFluor™ 660 (Cat. No. 65-0864), or eFluor™ 780 (Cat. No. 65-0865)
- Directly conjugated antibodies
- Intracellular Fixation & Permeabilization Buffer Set (Cat. No. 88-8824)
- Flow Cytometry Staining Buffer (Cat. No. 00-4222)
- Cell Stimulation Cocktail (plus protein transport inhibitors) (500X) (Cat. No. 00-4975) or
  - Protein Transport Inhibitor Cocktail (500X) (Cat. No. 00-4980) or
  - Brefeldin A Solution (Cat. No. 00-4506) or
  - Monensin Solution (Cat. No. 00-4505)

Buffer and solution preparation

- Prepare a 1X working solution of Permeabilization Buffer by mixing 1 part 10X concentrate with nine parts distilled water. Each sample will require 8.5 mL of 1X Permeabilization Buffer.

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Staining intracellular antigens for flow cytometry, cont.

Experimental procedure in 12 x 75 mm tubes

1. Prepare a single cell suspension. Refer to Best Protocols Cell Preparation for Flow Cytometry at **thermofisher.com/cellpreparation**.

2. **Optional** Stain cells with a Fixability Viability Dye. Refer to Best Protocols Viability Dye Staining Protocols, Protocol C at **thermofisher.com/viabilitystaining**.

3. Stain cell surface markers. Refer to Best Protocols Staining Cell Surface Targets, Protocol A at **thermofisher.com/surfacetargetsflow**.

4. After the last wash, discard the supernatant and pulse vortex the sample to completely dissociate the pellet. Typically about 100 µL residual volume remains.

5. Fix the cells by adding 100 µL of IC Fixation Buffer and pulse vortex to mix.

6. Incubate 20–60 minutes at room temperature. **Protect from light**.

7. Add 2 mL of 1X Permeabilization Buffer and centrifuge at 400–600 x *g* for 5 minutes at room temperature. Discard the supernatant.

8. Repeat Step 7.

9. Resuspend the cell pellet in 100 µL of 1X Permeabilization Buffer. Add the recommended amount of directly conjugated primary antibody for detection of intracellular antigen(s) to cells and incubate for 20–60 minutes at room termperature. **Protect from light**.

10. Add 2 mL of 1X Permeabilization Buffer and centrifuge at 400–600 x *g* for 5 minutes at room temperature. Discard supernatant.

11. Repeat Step 10.

12. Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer.

13. Analyze samples by flow cytometry.

This protocol is continued on page 39

Experimental procedure in 96-well plate

1. Prepare a single cell suspension. Refer to Best Protocols Cell Preparation for Flow Cytometry at **thermofisher.com/cellpreparationflow**.

2. **Optional** Stain cells with a Fixability Viability Dye. Refer to Best Protocols Viability Dye Staining Protocols, Protocol C at **thermofisher.com/viabilitystaining**.

3. Stain cell surface markers. Refer to Best Protocols Staining Cell Surface Targets, Protocol A at **thermofisher.com/surfacetargetsflow**.

4. After the last wash, discard the supernatant and pulse vortex the sample to completely dissociate the pellet. Typically about 100 µL residual volume remains.

5. Fix the cells by adding 200 µL of IC Fixation Buffer to each well. It is ideal to add the solution such that the cells are fully resuspended in the solution. Pipetting is an option.

6. Incubate 20–60 minutes at room temperature. **Protect from light**.

7. Centrifuge samples at 400–600 x *g* at room temperature for 5 minutes. Discard the supernatant.

8. Add 200 µL 1X Permeabilization Buffer to each well and centrifuge samples at 400–600 x *g* at room temperature for 5 minutes. Discard the supernatant.

9. Resuspend pellet in residual volume and adjust volume to about 100 µL with 1X Permeabilization Buffer.

10. **Optional** Block with 2% normal mouse/rat serum by adding 2 µL directly to the cells. Incubate at room temperature for 15 minutes.

11. **Without washing**, add the recommended amount of directly conjugated antibody for detection of intracellular antigen(s) to cells and incubate for at least 30 minutes at room temperature. **Protect from light**.

12. Add 200 µL of 1X Permeabilization Buffer to each well and centrifuge samples at 400–600 x *g* at room temperature for 5 minutes. Discard the supernatant.

This protocol is continued on page 40

Staining intracellular antigens for flow cytometry, cont.

13. Repeat Step 12.

14. Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer.

15. Analyze by flow cytometry.

Protocol B: One-step protocol: intracellular (nuclear) proteins

The following protocol allows the simultaneous analysis of cell surface molecules and intracellular antigens, including nuclear antigens, at the single-cell level. This protocol combines fixation and permeabilization into a single step. It is recommended for the detection of nuclear antigens such as transcription factors but is also useful for the detection of many cytokines. For compatibility of the Foxp3/Transcription Factor Staining Buffer Set (Cat. No. 00-5523) with cytokine antibodies, please refer to Antibody Fixation Considerations for antibody clone performance at **thermofisher.com/antibodyfixation**.

Materials

- 12 x 75 mm round bottom test tubes or 96-well V- or U-bottom microplates
- **Optional** Fixable Viability Dyes (FVD) eFluor 455UV, 450, 506, 520, 660 and 780 (Cat. Nos. 65-0868, 65-0863, 65-0866, 65-0867, 65-0864, 65-0865, respectively)
- **Optional** Normal Mouse Serum (Cat. No. 24-5544)
- **Optional** Normal Rat Serum (Cat. No. 24-5555)
- Directly conjugated antibodies
- Foxp3/Transcription Factor Staining Buffer Set (Cat. No. 00-5523)
- Flow Cytometry Staining Buffer (Cat. No. 00-4222)

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Buffers and solution preparation

- Prepare fresh Foxp3 Fixation/Permeabilization working solution by mixing one part of Foxp3 Fixation/Permeabilization Concentrate with three parts of Foxp3 Fixation/Permeabilization Diluent. One mL of the working solution is required for each sample, if staining in tubes.
- Prepare a 1X working solution of Permeabilization Buffer by mixing one part of 10X Permeabilization Buffer with nine parts of distilled water. 8.5 mL of the working solution is required for each sample, if staining in tubes.

Experimental procedure in 12 x 75 mm tubes

1. Prepare a single-cell suspension. Refer to Cell Preparation Protocols for Flow Cytometry at **thermofisher.com/cellpreparation**.
2. **Optional** Stain cells with a Fixable Viability Dye. Refer to Viabilty Dye Staining, Protocol C for more details visit **thermofisher.com/viabilitystaining**.
3. Stain cell surface markers. Refer to Staining Cell Surface Targets, Protocol A for details visit **thermofisher.com/surfacetargetsflow**.
4. After the final wash, discard the supernatant and pulse vortex the sample to completely dissociate the pellet. Typically about 100 µL of residual volume remains.
5. Add 1 mL of Foxp3 Fixation/Permeabilization working solution to each tube and pulse vortex.
6. Incubate for 30–60 minutes at 2–8°C or room temperature. **Protect from light**. (Mouse samples can be incubated for up to 18 hours at 2–8°C in the dark).
7. Add 2 mL of 1X Permeabilization Buffer to each tube and centrifuge samples at 400–600 x *g* for 5 minutes at room temperature. Discard the supernatant.
8. **Optional** Repeat Step 7.
9. Resuspend pellet in residual volume of 1X Permeabilization Buffer. This is typically 100 µL after decanting.

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Staining intracellular antigens for flow cytometry, cont.

10. **Optional** Block with 2% normal mouse/rat serum by adding 2 µL directly to the cells. Incubate for 15 minutes at room temperature.

11. **Without washing**, add the recommended amount of directly conjugated antibody for detection of intracellular antigen(s) to cells and incubate for at least 30 minutes at room temperature. **Protect from light**.

12. Add 2 mL of 1X Permeabilization Buffer to each tube and centrifuge samples at 400–600 x *g* for 5 minutes at room temperature. Discard the supernatant.

13. Repeat Step 12.

14. Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer.

15. Analyze samples by flow cytometer.

Note

If needed, surface staining and intracellular phospho-staining can be performed simultaneously. As not all antibody clones will bind to a fixed epitope, refer Antibody Clone Performance Following Fixation/Permeabilization table at **thermofisher.com/antibodyfixation**.

Experimental procedure in 96-well plate

1. Prepare a single-cell suspension. Refer to Cell Preparation Protocols for Flow Cytometry at **thermofisher.com/cellpreparation**.

2. **Optional** Stain cells with a Fixable Viability Dye. Refer to Viabilty Dye Staining, Protocol C for more details visit **thermofisher.com/viabilitystaining**.

3. Stain cell surface markers. Refer to Staining Cell Surface Targets, Protocol A for details visit **thermofisher.com/surfacetargetsflow**.

4. After the final wash, discard the supernatant and pulse vortex the sample to completely dissociate the pellet.

5. Add 200 µL of Foxp3 Fixation/Permeabilization working solution to each well. It is ideal to add the solution such that the cells are fully resuspended in the solution. Pipetting is an option.

6. Incubate for 30–60 minutes at 2–8°C or room temperature. **Protect from light**. (Mouse samples can be incubated for up to 18 hours at 2–8°C in the dark).

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7. Centrifuge samples at 400–600 x *g* for 5 minutes at room temperature. Discard the supernatant.

8. Add 200 µL 1X Permeabilization Buffer to each well and centrifuge samples at 400–600 x *g* for 5 minutes at room temperature. Discard the supernatant.

9. Repeat Step 8.

10. Resuspend pellet in residual volume and adjust volume to about 100 µL with 1X Permeabilization Buffer.

11. **Optional** Block with 2% normal mouse/rat serum by adding 2 µL directly to the cells. Incubate for 15 minutes at room temperature.

12. **Without washing**, add the recommended amount of directly conjugated antibody for detection of intracellular antigen(s) to cells and incubate for at least 30 minutes at room temperature. **Protect from light**.

13. Add 200 µL of 1X Permeabilization Buffer to each well and centrifuge samples at 400–600 x *g* for 5 minutes at room temperature. Discard the supernatant.

14. Repeat Step 13.

15. Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer.

16. Analyze by flow cytometer.

This protocol is continued on page 44

Staining intracellular antigens for flow cytometry, cont.

Protocol C: Two-step protocol for fixation/methanol

The following protocol allows for the simultaneous analysis of cell surface molecules and some intracellular phosphorylated signaling proteins. In this protocol, fixation is followed by treatment of cells with methanol. For phospho-protein detection, the appropriate stimulation conditions and kinetics of phosphorylation will vary depending on the cell type and the particular signaling event being assayed. For example, to induce phospho-STAT1 (Y701) phosphorylation, macrophages can be activated with IFNγ, while phospho-ERK1/2 (T202/Y204) is induced in T cells in response to PMA (a phorbol ester and protein kinase C activator) or anti-CD3 crosslinking.

General notes for Protocol C

- Fluorochrome-conjugated antibodies can be used to stain surface proteins for the purpose of immunophenotyping cells that will be further analyzed for phosphorylated proteins; however, additional considerations for staining are warranted.
  - Antibody staining for surface markers on live cells has been shown to alter expression of signaling proteins due to possible stimulation/suppression of signaling events. Because of this, surface staining is not recommended prior to cell stimulation. Instead, stain surface proteins at the same step as the intracellular protein staining. Please note that some proteins will also have intracellular pools, in addition to surface localization, which should be considered. Antibody clones to surface proteins that will recognize fixed cells/epitopes will need to be evaluated and used. Please refer to Antibody Fixation Considerations for antibody clone performance at **thermofisher.com/antibodyfixation**.
  - If surface staining is required before fixation (due to epitope destruction caused by fixation), cells may be stained with fluorochrome-conjugated antibodies before the Fixation/Methanol steps only if the conjugated fluorochromes are resistant to methanol exposure (refer to the table below).

MeOH resistant fluorochromes	MeOH sensitive fluorochromes
Alexa Fluor 488	PE
eFluor 660	PE-tandems
Alexa Fluor 647	PerCP
eFluor 450	PerCP-tandems
FITC	APC
	APC-tandems

- For adherent cells, we recommend fixing the cells in the plates/well. After fixation, scrape cells or treat with EDTA solution to harvest and continue with protocol. Trypsin can be used if surface staining is not needed or if the surface staining protein is resistant to trypsin digestion.

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Materials

- 12 x 75 mm round bottom test tubes or 96-well V- or U- bottom microplates
- Tissue culture media of choice
- Cell stimulants of choice
- Primary antibodies (directly conjugated)
- Flow Cytometry Staining Buffer (Cat. No. 00-4222)
- IC Fixation Buffer (Cat. No. 00-8222)
- 90–100% methanol (HPLC grade)
- **Optional** Fc Block: Anti-Mouse CD16/CD32 Purified (Cat. No. 14-0161) or Human Fc Receptor Binding Inhibitor Purified (Cat. No. 14-9161)

Experimental procedure

1. Prepare cells of interest for stimulation in appropriate media.
2. Count cells and resuspend in appropriate media at 1–5 x 10^6 cells/mL.
3. Stimulate cells at 37°C with appropriate treatment for desired time point(s). Remember to incubate untreated cells at 37°C as a negative control.
4. **Optional** If surface staining is needed prior to fixation (in Step 5), stain cell surface antigen(s) as described in Staining Cell Surface Targets for Flow Cytometry Protocols at **thermofisher.com/surfacetargetsflow**, with antibodies conjugated to methanol-resistant fluorochromes.
5. At the end of the stimulation period, fix cells to stop stimulation by adding an equal volume of IC Fixation Buffer directly to cells and vortex to mix.
6. Incubate samples for 10–60 minutes at room temperature. **Protect from light**.
7. Centrifuge samples at 400–600 x *g* for 5 minutes at room temperature. Discard the supernatant.
8. Resuspend the cell pellet in residual volume and add 1 mL of ice-cold 90–100% methanol. Vortex to mix and incubate for at least 30 minutes at 2–8°C.

Note

Once in methanol, cells can be stored at < –20°C for up to 4 weeks.

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Staining intracellular antigens for flow cytometry, cont.

9. Wash cells with an excess volume of Flow Cytometry Staining Buffer.

10. Centrifuge cells at 400–600 x *g* for 5 minutes at room temperature. Discard the supernatant.

11. Resuspend cells at 1 x 10^7 cells/mL in Flow Cytometry Staining Buffer and aliquot 1 x 10^6 cells (100 µL) into separate flow tubes.

12. **Optional** Cells can be blocked for nonspecific Fc-mediated binding using Anti-Mouse CD16/CD32 Purified or Human Fc Receptor Binding Inhibitor Purified before staining.

13. Add the recommended amount of directly conjugated antibody to each tube and incubate for 30–60 minutes at room temperature. **Protect from light**.

14. Add 2 mL of Flow Cytometry Staining Buffer and centrifuge at 400–600 x *g* for 4–5 minutes at room temperature. Discard supernatant.

15. Repeat Step 14.

16. Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer.

17. Analyze samples by flow cytometer.

Note

If needed, surface staining and intracellular phospho-staining can be performed simultaneously. As not all antibody clones will bind to a fixed epitope, refer to the "Antibody Clone Performance Following Fixation/Permeabilization" table at **thermofisher.com/antibodyfixation**.

Experimental procedure in 96-well plate

1. Prepare cells of interest for stimulation in appropriate media.

2. Count cells and resuspend in appropriate media at 1–5 x 10^6 cells/mL.

3. Add 100 µL media containing appropriate stimulants to wells.

4. Add 100 µL cells to wells and incubate at 37°C for desired time point(s). Remember to incubate untreated cells at 37°C as a negative control.

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5. **Optional** If surface staining is needed prior to fixation (in Step 6), stain cell surface antigen(s) as described in Staining Cell Surface Targets for Flow Cytometry Protocols at **thermofisher.com/surfacetargetsflow**, with antibodies conjugated to methanol-resistant fluorochromes.

6. At the end of the stimulation period, fix cells to stop stimulation by adding 200 µL of IC Fixation Buffer directly to wells.

7. Incubate plate 10–60 minutes at room temperature in the dark. **Protect from light**.

8. Centrifuge plate at 600 x *g* for 4–5 minutes at room temperature. Discard the supernatant.

9. Resuspend the cell pellets in residual volume and add 100 µL ice-cold 90–100% methanol to wells. Vortex to mix and incubate plate for at least 30 minutes at 2–8°C or on ice.

Note

Once in methanol, cells can be stored at < –20°C for up to 4 weeks.

10. Add 200 µL Flow Cytometry Staining Buffer and centrifuge cells at 600 x *g* for 4–5 minutes at room temperature. Discard the supernatant.

11. Repeat Step 10.

12. **Optional** Cells can be blocked for nonspecific Fc-mediated binding using Anti-Mouse CD16/CD32 Purified or Human Fc Receptor Binding Inhibitor Purified before staining.

13. Add the recommended amount of directly conjugated antibody to each well and incubate for 30–60 minutes at room temperature. **Protect from light**.

Note

If needed, surface staining and intracellular phospho-staining can be performed simultaneously. As not all antibody clones will bind to a fixed epitope, refer to the "Antibody Clone Performance Following Fixation/Permeabilization" table at **thermofisher.com/antibodyfixation**.

14. Add 200 µL Flow Cytometry Staining Buffer and centrifuge at 600 x *g* for 4–5 minutes. Discard the supernatant.

15. Repeat Step 14.

16. Resuspend stained cells in an appropriate volume of Flow Cytometry Staining Buffer.

17. Analyze samples by flow cytometer.

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Intracellular staining guide for human and mouse cytokines

- See below for intracellular staining quick guides for mouse and human cytokines as well as for non-cytokine proteins.
- See step-by-step Intracellular Staining Protocols.

Mouse cytokines: intracellular staining quick guide for flow cytometry

Mouse cytokine	Cell source	Activation	Incubation time	Restimulation	Intracellular block	Antibody
GM-CSF	mouse spleen	ConA (3 µg/mL) (2d)/IL-2 (20 ng/mL) + IL-4 (20 ng/mL) (3d)	2d/3d	anti-CD3 (10 µg/mL immobilized) + anti-CD28 (2 µg/mL soluble) 5hr	Brefeldin A	MP1-22E9
IFN-gamma	mouse spleen	ConA (3 µg/mL) (2d)/IL-2 (20 ng/mL) + IL-4 (20 ng/mL) (3d)	2d/3d	anti-CD3 (10 µg/mL immobilized) + anti-CD28 (2 µg/mL soluble) 5hr	Brefeldin A	XMG1.2
IL-1 alpha	mouse PEC	mIFNγ (100 ng/mL) (2hr)/LPS (100 ng/mL)(22hr)	2hr/22hr	—	Brefeldin A	ALF-161
IL-1 beta	mouse PEC	LPS (100 ng/mL) (22hr)	22hr	—	Monensin	NJTEN3
IL-2	mouse spleen	ConA (3 µg/mL) (2d)/IL-2 (20 ng/mL) + IL-4 (20 ng/mL) (3d)	2d/3d	anti-CD3 (10 µg/mL immobilized) + anti-CD28 (2 µg/mL soluble) 5hr	Brefeldin A	JES6-5H4
IL-4	mouse spleen	Th2 polarized	6d	PMA (50 ng/mL) + Ionomycin (1 µg/mL) 5hr	Brefeldin A	BVD6-24G2, 11B11
IL-5	mouse splenic CD4	ConA (3 µg/mL) (2d)/IL-2 (20 ng/mL) + IL-4 (20 ng/mL) (3d)	2d/3d	immobilized) + anti-CD28 (2 µg/mL soluble) 5hr	Brefeldin A	TRFK5
IL-6	mouse PEC	LPS (100 ng/mL) (22hr)	22hr	—	Monensin	MP5-20F3
IL-10	mouse spleen	ConA (3 µg/mL) (2d)/IL-2 (20 ng/mL) + IL-4 (20 ng/mL) (3d)	2d/3d	anti-CD3 (10 µg/mL immobilized) + anti-CD28 (2 µg/mL soluble) 5hr	Brefeldin A	JES5-16E3, JES5-2A5
IL-12/IL-23 (p40)	mouse PEC	LPS (100 ng/mL) (22hr)	22hr	—	Brefeldin A	C17.8
IL-13	mouse spleen	Th2 polarized	6d	PMA (50 ng/mL) + Ionomycin (1 µg/mL) 5hr	Brefeldin A	eBio13A
IL-17A	mouse spleen	Th17 polarized	6d	PMA (50 ng/mL) + Ionomycin (1 µg/mL) 5hr	Monensin	eBio17B7
IL-17F	mouse spleen	Th17 polarized	6d	PMA (50 ng/mL) + Ionomycin (1 µg/mL) 5hr	Monensin	eBio18F10
IL-21	mouse spleen	Th17 polarized	9d	PMA (50 ng/mL) + Ionomycin (1 µg/mL) 5hr	Monensin	FFA21
IL-22	mouse spleen	Th17 polarized	12d	PMA (50 ng/mL) + Ionomycin (1 µg/mL) 5hr	Brefeldin A	IL22JOP
IL-23 p19	mouse bone marrow	mGM-CSF (40 ng/mL)	8d	LPS (1 µg/mL) (24 hr)	Monensin	fc23cpg
MCP-1/CCL2	mouse PEC	LPS (100 ng/mL) (24hr)	24hr	—	Brefeldin A	2H5
TNF-α	mouse spleen	ConA (3 µg/mL) (2d)/IL-2 (20 ng/mL) + IL-4 (20 ng/mL) (3d)	2d/3d	anti-CD3 (10 µg/mL immobilized) + anti-CD28 (2 µg/mL soluble) 5hr	Brefeldin A	MP6-XT22, TN3-19

Annotations: mouse PEC = mouse thioglycolate-elicited peritoneal macrophages; ConA = Concanavalin A; Iono = Ionomycin; LPS = Lipopolysaccharide; PMA = Phorbol Myristate Acetate; 2d = 2-day culture; 5hr = 5-hour culture

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Human cytokines: intracellular staining quick guide

Human cytokine	Cell source	Activation	Incubation time	Restimulation	Intracellular block	Antibody
G-CSF	PBMC	LPS (1 µg/mL)	24hr	—	Monensin	8F5CSF
GM-CSF	PBMC	PMA (30–50 ng/mL)/Iono (1 µg/mL)	5hr	—	Monensin	BVD2-21C11
IFN gamma	PBMC	PMA (30–50 ng/mL)/Iono (1 µg/mL)	5hr	—	Brefeldin A	4S.B3
IL-1 alpha	PBMC	LPS (1 µg/mL)	24hr	—	Monensin	364/3B3-14, CRM8
IL-1 beta	PBMC	LPS (100 ng/mL)	4hr	—	Brefeldin A	CRM56
IL-1RA	PBMC	LPS (100 ng/mL)	24hr	—	Brefeldin A	CRM17
IL-2	PBMC	PMA (30–50 ng/mL)/Iono (1 µg/mL)	4–6hr	—	Brefeldin A	MQ1-17H12
IL-4	PBMC	PMA (30–50 ng/mL)/Iono (1 µg/mL)	4–6hr	—	Brefeldin A	8D4-8
IL-5	CD4	Th2 polarizing cultures	6d	PMA (50 ng/mL) + Ionomycin (1 µg/mL) (5hr)	Brefeldin A	TRFK5, JES1-5A10
IL-6	PBMC	LPS (100 ng/mL)	24hr	—	Brefeldin A	MQ2-13A5
IL-9	CD4	Th2 polarizing cultures	6d	PMA (50 ng/mL) + Ionomycin (1 µg/mL) (5hr)	Monensin	MH9A4
IL-10	CD4	Th2 polarizing cultures	6d	PMA (50 ng/mL) + Ionomycin (1 µg/mL) (5hr)	Monensin	JES3-9D7
IL-12/IL-23 (p40)	PBMC	hIFN gamma (100 ng/mL) (2hr)/LPS (100 ng/mL) (22hr)	2hr/22hr	-	Brefeldin A	C8.6
IL-13	CD4	anti-CD3 (10 µg/mL, immobilized) + anti-CD28 (2 µg/mL, soluble) + IL-2 (10 ng/mL) + IL-4 (20 ng/mL) (2d); IL-2 (10 ng/mL) + IL-4 (20 ng/mL) (3d)	2d/3d	PMA (5 ng/mL) + Ionomycin (500 ng/mL) (4hr)	Brefeldin A	PVM13-1
IL-17A	PBMC	Th17 polarizing cultures	6d	PMA (50 ng/mL) + Ionomycin (1 µg/mL) (5hr)	Brefeldin A	eBio64CAP17, eBio64DEC17
IL-21	PBMC	PMA (30–50 ng/mL)/Iono (1 µg/mL)	4–7hr or 12–18hr	—	Brefeldin A	eBio3A3-N2
IL-22	CD4	Th17 polarizing cultures	6d	PMA (50 ng/mL) + Ionomycin (1 µg/mL) (5hr)	Brefeldin A	IL22JOP
IL-23 p19	PBMC	hGM-CSF (40 ng/mL) + hIL-4 (40 ng/mL)	6d	LPS (1 µg/mL) (24hr)	Monensin	23dcdp
MCP-1/CCL2	PBMC	LPS (1 µg/mL)	24hr	—	Monensin	2H5, 5D3-F7
RANTES/CCL5	PBMC	LPS (1 µg/mL)	24hr	—	Monensin	VL1
TNF-α	PBMC	PMA (30–50 ng/mL)/Iono (1 µg/mL)	5hr	—	Brefeldin A	MAb11
TNF-β	PBMC	Th1 polarizing cultures	6d	PMA (50 ng/mL) + Ionomycin (1 µg/mL) (5hr)	Monensin	359-81-11

Annotations: Iono = Ionomycin; PMA = Phorbol Myristate Acetate; LPS = Lipopolysaccharide; 2d = 2-day culture; 5hr = 5-hour culture; LPS for activation of human PBMC obtained from Sigma (#L-8274)

This protocol is continued on page 50

Intracellular staining guide for human and mouse cytokines, cont.

Non-cytokine proteins: intracellular staining quick guide

Antigen	Antibody
Mouse/Rat Bcl-2	10C4
Mouse CTLA-4 (CD152)	UC10-4B9
Human CTLA-4 (CD152)	14D3
Human/Mouse/Rat Cytochrome C	6H2
Human Foxp3	PCH101
Mouse Foxp3	FJK-16s
GATA3	
Mouse/Human Granzyme B	eBioGrB
House Langerin (CD207)	eBioRMUL.2
Human Nanog	hNanog.1
Human PCNA (Proliferating Cell Nuclear Antigen)	PC10
Mouse Perforin	eBioOMAK-D
Human Perforin	dG9
ROR gamma	
Mouse SLP-76	MS76
Human SLP-76	HS76
Human/Mouse T-bet	eBio4B10 (4B10, 4-B10)
Human TLR3	TLR3.7
Mouse TLR9	M9.D6
Human TLR9	eB72-1665
Mouse/Human ZAP-70	1E7.2

For Research Use Only. Not for use in diagnostic procedures.

Compensation

Introduction

Invitrogen™ UltraComp eBeads™ react with antibodies of human, rabbit, mouse, rat and hamster origin, and are immunoglobulin light chain independent. Each drop of beads contains two populations: a positive population that will capture any mouse, rat, or hamster antibody; and a negative population that will not react with antibodies. When a fluorochrome-conjugated antibody is added to the beads, both positive and negative populations result. This bimodal distribution can be used for single-color compensation controls in multicolor flow cytometry experiments.

UltraComp eBeads are compatible with all fluorochromes excited by blue (488 nm), green (532 nm), yellow-green (561 nm), red (633–635 nm), ultraviolet (355 nm) or violet (405 nm) lasers.

Ultra Comp ebeads Plus are broadly in use for many fluorophores and animal species, including all fluorochromes excited by ultraviolet (355 nm) violet (405 nm), blue (488 nm), green (532 nm), yellow-green (561 nm), and red (633-640 nm) lasers. UltraComp eBeads are optimized for Brilliant Violet 785/786-, Brilliant Violet 711-, Super Bright 780- and Super Bright 702-conjugated antibodies.

Note

This protocol is intended for use with the specific products mentioned within it. Substituting different products is not recommended.

Note

This protocol can be used for OneComp eBeads (Cat. No. 01-1111).

Protocol

Materials

- Compensation beads: UltraComp eBeads (Cat. No. 01-2222) or UltraComp eBeads Plus (Cat. No. 01-3333-41)
- Unstained cells
- Primary antibodies (directly conjugated)
- Flow Cytometry Staining Buffer (Cat. No. 00-4222)
- 12 x 75 mm round-bottom test tubes

This protocol is continued on page 52

Compensation, cont.

Experimental procedure

Step I: Preparation of single-color compensation controls

1. Label a tube for each fluorochrome that will be used in the experiment.
2. Mix beads by vigorously inverting at least 10 times or pulse-vortexing.
3. Label each tube and pulse vortex 10 times.
4. Add 1 drop of UltraComp eBeads to each tube.
5. Add 1 test or less of antibody conjugate to each tube and mix.
6. Mix well by flicking, inverting vigorously, or pulse vortexing.
7. Incubate at 2–8°C for 15–30 minutes in the dark.
8. Add 2 mL of Flow Cytometry Staining Buffer to each tube and centrifuge at 400–600 x *g* for 3–5 minutes.
9. Decant supernatant and add 0.2-0.4 mL of Flow Cytometry Staining Buffer to each tube.
10. Mix briefly by flicking or pulse vortexing before analysis.

Note

UltraComp eBeads are compatible with standard staining buffers that contain PBS or HBSS, proteins such as bovine serum albumin (BSA) or FBS, and sodium azide. No other additives should be used. For more information, please contact Technical Support.

Note

A test is defined as the amount (µg) of antibody that will stain a cell sample in a final volume of 100 µL. If high background is observed on the negative-bead population, less antibody can be used. For these cases, it is recommended to use 0.125 µg or less. Because the binding of the antibody to the positive bead is not dependent on the antibody's specificity, it is not necessary to use the antibody at its optimal concentration. For most antibodies, appropriate compensation values will result when 0.03–1.0 µg of antibody is used in a test.

This protocol is continued on page 53

Step II: General compensation setup principles

1. Run unstained cells on cytometer. Determine appropriate Forward scatter (FSC) and Side scatter (SSC) settings and fluorescence detector (photomultiplier tube, or PMT) voltages for the cells.

2. Run a sample of beads to adjust FSC/SSC to visualize beads (this can even be a single color–stained bead). It is acceptable to adjust the FSC/SSC to get the beads in view.

3. Run each single-stained bead sample to assure the positive peaks are on scale. PMT voltages should be decreased (as minimally as possible) for any positive bead peak that is off-scale. Do not record any data until all single color–stained beads have been reviewed.

4. Run each single-stained bead sample to perform compensation setup and record files for compensation controls.

5. Readjust FSC/SSC settings for cell samples and acquire experimental samples.

6. Collect and record experimental samples.

Note

Goat and sheep host species should use single color cell and FMO controls, not beads.

Panel design

Introduction

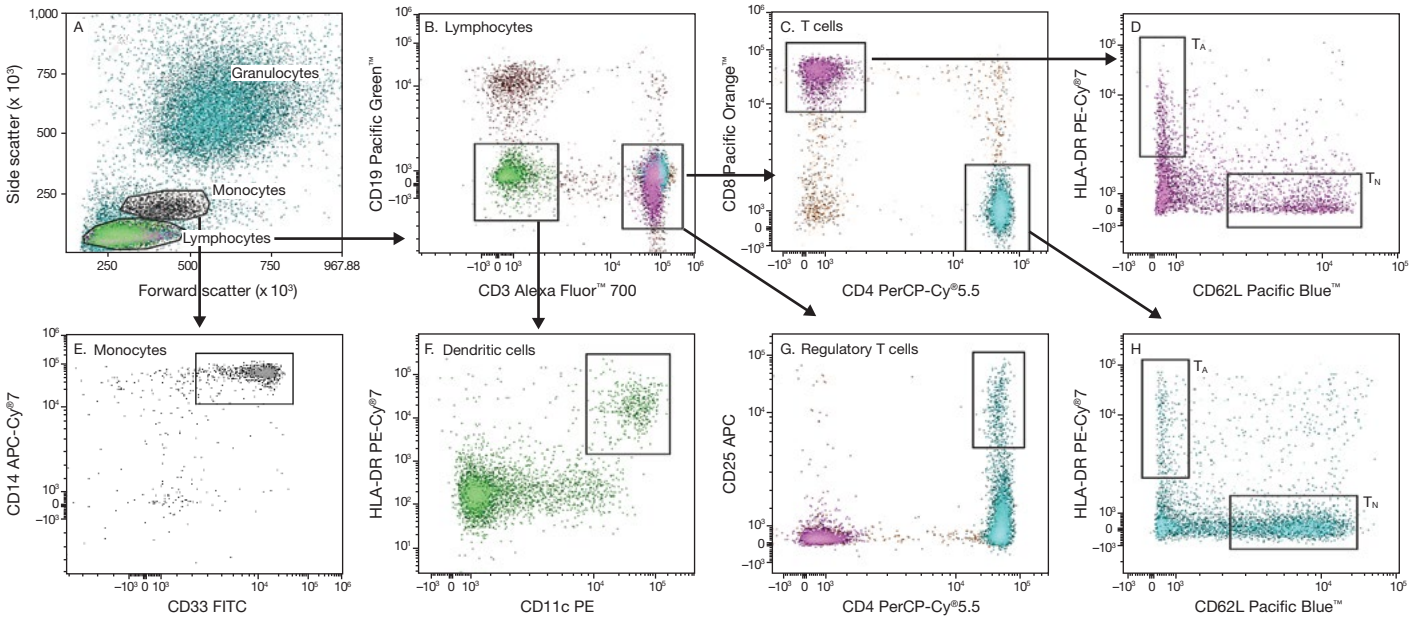
Advances in both flow cytometry reagents and instrumentation allow researchers to run increasingly complex multicolor experiments. The advantages of multiparameter flow cytometry include the ability to perform single-cell interrogation with multiple markers, to correlate cell data using multiple analytes, and ultimately to more accurately define cell populations (Figure 1). Additionally, multiparameter experiments can improve efficiency by requiring fewer samples and smaller sample volumes and by increasing sample throughput. Increasing the number of colors and antigens detected, however, increases the complexity of the experimental design, requiring significantly higher attention to optimization, controls, and other details [1].

One of the biggest challenges in multiparameter flow cytometry is selecting the right combination of fluorophores and antibody conjugates so that the need for compensation and spillover adjustments is kept to a minimum while the quality and accuracy of the data are not compromised. There are some excellent resources available for the beginner, including the Molecular Probes flow cytometry webinar "Multicolor Flow Cytometry Panel Design" by Dr. Holden Maecker of Stanford University. Additional publications on this topic are available [2–4].

When designing a multicolor flow cytometry panel, there are several key points to consider:

- Know the configuration of the instrument being used (laser and filters) before you begin.
- Use a tool like the Molecular Probes Fluorescence SpectraViewer to visualize the spectral overlap of fluorophores.
- Titrate and optimize each antibody; building the right panel is an iterative process.
- Use bright fluorophore labels on antibodies for low-abundance antigens and dim fluorophore labels on antibodies for highly expressed antigens.
- Use fluorophores that are spectrally similar for different cell subpopulations that will be gated and analyzed separately.
- Include a cell viability dye in the panel to exclude dead cells and debris from the data.

This protocol is continued on page 55



Determining fluorophore brightness

In flow cytometry, fluorophore brightness is a function not only of the quantum yield and extinction coefficient of the fluorophore itself, but also of the effects of background contributions. Background fluorescence—e.g., from nonspecific staining, cellular autofluorescence, and instrument noise—can affect the ability to resolve the fluorescence of the antibody conjugate–stained cell population (positive) from that of the unstained cell population (negative).

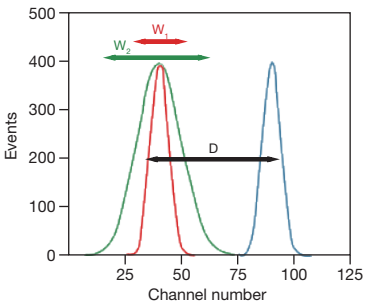
The signal-to-noise ratio (S/N) is one measure of the sensitivity of an assay and its ability to detect differences between stained and unstained populations. To calculate a simple S/N, divide the median fluorescence intensity (MFI) of the positive cells by that of the negative cells (Figure 2).

However, the relative brightness of a fluorophore-conjugated antibody is determined not only by the intensity difference between stained and unstained cells, but also by the intensity distribution spread of the unstained cell population. Proposed by Maecker *et al.* [2], the Stain Index (SI) takes these two parameters into account (Figure 2). The SI can be useful for comparing histograms of cell populations stained with different fluorescent conjugates of the same antibody (Table 1, Figure 3).

Figure 1. Ten-parameter immunophenotyping of human peripheral blood mononuclear cells (PBMCs) with the Attune NxT Acoustic Focusing Cytometer. Lymphocytes and monocytes were gated based on forward- and side-scatter profiles (A). Within the lymphocyte gate, T cells can be isolated based on their expression of CD3 (B), and further subdivided into CD4⁺ and CD8⁺ subpopulations (C). In addition, regulatory T cells express CD4 and CD25 (G) and are important mediators of dominant peripheral tolerance. CD62L identifies naive CD4⁺ and CD8⁺ T cells (TN), whereas HLA-DR is expressed by activated T cells (TA) (D, H). Conventional dendritic cells found in peripheral blood are generally negative for T and B cell lineage markers and co-express the integrin CD11c and HLA-DR (F). Monocytes are located just above lymphocytes in the scatter profile (A), and express both CD14 and CD33 (E).

This protocol is continued on page 56

Panel design, cont.



Stain Index = D / W

Where:
D is the difference between positive and negative peak medians.
W is the spread of the negative peak and is equal to 2 x rSD.
rSD is the robust standard deviation.

Signal-to-noise ratio = MFI (positive cells) / MFI (negative cells)

Where:
MFI is the median fluorescence intensity.

Figure 2. Comparison of Stain Index (SI) and signal-to-noise ratio (S/N). An illustration of two fluorophores with the same S/N but different SI due to different widths of the negative peak (narrow W1 vs. wide W2). Because the width of the negative peak affects the separation of the positive and negative signals, SI is the preferred statistic when comparing fluorophore brightness.

Table 1. Staining Index for different fluorophore conjugates of an anti-CD4 antibody (clone 53.5).

Brightness	Fluorophore component of conjugate (Conjugate Cat. No.)	Ex max*	Em max*	Laser line	BP Em filter†	Stain Index
High	APC (MHCD0405)	645	660	633	660/20	200.31
	PE (MHCD0404)	496, 565	575	488	585/42	158.46
	APC-Cy5.5 (MHCD0419)	650	690	633	710/50	108.97
	PE-Cy5.5 (MHCD0418)	496, 565	690	488	695/40	105.91
	Alexa Fluor 488 dye (MHCD0420)	495	519	488	525/50	91.72
Medium	PE–Alexa Fluor 610 dye (MHCD0422)	488	628	488	620/10	70.71
	FITC dye (MHCD0401)	493	525	488	525/50	56.40
	PE-Cy7 (MHCD0412)	496, 565	774	488	780/60	53.70
	PE–Alexa Fluor 700 dye (MHCD0424)	496, 565	723	488	720/30	52.45
	TRI-COLOR dye (PE-Cy5) (MHCD0406)	496, 565	670	488	695/40	50.31
	PE–Texas Red dye (MHCD0417)	496, 565	613	488	695/40	40.85
	Qdot 605 nanocrystal (Q10008)	350	605	405	605/20	35.17
	APC–Alexa Fluor 750 dye (MHCD0427)	645	775	633	780/60	31.91
	Alexa Fluor 700 dye (MHCD0429)	696	719	633	710/50	24.85
Low	Qdot 655 nanocrystal (Q10007)	350	655	405	655/20	20.62
	Qdot 705 nanocrystal (Q10060)	350	720	405	720/20	18.38
	Pacific Blue dye (MHCD0428)	410	455	405	450/50	14.61
	Alexa Fluor 405 dye (MHCD0426)	401	421	405	450/50	10.01
	PerCP (MHCD0431)	482	675	488	695/40	8.75
	Pacific Orange dye (MHCD0430)	400	551	405	585/42	6.06

* Approximate fluorescence excitation (Ex) and emission (Em) maxima for conjugates, in nm.
† BP Em filter = bandpass emission filter, in nm. Staining Index was determined on the BD™ LSR II Flow Cytometer with FACSDiva™ version 6.1 software.

This protocol is continued on page 57

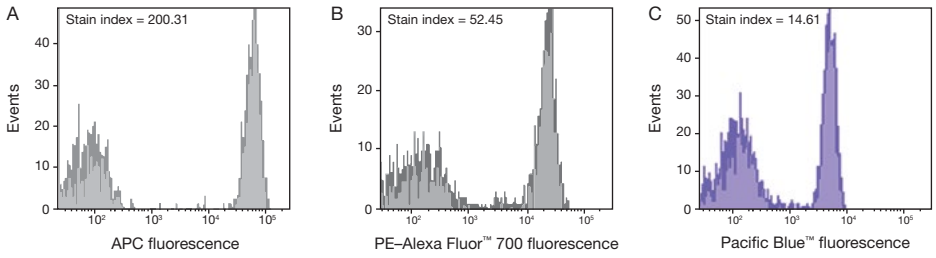


Figure 3. Representative histograms for cells stained with anti-CD4 antibody conjugates. Ammonium chloride-lysed human whole blood was used to evaluate the performance of 20 different mouse anti-human CD4 antibody direct conjugates (see Table 1). Each conjugate was titrated and optimized to produce a maximum signal-to-noise ratio. Cells were analyzed on a BD™ LSR II Flow Cytometer with FACSDiva™ version 6.1 software. Histograms represent 10,000 cells collected in a lymphocyte gate: (A) high brightness from anti-CD4 antibody, APC conjugate; (B) medium brightness from anti-CD4 antibody, PE-Alexa Fluor 700 conjugate; (C) low brightness from anti-CD4 antibody, Pacific Blue conjugate. The Stain Index (SI) for each conjugate is listed in the left corner of the plot.

Online tools:
Fluorescence SpectraViewer and Flow Cytometry Panel Builder

The Molecular Probes Fluorescence SpectraViewer is an online tool that displays the excitation and emission spectra for fluorescent dyes and proteins, facilitating selection of appropriate dyes for your multicolor experiment. You can enter your instrument laser and filter configuration (Figure 4), then select the fluorophores under consideration. Figure 5 shows an example of a five-color panel. An additional feature of the SpectraViewer is the spillover table function, which shows fluorescence overlap (or spillover) for each dye in each channel (Table 2). You can find the Fluorescence SpectraViewer at **thermofisher.com/spectraviewer**.

Our Invitrogen Flow Cytometry Panel Builder can help you choose fluorescent antibody conjugates for your flow cytometry panel. This online tool guides you through flow cytometry panel design, providing a simplified, customizable experience to fit your flow cytometry panel design needs. Similar to the SpectraViewer it displays the excitation and emission spectra for fluorescent dyes and proteins, but also shows available antibody conjugates to facilitate selection of appropriate antibodies for your multicolor experiment.

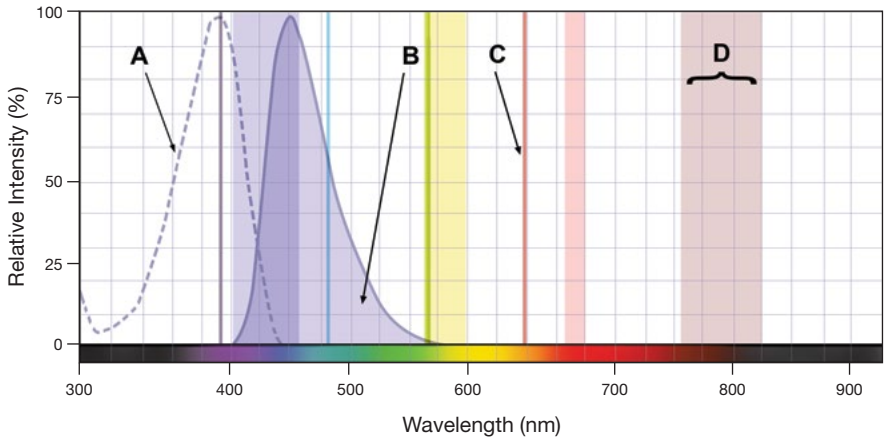


Figure 4. Overview of Molecular Probes SpectraViewer components. (A) Excitation spectrum and (B) emission spectrum for the same fluorophore. (C) Laser excitation wavelength. (D) Bandpass emission filter wavelengths.

This protocol is continued on page 58

Panel design, cont.

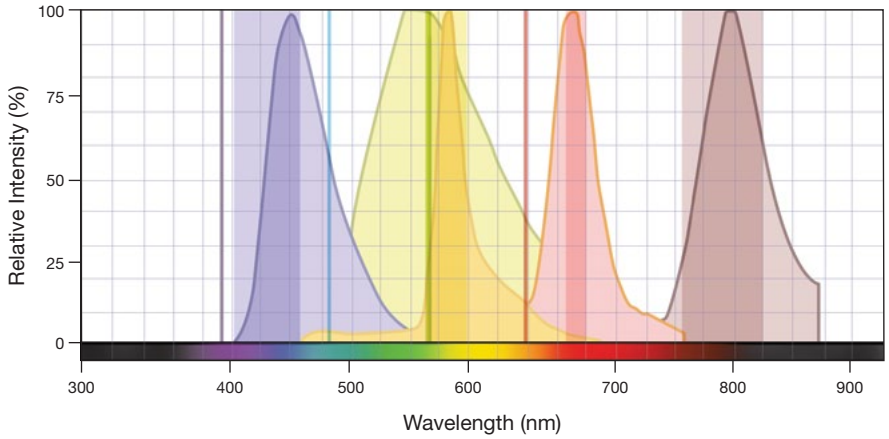


Figure 5. Five-color panel depicted on the Molecular Probes SpectraViewer. Emission curves for Pacific Blue dye (purple) excited by the 405 nm laser, LIVE/DEAD Fixable Yellow stain (yellow) excited by the 405 nm laser, R-phycoerythrin (PE, orange) excited by the 488 nm laser, Alexa Fluor 647– PE (red) excited by the 561 nm laser, and Alexa Fluor 750–allophycocyanin (brown) excited by the 633 nm laser. Lasers shown are 405 nm (violet), 488 nm (blue), 561 nm (yellow), and 633 nm (red).

Table 2. SpectraViewer spillover table for five fluorophores (Figure 5) with emission filter configurations found on the Attune NxT cytometer.

Fluorophore	Bandpass (BP) emission filter (nm)			
	440/50	574/26	670/14	780/60
Pacific Blue dye	48.2%	0.9%	0.0%	0.0%
LIVE/DEAD Fixable Yellow stain	0.2%	20.3%	2.2%	0.0%
PE (R-phycoerythrin)	0.0%	53.7%	1.2%	0.0%
Alexa Fluor 647–PE	0.0%	3.3%	28.9%	0.1%
Alexa Fluor 750–allophycocyanin	0.0%	0.0%	2.9%	66.1%

Percentages represent the relative fluorescence signal detected with the indicated emission filters (independent of excitation light source). The 574/26 nm BP filter collects 20.3% and 53.7% of the LIVE/DEAD Fixable Yellow stain and PE fluorescence, respectively. However, there should be no actual spillover because the LIVE/DEAD stain is excited at 405 nm but not 488 nm, and PE is excited at 488 nm but not 405 nm; i.e., they will be excited using different lasers.

This protocol is continued on page 59

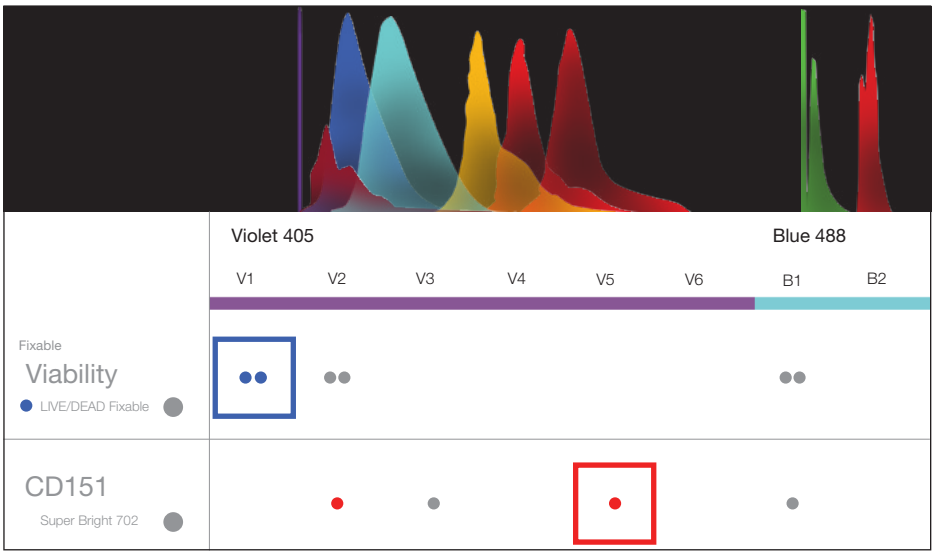


Figure 6. Screen shot of the Flow Cytometry Panel Builder.

References

1. *Nat Rev Immunol* 4:648 (2004).
2. *Cytometry A* 62:169 (2004).
3. *Cytometry A* 69:1037 (2006).
4. *Clin Lab Med* 27:469 (2007).

Blocking non-specific binding with CellBlox™ Blocking Buffer

Product description

The Invitrogen™ CellBlox™ Blocking Buffer is formulated to block nonspecific binding of Invitrogen™ NovaFluor™ labels with cells. These nonspecific interactions can result in higher background labeling. CellBlox Blocking Buffer is a non-antibody, non-protein-based blocking solution, and should be used every time a NovaFluor dye is used for labeling any cell type to minimize background labeling.

CellBlox Blocking Buffer is also recommended for use with cyanine-based dyes or cyanine-based tandem dyes to block non-specific interactions with monocytes, macrophages and other cell types to minimize background labeling.

Use of CellBlox Blocking Buffer requires minimal change to most flow cytometry staining protocols. Add 5 µL CellBlox Blocking Buffer directly to a cell suspension containing 10^3–10^8 cells prior to the addition of an antibody, with 100 µL as a final staining volume. CellBlox Blocking Buffer may instead be added to an antibody mixture prior to labeling cells, by adding 5 µL CellBlox Blocking Buffer for every stained sample to be labeled with the antibody mixture, with 100 µL as a final staining volume.

Experimental protocol

Materials

- CellBlox Blocking Buffer (Cat. No. B001T02F01, B001T03F01, B001T06F01)
- eBioscience Flow Cytometry Staining Buffer (Cat. No. 00-4222-26)
- Primary conjugated antibodies
- 12 x 75 mm round-bottom polystyrene test tubes or U- or V-bottom polystyrene microplates

Critical notes

This protocol is intended for use with the specific products mentioned within it. Substituting different products is not recommended.

Note

Cellblox Blocking Buffer should be used any time NovaFluor dyes are used with cells.

Tips for Success

- Always use CellBlox Blocking Buffer with NovaFluor dyes when labeling cells for best background reduction.
- CellBlox Blocking Buffer is compatible with all fluorophores and with Invitrogen™ LIVE/DEAD™ Fixable Dead Cell Stains.
- CellBlox Blocking Buffer can be used with any fluorophore-antibody conjugate as a high-performance monocyte and macrophage blocking solution.
- CellBlox Blocking Buffer is compatible with other blocking reagents, such as Fc Block, blocking proteins, Brilliant Stain Buffer, and Super Bright Complete Staining Buffer.
- CellBlox Blocking Buffer is not required when labeling antibody-capture beads.

This protocol is continued on page 61

Protocol when adding CellBlox Blocking Buffer to antibody mixture (preferred protocol)

1. Prepare single cell suspension as described in Cell Preparation for Flow Cytometry Protocols at **thermofisher.com/cellpreparation**.
2. Aliquot cell suspension containing 10^3–10^8 cells to each sample tube or well.
3. Prepare an antibody mixture of conjugated antibodies, at predetermined optimal concentrations of each antibody conjugate. Mix well after addition of each antibody.
4. Add 5 µL CellBlox Blocking Buffer for every sample to be labeled directly into the antibody mixture, to a final staining volume of 100 µL per sample. For example, if preparing enough antibody mixture for use with 10 samples, add 50 µL CellBlox Blocking Buffer to the antibody mixture.
5. Add volume of antibody mixture containing CellBlox Blocking Buffer to aliquoted cell samples, with 100 µL as a final staining volume per sample.
6. Incubate samples for 30 minutes at 2–8°C, protected from light.
7. Wash the cells by adding 2 mL Flow Cytometry Staining Buffer per sample. Centrifuge at 400–600 x *g* for 5 minutes. Discard supernatant.
8. Repeat Step 7.
9. Resuspend cells in an appropriate volume of Flow Cytometry Staining Buffer.
10. Analyze samples by flow cytometry, or if staining for intracellular targets, proceed with Best Protocols: Staining Intracellular Antigens for Flow Cytometry at **thermofisher.com/intracellularstaining**.

This protocol is continued on page 62

Blocking non-specific binding with CellBlox™ Blocking Buffer, cont.

Protocol when adding CellBlox Blocking Buffer to bulk cell samples (alternate protocol 1)

1. Prepare single cell suspension as described in Cell Preparation for Flow Cytometry Protocols at **thermofisher.com/cellpreparation**.
2. Prepare bulk cell suspension containing 10^3–10^8 cells for every sample to be labeled.
3. Add 5 µL CellBlox Blocking Buffer to the bulk cell suspension for every sample to be labeled. For example, if preparing enough bulk cells for use with 10 samples, add 50 µL CellBlox Blocking Buffer to the bulk cell suspension.
4. Aliquot cell suspension containing CellBlox Blocking Buffer to each sample tube or well.
5. Add appropriate amount of each antibody conjugate to cell suspension aliquot containing CellBlox Blocking Buffer, with 100 µL as a final staining volume per sample.
6. Incubate samples for 30 minutes at 2–8°C, protected from light.
7. Wash the cells by adding 2 mL Flow Cytometry Staining Buffer per sample. Centrifuge at 400–600 x *g* for 5 minutes. Discard supernatant.
8. Repeat Step 7.
9. Resuspend cells in an appropriate volume of Flow Cytometry Staining Buffer.
10. Analyze samples by flow cytometry, or if staining for intracellular targets, proceed Best Protocols: Staining Intracellular Antigens for Flow Cytometry at **thermofisher.com/intracellularstaining**.

This protocol is continued on page 63

Protocol when adding CellBlox Blocking Buffer to individual cell samples (alternate protocol 2)

1. Prepare single cell suspension as described in Cell Preparation for Flow Cytometry Protocols at **thermofisher.com/cellpreparation**.
2. Aliquot cell suspension containing 10^3–10^8 cells to each sample tube or well.
3. Add 5 µL CellBlox Blocking Buffer directly to each sample, prior to staining cells.
4. Add appropriate amount of each antibody conjugate to cell suspension containing CellBlox Blocking Buffer, to a final staining volume of 100 µL per sample.
5. Incubate samples for 30 minutes at 2–8°C, protected from light.
6. Wash the cells by adding 2 mL Flow Cytometry Staining Buffer per sample. Centrifuge at 400–600 x *g* for 5 minutes. Discard supernatant.
7. Repeat Step 6.
8. Resuspend cells in an appropriate volume of Flow Cytometry Staining Buffer.
9. Analyze samples by flow cytometry, or if staining for intracellular targets, proceed Best Protocols: Staining Intracellular Antigens for Flow Cytometry at **thermofisher.com/intracellularstaining**.

Flow cytometry

Cell viability and apoptosis

Cell viability

Introduction

Cell viability assays can be used to distinguish between live and dead cell populations, to correlate with other cell functions or treatments, or to exclude dead cell populations from analyses. Distinguishing dead cells and removing artifacts is a critical step to help ensure accurate results in all flow cytometry assays because exclusion of the dead cells from the data allows cleaner separation and identification of cell populations.

The process of apoptosis is characterized by specific changes in cellular features, including reduced DNA content, loss of mitochondrial membrane potential, and disruption of lipid asymmetry in the cell membrane. Each of these can be measured by flow cytometry using dyes and reagents that are sensitive and specific for these changes.

Membrane integrity-based viability assay

The Invitrogen LIVE/DEAD fixable dead cell stains distinguish between live and dead cells in flow cytometry. In cells with compromised membranes, the dye reacts with free amines both in the cell interior and on the cell surface, yielding intense fluorescent staining. In viable cells, the dye's reactivity is restricted to the cell-surface amines, resulting in less intense fluorescence. The difference in intensity between the live and dead cell populations is typically greater than 50 fold, and this fluorescence intensity discrimination is completely preserved following formaldehyde fixation. The ability to stain live cells with a viability dye and preserve that staining pattern after fixation is critical for intracellular immunophenotyping.

These single-color assays use only one channel of a flow cytometer, making the reactive dyes in the LIVE/DEAD Fixable Dead Cell Stain Kits compatible with multiparameter staining experiments (see table on page 67); appropriate flow cytometer channels may vary depending on the instrument.

This protocol can be used for:

- Identifying live and dead cells using a flow cytometer

You will need the following for this protocol:

- Cells growing in culture
- One of the LIVE/DEAD fixable dead cell stains (e.g. LIVE/DEAD Fixable Green Dead Cell Stain Kit (Cat. No. L23101))
- Flow cytometer

This protocol is continued on page 66

Note

ArC Amine-Reactive Compensation Bead Kit: Optimized for use with the LIVE/DEAD Fixable Dead Cell Stain Kits, the Invitrogen™ ArC™ Amine Reactive Compensation Bead Kit (Cat. No. A10346) is a tool designed to remove spectral overlap of the fixable dead-cell stains with other standard fluorophores. This kit provides two polystyrene microsphere samples: ArC Reactive Beads, which are reactive to all dyes in the LIVE/DEAD Fixable Dead Cell Stain Kits, and Negative Control Beads, which have no reactivity. The two components provide negative and positive populations that can be used to accurately set compensation when using the LIVE/DEAD Fixable Dead Cell Stains.

Cell viability, cont.

Protocol

1. Thaw vial of dye.
2. Dilute LIVE/DEAD fixable dead cell stain by adding 50 µL DMSO to vial.
3. Centrifuge a sample of cells in suspension containing at least 1 × 10^6 cells. Discard the supernatant.
4. Wash the cells once with 1 mL of PBS.
5. Resuspend the cells in 1 mL of PBS.
6. Count the cells and adjust the density with PBS to 1 x 10^6 cells in a 1 mL volume.
7. Add 1 µL of diluted stain to cells and mix well.
8. Incubate 30 minutes at room temperature or on ice for 30 minutes, protected from light.
9. Wash the cells once with 1 mL of PBS and resuspend the cells in 900 µL of PBS.
10. Add 100 µL of 37% formaldehyde.
11. Incubate at room temperature for 15 minutes.
12. Wash once with 1 mL of PBS with 1% bovine serum albumin and resuspend the cells in 1 mL of PBS with 1% bovine serum albumin.
13. Analyze on flow cytometer using the appropriate excitation and detection channel (see table on page 67).

Note

If fixation is not required, then you can skip the steps 9–12 below. Instead, wash the cells twice with 1 mL of PBS with 1% bovine serum albumin, and resuspend in 1 mL of PBS with 1% bovine serum albumin.

This protocol is continued on page 67

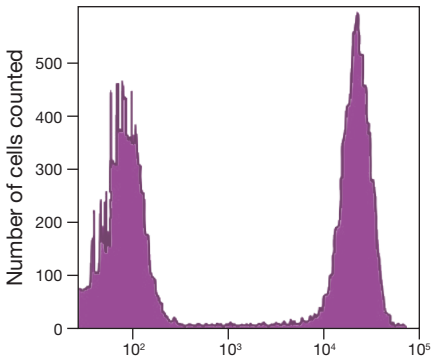
Emission specifications

LIVE/DEAD dye	Excitation source	Ex*	Em*
LIVE/DEAD Fixable Blue stain	UV	350	450
LIVE/DEAD Fixable Violet stain	405	416	451
LIVE/DEAD Fixable Lime stain	405	405	506
LIVE/DEAD Fixable Aqua stain	405	367	526
LIVE/DEAD Fixable Yellow stain	405	400	575
LIVE/DEAD Fixable Green stain	488	495	520
LIVE/DEAD Fixable Olive stain	488	480	557
LIVE/DEAD Fixable Orange stain	561	580	602
LIVE/DEAD Fixable Red stain	561	595	615
LIVE/DEAD Fixable Far Red stain	633/635	650	665
LIVE/DEAD Fixable Scarlet stain	633/635	700	775
LIVE/DEAD Fixable Near-IR (775) stain	633/635	750	775
LIVE/DEAD Fixable Near-IR (780) stain	633/635	750	780
LIVE/DEAD Fixable Near IR (876) stain	808	840	876

*Approximate fluorescence excitation (Ex) and emission (Em) maxima, in nm.

Protocol tip

- Use the solution of reactive dye as soon as possible, ideally within a few hours of reconstitution.
- Cell concentration should be 1 x 10^4–1 x 10^6 cells per mL
- Washing is optional after staining
- Cell staining is preserved after fixation, but fixation is not required
- Protein concentration in buffer should be <1%



Live and dead cells distinguished by flow cytometry using the LIVE/DEAD Fixable Violet Dead Cell Stain Kit.

Annexin V staining for apoptosis detection

Introduction

The use of the Annexin V apoptosis assay protocol is a common method for detecting apoptotic cells. The below protocols are recommended for use with the specific flow cytometry kits mentioned. Please see the Annexin V Staining page for a discussion about general experimental conditions and avoiding false positives, or to review a selection guide for all of our Annexin V products.

Note

This protocol is intended for use with the specific products mentioned within it. Substituting different products is not recommended.

Annexin V flow cytometry protocols

- Annexin V staining protocol
- Annexin V staining protocol with Fixable Viability Dyes
- Annexin V staining protocol with surface and intracellular staining

Annexin V staining protocol

Materials

- 12 x 75 mm round-bottom tubes
- 1X Gibco™ PBS (Phosphate-buffered saline), pH 7.4, (Cat. No. 10010023)
- Annexin V Apoptosis Detection kit (any one of the kits listed). Each kit includes an Annexin V conjugate.
  - eFluor 450 (Cat. Nos. 88-8006-72, 88-8006-74)
  - FITC (Cat. Nos. 88-8005-72, 88-8005-74)
  - PerCP-eFluor 710 (Cat. Nos. 88-8008-72, 88-8008-74)
  - PE (Cat. Nos. 88-8102-72, 88-8102-74)
  - PE-Cyanine7 (Cat. Nos. 88-8103-72, 88-8103-74)
  - APC (Cat. Nos. 88-8007-72, 88-8007-74)
- 10X Binding buffer

General notes

- Due to the calcium dependence of the Annexin V:PS interaction, it is critical to avoid buffers containing EDTA or other calcium chelators during Annexin V experiments.
- Annexin V can only be used as a marker of apoptosis in cells where the plasma membrane is intact. Destroying the integrity of the plasma membrane will allow binding of Annexin V to PS inside the cell.

Note

In early-stage apoptosis, the plasma membrane excludes viability dyes such as propidium iodide (PI), 7-AAD, or Fixable Viability Dyes such as LIVE/DEAD™ Fixable Dead Cell Stain Kits. These cells will stain with Annexin V but not a viability dye, thus distinguishing cells in early apoptosis. However, in late stage apoptosis, the cell membrane loses integrity thereby allowing Annexin V to also access PS in the interior of the cell. A viability dye can be used to resolve these late-stage apoptotic and necrotic cells (Annexin V, viability dye-positive) from the early-stage apoptotic cells (Annexin V positive, viability dye-negative).

This protocol is continued on page 69

Experimental procedure

1. Prepare 1X binding buffer by mixing 1 part of 10X binding buffer with nine parts of distilled water.
2. Harvest cells.
3. Wash cells once in 1X PBS, then once in 1X binding buffer.
4. Resuspend cells in 1X Binding Buffer at 1–5 x 106 cells/mL.
5. Add 5 µL of fluorochrome-conjugated Annexin V to 100 µL of the cell suspension.
6. Incubate 10–15 minutes at room temperature. **Protect from light**.
7. Add 2 mL 1X binding buffer and centrifuge at 400–600 x *g* for 5 minutes at room temperature. Discard supernatant.
8. Resuspend cells in 200 µL of 1X binding buffer.
9. Add 5 µL of Propidium Iodide Staining Solution or 7-AAD Viability Staining Solution and incubate 5–15 minutes on ice or at room temperature.
10. Analyze by flow cytometry.

Note

Propidium iodide and 7-AAD must remain in the buffer during acquisition.
Do not wash cells after the addition of propidium iodide or 7-AAD.

Note

Cells should be analyzed within 4 hours after the initial incubation period due to adverse effects on the viability of cells left in the presence of propidium iodide or 7-AAD for prolonged periods. Store at 2–8°C and **protect from light** until ready for analysis.

This protocol is continued on page 70

Annexin V staining for apoptosis detection, cont.

Annexin V staining protocol with Fixable Viability Dyes

Materials

- 12 x 75 mm round-bottom tubes
- 1X Gibco™ PBS (Phosphate-buffered saline), pH 7.4, (Cat. No. 10010023)
- Annexin V Apoptosis Detection kit (any one of the kits listed). Each kit includes an Annexin V conjugate.
  - eFluor 450 (Cat. Nos. 88-8006-72, 88-8006-74)
  - FITC (Cat. Nos. 88-8005-72, 88-8005-74)
  - PerCP-eFluor 710 (Cat. Nos. 88-8008-72, 88-8008-74)
  - PE (Cat. Nos. 88-8102-72, 88-8102-74)
  - PE-Cyanine7 (Cat. Nos. 88-8103-72, 88-8103-74)
  - APC (Cat. Nos. 88-8007-72, 88-8007-74)
- 10X Binding buffer
- Flow Cytometry Staining Buffer (Cat. No. 00-4222)
- Gibco™ PBS (Phosphate-buffered saline), pH 7.4, (Cat. No. 10010023) (azide- and serum/protein-free PBS)
- Fixable Viability dye such as LIVE/DEAD™ Fixable Dead Cell Stain Kits (see table on page 67)

This protocol is continued on page 71

Experimental procedure

1. Prepare 1X binding buffer by mixing 1 part of 10X binding buffer with nine parts of distilled water.
2. Wash cells twice in azide-free and serum/protein-free PBS.
3. Resuspend cells at 1–10 x 10^6 cells/mL in azide-free and serum/protein-free PBS.
4. Add 1 µL of Fixable Viability Dyes (FVD) per 1 mL of cells and vortex immediately.
5. Incubate for 30 minutes at 2–8°C. **Protect from light**.
6. Wash cells twice with Flow Cytometry Staining Buffer or equivalent.
7. Wash cells once with 1X Binding Buffer.
8. Resuspend cells in 1X Binding Buffer at 1–5 x 10^6 cells/mL.
9. Add 5 µL of fluorochrome-conjugated Annexin V to 100 µL of the cell suspension.
10. Incubate 10-15 minutes at room temperature. **Protect from light**.
11. Add 2 mL of 1X binding buffer and centrifuge at 400–600 x *g* for 5 minutes at room temperature. Discard supernatant.
12. Resuspend cells in 200 µL of 1X Binding Buffer.
13. Analyze by flow cytometry.

This protocol is continued on page 72

Annexin V staining for apoptosis detection, cont.

Annexin V staining protocol with surface and intracellular staining

Materials

- 12 x 75 mm round-bottom tubes
- 1X Gibco™ PBS (Phosphate-buffered saline), pH 7.4, (Cat. No. 10010023) (azide- and serum/protein-free PBS)
- Flow Cytometry Staining Buffer Set (Cat. No. 00-4222)
- Foxp3/Transcription Factor Staining Buffer Set (Cat. No. 00-5523) or Intracellular Fixation & Permeabilization Buffer Set (Cat. No. 88-8824)
- Annexin V Apoptosis Detection kit (any one of the kits listed). Each kit includes an Annexin V conjugate.
  - eFluor 450 (Cat. Nos. 88-8006-72, 88-8006-74)
  - FITC (Cat. Nos. 88-8005-72, 88-8005-74)
  - PerCP-eFluor 710 (Cat. Nos. 88-8008-72, 88-8008-74)
  - PE (Cat. Nos. 88-8102-72, 88-8102-74)
  - PE-Cyanine7 (Cat. Nos. 88-8103-72, 88-8103-74)
  - APC (Cat. Nos. 88-8007-72, 88-8007-74)
- 10X Binding buffer
- Fixable Viability Dyes (FVD) eFluor 660 (Cat. Nos. 65-0864-14, 65-0864-18), FVD eFluor 506 (Cat. Nos. 65-0866-14, 65-0866-18) or FVD eFluor 780 (Cat. Nos. 65-0865-14, 65-8065-18)

Note

FVD eFluor 450 is not recommended for use with the Annexin V Apoptosis Detection Kits.

This protocol is continued on page 73

Experimental procedure

1. Prepare 1X binding buffer by mixing 1 part of 10X binding buffer with nine parts of distilled water.
2. Stain cell surface antigen(s). Refer to Staining cell surface targets for flow cytometry, Protocol at **thermofisher.com/surfacetargetsflow**.
3. Wash cells twice in azide-free and serum/protein-free PBS.
4. Resuspend cells at 1–10 x 10^6 cells/mL in azide-free and serum/protein-free PBS.
5. Add 1 µL of Fixable Viability Dye per 1 mL of cells and vortex immediately.
6. Incubate for 30 minutes at 2–8°C. **Protect from light**.
7. Wash cells twice in Flow Cytometry Staining Buffer or equivalent.
8. Wash cells once with 1X binding buffer.
9. Resuspend cells in 1X binding buffer at 1–5 x 10^6 cells/mL.
10. Add 5 µL of fluorochrome-conjugated Annexin V to 100 µL of the cell suspension.
11. Incubate 10–15 minutes at room temperature. **Protect from light**.
12. Wash cells once with 1X binding buffer.
13. Stain intracellular antigen(s). Refer to the Staining cell surface targets protocol at **thermofisher.com/surfacetargetsflow** or the Staining intracellular antigens protocol at **thermofisher.com/intracellularstaining**.
14. Analyze by flow cytometry.

Camptothecin induced apoptosis protocol

Introduction

Cell death cascades associated with apoptosis are complex and dynamic processes that can be assessed by a variety of assay types. The use of a positive experimental apoptosis control provides a level of confidence in the data and is used to ensure the experiment was performed correctly. A positive apoptosis control is not always necessary, but it is good laboratory practice to include. Described is a method of apoptosis induction as a positive control for cell death assays, specifically a method for inducing apoptosis using camptothecin.

Note

This protocol is intended for use with the specific products mentioned within it. Substituting different products is not recommended.

Camptothecin apoptosis induction protocol

Materials

- A cell line or primary cells susceptible to apoptosis induction.
- RPMI-1640 medium supplemented with 10% FBS
- 1 mM stock solution of camptothecin prepared in DMSO
- Tissue culture flasks or tissue culture plates

Experimental procedure

1. Prepare cells in fresh RPMI-1640 medium with 10% FBS at a concentration of 0.5 x 10^6 cells/mL in desired tissue culture flasks or tissue culture plates.
2. Add an appropriate amount of 1 mM Camptothecin to the cell suspension to achieve a final concentration of 4–6 µM. The negative control should consist of cells maintained in medium with an equivalent dilution of DMSO only.
3. Incubate cells for the amount of time optimal for your cell type in a humidified, 5% CO_2 incubator at 37°C. It is recommended that you first do a time course to get an idea of how sensitive your cells are to undergo apoptosis.
4. Harvest cells by centrifugation and proceed with appropriate assay to evaluate the induction of apoptosis.

Note

Other pharmacological reagents that have been shown to induce apoptosis include: Actinomycin D, Aphidocolin, Cycloheximide, Dexamethasone, 5-Fluorouracil, Hydroxyurea, and Staurosporine.

Flow cytometry

Cell cycle

DNA content analysis in living cells

Introduction

Live cell studies of cellular DNA content and cell cycle distribution are useful to detect variations of growth patterns due to a variety of physical, chemical, or biological means, to monitor apoptosis, and to study tumor behavior and suppressor gene mechanisms. In a given population, cells are distributed among three major phases of cell cycle: G_0/G_1 phase (one set of paired chromosomes per cell), S phase (DNA synthesis with variable amount of DNA), and G_2/M phase (two sets of paired chromosomes per cell, prior to cell division) [1–4]. DNA content can be measured using fluorescent, DNA-selective stains that exhibit emission signals proportional to DNA mass. Flow cytometric analysis of these stained populations is then used to produce a frequency histogram that reveals the various phases of the cell cycle. This analysis is typically performed on permeabilized or fixed cells using a cell-impermeant nucleic acid stain, but is also possible using live cells and a cell-permeant nucleic acid stain. While the choices for fixed cell staining are varied, there are only a few examples of useful cell-permeant nucleic acid stains.

The Vybrant™ DyeCycle™ Violet stain is a DNA-selective, cell membrane-permeant, and nonfluorescent stain that uses the violet laser for DNA content analysis in living cells. The Vybrant DyeCycle Violet stain is fluorescent upon binding to double-stranded DNA. Well suited for the popular violet laser line (Figure 1), Vybrant DyeCycle Violet stain can also be used with UV excitation, having emission at ~440 nm.

The staining protocol is simple and includes incubating suspended cells in the presence of Vybrant DyeCycle Violet stain and directly measuring the fluorescence without the need for any additional treatment or centrifugation steps. This live cell stain allows the simultaneous co-staining of the cell population for other parameters, and allows for the possibility of cell sorting based on DNA content. Vybrant DyeCycle Violet stain does efflux in rodent and human stem cells, making the Side Population (SP) technique now available with violet excitation [5].

Spectral Characteristics

The fluorescence excitation and emission spectra of the stain are shown in Figure 1. The spectra were obtained from samples of the Vybrant DyeCycle Violet stain bound to DNA. The Vybrant DyeCycle Violet stain/DNA complex has fluorescence excitation and emission maxima of 369/437 nm, respectively.

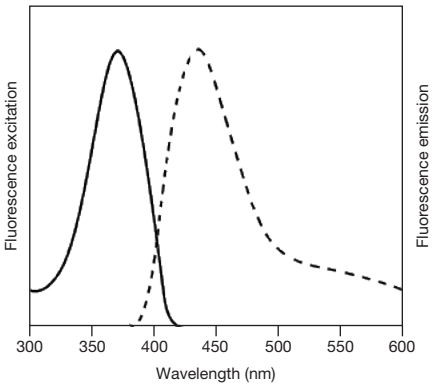


Figure 1. Fluorescence excitation and emission spectra for the Vybrant DyeCycle Violet stain bound to DNA in TBE, pH 8.3.

This protocol is continued on page 76

DNA content analysis in living cells, cont.

Materials

Table 1. Contents and storage information.

Material	Amount	Concentration	Storage*	Stability
Vybrant DyeCycle Violet stain	200 µL	5 mM solution in deionized water	• 2–6°C • DO NOT FREEZE • **Protect from light**	When stored as directed, this kit is stable for at least 6 months.

Number of assays: Sufficient material is supplied for approximately 200 flow cytometry assays based on a 1 mL test volume.
Approximate fluorescence excitation/emission maxima:Vybrant DyeCycle Violet stain: 369/437 in nm bound to DNA.

Materials required but not provided

- Cells and culture medium
- Flow cytometer tubes

Caution

The hazards posed by this stain have not been fully investigated. Since Vybrant DyeCycle Violet stain is known to bind to nucleic acids, treat the stain as a potential mutagen and use with appropriate care. The stain is supplied as a solution in DMSO, which is known to facilitate the entry of organic molecules into tissues. Use the stain using equipment and practices appropriate for the hazards posed by such materials. Dispose of the reagents in compliance with all pertaining local regulations.

Protocol

The following staining protocol was optimized using Jurkat cells, a human T cell leukemia line, in complete RPMI medium containing 10% fetal bovine serum with staining at 37°C, but can be adapted to most cell types. Test samples comprise of 1 x 10^6 cells per 1 mL. Growth medium or buffer used, cell density, cell type variations, and other factors may influence staining. In initial experiments, try a range of dye concentrations to determine the one that yields optimal staining for the given cell type, buffer, and experimental condition. For a given experiment, each flow cytometry sample should contain the same number of cells, as sample-to-sample variation in cell number leads to significant differences in fluorescence signal.

If Vybrant DyeCycle Violet stain is used in combination with other stains for multicolor applications, apply the other stain(s) to the sample first, following all manufacturers' instructions, including wash steps. Vybrant DyeCycle Violet stain should be the last stain applied to the sample, and do not wash or fix samples prior to flow cytometric analysis.

This protocol is continued on page 77

General Guidelines

For optimal DNA content cell cycle analysis, follow these guidelines:

- Eliminate cell clumps and aggregates from the cell suspension before staining
- Use 37°C for incubation with the Vybrant DyeCycle Violet stain
- Hanks' Balanced Salt Solution (HBSS) is recommended if media is not desired, however phosphate buffers are not recommended
- Do not use glass containers with this stain
- Do not wash or fix cells after staining cells with Vybrant DyeCycle Violet stain
- Validate flow cytometry instrument performance on the day of use
- Use linear amplification for DNA content
- Use low flow rate for acquisition
- Collect adequate numbers of events for the intended application
- Eliminate dead cells from the DNA content analysis of living cells using a dead cell discriminating stains such as SYTOX™ Green, SYTOX™ Red or SYTOX™ AADvanced™ dead cell stains or LIVE/DEAD™ Fixable Dead cell stains such as Green, Red, Far Red, or Near-IR kits
- Compensation may be required for Alexa Fluor 488 and PE channels
- Eliminate or correct for cell aggregates during data analysis using gating or modeling software
- Human and rodent stem cells efflux Vybrant DyeCycle Violet stain, and this is the basis for the Side Population (SP) technique; the efflux can be blocked with verapamil, fumitermorgin C, or other such blocking agents, to prevent dye efflux for accurate DNA content analysis in these stem cells

This protocol is continued on page 78

DNA content analysis in living cells, cont.

Vybrant DyeCycle Violet Staining Protocol

This basic protocol is optimized using Jurkat cells suspended in complete medium (RPMI/10% fetal bovine serum) and stained with Vybrant DyeCycle Violet stain at 37°C.

1. Remove the Vybrant DyeCycle Violet stain from the refrigerator and allow the vial to equilibrate to room temperature.

2. Prepare flow cytometry tubes each containing 1 mL of cell suspension in complete media at a cell concentration of 1 x 10^6 cells/mL.

3. To each tube, add 1 µL of Vybrant DyeCycle Violet stain and mix well. Final stain concentration is 5 µM.

4. Incubate at 37°C for 30 minutes, protected from light. Keep cells at 37°C until acquisition.

5. Analyze samples **without washing** or fixing on a flow cytometer using ~405 nm excitation and ~440 nm emission (Figure 2). Vybrant DyeCycle Violet stain may also be excited with a UV light source.

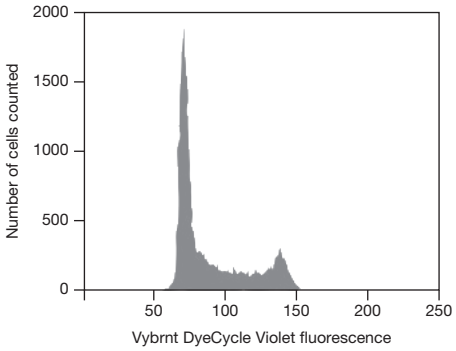


Figure 2. Histogram of live Jurkat cells stained with Vybrant DyeCycle Violet stain showing DNA content distribution. G_0/G_1 and G_2/M phase histogram peaks are separated by the S-phase distribution. Violet 405 nm excitation was used with a 440/40 nm bandpass filter.

References

1. Current Protocols in Cytometry, 7.0.1–7.27.7 (2004).
2. Practical Flow Cytometry, 4th Ed., Shapiro HM, Ed. (2003).
3. *Methods Mol Biol* 281:301 (2004).
4. *Cytometry A* 58:21 (2004).
5. *Stem Cells* 25:1029 (2007).

DNA content in fixed cells

Introduction

Analysis of nucleic acids is a common application of flow cytometry. Measurement of DNA content allows the study of cell populations in various phases of the cell cycle as well as analysis of DNA ploidy. In a given population, cells are distributed among three major phases of cell cycle: G_0/G_1 phase (one set of paired chromosomes per cell), S phase (DNA synthesis with variable amount of DNA), and G_2/M phase (two sets of paired chromosomes per cell, prior to cell division) [1–4]. DNA content can be measured using fluorescent DNA stains that exhibit emission signals proportional to the DNA mass. Flow cytometric analysis of these stained populations is then used to produce a frequency histogram that reveals the various cell cycle phases.

Univariate DNA content analysis is an established assay method and is widely used for studies in oncology, cell biology, and molecular biology. Using flow cytometry, multicolor cell cycle studies are possible, and it is advantageous to analyze DNA content on alternative lasers to preserve the common 488 nm laser for other markers. Well suited for the popular violet laser line, FxCycle™ Violet stain (4', 6-diamidino-2-phenylindole, dihydrochloride) can also be used with UV excitation. With DNA content measurement on the violet laser, other parameters such as cyclins, cyclin-dependent kinases, cell cycle checkpoints, nuclear proteins, and proliferation markers can be measured on the familiar 488 nm laser. FxCycle™ Violet stain preferentially stains dsDNA; it appears to associate with AT clusters in the minor groove [5]. Binding of FxCycle™ Violet stain to dsDNA produces a ~20-fold fluorescence enhancement [6].

For long-term storage, the dye is supplied as five vials of 100 µg solid dye and stored at 2–6°C; individual vial stock solution in deionized water is also stored at 2–6°C for convenience.

Caution

The hazards posed by FxCycle™ Violet stain has not been fully investigated. The stain is a known mutagen and may cause sensitization by inhalation and skin contact, and is irritating to eyes, respiratory system, and skin. Do not breath dust. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing, safety glasses, and gloves. Avoid contact with skin and eyes. Dispose of the reagents in compliance with all pertaining local regulations.

Before starting

Materials required but not provided

- Deionized water
- Reagents for fixing cells such as alcohol or formaldehyde
- Reagents for permeabilizing cells such as Triton® X-100
- Buffer such as Gibco™ PBS (Phosphate-buffered saline), pH 7.4, (Cat. No. 10010023)

This protocol is continued on page 80

DNA content in fixed cells, cont.

Preparing stock solution

To make a 1 mg/mL stock solution of FxCycle™ Violet stain, add 100 µL deionized water to one vial of the stain. Mix well. Store this solution at 2–6°C, protected from light. When stored as directed, this FxCycle™ Violet stock solution is stable for at least six months.

Spectral characteristics

The fluorescence excitation and emission spectra of the FxCycle™ Violet stain are shown in Figure 1. The spectra were obtained from samples of the stain bound to DNA with fluorescence excitation and emission maxima of 358/461 nm respectively. FxCycle™ Violet stain may be used with the violet 405 nm excitation laser commonly found on flow cytometers, as well as UV excitation.

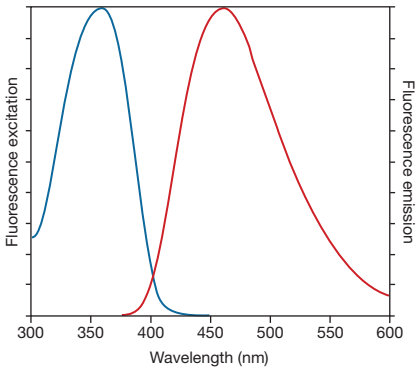


Figure 1. Fluorescence excitation and emission spectra of FxCycle™ Violet stain bound to dsDNA.

Experimental protocol

The following staining procedure was developed using the Jurkat T cell leukemia cell line, but can be adapted for any cell type. Fixative, permeabilization reagent, cell density, cell type variations, and other factors may influence staining.

In initial experiments, try a range of stain concentrations to determine the concentration that yields optimal staining for the given cell type and experimental conditions. All fixative should be removed from cells before proceeding with cell staining, however staining with FxCycle™ Violet stain may be done concurrent with the addition of a permeabilization reagent if desired. For a given experiment, each flow cytometry sample should contain the same number of cells, as sample-to-sample variation in cell number leads to significant differences in fluorescence signal.

If FxCycle™ Violet stain is used in combination with other stains for multicolor applications, apply the other stain(s) to the sample first, following all manufacturers instructions, including wash steps. FxCycle™ Violet stain should be the last stain applied to the sample, and do not wash samples prior to flow cytometric analysis.

This protocol is continued on page 81

General guidelines

For optimal DNA content cell cycle analysis, follow these general guidelines:

- Eliminate cell clumps and aggregates from the cell suspension before staining
- Validate flow cytometry instrument performance on the day of use
- Use linear amplification for DNA content
- Use low flow rate for acquisition
- Collect adequate number of events for the intended application
- Cells must be fixed before staining with FxCycle™ Violet stain for DNA content cell cycle

Staining procedure

1. Harvest the cell sample(s).
2. Fix cells according to your preferred protocol.
3. Wash cells. Remove all fixative from cells before proceeding with the cell staining.
4. Using an appropriate buffer, adjust the sample cell concentration to be 1 x 10^6 cells/mL.
5. Prepare flow cytometry samples each containing 1 mL cell suspension. Optional permeabilization reagent may be added.
6. Add 1 µL of FxCycle™ Violet stain to each flow cytometry sample and mix well.
7. Incubate flow cytometry tubes for 30 minutes at room temperature or 2–6°C, **protected from light**.
8. Analyze samples **without washing** in a flow cytometer, using 405 nm excitation and emission collected in a 450/50 bandpass or equivalent.
9. Example of results obtained using FxCycle™ Violet stain for DNA content cell cycle analysis is shown in Figure 2.

Caution

Do not wash cells after staining with FxCycle™ Violet stain

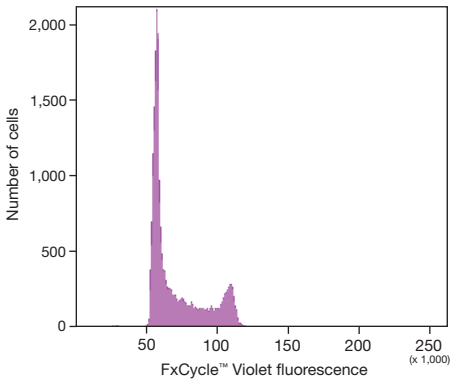


Figure 2. Histogram of HL-60 promyeloblast cells stained with FxCycle™ Violet stain showing DNA content distribution. HL-60 cells were fixed overnight with alcohol, washed, and then resuspended in 0.1% Triton® X-100/PBS/1% BSA before staining with FxCycle™ Violet stain for 30 minutes at room temperature. G_0/G_1 and G_2/M phase histogram peaks are separated by the S-phase distribution. Analysis was performed at 405 nm excitation with a 450/50 bandpass filter.

References

1. Current Protocols in Cytometry, 7.0.1–7.27.7 (2004).
2. Practical Flow Cytometry, 4th Ed., Shapiro HM, Ed. (2003).
3. *Methods Mol Biol* 281:301 (2004).
4. *Cytometry A* 58:21 (2004).
5. *Biochemistry* 26:4545 (1987).
6. *Biochem Biophys Res Commun* 170:270 (1990).

Flow cytometry

Cell proliferation

Cell proliferation assay

Introduction

Measuring a cell's ability to proliferate is a fundamental method for assessing cell health, determining genotoxicity, and evaluating anti-cancer drugs. The most accurate method is by directly measuring DNA synthesis. Initially, this was performed by incorporation of radioactive nucleosides, i.e., ^{3}H-thymidine. This method was replaced by antibody-based detection of the nucleoside analog bromo-deoxyuridine (BrdU). The Click-iT™ Plus EdU Flow Cytometry Assay Kits are novel alternatives to the BrdU assay. EdU (5-ethynyl-2'-deoxyuridine) is a nucleoside analog to thymidine and is incorporated into DNA during active DNA synthesis. Detection is based on a click reaction, a copper catalyzed covalent reaction between a picolyl azide and an alkyne. In this application, the alkyne is found in the ethynyl moiety of EdU, while the picolyl azide is coupled to Alexa Fluor™ 350 dye, Alexa Fluor™ 488 dye, Alexa Fluor™ 594 dye, Alexa Fluor™ 647 dye, or Pacific Blue™ dye. Standard flow cytometry methods are used for determining the percentage of S-phase cells in the population (Figure 1).

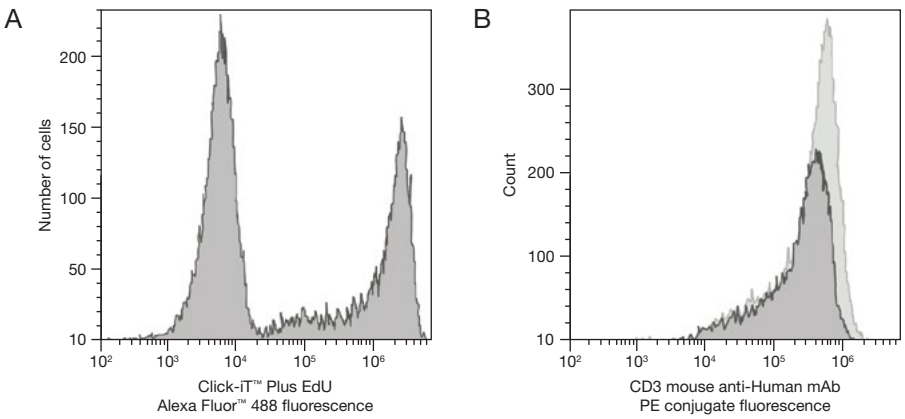


Figure 1. Fluorescence signal from Alexa Fluor™ 488 Click-iT™ Plus EdU Flow Cytometry Assay Kits and CD3 mouse anti-Human mAb PE conjugate Jurkat (human T-cell leukemia) cells were treated with 10 µM EdU for 2 hours, stained with CD3 mouse anti-Human mAb PE conjugate (Cat. No. MHCD0304) and detected according to the recommended staining protocol. The figures show a clear separation of proliferating cells which have incorporated EdU and nonproliferating cells which have not. Panel A shows data from cells labeled with Alexa Fluor™ 488 picolyl azide analyzed on an Attune™ Acoustic Focusing Cytometer using 488 nm excitation and a 530/30 nm bandpass emission filter; Panel B shows the same cells using 635 nm excitation and a 574/26 nm bandpass emission filter. The black outlined histogram is the cells stained with CD3 mouse anti-Human mAb PE conjugate and Click-iT™ Plus EdU Alexa Fluor™ 488 picolyl azide. The gray outlined histogram is the CD3 mouse anti-Human mAb PE conjugate positive control cells treated the same but without copper in the reaction.

This protocol is continued on page 84

Cell proliferation assay, cont.

The advantage of Click-iT™ Plus EdU labeling is that the small size of the picolyl dye azide allows for efficient detection of the incorporated EdU using mild conditions. Standard aldehyde-based fixation and detergent permeabilization are sufficient for the Click-iT™ Plus detection reagent to gain access to the DNA. This is in contrast to BrdU assays that require DNA denaturation (using acid, heat, or digestion with DNase) to expose the BrdU so that it may be detected with an anti-BrdU antibody. Sample processing for the BrdU assay can result in signal alteration of the cell cycle distribution as well as the destruction of antigen recognition sites when using the acid denaturation method. In contrast, the Click-iT™ Plus EdU cell proliferation kit is compatible with cell cycle dyes, R-PE, R-PE tandems, and fluorescent proteins such as GFP, RFP, and mCherry. The EdU assay can also be multiplexed with antibodies against surface and intracellular markers (Figure 2).

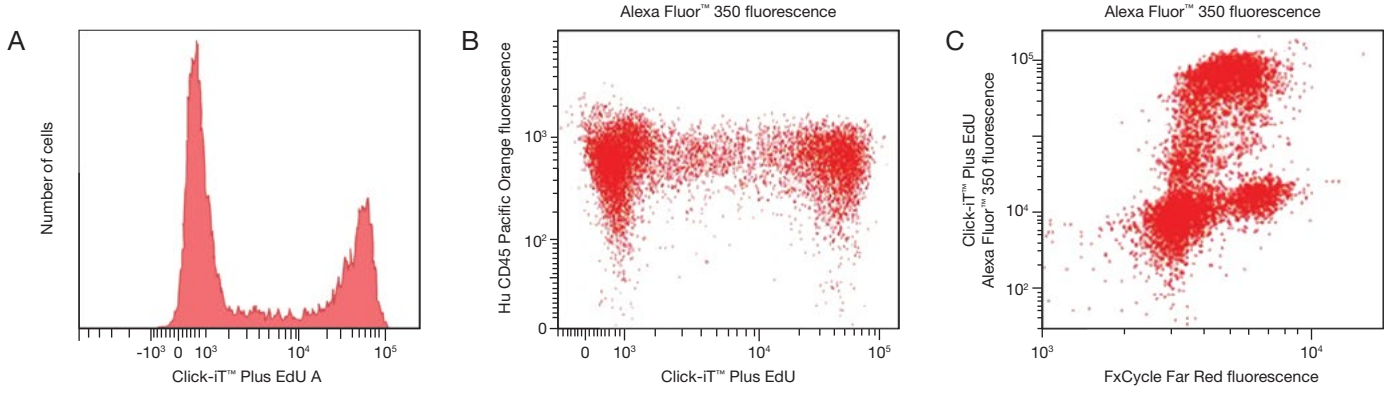


Figure 2. Fluorescence signal and dual parameter plots from Alexa Fluor™ 350 Click-iT™ Plus EdU Flow Cytometry Assay Kit, CD45-Pacific Orange™ and FxCycle™ Far Red Jurkat (human T-cell leukemia) cells were treated with 10 µM EdU for 2 hours, stained with CD45-Pacific Orange™ (Cat. No. MHCD4530), and detected according to the recommended staining protocol. The figures show a clear separation of proliferating cells which have incorporated EdU and non-proliferating cells which have not. Panel A shows data from cells labeled with Alexa Fluor™ 350 picolyl azide analyzed on a BD™ LSRII flow cytometer using UV excitation and a 450/50 nm bandpass emission filter; Panel B shows the same cells using 488 nm excitation and a 530/30 nm bandpass emission filter for detection of the CD45-Pacific Orange™ and UV excitation, and a 450/50 nm bandpass emission filter for detection of the Alexa Fluor™ 350 picolyl azide; Panel C shows the dual parameter plot of the Click-iT™ Plus EdU Alexa Fluor™ 350 and FxCycle™ Far Red. Data were collected and analyzed using a BD™ LSRII flow cytometer using UV excitation and a 450/50 nm bandpass.

This protocol is continued on page 85

Materials

Materials provided in kit

Contents	C10632	C10634	C10636	C10645	C10646	Storage
EdU (Component A)	—	—	10 mg	—	—	• 2-8° C • Desiccate • Protect from light • Do no freeze
Alexa Fluor™ 350 picolyl azide; in DMSO (Component B)	—	—	—	130 µL	—	
Alexa Fluor™ 488 picolyl azide; in DMSO (Component B)	130 µL	—	—	—	—	
Alexa Fluor™ 594 picolyl azide; in DMSO (Component B)	—	—	—	—	130 µL	
Alexa Fluor™ 647 picolyl azide; in DMSO (Component B)	—	130 µL	—	—	—	
Pacific Blue™ picolyl azide; in DMSO (Component B)	—	—	130 µL	—	—	
Dimethlysulfoxide (DMSO) (Component B)			4.5 mL			
Click-iT™ fixative (4% paraformaldehyde in PBS) (Component D)			5 mL			
Click-iT™ fixative saponin-based permeabilization and wash reagent, 10X (Component E)			50 mL			
Copper protectant, 100 mM aqueous solution (Component F)			0.5 mL			
Click-iT™ EdU buffer additive (Component G)			400 mg			

Materials required but not provided

- 1% bovine serum albumin (BSA) in Gibco™ PBS (Phosphate-buffered saline), pH 7.4, (Cat. No. 10010023)
- Buffered saline solution, such as PBS, D-PBS, or TBS
- Deionized water or 18 MΩ purified water
- 12 x 75 mm tubes, or other flow cytometry tubes

This protocol is continued on page 86

Cell proliferation assay, cont.

Protocol

Preparing reagents

1. Allow vials to warm to room temperature before opening.

2. To prepare a 10 mM solution of EdU, add 4 mL of DMSO (Component C) or aqueous solution (PBS) to Component A and mix well. After use, store any remaining stock solution at ≤–20°C. When stored as directed, the stock solution is stable for up to 1 year.

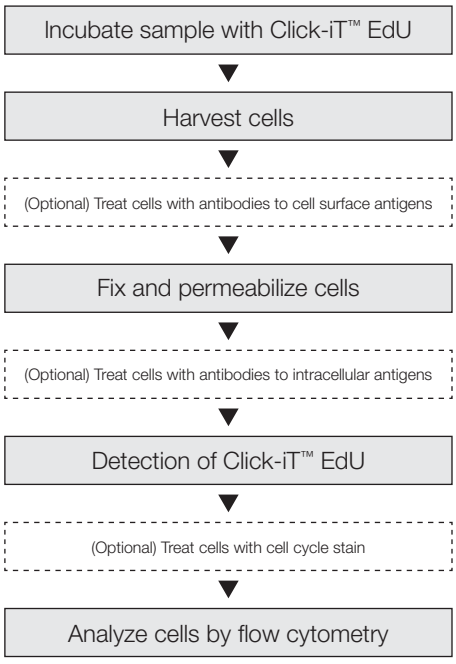
3. To prepare a working solution of Pacific Blue™ azide (Cat. No. C10418), Alexa Fluor™ 647 azide (Cat. No. C10424), or Alexa Fluor™ 488 azide (Cat. No. C10425), add 130 µL of DMSO to Component B and mix well. After use, store any remaining working solution at ≤–20°C. When stored as directed, this working solution is stable for up to 1 year.

4. To prepare 500 mL of 1X Click-iT™ saponin-based permeabilization and wash reagent, add 50 mL of Component E to 450 mL of 1% BSA in PBS. Smaller amounts can be prepared by diluting a volume of Component E 1:10 with 1% BSA in PBS. After use, store any remaining solutions at 2–6°C. When stored as directed, the 1X solution is stable for 6 months and the 10X solution is stable for 12 months after receipt. Note: Component E contains sodium azide (see Cautions).

5. To make a 10X stock solution of the Click-iT™ EdU buffer additive (Component G), add 2 mL of deionized water to the vial and mix until the Click-iT™ EdU buffer additive is fully dissolved. After use, store any remaining stock solution at ≤–20°C. When stored as directed, the stock solution is stable for up to 1 year.

Cautions

- DMSO (Components B and C), provided as a solvent in this kit, is known to facilitate the entry of organic molecules into tissues. Handle reagents containing DMSO using equipment and practices appropriate for the hazards posed by such materials. Dispose of the reagents in compliance with all pertaining local regulations.
- Click-iT™ fixative (Component D) contains paraformaldehyde, which is harmful. Use with appropriate precautions.
- Click-iT™ saponin-based permeabilization and wash reagent (Component E) contains sodium azide, which yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.



Click-iT EdU Flow Protocol Schematic

This protocol is continued on page 87

Labeling cells with EdU

The following protocol was developed with Jurkat cells, a human T cell line, and using an EdU concentration of 10 µM, and can be adapted for any cell type. Growth medium, cell density, cell type variations, and other factors may influence labeling. In initial experiments, we recommend testing a range of EdU concentrations to determine the optimal concentration for your cell type and experimental conditions. If currently using a BrdU based assay for cell proliferation, a similar concentration to BrdU is a good starting concentration for EdU. If using whole blood as the sample, we recommend heparin as the anticoagulant for collection.

6. Suspend the cells in an appropriate tissue culture medium to obtain optimal conditions for cell growth. Disturbing the cells by temperature changes or washing prior to incubation with EdU slows the growth of the cells during incorporation.

7. Add EdU to the culture medium at the desired final concentration and mix well. We recommend a starting concentration of 10 µM for 1–2 hours. For longer incubations, use lower concentrations. For shorter incubations, higher concentrations may be required. For a negative staining control, include cells from the same population that have not been treated with EdU.

8. Incubate under conditions optimal for cell type for the desired length of time. Altering the amount of time the cells are exposed to EdU or subjecting the cells to pulse labeling with EdU allows the evaluation of various DNA synthesis and proliferation parameters. Effective time intervals for pulse labeling and the length of each pulse depend on the cell growth rate.

9. Harvest cells and proceed immediately to step 10 if performing antibody surface labeling; otherwise continue to step 16.

This protocol is continued on page 88

Cell proliferation assay, cont.

Staining cell-surface antigens with antibodies (optional)

10. Wash cells once with 3 mL of 1% BSA in PBS, pellet cells by centrifugation, and remove supernatant.

11. Dislodge the pellet and resuspend cells at 1 x 10^7 cells/mL in 1% BSA in PBS.

12. Add 100 µL of cell suspension or whole blood sample to flow tubes.

13. Add surface antibodies and mix well (Table 1).

14. Incubate for the recommended time and temperature, protected from light.

15. Proceed to step 16 for cell fixation.

Note

Do not use PE, PE-tandem, or Qdot™ antibody conjugates before performing the click reaction; wait until steps 21–23 for labeling with these fluorophores.

Table 1. Click-iT™ Plus EdU detection reagent compatibility.

Fluorescent molecule	Compatilbility*
R-phycoerythrin (R-PE) and R-PE based tandems (i.e., Alexa Fluor™ 610-RPE)	Compatible, except Alexa Fluor™ 594 with R-PE-Cy7™ tandems
Fluorescent proteins (GFP)	Compatible
PerCP, allophycocyanin (APC) and APC-based tandems (i.e., Alexa Fluor™ 680-APC)	Compatible
Organic dyes such as Alexa Fluor™ dyes, fluorescein (FITC)	Compatible
Qdot™ nanocrystals	Use Qdot™ nanocrystals after the Click-iT™ Plus detection reaction

* Compatibility indicates whether the fluorescent molecule itself or the detection methods involve components that are unstable in the presence of the copper catalyst used for the Click-iT™ Plus EdU detection reaction.

Fixation and permeabilization

The Click-iT™ saponin-based permeabilization and wash reagent can be used with whole blood or cell suspensions containing red blood cells, as well as with cell suspensions containing more than one cell type. This permeabilization and wash reagent maintains the morphological light scatter characteristics of leukocytes while lysing red blood cells.

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16. Wash the cells once with 3 mL of 1% BSA in PBS, pellet the cells, and remove the supernatant.

17. Dislodge the pellet, add 100 µL of Click-iT™ fixative (Component D), and mix well.

18. Incubate the cells for 15 minutes at room temperature, protected from light.

19. Wash the cells with 3 mL of 1% BSA in PBS, pellet the cells, and remove the supernatant. Repeat the wash step if red blood cells or hemoglobin are present in the sample. Remove all residual red blood cell debris and hemoglobin before proceeding.

20. Dislodge the cell pellet and resuspend the cells in 100 µL of 1X Click-iT™ saponin-based permeabilization and wash reagent (prepared in step 4), and mix well. Incubate the cells for 15 minutes or proceed directly to step 24 for click labeling.

Note

This step can be performed either before or after the Detect Click-iT™ EdU step.

Staining intracellular or surface antigens (optional)

21. Add antibodies against intracellular antigens or against surface antigens that use Qdot™ antibody conjugates. Mix well.

22. Incubate the tubes for the time and temperature required for antibody staining, protected from light.

23. Wash the cells according to the following conditions, depending on whether you have already performed the Detect Click-iT™ EdU step:

Click-iT™ EdU detection	Action
Already performed the Detect Click-iT™ EdU step	1. Wash each tube with 3 mL of 1X Click-iT™ permeabilization and wash reagent. 2. Centrifuge the cells, and remove the supernatant. 3. Dislodge the cell pellet and resuspend the cells in 500 µL of 1X Click-iT™ permeabilization and wash reagent. 4. Proceed to "Stain cells for DNA content" (step 29) or "Analyze cells by flow cytometry" (step 31)
Have not yet performed the Detect Click-iT™ EdU step	1. Wash the cells once with 3 mL of 1% BSA in PBS, pellet the cells, and remove the supernatant. 2. Dislodge the cell pellet and add 100 µL of 1X Click-iT™ permeabilization and wash reagent and mix well. 3. Proceed to "Detect Click-iT™ EdU" (step 24)

This protocol is continued on page 90

Cell proliferation assay, cont.

Click-iT™ reaction

24. Prepare 1X Click-iT™ EdU buffer additive by diluting the 10X stock solution (prepared in step 5) 1:10 in deionized water.

25. Prepare the Click-iT™ reaction cocktail according to Table 2.

Note

Use the Click-iT™ reaction cocktail within 15 minutes of preparation.

Table 2. Click-iT™ Plus EdU Reaction Cocktails

	Number of reactions						
Reaction components	1	2	5	10	15	30	50
PBS, D-PBS, or TBS	438 µL	875 µL	2.19 mL	4.38 mL	6.57 mL	13.2 mL	21.9 mL
$CuSO_4$ (Component F)	10 µL	20 µL	50 µL	100 µL	150 µL	300 µL	500 µL
Fluorescent dye azide	2.5 µL	5 µL	12.5 µL	25 µL	37.5 µL	75 µL	125 µL
Reaction Buffer Additive (prepared in step 24)	50 µL	100 µL	250 µL	500 µL	750 µL	1.5 mL	2.5 mL
Total reaction volume	500 µL	1 mL	2.5 mL	5 mL	7.5 mL	15 mL	25 mL

26. Add 0.5 mL of Click-iT™ reaction cocktail to each tube and mix well.

Note

The total volume for each reaction mixture is 600 µL.

27. Incubate the reaction mixture for 30 minutes at room temperature, protected from light.

28. Wash the cells once with 3 mL of 1X Click-iT™ saponin-based permeabilization and wash reagent (prepared in step 4), pellet the cells, and remove the supernatant.

    If proceeding with "Staining intracellular or surface antigens" dislodge the cell pellet and resuspend the cells in 100 µL of 1X Click-iT™ saponin-based permeabilization and wash reagent.

    If proceeding to "Staining cells for DNA content" add 500 µL of 1X Click-iT permeabilization and wash reagent.

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Staining cells for DNA content (optional)

29. If necessary, add Ribonuclease A to each tube and mix (Table 3).

30. Add the appropriate DNA stain to each tube, mix well, and incubate as recommended for each DNA stain.

Table 3. Click-iT™ Plus EdU compatibility with DNA content stains

DNA content stain	RNase required?	Click-iT™ Plus EdU stain compatibility				
		Alexa Fluor™ 350 picolyl azide	Alexa Fluor™ 488 picolyl azide	Alexa Fluor™ 594 picolyl azide	Alexa Fluor™ 647 picolyl azide	Pacific Blue™ picolyl azide
FxCycle™ PI/RNase	No	Yes	Yes	No	Yes	Yes
FxCycle™ Violet	No	No	Yes	Yes	Yes	No
FxCycle™ Far Red	Yes	Yes	Yes	Yes	No	Yes
SYTOX™ AADvanced™ stain	Yes	Yes	Yes	Yes	Yes	Yes
Propidium iodide (PI)	Yes	Yes	Yes	No	Yes	Yes

Analysis by flow cytometry

If measuring total DNA content on a traditional flow cytometer using hydrodynamic focusing, use a low flow rate during acquisition. If using the Attune™ Acoustic Focusing Cytometer, all collection rates may be used without loss of signal integrity if the event rate is kept below 10,000 events per second. However, for each sample within an experiment, the same collection rate and cell concentration should be used. The fluorescent signal generated by DNA content stains is best detected with linear amplification. The fluorescent signal generated by Click-iT™ Plus EdU labeling is best detected with logarithmic amplification.

31. Analyze the cells using a flow cytometer.

For detection of EdU with:	Use:
Alexa Fluor™ 350 picolyl azide	350 nm excitation with a 440 emission filter (450/50 nm or similar)
Alexa Fluor™ 488 picolyl azide	488 nm excitation with a green emission filter (530/30 nm or similar)
Alexa Fluor™ 594 picolyl azide	532 or 561 nm excitation with a 620 emission filter (620/15 nm or similar)
Alexa Fluor™ 647 picolyl azide	633/635 nm excitation with a red emission filter (660/20 nm or similar)
Pacific Blue™ picolyl azide	405 nm excitation with a violet emission filter (450/40 nm or similar)

References

1. *Chembiochem* 4:1147 (2003).
2. *J Am Chem Soc* 125:3192 (2003).
3. *Angew Chem Int Ed Engl* 41:2596 (2002)
4. *Angew Chem Int Ed Engl* 40:2004 (2001).
5. *BioTechniques* 44:927 (2008).
6. *Curr Protoc Cytom* 55:7.38.1 (2011.)

Generational analysis of T cell proliferation

Flow cytometric visualization of cell generations

The CellTrace Violet kit is used to monitor distinct generations of proliferating cells by dye dilution. Live cells are covalently labeled with a very bright, stable dye. Every generation of cells appears as a different peak on a flow cytometry histogram.

You will need the following for this protocol:

- 10 mL of heparinized peripheral whole blood
- CellTrace Violet Cell Proliferation Kit (Cat. No. C34557)
- OpTmizer T Cell Expansion SFM (Cat. No. A10485-01)
- Penicillin-Streptomycin-Glutamine (100X) (Cat. No. 10378-016)
- Gibco™ PBS (Phosphate-buffered saline), pH 7.4, (Cat. No. 10010023)
- GE Ficoll-Paque™ Plus
- Cell counter such as the Countess II FL (Cat. No. AMQAF1000)
- Dynabeads Human T-Activator CD3/CD28 (Cat. No. 11161D)

Protocol

Culture medium preparation

1. To 1 L OpTmizer T Cell Expansion SFM, add the following:
   – 26 mL of T Cell Expansion Supplement (supplied in Cat. No. A10485-01)
   – 10 mL of Penicillin-Streptomycin-Glutamine

2. Complete medium is stable for 4 weeks when stored at 2–8°C in the dark.

Note

This protocol can be used for detecting cell proliferation using flow cytometry

Caution

This protocol should not be used for fluorescence microscopy or microplate readers

Protocol tip

- Reserve 1 mL of cells for unstained control and 1 mL of cells for a stained, but unstimulated control
- Dynabeads stimulation typically results in T cell division every 18–20 hr
- Analyze as many cells as possible from each sample
- Use a viability dye and gate on live cells

This protocol is continued on page 93

Mononuclear cell isolation from whole blood

1. Dilute 10 mL whole blood in 10 mL PBS and mix well.
2. Add 15 mL Ficoll-Paque™ Plus to a 50 mL centrifuge tube and gently layer 20 mL diluted whole blood on top.
3. Centrifuge for 30 minutes at 400 x *g*.
4. Carefully remove lymphocyte layer and transfer to a new tube.
5. Resuspend cells in 25 mL DPBS buffer in a 50 mL conical tube.
6. Centrifuge for 5 minutes at 300 x *g*, pour off supernatant, and resuspend in 25 mL DPBS.
7. Repeat wash step and resuspend in 10 mL DPBS.
8. Count cells on the Countess Automated Counter or by another method; adjust concentration to 10^6 cells/mL.

Cell staining

1. Add 20 µL DMSO to a vial of CellTrace Violet staining solution.
2. Dilute this stock solution into 20 mL of PBS (warmed to 37°C) for a 5 µM staining solution.
3. Add 10 mL of cells to a 50 mL centrifuge tube.
4. Centrifuge cells for 5 minutes at 300 x *g* and carefully pour off supernatant.
5. Resuspend cells in 10 mL of CellTrace Violet staining solution.
6. Incubate cells for 20 minutes in a 37°C water bath.

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Generational analysis of T cell proliferation, cont.

7. Add 40 mL OpTmizer T Cell Expansion SFM to the cells to absorb any unbound dye.

8. Incubate cells for for 5 minutes.

9. Centrifuge cells for 5 minutes at 300 x *g* and resuspend the cell pellet in pre-warmed OpTmizer T Cell Expansion SFM.

Stimulation and analysis

1. Distribute aliquots of stained cells into culture plates or flasks.

2. Stimulate with 50 µL Dynabeads Human T-Activator CD3/CD28 per 1 mL of cells, or other stimulus.

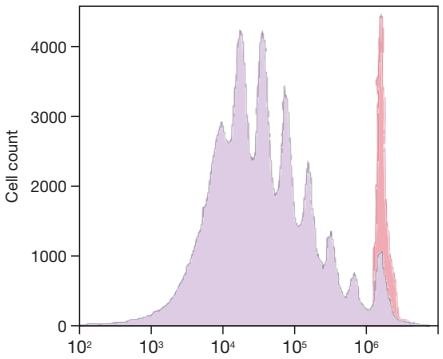
3. Incubate for desired length of time under growth conditions.

4. Harvest cells and stain for other markers if appropriate.

5. Analyze using a flow cytometer with 405 nm excitation and a 450/40 bandpass emission filter.

Spectral information and storage

	CellTrace Violet
Excitation/Emission (in nm)	405/450
Standard filter set	Pacific Blue
Storage conditions	≤ –20°C



Human PBMCs stained with CellTrace Violet reagent and stimulated in culture for seven days. The discrete peaks in this histogram represent successive generations of live cells. Analysis was completed using the Attune Acoustic Focusing Cytometer with 405 nm excitation and a 450/40 nm bandpass emission filter. The unstimulated parent generation is indicated in red.