Preliminary Notes:

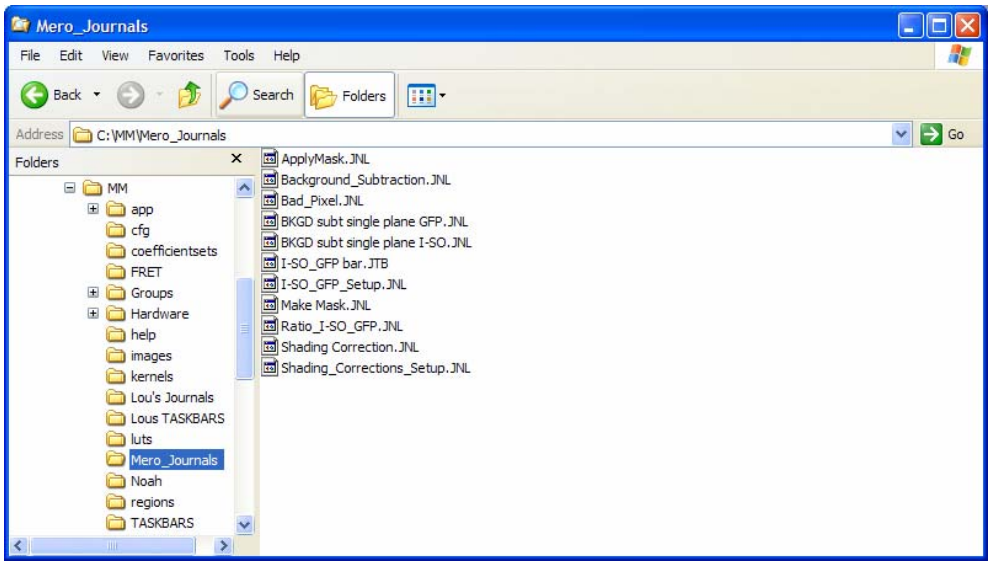
These Journals are configured to look for certain names; please see table at the end of this document for the specific Input and Output parameters.

Metamorph version 6.3r2 was used in preparing this document

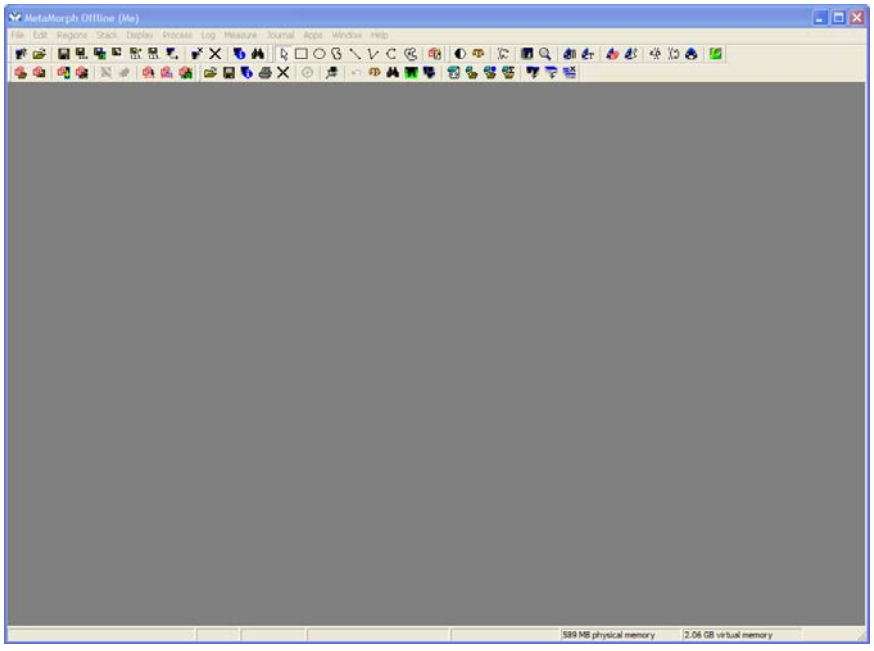
Important Note: These Journals will **NOT Automatically Save Processed Images**. If the user wants to save image processing intermediates, please do so in between the running of these Journals. An asterisk at the left of an image name signifies that the image has not been saved.

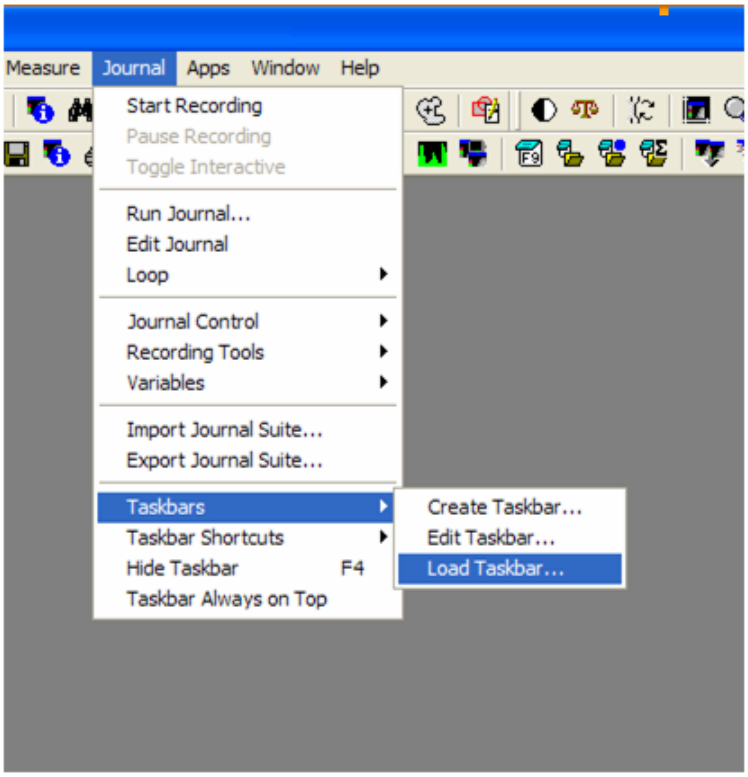
Installing Journals and Opening up Taskbar in Metamorph Setup

Download and place Mero_Journals subdirectory in *C:/MM/*



Open the *Metamorph* Software





Loading Mero-CBD Taskbar

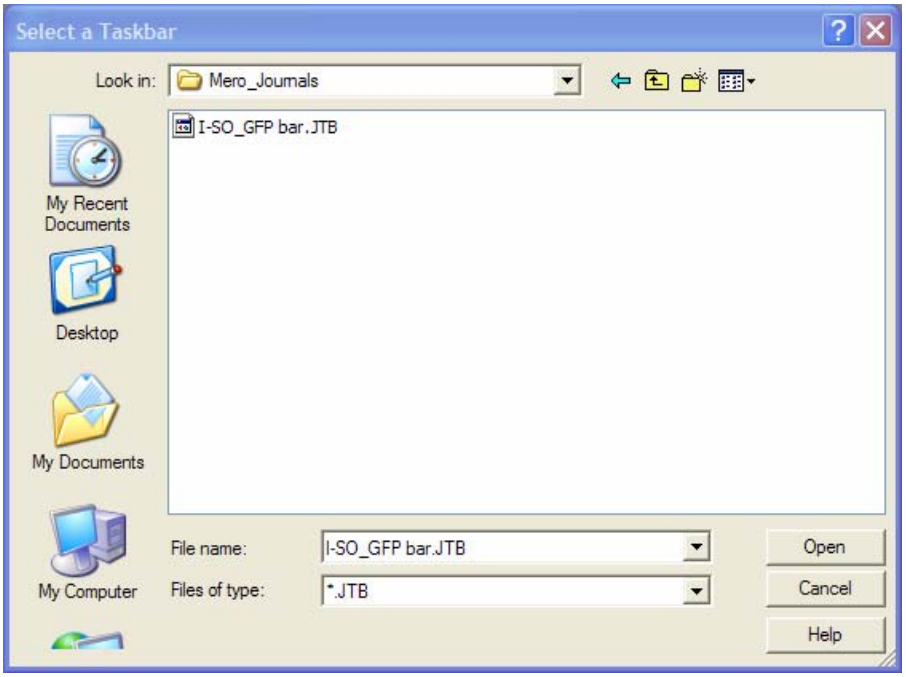
Within Metamorph Software Environment

Select:

→ *Journal* Menu

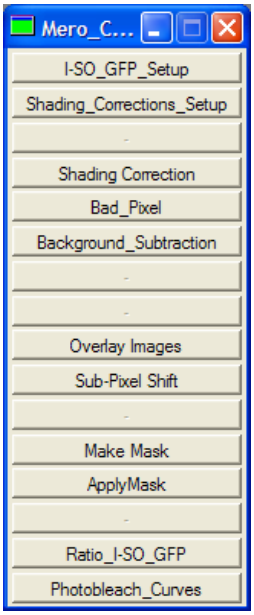
→ *Taskbars*

→ *Load Taskbars*



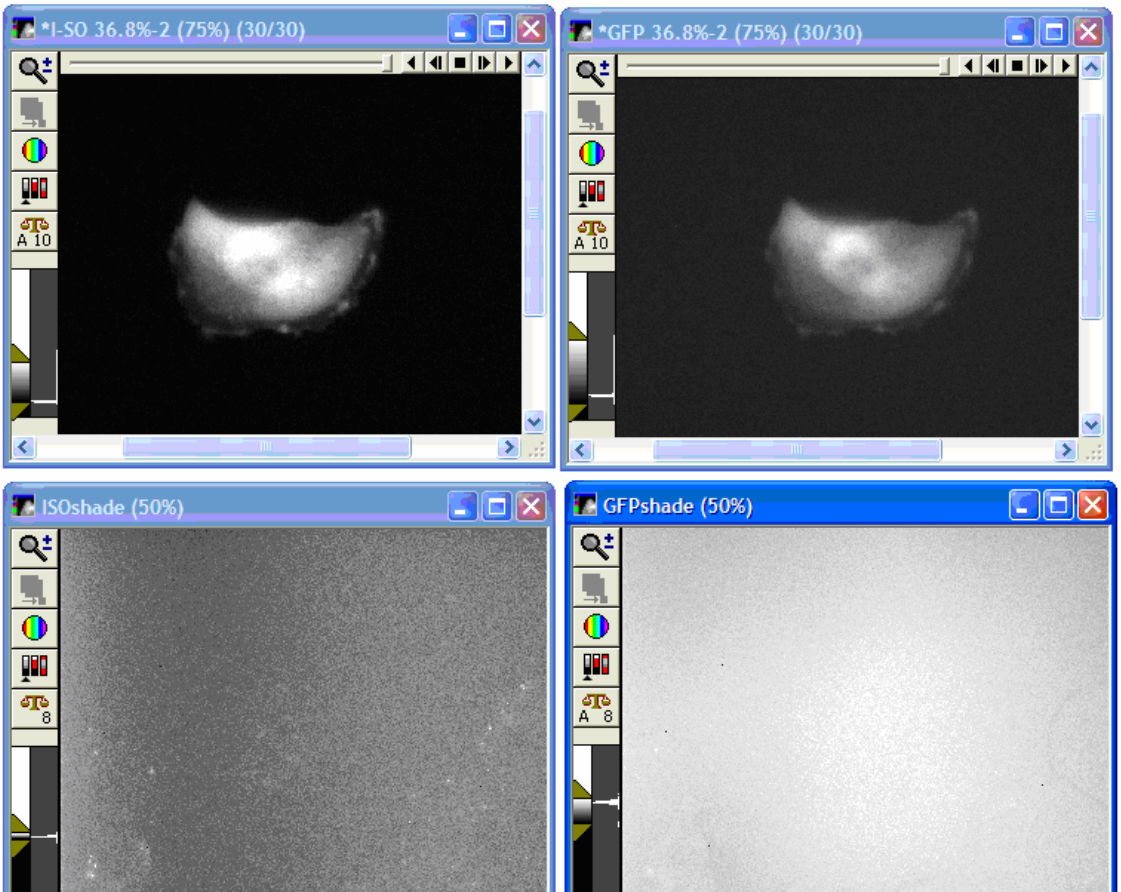
Look In:
C:\MM\Mero-Journals directory

Highlight & Open
I-SO_GFP.bar.JTB



Open MERO-CBD Taskbar in Metamorph Window (above)

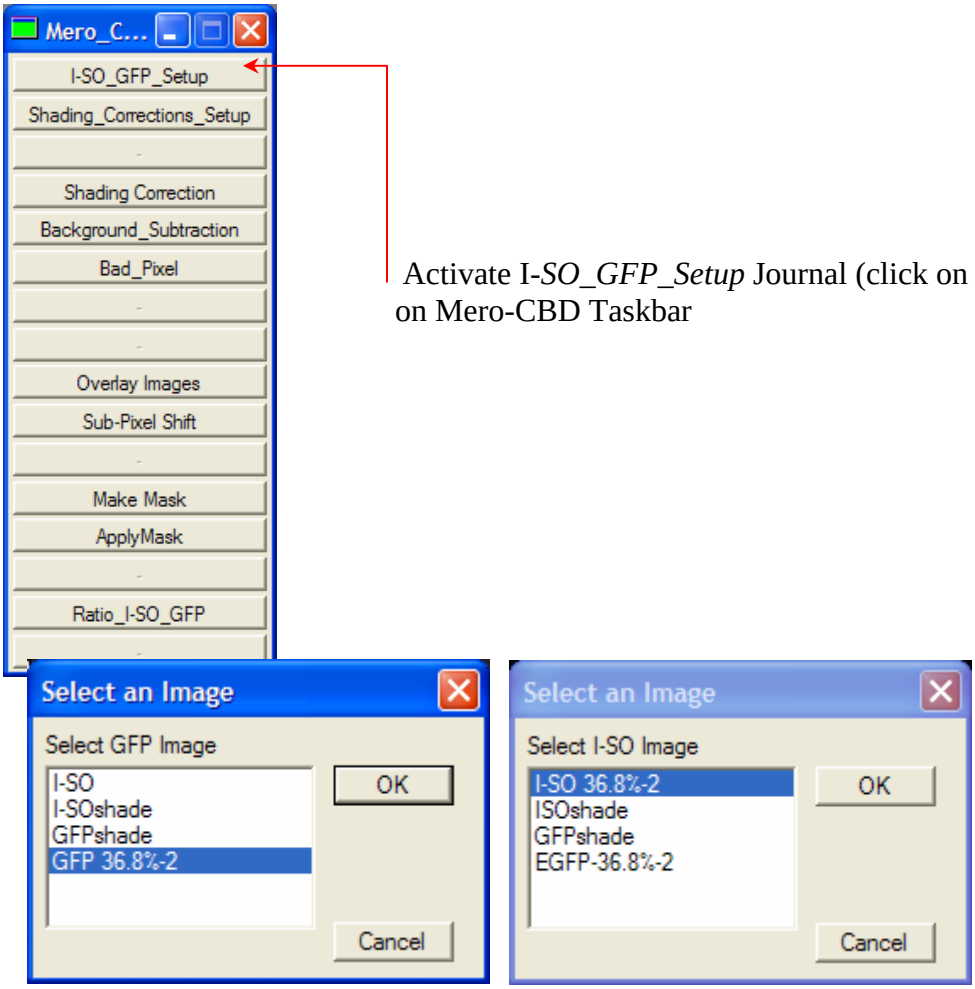
Opening up Image Sets



Open I-SO and GFP Image or Image Stacks in *Metamorph.*

Open appropriate Shading Correction Images for each Set.

I-SO_GFP_SETUP Journal



Activate I-*SO_GFP_Setup* Journal (click on button) on Mero-CBD Taskbar

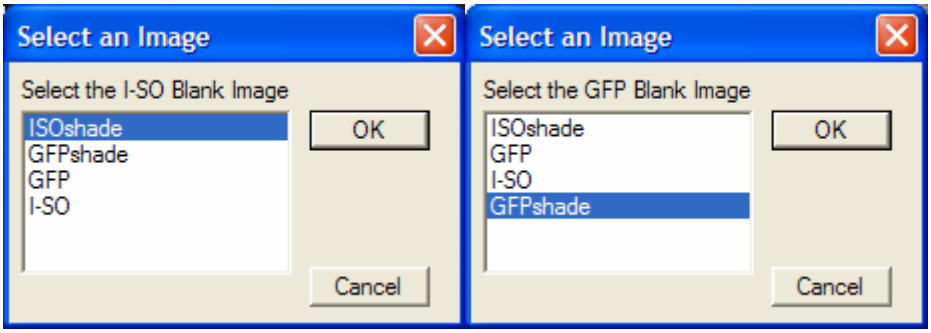
Select the I-SO and GFP Image(s) respectively

Note: the image names highlighted below within the screenshots of the journal dialog boxes below (and within the rest of this document) will not necessarily correspond to your choice of images.

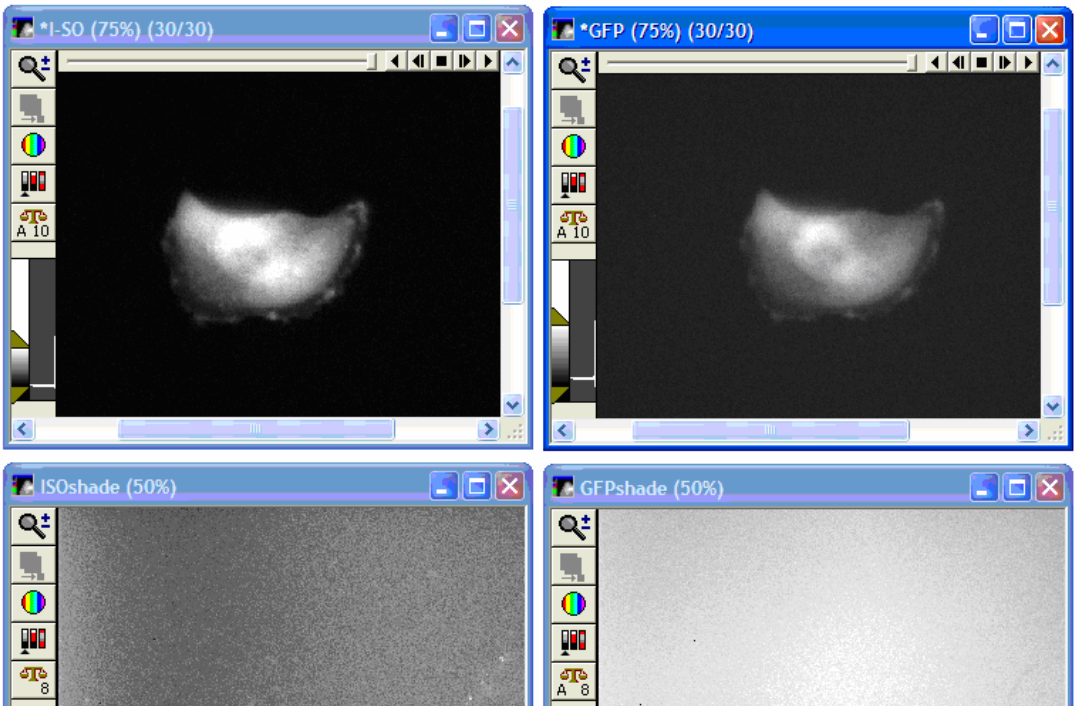
Shading_Corrections_Setup Journal

Click on Taskbar button entitled *"Shading_Corrections_Setup"*

In response to the Dialog boxes (below); select your I-SO and GFP Blank (Shading Correction) Images respectively.



Note: The image processor can continue to use the same set of background images for the cell images from a particular cover slip without running *Shading_Corrections_Setup Journal* each time (as long as the journal was run once during current present image processing).

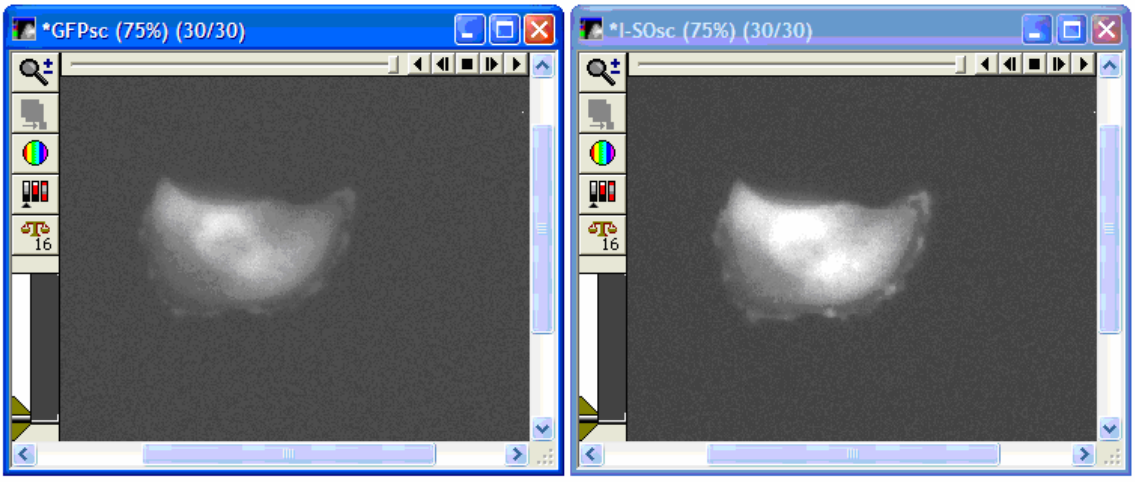


After running the Setup Journals, the I-SO and GFP Image(s) have been renamed and the Shade Correction Images have been identified but their names have been unaltered (above).

Shading Correction Journal

Click on *Shading Correction* button on Taskbar.

This Journal will automatically correct the images for uneven illumination.



The Shade Corrected images (above) now have the respective names "*GFPsc*" and "*I-SOsc*" where "sc" stands for shading-corrected.

Bad Pixel Journal

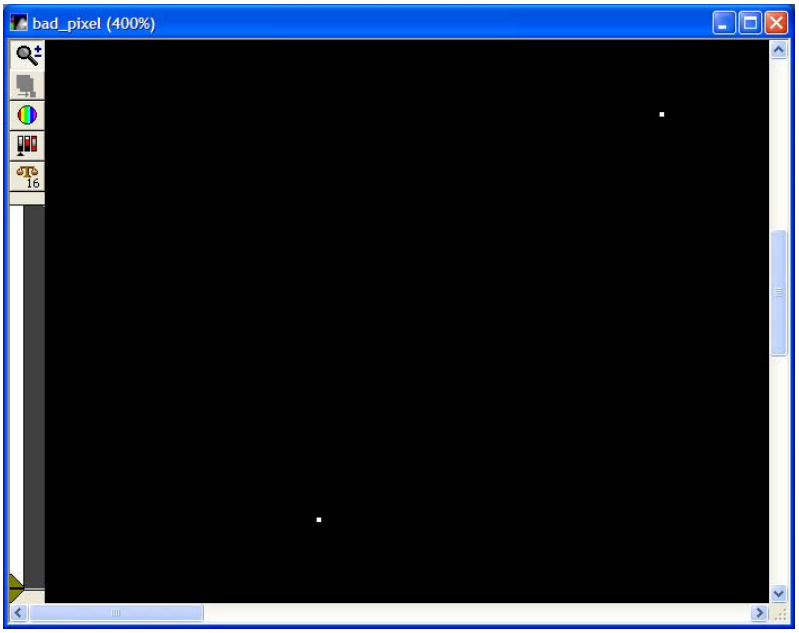
A Bad-Pixel Correction image is a binary mask having values of “0” and “65535”. The image is collected using the same binning and image sizes as the I-SO and GFP image sets.

A Bad_Pixel Correction Image can be created by thresholding a shading-correction image for the highest values or by collecting an image while the CCD camera shutter is closed.

Note: The *Bad_Pixel Correction Image* can be used repeatedly.

Note: If the Bad Pixels are few in number or located towards a corner of the field you can skip using the following journal.

Note: Bad Pixel correction should be done prior to Background Subtraction or a “hot” pixel’s value could impact the Background pixel Intensity calculations. The Bad Pixel image below has been magnified to emphasize two bad pixels (white dots).



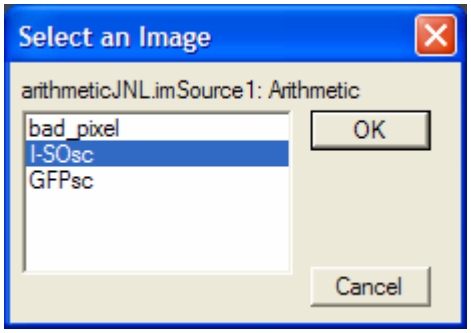
Open the Bad Pixel correction image.

Rename to “*bad_pixel”* if necessary

Click on *Bad Pixel* Button on Taskbar.

Select Image to Correct

Run once for GFPsc and I-SOsc respectively (below)

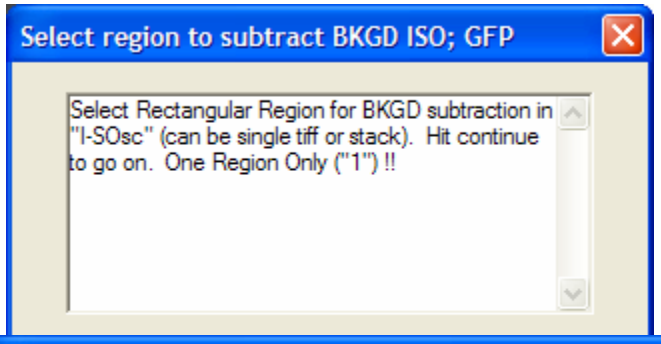


This Journal will correct and overwrite these images without changing their name.

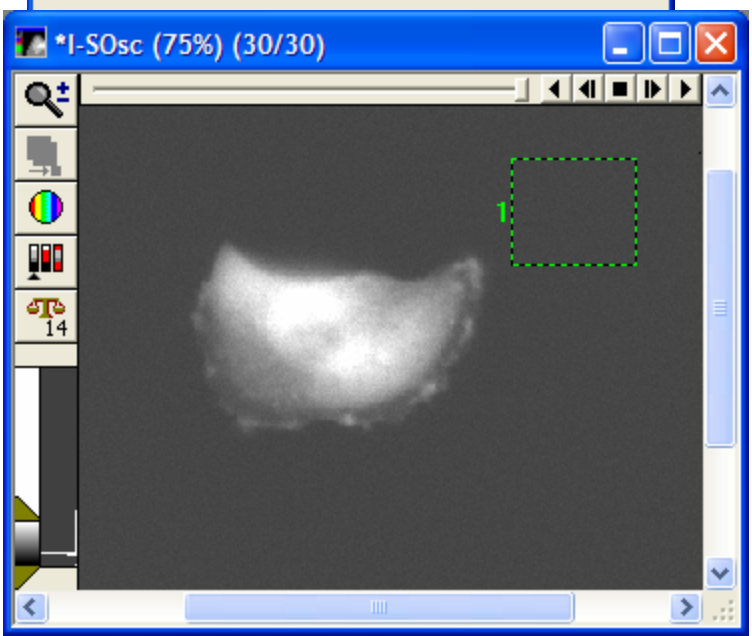
Close the Bad_Pixel Image when finished using this Journal

Background Subtraction

Click the button entitled *Background Subtraction*



A Dialog Box will pop up

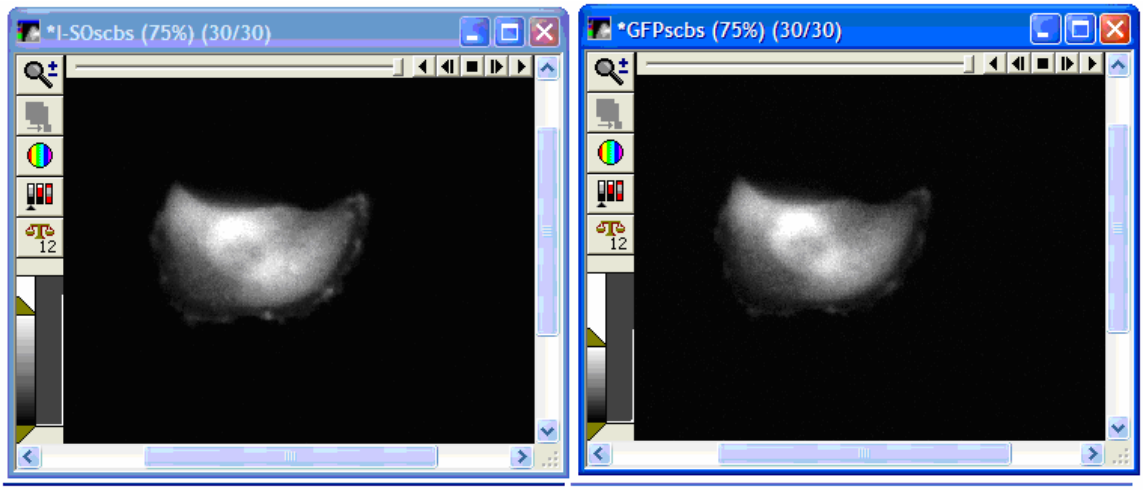


Select Rectangle Icon from Toolbar (see below)

Create a Rectangle on a background region of I-SOsc.

Note: Scan through all planes to ensure that debris did not float through the ROI (Region of Interest) during Image Capture.

Click on *Continue* on the Dialog Box (shown above)



These Images now have been corrected for uneven illumination, background, and for the CCD's bad pixels (Above).

Registration

Registration offsets between the I-SO and GFP Channels can be empirically defined in Metamorph by using two Function calls (as opposed to Journals). These Functions (*Overlay Images* and *Subpixel Shift*) have been placed on our taskbar for your convenience; they are also available from *Metamorph* Menus

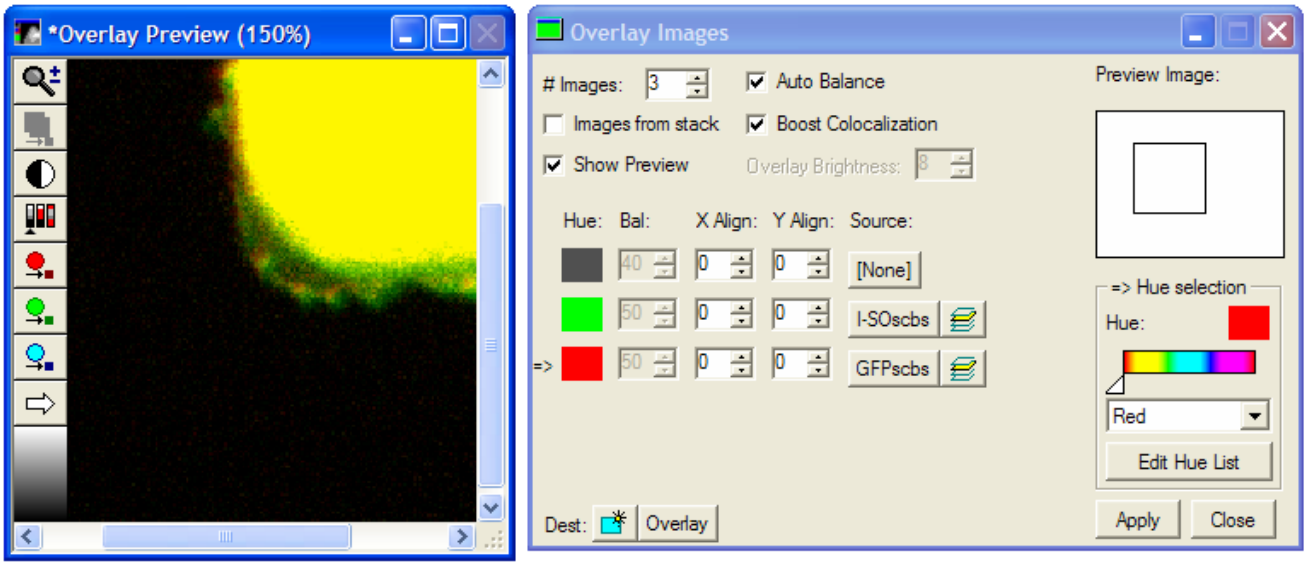
Note: If these functions do not work; check to see if the Drop-Ins have been Activated (see Metamorph Help for details).

Overlay Images Function

Click on the Taskbar button entitled *Overlay Images*

Verify that the Identical Plane (time point) is selected for each Image Set

Adjust Image Contrast so images display is saturated in cell centers (very bright)



Set the Settings for *Overlay Images* as shown above.

Move the Rectangle around the *Preview Image* Inset (upper right) to scan for unique features at the cell perimeter.

Change the *X Align* and *Y Align* values for only one of the Images.

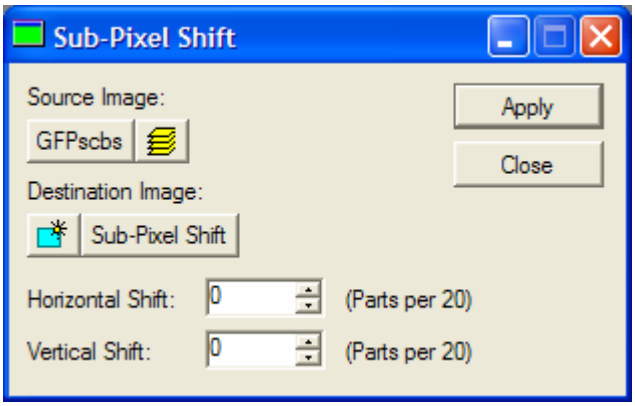
Note: The *Overlay Preview* Function will give the **Integer** Values for the X,Y Offsets (here we are using this function as a diagnostic tool).

Click on the *Apply* button to examine effect of moving one image (X,Y) with respect to the other.

Click on the *Close* button to Exit *Overlay Images* Dialog box

Sub-Pixel Shift Function

Click on the *Sub-Pixel Shift* Button on Taskbar



The Sub-Pixel Shift dialog box

The Sub-Pixel Shift Function can be used to move one of the stacks from 1/20 to 19/20 pixels in the X or Y direction. This function may be used to correct for the Integer Offsets estimated by using Overlay Images and refine the registration to precision at the Sub-Pixel level.

Fill in the derived values for *Horizontal* and *Vertical Shift.*

Click on the *Apply* button to Shift the Selected *Source Images*

Click on the *Close* button to end Function Call to *Sub-Pixel Shift*

Note: The processor will have to run the function multiple times to compensate for pixel shifts of 1 (1 = 1/20 + 19/20) or above.

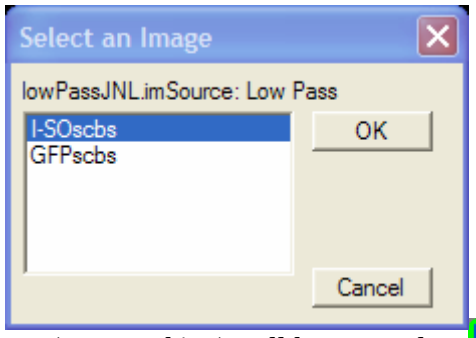
Note: A more precise approach to Image Registration is available at this website using MatLab Routines. After using these routines please make sure the names of the images conform to the naming scheme shown in the table at the end of this document. In *Metamorph* the *rename* function can rename images.

Note: Registration Offset values should be determined rigorously; however once they are determined they should be constant for a give set of Image Acquisition Settings as long as the Microscope has not been re-aligned.

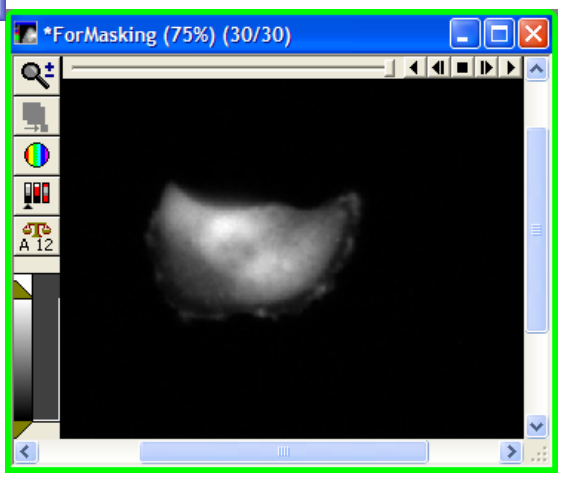
Masking

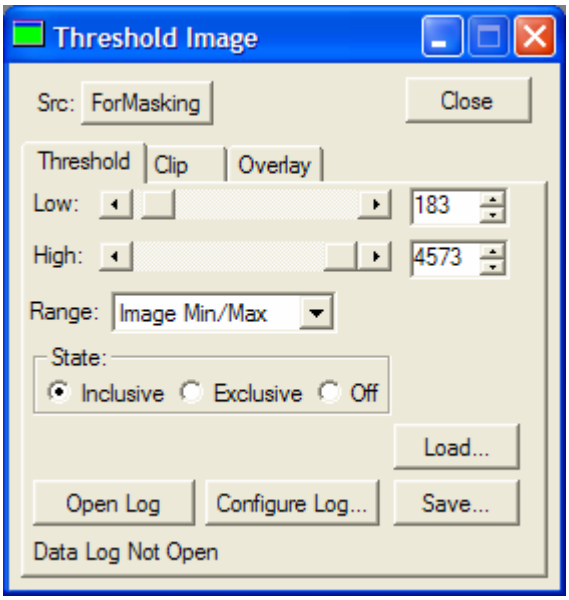
Click on the *Make Mask* Button on Taskbar

Select the Image set that has higher intensity values (see dialog box below)

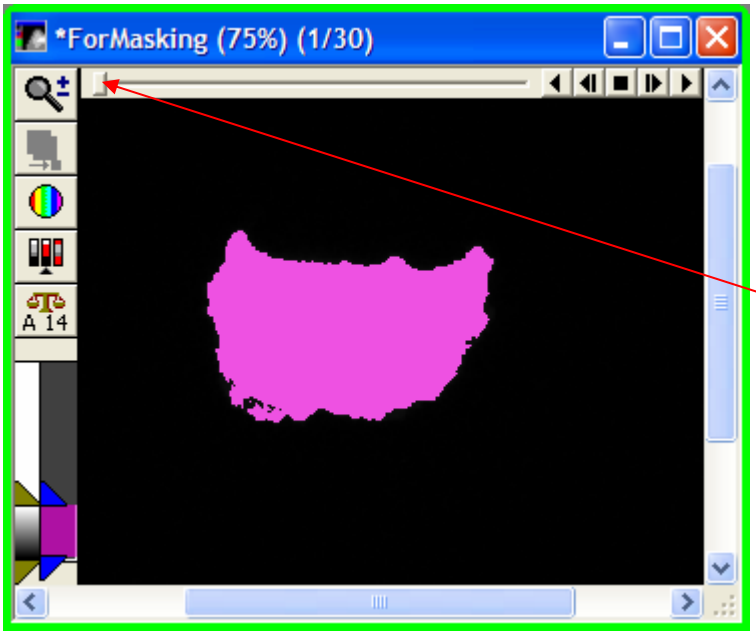


A smoothed Image (*ForMasking*) will be created for the purpose of thresholding and creating a 1-bit Mask. The first plane of this image will be displayed





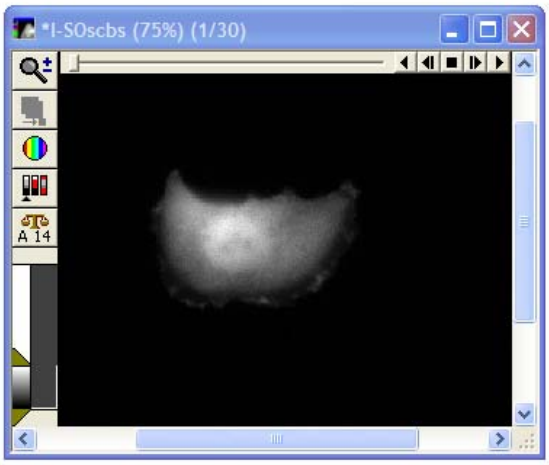
- Set the Thresholding *Range* to *Min/Max*.
- Check *Inclusive* Thresholding.
- Move Low Value bar towards the right the Cell Area will be segmented (Pink Overlay Figure Below).

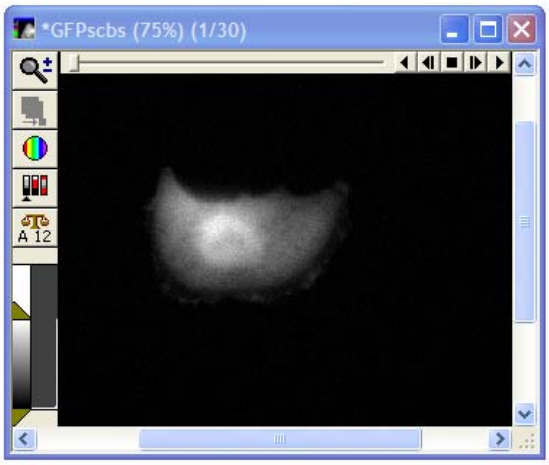


The Segmented Image is shown with a Pink Overlay. This shows that the Thresholding is appropriate for this plane.

At this point scan through the planes and ensure that the chosen Low and High Intensity Thresholds were appropriate for this image series.

To finish Thresholding click on the *Close* button (upper left of Thresholding dialog box)





The Open Images should be: I-SOscbs, GPFscbs, and Mask (above)

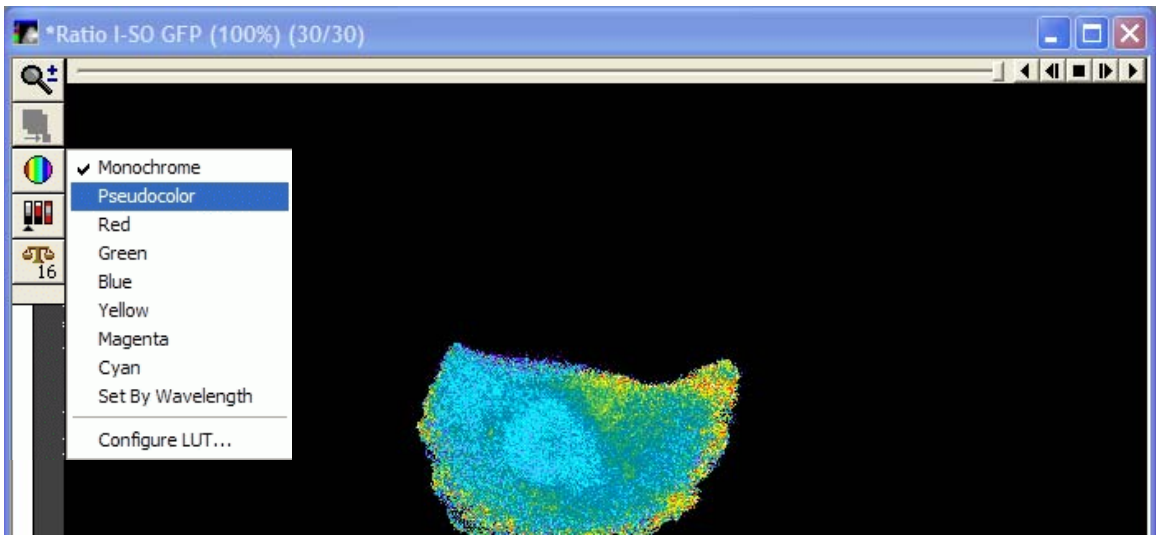
Click on the *ApplyMask* Button on Mero-CBD Taskbar.

This Journal will apply the mask to the images so that only cell intensity values remain.

After running the journal the open images will be: I-SOmk, GFPmk and Mask (not shown).

Ratio I-SO_GFP Journal

Click on the *Ratio_I-SO_GFP* Button on Taskbar



A Ratio Image (I-SO/GFP) will be created in a Black and White Look up table: Select the Pseudocolor LUT (above)

Note: Sliding the Brown Triangles (left of image) adjusts the color look up table (LUT) highlighting the CDC42 Distribution in Red and Yellow while the Cell remains mostly Blue, Green, and Purple.

Photobleach Corrections

Click on the *Photobleach Curves* button on Taskbar

This journal will automatically create an Excel File for further Image Processing.

	A	B	C
1	plane#	ISO	GFP
2	1	1799.76	884.545
3	2	1765.74	878.31
4	3	1748.69	872.709
5	4	1756.65	885.731
6	5	1740.91	888.23
7	6	1754.88	901.714
8	7	1720.38	890.4
9	8	1710.71	887.779
10	9	1691.16	885.594
11	10	1697.41	897.489
12	11	1652.93	889.005
13	12	1625.46	881.204
14	13	1614.9	884.729
15	14	1607.06	885.667
16	15	1620.51	901.597
17	16	1624.33	903.577
18	17	1584.96	885.671
19	18	1547.66	874.891
20	19	1546.4	882.418
21	20	1553.81	898.635
22	21	1536.15	894.079
23	22	1547.13	912.649
24	23	1533.67	909.543
25	24	1514.64	906.056
26	25	1489.33	896.531
27	26	1496.9	913.503
28	27	1492.96	916.062
29	28	1474.55	912.177
30	29	1478.99	923.088
31	30	1460.17	918.535

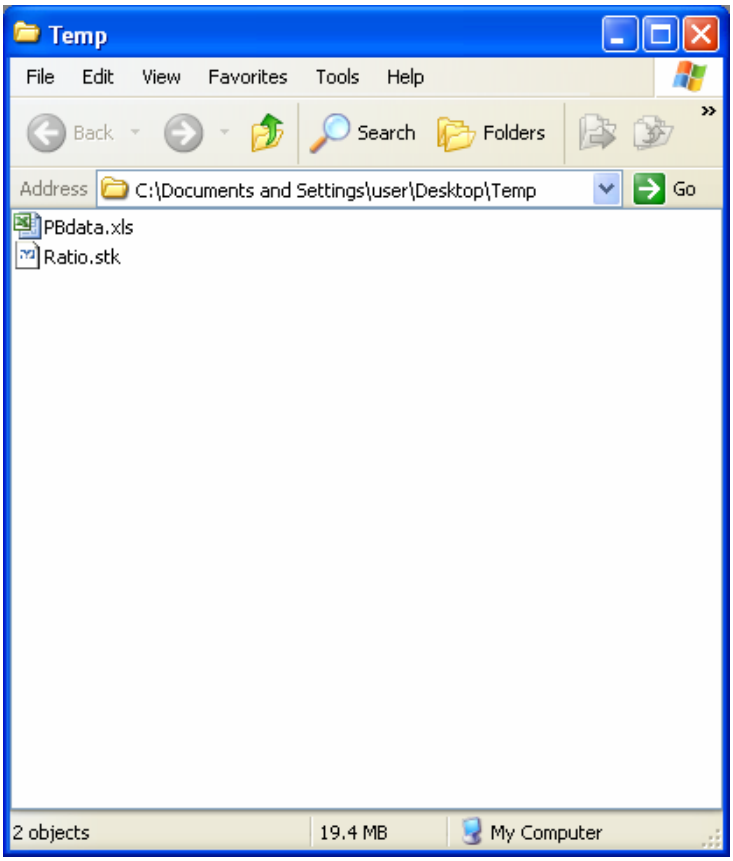
Changed the Label “Image Plane” to “Plane#”

Changed the Label “Average Intensity” in (B1) to “ISO”

Changed the Label “Average Intensity” in (C1) to GFP”

Note: Depending on your Metamorph Log settings your Excel File might look slightly different prior to adjustments.

Saving Images and Data for use with MatLab

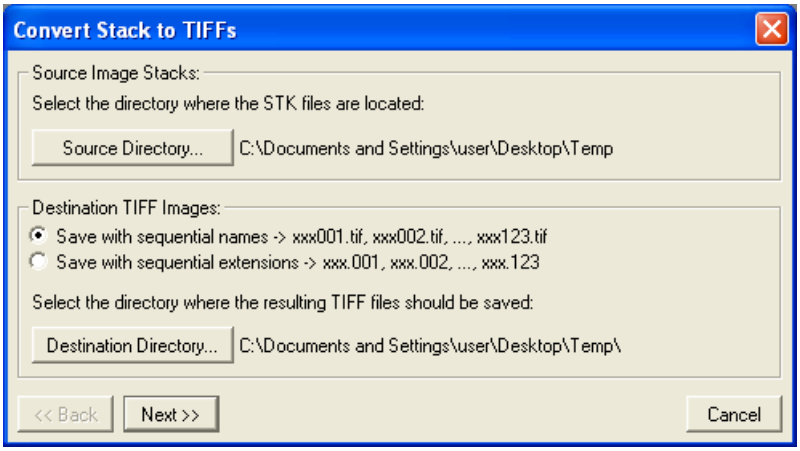


In Excel save the Spread Sheet with meaningful name to a directory

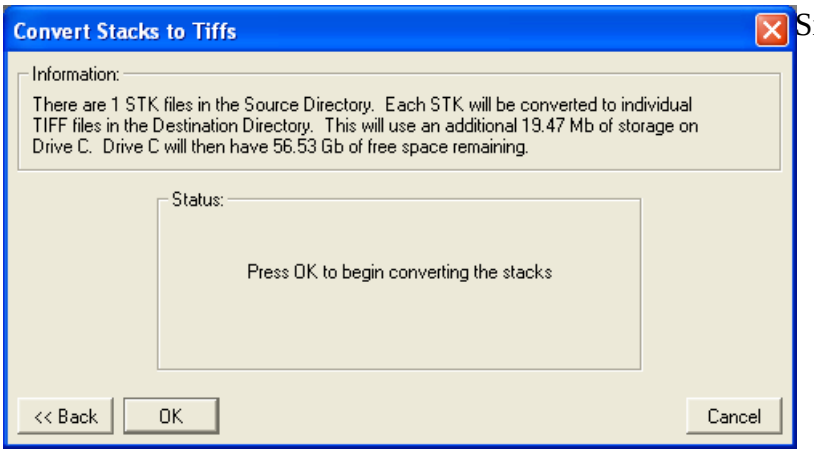
In Metamorph: Save a copy of the Ratio file to the same directory on your computer.

Convert Ratio Image Stack to Tiff Series

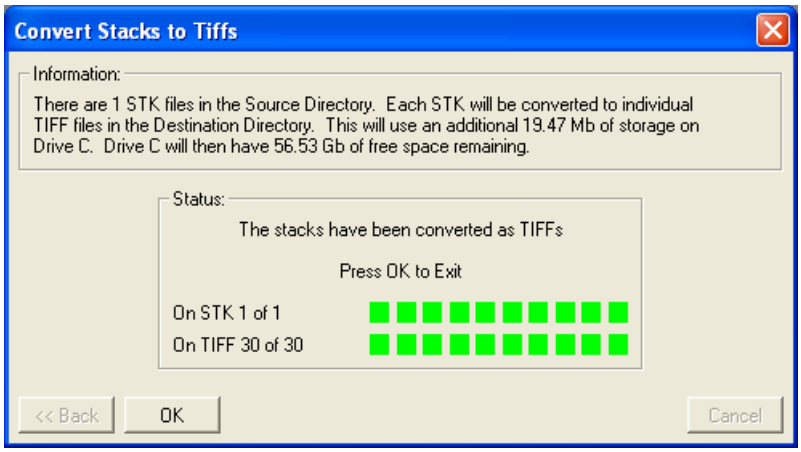
Choose **File** Menu (in Metamorph) and then select **Convert Stack to Tiffs.**



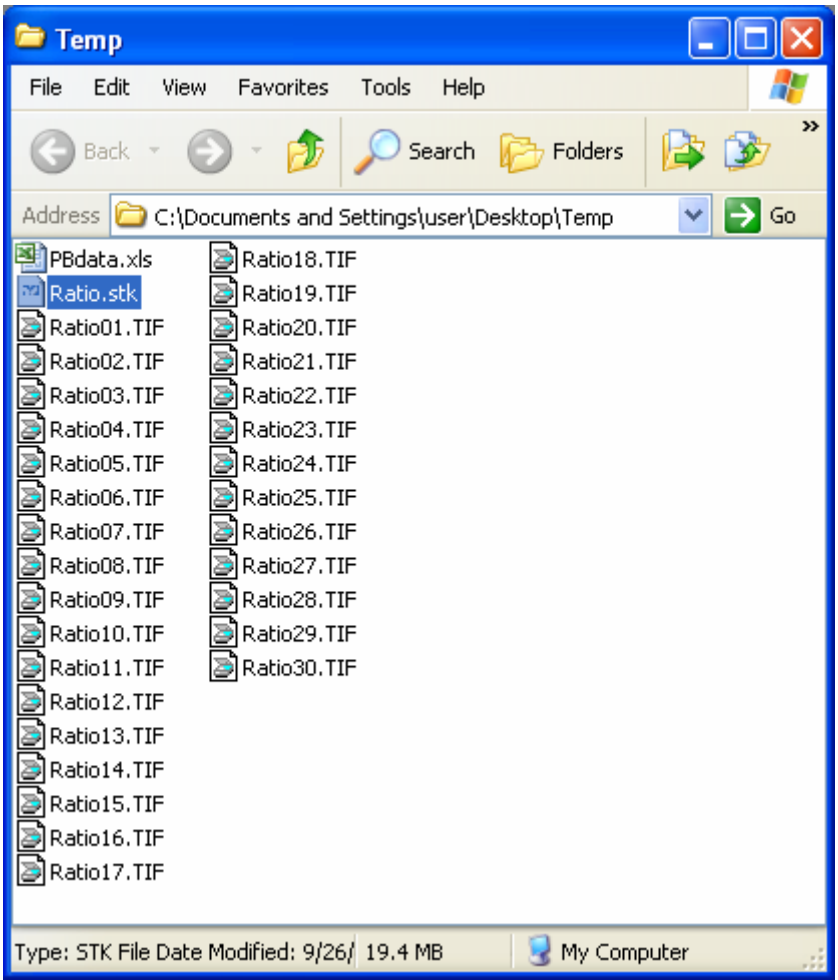
Setup as shown in figure at Left.



Read and Press OK



Select OK to Convert Stack to Image (open Image will Close)



Your Directory should look something like this.

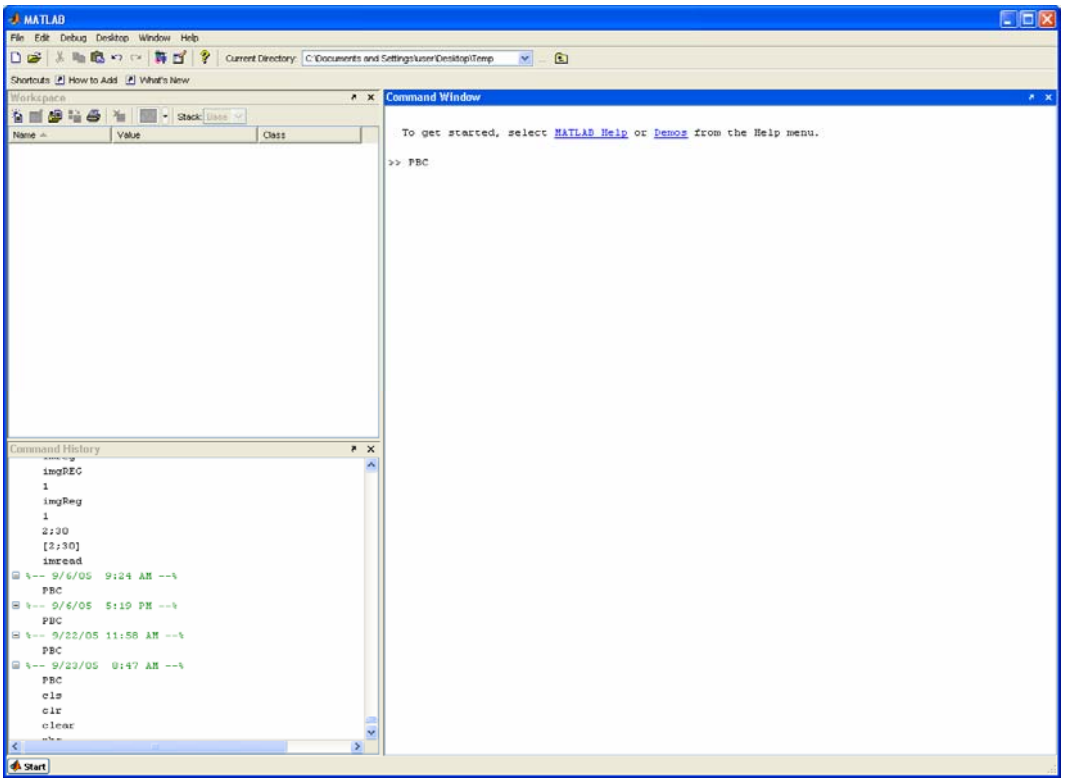
The Ratio Image planes and the Excel photobleaching data will be used by MatLab Routine to create a set of Photobleach-Corrected Ratio Images.

Matlab (version 7.0 was used for this demonstration)

Installation: Place the files PB3.m, PBC.M and PBC.fig into active Matlab folder.

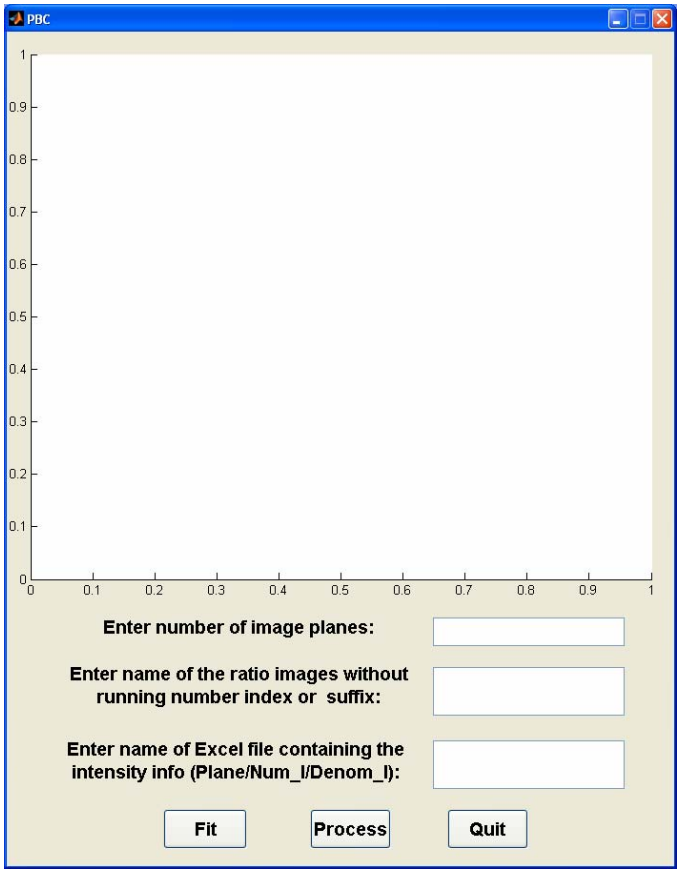
Open Matlab (version 7.0 was used for this demonstration)

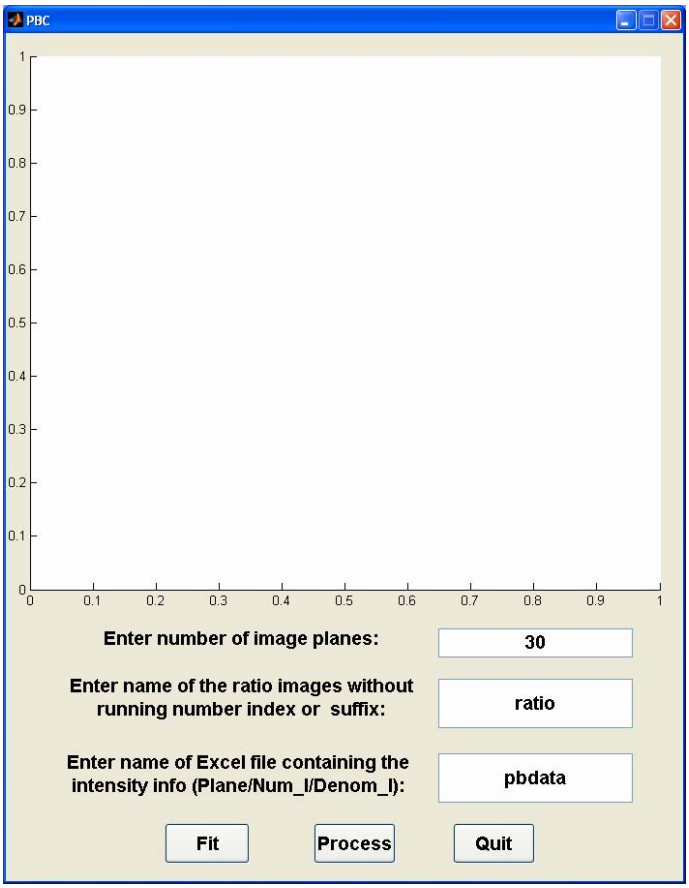
Set Current Directory to directory containing Ratio Images and Photobleaching Data



Enter **PBC** into Command Window and hit Enter.

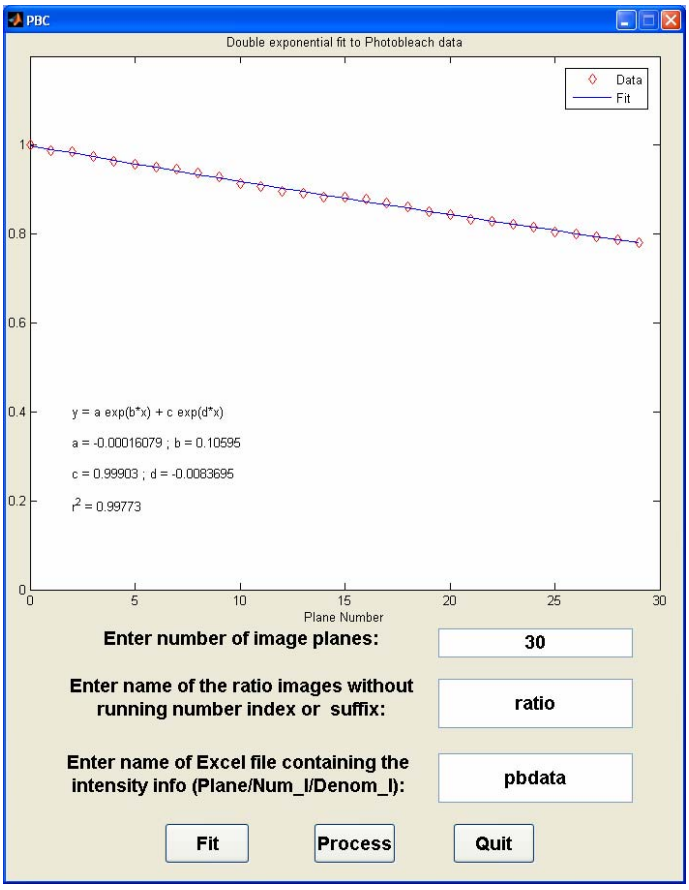
The Matlab Photobleaching Dialog Box will open up (below).





Enter the number of image planes, the base name of the tiff image set (i.e. in this case “ratio”) and the name of the Excel Data file.

Click on the Button entitled “Fit”

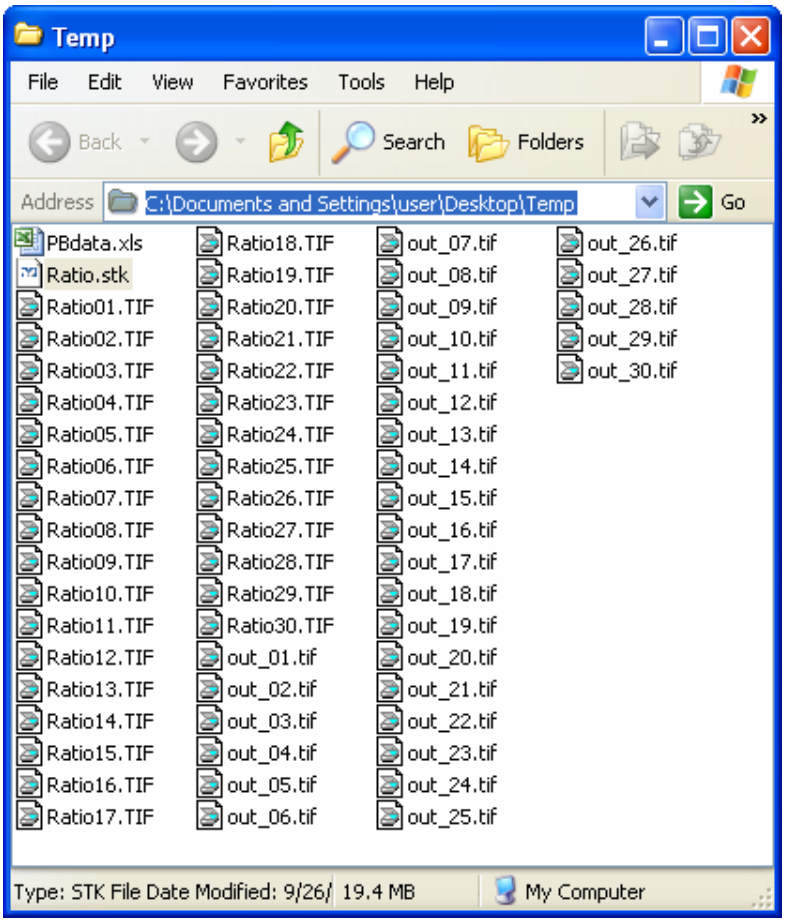


The Routine will fit the line with a double-exponential fit correlating to photobleaching corrections for both channels.

The R value is a statistical metric correlating to assay quality.

Note: This routine will print out a copy of the graph automatically.

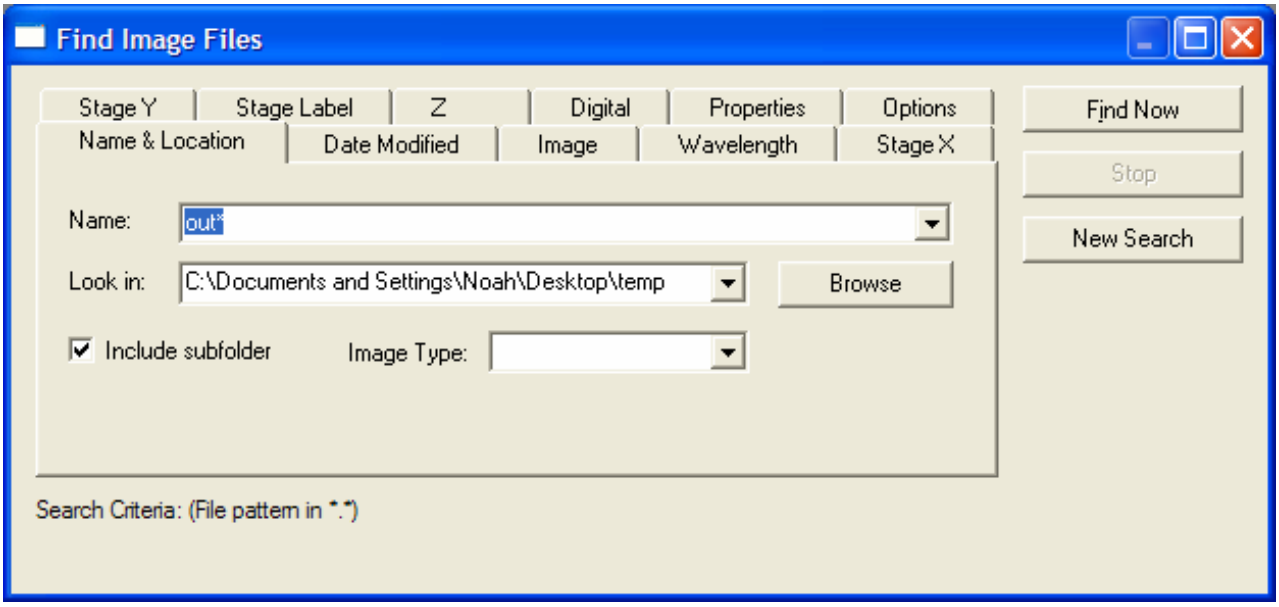
Click on the **Process** button; MatLab will now create a set of Photobleach-corrected Tiff files which correlate to the Input Ratio Tiff set.



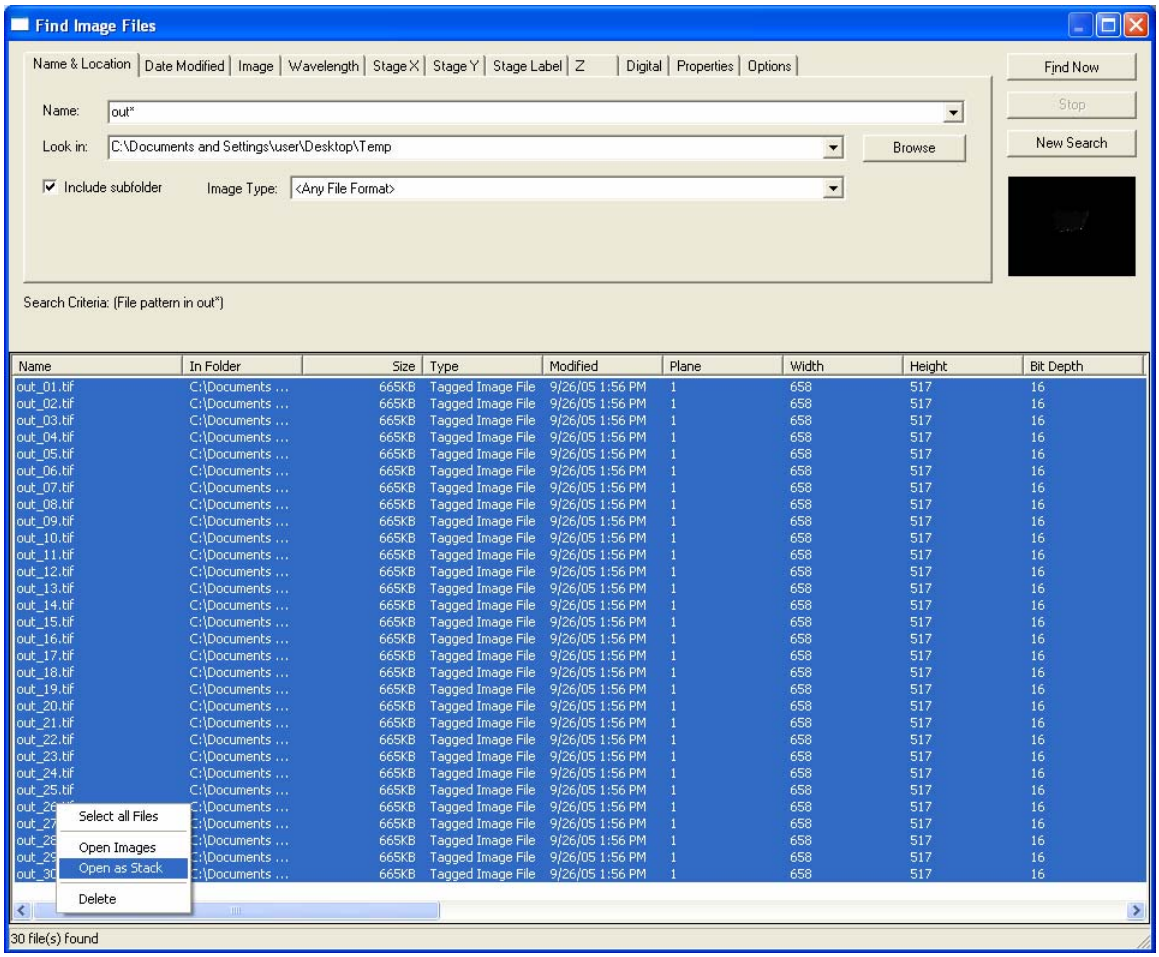
The Matlab-created Image files are written to the same directory containing your data and input image set. They are entitled “out” for Output.

Assembling Photobleach-Corrected Ratio Image Stack in Metamorph.

Open up Metamorph (or return to Metamorph Software)



In **File** menu select **Find File:** point dialog box towards output Image Set (see figure). Click on the “Find Now” button.



Highlight all Image Names, Right-Click and select “Open as Stack” (above).

Metamorph will open a stack of the Photobleach-Corrected Ratio Image Set. Choose Pseudocolor and adjust the display contrast as for the I-SO_EGFP Ratio Image.

Input and Output Images for Metamorph Mero-CBD Journals and Function Calls.

Journal Name (or Metamorph Function*)	Input Images	Output Images/Files	Purpose of Journal
I-SO_GFP_Setup	(Select I-SO Image or Stack) (Select GFP Image or Stack)	I-SO GFP	Select and Rename I-SO Image Select and Rename GFP Image
Shading_Corrections_Setup	(Select I-SO Shading Correction Image (Select GFP Shading Correction Image	Input (I-SO) Input (GFP)	Selects I-SO Shading Correction (as Variable) Selects GFP Shading Correction (as Variable)
Shading_Correction	I-SO GFP (I-SO Shading Correction Image) (GFP Shading Correction Image)	I-SOsc GFPsc	Equals (1000*I-SO)/(I-SO correction Image) Equals (1000*GFP)/(GFP correction Image)
Bad_Pixel	Bad Pixel Input Image (I-SOsc or GFPsc)	 I-SOsc	 Equals (I-SOsc - Bad_Pixel)
Background_Subtraction	I-SOsc GFPsc	I-SOscbs GFPscbs	Equals (I-SOsc - Avg Pixel Int per ROI per plane) Equals (GFPsc - Avg Pixel Int per ROI per plane)
Overlay Images*	*I-SOscbs* *GFPscbs*	*I-SOscbs* *GFPscbs*	*No Change just determine X,Y Offsets (Integer)*
Sub_Pixel_Shift*	*I-SOscbs OR GFPscbs*	*I-SOscbs OR GFPscbs*	*Moves X and/or Y 1/20 to 19/20 of Pixel per run*
Make Mask	*I-SOscbs* *GFPscbs*	*I-SOscbs* *GFPscbs* Mask	 One Bit Mask: values of "1" (white) and "0" (black)
ApplyMask	*I-SOscbs*	I-SOmk	Masked I-SO Image

	GFPscbs Mask	GFPmk Mask	Masked GFP Image
I-SO_GFP_Ratio	I-SOmk GFPmk Mas	I-SO_GFP_Ratio (and masked input images)	Pseudo-Colored I-SO/GFP Image
Photobleach Curves	GFPmk I-SOmk	Excel File (and masked input images)	