A Practical Guide to Measuring Intracellular Ca^{2+} for Beginners

I. Introduction

In the late 19th century, Ca^{2+} was proposed to be involved in muscle contraction by Ringer et al. Subsequent studies have demonstrated that Ca^{2+} plays a critical role in a wide range of biological functions. However, the intracellular Ca^{2+} concentration is extremely low (typically several hundred nmol/l), making its measurement technically challenging.

In 1980, Tsien et al. at the University of California published a method for measuring intracellular Ca^{2+} using Quin 2[1)]. The detection reagent (Ca^{2+} probe) they developed had excellent features: it could be taken into cells simply by incubating with cells, and the fluorescence intensity changed according to the Ca^{2+} concentration. Subsequent technological advances in Ca^{2+} probes and optical instruments have made intracellular Ca^{2+} imaging an accessible experimental technique, even for researchers with no prior experience in Ca^{2+} measurement.

Nevertheless, we frequently receive inquiries from researchers who encounter difficulties when attempting intracellular Ca^{2+} measurements. In fact, it is a fact that it is difficult to obtain experimental results as imagined without a good understanding of the properties of Ca^{2+} probes.

In this paper, we would like to introduce the solutions, experimental essentials, and troubleshooting methods for beginners based on the consultation on Ca^{2+} concentration measurement frequently received by our company, which handles Ca^{2+} probes.

Ⅱ Selection of Ca^{2+} probe

There are many different types of Ca^{2+} fluorescent probes. Therefore, many novice researchers are unsure of what to choose. Below are some key points to consider when selecting a Ca^{2+} probe.

1. AM derivatives?

Ca^{2+} fluorescent probes have the name AM, as in Fluo 4-AM, Fura 2-AM. “AM” refers to an acetoxymethyl ester, which temporarily masks the Ca^{2+}-chelating carboxyl groups of the probe.

Why do we use AM groups for protection? The reason is to make the probe permeable to the cell. In other words, probes whose carboxyl groups are not protected by AM groups have extremely low permeability to cells, so if you want to measure intracellular Ca^{2+} concentration, please be sure to use AM bodies. Once the probe enters the cell, intracellular esterases hydrolyze the AM group, converting the probe into its active, Ca^{2+}-chelating form and effectively trapping it inside the cell. (Fig. 1).

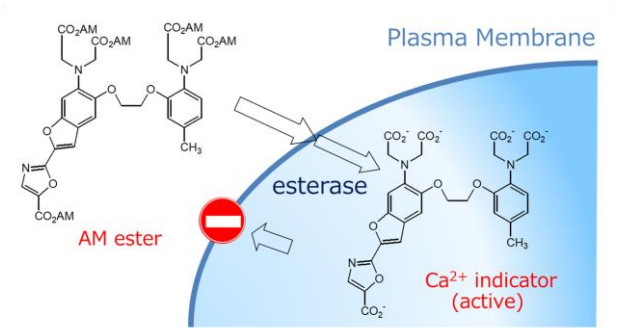

Figure 1. Cell transduction of AM ester bodies

2. Compatibility with Available Instrumentation

Each fluorescent substance has a unique maximum excitation wavelength and maximum fluorescence wavelength. If a fluorescent substance is not excited by light near the wavelength of the maximum excitation wavelength, it will not emit fluorescence efficiently. Although it would be ideal to have a device that can irradiate a wide range of excitation wavelengths, most devices usually have filters or lasers that limit the wavelengths of excitation light that can be irradiated. Therefore, it is necessary to determine whether the device on hand can excite the target fluorescent substance at the appropriate wavelength and efficiently measure the emitted fluorescence.

Fluo 3 and Fluo 4 are recommended for first-time Ca^{2+} probe users because they can be measured with general-purpose filters (B excitation for microscopes, filters around 485 nm).

Fura 2, on the other hand, requires almost simultaneous excitation at two wavelengths (fluorescence measurement

is at one wavelength). The advantage of this is discussed below, but the need for simultaneous excitation at two wavelengths (e.g., 340 nm and 380 nm) requires somewhat specialized equipment.

3. Does the dissociation constant (K_d value) of the fluorescent probe match the measurement target?

The dissociation constant (K_d value) reflects the affinity of the probe for Ca^{2+} and must be matched to the expected Ca^{2+} concentration range of the target compartment. It is necessary to select a probe with a K_d value that is appropriate for the Ca^{2+} concentration to be measured, although many people are surprisingly unaware of this. For example, for cytoplasm, Fura 2 with a $K_{(d)}$ value of about 0.2 μmol/l or Fluo 4 $_{(d)}$ value about 0.3 μmol/l are suitable. Using unsuitable probes will result in only small signal changes (Fig. 2).

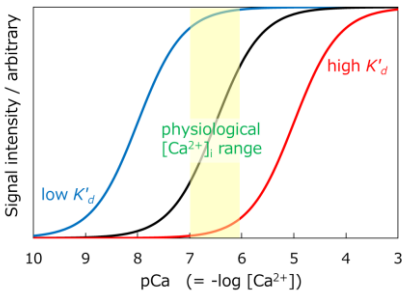

Figure 2. Relationship between Ca^{2+} concentration and signal intensity

Table 1 Ca^{2+} fluorescence probe types and fluorescence properties

name of product	Excitation wavelength	Fluorescence wavelength	Ca complex dissociation constant (K_d)	literature
Quin 2	339 nm	492 nm	115 nmol/l	1)
Fura 2	340 nm/380 nm	510 nm	224 nmol/l	2)
Fluo 3	508 nm	527 nm	0.4 μmol/l	3)
Indo 1	330 nm	Ca free : 485 nm Ca bound : 410 nm	250 nmol/l	2)
Rhod 2	553 nm	576 nm	1.0 μmol/l	3)
Fluo 4	495 nm	518 nm	345 nmol/l	4)

"Considerations Regarding Probe Concentration"

In addition to the K_d and Ca^{2+} concentrations (which usually cannot be changed), the concentration of the probe also affects the ability of the probe to sufficiently bind Ca^{2+} ions.

$K_d = [Ca^{2+}][probe] / [Ca^{2+}, probe]$.

Kd is the equilibrium constant for the dissociation equilibrium. Thus, in addition to Kd, the concentration of the probe also influences the formation of Ca^{2+}-probe complexes.

If the probe concentration is increased too much, Ca^{2+} of intracellular Ca^{2+}-binding proteins will also bind to the probe (this is affected by the $K_{(d)}$ for Ca^{2+} of the probe and K_d for Ca^{2+} of the protein). For cultured cells, a concentration of 1-5 μmol/l and an introduction time of 30 to 60 minutes is generally used.

4. Is Quantitative Determination of Intracellular Ca^{2+} Necessary?

You may be thinking, "Isn't the probe used to determine Ca^{2+} concentration in the first place?" However, what is obtained by the measurement is only the fluorescence intensity, not the Ca^{2+} concentration. The fluorescence intensity must be converted to Ca^{2+} concentration by means of calibration. Broadly speaking, there are two calibration methods: **in vitro calibration**, in which fluorescence is measured at different Ca^{2+} concentrations in a salt solution mimicking the intracellular ionic environment to generate a calibration curve correlating fluorescence intensity with Ca^{2+} concentration. The other method is ***in vivo* calibration**, in which probe-transfected cells are treated with Ca^{2+} ionophores (ionomycin or Br-A23187) to increase the Ca^{2+} permeability of the cell membrane, and fluorescence is measured by changing the
extracellular solution. The intracellular Ca^{2+} concentration is calculated by a formula from the K_d value for Ca^{2+} of the probe, the maximum fluorescence value, and the minimum fluorescence value. In general, when calculating intracellular Ca^{2+} concentration, Fura 2-AM with two-wavelength excitation is often selected, which is less susceptible

to cell thickness and probe leakage.

However, these calibration methods do not always provide accurate absolute values of ion concentrations. Although dissociation constants of 135 nmol/l or 224 nmol/l reported by Tsien et al.2) are commonly used for Fura 2, actual Kd values vary depending on intracellular conditions. For example, it is known that the K_d value of Fura 2 increases when the intracellular protein binds to Fura 2[5]. However, it is extremely difficult to accurately determine the extent to which intracellularly incorporated Fura 2 binds to proteins, and the exact dissociation constant is unfortunately unknown. Thus, it is virtually impossible to determine the exact intracellular Ca^{2+} concentration. Perhaps because of this background, many publications describe the value of ratiometry (ratio of fluorescence intensity of two-wavelength measurement) as it is in the case of two-wavelength excitation such as Fura 2. In the case of one-wavelength excitation such as Fluo 4, it is sometimes expressed as the percentage change in fluorescence compared to the beginning of measurement.

5. Single-Wavelength versus Dual-Wavelength Excitation

If you only need to discuss the rate of change in fluorescence intensity, we recommend single-wavelength excitation probes such as Fluo 3-AM and Fluo 4-AM. These probes, whose fluorescence intensity changes according to intracellular Ca2+ concentration, have fixed excitation and emission wavelengths, making them easier for beginners to use.

It is possible to obtain data such as, "If the fluorescence intensity (relative value) before the addition of the drug you want to measure is 5000, the fluorescence intensity (relative value) became 20,000 after the addition of the drug. However, a point to note about probes with single-wavelength excitation is that the fluorescence intensity changes according to the amount of probe taken into the cell, the thickness of the cell, and the fading of the probe (Figure 3). In other words, the probe is susceptible to variations depending on the measurement conditions. Fluorescent probes with two-wavelength excitation, such as Fura 2, can solve these problems: the fluorescence intensity of

Fura 2 increases with 340 nm excitation but decreases with 380 nm excitation as Ca^{2+} concentration increases (Figure 4). By discussing these ratios (ratiometry), even if the amount of Fura 2 taken up by cells differs, the ratios will remain constant, allowing data to be acquired with little variation. Such probes with two-wavelength excitation are often used to calculate intracellular Ca^{2+} concentrations.

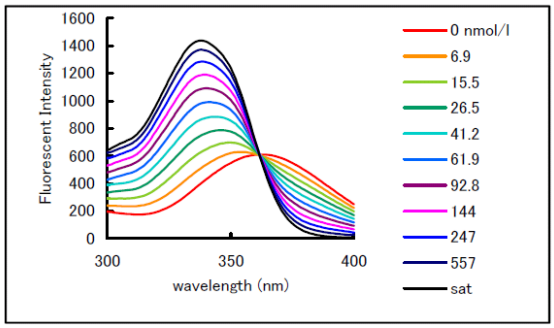

Figure 3. Variation of excitation spectrum of Fura 2

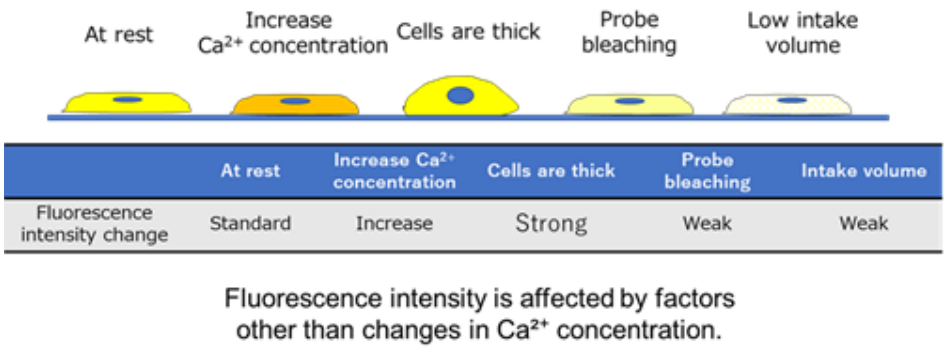

Figure 4. Signal increase or decrease with probe

III Selection of measurement device

Fluorescence plate readers and fluorescence microscopes are probably the most familiar fluorescence measurement devices for users of cultured cells. We often receive inquiries such as, "I have been using a fluorescent plate reader and a 96-well plate to measure Ca^{2+} concentration, but I have been having trouble. Here, we will introduce a protocol using a fluorescent plate reader for those who would like to measure Ca^{2+} with a fluorescent plate reader for ease of use.

① Fluorescent Plate Reader

When measuring with a fluorescent plate reader, a type of device with an injector function is desirable. The reason is that the Ca^{2+} concentration in cells due to drug response often occurs instantaneously after addition. However, depending on the type of drug, the intracellular Ca^{2+} concentration may gradually increase after addition and remain constant at a certain level. With such agents, it is possible to follow the change in fluorescence intensity if the measurement is made immediately after addition. However, if the Ca^{2+} concentration rises for a moment (a few seconds) and then immediately decreases, the fluorescence intensity may have decreased by the time of measurement. Also, when measuring with a fluorescent plate reader, it is important to note that fluorescence plate readers measure population-averaged signals rather than single-cell responses. As is obvious when adding a drug such as ionomycin while observing with a fluorescence microscope, cells do not all undergo the same change in fluorescence intensity. Some cells emit fluorescence even before the drug is added, while others show almost no change in fluorescence intensity after the drug is added. The fluorescence intensity changes of cells in the excitation light path in the plate reader are averaged and the values appear as fluorescence intensity changes. Therefore, it is important to note that depending on cell conditions, the fluorescence intensity may change slowly, or significant changes may occur in only a small subset of cells, causing minimal changes in measured average fluorescence. In addition, if there are no cells in the area where the excitation light hits, no increase in fluorescence intensity can be observed.

② fluorescence microscope

Many types of this system are inverted and can use gradient fluorescence. The Fura 2 requires a device to switch between two wavelengths alternately. filters.

If fluorescence generated from cells is to be quantified, an image processing system with a CCD camera is required. For details on the principles and fundamentals of each system, please contact the manufacturer of each system. Depending on the type of imaging system, it may be possible to follow changes in fluorescence intensity in a single cell.

IV Probe Dissolution Method

Due to their lipophilic nature, AM ester probes exhibit poor solubility in aqueous solutions. Therefore, it is necessary to dissolve them in a solvent such as DMSO and then mix them with the buffer solution for measurement. However, AM bodies form granules in water and float in the buffer solution. In such suspended solution, the efficiency of cellular uptake is greatly reduced. Therefore, it is necessary to use a small amount of surfactant (Pluronic F-127 or Cremophor EL) or ultrasonic treatment to increase the uptake efficiency.

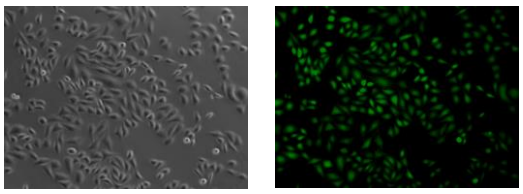

Figure 5. Images of cells after Fluo 4-AM incorporation (left: bright field, right: fluorescence)

V Example of measurement of intracellular Ca^{2+} concentration in a plate reader using cells

Dojindo offers a Calcium Kit that includes reagents necessary for measuring intracellular Ca^{2+} concentration. In addition to the Ca^{2+} probe, the kit includes DMSO for dissolution, surfactant Pluronic F-127, and an anion transporter inhibitor Probenecid, and is recommended for those who are measuring intracellular Ca^{2+} for the first time. Here we introduce two examples of experiments, one using the kit and the other using the reagent (Fluo 4-AM).

[Experimental Example] Using a fluorescent plate reader equipped with a Calcium Kit and an injector function (Infinite M200)

1. Reagent
Calcium Kit - Fluo 4 (product code: CS22)
Drug
Cells CHO cells (Chinese hamster ovary-derived cells)
PBS (phosphate buffered saline: NaCl 8 g/l, KCl 0.2 g/l, KH_2PO_4 0.2 g/l, Na_2HPO_4 1.15 g/l)
Plate Clear bottom plate (Nunc)
*Although measurement is possible with models that can measure upward fluorescence with upward excitation without using a clear bottom plate, a clear bottom plate is easier to use because it allows measurement while observing the state of the cells with an optical microscope.

2. Reagent preparation (for one 96 well plate)
1) Preparation of Fluo 4-AM DMSO solution
Add 50 µl of DMSO to 50 µg (1 bottle) of Fluo 4-AM and dissolve by pipetting.

2) Loading Buffer preparation
Add 50 µl of Fluo 4-AM DMSO solution to 5 ml of Recording Medium (2×). Add Pluronic F-127 (final concentration 0.04 % (w/v)), a surfactant to facilitate uptake of the Ca^{2+} probe into the cells, and Probenecid (final concentration 1.25 mmol/l) to prevent leakage of the Ca^{2+} probe from the cells, if necessary, and pure water to bring the total volume to 10 ml. Add pure water to make the total volume 10 ml. Mix well using a vortex mixer or ultrasonic waves.

3) Preparation of Recording Medium (1×)
Add 50 µl of probenecid (1.25 mmol/l) to 5 ml of Recording Medium (2×), mix well after adding pure water to bring the total volume to 10 ml. 37 Keep warm at℃ .

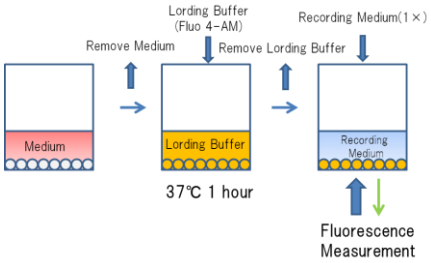

Figure 6. Intracellular Ca^{2+} measurement method

3. Assay protocol (for one 96 well plate)
1) Prepare a suspension of cells, dispense into plates at 40,000 cells/100 µl per well, and incubate overnight in a CO_2 incubator.
*Too few cells may cause cells to be biased to the edge of the well. To ensure that cells are cultured in the central light path, the cell density should be 80% to 90% confluent.

2) Remove the medium so as not to damage the cells. Wash cells several times gently with PBS preheated to 37℃. If cells detach easily, avoid washing. Residual serum components can degrade Fluo 4-AM.

3) Add 100 µl of Loading Buffer to each well.

4) Incubate at 37℃ for 1 hour.
*Incubation for more than 1 hour is not recommended because it may cause probe localization and leakage.

5) Remove Loading Buffer so as not to damage the cells. Wash cells several times in PBS heated to 37℃ (do not wash if cells are easily detached), as hydrolyzed probes can cause background elevation.

6) Add 100 µl/well of Recording Medium (1×) that has been preheated to 37℃ .

7) Measure the change in fluorescence intensity with the addition of the drug (ATP) using a plate reader.

Example: Setting conditions for Infinite M200 (fluorescent microplate reader)

Plate Definition	96well Flat Black microplate
Part of Plate	Select rows
Kinetic Cycle	100 cycles
Kinetic condition	Handling for cycle 10
Injection	Injector A injects 20 µl with speed 100 µl/sec.
Fluorescence Intensity	Ex.485 nm/Em.535 nm, gain 100

4. Results

The fluorescence intensity increased when ATP was added 10 seconds later, indicating that the intracellular Ca^{2+} concentration increased.

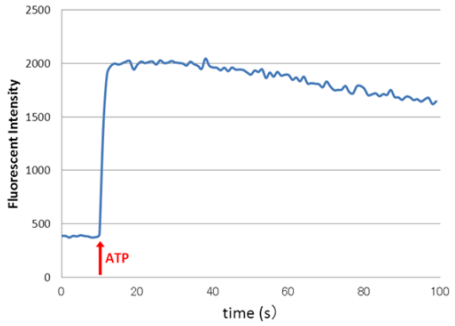

Figure 7. CHO cells are stimulated with ATP (arrows).

5. points to note when measuring

Fluo 4-AM is stored frozen. Repeated long-term storage or freezing and thawing of Fluo 4-AM dissolved in DMSO may cause degradation of the Ca^{2+} probe. If not used up at once, divide into small portions and store frozen. Loading Buffer should be prepared as needed. Stored Fluo 4-AM is hydrolyzed, and the efficiency of introduction into cells is significantly reduced.

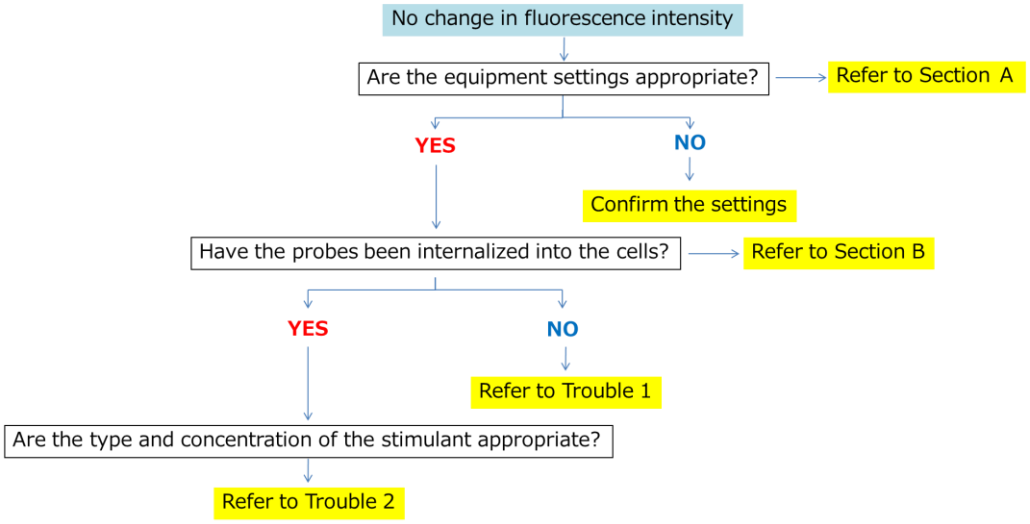

Figure 8. Troubleshooting flowchart

VI Troubleshooting

Although successful Ca^{2+} responses are often observed, many researchers have trouble obtaining reproducible fluorescence changes. Below are some fundamental troubleshooting guidelines.

If fluorescence intensity changes are not obtained, there are three main possible causes

1. There is a problem with the setup conditions of the device (e.g., detection system such as filters, direction of excitation light and fluorescence detection, etc.).
2. Ca^{2+} probe has not been introduced into the cell.
3. Drugs are not suitable (e.g., concentration and type).

To solve the problem, although it may be tedious, we recommend that the above three key points be solved one by one from the top.

The following are ways to check each of these.

Section A. How to check if the equipment setup conditions and filters are suitable for Ca^{2+} probing

Force hydrolysis of Fluo 4-AM and check for increase in fluorescence intensity.

<Procedure>

1) Add 100 µl each of PBS, medium with 10% serum, and 0.1 mol/l NaOH to each well of a 96-well plate (without cells) (Fig. 11 top).
2) Then add 100 µl of Loading Buffer and incubate at 37℃ for 1 h. Fluo 4-AM is hydrolyzed by alkali and serum.
3) Measure with a fluorescent plate reader.

If the fluorescence intensity of the wells filled with NaOH or medium containing serum does not change compared to the fluorescence intensity of the wells filled with PBS+Loading Buffer, there is a problem with the device or filter, and the settings of the device should be checked again. Since the change in fluorescence intensity in cells is very small, the increase in fluorescence intensity may not be detected if a plate reader with reduced fluorescence detection sensitivity is used.

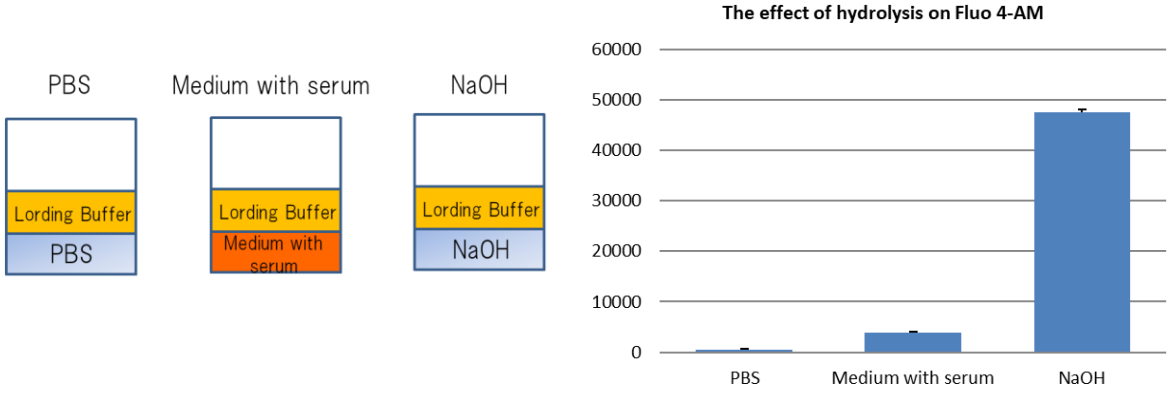

Figure 9. Schematic of addition to each well (top) and Fluo 4-AM hydrolysis results (bottom)

Section B. How to confirm that the Ca^{2+} probe has been introduced into the cell

Observe directly with a fluorescence microscope or treat with Ca^{2+} ionophores (ionomycin or Br-A23187) to increase the Ca^{2+} permeability of the cell membrane, then check for increased fluorescence intensity.

<Procedure>

1. If a fluorescence microscope is available, observe the cells.

In the case of Fluo 4, slight fluorescence is observed when the Ca^{2+} probe is introduced into the cells; Fura 2 and Fluo 3 are less sensitive than Fluo 4 and may not be observed.

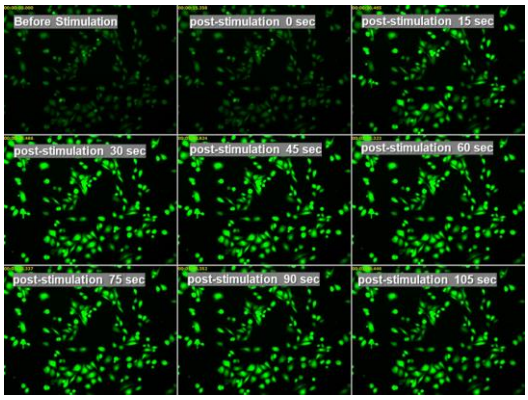

Figure 10. After loading CHO cells with Fluo 4-AM Drug (ionomycin) stimulated and observed every 15 seconds

If a fluorescence microscope is not available, check by either of the following methods

[Method 1: Method using Ca^{2+} ionophore and GEDTA (EGTA)]

<Procedure>

1) Prepare 10 µmol/l ionomycin solution by adding 990 µl of PBS to 10 µl of 1 mmol/l ionomycin solution.
   A stock solution of 1 mmol/l of ionomycin free acid may be prepared by dissolving it in DMSO. Depending on the cell type, ionophores such as Br-A23187 should be used.

2) Instead of the drug, add 10 µl of ionomycin solution to a final concentration of about 1 µmol/l and quickly measure the fluorescence intensity change. If uptake into the cell has occurred, a change in fluorescence intensity will occur.
3) Next, add 12 µl of 100 mmol/l GEDTA solution (final concentration 10 mmol/l), incubate at 37℃ for about 5 minutes, and measure the change in fluorescence intensity.
   Preparation of 100 mmol/l GEDTA solution
   Dissolve 380 mg of GEDTA (Tong Ren product code: G002) in 10 ml of PBS or Recording Medium (x1).

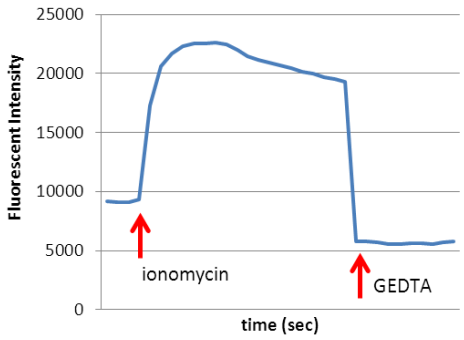

Figure 11. Fluorescence intensity changes when ionomycin and GEDTA are added to CHO cells.

*Because Ca^{2+} ionophores such as ionomycin are agents that force Ca^{2+} permeability of the cell membrane, an increase in fluorescence intensity is observed if the Ca^{2+} probe is incorporated into the cell. On the other hand, GEDTA (EGTA) is a Ca^{2+} chelating agent, so when GEDTA is added outside the cell with ionomycin, the intracellular Ca^{2+} concentration decreases and the fluorescence intensity decreases.

If no change in fluorescence intensity is observed after addition of Ionomycin solution and GEDTA, it is possible that the Ca^{2+} probe is not incorporated into the cells. Refer to the following troubleshooting [Trouble 1] to try to solve the problem.

[Method 2: Method of lysing cell membranes and checking for the presence of probes in the cells]

<Procedure>

1) Prepare 10% Triton X-100 (surfactant that dissolves cell membranes) in PBS.
2) Add 10 µl of Triton X-100 solution to a final concentration of about 1% in place of the drug, and measure the change in fluorescence intensity after several minutes. If uptake into the cell has occurred, the Ca^{2+} probe leaks out as the cell membrane dissolves, and the probe binds to the Ca^{2+} in the recording medium, and a change in fluorescence intensity is observed.
   Extracellular Ca^{2+} probes should be washed off before adding Triton X-100 solution.

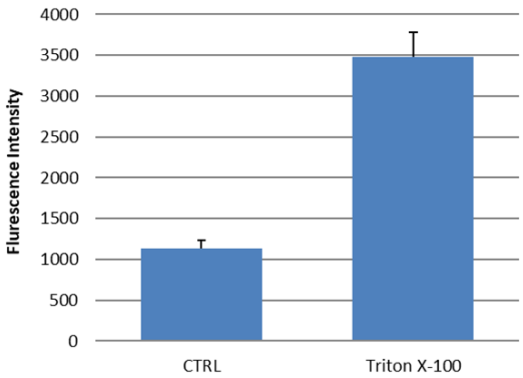

Figure 12. Changes in fluorescence intensity are observed when the cell membrane of a cell incorporating the probe is lysed.

If there is no significant change in fluorescence intensity when 1% Triton X-100 is added, it is possible that the Ca^{2+} probe is not incorporated into the cells (assuming that the measurement system is intact).

Refer to the following troubleshooting [Trouble 1] to try to solve the problem.

Trouble 1: Ca^{2+} probe is not incorporated into the cell

Possible Factors	Recommended Solutions
Ca^{2+} probe is hydrolyzed and not introduced into the cell.	Prepare Loading Buffer with the new Ca^{2+} probe. Ca^{2+} probes are also hydrolyzed by water in DMSO. Avoid using DMSO that has been opened for a long time.
Intracellular Ca^{2+} probes are leaking from cells.	① Increase the concentration of probenecid added when preparing the Loading Buffer from 1.25 mmol/l to about 2 mmol/l. ② Probenecid solution is added to Recording Medium to prevent leakage.
DMSO solution of Ca^{2+} probe and Recording Medium are not well mixed.	① If Pluronic F-127 is not used, add it. ② When the Ca^{2+} probe DMSO solution and Recording Medium are mixed, ultrasound is applied for a few seconds.
Extremely low intracellular esterase activity.	Because of the nature of the cells, it is difficult to measure them as they are. Change the cell type if possible.

If a change in fluorescence intensity is observed with the Ionomycin solution, but no change in fluorescence intensity is observed with the target drug or the change in fluorescence intensity is not obtained as desired, refer to [Trouble 2] and [Trouble 3] below to solve the problem of drug stimulation.

Trouble 2: Assuming that a change in fluorescence intensity is observed with ionomycin, no change in fluorescence intensity is observed with the target drug.

Possible Factors	Recommended Solutions
Drug concentration is too high or too low. The drug might not induce a Ca^{2+} response.	Consider varying the concentration of the agent used. If the drug concentration is too high, the cells may die as a result and no change in fluorescence intensity may be observed. The low concentration side of the drug also needs to be considered.

Trouble 3: A weak cellular Ca^{2+} response signal and a slow cellular Ca^{2+} response.

Possible Factors	Recommended Solutions
Drug concentration is too high or too low.	Consider varying the concentration of the agent used. If the drug concentration is too high, the cells may die as a result and no change in fluorescence intensity may be observed. The low concentration side of the drug also needs to be considered.
The presence of hydrolysed Ca^{2+} probes in the recording medium raises the background.	After removing the Loading Buffer, wash several times with PBS heated to 37℃. If cells are easily detached and cannot be washed, the use of a non-wash type Calcium Kit (product code: CS32) may be a solution.
Poor cell condition. A slow increase in fluorescence intensity may be observed due to a mixture of cells, some of which undergo a change in fluorescence intensity due to the addition of drugs, while others do not.	① Take a longer time for pre-culture and measure the cells when their condition has recovered. ② Reduce the incubation time from 1 hour to around 30 minutes, as prolonged incubation can cause cell damage. ③ Reduce DMSO content and Fluo 4-AM concentration (reduce toxicity of DMSO and probes). ④ Add Loading Buffer without removing the medium containing serum. In some cases, the inclusion of serum may reduce the effect on cells.

VII Related Products

<Kit Products

Product name	capacity	item code	remarks
Calcium Kit-Fluo 4	10 plates	CS22	Wash type Recommended for beginners
Calcium KitⅡ-Fluo 4	10 plates	CS32	Non-wash type Convenient for measurement of cells that easily peel off
Calcium Kit-Fura 2	10 plates	CS23	Wash type Ratiometry measurement is available
Calcium KitⅡ-Fura 2	10 plates	CS33	Non-wash type Ratiometry measurement is available

<Other related reagents

Product name	capacity	item code	remarks
GEDTA (EGTA)	5 g	G002	Ca^{2+} chelating agent Used during calibration
HEPES	25 g	GB10	Used for recording medium

VIII References

1) R. Y. Tsien*, J. Biol. Chem.* **1980**, *19*, 2396.
2) G. Grynkiewicz, M. Poenie, R. Y. Tsien, *J. Biol. Chem.* **1985**, *260,* 3440.
3) A. Minta, J. P. Y. Kao, R.Y. Tsien*, J. Biol. Chem.* **1989**, *264*, 8171.
4) K. R. Gee, K. A. Brown, W-N. U. Chen, J. Bishop-Stewart, D. Dray, I. Johnson*, Cell Calcium*, **2000**, *27*, 97.
5) M. Konishi, A. Olson, S. Hollingworth, S. M. Baylor, *Biophys. J.***, 1988**, *54*, 1089.