Calcium monitoring with the Mithras LB 940 multimode plate reader

Introduction

Ca^{2+} 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. Intracellularily, 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.

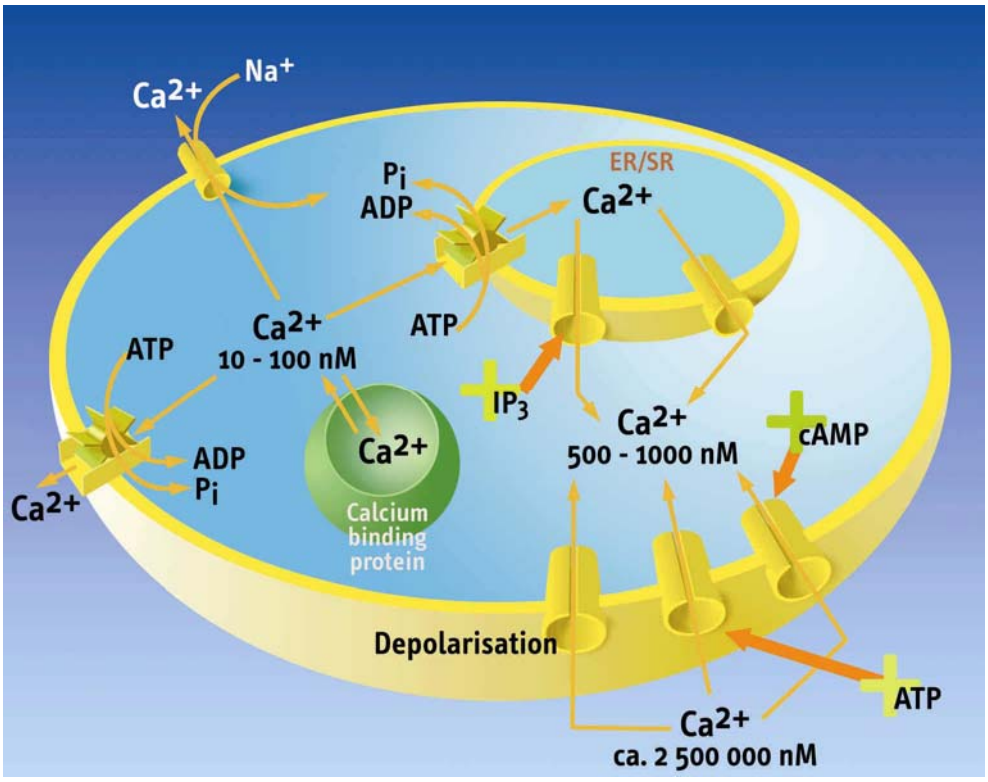


Figure 1. Ca^{2+}-transport: Cytosolic Ca^{2+}-concentrations are raised in response to stimuli, which open Ca^{2+}-channels in the cell membrane or in membranes of the ER/SR. Ca^{2+} is then bound to different target proteins; thereby modulating its effectors. As high Ca^{2+}-concentrations are toxic; 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 (351 – 364 nm spectra of the argon laser) 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+}.

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

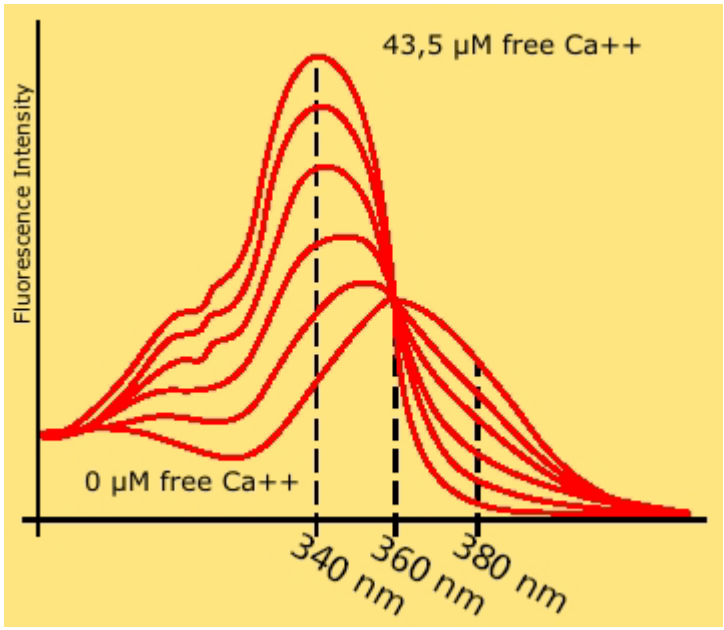


Figure 2. Correlation between fluorescence intensity and Ca^{2+}-concentration: The shift of the absorption maximum of Fura-2 from 380nm to 340 nm (green arrows) depends on the Ca^{2+}-concentration.

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 acetomethylesters (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-impermeant 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 from multiplate formats including 96-well plates. Thus, small cell numbers are applicable and the throughput is high.

Assay protocol

Ca^{2+}-flux was analysed 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 analysed. 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

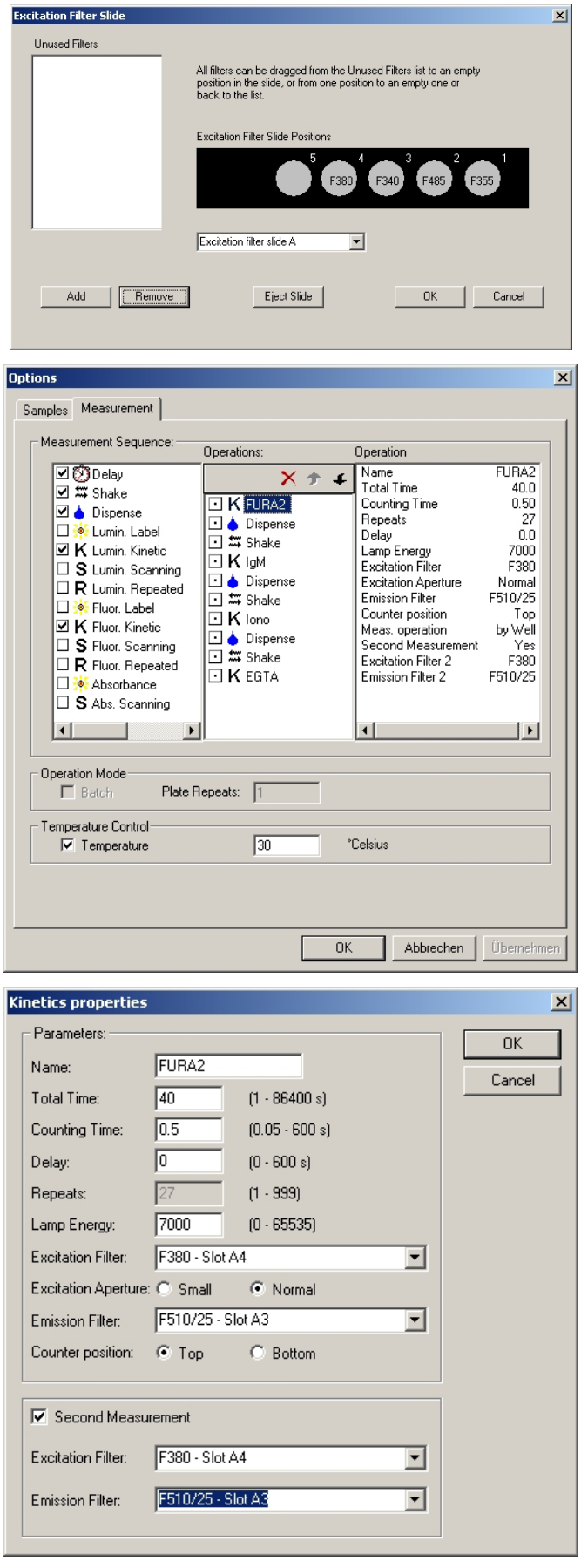


Figure 4. Instrument settings

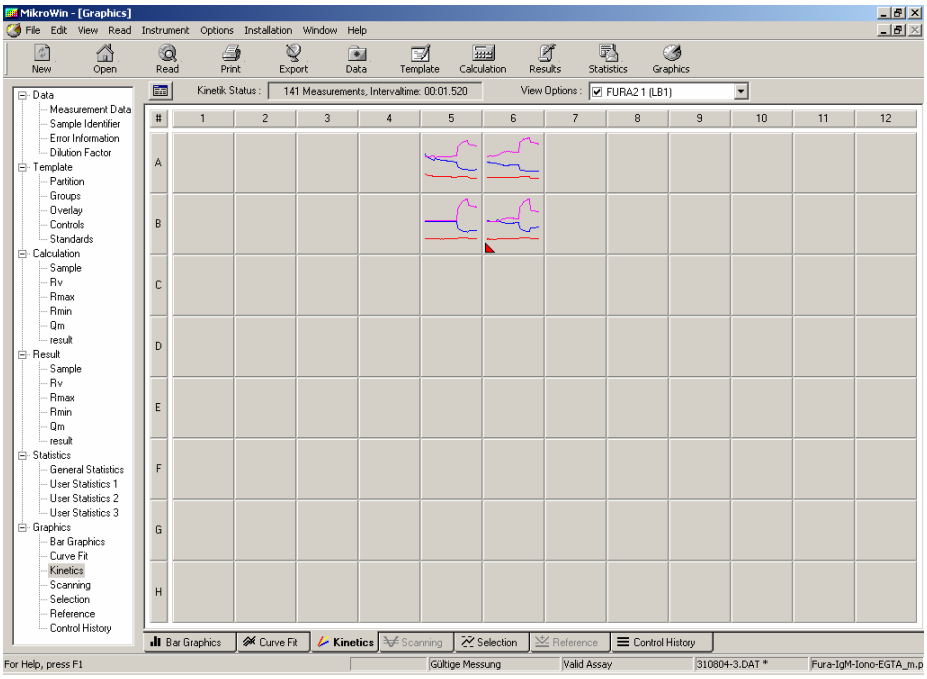
Upper panel: The excitation filters are placed next to each other in order to ensure minimum delay for filter change (upper panel).

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 on line.



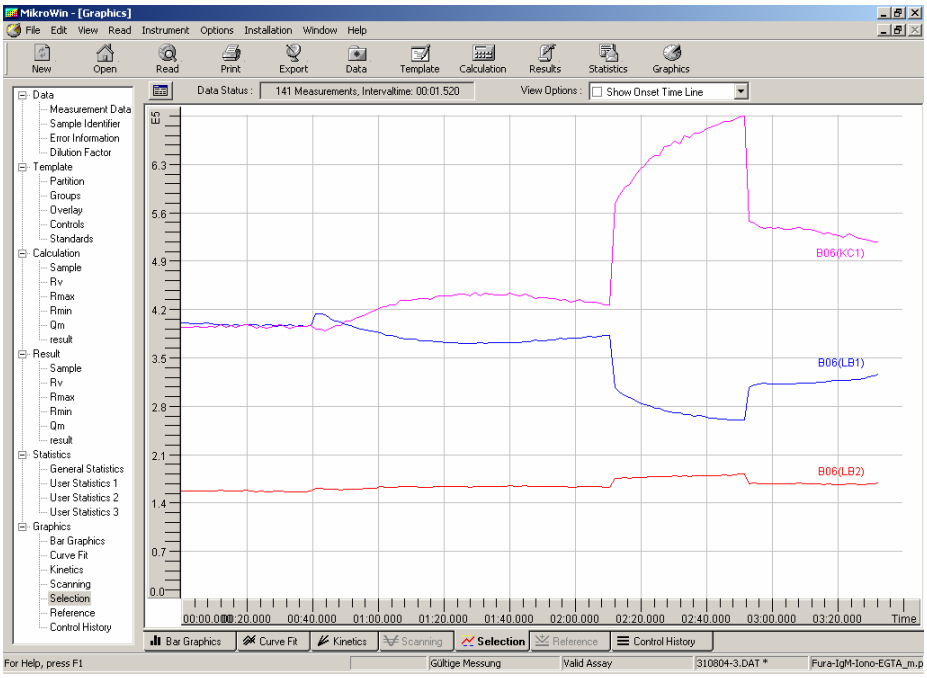


Figure 5. Graphic display of kinetic curves: red graph represents excitation at 340 nm, blue graph represents excitation at 380 nm, pink graph represents the ratio.

Upper panel: View of total microplate

Lower panel: Zoomed view of selected wells

For further data evaluation Mikrowin 2000 offers various possibilities which will be discussed later. In addition it is possible to analyse 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.

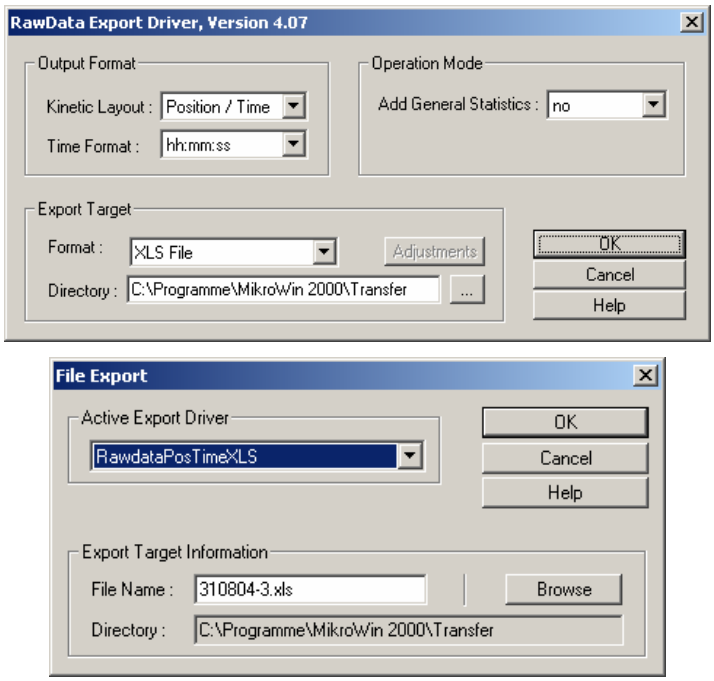


Figure 6. Usage of Rawdata Export Driver to transfer all readings into a sread-sheet readable format.

Upper panel: 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 is calculated. These data are then 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 CaEGTA buffers with given Ca^{2+}-concentrations are measured at 340 nm and 380 nm. These data are plotted as the log of the $[Ca^{2+}]_{free}$ (x-axis) versus the $\log\{(R-R_{min})/(R_{max}-R)\}$ (y-axis). The MikroWin2000 software (Parameterdatei XY) calculates the K_d of EGTA, defined as the concentration at which the function reaches the half saturation point.

Figure 7. Standard Curve Ca

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+}.

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

Conclusion

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 may be injected automatically.

Materials

- $1x10^6$ 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 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)