

## Measurement of $[Ca^{2+}]_i$ in Whole Cell Suspensions Using Fura-2

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### 1. Introduction

An elevation in intracellular calcium concentration ( $[Ca^{2+}]_i$ ) acts to trigger a range of cellular events including neurotransmitter release, muscle contraction, and oocyte fertilization (**1,2**). The pattern of elevation in  $[Ca^{2+}]_i$  and response to that elevation is dependent on the agonist and the cell type.

The introduction of the calcium-sensitive dye fura-2 (**3**) revolutionized the measurement of  $[Ca^{2+}]_i$  in whole cell suspensions, populations of adherent cells, single cells, and in subcellular regions (*see ref. 4*). Fura-2 is a ratiometric dye in that when  $Ca^{2+}$  binds, the excitation spectrum shifts rightward. In the presence of  $Ca^{2+}$ , maximum fura-2 fluorescence (at 510 nm emission) is observed at a wavelength of 340 nm and in  $Ca^{2+}$ -free conditions at 380 nm. Therefore, it follows that the concentration of free intracellular  $Ca^{2+}$  is proportional to the ratio of fluorescence at 340/380. The Grynkiewicz equation describes this relationship (**3**).

$$[Ca^{2+}]_i \text{ (nM)} = K_d \times [(R - R_{\min}) / (R_{\max} - R)] \times Sfb$$

where  $K_d$  (for  $Ca^{2+}$  binding to fura-2 at 37°C) = 225 nM,  $R$  = 340/380 ratio,  $R_{\max}$  = 340/380 ratio under  $Ca^{2+}$ -saturating conditions,  $R_{\min}$  = 340/380 ratio under  $Ca^{2+}$ -free conditions, and  $Sfb$  = ratio of baseline fluorescence (380 nm) under  $Ca^{2+}$ -free and -bound conditions. The  $K_d$  for  $Ca^{2+}$  binding to fura-2 decreases with decreasing temperature.

As noted in Chapter 1, fura-2-free acid is  $Ca^{2+}$  sensitive but membrane impermeant. To effect cell loading, cells are incubated with fura-2 pentaacetoxymethyl

(AM) ester; this form of the dye is  $\text{Ca}^{2+}$  insensitive. Once inside the cell, esterase enzymes sequentially cleave the AM groups to leave fura-2-free acid trapped inside the cell, where it is able to bind  $\text{Ca}^{2+}$ .

In this chapter the authors will describe the use of fura-2 to measure  $[\text{Ca}^{2+}]_i$  in suspension of several different cell types (*see* **ref. 4**). The technique is quite straightforward and involves incubating cells with fura-2/AM, a postincubation period to allow full de-esterification and extensive washing.

In cell suspensions, an estimate of global changes in  $[\text{Ca}^{2+}]_i$  can only be made. This is useful in combination with the currently available pharmacological agents to study sources of  $\text{Ca}^{2+}$  (intracellular vs extracellular) in a given response and to screen for  $\text{Ca}^{2+}$  mobilizing drugs and receptors. However, detailed information regarding subcellular localization requires more sophisticated measurements using standard subcellular imaging (*see* Chapter 5) or confocal microscopy (*see* Chapter 6).

## 2. Materials

### 2.1. Cell Culture

1. Undifferentiated SH-SY5Y human neuroblastoma cells (gift from Dr. J. L. Beidler, Sloane Kettering Institute, Rye, NJ).
2. Culture medium for SH-SY5Y cells: minimum essential medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU/mL penicillin, 100 IU/mL streptomycin, and 2.5  $\mu\text{g/mL}$  fungizone (*see* **Note 1**).
3. NG108-15 neuroblastoma X glioma hybrid cells (*see* **Note 2**).
4. Culture medium for NG108-15 cells: Dulbecco's minimum essential medium supplemented with 10% FCS, 2 mM glutamine, 100 IU/mL penicillin, 100 IU/mL streptomycin, 2.5  $\mu\text{g/mL}$  fungizone, and HAT (hypoxanthine [0.1 mM], aminopterin [0.4  $\mu\text{M}$ ], thymidine [16  $\mu\text{M}$ ]) (*see* **Note 1**).
5. Chinese hamster ovary (CHO) cells expressing the recombinant  $\delta$  opioid receptor (gift from Dr. L. A. Devi, Department of Pharmacology, New York University, NY).
6. Culture medium for CHO cells: HAMS F12 medium supplemented with 10% FCS, 100 IU/mL penicillin, 100 IU/mL streptomycin, 2.5  $\mu\text{g/mL}$  fungizone, and 100  $\mu\text{g/mL}$  geneticin (*see* **Notes 1 and 3**).

### 2.2. Buffers

1. Krebs HEPES buffer (for loading and washing): 143.3 mM  $\text{Na}^+$ , 4.7 mM  $\text{K}^+$ , 2.5 mM  $\text{Ca}^{2+}$ , 1.3 mM  $\text{Mg}^{2+}$ , 125.6 mM  $\text{Cl}^-$ , 25 mM  $\text{HCO}_3^-$ , 1.2 mM  $\text{H}_2\text{PO}_4^-$ , 1.2 mM  $\text{SO}_4^{2-}$ , 11.7 mM glucose, and 10 mM HEPES, pH 7.4 titrated with 10M NaOH.
2. Nominally  $\text{Ca}^{2+}$ -free Krebs HEPES buffer, pH 7.4, as in **item 1** omitting  $\text{Ca}^{2+}$  and adding 0.1 mM EGTA. This should be made in plastic beakers as glass leaches significant amounts of  $\text{Ca}^{2+}$ .
3. Low  $\text{Na}^+$  Krebs HEPES buffer, pH 7.4, for depolarization: 43.3 mM  $\text{Na}^+$ , 2.5 mM  $\text{Ca}^{2+}$ , 1.3 mM  $\text{Mg}^{2+}$ , 125.6 mM  $\text{Cl}^-$ , 25 mM  $\text{HCO}_3^-$ , 1.2 mM  $\text{H}_2\text{PO}_4^-$ , 1.2 mM

$\text{SO}_4^{2-}$ , 11.7 mM glucose, and 10 mM HEPES. With this buffer, 100 mM  $\text{K}^+$  is added (*see Note 4*).

4. Cell harvest buffer: 10 mM HEPES-buffered 0.9% saline plus 0.05% EDTA, pH 7.4 (with 10 M NaOH).

### 2.3. General Reagents

1. Fura-2/AM (Sigma, Dorset, UK). Make up as a stock (1 mM) solution by dissolving in dimethylsulfoxide and storing aliquots (10  $\mu\text{L}$ ) at  $-20^\circ\text{C}$  until required.
2. Triton X-100 (Sigma). Make a stock (4%) solution in warmed water.
3. EGTA (Sigma). Make a stock (90 mM) solution in 1 M NaOH.
4. Probenecid (Sigma). Dissolve at 50 mg/mL (175 mM) stock in 1 M NaOH. Use at 2.5 mM in buffer (*see Note 5*).

## 3. Methods

### 3.1. Tissue Culture and Monolayer Harvesting

1. Maintain confluent monolayers (75  $\text{cm}^2$ ) of cells in the appropriate media.
2. Split one flask of confluent cells using trypsin (0.5g/L)-EDTA (2g/L, 5 mL) solution as supplied (*see Note 1*) into nine other flasks each containing 20 mL of supplemented media. After 2 d of incubation ( $37^\circ\text{C}$ , 5%  $\text{CO}_2$  incubator), remove the media and replace with 25 mL of fresh supplemented media.
3. Culture cells (feed 24 h before use with fresh medium) until confluent (use cells up to 3 d post confluency).
4. On the day of the experiment, remove medium and add 5 mL of harvest buffer to cell monolayer.
5. Remove 5 mL of harvest buffer immediately, and add a further 5 mL of fresh harvest buffer and incubate at  $37^\circ\text{C}$  for  $\sim 5$  min.
6. Gently tap the side of the flask to dislodge the adherent cell monolayer.
7. When all the cells are in suspension, transfer it to a centrifuge tube. Rinse the cells out of the flask by adding approx 15 mL of experimental buffer. Transfer this to the centrifuge tube.
8. Sediment at 1000g in a low-speed centrifuge for 3 min.
9. Remove supernatant and resuspend the pellet into 30 mL of fresh experimental buffer. Invert the tube three times and resediment at 500g for 3 min.
10. Repeat **step 9** once more, and finally resuspend the pellet in 3 mL of experimental buffer.

### 3.2. Fura-2 Loading and Measurement of Intracellular Calcium

Optimal fura-2 loading time and de-esterification time may vary depending on the cell type used, and hence it is recommended that these times should be adjusted accordingly. The protocol used by the authors in a range of cell types is as follows:

1. Incubate cell suspensions with 3  $\mu\text{M}$  of fura-2/AM in 3 mL (10  $\mu\text{L}$  of 1 mM fura-2/AM) for 30 min at  $37^\circ\text{C}$  in the dark.

2. Sediment the cells (500g for 2 min) and resuspend in 30 mL of Krebs/HEPES buffer.
3. Incubate a further 15 min at room temperature in the dark to allow for de-esterification of the fura-2/AM.
4. Sediment the cells (500g for 2 min) and resuspend in 30 mL of Krebs/HEPES buffer twice more.
5. Sediment (500g for 2 min) and resuspend in buffer allowing 2 mL per determination (*see Note 6*).
6. Place cell suspensions (2 mL) in a quartz cuvet containing a magnetic stirrer and place in the cuvet holder, which is maintained at 37°C with a water jacket.
7. Simultaneously monitor and, if possible, display 340 and 380 excitation intensity (at 510 emission). Signal sampling should be set according to the kinetics of the changes in  $[Ca^{2+}]_i$ ; the authors routinely make one ratio measurement per second (*see Note 7*).
8. Following establishment of stable 340 and 380 recordings, add compounds to be tested (*see Note 8*).
9. Maintain stock of loaded cells on ice (*see Note 9*).
10. Calibrate the fluorescence signal as follows (*see Note 10*):
  - a. Add 0.1% Triton X-100 to the cuvet to produce cell lysis and liberate fura-2 into a  $Ca^{2+}$ -containing buffer. Under these conditions, fura-2 saturates with  $Ca^{2+}$  and maximum fluorescence ratio ( $R_{max}$ ) is determined (*see Note 11*).
  - b. 4.5 mM EGTA, pH >8.0, to chelate  $Ca^{2+}$  and determine minimum fluorescence ratio ( $R_{min}$ ) (*see Note 12*).
  - c. Substitute  $R_{max}$ ,  $R_{min}$ , and the derived Sfb along with measured  $R$  values from cell suspensions into the Grynkiewicz equation (3) and estimate  $[Ca^{2+}]_i$ . This can be accomplished using a spreadsheet type program, although the authors use FLDM software associated with the fluorimeter (*see Note 7*).

### 3.3. Examples of $[Ca^{2+}]_i$ Measurements Made in Cell Suspensions

#### 3.3.1. Carbachol Stimulation in SH-SY5Y Cells

SH-SY5Y cells express a homogenous population of  $M_3$  muscarinic receptors that are coupled to phospholipase C and increased  $[Ca^{2+}]_i$ . The authors have shown that this  $[Ca^{2+}]_i$  is biphasic, with a peak phase mediated by release from intracellular stores and a plateau phase resulting from  $Ca^{2+}$  entry across the plasma membrane (4,5). A typical experiment is described below:

1. Cells are harvested (*see Subheading 3.1., steps 4–10*).
2. Suspensions are loaded with fura-2 as described in **Subheading 3.2**.
3. Following de-esterification and washing, cells are placed into a cuvet and 340/380 nm fluorescence monitored.
4. Stocks of loaded cells are kept on ice.
5. As can be seen in **Fig. 1**, the response to 10  $\mu M$  carbachol was biphasic (**Fig. 1A**). Also shown for comparison is a typical 340/380 nm recording (**Fig. 1B**) and the derived 340/380 ratio (**Fig. 1C**).

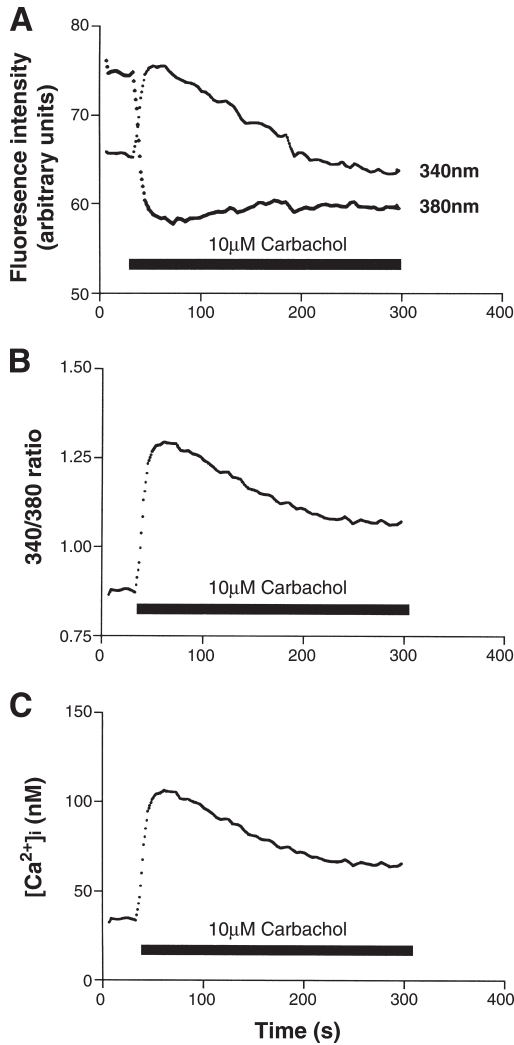


Fig. 1. Carbachol increases  $[Ca^{2+}]_i$  in suspensions of SH-SY5Y cells. (A) Emission at 340 and 380 nm excitation. Note the antiparallel movement of both traces. (B) Derived 340/380 ratio and (C)  $[Ca^{2+}]_i$  after calibration. In these studies  $R_{max}$ ,  $R_{min}$ , and Sfb were 4.61, 0.64, and 2.39, respectively. Autofluorescence at 340 and 380 were 1.67 and 3.18 arbitrary units  $\leq 2\%$  of cell signal.

### 3.3.2. $K^+$ Stimulation in NG108-15 Cells

The authors have previously reported a nifedipine sensitive increase in  $[Ca^{2+}]_i$  in NG108-15 cells in response to depolarization with high  $K^+$  (6). A typical experiment is described next:

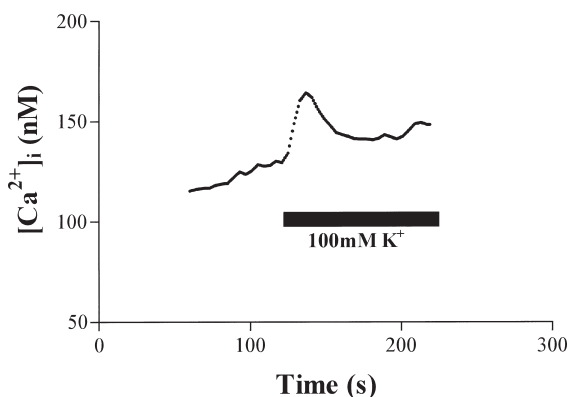


Fig. 2.  $K^+$  depolarization (100 mM, bar) of NG108-15 cells results in a monophasic increase in  $[Ca^{2+}]_i$ . Representative trace modified from ref. 6.

1. Cells are harvested (*see Subheading 3.1., steps 4–10*).
2. Suspensions are loaded with fura-2 as described in **Subheading 3.2.**
3. Following de-esterification and washing in low  $Na^+$  buffer (**Subheading 2.2., item 3**), cells are placed into a cuvet and 340/380 nm fluorescence monitored.
4. Cells are challenged with 100 mM  $K^+$ .
5. Stocks of loaded cells are kept on ice
6. As can be seen in **Fig. 2**, depolarization with  $K^+$  produces a monophasic increase in  $[Ca^{2+}]_i$ . This response is mediated by L-type, voltage-sensitive  $Ca^{2+}$  channels (6).

### 3.3.3. *D*-[Pen<sup>2,5</sup>]enkephalin and Adenosine Triphosphate Stimulation in CHO Cells

CHO cells have been shown to express low levels of the multidrug-resistance efflux pump, P-glycoprotein (7). It is possible that this pump is responsible for extrusion of fura-2 from the cell and, hence, increasing baseline measurements. Probenecid is an organic anion transport inhibitor, originally developed to prevent excretion of penicillin from the kidney, that has been shown to block efflux of fura-2 (7,8). The authors have noted that with the use of CHO cells expressing recombinant opioid receptors (and endogenous purinergic receptors [9]), high rates of fura-2 leakage that can be reduced by inclusion of probenecid (**Fig. 3A**). A typical experiment is described below.

1. Cells are harvested (*see Subheading 3.1., steps 4–10*).
2. CHO cell suspensions are loaded, washed, and then de-esterified in the presence of 2.5 mM probenecid as noted in **Subheading 3.2.**
3. Cells are challenged with either 1  $\mu M$  DPDPE or 100  $\mu M$  adenosine triphosphate (ATP).
4. Between determinations, the stock of loaded cells is kept on ice.

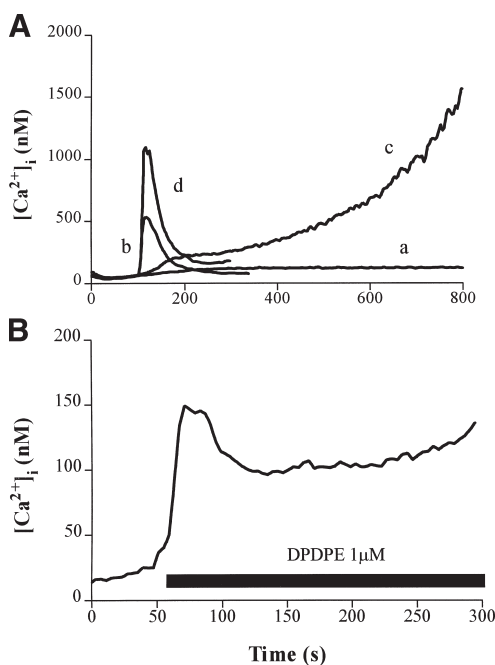


Fig. 3. (A) Representative time course showing effects of 2.5 mM probenecid in CHO cells expressing the recombinant  $\delta$  opioid receptor. Probenecid reduced fura-2 leakage in unstimulated cells (A) when compared to unstimulated control (C) and reduced the peak and plateau phases of 100  $\mu$ M ATP-stimulated (B) when compared to stimulated control (D). (B) 1  $\mu$ M DPDPE increased  $[Ca^{2+}]_i$  in CHO cells expressing the recombinant  $\delta$  opioid receptor. Probenecid was not included in this experiment.

5. As can be clearly seen in **Fig. 3A**, fura-2 leakage was significantly reduced in the presence of probenecid. However, the peak phase response to ATP was also reduced. Careful characterization of the effects of probenecid on the signaling process under study should always be made (*see Note 5*).

#### 4. Notes

1. All tissue culture media and reagents are supplied by Life Technologies, Paisley, Scotland.
2. From European Collection of Animal Cell Cultures (web site <http://www.biotech.ist.unige.it/cldb/cname-dh.html>).
3. Where geneticin (G418) or similar is included in cells expressing recombinant receptors and so on as a selection agent, only the stock cultures should be treated. Experimental cultures should be free of selection medium as G418 may inhibit phospholipase C-mediated responses.

4. For varying levels of  $K^+$ , adjust  $Na^+$  accordingly.
5. Probenecid is insoluble at millimolar concentrations in Krebs HEPES buffer. Therefore, a stock solution was made at 50 mg/mL (175 mM) in 1 M NaOH. This was then diluted in Krebs HEPES buffer prior to addition of  $CaCl_2$  (2.5 mM). The Krebs HEPES buffer containing probenecid (NaOH) was set at pH 7.4 by the addition of HCl (10 M, ~100  $\mu$ L). Caution should be used when using probenecid to reduce fura-2 leakage as the authors have shown that agonist-induced increases in  $[Ca^{2+}]_i$  could be inhibited by this agent (*see Fig. 2*).
6. One confluent 75-cm<sup>2</sup> flask of SH-SY5Y cells is sufficient to give five determinations (i.e., resuspend in 10 mL of buffer). For larger numbers of determinations, load more flasks. However, remember that as the loaded cells stand they leak fura-2, leading to a time-dependent increase in basal. This can be overcome to some extent by sedimenting and resuspending aliquots of the loaded suspension periodically. Some cells leak fura-2 more than others, notably CHO cells (*see below*).
7. The authors routinely use a Perkin-Elmer LS50B fluorimeter (Beaconsfield, UK) equipped with the software FLDM. Files are saved to disk and 340/380 ratios can be converted to  $[Ca^{2+}]_i$  following calibration. It is always advisable to be familiar with the software that controls the configuration and experimental settings of the fluorimeter. Different software packages are available, and for information and troubleshooting the reader is advised to consult the software supplier.
8. For drugs make up 100 times more concentrated so that when 20  $\mu$ L is added to 2 mL of buffer + cells, the desired concentration is achieved. Additions are made as swiftly as possible to avoid light entering the fluorimeter. All agents used should be tested for fluorescence properties. This can be accomplished by adding to a cuvet containing nominally  $Ca^{2+}$ -free buffer (containing several micromolar  $Ca^{2+}$ ) and fura-2-free acid (0.5  $\mu$ M).
9. The authors have noted that de-esterified cells that extrude fura-2 should be maintained on ice between experiments as this reduces the loss of fura-2. In addition, care should be taken to ensure that fura-2-loaded cells are used for experiments immediately after de-esterification.
10. For cells loaded from a single batch of cells, the authors make a single calibration (i.e., they do not calibrate each cuvet of cells), normally the last cuvet used. This needs to be checked for all cell lines and they recommend a comparison of individually calibrated data with all data calibrated from the first and last run of the batch.
11. Addition of Triton X-100 causes complete cell lysis and an increase in 340 and a decrease in 380 nm fluorescence. A globular residue remains in the cuvet, and, therefore, the reusable quartz cuvet should be thoroughly rinsed between experiments using deionized water.
12. Autofluorescence is an important issue for many cell types. This is the fluorescence produced from unloaded cells and can be determined in two ways. First, place an aliquot of unloaded cells into the fluorimeter and measure the fluorescence at 340 and 380 nm (FLDM software has this capability). The main drawback with this method is that the density of unloaded cells should be identical to



the density of cells used for  $\text{Ca}^{2+}$  measurements. The second method is to add 0.1 mM  $\text{Mn}^{2+}$  to the lysed cell suspension after determination of  $R_{\min}$ . In this protocol, the quenching properties of  $\text{Mn}^{2+}$  are utilized. In the authors' studies using SH-SY5Y, NG108-15, and CHO cells, they have found the autofluorescence to be negligible when compared to the signal from loaded cells and, therefore, do not routinely subtract autofluorescence (e.g., see **Fig. 1**). However, they recommend that whenever using a new cell line, autofluorescence should be assessed.

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<http://www.springer.com/978-1-58829-442-5>

Calcium Signaling Protocols

Lambert, D.G. (Ed.)

2005, X, 359 p. 61 illus., Hardcover

ISBN: 978-1-58829-442-5

A product of Humana Press