International Journal of the Physical Sciences Vol. 6(33), pp. 7435 - 7440, 9 December, 2011
Available online at http://www.academicjournals.org/IJPS
DOI: 10.5897/IJPS11.482
ISSN 1992 - 1950 ©2011 Academic Journals

Full Length Research Paper

Using two-photon excitation methods for determination of Ca^{2+} in contamination with protein

A. S. M. Noor[1]*, A. Miyakawa[2], W. Inami[3] and Y. Kawata[2]

[1]Wireless and Photonics Network Centre of Excellent (WiPNet), Department of Computer and Communication System Engineering, Faculty of Engineering, Universiti Putra Malaysia, 43400 UPM Serdang, Malaysia.
[2]Department of Mechanical Engineering, Faculty of Engineering, Shizuoka University, Johoku 3-5-1, Naka-ku, Hamamatsu, Shizuoka 432-8561, Japan.
[3]Division of Global Research Leaders, Shizuoka University, Johoku 3-5-1, Naka-ku, Hamamatsu, Shizuoka 432-8561, Japan.

Accepted 8 September, 2011

Three emission wavelengths from two-photon excitation techniques were use to determine free Ca^{2+} using a new analytical calculation. This was achieved by considering the interaction of protein with free Ca^{2+} and indo-1 in protein contaminated sample. The emissions obtained from the excitation with the dissociation constants were used in the calculation. Agreement values of the Ca^{2+} from the calculation with the known Ca^{2+} buffer solution used for the measurement was obtained for various Ca^{2+} and protein concentration. The analytical analysis shows that dissociation constants for each binding's deviation errors are small to affect free Ca^{2+} determination. This would provide an accurate measurement of free Ca^{2+} in biological cells than the typical two emission wavelength processes. Furthermore, using two-photon excitation methods, deep inside cells observation of free Ca^{2+} are possible using the methods introduced.

Key words: Free Ca^{2+}, indo-1, two-photon excitation methods.

INTRODUCTION

The calcium ion (Ca^{2+}) indicator indo-1 is widely used to estimate free intracellular calcium ion ($[Ca^{2+}]_i$) in various type of cells. Attempts to make *in situ* Ca^{2+} measurements using Ca^{2+} indicator is often met with difficulties due to its unstable and uncontrolled bindings of both Ca^{2+} and indicator. The estimation of $[Ca^{2+}]_i$ is assumed to be same as in calibration solutions (Williams and Fay, 1997; Ikenouchi et al., 1991; Miyakawa et al., 1989). These difficulties are caused by protein in extracellular and intracellular cells, thus bindings of Ca^{2+} indicator and protein is highly antici-pated. It has been reported that protein bindings altered the fluorescence characteristics of indo-1 during the measurement, thus affected the quantitative measurements of $[Ca^{2+}]_i$ in cells (Konishi et al., 1998; Blatter and Blinks, 1991; Kurebayashi et al., 1993).

A report by Miyakawa et al. (1989) shows that alteration occurred on excitation intensity of Ca^{2+} indicator fura-2 in appearance of protein serum albumin concentration in calibrated solution. This alteration induced properties changes for the indicator, such as dissociation constant, k_d which is vital in estimating the $[Ca^{2+}]_i$ concentration.

Hove-Madsen and Bers (1992) showed that using indo-1 binding to protein in permeabilized ventricular myocytes is calculated to be 72% at a protein concentration of 100 mg/ml in saturating Ca^{2+} when compared with lower Ca^{2+} concentration. This resulted in an alteration to k_d which mount up to 3 fold from only Ca^{2+} with indo-1 bindings. This report aims to analyze the effects of Ca^{2+}, protein and Ca^{2+}-protein with indo-1.

*Corresponding author. E-mail: ashukri@eng.upm.edu.my.

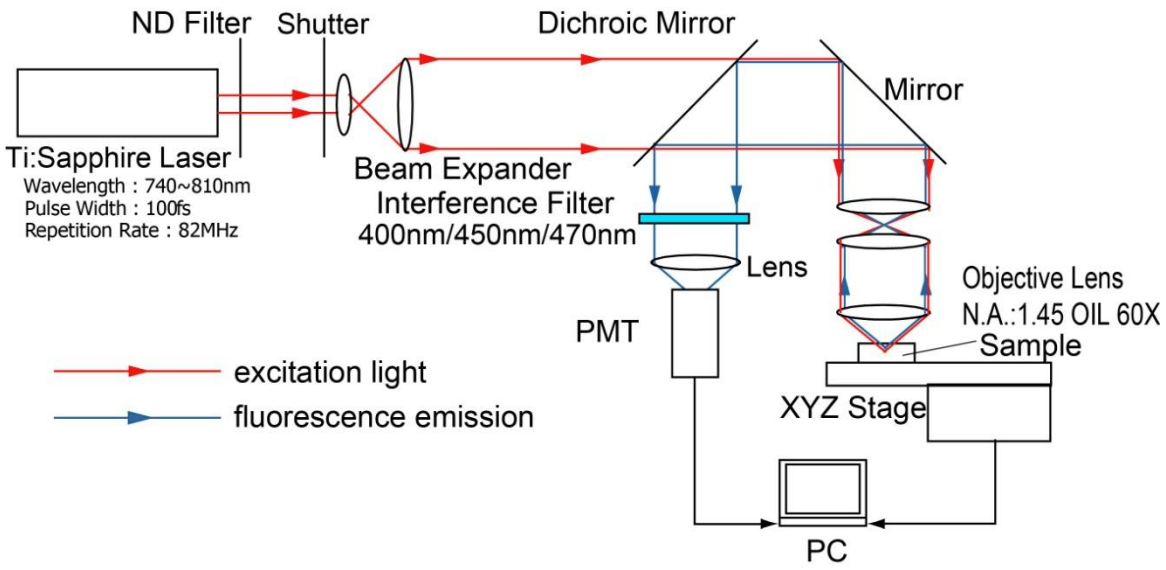

Figure 1. Experimental measurement setup for two-photon excitation emission method.

MATERIALS AND METHODS

The measurement of emission from each interaction measured is quantified into a single analytical equation. The analytical calculation was then used to quantitatively measure free Ca^{2+}. The primary sample used in this study is Ca^{2+} calibrated buffer solution which varies from 0 to 39.0 µM free Ca^{2+} solution. This allows comparison between the measurement and the calculated free Ca^{2+} from the analytical measurement. Soluble proteins concentrations consist of bovine serum albumin (BSA), grain powder, heat-shocked fractionate and agarose gel electrophoresis (Sigma-Aldrich) diluted in distilled water prepared ranging from 10 to 100 mg/ml concentration. The solution of Ca^{2+}-protein is achieved by adding both concentrations, using dialysis process. This is to that ensure equilibrium is achieved for the Ca^{2+}-protein solution binds for various free Ca^{2+} and protein solution.

Indo-1 (20 µl, 1.19 mM) is added to the sample (buffer solution) just before the measurement. For the measurement, 40 µl of each samples solution will make the final indo-1 concentration to 0.40 mM. Respective samples (Ca^{2+}, protein and Ca^{2+}-protein) are independently excited with two-photon excitation methods as shown in Figure 1. The light source used in this measurement is Ti: Sapphire laser (Tsunami, SpectraPhysics) wavelength at 770 nm with an average power fix at 120 mW. The two-photon excitation laser is then incident on the sample place on an XYZ axis stage and focused by an oil emission objective lens (Numerical aperture = 1.45, 60X, UPlanApo, Olympus). The average power detected at the output of the objective lens is 12 mW. The emission from the sample is then picked by the same objective and reflected onto a detector by a set of dichroic mirror and interference filter. Interference filter used for this measurement are 420, 450 and 470 nm to selectively select the emission wavelength before the photomultiplier tube (PMT). Difference in indo-1 concentration value for single- and two-photon excitation can be ignored for two-photon excitation, since the scattering and absorption by the sample is predicted to be small.

Two-photon excitation is used in this experiment, since its advantages of reducing autofluorescence and photobleaching, due to the fact that its excitation only confine to its focal volume. Furthermore, the wavelength used is twice as long as in usual ultraviolet (UV) typical excitation. This is an added advantage for two-photon excitation since UV excitation light is known to be harmful to the live sample. Thus, using here in this measurement will set a benchmark for the usage of two-photon excitation in real live sample.

EXPERIMENTAL RESULTS

Two photon excitation emissions achieved at wavelength of 420, 450 and 470 nm for Ca^{2+} binding with indo-1 are shown in Figure 2a for various free Ca^{2+} solution. From the graph, the dissociation constant (k_d) for the bindings is shown in Figure 2b. The k_d is evaluated from the ratio between 420/450 nm and 420/470 nm and were plotted using the least square method. From the graph, k_d value is 240 nm and it agreed with the measurement done using typical one-photon excitation (Hove-Madsen and Bers, 1992; Gelamo et al., 2002; Sako et al., 1995; Szmanciski et al., 1996). Fluorescence emission at 420 nm shows an increment at higher concentration for higher free Ca^{2+} samples, while at 470 nm shows a reduction of intensity which has similar results with the single-photon excitation. No obvious increment or decrement was observed at 450 nm since it resembles the isosbestic point of Ca^{2+}-indo-1 interaction at this particular wavelength.

Protein-indo-1 interaction is shown in Figure 3a. This was observed after the protein was dialyzed with zero free Ca^{2+} buffer solution before indo-1 was added for the measurement. The figure shows a decrease in intensity for higher concentration of protein at 420 nm. This is due

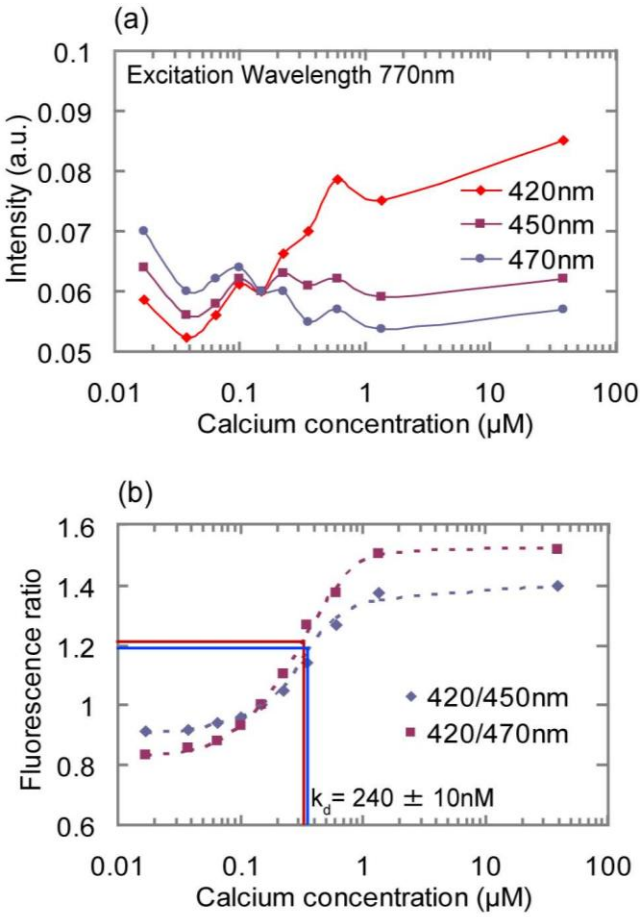

Figure 2. (a) Emission intensity obtained for various free Ca^{2+} solutions with indo-1 using two-photon excitation and (b) its k_d measured.

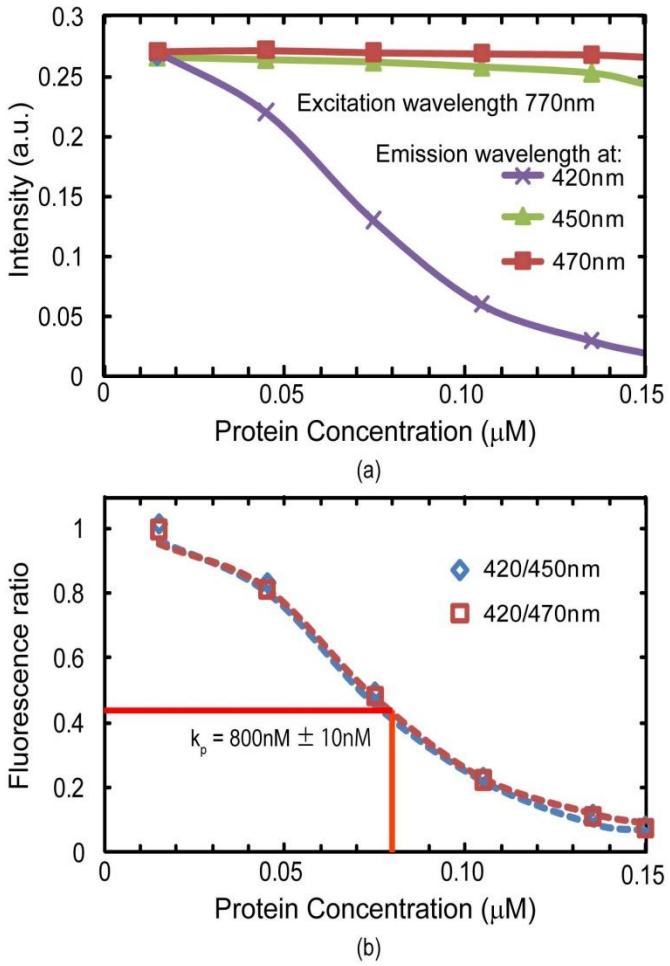

Figure 3. (a) Two-photon emission intensity for emission at 420, 450 and 470 nm for various indo-1-protein concentration samples using two-photon excitation method, (b) k_p determination of indo-1-protein interactions.

to the emission which is highly absorbable by the high protein solution concentration. No changes are detected at 470 nm, however a slight absorption occurs at 450 nm at higher concentration. From this curve, the dissociation constant of protein-indo-1 interaction (k_p) is determined by taking the ratio at 420/450 nm and 420/470 nm. The result is shown in Figure 3b. The curve was plotted using the least square method, and the k_p achieved is 800 nm with a deviation error of 10 nm. The values show that indo-1 is more to bound/unbound with protein as compared to free Ca^{2+}, thus it is important to evaluate each bounds before being able to analytically investigate the free Ca^{2+} in a real sample.

Interaction for Ca^{2+}-indo-1-protein binding's fluorescence spectrum is shown in Figure 4a for two-photon excitation methods. It shows the intensity obtained at fluorescence wavelength of 420, 450 and 470 nm for low to high protein concentrations with a fix 20.6 μM free Ca^{2+} buffer solutions. The curves decreased linearly along with the protein concentration becoming high, but for 450 nm fluorescence intensity which increase with increasing concentration of protein. This shows that the increment or decrement of intensities at any particular wavelength is due to its emission property, not due to the absorption of emission by the protein or Ca^{2+}. From the curve, k_{dp} was determined by taking the ratio between 420/450 nm and 420/470 nm, and the results are shown in Figure 4b. The k_{dp} achieved was 930 nM with a deviation error of 15 nM. This value agrees with the values reported in (Hove-Madsen and Bers, 1992), however in that report, single-photon excitation was used in a real biological sample while in this study, two-photon excitation is used in Ca^{2+}-protein equilibrium solution with indo-1. This agreement verify that two-photon excitation methods can be applied to determine the $[Ca^{2+}]_I$ when it comes to real live sample.

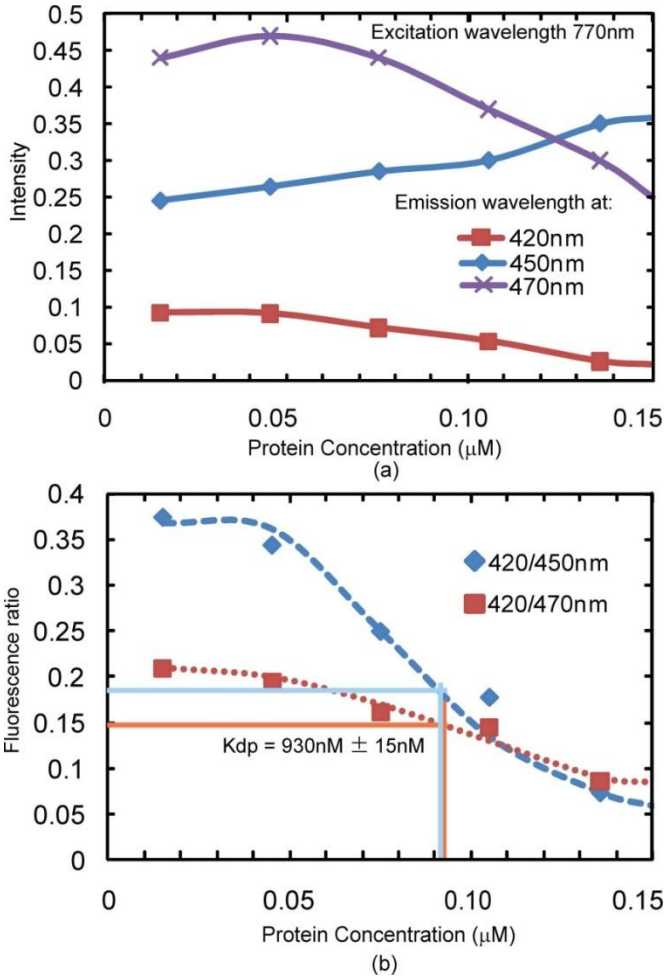

Figure 4. (a) Two-photon emission spectrum of Ca^{2+}-protein-indo-1 interactions measured at wavelength of 420 and 450 nm, (b) k_{dp} for Ca^{2+}-indo-1-protein.

DISCUSSION

The respective bindings to indo-1 for free Ca^{2+} and protein is in visible form in single sample during measurements, the emission detected must consider three respective emissions, and this is shown as (Kudo, 1996):

$$I_\lambda = S_\lambda [\text{indo-1}] + S'_\lambda [Ca^{2+}\text{-indo-1}] + S''_\lambda [\text{indo-1-protein}] + S'''_\lambda [Ca^{2+}\text{- indo-1-protein}] \quad (1)$$

where I_λ is the emission detected at a wavelength λ and $S_\lambda S'_\lambda$ S''_λ and S'''_λ reflects the emission intensity constant functions at that wavelength which is the value of respective saturated bindings becoming constant. The emission detected consist of emissions from Ca^{2+} indicator indo-1 binding [Ca^{2+}-indo-1], protein-indo-1 binding[indo-1-protein] and the Ca^{2+}-indo-1-protein binding[Ca^{2+}-indo-1-protein], respectively. [Ca^{2+}-indo-1], [indo-1-protein] and [Ca^{2+}-indo-1-protein] can be expressed as:

$$[Ca^{2+}\text{-indo-1}] = \frac{[Ca^{2+}][\text{indo-1}]}{k_d}, \quad (2a)$$

$$[\text{indo-1-protein}] = \frac{[\text{Protein}][\text{indo-1}]}{k_p}, \quad (2b)$$

$$[Ca^{2+}\text{-indo-1-protein}] = \frac{[Ca^{2+}][\text{indo-1-protein}]}{k_{dp}} \quad (2c)$$

Inserting Equation 2a to 2c into Equation 1 thus:

$$I_\lambda = S_\lambda [\text{indo-1}] + S'_\lambda [Ca^{2+}][\text{indo-1}]/k_d + S''_\lambda [\text{protein}][\text{indo-1}]/k_p + S'''_\lambda [Ca^{2+}][\text{protein}][\text{indo-1}]/k_p k_{dp} \quad (3)$$

However, Equation 3 consists of three unsolved parameters which are the concentrations indo-1, Ca^{2+} and protein. The equation resembles the emission intensity for Ca^{2+}-indo-1-protein interaction bindings. In order to solve the Ca^{2+} concentration that requires emission determination at three different wavelengths, emission intensities at 420, 450 and 470 nm were chosen to determine Ca^{2+} and are shown as:

$$I_{420} = S_{420} [\text{indo-1}] + S'_{420} [Ca^{2+}][\text{indo-1}]/k_d + S''_{420}[\text{protein}][\text{indo-1}]/k_p + S'''_{420}[Ca^{2+}][\text{protein}][\text{indo-1}]/k_p k_{dp} \quad (4)$$

$$I_{450} = S_{450} [\text{indo-1}] + S'_{450} [Ca^{2+}][\text{indo-1}]/k_d + S''_{450}[\text{protein}][\text{indo-1}]/k_p + S'''_{450}[Ca^{2+}][\text{protein}][\text{indo-1}]/k_p k_{dp} \quad (5)$$

$$I_{470} = S_{470} [\text{indo-1}] + S'_{470} [Ca^{2+}][\text{indo-1}]/k_d + S''_{470}[\text{protein}][\text{indo-1}]/k_p + S'''_{470}[Ca^{2+}][\text{protein}][\text{indo-1}]/k_p k_{dp} \quad (6)$$

Equations 4 to 6 were analytically solved using mathematical (Wolfram Research). The equation resulted

Table 1. Respective measurement of emission constant at 420, 450 and 470 nm.

Emission wavelength (nm)	420	450	470
S	1.3×10^2	1.4×10^2	1.5×10^2
S'	0.10	0.23	0.44
S''	0.020	0.25	0.27
S'''	0.020	0.36	0.24
k_d (nM)		240	
k_p (nM)		805	
k_{dp} (nM)		930	

in two outcomes (plus and minus value) for quantitative Ca^{2+} equation as shown in Equation 7. The Ca^{2+} solving equation shows that the Ca^{2+} only depends on the dissociation constant of Ca^{2+}-indo-1 and Ca^{2+}-indo-1-protein interactions binding with the emission intensity constant resolved at each wavelength of interest.

Proving Equation 7 is done by taking all the necessary measurement from the respective emission intensity

$$
\begin{aligned}
Ca^{2+} = \Bigg(& -\frac{1}{2(I_{420}S'_{450}S'''_{470} - I_{420}S'''_{450}S'_{470} + S'''_{420}I_{450}S'_{470} - S'_{420}I_{450}S'''_{470} + S'_{420}S'''_{450}I_{470} - S'''_{420}S'_{450}I_{470})} \\
& \times (S'''_{420}I_{450}S_{470}k_d - S_{420}I_{450}S'''_{470}k_d + I_{420}S_{450}S'''_{470}k_d - I_{420}S'''_{450}S_{470}k_d + S_{420}S'''_{450}I_{470}k_d - S'''_{420}S_{450}I_{470}k_d \\
& + S''_{420}I_{450}S'_{470}k_{dp} - S'_{420}I_{450}S''_{470}k_{dp} + I_{420}S''_{450}S'_{470}k_{dp} - I_{420}S'_{450}S''_{470}k_{dp} + S'_{420}S''_{450}I_{470}k_{dp} - S''_{420}S'_{450}I_{470}k_{dp} \\
& \pm \sqrt{(-4k_dk_{dp}(I_{420}S_{450}S''_{470} - I_{420}S''_{450}S_{470} + S''_{420}I_{450}S_{470} - S_{420}I_{450}S''_{470} + S_{420}S''_{450}I_{470} - S''_{420}S_{450}I_{470})} \\
& \overline{(I_{420}S'_{450}S'''_{470} - I_{420}S'''_{450}S'_{470} + S'''_{420}I_{450}S'_{470} - S'_{420}I_{450}S'''_{470} + S'_{420}S'''_{450}I_{470} - S'''_{420}S'_{450}I_{470})} \\
& + [I_{450}(S'''_{420}S_{470}k_d - S_{420}S'''_{470}k_d + S''_{420}S'_{470}k_{dp} - S'_{420}S''_{470}k_{dp}) \\
& + k_{dp}(I_{420}S'_{450}S''_{470} - I_{420}S''_{450}S'_{470} + S'_{420}S''_{450}I_{470} - S''_{420}S'_{450}I_{470}) \\
& + k_d(I_{420}S_{450}S'''_{470} - I_{420}S'''_{450}S_{470} + S_{420}S'''_{450}I_{470} - S'''_{420}S_{450}I_{470})]^2)\Bigg)
\end{aligned}
\qquad (7)
$$

constant. Emission intensity, I_0 at 0 free Ca^{2+} solution buffer with indo-1 is taken at all respective emission wavelength. The equation is shown as

$$I_{0\lambda} = S_\lambda[\text{indo-1}] \qquad (8)$$

Equation 8 relies on the emission of the indo-1 without any influence or binds to protein or Ca^{2+}. In other words, few of indo-1 will emit autofluorescence which will have influence in determining the absolute Ca^{2+} concentration. Indo-1 is used for the two-photon excitation values of 0.397 mM and thus, respective emission intensity at 420, 450 and 470 nm are shown in Table 1.

S, S', S'' and S''' are the emission intensity constant functions determined by plotting the intensity at different protein concentrations and taking the value of constant emission in Figures 3a and 4a. S' is the emission constant for Ca^{2+}-indo-1 binding, S'' is the emission constant for indo-1-protein while S''' is the emission constant for Ca^{2+}-indo-1-protein binding. Inserting all the values in Table 1 to Equation 7 and the values of emission at 420, 450 and 470 nm for any Ca^{2+}-protein-indo-1 samples, calculation of free Ca^{2+} can be obtained. Examples are shown in Table 2 for two types of free Ca^{2+} solution in 100 mg/ml protein which shows the calculated value consistent with the know free Ca^{2+} solution. Further analysis of different protein concentration at 21.0 µM free samples also resulted in a consistent and similar value to the free Ca^{2+} buffer solution for all protein concentration. Different values of dissociation constants for each binding due to the deviation error were also investigated for the calculated free Ca^{2+}. However, it was found that no discrepancy value was obtained when the deviation value was taken into account. This proved that any small deviation of dissociation constants will not interfere with the values of free Ca^{2+} calculated.

CONCLUSION

In this paper, achievement on determining Ca^{2+} based on calculation methods of three different emission wavelengths, the value of emission constant at selective wavelength and dissociation constant for each binding of Ca^{2+}, indo-1 and protein has been introduced and discussed. BSA is used as a protein sample since the binding site to Ca^{2+} indicator is similar as in human serum. However, further investigation on real human serum is

Table 2. Different types of free Ca^{2+} concentration and protein concentration. The positive calculated equation values are within agreement with the measurement values.

At 100 mg/ml protein concentration					At 21.0 µM free Ca^{2+} sample				
Free Ca^{2+} solution (µM)	0.017		0.150		BSA (mg/ml)	10		70	
Emission detected, I_{420}	0.052		0.06		Emission detected, I_{420}	0.092		0.051	
Emission detected, I_{450}	0.064		0.061		Emission detected, I_{450}	0.25		0.30	
Emission detected, I_{470}	0.070		0.066		Emission detected, I_{470}	0.44		0.37	
Calculated free Ca^{2+} (µM)	0.0174 (Accepted)	-1.38	0.155 (Accepted)	-0.116	Calculated free Ca^{2+} (µM)	22.0 (Accepted)	-0.289	27.8 (Accepted)	-0.672

needed as in real cells sample. This would lead to more accurate results which have near resemblance to real cells. Other type of proteins such as kinase, leads to more understanding of the calcium interaction at the cytoplasm. However, it can be assumed that when biological sample is used to determine the absolute Ca^{2+}, two-photon excitation has an advantage when compared with single-photon excitation since the excitation only occur at the focal volume.

ACKNOWLEDGEMENTS

Special thanks to Professor A. Okazaki of Hamamatsu University School of Medicine and Professor C. Egami of Shizuoka University for their laboratory equipments.

REFERENCES

Blatter LA, Blinks JR (1991). Simultaneous measurement of Ca^{2+} in muscle with Ca electrodes and aequorin. Diffusible cytoplasmic constituent reduces Ca^{2+}-independent luminescence of aequorin. J. Gen. Physiol., 98: 1141-1160.

Gelamo EL, Silva CHTP, Imasato H, Tabak M (2002). Interaction of bovine (BSA) and human (HSA) serum albumins with ionic surfactants: spectroscopy and modeling. Biochim. Biophys. Acta,, 1594: 84-99.

Hove-Madsen L, Bers DM (1992). Indo-1 binding to protein in permeabilized ventricular myocytes alters its spectral and Ca binding properties. Biophys. J., 63(1): 89-97.

Ikenouchi H, Peeters GA, Barry WH (1991). Evidence that binding of Indo-1 to cardiac myocyte protein does not markedly change K_d for Ca^{2+}. Cell Calcium, 12(6): 415-422.

Konishi M, Olson A, Hollingworth S, Baylor SM (1998). Myoplasmic binding of fura-2 investigated by steady-state fluorescence and absorbance measurements. Biophys J., 54(6): 1089-1104.

Kudo K (1996). *Biomanual up series textbook*, *Youdosya Publisher*, Tokyo, Japan, pp. 19-45.

Kurebayashi N, Harkins AB, Baylor SM (1993). Use of fura red as an intracellular calcium indicator in frog skeletal muscle fibers. Biophys J., 64(6): 1934-1960.

Miyakawa A, Makino T, Tamakawa A, Ozaki K (1989). Determination of intracellular free calcium ion distribution with fluorescent calcium reagent Fura-2 using video image processing. Bunseki Kagaku, 38: 643-649.

Sako Y, Sekihata A, Yanagisawa Y, Yamamoto M, Shimada Y, Ozaki K, Kusumi A (1995). Comparison of two-photon excitation laser scanning microscopy with UV-confocal laser scanning microscopy in three-dimensional calcium imaging using the fluorescence indicator Indo-1. J. Micros., 185(1): 9-20.

Szmanciski H, Gryczynski I, Lakowicz JR (1996). Three-photon induced fluorescence of the calcium probe Indo-1. Biophys J., 70**(**1): 547-555.

Williams DA, Fay FS (1997). Intracellular calibration of the fluorescent calcium indicator Fura-2. Cell Calcium, 11(2): 75-83.