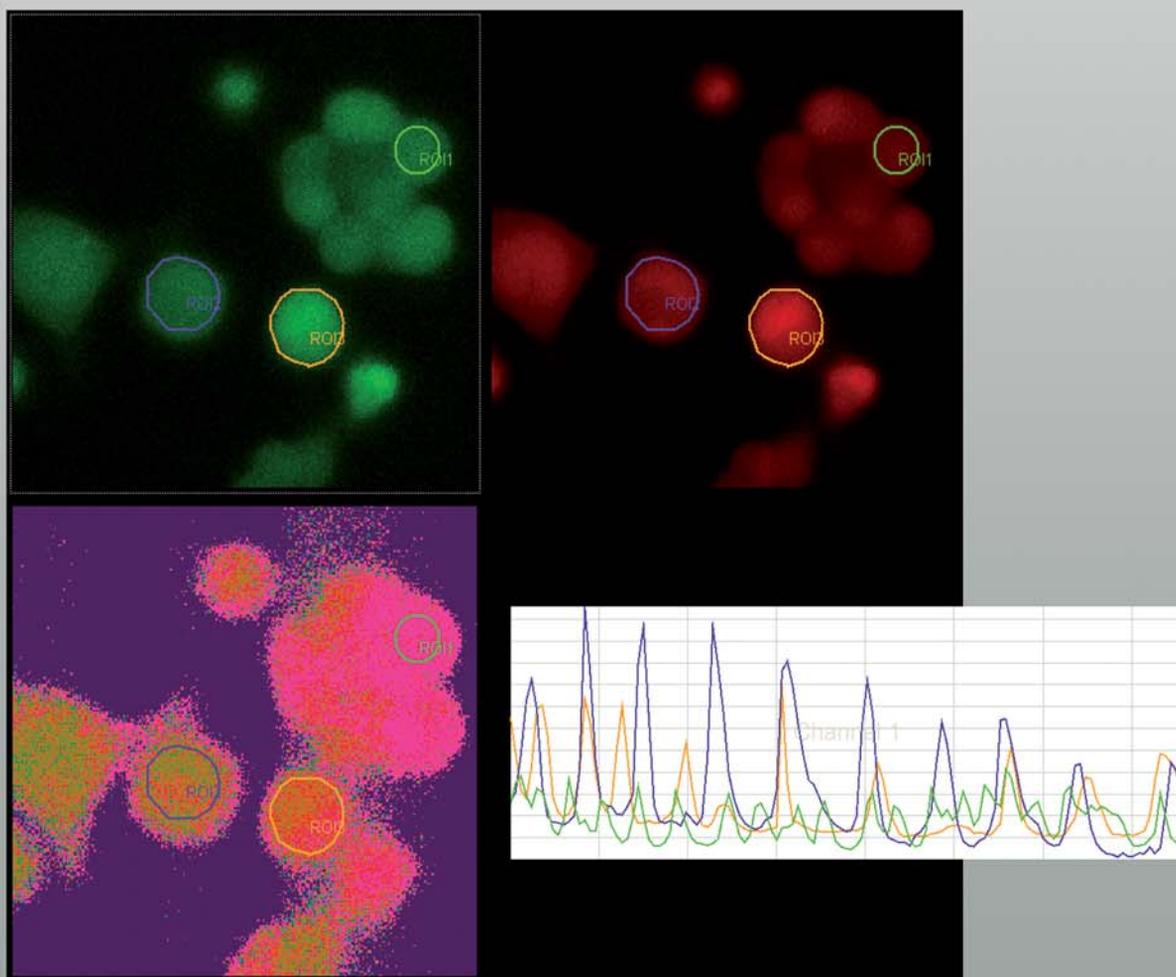


WIDEFIELD APPLICATION LETTER

Widefield Calcium Imaging with Calcium Indicator Fura2

reSOLUTION



Widefield Calcium Imaging with Calcium Indicator Fura2

In eukaryotic cells Ca^{2+} is one of the most widespread second messengers used in signal transduction pathways. Intracellular levels of Ca^{2+} are usually kept low, as Ca^{2+} often forms insoluble complexes with phosphorylated and carboxylated compounds. Typically cytosolic Ca^{2+} concentrations are in the range of 100 nM. In response to stimuli Ca^{2+} may either be released from external medium or internal stores to raise the Ca^{2+} concentration.

As Ca^{2+} cannot be visualized directly in living cells, it may be imaged indirectly by fluorescent Ca^{2+} indicators.

References:

Grynkiewicz, G., M. Poenie, and R. Y. Tsien
A new generation of Ca^{2+} indicators with greatly improved fluorescence properties.
J. Biol. Chem. 260: 3440–3450, 1985.

Takahashi A., P. Camacho, J.D. Lechleiter and B. Herman
Measurement of Intracellular Calcium
Physiological Rev 79, 1089 (1999).

<http://probes.invitrogen.com/handbook/>

Ca^{2+} dyes

Ion sensitive dyes are fluorescent molecules which reversibly bind to specific ions. These dyes are sensitive to changes in ion concentration either by increase or decrease of fluorescence. A measure for the affinity of ion binding to the dye is the dissociation constant (K_d). The lower the Ca^{2+} binding affinity the higher the K_d (Ca^{2+}).

There are two major groups of ion-sensitive dyes:

- Single wavelength dyes, like Fluo3 and Calcium Green dyes are suitable for non-ratiometric measurements. The intensity of the emitted fluorescence light increases proportional to free ion concentration.
- Dual wavelength dyes are likely for ratiometric measurements. These indicators can also be divided in two groups:

Dual emission ion indicators:

These dyes are excited at one wavelength and show a shift in their emission spectra upon binding specific ions. A well-known and often applied indicator of this group is Indo1.

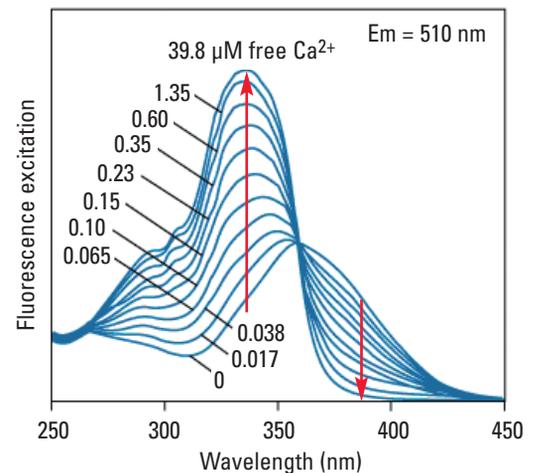
Dual excitation ion indicators:

These dyes are excited at two different wavelengths. The intensity of the emitted fluorescence light changes depending on the ion concentration. Fura2 is one of the most frequently used ratiometric Ca^{2+} indicator.

Fura2

The Ca^{2+} unbound form of Fura2 gets excited at 380 nm and the Ca^{2+} bound form at 340 nm. The emitted light is measured around 510 nm.

The fluorescence intensity increases at 340 nm with increasing Ca^{2+} concentration and decreases at 387 nm for the unbound form.



Fluorescence excitation spectra of Fura2 at different calcium concentrations. Reference: Molecular Probes

There are some techniques to introduce the fluorescent Ca^{2+} indicator into cells, like ester loading, microinjection, chemical loading techniques or diffusion from patch-clamp pipettes.

For further information on different Ca²⁺ indicators, handling of the dye, labeling of the cells and cell loading, please refer to the Molecular Probes Handbook and Takahashi et al. (see References).

Benefit of ratiometric measurement

Artifacts due to uneven dye distribution, leakage of dye, photo bleaching and unequal cell thickness may be avoided by ratioing methods.

In a typical experiment where you detect Ca²⁺ changes over time a calibration must be done. Calibration is possible in vitro or in vivo.

For in vivo calibration the ratio of maximum and minimum Ca²⁺ concentrations is measured. These values may be obtained within the same experiment.

For the maximum amount of bound Ca²⁺ indicator you may add an ionophore like Ionomycin or Bromo-A2318 to equilibrate the Ca²⁺ concentration. To get the minimum Ca²⁺ concentration you may apply an EGTA buffered solution to chelate all Ca²⁺.

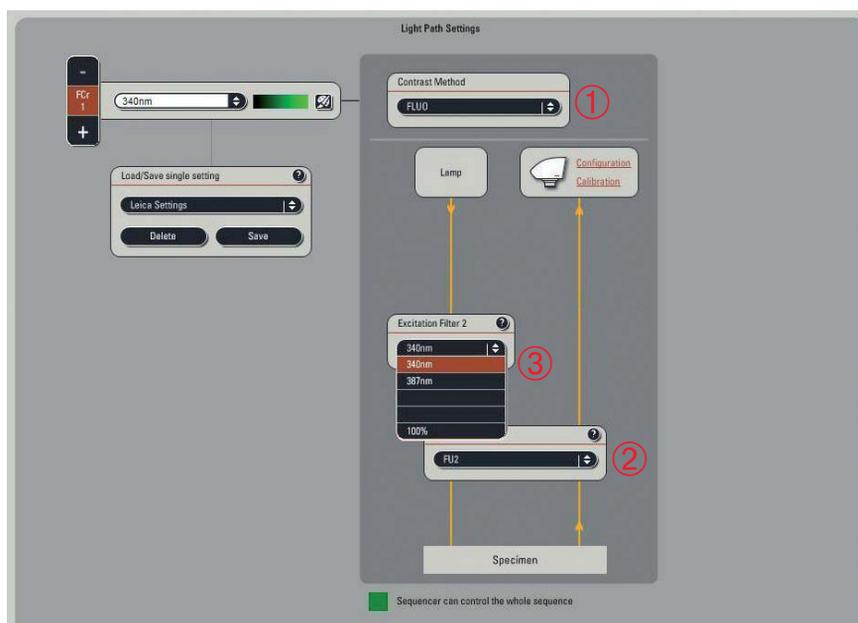
Leica Systems Configuration

The system must be equipped with external filter wheels and EL6000 light source. The external filter wheels contain special excitation filters for Fura2 at 340 nm and 387 nm. Additionally you need the according filter cube consisting of a dichroic beamsplitter and an emission band pass filter for selection of the wavelength with the maximum at 510 nm.

Experimental setup

Setting of experimental conditions for imaging

1. Define imaging conditions for Fura2-bound Ca²⁺ (Excitation at 340 nm, Emission at 510 nm).



In the **Light Path Settings** window choose from the drop-down menu contrast method Fluo ① for fluorescence imaging.

Then select Fura2 ② in the **Filter Cube** list box.

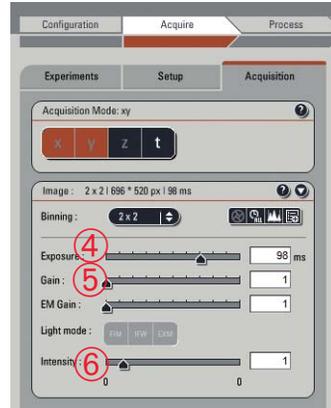
Choose **Excitation Filter 340 nm** ③.

If your system is equipped with additional filter wheels like a second **Excitation Filter** or **Emission Filter**, these will be automatically set to 100% transmission.

Begin with **Live image display** by pressing the **Live** button.

For image optimization define the appropriate exposure time ④ and camera gain and/or EM gain ⑤ (depending on which camera you are using).

You may change the light intensity by means of the intensity slider ⑥.



Note:

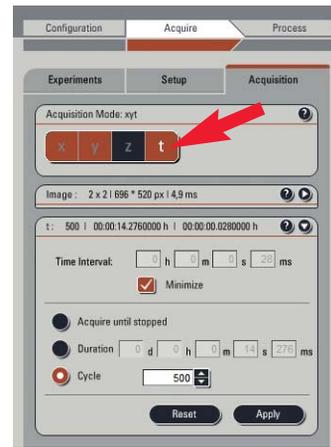
It is possible to define different values for exposure time, gain/EM gain as well as intensity for both channels. For channel 1 it is essential to be well below saturation as a calcium increase will result in an increase of fluorescence intensity.

Continue defining imaging parameters analog to step 1. Use the **Excitation Filter** 387 nm ②.

Note:
In a first step camera settings such as binning, speed or resolution may also be defined. This will always affect both channels.

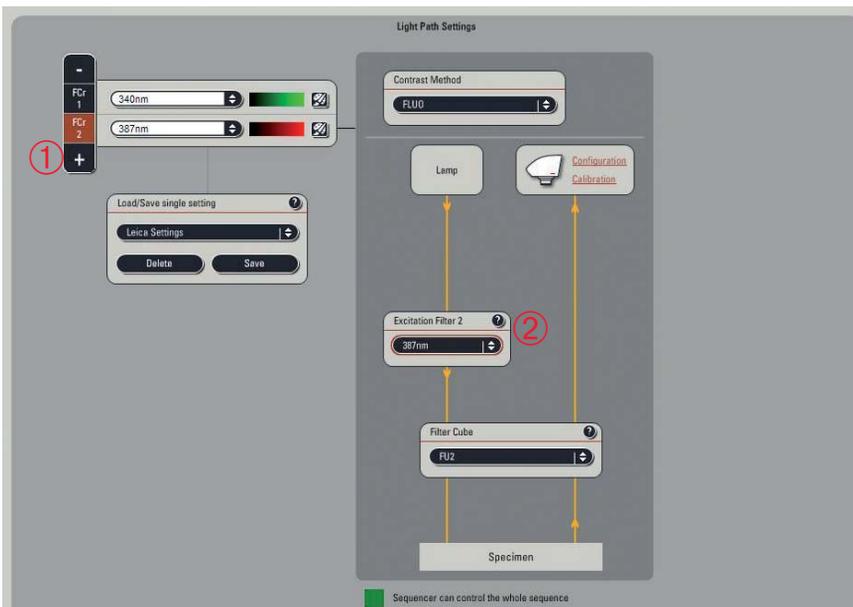
Define your time lapse experiment

For time lapse experiments use the time window and define your parameters as desired. To get there activate the **t** button.



2. Define imaging conditions for Fura2-unbound Ca²⁺ (Excitation at 387 nm, Emission at 510 nm).

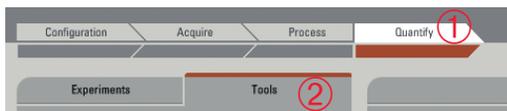
Press the button **+** to create the second imaging set-up ①.



Run your time series experiment with the **Start** button.

Calculation/Quantification

For measurements go to **Quantify** ① click the arrow symbol, then select the tab **Tools** ②. Here you have access to all quantification tools, graphs and statistics.



The upper formula ④ is used for calculation of the **Fluorescence Intensity Ratio**.

$$R = F_{(340 \text{ nm})} / F_{(380 \text{ nm})}$$

In the **Current Calculation Results** window press the **Calcium Imaging** button ③.

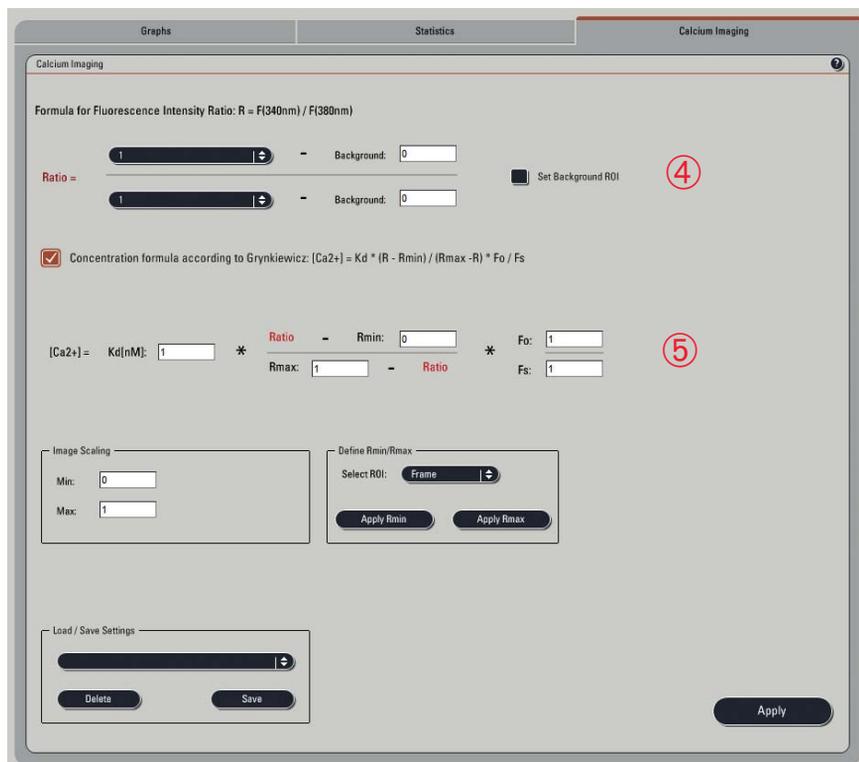


The second formula ⑤ is used for evaluation of the **Calcium Concentration** according to Grynkiewicz (see Reference).

The Ratio (**R**) values used in this formula are calculated by formula 1.

$$[Ca^{2+}] = Kd * R - R_{min} / R_{max} - R * F_0 / F_s$$

A new tab **Calcium Imaging** appears with two formulas for calcium calculations.



A click on the question mark in the upper right corner of the window reveals important information on the formulas used.

Press **Apply** ③ for calculating the Ratio Image, Ratio Statistics and Ratio Graph respectively.

Note:

Any change needs to be verified by **Apply**.

Grynkiewicz Formula Help

R = Fluorescence intensity ratio (F(340) / F(380)). Please define channel 1 for 340 nm excitation and channel 2 for 380 nm excitation.

KD = dissociation of Fura 2

Rmin = the value that R has at zero Ca²⁺.

Rmax = value that R has at Ca²⁺ saturation.

Fo = Fmin(380) (Ca free) proportionality coefficient for free dye at 380nm.

Fs = Fmax(380) (Ca bound) proportionality coefficient for Ca²⁺ bound dye at 380nm.

Reference: Grynkiewicz G./M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. J. Biol. Chem. 260: 3440-3450

OK

In the Viewer additionally to channel 1 and channel 2 a Ratio Image ④ is displayed as a separate channel with a special LUT ⑤ representing the ratio values from 0 to 1.

2. Background subtraction

In most cases for correct measurements it is additionally important to perform a background subtraction. To do so check **Set Background ROI** ⑥, and draw a squared region of interest (ROI) in the viewer. Choose an area in the image where only background signal is found. The intensity values will be automatically included into the formula ⑦ for both channels. These background values are fixed values and will not change over the course of the experiment.

Fluorescence Intensity Ratio

1. Ratio measurement

For Ratio measurements choose **Channel 1** ① in the numerator list and **Channel 2** ② in the denominator list.

You may also type in values to match background variations more precisely. First deactivate **Set Background ROI** and the ROI will disappear in the viewer. Now the edit fields are ready to type in a number.

Click **Apply** once again for recalculation.

The screenshot shows the 'Calcium Imaging' window with the following elements highlighted by red circles:

- ① Channel 1 dropdown menu
- ② Channel 2 dropdown menu
- ③ Apply button at the bottom right
- ④ Ratio image displayed in the viewer window
- ⑤ LUT (Look Up Table) for the ratio image
- ⑥ Set Background ROI checkbox
- ⑦ Background input field for Channel 1

3. Graphs and statistics

To see the Ratio values and Fluorescence Intensity values displayed in a graph or the according statistics go to tab **Graphs** or tab **Statistics**. Uncheck **Set Background ROI** to get access to defining ROIs. Draw one or multiple ROIs in the image viewer to see the values displayed in the graphs or the statistic tables.

Switching between tabs and drawing, changing or deleting ROIs is also possible during the running experiment.



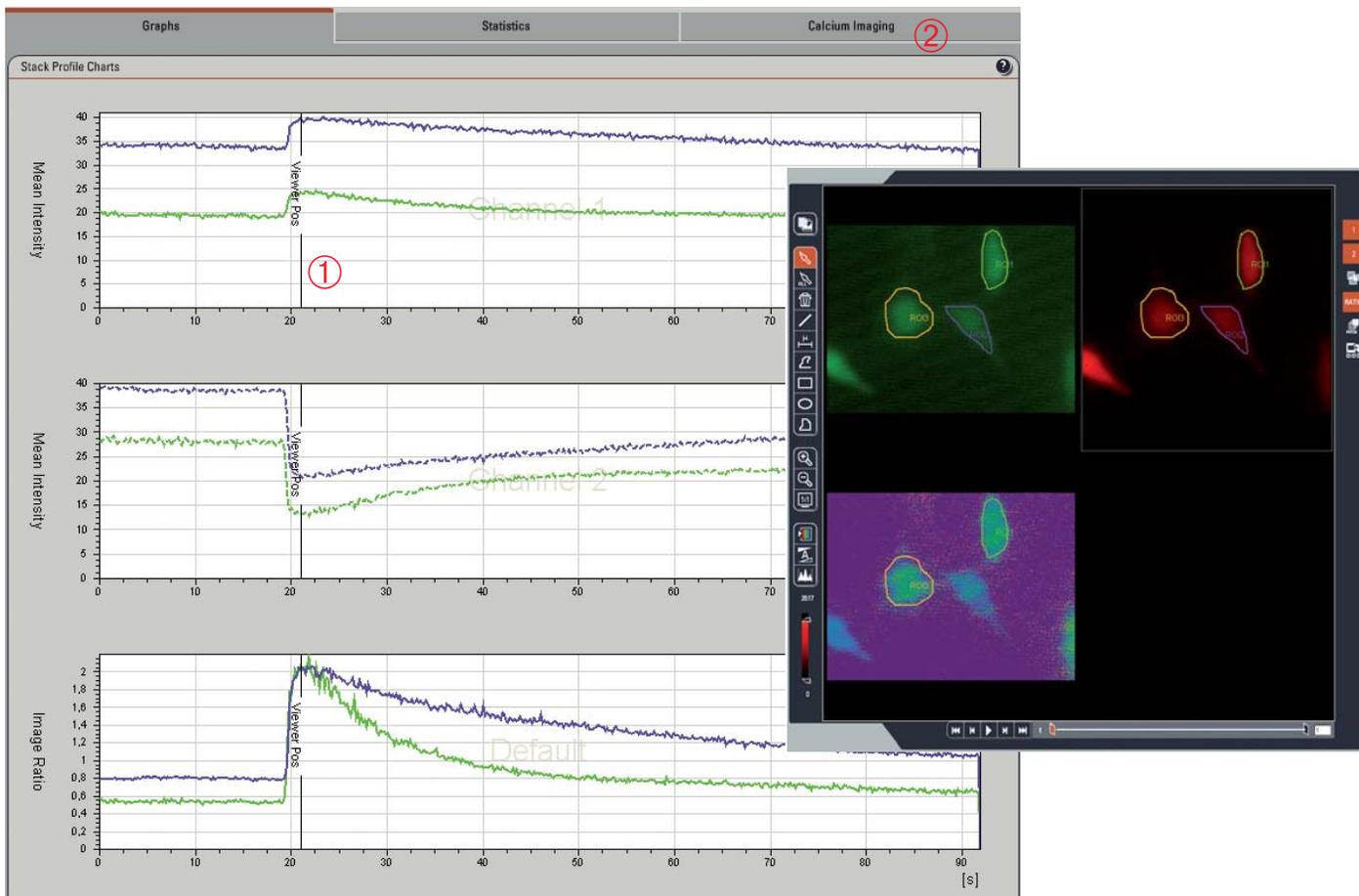
Calculation of the calcium concentration according to Grynkiewicz

Click the checkbox to enable the formula.

1. Determining R_{max}

Go to the image where the maximum Ca^{2+} concentration is displayed. If this image is in a time course series pass through the images of the series within the viewer by using the movie tool. Find in the graphs the actual viewer position indicated by a marker in

the chart ①. This way the image with the highest ratio value (= maximum in channel 1 and minimum in channel 2) can be identified. Memorize the ROI which represents best the maximal Ca^{2+} concentration.



Graphs Statistics **Calcium Imaging** ②

Calcium Imaging

Formula for Fluorescence Intensity Ratio: $R = F(340nm) / F(380nm)$

Channel 1 - Background: 127
 Channel 2 - Background: 146

Ratio = Set Background ROI

Concentration formula according to Grynkiewicz: $[Ca^{2+}] = K_d * (R - R_{min}) / (R_{max} - R) * F_0 / F_s$

[Ca²⁺] = K_d[nM]: 224 ④ * Ratio - R_{min}: 0.18 * F₀: 1245
 R_{max}: 0.43 - Ratio F_s: 814

Image Scaling: Min: 0 Max: 2E-07 ⑤

Define R_{min}/R_{max}: Select ROI: ROI1 ③
 Apply R_{min} Apply R_{max}

Load / Save Settings: Delete Save

Apply

Now go back to the **Calcium Imaging** tab ② and select the ROI number you choose in the field **Define R_{min}/R_{max}** ③.

Press **Apply R_{max}** button. The R_{max} value as well as the F_s value will be automatically added to the formula. F_s is the fluorescence intensity at calcium saturation at 387 nm excitation.

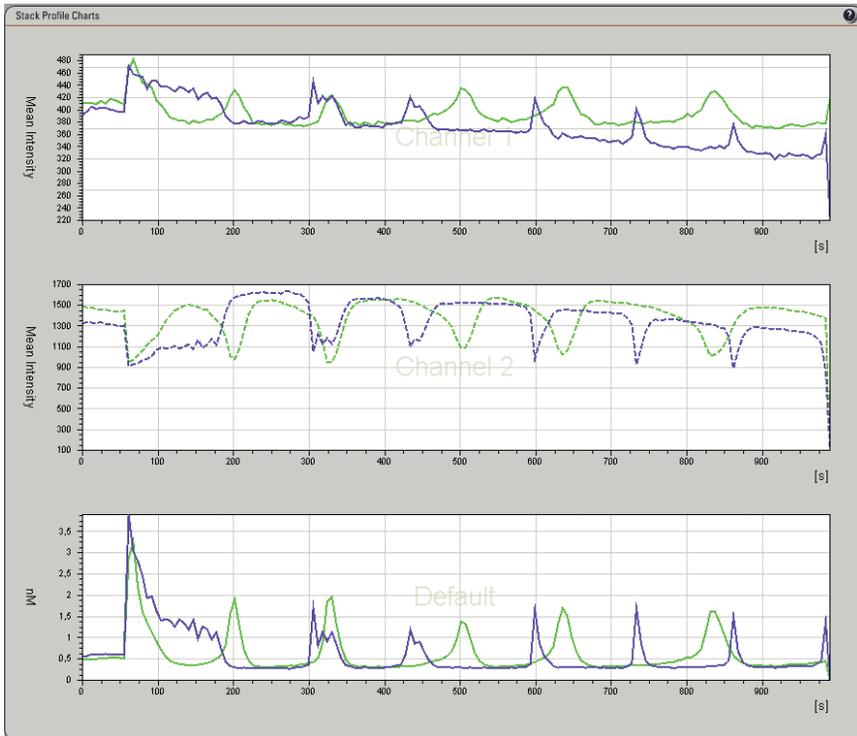
2. Determining R_{min}

Continue likewise for determination of R_{min}. Find the image with the lowest ratio value (= minimum in channel 1 and maximum in channel 2), select an appropriate ROI and click **Apply R_{min}** to add R_{min} and the F₀ value to the formula. F₀ is the fluorescence intensity at minimum calcium concentration at 387 nm excitation.

3. K_d

The K_d value (Ca²⁺ dissociation constant of Fura 2) may be determined experimentally or you may use a K_d value from the literature. Type the according number into the **K_d-field** ④. A typical value for Fura2 is around 224 nM (Reference: Takahashi et al. and Grynkiewicz et al.).

Now press **Apply** to activate the concentration image in the viewer as well as the calculated Calcium concentrations within the Statistics and Graph.



Note:
 The concentration in the UI is displayed in μm . So the ratio/concentration image can be empty due to wrong scaling. Change the Max Value to 0.0001 or below in the **Image Scaling** field ⑤.

Again click **Apply** for recalculation.

Software version: 1.6.2. build 1110

www.leica-microsystems.com

