Imaging the Tumor Microenvironment Using 1,2-Dioxetane Chemiluminescence Agents

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IMAGING THE TUMOR MICROENVIRONMENT USING 1,2-DIOXETANE CHEMILUMINESCENCE AGENTS

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IMAGING THE TUMOR MICROENVIRONMENT USING 1,2-DIOXETANE CHEMILUMINESCENCE AGENTS

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in
Partial Fulfillment of the Requirements
for the degree of
Doctor of Philosophy
with a
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by
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Lucas S. Ryan

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As complex organisms, animals and humans rely on a delicate interplay between many connected systems to maintain proper cellular and whole-body function. Oxygen regulation and homeostasis are vital for proper function in healthy cells, as are core components of aerobic metabolism and efficient ATP generation to supply our energy needs. Maintaining pH homeostasis is also of primary importance; cells maintain pH homeostasis through a host of active and passive transporters to maintain pH balance both inside and outside of the cell.

Events of uncontrolled cellular proliferation, also known as cancer, survive and thrive under conditions that perturb the delicate balance of both oxygen and pH homeostasis. Because tumors rapidly divide, they outgrow the vasculature that supplies them with the nutrients they require, including $O_2$. This causes the hypoxia dependent expression of enzymes that shifts the tumor microenvironment into anaerobic metabolic states, resulting in the overproduction of lactic acid and concomitant decrease in extracellular pH within the tumor relative to that of healthy cells.
This thesis is focused on the development of chemical tools to image oxygen and pH (mis)regulation within the tumor microenvironment. To accomplish this, we use chemiluminescent 1,2-dioxetane compounds that emit light in response to analytes within their chemical environment. Specifically, we developed an activity-based hypoxia sensing chemiluminescence probe that emits light under low O2 conditions. HyCL-4-AM provides a selective 60,000-fold increase in luminescence emission in the presence of rat liver microsomes (RLM), and provides highly sensitive and reproduceable O2 dependent emission in cells. Whole animal imaging experiments in muscle tissue and tumor xenografts show that HyCL-4-AM can differentiate between well oxygenated muscle tissue and hypoxic tumors, demonstrating potential for monitoring tumor reoxygenation via hyperoxic treatment.

Furthermore, we report a chemiluminescence resonance energy transfer (CRET) probe Ratio-pHCL-1, that transfers energy from the dioxetane substrate to a pH responsive fluorophore. The probe provides an accurate measurement of pH between 6.8 and 8.4, making it a viable tool for measuring pH in biological systems. Using an IVIS Spectrum, pH can be measured through tissue with Ratio-pHCL-1, which is shown in vitro and calibrated in sacrificed mouse models. Intraperitoneal injections of Ratio-pHCL-1 into live mice show high photon outputs and consistent increases in flux ratio when measured at pH 6, 7, and 8. This design could ultimately be used to study pH heterogeneity within the tumor microenvironment.

Finally, we report UVC-454, UVA-454, and Spiro-CL as photoactivatable chemiluminescence compounds. UVC-454 and UVA-454 are protected with ortho-nitrobenzyl protecting groups that provide irreversible photochemical uncaging of the chemiluminophore species through UV light irradiation. UVC-454 and UVA-454 can be selectively activated based on uncaging wavelength, and demonstrate ability to be photoactivated in water. Spiro-CL is a
novel chemiluminescent spiropyran that can reversibly interconvert from its stable spiropyran form to a metastable merocyanine form through UV or visible light irradiation, respectively. Further, this compound exhibits chemiluminescence in its open form upon irradiation with UV light in DMSO.
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<tr>
<td>A549</td>
<td>Adenocarcinomic human alveolar basal epithelial cells</td>
</tr>
<tr>
<td>ABPP</td>
<td>Activity based protein profiling</td>
</tr>
<tr>
<td>Acetone-d6</td>
<td>Deuterated acetone</td>
</tr>
<tr>
<td>AcOH</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>ACT</td>
<td>Activator</td>
</tr>
<tr>
<td>AM</td>
<td>Acetoxymethyl</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosyl monophosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-bis(o-aminophenoxy)ethane-N,N,-N’,N’-tetraacetic acid</td>
</tr>
<tr>
<td>BODIPY</td>
<td>Boron dipyrromethene</td>
</tr>
<tr>
<td>BOLD MRI</td>
<td>Blood oxygen level dependent magnetic resonance imaging</td>
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<tr>
<td>BRET</td>
<td>Bioluminescence resonance energy transfer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CAF</td>
<td>Cancer associated fibroblasts</td>
</tr>
<tr>
<td>CEST</td>
<td>Chemical exchange saturation transfer</td>
</tr>
<tr>
<td>CIEEL</td>
<td>Chemically initiated electron exchange luminescence</td>
</tr>
<tr>
<td>CDCl₃</td>
<td>Deuterated chloroform</td>
</tr>
<tr>
<td>CF₄</td>
<td>Carbon tetrafluoride</td>
</tr>
<tr>
<td>CIAKI</td>
<td>Contrast-induced acute kidney injury</td>
</tr>
<tr>
<td>CL</td>
<td>Chemiluminescence or Chemiluminescent</td>
</tr>
<tr>
<td>CRET</td>
<td>Chemiluminescence resonance energy transfer</td>
</tr>
<tr>
<td>CT26</td>
<td>N-nitroso-N-methylurethane-(NNMU) induced, undifferentiated colon carcinoma cell line.</td>
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<tr>
<td>CTAB</td>
<td>Cetyltrimethylammonium bromide</td>
</tr>
<tr>
<td>Abbr.</td>
<td>Name</td>
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<tr>
<td>-------</td>
<td>-----------------------------</td>
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<tr>
<td>CyP450</td>
<td>Cytochrome P450 oxidase</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DCMC</td>
<td>Dicyanomethylchromone</td>
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<tr>
<td>DEAD</td>
<td>Diethyl azodicarboxylate</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-Diisopropylethylamine</td>
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<tr>
<td>DMAP</td>
<td>4-Dimethylaminopyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-Dimethylformamide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPI</td>
<td>Diphenyl iodonium cation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethyl-enediaminetetraacetic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMSO-d6</td>
<td>Deuterated dimethyl sulfoxide</td>
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<tr>
<td>ECL</td>
<td>Electrochemiluminescence</td>
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<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
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<td>Et$_3$N</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>F12K</td>
<td>a modification of Ham's F-12 Nutrient Mixture. Ham's F-12K (Kaighn's) Medium</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FOV</td>
<td>Field of view</td>
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<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
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<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas Chromatography-Mass Spectrometry</td>
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</table>
GFP  Green fluorescent protein

GSH  Glutathione

HBTU  1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HIF-1α  Hypoxia-inducible factor 1-alpha

HIF-1β  Hypoxia-inducible factor 1-beta

HPbCD  (2-hydroxypropyl)-b-cyclodextrin

MeOH  Methanol

Me₂S  Dimethyl sulfide

MeSH  Methyl mercaptan

IM  Intramuscular

IP  Intraperitoneal

MRI  Magnetic resonance imaging
MS  Mass spectrometry

MTT  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADH  Nicotinamide adenine dinucleotide

NADPH  Nicotinamide adenine dinucleotide phosphate
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>n-BuLi</td>
<td>n-butyllithium</td>
</tr>
<tr>
<td>Pd(OAc)$_2$</td>
<td>Palladium (II) acetate</td>
</tr>
<tr>
<td>NIR</td>
<td>Near-infrared</td>
</tr>
<tr>
<td>NIST</td>
<td>National Institute of Standards and Technology</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NQO1</td>
<td>NAD(P)H dehydrogenase protein that reduces quinones to hydroquinones</td>
</tr>
<tr>
<td>NTR</td>
<td>Bacterial nitroreductase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PET/CT</td>
<td>Positron emission tomography/computed tomography</td>
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<tr>
<td>PHD</td>
<td>Prolyl hydroxylase domain</td>
</tr>
<tr>
<td>PLE</td>
<td>Porcine liver esterase</td>
</tr>
<tr>
<td>PPh$_3$</td>
<td>Triphenylphosphine</td>
</tr>
<tr>
<td>P(o-tolyl)$_3$</td>
<td>Try(o-tolyl)phosphine</td>
</tr>
<tr>
<td>PTSA</td>
<td>$p$-Toluenesulfonic acid</td>
</tr>
<tr>
<td>RET</td>
<td>Resonance Energy Transfer</td>
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<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>RLM</td>
<td>Rat Liver Microsomes</td>
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<tr>
<td>RSON</td>
<td>Reactive sulfur, oxygen, and nitrogen</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>SCID/BALB-C</td>
<td>Severe combined immunodeficiency / an albino, laboratory-bred strain of the house mouse</td>
</tr>
<tr>
<td>SNARF</td>
<td>Seminapthorhodafluor</td>
</tr>
<tr>
<td>TBS</td>
<td>Tert-butyldimethylsilyl</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TOLD</td>
<td>Tissue oxygen level dependent</td>
</tr>
<tr>
<td>TPrA</td>
<td>Tri-n-propylamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane hydrochloride</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet light</td>
</tr>
<tr>
<td>UV/VIS</td>
<td>UV/Visible light absorption spectroscopy</td>
</tr>
<tr>
<td>VHL</td>
<td>Von Hippel Lindau</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
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LIST OF PUBLICATIONS


ACKNOWLEDGMENTS

Graduate school has been without a doubt the best years of my life so far. Looking back on my time here at Southern Methodist University, I see a period of my life in which I grew immensely in mind, body, and spirit. The challenge of obtaining a Ph.D. was by no means easy, but I had so many people that supported me through this process that I express my gratitude for, starting with my parents. To preface, I think I always wanted to become a scientist since I was a kid. My parents used to catch me running “experiments” by immersing ping pong balls in water and trying to freeze them. Fast-forward to my senior year of undergrad, I was graduating with a B.S. in biochemistry, but I ultimately felt lost on what I actually wanted to do. I begrudgingly plodded through graduate school applications, knowing that I didn’t really want a menial job out of college. At the same time, I didn’t quite know where I wanted to go, or even if grad school was right for me at the time. Dad insisted that I apply SMU because he loved the school so much when he and my brother visited, so I did. I would have never applied here had he not told me to do so. A few months later, after finding Alex’s invitation to interview in my junk email box, quickly interviewed and accepted Alex’s offer.

Mom, Dad, I want to thank you with all of my heart for helping me through that period of my life. It was difficult for all of us, but you never stopped supporting me. Thank you for taking me to basketball practice, seeing my band concerts, staying on my case about grades, teaching me how to be a good and decent human being, and pushing me toward my fullest potential.
Next, I need to thank my professor, Alex Lippert, for helping me realize my dreams of becoming a scientist. After my first semester in the laboratory, Alex took the lab out for dinner and drinks to celebrate. At one point in the night, I sat down next to him at the bar and asked him: “What does it take to be the idea man? How do you go from a lowly graduate student becoming a professor, and where do all the ideas come from?” His response was something I will remember for the rest of my life: “You need to sit down and let anything come to you. Let the craziest things flow through your head, anything and everything. Once you found an idea, ask questions, and use the tools at your disposal to answer those questions.” I think this stuck with me because it made me realize that I needed more tools. If I put my head down and worked at it hard enough, that I too could contribute to the idea process and bring those ideas to fruition. In the first couple of years working, I came to Alex with new “ideas” almost every week. To be honest, I think he got a little tired of me knocking on his door all the time. But every time he listened, and asked me to vet those ideas and figure out their conceptual pitfalls. As my lab skills honed over the course of my graduate career, my creativity and critical thinking followed suit.

After our 2019 ACS Sensors publication, Alex set me loose and told me to figure out how to do the pH sensor project. With his guidance, I was able to design, synthesize, and run all the experimental procedures, and write the manuscript for the paper. We definitely had some hurdles along the way, but I’ve never had a more gratifying feeling to see my own idea come to fruition. Thank you Alex, for letting me be creative and take ownership of my work, thank you for not getting too mad with me after breaking the rotavap, or the HPLC, or making you buy an ozonator that only proved useful years after we bought it. Most of all, thank you for teaching me how to be a real scientist. I know that wherever this career takes me, I will always remember that it started here at SMU messing with glow sticks.
Next I want to thank my friends, starting with Stephen Sattler. Over the last two years you’ve become one of my closest friends. On top of that you’ve played a huge part in helping me grow as a person. Thank you for teaching me how to cook, being my gym partner, keeping me accountable, and growing with me over the last two years. You have impacted my life more than you could ever know. On top of that, I need to give thanks to others who have been there for me. Jian and Weiwei, thank you for your friendship during my first years here in Dallas, and showing me the ropes when I started grad school. I miss all the trash talking we used to do. You guys made it easy to come to work every day, and I think the three of us really played a part in creating the social atmosphere that the lab benefits from to this day. I’d also like to thank other current and former lab mates including Uroob, Husain, Bo, Maha, Briley, and all of the former and current members of the Lippert Lab. I’d also like to thank my brother, Alex Ryan, for helping me with the interview process, and getting much closer over the last few years playing games together over the internet, Mitchell Hechsel, Tyler San Agustin, Zach Martens, Jordan Krueger, Mike Ferrera, Erik Svensson, Alex Gross, Blair Henning, I love all of you guys.

I’d like to give special recognition to Prof. Ralph Mason and Jeni Gerberich at UTSW for all of their assistance with animal imaging over the last few years. You have been fantastic coworkers and mentors. Thank you to my committee, including Brian Zoltowski, Nicolay Tsarevsky, John Buynak, and Ralph Mason for being a part of my examination process. Finally, thank you to everyone who has been here for me. It’s been an amazing journey, and I couldn’t have done it without you.
Light is one of the most fundamental aspects of the natural world. Plants process light using photosynthesis to create energy, ultimately supplying us with both food to eat and oxygen to breathe. Animals, bacteria, and plants have developed advanced photoreceptors to enable them to respond to light stimuli. Light is prevalent all around us, and the ability to create, manipulate, and harness light has been integral to the development of the human species.

Light comes in many forms; The most common form is the generation of light through combustion or heating, also known as incandescence. Incandescence arises as a byproduct of heating a substance, where molecules will give off energy in the form of a photon as a means of reducing their energetic state. These are known as “hot light” sources. Fire is the main example of combustion-based light production where cellulose in the presence of oxygen will combust, releasing energy stored in the wood to create light, heat, char, carbon monoxide, carbon dioxide, and water. Light bulbs are also a source of incandescence, where a carbon filament inside of the bulb acts as a resistor in an electrical current, and is heated to a point where a small percentage of energy put into the system is released as light.

1.1 Chemiluminescence

Light, however, does not just occur from “hot light” sources. There are many natural examples of “cold light” generation, where photonic emission arises from sources other than
heat. One such process is the result of chemiluminescence, where a chemical reaction causes the excitation and concomitant emission of a photon. A Jablonski diagram of a chemiluminescence reaction is given in Scheme 1-1, showing the requirements of a chemiluminescence reaction.\(^8\) The reaction needs to be sufficiently exothermic to excite the product formed during the reaction into an excited state. Upon relaxation back to the ground state, the energy can be released either in the form of a quantized emission of a photon, or through heat in the process of thermal relaxation. This dissertation will focus on light production through “cold” luminescence sources and their applications in science and technology.

**Scheme 1-1.** Potential energy diagrams for (a) an exothermic reaction and (b) a chemiluminescent reaction. (S = starting material, I = reactive intermediate or transition state, P = product).

Numerous methods have been shown to produce chemiluminescence, including light generation from gas phase reactions between nitric oxide and ozone, sulfur and ozone, and fluorine-based chemiluminescence systems.\(^9\)–\(^13\) Other common systems include electrochemiluminescence,\(^14\)–\(^19\) decomposition of dioxetanes, dioxetanones and dioxins, and bioluminescence.\(^20,21\)
**1.1.1 Gas phase chemiluminescence**

A few interesting gas phase reactions involving ozone and other species can also generate chemiluminescence. Sulfur compounds such as H$_2$S, Me$_2$S, MeSH and others can be oxidized to form SO radical, which can be reacted with ozone in the following reaction to form excited SO$_2$ (Scheme 1-2).$^{9,10}$ Gas phase detection of NO$_x$ can also be detected in a similar manner, where generation of NO combined with ozone also creates chemiluminescent emission. These reactions have been utilized for detection and quantification of sulfur and nitrogen compounds through GC analysis.$^{11,12}$ Atomic fluorine or CF$_4$ can be reacted with both organic and inorganic hydrides, and some hydrocarbons in the gas phase to produce fluorine-based chemiluminescence.$^{13}$

**Scheme 1-2.** Mechanism of gas phase SO$_2^*$ and NO$_2^*$ generation and luminescence.

\[
\begin{align*}
\text{SO} + \text{O}_3 & \rightarrow \text{SO}_2^* + \text{O}_2 \rightarrow \text{SO}_2 + \text{hv} \\
\text{NO} + \text{O}_3 & \rightarrow \text{NO}_2^* + \text{O}_2 \rightarrow \text{NO}_2 + \text{hv}
\end{align*}
\]

**1.1.2 Electrochemiluminescence**

Electrochemiluminescence, or ECL is luminescence emission caused by the generation of excited state species on electrode surfaces, and is a means of converting electrical energy into photonic emission.$^{14}$ Perhaps the most utilized method for ECL generation is through alternating voltage potentials on Ru(bpy)$_3$$^{2+}$ to produce electrochemiluminescence emission from the excited state Ru(bpy)$_3$$^{2+}$ species (Scheme 1-3.). Ru(bpy)$_3$$^{2+}$ is an example of annihilation induced ECL, where an electron transfer reaction occurs between an excited and reduced species generated by pulsing voltage potentials at the electrode react, “annihilating” both the oxidized and reduced form to produce the emitting compound in the excited state.$^{15}$
Scheme 1-3. Mechanism of electrogenerated chemiluminescence emission from Tris(2,2’-bipyridine)ruthenium dichloride.

\[
\begin{align*}
\text{Ru(bpy)}_3^{2+} - \text{e}^- &\rightarrow \text{Ru(bpy)}_3^{3+} \quad \text{E}^{\circ}_{\text{Ru}^{2+/3+}} = +1.2 \text{V} \\
\text{Ru(bpy)}_3^{2+} + \text{e}^- &\rightarrow \text{Ru(bpy)}_3^{1+} \quad \text{E}^{\circ}_{\text{Ru}^{2+/1+}} = -1.4 \text{V} \\
\text{Ru(bpy)}_3^{2+} + \text{Ru(bpy)}_3^{1+} &\rightarrow \text{Ru(bpy)}_3^{\cdot2\cdot} + \text{Ru(bpy)}_3^{2+} \\
\text{Ru(bpy)}_3^{\cdot2\cdot} &\rightarrow \text{Ru(bpy)}_3^{2+} + \text{hv}
\end{align*}
\]

Other forms of ECL generation have been successfully utilized. Coreactant ECL is one such form, where a redox active species will be introduced to the system to react with the emitter. Common redox active compounds employed as coreactants include the oxalate\textsuperscript{16,17} and per oxydisulfate ions,\textsuperscript{18} and tri-\textit{n}-propylamine (TPrA),\textsuperscript{19}

Electrochemiluminescence has proven useful in many analytical techniques including electrochemical assays for analyte detection. Multitudes of analytes have been successfully detected and quantified through ECL including small molecules, drugs, explosives, and viral and bacterial DNA and RNA,\textsuperscript{14} showing extensive utility through accurate quantification methods and sensitivity of analytes of down to 10\textsuperscript{-11}M.

1.1.3 1,2-Dioxetanones

Classical chemiluminescence systems are derived from the breaking of highly energetic chemical bonds. Notably, decomposition of strained peroxide bonds including dioxetanes, dioxetanones, dioxetanediones, and dioxins, are commonly utilized reactions for chemiluminescence emission, and examples of these reactions have been produced both synthetically and in the natural world. These small molecule chemiluminescence generating compounds share a common endoperoxide motif with either $\alpha$-carbonyl or CR$_2$ groups.
Dioxetanones and dioxetanediones are key players in chemiluminescence generation. Dioxetanones specifically have an important role to play in nature, as they are the most common bioluminescence substrates in luciferin/luciferase pairs.\textsuperscript{21} D-luciferin derived from the north American firefly \textit{Photinus pyralis} has been the most extensively studied of all luciferins. The structure and mechanism of chemiluminescence from D-luciferin are given in Scheme 1-4.\textsuperscript{22} D-luciferin is activated to luciferyl adenylate, and will react with superoxide in a single electron transfer mechanism\textsuperscript{23} and displacement of the AMP ester to obtain the dioxetanone intermediate. Decomposition of the dioxetanone releases CO\textsubscript{2} to generate oxyluciferin in the excited state.

\textbf{Scheme 1-4.} Mechanism of the D-luciferin bioluminescence reaction.\textsuperscript{21}

While oxyluciferin serves as the luminophore, many factors play into determining its emission profile. The primary cause of changes in emission is the luciferase itself, where both structural differences from protein to protein enact changes in the microenvironment where the luminescence reaction is catalyzed, and ultimately influences the electronics of the emitter.\textsuperscript{24} Oxyluciferin exists in up to 6 different forms depending on its microenvironment, and each of those forms have varying emission profiles ranging from 420–620 nm.\textsuperscript{25}

\subsection*{1.1.4 1,2-Dioxetanediones}

Dioxetanediones represent another key intermediate generated for chemiluminescence emission in peroxoxygenalate systems. In the late 1960’s, Rauhut initiated research into
peroxyoxalate, finding that some oxalyl esters\textsuperscript{26} and oxalyl chloride\textsuperscript{27} could be reacted with H\textsubscript{2}O\textsubscript{2} and base (Scheme 1-5) to generate chemiluminescence with quantum yields of up to 23\% in aqueous solution with the addition of rubrene as the fluorescer.

**Scheme 1-5.** Mechanism of per oxyoxalate decomposition.

1.1.5 1,2-Dioxins

1,2-dioxins are unstable intermediates that can provide the energetic requirements for chemiluminescence emission. Perhaps the most widely recognized form of chemiluminescence detection, luminol, is often utilized for the detection of blood samples because heme iron found in blood catalyzes luminescent emission from the molecule in the presence of molecular oxygen. Luminol first reacts with base to form the dianionic intermediate, followed by the interaction with triplet oxygen in the presence of heme iron to form a 1,2-dioxin upon the release of molecular nitrogen. Cleavage of the dioxin will excite the luminophore giving blue emission upon relaxation (Scheme 1-6).\textsuperscript{28,29}
**Scheme 1-6.** Chemical structure of luminol and mechanism of heme iron-catalyzed chemiluminescence emission from luminol with base and O₂.

![Chemical structure of luminol and mechanism of heme iron-catalyzed chemiluminescence emission from luminol with base and O₂.](image)

1.1.6 1,2-Dioxetanes

1,2-dioxetanes were first synthesized in 1969 in the form of 3,3,4-trimethyl-1,2-dioxetane\(^\text{30}\) as a confirmation of theoretical reports stating that the decomposition of such compounds should yield an excited state carbonyl product. This was indeed the case, and thermal decomposition of the compound yielded chemiluminescence emission near 435 nm. Other early dioxetanes were also prepared,\(^\text{31}\) but had short half-lives. In 1972, Wieringa and coworkers postulated that bulky groups could stabilize the dioxetane compounds, and installation of an endoperoxide onto adamantylideneadamantane via triplet photosensitization of oxygen provided the most stable 1,2-dioxetane to date, exhibiting a half-life of approximately 20 years.\(^\text{32}\)

1.2 Mechanisms of decomposition and luminescence

1.2.1 Thermal decomposition of 1,2-dioxetanes and relatives

The thermal stability of 1,2-dioxetanes is determined by the activation energy required to initiate uncatalyzed decomposition of the dioxetane, and the mechanism of this decomposition has been hotly debated in the literature. Through experimental and theoretical mechanistic studies, three separate mechanisms have been proposed, including concerted cleavage, stepwise biradical cleavage, and asynchronous-concerted mechanisms (Scheme 1-7).
Scheme 1-7. Proposed mechanisms for unimolecular and uncatalyzed 1,2-dioxetane decomposition. From top to bottom: the concerted mechanism, the merged (asynchronous-concerted), and the biradical (stepwise-biradical) mechanism.\textsuperscript{20}

The concerted mechanism indicates that both C-C and O-O bond cleavage occur at the same time through simultaneous bond lengthening and breaking processes. However, this is likely a simplistic mechanistic view, as it does not take into account the inherent weakness of O-O bonds. The proposed biradical mechanism first involves O-O bond cleavage, leaving a biradical intermediate. This intermediate will then rotate 180° about the O-C-C-O dihedral angle to the anti-conformer, followed by C-C bond cleavage to generate both excited and ground state carbonyl products. The biradical mechanism better fits experimental data exploring the topic,\textsuperscript{33,34} however, the concerted mechanism better explains formation of singlet and triplet excited states upon decomposition. A combination of the two mechanisms, the asynchronous-concerted, or merged mechanism, has been proposed to account for these discrepancies. The asynchronous-concerted mechanism includes lengthening of both C-C and O-O bonds, along with simultaneous torsion about the O-C-C-O dihedral angle to approximately 44° to initiate O-O bond cleavage, serving as the rate limiting step for the reaction.\textsuperscript{35} The C-C bond shortly breaks thereafter, with no true formation of a dioxybiradical species.\textsuperscript{36}
1.2.2 Activated decomposition of 1,2-dioxetanes and relatives

It was found during the study of 1,2-dioxetanes, dioxetanones, and dioxetanediones, that decomposition was catalyzed by aromatic compounds.\textsuperscript{26,37,38} This phenomenon was investigated by Koo and Schuster in 1977.\textsuperscript{39} They uncovered that chemiluminescence intensity given off by diphenoyl peroxide was proportional to the concentration of added aromatic hydrocarbons, indicating that a bimolecular reaction was occurring to induce chemiluminescence. They proposed a mechanism, termed chemically initiated electron exchange luminescence (CIEEL), where the aromatic hydrocarbon serves as an activator in a bimolecular electron transfer reaction. The mechanism, given in Scheme 1-8 shows that an electron from the activator is donated into peroxide bond. The bond is then cleaved and a strongly reducing radical anion species is formed in solvent cage with the activator. An electron back transfer occurs, annihilating both radical species, and rendering the initial electron donor (activator) in the excited state and able to emit a photon through relaxation.

\textbf{Scheme 1-8.} Activated decomposition mechanism of diphenoyl peroxide.
Chemiluminescence generation in the presence of activators through the bimolecular CIEEL mechanism provided a means to generate chemiluminescence emission at a much faster rate, and has been studied extensively with various aromatic compounds. Bioluminescent substrates such as D-luciferin, however, are unimolecular emitters with a 1,2-dioxetanone intermediate. Researchers thus expected that D-luciferin proceeds via a unimolecular activation CIEEL process.\textsuperscript{40} It was also known that bioluminescence emission wavelength and intensity from D-luciferin varied with pH. E.H. White and coworkers in 1966 synthesized analogs of luciferin to examine the role of the 6’-OH group on D-luciferin bioluminescence. They found that only 6’-OH and 6’-NH\textsubscript{2} groups were capable of bioluminescence, and deprotonation of the 6’-OH yielded a much brighter luminescent species when subjected to luciferase, suggesting that the increased electron density of the amino and phenolate groups served as particularly strong activators in the CIEEL mechanism.\textsuperscript{41} Inspired by this work, Schaap and coworkers synthesized a model compound of D-luciferin bearing a phenol covalently appended to a 1,2-dioxetane scaffold in 1982.\textsuperscript{42} The protonated dioxetane species exhibited a half-life of 17 years, and low singlet chemiexcitation efficiency. However, deprotonation of the phenol group to the phenolate exhibited an over 4x10\textsuperscript{6}-fold increase in decomposition kinetics, a sharp increase in singlet chemiexcitation efficiency, and showed similar flash luminescence properties to that firefly bioluminescence.

In a series of three flagship papers, Schaap and coworkers reported chemical and enzymatic triggering of 1,2-dioxetanes using adamantylidene-substituted phenoxy-dioxetane chemiluminescent substrate. These papers had two-fold importance: 1. By protecting the phenolate of the dioxetane with selective trigger, one could cage the chemiluminescence substrate from reacting until it reacted with analytes of interest (commonly known as activity-
based reactions in the probe world) 2. The newly synthesized chemiluminescent scaffold combined the excellent dioxetane-stabilizing properties of the adamantylidene group along with the flash luminescence capabilities derived from D-luciferin. This design strategy has proven to be extremely prolific, as almost all synthesized 1,2-dioxetane chemiluminescence agents can be derived from this core structure.

1.3 1,2-Dioxetane Chemiluminescence Enhancement Strategies

A clear trend in the development of chemiluminescence has been to create brighter chemiluminophores with red-shifted emission. Increasing the brightness ultimately increases the signal to noise response, providing lower detection limits of analytes relative to the concentration of the dioxetane. Red-shifted emission has two-fold importance. 1. The use of visible-light/ NIR-light emission window greatly reduces risk of cellular damage by irradiation. 2. NIR light can pass through tissue to a much greater extent than UV and visible light, allowing for much higher depth penetration by compounds that possess NIR emission capabilities. Brighter, red-shifted chemiluminescence substrates have so far been achieved through multiple methods including encapsulation via luminescence enhancers, chemiluminescence self-amplification mechanisms, and direct scaffold modification, and creation of dioxetane-fluorophore conjugates.

1.3.1 Self-amplification

An interesting method to obtain chemiluminescence enhancement is through chemiluminescence self-amplification mechanisms. Gnaim and Shabat provided two such examples of auto-amplifying chemiluminescence substrates for fluoride (Scheme 1-9.) and \( \text{H}_2\text{O}_2 \). The general mechanism of these reactions follows that the chemiluminescence probe will react selectively with an analyte to generate luminescence emission while simultaneously...
releasing the analyte it was reacted with to generate a chain reaction. Low concentrations of analyte are required for these systems, but the signal/noise is ultimately low.

**Scheme 1-9.** Mechanism of fluoride induced chemiluminescence autoamplification.\(^{43}\)

1.3.2 Encapsulation

Methods of chemiluminescence encapsulation effectively enhance chemiluminescence by creating a hydrophobic environment for the chemiluminescence reaction to occur, thus reducing aqueous quenching effects. Sapphire II™, Emerald II™, and Ruby™ are commercial polymeric enhancers developed by Applied Biosystems to increase sensitivity via encapsulation, while providing luminescence emission at 475 nm, 542 nm, and 620 nm, respectively. Both Emerald and Ruby enhancers contain fluorescent compounds that redshift the emission wavelength of the chemiluminophore through an energy transfer mechanism (Scheme 1-10). Other methods of encapsulation include the use of cetyltrimethylammonium bromide (CTAB)\(^{42a}\) that create reverse
micelles, as well as luminescence enhancement via supramolecular complexation with β-cyclodextrin. While these enhancement strategies are important findings, they are not ideal for cellular studies due to issues with cytotoxicity and low cell permeability.

**Scheme 1-10.** Mechanism of luminescence enhancement through polymeric encapsulation.

1.3.3 Fluorophore conjugation

This method of chemiluminescence enhancement involves the direct conjugation of highly fluorescent compounds to the dioxetane structure, providing chemiluminescent emission through the appended fluorophore via resonance energy transfer. Schaap and Arkhavan-Tafti first adapted this methodology in a patent issued in 1997, where they conjugated a fluorescein or 6-hydroxybenzothiazoate to the CL scaffold by tethering through the enol ether position. This strategy provided an over 300-fold increase in chemiluminescence quantum yield for detection.
their aryl esterase-cleaved dioxetanes. This was later studied by Hananya and others in 2016, showing up to 114-fold improvement in $\phi_{\text{CL}}$ through attaching fluorescein via a benzylamine linker directly bonded to the phenol of the CL scaffold, as well as NIR CL emission when a cyanine was appended to the structure instead.\(^{47}\)

1.3.4 Direct modification

Perhaps the most important methods to increase brightness in chemiluminescence agents lie in direct modification of the chemiluminescent scaffold (Scheme 1-11). In the development of a viable in vivo chemiluminescence probe for hydrogen sulfide, Cao et al. in 2015 studied the effect of halogen substitution on the dioxetane phenol substrate, finding that ortho-chlorination relative to the phenol had the greatest effect in lowering the $pK_a$ of the phenol relative to hydrogen and fluorine substitution at the same position, ultimately increasing brightness by increasing concentration of the phenolate-dioxetane (and therefore rate of CIEEL) at biological pH.\(^{48}\) This was later confirmed to be the case as well with acrylate-substituted dioxetanes.\(^{49}\)

**Scheme 1-11.** Advances in direct structural modification of 1,2-dioxetane compounds.

Another major advancement in chemiluminescence technology came in 2017, where Green and coworkers attached electron withdrawing acrylate and acrylonitrile groups to the CL
scaffold, further decreasing the pK\textsubscript{a} of the phenol and generating much greater degree of electronic push-pull in the chemiluminophore \(\pi\) system.\textsuperscript{50} This red-shifted the wavelength of chemiluminescence emission to 530 nm, and increased the chemiluminescence quantum yield to up to 9.8\% with acrylonitrile substitution, which is an approximate 3000-fold increase in quantum yield over the unsubstituted dioxetane. Styryl-substitution at the same position provided improvement in chemiexcitation efficiency, which the authors attribute to stabilization of the key diradical species generated during CIEEL.\textsuperscript{51} Other examples of scaffold modification include the production of NIR chemiluminescence emission from a dicyanomethylchromone (DCMC) substituted dioxetane by further increasing \(\pi\) conjugation within the chemiluminophore,\textsuperscript{52} as well as the development of a chemiluminescent coumarin-based scaffold with extremely high chemiluminescence quantum yield in aqueous media.\textsuperscript{53}

1.4 Activity-based sensing

The field of activity-based sensing is based upon the design of chemoselective reactions to determine the concentration, reactivity, and activity of biomolecules in living systems.\textsuperscript{54} This is accomplished by exploiting inherent reactivity of these compounds to undergo selective transformations, which are then quantified by reporter compounds linked to the sensor. This process is often referred to a lock and key mechanism, where the lock is a caged reporter that will selectively react with a specific analyte, undergo chemical change, and ultimately unlock properties from the reporter that can be used to detect or quantify the analyte it reacted with. Because activity-based sensing is founded upon the detection of biomolecules, reporters must be biocompatible and exhibit ability to detect through tissue. Furthermore, the reaction should be selective with only one analyte to ensure accurate data. Finally, the reaction kinetics need to take place under reasonable time frames in physiological conditions to be considered true and
accurate. Therefore, tuning the reactivity, biocompatibility and reporting properties of the probe are paramount to the process of developing sophisticated ABS compounds.

The history of activity-based sensing begins with Roger Tsien’s group in the 1980’s as they pioneered binding-based sensing through their study on a close relative of ethyl-enediaminetetraacetic acid EDTA. They designed 1,2-bis(o-aminophenoxy)ethane-N,N,-N’,N’-tetraacetic acid (BAPTA) which they could use to selectively bind Ca$^{2+}$. They later attached a fluorophore to the chelator, realizing both fluorescence intensity-based and ratiometric detection of Ca$^{2+}$, which they later adapted to chelators for Mg$^{ll}$, Mn$^{ll}$, and Zn$^{ll}$. This work was succeeded by the discovery of chemodosimeters by both Czarnik who devised reactive fluorescent sensors for heavy metals, and Nagano, who developed selective biosensors for nitric oxide.

Chang and coworkers set out to design fluorescence turn-on probes for other RSON species. By using a selective oxidation of boronate esters by H$_2$O$_2$, they established use of turn-on fluorescence probes with fluorescein and lanthanide-based fluorescers. Since these initial findings, the field of activity-based sensing has flourished, inspiring the development of new, selective reactions to study biomolecules and enzyme activity in living systems, as well as the development of sophisticated fluorescent, chemiluminescent, photoacoustic, and NMR-based reporters to elicit their activity in biological settings. Furthermore, this area has expanded into the drug discovery sector with proteomics and activity-based protein profiling (ABPP) through the exploration of native reactive amino acid residues such as lysine, and cysteine, and methionine on proteins, Cravatt and others have developed selective probes to interrogate global protein activity and cellular signaling mechanisms.
1,2-dioxetanes have played a key role in the development and advancement of activity-based sensing. Since the discovery of protected phenoxy-dioxetanes, many different analyte selective triggers have been utilized for development of sensitive and selective detection of analytes and enzyme activity with 1,2-dioxide compounds, providing ultrasensitive methods to detect, and in some cases, quantify analytes of interest in in vitro and in vivo. Furthermore, the high depth penetration associated with these agents allows for photon detection through tissue in animal models.

1.4.1 Chemiluminescence-based ABS detection of enzymes

Applied Biosystems supplies chemiluminescence assay agents based on the phenoxy 1,2-dioxetanes including substrates for alkaline phosphatase (Scheme 1-12A), β-glucuronidase (Scheme 1-12B), β-Glucosidase (Scheme 1-12B), Neuraminidase (Scheme 1-12D), and β-galactosidase (Scheme 1-12E) with Galacton Star®. In 2010, Liu and Mason established the use of Galacton-star® for detection of the enzyme in tumor models, providing the first instance of in vivo analyte detection using 1,2-dioxetanes. This was further investigated by Eilon-Shaffer and coworkers using probe 2a (Scheme 1-12G), an acrylamide substituted dioxetane masked with a β-galactose group, and bearing a tumor-homing peptide to measure endogenous β-galactosidase activity in tumor bearing mice. Cao and coworkers established chemiluminescence-based detection of bacterial nitroreductase and CyP450 nitroreductase activity in live animals with HyCL-2 (Scheme 1-12I), improved upon by Sun et. al. (Scheme 1-12J) and Ryan et. al. (Scheme 1-12K) in separate publications in 2019. Roth-Konforti and others provided an ultrasensitive measurement of Cathepsin-B activity in cellulo using a chemiluminescent substrate bound to a self-immolative linker bearing the peptide responsive to cleavage by the cysteine protease (Scheme 1-12H). Das and collaborators established a chemiluminescence substrate
for carbapenemase detection, providing a detection limit with 10 µM **CPCL** of carbapenemase to 0.06 mU mL⁻¹ *in vitro* (Scheme 1-12F). An interesting work provided fast and sensitive detection of *Salmonella* (Scheme 1-12M) and *Listeria monocytogenes* (Scheme 1-12L) with approximately 600-fold better detection limits than their fluorescent counterparts. Recently, An and collaborators devised a chemiluminescent probe for detection of γ-glutamyl transpeptidase in both cells and animals (Scheme 1-12N).
Scheme 1-12. Activity-based chemiluminescence agents for enzyme detection

A. CPD-Star

B. Glucaron

C. Glucon

D. NA-Star

E. Galacton Star

F. CPCL

G. Probe 2a

H. Probe 4
1.4.2 Chemiluminescence-based ABS detection of small molecules and reactive species

Activity-based sensing using 1,2-dioxetane substrates has been particularly effective for studying reactive species in biological environments (Scheme 1-13). Particular attention has been put forth by the Lippert group to utilize chemiluminescence 1,2-dioxetanes to detect small molecules and reactive sulfur, oxygen, and nitrogen (RSON) species. Cao et. al. provided the first demonstration chemiluminescence detection of the H_2S in live cells and animals with CHS-3 (Scheme 1-13B) in 2015 utilizing an aryl azide trigger. This work continued in this area with the chemiluminescent probes for the detection of peroxynitrite with PNCL with utilizing a selective isatin trigger (Scheme 1-13E). Utilization of a phosphine trigger attached to an acrylonitrile substituted dioxetane with HNOCL-1 (Scheme 1-13D) provided the means to quantitatively measure HNO concentrations cells and in vitro, and could detect bolus injections of Angeli’s salt in live animals. Shabat and coworkers provided chemiluminescent substrates for H_2O_2 via a chain reaction amplification mechanism, as well as green and NIR emitting
chemiluminescent substrates to detect H$_2$O$_2$ in cells and animals (Scheme 1-13C). Bruemmer et al. developed CPAF700 for sensitive NIR detection of formaldehyde using an aza-cope reaction mechanism. Furthermore, both green and red-emitting 1,2-dioxetanes for singlet oxygen detection have been developed (Scheme 1-13F,G).
Scheme 1-13. Activity-based chemiluminescence agents for small molecule and reactive species detection.
1.4.3 Chemiluminescence-based ABS detection of miscellaneous analytes

A few works based on detection of analytes that fall outside the area of small molecules and enzymes. An et. al. developed the first instance of ratiometric imaging of pH using a 1,2-dioxetane scaffold by utilizing an energy transfer mechanism initiate fluorescence in the pH sensitive fluorophore seminaphthorhodafluor (Scheme 1-14A). Ryan and coworkers advanced this technology with a pH sensitive carbofluorescein moiety appended directly to the 1,2-dioxetane through a piperazine linker, providing quantitative pH measurements in vitro and in live animals (Scheme 1-14B). Gnaim and coworkers established the use of a chemiluminescent substrate to monitor the release of monomethyl auristatin E through a self-immolative prodrug release mechanism in cells and mice grown with CT26-LacZ tumors (Scheme 1-14C). Huang et. al. designed a duplexed imaging agent of contrast induced acute kidney injury by attaching green emitting dioxetane responsive to superoxide to a NIR fluorescence emitter responsive to lysosomal damage through release of N-acetyl-β-D-glucosaminidase (Scheme 1-14D). Both the NIR fluorophore and chemiluminophore were attached together by a (2-hydroxypropyl)-β-cyclodextrin (HPbCD) linker to promote renal clearance, allowing them to detect CIAKI in vivo before significant decline in kidney function occurred.
1.5 Resonance Energy Transfer

Resonance energy transfer is a physical process in which atoms or molecules can transfer energy between each other through a non-radiative dipole-dipole coupling mechanism. This process was first discovered by Cario and Franck in 1922, where excitation of a mercury and thallium mixture in the excitation range of mercury gave luminescence emission from thallium. This process was later explained by Theodor Förster, who made the key discovery that the efficiency of energy transfer is dependent upon two conditions: (1) spectral overlap of the donor and acceptor (2) distance between the donor and acceptor (Scheme 1-15). The equation for energy transfer efficiency ($K_{ET}$) is given in Equation 1. $R_0$ is the Förster radius, an empirically determined distance at which the energy transfer efficiency is 50%. $\tau_D$ is the fluorescence lifetime of the donor species, and $d_{D-A}$ is the distance between the donor and acceptor. The distance between the donor and acceptor dominates this phenomenon, because the efficiency is proportional to $(1/d_{D-A})^6$. In practical terms, RET efficiency falls off very quickly the further the distance the donor and acceptor are from one another.

$$K_{ET} = \frac{1}{\tau_D} \left( \frac{R_0}{d_{D-A}} \right)^6$$

Resonance energy transfer has particularly found uses in the field of biology to study processes that involve proximity. To this end, a vast array of biological and chemical luminophores have been developed to study biomolecular interactions. Some key research within this field is based on the use of Green Fluorescent Protein (GFP) and its multicolored mutant relatives. Furthermore, bioluminescence resonance energy transfer has also been established as an advantageous reporter in some cases over FRET-based detection due by attenuating photobleaching and autofluorescence effects, as well as unintentional excitation of both fluorophores.
**Scheme 1-15.** Plot of spectral overlap between FRET donor and acceptor.

### 1.6 Research Objectives

This research aims to utilize the chemiluminescence imaging modality to create low-cost preclinical methods for detection and quantification of analytes within the tumor microenvironment. Bearing this in mind, we developed a bright and selective chemiluminescence probe for hypoxia detection with **HyCL-4-AM**, which offers the unique ability to monitor native O₂ dependent nitroreductase activity in cells and animals through monitoring its reaction kinetics. Furthermore, we developed **Ratio-pHCL-1**, a chemiluminescence resonance energy transfer sensor that can be used to quantify pH *in vitro* and in living systems that can reach a photon-flux through animal tissue of up to $10^{10}$ p/s, providing exceptionally bright luminescence and pH detection in animals.


46. Schaap, A. P.; Arkhavan-Tafti, H. Enhanced Chemiluminescence From 1,2-Dioxetanes Through Energy Transfer to Tethered Fluorescers. 56168729, 1997.


CHAPTER 2
KINETICS BASED MEASUREMENT OF HYPOXIA IN LIVING CELLS AND ANIMALS
USING AN ACETOXYMETHYL ESTER CHEMILUMINESCENT PROBE

2.1 Introduction

Cancer is defined as the uncontrolled proliferation of cells in organisms, and occurs due to genetic mutations that arise in affected cells.\(^1\) Normally, these mutations are recognized and eliminated by five major DNA damage repair pathways, including base excision repair, nucleotide excision repair, homologous recombination, and nonhomologous end joining.\(^1\) In the case that mutations persist beyond the repair of the cell, apoptosis can be induced to isolate uncontrolled expression. These regulation and repair mechanisms are often disrupted in malignant cells, allowing the potential for more stochastic DNA damage events within a cell before the DNA is repaired. It is estimated that 2–8 mutations need to occur in succession before uncontrolled cell division occurs, and also depends on cell type, cancer type, and age of the afflicted individual.\(^2\)

Development of the tumor microenvironment is paramount to its ability to proliferate. Mutated epithelial cells begin to secrete proteases that break down the extracellular matrix (ECM) in higher concentration than expressed protease inhibitors, thus allowing for breakdown and remodeling of the ECM and creating room for tumor growth.\(^3\) Cancer associated fibroblasts (CAF’s) also play an important role in tumorigenesis, providing essential growth factors for proliferation, as well as another source for ECM degrading proteases.\(^4\)
Unchecked cellular proliferation often leads to hypoxic tumors, where the oxygen demand within the tumor exceeds the oxygen supply. Hypoxia is self-propagating within the tumor, as uncontrolled expansion ultimately increases distance between vasculature and the cells that require oxygen, thus lowering the diffusion rate of $O_2$ into those cells and creating an even more hypoxic environment. Chronic hypoxia within tumors leads to long-term cellular changes associated with increased tumorigenesis, including increased susceptibility to DNA damage coinciding with reduced efficacy of DNA repair mechanisms. 5,6

Changes in protein expression and regulation also occur in hypoxic environments. The HIF transcription pathway (responsible for expression of over 100 genes) is regulated in physoxic conditions through ubiquitination and proteosomal degradation of the HIF-α mediated by prolyl hydroxylase (PHD) and the Von Hippel Lindau (VHL) E3 ligase. 7 Proteosomal degradation of HIF-1α is therefore inhibited in low $O_2$, allowing for the dimerization of HIF-1β. This heterodimer is the active transcription factor that is responsible for the expression of a host of genes that promote tumor survival, including the upregulation proteins such of vascular epithelial growth factor (VEGF), inhibition of adhesion proteins, as well as upregulating glucose transporters and glycolytic proteins to shift cells into anaerobic energy production (the Warburg effect). 8,9 Hypoxia is a critical component of tumor physiology, and viable scientific tools must be developed to understand hypoxia in both clinical and preclinical settings.

Current implemented methods for hypoxia detection in clinical settings include oxygen electrodes, 10 blood oxygen and tissue oxygen level dependent magnetic resonance imaging (BOLD and TOLD), 11,12 and positron emission tomography/computed tomography (PET/CT). These methods are excellent for clinical use, but are either invasive in the case of electrodes or require expensive instrumentation and expertise in the case of MRI and PET. Preclinical methods
for hypoxia detection have thus gravitated toward less expensive and easy to implement optical imaging methods. Many optical, PET and immunohistochemical approaches utilize O₂ dependent nitroaromatic reduction to both monitor and treat hypoxia, and occurs through a series of 1 and 2 electron reductions mediated by cytochrome P450, cytochrome c, xanthine oxidase, and lipoamide dehydrogenase (Scheme 2-1). The first step in this reduction process forms the nitroaromatic radical anion (RNO₂⁻). Triplet oxygen, however, renders this step as a futile cycle by re-oxidizing the radical anion with concomitant formation of superoxide. Therefore, this reaction can only proceed in hypoxic conditions. This mechanism has been employed to develop sensitizers for radiation and chemotherapy, as well as compounds to aid in hypoxia imaging and detection. Fluorescent, photoacoustic, bioluminescent, and chemiluminescent imaging agents based on this approach have also been reported.

Scheme 2-1. Mechanism of nitroaromatic reduction.

It is, however, important to note that use of nitroaromatic groups and their redox byproducts present considerable hazards to human health, as they exhibit carcinogenic behavior. Most of their cytotoxicity revolves around generation of oxidative stress in cells. Redox cycling between the aforementioned nitroaromatic group and the nitroradical anion catalyzes the production of superoxide, a well-established carcinogen that oxidizes DNA, lipids, and proteins. Nitroaromatic compounds such as 2,4,6-trinitrotoluene, o-nitrotoluene, nitrotyrosine, nitrobenzene, and others have been extensively linked in relation to oxidative stress and carcinogenesis. Redox cycling between nitroso and hydroxylamine species can also
generate reactive oxygen species\textsuperscript{33} are also highly carcinogenic, and presence of nitroso-
compounds in the diet lead to significant increases in colorectal cancer.\textsuperscript{34} It has also been shown
that aromatic hydroxylamine and amino compounds form covalent adducts with DNA, thus
leading to mutagenesis through DNA damage.\textsuperscript{32}

Research thus far shows that nitroimidazole agents exhibit unacceptable toxicity for
therapeutic use, but show clinical potential for hypoxia imaging at lower concentrations. There
has been extensive research in using nitroimidazoles as oxygen mimetic radiosensitizers with the
development and clinical testing of metronidazole, misonidazole, and etanidazole.\textsuperscript{35}
Metronidazole showed promise for both in vitro and clinical radiosensitization, but repeated
doses of up to 2.5 g/m\textsuperscript{2} were not tolerated, as patients exhibited neuropathy 15-26 days post-
administration.\textsuperscript{36} Misonidazole and Etanidazole were developed as more polar amide-containing
derivatives of metronidazole to decrease neural uptake,\textsuperscript{35} but were also unable to pass clinical
trials for therapeutic use.\textsuperscript{37,38} Pimonidazole exhibited a similar fate through clinical trials,\textsuperscript{38} but
has since been repurposed as a hypoxia imaging agent.\textsuperscript{39} A recent clinical study involving
patients with prostate cancer showed effective tumor hypoxia staining in patients with one 500
mg/m\textsuperscript{2} dose administered intravenously the day before their biopsy without significant side
effects post-administration.\textsuperscript{40}

Chemiluminescence has proven to be advantageous for molecular imaging by providing
bright luminescence output without the need for an external light source or genetic
modification,\textsuperscript{41} ultimately increasing sensitivity and tissue imaging depth by drastically
attenuating autofluorescence and light scattering effects.\textsuperscript{42,43} Indeed, recent advances in triggered
spiro adamantane 1,2-dioxetane chemiluminescence technology have allowed for high quantum
yields in aqueous systems,\textsuperscript{44,45} and have been applied for the detection of peroxynitrite.\textsuperscript{46}
nitroxyl, formaldehyde, prodrug release, cathepsin B, NQO1, transition metals, and other analytes.

Here, we use hypoxia mediated nitroaromatic reduction of a caged chemiluminophore **Hypoxia ChemiLuminescence probe 3 (HyCL-3) and HyCL-4-AM** (Scheme 1). While previously reported **HyCL-2** and **CL-NTR** were demonstrated to be responsive to tissue oxygenation in vitro and in animal experiments, these probes were not studied in cellular milieu, and had limited animal imaging data. In this study, we evaluate **HyCL-3** and **HyCL-4-AM** as chemiluminescent reporters for hypoxia in vitro, in cellulo, and in vivo. We demonstrate that incorporation of an acetoxymethyl (AM) ester dramatically increases probe performance compared to **CL-NTR**, and can be used as a tool to directly monitor in vivo oxygen dependent nitroreduction activity.

**Scheme 2-2.** Probe designs and sensing reactions for **HyCL-3**, **HyCL-4-AM**, and **HyCL-4-AM-Cont**.
2.2 Results and Discussion

2.2.1 Design and synthesis of HyCL-3 and HyCL-4 AM

In previous work, our lab has shown the efficacy of reaction-based hypoxia probes with HyCL-2. This features a chemiluminescent 1,2-dioxetane scaffold masked by a para-nitrobenzyl group through an ether linkage. This para-nitrobenzyl group undergoes self-immolative cleavage in response to enzymatic reduction from nitroreductase enzymes. The released phenolate-dioxetane species undergoes decomposition and concomitant luminescence emission via a chemically initiated electron exchange luminescence (CIEEL) mechanism. In continuation of our efforts to develop chemiluminescent probes for tumor hypoxia imaging, we sought methods to increase probe sensitivity for cellular measurements. We took a multi-faceted approach to this challenge and designed HyCL-3 and HyCL-4-AM, which we hypothesized would increase sensitivity through increased chemiluminescence quantum yields, as well as using an acetoxymethyl (AM) ester to improve cellular uptake (Scheme 1). A control compound HyCL-4-AM-Cont without the nitroaromatic trigger was also designed to control for effects unrelated to nitroaromatic group reduction.

HyCL-3 was prepared by Mitsunobu coupling of acrylonitrile phenol 1 and para-nitrobenzyl alcohol, followed by subsequent [2+2] cycloaddition with photogenerated singlet oxygen using Rose bengal as a photosensitizer (Scheme 2). Synthesis of HyCL-4-AM began with an S_N2 reaction of phenol 2 and para-nitrobenzyl tosylate, followed by hydrolysis of the methyl ester to yield carboxylic acid 3. We then appended the acetoxymethyl ester through an S_N2 reaction with bromomethyl acetate. Finally, we subjected this precursor to a [2+2] cycloaddition with singlet oxygen to achieve HyCL-4-AM. The control compound, HyCL-4-AM-Cont was prepared using analogous procedures using benzyl bromide in place of para-nitrobenzyl tosylate.
2.2.2 HyCL-3,4-AM Response to Nitroreductase and NADH

Upon synthesis of HyCL-3 and HyCL-4-AM, their responses towards bacterial nitroreductase and cofactor reduced nicotinamide adenosine dinucleotide (NADH) were
examined (Figure 2-1). Luminescence emission spectra for **HyCL-3** and **HyCL-4-AM** were collected using a F-7000 Hitachi spectrophotometer by treating 10 μM **HyCL-3** with 1 μg mL⁻¹ nitroreductase (NTR) and 0.2 mM NADH in 20 mM PBS buffer, revealing an emission peak centered at 525 nm (Figure 2-1A), whereas 10 μM **HyCL-4-AM** shows an emission maximum at 516 nm when treated with 1 μg mL⁻¹ NTR, 0.2 mM NADH, and 1 U mL⁻¹ esterase to cleave the AM ester (Figure 2-1B). The dose dependence of the luminescence emission with regards to nitroreductase was evaluated from 0–1 μg mL⁻¹ NTR for **HyCL-3** and **HyCL-4-AM** using a luminescence plate reader (Figure 2-1C). While **HyCL-4-AM** showed a clear dose dependence under these conditions, the relative emission intensity for **HyCL-3** was much lower and higher enzyme concentrations were required to observe a dose-dependence (Figure 2-2). The time-course of the chemiluminescence emission of **HyCL-4-AM** reached a maximum at around 15 minutes (Figure 2-1D). The reaction products of **HyCL-3** and **HyCL-4-AM** were analyzed by GC/MS and a peak definitively assigned to 2-adamantanone (m/z = 150.1, M⁺) as well as one tentatively assigned to (4-(hydroxyamino)phenyl)methanol (m/z = 138.8, M⁺) were found for both probes (Figure 2-3 – 2-6).
Figure 2-1. Response of HyCL-3 and HyCL-4-AM. Chemiluminescence emission spectra of (A) 10 µM HyCL-3 (blue trace) before and (red trace) after treatment with 1 µg mL⁻¹ NTR and 0.2 mM NADH and (B) 10 µM HyCL-4-AM (blue trace) before and (red trace) after treatment with 1 µg mL⁻¹ NTR, 0.2 mM NADH, and 1 U mL⁻¹ PLE. (C) Integrated emission intensity of 10 µM HyCL-4-AM (red trace) or HyCL-3 (blue trace) alone or with 200–1000 ng mL⁻¹ NTR and 0.2 mM NADH. PLE (1 U mL⁻¹) was added for HyCL-4-AM. (D) Time-course of the chemiluminescence emission of 10 µM HyCL-4-AM alone (blue trace) or with 200, 400, 600, 800, and (red trace) 1000 ng mL⁻¹ NTR, 0.2 mM NADH, and 1 U mL⁻¹ PLE. All experiments were performed in 20 mM PBS (pH 7.4) containing ≤ 1% DMSO. Error bars are ± S.D.

Figure 2-2. Response of HyCL-3. (A) Time-course and (B) integrated emission intensity of the chemiluminescence emission of 10 µM HyCL-3 alone (blue trace) or with 2.5, 5, 7.5, 10, and (red trace) 12.5 mg mL⁻¹ NTR, and 0.4 mM NADH. All experiments were performed in 20 mM PBS (pH 7.4) containing ≤ 1% DMSO. Values are the average of n = 3 technical replicates. Error bars are ± S.D. Data collected by Jian Cao.
Figure 2-3. Mass spectrum of the peak assigned to adamantane from HyCL-3. Inset is the NIST standard.

Figure 2-4. Mass spectrum of the peak assigned to hydroxylamine for HyCL-3. Data collected by Becky Jenkins.
Figure 2-5. Mass spectrum of the peak assigned to adamantanone for **HyCL-4-AM**. Inset is the NIST standard.

Figure 2-6. Mass spectrum of the peak assigned to the hydroxylamine for **HyCL-4-AM**.

2.2.3 **HyCL-3,4-AM Response to rat liver microsomes, selectivity, and pH dependence**

We next studied the *in vitro* response to rat liver microsomes (RLM), a more relevant model to mammalian hypoxia due to the prevalence of cytochrome P450 reductases in the RLM (Figure 2-7). Solutions of 20 µM **HyCL-3** with 50 µM cofactor reduced nicotinamide adenine
dinucleotide phosphate (NADPH) and 200 μg mL\(^{-1}\) RLM in 20 mM PBS (pH = 7.41) were incubated for 4 hours at 37 °C and compared against solutions of only 20 μM HyCL-3 in 20 mM PBS (pH = 7.4) as a negative control in both 1% and 20% O\(_2\). Under these conditions, HyCL-3 showed a 37-fold increase in peak luminescence at 80 minutes versus the control, and a glow that persisted for more than 4 hours (Figure 2-7A). The local maximum seen at 80 minutes was not seen in the 20% oxygen condition. By comparison, the same experiment with 20 μM HyCL-4-AM, which showed a more rapid onset of the peak intensity after 20 min, with a phenomenal 60,000-fold increase in luminescence intensity versus the probe alone (Figure 2-7B).\(^{53}\) The reaction curve showed a similar small increase in luminescence intensity around 80 minutes for the 1% condition as compared to 20%. Knowing that HyCL-3 and HyCL-4-AM were sensitive to enzymatic reduction by RLM and NTR, we performed selectivity studies with other biologically relevant reductive species, including metal cations and nucleophilic thiols. The response of 20 μM HyCL-3 or HyCL-4-AM to RLM was monitored against analytes in PBS buffer (20 mM, pH = 7.4) by adding 200 μM CuI, 200 μM Cu(OTf)\(_2\), 200 μM Fe(OTf)\(_2\), 200 μM Fe(OTf)\(_3\), 200 μM Mn(OTf)\(_2\), 200 μM Co(OAc)\(_2\), 200 μM Ni(OTf)\(_2\), 200 μM Zn(OAc)\(_2\), 5 mM reduced glutathione (GSH), 1 mM L-cysteine, or 200 μM Na\(_2\)S (Figure 2-7C,D). Both HyCL-3 and HyCL-4-AM showed good selectivity and no significant increase in luminescence intensity towards any of the selected species over the blank control.
Figure 2-7. Selectivity of HyCL-3 ad HyCL-4-AM. (A) Response of 20 μM HyCL-3 in the presence of 200 μg mL⁻¹ rat liver microsomes (RLM), 50 μM NADPH at 1% (red trace) and 20% (black trace) O₂. (B) Response of 20 μM HyCL-4-AM in the presence of 200 μg mL⁻¹ rat liver microsomes (RLM), 50 μM NADPH at 1% (red trace) and 20% (black trace) O₂. Response of (C) 20 μM HyCL-3 and (D) 20 μM HyCL-4-AM to the indicated analytes from 0 to (red bars) 50 min at 10-minute increments. All experiments were performed in 10 mM PBS (pH 7.4) containing ≤ 1% DMSO. Error bars are ± S.D.

Finally, we examined the pH dependence of the acrylic acid dioxetane species 13, which is the pre-chemiluminescence product generated through nitroaromatic reduction and consumption of the compound’s AM ester (Figure 2-8). Responses from 2 μM acrylic acid dioxetane in 20 mM buffer ranging from pH 2.98-10.04 at 37 °C were taken over 40 min. A clear dependence in chemiluminescence intensity maxima is seen over this pH range, as photon counts from pH 8.04-10.04 were maxed out in the first 3 measurements, and exhibited luminescence half-lives around \( t_{1/2} = 140 \) seconds. This indicates near full deprotonation of the dioxetane phenol above pH 8.0. Maximum luminescence intensity generated from the dioxetane is noticeably depreciated at pH 6.98 to 2.94x10⁶ counts paired with an increase in luminescence half-life to approximately to \( t_{1/2} = 220 \) seconds. pH 5.98 shows an intensity maximum at 4.63x10⁶ counts with a near flatlined-luminescence emission output with a half-life that could
not be measured at the experimental time scale. No appreciable luminescence is seen from acrylic acid dioxetanes from pH 2.98-5.02, indicating that deprotonation of dioxetane phenol at this pH range is minimal. These values are fairly consistent with the reported pK\(_a\) values of the methyl acrylate benzoate ester (pK\(_a\) = 6.14) generated after luminescence emission.\(^{49}\) However, it is likely that the generated acrylate methyl ester has a lower pK\(_a\) due to increased electron-withdrawing nature of the negatively charged oxygen as compared to the previously reported methyl ester species. Since 1,2-dioxetane agents are used as biological tools in the pH range of 6.8 to 7.4, these results show there is a small pH dependences within this pH range.

![Figure 2-8. pH dependence of acrylate dioxetane phenol.](image)

**Figure 2-8. pH dependence of acrylate dioxetane phenol.** Response to 2 µM acrylate dioxetane phenol in the presence of 20 mM carbonate, PBS, or Tris buffer (pH 2.98-10.04) containing 5% DMSO.

### 2.2.4 HyCL-3, HyCL-4-AM biological studies

Given that **HyCL-4-AM** exhibited higher sensitivity than **HyCL-3**, we decided to proceed with **HyCL-4-AM** as our primary agent for hypoxia detection in cellular models (see Figure 2-9 for **HyCL-3** cellular data). We first tested the cellular toxicity of **HyCL-4-AM** using an MTT assay, which showed no significant reduction to cell growth at the tested concentrations (Figure 2-9). Next we tested whether **HyCL-4-AM** could distinguish hypoxic and normoxic conditions in A549 cells. Cells in 12-well plates were treated with 40 µM **HyCL-4-AM** or 40 µM **HyCL-4-AM** and 500 µM diphenyleneiodonium chloride (DPI, a broad-spectrum inhibitor
of cytochrome P450 reductase that acts by phenylating the flavin ring through a radical mechanism, rendering it redox inactive) and directly placed into a luminescence plate reader at 37 °C with 5% CO₂ and either 1%, 5%, or 20% O₂ conditions for 20 hours. In all conditions, HyCL-4-AM showed an emission maximum after approximately one hour (Figure 2-10A,B). Interestingly, HyCL-4-AM exhibited a sharp decline from intensity maximum over 7 hours to near baseline values when incubated at 1% O₂ (Figure 2-10A), whereas a slower decrease in chemiluminescence intensity over 16 and 19 hours was observed in cells that were monitored under 5% or 20% O₂ atmosphere, respectively (Figure 2-10B). Comparison to the cellular response of the carboxylate HyCL-4 demonstrated the dramatic effect of the acetoxy methyl ester for efficient cellular operation (Figure 2-11).

**Figure 2-9. HyCL-3 Cellular Data.** (A) A549 cells were incubated with 0–100 µM HyCL-3 for 16 hours and then viability was evaluated using the MTT assay. Error bars are ± S.D. from n = 3 replicates. (B) Time-course of the chemiluminescence emission of A549 cells incubated with 40 µM HyCL-3 at (red trace) 1% O₂ or (blue trace) 20% O₂. (C) Luminescence intensity of 40 µM HyCL-3 at t = 30 min in 1% O₂ and 20% O₂ in A549 cells. Error bars are ± S.D. from n = 9 wells across 3 biological replicates. Statistical significance was assessed using a Student's two-tailed t-test. **** p<5x10⁻⁶.
Figure 2-10. **HyCL-4-AM MTT assay.** A549 cells were incubated with 0–100 µM HyCL-3 for 16 hours and then viability was evaluated using the MTT assay. Error bars are ± S.D. from n = 3 technical replicates.

Figure 2-11. **Measuring hypoxia in living A549 cells.** (A)–(C) Time-course of the chemiluminescence emission of A549 cells incubated with 40 µM HyCL-4-AM at 1% O₂, 5% O₂ or 20% O₂ in the presence or absence of DPI. (D) Chemiluminescence emission intensity at 300 min of A549 cells incubated with 40 µM HyCL-4-AM at 1% O₂, 5% O₂, and 20% O₂. (E) Measured rate constants for the cellular response of HyCL-4-AM. (F) Time-course of the chemiluminescence emission of A549 cells incubated with 40 µM HyCL-4-AM-Cont at 20% O₂ in the presence or absence of DPI. All experiments were performed in F12K containing 10% FBS and ≤ 1% DMSO. Error bars are ± S.D. Statistical significance was assessed using a two-tailed Student's t-test. *p<0.05 (n= 1-4 biological replicates) *****p<5x10⁻⁷ (n = 1–4 biological replicates), ******p<5x10⁻⁹ (n = 9–12 wells across 3–4 biological replicates).
Figure 2-12. Comparison of the cellular response of HyCL-4-AM and HyCL-4. (A) Time-course of the chemiluminescent emission of 40 µM HyCL-4-AM in the (red trace) presence and (blue trace) absence of A549 cells at 1% O₂ and 37 °C. (B) Time-course of the chemiluminescent emission of 40 µM HyCL-4 in the (red trace) presence and (blue trace) absence of A549 cells at 1% O₂ and 37 °C. Values are the average of n = 3 technical replicates, except for the red trace in Figure 2-12A, which are the average of n = 9 wells across three biological replicates.

While HyCL-4-AM showed a clear response over four hours in the presence of A549 cells, with no response from cell culture media in the absence of cells (Figure 2-11A), HyCL-4 showed only a very bright background that rapidly decays with no clear difference in the presence or absence of cells (Figure 2-11B). Furthermore, treatment with 500 µM DPI at 1% O₂ slows the decay and yields a result indistinguishable from the response at 20% O₂ (Figure 2-10A,B), demonstrating that DPI has an effect similar to that of O₂. Comparison of the cellular response of HyCL-4-AM at 1% versus 20% O₂ showed a highly significant and reproducible reduction in luminescence emission under hypoxic conditions (Figure 2-10C,D). This, at first, appears contradictory to established literature on biological nitroaromatic reduction chemistry, but can be understood when considering the kinetics of chemiluminescence emission. The rate of chemiluminescence decomposition of the acrylic acid dioxetane phenol released by HyCL-4-AM was measured to be 5.47 x 10⁻³ s⁻¹ and completely decayed within 15 minutes at pH 7.4 (Figure 2-12A), approximately ten times more rapidly than the acrylonitrile dioxetane phenol (Figure 2-12B). In cells, it takes more than 150 minutes for the signal decay, indicating that nitroaromatic group reduction is the rate-limiting step for producing chemiluminescence.
Figure 2-13. Rate of chemiluminescence decomposition of dioxetane phenols at biological pH. (A) Time-course of the chemiluminescent emission of 0, 0.5, 1.0, 1.5, 2.0, 2.5, and (red trace) 3.0 µM acrylic acid phenol dioxetane in 20 mM PBS (pH = 7.4) and ≤ 1% DMSO. (B) Time-course of the chemiluminescent emission of 0, 0.5, 1.0, 1.5, 2.0, 2.5, and (red trace) 3.0 µM acrylonitrile phenol dioxetane in 20 mM PBS (pH = 7.4) and ≤ 1% DMSO.

2.2.5 HyCL-4-AM kinetic modeling in A549 cells

We modeled the luminescence output from the probe in A549 cells as an X to Y to Z consecutive reaction (Figure 2-13) for . \( k_1 \) represents cellular uptake and intracellular esterase activity, and \( k_2 \) includes both nitroaromatic reduction and light emission through CIEEL. This is modeled by the rate equation (Figure 2-13B), where \( y \) is the relative emission intensity, \( k_1 \) a rate of cell uptake and ester cleavage, \( k_2 \) is the rate of nitroaromatic group reduction, \( A \) is a parameter proportional to the initial concentration of the probe, and \( B \) is a parameter to fit the background. Fitting the cellular data to this model (Figure 2-13C,D) shows that the rate constant \( k_1 \) does not significantly change, but \( k_2 \) significantly decreases from 2.82 x 10⁻⁴ M⁻¹ s⁻¹ at 1% O₂ to 1.79 x 10⁻⁵ M⁻¹ s⁻¹ at 20% O₂ (Figure 2-13E), consistent with O₂ inhibition of nitroreductase activity. This model demonstrates that HyCL-4-AM can provide a numerical measure of the inhibition of endogenous nitroreductase activity by O₂ by tracking changes in \( k_2 \). By comparison, 40 µM HyCL-4-AM-Cont in A549 cells showed only baseline luminescence emission over the same conditions and time span (Figure 2-13F), confirming the requirement of nitroreductase activity for the chemiluminescence response.
Figure 2-14. Kinetic model. (A) Reactions used to model the chemiluminescence emission. (B) Rate equation for the chemiluminescence emission. y-value represents [HyCL-4AM] generated after $k_1$. (C) Example fit of the chemiluminescence response of 40 µM HyCL-4-AM in A549 cells at 1% O$_2$. (D) Example fit of the chemiluminescence response of 40 µM HyCL-4-AM in A549 cells at 20% O$_2$. See Appendix 2 for equation derivation and alternate fits.

2.2.6 HyCL-4-AM in vivo imaging

After demonstrating that HyCL-4-AM displayed an alteration in chemiluminescence kinetics dependent on O$_2$ levels, we sought to investigate its behavior in animal models. We subcutaneously implanted the flanks of athymic nude mice with MDA-MB-231 breast cancer cells and allowed the tumors to grow to appropriate size for hypoxia testing. The mice were set to breathe 16% O$_2$ with 2.5% isoflurane for 10 minutes and then treated with 30 µL of 120 µM HyCL-4-AM via intratumoral (IT) injection. These were compared with mice administered with the same amount of HyCL-4-AM via intramuscular (IM) injection. Images were acquired every minute for 100 minutes using an IVIS Spectrum. HyCL-4-AM showed strong luminescence through tissue that was dependent on O$_2$ level the mouse was breathing (Figure 2-14). By comparing the response of chemiluminescence emission over 100 minutes, the rate of signal
decay in hypoxic tumor tissue at 16% O$_2$ was faster than in the well oxygenated muscle tissue (Figure 2-14A), consistent with the results of our cellular studies.

Figure 2-15. Measuring oxygenation in healthy muscle tissue and tumor xenografts. (A)–(C) Time course of the chemiluminescence emission of 30 µL of 120 µM HyCL-4-AM in 20 mM PBS (pH 7.4) containing 2.4% DMSO in athymic nude mice after intramuscular (IM) injection into the flank, intratumoral injection while mice were breathing 16% O$_2$ (IT, 16% O$_2$), and intratumoral injection while mice were breathing 100% O$_2$ (IT, 100%). (D) Photon flux and (E) chemiluminescence images of athymic nude mice 60 minutes after IM injection, IT injection while mouse breathed 16% O$_2$, and after IT injection while mouse breathed 100% O$_2$. Error bars are ± S.E. from n = 3–6 mice. Statistical significance was assessed using a two-tailed Student's t-test. *p<0.05.

It has been shown that hyperoxic treatment of tumors can be used to both characterize and resensitize tumors to support aerobic metabolism.$^{12,55}$ When mice were set to breath 100% O$_2$, the response of HyCL-4-AM showed clear changes in $k_2$ similar to trends seen in IM
injection, showing that hyperoxic treatment can re-oxygenate tumors and this tumor response can be monitored using **HyCL-4-AM** (Figure 2-14B). Comparison between IT injections with mice breathing 16% O₂ versus 100% O₂ showed that, on average, signal decayed more rapidly when mice breathe 16% O₂ (Figure 2-14C). It was observed, however, some tumors responded differently to hyperoxic treatment, indicating potential for **HyCL-4-AM** to differentiate between tumors that respond to hyperoxic treatment from those that don't. Representative images from mice 60 minutes after injection showed differentiation between hypoxic and well oxygenated tissues (Figure 2-14E). Overall, these results show that **HyCL-4-AM** can effectively distinguish hypoxic tumors from healthy tissue *in vivo*.

### 2.4 Conclusions

In conclusion, we have synthesized **HyCL-3** and **HyCL-4-AM** as chemiluminescent indicators of hypoxia in cellular and animal models. Both probes provide a luminescence response to nitroreduction by bacterial nitroreductase or RLM with added cofactors. Due to increased sensitivity, **HyCL-4-AM** was further tested in cells, where it exhibited luminescence emission kinetics that were highly dependent on oxygen concentrations within the cell. Furthermore, robust animal studies demonstrated the ability of **HyCL-4-AM** to measure and image hypoxia in tumor xenograft models. The observed inhibition of reductase activity in oxygenated tissue is consistent with the response expected from one-electron mammalian reductase enzymes and is unlikely to be due to oxygen-independent bacterial nitroreductase activity. In comparison to the recently published **CL-NTR**, the inclusion of the AM ester moiety provides a drastic increase in sensitivity and response due to increased cell uptake. This opens up the possibility of high throughput cell experiments that have thus far not been reported for previous chemiluminescent probes **HyCL-2** and **CL-NTR**. **HyCL-4-AM** provides a
maximum photon flux of more than $1 \times 10^7$ photons s$^{-1}$ in tumor xenografts, which is ca. 1000-fold greater mole for mole than previously reported chemiluminescent hypoxia probes.\textsuperscript{25,40} This kinetics-based approach to monitoring cellular hypoxia is a significant advance in chemiluminescence detection technology since the reaction kinetics are less dependent on tumor size and depth of injection, which can vary greatly between experiments, and could explain potential differences in results from less sensitive probes. This study establishes that \textbf{HyCL-4-AM} is a highly reproducible tool to investigate hypoxia in cells and has efficacy in monitoring tumor oxygenation in animal models. More generally, we showed that installation of an AM ester provides a versatile strategy for using 1,2-dioxetane probes in cellular systems.

\textbf{2.4 Experimental section}

\textbf{2.4.1 General synthetic methods and materials}

All reactions were performed in dried glassware under an atmosphere of dry N$_2$. 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 or by staining with iodine. Other reagents were purchased from Sigma-Aldrich (St. Louis, MO), Alfa Aesar (Ward Hill, MA), EMD Millipore (Billericia, MA), Oakwood Chemical (West Columbia, SC), and Cayman Chemical (Ann Arbor, MI) and used without further purification. $^1$H NMR for compounds 13, 14, 15, 3, 16, \textbf{HyCL-4-AM}, 17, 4, 18, \textbf{HyCL-4} and \textbf{HyCL-4-AM-Cont} and $^{13}$C NMR for compounds 13, 15, 3, 16, 4, and 8 were collected on a Bruker 400 MHz spectrometer in the Department of Chemistry at Southern Methodist University. $^1$H NMR for compound \textbf{HyCL-3} and $^{13}$C NMR for compounds 14, \textbf{HyCL-3}, \textbf{HyCL-4-AM}, 17, \textbf{HyCL-4} and \textbf{HyCL-4-AM-Cont} were measured on a JEOL 500 MHz spectrometer in the Department of Chemistry at Southern Methodist University.
University. All $^1$H and $^{13}$C NMR spectra for characterization of new compounds and monitoring reactions were collected in CDCl$_3$ (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 2-4. Full synthesis of HyCL-3, HyCL-4-AM, HyCL-4, HyCL-4-AM-Cont, and dioxetane phenols.
2-chloro-1-(dimethoxymethyl)-3-methoxy-benzene (6). 2-chloro-3-methoxybenzaldehyde 5 (10 g, 59 mmol, 1.0 equiv) was dissolved in 100 mL anhydrous methanol in a round bottom flask under a nitrogen atmosphere at room temperature. *p*-toluenesulfonic acid (1115 mg, 6.475 mmol, 0.1 equiv) and trimethyl orthoformate (6.41 mL, 58.6 mmol, 1.0 equiv) were then added. The reaction was stirred at room temperature for 24 hr. Upon completion, the crude mixture was poured into a separatory funnel and washed with saturated NaHCO$_3$ and brine mixture. The organic layer was extracted with 3 x 50 mL EtOAc. The combined organic layers were collected and dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure to give 6 as a colorless oil without further purification. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.25 (m, 2H), 6.93 (m, 1H), 5.65 (s, 1H), 3.90 (s, 3H), 3.38 (s, 6H).

Diethyl ((2-chloro-3-methoxyphenyl)(methoxy)methyl)phosphonate (7). Acetal 6 (19 g, 88 mmol, 1.0 equiv) was dissolved in 100 mL anhydrous DCM in a dry round bottom flask under N$_2$ atmosphere and cooled to 0 °C. Boron trifluoride diethyl etherate (11.4 mL, 90.9 mmol, 1.0 equiv) was added dropwise and allowed to stir for 10 min. Then, triethyl phosphite (15.6 mL, 90.9 mmol, 1.03 equiv) was also added dropwise and stirred for 10 min. The reaction was then heated to 45 °C and refluxed for 1.5 hr and monitored by TLC. Upon completion, the reaction was transferred into a separatory funnel, washed with brine, extracted with 3 x 50 mL DCM. The
combined organic layer was dried with Na₂SO₄, filtered, and concentrated under reduced pressure. Purification by column chromatography (50% EtOAc/ hexanes) yielded 7 as a beige oil. ¹H NMR (400 MHz, CDCl₃) δ 7.32 (m, 2H), 6.94 (m, 1H), 5.23 (d, 1H, J = 16.0 Hz), 4.17–4.21 (m, 4H), 4.16 (s, 3H), 3.37 (s, 1H), 1.31–1.35 (t, 3H, J = 6.8 Hz), 1.20–1.24 (t, 3H, J = 6.8 Hz).

(1r,3r,5R,7S)-2-((2-chloro-3-methoxyphenyl)(methoxy)methylene)adamantane (8).

Phosphonate 7 (4.33 g, 13.4 mmol, 1.0 equiv) was dissolved in 50 mL anhydrous THF in an oven-dried round bottom flask under N₂ atmosphere at −78 ºC. 2.6 M n-BuLi (6.2 mL 16. mmol, 1.2 equiv), was added dropwise over 10 min and allowed to stir for 20 min. Then, 2-adamantanone (2420 mg, 16.12 mmol, 1.2 equiv) dissolved in 15 mL anhydrous THF was added dropwise and allowed to stir for 5 min. Then, the reaction was taken out of the −78 ºC bath and allowed to stir for 2 hr. The temperature was raised to 90 ºC and the reaction was refluxed for 1 hr. Upon completion as determined by TLC, the reaction was transferred to a separatory funnel and quenched with sat. NH₄Cl. The organic layer was extracted with 3 x 30 mL EtOAc, dried with Na₂SO₄, and concentrated under reduced pressure. Column chromatography (5% EtOAc/ hexanes) yielded 8 (3.56 g, 11.2 mmol, 83%) as an amber oil. ¹H NMR (400 MHz, CDCl₃) δ 7.25 (t, 1H, J = 7.6 Hz), 6.89–6.94 (m, 2H), 3.95 (s, 3H), 3.36 (s, 3H), 3.30 (s, 1H), 1.34–2.20 (m, 12H).
3-(((1R,3R,5R,7S)- adamantan-2-ylidene)(methoxy)methyl)-2-chlorophenol (9). Ether 8 (4.5 g, 14 mmol, 1.0 equiv) was dissolved in 50 mL anhydrous DMF in an oven-dried round bottom flask under N\textsubscript{2} atmosphere. Sodium ethanethiolate (1430 mg, 17.00 mmol, 1.2 equiv) and Cs\textsubscript{2}CO\textsubscript{3} (5540 mg, 17.00 mmol, 1.2 equiv) were added to the solution. The reaction was heated to 90 °C and stirred for 12 hr. Upon completion as determined by TLC, the crude reaction was washed with sat. NH\textsubscript{4}Cl and brine, and extracted with 3 x 30 mL EtOAc. The organic layer was dried with Na\textsubscript{2}SO\textsubscript{4}, filtered, and concentrated under reduced pressure. Column chromatography (10% EtOAc/ hexanes) yielded compound 9 (3.68 g, 12.06 mmol, 85%) as an off-white solid. \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) δ 7.05 (t, 1H, \textit{J} = 7.5 Hz), 6.92 (d, 1H, \textit{J} = 6.0 Hz), 6.73 (d, 1H, \textit{J} = 6.0 Hz), 3.22 (s, 3H), 3.19 (s, 1H), 1.72–2.20 (m, 12H).

3-(((1R,3R,5R,7S)- adamantan-2-ylidene)(methoxy)methyl)-2-chloro-6-iodophenol (10). Compound 9\textsuperscript{1} (3677 mg, 12.06 mmol, 1.0 equiv) was dissolved in 50 mL anhydrous toluene under N\textsubscript{2} atmosphere at 0 °C. N-Iodosuccinimide (2714 mg, 12.06 mmol, 1.0 equiv) was then added in 1 portion. The reaction was stirred for 1 hr. Upon completion of the reaction as determined by TLC, the reaction was transferred into a separatory funnel and washed with brine. A few crystals of sodium thiosulfate pentahydrate were added and the separatory funnel was shaken vigorously to quench any remaining iodine. Upon quenching, the organic layer changed...
from pink to colorless. The organic layer was then extracted with 3 x 30 mL EtOAc, dried with Na₂SO₄, and concentrated under reduced pressure. Washing the crude residue with hexanes yielded compound 10 (1.68 mg, 3.9 mmol, 30%) as a white solid. ¹H NMR (400 MHz, CDCl₃) δ 7.64 (d, 1H, J = 8.0 Hz), 6.65 (d, 1H, J = 8.0 Hz), 6.16 (s, 1H), 3.33 (s, 3H), 3.28 (s, 1H), 1.63–2.11 (m, 12H).

\[(E)-3-(4-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chloro-2-hydroxyphenyl)acrylonitrile\ (1).\]

Iodophenol 10¹ (500 mg, 1.16 mmol, 1.0 equiv) was dissolved in 3 mL anhydrous MeCN under N₂ atmosphere in a 10 mL microwave flask. Acrylonitrile (0.22 mL, 3.5 mmol, 3.0 equiv), anhydrous Et₃N (0.24 mL, 1.7 mmol, 1.5 equiv), and Pd(OAc)₂ (13.0 mg, 0.058 mmol, 0.05 equiv) were added to the solution. The reaction was capped and microwaved at 120 °C for 70 min. Upon completion, the reaction was transferred to a separatory funnel and washed with NH₄Cl and brine mixture. The organic layer was extracted with 3 x 30 mL EtOAc, dried with Na₂SO₄, filtered, and concentrated under reduced pressure. Column chromatography (100 % DCM) yielded compound 1 (201 mg, 0.56 mmol, 48%) as a yellow solid. ¹H NMR (400 MHz, CDCl₃) δ 7.61 (d, 1H, J = 16.8 Hz), 7.29 (d, 1H, J = 8.0 Hz), 6.90 (d, 1H, J = 8.0 Hz), 6.21 (d, 1H, J = 16.8 Hz), 3.33 (s, 3H), 3.28 (s, 1H), 1.63–2.18 (m, 12H).
Compound (11). Compound 1 (112 mg, 0.341 mmol, 1 equiv) and Rose bengal (18.1 mg, 0.0186 mmol, 0.0545 equiv) added to a 2-neck round bottom flask and dissolved in 5 mL THF. Oxygen was bubbled through the reaction mixture, while irradiating with a 120 W light bulb (Home Depot, Dallas, TX) at 0 °C. After 2 hr of reaction, TLC showed no starting material left and the mixture was then concentrated under vacuum at 0 ºC. The residue was purified by column chromatography (5% EtOAc/ hexanes) to deliver compound 10 (74.8 mg, 0.129 mmol, 41%) as a white solid. H NMR (400 MHz, CDCl3) δ 7.72 (d, 1H, J = 8.0 Hz), 7.60 (d, 1H, J = 16.8 Hz), 6.44 (d, 1H, J = 8.0 Hz), 6.23 (d, 1H, J = 16.8 Hz), 3.24 (s, 3H), 3.04 (s, 1H), 1.22–2.26 (m, 12H).

(E)-3-(4-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chloro-2-hydroxyphenyl)acrylic acid (2). In an oven-dried round bottom flask, iodophenol 10 (431 mg, 1.00 mmol, 1.0 equiv) was dissolved in 5 mL anhydrous MeCN under N2 atmosphere. Methyl acrylate (0.26 mL, 3.0 mmol, 3.0 equiv), Et3N (0.20 mL, 1.5 mmol, 1.5 equiv), Pd(OAc)2 (11 mg 0.05 mmol, 0.05 equiv), and tris(ortho-tolyl)phosphine (3 mg, 0.01 mmol, 0.01 equiv) were added into the solution. The reaction was heated to 120 ºC and refluxed for 2 hr. Upon completion of the reaction as determined by TLC, the crude mixture was transferred to a separatory funnel and
washed with NH₄Cl. The organic layer was extracted with 3 x 30 mL EtOAc, dried with Na₂SO₄, filtered, and concentrated under reduced pressure. Purification by column chromatography (15% EtOAc/ hexanes) yielded compound 2 (215 mg, 0.55 mmol, 55%) as a yellow solid. ¹H NMR (400 MHz, CDCl₃) δ 7.98 (d, 1H, J = 16.0 Hz), 7.41 (d, 1H, J = 8.0 Hz), 6.90 (d, 1H, J = 8.0 Hz), 6.67 (d, 1H, J = 16.0 Hz), 6.22 (s, 1H), 3.84 (s, 3H), 3.33 (s, 3H), 3.29 (s, 1H), 1.63–2.20 (m, 12H).

(E)-3-(4-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chloro-2-hydroxyphenyl)acrylic acid (12).¹ Acrylic methyl ester phenol 2¹ (0.668 mmol, 260 mg 1 equiv) was dissolved in 5.0 mL THF in a dry round bottom flask flushed with nitrogen. 5 mL of 1.0 M LiOH in H₂O was added to the solution. The reaction was then heated to 80 ºC and stirred under reflux for 4 hr. Upon completion of the reaction as determined by TLC, the crude was washed with 1 M HCl and extracted with 3 x 30 mL EtOAc. The organic layer was collected, dried with Na₂SO₄, and evaporated under reduced pressure, yielding compound 12 (257 mg, 0.668 mmol, quantitative) without further purification. ¹H NMR (400 MHz, CDCl₃) δ 8.00 (d, 2H, J = 16.1 Hz), 7.49 (d, 2H, J = 8.0 Hz), 6.83 (d, 2H, J = 8.0 Hz), 6.59 (d, 2H, J = 16.1 Hz), 3.30 (s, 3H), 3.24 (s, 1H), 1.40–2.22 (m, 12H).
(E)-3-(3-chloro-2-hydroxy-4-((1r,3r,5r,7r)-4'-methoxyspiro[adamantane-2,3'-[1,2]dioxetan]-4'-yl)phenyl)acrylic acid (13). Enol ether 12 (20 mg, 0.053 mmol, 1 equiv) and methylene blue (5.0 mg, 0.015 equiv, 0.028 equiv) were added into a dry flask and dissolved in 5 mL THF. Oxygen was bubbled through the reaction mixture, while irradiating with a 120 W light bulb (Home Depot, Dallas, TX) at 0 °C. After 1.5 hr of reaction, TLC showed no starting material left and the mixture was then concentrated under vacuum at 0 °C. The residue was purified by column chromatography (50% EtOAc/ hexanes) to deliver 13 (19.5 mg, 97%) as a white solid.

$^1$H NMR (400 MHz, CDCl$_3$) δ 8.06 (d, 1H, $J = 16.4$ Hz), 7.72 (d, 1H, $J = 8.0$ Hz), 7.58 (d, 1H, $J = 8.0$ Hz), 6.70 (d, 1H, $J = 16.4$ Hz), 6.65 (br s, 1H), 3.26 (s, 3H), 3.04 (s, 3H), 1.27-2.26 (m, 12H); $^{13}$C NMR (100 MHz, CDCl$_3$) 171.80, 151.04, 140.73, 134.25, 127.16, 124.76, 123.49, 120.37, 96.32, 49.72, 39.28, 36.51, 34.07, 33.45, 32.82, 32.16, 31.56, 26.12, 25.78; LRMS data for C$_{21}$H$_{23}$ClO$_6$ [M-H]$^-$ found 405.3.

(E)-3-(4-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chloro-2-((4-nitrobenzyl)oxy)phenyl)acrylonitrile (14). Acrylonitrile phenol 1 (85 mg, 0.22 mmol, 1.0 equiv), triphenyl phosphite (69 mg, 0.26 mmol, 1.2 equiv) and 4-nitrobenzyl alcohol (33 mg, 0.22 mmol, 1.0 equiv) were added to a dry round bottom flask flushed with nitrogen. The
reaction contents were dissolved in 3.0 mL THF, and the reaction was cooled to 0 °C. Diethyl azodicarboxylate (41 µL, 0.26 mmol, 1.2 equiv) was added dropwise to the solution, and stirred for 1 hr. The crude mixture was combined with 30 mL brine solution, and mixture was washed with 2 x 20 mL EtOAc. The combined organic layers were collected and dried over Na₂SO₄, filtered, and concentrated under reduced pressure. Purification by column chromatography (10% EtOAc/Hexanes) yielded 14 (99 mg, 92%) as a pale yellow solid. ¹H NMR (400 MHz, CDCl₃) δ 8.31 (d, 2H, J = 8.8 Hz), 7.69 (d, 2H, J = 8.8 Hz), 7.60 (d, 1H, J = 16.8 Hz), 7.41 (d, 1H, J = 8.0 Hz), 7.16 (d, 1H, J = 8.0 Hz), 6.98 (d, 1H J=16.8 Hz), 5.13 (d, 2H, J = 6.8 Hz), 3.32 (s, 3H), 3.30 (s, 1H), 1.61–2.08 (m, 12H); ¹³C NMR (125 MHz, CDCl₃) δ 153.07, 148.03, 144.38, 143.05, 139.69, 139.07, 133.62, 129.87, 128.53, 128.38, 124.01, 117.91, 99.12, 77.86, 76.86, 57.69, 38.74, 37.02, 33.10, 29.84, 28.36, 28.19; HRMS calcd for C₂₈H₂₇ClN₂O₄ [M-H]⁻ 489.1587, found 489.1592.

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\text{(E)-3-(3-chloro-4-((1\text{r},3\text{r},5\text{r},7\text{r})-4'-methoxyspiro[adamantane-2,3'-(1,2)dioxetan]-4'-yl)-2-((4-nitrobenzyl)oxy)phenyl)acrylonitrile (HyCL-3). Enol ether 14 (40 mg, 0.081 mmol, 1.0 equiv) and Rose bengal (8.5 mg, 0.0087 mmol, 0.11 equiv) were added into a dry flask and dissolved in 5 mL THF. Oxygen was bubbled through the reaction mixture, while irradiating with a 120 W light bulb (Home Depot, Dallas, TX) at 0 °C. After 3.5 h of reaction, TLC showed no starting material left. The mixture was then concentrated under vacuum at 0 °C and the residue was purified by column chromatography (10% EtOAc/ hexanes) to deliver HyCL-3.}
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(56.4 mg, 87%) as a white solid. $^1$H NMR (500 MHz, CDCl$_3$) δ 8.30 (d, 2H, $J = 6.25$ Hz), 7.99 (d, 1H, $J = 7.45$ Hz), 7.67 (d, 2H, $J = 6.25$ Hz), 7.57 (d, 1H, $J = 17.2$ Hz), 7.25 (d, 1H, $J = 7.45$ Hz), 6.02 (d, 1H, $J = 17.2$ Hz), 5.02 (s, 2H), 3.22 (s, 3H), 3.02 (s, 1H) 1.71–2.28 (m, 12H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 153.53, 148.11, 143.86, 142.69, 136.79, 130.33, 129.77, 128.36, 127.93, 124.91, 124.07, 117.55, 111.59, 100.59, 96.44, 72.14, 49.87, 36.58, 33.73, 32.23, 31.61, 26.16, 25.86; LRMS data for C$_{28}$H$_{27}$ClN$_2$O$_6$ [M+CH$_3$CN+H]$^+$ found 564.8.

![Methyl (E)-3-((4-(((1R,3R,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chloro-2-((4-nitrobenzyl)oxy)phenyl)acrylate (15).](image)

Methyl (E)-3-((4-(((1R,3R,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chloro-2-((4-nitrobenzyl)oxy)phenyl)acrylate (15). Methyl acrylate phenol 2$^1$ (215 mg, 0.55 mmol, 1.0 equiv) was dissolved in 5.0 mL anhydrous acetone in a dry round bottom flask flushed with nitrogen. $p$-Nitrobenzyl tosylate (253 mg, 0.825 mmol, 1.5 equiv) and K$_2$CO$_3$ were added in 2 separate portions. The reaction was stirred at room temperature for 12 hr, at which time TLC inspection showed complete consumption of starting material 2. The crude mixture was combined with 30 mL brine solution, and mixture was washed with 2 x 20 mL EtOAc. The combined organic layers were collected and dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. Purification by column chromatography (10% EtOAc/ hexanes) yielded 15 (228 mg, 92%) as an off-white solid. $^1$H NMR (400 MHz, CDCl$_3$) δ 8.28 (d, 2H, $J = 8.8$ Hz), 7.90 (d, 1H, $J = 16.0$ Hz), 7.70 (d, 2H, $J = 8.8$ Hz), 7.48 (d, 1H, $J = 8.0$ Hz), 7.13 (d, 1H, $J = 8.0$ Hz), 6.47 (d, 1H, $J = 16.0$ Hz), 5.11 (d, 2H, $J = 5.2$ Hz), 3.81 (s, 3H), 3.35 (s, 3H), 3.30 (s, 1H), 1.77–2.10 (m, 12H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 166.95, 153.32, 147.79, 143.40, 139.28,
128.26, 132.87, 129.42, 128.63, 128.28, 125.26, 123.76, 123.38, 74.40, 57.36, 51.88, 39.17, 39.02, 38.67, 36.99, 32.95, 29.71, 28.31, 28.16; HRMS calcd for C$_{29}$H$_{30}$ClNO$_6$ [M+Na]$^+$ 546.1654, found 546.1642.

(E)-3-(4-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chloro-2-((4-nitrobenzyl)oxy)phenyl)acrylic acid (3). Acrylate phenol 15 (228 mg, 0.49 mmol, 1.0 equiv) was dissolved in 5 mL THF in a 20 mL round bottom flask. 5 mL of 1 M LiOH was then added to the mixture. The reaction was placed under an inert atmosphere, heated to 80 ºC, and refluxed for 2 hr. Upon completion, the reaction was transferred into a separatory funnel. The organic layer was washed with 1 M HCl and extracted with 2 x 20 mL EtOAc. The combined organic layers were collected and dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. Purification by column chromatography (50% EtOAc/ hexanes) yielded 3 (142 mg, 57%) as a yellow solid. $^1$H NMR (400 MHz, CDCl$_3$) δ 8.27 (d, 2H, $J = 8.4$ Hz), 7.99 (d, 1H, $J = 16.0$ Hz), 7.70 (d, 2H, $J = 8.4$ Hz), 7.52 (d, 1H, $J = 8.0$ Hz), 7.15 (d, 1H, $J = 8.0$ Hz), 6.47 (d, 1H, $J = 16.0$ Hz), 5.14 (d, 2H, $J = 5.2$ Hz), 3.36 (s, 3H), 3.30 (s, 1H), 1.73–2.19 (m, 12H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 171.65, 153.60, 147.89, 143.26, 140.44, 139.20, 138.88, 133.16, 129.63, 129.11, 128.72, 128.33, 125.43, 123.83, 119.85, 74.61, 57.41, 39.20, 39.05, 38.67, 38.58, 37.00 33.01, 29.76, 28.32, 28.17; HRMS calcd for C$_{28}$H$_{28}$ClNO$_6$ [M-H]$^-$ 508.1532, found 508.1524.
(E)-3-(3-chloro-4-(((1r,3r,5r,7r)-4'-methoxyspiro[adamantane-2,3'-[1,2]dioxetan]-4'-yl)-2-((4-nitrobenzyl)oxy)phenyl)acrylic acid (HyCL-4). Compound 3 (13 mg, 0.048 mmol, 1.0 equiv) and methylene blue (5.0 mg, 0.015 mmol, 0.31 equiv) were added into a dry flask and dissolved in 5 mL THF. Oxygen was bubbled through the reaction mixture, while irradiating with a 120 W light bulb (Home Depot, Dallas, TX) at 0 °C. After 2 h of reaction, TLC showed no starting material left and the mixture was then concentrated under vacuum. The residue was purified by column chromatography (15% EtOAc/hexanes) to deliver HyCL-4 as a white solid (2.6 mg, 20 %). $^1$H NMR (400 MHz, CDCl$_3$) δ 8.29 (d, 2H, $J = 8.8$ Hz), 8.00 (d, 1H, $J = 8.0$ Hz), 7.96 (d, 1H, $J = 16.4$ Hz), 7.66 (d, 2H, $J = 8.0$ Hz), 5.07 (s, 2H), 3.25 (s, 3H), 3.05 (s, 1H), 1.67–2.11 (m, 12H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 170.42, 154.10, 148.06, 142.96, 139.92, 136.04, 131.08, 129.62, 128.82, 125.71, 124.01, 121.02, 111.70, 96.45, 74.04, 49.86, 36.62, 33.70, 32.27, 31.64, 26.20, 25.90.

Acetoxymethyl (E)-3-(4-(((1r,3r,5S,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chloro-2-((4-nitrobenzyl)oxy)phenyl)acrylate (16). Acrylate 3 (98 mg, 0.19 mmol, 1.0 equiv) was dissolved in 1 mL anhydrous DMF and quantitatively transferred into a dry 10 mL round bottom
flask flushed with nitrogen. Anhydrous DIPEA (0.12 mL, 0.69 mmol, 3.6 equiv) was added and allowed to mix for 2 min. Bromomethyl acetate (0.08 mL, 0.79 mmol, 4.1 equiv) was then added dropwise. The reaction was stirred for 24 hr at RT, upon which the starting material was fully consumed as determined by TLC. The crude reaction was then diluted with 4 mL EtOAc and transferred into a separatory funnel. The organic layer was washed with 1 M HCl and extracted with 2 x 20 mL EtOAc. The combined organic layers were collected and dried over Na₂SO₄, filtered, and concentrated under reduced pressure. Purification by column chromatography (10–30% EtOAc/ hexanes gradient) yielded 16 (33 mg, 30%) as a pale yellow solid. ¹H NMR (400 MHz, CDCl₃) δ 8.29 (d, 2H, J = 8.8 Hz), 7.98 (d, 1H, J = 16.0 Hz), 7.69 (d, 2H, J = 8.8 Hz), 7.49 (d, 1H, J = 8.0 Hz), 7.14 (d, 1H, J = 8.0 Hz), 6.47 (d, 1H, J = 16.0 Hz), 5.87 (s, 2H), 5.12 (d, 2H, J = 5.6 Hz), 3.36 (s, 3H), 3.30 (s, 1H), 2.16 (s, 3H), 1.62–2.14 (m, 12H); ¹³C NMR (100 MHz, CDCl₃) δ 169.73, 165.05, 153.53, 147.94, 143.13, 140.13, 134.21, 138.99, 133.16, 129.65, 129.03, 128.79, 128.33, 125.26, 123.86, 119.16, 57.44, 39.20, 39.20, 39.05, 38.69, 38.59, 37.00, 33.00, 31.59, 29.76, 28.32, 28.16, 22.66, 20.75, 14.13; HRMS calcd for C₃₁H₃₂ClNO₈ [M-H]⁻ 580.1744, found 580.1742.

Acetoxymethyl (E)-3-(3-chloro-4-((1r,3r,5r,7r)-4'-methoxyspiro[adamantane-2,3'-[1,2]dioxetan]-4'-yl)-2-((4-nitrobenzyl)oxy)phenyl)acrylate (HyCL-4-AM). Enol ether 16 (22 mg, 0.038 mmol, 1.0 equiv) and Rose bengal (9.8 mg, 0.0087 mmol, 0.107 equiv) were added into a dry flask and dissolved in 5 mL THF. Oxygen was bubbled through the reaction mixture,
while irradiating with a 120 W light bulb (Home Depot, Dallas, TX) at 0 °C. After 3.5 h of reaction, TLC showed no starting material left and the mixture was then concentrated under vacuum. The residue was purified by column chromatography (10% EtOAc/ hexanes) to deliver **HyCL-4-AM** as a pale-yellow solid (20 mg, 85%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.30 (d, 2H, 8.8 Hz), 7.99 (d, 1H, $J = 8.4$ Hz), 7.97 (d, 1H, $J = 16.0$ Hz), 7.71 (d, 1H, $J = 8.4$ Hz), 7.69, (d, 2H, $J = 8.8$ Hz) 6.53 (d, 1H, $J = 16.0$ Hz), 5.87 (s, 2H), 5.05 (s, 2H), 3.25 (s, 3H), 3.05 (s, 1H), 2.16 (s, 3H), 1.60–2.14 (m, 12H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 169.76, 164.84, 148.11, 142.82, 139.60, 129.60, 128.92, 125.55, 124.01, 120.57, 111.68, 96.42, 49.84, 36.62, 33.71, 32.26, 31.64, 26.20, 25.90, 20.80; LRMS for C$_{31}$H$_{32}$ClNO$_{10}$ [M-H]$^-$ found 613.0.

**Methyl (E)-3-4-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-2-(benzyloxy)-3-chlorophenyl)acrylate (17).** Acrylate phenol 2 (90 mg, 0.231 mmol 1.0 equiv) was dissolved in anhydrous acetone and transferred into a dry round bottom flask flushed with nitrogen. K$_2$CO$_3$ (64 mg, 0.46 mmol, 2.0 equiv) was added in one portion and stirred for 2 min. Then, benzyl bromide (0.04 mL, 0.34 mmol, 1.2 equiv) was added in one portion and the reaction was monitored for 3 hr. Additional benzyl bromide (0.03 mL, 0.25 mmol, 1.0 equiv) was added to drive the reaction to completion. The reaction was stirred for 16 hr, then transferred into a separatory funnel. The reaction was then washed with brine and extracted with 3 x 15 mL EtOAc. The combined organic layers were collected and dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. Purification by column chromatography (10–30% EtOAc/
hexanes) yielded 6 (97 mg, 88%) as a pale-yellow solid. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.94 (d, 1H, $J$ = 16.0 Hz), 7.53 (m, 2H), 7.45 (d, 2H, $J$ = 8.0 Hz), 7.39–7.44 (m, 3H), 6.45 (d, 1H, $J$ = 16.0 Hz), 5.03 (d, 2H, $J$ = 5.6 Hz), 3.83 (s, 3H), 3.35 (s, 3H), 3.30 (s, 1H), 1.72–2.10 (m, 12H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 167.08, 153.66, 139.50, 138.94, 132.51, 129.76, 128.89, 128.63, 127.86, 125.11, 119.96, 57.35, 51.88, 38.73, 37.14, 33.01, 29.79, 28.44, 28.28; LRMS for C$_{28}$H$_{29}$ClO$_4$ [M+H]$^+$ found 479.4.

(E)-3-((4-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chloro-2-((4-nitrobenzyl)oxy)phenyl)acrylic acid (4). Methyl acrylate 17 (90 mg, 0.19 mmol, 1.0 equiv) was dissolved in 5 mL THF in a 20 mL round bottom flask. 5 mL of 1 M LiOH was then added to the mixture. The reaction was placed under an inert atmosphere, heated to 80 °C and refluxed for 6 hr. Upon completion, the reaction was transferred into a separatory funnel. The organic layer was washed with 1 M HCl and extracted with 2 x 20 mL EtOAc. The combined organic layers were collected and dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. Purification by column chromatography (50% EtOAc/ hexanes) yielded 4 (77 mg, 86%) as a pale-yellow solid. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.05 (d, 1H, $J$ = 16.0 Hz), 7.51 (m, 3H) 7.38–7.49 (m, 3H), 5.06 (d, 2H, $J$ = 5.6 Hz), 3.37 (s, 3H), 3.32 (s, 1H), 1.73–2.21 (m, 12H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 171.92, 153.92, 140.98, 139.36, 138.62, 135.83, 132.59, 129.96, 129.43, 128.93, 128.64, 128.62, 127.85, 125.23, 119.32, 57.29, 39.22, 39.06, 38.65, 37.07, 32.97, 30.94, 29.74, 28.37, 28.21; HRMS calcd for C$_{28}$H$_{29}$ClO$_4$ [M-H]$^-$ 463.1682, found 463.1675.
Acetoxymethyl (E)-3-(4-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-2-(benzyloxy)-3-chlorophenyl)acrylate (18). Acrylic acid 4 (72 mg, 0.15 mmol, 1.0 equiv) was dissolved in 1 mL anhydrous DMF and quantitatively transferred into a dry 10 mL round bottom flask flushed with nitrogen. Anhydrous DIPEA (0.096 mL, 0.55 mmol, 3.6 equiv) was added and allowed to mix for 2 min. Bromomethyl acetate (0.08 mL, 0.79 mmol, 4.1 equiv) was then added dropwise. The reaction was stirred for 24 hr at RT, upon which the starting material was consumed as determined by TLC. The crude reaction was then diluted with 4 mL EtOAc and transferred into a separatory funnel. The organic layer was washed with saturated NH₄Cl and extracted with 2 x 20 mL EtOAc. The combined organic layers were collected and dried over Na₂SO₄, filtered, and concentrated under reduced pressure. Purification by column chromatography (10–30% EtOAc/ hexanes gradient) yielded 6 (33 mg, 71%) as a pale-yellow solid. ¹H NMR (400 MHz, CDCl₃) δ 8.02 (d, 1H, J = 16.4 Hz), 7.37–7.51 (m, 6H), 7.10 (d, 1H, J = 8.0 Hz), 6.44 (d, 1H, J = 16.4 Hz), 5.90 (s, 2H), 5.03 (d, 2H, J = 5.6 Hz), 3.53 (s, 3H), 3.31 (s, 1H), 1.68–2.19 (m, 15H); ¹³C NMR (100 MHz, CDCl₃) δ 169.72, 165.18, 153.92, 140.76, 139.37, 138.71, 135.79, 132.57, 129.96, 129.35, 128.94, 128.63, 128.61, 127.84, 125.08, 118.59, 57.30, 39.21, 39.05, 38.64, 37.06, 32.96, 30.94, 29.73, 28.36, 28.21, 20.80; HRMS calcd for C₃₁H₃₃ClO₆ [M+Na]⁺ 559.1858, found 559.1844.
Acetoxymethyl (E)-3-(3-chloro-4-((1r,3r,5r,7r)-4'-methoxyspiro[adamantane-2,3'-[1,2]dioxetan]-4'-yl)-2-((4-nitrobenzyl)oxy)phenyl)acrylate (HyCL-4-AM-Control). Enol ether 18 (29 mg, 0.081 mmol, 1.0 equiv) and Rose bengal (7.9 mg, 0.0081 mmol, 0.1 equiv) were added into a dry flask and dissolved in 5 mL THF. Oxygen was bubbled through the reaction mixture, while irradiating with a 120 W light bulb (Home Depot, Dallas, TX) at 0 °C. After 3.5 h of reaction, TLC showed no starting material left and the mixture was then concentrated under vacuum. The residue was purified by column chromatography (20% EtOAc/hexanes) to deliver HyCL-4-AM-Cont as a pale-yellow solid (25 mg, 82%). $^1$H NMR (400 MHz, CDCl$_3$) 7.99 (d, 1H, $J = 16.0$ Hz), 7.94 (d, 1H, $J = 8.0$ Hz), 7.58 (d, 1H, $J = 8.0$ Hz), 7.48 (m, 2H), 7.38–7.44 (m, 3H), 6.48 (d, 1H, $J = 16.0$ Hz), 5.90 (s, 2H), 4.97 (s, 2H), 3.25 (s, 3H), 3.05 (s, 1H), 1.75–2.36 (m, 15H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta 169.77, 164.98, 154.42, 140.22, 135.77, 135.53, 131.33, 129.08, 128.88, 128.79, 125.34, 119.94, 111.81, 86.46, 49.84, 33.68, 32.30, 33.68, 32.30, 31.65, 29.80, 26.24, 25.89, 20.87, 14.22; HRMS calcd for C$_{31}$H$_{33}$ClO$_8$ [M+Na]$^+$ 591.1756, found 591.1702.

$p$-Nitrobenzyl tosylate (19).$^{56}$ In a 100 mL round bottom flask under nitrogen atmosphere, $p$-nitrobenzyl alcohol (2002 mg, 13.07 mmol, 1.1 equiv) and $p$-toluenesulfonyl chloride (2265 mg
11.88 mmol, 1.0 equiv) were dissolved in 25 mL THF. In a separate 50 mL roundbottom flask, NaOH (713 mg, 17.82 mmol, 1.5 equiv was dissolved in 25 mL H₂O and poured into the flask. The reaction was stirred at rt for 3 hr. Upon completion as determined by TLC, the reaction was transferred into a separatory funnel and washed with sat. NH₄Cl, brine and EtOAc. The organic layer was then extracted with 3 x 50 mL EtOAc. The combined organic layer was dried with Na₂SO₄, filtered, and concentrated under reduced pressure. Product was recrystallized in hot EtOH to give p-nitrobenzyl tosylate (3655 mg, 91%) as a burnt orange solid. 'H NMR (400 MHz, CDCl₃) δ 6.89–6.94 (m, 2H), 3.95 (s, 3H), 3.34 (s, 3H), 3.30 (s, 1H), 1.34–2.20 (m, 12H).

2.4.2 Chemiluminescence response of HyCL-3 to NTR and NADH

Dose dependent responses to NTR and NADH for HyCL-3 were acquired using a Hitachi F-7000 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan) using the luminescence detection module and setting luminescence emission at 525 nm. 10 μM HyCL-3 was treated with 400 μM NADH and 0, 2.5, 5, 7.5, 10, and 12.5 μg mL⁻¹ NTR. Scans were started immediately after addition of all agents, and luminescence was measured over 30 min. The integrated emission intensity was measured on a Cytation 5 BioTek plate reader via luminescence detection mode at 37 ºC using a gain set to 135. Measurements were taken every 20 minutes over 4 hours. Reported values are the average of 3 technical replicates.

2.4.3 Chemiluminescence response of HyCL-3 and HyCL-4-AM to NTR and NADH and rat liver microsomes (RLM)

Chemiluminescent emission wavelength spectra were acquired using a Hitachi F-7000 fluorescence spectrophotometer via the luminescence detection module and scanning luminescence emission from 400–700 nm in response to NTR and NADH. Final concentrations of 200 μM NADH, 1 μg mL⁻¹ nitroreductase from Escherichia coli (NTR, Sigma-Aldrich
N9284-1MG) in DI-H$_2$O, and 10 µM HyCL-3 or HyCL-4-AM were added to a solution of 20 mM PBS buffered to pH 7.41. A final concentration of 1 U mL$^{-1}$ pig liver esterase (PLE) was also added for HyCL-4-AM reads. Scans were acquired using the wavelength scan module 10 min after addition of the probe to the solution. Dose dependent responses to NTR and NADH for HyCL-3 and HyCL-4-AM from 0–1000 ng mL$^{-1}$ NTR were acquired using a Cytation 5 BioTek plate reader via 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, 10 µM HyCL-3 or HyCL-4-AM in 20 mM PBS buffered to pH 7.4, containing ≤ 1% DMSO were treated 0–1000 ng mL$^{-1}$ NTR and 200 µM NADH. A final concentration of 1 U mL$^{-1}$ PLE was used for HyCL-4-AM reads. Luminescence emission was measured for 4 hours. Rat liver microsome experiments were conducted in a 96 well plate using final concentrations of 200 µg mL$^{-1}$ RLM, 50 µM NADPH, and 20 µM HyCL-3 or HyCL-4-AM in 20 mM PBS (pH 7.4). The plate was then placed into a Cytation 5 BioTek plate reader. The luminescence was recorded using the luminescence detection mode, end point read type, and setting gain to 135 and temperature to 37 °C for 4 hr. Control experiments with probe alone were performed in a separate plate to avoid increased background due to light leakage. Reported values are the average of 3 technical replicates.

2.4.4 GC/MS determination of HyCL-3 and HyCL-4-AM products

In a 20 mL vial, 1.5 mg of HyCL-3 or HyCL-4-AM was dissolved in 10 mL acetone. Then, 9.9 mL of 100 µM NADPH were added to the vial and 100 µL of 20 mg mL$^{-1}$ RLM were added for a final concentration of 122 µM HyCL-4-AM or 144 µM HyCL-3, 50 µM NADPH and 200 µg mL$^{-1}$ RLM. The reaction was capped and allowed to stir for 12 hr. Upon completion, the reaction was transferred to a separatory funnel and washed with sat. NH$_4$Cl. The organic layer was
extracted with 3 x 10 mL EtOAc. The organic layer was then dried with Na₂SO₄, filtered, and concentrated under reduced pressure, reconstituted with 1.5 mL DCM, and transferred into a 2 mL GC/MS vial. GC/MS was conducted immediately using a 6850 Series GC/MS (Agilent Technologies, Santa Clara, CA). Mass spectra were averaged across the major peaks in the chromatogram and molecular ions for m/z = 150.1 (Figure S1) and m/z = 138.8 (Figure S2) were found. The spectrum of 2-adamantanone was matched against the NIST standard, which can be found via web at:

2.4.5 Selectivity studies.

Selectivity for HyCL-3 was measured by monitoring the time-dependent full-spectrum chemiluminescence emission using a Cytation 5 BioTek plate reader (Winooski, VT) by using the luminescence detection method, endpoint read type, and setting gain to 135 and temperature to 37 °C. All assays were performed in 20 mM PBS buffered to pH 7.4. All analytes were tested with a final concentration of 200 µM for all metal species, 5 mM for GSH, 1 mM for cysteine, and 200 µM for Na₂S. Values are the average of 3 technical replicates.

Cu²⁺ (200 µM): 1 µL of 50 mM Cu(OTf)₂ in DI H₂O was added to a solution of 245 µL 20 mM PBS (pH 7.43) buffer and 1 µL of 5 mM HyCL-3 or HyCL-4-AM (20 µM final concentration) in DMSO.

Fe²⁺ (200 µM): 1 µL of 50 mM Fe(OTf)₂ in DI H₂O was added to a solution of 245 µL 20 mM PBS (pH 7.43) buffer and 1 µL of 5 mM HyCL-3 or HyCL-4-AM (20 µM final concentration) in DMSO.
Fe$^{3+}$ (200 µM): 1 µL of 50 mM Fe(OTf)$_3$ in DI H$_2$O was added to a solution of 245 µL 20 mM PBS (pH 7.43) buffer and 1 µL of 5 mM HyCL-3 or HyCL-4-AM (20 µM final concentration) in DMSO.

Mn$^{2+}$ (200 µM): 1 µL of 50 mM Mn(OTf)$_2$ in DI H$_2$O was added to a solution of 245 µL 20 mM PBS (pH 7.43) buffer and 1 µL of 5 mM HyCL-3 or HyCL-4-AM (20 µM final concentration) in DMSO.

Co$^{2+}$ (200 µM): 1 µL of 50 mM Co(OAc)$_2$ in DI H$_2$O was added to a solution of 245 µL 20 mM PBS (pH 7.43) buffer and 1 µL of 5 mM HyCL-3 or HyCL-4-AM (20 µM final concentration) in DMSO.

Ni$^{2+}$ (200 µM): 1 µL of 50 mM Ni(OTf)$_2$ in DI H$_2$O was added to a solution of 245 µL 20 mM PBS (pH 7.43) buffer and 1 µL of 5 mM HyCL-3 or HyCL-4-AM (20 µM final concentration) in DMSO.

Zn$^{2+}$ (200 µM): 1 µL of 50 mM Zn(OAc)$_2$ in DI H$_2$O was added to a solution of 245 µL 20 mM PBS (pH 7.43) buffer and 1 µL of 5 mM HyCL-3 or HyCL-4-AM (20 µM final concentration) in DMSO.

GSH (5 mM): 10 µL of 125 mM GSH in 0.01 M NaOH was added to a solution of 236 µL 20 mM PBS (pH 7.43) buffer and 1 µL of 5 mM HyCL-3 or HyCL-4-AM (20 µM final concentration) in DMSO.

Cysteine (1 mM): 1 µL of 250 mM cysteine in 0.01 M NaOH was added to a solution of 245 µL 20 mM PBS (pH 7.43) buffer and 1 µL of 5 mM HyCL-3 or HyCL-4-AM (20 µM final concentration) in DMSO.

Na$_2$S (200 µM): 1 µL of 50 mM Na$_2$S in 0.01 M NaOH was added to a solution of 245 µL 20 mM PBS (pH 7.43) buffer and 1 µL of 5 mM HyCL-3 or HyCL-4-AM (20 µM final concentration) in DMSO.
2.4.6 Biological studies

**Cell culture.** 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. Chemiluminescence responses were measured using a Cytation 5 BioTek plate reader (Winooski, VT).

**Cellular Hypoxia Studies.** Chemiluminescence responses were measured using a Cytation 5 BioTek plate reader. Before imaging, the medium was removed upon 90%–95% confluency and the cells were washed with 1 mL PBS. Each well was filled with 996 µL F-12K media. Then, 4 µL of 10 mM **HyCL-4-AM** or **HyCL-3** in DMSO (40 µM final concentration) was added to each well and immediately placed into a Cytation 5 BioTek plate reader. The luminescence was recorded using the luminescence detection mode, end point read type. The temperature was set at 37 °C, O₂ level was set to 0.5%–1% or 20%, and CO₂ was set at 5%. Each experiment consisted of three technical replicates for each condition, and each experiment was repeated with three or four biological replicates on separate days. The reported chemiluminescence intensity values are the average of a total of nine to twelve wells across three or four biological replicates.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.** Human lung adenocarcinoma epithelial cells (A549) were purchased from ATCC and were seeded in a 96-well plate to a total volume of 120 µL/ well. The plate was maintained at 37 °C with 5% CO₂ for 12 h. Then the medium was removed upon reaching 70%–80% confluency and the cells were washed with PBS. The cells were then incubated for 18 h after adding **HyCL-3** or **HyCL-4-AM**
at 0, 0.1, 1, 10, and 100 µM respectively in 125 µL completed F12K media. 10 µL of the MTT reagent (Cayman Chemical, Ann Arbor, MI) was then added to each well, and mixed gently. After 4 h incubation, 100 µL of crystal dissolving solution was added to each well to dissolve the formazan crystals. Absorbance was measured at 570 nm in a Cytation 5 BioTek plate reader and cell viability was expressed as a percent of the control.

Hypoxia detection in A549 cells. Human lung adenocarcinoma epithelial cells (A549) were seeded in a 12-well plate to a total volume of 1 mL per well. Before imaging, the media was removed upon 70%–80% confluency and the cells were washed with 1 mL PBS. Each well was filled with 996 µL F-12K media. Then, 4 µL of 10 mM HyCL-3 in DMSO (40 µM final concentration) was added to each well and immediately placed into a Cytation 5 BioTek plate reader. Then, the luminescence was recorded using the luminescence detection mode, end point read type. The temperature was set at 37 °C, O₂ level was set to 1% or 20%, and CO₂ was set at 5%. Each experiment consisted of three technical replicates for each condition, and each experiment was repeated with three biological replicates on three separate days. The reported integrated chemiluminescence intensity values are the average of a total of nine wells across three biological replicates. Control experiments with HyCL-4-AM and HyCL-4 were performed in F12K media in the absence of cells and reported values are the average of three technical replicates.

2.4.7 Kinetic modeling.

Measurement of chemiluminescent decomposition of acrylonitrile and acrylic acid dioxetane phenols. The chemiluminescence decomposition rate of the acrylonitrile and acrylic acid dioxetane phenols were obtained using a Cytation 5 BioTek plate reader (Winooski, VT) by using the luminescence detection method, endpoint read type, and setting gain to 135 and
temperature to 25–28 °C. 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 µM 11 or 13 were dissolved in 20 mM PBS buffered to pH 7.4 and placed immediately into the plate reader for measurement. The procedure was timed from the initial addition of probe into solution until the first read to account for time differences for kinetic modeling.

**Kinetic fits for the cellular response of HyCL-4-AM.** A549 cells were incubated with 40 µM HyCL-4-AM and the chemiluminescence response was immediately measured in a Cytation 5 BioTek plate reader using the luminescence detection mode at 37 ºC, gain set to 135, and either 0.5–1% O₂ or 20% O₂. The average of 3 technical replicates in a single plate were fit to the rate equation given in Figure 2-14B, based on the reactions given in Figure 2-14A using Mathematica 11.0.0.0 for Mac OS X. The values reported in the manuscript, Figure E are the average values of fits from three or four biological replicates. Example fits are given in Figure 2-14C,D.

### 2.4.8 Animal Imaging

**Hypoxia imaging in living mice.** The UT Southwestern Institutional Animal Care and Use Committee approved these investigations under Animal Protocol Number (APN #2017-102329). Tumor xenografts were established by subcutaneous injection of MDA-MB-231 cells into the flanks of athymic nude mice. Mice were placed on a breathing regimen of 16% or 100% O₂ for 10 minutes, then anesthetized and maintained using inhalation of 2.5% isoflurane, while breathing 16% or 100% O₂. The anesthetized mice were administered intratumoral (IT) or intramuscular (IM) injections of 30 µL of 120 µM HyCL-4AM (20 mM PBS buffer containing 2.4% DMSO). Images were acquired every 60 seconds using a Caliper Xenogen IVIS Spectrum® (PerkinElmer, Santa Clara, CA) with auto exposure, medium binning, f-stop set to 1, blocked excitation, open emission, FOV set to C, and height set to 1.5. Reported values are the average photon flux from three to six mice.
CHAPTER 2 REFERENCES


53. We note that these high turn-on values are achieved using freshly purified HyCL-4-AM and performing the control in a separate plate to avoid light leakage from adjacent wells. Light leakage from experiments in adjacent wells and impurities can increase background signal by ca. 100-fold.


CHAPTER 3
RATIOMETRIC pH IMAGING USING A 1,2-DIOXETANE CHEMILUMINESCENCE RESONANCE ENERGY TRANSFER SENSOR

3.1 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, ammonia excretion, CO₂ respiration, and excretion of non-volatile acids obtained from the diet. These processes are controlled at a subcellular level through active transport by H⁺-ATPase, and passive transport by Na⁺/HCO₃⁻ coporters and Cl⁻/HCO₃⁻ and Na⁺/H⁺ antiporters. 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. Diseases associated with chronic inflammation display decreased pH levels, and are positively correlated with increased cancer risk. 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. Because of their highly heterogenous microenvironments, tumors often display “hot spots” of increased acidity. The ability to accurately quantify pH in a pre-clinical and clinical setting is critical to understand its impact on human health.
Current clinically relevant methods to measure pH include magnetic resonance imaging (MRI) techniques such as intra and extracellular $^{31}$P MRI,$^{12}$ chemical exchange saturation transfer (CEST) MRI,$^{13-15}$ and other infused reporter molecules, notably $^{19}$F-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 in 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 1,2-dioxetane ratiometric chemiluminescence agents for pH imaging 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,$^{40}$ $\beta$-galactosidase,$^{41,42}$
formaldehyde, and others. Chemiluminescence provides many advantages compared to other optical methods in vivo by providing its own light source, thus attenuating autofluorescence and light scattering effects. Chemiluminescent probes have also been shown to achieve much higher fold turn-on than comparable fluorophores. Furthermore, they do not require expression of an enzyme for use, making them valuable standalone optical imaging tools as opposed to bioluminescence methods for monitoring pH.

A major need 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, xanthene, and cyanine 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. A dicyanomethylchromone-substituted scaffold has also been reported, providing the first 1,2-dioxetane to emit in the near-infrared (NIR) region, and has been successfully implemented in the detection of formaldehyde and β-galactosidase. 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. FRET also allows for fluorophores to be paired to dyes with sensing capabilities, thus providing ratiometric detection of analytes with the FRET pair. It is currently known that 1,2-dioxetanes exhibit energy transfer capabilities, but to our knowledge, no single-molecule 1,2-dioxetane ratiometric chemiluminescent probes for imaging pH have been developed. Here, we report the first single
molecule ratiometric chemiluminescence resonance energy transfer (CRET) sensor for pH imaging in living systems.

3.2 Results

3.2.1 Design and synthesis of Ratio-pHCL-1

In previous work, we demonstrated ratiometric quantification of pH in vitro using the light emission from 1,2-dioxetane chemiluminophores. These studies, however, required the addition of the pH sensitive dye seminaphthalorhodafluor (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 fluorescence emissions near 580 nm that interconvert between an open, fluorescent form at high pH and a colorless, non-fluorescent closed form under acidic conditions, we postulated that conjugation of this fluorophore to the dioxetane scaffold could provide a ratiometric measure of pH (Scheme 3-1.).
Synthesis of Ratio-pHCL-1 began with the with synthesis of tert-butyl-dimethylsilyl ether (TBS)-protected anthrone 20 from reported literature procedures (Scheme 3-2). Due to difficulties in conjugation of the top segment of the fluorophore to 20 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, so we proceeded to synthesize 2-iodo-tert-butyl-isophthalate 21 from commercially available 2-aminoisophthalic acid (Scheme 3-3). TBS-protected carbofluorescein 22 was successfully formed in 35% yield by premixing 20 and 21, purging the mixture three times under vacuum, addition of dry THF, then addition of 1 equivalent 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 3-3). 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 22 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 24, 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 triethylamine and five equivalents of pyridine in a 1:1 THF/H$_2$O mixture to produce 24 in a 64% yield. We then appended tert-butyl piperazine-1-carboxylate to 24 and subsequently deprotected the tert-butyloxy carbamate with TFA to obtain amine 26.

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 27 from literature reported compound 12 in 64% yield (Scheme 3-3). This was advantageous to preparation of the final probe because all three acetates could be deprotected in a single step. We proceeded to conjugate 26 and 27 together via 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethylyuronium hexafluorophosphate (HBTU) coupling to form triacetate 28, which was subsequently deprotected with 1 M LiOH to form precursor 29 in quantitative yield. 29 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.

3.2.2 Ratio-pHCL-1 in vitro characterization

Upon synthesis of Ratio-pHCL-1, we proceeded to characterize its properties in vitro. We verified the absorbance spectrum of 100 µM Ratio-pHCL-1 (100 mM PBS, pH 7.45, 5% DMSO) against the chemiluminescence emission spectrum of the reported methyl acrylate dioxetane (20 µM, 100 mM PBS, pH 7.45, 5% DMSO) (Figure 3-1). The emission wavelength of the methyl acrylate dioxetane centered at 530 nm coincides with an absorbance maximum of
**Ratio-pHCL-1** at 544 nm, indicating strong spectral overlap between the chemiluminescence emission profile of the dioxetane donor and absorption of the carbofluorescein acceptor. 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 3-2A). 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 emission intensities at 580 nm over 530 nm from pH 6.81–8.42 (Figure 3-2B), demonstrating that the emission spectra of the **Ratio-pHCL-1** is pH dependent. We then measured decomposition kinetics of 1.0 µM **Ratio-pHCL-1** (100 mM PBS, pH 7.45, 5% DMSO, 37 °C). Figure 3-3 shows an observed peak emission at the initial time point followed by a steady decline to baseline values over 50 minutes.

**Figure 3-1.** (black trace) Absorbance of 100 µM **Ratio-pHCL-1** (100 mM PBS, pH 7.45, 5% DMSO) and (red trace) chemiluminescence emission spectra of 20 µM methyl acrylate dioxetane phenol67 (100 mM PBS, pH 7.45, 5% DMSO).
Figure 3-2. The pH dependent (A) chemiluminescence emission spectra of 20 µM 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 µM 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 ± SD from n = 3 independent experiments.

Figure 3-3. Kinetic emission plot of 1.0 µM Ratio-pHCL-1 in 100 mM PBS, pH 7.44, 5% DMSO, 37 ºC.

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 3-4). We tested 10, 20, and 40 µM 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 3-4B). 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 3-4B), only showing a slight downward trend with increasing concentration at pH 7.45. We then subjected 20 µM 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 chemiluminescence quantum yield (Figure 3-5A). 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 3-5B).

![Figure 3-4](image1)

**Figure 3-4.** 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 µM **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 ± SD from n = 3 independent experiments.

![Figure 3-5](image2)

**Figure 3-5.** 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 µM **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 ± SD from n = 3 independent experiments.
We then measured 20 µM **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 3-6A 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 3-6B), 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.

![Figure 3-6](image)

**Figure 3-6.** 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 µM **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 ± SD from n = 3 independent experiments.

Next, we examined the behavior of **Ratio-pHCL-1** in biological systems. Cellular uptake of 20 µM **Ratio-pHCL-1** in A549 cells was examined over an 80 min period (Figure 3-7), and changes in fluorescence were monitored using a fluorescence microscope. After washing the cells, increased intracellular fluorescence could be seen from the acrylamide and carbofluorescein motifs by using GFP and RFP filter sets, respectively. These results show that **Ratio-pHCL-1** can cross the cellular membrane and accumulate inside cells. We proceeded to
measure cell viability of Ratio-pHCL-1 (Figure 3-8) using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells maintained at least 67% viability when exposed to 1–250 µM Ratio-pHCL-1.

**Figure 3-7.** (A–B) Fluorescence microscopy images using a GFP filter set of A549 cells with (A) no probe and (B) 20 µM Ratio-pHCL-1 incubated in cells for 80 min, followed by wash step. (C–D) Fluorescence microscopy images using an RFP filter set of A549 cells with (A) no probe and (B) 20 µM Ratio-pHCL-1 incubated in cells for 80 min, followed by wash step.

**Figure 3-8.** Ratio-pHCL-1 MTT assay. A549 cells were incubated with 0–250 µM Ratio-pHCL-1 for 16 hours and then viability was evaluated using the MTT assay. Error bars are ± S.D. from n = 3 technical replicates.

### 3.2.3 *In vitro* ratiometric pH imaging

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 sec$^{-1}$ cm$^{-2}$ sr$^{-1}$ and $10^8$ photons sec$^{-1}$ cm$^{-2}$ sr$^{-1}$ for the 540 nm filter (Figure 3-9A, 3-9B, 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. The imaging data, as shown in Figure 3-9, were used to determine the observed pK$_a$ of the response. It was found that a modified Henderson-Hasselbalch equation with the inclusion of a Hill coefficient$^{78}$ was needed to fit the data. The observed pK$_a$ of the response was determined to be 7.70 ± 0.03 with a Hill coefficient of 2.21±0.32 (S.D., n = 12) indicating cooperativity between multiple protonation sites. 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 3-10), which clearly showed penetration through this tissue surrogate.
Figure 3-9. 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. Rows represent technical repeats. (C) Ratio of total flux (p/s) at 580 nm and 540 nm. n = 12 technical replicates across 4 independent experiments, error bars are ± SD.

Figure 3-10. Chemiluminescence intensity images of the pH dependent emission of 20 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 with bologna slice (2.8 mm thickness) covering 96 well plate. Rows represent technical repeats (C) Ratio of total flux (p/s) at 580 nm and 540 nm. n = 9 technical replicates across 3 independent experiments, error bars are ± SD.

3.2.4 Ratiometric pH imaging in mouse models

Finally, we investigated the response of Ratio-pHCL-1 in animal models. First, we did a proof-of-principle experiment in recently sacrificed C57BL6 mice, which 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 $10^9$ p sec$^{-1}$ cm$^{-2}$ sr$^{-1}$ (Figure 3-11A, B), 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 each filter set with increasing pH (Figure 3-11C), demonstrating that **Ratio-pHCL-1** can discern changes in pH in the peritoneal cavity of mice. Confident with these results, we moved to *in vivo* measurement of pH in the peritoneal cavity of mice. Live male BALB-C mice aged 4.5 months were given 475 µL injections of 20 µM **Ratio-pHCL-1** (1.0 M PBS, pH 5.99–7.99, 5% DMSO), and immediately imaged with a 580 nm and 540 nm filter. With radiance heat maps set to $10^8$ p sec$^{-1}$ cm$^{-2}$ sr$^{-1}$ for both images, there are pronounced increases in overall intensity through each filter sets across the pH gradient, and discernable changes in flux output in each mouse from the 580 nm filter over the 540 nm filter images (Figure 3-12A,B). Indeed, the flux ratio between these filter sets increases with increasing pH (Figure 3-12C). This result was consistent across three independent experiments, demonstrating the robustness of using **Ratio-pHCL-1** for *in vivo* chemiluminescence pH imaging.
Figure 3-11. 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 ± SD.

Figure 3-12. Chemiluminescence images of the pH dependent emission of 475 µL of 20 µM Ratio-pHCL-1 (1.0 M PBS, pH 5.99–7.99, 5% DMSO) injected into the peritoneal cavity of male BALB-C mice aged to 4.5 months 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 ± SD. Statistical significance was assessed using a two-tailed Student’s t test. **p < 0.005 (n = 3 biological replicates).

3.3 Discussion

While Ratio-pHCL-1 clearly shows a pH dependent flux ratio when measured in vitro and in animal models, the question arises as to why the ratiometric signal differs from experiment to experiment. When comparing the in vitro ratiometric output in Figure 3-9 to the in vitro signal through bologna seen in Figure 3-10, the flux ratio from Figure 3-9 changes
approximately 13-fold over the experimental pH range, whereas flux ratio measurements through
bologna are much higher. Figure 3-10 shows approximately 98-fold change in flux ratio over the
same pH range. It is likely that this change is largely due to high absorption of the 540 nm
emission by hemoglobin present in the bologna slices, as both oxygenated and deoxygenated
hemoglobin exhibit high extinction coefficients in this region of the visible spectrum. This can
be corroborated with differences in flux seen from the contribution from the 540 nm images
(Figures 3-9B, 3-10B). However, when comparing the pH dependent flux ratios of Ratio-pHCL-
I in animal models (Figure 3-11, 12) to in vitro (Figure 3-9) there is an overall decrease in the
flux ratio, making the analysis more complex. These results likely exhibit contribution from
multiple factors. First, increased spectral coloring through absorbance in the 580 nm region may
arise from a higher contribution of oxygenated hemoglobin vs deoxygenated hemoglobin, which
exhibits a local maximum in its extinction coefficient near 580 nm. Second, we believe that a
decreased contribution from the carbofluorescein unit may arise to solvatochromism, where a
change in the solvent (i.e., biological milieu) may cause changes in the pKₐ of the dye.
Solvatochromism has been shown in fluoresceins, and it is possible that the related structure of
the carbofluorescein exhibits similar properties. It is also seen that there are small differences
between the flux ratio in the sacrificed animal imaging experiment (Figure 3-11) which exhibited
a 5-fold change in flux ratio from pH 6-8 as compared to the live animal imaging (Figure 3-12),
which exhibited an approximate 4-fold change over the same pH range. Differences may arise
from a higher proportion of deoxygenated hemoglobin vs. oxygenated hemoglobin in the
sacrificed mice as compared to live mice. A higher proportion of spectral coloring may be
exhibited in the sacrificed mice due to an increase in melanin content in their skin as compared
to white mice used in the in vivo studies.
3.4 Conclusion

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.

3.4 Experimental Procedure

3.4.1 General synthetic methods and materials

All reactions were performed in dried glassware under an atmosphere of dry N$_2$. 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. $^1$H NMR for compounds and $^{13}$C 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. $^1$H and $^{13}$C NMR spectra for characterization of new compounds and monitoring reactions were collected in CDCl$_3$, acetone-d$_6$, methanol-d$_4$, or DMSO-d$_6$ (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$^1$ CMS (ESI source) at Southern Methodist University.

![Structure of 31]

**5-iodoisophthalic acid (31).** In a 1L roundbottom flask equipped with a stir bar, 2-aminoisophthalic acid (50.0 mmol, 9.06 g, 1.0 equiv) was suspended in 300 mL 5.8 M HCl at 0°C under N$_2$ atmosphere. Sodium nitrite (125 mmol, 8.63 g, 2.5 equiv) was dissolved in 100 mL H$_2$O 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.

\[
\begin{align*}
\text{di-tert-butyl 5-iodoisophthalate (21).} \quad &\text{in a 100 mL roundbottom flask equipped with a stir bar under N}_2 \text{ atmosphere, 2-iodoterephthalic acid (7.23 mmol, 2111 mg, 1.0 equiv) and DMAP (1.44 mmol, 176 mg, 0.2 equiv) were dissolved in 2 mL DMF and 20 mL toluene. Di-tert-butyl dicarbonate (21.69 mmol, 4734 mg, 3.0 equiv) was added. The reaction was heated to 80 °C and stirred for 30 min. 1 equiv Boc anhydride was added to drive the reaction to completion and stirred for 30 more min. Upon completion, the reaction was allowed to cool to RT, washed with sat. NH}_4\text{Cl, extracted with 3x 30 mL EtOAc, dried with Na}_2\text{SO}_4, \text{ and concentrated under reduced pressure. Column chromatography in 5% EtOAc/Hexanes yielded compound 2 (5.35 mmol, 74%) as a yellow oil.} \quad &\text{1H NMR (400 MHz, CDCl}_3\text{) } \delta 8.52 \text{ (d, 1H, } J = 1.2 \text{ Hz), 7.97 \text{ (dd, 1H, } J_1 = 8.0 \text{ Hz, } J_2 = 1.2 \text{ Hz), 7.67 \text{ (d, 1H, } J = 8.0 \text{ Hz), 1.65 \text{ (s, 9H), 1.62 \text{ (s, 9H).}}}
\end{align*}
\]
In a 25 mL oven-dried roundbottom flask equipped with a stir bar, compound 20 (0.46 mmol, 220 mg, 1.0 equiv) and compound 21 (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 ºC. 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, extracted 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).
tert-butyl 3-iodo-4-methylbenzoate (33). 3-Iodo-4-methylbenzoic acid (32) (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, 186 mg, 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.

N-(10-(5-carboxy-2-methylphenyl)-7-(dimethylamino)-5,5-dimethyldibeno[b,e]silin-3(5H)-ylidene)-N-methylmethanaminium (35). Compound 33 (4.93 mmol, 1.57 g, 4.0 equiv) and
silicon anthrone 34\textsuperscript{71} (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 is an intense blue solution of pH = 1. Saturated aqueous NaHCO\textsubscript{3} was added to neutralize the reaction, which was then extracted with DCM. The blue organic layer was washed with brine, dried over Na\textsubscript{2}SO\textsubscript{4} 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%). \textsuperscript{1}H NMR (500 MHz, CD\textsubscript{3}OD) \(\delta\) 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); \textsuperscript{13}C NMR (125 MHz, CD\textsubscript{3}OD) \(\delta\) 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.
3,6-dihydroxy-10,10-dimethyl-3'-oxo-3'H,10H-spiro[anthracene-9,1'-isobenzofuran]-5'-carboxylic acid (23). In a 20-dram vial equipped with a stir bar, Compound 22 (0.278 mmol, 191 mg) was dissolved in 10 mL DCM. 2 mL TFA was added, and the reaction was stirred at RT under N$_2$ 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 23 (0.189 mmol, 76 mg, 68%) as a red-orange solid. $^1$H NMR (400 MHz, Acetone-D6) $\delta$ 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); $^{13}$C NMR (100 MHz, Acetone-D6) $\delta$ 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 C$_{24}$H$_{18}$O$_6$ [M+H]$^+$ 403.1176, found 403.1163.
3,6-diacetoxy-10,10-dimethyl-3'-oxo-3'H,10H-spiro[anthracene-9,1'-isobenzofuran]-5'-carboxylic acid (24). Compound 23 (0.189 mmol, 76.0 mg, 1.0 equiv) was dissolved in 2 mL Ac₂O in a 20 mL roundbottom flask equipped with a stir bar. Pyridine (0.98 mmol, 76. µL, 5.0 equiv) was added. The reaction was heated to 80 ºC 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, extracted 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 (0.98 mmol, 76. µL, 5.0 equiv) and Et₃N (0.19 mmol, 30. µL, 1.0 equiv) was added. The reaction was heated to 80 ºC 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 24 (0.12 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.
5′-(4-(tert-butoxycarbonyl)piperazine-1-carbonyl)-10,10-dimethyl-3′-oxo-3′H,10H-spiro[anthracene-9,1′-isobenzofuran]-3,6-diyl diacetate (25). In a 25 mL roundbottom flask equipped with a stir bar under N₂ atmosphere, Compound 24 (0.119 mmol, 58 mg, 1.0 equiv) was dissolved in 8 mL DCM. The reaction was cooled to 0 °C. DIPEA (0.16 mmol, 27 µL, 1.3 equiv) and N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU) (0.14 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 (0.14 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, extracted with 2x 20 mL DCM, dried with Na₂SO₄, filtered, and concentrated under reduced pressure. Column chromatography (50–75% EtOAc/Hexanes) yielded Compound 25 (0.10 mmol, 66 mg, 85%) as a white foam. \(^1\)H 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); \(^1^3\)C 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 C_{37}H_{38}N_{2}O_{9} [M+Na]^+ 677.2470, found 677.2468.
10,10-dimethyl-3′-oxo-5′-(piperazine-1-carbonyl)-3'H,10H-spiro[anthracene-9,1′-isobenzofuran]-3,6-diyl diacetate (26). In a 20 mL vial, compound 25 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 26 (0.098 mmol, 54 mg, 98%) as a tan foam. \(^1\)H 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); \(^{13}\)C 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.
(E)-3-(2-acetoxy-4-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chlorophenyl)acrylic acid (27). Compound 12\(^\text{22}\) (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, extracted with 2x 10 mL DCM, dried with Na\(_2\)SO\(_4\), and concentrated under reduced pressure. The crude was then dissolved in 10 mL 1:1 THF H\(_2\)O mixture. Pyridine (1.84 mmol, 0.148 mL, 5.0 equiv) and Et\(_3\)N (0.37 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 27 (116 mg, 76%) as a yellow foam. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 10.58 (bs, 1H), 7.74 (d, 1H, \(J = 16.0 \text{ Hz}\)), 7.57 (d, 1H, \(J = 8.0 \text{ Hz}\)), 7.24 (d, 1H, \(J = 8.0 \text{ Hz}\)), 6.49 (d, 1H, \(J = 16.0 \text{ Hz}\)), 3.34 (s, 3H), 3.30 (s, 1H), 2.47 (s, 3H) 1.61–2.13 (m, 12H); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 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 C\(_{23}\)H\(_{25}\)ClO\(_5\) [M+Na]\(^+\) 439.1283, found 439.1287.
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-spiroanthracene-9,1′-isobenzofuran]-3,6-diyl diacetate (28). In a 25 mL roundbottom flask equipped with a stir bar under N₂ atmosphere, Compound 27 (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 (0.14 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 26 (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/NH₄Cl, extracted with 2x 20 mL DCM, dried with Na₂SO₄, filtered, and concentrated under reduced pressure. Column chromatography (75–100% EtOAc/Hexanes) yielded Compound 28 (0.10 mmol, 8 mg, 85%) as a white solid. ¹H NMR (400 MHz, CDCl₃) 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); ¹³C NMR (100 MHz, CDCl₃) 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 C₅₅H₅₃ClN₂O₁₁ [M+H]⁺ 953.3411, found 953.3459.
In a 20-dram vial, compound 28 (0.021 mmol, 20 mg) was dissolved in 2 mL THF, and 2 mL 10 M LiOH was added under N₂ 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 extracted with 2x 10 mL EtOAc, then 2x 10 mL DCM, dried with Na₂SO₄, filtered, and concentrated under reduced pressure, yielding 29 (18 mg, 0.021 mmol, quantitative) as a red solid without further purification. \(^1\)H NMR (400 MHz, Acetone-d₆) 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); \(^1^\)C NMR (100 MHz, CDCl₃) 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 29 (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. 

\[^1\text{H} \text{NMR (400 MHz, Acetone-d6)}\] \(\delta\) 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); \[^{13}\text{C NMR (100 MHz, Acetone-d6)}\] \(\delta\) 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 C\(_{49}\)H\(_{47}\)ClN\(_2\)O\(_{10}\) [M+H]\(^+\) 859.2992, found 859.3017.
3.4.2 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.

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.
3.4.3 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.

3.4.4 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 µM **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 µM **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.

**Sacrificed mouse imaging.** A solution of 950 µL 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 µL of 400 µM Ratio-pHCL-1 in DMSO was injected shortly after to achieve an approximate final concentration of 20 µM 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)
CHAPTER 3 REFERENCES


CHAPTER 4
PHOTOACTIVATABLE 1,2-DIOXETANE CHEMILUMINOPHORES

4.1 Introduction

Light and chemistry have a uniquely intertwined relationship, where light can be used to enact and enable control of chemical phenomena.\(^1\) Photoswitches are one such class of molecules that can be precisely manipulated by light.\(^2\) Light interacts with photoswitch structures by inducing reversible photochemical isomerization or bond formation/cleavage to metastable intermediates.\(^3\) Rhodopsin is one such naturally occurring photoswitch that enables vision in animals\(^4\) and photosynthesis for some archaea.\(^5\) Furthermore, synthetically devised photoswitches such as azobenzenes and stilbenes have been implemented for controlling both structure and expression of biomacromolecules.\(^6-8\) Spiropyrans are another type of photoswitch that has unique properties as compared to others in its class.\(^9\) The spiropyran converts from a relatively non-polar, non-fluorescent spiropyran (SP) form to an open merocyanine (MC) chromophore, coinciding with a large shift in the molecule’s polarity in zwitterionic form. While the MC chromophore shows higher absorbance than the closed SP form, it exhibits moderate fluorescence in polar solvents and is virtually non-fluorescent in water. However, encapsulation\(^10\) or incorporation into polymers,\(^11-13\) nanodots,\(^14\) and thin films\(^15\) has granted major improvements in fluorescence efficiency. Spiropyrans have been largely implemented for dynamic fluorescence due to increased fluorescence quantum yield that arises from conformational restraint and
minimization of solvent-based quenching, as well as innate thermochromic,\textsuperscript{16} acidochromic,\textsuperscript{17} solvatochromic,\textsuperscript{18} electrochromic,\textsuperscript{19} and mechanochromic\textsuperscript{12,20,21} properties.

Light can also initiate irreversible photochemical processes. Photolabile protecting groups (PPGs) are an established method to cage compound activity through covalent attachment of a photolabile group, allowing for buildup of the substrate in regions of interest and high spatiotemporal control for the release of a substrate through photochemical bond cleavage. Development of photoremovable groups has been of particular interest over the last half century, and its study has garnered a diverse array of new PPGs exhibiting a wide range of uncaging mechanisms, uncaging wavelengths, and functional group compatibility. Some PPGs include phenacyl,\textsuperscript{22-24} nitroaryl,\textsuperscript{25-29} coumarinyl,\textsuperscript{30,31} BODIPY,\textsuperscript{32-37} and cyanine\textsuperscript{38} derivatives. Of this class, ortho-nitrobenzyl protecting groups exhibit uncaging capability in both the UV and NIR regions through single and two photon\textsuperscript{28,29} mechanisms, respectively, and have been implemented in a wide range of applications, including natural product synthesis,\textsuperscript{39,40} caged ATP,\textsuperscript{41} RNA,\textsuperscript{42} DNA,\textsuperscript{42} and peptide\textsuperscript{43} synthesis, prodrug release,\textsuperscript{44-46} and others.

Activity-based sensing (ABS) relies on the use of caged luminophores that are uncaged based on selective cleavage of protecting groups to obtain a readout.\textsuperscript{47} Common ABS detection techniques use optical readouts including absorption, NMR, optoacoustic,\textsuperscript{48-50} fluorescence,\textsuperscript{51-53} and chemiluminescence-based agents.\textsuperscript{54} Chemiluminescence provides many advantages to other optical reporters, including high turn-on and increased depth penetration, while obviating the need for external excitation.\textsuperscript{55} Specifically, 1,2-dioxetanes have been established as critical chemiluminescence-based ABS reporter substrates, as they provide a facile and functional means of caging luminescent emission through protection of the dioxetane phenol with a reactive handle. Subsequent interaction with the analyte of interest will uncage the dioxetane phenol,
initiating an electron exchange luminescence (CIEEL) process to generate photonic emission. This strategy has been used for activity-based detection and quantification of various substrates.

A key challenge for in vivo chemiluminescence imaging is attaining good spatiotemporal control of the luminescent signal for localization and maximizing total luminescent output. Utilization of photocaged chemiluminescent compounds can ultimately circumvent this issue by localizing chemiluminescence signal to the point of illumination. Herein, we report the use of both irreversible and reversible photoinitiated uncaging of 1,2-dioxetane chemiluminophores as a fundamental step towards this goal. We synthesized ortho-nitrobenzyl protected dioxetanes UVC-454 and UVA-454 that can be irradiated by UVC (254 nm) and UVA (365 nm) light, respectively to emit luminescence at 454 nm. We also report the first chemiluminescent spiropyran compound Spiro-CL that undergoes reversible opening to its MC form under UV light, and exhibits chemiluminescence in DMSO (Scheme 4-1).

Scheme 4-1. Design of photoactivatable chemiluminescence compounds.
4.2 Results

4.2.1 Synthesis and *in vitro* response of UVC-454 and UVA-454

Both **UVC-454** and **UVA-454** were synthesized from literature reported precursor, 5-(((1r, 3r, 5R, 7S)-adamantan-2-ylidene) (methoxy) methyl)-2-chlorophenol\(^{71,73}\) through Mitsunobu couplings with 2-nitrobenzyl alcohol (**UVC-454**) and 4,5-dimethoxy-2-nitrobenzyl alcohol (**UVA-454**) to make ethers 36 and 37, followed by a [2+2] cycloaddition with \(^1\)O\(_2\) via sensitization with Rose bengal to make the final photocaged dioxetane compounds (Scheme 4-2).

**Scheme 4-2. Synthesis of UVC-454 and UVA-454.**

Upon synthesis of the photocaged compounds, we proceeded to test their photoactivation capabilities (Figure 4-1). Solutions of 10 mM concentrations of **UVC-454** or **UVA-454** in DMSO were irradiated with 254, 365, and 488 nm light for 180 minutes, and aliquots were transferred into 20 mM PBS pH 7.4 buffer containing 10% Emerald II® enhancer solution to give a final concentration of 50 \(\mu\)M. Figure 4-1A and 4-1C show the luminescence emission response of **UVC-454** and **UVA-454** to uncaging with UVC and UVA light, respectively. Both give off bright luminescence, with emission peaks centered at 454 nm from the chemiluminescent scaffold, and 540 nm through energy transfer to Emerald II®. **UVC-454** gives an overall brighter response than **UVA-454** at 254 nm and 365 nm uncaging wavelengths, however, **UVA-454** showed statistically
significant uncaging response at its respective uncaging wavelength of 342 nm over 180 min as compared to 254 nm and 488 nm. Utilizing 254 nm light over 365 nm light with UVC-454 did not show a statistically significant response, however the uncaging response was statistically significant as compared to photoinitiated cleavage using 488 nm light.

Figure 4-1. Photoactivation of UVC-454 and UVA-454 in DMSO with 254 nm, 365 nm, and 488 nm irradiation (A) Plot of relative emission intensities of 50 µM UVC-454 in PBS 7.4 buffer and 10% Emerald II® irradiated with 254 nm for 0–180 min. Inlay denotes emission intensity at 545 nm over 180 min. (B) Integrated emission intensities at 545 nm of 50 µM UVC-454 in PBS 7.4 buffer and 10% Emerald II irradiated with 254 nm (red), 365 nm (black), and 488 nm (gray) light over 180 min. (C) Plot of relative emission intensities of 50 µM UVA-454 in PBS 7.4 buffer and 10% Emerald II® irradiated with 365 nm for 0–180 min. Inlay denotes emission intensity at 545 nm over 180 min. (D) Integrated emission intensities at 545 nm of 50 µM UVC-454 in PBS 7.4 buffer and 10% Emerald II® irradiated with 254 nm (red), 365 nm (black), and 488 nm (gray) light over 180 min. Error bars are ± SD. Statistical significance was assessed using a two-tailed Student’s t test. *p < 0.05 (n =3 technical replicates), **p<0.005 (n = 3 technical replicates), ***p <0.0005 (n = 3 technical replicates), ****p < 0.00005 (n = 3 technical replicates).

We also controlled for uncaging due to thermolysis with UVC-454 (Figure 4-2). The agent showed a small but steady luminescence output due to heating of the sample. We also tested the uncaging response UVC-454 directly in aqueous media (Figure 4-3), where 100 µM to 1 mM solutions of UVC-454
in 20 mM PBS pH 7.4 buffer containing 10% Emerald II® solution were prepared and irradiated with 254 nm light for 180 min. Relatively low luminescence output was seen from both the 100 µM and 500 µM concentrations over the 180 min time period, but could be readily seen at the 750 µM and 1 mM concentrations.

**Figure 4-2. UVC-454 thermolysis.** Plot of relative emission intensities of 50 µM UVC-454 in PBS 7.4 buffer and 10% Emerald II®. 10 mM UVC-454 in DMSO was irradiated at 254 nm in a glass vial wrapped in aluminium foil over 180 min. Inlay is emission intensity measured at 545 nm.

**Figure 4-3. UVC-454 photoactivation in water.** Plot of relative emission intensities measured 545 nm of 1 mM (red), 750 µM (black), 500 µM (dark gray), and 100 µM (light gray) UVC-454 in 20 mM PBS pH 7.4 buffer and 10% Emerald II® irradiated at 254 nm in aqueous solution over 180 min. Measurements were taken in 30 min intervals.

### 4.2.2 Synthesis and *in vitro* response of Spiro-CL

Spiropyrans are synthesized through condensation of indoline compounds to salicylaldehydes. Due to the structural similarities between the spiropyrans and reported chemiluminescent salicylaldehyde precursor,65 we decided to explore the possibility of developing a chemiluminescence photoswitch, where we utilize the CL scaffold as the chromene
portion of the spiropyran. We first synthesized aldehyde 38 via a palladium catalyzed formylation method in 85% yield (Scheme 4-3). We then appended 1,3,3-trimethyl-2-methyleneindoline to this structure through condensation to form the spiropyran precursor 39 in 30% yield. Finally, the enol ether underwent a [2+2] cycloaddition with $^1$O$_2$ generated by photosensitizer methylene blue under white light to generate Spiro-CL in 35% yield.

**Scheme 4-3. Synthesis of Spiro-CL.**

Upon synthesis of Spiro-CL, we began to examine its photoswitching capabilities. We first monitored changes in its absorbance to examine the effect of UV light irradiation on the sample. A 100 µM sample of Spiro-CL was irradiated for 20 min with a 254 nm light source and absorbance was measured every two minutes during that time period (Figure 4-4). The absorbance spectra show clear increases in absorbance with the growth of a peak from 0 minutes to 20 minutes centered at 418 nm, indicating changes in the absorbance profile of the compound upon UV radiation. After the 20-minute UV irradiation period, the solution was irradiated for 10 minutes with a white light source and absorbance was monitored every two minutes. Absorbance at 418 nm decreased down to a steady state of around 0.06 absorbance units for the sample.

We then monitored changes in the fluorescence and chemiluminescence profiles of Spiro-CL due to UV light exposure (Figure 4-5). A 10 µM solution of Spiro-CL in EtOH was subjected to fluorescence excitation and emission measurements before and after irradiation with UV light (Figure 4-5A). Before irradiation, Spiro-CL exhibits a fluorescence excitation and emission...
peaks centered at 290 nm and 350 nm, respectively. After 20 minutes of irradiation with the UV source, the excitation profile of the spiropyran broadens, with excitation maxima located at 260, 350, and 450 nm. Fluorescence emission spectra after irradiation red-shifted to a peak centered around 540 nm. Finally, we tested the ability of the compound to exhibit chemiluminescence due to UV light-mediated opening of the luminophore (Figure 4-5B). We subjected 10 µM Spiro-CL in DMSO to irradiation with a 254 nm light source and monitored chemiluminescence emission from the sample for 16 minutes. After 16 minutes, a small peak centered at 510 nm arose, indicating that the ring opening process mediated by UV irradiation enabled luminescence emission from the open MC form.

Figure 4-4. UV monitoring of Spiro-CL photoswitching. (A) Absorbance of 100 µM Spiro-CL in EtOH upon irradiation with 254 nm light from t = 0–20 min. (B) Absorbance at 418 nm of 100 µM Spiro-CL in EtOH in response to 254 nm irradiation from t = 0–20 min and white light irradiation from t = 22–30 min.

Figure 4-5. Fluorescence and chemiluminescence monitoring of Spiro-CL photoswitching. (A) Fluorescence excitation and emission spectra before (red traces) and after (blue traces) 254 nm irradiation. (B) Chemiluminescence emission spectra of 10 µM Spiro-CL in DMSO at t = 0 (black) and 16 min (red).

4.3 Conclusion
In conclusion, we synthesized and conducted *in vitro* characterization of photoactivatable chemiluminescence agents UVC-454, UVA-454, and Spiro-CL. UVC-454 and UVA-454 undergo irreversible uncaging processes to emit light, and Spiro-CL acts under a reversible photoswitching mechanism governed by UV light irradiation to generate its chemiluminescent merocyanine form. Photoactivated chemiluminescence provides an attractive target for targeted imaging agents. We envision that 1,2-dioxetane probes outfitted with targeting capability such as homing peptides\textsuperscript{75} or antibodies promote the use of *in vivo* tail vein injections that localize and build up in areas of interest. Uncaging of the chemiluminophore system using directed light excitation provides extremely high spatiotemporal control over imaging agent activity. With the production of new chemiluminescence scaffolds that emit in green\textsuperscript{76} and red\textsuperscript{77} regions, along with a myriad of PPGs that have uncaging abilities across the spectrum, a wide variety of photocaged chemiluminescence agents could be devised with catered uncaging and emission wavelengths. This technology could be applied for formal energy upconversion applications\textsuperscript{78} by use of PPGs that are uncaged in the NIR to far red regions to formally generate anti-Stokes emission from the photocaged chemiluminescent systems.

To our knowledge, Spiro-CL represents the first example of photoswitch – enabled chemiluminescence. While most 1,2-dioxetanes exhibit turn-on luminescence by caging the 1,2-dioxetane species with an activity-based reactive handle, this unique strategy enables the chemiluminophore to be self-caged, and uncaging the chemiluminescent photoswitch species allows for emission at longer wavelength than the initial input. While Spiro-CL represents a novel chemiluminescent scaffold modality with a unique photoswitching mechanism to initiate luminescence, due to the low chemiluminescence emission seen from Spiro-CL, we believe that further improvement on the photoswitching and chemiluminescence emission properties of this
compound need to be explored, potentially including aforementioned encapsulation or incorporation into polymers.

4.4 Experimental Procedure

4.4.1 General synthetic materials and methods

All reactions were performed in dried glassware under an atmosphere of dry \( \text{N}_2 \). 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), Beantown Chemical (Hudson, NH), and Cayman Chemical (Ann Arbor, MI) and used without further purification. \(^1\)H NMR for compounds and \(^{13}\)C 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. \(^1\)H and \(^{13}\)C NMR spectra for characterization of new compounds and monitoring reactions were collected in CDCl\(_3\) (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. Low resolution mass spectrometry was performed on an Advion Expression\(^1\) CMS (ESI source) at Southern Methodist University.
(1r,3r,5R,7S)-2-((4-chloro-3-((2-nitrobenzyl)oxy)phenyl)(methoxy)methylene)adamantane (36). To an oven-dried 50 mL roundbottom flask, 5-(((1r, 3r, 5R, 7S)-adamantan-2-ylidene)(methoxy)methyl)-2-chlorophenol\(^{72}\) (441 mg, 1.45 mmol, 1.0 equiv) and 24 mL anhydrous THF were added and cooled to 0 °C. Diethyl azodicarboxylate (0.54 mL, 3.5 mmol, 2.4 equiv) was added. Triphenyl phosphine (663 mg, 1.74 mmol, 1.2 equiv) was dissolved in 3 mL anhydrous THF was added dropwise over 5 min. The reaction was stirred for 2 hr until completion as determined by TLC. The crude contents were washed with saturated NH\(_4\)Cl, extracted with 3 x 25 mL EtOAc, dried with Na\(_2\)SO\(_4\), filtered, and concentrated under vacuum. Column chromatography (1% EtOAc/Hexanes) yielded 36 (310 mg, 0.70 mmol, 48%). \(^1\)H NMR \(\delta \) 8.23 (d, 1H, \(J = 7.6 \) Hz), 8.09 (d, 1H, \(J = 7.6 \) Hz) 7.74 (t, 1H, \(J = 7.6 \) Hz), 7.52 (t, 1H, \(J = 7.6 \) Hz), 7.39 (d, 1H, \(J = 8.0 \) Hz), 6.98 (s, 1H), 6.93 (m, 1H), 5.59 (s, 2H), 3.30 (s, 3H), 2.25 (s, 1H), 1.76–1.99 (m, 11H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta \) 153.2, 142.4, 135.5, 134.3, 133.6, 132.9, 130.0, 128.4, 128.4, 125.1, 123.1, 121.9, 114.5, 67.5, 39.1, 39.0, 37.1, 32.4, 30.3, 28.2. LRMS for C\(_{25}\)H\(_{26}\)ClNO\(_4\) [M+H]\(^+\) found 440.2.
(1R,3r,5R,7S)-2-((4-chloro-3-((4,5-dimethoxy-2-nitrobenzyl)oxy)phenyl)(methoxy)methylene)adamantane (37). To an oven-dried 50 mL roundbottom flask, 5-(((1R, 3r, 5R, 7S)-adamantan-2-ylidene) (methoxy) methyl)-2-chlorophenol\(^{72}\) (158 mg, 0.518 mmol, 1.0 equiv) and 5 mL anhydrous THF were added and cooled to 0 °C. Diethyl azodicarboxylate (0.09 mL, 0.6 mmol, 1.2 equiv) was added. Triphenyl phosphine (164 mg, 0.624 mmol, 1.2 equiv) dissolved in 1 mL anhydrous THF was added dropwise over 5 min. The reaction was stirred for 2 hr until completion as determined by TLC. The crude contents were washed with saturated NH\(_4\)Cl, extracted with 3 x 25 mL EtOAc, dried with Na\(_2\)SO\(_4\), filtered, and concentrated under vacuum. Column chromatography (1–5% EtOAc/Hexanes) yielded 37 (170 mg, 0.34 mmol, 64%). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.82 (s, 1H), 7.71 (s, 1H), 7.40 (d, 1H, \(J = 8.0\) Hz), 7.06 (d, 1H, \(J = 1.2\) Hz), 6.93 (dd, 1H, \(J_1 = 8.0\) Hz, \(J_2 = 1.2\) Hz), 5.57 (s, 2H), 4.04 (s, 3H), 4.00 (s, 3H), 3.32 (s, 3H), 3.26 (s, 1H), 2.62 (s, 1H) 1.79–2.00 (m, 11H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 154.1, 153.2, 147.8, 142.4, 138.7, 135.7, 133.0, 129.8, 129.2, 123.1, 121.7, 114.6, 109.5, 107.9, 67.6, 56.5, 56.4, 39.1, 39.0, 37.1, 32.4, 30.3, 28.2. LRMS for C\(_{27}\)H\(_{23}\)ClNO\(_6\) [M+H]\(^+\) found 500.2.

![UVC-454](image)

UVC-454

(1R,3r,5r,7r)-4'-(4-chloro-3-((2-nitrobenzyl)oxy)phenyl)-4'-methoxyspiro[adamantane-2,3'-[1,2]dioxetane] (UVC-454). In a 2-neck roundbottom flask, Compound 36 (50 mg, 0.12 mmol) was dissolved in 3 mL THF. Rose bengal (8 mg, 0.0082 mmol) was added. Upon cooling to 0 °C, O\(_2\) was bubbled into the solution the solution and the flask was irradiated under white light
for 1 hr. Upon completion as determined by TLC, the crude was evaporated onto silica and loaded for column chromatography in 5% EtOAc/Hexanes, yielding **UVC-454** (4.4 mg, 0.008 mmol, 35%) as a mixture of diastereomers. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta 8.12\) (d, 1H, \(J = 1.1\) Hz), 7.92 (d, 1H, \(J = 1.1\) Hz), 7.63 (m, 2H) 7.40–7.45 (m, 4H), 5.54 (s, 2H), 3.12 (s, 3H), 2.93 (s, 1H), 1.71–1.91 (m, 12H). \(^1^3\)C NMR (125 MHz, CDCl\(_3\)) \(\delta 146.8, 135.0, 134.1, 133.0, 130.3, 128.6, 125.1, 111.4, 95.4, 67.6, 49.9, 36.3, 34.7, 33.1, 31.6, 25.9, 25.8, 14.2.

![UVA-454](image)

**UVA-454**

\((1r,3r,5r,7r)-4'-(4-chloro-3-((4,5-dimethoxy-2-nitrobenzyl)oxy)phenyl)-4'-methoxyspiro[adamantane-2,3'-[1,2]dioxetane](UVA-454)**. In a 2-neck roundbottom flask, Compound 37 (45 mg, 0.09 mmol) was dissolved in 3 mL THF. Rose bengal (12 mg, 0.012 mmol) was added. Upon cooling to 0 °C, \(O_2\) was bubbled into the solution the solution and the flask was irradiated under white light for 3 hr. Upon completion as determined by TLC, the crude was evaporated onto silica and loaded for column chromatography in 5% EtOAc/Hexanes, yielding **UVA-454** (30 mg, 0.056 mmol, 63%). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta 7.73\) (s, 1H), 7.52 (s, 1H), 7.40 (s, 1H), 7.19 (m, 2H), 3.93 (s, 3H), 3.91 (s, 3H), 3.15 (s, 3H), 2.93 (s, 1H), 1.61–1.93 (m, 12H).
4-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-3-chloro-2-hydroxybenzaldehyde (38).\textsuperscript{65} In an oven-dried 3 mL pressure flask, 3-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-2-chloro-6-iodophenol\textsuperscript{64} (600 mg, 1.39 mmol, 1 equiv), N-formylsaccharine (441 mg, 2.09 mmol, 1.5 equiv), Na\textsubscript{2}CO\textsubscript{3} (221 mg, 2.09 mmol, 1.5 equiv), Pd(OAc)\textsubscript{2} (9.4 mg, 0.042 mmol, 0.03 equiv), and 1,4-bis(diphenylphosphino)butane (26 mg, 0.062 mmol, 0.045 equiv) were added, and purged 3x with N\textsubscript{2}. 4 mL of anhydrous DMF was added, followed by anhydrous triethylsilane (0.29 mL, 1.8 mmol, 1.3 equiv). The reaction was capped and pre-stirred for 10 min, then brought up to 80 °C and stirred for 16 hr. The reaction was taken off heat and allowed to cool to RT, washed with saturated NH\textsubscript{4}Cl, extracted with 3 x 25 mL EtOAc, dried with Na\textsubscript{2}SO\textsubscript{4}, filtered, and concentrated under vacuum. Column chromatography (10% EtOAc/Hexanes) yielded 38 (250 mg, 0.76 mmol, 75%) as a yellow solid. H\textsuperscript{1} NMR (500 MHz, CDCl\textsubscript{3}) δ 11.63 (s, 1H), 9.90 (s, 1H), 7.48 (d, 1H, J = 7.4 Hz), 6.97 (d, 1H, J = 7.4 Hz), 3.33 (s, 3H), 3.30 (s, 1H), 1.6–2.12 (m, 12H).

7-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-8-chloro-1',3',3'-trimethylspiro[chromene-2,2'-indoline] (39). In a 2-neck roundbottom flask, Compound 38 (32 mg, 0.097 mmol, 1.0 equiv) and 1,3,3-trimethyl-2-methyleneindoline (0.02 mL, 0.1 mmol, 1.0
equiv) were dissolved in 4 mL pure EtOH under nitrogen atmosphere. The reaction was heated to 90 °C and refluxed for 5 hr. Upon completion as determined by TLC and ESI-MS, the reaction was washed in brine, extracted with 3 x 10 mL EtOAc, and concentrated under vacuum. Column chromatography (2% EtOAc/hexanes) yielded 39 (14 mg, 0.029 mmol, 30%). $^1$H NMR (400 MHz, CDCl$_3$) δ 7.17 (t, 1H, $J$ = 7.2 Hz), 7.08 (d, 1H, $J$ = 7.2 Hz), 6.95 (d, 1H, $J$ = 7.6 Hz), 6.85 (m, 2H), 6.75 (d, 1H, $J$ = 8.0 Hz), 6.55 (d, 1H, $J$ = 7.6 Hz), 5.76 (d, 1H, $J$ = 10.0 Hz), 3.15–3.32 (m, 6H), 2.77 (d, 3H, $J$ = 7.2 Hz), 2.20 (s, 6H), 1.21–1.89 (m, 12H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 136.6, 135.8, 131.1, 128.7, 127.5, 123.9, 122.9, 121.4, 121.1, 120.7, 119.6, 119.2, 106.8, 56.9, 52.0, 51.8, 39.2, 39.1, 38.6, 37.2, 34.7, 32.9, 30.9, 29.7, 29.6, 29.0, 28.5, 28.3, 26.9, 25.7, 25.3, 22.7, 20.3, 14.4. LRMS for C$_{31}$H$_{34}$ClNO$_2$ [M+H]$^+$ found 488.3.

Spiro-CL

8-chloro-7-((1r,3r,5r,7r)-4'-methoxyspiro[adamantane-2,3'-(1,2)dioxetan]-4'-yl)-1',3',3'-trimethylspiro[chromene-2,2'-indoline] (Spiro-CL). In a 2-neck roundbottom flask, Compound 39 (12.1 mg, 0.024 mmol) was dissolved in DCM. Methylene blue (3.5 mg, 0.011 mmol) was added. Upon cooling to 0 °C, O$_2$ was bubbled into the solution and the flask was irradiated under yellow light for 1 hr. Upon completion as determined by ESI-MS, the crude was evaporated onto silica and loaded for column chromatography in 2% EtOAc/Hexanes, yielding Spiro-CL (4.4 mg, 0.008 mmol, 35%) as a mixture of diastereomers $^1$H NMR (400 MHz, CDCl$_3$) δ 7.58 (d, 1H, $J$ = 7.6 Hz), 7.18 (t, 1H, $J$ = 7.6 Hz), 7.07 (m, 2H), 6.87 (m, 2H), 6.56 (dd, 1Hz, $J_1$ = 7.6 Hz, $J_2$ =5.2 Hz), 5.82 (t, 1H, $J$ = 11.8 Hz), 3.22 (d, 3H, $J$ = 12.4 Hz), 2.98
(s, 1H), 2.69-2.81 (d, 3H), 2.28 (d, 1H), 2.03 (d, 1H) 1.84 (m, 2H), 1.71 (m, 2H), 1.63 (s, 6H) 1.3–1.8 (m, 4H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 150.7, 150.5, 148.1, 147.7, 136.5, 136.3, 132.7, 128.5, 127.6, 124.1, 122.7, 121.7, 121.4, 121.4, 121.2, 119.4, 119.3, 118.9, 122.1, 106.9, 106.8, 106.1, 105.4, 96.3, 42.4, 51.8, 49.7, 49.6, 36.7, 33.7, 33.5, 32.3, 32.2, 31.6, 31.4, 29.7, 29.1, 28.7, 26.2, 25.9, 25.8, 25.5, 20.2, 20.0, 14.1.

4.4.2 In Vitro characterization of UVC-454 and UVA-454

**UVC-454 and UVA-454 irradiation in DMSO.** Samples of UVC-454 and UVA-454 were dissolved to a concentration of 10 mM in DMSO in a 20-dram vial. The vial contents were irradiated by placing the vial directly in front of a Hitachi F-7000 fluorescence spectrophotometer Xe lamp source with 20 nm slit widths, and irradiated with 254, 365, and 488 nm light. A portion of the irradiated sample was then transferred into a cuvette containing 20 mM PBS pH 7.4 buffer with 10% Emerald II® to make a final concentration of 50 µM UVC-454 or UVA-454. Luminescence was measured using a Hitachi F-7000 fluorescence spectrophotometer from 0–180 min, and readings were taken every 20 min

**In vitro chemiluminescence response of UVC-454 to irradiation in aqueous media.** Samples of UVC-454 and UVA-454 were dissolved to give 100 mM solutions in DMSO. Aliquots of UVC-454 and UVA-454 were then transferred into a cuvette containing 20 mM PBS pH 7.4 buffer containing 10% Emerald II® solution to give a final concentration of 100–1000 µM UVC-454 and 5% DMSO. The solution was irradiated by placing the cuvette in the cuvette holder of a Hitachi F-7000 fluorescence spectrophotometer Xe lamp source with 20 nm slit widths, and irradiated for 180 min. Chemiluminescence readings were taken every 20 min.
4.4.3 *In Vitro* characterization of Spiro-CL

Monitoring **Spiro-CL** photoswitching using absorption spectroscopy. Absorbance spectra of **Spiro-CL** were collected using a Beckman Colter DU 800 spectrophotometer scanning from 200–800 nm, wavelength interval of 1.0 nm, and scan speed of 1200 nm/min. For solutions in EtOH, 100 µM **Spiro-CL** were dissolved in 200 proof EtOH and added to a cuvette. An initial absorbance read at t = 0 was taken, then the cuvette was taken out of the spectrophotometer and irradiated with 254 nm light from a Spectroline® ENF-240C UV lamp for 2 min. This process was repeated until t = 20 min. Then, the solution was irradiated with white light using a 120 W lamp (Home Depot, Dallas, TX) for 2 min, and an absorbance read was taken, and this process was repeated until t = 30 min. For solutions in DMSO, 10 µM **Spiro-CL** was dissolved in DMSO and added to a cuvette. The cuvette was then irradiated with 254 nm light from a Spectroline® ENF-240C UV lamp for 2 min, and repeated for 20 min.

**Monitoring fluorescence of Spiro-CL before and after UV irradiation.** Fluorescence measurements were taken using a Hitachi F-7000 fluorescence spectrophotometer via the fluorescence detection mode. Fluorescence excitation and emission spectra were acquired with a slit widths of 5.0 nm. 10 µM **Spiro-CL** was dissolved in 200 proof EtOH, and fluorescence excitation and emission spectra were taken before irradiation of the sample with UV light. For the t = 0 fluorescence excitation read, the emission wavelength was set to 330 nm, and the excitation range was scanned from 200–320 nm. For the t = 0 fluorescence emission read, the excitation wavelength was set to 269 nm, and emission range was set from 279–600 nm. Then, the sample was taken out of the fluorometer and irradiated with 254 nm light from a Spectroline® ENF-240C UV lamp for 20 min. After irradiation, fluorescence excitation and emission reads were retaken. For the t = 20 min fluorescence excitation read, the emission wavelength was set to
525 nm, and the excitation range was scanned from 200–515 nm. For the t = 20 min fluorescence emission read, the excitation wavelength was set to 410 nm, and emission range was set from 420–600 nm.

**Chemiluminescence of Spiro-CL.** Chemiluminescence measurements were taken using a Hitachi F-7000 fluorescence spectrophotometer via the fluorescence detection mode. Fluorescence excitation and emission spectra were conducted with slit widths of 20 nm. solutions of 10 µM Spiro-CL were dissolved in 200 proof EtOH or pure DMSO and transferred into a cuvette. Then, the solutions were irradiated for t = 16 min using the 254 nm light source from a Spectroline® ENF-240C UV lamp. chemiluminescence measurements were taken at t = 0 and 16 min.
CHAPTER 4 REFERENCES


CHAPTER 5
FUTURE DIRECTIONS

5.1 Future hypoxia quantification strategies

**HyCL-4-AM** served as a highly sensitive chemiluminescence agent that utilized activity-based nitroaromatic reduction to quantify enzyme activity within the native reductive environment in tumors. Its advancements are numerous as compared to previous hypoxia detection probes, however, to truly *quantify* hypoxia, it is more viable to directly measure oxygen content within cells and tissues rather than use enzymatic activity as a proxy for the same measurement. To accomplish this task, we plan to employ a ratiometric quantification strategy similar to that of **Ratio-pHCL-1**. We will append an oxygen sensitive iridium-based phosphorescence complex whose excitation overlaps with the emission wavelength of the chemiluminescent scaffold. The phosphorescence emission of the iridium complex is quenched in the presence of oxygen through triplet quenching. When exposed to an aqueous environment, the chemiluminescent scaffold will undergo CIEEL to generate chemiluminescence emission at 530 nm. Some of the energy from CIEEL can undergo resonance energy transfer into the iridium complex, which can then emit oxygen-dependent phosphorescence emission wavelength based on the degree of oxygen quenching exhibited on the iridium complex. By taking the ratio of emissions between the donor and acceptor, oxygen content can be quantified in a ratiometric manner. Examples of some target compounds being researched by my coworker Husain Kagalwala is shown in Scheme 5-1.
This strategy provides many advantages to the currently implemented activity-based nitroreduction method. First, it has the potential to quantify oxygen through ratiometric analysis, which is often a more accurate measurement for \textit{in vivo} studies because ratiometric sensors are less sensitive to spectral coloring. Second, this strategy allows for the use of an unprotected
dioxetane phenol, which we have previously shown with Ratio-pHCL-1 greatly increases the brightness of the probe. It may also allow for NIR hypoxia detection through NIR phosphorescence emission from the iridium phosphor. Granted we are successful in the development of a ratiometric oxygen sensor, we will provide a great advancement for chemiluminescence quantification of hypoxia in cells and animals.

5.2 Future studies based on Ratio-pHCL-1

Ratio-pHCL-1 implemented many novel strategies to ultimately quantify pH in vitro and in animal models, and we are very excited by the accurate pH dependent response and sheer brightness of the sensor. While Ratio-pHCL-1 gave highly accurate outputs in vitro, it is unclear whether the sensor quantified pH in our animal studies, because we did not truly know the pH of the peritoneal fluid after injection of the buffer. Peritoneal fluid normally exhibits a pH buffered to the range of 7.6-8.0, and while we used highly concentrated (1.0 M) buffer to change the intraperitoneal pH as close as possible to the pH of the injected buffer, it may have been buffered toward the physiological pH by the peritoneal fluid. This could be examined in future studies by obtaining a secondary pH measurement of the peritoneal cavity using pH microelectrodes before and after injection of the buffer solution, and would give us a more accurate calibration of Ratio-pHCL-1 in animal tissue.

Further studies may also be relevant to test with Ratio-pHCL-1 in the future. The focus of this thesis is to develop imaging agents to study the tumor microenvironment. Unfortunately, due to the current COVID-19 pandemic, we were unable to proceed with further animal testing with Ratio-pHCL-1 in animal tumor models. One possible experiment that could see success using Ratio-pHCL-1 in tumors is to conduct injections of Ratio-pHCL-1 into tumor mouse models, and through detection of its pH dependent luminescence output in various regions of the
tumor using an IVIS spectrum, we could detect heterogenous pH expression throughout the tumor microenvironment. This would ultimately show regions in the tumor that are more acidic, and therefore more reductive and less responsive to chemo and radiotherapy.

One major drawback of Ratio-pHCL-1 was that its response pK$_a$ of 7.7 was too high to be seen as a truly viable agent for biological pH studies. The agent would be more impactful if the response pK$_a$ were to be reduced to a range between 6.8-7.4. This could be readily achieved through synthetic modification of the carbofluorescein unit. It has been previously shown that fluorine substitution on the carbofluorescein’s xanthene core lowers the pK$_a$ of the fluorophore from 7.5 (no fluorine substitution) to 6.75 (difluoro substitution). I postulate that a monofluorinated carbofluorescein would exhibit a pK$_a$ between these two values. Ultimately, it would be wise to synthesize both the monofluorinated and difluorinated derivatives of Ratio-pHCL-1 to ensure the response pK$_a$ of the final probe is within the designated biological range.

Surprisingly, we also found that Ratio-pHCL-1 can penetrate cells even though it is a high MW compound. Especially for intratumoral studies, it would be beneficial to attach a charged functional group on the compound such as a sulfonate to ensure that the compound remains in the extracellular matrix and does not penetrate cells. This would give a much more accurate representation of the acidity of the tumor microenvironment. Examples of potential future ratiometric sensors are listed in Scheme 5-2.
**Scheme 5-2.** Design of future **Ratio-pHCL** compounds.

We can also use the general strategy of fluorophore conjugation to provide ratiometric detection of reactive species, enzymes, and small molecules. Fluorophores appended to the chemiluminescent scaffold could be protected with reactive triggers, such as our current handles for H$_2$S, HNO, ONOO$^-$, or hypoxia. Such probes would then undergo selective reactions with their respective analytes to generate the open fluorophore, and the ratio of emissions between the open fluorophore vs. the chemiluminescent scaffold could provide ratiometric measurement of the analyte. Potential structures are shown in Scheme 5-3.
Scheme 5-3. Potential structures of ratiometric chemiluminescence probes for reactive species and enzyme detection
5.3 Future studies in photoactivatable chemiluminescence

Photoactivated chemiluminescence has huge potential for \textit{in vivo} molecular imaging. A major drawback for currently utilized probes and sensors, is they have no targeting capability. All of the agents must be directly administered to the imaging site. This may be harmful for studies such as intratumoral imaging, where direct intratumoral injections may affect tumor structure and biology. A more advantageous option would be to have photoactivatable chemiluminescence agents that are administered via intravenous injection, and can localize to their target through homing strategies.\textsuperscript{14,15} Once the agent has localized, the compound can be uncaged using NIR light\textsuperscript{16,17} through tissue to elicit a chemiluminescence response. An example of a tumor homing photocaged ratiometric pH probe is shown scheme 5-4.

Scheme 5-4. Design of photoactivatable homing chemiluminescence agents.
CHAPTER 5

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Morrow, J. R. Modulating the Properties of Fe(III) Macrocyclic MRI Contrast Agents by 

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APPENDIX I

$^1$H AND $^{13}$C NMR SPECTRA

Figure A-1. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 13.
Figure A-2. $^{13}$C NMR spectrum (100 MHz, CDCl$_3$) of 13.
Figure A-3. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 14.
Figure A-4. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 14.
Figure A-5. $^1$H NMR spectrum (500 MHz, CDCl$_3$) of HyCL-3.
Figure A-6. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of HyCL-3.
Figure A-7. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 15
Figure A-8. $^{13}$C NMR spectrum (100 MHz, CDCl$_3$) of 15.
Figure A-9. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 3.
**Figure A-10.** $^{13}$C NMR spectrum (100 MHz, CDCl$_3$) of 3.
Figure A-11. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 16.
Figure A-12. $^{13}$C NMR spectrum (100 MHz, CDCl$_3$) of 16.
Figure A-13. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of HyCL-4-AM.
Figure A-14. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of HyCL-4-AM.
Figure A-15. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 17.
Figure A-16. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 17.
Figure A-17. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 4
Figure A-18. $^{13}$C NMR spectrum (100 MHz, CDCl$_3$) of 4.
Figure A-19. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 18.
Figure A-20. $^{13}$C NMR spectrum (100 MHz, CDCl$_3$) of 18.
Figure A-21. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of HyCL-4-AM-Cont.
Figure A-22. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of HyCL-4-AM-Cont.
Figure A-23. $^1$H NMR spectrum (400 MHz, Acetone-D6) of 23.
Figure A.2. 1^3C NMR spectrum (100 MHz, Acetone-D6) of 23.
Figure A-25. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 24.
Figure A-26. $^{13}$C NMR spectrum (100 MHz, CDCl$_3$) of 24.
Figure A-27. $^1$H NMR spectrum (500 MHz, CDCl$_3$) of 25
Figure A-28. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 25.
Figure A-29. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 26.
Figure A-30. $^{13}$C NMR spectrum (100 MHz, CDCl$_3$) of 26.
Figure A-31. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 27.
Figure A-32. $^{13}$C NMR (100 MHz, CDCl$_3$) of 27.
Figure A-33. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 28.
Figure A-34. $^{13}$C NMR (100 MHz, CDCl$_3$) of 28.
Figure A-35. $^1$H NMR spectrum (400 MHz, Acetone-D6) of 29.
Figure A-36. $^{13}$C NMR spectrum (100 MHz, Acetone-D6) of 10.
Figure A-37. $^1$H NMR spectrum (400 MHz, Acetone-D6) of Ratio-pHCL-1.
Figure A-38. $^{13}$C NMR spectrum (100 MHz, Acetone-D6) of Ratio-pHCL-1.
Figure A-39. $^1$H NMR spectrum of 36 (400 MHz, CDCl$_3$).
Figure A-40. $^{13}$C NMR spectrum of 36 (100 MHz, CDCl$_3$).
Figure A-41. $^1$H NMR spectrum of 37 (400 MHz, CDCl$_3$).
Figure A-42. $^{13}$C NMR spectrum of 37 (100 MHz, CDCl$_3$).
Figure A-43. $^1$H NMR spectrum of UVC-454 (500 MHz, CDCl$_3$).
Figure A-44. $^{13}$C NMR spectrum of UVC-454 (125 MHz, CDCl$_3$).
Figure A-45. $^1$H NMR spectrum of UVA-454 (400 MHz, CDCl$_3$).
**Figure A-46.** $^1$H NMR spectrum of 38 (500 MHz, CDCl$_3$).
Figure A-47. $^1$H NMR spectrum of 39 (400 MHz, CDCl₃).
Figure A-48. $^{13}$C NMR spectrum of 39 (100 MHz, CDCl$_3$).
Figure A-49. $^1$H NMR spectrum of Spiro-CL (400 MHz, CDCl$_3$).
Figure A-50. $^{13}$C NMR spectrum of Spiro-CL (100 MHz, CDCl$_3$).
APPENDIX 2

HYCL-4-AM KINETICS CONTINUED

A2-1. Deriving $[A_t]$, $[B_t]$, $[C_t]$ in a linear reaction

$A \rightarrow B \rightarrow C$ reaction

Where $x=[A]$, $y=[B]$, $z=[C]$

Rate of formation of $A$:

(1) $\frac{dx}{dt} = -k_1 x$ integrate $0 \rightarrow t$

(2) $-\ln(x_t) = k_1 t + C$ when $t = 0, x = a$

(3) $-\ln(x) = k_1 t - \ln(a)$ rearrange

(4) $\ln(x) - \ln(a) = -k_1 t$ rearrange

(5) $\ln\left(\frac{x}{a}\right) = -k_1 t$ take exponential rearrange

(6) $x = ae^{-k_1 t}$

Rate of formation of $C$:

(7) $\frac{dz}{dt} = k_2 y$

Rate of formation of $B$:

(8) $\frac{dy}{dt} = -\frac{dx}{dt} - \frac{dz}{dt}$ substitute (1) for $\frac{dx}{dt}$ and (7) for $\frac{dz}{dt}$

(9) $\frac{dy}{dt} = k_1 x - k_2 y$ substitute (6) for $x$

(10) $\frac{dy}{dt} = k_1 ae^{-k_1 t} - k_2 y$ rearrange
Differentiation:

(11) \( \frac{dy}{dt} + k_2y = k_1ae^{-k_1t} \) multiply both sides by \( e^{-k_2t} \)

(12) \[ \frac{dy}{dt} + k_2y(e^{-k_2t}) = k_1a(e^{-k_1t})(e^{-k_2t}) \quad \frac{d}{dt}(x \times y) = xy + ydx \]

(13) \( \frac{d}{dt}[y \times e^{k_2t}] = k_1ae^{(k_2-k_1)t} \) rearrange

(14) \( d[y \times e^{k_2t}] = k_1ae^{(k_2-k_1)t}dt \) integrate \( 0 \rightarrow t \)

(15) \( ye^{k_2t} = k_1a \frac{e^{(k_2-k_1)t}}{k_2-k_1} + C \) at initial conditions when \( t = 0, y=0 \), substitute for \( t \) and \( y \)

(16) \( 0 = k_1a \frac{e^0}{k_2-k_1} + C \) rearrange

(17) \( C = \frac{-k_1a}{k_2-k_1} \) substitute into (15)

(18) \( ye^{k_2t} = k_1a \frac{e^{(k_2-k_1)t}}{k_2-k_1} - \frac{k_1a}{k_2-k_1} \) simplify and divide expression by \( e^{k_2t} \)

(19) \( y = \frac{k_1a}{k_2-k_1} [e^{k_2t} \times e^{-k_1t} \times e^{-k_2t} - e^{-k_2t}] \) \( e^{k_2t} \) and \( e^{-k_2t} \) cancel, leaving:

(20) \( y = \frac{k_1a}{k_2-k_1} [e^{-k_1t} - e^{-k_2t}] \)
**A2-2. Alternate kinetic fits based on derived rate equation**

**Figure A2-1. Kinetic model.** (A) Reactions used to model the chemiluminescence emission. (B) Rate equation for the chemiluminescence emission. y-value represents [HyCL-4] generated after $k_1$. (C) Example fit of the chemiluminescence response of 40 µM HyCL-4-AM in A549 cells at 1% O$_2$. (D) Example fit of the chemiluminescence response of 40 µM HyCL-4-AM in A549 cells at 20% O$_2$. (E) Alternate Rate equation for the chemiluminescence emission. y-value represents [HyCL-4] generated after $k_1$. (F) Example fit of the chemiluminescence response of 40 µM HyCL-4-AM in A549 cells at 1% O$_2$. (G) Example fit of the chemiluminescence response of 40 µM HyCL-4-AM in A549 cells at 20% O$_2$. 

\[ y = A \frac{k_f}{k_2 - k_f} \left( e^{-k_f t} - e^{-k_1 t} \right) + B \]

\[ y = A \frac{k_2 \cdot k_1}{k_2 - k_1} \left( e^{k_1 t} - e^{k_2 t} \right) + B \]

\[ r^2 = 0.996 \]

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