Visualisation of mitochondria in living neurons with single- and two-photon fluorescence laser microscopy

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Abstract

In this work we investigated the relative merits of conventional single-photon confocal laser scanning fluorescence microscopy (CLSM) and two-photon laser scanning fluorescence microscopy (2p-LSM) for the study of mitochondria in living neurons. Dorsal root ganglion neurons were loaded with the mitochondrion-specific fluorescent dye JC-1, the ratio between red (J-aggregates) and green (monomer) fluorescence of which reflects mitochondrial membrane potential. Cells were illuminated at 488 nm for single-photon excitation or at 870 nm for two-photon excitation. In both modalities we found that mitochondria showed: (i) similar appearance; (ii) similar fluorescence ratio values over both whole cell bodies and individual mitochondria; and (iii) similar responses to mitochondrial uncoupler, which dropped the ratio values by 50%. However, 2p-LSM exhibited several advantages over CLSM: (i) better signal/noise ratio in the green emission channel; (ii) less phototoxicity upon repetitive scanning in the focal plane; and (iii) no significant loss of image quality upon repetitive scans in the *z* direction. We conclude that, while both techniques enable visualisation of individual mitochondria in living cells, 2p-LSM has significant advantages for physiological work requiring time-lapse experiments or four-dimensional reconstructions of mitochondria. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Mitochondria; Confocal laser microscopy; Two-photon laser microscopy; Sensory neurons; JC-1

1. Introduction

Mitochondria are semi-autonomous organelles with a large variety of functions in cellular metabolism (for review see Skulachev, 1988). A major role of mitochondria is ATP production, which depends on mitochondrial membrane potential (MMP) and determines cell survival and specific cell functions. A number of microscopic techniques have been used for the study of mitochondria in living cells including bright field, phase contrast and video enhanced microscopy. However, a current technique of choice is fluorescence microscopy. Fluorescent probes can, in appropriate circumstances, enable the detection of spatial and temporal changes of the inner compartments which lie below the resolution limit of light microscopy (Bereiter-Hahn and Voth, 1994).

A number of fluorescent probes have been introduced for mitochondria, including rhodamines and cyanine derivatives (Bereiter-Hahn and Voth, 1994). All of them are positively charged lipophilic molecules, which are accumulated in mitochondria driven by MMP. However, the most reliable fluorescent dye reflecting MMP in living cells is JC-1 (5,5$'$,6,6$'$-tetrachloro-1,1$'$,3,3$'$-tetraethylbenzimidazolo carbocyanine iodide) (Salvioli et al., 1997). This is a lipophilic compound with delocalised positive charge and high internal negativity, which is taken up by mitochondria to a greater extent than by other organelles, including the plasma membrane. JC-1 exists as a monomer at low concentration and as aggregates, called J-aggregates, at high concentration. JC-1 monomer is maximally excited at 490 nm and emits at around 530 nm. Upon aggregation, fluorescent emission shifts to 590 nm. As a consequence, mitochondria with a low membrane potential will accumulate low concentrations of JC-1 and will fluoresce in green; in more highly polarised regions within mitochondria fluorescence, will be orange–red. It was estimated that mitochondria with MMP exceeding 140 mV form J-aggregates (for review see Reers et al., 1995).

JC-1 was used for the measurement of MMP by cytofluorometry and confocal microscopy in living cells (Di Lisa et al., 1995; Salvioli et al., 1998) as well as in isolated mitochondria at the single organelle level (Cossarizza et al., 1996). Confocal microscopy enables measurement of MMP

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changes within a population of cultured central neurons (White and Reynolds, 1996). However, a major limitation of single-photon confocal laser scanning fluorescence microscopy (CLSM) in living cells is the phototoxicity of high-intensity short wavelength light on living cells. In confocal microscopy the area above and below the focal plane is equally subject to bleaching, and the situation is exacerbated by the high laser power required to compensate for the relatively poor sampling statistics, compared to widefield microscopy (for review see Pawley, 1995).

Two-photon excitation laser scanning fluorescence microscopy (2p-LSM) could circumvent these problems (Cox and Sheppard, 1999). In 2p-LSM a fluorescent molecule is excited by the simultaneous absorption of two photons, each with approximately half the energy required to stimulate emission. Only fluorophores that absorb two photons simultaneously can be excited; the excitation probability decreases rapidly away from the focal point, and thus the out-of-focus volume effectively receives no excitation. This obviates the requirement for a pinhole, thereby improving the collection statistics (for theoretical background, instrumentation and technical aspects see Konig et al., 1996; Pawley, 1995).

Several previous studies showed the practical advantages of 2p-LSM over CLSM. 2p-LSM provides unprecedented capabilities for three-dimensional (3D), spatially resolved photochemistry, particularly photolytic release of caged effector molecules (Denk et al., 1990). This technique has been used for various applications, specifically, imaging of vital DNA stains in developing cells and embryos, imaging of cellular metabolic activity from NADH autofluorescence, spatially resolved measurements of cytoplasmic calcium ion activity and optically induced micropharmacology using caged bioeffector molecules (Williams et al., 1994). Two-photon excitation of the ultraviolet-absorbing fluorescent calcium indicator Indo-1 in laser scanning microscopy makes possible a quantitative, 3D recording of intracellular free calcium activity distributions and dynamics with low background and minimal photobleaching (Sako et al., 1997). Two-photon excitation scanning laser microscopy with near-infrared excitation provides high fluorescence collection efficiency, reduced photodamage and eliminates ultraviolet chromatic aberration, all of which have previously degraded the visualisation of pyridine nucleotide fluorescence (Piston et al., 1995).

Previously we have applied CLSM to 3D reconstructions of mitochondrial JC-1 fluorescence in living neurons (Dedov and Roufogalis, 1999b). We now compare CLSM with 2p-LSM in the same system.

2. Materials and methods

2.1. Culture of dorsal root ganglion neurons

This study was approved and carried out in accordance with the guidelines of the Animal Ethics Committee of Sydney University, as described previously (Dedov and Roufogalis, 1999a). Briefly, dorsal root ganglia (DRG) from neonatal (3–5 day old) Sprague–Dawley rats killed by decapitation were incubated in Hanks CMF saline with 0.05% collagenase and 0.25% trypsin for 25 min at 37°C. Individual cells were obtained by trituration with fire polished Pasteur pipettes of diminishing diameters. Neurons were isolated from the cell suspension in 30% Percoll and then plated on collagen coated coverslips, then cultured in neurobasal medium with B27 supplement, 50 ng/ml 2.5 S nerve growth factor (NGF), 2 mM 1-glutamine and 100 U/ml penicillin/streptomycin. Cultures were maintained at 37°C with 5% CO_2 for 1–3 days.

2.2. Instrumentation

The microscope used was a Leica TCS NT (Leica Microsystems Heidelberg GMBH, Germany) confocal system on an inverted microscope equipped with 63× water immersion objective (NA 1.2). A 75 mW argon–krypton Innova 310 laser was used for CLSM. For 2p-LSM a Mira-900 femtosecond pulsed Ti-sapphire laser pumped by a 5 W VERDI was used (Coherent Scientific, Santa Clara, USA). This system delivers 500 mW at 800 nm falling to around 200 mW towards the ends of the tuneable range. The two lasers were connected in parallel to the inverted Leica TCS NT system (the Ar/Kr laser by an optical fibre and the Ti-sapphire laser coupled directly), thereby enabling alternation of single- and two-photon excitation delivered to the cells. Magnification of the final image was controlled by varying the size of the area scanned (zoom factor) as stated in the figure legends. In most cases scanned areas were either 80 × 80 μm (zoom 2) or 40 × 40 μm (zoom 4).

2.3. Dye loading and image acquisition

Primary cultured dorsal root ganglion (DRG) neurons, adhering to coverslips, were incubated with 3 μM JC-1 in physiological solution for 15 min in 37°C. The coverslips were then mounted on a chamber filled with physiological solution consisting of 140 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 20 mM HEPES, 10 mM glucose, pH 7.4 (Dedov and Roufogalis, 1999a). Experiments were performed at room temperature in a darkened room. JC-1 was excited either in single-photon mode with the 488 nm laser line or in two-photon mode with the Ti-sapphire laser tuned to 870 nm and images were collected at red (TRITC, LP590 nm) and green (FITC, BF530/30) channels in parallel. Neutral density filters of 10 and 50% were applied for CLSM and 2p-LSM, respectively. Maximum voltage of photomultipliers was used to decrease the required laser power as much as possible. A pinhole of 1 Airy unit was used for CLSM; for 2p-LSM the pinhole was opened to its maximum value (about 5 Airy units). Non-descanned detection was limited to a single channel on this system and could not therefore be applied to ratiometric imaging.
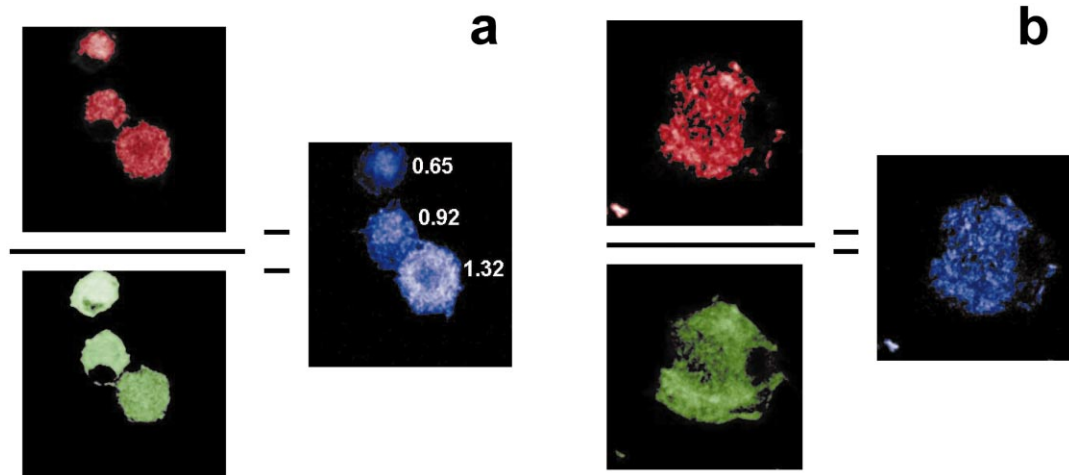
Fig. 1. Fluorescence images of DRG neurons loaded with JC-1 and excited by single-photon (CLSM) of 490 nm with (a) a zoom factor of 2 (scanned area 80 × 80 μm) and (b) a zoom factor of 4 (scanned area 40 × 40 μm). Emission fluorescence was filtered and taken by PMT simultaneously as red (TRITC, LP590 nm, left top panel) and green (FITC, BF530/30, left bottom panel) fluorescence. Background fluorescence was subtracted and ratio images (right panel) were obtained and visualised following multiplication by a factor of 100.

2.4. Image collection and analysis

Images were collected and processed using the imaging software provided by the Leica TCS NT system. Emission fluorescence was collected in red (>580 nm) and green (500–560 nm) channels simultaneously. Background fluorescence was subtracted and ratios between fluorescence intensity in the red and green channels were calculated for both whole neurons and individual mitochondria. Data are the mean + standard deviation (S.D.).

2.5. Chemicals

All reagents were of analytical grade. JC-1 was obtained from Molecular Probes Inc. (Eugene, OR), neurobasal medium with B27 supplement from GIBCO (Gaithersburg, MD), NGF from ICN Biochemicals (Costa Mesa, CA). Other agents were obtained from Sigma (St. Louis, MO).

3. Results

3.1. Visualisation of mitochondria with CLSM

Observed with phase contrast, DRG neurons were round, 10–30 μm in diameter, with significant neurite outgrowths that are indicative features for developing DRG neurons from neonatal rats (not shown).

First, we used CLSM to evaluate a model for the visualisation of mitochondria in living neurons. Fig. 1(a) represents neurons loaded with JC-1 for 15 min and excited in single-photon mode at 488 nm. Here and below (Figs. 1–4),
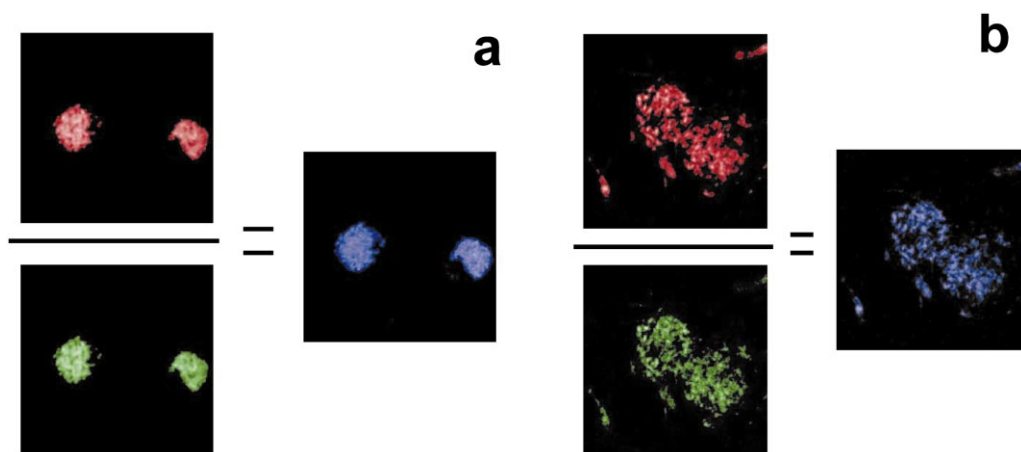

Fig. 2. Fluorescence images of DRG neurons loaded with JC-1 and excited by two photons (2p-LSM) of 870 nm with (a) a zoom factor of 2 (scanned area 80 × 80 μm) and (b) a zoom factor of 4 (scanned area 40 × 40 μm). Emission fluorescence was filtered and taken by PMT simultaneously as red (TRITC, LP590 nm, left top panel) and green (FITC, BF530/30, left bottom panel) fluorescence. Background fluorescence was subtracted and ratio images (right panel) were obtained and visualised following multiplication by a factor of 100.
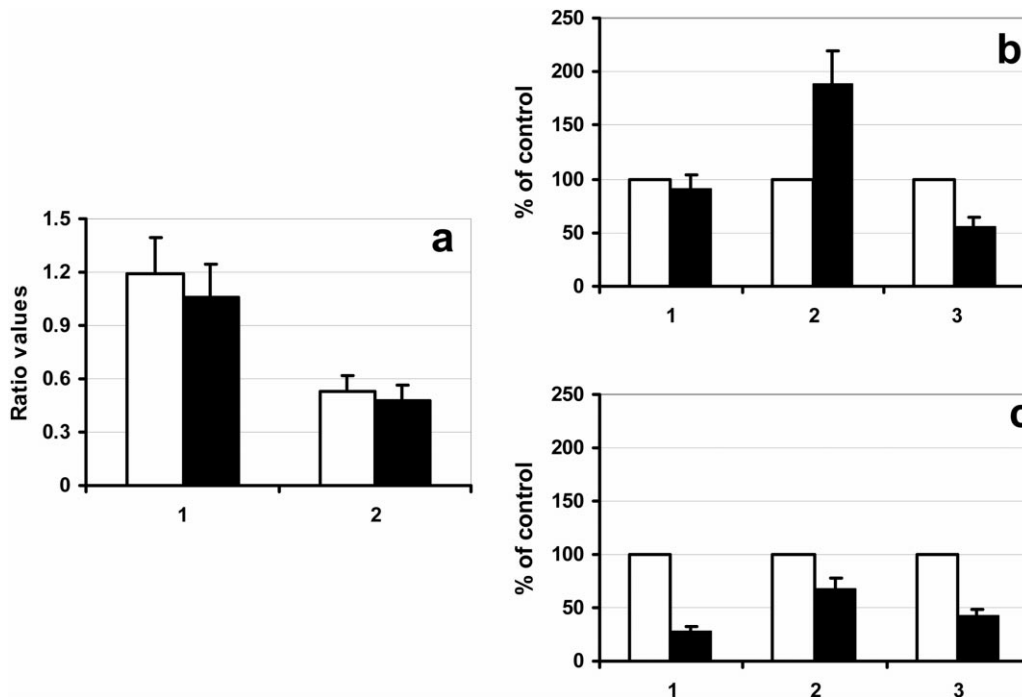
Fig. 3. Effect of mitochondrial uncoupler on JC-1 fluorescence: (a) ratio changes upon application of 2 mM FCCP to DRG neurons loaded with JC-1 detected by CLSM (white bars) and 2p-LSM (black bars) where (1) control ratio values and (2) ratio values after FCCP application; (b) relative changes of (1) red and (2) green fluorescence intensity, and (3) ratio upon FCCP application (black bars) detected by CLSM; and (c) relative changes of (1) red and (2) green fluorescence intensity, and (3) ratio upon FCCP application (black bars) detected by 2p-LSM.

top left images represent fluorescence at the TRITC (red) channel whereas bottom left images represent fluorescence at the FITC (green) channel. The right images reflect the ratio between intensities at the red and green channels. As long as the ratio values were within 1.5–0.4 (see below), they were visualised by multiplying the ratio image by a factor of 100, producing the images seen in the right panels.

Fig. 1 represents three neurons with different MMP, reflecting a range of ratios that may occur in cultured DRG neurons. JC-1 emits red fluorescence when MMP is above 140 mV because of J-aggregates, whereas green fluorescence of JC-1 monomers is less potential dependent and is present when MMP is lower than 140 mV. Therefore, the ratios between red and green fluorescence should reflect MMP (Cossarizza et al., 1996). In the neuron in the top left, green fluorescence over the whole cytoplasm prevailed, resulting in a low ratio of 0.65. By contrast, the neuron in the bottom fluoresced with higher intensity in red than in green, resulting in a high ratio of 1.32. Images taken with zoom factors of 2 (80 μm field width) or unity (160 μm field width) allowed us to measure and calculate ratios over a number of cells simultaneously; however, they did not allow us to distinguish clearly individual mitochondria (Fig. 1(a)). This zoom range was used to calculate ratios in three separate experiments in a total of 16 neurons. Neurons expressed ratios of 1.19 $\pm$ 0.23.

Next we attempted to visualise individual mitochondria. We focused on a single neuron and used a zoom factor of 4 (40 μm field width) (Fig. 1(b)). Bright spots of about 1 μm

diameter were seen at the red channel (top left panel), reflecting J-aggregates of JC-1 in mitochondria (Fig. 1(b)). However, mitochondria were less sharp at the green channel (left bottom panel). Nevertheless, we were able to calculate ratios over 29 random mitochondria, as represented for a typical case on the right panel. The average ratio taken over individual mitochondria was 1.08 $\pm$ 0.16, while that taken over this whole cell was 0.88. The difference is likely to reflect concomitant JC-1 fluorescence from the cytoplasm when the ratio is taken over the whole cell (see Section 4).

Only single plane images were taken, approximately in the mid-plane of the cell, so it cannot be assumed that individual mitochondrial sections actually represent single mitochondria. 3D reconstruction of mitochondria, which would be required to assess their true shape and extent, lay outside the scope and objectives of this work.

3.2. Visualisation of mitochondria with 2p-LSM

There are no data available on two-photon excitation of JC-1, and to the best of our knowledge this has not been attempted hitherto. We therefore reproduced the experiments described above for CLSM using 2p-LSM. Fig. 2(a) shows neurons loaded with JC-1 for 15 min and excited at 870 nm, where an image on the right represents ratios between red (left top panel) and green (left bottom panel) channels (see above for details). Average ratios over whole neurons were 1.06 $\pm$ 0.15 ($n = 11$), which is close to that obtained by CLSM (see above).
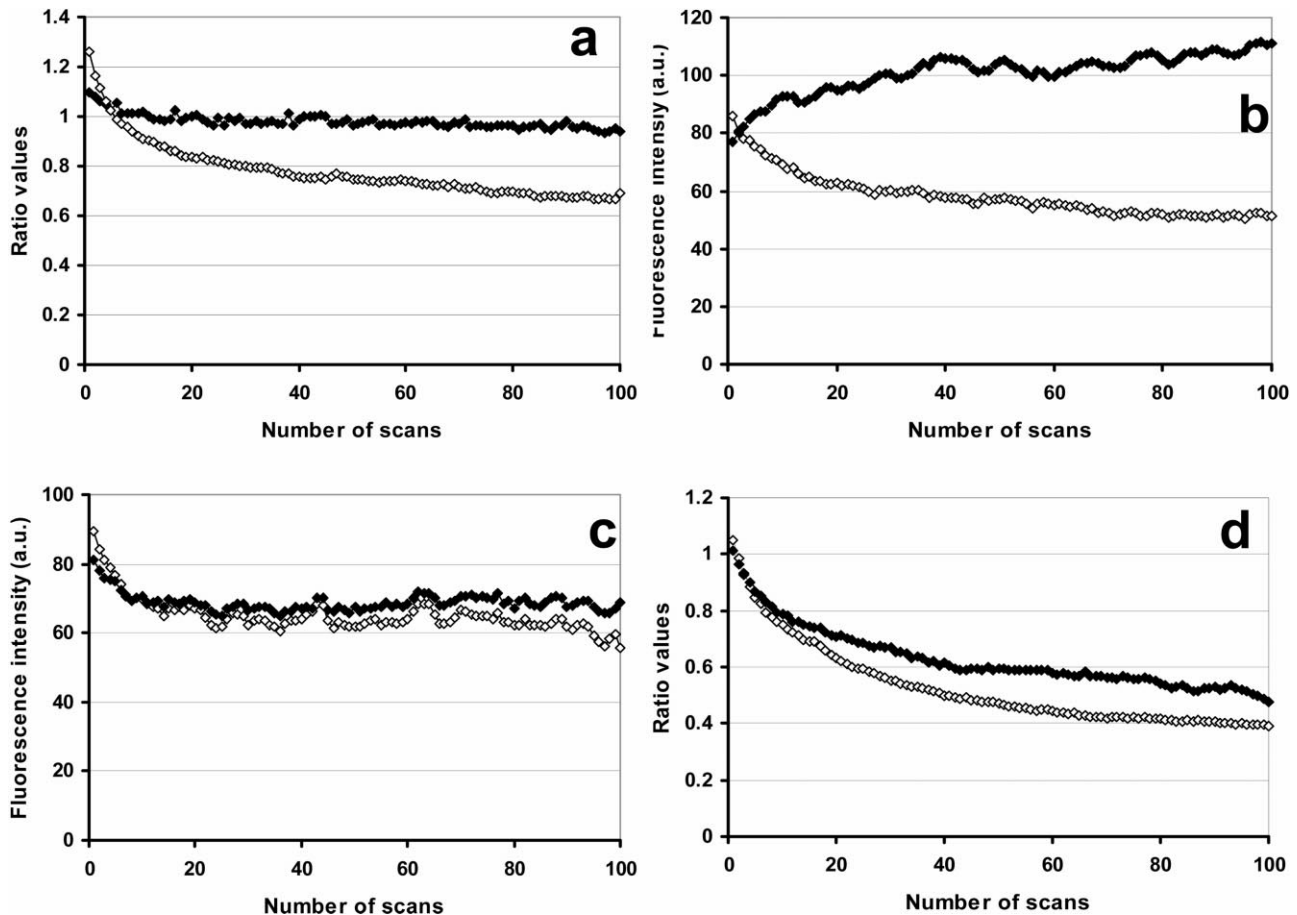
Fig. 4. Effect of repetitive scanning in the focal plane on JC-1 fluorescence: (a) ratio changes during repetitive scanning by CLSM (white) and 2p-LSM (black); (b) relative changes of red (white squares) and green (black squares) fluorescence intensity during repetitive scanning by CLSM; (c) relative changes of red (white squares) and green (black squares) fluorescence intensity during repetitive scanning by 2p-LSM; and (d) ratio changes during repetitive scanning by CLSM (white squares) and 2p-LSM (black squares) when 2× zoom was applied.

Increasing the zoom to 4 enabled us to visualise individual mitochondria in two adjacent neurons (Fig. 2(b)), as we did for a single neuron by CLSM (Fig. 1(b)). However, in contrast to CLSM, 2p-LSM showed a better signal/background ratio in the green channel (Fig. 2(b), right bottom panel), resulting in better correspondence between channels. The average ratios of 19 mitochondria were 1.11 ± 0.13 and 1.13 ± 0.17, matching the ratios obtained over the corresponding whole cells (1.11 and 1.09, respectively). Thus, 2p-LSM produces a closer correlation between mitochondrial ratios and those of whole cells, indicating less influence from non-mitochondrial green fluorescence background.

3.3. Effect of mitochondrial uncoupler on JC-1 fluorescence

The mitochondrial uncoupler FCCP (2 μM carbonyl cyanide p-(trifluoromethoxy) phenylhydrasone) transports protons across the inner mitochondrial membrane and dissipates MMP. It has been shown that uncoupler applied to central neurons decreases the JC-1 ratio from above 1 (fully charged mitochondria) to about 0.5 (fully uncoupled mitochondria) (White and Reynolds, 1996), thereby

confirming the mitochondrial localisation of JC-1 fluorescence and that the ratio obtained reflects MPP in neurons.

In our experiments the effect of 2 mM FCCP on ratio values was almost the same when using either CLSM or 2p-LSM (Fig. 3(a)). In both cases, the uncoupler halved the ratio values from above 1 to about 0.5, agreeing with the literature (see above). However, the relative input of changes in green and red fluorescence upon uncoupler application for CLSM or 2p-LSM was different. In experiments where CLSM was used, red (590 nm) fluorescence was diminished by uncoupler by just 10%, whereas the green (530 nm) fluorescence was augmented by about 90% (Fig. 3(b)). When 2p-LSM was used in the same experimental conditions, red fluorescence dropped by 75%, while green fluorescence also dropped by about 25% (Fig. 3(c)).

3.4. Multiple scans in focal plane by CLSM and 2p-LSM

To evaluate the longer term effects of single- and two-photon laser illumination on MMP, we studied JC-1 fluorescence when neurons were scanned in the same focal plane up to 100 times at different zoom settings.

Even at zoom 1 (160×160 μm), repeated scans in CLSM caused a significant drop in the ratio. During the first 10 scans, the ratio dropped by 0.37 (from 1.29 to 0.92), that is approximately 60% of the uncoupler effect (Fig. 4(a)). This ratio drop reflected both an increase in green fluorescence and a decrease in red fluorescence (Fig. 4(b)). Using a zoom setting of 2 (80×80 μm) concentrated the exciting light in 1/4 of the volume and thereby intensified the damaging effect of CLSM laser illumination (Fig. 4(d)).

When 2p-SLM was used in the same experimental set-up at zoom 1, the ratio dropped by 10% after the first six scans, but then stabilised at a value of 1 (Fig. 4(a)). Both red and green fluorescence were diminished in parallel during the early scans, but then stabilised (Fig. 4(c)). When the effective laser power was increased by the selection of $2 \times$ zoom the ratio values also dropped although, again, more slowly than was observed with CLSM (Fig. 4(d)).

3.5. Four-dimensional (4D) study of mitochondria by CLSM and 2p-LSM

Finally, we explored the possibility of using CLSM and 2p-LSM for repetitive 3D (4D) reconstructions of mitochondria loaded with JC-1. We took 32 optical slices at 0.5 mm increments in the z direction and compared the quality of images in the same focal plane when neurons were scanned up to four times. CLSM was not suitable for 4D experiments because, even after the first scan, red fluorescence was greatly diminished, reflecting significant deterioration of mitochondria (not shown). However, with 2p-LSM we scanned in the z direction at least three times without significant changes in red (Fig. 5) or green fluorescence (not shown). Less than a 10% decrease of ratios taken over the whole cell was observed between the first and third scans (Fig. 5). Previously we had used CLSM for 3D reconstruction of mitochondria in DRG neurons (Dedov and Roufogalis, 1999b; Dedov et al., 2000); however, in the present work we did not attempt to undertake actual 3D reconstructions.

4. Discussion

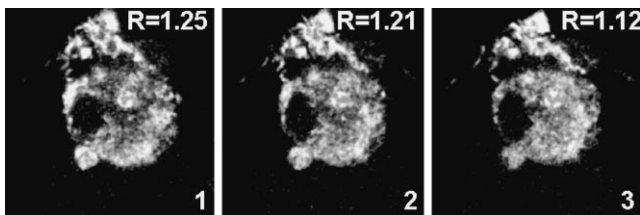Visualisation of mitochondria in living cells is limited by the resolution of light microscopy and by some other general limitations of confocal microscopy (for review see (Pawley, 1995). Nevertheless, the best lateral (xy) resolution in CLSM is about 0.2 μm, enabling the visualisation of individual mitochondria whose size is about 1 μm. While confocal imaging offers higher resolution than wide-field fluorescence, the poorer sampling statistics require that in order to obtain an adequate signal *in a given time* period, a higher excitation power must be used, and this inevitably results in photodamage of the mitochondria. Decreasing the area scanned (increasing the zoom factor) supplies additional magnification, increasing the resolution (provided that sampling does not exceed the Nyqvist value), but the concentration of light in a small area results in more photodamage (Pawley, 1995). Mitochondria are particularly vulnerable to light illumination, especially to blue light, absorbed by the strong Soret bands of cytochromes, although even green light induces pathological changes during long-term observation. Illumination through a long pass filter ($T > 580$ nm) avoids these problems (Bereiter-Hahn and Voth, 1994). A concentrated laser beam focused on mitochondria in living fibroblasts evoked a rapid drop in MMP (Amchenkova et al., 1988).

The alternative to CLSM is 2p-LSM, which uses a long excitation wavelength and thus produces less photodamage to the cells. The elimination of the pinhole in 2p-LSM also improves collection statistics, particularly in thick or turbid specimens. However, the lateral resolution of 2p-LSM is lower than that of the CLSM by a factor of two (Cox and Sheppard, 1999) and will not be better than 0.3 μm (Konig et al., 1996).

A large amount of data exists on the application of confocal microscopy to the study of living cells, including mitochondria in whole living cells (for review see (Loew, 1993; Loew et al., 1993), but much less information is available on applications of 2p-LSM. In most papers 2p-LSM was used for fluorophores normally excitable at UV wavelengths, such as Indo-1 and DAPI (see Section 1). The possibility of exciting visible transitions of intracellular fluorophores, such as Calcium Green and Rhodamine 123, was investigated using simultaneous absorption of two 780 nm photons (Konig et al., 1996).

In this work we used previous knowledge of the properties of JC-1 as a potentiometric and ratiometric fluorescent dye to visualise individual mitochondria in living cells by CLSM and calculate their relative MMP. Our data agree with previous data (White and Reynolds, 1996), revealing similar ratio values in neuronal cell populations. The effect of mitochondrial uncoupler serves also to prove mitochondrial localisation and dependence of the ratio on MMP. Using a zoom factor of 4, we were able to visualise individual mitochondria and calculate their ratios. However, we found a lower signal/background ratio in the green than in the red channel. This is likely to reflect concomitant fluorescence of JC-1 from the cytoplasm, resulting in a lower ratio over the whole cells than the aggregate ratios taken over individual mitochondria.

Fig. 5. Images of red JC-1 fluorescence after (1) first, (2) second, and (3) third scanning in the z direction by 2p-LSM, where R denotes the respective ratio values. The same cell was scanned in 32 focal planes with 0.5 mm increments consecutively for three times.

To investigate the accuracy of 2p-LSM in reflecting MMP we used the same cell cultures, loading procedures and set-up of the microscope as we did for CLSM. Moreover, most experiments were performed on the same cell preparations by switching between single- and two-photon lasers attached to the microscope. 2p-LSM images were quite similar to those obtained by CLSM, including the appearance of individual neurons and mitochondria (especially in the red channel), the intensity of fluorescence signal in both channels, the ratios over whole cells and over individual mitochondria and the sensitivity to mitochondrial uncoupler.

The sensitivity of a mitochondrion-selective fluorescent dye to mitochondrial uncoupler is strong evidence that the probe used is suitable for the assessment of MMP (Skulachev, 1988). Previously it was shown that dissipation of MMP with uncoupler results in the disappearance of red fluorescence (J-aggregates dissociation) and the increase of green (monomer) fluorescence (Reers et al., 1995).

Using CLSM and DRG neurons, we reproduced the experiments carried out by White and Reynolds (1996) on central neurons and, like them, we found that the uncoupler diminished the ratio by about 50%. 2p-LSM revealed the same drop in ratio value upon uncoupler application. However, the components of the ratio decrease were distinct for CLSM and 2p-LSM. An increase of green fluorescence was a major determinant of the ratio drop measured by CLSM, whereas a decrease of red fluorescence was a major component of the ratio drop in 2p-LSM (Fig. 3). JC-1 monomers were better excited by single-photon than by the two photons (see above), resulting in better detection of green fluorescence increase upon uncoupler application by CLSM, whereas 2p-LSM truly reflected J-aggregate dissociation.

There are two major approaches in the use of fluorescence confocal microscopy in living cells: kinetic study of intracellular processes in time-lapse experiments and 3D reconstructions of intracellular structures. The feasibility of the former depends on the number of laser scans to be taken in the focal plane without significant cell damage. When the neurons loaded with JC-1 were scanned up to 100 times we found that CLSM resulted in significant damage of the mitochondria. The decrease in JC-1 ratio value had an exponential character, with a maximum comparable with the uncoupler effect (Fig. 4). Analysis of changes in red and green fluorescence confirmed that CLSM scanning in the focal plane dissipates MMP and therefore should be used for kinetic studies with extreme caution. In contrast to CLSM, 2p-LSM was less detrimental. After an initial ratio drop (less than 10%), the ratio stabilised at a level of 1 for at least up to 90 scans (Fig. 4). Therefore, 2p-LSM is preferred to CLSM for kinetic studies in living cells. However, one should be aware that concentrating the laser light, by reducing the area scanned, results in significant mitochondrial uncoupling both with CLSM and 2p-LSM (Fig. 4(d)). An increase in the zoom factor beyond the Nyqvist value (2.4

pixels per minimum resolved unit) will increase the damage without any increase in the information content of the image (Pawley, 1995).

As we have shown previously, CLSM can be used for 3D reconstruction of mitochondria loaded with JC-1 (Dedov and Roufogalis, 1999b; Dedov et al., 2000). However, the 4D study of mitochondria by CLSM is impossible because of the detrimental effect of repeated scans in depth, but 2p-LSM is suitable for this task. In contrast to CLSM, 2p-LSM minimises excitation in the out of focal plane, crucially reducing the total illumination time for the whole cell (see Section 1), and at least three scans in the z direction did not change significantly the image quality in the same focal plane (Fig. 5).

5. Conclusions

We have been able to monitor individual mitochondria and measure relative membrane potential over single mitochondria and over whole living neurons with the ratiometric fluorescent dye JC-1. Comparison of two currently commercially available fluorescence techniques, a conventional CLSM and a relatively new 2p-LSM, revealed similar *xy* resolution of mitochondrial fluorescence. However, 2p-LSM excites JC-1 monomers to a lesser extent than CLSM, resulting in lesser background fluorescence. Dissipation of MMP by mitochondrial uncoupler was reflected similarly by both techniques, but CLSM mostly detected an increase of monomer concentration whereas 2p-LSM more truly reflected J-aggregate dissociation. 2p-LSM has significant advantages for time-lapse kinetic studies of mitochondria, including 4D studies, because of less uncoupling effects of this technique on MMP, whereas CLSM should be used cautiously for this kind of experiment.

Acknowledgements

References

Amchenkova, A.A., Bakeeva, L.E., Chentsov, Y.S., Skulachev, V.P., Zorov, D.B., 1988. Coupling membranes as energy-transmitting cables. I. Filamentous mitochondria in fibroblasts and mitochondrial clusters in cardiomyocytes. Journal of Cell Biology 107, 481–495.
Bereiter-Hahn, J., Voth, M., 1994. Dynamics of mitochondria in living cells: shape changes, dislocations, fusion, and fission of mitochondria. Microscopy Research and Technique 27, 198–219.

Cossarizza, A., Ceccarelli, D., Masini, A., 1996. Functional heterogeneity of an isolated mitochondrial population revealed by cytofluorometric analysis at the single organelle level. Experimental Cell Research 222, 84–94.

Cox, G.C., Sheppard, C.J.R., 1999. Multiphoton fluorescence microscopy. In: Mason, W.T. (Ed.). Fluorescent and Luminescent Probes for Biological Activity. Academic Press, New York, pp. 331–336.

Dedov, V.N., Roufogalis, B.D., 1999a. Mitochondrial Ca2 + accumulation in DRG neurones following activation of capcaicin receptors in DRG neurones. Neuroscience 95, 183–188.

Dedov, V.N., Roufogalis, B.D., 1999b. Three-dimensional organisation of mitochondrial network in living neurons. FEBS Letters 456, 171–174.

Dedov, V.N., Armati, P.J., Roufogalis, B.D., 2000. Three-dimensional organisation of mitochondrial clusters in regenerating dorsal root ganglion (DRG) neurons from neonatal rats: evidence for mobile mitochondrial pools. Journal of the Periferal Nervous System 5, 3–10.

Denk, W., Strickler, J.H., Webb, W.W., 1990. Two-photon laser scanning fluorescence microscopy. Science 248, 73–76.

Di Lisa, F., Blank, P.S., Colonna, R., Gambassi, G., Silverman, H.S., Stern, M.D., Hansford, R.G., 1995. Mitochondrial membrane potential in single living adult rat cardiac myocytes exposed to anoxia or metabolic inhibition. Journal of Physiology 486, 1–13.

Konig, K., Simon, U., Halbhuber, K.J., 1996. 3D resolved two-photon fluorescence microscopy of living cells using a modified confocal laser scanning microscope. Cellular and Molecular Biology 42, 1181–1194.

Loew, L.M., 1993. Confocal microscopy of potentiometric fluorescent dyes. Methods in Cell Biology 38, 195–209.

Loew, L.M., Tuft, R.A., Carrington, W., Fay, F.S., 1993. Imaging in five dimensions: time-dependent membrane potentials in individual mitochondria. Biophysical Journal 65, 2396–2407.

Pawley, J., 1995. Handbook of Biological Confocal Microscopy. Plenum Press, New York/ London.

Piston, D.W., Masters, B.R., Webb, W.W., 1995. Three-dimensionally resolved NAD(P)H cellular metabolic redox imaging of the in situ cornea with two-photon excitation laser scanning microscopy. Journal of Microscopy 178, 20–27.

Reers, M., Smiley, S.T., Mottola-Hartshorn, C., Chen, A., Lin, M., Chen, L.B., 1995. Mitochondrial membrane potential monitored by JC-1 dye. Methods in Enzymology 260, 406–417.

Sako, Y., Sekihata, A., Yanagisawa, Y., Yamamoto, M., Shimada, Y., Ozaki, K., Kusumi, A., 1997. Comparison of two-photon excitation laser scanning microscopy with UV-confocal laser scanning microscopy in three-dimensional calcium imaging using the fluorescence indicator Indo-1. Journal of Microscopy 185, 9–20.

Salvioli, S., Ardizzoni, A., Franceschi, C., Cossarizza, A., 1997. JC-1, but not DiOC6(3) or rhodamine 123; is a reliable fluorescent probe to assess delta psi changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS Letters 411, 77–82.

Salvioli, S., Maseroli, R., Pazienza, T.L., Bobyleva, V., Cossarizza, A., 1998. Use of flow cytometry as a tool to study mitochondrial membrane potential in isolated, living hepatocytes. Biochemistry 63, 235–238.

Skulachev, V.P., 1988. Membrane Bioenergetics. Springer, Berlin/New York.

White, R.J., Reynolds, I.J., 1996. Mitochondrial depolarization in glutamate-stimulated neurons: an early signal specific to excitotoxin exposure. Journal of Neuroscience 16, 5688–5697.

Williams, R.M., Piston, D.W., Webb, W.W., 1994. Two-photon molecular excitation provides intrinsic 3-dimensional resolution for laser-based microscopy and microphotochemistry. FASEB Journal 8, 804–813.