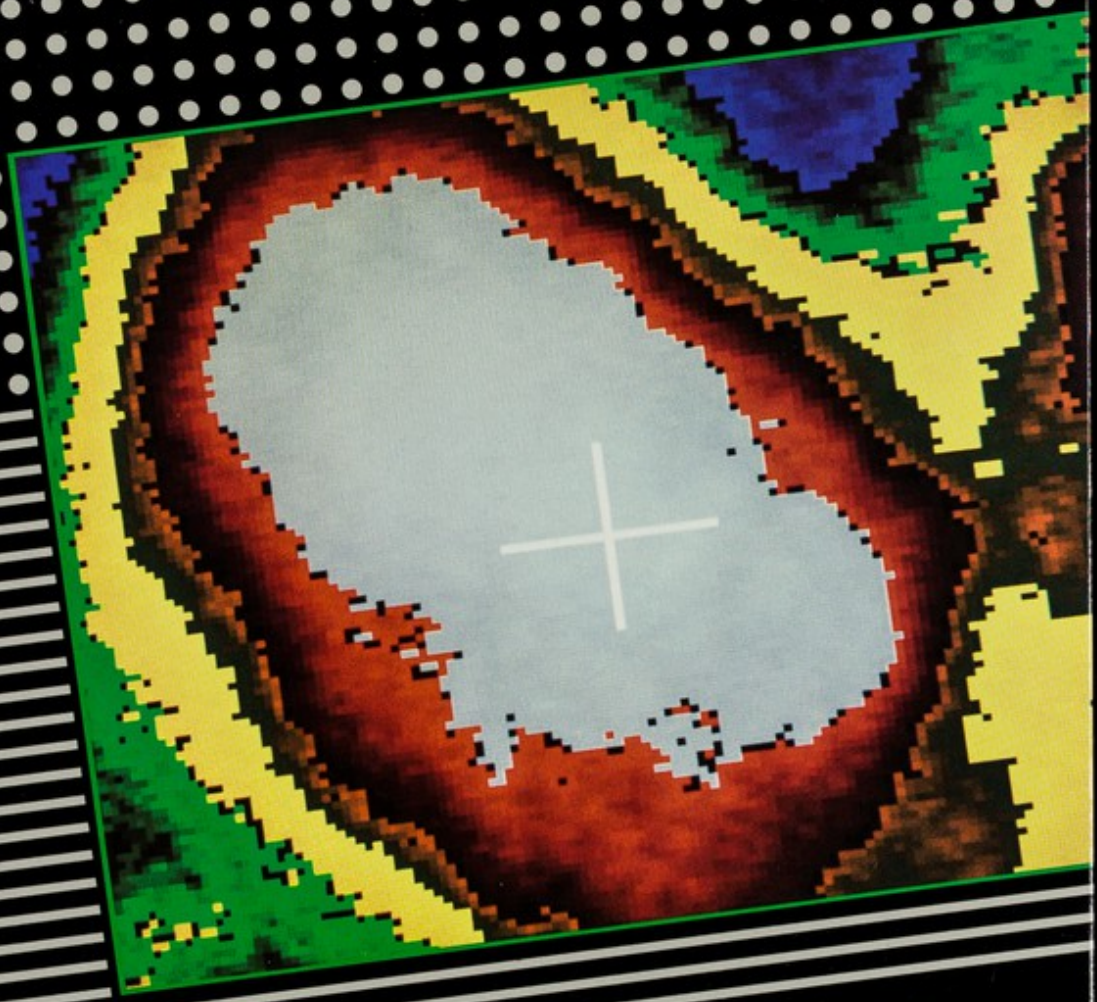
A unique way to measure calcium in living cells / SPEX Industries, Inc.

A Unique Way to Measure Calcium in Living Cells



SPEX

Introducing . . .

The SPEX Cation Measurement System

Meeting a Critical Measurement Need

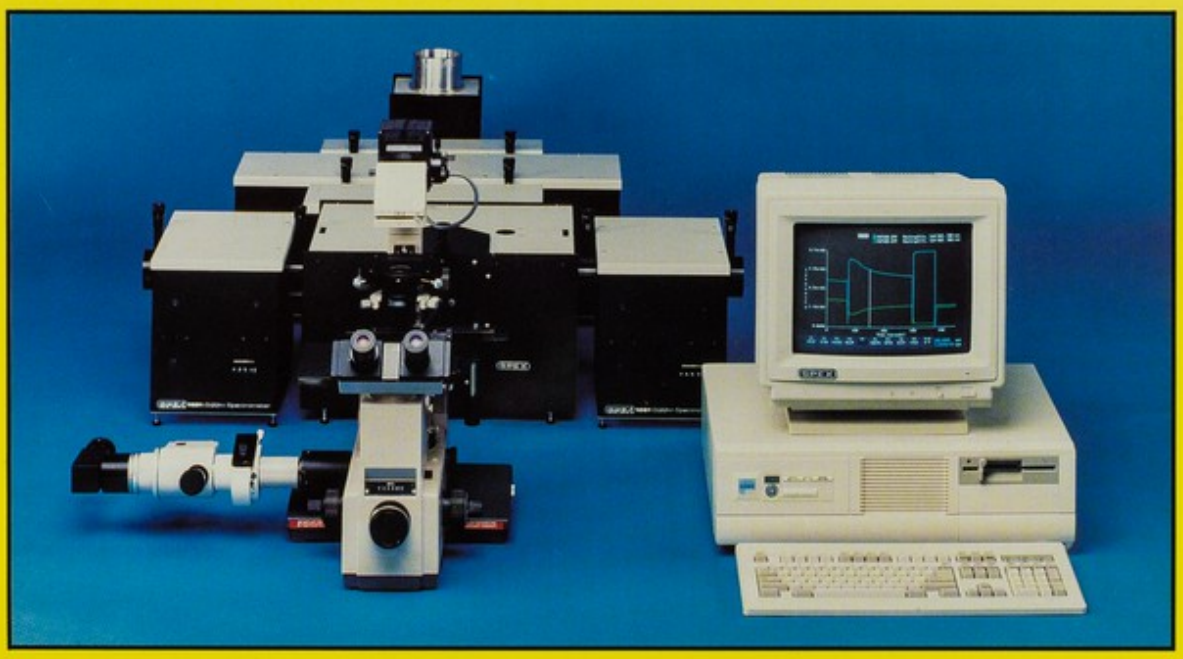
Changes in the concentration of calcium ions within cells are basic to a wide range of physiological events, including muscle contraction, nerve impulse transmission, drug responses, vision, fertilization and tumor growth. Until recently, however, better understanding of the role played by the calcium cation Ca^{2+} was prevented by the difficulty of making intracellular cation measurements, creating the need for an accurate and convenient way to collect such data.

Responding to this need, SPEX developed a spectrofluorometer system that can monitor Ca^{2+} as well as other cations in ***living*** cells with unparalled ease and accuracy. When used with the fluorogenic dye fura-2, the SPEX Cation Measurement System offers:

- specificity for intracellular Ca^{2+}
- absolute measurement of free calcium concentrations
- sensitivity to 10 picomoles
- detection of changes in fast-response cells
- choice of macroscopic or microscopic measurements
- isolation of small cells on the microscopic stage

The SPEX CM System is available in a number of versions to accomodate individual research needs and the requirements of working with fluorogenic probes that have either distinctive dual-excitation or dual-emission characteristics.

- Our macro-sampling system with two excitation monochromators is ideal for studies using dual-excitation probes to measure average cation concentration for a cell population in a cuvette and monitoring changes over time.
- Our dual-emission system incorporates two emmission monochromators and the T-format sample compartment required for dual-emission probes.
- Adding our optical interface kit and auxilary detection module allows you to attach an epifluorescence microscope to your CM System for measuring the concentration of cations in single cells.
- For maximum versatility with probes like fura-2, our fully integrated macro/micro-sampling system includes an epifluorescence microscope and illuminator in addition to a variable-aperture photometer for isolating individual cells on microscope stage.



The Fura-2 Calcium Measurement Technique

Other techniques for measuring intracellular Ca^{2+} are subject to many technical problems. Micro-electrodes, although highly sensitive, cause damage to small cells and measure only local responses in large cells. Calcium-specific photoproteins are slow to disperse and yield accurate measurements only when the intracellular ionic condition is known.

Fortunately, the introduction of fluorescent dyes that bind specifically to calcium greatly reduced the difficulty of measuring intracellular Ca^{2+}. These dyes make it possible to employ fluorescence spectroscopy for determining absolute concentrations of free Ca^{2+} without damage to living cells. Fura-2, the most effective of these calcium indicators, offers the best selectivity and superior fluorescence intensity.

Fura-2 is widely available and convenient to use. The form fura-2 AM is easily loaded into living cells, where it diffuses rapidly and causes little or no damage. Possible interference due to the binding of dye molecules to external Ca^{2+} can be eliminated by adding a buffer such as EGTA to the cell suspension or cell culture.

Differential Excitation

Although free and calcium-bound fura-2 molecules fluoresce strongly at about the same wavelength, they are excited by light at different wavelengths. It is this differential sensitivity that ultimately establishes the ratio which is the key to determining actual concentrations of calcium.

As shown by the excitation spectra in Figure 1, free molecules of fura-2 are excited by radiation over a fairly broad range, from about 350 nanometers to 400 nanometers. The narrower excitation peak for calcium-bound fura-2 is centered at 336 nanometers.

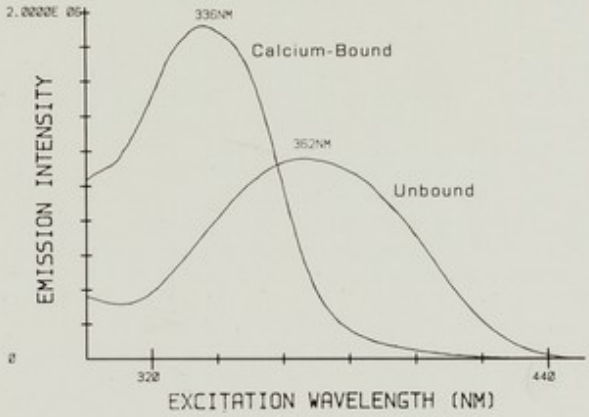


Figure 1
Excitation spectra for calcium-free fura-2 and calcium-bound fura-2. The distinct wavelength shift makes it possible to distinguish between bound and unbound dye molecules.

The specially designed SPEX CM System measures the fluorescence of bound and unbound fura-2 by rapidly alternating the exciting radiation between two selected wavelengths and separating the resulting signals electronically. The ratio of the two signals can be used to calculate the absolute concentration of intracellular Ca^{2+}. The ratio measurement inherently corrects for cell-to-cell variations in dye concentration, changes in concentration due to bleaching or leakage, and time or spatial effects on instrumental efficiency. Furthermore, the macro/micro system can be readily used in either the macro or micro mode to obtain average calcium concentration information from a cell population in suspension or from a cell monolayer on a microscope slide.

The CM System In Use

Figures 2a-2c illustrate how the SPEX CM System determined the effects of ATP and thrombin on cytosolic free calcium in a suspension of human umbilical vein endothelial cells loaded with 1 μM fura-2 AM.

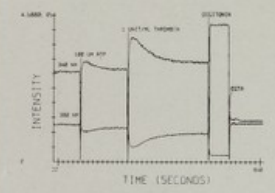


Figure 2a
Fluorescence intensity of the sample plotted at 340 and 380 nanometers.

Figure 2b
Automatically calculated ratio of the data shown in Figure 2a.

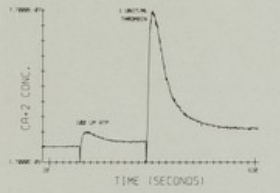


Figure 2c
The 340/380 ratio correlated with the absolute concentration of calcium in the sample.

Equipped with an epifluorescence microscope, the CM System can measure the amount of calcium in single cells as small as 5 micrometers in diameter. Figures 3a-3c involve a monolayer of smooth vascular muscle cells from the aorta of a rat.

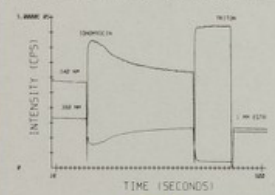


Figure 3a
Effect of ionomycin on a single cell in the monolayer.

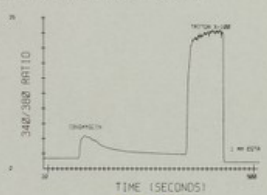


Figure 3b
The 340/380 ratioed trace after subtraction of auto-fluorescence.

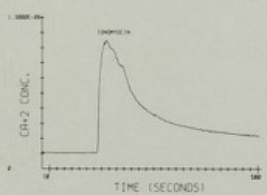


Figure 3c
Final Ca^{2+} concentration.

References

The following references provide detailed discussion of relevant theory and research.

1. T.J.A. Allen and P.F. Baker. Intracellular Ca indicator quin-2 inhibits Ca^{2+} inflow via Na/Ca exchange in squid axon. Nature 315: 755-756 [1985].
2. F. Fay. Calcium gradients in single smooth muscle cells revealed by digital imaging microscope using fura-2. Nature 318: 6046 [1985].
3. G. Grynkiewicz, M. Poenie and R.Y. Tsien. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260: 3440-3450 [1985].
4. T.R. Hesketh, G.A. Smith, J.P. Moore, M.V. Taylor and J.C. Metcalfe. Free cytoplasmic calcium concentration and the mitogenic stimulation of lymphocytes. J. Biol. Chem. 258: 4876-4882 [1983].
5. I. Kurtz and R.S. Balaban. Fluorescence emission spectroscopy of 1,4-dihydroxyphthalonitrile: a method for determining intracellular pH in cultured cells. Biophys. J. 48: 499-508 [1985].
6. A.M. Paradiso, R.Y. Tsien and T.E. Machen. Na^+ - H^+ exchange in gastric glands as measured with a cytoplasmic-trapped fluorescent pH indicator. Proc. Nat. Acad. Sci. 81: 7436-7440 [1985].
7. M. Poenie, J. Alderton, R.Y. Tsien and R.A. Steinhardt. Changes of free calcium levels with stages of the cell division cycle. Nature 315: 147-149 [1985].
8. M. Poenie, J. Alderton, R. Steinhardt and R.Y. Tsien. Calcium rises abruptly and briefly throughout the cell at the onset of anaphase. Science 233: 886-889 [1986].
9. T.J. Rink, R.Y. Tsien, and T. Pozzan. Cytoplasmic pH and free magnesium in lymphocytes. J. Cell Biology 95: 189-196 [1982].
10. J. Rogers, R. Hesketh, G.A. Smith, M.A. Beaven, J.C. Metcalfe, P. Johnson and P.B. Garland. Intracellular pH and free calcium changes in single cells using quin-1 and quin-2 probes and fluorescence microscopy. FEBS 161: 21-27 [1983].
11. T. Tiffert, J. Garcia-Sancho and V.L. Lew. Irreversible ATP depletion caused by low concentrations of formaldehyde and of calcium-chelator esters in intact human red cells. Biochem. Biophys. Acta 773: 143-156 [1984].
12. R.Y. Tsien. New calcium indicators and buffers with selectivity against magnesium and protons: Design, synthesis, and properties of prototype structures. Biochemistry 19: 2396-& [1980].
13. R.Y. Tsien. A non-disruptive technique for loading calcium buffers and indicators into cells. Nature 290: 527-537 [1981].

Figures 2a-2c show how the agonists ATP and thrombin affect cytosolic free calcium in a suspension of cultured human umbilical vein endothelial cells buffered with EGTA. Lysed with digitonin, the cells were loaded with 1 μM fura-2 AM.

Figure 2a is a time plot of the sample's fluorescence intensity at excitations of 340 and 380 nanometers. As illustrated by Figure 2b, the SPEX CM System will automatically ratio this data in order to provide information about the effects of ATP and thrombin on the absolute concentration of Ca^{2+}. Figure 2c plots the concentration of free Ca^{2+} calculated by determining F, F-min and F-max at the same instrumental sensitivity, optical path length and effective total concentration of dye.

With an attached epifluorescence microscope, the concentration of Ca^{2+} in single cells as small as 5 micrometers in diameter can be measured. Accordingly, Figures 3a-3c depict the results of adding ionomycin to a monolayer of smooth vascular muscle cells from the aorta of a rat. The cells were grown to confluence on a cover slip, loaded with fura-2 AM and viewed on the microscope stage. EGTA and the lysin Triton X-100 were added to facilitate determination of absolute calcium concentration, plotted in Figure 3c.

Expanding Cation Measurement Potential

The sensitivity and selectivity of the SPEX Cation Measurement System used in conjunction with fura-2 make it the preferred technique for monitoring intracellular free calcium, especially in single cells, adherent cell layers or bulk tissues. The fura-2 technique has already been employed to investigate the role of cell calcium in muscle contraction, neural response and cell permeability, as well as to study cell division in sea urchins.

What's more, continuing research has demonstrated that the SPEX CM concept is not limited to a specific type of tissue preparation or to the measurement of calcium exclusively with dual-excitation dyes like fura-2. Indo-1, a dye having unique dual-emission characteristics, has produced promising results in measuring Ca^{2+} with a CM System which features two emission monochromators and the T-format sample compartment required for such analyses.

Going beyond calcium, the CM System has been proven in determining cytosolic pH for individual cells using the fluorescein derivative BCECF with excitation at 490 nanometers and 438 nanometers (Reference 10). The dual-emission pH probe 1,4-dihydroxyphthalonitrile has also been utilized successfully with the versatile SPEX CM System. And sodium experiments which appear to document shifts in excitation wavelength suggest that the dual-excitation method may be applicable to the measurement of intracellular free sodium.

CM System Architecture

Essentially, each CM System model is built around a research spectrofluorometer with photon-counting detection and dedicated electronics support. Models configured for dual-excitation work include a high-intensity radiation source, a beam divider, two excitation monochromators, a variable-speed light chopper, a macro-sample compartment, an emission monochromator and a photomultiplier tube. The CM System with two emission monochromators and T-format sample compartment which facilitate the use of dual-emission probes can also be employed for all types of polarization experiments.

Our optical interface kit and auxiliary detection module allow the utilization of an inverted epifluorescence microscope of your choice. In addition to being a dual-excitation macro-sampling system, our macro/micro model is equipped with a fully integrated microscope, illuminator, photometer and detection module. An appropriately configured SPEX DM3000 spectroscopy computer provides the electronics for completely automated instrument control, signal acquisition and data processing for all CM System models.

Thus, along with offering maximum versatility for present and future cation measurement techniques, all SPEX CM Systems are at heart spectrofluorometers that can utilize the full line of SPEX accessories for general fluorescence spectroscopy. The monochromators can be preset to a particular wavelength or scanned across a spectral region. Controlled by the DM3000-CM, which includes all necessary power supplies, the variable-speed chopper allows analysis of events as fast as milliseconds or as slow as minutes in duration.

Dual Excitation

Dual-wavelength excitation is achieved via an optical design which feeds the output from a xenon lamp into two monochromators. The two beams of monochromatic radiation are then alternately directed into the sample compartment. A quantum counter monitors the intensity of the radiation to compensate for lamp fluctuations and spectral variations.

Macro and Micro Options

In the macro-sampling system, the excitation beam is focused onto a cuvette containing a cell suspension. Depending on the optical density of the sample, fluorescence can be collected either at right angles to the exciting radiation or "front face" at 22.5 degrees, after which it is passed through a monochromator to a photomultiplier tube.

The micro-sampling system offers two alternative sample setups. The exciting radiation can be focused onto the sample cuvette or directed through a port into a microscope for excitation of single-cell samples. A variable-aperture disk in the photometer makes it possible to focus precisely on an individual cell only a few microns in diameter, screening out interference from other cells or from impurities in the medium. Fluorescence from the selected cell is then passed through a filter to a photomultiplier tube or to the digital imaging camera which can be attached to the microscope.

System Control and Data Acquisition

The SPEX DM3000-CM spectroscopy computer supplied with the Cation Measurement System provides full electronics support for all your analytical work. This IBM® PC/AT compatible unit runs unique menu-driven software designed to make data acquisition as easy and accurate as possible. The DM3000-CM controls the scanning monochromators, the light chopper and the PMT high-voltage sources, and it offers convenient selection of experimental parameters.

To record fluorescence signals associated with the two excitation wavelengths, the DM3000-CM automatically synchronizes signal acquisition with the alternating excitation beams. The signals can be stored as separate data files or plotted as a real-time ratio. The acquired signals are automatically corrected for variations in lamp intensity, and DM3000-CM software routines permit easy manipulation of the stored data.

Cation measurement data obtained with the DM3000-CM spectroscopy computer can be displayed on the non-glare color monitor and printed on the system's dot matrix printer. A 1.2 megabyte floppy disk drive and 30 megabyte hard disk drive afford ample storage capacity for parameter files and experimental data.

Automated Experiments

The exclusive SPEX software package supplied with each CM System model completely automates the fura-2 calcium measurement technique. The methods routine makes it easy to customize experiments, and methods can be stored on disk so that subsequent work involving established parameters will be fully automated. This exceptional versatility also allows a number of people to pursue independent lines of research with the *same* system, permitting individuals to recall and apply personal experimental parameters at any time.

Acknowledgement

The data on umbilical vein endothelial cells illustrated in Figures 2a-2c was provided by Dr. Tom Brock of the Department of Pathology, Brigham and Women's Hospital, Boston, Massachusetts.

AR-CM Series

Filter Cation Measurement Systems for Applied Research

Fluorometric measurement of calcium and other cations in living cells has become a widely accepted biomedical research tool. SPEX Industries, a pioneer in cation instrumentation, now places this technique in the service of an even broader range of users with the introduction of the filter-based systems in the SPEX AR-CM Series.

Compact and economical AR-CM systems are ideal for routine analyses of calcium , Mg^{2+}, Na^{+}, K^{+} and pH in single living cells or cell suspensions. Employing interference filters instead of scanning monochromators, AR-CM systems are configured for time-based cation studies utilizing single-wavelength and dual-wavelength biological probes for which detailed fluorescence profiles have been established. Thus, an AR-CM makes it easy to acquire cation concentration data from a large number of samples with minimal operator attention. For more information, contact SPEX today!

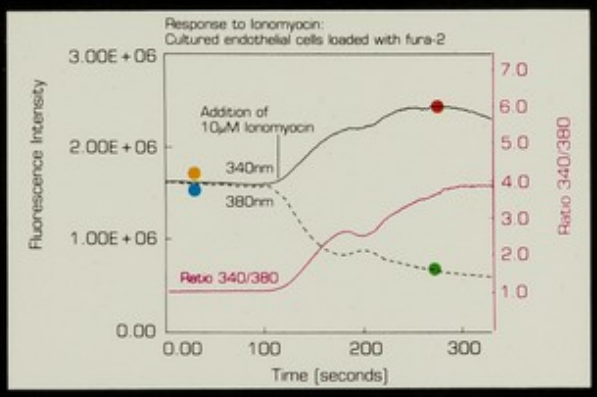
- Rapid Sampling
- Photon-Counting
- Application-Specific Filter Sets
- Microscope Interfaces
- Simultaneous Dual-Emission Detection
- Upgrading with Image Processing
- Completely Automated Experiments
- Totally Integrated, Pretested Systems

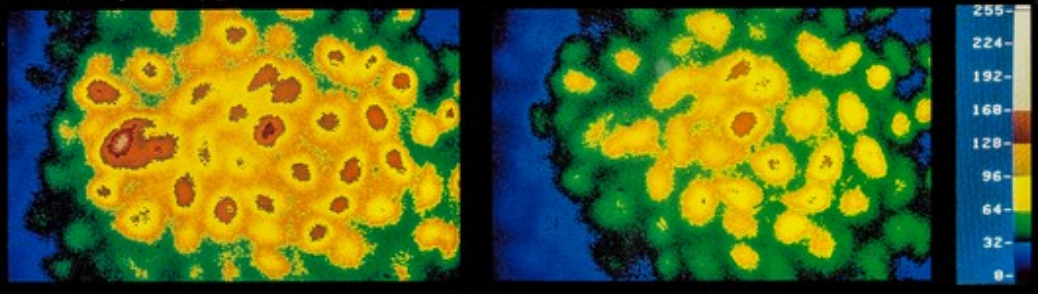
Map Ca^{2+} Migration and Other Ions in Living Cells with Digital Fluorescence Imaging

Whether your work involves cell division, vascular muscle contraction or neurotransmission, you'll want to evaluate the role of intracellular Ca^{2+}. And the best route to these data is a SPEX CM/IM System automated by our exclusive menu-driven software.

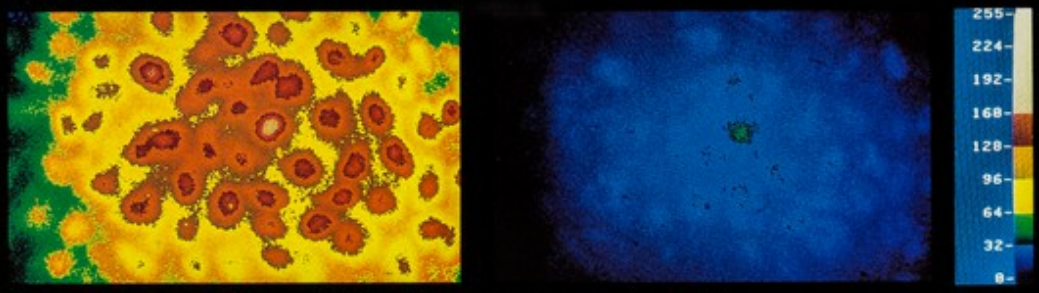
Just as a CM system will ratio fluorescence intensities to determine Ca^{2+} concentration, a CM/IM system will, in addition ratio fluorescence images providing a map of Ca^{2+} concentration across a single cell or group of cells.

These pseudo-color images of cultured bovine aortic endothelial cells loaded with the dual-excitation probe fura-2 reveal the distinctive Ca^{2+} influx for cells stimulated by ionomycin.





• 340nm **Resting Calcium** • 380nm



• 340nm **Maximum Response to Ionomycin** • 380nm

Brightfield Image of Endothelial Cells

By comparing pseudo-color images with the corresponding brightfield image, the investigator can identify individual active cells and the level of activity.

The SPEX CM/IM system integrates real-time ratio imaging with extensive feature analysis capabilities, offering a powerful tool for characterizing metabolic behavior in relation to the morphology of individual cells.