Immuno-Staining and Microscope Slide Mounting

Media:

1. 4% Paraformaldehyde (PFA)
    a. 100ml deionized water + 8g Paraformaldehyde powder
    b. Heat (hotplate or water bath) until dissolve
    c. Add 100ml 2xPHEM buffer (now in total 200ml)
    d. Filter with syringe and 0.45um syringe filter
    e. Separate into centrifuge tubes and seal. Store at -20C freezer

2. 2xPHEM buffer (2x 500ml, for use in 4% PFA)
    a. 18.14g PIPES (min 99% titration, Sigma P6757)
    b. 6.5g HEPES
    c. 3.8g EGTA (Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N',-tetraacetic acid
    d. 0.99g $MgSO_4$

3. 0.3% triton X = 10ml PBS + 30µl Triton X. Mix well

4. 5% Bovine Serum Albumin (BSA) = 10ml PBS + 0.5g BSA (powder). Mix well

<u>Immuno-Staining</u>:

1. Wash cells with 1ml 1xPBS
2. Aspirate PBS, then fix with 300µl 4% PFA, incubate 20 mins at RT
    - After fixing, the PFA can be aspirated, 1ml 1xPBS added, and the cells can be kept at 4C for weeks
3. Aspirate 4% PFA. Add 300µl 0.3% triton X, incubate 10 mins at RT
4. Aspirate 0.3% triton X. Wash with 1ml 1xPBS, three times
5. Add 300 µl 5% BSA, incubate for 1hr at RT
    - During the waiting time, prepare and dilute (with 5% BSA) the primary Antibodies
    - A table containing the recommended dilution of primary Antibodies for CLS1 cells can be found at the bottom of this protocol
6. Aspirate 5% BSA. Add 100µl primary Antibodies, incubate overnight at 4C or 1hr at RT
7. Aspirate primary Antibodies. Wash with 1ml 1xPBS, incubate 10 mins at RT. Repeat wash three times
    - During the waiting time, prepare and dilute (with 5% BSA) the secondary Antibodies
    - A table containing the recommended dilution of secondary Antibodies for CLS1 cells can be found at the bottom of this protocol
8. Add 100µl secondary Antibodies, incubate 1hr at RT in the dark (wrap with aluminum foil)
9. Aspirate secondary Antibodies. Wash with 1ml 1xPBS, incubate 5 mins at RT. Repeat wash three times
10. Keep cells in PBS, store at 4C. Image/mount within one week

Microscope Slide Mounting

1. Apply ~ 5µl mounting medium/immersion oil of choice to the microscope slide
    - Allow mountant to warm to room temperature for 1 hour before use
    - For mountant in squeeze dropper bottles, one drop is enough
    - Be careful not to introduce any air bubbles when placing the mountant on the microscope slide
2. Remove coverslip from the well using needle and forceps.
    - Take note which side of the coverslip contains the specimen
3. Place a piece a kimwipe at the corner of the coverslip to remove any excess liquids. Take care to not touch the specimen with the kimwipe.
4. Slowly and carefully lower the coverslip (with the specimen faced down) onto the mountant to avoid trapping any air bubbles.
   Do not press down on the coverslip, instead let it spread.
5. Seal the coverslip edge with nail polish, leave at room temperature in the dark for 2hrs.
    - When using Invitrogen Prolong Glass Antifade Mountant, nail polish sealing is not needed as it is a hard-setting mountant. Let it cure on a flat surface, in the dark at room temperature for 24 - 48hrs, for best results.
6. Keep slides at 4C in dark. Image within one week

Notes:

1. The volumes stated in this protocol are recommended for 24 well plates.
   For BSA, Triton-X, PFA, and Antibodies, add enough to cover the bottom of the well, ensuring the coverslip/specimen is submerged
2. When aspirating reagents, place the pipette tip at the corners of the well to minimize contact with specimen
3. Gently pipette solutions into the well, taking care to not directly pipette onto the specimen
4. The antibodies should always be kept on ice, and only diluted immediately before being added
5. If the signal is weak, incubation with primary antibodies at 4C overnight might improve the signal
6. After secondary Antibodies have been added, minimize exposure to light by wrapping in aluminum foil
7. For recommended dilution of Antibodies for CLS1 cells, refer to the table below.
   Take note the volumes is for **each well!**

		Ratio to 5% BSA	Amount to add	Amount of BSA to add	Total volume in **each well**
Primary Antibody	Mouse Anti-Paxillin	1:100	3	298.5µl	300µl
	Rabbit Anti-Vinculin	1:200	1.5µl		
Secondary Antibody	Hoechst	1:1000	0.3µl	296.1µl	300µl
	Alexa Fluor 546 Phalloidin	1:250	1.2µl		
	Alexa Fluor 647 Anti-mouse	1:250	1.2µl		
	Alexa Fluor 488 Anti-rabbit	1:250	1.2µl		