Systems *Bioengineering* Laboratory

General Immunofluorescent Staining Protocol

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Version: 1.0
Date: 07-Jul-2017

Materials

- 4% Paraformaldehyde Fixative
  - PFA Stock, 16% Paraformaldehyde (EMS, Cat: 15710)
  - Sucrose (Sigma, Cat: S7903)
  - 10X PBS (Gibco, Cat: 70011-044)
  - Mix 26 mL of ddH_2O, 4 mL of 10X PBS, 10mL PFA stock and 1.6 g sucrose in an amber conical tube, and vortex mix. We keep this solution at 4ºC for up to two weeks.
- Blocking Buffer (Antibody Diluent)
  - Bovine serum albumin (Sigma, Cat: A8806)
  - Normal goat serum (Vector, Cat: S-1000)
  - Prepare and aliquot sterile filtered 10% BSA stock in ddH_2O. Dilute 1mL stock with 500 µL 10X PBS, 250 µL normal goat serum and 3.25 mL of ddH_2O. We keep this solution at 4ºC for up to two weeks.
- Permeabilization Solution
  - 0.5% Triton-X 100 (Sigma, Cat: X100) diluted in PBS
- Phalloidin/ActinGreen (Life Technologies, Cat: R37110)
- Hoechst 33342 (Life Technologies, Cat: H3570)
- ProLong Diamond Anti-fade Solution (Life Technologies, Cat: P36970)
- Microscope slides (Fisher Scientific, Cat: 12-550-15)

Methods

1. Prepare the slides/dishes by washing twice with 37°C PBS inside the biological hood.
2. Fix the cells with *4% paraformaldehyde fixative* at room temperature (RT) for 15 min.
3. Prepare a moisture chamber for staining
   - 3.1. Line up water-saturated Kimwipes around the edges of a 150 mm culture dish
   - 3.2. Mount a 2 x 2 in^2 piece of Parafilm at the center.
4. Wash cells with PBS at RT three times. Suggested volumes for different slide geometries:
   - 4.1. 75-125uL solution for D12-15mm coverslips
   - 4.2. 150-200uL solution for D18-25mm coverslips
   - 4.3. Titrate >250uL for larger (25x25, 25x40) coverslips or dishes. For large surfaces such as 60 mm culture dishes, consider demarcating a smaller region of interest using a hydrophobic pen.
5. Replace PBS with *permeabilization solution* and incubate at RT for 12 min.

   Note: Replacing a solution refers to gentle aspiration from one side and immediate addition of the next solution from the other side with a micropipette. Do not let samples to dry. Replace thrice refers to back-to-back three washes without interruptions.
6. Replace thrice with PBS, wait 1 min incubation and repeat
7. Replace PBS with *blocking buffer* and incubate in RT for 2 hrs.
8. Prepare primary antibody by diluting antibody of choice with *blocking buffer* at the recommended ratio

9. Replace *blocking buffer* with primary antibody and incubate for overnight at 4°C

    Note: This step could also be performed at RT for 2 hrs.

10. Replace thrice with PBS.
11. Wait for 1 min and repeat step 10 four more times.

    Note: Washing off the primary antibody is the most important step in immunostaining; inadequate removal of the primary may result in poor images.

12. Replace PBS with secondary antibody that is diluted at the recommended ratio with the *blocking buffer*, and incubate for 30 min at RT.

    Note: We use AlexaFluor secondary antibodies at 1:250 and Phalloidin/ActinGreen at 1:25 dilution.

13. Replace thrice with PBS supplemented with Hoechst 33342 (1:5,000)
14. Wait for 1 min and repeat step-13 two more times.
15. Replace thrice with PBS
16. Gently aspirate until dry and rapidly mount the coverslip onto a microscope slide
    - 16.1. Add the anti-fade solution on a microscope slide. Since anti-fade solutions are oil-based, failure to remove PBS completely from the coverslip may result in poor image quality.
        - 16.1.1. Use 10-15uL mounting solution for D12-15mm coverslips
        - 16.1.2. Use 20-25uL mounting solution for D18-25mm coverslips
    - 16.2. Pick up the coverslip carefully and use a Kimwipe to dry the back by gentle contact.
    - 16.3. Slowly place the coverslip upside down over the microscope slide without introducing air bubbles.
17. Wait for 15 min for the mounting solution to dry, and then coat the edges with nail polish.