Comparative analysis of mitochondria selective dyes in different cell types detected by Confocal Laser Scanning Microscopy: methods and applications

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Mitochondria are organelles playing a pivotal role in controlling the cellular bioenergetic homeostasis. They are pleomorphic organelles with structural variations depending on cell type, cell-cycle stage and intracellular metabolic state. In this work, we have examined the potency and limitation of the confocal laser scanning microscopy (CLSM) technique in combination with specific fluorescent probes to assess the morpho-functional features of mitochondria in different living cell types.

Keywords confocal laser scanning microscopy, probes, mitochondria

1. Introduction

Mitochondria are subcellular organelles found in eukariotes, where they make up as much as 10% of the cell volume. In their primary role as cellular energy generators, by the oxidative phosphorylation system (OXPHOS), mitochondria are responsible for between 50 and 90 % of cellular ATP generation. Several other metabolic functions are performed by mitochondria mainly correlated to the terminal steps of catabolism as well as production of heme and steroid biogenesis. Mitochondria also play a pivotal role in apoptosis, a process by which unneeded cells are removed during development, and defective cells are selectively destroyed; a number of apoptotic factors are indeed "store housed" in mitochondria and poised for eventual release and action in the cytoplasm; the homeostasis of intracellular Ca^{2+} is also controlled by these organelles [1,2]. Mitochondria harbour their own genome (mtDNA) consisting in 2 to 10 copy number/mitochondrial unit of a 16.5 Kb circular DNA coding for 13 genes of the OXPHOS as well as 2 rRNAs and the set of tRNAs for translation of mitochondrial mRNAs. Being the intracellular compartment where about 95% of the O_2 is consumed, mitochondria, as side consequence of their oxidative activity, are the major potential source for reactive oxygen species (ROS) production. The absence of histones and efficient DNA repair mechanisms makes the mtDNA, given the close proximity to a ROS source, prone to a 10-20 higher mutational frequency than the nDNA. The cellular oxidative stress to which mitochondria contribute, strongly support their involvement in ageing and degenerative diseases [3-5].

Each mitochondrion is surrounded by two highly specialized membranes that play a crucial part in its activities. The outer membrane contains many copies of a transport protein called porin, which forms large aqueous channels through the lipid bilayer. The major working part of the mitochondrion is the matrix space and the inner membrane that contains it. The inner membrane exhibits a peculiar structure with deep invaginations (cristae) whose structural features and connections to the outer membranes have been resolved very recently by EM tomography [6]. Mitochondrial cristae host the tools of the OXPHOS (respiratory complexes of the electrons transport chain and the FoF1 ATPase) as well as the adenine nucleotide transporter (ANT), which regulates ADP/ATP exchange across the membrane, as well as a number of other ions and metabolites carriers [7]. Accordingly to the chemiosmotic theory the mitochondrial respiratory chain converts the redox free energy made available by "down-hill" electron transfer from reduced substrates to O_2, in active H^+ translocation from the matrix toward the intermembrane

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space. This generates a transmembrane electrochemical potential $\Delta\mu H^+$ contributed mainly by an electrical component ($\Delta\Psi$, negative inside) and by a ΔpH (alkaline inside) [7]. The protonmotive force so generated (estimated to rise up to 200-250 mV) cannot collapse because of the low H^+-conductance of the inner mitochondrial membrane and is instead utilised to drive up-hill reactions, first of all the ATP synthesis by the H^+-ATP synthase [7]. The inner membrane is highly specialized: it contains a high proportion of the anionic "double" phospholipid cardiolipin whose specific function is still to be completely elucidated [8]. It may be essential in maintaining the activity of a number of membrane-embedded proteins as well as in conferring to the membrane the low permeability to ions. The matrix enzymes include those that metabolise pyruvate and fatty acids to produce AcetylCoA and those that oxidize acetylCoA in the citric acid cycle. The end products of these oxidations are CO_2, which is released from the cell as waste, and NADH, which is the main source of electrons for transport along the respiratory chain.

Mitochondria are pleomorphic organelles with structural variations depending on cell type, cell cycle stage and intracellular metabolic state [9,10]. They are usually depicted, based on electron microscopy analysis, as stiff, elongated cylinders with a diameter of 0,5 to 1 μm, resembling bacteria. Time-lapse micro-cinematography of living cells shows that mitochondria are remarkably mobile and plastic organelles, constantly changing their shape and even fusing with one another and then separating again [11]. As they move about in the cytoplasm, they often appear to be associated with microtubules, which may determine the unique orientation ant distribution of mitochondria in different types of cells. Thus the mitochondria in some cells form long moving filaments or chains, while in others they remain fixed in one position where they provide ATP directly to a site of unusually high ATP consumption. Examples of this are mitochondria packed between adjacent myofibrils in cardiac muscle cells or wrapped tightly around the flagellum in a spermatocyte. In multicellular organisms mitochondria are likely to have a more constant morphology in a given tissue, but different tissues have distinct energy needs. Furthermore recent observations in certain cell types suggest a tight contiguity of mitochondria (perhaps functional) with other membranous subcellular compartments like endoplasmic reticulum [12,13].

The number of mitochondria per cell is a parameter that appears to vary significantly from cell type to cell type, and this will become an important consideration when mitochondrial biogenesis is considered in the context of cell differentiation and specialization [10,14]. Estimates from serial sections of cells yield values in the range of a few hundred to a few thousand per cell. Specialized vertebrate cells show significant variations. The abundance of mitochondria varies with cellular energy level and is a function of cell type, cell-cycle stage, and proliferative state. For example, brown adipose tissue cells, hepatocytes and certain renal epithelial cells tend to be rich in active mitochondria, whereas quiescent immune system progenitor or precursor cells show little staining with mitochondrion-selective dyes. In addition there is emerging evidence of functionally significant heterogeneity of mitochondrial forms within individual cells [10,14].

Although mitochondria are large enough to be seen in the light microscope and were first identified in the nineteenth century, real progress in understanding their function has depended on procedures developed for isolating intact mitochondria. For technical reasons, many biochemical studies have been carried out with mitochondria purified from liver; each liver cell contains 1000 to 2000 mitochondria, which in total occupy roughly a fifth of the cell volume. Confocal laser scanning microscopy (CLSM) has recently emerged as a technique that offers several advantages over conventional fluorescence microscopy [15]. The out-of-focus blur is virtually absent from confocal images, giving the capability for serial optical sectioning of intact specimens and subsequent three-dimensional reconstruction and allowing a precise analysis of stereo-spatial relationships of different cellular organelles in the cell. Three-dimensional reconstructions from serial sections by confocal microscopy (even in live cells) have removed some of the last ambiguities of interpretation of thin sections used in electron microscopy. A general correlation of the total area of inner membrane (density of cristae) with the capacity for oxidative phosphorylation is apparent but some of the more detailed aspects of cristae morphology in relation to function and activity are still relatively obscure [6].

Despite the long history of research, there is yet to be a consensus on the nature of mitochondrial structure. Several lines of evidence suggest that mitochondria are physically interconnected and functionally homogeneous. Since mitochondrial structure is a dynamic balance between fission and fusion its intra-

cellular morphology might be the result of what in a given moment is prevailing (i.e. fusion resulting in an extended network or fission to form discrete organelles) [14, 16, 17]. Mitochondrial functions, including ATP synthesis, Ca^{2+} homeostasis and apoptosis signalling, could be profoundly affected by whether the mitochondria exist as a continuous network or discrete individuals [14]. Even less is known about the control of this morphology by nuclear genes in the course of development and differentiation. Moreover mitochondria shape and distribution within a cell may be controlled by extramitochondrial cytoskeletal elements, but the establishment of the particular cell-specific interior morphology remains mysterious and therefore a challenge for the future.

2. Mitochondria staining

When the nineteenth century morphologists, with their light microscopes, discovered grains (chondria) and filaments (mito), they could not be sure that they were all looking at the same functionally distinct structure in different cells, and undoubtedly they were not. Nevertheless, variability in number and shape must have been apparent. A more systematic approach became possible when selective staining methods were developed. The capacity of mitochondria to reduce janus green, an indicator of oxidation-reduction reaction, should have been a major clue, which was evidently missed at the time or, rather, not interpreted fully. Major progress in the characterization and understanding of the morphology and ultrastructure of mitochondria was not possible until the techniques for electron microscopy and specimen preparation were perfected, but the use of the light microscope in the study of mitochondria was revived in the past few decades by two technical innovations. First, mitochondria could be viewed with the fluorescence microscope in permeabilised cells after staining with specific antibodies and secondary antibodies conjugated to fluorescent dyes. Another very important discovery was the observation that certain rhodamine derivatives [18] and other lipophilic "vital dyes" would stain mitochondria because the dye became concentrated in these organelles by an uptake mechanism driven by the membrane potential [19, 20]. The basic mechanism for all the "functional" mitochondrial probes relies on their chemical structure consisting in highly conjugated moieties that extensively delocalises a positive charge thus allowing electrophoretic uptake toward the negatively charged matrix phase of the polarised inner mitochondrial membrane (accumulation of the dye in the

Probe	Ex(nm)	Em(nm)
MitoTracker green	490	516
MitoTracker red 580	581	644
MitocaptureTR	510-543	527-590
NAO	495	519
TOPRO-1	515	531
TOPRO-3	642	661

Table 1. Spectroscopic features of the fluorescent probes used in this work.

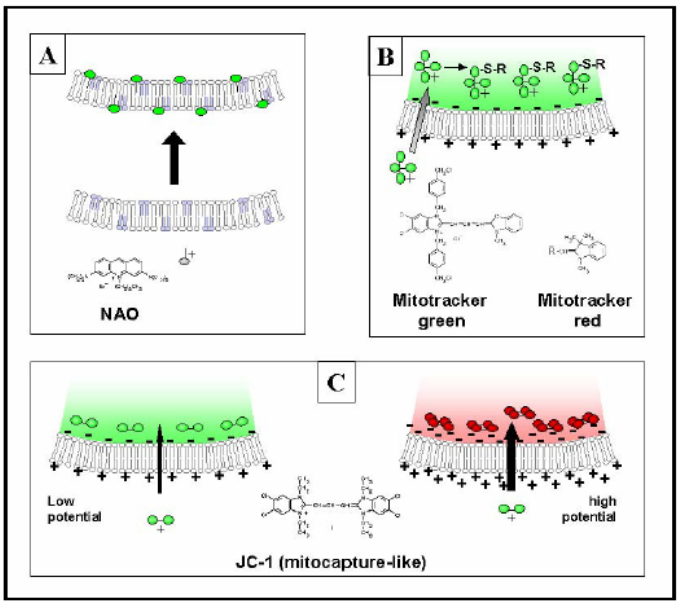

Fig.1. Schematic representation of the mechanisms of action of mitochondrial probes.

cytoplasm from the extra-cellular space because of the plasma membrane potential is usually negligible). A variety of cell-permeant mitochondrion-selective dyes are commercially available, to enable researchers to probe mitocondrial localization and abundance, as well as to monitor mitochondria morphology and organelle functioning [19, 20]. Although conventional fluorescent stains for mitochondria, such as rhodamine 123 and tetramethylrosamine, are readily sequestered by functioning mitochondria, they are subsequently washed out of the cells once the mitochondrion membrane potential is lost. This characteristic limits their use in experiments in which cells must be treated with aldeyde-based fixative or other agents that affect the energetic state of mitochondria. To overcome this limitation recently selective stains, *MitoTracker* probes (Molecular probes), have been developed [21]: this family of dyes are able to concentrate by active mitochondria and to be retained during cell fixation and also following permeabilization: the sample retains the fluorescent staining pattern characteristic of live cells. *MitoTracker* probes are cell-permeant mitochondrion-selective dyes that contain a mildly thiol-reactive chloromethyl moiety (fig.1 B). The chloromethyl group appears to be responsible for keeping the dye associated with the mitochondrial tyols after fixation. Once it is introduced to the cell, the cell-permeable *MitoTracker* freely diffuses across plasma membrane into the cytosol and accumulates electrophoretically into the matrix of metabolically active mitochondria driven by the proton motive force generated by the electron transfer chain localised at the inner mitochondrial membrane; within the matrix, some variants of mitotrackers become oxidized converting to a photostable fluorescent product which stains the cells upon excitation by blue light. This property makes it suitable for live cell microscopy, but it is also fixable using aldehydes, facilitating double-label immunofluorescence. The spectral characteristics of two mitotrackers are shown in Table 1.

The green-fluorescent MitocaptureTR (Biovision) is another example of "live cells dye". In detecting variations in $\Delta\Psi$, MitocaptureTR is more advantageous over *MitoTracker* dye: it's capable of entering selectively into mitochondria and it changes reversibly its colour from green to orange as membrane potential increases (dual-emission potential probe). This property is due to the reversible formation of aggregates upon membrane polarization that causes shifts in emitted light from 530nm (i.e emission of monomeric form) to 590 nm (i.e. emission of the aggregate form) when excited at 490 nm (Table 1). As a consequence, mitochondria having a low membrane potential will accumulate low concentrations of Mitocapture and will fluoresce in green; in more highly polarised mitochondria ($\Delta\Psi$ exceeding 140 mV), fluorescence will be orange-red. The main advantage Mitocapture staining is that it can be both qualitative, considering the shift from green to orange fluorescence emission, and quantitative, considering the pure fluorescence intensity detected. Various types of ratio measurements are possible by combining signals from the Mitocapture green-fluorescent monomer and the orange(red)-fluorescent aggregate. The ratio of red-to-green Mitocapture fluorescence is dependent only on the membrane potential and not on other factors that may influence single-component fluorescence signals such as mitochondrial size, shape and density. Although the molecular structure of mitocapture is unavailable it is alike to be close to that of JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine) another very efficient mitochondrial tracker with which it shares the same features [19] (see fig.1 C).

In addition to the "functional" staining dyes, other "structural" probes are available and used to evaluate the mitochondrial mass; accurate measurement of mitochondrial mass require a probe that will accumulate in mitochondria regardless of the mitochondrial membrane potential. The hydrophobic fluorescence dye *10-n-nonyl-acridinium-orange-chloride* (NAO) specifically stains mitochondria of living cells and it is not sensitive to the uncoupler CCCP. Mitochondrial staining by NAO is attributed to binding of cardiolipin in the inner mitochondrial membrane, suggesting that the observed fluorescence signal may not correlate with the "mass" of the entire mitochondria but instead may measure the amount of inner membrane present [22] (see Fig. 1 A).

3. Nucleus staining

DNA in cells is usually stained with DAPI (4'6-diamidino-2-phenylindol) for fluorescence microscopy [23]. When stained with DAPI, the DNA appears as blue-white fluorescence under ultraviolet (UV) illumination. For example, if a simultaneous analysis of membrane antigens with FITC and/or TRITC is performed, nuclei should be stained with a UV-excited, blue-emitting fluorochrome such as DAPI (excitation 358 nm, emission 461 nm). Most laser confocal microscopes, however, do not have a UV laser illumination system and thus the use of DAPI is restricted to specialized systems. Recently, a variety of nuclei acid binding dyes have been developed suitable for analysis with commoner laser. The TO-PRO family comprises single cyanine and a cationic side chain dyes [23]. They exhibit reduced affinity for nuclei acids than DAPI and, like this, they are impermeant to cells. The spectral characteristics of TOPRO-1 and -3 one are shown in Table 1.

4. Experimental procedures

The cell lines used were the following: HepG2 (derived from human hepatic carcinoma), NIH3T3 (mouse fibroblasts), BSPRC20 (huke human keratinocytes), RD (human myoblasts derived from rhabdomyosarcoma), EA (human endothelial like cell line derived from the fusion of HUVEC, human umbilical vein endothelial cells, with the lung carcinoma cell line A549, pulmonary epithelial cells. Cells were seeded at low density onto fibronectin coated 35 mm glass bottom dishes (WillCo Wells). After adhesion, living cells were incubated for 20 min at 37 °C in culture medium containing specific probe (MitoTracker Green - MitoTracker Red 580 - NAO , 500 nM each, from Molecular Probes). Mitocapture probe was used according to the manufactory protocol. Nuclei were stained using TOPRO-1 or TOPRO-3 as follows: after mitochondria staining, cells were washed twice with PBS and softly permeabilized by 0.02 % Triton X-100 treatment for 5 minutes, to allow the dye to enter the nucleus; then cells were washed and incubated with TOPRO-1 or TOPRO-3 (1μM) for 10 minutes at room temperature. At the end, cells were washed twice with PBS and examined using a Nikon TE 20000 microscope coupled to a Radiance 2100 dual laser scanning confocal microscopy system (Biorad). Images were acquired, stored and analysed using LaserSharp and LaserPix software from Biorad.

5. Results and discussion

Fig. 2 shows the image reconstruction, obtained by CLSM of three different cultured cell lines treated with the fluorophore NAO (Fig.1). NAO is a cationic probe that binds to anionic cardiolipin [22]. The interaction seems to depend on the stacking of the probe between ordered arrays of cardiolipins with a defined stoicheiometric ratio [24]. The stacking of the aromatic rings of the NAO molecules because of the π–π interaction results in alteration of the emission features when exited with blue light resulting in green fluorescence. Given that cardiolipin is dis-homogeneously distributed within the cell membrane, with a much higher if not exclusive content in the inner mitochondrial membranes, NAO is widely used as a mitochondrial cardiolipin specific probe. Although the affinity of NAO to cardiolipin is about 100 times greater than that measured with other anionic phospholipids, the possibility that NAO binds to other membranous cellular compartment cannot be excluded. Given this limitation, the concentration of NAO must be kept as low as possible in order to avoid "unspecific" staining. Accordingly to the mechanism of action proposed, the binding of NAO is not affected by the presence of a membrane potential across the membrane thus behaving as a reporter of the mitochondrial mass independently of its functioning. From the examination of the pictures in fig.2, given the features and limitations of NAO, the following can be inferred:

- the NAO staining is spreadly diffused throughout the cell interior with localized areas showing more intense signals than others; this is particularly evident observing the picture of confocal median planes (Fig.2 D) rather then those obtained by the superimposition of all the confocal planes recorded (Fig. 2 A-C).

- Although the signal shows smearing, likely due to the not absolute specificity of the dye, a more compact structure of the mitochondrial network in the perinuclear zone can be clearly noticed with a more grain-like structure in the sub-plasma membrane zone.

Fig. 3 shows the staining of cells with two functional probes, Mitotracker green and Mitotracker red (Fig.1). In both cases the overall stained texture is comparable with that observed staining the same cell type with the "structural" probe NAO, providing evidence that the entire mitochondrial network is functional (i.e able to generate and maintain a transmembrane electrical potential). To prove this conclusion, a number of controls are recommended. First of all the Mitotracker signal must vanish when the cells are pre-treated with CCCP (or other uncouplers) that by its protonophoric activity collapses the transmembrane potential. The same fading effect must be obtained by pre-treating the cells with inhibitors of any of the respiratory complexes chain that in turn will block the proton pumping activity. Conditions, which affect the staining by Mitotracker, are ineffective toward the NAO staining. Although the Mitotracker staining is widely spread out, with more intense and crowded fluorescence signal going from the periphery to the perinuclear region of the cell, a quantification of the signal cannot unambiguously be carried out. To address this last point the Mitocapture probe can be used. Given the peculiar mechanism of action (described in a previous section) of this dual-emission potential probe (resembling JC-1) it behaves more or less like Mitotrackers when the "$\Delta\Psi_m$ has a value below 150 mV. When the "$\Delta\Psi_m$ exceeds this threshold value Mitocapture not simply accumulates in the interior of mitochondria but changes its aggregation state. As a consequence of this, the frequency of the emitted fluorescence changes from green to bright red. This is shown in Fig 4 where the functional heterogeneity of mitochondria is illustrated very clearly. If the larger membrane potential in red-stained mitochondria is the expression of an higher electron transfer rate, (*versus* the green-stained low-potential mitochondria)

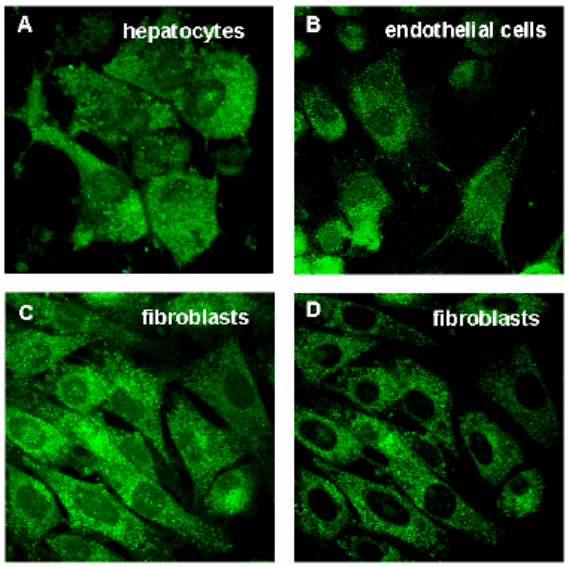

Fig. 2. Staining of mitochondrial mass in three different cultured cell types by NAO.
A, B, C are the CLSM images obtained by Superimposition of the confocal planes examined along the z-axes, going from the top to the bottom of the cells. D shows the imaging of the median plane.

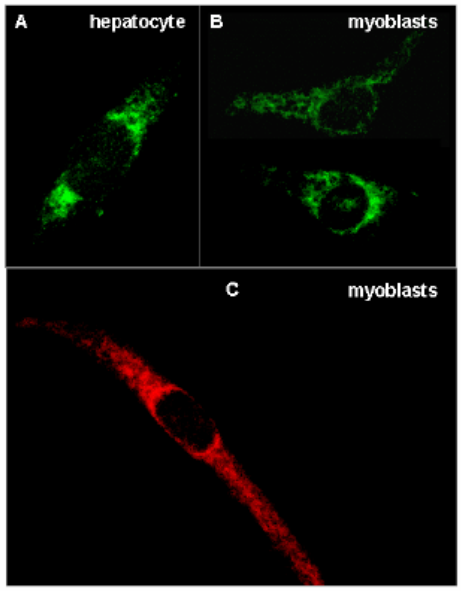

Fig. 3 CLSM imaging of cultured cells with the mitochondria specific probes Mitotracker green (A and B) and Mitotracker red (C).

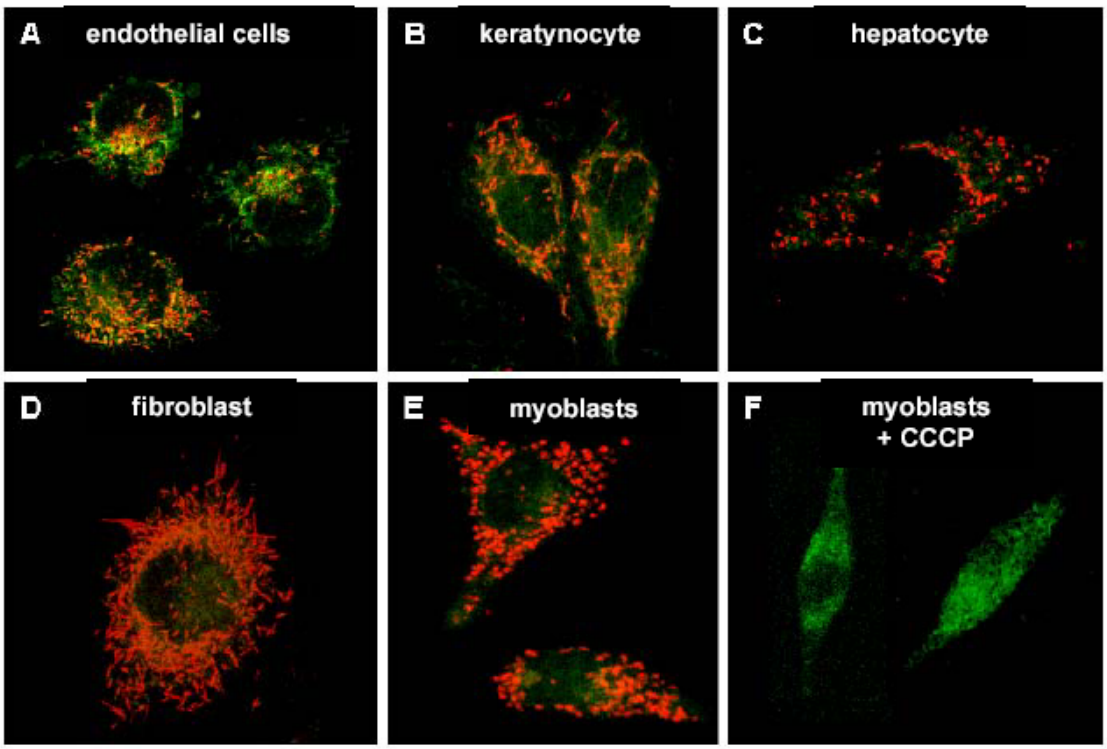

Fig. 4. CLSM imaging of cultured cells with the mitochondria specific probe Mitocapture. Staining treatment was carried out under coupled (A to E) and uncoupled (plus CCCP, F) conditions.

yhus the bichromatic texture of the mitochondrial network obtained is likely to be the visualisation of the intracellular bioenergetic map. It can be clearly noticed, comparing the staining of different cell types, that the mitochondrial functionality is not homogenously distributed within the network but instead high- and low-potential generating mitochondria coexist in a given intracellular space with a relative distribution which depends on the bioenergetic cell phenotype (compare Figs. 4 A and D).

Another advantage, in the use of mitocapture, comes from his property to retain its staining ability even after treatment of the cell sample with detergents like Triton X-100. This makes mitocapture more suitable than other mitochondrial probes for double staining protocols. Fig. 5 shows an example of double staining of the nuclei with TOPRO probes and mitochondria with functional and mass-detecting probes. It can be seen that differently from mitotracker and NAO (which diffuses or vanishes respectively) mitocapture maintains fully its well defining bright staining ability.

Given the high contrasted mitocapture red stained mitochondria a closer look at the reconstructed images reveals, within the limitation of the approach, a number of clues. When the same cell type is stained with NAO and mitocapture (see Fig. 6) it is evident that despite of the diffused population of mitochondrial cardiolipin-rich membranes within the cell not all of them exhibit the same bioenergetic properties. Most of the mitochondria located at the periphery of the cell do not seem to be able to generate appreciable protonmotive force. Moving closer to the interior of the cell, to the perinuclear region, the extent of the generated $\Delta\Psi_m$ increases, as detectable by mitocapture staining, with subsets of differently polarised mitochondria coexisting and with some of them showing a very high $\Delta\Psi_m$. The relative proportion and localization of these mitochondrial subsets depend on the cell type. It is likely that this variegated intra-

cellular bioenergetic map might reflect different energy requirements within the cell. The conclusion that can be drawn from this analysis, however, is not so straightforward. The presence of ipo-polarised

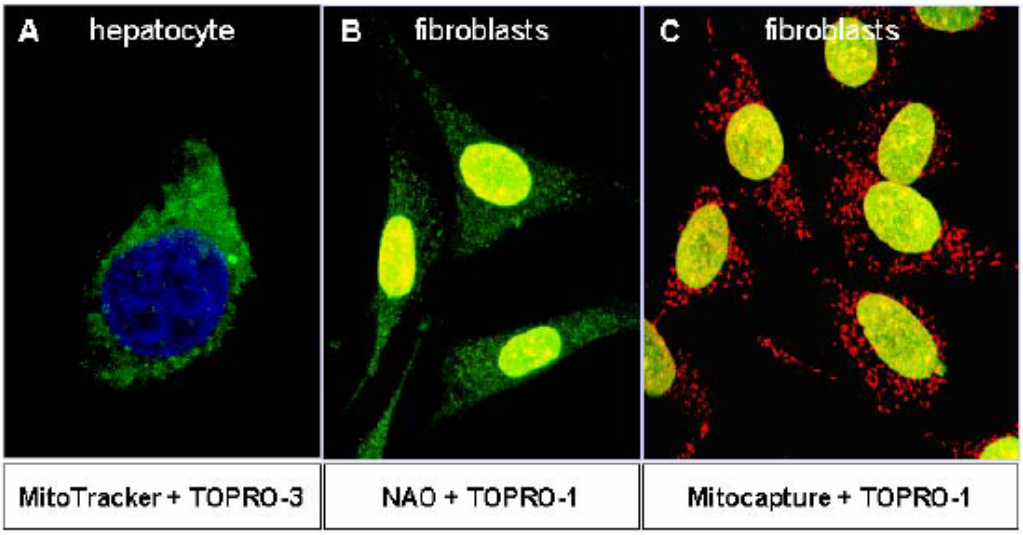

Fig. 5. Double staining of cultured cells with nuclei and mitochondria specific probes.

mitochondria could be due, in principle, to a number of reasons: i) low respiratory activity of the respiratory chain; ii) high activity of the $\Delta\Psi_m$ consuming reaction (i.e. higher activity of the H^+ ATP-synthase); iii) decoupling between respiratory activity and protonmotive force generation due either to higher H^+ leaks across the membrane and/or to slip of the redox driven proton pumping complexes [25-27]. The opposite can apply for each of these points to the iper-polarised mitochondria. Given the commonly accepted link between mitochondrial reduced membrane potential and activation of the apoptotic program understanding the functional meaning of the presence of a variable amount of potentially apoptogenic mitochondria deserves attention to elucidate the overall controlling mechanism underlying the fate of cell (i.e. survival *vs* death) [2]. As far as the structure of the mitochondrial network is concerning our analysis cannot address directly the problem of its possible functional connectivity. In all the cell type analysed the NAO staining reveals a mitochondrial population composed mainly by isolated granular rather than by interconnected filamentous entities (more clustered or packed in the perinuclear region). The functional staining by mitotrackers reveals instead a more diffuse (interconnected ?) mitochondrial texture which is more clearly observable with the mitocapture green-stained mitochondria whereas the red- stained mitochochondria appear mainly like individual grain (more or less elongated) without evident connectivity. It has been proposed that the interconnecting mitochondria can represent electrically united systems, which can facilitate energy delivery in the network by extensive delocalisation of the membrane potential [28]. Within the frame of this hypothesis specialised intermitochondrial junctions could serve to facilitate electrical transmission. Under certain conditions these junction could be switched off blocking the intermitochondrial connections. It would be tempting to propose on the basis of the results presented that the opening/closure of these junctions could be voltage dependent. When the membrane potential in a cluster of interconnected mitochondria is below a threshold value the junctions are open and the system is electrically communicating, when the $\Delta\Psi$ value in a given mitochochondria exceeds a certain value the junctions close and that mitochondria became isolated. Although very speculative the proposal of this putative voltage-dependent mechanism controlling the actual degree of the local functional interconnection in the mitochondrial network is intended to stimulate more extensive investigation.

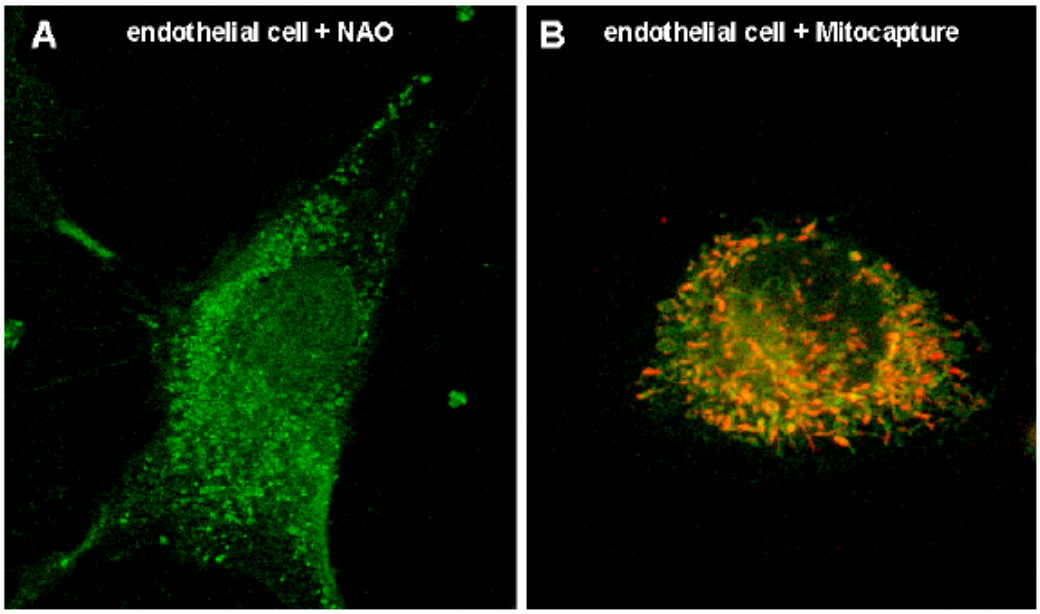

Fig. 6. Comparison between staining of the the same cell type by mitochondrial mass- and potential-specific probes.

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