Icahn School of Medicine at Mount Sinai LINCS Center for Drug Toxicity Signatures

Standard Operating Procedure: Immunofluorescence Staining

DToxS SOP Index: A-4.0
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Quality Assurance/Control (QA/QC) steps are indicated with green highlight.

Metadata recording is highlighted with yellow highlight and superscript indices.

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1. This protocol was optimized for cardiomyocytes on laminin-coated 12 mm diameter (D12) coverslips, cultured individually in wells of a 24-well plate. This SOP assumes that the cells have been treated as desired and are ready for fixation. We process these samples in the multiwell plate until Step 6.
    a. The protocol was also used with 96-well plates (Corning, Cat: 3603) and 25 mm coverslips with no changes except for volumes, which are noted where appropriate.
    b. Laminin coating is not necessary for this SOP.
2. Prepare the cells for fixation.
    a. Aspirate growth media and wash twice with 37°C phosphate buffered saline (PBS) inside the biological hood. Remove PBS by aspiration.
    b. We use 10x concentrated PBS stock (Boston Bioproducts, Cat: BM-221) diluted 1:10 with distilled deionized water.
3. Fix the cells with 4% paraformaldehyde in PBS supplemented with 4% sucrose at room temperature (RT) for 15 min
    a. We prepare 4% paraformaldehyde fixative fresh before each use by mixing 10 mL of 16% paraformaldehyde stock (EMS, Cat: 15710) with 4 mL of 10X concentrated PBS stock, 1.6 g of sucrose (Sigma, Cat: S7903) and 26 mL of distilled deionized water, and vortex mixing. All solutions are at RT.
    b. For D12 coverslips we use a volume of 0.5 mL per well of the 24-well plate.
    c. For 96-well plates, we use a volume of 100 µL per well.
4. Wash cells with PBS at RT three times (triple wash)
    a. Unless noted otherwise, all washes are done with no wait in between
    b. Same volumes from step 3 are used for washes
    c. Ensure that the samples are never allowed to dry completely; the solution is left on the coverslips after the last wash.

5. If staining coverslips, prepare a moisture chamber by placing water-saturated Kimwipes around the edges of a 150mm culture dish and placing a piece of Parafilm at the center (roughly 8x8 cm)
6. Move the coverslips into the moisture chamber on top of the Parafilm.
7. Add PBS on top of the coverslips. For this step and all the subsequent steps (excluding mounting at step 19), the recommended volumes for solutions are:
    a. 100-150 µL solution for each D12-15mm coverslips
    b. 150-200 µL solution for each D18-25mm coverslips
    c. For 96-well plates, the volume of 100 µL per well applies for the entire protocol
8. Permeabilize cells: Aspirate PBS and immediately add 0.5% Triton-X (Sigma, Cat: X100) in PBS (vol/vol). Incubate at RT for 10 min.
    a. It is critical to not let the sample dry out during solution changes.
9. Aspirate solution and triple wash with PBS. Repeat triple washing two more times with 1 min intervals in between (9 total solution changes)
10. Aspirate PBS and immediately add blocking buffer: 2% BSA (Sigma, Cat: A8806) + 5% goat serum (Vector, Cat: S-1000) in PBS (vol/vol) and incubate at RT for 1-2 hrs.
11. Aspirate blocking solution and immediately add primary antibody. Incubate overnight at 4°C.
    a. We use mouse monoclonal anti-α-sarcomeric actinin (Sigma, Cat: A7811) diluted in blocking buffer 1:250 (vol/vol) OR mouse monoclonal anti-troponin-T (Thermo Scientific, Cat: MS295) diluted in blocking buffer 1:200 (vol/vol) as the primary antibody.
    b. As we validate other antibody dilutions/cell line combinations their information will become available via the DToXS website.
12. Aspirate primary antibody and triple wash with PBS.Repeat triple washing two more times with 1 min intervals in between (9 total solution changes)
13. Aspirate PBS and immediately add secondary antibody. Incubate for 30 min at RT.
    a. Secondary Alexa Fluor 488 anti-mouse antibody (Invitrogen, Cat: A-1101) diluted in blocking buffer 1:250 (vol/vol)
14. Aspirate secondary antibody solution and triple wash with PBS.Repeat triple washing two more times with 1 min intervals in between (9 total solution changes)
15. Aspirate PBS and add Alexa Fluor 647 phalloidin (Invitrogen, Cat: A-22287) that is diluted 1:30 in PBS (vol/vol) and incubate for 20 min at RT
16. Aspirate phalloidin and triple wash with PBS.Repeat triple washing two more times with 1 min intervals in between (9 total solution changes)
17. Aspirate PBS and add Hoechst 33342 counterstain (Invitrogen, Cat: H-3570) diluted 1:10,000 (vol/vol) in PBS and incubate for 10 min at RT
18. Aspirate Hoechst and triple wash with PBS. Repeat triple washing two more times with 1 min intervals in between (9 total solution changes)
19. For coverslips only, completely aspirate PBS until dry and rapidly add ProLong Gold antifade mounting solution (Invitrogen, Cat: P10144).
    a. Since mounting solutions are not water-based, failure to remove PBS completely will result in poor image quality.
    b. 10-15 µL mounting solution for each D12-15mm coverslip
    c. 15-20 µL mounting solution for each D18-25mm coverslip
    d. 96-well plates are not mounted or sealed; they remain in PBS
20. Mount and seal coverslips onto a microscope slide
    a. Using fine-tipped forceps, pick up the coverslip carefully (by the edge) and use a Kimwipe to dry the underside (side without cells) by gentle contact
    b. Slowly place the coverslip cell-side down over a microscope slide without introducing air bubbles
    c. Wait for 2 min and seal the coverslip edges with clear nail polish (Fisher Scientific, Cat: NC0516334).