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The Kinetics and Cellular Imaging of Multi-color Fluorescent and Chemiluminescent Compounds

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The Kinetics and Cellular Imaging of Multi-color Fluorescent and Chemiluminescent Compounds

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The Kinetics and Cellular Imaging of Multi-color Fluorescent and Chemiluminescent Compounds

A Dissertation Presented to the Graduate Faculty of the
Dedman College
Southern Methodist University
in
Partial Fulfillment of the Requirements
for the degree of
Doctor of Philosophy
with a
Major in Chemistry
by
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B.S in Chemistry, Nankai University, China
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August 3, 2022
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The Kinetics and Cellular Imaging of Multi-color Fluorescent and Chemiluminescent Compounds

Advisor: Professor Alex Lippert

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A three-dimensional display is a useful method which can provide spatially accurate representations of high-resolution images with a 360° view. Therefore, it has potential to be applied widely in the medical area, entertainment industry and for military applications. According to the first-generation 3D digital light photoactivatable dye display (3D Light PAD), \textbf{N-phenyl} spirolactam rhodamine B was used as photoactivatable compound that could be switched from non-fluorescent to fluorescent using ultraviolet light. However, the detailed mechanism, how other rhodamine derivatives would perform in 3D Light PAD, and what other factors in this system could improve the imaging quality remained unclear. In order to solve these problems and understand the properties of rhodamine derivatives, we synthesized a series of \textbf{N-aryl} spirolactam rhodamine photoswitches and tested their spectroscopic characterizations such as irradiation wavelength and absorbance, duration of switching from off-state to on-state and irradiation wavelength dependence. Also, extinction coefficients and fluorescent quantum yields of the fluorescent form of these compounds was measured by using trifluoroacetic acid to drive the equilibrium to the open form. According to these results, N-aryl spirolactam rhodamine derivatives with fluorinated aminoalkyl groups (SRCF3) that have green (518 nm) emission wavelengths were
synthesized by a new procedure and their spectroscopic characterizations including extinction coefficients and fluorescent quantum yields were measured by the same methods as the red dyes. These results helped us better understand the mechanism of photoswitching reactions more clearly and were used to set the foundation for a multi-color volumetric 3D display.

Different with fluorescence, chemiluminescence is the light production from exothermic chemical reactions, where the excited state is accessed during the reaction and then the light will be generated by relaxation from excited state to the ground state. As an imaging modality, chemiluminescence has many advantages compared with current imaging methods, including fluorescence, bioluminescence and phosphorescence. The most important thing is chemiluminescence could work well both in vivo and in vitro without light excitation or genetic modification like fluorescence and bioluminescence. Based on it, several chemiluminescent probes for different reactive species were designed, synthesized, characterized, and applied for cellular and in vivo studies. CHS-4 and CL-DNP are chemiluminescent probes for hydrogen sulfide (H₂S) with azide and dinitro phenyl group triggers. According to the sensitivity and selectivity studies of these probes during in vitro tests, finally the cellular studies of them were designed in A549 cells.
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2. Li, B.; Kim, Y. L.; Lippert, A. R. Antioxidants & Redox Signal 2021, 36, 4-6.


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Name</th>
<th>Structure</th>
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<tr>
<td>A549</td>
<td>Human lung adenocarcinoma epithelial cells</td>
<td>-</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
<td>H(\text{-C-C\equiv N})</td>
</tr>
<tr>
<td>ACT</td>
<td>Activator</td>
<td>-</td>
</tr>
<tr>
<td>BET</td>
<td>Back Electron Transfer</td>
<td>-</td>
</tr>
<tr>
<td>CBS</td>
<td>Cystathionine beta synthase</td>
<td>-</td>
</tr>
<tr>
<td>CCD</td>
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</tr>
<tr>
<td>CHS-3 X = Cl</td>
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<td>-</td>
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<tr>
<td>CIEEL</td>
<td>Chemically initiated electron exchange luminescence</td>
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<tr>
<td>CSE</td>
<td>Cystathionine gamma-lyase</td>
<td>-</td>
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<td>Diethyl azodicarboxylate</td>
<td><img src="image" alt="Diethyl azodicarboxylate" /></td>
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<tr>
<td>DCM</td>
<td>Dichloromethane</td>
<td>Cl(\text{-C-H})</td>
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xviii
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>DFP</td>
<td>Density Functional Theory</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-Diisopropylethylamine</td>
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<tr>
<td>DLP</td>
<td>Digital Light Processing</td>
</tr>
<tr>
<td>DMAP</td>
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<tr>
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<td>Fluorescence Resonance Energy Transfer (FRET)</td>
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<tr>
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<td>Gas Chromatography-Mass Spectrometry</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>H2S</td>
<td>Hydrogen Sulfide</td>
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</table>
HPLC  High Performance Liquid Chromatography
LDBC  linear-dendritic block copolymer
Light PAD  Light photoactivatable dye display
MB  Methylene Blue

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

NIS  Ammonium Chloride

pTSA  p-toluenesulfonic acid

P (o-tolyl)$_3$  Tri (o-tolyl) phosphine

RhB  Rhodamine B
SRCF3  fluorinated N-aryl spiro lactam rhodamine

SiR  silicon rhodamine

THF  Tetrahydrofuran

TsCl  4-Toluenesulfonyl Chloride
For my parents and best friends.
CHAPTER 1
SPIROLACTAM RHODAMINE PHOTOSWITCHES FOR VOLUMETRIC 3D DISPLAYS

1.1 Introduction

1.1.1 Spirolactam rhodamine photoswitches

Photoswitches are a series of molecules which can achieve reversible photoactivation processes by chemical reactions. Based on the properties of these molecules, many applications such as molecular machines, 3D printing, advanced materials have been explored in the last decades. With more research in this area, there are various classes of photoswitches being developed, including azobenzenes, diarylethenes, oxazines, acyl hydrazones, spiropyrans, dihydropyrenes and spirolactam rhodamines and the mechanism of photoactivation is becoming more clearly understood.

As representative fluorescent photoswitches, spirolactam rhodamines can be used for numerous applications like single-molecule microscopy and volumetric 3D digital light photoactivatable dye displays. These applications depend on the mechanisms of photoactivation process as described in Scheme 1-1. Spirolactam rhodamines could be transformed from a non-fluorescent state into a fluorescent state with a specific wavelength of light irradiation. Not only this fluorescent state can be excited to the emissive excited state by illumination but also the on-state molecule could achieve relaxation to the non-fluorescent state by a thermal fading process.
Scheme 1-1. Mechanism of single molecule photoactivation. (Adapted with permission from reference 17. Copyright 2009 John Wiley and Sons)

1.1.2 Volumetric 3D display

As a novel and useful technology, volumetric 3D displays can be used in various applications, such as medical imaging, engineering, education, and other areas, which exists everywhere in our daily life.\textsuperscript{15,16} Compared with traditional 2D imaging, volumetric 3D displays can show the real structure in specific condition and the detailed differences of some physical factors, like the eyes’ activities\textsuperscript{17}, motion parallax\textsuperscript{18} and binocular disparity\textsuperscript{19}. In addition, volumetric 3D displays can be characterized not only into swept-volume displays,\textsuperscript{20} such as high-speed scanning and rotating light imaging,\textsuperscript{21,22,23,24} but also static volume displays\textsuperscript{25} like active matrix displays\textsuperscript{26} and beam-addressable displays.\textsuperscript{27,28,29}

A chemically enabled volumetric 3D digital light photoactivatable dye display (3D Light PAD) is an effective method for 3D image generation.\textsuperscript{30,31} (Figure 1-1) There are two important factors in this system, one is a special class of photoactivatable molecules, the other is digital light processing (DLP) technology to generate structured light in three dimensions. Spirolactam rhodamine photoswitches are specific molecules that have found widespread use in volumetric 3D displays. DLP projector technology consists of two personal computers which control a
Miroir 720p picoprojector, and a Pro4500 projector equipped with a 385 nm ultraviolet LED and a 525 nm green LED. The ultraviolet light is used to complete the photoactivation and green LED is used to project a 2D visible light with ultraviolet pattern projected from the Pro4500. Finally, high-resolution images were displayed from both Pro4500 projector and green LED to generate 3D image at the intersection. (Figure 1.2)

Figure 1. Digital light processing (DLP) for volumetric 3D display. (Adapted with permission from reference 32. Copyright 2017 Nature communication)

Figure 1.2. High-resolution images generation by DLP system. (Adapted with permission from reference 32. Copyright 2017 Nature communication)
1.1.3 Photoswitching kinetics

As a specific class of photoswitches, the photoswitching mechanisms of spirolactam rhodamine derivatives has been investigated in past years. As the Figure 1-3 shows, spirolactam rhodamine has a colorless and non-fluorescent closed form in DCM solvent\textsuperscript{33} which could achieve a photochemical reaction that switch the close-form molecule into an open fluorescent form.\textsuperscript{34} According to this, Gleiter group made more research about the kinetics of this photoactivation process and thermal fading in other solvents such as water and alcohols.\textsuperscript{35} Besides that, Bossi developed another experiment that demonstrated the kinetics of isomerization and acid induced changes with addition of trifluoroacetic acid for N-phenyl spirolactam rhodamine photoswitches.\textsuperscript{36} After that, they set up a four-state mechanism that described this photoswitching process with a first order reaction model.\textsuperscript{37} Scott et.al explored the acid-dependent properties of these derivatives based on the previous results,\textsuperscript{38,39} that were valuable for us. In the summary, these studies provided us with more kinetics and synthetic methodology knowledge of spirolactam rhodamine photoswitches and spectroscopy measurements of this series of derivatives,\textsuperscript{43,44} for example absorption and emission intensity, photoswitching kinetics\textsuperscript{45} and quantum yields\textsuperscript{46} are useful for us to make more comprehensive understanding of the mechanisms of photoactivation.
1.2 Research objectives

In our previous work, we used N-phenyl spirolactam rhodamine B as a fluorescent photoswitch to generate 3D images in a solution of dichloromethane in a 3D display. According to this result, we design and synthesize various of photoswitches with different colors, spirolactam rhodamine B (SRB) with red emission wavelengths (585 nm) and spirolactam rhodamine derivatives with fluorinated aminoalkyl groups (SRCF3) with green emission wavelengths (518 nm). In this project, we developed a new procedure to prepare fluorinated spirolactam rhodamine derivatives. Additionally, we completed spectroscopy measurements for these compounds such as absorption and emission spectrum, as well as excitation time scanning. Furthermore, we measured the kinetics and quantum yields of every fluorescent derivative, which is useful for us.
to understand the photoactivation mechanisms and achieve multi-color 3D display by DLP technology in the future.

1.3 Results and discussions

1.3.1 Design and synthesis of spirolactam rhodamine B (SRB) and fluorinated spirolactam rhodamine (SRCF3) derivatives

In our project, we synthesized nine spirolactam rhodamine B (SRB) derivatives (red dye) and eight fluorinated spirolactam rhodamine (SRCF3) derivatives (green dye). The SRB derivatives were synthesized by rhodamine B coupling with a library of anilines in phosphorus oxychloride condition. Derivatives were designed with electron donating groups (compounds 2, 3, 7–9), electron withdrawing groups (compounds 4–6), and steric hindrance photoswitches (compounds 7–9). The SRCF3 compounds were synthesized starting from fluorescein and the bis-triflate was prepared with high yield. The final precursor product was produced by palladium catalysis with the XPhos for generating the fluorinated rhodamine with trifluoro ethyl-amino groups. In the next step, we prepared derivatives with similar method as SRB derivatives, which reacted with electron donating groups (compounds 12–16), electron withdrawing groups (compounds 11, 17), and sterically-hindered groups (compounds 12, 13, 16).
Scheme 1-2. Synthesis of SRB derivatives (red dye). (Adapted with permission from reference 17. Copyright 2009 John Wiley and Sons)
1.3.2 Tuning and photophysical properties experiments

According to the absorption and emission spectrum results, we first measured the extinction coefficients ($\varepsilon_{556\text{nm(on)}}$, $\varepsilon_{495\text{nm(on)}}$) and fluorescence quantum yields ($\Phi_{\text{fl}}$) of the fluorescent state of the dyes that could describe how strongly a species absorbs radiation or light at a particular wavelength. According to some studies about visible-light photoswitching and acid-catalyzed isomerization, the researchers found that TFA addition could provide the proton combining with the nitrogen of N-aryl group for achieving the intermolecular proton transfer of photoswitching
derivatives which could complete similar photoactivation process with UV light irradiation. Based on it, we attempted to add trifluoroacetic acid with different equivalents for achieving the complete conversion for them to the on-state. As the results show, fully turn-on state happened with the addition of 50 equivalents of trifluoroacetic acid for the SRB derivatives and 500 equivalents of trifluoroacetic acid for SRCF3 derivatives. (Figure 1-4) Then we obtained extinction coefficients at 556 nm for SRB compounds (Figure 1-5) and at 495 nm for SRCF3 compounds (Figure 1-6). As these results, \( \varepsilon_{556\text{nm(on)}} \) were found to be between 84,700 M\(^{-1}\) cm\(^{-1}\) and 132,100 M\(^{-1}\) cm\(^{-1}\) for the SRB series (Table 1-1) and \( \varepsilon_{495\text{nm(on)}} \) were found to be between 54,400 M\(^{-1}\) cm\(^{-1}\) and 94,900 M\(^{-1}\) cm\(^{-1}\) for SRCF3 series (Table 1-2).

Similarly, the fluorescence quantum yields (\( \Phi_f \)) measurements were also determined by trifluoroacetic acid addition to drive the equilibrium to the complete fluorescent state. Finally, we found that derivatives with electron-rich aryl groups (compounds 2, 3, 7, 13–15) had lower quantum yields. The reason of this result was probably because donating group could enhance the efficiency of photoinduced electron transferring (PET) in fluorescence quenching, which led to decrease the slope values for calibration curve of absorption verse fluorescence emission. Furthermore, the other derivatives with high steric hindrance (compounds 8, 9, 12, 16) performed similar fluorescence quantum yields with original product.
Figure 1-4. TFA addition experiments. (A) Fluorescence emission intensity at 585 nm of 5 µM 1 incubated with 50 equivalents of TFA for 0–60 min. (B) Fluorescence emission intensity at 585 nm of 5 µM 1 with 0–100 equivalents of TFA after incubating for 40 min. (C) Fluorescence emission intensity at 518 nm of 5 µM 10 incubated with 500 equivalents of TFA for 0–60 min. (D) Fluorescence emission intensity at 518 nm of 5 µM 10 with 0–1000 equivalents of TFA after incubating for 40 min. Indicate permissions – these figures are in the SI, so it is still safer to indicate the permissions. (Adapted with permission from reference 45. Copyright 2021 Elsevier)
Figure 1-5. Extinction coefficient linear fits of absorbance at 556 nm versus concentration of compounds 1–9. (Adapted with permission from reference 45. Copyright 2021 Elsevier)

Figure 1-6. Extinction coefficient linear fits of absorbance at 495 nm versus concentration of compounds 10–17. (Adapted with permission from reference 45. Copyright 2021 Elsevier)
Table 1-1. Fluorescence quantum yields and extinction coefficients of the on-state of N-aryl spirolactam rhodamine B (SRB) derivatives in dichloromethane.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\Phi_{fl}$ / %</th>
<th>$\varepsilon_{566\text{nm(on)}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>52</td>
<td>84,700</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>122,400</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>110,200</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
<td>96,000</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>118,900</td>
</tr>
<tr>
<td>6</td>
<td>55</td>
<td>132,100</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>117,500</td>
</tr>
<tr>
<td>8</td>
<td>28</td>
<td>103,500</td>
</tr>
<tr>
<td>9</td>
<td>33</td>
<td>112,200</td>
</tr>
</tbody>
</table>

Table 1-2. Fluorescence quantum yields and extinction coefficients of the on-state of fluorinated N-aryl spirolactam rhodamine (SRCF3) derivatives in dichloromethane.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\Phi_{fl}$ / %</th>
<th>$\varepsilon_{495\text{nm(on)}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>58</td>
<td>94,900</td>
</tr>
<tr>
<td>11</td>
<td>24</td>
<td>91,400</td>
</tr>
<tr>
<td>12</td>
<td>41</td>
<td>94,200</td>
</tr>
<tr>
<td>13</td>
<td>6</td>
<td>90,900</td>
</tr>
<tr>
<td>14</td>
<td>3</td>
<td>65,900</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>58,900</td>
</tr>
<tr>
<td>16</td>
<td>31</td>
<td>70,300</td>
</tr>
<tr>
<td>17</td>
<td>13</td>
<td>54,400</td>
</tr>
</tbody>
</table>

Besides the extinction coefficient and fluorescence quantum yields, we determined the on-rates ($k_{on}$) and photochemical quantum yields ($\Phi_{pc}$) of the photoswitching reactions which quantify the relationship between reaction rate and light quantity. Both of these two characterizations are related by equation 3 (All equations are showed in Methods sections 1.4.3 and Figure 1-8A) and
depend on the extinction coefficients of the closed form ($\varepsilon_{254nm(\text{off})}$) (Figure 1-7), and the excitation light intensity ($I_0$). We measured the maximum fluorescence emission intensity of the open form with continuous excitation at 254 nm and then fit this data to the rate equation for a reaction at equilibrium. For confirming the robust of results, we choose two methods of photochemical quantum yields measurement. First, we measured $N$-phenyl spirolactam rhodamine B (compound 1) emission spectrum and found that its fluorescence emission at 585 nm with continuous excitation at 254 nm provided a smooth time-scan curve and finally reached an equilibrium state after about 1200 seconds (Figure 1-8A).
Figure 1-7. Extinction coefficient linear fits of absorbance at 254 nm versus concentration of compounds 1–17. (Adapted with permission from reference 45. Copyright 2021 Elsevier)

We fitted this data with equation 3 using Mathematica software and the rate constants were determined as $k_{on} = 1.53 \times 10^{-3} \text{ s}^{-1}$, $k_{off} = 1.14 \times 10^{-3} \text{ s}^{-1}$ after fitting. (Figure 1-8B) The equilibrium constant $K_{eq}$ was determined directly by using $k_{on}$ and $k_{off}$ values, which described the photoactivation process in more detail and demonstrated the reaction trends and speed of this mechanisms for every derivative. $k_{on}$ is the rate for the light-mediated reaction that converts the closed form to the open form. $k_{off}$ is the rate for this reaction that returns to the close form. Also, $K_{eq}$ is valuable for us to describe the final state at equilibrium and understand the relationship between electron inductive groups and the kinetics. For confirming these fits result, we used another approach to compare the equilibrium constant evaluated with this method to that determined by obtaining the maximum emission of fluorescent state by driving the equilibrium to the on-state with addition of trifluoracetic acid (Figure 1-9A). For example, we found that the value of 5 µM concentration on this calibration curve performs the maximum fluorescence ($I_{max}$)
in the open fluorescent form in Figure 1-8A. The calibration curve of it could be used to
determine the concentration of the fluorescent state and nonfluorescent state when the
photochemical reaction reached at equilibrium, then $K_{eq}$ was determined with them. Comparing
the results using these two methods, the equilibrium constant determined by different approaches
is similar (Figure 1-9B).

Figure 1-8. Methodology to measure photoswitching rates (Method 1). (A) Emission intensity at
585 nm of 5 µM 1 in CH$_2$Cl$_2$ irradiated with 50.8 µW cm$^{-2}$ of 254 nm light (black trace) and the
fit to equation 3 (red trace). (B) Equation and parameters found for the trace shown in (A).
(Adapted with permission from reference 45. Copyright 2021 Elesvier)
Another method of photoswitching rates measurement (Method 2). (A) Calibration curve generated from increasing concentrations of 1 in CH$_2$Cl$_2$ 40 min after addition of 50 equivalents of TFA. Data are average values from n = 6 independent experiments. Error bars are ± S.D. (B) Comparison of $K_{eq}$ determined from Method 1: $k_{off}$ and $k_{on}$ found from equation 1 and Method 2: Determined by evaluating the equilibrium concentration of the on-state using the equilibrium fluorescence emission in (Figure 1-7A) and the calibration curve in (A). (Adapted with permission from reference 45. Copyright 2021 Elsevier)

Next, we summarized the fluorescence turn-on data and determined photochemical quantum yields for the series of SRB photoswitches (Table 1-3). Extinction coefficients ($\varepsilon_{254nm(\text{off})}$) on the closed form were found to be between 34,500 M$^{-1}$cm$^{-1}$ and 47,000 M$^{-1}$cm$^{-1}$ and the off-rates $k_{off}$ values were determined to be in the range of 0.38–6.1 x 10$^{-3}$ s$^{-1}$ for these derivatives. According to these results, we found that SRB photoswitches with electron withdrawing groups (compounds 4–6) and the sterically hindered group compound 9 had lower values for $k_{off}$ compared with compound 1. The photoswitches with electron donating groups (compounds 2, 3, 7, and 8) had similar $k_{off}$ values which were found to be between 0.93 x 10$^{-3}$ s$^{-1}$ and 6.1 x 10$^{-3}$ s$^{-1}$. As the difference of nucleophilicity for every derivatives, compound 4-6 have stronger electron withdrawing effect to the nitrogen negative part which makes the converse from open form to close form more difficult, that caused there are higher $k_{on}$ and lower $k_{off}$. In the other hand, compound 2 and 3 with electron donating groups prefer to push the photoactivation equilibrium to the closed form and prevent the reaction switching to the fluorescent state which leads to increase $k_{off}$ value.

We next evaluated the $k_{on}$ values for using these to determine the equilibrium constant ($K_{eq}$) and the photochemical quantum yield ($\Phi_{pc}$). Although the 4-methoxy and 3,4-dimethoxy electron-rich derivatives 2 and 3 have lower $k_{on}$, $\Phi_{pc}$, and $K_{eq}$, the effect of electron withdrawing and
sterically hindered substituents on $k_{\text{on}}$ was not increasing obviously. Compared to the -methoxy compounds, we found that the phenyl, 2-fluoro, 2-methyl, 2,6-dimethyl, and 2,6-diisopropyl compounds had higher $k_{\text{on}}$ and $\Phi_{\text{pc}}$, which indicated that steric hindrance in the spirolactam structure would increase the turn-on rate and fluorescence quantum yield. On the other hand, the 2-fluoro, 3-chloro, and 3-fluoro derivatives performed slightly lower $k_{\text{on}}$ and $\Phi_{\text{pc}}$ values than that of the original derivative, that indicated that the photochemical reaction rate $k_{\text{on}}$ is not completely influenced by the electronic inductive effect of the $N$-aryl substituent. Compared with SRB derivatives, the green dye SRF3 photoswitches had much higher $k_{\text{off}}$ value compared with the SRB derivatives for the same measurement (Table 1-4). This result demonstrated that the fluorination of the amino groups prefer to pushing the equilibrium forward to the closed form of the dye. Also this effect could be affected strongly by electron-withdrawing groups (compound 11 and 17) or steric hinderance at the spirolactam position (compounds 12, 13, 16), which performed much lower $k_{\text{off}}$ values compared with the original N-phenyl compound.
<table>
<thead>
<tr>
<th>Compound</th>
<th>( \varepsilon_{254\text{nm}(0)} )</th>
<th>( K_{eq} )</th>
<th>( k_{off}^{[1]} )</th>
<th>( k_{on}^{[1]} )</th>
<th>( \Phi_{pc} / % )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40,300</td>
<td>1.36 ±0.05</td>
<td>1.2 ±0.3</td>
<td>1.6 ±0.4</td>
<td>6.0 ±1.5</td>
</tr>
<tr>
<td>2</td>
<td>42,600</td>
<td>0.044 ±0.023</td>
<td>6.1 ±3.5</td>
<td>0.31 ±0.26</td>
<td>1.1 ±0.93</td>
</tr>
<tr>
<td>3</td>
<td>41,800</td>
<td>0.17 ±0.056</td>
<td>1.4 ±0.44</td>
<td>0.25 ±0.14</td>
<td>0.82 ±0.45</td>
</tr>
<tr>
<td>4</td>
<td>40,400</td>
<td>1.54 ±0.12</td>
<td>0.72 ±0.16</td>
<td>1.1 ±0.2</td>
<td>4.1 ±0.83</td>
</tr>
<tr>
<td>5</td>
<td>47,000</td>
<td>1.23 ±0.15</td>
<td>0.38 ±0.24</td>
<td>0.49 ±0.34</td>
<td>1.6 ±1.1</td>
</tr>
<tr>
<td>6</td>
<td>42,300</td>
<td>1.93 ±0.62</td>
<td>0.57 ±0.45</td>
<td>0.97 ±0.54</td>
<td>3.1 ±1.8</td>
</tr>
<tr>
<td>7</td>
<td>41,300</td>
<td>1.64 ±0.13</td>
<td>0.93 ±0.56</td>
<td>1.5 ±1.0</td>
<td>6.7 ±3.7</td>
</tr>
<tr>
<td>8</td>
<td>34,500</td>
<td>0.68 ±0.14</td>
<td>2.7 ±1.1</td>
<td>1.8 ±1.0</td>
<td>8.0 ±4.2</td>
</tr>
<tr>
<td>9</td>
<td>38,900</td>
<td>1.37 ±0.46</td>
<td>0.60 ±0.1</td>
<td>0.84 ±0.39</td>
<td>3.3 ±1.5</td>
</tr>
</tbody>
</table>

Table 1-3. Kinetics and photochemical quantum yields of 5 µM N-aryl spirolactam Rhodamine B (SRB) derivatives in dichloromethane.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( \varepsilon_{254\text{nm}(0)} )</th>
<th>( K_{eq} )</th>
<th>( k_{off}^{[1]} )</th>
<th>( k_{on}^{[1]} )</th>
<th>( \Phi_{pc} / % )</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>41,300</td>
<td>0.016 ±0.0011</td>
<td>182 ±26</td>
<td>2.9 ±0.29</td>
<td>11 ±1</td>
</tr>
<tr>
<td>11</td>
<td>37,600</td>
<td>0.28 ±0.051</td>
<td>4.7 ±1.4</td>
<td>1.3 ±0.26</td>
<td>5.3 ±1.0</td>
</tr>
<tr>
<td>12</td>
<td>36,500</td>
<td>0.75 ±0.038</td>
<td>2.8 ±1.1</td>
<td>2.1 ±0.79</td>
<td>8.8 ±3.3</td>
</tr>
<tr>
<td>13</td>
<td>40,400</td>
<td>0.094 ±0.064</td>
<td>3.2 ±1.2</td>
<td>0.25 ±0.066</td>
<td>0.94 ±0.002</td>
</tr>
<tr>
<td>14</td>
<td>36,300</td>
<td>0.0095±0.0013</td>
<td>287 ±16</td>
<td>2.7 ±0.47</td>
<td>11 ±2.0</td>
</tr>
<tr>
<td>15</td>
<td>41,100</td>
<td>0.0094±0.0023</td>
<td>356 ±88</td>
<td>3.3 ±0.9</td>
<td>18 ±5.0</td>
</tr>
<tr>
<td>16</td>
<td>36,800</td>
<td>0.78 ±0.10</td>
<td>1.9 ±0.086</td>
<td>1.5 ±0.14</td>
<td>6.1 ±0.56</td>
</tr>
<tr>
<td>17</td>
<td>43,400</td>
<td>0.069 ±0.0079</td>
<td>46 ±19</td>
<td>3.1 ±0.89</td>
<td>11 ±3.1</td>
</tr>
</tbody>
</table>

Table 1-4. Kinetics and photochemical quantum yields of 5 µM fluorinated N-aryl spirolactam rhodamine (SRCF3) derivatives in dichloromethane.
1.3.3 Volumetric 3D display imaging

Finally, based on the results of the spectroscopy measurements, we attempted to choose a proper photoswitching molecule compound 10 which has a good performance of fluorescence quantum yields and high value of $K_{eq}$, that demonstrates the probe could achieve the photoactivation efficiently and controllably and make preliminary volumetric 3D display using the 3D Light PAD. For optimizing the imaging system and increasing the imaging quality, we found that the addition of triethylamine had a significant effect on the $k_{off}$ value, which increases from $1.2 \times 10^{-3}$ s$^{-1}$ in the absence of triethylamine to $30 \times 10^{-3}$ s$^{-1}$ in the presence of triethylamine. This result was useful for us to achieve the high-resolution display with better quality observed. Therefore, we used the $5 \mu$M 2-fluoro SRCF3 as photoswitchable molecule in dichloromethane with $7.2 \mu$M triethylamine in a $2.5 \text{ cm} \times 2.5 \text{ cm} \times 5 \text{ cm}$ cuvette and a 254 nm excitation source for making 3D displays. A 254 nm TLC lamp was projected as UV light source and a blue light was projected from the side using a LightCrafter 4500 projector. A green light display can be observed (Figure 10B), that provide a useful demonstration for new synthetic green dye SRCF3 photoswitches using in DLP technology.
Figure 1-10. Demonstration of 3D Voxel Formation. (A) Schematic and (B) photograph of the setup for 3D voxel formation using a 254 nm TLC lamp, the blue LED of a LightCrafter 4500 projector, and a cuvette filled with 5 µM 11 and 7.2 µM triethylamine. (Adapted with permission from reference 45. Copyright 2021 Elsevier)

1.4 Experimental Section

1.4.1 General materials and methods

All reactions were performed in dried glassware under an atmosphere of dry N₂. Silica gel P60 (SiliCycle) was used for column chromatography and SiliCycle 60 F254 silica gel (precoated sheets, 0.25 mm thick) was used for analytical thin layer chromatography. Plates were visualized by fluorescence quenching under UV light or by staining with iodine. Other reagents were purchased from Sigma-Aldrich (St. Louis, MO), Alfa Aesar (Ward Hill, MA), EMD Millipore (Billerica, MA), or Oakwood Chemical (West Columbia, SC) and used without further purification. ¹H NMR and ¹³C NMR spectra for characterization of new compounds and monitoring reactions were collected in CDCl₃ (Cambridge Isotope Laboratories, Cambridge, MA) on a JEOL 500 MHz spectrometer or Bruker 400 MHz spectrometer in the Department of Chemistry at Southern Methodist University. 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. Low resolution mass spectrometry was performed using an Advion ExpressionL CMS (ESI source) at Southern Methodist University.
Fluorescence responses were acquired with a Hitachi F-7000 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan) using the fluorescence detection mode. Fluorescence emission scans were acquired in wavelength scan mode with emission wavelength range from 400 nm to 700 nm, and scan speed set to 1200 nm/min. Response time was set to 2.0 s. Absorbance responses were acquired with a DU 800 spectrometer (Beckman Coulter, USA) using the absorbance wavelength scan mode with the absorbance wavelength range from 200 nm to 850 nm, and scan speed set to 1200 nm/min. Compounds were purified by recrystallization before spectroscopic experiments. All measurements were obtained in a clean, 1.0 cm pathlength quartz cuvette with screw cap on (Starna Cells, USA). Dichloromethane and trifluoroacetic acid were purchased from Fisher Scientific.

1.4.2 Synthesis procedure

\[
\begin{align*}
3',6'-\text{bis(diethylamino)-2-phenylspiro[isoindoline-1,9'-xanthen]-3-one (1).}^1
\end{align*}
\]

Rhodamine B (2.99 g, 6.24 mmol, 1.0 equiv) and aniline (1.7 mL, 19 mmol, 3.0 equiv) were dissolved in dichloromethane (50 mL). The mixture was cooled to 0 °C, then POCl\textsubscript{3} (0.70 mL, 7.5 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 50 mL dichloromethane,
and washed with 1 M HCl (3 x 50 mL), 1 M NaOH (3 x 50 mL), and brine. The organic layer was collected, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate: hexane (1:4) as the eluent, affording the product as a white solid (2.4 g, 75% yield). The compound can be additionally purified by recrystallization from ethyl acetate: hexanes (1:2). ¹H NMR (500 MHz, CDCl₃) 7.87 (dd, J = 6.9 Hz, 1.2 Hz, 1H), 7.50–7.58 (m, 2H), 6.80–6.93 (m, 6H), 7.61 (d, J = 8.6 Hz, 2H), 6.40 (d, J = 8.6 Hz, 2H), 6.27 (d, J = 2.3 Hz, 2H), 3.34 (q, J = 6.9 Hz, 8H), 1.11 (t, J = 6.9 Hz, 12H). ¹³C NMR (125 MHz, CDCl₃) 166.9, 153.9, 152.9, 148.8, 137.7, 132.9, 130.7, 128.7, 128.2, 126.6, 126.1, 123.3, 122.4, 108.2, 106.3, 97.7, 67.3, 44.3, 12.5.

![Chemical Structure](image)

[2]

3',6'-bis(diethylamino)-2-phenyl-4-methoxyspiro[isoindoline-1,9'-xanthen]-3-one (2).

Rhodamine B (2.95 g, 6.22 mmol, 1.0 equiv) and 4-methoxy aniline (2.2 mL, 18.6 mmol, 2.9 equiv) were dissolved in dichloromethane (50 mL). The mixture was cooled to 0 °C, then POCl₃ (0.70 mL, 7.5 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 50 mL dichloromethane and then washed with 1 M HCl (3 x 50 mL), 1 M NaOH (3 x 50 mL) and brine. The organic layer was collected, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate: hexane (1:4) as the eluent, affording the product as a white solid (2.2 g, 71% yield). The compound can
be additionally purified by recrystallization from ethyl acetate: hexanes (1:2). $^1$H NMR (500 MHz, CDCl$_3$) 8.03 (d, $J = 6.3$ Hz, 1H), 7.52 (t, $J = 6.4$ Hz, 2H), 7.19 (d, $J = 6.4$ Hz, 1H), 6.65 (q, $J = 8.5$ Hz, 6H), 6.34 (d, $J = 8.8$ Hz, 2H), 6.27 (s, 2H), 3.80 (s, 3H), 3.71 (s, 3H), 3.34 (q, 8H), 1.17 (t, $J = 6.9$ Hz, 12H). $^{13}$C NMR (125 MHz, CDCl$_3$) 167.6, 158.2, 153.2, 153.0, 148.7, 132.6, 131.4, 129.0, 128.9, 128.1, 124.0, 123.3, 122.0, 113.9, 108.0, 106.3, 97.7, 67.3, 55.1, 44.3, 12.5.

![Structure](image)

3',6'-bis(diethylamino)-2-phenyl-3,4-dimethoxyspiro[isoindoline-1,9'-xanthen]-3-one (3).

Rhodamine B (2.97 g, 6.24 mmol, 1.0 equiv) and 3,4-dimethoxy aniline (2.74 g, 18.7 mmol, 3.0 equiv) were dissolved in dichloromethane (50 mL). The mixture was cooled to 0 °C, then POCl$_3$ (0.70 mL, 7.5 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 50 mL dichloromethane and then washed with 1 M HCl (3 x 50 mL), 1 M NaOH (3 x 50 mL) and brine. The organic layer was collected, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate: hexane (1:4) as the eluent, affording the product as a white solid (2.07 g, 54% yield). The compound can be additionally purified by recrystallization from ethyl acetate: hexanes (1:2). $^1$H NMR (500 MHz, CDCl$_3$) 8.03 (d, $J = 6.3$ Hz, 1H), 7.52 (t, 2H), 7.19 (d, $J = 6.4$ Hz, 1H), 6.65 (q, 6H), 6.34 (d, $J = 8.8$ Hz, 2H), 6.27 (s, 1H), 3.71 (s, 3H), 3.34 (q, 8H), 1.17 (t, $J = 6.9$ Hz, 12H). $^{13}$C NMR
(125 MHz, CDCl$_3$) 167.6, 158.2, 153.2, 153.0, 148.7, 132.6, 131.4, 129.0, 128.9, 128.1, 124.0, 123.3, 122.0, 113.9, 108.0, 106.3, 97.7, 67.3, 55.1, 44.3, 12.5.

**4**

3',6'-bis(diethylamino)-2-phenyl-2-fluorospiro[isoindoline-1,9'-xanthen]-3-one (4).ii

Rhodamine B (1.02 g, 2.09 mmol, 1.0 equiv) and 2-fluoro aniline (0.60 mL, 6.30 mmol, 3.1 equiv) were dissolved in dichloromethane (20 mL). The mixture was cooled to 0 °C, then POCl$_3$ (0.23 mL, 2.5 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 20 mL dichloromethane and then washed with 1 M HCl (3 x 20 mL), 1 M NaOH (3 x 20 mL) and brine. The organic layer was collected, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate: hexane (1:3) as the eluent, affording the product as a white solid (0.96 g, 85% yield). The compound can be additionally purified by recrystallization from ethyl acetate: hexanes (1:2). $^1$H NMR (500 MHz, CDCl$_3$) 8.02 (m, 1H), 7.50–7.46 (m,2H), 7.14–7.12 (m, 1H), 7.05 (s, 1H), 6.98-6.94 (m, 2H), 6.64–6.60 (d, J = 8.0 Hz, 4H), 6.32–6.30 (d, J = 7.6 Hz, 2H), 6.21 (s, 2H), 3.30–3.26 (q, J = 7.0 Hz, 8H), 2.18 (d, J = 1.2 Hz, 3H), 1.17–1.13 (t, J = 7.0 Hz, 12H); $^{13}$C NMR (125 MHz, CDCl$_3$) 167.6, 153.2, 153.1, 148.6, 136.3, 133.7, 132.6, 131.1, 129.2, 128.9, 128.0, 127.3, 123.9, 123.3, 108.0, 106.4, 97.7, 67.2, 44.3, 21.1, 12.5.
3',6'-bis(diethylamino)-2-phenyl-4-chlorospiro[isoindoline-1,9'-xanthen]-3-one (5).²

Rhodamine B (1.01 g, 2.08 mmol, 1.0 equiv) and 3-chloro aniline (0.66 mL, 6.25 mmol, 3.0 equiv) were dissolved in dichloromethane (20 mL). The mixture was cooled to 0 °C, then POCl₃ (0.23 mL, 2.5 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 20 mL dichloromethane and then washed with 1 M HCl (3 x 20 mL), 1 M NaOH (3 x 20 mL) and brine. The organic layer was collected, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate: hexane (1:3) as the eluent, affording the product as a white solid (0.73 g, 71% yield). The compound can be additionally purified by recrystallization from ethyl acetate: hexanes (1:2).¹H NMR (500 MHz, CDCl₃) 8.02–7.99 (m, 1H), 7.50–7.46 (m, 2H), 7.15–7.12 (m, 1H), 7.08–7.06 (d, J = 7.0 Hz, 1H), 7.04–7.02 (m, 2H), 6.66–6.64 (d, J = 8.2 Hz, 4H), 6.33–6.30 (d, J = 8.6 Hz, 2H), 6.26 (s, 2H), 3.34–3.29 (q, J = 7.0 Hz, 8H), 2.21 (d, J = 1.2 Hz, 3H), 1.17–1.13 (t, J = 7.0 Hz, 12H).¹³C NMR (125 MHz, CDCl₃) 167.6, 153.2, 153.1, 148.6, 136.3, 133.7, 132.6, 131.1, 129.2, 128.9, 128.0, 127.3, 123.9, 123.3, 108.0, 106.4, 97.7, 67.2, 44.3, 21.1, 12.5.
3',6'-bis(diethylamino)-2-phenyl-3-fluorospiro[isoindoline-1,9'-xanthen]-3-one (6).²

Rhodamine B (1.02 g, 2.09 mmol, 1.0 equiv) and 3-fluoro aniline (0.61 mL, 6.33 mmol, 3.1 equiv) were dissolved in dichloromethane (20 mL). The mixture was cooled to 0 °C, then POCl₃ (0.23 mL, 2.5 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 20 mL dichloromethane and then washed with 1 M HCl (3 x 20 mL), 1 M NaOH (3 x 20 mL) and brine. The organic layer was collected, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate: hexane (1:3) as the eluent, affording the product as a white solid (0.77 g, 71% yield). The compound can be additionally purified by recrystallization from ethyl acetate: hexanes (1:2). ¹H NMR (500 MHz, CDCl₃) 8.02 (m, 1H), 7.50–7.46 (m, 2H), 7.14–7.12 (m, 1H), 7.08–7.06 (d, J= 7.0 Hz, 1H), 7.02–7.0 (m, 2H), 6.63–6.61 (d, J = 7.6 Hz, 4H), 6.33–6.30 (d, J = 8.4 Hz, 2H), 6.23 (s, 2H), 3.30–3.26 (q, J = 7.0 Hz, 8H), 2.18 (d, J = 1.2 Hz, 3H), 1.17–1.13 (t, J = 7.0 Hz, 12H). ¹³C NMR (125 MHz, CDCl₃) 167.6, 153.2,153.1, 148.6, 136.3, 133.7, 132.6, 131.1, 129.2, 128.9, 128.0,127.3, 123.9, 123.3, 108.0, 106.4, 97.7, 67.2, 44.3, 21.1, 12.5.
3',6'-bis(diethylamino)-2-phenyl-2-methylspiro[isoindoline-1,9'-xanthen]-3-one (7).ii

Rhodamine B (0.997 g, 2.07 mmol, 1.0 equiv) and 2-methyl aniline (0.61 mL, 5.54 mmol, 2.7 equiv) were dissolved in dichloromethane (20 mL). The mixture was cooled to 0 °C, then POCl₃ (0.23 mL, 2.5 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 20 mL dichloromethane and then washed with 1 M HCl (3 x 20 mL), 1 M NaOH (3 x 20 mL) and brine. The organic layer was collected, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate: hexane (1:3) as the eluent, affording the product as a white solid (0.56 g, 43% yield). The compound can be additionally purified by recrystallization from ethyl acetate:hexanes (1:2). ¹H NMR (500 MHz, CDCl₃) 8.02–7.99 (m, 1H), 7.50–7.46 (m, 2H), 7.16–7.15 (m, 1H), 6.93–6.91 (d, J = 7.0 Hz, 2H), 6.66–6.64 (d, J = 8.2 Hz, 4H), 6.33–6.30 (d, J = 8.6 Hz, 2H), 6.26 (s, 2H), 3.34–3.29 (q, J = 7.0 Hz, 8H), 2.21 (d, J = 1.2 Hz, 3H), 1.17–1.13 (t, J = 7.0 Hz, 12H). ¹³C NMR (125 MHz, CDCl₃) 167.6, 153.2,153.1, 148.6, 136.3, 133.7, 132.6, 131.1, 129.2, 128.9, 128.0,127.3, 123.9, 123.3, 108.0, 106.4, 97.7, 67.2, 44.3, 21.1, 12.5.
Rhodamine B (1.05 g, 2.10 mmol, 1.0 equiv) and 2,6-dimethyl aniline (0.78 mL, 6.32 mmol, 3.0 equiv) were dissolved in dichloromethane (20 mL). The mixture was cooled to 0 °C, then POCl₃ (0.23 mL, 2.5 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 20 mL dichloromethane and then washed with 1 M HCl (3 x 20 mL), 1 M NaOH (3 x 20 mL) and brine. The organic layer was collected, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate: hexane (1:3) as the eluent, affording the product as a white solid (0.85 g, 62% yield). The compound can be additionally purified by recrystallization from ethyl acetate: hexanes (1:2). \(^1\)H NMR (500 MHz, CDCl₃) 8.08 (d, J = 7.4 Hz, 1H), 7.70–7.62 (m, 2H), 7.42 (d, J = 7.4 Hz, 1H), 7.03 (t, J = 7.4 Hz, 1H), 6.83 (d, J = 7.4 Hz, 2H), 6.56 (d, J = 9.0 Hz, 2H), 6.28 (dd, J = 8.8 Hz, 2.6 Hz, 2H), 6.23 (d, J = 2.4 Hz, 2H), 3.31 (q, J = 7.0, 8H), 1.35 (s, 6H), 1.12 (t, J = 7.1, 12H). \(^{13}\)C NMR (125 MHz, CDCl₃) 166.0, 155.9, 149.0, 148.6, 139.0, 133.6, 132.4, 132.1, 129.4, 128.7, 127.9, 127.7, 124.8, 123.9, 109.0, 107.4, 98.4, 69.4, 44.5, 18.3, 12.4.
3',6'-bis(diethylamino)-2-phenyl-2,6-diisopropylspiro[isoindoline-1,9'-xanthen]-3-one (9).\(^{ii}\) Rhodamine B (1.01 g, 2.08 mmol, 1.0 equiv) and 2,6-diisopropyl aniline (1.11 g, 6.26 mmol, 3.0 equiv) were dissolved in dichloromethane (20 mL). The mixture was cooled to 0 °C, then POCl\(_3\) (0.23 mL, 2.5 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 20 mL dichloromethane and then washed with 1 M HCl (3 x 20 mL), 1 M NaOH (3 x 20 mL) and brine. The organic layer was collected, dried over Na\(_2\)SO\(_4\), filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate: hexane (1:3) as the eluent, affording the product as a white solid (0.66 g, 53% yield). The compound can be additionally purified by recrystallization from ethyl acetate: hexanes (1:2). \(^1\)H NMR (500 MHz, CDCl\(_3\)) 8.08 (d, J = 7.4 Hz, 1H), 7.68-7.66 (m, 2H), 7.42 (d, J = 7.0 Hz, 1H), 7.02 (t, J = 7.0 Hz, 1H), 6.80 (d, J = 7.2 Hz, 2H), 6.56 (d, J = 9.0 Hz, 2H), 6.28 (d d, J = 9.0 Hz, 2.6 Hz, 2H), 6.23 (d, J = 2.8 Hz, 2H), 3.31 (q, J = 7.0, 8H), 1.72 (s, 12H), 1.12 (t, J = 7.1, 12H). \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) 166.0, 155.9, 149.0, 148.6, 139.0, 133.6, 132.4, 132.1, 129.4, 128.7, 127.9, 127.7, 124.8, 123.9, 109.0, 107.4, 98.4, 69.4, 44.5, 18.3, 12.4.
3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl bis(trifluoromethanesulfonate).

Fluorescein (2.79 g, 8.42 mmol, 1.0 equiv) and bis(trifluoromethanesulfonyl) aniline (7.52 g, 21.1 mmol, 2.5 equiv) were dissolved in dichloromethane (20 mL). Then diisopropylethylamine (4.31 mL, 25.2 mmol, 3.0 equiv) was added into the mixture. The reaction mixture was allowed to stir at 25 °C for 18 h. The reaction was monitored by TLC. Upon completion, the mixture was diluted with 20 mL dichloromethane and then washed with 1 M HCl (3 x 20 mL), 1 M NaHCO₃ (3 x 20 mL) and brine. The organic layer was collected, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with dichloromethane:methanol (20:1) as the eluent, affording the product as a light yellow solid (3.56 g, 71% yield). ¹H NMR (500 MHz, CDCl₃) 8.05 (d, J = 7.0 Hz, 1H), 7.73-7.69 (m, 2H), 7.29 (s, 2H), 7.19 (d, J = 6.6 Hz, 1H), 7.01-6.97 (m, 4H). ¹³C NMR (125 MHz, CDCl₃) 168.7, 152.1, 151.4, 150.2, 131.0, 130.0, 125.8, 123.7, 119.3, 117.7, 110.7, 80.1.
**3',6'-bis((2,2,2-trifluoroethyl)amino)-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one.** The pure bis-triflate product (2.98 g, 5.04 mmol, 1 equiv) was added into a 50 mL pressure flask with Pd₂dba₃ (462 mg, 0.504 mmol, 0.1 equiv), XPhos (720 mg, 1.51 mmol, 0.3 equiv) and Cs₂CO₃ (4.60 g, 14.1 mmol, 2.8 equiv). The flask was vacuumed and filled with N₂ for 10 min. Then, the mixture was dissolved in 20 mL dioxane and stirred for 15 min under N₂. 2,2,2-trifluoroethylamine (0.92 mL, 12.9 mmol, 2.4 equiv) was added into the mixture by dropwise addition. The flask was capped and heated at 100 °C for 20 h. The mixture was then transferred into round bottle and evaporated all the solvent. The residue was poured into brine (50 mL), 1 M HCl (10 mL) was added, and the mixture was extracted with ethyl acetate (3 × 25 mL). The organic layer was collected, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with dichloromethane: methanol (30:1) as the eluent, affording the product as a red solid (1.59 g, 64% yield). ¹H NMR (500 MHz, CDCl₃) 7.99 (m, 1H), 7.65 (m, 1H), 7.59 (m, 1H), 7.17 (d, 1H), 6.58 (s, 2H), 6.51 (m, 2H), 6.36 (t, J = 8.3 Hz, 2H), 4.23 (s, 2H), 3.78 (m, 4H). ¹³C NMR (125 MHz, CDCl₃) 167.7, 154.8, 154.0, 133.3, 131.9, 130.6, 128.7, 123.9, 108.7, 98.1, 69.3, 44.6, 37.9, 30.0, 14.9, 12.6. HRMS calculated for C₂₄H₁₆N₂O₃F₆ [M+H]+ 495.1121, found 495.1119.

![Chemical Structure](image)

**2-phenyl-3',6'-bis((2,2,2-trifluoroethyl)amino)spiro[isoindoline-1,9'-xanthen]-3-one (10).**
Rhodamine-CF3 (305 mg, 0.607 mmol, 1.0 equiv) and aniline (0.17 mL, 1.82 mmol, 3.0 equiv) were dissolved in dichloromethane (10 mL). The mixture was cooled to 0 °C, then POCl₃ (0.07 mL, 0.73 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 10 mL dichloromethane and then washed with 1 M HCl (3 x 10 mL), 1 M NaOH (3 x 10 mL) and brine. The organic layer was collected, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate:hexane (1:4) as the eluent, affording the product as a white solid (141.2 mg, 40% yield). The compound can be additionally purified by recrystallization from ethyl acetate: hexanes (1:2).

**1H NMR** (500 MHz, CDCl₃) 8.01 (d, J = 7.1 Hz, 1H), 7.54–7.50 (m, 2H), 7.14–7.08 (m, 4H), 6.54 (d, J = 8.0 Hz, 4H), 6.30 (d, J = 8.6 Hz, 4H), 3.73 (q, J = 6.6 Hz, 4H).

**13C NMR** (125 MHz, CDCl₃) 167.6, 152.7, 147.0, 135.9, 132.9, 131.3, 129.1, 127.3, 126.2, 123.7, 110.3, 99.3, 67.1, 45.9, 29.7.

HRMS calculated for C₃₀H₂₁N₃O₂F₆ [M+H]⁺ 570.1533, found 570.1531.

![Structure](image_url)

**11**

2-(2-fluorophenyl)-3',6'-bis((2,2,2-trifluoroethyl)amino)spiro[isoindoline-1,9'-xanthen]-3-one (11). Rhodamine-CF3 (311 mg, 0.607 mmol, 1.0 equiv) and 2-fluoro aniline (0.18 mL, 1.83 mmol, 2.9 equiv) were dissolved in dichloromethane (10 mL). The mixture was cooled to 0 °C, then POCl₃ (0.07 mL, 0.73 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted
with 10 mL dichloromethane and then washed with 1 M HCl (3 x 10 mL), 1 M NaOH (3 x 10 mL) and brine. The organic layer was collected, dried over Na2SO4, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate:hexane (1:2) as the eluent, affording the product as a white solid (221.4 mg, 62% yield).

The compound can be additionally purified by recrystallization from ethyl acetate: hexanes (1:2).

1H NMR (500 MHz, CDCl3) 8.01−8.05 (m, 1H), 7.54 (t, J = 4.9 Hz, 2H), 7.12−7.16 (m, 2H), 6.77 (t, J = 10.3 Hz, 1H), 6.61 (d, J = 8.6 Hz, 2H), 6.57 (m, 2H), 6.31 (dd, J = 2.3, 8.6 Hz, 4H), 4.12 (t, J = 8.8 Hz, 2H), 3.73 (p, J = 13.3 Hz, 4H).

13C NMR (125 MHz, CDCl3) 166.8, 159.6, 152.9, 152.6, 147.6, 133.1, 130.9, 129.8, 129.5, 129.0, 128.7, 125.9, 124.0, 123.9, 123.7, 116.4, 116.2, 110.2, 109.6, 99.0, 66.9, 53.4, 45.9, 45.6, 29.7. HRMS calculated for C30H20N3O2F7 [M+H]+ 588.15, found 588.15.

![Chemical Structure](image)

2-(2,6-diisopropylphenyl)-3',6'-bis((2,2,2-trifluoroethyl)amino)spiro[isoindoline-1,9'-xanthen]-3-one (12). Rhodamine-CF3 (152 mg, 0.307 mmol, 1.0 equiv) and 2,6-diisopropyl aniline (0.16 g, 0.906 mmol, 2.9 equiv) were dissolved in dichloromethane (10 mL). The mixture was cooled to 0 °C, then POCl3 (0.04 mL, 0.36 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 10 mL dichloromethane and then washed with 1 M HCl (3 x 10 mL), 1 M NaOH (3 x 10 mL) and brine. The organic layer was collected, dried over Na2SO4, filtered,
and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate: hexane (1:3) as the eluent, affording the product as a white solid (120.1 mg, 62% yield). The compound can be additionally purified by recrystallization from ethyl acetate: hexanes (1:2). $^1$H NMR (500 MHz, CDCl$_3$) 8.06 (d, J = 6.9 Hz, 1H), 7.63 (m, 1H), 7.03 (d, J = 7.6 Hz, 2H), 6.97 (m, 2H), 6.82 (m, 2H), 6.59–6.57 (m, 1H), 6.33 (d, J = 6.8 Hz, 2H), 6.26 (d, J = 5.4 Hz, 2H), 3.72 (m, 4H), 2.30 (m, 2H), 1.16 (s, 2H), 0.88 (d, J = 6.9 Hz, 6H), 0.41 (d, J = 6.3 Hz, 6H). $^{13}$C NMR (125 MHz, CDCl$_3$) 167.6, 157.0, 150.3, 133.8, 130.3, 129.7, 127.4, 123.6, 123.2, 112.0, 110.3, 100.2, 70.0, 45.4, 28.6, 21.4. HRMS calculated for C$_{36}$H$_{33}$N$_3$O$_2$F$_6$ [M+H]$^+$ 654.2550, found 654.2548.

![Image of the molecule](image_url)

**13**

2-(o-tolyl)-3',6'-bis((2,2,2-trifluoroethyl)amino)spiro[isoindoline-1,9'-xanthen]-3-one (13). Rhodamine-CF$_3$ (201.5 mg, 0.40 mmol, 1.0 equiv) and o-toluidine (0.13 mL, 1.21 mmol, 3.0 equiv) were dissolved in dichloromethane (10 mL). The mixture was cooled to 0 °C, then POCl$_3$ (0.04 mL, 0.48 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 10 mL dichloromethane and then washed with 1 M HCl (3 x 10 mL), 1 M NaOH (3 x 10 mL) and then washed with brine. The organic layer was collected, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate: hexane (1:4) as the eluent, affording the product as a white
solid (134 mg, 37% yield). The compound can be additionally purified by recrystallization from ethyl acetate: hexanes (1:2). $^1$H NMR (500 MHz, CDCl$_3$) 8.01 (d, $J = 7.1$ Hz, 1H), 7.54–7.50 (m, 2H), 7.14–7.08 (m, 4H), 6.62 (q, $J = 8.5$ Hz, 6H), 6.30 (d, $J = 8.0$ Hz, 2H), 6.27 (s, 2H), 3.73 (q, $J = 6.6$ Hz, 4H). $^{13}$C NMR (125 MHz, CDCl$_3$) 165.9, 154.2, 153.7, 152.9, 150.9, 147.8, 138.1, 133.7, 132.5, 130.6, 130.1, 129.1, 128.2, 125.7, 123.6, 116.4, 111.5, 110.6, 109.1, 99.5, 67.4, 45.6, 18.2. HRMS calculated for C$_{31}$H$_{28}$N$_3$O$_2$F$_6$ [M+H]$^+$ 584.1783, found 584.1767.

![Chemical Structure](image)

14

2-(3,4-dimethoxyphenyl)-3',6'-bis((2,2,2-trifluoroethyl)amino)spiro[isoindoline-1,9'-xanthen]-3-one (14). Rhodamine-CF3 (201.7 mg, 0.40 mmol, 1.0 equiv) and 3,4-dimethoxy aniline (184 mg, 1.21 mmol, 3.0 equiv) were dissolved in dichloromethane (10 mL). The mixture was cooled to 0 °C, then POCl$_3$ (0.04 mL, 0.48 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 20 mL dichloromethane and then washed with 1 M HCl (3 x 10 mL), 1 M NaOH (3 x 10 mL) and brine. The organic layer was collected, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate:hexane (1:3) as the eluent, affording the product as a white solid (382 mg, 75% yield). The compound can be additionally purified by recrystallization from ethyl acetate: hexanes (1:2). $^1$H NMR (500 MHz, CDCl$_3$) 8.02 (d, $J = 7.1$ Hz, 1H), 7.57–7.55 (m,
2H), 7.17–7.15 (m, 1H), 6.65 (q, J = 7.8 Hz, 2H), 6.59 (d, J = 6.6 Hz, 1H), 6.34 (d, J = 8.0 Hz, 2H), 6.29 (s, 2H), 6.21 (m, 1H), 6.04 (s, 1H), 3.79 (q, J = 6.6 Hz, 4H), 3.45 (s, 6H). $^{13}$C NMR (125 MHz, CDCl$_3$) 167.3, 153.7, 151.7, 147.9, 147.5, 132.9, 131.4, 129.3, 128.2, 124.1, 123.5, 120.7, 110.9, 109.9, 99.2, 67.0, 55.8, 45.6. HRMS calculated for C$_{32}$H$_{25}$N$_3$O$_4$F$_6$ [M+H]$^+$ 630.1824, found 630.1822.

2-(4-methoxyphenyl)-3',6'-bis((2,2,2-trifluoroethyl)amino)spiro[isoindoline-1,9'-xanthen]-3-one (15). Rhodamine-CF3 (203 mg, 0.41 mmol, 1.0 equiv) and 4-methoxy aniline (0.10 mL, 1.23 mmol, 3.0 equiv) were dissolved in dichloromethane (10 mL). The mixture was cooled to 0 °C, then POCl$_3$ (0.04 mL, 0.50 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 10 mL dichloromethane and then washed with 1 M HCl (3 x 10 mL), 1 M NaOH (3 x 10 mL) and brine. The organic layer was collected, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate:hexane (1:3) as the eluent, affording the product as a white solid (158 mg, 45% yield). The compound can be additionally purified by recrystallization from ethyl acetate: hexanes (1:2).

$^1$H NMR (500 MHz, CDCl$_3$) 8.01 (d, J = 7.1 Hz, 1H), 7.54–7.50 (m, 2H), 7.14–7.08 (m, 4H), 6.62 (q, J = 8.5 Hz, 6H), 6.30 (d, J = 8.0 Hz, 2H), 6.27 (s, 2H), 3.73 (q, J = 6.6 Hz, 4H), 3.68 (s, 3H). $^{13}$C NMR (125 MHz, CDCl$_3$) 166.9, 159.2, 153.9, 152.9, 147.8, 133.1, 131.0, 129.3, 128.2,
126.6, 124.1, 123.7, 116.4, 115.6, 114.1, 110.2, 98.7, 67.3, 55.4, 45.6. HRMS calculated for C$_{31}$H$_{23}$N$_3$O$_3$F$_6$ [M+H]$^+$ 600.1725, found 600.1716.

![Chemical Structure](image)

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2-(2,6-dimethylphenyl)-3',6'-bis((2,2,2-trifluoroethyl)amino)spiro[isoindoline-1,9'-xanthen]-3-one (16). Rhodamine-CF$_3$ (302.4 mg, 0.61 mmol, 1.0 equiv) and 2,6-dimethyl aniline (0.22 mL, 1.82 mmol, 3.0 equiv) were dissolved in dichloromethane (10 mL). The mixture was cooled to 0 °C, then POCl$_3$ (0.07 mL, 0.73 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 10 mL dichloromethane and then washed with 1 M HCl (3 x 10 mL), 1 M NaOH (3 x 10 mL) and brine. The organic layer was collected, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate: hexane (1:4) as the eluent, affording the product as a white solid (241 mg, 67% yield).

The compound can be additionally purified by recrystallization from ethyl acetate: hexanes (1:2). $^1$H NMR (500 MHz, CDCl$_3$) 8.06 (d, J = 7.4 Hz, 1H), 7.66–7.62 (m, 2H), 7.37 (m, 1H), 7.02–6.96 (m, 2H), 6.82 (q, J = 8.4 Hz, 2H), 6.57 (d, J = 6.6 Hz, 2H), 6.28 (d, J = 8.0 Hz, 4H), 4.08 (q, J = 6.8 Hz, 2H), 3.73 (q, J = 6.6 Hz, 4H), 2.09 (s, 6H). $^{13}$C NMR (125 MHz, CDCl$_3$) 167.1, 152.2, 147.8, 138.1, 133.7, 132.5, 130.6, 130.1, 129.1, 128.2, 125.7, 123.6, 116.4, 111.5, 110.6, 109.1, 99.5, 67.4, 45.6, 18.2. HRMS calculated for C$_{32}$H$_{25}$N$_3$O$_3$F$_6$ [M+H]$^+$ 598.1944, found 598.1935.
2-(3-chlorophenyl)-3',6'-bis((2,2,2-trifluoroethyl)amino)spiro[isoindoline-1,9'-xanthen]-3-one (17). Rhodamine-CF3 (172 mg, 0.347 mmol, 1.0 equiv) and 3-chloro aniline (0.10 mL, 0.86 mmol, 2.5 equiv) were dissolved in dichloromethane (10 mL). The mixture was cooled to 0 °C, then POCl₃ (0.04 mL, 0.34 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 10 mL dichloromethane and then washed with 1 M HCl (3 x 10 mL), 1 M NaOH (3 x 10 mL) and brine. The organic layer was collected, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate: hexane (1:2) as the eluent, affording the product as a white solid (117 mg, 68% yield). The compound can be additionally purified by recrystallization from ethyl acetate: hexanes (1:2).

H NMR (500 MHz, CDCl₃) 8.00 (d, J = 6.8 Hz, 1H), 7.54–7.50 (m, 2H), 7.11–7.02 (m, 3H), 6.81 (s, 1H), 6.60 (q, J = 8.3 Hz, 3H), 6.33 (d, J = 8.2 Hz, 4H), 4.1 (m, 2H), 3.75 (q, J = 6.6 Hz, 4H). C NMR (125 MHz, CDCl₃) 167.3, 147.6, 137.6, 134.1, 133.2, 130.6, 130.1, 129.7, 128.9, 126.7, 125.8, 124.8, 110.6, 109.7, 99.0, 67.2, 45.9. HRMS calculated for C₃₀H₂₀N₃O₂F₆Cl [M+H]⁺ 604.1221, found 604.1221.
1.4.3 Extinction coefficient measurements of on-state and off-state

1.4.3.1 Off-state measurements ($\varepsilon_{254\text{nm}(\text{off})}$)

A 0.5 mM stock solution of recrystallized photoswitch was prepared in dichloromethane and diluted to 1.0 mL solutions of five different concentrations between from 2 µM and 20 µM of each photoswitch. 300 µL of each solution was transferred into a cuvette and absorbance scans were acquired for each concentration. The concentrations were selected to ensure that absorbances at 254 nm ranged between 0.10 and 1.00 absorbance units. Plots of photoswitch concentration versus absorbance at 254 nm were generated and extinction coefficients were calculated as the slope of the linear plot based on the Beer-Lambert law:

$$\varepsilon = \frac{A}{c \times l}$$

where $\varepsilon$ is the extinction coefficient, $A$ is the absorbance of samples at 254 nm, $c$ is the concentration of the photoswitch, and $l$ is the pathlength of the cuvette. All values are the average of $n = 3$ independent experiments ± standard deviation.

1.4.3.2 On-state measurements ($\varepsilon_{495\text{nm}(\text{on})}$, $\varepsilon_{556\text{nm}(\text{on})}$)

0.5 mM stock solutions of every SRB derivative and 25 mM stock solution of trifluoroacetic acid was prepared in dichloromethane, which were diluted with final concentrations between 1 µM and 30 µM of the photoswitches containing 50 equivalents of trifluoroacetic acid. The solutions were capped and put into dark condition for 40 minutes to drive the photoswitches to the fully fluorescent on-form. After incubation, 300 µL of the sample was transferred to a cuvette and UV/Vis absorption spectra were obtained for each compound. The extinction coefficient was determined according to the slope of the calibration curve of concentration/absorbance at 556 nm for the SRB derivatives based on Lamber-Beer's law. For the SRF3 probes, A similar protocol
was used except using a 250 mM stock solution of trifluoroacetic acid for using with the SRCF3 derivatives.

### 1.4.4 Fluorescence quantum yield (Φₙ) measurements

Based on the TFA adding experiments, fluorescence quantum yields of SRB derivatives were determined with the addition of 50 equivalents of trifluoroacetic acid and SRCF3 derivatives were determined with the addition of 500 equivalents of trifluoroacetic acid, both of them were using rhodamine B as a standard. Five concentration spots of the standard rhodamine B were prepared in ethanol that make sure all of the solution performed absorbance values between 0.01 and 0.1. A same method was used to prepare the SRB and SRCF3 derivatives solutions with measuring the absorbance and exciting at 556 nm for the SRB derivatives and the absorbance and exciting at 495 nm for the SRCF3 derivatives.

The fluorescence quantum yield was determined by using equation 1, where G is the slope of the calibration curve of fluorescence emission intensity/absorbance, Gₘₘ is the slope of the same plot for the rhodamine b, η is the index of refraction for the sample solutions (dichloromethane=1.42), ηₘₘ is the index of refraction of the standard solution (ethanol= 1.36). Φₙ is the fluorescence quantum yield of the sample and Φₘₘ is the fluorescence quantum yield of the standard.

\[ Φₙ = Φₘₘ \times \frac{G}{Gₘₘ} \times \frac{η^2}{ηₘₘ} \] (1)

### 1.4.5 Determination of kₖoff, kₖon, and Kₖeq

Besides that, SRB and SRCF3 samples were prepared at 5 µM in dichloromethane in a quartz cuvette and placed in a Hitachi F-7000 fluorescence spectrophotometer for measuring the kinetics. For the SRB derivatives, the following parameters were used: \( λ_{ex} = 254 \text{ nm}, \lambda_{em} = 585 \)
nm, excitation slits = 5 nm, emission slits = 2.5 nm and for the SRF3 derivatives, the following parameters were used: \( \lambda_{\text{ex}} = 254 \text{ nm}, \lambda_{\text{em}} = 518 \text{ nm}, \) excitation slits = 5 nm, emission slits = 5 nm. Time scans were performed with continuous excitation at 254 nm until an equilibrium was reached (around 30 min). After that, the curves were fit to equation 2 for the first-order reaction at equilibrium, where \( I_{\text{fl}} \) is the fluorescence emission intensity, \( I_{\text{max}} \) is the theoretical maximum emission on complete turn-on state of sample, \( k_{\text{on}} \) is the rate constant for the photoactivation process, \( k_{\text{off}} \) is the rate constant for the photoactivation process, B is a parameter to account for background signal. Fitting result was performed using Mathematica 12.0 and values for \( I_{\text{max}} \) were estimated by measuring the fluorescence emission with addition of excess trifluoroacetic acid, which were used to confirm the value of \( I_{\text{max}} \). This constraint could provide consistent values of \( k_{\text{on}} \) and \( k_{\text{off}} \). All values are the average of \( n = 3 \) independent experiments ± standard deviation. 

\[ K_{\text{eq}} = \frac{k_{\text{on}}}{k_{\text{off}}} \]  

(2)

1.4.6 Determination of the Photochemical Quantum Yield for Photoswitching to the On-State, \( \Phi_{\text{pc}} \)

The photochemical quantum yields were determined using equation 3, where \( k_{\text{on}} \) is the rate of photochemical reaction determined by equation 2, \( \varepsilon_{254\text{nm}} \) is the extinction coefficient of the off-state at 254 nm, \( V \) is the total volume of the testing sample, \( I_0 \) is the intensity of the excitation light at 254 nm (50.8 \( \mu \)W cm\(^{-2} \), measured with a Digital Light Meter, ThorLabs PM100D), and \( l \) is the path length of the cuvette (1 cm). All values are the average of \( n = 3 \) independent experiments ± standard deviation.
\[ \Phi_{pc} = \frac{k_{on} V}{k_0 \epsilon_{254 \text{nm}} \ln 10} \]  \hspace{1cm} (3)

1.5 Conclusions

In summary, we have successfully synthesized two series of fluorescent spirolactam rhodamine photoswitches SRB and SRCF3 photoswitches with fluorescence emission in the red (585 nm) or green (518 nm) of the spectrum. Then we obtained their important measurements such as extinction coefficients, fluorescence and photochemical quantum yields, and kinetics of photoactivation. In addition, we reported a novel synthetic method to synthesize SRCF3 photoswitches and using two different methods to evaluate \( k_{on} \) and consequently quantum yields \( \Phi_{pc} \), one of which was depended on the constraints for the \( I_{\text{max}} \) parameter. The other one related with the maximum emission intensity of the photoswitch on its fully open form. At the same time, we performed experiments using excess trifluoroacetic acid to drive the photoreaction equilibriums to the fully on-state. Finally, volumetric 3D imaging is demonstrated in dichloromethane with triethylamine addition by using DLP technology, that provides a valuable example for exploring other applications like 3D printing.
1.6 References


CHAPTER 2
CHEMILUMINESCENT PROBES FOR CELLULAR HYDROGEN SULFIDE DETECTION

2.1 Introduction

Reactive sulfur and nitrogen species like hydrogen sulfide and nitric oxide are cellular signaling molecules which play important roles in physiology and medical science.\textsuperscript{1,2,3} Upon their chemical reactions like oxidation and reduction, the detection and measurements of sulfur and nitrogen species have been developed. Different with fluorescent probes, chemiluminescent probes can detect specific species by chemiluminescent reactions that generate the light from unique mechanisms without any light irradiation.\textsuperscript{4} In recent years, chemiluminescence probes are widely used in detecting and measuring some biological reactive species like hydrogen sulfide in \textit{in vitro} and \textit{in vivo} conditions.\textsuperscript{6,7} In this dissertation, we mainly discuss the 1,2-dioxetane chemiluminescent probes for detecting, measuring, and imaging reactive species \textit{in vivo} and \textit{in vitro}. We do the synthesis and measurements of \textbf{CHS-4} and \textbf{CL-DNP} probes for hydrogen sulfide. After doing the selectivity and kinetics tests of them \textit{in vitro}, \textit{in vivo} studies for monitoring and imaging reactive sulfur and oxygen species in A549 cells are explored to demonstrate that chemiluminescence holds great potential for \textit{in vivo} imaging.

2.1.1 Chemiluminescence

Chemiluminescence is observed easily in our daily life in various formats, such as glow sticks, bioluminescent bays, fireflies and so on.\textsuperscript{8,9} Chemiluminescence was first defined by Eilhard
Wiedemann in 1888, which could be described as light emission from a chemical reaction.\textsuperscript{10} With more understanding of chemiluminescence, the mechanisms of chemiluminescence light generation has been understood more clearly which provides us more useful applications in biochemistry and medical areas.

Chemiluminescence is the light generated from a thermal reaction that is observed when the product in an excited state relaxes to its ground state with emission of photons. It is normally defined as a reaction that produces the energy to achieve the transition of an electron from its ground state to an excited electronic state in chemiluminescent reaction.\textsuperscript{11} In chemistry and biology research, chemiluminescence applications have various potential advantages such as high sensitivity, wide linear range, simple and inexpensive instrumentation compared with traditional fluorescence methods.\textsuperscript{12} The most important advantage of chemiluminescence is that low background noise is produced from the elimination of an external light source, which could improve sensitivity in large extent and provide more opportunities for \textit{in vivo} imaging.

\textbf{Figure 2-11} Potential energy diagrams. (A) an exothermic reaction (B) a chemiluminescent reaction. (S = starting material, I = reactive intermediate or transition state, P = product).
2.1.2 Reactive sulfur and nitrogen species

Reactive sulfur and nitrogen species like hydrogen sulfide and nitric oxide as representative signaling molecules in medical area\textsuperscript{13} participate in various important activities \textit{in vivo}. How to complete accurate measurement of these species becomes a meaningful work, which need us to obtain a deep understanding of their chemical and biological characterizations.

Hydrogen sulfide (H\textsubscript{2}S) as a representative chemical of reactive species, has been regarded as a toxic gas to physiology systems for a long time, that could cause various diseases such as cancer, Down’s syndrome and diabetes.\textsuperscript{14,15,16} However, accumulating evidence performs that except being a harmful chemical and environmental pollutant, H\textsubscript{2}S also plays and important role in mammalian systems,\textsuperscript{17} including some important biological functions like protecting against cardiovascular disease and promoting vasorelaxation in animal’s body.\textsuperscript{18,19} As more studies about hydrogen sulfide generation, there are two main pathways of H\textsubscript{2}S production in physiological conditions. One is described as non-enzymatic processes, which can be directly generated from some sulfur containing species such as glucose, glutathione, inorganic and organic polysulfides,\textsuperscript{20} the other is enzymatic processes with cystathionine gamma lyase (CSE)\textsuperscript{21}, cystathionine beta synthase (CBS)\textsuperscript{22} and 3-mercaptopyruvate sulfurtransferase (3-MST)\textsuperscript{23} that have been reported in some human organs. Therefore, H\textsubscript{2}S has been found that it has large influence in most tissues of living systems, such as vasculature, brain, lungs, liver, and pancreas in living systems.\textsuperscript{59,60,61,62}
As a signaling nitrogen reactive molecule, nitric oxide was firstly discovered that it could achieve the activation of cyclase and help muscle cells complete relaxation in 1998. Similar with hydrogen sulfide, some results are reported that nitric oxide could be produced in physiological conditions which is generated from three isoforms of the nitric oxide synthase (NOS) enzyme - neuronal NOS (nNOS, NOS-1), inducible NOS (iNOS, NOS-2), and endothelial NOS (eNOS, NOS-3), all of them use L-arginine as precursor. The bacterial nitrate reduction in the microbiome is discovered, which could also produce nitric oxide by nitrite reduction. According to these studies, nitric oxide plays an important and central roles in the cardiovascular system as a small, diffusible, and reactive molecule. Additionally, nitric oxide can also participate in cellular signaling, that use small molecules as reactive nitrogen species donors, including peroxynitrite and decomposition products, nitroxyl, and nitrite/nitrate.
Scheme 2-5 The nitric oxide synthase (NOS) reactions with L-Arginine.

2.1.3 Chemiluminescent 1,2-dioxetane probes for reactive sulfur and nitrogen species

Except the traditional detecting methods for reactive sulfur and nitrogen species like HPLC and methylene blue assay, reaction-based fluorescent probes have received enormous attention over past decade, which is available to detect target molecule in live cells with higher emission intensity and greater selectivity. These reaction-based methods take advantage of the chemical reactivity of H$_2$S, including nucleophilicity, metal precipitation and reductivity. (Scheme 2-6) However, there are some drawbacks of fluorescence imaging during in vivo measurements, such as light scattering, phototoxicity, which affect the probe kinetics and sensitivity. Compared with it, a reaction-based chemiluminescent methods for reactive species could provide a valuable choice to solve these problems. Several chemiluminescent systems have been reported for reactive sulfur and nitrogen species in physiology, including luciferin/luciferase, luminol, acridinium esters, peroxoxyxalates, and 1,2-dioxetanes. In this dissertation, we mainly discuss the 1,2-dioxetane triggered chemiluminescent probes for reactive sulfur and nitrogen molecules. Compared with other chemiluminescent probes, spiroadamantane 1,2-dioxetanes could achieve the CIEEL mechanisms more stably as it is easily forming the “solvent cage”
structure as protecting group and steric hinderance group of this structure could reduce the proton activity of phenyl which improves the selectivity and sensitivity of chemiluminescence generation. In recent years, with more studies about 1,2-dioxetane mechanisms, this class of chemiluminescent probes was realized that it could be used for cell studies and \textit{in vivo} imaging. Furthermore, several new chemiluminescent probes have now been developed, including chemiluminescent 1,2-dioxetane probes for reactive sulfur and nitrogen species.

In earlier years, some groups reported a novel dioxetane probe with dinitrophenyl group of detecting H$_2$S, that could produce the dioxetane phenol with nucleophilic aromatic substitution reaction finally$^{46}$. In addition, Baader reported that electron donating groups with the dioxetane compound could obviously increase the chemiluminescent intensity$^{48}$, that was further demonstrated by Schaap and Gagnon.$^{49,50}$ According to this result, another group indicated that chemiluminescence emission was significantly increased with the phenolic proton elimination and intramolecular electron transfer from an electron rich group, such as a phenolate$^{50}$. It was found that the excited species could be produced by a back-electron transfer step, which released the light emission in this process. In addition, the other energy transfer method of this excited intermediate could be achieved, for example fluorescent dye could be regarded as energy acceptor, which results in a fluorescent emission. The detailed process has been outlined with the example of spiroadamantane-1,2-dioxetane$^{47}$.(\textbf{Scheme 2-7}) Although many recent advances have been obtained about 1,2 dioxetanes mechanistic studies, the exact mechanism of light emission has not yet been well understood.
Scheme 2-6 Examples of reaction-based detection strategies for hydrogen sulfide fluorescent scaffolds.
Figure 2-12 Some examples of 1,2-dioxetane chemiluminescent probes. (A) 1,2-dioxetane H$_2$S probes. (B) 1,2-dioxetane ONOO$^\cdot$ probes. (C) 1,2-dioxetane HNO probe.
Scheme 2-7 The catalyzed intramolecular decomposition process of spiro adamantane-1,2-dioxetane.

2.1.4 Triggered 1,2-dioxetane chemiluminescent probes

Triggered 1,2-dioxetanes chemiluminescent probes were firstly reported by Schaap and his coworkers in 1987\textsuperscript{49,50}. Later, the same group developed some other adamantane 1,2-dioxetane derivatives containing masked phenolate groups and they found that chemiluminescence emission can be triggered from the method of specific chemical deprotection of the phenolate masking group. For example, chemiluminescence could be produced by converting tetra-butyl ammonium fluoride to the tert-butylsilyl from a phenolate\textsuperscript{51}. Later, the same group reported other masking groups, such as acetate and phosphate\textsuperscript{52}. These strategies of the phenolate chemical decomposition provide the evidence of basic conception that efficient chemiluminescence could be performed by using target molecule triggering the chemiluminescent probes with high specificity.
In our previous research, we successfully synthesized and characterized adamantane 1,2-dioxetane chemiluminescent probes CHS-1, CHS-2, and CHS-3, that used an azide trigger strategy for the chemiluminescence detection of H$_2$S$^{53}$. The mechanism of this H$_2$S mediated azide reduction has been reported by the Pluth group$^{54}$. This reaction is regarded as a first order reaction for both reactants and hydrogen sulfide. Then chemiluminescence emission is triggered upon the decomposition of this phenolate through CIEEL mechanism as **Scheme 2-9**.

**Scheme 2-8** 1,2-Dioxetane derivatives with masked phenolate groups (MG = masking group).

**Scheme 2-9** Chemically Induced Electron Exchange Luminescence (CIEEL) mechanisms of spiroadamantane-1,2-dioxetane.
Scheme 2-10 Mechanisms of spiro adamantane 1,2-dioxetanes (CHS-1, CHS-2, and CHS-3) for H₂S detection. (Adapted with permission from reference 53. Copyright 2015 RSC)

2.1.5 In vivo imaging

Based on the properties of reactive hydrogen and nitrogen species in previous research, traditional methods for reactive hydrogen and nitrogen species detection are difficult to achieve the real-time in vivo measurement with high sensitivity, including the methylene blue assay, high performance liquid chromatography, mass spectrometry, electrochemical methods and so on\(^{55,56}\). Especially, many advanced reports of reaction-based chemiluminescent probes for H₂S detection have been performed in recent years, which enables us to detect H₂S in living organisms with higher sensitivity and selectivity.

As the advantages of chemiluminescence imaging, reaction-based chemiluminescent platform could avoid some problems in fluorescence imaging, including autofluorescence, light scattering and phototoxicity. Direct light generation from chemiluminescent molecules without any external light source provides us with some unique advantages such as signaling the production
and intensity of the chemiluminescence emission. It is available for us to determine the presence and concentration of the reactive species more accurately and provides a brilliant sensitive and selective performance. Many reports about fluorescent and bioluminescent probes for living animal imaging\textsuperscript{57,58} indicated that chemiluminescent probes also have widespread potential value for \textit{in vivo} imaging.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2-13.png}
\caption{Imaging H\textsubscript{2}S in SCID/BALB-C mouse carcasses using CHS-3. (Adapted with permission from reference 53. Copyright 2015 RSC)}
\end{figure}
2.2 Research Objectives

In this project, according to the previous study about adamantane 1,2-dioxetane chemiluminescent probes for H₂S (CHS-1, CHS-2 and CHS-3), we designed and synthesized two new chemiluminescent probes CHS-4 and CL-DNP and completed the measurements of selectivity and sensitivity for in vitro and in vivo conditions. The purpose of new probes synthesis in this project is to improve the quantum yield and selectivity in aqueous solution compared with other CHS probes we made. Furthermore, that could help us understand the CIEEL mechanisms clearly and demonstrate how much influence of probes with different trigger and linker have. In this project, we reported that new probes made some excited performances in imaging process with different substituted groups of trigger and linker. Also, we explored that the experimental conditions would significantly affect the measurements results for H₂S probes.

2.3 Results and Discussion

2.3.1 Synthesis of Chemiluminescent Hydrogen Sulfide probes CHS-4 and CL-DNP
**Scheme 2-11** Synthesis of probe precursor adamantane acrylonitrile phenol.

The synthesis of this precursor started with 2-chloro-3-methoxy benzaldehyde, that was reacted with trimethyl orthoformate and p-toluenesulfonic acid in MeOH. The product compound 2 was reacted with triethyl phosphite and boron trifluoride diethyl etherate in DCM to obtain the diethyl methoxy phosphonate compound 3. Next step, n-butyllithium and 2-adamantanone was added to compound 3 in anhydrous THF to yield the enol ether product compound 4. An substitution reaction was obtained by reacting compound 4 with sodium ethanethiolate and cesium carbonate in anhydrous DMF. The product 5 was treated with N-iodosuccidimide (NIS) in toluene to yield iodophenol product (compound 6). Compound 6 was then reacted with addition of methyl acrylate, tri(o-toly)-phosphine, palladium (II) acetate by Heck reaction, which produce the important probe precursor compound 7, that was used for combining with multiple group triggers for detecting H\(_2\)S in the future.
Scheme 2-12 Synthesis of CHS-4.

For doing the synthesis of CHS-4, we firstly synthesized the hydrogen sulfide azide trigger reagents. The procedure started with 4-aminobenzyl alcohol (compound 8), which was first reacted with sodium nitrite and sodium azide in HCl to yield compound 9. Then compound 10 was obtained by adding p-toluenesulfonyl chloride and sodium hydroxide in DCM to compound 9. After obtaining this azide trigger product, there was two steps from compound 6 for completing the synthesis of CHS-4. First, the hydrogen sulfide azide trigger, compound 10, was reacted with adamantane acrylonitrile phenol (compound 7) in DMF with potassium carbonate to yield compound 11. Finally, CHS-4 (compound 12) was obtained through an ozone reaction, which was
dissolving compound 11 in DCM and adding the triplet sensitizer methylene blue hydrate with bubbling oxygen through and shining light. (Scheme 2-2)

Scheme 2-13 Synthesis of CL-DNP.

Compared with CHS-4, there is no need of doing the trigger synthesis for the synthesis of CL-DNP firstly. The CL-DNP precursor, compound 13 was obtained through the addition of 2-bromo-1,4-dinitrobenzene and potassium carbonate in DMF. Then CL-DNP was obtained through the similar method as CHS-4, that achieves the dioxetane reaction dissolving the scaffold in DCM with adding methylene blue hydrate, bubbling oxygen and shining yellow light.
2.3.2 Response and selectivity of CHS-4 and CL-DNP

![Graph A: Time scan for 20 μM CHS-4 and 0, 50, 100, 200, 400, 800, 1000 μM Na$_2$S.](image)

![Graph B: The integrated emission intensity of the chemiluminescent response for CHS-4.](image)

![Graph C: Selectivity test of 200 μM CHS-4 to the indicated analytes from 0 to 20 min.](image)

**Figure 2-14** Hydrogen sulfide dose dependence in PBS buffer (pH=7.41). (A) Time scan for 20 μM CHS-4 and 0, 50, 100, 200, 400, 800, 1000 μM Na$_2$S. (B) The integrated emission intensity of the chemiluminescent response for CHS-4. (C) Selectivity test of 200 μM CHS-4 to the indicated analytes from 0 to 20 min. All experiments were performed in 10 mM PBS (pH 7.4) containing ≤ 1% DMSO. Error bars are ± S.D. (1. 1 mM Na2S 2. 10 mM GSH 3. 1 mM cystein 4. 1 mM Na2SO3 5. 1 mM H2O2 6. 1 mM NaClO 7. 1 mM NaNO3 8. t-BuOOH 9. 1 mM H2S+10 mM GSH 10. blank)

Based on the other hydrogen sulfide chemiluminescent probes (CHS-1, 2 and 3) measurements, the chemiluminescent response of CHS-4 was first tested using 20 μM probe
solution and different concentration Na$_2$S aqueous solution (0, 50, 100, 200, 400, 800, 1000 µM) in PBS buffer (pH = 7.41). CHS-4 performed immediately light emission in this animal relevant conditions with H$_2$S dose dependence. After demonstrating the chemiluminescent response of CHS-4, we next tested the selectivity of this probe for H$_2$S against other biologically relevant RSON species such as cysteine, glutathione, Na$_2$SO$_3$ and so on. The selectivity test was carried out by treating 200 µM CHS-4 with different amount of RSON species in 10 mM PBS buffer (pH 7.4). 10 mM reduced GSH, 1 mM L-cysteine and Hcy, and 1mM sulfite (SO$_3^{2-}$), hydrogen peroxide (H$_2$O$_2$), hypochlorite (OCl$^-$), tert-butyl hydroperoxide (tBuOOH) were used for evaluation. There is no significant luminescence intensity increase of the other species over the blank control after 30 min test, which demonstrates that CHS-4 has good selectivity and sensitivity for H$_2$S detection in vitro.
Figure 2-15 Hydrogen sulfide dose dependence in PBS buffer (pH=7.41). (A) Time scan for 20 μM CL-DNP and 0, 0.2, 1, 2, 4 mM NaHS. (B) The integrated emission intensity of the chemiluminescent response for CL-DNP. (C) Selectivity test of 200 μM CL-DNP to the indicated analytes from 0 to 20 min. All experiments were performed in 10 mM PBS (pH 7.4) containing 5% DMSO. Error bars are ± S.D. (1. 1 mM Na₂S 2. 10 mM GSH 3. 1 mM cystein 4. 1 mM Na₂SO₃ 5. 1 mM H₂O₂ 6. 1 mM NaClO 7. 1 mM NaNO₃ 8. t-BuOOH 9. 1 mM H₂S+10 mM GSH 10. blank)

The chemiluminescent response measurement for the other hydrogen sulfide probe CL-DNP was also tested using 20 μM probe solution. According to Pluth’s report, CL-DNP probe has better response performance and sensitivity with NaHS dose dependence compared with Na₂S, that is used for azide trigger probes. Therefore, different with CHS-4, we chose different concentration NaHS aqueous solution as the H₂S donor. Additionally, the response test temperature for CL-DNP was also changed from room temperature to 37 °C and 5% DMSO was added in test solvent. The result of its response performed well as CHS-4 in the proper conditions with H₂S dose dependence. Compared with CHS-4, dinitrophenyl probe displayed a lower background emission and better sensitivity with H₂S donor dose dependence. In the other hand, we also tested selectivity of this probe for H₂S against other biologically relevant RSON species same as CHS-4. The selectivity test was carried out by treating 200 μM CHS-4 with different amount of RSON species in 10 mM PBS buffer (pH 7.4). Same as CHS-4, CL-DNP displayed strongest emission intensity compared with the other species over the blank control after time scan. However, there was an obviously intensity increase of this probe detecting some other reactive species such as L-cysteine, sulfite (SO₃²⁻) and hydrogen peroxide (H₂O₂), which indicated that CL-DNP performed not really good selectivity of H₂S as CHS-4.
Figure 2-16 The chemiluminescent response of CHS-4 and CL-DNP in different conditions. (A) Time scan for 20 μM CHS-4 and 0, 0.2, 1, 2, 4 mM NaHS in 10 mM PBS buffer (pH=7.41) with 5% DMSO at 37 °C. (B) Time scan for 20 μM CL-DNP and 0, 50, 100, 200, 400, 800, 1000 μM Na₂S in 10 mM PBS buffer (pH=7.41) with ≤ 1% DMSO at 25 °C.

An interesting result we found that the chemiluminescence response performed significant changes when we switched testing condition of CHS-4 and CL-DNP each other. For CHS-4, after using NaHS as hydrogen sulfide donor with 5% DMSO at 37 °C, the time scan curves displayed lower sensitivity compared with the testing result in original condition. On the other hand, there was almost no response for CL-DNP probe with Na₂S solution at room temperature. Compared with the results we reported before, the emission intensity strength and sensitivity had an obvious decrease, which demonstrated that Na₂S was not a proper H₂S donor for CL-DNP probe. According to the different chemiluminescent response performance, we concluded that some important factors like reactive species donor, testing temperature and solvent would affect the emission intensity for chemiluminescent probes with different triggers, for example the azide-based probe CHS-4 and dinitrophenyl probe CL-DNP.
2.3.3 Cell studies of CHS-4 and CL-DNP

Next, we applied our probes CHS-4 and CL-DNP to detect cellularly generated H₂S in human lung adenocarcinoma epithelial cells (A549) due to their robust luminescence emission intensity in biologically condition. As the *in vitro* measurements, the luminescent intensity increased with increasing concentration of H₂S for both of CHS-4 and CL-DNP, demonstrating that they were available to detect Na₂S/NaHS sensitively in a multi-well plate reader format.

Detection of endogenous hydrogen sulfide *in vivo* was then tested in F-12K media with CHS-4. The cells were first incubated with black, 0, 400, 800 and 1000 μM Na₂S for 1 hour. Then the cells were washed by PBS buffer to remove any extracellular Na₂S and dead cells. Next, the cells were treated with 20 μM CHS-4 and tested immediately with the plate reader at 37 °C. After running for 4 h, the instantaneous luminescence emission figure was obtained with Na₂S dose dependence. The cellular study of CL-DNP probe had same protocols with CHS-4, including the initial incubation, cells washing and play reader running steps. Different with CHS-4, different concentration NaHS aqueous solution with 5% DMSO was added in culture before the incubation step instead of Na₂S solution. Compared with the luminescence response *in vitro*, both of CHS-4 and CL-DNP performed similar sensitivity trends of H₂S dose dependence and the peak emissions occurred around 15 minutes. In the future direction, we would explore the cellular toxicity of these two probes using MTT assay and combine it with the cellular response results to find the proper experimental condition for *in vivo* tests.
2.3.4 Conclusion

In this project, we reported two chemiluminescent probe CHS-4 and CL-DNP for hydrogen sulfide detection. CHS-4 has better performance of chemiluminescence emission and selectivity compared with CHS-1, 2 and 3 as adamantane azide trigger probe. CL-DNP is another chemiluminescent probe for hydrogen sulfide with a novel trigger, dinitrophenyl. After obtaining the same measurements with CHS-4, we demonstrate that there are some different characterizations with trigger changing, including hydrogen sulfide donor species, solvent system and experimental conditions. Cellular studies provide us robust evidence for exploring these chemiluminescent probes potential approaches of in vivo imaging in the future.
2.4 Experimental Section

2.4.1 General Materials and Methods

All reactions were performed in dried glassware under an atmosphere of dry N\textsubscript{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 (Billerica, MA), Oakwood Chemical (West Columbia, SC), and Cayman Chemical (Ann Arbor, MI) and used without further purification. \textsuperscript{1}H NMR and \textsuperscript{13}C NMR spectra for characterization of new compounds and monitoring reactions were collected in CDCl\textsubscript{3} (Cambridge Isotope Laboratories, Cambridge, MA) on a JEOL 500 MHz spectrometer or Bruker 400 MHz spectrometer in the Department of Chemistry at Southern Methodist University. 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. Low resolution mass spectrometry was performed using an Advion ExpressionL CMS (ESI source) at Southern Methodist University.
2.4.2 Synthesis Procedure

\[ \text{Diethyl ((2-chloro-3-methoxyphenyl)(methoxy)methyl)phosphonate (3).} \]

\[ \text{Diethyl ((2-chloro-3-methoxyphenyl)(methoxy)methyl)phosphonate (3).} \]

Diethyl ((2-chloro-3-methoxyphenyl)(methoxy)methyl)phosphonate (3). The acetal compound (2) (6.51 g, 29.8 mmol, 1.00 equiv.) was added to an oven-dried round-bottom flask, flushed with N\textsubscript{2}, then dissolved it with 10 mL anhydrous DCM. The mixture was then cooled to 0 °C in an ice bath and stirred it for 15 min. Boron trifluoride diethyl etherate (3.93 mL, 30.8 mmol, 1.02 equiv.)
was then added dropwise to the flask. Then triethyl phosphate (5.25 mL, 30.6 mmol, 1.01 equiv.) was added dropwise to the mixture, and the reaction was stirred for 20 minutes at 0 °C. The reaction mixture was then heated at 40 °C under refluxed for 2 hours while being monitored by TLC. Upon completion, the reaction was cooled to room temperature and quenched with 30 mL of saturated NH₄Cl. After transferring to separated funnel, the organic layer was then eluted with 3 x 30 mL of EtOAc. Then the organic layers were combined and dried with Na₂SO₄, filtered, and concentrated under reduced pressure. Column chromatography (10:1 hexanes: EtOAc) yielded compound (3) (7.55 g, 23.2 mmol, 80 %) as a yellow oil. \(^1\)H NMR (500 MHz, CDCl₃) \(\delta\) 7.25 (s, 1H), 6.87 (s, 1H), 5.15 (d, 1H, \(J = 14\) Hz), 4.13 – 3.90 (m, 5H), 3.87 (s, 3H), 3.30 (s, 3H), 2.00 (s, 1H).

\(\text{(1r,3r,5R,7S)-2-((2-chloro-3-methoxyphenyl)(methoxy)methylene)adamantine} \quad (4)\).

Compound (3) (1.02 g, 3.09 mmol, 1.00 equiv.) was added to an oven-dried round-bottom flask, flushed with N₂, and dissolved in 10 mL anhydrous THF. The temperature was then brought down to –78 °C in a dry-ice with acetone bath. 2.5 M n-BuLi (1.51 mL, 3.72 mmol, 1.20 equiv.) was then added slowly and dropwise over 10 minutes. Then 2-adamanatone (0.581 g, 3.73 mmol, 1.20 equiv.) was dissolved in 5 mL anhydrous THF in a separate round bottom flask and then added to the reaction mixture. The temperature is then raised to 35 °C in an oil bath and stirred for 2 hours. Subsequently, the temperature was raised to 90 °C and put under reflux for 1 hour. After reaction completion, the reaction was cooled to room temperature and quenched with 30 mL of saturated
NH₄Cl. The mixture was transferred to a separatory funnel and the organic layer was eluted with 3 x 30 mL EtOAc. The combined organic layers were then dried with Na₂SO₄, filtered, and concentrated under reduced pressure. Column chromatography (15: 1 hexanes: EtOAc) yielded compound (4) (0.71 g, 2.15 mmol, 72 %) as an amber oil. ¹H NMR (500 MHz, CDCl₃) δ 7.18 (t, 1H, J = 17 Hz), 6.90 – 6.87 (m, 2H), 3.88 (s, 3H), 3.31 (s, 3H), 3.26 (s, 1H), 1.67 – 1.29 (m, 15H).

3-(((1r,3r,5R,7S)-adamantan-2-ylidene)(methoxy)methyl)-2-chlorophenol (5). The ether compound (4) (13.1 g, 40.9 mmol, 1.00 equiv.) was added to a dry flask and flushed with N₂ and dissolved in 50 mL of anhydrous DMF. Sodium ethanethiolate (4.13 g, 49.1 mmol, 1.20 equiv.) and cesium carbonate (33.1 g, 102 mmol, 2.40 equiv.) were then added to the flask. The temperature was raised to 120 °C and stirred for 24 hours under reflux. The reaction was monitored by TLC. After the completion of the reaction, the reaction was quenched with 200 mL saturated NH₄Cl. Then the reaction mixture was transferred to a separatory funnel, and the organic layer was eluted with 3 x 200 mL of EtOAc. Then the organic layer was washed with 3 x 250 mL of brine. The organic layers were combined and dried with Na₂SO₄, filtered, and concentrated under reduced pressure. Column chromatography (5:1 hexanes:EtOAc) was used for purification to give compound (5) (10.8 g, 35.1 mmol, 89%) as an off-white solid. ¹H NMR (500 MHz, CDCl₃) δ 7.04 (t, 1H, J = 14 Hz), 6.93 (d, 1H, J = 4 Hz), 6.71 (d, 1H, J = 6 Hz), 3.22 (s, 3H), 3.16 (s, 1H), 2.00 – 1.81 (m, 16H).
Compound (5) (1.00g, 3.28 mmol, 1 equiv.) and N-iodosuccinimide (0.75 g, 3.3 mmol, 1.0 equiv.) were added to a round-bottom flask, flushed with N₂, and then dissolved in 20 mL of toluene. The reaction was cooled to 0 °C in an ice bath for 20 minutes. The mixture color changes from light orange to red. Upon completion, the reaction mixture was transferred to a separatory funnel and washed with brine. Some sodium thiosulfate pentahydrate crystals were added to the separatory funnel to quench any remaining iodine. After shaking the separatory funnel, there was a color change from red to clear. If the color did not become clear, more crystals were added. The organic layer was eluted with 3 x 30 mL of EtOAc. Then the organic layers were combined, dried with Na₂SO₄, and concentrated under reduced pressure. For purification, the crude product was rinsed multiple times with hexanes. A solid white powder crashed out giving compound (6) (0.41 g, 1.09 mmol, 28%). ¹H NMR (500 MHz, CDCl₃) δ 7.62 (d, 1H, J = 6 Hz), 6.63 (d, 1H, J = 6 Hz), 3.25 (s, 3H), 3.23 (s, 1H), 2.04 – 1.65 (m, 15H).

(E)-3-{(1r,3r,5R,7S)-adamantan-2-ylidene}(methoxy)methyl)-2-chloro-3-iodo-2-hydroxyphenyl)acrylonitrile (7). Compound (6) (0.22 g, 0.46 mmol, 1.00 equiv.) and palladium (II) acetate (0.011 g, 0.046 mmol, 0.10 equiv.) were added to an oven-dried microwavable flask,
flushed with N₂, and dissolved in 1 mL of acetonitrile. Then acrylonitrile (0.11 mL, 1.4 mmol, 3.0 equiv.) and triethylamine (0.11 mL, 0.70 mmol, 1.50 equiv.) were added to the reaction mixture. The flask was then capped and stirred in the microwave for 70 minutes at 120 °C. Upon completion, the reaction was cooled to room temperature and quenched with 15 mL of NH₄Cl and transferred to a separatory funnel. The organic layer was then eluted with 3 x 20 mL of EtOAc. The organic layers were then combined, dried with Na₂SO₄, filtered, and concentrated under reduced pressure. Column chromatography (9:1 hexanes: EtOAc) yielded compound 7 (0.08 g, 0.24 mmol, 46 %) as a brown oil. ¹H NMR (500 MHz, CDCl₃) δ 7.54 (d, 1H, J = 14 Hz), 7.26 (d, 1H, J = 6 Hz), 6.83 (d, 1H, J = 8 Hz), 6.32 (s, 1H), 6.14 (d, 1H, J = 18 Hz), 3.30 (s, 3H), 3.24 (s, 1H), 2.01 – 1.17 (m, 15H).

(4-azidophenyl)methanol (9) 4-aminobenzyl alcohol (1.02 g, 8.1 mmol, 1.0 equiv) was dissolved in 20 mL 10% HCl solution in a round bottom flask under N₂ atmosphere and cooled to 0 °C. Sodium nitrite (0.68 g, 9.75 mmol, 1.2 equiv.) was dissolved in 10 mL water, added slowly and dropwise, and allowed to stir for 30 min. Then sodium azide (0.83 g, 9.75 mmol, 1.2 equiv.) was dissolved in 10 mL water, added slowly and dropwise, and stirred for an additional 2 hrs. The reaction was monitored by TLC. Upon completion, the reaction was transferred into a separatory funnel, washed with brine, eluted with 3 x 50 mL EtOAc. The combined organic layer was dried with Na₂SO₄, filtered, and concentrated under reduced pressure. Purification by column
chromatography (5:1 hexanes:EtOAc) yielded compound 9 (1.10 g, 6.82 mmol, 87%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.39 (d, 2H, $J = 20$ Hz), 7.01 (d, 2H, $J = 18$ Hz), 4.67 (s, 2H), 1.91 (s, 1H).

(E)-3-(4-(((1r,3r,5r,7S)-adamantan-2-ylidene)(methoxy)methyl)-2-((4-azidobenzyl)oxy)-3-chlorophenyl)acrylonitrile. (11) The precursor phenol, compound (6) (0.04 g, 0.11 mmol, 1 equiv.) was dissolved in 3 mL anhydrous THF under a N$_2$ atmosphere and cooled to 0°C. Compound 7 (0.016 g, 0.10 mmol, 1 equiv.) and triphenylphosphine (0.036 g, 0.12 mmol, 1.2 equiv.) were added to the reaction mixture and allowed to cool for 5 min. Then DEAD (0.02 mL, 0.1 mmol, 1.2 equiv.) was added and stirred for 1 hr while being monitored by TLC. Upon completion, the reaction was transferred to a separatory funnel and quenched with sat. NH$_4$Cl. The organic layer was eluted with 3 x 25 mL EtOAc, dried with Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. Column chromatography (10:1 hexanes:EtOAc) yielded (10.4 mg, 0.36 mmol, 33%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.55 (d, 1H, $J = 14$ Hz), 7.41 (d, 4H, $J = 20$ Hz), 7.32 (d, 2H, $J = 20$ Hz), 7.16 – 7.02 (m, 5H), 5.92 (d, 2H, $J = 14$ Hz), 5.44 (s, 1H), 4.99 (s, 3H), 4.54 – 4.53 (m, 2H), 3.34 (s, 3H), 3.31 (s, 1H), 2.20 – 0.92 (m, 21H).

(E)-3-(2-((4-azidobenzyl)oxy)-3-chloro-4-(((1r,3r,5r,7r)-4'-methoxyspiro[adamantane-2,3'-[1,2]dioxetan]-4'-yl)phenyl)acrylonitrile (CHS-4). Compound 11 (10.2 mg, 0.02 mmol, 1
equiv.) and methylene blue hydrate (8 mg, 0.02 mmol, 1 equiv.) were added to a dry flask and dissolved in 2 mL DCM. 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 hours, TLC indicated no starting material remained, the reaction mixture was concentrated under reduced pressure. The product was purified by column chromatography (8:1 hexanes:EtOAc) yielding compound CHS-4 (6.8 mg, 0.01 mmol, 51%). ¹H NMR (400 MHz, CDCl₃) δ 7.98 (d, 1H, J = 13 Hz), 7.53 (s, 1H), 7.49 (s, 1H), 7.44 (d, 2H, J = 6 Hz), 7.08 (d, 2H, J = 4 Hz), 5.96 (d, 1H, J = 18 Hz), 5.02 (s, 1H), 4.96 (s, 2H), 3.26 (s, 3H), 3.07 (s, 1H), 2.17 − 0.90 (m, 20H).

![Chemical Structure](CL-DNP)

(E)-3-(2-((4-dinitrophenyl)oxy)-3-chloro-4-((1r,3r,5r,7r)-4'-methoxyspiro[adamantane-2,3'-[1,2]dioxetan]-4'-yl)phenyl)acrylonitrile (CL-DNP). Compound 6 (103 mg, 0.28 mmol, 1.0 equiv.) was dissolved in 5 mL anhydrous DMF with addition of 2,4-dinitrobromobenzene (74 mg, 0.29 mmol, 1.0 equiv.) and potassium carbonate (80 mg, 0.57 mmol, 2.0 equiv.). The reaction mixture was then stirred under N₂ for 24 h at room temperature. After completion, the reaction was quenched with 5 mL brine and extracted with 3 x 10 mL EtOAc. The organic layer was washed with 3 x 10 mL 5% aqueous LiCl and dried Na₂SO₄, filtered, and concentrated under reduced pressure. The product was purified by column chromatography (2:1 hexanes:EtOAc) to yielding compound 13 110 mg of white solid. This enol ether product (10.4 mg, 0.02 mmol, 1.0 equiv.) was then added to a round-bottom flask and dissolved in 15 mL DCM with addition of methylene blue hydrate (4 mg, 0.01 mmol, 0.5 equiv.). 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 hours, TLC
indicated no starting material remained, the reaction mixture was concentrated under reduced pressure. The product was purified by column chromatography (1:1 Hexane: EtOH) to yield **CL-DNP** (9.8 mg, 0.018 mmol, 77%). $^1$H NMR (500 MHz, CDCl$_3$) δ 8.93 (d, 1H, $J = 2.8$ Hz), δ 8.24 (bs, 1H), 8.23 (d, 1H, $J = 7.8$ Hz), 7.70 (d, 1H, $J = 7.4$ Hz), 7.50 (d, 1H, $J = 14.8$ Hz), 6.48 (bs, 1H), 6.16 (d, 1H, $J = 14.8$ Hz), 3.25 (s, 3H), 3.06 (s, 1H), 2.09 – 1.08 (m, 14H).

### 2.4.3 Chemiluminescent response

The chemiluminescent response and time scans of CHS-4 were obtained with a Cytation 5 Bio Tek plate reader using the luminescence detection method under room temperature (25°C). A 5 mM stock solution of each probe was diluted to a 20 μM before testing. A 50 mM stock solution of Na$_2$S was prepared and diluted to the final concentration of 0, 50, 100, 200, 400, 800, 1000 μM. Time scans were obtained immediately following adding the reagents, including PBS buffer, probe, and Na$_2$S.

The chemiluminescent response and time scans of **CL-DNP** were obtained with a Cytation 5 Bio Tek plate reader using the luminescence detection method at 37 °C. A 5 mM stock solution of each probe was used with a 20 μM final concentration. A 50 mM stock solution of NaHS was prepared for each experiment and diluted to the final concentration to 0, 0.2, 1, 2, 4 mM. Time scans were obtained immediately following adding the reagents, including PBS buffer, probe, and NaHS.

### 2.4.4 Selectivity tests

Selectivity for **CHS-4** and **CL-DNP** was measured by monitoring the time-dependent chemiluminescence emission at 545 nm. All assays were performed in 10 mM PBS buffered to pH 7.4.
H$_2$S: 5 µL of a 20 mM stock solution of Na$_2$S/NaHS in DI-H$_2$O was added to a solution of 393 µL PBS and then 2 µL of 10 mM CHS-4/CL-DNP in DMSO was added into this mixture.

GSH: 25 µL of a 100 mM stock solution of GSH in 20 mM HEPES buffer was added to a solution of 373 µL PBS and then 2 µL of 10 mM CHS-4/CL-DNP in DMSO was added into this mixture.

Cysteine: 5 µL of a 100 mM stock solution of L-cysteine in DI-H$_2$O was added to a solution of 393 µL PBS and then 2 µL of 10 mM CHS-4/CL-DNP in DMSO was added into this mixture.

NaNO$_2$: 1 µL of a 100 mM stock solution of NaNO$_2$ in DI-H$_2$O was added to a solution of 397 µL PBS and then 2 µL of 10 mM CHS-4/CL-DNP in DMSO was added into this mixture.

Na$_2$SO$_3$: 1 µL of a 100 mM stock solution of Na$_2$SO$_3$ in DI-H$_2$O was added to a solution of 397 µL PBS and then 2 µL of 10 mM CHS-4/CL-DNP in DMSO was added into this mixture.

H$_2$O$_2$: 0.5 µL of a 200 mM stock solution of H$_2$O$_2$ in DI-H$_2$O was added to a solution of 397.5 µL PBS and then 2 µL of 10 mM CHS-4/CL-DNP in DMSO was added into this mixture.

OCl$: 1 µL of a 100 mM stock solution of OCl$^-$ in DI-H$_2$O was added to a solution of 397 µL PBS and then 2 µL of 10 mM CHS-4/CL-DNP in DMSO was added into this mixture.

tBuOOH: 1 µL of a 100 mM stock solution of tBuOOH in DI-H$_2$O was added to a solution of 397 µL PBS and then 2 µL of 10 mM CHS-4/CL-DNP in DMSO was added into this mixture.

Blank: 2 µL of 10 mM CHS-4/CL-DNP in CH$_3$CN was added to a solution of 398 µL PBS buffer.
2.4.5 Cellular Experiments

Human lung adenocarcinoma epithelial cells (A549) were purchased from ATCC. The cells were cultured in Ham’s F-12K (Kaighn’s) medium with 10% Fetal Bovine Serum (FBS) and 1% antibiotics (penicillin/streptomycin, 100 U/mL). Cells were kept in a humidified incubator at 37 °C with 5% CO₂. One or two days before a cellular experiment was conducted, cells were plated onto a sterile multi-well plate and allowed to reach a confluence of 80–95% by the time of testing. All chemiluminescent responses for cellular testing were obtained using the Cytation 5 Bio Tek plate reader (Winooski, VT) at 37 °C.

Stock solutions of 20 mM Na₂S/NaHS were prepared in DI-H₂O and 10 mM CHS-4/CL-DNP was prepared in DMSO. According to the H₂S dose dependence, 2 µL probe stoke solutions and different volume Na₂S/NaHS stoke solutions were added in every well. After another 40 min incubation, cells were washed with 2 x PBS. Then 500 µL PBS media was added into each well and the luminescent intensity was measured every five minutes for 240 minutes. The experiment was repeated with three independent well plates and the peak value for the luminescence emission for each well at 12–15 minutes was normalized to the average luminescence emission at 12–15 minutes for the control replicates of each plate. A single outlier was rejected according to the extreme studentized deviate method (p < 0.01).
2.5 References


CHAPTER 3

CELLULAR STUDIES OF VARIOUS FLUORESCENT COMPOUNDS

3.1 Introduction

Besides the chemiluminescent probes research, more cellular studies of other compounds are obtained, including LIVE/DEAD assay, photothermal experiments, and cells imaging with luminescent nanoclusters. First, we cooperated with Dr. Watkins (Associate Professor from University of Mississippi) for an interesting project named “Crosslinking PCL-PAMAM Linear Dendritic Block Copolymers (LDBC) for Theranostic Nanomedicine”. In this project, we completed the in vitro photothermal tests, which determined how much toxicity of nanoparticle treatment and irradiation time for A549 cells was. Additionally, another project “Visible Light Chemical Micropatterning Using a Digital Light Processing Fluorescence Microscope” in our group was completed last year and cell labelling experiments of photoluminescent dyes was displayed using visible light mediated chemistry and the DLP microscope. Furthermore, we attempted the Cy5 and Dapi cellular imaging for a series of metal organic polymer clusters with Dr. Das (Assistant Professor from SMU). In this project, we explored these clusters and performed characterizations such as emission intensity and phototoxicity could be affected by cell culture and incubation time.
3.2 Photothermal cellular test of LDBC

In this study, Dr. Watkins group designed and synthesized novel nanoparticles formed from LDBC.\textsuperscript{1,2,3} This crosslinked nanoparticle demonstrated a significant increase of hydrophobic loading efficiency.\textsuperscript{4,5,6} In addition, when nanoparticles loaded with a potential photothermal agent (C5), a high photothermal efficiency was performed. In cells studies, these nanoparticles performed efficiently into HEK293 cells with proper condition. Our group supported another cellular phototoxicity test for exploring the relationship between illumination with red light and cell death in A549 cells.

\textbf{Figure 3-18} Graphical representation of PhPCL-G3 LDBCs forming polymersome-like nanoparticles in water via physical core-crosslinking induced by $\pi-\pi$ interactions. (Adapted with permission from reference 37. Copyright 2022 ACS)
3.2.1 Photothermal Efficiency

The photothermal efficiency described the property of the dispersion temperature increase of nanoparticle upon irradiation with an 808 nm laser. The photothermal efficiency, $\eta$, was calculated by:

$$\eta = \frac{hA(\Delta T_{\text{max}} - \Delta T_{\text{sol}})}{I(1 - 10^{-A_\lambda})}$$

where $h$ is the heat transfer coefficient, $A$ is the surface area of the container, $\Delta T_{\text{max}}$ is the maximum temperature change of the dispersion, $\Delta T_{\text{sol}}$ is the maximum temperature change for the negative control MilliQ water under same condition, $I$ is the laser irradiation power and $A_\lambda$ is the absorbance of nanoparticles with photothermal agents at this light condition. After the irradiation with a visible light irradiation, these nanoparticles performed an obvious increase the temperature of the system from 21.6 °C to 66 °C in 20 minutes.

<table>
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<th>Sample</th>
<th>$\eta$ (%)</th>
<th>$hA$</th>
<th>$k_t$ (s)</th>
<th>mc$_p$ (J/K)</th>
<th>$\Delta T_{\text{max}}$ (K)</th>
<th>$\Delta T_{\text{sol}}$ (K)</th>
<th>$I$ (J/sec)</th>
<th>$A_\lambda$ (estimated)</th>
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</table>
Figure 3-19 (A) Dispersion of C5 in water; (B) C5 loaded 10-PhPCL-G3 (C) photothermal activity of PhPCL-G3 nanoparticles. (Adapted with permission from reference 37. Copyright 2022 ACS)

3.2.2 Nanoparticles in Biological Imaging and Phototherapy

Additionally, we designed and completed the cellular imaging and phototoxicity tests of C5-loaded nanoparticles in human lung cancer (A549) cells. According to the properties of this nanoparticles, we designed two experiments by treating cells with different concentrations of the nanoparticle and different irradiation time with 100W red LED lamp. Compared to cells treated with two negative controls (no dye-loaded nanoparticle and no light irradiation) (Figure 3-20), positive samples indicate an obvious increase in cell death after irradiation and increase with time (Figure 3-21). As the figure shows, the difference in the number of dead cells versus live cells (red versus green) was related to nanoparticle treatment and irradiation time, which performed a close relationship with increasing illumination time, and provide valuable evidence for using these nanoparticles for in vivo experiments in the future.9,10
Figure 3-20 Representative trial experiments: cells treated with varying concentrations of the nanoparticle suspension where either irradiated with a 100W red LED lamp for 0, 8, or 80 mins.37 (Adapted with permission from reference 37. Copyright 2022 ACS)
3.2.3 Experimental Section

In this project, our group mainly focused on the in vitro photothermal assay,\textsuperscript{11,12} that used Human lung cancer (A549) cells for it. A549 cells were prepared in three 12-wells plates and grown at 37 °C, 5% CO\textsubscript{2}, F-12K media with 10% FBS. After the confluency reaching around 90%, cells were washed with 10 mM PBS buffer for three times and treated with different concentrations of polymers in 1 mL cells media. Then the cells were incubated overnight under 5% CO\textsubscript{2} at 37 °C. After that, one plate of cells was placed in the dark for 8 min, the other two plate cells were irradiated with the red LED (100W RGB LED Flood Light), one for 8 min and the other one for 80 min, followed by incubation for 2 h. The light intensity was measured with a ThorLabs digital light meter and sensor and was found to be 9.7 ± 2.9 mW/ cm\textsuperscript{2}. After incubation, cells of each well were washed with 10 mL PBS buffer and then 200 mL LIVE/DEAD Cell Imaging assay solution.
was added in every well. Cell death was evaluated with a LIVE/DEAD Cell Imaging KitTM Invitrogen following the manufacturer’s protocols, that was evaluated by counting the live cells indicated by green fluorescence and the dead cells indicated by red fluorescence (GFP filter cube: 470/22 nm excitation, 525/50 nm emission. RFP filter cube: 531/40 nm excitation, 593/40 nm emission) using an EVOS-fl fluorescence microscope.

3.2.4 Conclusions

In this project, Watkins group successfully synthesized crosslinking LDBC nanoparticles and analyze some important characterizations about loading efficiency and photothermal stability. It’s my pleasure to participate and complete the photothermal experiments for this project. As the results of LIVE/DEAD assay, C5-loaded nanoparticles were able to cause cell death in cancer cells under exposure to red light irradiation. This photothermal test results provided us valuable evidence for improving the potential of LDBCs in phototherapy applications.
3.3 Visible Light Chemical Micropatterning Using DLP

High-resolution patterning chemical reactivity\(^{13}\) plays an important role in advanced area including nanorobotics\(^{14}\), bioprinting\(^{15}\), and photopharmacology\(^{16}\). It’s necessary for us to develop novel techniques that could be widely used and compatible with various chemical properties. Our group reports the development of a digital light processing (DLP) fluorescence microscope that could achieve structuring of visible light (465–625 nm) for high-resolution photochemical patterning and fluorescence imaging\(^{17}\). Visible light photochemical systems can be performed by various specific reactions, such as thiol-ene photoclicking\(^{18}\) and Wolff rearrangements of diazoketones\(^{19}\). Therefore, we designed and achieved the patterning of this functional chemical reaction onto microscopic polymer beads and films\(^{21,22}\). Also, target labelling in living cells is demonstrated by photoactivation of RhBNN. That could help us explore new approaches for novel areas such as 3D printing and new generation nanorobots.

3.3.1 DLP Microscope Design and Fabrication

For achieving high-resolution photochemical imaging, we designed and projected a digital light processing (DLP) fluorescence microscope for projecting patterned visible light with microscale substrates and fluorescence imaging (in chapter 1). DLP system was accomplished using a digital micromirror and projection device in the LightCrafter 4500 with the projection optics removed (Texas Instruments, USA). (Figure 3-22)
Figure 3-22 (A) Design scheme and (B) photograph of the DLP microscope setup composed of: 1. DLP LightCrafter 4500 as the patterned light source, 2. An adjustable iris diaphragm, 3. A mounted achromatic doublet collimation lens with 100 mm focal length, 4. Zoom housing with 4.1 mm linear travel, 5. A 650 nm shortpass excitation filter, 6. A 660 nm dichroic beamsplitter, 7. Zoom housing with 4.1 mm linear travel, 8. A 4x objective (0.10 NA, 18.5 mm WD), a 40x objective (0.60 NA, 1.2 mm WD), or a 100x oil objective (1.30 NA, 0.17 mm WD), 9. An XY translational stage, rigid stand, and slide holder, 10. A 692/40 nm bandpass emission filter, 11. A mounted achromatic doublet lens with 100 mm focal length and 2.5” lens tube, and 12. Chameleon3 Monochrome sCMOS camera. (C) Checkerboard pattern and one-pixel width diagonal lines projected onto a target resolution slide using 4x, 40x, and 100x objectives. Inset: projection resolution determined as the FWHM of the Gaussian fit to imaged pixel intensities across a projected one-pixel line. Error on FWHM is ± S.D. with n = 3 different lines measured. (Adapted with permission from reference 36. Copyright 2021 ACS)
3.3.2 Visible Light Mediated Reaction

In this study, thiol-ene photoclick reaction was investigated and initiated by a visible light photoinitiator\textsuperscript{24,25}, which has some specific photochemical properties. According to it, our group developed and synthesized some observable thiol covalent modification containing polymer constructs, that were combined with the fluorescent silicon rhodamine. Thiol-ene reactions was reported in previous studies last decades, that was useful to accomplish photochemistry patterning\textsuperscript{26}. Based on it, we firstly prepared the fluorescent silicon rhodamine-styrene (\textit{SiR-sty}) as alkene reaction partner, that increased fluorescence by achieving thiol-ene ligation on the SH-resin beads. The reactant \textit{SiR} was synthesized by using the silicon anthrone and tert-butyl protected 3-iodo-4-methylbenzoic acid with n-butyllithium, and the thiol-ene partner \textit{SiR-sty} was yielded with an amide coupling of \textit{SiR} with 4-amino styrene. The other part of thiol-ene reaction, SH-resin was obtained by coupling the amino resin with fmoc- and mmt- protected cysteine, then deprotected the mmt-cysteine group with 1% trifluoroacetic acid. Finally, the thiol-modified SH-resin was reacted with \textit{SiR-sty} and eosin y in DMSO under blue light irradiation overnight. As the results in \textbf{Scheme 3-16}, strong fluorescence emission from beads was observed when the reaction was irradiated under blue light. In the other hand, there was extreme weak emission observed when the reaction was conducted in the dark.
Scheme 3-14 Examples of thiol-ene reaction with eosin y.

Scheme 3-15 Synthesis of fluorophore-tagged alkene, SiR-sty. (Adapted with permission from reference 36. Copyright 2021 ACS)
Besides that, we designed another fluorescent probe that could achieving for photochemical patterning with diazo compounds, Rhodamine B based photocaged dye RhBNN\textsuperscript{27,28}. When the diazoketone group on RhBNN is irradiated with blue light, a carbene is generated, which could form a ketene intermediate immediately by Wolff rearrangement. Then the ketene could be reacted with nucleophilic species such as alcohols\textsuperscript{29} and amines\textsuperscript{30}, that performs strong fluorescence emission of rhodamine B. In this study, we irradiated polyvinylalcohol (PVA)\textsuperscript{31} films containing 50 µm Rhodamine BNN (RhBNN-PVA) covered by glass microscope slides with blue light. After 22 hours irradiation, the films performed strong red fluorescence compared to those that were kept in the dark condition (Scheme 3-17).
**Scheme 3-17** Reaction scheme and EVOS-fl fluorescence images (Ex: 542/20 Em: 593/40) of PVA films doped with 20 µM Rhodamine BNN with and without 22 h blue light irradiation from a 100 W LED lamp at 0.04 W cm$^{-2}$. (Adapted with permission from reference 36. Copyright 2021 ACS)

**3.3.3 RhBNN Patterned Labelling of Living Cells**

Next, based on the DLP microscope and Rhodamine B insertion studies, we attempted visible light driven targeted labelling experiment of live cells with RhBNN. We incubated A549 cells in T25 cell culture flasks with 20 µM RhBNN in PBS buffer and 2% DMSO for 30 mins for uptake. After washing the cells with PBS buffer, the cells were displayed on the DLP microscope and irradiated with blue light in the split field pattern. After blue light irradiation, the fluorescence emission of cells was performed by using green light at time points from 0–30 seconds (**Figure 3-23**). The result confirmed that cells displayed obvious stronger fluorescence emission with increasing irradiation time on the irradiated field. Compared to it, cells with no blue light on the right side of the field remained non-fluorescent, which demonstrated that living cells can be accurately labelled and selectively functionalized with fluorescent dye using RhBNN patterning chemistry and the DLP microscope.
Figure 3-23 DLP Micropatterning of live A549 cells using visible light. (A) DLP fluorescence image of cells incubated for 30 mins with 20 µM RhBNN under a 40x objective before blue light irradiation and (B) after 30 s irradiation with blue light at 9.4 W cm$^{-2}$ with a split field pattern which irradiates the left half of the field. (C) Plot of average pixel intensities of all cells in irradiated and non-irradiated half of field with increasing irradiation times. Error bars are ± S.D. with n = 3 independent replicates. Images A,B are displayed with equally brightness and contrast; raw images available in ESI. (Adapted with permission from reference 36. Copyright 2021 ACS)

3.3.4 Experimental Section

In cellular experiments, cells were passaged by seeding in T25 cell culture flasks (Falcon,29185-298) and covering with 5.0mL of media. These experimental flasks were allowed to grow for 2 to 3 days, or until they had reached between 70% and 90% confluence. A 1mM RhBNN stock solution in DMSO was prepared by dissolving 3.4 mg RhBNN into 6.0 mL DMSO and stored in a foil-wrapped scintillation vial. To each flask of confluent cells, 50µL of RhBNN stock solution was added to the T25 flask without replacement or removal of media (final concentration:10 µM RhBNN,1% DMSO). The flasks were gently agitated, and then allowed to incubate for 30 min to uptake the RhBNN. Medium was then removed, and cells were washed with 2x5 mL PBS buffer to remove any RhBNN not uptaken by the live cells. The cells were submersed in PBS for micropatterning experiments.
A549 cells were incubated with RhBNN and prepared for imaging as described above. A field of cells was focused under a 40x objective and imaged under uniform green light (2.8W cm\(^{-2}\)) prior to patterning. Using the Parallel RGB input of the Light Crafter 4500, the coordinates of the desired cell were mapped onto a PowerPoint slide, where a white square was placed on top of a black background to illuminate one and only one cell. The cells were irradiated with this powerpoint slide for 0s, 30s, 60s, and 90s with high intensity blue light (9.4W cm\(^{-2}\)) and imaged under weak green light (2.8W cm\(^{-2}\)). The selected cell that was irradiated showed a significant increase in fluorescent intensity, whereas cells not irradiated showed no significant increase in fluorescent intensity.

3.3.5 Conclusion

In summary, we design a chemical micropatterning technique using digital light processing fluorescence microscope (DLP) and photochemical reaction systems. Based on it, visible-light based thiol-ene reactions and diazoketone Wolff rearrangements of silicon rhodamine and rhodamine BNN onto solid resin beads and PVA films were achieved by this technology with high resolution. In addition, DLP micropatterning was performed for fluorescent labelling of selected single live cells, which demonstrated a significant potential for using in a wide range of applications like metamaterials.
3.4 Generation of fluorescent Au$_n$(Sdam)$_n$ Nanoclusters

Metal nanoclusters are novel compounds which have small size and special properties governed by their sub-nanometer dimensions.$^{32}$ As a representative class of polymers, metal nanoclusters have some advantages and unique fluorescent and luminescent properties compared with other polymers. Additionally, metal nanoclusters have better performance for labeling and imaging applications in vivo compared with some molecules which have larger size and toxic metal species such as cadmium and lead.$^{33,34}$ Hererin, Dr. Das and her group designed and several novel gold nanoclusters with multiple ligands that demonstrated excited photochemical properties. Next, we completed some cellular experiments in A549 cells including EVO-fi imaging and cluster toxicity test. These results provide valuable evidence for deeply understanding the characterizations of noble metal nanoclusters and developing the photoexcitation process of single oxygen production in the future.

3.4.1 Au$_2$Ag$_6$(dppm)$_2$(Sdam)$_6$ nanocluster

In this study, the noble metal nanocluster is designed as Figure 3-24, which is combined with gold and silver as center, dppm and Sdam as ligands. Dr. Das group successfully synthesized it and completed some photochemical measurements such as absorption and emission spectrum, quantum yield and so on. (Figure 3-25) According to these results, we continued to be setting up several in vivo experiments for confirming the fluorescence emission imaging and cellular toxicity.
Figure 3-24 Crystal structure of \( \text{Au}_2\text{Ag}_6 (\text{Ph}_3\text{P-CH}_2\text{-PPh}_3)_2(\text{SR}) \) (SR=1-Adamantanethiol (C\(_{10}\)H\(_{16}\)S), tert-Butylthiol) (Allowed to use by Dr. Das group)

Figure 3-25 (A) Absorption spectrum of T sample-1 and T-sample 2. (B) Emission under 365 nm UV light of T sample-1 and T sample-2 in solid and solution state. (Allowed to use by Dr. Das group)
To confirm the biocompatibility and photodynamic activity of \( \text{Au}_2\text{Ag}_6(\text{dppm})_2(\text{Sdam})_6 \) nanocluster toward cancer cells, we investigated the capability of the cluster to reduce the viability of cancer cells. Before applying \( \text{Au}_2\text{Ag}_6(\text{dppm})_2(\text{Sdam})_6 \) for the photodynamic killing of cancer cells, the cellular imaging and cytocompatibility of \( \text{Au}_2\text{Ag}_6(\text{dppm})_2(\text{Sdam})_6 \) was first examined using EVO-fi microscope and MTT assay. As shown in Figure 3-26, upon incubation with \( \text{Au}_2\text{Ag}_6(\text{dppm})_2(\text{Sdam})_6 \), the cellular viability of A549 cells remained above \( \sim 70\% \) in the range of 1–25 \( \mu \text{M} \) nanocluster for 1 h. With the concentration increasing above 50 \( \mu \text{M} \), the cellular viability decreased to 30-40\% immediately. As shown in Figure 3-27, the fluorescence imaging of \( \text{Au}_2\text{Ag}_6(\text{dppm})_2(\text{Sdam})_6 \) with 1-100 \( \mu \text{M} \) was performed after 1 h incubation. Also, combining with the nuclear localization test, the mean pixel average was showed a clear trend with concentration dependence. It’s meaningful for us to develop more applications such as singlet oxygen photoexcitation in the future.

![Figure 3-26](image)

**Figure 3-26** MTT assay of A549 cells in the presence of different concentrations of T-sample 2.
3.4.2 \( \text{Au}_2\text{Cu}_6(\text{F-phosphine})_2(\text{Sdam})_6 \) nanocluster

In addition, for deeply understanding the properties of these gold nanoclusters, Dr. Das group also synthesized other compounds to be characterized, for example \( \text{Au}_2\text{Cu}_6(\text{F-phosphine})_2(\text{Sdam})_6 \). Compared with the gold-silver cluster, this molecule used F-phosphine as ligands instead of dppm, which could bond with metal anions center. As the experiments of \( \text{Au}_2\text{Ag}_6(\text{dppm})_2(\text{Sdam})_6 \), cellular imaging and viability tests were completed with the same condition (final concentration of sample is in the range of 0-100 \( \mu \text{M} \)). Interestingly, the fluorescence cellular imaging of \( \text{Au}_2\text{Cu}_6(\text{F-phosphine})_2(\text{Sdam})_6 \) with 25-50 \( \mu \text{M} \) was performed well after 1 h incubation. However, we found that the cell toxicity would increase obviously with above 50 \( \mu \text{M} \) cluster, which was also confirmed by cell viability experiment using MTT assay.
3.4.3 Experimental Section

**Cellular fluorescence imaging.** A549 cells were prepared in three 12-wells plates and grown under standard conditions (37 °C, 5% CO₂, F-12K media with 10% FBS). A549 cells were washed with 10 mM PBS buffer and treated with 0 (A1-A2), 10 μM (B1-B3), 25 μM (C1-C3), 50 μM (D1-D3) polymers in 1 mL F-12K media. The cells were incubated for 1 h under 5% CO₂ at 37 °C. Then 50 μL nuclear localization assay solution was added into the cell culture. Plates were put in the dark condition for 30 mins. After incubation, the culture media of each well was removed and 200 mL 10 mL PBS buffer was added to each well. After washing out, the cells were indicated by red fluorescence (Cy5 filter cube: 531/40 nm excitation, 593/40 nm emission) and the cells nuclear indicated by blue fluorescence (Dapi filter cube: 357/44 nm excitation, 447/60 nm emission) using an EVOS-fl fluorescence microscope. Two independent experiments were performed to assure consistency. Statistical significance between dark control and 8 min irradiation test and statistical significance between dark control and 80 min irradiation test were calculated using a two-tailed Student’s t-test with n = 4 technical replicates across two independent experiments.

**Cell viability test.** RAW 264.7 macrophage cells (10⁶ cell/mL) were seeded in a 12-well plate to a total volume of 1 mL/well. The plate was maintained at 37 °C with 5% CO₂ for 12 h. Cells were then incubated for 1 h after adding different concentrations of nanocluster, 0, 1, 10, 100 and 1000 μM respectively. 50 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, that was used to calculate the cell viability percentage.
3.4.4 Prospective research

According to the promising results we have, some photoexcited study can be explored in the next step such as singlet oxygen generation. In recent advanced research, singlet oxygen can be efficiently produced upon the direct photosensitization by gold nanoclusters without any conventional photosensitizers under visible/near-IR irradiation. Based on it, we could develop the new clusters potential of $^1O_2$ generation using the multiple adamantane chemiluminescent probes as detector. In the future, this series of nanoclusters will be investigated for their potential use in targeting, imaging and other applications, where the organic-soluble or water-soluble clusters perform promise as heterogeneous $^1O_2$ catalysts in chemical reactions.

3.5 Summary

Cellular study is a meaningful and important part in the research of luminescent compounds. There are various in vivo experiments, including fluorescence imaging, chemiluminescence detection, photochemical properties measurements and so on. In this dissertation, we completed several cellular tests for different photochemical molecules. These experiments provide us more evidence to demonstrate the spectroscopy results of in vitro measurements. Furthermore, there are large potential use for developing more applications in medical areas such as disease detection and therapy.
3.6 Reference

1. Sumer, B.; Gao, J. *Nanomedicine* *2008*, 3, 137-140.


Cell Culture Protocol

Human lung adenocarcinoma epithelial cells (A549) were purchased from ATCC and is the only current cell line in the lab. Cells are kept in a humidified incubator at 37 °C with 5% CO₂.

The cells are cultured in Ham’s F-12K (Kaighn’s) medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotics (penicillin/streptomycin, 100 U/mL). To make complete F-12K medium for culturing A549 cells follow the following steps:

1. Turn on the UV for the hood 10 minutes prior to working in it.
2. Spray the hood down with the 70% alcohol mixture and wipe dry with paper towel.
3. Put the following items in the hood (after warming them up in the 37 °C water bath and then spraying with the alcohol mixture): a new bottle of incomplete F-12K (500 mL), FBS, and the penicillin.
4. Take out 55 mL of the incomplete F-12K media using a 55 mL pipette and transfer into a falcon tube. Label this falcon tube as “incomplete F-12K and the date”.
5. Using another 55 mL pipette, add 50 mL of FBS to the remainder of the incomplete F-12K bottle.
6. Then using a 5 mL pipette, add 5 mL of penicillin to the incomplete F-12K bottle.
7. Swirl gently to ensure mixing without causing bubbles.
8. Label the bottle as “Complete and date and ARL”. The mixture will be a red color at first and then turn to a pink color over time.

9. Store the complete media in the fridge.

10. The FBS is stored in Dr. Zoltowski’s lab in the −80 °C freezer. Make 4 aliquots of 50 mL FBS in falcon tubes to keep in the fridge before storing in the freezer.

To passage A549 cells, follow the following steps:

1. Check the confluence of the cells. The confluence should be around 80–95% before passaging. Otherwise, wait another day or two to allow the cells to proliferate more.

2. Turn on the UV for the hood 10 minutes prior to working in it.

3. Spray the hood down with the 70% alcohol mixture and wipe dry with paper towel.

4. Put the following items in the hood (after warming them up in the 37 °C water bath and then spraying with the alcohol mixture): the complete F-12K media, trypsin, and PBS.

5. To start, remove the cell flask from the incubator and aspirate off the old media.

6. Wash away the dead cells by rinsing the cells with 10 mL of PBS (use a 10 mL pipette). Then aspirate off the PBS.

7. Add 2 mL of trypsin to the flask. Move the plate around to ensure all the cells are coated with trypsin. Then incubate for 10 minutes to allow the trypsin to break the bonds between the cell surface and the flask.

8. Then bring the flask back to the hood and add 10 mL of complete F-12K media. Swirl the flask slightly to make sure all the cells are in the media.

9. Using the same 10 mL pipette, draw up the entire contents of the flask back into the pipette and transfer into a falcon tube.
10. Tightly cap the flacon tube and put it into the centrifuge for 10 minutes at 1000 rpm.
11. Aspirate off the supernatant. Then add about 1 mL of complete media to the falcon tube. Disperse the cells evenly throughout by using a 1000 μL pipette and pipetting up and down multiple times.
12. Add 20 mL of complete F-12K media to a new T75 flask.
13. Add 20 μL of the cells to the flask and gently rock the flask back and forth to disperse the cells. Check the confluence of the cells. If more cells are needed, continue to add cells and check the confluence.
14. Lastly, label the flask with the your initials, the date, the cell line (A549), and the passage and generation number. Store in the incubator.

The frozen cells are kept in a liquid nitrogen tank and a binder is kept nearby with the inventory of the frozen cells. To freeze cells, follow the following steps:

1. Freezing more cells should be done after 2 or 3 passages from newly thawed cells. This allows the cells to grow enough before freezing again.
2. The confluence should be around 80–90 % before freezing.
3. Prepare 10 mL the freezing media (10% cell-culture grade DMSO in complete media). This will make 10 vials for freezing.
4. Follow all cell passaging steps like normal up until the centrifuge step.
5. After the centrifuge step, aspirate off the media.
6. Add 1 mL of the cell freezing media. Disperse the cells throughout the media by pipetting up and down with a 1000 μL pipette.
7. Then add the rest of the freezing media to the falcon tube and mix.
8. Use the cryopreservation vials to freeze the cells. Add 1 mL of the cell mixture to each vial. Cap the vials tightly and label with the cell type, generation and passage number, date, and initials. Parafilm the lids of the vials.

9. Put the vials in the Mr. Frosty vial container and put into the –80 °C freezer in Dr. Zoltowski’s lab for 3 days.

10. After 3 days, transfer the vials into the liquid nitrogen container.

11. Fill out the cell inventory in the liquid nitrogen storage chart.

To thaw cells, follow the following steps:

1. Turn on the UV for at least 10 minutes prior to starting.

2. Put the complete F-12K media in the water bath and allow to reach 37 °C.

3. Check the inventory of the cells to locate the vials in the liquid nitrogen storage chart. Pull up the cells from the liquid nitrogen tank labeled as B. Let the liquid nitrogen drip back into the tank before pulling it all the way out. Use the thick orange gloves when handling the storage containers in the liquid nitrogen tank.

4. Take out 2 vials and allow them to thaw on the counter for about 5–10 minutes.

5. Spray down the hood with the 70% alcohol spray.

6. In the hood, draw up the cells from the vials and transfer into a falcon tube.

7. Add complete F-12K media to the falcon tube so the total volume is about 10 mL.

8. Centrifuge for 10 minutes at 1000 rpm.

9. Then remove the media from the falcon tube, leaving the pellet of cells.

10. Add 1 mL of complete F-12K media to the falcon tube and pipette up and down to mix.

11. Add about 20 mL of complete F-12K media to a new T75 flask.
12. Add ALL the cells from the falcon tube into the T flask.

13. Label the flask with your initials, the date, the cell line (A549), and the passage and generation number. (If the cell vial thawed was labeled “P_2G_2”, then this new flask would be labeled “P_1G_3”. Store the cells in the incubator.

14. The next day, remove the media and wash the cells with 10 mL PBS. Then aspirate off the PBS and add 20 mL of complete F-12K media. The purpose of this step is to remove the dead cells that did not survive the freezing and thawing cycle.
SCANNED 1H AND 13C NMR SPECTRA
**Figure S9.** $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 10.