Single-Cell Handling and Staining Protocol for Suspension and Adherent Cells Protocol-At-A-Glance

I. Purpose

This protocol-at-a-glance describes how to dissociate, stain, and dilute cells with Hoechst 33342 and propidium iodide in preparation to isolate, image, and analyze samples for applications on the Shasta™ Single Cell System (Takara Bio, Cat. No. 640282) or the ICELL8® cx Single-Cell System (Takara Bio, Cat. No. 640188 or 640189).

II. Materials Required

Reagents:

- 1X PBS (no Ca^{2+}, Mg^{2+}, or phenol red; pH 7.4; Thermo Fisher Scientific, Cat. No. 10010-023)
- ReadyProbes Cell Viability Imaging Kit, Blue/Red (contains Hoechst 33342 and propidium iodide; Thermo Fisher Scientific, Cat. No. R37610)
- TrypLE Express Enzyme (1X), no phenol red (for dissociation of adherent cells; Thermo Fisher Scientific, Cat. No. 12604-021)
- Complete cell culture medium appropriate for the cell type(s) being analyzed (for suspension of adherent cells)

Equipment:

- Personal protective equipment (PPE; e.g., powder-free gloves, safety glasses, lab coat, sleeve protector)
- Nuclease-free non-sticky 1.5 ml tubes
- 5 ml flip-cap tubes
- Centrifuge and rotor for 5 ml tubes
- Microcentrifuge for 1.5 ml tubes
- Vortex mixer
- Filter pipette tips: 2 µl, 20 µl, 200 µl, and 1,000 µl
- Wide-bore pipette tips: 20 µl, 200 µl, and 1,000 µl
- Serological pipettes and controller
- Wide-bore pipette tips: 200 µl and 1,000 µl
- Single-channel pipettes: 10 µl, 20 µl, 200 µl, and 1,000 µl
- MOXI Z Mini Automated Cell Counter Kit (ORFLO, Cat. No. MXZ001) or equivalent
- MOXI Z Cell Count Cassettes, Type M (25 pack; ORFLO, Cat. No. MXC001) or equivalent
- MOXI Z Cell Count Cassettes, Type S (25 pack; ORFLO, Cat. No. MXC002) or equivalent
- Microscope for monitoring dissociation of adherent cells

III. Protocols

- Start from Section III.A if working with cells in suspension.
- Start from Section III.B and then proceed to Section III.A if working with adherent cells.

Before You Start

- Prewarm 1X PBS (Ca^{2+} and Mg^{2+} free), TrypLE Express*, and cell culture medium* at 37°C
  *Required only if performing dissociation of adherent cells.

A. Protocol: Preparation of Suspension Cells

1. Prepare a mixture of Hoechst 33342 and propidium iodide. Combine 80 µl of each dye per ml of cells to be stained. An example using 2 ml of cells is described below (e.g., prepare 320 µl of premixed dye solution).
   NOTE: Protect this mixture from light until ready for use.
2. Transfer ~2.1 ml of suspension cells to a fresh 5 ml tube.
3. Determine cell concentration using a MOXI automated cell counter (or your preferred method).
4. Add 320 µl of the premixed Hoechst 33342 and propidium iodide dye mix to the cells. Mix gently by inverting the tube 5 times. Do not vortex or overagitate the cells.
5. Incubate cells at 37°C for 20 minutes.
6. Add an equal volume of 1X PBS (prewarmed to 37°C) to stained cells. For the example described here, 2 ml of prewarmed 1X PBS is added to the 5 ml tube containing the stained cell suspension.
7. Mix the combination of stained cell suspension and PBS by gently inverting the tube 5 times. Do not vortex or overagitate the cells.
8. Pellet the cells by centrifugation at 100*g* for 3 minutes at room temperature. Avoid over-centrifugation or pelleting into a firm pellet or clump.
   NOTE: Optimal centrifugation speed and time may vary depending on the cell type being analyzed. Examples:
   - K562 or 3T3 cells: 100*g* for 3 minutes
   - PBMCs or similarly sized cells: 500*g* for 3 minutes
9. Gently remove the tube from the centrifuge without disturbing the cell pellet.
10. Carefully decant the supernatant without disturbing the cell pellet.
11. Wipe remaining fluid from the tube top using a fresh Kimwipe such that the last remaining drop on the tilted tube top is gently removed.
12. Gently add 1 ml of 1X PBS (prewarmed to 37°C) to the tube side wall.
13. Use a wide-bore 1 ml pipette tip to gently mix the cell suspension by slowly pipetting up and down ~5 times. Do not vortex or overagitate the cells.
14. Count the cells using a MOXI automated cell counter (or your preferred method). Take two readings for each stained cell sample and average the results.
15. Proceed to your desired Shasta or ICELL8 cx application with the prepared sample.

B. Protocol: Preparation of Adherent Cells in a 75-cm Flask

1. To a 75-cm flask containing adherent cells, exchange cell media with 10 ml of 1X PBS prewarmed to 37°C by dispensing the PBS on the side walls of the flask.

   NOTE: Do not pour PBS directly on the cells.

2. Wash the cells by tilting the flask gently. Do not mix by pipetting.
3. Remove the PBS from the cells using a standard large-bore tissue culture pipette.
4. Add 3 ml of TrypLE Express prewarmed to 37°C to the flask to dissociate the cells.
5. The efficiency of cell dissociation may vary with cell type. Monitor the process visually using a microscope.
6. Once sufficient cell dissociation has occurred, neutralize the trypsinization reaction by gently adding 7 ml of complete media, prewarmed to 37°C. Do not vortex or overagitate the cells.
7. Follow the Preparation of Suspension Cells protocol (Section III.A, above), beginning from Step 1.

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