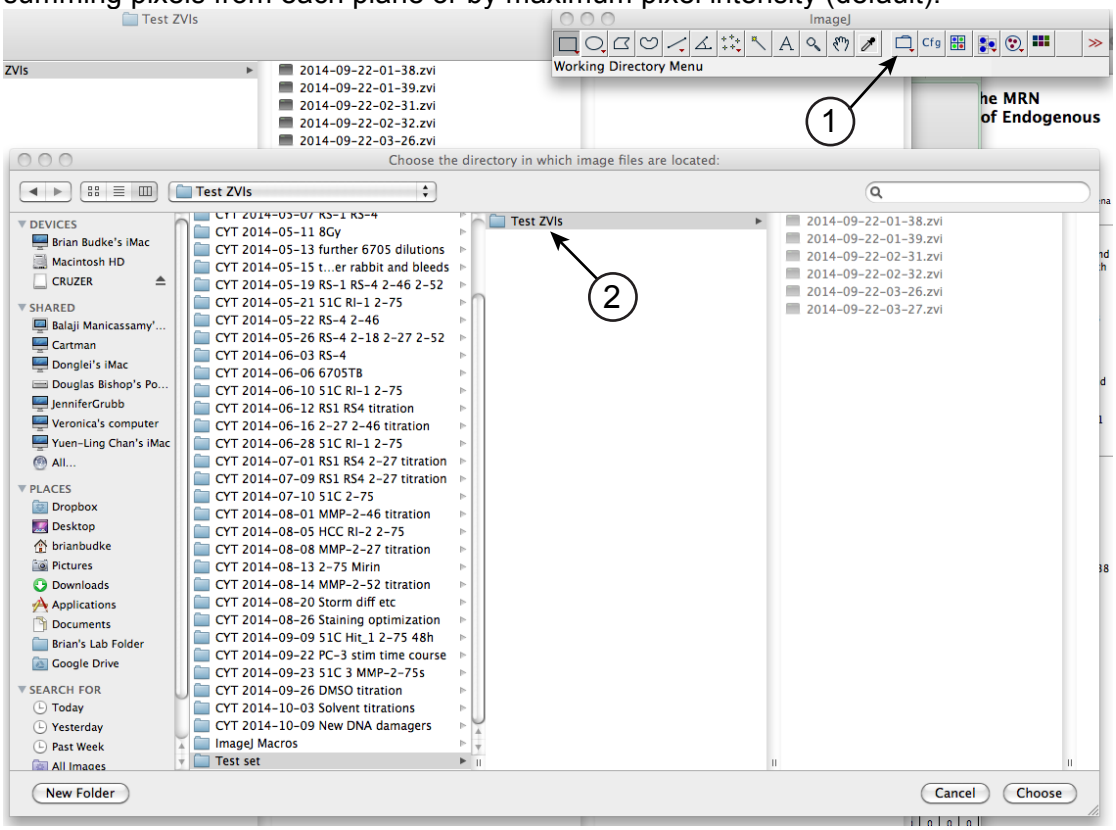
Documentation for
BB Macros: Cytology version 0.9
2014-10-13

Installation:

- Requires ImageJ 1.49g or later.
- Unpack 'BB Macros.zip' and place the 'BB Macros' folder into the 'plugins' folder located in the ImageJ program folder.

Using the macro set:

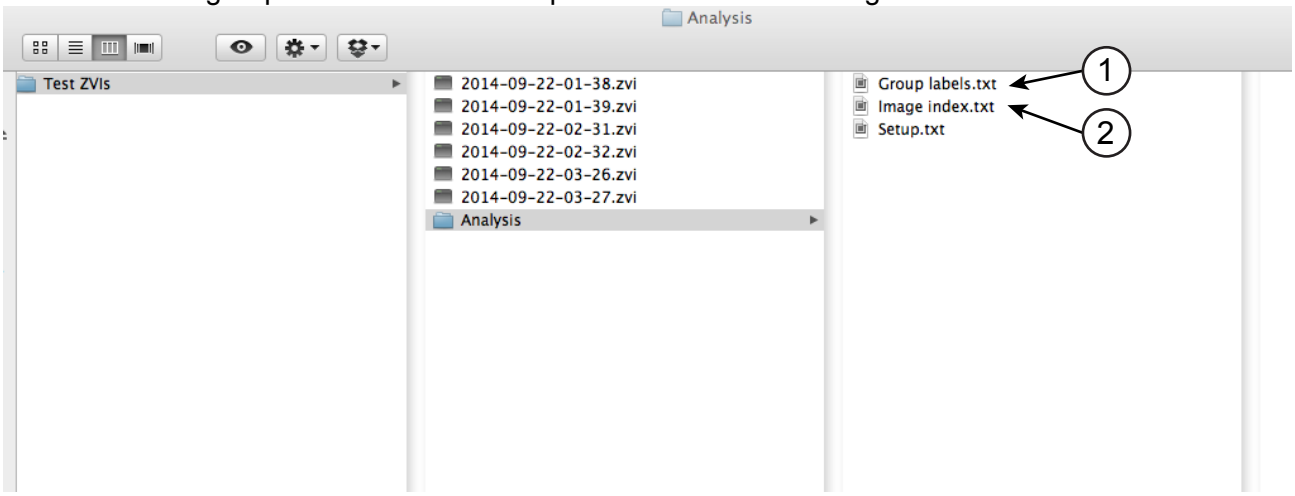
1. Initial setup
   1.1. Go to the 'Plugins' menu in ImageJ and find 'BB Macros'. Within this group, run the 'Cytology' macro to start up. (There is also another folder in 'BB Macros' called 'Cytology Modules'; these are components of the macro set that are run by the Cytology macro and are not intended to be run as stand-alone macros).
   1.2. The Cytology macro tool set should appear on the right-hand side of the ImageJ main menu. To start analyzing a new or previous set of pictures, click on the folder icon ("1" below) and select the folder containing the raw image files to analyzed. Currently, only .tif, Zeiss .zvi and Leica .lsm files are supported. If the folder contains multi-plane .lsm files, then the analysis images (the original data files are never modified) will be flattened to single-plane by either summing pixels from each plane or by maximum pixel intensity (default).



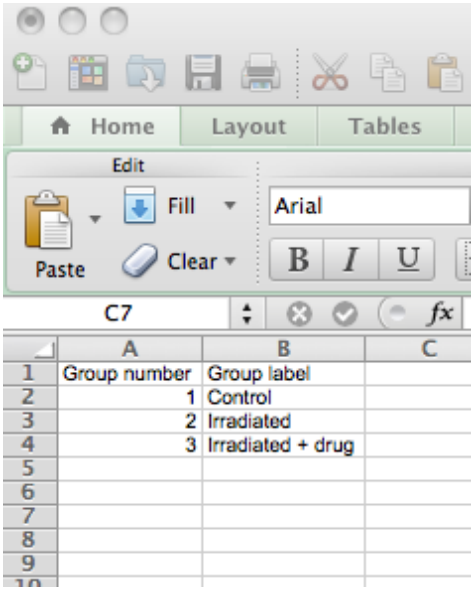
   1.3. Click the 'Cfg' button next to the folder icon to run the initial configuration. For first-time setup, a menu will appear in which the file type is selected (either .tif, .zvi., or .lsm files are supported). All image files must contain the same number of channels (blue, green, etc.). In the following dialog, descriptive labels are entered for each channel (i.e., RAD51, DAPI) and the 'Obs Unit/Submask Channel' is specified. The 'Obs Unit/Submask Channel' is the channel that will be used to delineate observational units, which are typically cells, nuclei, and spreads.

For nuclei and spreads, the Obs Unit/Submask channel is usually the channel that contains DAPI staining.

1.4. When the initial configuration is finished, an ‘Analysis’ directory is created in the raw images folder. The Analysis folder contains two tab-delimited text files that are used to index images. Both files can be easily edited using Excel or any other spreadsheet program, as long as they are saved as plain tab-delimited text files. The ‘Image index.txt’ file initially contains columns for the raw image filenames (no extension!) and an empty column in which group numbers must be entered. Each group typically corresponds to a set of images obtained from the same slide. The ‘Group labels.txt’ file initially contains empty columns for group numbers and group labels, which are usually descriptive names for each group. Both columns must be filled out with labels and group numbers that correspond to those in the Image Index.

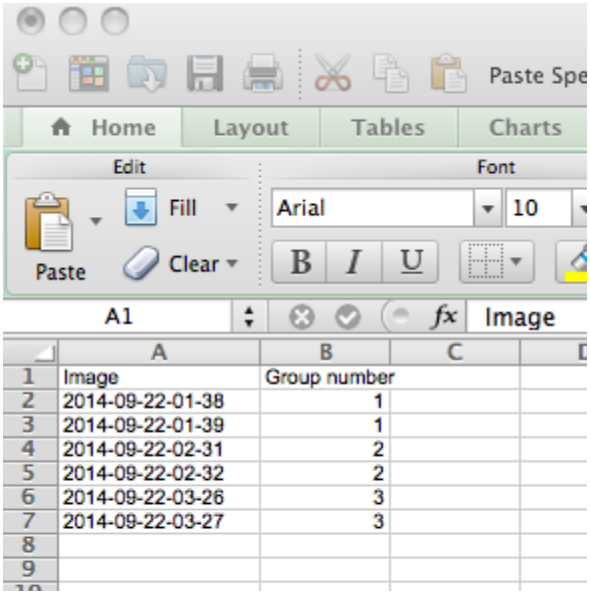


Group labels.txt



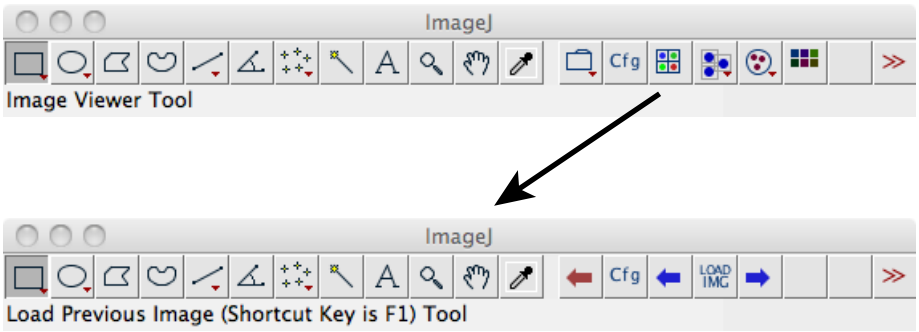
	A	B	C
1	Group number	Group label	
2	1	Control	
3	2	Irradiated	
4	3	Irradiated + drug	
5			
6			
7			
8			
9			

Image index.txt

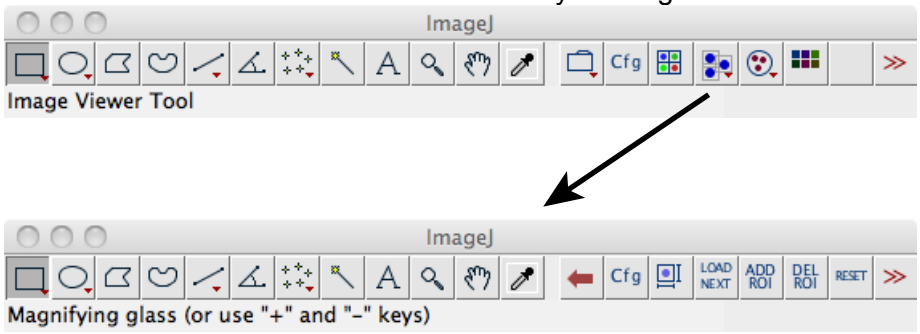


	A	B	C
1	Image	Group number	
2	2014-09-22-01-38	1	
3	2014-09-22-01-39	1	
4	2014-09-22-02-31	2	
5	2014-09-22-02-32	2	
6	2014-09-22-03-26	3	
7	2014-09-22-03-27	3	
8			
9			

2. Image viewer
   2.1. To open the image viewer, click on the ‘Image Viewer’ icon in the main tool set. This will bring up a new tool set for image viewing. You can return to the main tool set by clicking the red arrow to the left hand side.



   2.2. First, the image viewer configuration (‘Cfg’) must be run. At least one channel must have a display color that is not ‘Unused’.
   2.3. Blue arrows cycle forward and backward through images in the experiment directory and ‘Load Img’ allows you to go directly to a specific image.
3. Observational units
   3.1. Selecting observational units
      3.1.1. Click on the Observational Units Menu button and select ‘Select observational units’ from the drop-down menu. This will bring up a new tool set for selecting observational units. You can return to the main tool set by clicking the red arrow to the left hand side.



      3.1.2. An observational unit is defined as a square box encompassing a region of interest in an image. The first thing to do is to specify an appropriate box size that will nicely encompass the largest and smallest regions of interest. Clicking on the third icon in the tool set (just to the right of ‘Cfg’) will generate a montage of random sample images and a square selection in the upper-left corner of this montage. Drag the square selection around, resizing it so that it fits the largest region of interest, and then click ‘Ok’. The configuration (‘Cfg’) in this tool set contains settings for the montage generated in this step; these settings usually don’t need to be changed from their defaults.
      3.1.3. Click on ‘Load Next’ to start or continue selecting regions of interest. Each image in the data set is opened sequentially and observational units are added by moving the square box selection to regions of interest on each picture and clicking ‘Add ROI’ for each region of interest in the image. One or more ROIs can be added for each image, and ROIs that have been added are indicated by a green box in the image. If you make a mistake and need to delete the last ROI added, click ‘Del ROI’. Hitting ‘Reset’ deletes all the ROIs in the current image and reloads that image. You can exit from this program at any time and return to it later to continue. When going through many images, it is helpful to use the shortcut keys F1 and F2 to add ROIs and advance to the next image, respectively.
      3.1.4. Once all the images have ROI .zip files associated with them, a message will appear in the status bar of the ImageJ main window indicating that there are no more ROIs to add. Click the red arrow at the left of the tool set to return to the main menu.

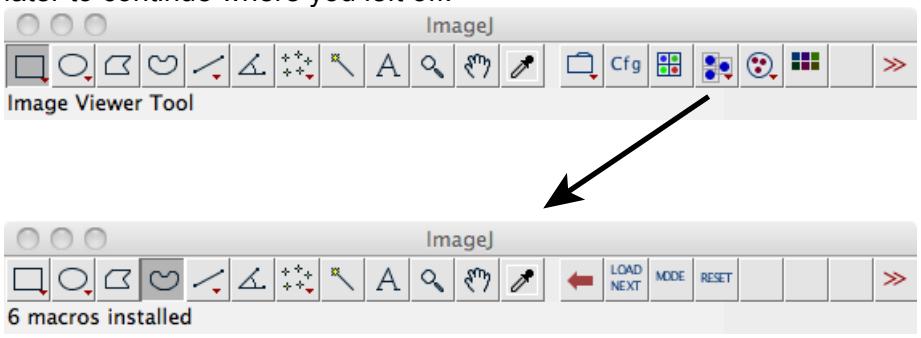
3.2. Global masking

3.2.1. Click on the Observational Units Menu button and select ‘Add global background masks’ from the drop-down menu. This automatically creates masks based on the staining intensity in the Obs Unit/Submask channel and may take a few minutes. This step is required to define submasks (below).

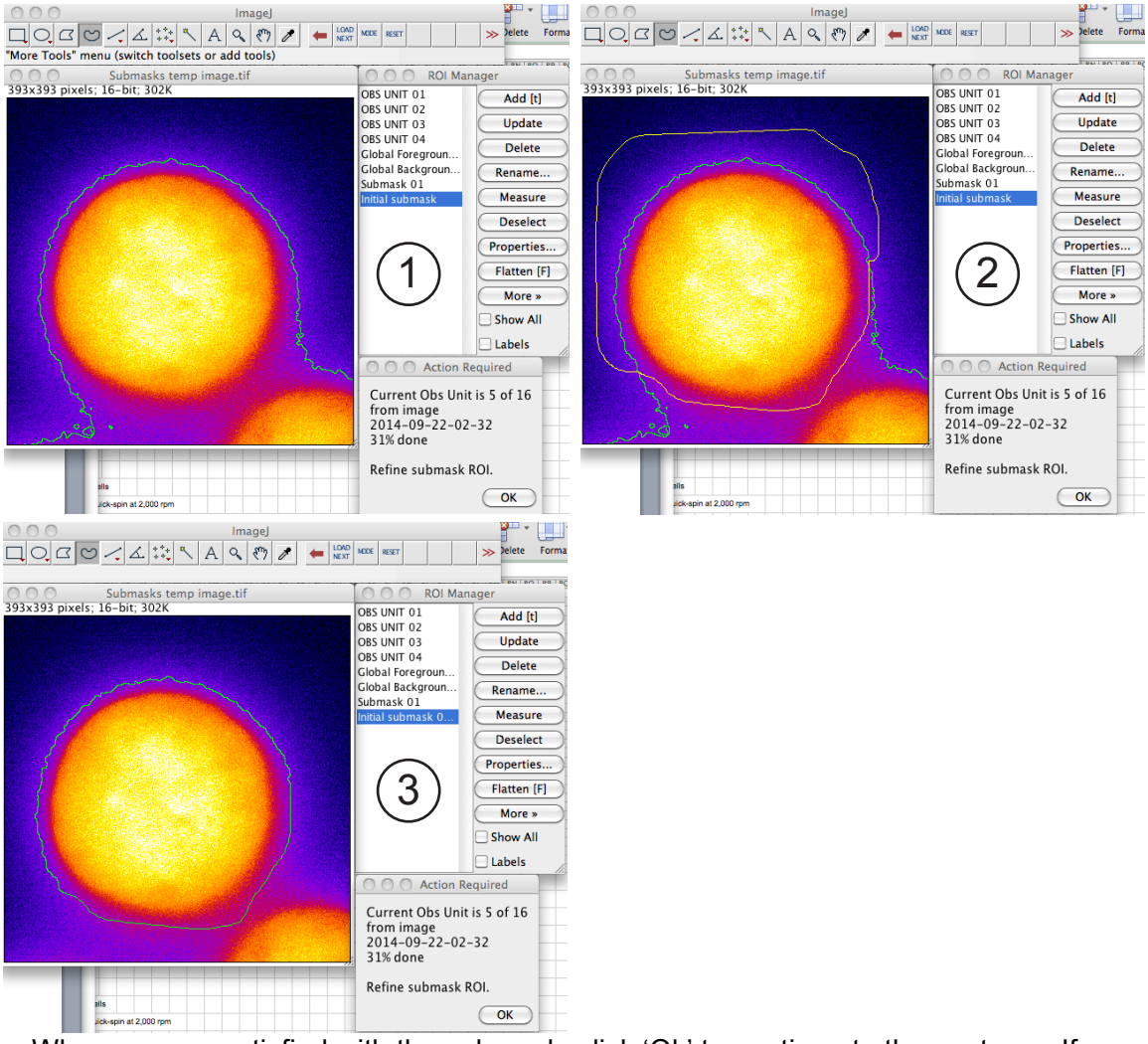
3.3. Submasks

3.3.1. The purpose of making a submask is precisely define the region of interest in an observational unit (i.e., the boundary of a single nucleus) so that downstream analyses such as staining intensity and focus counts are restricted to that area of the observational unit, rather than to the entire square area of the observational unit frame that was first selected.

3.3.2. Click on the Observational Units Menu button and select ‘Add submasks’ from the drop-down menu. This will bring up a new tool set for selecting observational units. You can return to the main tool set by clicking the red arrow to the left hand side. As with selecting observational units (Section 3.1), you can leave this program at any point and return to it later to continue where you left off.



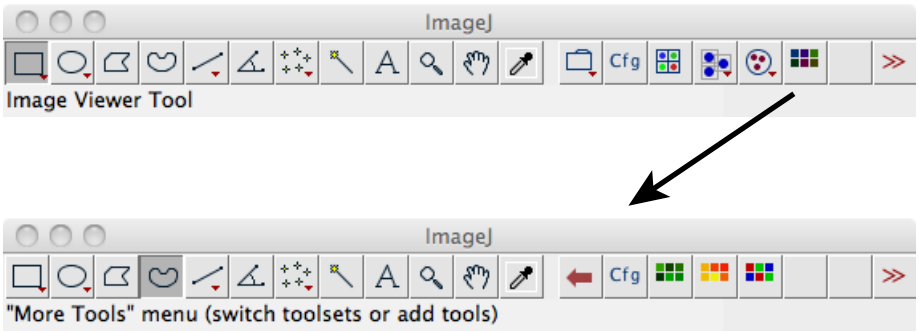
3.3.3. Click on ‘Load next’ to start or continue defining submasks. Each observational unit frame that was previously selected in Section 3.1 will be displayed with an initial submask ROI that is created in Section 3.2 based on the counterstain intensity (i.e., DAPI). This initial submask can be modified. Usually, this is not necessary with observational unit frames that contain a single intensely-stained body. If the observational unit frame contains many intensely-stained areas that are initially in the submask ROI (i.e., nuclei that are right next to each other), then it is necessary to manually refine the submask ROI. This is done using ImageJ’s freehand selection tool to draw around the single region of interest that should be outlined (see below). When you click ‘OK’, the resulting submask will only outline the area that is covered by the initial submask AND the manually drawn selection, so there is no need to be precise with the manual selection around boundaries that are already well-defined by the initial submask. In the rare case that you need to expand the submask boundary, click ‘Mode’ to switch between ‘Crop’ and ‘Expand’; when you click ‘Ok’ while ‘Mode’ is set to ‘Expand’, the submask will expand to include the outline that was just manually drawn. Remember to click on ‘Mode’ to set it back to ‘Crop’ when you are finished. Currently, only a single contiguous selection can be used as a submask.



3.3.4. When you are satisfied with the submask, click ‘Ok’ to continue to the next one. If you mess up too badly and want to start over on the current submask, click ‘Reset’. This only works before you click ‘Ok’ for the last time.

4. Focus counting
    4.1. Select channel to be measured
        4.1.1. Click the ‘Focus Counter Menu’ button to bring up the focus counter menu and select ‘Change channel to be measured’ to select the channel where you want to count foci. Multiple channels can be analyzed separately. Results are stored in separate folders under the ‘Analysis’ folder in the main image directory.
    4.2. Select calibration images
        4.2.1. Next, choose ‘Select calibration images’ from the focus counter menu to pick a few images that you will use to calibrate the focus counter. Ideally, the calibration images should contain at least one positive control image and one negative control image for focus counting (i.e., images of RAD51 staining from irradiated and unirradiated cells).
        4.2.2. Select a raw image file from the list. A heatmap image of the selected image for the selected channel will be displayed with an overlay showing the positions of the observational units within the image. Select an observational unit from the top list and click ‘Ok’ to add the selected area as a calibration image. Alternatively, you can select ‘Go to another image’ from the bottom list. If you hit ‘Ok’ while this is selected, this will open another raw image that you select without adding the selected observational unit as a calibration image. When you are done specifying calibration images, select ‘Finish and close’ from the bottom list to exit; this command only exits the program and does not add the currently selected observational unit as a calibration image.
    4.3. Calibrate focus counter
        4.3.1. Choose ‘Calibrate focus counter’ from the focus counter menu to begin optimizing the settings for determining foci. Each of the calibration images will be loaded in the order that they were selected in Section 4.2.
        4.3.2. The program will display graphical and numerical output from the first results of the focus counting using the default settings. Usually, the default settings provide a good starting point for Zeiss single-plane images, but are way too high for Leica confocal images.
        4.3.3. Adjust the parameters until the focus counting results look right. Sometimes, it is helpful to select ‘Inspect images’ to examine pixel values in the ‘Background subtracted.tif’ image in order to guide the manual optimization of focus counting parameters. It is also helpful to look at the ‘Thresholded.tif’ and ‘Foci mask.tif’ images. If there are not enough white spots appearing in the ‘Thresholded.tif’ image, then reduce the ‘Lower threshold value’ until a reasonable amount of foci appear in this image. If there are lots of grayed-out spots in the ‘Foci mask.tif’ image, then reduce the four values below ‘Lower threshold value’ until real foci appear as white spots in this image; these will also appear as outlines in the ‘Original image overlay.tif’ window. Hit ‘Ok’ to re-count foci in the calibration image using the new settings. Hitting ‘Ok’ will advance to the next calibration image if the settings remain unchanged.
    4.4. Batch focus counting
        4.4.1. Once the focus counter has been calibrated, select ‘Count foci and organize data’ from the focus counter menu. This will count foci in the selected channel for all images that have observational unit ROI files associated with them. This process can take a while, up to 1 minute per 10 observational units on slower computers, and can use a lot of memory. If you get an out-of-memory error before the batch run is finished, go into Edit -> Options -> Memory & Threads… in the ImageJ main menu and increase the amount of memory allocated to ImageJ.
        4.4.2. The output is deposited into a folder for the selected channel under the Analysis folder in the main image directory. The data are in plain text tab-delimited files that can be opened directly in Kaleidagraph.

5. Montages
   5.1. To open the auto-montager, click on the ‘Auto Montage Tool’ icon in the main tool set. This will bring up a new tool set for making montages. You can return to the main tool set by clicking the red arrow to the left hand side.



   5.2. Hitting ‘Cfg’ brings up the configuration window for montages. Here you can select the channel to show for single-channel montages, specify the width and height in panels, randomize panels, and enter the maximum overlap between panels.
   5.3. Montages can be automatically generated by clicking either of the three montage icons to the right of ‘Cfg’. The first two generate single-channel monochrome or heat map montages, respectively. The right-most icon generates RGB composite montages; for this to work, there must be at least one channel that is not marked as ‘Unused’ in the Image Viewer settings (Section 2.2). The brightness and contrast of the auto-generated montages can be adjusted in the Image Viewer settings by playing with the Min and Max display values; it is helpful to optimize these settings before running the auto-montager since each montage can take half a minute or more to generate, depending on the speed of your computer and the number of observational units in each image group. The montages are added to the ‘Montages’ subfolder under the Analysis folder in the main image directory.