Startup

1. Turn on Insight DeepSee Laser
    a. Open the Insight software icon on laptop
    b. Push and hold power button for 3 seconds to turn on
    c. Once in "Running Mode", the laser output should be shown to the right as a blue bar
    d. After laser comes on and stabilizes, push and hold Main Shutter for 3 seconds to open
    ***NOTE** – Do not open 1040 shutter, always use Main shutter
    e. Ensure that the wavelength is set to 890nm
2. Turn on the electronics rack - There is a large white power toggle at the top of the backside of the electronics rack – this turns on almost everything you need
    a. Nikon C-mount camera is controlled separately (most users don't need)
    b. Epifluorescent lamp is controlled separately (most users don't need)
3. Turn on the Computer power
4. Login as User (password: nhojk4)
5. Open PrairieView Software: There are two desktop versions of PrairieView
    a. STAGE XY – This is the we recommend to users because it has the most range of movement
    b. SCOPE XY
    ***NOTE** – the Configuration file icon looks similar to the PrairieView Software icons, but it should not be accessed by users

Pollen Grain Image

1. Once startup is completed, place the pollen grain slide on the stage (coverslip up) and insert the Nikon 10x Objective (0.5 NA).
2. Ensure that the microscope is set for brightfield (BF) settings:
    a. Turret: position 2
    b. Sliding trinocular pin: In
3. While looking through the eyepieces, focus the objective onto the pollen grain sample using the Z movement control knob on the left side of the microscope body. It is necessary to use a flashlight for this.
4. Turn off flashlight and room lights
5. Set the microscope to the laser scanning microscopy (LSM) settings:
    a. Turret: position 1
    b. Sliding trinocular pin: Out
6. Close the light box doors
7. Switch the PMT controller box for channel 1 to the ON position

    ***NOTE** - The top box controls PMT 1 and 2, the second box controls PMT 3. PMT 4, the hybrid detector, is controlled through separate software and should only be used for FLIM.
8. In the PrairieView software, set the following:
    a. Resolution: 512 x 512
    b. Dwell Time: 4us
    c. Optical Zoom: 1
    d. Scan Rotation: 0
    e. Objective Lens: Nikon 10x Air
    f. Pockel's Cell: 10
    g. Filter cube: None
    h. A starting point for PMT 1 can be approximately what the last user recorded on the sign in clipboard.
9. Using the Live Scan button, start raster scanning your sample
10. Adjust z focal plane using the remote controller. The BF and LSM images are not parfocal, so you will need to refocus through the software until you get the brightest focal plane (the plane with the most amount of saturated pixels). With these settings, it is roughly 40um down in Z (rotate wheel counterclockwise).
11. Adjust the PMT 1 value to a point where you have only a few saturated pixels.

    ***NOTE** - We recommend using the range check lookup table (blue = zero, red = saturated, gray scale = in between) as it is the easiest way to ensure that only a small amount of pixels are saturating (red).
12. Stop scanning and switch everything back to BF settings:
    a. Zero Pockel's cell

b. Zero PMT 1
c. Switch the PMT controller box to the OFF position
d. Open light box doors
e. Turret: position 2
f. Sliding trinocular pin: In

Focusing Z: Counterclockwise moves objective down, clockwise moves objective up

Single Image

1. Once you have successfully collected a pollen grain image and returned the microscope to BF settings, you should be confident that the laser and microscope are functioning as desired and can therefore place your sample on the microscope stage.

   ***NOTE** The microscope stage can be translated up and down to accommodate samples of all sizes including, but not limited to, slides, dishes, tissue blocks, and live animals.
2. Insert the desired objective.
3. Insert the desired filter(s) and dichroic(s).
4. Ensure that the microscope is set for brightfield (BF) settings:
   a. Turret: position 2
   b. Sliding trinocular pin: In
5. While looking through the eyepieces, focus the objective onto the sample using the Z movement control knob on the left side of the microscope body. It is necessary to use a flashlight for this.
6. Turn off flashlight and room lights
7. Set the microscope to the laser scanning microscopy (LSM) settings:
   a. Turret: position 1
   b. Sliding trinocular pin: Out
8. Close the light box doors
9. Switch the PMT controller box(es) for desired channel(s) to the ON position

   ***NOTE** - The top box controls PMT 1 and 2, the second box controls PMT 3. PMT 4, the hybrid detector, is controlled through separate software and should only be used for FLIM.
10. In the PrairieView software, set your save path at any of the three save path locations (shown as a […] button).
11. Adjust the following to the desired settings:
    a. Resolution – 512 x 512 is a good starting point
    b. Dwell Time – 4us is a good starting point
    c. Optical Zoom
    d. Scan Rotation
    e. Objective Lens
    f. Pockel's Cell – start low and go higher for best Signal to Noise
    g. PMT(s) – start high (around 650) and go lower for best Signal to Noise
12. Using the Live Scan button, start raster scanning your sample
13. Adjust z focal plane using the remote controller. The BF and LSM images are not parfocal, so you will need to refocus through the software. Adjust X and/or Y positions if necessary.

14. Adjust the Pockel's cell and PMT values to a point where you have good Signal to Noise ratio, and only a few saturated pixels.

    ***NOTE** - We recommend using the range check lookup table (blue = zero, red = saturated, gray scale = in between) as it is the easiest way to ensure that only a small amount of pixels are saturating (red).
15. When you get to a point where you would like to save an image, stop Live Scan.
16. Enter the file name in the Save Path field under the Live Scan button.

    ***NOTE** – The other Save Path fields, located in the Z Series and T Series tabs, can not be used for the naming of single image files, they can only be used for their respective series types.

    ***NOTE** – PrairieView will auto-increment the number in the field following the save path field. However, if you want a group of files to begin at a specific number (e.g. Filename-001), you must select that number for the first file.
17. Adjust the settings for your final image if desired.
    a. Higher resolution?
    b. Longer dwell time?
    c. Average Every?
18. Press Single Scan button
19. Repeat steps as necessary.
20. Stop scanning and switch everything back to BF settings:
    a. Zero Pockel's cell
    b. Zero PMT(s)
    c. Switch the PMT controller box(es) to the OFF position
    d. Open light box doors
    e. Turret: position 2
    f. Sliding trinocular pin: In

Z Series (Z Stack)

1. Follow the Single Image collection directions through step 14.
2. In the Z series tab, set the start and stop locations for the Z stack

   ***NOTE** – It does not matter which is top or bottom as long as you have the two extreme ends of your Z stack.

   ***NOTE** – Pockel's cell and PMT values used while live scanning will be recorded for the start and stop values.
3. If Pockel's cell power or PMT values are not constant throughout the stack, check the Adjust PMT & Laser box to the right of the panel.
   a. Default – linear interpolation between start and stop values
   b. Manual – enter values as desired
   c. Custom – non-linear interpolation between start and stop values
4. In the Z series tab, set the Step Size (um) between planes of your stack
5. When you get to a point where you would like to save your Z stack, stop Live Scan.
6. Enter the file name in the Save Path field at the bottom of the Z series tab.

   ***NOTE** – The other Save Path fields, located in the Scanning field and T Series tab, can not be used for the naming of Z Series files, they can only be used for their respective file types.

   ***NOTE** – PrairieView will auto-increment the number in the field following the save path field. However, if you want a group of files to begin at a specific number (e.g. Filename-001), you must select that number for the first file.
7. Adjust the settings for your final image if desired.
   a. Higher resolution?
   b. Longer dwell time?
   c. Average Every?
8. Press Start Z Series button
9. Repeat steps as necessary.
10. Stop scanning and switch everything back to BF settings:
    a. Zero Pockel's cell
    b. Zero PMT(s)
    c. Switch the PMT controller box(es) to the OFF position
    d. Open light box doors
    e. Turret: position 2
    f. Sliding trinocular pin: In

T Series (Time series)

Time series are extremely configurable, and as such these directions will be far from comprehensive. The following directions will go through the most common usage of the T series tab.

1. Follow the Single Image collection directions through step 14.
2. If desired, follow Z stack directions through step 5.
3. In the T Series tab, start by pressing the Clear All button at the upper right.
4. Add in any desired elements (rows) to your time series by clicking the buttons along the right side.
    a. Image Sequence will add a single scan image (create in the scanning field, see Single image directions)
    b. Z series will add a Z stack (create in the Z series tab, see Z Stack directions)
5. The elements (rows) in the T series list can be repeated in different ways:
    a. You can increase the number of iterations at the bottom right corner of the T series tab. This will repeat all elements in their listed order.
       Example: Three elements in T series, 3 iterations – 1,2,3,1,2,3,1,2,3
    b. Alternatively you can add repeats to each element by changing the # Rep column in each row of the T series. # Rep will only affect that element.
       Example: Three elements in T series, 3 rep for each element – 1,1,1,2,2,2,3,3,3
    c. If your T series only has one element, Iterations and # Rep will act similarly.
6. The time between image/stack collections can be changed in different ways:
    a. You can set the Iteration Period at the bottom right corner of the T series tab. This will set the amount of time between consecutive iterations.
       Example: Element 1 takes 5 sec to complete, element 2 takes 7 sec to complete, iteration period set to 20 sec – timing will be as follows
        - Element 1 (5 sec)
        - Element 2 (7 sec)
        - Waiting for 8 sec (20sec iteration period minus element 1 time & element 2 time)
    b. Alternatively, you can set the period for each element. This will set the amount of time between consecutive repetitions.

Example: Element 1 takes 5 sec to complete and has a 20 sec period, element 2 takes 5 sec to complete and has a 20 sec period – timing will be as follows

- Element 1 (5 sec)
- Waiting for 15 sec (20 sec period minus element 1 time)
- Element 2 (7 sec)
- Waiting for 13 sec (20 sec period minus element 2 time)

c. Wait can also be added as an element (row) to the T series. However, this is generally a more difficult method.

Example: If you wanted to repeat an element that takes 5 sec to complete every 2 minutes, you could not simply add a 120 sec wait time as this would actually repeat every 125 sec. For precision, you would have to calculate the wait time as 120 sec minus the 5 sec period of that element.

7. Enter the file name in the Save Path field at the bottom of the T series tab.
   ***NOTE** – The other Save Path fields, located in the Scanning field and Z Series tab, can not be used for the naming of T Series files, they can only be used for their respective file types.
   ***NOTE** – PrairieView will auto-increment the number in the field following the save path field. However, if you want a group of files to begin at a specific number (e.g. Filename-001), you must select that number for the first file.
8. Adjust the settings for your final image if desired.
   a. Higher resolution?
   b. Longer dwell time?
   c. Average Every?
9. Press Start T Series button
10. Repeat steps as necessary.
11. Stop scanning and switch everything back to BF settings:
    a. Zero Pockel's cell
    b. Zero PMT(s)
    c. Switch the PMT controller box(es) to the OFF position
    d. Open light box doors
    e. Turret: position 2
    f. Sliding trinocular pin: In

Montage

These directions will take you through an in depth workflow for montaging 2D or 3D images. It is not necessary to follow all the steps below, but it is recommended.

1. Follow the Single Image collection directions through step 14.
2. In the menu bar, navigate to Applications > Atlas Imaging. This will open up two windows:
   a. Atlas Overview – This is a large window in which you can preview a map view of your sample. The thumbnail image location will update as you move in X and Y. This is the window in which you will define your montage grid.
   b. Atlas Image – This functions as a normal image window, where you can select the channels and lookup tables that you would like to view in the Atlas imaging windows. You can also set the start and end of a Z stack in this window using the Set Z Top and Set Z Bottom buttons, as well as set the Z step size.
3. The easiest way to define the outer limits of your montage is to generate a large-scale preview of the sample. I recommend doing this at a low resolution, just to speed it up. Preview images are not saved.
   a. Find the approximate XY center location for your montage and press the Set as Center button in the Atlas Overview window.
   b. If you are collecting a 3D montage (Z stacks at multiple XY locations), select a location where you are able to preview the sample and its boundaries. This is usually at or near the middle of a Z stack that will be set up in subsequent steps.
   c. Define an Overview Size that you would like to preview (i.e. 10 Wide x 10 High). The software requires that this overview be a square.
   d. Temporarily reduce the image resolution and dwell time to speed up the preview process.
   e. Press the Preview All XY button. This will show non-overlapping images in the Atlas Overview window.
4. Once you have a preview of the tissue, you can find specific regions that you want to include in your montage. Click the Add Location / Update Z button when the thumbnail is over any XY location you want included.
   a. The easiest way to do this it to use the top left and bottom right corner locations (or top right and bottom left corners).
   b. However, you can add as many locations as you want. This may be useful if you have a sample with irregular borders and cannot easily envision the "corners" of a rectangle.

5. Set % Overlap for the final montage in the Overview Size field. I recommend 10% overlap.
6. Press the Generate Montage button to make a grid with overlapping XY locations that encompasses all locations you previously added. By default, this is a rectangular montage.
7. Once the grid is generated, you can choose to remove tiles if desired, using the Remove Locations button.
    a. You may want to remove an entire row or column
    b. You may not want to collect a rectangular grid

    ***NOTE** – if you do choose to remove locations, make sure that you press the button again once you have completed deleting tiles. It will stay "on" (highlighted green) until you turn it "off" and it is easy to accidently delete locations that you do not want to delete.
8. You can verify that all of your grid locations are saved by going to the XY Stage Tab in main window, and seeing them listed as rows.
9. If you are collecting a 2D montage (one image at each XY location), you can skip to step 13.
10. If you would like to collect a 3D montage, you will need to set up a Z stack. This can be done in the Atlas Image window or the Z Series tab. See Z stack instructions for more detail.

    ***NOTE** – The start and end locations of the Z stacks throughout a montage do not have to stay the same (although the number of images in the stack and therefore the depth of the stack must remain the same throughout). However, I recommend that you keep them constant by generating a Z stack that is appropriate for the entire XY area.
11. This step is confusing, so bear with me. When you previewed your sample in the Atlas overview window, and subsequently chose the XY locations for your montage grid, you were likely at a plane where you could easily image the tissue, and therefore somewhere in the middle of your desired Z stack. However, when you generate a montage, the Atlas Imaging module will record XY and Z positions at this middle location, and it will start a Z stack of a set depth at the Atlas Imaging Z position instead of starting at the Z-Series tab defined position where you really want it to start.

    Example: Preview sample at Z = 100um, generate a 2 x 2 grid of XY images, all four XYZ locations will appear in the XY Stage tab as rows.
        - Location 1: X = -500um, Y = 0um, Z = 100um
        - Location 2: X = 0um, Y = 0um, Z = 100um
        - Location 3: X = -500um, Y = 500um, Z = 100um
        - Location 4: X = 0um, Y = 500um, Z = 100um

    But … your desired Z stack ranges from a Z Start = 200um to Z stop = 0um

Therefore, you will have to manually reset the Z for all locations in the XY Stage tab to be the same as the Z start location (reset to be 200um). If you do not do this, your Z stack in this example would actually start at 100um, move down the Z stack depth of 200um, and end at -100um. This would not be the stack you want!

12. The easiest way to reset all Z positions is as follows:
    a. In the XY Stage tab, highlight a value in row 1, then click the Move Motors to Selected Location button
    b. Manually move Z until you reach the value which equals Z start, then press the Add Location / Update Z button in the Atlas Overview window.
       ***NOTE** – You do not have to be spot on, just within one "Z step" interval
    c. Repeat steps 12a and 12b for the last row
    d. After those two Z locations have been updated, press the Interpolate Z Ranges button in the Atlas Overview window.
    e. Unfortunately, this interpolation does not refresh until you then go to the XY Stage tab and click on the Z (um) column. At this point, you should scrool up and down through all locations to make sure that interpolation has taken place and that no values are zero (unless they are supposed to be zero).
13. Now you are ready to collect your montage! Adjust the settings for your final image if desired.
    a. Higher resolution?
    b. Longer dwell time?
    c. Average Every?
14. To collect the montage, we will run it through the T Series tab.
    a. There should only be one element in your T series list:
        i. For 2D montages, add Image Sequence
        ii. For 3D montages, add Z series
    b. Ensure that Run at all XYZ stage locations is checked in the bottom left corner and that iterations = 1 and iteration period = 0.
15. Enter the file name in the Save Path field at the bottom of the T series tab.
    ***NOTE** – The other Save Path fields, located in the Scanning field and Z Series tab, can not be used for the naming of T Series files, they can only be used for their respective file types.
    ***NOTE** – PrairieView will auto-increment the number in the field following the save path field. However, if you want a group of files to begin at a specific number (e.g. Filename-001), you must select that number for the first file.
16. Press Start T Series button
17. Repeat steps as necessary.
18. Stop scanning and switch everything back to BF settings:
    a. Zero Pockel's cell

b. Zero PMT(s)
c. Switch the PMT controller box(es) to the OFF position
d. Open light box doors
e. Turret: position 2
f. Sliding trinocular pin: In

Other notes:
The Clear All button in the Atlas Overview window will clear all previewed images as well as XY stage locations. If you would like to clear the XY stage locations, but keep the previewed image – go the The XY Stage tab, and press the Remove All button in the Add/Modify Locations field.

The Acquire Volume button in the Atlas Overview window will start your montage collection, but it will not allow for naming of files. I recommend that you do this in the T Series tab instead (steps 14 - 16).

Shutdown

1. Prior to the end of each imaging session, you need to collect a pollen grain image as instructed in the Pollen Grain Image tab.
2. On the sign-in clipboard, record the following:
    a. Username
    b. Start Date, Time
    c. End Date, Time
    d. PMT 1 value from pollen grain image (only a few pixels should be saturated at this value)
    e. Objectives used
    f. Comments
3. Shut down the PrairieView software
    a. zero all PMTs
    b. zero the Pockel's cell
    c. exit software
4. Copy any data that you need to an external or cloud based drive.
5. Shutdown the computer
6. After the computer completes shutdown, turn the power to the electronics rack off using the large white power toggle at the top of the backside of the electronics rack.

    ***NOTE** – if you have turned on the Nikon camera or the epifluorescent lamp, those will need to be turned off separately from the electronics rack.
7. Ensure that the microscope is set for brightfield (BF) settings:
    a. Turret: position 2
    b. Sliding trinocular pin: In
8. Shutdown the SpectraPhysics Insight DeepSee laser
    a. Press the Main shutter to close it
    b. Press the power button to turn power off
    c. Close the Insight software window, and when prompted – choose the Hibernate option

FAQ

1. **I am not getting an image in PrairieView – why?**

   There are several things to easily check before you should request assistance

   a. Pockel's cell is set to a reasonable value for your sample
   b. The correct PMT(s) are set to reasonable values for your sample
   c. Turret is in position 1 for LSM
   d. Sliding trinocular pin is out for LSM
   e. PMT controller box(es) are on
   f. Laser is on and at correct wavelength
   g. Main shutter on the laser is open
   h. Your filters are in the correct orientations

2. **What Pockel's cell and PMT values should I start at?**

   a. There is no good way to answer this question because it is sample and microscope configuration dependent.
   b. The general rule of thumb is to start with low Pockel's cell values, and to increase as necessary. The reason to start low is because too much laser power can harm your sample (burn, photobleach, phototoxicity).
   c. The opposite movement can be followed for PMTs; values can start higher, and decrease to achieve better signal to noise. "High" PMT values on Multi alkali PMTs (channels 1 through 3) are about 650-700. Note that channel 4, the hybrid PMT, is more sensitive and should be controlled more cautiously.

3. **How deep into my sample will a multiphoton image?**

   The laser wavelengths used for multiphoton imaging can penetrate deeper into samples than those used for single photon imaging techniques. However, there is no way to give a broadly applicable answer to this question because imaging depth is also sample dependent. Some tissues scatter light more than others, and subsequently the achievable imaging depth will be more shallow.

4. **My montaged images look like they have grid lines – what should I do?**

   a. Fluorescent probes that photobleach will show greater bleaching in areas that are scanned more than others (i.e. areas of image overlap). This is unavoidable, but can be minimized or possibly corrected for in post-processing. Note that SHG signals do not photobleach.
   b. If the alignment of the system is poor, the illumination of each field of view will be uneven. When multiple images are placed next to one another in a montage, the uneven illumination is easier to observe because of the

contrast between a dim area and a bright area in a repeated fashion. If this is the case, an administrator will need to re-align the system.

Stitching in FIJI

1/5/17 4:05 PM

1. Plugins > Stitching > **Grid/Collection stitching**
    a. Choose Type: **Positions from file**
    b. Choose Order: **Defined by image metadata**
    c. Click OK
2. A new window will pop up
    a. Choose multi series file: click Browse and select a file from your dataset (for PrairieView data, you should select the **first OME.TIF** file)
    ***NOTE** – the XML file will also work, but in this case, you will also have to check the "Invert Y Coordinates" box
    b. Uncheck the "Compute overlap" box
    c. Everything else can be left in the default settings.
    d. If your data is too large for computer memory, check the "Use virtual input images" box
    e. Click OK and wait

Save fused image as a TIF file