Standard Operating Procedure				
Title: Cholera Toxin B subunit (Lipid-raft marker) staining and Imaging of YFP-DAT cells.			**SOP No.: CTXB-01**	
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1.	Formating	08.15.2013
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PURPOSE
Staining and imaging of lipid rafts with Cholera Toxin Subunit B in YFP-DAT Cells

1.0 RESPONSIBILITY

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

2.0 PROCEDURE

2.1 Materials

Name	Catalog No.	Lot Number	Company
22x22 mm Cover Glasses	C9802	-	Sigma
35x10 mm style Petri Dish	-	2249665	Falcon
50 ml centrifuge tube	-	110608-060-1	Celltreat
5 ml pipettes	-	12101151	Cellstar
Ultra micro tips – 10 µl	1235A44	-	Thomas Scientific
Superfrost Microscopy Slides	12-550-15	14392	Fisher Scientific
37% Formaldehyde (PFA)	15682		Electron Microscopy Science
1X phosphate buffer saline (PBS)	-	-	Lab
Fluoromount-G	0100-01	E3710-R830	SouthernBiotech
Cholera Toxin subunit B Alexa Fluor 647 conjugate	-	1322300	Life technologies
Tweezers	-	-	-
Nail Polish	-	-	-
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
Bovine Serum Albumin (BSA)	SLBG1507V	-	Sigma Life Sciences

2.2 Preparations:

2.2.1 Recipe for 1x PBS solution:

3.2.1.1 Reagents

To prepare 1 L add:

1. 80 g sodium chloride (NaCl)
2. 2 g potassium chloride (KCl)
3. 14.4 g sodium phosphate dibasic (Na_2HPO_4)
4. 2.4 g potassium phosphate, monobasic (KH_2PO_4)
5. Add upto 950ml of deionized water
6. Adjust pH to 7.4
7. Filter with 0.22µ filters
8. Store at room temperature or 4ºC.

3.2.1.2 Equipment and disposables

9. Measuring cylinder
10. 2 liter Conical flask / Beaker
11. pH meter and/or pH paper
12. Magnetic stirrer

4.0 Cholera toxin B Staining Protocol:

1. Plate cells from a flask that is 80% confluent.
2. Your dishes will be ready for experiment, if it is 60-70% confluent.
3. Prepare your PBS solution. Refer to the PBS recipe for more information.
4. Prepare about 15 ml of PBS for every dish you are experimenting on.
5. Keep your prepared PBS in ice.
6. Prepare 2 ml of 3.7 % PFA for every dish you are experimenting on.
7. Keep your prepared PFA in ice.
8. Take our your dish from the incubator.
9. Put it on ice.
10. Wash it three times with 1ml of ice cold PBS.
11. Put 2 ml of PBS and add 4µl of Cholera toxin B in each dish
12. Incubate it in the 4^c fridge under a cover for 30 minutes.
13. Take out your dish from the fridge.
14. Pipette out the CTXB solution and save it for later use.
15. Wash your dish with 1ml of ice cold PBS for 3 times.
16. Put 2 ml of your ice cold PFA in the dish
17. Incubate it in the 4^c fridge under a cover for 10 minutes.
18. Pipette out the PFA.
19. Wash your dish with 1 ml ice cold PBS for 3 times.
20. Leave 2 ml of PBS in the dish.
21. Your dish is ready for imaging now.

If you are using coverslips stop at step 20 and continue with the following steps.

22 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.
23 Put a very small drop of fluoromount on the glass slide.
24 Take a sharp tweezers grab you coverslip from the dish
25 Flip the coverslip.
26 Dry in on the Kim wipe.
27 And put it directly on top of the fluoromount drop.
28 Wait 5 minutes for it to dry.
29 Put nail polish on the sides of your cover slip.
30 Wait 5 minutes for it to dry
31 Your slide is ready for imaging.

5.0 Cholera toxin B labeled YFP-DAT cells Imaging protocol:

1. Turn the Nikon Confocal Microscope on in the numbered order
2. You don't need to turn the heater on
3. Make sure the water chamber has enough water
4. Put a very small drop of oil on the lens
5. Put your glass slide on the slide holder
6. Bring the lens up until the drop of oil hits your coverslip
7. Click on the FITC laser to see your YFP tagged cells
8. Click on the TRITC laser to see the CTXB alexa 647
9. Choose a healthy cell with good expression of YFP and Alexa 647
10. Turn on the computer
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
24. Choose Alexa 647 2 channel
25. Click apply
26. Click live now
27. You should be seeing you Alexa 647 CTXB now
28. If you can not see a clear image, try changing the focus, Laser power, HV, offset until you get your ideal image
29. Save your image
30. Once you are done imaging, turn the computer off
31. Clean the lens very carefully with the lens paper
32. Bring the objective down
33. Turn the Confocal Microscope off in the numbered order

34. Put the cover on the microscope
35. If your cells are fixed, you can store them in the 4^{c} for future imaging

5.1 Cholera toxin B labeled YFP-DAT cells colocalization analysis protocol:

1. Choose the YFP DAT image and the CTXB image from you imaging folder.
2. Drag one of the images on the other so they overlap
3. Right click and choose analysis control
4. Next Choose colocalization analysis

5.1.1 For whole cell analysis

1. Choose to RIO option on the right menu
2. Choose draw Bezier ROI
3. Manually draw an ROI on the whole cell
4. You can also choose auto detect from the ROI menu
5. Click on the cell and let the microscope auto detect the whole cell
6. After drawing the ROI, look at the corresponding values on the colocalization analysis window
7. If interested in the data, choose the export to excel option on the top of the colocalization window
8. You can see the colocalization values in excel
9. When all the data is collected, run the proper statistical analysis on Excel

5.1.2 For ROI analysis

1. Choose to RIO option on the right menu
2. Choose draw Bezier ROI
3. Manually draw an ROI on the region of interest
4. After drawing the ROI, look at the corresponding values on the colocalization analysis window
5. If interested in the data, choose the export to excel option on the top of the colocalization window
6. You can see the colocalization values in excel
7. When all the data is collected, run the proper statistical analysis on Excel