Multispectral fluorescence imaging to assess pH in biological specimens

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Abstract. Simple, quantitative assays to measure pH in tissue could improve the study of complicated biological processes and diseases such as cancer. We evaluated multispectral fluorescence imaging (MSFI) to quantify extracellular pH (pHe) in dye-perfused, surgically-resected tumor specimens with commercially available instrumentation. Using a water-soluble organic dye with pH-dependent fluorescence emission (SNARF-4F), we used standard fluorimetry to quantitatively assess the emission properties of the dye as a function of pH. By conducting these studies within the spectroscopic constraints imposed by the appropriate imaging filter set supplied with the imaging system, we determined that correction of the fluorescence emission of deprotonated dye was necessary for accurate determination of pH due to suboptimal excitation. Subsequently, employing a fluorimetry-derived correction factor (CF), MSFI data sets of aqueous dye solutions and tissue-like phantoms could be spectrally unmixed to accurately quantify equilibrium concentrations of protonated (HA) and deprotonated (A−) dye and thus determine solution pH. Finally, we explored the feasibility of MSFI for high-resolution pHe mapping of human colorectal cancer cell-line xenografts. Data presented suggest that MSFI is suitable for quantitative determination of pH in ex vivo dye-perfused tissue, potentially enabling measurement of pH across a variety of preclinical models of disease. © 2011 Society of Photo-Optical Instrumentation Engineers (SPIE). [DOI: 10.1117/1.3533264]

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1 Introduction

In normal mammalian tissues, intracellular and extracellular pH (pHi and pHe, respectively) are dynamically regulated by a variety of sophisticated mechanisms.1 For example, pHi is maintained by both ion-exchange mechanisms and cytosolic buffering capacity. Within this context, pHi plays important roles in numerous physiological processes, such as protein synthesis and the regulation of cell cycles. Similarly, pHe is controlled by mechanisms that include vascular delivery of physiological buffers and removal of lactic acid. Although the observed pH values vary across tissues of differing origin and function, deviation from normal values for a given tissue coincides with a variety of important pathological states (e.g., renal failure, ischemia, chronic pulmonary disease, and cancer).1 In highly metabolic tissues, such as tumors, reduced pHi can result from elevated production and diminished removal of lactic acid combined with the reduced capability of tumor-associated vasculature to deliver blood-based pH buffers.2 Acidic tumor pH can impart significant consequences on cancer cells, such as increased potential for invasion and likelihood of metastasis, although further elucidation of the role of pH within this setting is needed.3 For these reasons, there is considerable interest in the development and validation of novel methods capable of measuring pH in biological specimens.

The majority of tools currently available for measurement of pH in tissues tend to be invasive (e.g., microelectrodes)3 and/or provide relatively modest spatiotemporal resolution (e.g., magnetic resonance imaging and magnetic resonance spectroscopy).1,5 Limitations of the former techniques result from the inability to determine spatially resolved pH gradients without significant damage to the specimen.2 The latter methods are noninvasive but significantly more costly and complicated to implement, limiting sample throughput and requiring highly specialized equipment and technical expertise.1,5 In contrast, optical imaging techniques offer an attractive alternative to the aforementioned techniques as they tend to be noninvasive, comparatively simple, analytically sensitive, cost-effective, and rapid.6,6 The most common optical methods rely on ratiometric fluorescence approaches. In typical assays utilizing exogenous organic dyes such as fluorescein as an indicator, a

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pH-sensitive excitation/emission combination is compared to a pH-insensitive reference combination.\textsuperscript{2,9,10} Alternately, pH-sensitive dyes such as members of the seminaphthorhodafluor (SNARF) family can be excited at a single wavelength and emission collected at multiple wavelengths corresponding to the discrete spectra of protonated (HA) and deprotonated (A\textsuperscript{−}) species in solution.\textsuperscript{11–15} Though ratiometric approaches are routine for solution analysis\textsuperscript{11} and flow cytometry,\textsuperscript{12–14} others have elegantly adapted ratiometric assays to intravital microscopy and demonstrated imaging of interstitial pH gradients in normal and neoplastic tissues in preclinical studies.\textsuperscript{2} An inherent limitation of these approaches, which is mitigated somewhat through emission filtering at the expense of signal rejection, is the difficulty of deconvolving multiple fluorescence emissions (i.e., signal versus reference or HA versus A\textsuperscript{−}) due to spectral overlap. Multispectral fluorescence imaging (MSFI) is a well-established technique that is suitable for the separation of multiple distinct, yet spectrally overlapping emissions.\textsuperscript{16} When utilized in preclinical animal studies, separation and quantification of multiple fluorescence emissions from imaging probes is feasible.\textsuperscript{17–20} Paired with pH-sensitive fluorochromes that generate unique emission spectra for HA and A\textsuperscript{−} species, MSFI potentially represents an ideal paradigm for measurement of pH in solution and/or in tissue. Additionally, MSFI systems suitable for use in preclinical animal studies or in conjunction with microscopy are now readily available and relatively inexpensive.

In these studies, we illustrate a simple, rapid approach to quantitatively assess pH\textsubscript{e} in dye-perfused biological tissues utilizing MSFI and commercially available instrumentation. Using the MSFI-based approach, we demonstrate the feasibility of mapping pH\textsubscript{e} gradients pertaining to the protonation and deprotonation equilibrium of the pH-sensitive dye SNARF-4F in tissue specimens. It is anticipated that this methodology will provide valuable information regarding \textit{ex vivo} tumor pH\textsubscript{e} and will ultimately aid in the study of the relationship between tumors and tumor microenvironments, as well as the effect of therapeutic regimens on tumor pH\textsubscript{e}.

2 Materials and Methods

2.1 Chemicals

All chemicals, reagents and solvents were purchased from indicated suppliers and used without further purification: SNARF-4F 5-(and-6)-carboxylic acid (Invitrogen; Carlsbad, California), American Chemical Society (ACS)-grade hydrochloric acid (HCl) (EMD; Gibbstown, New Jersey), ACS-grade sodium hydroxide (NaOH) (Fisher Scientific; Pittsburgh, Pennsylvania), laboratory-grade gelatin from porcine skin, type A (Sigma Aldrich; Milwaukee, Wisconsin), bovine hemoglobin (Sigma Aldrich; Milwaukee, Wisconsin), and intralipid 20% (Fresenius Kabi AB).

2.2 Aqueous pH Solutions

Solutions ranging from pH 3–10 in 0.5-pH increments were prepared from a 1.0-μM aqueous stock solution of SNARF-4F separated into 10-mL aliquots. The pH of each aliquot was adjusted via microadditions of concentrated HCl and/or NaOH. Solution pH adjustments were monitored with a calibrated pH meter (MP220 pH Meter, Mettler Toledo, Columbus, OH) under positive nitrogen pressure with vigorous stirring. Over the course of the experiment, the solutions were wrapped in aluminum foil to minimize light exposure. Spectroscopic analysis and/or MSFI of pH-adjusted samples were performed immediately following pH adjustment and stabilization.

2.3 Preparation of Biological Phantoms

To validate the ability of MSFI to measure pH in biological environments, tissue-like phantoms with the approximate photon scattering and absorption properties of biological tissues were prepared.\textsuperscript{21} First, a 10% gelatin solution was prepared by adding gelatin to heated deionized water (40–50°C) with constant stirring. The solution was then cooled to 30–40°C, at which the desired amounts of bovine hemoglobin and intralipid were added, resulting in a 42.5-μM hemoglobin and 1% intralipid phantom mixture. Three parts buffered dye solution [acetate buffer (pH 4.3, 5.6), phosphate buffer (pH 6.2, 7.3), tris-HCl buffer (pH 8.4), and carbonate buffer (pH 9.5)] and one part phantom mixture were combined and dispensed into a chilled well plate and refrigerated at 4°C until solid.

2.4 Fluorimetry

The spectroscopic characteristics of pH-adjusted aqueous SNARF-4F solutions were investigated using a Photon Technology International QuantaMaster\textsuperscript{TM} 50 fluorimeter (Birmingham, New Jersey) and a standard, 1-cm path-length quartz cuvette (NSG Precision Cells 517BES10). An excitation wavelength (λ\textsubscript{ex}) of 523 nm was used for single-wavelength studies, whereas an excitation range of 425–650 nm in increments of 25 nm was used for multiple-wavelength studies. Emission scans were collected every nanometer over the emission wavelength (λ\textsubscript{em}) range of 400–800 nm with an integration time of 0.1 s and a shutter width of 1.5 nm. Three acquisitions were averaged for each λ\textsubscript{em}. Excitation emission matrices of fluorescence emission as a function of excitation were generated in MATLAB (The MathWorks, Inc., Natick, Massachusetts). Fluorimetry data were used in conjunction with

\[
C_F = \frac{F_{\lambda_2}}{F_{\lambda_1}}
\]  

(1)

to generate a correction factor (C\textsubscript{F}) that was later applied to the A\textsuperscript{−} fluorescence intensity obtained in MSFI studies. In Eq. (1), \textit{F} refers to the fluorescence intensity at 653 nm when excited at 525 nm (λ\textsubscript{1}) or 575 nm (λ\textsubscript{2}).

2.5 MSFI: Aqueous Solutions

Aqueous SNARF-4F solutions ranging in pH from four to eight in increments of ~1.0 pH unit were imaged using the Maestro\textsuperscript{TM} Q FLEX In Vivo Imaging System from CRi, Inc. (Woburn, Massachusetts). Solutions were imaged in 1.5-mL microcentrifuge tubes that were prerinsed with pH-adjusted solution prior to addition of dye solution. All images were obtained using Maestro Q Filter Set B, which features a bandpass excitation filter with transmission centered at 525 nm (full width at half

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maximum = 47 nm) and a long-pass emission filter with 560 nm cut in. A spectral library was generated that included emission spectra for fully protonated/deprotonated dye species as well as autofluorescence from the microcentrifuge tube plastic. This library was used throughout the solution phase experiments for spectral unmixing. Quantitative analysis of the autofluorescence signal arising from the microcentrifuge tubes demonstrated that >95% of the autofluorescence could be removed by spectral unmixing (data not shown). Unmixed dye fluorescence intensity data were obtained from a manually drawn region of interest (ROI) shaped and sized to include the central region of the microcentrifuge tube. The data were fitted to a nonlinear sigmoidal model and plotted against the analytically measured solution pH. Uncorrected and corrected SNARF-4F pKₐ values were calculated.

2.6 MSFI—Biological Phantoms
Using a protocol similar to that used for the solution phase experiments, dye containing tissue-like phantoms ranging in a pH of 4–9.5 were imaged with the Maestro Q system. Spectral unmixing was achieved by applying the previously derived solution phase spectral library with the inclusion of autofluorescence from tissue-like phantom controls. Unmixed fluorescence intensity data were obtained from an ellipsoidal manually drawn ROI sized to include the entirety of a single well. The data were fit to a nonlinear sigmoidal model and plotted against the analytically measured solution pH. Uncorrected and corrected SNARF-4F pKₐ values were calculated.

2.7 Ex Vivo pH Mapping of Human Xenograft Tumor Tissue
All studies involving the use of animal models were conducted in accordance with Vanderbilt University Institutional Animal Care and Use Committee and applicable federal guidelines. To generate the human colorectal cancer (CRC) model used for tumor imaging, 200,000 DiFi human CRC cells were subcutaneously injected on the left flank of athymic nude mice, as previously described. Experiments were conducted two to three weeks postinoculation, when tumors reached ~250 mm³. For pH mapping, 1.0 nmol of SNARF-4F in 200 μL of saline was administered to xenograft-bearing mice via intravenous injection under inhalation of anesthesia (2% isofluorane). Following dye administration, mice were allowed to briefly recover from anesthesia and given access to food and water ad libitum during a 15-min uptake period. Mice were then sacrificed and tumor tissue harvested. Immediately following collection (<2 min), tumors were macerated into two equivalent hemispheres, positioned with the intratumoral facets facing toward the camera, and imaged using the Maestro Q system. Fluorescence images were collected from 560 to 850 nm and unmixed using the previously described spectral library (excluding autofluorescence from plastic). Unmixed fluorescence intensity data were obtained from a manually drawn ROI (~2% of the total area) that was applied to multiple areas of the tumor in order to ascertain pH heterogeneity. A gradient pHe map was generated in MATLAB from each MSFI unmixed image using

\[
pH = pK_a - \log \left( \frac{C_{FS}S_A}{S_{HA}} \right). \tag{2}
\]

Here, pKₐ is that of the dye while Sₐ and Sₐₐ correspond to the observed fluorescence intensity of each species, respectively.

3 Results and Discussion
3.1 Spectroscopy
The fluorescence spectroscopy of the SNARF family of organic dyes is dependent on the protonation state of the compound. Typically, the protonated and deprotonated states of the compound yield distinct, yet overlapping, emission peaks that may be separated by as much as 60 nm. For these studies, aimed at measuring pH in tissue, an important determinant was selection of a dye possessing a pKₐ near the anticipated pH of the specimens being assayed. These studies utilized SNARF-4F, with a known pKₐ of 6.4. Thus, at pH values well below its pKₐ, the dye exists predominantly in a protonated state and exhibits fluorescence emission at 580 nm when excited at 523 nm (Fig. 1). SNARF-4F exists in a predominately deprotonated state at pH values well above its pKₐ and exhibits fluorescence emission at 640 nm when excited at 523 nm (Fig. 1). Importantly, across the physiologically relevant pH range of 5–7, both HA and A⁻ species exist in equilibrium. Because of the unique emission spectroscopy of the HA and A⁻ species, spectral unmixing and quantitative measurement of each species facilitate determination of pH in accordance with a modified Henderson–Hasselbalch equation.

Conceptually, the simplest pH imaging study coupling SNARF-4F and the Maestro system would feature utilization of a single excitation band and simultaneous collection and spectral unmixing of both the HA and A⁻ emissions. However, an important assumption of this approach is the requirement for equivalent excitation and collection of both the HA and A⁻ species, which may be thought of as two separate fluorophores in this experiment. Because the emission of the A⁻ species is significantly redshifted (ca. 60 nm) from the HA species,
it follows that equivalent excitation of each species using a common excitation wavelength is unlikely. Therefore, we explored the relationships between excitation wavelength, pH, and the resulting emission spectroscopy of both the HA and A⁻ SNARF-4F species using standard fluorimetry. We found that the HA species exhibited maximum fluorescence emission at an excitation wavelength of \( \sim 525 \) nm (emission = 590 nm), while the A⁻ species exhibited maximum fluorescence emission at an excitation wavelength of \( \sim 575 \) nm (emission = 650 nm) (Fig. 2). Optimization of the imaging assay to utilize only a single filter set could be accomplished using a single filter set included with the Maestro system [denoted as “B”, bandpass excitation 525/25 nm, long-pass emission 560 nm, (Fig. 3)]. This filter set was ideally suited for exciting the HA species but was suboptimal for exciting the A⁻ species. We found the long-pass emission filter contained within the B filter set was suitable for simultaneous collection of both the HA and A⁻ emission, with minor attenuation (~5%) of the HA emission from 530 to 560 nm.

To compensate for the suboptimal excitation of the A⁻ species, a correction factor \( (C_F = 2.1 \pm 0.03) \) was derived from Eq. (1). Equation (1) defines the ratio of the observed emission intensity at 650 nm for the A⁻ species when excited at 525 nm \( (\lambda_1) \), the excitation wavelength in the Maestro, compared to the emission intensity at 650 nm when optimally excited at 575 nm \( (\lambda_2) \).

### Table 1 Observed (uncorrected) and calculated (corrected) pKₐ values for different SNARF-4F containing mediums.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Observed pKₐ</th>
<th>Calculated pKₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution</td>
<td>6.7 ± 0.3</td>
<td>6.4 ± 0.2</td>
</tr>
<tr>
<td>Phantom</td>
<td>6.7 ± 0.3</td>
<td>6.3 ± 0.4</td>
</tr>
</tbody>
</table>

3.2 **MSFI**

To validate \( C_F \) for use in MSFI assays, aqueous SNARF-4F solutions with pH values between 4 and 8 and tissue-like phantoms with pH values between 4 and 9.5 were imaged using filter set B and spectrally unmixed using the previously established spectral library. Following spectral unmixing, manual ROIs were drawn on unmixed images corresponding to the HA and A⁻ species [Figs. 4(a) and 4(b)]. Accordingly, the measured fluorescence intensities for each species were used to calculate the observed, uncorrected pKₐ for the dye using a modified Henderson–Hasselbalch equation, substituting the fluorescence emission intensity for each species in place of HA and A⁻ concentration. Prior to correction of the A⁻ intensity, fitting the unmixed HA and A⁻ intensities to a sigmoidal curve and plotting versus pH [Figs. 4(c) and 4(d)] resulted in intersection between the two curves (by definition, pKₐ) at 6.7 ± 0.3 for both the solution phase and biological phantom data (Table 1). Though these observed values are slightly higher than the known pKₐ of 6.4, application of the fluorimetry determined \( C_F \) to the A⁻ intensities of all data resulted in indistinguishable calculated pKₐ values of \( \sim 6.4 \) (Table 1) using Eq. (2), therefore validating \( C_F \) derived from the fluorimetry experiments in multiple mediums.

![Fig. 2](image-url) Excitation emission matrices of SNARF-4F fluorescence emission as a function of excitation wavelength for HA (a) pH 4.5 and A⁻ (b) pH 10.0. Red marks located along the excitation axis show the approximate bandpass transmission of the Maestro Q B excitation filter, while the red mark on the emission axis shows the cut-on point for the long-pass emission filter. The excitation-emission coordinates for the point at which maximum fluorescence emission intensity is achieved are (525, 587) for (a) and (575, 653) for (b).

![Fig. 3](image-url) Transmission spectrum for (a) bandpass excitation filter and (b) long-pass emission filter supplied as Filter Set B with the Maestro Q imaging system.

![Table 1](image-url) Observed (uncorrected) and calculated (corrected) pKₐ values for different SNARF-4F containing mediums.
Fig. 4 Multispectral fluorescence imaging of (a) aqueous SNARF-4F solutions ranging in pH from approximately 4 to 8 and (b) dye-containing tissue-like phantoms ranging in pH from approximately 4 to 9.5. Spectrally mixed, pseudocolor composite images are shown in row i of both (a) and (b). Spectrally mixed composite images were unmixed to visualize emission from the HA [row ii of both (a) and (b)] and A⁻ [row iii of both (a) and (b)] species. Pseudocolor, unmixed composite images are shown in row iv of both (a) and (b), where the HA fluorescence emission is pseudocolorized in green and the A⁻ fluorescence emission is pseudocolorized in red. Quantified average photon intensities from (a) are given in (c) while intensities from (b) are given in (d). HA (red) and A⁻ (blue) species are displayed across pH as assessed by MSFI. Nonlinear fits of MSFI data (solid colored lines) result in goodness of fits of 7 (c) and 33 (d) degrees of freedom. Observed pKa values (Table 1), the pH at which [HA] = [A⁻], are noted by solid black lines. Correction of the A⁻ intensity using Cf at each point (dotted blue line) results in calculated pKₐ values (noted by dashed black lines) that are equivalent within one standard deviation of one another (Table 1).

Table 2 MSFI-measured photon intensities and calculated pH values for DiFi CRC xenograft tumor. Location for each ROI is shown in Fig. 5(d).

<table>
<thead>
<tr>
<th>ROI</th>
<th>A⁻ Photon Intensity</th>
<th>Corrected A⁻ Photon Intensity</th>
<th>HA Photon Intensity</th>
<th>log(A⁻/HA)</th>
<th>Estimated pHₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>454</td>
<td>937</td>
<td>371</td>
<td>0.4</td>
<td>6.8</td>
</tr>
<tr>
<td>2</td>
<td>141</td>
<td>290</td>
<td>99</td>
<td>0.5</td>
<td>6.9</td>
</tr>
<tr>
<td>3</td>
<td>132</td>
<td>272</td>
<td>98</td>
<td>0.5</td>
<td>6.9</td>
</tr>
<tr>
<td>4</td>
<td>139</td>
<td>286</td>
<td>67</td>
<td>0.6</td>
<td>7.0</td>
</tr>
<tr>
<td>5</td>
<td>141</td>
<td>290</td>
<td>98</td>
<td>0.5</td>
<td>6.9</td>
</tr>
<tr>
<td>6</td>
<td>247</td>
<td>509</td>
<td>162</td>
<td>0.5</td>
<td>6.9</td>
</tr>
<tr>
<td>7</td>
<td>190</td>
<td>392</td>
<td>70</td>
<td>0.8</td>
<td>7.2</td>
</tr>
</tbody>
</table>

pHe: range = 6.80–7.2, mean = 6.9 ± 0.1
tissue. To quantify intratumoral pH, the fluorescence intensity tumor was heterogeneous with a mean pH of 6.9
studies where pH was assessed across multiple tumor types observations are in agreement with those reported by previous
size that the ratiometric nature of the proposed MSFI approach
renders pH measurement inherently concentration independent
concentration independent with respect to the dye, provided that detectable quantities are present within tissues of interest. Applying the previously established spectral library for the HA and A− species to the unmixed data, fluorescence emission of the HA [Fig. 5(b)] and A− [Fig. 5(c)] species could be visualized within tumor tissue. To quantify intratumoral pH, the fluorescence intensity for the HA and A− species was measured by manual ROI analysis [Fig. 5(d)]. The measured intensity of the A− species was corrected using $C_F$, and tumor pH$_T$ for each ROI was calculated using Eq. (2) (Table 2). We found that pH$_T$ within the tumor was heterogeneous with a mean pH of 6.9 ± 0.1. These observations are in agreement with those reported by previous studies where pH$_T$ was assessed across multiple tumor types by alternative techniques.23–26 Expanding on this, to generate a high-resolution pH map of tumor tissue, the HA and corrected A− pixel intensities were imported into MATLAB, where the pH was calculated, fit to a color bar, and displayed across each image pixel [Fig. 5(e)]. Although much of the tumor could be considered acidic with respect to normal tissue, we observed that tumor regions exhibiting the lowest pH tended to coincide with the highest degrees of necrosis in this model. These results suggest the feasibility of MSFI for ex vivo measurement of pH$_T$ in preclinical tissue specimens.

4 Conclusions

We report a novel method for simple, rapid measurement of pH$_T$ in biological tissue that utilizes MSFI. Given the ability to assess pH in a spatially resolved manner, this technique could be employed in preclinical research to further elucidate the relationship(s) between the pH of a tumor and its surrounding microenvironment, as well as the role of pH in other preclinical models of disease. We envision that this approach could be most useful when utilized in conjunction with other complementary molecular techniques, such as immunohistochemistry, and deployed immediately following tissue collection and prior to freezing or fixation.

Acknowledgments

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