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Calcium-dependent activation of mitochondrial metabolism in mammalian cells

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Abstract

Endogenous fluorophores provide a simple, but elegant means to investigate the relationship between agonist-evoked Ca^{2+} signals and the activation of mitochondrial metabolism. In this article, we discuss the methods and strategies to measure cellular pyridine nucleotide and flavoprotein fluorescence alone or in combination with Ca^{2+} -sensitive indicators. These methods were developed using primary cultured hepatocytes and neurons, which contain relatively high levels of endogenous fluorophores and robust metabolic responses. Nevertheless, these methods are amendable to a wide variety of primary cell types and cell lines that maintain active mitochondrial metabolism.

Keywords

Mitochondria; calcium; inositol 1,4,5-trisphosphate; pyridine nucleotides; flavoproteins; membrane potential; digital fluorescence imaging; confocal microscopy

Introduction

Circulating nutrient and hormone levels in the extracellular milieu are cues used by cells and tissues to regulate their physiological status. The information embedded in these external stimuli is converted into intracellular second messengers through a series of interconnected signal transduction pathways. One of the most common second messengers in these signaling networks is Ca^{2+} ions. Periodic fluctuations in cytosolic calcium concentration ($[\text{Ca}^{2+}]_i$) can be driven by electrical activation of voltage-sensitive Ca^{2+} channels or by hormone stimulation of plasma membrane receptors and the subsequent formation of Ca^{2+} -mobilizing second messengers, such as inositol 1,4,5-trisphosphate (InsP_3). The frequency, amplitude and/or duration of these $[\text{Ca}^{2+}]_i$ spikes can be detected and decoded by downstream Ca^{2+} -sensitive proteins providing a versatile pathway for extracellular stimuli to exert control over a wide range of metabolic pathways (1–3).

One important target for these Ca^{2+} signals is the activation of mitochondrial oxidative metabolism and the consequent increase in the formation of ATP. It has been proposed that mitochondria are strategically localized close to Ca^{2+} release sites, such that Ca^{2+} influx across the plasma membrane or Ca^{2+} released through InsP_3 or ryanodine receptors in the sarcoplasmic/endoplasmic reticulum is rapidly transported into the mitochondrial matrix (4–

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11). Mitochondrial Ca^{2+} ions bind directly to NAD^+ -isocitrate dehydrogenase and α -ketoglutarate dehydrogenase to decrease the K_m for their respective substrates, whereas an increase in the dephosphorylated and active form of pyruvate dehydrogenase is regulated by a Ca^{2+} -sensitive phosphatase (4,10–16). These studies have led to the concept that the oxidation state of the mitochondrial pyridine nucleotide pool is directly regulated by $[\text{Ca}^{2+}]_m$ and linked to changes in $[\text{Ca}^{2+}]_i$. Moreover, increases in mitochondrial Ca^{2+} can also augment the magnitude of the mitochondrial proton motive force (PMF) either directly or through increases in matrix NAD(P)H (10,11). PMF is an electrochemical gradient formed by the vectorial transport of protons from the mitochondria matrix to the extramitochondrial space by the electron transport chain and consists of pH gradient (ΔpH_m) and transmembrane potential ($\Delta\Psi_m$) components. The free energy stored in the electrochemical gradient is used for ion and substrate transport across the mitochondrial inner membrane (17,18), as well as providing the energy required to rotate the γ -subunit of the F_1 -ATP synthetase, which is the mechanism coupling PMF to the synthesis of ATP (19,20).

We have taken advantage of endogenous cellular fluorophores, such as NAD(P)H and mitochondrial flavoproteins to monitor changes in mitochondrial metabolism in real time. Moreover, careful selection of Ca^{2+} -sensitive indicators or dye loading protocols can be employed to simultaneously measure agonist-evoked increases in either $[\text{Ca}^{2+}]_i$ or $[\text{Ca}^{2+}]_m$ along with changes in mitochondrial function. These imaging techniques open up the ability to study the complex relationship between Ca^{2+} signal transduction and the control of Ca^{2+} -sensitive processes. This article will describe the methodology required to carry out these imaging techniques and some of the more common pitfalls that can be encountered.

Hardware overview

There are two main considerations when carrying out imaging measurements of endogenous fluorophores with wide-field excitation. First, the fluorescence intensities of endogenous fluorophores are typically low and, thus a sensitive camera for low light level imaging is required to carry out these measurements. For comparison sake, our standard fura-2 loading protocol in hepatocytes (10,21,22) results in between 20 and 100 fold higher fluorescence intensity values for the Ca^{2+} -sensitive indicator compared to pyridine nucleotides. The second issue to cope with is the photochemical destruction of the fluorophore or photobleaching. Resolving this issue is also related to the sensitivity of the imaging camera. Photochemical damage can be diminished by reducing the illumination intensity through the use of neutral density filters and/or decreasing the exposure time for acquiring an image. Neutral density filters are designed to attenuate the intensity of light over a broad spectral range. We typically use neutral density filters with optical densities of 0.6 to 1.0 OD (25-10% transmission) in our experiments, but this will depend on the optical properties of each microscope system and the strength of the fluorescence excitation source.

In our laboratory, we use cooled charge-coupled device (CCD) cameras from Photometrics (<http://www.roperscientific.com/>) that are mounted on inverted microscopes equipped with 20x and 40x high numerical aperture (NA) objectives. The cameras are equipped with back-illuminated, slow-scan CCD chips with 14 to 16 bit dynamic range. Comparable CCD cameras from other vendors can also be used. The objectives are infinity-corrected with high transmission rates in the ultra violet (UV) region and have a long working distance. High UV transmission is important for exciting pyridine nucleotides and the long working distance is particularly helping when imaging thick specimens or perfused tissues. The light source is a 75 watt xenon arc lamp. The arc lamp is connected to the microscope light path through a filter wheel/shutter assembly from Ludl (<http://www.ludl.com/>). Excitation, dichroic or emission sets and neutral density filters are acquired from either Omega Optical (<https://www.omegafilters.com/>) or Chroma Technology (<http://www.chroma.com/>). We

typically have all emission filters blocked in the infrared region (800–200 nm) to reduce stray light. See Table 1 for list of filter sets. Data acquisition is controlled with in-house written software.

Confocal images are acquired with a Bio-Rad Radiance 2002-MP confocal system using the manufacture's software. The Bio-Rad confocal system is equipped with a 50 mW argon laser and a 10 watt Mira/Verdi Ti:sapphire laser from Coherent™. The 488 nm argon laser line is utilized for single photon excitation of mitochondrial flavoproteins, while the Ti:sapphire laser is used for multi-photon excitation of pyridine nucleotides or the Ca^{2+} -sensitive indicator, fura-2. The fluorescence emitted by pyridine nucleotides or fura-2 is collected with an external direct detector system (DDS) when measured alone or descanned and measured with internal detectors when measured sequentially with flavoprotein fluorescence. See Table 2 for the filters and settings used to acquire confocal images. The sensitivity is lower with the internal detectors, but the fluorescence signals are still adequate for temporally and spatially resolved imaging. The confocal division of Bio-Rad was sold to by Carl Zeiss (<http://www.zeiss.com/>) and the above described system is no longer available.

Imaging measurements of pyridine nucleotides

The reduced forms of nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) have indistinguishable single photon excitation and absorption spectra with peaks centered at approximately 340 nm, whereas the oxidized forms display little to no fluorescence at this wavelength. Cellular pyridine nucleotides also have broad and overlapping fluorescence emission spectra with a peak ranging from 440 nm to 460 nm (23–25). Two-photon excitation of either pyridine nucleotide yields emission profiles that are identical to the single photon emission spectra (26). Moreover, NADH and NADPH have the same extinction coefficients and quantum yields, consequently these molecules cannot be spectrally separated by either one- or two-photon excitation. The composite pyridine nucleotide fluorescence signal is typically designated as NAD(P)H.

Cellular pyridine nucleotide fluorescence can be derived from both the cytosolic and mitochondrial compartments (27). We utilized 710 nm multi-photon excitation to image the cellular distribution of pyridine nucleotide fluorescence in cultured hepatocytes (Fig 1A) or hepatocytes within the intact perfused rat liver (Fig. 1B). The images show bright punctate structures surrounding the darker nucleus. The fluorescence intensity of the punctate structures changed in the correct direction upon the addition of acetoacetate or β -hydroxybutyrate to increase or decrease the mitochondrial NAD^+/NADH ratio, respectively. The ketone bodies, acetoacetate or β -hydroxybutyrate, are readily interconverted by the oxidation/reduction reactions catalyzed by mitochondrially localized β -hydroxybutyrate dehydrogenase (see **Calibration**). These data indicate that the punctate structures reflect pyridine nucleotides in the mitochondria, a conclusion consistent with the study by Nieminen et al. (28). NAD(P)H fluorescence was also predominately localized to the mitochondrial matrix in cardiomyocytes, pancreatic β -cells and neurons (26,29,30). The higher fluorescence intensity values in the mitochondria may reflect the large increase in fluorescence quantum yield for NAD(P)H in the matrix compared to aqueous solutions (24). It should be noted that some cells types also contain significant levels of cytosolic NAD(P)H due to high rates of glycolysis (30). Moreover, experimental manipulations could potentially perturb both the cytosolic and mitochondrial NAD(P)H pools. These possibilities need to be taken into consideration when interpreting changes in NAD(P)H fluorescence intensity (see **Calibration**).

Endogenous fluorophores other than pyridine nucleotide were also excited with 710 nm multi-photon and 360 nm single photon excitation. These fluorophores were predominately observed lining the sinusoids of the intact perfused liver (Figs 1B) and also found to varying degrees in

cultured hepatocytes (not shown). The fluorophore was randomly distributed across the entire liver lobule and appeared as bright specks in low magnification images (Fig 2B). The fluorescence intensity of these fluorophores did not change with the oxidation/reduction state of the mitochondria. The fluorescence intensity was stable with multi-photon excitation; where as wide-field UV excitation resulted in the rapid photobleaching (Fig 1C). We have not fully explored the nature of this endogenous fluorophore; however its lobular distribution and sensitivity to UV excitation suggests that the compound may be vitamin A (31). We have only observed these UV sensitive endogenous fluorophores in hepatocyte cultures and the intact liver.

Figure 1 C and D illustrate the measurement of cellular NAD(P)H using wide-field excitation. When cellular pyridine nucleotides are imaged, alone, cultures are illuminated with 360 ± 40 nm excitation and the emitted fluorescence collected with a broad band filter passing 420 to 600 nm. The broad band emission filter allows the collection of the entire NAD(P)H emission spectrum. The data are displayed as F/F_0 , where the F is the recorded 360 nm fluorescence intensity over time and F_0 is the fluorescence intensity at the start of data acquisition. Figure 1C illustrates the photobleaching of a portion of the endogenous autofluorescence upon UV excitation. Notice that once the photo-sensitive fluorophore is bleached the remaining fluorescence signal is stable. Since this fluorescence does not contribute to the NAD(P)H signal, we routinely illuminate the hepatocyte cultures with UV excitation until a stable fluorescent baseline is obtained prior to starting data collection. Figure 1D shows the NAD(P)H response to maximal ATP stimulation of purinergic receptors in a single hepatocyte that was prebleached. This agonist-induced increase in NAD(P)H fluorescence reflects a response to $[Ca^{2+}]_i$ signals that are transmitted to the mitochondria (see below and 7, 10, 16, 32)

Imaging measurements of mitochondrial flavoproteins

Flavin adenine dinucleotides (FAD) are prosthetic groups found in many enzymes catalyzing oxidation-reduction reactions. The majority of flavin-containing enzymes display weak to no fluorescence due to quenching of the coenzyme when bound to the protein (27,33,34). The enzymes contributing significantly to cellular flavoprotein fluorescence are localized in the mitochondrial matrix and have been assigned to lipoamide dehydrogenase and electron transfer flavoprotein (ETF) (27,33–8). Lipoamide dehydrogenase is an integral component of the Ca^{2+} -sensitive pyruvate and 2-oxoglutarate dehydrogenase complexes (39) and their flavin co-factors are in direct equilibrium with the mitochondrial $NAD^+/NADH$ pool. ETF is involved in the transfer of reducing equivalents to ubiquinone during β -oxidation of fatty acids (36). The oxidation-reduction state of this flavoprotein is not directly influenced by NAD^+ -linked reactions.

Flavoproteins have broad excitation and emission spectra with an excitation maxima around 470 nm and an emission peak close to 520 nm (37,38). Contrary to pyridine nucleotides, the oxidized flavin has a higher fluorescence intensity compared to the reduced form. Therefore, flavoproteins and NAD(P)H display opposite fluorescence intensity responses to changes in the mitochondrial metabolic state (Fig. 2). It has been estimated that lipoamide dehydrogenases comprise about 50% of the total flavoprotein fluorescence in isolated mitochondria, whereas the ETF contributes about 25%. The remaining flavoprotein fluorescence is only reducible by dithionite and is presumably not in redox contact with the electron transfer chain (34,38,40). The excitation and emission fluorescence spectra of lipoamide dehydrogenases are slightly red-shifted compared to ETF. The differences in the spectral properties of the flavoproteins have been utilized by other groups to monitor redox-dependent changes in the lipoamide dehydrogenase fluorescence (25,40).

Figure 2 shows the dual measurement of cellular pyridine nucleotides and mitochondrial flavoproteins using wide field excitation (Fig 2A) or a combination of multi-photon and single photon confocal microscopy (Fig 2B). The wide-field fluorescence images were acquired by alternating excitation at 360 ± 40 nm and 470 ± 35 nm using a computer-controlled filter wheel and shutter (Ludel). The emitted fluorescence was collected using a 515 nm long band pass beamsplitter and a 520 nm long band pass emission filter (Table 1). Notice that the optical setup is suboptimal for acquiring pyridine nucleotide fluorescence and this may preclude measurement of NAD(P)H in some cell types. An alternative approach would be to use an imaging system capable of switching between different dichroic mirror and emission filter sets (7). In this setup, the registration between the two fluorescence images has to be taken into consideration, because differences in the light pathway for image formation can lead to misalignment and dissimilar spatial aberrations. The confocal images shown in Fig 2B-D were acquired by sequentially scanning the intact perfused liver with 710-nm multi-photon excitation then 488-nm excitation from an argon laser. The emission fluorescence was descanned and detected with 450 ± 80 nm (pyridine nucleotides) or 500 nm long band pass filters (FAD).

The NAD(P)H and FAD traces in Fig 2A and 2D are displayed as raw fluorescence intensities (arbitrary units, a.u.) to show the reader the magnitude of the change in autofluorescence. The experiment shown in Fig 2A was digitized to 14 bits (16,383 gray levels), whereas the experiment in Fig 2D was digitized to 8 bits (256 gray levels). The addition of vasopressin, a Ca^{2+} -mobilizing hormone, evoked an increase in the NAD(P)H fluorescence and a decrease in the mitochondrial flavoprotein fluorescence that occurred with similar time courses (Fig 2A). In this experiment, hormone stimulation induced an approximately 20% reduction in the pyridine nucleotide and mitochondrial flavoprotein pools coupled to the electron transport chain. Oleic acid (30 μM) infusion into the intact perfused rat liver also induced parallel changes in NAD(P)H and FAD fluorescence intensities with similar time courses (Fig 2D). The fluorescence returned toward baseline values upon washout of the fatty acid.

Calibration

The relative oxidation state of the cellular pyridine nucleotide and mitochondrial flavoproteins was assessed by the addition of the mitochondrial uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 0.5–5 μM). CCCP induces a proton leak across the mitochondrial inner membrane which collapses the mitochondrial pH gradient and membrane potential. As a result, CCCP stimulates the electron transfer chain resulting in the oxidation of the pyridine nucleotides and subsequently the oxidation of all mitochondrial flavoproteins. Oligomycin (Oligo; 1–5 $\mu\text{g/ml}$) is included in this treatment to prevent uncoupler stimulated ATP hydrolysis and the potential for an ATP-driven proton gradient. The addition of rotenone (Rot; 1–5 μM) to block NADH dehydrogenase plus β -hydroxybutyrate, a substrate for β -hydroxybutyrate dehydrogenase (see below), causes the reduction of the pyridine nucleotide pool and NAD^+ -linked flavoproteins (Fig 2A). ETF can only be fully reduced by inhibiting the electron transport chain below ubiquinone with myxothiazole (1 to 5 μM) or cyanide (1 mM) to block cytochrome C reductase or cytochrome C oxidase, respectively (40). The effective concentration for each mitochondrial toxin should be determined empirically and may deviate from those concentrations listed in the text. Our media contains 0.1–0.25% (w/v) bovine serum albumin (BSA) which is known to bind these toxins and decrease their efficacy. To calibrate the redox signals in the intact perfused liver, it is more convenient to use anoxia to fully reduce NAD(P)H. This can be accomplished by saturating the perfusion buffer with nitrogen or argon gas. A mitochondrial uncoupler can be used to obtain the fully oxidized state in the perfused liver. However, tissue swelling and movement frequently occur under these conditions.

Cytosolic or mitochondrial pyridine nucleotide pools can also be sequentially oxidized or reduced by the addition of NAD-linked or NADP-linked substrates. These manipulations can give some insight into which pyridine nucleotide pool is perturbed and in which compartment. As stated above, the mitochondrial NAD^+/NADH ratio can be perturbed with the ketone bodies, acetoacetate and β -hydroxybutyrate, which are interconverted exclusively by the mitochondrial matrix enzyme β -hydroxybutyrate dehydrogenase utilizing mitochondrial NADH and NAD^+ , respectively (7,10,27,41,42). The addition of L-lactate or pyruvate can be used to set the cytosolic NAD^+/NADH ratio through the actions of lactate dehydrogenase (41). However, it should be pointed out that cell-type specific expression of monocarboxylate transporters or the activity of the malate/aspartate and glycerol-3-phosphate redox shuttles could alter the efficacy of these protocols to maximally increase or decrease the NAD^+/NADH ratio.

The mitochondrial and cytosolic NADPH pools can be distinguished from one another by the stimulating amino acid transamination in the mitochondrial matrix. The addition of NH_4Cl in the presence of lactate and ornithine causes a preferential oxidation of mitochondrial NADPH in hepatocytes and this effect can be enhanced by inhibiting NADP-specific isocitrate dehydrogenase (43,44). Under these conditions, mitochondrial NADPH is utilized for the reductive amination of α -ketoglutarate to aspartate, which is required for urea synthesis (43). While mitochondrial NADP-specific isocitrate dehydrogenase has a wide tissue distribution (45), we do not know if this manipulation can be carried out successfully in other cell types. The addition of *tert*-butyl hydroperoxide can be used to oxidize both cytosolic and mitochondrial NADPH pools through the actions of glutathione peroxidase (44). Caution should be used with *tert*-butyl hydroperoxide as it is toxic and prolonged exposure can cause rupture of the plasma membrane (46).

Multi-photon microscopy has been utilized to obtain direct spatial separation of cytosolic and mitochondrial NAD(P)H increases evoked by glucose stimulation of pancreatic β -cells (29) or by increased neural activity in hippocampal slices (30). While these are elegant and straightforward methods to carry out, it is difficult to completely separate mitochondrial derived pyridine nucleotide fluorescence from the cytoplasm. This is because the two-photon excitation volume is an ellipsoid sphere approximately $0.3\ \mu\text{m}$ by $1.0\ \mu\text{m}$ (47) and, thus the maximal resolution of the multi-photon microscopy is close to the size of mitochondria. Multi-photon excitation of pyridine nucleotides coupled with single photon excitation of NAD^+ -linked mitochondrial flavoproteins has been used to distinguish between nutrient-induced increases in NADH versus NADPH in pancreatic β -cells. For more details regarding this method see (25).

Simultaneous measurement of Ca^{2+} -sensitive indicators and mitochondrial autofluorescence

In this section, we will describe imaging techniques to simultaneously measure agonist-evoked increases in $[\text{Ca}^{2+}]_i$ along with mitochondria metabolism. Most Ca^{2+} -sensitive indicator dyes, including red-shifted versions, absorb some light at 360 nm (see Molecular Probes; <http://www.invitrogen.com/>) and, thus the NAD(P)H fluorescence intensity changes have to be corrected for this spectral overlap. We prefer to use fura-2/AM loaded cells in these experiments even though the absorbance spectra of NAD(P)H and fura-2 completely overlap. Fura-2 is advantageous over other Ca^{2+} -sensitive indicators because it can be used in the dual excitation ratiometric mode to measure $[\text{Ca}^{2+}]_i$ and fura-2 has an isofluorescence wavelength ($\sim 360\ \text{nm}$) where its fluorescence is independent of $[\text{Ca}^{2+}]$. Cell cultures are incubated on the microscope stage in the presence of $1\ \mu\text{M}$ fura-2/AM for 2 to 10 minutes while monitoring the fluorescence intensities at 360 nm and 380 nm. The objective is to obtain intracellular fura-2 fluorescence intensities at 380 nm that are less than or equal to the fluorescence intensities

measured at 360 nm or the NAD(P)H fluorescence signal. The dye loading protocol and imaging experiments are carried out in the presence of a competitive organic anion transport inhibitor, bromosulphophthalein (100 μ M, Fluka <http://www.sigmaaldrich.com>), to minimize dye loss (32) and N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN, 30 μ M) to prevent quenching of intracellular fura-2 by heavy metals (7).

Fura-2 and NAD(P)H fluorescence images were acquired by sequentially switching between excitation at 340 ± 10 nm, 380 ± 10 nm and 360 ± 10 nm and the fluorescence collected as described above for NAD(P)H (see also Table 1). Note that the amount of fura-2 fluorescence spill over into the NAD(P)H signal is reduced by using a narrower 360 nm excitation filter in these experiments. Figure 3A shows the fluorescence intensities for 360 nm (black trace), 380 nm (green trace) and 340 nm (red) excitation in a single hepatocyte stimulated with a low concentration of the α_1 -adrenergic receptor agonist, phenylephrine. The rapid and large increases in the F_{340} intensity and parallel decreases F_{380} signal represents oscillatory $[Ca^{2+}]_i$ spikes. The small downward deflections in the F_{360} trace that occur with the $[Ca^{2+}]_i$ spike are caused by the overlap of the Ca^{2+} -free form of fura-2 with the NAD(P)H signal. Note that the baseline fluorescence for both F_{340} and F_{380} signals increases after the onset of $[Ca^{2+}]_i$ spiking, which is due to a sustained rise in pyridine nucleotide fluorescence. At the end of the experiment, rotenone and β -hydroxybutyrate (Rot) were added to fully reduce mitochondrial pyridine nucleotides, which is used to normalize the NAD(P)H increases between different experiments (10,11).

The bottom traces (Fig. 3B) show the NAD(P)H response corrected for fura-2 spill over together with the 340nm/380nm fura-2 ratio for the same cell shown in panel A. The fura-2 fluorescence spill over was corrected with the following algebraic equation: $F_{360corr} = F_{360} - \alpha * F_{380}$. Where α represents the ratio of the fluorescence decrease in the 360 nm signal to the fluorescence decrease in the 380 nm signal ($\Delta F_{360}/\Delta F_{380}$) during an increase in $[Ca^{2+}]_i$ (10). To eliminate NAD(P)H increases during the calibration, mitochondrial NAD(P)H is oxidized with a mitochondrial uncoupler prior to the addition of high concentrations of agonist or ionomycin to evoke a rapid $[Ca^{2+}]_i$ increase. For an example, see Fig 3D in (7). A separate correction factor needs to be determined for each experiment.

Mitochondrial flavoprotein and fura-2 fluorescence are spectrally distinct allowing simultaneous measurement of both parameters without overlap. Thus, investigators can use their normal dye loading protocol to carry out these experiments. Fura-2 and flavoprotein fluorescence were excited as described above and emitted fluorescence was collected with a 515 long band pass dichroic reflector and a 520 nm long band pass emission filter (Table 1). The traces show representative single cells $[Ca^{2+}]_i$ and FAD responses in a hepatocyte (Fig. 4A) or a hypothalamic neuron (Fig 4B). The addition of acetoacetate (AcAc) to the liver cells oxidizes the mitochondrial matrix resulting in a stable increase in flavoprotein fluorescence. Subsequent vasopressin (100 pM) stimulation evoked baseline-separated $[Ca^{2+}]_i$ oscillations and a sustained decrease in mitochondrial flavoprotein fluorescence that was proportional to the increase induced by acetoacetate. The results in figures 3 and 4A indicate that Ca^{2+} -sensitive intramitochondrial dehydrogenases integrate low frequency of $[Ca^{2+}]_i$ spiking into a smooth continuous sustained activation of mitochondrial metabolism, see also (7,11).

The hypothalamus contains neurons that detect and respond to circulating nutrient levels in the brain, and a change in their neural activity is thought to control feeding behavior (48). Some neuronal subtypes are inactivated by low glucose concentrations whereas, in a different subpopulation the fall in glucose stimulates neuronal activity (49). Figure 4B shows periodic $[Ca^{2+}]_i$ spiking in a single hypothalamic neuron incubated with 0.5 mM glucose. Notice the striking difference between the flavoprotein responses in hypothalamic neurons versus hepatocytes. In these neurons, each $[Ca^{2+}]_i$ spike is closely paralleled by reduction then

oxidation of the mitochondrial flavoproteins. Indeed, the FAD response is almost a mirror image of the $[Ca^{2+}]_i$ spike with similar rates of rise and decline. These data suggest that neuronal mitochondria may not decode oscillatory Ca^{2+} signals into a time-averaged increase in metabolism, but rather maintain a digital output at the level of the metabolic signal.

Simultaneous measurement of Ca^{2+} -sensitive indicators and mitochondria membrane potential ($\Delta\Psi_m$)

Activation of intramitochondrial dehydrogenases and increased NAD(P)H formation is expected to stimulate flux through the electron transport chain (50,51) leading to an enhancement of mitochondrial PMF. Indeed, we have previously shown that Ca^{2+} -mobilizing hormones increase both components of the electrochemical gradient (10,11). Mitochondrial PMF can be monitored in real-time by co-loading hepatocyte cultures with fura-2/AM and either tetramethyl rhodamine ethyl ester (TMREE), a mitochondrial membrane potential-sensitive dye, or with the weak acid fluorescein (10,11,52,53). For $\Delta\Psi_m$ measurements, we incubate the cells in the presence of low concentrations of TMREE (≤ 10 nM) for extended periods to allow the dye to reach equilibrium across the mitochondrial inner membrane. The low TMREE loading protocol is used to avoid an inner filter effect which causes self quenching of the dye in the mitochondrial matrix. Self quenching is evident by an increase in TMREE fluorescence intensity upon depolarization of the mitochondrial membrane. In addition, we have found that prolonged fluorescence imaging of hepatocytes loaded with high levels of TMREE (≥ 100 nM) causes mitochondrial swelling and cytotoxicity.

The traces in figure 5 show the simultaneous measurement of vasopressin-evoked increases in $[Ca^{2+}]_i$ and $\Delta\Psi_m$. Fura-2 and TMREE fluorescence images were acquired by sequential excitation at 340 ± 10 nm, 380 ± 10 nm and 548 ± 20 nm and the emitted fluorescence was collected with a multi-band pass dichroic beamsplitter and emission filter set (Table 1). The dichroic beamsplitter reflects light below 395 nm, to excite fura-2, and between 545 nm and 565 nm for TMREE excitation. The dichroic reflector also transmits light between 507 ± 54 nm and ≥ 570 nm to pass fura-2 and TMREE fluorescence, respectively. The emitted fluorescence is further delimited by an emission filter with two band passes at 509 ± 30 nm and 600 ± 50 nm. For an example of this type of beamsplitter and emission filter see the 73000 filter set from Chroma Technology. In addition, note that this filter set will markedly decrease the fura-2 fluorescence intensities compared to a standard fura-2 filter set (Table 1) and, thus the investigator may need to increase the exposure time for image acquisition.

The data show that onset of each $[Ca^{2+}]_i$ spike is accompanied by a slow rise in TMREE fluorescence intensity indicating an increase in the magnitude of $\Delta\Psi_m$. Vasopressin-evoked increases in $\Delta\Psi_m$ peaked after the $[Ca^{2+}]_i$ spike had already turned to baseline values; then slowly recovered. Discrete oscillations in $\Delta\Psi_m$ fused into a sustained increase in PMF at higher frequencies of $[Ca^{2+}]_i$ spiking. A similar relationship was observed between $[Ca^{2+}]_i$ oscillations and increases in ΔpH_m . The relative changes in $\Delta\Psi_m$ can be calibrated with the K^+/H^+ ionophore, nigericin, to hyperpolarize the mitochondrial inner membrane followed by the addition of CCCP plus oligomycin to completely collapse the mitochondrial PMF (10, 11).

Genetically encoded Ca^{2+} -sensitive indicators and mitochondria function

We (7,10,54) and others (5,6,8,9,55–8) have shown that rhod-2 or mitochondrially-targeted aequorin can be used to monitor agonist-evoked increases in $[Ca^{2+}]_m$ in many different cells types including primary cultured hepatocytes. However, rhod-2 is a single wavelength dye and its fluorescence intensity changes are difficult to quantify. Rhod-2 is also subject to potential artifacts, such as movement, changes in the mitochondrial volume and it can buffer increases

in $[Ca^{2+}]_m$. Moreover, it is difficult to achieve complete mitochondrial localization, raising the possibility that a portion of the rhod-2 fluorescence signal may originate from extramitochondrial compartments; e.g. cytoplasm. Beside the mitochondria, rhod-2 also rapidly labels the nucleolus (55). We find that if the nucleolus is intensely labeled with rhod-2, then the indicator was already started to accumulate in the cytoplasm. Thus, one needs to use caution when interpreting agonist-evoked increases in rhod-2 fluorescence intensity. On the other hand, aequorin can be efficiently targeted to the mitochondrial matrix and is not expected to significantly buffer $[Ca^{2+}]_m$ increases. Aequorin measurements suffer from low signal output and, thus it is generally necessary to measure $[Ca^{2+}]_m$ responses in cell populations with a photomultiplier tube or use a photon counting device with little or no spatial resolution.

The cameleon family of proteins are a second generation of genetically encoded Ca^{2+} indicators that use calmodulin and a calmodulin binding protein as Ca^{2+} -responsive elements (59,60). These biosensors can potentially overcome the sensitivity and localization issues discussed above. The latest version of these biosensors have a large dynamic FRET emission ratio, low sensitivity to protons and that do not interact with endogenous calmodulin (60). See McCombs and Palmer in this issue for further details on these probes.

Figure 6 shows an experiment using mitochondrially-targeted cameleon to measure ATP-evoked increases in both $[Ca^{2+}]_m$ and cellular pyridine nucleotide fluorescence (4mtD3cpV kindly provided by Dr. A. Palmer, U. of Colo). The fluorescence of cameleons and pyridine nucleotides do not overlap spectrally, allowing simultaneous measurement of both parameters. In these experiments, we used electroporation techniques and Nucleofection reagents from Amaxxa (<http://www.amaxa.com/home/>) to express the fluorescent protein in hepatocyte cultures. We find that electroporation results in higher transfection efficiency (20–60%) in primary cultured cells compared to liposome-based transfection reagents (~1%). Moreover, electroporation does not interfere with the correct targeting of the fluorescent protein under optimal conditions (Fig. 6A). Nevertheless, we have observed that high levels of cameleon expression can result in significant buffering of intraorganelle Ca^{2+} responses and increase mistargeting of the biosensor to the cytosol. Careful titration of the plasmid concentration and/or selection cells with low levels of cameleon expression can overcome these issues. Another consideration for these experiments is to maintain cell physiology during the time period required to express the transgene. Hepatocytes are cultured for 18–24 hrs after the electroporation procedure and this time window can result in a substantial decrease in intercellular NAD^+ content and mitochondrial function (61). Supplementing the hepatocyte culture media with nicotinamide (1 mM), the precursor for pyridine nucleotide synthesis, prevents NAD^+ depletion and also helps to maintain a hepatocyte-like phenotype (61).

Hepatocytes transiently expressing 4mtD3cpV are sequentially illuminated with 436 ± 20 nm then 360 ± 40 nm excitation using a 455 nm long band pass dichroic (Table 1). For these dual parameter measurements, a dichroic mirror with high reflectance characteristics in the UV range is required. The emitted fluorescence is directed into a Dual-View image beamsplitter from Optical Insights™ (<http://www.optical-insights.com/>). Donor and acceptor fluorescence images were separated with a 505dcmr dichroic mirror then directed to 480 ± 30 nm (CFP) or 535 ± 40 nm (YFP) emission filters. The resultant acceptor emission image recorded at 535 nm is divided by the CFP donor emission image recorded at 480 nm to obtain the FRET emission ratio (Fig 6B, green trace). The cellular pyridine nucleotide responses are observed in both emission channels and these images are summed to increase the signal output (Fig. 6B, black trace).

The data indicate that maximal ATP stimulation evokes a transient increase in both NAD(P)H fluorescence and $[Ca^{2+}]_m$. These cellular responses are qualitatively similar to our previously published measurements of vasopressin-evoked $[Ca^{2+}]_m$ increases and parallel NAD(P)H

responses recorded in rhod-2 loaded hepatocytes (7,10,11). The transient nature of the $[Ca^{2+}]_m$ response is presumably due to the depletion of internal Ca^{2+} stores and dissipation of high localized Ca^{2+} gradients surrounding the $InsP_3$ receptor (5,8,11). It should be pointed out the $[Ca^{2+}]_m$ transient shown in figure 6B is more prolonged compared to Ca^{2+} response measured with aequorin, but similar to the Ca^{2+} increase measured in rhod-2 loaded hepatocytes (10). These data would suggest that rhod-2 and 4mtD3cpV may cause a modest buffering of the $[Ca^{2+}]_m$ spike. Alternatively, the light output from aequorin may not be able to accurately measure low matrix Ca^{2+} levels in hepatocytes.

Concluding Remarks

The measurement of cellular autofluorescence coupled with Ca^{2+} -sensitive indicators provides a relatively straightforward technique to investigate the regulation of mitochondrial metabolism by extracellular stimuli. These types of studies are expected to help clarify the role of mitochondria in normal cell physiology, as well as give insight into defects in mitochondrial energy metabolism which occur with the onset and progression of many diseases.

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Fig. 1. Measurement of cellular pyridine nucleotide fluorescence

NAD(P)H autofluorescence was imaged in primary cultured hepatocytes (A) or hepatocytes within the intact perfused liver (B) using 710 nm excitation from a pulsed Ti:sapphire laser. In panels C-D, pyridine nucleotides were illuminated with 360 ± 40 nm wide field excitation. Trace C shows the photo-bleaching of endogenous autofluorescence prior to ATP stimulation. The fluorophore sensitive to 360 nm illumination has been reported to be vitamin A (31). In D, hepatocyte cultures were prebleached (not shown) prior to data collection.

Fig. 2. Measurement of mitochondrial flavoproteins and pyridine nucleotides
 Measurement of NAD(P)H (360 ± 40 nm) and flavoproteins (470 ± 35 nm) fluorescence in hepatocyte cultures (**A**) stimulated with maximal vasopressin concentrations (100nM) followed by an *in situ* calibration. Emitted fluorescence was collected with a 515 nm long band pass dichroic and a 520 nm long band pass filter. Cellular NAD(P)H (**B**) and mitochondrial flavoprotein (**C**) fluorescence intensities in the intact perfused liver were sequentially imaged using 710 nm and 488 ± 10 nm excitation, respectively. The traces in **D** depict the mean metabolic response to oleic acid infusion ($30 \mu\text{M}$, black bar).

Fig. 3. Simultaneous measurement of hormone-evoked increases in cytosolic calcium and pyridine nucleotide fluorescence

Hepatocyte cultures were briefly loaded with fura-2/AM and then stimulated with submaximal phenylephrine (PE) concentrations (1 μ M). Experiments were carried out in the presence of TPEN to prevent the quenching of fura-2 fluorescence by endogenous heavy metals (7). **(A)** PE-evoked changes in the raw 360 nm (F360), 380 nm (F380) and 340 nm (F340) fluorescence intensities. Traces were arbitrarily adjusted on the y-axis for clarity. Rotenone (5 μ M) plus β -hydroxybutyrate (10 mM) were added at the end of the run to fully reduce the pyridine nucleotide pool (10). **(B)** The 360 nm response (black trace) corrected for the spillover of fura-2 and the fura-2 excitation ratio (red trace) are shown for the same cell as in panel A.

Fig. 4. Simultaneous measurement of cytosolic calcium and mitochondrial flavoproteins

Freshly cultured hepatocytes (**A**) or hypothalamic neurons (**B**) were loaded with fura-2/AM. Fura-2 and flavoprotein fluorescence images were acquired by sequential excitation at 340 ± 10 , 380 ± 10 and 470 ± 35 nm. Emitted fluorescence was collected with a 515 nm long band pass dichroic and a 520 nm long band pass filter. Calcium concentrations were calculated from the 340/380 nm fura-2 ratio after correcting for autofluorescence (62). Autofluorescence values were obtained at the end of each experiment by permeabilizing the cells with digitonin in an intracellular-like media containing a Ca^{2+} /EGTA buffer and Mg-ATP (62). The fura-2 calibration parameters were determined in vitro using a K_d value of 224 nM. In **A**, hepatocytes cultures were incubated with 5 mM acetoacetate (AcAc) prior to the stimulation with 200 pM

vasopressin. Neurons were loaded with fura-2/AM in the presence of physiologic glucose levels (2.5 mM), and then washed into a buffer containing low glucose (0.5 mM) to active glucose-inhibited neurons (**B**).

Fig. 5. Simultaneous measurement of cytosolic calcium and mitochondrial membrane potential ($\Delta\Psi_m$)

Hepatocyte cultures were loaded with fura-2/AM then transferred to a fresh buffer containing 10 nM TMREE for 40–0 min prior to data acquisition. Hepatocytes were stimulated with 200 pM vasopressin. Fura-2 and TMREE fluorescence images were acquired with 380 ± 10 nm and 548 ± 10 nm excitation, respectively. Emission was collected with a fura-2/rhodamine beamsplitter as described in the text.

Fig. 6. Simultaneous measurement of mitochondrial calcium with targeted cameleon and pyridine nucleotide fluorescence

The confocal image (A) depicts the fluorescence of mitochondrially-targeted cameleon transiently expressed in primary cultured hepatocytes. The traces (B) are the simultaneous measurements of ATP-evoked increases in NAD(P)H (black trace) and the emission ratio of 4mt-D3 cameleon (green trace). Pyridine nucleotides and cameleon fluorescence images were acquired with 360 ± 40 nm and 436 ± 20 nm excitation, respectively. Emission was collected with an image splitter from Optical Insights™ as described in the text.

Wide-field filters sets. A list of filter sets used to measure mitochondrial metabolism, bioenergetics and matrix calcium. The filters in the Optical Insights image splitter are listed in the main text.

Table 1

Fluorophore	Excitation	Dichroic	Emission
NAD(P)H	360 ± 40 nm or 360 ± 10 nm	400 DCLP	420 nm LP
Fura-2	340 ± 10 nm 380 ± 10 nm	400 DCLP	420 nm LP
Flavoproteins	470 ± 35 nm	510 nm LP	520 nm LP
Fura-2+ TMREE	Fura-2 (see above) 548 ± 20 nm	507 ± 54 nm and ≥570 nm	509 ± 30 nm and 600 ± 50 nm
Cameleon	436 ± 20 nm	455 DCLP	Optical Insights™ Image splitter

Confocal filter settings. The settings used to acquire confocal images of cellular pyridine nucleotides, mitochondrial flavoproteins or fura-2 on the Bio-Rad Radiance 2002-MP system. DDS stands for direct detector system and TPE stands for two-photon excitation. When measured together, NAD(P)H and mitochondrial flavoproteins were sequentially imaged.

Table 2

External Detection Fluorophore	Excitation	DDS	Emission
NAD(P)H	710 nm	670 UV DDS	450 ± 80 nm
Fura-2	810 nm	670 UV DDS	420 LP
Internal Detection Fluorophore	Excitation	Dichroic	Emission
NAD(H)P plus	710 nm (TPE)	100% Detector 1	450 ± 80 nm
Flavoproteins	488 nm (Argon)	100% Detector 1	500 LP