Intracellular calcium is related to many different physiological processes like neurotransmitters release, muscle contraction, ion channel gating, second messenger pathways, etc. For this reason, calcium quantification is performed in a variety of cellular studies to highlight molecular mechanism involved in different process.

Measuring intracellular calcium can be done using different techniques: the use of fluorescent indicators has become the most popular. Depending on their nature, they can be classified as **Fluorescent Dyes** (Fluo-4, Fura-2, calcium green, etc.) or **Fluorescent Proteins** (aequorin, derivatives of the green fluorescence protein (GFP) such as yellow camaleons, etc.). The main difference between them is the fact that fluorescent proteins can be easily targeted to different cell compartments while most dyes cannot.

Last modified: 18/1/05

**Fluorescent Dyes** - **Fluorescent Proteins**
Fluorescent Dyes: Introduction

Through there are different dyes used as calcium indicators, BAPTA based compounds are among the most popular for measuring free intracellular Ca²⁺. These compounds are able to chelate Ca²⁺ causing a change in their fluorescence spectra.

Fluorescent calcium indicators are normally used to measure intracellular calcium concentration. Only rhod-2 and its derivatives are used to quantify calcium inside an organelle owing to the fact that, in some cell lines, they are sequestered into mitochondria.

Quantification using fluorescent ion indicators can be done using ratiometric or non-ratiometric methods. In general, those compounds that only present an increase in their fluorescence intensity (intensity shift) are used for non-ratiometric measurements (Figure 1A). A ratio between two fluorescence intensity is calculated in ratiometric measurements: this is performed using ion indicators that, on binding calcium, present an emission spectral shift (Figure 1B) or excitation spectral shift (Figure 1C) or mixtures of indicators. Compounds which have a shift in their spectra on binding to ions are commonly referred as ratiometric indicators.

A. Intensity shift when bound/unbound to calcium: non-ratiometric measurements

B. Emission spectral shift: ratiometric measurements

C. Excitation spectral shift: ratiometric measurements
It is important to consider the advantages and drawbacks of ratiometric and non-ratiometric methods to design a calcium experiment. Other important parameters to evaluate for choosing an indicator are its dissociation constant ($K_d$) for calcium and/or other ions, excitation and emission wavelength, quantum yield, buffering capacity, loading requirements, etc.

Calcium quantification experiments can be performed in a quantitative or semi-quantitative way. If quantitative experiments are performed, a calibration is recommended.
Fluorescent Proteins - Introduction

Different fluorescent proteins can be found in natural organisms ranging from bacteria to insects or fish and in some cases they are responsible for bioluminescence. The emission of light can depend on a given conformation, the presence of a cofactor or the environment where the protein is placed (pH, ions, etc.).

*Aequorea victoria*, a jellyfish used in the 1960s to investigate bioluminescence, is one of those organisms capable of emitting light. Its bioluminescence is due to the presence of aequorin (sensitive to calcium) and green fluorescent protein (GFP, a fluorescent protein).

**Aequorin** has been used for calcium imaging during more than 30 years. It is a chemiluminescent protein: light is emitted on binding calcium, without the need of light irradiation. This makes the assay very sensitive but, due to the low signal, it requires long acquisitions, lowering the spatial and temporal resolution.

Although GFP is non-sensitive to calcium, it has been used as a basis for the development of many chimeras **GFP-based fluorescent calcium indicators**. The first derivatives were described in 1997 by R. Tsien et. al. and were based on a fluorescence resonance energy transfer (FRET) process. Since then, many other GFP-indicators have been described and most of them are based on calmodulin as calcium switch.

The main advantage of using fluorescent proteins in calcium quantification is the fact that recombinant proteins can be tagged to different organelles, so calcium can be measured specifically (i.e. endoplasmic reticulum, mitochondria, etc.). A drawback is that, in general, they are not commercially available.
Fluorescent Dyes - Quantifying calcium concentration

Changes in $[\text{Ca}^{2+}]$ are related to changes in fluorescence intensity (non-ratiometric indicators) or fluorescence intensity ratio (ratiometric indicators) of a chosen dye.

**Relative values of calcium concentration** (semi-quantitative values) can be obtained without too many cautions. Roughly, fluorescence intensity for each cell is measured during an experiment and values are normalized by their resting intensities. Relative values can only be compared with those obtained using the same experimental conditions (ideally in the same experiment set). These values depend not only on the indicator used but also on the loading protocol, microscope settings, cell line, temperature, etc.

**Absolute calcium concentration**, though being one of the most desired goals on calcium imaging, is very tricky. Equation 1 shows the relationship between fluorescence and absolute calcium concentration ($[\text{Ca}^{2+}]$).

$$[\text{Ca}^{2+}] = K_d \cdot (F - F_{\text{min}})/(F_{\text{max}} - F) \quad \text{Equation 1.}$$

$F_{\text{min}}$ and $F_{\text{max}}$ are values obtained at minimum and maximum calcium concentration (i.e. 0 and saturating values). These parameters should ideally be measured in each cell to obtain $[\text{Ca}^{2+}]$ accurately, but often this approximation is not viable.

**Calcium chelators** are used to obtain an accurate $F_{\text{min}}$, but this kind of compounds interferes in the value of $F$. Another problem is that $F_{\text{max}}$ value of a cell might be modified during the measurement of $F$. Acquisition of $F_{\text{max}}$ value requires the use of high intracellular calcium concentrations (values $>>10 \cdot K_d$), which disturbs intracellular environment.

$K_d$ is the **dissociation constant** for each indicator and should be established for each experimental condition. $K_d$ tabulated values usually correspond to the dye in water or buffer (not inside a cell). Differences in pH, protein concentration, ionic strength, lipid interaction, etc. have been reported to modify dramatically $K_d$ value (Molecular Probes), so a **calibration** is needed for each experimental conditions.
Definitions & Literature

Concepts

Some concepts are basic to understand and perform calcium quantification. Though they are quoted in the text, they are also listed here.

- Calcium chelators.
- Dissociation constant ($K_d$).
- Loading indicators.
- Microscope set-up (confocal vs. fluorescence).
- Macros.
- Non-ratiometric methods.
- Ratiometric methods.

Useful Web sites with Protocols and Definitions

- Molecular expressions Molecular Prime. It is a wide treatise on microscopy.
- Universidad de Oviedo Tutorials. Basic concepts on Confocal Microscopy, Flow Cytometry and other techniques (Spanish).
- LabVelocity. Basic protocols and a utility to search material, products. etc.
- Molecular Probes. Website of one of the most important providers of fluorescence-based detection products. It has utilities (like $K_d$ calculator) and protocols.

Other laboratories

- Roger Y. Tsien's laboratory
Definitions & Literature

**Literature**

**General Articles**


**General Books**


**Handbooks**

  - *Molecular Probes' Handbook of Fluorescent Probes and Research Products*.

**Fluorescent Proteins**

**Fluorescent Dyes**


  Fura-2 AM calcium green-1 dextran


Fluorescent Proteins
Material and solutions used in this tutorial are detailed here. Some tips to adapt them to each experiment have been included.

The following protocols are routinely used in our lab for dyes loading and were also used for the examples shown.

- Loading Fluo-4 AM in HeLa cells
- Loading Indo-1 AM in HeLa cells

Due to the high variability in calcium quantification experiments, these protocols can be used as a starting point, but they should be optimized for each particular experiment.

Some tips to chose and setup the microscope can be found here.
Fluorescent Dyes: Examples

Fluorescent Dyes - Examples

We have used the protocols described in this teaching module in different experiments. Some of these examples are available on-line to illustrate them.

Fluo-4 AM:

- Effect of ionomycin in HeLa cells:
  - A reference experiment.
  - Incomplete loading/de-esterification example.
  - Effect of serum in HeLa cells deprived of serum for 4h.
  - Performing a calibration.

Indo-1 AM (example of a ratiometric indicator):

- A reference experiment (ionomycin)

EAMNet Course on Calcium Imaging for Cell Biologists (6-8 Oct. 2004, Barcelona):

  Practical Workshops presentation (done by course participants)

Analysis of calcium experiments

- General tips

- Tips on Excel macros
Fluorescent Dyes - Choosing a dye

There are many commercially available calcium indicators, so it is important to choose the right one for each experiment. Some facts to be considered are listed below:

- **Range of calcium concentration.** In general, quantification is possible if calcium concentration is close to $K_d$ value. As a rule of thumb, $[\text{Ca}^{2+}]$ has to be between the following values during the experiment:

  $$0.1 \cdot K_d < [\text{Ca}^{2+}] < 10 \cdot K_d$$

- **Instruments.** Not only epifluorescence microscopes are suitable for ion imaging, but also confocal microscopes can be used and offer advantages.

  Illumination source and detection filter/settings have to be suitable for the fluorochrome: the optimum $\lambda_{\text{excitation}}$ for the fluorochrome should be very similar to our excitation light source. Very fast excitation/emission changes are required sometimes to follow fast biological processes.

- **Loading.** Many indicators are available as acetoxymethylester (AM), but some of them have to be microinjected or electroporated inside the cell. These compounds require a special equipment and training.

- **Compartmentalization.** Some dyes tend to accumulate inside intracellular reservoirs (endoplasmic reticulum, mitochondria, vesicles, etc.). This behaviour depends not only on the dye used, but also on the loading conditions and cell type. There is not a general rule, but sometimes it can be overcome if the loading protocol is modified.

- **Ratiometric/non-ratiometric methods.** Though non-ratiometric indicators are easier to measure and quantify, ratiometric indicators are more insensitive to bleaching, concentration differences, optical path, etc. giving robust measurements.

- **Quantum efficiency/photobleaching.** A high quantum efficiency gives a stronger fluorescence signal, allowing to work with lower laser intensities. It is important to use low excitation intensities in order to minimize photobleaching. If UV light is used, higher intensities can induce phototoxicity.
Fluorescent dyes: Introduction

**Introduction - Choosing a dye - Loading**

**Fluorescent Dyes - Loading**

Calcium indicators are unable to cross lipid membranes due to their nature, making necessary the use of physical or chemical methods to load them inside the cell.

**Loading Acetoxymethyl (AM) esters**: the protection of carboxylic groups as AM esters makes the dye neutral, so it can cross the cell membrane. Once inside the cell, esterases will cleave AM groups. This process gives place to charged compounds that are entrapped inside the cell. A scheme of this process is represented below (red balls represent AM protecting groups, while the big ball represents the fluorescent dye core).

Complete hydrolysis of the AM esters is very important to avoid artefacts. If the experiment begins before all AM ester dye is converted to free dye, total concentration increases during the experiment and gives place to false fluorescence variations (see an example).

Complete hydrolysis is still more crucial if the AM ester is already fluorescent. This is the case of Indo-1 AM and Fura-2 AM, which are fluorescent and calcium insensitive. If de-esterification is not completed, there will be a mixture of indicator (free and bounded to calcium) and indicator-AM.

When the three forms of the indicator are fluorescent, the signal depends not only on calcium levels, but also on the non de-esterificated indicator concentration.

An example of a protocol used for loading AM esters in HeLa cells is available for Fluo-4 AM and Indo-1 AM.

Though charged indicators cannot cross cell membrane, intracellular concentration might decrease during experiment due to photobleaching and/or active transport out of the cell.

A drawback of AM esters is that they can accumulate inside intracellular compartments, making indicator insensitive to calcium cytosolic levels. To avoid this behaviour, loading/
Fluorescent dyes: Introduction

de-esterification temperature can be decreased, which usually implies longer incubation periods.

**Loading of dextran conjugates and salt form**: these compounds are impermeable to cell membranes, so they are less prone to accumulate in intracellular compartments. As a drawback, they have to be loaded using reversible methods to make permeable large populations or procedures such as microinjection and electroporation (cells can be damaged during these processes).

An important advantage of dextran conjugates is that dextran moiety not only avoids compartmentalization, but also prevents conjugation of the indicator to proteins, membranes, etc. This prevents indicator from being sequestered and provides enhanced resistance to leakage.

Protocols for:

- Loading **Fluo-4 AM** in HeLa cells
- Loading **Indo-1 AM** in HeLa cells
Fluorescent dyes: Examples

**Calcium chelators**

Chelators are compounds that bind to metal ions forming a complex and some of them are also fluorescent. If the complex has fluorescence properties different from those of the free chelator, it can be used as an ion indicator.

Calcium chelators are able to bind calcium (usually in a relationship one to one) in a selective way (they have higher affinity for calcium than for any other metal ions). Binding to calcium is performed through carboxylic groups, so it can be affected by pH, other ions or co-ordination to proteins, lipids, etc.

This process is a reversible equilibrium:

\[
\text{[Chelator-Ca]} \leftrightarrow \text{Chelator + Ca}^{2+}
\]

Calcium, chelator and complex concentration are related by an equilibrium constant called disassociation constant, \(K_d\), that is defined as:

\[
K_d = ([Ca^{2+}] \cdot \text{[Chelator]} ) / ([\text{Chelator-Ca}])
\]

When \(K_d\) is very low, it is a **high-affinity chelator** (it has a high tendency to bind calcium). If \(K_d\) is high (µM or higher), we talk about **low-affinity chelators**.

Optimum **calcium concentration range** for an indicator is between \(0.1 \cdot K_d < [Ca^{2+}] < 10 \cdot K_d\). Nearly all chelator is forming the fluorescent complex at high calcium concentration, so no variations are observed in fluorescence intensity. At lower \([Ca^{2+}]\), there is a very low indicator response.

High-affinity chelators trap calcium very efficiently, so high intracellular chelator concentration can buffer calcium response. This attribute is used in the case of the non-fluorescent BAPTA (\(K_d = 190 \text{ nm if there is no Mg}^{2+}\)) to make intracellular calcium concentration zero and obtain \(F_{\text{min}}\).
high concentration of BAPTA is loaded along with the desired calcium indicator. In these conditions, all intracellular calcium is sequestered by BAPTA, so fluorescence in absence of calcium can be obtained.
Ratiometric methods

Ratiometric methods are based on the use of a ratio between two fluorescence intensities. This allows correction of artifacts due to bleaching, changes in focus, variations in laser intensity, etc. but makes measurements and data processing more complicated.

Ratiometric indicators show a shift in their emission or excitation spectra when they bind to calcium, therefore they can be classified as dual emission or dual excitation indicators. Measurement of calcium with these compounds is achieved by using two excitation lasers (if they are dual excitation indicators) or two detection ranges (if they are dual emission indicators).

If a so-called ratiometric indicator is used, intensity ratio is calculated at wavelengths were difference of fluorescence between bound and free indicator is maximum.

A ratiometric quantification can also be done using a mixture of an intensity shift indicator and a insensitive fluorescence compound (i.e. Fluo-3 and Fura-Red). In this case, it is important to adjust conditions to obtain a similar fluorescence intensity response from both compounds.

Cytosolic free calcium concentration is related with fluorescence as follows:

\[
[Ca^{2+}] = K_d \cdot \frac{(R - R_{\text{min}})/(R_{\text{max}} - R) \cdot (F_{\lambda_{\text{max}}}/F_{\lambda_{\text{min}}})}{R_{\text{max}} - R_{\text{min}}}
\]

Where:

- \( K_d \) is the dissociation constant (depends on the indicator, but also on pH, ionic strength, cell line, etc.).
- \( F \) is the observed fluorescence of our sample or cell.
- \( R \) is the fluorescence ratio at both wavelengths \( F_{\lambda_1}/F_{\lambda_2} \).
- \( R_{\text{max}} \) is the minimum ratio value (it can be at minimum or maximum calcium concentration, depending on the wavelength used in the denominator).
- \( R_{\text{max}} \) is the maximum ratio value.
- \( F_{\lambda_{\text{max}}}/F_{\lambda_{\text{min}}} \) is a scaling factor, also known as \( \beta \) (\( F_{\lambda_1} \) is the fluorescence used in the denominator at its maximum an minimum value).

**Advantages of ratiometric methods:** [Ca^{2+}] estimated with ratiometric compounds is not affected by probe loading (loading conditions is critical when mixtures are used), bleaching, optical path length, illumination intensity, etc. Therefore using ratios avoids many of the problems related to absolute fluorescence values.

**Drawbacks of ratiometric methods:** acquisition and data manipulation is more complex due to the use of fluorescence ratios. Not all microscopes are suitable for these measurements (changing excitation/emission wavelength at suitable rates is required) and many ratiometric indicators require the use of UV excitation.

Ratiometric calcium indicators are among the most popular due to their higher robustness than non-ratiometric indicators.

Some ratiometric calcium indicators are listed in the table below (data from Molecular Probes). The most suitable for each experiment will depend on the range of calcium
Ratiometric calcium indicators

concentration that has to be evaluated, instrumentation, loading requirements, etc (see our tips for choosing a dye).

<table>
<thead>
<tr>
<th>Indicator</th>
<th>$K_d$ (nM)</th>
<th>$\lambda_{\text{excitation}}$ (nm)</th>
<th>$\lambda_{\text{emission}}$ (nm)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Free</td>
<td>Bond</td>
<td>Free</td>
</tr>
<tr>
<td>Fura Red</td>
<td>140</td>
<td>472</td>
<td>436</td>
<td>657</td>
</tr>
<tr>
<td>Fura-2</td>
<td>145</td>
<td>363</td>
<td>335</td>
<td>512</td>
</tr>
<tr>
<td>Mag-fura-2</td>
<td>25000</td>
<td>369</td>
<td>329</td>
<td>511</td>
</tr>
<tr>
<td>Indo-1</td>
<td>230</td>
<td>346</td>
<td>330</td>
<td>475</td>
</tr>
<tr>
<td>YC2.1</td>
<td>100 and 4300</td>
<td>430</td>
<td>430</td>
<td>480</td>
</tr>
<tr>
<td>YC3.1</td>
<td>1500</td>
<td>430</td>
<td>430</td>
<td>480</td>
</tr>
</tbody>
</table>

Non-ratiometric calcium indicators

Non-ratiometric methods

On binding to calcium, indicators used for non-ratiometric measurements show a shift in their fluorescence intensity (the free indicator has usually a very weak fluorescence). In this case, cytosolic free calcium concentration is related with fluorescence intensity as follow:

\[ [\text{Ca}^{2+}] = K_d \cdot \frac{(F - F_{\text{min}})}{(F_{\text{max}} - F)} \]

Where:

- \( K_d \) is the dissociation constant (depends on the indicator, but also on pH, ionic strength, cell line, etc.).
- \( F \) is the observed fluorescence of our sample or cell.
- \( F_{\text{min}} \) is the lowest fluorescent value at fixed experimental conditions. It is usually obtained at \([\text{Ca}^{2+}] = 0\).
- \( F_{\text{max}} \) is the highest fluorescent value at the same experimental conditions. It is obtained at \([\text{Ca}^{2+}] \gg 10 \cdot K_d\).

Advantages of non-ratiometric indicators: an increase in fluorescence signal can be related directly to an increase in calcium concentration.

Drawbacks of non-ratiometric indicators: fluorescence intensity depends on many factors not related to calcium concentration. Some of these are acquisition conditions, probe concentration and optical path length.

There are many non-ratiometric calcium indicators, some of them are listed below (data from Molecular Probes). The most suitable for each experiment depends on the range of calcium concentration that has to be evaluated, instrumentation, loading requirements, etc (see our tips for choosing a dye).

<table>
<thead>
<tr>
<th>Indicator</th>
<th>( K_d ) (nM)</th>
<th>( \lambda_{\text{excitation}} ) (nm)</th>
<th>( \lambda_{\text{emission}} ) (nm)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quin2</td>
<td>60</td>
<td>332</td>
<td>498</td>
<td>High intracellular buffering. UV excitation required.</td>
</tr>
<tr>
<td>Fluo-3</td>
<td>390</td>
<td>506</td>
<td>526</td>
<td></td>
</tr>
<tr>
<td>Fluo-4</td>
<td>345</td>
<td>494</td>
<td>516</td>
<td>More sensitive than Fluo-3 due to its ( \lambda_{\text{exc}} ) closer to 488 nm excitation laser</td>
</tr>
<tr>
<td>Calcium Green-2</td>
<td>550</td>
<td>503</td>
<td>536</td>
<td></td>
</tr>
<tr>
<td>Calcium Orange</td>
<td>185</td>
<td>549</td>
<td>575</td>
<td></td>
</tr>
<tr>
<td>Fluo-4FF</td>
<td>9700</td>
<td>494</td>
<td>516</td>
<td>Derivative of Fluo-4 suitable for ([\text{Ca}^{2+}]) between 1 (\mu)M and 1 mM</td>
</tr>
</tbody>
</table>
Fluorescent Dyes - Calibration

Calibration process implies calculating $K_d$ value for our particular experimental conditions. This value depends on loading/measuring conditions (temperature, medium, concentration and volume used) and also on cell line. If fluorescence is represented vs. calcium concentration, a non-linear curve is obtained, so $K_d$ is not easily calculated.

If fluorescence intensity changes with calcium concentration for different $K_d$ values (an $F_{\text{max}} = 100$ and $F_{\text{min}} = 0$ are used in the graph) are analyzed, it is easy to see the non-linear relationship at high calcium values.

Note that high-affinity indicators get saturated very quickly, so very small changes in intensity are observed above $1 \mu M$ for $K_d = 100 \text{ nM} ([\text{Ca}^{2+}] = 10 \cdot K_d)$. This example also illustrates the importance of working at concentrations near $K_d$ value.

If equation 1 is used, equation 2 can be obtained using logarithms.

\[
[\text{Ca}^{2+}] = K_d \cdot \frac{(F - F_{\text{min}})}{(F_{\text{max}} - F)} \quad \text{Equation 1.}
\]

\[
\log \left(\frac{(F - F_{\text{min}})}{(F_{\text{max}} - F)}\right) = \log [\text{Ca}^{2+}] + \log K_d \quad \text{Equation 2.}
\]

Graphical representation of $\log \left(\frac{(F - F_{\text{min}})}{(F_{\text{max}} - F)}\right)$ vs. $\log [\text{Ca}^{2+}]$ can be adjusted with a linear regression ($y = a \cdot x + b$), where $b = \log K_d$. Automatic calculation can be done using Molecular Probes' $K_d$ calculator (this equation can also be applied to solution measured with a cuvette and a fluorometer).

To obtain $F$ values, different extracellular calcium concentrations are used and cells are treated with an ionophore that equilibrates extracellular and intracellular concentration and fluorescence or ratio is recorded. As calcium ion is very common in our environment (it is present in glassware, reagents, etc.), calcium buffers are recommended to avoid artefacts (it is more important at lower calcium concentrations) during calibration experiments.

Obtaining exact $F$ values is not as easy as it might seem at first glance, so some other details have to be considered to obtain an accurate $K_d$ for each particular experimental condition. Many factors can affect its value, so special care has to be taken to avoid misleading results.
See also more tips on calcium calibration.

* For ratiometric indicators, \( \log \left[ \frac{(R - R_{\text{min}})/(R_{\text{max}} - R) \cdot (F_{\lambda_{\text{max}}} / F_{\lambda_{\text{min}}})}{1} \right] \) has to be used instead.
Intracellular calcium is related to many different physiological processes like neurotransmitters release, muscle contraction, ion channel gating, second messenger pathways, etc. For this reason, calcium quantification is performed in a variety of cellular studies to highlight molecular mechanism involved in different process.

Measuring intracellular calcium can be done using different techniques: the use of fluorescent indicators has become the most popular. Depending on their nature, they can be classified as Fluorescent Dyes (Fluo-4, Fura-2, calcium green, etc.) or Fluorescent Proteins (aequorin, derivatives of the green fluorescence protein (GFP) such as yellow camaleons, etc.). The main difference between them is the fact that fluorescent proteins can be easily targeted to different cell compartments while most dyes cannot.

Fluorescent Dyes - Fluorescent Proteins
Fluorescent Proteins - Quantifying calcium

Quantification of fluorescent proteins can be done by using the same rules described for quantification of fluorescent dyes. Cameleons are usually employed as ratiometric indicators while other fluorescent proteins are used as non-ratiometric indicators.

In the case of cameleons, the ratio between the donor excitation wavelength and the acceptor emission wavelength allows an increase in the signal to noise ratio and also has the advantages of ratiometric methods. There is just an important difference between dyes and proteins: Hill coefficient has to be considered in the case of fluorescent proteins that present a cooperative mechanism.
Fluorescent Proteins: Introduction - Quantifying calcium - Definitions - Protocols & Examples

Material and solutions used for cell transfection and Fluorescent Protein experiments are detailed here.

The following protocols are routinely used in our lab for YCs transfection and were also used for the examples shown.

- YCs expressed in cytosol

- YCs expressed in endoplasmic reticulum

Due to the high variability in calcium quantification experiments, these protocols can be used as a starting point, but they should be optimized for each particular experiment.

Some tips to chose and setup the microscope can be found here.

Fluorescent Dyes Calcium Tutorial Home

Aequorin was originally obtained by purification from the jellyfish *Aequorea victoria*, but now it is prepared more efficiently using recombinant techniques. The active aequorin is formed by a complex between apoaequorin (APO), oxygen and coelenterazine. On binding calcium, the complex is broken emitting light and rendering the free apoaequorin and coeleteramide.

Active aequorin can be directly microinjected or obtained by reconstitution of apoaequorin with coelenterazine in transfected cells. In both cases, coelenterazine is needed to obtain the active complex (coelenterazine is burn out after light emission and is needed for reconstitution of aequorin). When using cells transfected with apoaequorin cDNA, the rate-limiting step in the reconstitution is the cell permeation of coelenterazine.

Reconstitution of aequorin can be done using the native coelenterazine or analogs (some analogs with quicker response and more intense signal have been described and are commercially available).

Although this protein was very popular for quantifying calcium in the past 30 years, it has been substituted by GFP derivatives.

New derivatives combining aequorin and GFP or RFP (red fluorescence protein) have been developed based on the chemiluminescence resonance energy transfer process (CRET or BRET).
Fluorescent proteins, GFP, adopts a beta-barrel structure with an alpha-helix inside. This structure is very rigid and the chromophore is in the centre, protected by the protein.

Specific mutations in GFP residues can modulate its fluorescent properties: different blue, cyan, green and yellow variants, have been described. Using these proteins as starting point, different calcium indicators have been developed by fusion of one or two GFP derivatives, a calcium binding protein, such as calmodulin, CaM, and/or a CaM binding peptide.

In general, those indicators can be classified depending on the process that causes calcium signal.

Cameleons are based on a fluorescent resonance energy transfer (FRET) process between two variants of GFP. Camgaroos and pericams can be classified as environment sensitive GFPs and there are also fusion proteins (fusion aequorin-GFP and aequorin-RFP, red fluorescent protein) that present chemiluminescence resonance energy transfer (CRET or BRET) on binding calcium to aequorin.
**Protocol for loading Fluo-4 AM in HeLa cells**

This protocol was used in the examples shown in this tutorial. Incubation times and temperatures have to be tested and adapted, if needed, to each cell line and experimental conditions. Material and solutions used can also be adjusted: just read some tips about the important facts.

**HeLa cells seeding:**

- Seed 25,000 cells per dish (1.5 mL) in Mat-Tek P35G-1.5-14-C dishes for 24 h (final confluence will be around 50%).

**Loading:**

- Wash cells three times with HBSS+.
- Incubate 45 min with 1 mL of Fluo-4 AM 2 μM at room temperature.

Note that variations in room temperature (23ºC in our case) might change slightly the required incubation period.

**De-esterification:**

- Remove the AM ester solution.
- Wash three times with HBSS+.
- Incubate 30 min with HBSS+ or serum-free medium at room temperature.

**Measurement:**

- If Ca²⁺ measurements are carried on at 37ºC (which is recommended), allow the cells to stabilize temperature for at least 5 minutes before starting the experiment.
- Check de-esterification process by monitoring fluorescence signal for a while (1-2 min). As Fluo-4 bleaches very little, fluorescence should be constant.

Image fluorescence should be homogeneous and cells have to be (and also look) healthy. If they do not look fine, discard the preparation.

Nucleus might look brighter. In some cases, this can be explained because of its thickness, but it can also be due to difference in calcium concentrations.
Some standard experiments that used this protocol are posted here. There is also an example of non-adequate loading.
Protocol for loading Indo-1 AM in HeLa cells

This protocol was used in the examples shown in this tutorial. Times and temperatures have to be tested and adapted, if needed, to each cell line and experimental conditions.

Material and solution used can be adjusted; just read some tips about the important facts.

HeLa cells seeding:

- Seed 25,000 cells per dish (1.5 mL) in Mat-Tek P35G-1.5-14-C dishes for 24 h (final confluence will be around 50%).

Loading period:

- Wash cells three times with HBSS+.
- Incubate 45 min with 1 mL of indo-1 AM 2 μM at 37ºC.

De-esterification period:

- Remove the AM ester solution.
- Wash three times with HBSS+.
- Incubate 45 min with HBSS+ or serum-free medium at 37ºC.

Measurement:

- Indo-1 AM ester is also fluorescent but insensitive to calcium concentration, therefore a poor response might indicate an inadequate de-esterification process.
- It is better to avoid UV light to locate cells in the microscope because indo-1 bleaches quickly and UV light causes cell damage.
Variations to material and solutions should be made for each experiment in order to obtain the best results. The following tips are intended to guide on choosing those variations.

Cells seeding tips:

- Cells confluence should be around 70% (do not use cells with more than 80% confluence).
- Optimize cell density for each experimental condition/cell line: 2500 cells/cm² normally renders good results with HeLa cells seed for 24h in Glass Bottom Culture Dish (Mat-Tek P35G-1.5-14-C). Other dishes, cell line or seeding time might require changes in cell density for optimum results.

Solutions:

- Extracellular medium used for loading, de-esterification and measuring calcium variations has to be chosen carefully to avoid artefacts.

Loading:

- AM indicators are very sensitive to esterases. Do not use medium with serum (heat-inactivated serum is still able to hydrolyze AM indicators partly) or other additives that might contain esterases.
- Use a medium composition or a buffered solution (e.g. HBSS) adequate to the chosen cell line. It is better to avoid the use of Phenol Red.
- Do not use Ca²⁺-free solutions. Cells do not feel comfortable for a long time in these conditions.

De-esterification:

- Once all the indicator AM form is inside the cell, complete medium can be used. If the incubation is done at room temperature, use buffered medium (HEPES can be used if there is no CO₂ chamber). It is better to avoid the use of Phenol Red.

Measurement:

- Phenol Red has to be avoided when measuring fluorescence. It increases background fluorescence and interferes in measurement.
- HEPES might interfere in fluorescence signal, so it is better to use carbonate buffer with a CO₂ chamber.
- HBSS without Ca²⁺ and Mg²⁺ should be used to prepare solutions of known calcium concentration. EGTA buffering can be affected by solutions containing calcium.

Indicator AM solution:
Fluorescent dyes: Examples

- Concentrated indicator AM solution is prepared in a minimum volume of DMSO to minimize DMSO final content. Use pluronic F-127 to enhance solubility.

- Dilutions of AM indicator have to be made in loading medium.

- Though 2µM concentration usually works fine, this value might be increased or decreased depending on the experiment. Be aware that intracellular concentration is usually much higher than extracellular one, so there is no linear relationship between loading and intracellular concentration.

- Total volume of AM indicator solution is important, so use always the same volume.

Drug/ionophore solution

- It might be better to dilute ionophores and drugs that induce calcium signalling in the same buffer used in the measurement or in a calcium free buffer. In this way, extracellular calcium concentration is controlled during all the experiment.

- If medium is used to make the measurements, you can dilute the ionophores/drugs in it. Just one caution: some compounds might be degraded by the medium, so prepare them fresh to avoid it.
**Fluorescent Dyes - Material and Solutions**

Material and solutions used are detailed below. Changes should be made to adapt them to your own needs (see material and solutions tips).

**Cells seeding:**

- ATCC HeLa cells grown in DMEM high glucose.
- Glass Bottom Culture Dish (Mat-Tek P35G-1.5-14-C).

**Solutions**

- Hanks buffer with Ca²⁺, Mg²⁺, without Phenol Red, 10 mM HEPES, 10 mM Glucose (HBSS+).
- Hanks buffer without Ca²⁺, Mg²⁺, without Phenol Red, 10 mM HEPES, 10 mM Glucose (HBSS-).
- 1 mM AM Ca²⁺ indicator: dissolve 50 µg of AM Ca²⁺ indicator in dimethylsulfoxide (DMSO) or 20% pluronic F-127/DMSO (Molecular Probes, P-3000) (the use of pluronic F-127 enhances solubility).
- 2 µM AM Ca²⁺ indicator: 10 µL of 1 mM AM Ca²⁺ indicator are diluted in 5 mL HBSS+ (prepare it fresh every day and keep it on ice during the experiment).

It is advisable to perform Calcium experiments on medium without Phenol Red (this compound interferes in fluorescence signal) and without serum. See material and solutions tips.
Example of incomplete loading/de-esterification

Though there was no variation in cell morphology during this example, an increase in fluorescence was observed after adding ionomycin (HeLa cells were loaded with Fluo-4 AM and an increase in intracellular [Ca^{2+}] was induced with 2µM ionomycin in HBSS+).

This graph indicate that the protocol used for loading cells has to be modified (different incubation conditions have to be tested in order to achieve an "ideal" behaviour: checking measurement conditions is also recomended).

Lower temperatures/less time in loading and de-esterification steps can produce an incomplete de-esterification. This problem causes an increase in the concentration of the free indicator during the experiment, which is reflected as a continual increase in fluorescence intensity. An insufficient de-esterification time can also produce variations in the fluorescence during the experiment (measurements are done at 37ºC, so samples incubated at room temperature need a time to equilibrate). Variations in cell morphology can also change mean fluorescence intensity.
Fluorescent dyes: Examples

### Microscope & Setup

Most experiments can be performed in a wide-field fluorescence microscope, though some experiments have particular requirements that might conditionate our choice. In general, any fluorescent microscope with the required excitation/emission lasers/filters can be adjusted for measuring intracellular calcium, but advantages and restrictions of each equip have to be considered for experimental setup.

**Confocal microscopes** can record series of thin xy planes to build xyz stacks and render subcellular resolution. Also measurements in xz plane can be performed. These measurements can be interesting for studying calcium distribution along z axis, which cannot be performed with wide-field fluorescence microscopes, and the use of a confocal microscope renders higher spatial resolution. Confocal microscopes can mimic wide-field fluorescence microscopes: opening the pinhole renders thicker sections, similar to those images obtained with CCD cameras, and minimizes the laser intensity required for acquisition of thinner sections. High pinhole values (around 4 or 5 times the optimum value) render good results with HeLa cells using a 63x/1.32 objective (this value changes depending on cell thickness and microscope settings).

Newer confocals microscopes are faster than older ones, so time resolution is usually more than enough. If standard adjustments are not enough for our experiment, some parameters can be modified to increase speed. Adjusting scanning mode (bi-directional), scanning speed (higher values), resolution (it is not always necessary a high resolution image to quantify calcium!), etc. usually renders enough temporal resolution for most experiments.

Studying very fast changes in [Ca^{2+}], as those observed in some cell lines, can be achieved by using an xt scanning mode. It consists on scanning just a line of the sample (x axis) while using the minimum dwell time. If xyt image are important, using a confocal microscope with a Nipkow disk system or a fast-scanning model allows capturing fast cell events without losing xy information.

xzt scanning mode gives a different point of view of the studied cell, so it can render interesting information about calcium concentration changes in z axis.

Not all fluorescent calcium indicators can be measured in a standard confocal microscope. An UV laser is not usually installed (many confocal microscopes only have visible lasers), so indicators requiring UV excitation are not suitable for those microscopes. Also ratiometric methods with an excitation spectral shift require to be excited at two close wavelengths (e.g. 335 and 363 nm for Fura-2) in a very short time (but not simultaneously), which makes many confocal microscopes not suitable for this kind of measurement.

**Fluorescence microscopes** do not have z resolution, but this is not a major problem for many calcium imaging experiments. Different brands and models of microscope offer different settings, so choose a fluorescent calcium indicator that suits yours (and also your experiment!).

Temporal resolution depends on the microscope, but tricks like those detailed for confocal microscopes can also be used (note that fluorescence microscopes normally use a...
Some laboratories have adapted their systems to improve their performance in a particular experiment (see a description of Monck's Pulsed Laser Imaging System for studying kinetics of Ca$^{2+}$ gradients or visit Monck's Lab web page) and suppliers have also developed high-speed filter wheels, fast shutters, specific software, etc. in order to satisfy the needs of most demanding experiments, including those with ratiometric methods.

Fluorescent Proteins
Protocols for transfection of YCs expressed in cytosol

HeLa cells seeding:

- Seed 15,000 cells per dish (1.5 mL) in Mat-Tek P35G-1.5-14-C dishes for 24 h.

Transfection (procedure for 1 dish):

- Prepare DNA solution: dilute 3 µL FuGENE in 100 µL Opti-MEM and mix thoroughly, add 1 µg of DNA, mix again and incubate 30 min at room temperature.
- Wash the cells gently with PBS x 3.
- Add 1400 µL of Opti-MEM.
- Add the DNA solution prepared previously.
- Incubated at 37°C overnight (8-10 h). Important: Opti-MEM does not contain antibiotic!
- Remove transfection mixture.
- Wash gently with PBS x 3.
- Add DMEM high glucose + 10 % FCS.
- Allow cells to express YCs proteins for 3-6 days (incubate at 37°C and change medium as usual).

Measurement:

- Use medium without phenol red or the corresponding buffer for measurements.
Folding of proteins expressed in endoplasmic reticulum might be problematic, so incubation at 30ºC for some hours is recommended before measurement.

**HeLa cells seeding:**

- Seed 15,000 cells per dish (1.5 mL) in Mat-Tek P35G-1.5-14-C dishes for 24 h.

**Transfection (procedure for 1 dish):**

- Prepare DNA solution: dilute 3 µL FuGENE in 100 µL Opti-MEM and mix thoroughly, add 1 µg of DNA, mix again and incubate 30 min at room temperature.
- Wash the cells gently with PBS x 3.
- Add 1400 µL of Opti-MEM.
- Add the DNA solution prepared previously.
- Incubated at 37ºC overnight (8-10 h). **Important: Opti-MEM does not contain antibiotic!**
- Remove transfection mixture.
- Wash gently with PBS x 3.
- Add DMEM high glucose + 10 % FCS.
- Allow cells to express YCs proteins for 3-6 days (incubate at 37ºC and change medium as usual).

**Measurement:**

- Incubate at 30ºC (4-8h) to allow correct folding.
- Use medium without phenol red or the corresponding buffer for measurements.
**Fluorescent Proteins - Material and solutions**

Material and solutions are detailed below. Changes should be made to adapt them to your own needs (see some tips here).

**Cells seeding:**

- ATCC HeLa cells grown in DMEM high glucose.
- Glass Bottom Culture Dish (Mat-Tek P35G-1.5-14-C).

**cDNA for expression of YCs**

All constructions were kindly provided by Roger Y. Tsien.

- YC2: shows a biphasic Ca\(^{2+}\) response (suitable for [Ca\(^{2+}\)] between 100 nM and 10 µM)
- YC3.1: shows a monophasic Ca\(^{2+}\) response (suitable for [Ca\(^{2+}\)] between 1 nM and 100 µM)
- YC3.1er: equivalent to YC3.1 but it has the calreticulin leading sequence in the N terminus and the retention signal KDEL in the C terminus. This YC is retained in the ER.
- YC4erQ69K: low affinity indicator (suitable for 10 - 1000 µM Ca\(^{2+}\)). It has the calreticulin leading sequence in the N terminus and the retention signal KDEL in the C terminus, so it is also retained in the ER.

**Solutions and reagents**

- FuGENE (Roche Diagnostics)
- Opti-MEM (Gibco-BRL)
- cDNA in ddH\(_2\)O
- DMEM high glucose (with and without Phenol Red)
- PBS

Calcium measurements should be performed in medium/buffer without Phenol Red (it can interfere in fluorescence signal).
Excel macros - Basic

Life is easier if we do not have to repeat the same thing over and over again. For this reason macros were invented.

One of the most popular software packages in the market is Microsoft Office®, which includes Excel for manipulating data. This program can be used to arrange and represent quantification data saved in text format, so some tips on how to make macros for Excel are detailed here. Software for acquiring and processing digital images usually also includes a self-built macro-recorder, but each trademark has its particular system.

Though writing VisualBasic macros for Microsoft Excel might look very complicated; using the self-built macro-recorder makes it quite easy. Just open Excel, click on Record macro icon (activate Visual Basic Applications, VBA, tool bar or choose Tools > Macro > Record), give a name to your new macro, choose a location to store it (your personal book is a good place) and make the sequence of commands that you want to record. Once finished, click stop recording. Playing the macro again is as easy as clicking “play macro”.

To visualize the code, just click on Visual Basic Editor. Each macro start by a Sub statement plus the macro name and finishes with End Sub. Comments start with a ’ sign (they are shown in green).

A macro or Sub statement called “MoveColumn” that cut the content of column G and H, paste it in column L and M and then eliminate the empty G and H column looks like this:

```vba
Sub MoveColumn()
    ' MoveColumn Macro
    ' Macro grabada el 11/06/2004 por Montse
    Columns("G:H").Select
    Selection.Cut
    Columns("L:M").Select
    ActiveSheet.Paste
    Columns("G:H").Select
    Selection.Delete Shift:=xlToLeft
End Sub
```

Comments about macro (additional comments can be added after a line just writing a ‘ sign)

Commands to execute: select the columns, cut them, select the destination columns, paste the previous ones, select the empty columns and delete them shifting the rest of the sheet to the left. This was written directly by the Macro recorder!

Though it does not look very useful at first glance, if you have a lot of files with column that need to be ordered, you will love a macro like this one (adapted to your needs, of course). Just record the macro while you order the first file and then apply it to the other files.

Unfortunately, not all macros are so easy to build. Modifications in the code allow introducing variables, loops, message box, etc. Tips on how to write code can be found in Excel help. Some useful tips and code for opening text files and ordering data are included here.

Be careful! different Excel version and/or different languages, can render different results (e.g. dots and comma are sometimes misunderstood depending on the “Regional
Settings). We use the Spanish version of Microsoft® Excel 2002 and Spanish settings.

If you are interested in learning a bit more, you can visit, for example, VBATutor for Office97.

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