# Flow Cytometric Measurement of Cellular Ionized Calcium Concentration

**Flow Cytometry**

**T Cell**

**Calcium**

**Indo-1**

**Lymphocyte**

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**Abstract**

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**COSATI Codes**

<table>
<thead>
<tr>
<th>FIELD</th>
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**Abstract**

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Flow Cytometric Measurement of Cellular Ionized Calcium Concentration
(with 1 color plate)

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Introduction

Considerable interest has been directed at measuring intracellular ionized calcium concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) in living cells. Calcium has an important role in a number of cellular functions, and perhaps most interestingly, can function to transmit information from the cell membrane to regulate diverse cellular functions. Until the early 1980s it was not possible to measure [Ca\textsuperscript{2+}]\textsubscript{i} in small intact cells, and attempts to measure cytosolic calcium were restricted mostly to large invertebrate cells where the use of microelectrodes was possible. A new family of fluorescent dyes was developed by Tsien et al. [80], and with quin2, it was possible for the first time to measure [Ca\textsuperscript{2+}]\textsubscript{i} in virtually any population of cells. In 1985, a second family of dyes was developed with the invention of fura-2 and indo-1 [23], and it became possible to measure [Ca\textsuperscript{2+}]\textsubscript{i} in intact single cells of nearly all types. The purpose of this review is to discuss the measurement of [Ca\textsuperscript{2+}]\textsubscript{i} using flow cytometry to analyze cells loaded with the fluorescent probe indo-1, and to survey the results to date. As can be seen in figure 1, the number of publications concerning indo-1 is increasing at an exponential rate, and therefore, this review must be selective in nature. For alternative techniques for the analysis of intracellular calcium concentration, several excellent reviews are available [10, 14].

1 This work was supported in part by Naval Medical Research and Development Command, Research Task No. M0095.003-1007, and by National Institutes of Health Grant AG01751. The opinions and assertions expressed herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

2 We thank Drs. J.A. Ledbetter and T.M. Chused for helpful comments, and D.S. Smoot and C. Kim for technical assistance.

Eukaryotic cells have an internal calcium ion concentration that is ~10,000-fold lower than the external medium. The level of Ca\textsuperscript{2+} is maintained at ~100 nM by regulation of calcium permeability across the plasma membrane and across intracellular calcium storage sites such as calciosomes.
endoplasmic reticulum and mitochondria [66, 84]. The transfer of calcium across the plasma membrane is regulated by calcium channels which may be voltage gated or receptor operated, the Na\(^+\)/Ca\(^{++}\) exchanger, and the Ca\(^{2+}\)-ATPase pump [63, 66]. Plasma membrane calcium influx can be initiated by membrane depolarization which opens voltage-gated channels or by the binding of ligands to receptor-operated channels. Cellular calcium homeostasis is maintained by the Na\(^+\)/Ca\(^{++}\) exchanger, which is a high capacity, low affinity system and the Ca\(^{2+}\)-ATPase pump, a low capacity, high affinity system; both extrude calcium in order to maintain \([\text{Ca}^{2+}]\) within a narrow range.

The mechanism by which the calcium concentration increases in cells involves a series of complex biochemical reactions of which the precise details remain speculative, and is not simply the result of the opening of channels that permit calcium to be transported down its electrochemical gradient. The binding of agonist to its specific membrane receptor causes the activation of phospholipase C which requires the intervening activation of a guanine nucleotide binding (G) protein [21]. Phospholipase C causes the hydrolysis of a membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P\(_2\)) which yields a water-soluble product, inositol 1,4,5-trisphosphate (IP\(_3\)) and a lipid, 1,2-diacylglycerol (DAG) [8]. IP\(_3\) then causes the release of calcium from intracellular stores while DAG in conjunction with calcium ions activates protein kinase C. Thus, a single agonist can result in the production of at least two 'second messengers' making the polyphosphatidylinositide pathway a unique, bifurcating system. Calcium is therefore a 'third messenger' that controls numerous cellular processes (table I).

While it appears clear that the initial elevation of ionized calcium is due to the release of intracellular calcium stores, little is known about regulation of the influx of calcium from extracellular sources that is necessary to sustain the response. Recent evidence indicates two additional products of IP\(_3\), inositol 1,2-cyclic 4,5-trisphosphate (cIns(1,2)P\(_3\)) and inositol 1,3,4,5-tetrakisphosphate (IP\(_4\)), also have second messenger function in that they are active in mobilizing cellular stores of calcium. The production of IP\(_4\) may be regulated in part by the cytosolic calcium concentration [91].

**Flow Cytometric Assay with indo-1**

**Loading of Cells with indo-1**

Uptake and retention of indo-1 ([1-[2-amino-5-[carboxylindol-2-yl]-phenoxy]-2'-amino-5'-methylphenoxy]ethaneN,N,N'N'-tetraacetic acid) is facilitated by the use of the acetoxymethyl ester of indo-1; this cell permeant form diffuses freely into the cyto-
Table I. Growth factors and agents that elevate cellular calcium in mammalian cells

<table>
<thead>
<tr>
<th>Factor</th>
<th>Receptor</th>
<th>Tissue/cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>M1 muscarinic receptor</td>
<td>nerve tissue, SMC</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td></td>
<td>adrenal cortex, SMC, hepatocytes</td>
</tr>
<tr>
<td>Antigen</td>
<td>IgM, IgD; TCR</td>
<td>B and T lymphocytes</td>
</tr>
<tr>
<td>Antigen</td>
<td>IgE</td>
<td>mast cells</td>
</tr>
<tr>
<td>ATP, ADP</td>
<td>P2 purinergic receptor</td>
<td>lymphocytes, platelets, epithelial cells</td>
</tr>
<tr>
<td>BCGF</td>
<td></td>
<td>B lymphocytes</td>
</tr>
<tr>
<td>Bradykinin</td>
<td></td>
<td>fibroblasts, carcinoma cells</td>
</tr>
<tr>
<td>Cholecystokinin</td>
<td></td>
<td>pancreas, SMC</td>
</tr>
<tr>
<td>EGF</td>
<td>c-erb-B</td>
<td>fibroblasts, hepatocytes, carcinoma cells</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>alpha-1-adrenergic receptor</td>
<td>SMC, hepatocytes</td>
</tr>
<tr>
<td>f-MLP</td>
<td></td>
<td>neutrophils</td>
</tr>
<tr>
<td>GnRH</td>
<td></td>
<td>pituitary</td>
</tr>
<tr>
<td>GRP, bombesin</td>
<td></td>
<td>lung carcinoma cells</td>
</tr>
<tr>
<td>Glucagon</td>
<td></td>
<td>liver</td>
</tr>
<tr>
<td>Glutamate</td>
<td></td>
<td>nerve tissue</td>
</tr>
<tr>
<td>Histamine</td>
<td>H1 histaminergic receptor</td>
<td>SMC, nerve tissue, endothelial cells</td>
</tr>
<tr>
<td>5-Hydroxytryptamine</td>
<td>S1c and S2 serotonin receptors</td>
<td>SMC, nerve tissue</td>
</tr>
<tr>
<td>Oxytocin</td>
<td></td>
<td>uterus, mammary epithelium</td>
</tr>
<tr>
<td>PAF</td>
<td></td>
<td>platelets, monocytes</td>
</tr>
<tr>
<td>PDGF</td>
<td>PDGFR</td>
<td>fibroblasts, SMC</td>
</tr>
<tr>
<td>Spermin</td>
<td></td>
<td>ovum</td>
</tr>
<tr>
<td>Substance P</td>
<td></td>
<td>nerve tissue, epithelial cells</td>
</tr>
<tr>
<td>Thrombin, collagen</td>
<td></td>
<td>platelets</td>
</tr>
<tr>
<td>TRH</td>
<td></td>
<td>pituitary</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>V1 receptor</td>
<td>SMC, fibroblasts, hepatocytes</td>
</tr>
</tbody>
</table>

BCGF = Low molecular weight B cell growth factor; EGF = epidermal growth factor; f-MLP = formylmethionyl-leucyl-phenylalanine; GnRH = gonadotropin-releasing hormone; GRP = gastrin-releasing peptide; PAF = platelet-activating factor; PDGF = platelet-derived growth factor; SMC = smooth muscle cells; TCR = T cell receptor; TRH = thyrotropin-releasing hormone.

Plasm where it serves as a substrate for esterases which hydrolyze the dye to the impermeant form [79]. After loading, cells must be washed to reduce extracellular indo-I concentrations, preferably to 10 nM or lower [64]. The efficiency of cellular dye uptake and trapping is ~20% in cells loaded under these conditions.

The lower limit of useful intracellular loading concentrations of indo-I is determined by the sensitivity of fluorescence detection of the flow cytometer and the upper limit is determined by avoidance of buffering of [Ca^{2+}] by the presence of the calcium chelating dye itself. Fortunately, indo-I has excellent fluorescence characteristics (30-fold greater quantum yield than quin2 at a given dye concentration [23]) and useful ranges of indo-I loading have been described by several authors. For human pe-
Fig. 2. Heterogeneity of calcium mobilization among the CD8 subset of T cells is related to expression of the CD16 antigen. A Peripheral blood lymphocytes were loaded with indo-1 and stained with PE-conjugated CD8 antibody G10-1 and FITC-conjugated CD16 antibody FC-2. B The cells were analyzed at 500 cells/s, and stimulated with 10 μg/ml CD3 antibody G19-4 at time = 2 min. The percent of responding cells that developed increased [Ca²⁺], as determined by changes in indo-1 fluorescence (not shown) is plotted versus time by electronically gating on the CD8<sup>dim</sup>/CD16<sup>+</sup>, CD8<sup>dim</sup>/CD16<sup>-</sup>, and CD8<sup>dim</sup>/CD16<sup>-</sup> subpopulations. The response of the CD8<sup>-</sup> cells is not shown.

Peripheral blood T cells, we have reported adequate detection at or above 1 μM loading concentrations (cells incubated for 40 min medium at 37 °C), conditions that achieve ~5 μM intracellular indo-1. Buffering of [Ca²⁺], was observed as a slight delay in the onset of the rise in [Ca²⁺], and a reduction in peak [Ca²⁺], seen with 20 μM loading (160 μM intracellular concentration), and a retarded rate of return of [Ca²⁺], to baseline values was seen at loading concentrations above 3 μM (22 μM intracellular concentration) [64]. In examination of murine B cells, Chu et al. [13] reported reduced peak and delayed re-equilibration of [Ca²⁺] at loading concentrations above 1 μM. We have confirmed that antigen receptor induced calcium transients appear to be more susceptible in B cells than in T cells to increased concentrations of indo-1, suggesting that the mechanism of calcium mobilization differs in the cells (fig. 2). For human platelets, a 2 μM loading concentration (15 min at 37 °C) has been reported [16].

Rates of loading of the indo-1 ester can be expected to vary between cell types, for example, as a consequence of variations in intracellular esterase activity. In human peripheral blood, visual inspection suffices to reveal more rapid rates of loading of platelets and monocytes than in lymphocytes. The T-cell line Jurkat loads more rapidly than peripheral blood T cells [64]. Even within one cell type, donor or treatment-specific factors may affect loading; for example, Miller et al. [55] observed reduced rates of
indo-1 loading in splenocytes from aged versus young mice.

The analysis of \([\text{Ca}^{2+}]\), using indo-1 is predicated upon distribution of the dye uniformly within the cytoplasm. In several cell types, the related dye fura-2 has been reported to be compartmentalized within organelles [18, 51]. In at least one case, that of bovine aortic endothelial cells, fura-2 is localized to mitochondria; however, indo-1 remains diffusely cytoplasmonic [76]. In lymphocytes, there appears to be no microscopic evidence of subcellular localization of indo-1, and concurrence of calibration experiments with predicted results (see below) makes such localization appear not to be a practical complication in analysis of this cell type. In each application of the flow cytometric technique with indo-1 it seems advisable to examine the cellular distribution of indo-1 microscopically, and to confirm the expected behavior of the dye in the presence of high cytoplasmic \([\text{Ca}^{2+}]\) levels as part of the calibration procedure described subsequently.

As an additional caveat, it has been suggested that both fura-2 and indo-1 may be incompletely deesterified within some cell types [48, 71]. Since the ester has little fluorescence spectral dependence upon \([\text{Ca}^{2+}]\), the presence of this dye form could lead to false estimates of \([\text{Ca}^{2+}]\). Again, results of calibration experiments are helpful to exclude this possibility.

Indo-1 has been found to be remarkably nontoxic to cells subsequent to loading. Analysis of the proliferative capacity of either human T lymphocytes [64] or murine B lymphocytes [13] has shown unaltered behavior of cells after loading with indo-1. This is especially pertinent to applications of sorting of cells based on \([\text{Ca}^{2+}]\), as described subsequently.

Finally, one incidental benefit of loading of indo-1 by use of its acetoxymethyl ester is that this procedure, like the more familiar use of fluorescein diacetate or carboxyfluorescein diacetate, discriminates between live and dead cells. The latter will not retain the hydrophilic impermeant dye and will be excluded during subsequent analysis.

**Instrumental Technique**

The absorption maximum of indo-1 is between 330 and 350 nm, depending upon the presence of calcium [23]; this is well suited to excitation at either 351–356 nm from an argon ion laser, or to 337–356 nm excitation from a krypton ion laser. Laser power requirements depend upon the choice of emission filters and optical efficiency of the instrument; however, it is our observation that although 100 mW is often routinely employed, virtually identical results can be obtained with 10 mW. This should allow, in principal, the use of less expensive helium-cadmium lasers. Stability of the intensity of the excitation source is less important in this application than most others, because of the use of the ratio of fluorescence emissions. Analysis with indo-1 has also been performed using excitation by a mercury arc lamp (FACS Analyzer, Becton Dickinson).

An increase in \([\text{Ca}^{2+}]\) is detected with indo-1 as a ratio of two emission wavelengths. The choice of filters used to select these wavelengths is dictated by the spectral characteristics of the shift in indo-1 emission upon binding to calcium [23]. Bandpass filters (multicavity, 20 nM bandwidth) can be chosen to be centered on the ‘violet’ peak emission of the calcium-bound indo-1 dye (400 nm) and free indo-1 dye ‘blue’ emission (485 nm). However, wavelengths nearer the isobestic point do not exhibit as large a
dependence upon calcium binding. This effect is summarized in table II by values of $R_{\text{max}}/R$, which indicates the range of change in indo-1 ratio observed from resting intracellular calcium to saturated calcium, the largest range of change ordinarily encountered in measurements of intact cells. As seen in this table, a larger dynamic range in the ratio of wavelengths is obtained if 'blue' emission below 485 nm is not collected and the center of the 'blue' emission bandpass filter is moved upward. Similarly, the 'violet' bandpass filter should be chosen to minimize the collection of wavelengths above 405 nm.

An increase in $[\text{Ca}^{2+}]$ will be evidenced by an increase in 'violet'/'blue' ratio. If displayed on the flow cytometer as a bivariate plot of 'violet' versus 'blue' signals, this change will be observed as a rotation around the axis through the origin. This method of ratio analysis is cumbersome, and fortunately commercial flow cytometers all have some provision for a direct calculation and display of the value of the fluorescence ratio itself. For some instruments, this is performed by analog circuitry (e.g., Coulter, Becton Dickinson) and on others by digital computation (e.g., Ortho 2150 computer, Becton Dickinson VAX computer). In principle, it should be of little concern how the ratio is calculated; in practice some analog ratio circuits are limited in their range of acceptable inputs; for example, that the 'violet' signal never be greater than the 'blue', yielding a ratio of greater than 1. This has prompted some users to reverse the ratio ('blue'/'violet') so that a rise in calcium results in a decline in ratio; this results in an inverted, counterintuitive display of calcium concentration. It is instead recommended that the 'violet'/'blue' ratio be used, but that the signal gains be initially set such that subsequent rises in the ratio will not exceed the permitted value. By either analog or digital calculation, it is important that no artifactual offset be introduced in the ratio, this can be quickly tested by altering the excitation

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### Table II. Effect of wavelength choice on calcium-sensitive indo-1 ratio shifts

<table>
<thead>
<tr>
<th>Wavelength pair, nM</th>
<th>$R_{\text{max}}$</th>
<th>$R_{\text{min}}$</th>
<th>$R$</th>
<th>$S_{\text{p}}/S_{\text{v}}$</th>
<th>$R_{\text{max}}/R_{\text{min}}$</th>
<th>$R_{\text{max}}/R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>475/395</td>
<td>2.33</td>
<td>0.040</td>
<td>0.352</td>
<td>3.3</td>
<td>58.2</td>
<td>6.62</td>
</tr>
<tr>
<td>475/405</td>
<td>2.38</td>
<td>0.100</td>
<td>0.410</td>
<td>3.3</td>
<td>23.8</td>
<td>5.80</td>
</tr>
<tr>
<td>495/395</td>
<td>3.51</td>
<td>0.048</td>
<td>0.429</td>
<td>4.2</td>
<td>73.1</td>
<td>8.18</td>
</tr>
<tr>
<td>495/405</td>
<td>3.59</td>
<td>0.119</td>
<td>0.501</td>
<td>4.2</td>
<td>30.2</td>
<td>7.17</td>
</tr>
<tr>
<td>515/395</td>
<td>5.75</td>
<td>0.070</td>
<td>0.644</td>
<td>4.63</td>
<td>82.1</td>
<td>8.93</td>
</tr>
<tr>
<td>515/405</td>
<td>5.88</td>
<td>0.176</td>
<td>0.752</td>
<td>4.63</td>
<td>33.4</td>
<td>7.82</td>
</tr>
<tr>
<td>530/395</td>
<td>9.68</td>
<td>0.117</td>
<td>1.073</td>
<td>4.68</td>
<td>82.7</td>
<td>9.02</td>
</tr>
<tr>
<td>530/405</td>
<td>9.89</td>
<td>0.292</td>
<td>1.252</td>
<td>4.68</td>
<td>33.9</td>
<td>7.90</td>
</tr>
</tbody>
</table>

$^1$ By spectrofluorometry, 2-nm slit width; uncorrected fluorescence.
power over a broad range of values in an analysis of a nonperturbed indo-1-loaded cell population—a correctly calculated ratio will not show any dependence upon excitation intensity. It can similarly be shown that loading of cells with a broad range of indo-1 concentrations results in a constant value of the 'violet'/blue' ratio [64].

The above approach results in a linear display of indo-1 blue and violet fluorescence. If cellular indo-1 loading is extremely heterogeneous, it may be desirable to work with a logarithmic conversion of 'violet' and 'blue' emission intensities in order to observe a broader range of cellular fluorescence. In this case, the hardware must permit the logarithm of the ratio to be calculated by subtraction of the log 'blue' from the log 'violet' signals [64].

Plotted as a histogram of the ratio values, unperturbed cell populations show narrow distributions of ratio. When cellular loading with indo-1 is very heterogeneous, and coefficients of variation of less than 10% are not uncommon [64, and shown below]. The effects of perturbation of \([\text{Ca}^{2+}]\), by agonists, can be noted by changes in the ratio histogram profile during sequential analysis and histogram storage. A more informative and elegant display is obtained by a bivariate plot of ratio versus time. The bivariate histogram can be stored, and subsequently subjected to further analysis, or presentation as 'isometric plots' in which the x-axis represents time, the y-axis \([\text{Ca}^{2+}]\), and the z-axis, number of cells (fig. 3). Alternatively, the data can be presented as color plots with time on the x-axis, \([\text{Ca}^{2+}]\), on the y-axis, and number of cells displayed by pseudocolor contouring (fig. 4). In the bivariate plot, kinetic changes in \([\text{Ca}^{2+}]\), are seen with much greater resolution, limited only by the number of channels on the time axis, and the interval of time between each channel. Changes in the fraction of cells responding, in the mean magnitude of response, and in the heterogeneity of the responding population, are best observed with these displays. Calculation of the mean y-axis value for each x-axis time interval allows presentation of the data as mean ratio versus time [64]. Conversion of the ratio to \([\text{Ca}^{2+}]\), (fig. 2) allows data presentation in the same manner as traditionally displayed by spectrofluorometric analysis, i.e., \([\text{Ca}^{2+}]\), versus time. While this presentation yields much of the information of interest in an easily displayed format, data relating to heterogeneity of the \([\text{Ca}^{2+}]\), response is lost. Some of this information can be displayed by a calculation of the 'proportion of responding cells'—if a threshold value of the resting ratio distribution is chosen, e.g., one at which only 5% of control cells are above, the proportion of cells responding by ratio elevations above this threshold versus time yields a presentation informative of the heterogeneity of the response [64]. Presentation of both the mean \([\text{Ca}^{2+}]\), versus time and the percent responding cells versus time allows easy visual comparison of results of different stimuli or treatment.

**Calibration of Ratio to \([\text{Ca}^{2+}]\).**

Prior to the development of indo-1 and fura-2, \([\text{Ca}^{2+}]\), determination by measurement of quin2 fluorescence was sensitive to changes in intracellular dye concentration as well as \([\text{Ca}^{2+}]\), and required calibration at the end of each individual assay by determination of the fluorescence intensity of the dye at zero and saturating \([\text{Ca}^{2+}]\). In contrast, with indo-1 use of the \([\text{Ca}^{2+}]\)-dependent shift in dye emission wavelength allows
Fig. 3. Effects of thrombin and fibronectin on [Ca\(^{2+}\)]. Human platelets were isolated by gel filtration and loaded with indo-1. Isoelectric plots of the time course of the indo-1 violet to blue fluorescence ratio are plotted where the x-axis represents time, the y-axis indo-1 fluorescence ratio, and z-axis represents cell number. The platelets were equilibrated at 37 °C and analyzed ~ 500/s. At 2 min, thrombin 1 U/ml (A) or fibronectin 1 µg/ml (B) was added. An indo-1 fluorescence ratio of 1.0 corresponds to an [Ca\(^{2+}\)], of ~ 130 nM, a ratio of 2.0 = 338 nM, a ratio of 3.0 = 680 nM and a ratio of 4.0 = 1,350 nM.

the ratio of fluorescence intensities of the dye at the two wavelengths [R] to be used to calculate [Ca\(^{2+}\)]:

\[
[Ca^{2+}] = \frac{K_d (R - R_{min}) S_D}{(R_{max} - R) S_B}
\]

where K_d is the effective dissociation constant (250 nM), R, R_{min}, and R_{max} are the fluorescence intensity ratios of violet/blue fluorescence at resting, zero, and saturating [Ca\(^{2+}\)], respectively, and S_D/S_B is the ratio of the blue fluorescence intensity of the calcium-free and bound dye, respectively [23]. Because this calibration is independent of total intracellular dye concentration and instrumental variation in efficiency of excitation or emission detection, it is not necessary to measure the fluorescence of the dye in the calcium-free and saturated states for each.
Fig. 4. Measurement of $[\text{Ca}^{2+}]$, in HIV-infected (B, D) and uninfected (A, C) T cells. CD4$^+$ T cells were purified, activated, and infected with HIV. On day 19 after infection, HIV-infected cells or uninfected cells from companion cultures were loaded with indo-1, stained with PE CD8 antibody, and stimulated with optimal amounts of CD3 monoclonal antibody (A, B) or CD2 monoclonal antibody (C, D). The indo-1 ratio of violet and blue fluorescence (proportional to $[\text{Ca}^{2+}]$) was digitally calculated in real time for each individual non-PE-fluorescent (i.e. CD4) cell and is shown on the y-axis versus time on the x-axis. The results are displayed on a $100 \times 100$ pixel grid on which the number of cells per pixel is represented by a 16-color progression following an exponential function where 0-1 cells are assigned a color of black, 2-10 cells shades of blue, 11-80 cells shades of green, and 81-600 cells shades of yellow to red.
individual assay. In principle, it is sufficient to calibrate the instrument once after set-up and tuning by measurement of the constants \( R_{\text{max}} \), \( R_{\text{min}} \), \( S_{2/2} \) and \( S_{bb} \), after which only \( R \) is measured for each subsequent analysis on that occasion.

One strategy to obtain the \( R_{\text{max}} \) and \( R_{\text{min}} \) values for indo-1 is to lyse cells in order to release the dye to determine fluorescence at zero and saturating \([\text{Ca}^{2+}]_{i}\), as carried out with quin2. However, this is not possible with flow cytometry, due to the loss of cellular fluorescence. Another strategy is the use of an ionophore to saturate or deplete \([\text{Ca}^{2+}]_{i}\), in order to allow approximation of the true endpoints without cell lysis. For this approach, the ionophore ionomycin is best suited, due to its specificity and low fluorescence. When flow cytometric quantitation of fluorescence from intact cells treated with ionomycin or ionomycin plus EGTA was compared with spectrofluorometric analysis of lysed cells in medium with or without EGTA, the indo-1 ratio of unstimulated cells (\( R \)) and the ratio at saturating \([\text{Ca}^{2+}]_{i}\), \( R_{\text{max}} \), were similar by both techniques [64]. The latter indicates that ionomycin-treated cells reach near-saturating levels of \([\text{Ca}^{2+}]_{i}\). The value of \( R_{\text{min}} \), which can be obtained by treatment of cells with ionomycin in the presence of EGTA, however, is substantially higher than either that predicted from the spectral emission curves [23] or that obtained by cell lysis and spectrofluorometry. Spectrofluorometric quantitation with either quin2 or indo-1 indicates that \([\text{Ca}^{2+}]_{i}\) remains at approximately 50 nM in intact cells treated with ionomycin and EGTA. Thus, due to the inability to obtain a valid flow cytometric determination with calcium-free dye, we have used for calibration the values of \( R_{\text{min}} \) and \( S_{2/2} \) or \( S_{bb} \) derived from spectrofluorometry, either of the indo-1 pentapotassium salt or after lysis of indo-1-loaded cells in the presence of EGTA. It is essential that the same optical filters be used for flow cytometry and spectrofluorometry, since the standardization is very sensitive to the wavelengths chosen. To illustrate typical values of \( R_{\text{max}} \), \( R_{\text{min}} \), \( R \) and \( S_{2/2} \), examples of these values are shown for different emission wavelength combinations in table II.

As an alternative to the use of a spectrofluorometer, a procedure which may be cumbersome or the instrument unavailable, Parks et al. [57] have proposed that the flow cytometer may be used as a spectrofluorometer with minor modification. In essence, the fluorescence of a steady stream of dye is measured by the photomultiplier and the photomultiplier voltage is analyzed as in a standard spectrofluorometer, or converted to a pulse for processing by the flow cytometer. With this technique it should be possible to determine all constants of indo-1 on the flow cytometer necessary for direct calibration.

Chused et al. [13] have suggested that a cocktail of ionomycin, nigericin, high concentrations of potassium, 2-deoxyglucose, azide and carbonyl cyanide m-chlorophenylhydrazone can be used to collapse the calcium gradient to zero, and therefore, that \([\text{Ca}^{2+}]_{i} = [\text{Ca}^{2+}]_{o}\). However, as mentioned above, it is not possible to obtain \( R_{\text{min}} \) using this technique, and therefore, calibration must be based upon a regression formula that relates \( R \) to ionomycin-treated cells suspended in a series of precisely prepared calcium buffers. Thus, this technique allows one to estimate \([\text{Ca}^{2+}]_{i}\) without the need to determine \( R_{\text{min}} \), \( S_{2/2} \) or \( S_{bb} \) although it is subject to the limitations that calcium concentrations that are less than those found in rest-
ing cells cannot be quantitated [13]. Accuracy of pr__lication of Ca^{2+} in buffer solutions is dependent upon a variety of interacting factors [10], so that care must be exercised in this aspect of this approach.

If, for a particular cell type loaded with indo-1, the values of R and R_{\text{max}} obtained by flow cytometry are in good agreement with the values obtained by spectrofluorometry, then it would be unlikely that the dye is in a compartment inaccessible to cytoplasmicCa^{2+}, in a form unresponsive to [Ca^{2+}]], (e.g., still esterified) or in a cytoplasmic environment in which the spectral properties of the dye were altered. With regard to the second condition, it has been proposed that since indo-1 fluorescence, but not that of the indo-1 ester, is quenched in the presence of mM concentrations of Mn^{2+}, that Mn^{2+} in the presence of ionomycin can be used as a further test of complete hydrolysis of the indo-1 ester within cells [48].

Even if careful calibration of the fluorescence ratio to [Ca^{2+}], is not being performed for a particular experiment, ordinary quality control should include a determination of the value of the indo-1 emission ratio in the presence and absence of ionomycin, i.e. R_{\text{max}}/R. Examples of the values of this parameter are shown in table II. unperturbed cells will usually be found to have a reproducible value of [Ca^{2+}], and day-to-day optical variations in the flow cytometer are usually minimal (with the same filter set), thus a limited range of R_{\text{max}}/R values should be obtained over a series of experiments.

**Simultaneous Analysis of [Ca^{2+}], and Other Fluorescence Parameters**

Although the broad spectrum of indo-1 fluorescence emission will likely preclude the simultaneous use of a second UV-excited dye, the use of two or more excitation sources allows additional information to be derived from visible light-excited dyes. Perhaps the most common application will be determination of cellular immunophenotype simultaneously with the indo-1 assay. This allows alterations in [Ca^{2+}], to be examined in, and correlated with, specific cell subsets.

Use of the most common fluorescent antibody conjugate, FITC, requires excitation at 488 nm. If indo-1 'blue' fluorescence is detected in a range which includes this wavelength, then scattered light from the 488-nm laser may interfere with accurate indo-1 quantitation, the extent of this problem varying with instrumental design. The problem can be avoided by using a higher wavelength for indo-1 'blue' detection, moving into the blue-green or even green, as shown in table II. Excellent results can, in fact, be obtained by using the same interference filter for FITC and indo-1 detection making use of the temporal and/or spatial separation of the respective emissions [64]. If phycoerythrin (PE)-conjugated antibody probes are used, then the user may have the choice of excitation at 488 nm, 514 nm, or even 531 nm (with a Krypton ion laser).

Combining the use of FITC, PE and indo-1 analysis allows determination of [Ca^{2+}], in complex immunophenotypic subsets. On instruments without provision for analysis of four separate fluorescence wavelengths, detection of both FITC and the higher indo-1 wavelength with the same filter element may allow successful implementation of these experiments. Gating the analysis of indo-1 fluorescence upon windows of FITC versus PE fluorescence allows information relating to each identifiable cellular subset to be derived from a single sample.
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There is an important caution in the use of simultaneous [Ca$^{2+}$], and immunofluorescence analysis relating to the possibility that use of the antibody probe can itself alter the results obtained in the indo-1 assay. It is becoming increasingly clear that binding of mAb to cell surface proteins can alter [Ca$^{2+}$], even when these proteins are not previously recognized as part of a signal transducing pathway [3, 29, 31, 40, 58, 59, 64]. For example, antibody binding to CD4 will reduce CD3-mediated [Ca$^{2+}$]$_i$ signals; if the anti-CD4 mAb is cross-linked, as with a goat-anti-mouse immunoglobulin, the CD3 signals are augmented [40, 42]. Antibody binding to CD8 has similar effects [unpubl data].

As a consequence of these concerns, we have used a reciprocal staining strategy whenever possible, so that the cellular subpopulation of interest is unlabelled while undesired cell subsets are identified and excluded from analysis by mAb staining. The CD4$^+$ subset in PBL may be identified, for example, by staining with a combination of CD8, CD20, and CD11 mAbs [64], and the CD5$^+$ subset can be identified by staining with CD16, CD20 and anti-HLA-DR [31]. Finally, it is important when staining cells with mAb for functional studies that the antibodies be azide-free, in order that metabolic processes be uninhibited. Commercial antibody preparations may thus require dialysis before use.

Using other probes excited by visible light, it may be possible to analyze additional physiologic responses in cells simultaneously with [Ca$^{2+}$]. Lazzari et al. [38] have used the membrane potential sensitive dye di-O-C$_3$(3) (excitation 488 nm, emission 562–588 nm) to monitor changes in membrane potential in neutrophils stimulated with the oligopeptide formyl-methionyl-leucyl-phenylalanine (FMLP), showing that the membrane potential changes occurred just as rapidly as changes in [Ca$^{2+}$], measured simultaneously with indo-1. By similar studies, it may be possible to analyze intracellular pH simultaneously with [Ca$^{2+}$].

The choice of medium in which the cell sample is suspended for analysis can be dictated primarily by the metabolic requirements of the cells, subject only to the presence of mM concentrations of calcium (to enable calcium agonist-stimulated calcium influx) and reasonable buffering. The use of phenol red as a pH indicator does not impair the detection of indo-1 fluorescence signals. Although the new generation of Ca$^{2+}$ indicator dyes are not highly sensitive to small fluctuations of pH over the physiologic range [23], unbuffered or bicarbonate-buffered solutions can impart uncontrolled pH shifts. If analysis of release of Ca$^{2+}$ from intracellular stores, independent of extracellular Ca$^{2+}$ influx is desired, addition of 5 mM EGTA to the cell suspension (final concentration) will reduce Ca$^{2+}$ from several mM to ~20 nM, thus abolishing the usual extracellular to intracellular gradient.

Regulation of the temperature of the cell sample is essential, as transmembrane signaling and calcium mobilization are temperature-dependent and active processes. Most applications will require analyses at 37 °C. If cells are allowed to cool before they flow past the laser beam, [Ca$^{2+}$], response will be affected, so that either the sample input tubing should be warmed, or narrow-gauge tubing and high flow rates (e.g., >50 µl sample/min) should be used to keep transit times from warmed sample to flow cell minimized.
Sorting on the Bases of \( [Ca^{2+}] \), Responses

The ability of the flow cytometric analysis with indo-I to observe small proportions of cells with different \( [Ca^{2+}] \), responses than the majority of cells suggests that the flow cytometer may be useful to identify and sort variants in the population for their subsequent biochemical analysis or growth. Results of artificial mixing experiments with Jurkat and K562 T- and myeloid leukemia cell lines indicated that subpopulations of cells with variant \( [Ca^{2+}] \), comprising <1% of total cells could be accurately identified \[64\]. Goldsmith and Weiss \[22\] have recently reported the use of sorting on the basis of indo-I fluorescence to identify mutant Jurkat cells which fail to mobilize \( [Ca^{2+}] \); in response to CD3 stimulus, in spite of the expression of structurally normal CD3/Ti complexes. The variant was cloned after four rounds of sorting cells with low \( [Ca^{2+}] \); after CD3 stimulation. These experiments suggest that sorting on the basis of indo-I fluorescence can be an important tool for the selection and identification of genetic variants in the biochemical pathways leading to \( Ca^{2+} \) mobilization and cell growth and differentiation.

Intracellular Applications

Calcium Homeostasis

An early study using indo-I and flow cytometry examined the distribution of \( [Ca^{2+}] \) in resting peripheral blood human lymphocytes \[64\]. A small coefficient of variation (often <10%) was found for the basal calcium level; this value appears to reflect physiologic variation because the instrumental variation was only 4.5%. It is not yet known if the basal calcium level remains constant or if the level oscillates about the mean of the resting level. In stimulated cells, there was heterogeneity in the responding cells. For example, after stimulation of peripheral blood lymphocytes with the mitogenic lectin PHA, only some cells responded. Some of this heterogeneity correlated with cell surface antigen expression, as CD4-positive T cells had the highest proportion of cells responding, with less response in the CD8 cells and least in large granular lymphocytes. Mechanisms of calcium homeostasis in T lymphocytes differ in several respects from B cells. Murine T cells have a \( Ca^{2+} \)-sensitive K+ channel that is not detectable in B cells \[26\], and thus, T cells develop membrane potential hyperpolarization after calcium ionophore treatment \[81\]. In contrast, large granular lymphocytes and B cells depolarize \[13, 28\]. There is evidence that the activity of the calcium extrusion pump increases with membrane depolarization and decreases with hyperpolarization, perhaps explaining the observation that depolarization decreases the \( Ca^{2+} \) response of calcium-ionophore-treated T cells \[26\]. Recent evidence indicates that human tonsillar B cells also have \( Ca^{2+} \)-sensitive K+ channels \[49\], thus raising the possibility that B cells have a functional channel only at certain stages of differentiation or that the disparate results are attributable to species differences.

Lymphocytes

B lymphocytes were the first cells to be studied with quin2. Pozzan et al. \[61\] showed that the binding of anti-immunoglobulin antibody to the antigen receptor of mouse and pig lymphocytes caused an elevation of \( [Ca^{2+}] \). A major limitation of quin2 in that study was the high concentrations of the indicator (\( \sim 1 \) mM) that were necessary
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...to load into the cells. Subsequent studies using indo-1 or fura-2 have shown that quin2 markedly affects both the magnitude and kinetics of calcium transients in B cells stimulated with antigen receptor cross-linking. In more recent studies [11, 13, 90], B cells loaded with indo-1 were shown to have transient calcium levels of approximately 1 μM and essentially 100% of mature murine splenic B cells responded to antigen receptor cross-linking; thus, the early studies that used quin2 [61, 65] underestimated the true change by 3-fold. In B cells loaded with small amounts of indo-1 the mean calcium peaks at ~10 s after stimulation and then rapidly declines to levels only slightly above resting whereas studies that used quin2 showed a delay in the onset of the reaction and a blunting of the response. Finally, a study using fura-2 with digital microscopy has shown that under certain circumstances, anti-immunoglobulin stimulation of B cells produces repetitive, transient oscillations of [Ca^{2+}]_{i}, rather than sustained elevations of [Ca^{2+}]_{i} [89].

One function of mature T cells is to kill foreign or virus-infected cells. Mentzer et al. [53] have used indo-1 and flow cytometry to show that calcium mobilization occurs in the effector T cells during the lytic stage of killing but not during the adhesion stage. Using fura-2 and digital imaging microscopy, PoeNie et al. [60] have surprising results which indicate that the calcium mobilization appears to initiate in the effector cell at a point in the cell that is located on the opposite pole of the cell with respect to the target cell.

The calcium mobilization in B cells is initiated by polyphosphoinositide degradation and is predominantly derived from intracellular stores; depletion of extracellular calcium from the medium has little effect on the peak response after antigen receptor ligation. The pattern of response varies depending on the type of receptor that is stimulated; in small resting murine B cells, cross-linking the IgD (delta) receptor causes a brief response onset at ~5 s that reaches high magnitude while stimulation of IgM (mu) receptors causes a reaction of later onset, lower magnitude and is more prolonged [13]. The molecular basis for this difference in calcium signalling by the two classes of B cell antigen receptors remains unexplained.

The cross-linking of surface immunoglobulin with Fc receptors for Ig on B cells inhibits phospholipid degradation and calcium mobilization when compared to that observed after ligation of the B cell receptor (by F(ab)'2 anti-immunoglobulin antibodies) that is independent on the Fc receptor [90]. Similarly, the binding of monoclonal antibodies to the CD19 ('B4') antigen inhibits proliferation induced by anti-immunoglobulin antibodies; the increase in [Ca^{2+}]_{i} that is induced by cross-linking the B cell antigen receptor is also inhibited by CD19 antibodies [58]. Phorbol esters, presumably through the activation of protein kinase C, can also inhibit anti-immunoglobulin-induced calcium signalling [68]. Current models propose that the antigen receptor is coupled to phospholipase C through G proteins, and that regulation ('desensitization') of this coupling accounts for the above differences in calcium signalling, and presumably, may serve to explain antigen-induced B cell proliferation and induction of B cell tolerance. Current evidence strongly indicates that calcium mobilization may be necessary for antigen-induced B cell activation. There is evidence, however, that very low concentrations of antigen may activate B cells to proliferate in the absence of detectable calcium mobilization [11], and that not all forms of...
lymphocyte activation involve measurable changes in $\text{Ca}^{2+}$ [39]. Stimulation of cells with IL-4 (BSF-1) and lipopolysaccharide (LPS) appears to be independent of the calcium signalling system [32], although the low molecular weight form of B cell growth factor (and not IL-1, IL-2, G-CSF or GM-CSF) is able to cause or potentiate antigen-receptor-induced calcium mobilization in human tonsillar B cells and in some leukemic cells [45].

The T cell antigen receptor consists of a heterodimeric complex composed of either alpha/beta chains or gamma/delta chains [reviewed in 27]. The receptor is noncovalently coupled to the CD3 complex, a nonpolymorphic structure that consists of at least 5 polypeptides. Antigen recognition occurs through the T cell receptor and it is thought that signal transduction occurs through the CD3 complex [reviewed in 87]. Initial studies indicate that the binding of monoclonal antibody to the gamma/delta T cell receptor is able to initiate signal transduction that is indistinguishable from alpha/beta T cells [56].

The mechanism by which perturbation of the CD3 complex by monoclonal antibodies generates signal transduction remains unknown. Elegant studies by van Lier et al. [82] using isotype switch variants of a CD3 antibody indicate that the isotype of the antibody influences calcium mobilization independent of the CD3 epitope, perhaps through altered binding affinities for the CD3 complex, although the importance of antigen cross-linking could not be evaluated in that study. Thus it is possible that it is not the isotype of the antibody per se that determines the efficiency of calcium mobilization, but rather the amount of cross-linking that is achieved by the antibody due to many variables, such as the amount of aggregates in the antibody preparation, valency of antibody binding, binding to Fc receptors, etc.

In studies using indo-1 in T cells, it was found that cross-linking of CD2, CD3, CD4, CD5, CD8, CD28, and MHC class I molecules all can cause calcium mobilization that involves phosphoinositide turnover, while perturbation of other surface molecules such as CD7, CD11, CD25 and CD45 does not cause calcium mobilization [20, 31, 40]. Most, if not all of the molecules that cause calcium mobilization are members of the immunoglobulin gene superfamily. This is a property that may confer some signalling capacity since the surface structures not associated with calcium mobilization are not related to immunoglobulin molecules.

In both B and T cells cross-linking of the antigen receptor enhances signal transduction. For example, in murine B cells, the binding of multimeric anti-delta immunoglobulin antibody results in a 3-log shift in the dose-response curve to elicit calcium mobilization compared to monomeric anti-delta immunoglobulin antibody [11]. In T cells, Fab'2 bivalent CD3 antibodies are required for protein kinase C membrane translocation whereas Fab (monovalent) antibodies are ineffective [41] and the binding of immobilized CD3 antibodies rather than fluid phase antibodies substantially prolongs membrane translocation of protein kinase C [52]. The mitogenic effects of monoclonal antibody ligation of antigen receptor is strongly enhanced by coupling the antibody to a solid support, presumably through prevention of receptor internalization and enhanced signal transduction [40-42]. There is some evidence that the cytoskeleton is involved in the regulation of cytoplasmic calcium. For example, treatment of lympho-
cytes with cytochalasins can either directly cause calcium transients or potentiate signals initiated by other ligands [4, 78].

Several molecules on the surface of murine and human T cells enhance signal transduction and may serve to augment or sustain proliferation initiated by the antigen receptor [reviewed in 87]. The CD5 molecule is found on human T cells and a subset of B cells and is defined by monoclonal antibodies such as OKT1 and Leu1, and on murine cells by Ly1. While not mitogenic themselves, the binding of monoclonal antibodies to the CD5 molecule augments CD3-induced T cell proliferation and increases both intracellular ionized calcium concentration and IP3 production over that caused by CD3 alone [31]. In contrast, antibodies to the CD2 (E-rosette receptor, TI1) molecule are mitogenic for T cells in the presence of accessory cells. CD2 antibodies cause calcium mobilization in T cells that is distinct from that after T cell receptor/CD3 stimulation [30]. The CD2 signal is delayed in onset and prolonged in duration when compared to CD3 stimulation, and furthermore, the CD2 signal also occurs in large granular lymphocytes, cells that are CD3 negative. The CD2 signal in T cells (but not LGL) requires cross-linking to be effective; either stimulation by two antibodies against distinct epitopes of the CD2 molecule [30] or by cross-linking of one CD2 antibody by use of an anti-immunoglobulin [40]. Interestingly, the binding of sheep red cells to T cells via interaction of CD2 and LFA-3 also increases calcium in T cells [44], suggesting a possible mechanism of T cell activation in the thymus where presumably the thymic epithelial cell would bind to CD2 [75].

The role of calcium signalling in T cell maturation is being explored. T cells acquire antigen specificity and selection for self major histocompatibility antigen reactivity ('restriction') by a complex, partially understood process that occurs in the thymus involving multiple antigen receptor genomic recombination events. Immature thymocytes (CD2+ CD3- CD4+ CD8+) progress to an intermediate stage of differentiation (CD2+ CD3+ CD4- CD8+) in the final stage of maturation, the double positive cells develop the mature T cell phenotype of either CD2+ CD3+ CD4+ CD8- or CD2+ CD3+ CD4- CD8+ single positive cells. Cells at the earliest stage of CD3 expression ('double positive' cells) may be capable of transmembrane signalling after ligation of the CD3 complex [19, 25, 86], however, consequences of the signal differ in that single positive cells express the IL-2 receptor, secrete IL-2 and proliferate after CD3 stimulation while double positive cells do not. There are qualitative differences between immature and mature thymocytes in the pattern of calcium mobilization. After antigen receptor stimulation, immature thymocytes have less transmembrane calcium influx than mature cells [19]. Intracellular calcium mobilization is similar in both immature and mature thymocytes [19]. These results suggest that immature T cells have fewer calcium channels or that the channels are less efficiently activated after antigen receptor ligation.

The use of flow cytometry with indo-1 has allowed the correlation of surface antigen expression with calcium mobilization. For example, after CD2 (TI1) stimulation of peripheral blood lymphocytes, it was found that both CD3+ T cells and CD16+ CD3+- large granular lymphocytes (LGL) mobilized calcium; and after CD3 (antigen-specific) stimulation, more than 90% of T cells responded, and as was expected, LGL did not
The pattern of the calcium signal after CD2 stimulation differed in that calcium mobilization in LGL was early in onset and low in magnitude while in T cells, the calcium signal was delayed and high in magnitude. After CD3 stimulation of T cells, the kinetics of calcium mobilization is correlated with surface antigen expression. Among the CD4 subset, the response is more rapid in the LFA-3-positive subset than in the CD45R+ (Leu18, 2H4) subset [64]. Subsequent studies have revealed that the LFA-3 subset is composed of T cells with memory function while the CD45R+ cells are naive T cells [69].

The heterogeneity of the response to CD3 stimulation of lymphocytes that express the CD8 antigen is shown in figure 2. In an experiment using four colors, peripheral blood lymphocytes were loaded with Indo-1 and then stained with PE-labelled CD8 and fluorescein-conjugated CD16 antibodies. The cells were stimulated with CD3 antibody and the calcium response correlated with surface phenotype. In cells expressing the CD8 antigen, the response to CD3 stimulation was confined to cells that are CD16 negative while the response of the CD16-CD8dim-positive and CD16-CD8bright-positive cells differed slightly in the rate of return to baseline [Ca^{2+}].

In contrast to T lymphocytes, in human B cells after stimulation by anti-immunoglobulin, only ~50% of cells respond. The CD22 antigen is expressed on a subset of 60–80% of mature B cells, and it was found that all cells responding to anti-immunoglobulin stimulation could be accounted for by cells having the phenotype of IgD+ IgM+ CD22+ while cells having the phenotype IgM+ CD22- did not respond [59]. Thus, the presence of CD22 rather than of antigen receptor correlates with calcium mobilization after anti-immunoglobulin stimulation, suggesting that the antigen receptor is in some way uncoupled from signal transduction in a subset of B cells. Heterogeneity in [Ca^{2+}], signals, as in the response of human lymphocytes to CD2 and anti-immunoglobulin stimulation, would have been impossible to discern in conventional assays carried out in a fluorometer where only the mean calcium response is recorded.

**Monocytes**

In studies using quin2, platelet-activating factor has been found to be a potent mediator of calcium transients in adherent macrophages [15]. On the other hand, other agents known to activate macrophages such as f-MLP, gamma interferon and LPS do not initiate rapid calcium transients [7, 15, 24] and only small changes in monocyte intracellular calcium concentration occur in response to the lectin concanavalin A (Con A) [73]. In contrast, large changes in calcium concentration occur in lymphocytes after Con A stimulation and in neutrophils after f-MLP stimulation, and since monocytes bind both Con A and f-MLP and exhibit biologic responses to these agonists, this implies that monocytes predominately utilize an alternative signalling pathway [24]. There are other examples where the same ligand can bind to receptors that are coupled to different signalling pathways; for example, vasopressin can bind to V1 receptors and activate a calcium pathway or it can bind to V2 receptors and cause the production of cAMP via activation of adenyl cyclase. In both human and murine macrophages, the binding of antibody to the receptor for the Fc portion of immunoglobulins can initiate rapid increases in cellular calcium, although this
area is controversial, in part perhaps because there are as many as four distinct Fc receptors on monocytes [50, reviewed in 24]. Thus, regulation of cellular calcium concentration in mononuclear phagocytes is complex and only partially understood. In studies using indo-1 and flow cytometry, leukotriene D4 has been shown to increase cytosolic calcium in differentiated HL-60 cells, a leukemic cell line that has characteristics of monocytes [5]. Studies of calcium metabolism in resting, nonadherent monocytes have not been reported, and could readily be accomplished using flow cytometry and indo-1.

**Neutrophils**

A study performed on bulk preparations of rabbit neutrophils loaded with indo-1 revealed a biphasic or triphasic calcium response after stimulation with the chemotactic agent f-MLP [12]. The initial response consisted of elevated calcium derived from intracellular sources, and as the cytoplasmic calcium concentration returns towards baseline levels, a second increase in calcium occurred that was dependent on the presence of external calcium in the medium. Davies et al. [38] reported the first application of flow cytometric analysis of neutrophils using indo-1. As mentioned in a preceding section, this analysis was performed simultaneously with measurement of membrane potential. These authors observed a rapid uniphasic [Ca$^{2+}$], response of neutrophils to f-MLP over a broad range of f-MLP concentrations. Interestingly, the membrane potential changes were biphasic at intermediate f-MLP concentrations, with hyperpolarization followed by depolarization. To reconcile the differences in the above observations of [Ca$^{2+}$], it will be important to exclude compartmentalization of indo-1 as an artifactual cause of the biphasic calcium response, given the known difficulty of loading neutrophils with fura-2 [71]. The growth factors GM-CSF and G-CSF do not appear to cause calcium mobilization in neutrophils [77].

**Platelets**

The indo-1-based flow cytometric assay of cells as small as platelets is eminently feasible. Davies et al. [16] found heterogeneity in the response of platelets to thrombin stimulation. At low doses of thrombin, the response consisted of a large Ca$^{2+}$ rise in a subpopulation of cells and a second population of platelets that failed to respond, whereas at high doses of thrombin, all cells responded. We have found that fibronectin is a potent calcium agonist for platelets, giving a different pattern of [Ca$^{2+}$] response than thrombin. In figure 3, isometric plots of indo-1-loaded platelets are shown. Fibronectin recruits essentially all platelets and the response is more sustained than after stimulation by thrombin [Nugent and Rabinovitch, unpubl. observations]. In the case of fibronectin, the addition of FITC-fibronectin showed that all platelets that had bound detectable amounts of FITC-fibronectin had elevated calcium levels, although not all cells with high calcium levels had detectable fibronectin bound, suggesting that the threshold for fibronectin-induced calcium mobilization is less than that detectable by FITC fluorescence. It remains to be determined whether a population of platelets exhibits differential thresholds for a response to an agonist based upon different requirements for receptor occupancy or whether all platelets respond and that the nature of the observed heterogeneity is due to oscillatory changes in Ca$^{2+}$.
Potential Clinical Applications

Several applications of cytosolic calcium measurement that have direct clinical relevance have been reported and many can be envisioned (table III). It is possible that the measurement of cytoplasmic-free calcium may be useful for the diagnosis of several diseases. For example, malignant hyperthermia (MH) is a genetic disorder that confers susceptibility to potentially lethal adverse reactions to certain anesthetics. Patients are generally asymptomatic until they require anesthesia, and therefore, a screening method to detect the disorder in family members of known patients with MH would be useful. It has recently been reported that lymphocytes from these patients exhibit exaggerated calcium flux after treatment with the anesthetic halothane [34, 35], and if these results are confirmed, the flow cytometric assay with indo-1 would be ideal to identify asymptomatic family members with MH. Cystic fibrosis is much more common than MH, and a defective chloride channel was reported in fibroblasts from patients with cystic fibrosis [46]. In studies using quin2, basal free calcium levels from lymphocytes of patients with cystic fibrosis have been reported to be lower than controls [72], and it is possible that a test for carriers could be devised using the more sensitive indo-1 technique [74, 83]. The Lambert-Eaton syndrome (LES) is an autoimmune disorder that results in muscle weakness due to antibodies against motor nerve terminals. It was recently reported in fura-2-loaded cells that serum from patients with LES could block voltage-gated calcium channels [33]. Manic-depressive illness is a heritable psychiatric illness. The primary agent for the treatment of manic depression is lithium chloride, and as lithium inhibits an enzyme in the phosphoinositide cycle, it has been proposed that manic depression is a disorder of phosphoinositide metabolism in the central nervous system [9].

One disorder of cellular signaling in the immune system has been described, congenital absence of the CD3 molecule [6], and it is likely that other disorders, perhaps involving G proteins, will be described in the near future.

The measurement of cellular calcium has proved useful in the investigation of the pathogenesis of certain acquired disorders of the immune system. There is a decline in immune function with aging, and in mice, calcium signaling in splenic lymphocytes after lectin stimulation or after anti-CD3 stimulation is substantially less vigorous in aged mice compared to young mice [55, 62]. Age-associated reductions in the [Ca\textsuperscript{2+}]\text{response} of human T cells to PHA or anti-CD3 stimulation are modest, however, and are present only in the CD4\textsuperscript{+} subset [Grossman and Rabinovitch, unpubl. data], and thus, the

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role of impaired signalling in senescence of the immune system requires further study.

We have recently found that infection with HIV-1, the pathogenic retrovirus that causes the acquired immunodeficiency syndrome (AIDS), causes a profound signalling defect after CD3 but not after CD2 stimulation (fig. 4) [47]. There is evidence that the CD4 (T4) molecule can transmit stimulatory or inhibitory signals to T cells. The binding of antibody to the CD4 molecule inhibits mobilization of \([Ca^{2+}]_i\), in response to simultaneous stimulation of the CD3 molecule. In contrast, when anti-CD4 and anti-CD3 are cross-linked together, calcium mobilization is substantially higher than after CD3 stimulation alone [42]. The above results provide further evidence that cellular activation can occur through distinct biochemical mechanisms and that one mechanism of immunosuppression in AIDS is through an impairment of antigen-specific T cell signalling. Similarly, patients with kidney failure appear to have a defect in CD3-induced T cell proliferation [54]. Cellular calcium levels have been reported to be elevated in patients after kidney transplantation [36], and thus the measurement of calcium may be useful in monitoring patients for allograft rejection. Interestingly, immunosuppressants such as ciclosporin and corticosteroids do not interfere significantly with calcium signalling in T cells [67], although corticosteroids do cause a moderate reduction of antigen receptor-induced calcium flux in murine B cells [17].

**Limitations**

There are several limitations to the flow cytometric assay of cellular calcium concentration. First, certain problems attributable to the use of fluorescent indicators have been mentioned. In some cells indo-1 will not load uniformly into cells, or may not be uniformly hydrolyzed to the calcium-sensitive moiety. Quin2 has been reported to block calcium efflux in squid axons [1], and it is possible that indo-1 may have similar effects, although this would be expected to be less of a problem due to the much lower concentrations of indo-1 that are required to attain a satisfactory fluorescent signal from cells. Similarly, quin2 has been reported to be mitogenic in certain cells and to alter certain cellular functions [37], although this has not as yet been observed in indo-1-loaded cells [13, 64]. Second, there are limitations imposed by the nature of the flow cytometric assay system. Flow cytometry is unable to detect heterogeneity of cellular calcium concentrations within a single cell, and there are reports from assays using digital video microscopy that in some situations, calcium transients may be compartmentalized [60, 88]. The use of the photoprotein aequorin may in some circumstances detect changes in cytosolic calcium not reported by indo-1 [85], although use of the two indicators is complimentary because aequorin cannot measure \([Ca^{2+}]_i\) in single cells. In addition, there is evidence that calcium elevations occurring after cellular stimulation may be oscillatory rather than sustained [2], thus raising the possibility that some cellular processes controlled by calcium may be frequency modulated as well as amplitude modulated. Since flow cytometry cannot measure the calcium concentration inside a single cell as a function of time, it is not possible to distinguish between a population of cells that is responding with an oscillatory response or alternatively, whether there are two subpopulations of cells, one that has ele-
vated calcium concentration and one that has basal levels.

In spite of these limitations, flow cytometric analysis with indo-1 offers great practical advantages and allows measurements of a kind not readily possible by alternative techniques.

References

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59 Pezzotto, A.; Rabinovitch, P.S.; Dorken, B.; Mol-denbauer, G.; Clark, E.A.: Role of the CD22 hu-
man B cell antigen in B cell triggering by anti-
(1988).

60 Peneie, M.; Tien, R.Y.; Schmitt-Verhulst, A.M.: 
Sequential activation and lethal hit measured by 
[Ca\textsuperscript{2+}], in individual cytolytic T cells and targets. 

61 Proust, J.J.; Filburn, C.R.; Harrison, S.A.; Buch-
hoit, M.A.; Nordin, A.A.: Age-related defect in 
signal transduction during lecithn activation of mu-
rine T lymphocytes. J. Immun. 139: 1472–1478

62 Putney, J.W.: A model for receptor-regulated 

63 Rabinovitch, P.S.; June, C.H.; Grossmann, A.; 
Ledbetter, J.A.: Heterogeneity of T cell intracellu-
lar free calcium responses after mitogen stimula-
tion with PHA or anti-CD3. Use of indo-1 and 
simultaneous immunofluorescence with flow cy-

64 Ransom, J.T.; DiGiusto, D.L.; Cambier, J.C.: Sin-
gle cell analysis of calcium mobilization in anti-
immunoglobulin-stimulated B lymphocytes. J. 

65 Rasmussen, H.: The calcium messenger system. 
(1986).

66 Redelman, D.: Cyclosporin A does not inhibit the 
PHA-stimulated increase in intracellular Ca\textsuperscript{2+} 
concentration but inhibits the increase in E-ros-
cite receptor (CD2) expression and appearance of 
interleukin-2 receptors (CD25). Cytometry 9: 

Backer, T.R.: Inhibition by phorbol esters of anti-
immunoglobulin-induced calcium signalling and 
B-cell activation. J. cell. Physiol. 129: 347–355
(1986).

68 Sanders, M.E.; Makoba, M.W.; Sharrow, S.O.; 
Stephan, D.; Springer, T.A.; Young, H.A.; Shaw, S.; 
Human memory T lymphocytes express in-
creased levels of three cell adhesion molecules 
(LFA-3, CD2, and LFA-1) and three other mole-
cules (UCHL1, CDw29, and PgP-1) and have en-
hanced IFN-gamma production. J. Immun. 140: 

69 Sanders, M.E.; Makoba, M.W.; Springer, T.A.; 
June, C.H.; Young, H.A.; Shaw, S.: Memory and 
naive T cell subsets differ in activation via CD2 or 
CD3 (submitted).

70 Scalon, M.; Williams, D.A.; Fay, F.S.: A Ca\textsuperscript{2+}- 
insensitive form of fura-2 associated with poly-
morphonuclear leukocytes. Assessment and accu-
rate Ca\textsuperscript{2+} measurement. J. biol. Chem. 262: 6308– 

Katz, S.: Intracellular free calcium levels in 
mononuclear cells of patients with cystic fibrosis 
and normal controls. Cell Calcium 8: 53–63

72 Scully, S.P.; Segel, G.B.; Lichtman, M.A.: Rela-
tionship of superoxide production to cytoplasmic 

73 Shapiro, B.L.; Lam, L.F.: Intracellular calcium in 
cystic fibrosis heterozygotes. Life Sci. 40: 2361– 

74 Singer, K.H.; Wolf, L.S.; Lobach, D.F.; Denning, 
S.M.; Tuck, D.T.; Robertson, A.L.; Haynes, B.F.: 
Human thymocytes bind to autologous and allo-

75 Simons, E.R.; Schafer, A.I.; Mchulam, T.; Fredette, J.P.; 
Maa, A.K.; Gadenne, A.S.; Leavitt, J.L.; Melnick, D.A.: Ef-
fects of recombinant human granulocyte and mac-
rophage colony-stimulating factors on signal 
transduction pathways in human granulocytes. J. 

76 Steinberg, S.F.; Bilezikian, J.P.; Al-Awqati, Q.: 
Fura-2 fluorescence is localized in mitochondria 
in endothelial cells. Am. J. Physiol. 253: C744– 

77 Sullivan, R.; Griffin, J.D.; Simons, E.R.; Schafer, 
A.I.; Mchulam, T.; Fredette, J.P.; Maa, A.K.; 
Gadenne, A.S.; Leavitt, J.L.; Melnick, D.A.: Ef-
fects of recombinant human granulocyte and mac-
rophage colony-stimulating factors on signal 
transduction pathways in human granulocytes. J. 

78 Treves, S.; Di Virgilio, F.; Vaselli, G.M.; Pazzan, 
T.: Effect of cytochalasins on cytosolic-free cal-
cium concentration and phosphoinositol metab-
olism in leukocytes. Exp Cell Res. 168: 283–298 

79 Tien, R.Y.: A non-disruptive technique for load-
ing calcium buffers and indicators into cells. Na-

80 Tien, R.Y.; Pazzan, T.; Rink, T.J.: Calcium ho-
meostasis in intact lymphocytes: cytoplasmic free 
calcium monitored with a new, intracellularly 
trapped fluorescent indicator. J. Cell Biol. 94: 

81 Tien, Y.R.; Pazzan, T.; Rink, T.J.: T-cell mito-


