

NIS-Elements: Ratio Acquisition

Configuration of a Fura-2 Ratio Acquisition

This document specifies the typical set up of a two wavelength timelapse in the NIS-Elements AR package with the 6D (MQS42560) and Ratio/ Fret Module (MQS42580). This configuration assumes that the ratio timelapse will be collecting 340 and 380^{1,2} at each timepoint with the ultimate goal of performing a calcium calibration post acquisition. The calcium calibration will determine the level of free calcium (in um) at each pixel in the dataset.

Note number: 0010
 Date: 6/2009
 Date Modified: 6/2009
 Software Version: 3.0
 Package: AR , 6D Module, Ratio/ FRET Module

There are many reasons that ratio acquisition is selected as an imaging method. In the case of observing levels of calcium calibration, Invitrogen's Product Information Sheet for their calcium indicators highlights the main advantages well:

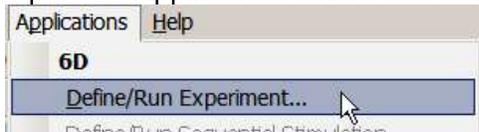
"Ratioing considerably reduces the effects of uneven dye loading, leakage of dye, and photobleaching, as well as problems associated with measuring Ca²⁺ in cells of unequal thickness."

Access to NIS-Elements Ratio Dialogs

Ratio Acquisition utilizes the following NIS-Elements dialogs

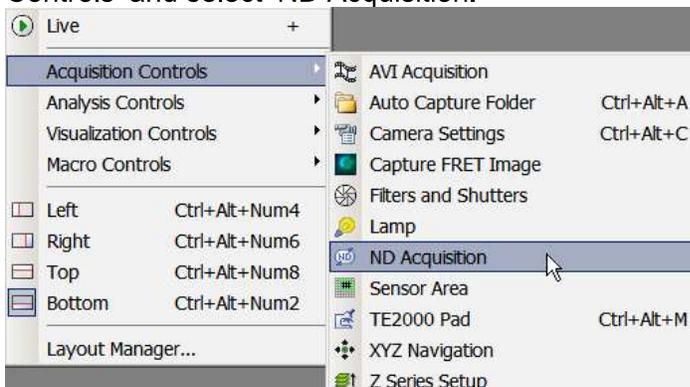
- **6D (ND Acquisition Dialog)**

- Open the Applications Menu and Select 'Define/ Run Experiment'



OR

- Right click on an open area of NIS-Elements desktop. Select 'Acquisition Controls' and select 'ND Acquisition.'



¹ Reference the Invitrogen's Product Information Sheet for 'Fura & Indo Ratiometric Calcium Indicators': <http://probes.invitrogen.com/media/pis/mp01200.pdf>

Protocols for dye preparation, concentration and loading are addressed in this document.

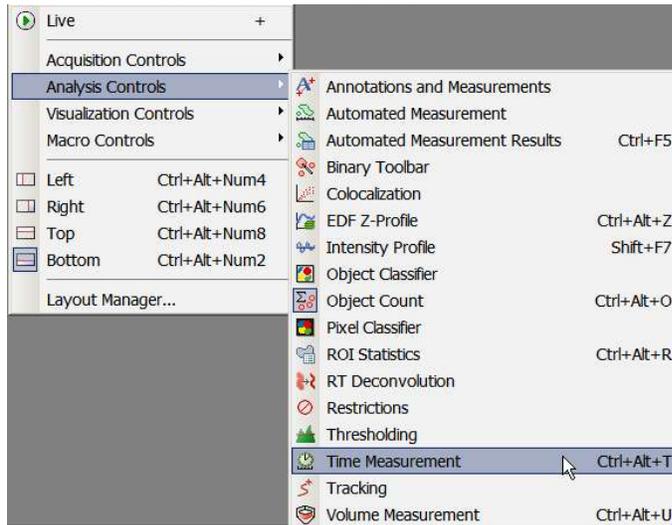
² This note describes set up of a Ratio timelapse using standard optical configurations. A different PowerPoint is available for details on the set up of a Ratio timelapse using Multi-Excitation (if utilizing the speed of a multi excitation device such as a Sutter DG4 or DG5).



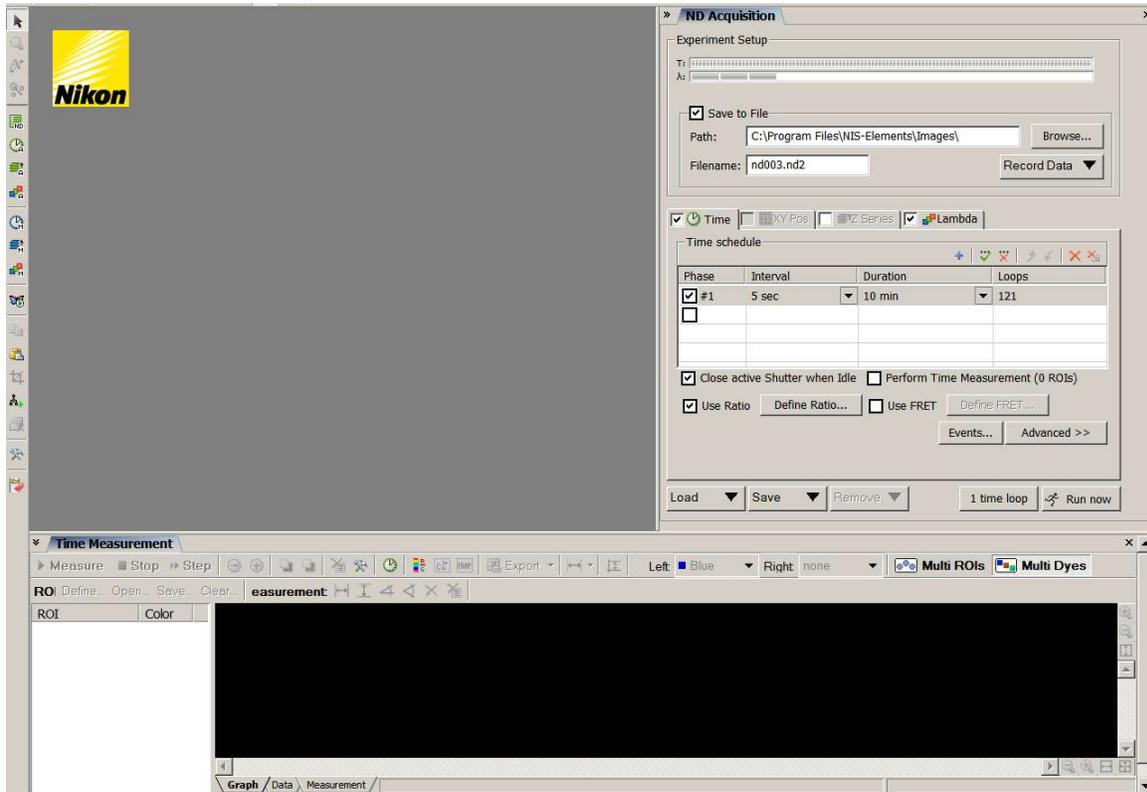


- **Time Measurement**

- Right click on an open area of NIS-Elements desktop. Select 'Analysis Controls' and select 'Time Measurement.'



Note: If desired, the ND Acquisition dialog can be docked (anchored) to the bottom or side docking panels. The Time Measurement dialog can be docked (anchored) to the bottom or top docking panels. Double click on the top frame of the dialog to quickly dock the dialog to a docking panel.



Steps for Configuring Ratio Acquisition

- 1) Create an Optical Configuration (microscope only) for 340 channel
- 2) Create an Optical Configuration (microscope only) for 380 channel

Notes:

- 1) If different camera settings (exposure, EM gain, etc) are desired for individual channels, then create microscope + camera settings optical configurations.
- 2) Active Shutter will most likely be the EPI Shutter.
- 3) The only difference between these two Optical Configurations may be the filter state of the excitation of 340 and excitation of 380.

Optical configurations

Name	Storage
340	Shared
380	Shared

Details

Objective: 1 - Plan Apochromat 4x

Camera setting:
 Assign current camera setting

Microscope setting:
 Assign current microscope setting

Options...
 Active Shutter

Property Values for Fast Mode:
 Property Values for Quality Mode:

Filter-Exc: 2 (EX340-380)
 Filter-Bar: 1 (EM400)
 FilterBlock1: 3 (UV-28)
 FilterBlock2: 1 (ANALY)

Comment:
 Shading: Not available
 Show on toolbar

Objectives

Name	Storage	Calibration	Working Dist...	Numerical Apert...	Z-Step (...)	Z-Ste...
Uncalibrated		Uncalibrated	NA	NA	NA	NA
Plan Apochromat 4x	Private	Not yet calibrat...	15700 µm	0.2	12.5 µm	25 µm
Plan Apochromat 10x	Private	Not yet calibrat...	4000 µm	0.5	2 µm	4 µm
Plan Apochromat 20x	Private	Not yet calibrat...	1000 µm	0.8	0.8 µm	1.6 µm
Plan Apochromat 40x	Private	Not yet calibrat...	100 µm	1	0.5 µm	1 µm
Plan Apochromat 60x Oil	Private	Not yet calibrat...	200 µm	1.4	0.3 µm	0.6 µm

Buttons: Insert, New..., Edit..., Duplicate..., Remove..., Recalibrate..., Backup..., Restore..., Close

2) Check on the Lambda Tab of the ND Acquisition Dialog:

Time XY Pos Z Series **Lambda**

Setup

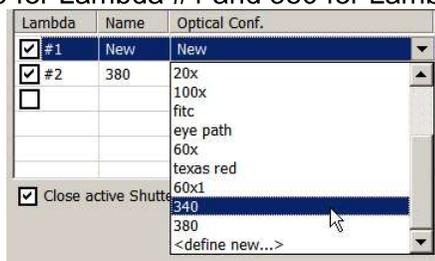
Lambda	Name	Optical Conf.	Comp.C...	T ph...
<input checked="" type="checkbox"/>	#1	340		All
<input checked="" type="checkbox"/>	#2	380		All
<input type="checkbox"/>				

Close active Shutter during Filter Change

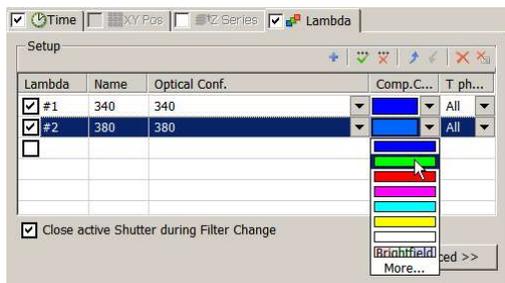
Advanced >>



3) If Lambda Tab is not already populated with the correct Optical Configurations, select the check box on 2 rows. Click on drop down menu of the Optical Configurations to select 340 for Lambda #1 and 380 for Lambda #2.



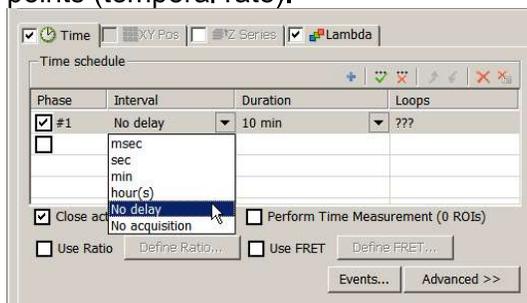
4) In the case of 340 and 380, this is a dual excitation experiment. These two channels will share the same emission (510nm). In this case, the component color (pseudocolor) of each of these channels will be the same because NIS-Elements uses the emission wavelength as the default to set the component color. If it is desired to have differing component colors for 340 and 380, then select the drop down list of component colors to change (or both) of the lambdas.



At this point, it is recommended (if not completed already) to optimize the correct camera exposures for 340 and 380.³

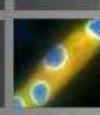
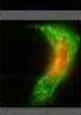
5) Configure the Time Tab of the ND Acquisition Dialog

a) Select the dropdown menu for the Interval and set the desired time between time points (temporal rate).

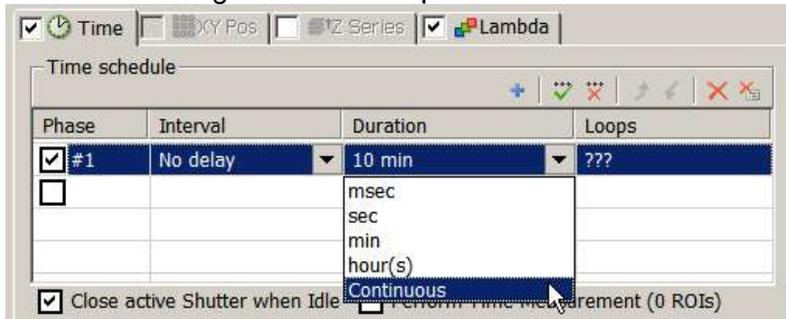


³ In a typical Fura-2 experiment, 380 emission may “appear” brighter than 340 emission at baseline. When establishing optimal exposure, be cognizant that in an experiment that induces a release of calcium, the emission of 340 may increase from baseline. Set exposures to match the dynamic range of the camera and also the full length of the timelapse and possible biological responses to drug additions.

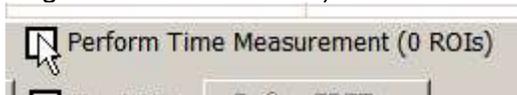




b) Select the dropdown menu for the Duration and set the desired length of the timelapse. In this case, 'Continuous' is selected, which indicates that the timelapse will continue until halted. If 'Continuous' is selected, the 'Loops' will display as '???' because the length of the timelapse is not known at onset.



c) If it is desired to graphically view intensity changes over time through the course of the timelapse, select the checkbox next to 'Perform Time Measurement.' Initially it will display '(0 ROIs)' until ROIs are drawn on the image (this is addressed on Page 7 of this document).



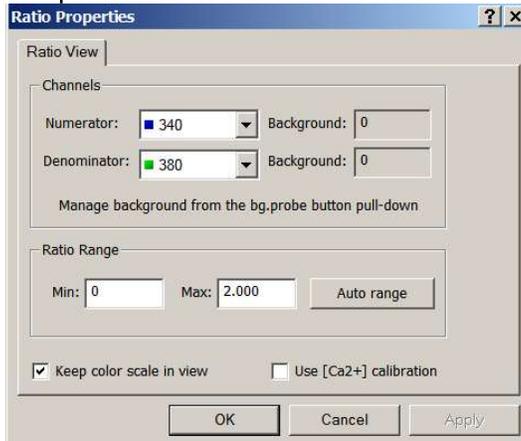
d) Select the checkbox next to 'Use Ratio.' The 'Define Ratio' button will become active.



e) Select the 'Define Ratio' button.

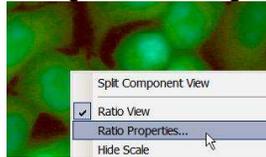


This is the location of the settings associated with the Ratio View and [Ca2+] Calcium Calibration parameters.

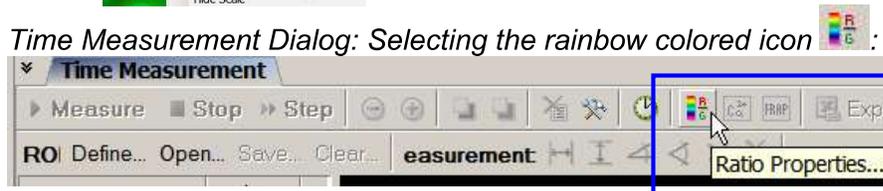


Note: It is possible to access this dialog from various locations:

- The Time Tab of the ND Acquisition Dialog
- Right clicking on the image and selecting 'Ratio Properties'



- Time Measurement Dialog: Selecting the rainbow colored icon



The Ratio Properties is the location to specify the numerator, the denominator, define a background offset (through the background probe- discussed later in this document on Page 9.), the ratio range, and further option for the Calcium Calibration, which is also discussed later in this document on Page 12.

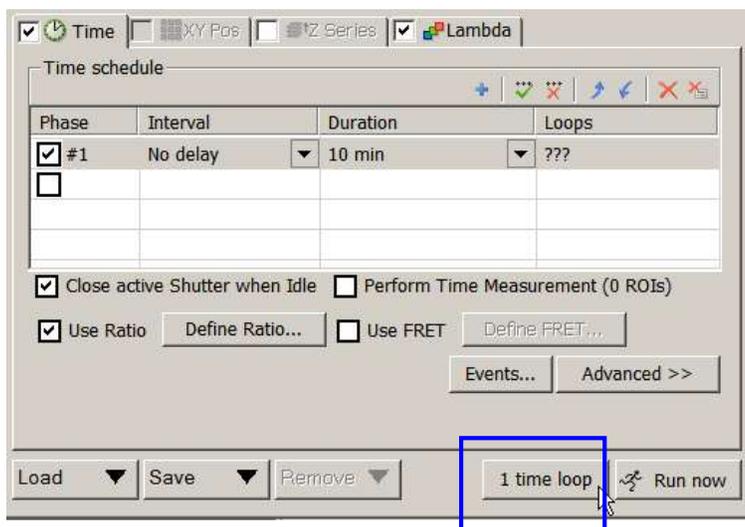
- The Ratio Range is a parameter that specifies the Intensity Modulation Display (IMD)^{4, 5}. Using the Auto range option will automatically scale the IMD based on ratios in the dataset.
 - 'Auto range' may not be available at the onset of an experiment/ timelapse because the data for the ratio has not yet been generated.
- 'Use [Ca2+] calibration' will be an important item to check at later time after acquisition. There are instances when [Ca2+] calibration can be specified before acquisition. Values from a previously run experiment can be inserted here, however, this will be addressed later in this document on Page 12.

Click on 'OK' to close the Ratio Properties dialog.

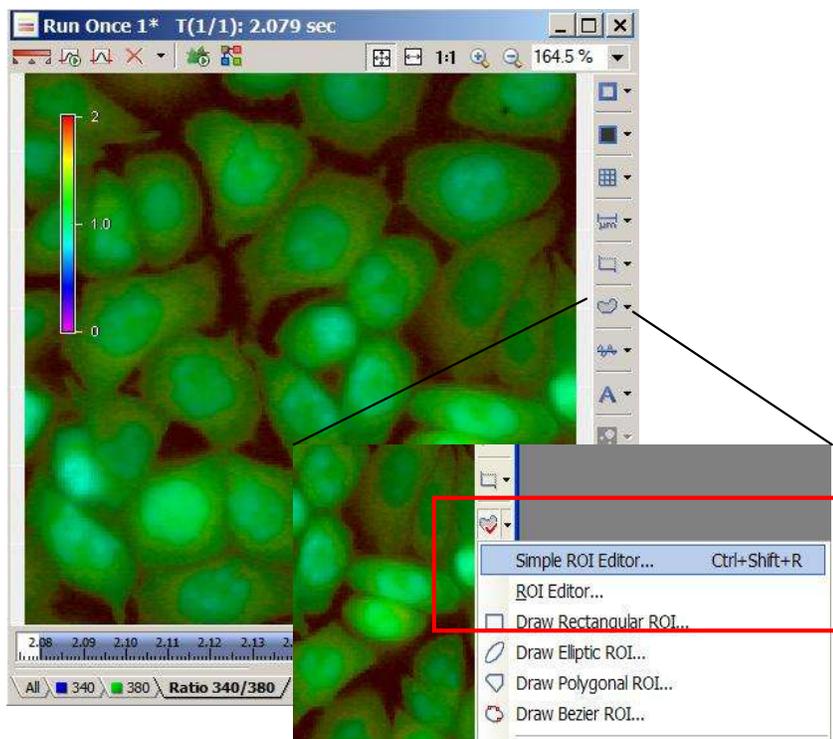
⁴ Intensity Modulation Display (IMD): Ratio images are constructed by displaying the ratio in a range of colors. (HOW MANY?) The display utilizes a combination of intensity and ratio level. (Live Cell Imaging: Robert D Goldman & David L Spector 2004 Cold Spring Harbor Press)

⁵ Tsien, R.Y., and Harootunian, A.T. 1990. Practical design criteria for a dynamic ratio imaging system. *Cell Calcium* **11**: 93-109.

6) Click on 'Run Once.' This will capture one iteration of the mutli-channel timelapse. The purpose of only one image is to be able to capture 1 frame and create regions of interest (ROIs) without having the EPI shutter open and consequently photobleaching.



7) Select the dropdown menu from the ROI tool  in the image window. There are various options for creating ROIs. In this case, select 'Simple ROI' editor to draw regions on the image⁶.

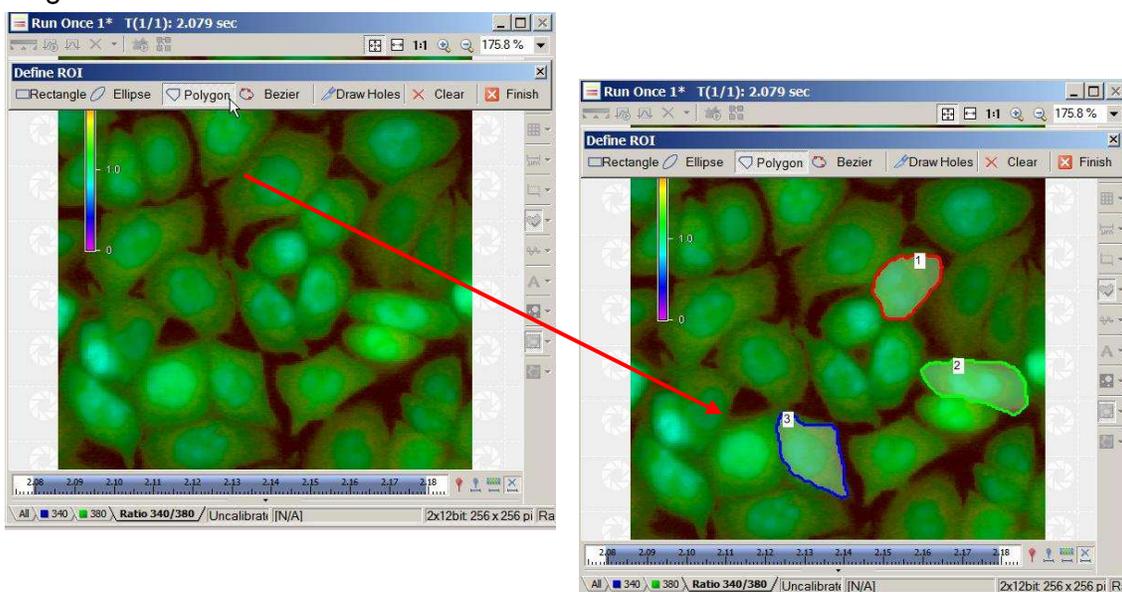


⁶Reference 'How To Note # 001' for a description of various aspects and details of ROI options and possibilities in NIS-Elements.

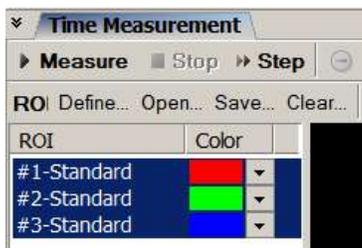
Note: It is also possible to access the same Simple ROI editor directly from the Time Measurement Dialog.



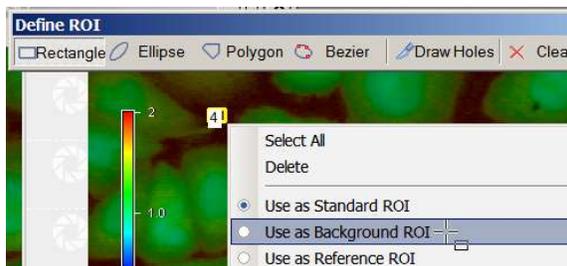
Select desired type of ROI definition tool and draw ROIs on image. To have ROI accepted, right click or double click and the cursor will release.



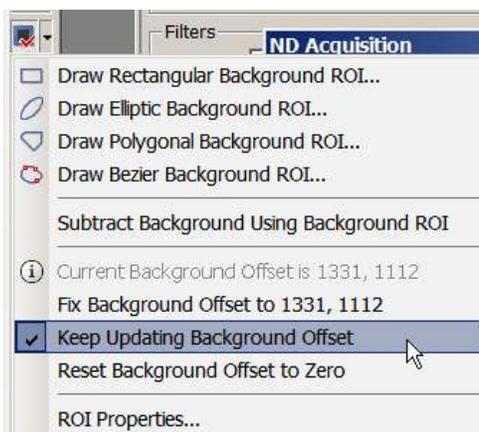
The list of ROIs will build in the Time Measurement Dialog:



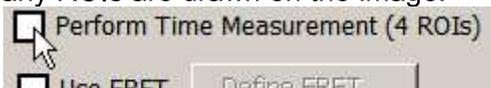
It is also possible to specify that an ROI be designated as a background ROI for background subtraction in the Time Measurement graphing and calculation.



Once a background ROI is specified, it immediately begins to perform a subtraction and it will be noticeable in the IMD display. The background probe icon on the image window  will also become active. This dropdown menu provides more options for how to utilize this background ROI. In this case, 'Keep Updating Background Offset' is selected.

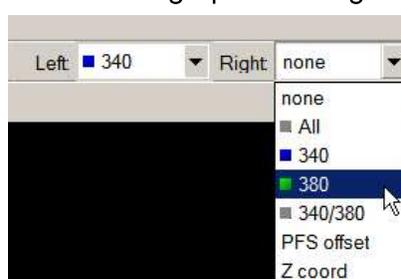
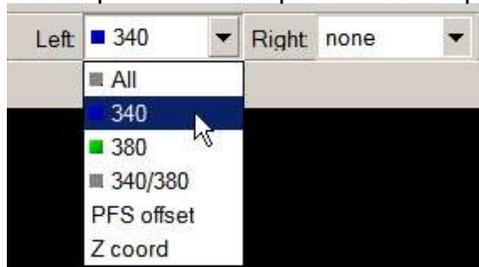


It is necessary at this time to recheck 'Perform Time Measurements' in the Time Tab of the ND Acquisition Dialog after ROIs are created on the image. Notice that NIS-Elements will read in how many ROIs are drawn on the image:



8) Time Measurement Graph Options

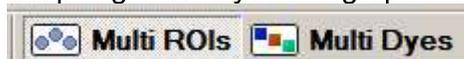
At this point in the experiment set up, notice what will be graphed during the timelapse.



Two channels or recorded data can be graphed in the Time Measurement display. In this case, select 340 for 'Left' and 380 for 'Right.' It is also possible to graph the ratio as well.

Graphing 'Multi ROIs' will graph each cell/ ROI individually over time.

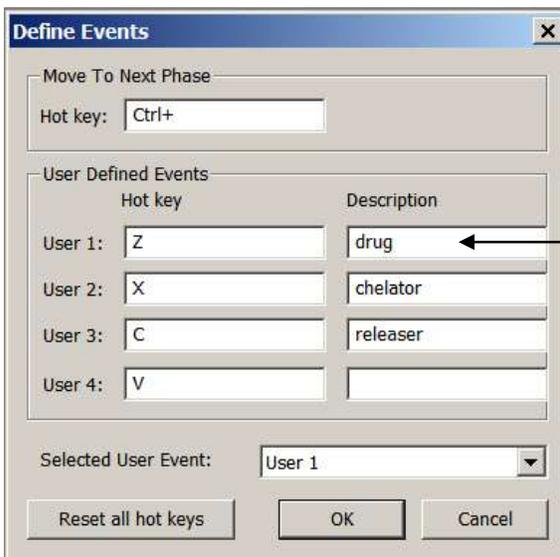
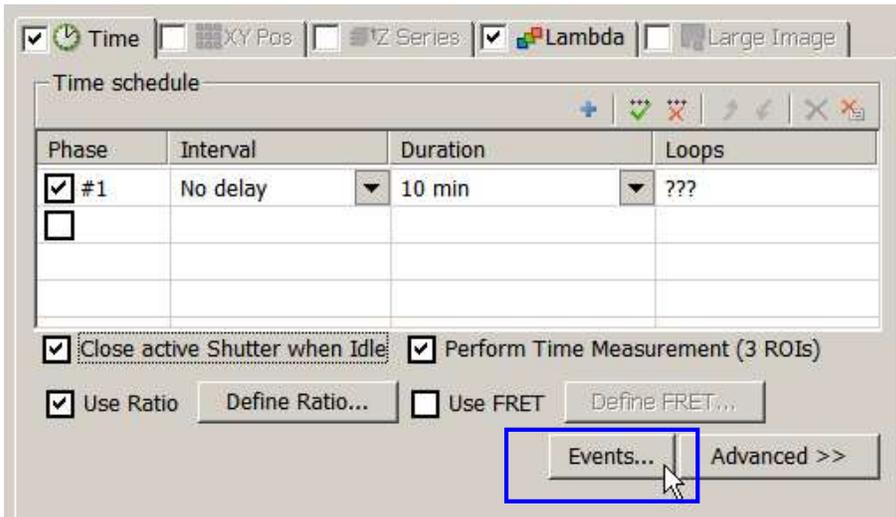
Graphing 'Multi Dyes' will graph the average intensity/ ratio of each cell/ ROI over time.



Note: These are graphing options for the during of the timelapse, but can be modified after acquisition. Choices made at this point in the experimental set up do not affect the data values or limit graph choices post acquisition.

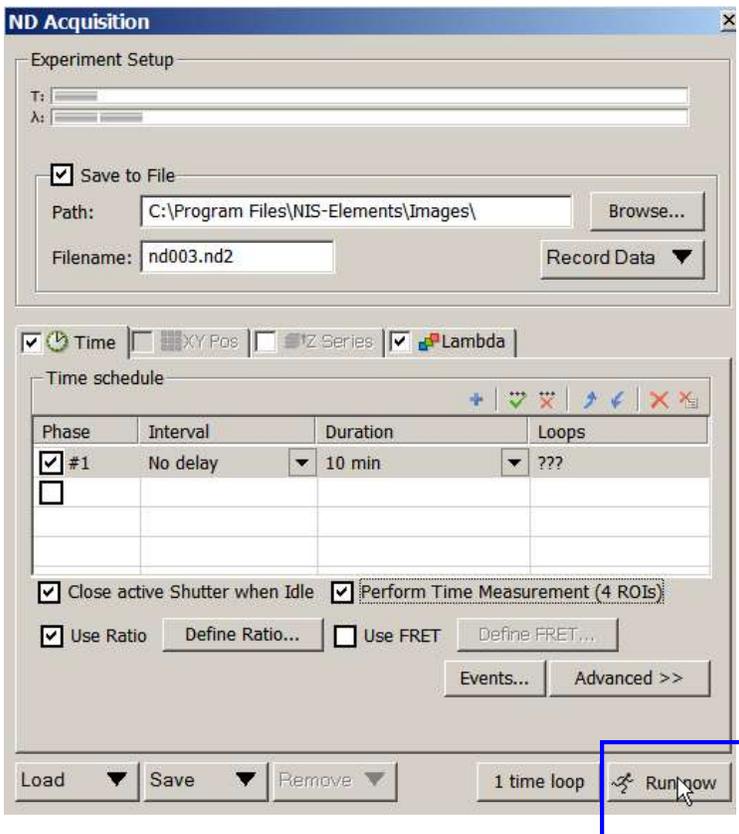


9) Inserting Event markers during acquisition are also an option. Click on 'Events...' in the ND Dialog.



Enter which keyboard Hot Key to use and then enter a description. In this case, when 'Z' is hit on the keyboard, 'drug' will be added to the image information and the display for this time point.

10) Start Acquisition by selecting 'Run Now' at the bottom right hand corner of the ND Acquisition Dialog.



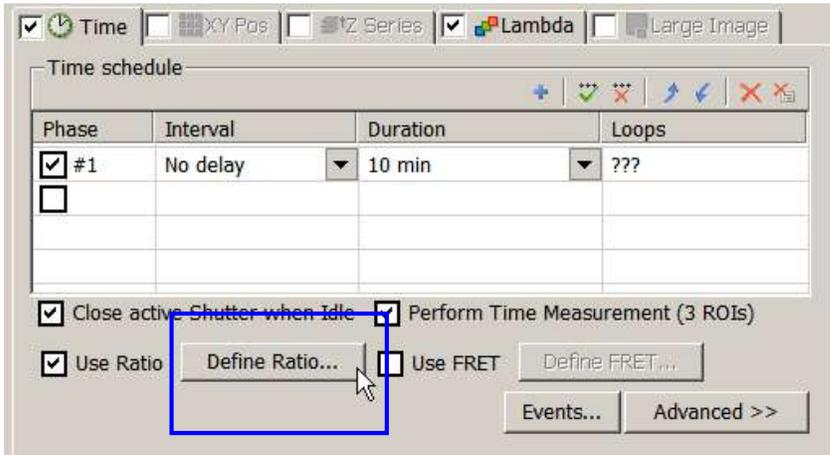
Experiment will progress and ROIs will graph in the Time Measurement Dialog.

After experiment/ data capture is complete...

11) Calculating the free Ca²⁺ concentration in nanomoles (um).

To calculate the free Ca²⁺, from the ratio graph, it is required that you have run through at least one full acquisition.

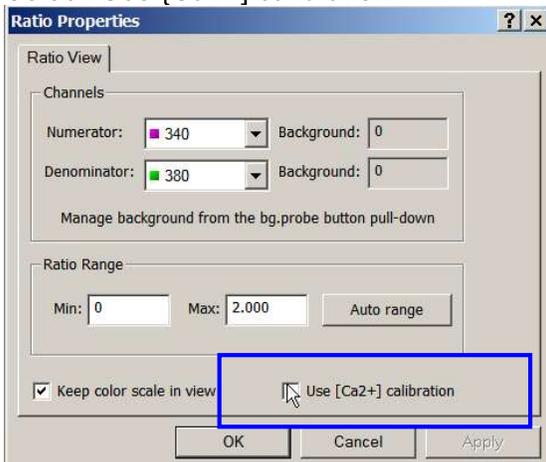
In the 'Time' tab of the ND dialog select 'Define Ratio...'



Defining Ratio parameters is also available from the  icon from the Time Measurement dialog:



Select 'Use {Ca²⁺} calibration':

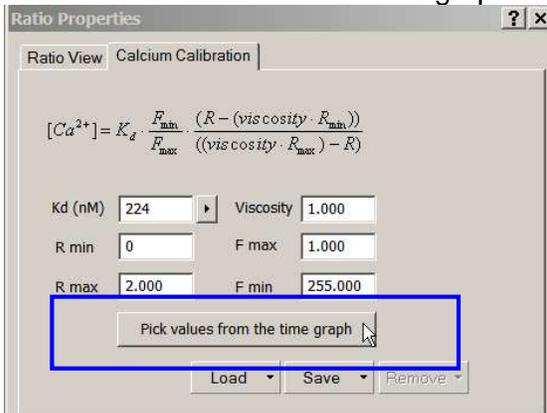


A new tab becomes available 'Calcium Calibration.'
Click on this tab.

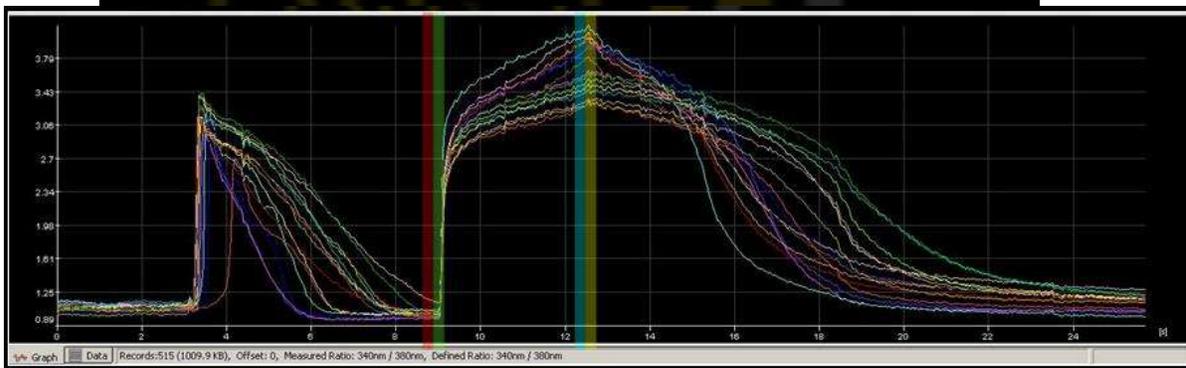
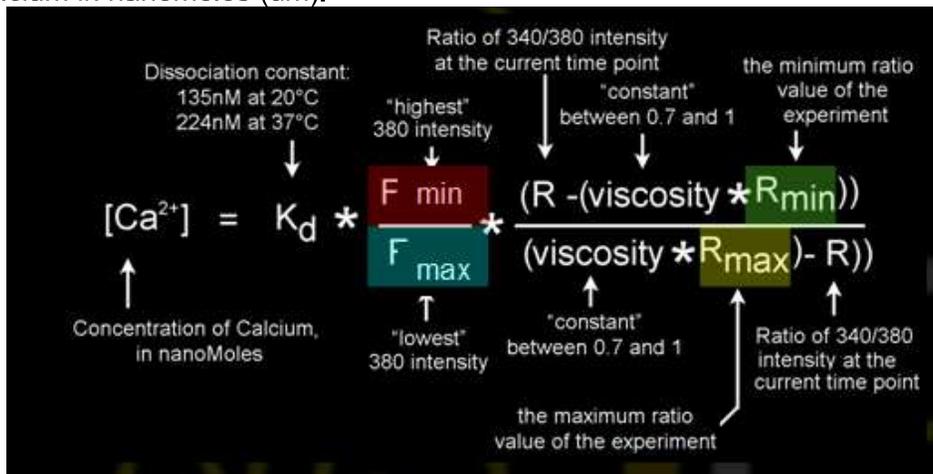




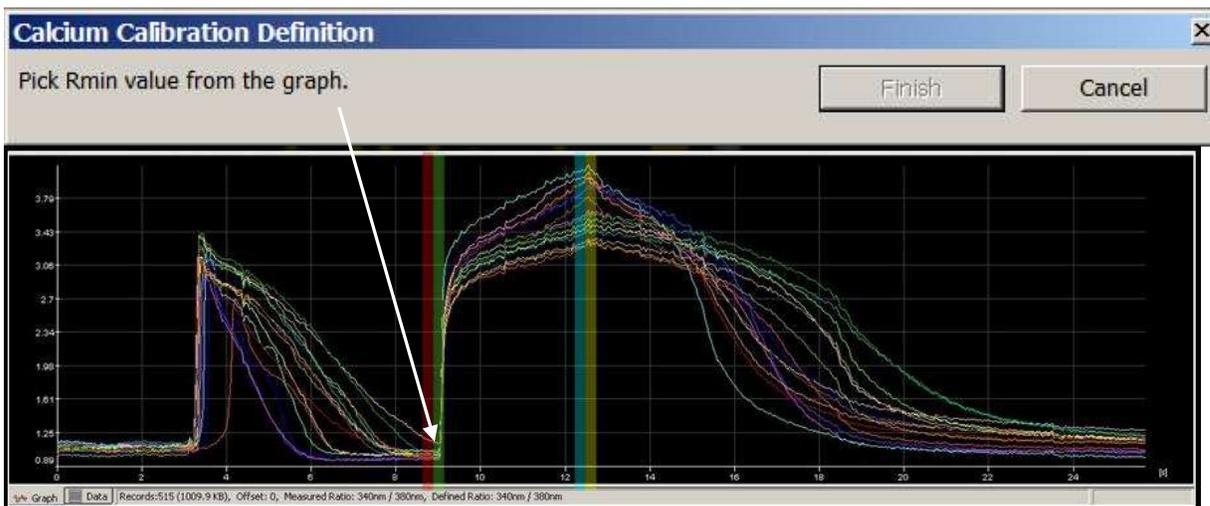
Select 'Pick values from the time graph.'



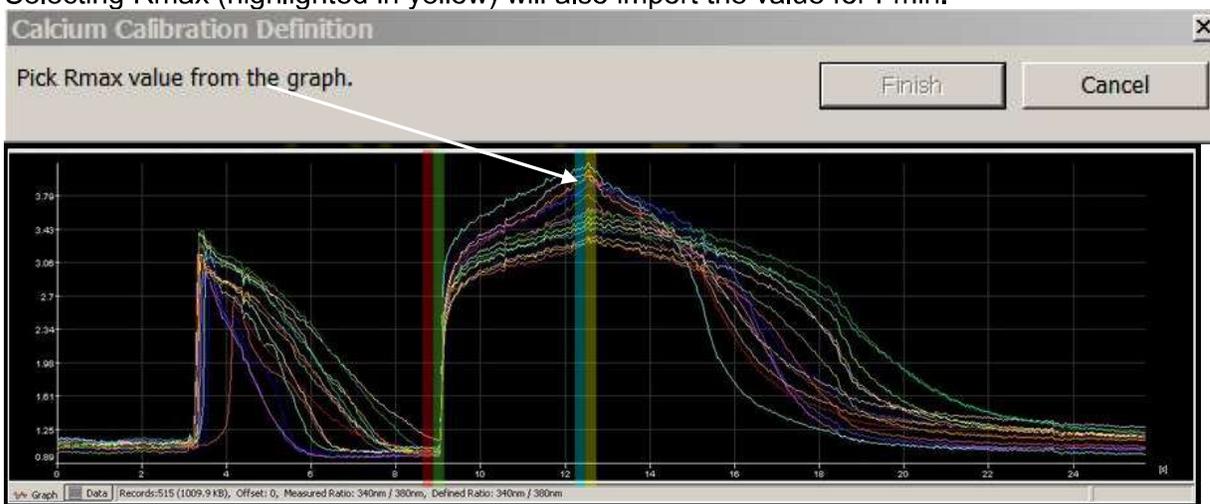
Using the NIS-Elements step by step wizard, it is easy to select values from the graph and import the respective R min, R max, F max and F min values. NIS-Elements will add the appropriate values from the dataset into the following equation for calculating concentration of free calcium in nanomoles (um).



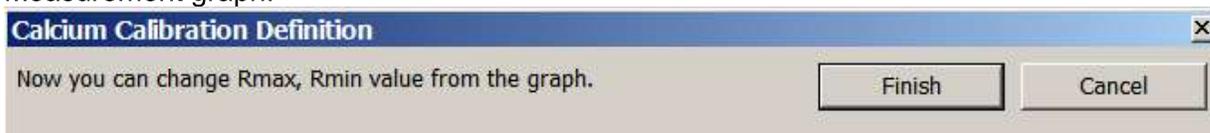
For graphical training purposes, selecting Rmin from the graph (highlighted in red) will also import the graph value for Fmax.



Selecting Rmax (highlighted in yellow) will also import the value for Fmin.



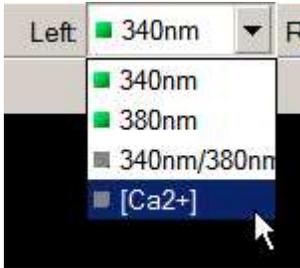
While in this process, it is possible to modify the Rmax and Rmin values on the Time Measurement graph:



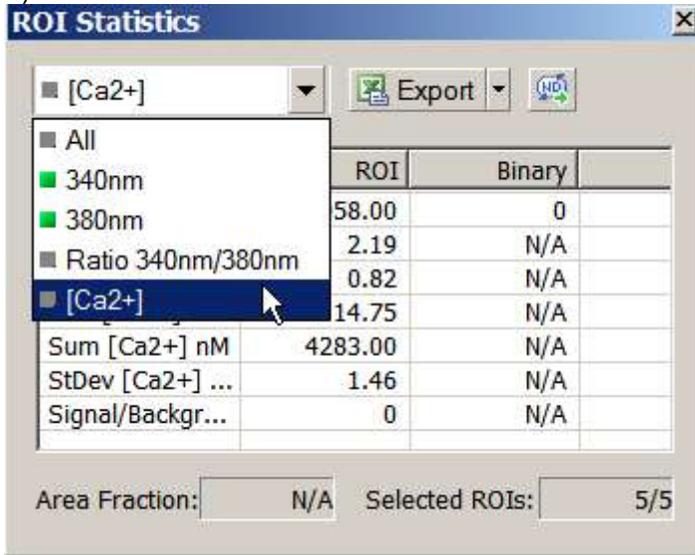
Once calibrated, NIS-Elements can display and utilize these calibrated values in various places within the image and software interface. Calcium calibrated values are available in the following locations:

- 1) The graph:

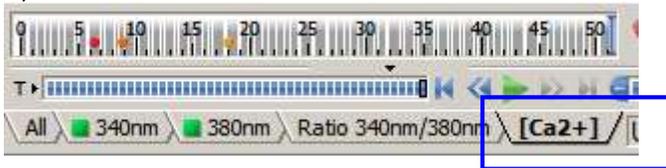




2) ROI Statistics



3) An additional channel tab with the Ca2+ values



4) Bottom of image window will display the Ca2+ value in nm of the cursor location

