UNIVERSITY of GUELPH

Advanced
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Molecular & Cellular Imaging Facility

Nikon Eclipse Ti2

Contents

March 2022

Nikon Eclipse Ti2

The inverted Nikon Eclipse Ti2 microscope, equipped with a high precision encoded stage and Perfect Focus System is specifically designed for automated acquisition of large image files (time lapse, stitched images, high-content screening), and fast ratiometric imaging. Furthermore, a laser engine makes the system capable of TIRF and FRET imaging.

Camera: Hamamatsu Orca Flash4.0 LT camera

LED excitation lines for fluorescence: 395 nm, 440 nm, 470 nm, 508 nm, 555 nm, 640 nm

Fluorescence filters for DAPI, CFP, GFP/FITC, YFP, RFP/TRITC, CY5

LED-based Fura-2 illuminator for ratiometric Ca^{2+} imaging: 340 nm and 380 nm

Laser engine with 488 nm and 561 nm lasers

Objectives: 4x, 10x (Air); 20x, 40x (Air; ELWD, long working distance); 60x, 100x (Oil), 100x TIRF (Oil)

Software: NIS Elements - with modules AR (Advanced Research) and HC (High Content), including high content analysis (HCA)

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User Guidelines

1. When you begin to use these facilities please provide a Billing Authorization Sheet including the supervisor's signature and the Trust Fund Account number.

2. Before any unsupervised access is granted, users must enroll in supervised training sessions during which they will review with the confocal manager how to operate the equipment properly and safely. The time required for the training sessions will vary depending upon the user's demonstrated competency with the equipment. Billing will be at the "Training" fee rate.

3. When using these facilities you must clearly write in the sign-up book the date, your name, department, log on and log off time, and total number of hours you used the equipment.

4. For any planned after hours use of the system, please make arrangements with the confocal manager for access to the hallways.

5. Users are expected to bring all their own supplies including pipets, slides, coverslips, computer disks, etc. However, the facility will provide immersion oil and lens paper.

6. Files saved to computer hard drives must be removed as soon as possible. All computer hard drives will be cleared on a regular basis - it is the users' responsibility to manage their own image files. USB keys are not allowed on any of the instrument computers! The facility offers a variety of other options for data transfer.

7. Please notify the confocal manager immediately of any problems that you encounter with the equipment - it is essential that we work together in taking care of the facility. Improper care of the equipment will result in rejection of access to the facility.

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Nikon Eclipse Ti2 Microscope

General Information & Care

Objectives

Lenses	Immersion	Refractive Index	Numerical Aperture (NA)	Coverslip µm (#)	Recommended Step Size for Image Stacks (µm)
4 x	Dry	1	0.13	< 1.2	29.6
10 x	Dry	1	0.3	< 1.2	5.6
20 x	Dry, ELWD	1	0.45	0-2	2.5
40 x	Dry, ELWD	1	0.6	0-2	1.4
60 x	Oil	1.52	1.4	0.17 (1.5)	0.3
100 x	Oil	1.52	1.3	0.17 (1.5)	0.3
100 x TIRF	Oil	1.52	1.49	0.17 (1.5)	0.3

Immersion oil

Use only the immersion oil (Type F) provided by the facility. Do not mix the immersion oil with other substances including immersion oil from another source. If you used any other immersion oil during previous observations with a different microscope, remove all traces with Windex.
Carefully apply the immersion oil to the lens.

Cover slip

Sealing the coverslip completely with nail polish or other sealing materials is highly recommended. This prevents mixing of immersion oil and embedding materials, movement of the coverslip when moving the stage, contamination of the objectives, and moving embedded materials during z-sectioning. Be sure that any nail polish is completely dried before you place the slide onto the stage.

Lens care

After use, clean the lenses with lens paper only. Fold the paper and hold both sides of the paper, keeping the folded edge straight. Draw the folded edge back and forth over the lens surface. Repeat with another piece of lens paper and continue until there are no more traces of immersion oil on the paper. Use Kim Wipes to remove excess oil on the objective (around the lens).

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Nikon Eclipse Ti2
Startup and Sample Setup

System Startup

1) Hamamatsu camera (Fig. 1, arrow)
2) Microscope (Fig. 2, arrow)
3) Computer, no password required
4) Start NIS Elements and log into your lab account, select 'Widefield'
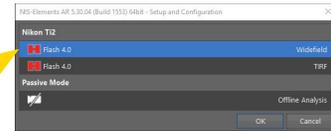
Accessory equipment

Lumencor: LED light source (Fig. 1B), currently with 4 excitation wavelengths: 395 nm, 470 nm, 550 nm, and 640 nm. The LEDs are constantly on standby. Please don't switch off the power supply.

Specialized LED-based Fura-2 Illuminator: The Fura-2 illuminator excites at 334 nm and 380 nm with a fast excitation wavelength switcher. It is used for ratiometric Ca^{2+} imaging.

Microscope Controller: Core Power Supply (Fig. 1C) – this is also permanently on standby, there's no need to switch it on or off.

Camera: The system has a monochrome Hamamatsu Orca Flash 4.0 for fluorescence or black/white transmission imaging (Fig. 1A).

Stage: The stage is fully motorized and the software gets the coordinates automatically. It is encoded for high precision but still fast. Two inserts are available: one for regular slides and petri dishes and one for well plates.

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Sample Setup and Observation With the Microscope

The microscope is fully automated and all functions can be controlled through the software. Therefore, you will likely not need to use the buttons on the microscope with the exception of setting the Köhler Illumination. Nevertheless, a quick overview of the controls available is given here (Fig. 3).

Please also refer to Fig. 9 on page 9 for setup using the NIS software controls (recommended!).
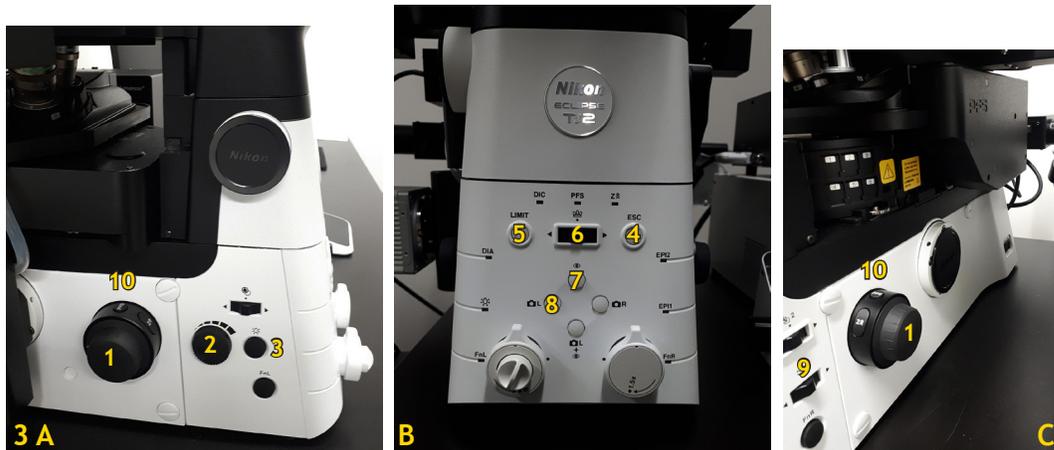
1 - Focus Wheel	6 - Change Objectives
2 - Light Intensity	7 - Ocular Observation
3 - Transmitted Light On/Off	8 - Camera Observation
4 - Escape Button	9 - Fluorescence Filter Wheel
5 - Limit (= upper threshold) Button	10 - PSF Button

Tilt the microscope pillar carefully back, place your slide, petri dish, or well plate on the stage and - if you use any of the oil immersion objectives - add the immersion oil (carefully place a drop of oil on the lens). Bring the pillar carefully back down. Select 'Eyepieces' as observation mode (Fig. 3B - 7; Fig. 9B).

If you use the 20x or the 40x ELWD (Long Working Distance) objectives (Fig. 4), you can not only image through a glass coverslip but also through relatively thin plastic (e.g., specific well plates). To achieve this, both objectives have a correction collar (Fig. 4, arrows) that needs to be adjusted for your sample (glass - setting closer to '0'; plastic - setting closer to '2').

The X/Y of the stage drive is the joystick on the controller box (Fig. 5A). Switching between fast and precise movement is done by a click on the 'XY' button (Fig. 5B) - the speed is indicated by the number of arrows on the controller display ($_\wedge$ $\hat{}$).

Using the focus wheels on the side of the Nikon controller box (Fig. 5C), move the objective nosepiece up until your sample is in focus (towards you = up, towards the wall = down). Switch to 'Fine Focus' by clicking on the 'Z' button on the controller box (Fig 5D). Fine and coarse focus are also indicated on the display ($_\wedge$ $\hat{}$).

Centre your sample. Switch the joystick to slow movement.

Observe through the oculars, find your ROI (region of interest) and change to the objective you will use for imaging (Fig. 3B - 6; Fig. 9A).

Adjust the focal plane again in your sample with the fine focus. Once your sample is in focus, press the 'Limit' button (Fig. 3B - 5) to set it as elevation limit. Pressing and holding the 'Limit' button will release the elevation limit setting. Press the 'Reset Z' button (Fig. 5E) on the on the controller box to reset the reative Z coordinates to Zero.

You now have saved your focal plane as 'upper threshold' and can move the nosepiece down quickly by pressing the 'ESC' (= Escape) button (Fig. 3B - 4; Fig. 9G), e.g., to change your sample or add immersion oil. To bring the objectives back up to your focal plane, press the 'ESC' button again.

Köhler Illumination

Köhler Illumination provides homogeneous illumination of your specimen without stray light. You get images with optimum contrast and resolution. Please do this in the BF setting!
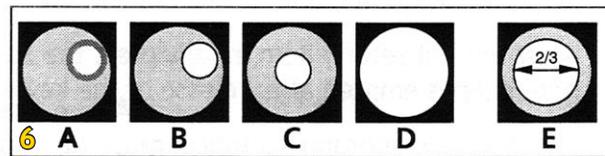

These are the steps (Figs. 6, 7):

    Focus the image

- A   Close the field diaphragm (Fig. 7, FD), adjust the aperture diaphragm (Fig. 7, AP) if necessary.
- B   Adjust condenser height with condenser focus wheel (Fig. 7, arrows) until the edge of the field diaphragm is in focus.
- C   Centre the field diaphragm with the screws on the condenser (Fig. 7, *).
- D   Open the field diaphragm (Fig. 7, FD) until the edge just disappears from the field of view.
- E   Adjust the aperture diapragm (Fig. 7, AP) so that it is two-thirds open.

Once you have adjusted these settings, you can continue with NIS Elements if you want to do transmitted light imaging.
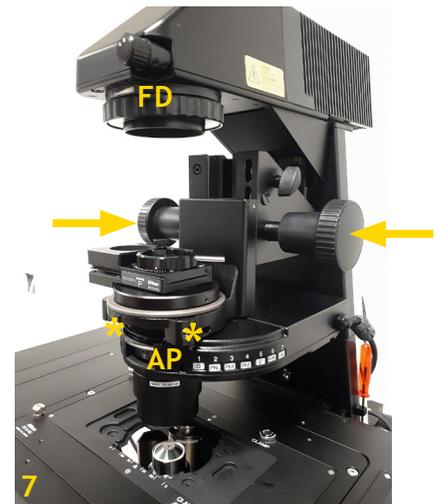
If you have fluorescently labeled samples, switch off the transmitted light (Fig. 3A - 3, Fig. 9E), select the excitation line and filter for your dye/fluorescent protein (Fig. 3C - 9) by pushing the Fluorescence Filter switch either towards the wall or towards you and open the Fluo Shutter by pressing on the Fluorescence Filter switch (Fig. 3C - 9). All this will be done automatically if you select one of the pre-configured fluorescent light paths (Fig. 11)! The installed fluorescence filters are for DAPI, CFP, GFP/FITC, YFP, RFP/TRITC, and CY5. Check whether the signal strength is sufficient, find your region of interest and adjust the focal plane if necessary.

If you used the microscope controls to set up fluorescence observation, make sure to close the Fluo Shutter again (Important!) and continue with NIS Elements.

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NIS Elements Acquisition Setup

Program Interface

All functions are accessible from the Main Menu at the top of the screen (Fig. 8C).

In the right tool area (Fig. 8A) you find the Main Tool Bar (E), the camera settings (Flash 4.0), the OC (Optical Configuration) panel, which has the pre-configured light paths, the Spectra pad with the LED emission controls, the microscope control panel, and the LUTs (look-up table) to adjust the image intensity display.

The main screen area (Fig. 8B) is used to display your images and has a top and a side tool bar (Fig. 8D) with display and annotation options. The icons in the tool bars change depending on your image (Z-stack, multichannel, etc).
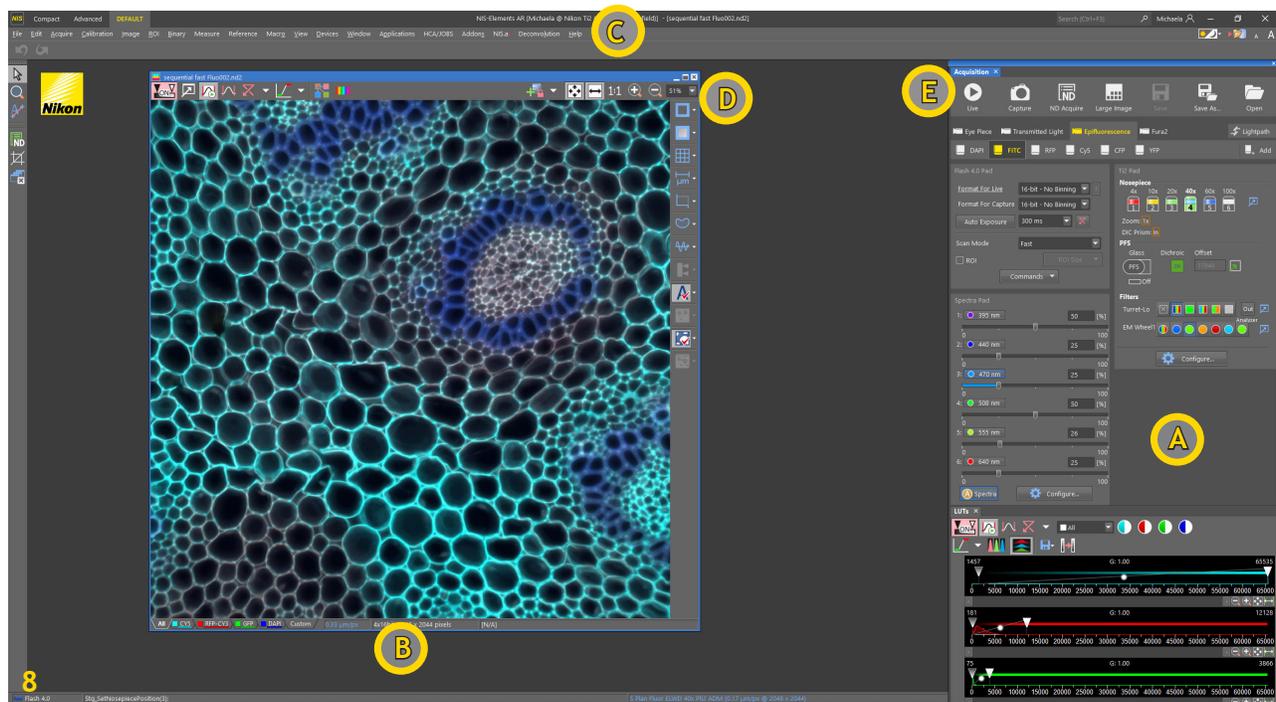

A - Right Tool Area
B - Main Screen Area
C - Menu Bar
D - Top and Right Display Tool Bars
E - Main Tool Bar

Basic Image Acquisition

Microscope Settings

Once the software is started, refer to the Optical Configuration (OC) Panel (Fig. 9A) and the 'Ti2 Pad' window (Fig. 9B) to set your light paths: you can select the objective, camera or ocular observation, and adjust light intensity. It is recommended to use the NIS controls during your initial sample setup (see pages 6-7).

Select the objective and 'Eyes Brightfield' (Fig. 9A, arrow) for your sample setup, all other parameters are saved with the light paths.
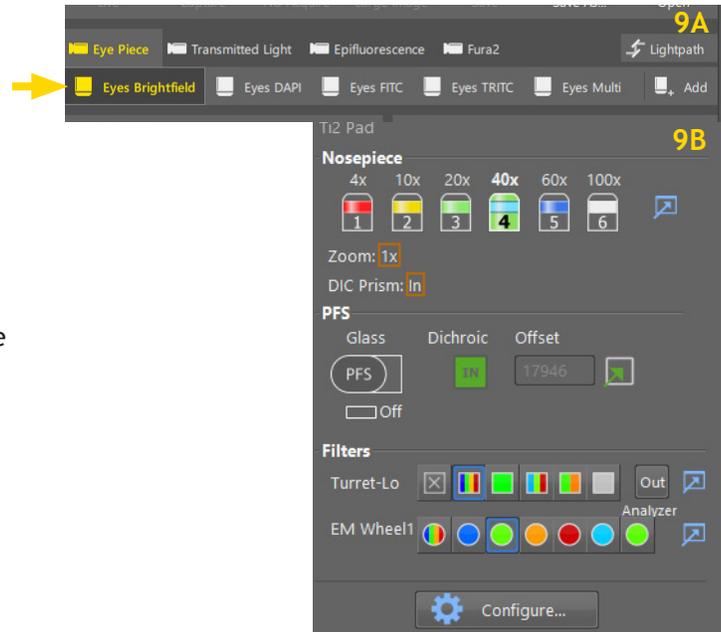
Acquisition
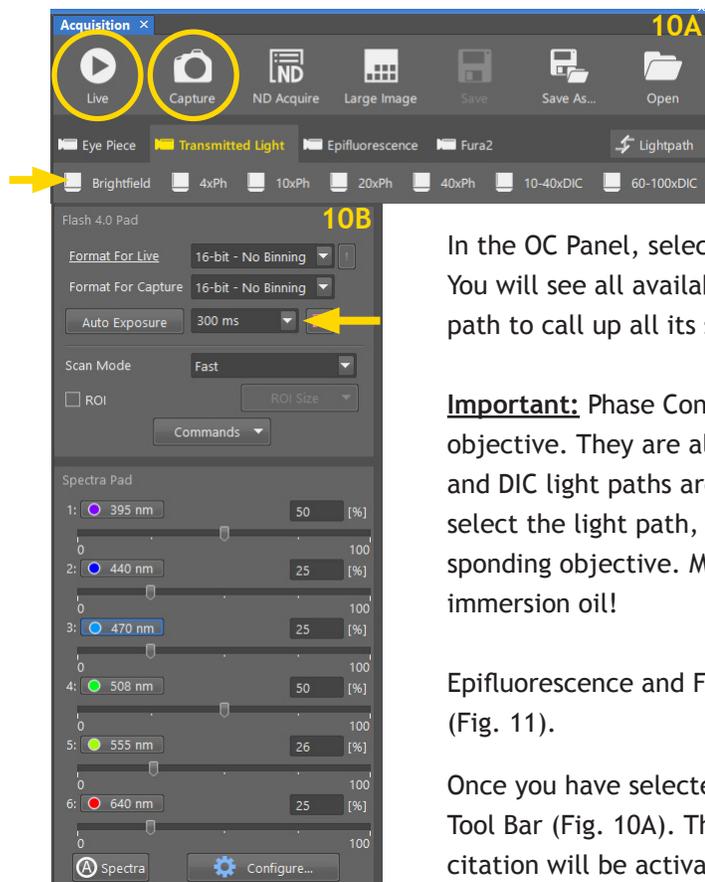
In the OC Panel, select 'Transmitted Light' to switch to camera observation. You will see all available pre-configured light paths. Click on the desired light path to call up all its settings (Fig. 10A, arrow).

Important: Phase Contrast and DIC require specific correction optics for each objective. They are also not available for all objectives. Therefore, the Phase and DIC light paths are individually programmed for each objective. If you select the light path, the microscope will automatically switch to the corresponding objective. Make sure you don't contaminate the dry objectives with immersion oil!

Epifluorescence and Fura2 light path settings are also selected in the OC panel (Fig. 11).

Once you have selected the light path, you need to click on 'Live' in the Main Tool Bar (Fig. 10A). The shutter will open and the transmitted light or LED excitation will be activated. Adjust the exposure time in the drop-down menu in the camera control panel (Fig. 10B, arrow).

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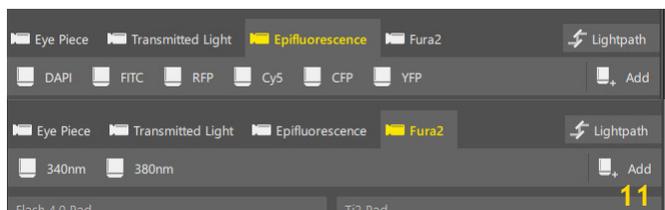
Since the camera is highly sensitive and has a range of 65,000 intensity values, you likely need to select 'Keep Auto Scale' in the 'LUTs' window (Fig. 12, arrow) in order to actually see your live image on the screen (most likely for fluorescence imaging). This will only change the display of your image on the screen, the camera settings will not be changed.

Focus your sample and find the visual location (i.e., ROI, region of interest).

Once your settings are optimized, select the camera icon ('Capture') in the top tool bar (Fig. 10A) to acquire a single channel image.
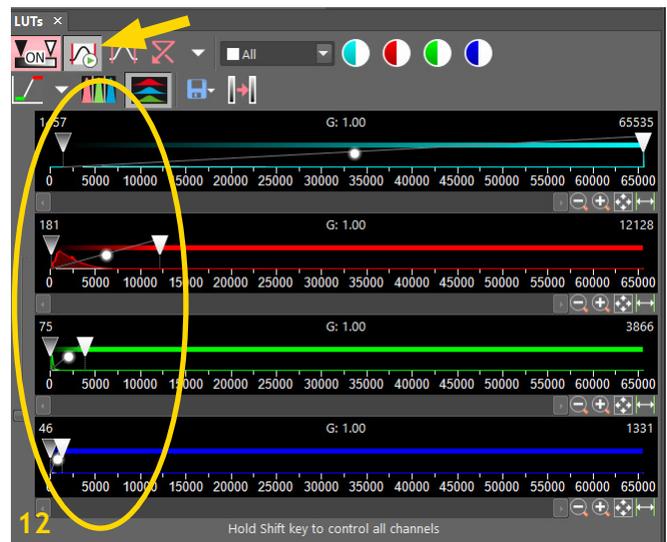
Advanced Image Acquisition

If you want to take a multichannel image or an image series like a Z-stack, time series, or stitched image, you need to define these actions in the ND Acquisition setup. Click on the 'ND' icon on the left (Fig. 13, circle) or in the Main Tool Bar (Fig. 10A, 13A) to open the 'ND Acquisition' setup window (Fig. 14). You can dock this window to the bottom centre area or keep it as separate window (Fig 13B).

Important: 'ND Acquire' in the Main Tool Bar (Figs. 10A, 13A) represents only a selected number of options with basic settings. Select the 'ND acquisition' on the left for full capability. Therefore, all acquisition steps are explained based on the windows for the left 'ND acquisition' icon (Fig. 13, circle). The simplified 'ND Acquisition Wizard' (Fig. 14) is always shown for reference.

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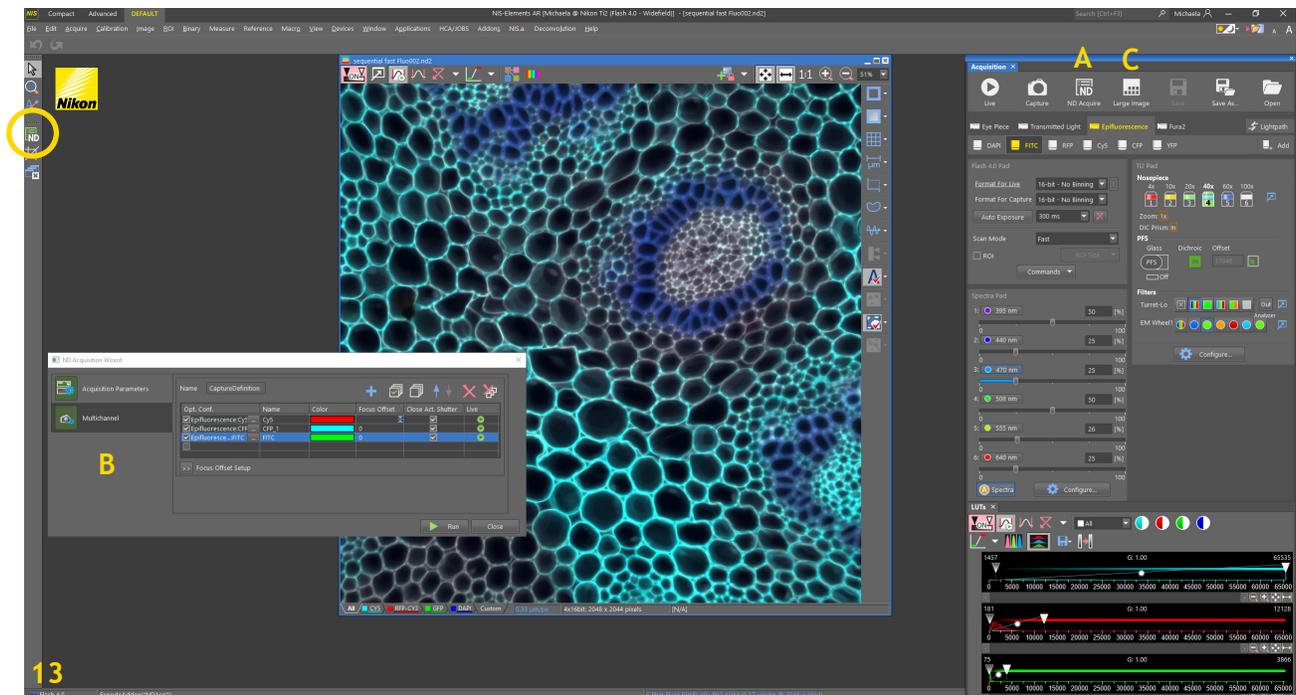
The options for ND Acquisition appear in the main 'ND Acquisition Wizard' window (Fig. 14) and at the top of the 'ND Acquisition' window (Figs 15-19) if selected.

They are:

Timelapse - Multipoint - Z-stack -

Multichannel - Large Image (left ND icon only and separate button in the Main Tool Bar (Fig. 10A).
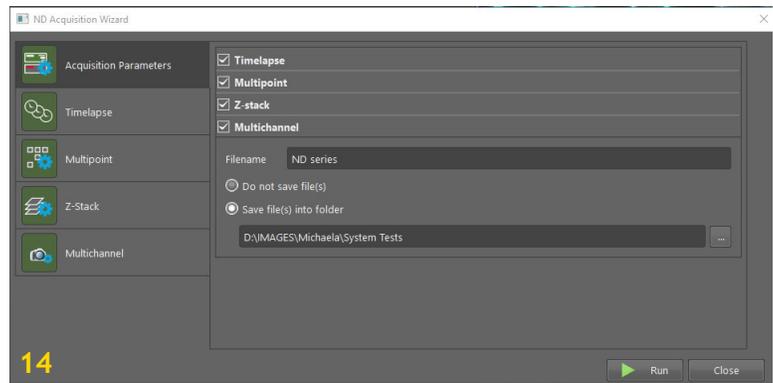
Multichannel

Fluorescent signals can be captured sequentially and merged into one overlay image. Transmitted light channels can also be included in the sequence.

Multichannel imaging is perfectly represented in the ND Acquisition Wizard on the Main Tool Bar.
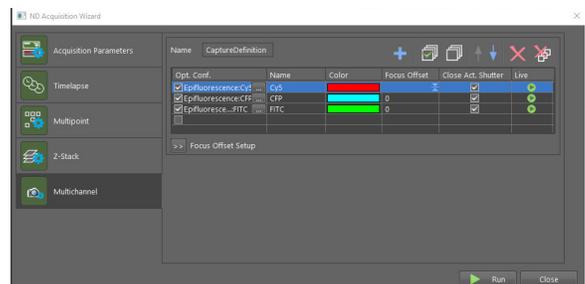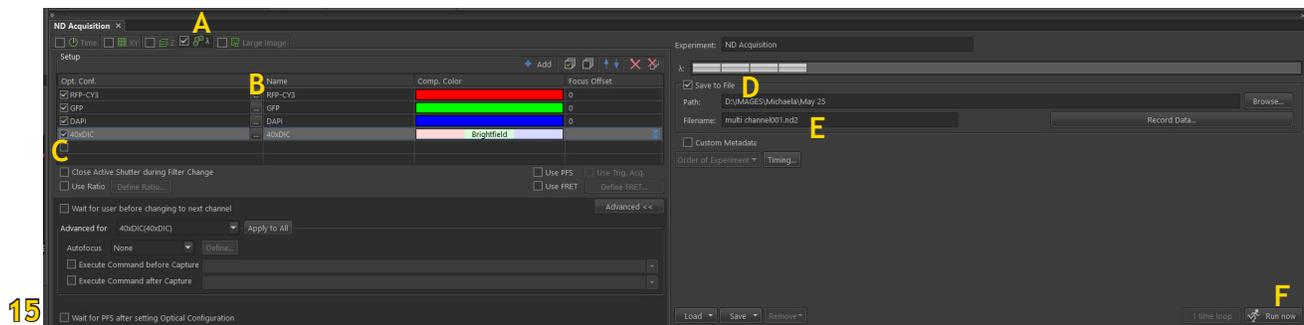
- Select the 'lambda' tab (or 'Multichannel') in the ND Acquisition window (Fig. 15A) and check-mark the box to activate this protocol.
- Select those channels you want to acquire ('...' and drop-down menu, Fig. 15B). Select the fluorescence channels in the order from longest to shortest wavelengths to minimize interferences!
- Make sure the box beside each channel you want to acquire is checked! (under optical configuration, Fig. 15C).
- Select 'Save to File' and define the path and folder where to save your image (Fig. 15D). This option is found under 'Acquisition Parameters' in the ND Acquisition Wizard.
- Define the file name (Fig. 15E) and press 'Run now' (Fig. 15F).

Z-Stack

A series of images from different focal planes in a sample can be acquired and, e.g., converted to a 3D model of the specimen.
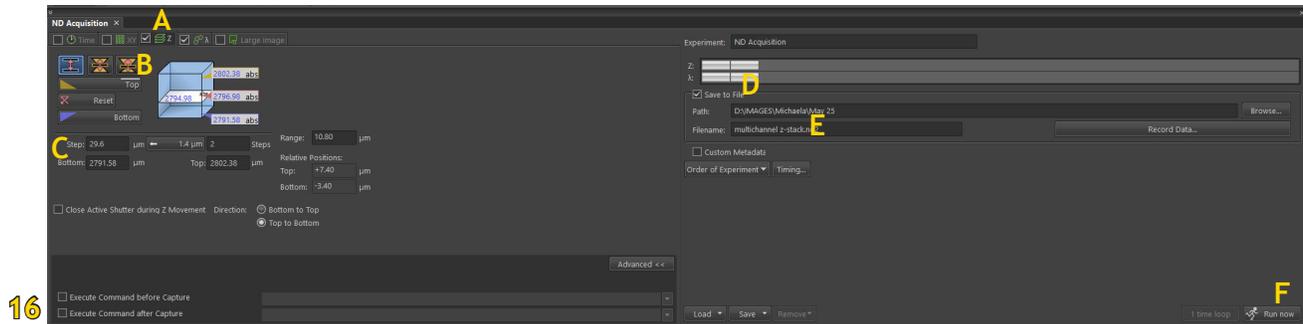
- Select the 'Z' tab (or 'Z-stack') in the ND Acquisition window (Fig. 16A) and check-mark the box to activate this protocol.
- Select how to define the range of your Z-Stack (Fig. 16B, there are 3 options - see below).
- Define the step size (Fig. 16C) - the system will automatically assign a predefined step size depending on the objective used. Recommended step sizes are listed in the table on page 4.
- Select 'Save to File' and define the path and folder where to save your image (Fig. 16D). This option is found under 'Acquisition Parameters' in the ND Acquisition Wizard.
- Define the file name (Fig. 16E) and press 'Run now' (Fig. 16F).
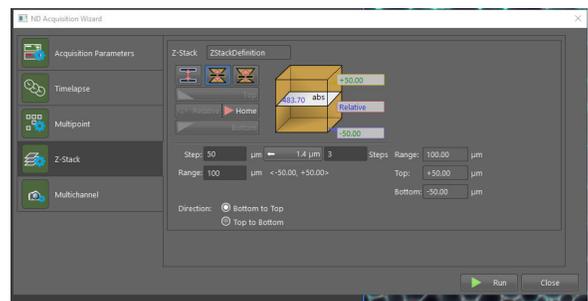
Define range by setting top and bottom plane

- Select 'Live Stream', focus and adjust exposure
- Move the Z drive to the top position of your stack and press the 'Top' button
- Move the Z drive to the bottom position of your stack and press the 'Bottom' button

Define range by symmetric distance from the current (= Home) position

- Select 'Live Stream', focus and adjust exposure
- Press the 'Home' button to define the home position
- Specify the Z-range in μm
- The software will acquire images in equal distances to the home position

## Define range by asymmetric distance from the current (= Home) position

- Select 'Live Stream', focus and adjust exposure
- Press the 'Home' button to define the home position
- Specify the Z-range with 2 values: one for 'Below' and one for 'Above' in μm
- The software will acquire images in those distances to the home position

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Large Image

   With the Large Image option, you can acquire a number of individual images (tiles) and merge them into a large image if the ROI exceeds the camera field of view. To do this, it is possible to define tile regions and positions.
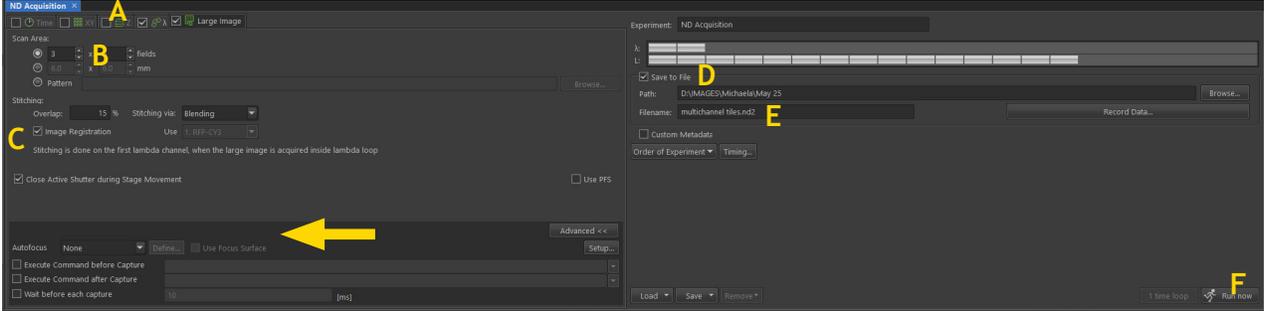
- Select the 'Large Image' tab in the ND Acquisition window (Fig. 17A) and check-mark the box to activate this protocol.
- Select how to define the area of your Large Image (Fig. 17B, there are 3 options - details see below).
- Define how the tiles are stitched together (Fig. 17C); a 10-15% overlap between the tiles is recommended.
- Select 'Save to File' and define the path and folder where to save your image (Fig. 17D).
- Define the file name (Fig. 17E) and press 'Run now' (Fig. 17F).
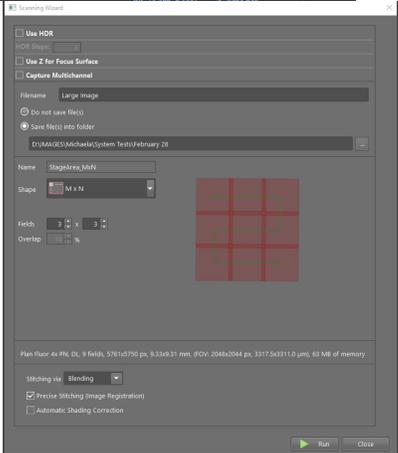
Depending on your sample and the size of your large image, it is recommended to create a focus surface. To do this, select 'Use Focus Surface' in the ND Acquisition Window (Fig. 17, arrow). Then define several positions across your large image region and focus the image there. Select 'Add' and they will be added with XYZ position information. The software will extrapolate these data to create a focus surface over the entire region.
Important: Large Image is not represented in the ND Acquisition Wizard - it has it's own icon, the 'Scanning Wizard' in the Main Tool Bar (Fig. 10C).

Define area by number of tiles in X and Y direction
Find the ROI - select the centre area – define the number of tiles in X and Y direction – this is simple and quick, but it is very general.

Define area by size
Find the ROI - select the centre area – define the area in millimetres - this is also simple and quick, but very general.

Define area by a specific pattern
Find the ROI - select the centre area – select the 'Pattern' button in the ND Acquisition window - click on the 'Browse' button - select one of the saved Large Image patterns.

Time Lapse

A sequence of images can be captured over a period of time to create a time lapse series.
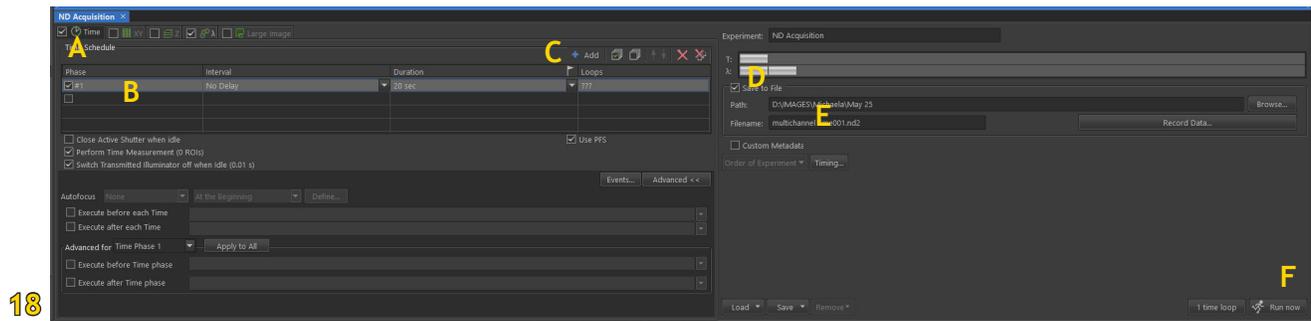
- Select the 'Time' tab (or 'Timelapse') in the ND Acquisition window (Fig. 18A) and check-mark the box to activate this protocol.
- Define the interval and duration of your image acquisition; make sure the box beside each time phase you want to acquire is checked (Fig. 18B).
- You can combine consecutive time acquisition protocols with different intervals and durations by clicking 'Add' (Fig. 18C).
- Select 'Save to File' and define the path and folder where to save your image (Fig. 18D). This option is found under 'Acquisition Parameters' in the ND Acquisition Wizard.
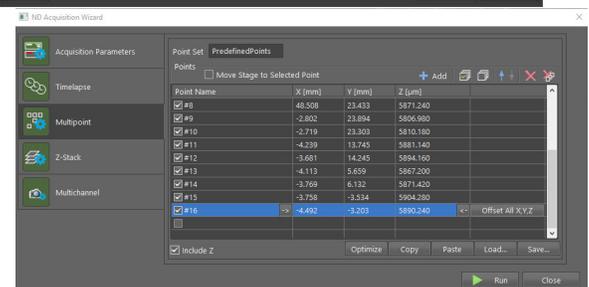- Define the file name (Fig. 18E) and press 'Run now' (Fig. 18F).
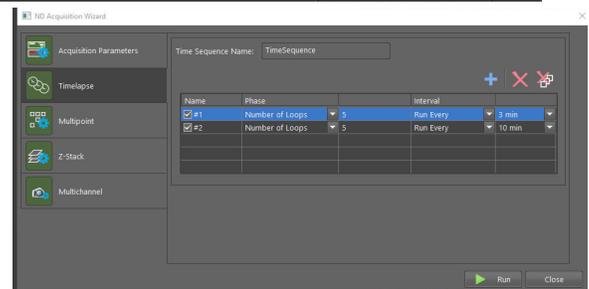
Multipoint Imaging
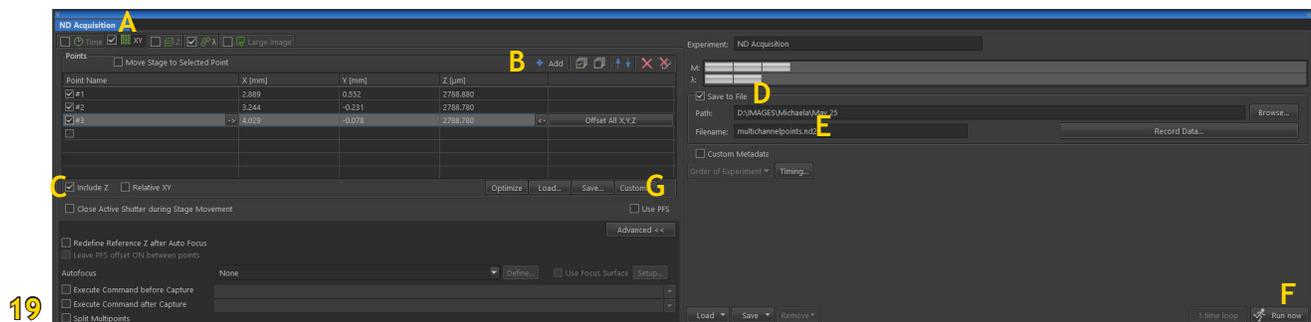
Images from different XY coordinates in your sample can be acquired, e.g. if you work with samples in well plates.

- Select the 'XY' tab (or 'Multipoint') in the ND Acquisition window (Fig. 19A) and check-mark the box to activate this protocol.
- Define your multiple positions - there are several options, see details below.
- Select whether to include the Z position (Fig. 19C) - it is recommended to include the Z position, especially in live cell imaging, as the focal planes might differ slightly.
- Select 'Save to File' and define the path and folder where to save your image (Fig. 19D). This option is found under 'Acquisition Parameters' in the ND Acquisition Wizard.
- Define the file name (Fig. 19E) and press 'Run now' (Fig. 19F).

Define points manually

Move the stage to the positions of interest, focus if necessary, and click 'Add' (Fig. 19B).

20A

20B
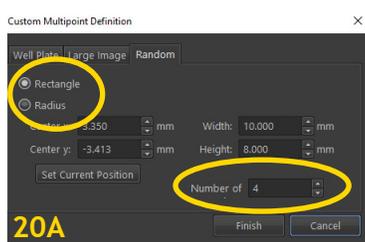
Define points randomly (Fig. 20A)

Click on the 'Custom' button (Fig. 19G) - select the 'Random' tab - select the size and shape of the area (rectangle or circle) to be filled with random points and define the reference XY coordinates needed - specify the number of random points - click 'Finish' .
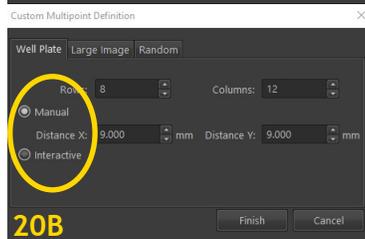
Define points in a carrier (Fig. 20B)

If your sample is in a wellplate, select 'Custom' (Fig. 19G), in the new window, select 'Manual' (if you know the distance between the wells) or 'Interactive'. This method lets you specify the number of fields to be scanned and starts a wizard in which you will define the top-left and bottom-right corner of the wellplate. The distances between the wells are then calculated automatically.

Working with carriers

NIS Elements has a large number of templates for commercially available carriers (Fig. 21A) that can be accessed through the Main Menu: 'View' - 'Acquisition Controls' - 'Wellplate and Slide Navigation'. Once you have selected the carrier, you can navigate within this template (Fig. 21B).

21A

21B
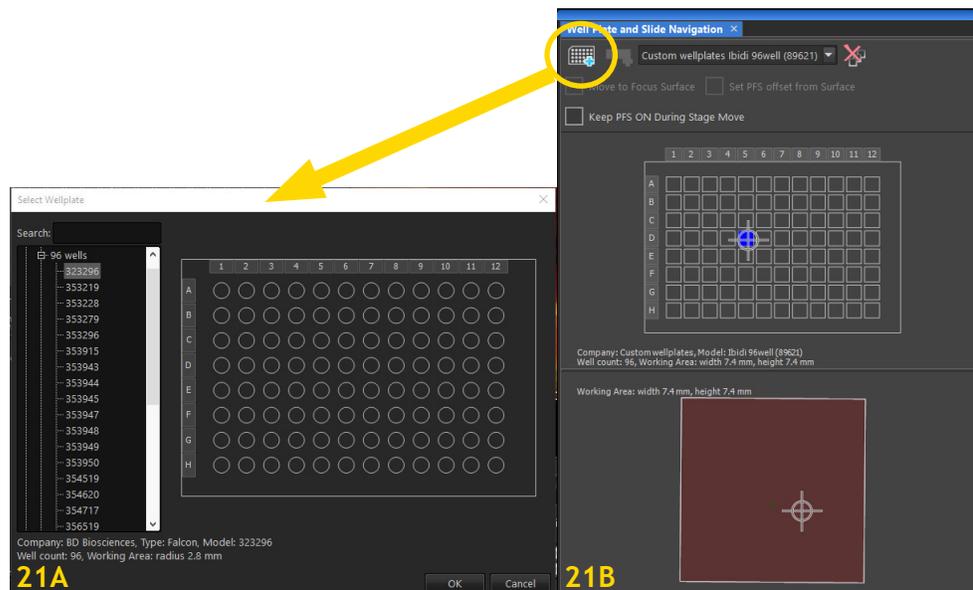
Combined ND Acquisition

Two or more of the ND Acquisition Dimensions can be combined. A typical example is Fura-2 imaging over a specific time.

If you use the left 'ND' icon:

Fig. 22

- Define all ND Acquisition protocols you want to include and make sure the checkbox in each tab is selected (Fig. 22, arrow).
- Based on those settings, an overview shows how the .nd2 file structure will look like (Fig. 22, A).
- Select 'Order of Experiment' to select the item which defines the desired order of loops in the .nd2 file (Fig. 22, B).
- Select 'Save to File', specify path and file name as described above (Fig. 22, B).
- Click 'Run now' (Fig 22, C).
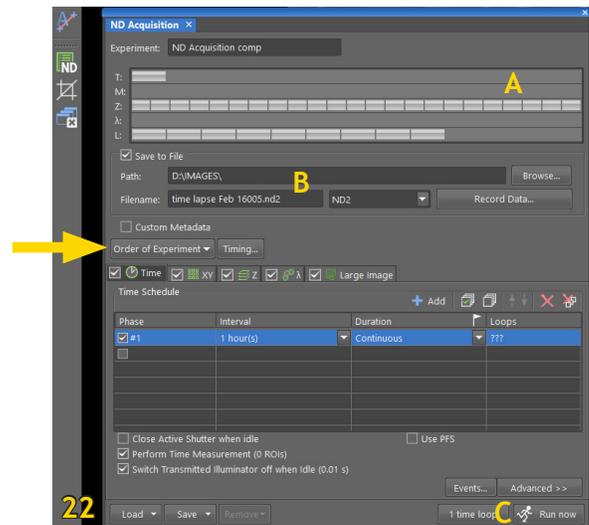
If you use the 'ND Acquisition wizard' in the main tool bar:

- Under 'Acquisition Parameters, check all ND Acquisition protocols (Timelapse, Multipoint, Z-stack, Multichannel) that you want to include (Fig. 14).
- Based on those settings, the relevant tabs will appear on the left of the window - you need to select each tab and set the parameters as described above
- Select 'Save to File', specify path and file name as described above (Fig. 14).
- Click 'Run' (Fig 14).

In order to combine the 'Scanning Wizard' and the 'ND Acquisition Wizard' you need to select 'Addons' in the Main Menu (Fig. 8C). There, you'll find the options:

Large Images:	Scanning Wizard (= icon in Main Tool Bar)
	Scanning Wizard EDF (combines the 'Scanning Wizard' with the option to take Z-Stacks)
ND Acquisition:	ND Acquisition Wizard (=icon in Main Tool Bar)
	ND Acquisition Wizard + Large Images (Timelapse, Z-Stack, Multichannel in the 'ND Wizard' can be combined with the 'Scanning Wizard')

For complex and long acquisition protocols an 'ND Progress Window' will appear which displays the overall progress, time elapsed and time remaining in the experiment as well as more details on status, remaining disk space, and the option to pause the acquisition.

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Perfect Focus System (PFS)

The PFS is a focus maintenance system that automatically corrects focus shift of the specimen or the stage due to temperature. It is most useful in long-term imaging sessions.

The PSF uses a mismatch in the refractive index to measure, track, and maintain the distance between the objective and the sample surface (i.e., cover glass, well plate bottom, etc.).

It has an optical offset function, which allows for manual adjustment of the focal plane position within the specific range for each objective.

With the exception of the 4X objective, all objectives are compatible with the PFS. The system will give an error message on the display of the controller when the objective can't be used with PSF.

Perfect Focus will be disabled when Z-Stack images are acquired!

The Perfect Focus System is not very useful with ...

- Fixed, mounted specimen and oil immersion objectives, since the mounting medium mostly has the same refractive index as glass and immersion oil.
- Thick samples or samples that generally strongly scatter light.
- Samples in plastic dishes other than the ones recommended.
- Samples which have the cover glass or well plate bottom contaminated with dust, oil, etc.

Starting the PSF

- Start focusing your sample. When the objective comes into the PSF range, you will hear several short beeps and the PSF indicator flashes.
- Continue to focus until you have the correct focal plane.
- If the PSF indicator flashes, press the PSF button either on the microscope focus wheel or on the Nikon controller box (Fig. 3A,C - 10, Fig. 5F).
- This PSF reference point is different for each objective.

The status of the PSF is displayed on the PSF indicator on the front panel of the microscope:

Indicator light

|	
On	PSF on - perfect focusing is in progress
Flashing in slow intervals	PFS on - waiting for interface detection
Flashing at fast intervals	PSF off - perfect focusing is off but position is within range
Off	PSF off - perfect focusing is off, position is out of range

Offset adjustment

To account for different focal planes within your sample (e.g., when imaging several wells of a plate), you can further focus your sample while the indicator light is on, i.e., the PSF is on and in progress. When reaching the upper limit of the allowable range, a beep tone sounds. If you leave this range, the PSF will remain on but the indicator will switch to slow flashing.

The offset range depends on the objective.

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Viewing Options

Depending on your image (Multichannel, Large Image, Z-Stack) you can change how the data of your image are displayed in the tool bars at the top and right side of the centre area (Fig. 23). The options will vary depending on your image data. Some of the defaults are shown here.

From top to bottom
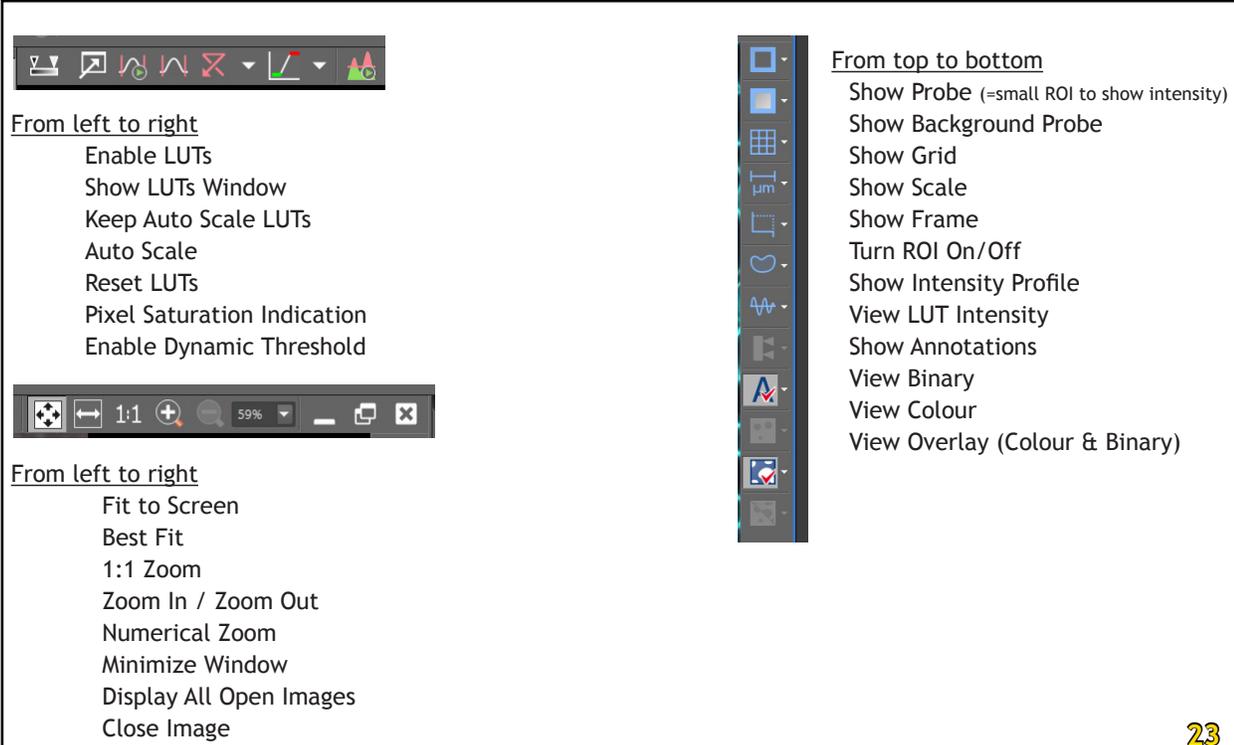
From left to right
- Enable LUTs
- Show LUTs Window
- Keep Auto Scale LUTs
- Auto Scale
- Reset LUTs
- Pixel Saturation Indication
- Enable Dynamic Threshold

Show Probe (=small ROI to show intensity)
Show Background Probe
Show Grid
Show Scale
Show Frame
Turn ROI On/Off
Show Intensity Profile
View LUT Intensity
Show Annotations
View Binary
View Colour
View Overlay (Colour & Binary)

From left to right
- Fit to Screen
- Best Fit
- 1:1 Zoom
- Zoom In / Zoom Out
- Numerical Zoom
- Minimize Window
- Display All Open Images
- Close Image

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If you right-click on the icons, or click on the arrow beside them, you will open the drop-down menu with more options for and properties of each tool.

Adding a scale bar to your image (Fig. 24)

Select the 'Show Scale' icon in the right panel. A scale bar will appear in your image. Click and hold the scale bar to move it around, right-click on the 'Show Scale' icon and select 'Properties' to change the length and appearance of the scale bar.

Switch view between the individual channels (Fig. 25)

The default display of a multichannel image is the overlay. Below your overlay image are the 'Channel tabs'. By selecting one of them, only this channel will be displayed.

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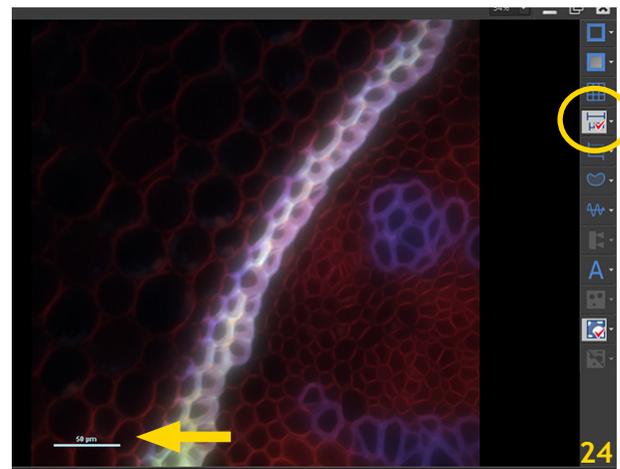
LUTs - Look-Up Tables (Fig. 26)

LUTs is a tool for image colour and brightness modifications. You can enhance the image for observation without changing any of the image data. If you select a specific LUT setting, it is saved along with the image file.

The best starting point is to select 'Keep Auto Scale' (Fig. 26A, applies auto scale permanently, also to live images) or 'Auto Scale' (Fig. 26B, adjusts the right slider position of all channels automatically).

But you may want to change the LUTs manually to equalize several images for observation:

Select ' Enable LUTs' (Fig. 26C)

The main parameters to change are

> Gamma Parameter - move the centre point of the gamma curve up or down (Fig. 26D) - although this is generally not recommended

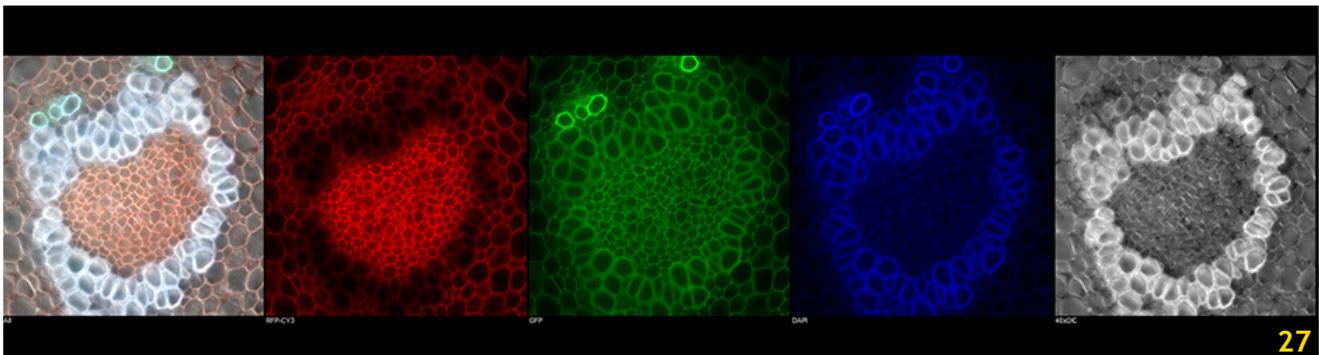> Intensity Range - move the sliders from the edges of the range towards the centre (Fig. 26E)

You can reset those values with a double-click on the graph or the 'Reset LUTs' button (Fig. 26F).

Up to 4 LUTs can be displayed simultaneously.
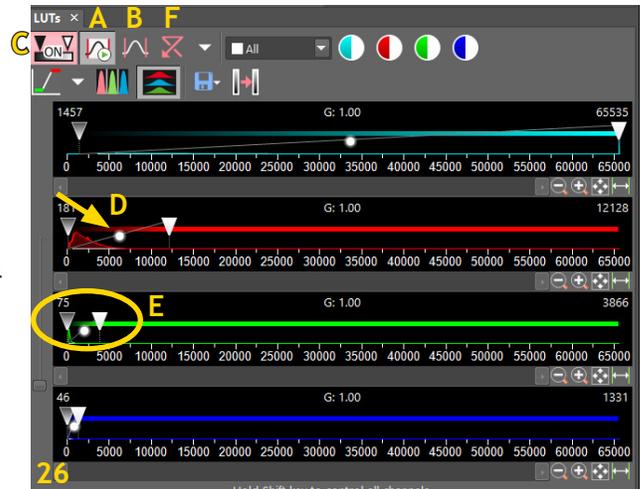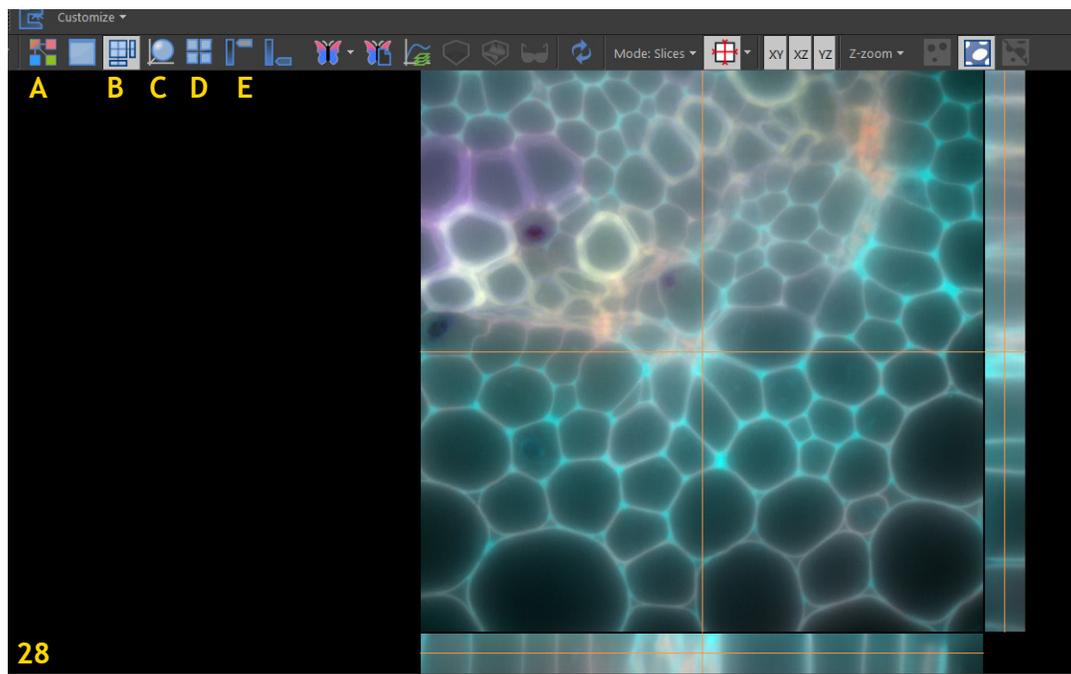

ND views

There are several ways to display your ND images. The most common options are listed below:

- Split Image View (Figs. 27, 28A) displays the individual channel images side by side in the display

- Slices View (Fig. 28B) - displays orthogonal XY, XZ, and YZ projections of the images sequence (Z-Stack or Time Lapse, then it's XY, XT, YT)

- Volume View (Figs. 28C, 29) - creates 3D model of the acquired object (Z-Stack)

- Tiled View (Fig. 28D) - displays individual frames of the selected dimension beside each other; one or two dimensions can be viewed at a time (Z-Stack, Time Lapse, or Multipoints)

- Maximum/Minimum Intensity Projection View (Fig. 28E) - analyzes all frames of one dimension and picks pixels with the maximum/minimum intensity values to be displayed in the resulting image (Z-Stack, Time Lapse)

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Volume Viewer

This module allows you to view and move your Z-Stack as a 3D model and navigate through it from all angles and sides (Fig. 29).

Select the 'Volume Viewer' icon in the top display tool bar, or

In the Main Menu, select 'View' - 'Image' - 'ND View' - 'Volume View'

The current Z-Stack is displayed as 3D model.

The top Display Window Toolbar will now show several options relating to viewing this model (Fig 29A: Free Rotation Control, X-, Y-, Z-Axis Rotation Control, View Plane, Blending options, 3D settings, etc.) and a new control panel with more functions will pop up, called 'Volume Options' (Fig. 29B).

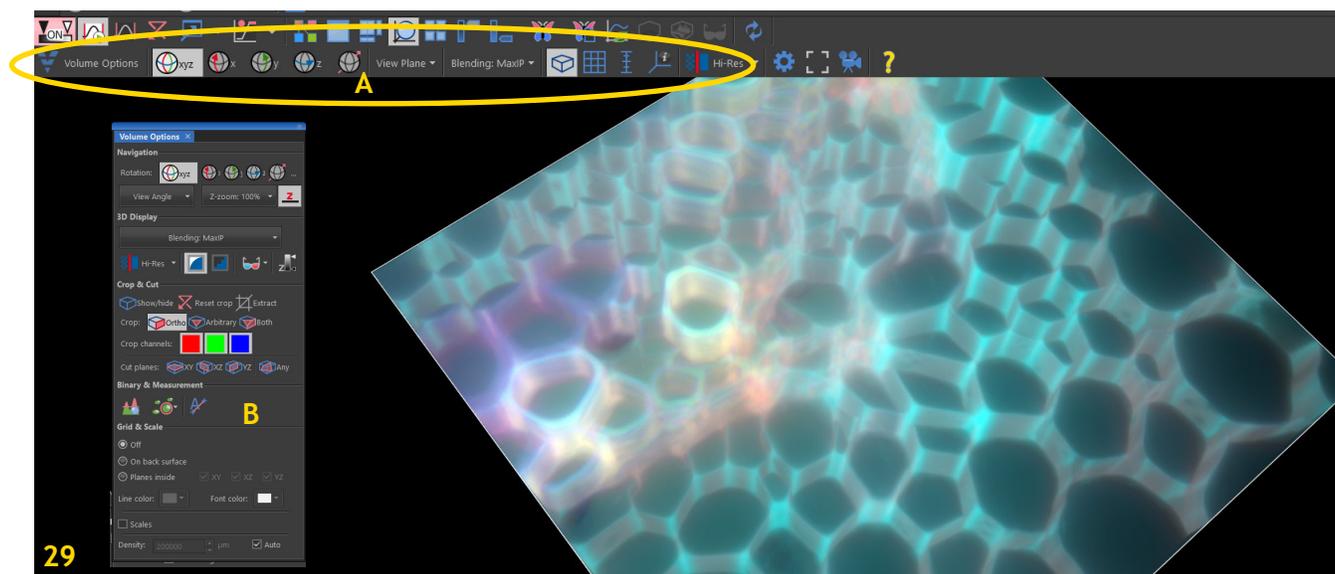You can also create an .avi movie of the 3D model.


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Image Processing

NIS Elements has a multitude of analysis options for individual images and high content batch processing. Some basic and quick examples are described below. Please refer to the online help or contact Michaela if you need a specific function not listed here.
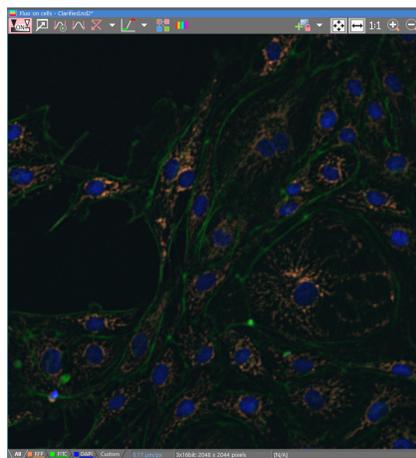
Clarify.ai and Denoise.ai

Clarify.ai is an AI-based image processing function that, by means of a neural network, has learned the characteristic components of fluorescent light emitted from out-of-focus planes. It can remove "blurred light" from fluorescence images, providing clear, high-contrast images and it processes images faster than conventional deconvolution algorithms. The application is simple - the parameters are obtained from the image metadata - just select 'Clarify.ai' in the main menu, check 'Create new document' and mark the channel(s) you want to improve - then select 'OK' (Fig. xxx).

Denoise.ai is another AI-based image processiong tool that improves the signal-to-noise ratio in your image, especially in samples with low signal intensity. Just like clarify.ai, the tool is simple to use and obtains the paramaters from the metadata of your images. Select 'Denoise.ai' in the top menu, check 'Create new document' and mark the channel(s) you want to improve - then select 'OK' (Fig. xxx).
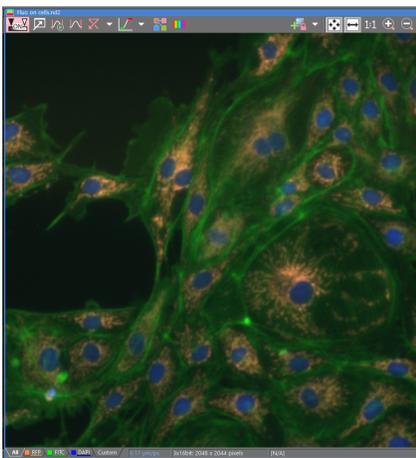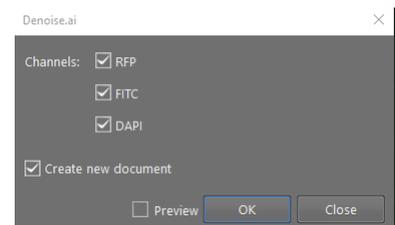
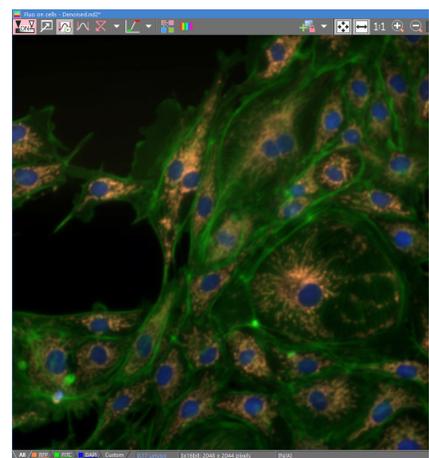Original Image   Clarify.ai   Denoise.ai

ND2 Files

You will find the options for ND2 file processing in the Main Menu under 'Image' - 'ND Processing'. Most of the image processing commands can be applied to one frame, all frames, or selected dimensions in the .nd2 file. You will select this in the command dialog window.

When an .nd2 file is opened, its structure is pictured at the bottom of the image window. There is a time line with all captured images indicated by gray markers. The blue-highlighted marker indicates the currently observed image.

The time dimension has a time line, loops of the other dimensions (e.g., number of channels or Z-stack planes) are shown as rectangles. The current selection in the loops/timeline is highlighted green. Browse the .nd2 file by clicking inside the timeline/loops.

You can play the selected sequence or go through the sequence frame by frame.

If you right-click the selection, a submenu will come up and you can adjust, delete, or crop your selection.

Examples

<u>Thresholding</u>

Specifying correct threshold limits is crucial in automated image analysis. NIS Elements has modes for thresholding available: RGB - HSI (hue, saturation, intensity) - Per channel - Intensity (Fig. 30A).
The mode depends on the type of image!
For Large Images, you have the option to display the threshold preview only on a limited area of your image to speed up the adjustment process - check the box 'Preview on selected area'.


Fig. 30

- Open your image
- In the Main Menu select 'View' - 'Analysis Controls' - 'Thresholding'
- The 'Thresholding' Control Panel will come up (Fig. 30)
- Select one of the Thresholding modes
- Define the thresholding limits:
    Select one of the pixel picking tools (Fig. 30B) and start clicking on objects in the image that you want to be detected. You can also directly define the thresholding limits by moving the vertical lines within the histogram (Fig. 30, arrows).

All pixels with numeric values within the threshold limits will be saved in the resulting binary layer.
If there are misdetected objects you may be able to eliminate them by morphology or size - these functions are available at the bottom of the control panel (Fig. 30C).

Basic operations on the binary layer can be performed before it is displayed on the screen (Fig. 30D):
    Clean (removes all small objects from the binary image)
    Smooth (smoothes the binary image contours)
    Fill Holes (fills holes with binary objects)
    Separate (separates objects)

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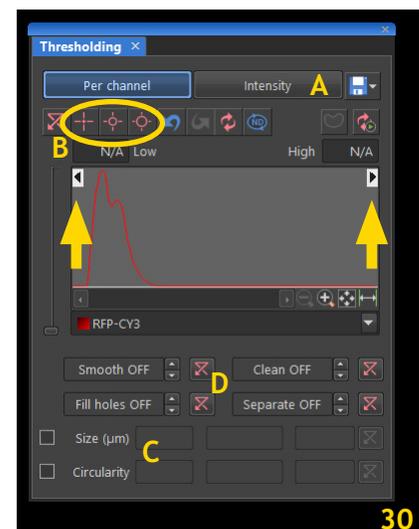
If you want to threshold for circular objects of similar size, you can select 'Spot Detection'.

In the Main Menu select 'Binary' - 'Spot Detection'

Select the method how the spots are detected (bright or dark, clustered or different sizes).

The 'Spot Detection' dialog window will come up (Fig. 31).

Select the channel on which you want to detect the objects (Fig. 31A).

You can display your objects either as circular areas, or as spots:

- Select 'Circular Area' (Fig. 31B).
- Check if you can see objects in the preview (if not, 'Reset' and start over).
- Adjust the values for 'Typical Diameter' and 'Contrast' to fit your objects tightly into the displayed circle and to remove unwanted spots (Fig. 31C).
- Choose where to apply the current settings (Fig. 31D) and click 'OK'.
- Save the new binary layer together with your file.
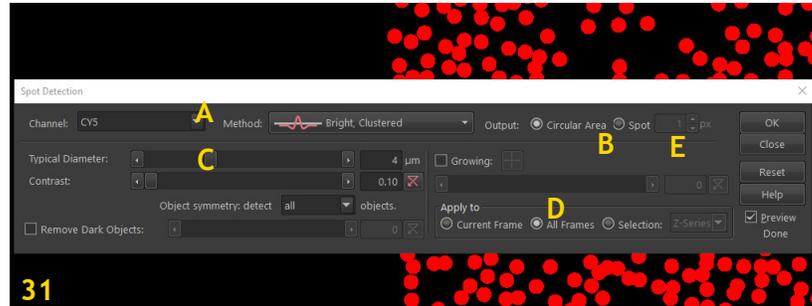
OR

- Select 'Spot' (Fig. 31B).
- Adjust the pixel size of the spots (Fig. 31E).
- Check if you can see objects in the preview (if not, 'Reset' and start over).
- Choose where to apply the current settings (Fig. 31D) and click 'OK'.
- Save the new binary layer together with your file.

Create an EDF (Extended Depth of Focus) image

If you have a Z-Stack, the EDF function combines this Z-Stack into one focused image by picking the focused regions from each frame and display the resulting data. You may want to create an EDF for your image analysis.

Open your image and select the EDF icon in the Top Display Tool Bar (Fig. 32). You have the choice to view the regular 2D image (Fig. 32A) or a 3D surface of this image (Fig. 32B).
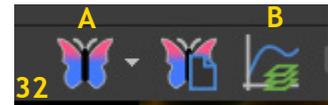
Create ROIs

ROIs can be created either in the Main Menu: select 'ROI', or by clicking on the 'Turn ROI On/Off' icon in the right Display Tool Bar.

You can change the properties of a selected ROI by right-clicking on it and select an option in the submenu that comes up. Many ROI controls can also be accessed through 'Ctrl'- Mouseclick in the image (Fig. 33).

If an .nd2 file has a time dimension, the ROI can be defined as 'Global' or 'Changing over time'.

If an .nd2 file has a multipoint dimension, the ROI can be defined 'Per Multi Point'; this means the ROIs in each point are entirely independent from the ROIs in other points. If the ROI is defined as 'Global' it will be applied to all points.

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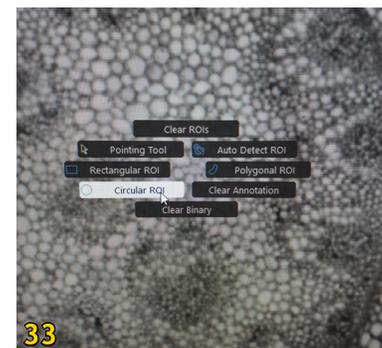
Deconvolution

Deconvolution might be necessary for some image stacks. To be able to do this, you need the PSFs (Point Spread Functions) for the corresponding objectives. Each objective has its own PSF for each channel. The software can automatically calculate the PSFs for each objective and wavelength.

Two deconvolution modules are available in the Main Menu:

  2D Deconvolution for standard 2D images (one focal plane)

  3D Deconvolution for Z-Stacks

- Open your image/image series and in the Main Menu select 'Deconvolution' and either '2D Deconvolution' or '3D Deconvolution' (Fig. 34A).
- In the control panel that comes up, check that the parameters (objective, wavelengths, NA, etc.) are correct - if not, change them (Fig. 34B).
- Select which channels to deconvolve (Fig. 34C) and click 'Deconvolve' (Fig. 34D).
- Depending on your image, this process might take quite a while. You can select to do the deconvolution only on a specific ROI (Fig. 34E).
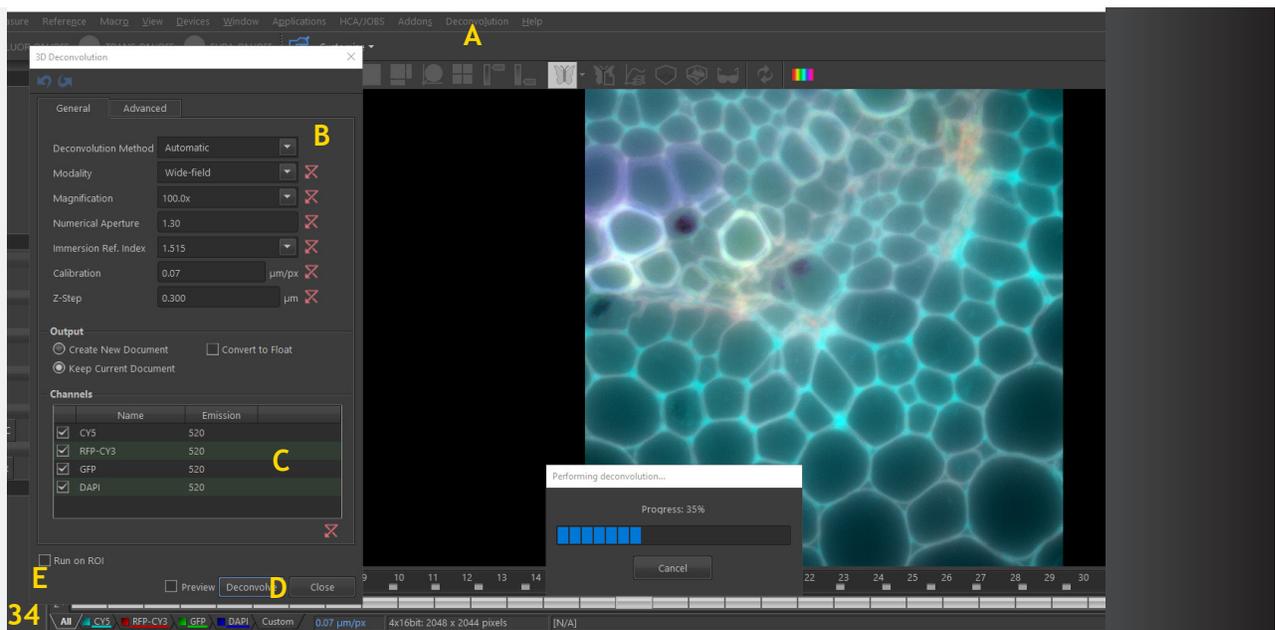
Colocalization

The Colocalization function under Main Menu - 'View' - 'Analysis Controls' - 'Colocalization' opens a new window (Fig. 35A) which automatically shows the scatter plot and Pearson Correlation for 2 channels in the entire image or for selected ROIs (Fig. 35B, C).

If you have more than 2 channels in your image, you need to select 2 of them in the drop down menues (Fig. 35, arrow). You can define one or more ROIs in your image, if you select all of them, the data for all ROIs will be displayed in the table below the scatter plot. You can then export the data into an Excel spread sheet or as report (Fig. 35D and see below, Data Management).
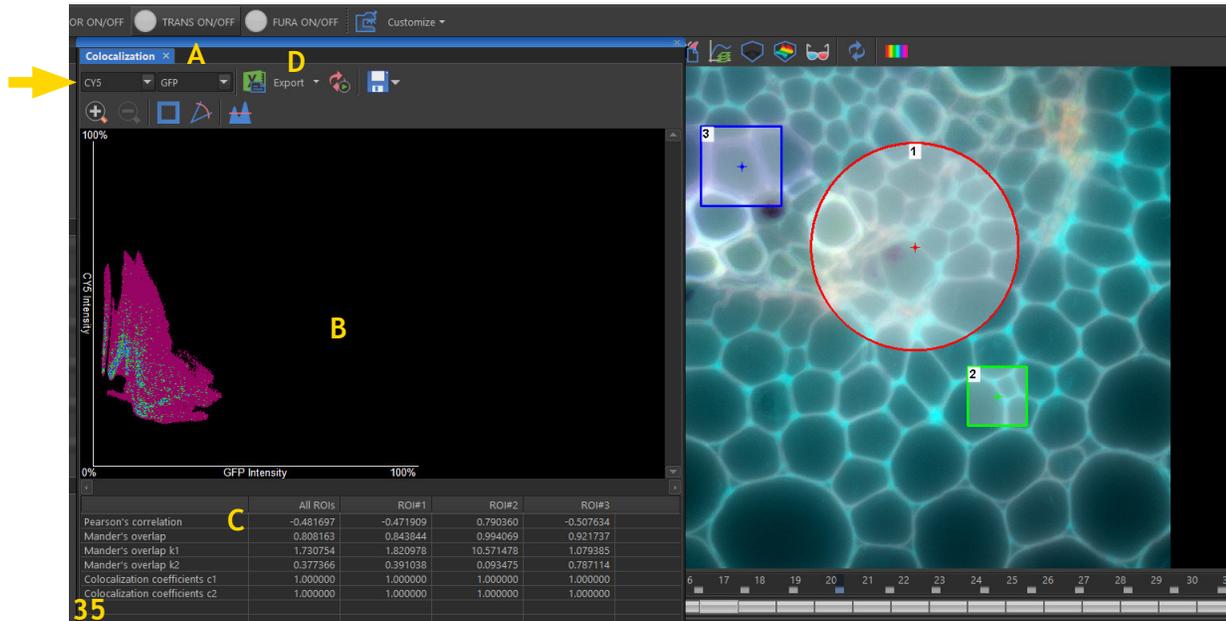
Measurements

Rough measurements can be performed utilizing graticules.

- To activate the graticules, press the 'Show Graticule' button (Fig. 23)
- Select the graticule type (grid, circle, cross, ruler) in the drop-down menu of this icon
- Define the graticule properties (shape, colour, line width)
- Define the graticule density (= closest distance between two line intersections)
- Measure

A binary layer can be created by 'Copy Graticules to Binary'

A graticule mask can be created, stored and loaded by 'Save Graticule Mask As' and 'Open Graticule Mask'

The following list describes all features that can be measured within NIS Elements:

Object features - measured within a binary layer (binary objects)

ROI features - measured within ROIs

Field features - measured over the entire image area

Manual - features available in manual measurement

EDF features - measured in an EDF (Extended Depth of Focus) image

3D features - measured on 3D binary objects within a thresholded Z-Stack data set)

Tracking features - measured on tracked binary objects or ROIs

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Manual measurements like length, length 3D, angle, area, radius can be measured over an image. The results are being recorded to a simple statistics table, which can be exported. The data can also be presented as graph.

- In the Main Menu select 'View' - 'Analysis Controls' - 'Annotations and Measurements'
- A new window will pop up (Fig. 36)
- Select a tool for your measurements
- Measure the objects in the image using the mouse
- Select where to export the results
- Export (see page 26)
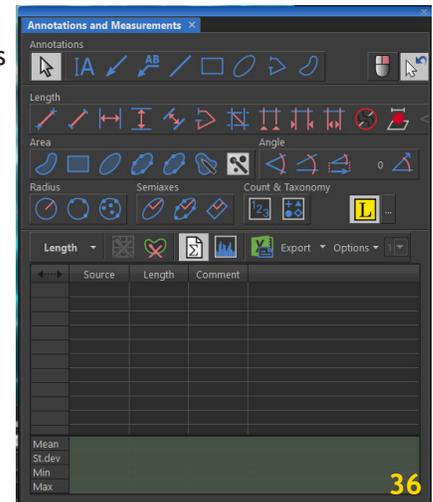
Automated measurements can be performed in combination with user-defined macros.

The key procedures are:

Optical system calibration (only necessary in specific cases, the system is calibrated)

Image acquisition

Define threshold, create the binary layer

Selection of measured features

Perform the automated measurement

Result presentation

The binary layer and the colour image can be used in the automated measurement. The binary layer is typically used for shape and size measurements while in the colour layer intensity or hue measurements are carried out. Measurements can be performed over the entire frame, in a measurement frame, or in a specific ROI.
NIS Elements distinguishes two types of automated measurements: Object Measurement and Field Measurement.

Object counts (Fig. 37):

Automated object detection can be performed after thresholding the image (see above).
Object count can also be performed on the Live image
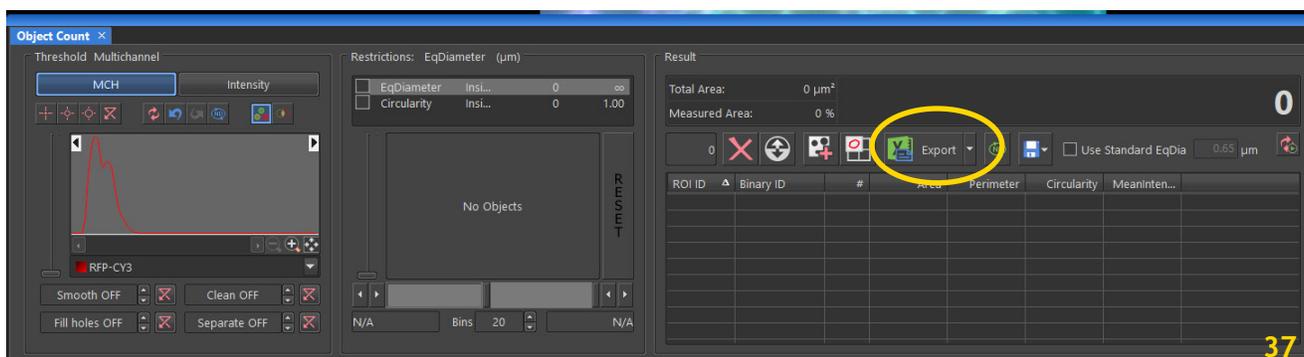
In the Main Menu, select 'View' - 'Analysis Controls' - 'Object Count'

The general workflow for object count is:

Thresholding   Use restrictions   Review the results - Export

Macros

A macro is an executable sequence of commands for image analysis that you can define using the NIS Elements Macro Recorder (recommended) or write yourself (programming knowledge required!!).

<u>Recording and saving a macro</u>

- In the Main Menu, select 'Macro' - 'Record'
- Perform the series of actions you would like to record
- Finish the recording by selecting again 'Macro' - 'Stop Recording'
- Check the macro in the Macro Editor before you save it: 'Macro' - 'Edit'
- Save the macro via 'Macro' - 'Save As'

<u>Running a macro</u>

If you just created your macro and it is currently loaded in NIS Elements, select 'Macro' - 'Run'
If you want to run a saved macro, select 'Macro' - 'Run from File'

The macro execution can be stopped anytime by pressing the 'Ctrl' + 'Break' key combination.

Data Management

<u>File format</u>

By default, none of the single snapped images is automatically saved!
All snapped images are kept open in the image window (you see tabs of the acquired images lined up at the top of the window) and they need to be saved as described below.
For the ND Acquisition, you will probably select the option 'Save File As'. The ND2 (= multi-dimensional) files contain a sequence of images.

<u>Image layers</u>

Each image can be saved with several layers
  Annotation Layer - stores vector objects like measurements, text labels and other annotations
  Binary Layer - is created when thresholding or spot detection was performed
  ROI Layer - stores created ROIs
  Colour Layer - contains image data captured by the camera

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Saving images

Some of the most common image file format are: TIFF, JPEG2000, JPEG, LIM, ome.TIFF, ND2, AVI, MP4

There are several ways to save your individual images:

In the main menu:

'File' - 'Save' or

'File' - 'Save As' (automatically called up for new images)

In the main toolbar:

- Select the 'Disk' icon to save your files as above.
- You can select the file format (tif, avi, etc.) in the drop-down menu.
- Check the 'Save Color Image' option if you want to save the image colours into your file!
- Check the 'Save Annotations' option if you want the scale bar or other annotations saved into your image file!
- When saving a multi-channel image as TIFF file, select 'Convert to RGB'.

To save an image with the scale bar, right-click on the 'Show Scale' icon (Fig. 23), select 'Burn Scale'. This will automatically convert the image to an RGB tif or jpeg format with the scale bar saved in it - a warning appears because some information of your Metadata may be lost - it's best to previously save your image in the original .nd2 or LIM format!!

Save (export) image sequences as .avi or .mov

Under the 'File' - 'Save As' function, .nd2 files can be exported as .avi or .mov files. If it's a time lapse, you can define the playback time (frame rate) and which video compression is applied.

If your nd2 file includeds several dimensions, you need to select the one that will be used to create the movie ('Use Time Loop' - 'Use Z-stack Loop' - 'Use Z-stack and Time Loop').

XY Points: when the ND document contains multiple points, you can decide how these are saved. Either only the current point can be save into the file, or all points can be saved as separate files. The different points can't be exported into one single file.

Export analysis data

Measurements and other analysis data can be exported from NIS Elements to an Excel spreadsheet or a tab de-limited text file.

In the Main Menu, select 'Export' and select the desired file type.

Create reports

Report Creator allows you to create a print-ready document which can contain measured data, images, graphics, etc.

Open the Report Creator under 'File' - 'Report' - 'New Blank Report'

A graphical editor appears, it works very much like any text editor - you can insert text fields, simple shapes, images, tables, or graphs.

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Shutdown

1. Lower the nosepiece, remove your sample, clean objective lenses with lens paper

2. Save your data, close NIS Elements, transfer your files to OneDrive (preferred) or the server

3. Switch off microscope

4. Switch off camera

5. Shut down computer

6. Cover the microscope

2-Hour Rule

If someone else has booked the microscope within the next 2 hours, please don't switch off the com-
puter, microscope and camera. Don't cover the microscope.

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