# Calcium monitoring with the Mithras LB 940 multimode plate reader

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## Introduction

Bivalent Calcium is an intracellular messenger in many eukaryotic signal transduction pathways. Most $Ca^{2+}$-signalling systems have one thing in common: they generate brief pulses of $Ca^{2+}$, thereby regulating cellular functions.

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 100 nM. In response to stimuli $Ca^{2+}$ is either released from external medium or internal stores to raise the $Ca^{2+}$-concentration. In the cell membrane the $Na^{+}/Ca^{2+}$ exchanger controls $Ca^{2+}$-flux following stimuli like membrane depolarisation or extracellular agonists. In the cell, $Ca^{2+}$ is stored in the endoplasmatic reticulum or in the sarcoplasmatic reticulum in muscles, respectively. $Ca^{2+}$-flux is triggered by an expanding group of messengers, like inositol-1,4,5,-trisphosphate ($IP_3$), cyclic ADP ribose (cADPR), nucleic adenine dinucleotide phosphate (NADP) and sphingosine-1-phosphate. The sarco(endo)plasmatic reticulum $Ca^{2+}$-ATPase pumps cytosolic $Ca^{2+}$ back into the lumenal space.

Most of the released $Ca^{2+}$ is bound to buffers like Calbindin D-28, Calretinin or Parvalbumin which function to fine-tune the $Ca^{2+}$-signals. Only a small proportion of $Ca^{2+}$ is bound to effector proteins like Calmodulin or Troponin C. Those effector proteins pass the information from $Ca^{2+}$ on to target proteins, a wide range of enzymes and pumps. Targets of Calmodulin may propagate the $Ca^{2+}$-signal and induce the synthesis and release of neurotransmitters. Alternatively, Calmodulin may abrogate the $Ca^{2+}$-signal by activating the $Ca^{2+}$-ATPase pump which restores the basal $Ca^{2+}$-level in the cell.

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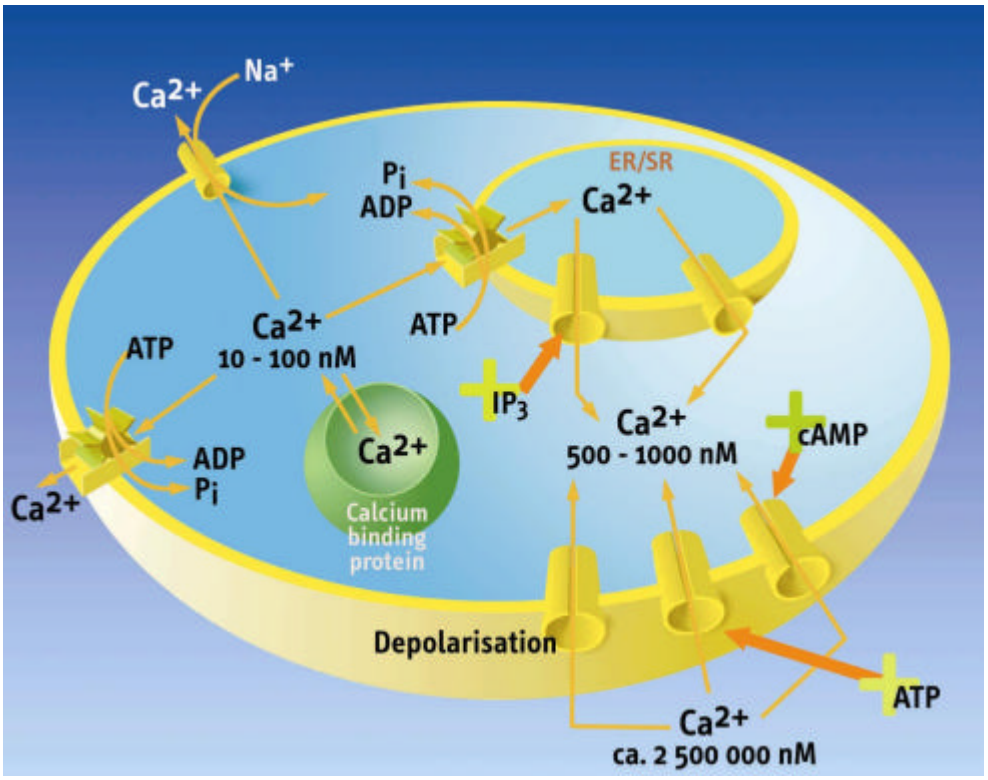


**Figure 1.** $Ca^{2+}$-transport.

Cytosolic $Ca^{2+}$-concentrations are raised in response to stimuli, which open $Ca^{2+}$-channels in the cell membrane or in membranes of the ER/SR. $Ca^{2+}$ is then bound to different target proteins; thereby modulating its effectors. As high $Ca^{2+}$-concentrations are toxic; $Ca^{2+}$ is removed from the cytosol by $Ca^{2+}$-ATPase pumps.

$Ca^{2+}$-signalling plays an important role in a number of physiological processes: For example nerves are triggered by $Ca^{2+}$-flux through voltage gated $Ca^{2+}$-channels and this subsequently leads to the release of neurotransmitters. In muscles the abrupt rise in cytosolic $Ca^{2+}$-levels culminates in contraction. In contrast, the rapid removal of $Ca^{2+}$ into the sarcoplasmatic reticulum results in muscle relaxation. In the visual system $Ca^{2+}$ controls the Guanylate cyclase after illumination. In the nucleus $Ca^{2+}$ regulates transcription by activating the Calmodulin kinase which phosphorylates, and thereby activates the transcription factor CREB.

## $Ca^{2+}$-measurement with Fura-2

The concentration of free $Ca^{2+}$ in intact cells can be monitored by using polycyclic chelators such as Fura-2 or Indo-1. The fluorescence properties of both dyes are markedly changed when $Ca^{2+}$ is bound and thus the direct detection of $Ca^{2+}$-fluxes in response to specific signal transduction pathways is possible.

Indo-1 is the preferred dye for flow cytometry where it is more practical to use a single laser for excitation (argon laser spectrum: 351 – 364 nm) and monitor two emissions. The

emission maximum shifts from ~475 nm in $Ca^{2+}$-free medium to ~400 nm when the dye is saturated with $Ca^{2+}$. Indo-1 is also applicable to microplate assays.

With Fura-2 the absorption maximum of Fura-2 shifts from 380 nm to 340 nm upon binding $Ca^{2+}$, while the emission remains constant at 510 nm (figure 2). This results in an opposite change of fluorescence intensity: an increase at 340 nm and a decrease at 380 nm.

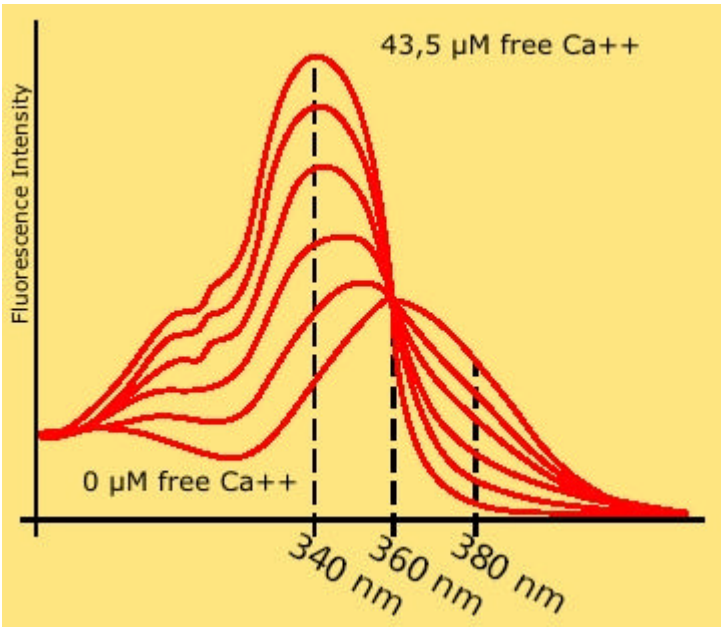


**Figure 2.** Correlation between fluorescence intensity and $Ca^{2+}$-concentration.
The shift of the absorption maximum of Fura-2 from 380nm to 340 nm (green arrows) depends on the $Ca^{2+}$-concentration.

Therefore Fura-2 is especially useful for $Ca^{2+}$-measurements regarding the correlation between fluorescence intensity and $Ca^{2+}$-concentration. The fluorescence intensity of a $Ca^{2+}$-loaded cell at 340 nm and an emission at 510 nm is $F_{340}$. It is determined by the dye concentration (c), the cell size (d) and a constant (K) which summarizes the optical characteristics of the device, and a function of the $Ca^{2+}$-concentration ($f[Ca^{2+}]$). Measuring $F_{340}$ and $F_{380}$ with short intervals, c, d and K are assumed not to change and the relative $Ca^{2+}$-concentration can be calculated by the ratio of both excitation wavelengths:

$$R=F_{340}/F_{380} = f'' ([\ Ca^{2+}])$$

Both Fura-2 and Indo-1 display high specificity for $Ca^{2+}$ relative to magnesium and the $K_d$-levels are close to 100 nM (the mammalian basal $Ca^{2+}$-concentration). As sodium and potassium salts of Fura-2 are cell impermeable these salts are useful as standards to calibrate $Ca^{2+}$-measurement. For loading of cells the acetomethyl esters (AM) of Fura-2

are applicable as they diffuse passively across the cell membranes. Inside the cell they are cleaved by intracellular esterases to yield cell impermeable fluorescent indicators.

### Mithras LB 940

The Mithras LB 940 is a multimode plate reader with a unique optical design (DOPS – Dedicated Optical Path System) to ensure optimized performance for the detection technologies implied. These are luminescence, BRET, fluorescence (top and bottom reading), absorbance, fluorescence polarization and AlphaScreen™. In addition accessory options, e.g. reagent injectors, temperature control and cooled PMT detection units are available. Especially the fact that at least one injector is located in the reading position fast reaction kinetics can be monitored.


**Figure 3.** Mithras LB 940 multimode reader for microplates.

Changes in $Ca^{2+}$-levels can be monitored by fluorescent microscopy, flow cytometry and fluorescent plate readers. The advantage of fluorescent microscopy is that $Ca^{2+}$-flux in single cells may be observed. The disadvantage however is that this method is very time consuming to yield significant results.

Using flow cytometry a large number of single cells may be monitored, but it only permits kinetic resolution of various cell populations, not a single one. With the Mithras multimode reader $Ca^{2+}$-flux of a distinct cell population is documented directly over time. Injection of stimulators or chelators is possible and the $Ca^{2+}$-concentration is displayed as a kinetic. Preheating of the measurement chamber at 37°C provides the cells with physiological conditions. This is especially important as $Ca^{2+}$-flux can only be measured in viable cells. An additional advantage is that the Mithras LB 940 reads multiple plate formats including 96-well plates. Thus, small cell numbers are applicable and the throughput is high. The

filter change in Mithras is extremely fast (~150 ms) enabling a high resolution of detection when fast changes in Calcium concentration are to be monitored.

## Assay protocol

$Ca^{2+}$-flux was analyzed in murine B-cells stimulated with IgM to induce the release of $Ca^{2+}$ from internal stores. The ionophore Ionomycin was used as a positive control since it depolarizes the membrane by its hydrophobic nature and thereby introduces $Ca^{2+}$ into the cells. Additionally, as the $Ca^{2+}$-specific chelator EGTA binds free $Ca^{2+}$ it can be used as the negative control. For each measurement 1 x $10^6$ murine B-cells per well of a 96-well plate were analyzed. Prior to the stimulation cells were loaded with 2.5 µM Fura-2-AM for 30 minutes at room temperature in the dark. Optional, a standard curve based on a calcium calibration buffer kit (e.g. Molecular Probes) may be generated to determine absolute $Ca^{2+}$-concentrations.

$Ca^{2+}$-flux was determined with the Mithras LB 940 heated to 37°C to provide physiological conditions. To minimize auto-fluorescence the assay was run in black 96 well assay plates. First, basal $Ca^{2+}$-levels were determined by calculating the ratio of the fluorescence intensity at 380 and 340 nm with an emission at 510 nm. Subsequently, 1 µg/mL IgM was injected and again the fluorescence intensity was measured. To determine the maximal $Ca^{2+}$-flux 2 µg/mL Ionomycin were injected. Finally, 2 mM EGTA were injected to bind free $Ca^{2+}$ and return to basal $Ca^{2+}$-levels.

## Instrument Settings

| | |
|---|---|
| Fluor. Kinetic | 380 nm excitation / 510 nm emission |
| | 340 nm excitation / 510 nm emission (second measurement) |
| | total time 40 sec, counting time 0.5 sec (time resolution) |
| | lamp energy 7000 |
| | temperature 37 °C |
| Dispense | Injector 4, 10 µL (IgM), medium speed |
| Shake | 2 s |
| Fluor. Kinetic 2 | total time 90 s, time resolution 0.5.s |
| Dispense | Injector 3, 10 µL (Ionomycin), medium speed |
| Shake | 2 s |
| Fluor. Kinetic 3 | total time 40 s, time resolution 0.5 s |
| Dispense | Injector 2, 10 µL (EGTA), medium speed |
| Shake | 2 s |
| Fluor. Kinetic 4 | total time 40 s, time resolution 0.5 s |

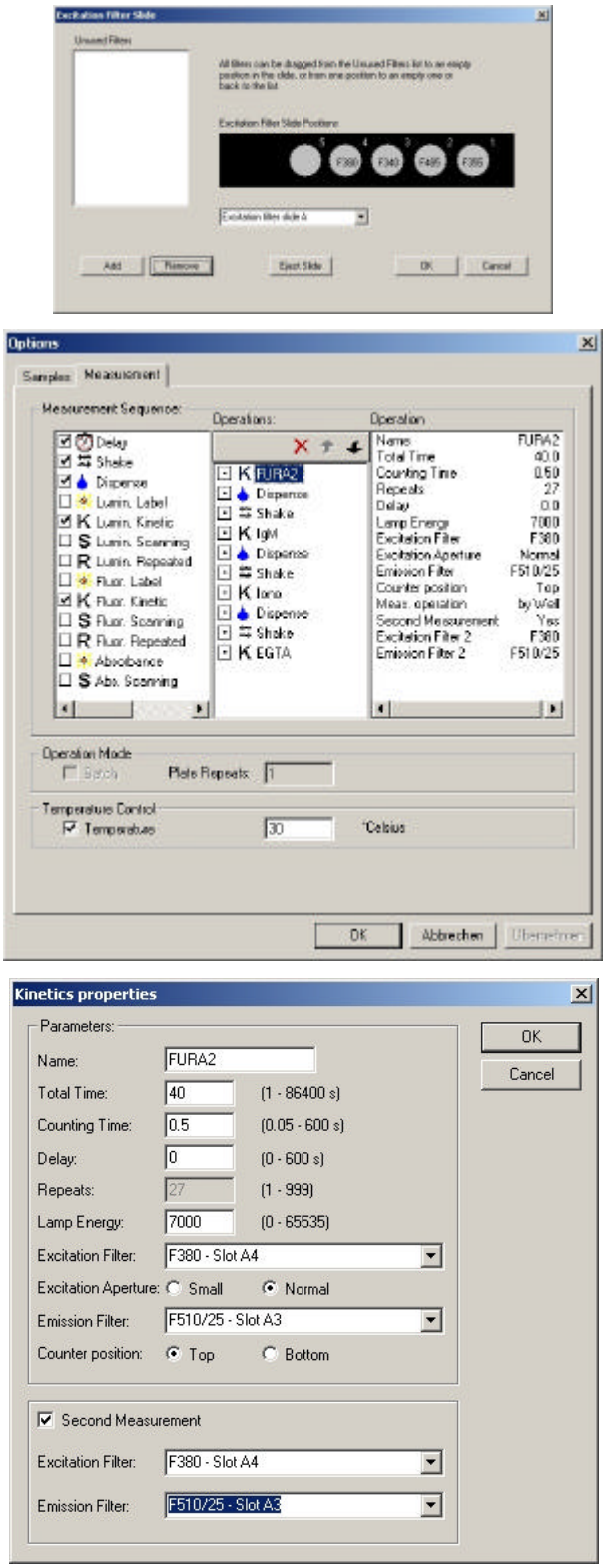


**Figure 4.** Instrument settings.

**Upper panel:** The excitation filters are placed next to each other in order to ensure minimum delay for filter change (upper panel).

**Middle panel:** Operation sequence shows the individual steps performed in the kinetic measurement. All operations are defined “by well”, i.e. all steps are performed completely per individual well before they are executed for the consecutive well.

**Lower panel:** Kinetic operation window displays the settings for reading times, lamp energy and filter selections.

## Results

The kinetic curves of the respective intensities at 340 and 380 nm as well as the ratio can be monitored online.

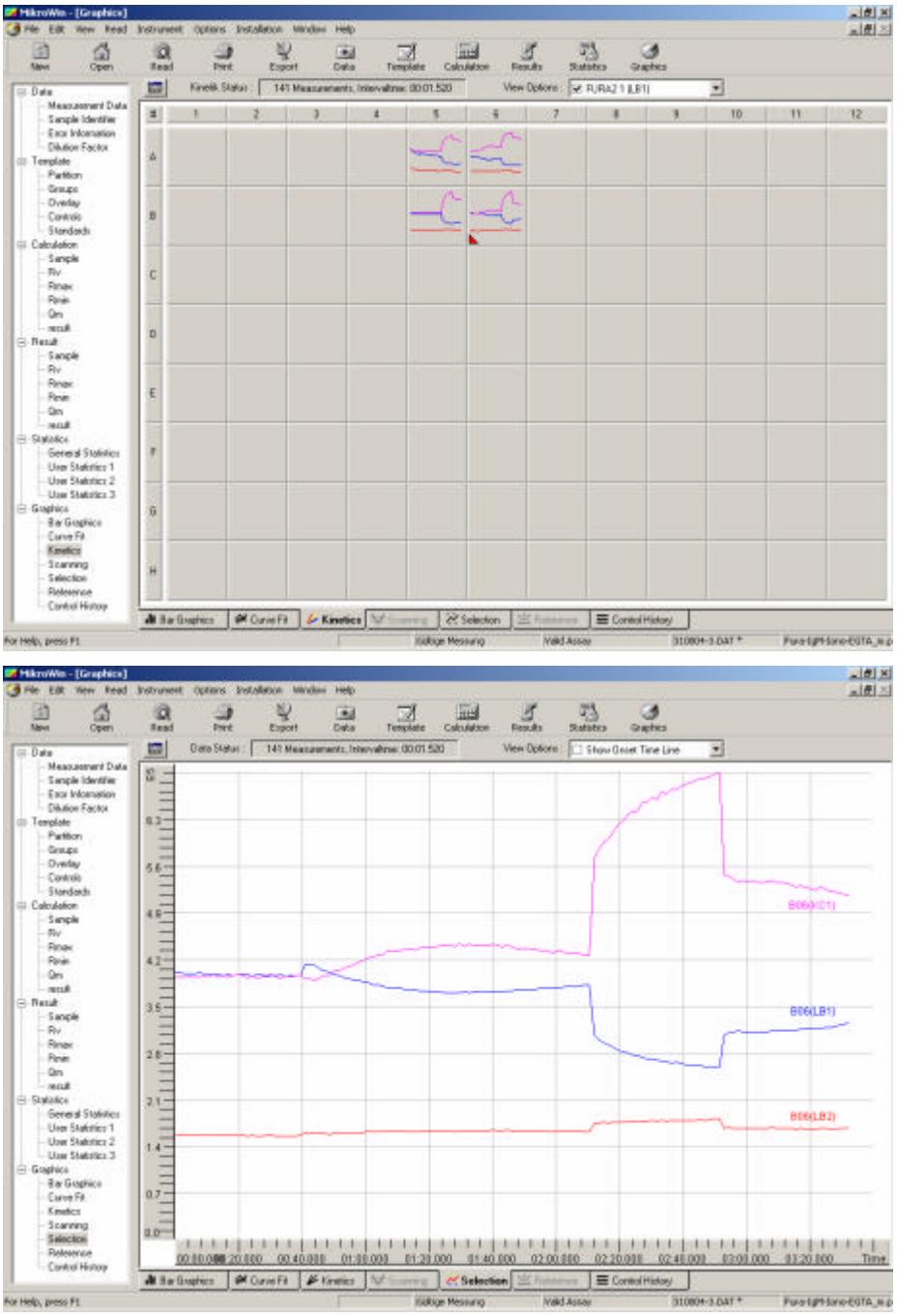

**Figure 5.** Graphic display of kinetic curves: red graph represents excitation at 340 nm, blue graph represents excitation at 380 nm, pink graph represents the ratio.
**Upper panel:** View of total microplate.
**Lower panel:** Zoomed view of selected well.

Figure 5 shows the kinetic curves of the excitation at 340 nm and 380 nm respectively and the ratio of these values. For an initial 40 s the baseline has been measured, followed by

the injection of IgM. The increase of the intracellular $Ca^{2+}$ concentration becomes visible by the increase of the signal at the excitation at 340 nm and a decrease at 380 nm. To get the numbers of the maximum $Ca^{2+}$ concentration, Ionomycin was added after another 90 s. Finally EGTA was injected driving the signals back to the baseline.

For data evaluation Mikrowin 2000 offers various possibilities which will be discussed later. In addition it is possible to analyze the fluorescence intensities in Microsoft Excel. All individual readings acquired with 340 and 380 nm excitation, i.e. raw data, are exported from Mikrowin 2000 using the ***RawData Export Driver***.

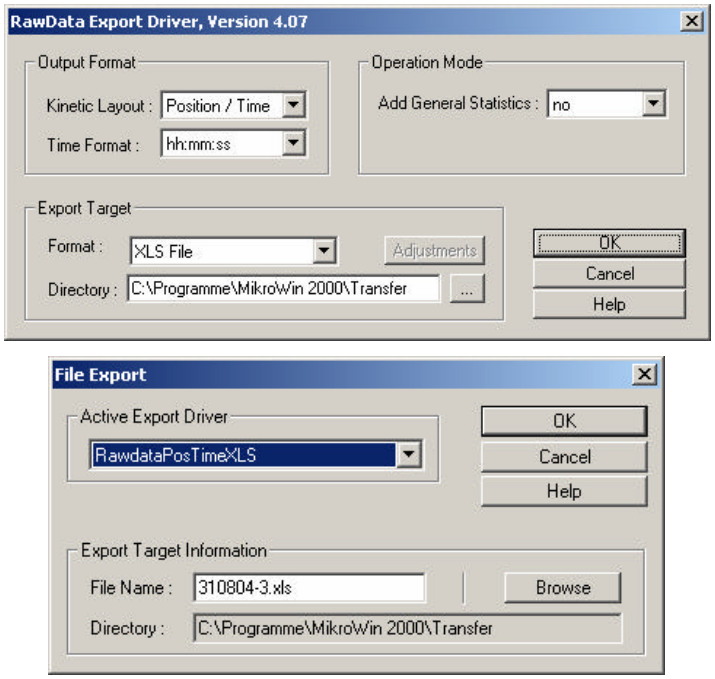


**Figure 6.** Usage of *Rawdata Export Driver* to transfer all readings into a spreadsheet readable format.
**Upper panel:** Export driver set-up.
**Lower panel:** User dialogue for execution of manual export including driver selection, file name and directory settings.

In Excel the $ratio_{340/380\ nm}$ of the fluorescence intensities at each time point can be calculated. These data are used to display the relative changes of free $Ca^{2+}$ as a graph.

The exact $Ca^{2+}$-concentration of distinct time points may be determined with a standard curve. To this end Ca-EGTA buffers with given $Ca^{2+}$-concentrations are measured at 340 nm and 380 nm. These data (Figure 7) are plotted as the log of the $[Ca^{2+}]_{free}$ {x-axis} versus the $\log((R-R_{min})/(R_{max}-R))$ {y-axis}. The MikroWin 2000 software (parameter file "***Fura2_Kd-Calibration***") calculates the $K_d$ of EGTA, defined as the concentration at which the function reaches a point where 50 % of the available $Ca^{2+}$ are bound and 50 % are free. Additionally the values for $R_{min}$, $R_{max}$ and Q (the latter representing the ratio of

the maximum and the minimum standard at 380 nm excitation $F^{380}_{max}/F^{380}_{min}$) are calculated (Figure 8).

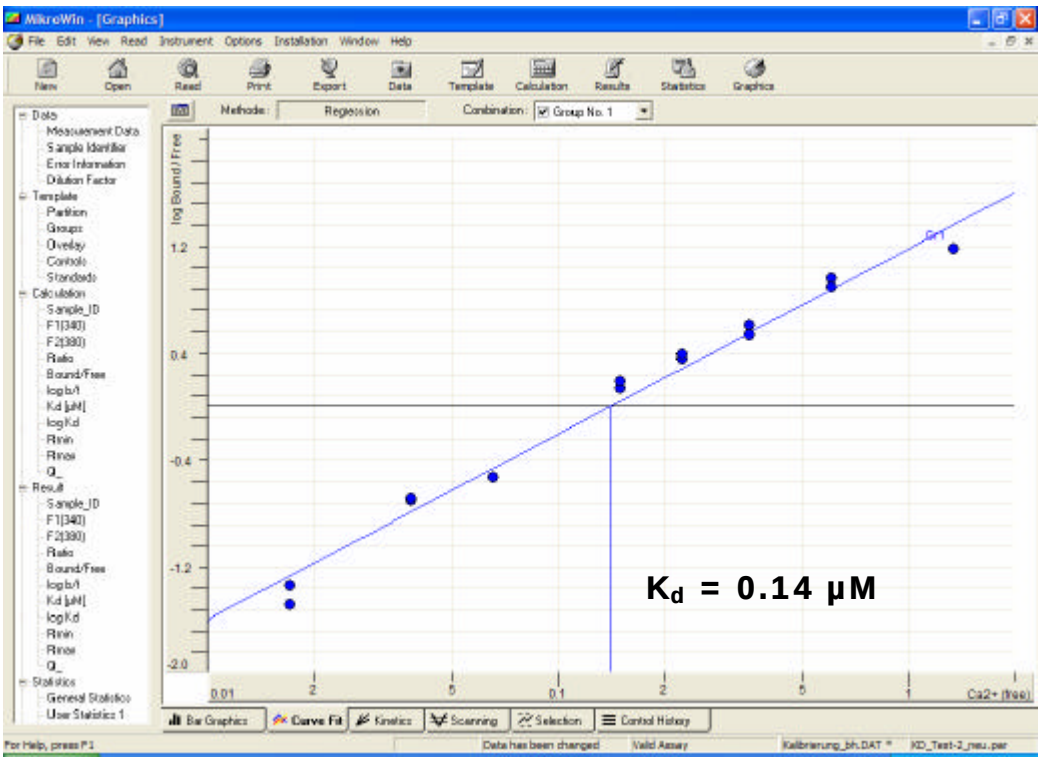


**Figure 7.** Standard Curve of bound/free $Ca^{2+}$ in relation to the free $Ca^{2+}$.

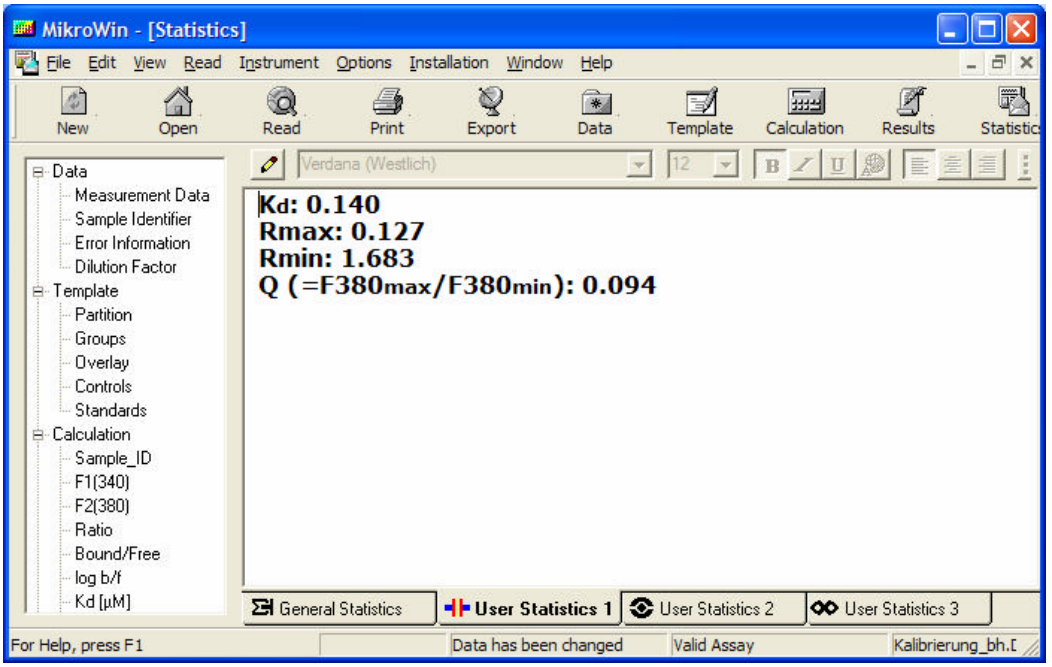


**Figure 8.** Calculated values of $K_d$, $R_{min}$, $R_{max}$ and **Q** displayed in the ***User Statistics*** window of Mikrowin 2000. **Q** is representing the ratio of the maximum and the minimum standard at 380 nm excitation $F^{380}_{max}/F^{380}_{min}$.

With the following formula free $Ca^{2+}$ at distinct time points may be calculated from the corresponding R value. $R_{min}$ is the ratio at zero $Ca^{2+}$ and $R_{max}$ is the ratio at saturating $Ca^{2+}$. $F^{380}{}_{max}$ is the fluorescence intensity at 380 nm for zero free $Ca^{2+}$ and $F^{380}{}_{min}$ is the fluorescence intensity at saturating free $Ca^{2+}$.

$$[Ca^{2+}]_{free} = K_d * (R-R_{min})/(R_{max}-R) * F^{380}{}_{max}/F^{380}{}_{min}$$

The values for **$K_d$**, **$R_{min}$**, **$R_{max}$** and **Q** are simply entered in the ***Options | Definitions*** dialogue of Mikrowin 2000. The calculation of the $Ca^{2+}$ concentrations can be performed for individual regions of the kinetic graph.

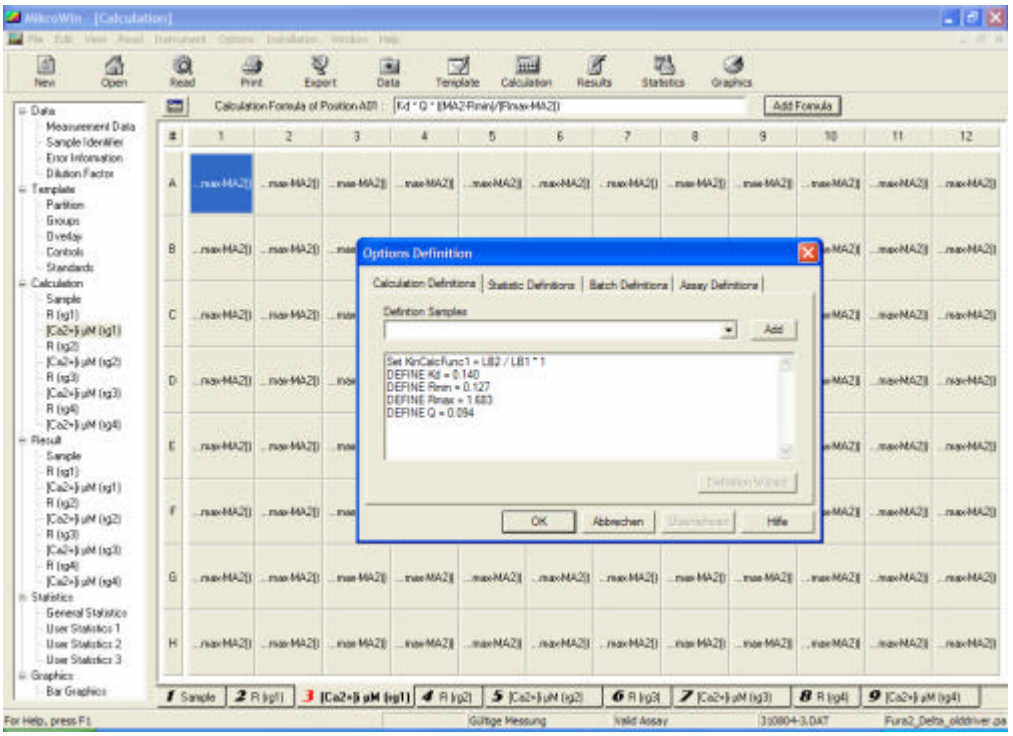


**Figure 9.** Graphic display of Calcium ratio kinetics with 4 regions set.

**Main panel:** Calculation matrices for Calcium concentrations of 4 regions containing the calculation formula ***Kd * Q * ((MAx-Rmin)/(Rmax-MAx))***; the "x" in the formula is to be replaced by the number of the matrix holding the respective experimental $F^{340}/F^{380}$ ratio of the region (see below).

**Inserted panel:** Menu for Definition of the variables **$K_d$**, **$R_{min}$**, **$R_{max}$** and **Q**.

For the calculation of the free $Ca^{2+}$ concentrations you may either use the $F^{340}/F^{380}$ ratio´s peak value (in Mikrowin 2000: ***KMAX(LB4; RGx)*** ) of the respective region or the ratio of the area-under-curve (in Mikrowin 2000: ***KITG(LB2; RGx)/KITG(LB1;RGx)***.).

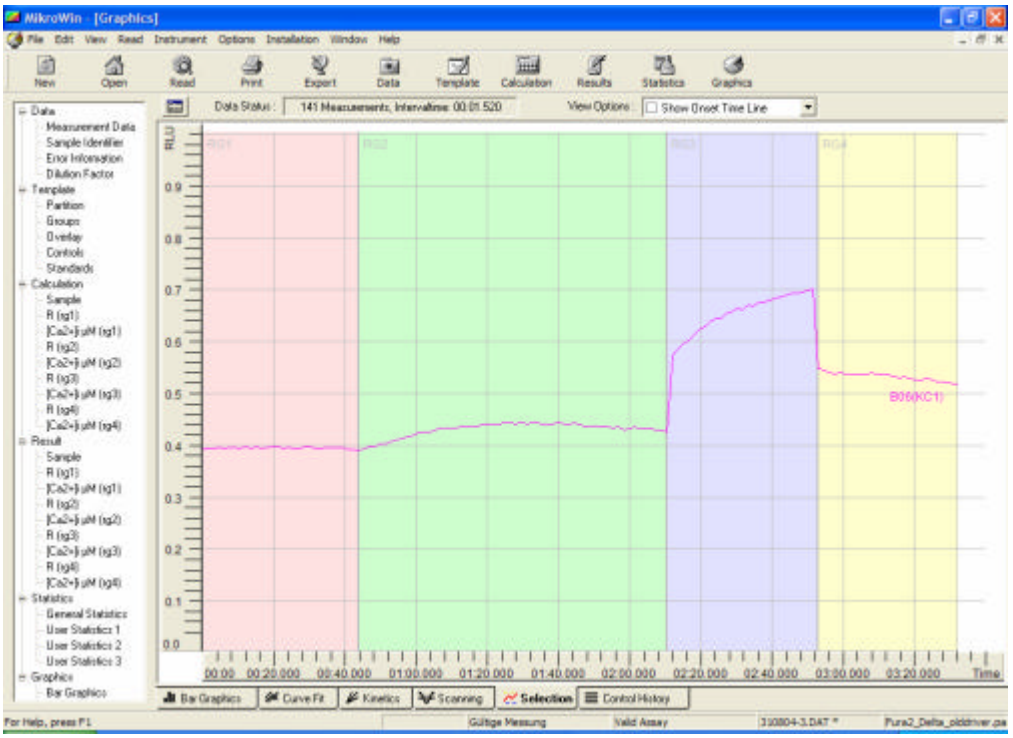


**Figure 10.** Graphic display of Calcium ratio kinetics with 4 regions set.

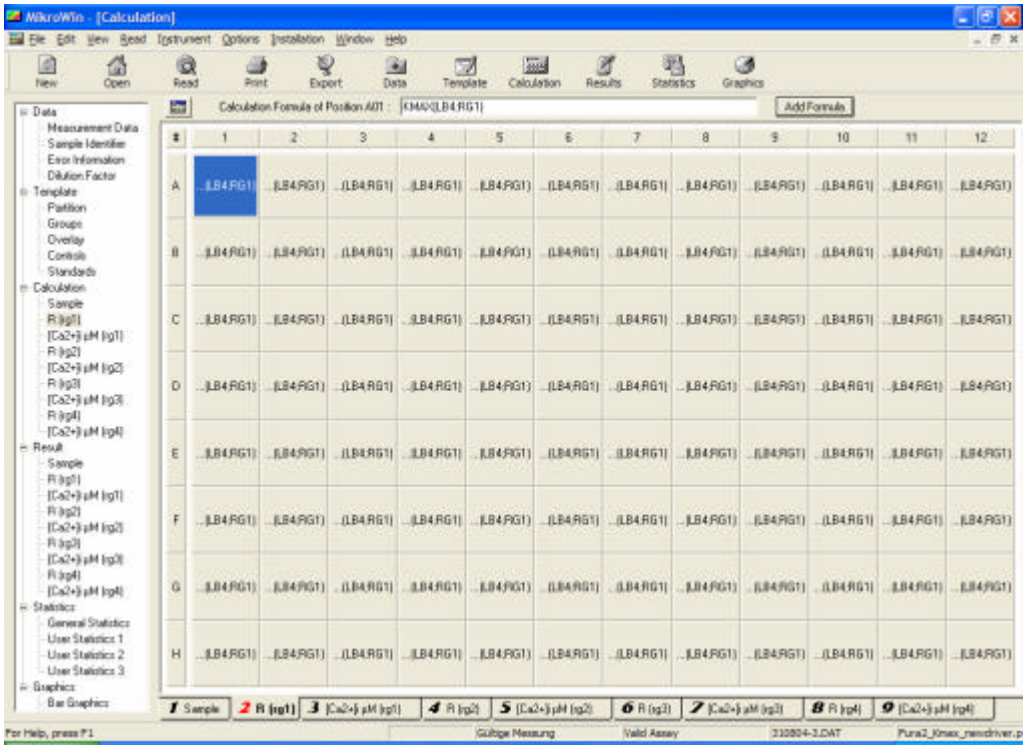


**Figure 11.** Calculation matrix for the experimental $F^{340}/F^{380}$ ratio which is determined by the peak value in this case . The Mikrowin 2000 formula is ***KMAX(LB4;RG1)***, LB4 representing the ratio of the second (i.e. 340 nm excitation) and the first (i.e. 380 nm excitation) reading at each time point which is automatically calculated by the instrument´s driver software.

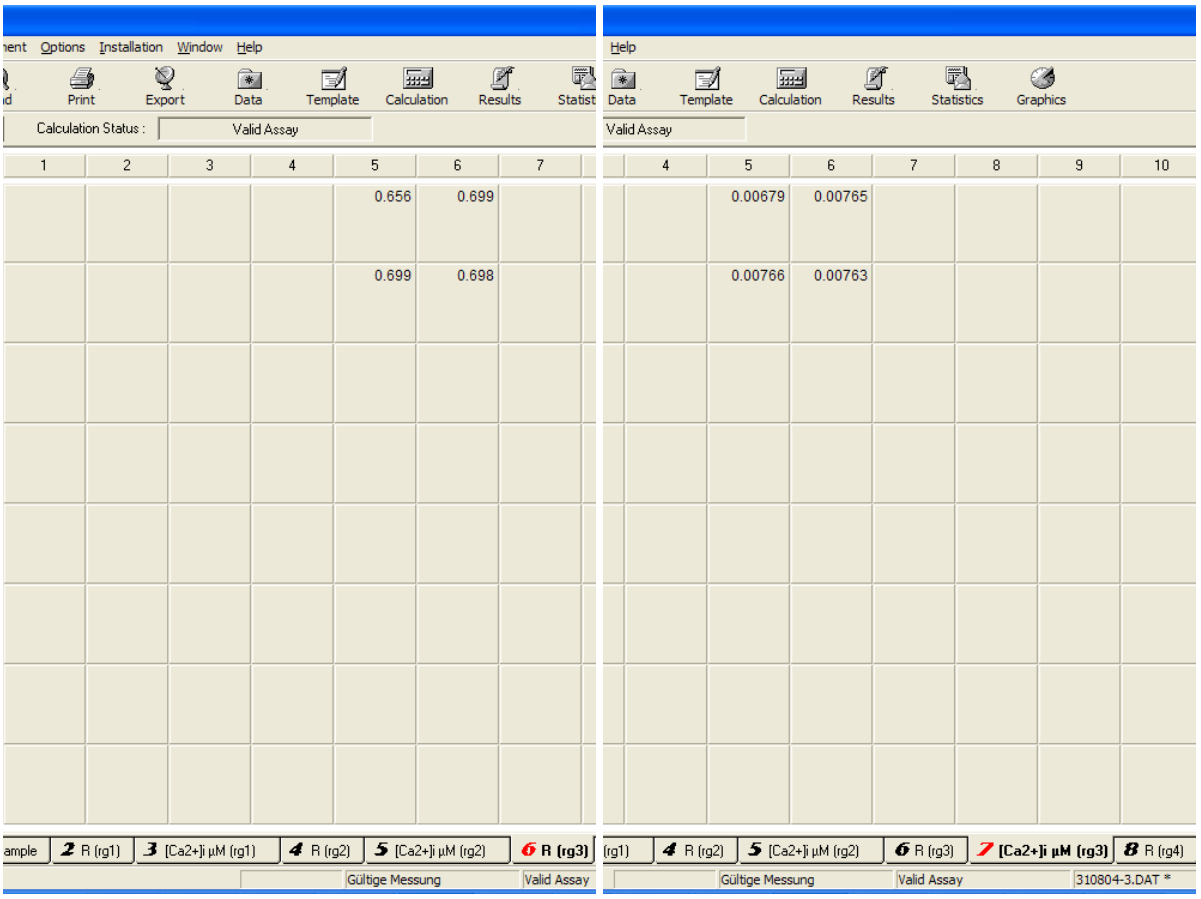


**Figure 12.** Result matrices.

**Left panel:** Matrix for the experimental $F^{340}/F^{380}$ ratio.

**Right panel:** Matrix for the corresponding $Ca^{2+}$ concentration.

## Conclusion

Calcium is an important intracellular messenger in many signal transduction pathways. For measuring $Ca^{2+}$-flux it is important to provide physiological conditions in order to keep the cells viable. For this purpose the Mithras LB 940 is equipped with a temperature control device. Furthermore live kinetic monitoring is possible. With 4 reagent injectors up to four different substances, e.g. agonist, antagonist, cell lysis reagent or $Ca^{2+}$-chelating reagent, may be injected automatically in an appropriate timely manner. Additionally, it is possible to calculate the exact $Ca^{2+}$ concentration of distinct time points and the area-under-curve with the MikroWin 2000 software.

Here we analyzed the $Ca^{2+}$-flux of B cells after stimulation with IgM and used ionomycin as positive and EGTA as negative control. But the measuring of intracellular $Ca^{2+}$ is not limited to the study of B cells, as we have also analyzed dendritic cells using the Mithras LB 940.

## Materials

- 1 x $10^6$ murine B cells/well
- Tyrode buffer for staining and measuring: 140 mM NaCl, 5 mM HEPES buffer, 10 mM glucose, 0,5 M KCl, 0,12 M $MgSO_4$, 1 M $CaCl_2$ and 1% BSA (prepare freshly)
- dye loading: Fura-2-acetomethylester (purchased from Invitrogen, Molecular Probes or Sigma)
- IgM, Ionomycin, EGTA or other stimulators/chelators
- black 96 well plate (Berthold Technologies, ID No. 23302)
- Mithras LB 940 multimode reader (Berthold Technologies)
- Filters (Berthold Technologies):
  excitation 340 nm (ID No. 40086) and 380 nm (ID No. 40087)
  emission: 510 nm (ID No. 40094)
- Optional: Calcium calibration buffer kit and Fura-2 (Molecular Probes)

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