

Nikon Deconvolution Microscope Quickstart Guide

For NIS v4.30.01 Guide V1.02, updated August 19, 2016.

Startup

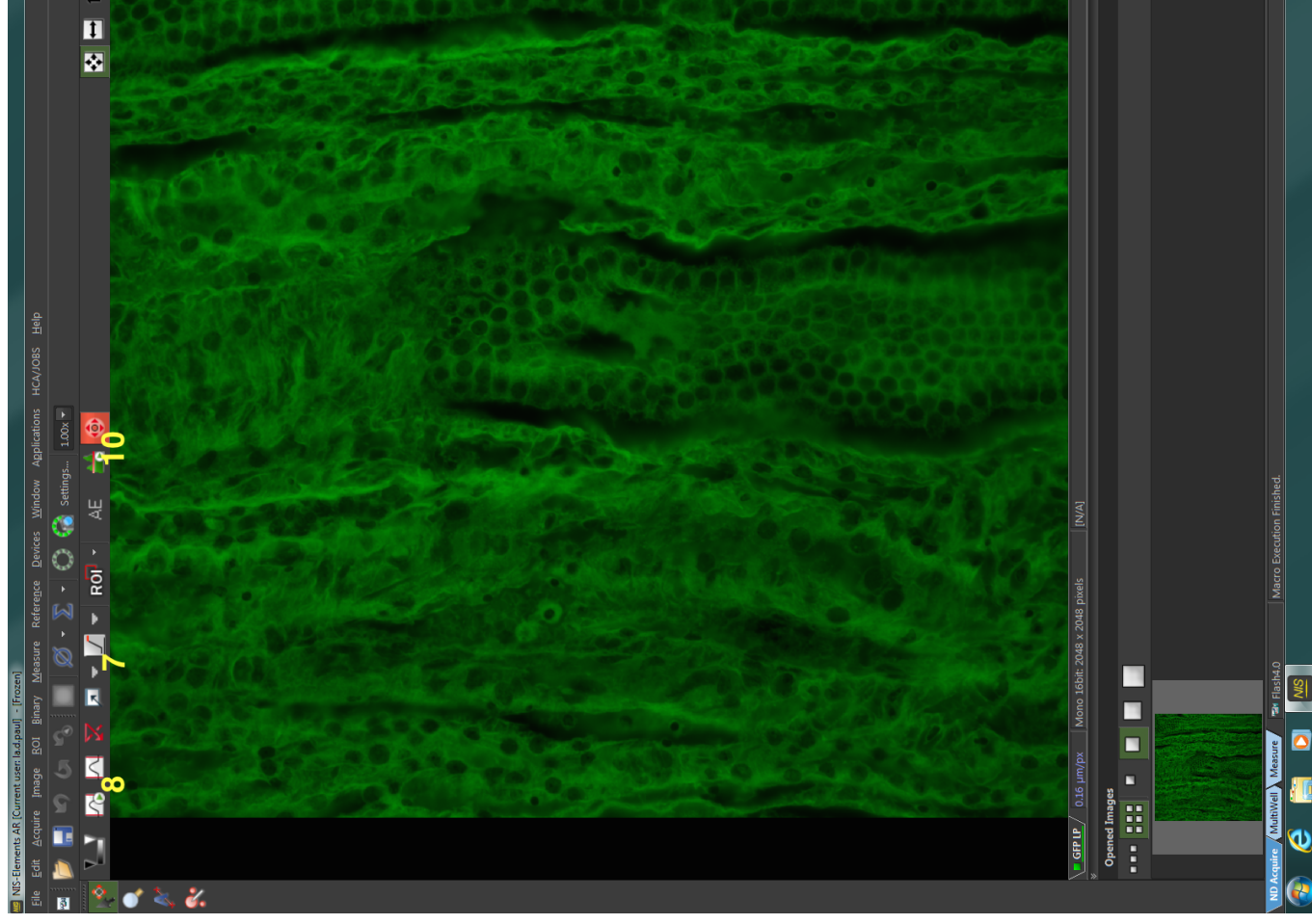
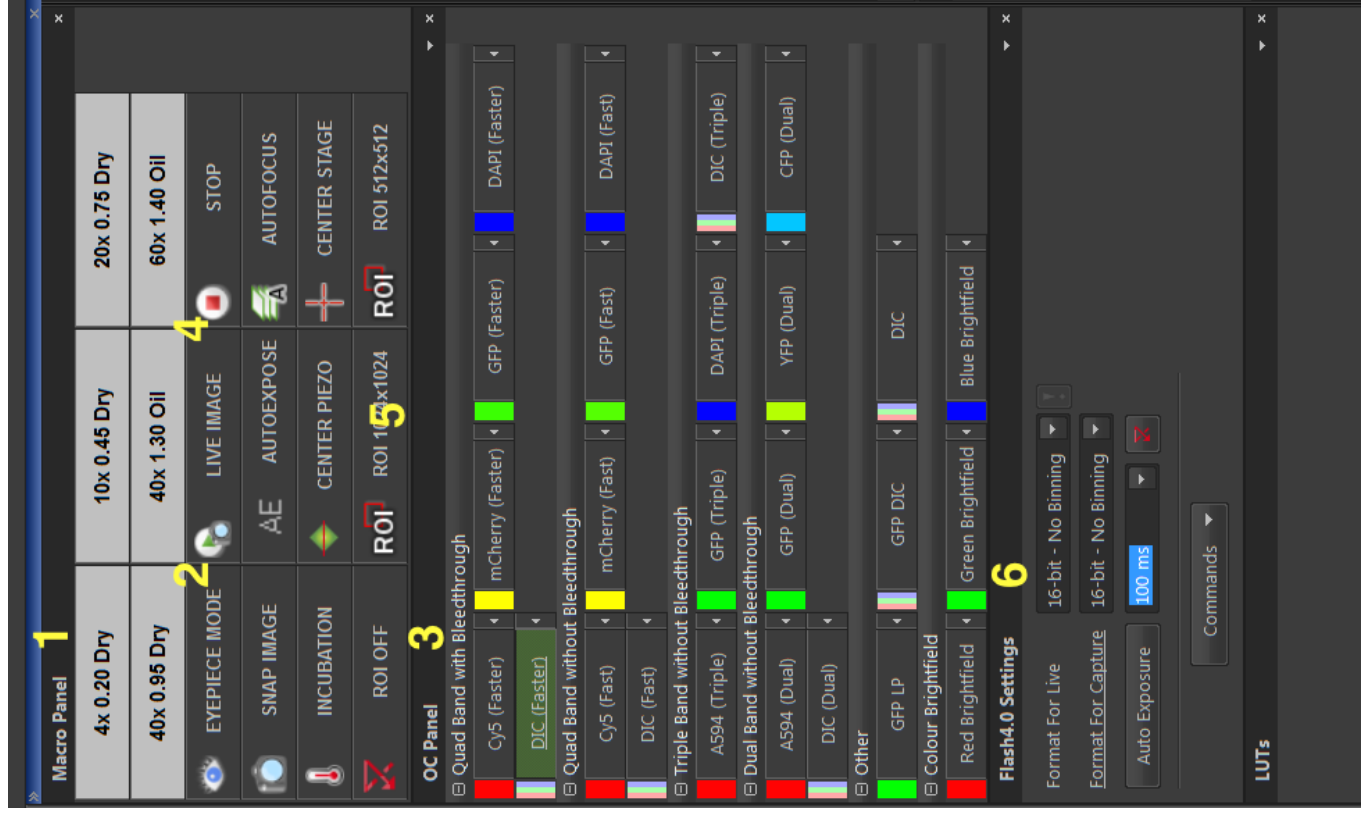
- Turn on the PC.
- Log onto the PC, wait until Windows has finished loading (~60 sec).
- The chamber is set to 37°C, measured via a thermocouple within a small dish on the microscope stage. The thermocouple must be immersed in water. Add water as necessary.
- If you require CO₂ Incubation, Double Click the “CO₂ On” icon on the desktop. Note that it may take one-half hour or more for the CO₂ level to stabilize.
- Double Click the NIS icon on the desktop to turn on the microscope and load the capture software. This will also configure your data file paths.

Basic Navigation

Note; there are three basic program screens (and modes). Tabs for these are presented in the lower left corner of the monitor display. The initial program screen is the **ND Acquire** screen. This is best used for imaging single or a few selected points in one or a few slides or dishes. The **Multiwell** screen brings up **JOBS** mode, which can run complex experiment macros, and is best used for repetitive, multiple point captures with multiwell plates, etc. The third screen is **Measure**, which provides access to a range of measurement and image analysis option during and post acquisition.

Basic Visualization and Setup (Common to both ND Acquire & JOBS modes)

- Select the objective you wish to use from the **Macro Panel**: the appropriate DIC or phase rings will move into place along with the objective **(1)**.
- If you are using an oil immersion objective, carefully clean the lens with a lens tissue. Place a drop of oil on the objective.
- Install the appropriate sample holder adaptor — i.e.: 3 place slide, 4 place petrie dish, etc. — and mount your sample.
- Set the XY stage speed to “Coarse” on the joystick by twisting the joystick knob and centre your sample over the objective.
- Select “Eyepiece Mode” **(2)** (on the **Macro Panel**).
- Select the Optical Configuration you wish to use from the **OC Panel (3)**, and ensure it is highlighted green. Choose either:
 - “Quad Band with Bleedthrough” – quad-band dichroic and quad-band emission filter.
 - “Quad Band w/o Bleedthrough” – quad-band dichroic and individual band-pass emission filters.
 - “Triple w/o Bleedthrough” – triple-band dichroic and individual band-pass emission filters.



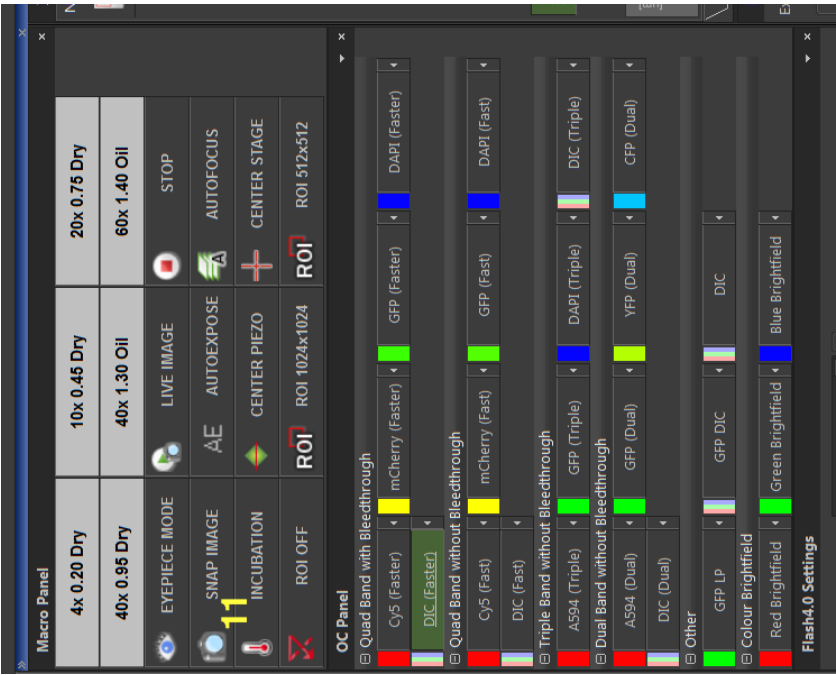
“Dual w/o Bleedthrough” – dual-band dichroic and individual band-pass emission filters.

“Other” – single dichroic and band-pass emission filters.

“Colour Brightfield” – false colour imaging of brightfield images. Permits assembly of realistic full colour overlays.

- If you wish to perform brightfield imaging, slide the polariser unit situated above the microscope condenser to the open position. The brightfield lamp is turned on via the push button on the small black box located to the left of the microscope. Adjust brightness there also, via the rotary knob.
- Bring the objective up using the microscope coarse focus knob until the oil touches the coverslip (if you are using immersion oil).
- Focus on your sample using the eyepieces, or via the Focus Light on the front of the microscope. Note: the LCD panel on the microscope displays the Z position. Correct focus is typically between 4000 & 6500µm.
- Set the XY stage speed to “Fine” or “Ex Fine” on the joystick, and centre an object of interest.
- Select “Live Image” (or “Snap Image” if you want to minimise bleaching) from the **Macro Panel**. **Note:** Selecting the “Stop” button will terminate the live image update and sample illumination **(4)**.
- Select the image size you would like: “ROI OFF” (2048x2048), “ROI 1024x1024” or “ROI 512x512” **(5)**.
- We recommend you adjust camera exposures manually via the drop down list on the **Flash4.0 Settings Panel (6)**. However, exposures can be set automatically using the “Auto-Exposure” button. The size and position of the automatic exposure calculation area can be set by selecting the “Probe” button on the right hand side of the image window).
- Turn on the “Pixel Saturation Indicator” **(7)** and the auto “Look Up Table” **(8)** in the toolbar above your image.
- If necessary, you can adjust the LED brightness (default = 75%) by clicking on the **OC Panel** title bar and then selecting **Spectra Pad (9)**. However, if you change the lamp LED brightness, you will notice when returning to the **OC Panel** that the currently selected OC bears a red exclamation mark. This is a warning that a setting has been modified. To proceed, save the change by clicking on the small arrow next to the red exclamation mark.
- Select the “Mouse XY” button **(10)** in the toolbar above your image, then click on your image, hold down the left mouse button and drag the mouse to move the motorised XY stage. Roll the mouse wheel to adjust the focus (total travel = 200µm).
- You can centre an object of interest by double clicking the left mouse button with the cursor on the object of interest.
- Focus on an object of interest. Either:

Adjust the focus automatically by pressing the “AutoFocus” button (#) (you can modify the auto-focus settings under the Devices > Auto Focus Setup menu),



OR

Adjust the focus manually by scrolling the mouse wheel, the microscope or joystick focus knob.

- Once the sample is in focus, adjust the exposure settings again either manually or using the “Auto-Expose” button, ensuring that the captured image is not over-exposed. **NB. Saturated pixels will NOT deconvolve, so it is much better to have a weak image than a bright one.**
- Repeat for all other desired Optical Configurations (fluorophores, channels, etc.). Exposure times are auto-saved when they are modified.

Note: at this stage it is possible to perform a simple one colour capture, if desired, by clicking Snap Image (11) on the **Macro Panel**.

The method of setting up a more complex experiment on the **ND Acquire** and **Multiwell (JOBS)** screens diverges at this point.

Perfect Focus (PFS)

The Perfect Focus System is a hardware based autofocus, that utilises an IR LED to focus on the sample side of the coverslip. The user may then tell the system how far away (offset) the sample is from the coverslip. PFS requires a refractive index change to detect the coverslip, such as a liquid sample mounting media, and may not work with fixed slides.

To set up:

- Focus on your sample.
- Press the PFS button on the front of the microscope. If viewing a live image, the sample may well appear to defocus. However, if the small recessed green FOCUS LED next to the PFS button lights up, PFS is working.
- Adjust the PFS focus controller or mouse wheel (not the microscope) to refocus your specimen (determine the offset).
- When configuring a multipoint ND acquisition, it is essential that PFS is turned on before you record each point. If not, PFS will not function during the experiment.
- If imaging more than one 35mm dish in an experiment, ensure that “Keep PFS On During Stage Move” in the XY Overview tab is NOT ticked, or PFS may fail when the metal stage insert moves over the objective.

Note: if the microscope triple beeps, or you don’t see the small FOCUS LED light up, try adjusting the microscope focus up and down until you get PFS to work.

You can make the microscope remember the PFS focus position for each objective by pressing the Memory Button once PFS focus lock is achieved, however this will be lost when the microscope is powered off.

ND Acquire screen, XYZ Overview Panel

The XYZ Overview Panel **(12)** contains a number of sub-panels, providing overall sample visualization, and for the configuration of some capture parameters. The sub-panels are accessed via the named tabs (listed below).

ND Acquisition Overview is the default tab **(13)**. Within this, one may navigate the sample and mark points of interest.

- Pressing the “Add Point” button **(14)** or the keyboard spacebar will record the XYZ coordinates of the current viewing position. Many such points of interest may be so defined. Once at least one point is recorded, pressing the “Adjust to Points” button **(15)** will zoom in the overview display zoom to encompass and display them. The mouse wheel or magnifier buttons may be used to zoom in manually.
- Double clicking anywhere within the preview window will move the stage to that point, or if “Move to Nearest point” button **(16)** is active the stage will move to the closest point to your click. Activating “live Image” will update the main image window. Clicking “Snap Image” will also show a preview image in the XYZ Overview window.
- Right click to bring up a menu for advanced features such as “Scan Large Image”, “Clear Preview Image” and “Use points for Focus Surface” etc.
- “Use points for Focus Surface” will use the Z-Height of each point to map out the focal surface of your sample, allowing the software to interpolate and predict the focal height of other areas on your slide.
- A Heat Map of the focal surface is displayed by pressing the “Show Focus Surface” button **(17)**.
- The focus surface can be used when scanning to keep the sample in focus over a slide or multiwell plate by ticking “Use Focus Surface” in the “Advanced” section of the “XY” tab in the “ND Acquisition” control (discussed below).

The Focus Surface and PFS Surface Tabs

The Focal Surface and PFS Surface Tab provide additional visualization of a focus surface derived from marked points and via the Perfect Focus automatic focus system, respectively.

Note that the focal surface can be mapped independently of the ND Overview points list if desired. Double clicking on the preview will to move the stage to that point. Focus, and, then click “Add Point” or press the space bar to record the Z heights.

The Document Overview Tab

This is used to visit XYZ points in previously acquired datasets.

Further details of the capabilities of the Focus Surface, PFS Focus Surface and Documents Overview Tabs may be obtained from the microscopy staff.

ND Acquire screen, ND Acquisition Panel (18)

- Enter a name for the experiment in the “Experiment” text box **(19)**, if desired. Note, this is not the image(s) filename but rather, a general description of your experiment.
- Checking the “Save to File” checkbox **(20)** will autosave the data as an .nd2 file. Autosaving may slow rapid capture sequences, but is safer.
- The “Path” textbox should be automatically populated with “D:\Data\your user name\ND\”. Enter a filename in the “Filename” textbox. Note; a checkbox permits files to be saved as a .tif series (rather than .nd2), if desired **(21)**. The software will not display images saved as .tif files as they are captured.
- Select the order in which capture functions will be executed via the “Order of Experiment” drop down box **(22)**. The last option displayed will be the fastest.
- The “Timing” button indicates the approximate total experiment time **(23)**.

The “**Time**”, “**XY**”, “**Large Image**”, “**λ**” (lambda), and “**Z**” function tabs may be checked to assign these functions to the capture **(24)**. Clicking a function tab allows configuration of the function options.

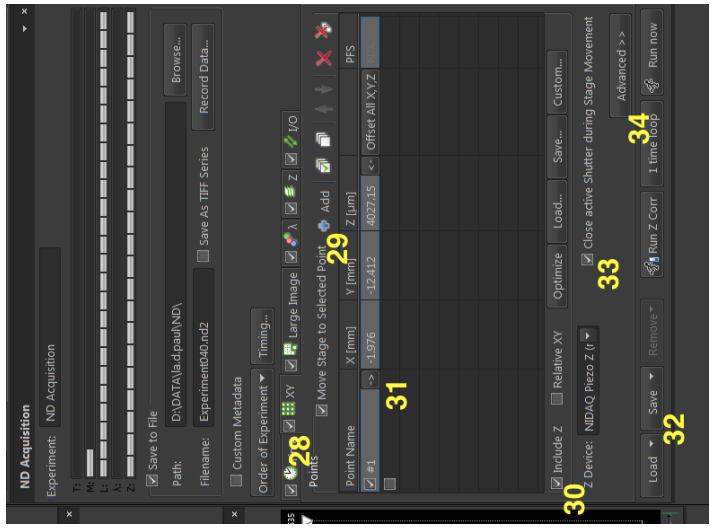
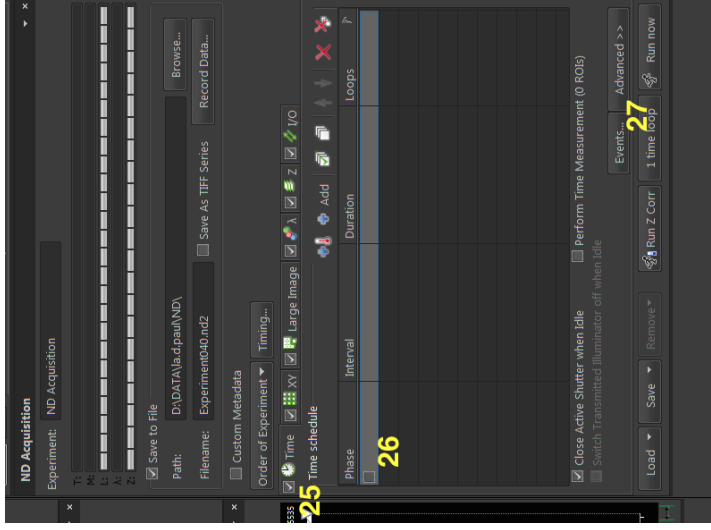
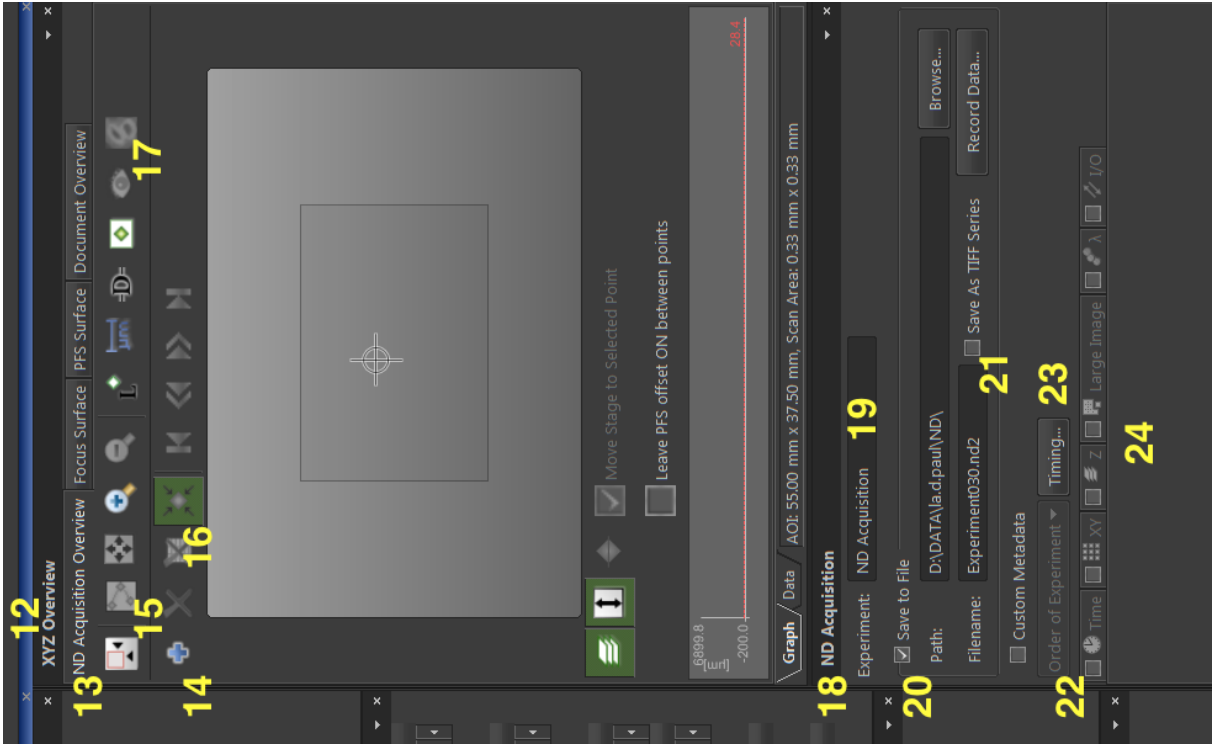
The Time tab (25)

- Click the checkbox in the “Phase” column **(26)** on the first line of the list display to add a time sequence.
- Set the Interval and Duration or the number of Loops (cycles) to be acquired. Note: you must never have “no delay” set in the Interval column. Note also: a number of “Phases” may be set with differing parameters, to run sequentially.

Note: Under the “Advanced Menu” **(27)**, “Keep Object in View” to have the software attempt to keep a live cell in the field of view of the camera.

The XY tab (28)

- Displayed is the list of points defined on the XYZ Overview Panel, above.
- Clicking on a point in the list will move the microscope stage to that point.
- Points may be added to the list by pressing the “Add New” button **(29)**, or pressing the keyboard space bar.
- Ticking “Include Z” **(30)** will set acquired images to be captured at their recorded focus positions.
- To update a point should you adjust the focus or XY position, press the horizontal arrows pointing to the value to be changed **(31)**. XY position and Z position may be updated individually. Note: Include Z must be ticked.
- Right clicking on the points list allows update of the Z position for all points.



- Point lists may be Saved and Loaded **(32)**, for example, for regular experiments using multiwell plates, etc.
- Clicking the “Close active Shutter during Stage Movement” check box **(33)** will close the shutter between points. This will reduce photobleaching, but again, it will also slow down image acquisition.
- The Advanced Menu **(34)** contains options to perform an Autofocus before/after imaging, and to use the Focus Surface derived in the XYZ Overview window.
- To image multiple slides or dishes within one experiment the system **MUST** be configured to lower the objective between samples. This is essential to prevent damage to the objective. Please contact microscopy staff for assistance in configuring the necessary macros.

Note: To use Perfect Focus (see later section) with points, you **MUST** ensure it is turned on before the points are recorded.

The Large Image tab **(35)**

This is equivalent to tiling on the Zeiss confocals.

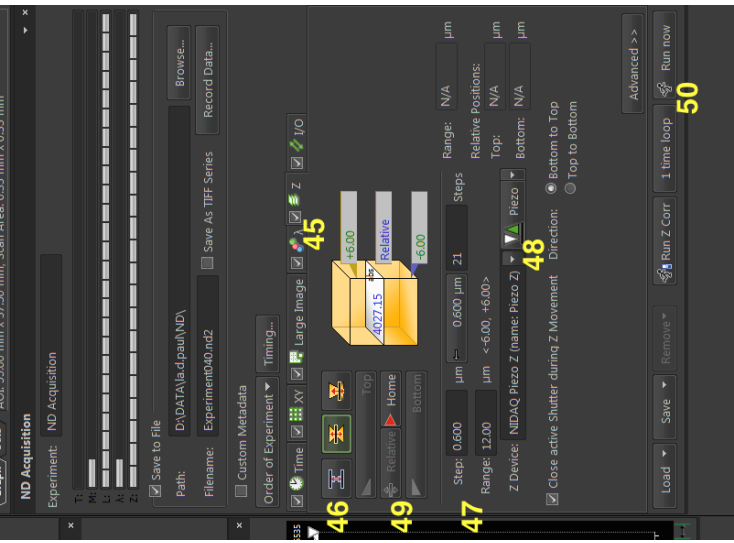
- Set the size of your image (“Scan Area”) **(36)** as a number of frames or as an area (mm²).
- Select Stitching if required, and set the image overlap to approximately 15% of the image size **(37)**.
- Select “All Channels” if you wish the software to stitch each channel individually, or a single channel if stitching may be performed on a “master” reference channel (faster).
- Clicking the “Close active Shutter during Stage Movement” check box **(38)** will close the shutter between images. This will reduce photobleaching, but will also slow down image acquisition.

The λ (Lambda) tab **(39)**

- Click the checkbox under “Optical Conf” to activate a line **(40)**, then use the textbox dropdown to choose and add your first Optical Configuration to the list **(41)**. Note that the exposure values applied to this configuration will be those determined during basic visualization and setup, above.
- Click the checkbox on the next line to add an additional optical configuration to the capture. The software will automatically select the next OC in the top to bottom order displayed in the OC Panel. Repeat as necessary.
- The “T Pos” and “Z Pos” columns and dropdown boxes **(42)** (visible if “Time” and “Z” tabs are checked) allow fine tuning of channel capture with respect to these additional parameters.

Note: if capturing a reference DIC image, set the “Z Pos” to “Home”, rather than “All”.

Note also: you have the option to close the shutter between Optical Configurations to reduce photobleaching (check “Close active Shutter during Filter Change”) **(43)**, but this will very



slightly slow down the acquisition. You may also need to place delays before and after the acquisition if you find you are missing images, or the channel colours are reversed, etc. These are configured under the “Advanced” dropdown (44).

The Z-Stack tab (45)

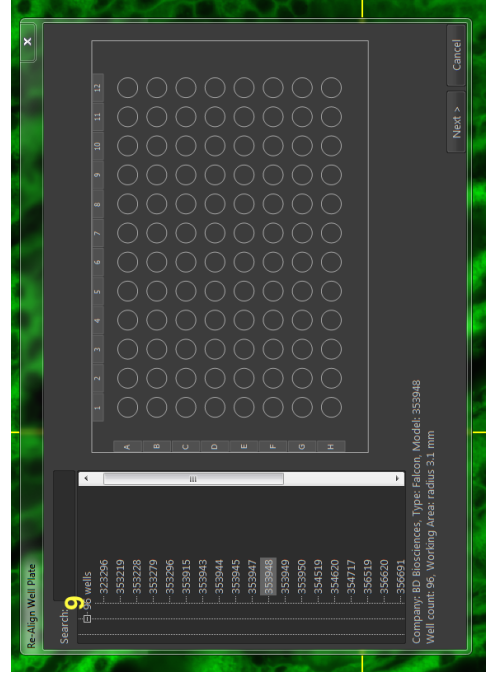
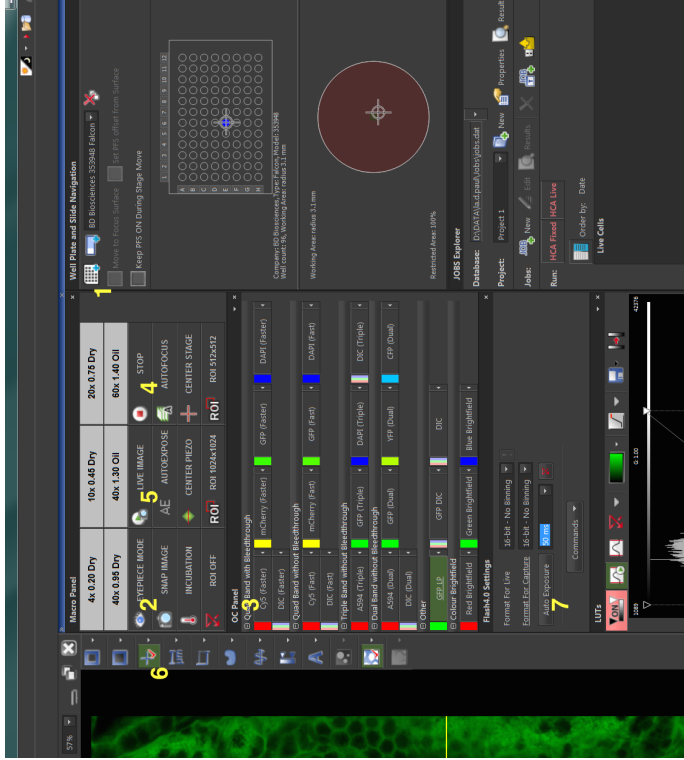
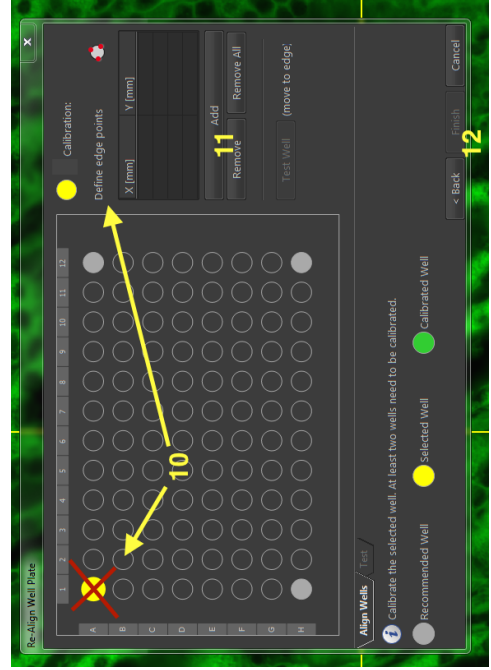
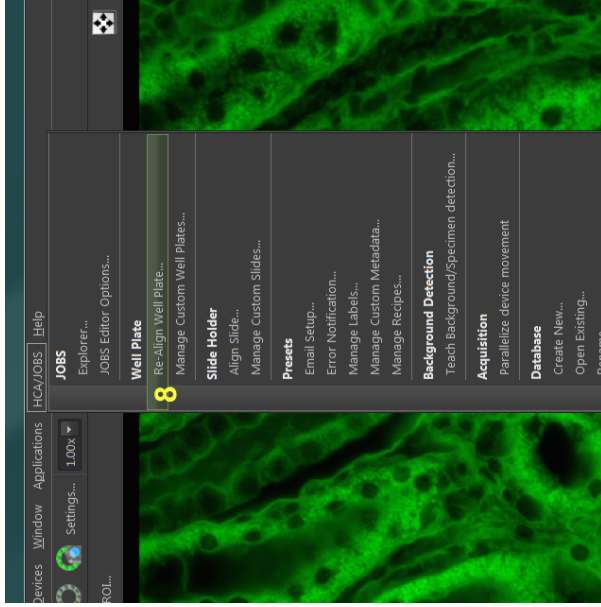
- The buttons toward the top left of this panel select the Z-series set up method (46). Options are;
 - Top – Bottom (same as First-Last on the confocals).
 - Symmetric (equal number of slices above and below a center position).
 - Asymmetric (unequal number of slices above and below a center position).
- Set an appropriate Step Size and Range (47).
- To use the faster piezo z drive, select “NIDAQ Piezo Z”, then press the “Piezo” button (48) to centre on the current Z position and maximise travel range (maximum = 200µm, ie: +/- 100µm from centre). If you require a Z-stack larger than 200µm you must use the Ti-Z Drive option.

Note: “Relative” (49) **MUST** be checked for Multipoint XY capture with Z stacks. This is only available with the Symmetric and Asymmetric set up options. The software will ask to convert to a relative stack should one attempt Multipoint XY with an absolute, Top – Bottom defined stack.

Note also; if Top-Bottom set up is selected, the exact start or end point can be locked in by right clicking on the relevant end button and selecting “Keep Exact (Bottom or Top) Position”, as necessary.

Once you have setup your experiment, press the “Run Now” button (50) to acquire your data.

NB. If your output data file size is too large, go to Image/Convert/Change depth to convert it to 8 bit, then save it again.



The Multiwell (JOBS) Screen

Setting up a Multiwell Plate

- Select the 10x objective, then wind it down so you don't hit it when mounting your plate.
- Multiwell plates insert directly into the microscope stage. Ensure that well A1 is positioned in the top-left corner, the plate is sitting flat, and is pushed into the lower right hand corner.
- Select the "Add Well Plate" button **(1)** at the top left of the "**Well Plate and Slide Navigation**" **Panel**, and choose the appropriate well plate from the pop up box & list. Click OK.
- **NB. You MUST NOT attempt to move to, view, or image the outer rows and columns of the plate as the objective will hit the stage and cause damage. Prepare your plate accordingly.**
- Double click on well B2. **Do not click on well A1.**
- Select "Eyepiece Mode" **(2)** and either "DIC" or a relevant fluorophore **(3)** from the **OC Panel**, and focus on your sample. **Note:** Selecting the "Stop" button **(4)** will terminate the live image update and sample illumination.
- Select "Live Image" **(5)** from the **Macro Panel**.
- Activate the crosshair overlay on the live image via the "Show Graticule" button, located on the right side of the image toolbar **(6)**.
- Adjust your exposure times manually or using "Auto Exposure" (on the **Flash4.0 Settings Panel**) **(7)**.
- Select "Re-Align Well Plate" from the **HCA / JOBS Menu** at the top of the screen **(8)**.
- The "Re-Align Well Plate" dialog will open **(9)**. Choose the plate you are using from the plate list, and click "Next".
- **Select well B2**, and the method of alignment (Centre, RHS or 3 points) **(10)**.
- Follow the prompts, using the joystick to move to the relevant points, and click "Add" **(11)** for each point.
- Click Finish **(12)**.

The Stage is now configured and aligned for your well plate.

Setting up a Job

The **Jobs Explorer Panel (13)** at the bottom right of the monitor is the hub of the Multiwell Screen. It is from here that configured jobs are usually run or rerun, by right clicking on the job name and selecting "Run" **(14)**, or by highlighting the job and clicking on the green arrow in the job list's left margin **(15)**. In both cases this opens the **Job Wizard** (described below), which must

be clicked through to run the job. Here also, one may return to the default view of results by clicking on the “Results” button (16), after exploring other options.

- Select your desired objective and focus.
- Go to the **Jobs Explorer Panel**, and ensure “D:\Data\your user name\Jobs\” is set as the path (17).
- Create and name a new “Project”, if desired (18).
- Jobs are defined by clicking on “Add New Jobs from Template” or “Add New Job” buttons (19 & 20) in the Jobs Explorer Panel’s button bar. These bring up the **Job Template Panel** and the more detailed, **Job Definition Panel**, respectively.
- As an example, click on the “Add New Jobs from Template” button. The **Job Template Panel** (21) opens with basic options for live and fixed plates. Selecting “Live Cells” and “Import” (for example) then establishes a basic capture framework, and shows a “Live” job in the Jobs Explorer jobs list (22). The **Jobs Wizard** popup (23) is opened.

The **Jobs Wizard** allows configuration of the experiment and capture parameters. The wizard interface is divided into two columns. The left column has basic parameter choices, and the right a series of subpanels with detailed options for each of these parameters (24). Work down the left column, checking the options or filling in the information required on the right. For example:

- The **Experiment Options** entry allows choice of capturing a single image or Z-stack, the use of autofocus via the built in Z-drive or Perfect Focus, and automated notifications. Selecting these options adds the relevant entry to the left column, with options then to be configured on the right.
- **Description & Notifications** provides options for an experiment description, and the configuration of notification parameters (if these were previously activated).
- **Acquisition Parameters** allows configuration of λ (Lambda) and (if applicable) Z-stack settings in similar manner to the equivalent ND Acquisition Panel tabs.
- **Sample Definition – Plate** allows selection of the well plate used and the specific wells to be imaged. **Remember**, the outer rows and columns of the plate **MUST NOT** be specified.
- **Sample Definition – Well** allows set up of imaging within the wells, as user defined points, randomised points, or sampling patterns. Large image settings may also be configured. If a shading (or flatfield) correction image has been defined, it may be applied here.
- **Timelapse Definition** allows definition of time series by number of loops (cycles), or interval between captures (as described in the ND Acquisition Time tab). **Note:** it is best to set a small delay between cycles, via the “Wait Between” option under “Speed of Acquisition” (25).
- **Data Analysis** presents “Cell Count Analysis”, “General Analysis”, and “Intensity Analysis” options. Configure by selecting an analysis type, clicking the “Define” button (26) and choosing desired options on the pop up settings panel. Analyses may be configured for each OC. Settings may be saved for future reuse via the “Save Recipe” button (27).

Upon reaching the end of the Jobs Wizard, the options to “Test Run” and “Run” the job will become available (28). Test Run executes a single cycle of the job; useful for verification. A completed job may be rerun from the **Jobs Explorer Panel**, as described above.

Job Definition - PFS ON, Multi-Point*

Experiment Options

Description and Notifications

Acquisition Parameters

Sample Definition - Plate

Sample Definition - Well

Time Sequence

Data Analysis

Summary of Assay

Summary of experiments: 96 well plate, 4 wells, 1 times, no delay, Center only, 1 Lambda (GFP (aster)), 1 Assay (Analysis), Estimated size: 32 MB

27

26

28

Job Definition - PFS ON, Multi-Point*

Print Job to Clipboard

Options

Job Templates

Export

Expand All

Collapse All

Show Deposit

Rows

Align

Stage XY Points

Generate Points

Preddefined Points

Points

Shift Point Set

New Point Set

Add Point

Remove Point

Add/Edit Points Manually

Export Point Set to ND

Import Point Set from ND

PFS On and Focus

TimeLapse : 2 times : wait 5 sec in between

WellLoop : for each well in WellLoop.CoverSheet

GeneratePoints : generate points in WellLoop.CoverSheet

PointLoop : for each point in WellLoop.CoverSheet

Run Macro1

Macro Na Macro1

Wait (0.250000) ;

34

35

36

Job Definition - PFS ON, Multi-Point

Print Job to Clipboard

Options

Job Templates

Export

Expand All

Collapse All

Show Deposit

Rows

Execution Flow

IF

Condition (if)

Condition (if-else)

Break

Question

Time Lapse

Redefine Time Lapse

Time Sequence

Repeat

Every nth

Well Plates

Define Plate

Select Wells

Loop Over Wells

Label Wells

Align Plate

Manual Plate List

Plates

Move to Well

More to Well Center

Sample Holder

Define Dish

Define Slide

Define Free Shape

Align Sample

Stage XY Points

Generate Points

Preddefined Points

Points

Shift Point Set

New Point Set

Add Point

29

30

31

33

32

Editing Jobs.

Jobs may be edited post deployment.

Right clicking on the job in the Jobs Explorer list and selecting “Edit This Job” will open the **Job Definition Panel (29)**. This allows comprehensive configuration of the job content, order and timing.

For example:

- The imaging surface of poorer quality (ie cheap) multiwell plates may vary in height or thickness by more than 50 micrometers. For such plates it may be necessary to turn off the PFS when moving between wells, and then turn it back on to image. To configure, drag the “PFS On and Focus” task **(30)** from the left column to just above the “Run Macro1” task **(31)** in the job, and drag the “PFS Off” task **(32)** to just after “Run Macro 2” **(33)**.
- Depending on the experiment, it may be found that images fail to acquire correctly unless a small delay is added before and after image capture, and at the end of the timelapse loop. This can be set by dragging the “Macro” task **(34)** from the left column, into the JOB, just before the “Capture” task **(35)**. Double click on the “Macro” task to expand it, and enter either “Wait (0.25)”, or press the “Fx” button **(36)** and search for the Wait command, edit the delay and press okay.

Consult the NIS Help Menu and contact Microscopy Support Staff for assistance with editing jobs.

Some Advanced Features

Many of the advanced features of NIS are available both under the “Applications” menu **(1)**, and via icons displayed above the image window. See, for example:

Averaging

Pushes image noise into the background by averaging pixel intensity over a number of images.

EDF

- Extended Depth of Field creates a single, focussed image from a previously acquired z-stack.
- The Z stack may be aligned first, if necessary, via the “Align Sequence” option of the EDF button dropdown box, or the “Applications” menu.
- The completed EDF may be viewed and exported in 3D.

Realtime EDF

Automatically acquire, align, HDR and process a z-stack to produce an EDF image.

HDR

Acquire a High Dynamic Range image from a stack of different camera exposure images. This can be useful for samples that contain both very bright and very dim structures.

FRET

Use the FRET application to acquire and analyse a FRET sample.

MultiWell Plate Imaging (as performed under ND Acquire).

It is possible to image multi-well plates under the **ND Acquire Screen**. However, this is best suited to a relatively simple (ie a few wells or points), one off experiment. For more complex or repeat experiments set up under the **Multiwell (JOBS) Screen**.

To set up under **ND Acquire Screen**:

- Click the down arrow at the right end of the **XYZ Overview Panel's** header bar **(2)**, and select "Well Plate and Slide Navigation". This will replace the panel.
- Select the "Add Well Plate" button **(3)** at the top left, and choose the appropriate well plate from the pop up box. Click OK.
- A map of the chosen well plate and of an individual well will be displayed on the **Well Plate and Slide Navigation Panel (4)**.
- **NB. You MUST NOT attempt to move to, view, or image the outer rows and columns of the plate as the objective will hit the stage and cause damage. Prepare your plate accordingly.**
- Clicking on a well in the plate map will move the stage to the centre of that well. If desired, clicking on positions within the enlarged map of the single well will move to those positions.
- Focus on the sample once more and set up the Perfect Focus System if required, as described previously.
- Points (ie wells or intrawell positions) may be marked via the **XY tab** of the **ND Acquisition Panel**, by activating the next available check box in the "Point Name" column **(5)**. This will record the current X, Y & Z positions as well as the PFS offset.
- Check the option "Include Z" **(6)**.
- Click "Keep PFS On During Stage Move" **(7)**, on the **Well Plate and Slide Navigation Panel**.
- Set up the rest of the experiment as usual.

Z intensity Correction

This can assist in the production of a uniformly bright z-stack. Set up is as follows:

- Right click on the “ND Acquisition Panel” header and select the “Acquisition Controls” submenu, then select “Z Intensity Correction” **(8)**. Note that in addition to switching to the Z intensity correction configuration panel, this puts the link to the panel into the header bar’s main menu. Clicking on the header thence provides easy switching between the “ND Acquisition” and “Z Intensity Correction” Panels.
- Under “ND Multipoint”, select “Relative” or “Absolute” correction as appropriate **(9)**. “Relative” offsets the exposure scaling to compensate for potential differences in z stack starting position across the sample, whereas “Absolute” applies the compensation only to those heights at which it was defined.
- The point list in this panel displays Z heights within a stack. To populate the list either:
 - Focus and click the “Add Point” button **(10)** to add a Z value to the list. Adjust the exposure (Auto Exposure is fastest) and click the horizontal arrow pointing to the exposure to update the stored value. Repeat to cover the height range of the Z stack.
 - Alternately, with a Z stack already defined under the “Z-Stack” tab of the “ND Acquisition Panel”, click “From ND” **(11)** to populate the list with the stack’s top, centre and bottom positions. Check “Move Z to Selected Point” **(12)** and click on each point to move to each point. Optimise and update exposure values as above.
 - Repeat for each channel.

The “Run Z Corr” **(13)** located at the bottom of the “ND Acquisition Panel” immediately executes the experiment with the configured correction values. Do not click the “Run Now” button, as this will not correctly apply the correction to multiple channels. **Note:** If the corrections have not been established, pressing “Run Z Corr” will generate an error.

The option “To ND” **(14)** pushes Z points defined here to the ND Acquisition Z-Stacks Tab, setting the stack top and bottom positions.

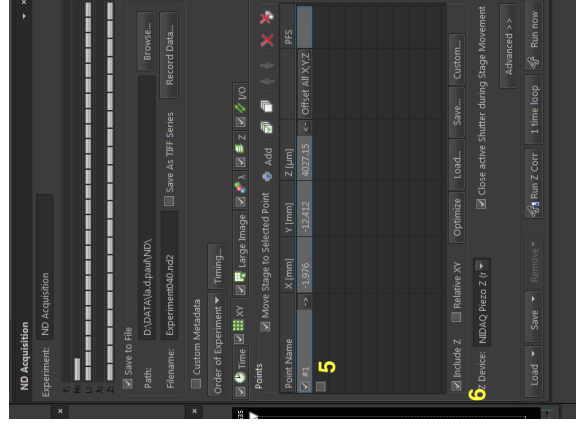
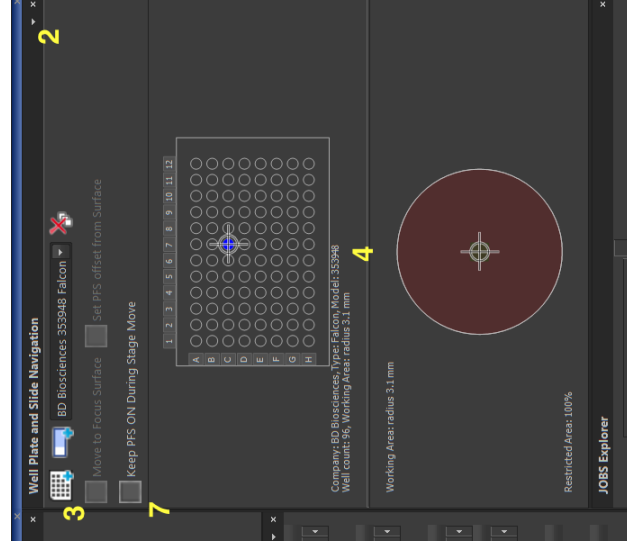
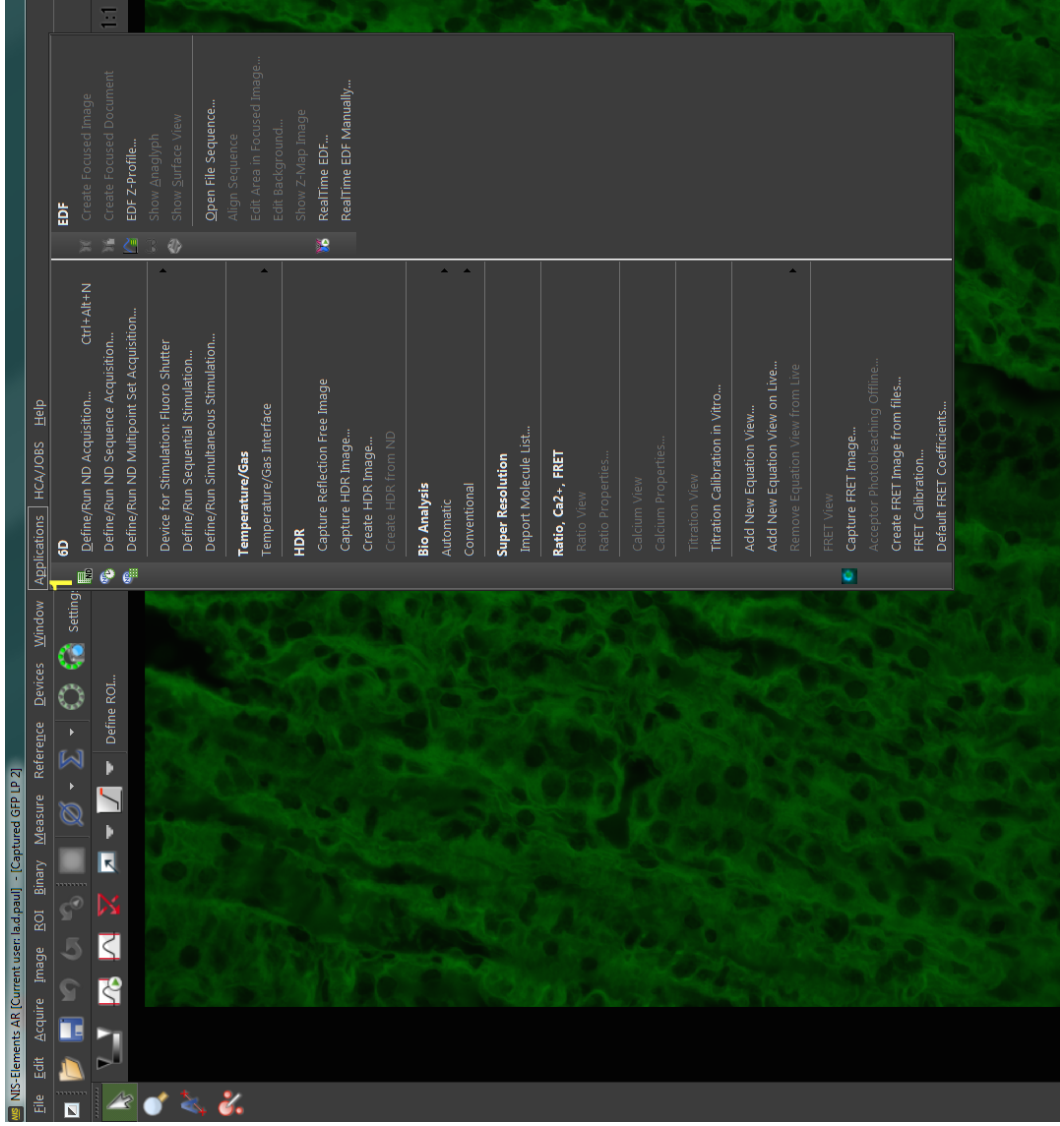
The “Use on Live” option **(15)** applies the defined correction to live imaging.

Shading (Flatfield) Correction

Shading correction applies a preacquired image of the pattern of illumination through the components of a given optical configuration to images acquired with that configuration, in order to correct illumination artifacts.

To set up:

- Click the tab at the bottom of the image screen to bring up the **Opened Images Panel (16)**, then click on the header bar and select “Shading Corrections”.
- Choose whether you would like to apply a single correction to all of your OC’s, or apply individual corrections to each OC (best).

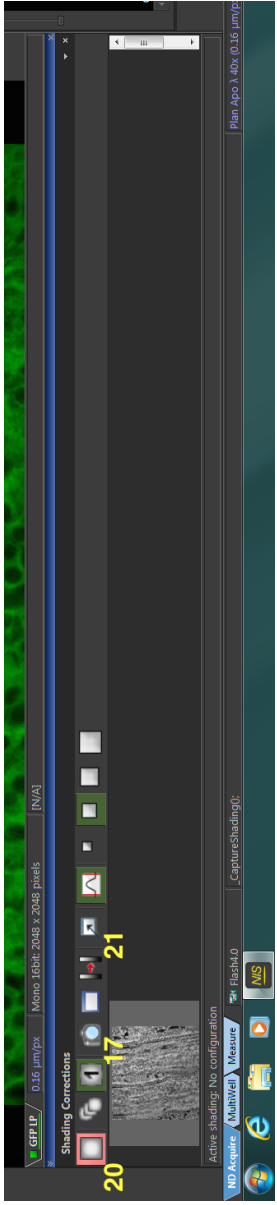
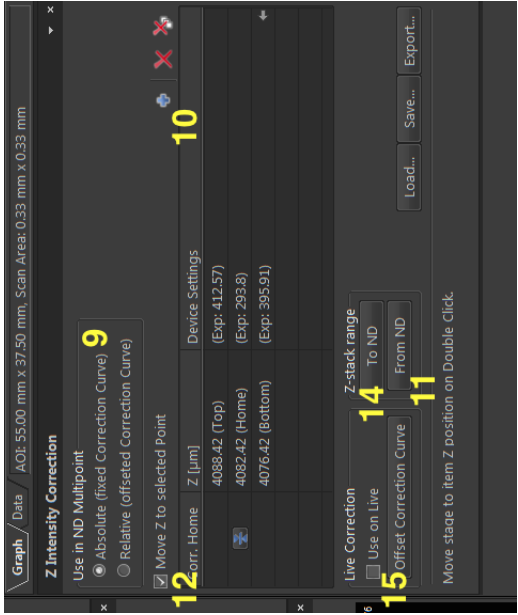
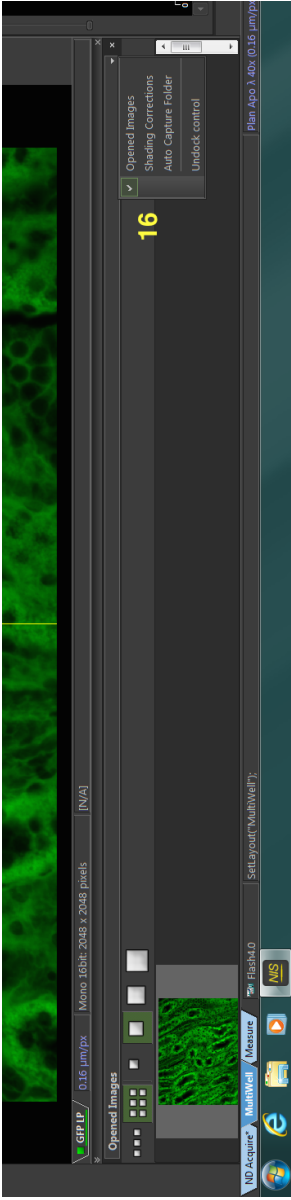
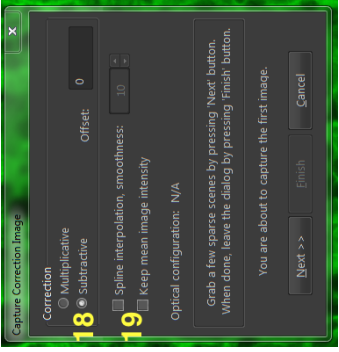
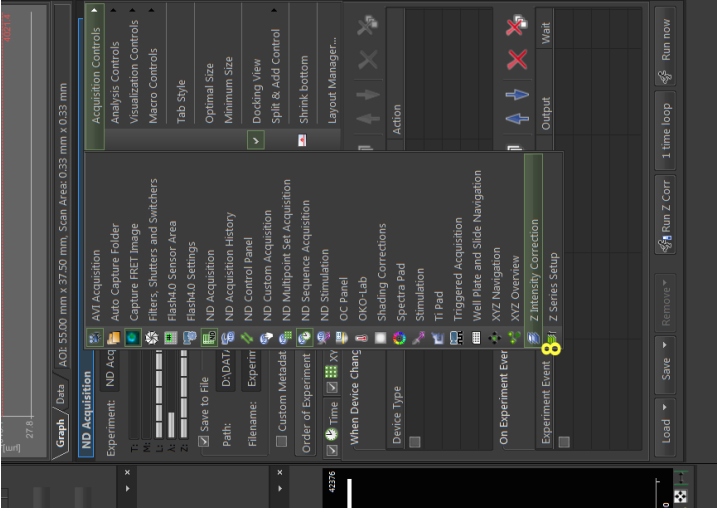


- Clicking the “Capture Correction Image” button will begin acquisition of your correction image (17).
- Choose “Subtractive” or “Multiplicative” as the “Correction” option (18). Subtractive is appropriate for correction images taken on a clear area of the sample plate or slide, while multiplicative is used for images derived from a uniformly fluorescent correction slide.
- Ensure the “Spline Interpolation, Smoothness” and “Keep Mean Image Intensity” options (19) are turned off.
- Follow the prompts.
- Click the “Shading Correction” button (20) to apply to live and subsequently captured images.
- To apply to a pre-existing image open the image and click the “Apply Shading Correction to Current Image” button (21).

Note: Right clicking on the shading correction image thumbnail also allows application, viewing and deletion of the shading image. Note also; clicking on the Opened Images Panel header bar also provides options for viewing an Intensity Profile, and specifying the Auto Capture Folder.

Shutdown Procedure

- Clean the oil immersion objectives, if used. Use the lens tissue provided, only, in the manner demonstrated by the microscopy staff. If a dry (air interface) objective is oil contaminated do not attempt to clean it; notify microscopy staff.
- Clean up any oil, sample or medium dripped on the microscope, within the chamber, or on the microscope and computer tables. Kimwipes are provided for that purpose. **NB.** You, the last user, are responsible for the condition of the microscope and ancillary equipment. If the equipment was found dirty, notify microscopy staff.
- If sample or sample medium has been spilled over any portion of the microscope please wipe it up, and then notify microscopy staff.
- Close the NIS software and wait at least 60 seconds before logging out or shutting down Windows.
- Copy the files you created to your network drive.
- Log out or shutdown Windows. This will shutdown the microscope and other hardware.
- Remove your samples, lab coat, and any other equipment you have brought with you.



Known Problems.

Extraneous light artefacts.

With dim samples, in particular, room lights and illumination light reflected from the brightfield lamp housing may swamp the sample image. It is best to switch off the room lights, including the wall mounted LED lamp. Reflection from the brightfield lamp can be minimized by switching in the blue/green filter, via the small lever at the rear of the lamp house. Ask microscopy staff if in doubt.

Software instability.

The Nikon capture software can be unstable, producing lock ups and crashes. Fortunately, these are now comparatively rare. Should a lock up or crash occur you must perform a full system restart (and NOT just a software restart). If the problem prevents Windows from responding also, hold down the computer power button for 10 seconds to force a system shutdown. Wait 30 seconds, then restart the computer.