# Calibration of Calcium Levels

The ratiometric method of determining cell calcium levels using fura-2 is simple and elegant. One measures the fluorescence emission excited by two separate wavelengths, subtracts the background signal and the ratio between the intensity levels is used to calculate the free calcium concentration. The mathematical relationship between the measured ratio (R) and the calcium ion concentration is expressed as follows[1]:

$$[Ca] = K_d * (R-R_{min}) / (R_{max} - R) * Sf2 / Sb2$$

The evaluation of this expression requires the values for the so-called calibration constants ($K_d$, $R_{max}$, $R_{min}$, Sf2, and Sb2) to be inserted. The values of $R_{max}$ and $R_{min}$ are the ratio values measured under conditions of saturating calcium levels and in the absence of calcium respectively. The values of Sb2 and Sf2 are proportional to the fluorescence excited by the denominator wavelength (normally 380nm) again under conditions of saturating calcium levels (the "b" referring to the bound state) and in the absence of calcium (the "f" referring to calcium free) respectively. A value of 225nM is normally used for the dissociation constant for the fura2-Calcium binding ($K_d$). The ratio equation then generates calcium values in nanomolar.

Careful determination of the calibration constants under conditions that closely match the actual experimental conditions is essential for obtaining reproducible calcium measurements. Since $R_{max}$ and $R_{min}$ derive from, and Sf2 and Sb2 essentially are fluorescence intensity values, their values depend on the characteristics specific optical elements in the system. These include the excitation and emission filters, dichroic mirrors and the microscope objective lens. Changing any optical element will have an effect on the calibration constants.

## in vivo *calibration*

There are two main strategies for determining the system-specific calibration constants. The conceptually simpler is the *in vivo* method. The notion here is to use the actual cells under investigation to assess the calibration values. To accomplish this one uses the normal loading procedure for fura2. It is then necessary to control the intracellular calcium concentration so that it can be set to essentially zero (to determine $R_{min}$) and to a level that saturates the indicator (to determine $R_{max}$). Controlling intracellular calcium levels is normally attempted using calcium ionophores (i.e. 8Bromo-A23187 or ionomycin). Optimal procedures for use of ionophores are tissue dependent and specific literature should be consulted for protocols.

The calibration constants require six measurements to be made: the first four are raw fluorescence intensities excited by wavelength 1 (numerator) and wavelength 2

[1] Grynkeivicz, Poenie and Tsien, A new generation of $Ca^{2+}$ indicators with greatly improved fluorescence properties, J Biol Chem 260, 1985 .

(denominator) from the fura2-loaded cells under conditions of zero and saturating intracellular calcium. The remaining values are the background (BG) signals obtained by moving the cells out of the measurement field and recording wavelength 1 and wavelength 2 values. Wavelength 1 and 2 BG signals are subtracted from the respective raw fluorescence values and then $R_{max}$ and $R_{min}$ are calculated. Sf2/Sb2 is the ratio between the background subtracted wavelength 2 excited fluorescence in zero and saturating calcium.

An elaboration of the background evaluation would be to obtain the signals from a cell that contains no fura2. Such measurements would include the autofluorescence of the cell itself. Under most circumstances the autofluorescence is a small fraction of the total signal and is inconsequential. Background levels that include autofluorescence can be obtained prior to loading the cells with fura2. Another approach that some investigators have employed is to bath fura2-loaded cells with manganese (in the presence of ionophore) to quench the indicator.

## in vitro *calibration*

*In vitro* calibration constants are obtained from samples of solutions of fura2 and a background solution containing no fura2. The calibration solutions are typically made to mimic the intracellular condition. The following recipes serve as examples:

| High Calcium | Zero Calcium |
|---|---|
| 150mM KCl | 150mM KCl |
| 10mM NaCl | 10mM NaCl |
| 3mM $MgCl_2$ | 3mM $MgCl_2$ |
| 10mM Hepes | 10mM Hepes |
| 1μM fura2 pentapotassium salt | 1μM fura2 pentapotassium salt |
| 1mM $CaCl_2$ | 10mM EGTA |
| pH set to 7.4 | pH set to 7.4 |

Fluorescence recordings are made from a volume of each solution typically placed in thin-walled capillary tubes. Background levels are obtained from a capillary tube containing a solution (i.e. $H_20$) containing no fura2. Background values with wavelength 1 (numerator) and wavelength 2 (denominator) are subtracted from the raw fluorescence signals in the two fura2-containing solutions. The fluorescence ratio $R_{max}$ is then calculated from the High Calcium values and $R_{min}$ is calculated from the Zero Calcium values. Sf2/Sb2 is the ratio between the background subtracted wavelength 2 excited fluorescence in Zero and High Calcium solutions respectively. Consistency in the droplet size, placement and the focusing level improve the reproducibility of the *in vitro* calibration process.