Measurement of Mitochondrial pH In Situ

BioTechniques 30:804-815 (April 2001)

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ABSTRACT

In this article, we describe the advantages and disadvantages of procedures for monitoring mitochondrial pH in situ using optical microscopic techniques. The first method employs the combination of the fluorescent pH-sensitive indicator carboxy-SNARF and laser scanning confocal microscopy. Manipulation of the loading and post-loading conditions enables relatively specific accumulation of carboxy-SNARF into mitochondria. With the use of a mitochondrial-specific marker, mitochondrial pH can be accurately monitored. More recently, mitochondria-targeted, pH-sensitive probes have been used to monitor mitochondrial pH. In particular, mitochondrial targeting of the yellow fluorescent protein (YFP) mutant of green fluorescent protein (GFP) combines the advantages of specific mitochondrial localization, high-fluorophore quantum yield, and extinction coefficient with an appropriate pKa for measuring mitochondrial pH. The use of dual-excitation ratiometry with mitochondrially targeted YFP increases the dynamic range of mitochondrial pH measurements and corrects for differences in the amount of expression of mitochondrially targeted YFP at the level of individual mitochondria.

INTRODUCTION

Mitochondria play a pivotal role in both the life and death of cells. On the one hand, they serve as the primary source of aerobic energy production in cells. The pH gradient (ΔpH_m) across the inner mitochondrial membrane (alkaline inside) and the inner mitochondrial membrane potential ($\Delta\phi_m$) make up the electrochemical gradient, which regulates the efficiency of ATP synthesis and other mitochondrial activity [e.g., $\text{Na}^+/\text{Ca}^{2+}$ exchange, K^+/H^+ antiport, transhydrogenation, etc. (35, 42)]. Alteration in mitochondrial pH has also been hypothesized to modulate mitochondrial apoptosis (programmed cell death). Cytochrome c is known to be released from mitochondria into the cytosol by several apoptotic stimuli, resulting in the activation of caspases that cause cell death via apoptosis (15,26, 39,41,90). The release of cytochrome c from mitochondria has been hypothesized to occur following the functional uncoupling of the mitochondria caused by the formation of a nonspecific pore in the inner mitochondrial membrane, termed the mitochondrial permeability pore (MPP) (5,29,32,46,47,62,85,89, 91). The opening of the MPP has been hypothesized to be regulated by mitochondrial pH. Accordingly, the measurement of mitochondrial matrix pH (pH_m) is essential for understanding the regulation of normal and pathological mitochondrial function.

One of the most popular methods for the analysis of pH_m has been to use isolated mitochondria (3,6,16,30,37,56, 65,74). This approach has contributed substantially to our understanding of the role of pH_m in mitochondrial function. However, this method requires isolated mitochondria; thus, it is difficult to observe and quantitate spatial and temporal alterations in pH_m as they relate to

mitochondrial and cellular physiology at the individual mitochondrial level in situ (33). In addition, it has been reported that experimental results obtained using isolated mitochondria can differ from results obtained in situ (7,43).

The distribution of non-metabolizable lipophilic cations and weak acids or bases has been used for the determination of the mitochondrial transmembrane potential ($\Delta\phi_m$) and ΔpH_m in situ (19,36,77). The weak acid 5,5'-dimethyloxazolidine-2,4-dione (DMO) distributes across the mitochondrial membrane according to ΔpH_m (1,34). Thus, ΔpH_m can be evaluated by quantitating the radiolabeled DMO in the mitochondrial compartment after separation from other cellular components. Unfortunately, because DMO requires at least 15 min to equilibrate throughout cells, this method can only be used to monitor steady-state changes in ΔpH_m (36,77). This approach also requires the isolation of mitochondria; thus, it is not possible to monitor pH_m in situ.

The most popular method for monitoring subcellular ion concentrations in situ in intact cells or tissues has been optical microscopy. Historically, Ca^{2+} was the first ion to be measured in cells with this approach, and such measurements allowed both spatial and temporal determination of cellular Ca^{2+} to be quantitated under a variety of normal and abnormal conditions (21). Over the past decade, refinements in these approaches have targeted measurements of Ca^{2+} dynamics to specific organelles including the nucleus, mitochondria, and endoplasmic reticulum—various techniques to monitor free Ca^{2+} concentration ($[\text{Ca}^{2+}]$) in these specific compartments in situ have been established. For measurements of mitochondrial $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_m$) specifically, investigators have used: (i) Rhod-2 or X-rhod-1, which accumulates into the

mitochondria as a function of $\Delta\phi_m$ (51,81); (ii) preferentially quenching Ca^{2+} -sensitive cytosolic (but not mitochondrial) reporter fluorescence with metal ions such as Mn^{2+} or Co^{2+} (53); (iii) co-staining cells with both a Ca^{2+} indicator and a mitochondrial marker to demonstrate which portion of the emitted Ca^{2+} -sensitive fluorescence is localized to mitochondria (11,63); (iv) loading Ca^{2+} indicators preferentially into mitochondria by manipulating the loading conditions [e.g., temperature, pre-loading and post-loading incubation times, and dye concentration (72,76)]; (v) targeting Ca^{2+} -sensitive reporter molecules such as the Ca^{2+} -sensitive photoprotein aequorin to mitochondria (66,67,69); and most recently, (vi) targeting Ca^{2+} -sensitive mutant GFP fluorescence resonance energy transfer (FRET) fusion protein constructs to mitochondria (54,55,61,73). Two such constructs have been produced: cameleons, which have been difficult to target specifically to mitochondria (they often mistarget to the cytoplasm), and Camgaros, which appear to target much more specifically to the mitochondria and to provide accurate assessment of mitochondrial Ca^{2+} .

While numerous approaches to the measurement of $[\text{Ca}^{2+}]_m$ have been developed, the technology for the measurement of mitochondrial pH has not progressed as rapidly. The approaches developed for the measurement of $[\text{Ca}^{2+}]_m$ can be applied to measurements of pH_m . Here, we describe some of the newer optical imaging-based approaches available to measure pH_m .

Measurement of Mitochondrial pH Using Laser Scanning Confocal or Multiphoton Microscopy

Fluorescein has been one of the most popular indicators for the measurement of pH in situ because its emission is pH-sensitive. Initially employed with widefield microscopes, fluorescein has more recently been used with confocal scanning laser microscopy (CLSM) because of the higher spatial resolution offered by this approach (70). Cells are normally loaded with a membrane-permeable form of fluorescein. Following loading, cells are incubated under conditions that allow the

membrane-permeable form of fluorescein to enter intracellular compartments (i.e., mitochondria). Subsequent hydrolysis of the fluorescein derivative results in the formation of a negatively charged fluorescein molecule, which is then trapped in the cytoplasm and any organelle into which it entered before hydrolysis. In many cells, negatively charged, cytosolically localized fluorescein can be pumped out of the cell via an anion transporter, leaving organelle-trapped (i.e., mitochondria) fluorescein behind. Because fluorescein distributes relatively freely in the cell according to the pH gradient (77,78,80), the concentration in the mitochondria is thought to be 80- to 100-fold higher than the concentration in the cytosol when the ΔpH_m is 1.0 (78). While this allows a large signal to be recorded from the mitochondria specifically, accurate measurement of pH_m is difficult because both its concentration and fluorescence change as a function of ΔpH_m .

This is one of the reasons why ratio-metric pH indicators have been a welcome addition to the armamentarium for accurate measurement of pH_m . The most popular fluorescent pH-sensitive probes for measuring pH_m in living cells and isolated mitochondria are the ratio-metric indicators such as BCECF and carboxy-SNARF (3,37). The combination of carboxy SNARF and CLSM or multiphoton laser scanning microscopy (MPLSM) has proven particularly useful in the measurement of pH_m (12,18,45). When using CLSM, dual-emission ratiometry is preferred over dual-excitation ratiometry because most CLSMs have at least two (and usually more) photomultiplier tubes and can detect fluorescence emission signals at different wavelengths simultaneously. It is very difficult for most CLSMs to perform dual-excitation ratiometry because they cannot change excitation wavelengths quick enough for physiological measurements.

Carboxy SNARF-1 is a pH-sensitive fluorophore with a pKa of approximately 7.5, which makes it suitable for measuring pH_m (2). The fluorescence-isobestic point of carboxy SNARF-1 is approximately 600 nm, and the ratio of fluorescence emission detected at wavelengths greater than 610 nm relative to that at 580 nm (when excited at

488 nm) undergoes a dramatic change as a function of pH. Carboxy SNARF is one of the most appropriate pH sensors for use with CLSM because it has a broad absorbance spectrum and displays pH-dependent alterations in its emission spectrum. For example, carboxy-SNARF can be excited at 488, 514, or 568 nm, making it suitable for use with CLSM argon, green HeNe, and krypton-argon lasers, respectively. When excited by 488 or 514 nm, carboxy SNARF exhibits an isosbestic point at 610 nm; it also exhibits higher emission intensity when excited at 514 nm compared to 488 nm. When excited at 568 nm, carboxy SNARF-1 demonstrates an isosbestic point at 585 nm and pH-dependent changes in emission intensity at 640 nm.

Successful use of carboxy-SNARF for measuring pH_m requires mitochondrial localization of the fluorophore. Because cells are normally loaded with the membrane-permeable acetoxymethyl (AM) ester form of carboxy SNARF, the ultimate intracellular distribution of the indicator is dependent on the activity of cytosolic and organelle esterases relative to the rate of uptake of the AM form of the dye into the cytosol and organelles (72).

The use of carboxy-SNARF has its drawbacks. One potential problem is if the pH of the mitochondria and the surrounding cytosol are similar, it may be difficult to distinguish mitochondria from cytosol, based on pH-dependent differences in the emission ratio image. As demonstrated in the studies of Chacon et al. (12), normal mitochondria can be easily distinguished from the surrounding cytosol. However, if pH_m decreases significantly, then the difference between the pH of the cytosol and the mitochondria becomes much smaller, making it more difficult to distinguish mitochondria from cytosol and accurately measure the pH_m . An additional concern is that mitochondria are motile organelles in cells, constantly moving and changing shape under physiological conditions (4). For both of these reasons, either information pertaining to mitochondrial localization must be obtained at the same time as pH-sensitive ratiometry is performed or loading of carboxy SNARF-1 must be mitochondria-specific.

To address the first of these concerns, it is possible to stain the mitochondria with a mitochondrial-specific fluorescent probe whose spectral properties are distinct from those of carboxy SNARF (11). Since the major emission of carboxy-SNARF occurs in the red wavelength, it is possible to use a green wavelength emitting mitochondrial-specific dye, such as DiOC₆(3), or MitoTracker Green FM™ (Molecular Probes, Eugene, OR, USA), as a specific marker of mitochondria. DiOC₆(3) is one of the most popular indicators for the measurement of $\Delta\phi_m$, whereas MitoTracker Green FM stains mitochondria independent of $\Delta\phi_m$ (27,83). Carboxy SNARF-1 and either DiOC₆(3) or MitoTracker Green FM can be excited with the 488-nm line of an argon laser or a multiphoton-excitation laser, allowing simultaneous acquisition of images for the morphological identification of mitochondria and the determination of $\Delta p H_m$ (Figure 1). To estimate mitochondrial pH, the image obtained at 560–600 nm (Figure 1B) would be divided (ratioed) by the image obtained at greater than 610 nm (Figure 1C). Pixels representing mitochondria would then be determined by overlaying the resultant ratio image with the DiOC₆(3) image and selecting those pixels in the ratio image that contained intensity in DiOC₆(3) image. Because the carboxy-SNARF is also local-

ized to the cytosol, an added benefit of this approach is that in addition to being able to measure mitochondrial pH and $\Delta\phi_m$, simultaneous measurement of cytosolic and mitochondrial pH is also possible. One caveat is that if MitoTracker dyes are used to identify mitochondria, these probes can be toxic to cells, so care is required in their usage.

Mitochondrial-specific loading of carboxy SNARF-1 takes advantage of the fact that many cells contain anion transporters in their plasma membrane. These plasma membrane proteins can transport negatively charged, cytosolically localized fluorescent dyes out of cells (81). Post-incubation of carboxy-SNARF (and cells loaded with other fluorescent probes) at 37°C or room temperature for a number of hours results in the loss of cytosolically localized fluorescent dyes but retains mitochondrial (and other organelle) accumulated probes intact (Figure 2).

Measurement of Mitochondrial pH Using GFP

GFP, isolated from the jellyfish *Aequorea victoria*, has been used extensively as a fluorescent label in molecular and cell biology. Over the past five years, numerous applications of GFP have been described in several reviews (23,25,28,44,52,64,82). The most im-

portant feature of GFP is that it can be expressed as a targetable fusion protein in cells, tissues, and whole animals, thereby providing an endogenous marker of the location and environmental context of specific proteins and components in intact living cells. Mutation of wild-type GFP has resulted in the development of spectrally distinct mutants of GFP that have further extended this molecule's usefulness.

It has been known for some time that the absorption and emission of various GFP mutants are pH-sensitive, although to differing degrees (86–88). The emission intensity of most GFP mutants decreases with lowered pH, while some GFPs, including the wild-type (88), have two major absorption peaks that demonstrate opposite pH-dependent effects upon the absorption of a photon. The pH sensitivity of the GFP emission is thought to be due to pH-dependent effects on the absorption process rather than any alternation in quantum yield (40). The pH-dependent change in the absorption/emission characteristics of GFP is caused by protonation of the chromophore portion of the protein (24,57), which occurs extremely rapidly and is reversible in the pH range between 5 and 12 (14,40,59,86, 87).

Recently, there have been reports that take advantage of the pH sensitivity of the different GFP variants to mon-

Figure 1. Multiphoton imaging of BHK cells co-stained with DiOC₆(3) and carboxy SNARF-1/AM. The excitation wavelength of 850 nm was provided by a Coherent 5 W Verdi pump laser used to excite a fs Ti:Sapphire laser (Coherent, Santa Clara, CA, USA). The fluorescence emitted was collected through appropriate dichroic mirrors and filters. (A) Fluorescent image detected via 505–525-nm bandpass filter for DiOC₆(3). (B) Fluorescent image detected via 560–600-nm bandpass filter for carboxy SNARF-1. (C) Fluorescent image detected via 610-nm longpass filter for carboxy SNARF-1.

itor local pH in mitochondria, the Golgi, and the secretory pathway (40,49,50). This approach may be the most ideal method for measuring organelle pH because pH-dependent GFP fusion proteins can be targeted to specific intracellular organelles without affecting organelle function. However, it is important with any fluorescent pH probe to consider the pKa of the particular GFP mutant being used, as it should be optimally matched to the pH to be monitored in the organelle. For example, the pKa of enhanced green fluorescent protein (EGFP) has been reported to be approximately 6.0 (Figure 3) (24,31,40,49). This makes this particular GFP variant a good monitor of the slightly acidic pH found in the

Golgi and secretory pathways (50) but precludes its use as a monitor of pH_m , which is much more alkaline. Cyan fluorescent protein (CFP) emission is reported to be relatively less pH-sensitive, while yellow fluorescent protein (YFP) displays more pH sensitivity than wild-type GFP (24,49). Moreover, the pKa of YFP has been reported to be approximately 7.0 (compared to approximately 6.0 for EGFP), making YFP potentially more useful for measuring pH_m (Figure 3A) (24,49,84).

While the fluorescent intensity of the GFP variants is pH-sensitive, the shape of the GFP fluorescent spectra is relatively pH-insensitive. This would appear to preclude the performance of the emission ratiometric measurements

similar to those previously described for carboxy-SNARF and other pH-sensitive fluorophores. To address this concern, Llopis et al. (49) have applied emission ratiometry to measure pH in organelles using a combination of YFP and CFP. However, during the course of our studies, we observed that, when excited at 380 nm, enhanced yellow fluorescent protein (EYFP) emission, while lower than that which occurs following excitation at 500 nm, still occurs in a pH-dependent manner (Figure 3B) (24,49). In addition, the pH sensitivity of the emission of YFP when excited at 380 nm is opposite to that when excited at 500 nm (Figure 3B), suggesting that the pH sensitivity of the YFP-emitted fluorescence when excited at 380 nm is mainly caused by the effects of pH on the photon absorption process (40).

Because of its appropriate pKa and its ability to perform excitation ratiometry, we explored the use of EYFP for precise measurement of pH_m (Figure 4, A and B). Resting pH_m was determined, and then the effect of the mitochondrial uncoupler, carbonyl cyanide m-chlorophenylhydrazone (CCCP), on pH_m was assessed. CCCP caused a rapid acidification of pH_m (Figure 4C). All of the pH data reported here were performed with either cytosolic or mitochondrial matrix-targeted (cytochrome c oxidase subunit VIII) YFP, and the resultant image data was calibrated using a high-potassium calibration buffer containing 10 μ M nigericin (13,79). We undertook this calibration *in vivo*, as previous studies have shown differences between *in vivo* and *in vitro* calibration of GFP pH indicators (71). This difference has been hypothesized to be due to the dimerization of GFPs, which can occur at high GFP protein concentrations (58).

The most significant drawback to using GFPs as indicators for the measurement of pH_m is their small dynamic range at alkaline pH values. It has been reported that the sensitivity of recombinantly purified YFP and/or YFP expressed in cells is limited at a pH greater than 8.0, relative to its pH sensitivity at neutral or slightly acidic pHs (49). One of the advantages of the excitation ratiometry approach we describe here is a larger dynamic range over which pH measurements can be per-

Figure 2. Selective loading of carboxy SNARF-1 into mitochondria. BHK cells were loaded with 10 μ M carboxy SNARF-1/AM for 10 min, followed by incubation for 4 h at room temperature. (A) Confocal image (488-nm excitation) of mitochondrial-specific loading of carboxy SNARF-1 visualized through a 560–600-nm bandpass filter. (B) Confocal image of the same cells as in A, but using a 605-nm dichroic mirror and a 610-nm longpass filter. (C) Ratio image (A and B) of mitochondria in cell pseudo-colored to represent different pH levels. (D) Change in mitochondrial pH following the addition of 10 μ M CCCP. Note that CCCP causes a decrease (acidification) of mitochondrial pH.

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formed. Since ΔpH_m is thought to be close to 8.0, the greater pH sensitivity at pHs over 8.0 provided by this excitation ratiometry approach allows accurate monitoring of mitochondrial matrix pH.

Another advantage of the ratiometric approach to ion measurement is that it can correct for differences in path length, accessible volume, and photobleaching, and thus correct for differences in YFP expression/concentration at the level of a single mitochondria (Figure 5). Ratiometry has been recognized as a useful method for correcting for differences in the concentration, pathlength, and accessible volume of several ion sensors (8,20,22,48,75). Although it may be desirable to use cell lines stably expressing the protein of interest, their production can be time consuming and difficult; if primary cell cultures are being used in an experiment, then the generation of stable cell lines is not possible. Transient transfection can be used for such studies, but the level of YFP expression in each cell or even in individual mitochondria

Figure 3. pH sensitivity of GFP variants determined using CLSM. (A) Cells expressing cytosolically localized GFP variants or loaded with carboxy SNARF-1/AM were incubated in buffer containing 10 μ M nigericin at various pHs. The normalized fluorescent intensity (the ratio of the measured fluorescence at pH 8.4 to that at 6.0) for (+) carboxy SNARF-1, 488-nm excitation; (●) EGFP, 488-nm excitation; (▲) EYFP, 514-nm excitation; (■) DsRed, 568-nm excitation as a function of pH is shown. Note that the dynamic range in terms of the pH sensitivity of the GFP mutants is not as great at alkaline pHs relative to that of carboxy SNARF-1. (B) Dual-excitation ratiometry with EYFP using widefield microscopy. pH calibration curves were obtained in cells cytosolically expressing EYFP incubated in buffer containing 10 μ M nigericin. Excitation wavelengths were 485–505 nm (●) and 370–390 nm (○). The ratio (X) represents the normalized fluorescent intensity when the EYFP is excited at 495 nm relative to that excitation at 380 nm. This ratiometric approach allows quantitation of pH between 6.0 and 8.5.

Figure 4. Measurement of pH_m with mitochondrially targeted EYFP. An image of a BHK cell expressing mitochondrially targeted EYFP. (A) The cell has also been co-stained with MitoTracker Red CM-H₂Xros. (B) Obtained using CLSM (488 and 568 nm; A and B). (C) Effect of 20 μ M CCCP on pH_m .

Figure 5. Correction for differences in expression level of EYFP in individual mitochondria by ratiometry. Mitochondria expressing different amounts of EYFP were incubated in calibration buffer containing 10 μ M nigericin. (A) Actual fluorescent intensities obtained with excitation at 495 nm. (B) Ratio of the fluorescent intensity excited at 495 nm to that at 380 nm in the same mitochondria as shown in A. Note that ratiometry can correct for differences in the level of YFP expression between individual mitochondria in a single cell.

within a single cell may not be the same. Moreover, the level of the expressed fusion protein changes as a function of time after transfection. High transfection efficiencies can be obtained using viral vectors (i.e., adenovirus) and may be very suitable when primary cultures of cells need to be employed, yet they are still subject to the same limitations as other transfection protocols. Ratiometry can correct for these potential problems and allow for in vivo calibration in the same cell as the experimental measurement is performed. Llopis et al. (49) demonstrated that emission ratiometry with Golgi-targeted EYFP and enhanced cyan fluorescent protein (ECFP) could correct for cell movement and focusing artifacts, but also reported that the ratio of EYFP to ECFP emission varied between cells because of differences in the level of expression of the two proteins (49). Dual-excitation ratiometry with YFP alone obviates this concern (Figure 5) and enables not only accurate estimation of pH_m in a single cell but also comparison of pH_m in several cells. Moreover, during normal or abnormal physiological functioning, mitochondrial shape, size, and volume can change drastically (10,38,60). In such

cases, even if the total concentration of YFP were kept constant, the relative concentration measured in the mitochondria would appear to change, resulting in misleading pH_m measurements. Ratiometry should be useful in such situations because it can correct for many of these potential artifacts.

While the use of YFP excitation ratiometry has many benefits for the measurement of pH_m , it does require transfection of cells with DNA containing mitochondrially targeted YFP DNA. Although numerous procedures for transfection have been established (e.g., lipofection, electroporation, and calcium phosphate co-precipitation), some cell lines are very sensitive to transfection, while other cell lines are resistant to transfection. Another potential problem of this approach is that the transfection process itself might alter cellular and mitochondrial function. Overexpression of various proteins (including GFPs) can be toxic to cells. Nevertheless, YFP excitation ratiometry is a valid approach for monitoring pH in not only the mitochondrial matrix but also other organelles (68). Numerous targeting sequences have been reported for use with GFP variants and other photoproteins (9,17), which should provide

an opportunity to monitor pH in a variety of different subcellular components. Moreover, because GFP fusion proteins often (although not always) maintain their functional status, (92) changes in the pH of the local environment of the GFP fusion protein can be evaluated during real-time physiological functioning of the protein.

Numerous variants of the GFPs with different spectra and pH sensitivities have already been produced, and it is expected that newly produced GFP variants will offer improvement to uses currently available and new ones in the very near future. For example, a new variant of GFP, which has a stronger emission when excited in the UV wavelength range than current GFP variants, has recently been reported (24). This particular modification is expected to decrease the cytotoxicity and autofluorescence of current UV-excited GFP variants, thereby increasing the accuracy and convenience of the ratiometry approach.

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