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Product Information

Yale
Center for High Throughput Cell Biology

Procedure for Fixation, Immunostaining, and Imaging in 384-well Plates

Reagents

- 384-well view plates (Aurora)
- HUVEC (pooled, Lonza)
- EBM-2 Medium (Lonza)
- Fixation Solution - 16% Paraformaldehyde (PFA) aqueous solution (diluted to 4% in wells)
- 1× PBS w/o Ca^{2+} and Mg^{2+} (prepared from powder)
- Donor Calf Serum (DCS)
- Triton® X-100
- Primary antibody (Sigma)
- Secondary Antibody (Alexafluor 647 goat anti-rabbit IgG–highly cross-adsorbed)
- Hoechst 33342

Precautions and Disclaimer

Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Triton X-100 Stock Solution - 10% (v/v) solution in PBS

Antibody Diluent - 10% DCS in PBS

Hoechst 33342 Stock Solution - 10 mg/ml in PBS

Permeabilization and Blocking Solution - 0.2% Triton X-100 and 10% Donor Calf Serum in PBS

Primary Antibody Solutions – Prepare 8 different primary antibody dilutions ranging from 1:50 to 1:6,400 using Antibody Diluent.

Secondary Antibody Solution – dilute secondary antibody 1:1,000 using Antibody Diluent. Add Hoechst 33342 Stock Solution to a final concentration of 1 µg/ml (10,000-fold dilution of Stock Solution).

Procedure

A. Cells preparation - HUVEC cells were cultured in EBM-2 medium and plated in 384-well plates at a density of 3,500 cells per well. The plates were then incubated for 24–48 hours until 80–90% confluent before fixation.

B. Fixation
   1. Dilute Fixation Solution (16% PFA) 4-fold in test wells (17 µl of Fixation Solution added to 50 µl of medium in wells).
      Note: Alternatively, Fixation Solution can be diluted to 4% PFA with PBS and added directly to wells after aspirating medium.
   2. Incubate for 20 minutes at room temperature.
   3. Wash once with 1× PBS.
   4. Store plates at 4 °C until ready to stain.

C. Immunostaining
   1. Wash once with 1× PBS and aspirate.
   2. Add 50 µl of Permeabilization and Blocking Solution to each well.
   3. Incubate for 20 minutes at room temperature.
   4. Aspirate Permeabilization and Blocking Solution and add 25 µl of Primary Antibody Solution.
   5. Incubate for 20 minutes at room temperature.
      Note: In some cases, the primary antibody incubation time can be extended to increase signal intensity.
   6. Aspirate Primary Antibody Solution, wash once in 1× PBS, and aspirate.
   7. Add 25 µl of Secondary Antibody Solution.
   8. Incubate for 20 minutes at room temperature.
   9. Wash 4 times with 1× PBS.
   10. Store plates in 50 µl of 1× PBS at 4 °C until ready to image

Results

Imaging

All images were acquired using the PerkinElmer® Opera® high-throughput confocal imager. All antibodies were imaged at 8 different dilutions ranging from 1:50 to 1:6400 with 2–3 images taken per well. Plates were imaged at both 20× (for data acquisition) and 40× (for high quality image generation). Antibody signal was measured using the 640 nm (red) laser and Hoechst intensity was measured using the 405 nm (violet) laser. In both cases, the exposures were taken separately for each channel to limit fluorescent bleedthrough between the channels. Exposure times varied for each channel with maximums of 2,000 ms for the 640 nm channel and 400 ms for the 405 nm channel.

Data Acquisition

Data was generated using the PerkinElmer Acapella® software package. Intensity values were obtained using the NFkB Translocation algorithm. Nuclear intensity was calculated from the antibody signal in the area overlaid with Hoechst staining. Cytoplasmic intensity was calculated from a cytoplasmic ring region defined by the area outside of the nuclear region. All values represent the average intensity per cell in each field.

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