Chapter 16

Imaging of Mitochondrial pH Using SNARF-1

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Abstract

Laser scanning confocal microscopy provides the ability to image submicron sections in living cells and tissues. In conjunction with pH-indicating fluorescent probes, confocal microscopy can be used to visualize the distribution of pH inside living cells. Here, we describe a confocal microscopic technique to image intracellular pH in living cells using SNARF-1, a ratiometric pH-indicating fluorescent probe. SNARF-1 is ester-loaded into the cytosol and mitochondria of adult cardiac myocytes. Using 568-nm excitation, emitted fluorescence longer and shorter than 595-nm are imaged and then ratioed after background subtraction. Ratio values for each pixel are converted to values of pH using a standard curve (lookup table). Images of the intracellular distribution of pH show cytosolic and nuclear areas to have a pH of ~ 7.1 , but in regions corresponding to mitochondria, pH is 8.0, giving a mitochondrial ΔpH of 0.9. During hypoxia, mitochondrial pH decreases to cytosolic values, signifying the collapse of ΔpH . These results illustrate the ability of laser scanning confocal microscopy to image the intracellular distribution of pH in living cells and to determine mitochondrial ΔpH .

Key words: Confocal microscopy, Cytosol, Mitochondria, Myocytes, pH, SNARF-1

1. Introduction

ATP is the source of energy for most biological reactions with mitochondria being the principal ATP generator in aerobic tissues like heart, brain, liver, and kidney. To synthesize ATP from ADP and phosphate via the mitochondrial F_1F_0 -ATP synthase, mitochondria must generate a protonmotive force (Δp) across the inner membrane. Δp in millivolts equals $\Delta\Psi - 60\Delta\text{pH}$, where $\Delta\Psi$ is the mitochondrial membrane potential (negative inside) and ΔpH is the mitochondrial pH gradient (alkaline inside) (1). Δp also supports other energy-requiring reactions, such as ion transport and the NAD(P) transhydrogenase reaction. The $\Delta\Psi$ component of Δp can

be visualized by confocal microscopy by any of several membrane permeant cationic fluorophores, such as rhodamine 123 and tetramethylrhodamine methylester that accumulate electrophoretically into polarized mitochondrial (2).

Several techniques have been developed to measure average mitochondrial ΔpH in isolated mitochondria, cell suspensions, and cell cultures (1). However, the magnitude of ΔpH of individual mitochondria in single cells has been much more difficult to assess, since mitochondria are too small to measure ΔpH using microelectrodes. Here, we illustrate the use of laser scanning confocal microscopy to visualize pH of individual mitochondria in living cardiac myocytes under normal and hypoxic conditions by ratio-metric imaging of SNARF-1 (3).

2. Materials

2.1. Buffers

1. Buffer A (5 mM KCl, 110 mM NaCl, 1.2 mM NaH_2PO_4 , 28 mM NaHCO_3 , 30 mM glucose, 20 mM butanedione monoxime, 0.05 U/ml insulin, 250 μM adenosine, 1 mM creatine, 1 mM carnitine, 1 mM octanoic acid, 1 mM taurine, 10 U/ml penicillin, 10 $\mu\text{g}/\text{ml}$ streptomycin, and 25 mM HEPES, pH 7.30) (see Note 1).
2. Buffer B (Joklik's medium and medium 199 (1:1 mixture) supplemented with 20 mM butanedione monoxime, 1 mM creatine, 1 mM taurine, 1 mM octanoic acid, 1 mM carnitine, 0.05 U/ml insulin, 10 U/ml penicillin, and 10 $\mu\text{g}/\text{ml}$ streptomycin).
3. Culture medium (Eagle's minimum essential medium supplemented with 5% newborn-calf serum, 0.5 U/ml penicillin G potassium salt, 0.05 mg/ml streptomycin sulfate, and 0.5 $\mu\text{g}/\text{ml}$ amphotericin B).
4. Krebs–Ringer–HEPES buffer (KRH) (110 mM NaCl, 5 mM KCl, 1.25 mM CaCl_2 , 1.0 mM Mg_2SO_4 , 0.5 mM Na_2HPO_4 , 0.5 mM KH_2PO_4 , and 20 mM HEPES, pH 7.4).

3. Methods

3.1. Preparation of Cardiac Myocytes

Adult rabbit cardiac myocytes are isolated by enzymatic digestion, as described in ref. 3, and plated at a density of 15,000/cm² on #1.5 glass coverslips coated with laminin (10 $\mu\text{g}/\text{cm}^2$). Experiments are conducted 1 day after initial plating. Cell lines or other primary cells (e.g., hepatocytes) may be substituted for myocytes.

3.2. Loading of SNARF-1

Intracellular pH is estimated with SNARF-1, a pH-sensitive fluorophore with a pK_a of about 7.5. To load SNARF-1, cultured myocytes are incubated with 5 μ M SNARF-1 acetoxymethyl ester (SNARF-1AM) for 45 min in culture medium at 37°C. During incubation, intracellular esterases release and trap SNARF-1 free acid in the cytoplasm. The cells are washed twice with KRH and placed on the microscope stage in KRH or other physiological medium like Buffer A or B. Unlike other ester-loaded fluorescent indicators, SNARF-1 loads well into mitochondria, although such loading may be cell specific. To promote better mitochondrial uptake, cells can be loaded with SNARF-1AM at a cooler temperature (4–12°C) for a longer time (4, 5).

3.3. Confocal Imaging of pH of Cardiac Myocytes

Confocal imaging of cells is performed using 568-nm excitation of an argon-krypton laser, which is near the absorbance maximum for the dye (3). At this excitation, SNARF fluorescence increases at >620 nm with increasing pH but remains unchanged at 585 nm (Fig. 1). Alternatively, excitation can be performed with the 543-nm line of a helium-neon laser. Emitted fluorescence is divided by a 595-nm long-pass dichroic reflector with the shorter wavelengths directed through a 585-nm (10-nm band pass) barrier filter and longer wavelengths through a 620-nm long-pass filter to separate detectors. Importantly, image oversaturation (pixels at highest gray level) and undersaturation (pixels with a zero gray level) should be kept to a minimum, and laser intensity should be kept at the lowest level possible consistent with an acceptable single-to-noise ratio (S/N). Because images are to be ratioed and background subtracted, S/N ratios higher than required for routine imaging are needed in both image channels. If necessary to improve S/N, binning or median filtering of pixels can be performed whereby each

Fig. 1. Fluorescence emission spectra of SNARF-1. Fluorescence emission spectra of SNARF-1 in KRH buffer at different pH. Excitation wavelength is 568 nm.

Fig. 2. Principle of background subtraction and ratio imaging. See text for details.

pixel is reassigned a value equal to the average of 2×2 or 3×3 groups of pixels or the median value of the pixel and its adjacent pixels. If instrumentation permits, images should be acquired using the multitrack option where each wavelength is acquired alternately on a line-by-line basis.

The intensity of fluorescence acquired at the two wavelengths must be corrected for background (background subtraction) (see Note 2) and then divided on a pixel-by-pixel basis (ratioing) (Fig. 2). The resulting ratios are converted to pH values based on an in situ pH calibration of SNAREF-1 through the microscope optics (see Note 3). An example of measured pH in a myocyte before and after chemical hypoxia (see Note 4) is shown in Fig. 3. pH is estimated at 7.0–7.2 in cytosolic (e.g., subsarcolemmal areas) and nuclear regions and 8.0 in mitochondria, yielding a mitochondrial Δ pH of ~ 0.9 . This gradient decreased to ~ 0.5 and 0 after 30 and 40 min of chemical hypoxia, respectively. After 42 min of hypoxia, the myocyte hypercontracted and died.

Fig. 3. Confocal SNARF-1 ratio images of intracellular pH. A 1-day cultured cardiac myocyte was loaded with SNARF-1-AM (5 μ M) for 45 min in culture medium at 37°C, and intracellular pH was measured by ratio imaging of SNARF-1 fluorescence before (*baseline*) and after 30, 40, and 42 min of chemical hypoxia.

4. Notes

1. All solutions should be prepared in deionized distilled water that has a resistance of 18.2 M Ω .
2. In confocal microscopy, detectors generate signals even in the absence of light. To quantify this dark signal, background images are collected by focusing the objective lens completely within the coverslip just underneath the cells using the same instrument settings as during acquisition of cell images. Average pixel intensity for each color channel of the background images is then determined and subtracted from each pixel of the fluorescence images of the cells at each of the two emission wavelengths. The resulting images are the background-subtracted fluorescence images (see Fig. 2).
3. For in situ calibration, SNARF-1 loaded myocytes are incubated with 5 μ M valinomycin and 10 μ M nigericin in modified KRH buffer in which KCl and NaCl are replaced by their corresponding gluconate salts to minimize swelling (6). Images are then collected as extracellular pH is varied. Instrument settings should be the same. Alternatively, the fluorescence of SNARF-1 free acid (100–200 μ M) in solution can be imaged through the microscope optics as pH is varied. After background subtraction, the >620-nm image channel is divided by the 585-nm channel on a pixel-by-pixel basis. Using thresholding to eliminate low pixel values of the extracellular space, a standard curve is created relating ratio values to pH. Lookup tables are then created assigning specific colors to different values of pH.
4. To simulate the ATP depletion and reductive stress of hypoxia, myocytes are exposed to 2.5 mM NaCN, an inhibitor of

mitochondrial respiration, and 20 mM 2-deoxyglucose, an inhibitor of glycolysis. This treatment is termed as chemical hypoxia (3).

Acknowledgment

This work was supported, in part, by grants 2-R01 DK37034, 1 R01 DK073336, and 1 R01 DK070195 from the National Institutes of Health. Imaging facilities were supported, in part, by NIH Center Grant 1P30 CA138313.

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