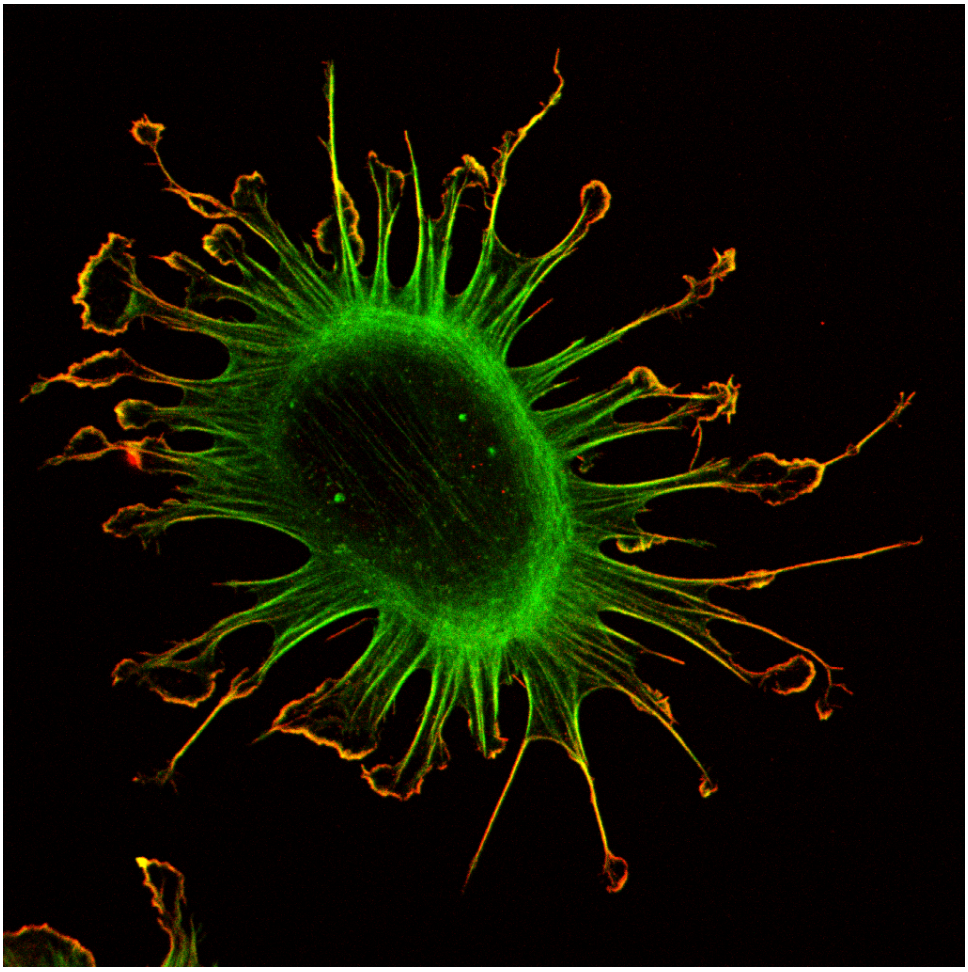
User – Manual

Nikon - A1Rsi+ Confocal

(11/27/2017)

From: Karl Ferdinand Ziegler

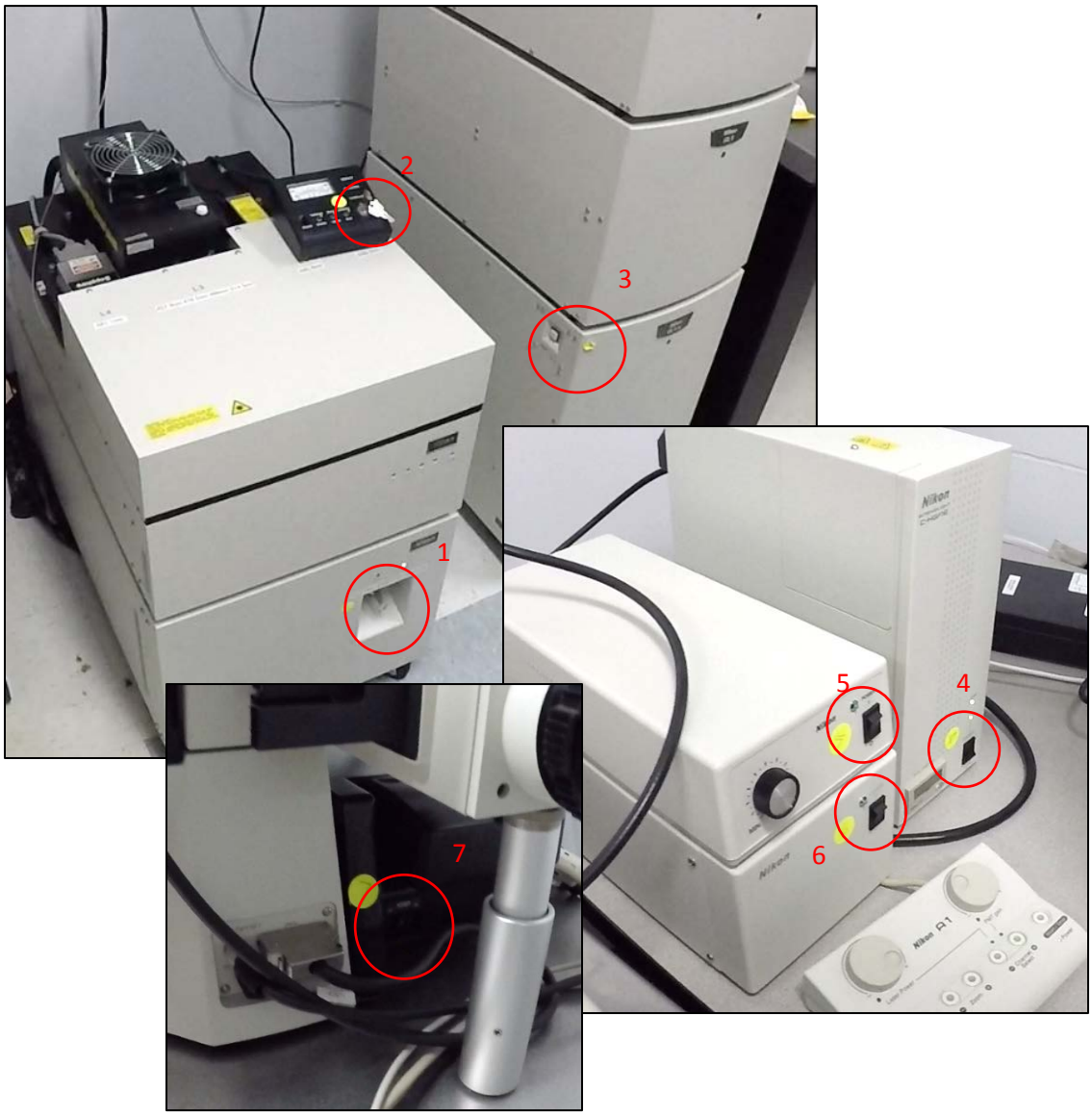


1 Contents

1. Turn On/Off Procedure

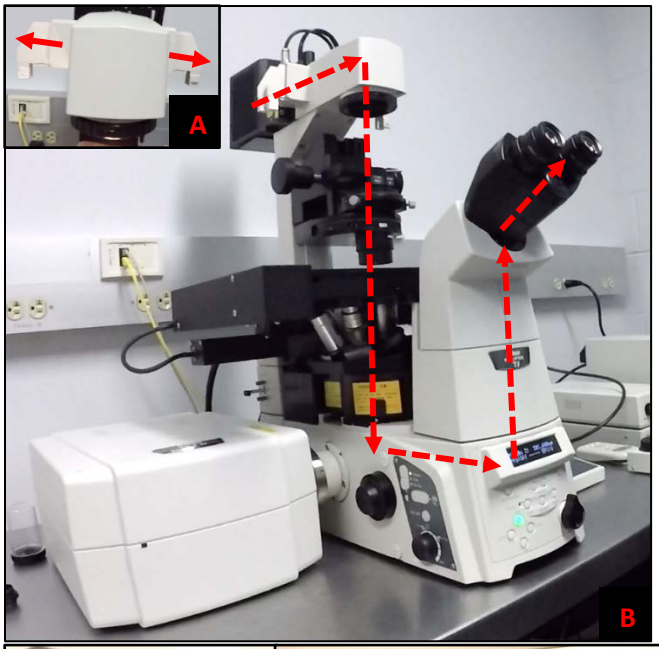
1. Power supply for Lasers
2. Key switch for laser
3. Power supply control unit
4. Metal halide lamp (Epi Fluorescence)
5. Halogen bulb
6. Power supply motorized stage
7. Power supply for micro-scope

Turn on Order 1 to 7 is important. For shutting down: Order in exactly opposite direction



Find the Sample

2.1. Step 1: Use Brightfield (transmitted light without DIC)



1. **After** turn on the Microscope (See 1. Turn On/Off Procedure) and don't start the Software
2. **Choose** an objective
3. Make sure objective is clean and **prepared** with Oil/water if necessary
4. **Place** sample on stage
5. Make sure **Filters** are out of light Path *[Panel A]*
6. **Turn On** the Transmitted light (Button) and change the intensity (Knob) *[Panel C]*
7. Switch the **Filter turret** to an empty position *[Panel D]* (dashed line) by using the button *[Panel E]*
8. Use the **Eye pieces** to find the Sample

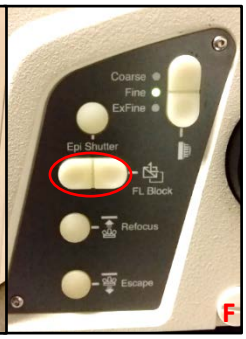
9. Control Stage (X,Y,Z) see Panel F:
   a. Rotate knob [1] change the moving speed for X,Y movement (current speed shown in Area 1b)
   b. Translate knob [1] move stage in X,Y position respectively
   c. Press button [2] change the moving speed for Z movement (current speed shown in Area 2b)
   d. Rotate knob [3] move stage in Z position

Comments:

1. **If contrast is not high enough use DIC (see description below)**
2. **To reduce photo bleaching turn the light off if don't needed and use only as much intensity as necessary.**

2.2. Step 2: Use transmitted light with DIC (for higher contrast)

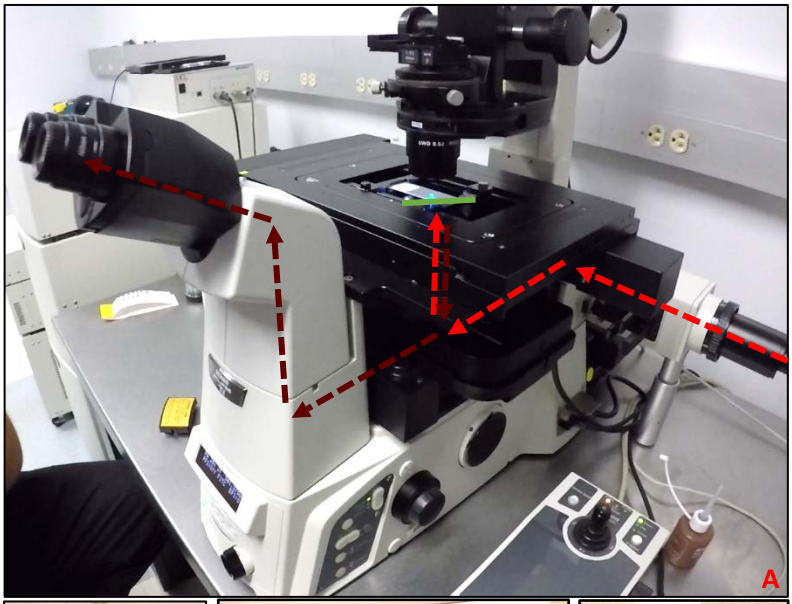
1. **Find** sample with bright field (2.1 Step: 1)
2. **Replace** Step 7 in 2.1 with the following 2 Steps
3. Make sure:
   a. **Polarizer** is in light path *[Panel B]*
   b. **Wollaston Prism** is in place *[Panel C]*
   c. Under the Objective is another **Wollaston Prism** *[Panel D]*
   d. Switch *[Panel F]* the Filter turret to **Analyzer** *[Panel E]*
4. Rotate the polarizer to change the DIC **contrast** *[Panel G]*



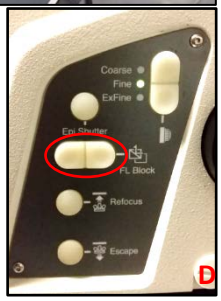
Comments:

1. **Some objectives are not available with Wollaston Prism. Both 20x + 60x will have it. If you want DIC for other objectives (e.g. 40x), please ask core facility.**

2.3. Step 3: Use Epi Fluorescence



1. **Step 1 – 4** from 2.1. (Step 1: Use transmitted light without DIC)
2. Open the **shutter** *[Panel B Button I]* for the metal halide lamp and **change** intensity *[Panel B Button II]*
3. Switch Filter turret to one of the Filters (**DAP, FITC or TxRed)** *[Panel C, D and E]* by pressing the button *[Panel D]*
4. Use the Eye pieces to find the sample



Comments:

1. **To reduce photo bleaching turn the light off if don't needed and use only as much intensity as necessary.**
2. **DIC is not available in this mode.**

3. Software (Confocal)

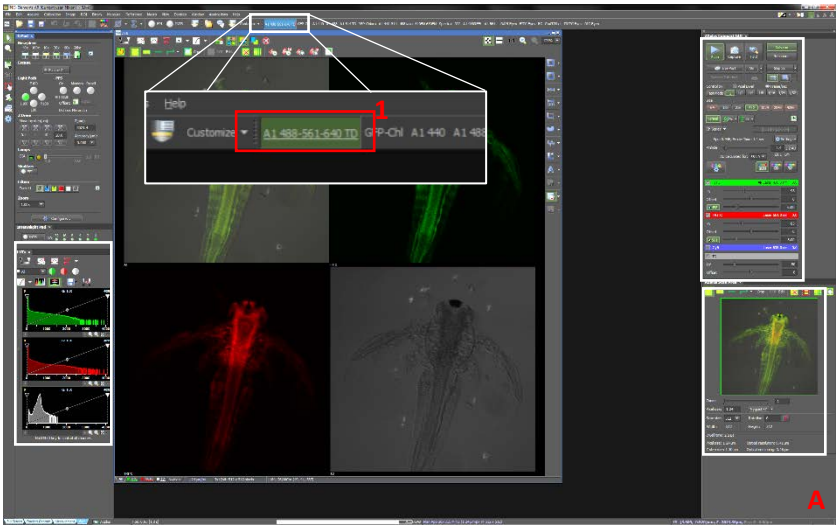
After the:

1. **Sample is in focus**
2. **Transmitted light is turned off (reduce photo bleaching).**
3. **Shutter of the metal halide lamp is closed (reduce photo bleaching).**

Now it's ready to start the Software and use confocal.

3.1. Start Software and Pre-setup

LUTs:
Look Up Table to see current intensity values and scaling (3.2. Setup the acquisition)



General Acquisition settings:
Change scan speed, laser power etc. (3.2. Setup the acquisition)

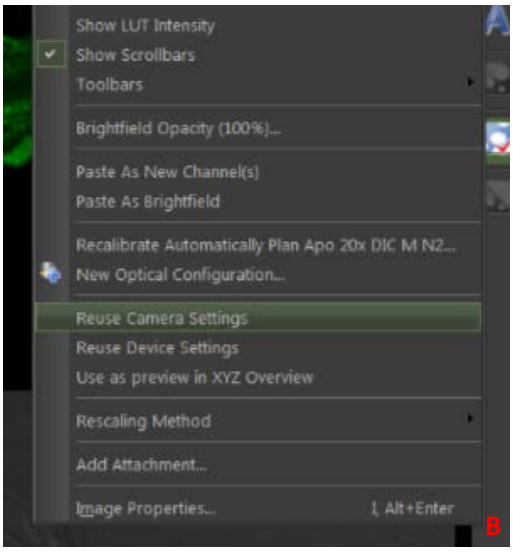
Optical Zoom:
To increase magnification but decrease the field of view (3.3. Optical Zoom)

Two ways to configure the confocal acquisition settings

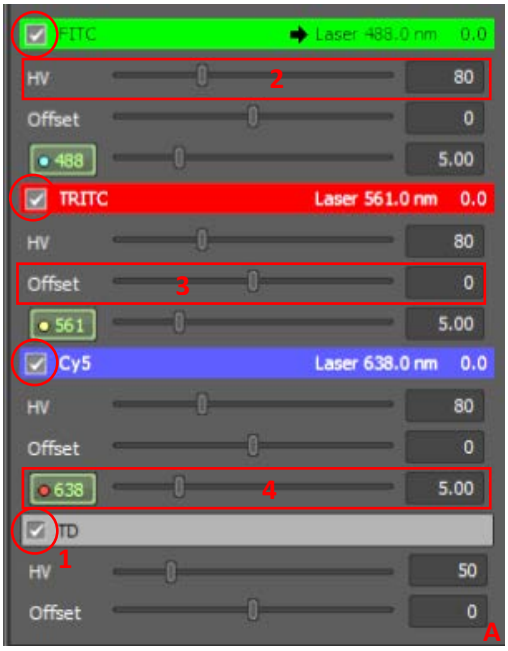
a) Select optical configuration *[Panel A, Button 1]* labeled "A1-488-561-640 TD

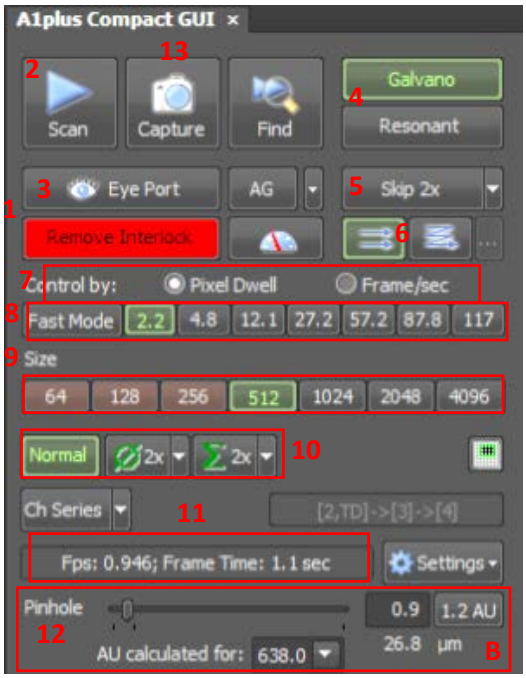
OR

b) Open a previously acquired image. Right click on the image → Reuse camera settings *[Panel B]*



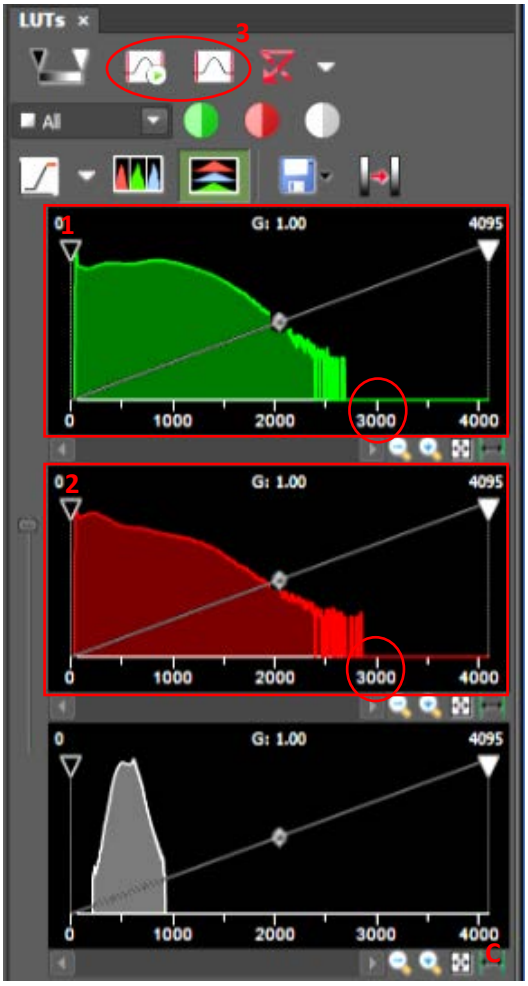
3.2. Setup the acquisition configuration and capture a 2D image





1. Choose **Light Sources** you want to use by checking/unchecking the boxes next to it *[Panel A, Button 1 respectively]*:
   a. Green Bar: Laser 488 nm and Detector
   b. Red Bar: Laser 561 nm and Detector
   c. Blue Bar: Laser 638 nm and Detector
   d. Transmitted light and Detector
2. If **Remove Interlock** [Panel B, Button 1] is red → press it to remove interlock. If the red color does not disappear then make sure L100 on the front of the microscope is selected
3. Press **Scan** *[Panel B, Button 2]* button to turn on the laser and get a **live image**. Press it again and you turn the laser off and freeze the image.
4. To prevent **pixel saturation** on the detector and achieve a good **signal/noise** ratio the intensity values in the LUTs *[Panel C, Field 1 and 2]* should be between 2000 and 3000 *[Panel C, marked value]*. You can achieve that on three different ways:
   a. Adjust the **gain** *[Panel A, Area 2 respectively for each la*
      Effect: Increase of gain increase signal and noise at the same time
   b. Adjust the **scan speed** *[Panel B, Area 8].*
      Effect: Reduce speed increase signal but increase photo bleaching (see also the following step 5)
   c. Adjust the **Laser power** *[Panel A, Area 4 respectively for each laser]*
      Effect: Increase of laser power increases photo bleaching

   (It's necessary to make compromise between these 3 settings above)
5. Choose the **Images Size** in Pixel *[Panel B, Area 9].* This is pixel density and contributes to resolution image quality. It is not possible to use all combinations of scan speed and image size. You have to take that in consideration in Step 4. A standard setting is 512 or 1024.



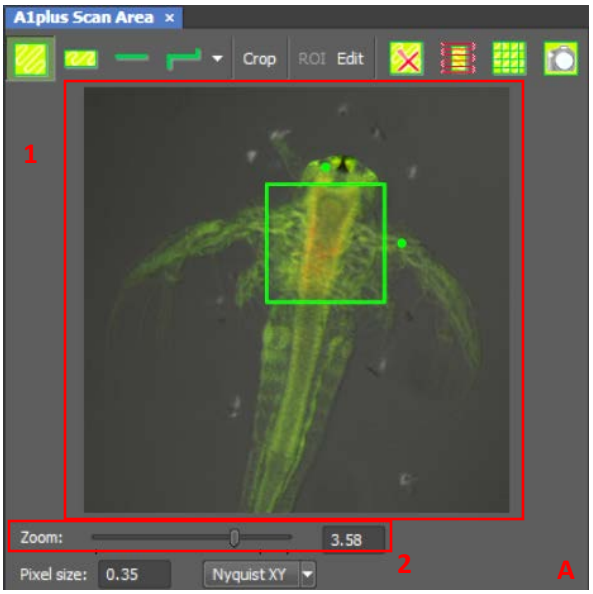
6. There are three additional settings (Step 4a) that has an effect on the **scan speed**:
   a. Switch between **Galvano** and **Resonant** scan mode *[Panel B, Area 4]*
      Effect: Resonant increase speed and noise as well.
   b. Switch between **uni- and bidirectional** scan *[Panel B, Area 6]*
      Effect: Bidirectional double speed but increase noise
   c. Change the **Averaging** *[Panel B, Area 10]* between normal, line and integral. It is only recommended to use Averaging only when capturing image and not during live.
      Effect: Line and integral reduce scan speed but increase signal/noise ratio (clean up but slow)
7. Change the **Pinhole size** (AU) in *[Panel B, Area 12]*. This value is wavelength dependent. It is recommended to use a value of 1.2 AU (next to the text box)
8. After you are done with all settings and you are ready to acquire an image the press **Capture** *[Panel B, Button 13]*

Comments:

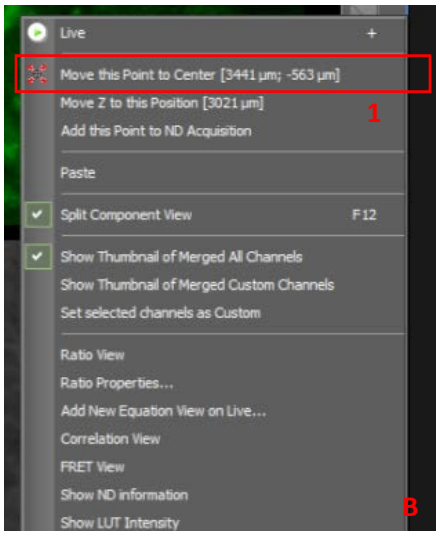
1. If you **lost your sample**: the button **Eye Port** *[Panel B, Button 3]* will turn off the laser and detector and switch to the Eye Port. Now it possible to use the transmitted light or the Epi-Fluorescence. After pressing the button again the old settings get restored.
2. **Unit** of scan **speed** *[Panel B, Area 7].* It is recommend to use Frame/sec.
3. **Shows** the current fps and Frame time *[Panel B, Area 11].*
4. To reduce **photo bleaching**: freeze the image as often as possible (that will turn of the laser light)
5. There are two ways to adjust image scaling to the monitor *[Panel C, Area 1 and 2]*
   a. Keep Auto scale *[Panel C, Area 3]*
   b. Auto scale *[Panel C, Area 3]*

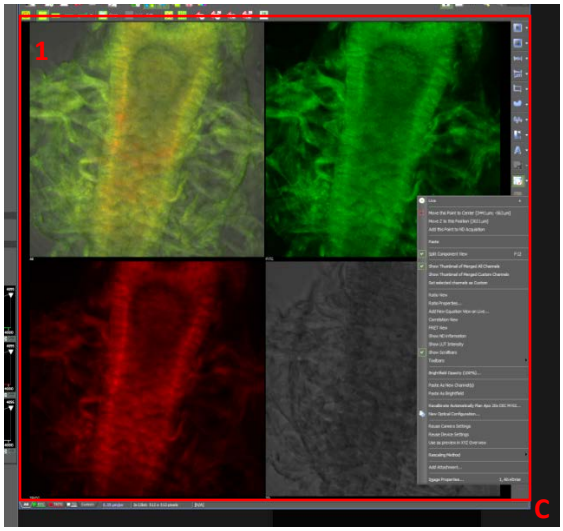
3.3. Optical Zoom and move the field of interest

Increase the magnification independent from the objective but decreases field of view as well.



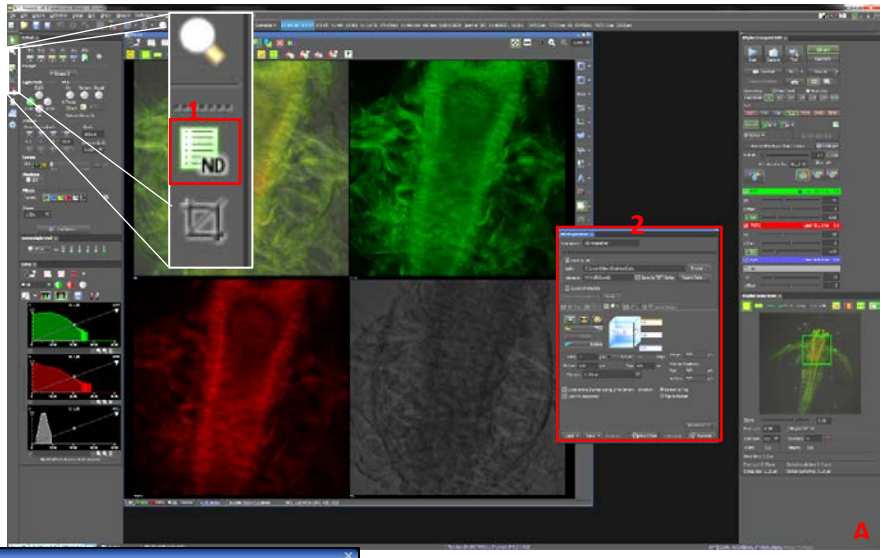
1. **Zoom** in a field of interest *[Panel A, area 1]* by enter a zoom value or move the slider*[Panel A, area 2]* or change the size of the field with the mouse
2. After you changed the size the green square *[Panel A, area 1]* becomes red. With right click in the area you can **accept** the new field and it turns into green again
3. You can also **move** the field per drag and drop in the entire scan field. But it is recommend to keep the field of interest in the middle of the scan area. Don't move it to far to the corners
4. If you want to **move** the center of your field of interest then make right click at this point in the window *[Panel C, Area 1]* and choose "Move this Point to Center" *[Panel B, Button 1]*

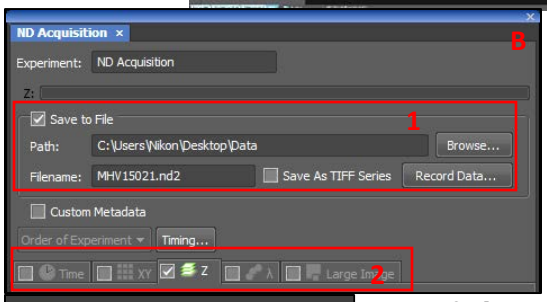




3.4. Acquire a "Z-Stack" Image (3D-Iamge)

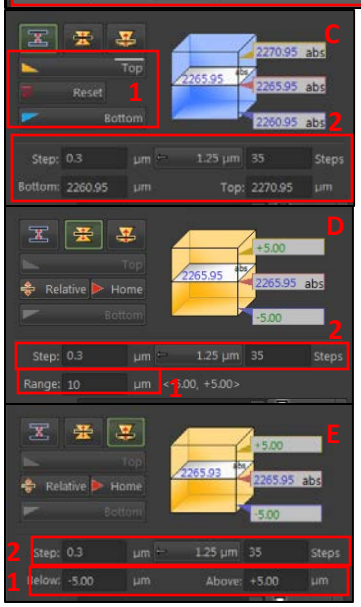
After you found the right acquisition settings (3.2) and the an area of interest (3.3) you can acquire a Z-Stack image (3D Image) by pressing the Button 1 in Panel A. After that the window shown in Panel A, Area 2 or in big, Panel B will appear.





1. Choose a Path to **save** the files and a Filename *[Panel B, Area 1]*
2. Uncheck all **boxes** *[Panel B, Area 2]* except for Z.
3. Click on **symbol** left beside Z *[Panel B, Area 2]* 

4. Now you have three **option** to acquire a Z-Stack image *[Panel B, Area 3]*: 



a. Left *[Panel C]*: Move the objective up and down by scrolling mouse wheel or using the stage control unit (see 2.1). When you found the lower limit of your region of interest then press the button "Bottom". For the upper limit press the button "Top" *[Panel C, Area 1]*. Choose your step size or your amount of *steps [Panel C, Area 2]. See comments.*

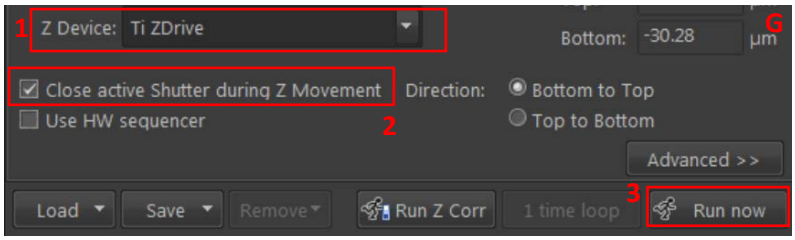
b. Middle *[Panel D]*: Enter the thickness of your Z-Stack image *[Panel D, Field 1]*. Your current position is symmetric in the middle of this value. Choose your step size or your amount of *steps [Panel D, Area 2]. See comments.*

c. Right *[Panel E]*: Enter the thickness of your Z-Stack image by typing a value relative below to your current position and a value relative above of your current position *[Panel E, Area 1]*. Thus, your current position is asymmetric in the middle of this two values. Choose your step size or your amount of *steps [Panel E, Area 2]. See comments.*

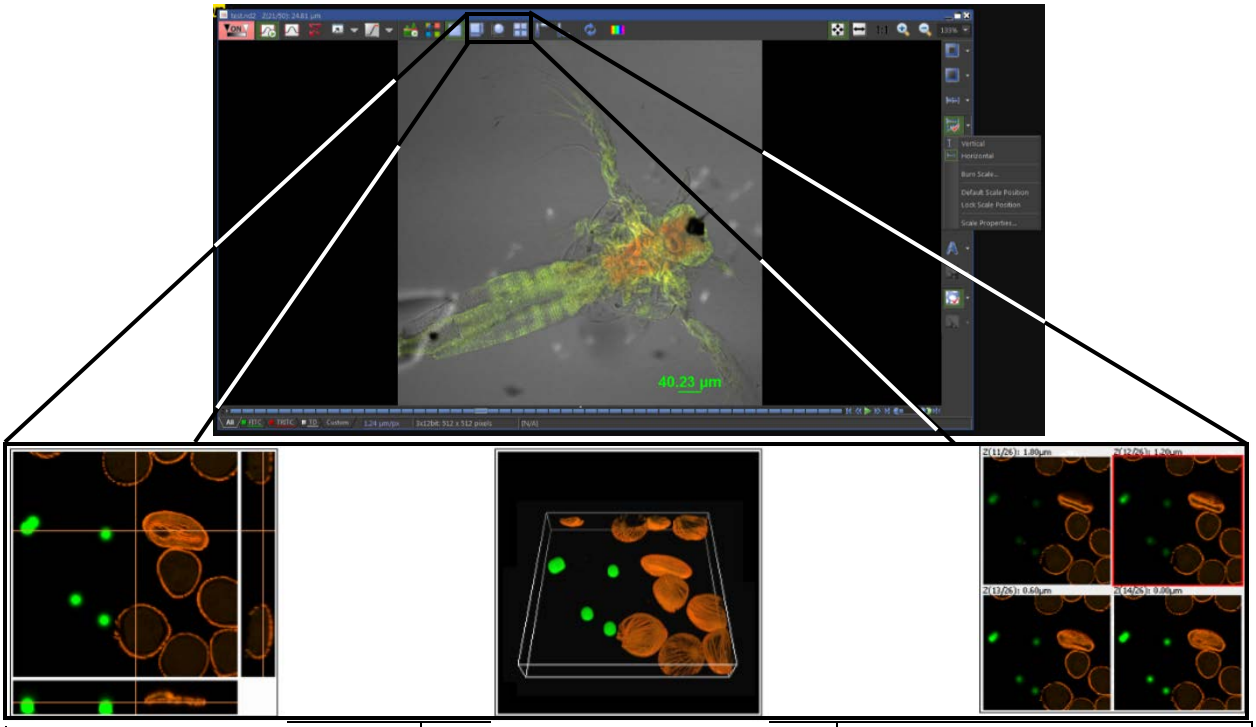
5. Press **Run** *[Panel G, Field 3 (next page)]* to acquire a Z-Stack image. Please pay attention for the comments on the next page.

Comments:

1. You can choose the value for Step size or the number of steps. If you enter a step size then the program calculate the amount of steps and opposite. But it is **recommended** to use always as step size the value between the field Step and Steps. You can set this value by clicking the field *[Panel F, Field 1]*
2. Make always sure that for Z-Device is "**Ti ZDrive**" chosen *[Panel G, Field 1].*
3. Make always sure that the **box** beside "Close active Shutter during Z Movement" is checked *[Panel G, Area 2].*



3.5. Different views



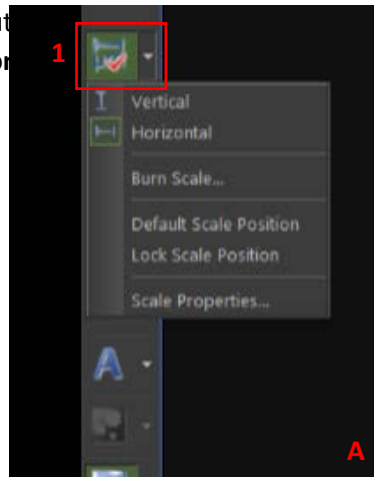
Slice View: This view displays orthogonal XY, XZ and YZ projections of the image sequence. (Requires Z or T dimension)	**Volume View:** This view creates a 3D model of the acquired object. (Requires Z dimension)	**Tiled View:** This view displays frames of the selected dimension arranged one next to other. (Requires Z, T or XY dimension)

Clicking the buttons open **new** windows with the selected view.

Drag and drop this buttons in the current window **changes** the view.

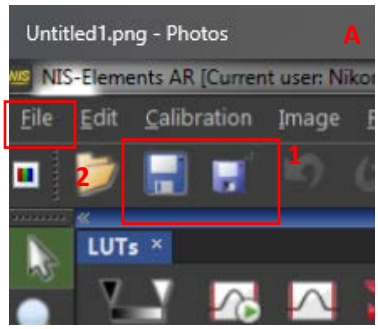
3.6. Scale bar

If you want to **add** a scale bar in your captured image then click on the but
right side of you acquired image and choose between **Vertical** and **Horizor**
burn the scale in your image or move the scale bar around.



3.7. Save data

3.7.1. For Analysis

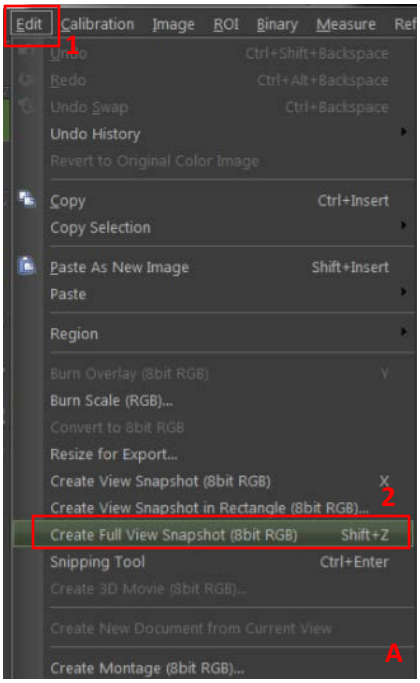


1. Two ways to **save** your data:
   a. **Press** one of the button *[Panel A; Area 1]*
   b. **Click** on File *[Panel A; Button 2]* and then select save as
2. Select always the Image File format **ND2** as shown in *Panel B, Area 1.*
3. After selecting the Image File Format ND2 press **save** *[Panel B; Button 2]*

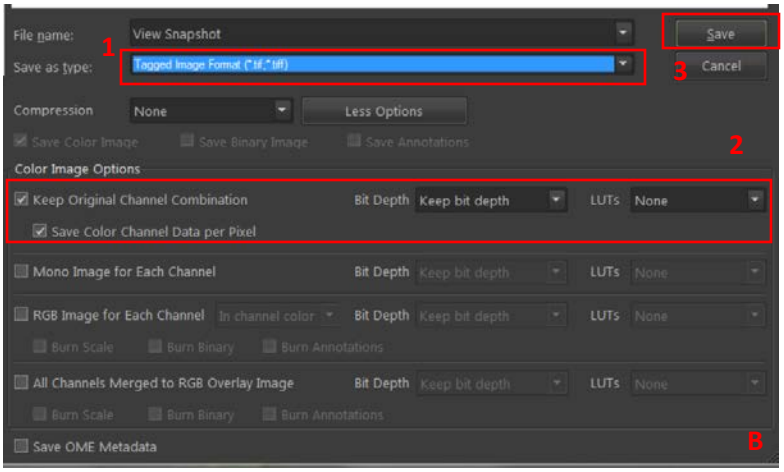
Comments:

1. This saves the image in a **raw format** and the microscope configuration as well
2. That allows you to **reuse the camera** settings as described in point 3.1. (Start Software and Pre-Setup) and to change your analysis after acquire an image.
3. The data format is quite too big for sending as **email** attachment. If you want to send images as email then follow the Section 3.7.2. below.

3.7.2. To send as email



1. Bring the image you want to send to the **front of the GUI** so it is visible
2. Now you have **two options** to generate an image:
   a. Option 1:
      i. Press the Edit Button in the **top left corner** *[Panel A; Button 1]* to open the drop down menu
      ii. Select "**Create Full View Snapshot** (8bit RGB)" *[Panel A; Area 2]* to generate a 8bit RGB image
   b. Option 2:
      i. Press the key **combination** "Shift + Z"
3. Now, **select File → Save as** in the top left corner like it is shown in 3.7.1. or press one of the two save symbols. 
4. Select the **Image File Format tiff** *[Panel B; Area 1]*
5. Make sure that the **boxes are checked** as shown in *Panel B; Area 2*
6. Press **save** *[Panel B; Button 3]*



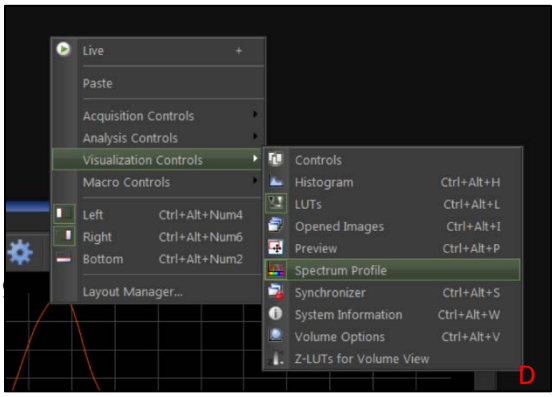
Comments:

1. The Settings shown in Panel B are only **recommendation**. Feel free to make your own choices.

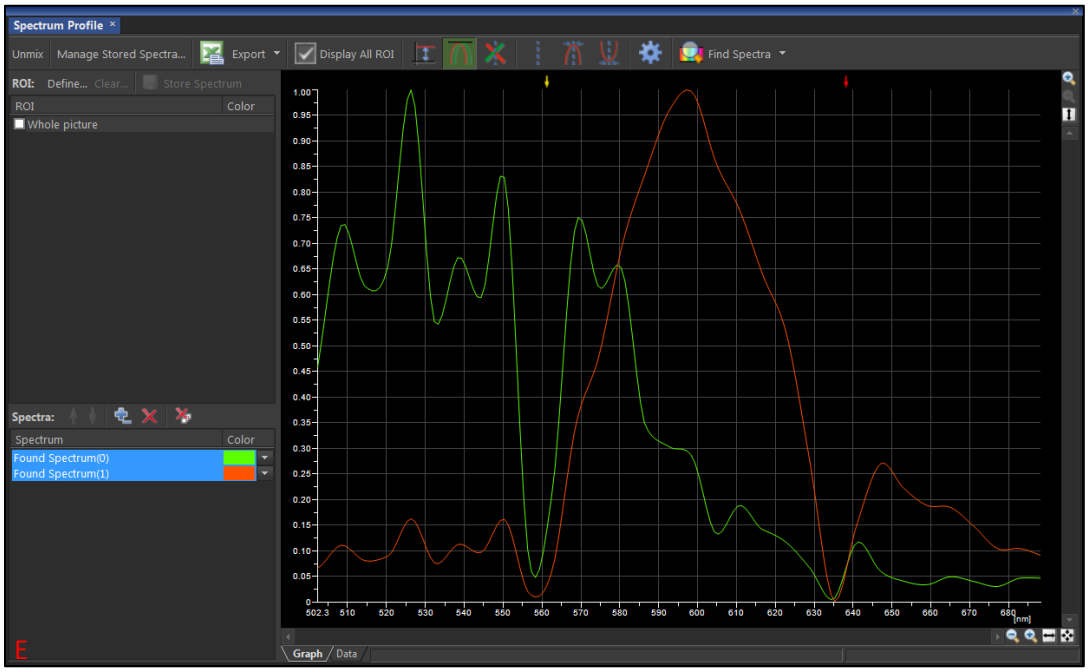
4. Spectral detector

4.1. Pre-setup

1. **Select** "Spectral" on top of the GUI *[Panel A; Area 1].*
2. After few seconds the Laser settings *[Panel B]* **appears** on the right side off the GUI.
3. **Hint:** The Spectral detector (SD) is selected *[Panel B; Area 1].*
4. To **change** the wavelength/Detector settings press the Button 2 *[Panel B].* A new window will appear *[Panel C, see Point 1.1 on next page].*
5. Now only one **gain setting** for all wavelength available *[Panel B; Area 3].* Change the value by moving the slider or type in a number.

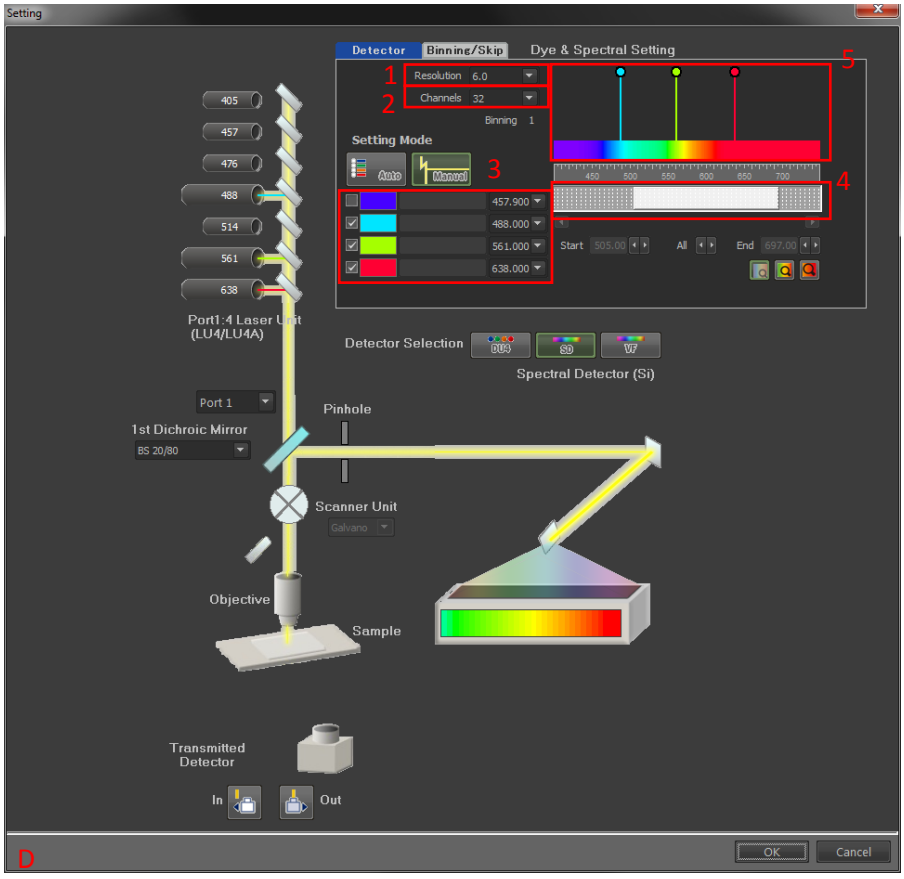


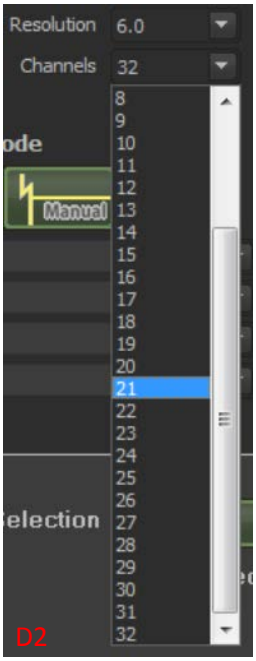
6. Change the **Laser power** by moving the slider or type a number (shown in %) in the box on the left side *[Panel B; Area 4].*
7. Select and unselect **different Laser** by clicking the button shown in Panel B; Area 5.
8. Click the **right mouse** button in the black screen → Visualization Controls → Spectrum Profile *[Panel D].*



4.1.1. Wavelength and detector configuration

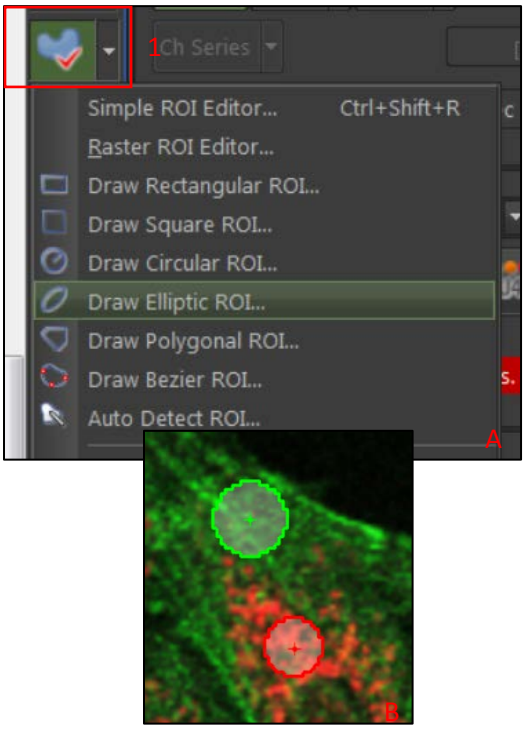
1. Change the **resolution** of the wavelength by clicking on the arrow shown in Area 1 Panel D. You can choose between three values: 2.5 nm, 6 nm and 10 nm *[Panel D1].* The resolution is inversely proportional to the bandwidth. With increasing the resolution you will lose bandwidth. The current size of the bandwidth is shown as a little white box underneath the spectra *[Panel D; Area 4].*
2. Change the number of **channels** you want to use by clicking the arrow shown in Area 2 Panel D. You can choose between 1 and 32 *[Panel D2].* The current size of the bandwidth is shown as a little white box underneath the spectra *[Panel D; Area 4].*
3. Select up to 4 different **excitation source** by checking/unchecking the boxes shown in Panel D Area 3.
4. Setup the **wavelength** for each source by clicking the arrows beside the checked boxes respectively *[Panel D; Area 3].* Your current excitation configuration is shown as long lines on top of the spectrum *[Panel D; Area 5].*
5. **Change the position** of the bandwidth in relation to the shown spectrum by moving the white box [Panel D; Area 4] left and right to fit with you excitation/emission.

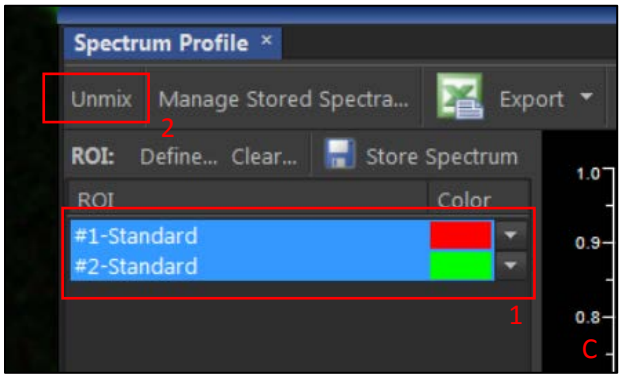




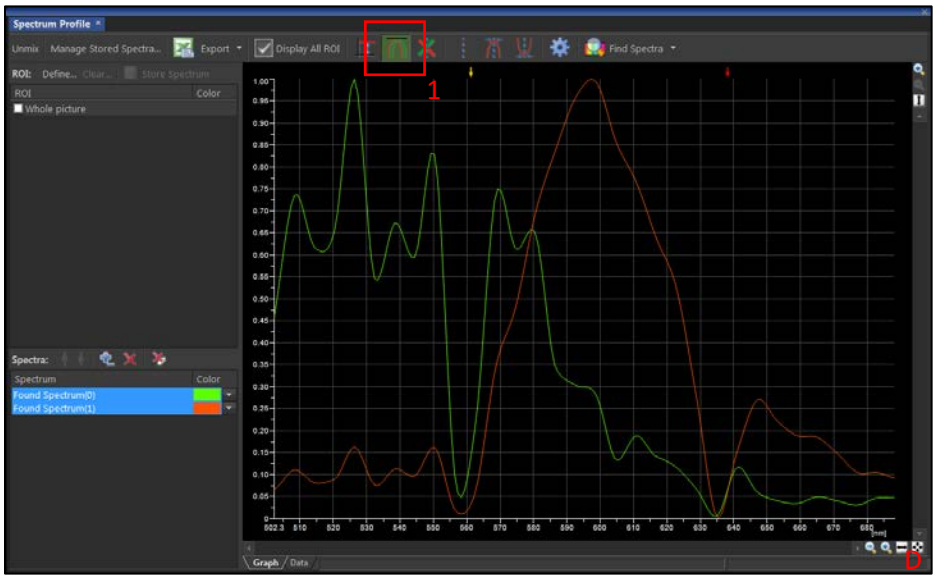
4.2. Method 1: Based on Region of Interest (ROI)

1. After Point 1 (Pre-setup) is done, configure the system (Laser power, Scanning speed, Averaging etc.) such that the **noise is on a low level** and the pixel are **not saturated** (According: Point 3.2)
2. **Capture** an Image (According: Point 3.2)
3. Click on the right side of the captured image the on Button shown in Panel A; Area 1 and select a tool to draw and **Region of Interest (ROI).**
   Hint: Select the button "Draw Polygonal ROI" to draw a ROI free hand.
4. **Repeat** Step 3. for all different fluorophores (channels). An example of possible ROIs is shown in Panel B. It is very important to be **accurate** (use zoom) by selecting the ROI and have as less overlap with other excitation as possible.





5. Change to "**Vertical Scale Normalization**" by pressing the Button 1 in Panel D.
6. **Select all ROIs** you want to unmix. An Example is shown in Panel C, Area 1
7. By pressing the Button "Unmix" [Panel C; Area 2] the program will separate the image based on the spectrum of the selected ROIs.



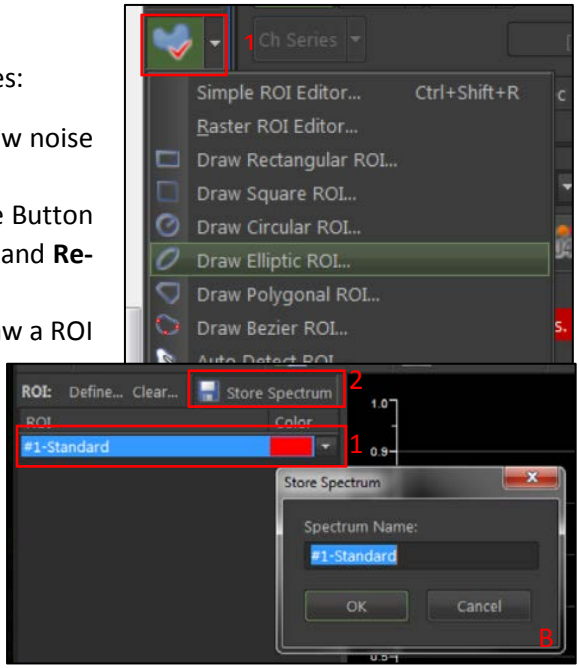
4.3. Method 2: Based on Control Spectrum

1. Make one **control sample** for each fluorophore

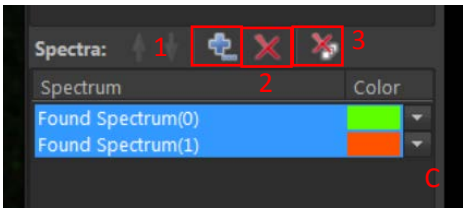
   Repeat the following Steps for each of these samples:

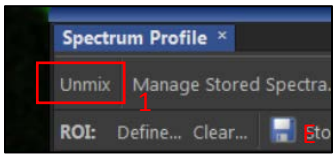
2. Configure the system and capture an image with low noise and no pixel saturation (According: Point 3.2)
3. Click on the right side of the captured image on the Button shown in Panel A; Area 1 and select a tool to draw and **Region of Interest (ROI).**
   Hint: Select the button "Draw Polygonal ROI" to draw a ROI free hand.
4. **Select** the ROI (as shown in Panel B; Area 1) and press the Button 2 ("Store Spectrum") to **save** the selected spectrum. In the new appeared window enter a **name** for the spectrum.

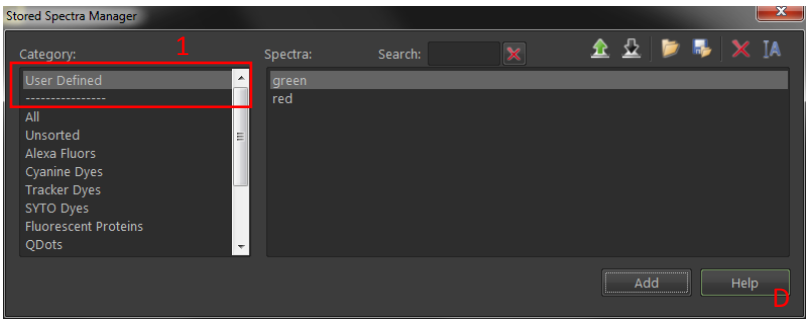
   Comment: Do this for each fluorophore, reduce the noise and eliminate the pixel saturation



5. After you have a control spectrum for each fluorophore you can **capture an image** of your real sample with all probes in one sample (Reduce noise and eliminate the pixel saturation).
6. **Add all Spectra** you need for this sample by clicking the Button 1 shown in Panel C. A new window will appear [Panel D]. Choose your spectra in the category "User Defined" *[Panel D; Area 1].*
7. To **delete a single** spectrum out of the selection click Button 2 shown in Panel C.
8. To **delete all spectra** out of the selection click Button 3 shown in Panel C.
9. After you added all Spectra you need (example in Panel C; Area 4) click the Button "Unmix" shown in Area 1 in Panel E.

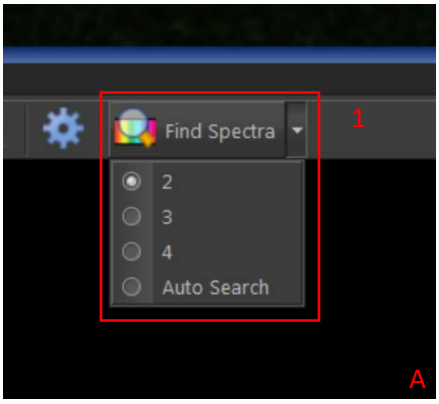






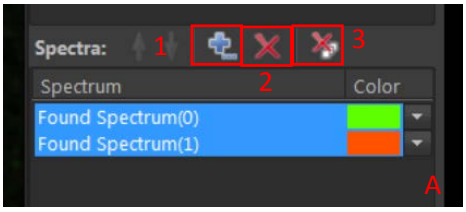
4.4. Method 3: Find Spectrum automatically

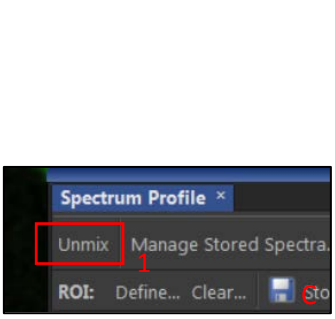
1. After Point 1 (Pre-setup) is done, configure the system (Laser power, Scanning speed, Averring etc.) such that the **noise is on a low level** and the pixel are **not saturated** (According: Point 3.2)
2. **Capture** an Image (According: Point 3.2)
3. Click on the arrow Button right beside "Find Spectra" and choose the **number of spectra** you want to find *[Panel A; Area 1]*
4. After that click on the button "Find Spectra" to analyze the image based on the setting in Step 3
5. The analyzed spectra will be **shown** in the Window named "Spectrum Profile"
6. If you agree with the analyzed spectra click the button "**Unmix**" to separate the spectra.

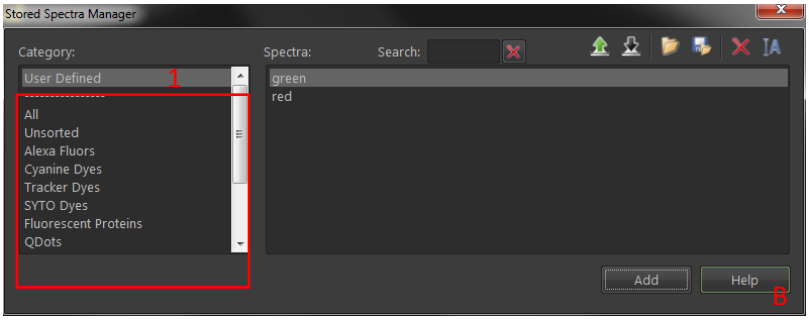


4.5. Method 4: Based on factory spectra database

1. After Point 1 (Pre-setup) is done, configure the system (Laser power, Scanning speed, Averring etc.) such that the **noise is on a low level** and the pixel are **not saturated** (According: Point 3.2)
2. **Capture** an Image (According: Point 3.2)
3. **Add all Spectra** you need for this sample by clicking the Button 1 shown in Panel A. A new window will appear [Panel B]. Choose your spectra out of the categories based on *[Panel B; Area 1].*
4. To **delete a single** spectrum out of the selection click Button 2 shown in Panel A.
5. To **delete all spectra** out of the selection click Button 3 shown in Panel A.
6. After you added all Spectra you need (example in Panel C; Area 4) click the Button "Unmix" shown in Area 1 in Panel C.







Comments:

1. The most precise method is "Method 2: Base on control spectrum" but it takes the most time.
2. A good compromise between precision and speed are "Method 1: Based on Region of interest (ROI)" or "Method 4: Based on factory spectra database"

5 Troubleshooting

If after starting the Software an error message appears and one or more of the device are not recognized by the software then follow the next steps:

1. Turn off the **Software** → Wait 30 sec.
2. Turn off the **control unit** *[Panel A, Field 1]* (Step 3 of the Turn On/Off procedure; Page 3) → Wait 1 min
3. Make sure all **7 power switches** are turned on except the control unit *[Panel A, Field 1]:*
    a. If that is the case **skip** to step 4
    b. If that is not the case: turn on **all missing device** except the control unit (Step 3 of the Turn On/Off procedure; Page 3) by following the turn on procedure (see 1. Turn On/Off procedure; Page 3).
    IMPORTANT: DON'T TURN EVERTHING OFF.
4. Wait 1 min
5. Turn on the **control unit** *[Panel A, Field 1]* → Wait 1 min
6. Start **Software**

Comments:

1. **If the problem still appears ask** Andy Schaber (Facility manager) or Ferdinand Ziegler (TA in Facility) for support.
2. If you coincidental **switch off the Metal halide lamp** (Step 4 of the Turn on Procedure; Page 3) when don't turn it immediately on again. That can damage the light source. Wait at least 15 min before you turn the lamp on again. You can use the Software without the metal halide lamp but you can't use the Epi fluorescence to find the sample.