Fluorescence-Based Calcium Ion Sensing at High Hydrostatic Pressures

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by

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ABSTRACT

FLUORESCENCE-BASED CALCIUM ION SENSING AT HIGH HYDROSTATIC PRESSURES

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Fluorescence-based calcium sensing can be extended to studies at biologically-relevant hydrostatic pressures by characterizing calcium fluorophores in the 1-500 atm range. Intensity measurements of the calcium-sensitive dye Fluo 4 (2 μM, λ(ex) = 500 nm, in EGTA/MOPS-based calcium buffer) indicate a reversible shift in dye/calcium equilibrium with pressure. An effective $\Delta V = -10.1 \pm 0.7 \text{ mL/mol}$ is found by modeling the relationship between dye $pK_D$ and pressure as Arrhenius. Knowledge of the $\Delta V$’s of EGTA and MOPS should enable extraction of the dye $\Delta V$. This will enable quantitative calcium sensing in future high pressure protein and cellular studies.
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Introduction

Background and Purpose

The average hydrostatic pressure within the biosphere is 380 atm, with a maximum pressure of 1100 atm [1]. Pressures in this range affect cellular properties such as morphology [2], substrate adhesion [3], and metabolism [4]. Yet, organisms have evolved which thrive at elevated pressures and even require them for survival. In recent years, entire ecosystems of so-called piezophiles have been discovered in high pressure environments previously thought barren, including Antarctic ice, deep-sea hydrothermal vents, and solid rock more than three kilometers deep [5]. These organisms have numerous potential applications to biomedicine and biotechnology [6]. Meanwhile, space exploration has revealed that extraterrestrial environments in our own Solar system (e.g. Mars, Europa) may be similar to terrestrial environments known to harbor piezophiles [5]. The evidence is tantalizing, and further investigation has the potential to answer fundamental questions regarding the origins, limits, and ubiquity of life. Understanding the biophysical effects of pressure on cells, therefore, is of both pure and applied interest.

In order to elucidate these effects, it is necessary to develop quantitative methods for studying cells and proteins under high pressure (HP). Calcium ion sensing is a highly successful method in ambient-pressure studies, as calcium is ubiquitous in many cells and tightly controlled for use in rapid intracellular signaling [7]. Fluctuating gradients of calcium ions play a critical role in many cellular processes, such cell division. Furthermore, it is known that pressures as low as 200-500 atm can abolish those processes [8]. Investigating the effects of pressure on intracellular calcium will therefore be important in understanding the biophysical mechanisms by which cells respond to pressure.
The use of fluorescence microscopy with a capillary-based pressure chamber/optical window has great potential in this regard, as this approach has already yielded quantitative, HP hydrogen ion (pH) sensing in vitro [9]. Salerno et al. achieved this by characterizing the effect of pressure on pH-sensitive fluorophores (fluorescent dyes) [9]. The purpose of this study is to extend fluorescent HP sensing to the calcium ion, utilizing a capillary-based fluorescence microscopy system. These newly-developed tools of HP fluorescence microscopy will enable future quantitative studies of living cells under high pressure.

**Calcium-Sensitive Fluorophores**

Most calcium-sensitive fluorophores are made of fluorescein (the fluorescent part) and BAPTA (the calcium-binding part), and can be described in solution by a two-state model:

\[ DCa \leftrightarrow D + Ca \]  \hspace{1cm} (1)

That is, the dye exists in solution only in a form that is singly-bound to calcium, DCa, or in the unbound form, D. All useful calcium-sensitive dyes exhibit some measurable change in fluorescence upon calcium binding (e.g. spectrum, lifetime, intensity). In the case of the Fluo class of dyes, this is expressed as a change in fluorescence emission intensity (as much as 70-fold in the case of some Fluo dyes) but no change in spectrum [10]. Since Fura Red exhibits a decrease, while the Fluo dyes exhibit an increase in emission intensity upon calcium binding, these two dyes can be used simultaneously for greater sensitivity in calcium measurements [11]. The dye Fluo 4 has a physiologically relevant sensitive range to calcium (~1 μM) and excites/fluoresces at visible wavelengths (500 nm excitation, fluorescence >500 nm) [10]. Because of these convenient and useful features, this dye has been selected for this study (see Fig. 1 for chemical structure).
Assuming the two-state model in equation (1), the intensity of fluorescence emission $F$ as measured at a single wavelength is

$$F = F_{\text{max}} \frac{[DCa]}{[D] + [DCa]} + F_{\text{min}} \frac{[D]}{[D] + [DCa]} \quad (2)$$

for a dye whose emission increases upon calcium binding, where $F_{\text{max}}$ is the intensity when the dye is saturated by calcium ($[D] = 0$) and $F_{\text{min}}$ is the intensity when there is zero calcium in solution ($[DCa] = 0$) [12]. The dissociation constant of the dye is

$$K_D = \frac{[D][Ca]}{[DCa]} \quad (3)$$

where $[Ca]$ is free calcium ion concentration in solution. Combining equations (2) and (3) yields a relationship between fluorescence intensity $F$ and the free calcium ion concentration:

$$pK_D = pCa + \log \left( \frac{F - F_{\text{min}}}{F_{\text{max}} - F} \right) \quad (4)$$

where the symbol $p$ stands for a negative logarithm in base ten, e.g., $pCa = -\log([Ca])$ [12]. The only constants in this equation which depend upon the particulars of the optical system being used (excitation intensity, etc.) are $F_{\text{max}}$ and $F_{\text{min}}$. These values can be measured by measuring the intensity of the dye in calcium-free and calcium-saturated solutions. This calibrates the dye for use at constant (ambient) pressure.

**Modeling the Effect of Pressure**

Assuming that the two-state model is valid at pressures of hundreds of atmospheres, pressure will cause a shift in dye-calcium equilibrium. As a first approximation, this can be modeled simply by an Arrhenius relation,

$$K_D = K' e^{-\Delta V (p - p')/RT} \quad (5)$$
where $K'$ is the dye dissociation constant at a reference pressure $P'$ (1 atm), $P$ is the pressure, $R$ is the gas constant and $T$ is temperature (in Kelvin). The parameter $\Delta V$ is a constant: it is the thermodynamic change in volume associated with dye/calcium binding. Taking the negative logarithm of equation (5) yields

$$pK_{D} = pK' + \frac{\Delta V (P - P')}{RT} \log(e)$$

(6)

Thus, pressure causes a linear change in $pK_{D}$, assuming the Arrhenius relation is valid. Since $pK_{D}$ can be measured by fitting data to equation (4), the only unknown constant in equation (6) is $\Delta V$, which is proportional to the slope of $pK_{D}$ vs. pressure. Measuring the slope of $pK_{D}$ vs. pressure, then, yields $\Delta V$, which fully characterizes the dye under pressure. By using calcium buffering solutions of varying $pCa$ to measure the dye fluorescence at several pressures between 1 and 500 atm, it should be possible to obtain fitted values of $pK_{D}$ at several pressures, and calculate $\Delta V$ from the slope of $pK_{D}$ vs. pressure. This will actually be an effective $\Delta V$, since pressure will cause a shift in equilibrium for all the constituent chemicals in solution, including the buffers. However, if the $\Delta V$ is known for every chemical in solution except the dye, it should be possible to extract the dye $\Delta V$.

**Summary**

The present study demonstrates the feasibility of the approach outlined above, by characterizing the calcium-sensitive fluorophore Fluo 4 on a capillary-based optical system; by obtaining precise intensity measurements with typical uncertainties less than 5%; and by acquiring pressure data on Fluo 4, which shows a reversible, measurable shift in calcium equilibrium, and using this data to estimate $\Delta V$. 
Materials and Methods

Calcium Buffering

Fluo 4 was purchased from Molecular Probes [10] in cell impermeant salt form, and characterized at pressures up to 7500 psi (500 atm). The dye were prepared in samples of the same dye concentration (typically 2 μM) in solutions of 18.2 mega-ohm-cm de-ionized water and Molecular Probes’ Calcium Calibration Buffer Kit #1 [10]. The kit consists of two solutions, which were mixed in different ratios to produce solutions of varying pCa.

Molecular Probes’ Calcium Calibration Kit #1 is based on the calcium chelator EGTA [10]. The kit includes two solutions which are prepared by methods developed by Roger Tsien [12]: solution A, with “zero” [Ca] and 10 mM [EGTA]; and solution B, with 39 μM [Ca] and 10 mM [CaEGTA] (both solutions contain 30 mM MOPS at pH 7.2, 100 mM KCl). The dissociation constant $K_D^{EGTA}$ of EGTA is related to the free calcium ion concentration [Ca] by

$$[Ca] = K_D^{EGTA} \times [CaEGTA]/[EGTA] \quad (7)$$

As shown by Takahashi and Camacho [13], the tiny amount of free calcium in solutions A and B (about six orders of magnitude less than the CaEGTA or EGTA) allows the approximation

$$[Ca] = K_D^{EGTA} \times [solutionA]/[solutionB] \quad (8)$$

Thus, knowing $K_D^{EGTA}$ allows solutions to be prepared with known free calcium ion concentration, [Ca], by mixing solutions A and B in different ratios. At 1 atm, $K_D^{EGTA}$ depends on ionic strength, pH, and temperature. At 20 degrees Celsius, pH = 7.2 and 100 mM KCl, $K_D^{EGTA} = 150.5$ nM [12]. Although temperature was not strictly controlled in this study, it was
measured to be 21 ± 1 degrees Celsius whenever data was taken. Also, the pH of solutions A and B after mixing with the dye were confirmed by pH meter to be equivalent (within 0.02 log units) and equal to 7.20 ± 0.03. When dye was added to calcium buffer solutions, it was added at 1-2 parts dye per 100 parts calcium buffer solution to maintain desired pCa.

**Fluorescence Measurement and Analysis**

The experimental setup is shown in Fig. 2 and described in detail by Salerno et al. in [9]. Fluorescence emission spectra were measured by a custom-built spectrofluorimeter utilizing a quartz capillary-based pressure chamber/optical window. A nitrogen-pumped, pulsed dye laser (λ=500 nm) excited the calcium-sensitive dye Fluo 4, which fluoresces maximally at 520 nm [10]. The standard settings for spectral acquisition: 60 μs gate width, accumulate over 25 pulses at 2 Hz, with 12 μJ reaching the sample per pulse. Intensities are averages of 5 nm around the wavelength of maximum emission as measured on the optical system (540 nm). Five or more such intensities for a given sample are averaged to calculate “the” intensity, F, of a sample. In cases where data was only taken at 1 atm, obviating the use of a pressure chamber, signal strength was improved by placing samples on a glass slide under a coverslip. The coverslip rested on two 1.5mm standoffs. This allowed the wetting of the sample to the coverslip to create a flat sample area of even thickness. Intensities under pressure were measured after waiting 15 minutes for system equilibrium, and pressure was increased or decreased in increments of at most 2000 psi over a period of one minute or longer. Nonlinear fits and parameter estimates were made using Origin software.
Cleaning Protocol

All glass/plastic-ware was soaked for several hours in detergent, and then rinsed at least five times in de-ionized water.

Results

Fig. 3 shows a few representative spectra for Fluo 4 at varying pCa, with peak measured emissions at ~540 nm. The intensity of emission decreases with increasing pCa for Fluo 4 mixed in EGTA/MOPS solutions of pCa 4 through 9. For a given pCa, intensities were measured for Fluo 4 over 450 – 455 nm and averaged over at least five trials, yielding intensity measurements with typically 5% error or less.

Fig. 4 shows normalized Fluo 4 spectra for different pCa (Fig. 4a), and for different samples prepared and measured at different times over three months (Fig. 4b). Dye spectra at varying time periods, pCa, and pressure were normalized and compared on a routine basis to check for any change in the shape of the spectrum. It should be noted that the spectra in Fig. 4b have been translated (< 5 nm) so that their peaks align, to better compare their shapes. Fig. 4 serves as a check for any spectral differences in samples at varying pCa. A second check is illustrated by Fig. 5, which shows the linearity of dye emission intensity vs. concentration for Fluo 4 over the used concentration range (around 2 μM). A line is fitted to the data by linear regression.

Fig. 6 is representative data showing the effect of pressure on Fluo-4 fluorescence intensity (pCa = 5.87 at 1 atm). Pressure causes a reversible decrease in intensity, which indicates a reversible shift in dye/calcium equilibrium towards dissociation. Intensity and uncertainty are found from the average and standard deviation of at least 5 measurements. Fig. 7
shows Fluo-4 emission intensity (uncertainties less than 2%) vs. pCa at (a) 14 psi (1 atm, ambient pressure); (b) 2000 psi; (c) 6500 psi; and (d) 7500 psi (500 atm). Fig. 7 is the result of several datasets like the one shown in Fig 6. Nonlinear curve fits to the two-state model are shown in red. At each pressure, the curve fit yields a fitted value for dye pK, and an uncertainty. These pK values and their uncertainties are plotted as a function of pressure in Fig. 8. A line of best fit (in red) is displayed which yields a fitted slope of $(-1.80 \pm 0.12) \times 10^{-9}$ Pa$^{-1}$.

Note that all of the spectra in this study are uncorrected. Wavelengths are estimated to be correct to within 10 nm based on measurements of fluorescence standards such as rhodamine.

Discussion

**Why Not Use an ISE?**

There are important reasons pCa was not measured directly with a calcium ion-selective electrode (ISE) in an attempt to confirm calculated pCa from equation (7). An ISE, like a standard pH meter, transduces the concentration of certain ions in solution into a voltage by way of a semi permeable membrane. Aside from being costly (up to $600 or more) an ISE would have limited use in high pressure (HP) studies. This is because in order to make a measurement, an ISE must come into contact with the sample; however, if that sample is in a pressurized chamber, this would mean exposing the ISE to pressure gradient of up to 7500 psi, which would surely crack the glass or plastic part of the probe. This could be avoided by pressurizing the ISE completely, but that is impractical. Also, ISE’s can only measure ion concentrations at the particular location of the probe, whereas fluorophores loaded into cells can be used in a fluorescence microscopy system to image ion concentrations and provide spatial information. Indeed, these are a few of the reasons that this study has pursued fluorescent (rather than ISE)
methods for HP ion sensing in the first place. In addition, [Ca] as low as 1 μM (pCa = 6) is outside the linear range of most ISEs. Furthermore, calibration of an ISE can only be done relative to calcium buffer solutions of known pCa, and it would be circular to verify the pCa of calcium buffer solutions using an ISE that is itself calibrated by calcium buffer solutions. Finally, the fact that the data is in good agreement with the two-state model (Fig. 7) indicates that any error in pCa is a systematic shift. Such a systematic shift, however, does affect ΔV, which depends only on the change in $pK_p$ over pressure, not its absolute value at any given pressure (see equation 6).

**Dye Linearity**

It is implicitly assumed in equation (2) that the emission intensity of the dye is linear with its concentration. That assumption is valid in dilute solutions where photons emitted by fluorescing dye molecules are not scattered or absorbed by other dye molecules. At high concentrations, however, these nonlinear effects become significant. To verify that the emission intensities of Fluo 4 and Fura Red are linear for dye concentrations used in this study (around 2 μM), intensities were measured at several concentrations from 1 to 10 μM. The results of this check are shown in Fig 5. The linearity of the data indicates that fluorescence emission intensity of Fluo 4 is linear at the dilute concentrations used in this study.

**Measurement of Fluo 4 Fluorescence Intensity**

The data shows the quality of spectra and precision of intensity measurements obtainable on this HP-adapted spectrofluorometer, both when sample is loaded onto a slide (Figs. 3-5) and
when it is loaded into a capillary (Figs. 6 and 7). Uncertainty in intensities is less than 5%, and often only 1-2%, as is the case for the capillary-based intensity measurements in Figs. 6 and 7.

The intensity of each spectrum is determined by the average number of counts 5 nm around a set wavelength, with bin sizes of 1 nm. Any wavelength can be chosen, since spectral shape does not change, but in order for data to be comparable this wavelength must be fixed. For the best signal-to-noise ratio, the fixed wavelength was chosen to be close to the maximum measured emission of the dye (~540 nm). The average of several such intensity measurements is called “the” intensity $F$, with the uncertainty determined by the standard deviation. Thus, each measurement of $F$—and each point in Figs. 5, 6, and 7—is effectively the result of 625 or more measurements (5 nm-sized bins $\times$ 25 pulses $\times$ 5 spectra). Yet, this method of measuring $F$ can be easy and quick on this fluorescence microscopy system: once a sample is on the microscope stage, only a single button need be pressed to acquire all the necessary data in less than 63 seconds (25 pulses / 2 Hz $\times$ 5 spectra).

**Pressure Data**

The reversible decrease in fluorescence emission with pressure indicates a shift in dye/calcium equilibrium toward dissociation. The shift toward dissociation rather than association may be explained physically by the effects of electrostriction. The fact that pressure causes a decrease in dye pK (Fig. 8) indicates a negative $\Delta V$. Assuming Arrhenius behavior, Eq. 6 yields an effective $\Delta V = -10.1 \pm 0.7$ mL/mol. (For comparison, the $\Delta V$ of water is -21 mL/mol). The uncertainties in fitted pK values are determined by the nonlinear curve-fitting software, ORIGIN. These uncertainties are somewhat arbitrary, and thus the uncertainty in the
slope of the line of best fit (Fig. 8) is somewhat arbitrary as well. The propagated uncertainty to
the calculated $\Delta V$ due to these errors, as well as the error in temperature $T$, is 7%.

In order to extract the dye $\Delta V$ from the effective $\Delta V$, the effect of pressure on the other
constituents in the solution (MOPS, EGTA, KCl) must be considered. First, it should be noted
that according to the two-state model (Eq. 4), the intensity $F$ depends only on two variables: dye
pK; and the free calcium ion concentration in solution, or pCa. The effect of pressure on dye pK
is modeled by an Arrhenius relation with an unknown dye $\Delta V$ term, as already discussed (Eq.
6).

The pCa of solution, on the other hand, is determined by the dissociation constant of
EGTA, $K_{\text{EGTA}}$. (Although the dye also binds calcium, its contribution to the pCa of solution is
negligible since $[\text{EGTA}] = 10 \text{ mM} >> [\text{DYE}] \sim 2 \mu \text{M}$). $K_{\text{EGTA}}$, in turn, depends on several
factors: ionic strength (determined by the 100mM KCl); temperature; pH (determined by the 30
mM MOPS); and pressure as mediated by $\Delta V_{\text{EGTA}}$.

As a first approximation under varying pressure, KCl can be assumed to be fully
dissociated and therefore the ionic strength is constant. The temperature is assumed to be
constant due to the small volume of sample in the heat bath of the laboratory environment. It is
assumed, then, that the pressure-dependence of $K_{\text{EGTA}}$ is dominated by $\Delta V_{\text{EGTA}}$ and the effect of
pressure on pH due to MOPS.

Modeling the effect of pressure on EGTA with an Arrhenius relation yields an expression
for the pressure-dependent change in pCa caused by $\Delta V_{\text{EGTA}}$ (Appendix A):

$$
\Delta pCa(P) = \frac{\Delta V_{\text{EGTA}}(P - P')}{RT} \log \frac{pCa}{pCa_{0}}
$$

(9)
The subscript “V” is simply to recall the fact that this pressure-dependent change in pCa is ultimately due to $\Delta V_{EGTA}$. Adjusting pCa by addition of this correction term (as in the pCa in Fig. 7, a-d) will enable a more accurate estimate of the dye $\Delta V$.

However, Eq. (9) does not take into account the pressure-dependent change in pH due to MOPS, which can be expressed as $\Delta pH/\Delta P = (1.5 \pm 0.5) \times 10^{-4} \text{ atm}^{-1}$ (estimated from data in [14]). Based on data in [10], $K_{EGTA}$ vs. pH can be well approximated by an exponential decay (see Appendix B, Fig. 1B), especially within pH of 7.2 ± 0.1, the maximum pH change expected at pressures less than 500 atm. The fitted parameters of this decay, combined with the estimated $\Delta pH/\Delta P$ of MOPS, can be used to write the pressure-dependent $K_{EGTA}$ due to MOPS-mediated changes in pH. This function can be substituted for $K_{EGTA}$’ in Eq. (A1) to get the pressure dependence of $K_{EGTA}$ due to both MOPS and $\Delta V_{EGTA}$. Analysis currently in progress will lead to further corrections of pCa and thus a more accurate estimate of the dye $\Delta V$.

Once a value for Fluo 4 $\Delta V$ has been calculated, this value should be confirmed. One way to do this is to repeat this study in other well-studied calcium or pH buffers (say, HEPES instead of MOPS) and try to reproduce the result.

**Error Analysis**

There are many possible sources of error to consider in these data, including pipetting error, lack of temperature control, contamination, variations in dye concentration from sample to sample, variations in laser firing and optical system alignment from day to day, and so on. Many of these sources of error can be neglected, either because they do not have a significant effect on the data, or because they are systematic errors that do not affect the measurement of the effective $\Delta V$. For example, variations in T, the temperature, will contribute to the uncertainty in the slope
of \( pK_D \) vs. pressure (see equation 6). Although temperature was not strictly controlled, it was measured to be 293 K and to vary less than 1 K, which contributes to a negligible uncertainty in temperature of 0.3%.

Noise picked up by the laser/optical system was the biggest source of random error, typically contributing a few percent uncertainty to intensity measurements, though usually less than 5%. This uncertainty is simply the standard deviation of the “intensities” of several spectra (at least five) taken repeatedly on the same sample, where each spectrum is the accumulation of fluorescence emission from 25 laser excitation pulses. The uncertainty is low in part because of the large amount of data that could be quickly acquired and averaged for each sample.

The spectra of the dye was normalized and compared across samples and days to ensure that the spectrum of the dye was not changing, which could be an indication of the degradation of the dye into new forms, or contamination. It is important that the spectral shape of the dye not change, because the analysis rests on intensity changes due only to shifts in calcium equilibrium. Fig. 4 shows a typical result: in Fig. 4 (a) are the normalized spectra of several Fluo 4 samples prepared together on the same day, in EGTA/MOPS buffer solutions of varying pCa. Note that the spectrum does not change. In Fig. 4 (b) are normalized Fluo 4 spectra for samples prepared of similar pCa (5.82, 5.82, and 5.87), but prepared and measured on different days. The spectrum is not changing, showing no signs of degradation or contamination.

The goodness of fit of fluorescence data (Fig. 7) to the two-state model (equation 4) indicates that certain sources of error can be neglected. That the data fits the model well indicates that propagation from sources of random error, such as pipetting error, is small. Systematic error, on the other hand, could cause the data in Fig. 7 to be shifted left or right along the pCa axis, while maintaining its functional shape. This form of systematic error will alter
apparent $pK_D$, but it will have no effect on calculating the effective $\Delta V$. That is because $\Delta V$ is determined only by the slope of $pK_D$ vs. pressure, while a systematic error in $pK_D$ would only affect the y-intercept of $pK_D$ vs. pressure (see equation 6).

Note that an ORIGIN nonlinear curve fit of the data to the two-state model yields an apparent Fluo 4 $pK_D$ of 5.76 ± 0.04 at 1 atm, though the literature value is 6.46 [10]. This could suggest a systematic error of 0.7 log units in pCa with respect to the Fluo 4 data. This error cannot be due to temperature or pH effects on $K_D^{\text{EGTA}}$. This is because, according to equation (7), such an error would require an increase in $K_D^{\text{EGTA}}$ of 600 nM, which would imply a decrease in pH to 6.85 or less, or an increase in temperature of over 15 degrees Celsius (based on $K_D^{\text{EGTA}}$ data in [10]): however, temperature was measured to be 21 ± 1 degrees Celsius whenever data was taken, and pH of dye/buffer solutions were confirmed by pH meter to be equivalent (within 0.02 log units) and equal to 7.20 ± 0.03. It is worth noting here that the measured values of $pK_D$ are apparent values, and equation (1) is a simple model of dye-calcium equilibrium in solution whose accuracy may depend upon experimental conditions. Whatever the cause of the discrepancy between the apparent $pK_D$ of Fluo 4 (at 1 atm) measured here and its literature value, this should not have any effect on calculating $\Delta V$, for reasons explained above.

**Conclusion**

This study takes steps towards extending a calcium-sensitive fluorescent dye to HP biophysical studies, using a capillary-based fluorescence microscopy system. Intensity measurements of Fluo 4, in a capillary-based pressure chamber at pressures up to 7500 psi (500 atm), have been made with precision (less than 2% error). A significant, reversible pressure
effect has been observed in the emission intensity of Fluo 4 in an EGTA/MOPS calcium buffer solution (pCa = 4 through 9). An effective $\Delta V = -10.1 \pm 0.7 \text{ mL/mol}$ has been measured for the Fluo 4/calcium buffer system. A formula for one of the most important complicating factors—the pressure-dependent change in pCa due to the $\Delta V$ of EGTA—has been found. In order to extract a more accurate estimate of the dye $\Delta V$, pressure-dependent changes in pCa due to MOPS/pH effects must also be taken into account. Future studies should test the robustness of the estimated dye $\Delta V$ by repeating this study, with MOPS or EGTA substituted with alternative pH/calcium buffers. Measuring a robust value for Fluo 4 $\Delta V$ will enable use of this dye in quantitative calcium sensing at high pressure. This capability should prove useful in future high pressure studies of calcium-binding proteins, and imaging the effects of pressure on calcium homeostasis in living cells.
References


Fig. 1: Chemical structure of the calcium-sensitive dye Fluo 4 ($K_D = 0.35 \, \mu M$, cell impermeant salt). The dye excites at 500 nm and fluoresces at visible wavelengths (520 nm peak intensity). $K_D$ is determined at 22 degrees Celsius in 100 mM KCl, 10 mM MOPS, pH 7.2. (Figure adapted and data acquired from Molecular Probes’ *Handbook* [10]).
Fig. 2: Experimental setup. A nitrogen-pumped pulsed dye laser excites the sample, which is mounted in a capillary-based pressure cell. A fiber optic feeds the fluorescence emission to a spectrograph coupled to an ICCD. Pressure is controlled with a piston pump. Setup is described in detail in [9].
Fig 3: Fluo-4 spectra in EGTA/MOPS solution of pCa 4.4, 5.87, 6.12, 6.41, 6.65, and 9 (top to bottom), 500 nm excitation, 1 atm. Intensity decreases with pCa while spectral shape remains constant.
Fig. 4: Representative normalized Fluo 4 spectra in EGTA/MOPS calcium buffers a) of varying [Ca]; and b) of samples prepared and measured on different days (pCa 5.82 or 5.87). Only the intensity, not spectral shape, is affected by [Ca] or variations in experimental conditions over time (compare to Fig. 3).
Fig. 5: Linearity of emission intensity with dye concentration for the calcium-sensitive dye Fluo 4 diluted in 18.2 mega-ohm-cm de-ionized water. A line has been fitted by linear regression. The linearity of intensity vs. concentration indicates that the nonlinear effects of self-interaction, such as a dye molecule emitting a photon which is scattered or absorbed by another dye molecule, can be neglected at the dilute concentrations used in this study (less than 3 μM).
Fig. 6: Representative intensity vs. pressure data for Fluo 4 in EGTA/MOPS solution (pCa 5.87 at 1 atm). The intensity decreases reversibly with pressure, indicating a shift towards calcium/dye dissociation. Intensity and uncertainty are found from the average and standard deviation of at least 5 measurements.
Fig. 7: Representative Fluo 4 intensity vs. pCa results at (a) 14 psi (ambient pressure, 1 atm); (b) 2000 psi; (c) 6500 psi; (d) 7500 psi (or 500 atm). A nonlinear curve fit to the two-state model is shown in red. Uncertainty in intensities is less than 2%.
Fig. 8: Graph of Fluo 4 pK vs. pressure. The pK values and uncertainties are determined from curve fits at constant pressures (Fig 3). A line (in red) is fitted to the data, with functional form $y = -1 \times 10^{-5} x + 5.791, R^2 = 0.962$. The fitted slope is $(-1.80 \pm 0.12) \times 10^{-9}$ Pa$^{-1}$. Assuming Arrhenius behavior yields an effective $\Delta V = -10.1 \pm 0.7$ mL/mol.
Appendix A

Assuming an Arrhenius relation yields

\[ K_{\text{EGTA}} = K'_{\text{EGTA}} \exp\left( -\frac{\Delta V_{\text{EGTA}}(P - P')}{RT} \right) \]  \hspace{1cm} (A1)

Or,

\[ pK_{\text{EGTA}} = pK'_{\text{EGTA}} + \frac{\Delta V_{\text{EGTA}}(P - P')}{RT} \log(e) \]  \hspace{1cm} (A2)

Now, equation [7] gives the EGTA-determined solution [Ca]:

\[ [\text{Ca}] = K_{\text{EGTA}} \times \frac{[\text{solution A}]}{[\text{solution B}]} \]  \hspace{1cm} (7)

Taking the negative logarithm, it is possible to write

\[ p\text{Ca} = -\log\left( \frac{[\text{solution A}]}{[\text{solution B}]} \times \frac{K_{\text{EGTA}}}{K'_{\text{EGTA}}} K'_{\text{EGTA}} \right) \]  \hspace{1cm} (A3)

Or, rearranging,

\[ p\text{Ca} = -\log\left( \frac{[\text{solution A}]}{[\text{solution B}]} \times K'_{\text{EGTA}} \right) - \log\left( \frac{K_{\text{EGTA}}}{K'_{\text{EGTA}}} \right) \]  \hspace{1cm} (A4)

According to Eq. 7, the first term on the right is simply the pCa at the reference pressure P’, which can be called pCa(P’). Substituting \( K_{\text{EGTA}} \) for Eq. A1 then yields

\[ p\text{Ca} = p\text{Ca}(P') + \frac{\Delta V_{\text{EGTA}}(P - P')}{RT} \log(e) \]  \hspace{1cm} (A5)

The second term on the right is the pressure-dependent change in pCa due to \( \Delta V_{\text{EGTA}} \), defined as:

\[ \Delta p\text{Ca}(P)_V \equiv \frac{\Delta V_{\text{EGTA}}(P - P')}{RT} \log(e) \]  \hspace{1cm} (A6)
Appendix B

![Graph of Kd of EGTA vs. pH](image)

The data is well approximated by an exponential decay. The fitted parameters of this decay, combined with the estimated $\Delta pH / \Delta P$ of MOPS, can be used to write the pressure-dependent $K_{EGTA}$ due to MOPS-mediated changes in pH.