pH\textsubscript{i} and pH\textsubscript{o} at different depths in perfused myocardium measured by confocal fluorescence microscopy

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Muller-Borer, Barbara J., Hua Yang, Sayed A. M. Marzouk, John J. Lemasters, and Wayne E. Cascio. pH\textsubscript{i} and pH\textsubscript{o} at different depths in perfused myocardium measured by confocal fluorescence microscopy. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H1937–H1947, 1998.—Confocal microscopy and the H\textsuperscript{+}\textsuperscript{-} sensitive fluorochrome carboxyseminaphthorhodafluor-1 (SNARF-1) were used to measure either intracellular pH (pH\textsubscript{i}) or extracellular pH (pH\textsubscript{o}) in isolated, arterially perfused rabbit papillary muscles. Single-excitation, dual-emission fluorescent images of the endocardial surface and underlying myocardium to a depth of 300 µm were simultaneously recorded from perfused cylindrical muscles suspended in a controlled atmosphere oriented oblique to the focal plane. Contraction was inhibited by the addition of butanedione monoxime. In separate muscles, pH\textsubscript{o} was measured during continuous perfusion of SNARF-1 free acid. pH\textsubscript{i} measurements were made after the muscle was loaded with SNARF-1/AM and the extracellular space was cleared of residual fluorophore. Initial experiments demonstrated the uniformity of ratiometric measurements as a function of pH\textsubscript{i}, image depth, and fluorophore concentration, thereby establishing the potential feasibility of this method for quantitative intramural pH measurements. In subsequent experiments, the method was validated in isolated, arterially perfused rabbit papillary muscle during normal arterial perfusion and as pH\textsubscript{i} and pH\textsubscript{o} were altered by applying CO\textsubscript{2} externally, exchanging HEPES and bicarbonate buffers, and changing pH\textsubscript{i} with NH\textsubscript{4}Cl washout. We conclude that in situ confocal fluorescent microscopy can measure pH\textsubscript{i} and pH\textsubscript{o} changes at the endocardial surface and deeper myocardium at arterially perfused ventricular myocardium. This method has the potential to study pH\textsubscript{i} regulation in perfused myocardium at boundaries where diffusion of gases, metabolites, and peptides are expected to modify processes that regulate pH\textsubscript{i}.

In this study, carboxyseminaphthorhodafluor-1 (SNARF-1), a single-excitation, dual-emission fluorescent pH indicator, was used in conjunction with confocal fluorescence microscopy to measure pH\textsubscript{i} and pH\textsubscript{o} in the superficial layers of an arterially perfused in situ rabbit papillary muscle suspended in a humidified atmosphere. Our studies show that ratiometric imaging of the pH-sensitive fluorophore permits quantitative measurements of pH\textsubscript{i} and pH\textsubscript{o} from the endocardial surface to a depth of ~300 µm. The absence of a significant inner-filter effect and the ability of ratiometric imaging to compensate for differences in fluorophore concentrations and attenuation of emitted light in the deeper cell layers made this technique possible. Although spatial resolution diminished beyond a depth of 100–125 µm and cellular structure could not be resolved below this depth, the mean pH values calculated from multicellular regions of interest remain valid.

In summary, this new method permits the determination of either pH\textsubscript{i} or pH\textsubscript{o} in situ arterially perfused ventricular myocardium at the endocardial surface and deeper endocardial layers. This method has the necessary spatial and temporal resolution to evaluate the effects of mobile, diffusible compounds such as CO\textsubscript{2} or vasoactive peptides on the regulation of pH at the blood-tissue boundaries. In addition, this method has the potential to examine the role of pH in microvascular regulation in the superficial layers of ventricular myocardium, i.e., within 50–100 µm of the surface of perfused in situ cardiac tissue.

MATERIALS AND METHODS

Perfusion of Papillary Muscle and Ventricular Septum

Arterially perfused intraventricular septum and right ventricular papillary muscles of rabbits were prepared in accordance with accepted guidelines for the care and treatment of experimental animals at the University of North Carolina.
School of Medicine. New Zealand White rabbits of either sex, weighing 2–3 kg, were anticoagulated with heparin (200 U/kg iv) and deeply anesthetized with thiopental sodium (30 mg/kg iv). The heart was rapidly excised and immediately immersed for 20 s in cold Tyrode solution (in mM: 149 Na$^+$, 4.5 K$^+$, 0.49 Mg$^{2+}$, 1.8 Ca$^{2+}$, 133 Cl$^-$, 25 HCO$_3^-$, 0.4 HPO$_4^{2-}$, and 2.5 glucose). The isolated heart was placed on a dissection tray, where the atria and left ventricular free wall were removed. The left ventricular surface of the septum was secured to a wax platform, and the septal artery was cannulated, secured with a purse-string suture, and perfused with a modified Tyrode (M-Tyrode) perfusate. This perfusate included the addition of insulin (1 U/l), heparin (400 U/l), albumin (2 g/l), and dextran [average mol wt 70,000; 40 g/l]. In each experiment the elapsed time between cross-clamping of the aorta and perfusion was <6 min.

The muscle and cannula were transported to a custom-made chamber (15) as shown in Fig. 1. The nonperfused portion of the right ventricle was removed, exposing two to three right ventricular papillary muscles. The wax platform with the septal preparation was lowered into the chamber, and one papillary muscle (diameter = 1.3 ± 0.5 mm, n = 16) was suspended in the chamber by securing its tendon to an adjustable pin. With the muscle attached to the pin, a 40–45° angle was formed between the muscle and the chamber base. The chamber was covered to maintain a constant humidified atmosphere around the muscle. A peristaltic pump (DigiStaltic, Masterflex, Barrington, IL) controlled perfusate flow to the chamber. Perfusate temperature was maintained at 36–37°C by passing the perfusate through a heated water bath in the base of the chamber before it reached the muscle. Intra-arterial pressure was monitored continuously with a pressure transducer (Millar, Houston, TX) and recorded on a strip-chart recorder. The intra-arterial pressure was maintained between 30 and 50 mmHg by adjustment of the perfusion flow rate (1.3–1.5 ml·min$^{-1}$·g tissue$^{-1}$). This is a normal perfusion pressure for the small arteries (i.e., diameters = 120–160 µm) of the rabbit heart. In rabbit, 40–50% of the peripheral coronary resistance is located in vessels with diameters >150 µm (6).

Control and Measurement of PO$_2$, PCO$_2$, and pH

During normal perfusion, the PCO$_2$ and PO$_2$ of the atmosphere surrounding the muscle were matched to the PCO$_2$ and PO$_2$ of the perfusate. The volume fractions of O$_2$ and CO$_2$ in the atmosphere of the chamber were frequently sampled —0.5 mm from the muscle surface with an O$_2$/CO$_2$ gas analyzer (Illinois Instruments, Ingleside, IL). The pH, PO$_2$, PCO$_2$, and [HCO$_3^-$] of the perfusate were measured with a blood gas analyzer (Instrumentation Laboratory, Lexington, MA). PO$_2$ and PCO$_2$ in the perfusate were adjusted in a membrane gas exchanger. A schematic drawing of the perfusion system is shown in the lower portion of Fig. 2. The perfusate pH was continuously monitored by a glass pH electrode connected to the perfusion line at the inlet to the chamber. The relative amount of CO$_2$ was adjusted to yield a perfusate pH of 7.40 ± 0.07 during control perfusion (PO$_2$ = 223 ± 35 mmHg, PCO$_2$ = 37 ± 3 mmHg). A major advantage of this method was the ability to precisely control and match the PCO$_2$ of the chamber and perfusate.

SNARF-1 Perfusion for pH$_o$ Measurements

pH$_o$ was measured in the perfused rabbit papillary muscle preparation during several interventions as the muscle was continuously perfused with M-Tyrode perfusate buffered with bicarbonate or HEPES. The constituents of the HEPES perfusate were (in mM) 149 Na$^+$, 4.5 K$^+$, 0.49 Mg$^{2+}$, 1.8 Ca$^{2+}$, 133 Cl$^-$, 0.4 HPO$_4^{2-}$, 10 HEPES, and 20 glucose and insulin (1 U/l), heparin (400 U/l), albumin (2 g/l), and dextran (average mol wt 70,000; 40 g/l). The perfusate contained SNARF-1 free acid (20 µM, Molecular Probes) and butanedione monoxime (BDM; 20 mM, Sigma). BDM was added to inhibit electromechanical coupling and contractions, to prevent motion artifact related to contraction. To validate pH$_o$ measurements acquired from SNARF-1 fluorescence a novel, custom-designed, solid-state electroplated iridium oxide pH electrode (18) was placed on the muscle surface. This pH$_o$ electrode continuously monitored pH$_o$ changes and recorded the pH$_o$ on a strip-chart recorder. Additionally, in several studies, after the muscle was perfused with SNARF-1, a fluorophore-free perfusate was used to eliminate SNARF-1 from the extracellular compartment. Extracellular washout of SNARF-1 was complete within 15 min, i.e.,

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**Fig. 1. Diagram of experimental chamber (A), shown with rabbit right ventricular septum (B) attached to a wax plate (C) and papillary muscle (D) suspended with apical end attached to an adjustable pin (E). Muscle is perfused through septal artery via a polyethylene cannula (F), with perfusion line (G) monitored for pressure and pH. Humidified atmosphere (H) enters chamber, where it is directed to muscle through multiple internal ports on chamber floor. This provides laminar flow around papillary muscle. Chamber atmosphere is monitored by withdrawing atmosphere close to the muscle (l) to an O$_2$/CO$_2$ analyzer. Chamber is maintained at 37°C by circulating heated water (inlets and outlets shown by arrows) through chamber base (A) and chamber top (J). Microscope objective (K) is wrapped with a heating element (L) and fitted with rubber seal (M).**
fluorescent emission intensities were equal to background emissions.

**SNARF-1/AM Loading for pH Measurements**

For pH measurements, muscles were loaded with the acetoxymethyl ester of SNARF-1 (SNARF-1/AM). SNARF-1/AM enters the cells and is converted to SNARF-1 free acid by intracellular esterases. SNARF-1/AM was dissolved in dimethyl sulfoxide at a concentration of 1 mg/ml. This stock solution was diluted in M-Tyrode perfusate for a final concentration of 10 µM. The papillary muscles were perfused with SNARF-1/AM perfusate for 30 min at 37°C. To improve intracellular SNARF-1 loading, the papillary muscles were simultaneously superfused by placing a single papillary muscle inside a piece of longitudinally cut plastic tubing filled with SNARF-1/AM perfusate. The superfusate in the tubing was replenished several times during SNARF-1 loading. In addition, a Grass stimulator (Grass Instruments, Quincy, MA) applied excitatory current pulses (0.5 ms at double threshold) to the apical end of the papillary muscle through an insulated platinum wire. While the muscle preparation spontaneously beat at 0.5–1.0 Hz, preliminary observations revealed improved SNARF-1 loading (i.e., higher fluorescence emission intensities) when the muscles were stimulated at 2 Hz. After SNARF-1 loading, the muscle preparation was perfused with fluorophore-free M-Tyrode containing FCS (10%, Life Technologies), BDM (20 mM), and a normal dextran concentration (40 g/l). The superfusion tubing was removed from the chamber, and electrical stimulation was discontinued. Results from initial studies indicated that the fluorophore was retained within the cells for a longer period of time when FCS was used during SNARF-1/AM washout. Conversely, when FCS and/or BDM was added to the perfusate during SNARF-1 loading very low-emission intensities were recorded, suggesting poor intracellular loading. All intracellular fluorophore concentrations were estimated after each experimental protocol. In 5 µM increments, SNARF-1 was added to HEPES-Tyrode (in mM: 149 Na⁺, 4.5 K⁺, 0.49 Mg²⁺, 1.8 Ca²⁺, 133 Cl⁻, 0.4 HPO₄²⁻, 10 HEPES, and 20 glucose) with the pH adjusted to five values ranging from 6.4 to 7.6 (e.g., 6.4, 6.7, 7.0, 7.3, and 7.6). The SNARF-1 solutions were placed in a single-well chamber slide covered with a coverslip. Emission fluorescence intensities from samples were recorded using identical system settings, objectives, and image acquisition times (i.e., total time to acquire emission image pairs) per experimental protocol. Intracellular fluorophore concentrations were estimated by comparing the magnitude of emission fluorescence intensities acquired in the samples with those acquired in the muscle. The SNARF-1 solutions with concentrations similar to the muscle preparation were subsequently used for pH calibrations (see pH Calibration).

**Confocal Fluorescence Microscopy and Image Analysis**

The chamber was placed on a modified stage of a Nikon microscope (Nikon, Melville, NY) equipped with a K2SBIO disk-scanning confocal attachment. A schematic drawing of the optical elements is shown in the top portion of Fig. 2. The 45-µm-wide curved slits in the scanning disk allowed uniform 10% light transmission and video rate confocal imaging. Light from a mercury-arc lamp (100 W) passed through a 546-nm excitation filter and was focused on the papillary muscle suspended inside the chamber. Emitted SNARF-1 fluorescence passed through a 570-nm long-pass dichroic reflector. Emission image pairs were collected serially through a 620-nm long-pass filter (Image620) and 585 ± 10-nm band-pass filter (Image585). Emission filters were located on a rotating filter wheel in the optical path. Filter position and image acquisition time were software controlled. A cooled charge-coupled device camera (Princeton Instruments, Trenton, NJ) with a 1,317 × 1,035-pixel array (6.8 × 6.8-µm pixel size) collected a 12-bit image at each emission wavelength (i.e., emission intensities ranged from 0 to 4,096 arbitrary units). A schematic of the image acquisition system is shown on the right side of Fig. 2. Before SNARF-1/AM loading, background autofluorescence images of the muscle in the chamber were acquired and stored at each emission wavelength (Image620,bkgd and Image585,bkgd, respectively).
biotically). To determine the SNARF-1 fluorescence ratio ($\text{Image}_{\text{ratio}}$), background autofluorescence was subtracted at each emission wavelength as calculated in Eq. 1.

$$\text{Image}_{\text{ratio}} = \frac{(\text{Image}_{420} - \text{Image}_{420,bkgd})}{(\text{Image}_{585} - \text{Image}_{585,bkgd})}$$

Equation 1

Image pairs were stored and processed on a 486 personal computer (Digital Equipment, Maynard, MA) with Meta-Fluor software (Universal Imaging, West Chester, PA).

SNARF-1 emissions were observed through an Olympus SPLAN10 air objective (0.3 NA). When the objective was lowered through the opening in the chamber cover, a rubber shield attached to the objective helped to contain the atmosphere within the chamber. To prevent condensation from the humidified atmosphere on the objective a flexible Kapton heater (Minco Products, Minneapolis, MN) was wrapped around the objective with the temperature maintained at 40°C. Figure 3 illustrates the muscle alignment used to acquire a ratiometric confocal image, simultaneously capturing the endocardial surface and deeper endocardial layers. To assist in muscle alignment, focus, and angle measurement, a drop of perfusate with a 1% concentration of 1.0-µm-diameter yellow-green fluorescent latex microspheres (excitation and emission wavelength 490 and 515 nm, respectively) was placed on the surface of the muscle. Microspheres that adhered to the surface of the muscle were focused, allowing accurate placement of the microscope stage, alignment of the muscle along its longitudinal axis, and focus at the muscle surface. The angle of the muscle was determined by measuring the depth of focus along the longitudinal axis as shown in Fig. 3. In this orientation using an air objective and no coverslip, all depth-of-focus measurements were scaled by a factor of 0.73 to adjust for the mismatch of refractive indexes (i.e., refractive index of air is 1, refractive index of vertebrate muscle is 1.38) (12).

Intracellular fluorescence emissions were evaluated by selecting two regions of interest (ROI) from within the ratioed image. Each ROI encompassed multiple cells within the confocal slice, providing an average pH measurement. The ROI were centered along the longitudinal axis of the muscle, with one ROI placed near the edge of the image where endocardial surface measurements were recorded and the second ROI placed near the edge of the image where fluorescence from the deeper endocardial layers was recorded. Centering the muscle to acquire images along its longitudinal axis reduced measurement variability produced by the cylindrical muscle geometry.

**pH Calibration**

To calibrate SNARF-1 emissions, an in vitro pH calibration was performed after each experimental protocol. SNARF-1 was added to HEPES-Tyrode with the pH adjusted to five values ranging from 6.4 to 7.6 for pH<sub>i</sub> measurements and from 6.4 to 8.0 for pH<sub>o</sub> measurements. The SNARF-1 concentration ranged from 15 to 30 µM, based on intracellular dye concentration estimates previously described (see SNARF-1/AM Loading for pH Measurements). The calibration solutions were placed in a single-well chamber slide covered with a coverslip. Emission fluorescence pairs were collected 100 µm below the surface of the liquid using identical system settings, objectives, and image pair acquisition times per experimental protocol. Background images were acquired with SNARF-1-free calibration solution. Background corrected ratios were created by division of the individual component fluorescence images as calculated from Eq. 1. Titration curves of the ratio vs. pH were calculated and used to convert tissue fluorescence emission ratios to pH.

**RESULTS**

Validation of Ratiometric Measurement Technique for Determination of pH at Different Depths

Experiments were designed to determine the effect of path length and fluorophore concentration on the acquired fluorescence emissions and resulting ratiometric measurements. These initial studies established conditions necessary to quantitatively measure pH from SNARF-1 fluorescence in papillary muscle.

Measurement of pH in a homogeneous aqueous volume, SNARF-1 fluorescence was measured in homogeneous aqueous solutions, with pH ranging from 6.5 to 7.5, as the fluorophore concentration was decreased from 50 to 25 µM and as the depth of focus was extended from the surface to 200 µm below the surface of the volume. The results, acquired 200 µm below the surface of the liquid, are shown in Fig. 4. In the 50 µM SNARF-1 solution, as the pH of the solution increased from 6.5 to 7.5, emission intensities recorded through the 620-nm long-pass filter increased, whereas the emission intensities recorded through the 585 ± 10-nm band-pass filter decreased. As the concentration of SNARF-1 decreased from 50 to 25 µM, the intensity of the emission fluorescence decreased significantly. However, at both SNARF-1 concentrations, the emission intensity ratios were the same, verifying that the fluorescence emission ratios were proportional to pH and independent of the fluorophore concentration. Fur-
Moreover, the difference in fluorescence emissions collected at 10 and 200 µm below the surface, although not shown in Fig. 4, was <2%.

Measurement of pH in a homogeneous semisolid volume. To simulate fluorophore measurements in thick tissue, SNARF-1 fluorescence was measured in a semiopaque solid. For this measurement, gelatin was dissolved in HEPES-Tyrode at 100°C. After the gelatin cooled to 25°C with a pH of 7.3, SNARF-1 (20 µM) was added to the gelatin, forming a semiopaque solid in which SNARF-1 was homogeneously distributed. The results, shown in Fig. 5, establish that SNARF-1 fluorescence can be measured at depths up to 300 µm without significant inner-filter effects. Furthermore, only a 2% decrease in fluorescence emission intensities occurred as the focal depth of the image plane moved from the surface to 300 µm below the surface. The observation that ratiometric imaging of SNARF-1 fluorescence compensated for differences in light attenuation caused by path length and fluorophore concentration supported the potential use of this method in tissue.

**In Vivo Measurement of pH**

The ability to image SNARF-1 fluorescence to quantitate relative changes in pH$_o$ was assessed by the simultaneous measurement of pH$_o$ with a solid-state electroplated iridium oxide pH electrode (18) placed on the muscle surface. Papillary muscles were continuously perfused with M-Tyrode at a pH of 7.45, containing SNARF-1 (20 µM) and BDM (20 mM). As shown in Fig. 6, during steady-state perfusion with the bicarbonate-buffered M-Tyrode perfusate and a PCO$_2$ of 40 mmHg, pH$_o$ at the muscle surface measured ~7.3 was measured by both fluorescence microscopy and the pH$_o$ electrode. This was slightly more acidic than the perfusate pH, measured by the glass pH electrode at the chamber inlet. A transient decrease in pH$_o$ was initiated by changing the perfusate to a non-bicarbonate-HEPES buffer, with CO$_2$ present. This decreased the pH of the perfusate to 6.7 within 3 min. The pH$_o$ at the muscle surface showed no initial change during the switch in perfusate but gradually decreased to a pH$_o$ of 6.8 and 6.75 as measured by the extracellular iridium oxide pH-sensitive electrode and SNARF-1 fluorescence, respectively. When CO$_2$ was removed from the atmosphere and perfusate the surface pH$_o$ returned to 7.2, slightly lower than the starting pH$_o$, a finding previously reported by Vanheel et al. (26). The difference in pH$_o$ measured by the surface electrode and SNARF-1 fluorescence during the switch to HEPES
and withdrawal of CO₂ is most likely caused by the surface effect of CO₂ withdrawal being sensed more rapidly by the surface electrode. However, at steady state both methods measured the same pHₒ. Figure 6 clearly shows the response of the SNARF-1 fluorescence measurement to changes in pH. The response to the detected change in H⁺ activity by SNARF-1 fluorescence was rapid and preceded the response of the extracellular iridium oxide pH electrode by ~1 min (i.e., response time of the extracellular iridium oxide pH electrode).

To further evaluate the relative changes in pHₒ at the surface and intramural layers, measurements of pHₒ were acquired as perfusate pH was decreased in a stepwise manner. In this series of experiments the papillary muscles were continuously perfused with HEPES perfusate, SNARF-1 (20 µM), and BDM (20 mM) at 100% O₂ saturation. Fluorescence images were recorded at 1-min intervals at the muscle surface and ~250 µm below the surface. Representative results are shown in Fig. 7 as the perfusate pH was decreased from 7.37 to 6.54. Initially, the pHₒ values both at the surface and below the surface of the muscle were more acidic than the perfusate pH. As the perfusate pH decreased, a corresponding decrease in pHₒ was observed. An interesting and consistent finding was a significant pHₒ difference between the muscle surface and deeper intramural layer that remained nearly constant as pHₒ decreased.

**pHₒ Changes Induced by CO₂**

Chamber PCO₂ was used to alter carbonic acid concentration and pHₒ in the superficial layers of the perfused papillary muscle and to assess ratiometric imaging as a means to measure pHₒ changes at the surface and deeper intramyocardial cell layers. Results are shown in Fig. 8. During continuous perfusion at constant PCO₂ and pH (perfusate PCO₂ = 33 mmHg, PO₂ = 256 mmHg, pH = 7.55) confocal images were acquired. The resultant ratiometric images provided pHₒ measurements from the muscle surface to a depth of ~200–260 µm. Initially, the PCO₂ values surrounding the muscle and perfusate were equal and measured pHₒ ranged from 7.40 at the surface to 7.58 in the deepest layer, consistent with the intrinsic pHₒ gradient found in this preparation with the HEPES-buffered perfusate. As the chamber PCO₂ was reduced from 33 to 7 mmHg, a superficial PCO₂ gradient was established (i.e., low PCO₂ on muscle surface compared with deeper layers). Consequently, the surface pHₒ rose more compared with pHₒ changes in the deeper layers and the initial pHₒ difference between the surface and deeper endocardial layers disappeared. When the superficial PCO₂ gradient was reversed by increasing the chamber PCO₂ from 7 to 340 mmHg, the initial pHₒ difference was reestablished and pHₒ decreased to 6.47 at the surface and 6.71 in the deeper layers. Returning the atmosphere PCO₂ to 33 mmHg restored the pHₒ to initial values.

**Measurement of pHᵢ**

After the assessment of SNARF-1 emission fluorescence in aqueous solutions, the validation of the pH calibration technique, and confirmed measurement of pHᵢ during CO₂ manipulation, our goal was to measure pHᵢ in the perfused rabbit papillary muscle preparation during normal perfusion. After SNARF-1 loading and SNARF-1/AM washout, fluorescence emission image pairs were collected at 1-min intervals until the fluorescence emission intensities decreased to the background noise level. The muscles (n = 9) were continuously perfused at a pH of 7.43 the average pHᵢ, recorded 100 µm below the surface, was 7.14 ± 0.01. However, the average fluorescence collected through the emission filters, >620 nm and 585 ± 10 nm, steadily decreased.

![Fig. 7. Change in pHₒ in perfused rabbit papillary muscle at endocardial surface (●) and deeper endocardial layers (200 µm, ○) as perfusate pH was decreased from 7.37 to 6.54. Muscle was perfused with SNARF-1 (20 µM) in HEPES perfusate with 100% O₂ saturation.](http://ajpheart.physiology.org/)

![Fig. 8. pHₒ response at endocardial surface (●) and deeper endocardial layers (250 µm, ○) in a perfused rabbit papillary muscle as PCO₂ of surrounding atmosphere was altered. Muscle was continuously perfused with SNARF-1 (20 µM) in M-Tyrode perfusate at a constant pH of 7.5.](http://ajpheart.physiology.org/)
Fig. 9. Fluorescence emissions collected 100 µm below papillary muscle surface at wavelengths > 620 nm (○) and 585 ± 10 nm (△) and estimated pH, (△). Rabbit papillary muscle was continuously perfused (●, pH = 7.45) over a 60-min period with M-Tyrode perfusate. Note decrease in fluorescence emissions with no significant change in pH.

over this same time period. Like the SNARF-1 ratiometric measurements in an aqueous solution, this study demonstrated that the emission intensity ratios correct for variability in the fluorophore concentration.

Modulation of pH I With NH₄Cl Washout

The recovery of pH I from acid loading induced by external NH₄Cl addition and removal was used to assess changes in myocardial pH I. In this study the papillary muscles (n = 4) were placed in the chamber and perfused with HEPES perfusate (pH = 7.40) saturated with 100% O₂. After 10 min of control perfusion, NH₄Cl (10 µM) was perfused to the muscle for 15 min, followed by 20 min of NH₄Cl-free perfusate. Figure 10 shows the pH I response measured ~100 µm below the surface of the papillary muscle during this intervention. As expected, NH₄Cl perfusion produced a transient alkalosis, with pH I increasing from 7.2 to 7.6 during the first 10 min and then decreasing to 7.4.

Conversely, when NH₄Cl was removed a transient acidosis was measured, decreasing to pH 6.7 before recovering to control levels.

Hypercapnic Acidosis

Analogous to the experiment where chamber P CO₂ was used to monitor pH o changes at the surface and deeper endocardial layers, a P CO₂ pulse was used to assess changes in pH I. Initially, fluorescence was collected from the muscle during control perfusion (n = 6). Hypercapnic acidosis was created by simultaneously increasing the P CO₂ in the perfusate and chamber atmosphere surrounding the muscle while adjusting the N₂ to maintain a constant P O₂. During control perfusion with P CO₂ at 37 ± 3 mmHg, pH I measured 7.17 ± 0.09. When P CO₂ was increased to 170 ± 33 mmHg, pH I decreased, reaching a steady state of 6.84 ± 0.05 within 10 min. Consequently, the perfusate pH decreased from 7.40 ± 0.07 to 6.65 ± 0.07. After acidosis, P CO₂ in the chamber and perfusate was returned to the initial levels and the muscle was allowed to recover. As expected with hypercapnic acidosis the overall decrease in pH I was 0.33 ± 0.03 pH units, ~44% of the 0.75 ± 0.05 decrease in perfusate pH. This change in pH I was within the physiological range of 6.68–6.95 as measured in cardiac myocytes by similar methods (21).

Representative pH I changes during hypercapnic acidosis are shown in Fig. 11. During control perfusion a pH I gradient was observed, with the surface pH I more acidic than the pH I of deeper endocardial layers ~200 µm below the muscle surface. When the P CO₂ in the chamber and perfusate were increased, pH I decreased ~0.3 pH units. Consequently, the pH I gradient between the surface and deeper layers was reversed as the change in pH I in the deeper layers was greater than that at the surface. This observation suggests more
active proton extrusion or buffering in the surface layers of the muscle. On return to control PCO\textsubscript{2}, the pHi returned to normal levels, with pH\textsubscript{i} of the deeper layers increasing more than the surface pH\textsubscript{i}.

**DISCUSSION**

Contemporary methods used to measure pH\textsubscript{i} in tissue include ion-selective microelectrodes and NMR. Although these methods contribute enormously to our understanding of cellular pH regulation, inherent technical restrictions of limited spatial resolution with microelectrodes and decreased temporal resolution with NMR preclude their practical application to the study of cellular pH regulation in the vicinity of boundaries between different ionic and/or metabolic environments or between cells in situ. Ratiometric fluorescent microscopy with the pH-sensitive fluorophore SNARF-1 has successfully measured pH\textsubscript{i} in isolated cardiac cells (3, 5, 21, 24). Confocal microscopy, when combined with ratiometric imaging of SNARF-1 in an arterially perfused papillary muscle, provided a novel means to measure dynamic intracellular and extracellular pH changes from a multicellular preparation in compact ventricular myocardium. The principal findings of this study show that quantitative measures of pH\textsubscript{i} and pH\textsubscript{o} are possible from both the surface and deep endocardial tissue layers in perfused ventricular tissue. The maximal depth at which pH\textsubscript{i} or pH\textsubscript{o} can be measured is limited by the working distance of the objective lens and the penetration of the excitation and emitted light. However, in practical terms the limit for pH measurements, using fluorescent emissions from SNARF-1, is \( \approx 300 \) µm below the muscle surface.

Initial experiments addressed the uniformity of ratiometric measurements as a function of pH, image depth, and fluorophore concentration, thereby establishing the potential feasibility of this method for quantitative intramural pH measurements. The method was validated in an isolated, arterially perfused rabbit papillary muscle during normal arterial perfusion as pH\textsubscript{o} was altered by applying CO\textsubscript{2} externally and by changing the perfuse buffer. Additionally, relative changes in pH\textsubscript{i} were evaluated during NH\textsubscript{4}Cl application and withdrawal and as CO\textsubscript{2} in the atmosphere and perfusate changed.

Confocal Microscopy, SNARF-1 Ratiometric Fluorescence Imaging, and Arterially Perfused Papillary Muscle Preparation

The determination of changes in intramural pH\textsubscript{o} and pH\textsubscript{i} in the perfused rabbit papillary muscle presented several technical challenges. Although confocal microscopy collects thin optical sections from thick specimens, acquisition of images from deep within the tissue required arterial perfusion to uniformly deliver the pH-sensitive fluorophore to the tissue. Additionally, confocal fluorescence microscopy allowed relatively fast image pair acquisition (e.g., ratiometric fluorescent images typically acquired in \(<20\) s). Acquiring focused images of the beating papillary muscle was not feasible; therefore, BDM was added to the perfusate to inhibit electromechanical coupling and abolish contractions. Although BDM reversibly abolishes cardiac contraction, the cardiac myocytes continue to propagate transmembrane action potentials (17, 29). A study by Liu et al. (16) concluded that BDM was acceptable in electrophysiological studies despite a small decrease in conduction velocity and membrane conductances. When the muscle was mechanically quiescent, thin optical sections were obtained with the muscle aligned oblique to the focal plane, providing simultaneous fluorescence emissions from the endocardial surface and deeper endocardial layers. This technique provided increased temporal resolution compared with NMR and a spatial resolution suitable for multicellular tissue preparations (i.e., less than whole heart resolution but greater than single-cell resolution).

Ratiometric Imaging of SNARF-1 Fluorescence

In comparison to other fluorophores available for the measurement of pH, SNARF-1 has several advantages. In the arterially perfused papillary muscle preparation, the acetoxyethyl ester of SNARF-1 distributes homogeneously and is readily taken up by rabbit myocardium. Cytosolic esterases convert the membrane-permeable SNARF-1/AM to SNARF-1, which is a useful indicator for measuring pH changes ranging from 6.3 to 8.6 (11). In addition, the spectral characteristics of SNARF-1 permit ratiometric imaging from deep within the muscle, i.e., SNARF-1 is excited and emits fluorescence at longer wavelengths compared with other commonly used fluorophores like 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein. This property of SNARF-1 fluorescence is advantageous in this application because the long wavelength of the excitation and emission spectrum enhances the excitation and transmission of emitted fluorescence from the subendocardial cell layers. The longer wavelengths are attenuated less than shorter-wavelength light, and the lower excitation energy wavelengths reduce the potential for photobleaching and photodamage. Finally, the use of a single-excitation, dual-emission fluorophore versus a dual-excitation, single-emission fluorophore eliminated effects of chromatic aberrations on the ratio of the emitted fluorescence.

A theoretical concern associated with the collection of emitted fluorescence from the deeper tissue layers is the loss of emitted light caused by absorption and scatter, which may severely restrict quantitative analysis of the image, i.e., the inner-filter effect. As shown in Figs. 4 and 9, emission wavelengths were attenuated as the concentration of the fluorophore decreased. Additionally, the measured intensity of the emitted light, from SNARF-1 homogeneously distributed in a semiopaque gel, decreased as the image depth increased (see Fig. 5). However, emission ratios of the fluorophore were independent of the concentration and focal plane depth, correcting for any inner-filter effects. Fluorescence ratios measured as a function of fluorophore concentration and path length resulted in a \(<2\%\) difference.
between the ratios. This is in agreement with previous assessments of SNARF-1 as a pH indicator (2, 23).

Loss of the intensity of the fluorescent emissions over time is observed in isolated cells (2, 4, 28) and in our arterially perfused tissue preparations. Not unexpectedly, loss of the intensity of SNARF-1 fluorescence over time was a significant limitation. A primary cause of the decrease in fluorescence intensity is related to the leakage of SNARF-1 from the cell. This was determined by the lack of any visible fluorophore in the muscle preparation (i.e., SNARF-1-loaded muscles were very pink) and low fluorescence emissions from multiple sites on the perfused rabbit papillary muscle 60–90 min after SNARF-1 loading. Although photobleaching contributes to some loss in emission intensity, Bassnnett et. al. (2) showed that at a pH < 7.0 the emission ratios of SNARF-1 appear to be independent of bleaching, whereas the rate of photobleaching increases with pH > 7.3. The pH, of the perfused rabbit papillary muscle under control conditions ranged from 7.04 to 7.23. Given the rate of loss of SNARF-1 from the intracellular space, fluorescence imaging was only practical for ~60–90 min after the cells were loaded. Studies show that probenecid, an anion-transport blocker, reduces fluorophore loss when added to a perfusing solution (1, 7). When probenecid (0.1 mM) was used in this preparation there was no measurable effect on the rate at which fluorescence intensity emissions decreased; however, the application of this technique requires further study.

With the loss of SNARF-1 from the cells, an obvious concern is the contribution of extracellular fluorescence to the pH measurement. Although we did not measure the concentration of extracellular SNARF-1 caused by cell loss, it is unlikely that cellular loss of SNARF-1 and accumulation into the extracellular space made any significant contribution to the fluorescence measurement. All muscles were continuously perfused with a fluorophore-free solution (i.e., a perfusion flow rate of 1.3–1.5 ml·min⁻¹·g tissue⁻¹), which served to constantly dilute any fluorophore leaking into the extracellular space.

Validation of pHᵢ Calibration Technique

In isolated cardiac myocytes calibration of the intracellular fluorescence emission ratio and its relationship to pH is accomplished by incubating the preparation with a variety of ionophores (monensin, nigericin, etc.) and equilibrating pHᵢ and pH₀ by perfusing with buffers of known pH (25). Studies show the intracellular calibration method to be superior to the in vitro method (2, 3, 20, 23), because the spectral characteristics of the dye are measured in the same environment during both the experiment and the calibration. Review of the literature suggests that because of the heterogeneity of the intracellular space and intracellular redistribution of SNARF-1 (5, 19, 20, 23), pHᵢ measurements with SNARF-1 may involve significant errors when an in vitro method is used to calibrate pHᵢ. These are important considerations when obtaining subcellular pHᵢ measures. Initially, an in vivo calibration was attempted in the arterially perfused papillary muscle. However, this approach caused an abrupt and irreversible increase in arterial vascular resistance. In addition, visible heterogeneity of perfusion, heterogeneous distribution of the ionophore, and pH calibration solution proved this technique to be unreliable and impractical in this muscle preparation. Consequently, an in vitro calibration was used to establish a ratio scale. The major focus of this study was to evaluate relative changes in pHᵢ from multicellular regions of the tissue rather than subcellular pHᵢ. Hence, the in vitro calibration and resulting ratio scale proved to be an adequate method to monitor relative pH changes. A HEPES-buffered Tyrode solution was used for the pH calibration standards. We choose this simplified approach rather than try to duplicate the cytosolic constituents of the different cell types in the papillary muscle. Previous studies with single cells indicates that individual cells often have unique calibration curves, and these differences may be caused by the cytosolic constituents (3, 19, 23). It is possible that our calibration technique overestimated pHᵢ. Nevertheless, using the in vitro calibration, when the muscle was perfused with M-Tyrode the average resting pHᵢ measured 7.13 ± 0.03, which is consistent with values obtained with intracellular microelectrodes in the same muscle preparation (i.e., pHᵢ of 7.03 ± 0.03; Ref. 30), thus validating this calibration technique. The relative changes in pHᵢ and pH₀ are reliable, whereas the absolute values may be limited by the technical limitations noted. It should be noted that if absolute pHᵢ is of prime importance an in vivo calibration should be used or the pHᵢ verified with microelectrodes as was shown with the pH₀ measurements.

Endocardial-to-Subendocardial pH Gradient

An initial finding during the development of the method was that pH₀ and pHᵢ of the superficial endocardial cells were consistently more acidic than the pH₀ and pHᵢ measured from cells ~200–300 µm below the surface. Although we can speculate why these differences exist, they are not likely to be related to a measurement artifact or an experimental artifact. The pH gradient was present in all experiments during steady state and was measured after the muscle preparations had reached equilibrium (i.e., 1 h after cannulation) and before any interventions. Indeed, the consistent measurement of pHᵢ by two separate methods was shown as the perfusate was switched from a bicarbonate perfusate to a HEPES-buffered perfusate. Second, in a HEPES-buffered perfusate the pH₀ of the muscle decreased coincident with a decrease in perfusate pH, yet the pHᵢ gradient remained fairly constant. Finally, with a bicarbonate-buffered perfusate the pHᵢ gradient could be altered by externally decreasing and increasing CO₂. Subsequently, the narrowing of pHᵢ gradient, and in some cases the reversal of the pH gradient, during the application of CO₂ suggests that the local differences in pH regulation can be detected at the endocardium and deeper endocardial layers. In this case the greater effect of CO₂ in the deeper layers...
indicates that the buffering capacity or the ability of the subendocardial cardiac myocytes to extrude protons is less than that of the superficial cells. Electron microscopy of the top 50 µm of the papillary muscle dissected from the right ventricle shows the surface of the papillary muscle to be covered with a layer of endothelial cells with underlying connective tissue measuring 20 ± 10 µm in depth (data not shown). The observed pH differences may relate to this structural difference and the different characteristics and properties of the endocardial endothelial cells compared with ventricular myocardium. It is possible that in vivo the endocardial pH has a different set point compared with the underlying ventricular myocardium. These differences may be related to local effects of endogenous vasodepressor peptide mediators or endothelium-derived relaxing factor or to differences in intrinsic buffering capacity. The absence of a potentiation of the gradient between the pH at the surface and that at deeper layers by externally applied CO2 is consistent with the known high diffusibility of CO2 and intracellular buffering capacity of myocardium.

Future Applications
Integration of confocal fluorescent microscopy and the ratiometric measurement of the emission spectra of the H+-sensitive fluorophore SNARF-1 provides a reliable method to measure dynamic changes in pH and pH, at the surface and deeper endocardial layers of the perfused ventricular myocardium. The high spatial and temporal resolution of this method makes it feasible to study the influence of diffusion of gases and metabolites on the regulation of ionic homeostasis in ischemic and reperfused myocardium bordering normal myocardium. Specifically, the role of the production, diffusion, and accumulation of CO2 for the regulation of H+ homeostasis in the ischemic border zone may be studied. The high spatial and temporal resolution of confocal microscopy used in conjunction with fluorophores sensitive to ions, metabolites, and other molecules may permit the study of intercellular signaling among endothelial cells, smooth muscle cells, and ventricular myocytes in tissue.

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