Masking Protocol for FISH

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1) Print out a few copies of the ProbeLog file. My computer/Local Disk/Microscope. Record the details of the Exp or profile on the top of the log and then under image record each image on a separate line eg #6.

2) Open the twelve sets of images on Image Pro Plus 7.0.

3) Under the Macro heading choose the macro management subtitle. A window will open up. Click on change and choose the Digital_Probe.scr file. This file should have macros suitable for masking.

4) Click on the active image. For DAPI images use DAPI. It depends on the background. I usually set the intensity range to 100-255. Basically the DAPI images can have an intensity range that is 5-25 over background. Use common sense as each image can differ. Just make sure all bacteria are counted and not any background contamination. To change the intensity range, under marcro click on edit macro. Scroll down to DAPI and under ret = IpBlbSetRange (100, 255) change 100 to what number works for your images.

5) Once the intensity range is set, then run the marco on the DAPI image. The resulting count file should have a window that states the actual count. Record the **in range** count on the ProbeLog under DAPI COUNT. In the Count/Size window, click on the image header and choose the make mask option. You should now have a black and white image of your count.

6) Close the count file and the DAPI image keeping the mask image open.

7) Click on the corresponding CY3 image. For CY3 images, use the CY3 acro. It depends on the background. I usually set the intensity range to 25-255. Basically the CY3 images can have an intensity range that is 1-5 over background. Use common sense as each image can differ. Just make sure all FISH bacteria are counted. Background contamination is excluded as it was not counted in the DAPI image. Thus if needed some can be included. SAR11 and Eubac tend to be rather faint so 0-2 over background is used while for everything else (esp Neg) 5 over back ground is sufficient. This can be done image by image using the select ranges button in the Count/Size window. Move the line to where you want to try, click OK and Count again. Your coutn will only change if you click on Count.

8) Once the intensity range is set, then run the marco on the CY3 image. The intensity range can be set on an image by image basis. **This can be done image by image using the select ranges button in the Count/Size window.** In the Count/Size window, click on the image header and choose the make mask option. You should now have a black and white image of your count.

9) Close the count file and the CY3image keeping the mask image open.

10) The two mask images should be in the forefront. In Image Pro, choose the process header and click on image overlay. A window opens up with transparency options. The blend control should be 50% for src (source) and dest (destination). Both the overlay and merging transparency should be ticked. Then choose the all option under the statement “apply blended image to:” Finally click on the overlay button. The images are now overlaid but they are not merged until you right click. Usually you need to move the image around to optimize the bacteria that have probe hits. This is done using the mouse and the bacteria with probe hits appear as white in the overlay. The two images (DAPI and CY3) are not perfectly overlaid as the stage can move during imaging.

11) Once you have the images overlaid, right click to merge and then in Image Pro click on the macro header and choose the MERGE macro. Record the in range count on the ProbeLog under MERGE.

12) My computer/Local Disk/Microscope - open the Probe Profile. Each sheet is a different probe. Change the probes to the ones you are using. Record the details of the Exp or profile on the top of the log and then under Input all the data including the counts. The negative control is the 338F nonsense probe and is needed for all probes as autoflourescence is subtracted out. The % is calculated and on the summary sheet is used to determine the probe count. The file is set-up for Eubac, SAR 11, Cyt and Roseo and a depth profile with counts but can be changed.