

Using Autofocus in NIS-Elements

Overview

This technical note provides an overview of the available autofocus routines in NIS-Elements, and describes the necessary steps for using the autofocus functions.

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Autofocus Z Function

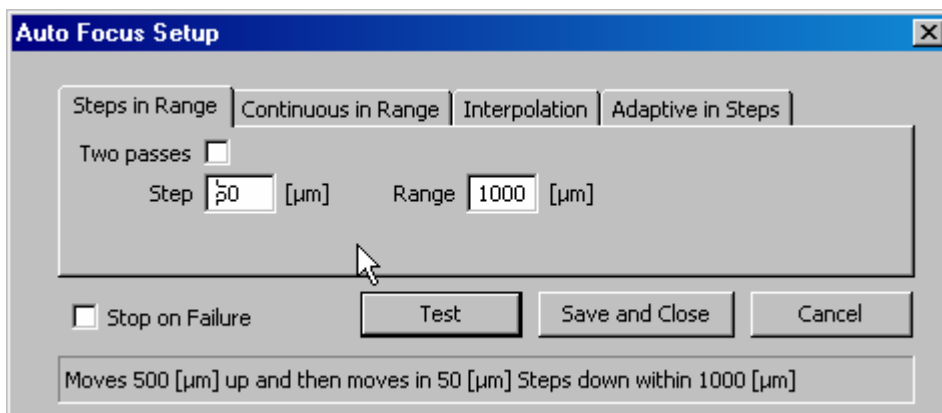
Autofocusing in Z with NIS-Elements is based primarily on recognizing contrast in an image, and finding which Z position contains the best contrast.

Because the autofocus is based on contrast, there are likely to be scenarios such that the autofocus routine does not perform with high efficiency. Careful experiment design and some basic protocols will help maximize the performance of the autofocus routines.

Autofocus can be achieved through several different techniques. The allowable techniques vary with which Z motor drive hardware is attached to the microscope. That is, different Z motors allow for different autofocus techniques. **Appendix A** at the end of this document lists some common Z motors and the available autofocus routines associated with them.

Setting up Autofocus in NIS-Elements

You can access the Autofocus Setup dialog box in NIS-Elements by selecting **Auto Focus Setup** from the DEVICES pull-down menu .



There are four Auto focus routines available in NIS-Elements, depending on the Z motor hardware installed on the system. Some routines are not available with certain Z devices.

All autofocus routines require the attached camera to actively acquire images in order to determine focus. Keep in mind this can greatly lengthen the amount of excitation energy on the specimen. Careful experimental design and camera settings can minimize this.

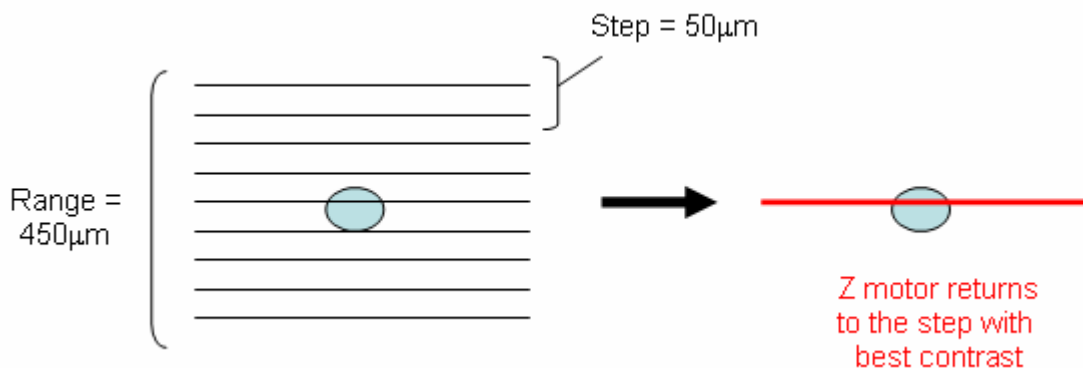
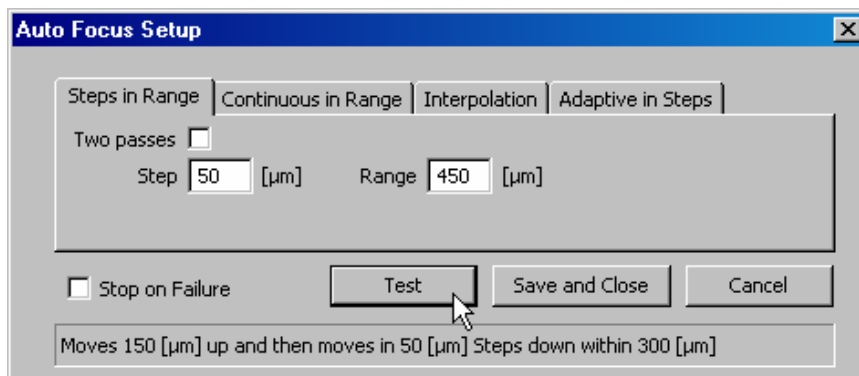
A description of how each routine works, and how to use each routine is described here:

Steps in Range method

Steps in Range method will start at the relative Z position you define (if you set this up in an ND experiment, see: ND section that follows), or the current Z position.

Steps in Range will move the Z motor in discrete steps that are defined by the user, for a range of travel defined by a user. The motor will move up by $\frac{1}{2}$ of the total range, and step down for the entire range, so that the current Z position (or Z position at the start of the autofocus routine) represents the “middle” of the range. At each step, the contrast ratio will be calculated and saved. After all steps have been completed, the position with the highest contrast ratio will be used as the “focused” position, and the Z motor will move to that position.

Autofocus success using **Steps in Range** is very sensitive to how coarse the step size is. This will depend on the microscope objective/depth of focus used. Example: lower magnification (e.g., 10x) objectives will typically use large coarse step sizes and larger ranges, and higher magnification (e.g., 60x) objectives will typically use small coarse step sizes and smaller ranges.



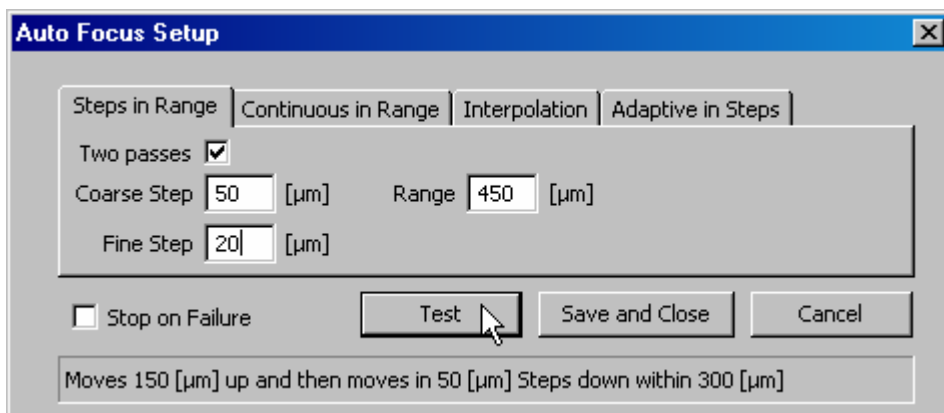
NOTE: This method may not return the best plane of focus – it only returns the step with the best focus. The best focus in the sample may have been somewhere between the steps that were taken in the autofocus routine.



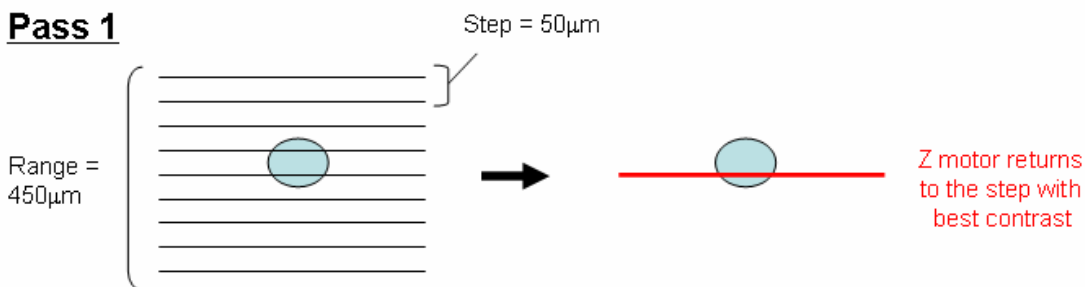
Steps in Range method- continued

The checkbox “**Two passes**” allows the user to select two independent step sizes for “fine tuning” the focus. In the first pass, the coarse step size will be used across the entire range that is defined.

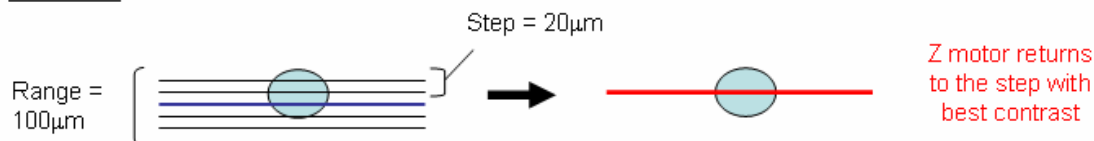
Once the Z position with best contrast is selected, the Z motor will move to that position, then make a second pass with smaller steps, using a new range that is 2x the size of the coarse step size in pass #1.



Pass 1



Pass 2



NOTE: This method may not return the best possible plane of focus – it only returns the step with the best focus. The best focus in the sample may have been somewhere between the steps that were taken in the autofocus routine.



Continuous in Range Method

Continuous in range method is designed for faster Z motor devices, and works particularly well with high frame rate acquisitions (e.g., video rate). Instead of taking discrete steps, continuous mode moves the Z motor continuously across the defined range. During that travel, the contrast ratio is calculated. The Z position corresponding to the best contrast ratio is

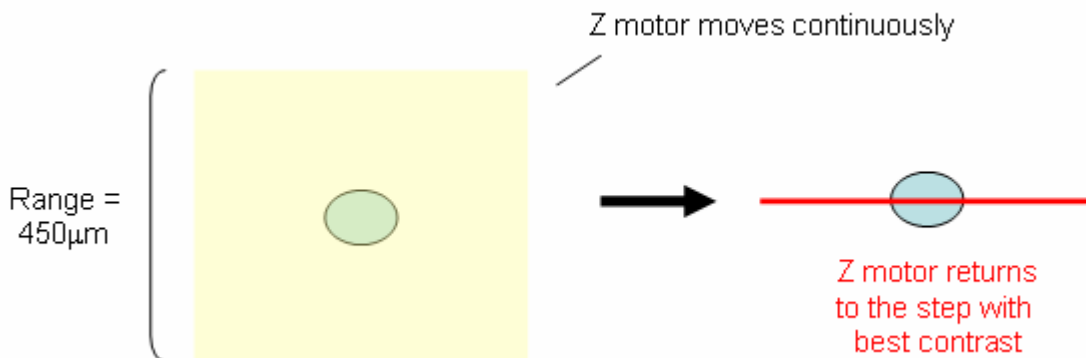
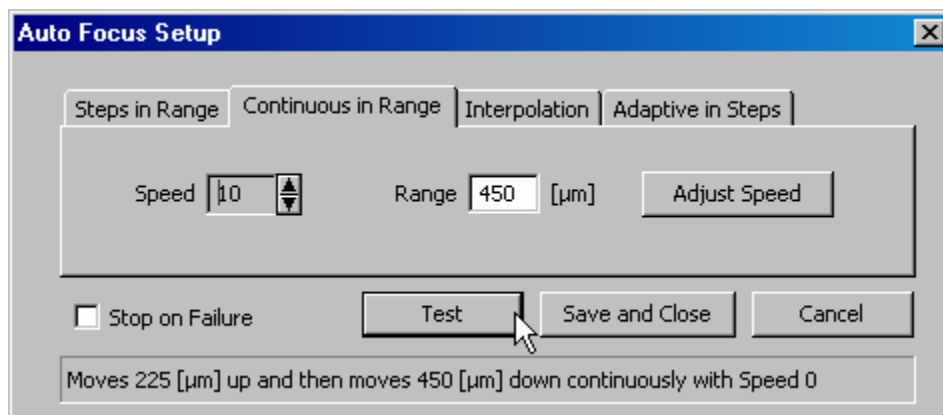
The shorter the delay between frames, and the faster the Z motor moves, the more successful the autofocus.

Continuous in Range Autofocus is best suited for piezoelectric Z motors and brightfield applications.

This method requires 2 basic settings:

Speed – relative speed of the Z motor, which should be related to the LIVE frame rate of the camera for best results.

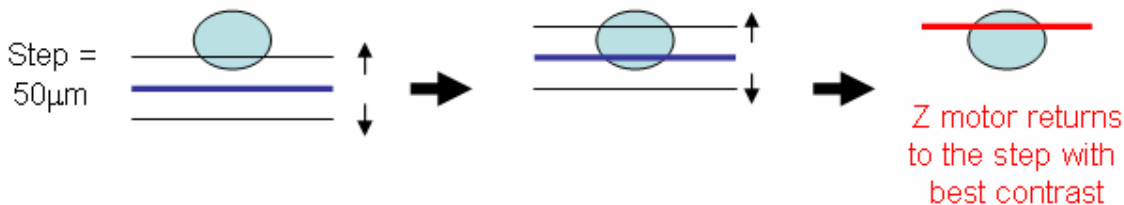
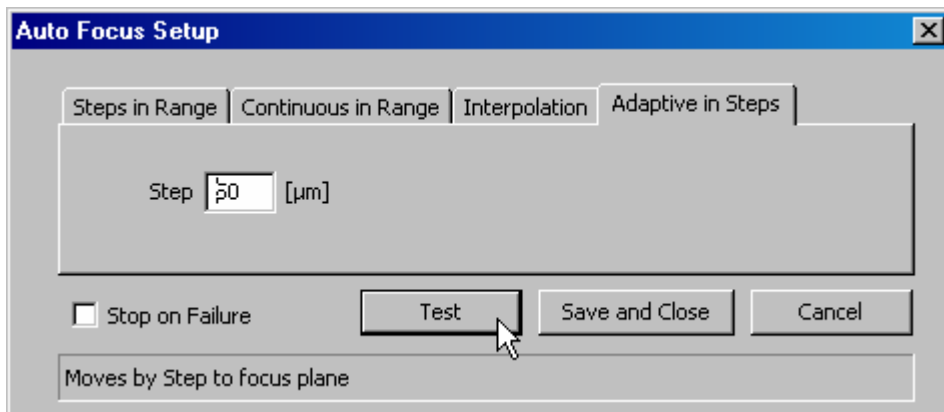
Range – the range of travel that the autofocus will use. The Z motor will move up $\frac{1}{2}$ of the total range, start LIVE, then move continuously down through the entire range of travel.



Adaptive in Steps Method

Adaptive in steps method is a robust autofocus method that does not require a range of travel to start. After inputting a **step size** for the Z motor to move, adaptive in steps method will step in each direction relative to the current Z position and determine which direction has the highest contrast ratio. This process is repeated until the highest contrast ratio is found. Here, the better contrast ratio position is chosen after each step, and that position is used as the home position for the next step.

As with *Steps in Range method*, Autofocus success using **Adaptive in steps** is very sensitive to how coarse the step size is. This will depend on the microscope objective/depth of focus used. Example: lower magnification (e.g., 10x) objectives will typically use large coarse step sizes and larger ranges, and higher magnification (e.g., 60x) objectives will typically use small coarse step sizes and smaller ranges.



NOTE: This method may not return the best possible plane of focus – it only returns the step with the best focus. The best focus in the sample may have been somewhere between the steps that were taken in the autofocus routine.

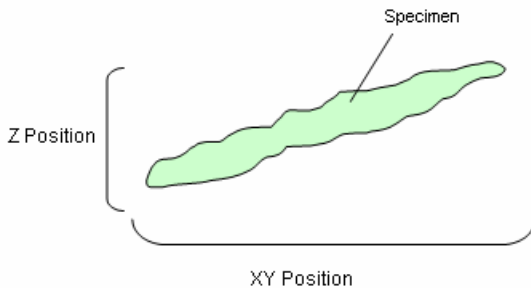
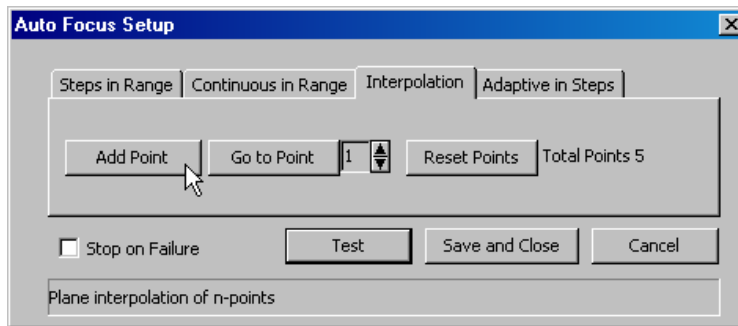


Interpolation Method

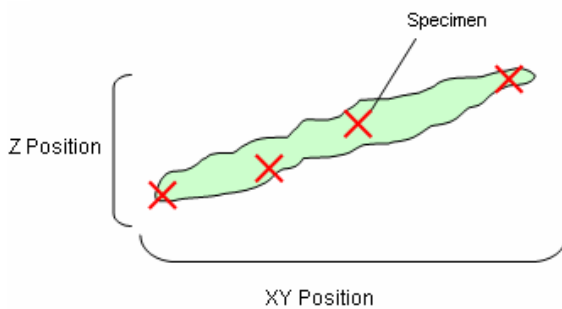
Interpolation method is uniquely different from the other autofocus methods. Interpolation does not actually perform an autofocus routine based on contrast ratio. Interpolation method requires an automated XY stage for operation.

Instead, the interpolation method is used primarily to account for changes in Z position across a specimen (in particular, larger specimens). The user teaches the software several XY positions along the specimen, and the corresponding Z position.

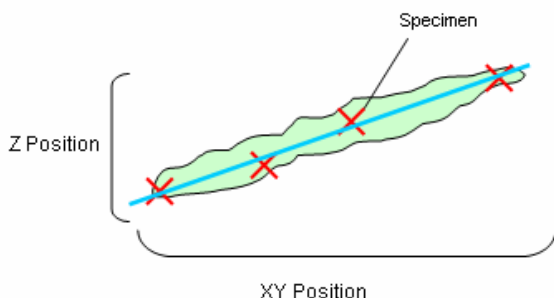
NIS-Elements uses the XYZ positions that are saved to develop a standard curve, to predict the most likely focused position along XY position.



For specimens with varying focus (z) positions, interpolation method can be used



At different XY positions, the user will focus on the specimen, then Add Points to represent where the specimen is in focus

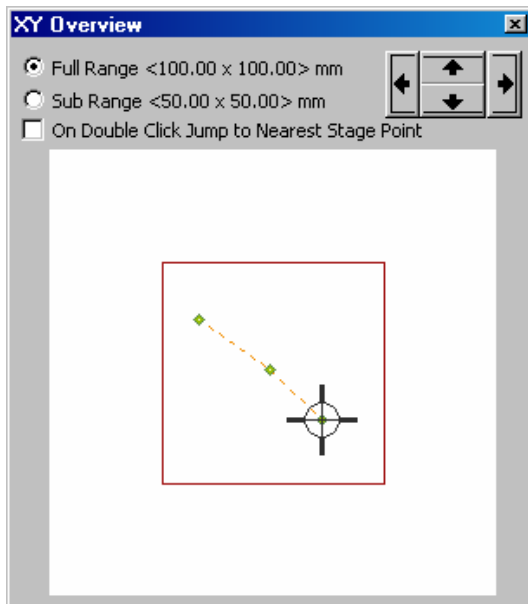
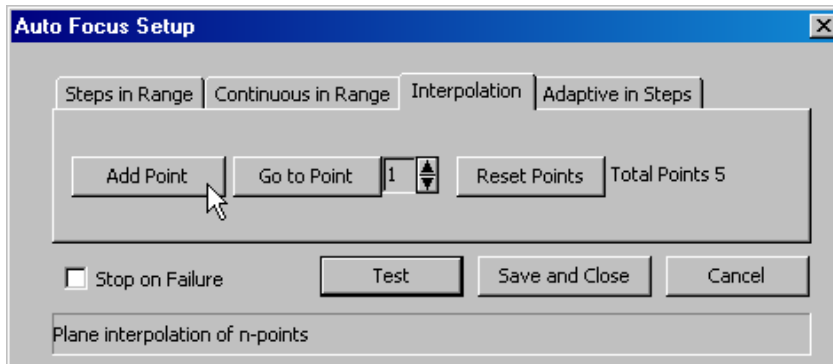


Interpolation method will determine a best-fit focus "line" between each of the memorized points. The "home" Z position at any XY position is interpolated and available during experiments.



Interpolation Method-continued

Interpolation method is most commonly used for specimens with a predictable change in focus, and is primarily used for creating Large Images.



To use the interpolation method, move to an XY position, then focus the specimen.

Then choose “add Point”

Next, move to a new XY position and refocus the specimen.

Repeat the process as needed. NIS-Elements will calculate and interpolate the “best” Z position to use at a given XY position.

At left, the navigation window “**XY Overview**” is used to help the user see what XY positions have been added.



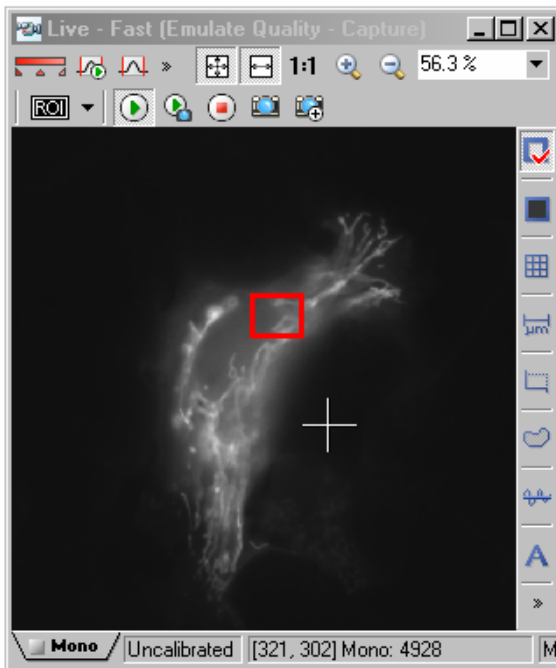
Fine-tune of Autofocus

There are various methods to fine-tune autofocus in NIS-Elements to help improve the rate of success. Apart from choosing appropriate step sizes, taking care as to the number of iterations (length of time) of focus, and choosing wavelengths or illumination techniques with the best possible contrast, there are some additional tools.

Probe Tool

Enable the Probe Tool on the image window, and positioning it over an area of high contrast. The autofocus routines and calculations will use the contrast ratio data from within the probe tool only, rather than the entire image, to calculate the autofocus.

This is useful for specimens where the user wishes to choose the portion of the specimen that should be critically focused.



Here, the probe tool is active and placed over an area of high contrast to improve autofocus.

Note: This method is useful for a single XY position, or with multiple stage positions only if the user ensures the probe is always selecting an area at all XY positions of contrast.

Relative Contrast Tool

A user can monitor the relative contrast of any live image by referencing the relative contrast tool found at the bottom-center of the NIS-Elements application window.

This tool, represented by a meter and relative number value, increases with increasing contrast. This can be monitored during an autofocus routine, or used manually.



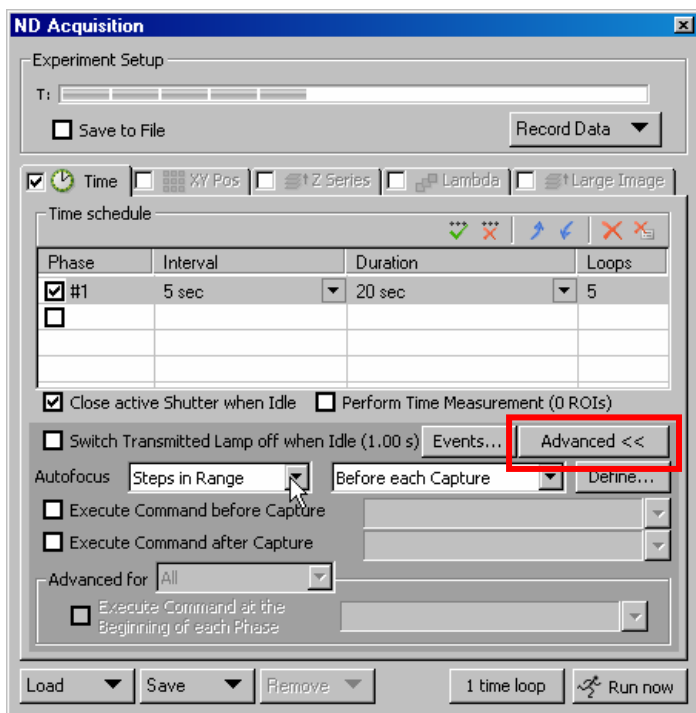
Using Autofocus in ND Experiments

Note: This is only available in Ar with 6D module and Br with 4D module

Autofocus can be enabled in ND experiments in various locations. Depending on the experiment setup, you may wish to autofocus at:

- different time points
- stage positions
- wavelengths (commonly, users use brightfield channels, or the most stable fluorescence channel, to use for autofocus)
- time phases.

Access to the autofocus controls is found in the **Advanced** submenu of the ND Acquisition dialog box, in the *Time*, *XY Position*, and *Lambda* tabs.



The available Autofocus routines for ND experiments are typically: **Steps in Range** method & **Adaptive** method.

Users can choose when to initiate the autofocus routine (e.g., before each capture, at the beginning of the experiment, at the beginning of the time phase, etc.)

Access to the autofocus function in ND Acquisition is by choosing the **Advanced>>** button, which expands the dialog box to display the Autofocus options.

Autofocus will use the currently set range, or alternatively a user can **Define...** the range used from within the ND Acquisition dialog box and define the autofocus parameters.



Appendix A: Z motor hardware and Autofocus routine compatibility

Motor	Steps in Range	Adaptive	Continuous
TE2000	●	●	
Ti-E	●	●	
Prior Z	●	●	●
Piezo Z	●	●	●
Nikon RFA	●	●	

