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Calcium concentrations in the cytoplasmic space and organelles of living cells

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Distribution of Ca²⁺ in mammalian cells

The concentration of free ionised Ca²⁺ in the cytoplasmic space is about 10⁻⁷M (0.1µM) at rest. A large proportion (99%) of total Ca²⁺ in the cytoplasmic space is bound to proteins and metabolites. Ca²⁺ is stored (accumulated) in the endoplasmic reticulum, sarcoplasmic reticulum, mitochondria and other intracellular organelles. For example, the total Ca²⁺ concentration in the ER can be 10mM with a free Ca²⁺ concentration of 10µM. Hormones, neurotransmitters and other extracellular signals increase [Ca²⁺]_{cyt} from 0.1µM to 1 - 5 µM. Intracellular fluorescent Ca²⁺ reporters provide one of the best techniques to measure the free Ca²⁺ concentration in the cytoplasmic space and organelles.

Principles of using Fura-2 as an intracellular fluorescent in Ca²⁺ reporter.

- Fura-2 is one of the best and most commonly used fluorescent intracellular Ca²⁺ reporters.
- Three COO⁻ groups can bind Ca²⁺.
- Affinity for Ca²⁺ is approximately 0.2µM, so suitable for [Ca²⁺]_{cyt} measurement.
- Excitation at 340nm leads to increase in fluorescence emission of fura-2 (at 510nm).
Excitation at 380nm leads to decrease in fluorescence emission of fura-2 (at 510nm).
Therefore the ratio of fluorescence emission 340nm:380nm can be measured.
- This ratiometric measurement reduces problems due to changes in amount fura-2 in the cell, and photo bleaching.

Introduction of Fura-2 into cells

- Commonly Fura-2 is esterified to form acetoxymethyl ester (AM). Cells are incubated with Fura-2 AM, which is lipid soluble and can diffuse across the plasma membrane (and intracellular membranes). Intracellular esterases cleave the ester group to release Fura-2 free acid.
- The intracellular localisation of the Fura-2 (or other dye) will depend on time and temperature of loading Fura-2 AM. Higher temperature (37°C) favours localisation, not in cytoplasmic space, but in organelles (ER, mitochondria) (see, too, below).
- Microinjection of Fura-2 free acid can be used.

Equipment Required for Fura-2 Studies

- Incubation chamber for cells. Cells usually grown on collagen – or poly-L-lysine – coated coverslip.
- Perfusion system.
- Epi fluorescence microscope, inverted.
 - Can use conventional fluorescence microscope (Xenon lamp) or confocal microscope (laser).
- Filters, and wavelength changer to generate alternating excitation light 340 and 380nm, and 510 barrier filter. Neutral density filters.
- Camera for fluorescence imaging.
- Computer and software, eg. Metafluor, Imaging Workbench.

Steps in measurement of $[Ca^{2+}]_{cyt}$ using Fura-2

1. Collect data of fluorescence as a function of time.
2. Calibrate. Use high $[Ca^{2+}]_o$ and ionomycin to give F_{max} , and EGTA or Mn^{2+} to give F_{min} .
3. Use equation and K_d value to convert observed fluorescence ratio to $[Ca^{2+}]_{cyt}$.

Potential problems in using Fura-2 in live cells

- Some Fura-2 can be located in organelles, especially ER. (Depends on loading protocol). (See above).
- Loss of Fura-2 from the cytoplasmic space, eg. exocytosis.

- Possible buffering of $[Ca^{2+}]_{cyt}$ by Fura-2 itself.
- Possible toxic effects of Fura-2.
- UV excitation light can damage cells.
- Photo bleaching should not be a problem with Fura-2 since ratiometric data are obtained.

Other Fluorescent Ca^{2+} reporters for $[Ca^{2+}]_{cyt}$

Non-ratiometric dyes

- Fluo-3.
- Fluo-4 (high fluorescence emission).
- Fura-red (when $[Ca^{2+}]$ increases, fluorescence emission decreases).
- Calcium green.
- Calcium orange.

Ratiometric dyes

- Indo-1 (K_d 0.23 μ M).
- Mag-Indo-1.
- Combination Fluo-3 and Fluo-red (when $[Ca^{2+}]$ increases, Fluo-3 emission increases, Fluo-red emission decreases).

Measurement of Concentration of free Ca^{2+} in Mitochondria using Rhod-2

1. Load Rhod-2 into mitochondria by incubation of cells with Rhod-2-AM (acetoxymethyl ester). Positive charge of Rhod-2 leads to preferential uptake Rhod-2 by mitochondria. K_d for Rhod-2 is 0.57 μ M.
2. Measure fluorescence emission as function time.
3. Account for/determine contribution from non-mitochondrial Rhod-2.
4. Calibrate and convert fluorescence to $[Ca^{2+}]_m$.

Strategies to measure free Ca^{2+} concentration in Endoplasmic Reticulum

- Fluorescent Ca^{2+} reporters include
Calcium green 5N

Mag Fura-2

Fura-2 FF

- Load as acetoxymethyl ester
- Dye needs to have an appropriate affinity for Ca^{2+} (eg. 20 μM).
- Need to account for/determine contribution from non-ER dye.
- Another strategy is to “wash out” any dye in cytoplasmic space after loading AM-dye and hydrolysis. Cells are gently treated with digitonin to selectively permeabilise plasma membrane but not ER, and incubated with medium which mimics ionic composition of cytoplasmic space.

Measurement of Ca^{2+} concentration in the ER using “cameleon” and FRET

- The cameleon sensors use changes in FRET between donor and acceptor variants of GFP separated by a ligand binding domain sensitive to Ca^{2+} (often calmodulin).
- D1ER (Palmer et al 2004) employs the fluorescent proteins CFP and citrine, and the Ca^{2+} sensing proteins calmodulin plus a calmodulin binding peptide from myosin light chain kinase.
- Ca^{2+} binding induces a conformational change and FRET.
- Transfect cells with cDNA encoding cameleon.
- Excite at 436 nm, emission ratio 475 nm (CFP) and 535 nm (citrine).

Measurement of intracellular concentrations of other ions

- pH : using BCECF.
 - Green emission. Calibrate using nigericin and gramicidin.
- Na^+ using Na^+ - binding benzofuran isophthalate.
 - Na^+ using SBFI.
 - Ratiometric 340/380 nm excitation.
 - 510 – 560 nm emission.
 - Calibrate by permeabilising with gramicidin and use standard $[\text{Na}^+]$.
- Na^+ : using sodium green.

References

Principles and applications of Fura-2

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Example of Measurement of Ca^{2+} in Mitochondria

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Information on fluorescent reporters for intracellular Ca^{2+} and other ions

Invitrogen Corporation (Molecular Probes) www.invitrogen.com

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