Quantitative Ratiometric pH Imaging Using a 1,2-Dioxetane Chemiluminescence Resonance Energy Transfer Sensor

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Regulation of physiological pH is integral for proper whole-body and cellular function, and disruptions in pH homeostasis can be both a cause and effect of disease. In light of this, many methods have been developed to monitor pH in cells and animals. In this study, we report a chemiluminescence resonance energy transfer (CRET) probe Ratio-pHCL-1, comprised of an acrylamide 1,2-dioxetane chemiluminescent scaffold with an appended pH-sensitive carbofluorescein fluorophore. The probe provides an accurate measurement of pH between 6.8-8.4, making it viable tool for measuring pH in biological systems. Further, its ratiometric output is independent of confounding variables. Quantification of pH can be accomplished both using common fluorimetry and advanced optical imaging methods. Using an IVIS Spectrum, pH can be quantified through tissue with Ratio-pHCL-1, which has been shown in vitro and precisely calibrated in sacrificed mouse models. Initial studies showed that intraperitoneal injections of Ratio-pHCL-1 into sacrificed mice produce a photon flux of more than 10^{10} photons per second, and showed a significant difference in ratio of emission intensities between pH 6.0, 7.0, and 8.0.

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Quantitative ratiometric pH imaging using a 1,2-dioxetane chemiluminescence resonance energy transfer sensor

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ABSTRACT: Regulation of physiological pH is integral for proper whole-body and cellular function, and disruptions in pH homeostasis can be both a cause and effect of disease. In light of this, many methods have been developed to monitor pH in cells and animals. In this study, we report a chemiluminescence resonance energy transfer (CRET) probe **Ratio-pHCL-1**, comprised of an acrylamide 1,2-dioxetane chemiluminescent scaffold with an appended pH-sensitive carbofluorescein fluorophore. The probe provides an accurate measurement of pH between 6.8–8.4, making it viable tool for measuring pH in biological systems. Further, its ratiometric output is independent of confounding variables. Quantification of pH can be accomplished both using common fluorimetry and advanced optical imaging methods. Using an IVIS Spectrum, pH can be quantified through tissue with **Ratio-pHCL-1**, which has been shown *in vitro* and precisely calibrated in sacrificed mouse models. Initial studies showed that intraperitoneal injections of **Ratio-pHCL-1** into sacrificed mice produce a photon flux of more than 10¹⁰ photons per second, and showed a significant difference in ratio of emission intensities between pH 6.0, 7.0, and 8.0.

Introduction

Regulation of physiological pH is an important factor for maintaining homeostasis. Whole body pH is regulated through numerous methods, including renal control of bicarbonate storage,¹ ammonia excretion,^{2,3} CO₂ respiration⁴, and excretion of non-volatile acids obtained from the diet.³ These processes are controlled at a subcellular level through active transport by H⁺-ATPase, and passive transport by Na⁺/HCO₃⁻ coporters,^{5,6} and Cl⁻/HCO₃⁻ and Na⁺/H⁺ antiporters.⁷ Deviations in pH homeostasis can have major implications on cellular and whole body function and can be both a cause and effect of disease states. For example, untreated type 1 diabetes can result in ketoacidosis (a lowering of blood pH from overproduction of ketone bodies in β -oxidation of fatty acids) due to insulin deficiency.⁸ Diseases associated with chronic inflammation display decreased pH levels, and are positively correlated with increased cancer risk.⁹ Extracellular pH is also altered in malignant tumors, as cancer cells generate excess lactate due to increased rates of glycolysis, even in the presence of sufficient O₂ needed for aerobic oxidation.¹⁰ Because of their highly heterogenous microenvironments, tumors often display “hot spots” of increased acidity.¹¹ The ability to accurately quantify pH in a pre-clinical and clinical setting is critical to understand its impact on human health.

Current clinical methods to detect pH in living systems include magnetic resonance imaging (MRI) techniques such as intra and extracellular ³¹P MRI,¹² chemical exchange saturation transfer (CEST) MRI,^{13–15} and other infused reporter molecules, notably ¹⁹F-based agents, which can provide a large chemical shift range, but often suffer from poor signal to noise.¹⁶ These methods, however, can be time consuming and often require extensive training and expertise. Preclinical techniques for pH

detection offer a more cost-effective approach to study pH a laboratory setting. Insertion of pH microelectrodes have been used for preclinical measurement of pH in tumors,^{17,18} but is invasive and may disturb tumor physiology. Optical imaging using luminescent pH reporters has been extensively developed in recent years,^{19–23} however, these compounds can be challenging to apply *in vivo* due to changes in luminescence intensity through tissue, hampering the ability to acquire accurate measurements. Ratiometric imaging agents circumvent this issue by providing an internal reference, and this technology has been utilized in GFP,²⁴ luciferase,²⁵ quantum dot,^{26,27} and nanoparticle-based^{28–30} agents for pH. However, no small molecule ratiometric chemiluminescence agents have been made for *in vivo* application.

Chemiluminescence is light generation through an exothermic chemical reaction, and is achieved through the breaking of chemical bonds. Specifically, 1,2-dioxetane compounds have garnered interest in this area due to their innate ability for triggered chemiluminescence emission via chemically initiated electron exchange luminescence (CIEEL).^{31,32} This technology has been employed for imaging analytes by protecting the chemiluminophore with an analyte selective trigger.³³ Upon deprotection of the dioxetane by the analyte, it will undergo CIEEL and luminesce. This strategy has been employed for detection of various analytes including hypoxia,^{34–36} hydrogen sulfide,³⁷ peroxynitrite,³⁸ nitroxyl,³⁹ cathepsin B,⁴⁰ β -galactosidase,^{41,42} formaldehyde,⁴³ and others.^{44–52} Chemiluminescence provides many advantages compared to other optical methods *in vivo* by providing its own light source, thus attenuating autofluorescence and light scattering effects.⁵³ Chemiluminescent probes have also been shown to achieve much higher fold turn-on than comparable fluorophores.³⁹ Furthermore, they do not

Scheme 1. Design and Mechanism of Ratio-pHCL-1.

require expression of an enzyme for use, making them valuable standalone optical imaging tools as opposed to bioluminescence methods for monitoring pH.²⁵

A major driver for both chemiluminescence and fluorescence *in vivo* imaging is the development of luminophores with high quantum yields and redshifted emission. Fluorophore development has flourished in this area, particularly with modifications to BODIPY,^{54–57} xanthene,^{58–63} and cyanine^{64–66} cores. However, red-shifting emission of 1,2-dioxetane chemiluminescent compounds has been difficult. Arguably the largest breakthrough in this area in recent years was the development of acryl-substituted phenoxy dioxetanes that greatly increased quantum yield and shifted emission to near 530 nm.⁶⁷ NIR chemiluminescent scaffolds have also been reported, but are less emissive.⁶⁸ One strategy to obtain bathochromic emission is by pairing donor and acceptor luminophores through resonance energy transfer mechanisms. Fluorescence resonance energy transfer (FRET) pairs have been rigorously vetted, and are often used to detect macromolecular interactions in biochemical applications.⁶⁹ FRET also allows for fluorophores to be paired to dyes with sensing capabilities, thus providing ratiometric detection of analytes with the FRET pair.^{70–73} It is currently known that 1,2-dioxetanes exhibit energy transfer capabilities,^{74–76} but to our knowledge, no single-molecule ratiometric chemiluminescent probes for imaging pH have been developed. Here, we report the first single molecule ratiometric chemiluminescence resonance energy transfer (CRET) sensor quantifying pH in living systems (Scheme 1).

Experimental Procedure

In vitro calibration of Ratio-pHCL-1. Chemiluminescent emission spectra were acquired using a Hitachi F-7000 fluorescence spectrophotometer via the luminescence detection module and scanning luminescence emission from 400–900 nm in response to exposure of **Ratio-pHCL-1** to pH buffered solutions. A solution of 20 μ M **Ratio-pHCL-1** in DMSO was added to a solution of 100 mM Tris, PBS or carbonate buffers ranging from pH 6.81–8.42. For concentration dependence, 10, 20, and 40 μ M **Ratio-pHCL-1** in 5% DMSO were subjected to 100 mM PBS buffered to pH 6.81, 7.45, and 8.02. For time dependence, 20 μ M **Ratio-pHCL-1** in 5% DMSO was subjected to 100 mM PBS buffered to pH 6.81, 7.45, and 8.02, and the chemiluminescence emission spectra was measured at $t = 0, 10, 20$ and 30 min. For DMSO dependence, 20 μ M **Ratio-pHCL-1** in a final concentration

of 1, 5, or 10% DMSO was subjected to 100 mM PBS buffered to pH 6.81, 7.45, or 8.02. Each experiment was independently conducted 3 times, and analysis of each experiment was conducted by dividing emission intensity values at 580 nm by emission intensity values at 530 nm from the emission plot.

Ratio-pHCL-1 kinetics. Kinetics data were acquired using a Biotek Cytation 5[®] plate reader using the luminescence detection mode, end point read type. The temperature was set at 37 °C under ambient atmosphere. The gain was set at 135 and the read height was set at 4.5 mm. In a 96-well plate, 1.0 μ L of 0.25 mM **Ratio-pHCL-1** was added to wells containing a solution of 237.5 μ L 100 mM PBS (pH = 7.45) and 11.5 μ L DMSO to create a final concentration of 1.0 μ M **Ratio-pHCL-1**, 5% DMSO. The plate was then immediately placed into the plate reader and allowed to stir for 1 min. Luminescence readings were acquired every 2 min for 50 min.

Ratiometric pH imaging. Chemiluminescence images were acquired with an IVIS Spectrum (Perkin Elmer, Waltham, MA) using the “Luminescent” and “Photograph” mode. For *in vitro* 96 well plate measurements, the exposure time was set as 0.5 seconds, binning was set to medium, F/stop was set as 4, FOV was set as C (12.9 cm), Excitation was blocked and the sequence was set for the emission mode, and images were acquired sequentially first using a 580 nm bandpass filter, then a 540 nm bandpass filter. A final concentration of 1 μ M **Ratio-pHCL-1** was added to 100 mM buffer ranging from pH 6.81–8.42. All images were analyzed using Living Image software. Image analysis was carried out by setting individual ROI’s to each well for both 580 and 540 nm filter images. The ROI’s were then measured, which gave a total flux (p/s) for each ROI, the flux value at 580 nm was divided by the flux at 540 nm for each well. For sacrificed mouse imaging, 950 μ L of 1.0 M PBS (pH 5.99–7.99) was injected into the peritoneal cavity of recently sacrificed CL6B57 mice aged 6–12 weeks old, then 50 μ L of 400 μ M **Ratio-pHCL-1** in DMSO was injected shortly after to achieve an approximate final concentration of 20 μ M **Ratio-pHCL-1** with 5% DMSO. The mice were imaged immediately after injection of **Ratio-pHCL-1** with a 580 nm and 540 nm filter with capture settings set to autoexposure and FOV set to C (12.9 cm).

Scheme 2. Synthesis of Ratio-pHCL-1

Results

In previous work, we have demonstrated ratiometric quantification of pH using the light emission from 1,2-dioxetane chemiluminophores.⁷⁶ These studies, however, required the addition of the pH sensitive dye seminaphtharhodafuor (SNARF) coupled with the addition of a chemiluminescence enhancer solution and were not viable for *in vivo* application. We sought a molecule with increased chemiluminescence quantum yields, red-shifted emission, and a pH sensitive fluorophore directly conjugated to the dioxetane scaffold for increased energy transfer efficiency and ease of use. Inspired by previously reported pH-sensitive carbofluoresceins with fluorescent emissions near 580 nm,⁵⁸ we postulated that conjugation of this fluorophore to the dioxetane scaffold could provide a ratiometric measure of pH (Scheme 1).

Synthesis of **Ratio-pHCL-1** began with the synthesis of *tert*-butyl-dimethylsilyl ether (TBS)-protected anthrone **1** from reported literature procedures (Scheme 2).⁵⁸ Due to difficulties in conjugation of the top segment of the fluorophore to **1** using reported Grignard metathesis to form the carbofluorescein unit, we decided to explore alternative methods. It has been shown that iodo compounds react faster than their bromo counterparts during lithium halogen exchange,⁷⁷ so we proceeded to synthesize 2-iodo-*tert*-butyl-isophthalate (compound **2**) from commercially available 2-aminoisophthalic acid.⁷⁸ TBS-protected carbofluorescein **3** was successfully formed in 35% yield by premixing **1** and **2**, purging the mixture three times under vacuum, addition of dry THF, then addition of 1 equiv of *n*-BuLi at 0 °C. This protocol proved to be general, and could be used for preparing other xanthene scaffolds, including silicon rhodamines (Scheme S1). We postulate that the faster rate of lithium-iodine exchange compared to nucleophilic addition of *n*-BuLi to the anthrone enables the use of *n*-BuLi as a safer alternative to *tert*-BuLi.

Compound **3** was further modified in order to conjugate it to the chemiluminescent scaffold. We simultaneously deprotected both the TBS and *tert*-butyl ester protecting groups with

trifluoroacetic acid (TFA). The xanthene phenols were protected with acetic anhydride to obtain **5**, but it was noticed that a less polar spot as shown by thin layer chromatography (TLC) also formed during the reaction, which was presumed to be the mixed anhydride product. This spot disappeared by subjecting the crude mixture to one equivalent of Et₃N and five equivalents of pyridine in a 1:1 THF/ H₂O mixture to produce **5** in a 64% yield. We then appended *tert*-butyl piperazine-1-carboxylate to **5** and subsequently deprotected the *tert*-butyloxy carbamate with TFA to obtain amine **7**.

The final steps were to conjugate the chemiluminescent scaffold and carbofluorescein together, deprotect to form the free phenol precursor, and form the dioxetane compound. We decided to also use an acetate protection strategy to form compound **8** from literature reported compound **S7** in 64% yield (Scheme S1). This was advantageous to preparation of the final probe because all three acetates could be deprotected in a single step. We proceeded to conjugate **7** and **8** together via 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) coupling to form triacetate **9**, which was subsequently deprotected with 1M LiOH to form precursor **10** in quantitative yield. **10** underwent a light mediated [2+2] cycloaddition with singlet oxygen generated with methylene blue as a photosensitizer to obtain **Ratio-pHCL-1** in 90% yield.

Upon synthesis of **Ratio-pHCL-1**, we proceeded to characterize its properties *in vitro*. We measured the chemiluminescence emission of 20 μM **Ratio-pHCL-1** (100 mM PBS or TRIS, pH 6.81–8.42, 5% DMSO). Analysis of the emission spectra over this pH range revealed one peak centered at 580 nm attributed to luminescence from the carbofluorescein scaffold, and a shoulder around 530 nm corresponding to CIEEL from the chemiluminescent scaffold (Figure 1A). As the pH of the system is increased, emission at both peaks increases due to increased concentration of phenolate over phenol, however, luminescence intensity from the carbofluorescein increases in this system to a greater extent than the luminescence from the chemiluminophore; analysis shows a 24-fold change in the ratio of

Figure 1. The pH dependent (A) chemiluminescence emission spectra of 20 μ M **Ratio-pHCL-1** and 5% DMSO in 100 mM PBS buffer from pH 6.81–7.63 and (B) ratio of chemiluminescence emission intensities at 580 nm and 530 nm of 20 μ M **Ratio-pHCL-1** and 5% DMSO in aqueous buffer (pH = 6.81–8.44). All measurements were taken in 100 mM PBS or 100 mM Tris, and measurements were taken 20 s after mixing. Error bars are $\pm$ SD from $n = 3$ independent experiments.

Figure 2. The dependence of chemiluminescence emission on the concentration of **Ratio-pHCL-1**. (A) Emission spectra at pH 7.45 and (B) ratio of the chemiluminescence emission intensity at 580 nm and 530 nm of 10, 20, and 40 μ M **Ratio-pHCL-1** in PBS buffer at pH 6.81 (white trace), 7.45 (gray trace), and 8.02 (black trace) containing 5% DMSO. All measurements were taken 20 s after mixing. Error bars are $\pm$ SD from $n = 3$ independent experiments.

emission intensities at 580 nm over 530 nm from pH 6.81–8.42 (Figure 1B), demonstrating that the emission spectra of the **Ratio-pHCL-1** is pH dependent. We then measured decomposition kinetics of 1.0 μ M **Ratio-pHCL-1** (100 mM PBS, pH 7.45, 5% DMSO, 37 $^{\circ}$ C). Figure S2 shows an observed peak emission at the initial time point followed by a steady decline to baseline values over 50 minutes.

We subjected **Ratio-pHCL-1** to variables to confirm its ratiometric response is independent of testing conditions. We first examined changes in emission spectra as compared with concentration of **Ratio-pHCL-1** (Figure 2). We tested 10, 20, and 40 μ M **Ratio-pHCL-1** (100 mM PBS, pH 6.81–8.02, 5% DMSO). **Ratio-pHCL-1** shows a dose dependent increase in emission intensity as concentration increases, however, both peaks increase proportionally (Figure 2A). Analysis of the ratio of emission intensities at 580 nm and 530 nm plotted versus the concentration of **Ratio-pHCL-1** reveals that the signal remains relatively consistent despite changes in its concentration (Figure 2B), only showing a slight downward trend with increasing concentration at pH 7.45. We then subjected 20 μ M **Ratio-pHCL-1** to varying DMSO concentrations (100 mM PBS, pH 6.81–8.02, 1–10% DMSO). **Ratio-pHCL-1** showed a dose dependent increase in overall emission intensity with increasing DMSO concentration, likely due to increases in

Figure 3. The dependence of chemiluminescence emission on the concentration of DMSO. (A) Emission spectra at pH 7.45 with 1, 5, or 10% DMSO and (B) ratio of the chemiluminescence emission intensity at 580 nm and 530 nm of 20 μ M **Ratio-pHCL-1** in PBS buffer at pH 6.81 (white trace), 7.45 (gray trace), and 8.02 (black trace) containing 1, 5 or 10% DMSO. All measurements were taken 20 s after mixing. Error bars are $\pm$ SD from $n = 3$ independent experiments.

Figure 4. Time dependent (A) emission spectrum at pH 7.45 and (B) ratio of the chemiluminescence emission intensities at 580 nm and 530 nm of 20 μ M **Ratio-pHCL-1** in 100 mM PBS at pH 6.81 (white trace), 7.45 (gray trace), and 8.02 (black trace) containing 5% DMSO. All measurements were taken 20 s after mixing. Error bars are $\pm$ SD from $n = 3$ independent experiments.

chemiluminescence quantum yield (Figure 3A). However, changes in DMSO concentration did not greatly change the ratio of emission intensities at 580 and 530 nm, albeit showing a slight upward trend in chemiluminescence emission at pH 7.45 (Figure 3B).

We then measured 20 μ M **Ratio-pHCL-1** (100 mM PBS, pH 6.81–8.02) from 0–30 min with 10 min intervals to test probe stability over time. Figure 4A reveals that the overall chemiluminescence intensity decreases over time, as was expected from free phenol dioxetane kinetics. Plotting the ratio of emission intensities at 580 nm over 530 nm does reveal changes in emission intensities over time, with a slight upward trend at pH 7.45 and a more pronounced downward trend at pH 8.02 over a 30 minute period (Figure 4B), likely due to more rapid decomposition kinetics at this pH. These variations indicate that care should be taken when using **Ratio-pHCL-1** at higher pH values. Imaging experiments should be performed quickly after injection to ensure equal comparisons, especially when measuring higher pH values. Next, we examined the behavior of **Ratio-pHCL-1** in biological systems. Cellular uptake of 20 μ M **Ratio-pHCL-1** in A549 cells was examined over an 80 min period (Figure S3), and changes in fluorescence were monitored using an EVOS fluorescence microscope. After washing the cells, increased intracellular fluorescence could be seen from the acrylamide and carbofluorescein motifs. These results show

Figure 5. Chemiluminescence intensity images of the pH dependent emission of 1 μM **Ratio-pHCL-1** in 100 mM PBS or Tris (pH 6.81–8.42) containing 5% DMSO using (A) 580 nm bandpass filter or (B) 540 nm bandpass filter in an IVIS Spectrum. (C) Ratio of total flux (p/s) at 580 nm and 540 nm. $n = 12$ technical replicates across 4 independent experiments, error bars are $\pm$ SD.

Figure 6. Chemiluminescence images of the pH dependent emission of 20 μM **Ratio-pHCL-1** in 1.0 M PBS (pH 5.99–7.99) containing 5% DMSO injected into the peritoneal cavity of sacrificed C57BL6 mice using a (A) 580 nm bandpass filter or (B) 540 nm bandpass filter in an IVIS Spectrum. (C) Ratio of total flux (p/s) at 580 nm and 540 nm. $n = 3$ biological replicates for each pH tested, error bars are $\pm$ SD.

that **Ratio-pHCL-1** can cross the cellular membrane and accumulate inside cells.

Upon completion of the *in vitro* spectrophotometry experiments and cellular studies, we confirmed the feasibility of quantitative pH imaging using **Ratio-pHCL-1** in an IVIS Spectrum. We subjected **Ratio-pHCL-1** to buffered solutions (100 mM PBS, pH 6.81–8.42, 5% DMSO) and took sequential images using 580 and 540 nm bandpass filters and 0.5 second exposure time. Four independent experiments were completed, and each buffered solution was measured with three technical replicates per experiment. A solution of 20 μM **Ratio-pHCL-1** was too bright and saturated the images at this setting, so the concentration was lowered to 1.0 μM . Images of the plate taken with average radiance heat maps set to 10^9 photons $\text{sec}^{-1} \text{ cm}^{-2} \text{ sr}^{-1}$ and 10^8 photons $\text{sec}^{-1} \text{ cm}^{-2} \text{ sr}^{-1}$ for the 540 nm filter (Figure 5A, 5B, respectively) show that average radiance increases with pH on both filter settings. Plotting the flux (photons/s) at 580 nm over flux at 540 nm for each well shows an increase in the ratio between the two wavelengths over increase in pH, and the generated curve trends well with our previous *in vitro* calibration. We then

confirmed that a ratiometric chemiluminescence response could be measured through a tissue surrogate by placing a 2.8 mm thick slice of bologna (Kroger, Dallas, TX) atop a 96 well plate loaded with 20 μM **Ratio-pHCL-1** in buffered solutions (100 mM PBS or Tris, pH 6.81–8.42, 5% DMSO) and took sequential 580 nm and 540 nm filtered images with 10.0 second exposure times (Figure S4), which clearly showed penetration through this tissue surrogate.

Finally, we investigated the response of **Ratio-pHCL-1** in animal models. Recently sacrificed C57BL6 mice were given 950 μL intraperitoneal (IP) injections of 1.0 M PBS (pH 5.99–7.99), then subsequently given 50 μL IP injections of 400 μM **Ratio-pHCL-1** to achieve a theoretical final concentration of 20 μM **Ratio-pHCL-1** with 5% DMSO in the injection volume, and imaged with a 580 nm and 540 nm filter. With radiance heat maps of both 580 and 540 nm images set to $10^9 \text{ p sec}^{-1} \text{ cm}^{-2} \text{ sr}^{-1}$ (Figure 6A, 6B) there is a clear increase in flux from the 580 nm image from pH 6.0–8.0, with a less pronounced increase in the 540 nm image over the same pH range. Plotting total flux at 580 nm over flux at 540 nm shows an increase in the ratio of total flux from both filter sets with increasing pH (Figure 6C), demonstrating that **Ratio-pHCL-1** can discern changes in pH in the peritoneal cavity of mice.

Conclusions

In summary, we have developed the first single molecule 1,2-dioxetane ratiometric chemiluminescence probe for quantitative imaging of pH in animal tissue. This was synthetically achieved by attaching a pH sensitive carbofluorescein to the chemiluminescent acrylamide dioxetane scaffold via a piperazine linker. **Ratio-pHCL-1** generates light through CIEEL decomposition, and concomitant pH dependent emission from the appended fluorophore through a CRET mechanism. **Ratio-pHCL-1** is highly accurate *in vitro*, providing measurement of pH in biological ranges. Its ratiometric output is stable to confounding variables, and provides a similar response when collected through optical imaging methods. A pH dependent ratiometric emission is also reported in the peritoneal cavity of sacrificed mice, and we plan to conduct *in vivo* quantification of pH in live mouse models in future experiments. We report flux outputs of more than 10^{10} p/s from 20 μM **Ratio-pHCL-1** through biological tissue, which exceeds the photon flux of most reported chemiluminescence agents. This increase in brightness is achieved through use of an unmasked phenoxy dioxetane that provides instantaneous decomposition and light output. This shows a clear advantage to chemiluminescence detection using unmasked dioxetanes – they have the potential for much higher sensitivity in biological settings. This methodology can be applied to highly sensitive quantitation of other analytes, and has provided the foundation for *in vivo* ratiometric imaging with 1,2-dioxetanes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare the following competing financial interest(s): A.R.L. discloses a financial stake in BioLum Sciences, LLC, a company developing chemiluminescence assays.

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REFERENCES

- (1) Hamm, L. L.; Nakhoul, N.; Hering-Smith, K. S. Acid-Base Homeostasis. *Clin. J. Am. Soc. Nephrol.* **2015**, *10*, 2232–2242.
- (2) Weiner, I. D.; Hamm, L. L. Molecular Mechanisms of Renal Ammonia Transport. *Annu. Rev. Physiol.* **2007**, *69*, 317–340.
- (3) Koeppen, B. M. The Kidney and Acid-Base Regulation. *Adv. Physiol. Educ.* **2009**, *33*, 275–281.
- (4) Ruffin, V. A.; Salameh, A. I.; Boron, W. F.; Parker, M. D. Intracellular PH Regulation by Acid-Base Transporters in Mammalian Neurons. *Front. Physiol.* **2014**, *5*, 1–11.
- (5) Boron, W. F. Regulation of Intracellular PH. *Adv. Physiol. Educ.* **2004**, *28*, 160–179.
- (6) Aalkjaer, C.; Boedtker, E.; Choi, I.; Lee, S. Cation-Coupled Bicarbonate Transporters. *Compr Physiol.* **2014**, *4*, 1605–1637.
- (7) Aoi, W.; Marunaka, Y. Importance of PH Homeostasis in Metabolic Health and Diseases: Crucial Role of Membrane Proton Transport. *Biomed Res. Int.* **2014**, *2014*.
- (8) Trachtenbarg, D. E. Diabetic Ketoacidosis. *Am Fam Physician* **2005**, *71*, 1705–1714.
- (9) Boedtker, E.; Pedersen, S. F. The Acidic Tumor Microenvironment as a Driver of Cancer. *Annu. Rev. Physiol.* **2020**, *82*, 103–126.
- (10) Heiden, M. G. V.; Cantley, L. C.; Thompson, C. B. Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science* **2009**, *329*, 1029–1033.
- (11) Rohani, N.; Hao, L.; Alexis, M. S.; Joughin, B. A.; Krismer, K.; Moufarrej, M. N.; Soltis, A. R.; Lauffenburger, D. A.; Yaffe, M. B.; Burge, C. B.; et al. Acidification of Tumor at Stromal Boundaries Drives Transcriptome Alterations Associated with Aggressive Phenotypes. *Cancer Res.* **2019**, *79*, 1952–1966.
- (12) Robey, I. F.; Baggett, B. K.; Kirkpatrick, N. D.; Roe, D. J.; Dosescu, J.; Sloane, B. F.; Hashim, A. I.; Morse, D. L.; Raghunand, N.; Gatenby, R. A.; et al. Bicarbonate Increases Tumor PH and Inhibits Spontaneous Metastases. *Cancer Res.* **2009**, *69*, 2260–2268.
- (13) Jin, T.; Wang, P.; Jin, T.; Kim, S. G.; Kim, S. G. Enhancing Sensitivity of PH-Weighted MRI with Combination of Amide and Guanidyl CEST. *Neuroimage* **2017**, *157*, 341–350.
- (14) Wu, Y.; Zhou, I. Y.; Igarashi, T.; Longo, D. L.; Aime, S.; Sun, P. Z. A Generalized Ratiometric Chemical Exchange Saturation Transfer (CEST) MRI Approach for Mapping Renal PH Using Iopamidol. *Magn. Reson. Med.* **2018**, *79*, 1553–1558.
- (15) Pavuluri, K. D.; McMahon, M. T. PH Imaging Using Chemical Exchange Saturation Transfer (CEST) MRI. *Isr. J. Chem.* **2017**, *57*, 862–887.
- (16) Mason, R. P. Transmembrane PH Gradients In Vivo: Measurements Using Fluorinated Vitamin B6 Derivatives. *Curr. Med. Chem.* **1999**, *6*, 491–499.
- (17) Jähde, E.; Rajewsky, M. F. Sensitization of Clonogenic Malignant Cells to Hyperthermia by Glucose-Mediated, Tumor-Selective pH Reduction. *J. Cancer Res. Clin. Oncol.* **1982**, *104*, 23–30.
- (18) Engin, K.; Leeper, D. B.; Thistlethwaite, A. J.; Tupchong, L.; Phil, D.; McFarlane, J. D. Tumor Extracellular PH as a Prognostic Factor in Thermoradiotherapy. *Int. J. Radiat. Oncol. Biol. Phys.* **1994**, *29*, 125–132.
- (19) Aggarwal, K.; Khurana, J. M. Indeno-Furan Based Colorimetric and on-off Fluorescent pH Sensors. *J. Photochem. Photobiol. A Chem.* **2015**, *307*–308, 23–29.
- (20) Li, Z.; Li, L. J.; Sun, T.; Liu, L.; Xie, Z. Benzimidazole-BODIPY as Optical and Fluorometric pH Sensor. *Dye. Pigm.* **2016**, *128*, 165–169.
- (21) Qi, J.; Liu, D.; Liu, X.; Guan, S.; Shi, F.; Chang, H.; He, H.; Yang, G. Fluorescent pH Sensors for Broad-Range pH Measurement Based on a Single Fluorophore. *Anal. Chem.* **2015**, *87*, 5897–5904.
- (22) Xu, X. Y.; Yan, B. An Efficient and Sensitive Fluorescent pH Sensor Based on Amino Functional Metal-Organic Frameworks in Aqueous Environment. *Dalt. Trans.* **2016**, *45*, 7078–7084.
- (23) Richardson, D. S.; Gregor, C.; Winter, F. R.; Urban, N. T.; Sahl, S. J.; Willig, K. I.; Hell, S. W. SRpHi Ratiometric pH Biosensors for Super-Resolution Microscopy. *Nat. Commun.* **2017**, *8*, 577.
- (24) Hanson, G. T.; McAnaney, T. B.; Park, E. S.; Rendell, M. E. P.; Yarbrough, D. K.; Chu, S.; Xi, L.; Boxer, S. G.; Montrose, M. H.; Remington, S. J. Green Fluorescent Protein Variants as Ratiometric Dual Emission pH Sensors. 1. Structural Characterization and Preliminary Application. *Biochemistry* **2002**, *41*, 15477–15488.
- (25) Zhang, Y.; Xie, Q.; Robertson, J. B.; Johnson, C. H. pHlash: A New Genetically Encoded and Ratiometric Luminescence Sensor of Intracellular PH. *PLoS One* **2012**, *7*, 43072.
- (26) Pratiwi, F. W.; Hsia, C. H.; Kuo, C. W.; Yang, S. M.; Hwu, Y. K.; Chen, P. Construction of Single Fluorophore Ratiometric PH Sensors Using Dual-Emission Mn²⁺-Doped Quantum Dots. *Biosens. Bioelectron.* **2016**, *84*, 133–140.
- (27) Jin, T.; Sasaki, A.; Kinjo, M.; Miyazaki, J. A Quantum Dot-Based Ratiometric pH Sensor. *Chem. Commun.* **2010**, *46*, 2408–2410.
- (28) Shi, W.; Li, X.; Ma, H. A Tunable Ratiometric pH Sensor Based on Carbon Nanodots for the Quantitative Measurement of the Intracellular pH of Whole Cells. *Angew. Chem. Int. Ed.* **2012**, *51*, 6432–6435.
- (29) Ma, T.; Hou, Y.; Zeng, J.; Liu, C.; Zhang, P.; Jing, L.; Shangguan, D.; Gao, M. Dual-Ratiometric Target-Triggered Fluorescent Probe for Simultaneous Quantitative Visualization of Tumor Microenvironment Protease Activity and pH in Vivo. *J. Am. Chem. Soc.* **2018**, *140*, 211–218.
- (30) Lei, J.; Wang, L.; Zhang, J. Ratiometric pH Sensor Based on Mesoporous Silica Nanoparticles and Förster Resonance Energy Transfer. *Chem. Commun.* **2010**, *46*, 8445–8447.
- (31) Augusto, F. A.; De Souza, G. A.; De Souza, S. P.; Khalid, M.; Baader, W. J. Efficiency of Electron Transfer Initiated Chemiluminescence. *Photochem. Photobiol.* **2013**, *89*, 1299–1317.
- (32) Vacher, M.; Fdez Galván, I.; Ding, B. W.; Schramm, S.; Berraud-Pache, R.; Naumov, P.; Ferré, N.; Liu, Y. J.; Navizet, I.; Roca-Sanjuán, D.; et al. Chemi- and Bioluminescence of Cyclic Peroxides. *Chem. Rev.* **2018**, *118*, 6927–6974.
- (33) Bezner, B. J.; Ryan, L. S.; Lippert, A. R. Reaction-Based Luminescent Probes for Reactive Sulfur, Oxygen, and Nitrogen Species: Analytical Techniques and Recent Progress. *Anal. Chem.* **2020**, *92*, 309–326.
- (34) Ryan, L. S.; Gerberich, J.; Cao, J.; An, W.; Jenkins, B. A.; Mason, R. P.; Lippert, A. R. Kinetics-Based Measurement of Hypoxia in Living Cells and Animals Using an Acetoxymethyl Ester Chemiluminescent Probe. *ACS Sens.* **2019**, *4*, 1391–1398.
- (35) Cao, J.; Campbell, J.; Liu, L.; Mason, R. P.; Lippert, A. R. In Vivo Chemiluminescent Imaging Agents for Nitroreductase and Tissue Oxygenation. *Anal. Chem.* **2016**, *88*, 4995–5002.
- (36) Sun, J.; Hu, Z.; Wang, R.; Zhang, S.; Zhang, X. A Highly

- Sensitive Chemiluminescent Probe for Detecting Nitroreductase and Imaging in Living Animals. *Anal. Chem.* **2019**, *91*, 1384–1390.
- (37) Cao, J.; Lopez, R.; Thacker, J. M.; Moon, J. Y.; Jiang, C.; Morris, S. N. S.; Bauer, J. H.; Tao, P.; Mason, R. P.; Lippert, A. R. Chemiluminescent Probes for Imaging H₂S in Living Animals. *Chem. Sci.* **2015**, *6*, 1979–1985.
- (38) Cao, J.; An, W.; Reeves, A. G.; Lippert, A. R. A Chemiluminescent Probe for Cellular Peroxynitrite Using a Self-Immolative Oxidative Decarbonylation Reaction. *Chem. Sci.* **2018**, *9*, 2552–2558.
- (39) An, W.; Ryan, L. S.; Reeves, A. G.; Bruemmer, K. J.; Mouhaffel, L.; Gerberich, J. L.; Winters, A.; Mason, R. P.; Lippert, A. R. A Chemiluminescent Probe for HNO Quantification and Real-Time Monitoring in Living Cells. *Angew. Chem. Int. Ed.* **2019**, *58*, 1361–1365.
- (40) Shabat, D.; Roth-Konforti, M.; Bauer, C. Unprecedented Sensitivity in a Probe for Detection and Imaging of Cathepsin B: Chemiluminescence Microscopy Cell Images of Natively-Expressed Enzyme. *Angew. Chem. Int. Ed.* **2017**, *56*, 15633–15638.
- (41) Liu, L.; Mason, R. P. Imaging β -Galactosidase Activity in Human Tumor Xenografts and Transgenic Mice Using a Chemiluminescent Substrate. *PLoS One*. **2010**, *5*, e12024.
- (42) Zhang, Y.; Yan, C.; Wang, C.; Guo, Z.; Liu, X.; Zhu, W. H. A Sequential Dual-Lock Strategy for Photoactivatable Chemiluminescent Probes Enabling Bright Duplex Optical Imaging. *Angew. Chem. Int. Ed.* **2020**, *59*, 2–10.
- (43) Bruemmer, K. J.; Green, O.; Su, T. A.; Shabat, D.; Chang, C. J. Chemiluminescent Probes for Activity-Based Sensing of Formaldehyde Released from Folate Degradation in Living Mice. *Angew. Chem. Int. Ed.* **2018**, *57*, 7508–7512.
- (44) Huang, J.; Lyu, Y.; Li, J.; Cheng, P.; Jiang, Y.; Pu, K. A Renal-Clearable Duplex Optical Reporter for Real-Time Imaging of Contrast-Induced Acute Kidney Injury. *Angew. Chem. Int. Ed.* **2019**, *58*, 17796–17804.
- (45) Gnaïm, S.; Scomparin, A.; Das, S.; Blau, R.; Satchi-Fainaro, R.; Shabat, D. Direct Real-Time Monitoring of Prodrug Activation by Chemiluminescence. *Angew. Chem. Int. Ed.* **2018**, *57*, 9033–9037.
- (46) Roth-Konforti, M.; Green, O.; Hupfeld, M.; Fieseler, L.; Heinrich, N.; Ihssen, J.; Vorberg, R.; Wick, L.; Spitz, U.; Shabat, D. Ultrasensitive Detection of Salmonella and Listeria Monocytogenes by Small-Molecule Chemiluminescence Probes. *Angew. Chem. Int. Ed.* **2019**, *131*, 10469–10475.
- (47) Shabat, D.; Das, S.; Ihssen, J.; Wick, L.; Spitz, U. Chemiluminescence Carbapenem-based Molecular Probe for Detection of Carbapenemase Activity in Live Bacteria. *Chem. Eur. J.* **2020**, *26*, 3647–3652.
- (48) Son, S.; Won, M.; Green, O.; Hananya, N.; Sharma, A.; Jeon, Y.; Kwak, J. H.; Sessler, J. L.; Shabat, D.; Kim, J. S. Chemiluminescent Probe for the In Vitro and In Vivo Imaging of Cancers Over-Expressing NQO1. *Angew. Chem. Int. Ed.* **2019**, *58*, 1739–1743.
- (49) An, R.; Wei, S.; Huang, Z.; Liu, F.; Ye, D. An Activatable Chemiluminescent Probe for Sensitive Detection of γ -Glutamyl Transpeptidase Activity in Vivo. *Anal. Chem.* **2019**, *91*, 13639–13646.
- (50) Hananya, N.; Press, O.; Das, A.; Scomparin, A.; Satchi-Fainaro, R.; Sagi, I.; Shabat, D. Persistent Chemiluminescent Glow of Phenoxy-Dioxetane Luminophore Enables Unique CRET-Based Detection of Proteases. *Chem. Eur. J.* **2019**, *25*, 14679–14687.
- (51) Miranda-Apodaca, J.; Hananya, N.; Velázquez-Campoy, A.; Shabat, D.; Arellano, J. B. Emissive Enhancement of the Singlet Oxygen Chemiluminescence Probe after Binding to Bovine Serum Albumin. *Molecules* **2019**, *24*.
- (52) Hananya, N.; Green, O.; Blau, R.; Satchi-Fainaro, R.; Shabat, D. A Highly Efficient Chemiluminescence Probe for the Detection of Singlet Oxygen in Living Cells. *Angew. Chem. Int. Ed.* **2017**, *56*, 11793–11796.
- (53) Ryan, L. S.; Lippert, A. R. Ultrasensitive Chemiluminescent Detection of Cathepsin B: Insights into the New Frontier of Chemiluminescent Imaging. *Angew. Chem. Int. Ed.* **2018**, *57*, 622–624.
- (54) Knox, H. J.; Hedhli, J.; Kim, T. W.; Khalili, K.; Dobrucki, L. W.; Chan, J. A Bioreducible N-Oxide-Based Probe for Photoacoustic Imaging of Hypoxia. *Nat. Commun.* **2017**, *8*.
- (55) Knox, H. J.; Kim, T. W.; Zhu, Z.; Chan, J. Photophysical Tuning of N -Oxide-Based Probes Enables Ratiometric Photoacoustic Imaging of Tumor Hypoxia. *ACS Chem. Biol.* **2018**, *13*, 1838–1843.
- (56) Zhou, E. Y.; Knox, H. J.; Liu, C.; Zhao, W.; Chan, J. A Conformationally Restricted Aza-BODIPY Platform for Stimulus-Responsive Probes with Enhanced Photoacoustic Properties. *J. Am. Chem. Soc.* **2019**, *141*, 17601–17609.
- (57) McDonnell, S. O.; O'Shea, D. F. Near-Infrared Sensing Properties of Dimethylamino-Substituted BF₂-2-Azadipyrromethenes. *Org. Lett.* **2006**, *8*, 3493–3496.
- (58) Grimm, J. B.; Sung, A. J.; Legant, W. R.; Hulamm, P.; Matlosz, S. M.; Betzig, E.; Lavis, L. D. Carbofluoresceins and Carborhodamines as Scaffolds for High-Contrast Fluorogenic Probes. *ACS Chem. Biol.* **2013**, *8*, 1303–1310.
- (59) Grimm, J. B.; Gruber, T. D.; Ortiz, G.; Brown, T. A.; Lavis, L. D. Virginia Orange: A Versatile, Red-Shifted Fluorescein Scaffold for Single- And Dual-Input Fluorogenic Probes. *Bioconjug. Chem.* **2016**, *27*, 474–480.
- (60) Grimm, J. B.; English, B. P.; Choi, H.; Muthusamy, A. K.; Mehl, B. P.; Dong, P.; Brown, T. A.; Lippincott-Schwartz, J.; Liu, Z.; Lionnet, T.; et al. Bright Photoactivatable Fluorophores for Single-Molecule Imaging. *Nat. Methods* **2016**, *13*, 985–988.
- (61) Grimm, J. B.; Brown, T. A.; Tkachuk, A. N.; Lavis, L. D. General Synthetic Method for Si-Fluoresceins and Si-Rhodamines. *ACS Cent. Sci.* **2017**, *3*, 975–985.
- (62) Zhou, X.; Lai, R.; Beck, J. R.; Li, H.; Stains, C. I. Nebraska Red: A Phosphinate-Based near-Infrared Fluorophore Scaffold for Chemical Biology Applications. *Chem. Commun.* **2016**, *52*, 12290–12293.
- (63) Fang, Y.; Good, G. N.; Zhou, X.; Stains, C. I.; Li, R. Phosphinate-Containing Rhodol and Fluorescein Scaffolds for the Development of Bioprobes. *Chem. Commun.* **2019**, *55*, 5962–5965.
- (64) Michie, M. S.; Götz, R.; Franke, C.; Bowler, M.; Kumari, N.; Magidson, V.; Levitus, M.; Loncarek, J.; Sauer, M.; Schnermann, M. J. Cyanine Conformational Restraint in the Far-Red Range. *J. Am. Chem. Soc.* **2017**, *139*, 12406–12409.
- (65) Cosco, E. D.; Caram, J. R.; Bruns, O. T.; Franke, D.; Day, R. A.; Farr, E. P.; Bawendi, M. G.; Sletten, E. M. Flavylum Polymethine Fluorophores for Near- and Shortwave Infrared Imaging. *Angew. Chem. Int. Ed.* **2017**, *56*, 13126–13129.
- (66) Pengshung, M.; Kwon, J.; Sletten, E.; Pengshung, M.; Kwon, J.; Sletten, E. M. Bathochromically Shifted Polymethine Dyes Through Silicon Incorporation. *Chemrxiv* **2019**.
- (67) Green, O.; Eilon, T.; Hananya, N.; Gutkin, S.; Bauer, C. R.; Shabat, D. Opening a Gateway for Chemiluminescence Cell Imaging: Distinctive Methodology for Design of Bright Chemiluminescent Dioxetane Probes. *ACS Cent. Sci.* **2017**, *3*, 349–358.
- (68) Green, O.; Gnaïm, S.; Blau, R.; Eldar-Boock, A.; Satchi-Fainaro, R.; Shabat, D. Near-Infrared Dioxetane Luminophores with Direct Chemiluminescence Emission Mode. *J. Am. Chem. Soc.* **2017**, *139*, 13243–13248.
- (69) Rainey, K. H.; Patterson, G. H. Photoswitching FRET to Monitor Protein–Protein Interactions. *Proc. Natl. Acad. Sci.* **2019**, *116*, 864–873.
- (70) Chung, C. Y. S.; Posimo, J. M.; Lee, S.; Tsang, T.; Davis, J. M.; Brady, D. C.; Chang, C. J. Activity-Based Ratiometric FRET Probe Reveals Oncogene-Driven Changes in Labile Copper Pools Induced by Altered Glutathione Metabolism. *Proc. Natl. Acad. Sci.* **2019**, *116*, 18285–18294.
- (71) Albers, A. E.; Okreglak, V. S.; Chang, C. J. A FRET-Based Approach to Ratiometric Fluorescence Detection of Hydrogen Peroxide. *J. Am. Chem. Soc.* **2006**, *128*, 9640–9641.
- (72) Zhang, X.; Xiao, Y.; Qian, X. A Ratiometric Fluorescent Probe Based on FRET for Imaging Hg²⁺ Ions in Living Cells. *Angew. Chem. Int. Ed.* **2008**, *47*, 8025–8029.
- (73) Ai, H. W.; Hazelwood, K. L.; Davidson, M. W.; Campbell, R. E. Fluorescent Protein FRET Pairs for Ratiometric Imaging of Dual Biosensors. *Nat. Methods* **2008**, *5*, 401–403.
- (74) Hananya, N.; Eldar Boock, A.; Bauer, C. R.; Satchi-Fainaro, R.; Shabat, D. Remarkable Enhancement of Chemiluminescent

- Signal by Dioxetane-Fluorophore Conjugates: Turn-ON Chemiluminescence Probes with Color Modulation for Sensing and Imaging. *J. Am. Chem. Soc.* **2016**, *138*, 13438–13446.
- (75) Hananya, N.; Press, O.; Das, A.; Scomparin, A.; Satchi-Fainaro, R.; Sagi, I.; Shabat, D. Persistent Chemiluminescent Glow of Phenoxy-Dioxetane Luminophore Enables Unique CRET-Based Detection of Proteases. *Chem. Eur. J.* **2019**, *25*, 14679–14687.
- (76) An, W.; Mason, R. P.; Lippert, A. R. Energy Transfer Chemiluminescence for Ratiometric PH Imaging. *Org. Biomol. Chem.* **2018**, *16*, 4176–4182.
- (77) Lumpi, D.; Wagner, C.; Schöpf, M.; Horkel, E.; Ramer, G.; Lendl, B.; Fröhlich, J. Fibre Optic ATR-IR Spectroscopy at Cryogenic Temperatures: In-Line Reaction Monitoring on Organolithium Compounds. *Chem. Commun.* **2012**, *48*, 2451–2453.
- (78) Kommreddy, A.; Bowsher, M. S.; Gunna, M. R.; Botha, K.; Vinod, T. K. Expedient Synthesis and Solvent Dependent Oxidation Behavior of a Water-Soluble IBX Derivative. *Tetrahedron Lett.* **2008**, *49*, 4378–4382.
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Supporting Information for: Quantitative ratiometric pH imaging using a 1,2-dioxetane chemiluminescence resonance energy transfer sensor

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Supporting Information:

1. General synthetic materials and methods	Page S1
2. Ratio-pHCL-1 <i>in vitro</i> characterization	Page S11
3. Cell culture and biological studies	Page S13
4. Ratiometric pH imaging	Page S14
5. Supplementary references	Page S15
6. Reported Spectra	Page S16

- 1. General synthetic materials and methods** All reactions were performed in dried glassware under an atmosphere of dry N₂. Silica gel P60 (SiliCycle) was used for column chromatography and SiliCycle 60 F254 silica gel (precoated sheets, 0.25 mm thick) was used for analytical thin layer chromatography. Plates were visualized by fluorescence quenching under UV light. Other reagents were purchased from Sigma-Aldrich (St. Louis, MO), Alfa Aesar (Ward Hill, MA), EMD Millipore (Billerica, MA), Oakwood Chemical (West Columbia, SC), and Cayman Chemical (Ann Arbor, MI) and used without further purification. ¹H NMR for compounds and ¹³C NMR for compounds were collected on a Bruker 400 MHz or a JEOL 500 MHz spectrometer in the Department of Chemistry at Southern Methodist University. ¹H and ¹³C NMR spectra for characterization of new compounds and monitoring reactions were collected in CDCl₃, acetone-d₆, methanol-d₄, or DMSO-d₆ (Cambridge Isotope Laboratories, Cambridge, MA). All chemical shifts are reported in the standard notation of parts per million using the peak of residual proton signals of the deuterated solvent as an internal reference. Coupling constant units are in Hertz (Hz) Splitting patterns are indicated as follows: br, broad; s, singlet; d, doublet; t,

triplet; q, quartet; m, multiplet; dd, doublet of doublets; dt, doublet of triplets. High resolution mass spectroscopy was performed on a Shimadzu IT-TOF (ESI source) at the Shimadzu Center for Advanced Analytical Chemistry at the University of Texas, Arlington, and low-resolution mass spectrometry was performed on an Advion Expression^L CMS (ESI source) at Southern Methodist University.

Scheme S1. Synthetic procedures for compounds **2**, **S6**, and **8**.

5-iodoisophthalic acid (S2).¹ In a 1L roundbottom flask equipped with a stir bar, 2-aminoterephthalic acid (50 mmol, 9.06 g, 1.0 equiv) was suspended in 300 mL 5.8 M HCl at 0 °C under N₂ atmosphere. Sodium nitrite (125 mmol, 8.63 g, 2.5 equiv) was dissolved in 100 mL H₂O and added dropwise via addition funnel over 1 hr. In a 2L roundbottom flask, sodium iodide (300 mmol, 45 g, 6.0 equiv) was dissolved. The contents of the 1L flask were then added to the sodium

iodide solution, upon which the reaction smoked and turned black. The reaction was stirred for another 18 hr. The reaction was then quenched with sodium bisulfite, and 450 mL DCM was added to the mixture. The solution was then filtered with filter paper, and the resulting brown solid was left to dry overnight, yielding 2-iodoterephthalic acid (48.94 mmol 14.29 g, 98%), which was used in the next step without further purification.

2

di-*tert*-butyl 5-iodoisophthalate (2).¹ in a 100 mL roundbottom flask equipped with a stir bar under N₂ atmosphere, 2-iodoterephthalic acid (7.23 mmol, 2111 mg, 1.0 equiv) and DMAP (1.44 mmol, 176 mg, 0.2 equiv) were dissolved in 2 mL DMF and 20 mL toluene. Di-*tert*-butyl dicarbonate (21.69 mmol, 4734 mg, 3.0 equiv) was added. The reaction was heated to 80 °C and stirred for 30 min. 1 equiv Boc anhydride was added to drive the reaction to completion and stirred for 30 more min. Upon completion, the reaction was allowed to cool to RT, washed with sat. NH₄Cl, eluted with 3x 30 mL EtOAc, dried with Na₂SO₄, and concentrated under reduced pressure. Column chromatography in 5% EtOAc/Hexanes yielded compound **2** (5.35 mmol, 74%) as a yellow oil. ¹H NMR (400 MHz, CDCl₃) δ 8.52 (d, 1H, *J* = 1.2 Hz), 7.97 (dd, 1H, *J*₁ = 8.0 Hz, *J*₂ = 1.2 Hz), 7.67 (d, 1H, *J* = 8.0 Hz), 1.65 (s, 9H), 1.62 (s, 9H).

3

***tert*-butyl-3,6-bis((*tert*-butyldimethylsilyl)oxy)-10,10-dimethyl-3'-oxo-3'H,10H-**

spiro[anthracene-9,1'-isobenzofuran]-5'-carboxylate (3**).²** In a 25 mL oven-dried roundbottom flask equipped with a stir bar, compound **1** (0.46 mmol, 220 mg, 1.0 equiv) and compound **2** (1.6 mmol, 647 mg, 3.5 equiv) were added, and purged 3x with N₂. 6 mL anhydrous THF was then added, and the reaction contents were cooled to 0 °C. 2.6 M *n*-BuLi (1.76 mmol, 0.68 mL, 3.85 equiv) was added dropwise, over which period the reaction changed from light yellow to deep purple in color. The reaction was stirred 10 min, then allowed to warm to RT and stirred for 2 hr. The reaction was then quenched with 5 mL H₂O, washed with brine/1M HCl, eluted 2x with 20 mL EtOAc, dried with Na₂SO₄, filtered, and concentrated under reduced pressure. Column chromatography (3–5% EtOAc/Hexanes) yielded compound **3** (0.231 mmol, 109 mg, 35%) as a white solid. ¹H NMR (400 MHz, CDCl₃) δ 8.17 (dd, 1H, *J* = 8.0 Hz, *J* = 1.2 Hz), 8.04 (d, 1H, *J* = 8.0 Hz), 7.64 (s, 1H), 7.10 (s, 2H), 6.62 (d, 4H, *J* = 1.2 Hz), 1.83 (s, 3H), 1.74 (s, 3H), 1.56 (s, 9H), 1.01 (s, 18H), 0.24 (s, 12H).

S4

***tert*-butyl 3-iodo-4-methylbenzoate (**S4**).** 3-Iodo-4-methylbenzoic acid (**S3**) (7.63 mmol, 2.00 g, 1.0 equiv), di-*tert*-butyl dicarbonate (19.0 mmol, 4.10 g, 2.5 equiv), and DMAP (1.53 mmol, 0.19 g, 0.2 equiv) were combined in a roundbottom flask and dissolved in 20 mL anhydrous THF. The reaction was heated to 60 °C and stirred under reflux for 20 hours. After cooling the reaction to RT, the solvent was evaporated off under reduced pressure and the residue was dissolved in Et₂O, which was then washed with sat. aq. NaHCO₃ (x3), H₂O, and brine. The organic layer was dried over Na₂SO₄ and concentrated. Purification via column chromatography with 10% EtOAc/Hexanes yielded the product as a golden oil (6.82 mmol, 2.17 g, 89%). ¹H NMR (500 MHz, CDCl₃) δ 8.36 (d, *J* = 1.7 Hz, 1H), 7.81 (dd, *J* = 1.7, 8.0 Hz, 1H), 7.22 (d, *J* = 8.0 Hz, 1H), 2.42 (s, 3H), 1.55 (s, 9H); ¹³C NMR (125 MHz, CDCl₃) δ 164.23, 146.18, 139.90, 131.25, 129.40, 129.23, 100.51, 81.41, 81.02, 28.40, 28.24, 27.97.

S6

N-(10-(5-carboxy-2-methylphenyl)-7-(dimethylamino)-5,5-dimethyldibenzo[b,e]silin-3(5H)-ylidene)-N-methylmethanaminium (S6). Compound **S4** (4.93 mmol, 1.57 g, 4.0 equiv) and silicon anthrone **S5**³ (1.23 mmol, 0.400 g, 1.0 equiv), prepared according to literature procedure, were added to an oven dried flask, which was evacuated and flushed with nitrogen. Dry THF (25 mL) was added to the flask and the reaction was stirred until starting materials dissolved. The reaction was then cooled to 0 °C, and 1.5M *n*-BuLi in hexanes (5.25 mmol, 3.5 mL, 4.2 equiv) was added dropwise, and a color change from yellow to orange was observed. The reaction was stirred for an additional 3 hours, allowing the temperature to rise to RT. TLC in 15% EtOAc/Hexanes indicated disappearance of the anthrone starting material and formation of a deep blue colored product. 1M HCl was then added to quench the reaction, resulting in an intense blue solution of pH = 1. Saturated aqueous NaHCO₃ was added to neutralize the reaction, which was then extracted with DCM. The blue organic layer was washed with brine, dried over Na₂SO₄ and concentrated to a blue solid. This crude *tert*-butyl ester product was dissolved in DCM (15 mL) and trifluoroacetic acid (3 mL) was added. The reaction was stirred at RT for 3.5 hours, while monitoring product formation via mass spectroscopy. The volatiles were then evaporated under reduced pressure. The product was isolated using column chromatography with 10% MeOH/DCM to obtain **S6** as a blue solid (0.851 mmol, 0.377 g, 69%). ¹H NMR (500 MHz, CD₃OD) δ 8.10 (dd, *J* = 2.3, 8.0 Hz, 1H), 7.72 (d, *J* = 1.7 Hz, 1H), 7.53 (d, *J* = 8.0 Hz, 1H), 7.37 (d, *J* = 2.3 Hz, 2H), 7.02 (d, *J* = 9.7 Hz, 2H), 6.77 (dd, *J* = 2.9, 9.7 Hz, 2H), 3.34 (s, 12H), 2.10 (s, 3H), 0.62 (s, 3H), 0.60 (s, 3H); ¹³C NMR (125 MHz, CD₃OD) δ 169.05, 168.86, 155.78, 149.51, 142.60, 141.93, 140.34, 131.72, 131.23, 131.14, 129.78, 128.28, 122.35, 115.41, 40.94, 19.65, -1.10, -1.31.

4

3,6-dihydroxy-10,10-dimethyl-3'-oxo-3'H,10H-spiro[anthracene-9,1'-isobenzofuran]-5'-carboxylic acid (4).² In a 20 dram vial equipped with a stir bar, Compound **3** (0.278 mmol, 191 mg) was dissolved in 10 mL DCM. 2 mL TFA was added, and the reaction was stirred at RT under N₂ atmosphere for 6 hr. Upon completion of the reaction as determined by TLC, the reaction was evaporated onto silica gel. Column chromatography (5% MeOH/DCM, 1% AcOH) afforded compound **4** (0.189 mmol, 76 mg, 68%) as a red-orange solid. ¹H NMR (400 MHz, Acetone-D₆) δ 8.29 (d, 1H, *J* = 8.0 Hz), 8.10 (d, 1H, *J* = 8.0 Hz), 7.67 (s, 1H), 7.22 (d, 2H, *J* = 2.4 Hz), 6.71 (dd, 2H, *J* = 8.8 Hz, *J* = 2.4 Hz), 6.65 (d, 2H, *J* = 8.8 Hz); ¹³C NMR (100 MHz, Acetone-D₆) δ 171.8, 169.0, 158.1, 155.7, 147.1, 130.5, 129.8, 129.3, 125.0, 124.7, 122.3, 114.9, 112.8, 86.5, 38.0, 34.2, 32.6, 19.7; HRMS calcd for C₂₄H₁₈O₆ [M+H]⁺ 403.1176, found 403.1163.

5

3,6-diacetoxy-10,10-dimethyl-3'-oxo-3'H,10H-spiro[anthracene-9,1'-isobenzofuran]-5'-carboxylic acid (5).² Compound **4** (0.189 mmol, 76 mg, 1.0 equiv) was dissolved in 2 mL Ac₂O in a 20 mL roundbottom flask equipped with a stir bar. Pyridine (0.982 mmol, 76 μL, 5.0 equiv) was added. The reaction was heated to 80 °C and stirred for 1 hr until TLC showed disappearance of starting material, and appearance of two spots, indicating a mixture of the generated mixed anhydride and free acid. The crude mixture was washed in 1 M HCl, eluted with 2x 10 mL DCM, dried with Na₂SO₄, and concentrated under reduced pressure. The crude was then dissolved in 10

mL 1:1 THF H₂O mixture. Pyridine (0.982 mmol, 76 μ L, 5.0 equiv) and Et₃N (0.189 mmol, 30 μ L, 1.0 equiv) was added. The reaction was heated to 80 °C and stirred for 1 hr, at which point TLC showed disappearance of the mixed anhydride. Column chromatography in 50% EtOAc/Hexanes and 1% AcOH yielded **5** (0.121 mmol, 59 mg, 64%) as a white foam. ¹H NMR (400 MHz, CDCl₃) δ 8.30 (d, 1H, *J* = 8.0 Hz), 8.12 (d, 1H, *J* = 8.0 Hz), 7.75 (s, 1H), 7.40 (d, 1H, *J* = 1.6 Hz), 7.40 (s, 1H), 6.92 (dd, 2H, *J* = 8.8 Hz, *J* = 2.4 Hz), 6.76 (d, 1H, *J* = 8.8 Hz), 2.33 (s, 6H), 1.87 (s, 3H), 1.77 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 169.4, 169.2, 169.2, 154.7, 141.5, 146.7, 135.8, 131.3, 130.1, 129.1, 128.1, 125.7, 125.6, 120.7, 119.5, 85.7, 38.6, 34.9, 32.8, 29.1, 21.2; HRMS calcd for C₂₈H₂₂O₈ [M+Na]⁺ 509.1207, found 509.1218.

6

5'-(4-(tert-butoxycarbonyl)piperazine-1-carbonyl)-10,10-dimethyl-3'-oxo-3'H,10H-spiro[anthracene-9,1'-isobenzofuran]-3,6-diyl diacetate (6). In a 25 mL roundbottom flask equipped with a stir bar under N₂ atmosphere, Compound **5** (0.119 mmol, 58 mg, 1.0 equiv) was dissolved in 8 mL DCM. The reaction was cooled to 0 °C. DIPEA (0.155 mmol, 27 μ L, 1.3 equiv) and *N,N,N',N'*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU) (0.142 mmol, 54 mg, 1.2 equiv) were then added and stirred for 10 min, upon which the HBTU activated ester was visible by TLC. *tert*-Butyl piperazine-1-carboxylate (0.143 mmol, 27 mg, 1.2 equiv) was then added, and the reaction was allowed to stir overnight. Upon completion, the reaction was washed in brine/NH₄Cl, eluted with 2x 20 mL DCM, dried with Na₂SO₄, filtered, and concentrated under reduced pressure. Column chromatography (50–75% EtOAc/Hexanes) yielded Compound **6** (0.10 mmol, 66 mg, 85%) as a white foam. ¹H NMR (500 MHz, CDCl₃) δ 8.01 (d, 1H, *J* = 8.0 Hz), 7.56 (d, 1H, *J* = 8.0 Hz), 7.33 (s, 2H), 7.02 (s, 1H), 6.86 (d, 2H, *J* = 4.0 Hz), 6.73 (d, 2H, *J* = 4.0 Hz), 3.18-3.76 (m, 8H), 2.25 (s, 6H), 1.79 (s, 3H), 1.78 (s, 3H), 1.38 (s, 9H); ¹³C

NMR (125 MHz, CDCl₃) δ 169.11, 168.5, 155.1, 154.4, 151.6, 146.7, 142.0, 129.1, 128.2, 122.7, 120.8, 119.6, 85.5, 80.5, 69.3, 53.9, 38.6, 35.0, 32.6, 29.3, 28.4, 21.2; HRMS calcd for C₃₇H₃₈N₂O₉ [M+Na]⁺ 677.2470, found 677.2468.

7

10,10-dimethyl-3'-oxo-5'-(piperazine-1-carbonyl)-3'H,10H-spiro[anthracene-9,1'-isobenzofuran]-3,6-diyl diacetate (7). in a 20 mL vial, compound **6** was dissolved in 5 mL DCM. 1 mL TFA was added under N₂ atmosphere at RT and stirred for 2 hr. Upon completion as monitored by TLC, 5 mL toluene was added, and the reaction contents were evaporated. The crude product was triturated 2x with 5 mL MeOH to obtain compound **7** (0.098 mmol, 54 mg, 98%) as a tan foam. ¹H NMR (400 MHz, CDCl₃) δ 8.04 (d, 1H, *J* = 7.2 Hz), 7.59 (d, 1H, *J* = 7.2 Hz), 7.38 (d, 2H, *J* = 1.6 Hz), 7.073 (s, 1H), 6.90 (dd, 2H, *J* = 8.8 Hz, *J* = 1.6 Hz), 6.76 (d, 2H, *J* = 8.8 Hz), 3.42-3.96 (m, 4H), 3.13 (bs, 4H), 2.29 (s, 6H), 1.84 (s, 3H) 1.75 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 169.5, 169.3, 168.6, 155.5, 151.6, 146.5, 140.3, 128.9, 128.3, 127.7, 127.4, 126.1, 122.8, 120.9, 119.7, 85.6, 43.3, 38.4, 34.7, 33.1, 21.1; HRMS calcd for C₃₂H₃₀N₂O₇ [M+Na]⁺ 577.1945, found 577.1927.

8

(E)-3-(2-acetoxy-4-(((1R,3R,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chlorophenyl)acrylic acid (8). Compound **S3**⁴ (0.368 mmol, 138 mg, 1.0 equiv) was dissolved in

5 mL acetic anhydride in a 20 mL roundbottom flask equipped with a stir bar. Pyridine (1.84 mmol, 0.148 mL, 5.0 equiv) was added. The reaction was heated to 80 °C and stirred for 30 min until TLC showed disappearance of starting material, and appearance of two spots, indicating a mixture of the generated acrylic anhydride and free acid. The crude mixture was washed in 1 M HCl, eluted with 2x 10 mL DCM, dried with Na₂SO₄, and concentrated under reduced pressure. The crude was then dissolved in 10 mL 1:1 THF H₂O mixture. Pyridine (1.84 mmol, 0.148 mL, 5.0 equiv) and Et₃N (0.368 mmol, 51 µL, 1.0 equiv). was added. The reaction was heated to 80 °C and stirred for 20 min, at which point TLC showed disappearance of the mixed anhydride. Column chromatography in 50% EtOAc/Hexanes and 1% AcOH yielded **8** (116 mg, 76%) as a yellow foam. ¹H NMR (400 MHz, CDCl₃) δ 10.58 (bs, 1H), 7.74 (d, 1H, *J* = 16.0 Hz), 7.57 (d, 1H, *J* = 8.0 Hz), 7.24 (d, 1H, *J* = 8.0 Hz), 6.49 (d, 1H, *J* = 16.0 Hz), 3.34 (s, 3H), 3.30 (s, 1H), 2.47 (s, 3H) 1.61–2.13 (m, 12H); ¹³C NMR (100 MHz, CDCl₃) δ 171.4, 168.1, 146.4, 139.5, 138.9, 138.3, 133.3, 129.5, 129.1, 128.5, 125.1, 120.6, 60.5, 57.4, 39.2, 39.0, 38.6, 37.0, 32.9, 29.8, 28.3, 28.3, 20.6, 20.6, 20.5, 14.9; HRMS calcd for C₂₃H₂₅ClO₅ [M+Na]⁺ 439.1283, found 439.1287.

9

5'-((4-((E)-3-(2-acetoxy-4-(((1R,3R,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chlorophenyl)acryloyl)piperazine-1-carbonyl)-10,10-dimethyl-3'-oxo-3'H,10H-

spiro[anthracene-9,1'-isobenzofuran]-3,6-diyl diacetate (9). In a 25 mL roundbottom flask equipped with a stir bar under N₂ atmosphere, Compound **8** (0.055 mmol, 23 mg, 1.0 equiv) was dissolved in 2 mL DCM. The reaction was cooled to 0 °C. DIPEA (0.072 mmol, 13 µL, 1.3 equiv) and HBTU (0.142 mmol, 54 mg, 1.2 equiv) were then added and stirred for 10 min, upon which the HBTU activated ester was visible by TLC. Compound **7** (0.055 mmol, 30 mg, 1.0 equiv) was then added, and the reaction was allowed to stir overnight. Upon completion, the reaction was

washed in brine/ NH_4Cl , eluted with 2x 20 mL DCM, dried with Na_2SO_4 , filtered, and concentrated under reduced pressure. Column chromatography (75–100% EtOAc/Hexanes) yielded Compound **9** (0.10 mmol, 8 mg, 85%) as a white solid. ^1H NMR (400 MHz, CDCl_3) δ 8.10 (d, 1H, $J = 7.6$ Hz) 7.65 (m, 2H), 7.53 (d, 1H, 8.0 Hz), 7.38 (s, 1H), 7.18 (d, 1H, $J = 8.0$ Hz), 6.79–6.94 (m, 6H), 4.52–4.85 (m, 6H) 3.25–3.36 (m, 6H), 2.41 (s, 3H), 2.31 (s, 6H), 1.61–2.16 (m, 12H); ^{13}C NMR (100 MHz, CDCl_3) δ 169.1, 168.5, 168.2, 164.9, 151.5, 146.1, 141.5, 139.0, 137.5, 136.4, 133.0, 129.3, 129.0, 128.6, 128.1, 126.1, 124.5, 120.8, 119.6, 60.4, 57.3, 56.0, 39.22, 38.5, 37.0, 34.9, 32.9, 29.7, 28.3, 28.1, 21.2, 21.1, 20.6, 14.2; HRMS calcd for $\text{C}_{55}\text{H}_{53}\text{ClN}_2\text{O}_{11}$ $[\text{M}+\text{H}]^+$ 953.3411, found 953.3459.

10

5'-((E)-3-(4-(((1R,3R,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chloro-2-hydroxyphenyl)acryloyl)piperazine-1-carbonyl)-3,6-dihydroxy-10,10-dimethyl-3'H,10H-spiro[anthracene-9,1'-isobenzofuran]-3'-one (10**).** In a 20-dram vial, compound **9** (0.021 mmol, 20 mg) was dissolved in 2 mL THF, and 2 mL 10 M LiOH was added under N_2 atmosphere at RT. The reaction was stirred for 2.5 hr. upon completion as determined by TLC, the reaction was washed in brine, and 30 mL 1M HCL was then added. The organic layer was eluted with 2x 10 mL EtOAc, then 2x 10 mL DCM, dried with Na_2SO_4 , filtered, and concentrated under reduced pressure, yielding **10** (18 mg, 0.021 mmol, quantitative) as a red solid without further purification. ^1H NMR (400 MHz, Acetone- d_6) δ 8.06 (d, 1H, $J = 7.6$ Hz), 7.96 (d, 1H, $J = 15.6$ Hz), 7.21(m, 1H), 6.86 (d, 4H, $J = 8.0$ Hz), 6.72 (d, 1H, $J = 2.0$ Hz) 6.70 (d, 2H, $J = 2.0$ Hz), 6.63 (d, 1H, $J = 8.8$ Hz), 3.50–3.92 (m, 6H), 3.28 (s, 6H), 2.65–3.15 (m, 12H); ^{13}C NMR (100 MHz, CDCl_3) δ 171.2, 169.0, 168.1, 165.1, 158.2, 155.5, 151.4, 147.4, 140.3, 136.5, 136.2, 130.3, 129.2, 128.2, 125.7, 125.2, 125.0, 124.0, 123.1, 122.8, 122.5, 119.8, 118.9, 114.8, 112.7, 86.6, 56.1, 38.7, 38.5,

38.1, 37.9, 36.8, 34.6, 32.9, 19.6; HRMS calcd for C₄₉H₄₇ClN₂O₈ [M+H]⁺ 827.3094, found 827.3089.

Ratio-pHCL-1

5'-(4-((E)-3-(3-chloro-2-hydroxy-4-((1r,3r,5r,7r)-4'-methoxyspiro[adamantane-2,3'-[1,2]dioxetan]-4'-yl)phenyl)acryloyl)piperazine-1-carbonyl)-3,6-dihydroxy-10,10-dimethyl-3'H,10H-spiro[anthracene-9,1'-isobenzofuran]-3'-one (Ratio-pHCL-1). In a 2-neck roundbottom flask, compound **10** (0.021 mmol), was dissolved in 2 mL THF at 0 °C. 2 mg methylene blue was added. The reaction was irradiated under yellow light for 1 hr and monitored by LRMS. Upon completion, the crude was evaporated onto silica gel and loaded for column chromatography in 5% MeOH/DCM, yielding **Ratio-pHCL-1** (16 mg, 90%) as a white solid. ¹H NMR (400 MHz, Acetone-d₆) δ 8.06 (d, 1H, *J* = 8.0 Hz), 7.94 (d, 1H, *J* = 16.0 Hz), 7.76 (dd, 1H, *J* = 8.0 Hz, *J* = 1.2 Hz), 5.64 (d, 1H, *J* = 7.6 Hz), 7.35 (bs, 1H), 7.20 (m, 4H) 6.70 (dd, 2H, *J* = 8.8 Hz, *J* = 2.4 Hz), 3.50–4.00 (m, 4H), 3.19, (s, 3H), 2.96 (s, 1H), 2.75 (s, 4H), 2.062 (s, 3H), 2.71 (s, 3H); ¹³C NMR (100 MHz, Acetone-d₆) δ 168.9, 168.1, 164.8, 158.1, 155.5, 152.6, 150.5, 147.3, 142.7, 140.5, 136.5, 136.9, 133.2, 129.3, 128.2, 127.5, 125.8, 125.0, 123.9, 122.8, 122.5, 120.2, 114.7, 112.6, 112.0, 111.5, 95.5, 86.5, 67.17, 48.8, 38.1, 37.8, 36.3, 34.6, 33.5, 32.4, 31.9, 26.2, 25.9, 25.3; HRMS calcd for C₄₉H₄₇ClN₂O₁₀ [M+H]⁺ 859.2992, found 859.3017.

2. Ratio-pHCL-1 *in vitro* characterization

Calibration of Ratio-pHCL-1. Chemiluminescent emission spectra were acquired using a Hitachi F-7000 fluorescence spectrophotometer via the luminescence detection module and scanning luminescence emission from 400–900 nm in response to exposure of **Ratio-pHCL-1** to pH buffered solutions. **Ratio-pHCL-1** in DMSO was added to a solution of 100 mM Tris, PBS or

carbonate buffers ranging from pH 6.81–10.02. For concentration dependence, 10, 20, and 40 μM **Ratio-pHCL-1** in 5% DMSO were subjected to 100 mM PBS buffered to pH 6.81, 7.45, and 8.02. For time dependence, 20 μM **Ratio-pHCL-1** in 5% DMSO was subjected to 100 mM PBS buffered to pH 6.81, 7.45, and 8.02, and the chemiluminescence emission spectra was measured at $t = 0, 10, 20$ and 30 min. For DMSO dependence, 20 μM **Ratio-pHCL-1** in a final concentration of 1, 5, or 10% DMSO was subjected to 100 mM PBS buffered to pH 6.81, 7.45, or 8.02. Each experiment was independently conducted 3 times, and analysis of each experiment was conducted by dividing emission intensity values at 580 nm over emission intensity values at 530 nm from the emission plot.

Figure S1. Ratio of chemiluminescence emission intensities at 580 nm and 530 nm of 20 μM **Ratio-pHCL-1** and 5% DMSO in aqueous buffer (pH = 6.81–10.02). All measurements were taken in 100 mM PBS, 100 mM Tris, or 100 mM carbonate buffer, and measurements were taken 20 s after mixing. Error bars are $\pm$ SD from $n = 3$ independent experiments.

Ratio-pHCL-1 kinetics. Kinetics data were acquired using a Biotek Cytation 5[®] plate reader using the luminescence detection mode, end point read type. The temperature was set at 37 °C under ambient atmosphere. The gain was set at 135 and the read height was set at 4.5 mm. In a 96-well plate, 1.0 μL of 0.25 mM **Ratio-pHCL-1** was added to wells containing a solution of 237.5 μL 100 mM PBS (pH = 7.45) and 11.5 μL DMSO to create a final concentration of 1.0 μM , 5% DMSO. The plate was then immediately placed into the plate reader and allowed to stir for 1 min. Luminescence data was acquired every 2 min for 4 hr.

Figure S2. Kinetic emission plot of 1.0 μM **Ratio-pHCL-1** in 100 mM PBS, pH 7.44, 5% DMSO, 37 °C.

3. Cell culture and biological studies. Human lung adenocarcinoma epithelial cells (A549) cells were purchased from ATCC and cultured in Ham's F-12K (Kaighn's) Medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotics (penicillin/streptomycin, 100 U/mL). Cells were maintained in a humidified incubator at 37 °C with 5% CO₂. One or two days before the experiment, cells were passaged and plated on Costar® 12-well plates by adding 150K–200K of cells per well, filling each well up to 1 mL of media.

Ratio-pHCL-1 cell penetration A549 cells were seeded in a 12 well plate, and maintained at 37 °C with 5% CO₂, and monitored until the plate reached 80% confluency. The cells were then washed with 1x PBS, and 996 μL complete F12k was added. Next, 4.0 μL of 5 mM **Ratio-pHCL-1** in DMSO was added to obtain a final concentration of 20 μM **Ratio-pHCL-1** incubated for $t = 80$ min. Upon completion of incubation, cells were washed twice with 1x PBS and reconstituted with 1000 μL incomplete F12K, or were given no wash, and the plate was imaged with an EVOS microscope, and images were taken using GFP and RFP filters with the 20x objective. Images taken with the GFP filter were uniformly processed using Image J with contrast changed to min = 18, max = 112, and RFP images were uniformly processed using Image J with contrast changed to min = 66, max = 135.

Figure S3. (A–B) Fluorescence microscopy images using a GFP filter set of A549 cells with (A) no probe and (B) 20 μ M **Ratio-pHCL-1** incubated in cells for 80 min, followed by wash step. (C–D) Fluorescence microscopy images using a RFP filter set of A549 cells with (A) no probe and (B) 20 μ M **Ratio-pHCL-1** incubated in cells for 80 min, followed by wash step.

4. Ratiometric pH imaging. Chemiluminescent images were acquired with an IVIS Spectrum (Caliper, Waltham, MA) using the “Luminescent” and “Photograph” mode. For *in vitro* 96 well plate measurements, the exposure time was set as 0.5 seconds, binning was set to medium, F/stop was set as 4, FOV was set as C (12.9 cm), Excitation was blocked and the sequence was set for the emission mode, and images were acquired sequentially first using a 580 nm bandpass filter, then a 540 nm bandpass filter. A final concentration of 1 μ M **Ratio-pHCL-1** was added in a snake-like fashion (left to right across pH gradient, then down one row, right to left across pH gradient, etc.) to 100 mM buffer ranging from pH 6.81–8.42. Image analysis was carried out by setting individual ROI’s to each well for both 580 and 540 nm filter images. The ROI’s were then measured, which gave a total flux (p/s) for each ROI, the flux value at 580 nm was divided by the flux at 540 nm for each well.

***In vitro* pH measurement through tissue surrogate.** For measurements through tissue surrogate (bologna, Kroger, Dallas, TX), the exposure time was set as 10.0 seconds, binning was set to medium, F/stop was set to 1, FOV was set to C (12.9 cm), Excitation was blocked and the sequence was set for the emission mode. Images were acquired sequentially first using a 580 nm bandpass filter, then a 540 nm bandpass filter. A final concentration of 20 μ M **Ratio-pHCL-1** was added in a snake-like fashion (left to right across pH gradient, then down one row, right to left across pH gradient, etc.) to 100 mM buffer ranging from pH 6.81–8.42. Then, 1 slice of bologna measuring 2.8 mm thickness (Oscar Meyer) was placed on top of the 96-well plate, and luminescence was

measured through the meat sample. All images were analyzed using Living Image software. Image analysis was carried out by setting individual ROI's to each well for both 580 and 540 nm filter images. The ROI's were then measured, which gave a total flux (p/s) for each ROI, the flux value at 580 nm was divided by the flux at 540 nm for each well.

Figure S4. Chemiluminescence intensity images of the pH dependent emission of 20 μ M **Ratio-pHCL-1** in 100 mM PBS or Tris (pH 6.81–8.42) containing 5% DMSO using (A) 580 nm bandpass filter or (B) 540 nm bandpass filter in an IVIS Spectrum with bologna slice (2.8 mm thickness) covering 96 well plate. (C) Ratio of total flux (p/s) at 580 nm and 540 nm. $n = 9$ technical replicates across 3 independent experiments, error bars are $\pm$ SD.

Sacrificed mouse imaging. A solution of 950 μ L of 1.0 M PBS (pH 5.99–7.99) was injected into the peritoneal cavity of recently sacrificed CL6B57 mice aged 6–12 weeks old, then 50 μ L of 400 μ M Ratio-pHCL-1 in DMSO was injected shortly after to achieve an approximate final concentration of 20 μ M Ratio-pHCL-1 with 5% DMSO. The mice were imaged immediately after injection of the probe with a 580 nm and 540 nm filter with capture settings set to autoexposure and FOV set to C (12.9 cm).

5. Supplementary References

- (1) Kommreddy, A.; Bowsher, M. S.; Gunna, M. R.; Botha, K.; Vinod, T. K. Expedient Synthesis and Solvent Dependent Oxidation Behavior of a Water-Soluble IBX Derivative. *Tetrahedron Lett.* **2008**, 49, 4378–4382.
- (2) Grimm, J. B.; Sung, A. J.; Legant, W. R.; Hulamm, P.; Matlosz, S. M.; Betzig, E.; Lavis, L. D. Carbofluoresceins and Carborhodamines as Scaffolds for High-Contrast Fluorogenic Probes. *ACS Chem. Biol.* **2013**, 8, 1303–1310.

- (3) Pastierik, T.; Šebej, P.; Medalová, J.; Štacko, P.; Klán, P. Near-Infrared Fluorescent 9-Phenylethynylpyronin Analogues for Bioimaging. *J. Org. Chem.* **2014**, *79*, 3374–3382.
- (4) Ryan, L. S.; Gerberich, J.; Cao, J.; An, W.; Jenkins, B. A.; Mason, R. P.; Lippert, A. R. Kinetics-Based Measurement of Hypoxia in Living Cells and Animals Using an Acetoxymethyl Ester Chemiluminescent Probe. *ACS Sensors* **2019**, *4*, 1391–1398.

6. Reported Spectra

Figure S5. ¹H NMR spectrum (400 MHz, Acetone-D₆) of **4**.

Figure S6. ^{13}C NMR spectrum (100 MHz, Acetone-D₆) of **4**.

Figure S7. ^1H NMR spectrum (400 MHz, CDCl_3) of **5**

Figure S8. ^{13}C NMR spectrum (100 MHz, CDCl_3) of **5**

Figure S11. ¹H NMR spectrum (400 MHz, CDCl₃) of **7**

Figure S12. ¹³C NMR spectrum (100 MHz, CDCl₃) of **7**.

Figure S13. ¹H NMR spectrum (400 MHz, CDCl₃) of **8**.

Figure S14. ¹³C NMR (100 MHz, CDCl₃) of **8**.

Figure S15. ¹H NMR spectrum (400 MHz, CDCl₃) of **9**.

Figure S16. ^{13}C NMR (100 MHz, CDCl_3) of **9**.

Figure S17. ^1H NMR spectrum (400 MHz, Acetone- D_6) of **10**.

Figure S18. ^{13}C NMR spectrum (100 MHz, Acetone- D_6) of **10**.

Figure S19. ^1H NMR spectrum (400 MHz, Acetone- D_6) of **Ratio-pHCL-1**.

Figure S20. ^{13}C NMR spectrum of **Ratio-pHCL-1**.

LRyan et al ChemRiv Ratio-pHCL-1 SI.pdf (2.67 MiB)

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