AntWeb.org's
Imaging Manual for LAS 3.8

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AntLab Setup

From left to right: **Leica Z6 APO** (No : 10447174), **Objective 2.0x Apo, Z6/Z16, f = 39 mm** (No: 10447178), **Leica Z16 APO** (No: 10447173), **Objective 0.5x Apo, Z6/Z16,**

f = 187 mm (No: 10447177), **Objective 1.0x Apo, Z6/Z16, f = 97 mm** (No: 10447176). Note that the objectives include a plastic spacer to fit the LED Dome light.

Equipment used at AntLab:

Gliding stage 120 mm
No : 10446301

Leica Z6 APO
No : 10447174

Leica Z16 APO
No : 10447173

A-tube for Z6 APO/ Z16 APO
No : 10447128

Video Objective 0.63x
No : 10447367

Objective 0.5x Apo, Z6/Z16, f = 187 mm
No : 10447177

Objective 1.0x Apo, Z6/Z16, f = 97 mm
No : 10447176

Objective 2.0x Apo, Z6/Z16, f = 39 mm
No : 10447178

Inc. light base, large w. AntiShock feet
No : 10450049

1000 Leica LED5000 HDI Dome Illuminator
No : 10450062

1200 Handwheel-Motor Focus for M-Series
No : 10450298

1300 Motor Focus drive long 620 mm
No : 10450503

1400 Carrier for S-Series on M-Series Column
No : 10450106

1600 Power cable, 2 m, USA
No : 10445661

1700 LAS Montage MultiFocus
No : 12730070

1800 LAS Hardware USB Dongle
No : 12730205

Leica DFC450, 5 MPixel, Firewire-b
2000 Leica DFC450 Digital Camera & SW Kit
No : 12730411

Before you Begin

Before you begin imaging, confirm the following LAS settings:

LAS Setup:

1. Double-click the LAS v3.8 icon on desktop
2. Confirm hardware setup under Setup tab
   - Optics carrier: Z6APO or Z16APO (see below for instructions on toggling between the two)
   - Eyepiece: 10x/23B
   - Tube: tube
   - Camera adapter: 0.63x (2/3") **#10447367** (not to be confused with the other 0.63x adaptor)
   - Main objective: 0.5x, 1.0x, or 2.0x (depending on which objective is in use)

If you are changing to a different optics carrier (e.g. from the Z6APO to the Z16APO), see the following instructions on how to select the hardware configuration. If you are not making any changes, skip to the next step.

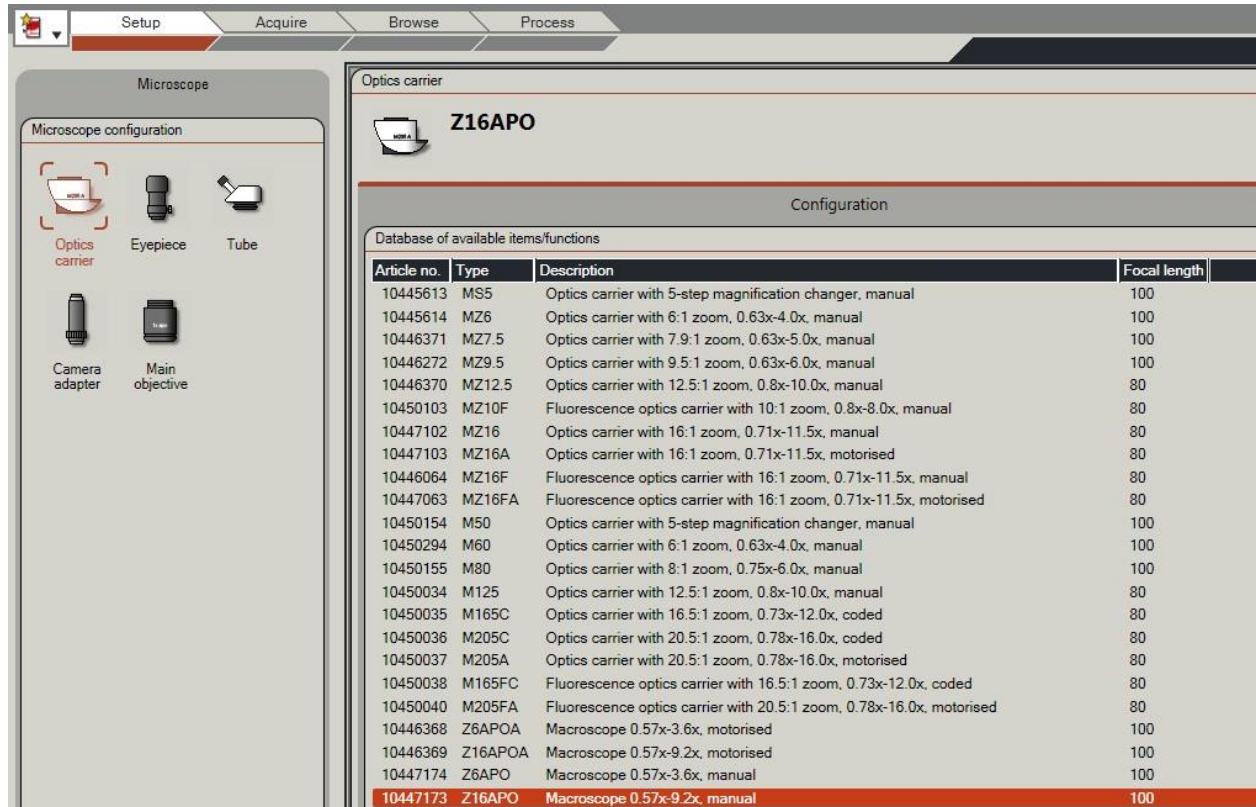## Changing the Optics Carrier:

1. Close LAS
2. Shift + Double click LAS v3.8 icon on the desktop
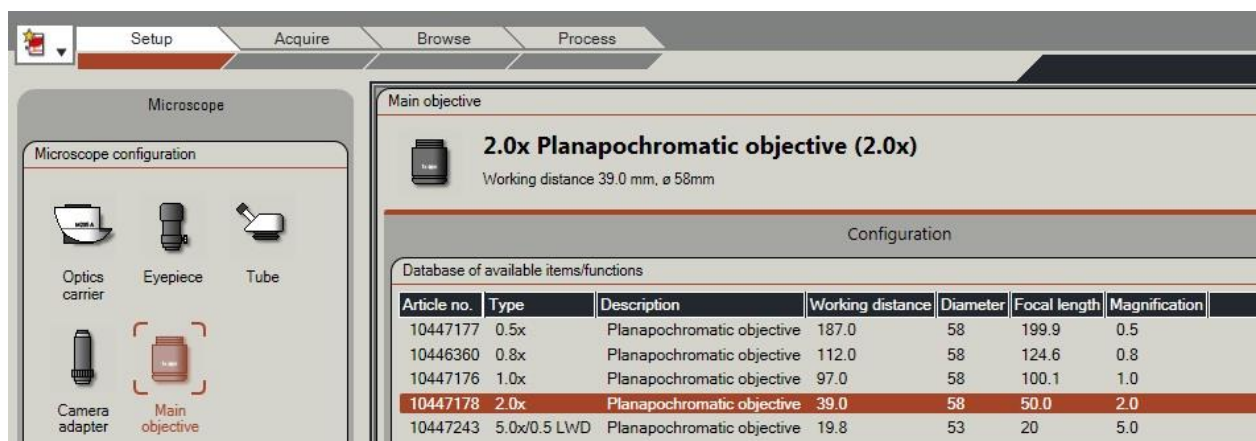3. Click Options-->Select Hardware Configuration

4. Click Leica Application Suite-->LAS Core to open software
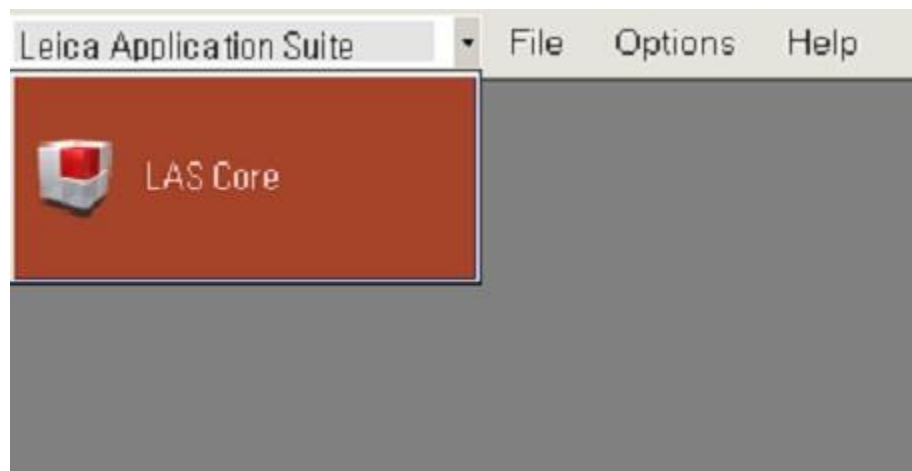
5. Click on Setup-->Microscope

6. Optics carrier: Choose the appropriate microscope lens (at AntLab, Z16APO or Z6APO)
7. Main Objective: Choose the appropriate objective (0.5x, 1.0x or 2.0x)

Note: Each time you change the optics carrier, you must exit the program and follow the above procedure.

Setting the Capture Location:

You will need to choose a capture location for the image stacks under
New Volume (D)-->Ant Images.
Go to the Browse tab-->Navigator. Select the fifth icon from the left, Create a 'New Folder.'
Name the folder with your name and the word "capture", as in "Brian Fisher capture".
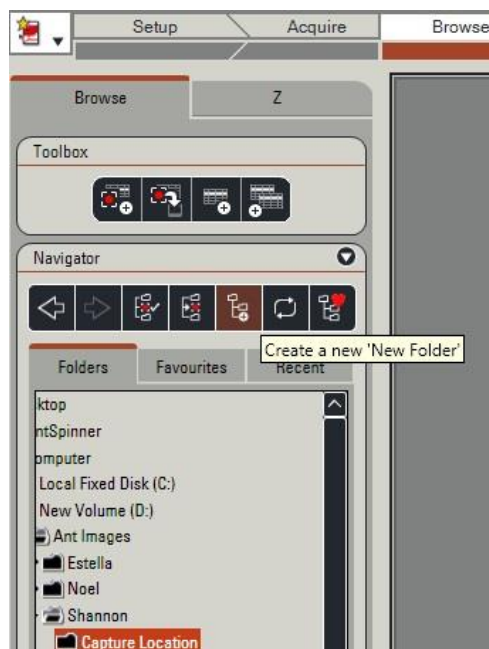
Click once on this designated capture folder to highlight it. Now, select the third icon from the left, "Set capture location." This folder is now the default capture location for all of the images you collect on LAS.
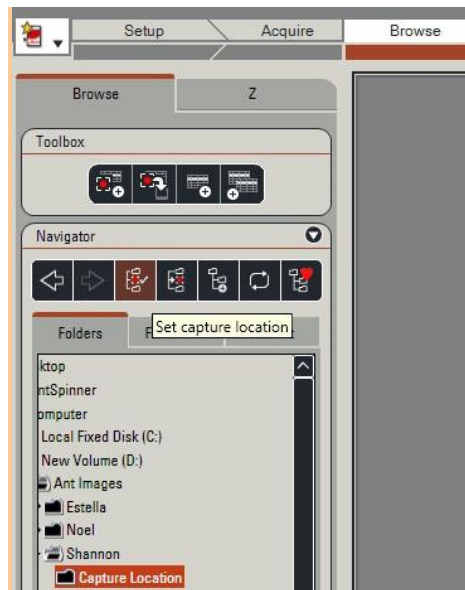
Setting Options:

1. Acquire-->Z Tab-->Options
2. Configuration: Last Used
3. Check the following boxes:
    - "Create Multifocus after stack acquire"
    - "Save sub-images"
    - "Align images before combining"
    - Do NOT check "Perform manual focus"
4. Select 500 or 1000 as the "ms Wait before acquire"

Image Format Settings:

Acquire-->Camera-->Image Formats

1. Captured image: Full Frame HQ. (Click on the Captured Format box to select show all modes)
2. Live format: 2x2 Color Binning
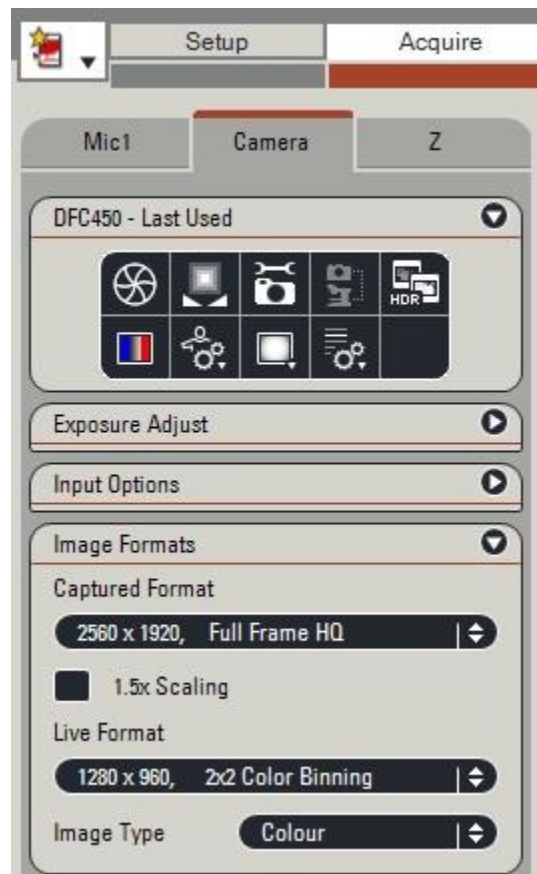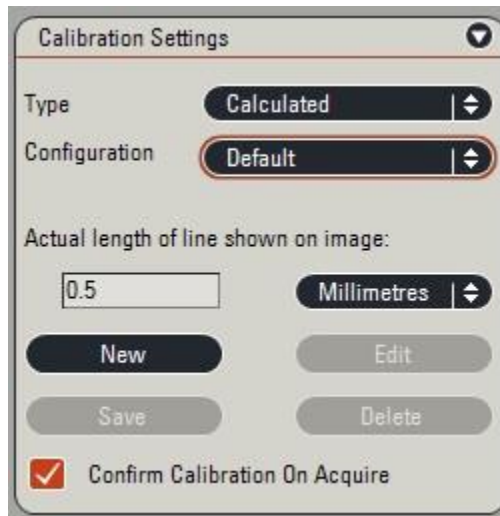
Calibration Settings:

Acquire-->Camera-->Calibration Settings

Type: Calculated
Configuration: Default

Confirm Calibration on Acquire: Ensure this box is checked or you will NOT be able to calibrate your images.

Gain, Saturation and Gamma:

Acquire-->Camera-->Exposure Adjust

> Gain: 1.0x
> Saturation: 0.65
> Gamma: 0.60

Instructions to determine the correct exposure follow.
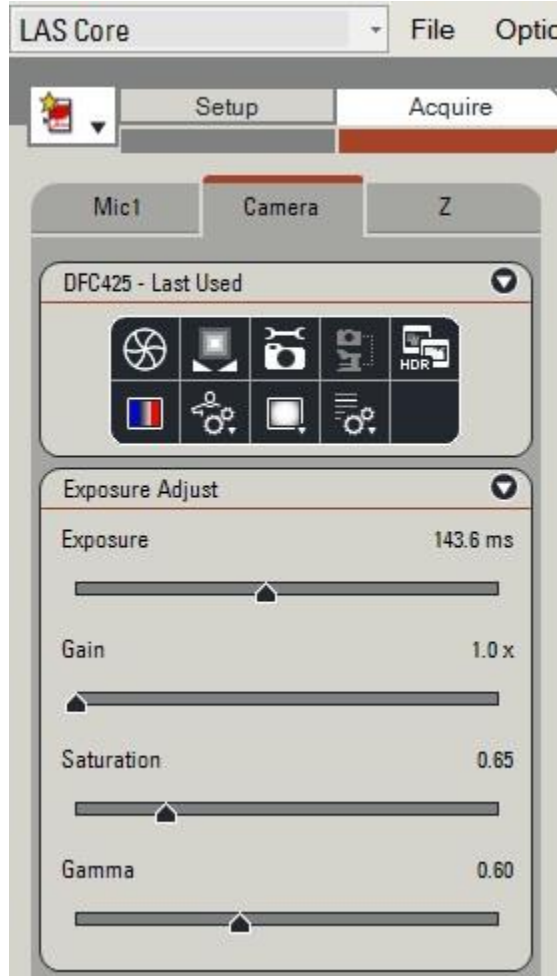
Note: These are the settings that work for our system. Leica states the default settings should be:

See which settings work best on your system by checking the true specimen color under a microscope.

Increasing the Gain can lead to grainy images, while increasing the Saturation can add too much color to the specimen.

White Balance:

Acquire-->Camera-->DFC425 – Last Used

Place a white sheet of paper on the microscope stage just as you would place a specimen. Increase or decrease the exposure to the appropriate level (See "Setting Exposure). Select the second icon from left on top (see screenshot below) which will set the automatic white balance. If the image is overexposed, the white balance cannot be captured. Reduce the exposure and try again.
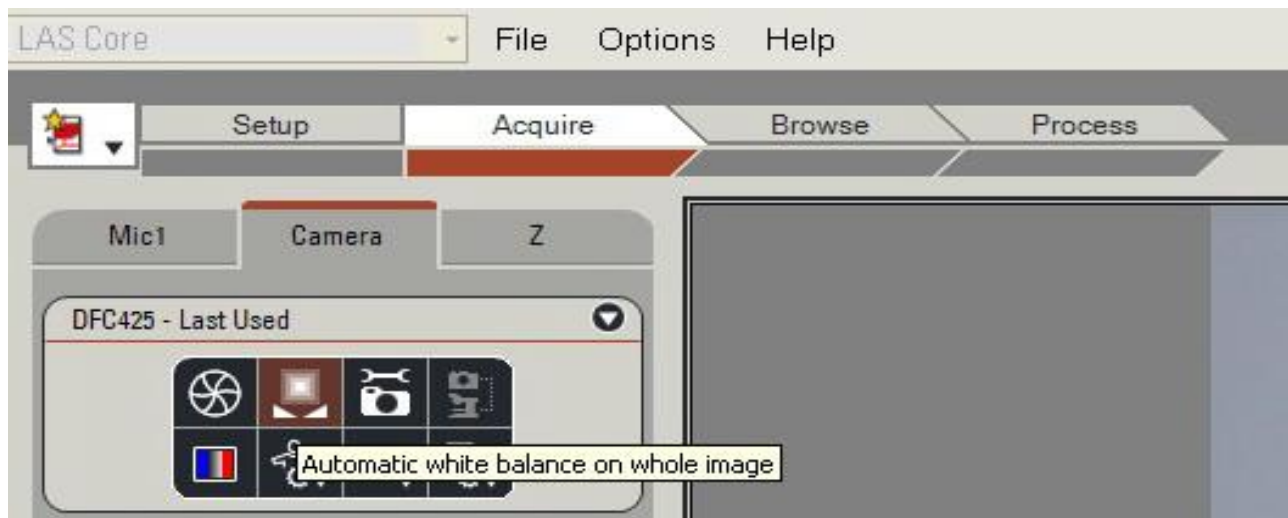
Note: If lighting conditions are kept constant, it should not be necessary to reset the white balance frequently. However, switching off the top or bottom dome lights or substantially changing the brightness setting on the dome may require more frequent use of the white balance. Leaving the dome source with both top and bottom LEDs at maximum brightness, and adjusting the exposure entirely within LAS, will minimize the need to reset white balance, keeping the dome source as the dominant light source.

Choosing the Appropriate Microscope Configuration:

AntLab uses the Leica DFC425 camera with multiple microscope lenses and objective configurations. The lenses and configurations used depend on the size of the specimen.

There are two microscope lenses, the Z6 and the Z16, and three objectives, the 0.5X, 1X, and 2X. Generally, the Z6 is used for larger ants and the Z16 for smaller ants. The 2x objective produces the best images; use this whenever possible.

Before imaging, you must determine which microscope configuration to use. Use the following as a guideline:

Z16 + 2X: 1 mm – 3 mm (e.g. *Solenopsis*, *Tetramorium*, and *Carebara*)
Z6 + 2X: 3 mm – 5 mm (e.g *Cerapachys*, *Hypoponera*)
Z6 + 1X: 5 mm – 13 mm (e.g. *Camponotus*)
Z6 + 0.5X: 13 mm+ (e.g *Dinoponera*)

See "Changing the Microscope Lens and Objective" for instructions on how to change configurations.

Acquiring Images_____

Imaging will be improved with the use of a grey card (60%) as a backdrop for specimens. Using black pins and black or grey points will also reduce the incidence of overexposure, high contrast, and unnecessary background information.

To begin imaging:

1. Click on the Acquire Tab
2. Turn on the dome lights on the microscope
3. Open the aperture to 5 for ease of focus
4. Increase the exposure until you can see the specimen well enough to position it and set the focus field

It is not important to obtain the proper exposure at this point. For now, focus on positioning and framing the specimen.

Positioning Specimens:

Dorsal (_D):
Think of the thorax as a box, and focus on it alone, ignoring the position of the head and the gaster. Make sure it is not tilted too far forward or backward, and that the top plane of focus is in the middle. Can you see the same amount of coxae on either side of the ant? If not, adjust until both sides appear equal.
AntWeb example:
http://www.antweb.org/bigPicture.do?name=casent0235489&shot=d&number=1

Profile (_P):
Think of the thorax as a box, and focus on it alone, ignoring the position of the head and gaster. First, look at the two hind coxae and make sure that they are in a horizontal line.

Next, look at the top and bottom of the thorax and make sure you have a dead-on lateral view (you should not see the underside of the ant). Finally, look at the front and rear of the thorax to make sure both are in the same plane and that the thorax is not spun towards or away from you. If there are spines present, they should be aligned.
AntWeb example:
http://antweb/bigPicture.do?name=casent0235489&shot=p&number=1

Head (_H):
Think of the head as a box. Make sure the top of the head and the clypeus are in the same plane of focus. Open Profile image in ACDSee and look at this, drawing a line

through the side of the head so that you get an idea where the top of the head that you would focus in on is. The sides of the face should be even. If eyes are present, both should come into focus at the same time and you should see the same amount of each eye on each side of the head. If there are frontal lobes, make sure that they both come into focus at the same time. Use the line tool for the scale bar in LAS software to draw a straight line across the top of the eyes to see if they line up. Right-click the mouse while in Acquire mode to access the line tool. Next, check straight down the center of the face to see if you need to rotate the specimen clockwise or counter-clockwise. Several markers on the head can be used to ensure everything is correct and symmetrical.

AntWeb example:
http://www.antweb.org/bigPicture.do?name=casent0172174&shot=h&number=1

Positioning the head can be difficult. Consult AntWeb or "Bolton's Catalogue of Ants of the World" if you have questions about positioning.

Wings (P_2):
From profile position, zoom in on the wings. Generally, you can get a great wing image from this position. However, there will be times when taking the wing image works better from the dorsal position.

AntWeb example:
http://www.antweb.org/bigPicture.do?name=casent0082732&shot=p&number=2

Male genitalia (P_3):
From the profile position, turn the specimen so that its genitalia are coming towards the camera at a 45 degree angle. The gaster should be in a horizontal line, and if possible, the paramere should also line up horizontally. Please refer to examples in AntWeb. It may be necessary to run your LAS stacks through an external processor such as Zerene to get a clear, detailed image.

AntWeb example:
http://www.antweb.org/bigPicture.do?name=casent0082732&shot=p&number=3

Framing Specimens:

Keep a half-inch to one inch margin of buffer space around the specimen. Cropping too closely can cut off hairs and stingers and result in the overall image appearing cramped. All parts of the specimen within the frame should be in focus, especially within the profile and dorsal shots.

Exceptions:
Only the head of the specimen need be in focus within the head shot.
Only the wings of the specimen need be in focus within the wing shot.
Only the male genitalia need be in focus within the male genitalia shot.

Framing Tips:

- **Dorsal:** Do not worry about including both antennae and all of the legs when framing the dorsal shot, especially if the antennae or legs are extremely long. Frame the specimen including all hairs on/around the head and the whole gaster including stinger when present.
- **Profile:** Frame the specimen including at least one full leg and one full antenna in the profile shot. Do not worry about including both antennae and all of the legs when framing, especially if the antennae or legs are extremely long. All visible hairs should be in focus. Petiole should be in focus, especially if not obscured by the specimen's legs.
- **Head:** If you were unable to capture a full antenna in the profile shot, include one or both antennae in the head shot. Include the palps, if they are visible. The mandibular teeth should be in sharp focus. Do not cut off mandible hairs or hairs on the top of the head while framing the specimen.
- **Wings:** Ensure that wing venation is clearly visible.

Setting the Focus Field and Number of Steps:

Once you have your specimen properly positioned and framed, set the focus field.
1. Click Acquire-->Z-->Define Stack to set the stack parameters.
2. Focus up (turn knob counter-clockwise) until the topmost part of the specimen begins to come into focus.
3. Double click left arrow "Start" to set the first image. A red arrow means the start point is set.
4. Focus down (turn knob clockwise) until the ant is no longer in focus, then focus up until the bottom of the specimen begins to come into focus.
5. Double click right arrow "End" to set the last image. A red arrow means the end point is set.
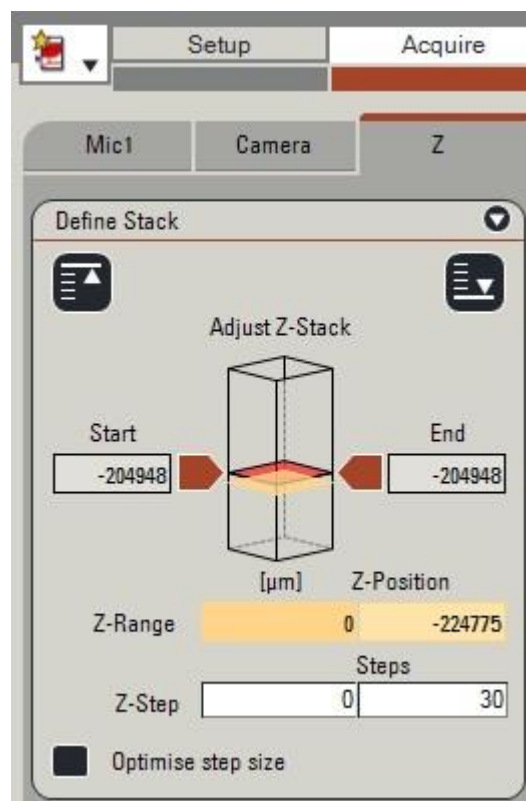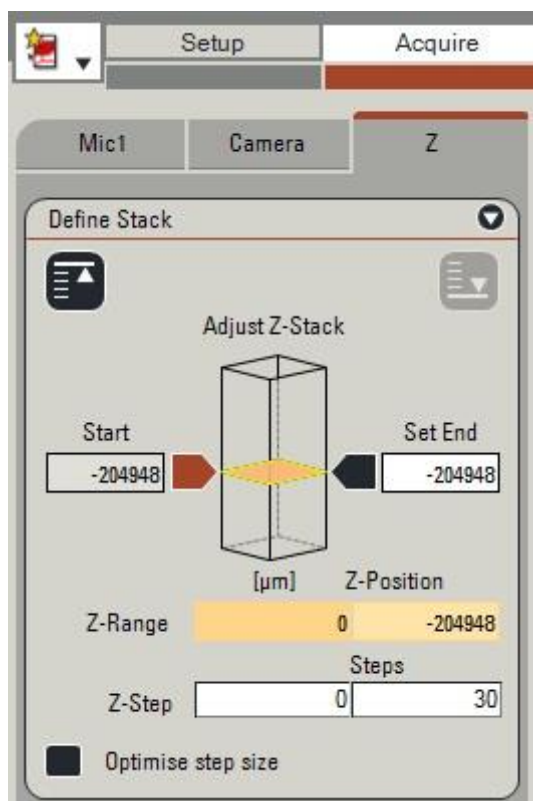
Closing Down the Aperture (Not required):

The aperture ring is located **on the optics carrier**. In the AntLab, the settings range from 1-5. Lowering the aperture setting reduces the chances of your final image having a "halo" or other distortions where the specimen and the background meet. These problems occur most often when imaging small or shiny specimens. You may be able to image your specimen with the aperture set at 4 or 5. These settings are ideal and provide the highest quality images. If necessary, lower the aperture setting **on the optics carrier** as low as 2.5.

Note: Lowering the aperture can result in a loss of detail in your image.
The reason for lowering the aperture **after** setting the focal parameters is that it can be difficult to set the focus at the lower aperture setting. Be careful not to disturb the specimen at this stage, as the focal parameters, framing, and positioning have already been set.

Setting the Exposure:

Keep the light dome fully lit and click under Acquire-->Camera-->Exposure Adjust to change the exposure level.
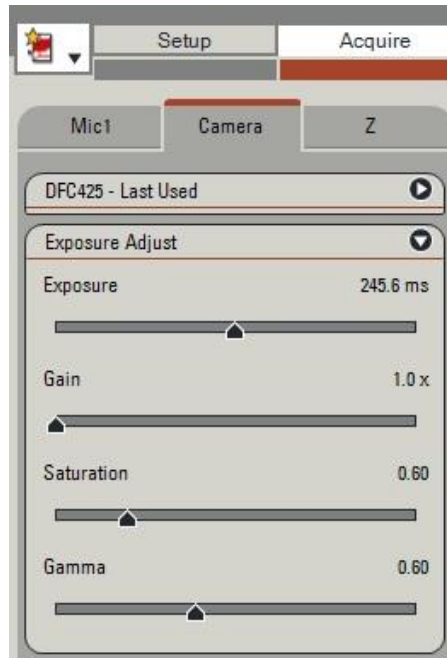
To determine the proper exposure, pay attention to the brightest and darkest portions of your specimen. The antennae, eyes, hairs, and legs of many of the specimens tend to get overexposed, resulting in a loss of detail in these areas. Focus on these areas while setting your exposure, but be careful not to underexpose the main body segments in the process. You will have an opportunity to adjust white/black levels during image processing, but it is essential to correct lighting before you take the image.

There are two tools for helping determine the proper exposure settings:

1. "Show under/over exposure in the viewer"
   - Acquire-->Camera-->DFC425 – Last Used
   - Select the icon on the bottom left. Once enabled, this tool will show underexposed pixels in the image as blue and overexposed pixels as red.

Ideally, neither red nor blue pixels should be present when the exposure is correct. But, if this cannot be avoided (i.e. you are imaging a specimen with high contrast features) it is better to underexpose some portions of the body while avoiding overexposure all together.
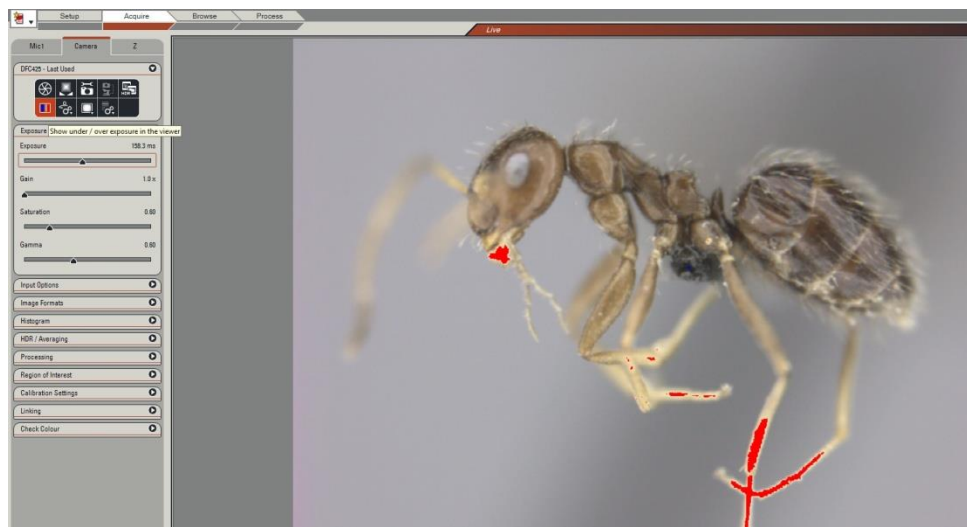
2. "Histogram"
- Acquire-->Camera-->Histogram
  Right-click on the Histogram box and select "Histogram"
- Options-->Average Only
  Ideally, the histogram should have a plateau shape, indicating that most of the information is being captured. Spikes in the histogram indicate a loss of information. Increase or decrease your exposure to correct for spikes.

Note: The histogram is best used in conjunction with the Average Only tool. Often the over/underexposure tool will leave you with a wide acceptable range of exposure. Use the histogram to narrow this range to a more ideal level of exposure. To do this, lower the exposure until there are no more overexposed (red) pixels. Continue to lower the exposure until the spikes in the histogram diminish somewhat. Be careful not to underexpose the image in the process.
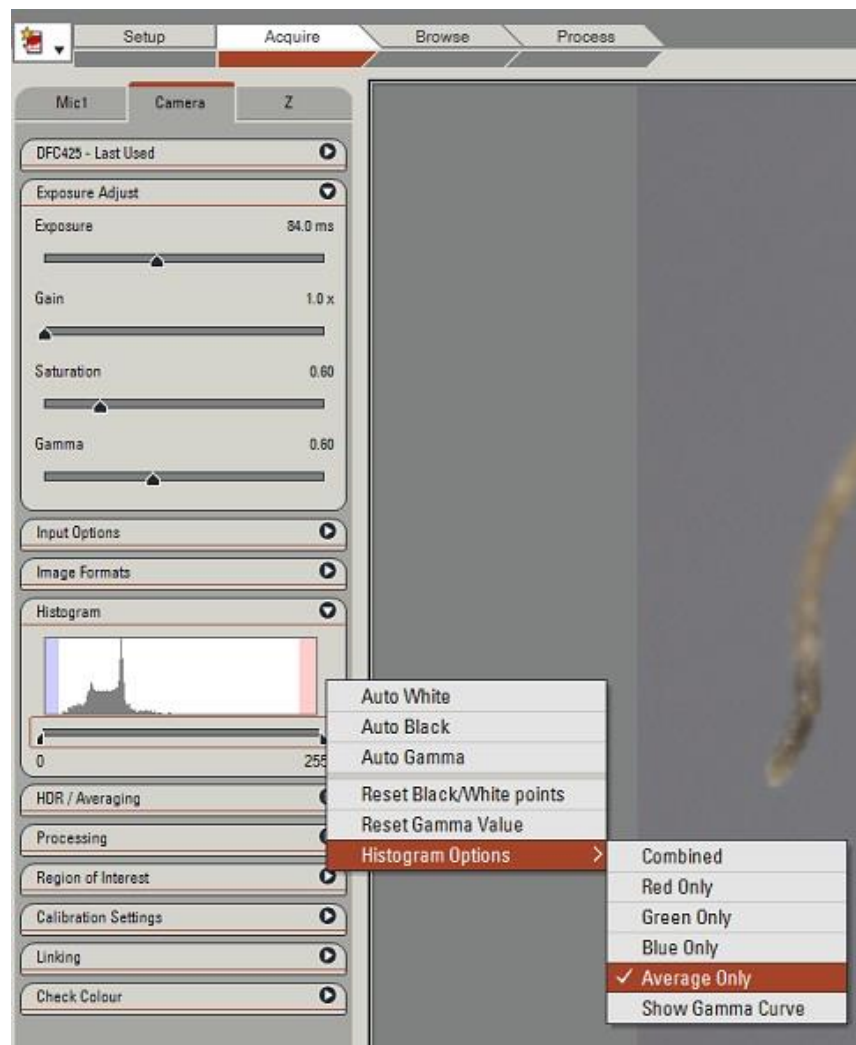
Example of overexposure:

Example of proper exposure:

HDR:

In cases where the specimen has extremely high contrast features (i.e. a dark black ant with bright white hairs or black ant on a white point), the HDR function may be needed.

1. From Acquire-->Camera-->DFC425 – Last Used
2. Right click on the HDR icon and choose HDR from the drop-down menu
3. From Acquire-->Camera-->HDR/Averaging, select "Automatic" or uncheck this box to manually adjust and experiment with contrast and brightness settings

For more information about using HDR and averaging, see LAS help.

Note: Using HDR will significantly increase capture times (by as much as five-fold, possibly more); use only when absolutely necessary.
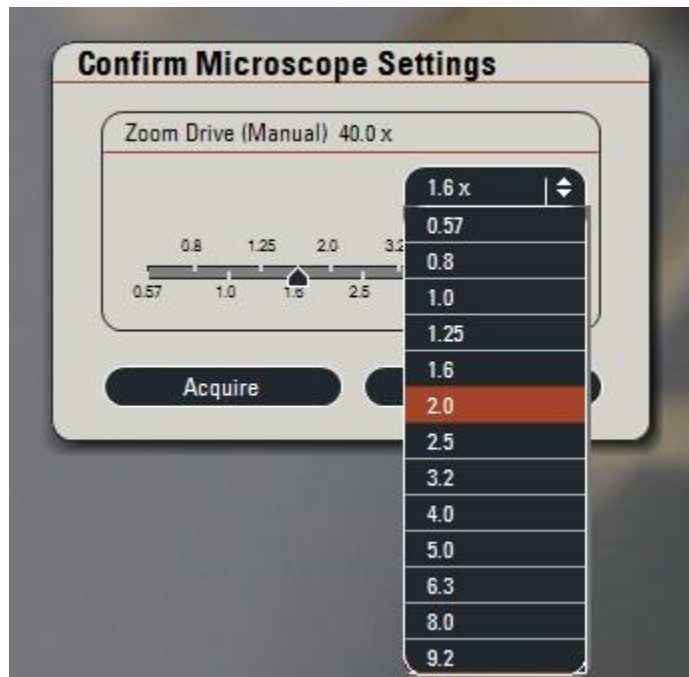
Acquiring the Multifocus Image

Return to Acquire-->Z-->Define Stack. The focal parameters that you set earlier are still saved here. Now that the aperture has been lowered and exposure has been set, the image is ready to be captured. Follow these instructions:

1. Define the number of Steps (images) you want to take. Suggested starting points:
   - Dorsal: 30 steps
   - Profile: 30 steps
   - Head: 20 steps
   - Wings: 20 steps
   - Male genitalia: 20 steps
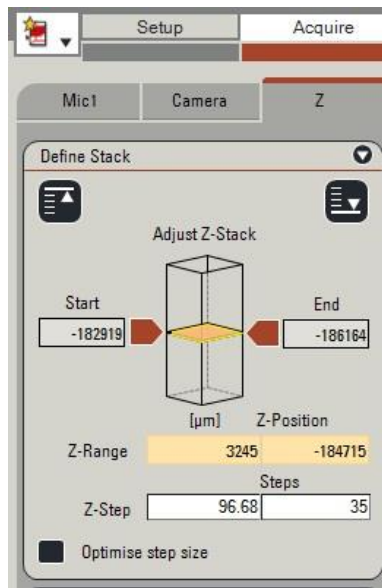
2. Double check that your "start" and "end" levels are set and that the number of steps is correct. Make sure "Optimise step size" is not checked.
3. It is helpful to let the system pause between the capture of each image so that vibrations from the movement of the motor column will not blur the images. Proper settings will vary and are dependent upon the setup you are using. Try a 500 ms or 1000 ms "wait before acquire"
4. Click Acquire Multifocus
5. The "Confirm Microscope Settings" window will pop up. Select your magnification from the sliding bar or by right-clicking on the box in the upper right corner.

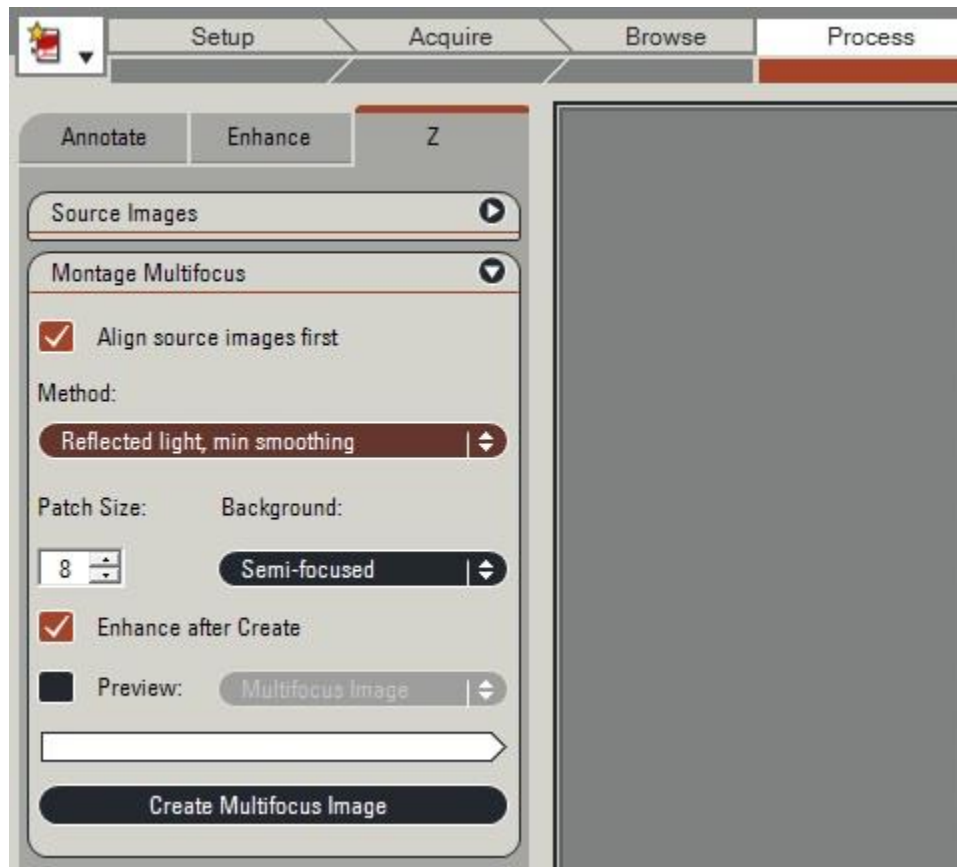6. Click "Acquire" to begin the capture process.

Processing the Image

Process Settings:

1. "Montage Multifocus"
   After imaging a specimen, change the method under Process-->
   Z-->Montage Multifocus to: Reflected light, min smoothing **or** Med
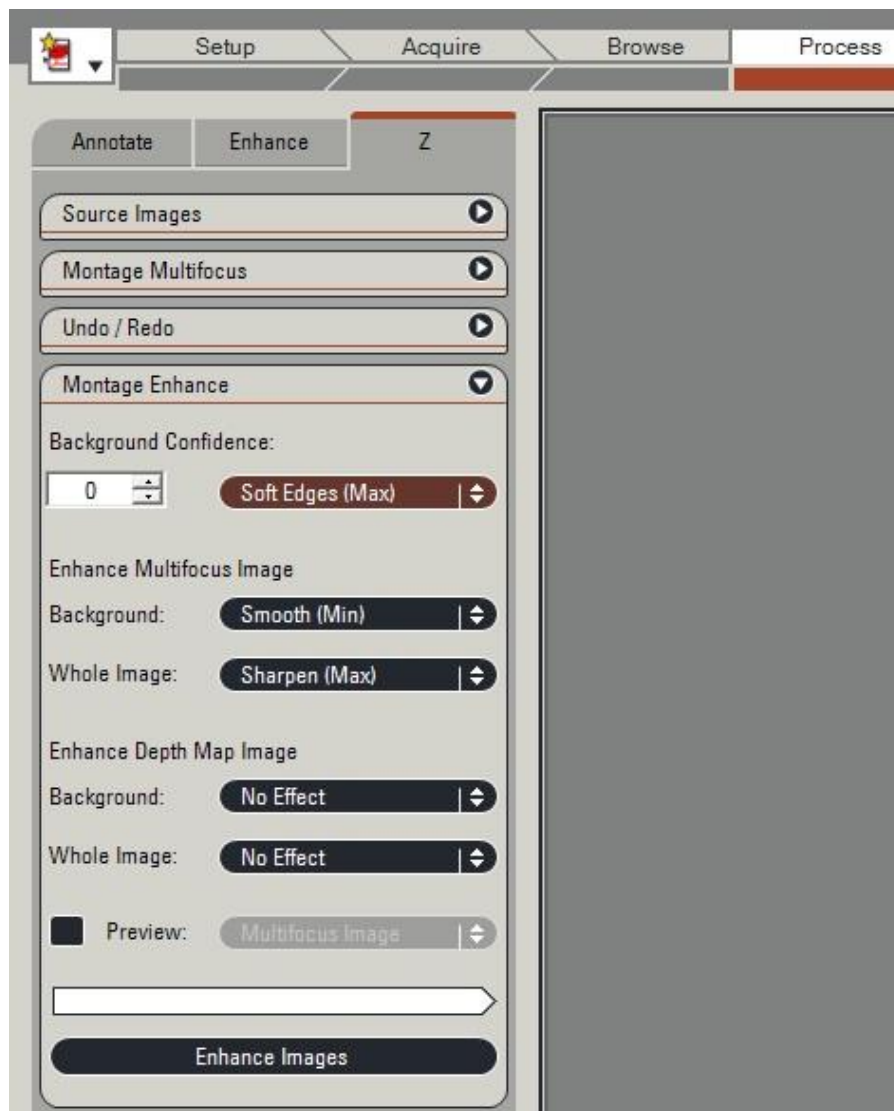   smoothing
   - Patch size: 8
   - Background: Semi-focused
   - The "Enhance after Create" box should be checked

2. "Montage Enhance"
   In Process -->Z -->Montage Enhance, use the following settings:
   - Background Confidence: 0, Soft Edges Max
   - Enhance Multifocus Image
       Background: Smooth Min
       Whole Image: Sharpen Max
   - Enhance Depth Map Image
       Background: No effect
       Whole Image: No effect

Carefully look over your montage image. If the lighting is good and the image does not need to be edited, skip ahead to the Scale Bar Section.

The below image illustrates the standard "Montage Multifocus" settings for image processing:
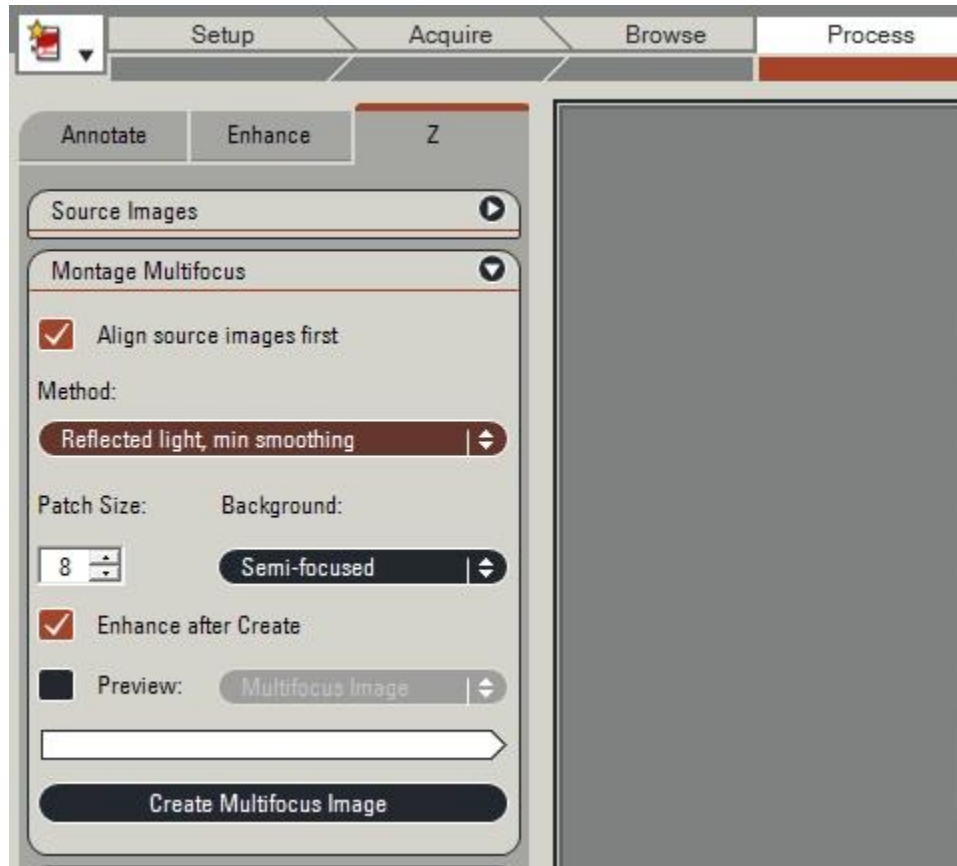
Note: These are the standard LAS v3.8 settings which we and our colleagues use. These settings should yield good results for majority of the ants you will image. If you are having trouble with particular ants, try the following changes to the Montage Multifocus settings:

    Patch Size settings:

- For specimens with lots of detail/hairs, try setting the patch size as low as 5.
- For shiny, smooth specimens with less detail, try setting the patch size as high as 20. We have found that 8 -10 works for most of the ants we image.

It is possible to run the same source images through a different algorithm to produce a better montage. You can experiment with changing both the method and the background settings. Useful methods are:

- Reflected Light-No Smoothing
   or
- Reflected Light-Med. Smoothing

If the image has any of the following problems, it may need to be re-imaged.

Problem	Solution
The specimen has moved during imaging and is either blurry or out-of-frame.	Try re-starting LAS.
The specimen was not framed properly and an important part of the specimen is not included in the image.	Return to the section on framing and see examples on AntWeb. Capture a new image.
The image is overexposed or underexposed, resulting in a loss of detail in the specimen.	Return to the section on exposure. Use all of the available tools to ensure proper exposure. As a last resort for high-contrast specimens, turn to the section on HDR.
There is a halo around the specimen.	Try decreasing the aperture from 5 to 2.5 and take another image or try processing the image using the Reflected Light, Medium Smoothing algorithm.
The positioning is off (e.g. the specimen is tilted too far forward, the spines are not lined up, mandibles are tucked).	Return to the section on positioning. Use AntWeb and "Bolton's Catalogue of Ants of the World" for reference.

The following problems may be fixed using editing tools:

- Slight halo
- Missing portions of specimen in montage image that are present in the stacked images

Enhancing:

Try the following settings to enhance your images:
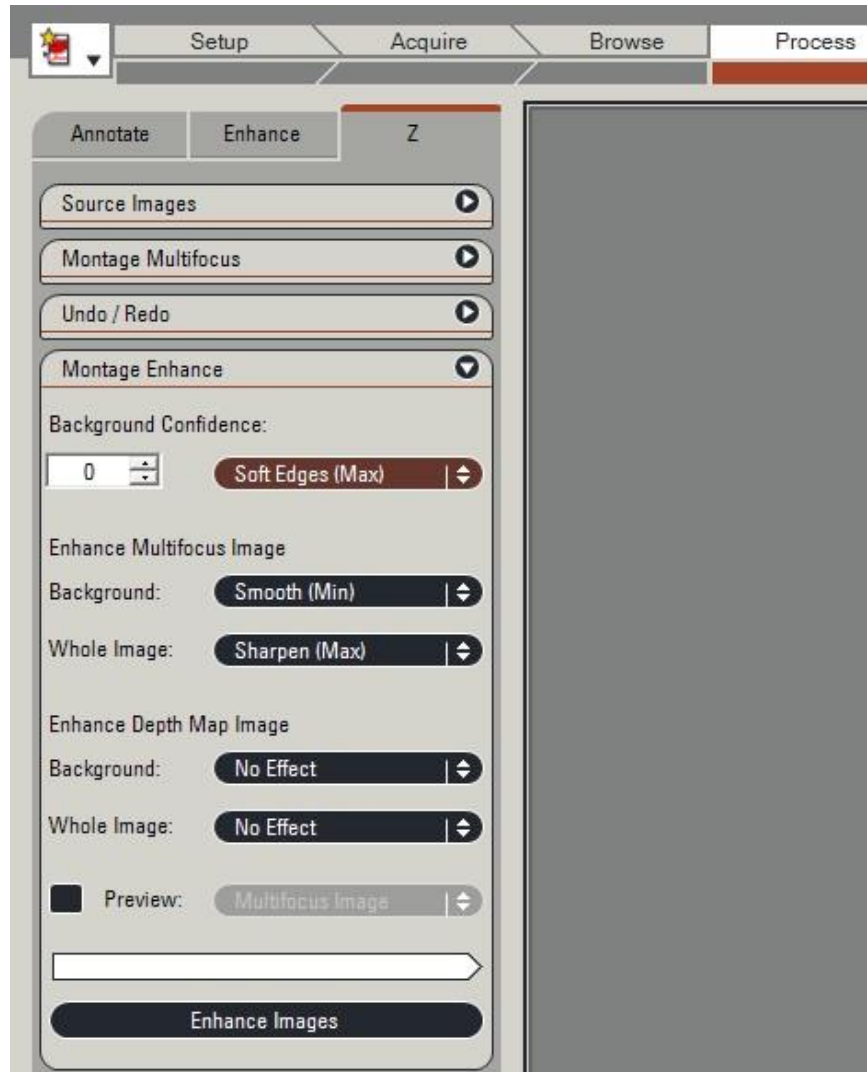
If you are still dissatisfied with the results, experiment with other settings, but first generate a copy by merging the scale bar and selecting "create copy" so you can compare the results of your efforts and choose the best image.

Note: If you return to a previously generated and enhanced stack after navigating out of its folder, be **very careful** when running further "Montage Enhance" combinations using LAS v3.8, as any sharpening applied will be added to the previous sharpening. It is safest to reset all enhancements by regenerating the montage from the image stack first. Other montage enhance settings do not appear to be additive after returning to a previously enhanced stack. (If the background becomes grainy, the image has been over-sharpened).

Editing:

If portions of the specimen are out of focus or missing in the montage image, use the edit tool to grab the necessary elements from any of the source files to correct the montage image.

1. Click on the "Show Viewer Options" icon near the bottom of the toolbar on the right side of the screen (see image below).
2. Select "Dual Viewer." This will allow you to look at the montage image and the individual source images simultaneously.
3. Check the box for "Lock View." This will synchronize all movement of the montage image and source images while editing.
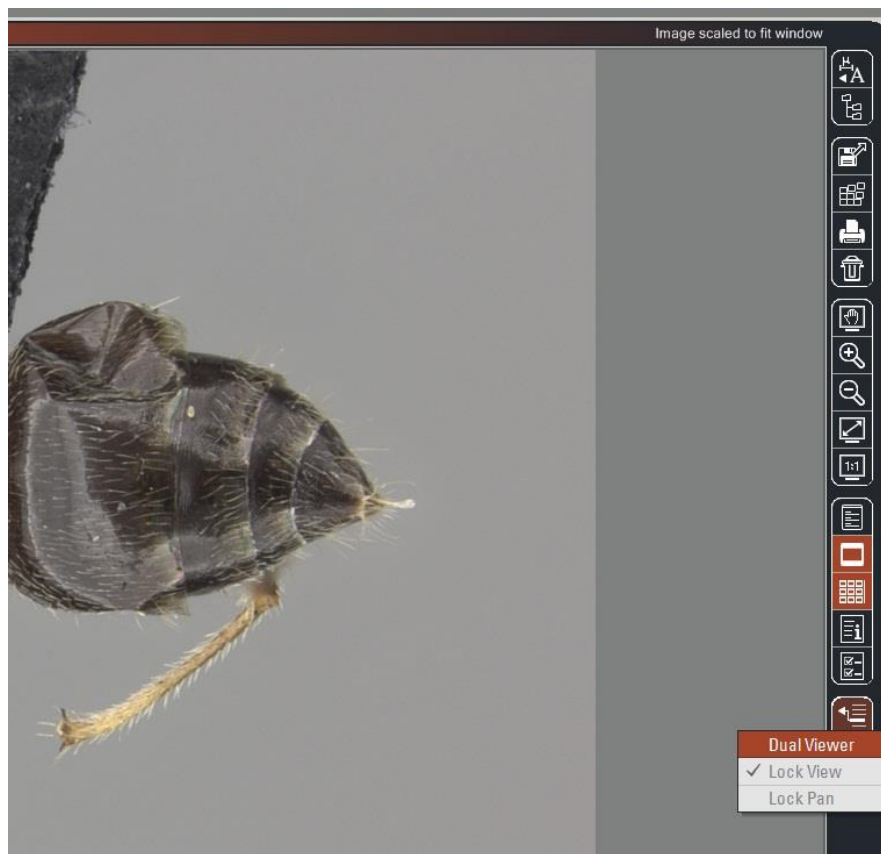
4. Under Process-->Z Tab-->Montage Edit, check the black box for "Edit Mode." Under "Region Selection Style," click the first icon. Choose the "Region Edge Style" that is appropriate for the editing you need to do.

You can change this setting as needed throughout the editing process. Use the icon on the far left for editing fine details such as hairs. Use the icon on the far right as a blurring tool.
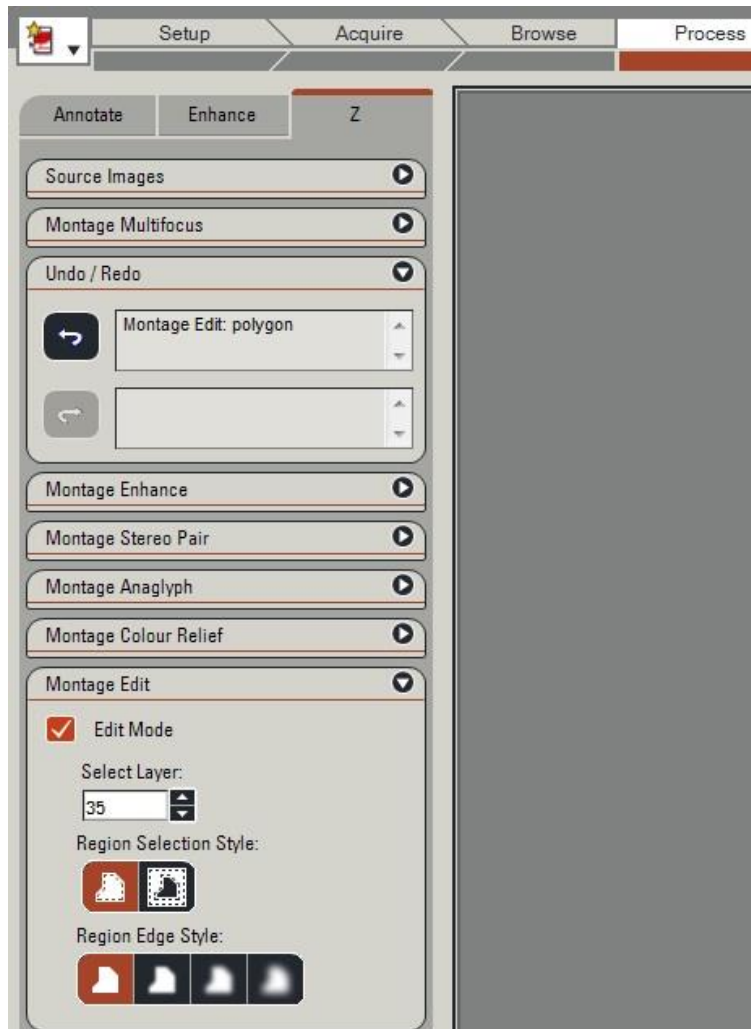
The montage image is automatically placed on the left side of the screen. Click on the right side of the dual screen and choose a source image to work from. A red line at the top and bottom of the dual viewer indicates the screen you are working from. Remember to click on the right viewer to scroll through the source images. Find the source file that you need for editing and click on it; the selected image will then appear on the right side of the viewer

It is quicker to select the area you want replaced on the montage image itself. Selecting on the source image tends to be slower and less reliable. Begin making the selection by clicking the mouse. There are two selection methods:

1. Hold the left clicker of the mouse down while selecting the area to be replaced. When you are finished, double-click to complete the edit.
2. Click the mouse then release, clicking once each time you reach a point where you would like to change the direction of the tool

When you are finished making changes

1. Click on the montage image
2. Select the "Show Viewer Options" icon on the toolbar on the right side of the screen.
3. Unselect "Dual Viewer"
4. Unselect the "Edit Mode" box
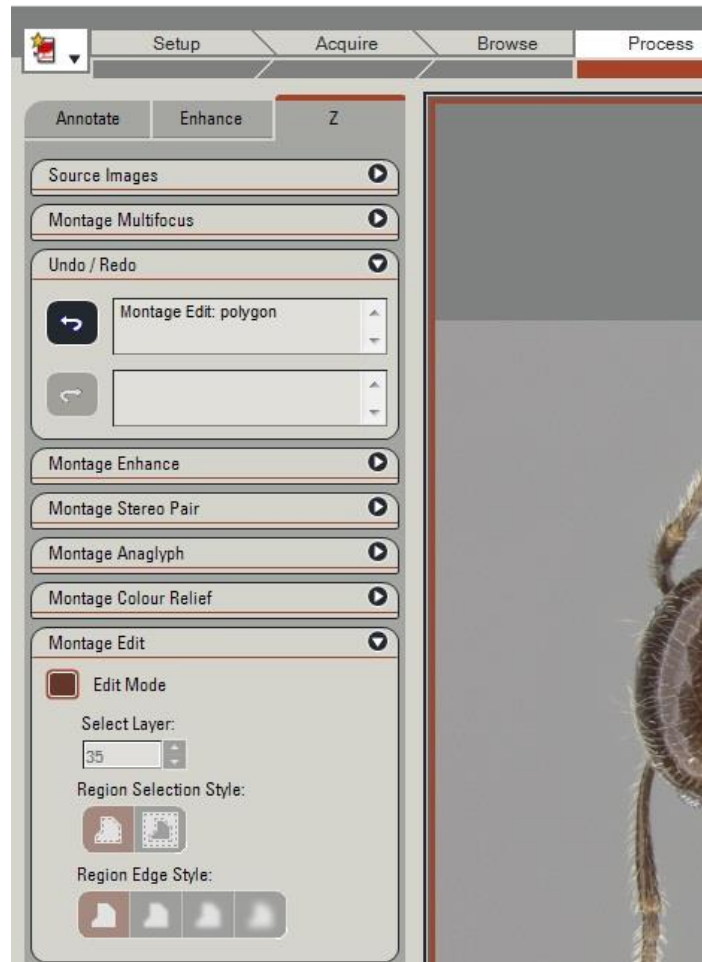
Note: Due to a software glitch in LASv3.8, you will need to click out of the Process tab to the Acquire tab and then back to Process tab so that you can enable the Annotate tab and add a scale bar to your final image.

Adjusting Black/White Levels:

Process-->Enhance-->Levels

This is the best way to brighten and sharpen your final image if such image enhancements are needed. Use subtle adjustments only. Try 1-5 for black levels and 238-255 for white levels. Level adjustments can be accomplished more precisely in image editing software such as Photoshop.
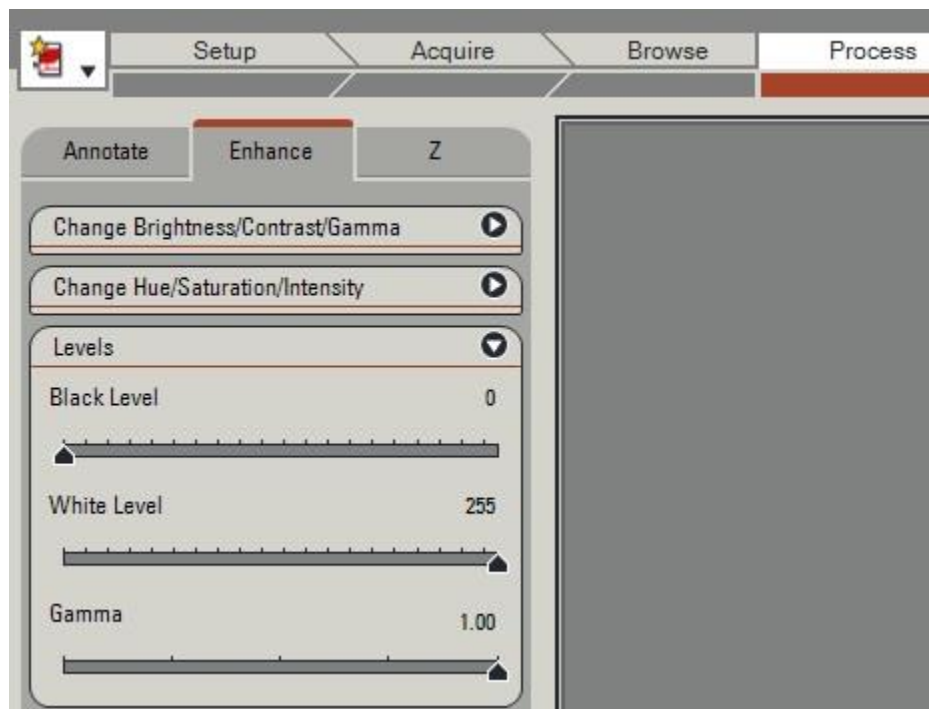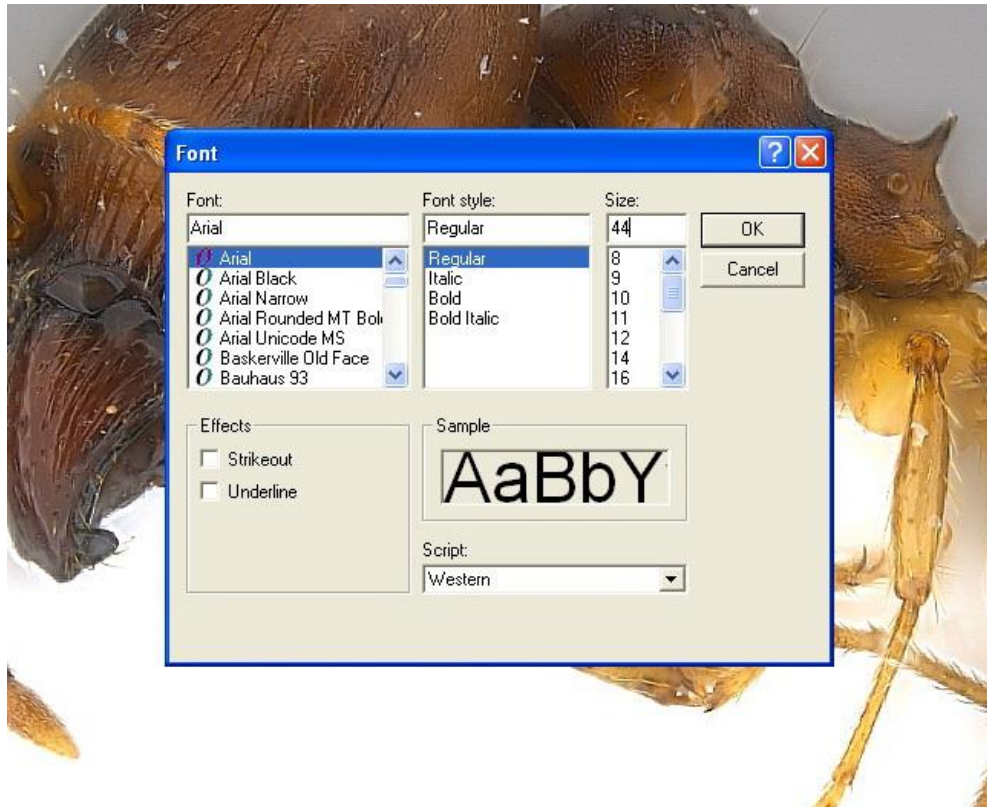

Scale bar:

Adding a scale bar is the last step before exporting your image. To create scale bar settings for the first time, use the following procedure.

1. Navigate to: Process-->Annotate-->Scale Bar
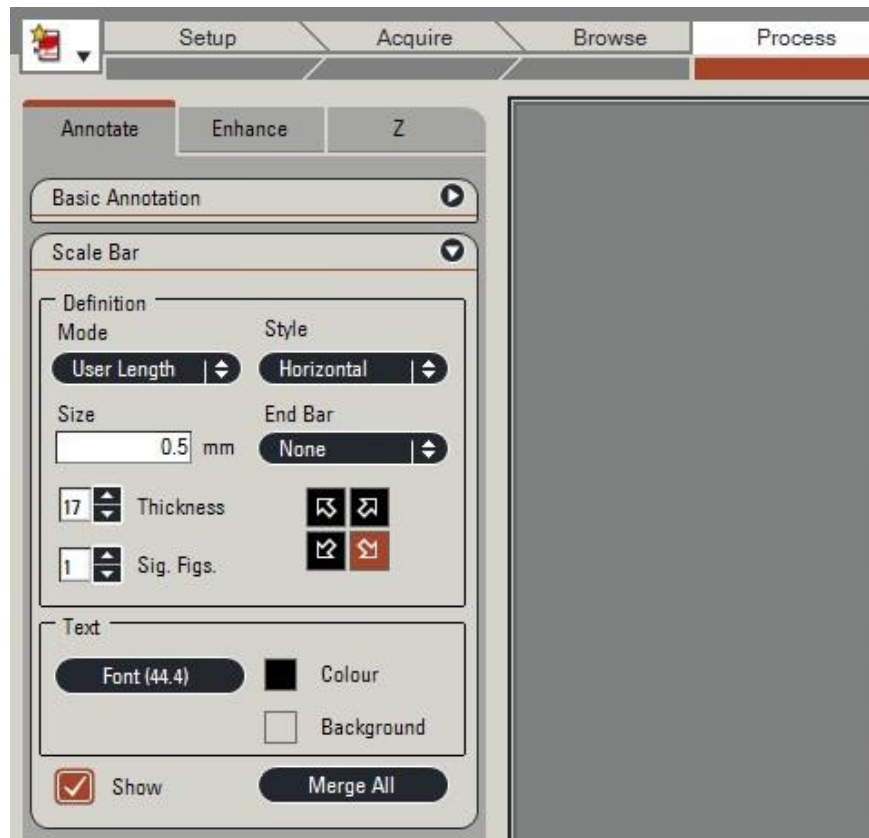
2. Check the "Show" box
3. Under "Mode," select User Length
4. Under "Style," select Horizontal
5. Under "End Bar," select None
6. Choose the appropriate size (you can change this for each image)
   AntWeb uses 0.1, 0.2, 0.5, 1, or 2
7. Select the directional arrow on the lower right, indicating the scale bar
   will be placed on the bottom right of the image
8. Set "Thickness" to 17
9. Set "Sig. Figs." to 1

10. Font is Arial (see following screenshot)
11. Font Style is Regular
12. Font Size is 44

13. Click on the box next to "Colour" and select Black
14. Click on the box next to "Background" and select white, then set
    "Transparency" to 0
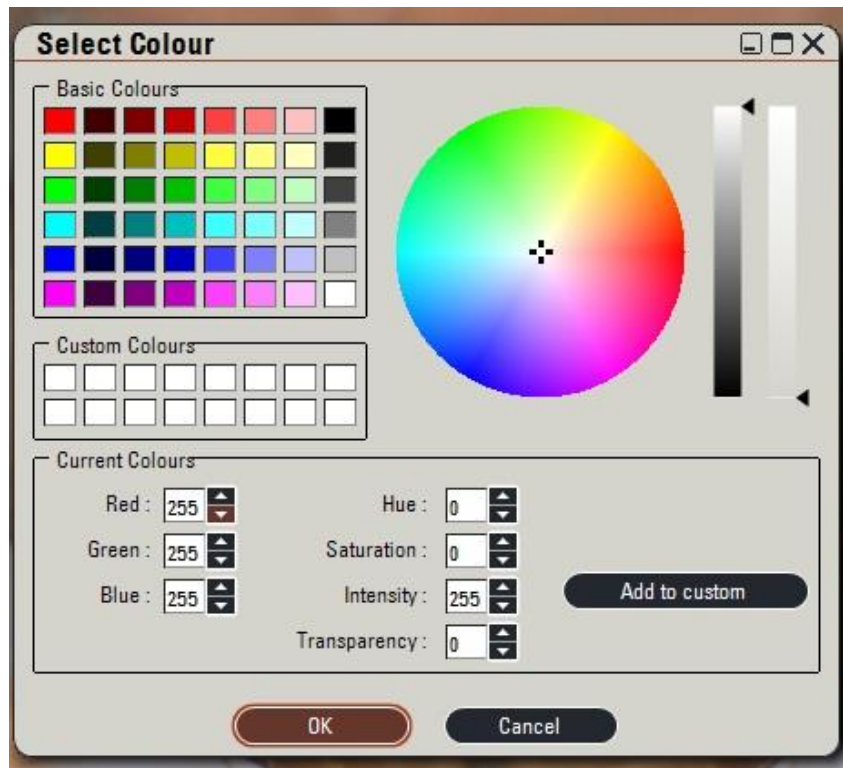
15. Process-->Annotate-->Basic Annotation
16. Check the "Show" box
17. Click on "Set" under "Defaults Including Scale bar." This will save all of
    the settings you have just created as default settings

Note: From now on, when you add a scale bar, you will only need to change its "size." When you are ready to add a scale bar to another image, go to Process-->
Annotate-->Basic Annotation and click on "Recall." You will only need to change the size and placement of the scale bar.
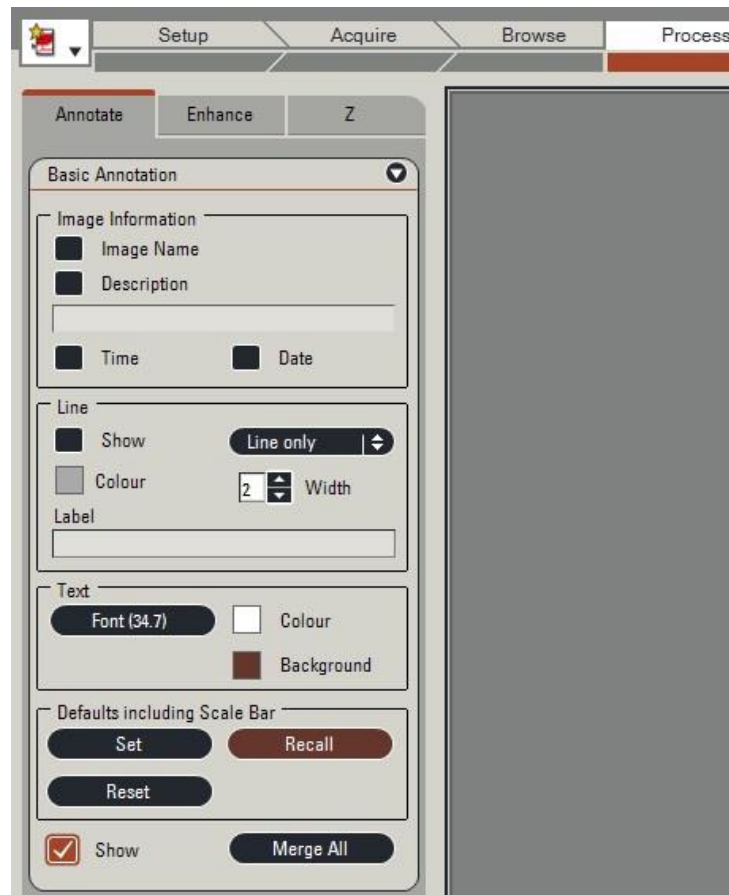
18. Place the scale bar on the lower right side of your image
19. Whenever possible, place the scale bar on a clear background area and not on the specimen's legs or antennae
20. When you are happy with the placement of the scale bar, select "Merge All." Next, select "Create Duplicate".

Note: Use only 0.1, 0.2, 0.5, 1, 2, 5 for scaling.
You can also access these areas via the toolbar on the right side of the screen. Select "Show Annotation Options" and select "Scale Bar" and "Basic Annotation." You may only use one option at a time.

Exporting:

In the Gallery at the bottom of the screen, right-click on the left-most color image "Copy of montage." This is the duplicate you just created in the above step. Select "Export." The "Export Images" box will pop-up.
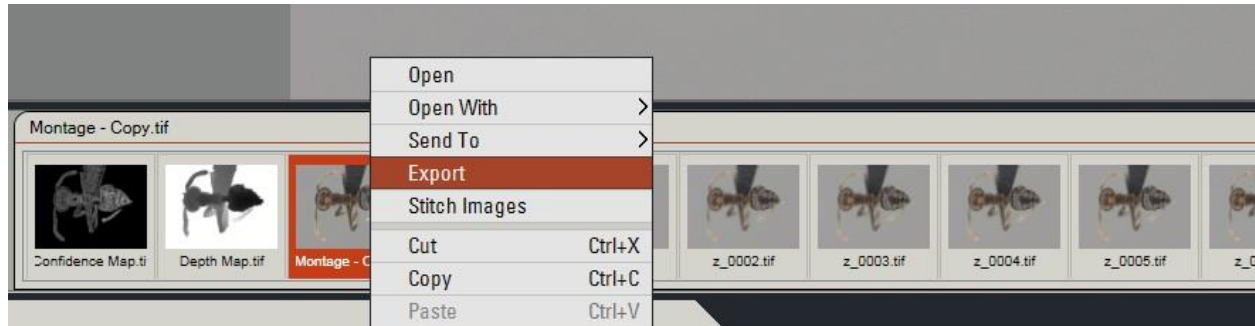

Select the export folder by clicking on the box to the right of "Select Export – Folder" and find the folder or create a folder for saving the final image.

If you are imaging at AntLab at CAS, save your image in a user folder in the following location: D-->Ant Images-->User Folder. Eventually your images will be uploaded to the CAS I-Drive (I:|\entomology\AntImages\Imaging in Progress\Original).

Check the "Rename File" box to name your image.

Each AntWeb specimen has a unique identifier associated with the imaged specimen. A seven digit CASENT specimen code or another unique identifier is used for identification in the database. Please double check that you have entered the correct CASENT code. Use the following naming format and corresponding suffixes:

- **Dorsal:** CASENT1234567_D
  - Additional dorsal shots: _D_2, D_3, etc.

- **Head:** CASENT1234567_H
  - Additional head shots: _H_2, H_3, etc.

- **Profile:** CASENT1234567_P
  - Additional profile shots: P_2, P_3, etc.

- **Wing:** CASENT1234567_P_2
- **Male genitalia:** CASENT1234567_P_3
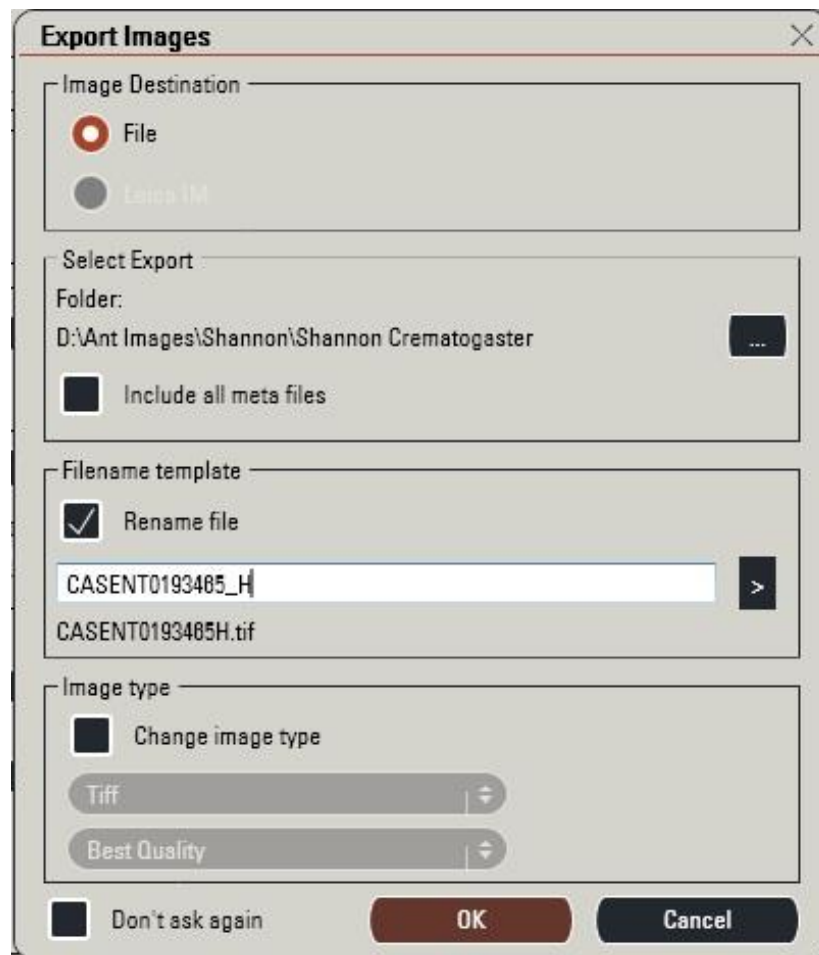
Saving and Naming Stacks:

Name each corresponding stack with the same CASENT as the final image.

1. Go to Browse-->Browse
2. Click on the stack which corresponds to the image you just exported
3. Click "Rename" to paste the CASENT number. If you are imaging at AntLab, temporarily save these stacks to your folder on the external hard drive. After you are certain your images have been accepted for upload to AntWeb, you may transfer these files to the network I-Drive.
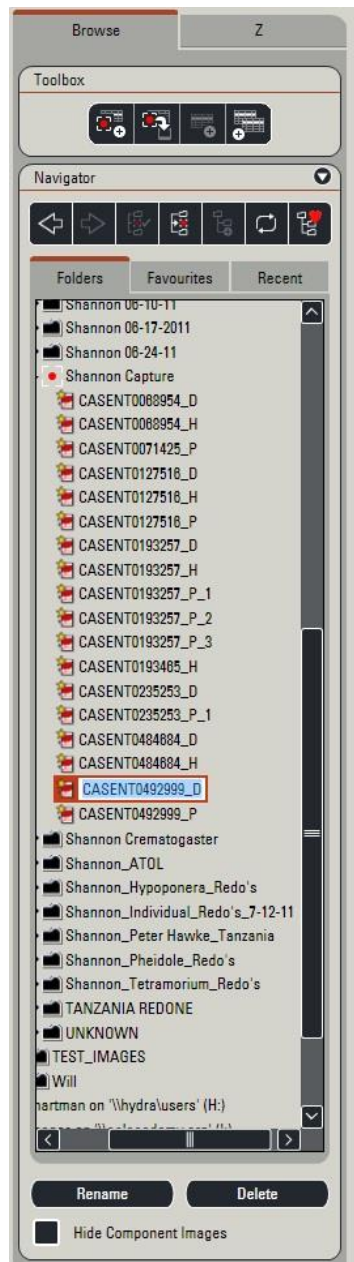
Note: Save all of your final stacks. This consumes a lot of storage space, but offers many benefits:

- You can process the stacks again with improved versions of the software.
- If you spot problems in the image, you can go back and edit the stacks.
- If you lose the final image, you can create another from the stacks.

After you have exported, click back to the Acquire tab to begin the process again…and again…and again…