Imaging mitochondrial calcium fluxes with fluorescent probes and single or two photon confocal microscopy

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Summary
The concentration of calcium ions in the mitochondria has been shown to affect its function, modulating respiratory activity at low levels, and causing lethal damage at high concentrations. The rhodamine series of dyes can be used to measure mitochondrial calcium concentration, but the reliability of measurements depends upon correct partitioning of dye within to the mitochondria. Methods are described to aid verification and quantification of the mitochondrial calcium concentration using single or two photon confocal microscopy. The method of linear unmixing to separate fluorescent signals based on either differing excitation or emission spectra is outlined and for the purposes of illustration is applied to the separation of rhod-2 signals originating from dye within the mitochondrial and nucleoli.

Key words
Calcium, mitochondria, rhod-2, confocal microscopy, multiphoton microscopy

1. Introduction

Ca^{2+} is not distributed evenly throughout the cell, but concentrated in microdomains, some of which are membrane-bound, such as the mitochondria [1]. Ca^{2+} enters mitochondria via the Ca^{2+} uniporter in accordance with its electrochemical gradient and accumulates in the mitochondrial matrix [2]. The concentration of Ca^{2+} in the mitochondria can affect mitochondrial respiration, since the rate-limiting enzymes of the citric acid cycle of mitochondria are activated by Ca^{2+}[2]. Physiological stimulation can increase $[Ca^{2+}]_{mito}$ in numerous cell types. For example, $[Ca^{2+}]_{mito}$ changes during with each contraction in cardiomyocytes. Mitochondria may act as a spatial buffer in normal cellular calcium signalling [1,3,4].

Mitochondrial calcium uptake is an important determinant of cell death particularly in the heart and the brain [4]. In particular, mitochondrial calcium overload causes opening of a large non-specific pore in the mitochondria - the mitochondrial permeability transition pore (mPTP) [4]. This results in rapid depolarization of the mitochondrial membrane and cessation of mitochondrial respiration. Prevention of mPTP opening can prevent cells from dying during ischaemia and reperfusion injury [5]. Given its pathological importance and the importance of understanding how it is regulated it is therefore necessary to be able to measure $[Ca^{2+}]_{mito}$ accurately.

Direct measurement of intramitochondrial Ca^{2+} using electron probe X-ray microanalysis showed that, under physiological conditions, the concentration of free matrix Ca^{2+} in liver mitochondria in vivo is fairly low, at ~ 0.3 mM [6], though it should be kept in mind that up to 2,500 times more calcium may be present in a non-ionised or bound form [7]. The development of dyes which increase fluorescence when they bind Ca^{2+} has greatly simplified the measurement of intracellular Ca^{2+}. Detection of mitochondrial Ca^{2+} was enabled in 1989 with the development of indicators based on rhodamine [8]. A range of these dyes are available with different Ca^{2+}-binding affinities (Table). With a K_D of 570 nM, rhod-2 is suitable for most studies. Intracellular accumulation is enhanced with the AM ester form of the dye (available from Invitrogen / Molecular Probes). Rhod-2/AM partitions in the mitochondria, but depending on the loading conditions used, there will normally be some dye present in the cytosol. With careful analysis however it can still be used to measure mitochondrial calcium.

There are other potential limitations with the use of rhod-2 that one should be aware of. Firstly, it is not ratiometric. i.e.: since the emission spectrum does not change on binding to Ca^{2+}, there is no way to control for efficiency of loading of the dye by comparing Ca^{2+}-responsive and Ca^{2+} non-responsive emitted fluorescence. Secondly, the reaction products of AM ester hydrolysis are acetate and formaldehyde, which may affect cellular functioning. Thirdly, the presence of rhod-2 may affect Ca^{2+} buffering itself, since it binds to calcium, so the concentration of rhod-2/AM used should not be increased above that recommended here. Lastly, the dye may be sensitive to pH, and may also accumulate in other cellular structure such as lysosomes [9]. In many cells it also accumulates within nucleolar regions, appearing as bright regions within the nucleus, though this appears to be artefactual and there is no evidence it corresponds to Ca^{2+}. It may be possible to remove some of this contaminating signal by computational methods such as linear unmixing as described in the method below, using for the purposes of illustration, the example of separating

the fluorescent signal originating from the mitochondria from that originating from the nucleoli.

By being aware of these precautions and by following the steps outlined in the protocols below, it is possible to take accurate measurements of free mitochondrial calcium.

2.Materials

1. Rhod-2/AM (Molecular Probes / Invitrogen) is supplied in aliquots. Add 50 ul DMSO to a tube before use, and store at -20 °C for up to 1 mo.
2. Imaging dish to contain coverslip and buffer while imaging cells
3. Confocal microscope equipped with HeNe (543 nm wavelength) laser and inverted objective[i]
4. Imaging buffer: 156 mM NaCl, 3 mM KCl, 2 mM MgSO4.7H2O, 1.25 mM K2HPO4, 2 mM CaCl2, 10 mM HEPES, 10 mM D-glucose, adjust to pH 7.4 with NaOH
5. Ca^{2+}-free buffer : Imaging buffer + 5 µM A23187[ii] + 5 mM EGTA to obtain the minimum (Rmin) fluorescence value.
6. High Ca^{2+} buffer : Imaging buffer + 5 µM A23187[iii] in presence of 3 mM Ca^{2+} to obtain the maximum (Rmax) fluorescence value.

Optional

1. Intracellular buffer: 130 mM KCl, 80 mM aspartate, 10 mM HEPES, 3 mM $MgCl_2.6H_2O$, 4 mM Na.pyruvate, 0.5 mM EGTA, 3 mM Na_2.ATP, 0.12 mM $CaCl_2$. Adjust pH to 7.3 using KOH.
2. Permeabilization buffer: intracellular buffer containing either 50 µg/ml saponin or 2.5 µM digitonin

3.Methods

Rhod-2/AM is taken up into cells and the AM ester is cleaved by cellular esterases. Various loading protocols can be used in order to increase the extent of dye loading into the mitochondria. A common technique is to load the dye at low temperature (RT, or even 4 °C), which inhibits esterase activity and allows the dye to localize into mitochondria, then move the cells to 37 °C where the dye is de-esterified and activated [9,10]. The optimum protocol depends on the cell type and should be determined empirically. If loading of dye can be achieved such that it is predominantly mitochondrial then it may be possible to image fluorescence using a fluorescent microscope. If not, in order to reliably distinguish the mitochondrial signal from the cytosolic signal, it will be necessary to use a confocal microscope to obtain the images. Note that an “inverted microscope” with the objective approaching the coverslip from below is required so that the live cells can be imaged while bathed in the imaging buffer. However, even after optimization there may still be some mis-localization of rhod-2 to intracellular structures such as lysosomes [9], and nucleoli, which it may be possible to distinguish by using dye separation techniques such as linear unmixed. This involves taking a series of images of the same field over a range of excitation or emission wavelengths, then digitally separating the fluorescence into the two (or more) components, and is described below.

Various approaches may be used to verify the extent of mitochondrial loading, including: ensuring the majority of the dye remains in the mitochondria after plasma membrane permeabilization (Fig 1D); loading cells with a cytosolic dye such as fluo-4/AM to confirm that the mitochondrial signal is distinct from that of the cytosol (Fig 2); and addition of 1 µM FCCP to depolarize mitochondria causing a decrease in $[Ca^{2+}]_{mito}$.

It is very important to bear in mind that after loading cells with rhod-2, mitochondrial localization may not be immediately obvious, as the signal will be weak at resting levels of $(Ca^{2+})_m$. It may be necessary to stimulate the cells with an agonist to raise $(Ca^{2+})_m$ before the mitochondrial localization becomes evident (Fig. 2A (i and ii), b (i and ii)). Note also that calcium-independent variations in signal intensity through the cell – staining of lysosomes or the nucleoli, for example – disappear if the images are ratioed against a first “resting” image. This means extracting a baseline image from an image series and dividing the whole of the rest of the series by that first image (Fig. 2B). Under these conditions, any nonuniformities of labelling that do not change when calcium signals change disappear. To prevent the background between cells becoming very noisy (as here you are dividing very small numbers by other very small numbers and so there will be a lot of noise), one can multiply the image series by a binary mask. To achieve the binary mask is trivial in most imaging software – it requires selecting a threshold and setting all signals above the threshold to a value of unity and all pixels below the threshold to zero. Multiplying the image series by the mask makes all the signals below the threshold disappear as set to zero, while the image data of interest above threshold remains unchanged.

In the example in Fig. 2, HL-1 cells – a cardiac-derived cell line that shows spectacular spontaneous calcium signals – were dual loaded with rhod-2/AM and fluo-4/AM as described in the protocol above. The sequence of Fig. 2A shows the raw rhod-2 images, in Fig. 2B the ratioed rhod-2 images while Fig. 2C shows the sequence of the fluo-4 signals (ratioed against the first image). Figure 2D shows plots of the intensity with time for each fluorophore from two regions of interest as marked (arrowhead and asterisk). Spontaneous cytosolic signals can be seen (i) and only when the cytosolic signals reached a high enough level did mitochondrial calcium rise. There are several important points illustrated here (1) at the start of the imaging, no mitochondrial signal is detectable, but mitochondria appear when calcium rises; (2) significant cytosolic rhod-2 signal remains but is distinguishable from the mitochondrial signal as the latter shows a distinct time course which is quite different to the cytosolic signal – which is in turn confirmed by the fluo-4 signal;(3) in the ratioed image local variations in signal disappear, highlighting only the areas in which the calcium-specific signal is changing.

It is possible to convert the fluorescent values obtained from the microscope into actual Ca^{2+} concentration. This requires calibration of the fluorescence values obtained when the dye is saturated (ie: in the presence of high $[Ca^{2+}]$)(Fig 1C) and background (ie: in the presence of a chelator of Ca^{2+} such as EGTA)(Fig 1B). These measurements are performed in the presence of a Ca^{2+} ionophore which equalizes $[Ca^{2+}]$ inside and outside the cell.

The following procedure describes the use of a Leica SP5 confocal microscope, but the procedure is similar on any similar confocal microscope.

3.1 Preparing the cells

1. Place glass coverslips in the bottom of 6-well tissue culture dishes or plates.[iv]

2. Trypsinize the cells and plate them in the wells containing coverslips at a density sufficient to achieve ~70% confluence when they are imaged.
3. Return cells to tissue culture incubator for at least 24 h to allow them to attach and recover[v].

3.2 Loading rhod-2/AM into the cells.

1. In a 1.5 ml eppendorf tube, add 5 µM rhod-2/AM + 5 µl 20% pluronic, then add 1 ml imaging buffer.
2. Replace the buffer on the cells with imaging buffer containing rhod-2/AM and pluronic
3. Leave cells 30 min RT to take up the dye.
4. Place cells in tissue culture incubator for 20 min for de-esterification of dye.
5. Leave the cells 20 min RT to complete dye de-esterification[vi].

3.3 Confocal imaging of rhod-2/AM fluorescence.

1. Transfer the coverslip containing the cells to the imaging chamber
2. Wipe any liquid from underneath the coverslip using a tissue and place the chamber on the objective of a microscope, adding a drop of oil to the objective if necessary
3. Using phase contrast or transmitted light, adjust the focus until the cells are clearly visible[vii].
4. In the confocal microscope software, choose an appropriate default imaging option for imaging of a red fluorescent dye[viii]. i.e: excitation using the 543nm line of the laser, and emitted light collected between 560-630nm (or if a bandpass filter is not available, use a longpass filter of 560nm).
5. Decrease the laser power to ~5% to avoid damaging the cells and increase the gain setting to maximum.
6. Start the continuous scan, and adjust the focus until the mitochondria in the cell are clearly visible[ix]
7. Open the pinhole setting in the software to approximately 3 Airey Units (AU)[x] and decrease the gain until a signal of ~50% saturated intensity is obtained[xi].
8. Stop continuous scanning.
9. Increase the number of images averaged to 4 and/or decrease the scan speed to obtain a higher quality image.
10. Start the scan to obtain the image of rhod-2 fluorescence in the cells.

3.4 Calibrating the fluorescence to calcium concentration.

Incubating the cells with a calcium ionophore will cause [Ca^{2+}] in all cellular compartments to equalize to the concentration in the buffer. In a buffer containing no Ca^{2+} and EGTA to chelate all Ca^{2+}, the minimum rhod-2 fluorescence value can be obtained (Fig 1B). In a buffer containing a saturating concentration of Ca^{2+}, the maximum rhod-2 fluorescence value can be obtained (Fig 1C). Using these images and the Kd for the dye being used it is possible to estimate the organellar concentration of calcium (Fig 1A). However, this will necessarily be an estimate, since intracellular components, particular pH and the presence of heave metals can influence the dye response.

1. Replace the buffer on the cells with calcium-free buffer and wait 2 min.[xii][xiii]

2. Image the cells using the same parameters as above[xiv].
3. Replace the buffer on the cells with high calcium buffer and wait 2 min.
4. Image the cells using the same parameters as above.
5. Using the analysis part of the software, draw a region of interest (ROI) containing a single mitochondrion or close group of mitochondria. Measure the average fluorescence intensity in the ROI to obtain F_{min} (calcium-free image) and F_{max} (high calcium image).
6. To calculate free $[Ca^{2+}]_{mito}$, use F_{min}, F_{max} and the Kd for the dye being used (see table 1), in the following formula from Grynkiewicz et al. [11]

$$[Ca^{2+}]_{free} = Kd\ [F-F_{min}]/[F_{max}-F]$$

3.5 Assessing the extent of dye compartmentalization by plasma membrane permeabilization

One approach to estimate the extent of dye that is compartmentalized in the mitochondria is to permeabilized the plasma membrane, resulting in the loss of cytosolic dye into the buffer (Fig 1D) and calculate the proportion remaining. This does not account for mitochondria volume however. An alternative approach is to determine the relative effective concentration of rhod-2 in the mitochondria compared to in the cytosol by determining the ratio of rhod-2 fluorescence in the mitochondria compared to the cytosol in the presence of saturating (high) calcium.

1. Use the image of the cells in high calcium from step 4 of section 3.4.
2. The relative effective concentration of rhod-2 in the mitochondria compared to in the cytosol can be determined by drawing one ROI in the cytosol and one ROI around the perimeter of a mitochondria, and a third ROI in a region with no cell (“background”). Calculate the ratio according to: (mitochondria-background)/(cytosol-background).
3. Continuing from step 4 of section 3.4, replacing the buffer on the cells with permeabilization buffer, which permeabilizes plasma membrane while leaving organelle membranes intact.
4. Start continuous scanning.
5. After several minutes[xv], the cytosolic signal will rapidly disappear over 10-20 s. This will happen at approximately the same time in cells. Wait until all cells in the field are permeabilized and signal intensity is constant.
6. Image the cells using the same parameters as step 1 (“Organelle fluorescence”)
7. Add 1 mM $MnCl_2$ + 5 μM A23187 to the buffer to quench all remaining dye fluorescence from the mitochondria and other organelles.
8. Image the cells using the same parameters as step 1 (“background”)
9. Using a region of interest surrounding an entire cell, calculate the average fluorescence intensity of each image.
10. Express (“Organelle fluorescence”-“background”)/(Total fluorescence”-“background”) as a percentage. This represents the extent of dye compartmentalization (i.e: the percentage of rhod-2 in the cell localized to mitochondria).

3.6 Using linear unmixing of emitted wavelengths to remove unwanted fluorescence artefacts

There can be occasions when it is useful to improve the image by removing unwanted fluorescence originating from cellular autofluorescence or other artefacts. It is also possible to remove or separate the fluorescent signal of other added dyes even if they have very similar or largely overlapping fluorescent emission spectra (see [12] for an example of separating rhod-2 from TMRM signal) (Fig 3B). To achieve this it is necessary to have a confocal microscope equipped with a finely graded emission filter. For example, the Leica SP5 includes as standard the ability to perform a "lambda" scan – collecting fluorescence over a range of wavelengths. The Zeiss META addition confers a similar capability. The software then allows "linear unmixing" in which the signal is separated into that originating from the different dyes. The basic principle that follows is for a Zeiss 510 META confocal, and demonstrates how the non-specific rhod-2 fluorescence from the nucleoli can be removed from the image (Fig 4A).

1. Perform the steps outlined in sections 3.1 to 3.2 to obtain a standard image of rhod2 fluorescence.
2. In lambda-mode (selected under "configuration control" button), using the 543nm laser, set the start and end of the lambda stack at 550nm and 650 nm respectively, and the interval to 10.7 nm. Set the color palette to "Range indicator" and using continuous scan, adjust the gain so that the signal does not saturate at any pixels.
3. View the image by clicking the "mean of ROIs" display method.
4. Using the region of interest (ROI) tools, draw a ROI exactly surrounding the rhod2 fluorescence in a nucleolus, a second ROI around a region where the mitochondria are bright, and a third, large ROI in a region outside the cell where there is background signal.
5. Click on "Linear unmixing" and the software will separate the image into a new image with four panels. Panel one contains all the non-specific and nucleolar fluorescence, panel two contains the mitochondrial rhod2 signal, panel three contains the background (which should be dark), and panel four contains an overlay of the other three panels. Adjust the colors a desired (eg: red for mitochondria, green for non-specific) (Fig 4C,D,E).

4.1 Multiphoton confocal imaging of rhod-2 fluorescence

Multiphoton confocal imaging is similar to confocal imaging except it is designed to use a tuneable laser in the infrared range of wavelengths, and excitation of the fluorophore (ie: rhod2 in this case) occurs only when 2 independent photons arrive simultaneously at the focal point. This design confers a number of advantages including lower toxicity, less photobleaching and greater depth penetration in the case where imaging is performed in whole tissues. The microscope must be designed or adapted specifically for multiphoton imaging, usually including a non-descanned detector for greater light sensitivity. When using the non-descanned detector it is necessary to exclude all extraneous lights by covering the microscope in a light-proof sheet or cover, and normally by turning out the room lights as well. To image rhod2 fluorescence in cells, load the dye as per the procedure above, but instead of the instructions in section 3.3, perform the following steps (described for a Zeiss LSM

NLO microscope). Rhod2 can be excited by any of a range of infrared excitation wavelengths (Fig 3A), though usually 840nm is appropriate.

1. Transfer the coverslip containing the cells to the imaging chamber
2. Place the chamber on the objective of a microscope.
3. Using phase contrast or transmitted light, adjust the focus until the cells are clearly visible[xvi].
4. In the confocal microscope software, adjust the excitation wavelength to an appropriate value (eg 840nm).
5. Collect emitted light using a bandpass filter such as 575-640nm (or if a bandpass filter is not available, use a longpass filter of 560nm).
6. Using the least power possible, adjust the gain and power to obtain a satisfactory image.
7. Start the continuous scan, and adjust the focus until the mitochondria in the cell are clearly visible[xvii]
8. Decrease the gain until a signal of ~50% saturated intensity is obtained[xviii].
9. Stop continuous scanning.
10. Increase the number of images averaged to 4 and/or decrease the scan speed to obtain a higher quality image.
11. Start the scan to obtain the image of rhod-2 fluorescence in the cells.

4.2 Using linear unmixing of excitation wavelengths to remove unwanted fluorescence artefacts

As describe in section 3.5, it is possible to separate out the fluorescence from different dyes, not only by their emitted spectra, but by their excitation spectra. Since it is possible to tune the excitation wavelength of the infrared laser, using a multiphoton microscope one can also perform linear unmixing of the images as described below for the Zeiss 510 NLO.

1. Begin by obtaining a standard image at 840nm as described in section 4.1.
2. Configure the software so that it is imaging relatively fast over the entire area of the cell (<4 s per scan) and so that there are no saturated pixels.
3. Select and run the macro “XPrint”[xix]. Select the “Excitation lambda stack” tab”.
4. Load the data table for the appropriate objective[xx]
5. Set the start and end wavelengths to 550nm to 650nm respectively, at 10nm intervals.
6. Click “Start”.
7. Using the region of interest (ROI) tools, draw a ROI exactly surrounding the rhod2 fluorescence in a nucleolus, a second ROI around a region where the mitochondria are bright, and a third, large ROI in a region outside the cell where there is background signal.
8. Click on “Linear unmixing” and the software will separate the image into four panels. Panel one contains all the non-specific and nucleolar fluorescence, panel two contains the mitochondrial rhod2 signal, panel three contains the background (which should be dark), and panel four contains an overlay of the other three panels. Adjust the colors a desired (eg: red for mitochondria, green for non-specific) (Fig 4F,G,H).

Figure Legends

Figure 1
Cells loaded with rhod-2/AM according to the standard protocol. Some fluorescence is visible originating from the mitochondria, though there is also some dye present throughout the cytosol, and in the nucleoli (small spots within the nucleus)(A). By measuring the fluorescence on changing to the Ca^{2+}-free buffer (B) and then to high Ca^{2+} buffer (C), it is possible to calculate the actual Ca^{2+} concentration, in this case, using Kd of 570nM, we obtain a $[Ca^{2+}]_{mito}$ of 196 nM. From panel C, the relative concentration of rhod2 in the mitochondria is determined to be 2.7 fold that in the cytosol. Finally, plasma membrane permeabilization (D) eliminates all but mitochondrial staining, allowing estimation of the proportion of dye localized to mitochondria (here, ~30%).

Fig. 2. HL1 cells were dual loaded with fluo-4AM and rhod-2AM (5 uM each for 20 min at room temperature) followed by washing. They were mounted on the stage of a Zeiss 700 confocal microscope and images acquired sequentially exciting at 488 and 555 nm, measuring emitted fluorescence at 505–550 nm (fluo-4) and at >570 nm (rhod-2). Both localized and global spontaneous calcium signals occur in these cells. Images extracted from the time sequence show a “resting” signal showing small local calcium signals in two cells (i), the peak global cytosolic signal (ii), and the residual mitochondrial signal after the recovery of the cytosolic signal (iii). The sequence (a) shows the raw rhod-2 data, in (b) the rhod-2 images ratioed against the resting signal and in (c) the corresponding fluo-4 images ratioed against the resting images. In (d) two plots are shown with the points indicated arrowhead and asterisk showing the divergence of the time course of the cytosolic calcium signal (green) and the mitochondrially localized calcium signal (rhod-2, red).

Fig.3. The infrared excitation spectra (**a**) and emission spectra (**b**) for rhod-2 localized to the mitochondria (*solid line*) and nucleoli (*dashed line*). Despite the spectra for rhod-2 being very similar in both compartments, they are sufficiently different to allow separation of the signals when using multiphoton microscopy.

Figure 4
Either single photon confocal microscopy (A) or two photon confocal microscopy (B) can be used to visualize rhod-2/AM fluorescence. The mitochondrial signal (arrowheads) can be separated from other contributing fluorescence sources such as nucleoli (arrows), by using linear unmixing of a series of images taken using 800-950 nm excitation wavelengths (C-E), or by linear unmixing of a series of images taken with 840nm excitation wavelength and a lambda series of emission wavelengths ranging from 506-635 nm (F-H).

Table

Dye	Kd
Rhod-2	570 nM
X-rhod-1	700 nM
Rhod-FF	19 μM
Rhod-5N	320 μM
X-rhod-FF	17 μM
X-rhod-5F	1.6 μM
X-rhod-5N	350 μM

Table 1. Kd (dissociation constant) values of a number of different mitochondrial Ca^{2+}-sensitive dyes[xxi].

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4. Notes

[i] An inverted microscope is more convenient for imaging live cells in buffer since the objective approaches from below the coverslip, however it may be possible to use a microscope in a standard orientation by using an appropriate imaging chamber such as a perfusion chamber in which the cells on the coverslip are placed at the top (facing in towards the buffer in the chamber).
[ii] Ionomycin may be used instead of A23187
[iii] Ionomycin may be used instead of A23187
[iv] In order to obtain images on sufficient resolution to distinguish mitochondria, it is essential to image the cells grown on a glass coverslip rather than imaging directly in the plastic tissue culture dish. An alternative is to grow cells on glass bottom tissue culture dishes (MatTek corporation, MA USA).
[v] If the cells are not well flattened the mitochondria will tend to remain around the nucleus making them difficult to image. It may be necessary to allow a longer time in order for some cell types to spread out on the cover slip, or to pre-coat the coverslip with 0.1% gelatin or fibronectin.
[vi] Over longer periods, the dye will be gradually extruded from the cytosol but remains in the mitochondria. It can be advantageous at this stage to return the cells to normal medium overnight, replacing the imaging buffer the following day, resulting in the fluorescence being close to 100% mitochondrial in origin.
[vii] Do not use a fluorescent lamp to focus the cells as this tends to cause oxidative damage and a progressive increase in dye fluorescence.
[viii] On the Leica confocal "texas red" is suitable. Other equivalent dyes are TMRE, or Mitotracker red.
[ix] It is usually easiest to focus on the mitochondria spread out around the middle of the cell, rather than those clustered around the nucleus.
[x] This increases the "thickness" of the optical slice being imaged, thus decreasing the likelihood that mitochondria will move up or down out of the imaging plane.
[xi] The presence of saturated (ie: maximum intensity) pixels is determined by changing the color scale to one indicating saturated pixels as blue. ~50% intensity is selected to that there is "overhead" room for the signal to increase without saturating the measurement.
[xii] To replace the buffer without moving the imaging field, use a 2 ml syringe with a 20 cm piece of flexible narrow tubing slipped tightly over the needle to remove the buffer, then, before cells dry out, rapidly replace the buffer with a 1 ml Gilson pipette.
[xiii] Some cells such as muscle cells may contract when calcium is increased, making analysis difficult. To prevent morphological alterations during calibration, cells can be depleted of ATP by 10 minute pre-treatment with 1 mM cyanide.
[xiv] In order to make valid comparisons and calculations between the different treatments it is essential that the imaging parameters (laser power, gain, objective, zoom, etc) remain the same.
[xv] The exact time will depend upon the cell type
[xvi] Do not use a fluorescent lamp to focus the cells as this tends to cause oxidative damage and a progressive increase in dye fluorescence.
[xvii] It is usually easiest to focus on the mitochondria spread out around the middle of the cell, rather than those clustered around the nucleus.
[xviii] The presence of saturated (ie: maximum intensity) pixels is determined by changing the color scale to one indicating saturated pixels as blue. ~50% intensity is

selected to that there is “overhead” room for the signal to increase without saturating the measurement.

[xix] If not printed on one of the macro buttons it may be necessary to install the macro first (refer to the Zeiss LSM software manual).

[xx] This data table contains a list of laser attenuations necessary to ensure that the power is the same at each laser wavelength (since power varies with wavelength), and must be calibrated to each objective normally used.

[xxi] The Kd of dyes in vivo may be different from these values determined in vitro, largely due to interfering interactions with cellular components (e.g. [13]). For example the *K*d for rhod-2 is 720 nM in intact cardiomyocytes [14].

Fig 1

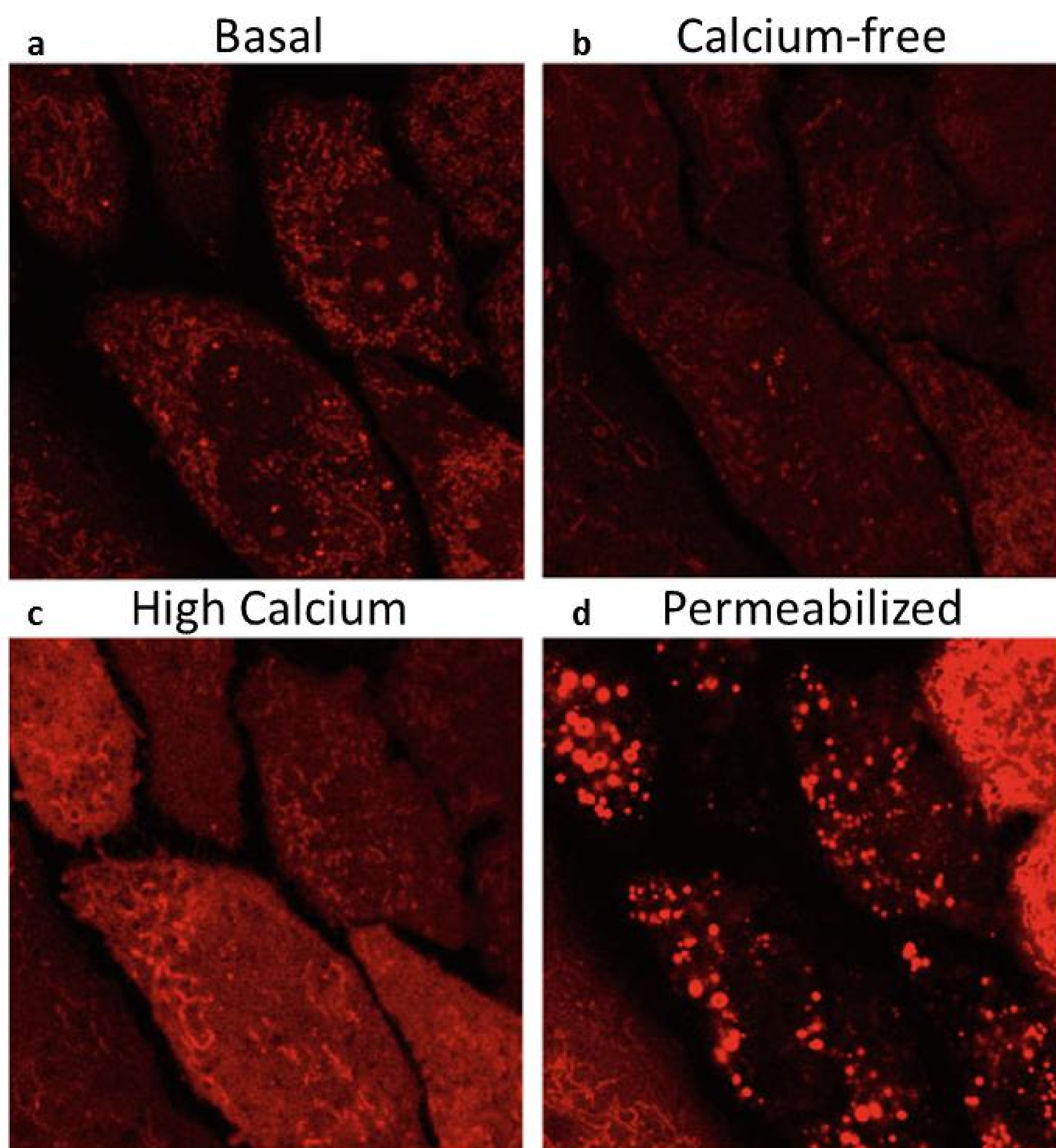


Fig 2

a

i ii iii

b

i ii iii

c

i ii iii

c

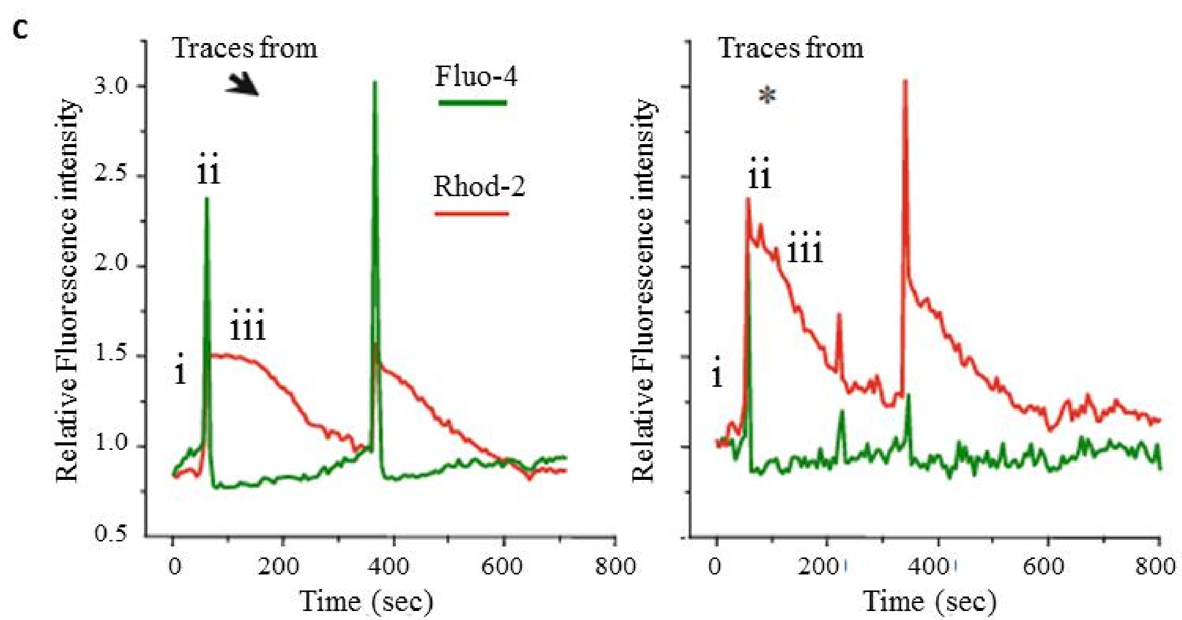
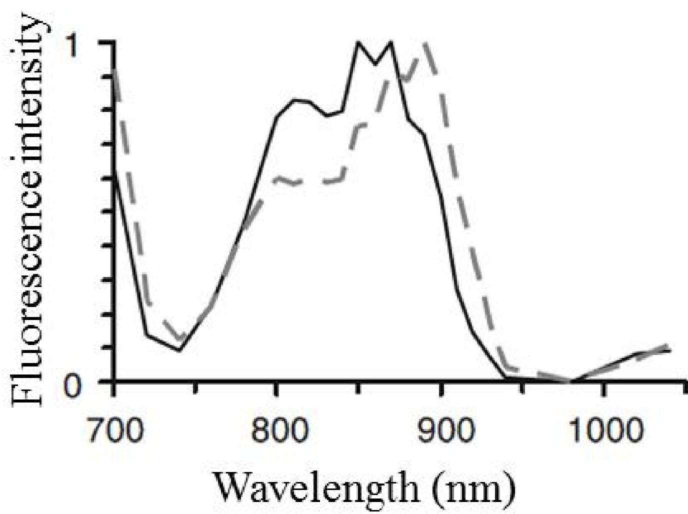


Fig 3



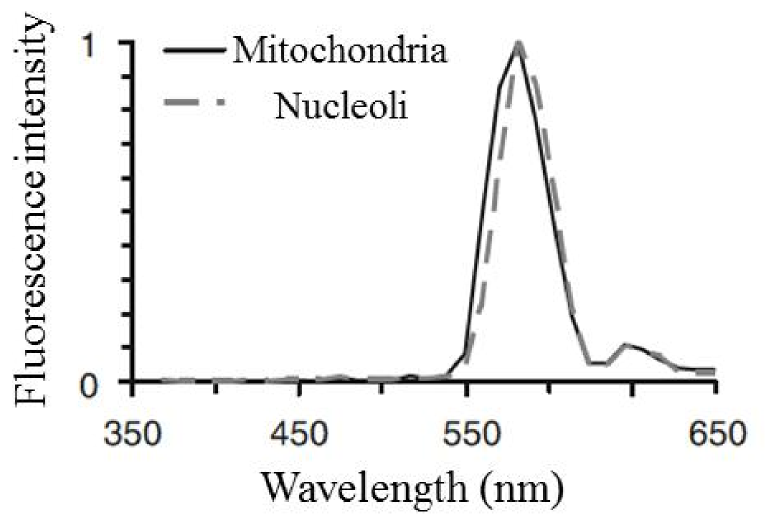


Fig 4