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Introduction to Functional Cell Assays

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The flow cytometer can be used to measure a variety of functional parameters that are of increasing interest to cell biologists. Some of these parameters are already of interest in the clinical laboratory and some will become clinically relevant in the near future. The recent development of a number of new fluorescent probes now permits the measurement of various intracellular free ion concentrations, membrane characteristics, indicators of cellular metabolism, and intracellular glutathione. Most previously available techniques to measure these cellular parameters determined the mean value for a mixed population of cells. The flow cytometer has the unique capacity to permit the measurement of physiologic parameters in large numbers of living single cells; it allows correlation with other parameters such as immunophenotype and cell cycle; and, finally, it reveals heterogeneity within the cell population.

As an introduction to several more detailed presentations elsewhere in this volume that describe the clinical application of probes of cellular physiology, this discussion will introduce several measurements of the greatest current interest.

INTRACELLULAR IONIZED CALCIUM

Ionized calcium has an important role as a mediator of transmembrane signal transduction, and elevations in intracellular ionized calcium concentration ([Ca^{2+}]) regulate diverse cellular processes. Measurement of [Ca^{2+}] in living cells is thus of considerable interest to a broad range of investigators.

Calcium influx is thought to be initiated either by membrane depolarization that opens voltage-gated channels or by the binding of ligands to receptor-operated channels. The binding of agonist to its specific membrane receptor activates enzymatic processes that result in the activation of phospholipase C. Phospholipase C causes the hydrolysis of a membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2), which yields a water-soluble product, inositol 1,4,5-trisphosphate (IP3), and a lipid, 1,2-diacylglycerol (DAG). IP3 then causes the release of calcium from intracellular stores, whereas 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 this pathway a unique, bifurcating system. Calcium is therefore a "third messenger" that controls numerous cellular processes, activating a broad variety of enzyme systems, both as a cofactor and in conjunction with the calcium-binding protein, calmodulin.

**Indicators of Intracellular Ionized Calcium Concentration**

Until 1982, it was not possible to measure \([\text{Ca}^{2+}]\), in small intact cells; measurements were restricted mostly to large invertebrate cells where the use of microelectrodes was possible. \([\text{Ca}^{2+}]\) was first measured in diverse populations of cells with the development of quin2. The indicator was easily loaded into small intact cells using the acetoxymethyl ester of the dye. This uncharged form diffuses freely into the cytoplasm where it is hydrolyzed by esterases to yield the tetraanionic form of the dye, which is trapped inside the cell. Unfortunately, quin2 has several disadvantages that limit its application to flow cytometry. A relatively low extinction coefficient and quantum yield have made detection of the dye at low concentrations difficult; at higher concentrations, quin2 itself buffers the \([\text{Ca}^{2+}]\). Subsequently, Grynkiewicz et al. described a new family of highly fluorescent calcium chelators that overcome most of the aforementioned limitations. One of these dyes, indo-1 (1-(1-[2-amino-5-((6-carboxyindol-2-yl)-phenoxy]-2-[2'-amino-5'-methylphenoxy]ethane N,N,N',N'-tetraacetic acid), has spectral properties that make it especially useful for analysis with flow cytometry. In particular, indo-1 exhibits large changes in the fluorescent emission wavelength upon calcium binding. Use of the ratio of the intensities of fluorescence at two wavelengths (approximately 400 nm and 500 nm) allows calculation of \([\text{Ca}^{2+}]\), independent of variability in cellular size or intracellular dye concentration. The ratiometric approach thus gives considerable accuracy to this analysis, allowing, for example, the basal \([\text{Ca}^{2+}]\), to be seen to be constrained within narrow limits (FIGURE 1). The only significant drawback to the use of indo-1 is the requirement for ultraviolet (UV) excitation.

A practical alternative to indo-1 became available upon the description of a fluorescein-based, calcium-sensitive probe, fluo-3. This dye exhibits an increase in fluorescence intensity with increasing \([\text{Ca}^{2+}]\). The use of fluo-3 permits, for the first time, the simultaneous use of other UV-excitable probes for flow cytometry, such as those used for cell cycle analysis or measurement of intracellular glutathione, allowing correlation of these parameters with calcium responses. The primary disadvantage of fluo-3 is that it does not have fluorescence properties that allow ratiometric determinations. Therefore, calibration on a flow cytometer is more complicated because the signal is proportional to cell size and dye concentration as well as to \([\text{Ca}^{2+}]\). In addition, the ability to measure responses in subsets of cells is more limited because the broad distribution of fluorescence intensities of unstimulated cells often results in an overlapping distribution of the values from stimulated and unstimulated cells (FIGURE 1). This problem can be minimized by the simultaneous use of a second dye that serves as an indicator of the magnitude of dye loading in an individual cell.

FIGURE 1. Comparison of indo-1 and fluo-3 measurement of $[Ca^{2+}]_{i}$ and methods of display of kinetic data. Human peripheral blood lymphocytes (PBL) were loaded simultaneously with 3 μg/mL indo-1 and 3 μg/mL fluo-3, stained with PE-CD4 mAb, and then stimulated with anti-CD3 antibody (10 μg/mL G19-4, Jeff Ledbetter, Oncogen, Seattle, Washington) at approximately 1 minute after the start of analysis (note the gap in data acquisition). Violet and green indo-1 emission (UV* excited) and green fluo-3 and orange PE emission (488-nm excited) were collected simultaneously for each cell. Results were gated from PE-CD4- cells. In panels A and B, the results are displayed as a "dot plot" where the x-axis is time and the y-axis is the indo-1 ratio or the fluo-3 fluorescence intensity. The mean indo-1 ratios versus time is shown in panel C and the fluo-3 intensity versus time is shown in panel D. The data were converted to calcium concentration versus time by calibration using measured constants for indo-1 (panel E) and by calibration with buffer solutions for fluo-3 (panel F). Whereas the shapes of the curves for the mean indo-1 ratio (C) and the fluo-3 intensity (D) are different (due to the different values of $K_{eq}$ for indo-1 and fluo-3), the two measurements are essentially identical once converted to $[Ca^{2+}]_{i}$. The percentages of cells responding with $[Ca^{2+}]_{i}$, elevated beyond two standard deviations above the mean of the cells before antibody stimulation ("percent cells above threshold") are plotted for indo-1 (panel G) and fluo-3 (panel H). The percentages of responding cells calculated by cumulative curve subtraction are plotted in panels I and J for indo-1 and fluo-3, respectively. Data analysis was performed with software written by one of the authors (P. S. Rabinowitch) ("MultiTime", Phoenix Flow Systems, San Diego, California).
Applications of the Flow Cytometric Analysis of [Ca\textsuperscript{2+}],

A common extension of analysis of [Ca\textsuperscript{2+}], is the simultaneous use of additional fluorochromes for the determination of cellular immunophenotype. This allows alterations in [Ca\textsuperscript{2+}], to be examined in specific immunophenotypic subsets (FIGURE 2). Fluorescein isocyanate (FITC)- and phycoerythrin (PE)-conjugated antibodies can be used with indo-1, and PE and allophycocyanin (APC) (or other red-excited dyes) can be used with fluo-3. Numerous examples of the analysis of [Ca\textsuperscript{2+}], in immunophenotypically defined subsets have been described (reviewed in reference 7).

**FIGURE 2.** Analysis of [Ca\textsuperscript{2+}] in human PBL stained with indo-1 and a cocktail of PE-labeled antibodies that stain all cells but CD4\textsuperscript{+} (A,C) or CD8\textsuperscript{+} (B,D). Analyses were gated to exclude all PE-labeled cells, a reciprocal staining strategy that avoids antibody binding to CD4 and CD8 receptors. Cells were stimulated with 30 µg/mL PHA (A,B) or 10 µg/mL CD3 (G19-4) (C,D). The indo-1 ratio is displayed versus time as an isometric plot where the z-axis is the cell number. Note that [Ca\textsuperscript{2+}], responses in CD4\textsuperscript{+} cells are of higher magnitude than those of CD8\textsuperscript{+} cells.

The flow cytometric assay of cellular calcium concentration has already been applied to a wide variety of cells, providing interesting and sometimes unexpected results. Examples of the initial applications of the technique are presented in recent reviews. One of the first observations that was made readily quantifiable by the flow cytometric analysis was that there is great heterogeneity in the response of lymphocytes to mitogenic agonists. Using simultaneous immunofluorescence, some of this heterogeneity can be shown to be related to immunophenotypic subsets; for instance, CD4\textsuperscript{+} cells show more vigorous responses to lectins and CD3 than do CD8\textsuperscript{+} cells (FIGURE 2). Considerable use of this approach has been made in the demonstration of differences between [Ca\textsuperscript{2+}], activation requirements of different cell subsets.
and subset specificities of activation pathways. The effects of antibody binding to cell surface molecules, sometimes a complication in labeling experiments (see legend to FIGURE 2), have been extensively employed to analyze signaling mechanisms, and augmented or even new relationships have been probed by cross-linking antibodies on the cell surface.\textsuperscript{11,12} Flow cytometric measurements with indo-1 have been performed to date with all nucleated blood cell types. Applications of fluo-3 have been reported with most types, with reports appearing at a rapidly increasing pace.

The combination of sensitivity, reliability, and ability to analyze large numbers of cells within cell subsets has made the flow cytometric assay of \([\text{Ca}^{2+}]\), the preferred technique for a broad spectrum of research applications. As a consequence of the success of this research, there are now many exciting potential clinical applications of the flow cytometric assay of cellular calcium concentration. The contribution by June et al. in this volume describes some of these developing clinical applications.

**INTRACELLULAR pH**

The pH\textsubscript{r} of mammalian cells is ~7.2 and it appears to be closely regulated. In mammalian cells, pH\textsubscript{r} is controlled by at least three mechanisms, including \(\text{Na}^+ /\text{H}^+\) exchange, sodium-dependent \(\text{Cl}^- /\text{HCO}_3^-\), and \(\text{HCO}_3^- /\text{Cl}^-\) exchange. Acid extrusion is primarily accomplished by the \(\text{Na}^+ /\text{H}^+\) antiport and by sodium-dependent \(\text{Cl}^- /\text{HCO}_3^-\), whereas \(\text{HCO}_3^- /\text{Cl}^-\) exchange has the major role for base extrusion. All of these mechanisms appear to be stimulated by a variety of growth factors and by phorbol esters, presumably through the activation of protein kinase C.

**Indicators of Intracellular pH**

Until recently, the most commonly used probes were modifications of fluorescein, with fluorescein diacetate being the first-generation pH probe, followed by carbosulfur-fluorescein diacetate (COFDA). Both of these dyes are limited by relatively poor retention inside loaded cells. An improved fluorescein probe is 2,7'-bis-carboxyethyl-5(6)-carboxyfluorescein (BCECF). As with indo-1 and fluo-3, BCECF is loaded into cells using the acetoxymethyl ester; after hydrolysis, it has a negative charge of ~4 or ~5 and therefore leaks more slowly than COFDA. The \(pK_a\) of BCECF, 6.98, is near the \(pH\) of resting cells and there is a \(pH\)-dependent shift in the excitation wavelength, making it possible to use the ratio of fluorescence signals to correct for differences in loading and cell size. In addition, BCECF fluorescence excited at 450 nm is \(pH\)-independent, whereas fluorescence at 500 nm is \(pH\)-dependent, allowing ratiometric fluorescence emission analysis (TABLE 1). In both cases, the magnitude of \(pH\)-dependent ratio shifts is relatively modest\textsuperscript{13} and these dyes are largely supplanted by newer probes. The most useful of several UV-excited pH probes that have been developed is 1,4-diacytov-2,3-dicyanobenzene (ADB). The cell-permeant ADB is hydrolyzed and trapped intracellularly to yield 2,3-dicyanoquinone (DCH).\textsuperscript{14} Ratiometric determinations of pH are possible using a single excitation source by measuring the fluorescence emission at 429 nm and 477 nm (TABLE 1). As with the measurement of \([\text{Ca}^{2+}]\), many laboratories will find the requirement for UV excitation to be the primary limitation for the use of this probe.
Recently, a useful probe for pH measurement named SNARF-1 (SemiNaphtho-Fluor) has been introduced. SNARF-1 has convenient excitation spectra (488 or 514 nm) and exhibits large changes in pH-dependent fluorescence. The emission of SNARF-1 in acid is maximal at 587 nm and the basic form emits maximally at 636 nm; there is an isosbestic point at 610 nm. The ratio of orange and red emission is used as the pH indicator. This provides the advantages of the ratiometric determination, as described previously.

Applications of Analysis of pH

As previously mentioned, the fluorescence emission properties of SNARF-1 allow simultaneous excitation of FITC probes using the same 488-nm laser. Analysis of pH in immunophenotypically defined cell subsets using FITC-conjugated mAb is thus very straightforward. Similarly, single-laser [Ca²⁺] measurements with fluo-3 are easily performed simultaneously with measurements of pH. When a second UV laser is available, the [Ca²⁺] measurement with indo-1 is optimal.

<table>
<thead>
<tr>
<th>Probe</th>
<th>$pK_a$</th>
<th>Excitation</th>
<th>Emission</th>
</tr>
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<tbody>
<tr>
<td>bis-carboxyethyl-carboxyfluorescein acetoxy-methyl ester (BCECF AM)</td>
<td>6.98</td>
<td>488</td>
<td>535</td>
</tr>
<tr>
<td>diacetoxy-dicyanobenzene (ADB); yields dicyanohydroquinone (DCH) after deesterification</td>
<td>8.0</td>
<td>~350</td>
<td>425, 540</td>
</tr>
<tr>
<td>carboxy-SNARF-1-acetoxyethyl acetate</td>
<td>7.50</td>
<td>488, 514</td>
<td>ratio 575, 670</td>
</tr>
</tbody>
</table>

Because of the large shifts in pH that take place upon activation of granulocytes, studies of intracellular pH in the analysis of granulocyte function are probably the most interesting in the clinical laboratory today. These are discussed by G. Valet in this volume.

INTRACELLULAR GLUTATHIONE

Glutathione (glutamylcysteinylglycine, GSH) is an important antioxidant tripeptide thiol that is involved in the scavenging of toxic oxygen products. It is also important for the maintenance of cellular thiol redox status, and its oxid state has been proposed to be a major determinant of the functioning of a number of enzymes and in the integrity of cytoskeletal proteins. These roles for GSH
have important consequences upon cell physiology and mitogenic activation. In general, augmenting cellular GSH with cysteine delivery agents, such as N-acetylcysteine or oxathiazolidine carbosulfonic acid, or with glutathione esters has a positive effect on cell growth, whereas depletion of GSH inhibits many cellular functions. GSH is known to influence cell growth and replication at various levels, including G1/S-phase transition and very early events in mitogen-induced cell activation. GSH has also been shown to affect the responsiveness of cells to various cytokines, for example, the action of IL-2 in lymphocytes.21-22

The activity of redox-responsive oncogene products and DNA-binding factors responsible for gene regulation may also be affected by the GSH status of the cell. For instance, the interactions of c-fos and c-jun products are known to be redox-sensitive.23 Another example is that of NF-kB. Roederer and colleagues24 have shown that this nuclear binding factor can be inhibited from binding to regulatory DNA-binding domains by N-acetylcysteine, a drug that increases the GSH level of cells.

Indicators of Intracellular GSH

Several years ago, Durand and Olive25 published a review of fluorescent indicators for thiols, including GSH, that might prove useful for flow cytometric purposes. The problem with all of the dyes reviewed was one of specificity for GSH. A major advance in the measurement of GSH by flow cytometry was made by Rice et al.26 who used monochlorobimane (MCB) to detect changes in the GSH status of individual normal and tumor cells. This dye owes its specificity for GSH (see Figure 3) to the fact that it is conjugated to GSH by glutathione-S-transferases and has

![Graph](image-url)

**FIGURE 3.** Correlation between GSH content measured by HPLC and MCB flow cytometry. Chinese hamster V79 cells were treated with 0, 1, 3, 6, 9, or 12 mM BSO for 12 hours. Cells were then processed for flow cytometry (45 μM MCB for 10 minutes at 37 °C) or for HPLC determination of the total reduced GSH content (from Reference 44, with permission from the publisher).
FIGURE 4. Proliferation of human CD4+ lymphocytes sorted on MCB fluorescence intensity. Human lymphocytes isolated from peripheral blood by density gradient centrifugation were stained with MCB (60 μM for 15 minutes at 37°C) and PE anti-CD4 mAb. CD4+ cells were then sorted according to their MCB fluorescence for the lowest 10%, the 10th to 20th percentile, the 40th to 60th percentile, and the highest 20% of the fluorescence histogram. Cells were then plated into 96-well plates precoated with anti-CD3 mAb in BrdU-containing medium. After three days, cells were harvested and assessed for proliferation by the BrdU/Hoechst method. There is a direct correlation between the MCB fluorescence intensity of the unstimulated cells and their subsequent ability to proliferate.

relatively low nonenzymatic reactivity towards GSH and other thiols. As examples of the application of the measurement of GSH in viable cells using MCB, FIGURES 4 and 5 show that the proliferation and [Ca2+]i responses of sorted CD4+ T cells are proportional to their basal GSH content.

Another reagent, chloromethylfluorescein diacetate (CMFDA), has recently been described for measuring intracellular GSH. Intracellular CMF is presumably metabolized in a manner similar to MCB, yielding a GSH-MF fluorescent conjugate. The advantage provided by this dye is that the excitation and emission characteristics are similar to fluorescein once conjugated to GSH, allowing the use of 488-nm excitation. The principal disadvantage with this dye is that it is apparently less specific for GSH than MCB.

Applications of Analysis of GSH

In the clinical laboratory, GSH has recently received attention because of its role in a number of pathological disease states including tumor cell resistance to chemotherapeutic agents (see the contribution by D. W. Hedley in this volume) and idiopathic pulmonary fibrosis. In addition, considerable recent attention has been
directed towards the analysis of GSH in acquired immune deficiency syndrome.\textsuperscript{25,26,33} This subject is addressed in detail by Roeder et al. in this volume.

**ANALYSIS OF MEMBRANE POTENTIAL.**

Resting cells maintain large gradients between the intracellular and extracellular concentrations of a variety of ions, including Ca\textsuperscript{2+}, K\textsuperscript{+}, Na\textsuperscript{+}, and Cl\textsuperscript{-}. Potassium ions, for example, are concentrated within cells by the action of the Na-K-ATPase. The relative permeability of the membrane to K\textsuperscript{+} ions is greater than that of other ions; the leakage of K\textsuperscript{+} ions establishes an electron countergradient and the cytoplasm becomes electronegative with respect to the external medium. This K\textsuperscript{+} electrochemical gradient is the most significant contribution to the negative membrane potential of most mammalian cells. Maintenance of a large negative trans-membrane potential has been postulated to be a control mechanism to arrest cells in an inactive stage and changes in cell membrane potential that occur in various cell types rapidly after
binding of ligands to transmembrane receptors have been suggested to be mediators of subsequent physiologic cellular responses. Detailed investigation of the membrane potential in small cells has been made feasible by the development of potential sensitive indicator probes. These probes are charged lipophilic molecules; they partition between the cell and the surrounding medium according to the Nernst equation: $C_e/C_i = e^{-nF\theta/RT}$, where $C_e$ and $C_i$ are the extracellular and intracellular indicator concentrations; $n$ is the charge of the indicator; $\theta$ is the membrane potential; and $F$, $R$, and $T$ are the Faraday and gas constants and temperature. For a cationic indicator, the cellular concentration falls as the membrane potential declines toward zero and it rises if the cell hyperpolarizes (i.e., the cytosol becomes more electronegative with respect to the medium).

**Cyanine Dyes**

A family of cationic cyanine dyes were described by Hoffmann and Laris to be useful indicators for cells in solution, in the manner described earlier. These fluorescent dyes have a single negative charge delocalized over an extensive pi-electron system in a highly symmetric molecule. Sims et al. introduced the shorthand nomenclature DiYC,(2m+1) for these dyes, where the "Y" member of the ring structure may be oxygen (O), sulfur (S), or isopropyl (I). The length of the alkyl side chains, "n", affects the lipid solubility and "m", the number of methylene groups, affects the fluorescence spectral characteristics. The use of the highly fluorescent dyes shown in TABLE 2 with flow cytometry allows adequate fluorescence signal detection of single cells with dye concentrations below $10^{-7}$ M. Under these conditions, hyperpolarization is accompanied by increased cellular fluorescence and depolarization is accompanied by decreased fluorescence. These low dye concentrations also help to minimize the toxicity of the cyanine dyes.

**Oxonol Dyes**

The oxonol dyes are chemically unrelated to the cyanine dyes, but are similarly symmetric, membrane-permeant molecules with a highly delocalized charge. This charge is negative, in contrast to the cyanine dyes, so that the changes in partitioning

<table>
<thead>
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<th>TABLE 2: Dyes for Flow Cytometric Analysis of Membrane Potential</th>
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<tr>
<td><strong>Excitation (nm)</strong></td>
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<tr>
<td><strong>Cyanine Dyes</strong></td>
</tr>
<tr>
<td>DiOC.(3)</td>
</tr>
<tr>
<td>DiOC.(3)</td>
</tr>
<tr>
<td>DiOC.(5)</td>
</tr>
<tr>
<td>DiIC.(3)</td>
</tr>
<tr>
<td><strong>Oxonol Dyes</strong></td>
</tr>
<tr>
<td>DiIO.C.(3)</td>
</tr>
<tr>
<td>DiIO.C.(5)</td>
</tr>
<tr>
<td>DiSO.C.(3)</td>
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in response to altered membrane potential are in the opposite direction to cyanine
dyes: depolarization of the membrane transfers the anion from the external medium
onto binding sites within the cell and hyperpolarization results in dye exclusion and
decreased cellular fluorescence. For flow cytometry, the properties of the oxonol
dyes are especially attractive: analysis of cells takes place almost completely apart
from the dye in the external medium (increasing the signal-to-noise ratio). In
addition, the lower proportion of bound dye increases the buffering of the external
dye concentration and the negative charge of the dye forces exclusion from the highly
negatively charged mitochondria, minimizing a complication encountered with cyanine dyes.

Application of the Study of Membrane Potential by Flow Cytometry

An increasing fraction of studies of membrane potential are being performed by
flow cytometry, taking advantage of the sensitivity of this methodology, the ability to
recognize heterogeneity in cellular responses, and the opportunities for multi-param.
eter analysis. These applications have been recently reviewed. 

Flow cytometric analysis of membrane potential has been useful in studying
neutrophil activation and can demonstrate subpopulations of cells with different
polarization following stimulation. Alterations in membrane potential can also be
correlated with other tests of neutrophil function, such as chemotaxis, and may have
potential clinical usefulness. Membrane potential measurements may also have
utility in monitoring drug effects on normal and tumor cells.

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among T cells in intracellular free calcium responses after mitogen stimulation with
PHA or anti-CD3. Simultaneous use of Indo-1 and immunofluorescence with flow


