Standard Operating Procedure				
Title: Staining and Imaging of Flotillin in HEK cells.			**SOP No.: Flot-01**	
			Page No.: 1 of 5	
			Effective Date: 08/15/13	
AREA: Immunofluorescence			**Review Period:**	
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REVISION NO.	REASON FOR REVISION	REVISION DATE
1	Structure and Formatting	08/20/13

1.0 PURPOSE

Immunofluorescence staining of Flotillin (raft-marker) in HEK cells

2.0 RESPONSIBILITY

All research workers involved in this experiment are responsible for implementation of this SOP.

3.0 MATERIALS

Name	Catalog Number	Company
50ml Centrifuge Tubes	229421	Celltreat Scientific Products
1.7ml Microtubes	MCT-175-C	Midsci
0.5ml Microcentrifuge tubes	14231-060	VWR
1000uL, 200uL, 20uL, 10uL, 2uL Pipettes	-	Gilson
1000uL, 200uL, 20uL, 10uL, 2uL Redi-Tip	21-197-8A	Fischerbrand
VWR Micro-Slides SuperFrost Plus	48311-703	VWR International
Fluoromount-G	0100-01	Southern Biotech
Anti-Flotillin 1 antibody (produced in rabbit)	102M4794V	Sigma Life Science
AlexaFluor 568 Anti-rabbit (produced in donkey)	1235798	Life Technologies
Sodium Chloride	SLBD2348V	Sigma Life Sciences
Potassium Chloride	015128	Fisher Scientific
Sodium Phosphate, Dibasic	S-3264	Sigma Chemical Company
Potassium Phosphate Monobasic Anhydrous	P-5379	Sigma
Albumin from Bovine Serum (BSA)	SLBG1507V	Sigma Life Sciences
37% Formaldehyde (PFA)	15682	Electron Microscopy Science
22x22 mm Cover Glasses		
35x10 mm style Petri Dish		
Kim Wipes		
Tweezers		
Nail polish		

3.1 Preparation of Reagents

3.1.1. Phosphate Buffered Saline 10X (PBS)

To prepare 1 L add:

- 80 g sodium chloride (NaCl)
- 2 g potassium chloride (KCl)
- 14.4 g sodium phosphate dibasic (Na_2HPO_4)
- 2.4 g potassium phosphate, monobasic (KH_2PO_4)
- Adjust to 1 L dH_2O

3.1.2. Blocking Buffer (3%)

To prepare 25mL add:

- 0.75 g Albumin from bovine serum (BSA)
- Adjust to 25 mL using cold PBS

3.1.3. Washing buffer (0.6%) (Prepare only amount needed according to your experiment)

To prepare 50mL add:

- 0.3 g Albumin from bovine serum (BSA)
- Adjust to 50 mL using cold PBS

3.1.4. Paraformaldehyde 3.7% (PFA)

To prepare 10mL add:

- 1mL of 37% stock Paraformaldehyde
- 9mL of cold PBS

4.0 PROCEDURE

4.1 PREPARATIONS

1. Have all solutions prepared and in ice bucket before commencing
2. Take out your individual dishes from cell culture and place in ice bucket
3. Label each dish accordingly if you have not done so

4.2 FIXING

4. Wash each dish 3x with 1mL cold PBS
5. Put 2 mL of ice cold 3.7% PFA
6. Incubate in -4ºC for 10minutes with aluminum foil protection
7. Take out dish from fridge and dispose of the PFA
8. Wash 3x with cold PBS

4.3 ANITIBODY TREATMENT

9. Put 1mL of cold blocking buffer (BSA/PBS) in each dish
10. Add 2 uL of primary antibody: Anti-Flotillin 1, produced in rabbit
11. Incubate at RT for 1 hour with aluminum foil protection (in a rocker)
12. Take out antibody and pipette it into separate vial if recycling
13. Wash 3x with washing buffer (BSA/PBS) on rocker with 10min intervals
14. Put 1mL of cold blocking buffer (BSA/PBS) in each dish
15. Add 2uL of secondary antibody: Anti-rabbit AlexaFluor 568
16. Incubate at RT for 1 hour with aluminum foil protection (in a rocker)
17. Take out antibody
18. Wash 3x with washing buffer (BSA/PBS) on rocker with 10min intervals
19. Leave 1mL of washing buffer in the dish after last wash

4.4 MOUNT COVERSLIPS ON GLASS SLIDES FOR IMAGING

20. Take a glass slide, put it on a Kim wipe and write your initials, date and your experimental data on the side of the slide.
21. Put a very small drop of fluoromount on the glass slide (1 per coverslip)
22. Take a sharp tweezer to retain your coverslip from the dish
23. Hold coverslip perpendicular to dish with cell side facing you
24. Tap the bottom of coverslip to the Kim wipe to remove excess liquid.
25. Flip coverslip onto fluoromount drop (Cell side facing down on fluoromount)
26. Wait 5 minutes for it to air dry
27. Put nail polish on the sides of your cover slip.
28. Wait 5 minutes for it to air dry
29. Your slide is now ready for imaging.

4.5 IMAGING

1. Turn the Nikon Confocal Microscope on in the numbered order

2. Incubator and/or heating system does not need to be turned on
3. Check if the appropriate slide holder is already in place
4. Put a very small drop of immersion oil on the lens
5. Put your glass slide on the slide holder
6. Bring the lens up until the oil on lens comes in contact with your coverslip
7. Select the FITC laser to see your YFP tagged cells
8. Choose a healthy cell with good expression of YFP
9. Turn on the computer
10. Open NIS Elements AR program
11. Click on the Nikon A1 Application icon
12. Load your personal configuration
13. Make sure to choose what folder you want your images to be automatically saved in.
14. On the top tool bar, go to optical configuration
15. Choose YFP-CFP-RFP
16. Uncheck CFP and RFP laser
17. Click Laser interlocked
18. Click Live now
19. You should be seeing your YFP tagged cells with this channel
20. If you can not see a clear image, try changing the focus, Laser power, HV and offset until you get your ideal image
21. Don't turn the laser power too high because you might bleach your cells.
22. Save your image
23. Go to optical configuration again
24. Choose the 4 color configuration channel
25. Check laser 561 and uncheck lasers 405 and 488,
26. Click apply
27. Click live now
28. You should be seeing your Alexa Fluor 568 with your 561 laser
29. If you can not see a clear image, try changing the focus, Laser power, HV, offset until you get your ideal image
30. Capture image and merge with your previous YFP image
31. Save your image in appropriate folder and naming
32. Once you are done imaging, turn the computer off
33. Bring the objective down
34. Clean the lens very carefully with the lens paper
35. Turn the Confocal Microscope off in the numbered order
36. Put the cover on the microscope
37. If your cells are fixed, you can store them in the 4ºC for future imaging