# CONFOCAL MICROSCOPY OF LIPID RAFT CLUSTERING

## Reagents

- Alexa Fluor 488-conjugated cholera toxin B subunit (Invitrogen)
    - May be part of a Lipid Raft Labeling Kit (Invitrogen)
- Alexa Fluor 555-conjugated cholera toxin B subunit (Invitrogen)
- Anti-gp91 phox (Santa Cruz)
- Mab to ceramide (Alexis biochemicals)
- FITC-conjugated or Texas Red-conjugated anti-mouse secondary antibody

## Solutions

1. 4% paraformaldehyde (PFA) solution in PBS
    - Mix 0.4 g PFA in 1.0 mL $dH_2O$ and add 100 µL 1N NaOH.
    - Heat this mixture until the PFA has dissolved.
    - Dilute the solution to 10mL with PBS.
2. 100 mL 0.05% (v/v) Tween-20 in PBS (PBT)
3. 10 mL 1% (w/v) BSA in PBT
4. 20 mL 0.1% BSA in PBT (diluted from the 1% solution)
5. Working dilutions of all conjugated, primary, and secondary antibodies (determined through experience, diluted in 0.1% BSA)

## Microscopes, other hardware, and software

- Olympus FV-300 FluoView Confocal Microscope Workstation (McGuire)
- Leica TCS-SP2 AOBS inverted confocal laser scanning microscope and workstation (Anatomy Lab, Sanger)

## Protocol

### *Confocal Slide Preparation*

1. Plate cells at about 50% confluence on a 4-chamber glass slide (Nunc, Lab-Tek) in 15% FBS medium for at least 1hour at 37°C.

*All remaining steps are performed at room temperature with the slides being rocked during incubations.*

2. While still in growth media, remove all but 500 µL medium and add stock treatment to the appropriate chambers for the appropriate times (i.e. 5 µL of 10ng/ µL stock FasL for a final concentration of 10ng/mL for 10 min.).
3. Pour off the media, wash each chamber twice quickly with 0.5 mL PBS, and then fix the cells by adding 0.5 mL of 4% PFA to each chamber and incubating for 15 min.
4. Wash the cells three times in 0.5 mL PBT (per chamber), 5 min each, and then incubate the cells for 30 min. in 0.5 mL of 1% BSA.

*All remaining steps are performed in the dark to protect fluorescent markers.*

5. Wash cells in PBT three times as previously described, then incubate cells in 0.5 mL of the working concentration of the rabbit anti-gp91 antibody (found through literature and/or response curve experiments), diluted in 0.1% BSA, for 45 minutes.
6. Repeat step 5 using Texas Red or FITC-conjugated anti-rabbit working solution.
7. Repeat step 5 using the Alexa 488- or Alexa 555-conjugated CTX or Mab to ceramide working solutions.
8. Repeat step 5 using the Texas Red- or FITC-conjugated anti-mouse secondary antibody working solution.
    - NOTE: Green fluorescence (Alexa 488/FITC) should only be paired with Red/Orange fluorescence (Texas Red/TRITC/Alexa 555), and vice versa.
9. Once again fix cells for 15 min. in 0.5 mL of 4% PFA.
10. Allow the slide to dry and remove the plastic chamber piece and sealer holding it in place completely.
11. Place one drop of Vectashield Mounting Media on each sheet of cells (1 for each chamber), and cover with a No. 1.5 thickness cover slip. Gently push out any air bubbles that form underneath the cover slip and seal the edges with clear nail polish.
12. Store slides at 4°C in the dark before and in between viewing under fluoresence.

***Fluorescence Visualization***

1. Capture digital images of both the green, red/orange, and overlay fluorescent patterns.
    - Begin with low-magnification images of all treatments, adjusting gain/offset according to the brightest group of cells and keeping these settings constant for all other groups
    - Switch to higher magnification, readjust gain/offset according to the brightest group of cells, and capture representative single cell images in all groups
        - Name files with slide number, magnification, treatment, and fluorescent label. Save in a folder named as today's date
2. After defining "activated" and "non-activated" cells, tally a total of 200 cells in all groups according to these definitions.