

Immunofluorescence

Project: Mai's Folder

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Materials:

- PBS (buffer solution that keeps the cells hydrated)
- PFA (paraformaldehyde)
- Triton: detergent which breaks down the membrane to allow for staining
- Tween: neutralizes triton
- Goat serum PBS OR BSA
- Antibodies

Procedure:

Preparing 8 well plates:

- Passage cells as usual
- Label 8 well lid columns with the cell type and dilution
- Label the side with the date, passage, and initials
- Aliquot cells according to dilution - cells + DMEM = 1200 microliters in the small tubes
 - Varies on the confluency and initial dilution
 - (06's & 07's) 1/4 = 300 μ l cells
 - (01's) 1/6 = 200 μ l cells
- Add 350 μ l cells to each well
- Place in incubator once complete
- Drug treatment is done the next day
 - OR you can create your 8 well first thing in the morning and very gently treat your cells at the end of the day

Fixation:

- Obtain a PFA ampoule and snap it to open
- Use the pipette to transfer all of the contents into a new test tube labeled 16% PFA along with the date
- Wrap the empty ampoule with your glove and dispose of them in the biohazard bin
- Wrap the tube with foil and the top with parafilm to prevent air and light from entering
- Fixation must go from 16% to 4% (1/4 the expected amount)
- In a new test tube labeled 4% PFA, add 1.8 ml PBS and 0.6 ml PFA
- For an 8 well = 300 μ l \rightarrow 240 μ l
- 8 well:
 - Aspirate all the media from the 8 well
 - Add 0.3 ml of PBS into each well before aspirating to ensure that all the media has been removed
 - Transfer the well to the other hood
 - Add 0.3 ml of PFA to each well and let it sit for 15 minutes
 - Aspirate out the PFA
 - Add 0.3 ml PBS and let it sit for 5 minutes before aspirating
 - Repeat the process with PBS 2-3 more times
 - After fixing, wrap the well in foil and place in the 4 degree celsius fridge until ready to be used (can be stored in PBS for up to 3 months, but be careful of the cells drying out)

NOTE: After fixation, the remaining steps can be done outside the hood if necessary

Permeabilization:

- When ready to stain, spray ethanol on the bottom of the 8 well to make sure it is sterile
- Aspirate the PBS in the wells
- Add 0.3 ml of Triton into each well before allowing it to sit for 15 minutes
- After 15 minutes, aspirate the triton
- Add 0.3 ml of Tween to the wells and let sit for 5 minutes
- Aspirate out the Tween
- Add 0.3 ml of PBS and let sit for 5 minutes
- Repeat the PBS rinse one more time

Blocking: **For this step you may use Goat PBS (goat serum) OR BSA (Bovine Serum Albumin)**

- Making Goat PBS: 10x dilution
 - If your supplies are not sterile, you will have to heat and filter your goat serum beforehand
 - Add 10 mL of goat serum to 100 mL of PBS
 - Store at 4°C
- Making BSA: 2%
 - Fill a 50 mL conical tube with 25 mL of PBS
 - Measure out 500mg (0.5g) of BSA powder and place into the 50 mL conical tube
 - Mix briefly and gentle
 - Place in the 4°C degree fridge for 10-15 minutes to allow the powder to fully dissolve
 - Store at 4°C for up to one week
- Aspirate out the PBS before adding in 3 ml of Goat PBS OR BSA, which will "block" the cells, preventing the antibodies from sticking to everything
- Allow the Goat PBS/BSA to sit for one hour

Primary/Conjugate Antibodies:

- Aspirate out the Goat PBS
- Add an additional 0.3 ml Goat PBS
 - H2AX 1:300 dilution with goat serum
 - 647
 - 1,440 ul total -> 180 µl in each well
 - Add 4.8 ul of H2AX and 1,435 µl Goat serum
 - Heterochromatin: H3K9me2,3 + H3K27me3
 - 6.5 µl H3K9me2,3 + 6.5 µl H3K27me3 + 637 µl Goat serum
 - For 1 8 well, double if for 2
 - Euchromatin: H3K9ac + Lamin A/C
 - H3K9ac - 1.6 µl + Lamin A/C 3.25 µl + 645 µl Goat serum
 - Both of these dilutions are for 1 8 well
 - Lamin A/C, Lamin B1, and Lamin B2 can all use a 1:200 dilution (3.25 µl + 645 µl Goat serum)
 - PMLC: 14 µl PMLC + 1426 ul GPBS for one 8 well
- Create the dilution in a centrifuge tube
- After placing the antibodies and goat serum in the eight well, wrap it in foil and return it to the fridge for overnight storage OR allow them to sit at room temperature for 2 hours (make sure they're still covered in foil)

Secondary Antibodies:

- Aspirate out the primary antibodies and wash 3 times with PBS, 5 mins each
- Add an additional 0.3 ml Goat PBS
- Create your dilutions for the secondary antibodies
 - 1, 8 well: 1440 -> 1.44 µl each into a tube with 1438 µl Goat serum
 - 2, 8 wells: 2880 -> 2.88 µl each into a tube with 2877 µl Goat serum
- Add 180 µl of the dilution into each well
- Foil and allow to sit on the rocker at room temperature for 1 hour

Staining (optional):

- Aspirate out the conjugate/secondary antibodies and wash with PBS for 5 minutes, 3 times
- Add 0.3 ml of PBS into each well
- Stain with Hoechst
 - Dilution is 1 μ l for 1 ml PBS
 - Take 1 ml of PBS add 1 μ l Hoechst (use red pipette for hoechst)
 - Take 30 μ l from your new dilution and add to each well
 - Aspirate out the hoechst after 10 - 15 minutes
- Wash with PBS for 5 minutes 3 times
- Add 0.3 ml of PBS to the wells
- Now the cells can be imaged- mounting is for long term storage

Mounting:

- Aspirate out the PBS
- Add 1 drop (or 20 μ l) of Antifade Gold Reagent into each well
- Parafilm (before adding the cover) and place in a drawer to cure overnight

Imaging:

- Set up the microscope as usual, make sure the 40x oil objective is in place
- Turn on the Nikon software
- Before starting, hit ESC on the microscope
- Make sure the objective is all the way down before placing a small amount of oil on top
 - Oil should be changed every 2 wells
- Place your wells onto the microscope and stabilize it with the magnetic holder
- Use the joystick to make sure your objective is beneath the well that you want to look at
- Carefully raise your objective- when the oil touches the glass, it should form a slightly larger circle (make sure the setting is on Trans and paused)
- Use the knob to adjust until it begins to beep- click PFS (perfect focus) which will zoom in
- Use the joystick to find a good field of view and hit stop
- Choose the folder it will go into by hitting Browse
 - Select your folder
 - Create a folder with the experiment ID with your initials and number (ex. MPP001)
 - Create another folder with the date (YYMMDD)
- Edit the file name
 - IF_cell line_conditions_added-labels
- For IF, make sure everything is WF
- In applications, open ND Aquisition
- Within these tabs, make sure you have the correct parameters:
 - XY:
 - Delete your previous parameters
 - Go to custom and hit large image
 - Create a 4 x 4 field
 - Select the 40x oil objective
 - 5% overlap
 - Z: 0.5 μ m steps for 13 steps
 - λ : select the proper wavelengths for your labels and stains

Wavelength settings for stains

	DAPI	FITC	TRITC	CY5
1	Hoechst	NLS-GFP	H2B-RFP	SiR-Hoechst (SiR-DNA)
2	PA-GFP(-H2A)	H2AX-488	53-BP1 (mCherry)	H2AX-647

- Hit "run" once you've adjusted your settings
- If it's been idle for a while, the laser may be off. To check you can take a picture with any one of your stains. If the screen is black when you run it, then the laser is off. Hit abort, exit the Nikon software, and log back in. Check one more time to make sure that your parameters are correct
- When you're moving to the next well, lower the objective completely before moving it. Add more oil if needed.
- Repeat steps for ND acquisition
- When you've completed all your imaging, dispose of your cells in the biohazard bin or store them in the fridge for later reference.