Development of Ultra-Sensitive Fluorescence Photoamplification Assays for the Detection of Molecular Recognition Events

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DEVELOPMENT OF ULTRA-SENSITIVE FLUORESCENCE PHOTOAMPLIFICATION ASSAYS FOR THE DETECTION OF MOLECULAR RECOGNITION EVENTS

A Dissertation

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In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

by

Tiffany Priscilla Gustafson

June 2010

Advisor: Andrei G. Kutateladze
Abstract

During the course of this research a novel method which couples the molecular recognition-triggered photoamplification chain in diaryl ketone adducts of dithiane with a "turn-off" or “turn-on” fluorescence-based assay for the detection of biological targets and ligands, regardless of their nature, through a molecular recognition event has been developed. This research has included several key steps, the most significant being: (1) the design of fluorophore adducts or dyads which recover fluorescence upon photocleavage for a “turn-on” assay and the identification of fluorophores which are quenched upon the photochemical release of a quencher for a “turn off” assay; (2) Optimization of the photochemistry and photoamplification of dithiane adducts of benzophenone as it applies to the development of an ultra-sensitive photoamplified fluorescence assay; (3) implementation of the method in the design and fabrication of chips for ultra-sensitive screening of microarrays, and (4) integration of this microchip assay into a fluorescence based signal transducer for ultra-sensitive detection of molecular recognition events. Proof of concept utilized biotin-avidin recognition, where avidin is coupled to the photochemical sensitizer.
Acknowledgements

There are many people who have helped me on my way to writing this dissertation. There are a few though, that deserve special thanks, as without their help I would not be where I am today. First I must thank my advisor, Dr. Andrei Kuateladze, who has offered his guidance during the course of this research and helped me to develop the skill necessary to move forward as a chemist and scientist. Next, I need to thank the faculty and staff of the Department of Chemistry and Biochemistry, especially my committee members: Dr. Sandra Eaton, Dr. Joseph Hornback, Dr. Keith Miller, and Dr. Michelle Knowles for their support and assistance during the course of this research. I also thank Dr. Joe Angleson who has graciously agreed to chair my defense committee. I would also like to thank all the people who have worked with me in the laboratory, especially Greg Metzel who has worked on this project with me over the course of the last three years.

Finally I need to thank my family, and especially my parents Rhodes and Shelley Gustafson. Without their support and their belief in me, at a time in my life when completing my bachelor’s degree let alone my Ph.D. was considered unlikely, I would never have had the opportunity to be here today. For this, their continuing encouragement, and many other things I thank them.
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<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>$A_{\text{std}}$</td>
<td>absorbance of the standard (OD) @ $\lambda_{\text{max}}$</td>
</tr>
<tr>
<td>$A_{\text{unk}}$</td>
<td>absorbance of the unknown (OD) @ $\lambda_{\text{max}}$</td>
</tr>
<tr>
<td>BET</td>
<td>back electron transfer</td>
</tr>
<tr>
<td>BP</td>
<td>benzophenone</td>
</tr>
<tr>
<td>PyBOP</td>
<td>benzotriazol-1-yl-oxytrypyrrolidinophosphonium hexafluorophosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>biobarcode assay</td>
</tr>
<tr>
<td>biotin capped lipid</td>
<td>1,2-Dipalmitoyl-\textit{sn}-Glycero-3-Phosphoethanolamine-N-(Cap Biotinyl)(Sodium Salt)</td>
</tr>
<tr>
<td>$k_B$</td>
<td>Boltzmann’s constant ($1.38 \times 10^{-23} \text{ J K}^{-1}$)</td>
</tr>
<tr>
<td>bp</td>
<td>boiling point</td>
</tr>
<tr>
<td>CCl$_4$</td>
<td>carbontetrachloride</td>
</tr>
<tr>
<td>CCD</td>
<td>charge coupled device</td>
</tr>
<tr>
<td>C6</td>
<td>coumarin 6</td>
</tr>
<tr>
<td>1,4-DCB</td>
<td>1,4-dichlorobutane</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DIPEA</td>
<td>Diisopropyl ethyl amine</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DPPC</td>
<td>dipalmitoylphosphatidylcholine</td>
</tr>
<tr>
<td>DT-BP adduct</td>
<td>1,3-dithiane benzophenone adduct</td>
</tr>
<tr>
<td>DT-EBP adduct</td>
<td>1,3-dithiane ethylbenzophenone adduct</td>
</tr>
<tr>
<td>DT-OBP adduct</td>
<td>1,3-dithiane octylbenzophenone adduct</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ET</td>
<td>electron transfer</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbant assay</td>
</tr>
<tr>
<td>ε</td>
<td>extinction coefficient</td>
</tr>
<tr>
<td>FL</td>
<td>fluorescence</td>
</tr>
<tr>
<td>$\lambda_{em}$</td>
<td>fluorescence emission maximum</td>
</tr>
<tr>
<td>$\lambda_{ex}$</td>
<td>fluorescence excitation maximum</td>
</tr>
<tr>
<td>$\tau_F$</td>
<td>fluorescence lifetime</td>
</tr>
<tr>
<td>ν</td>
<td>frequency</td>
</tr>
<tr>
<td>$R$</td>
<td>gas constant (8.314 J K$^{-1}$ mol$^{-1}$)</td>
</tr>
<tr>
<td>$\Delta G^\pm$</td>
<td>Gibbs energy of activation</td>
</tr>
<tr>
<td>$S_0$</td>
<td>ground state energy</td>
</tr>
<tr>
<td>HTS</td>
<td>high through-put screening</td>
</tr>
<tr>
<td>HOMO</td>
<td>highest occupied molecular orbital</td>
</tr>
<tr>
<td>ID</td>
<td>inner diameter</td>
</tr>
<tr>
<td>IL</td>
<td>ionic liquid</td>
</tr>
<tr>
<td>LFP</td>
<td>laser flash photolysis</td>
</tr>
<tr>
<td>τ</td>
<td>lifetime</td>
</tr>
<tr>
<td>LED</td>
<td>light emitting diode</td>
</tr>
<tr>
<td>LMGO’s</td>
<td>low molecular weight organogelators</td>
</tr>
<tr>
<td>LUMO</td>
<td>lowest unoccupied molecular orbital</td>
</tr>
<tr>
<td>MMP</td>
<td>magnetic microparticle probe</td>
</tr>
<tr>
<td>MDT-BP adduct</td>
<td>2-methyl-1,3-dithiane benzophenone adduct</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>μARCS</td>
<td>microarrayed compound screening</td>
</tr>
<tr>
<td>M</td>
<td>molarity (moles/L)</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar (millimoles/L)</td>
</tr>
<tr>
<td>np</td>
<td>nanoparticle</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinamide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>OD</td>
<td>outer diameter</td>
</tr>
<tr>
<td>ppb</td>
<td>parts per billion</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>PA&lt;sub&gt;std&lt;/sub&gt;</td>
<td>peak Area of the standard ( \lambda_{em} )</td>
</tr>
<tr>
<td>PA&lt;sub&gt;unk&lt;/sub&gt;</td>
<td>peak Area of the unknown at ( \lambda_{em} )</td>
</tr>
<tr>
<td>( h )</td>
<td>planck’s constant (6.63 ( \times ) 10&lt;sup&gt;-34&lt;/sup&gt; J s)</td>
</tr>
<tr>
<td>PCX</td>
<td>plano-convex</td>
</tr>
<tr>
<td>POPS</td>
<td>L-( \alpha )-phosphatidylcholine (egg, chicken)</td>
</tr>
<tr>
<td>PMT</td>
<td>photomultiplier tube</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PVA</td>
<td>polyvinyl alcohol</td>
</tr>
<tr>
<td>p-QPh</td>
<td>p-quaterphenyl</td>
</tr>
<tr>
<td>PFG</td>
<td>pulse field gradient</td>
</tr>
<tr>
<td>( \Phi_F )</td>
<td>quantum yield of fluorescence</td>
</tr>
<tr>
<td>( \Phi_{F, std} )</td>
<td>quantum yield of fluorescence for the standard</td>
</tr>
<tr>
<td>( \Phi_{sens} )</td>
<td>quantum yield of sensitized cleavage</td>
</tr>
<tr>
<td>( \Phi_{self} )</td>
<td>quantum yield of self cleavage</td>
</tr>
</tbody>
</table>
\( k \) reaction rate constant

SAAC signal amplification by allostERIC catalysis

SET single electron transfer

\( S_1 \) singlet energy state

SMM small-molecule microarray

c speed of light (3.0 x 10^8 m/s)

\( K_{SV} \) Stern-Volmer Quenching Constant \((I_0/I = 1 + K_{sv} [Q])\)

SA surface area

T temperature

p-TPh p-terphenyl

t-BuPh tert-butylbenzene

THF tetrahydrofuran

Pristane 2,6,10,14-tetramethylpentadecane

TX thioxanthone

TXA thioxanthoneamide

TMS trimethylsilane

\( T_1 \) triplet energy state

\( \lambda \) Wavelength
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Chapter 1: Introduction

During the past several years the Kutateladze research group has systematically studied the photochemistry of dithiane adducts of various aldehydes and ketones. More recently their use in applications such as the design of photolabile lipids [1,2] and photoactive barbiturate receptors,[3] the screening of solution phase combinatorial libraries,[4] and the design of bioanalytical molecular recognition devices has been explored.[5] My research began with a study of the sensitized mesolytic fragmentation of dithiane-ketone adducts [6] which supported the mechanism for adduct cleavage previously reported [7,8] and progressed toward the major topic of this thesis - ultra-sensitive photoamplified fluorescence detection of molecular recognition events.

My task was to develop a novel method which couples the molecular recognition-triggered photoamplification chain in diaryl ketone adducts of dithiane with a "turn-off" or “turn-on” fluorescence-based assay for the detection of biological targets and ligands, regardless of their nature, through a molecular recognition event. This research has included several key steps, the most significant being: (1) optimization of photochemistry as it applies to the development of an ultra-sensitive photoamplified fluorescence turn-off assay, (2) implementation of the method in the design and fabrication of chips for ultra-sensitive screening of microarrays, and (3) integration of this microchip assay into a fluorescence based signal transducer for ultra-sensitive detection of molecular recognition events. Before presenting my own research it is important to understand the
general concepts which are prevalent in it, and the work of others, both in the Kutateladze group and outside of it, which explains the relevance and importance of this research along with its broader impact in science.

A. Chemical Microarrays

The end goal of my research is to develop a bioanalytical amplification assay which is compatible with high throughput screening (HTS) of small molecules on a microarray chip. Gene arrays for HTS will not be discussed as they do not concern this project. HTS is currently the quickest and most economical method for identifying lead compounds in the development of new drugs. However, the development of HTS requires the miniaturization and automation of biological assays. This miniaturization has lead to the search for new assays, such as those discussed in the next section, which can be made into chemical microarrays. A key problem with developing these arrays has been that they must screen a diverse group of compounds with different structures, properties, and characteristics while each individual reaction is isolated in an individual spot or well.[9] Their inability to do this leads to the need to run multiple assays for different compound types and does not allow for the screening of compounds which cannot be immobilized onto these microarrays. This means there is a need for assays which can account for diverse groups of compounds being screened simultaneously.

There are currently three common classifications into which HTS microarrays for small molecule screening fall: (1) chemical microarrays with immobilization technology [10,11]; (2) dry chemical microarrays [12,13]; and (3) solution-phase chemical microarrays.[14,15] Of these three classifications only the first two have been broadly
developed. For example, assays of classification 2 were developed by Abbot Laboratories™ and have been licensed by Discovery Partners International™. Assays of classification 1 are marketed by Graffinity™ which works with several drug companies (Elan™, AstraZeneca™, Rigel™, etc.) to provide HTS for drug discovery. On the other hand, there are very few assays in classification 3 that have been successfully designed and even these tend to have drawbacks.

A.1. Chemical microarray with immobilization technology

The first chemical microarrays were developed in the late 1990’s when many of the original studies were published by Schreiber’s lab at Harvard.[10,11] These small-molecule microarrays (SMM’s) (Figure 1.1) can utilize a diverse number of linking techniques to immobilize ligands onto a surface such as gold or glass. Once ligands are immobilized biological targets are applied to the surface which is then washed to prevent non-specific and weak binding. Lead compounds are then identified by immunoassays which rely upon fluorescence to identify the lead compounds.

While these arrays allow for the screening of thousands of compounds or pharmacophores at once, and are useful in identifying receptors binding lead compounds, they do have their limitations. Not only does the immobilization of a large diversified chemical library onto the array pose a challenge, but factors which can effect target binding - linker length and flexability, ligand orientation, spatial hindrance on the surface, etc. - must also be optimized and may be different from one target to the next.[9]
A.2. Dry chemical microarray

The dry chemical microarray (Figure 1.2) was originally designed by Abbott Laboratories™ as a way to circumvent the limitations imposed by immobilization in the design of SMM’s. This process, called microarrayed compound screening (μARCS),[12,13] arrays chemical compounds (analytes) onto dried polystyrene sheets in DMSO which is removed by drying. The enzyme and substrate are imbedded into an agarose gel which is applied to the array surface and a second gel is applied which contains radioactive ATP, the substrate, which initiates a biological reaction. These reactions can then be detected using a phosphorimager.

A major drawback of this technology is that the compounds on the surface must be resolubilized. Because the rate of diffusion and capability for resolubilization of each molecule varies, the outcome of the array is affected. A time differential in the rate at which a positive binding event takes place during HTS results, making the inhibition profiles of the compounds screened incomparable on a single array.[9]
Figure 1.2. Dry chemical microarray taken from reference. [9].

A.3. Solution-phase chemical microarrays

One of the first attempts to create a solution-phase microarray was by Houseman in 2002.[16] This array utilized a 384 well plate for the mixing of reagents before applying them to a substrate chip, making it only partially a solution-phase microarray. In 2003 Diamond’s group at the University of Pennsylvania published one of the first successful “true” solution-phase arrays (Figure 1.3).[15] In this assay glycerol is added to the solution of potential drug molecules to prevent evaporation from the surface of the substrate chip, keeping the molecules in solution at all times once they are printed onto the microarray. After the potential drugs are printed the biological targets are added by aerosol deposition. When a substrate is necessary to catalyze a reaction it can be immobilized before addition of the analytes in solution are sprayed on following application of the target. The reactions are then detected by a traditional enzyme-linked immunosorbant assay (ELISA) techniques.

While this is a solution-phase microarray it is still designed like a traditional chip based microarray in that the analytes are spotted onto the substrate chip in “pools” of
solvent. There are still no walls which delineate individual pores or wells for each reaction, which would be ideal to guarantee that there is no mixing of reaction “pools”. Additionally, this technique and others like it require several expensive, time consuming, and labor intensive steps, which cannot be carried out in the field, to detect binding.

Due to the drawbacks of currently developed HTS methods, such as those described above, and because it is currently the quickest and most economical route to the identification of lead compounds in the development of new drugs, there is a substantial need to develop better HTS arrays. However, because the cost of replacing old systems, such as microtiter-plate-based assays, is large it would be ideal if new solution-phase arrays could be inexpensive and rely upon inexpensive equipment to determine results.[9]

Figure 1.3. Solution-phase chemical microarray taken from reference. [9].
B. Previous Developments in the Design of Bioanalytical Amplification Assays

My goal is to develop a bioanalytical amplification assay. This assay couples a photoamplification chain with a "turn-off" or “turn-on” fluorescence-based assay for the detection of biological targets and ligands, regardless of their nature, through a molecular recognition event. Thus, it is important to understand previous strides in the literature which utilize amplification for the detection of similar events. At the same time drawbacks of these methods will be explored to provide an understanding of why new methods are currently needed.

B.1. The original use of amplification in bioanalytical assays

The use of amplification assays has long been an area of primary focus in bioanalytical sciences, especially since modern methods for drug discovery require testing of small molecules for binding to molecular targets via high throughput screening. The first bioanalytical amplification assays, developed by A. Coons,[17,18] were based upon antigen-antibody interactions where the amplification systems correspond to an indirect or “sandwich” procedure for immunofluorescence detection, upon which today’s ELISA’s are based. These assays rely upon the specific interaction between antigens and antibodies. Generally the surface is coated with an antibody that binds to a specific antigen in the sample. The sample is exposed to a second antibody which is covalently attached to a florophore or enzyme that eventually relays a signal indicating detection of the antigen. While the original techniques have been largely refined over the years to include variations such as time resolved immunoassays, energy-transfer immunoassays, and fluorescence polarization immunoassays, they are still restricted to screening of
antibodies or antigens.[19] Even though current detection limits of ELISA can be below 10 ng/mL of analyte they are not applicable in the HTS of potential drug candidates, unless coupled to other detection assays.[20-23]

**B.2. Recently developed bioanalytical amplification techniques**

More recent developments in bioanalytical techniques which utilize signal amplification include PCR amplification,[24,25] the bio-bar-code based detection system developed by C. Mirkin,[23,26] massive liquid crystal reorientation,[27] allosteric catalysis,[28] and visible-light-polymerization based amplification.[29-32] PCR has become the leading amplification technique in molecular biology due to its ability to amplify very few copies, or a specific region, of DNA into several million. The original technique was developed in 1984 by K. Mullis [33,34] and has been modified over the years, creating new techniques such as (A) Multiplex-PCR,[35] which allows for the simultaneous use of multiple primers in a single PCR mixture to produce amplicons specific to different DNA sequences, (B) Hot-start PCR,[36] which reduces non-specific amplification at lower temperatures, and (C) Quantitative PCR,[37] which uses fluorophore-containing DNA probes to measure the amount of amplified product as amplification progresses. Today several microarrays based upon the use of PCR have been created.[23,25] These microarrays rely upon hybridization of oligonucleotides carrying a fluorophore, pre-amplified by PCR techniques, to the immobilized ligand on a microarray chip. After hybridization the assay can be screened by a fluorescence chip reader. The problem with this type of amplification and screening is that it is specific to DNA, expensive, time consuming, skilled-labor intensive, and not possible in settings
outside of a laboratory. Thus, it cannot be applied to amplifying the signal of a positive hit from a lead compound in a drug detection assay, nor can it be used as a portable screening method.

The biobarcode amplification (BCA) assay (Figure 1.4) was developed by C. Mirkin and coworkers for the detection of proteins and nucleic acids.[23,38] The original assay used a magnetic microparticle probe (MMP) containing an antibody specific for the target protein or antigen. After the target binds to the MMP a second nanoparticle (np) is introduced outfitted heavily with hybridized barcode oligonucleotides and a second antibody to recognize the antigen on the MMP. The MMP’s, bound to the barcode containing np’s, are then collected using a magnetic field, and the barcode DNA is dehybridized from the np’s, which are then removed. These barcode strands can be analyzed by traditional DNA detection methods, or by PCR-less detection which was also developed in the Mirkin lab.[38]

In 2007 Mirkin’s group introduced two new adaptations of this assay: (1) it was adapted for use with ELISA for the detection of HIV-1, showing a detection limit which is 100 times greater than conventional PCR assays and 150 times greater than traditional ELISA’s[39]; (2) the BCA assay was modified for use with fluorescent probes to detect double stranded bacterial genomic DNA with a detection limit of 2.5 fM (7.5 x 10^4 copies/50 uL).[40] While the BCA assays provide excellent amplification of a target signal with very low detection limits, they are still target specific to proteins, nucleic acids, and DNA and cannot currently be used to find lead compounds in high through-put (HTS) drug screening assays. They also require several steps to obtain the result and, in
some cases, rely upon PCR amplification to increase the concentration of barcode DNA after target detection.

Figure 1.4. BCA assay developed by C. Mirkin and coworkers for the detection of proteins and nucleic acids taken from reference. [38].

Two additional bioanalytical screening methods which employ amplification techniques for the screening of proteins and enzymes are (A) liquid crystal reorientation and (B) allosteric catalysis. (A) relies upon a change in the chemical or topographical structure at an interface where, as a result of a chemical or biological species, a new orientation of liquid crystals in contact with the surface is achieved (Figure 1.5). [27] While the use of liquid crystals to amplify an event at an interface does seem feasible for the detection of biomolecular binding events it does have the drawback of being target specific. In this array, for example, the authors concluded that the liquid crystals used in this study could be used to report the presence of phosphorylated peptides at interfaces, which would be useful in the study of protein kinase enzymes. Although it would be possible to screen multiple enzymes or proteins at one time, what could be adhered to the
surface and still allow for a change in liquid crystal orientation upon binding to its target would limit the assays’ usefulness in HTS for lead compounds.

Figure 1.5. Liquid crystal orientation reports biomolecule binding events. 4-pentyl-4’-cyanobiphenyl (A). The binding of protein changes the ordering of the liquid crystal near the interface (B). Images of samples (before and after protein binding) viewed using a polarizing microscope (C) taken from reference. [27].

Method (B), signal amplification by allosteric catalysis (SAAC), mimics a biological signal transduction pathway where a molecular recognition event between an external signal and a protein receptor is allosterically transduced into catalytically amplified chemical information via second messengers.[28] Two major areas where SAAC is applied are biocatalysis for the detection of DNA (Figure 1.6),[41] RNA, and ATP, and signal amplification by supramolecular and organometallic catalysis [42] to sense halides and metal ions.[28] Although SAAC is not limited to biological targets it is restrained by the fact that each assay is designed for a specific target, making it an unlikely choice for HTS.
Figure 1.6. Biocatalysis signal amplification. Binding of miRNA with the loop sequence C allowing fluorogenic substrate to bind A, which cleaves the substrate. This cleavage results in fluorescent products as a result of the termination of fluorescence resonance energy transfer (FRET). The fluorescent signal intensity reflects the concentration of the target miRNA molecules taken from reference. [28].

B.3. Visible-light-polymerization for the detection of molecular recognition events

The final amplification technique to be discussed, and the method most similar to the one developed in this body of research, is the use of visible-light-polymerization for the detection of molecular recognition events. In 2007 C.N. Bowman at the University of Colorado published the original assay (Figure 1.7) showing that the use of polymeric materials could generate an amplified response to a molecular recognition event, specifically the binding of the biotin-avidin pair. The assay works by incubating a surface containing the biotin-labeled species of interest with a macrophotoinitiator (a high molecular weight copolymer of acrylic acid, acrylamide, and lysine containing one or two pendant avidins and ~100 photoinitiator molecules) coupled to avidin. Biotin-
avidin recognition and photoinitiation of polymerization lead to formation of a polymer, giving a positive binding hit.[29,32]

Photoinitiation upon biotin-avidin recognition is caused by a radical initiator, (a derivative of 2-hydroxy-2-methylpropiophenone or eosin-5-isothiocyanate) which is part of the macrophotoinitiator (it is bound to the –COOH groups the copolymer). This macrophotoinitiator is coupled to avidin via an amide linkage to its lysines. Photoinitiation occurs upon the application of 365 nm UV light allowing for the formation of white polymers which can be easily viewed under a microscope (570 um diameter, 0.14 um thick). By using eosin, which is fluorescent, as the radical initiator the chips can be analyzed using a fluorescence scanner. With the use of an optical microscope and CCD camera 0.4 attomoles of biotin have been detected on the chip. This is approximately 10 biotinylated species per μm². The maximum amplification factor achieved by this assay is 10⁷, due to non-specific interactions that limit the sensitivity of the system.[29,32]

Unlike the previously discussed amplification methods, visible-light-polymerization has the ability to detect binding events regardless of their nature. To show the universality of this technique C.N. Bowman’s group has used it in the detection of nucleic acids,[31] oligonucleotides,[29] and antigens.[30] While this method is promising for use in HTS due to its ability to detect various target types there are a few drawbacks: (1) large spot sizes can limit the number of analytes on a chip; (2) amplification cannot be stopped once it has begun; (3) there is no separation between the radical polymerization and the biological analytes (they are in the same solution and linked together) and thus the radicals can react freely with the biological sample; and (4)
to screen various proteins in a library, all of them would have to be coupled to the macrophotoinitiator (a large copolymer described above), which, due to its large size, is likely to affect the proteins’ folding and its ability to bind to the analytes on the surface of the chip more than a few small molecules.

**Figure 1.7.** Visible-light-polymerization for the detection of molecular recognition events taken from reference. [32].

### C. Photochemistry

The chemistry upon which this newly designed bioanalytical amplification assay is based is photochemical and has originated in the Kutateladze laboratory. The following is a basic explanation of photochemistry and its importance for providing energy to chemical reactions. Most reactions require the input of external energy in order to overcome the activation barrier which leads to product formation. Generally this energy is supplied by heat (thermal reactions) or light (photochemical reactions).
Photochemical reactions differ from their thermal counterpart in several key ways: (A) the activation energy for the reaction is provided by light in place of heat, (B) the electronic distribution and nuclear configuration of a photochemically activated molecule are such that the excited molecule is an electronic isomer of the ground state molecule and differs greatly from its thermally activated form, and (C) because the molecule is in a photoexcited state the number of products accessible to it are larger than in the ground-state of a thermally activated reaction, as the excited molecule possesses excess energy as a result of photon absorption.

In order to compare the efficiency of these two energy types let us consider a reaction which requires \( \Delta G^\ne \approx 78 \text{ kcal/mol} \) of energy to overcome its activation barrier. The amount of energy necessary to achieve this barrier photochemically can be found in one mole of 365 nm light. To estimate the amount of heat required to reach this barrier in the ground state the Eyring-Polanyi equation was used \( k = A e^{-\Delta G^\ne /RT} \), where \( A = k_B T / h = 6 \times 10^{12} \text{ s}^{-1} \) at room temperature. For a reaction to proceed in a matter of minutes (\( \tau = 100 \text{ s} \)) the reaction would have to reach \( \sim 900 \text{ °C} \). It is fairly obvious that the use of light provides the only easily accessible route to this amount of energy.

In photochemical reactions the absorption of a photon by a molecule transforms light energy into electronic excitation energy, \( E = \frac{hc}{\lambda} \). The absorption of this light excites an electron from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO). This excitation leads the molecule from the ground state \( (S_0) \) to its excited singlet state \( (S_1) \). From the \( S_1 \) state the molecule can either (1) take part in a reaction, (2) fluoresce, (3) undergo internal conversion to return to the ground state, or (4) undergo intersystem crossing to the triplet state \( (T_1) \). It is the
T$_1$ state from which most of the photochemical reactions described in this body of research take place. If a reaction does not occur from T$_1$ then the molecule will decay to the ground state via phosphorescence or intersystem crossing (see Jablonski diagram Figure 1.8).[43]

**Table 1.1.** Energy conversion table taken from reference [43].

<table>
<thead>
<tr>
<th>Region</th>
<th>$\lambda$</th>
<th>nm</th>
<th>Wavenumber</th>
<th>$\Delta E$</th>
<th>$\nu$ sec$^{-1}$</th>
<th>Structure and motion involved in absorption or emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultraviolet</td>
<td>2,000</td>
<td>200</td>
<td>50,000</td>
<td>143</td>
<td>$1.5 \times 10^{15}$</td>
<td>Electrons-orbital motion</td>
</tr>
<tr>
<td></td>
<td>2,500</td>
<td>250</td>
<td>40,000</td>
<td>114.4</td>
<td>$1.2 \times 10^{15}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3,000</td>
<td>300</td>
<td>33,333</td>
<td>95.3</td>
<td>$1.0 \times 10^{15}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3,500</td>
<td>350</td>
<td>28,571</td>
<td>81.7</td>
<td>$8.7 \times 10^{14}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4,000</td>
<td>400</td>
<td>25,000</td>
<td>71.5</td>
<td>$7.5 \times 10^{14}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4,500</td>
<td>450</td>
<td>22,222</td>
<td>63.5</td>
<td>$6.6 \times 10^{14}$</td>
<td></td>
</tr>
<tr>
<td>Visable</td>
<td>5,000</td>
<td>500</td>
<td>20,000</td>
<td>57.2</td>
<td>$6.0 \times 10^{14}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5,500</td>
<td>550</td>
<td>18,182</td>
<td>52</td>
<td>$5.4 \times 10^{14}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6,000</td>
<td>600</td>
<td>16,666</td>
<td>47.7</td>
<td>$5.0 \times 10^{14}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6,500</td>
<td>650</td>
<td>15,385</td>
<td>44</td>
<td>$4.6 \times 10^{14}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7,000</td>
<td>700</td>
<td>14,286</td>
<td>40.8</td>
<td>$4.2 \times 10^{14}$</td>
<td></td>
</tr>
<tr>
<td>Infrared</td>
<td>10,000</td>
<td>1,000</td>
<td>10,000</td>
<td>28.6</td>
<td>$3 \times 10^{14}$</td>
<td>Nuclei-vibrational motion</td>
</tr>
<tr>
<td></td>
<td>50,000</td>
<td>5,000</td>
<td>2,000</td>
<td>5.8</td>
<td>$6 \times 10^{13}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100,000</td>
<td>10,000</td>
<td>1,000</td>
<td>2.86</td>
<td>$3 \times 10^{13}$</td>
<td></td>
</tr>
<tr>
<td>Microwave</td>
<td>1.00E+09</td>
<td>1.00E+08</td>
<td>10</td>
<td>$3 \times 10^{-2}$</td>
<td>$3 \times 10^{11}$</td>
<td>Electron spin-precessional motion</td>
</tr>
<tr>
<td></td>
<td>1.00E+11</td>
<td>1.00E+10</td>
<td>0.1</td>
<td>$3 \times 10^{-4}$</td>
<td>$3 \times 10^{9}$</td>
<td>Nuclei spin-precessional motion</td>
</tr>
<tr>
<td>Radiowave</td>
<td>1.00E+13</td>
<td>1.00E+12</td>
<td>0.001</td>
<td>$3 \times 10^{-6}$</td>
<td>$3 \times 10^{7}$</td>
<td>Nuclei spin-precessional motion</td>
</tr>
</tbody>
</table>
Photolabile groups are commonly used as protecting groups in organic synthesis as their removal does not require the use of a reagent(s) which interfere with other functional groups and must be removed from the product via a purif ication step, thus adding complexity to the total synthesis. By utilizing photolabile protecting groups deprotecting reagents can be eliminated, as light causes deprotection to occur.[44] For several photolabile protecting groups the deprotection mechanism and procedures are well developed and understood. These groups include: (a) o-nitrobenzyl protection of alcohols, carboxylic acids,[44,45] and thiols, (b) benzyl alcohol protection of
amines,[46,47] and most importantly to this research (c) dithiane protection of ketones and aldehydes.[6,7,48]

C.2. Dithiane protection of aldehydes and ketones

The addition of dithiane to aldehydes and ketones, via 2-lithio-1,3-dithiane, was shown to have excellent yields by Corey and Seebach in 1965 (Scheme 1).[49] It has since been shown that dithiane can be treated as a photoprotecting group for ketones and aldehydes. Traditionally, it is deprotected via photooxidation as a dithiacetal, but in the Kutateladze group a completely orthogonal method has been developed. They have found that the entire dithiane group can be photocleaved via single electron transfer (SET) initiated C-C bond fragmentation when irradiated in the presence of a sensitizer molecule.[48] This C-C fragmentation generally occurs when a donor molecule is irradiated in the presence of an acceptor molecule resulting in SET leading to the formation of a radical ion. The radical ion can then take part in several different reactions (cyclodimerization, isomerization, addition and elimination reactions, etc.), the most common being hydrogen abstraction which leads to C-C bond fragmentation.[50]

Scheme 1.1. Dithiane addition

This SET initiated photocleavage of dithiane adducts (dithiane protected ketone or aldehyde which is an electron donor) requires a triplet sensitizer (electron acceptor)
(Scheme 2) and has a general mechanism as follows: (1) benzophenone (BP) (sensitizer) is excited to its triplet state by irradiation with 365 nm light, (2) SET occurs from adduct to BP forming the cation and anion radical pair, (3) hydrogen abstraction from the adduct, by BP, leads to intramolecular electron transfer to form the O radical, (4) the formation of the O radical induces photochemical cleavage of the adduct leading to (5) the reformation of dithiane and a deprotected ketone or aldehyde.[6,7,48]

**Scheme 1.2. Photocleavage of dithiane adducts**

C.3. Utilizing dithiane protected ketones and aldehydes (adducts) as photolabile scaffolds for molecular recognition

In recent years the use of dithiane protected ketones and aldehyde adducts as photolabile scaffolds for molecular recognition has been explored. These molecular scaffolds are capable of efficient photoinduced fragmentation, but only in the presence of an external electron transfer (ET) sensitizer. The critical feature of such systems is that they are not photolabile until an external event brings its respective parts (sensitizer and adduct) into the proximity of each other, arming the system and making it photolabile. Molecular recognition can be such an external event.
In previous years the K-group has focused on the design of a photolabile system based upon the concept of conditional photofragmentation in artificial barbiturate receptors.[3] Photofragmentation in this system was based upon the docking of the barbiturate carrying sensitizer (the “ligand”) to the receptor (containing adduct). The result was photofragmentation of the adduct (Figure 1.9).

![Figure 1.9. Photoactive barbiturate receptor taken from reference. [3].](image)

The group then applied the idea of photolabile scaffolds for molecular recognition towards triggering an observable photochemical effect due to the molecular recognition of the biotin-avidin pair. This research has been developed into a novel methodology for the direct screening of solution-phase libraries encoded with photolabile tags.[4] Figure 1.10 gives a general outline for the screening of micelle-solubilized libraries of ligands L, encoded with the tethered tags T. The receptor R is outfitted with an ET-sensitizer S, and incubated with the library (A). The host-guest binding brings the sensitizer into the vicinity of the adduct (B), which upon irradiation (C), triggers the release of dithiane tags (D) into the solution, where they are analyzed revealing the identity of the lead
compound. The next logical question was whether such a molecular recognition event could trigger not just one fragmentation, but set off a progression of photochemical events (photoamplification).

Figure 1.10. Screening of micelle-solubilized solution phase libraries encoded with photolabile tags taken from reference. [4].

C.4. Photoamplification and its use with photolabile scaffolds for molecular recognition

Photoamplification is a unique case where the adduct used is also a masked sensitizer. During BP (or xanthone) sensitized mesolytic fragmentations of diarylketones (masked sensitizers) a sensitizer itself is released (Scheme 1.2, page 19, where $R^1$ is aromatic). This results in continued generation of sensitizer that is capable of carrying the photofragmentation chain, which we call photoamplification. Figure 1.11 illustrates a typical result obtained when the photoamplification is monitored. 3 mol % of 4,4-dimethoxybenzophenone was added to its dithiane adduct in acetonitrile and irradiated, while UV absorption of the solution was monitored at 360 nm. An autocatalytic curve
was obtained, indicating that photorelease of the masked ketone accelerated this bimolecular fragmentation.[51]

**Figure 1.11.** Diarylketone sensitizing its own unmasking as monitored by UV absorption at 360 nm (Ar = MeOPh) taken from reference. [51].

We must remember though that ultimately, at a shorter wavelength, any adduct is capable of self-cleavage. Thus photoamplification is feasible only if, at a given wavelength, the sensitized fragmentation is overwhelmingly more efficient than self-cleavage, i.e., \( \varepsilon_{ket} \Phi_{sens} \gg \varepsilon_{add} \Phi_{self} \) where \( \varepsilon_{ket} \) and \( \varepsilon_{add} \) are the extinction coefficients of the free sensitizer and its adduct, and \( \Phi_{sens} \) and \( \Phi_{self} \) are the quantum yields of the sensitized process and self-cleavage. The addition of dithianes to aromatic ketones disrupts conjugation, causing large blue-shifts in absorbance. Yet, some adducts retain significant absorptions at irradiation wavelengths - in thioxantheres this is due to the surviving diphenylsulfide moieties and quantum yields of self-cleavage remain rather high.[52] The adducts of BP and xanthone which are either unsubstituted or substituted with groups which are not electron donating, for example benzophenone-3- and xanthone-2-carboxamides, generally show no detectable self-cleavage when irradiated for 5 h above 320 nm at 9.5 \( \times \) 10\(^{19} \) photons/h. The sensitized reaction in solution is fully completed within 1.5 h at this flux.
Based upon this data the next logical question was whether a molecular recognition event, could trigger not just one fragmentation, but set off a progression of photochemical events (photoamplification), enhancing the sensitivity of detection (Figure 1.12). Recently, this concept was tested by planting 5% of free sensitizer on polystyrene high loading beads (PS-NH₂ 1.86 mmol/g, 200 μm) to emulate a small amount of the initiator, brought in by a molecular recognition event. The rest of the surface material was the masked sensitizer. The beads were suspended in MeCN, purged with argon, and irradiated with a 320 nm long pass filter, and dithiane release was monitored by GC/MS. Figure 1.13 shows a typical release profile: dithiane concentration increases, reaching a maximum, followed by a slow decrease due to secondary photodegradation. Control experiments were also performed, ruling out photocleavage due to interbead sensitization and self-cleavage.[51]

![Figure 1.12.](image)

**Figure 1.12.** Photoamplification initiated by molecular recognition. Incubation with a receptor, modified with a tethered sensitizer, brings the sensitizer into the vicinity of adducts (A, red arrow). As irradiation commences, the initiator unMASKS proximal sensitizers on the surface which, in turn, can sensitize the unmasking of their neighbors (B, red arrows) yielding more sensitizer taken from reference. [51].

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The next question brings me to the topic of my dissertation research: can the photoamplification of these adducts be utilized in an ultra-sensitive fluorescence assay for the detection of molecular recognition events. In order to design such an assay the following must be undertaken: (1) optimize photochemistry of the adduct; (2) identify fluorophores which are quenched by one or both product(s) of photocleavage (2-alkyl-1,3-dithiane and/or benzophenone) but not the adduct, and prove that photoamplification can result in quenching of these fluorophores; (3) optimize the assay in bulk solvent (including the optimization of the adduct for solubility); (4) design and fabricate a microarray chip and imager for the detection of molecular recognition events; and (5) demonstrate that the designed bioanalytical microarray works using the biotin-avidin binding pair.
Chapter 2: Optimization of Photochemistry for the Adducts of Dithiane with Diarylketones

The first step in applying the photoamplification of di/tri-thiane adducts of ketones (sensitizers) to the development of a fluorescence assay was to obtain a better understanding of the reaction mechanism for photocleavage and optimize the quantum yield of sensitized cleavage ($\Phi_{sens}$). In order to increase $\Phi_{sens}$ of the adducts we utilized an alkyl tail at $R_1$ or $R_2$ of dithiane (Figure 2.1.).

Figure 2.1. Dithiane which can be substituted at Carbon 2 or 5.

![Figure 2.1](image)

A. The Mechanism for Photocleavage of Dithiane Adducts of Ketones[6]¹

The Kutateladze group has previously found that dithianes are extremely suitable for use in reactions involving ET-induced fragmentations as they are easily synthesized and cleave efficiently upon photoinduced fragmentation. The mechanism of cleavage in these adducts has been investigated using classical physical organic methods such as determining the Hammett substituent effect, the kinetic isotope effect,[8] and laser flash photolysis (LFP).[7] While initial mechanistic findings were in keeping with the

¹ The research described in A has largely been taken or adapted from reference [6].
recognized ‘Grobe-like’ mechanism it was later established, by studying photoinduced fragmentation of the dithiane adduct of pivalaldehyde (A, Scheme 2.1.), that this mechanism needed refinement. Adduct A cleaved to produce the expected product, pivalaldehyde, and dithiane-2-carboxaldehyde as the major product in a 1:6.2 ratio.[53] As the ‘Grobe-like’ mechanism could not explain this product further investigation was undertaken, and suggested that deprotonation of the hydroxy group in the initially generated cation radical does not result in the formation of a charge separated ‘Grob-like’ precursor. Instead it produces a neutral oxygen-centered radical (or a species behaving as one) via intramolecular electron transfer. The O-centered radical undergoes subsequent fragmentation in either direction and the partitioning correlates with stability of the produced radicals (Scheme 2.2)[54]

**Scheme 2.1.** Photocleavage of pivalaldehyde adduct of dithiane, from reference [6].

**Scheme 2.2.** Proposed mechanism for cleavage of dithiane adducts which bypasses the ‘grobe-like’ mechanism’s charge separated species, from reference [6].
The case of tert-butyl derivatives, such as A, is unique in a sense that it allowed the Kutateladze group to discover this new channel in the mechanism of fragmentation simply by product analysis. For dithiane adducts of aromatic aldehydes and ketones the barrier for the aryl radical departure is prohibitively high, i.e. it is the dithiane radical which is always departing. Therefore, it was much more difficult to ascertain whether or not the original ‘Grob-like’ mechanism for cleavage competes with the anomalous O-centered radical mechanism (or either one of them is operating exclusively) in the case of aromatic adducts. Because of the presence of sulfur it is synthetically challenging, if not impossible, to generate the O-radical from an alternative precursor, for example, a peroxide, and study the effect of substituents on the rate of its degradation.

In order to further refine the mechanism of fragmentation in adducts of 2-alkyl substituted dithianes with benzophenone (BP) I began my work in the lab by carrying out an experimental and computational study of the mechanism of their photoinduced cleavage. BP-sensitized mesolytic fragmentations, in adducts of the sensitizer itself, result in generation of more BP and, as such, constitute its amplification. Hence, our particular interest in this system, which I plan to utilize in developing a fluorescence photoamplification assay.

A.1 Experimental study of adducts of 2-alkyl substituted dithianes with benzophenone

BP adducts 2a–e of unsubstituted (1a), 2-methyl- (1b), 2-ethyl- (1c), 2-hexyl (1d), and 2-decyl (1e) dithiane (Scheme 2.3) were synthesized according to a modified Corey–Seebach procedure,[55] and their photoinduced fragmentation was studied over a
temperature range from -40°C to +40°C in acetonitrile upon BP sensitization. The
driving force for the oxidative electron transfer from the dithiane moiety to the triplet
state of BP in acetonitrile is rather large; the one electron reduction potential of triplet BP
is -1.68 V (vs SCE in acetonitrile),[56] whereas various 2-substituted dithianes oxidize in
the range of +0.73 to +1.18 V in the same solvent.[57] During the course of a previously
carried out laser flash photolysis study[7] it was found that the rate of initial electron-
transfer quenching of triplet BP with dithiane-benzophenone adduct in dry acetonitrile
was near the diffusion limit, $8.4 \pm 0.7 \times 10^8 \text{M}^{-1} \text{s}^{-1}$. During this experimental study I
determined the dependence of the quantum efficiency of fragmentation on temperature,
with the goal of determining the apparent enthalpy of activation.

**Scheme 2.3.** Synthesis of benzophenone adducts of 2-alkyl-1,3-dithianes taken from reference [6].

Photolyses were carried out in a Rayonet carousel photoreactor with a Pyrex heat
exchange jacket cooled by ethanol circulating through a FTS Systems Multi-Cool
refrigeration unit. The quantum yield determinations for adduct disappearance were done
using the classic benzophenone – benzhydrol actinometer system as a standard to
improve the accuracy of quantum yield determinations.[58] Another important parameter determined was the percent recovery of the cleaved dithiane. Remember that both the ‘Grob-like’ mechanism and the O-radical mechanism, which Kutateladze’s group has proposed, give the dithiane-2-yl radical as the immediate fragmentation product. The fate of this radical is dependent upon multiple factors, including: (a) reactivity of the radical towards oxygen; (b) dimerization; and (c) disproportionation. Thus, the recovery of dithiane depends on the above factors and its rate of degradation due to secondary photooxidation by benzophenone.

We found that more substituted dithianes showed better recovery. This is in agreement with the overall trends in reactivity of the dithian-2-yl radicals (i.e., unsubstituted dithianyl radicals react/degrade faster) and of the final products – dithianes (i.e., less substituted dithianes undergo benzophenone-sensitized oxidative photodegradation faster). The quantum efficiency of cleavage also steadily increases for adducts of dithianes substituted with longer alkyl chains (Table 2.1). On the other hand, previous LFP experiments [7] showed that the initial electron transfer quenching rate increases with the decreased substitution, even though the adduct of unsubstituted dithiane shows poor efficiency of cleavage. Thus, it is difficult to determine the partitioning (between wasteful back electron transfer (BET) and the productive deprotonation of the adduct) of the initially formed dithiane cation radical – benzophenone anion radical pair.
Table 2.1. Quantum efficiencies and dithiane recovery rates at +20 °C for adducts 2b-e, taken from reference [6].

<table>
<thead>
<tr>
<th>Adduct</th>
<th>$\phi$</th>
<th>Dithiane recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl- (2b)</td>
<td>0.20</td>
<td>32.8</td>
</tr>
<tr>
<td>Ethyl- (2c)</td>
<td>0.23</td>
<td>35.3</td>
</tr>
<tr>
<td>Hexyl- (2d)</td>
<td>0.25</td>
<td>41.0</td>
</tr>
<tr>
<td>Decyl- (2e)</td>
<td>0.27</td>
<td>47.8</td>
</tr>
</tbody>
</table>

Whitten makes the assumption [59] that the only temperature-dependent process in a reaction of the geminate radical ion pair is fragmentation. Therefore we obtained the enthalpy of activation by plotting the data shown in Table 2.2 as $\ln(\phi/T)$ vs $1/T$ for the reaction of 2b and 2c (Figure 2.2). The activation enthalpies, $\Delta H^\neq$, are obtained from the slopes: 4.7 kcal/mol for the methyl derivative 2b and 1.5 kcal/mol for the ethyl derivative 2c. These values are very similar to the activation energies obtained by Whitten for the thioindigo sensitized fragmentation in vicinal amino alcohols.[59]

Table 2.2. Quantum yields of fragmentation and % dithiane recovery as a function of temperature, taken from reference [6].

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>Ethyl adduct 2c</th>
<th>Methyl adduct 2b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\phi$</td>
<td>Dithiane recovery, %</td>
</tr>
<tr>
<td>-40</td>
<td>0.04</td>
<td>0.7</td>
</tr>
<tr>
<td>-20</td>
<td>0.13</td>
<td>6.2</td>
</tr>
<tr>
<td>0</td>
<td>0.18</td>
<td>17.8</td>
</tr>
<tr>
<td>20</td>
<td>0.21</td>
<td>14.8</td>
</tr>
<tr>
<td>40</td>
<td>0.32</td>
<td>37.6</td>
</tr>
</tbody>
</table>
Figure 2.2. 1/T dependence of fragmentation quantum yields for ethyl (2c) and methyl (2b) adducts, taken from reference [6].

We propose that substitution at position 2 of dithiane accelerates fragmentation in the deprotonated species, the oxygen-centered radical. Walling and Padwa[58] studied the substitution effect in decomposition of hypochlorites of alkylidimethylcarbinols, R-C(Me)\(_2\)-O-Cl, by comparing the differences in activation energies for decomposition and hydrogen abstraction (Scheme 2.4). They reported that while the difference was 10 kcal/mol for tert-butoxy radical (i.e. \(R = \text{Me}\)), it decreased to 1.7 for \(R = \text{i-Pr}\), 0.7 for \(R = \text{benzyl}\), and was negligibly small for \(R = \text{t-Bu}\). The absolute value for the activation energy of fragmentation in tert-butoxy radical is 11-13 kcal/mol.[60] If we assume the rate of hydrogen abstraction does not change much, introduction of the tert-butyl group in place of methyl should lower the activation energy of fragmentation in these alkoxy radicals by about 10 kcal/mol, to approx. 1-3 kcal/mol. 2-Methyl-1,3-dithian-2-yl and 2-ethyl-1,3-dithian-2-yl are very stable (and bulky) radicals. Judging by previous observations the 2-methyldithianyl radical is more stable than tert-butyl, which in turn is more stable than the unsubstituted dithiane-2-yl radical.[53] Thus, it is not unreasonable...
to assume that the apparent $\Delta H^\circ$ of 4.7 and 1.5 kcal/mol correspond to fragmentation of the oxygen-centered radicals, releasing methyldithianyl and ethyldithianyl radicals respectively. These results provide evidence that the reaction takes place via the O-centered radical mechanism and not via the original ‘Grob-like’ mechanism in the case of adducts of benzophenone.

**Scheme 2.4.** Decomposition and hydrogen abstraction of alkylidimethylcarbinols.

\[
\begin{align*}
R_3C-O^\cdot & \rightarrow R_2CO + R^\cdot \\
R^\cdot + R(CH_3)_2OC & \rightarrow R(H_3C)_2C-O^\cdot + RCl \\
 & \quad \text{k_d} \quad CH_3COCH_3 + R^\cdot \rightarrow RCl \\
 & \quad \text{k_a} \quad R^\cdot + R(CH_3)_2OH \rightarrow RCl \\
\end{align*}
\]

**A.2. Computational study of adducts of 2-alkyl substituted dithianes with benzophenone**

In order to support my findings from section A.1, a computational study of the same dithiane adducts was undertaken. The computations for both the starting and transition states were run at the DFT, B3LYP/6-31G(d), level using Gaussian 03, revision C.02.[61] The respective oxygen centered radicals ($2b^\cdot$, $2c^\cdot$) were generated in Chem3D and pre-optimized at the AM1 level. Vibrational analysis of the DFT optimized geometries shows no imaginary frequencies for the computed ground states and only one imaginary frequency for the transition states corresponding to the reaction coordinate (i.e. C-C stretch). Due to the importance of conformational considerations, the conformational space was scanned and the relative energies of the starting alkoxy radicals and their respective fragmentation transition states were analyzed. The four major
conformers are: (i) equatorial 2-alkyl with the CO bond in anti conformation to this alkyl, denoted eMaO, i.e. equatorial Methyl anti Oxygen (the oxygen is anti to the methyl group), and eEaO for Ethyl; (ii) equatorial 2-alkyl – gauche CO bond, eMgO/eEgO; (iii) axial 2-alkyl – anti CO bond; aMaO/aEaO; (iv) axial 2-alkyl – gauche CO bond, aMgO/aEgO. The other two sets of gauche conformers are enantiomers of eMgO/eEgO and aMgO/aEgO. The relative energies of the starting oxy-radicals and their respective transition states are listed in Table 2.3 with the calculated $\Delta H^\ne$ summarized in the third column.

**Table 2.3.** ZPE corrected relative energies of the starting oxy-radicals and their transition states, taken from reference [6].

<table>
<thead>
<tr>
<th></th>
<th>Ethyl-derivative 2c• (O-radical)</th>
<th>Methyl derivative 2b• (O-radical)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS (kcal/mol) TS (kcal/mol) $\Delta H^\ne$ (kcal/mol)</td>
<td>SS (kcal/mol) TS (kcal/mol) $\Delta H^\ne$ (kcal/mol)</td>
</tr>
<tr>
<td>aEaO-2c•</td>
<td>5.4 10.0 4.8</td>
<td>aMgO-2b•</td>
</tr>
<tr>
<td>aEgO-2c•</td>
<td>4.5 23.0 18.0</td>
<td></td>
</tr>
<tr>
<td>eEaO-2c•</td>
<td>1.1 a –</td>
<td></td>
</tr>
<tr>
<td>eEgO-2c•</td>
<td>0.0 1.7 1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>a computations did not converge</td>
</tr>
<tr>
<td>aMgO-2b•</td>
<td>2.8 5.1 2.2</td>
<td></td>
</tr>
<tr>
<td>aMgO-2b•</td>
<td>0.9 a –</td>
<td></td>
</tr>
<tr>
<td>eMgO-2b•</td>
<td>0.0 1.7 1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The lowest energy conformations for both methyl and ethyl derivatives, the eMgO/eEgO conformers (Figure 2.3), had the smaller alkyl substituent in the equatorial position, whereas the bulky benzhydryl group was axial, with oxy-radical being *gauche* to methyl/ethyl (that is, *anti* to one of sulfur atoms). These conformers have the lowest
energies at both the ground and transition states. This is also in keeping with the computed conformational energies of the parent alcohols that showed at least a 2 kcal/mol preference for the eRgO conformers (Table 2.4). Conversely, the lowest energy conformer for the C-2 unsubstituted dithiane adduct of BP were those with an axial-H (aHaO, aHgO), being more stable by approximately 3 kcal/mol.

![Figure 2.3. The lowest energy conformers of the respective O-centered radicals: eMgO-2b• (left) and eEgO-2c• (right), taken from reference [6].](image)

**Table 2.4.** DFT relative energies for the conformers of alcohols 2a-c.

<table>
<thead>
<tr>
<th>Conformers</th>
<th>Rel. energy, kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>aMaO-2b</td>
<td>3.2</td>
</tr>
<tr>
<td>aMgO-2b</td>
<td>3.5</td>
</tr>
<tr>
<td>eMaO-2b</td>
<td>2.9</td>
</tr>
<tr>
<td>eMgO-2b</td>
<td>0.0</td>
</tr>
<tr>
<td>aEaO-2c</td>
<td>5.5</td>
</tr>
<tr>
<td>aEgO-2c</td>
<td>1.9</td>
</tr>
<tr>
<td>eEaO-2c</td>
<td>3.2</td>
</tr>
<tr>
<td>eEgO-2c</td>
<td>0.0</td>
</tr>
<tr>
<td>aHaO-2a</td>
<td>0.01</td>
</tr>
<tr>
<td>aHgO-2a</td>
<td>0.0</td>
</tr>
<tr>
<td>eHaO-2a</td>
<td>3.3</td>
</tr>
<tr>
<td>eHgO-2a</td>
<td>2.8</td>
</tr>
</tbody>
</table>
A.3. An NMR study of adducts of 2-alkyl substituted dithianes with benzophenone

In order to confirm the structural information obtained during the computational study in A.2, a low temperature NMR study of the adducts (parent alcohols to the oxy-radical) was undertaken. Initial analysis of the $^1$H NMR for each of the three adducts ($2a$, $2b$, and $2c$) shows a systematic upfield shift of the dithiane's $H_2C^{(3)}$ and $H_2C^{(5)}$ protons upon introduction of the methyl and then ethyl group (Figure 2.4). The downfield multiplet corresponds to the two axial $H_2C^{(3)}$ and $H_2C^{(5)}$ protons and the upfield two equatorials. Significant differences in the conformational behavior between the substituted and unsubstituted adducts becomes obvious when analyzing the low temperature NMR data.

Figure 2.4. 1D proton NMR spectra of $2a$-$c$ at 25 °C.
Figure 2.5 shows the temperature dependent changes in the \(^1\text{H}\) NMR spectra from -22 °C to -95 °C. In the methyl and ethyl derivatives of the adduct the multiplets for the axial and equatorial protons H\(_2\text{C}^{(3)}\) and H\(_2\text{C}^{(5)}\) split into two sets. If the rotation of the benzhydrol group has been stopped at this temperature this would indicate that the most stable isomer is unsymmetrical. The unsubstituted adduct does not show this divergence of the peaks for the axial and equatorial protons H\(_2\text{C}^{(3)}\) and H\(_2\text{C}^{(5)}\), indicating that its most stable isomer is likely symmetrical. Both of these findings are in agreement with our DFT calculations.

In order to prove that the split signals of the equatorial and axial protons for the ethyl and methyl derivatives belong to the same conformer, a COSY experiment was run at -95 °C. This experiment showed definitive cross-peaks for the respective split pairs of signals leading us to conclude that below -40 °C the equatorial (H\(_{eq}\text{C}^{(3)}\) and H\(_{eq}\text{C}^{(5)}\)) and axial (H\(_{ax}\text{C}^{(3)}\) and H\(_{ax}\text{C}^{(5)}\)) protons are not equivalent to each other. This result can only be explained if the adducts conformation is unsymmetrical, further confirming our computational findings.
While the findings in sections A.1., A.2., and A.3 do not rule out the ‘Grobe-like’ mechanism for photofragmentation they do provide further evidence that supports the alternative oxygen-centered radical mechanism. Both the experimental studies done on the parent adducts (alcohols), and computational studies of the oxygen centered radicals, showed apparent enthalpies of activation for photoinduced fragmentation which were in agreement. Additionaly an NMR study confirmed the computational results that the most stable conformation of the ethyl and methyl adducts of BP are unsymmetrical, while that of the unsubstituted adduct is symmetrical.
B. Increasing $\Phi_{\text{sens}}$ for Adducts of Dithiane with a Diarylketone Through 2- and 5-Alkyl Substitution in Dithianes.

Synthesis of 2-alkyl substituted dithianes is relatively straightforward and simple. It is accomplished via the addition of 1,3-propanedithiol to an aldehyde (Scheme 2.5). The resulting 2-alkyl-1,3-dithiane is then added to a ketone via our modified Corey–Seebach procedure[55] to produce the desired adduct $2a-e$ of unsubstituted ($1a$), 2-methyl- ($1b$), 2-ethyl- ($1c$), 2-hexyl ($1d$) and 2-decyl ($1e$) dithiane.

Scheme 2.5. Synthesis of 2-alkyl-1,3-dithianes.

Based upon the previous findings that (1) the rate of SET quenching of triplet benzophenone with dithiane-benzophenone adduct in dry acetonitrile was near the diffusion limit, $8.4 \pm 0.7 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, and (2) the rate of SET is slower for more substituted adducts due to triplet BP decaying before ET takes place, [7] one might think the $\Phi_{\text{sens}}$ would decrease when an alkyl chain is added to position 2 of dithiane. Instead the results from section A show the quantum efficiency of cleavage steadily increases for adducts of dithianes substituted with longer alkyl chains at position 2 (Table 2.1., $\Phi_{\text{sens}}$ for unsubstituted adduct is 0.12). This increase in $\Phi_{\text{sens}}$ is explained in two ways. First, because the rates of SET and BET should be similarly affected by the size of the alkyl group fewer adducts containing a large 2-alkyl substituent on dithiane may undergo SET (as compared to the unsubstituted dithiane or a dithiane with smaller alkyls), even fewer
of these adducts will participate in BET, leading to an increase in fragmentation of the adducts. Second, we believe that the 2-alkyl substituent sterically inhibits the recombination of the dithiane radical with its own cleaved ketone, slowing reformation of the oxygen centered radical. These two factors combined lead to an increase in the $\Phi_{\text{sens}}$ for adducts of 2-alkyl-1,3-dithianes with benzophenone which is based upon the length/size of the 2-alkyl substituent.

In order to increase adducts solubility (a topic to be discussed in more detail later) we pursued the design of dithianes substituted at position 5 with two alkyl chains with the hope that such substitution would either further increase or not effect $\Phi_{\text{sens}}$. Synthesis of 5-alkyl-1,3-dithianes (3a-d, Scheme 2.6) involves a 4 step reaction, as the starting 1,3-propanedithiols are not readily available. The adducts of 3a-d with benzophenone were then synthesized via the previously mentioned modified Corey–Seebach procedure.[55] Unfortunately, we discovered that the $\Phi_{\text{sens}}$ is lowered as a result of 5-alkyl groups. The relative $\Phi_{\text{sens}}$ of benzophenone adducts with 3b-c to adduct 2a and 2b are 37% and 53% respectively. We believe that this results is due to the 5-alkyl substituents “shielding” of the dithiane sulfurs which inhibits SET. This would be in agreement with previous results, mentioned above, which showed that SET slows for more substituted adducts.
Scheme 2.6. Synthesis of 5-alkyl-1,3-dithaines.

C. Experimental

C.1. General

Common solvents were purchased from Pharmco and used as is, except for THF, which was refluxed over and distilled from potassium benzophenone ketyl prior to use. n-BuLi (as a 1.6 M solution in hexanes), 1,3-dithiane, 2-methyl-1,3-dithiane, benzophenone, and benzhydrol were purchased from Aldrich. 1,3-Dithiol was purchased from Acros. Propanal, decanal, and heptanal were purchased from Alfa Aesar. All reagents were used without purification. $^1$H NMR spectra were recorded at 25 °C on a Varian Mercury 400 MHz instrument in CDCl$_3$ with TMS as an internal standard (unless noted otherwise). Low temperature NMR was carried out in CD$_3$OD using a Varian Mercury VT system. Temperature was controlled using dry nitrogen flow through a liquid nitrogen Dewar. Column chromatography was performed on silica gel, 70-230 mesh ASTM, using ethyl acetate-hexane mixtures as eluent. Photoreactions were carried out in a carousel Rayonet photoreactor outfitted with a jacketed Pyrex reaction vessel.
connected to a FTS Systems Multi-Cool refrigeration unit with a peristaltic pump, using ethanol as a coolant.

Ab initio computations were performed on a Linux workstation using Gaussian 03, Revision C.02.[61] Input geometries were created and preoptimized using a force field geometry optimization as implemented in Chem3D (Cambridgesoft). The geometries were further pre-optimized at the Austin model 1, semi-emperical (AM1) level. Full geometry optimizations were performed using density functional theory (DFT) at the B3LYP/6-31G(d) level of theory (the Becke three-parameter hybrid functional combined with Lee, Yang, and Parr correlation functional).


1,3-Propanedithiol (8 g, 0.07 mol) and the appropriate aldehyde (0.06 mol) were dissolved in 250 mL CH₂Cl₂. BF₃•Et₂O (33 mL, 0.26 mol) was added to the solution. The reaction mixture was then stirred overnight at room temperature. The mixture was washed with NaOH (5% aq. soln.) and water. The organic layer was dried over anhydrous NaSO₄ and the solvent was removed by rotary evaporator and the resulting product was distilled under vacuum.

2-Ethyl-1,3-dithiane (1c): (8.7g, 59 mmol), 86%; b.p. 38 °C @ 63 mTorr; ¹H NMR (CDCl₃, 400 MHz): δ 4.0 (t, 1H, J=6.8Hz), 2.80-2.92 (m, 4H), 2.09-2.16 (m, 1H), 1.84-1.92 (m, 1H), 1.77-1.92 (m, 2H), 1.09 (t, 3H). (NMR tpg17001_pure)
2-Hexyl-1,3-dithiane (1d): (5.4g, 26 mmol), 54%; b.p. 125 °C @ 72 mTorr; $^1$H NMR (CDCl$_3$, 400 MHz): δ 4.03 (t, 1H, J=6.9Hz), 2.76-2.90 (m, 4H), 2.06-2.13 (m, 1H), 1.83-1.92 (m, 1H), 1.68-1.74 (m, 2H), 1.44-1.52 (m, 2H), 1.22-1.35 (m, 6H), 0.87 (t, 3H). (NMR tpg17141_pure)

2-Decyl-1,3-dithiane (1e): (12.56g, 48 mmol, 63%; b.p. 142 °C @ 69 mTorr; $^1$H NMR (CDCl$_3$, 400 MHz): δ 4.07 (t, 1H, J=6.9Hz), 2.81-2.94 (m, 4H), 2.10-2.17 (m, 1H), 1.85-1.94 (m, 1H), 1.73-1.79 (m, 2H), 1.48-1.55 (m, 2H),1.25-1.33(m, 14H), 0.90 (t, 3H). (NMR tpg17152_pure)


A generic method by Corey and Seebach was modified and used to prepare the desired dithiane-benzophenone adducts.[55] The desired dithiane (5.1 mmol) was dissolved in freshly distilled THF (30 mL) and placed under nitrogen. n-Butyllithium (4.3 mL, 6.8 mmol, 1.6 M solution in hexanes) was added at room temperature upon stirring and the resulting mixture was stirred for 10 more minutes. The appropriate benzophenone (3.4 mmol) was dissolved in freshly distilled THF (10 mL) and added to the anion mixture while stirring. The reaction was left for 2 hours at room temperature. The reaction mixture was quenched with a saturated solution of ammonium chloride and the aqueous layer was extracted twice with ethyl acetate. The organic layer was dried over anhydrous sodium sulfate. The solvent was removed under vacuum, and the residue
was purified by column chromatography (silica-gel, ethyl acetate/hexanes) or recrystallization (DCM in hexanes).

**(2-Methyl-[1,3]-dithian-2-yl)-diphenyl-methanol (2b):** 0.80 g, 2.7 mmol, 80 %; $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 7.85-7.87 (m, 4H), 7.21-7.30 (m, 6H), 2.82 (ddd, 2H, J= 3.5, J=10.5, J=14.6 Hz), 2.53 (ddd, 2H, J=3.9, J=5.7, J=14.7 Hz), 1.80-1.97 (m, 2H), 1.894 (s, 3H). (NMR tpg17768_pure)

**(2-Ethyl-[1,3]-dithian-2-yl)-diphenyl-methanol (2c):** 0.94 g, 2.8 mmol, 93 %; $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 7.93-7.96 (m, 4H), 7.28-7.33 (m, 4H), 7.22-7.26 (m, 2H), 2.52 (ddd, 2H, J=4.8, J=7.0, J=14.2 Hz), 2.25 (ddd, 2H, J=4.8, J=8.1, J=13.0 Hz), 1.97-2.04 (m, 2H), 1.67-1.73 (m, 2H), 1.12-1.16 (m, 3H). (NMR tpg17002_fr2 and 17002_cosy)

**(2-Hexyl-[1,3]-dithian-2-yl)-diphenyl-methanol (2d):** 0.61 g, 1.6 mmol, 49 %; $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 7.91-7.94 (d, 4H), 7.20-7.31 (m, 6H), 2.49 (td, 1H, J=4.8Hz, J=10.3Hz), 2.22 (ddd, 1H, J=5.9Hz, J=8.0Hz, J=14.1Hz), 1.91-1.96 (m, 2H), 1.60-1.73 (m, 4H),1.10-1.31(m, 6H), 0.81-0.85 (t, 3H). (NMR tpg17154_column2)

**(2-Decyl-[1,3]-dithian-2-yl)-diphenyl-methanol (2e):** 0.62 g, 1.4 mmol, 52 %; $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 7.92-7.94 (dd, 4H), 7.20-7.33 (m, 6H), 2.49 (td, 2H, J=5.4Hz,
J=14.1Hz), 2.23 (dd, 1H, J=5.7Hz, J=8.2Hz, J=13.9Hz), 1.91-1.96 (m, 2H), 1.60-1.77 (m, 6H), 1.10-1.34 (m, 12H), 0.81-0.85 (t, 3H).  (NMR tpg17153_column)

C.4. 5-alkyl-1,3-dithiane preparation.


To a suspension of NaH (1.0 g, 43 mmol) in THF (115 mL) was added diethyl malonate (5.30 g, 33 mmol) dropwise. The mixture was stirred at room temperature for 15 min and then 1-bromoalkyl (33 mmol) was added in one portion. The reaction was refluxed 5 hours, quenched with water (200 mL), and extracted with diethyl ether (3 x 100 mL). The organic layers were combined, dried over anhydrous sodium sulfate, and removed under vacuum to give the product which was used without purification.

Diethyl 3-pentylpentanedioate (precursor for 3c): 5.42 g, 68 %; \(^1\)H NMR (CDCl\(_3\), 400 MHz): \(\delta\) 4.14-4.22 (dq, 4H, J=7.16 Hz, J=14.19Hz), 3.29-3.32 (t, 1H, J=7.53), 1.82-1.97 (m, 4H), 1.21-1.34 (m, 10H), 1.11-1.18 (m, 4H), 0.85-0.89 (t, 3H).  (NMR tpg27049_pure)

Diethyl 3-octylpentanedioate (precursor for 3d): 6.34 g, 64%; \(^1\)H NMR (CDCl\(_3\), 400 MHz): \(\delta\) 4.16-4.21 (dq, 4H, J = 0.79, 7.07, 7.09 Hz), 3.28-3.32 (t, 1H, J = 7.56 Hz), 1.83-1.90 (dt, 2H, J = 0.76, 15.20 Hz), 1.24-1.28 (m, 20H), 0.85-0.88 (t, 3H, J = 6.81 Hz).  (NMR mpc24108_rm)
C.4.2. General Method for the Preparation of Diethyl 3,3-dialkylpentanedioate.

To a suspension of NaH (0.72 g, 30 mmol) in THF (100 mL) was added the appropriate diethyl 3-alkylpentanedioate (22 mmol) dropwise. The mixture was stirred at room temperature for 15 min and then 1-bromoalkyl (22 mmol) was added in one portion. The reaction was refluxed for 8 hours, quenched with water (200 mL), and extracted with diethyl ether (3 x 100 mL). The organic layers were combined, dried over anhydrous sodium sulfate, and removed under vacuum to give the product which was used without purification in the next step.

Diethyl 3,3-dipentylpentanedioate (precursor for 3c): 4.55 g, 80 %; \(^1\)H NMR (CDCl\(_3\), 400 MHz): \(\delta\) 4.14-4.20 (q, 4H, \(J = 7.16\)), 1.83-1.87 (m, 4H), 1.24-1.32 (m, 12 H), 1.21-1.25 (t, 6H, \(J = 7.16\) Hz), 1.14-1.18 (m, 4H), 0.85-0.89 (t, 6H, \(J = 7.02\)). (NMR tpg27051_pure)

Diethyl 3,3-dioctylpentanedioate (precursor for 3d): 6.83 g, 99 %; \(^1\)H NMR (CDCl\(_3\), 400 MHz): \(\delta\) 4.14-4.19 (q, 4H, \(J = 7.11, 7.12\) Hz), 1.83-1.87 (m, 4H), 1.21-1.25 (m, 30H), 0.85-0.89 (t, 6H, \(J = 6.81\) Hz). (NMR mpc24112_rm)

C.4.3. General Method for the Preparation of 2,2-dialkylpropane-1,3-diol (3c-d).

LiAlH\(_4\) (1.33 g, 35 mmol) was suspended in THF (50 mL). Diethyl 3,3-dialkylpentanedioate (15 mmol) in THF (40 mL) was added dropwise to the suspension over 10 minutes. The mixture was refluxed with stirring for 1 h. 5% aq. HCl (150 mL)
was added dropwise to quench the reaction, which was then extracted with ether (3x 100 mL). The organic layer was dried over anhydrous sodium sulfate and removed under vacuum to give the product which was used without further purification.

2,2-dipentylpropane-1,3-diol (3c): 3.28 g, 100 %; $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 3.57 (s, 4H), 2.17 (s, 2H), 1.17-1.35 (m, 16 H), 0.86-0.90 (t, 6H). (NMR tpg27052_pure)

2,2-dioctylpropane-1,3-diol (3d): 3.34 g, 74 %; $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 3.57 (s, 4H), 1.22-1.27 (m, 28H), 0.86-0.90 (t, 6H, J = 6.75 Hz). (NMR mpc24110_rm)

C.4.4. General method for preparation of 3,3-bis(iodomethyl)alkanes:

To triphenylphosphine (8.65 g, 33 mmol) dissolved in benzene (50 mL), was added pyridine (8 mL) and iodine (11.4 g, 45 mmol), in this order. The reaction was stirred for 15 minutes at room temperature. The appropriate 2,2-dialkylpropane-1,3-diol (15 mmol) was added and the reaction was refluxed with stirring overnight. The precipitate was filtered off and the solvent was evaporated. The resulting residue was purified by gel filtration (silica gel eluted with hexane:EtOAc 10:1) to give the desired product.

3,3-bis(iodomethyl)pentane (4a): 4.17 g, 79 %; $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 3.20 (s, 4H), 1.43-1.49 (q, 4H, J = 7.5 Hz), 0.81-0.85 (t, 6H, J = 7.5 Hz). (NMR mpc24025_DEIodo_pure)
3,3-bis(iodomethyl)nonane (4b): 5.2 g, 85 %; $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 3.20 (s, 4H), 1.30-1.37 (m, 8H), 1.10-1.15 (m, 4H), 0.90-0.93 (dd, 6H, $J = 6.3$ Hz, 13.6 Hz). (NMR mpc24141_pure)

3,3-bis(iodomethyl)undecane (4c): 3.20 g, 82 %; $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 3.20 (s, 4H), 1.23-1.40 (3, 12H), 1.14-1.20 (m, 4H), 0.88-0.92 (t, 6H). (NMR tpg27054_pure)

3,3-bis(iodomethyl)heptadecane (4d): 6.79 g, 87 %; $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 3.20 (s, 4H), 1.23-1.40 (3, 22H), 1.07-1.21 (m, 2H), 0.87-0.90 (t, 6H). (NMR mpc24116_rm)

C.4.5. General method for preparation of S,S'-2,2-dialkylpropane-1,3-diyl diethanethioates:

Bis(iodomethyl)alkane (12 mmol) was dissolved in a mixture of DMF:THF (1:1) (100 mL). Potassium thioacetate (3.1 g, 27 mmol) was added and the reaction was refluxed with stirring for 3 hours. The mixture was poured into water, extracted with ether (3 x 100 mL), and the organic layer was collected, dried over anhydrous sodium sulfate, and removed under vacuum. The product was used directly in the next reaction.

S,S'-2,2-diethylpropane-1,3-diyl diethanethioate (5a): 2.77 g, 93 %; $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 2.93 (s, 4H), 2.34 (s, 6H) 1.31-1.37 (q, 4H, $J = 7.5$ Hz), 0.79-0.83 (t, 6H, $J = 7.5$ Hz). This product was used directly to make 7a. (NMR mpc24030_DEDTrm)
S,S’-2,2-dibutylpropane-1,3-diyl diethanethioate (5b): 3.65 g, 100 %; $^1$H NMR (CDCl$_3$, 400 MHz): δ δ 2.93 (s, 4H), 2.33 (s, 6H) 1.24-1.28 (m, 8H), 1.16-1.22 (m, 4H), 0.87-0.91 (s, 6H). (NMR mpc24144_rm)

S,S’-2,2-dipentylpropane-1,3-diyl diethanethioate (5c): 4.14 g, 100 %; $^1$H NMR (CDCl$_3$, 400 MHz): δ 2.93 (s, 4H), 2.33 (s, 6H), 1.19-1.32 (m, 16 H), 0.85-0.89 (t, 6H). (NMR tpg27056_rm)

S,S’-2,2-dioctylpropane-1,3-diyl diethanethioate (5d): 5.00 g, 100 %; $^1$H NMR (CDCl$_3$, 400 MHz): δ δ 2.93 (s, 4H), 2.33 (s, 6H) 1.20-1.37 (m, 24H), 0.79-0.83 (t, 6H). This product was used directly to make 7d. (NMR mpc24125_rm)

C.4.6. General method for preparation of 2,2-dialkylpropane-1,3-dithiols:

LiAlH$_4$ (1.1 g, 28 mmol) was suspended in THF (40 mL). S,S’-2,2-dialkylpropane-1,3-diyl diethanethioate (12.3 mmol) dissolved in THF (40 mL) was added to the suspension dropwise over 10 minutes. The mixture was refluxed with stirring for 1 h. 5% aq. HCl (100 mL) was added dropwise to quench the reaction, which was extracted with ether (3x 100 mL). The organic layer was dried over anhydrous sodium sulfate and removed under vacuum to give pure product.
2,2-dibutylpropane-1,3-dithiol (6b): 2.44 g, 90 %; \(^1\)H NMR (CDCl\(_3\), 400 MHz): \(\delta\) 2.51-2.53 (d, 4H, J = 8.5 Hz), 1.27-1.33 (m, 8H), 1.07-1.15 (m, 4H), 0.90-0.93 (t, 6H, J = 7.2 Hz). (NMR mpc24148_rm)

2,2-dipentylpropane-1,3-dithiol (6c): 2.83 g, 93 %; \(^1\)H NMR (CDCl\(_3\), 400 MHz): \(\delta\) 2.51-2.53 (d, 4H), 1.11-1.35 (m, 16H), 1.06-1.11 (t, 2H, J=8.57 Hz), 0.87-0.91 (t, 6H). (NMR tpg27057_pure)

C.4.7. General method for preparation of 2,2-dialkylpropane-1,3-dithianes:

A solution of dimethoxymethane (1.24 mL, 14 mmol) and 2,2-dialkylpropane-1,3-dithiol (11.4 mol) were dissolved in CH\(_2\)Cl\(_2\) (100 ml). BF\(_3\)\(\cdot\)Et\(_2\)O (7.22 ml, 57 mol) was slowly added and stirred overnight at room temperature. The reaction was quenched with 30% aq. NaOH (20 ml, added slowly), washed with 5% aq. NaOH (200 mL), and H\(_2\)O (100 ml). The organic layer was collected and dried over anhydrous Na\(_2\)SO\(_4\). The solvent was removed under vacuum and the residue was purified by gel filtration (silica gel eluted with 10:1 Hexane:EtOAc) to give the desired product which was used to make the adducts of benzophenone as per C.3 above. The benzophenone adducts containing these dithianes will be reported in Chapter 4.

2,2-diethylpropane-1,3-dithiane (7a): 5a (59 mmol) and concentrated HCl (48 mL) were added to formaldehyde (466 mL, 36% aq. solution stabilized by 10-15% MeOH) and refluxed overnight with stirring. The reaction mixture was brought to neutral pH
with saturated aqueous sodium bicarbonate, extracted with DCM (2 x 100 mL), and the organic layer was collected and removed under vacuum to give the product (7.49 g, 72%). $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 3.64 (s, 2H), 2.57 (s, 4H), 1.57-1.63 (q, 4H, J = 7.5 Hz), 0.79-0.82 (t, 6H, J = 7.5 Hz). (NMR mpc24033_4)

**2,2-dibutylpropane-1,3-dithiane 7b):** 8.77 g, 64 %; $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 3.64 (s, 2H), 2.56 (s, 4H), 1.32-1.35 (m, 8H), 1.13-1.19 (m, 4H), 0.91-0.94 (t, 6H). (NMR mpc24150_53)

**2,2-dipentylpropane-1,3-dithiane (7c):** 11.83 g, 77 %; $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 3.63 (s, 2H), 2.57 (s, 4H), 1.50-1.55 (m, 4H), 1.25-1.34 (m, 8H), 1.14-1.21 (m, 4H), 0.87-0.91 (t, 6H). (NMR tpg27058_wup)

**2,2-dioctylpropane-1,3-dithiane (7d):** To a solution of S,S’-2,2-dioctylpropane-1,3-diyl diethanethioate (6.88 mmol) in ethanol (20 mL) dimethoxymethane (1.2 g, 15.5 mmol) was added. HCl (3.0 mL, 4.0 M in Dioxane) was then added dropwise and the reaction was refluxed overnight. The solvent was removed, the residue was dissolved in DCM (100 mL) and washed with 5% aq. NaOH (2 x 100 mL). The organic layer was collected, dried over anhydrous sodium sulfate, and removed under vacuum to give the product. (13.8 g, 68 %). $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 3.64 (s, 2H), 2.58 (s, 4H), 1.25-1.34 (m, 22H), 1.09-1.22 (m, 2H), 0.87-0.90 (t, 6H). (NMR mpc24136_33)
Chapter 3: Identification of Fluorophores for a “Turn On” or “Turn Off” Photoamplification Assay

The purpose of this research is to develop a novel method which couples the molecular recognition-triggered photoamplification chain in diaryl ketone adducts of dithiane with a "turn-off" or “turn-on” fluorescence-based assay for the detection of biological targets and ligands, regardless of their nature, through a molecular recognition event. Integral to this assay are three components to the development of a new photoinduced methodology for an ultra-high sensitivity fluorescence “turn-on” or “turn-off” assay: (1) the fluorophore, (2) the masked sensitizer (adduct), and (3) the solvent. All of these components have to be optimized, both independently and in combination, for use with this new methodology. Before (2) and (3) can be optimized a fluorophore(s) must be identified.

Both the fluorescence “turn-on” and “turn-off” assays have advantages and disadvantages which are unique to themselves. A fluorescence turn-on assay will provide a better signal to noise ratio, as it is easier to locate one bright spot in a field of black than to locate one dark spot in a field of light. The difficulty with this type of assay (turn-on) is identifying a fluorophore that will be quenched when incorporated as part of an adduct but not inhibit the adducts photoamplification. If the adduct cannot participate in photoamplification then the “turn-on” assay becomes more complex, as an adduct which can carry the photoamplification chain must be added separately. On the other hand, our
1,3-dithiane adducts of benzophenone are known to participate in photoamplification and might be ideal for a fluorescence turn-off assay due to the release of two potential quenchers – 1,3-dithiane and benzophenone.

**A. Towards the Development of a Fluorescence Recovery (“turn-on”) assay.**

I have considered three approaches to the design of a fluorescence “turn-on” assay (Figure 3.1). First, we could utilize a fluorescent diaryl ketone to which 1,3-dithiane can be added via the modified Corey-Seebach addition.[49] This should effectively mask the fluorophore by disrupting the conjugation at the core of the fluorophore’s π-system via the formation of a covalent C-C bond, leading to quenching of fluorescence. Upon photocleavage of the dithiane, conjugation is restored and fluorescence is recovered. Second, a fluorescent dye could be attached to an adduct which contains a quencher. When the fluorophore and quencher are held in close proximity, as part of the adduct, fluorescence should be significantly diminished. Upon photocleavage of the adduct the quencher would be released and fluorescence will recover. The third option is to identify an organic dye that self quenches when in close proximity to itself. Utilizing trithiane a bis or tris adduct of this dye could be synthesized, giving a product with a significantly lowered fluorescence intensity as the dyes are held in close proximity. Once photocleavage takes place the dye would be released into solution leading to fluorescence recovery.
Figure 3.1. Three approaches to the design of a fluorescence “turn-on” assay. (1) A fluorophore masked with dithiane which recovers fluorescence upon cleavage. (2) A fluorophore conjugated to an adduct carrying a quencher which upon cleavage releases the quencher allowing for fluorescence recovery. (3) An adduct carrying self quenching fluorophores that recover fluorescence upon their release due to photocleavage.
A.1. Fluorescence recovery by photocleavage of 1,3-dithaine adduct

In addition to the fluorophore meeting the requirements inherent in the three requirements above for a “turn on” assay, it must also meet the following requirements:

1. The quantum yield of fluorescence ($\Phi_F$) must be high, or if low offset by a high extinction coefficient ($\varepsilon$). This is because $\Phi_F \cdot \varepsilon$ is a quality parameter which describes how efficient a fluorophore is. If $\Phi_F$ is low but the $\varepsilon$ is high then the fluorophore is absorbing most of the light that is put into it, which may allow it to overcome its low $\Phi_F$. On the other hand if the $\Phi_F$ is high but the $\varepsilon$ is low then the fluorophore may not absorb enough light to be useful.

2. Irreversible photobleaching must not occur when the adduct is irradiated for photocleavage ($\lambda$ from 340 nm to 375 nm).

3. The fluorophore must not interfere with photofragmentation in dithiane adducts and therefore should not absorb light considerably at the irradiation wavelength – 365 nm.

4. Both product(s) of photocleavage (2-alkyl-1,3-dithiane and/or benzophenone) must not quench the fluorophore.

Initial research had shown that the fluorescence of 2-amidothioxanthones (Figure 3.2) were effectively quenched by two orders of magnitude upon addition of 1,3-dithiane to form an adduct. Upon photocleavage of these adducts fluorescence of thioxanthone (TX) is recovered (Figure 3.3).[52] Unfortunately, there is one significant problem with the use of TX adducts, they are self-cleavable systems ($\Phi_{self} \geq \Phi_{sens}$), making them inappropriate for a photoamplification assay where $\Phi_{self}$ should not exist or be $<< \Phi_{sens}$. As this rendered adducts of TX unusable for this application I explored the use of
alternative fluorescent ketones in which the fluorophore’s $\pi$-system could be disrupted via the formation of a covalent C-C bond with dithiane.

![Image](image.png)

**Figure 3.2.** 2-Amidothioxanthones and their adducts for which fluorescence recovery takes place upon photofragmentation. $\Phi_F$, $\lambda_{\text{ex}}$, $\lambda_{\text{em}}$ are all in acetonitrile. Adapted from reference. [52].

![Image](image.png)

**Figure 3.3.** Fluorescence monitoring of release of dithiane from ATX adduct bound to tantalgel beads: (a) overall curve of fluorescence recovery in 20 mg of TantaGel beads irradiated with a U-360 nm broadband filter, arbitrary units; (b) pseudocolor image of the beads (excited at 405 nm), adapted from reference. [52].

Two well known diaryl ketones, which also fluoresce, are fluorenones ($3a \lambda_{\text{ex}} = 293 \text{ nm, } \lambda_{\text{em}} = 470 \text{ nm}$, $3c \lambda_{\text{ex}} = 312 \text{ nm, } \lambda_{\text{em}} = 450 \text{ nm}$, all $\lambda$ measurements taken in ethanol) and 10-methyl-9-acridone (5) (Figure 3.4). The dithiane adducts of both ketones were prepared via the previously described Corey-Seebach addition[49] to give 4a-c and 6. Fluorenone adducts 4a-c did not fluoresce and cleavage of the adduct, when irradiated
with a sensitizer (BP @ 365 nm or anthraquinone at 405 nm), was confirmed by $^1$H NMR. When fluorenones 3a-c were used as the sensitizer it was not possible to photoamplify the release of fluorenone. This is probably because the excitation of fluorenone is $\pi \rightarrow \pi^*$, not $n \rightarrow \pi^*$, making it incapable of sensitizing photocleavage. The inability of the adducts to photoamplify did not alone rule out there use. It is possible to add a photoamplifiable adduct which could release BP capable of fragmenting the fluorenone adduct. During fluorescence studies there was no fluorescence recovery upon cleavage of the adduct. It was found that fluorenone is quenched, at a diffusion controlled rate, by dithiane adduct which is in solution in bulk ([adduct] > 10 mM).

While we were able to form the acridone adduct 6 it did not undergo sensitized fragmentation in the presence of acridone, benzophenone, or anthraquinone, but rather underwent self cleavage when irradiated at either 365 or 405 nm. This is likely due to stabilization of the charge on acridone after C-C bond fragmentation by the N heteroatom. Because diaryl ketones with a heteroatom containing a lone pair in resonance with the cleavable C-C bond have a high tendency to undergo self cleavage we decided to direct our efforts in a different direction.

![Figure 3.4. Fluorenones and acridone used to design fluorescence recovery adducts.](image)
A.2. Intramolecular quenching of a fluorophore by linkage to a quencher containing dithiane adduct

Knowing that coumarins are highly fluorescent, biologically compatible, readily available or easily synthesized fluorescent tags, they seemed to be excellent choices for use as fluorescent markers in this application. In addition they have been shown to be quenched by donors with oxidation potentials less positive than 1.0 V and more positive than 1.50 V vs. SCE.[62] This led us to believe that dithiane itself might quench coumarin as its oxidation potential is between 0.73 to 1.18 V vs. SCE.[57], resulting in the synthesis of coumarin directly coupled to dithiane (Figure 3.5) which unfortunately showed no decrease in fluorescence intensity as compared to the parent coumarin. As a result a plan to incorporate a quencher into the adduct itself was devised.

Figure 3.5. N-(3-(1,3-Dithian-2-yl)propyl)-7-(diethylaminocoumarin)-3-carboxamide

To design such an adduct an appropriate quencher had to be identified which (1) can be linked to the adduct and (2) would not interfere with linking the adduct to the fluorophore (example in Figure 3.6). Aniline is noted in the literature as a commonly available quencher of coumarins which works via an electron transfer from aniline to the coumarin dye.[63]
Figure 3.6. An example of a fluorophore linked to a quencher containing dithiane adduct.

Because the synthesis of 2-alkyl-1,3-dithianes is relatively straightforward, it was decided to incorporate the quencher as part of the alkyl tail of dithiane resulting in 13 (Scheme 3.1). 13 was then added to 4-carboxybenzaldehyde via the previously described Corey Seeback addition[49] to give the adduct which was turned into its N-hydroxysuccinamide (NHS) ester (16) and coupled to 1,6-diaminohexane extended 7-diethylaminocoumarin-3-carboxylic acid (15) to give 8 (Scheme 3.2). A control coumarin (17), which is coupled to formylbenzoic acid NHS ester (18) was also synthesized.
Scheme 3.1. Synthesis of dithiane with an aniline containing 2-alkyl tail (13).

Scheme 3.2. Synthesis of 8, 7-diethylaminocoumarin-N-(6-(4-(hydroxy(2-(10-(methyl(phenyl)amino)decyl)-1,3-dithian-2-yl)methyl)benzamido)hexyl)-3-carboxamide.

The Stern-Volmer quenching constant ($K_{SV}$) for quenching of the control coumarin with dithiane 8 was experimentally determined to be 14,000 M$^{-1}$s$^{-1}$. When the fluorescence of 7 was compared to the control coumarin there was no difference in
intensity, indicating that the attached aniline was not acting as a quencher. Additionally, 7 showed no evidence of photofragmentation to release dithiane under both self and sensitized cleavage condition. To confirm that the distance between aniline and the coumarin was not leading to ineffectual quenching 19 was prepared (Figure 3.7). Fluorescence results for 19, when compared to the coumarin control, did not exhibit a difference in fluorescence intensity.

![Figure 3.7](image)

**Figure 3.7.** 7-(Diethylaminocoumarin-N-(3-(methyl(phenyl)amino)propyl)-chromene-3-carboxamide (19).

### A.3. Trithiane adducts of self quenching fluorescent dyes

The final effort to prepare a fluorescence recovering adduct was to use highly fluorescent well known dyes that self quench while also meeting our previously noted requirements. For this we chose to use Cy3 and coumarin dyes which could be coupled to a trithiane adduct (Figure 3.8 and Figure 3.9). While both dyes do have a small amount of absorption at 365 nm (the excitation wavelength for the sensitizer BP) they do not interfere with cleavage of dithiane adducts when present in solution. In addition they both showed self quenching at concentrations above $10^{-5}$ M
Figure 3.8. Excitation and emission spectra, in DCM, for 20, 2-((1E,3E)-3-(3,3-dimethyl-1-propylindolin-2-ylidene)prop-1-enyl)-1-carboxyhexyl-3,3-dimethyl-3H-indolium chloride salt

Figure 3.9. Excitation and emission spectra, in DCM, for 21, N-butyl-7-diethylaminocoumarin-3-carboxamide
The trithiane adduct (22, Scheme 3.3) was designed with three free amines to which the dyes could be coupled. Once prepared both tris adducts of trithiane, containing Cy3 (23 confirmed by NMR analysis Figure 3.10) or coumarin (24), showed little to no decrease in fluorescence intensity compared to the free dye. It is possible that the dyes are too far apart to self quench, but when synthesis of the dye triads was attempted without the glycine spacer coupling did not take place. In addition the adducts showed little evidence of cleaving the C-C bond of the trithane adduct. It is possible that the dyes shield the adduct from triplet benzophenone preventing sensitization and consequently photocleavage.

Scheme 3.3. Trithiane adduct for attachment of Cy3 and coumarin dyes.
Figure 3.10. NMR identification of 23.
B. Towards the Development of a Fluorescence Quenching ("Turn-off") Assay:

While the “turn-on” assay requires the use of a quenched fluorophore which recovers fluorescence upon cleavage of the adduct, the “turn-off” assay requires a fluorophore which is fluorescent until a quencher is introduced to, or produced within, the assay. In our case the adduct cleaves into two parts upon photofragmentation, BP and dithiane. If one or both of these parts is capable of quenching the fluorophore, which is separate from the adduct in solution, this could constitute a fluorescence “turn-off” event. If this event can be utilized to detect a molecular recognition event then a “turn-off” assay has been developed.

The requirements for a fluorophore to be used in a fluorescence quenching “turn-off” assay are similar to those for the “turn on” assay and are as follows: (1) the quantum yield of fluorescence (\( \Phi_F \)) must be high; (2) irreversible photobleaching must not occur when irradiated for photocleavage (\( \lambda \) from 340 nm to 375 nm); (3) the fluorophore must not interfere with photofragmentation in dithiane-ketone adducts and therefore should not absorb light considerably above 340 nm; (4) one or both product(s) of photocleavage (2-alkyl-1,3-dithiane and/or benzophenone), but not the adduct (i.e. masked benzophenone), must quench the fluorophore. There are two approaches which can be taken to this type of “turn-off” methodology - quenching of the fluorophore via energy transfer (dexter quenching) or quenching of the fluorophore by a reaction which leads to photobleaching.
B.1. Identifying a fluorophore quenched by energy transfer

Initial experiments towards the development of the “turn-off” assay utilized fluorene due to its high $\Phi_F$ of 0.68 and a fluorescence lifetime $\tau_F$ of 10 ns in non-polar solvents.\textsuperscript{[3]} Initial studies (Figure 3.11) showed benzophenone to be a very good quencher for fluorene ($K_{SV} = 1100 \text{ M}^{-1}\text{s}^{-1}$ in CH$_2$Cl$_2$). Unfortunately the adduct proved to be an even more efficient quencher ($K_{SV} = 2300 \text{ M}^{-1}\text{s}^{-1}$), rendering it unsuitable because the fluorophore $\tau_F$ is too long for this application. We then looked for a fluorophore with a shorter lifetime (less prone to quenching by the masked sensitizer) and focused on p-oligophenylenes (Figure 3.12),\textsuperscript{[65]} particularly $p$-terphenyl ($\Phi_F = 0.84$, $t_f = 0.95$ ns), which strongly absorbs at $\lambda_{\text{max}} \sim 280$nm ($\varepsilon \sim 3.5 \times 10^4$) with no absorption beyond 330nm, and p-quaterphenyl ($\Phi_F = 0.81$, $t_f = 0.92$ ns).

![Figure 3.11. Stern-Volmer plot for fluorescence quenching of fluorene (10$^{-5}$ M), in CH$_2$Cl$_2$, by benzophenone, benzhydrol, 2-methyl-1,3-dithiane, and 2-methyl-1,3-dithiane benzophenone adduct (MDT BP Adduct) to determine $K_{SV}$ for each quencher taken from reference. [5].](image)

\textsuperscript{2}Much of the data presented in this section has been taken or adapted from references [5] and [64]
p-Oligophenylenes are well known as durable fluorophores, and commonly used as a dye in dye lasers due to their resistance to photobleaching,[66,67] a desirable characteristic for this application. Quenching results for p-terphenyl (p-TPh) and p-quaterphenyl (p-QPh) show them to be very efficiently quenched by benzophenone (p-TPh: $K_{SV} = 3000 \text{ M}^{-1}\text{s}^{-1}$ in DCM, 1100 \text{ M}^{-1}\text{s}^{-1}$ in t-BuPh; and p-QPh: $K_{SV} = 1250 \text{ M}^{-1}\text{s}^{-1}$ in DCM, 1100 \text{ M}^{-1}\text{s}^{-1}$ in t-BuPh) whereas the effect of the adduct, 2-methyl-1,3-dithiane benzophenone adduct (MDT BP adduct) is significantly smaller ($K_{SV} = 80 \text{ M}^{-1}\text{s}^{-1}$, 40 \text{ M}^{-1}\text{s}^{-1}$ in t-BuPh) and only relevant at low concentrations (< 10 mM). Quenching by free alkyl dithianes was found to be insignificant. Compared to p-TPh (in DCM $\lambda_{\text{ex max}} = 280$ nm, $\lambda_{\text{em max}} = 345$ nm) the excitation and emission of p-QPh (in DCM $\lambda_{\text{ex max}} = 297$ nm, $\lambda_{\text{em max}} = 376$ nm) has a bathochromic shift, with some emission in the visible region (Figure 3.12). For this reason p-QPh was chosen as the best fluorophore to test a
fluorescence “turn-off” assay via collisional quenching. We found that the optimum fluorescence of p-QPh is achieved at a concentration of $2 \times 10^{-5}$ M (Figure 3.13).

![Figure 3.13. Determination of the concentration of p-QPh to achieve maximum fluorescence intensity.](image)

The next step was to verify that enough benzophenone can be produced during photoamplification to effectively quench the fluorescence of p-QPh. At first a $^1$H NMR experiment was run containing equimolar concentrations of p-TPh and BP to rule out a reaction between the two molecules. NMR’s before and after irradiation at 365 nm showed no changes. A second $^1$H NMR experiment was run to show that the production of BP, as a result of photocleavage, takes place in the presence of p-TPh (Figure 3.14). The NMR samples contained $1 \times 10^{-5}$ M p-TPh, 10 mM MDT-BP adduct, and $1 \times 10^{-6}$ M BP (the concentrations of pTPh and BP are too low to detect by NMR). This experiment verified that the fluorophore does not interfere with photocleavage of the adduct, nor does it react with the adduct or any products produced during the cleavage reaction.
In order to prove that the assay works it was necessary to show that fluorescence quenching occurs due to photocleavage of the adduct. A solution containing 30 mM DT-BP adduct, $10^{-5}$ M BP, and $10^{-5}$ M p-QPh was prepared in DCM along with a matching solution lacking BP. The solutions’ fluorescence intensities were recorded and they were irradiated using an in house built 365 nm LED reactor. The results, for each sample, were normalized to their initial fluorescence intensity and plotted to show an autocatalytic curve for fluorescence quenching which is typical of photoamplification (Figure 3.15), proving that the use of p-QPh in a photoamplified fluorescence turn off assay is possible.
Figure 3.15. Autocatalytic curve for the quenching of p-QPh due to photoamplified release of BP from DT-BP adduct in DCM. 30 mM DT-BP adduct + 10⁻⁵ M BP + 10⁻⁵ M p-QPh (blue), 30 mM DT-BP adduct + 10⁻⁵ M p-QPh (red)

B.2. Identifying a fluorophore quenched via a chemical reaction with benzophenone

While quenching of fluorescence generally refers to either the Dexter or Förster resonance energy transfer mechanisms it can also be applied to any situation where the fluorescence of a fluorophore is turned off. In coumarins it is well documented that electron transfer reactions, when coumarins are one half of a donor-acceptor pair, not only take place but also result in the dealkylation of the diethylamino group.[62,68,69] In the case of this assay fluorescence is quenched via a reaction of the fluorophore, 3-(benzo[d]thiazol-2-yl)-7-diethylaminocoumarin, commonly known as coumarin 6 (C6), catalyzed by BP, which leads to de-ethylation of C6 to give a secondary fluorophore that photobleaches quickly upon formation. The two possible reaction mechanisms are shown in Scheme 3.4 and Scheme 3.5. Both reactions give the same product, 3-(benzo[d]thiazol-2-yl)-7-ethylaminocoumarin (25).
Scheme 3.4. Mechanism 1 for the de-ethylation of Coumarin 6 sensitized by benzophenone.

Scheme 3.5. Mechanism 2 for the de-ethylation of Coumarin 6 sensitized by benzophenone.
The dealkylation reaction of diethylaminocoumarins was verified by $^1$H NMR (Figure 3.16) experiments using 7-diethylaminocoumarin-3-(N-butyl)-carboxamide. The $^1$H NMR shows both the appearance of acetaldehyde and the loss of one ethyl group, while the coumarin itself remains intact. Upon isolation of the dealkylated product its emission was compared with that of the starting coumarin (Figure 3.17) and found to have a relative $\Phi_F$ (ethyl/diethyl) of 1.60. This was determined by the following formula $\Phi_F=\frac{(A_{\text{std}}*PA_{\text{unk}})}{(A_{\text{unk}}*PA_{\text{std}})}*\Phi_F_{\text{std}}$ (see list of abbreviations for definition of variables). Unfortunately, further investigation of the dealkylated coumarin showed that its fluorescence was quenched within 20 minutes upon its direct irradiation or its irradiation with $10^{-5}$ BP using 365 nm light (the wavelength at which the photoamplification reaction takes place). As a result we decided to utilize this dealkylation reaction to develop a second fluorescence “turn-off” assay, as the diethylamino starting coumarin retains more than 80% of its fluorescence after irradiation at 365 nm for 1 h, but in the presence of $10^{-5}$ M BP it fluorescence is quenched by $>50\%$ in less than 20 minutes.
Figure 3.16. NMR of dealkylation reaction of 7-diethylaminocoumarin-3-(N-butyl)-carboxamide (20) to give 7-ethylaminocoumarin-3-(N-butyl)-carboxamide (25).

Figure 3.17. Excitation and emission spectra comparison for 7-diethylaminocoumarin-3-(N-butyl)-carboxamide (20) and its dealkylated product 7-ethylaminocoumarin-3-(N-butyl)-carboxamide (25)
The final step was to verify 7-diethylaminocoumarins as suitable fluorophores for this fluorescence “turn-off” assay by showing that photoamplification of benzophenone does result in its quenching, and that the fluorophore does not interfere with photoamplification. Coumarin 6 became the preferred fluorophore to carry out these experiments as its excitation and emission maxima are shifted farther into the visible range than its carboxamide counterparts (Figure 3.18). Additionally, its extinction coefficient at 365 nm (the irradiation wavelength for photochemistry) is lower than for the coumarin carboxamides.

![Figure 3.18. Excitation and emission spectra of coumarin 6.](image)

A 30 mM solution of DT-BP adduct containing $10^{-6}$ M BP, and $10^{-6}$ M C6 was prepared in DCM and 0.05 M PBS (pH 7.5) was added. A matching solution lacking BP was also prepared. The solutions’ fluorescence spectra were recorded ($\lambda_{ex} = 450$ nm) and
they were irradiated using an in house built 365 nm LED reactor. In Figure 3.19 the decrease in emission over an irradiation time of 35 min can be seen.

![Fluorescence decline over 35 minutes for a sample containing 30 DT-BP adduct + 10⁻⁶ M BP + 10⁻⁶ M C6 in DCM under 0.05 M PBS (pH7.5).](image)

**Figure 3.19.** Fluorescence decline over 35 minutes for a sample containing 30 DT-BP adduct + 10⁻⁶ M BP + 10⁻⁶ M C6 in DCM under 0.05 M PBS (pH7.5).

The fluorescence intensity at 495 nm for the scan at each irradiation interval was normalized to the starting intensity at time zero and plotted to show an autocatalytic curve for fluorescence quenching which is typical of photoamplification (Figure 3.20). The resulting autocatalytic curve verifies that the fluorescence quenching is due to the photoamplification of benzophenone from cleavage of DT-BP adduct which sensitizes the dealkylation of C6 to its quickly photobleached counterpart. In addition ¹H NMR results showed no interference by coumarin in the photocleavage reaction. These results lead us to believe that coumarin 6 would be a second acceptable fluorophore for this fluorescence “turn-off” assay. The next step in this assays design was to find conditions to achieve the lowest possible detection limit.
Figure 3.20. Autocatalytic curve for the quenching of C6 due to photoamplified release of BP from DT-BP adduct in DCM under 0.05 M PBS (pH 7.5). 30 mM DT-BP adduct + 10^-6 M BP + 10^-6 M C6 (blue), 30 mM DT-BP adduct + 10^-6 M C6 (red).

C. Experimental

C.1. General

Common solvents were purchased from AApier Alcohol and used as is, except for THF and hexanes. THF was refluxed over and distilled from potassium benzophenone ketyl prior to use. Hexanes was distilled over calcium hydride before use. All reagents for which a synthesis is not referenced in the text or described here were purchased from Alfa Aesar, TCI America, Fisher Scientific, AK Scientific, ChemImpex, or Aldrich. All reagents were used without purification unless otherwise noted. NMR spectra were recorded at 25 °C on either a Varian Mercury 400 MHz instrument, or a Bruker Biospin 500 MHz instrument, in CDCl3 with TMS as an internal standard (unless noted otherwise). Column chromatography was performed on silica gel, 32-63μ mesh, and the eluent is noted in the procedure.
C.2. General Information regarding photoreactions

Photoreactions were carried out using an in house built 365 nm five LED reactor outfitted with a 300-400 nm long pass filter. For each photoreaction discussed a bulk solution of fluorophore and adduct was prepared and divided to the appropriate number of samples. Benzophenone was added to each sample, from a stock solution, at the appropriate concentration. The concentrations of each reagent in the photoreactions are stated in the text where the reaction is presented. All bulk solution photoreactions were carried out in quartz fluorescence spectroscopy cells from NSG precision cells.

C.3. General method for the synthesis of dithiane adducts

A generic method by Corey and Seebach was modified and used to prepare the desired dithiane adducts.[55] Dithiane (5.1 mmol) was dissolved in freshly distilled THF (30 mL) and placed under nitrogen. n-Butyllithium (4.3 mL, 6.8 mmol) was added at room temperature with stirring and the resulting mixture was stirred for 10 minutes. Benzophenone (3.4 mmol) was dissolved in freshly distilled THF (10 mL) and added to the anion mixture while stirring. The reaction was left 2 hours at room temperature. The reaction mixture was quenched with a saturated solution of ammonium chloride and the aqueous layer was extracted twice with ethyl acetate. The combined organic layer was dried over anhydrous sodium sulfate. The solvent was removed under vacuum, and the residue was purified by column chromatography (silica-gel, ethyl acetate/hexanes) or recrystallization (DCM in hexanes).
C.4. Synthetic methods

N-butyl-9-oxo-9H-fluorene-2-carboxamide (3b): A mixture of 9-fluorenone-2-carboxylic acid (0.40 g, 1.8 mmol), N-hydroxysuccinimide (0.311 g, 2.7 mmol), and EDC (1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride, 0.414, 2.2 mmol) was dissolved in CH$_2$Cl$_2$ (20 mL) and stirred for 24 hours. n-Butylamine (0.27 mL, 2.7 mmol) and NEt$_3$ (5 drops) were added to the mixture which was refluxed for 6 hours. The solution was left overnight with stirring at room temperature. It was washed with water (40 mL) and saturated NaHCO$_3$ (40 mL) followed by NaCl (20 mL). The organic layer was dried over anhydrous sodium sulfate and removed under vacuum. The resulting solid was purified using column chromatography (silica gel with 20 % ethyl acetate in Hexane increased to 40 % ethyl acetate in hexane as the eluent) giving the product (0.380 g, 76% yield). $^1$H NMR (CDCl$_3$, 400 MHz): δ 8.06-8.08 (d, 1H), 7.91 (s, 1H), 7.69-7.71 (d, 1H), 7.57-7.62 (t, 2H), 7.51-7.55 (dt, 1H, J=1.1, 7.4Hz), 7.34-7.38 (dt, 1H, J=1.2, 7.4Hz), 6.15 (s, 1H), 3.45-3.50 (dt, 2H, J=5.7, 7.2Hz), 1.58-1.66 (td, 2H, J=7.4Hz, J=14.8Hz), 1.39-1.48 (qd, 2H, J=7.2, 14.3Hz), 0.95-0.99 (t, 3H, J=7.34 Hz). (NMR tpg17390_pure)

N-(9-oxo-9H-fluoren-2-yl)butyramide (3c): 2-amino-9-fluorenone (0.50 g, 2.1 mmol), butyrylchloride (0.41 mL, 3.8 mmol) and a catalytic amount of NEt$_3$ were combined in dichloromethane and stirred overnight. The organic layer was washed with water, dried over sodium sulfate, and removed under vacuum. The resulting solid was recrystallized in toluene to give the desired product (150 mg, 27 %). $^1$H NMR (CDCl$_3$, 400 MHz): δ
7.89-7.92 (d, 1H), 7.61-7.64 (d, 1H), 7.58-7.63 (s, 1H), 7.46-7.48 (m, 3H), 7.26-7.27 (m, 1H), 2.35-2.38(t, 2H), 1.75-1.80 (dd, 2H, J=7.32, 14.88Hz.), 1.00-1.04 (t, 3H, J=7.37 Hz).  (NMR tpg17384_rc)

9-(2-methyl-1,3-dithian-2-yl)-9H-fluoren-9-ol (4): To a solution of 2-methyl-1,3-dithiane (1.34 mL, 11.2 mmol) in freshly distilled THF (10 mL) was added n-butyllithium (1.6M solution in hexanes, 5.3 mL, 8.4 mmol). The resulting mixture was stirred 10 minutes at room temperature. 9-Fluorenone (0.50 g, 2.8 mmol) in freshly distilled THF (25 mL) was added slowly. The mixture was stirred overnight at room temperature. The reaction was quenched with a saturated solution of ammonium chloride, extracted with dichloromethane (30 mL) and dried over anhydrous NaSO4. The mixture was washed with hexane to remove dithiane giving the product (0.161g, 20% yield). ¹H NMR (CDCl3, 400 MHz): δ 8.04-8.06 (d, 2H, J=7.6 Hz), 7.56-7.58 (d, 2H, J=7.5 Hz), 7.35-7.40 (t, 2H, J=11.6Hz), 7.23-7.27 (t, 2H, J=7.5Hz), 3.26-3.29 (m, 2 H), 2.82-2.89 (m, 3H), 1.95-2.07 (m, 2H), 1.23 (s, 3H).  (NMR tpg17361_pure)

10-methyl-9-(2-methyl-1,3-dithian-2-yl)-9,10-dihydroacridin-9-ol (6): To a solution of 2-methyl-1,3-dithiane (0.67 mL, 5.6 mmol) in freshly distilled THF (10 mL) was added n-butyllithium (1.6M solution in hexanes, 2.6 mL, 4.2 mmol) and the reaction was stirred at room temperature for 10 minutes.. 10-methyl-9-acridone (0.30 g, 1.4 mmol) in freshly distilled THF (25 mL) was slowly added. The mixture was left overnight at room temperature. The reaction was quenched with a saturated solution of ammonium
chloride, extracted with dichloromethane (30 mL) and dried over anhydrous NaSO₄. Trituration with hexane was utilized to remove dithiane giving the product (0.161g, 20% yield) 

\[ ^1H \text{NMR (CDCl}_3, 400 \text{ MHz): } \delta 7.92-7.95 (d, 2H, J=7.8 \text{ Hz }), 7.34-7.38 (dd, 2H, J=7.24, 8.26 \text{ Hz}), 7.06-7.11 (t, 2H), 6.96-6.98 (d, 2H, J=7.25Hz), 3.46 (s, 1 H), 2.89-2.95 (m, 2H), 2.64-2.71 (m, 2H), 1.86-1.92 (m, 2H), 1.27 (s, 3H). \] (NMR tpg17362_pure)

**N-(3-(1,3-dithian-2-yl)propyl)-7-(diethylaminocoumarin)-3-carboxamide (7):**

Triethylamine (0.20 mL) and DMAP (catalytic) were added to a solution of (14) (0.30 g, 0.84 mmol) and 3-(1,3-dithian-2-yl)propan-1-amine (0.163 g, 0.92 mmol) in dichloromethane and stirred for 5 hours while the reaction was monitored by TLC. The reaction mixture was washed with water which was extracted with dichloromethane (100 mL). The organic solvent was concentrated, gel filtration was carried to remove remaining dithiane using hexane and then 10% ethyl acetate in hexane (0.230 g, 65 % yield). 

\[ ^1H \text{NMR (CDCl}_3, 400 \text{ MHz): } \delta 8.79-8.81 (t, 1H, J=5.9Hz), 8.69 (s, 1H), 7.41-7.43 (d, 1H, J=8.97 Hz), 6.3-6.65 (dd, 1H, J=2.5Hz, J=8.9Hz), 6.49 (d, 1H, J=2.20 Hz), 4.06-4.09 (t, 1H, J=6.40 Hz), 3.43-3.47 (q, 4H, J=7.3Hz, J=14.29 Hz), 2.80-2.90 (m, 4H), 2.07-2.13 (m, 1H), 1.82-1.90 (m, 5H). \] (NMR tpg17501_082406)

**(11-bromoundecyl)(tert-butyl)dimethylsilane (9):** 11-Bromoundecanol (10.0 g, 40 mmol) and t-butyldimethylsilyl chloride (TBDMScI) (7.84 g, 52 mmol) were dissolved in THF (125 mL). Triethylamine (20 mL) and a catalytic amount of DMAP were added and the reaction was stirred at room temperature overnight. The reaction mixture was
washed with 5% aq. HCl (100 mL) and water (100 mL). The organic layer was collected, dried over anhydrous sodium sulfate, and removed under vacuum. The excess TBDMSCl was removed by using a high vacuum pump to give (11-Bromo-undecyloxy)-tert-butyl-dimethyl-silane (12.78 g, 87% yield). \(^1\)H NMR (CDCl\(_3\), 400 MHz): \(\delta\) 3.58-3.61 (t, 2H, \(J=6.6\)Hz), 3.38-3.58 (t, 2H, \(J=6.9\)Hz), 1.82-1.89 (m, 2H), 1.80-1.91 (m, 2H), 1.38-1.55 (m, 4H), 1.45-1.55 (m, 4H), 1.27-1.32 (m, 12H), 0.89 (s, 9H), 0.05 (s, 6H).

(11-(1,3-dithian-2-yl)undecyl)(tert-butyl)dimethylsilane (10): To a solution of 1,3-dithiane (3.8 g, 32 mmol) in freshly distilled THF (100 mL), at 0 °C and under nitrogen atmosphere, was added n-butyllithium (1.6M solution in hexanes, 21 mL, 34 mmol). The resulting mixture was stirred 15 min at 0 °C and then the temperature was lowered to -70 °C. (9) (6.40 g, 17.0 mmol) in THF (40 mL) was added and the reaction was slowly warmed to room temperature and kept overnight. The reaction mixture was then poured into saturated aq. NH\(_4\)Cl (100 mL), extracted with ethyl acetate (100 mL), and washed with brine (40 mL). The organic layer was collected, dried over anhydrous sodium sulfate, and removed under vacuum to give the product (13.6 g with dithane present).\(^1\)H NMR (CDCl\(_3\), 400 MHz): \(\delta\) 4.02-4.06 (t, 1H, \(J=6.9\)Hz, \(J=13.8\)Hz), 3.57-3.61 (m, 2H, \(J=6.6\)Hz, \(J=13.3\)Hz), 2.78-2.91 (m, 4H), 2.08-2.15 (m, 1H), 1.80-1.91 (m, 1H), 1.70-1.77 (m, 2H), 1.46-1.60 (m, 4H), 1.26-1.30 (s, 14H), 0.89 (s, 9H), 0.05 (s, 6H).
11-(1,3-dithian-2-yl)undecan-1-ol (11): To (10) (6.39 g, 17 mmol) in THF was added TBAF (5.91 g, 19 mmol) and the reaction was stirred overnight. The reaction was poured into NH₄Cl (100 mL), extracted with EtOAc (100 mL), and washed with water (25 mL). The organic layer was dried over anhydrous sodium sulfate and removed under vacuum. High vacuum was used to remove the silyl group and the product was purified by column chromatography (silica gel eluted with 10% EtOAc in hexane to remove dithiane and 45% EtOAc in hexane to remove the product) giving 11-[1,3]Dithian-2-yl-undecan-1-ol (7.86 g, 68% yield starting from 11-bromoundecanol). ¹H NMR (CDCl₃, 400 MHz): δ 4.02-4.06 (t, 1H, J=6.9, 13.8Hz), 3.62-3.65 (t, 2H, J=6.2, 12.6Hz), 2.79-2.91 (m, 4H), 2.07-2.17 (m, 1H), 1.80-1.91 (m, 1H), 1.71-1.76 (m, 2H), 1.44-1.60 (m, 4H), 1.23-1.36 (s, 14H). (NMR tpg17614_column)

2-(11-Bromo-undecyl)-1,3-dithiane (12): (11) (7.83 g, 27 mmol) and carbon tetrabromine (10.7 g, 32 mmol) were dissolved in THF (200 mL). Triphenylphosphine (8.5 g, 32 mmol) was added and the reaction was stirred overnight. The precipitate was filtered and washed with hexane. The solvent was removed under vacuum and the resulting residue was dissolved in hexane. The solid was subsequently filtered and washed with hexane and the solvent was removed under vacuum to give the desired product with PPh₃ present (13.12 g) which was used in the next step. ¹H NMR (CDCl₃, 400 MHz): δ 4.02-4.06 (t, 1H, J=6.9, 13.8Hz), 3.38-3.41 (t, 2H, J=6.9, 13.8Hz), 2.79-2.91 (m, 4H), 2.07-2.14 (m, 1H), 1.79-1.92 (m, 3H), 1.71-1.76 (m, 2H), 1.34-153 (m, 4H), 1.27-1.31 (s, 12H). (NMR tpg17616_rm)
N-(11-(1,3-dithian-2-yl)undecyl)-N-methylaniline (13): To a solution of N-methylaniline (3.33 g, 31 mmol) in freshly distilled THF (30 mL) at -20 °C, under nitrogen atmosphere, was added n-butyllithium (1.6M solution in hexanes, 21.4 mL, 34 mmol). The resulting mixture was stirred 20 min at this temperature and then crude (12) (6.56 g, 19 mmol) in THF (20 mL) was added and the reaction was allowed to warm to room temperature with stirring overnight. The reaction mixture was then purged into 5% HCl (100 mL), extracted with dichloromethane (50 mL), and washed with aq. sodium bicarboate (40 mL). The organic layer was collected, dried over saturated sodium sulfate, and removed under vacuum to give a yellow oil. The product was purified using column chromatography (silica gel eluted with 5%EtOAc in hexane) to give the product (6.7 g, 67% yield from 11-[1,3]Dithian-2-yl-undecan-1-ol).  

\[ \text{H NMR (CDCl}_3, 400 \text{ MHz):} \delta 7.19-7.24 (dd, 2H, J=7.2, 8.9Hz), 6.64-6.70 (m, 3H), 4.02-4.06 (t, 1H, J=6.9Hz), 3.27-3.31 (m, 2H, J=7.5, 15.1Hz), 2.91 (s, 3H), 2.79-2.89 (m, 4H), 2.08-2.15 (m, 1H), 1.80-1.91 (m, 1H), 1.71-1.77 (m, 2H), 1.46-1.60 (m, 4H), 1.26-1.30 (s, 14H). \]

\[ \text{C NMR (CDCl}_3, 400 \text{ MHz):} \delta 149.55, 129.33, 115.96, 112.26, 53.03, 47.91, 38.51, 35.70, 30.73, 29.86, 29.77, 29.72, 29.59, 29.46, 27.42, 26.86, 26.84, 26.30. \] (NMR tpg17612_pure and tpg17583_13C)
7-diethylaminocoumarin-3-carboxylic acid (26): A mixture containing ethanol (20 mL), 4-diethylamino salicylaldehyde (5.0 g, 26 mmol), Meldrum's Acid (3.7 g, 26 mmol), piperidine (0.40 mL), and acetic acid (0.80 mL) was allowed to react at room temperature for 30 min and then refluxed for 4 hours. The mixture was cooled to room temperature and chilled in an ice bath for 1.5 h to precipitate the product, a bright orange solid. The solid was filtered and washed with ice cold ethanol to give pure coumarin (5.10 g, 75% yield). ¹H NMR (CDCl₃, 400 MHz): δ 8.66 (s, 1H), 7.44-7.46 (d, 1H, J=9.08 Hz), 6.69-6.72 (dd, 1H, J=2.48, 9.03 Hz), 6.53 (d, 1H, J=2.47 Hz), 3.46-3.51 (q, 4H, J=7.16, 14.30 Hz), 1.24-1.28 (t, 6H, J=7.15 Hz). (NMR tpg17472)

7-diethylaminocoumarin-3-carboxylic acid N-hydroxysuccinamide ester (14): (26) (1.0 g, 3.8 mmol) was dissolved in dichloromethane (50 mL), N-hydroxysuccinimide (0.66 g, 5.7 mmol) and EDC (0.87 g, 4.6 mmol) were added. The reaction mixture was stirred overnight at room temperature. The solution was diluted with water (30 mL), extracted with ethyl acetate (2 x 30 mL), washed with sodium bicarbonate solution (2x30 mL), and brine (30 mL). The organic layer was collected and evaporated under vacuum to give a bright yellow solid (1.41 g, 99% yield). ¹H NMR (CDCl₃, 400 MHz): δ 8.58 (s, 1H), 7.36-7.38 (d, 1H, J=9.04 Hz), 6.62-6.64 (dd, 1H, J=2.4, 9.0 Hz), 6.53 (d, 1H, J=2.47 Hz), 3.46-3.51 (q, 4H, J=7.16, 14.30 Hz), 1.24-1.28 (t, 6H, J=7.15 Hz). (tpg17497_082406)
tert-butyl 6-(7-diethylaminocoumarin-3-carboxamido)hexylcarbamate (27): N-boc-1,6-diaminohexane (0.814 g, 3.8 mmol) was dissolved in DMF (15 mL), triethylamine (1 mL) was added and the mixture was stirred for 5 minutes. (14) (1.0 g, 2.7 mmol) in DMF (25 mL) and a catalytic amount of DMAP were added the reaction which was stirred overnight at room temperature. The reaction mixture was poured into sodium bicarbonate (50 ml), extracted with ethyl acetate (2 x 40 mL), and washed with brine (50 mL). The organic layer was dried over anhydrous sodium sulfate and removed under vacuum. The resulting solid was purified on a silica gel column (80% EtOAc:20% hexane as eluent) to give pure product (0.85 g, 69 % yield). $^1$H NMR (CDCl$_3$, 500 MHz): $\delta$ 8.78 (s, 1H), 8.70 (s, 1H), 7.41-7.43 (d, 1H, $J=8.96$ Hz), 6.63-6.65 (dd, 1H, $J=2.49, 8.49$), 6.49-6.50 (d, 1H, $J=2.40$ Hz), 4.54 (s, 1H), 3.40-3.48 (m, 4H), 3.08-3.12 (m, 4H), 1.58-1.64 (q, 2H, $J=7.09, 14.03$Hz), 1.35-1.51 (m, 15H), 1.22-1.25 (t, 6H, $J=7.13$ Hz). (NMR tpg17475_081406)

N-(6-aminohexyl)-7-diethylaminocoumarin-3-carboxamide (15): (27) (0.85 g, 1.8 mmol) was dissolved in dichloromethane (25 mL) and trifluoroacetic acid (2.11 mL, 18.5 mmol) was added. The reaction was stirred overnight and then poured into water. The water was made basic and extracted with ethyl acetate. The organic layer was removed under vacuum to give the product (0.453 g, 70% yield). $^1$H NMR (CDCl$_3$, 500 MHz): $\delta$ 8.79 (s, 1H), 8.70 (s, 1H), 7.42-7.44 (d, 1H, $J=8.9$ Hz), 6.63-6.65 (dd, 1H, $J=2.4$, 9.0 Hz),
6.49-6.50 (d, 1H, J=2.4 Hz), 3.41-3.47 (m, 6H), 2.69-2.73 (t, 2H, J = 6.84 Hz), 1.58-1.65 (m, 2H), 1.35-1.51 (m, 6H), 1.22-1.25 (t, 6H, J=7.13 Hz). (NMR tpg17493_082106)

4-(hydroxy(2-(11-(methyl(phenyl)amino)undecyl)-1,3-dithian-2-yl)methyl)benzoic acid (28): To a solution of (13) (1.0 g, 2.7 mmol) in freshly distilled THF (40 ml), under nitrogen, was added n-butyllithium (1.6M solution in hexanes, 2.2 mL, 3.5 mmol). The solution was stirred at room temperature for 15 min. to generate the anion. Formylbenzoic acid (0.210 g, 1.4 mmol) in THF (20 mL) was added and the reaction was continued at room temperature for 20 min. before quenching with 5% aq. HCl (20 mL). The reaction mixture was extracted with dichloromethane (50 mL), washed with NaHCO₃ (10 mL) and brine (30 mL). The organic layer was collected, dried over anhydrous sodium sulfate, and removed under vacuum. The orange oil was purified using column chromatography (silica gel with an eluent of 7% EtOAc in hexane to remove dithiane and 45% EtOAc in hexane to remove the product) giving the product (0.50 g, 68 % yield). ¹H NMR (CDCl₃, 400 MHz): δ 8.04-8.06 (d, 2H, J=8.5Hz), 7.63-7.65 (d, 2H, J=8.4Hz), 7.19-7.24 (dd, 2H, J=7.2, 8.9Hz), 6.66-6.72 (m, 3H), 5.24 (s, 1H), 3.36 (s, 1H), 3.26-3.30 (m, 2H, J=7.5, 15.2 Hz), 3.18-3.25 (ddd, 1H, J=2.8, 11.9, 14.5Hz), 3.01-3.09 (ddd, 1H, J=2.6, 11.9, 14.5Hz), 2.91 (s, 3H), 2.66-2.78 (tdd, 2H, J=3.3, 14.2, 18.3 Hz), 2.11-2.20 (m, 1H), 1.83-1.96 (m, 1H), 1.73-1.81 (ddd, 1H, J=4.8, 12.1, 14.4 Hz), 1.43-1.56 (m, 3H), 1.13-1.31 (m, 16H). ¹³C NMR (CDCl₃, 400 MHz): δ 172.02,
4-(hydroxy(2-(11-(methyl(phenyl)amino)undecyl)-1,3-dithian-2-yl)methyl)benzoic acid N-hydroxysuccinamide ester (16): (28) (0.50 g, 0.944 mmol) was dissolved into dichloromethane (25 mL) and N-Hydroxysuccinimide (0.163 g, 1.4 mmol) and EDC (0.217 g, 1.1 mmol) were added. The reaction mixture was stirred overnight at room temperature. The solution was diluted with water (30 mL) extracted with ethyl acetate (2 x 30 mL), washed with sodium bicarbonate solution (2x30 mL), and brine (30 mL). The organic layer was evaporated under vacuum to give the desired product (0.50 g, 84% yield). $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 8.08-8.10 (d, 2H, $J = 8.52$ Hz), 7.67-7.69 (d, 2H, $J = 8.45$ Hz), 7.19-7.23 (m, 2H), 6.64-6.69 (m, 2H), 5.24 (s, 1H), 3.48 (s, 1H), 3.26-3.30 (m, 2H), 3.15-3.25 (t, 1H, $J = 11.91$ Hz), 2.98-3.11 (t, 1H, $J = 11.93$ Hz), 2.91 (m, 7H), 2.64-2.79 (t, 2H, $J = 14.90$, 18.97 Hz), 2.15-2.19 (m, 1H), 1.84-1.94 (m, 1H), 1.71-1.79 (m, 1H), 1.45-1.60 (m, 5H), 1.05-1.35 (m, 14H). (NMR tpg17643)

7-diethylaminocoumarin-N-(6-(4-(hydroxy(2-(10-(methyl(phenyl)amino)decyl)-1,3-dithian-2-yl)methyl)benzamido)hexyl)--3-carboxamide (8): (15) (0.440 g, 0.70 mmol) was added to a solution of (16) (0.302 g, 0.84 mmol) and NEt$_3$ (3 mL) dissolved in DMF (25 mL), then a catalytic amount of DMAP was added. The mixture was stirred overnight. The reaction mixture was decanted into saturated aq. bicarbonate solution,
washed with brine, and extracted with EtOAc. The organic layer was dried over anhydrous sodium sulfate and removed under vacuum to give an orange residue which was purified by column chromatography (silica gel eluted with EtOAc) to give 8 (100 mg, 17.4% yield). ¹H NMR (CDCl₃, 400 MHz): δ 8.80-8.20 (t, 1H, J = 5.42 Hz), 8.66 (s, 1H), 7.75-7.77 (d, 2H, J = 8.24 Hz), 7.57-7.59 (d, 2H, J = 8.22) 7.38-7.40 (d, 1H, J = 8.95 Hz), 7.19-7.23 (dt, 2H, J = 8.65, 7.33, 1.36 Hz), 6.67-6.69 (d, 2H, J = 7.91 Hz), 6.62-6.65 (m, 2H), 6.49 (d, 1H), 6.38-6.41 (t, 1H, J = 5.82 Hz), 5.20 (s, 1H), 3.40-3.47 (m, 10H), 3.26-3.30 (m, 2H), 3.16-3.22 (t, 1H, J = 11.90 Hz), 2.99-3.05 (t, 1H, J = 12.17 Hz), 2.91 (s, 3H), 2.65-2.73 (ddt, 2H, J = 18.13, 14.96, 3.67 Hz), 2.12-2.15 (m, 1H), 1.73-1.92 (m, 2H), 1.62-1.65 (m, 5H), 1.45-1.56 (m, 8H), 1.17-1.28 (m, 16H). ¹³C NMR (CDCl₃, 400 MHz): δ 167.48, 163.33, 162.97, 157.74, 152.64, 149.51, 148.17, 141.52, 134.40, 131.28, 129.27, 129.02, 126.04, 115.88, 112.20, 110.49, 110.09, 108.53, 96.69, 73.48, 59.32, 52.96, 45.23, 39.84, 39.36, 38.44, 34.77, 30.18, 29.78, 29.70, 29.66, 29.60, 27.35, 26.79, 26.73, 26.50, 25.63, 24.50, 24.32. (NMR tpg17647_pure, tpg17647_13C, and tpg17647_cosy)
Cosy for (8).
7-Diethylaminocoumarin-3-carboxylic acid [6-(4-formyl-benzoylamino)-hexyl]-amide (17): (15) (0.094 g, 0.26 mmol), (18) (0.065 g, 0.26 mmol), NEt3 (0.20 mL) and DMAP (catalytic) were put into DMF and stirred at room temperature overnight. This solution was poured over ice and made basic. The water was then extracted with DCM and the organic solvent was dried over anhydrous sodium sulfate. The solvent was removed under vacuum to give pure product (60 mg, 47% yield). ¹H NMR (CDCl₃, 400 MHz): δ 10.06 (s, 1H), 8.81-8.83 (t, 1H), 8.62 (s, 1H), 8.14-8.17 (s, 1H), 7.99-8.00 (d, 2H, J=7.83 Hz), 7.93-7.95 (d, 2H, J=8.17 Hz), 7.36-7.38 (d, 1H, J=8.95), 6.62-6.64 (m, 1H), 6.49 (s, 1H), 3.43-3.49 (m, 8H), 1.62-1.67 (dt, 4H, J=6.6, 13.8Hz), 1.37-1.52 (m, 4H), 1.22-1.25 (t, 6H, J=7.23 Hz).  (NMR tpg17493_082106)

Formylbenzoic acid N-Hydroxysuccinamide ester (18): Formylbenzoic acid (0.5 g, 3.33 mmol), N-hydroxysuccinimide (0.57 g, 4.9 mmol), and EDC (0.48 g, 4.0 mmol) were dissolved in a mixture of DCM:THF (1:1) and stirred overnight. The solution was washed with water (30 mL), NaHCO₃ (30 mL) and brine (30 mL). The organic layer was dried over anhydrous sodium sulfate and removed via vacuum filtration to give the product formylbenzoic acid N-Hydroxysuccinamide ester (0.550 g, 68% yield). ¹H NMR
7-Diethylaminocoumarin-3-carboxylic acid [3-(methyl-phenyl-amino)-propyl]-amide (19): To a solution of N-(3-aminopropyl)-N-methylaniline (0.28 g, 1.7 mmol) in DMF (25 mL) was added triethylamine (2 mL), (14) (0.5 g, 1.1 mmol) and DMAP (catalytic amount). The reaction was stirred overnight at room temperature. The mixture was poured over ice and extracted with ethyl acetate (3 x 30 mL), washed with brine (25 mL), dried over anhydrous sodium sulfate, and removed under vacuum to give the product containing the starting amine. The residue was purified by column chromatography (silica gel eluted with ethyl acetate) to give the product (0.360 g, 63% yield). $^1$H NMR (CDCl$_3$, 400 MHz): δ 8.86-8.89 (t, 1H, J = 5.68 Hz), 8.70 (s, 1H), 7.42-7.44 (d, 1H, J = 8.97), 7.21-7.25 (m, 2H), 6.72-6.74 (dd, 2H, J = 0.93, 8.83 Hz), 6.66-6.70 (m, 1H), 6.63-6.65 (dd, 1H, J = 2.54, 9.04 Hz), 3.39-3.51 (m, 8H), 2.95 (s,3H), 1.88-1.95 (m, 2H, J = 7.14, 14.25 Hz), 1.22-1.26 (t, 6H, J = 7.12 Hz). (NMR tpg17662_rm)

Synthesis of cyanine dye (20):

2,3,3-trimethyl-1-propyl-3H-indolium bromide: A mixture of 2,3,3-trimethylindolenine (2.0 mL, 13.0 mmol) and 1-bromopropane (1.42 mL, 16 mmol) in 1,2-dichlorobenzene (10 mL) was heated in a pressure vessel at 110 °C with stirring for
24 h. The solution was cooled to room temperature, chilled in the fridge (4 °C) overnight. The solvent was decanted, and the glassy red/brown residue which remained was dissolved in methanol. Methanol was removed under vacuum to give the crude product which was purified by column chromatography (silica gel eluted with 10:1 DCM:MeOD) to give pure product (2.06 g, 52% yield). 1H NMR (CD3OD, 500 MHz): δ 7.87-7.91 (m, 1H), 7.77-7.80 (m, 1H), 7.64-7.68 (m, 1H), 4.48-4.52 (m, 2H), 2.15-2.16 (dq, 2H, J = 7.94, 7.96, 15.87 Hz), 1.62 (s, 6H), 1.30 (s, 3H), 1.08-1.12 (t, 3H, J = 7.42 Hz). (NMR tpg27025_fr30)

(E)-3,3-dimethyl-2-(2-(N-phenylacetamido)vinyl)-1-propyl-3H-indolium bromide: A mixture of N,N-diphenylformamidine (0.59 g, 3.0 mmol) and 3,3-dimethyl-2-methylene-1-propylindoline (0.7 g, 2.5 mmol) was refluxed in acetic anhydride (10 mL) for 30 min. The solution was cooled to room temperature and the solvent was removed to give an impure product which was used in the next step.

1-(5-carboxypentyl)-2,3,3-trimethyl-3H-indolium bromide: A mixture of 2,3,3-trimethylindolenine (6.0 mL, 39 mmol) and 1-bromohexanoic acid (9.36 g, 48 mmol) in
1,2-dichlorobenzene (50 mL) was heated in a pressure vessel at 110 °C with stirring for 72 h. The solution was cooled to room temperature, chilled in a fridge (4 °C) 2 h, and then the solvent was decanted, and the residue which remained was dissolved in ethanol. Ethanol was removed under vacuum to give the crude product which was purified by column chromatography (silica gel eluted with 10:1 DCM:MeOD) to give pure product (9.79 g, 57% yield). 1H NMR (CDCl3, 500 MHz): δ 7.71-7.73 (m, 1H), 7.56-7.61 (m, 3H), 4.68-4.72 (t, 2H), 3.15 (s, 3H), 2.42-2.46 (t, 2H, J=6.92 Hz), 1.98-2.07 (td, 2H, J=16.26, 8.33 Hz), 1.70-1.78 (m, 2H), 1.65 (s, 6H), 1.58-1.63 (m, 2H). (NMR tpg27228, 11)

1-(5-carboxypentyl)-2-((1E,3E)-3-(3,3-dimethyl-1-propylindolin-2-ylidene)prop-1-enyl)-3,3-dimethyl-3H-indolium chloride (20 abbreviated Cy3): A mixture of 2-Methyl-3-(3-carboxyhexyl) benzooxazole Iodine salt (1.3 g, 3.5 mmol), acetonilide derivative (1.52 g, 2.96 mmol), and dry triethylamine (1 mL) in absolute ethanol (90 mL) was refluxed for 2 h. The solvent was removed under vacuum and the crude product was purified twice by column chromatography using silica gel (column 1; 7% MeOD in DCM, column 2; 20% Hexane in EtOAc then 7% MeOD in DCM) to give the pure product (0.72 g, 40% yield). 1H NMR (CDCl3, 500 MHz): δ 8.55-8.61 (t, 1H, J = 13.46 Hz), 7.93 (s, 1H), 7.56-7.58 (dd, 2H, J = 2.16, 7.14 Hz), 7.45-7.49 (t, 2H, J = 8.16), 7.37-7.40 (dd, 2H, J = 4.42, 7.92 Hz), 7.32-7.36 (t, 2H, J = 7.48 Hz), 4.14-4.20 (dt, 4H, J = 7.62, 11.91 Hz), 2.33-2.36 (t, 2H, J = 7.28 Hz), 1.86-1.96 (m, 4H), 1.78 (s, 12H), 1.70-1.76 (m, 2H), 1.52-1.58 (m, 2H), 1.09-1.12 (t, 3H, J = 7.42 Hz). (NMR tpg27293_column1, 60)
N-butyl-7-diethylaminocoumarin-3-carboxamide (21): (14) (0.30 g, 0.84 mmol) was added to a solution of n-butylamine (0.11 mL, 1.1 mmol) and NEt₃ (0.15 mL) in dichloromethane (15 mL). The reaction was left overnight at room temperature before being poured into 5% aq. HCl (25 mL), extracted with dichloromethane (2 x 30 mL), and washed with saturated aq. NaHCO₃ (25 mL). The organic layer was collected, dried over anhydrous sodium sulfate, and removed under vacuum to give the product (0.20 g, 75% yield). ¹H NMR (CDCl₃, 500 MHz): δ 8.79-8.83 (t, 1H, J = 5.86 Hz), 8.73 (s, 1H), 7.45-7.46 (d, 1H, J = 8.95 Hz), 7.29 (s, 1H), 3.44-3.53 (m, 6H), 1.60-1.66 (m, 2H), 1.41-1.48 (dq, 2H, J = 7.31, 7.35, 14.66 Hz), 1.25-1.28 (t, 6H, J = 7.13 Hz), 0.96-0.99 (t, 3H, J = 7.36 Hz). ¹³C NMR (CDCl₃, 400 MHz): δ 163.20, 162.98, 157.63, 152.63, 148.14, 131.25, 110.78, 110.07, 108.61, 96.78, 45.25, 39.58, 31.85, 20.44, 14.00, 12.63. (NMR tpg17714_pure and tpg17714_13C)

Synthesis of trithiane adduct (22)

N-benzylidene-1,1,1-trimethylsilanamine: To the solution of hexamethyl disilazane (46 mL, 0.22mol) in THF (150 mL), BuLi (1.6 M, 125 mL, 0.2mol) was added at 0 °C and stirred for 1h at this temperature. Then benzaldehyde (20 mL ) was added at 0 °C and stirred for 10h at 20 °C. Distilled Me₃SiCl (25 ml) was added, the formed precipitate was filtered off, the solvents were removed, the residue was distilled on a high vacuum pump to give the product (30 g, 77% yield) ¹H NMR (CDCl₃, 400 MHz): δ 8.98 (s, 1H), 7.79-7.81 (m, 2H), 7.40-7.47 (m, 3H), 0.26 (s, 9H). (NMR mpc24166_disfrac)
(1,3,5-trithiane-2,4,6-triyl)tris(phenylmethanamine): To a suspension of 1,3,5-trithiane (2.0 g, 14.4 mmol) in 20 mL THF, t-BuLi (1.7 M in pentane, 34 mL, 57.8 mmol) was added at -20°C and allowed to stir at this temperature for 1 hour. N-trimethylsilylimine (8.46 g, 47.7 mmol) solution was then added and the reaction mixture was allowed to stir for 3 hours at room temperature. The reaction was quenched with saturated NH₄Cl solution, the organic layer was separated and the compound was extracted with DCM. The organic layer was washed with 10% aq. HCl. The organic layer was removed and the aqueous layer was made basic with 20% NaOH and washed with CH₂Cl₂. The organic layer was extracted, dried, and removed to give the product. (Yield: 5.5 g, 84 %)

$^1$H NMR (Acetone, 400 MHz): δ 7.22-7.39 (m, 15H), 4.56-4.74 (m, 3H), 4.21-4.24 (m, 3H), 3.22-3.30 (m, 6H). (NMR mpc24168_rm)

tert-butyl-(2,2',2''-(1,3,5-trithiane-2,4,6-(riyl)tris(phenylmethylene)tris(azanediyl)

tris(2-oxoethane-2,1-diyl)tricarbamate: Boc-Gly-OH (1.35 g, 7.72 mmol), EDC-HCl (1.48 g, 7.72 mmol), 1-Hydroxy-benzotriazole hydrate (1.04 g, 7.72 mmol) were added to
a solution of trithiane adduct (1.0 g, 2.20 mmol) in DMF (30 mL) and allowed to stir overnight at room temperature. DMF was removed under vacuum, the compound was extracted with ethyl acetate, washed with 5% aq. NaOH solution and brine. The organic layer was dried over anhydrous Na$_2$SO$_4$ and concentrated. The compound purified by column chromatography. (Yield 1.55 g, 76 %) $^1$H NMR (CD$_3$OD, 400 MHz): δ 7.53 (s, 3H), 7.20-7.30 (m, 15H), 5.31-5.32 (t, 3H, J = 5.74 Hz), 4.67-4.71 (m, 3 H), 3.66-3.74 (m, 6H), 1.40 (s, 27H). (NMR mpc24170_68)

N,N',N''-(1,3,5-trithiane-2,4,6-triyl)tris(phenylmethylene)tris(2-aminoacetamide)

(22): To N-Boc protected (1,3,5-trithiane-2,4,6-triyl)tris(phenylmethanamine) (1.1 g, 1.12 mmol), HCl (12 mL, 4.0 M in dioxane) was added and stirred for 30 min at room temperature. The reaction mixture was quenched with 5% aq. NaOH solution. Compound was extracted with EtOAc, dried over anhydrous Na$_2$SO$_4$ and concentrated to give the product (Yield: 0.711 g, 93 %). $^1$H NMR (DMSO, 400 MHz): δ 8.45 (s, 3H), 7.27-7.32 (m, 15H), 5.22-5.23 (m, 3H), 4.95-5.10 (m, 3H), 3.29 (s, 6H), 3.07-3.12 (m, 6H). (NMR mpc24179_rm)

1,1',1''-(6,6',6''-(2,2',2''-(1,3,5-trithiane-2,4,6-triyl)tris(phenylmethylene)tris(azanediyl)tris(2-oxoethane-2,1-diyl))tris(azanediyl)tris(6-oxohexane-6,1-diyl))tris(2-((1E,3E)-3-(3,3-dimethyl-1-propyldolin-2-ylidene)prop-1-enyl)-3,3-dimethyl-3H-indolium) chloride (23): (20) (0.243 g, 0.47 mmol) and DIEA (0.175 mL, 1.0 mmol) were dissolved in DMF (6 mL). PyBOP (0.27 g, 0.52 mmol) was added in one portion
and the reaction mixture was stirred for 5 min at room temperature. A solution of (22) (0.100 g, 0.16 mmol) in DMF (1 mL) was added and the reaction was left overnight with stirring. The mixture was diluted with chloroform (50 mL), washed with water (2 x 40 mL) and brine (40 mL), dried over anhydrous Na₂SO₄, and the solvent was removed under vacuum. Ethanol (10 mL) was used to dissolve residue, diethyl ether was added (15 mL) to precipitate the product. This was repeated twice. Diethyl ether (10 mL) was then added, the mixture was heated to boiling, and the solid was decanted. The solid was then purified by column chromatography (silica gel eluted with ethanol in chloroform gradient 0-8%) to give the pure reddish product which was dried under high vacuum (100 mg, 10 % yield). ¹H NMR (CDCl₃, 500 MHz) 8.52-8.57 (t, 3H, J = 13.52 Hz), 7.93 (s, 3H), 7.53-7.57 (t, 6H, J = 8.23 Hz), 7.41-7.48 (dt, 6H, J = 19.24, 7.87 Hz), 7.21-7.35 (m, 27H), 6.45-6.52 (m, 6H), 5.29-5.35 (m, 3H), 4.80-4.86 (m, 3H), 4.05-4.16 (m, 12H), 3.84-3.88 (m, 6H), 2.27-2.31 (ddd, 6H, J = 10.23, 7.10, 2.85 Hz), 1.80-1.88 (m, 12H), 1.76 (s, 36 H), 1.70-1.74 (m, 6H), 1.49-1.56 (m, 6H), 1.03-1.06 (td, 9H, J = 7.36, 7.32, 1.07 Hz). (NMR tpg27307_column1, 50, and tpg27307_cosy)

N,N',N''-(2,2',2''-(1,3,5-trithiane-2,4,6-triyl)tris(phenylmethylene)tris(azanediyl)tris(2-oxoethane-2,1-diyl))tris(7-diethylaminocoumarin-3-carboxamide) (24):

(26) (0.173 g, 0.66 mmol) and DIEA (0.24 mL, 1.3 mmol) were dissolved in DMF (3 mL). PyBOP (0.345 g, 0.66 mmol) was added in one portion and the reaction mixture was stirred for 5 min at room temperature. A solution of the 4-fluorophenyl version of (22) (0.15 g, 0.22 mmol) in DMF (1 mL x 2) was added and the reaction was left
overnight with stirring. The mixture was poured over ice and a yellow solid was collected by vacuum filtration. The solid was then purified by column chromatography (silica gel eluted with ethanol in chloroform gradient of 0 to 6%) to give pure product (0.140 g, 15% yield) $^1$H NMR (CDCl$_3$, 500 MHz): δ 9.30-9.33 (t, 1H, J = 5.90 Hz), 9.25-9.27 (t, 2H, J = 5.42 Hz), 8.62-8.67 (m, 3H), 7.42-7.46 (m, 3H), 7.23-7.30 (m, 6H), 6.91-6.95 (td, 3H, J = 3.89, 8.54, 8.56 Hz), 6.86-6.89 (t, 3H, J = 8.61 Hz), 6.63-6.68 (ddd, 3H, J = 2.90, 7.70, 9.02 Hz), 6.48-6.51 (dd, 3H, J = 2.11, 11.90 Hz), 5.42-5.44 (dd, 1H, J = 5.94, 8.27 Hz), 5.32-3.39 (m, 2H), 4.71-4.72 (d, 1H, J = 5.09 Hz), 4.66-4.68 (t, 2H, J = 5.41 Hz), 4.07-4.38 (m, 6H), 3.44-3.53 (m, 12H), 1.13-1.39 (td, 18H, J = 2.77, 7.04, 7.05 Hz). (NMR tpg27363_column, 10, and tpg27363_cosy)
Cosy of (24):
Chapter 4: Optimizing a Fluorescence “Turn-off” Assay

In Chapter 3 two fluorophores were identified for use in this assay: p-QPh and C6. The next step in developing this new photoinduced methodology for an ultra-high sensitivity fluorescence “turn-off” assay is to optimize the other two integral components: (1) the masked sensitizer (adduct) and (2) the solvent. What is important to understand about these optimizations is that they are an iterative process, meaning that to optimize one we must optimize the other.

To choose a dithiane-benzophenone adduct we must always consider two factors, solubility and purity. As far as the solvent is concerned we know that the assay works in DCM. While DCM is an excellent solvent from a reactivity and solubility standpoint it is not ideal in regards to evaporation, with a boiling point of 40 °C. But, before the adduct can be modified to improve solubility some requirements for the solvent must be laid out. The solvent should: (1) be hydrophobic with a high boiling point and low viscosity; (2) dissolve the masked sensitizer (a dithiane adduct of benzophenone) which will be used; (3) allow for the use of an adduct which can be chromatographed or crystallized to high purity; (4) allow for high quantum yields of fragmentation; (5) not interfere with photochemistry or emission of the fluorophore; (6) be immiscible with aqueous physiological buffers which will hold the target (“receptor”); and (7) allow for
benzophenone to diffuse through solution with a diffusion coefficient similar to DCM (1.5 x 10^{-5} \text{ cm}^2 \text{ s}^{-1}).

**A. Improving Solubility of the Masked Sensitizer (Adduct)**

Before we can improve the adducts solubility we must consider the solvent requirements to determine the types of solvent into which the adduct must dissolve. The solvent should be hydrophobic with a high boiling point and low viscosity. The requirement for hydrophobicity is mandated by the need for high quantum yields of fragmentation. The use of hydrophobic solvents increases the quantum yield of sensitized fragmentation ($\Phi_{\text{sens}}$). In non-polar/hydrophobic solvents the rate of electron transfer (ET) is slower than in polar/hydrophilic solvents. This also means that the rate of back electron transfer is slowed, meaning that once electron transfer occurs there is more time for the sensitizer and adduct to diffuse away from one another before BET takes place. The result is an overall increase in $\Phi_{\text{sens}}$. A hydrophobic solvent will also be immiscible with aqueous physiological buffers which will hold the target ("receptor").

Because the final application of this assay is a chip, the solvent needs to be high boiling in order to prevent rapid evaporation at room temperature. In order to be high boiling and hydrophobic it is likely that the ideal solvent will be an alkane, such as decane, or incorporate an alkane chain into its structure. Contradictory to this requirement of a high boiling solvent is the need for low viscosity. Viscosity is an issue as the solvent must allow for benzophenone to diffuse through solution with a diffusion coefficient similar to DCM (1.5 x 10^{-5} \text{ cm}^2 \text{ s}^{-1}). The experimentally determined diffusion
coefficients for 4-trifluoromethylbenzophenone, which I measured by $^{19}$F NMR in several solvents, either purchased or synthesized, are shown in Table 4.1.

The first step in determining which solvent to move forward with was to design and synthesize adducts which would be soluble in more hydrophobic solvents such as decane and pristane. If we are to follow the principle that like dissolves like then the adduct itself must be made more hydrophobic. To implement this we can substitute the adducts protons at $R_1$, $R_2$, $R_3$, or $R_4$ with an alkyl tail (Figure 4.1). In Chapter 2 I reported that the $\Phi_{\text{sens}}$ for DT-BP adduct is 0.12, and that by introducing an alkyl chain as $R1$ (2-alkyl-1,3-dithiane benzophenone adduct) $\Phi_{\text{sens}}$ can be increased to $\geq 0.23$ depending on the length of the alkyl chain.[6] It was found that such adducts undergo small amounts of ground state cleavage (thermally induced) and unsensitized cleavage, and are therefore not suitable. Thioxanthone adducts undergo thermal cleavage at 30 Kcal/mol which will result in the production of nanomolar amounts of thioxanthone in minutes at room temperature. This is most likely due to the increased steric effect placed upon the cleaving C-C bond when a substituent is present. This rules out modification at $R_1$.

**Figure 4.1.** 2-Alkyl-1,3-dithaine adduct of BP showing all possible modification points
Table 4.1. Diffusion coefficients of 4-trifluoromethylbenzophenone in various solvents measured by $^{19}$F PFG NMR

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Diffusion Coefficient (cm$^2$s$^{-1}$)</th>
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<tbody>
<tr>
<td>Adiponitrile</td>
<td>1.26E-06</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>1.50E-05</td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>1.48E-05</td>
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<tr>
<td>Pristane (2,6,10,14-tetramethylpentadecane)</td>
<td>4.30E-06</td>
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<tr>
<td>t-Butyl Benzene</td>
<td>6.50E-06</td>
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<tr>
<td>Tetradecane</td>
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<td>2,2,4,4,6,8,8-heptamethylnonane</td>
<td>6.00E-06</td>
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<td>2-Phenylethyl Priopionate</td>
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<td>3-Phenylpropyl Acetate</td>
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<td>Propionic acid 3-chloro-2,2-dimethyl-propyl ester (9)</td>
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<td>1.28E-06</td>
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<tr>
<td>Methyltriocytalammonium bis(trifluoromethanesulfonyl)imide (11)*</td>
<td>9.07E-07</td>
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*Ionic Liquid
The second option was to prepare adducts modified with $C_n$ (alkyl) tails at $R_2$, $R_3$, and $R_4$ (refer to Figure 4.1). Adducts 1b, have a $C_n$ substituent at $R_4$, $n = 2, 4$ or 8. Adducts 1c, have a $C_n$ substituent at $R_2$ or $R_3$, $n = 2, 3, 4, 5, 6, 8$, or 12, and adducts 1d, have a $C_n$ substituent at $R_2 n=8$ and $R_4$, $n=4$, or 5. Adducts substituted with alkyl chains at both $R_2$ and $R_3$ were also created, but they did not further improve solubility. Before testing these adducts for solubility their ability to take part in a sensitized photofragmentation reaction was evaluated. The $\Phi_{\text{sens}}$ range from 0.12 for adducts 1b (adducts modified at $R_4$) to 0.18 for adducts 1c (adducts modified at $R_2$ or $R_3$). Additionally, no unsensitized cleavage was detected by $^1$H NMR when irradiated at 365 nm for up to 4 hours.

Next the solubility of these adducts was tested in three hydrophobic, high boiling solvents – decane, dodecane, and pristane (Table 4.2).[64] From these results a direct correlation between the length of the $C_n$ tail, on the adducts, and its solubility in higher alkanes can be made. In Table 4.2 there is also a column indicating whether the adduct is recrystallizable or not. This has to do with purification of the adduct which will be discussed in the next section of this chapter.
Table 4.2. Representative results for the solubility of adducts (Figure 4.1). $R_n = R$ substituted on the adduct and $C_n = \text{tail length}$. If the adduct eventually crystallized from the solvent the time for this to occur is indicated.

<table>
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<th>$R_n$</th>
<th>$C_n$</th>
<th>Recrystallizable</th>
<th>DCM</th>
<th>Decane</th>
<th>Dodecane</th>
<th>Pristane</th>
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</table>

*Unsubstituted DT-BP Adduct

B. Increasing the Purity of the Masked Sensitizer (Adduct)

Initially purity of the adducts was determined by $^1\text{H NMR}$. It was realized that this was not a good measure of purity when the crude synthesized adduct showed no BP to be present and the fluorescence assay results, at this time in DCM, were not reproducible from one batch of the adduct to the next. We found the assay we were developing to be more sensitive to the presence of trace amounts of BP (less than $10^{-5}$ M or 1.82 ppm) than NMR, and thus, had to rely upon it (the assay being developed) to determine the purity of each batch of adduct and the overall detection limit of BP. Because there currently is no method to differentiate between a minute impurity of BP and very small amounts of direct (unsensitized) cleavage of the adduct we cannot
determine whether the BP present is truly an impurity or if it is a product of cleavage and therefore will always determine our detection limit.

In Table 4.2 the ability of the adduct to be recrystallized is noted. The adducts can be purified by one of two methods, recrystallization or column chromatography with silica gel. Recrystallization is generally accepted as a better method for purification when possible. This is because silica gel can catalyze minute degradation of the product being purified. In our case, it is highly likely that silica gel, which is acidic in nature, can cause very small amounts of cleavage of the adduct. The result would be that the same purity achieved during recrystallization cannot be achieved as a trace amount of BP, not detectable by NMR, is always produced during chromatographic purification. Therefore, the method by which purification takes place has a large effect on the detection limit of the assay.

Initial studies were done in DCM, as the recrystallizable adducts were all soluble in it. A solution containing 2x10^{-5} M p-QPh and 30 mM DT-BP adduct recrystallized 8 times was prepared and divided into 3 samples. The sensitizer, BP, was added to samples 1 and 2 at 10^{-6} and 10^{-7} M respectively. The third sample did not contain any added sensitizer. The initial fluorescence intensity of each sample was recorded and then they were all irradiated at 365 nm using a carousel reactor. The fluorescence intensity of each sample was recorded at regular intervals to give the results in Figure 4.2.
Figure 4.2. Normalized fluorescence emission to determine the detection limit for photoamplified fluorescence quenching of p-QPh by benzophenone in DCM using recrystallized DT-BP adduct.

The adducts must have alkyl substituents to afford solubility in the best hydrophobic solvents. In decane (bp = 174 °C) it takes 2-3 hours to evaporate from a capillary chip at room temperature, while in pristane (bp = 68 °C @ 0.001 mmHg) it takes >5 hours to evaporate from a capillary chip. This means that adducts which are soluble in higher boiling alkanes will require purification by column chromatography, as they have an alkyl substituent(s) present. The best results for these adducts were achieved in pristane. The samples were prepared, and the experiment carried out, identical to the experiment described in DCM. The results are shown in Figure 4.3.
Figure 4.3. Normalized fluorescence emission to determine the detection limit for the photoamplified fluorescence quenching of p-QPh by benzophenone in pristane using column purified DT-OBP adduct.

The detection limit in DCM ($10^{-7}$ M BP – 18 ppb, 18 μg/L), using the recrystallized adduct is clearly much lower than that for pristane ($10^{-6}$ M BP – 180 ppb, 180 μg/L) where a column purified adduct was used. The maximum difference in normalized fluorescence intensity between a sample with $10^{-6}$ M BP and no BP in DCM is 0.84 while in pristane it is 0.23. Although both results are reproducible, within 20% of those shown, they are clearly better in DCM. This is especially true when one considers that $10^{-7}$ M BP is reproducibly detectable in DCM with an intensity difference of 0.61, but that it is not detectable in pristane. Thus, the conclusion is drawn that in order to achieve higher sensitivity a recrystallizable adduct should be used.

I must point out that the assay being developed is an amplified assay, meaning that a small amount of analyte produces a large response as the presence of BP is photoamplified. In the case of DCM (Figure 4.2 above) $10^{-7}$ M BP (18 ppb) was
reproducibly detected. This translates into a 100,000 fold amplification of the original sensitizer. This estimate is determined by the fact that there is 30 mM adduct in solution, and that at least 33% of this adduct undergoes photocleavage to produce benzophenone during the irradiation period, producing the maximum difference in fluorescence intensity. The result is the generation of minimum of 10 mM BP, giving us 100,000 times its starting concentration. This is significant as chemical preamplification takes place before the use of scientific instrumentation/hardware, such as spectrophotometers or chip readers, to “read” the results. This signal amplification affords scientists the ability to detect a wide range of molecular binding events. It not only improved the signal strength from those events which are already detectable at reasonable limits, but also affords one the ability to identify binding events which, without this amplification, would be difficult to detect or undetectable.


The end product of this research will be a chip which is easy to use. Thus, it would be ideal to utilize a solvent that is more resistant to evaporation than DCM but offers a better detection limit than pristane. To do this the reaction was studied in various solvents to identify a solvent “type” which may allow for the use of a recrystallizable adduct. The results of these studies (Table 4.3) show that the largest difference in normalized fluorescence intensity between a sample containing the fluorophore (p-TPh or p-QPh) and 30 mM DT-BP adduct with 10⁻⁷ M BP and one without BP is achieved in DCM. The next best solvents, which do not affect the photochemistry of the adduct,
appeared to be ethyl acetate, benzene, and cyclohexane. All of these solvents have similar rates of evaporation to DCM. As a result the use of solvents with similar functional groups and properties were explored as possible solvent choices for implementation onto a chip.

Table 4.3. p-QPh fluorescence “turn-off” photoamplification assay results in selected solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Difference*</th>
<th>Photoamplification Negatively Effected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipodinitrile</td>
<td>0.42</td>
<td>yes</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>0.61</td>
<td>no</td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>0.38</td>
<td>no</td>
</tr>
<tr>
<td>t-Butyl Benzene</td>
<td>0.18†</td>
<td>no</td>
</tr>
<tr>
<td>1,2-Dichlorobenzene</td>
<td>0.04†</td>
<td>yes</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>0.31</td>
<td>no</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.08</td>
<td>no</td>
</tr>
<tr>
<td>t-Butanol</td>
<td>0.12</td>
<td>no</td>
</tr>
<tr>
<td>Benzene</td>
<td>0.32</td>
<td>no</td>
</tr>
<tr>
<td>Pristane</td>
<td>0.23‡</td>
<td>no</td>
</tr>
</tbody>
</table>

*Difference maximum between normalized intensity values for a sample with 10⁻⁷ M BP and No BP
†10⁻⁶ M BP was used
‡A BP adduct with an alkyl tail was used for solubility

C.1. The design and use of esters as a solvent for photoamplification

Due to the favorable result in Table 4.3 for ethyl acetate the use of an ester as a solvent for photoamplification was explored. It was necessary to make the solvent denser, increase the boiling point, and not compromise the viscosity. Figure 4.4 shows several examples of the solvents which were prepared and purified by distillation, for which the experimental boiling points and densities are reported in Table 4.4. While increasing the ester tail length successfully increased the solvents boiling point it did not
always provide a solvent with a density higher than 1 g/mL (2b), which is necessary as the analyte (protein) to be use in the assay will be in an aqueous buffer. By introducing bromine to the molecule (compare 2b to 3b) both the boiling point and density of the solvent were increased. The problem with bromine substitution is that the solvent becomes more viscous. This led to the use of fluorocarbon groups which are expected to make the solvents less viscous (but can also compromise the solubility of the adduct) and could also increase the boiling point and density (compare 2b to 6 and 8 to 9).

Figure 4.4. Synthesized esters tested as solvents for photoamplification
Table 4.4. Boiling points and densities for compounds in Figure 4.4.

<table>
<thead>
<tr>
<th>Compound #</th>
<th>Boiling Point °C</th>
<th>Distillation Pressure</th>
<th>Density (g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2b</td>
<td>95</td>
<td>290 mTorr</td>
<td>0.94</td>
</tr>
<tr>
<td>3a</td>
<td>95</td>
<td>150 mTorr</td>
<td>1.58</td>
</tr>
<tr>
<td>3b</td>
<td>143</td>
<td>200 mTorr</td>
<td>1.64</td>
</tr>
<tr>
<td>3c</td>
<td>148</td>
<td>500 mTorr</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>152</td>
<td>200 mTorr</td>
<td>1.08</td>
</tr>
<tr>
<td>5</td>
<td>110</td>
<td>140 mTorr</td>
<td>1.84</td>
</tr>
<tr>
<td>6</td>
<td>61</td>
<td>500 mTorr</td>
<td>1.45</td>
</tr>
<tr>
<td>7</td>
<td>70</td>
<td>200 mTorr</td>
<td>1.65</td>
</tr>
<tr>
<td>8</td>
<td>60</td>
<td>18 mmHg</td>
<td>1.38</td>
</tr>
<tr>
<td>9</td>
<td>90</td>
<td>18 mmHg</td>
<td>0.97</td>
</tr>
<tr>
<td>10</td>
<td>75</td>
<td>18 mmHg</td>
<td>-</td>
</tr>
</tbody>
</table>

Once solvents that have a high boiling point, and a density higher than 1 g/mL were identified, they were tested for the photoamplification assay using p-QPh. Unfortunately, the reasonable fluorescence intensity difference between a sample with BP and without seen in ethyl acetate (0.38) did not translate into similar results for any of these solvents, which all had differences less than 0.10 for samples with 10⁻⁶ M BP vs. no BP. As a result the use of these solvents was abandoned.

C.2. Evaluation of ionic liquids as solvents for photoamplification

In recent years ionic liquids (IL’s) have become popular as a new class of solvents for various organic reactions,[70,71] including photochemical reactions.[72,73] These unique solvents are commonly referred to as designer solvents as their unique properties – a large liquidus range, lack of measurable vapor pressure, tunable density, viscosity and salvation strengths, and high thermal, chemical and electrochemical stability – allow them to be optimized for specific applications.[70,71] When one considers the influence of solvent on photochemical reactions, such as the ratio between homolytic vs. heterolytic
bond cleavage in alkyl halides, the cage effect on radical recombination, and the competition between energy vs. electron transfer processes, it would be ideal to identify one which has tunable properties. For this reason, and the fact that identifying a conventional hydrophobic, low viscosity solvent, which would not compromise the detection limit, appeared to be unattainable, the use of IL’s as a solvent for this assay was considered.

In 2002 Narayana and co-workers explored the use of IL’s as a medium for photochemical reactions, including energy and electron transfer reactions.[72] They utilized a 1-alkyl-3-methylimidazolium salt and determined that the major features of carrying out photochemical reactions in IL’s was (1) they have remarkable low oxygen solubility ([O₂] < 0.2 mmol/L compared to DCM [O₂] < 10.7 mmol/L), (2) triplet states live longer in IL’s, (3) they decrease the association time for charge transfer complexes, and (4) diffusion is slowed making diffusion controlled processes about two times slower in IL’s. While points (1-3) could be helpful for our application, (4) may hinder BP ability to quench the fluorophore, which has to be tested experimentally. Six IL’s were prepared (Figure 4.5) to test their ability as a solvent for this assay.
Figure 4.5. Ionic liquids tested as solvents for photoamplification

Pulse field gradient $^{19}$F NMR was used to determine the diffusion coefficient (see Table 4.1 for representative measurements) of 4-trifluoromethylbenzophenone in each solvent. 13 and 15 quenched the fluorophores, p-QPh and p-TPh, without BP being present, eliminating their use. In solvents 11 and 12 the diffusion of BP was found to be too slow to effectively quench the fluorophore even at concentrations of 0.01 M BP. The diffusion of BP was found to be slower in 14 and 16, in keeping with Narayana’s findings. Experimental results showed that at a concentration of 0.01 M BP 50% of p-QPh fluorescence was quenched. Additionally, photocleavage experiments indicated that after 15 min of irradiation at 365 nm more than 50% of DT-BP adduct had cleaved, but that the production of benzopinacole is higher in the IL’s than in other standard organic
solvents. These findings lead to the conclusion that while IL’s may help facilitate the photocleavage reaction they cannot be used because (1) they quench the fluorophore and (2) they slow down diffusion of BP, lowering the detection limit of the assay.

C.3. Revisiting the use of DCM

Based upon the results presented above it became quite obvious that DCM was going to be our gold standard as far as the detection limit of this assay was concerned. Using DCM provided us with the ability to use a recrystallizable adduct – DT-BP adduct – and a detection limit on the order of $10^{-7}$ M (18 ppb) BP, with the possibility of going even lower to $10^{-8}$ M (1.8 ppb) of BP. Unfortunately, we continued to return to the problem of fast evaporation at room temperature. The one thing not probed so far was running the assay under aqueous buffer, and thus if this prevented evaporation we may still be able to utilize DMC as the solvent.

DCM was placed in a capillary under water and left to evaporate overnight, the layers stayed in place with water on top. No evaporation was seen when there was at least 5 mm of water between DCM and air. When DCM was placed in a test tube and water was added it seemed to create two vertically divided columns (Figure 4.6 gives a visual representation). This partitioning was even more apparent when a buffer (0.02 M PBS, pH 7.5) was added, even though DCM has a density of 1.33 g/mL. This lead to the use of carbon tetrachloride (CCl₄) ($bp = 77 \degree C$, $D = 1.59$ g/mL). CCl₄ did not show partitioning problems with the buffer and gave similar results to DCM when used as the solvent for the fluorescence assay. Therefore, by running the assay under water we allow
for the use of solvents which evaporate quickly in air, making it possible to run the assay in DCM and CCl₄.

![Visual representation of the partitioning between DCM and water observed in a test tube.](image)

**Figure 4.6.** Visual representation of the partitioning between DCM and water observed in a test tube.

**D. Application of Adduct and Solvent Optimization Studies to the Coumarin 6 “Turn-Off” Assay**

While the C6 fluorescence “turn-off” assay is different from the one utilizing p-oligophenylenes as far as the method of quenching, it is very much the same in its solvent requirements. Initial results in DCM, for solutions containing C6 at 10⁻⁵ M and 30 mM DT-BP adduct, showed an astounding normalized fluorescence intensity difference between samples containing 10⁻⁸ M BP and no BP of 0.65 (Figure 4.7). We later learned that coumarins have a large bathochromic shift in fluorescence upon the buildup of HCl in solvents such as CCl₄ and DCM (Figure 4.8), something that happens during irradiation at 365 nm (Figure 4.9). This build-up of HCl leads to non-reproducible results which could not be solved by using a wet solvent. As a result the solvent needed to be changed to one which retained the properties of DCM without building up excessive amounts of HCl.
Figure 4.7. Detection limit for the photoamplified fluorescence quenching of C6 by benzophenone in DCM using recrystallized DT-BP adduct.

Figure 4.8. Control experiment to determine the effect of HCl on coumarin 6 \([10^{-5} \text{ M}]\) fluorescence emission in DCM.
Figure 4.9. Bathochromic shift of C6 fluorescence in DCM due to the build up of HCl during irradiation at 365 nm in the presence of 30 mM DT-BP adduct and $10^{-6}$ M BP.

D.1. Towards the use of chlorinated alkyls as solvents for the photoamplification assay

We set out to identify other chlorinated solvents which may have the same solubilizing abilities as DCM, are commercially available, have densities of about 1 g/mL, do not produce an abundance of HCl upon irradiation at 365 nm, and have higher boiling points ($\geq 100$ °C). We initially identified 1-chlorocyclohexane, 1,3-dichlorobutane, 1,4-dichlorobuante (1,4-DCB), and 1,5-dichloropentane (see Table 4.5 for properties).

After distilling all solvents the fluorescence quenching assay was carried out. Each solvent was vortexed with water for 30 seconds and then a solution containing $2\times10^{-5}$ M p-QPh and 30 mM DT-BP adduct recrystallized 8 times was prepared and
divided into 2 samples. The sensitizer, BP, was added to one of them at a concentration of $10^{-5}$ M. The other sample did not contain any added sensitizer. The initial fluorescence intensity of each sample was recorded and then they were all irradiated at 365 nm using a carousel reactor. The emission intensity of each sample was recorded at regular intervals and normalized to give the maximum difference shown in Table 4.5.

**Table 4.5.** Coumarin fluorescence “turn-off” photoamplification assay results in chlorinated alkanes

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Difference*</th>
<th>Boiling Point °C</th>
<th>Density (g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichloromethane (wet)</td>
<td>0.90</td>
<td>40</td>
<td>1.32</td>
</tr>
<tr>
<td>1-Chlorocyclohexane</td>
<td>0.70</td>
<td>142</td>
<td>1.00</td>
</tr>
<tr>
<td>1,3-Dichlorobutane</td>
<td>0.57</td>
<td>134</td>
<td>1.12</td>
</tr>
<tr>
<td>1,4-Dichlorobutane</td>
<td>0.50</td>
<td>162</td>
<td>1.16</td>
</tr>
<tr>
<td>1,5-Dichloropentane</td>
<td>0.14</td>
<td>65/10 mmHg</td>
<td>1.06</td>
</tr>
</tbody>
</table>

*Difference maximum between normalized emission intensity values for a sample with $10^{-5}$ M BP and No BP

The results show that 1-chlorocyclohexane, 1,3-dicholobutane, and 1,4-DCB are reasonable solvents for this application. Because the density of 1,4-DCB is higher than for the other two solvents it is preferred for this assay. The detection limit of BP in bulk solution is $10^{-6}$ M (180 ppb) in 1,4-DCB. Based upon these results I believe C6 is a better choice as the fluorophore for this assay, as the excitation and emission maxima are both in the visible region while those of p-QPh are in the UV. In addition, the time it takes the assay to complete is sixty minutes or less, whereas the same assay using p-QPh takes up to three hours to complete in some cases.
E. Experimental

E.1. General

Common solvents were purchased from AAper Alcohol and used as is, except for THF and hexanes. THF was refluxed over and distilled from potassium benzophenone ketyl prior to use. Hexanes was distilled over calcium hydride before use. All reagents for which a synthesis is not referenced in the text or described here were purchased from Alfa Aesar, TCI America, Fisher Scientific, AK Scientific, ChemImpex, or Aldrich. All reagents were used without purification unless otherwise noted. All NMR spectra were recorded at 25 °C on either a Varian Mercury 400 MHz instrument, or a Bruker Biospin 500 MHZ instrument, in CDCl₃ with TMS as an internal standard (unless noted otherwise). Column chromatography was performed on silica gel, 32-63μ mesh, the eluent is noted in the procedure.

E.2. Pulse field gradient (PFG) NMR

¹⁹F PFG NMR was used to determine all diffusion coefficients for solvents in Table 4.1. Each sample contained 4-trifluoromethylbenzophenone (50 mg) and the solvent to be analyzed (0.6 mL). In cases where the benzophenone used was not soluble, CDCl₃ was used to dissolve it (0.1 mL), before adding it to the solvent for which the diffusion coefficient was being determined.

The diffusion measurements (PFG-LED technique) were carried out with Varian Mercury 400MHz spectrometer equipped with Performa I PFG module and PFG-capable, 4 nuclei autoswitchable probe. The PFG module is capable of forming PFG pulses up to
21 G/cm strength, which was sufficient to drive the signal amplitude of free probe molecules to zero. We used slightly modified watersLED sequence (with water-suppression turned off completely). This is a stimulated spin-echo technique. The echo amplitude is related to the diffusion coefficient by the following expression:

\[ A = A_0 \exp\left[-\left(\gamma \delta G\right)^2 \frac{(\Delta-\delta/3)}{\Delta S} G s\right] \]

\( \gamma \) - magnetogyric ratio, \( 2.5181 \times 10^4 \) 1/G s
\( \delta \) - length of pulsed field gradient (s)
\( G \) - gradient strength (G)
\( \Delta \) - diffusion time = \( \delta + g\text{stab}1 + \text{pulse width} + \text{diff} \)
\( \Delta S \) - diffusion coefficient

For each NMR the PFG strength (variable gzlvl 1) was arrayed from 100 to 2000 while other relevant parameters were held constant: \( gt1 = 0.005 \) ms ( = \( \delta \), \( pw = 20 \) \( \mu s \), \( led = 0.017 \) ms, \( diff = 0.10 \) to 0.20 ms depending on viscosity of the solvent, \( g\text{stab}1 = 0.0005 \) ms, \( gt2 = 0.003 \) ms.

**E.3. Solubility tests**

All solubility tests in Table 4.2 were carried out by dissolving a quantity of adduct equal to 30 mM in 1 mL of the solvent used. Heat was used to try and dissolve adducts which were not soluble at room temperature. Each sample was then left sitting for 1 week to determine whether the adduct crystallized from the solvent or remained in solution.
E.4. General information regarding photoreactions

Photoreactions were carried out using an in house built 365 nm five LED reactor outfitted with a 300-400 nm long pass filter. For each photoreaction discussed a bulk solution of fluorophore and adduct was prepared and divided to the appropriate number of samples. Benzophenone was added to each sample, from a stock solution, at the appropriate concentration. The concentrations of each reagent in the photoreactions are stated in the text where the reaction is presented. All solvents were evaluated using two samples containing fluorophore and adduct as stated above. One contained $10^{-5}$ M BP and one no BP. All bulk solution photoreactions were carried out in quartz fluorescence spectroscopy cells from NSG precision cells.

E.5. General method for the synthesis of 4-alkylbenzophenones

Method 1 – Suzuki coupling: In a pressure vessel to the appropriate 1-alkene (20 mmol) was added 9-BBN (0.5 M in THF, 52 mL, 26 mmol). The reaction mixture was heated at 90 °C with stirring for 18 hrs. Water (0.4 mL) was added and this mixture was pured into a flask containing 4-bromobenzophenone (2.61 g, 10 mmol), Pd(PPh₃)₄ (0.1 eq.) and K₃PO₄ (6 eq). The reaction mixture was refluxed 18 h with stirring and then filtered. The filtrate was removed under vacuum to give the crude product which was purified by gel filtration (silica gel eluted with 5% EtOAc in hexane). After removing the solvent under vacuum the small amount of remaining alkene was removed by high vacuum pump to give pure 4-alkylbenzophenone.
**Method 2 – Grignard reaction:** In a three neck RB flask, with a condenser and addition funnel attached, under nitrogen 3 crystals of iodine were added to Mg turnings (1.7 g, 42 mmol) in 15 mL of dry THF and stirred for 5 min. Neat 1-bromo-4-alkylbenzene (4 mmol) was added and stirred until the iodine color faded and heat began to evolve from the reaction. The remaining 1-bromo-4-alkylbenzene (36 mmol) in dry THF (15 mL) was added dropwise over 5 minutes. After reflux stopped the reaction mixture was heated slightly until all Mg dissolved. The mixture was then cooled to 0 °C and 4-alkylbenzaldehyde (40 mmol) in THF (15 mL) was added dropwise over 5 minutes. A slightly purple color prevailed until the addition was complete. The reaction was removed from the ice bath and reacted at room temperature with stirring for 3.5h and then refluxed for 30 min. Ethanol (10 mL) was added to react with any remaining Grignard reagent and then the reaction was quenched with saturated NH₄Cl (10 mL), and washed with DCM (2 x 40 mL). The organic solvent was collected and dried over sodium sulfate and removed under vacuum. Impurities were removed on high vacuum to give the benzhydrol containing 20% benzophenone which was dissolved in DCM (100 mL) and PCC (8.1 g, 37.5 mmol) was added. The reaction was stirred overnight at room temperature. Ether (50 mL) was added to the reaction mixture and the precipitate was filtered off. The organic layer was removed under vacuum and the resulting residue was dissolved in hexane:ether (1:1). The precipitate was filtered off and the organic solvent was removed. The residue was dissolved in hexane:ethyl acetate (20:1 - 25 mL) and purified by gel filtration using this solvent as the eluent (500 mL). Impurities were removed under high vacuum with a heat gun to give pure 4,4-dialkylbenzophenone.
4-propylbenzophenone (17a): 12.8 g, 89% yield, $^1$H NMR (400 MHz, CDCl$_3$) δ 7.79-7.81 (ddd, 2H, $J = 1.6$, 2.9, 7.1 Hz), 7.71-7.76 (m, 2H), 7.54-7.62 (m, 1H), 7.46-7.50 (tt, 2H, $J = 1.3$, 6.6 Hz), 7.26-7.31 (d, 2H, $J = 8.5$ Hz), 2.66-2.69 (m, 2H), 1.58-1.74 (m, 2H), 0.92-0.99 (t, 3H, $J = 7.3$ Hz). (NMR tpg27231_product)

4-butylbenzophenone (17b): $^1$H NMR (400 MHz, CDCl$_3$) δ 7.79-7.82 (ddd, 2H, $J = 1.9$, 3.3, 7.1 Hz), 7.73-7.76 (m, 2H), 7.56-7.60 (m, 1H), 7.46-7.50 (tt, 2H, $J = 1.3$, 6.6 Hz), 7.26-7.31 (d, 2H, $J = 8.5$ Hz), 2.68-2.72 (m, 2H), 1.61-1.69 (m, 2H), 1.34-1.44 (td, 2H, $J = 7.3$, 14.7), 0.93-0.97 (t, 3H, $J = 7.3$ Hz). (NMR tma25059_c1fr5)

4-pentylbenzophenone (17c): $^1$H NMR (400 MHz, CDCl$_3$) δ 7.79-7.81 (d, 2H, $J = 7.1$ Hz), 7.73-7.75 (d, 2H, $J = 7.9$ Hz), 7.55-7.60 (t, 1H, $J = 7.4$ Hz), 7.46-7.50 (t, 2H, $J = 7.8$ Hz), 7.25-7.27 (d, 2H, $J = 8.0$ Hz), 2.69-2.75 (dd, 1H, $J = 6.3$, 13.3 Hz), 2.43-2.48 (dd, 1H, $J = 8.2$, 13.3 Hz), 1.20-1.85 (m, 6H), 0.91-0.95 (t, 3H, $J = 7.5$ Hz). (NMR tma25074_fr5)

4-hexylbenzophenone (17d): $^1$H NMR (400 MHz, CDCl$_3$) δ 7.73-7.81 (d, 2H, $J = 8.1$ Hz), 7.73-7.75 (d, 2H, $J = 8.1$ Hz), 7.56-7.60 (t, 1H, $J = 7.4$ Hz), 7.46-7.50 (t, 2H, $J = 7.7$ Hz), 7.26-7.30 (d, 2H, $J = 8.0$ Hz), 2.67-2.71 (m, 2H), 1.56-1.67 (m, 2H), 1.26-1.40 (m, 6H), 0.88-0.91 (t, 3H, $J = 6.7$ Hz). (NMR gam26002_fr15)
4-octylbenzophenone (17e): 1.85 g, 50% yield, $^1$H NMR (400 MHz, CDCl$_3$) δ 7.79-7.80 (d, 2H, J = 6.9 Hz), 7.73-7.76 (d, 2H, J = 8.2 Hz), 7.56-7.60 (t, 1H, J = 7.4 Hz), 7.46-7.50 (t, 2H, J = 7.5 Hz), 7.26-7.30 (m, 2H), 2.68-2.71 (m, 2H), 1.62-1.69 (dt, 2H, J = 7.5, 15.1 Hz), 1.28-1.33 (m, 10H), 0.87-0.90 (t, 3H, J = 6.8 Hz). $^{13}$C NMR (400 MHz, CDCl$_3$) δ 196.51, 148.21, 137.96, 135.04, 132.11, 130.31, 129.93, 128.31, 128.18, 36.03, 31.85, 31.17, 29.42, 29.29, 29.22, 22.65, 14.09. (NMR tpg27047_pure and tpg27047_13C)

4-dodecylbenzophenone (17f): 2.69 g, 77% yield, $^1$H NMR (400 MHz, CDCl$_3$) δ 7.73-7.80 (d, 2H, J = 7.6 Hz), 7.73-7.75 (d, 2H, J = 8.1 Hz), 7.56-7.59 (t, 1H, J = 7.5 Hz), 7.46-7.49 (t, 2H, J = 7.6 Hz), 7.26-7.29 (d, 2H, J = 8.2 Hz), 2.66-2.70 (m, 2H), 1.59-1.65 (m, 2H), 1.25-1.42 (m, 18H), 0.86-0.89 (t, 3H, J = 6.7 Hz). (NMR tpg27026_pure)

4,4-diethylbenzophenone (17g): 5.85 g, 62% yield, $^1$H NMR (400 MHz, CDCl$_3$) δ 7.70-7.73 (d, 4H, J = 8.1 Hz), 7.23-7.28 (d, 2H, J = 8.0 Hz), 2.58-2.74 (q, 4H, J = 7.6 Hz), 1.24-1.28 (t, 6H, J = 7.6 Hz). (NMR tpg27168_pure)

4-ethyl-4’-propylbenzophenone (17h): 5.6 g, 63% yield, $^1$H NMR (400 MHz, CDCl$_3$) δ 7.72-7.75 (dd, 2H, J = 3.1, 8.0 Hz), 7.27-7.31 (t, 2H, J = 8.6 Hz), 2.71-2.77 (q, 2H, J = 7.6 Hz), 2.65-2.69 (m, 2H), 1.61-1.73 (m, 2H), 1.26-1.31 (t, 3H, J = 7.6 Hz), 0.91-0.99 (t, 3H, J = 7.3 Hz). (NMR tpg27175_rm)
**E.6. General method for the synthesis of dithiane adducts**

A generic method by Corey and Seebach was modified and used to prepare the desired dithiane adducts.[55] The appropriate dithiane (5.1 mmol) was dissolved in freshly distilled THF (30 mL) and placed under nitrogen. n-Butyllithium (4.3 mL, 6.8 mmol) was added at room temperature while stirring and the resulting mixture was stirred for 10 minutes. The desired benzophenone (3.4 mmol) was dissolved in freshly distilled THF (10 mL) and added to the anion mixture with stirring. The reaction was left 2 hours at room temperature. The reaction mixture was quenched with a saturated solution of ammonium chloride and the aqueous layer was extracted twice with ethyl acetate. The combined organic layer was dried over anhydrous sodium sulfate. The solvent was removed under vacuum, and the residue was purified by column chromatography 6 times (silica-gel, ethyl acetate/hexanes) or recrystallization 8 times (DCM in hexanes). The 2-alkyl-1,3-dithiane adducts were described in chapter 2, (2a-e) along with the 5-alkyl-1,3-dithianes which are used here (Chapter 2 compounds 3-7).

**(1,3-dithian-2-yl)diphenylmethanol (1a):** 1.8 g, 60% yield, $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.56-7.63 (d, 4H, J = 7.2 Hz), 7.30-7.37 (d, 4H, J = 7.5 Hz), 7.22-7.27 (m, 2H), 3.28 (s, 1H), 2.84-2.99 (m, 4H), 2.04-2.12 (m, 1H), 1.82-1.90 (m, 1H). (NMR tpg17851_rc_2)

**(5,5-diethyl-1,3-dithian-2-yl)diphenylmethanol (1b):** $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.55-7.57 (d, 4H, J = 7.4 Hz), 7.30-7.33 (t, 4H, J = 7.6 Hz), 7.21-7.25 (t, 2H, J = 7.3 Hz),
1.64-1.70 (q, 2H, J = 7.5 Hz), 1.35-1.41 (q, 2H, J = 7.5 Hz), 0.83-0.80 (t, 3H, J = 7.5 Hz), 0.74-0.78 (t, 3H, J = 7.5 Hz). (NMR mpc24059_clm)

(5,5-dibutyl-1,3-dithian-2-yl)diphenylmethanol (1bₙ=4): ¹H NMR (400 MHz, CDCl₃) δ 7.55-7.57 (d, 4H, J = 8.0 Hz), 7.30-7.33 (t, 4H, J = 7.7 Hz), 7.21-7.25 (m, 2H), 1.53-1.62 (m, 2H), 1.26-1.34 (m, 6H), 1.04-1.21 (m, 4H), 0.88-0.90 (m, 6H). (NMR mpc24107_II48)

(5,5-dipentyl-1,3-dithian-2-yl)diphenylmethanol (1bₙ=5): 0.12 g, 27% yield, ¹H NMR (400 MHz, CDCl₃) δ 7.55-7.57 (d, 4H, J = 8.4 Hz), 7.30-7.33 (t, 4H, J = 7.8 Hz), 7.21-7.25 (t, 2H, J = 7.3 Hz), 1.07-1.33 (m, 16H), 0.86-0.90 (t, 6H, J = 6.9 Hz). (NMR tpg27059_pure)

(5,5-dioctyl-1,3-dithian-2-yl)diphenylmethanol (1bₙ=8): ¹H NMR (400 MHz, CDCl₃) δ 7.54-7.56 (d, 4H, J = 8.0 Hz), 7.29-7.33 (t, 4H, J = 7.6 Hz), 7.21-7.24 (t, 2H, J = 6.9 Hz), 1.05-1.35 (m, 20H), 0.87-0.90 (t, 6H, J = 6.6 Hz). (NMR mpc24149_66)

(1,3-dithian-2-yl)(4-ethylphenyl)(phenyl)methanol (1cₙ=2): ¹H NMR (500 MHz, CDCl₃) δ 7.55-7.58 (d, 2H, J = 7.2 Hz), 7.46-7.49 (d, 2H, J = 8.3 Hz), 7.30-7.33 (t, 2H, J = 7.5 Hz), 7.22-7.25 (t, 1H, J = 7.3 Hz), 7.14-7.16 (d, 2H, J = 8.1 Hz), 3.25 (s, 1H), 2.86-2.92 (m, 4H), 2.59-2.64 (q, 2H, J = 7.5 Hz), 2.05-2.11 (m, 1H), 1.79-1.90 (m, 1H), 1.18-1.21 (t, 3H, J = 7.6 Hz). (NMR tpg27203_rm)
(1,3-dithian-2-yl)(phenyl)(4-butylphenyl)methanol ($1c_{n=4}$): $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.58-7.60 (d, 2H, $J = 7.3$ Hz), 7.48-7.50 (d, 2H, $J = 8.2$ Hz), 7.34-7.37 (t, 2H, $J = 6.0$ Hz), 7.25-7.29 (m, 1H), 7.14-7.16 (d, 2H, $J = 8.0$ Hz), 3.31 (s, 1H), 2.83-2.95 (m, 4H), 2.59-2.64 (m, 2H), 2.03-2.10 (m, 1H), 1.80-1.91 (m, 1H), 1.55-1.64 (m, 2H), 1.29-1.42 (m, 2H), 0.91-0.95 (t, 3H, $J = 7.3$ Hz). (NMR tma25063_evapdithiane)

(1,3-dithian-2-yl)(phenyl)(4-pentylphenyl)methanol ($1c_{n=5}$): $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.78-7.81 (dd, 2H, $J = 1.4$, 8.4 Hz), 7.73-7.75 (d, 2H, $J = 8.4$ Hz), 7.56-7.60 (m, 2H), 7.46-7.50 (t, 1H, $J = 7.5$ Hz), 7.25-7.27 (d, 2H, $J = 8.4$ Hz), 2.67-2.74 (m, 4H), 2.42-2.48 (dd, 2H, $J = 8.2$, 13.3 Hz), 2.03-2.10 (m, 1H), 1.16-1.73 (m, 8H), 0.91-0.95 (t, 3H, $J = 7.4$ Hz). (NMR tma25070_fr50)

(1,3-dithian-2-yl)(phenyl)(4-octylphenyl)methanol ($1c_{n=8}$): 0.49 g, 70% yield, $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.55-7.57 (d, 2H, $J = 7.4$ Hz), 7.45-7.47 (d, 2H, $J = 8.2$ Hz), 7.30-7.33 (t, 2H, $J = 7.8$ Hz), 7.22-7.26 (t, 2H, $J = 7.2$ Hz), 7.11-7.13 (d, 2H, $J = 8.1$ Hz), 3.24 (s, 1H), 2.83-2.95 (m, 4H), 2.53-2.57 (m, 2H), 2.05-2.12 (m, 1H), 1.78-1.90 (m, 1H), 1.54-1.60 (m, 2H), 1.20-1.35 (m, 10H), 0.85-0.88 (t, 3H, $J = 6.4$ Hz). (NMR tpg27067_pure)

(1,3-dithian-2-yl)(phenyl)(4-dodecylphenyl)methanol ($1c_{n=12}$): 0.45 g, 68% yield, $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.58-7.60 (d, 2H, $J = 7.3$ Hz), 7.48-7.50 (d, 2H, $J = 8.2$ Hz), 7.34-7.37 (t, 2H, $J = 6.0$ Hz), 7.25-7.29 (m, 1H), 7.14-7.16 (d, 2H, $J = 8.0$ Hz), 3.24 (s,
1H), 2.84-2.93 (m, 4H), 2.53-2.57 (m, 2H), 2.05-2.10 (m, 1H), 1.84-1.87 (dt, 1H, J = 4.7, 10.3, 20.1 Hz), 1.54-1.57 (m, 2H), 1.20-1.35 (m, 18H), 0.83-0.88 (t, 3H, J = 6.9 Hz).

(NMR tpg27031_pure)

(5,5-dipentyl-1,3-dithian-2-yl)(4-octylphenyl)(phenyl)methanol (1d R2n=8, R4n=5):
0.43g, 78% yield, 1H NMR (400 MHz, CDCl3) δ 7.54-7.56 (d, 2H, J = 7.8 Hz), 7.43-7.45 (d, 2H, J = 8.1 Hz), 7.29-7.32 (t, 2H, J = 7.7 Hz), 7.20-7.24 (t, 1H, J = 7.0 Hz), 7.10-7.12 (d, 2H, J = 8.1 Hz), 3.38 (s, 1H), 2.53-2.61 (m, 4H), 1.53-1.60 (m, 4H), 1.12-1.28 (m, 22H), 0.85-0.90 (m, 9H). (NMR tpg27060_product)

(5,5-dibutyl-1,3-dithian-2-yl)(4-octylphenyl)(phenyl)methanol (1d R2n=8, R4n=4):
1H NMR (400 MHz, CDCl3) δ 7.54-7.56 (d, 2H, J = 7.5 Hz), 7.43-7.45 (d, 2H, J = 8.0 Hz), 7.29-7.33 (t, 2H, J = 7.7 Hz), 7.20-7.24 (t, 1H, J = 7.3 Hz), 7.10-7.12 (d, 2H, J = 8.1 Hz), 3.38 (s, 1H), 2.53-2.65 (m, 4H), 1.54-1.61 (m, 4H), 1.10-1.32 (m, 18H), 0.85-0.94 (m, 9H). (NMR mpc24152_C11)

(1,3-dithian-2-yl)bis(4-ethylphenyl)methanol (1e R2n=2, R3n=2): 1H NMR (400 MHz, CDCl3) δ 7.46-7.48 (d, 4H, J = 8.1 Hz), 7.14-7.16 (d, 4H, J = 8.1 Hz), 3.21 (s, 1H), 2.84-2.94 (m, 4H), 2.59-2.64 (q, 4H, J = 7.6 Hz), 2.06-2.17 (m, 1H), 1.80-1.89 (m, 1H), 1.18-1.22 (t, 6H, J = 7.6 Hz). (NMR tpg27180_rc3x)
E.7. Synthesis of Acetates as Solvents

E.7.1. 2,2-Diethylpropane-1,3-diyl dialkyl esters

2,2-Diethyl-1,3-propanediol (10.0 g, 0.076 mol) was added to pyridine (50 mL). The desired anhydride (0.17 mol) was added and the reaction was stirred at reflux for 4 hours. The reaction was poured into 10% aq. HCl (150 mL), extracted with dichloromethane (2 x 100 mL), the organic layer was collected, dried over anhyd. sodium sulfate, and removed under vacuum. The clear oil was then distilled under vacuum to give the dialkyl ester.

2,2-diethylpropane-1,3-diyl diacetate (2a): 27.83 g, 62% yield, distilled 75 °C, 380 mTorr, density: 1.05 g/mL, \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 2.05 (s, 6H), 1.32-1.37 (q, 4H, \(J = 7.6\) Hz), 0.81-0.85 (t, 6H, \(J = 7.6\) Hz). (NMR tpg17927_ds2)

2,2-diethylpropane-1,3-diyl dipropionate (2b): 7.57 g, 40% yield, distilled 95 °C, 290 mTorr, density: 0.94 g/mL, \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 2.31-2.36 (q, 4H, \(J = 7.6\) Hz), 1.33-1.38 (q, 4H, \(J = 7.6\) Hz), 1.12-1.16 (t, 6H, \(J = 7.6\) Hz), 0.82-0.85 (t, 6H, \(J = 7.5\) Hz). (NMR tpg17902_ds2)

2,2-diethylpropane-1,3-diyl bis(2,2,3,3,3-pentafluoropropanoate) (6): 6.5 g, 40% yield, distilled 61 °C, 500 mTorr, density: 1.45 g/mL, \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.39-1.44 (q, 4H, \(J = 7.5\) Hz), 0.86-0.90 (t, 6H, \(J = 7.6\) Hz). \(^{19}\)F NMR (376 MHz, CDCl\(_3\))
2,2-diethylpropane-1,3-diyl bis(2,2,3,3,4,4,4-heptafluorobutanoate) (7): 6.27 g, 64% yield, distilled 70 °C, 200 mTorr, density: 1.65 g/mL, \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.39-1.44 (q, 4H, J = 7.5 Hz), 0.86-0.90 (t, 6H, J = 7.6 Hz). \(^{19}\)F NMR (376 MHz, CDCl\(_3\) with C\(_6\)F\(_6\) as standard) \(\delta\) -88.6 (m, 6F), -126.2 (m, 2F), -127.78 (m, 2F), -134.8 (s, 4F). (NMR tpg17915_pure and tpg17915_19F_rf)

E.7.2. 2,2-bis(bromomethyl)propane-1,3-diyldialkyl esters

The appropriate anhydride or acid chloride (0.12 mol) and 2,2-bis(bromomethyl)-1,3-propanediol (15.0 g, 0.057 mol) were added to pyridine (65 mL) and stirred at reflux overnight. The salt was filtered and washed with dichloromethane (2 x 100 mL). The filtrate was washed with 10% aq. HCl (150 mL) and the organic layer was collected, dried over anhyd. sodium sulfate and removed under vacuum. The resulting oil was distilled under vacuum to give the product.

2,2-bis(bromomethyl)propane-1,3-diyldiacetate (3a): 4.3 g, 33% yield, density: 1.58 g/mL, \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 4.15 (s, 4H), 3.64 (s, 4H), 2.09 (s, 6H). (NMR tpg17906_ds2)
2,2-bis(bromomethyl)propane-1,3-diyol dipropionate (3b): 8.2 g, 58% yield, density: 1.64 g/mL, $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 4.16 (s, 4H), 3.51 (s, 4H), 2.34-2.39 (q, 4H, $J = 7.6$ Hz), 1.14-1.17 (t, 6H, $J = 7.6$ Hz). (NMR tpg17907_ds2_pure)

2,2-bis(bromomethyl)propane-1,3-diyol dibutyrate (3c): 15 g, 52% yield, distilled 75 °C, 18 mmHg, $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 4.01 (s, 6H), 2.31-2.37 (q, 6H, $J = 7.6$ Hz), 1.12-1.16 (t, 9H, $J = 7.6$ Hz), 1.02 (s, 3H). (NMR tpg17895_product)

E.7.3. Synthesis of 3-chloro-2,2-dimethylpropyl alkyl esters

3-chloro-2,2-dimethyl-1-propanol (12.0 g, 0.098 mol) was added to pyridine (40 mL). The appropriate anhydride (0.12 mol) was added and the reaction was stirred at reflux for 4 hours. The reaction was poured into saturated aq. sodium bicarbonate (200 mL) and extracted with dichloromethane (100 mL). The organic layer was washed with 10% aq. HCl (150 mL x 2), sat. sodium bicarbonate (50 mL), and collected and dried over anhydrous sodium sulfate. The solvent was removed under vacuum and the resulting oil was distilled under vacuum to give the product.

3-chloro-2,2-dimethylpropyl 2,2,3,3,3-pentafluoropropanoate (8): 2.27 g, 26% yield, distilled 60 °C, 17 mmHg, density: 1.34 g/mL, $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 4.23 (s, 2H), 3.42 (s, 2H), 1.08 (s, 6H). $^{19}$F NMR (376 MHz, CDCl$_3$) $\delta$ 19.46 (d, 6F), -18.67 (s, 4F). (NMR tpg17913_pure and tpg17913_19F_rm1)
3-chloro-2,2-dimethylpropyl propionate (9): 8.72 g, 50% yield, density: 0.97 g/mL, 
$^1$H NMR (400 MHz, CDCl$_3$) δ 3.93 (s, 2H), 3.43 (s, 2H), 2.33-2.39 (q, 2H, J = 7.6 Hz), 1.14-1.17 (t, 3H, J = 7.6 Hz), 1.03 (s, 6H). (NMR tpg17909_ds3_pure)

E.7.4. Synthesis of all other esters as solvents

2-methyl-2-(propionyloxymethyl)propane-1,3-diyl dipropionate (4): 1,1,1-Tris(Hydroxymethyl)ethane (10 g, 0.083 mol) was added to pyridine (60 mL). Propionic anhydride (12.2 mL, 0.29 mol) was added and the reaction was stirred at reflux for 4 hours. The reaction was poured into 10% aq. HCl (150 mL x 2) and extracted with dichloromethane (200 mL). The organic layer was collected, dried over anhydrous sodium sulfate, and removed under vacuum. The crude oil was distilled under vacuum (152 °C, 200 mTorr) to give the product as a clear oil (14.62 g, 61% yield, Density:1.08 g/mL). $^1$H NMR (400 MHz, CDCl$_3$) δ 4.01 (s, 6H), 2.31-2.37 (q, 6H, J = 7.6 Hz), 1.12-1.16 (t, 9H, J = 7.6 Hz), 1.02 (s, 3H). (NMR tpg17903_ds2)

3-bromo-2,2-bis(bromomethyl)propyl propionate (5):

Pentaerythritol Tribromide (10 g, 0.030 mol) was added to pyridine (40 mL). Propionic anhydride (12.2 mL, 0.12 mol) was added and the reaction was stirred at reflux for 4 hours. The reaction was poured into saturated aq. sodium bicarbonate (200 mL) and extracted with dichloromethane (100 mL). The organic layer was washed with 10% aq. HCl (150 mL x 2) and sat. sodium bicarbonate (50 mL) before being collected, dried over anhydrous sodium sulfate, and removed under vacuum. The crude oil was distilled under
vacuum to give the product as a clear oil (6.43 g, 56 % yield, 1.84 Density:). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 4.20 (s, 2H), 3.54 (s, 6H), 2.36-2.41 (q, 2H, $J = 7.6$ Hz), 1.15-1.17 (t, 3H, $J = 7.6$ Hz). (NMR tpg17908_ds3_pure)

**Neopentyl pivalate (10):** Pivalyl chloride (21 mL, 0.17 mol) and neopentyl alcohol (15 g, 0.17 mol) were added to pyridine (35 mL) and stirred at reflux overnight. The salt was filtered and washed with dichloromethane. The filtrate was washed with 10% aq. HCl (150 mL) and the organic layer was collected, dried over anhydrous sodium sulfate and removed under vacuum. The crude oil was distilled under vacuum (75 °C, 18 mmHg) to give the pure product (15 g, 52% yield). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 3.57 (s, 2H), 1.21 (s, 9H), 0.94 (s, 9H). (NMR tpg17892_product)

### E.8. Synthesis of ionic liquids

**General synthesis of bis(trifluoromethanesulfonyl)imides:** The appropriate tetraalkylammonium bromide (8.9 mmol) and LiNTF$_2$ (2.56 g, 8.9 mmol) were dissolved in acetone (50 mL) and reacted at room temperature for 24 hours. The reaction mixture was filtered through Celite 512, and the solvent was removed under vacuum. The resulting residue was extracted with dichloromethane and filtered to remove the solid. The organic layer was filtered 2 times and the solvent was evaporated. The clear lightly yellow liquid was dissolved in acetonitrile and stirred with carbon for 1 h. The carbon was filtered off and the filtrate was purified by gel filtration (neutral alumina, eluted with 100 mL acetonitrile) to give the desired product.
methyltrioctylammonium bis(trifluoromethanesulfonyl)imide (11): 4.25 g, 74% yield, $^1$H NMR (400 MHz, Acetone) $\delta$ 3.46-3.50 (m, 6H), 3.23 (s, 3H), 1.80-1.92 (m, 6H), 1.27-1.39 (m, 30H), 0.85-0.89 (t, 9H, J = 6.9 Hz). (NMR tpg17791_rm and tpg17791_13C)

dimethyldioctylammonium bis(trifluoromethanesulfonyl)imide (12): 6.8 g, 95% yield, $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 3.19-3.22 (m, 4H), 3.02 (s, 6H), 1.60-1.75 (m, 4H), 1.27-1.35 (m, 20H), 0.87-0.90 (t, 6H, J = 6.8 Hz). $^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ 124.56, 121.37, 118.18, 116.18, 115.00, 64.72, 50.96, 31.68, 29.03, 29.00, 26.09, 22.64, 14.08, 1.68. (NMR tpg17806_1H and tpg17806-C13)

tetrabutylammonium bis(trifluoromethanesulfonyl)imide (14): 6.22 g, 99% yield, $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 3.18-3.22 (m, 8H), 1.61-1.69 (m, 8H), 1.42-1.51 (m, 8H), 1.03-1.07 (t, 12H, J = 7.3 Hz). (NMR tpg17821_product)

1-hexyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide (15): See reference [70].

tetrahexylammonium bis(trifluoromethanesulfonyl)imide (16): 5.64 g, 97% yield, $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 3.15-3.19 (m, 8H), 1.59-1.69 (m, 8H), 1.31-1.47 (m, 24H), 0.92-0.96 (t, 12H, J = 6.9 Hz). $^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ 124.85, 121.66, 118.46,
116.72, 115.26, 58.77, 31.14, 25.86, 22.43, 21.86, 13.86, 1.86. (NMR tpg17820_product and tpg17820_13C)
Chapter 5: Design and Fabrication of a “Chip” and Imager for the Detection of Photoamplified Fluorescence Quenching.

In Chapters 3 and 4 two versions of the fluorescence “turn-off” assay were successfully developed. The first uses either the p-oligophenylene p-terphenyl (p-TPh) or p-quaterphenyl (p-QPh) as the fluorophore, which is quenched by the release of benzophenone (BP) during photoamplification. The second method relies upon the conversion of a robust fluorophore, 7-diethylaminocoumarin, to one which is short lived and quickly photobleached, 7-ethylaminocoumarin, via a reaction catalyzed by BP. The coumarin reaction was identified only recently, within the last 8 months, therefore much of the work to design and fabricate a chip was carried out using the first quenching methodology which utilizes p-oligophenylenes as the fluorophore. Thus, when we began looking for an imager we were planning to image the fluorescence of p-TPh or p-QPh, for which $\lambda_{ex} = 280$ nm or 297 nm, and $\lambda_{em} = 345$ nm or 376 nm respectively. The use of fluorophores which emit in the UV (below 400 nm) thus limited our imager design to devices which were sensitive to these wavelengths. The first attempt at designing an imager was to build a scanner which utilized a photomultiplier tube (PMT) as the detector. Next we considered the use of a charge-coupled device (CCD) camera on which the “chip” is imaged from above. Once the coumarin quenching reaction was discovered we created our own FL imager, for which the design is similar to a FL microscope.
A. Identifying a “Chip”

Before evaluating a scanner/imager a “chip” must be designed. The most expedient way to obtaining a “chip” is to utilize a substrate or material which is cheap and quick. The cheapest and quickest way to make your own “chip” is to utilize a glass microscope slide and create spots or wells for analysis. Each spot should contain all the necessary elements for photoamplification, except the sensitizer. The “chip” should also provide a compartmentalization strategy which (1) allows for free 3-dimensional collisional quenching in solution and (2) provides a structural element suitable for a pixilated spatially addressable array. In addition, we must be able to immobilize the ligands onto the surface of the chip with existing technology. Currently the printing of a bioanalytical parallel library onto chip surfaces is carried out by contact or piezo activated ink-jet printing to provide a uniform distribution of the library onto the “chip”.

A.1. Utilizing an organogelator

In order to provide a structural element, which compartmentalizes individual wells resulting in a spatially addressable array, but does not interfere with diffusion of the quencher in solution, organogels were utilized. Dialkylureas (Figure 5.1) were utilized for this purpose and are well described as low molecular weight organogelators (LMOG’s) in the literature.[74,75] These urea based gelators self-assemble through intermolecular hydrogen bonds to form a helical lattice into which guest molecules, like n-alkanes, fit to form aggregates which provide the structural lattice of the gel. The nature of these aggregates depends on the cooling temperature, type of gelator, and its
alkyl chain length (Figure 5.2). These gelators are also reported to form organogels in multiple solvent types, such as hexane, n-octane, silicone oil, ethanol, toluene, DMSO, CCl₄, etc.[74]

![Figure 5.1. Dialkyureas tested as organogelators.](image)

![Figure 5.2. Polarizing optical micrographs of gels comprised of dipropylurea (top row), and dipropylthiourea (bottom row) in silicone oil. Different cooling methods were used (a) fast cooling, (b) moderate cooling, and (c) slow cooling. Black space bars are 200 μm. These images were taken from reference [74].](image)

3-5% of N-n-octadecylurea (1b) produced stable organogels in t-butylbenzene (t-BuPh) (Figure 5.3a) which did not affect fluorescence, nor greatly diminish the diffusion of benzophenone, as measured by PFG NMR (Table 5.1). The photoamplified
fluorescence quenching of p-terphenyl by benzophenone was studied (Figure 5.3b and 5.4). The results mimicked those seen in our previous solution studies (Chapter 4), showing successful amplified quenching of the fluorophore which is not affected by the organogel.

**Figure 5.3.** (a) Stable 4% N-n-octadecylurea organogel prepared in t-BuPh containing $10^{-5}$ M p-TPh, $10^{-6}$ M benzophenone, and $10^{-2}$ M MDT BP Adduct. (b) Organogels irradiated for 100 minutes (left) control with only $10^{-5}$ M p-terphenyl, $10^{-6}$ M benzophenone, (right) photoamplified fluorescence quenching with $10^{-5}$ M p-terphenyl, $10^{-6}$ M benzophenone, and $10^{-2}$ M MDT BP Adduct.

**Table 5.1.** Diffusion coefficients of 4-trifluoromethyl-benzophenone for organogels made in t-BuPh as determined by PFG $^{19}$F NMR.

<table>
<thead>
<tr>
<th>% N-n-Octadecylurea</th>
<th>Diffusion Coefficient (cm$^2$ s$^{-1}$)</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td>$6.50 \times 10^{-6}$</td>
</tr>
<tr>
<td>2</td>
<td>$5.32 \times 10^{-6}$</td>
</tr>
<tr>
<td>4</td>
<td>$5.60 \times 10^{-6}$</td>
</tr>
<tr>
<td>6</td>
<td>$5.34 \times 10^{-6}$</td>
</tr>
</tbody>
</table>
Figure 5.4. Normalized fluorescence quenching of p-terphenyl (2 x 10^{-5} M) due to photoamplification of quencher – benzophenone (BP) - in 4% N-n-octadecylurea organogels, made in t-BuPh containing 10^{-2} M MDT BP adduct, with no BP, 10^{-8} M BP, and 10^{-6} M BP. Controls of p-terphenyl (2 x 10^{-5} M) alone and with BP (10^{-6} M) showed no interference between the gel and fluorescence as well as no significant fluorescence quenching without photoamplification.

The problems with the commercially available organogelator (1b) were (1) the opacity led to all spots looking dark on an imager and (2) the stability of the gels. Over the course of one or two days the gels separated into two phases, the fibers of the gel and the solvent. To circumvent these problems we synthesized and tested various urea based gelators (1c-h) only to find that they either did not form gels in the desired solvents, Chapter 4, or that these gels separated in the same manner as those prepared from (1b). This lead to a search for a new organogelator which would form clear transparent gels in CCl_{4}, a dichloroalkane, or an alkane.

A new dicholesteryl-based gelator was identified (Figure 5.5)[76] and two variations were synthesized. (2) utilized an alanine spacer between succinic acid and cholesterol while (3) utilized phenylalanine. This new dicholesteryl-based gelator forms
a stable and transparent organogel in CCl₄ (Figure 5.6) and decane which remains intact and is stable for at least a week. In addition the gelator does not interfere with the photoamplified fluorescence quenching assay in bulk organogel (Figure 5.7). The gelator was tested for evaporation from a microscope slide in CCl₄ and dodecane. CCl₄ evaporated from the gel within four minutes while dodecane did not evaporate after 1 hour. When the chip was placed under water, to prevent evaporation of the solvent from the gel spots, the gel did not always remain adhered to the glass surface and at the interface with water turned white, probably due to crystallization of the gelator or intercalation of water into the gel structure. As a result it was deemed necessary to provide an additional structural element as part of the chip.

Figure 5.5. Dicholesteryl-L-alininate succinic amide gelator (2).

(3) phenylalnine in place of alanine
Figure 5.6. Organogel of (2) in CCl₄.

![Figure 5.6. Organogel of (2) in CCl₄.](image)

Figure 5.7. Photoamplification of benzophenone in a CCl₄ dichloesteryl-based organogel. The organogel was prepared with 30 mM DT-BPA and 2x10⁻⁵ M p-QPh. To one sample was added 10⁻⁵ M (1.8 ppm) BP. Both samples were irradiated using five 365 nm Nichia LED’s.

![Figure 5.7. Photoamplification of benzophenone in a CCl₄ dichloesteryl-based organogel.](image)

A.2. Anodiscs™

In order to continue using a commercially available product Anodiscs, sold as Anapore™ aluminum oxide membranes (Figure 5.8) were tested. Anodiscs come in three diameters: 47, 25, and 13 mm each. They are 60 µm thick and have one of three pore sizes: 200, 100, and 20 nm. Our initial studies were done with Anodiscs having 200
nm pores, which is a pore density of 10 pores per \( \mu m^2 \). Each pore is calculated to hold 1.88 \( \mu m^3 \). Anodiscs provide a unique opportunity for massive 3D amplification (within the pore) without sacrificing the surface area of the chip (the exposed surface area of solvent per pore is \( \sim 0.063 \mu m^2 \)). In addition they have a fluorescence background 7 times lower than flat glass surfaces.[77] The use of anodiscs should enable miniaturization of the chip design while improving the statistical sampling and mechanical properties of the chip.

**Figure 5.8.** Anodisc\textsuperscript{TM} images: (left) full Anodisc shown, (right) electron microscope image of the anodisc pore structure. Taken from reference [77].

Before the Anodiscs could be utilized as a “chip” diffusion of BP had to be tested. An Anodisc\textsuperscript{TM} was loaded with decane containing 2x10\(^{-5}\) M p-QPh by dipping it into the solution and 0.1 M BP was spotted onto the disc using a capillary. The diffusion of BP was then visually monitored under UV light over the course of four hours. When a single spot is placed in the center of the Anodiscs it doubles in size. When 8-10 spots of BP were placed around the Anodiscs they diffused to cover the entire disc after 3 hours. The use of anodiscs was thus ruled out as the pores do not appear to be sealed and BP diffuses easily between them.
A.3. Glass microcapillary arrays

Due to the facile diffusion of BP through the pores in Anodiscs a more sound pore structure was needed to prevent amplification of one pixel from spilling over into adjacent pixels. Currently there are a few companies which can provide microcapillary arrays made of glass. One such company is INCOM USA ©. These arrays can be cut to almost any dimensions and come with one of several pore sizes (diameters of 25, 50, 100, 200 μm, etc.) In addition they can be made from different glass types, including clear or black doped glass. INCOM provided us with several arrays having a pore diameter of 100 μm (Figure 5.9). Pores are hexagonally packed and have a pore density of 780 pores per mm², each pore holds ~0.04 μL. The glass array provides a unique opportunity for massive 3D amplification (within the pore) without sacrificing the surface area of the chip (the exposed surface area of solvent per pore is ~ 0.008 mm³).

![Figure 5.9. Photograph of a microcapillary array chip provided by INCOM USA © (left). Image of an empty microcapillary array taken on a Mead Deep Sky Imager 2 Pro CCD camera (right).](image)

The microcapillary arrays can be quickly filled by capillary action. Several attempts were made to fill the arrays with dicholesteryl organogel in decane. It was
found that when the critical gelation temperature (temperature at which all gelator
dissolves in solution) was reached the pores were filled by simply placing the chip into
solution and heating it for several minutes. Upon removing the array from the hot gel
solution it was washed with hot solvent to remove excess gelator from the exterior. The
array was then viewed under a stereoscope to verify that all pores were filled. We found
that when the gelator was present above 0.5% in solution it crystallized out of the gel,
inside the pores of the array, upon cooling to form the organogel. When the gelator was
present below 0.5% in solution no organogels was formed upon cooling in the array. At
this point the use of organogels was abandoned in favor of using solvent, as the
microcapillary array alone could provide the structural element necessary for a pixilated
array.

The micro arrays were filled via capillary forces by simply placing the array into a
solution of solvent without immersing the top of the array. Upon removal from the
solvent the arrays were then placed upon a glass microscope cover slip to “read” them.
This proved problematic as the solvent seemed to leave the pores due to reverse capillary
action as a result of the array coming into contact with the glass cover slip. To prevent
this it became necessary to seal one end of the arrays once they had been filled. We
explored two options to accomplish this: (1) sodium silicate, and (2) polyvinyl alcohol
(PVA).

In order to prevent destroying the arrays provided by INCOM, simple capillaries
were used to test their ability to seal one end onto a glass cover slip with sodium silicate.
Upon heating, sodium silicate forms a glass which has a high tendency to bubble if it
becomes too hot. We found that it was very difficult to prevent bubbles and fractures in sodium silicate and thus decided to concentrate on using PVA.

Powdered PVA was dissolved in hot water and boiled until a thick syrup formed. When spread onto a microscope cover slip and allowed to dry the syrup forms a thin transparent film. This film is capable of sealing the end of a capillary against the cover slip and is resistant to organic solvents, but will dissolve in the presence of water. While this film does seal the pores of the microcapillary array from INCOM, it is hard to remove from the ends of all pores upon cleaning of the array. For this reason a disposable (single use) “chip” was prepared.

A.4. Designing an inexpensive and disposable array

These arrays or “chips” (Figure 5.10) were assembled by filling a glass rod with cement epoxy and then placing capillaries within (4 in x 1.5 mm OD x 0.86 mm ID). The rods were then cut to smaller “chips” of which the ends could be polished to 0.3 μ. Each array could be reused up to 5 times before the epoxy began to visibly decay. These arrays were sealed onto a glass microscope cover slide by a layer of PVA. This was confirmed by inspection under a stereoscope. The use of these “chips” will be further discussed during development of the imager.
B. Designing an Imager

B.1. The design of an LED scanner which utilizes a PMT detector

A LED scanner was built (Figure 5.11) which utilized a PMT, outfitted with an appropriate filter to collect light at/near the emission maximum of the fluorophore, as the detector. The voltage should directly reflect the intensity of emission at the maximum. The excitation source is a 300 nm LED purchased from Sensor Electronic Technology, Inc. To direct the light a 310 nm 45° single edge dichroic mirror from Semrock© was used which has a reflection band from 255-295 nm and a transmission band from 315-600 nm. The beam then passed thru a focusing lens and a pinhole to make it narrower. The scanner saved three numbers for each “point” it recorded: the x and y coordinate and voltage signal.
To test the scanner two spots were applied to a microcapillary array chip, one contained p-QPh (2x10^{-5} M) and 1,3-dithiane ethylbenzophenone adduct (DT-EBP adduct) (30 mM) in decane and the other was exactly the same with BP (10^{-5} M). The spots were placed on separate sides of the chip so that they did not meet. The scanner was aligned so the excitation source was aimed through the spot without BP and the voltage was recorded. They x,y-coordinates were then used to move the chip so that the light excited the spot containing BP, the spots voltage was recorded. The chip was then irradiated at regular intervals, and the voltage of each spot was recorded by moving between them with the x,y-coordinates at the end of each irradiation period. The results (Figure 5.12) show a difference of FL intensity, which is equivalent to the voltage intensity, of 0.1 V, indicating that the scanner is capable of reading the “chip”.

Figure 5.11. Picture and schematic diagram of the LED scanner.
Figure 5.12. Photoamplified FL quenching on a microcapillary array where each spot has p-QPh (2x10⁻⁵ M) and DT-EBP adduct (30 mM) in decane one contains BP (10⁻⁵ M) (black) and one does not (red) (left). Difference in the normalized intensity between the spot with no BP and the one with BP (No BP- BP) (right). \( \Delta I_{\text{norm}} = (I-I_{\text{final}})/(I_{\text{initial}}-I_{\text{final}}) \).

While this scanner was fully capable of reading the difference in voltage between two large spots on the microarray, the resolution became a problem when smaller “spots” were used. The resolution of the scanner was limited by how small the excitation source could be made. We purchased copper apertures from Edmund optics© with diameters of 10, 20, and 50 \( \mu \)m to try and narrow the excitation beam. The result was a lack of photons to excite a small spot of fluorophore. In order to increase the number of photons present a 266 nm laser was utilized as the excitation source, as the initial excitation beam would be much smaller and provide an excess of photons.

**B.2. Design of an laser scanner which utilizes a PMT detector**

A laser scanner was built (Figure 5.13) which utilized a PMT, outfitted with an appropriate filter to collect light at/near the emission maximum of the fluorophore, as the
detector. The voltage should directly reflect the intensity of emission at the maximum. The excitation source is a 266 nm solid state laser which uses the second harmonic of 532 nm. A 45° 266 nm laser line mirror from Edmund optics© was used to direct the beam thru a focusing lens and onto the chip above the PMT lens. The scanner saved three numbers for each “point” it recorded: the x and y coordinate and the voltage signal. An “in house” program then converted the voltage to a color map to visualize the spots on the chip. Each progression of the x,y-coordinate in one direction is equal to 2 μm. An image of the laser beam was then taken using a CCD camera and the lens over the PMT tube. By measuring the number of pixels equal to the crosswidth of the beam we determined the width to be ~100 μm (Figure 5.14).

**Figure 5.13.** Schematic diagram of 266 nm laser scanner

**Figure 5.14.** CCD image of the laser beam.
We used the scanner to “image” a microscope cover slip containing small spots of p-TPh in adipodinitrile (used for its extremely slow evaporation at room temperature), applied with a capillary. The sample was placed on the laser scanner which was set to read the area where the spots were applied, giving Figure 5.15. While the imaging of the “chip” was successful it was extremely slow, taking more than 1 hour. We needed a faster way to image the chips for which the resolution would be high enough to image spots 100 μm or smaller if the INCOM arrays were to be used.

![Figure 5.15. Color map of spots containing 2x10^{-5} M p-TPh in adipodinitrile on a glass microscope cover slip.]

**B.3. Using a CCD camera to image the microarray**

A Mead Deep Sky Imager 2 Pro monochromatic CCD camera was purchased and set up according to Figure 5.16, with the excitation source above the “chip”. To test the imager for its ability to detect fluorescence and to detect quenching by BP the INCOM chip was loaded with a solution containing 2x10^{-5} M p-QPh and a picture was taken. A capillary was then used to spot 1 mM BP onto the surface. The results (Figure 5.17) show that quenching of the fluorophore can be easily detected by the CCD camera.
Figure 5.16. CCD imager which excites fluorophore from above.

Figure 5.17. CCD image of microcapillary array filled with CCl₄ + 2x10⁻⁵ M p-QPh (right) and then spotted with 10⁻⁵ M BP (left).

We then utilized the single use “chips” prepared above (A.4.) to show that FL quenching takes place in a solution and that the FL difference between a solution seeded with BP and one without BP could be detected (Figure 5.18). The chip was prepared so that all three pores contained 2x10⁻⁵ M p-QPh and 30 mM DT-BP adduct, but only one of these pores contained 10⁻⁵ M BP. The fluorophore was excited from above with a 300 nm LED and an initial image was captured. The chip was then irradiated using one 365 nm LED and at various time intervals a new image was captured to monitor the quenching of fluorescence. While the results do show that the pore seeded with BP lost
fluorescence faster than the other two, they also show uneven pore sizes and non-uniform excitation of the pores. In addition the results were not reproducible. We believe that this may be because we are exciting the fluorophore from above and imaging from below. Therefore, much of the light we are using to image the chip is affecting the image that we take. Thus, it would be better to excite the fluorophore from below and image it from below, as is done on a FL microscope.

Figure 5.18. FL quenching of p-QPh by photoamplification on a single use chip. Pores were loaded according to the guide (left). All pores contained $2 \times 10^{-5}$ M p-QPh and 30 mM DT-BP adduct. Only one pore was seeded with $10^{-5}$ M BP.

B.4. The design and use of a fluorescence imager based upon the fluorescence microscope

Fluorescence microscopes have become an important tool in fluorescence imaging, but they remain extremely expensive. It is our goal to develop a low cost imager so utilizing a FL microscope is not possible. For our purposes the three integral
parts of the microscope are the CCD, the lenses, and the fluorescence filter block, which contains a dichroic mirror and the excitation and emission filters (Figure 5.19). It is this block that allows one to excite the fluorophore and image from the same direction. An empty block was purchased along with the necessary dichroic mirror and filters. In addition, inexpensive plano-convex (PCX) lenses were added to the filter block. A spacer was then added to keep the sample (chip) at the focal length of the lens. The previously purchased Mead Deep Sky Imager 2 Pro CCD camera was mounted below the fluorescence filter block and an LED was added as the excitation source to create the imager (Figure 5.20)

![Fluorescence Interference Filter Block](image)

**Figure 5.19.** Fluorescence filter block taken from reference [78].
Figure 5.20. Inexpensive FL imaging device which utilizes a fluorescence filter block and CCD camera. The figure shows placement of the chip which will be irradiated from above with an irradiation LED. The image at right is the filter block built in the lab.

In order to determine the most effective focal depth (volume) of fluorophore in the chip, to maximize fluorescence intensity, increasing amounts of a solution of C6 at $10^{-5}$ M was added to each capillary (1 mm $\approx$ 1 μL). The results (Figure 5.21) show that increasing the path length up to 5 mm does improve the fluorescence intensity of the image, but beyond this the intensity is not affected. This also shows that amplification should improve the signal to noise ratio, because upon photoinitiation the path length of solution containing fluorophore should begin to decrease due to quenching, making the wells where sensitizer is present less intense.
Figure 5.21. Effect of path length on FL intensity as imaged by the CCD camera. The top number is the average pixel intensity per spot while the bottom number is the number of μL C6 solution added to each pore. Maximum intensity is reached at about 5 μL.

Initial photoamplification experiments were run using the single use chip described above. Using PVA a chip was sealed onto a holder made of a glass rod (22 mm diameter x 1 in tall) glued to a glass microscope cover slip. Each well was then filled with a solution of either (A) C6 (10^{-5} M), (B) C6 (10^{-5} M) with DT-BP adduct (30 mM) and BP (10^{-5} M), or (C) solution B without BP, and the chip was covered with DI water to prevent evaporation. The chip was then placed on top of the imager, a picture was taken, and it was irradiated. Images were taken at equal time intervals to monitor the progression of the quenching due to photoamplification (Figure 5.22).
Figure 5.22. Photoamplification of benzophenone in 1,4-dichlorobenzene on a single use chip irradiated from above with a 365 nm LED. (A) C6 (10^{-5} M), (B) C6 (10^{-5} M) with DT-BP adduct (30 mM) and BP (10^{-5} M), or (C) solution B without BP. Before irradiation (left), after 150 minutes irradiation (right).

The results show that the pores containing solution (B) went dark faster than A and C as a result of fluorescence quenching by benzophenone produced during photoamplification. This shows that a sample with BP is distinguishable from a sample without it. Irradiation to obtain this result took 150 minutes, compared to 30-40 in bulk solution. Additionally, this result was not reproducible in the majority of experiments. In those where it could be reproduced the time it took to “turn-off” pores where BP was seeded varied from 45 to more than 200 minutes. We believe that this is due to uneven irradiation of the chip from above. When irradiating fluorescence cells during bulk irradiation they are irradiated from the side while rotating the samples to ensure an even distribution of photons. Irradiation from above seems to cause a new problem in providing this even distribution.
To correct this problem we returned to irradiating the sample from the side by creating a “mini” photoreactor above the imager (Figure 5.23). Doing this also necessitates leaving the sides of the capillaries exposed (they could not be covered by epoxy) in order to irradiate. We also returned to using closed capillary tubes to assemble the “chip” (Figure 5.24), believing it would be easier. The chip is now assembled around a rod by using heat shrink tubing to hold it all together. The rod can then be screwd into the motor to load the chip onto the scanner.

Figure 5.23. FL imager modified to include a “mini” photoreactor on top and its schematic diagram. Top right shows the “mini” reactor made on top of the imager.
To test this new imager, and determine its detection limit, photoamplified quenching of C6 was carried out in 1,4-DCB. Each pore contained either (A) $10^{-5}$ M C6, (B) $10^{-5}$ M C6 and 30 mM DT-BP adduct, (C) $10^{-5}$ M C6, 30 mM DT-BP adduct, and $10^{-4}$ M BP, (D) $10^{-5}$ M C6, 30 mM DT-BP adduct, and $10^{-5}$ M BP, (E) $10^{-5}$ M C6, 30 mM DT-BP adduct, and $10^{-6}$ M BP, or (F) $10^{-5}$ M C6, 30 mM DT-BP adduct, and $10^{-7}$ M BP. The chip was then loaded onto the motor, imaged, and rotated while being irradiated with one 365 nm LED outfitted with a 300-400 nm long pass filter. At various time intervals irradiation was stopped and another image was taken. The results (Figure 5.25) show that solutions seeded with $10^{-4}$ M BP and $10^{-5}$ M BP are different from those with $10^{-6}$ or $10^{-7}$ M BP and no BP. Thus, the visual detection limit is $10^{-5}$ M BP, while intensity analysis shows a difference in normalized fluorescence intensity between a pore containing $10^{-4}$ M, $10^{-5}$ M, or $10^{-6}$ M BP and one without BP, after 25 minutes of irradiation, or 80%, 65%, and 25% respectively.
Figure 5.25. Photoamplified fluorescence quenching of C6, using seeded BP solutions, in a mini reactor using capillaries sealed at one end. (A) $10^{-5}$ M C6, (B) $10^{-5}$ M C6 and 30 mM DT-BP adduct, (C) $10^{-5}$ M C6, 30 mM DT-BP adduct, and $10^{-4}$ M BP, (D) $10^{-5}$ M C6, 30 mM DT-BP adduct, and $10^{-5}$ M BP, (E) $10^{-5}$ M C6, 30 mM DT-BP adduct, and $10^{-6}$ M BP, or (F) $10^{-5}$ M C6, 30 mM DT-BP adduct, and $10^{-7}$ M BP.

The image taken before irradiation (Figure 5.25) shows significant differences between the fluorescence intensity of some of the individual pores, i.e.: they are not all equal. This is likely due to an unequal distribution of the four LED’s used as the excitation source. This was solved by using a lens to focus the LED’s.

In this chapter an inexpensive imaging method, which we have developed, based upon a FL microscope and a “mini” reactor chip in which to carry out our biotin-avidin molecular recognition studies, has been described. The imager easily allows for the
visible detection of seeded $10^{-5}$ M BP (1.80 ppm, 50 pmoles, 9.1 ng), while carrying out an intensity analysis allows for the detection of $10^{-4}$ M (500 pmoles), $10^{-5}$ M (50 pmoles), or $10^{-6}$ M (5 pmoles) BP after 25 minutes of irradiation (Figure 5.25 above).

**C. Experimental**

**C.1. General**

Common solvents were purchased from AAper Alcohol and used as is, except for THF and hexanes. THF was refluxed over and distilled from potassium benzophenone ketyl prior to use. Hexanes was distilled over calcium hydride before use. All reagents for which a synthesis is not referenced in the text or described here were purchased from Alfa Aesar, TCI America, Fisher Scientific, AK Scientific, ChemImpex, or Aldrich. All reagents were used without purification unless otherwise noted. All NMR spectra were recorded at 25 °C on either a Varian Mercury 400 MHz instrument, or a Bruker Biospin 500 MHz instrument, in CDCl$_3$ with TMS as an internal standard (unless noted otherwise). Column chromatography was performed on silica gel, 32-63μ mesh and the eluent is noted in the procedure.

**C.2. Pulse field gradient (PFG) NMR**

$^{19}$F PFG NMR to determine all diffusion coefficients for solvents in Table 5.1 was done on a Varian Mercury 400 MHz instrument using a PFG sequence adapted from the WaterSLED experiment. Each sample contained 4-trifluoromethylbenzophenone (50 mg), the noted % gelator and the solvent to be analyzed (0.6 mL).
**C.3. General information regarding photoreactions**

Photoreactions in fluorescence cells were carried out using an in house built 365 nm five LED carousel reactor outfitted with a 300-400 nm long pass filter. For each photoreaction discussed a bulk solution of fluorophore (10^{-5} M) and adduct (30 mM) was prepared and then divided to the appropriate number of samples. Benzophenone was then added to each sample, from a stock solution, at the appropriate concentration. The concentrations of each reagent in the photoreactions are stated in the text where the reaction is presented if they are not stated here. All bulk solution photoreactions were carried out in quartz fluorescence spectroscopy cells from NSG precision cells.

**C.4. General information regarding scanners and imagers**

Photoreactions carried out on chips were irradiated using one or two 365 nm LED’s from Nichia Corporation. The excitation source for the LED scanner was a 295 nm UVTOP LED purchased from Sensor Electronic Technology, Inc. (UVTOP295TO39HS) and utilized a 45° single-edge dichroic beamsplitter centered at 310 nm with a reflection band from 255 – 295 nm and a transmission band from 315-600 nm (FF310-DiO1-25x36) to direct the excitation source. The laser scanner was built with an Opto Engine LLC 5 mW 266 nm solid state laser (MPL-F-266nm) run by a DPSSL driver. A TECHSPEC® ND:YAG laser line 45° mirror from Edmund optics was used to direct the laser beam onto the sample (NT47-980). The PMT for both scanners was connected to a solid state electrometer, which acted as the amplifier, and a high volt
power supply. The x,y-coordinates were Vextra model PK245-01AA 2 phase coordinates run by a Velmex VMX stepping motor controller.

The CCD camera used for all imagers was a Mead Deep Sky Imager 2 Pro with a Sony ICX429ALL interline monochromatic CCD solid state image sensor. The effective pixels were 7.4x5.95 mm with a pixel size of 8.6x8.3 μm. The fluorescence filter block was from a Zeiss Axiovert 100 microscope. The filters and dichroic mirror for C6, p-QPh, and 7-diethylaminocoumarin-3-carboxyamide were purchased from Semrock. For C6 a 45° single-edge dichroic beamsplitter centered at 495 nm with a reflection band from 442 – 488 nm (reflects all light at wavelengths below this band) and a transmission band from 502-730 nm was used (FF495-DiO2-25x36). The excitation source was four 400 nm Ultraviolet LEDs purchased from LED Supply (L3-0-U5TH15-1) which were used without an excitation filter. A 515 nm short pass emission filter was used. For p-QPh, a 45° single-edge dichroic beamsplitter centered at 310 nm with a reflection band from 255 – 295 nm (reflects all light at wavelengths below this band) and a transmission band from 315-600 nm was used (FF310-DiO1-25x36). The excitation source was a 295 nm UVTOP LED purchased from Sensor electronic Technology, Inc. (UVTOP295TO39HS), which was used without an excitation filter. A 295 nm short pass emission filter was used (FF01-292/15-25). For 7-diethylaminocoumarin-3-carboxyamide, a 45° single-edge dichroic beamsplitter centered at 409 nm with a reflection band from 344 – 404 nm (reflects all light at wavelengths below this band) and a transmission band from 415-570 nm was used (FF409-DiO2-25x36). The excitation source was a 365 nm Nichia LED (used for irradiation previously), which was used
without an excitation filter. A 465 nm short pass emission filter was used (FF01-465/30-25). The lenses for all filter bocks were purchased from Edmund Optics®. For both coumarins TECHSPEC® PCX uncoated lenses were used that were 25x40 mm (NT45-278) and 25x25 mm (NT45-097). For p-QPh the same 25x25 mm lens (NT45-097) was used on the emission side but a fused silica TECHSPEC® PCX 25x38 mm lens (NT48-273) was used on the excitation end.

C.4.1. General information regarding the fluorescence analysis of images obtained on the CCD imaging device.

Each image was opened in Adobe Photoshop and a circle was drawn around the image of the first pore using the marquee tool. The mean pixel intensity value (from 0 to 255) was determined by the histogram tool for all pixels inside the marquee tool circle. The mean pixel intensity was recorded in Excel where the fluorescence was normalized as $I_{\text{norm}} = (I/I_{\text{std}})/I_0$, where $I$ is the pixel intensity of the pore being normalized, $I_{\text{std}}$ is the intensity of the pore containing fluorophore only in a given image, and $I_0$ is $(I/I_{\text{std}})$ for the same pore from the image at $t=0$. Following normalization the intensity difference between samples could be determined.

C.5. Preparation of PVA

Poly(vinyl alcohol) with a typical molecular weight of 89,000-98,000 was purchased from Aldrich (Item # 341584). It was then dissolved in water with heating and
water was removed until the weight of solution was equal to 0.3 g PVA/1 g water. This solution was then stored at room temperature in a sealed container.

C.6. Preparation of chips

Single use chips were assembled by filling a glass rod with cement epoxy and then filling them with capillaries (4 in x 1.5 mm OD x 0.86 mm ID). The rods were then cut to smaller “chips” and the ends were polished to 0.03 μ by Brian Burks in the Department of Engineering. A layer of PVA was painted onto the surface of a glass microscope cover slip and the chip was pushed into this PVA layer and left to dry overnight. After inspection under a stereoscope, to verify that all pores were sealed, each pore was filled with the solution of interest using a Hamilton 10 μL gas-tight syringe.

Sealed capillary “mini” reactor chips were prepared by drawing OD: 1.5 x ID: 0.86 mm capillary tubes from AM Systems glass to a 15 m point on a Sutter Instrument Co. flaming brown micropipette puller model #P97 using a 3 mm box filament with program P=500 (Heat = 515, Vel = 20, Del = 1). The 15 m end was then flame sealed for approximately 20 seconds and the capillaries were cut to 2.5 cm in length. 10 sealed capillaries were assembled around a center rod and then bound by heat shrink tubing to create a chip. Each pore of the chip was filled with the solution of interest by hand using a Hamilton 10 μL gas-tight syringe. The buffer was then added to the top of the solution by putting the tip of the syringe just below the solvent line and then injecting the buffer solution.
C.7. Preparation of dithiane adducts of benzophenone

All dithiane adducts of benzophenone used in this chapter were previously described in either Chapter 3 or Chapter 4.

C.8. Preparation of organogelators

N,n-octadecylurea and N-butylurea were purchased from TCI America. All other gelators were synthesized.

C.8.1. Urea based organogelators

To alkylamine (5 mmol) dissolved in THF (50 mL), NEt3 (1 mL) was added. This mixture was stirred at room temperature for 10 minutes before adding alkyl isocyanate (5 mmol) in THF (20 mL). Additional THF (25 mL) was added. The reaction was then left for 6h with stirring at room temperature. 5% aq. HCl (60 mL) was added and the product was collected by vacuum filtration. After drying at ambient temperature overnight the remaining water and triethylamine were removed under high vacuum to give the product which was tested as a gelator.

1,3-dioctadecylurea (1c): $^1$H NMR (500 MHz, CDCl$_3$) δ 3.12-3.17 (q, 4H, $J = 7.1$ Hz), 1.47-1.53 (m, 12H), 1.24-1.29 (m, 52H), 0.86-0.90 (t, 6H, $J = 6.9$ Hz). (NMR tpg27213_rm)
1-hexyl-3-octylurea (1d): $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 3.14-3.19 (q, 4H, J = 6.2 Hz), 1.47-1.53 (m, 4H), 1.20-1.34 (m, 16H), 0.89-0.91 (t, 3H, J = 6.9 Hz), 0.88-0.90 (t, 3H, J = 7.0). (NMR gam26026)

1-hexyl-3-dodecylurea (1e): $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 3.12-3.16 (q, 4H, J = 7.1 Hz), 1.45-1.51 (m, 4H), 1.20-1.34 (m, 24H), 0.87-0.89 (t, 3H, J = 6.9 Hz), 0.86-0.989 (t, 3H, J = 7.0). (NMR gam26025)

1-octyl-3-tetradecylurea (1f): $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 3.12-3.16 (t, 4H, J = 7.1 Hz), 1.47-1.51 (m, 4H), 1.20-1.34 (m, 36H), 0.86-0.89 (t, 6H, J = 6.7 Hz). (NMR tpg27155_pure)

1-octyl-3-hexadecylurea (1g): $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 3.15-3.19 (q, 4H, J = 7.0 Hz), 1.48-1.62 (m, 4H), 1.20-1.34 (m, 40H), 0.89-0.92 (dt, 6H, J = 3.4, 7.0 Hz). (NMR gam26029)

1-tetradecyl-3-dodecylurea (1h): $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 3.15-3.15 (q, 4H, J = 7.0 Hz), 1.48-1.52 (m, 4H), 1.20-1.34 (m, 44H), 0.89-0.92 (t, 6H, J = 7.0 Hz). (NMR gam26023)
C.8.2. Dicholesteryl succinic amide gelators:

**cholesteryl-ala-Boc (2i):** Boc-ala-OH (13 mmol) and Cholesterol (13 mmol) were dissolved in DCM (250 mL dried over CaH). The solution was maintained at 0° C. DCC (13 mmol) and DMAP (1.3 mmol) were added and the reaction mixture was stirred at 0° C for 4 h. The mixture was filtered and the filtrate was washed with 5% aq. HCl (2 x 100 mL), 5% aq. NaOH (2 x 100 mL), and water (100 mL). The organic layer was removed under vacuum and the product was purified by column chromatography (silica gel eluted with THF:Hexane (1:6)) to give a white solid as the product (4.43 g, 62% yield). 1H NMR (500 MHz, CDCl$_3$) δ 5.39-5.40 (d, 1H, J = 4.7 Hz), 5.08-5.09 (d, 1H, J = 7.4 Hz), 4.64-4.70 (tdd, 1H, J = 4.2, 7.1, 11.5 Hz), 4.25-4.32 (m, 1H), 2.32-2.34 (d, 2H, J = 7.0 Hz), 1.96-2.05 (m, 2H), 1.81-1.88 (m, 2H), 1.70-0.95 (m, 22H), 1.46 (s, 9H), 1.38-1.40 (d, 3H, J = 7.2 Hz), 1.04 (s, 3H), 0.93-0.94 (d, 3H, J = 6.5 Hz), 0.87-0.89 (dd, 6H, J = 2.3, 6.6 Hz), 0.70 (s, 3H). (NMR tpg27280, 11)

**cholesteryl-ala (2ii):** (2i) (14.4 mmol) was dissolved in dioxane (40 mL) and lowered to 0° C. 4M HCl in Dioxane (64 mL) was added and the reaction was stirred at 0° C warming to room temperature. The reaction was poured over ice after 4 hours. The product was collected by filtration and dried to give the product (4.6 g, 70% yield). 1H NMR (500 MHz, CDCl$_3$) δ 5.39-5.40 (d, 1H, J = 4.7 Hz), 5.40 (s, 1H), 4.62-4.69 (m, 1H), 3.50-3.54 (m, 1H), 2.33-2.34 (d, 2H, J = 8.0 Hz), 1.98-2.04 (m, 2H), 1.81-1.91 (m, 2H), 1.70-0.95 (m, 22H), 1.34-1.35 (d, 3H, J = 7.0 Hz), 1.04 (s, 3H), 0.93-0.94 (d, 3H, J = 6.5 Hz), 0.87-0.89 (dd, 6H, J = 2.3, 6.6 Hz), 0.70 (s, 3H). (NMR tpg27286, 31)
**dicholesteryl-L- alinate succinic amide gelator (2):** (2ii) (11.4 mmol) and succinic acid (5.70 mmol) were dissolved in dry THF (150 mL) at 0°C. DCC (11.4 mmol) and DMAP (1.14 mmol) were added and the reaction was left for 3 hours at 0°C and then warmed to room temperature over 1 h. The mixture was filtered and the filtrate was removed under vacuum. The resulting white solid was washed with hot methanol while stirring 3 times and collected by filtration to give the product (1.36 g, 11.5% yield). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta 6.37-6.38 \text{ (d, 2H, J = 7.6 Hz)}, 5.40 \text{ (s, 2H)}, 4.64-4.67 \text{ (ddd, 1H, J = 5.3, 10.9, 16.5 Hz)}, 4.56-4.61 \text{ (m, 1H)}, 2.24-2.35 \text{ (m, 8H)}, 1.98-2.05 \text{ (m, 4H)}, 1.81-1.91 \text{ (m, 6H)}, 1.70-0.95 \text{ (m, 42H)}, 1.40-1.42 \text{ (d, 3H, J = 7.2 Hz)}, 1.04 \text{ (s, 3H)}, 0.93-0.94 \text{ (d, 3H, J = 6.4 Hz)}, 0.88-0.89 \text{ (dd, 6H, J = 2.1, 6.6 Hz)}, 0.70 \text{ (s, 3H)}. \) (NMR tpg27288, 11)

**cholesteryl-phe-Boc (3i):** Prepared the same as (2i) (11.2 g, 67% yield). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta 7.27-7.30 \text{ (m, 3H)}, 7.15-7.16 \text{ (d, 2H, J = 6.9 Hz)}, 5.36-5.37 \text{ (m, 1H)}, 4.97-4.99 \text{ (d, 1H, J = 8.2 Hz)}, 4.58-4.64 \text{ (ddd, 1H, J = 4.9, 11.3, 16.2 Hz)}, 4.51-4.55 \text{ (dd, 1H, J = 6.1, 14.0 Hz)}, 3.04-3.11 \text{ (m, 2H)}, 2.19-2.29 \text{ (m, 2H)}, 1.99-2.02 \text{ (m, 2H)}, 1.81-1.86 \text{ (m, 4H)}, 1.70-0.95 \text{ (m, 23H)}, 1.42 \text{ (s, 9H)}, 1.00 \text{ (s, 3H)}, 0.91-0.92 \text{ (d, 3H, J = 6.5 Hz)}, 0.85-0.87 \text{ (dd, 6H, J = 2.3, 6.6 Hz)}, 0.67 \text{ (s, 3H)}. \) (NMR tpg27290, 11)

**cholesteryl-phe (3ii):** Prepared the same as (2ii) (0.77 g, 90% yield). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta 7.29-7.32 \text{ (dd, 2H, J = 4.5, 10.0 Hz)}, 7.20-7.26 \text{ (m, 3H)}, 5.36-5.37 \text{ (d, 1H, J = 5.1 Hz)}, 4.9-4.65 \text{ (ddd, 1H, J = 5.4, 11.1, 16.6 Hz)}, 3.67-3.70 \text{ (dd, 1H, J = 5.6, 7.7 Hz)}.

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Hz), 3.04-3.08 (dd, 1H, J = 5.6, 13.5 Hz), 2.85-2.89 (dd, 1H, J = 7.8, 13.5 Hz), 2.19-2.28 (m, 2H), 1.95-2.02 (m, 2H), 1.79-1.87 (m, 4H), 1.70-0.95 (m, 23H), 1.01 (s, 3H), 0.90-0.92 (d, 3H, J = 6.5 Hz), 0.85-0.87 (dd, 6H, J = 2.3, 6.6 Hz), 0.67 (s, 3H). (NMR tpg27294)

dicholesteryl-L-phenylalininate succinic amide gelator (3): Prepared the same as (2) (0.41 g, 50% yield). $^1$H NMR (500 MHz, CDCl$_3$) δ 7.25-7.32 (m, 3H), 7.16-7.18 (d, 2H, J = 6.9 Hz), 6.39-6.40 (d, 2H, J = 7.8 Hz), 4.79-4.83 (dd, 1H, J = 6.2, 13.8 Hz), 3.51-3.52 (d, 2H, J = 5.5 Hz), 3.06-3.14 (m, 4H), 2.50-2.51 (m, 4H), 2.21-2.31 (m, 4H), 1.98-2.05 (m, 4H), 1.84-1.88 (m, 6H), 1.70-0.95 (m, 48H), 1.02 (s, 3H), 0.93-0.94 (d, 3H, J = 6.5 Hz), 0.88-0.90 (dd, 6H, J = 2.3, 6.6 Hz), 0.67 (s, 3H). (NMR tpg27295, 31)
Chapter 6: Detection of a Molecular Recognition Event by Photoamplified Fluorescence Quenching

In Chapters 1 – 5 a new photoinduced amplified fluorescence quenching methodology for ultra-high sensitivity fluorescence "turn-off" assays has been described. At the core of this approach is the self-amplified release of a sensitizer from its masked state (DT-BP adduct). Each component of this assay – the fluorophore, solvent, and masked sensitizer – has been optimized both independently and jointly. We have shown that the assay can achieve a detection limit of $10^{-7}$ M (18 ppb) of sensitizer in bulk solution, a 100,000 fold amplification.[5,64]

I have also designed and fabricated an imager and microcapillary chip for use with this methodology. This imager is inexpensive and is adapted from current technology which is widely accepted for fluorescence imaging. We have shown that the visible detection of 50 pmoles BP (9.1 ng, $10^{-5}$ M) is possible with this new imager, and that 5 pmoles of BP (910 picograms, $10^{-6}$ M) can be detected by analyzing the fluorescence intensity of a chips pores.

The Kutateladze group has previously shown that a molecular recognition event can trigger photofragmentation, and that a photofragmentation event can set off a progression of photochemical events (photoamplification).[4,51] Thus, we must now ask if a molecular recognition can set off not just one photofragmentation, but set off a cascade of fragmentations leading to photoamplification, detecting a molecular
recognition event through the use of our new photoinduced amplified fluorescence quenching methodology. If such a binding event does trigger photoamplification it will provide a yes or no signal for the presence of the receptor’s ligand in the pore where quenching takes place (Figure 6.1).

Figure 6.1. The pores are loaded with organogel containing fluorophore (blue) and the masked sensitizer (30 mM). The ligands are printed on the gel surface (A). The target ("receptor"), carrying the sensitizer (red) is applied. Binding of the receptor to the ligand leads to penetration of the lipophilic sensitizer through the gel surface (B). Irradiation at 365 nm results in initiation of photofragmentation by the sensitizer, unmasking benzophenone in its immediate vicinity (C). The unmasked benzophenone is free to diffuse throughout the pore volume, leading to the release of more benzophenone, resulting in massive photoamplification and quenching. Taken from reference [5,64].
A. Biotin-Avidin Molecular Recognition Pair

The biotin-avidin recognition pair is widely used in bioanalytical and biotechnological applications because of its high affinity constant \((7 \times 10^{14} \text{ M}^{-1})\), which leads to a stable biomolecular construct. Each avidin is tetrameric providing four binding sites for biotin.[79] Biotin-avidin binding is commonly employed to prove that molecular recognition based sensors \([80,81]\) and immunoassays \([82]\) work. This, in addition to the fact that avidin is easily conjugated to a sensitizer capable of initiating photoamplification, is why we have chosen to utilize this pair for the proof of concept experiments for this assay.

The affinity constant of \(7 \times 10^{14} \text{ M}^{-1}\) is for biotin-avidin binding in solution. At interfaces the reported affinity constant is between \(10^{10}\) and \(10^{11} \text{ M}^{-1}\).[79] In previous studies, in aqueous buffers,[83] on vesicle surfaces,[84] on alkylthiol monolayers,[85] on optical fiber tips,[86] and for use in single molecule imaging,[79] the ratios of biotin to avidin used range from 10:1 to 1:10. The actual concentrations of biotin vary from \(2.0 \times 10^{-7} \text{ M}\) to \(1 \text{ mM}\), with avidin concentrations for binding ranging from \(9.0 \times 10^{-7} \text{ M}\) to \(9 \times 10^{-4} \text{ M}\). Thus, the amounts of biotin and avidin needed vary widely by application. In our case we will be trying to detect avidin, thus it would be appropriate to utilize an excess of biotin, while minimizing the avidin concentrations.

A.1. Determination of appropriate biotin-avidin concentrations for binding.

Calculations were carried out to determine the number of lipid molecules needed to cover the surface at the interface of organic solvent and buffer. The capillaries being
used have an ID of 0.86 mm, which would give a surface area (SA) of the solvent/buffer interface equal to 0.58 mm$^2$. It is reported that the approximate area of dipalmitoylphosphatidylcholine (DPPC), the lipid which biotin is capping during these studies, is 65 Å$^2$ or 6.5x10$^{-13}$ mm$^2$.\[87] This translates to 8.93x10$^{11}$ molecules per pore to cover the surface, which is a total concentration of 2.97x10$^{-10}$ M in 5 μL of solution. 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl)(sodium salt) (biotin capped lipid) was thus at a concentration capable of forming a monolayer at the solvent/buffer interface.

The first step towards using biotin-avidin binding to initiate photoamplification is to determine the necessary starting concentrations for binding to occur. To do this avidin-fluorescein conjugate was purchased and diluted to a concentration of 3x10$^{-8}$ M in 0.02 M PBS buffer, pH 7.5. The biotin-capped lipid was then diluted in 1,4-DCB at varying concentrations from 9.5x10$^{-9}$ to 9.5x10$^{-5}$ M. The solution containing biotin was injected into a capillary sealed at one end (OD 1.5 x ID 0.86 mm) and then avidin in buffer was loaded on top of the biotin solution using a Hamilton syringe. The capillary was then placed sideways above the CCD imager (described in Chapter 5) and images were taken at regular time intervals to determine the incubation time needed for biotin-avidin binding to occur (results for 9.5x10$^{-5}$ M biotin in Figure 6.2). The results showed that when 9.5x10$^{-5}$ M biotin capped lipid was present avidin bound to it at detectable levels within 2 hours. The FL intensity of the bound avidin-fluorescein conjugate band continued to increase up to 20 hours.
Next, we needed to determine whether avidin was capable of “pulling” the biotin capped lipid through the aqueous layer. Thus a similar experiment to the one described above was set up, except that a second layer of 1,4-DCB was added above the aq. buffer containing biotin. The results (Figure 6.3) show that no detectable amount of bound biotin-avidin is found at the second solvent-buffer interface after 2 hours. After 18.5 hours there appears be a small amount of avidin present at this interface, but this is insignificant as the entire assay (including incubation) takes no more than 6 hours.

Finally, before moving on to utilizing biotin-avidin recognition for initiating photoamplified fluorescence quenching, it was necessary to determine whether a lower concentration of avidin could be detected. The same experiment performed above (Figure 6.3) was repeated with 3.0x10⁻⁹ M avidin, a concentration ten times lower. The results in (Figure 6.4) show that bound avidin is detectable at this concentration. At this
lower avidin concentration there is also no detectible avidin at the second solvent/buffer
interface after 18.5 hours. Hopefully, this concentration of avidin is an indicator for the
detection limit of biotin-avidin binding to initiate photoamplification in our assay.

Figure 6.3. CCD images of a capillary containing 1,4-DCM with 9.5x10^{-5} M biotin
capped lipid below 0.02 M PBS pH 7.5 with 3.0x10^{-8} M avidin-fluorescein conjugate
covered with empty 1,4-DCB to determine if avidin can pull the biotin capped lipid thru
solution. Incubation times are (A) 0 minutes, (B) 60 minutes, (C) 120 minutes, and (D)
18.5 hours.

Figure 6.4. CCD images of a capillary containing 1,4-DCM with 9.5x10^{-5} M biotin
capped lipid below 0.02 M PBS pH 7.5 with 3.0x10^{-9} M avidin-fluorescein conjugate
covered with empty 1,4-DCB to determine if avidin can pull the biotin capped lipid thru
solution. Incubation times are (A) 0 minutes, (B) 60 minutes, (C) 120 minutes, and (D)
18.5 hours.
The use of excess biotin capped lipid (more than calculated for a single lipid monolayer) appears to be necessary, as concentrations below 9.5x10⁻⁵ M failed to show detectable binding of avidin-fluorescein conjugate. This is likely due to the solubility of the biotin capped lipid in 1,4-DCB, which results in the need for more lipid to cover the surface. The solubility of the biotin capped lipid was confirmed by ¹H NMR where the concentration of lipid did not decrease in the bulk NMR solvent (CDCl₃) over 18.5 hours in the presence of water. If the lipid preferred to form a monolayer then the integration vs. a standard should have decreased, instead it remained the same. Taking this into account we moved toward utilizing this molecular recognition pair to initiate photoamplified fluorescence quenching.

B. Optimizing Photoamplified Fluorescence Quenching Initiated by the Binding of the Biotin-Avidin Molecular Recognition Pair.

The first step in using biotin-avidin molecular recognition to initiate photoamplification is to outfit avidin with a sensitizer. Because xanthone is less reducible than BP it is longer lasting and a better sensitizer for this purpose. 2-Bromo-9H-xanthen-9-one (1) was synthesized, and methyl-10-undecenoate was added via Suzuki-Miyaura coupling to give (3). (3) was hydrolyzed to give the free acid (4) which was converted to its NHS ester (5) (Scheme 6.1). The NHS ester (5) was then coupled to avidin to give (Av1) (Scheme 6.2) which was purified by a sephadex G-25 column, and quantified by UV/Vis.

![Synthesis Scheme](image)

Scheme 6.2. Coupling of (5) to avidin to make (Av1).

![Coupling Scheme](image)
Conjugation of xanthone to avidin was verified by UV/Vis spectroscopy by identifying xanthone in a fraction of purified avidin conjugate (Figure 6.5). The Solver in Excel was used to carry out a least squares analysis between a model system (a fit of the sum of OD’s for free ketone and protein, at known concentrations) and the actual UV/Vis spectrum of a fraction of the conjugated protein. The Solver determined the relative contributions to the conjugate spectrum of the UV/Vis spectra of free ketone and avidin, from which the ratio could be taken to determine the number of coupled xanthone molecules per avidin. Each avidin generally carried between 3 and 5 xanthones, depending on the experiment and the fraction. Using the absorbance of xanthone at 365 nm the actual concentration of xanthone ($\varepsilon_{365} = 635$) was determined and from this the approximate concentration of avidin was calculated for each fraction. Avidin for each binding experiment was then diluted from these fractions.

![UV/Vis spectra of avidin-xanthone conjugate](image)

**Figure 6.5.** UV/Vis spectra of avidin-xanthone conjugate showing the presence of xanthone absorption at 350 nm. This sample was calculated to have 5 xanthone molecules/avidin.
The results from the above fluorescent binding study provided a starting point for photoamplified quenching experiments. The same concentrations of biotin were used with a higher concentration of avidin to be sure that plenty of sensitizer would be present upon binding. The prepared avidin was then tested for initiation of photocleavage. Using a sealed end capillary chip comparing pores containing C6 (10^{-5} \text{ M}) and DT-BP adduct (30 \text{ mM}) in 1,4-DCB with either biotin capped lipid (9.5\times10^{-5} \text{ M}) or POPS (9.5\times10^{-5} \text{ M}) as a control. The results showed no quenching of fluorescence after 2 hours indicating that no photocleavage had taken place. This is likely due to the length of the linker (11-aminoundecanoic acid) not being long enough for the sensitizer to penetrate the lipid monolayer (the tails on the lipids are 16 carbons long). To extend the linker a second 11-aminoundecanoic acid molecule was added giving (6), which was made into its NHS ester (7) and coupled to avidin to give (Av2) (Scheme 6.3). (Av2) was then used for photoamplification experiments.
Scheme 6.3. Synthesis of 11-(11-(9-oxo-9H-xanthen-2-yl)undecanamido)undecanoic acid and its coupling to avidin to give (Av2).

Using the new xanthone, with an extended linker, experiments were carried out using the CCD imager and a sealed capillary “chip”. They show (Figure 6.6) that (Av2) “sticks” to the solvent layer, in the absence of biotin, preventing detection of binding in the absence of a lipid layer to keep avidin away from the solvent surface – pores B. This “sticking” is likely due to an exposed lipophilic surface on avidin. In the presence of L-α-phosphatidylcholine (POPS) “sticking” appears to be prevented as the solvent layer is no longer easily accessible – pores C. Pores A show that in the presence of both biotin and avidin photoamplified quenching does take place. While this is likely due to molecular recognition, as the controls in the absence of avidin did not go dark, further studies were needed in order to confirm the results.
Figure 6.6. All solutions used in pores (A-E) contained C6 (10^{-5} M) and DT-BP adduct (30 mM) in 1,4-DCB. To some pores was added the following: (A) biotin capped lipid (9.5x10^{-5} M), (C) POPS (9.5x10^{-5} M), (E) POPS (9.5x10^{-5} M). Pores A, B, and C were incubated with 0.01 M PBS pH 7.5 containing (Av2) (10^{-5} M), while all others were incubated with 0.01 M PBS pH 7.5, for 4 hours prior to irradiation. Irradiation time is noted on each image.

Experiments to confirm that biotin-avidin molecular recognition initiate photoamplified fluorescence quenching were carried out in 0.7 mL fluorescence cells (1 cm x 2 mm). The amount of biotin capped lipid necessary, in 5 μL of solvent, to form a monolayer over the solvent surface area in this cell is 1.7x10^{-6} M. Therefore, the use of 9.5x10^{-5} M biotin capped lipid should still provide more than enough biotin to cover the surface of the solvent/buffer interface. These experiments should confirm: (1) biotin-avidin recognition does initiate photoamplified quenching; (2) POPS prevents avidin from sticking to the organic layer and initiating photoamplification in the absence of biotin; (3) biotin, in the absence of avidin, cannot initiate photoamplification; and (4) the avidin initiated photocleavage is detectable from photocleavage initiated by either self-cleavage or trace amounts of BP which may be present in the purified adduct.
These experiments were carried out by preparing a solution of C6 (10^{-5} M) containing DT-BP adduct (30 mM) in 1,4-DCB. To one half of this solution was added biotin (9.5 \times 10^{-5} M) and to the other POPS (9.5 \times 10^{-5} M). Four samples were then prepared, (A) and (B) contained biotin while (C) and (D) contained POPS. (A) and (C) were incubated with 0.01 M PBS pH 7.5 containing (Av2) (10^{-5} M) while (B) and (D) were incubated with 0.01 M PBS pH 7.5. After taking an initial FL spectrum the samples were then irradiated using five 365 nm LED’s from Nichia Corporation. Additional spectra were taken at regular intervals during irradiation. The results (Figure 6.7) are as expected. Biotin-avidin recognition does initiate photoamplification, giving a difference in normalized intensity between (A) and (B) of at least 0.50 after 90 minutes of irradiation. Additionally, POPS prevents avidin from sticking to the organic layer and initiating photoamplification in the absence of biotin, but it does not prevent all insertion of the sensitizer into the solvent layer. In the absence of avidin minimal photoamplified quenching takes place, making avidin initiated photocleavage detectable from photocleavage initiated by either self-cleavage or trace amounts of BP which may be present in the purified adduct.
Figure 6.7. (A-D) contain C6 (10^-5 M) and DT-BP adduct (30 mM) in 1,4-DCB. To (A) and (C) was added biotin capped lipid (9.5x10^-5 M) while (B) and (D) contained POPS (9.5x10^-5 M). (A) and (B) were incubated with 0.01 M PBS pH 7.5 containing (Av2) (10^-5 M) while (C) and (D) were incubated with 0.01 M PBS pH 7.5 for 4 hours before irradiation. (A) was the only sample which contained both biotin and avidin.

The above experiment was repeated using the capillary chip (Chapter 5) to show that biotin-avidin molecular recognition can be detected on the CCD imager developed in Chapter 5. The results (Figure 6.8) are very similar, showing that biotin-avidin binding does result in photoamplified fluorescence quenching (pores A, Figure 6.8) detectable from pores in which biotin and avidin were not incubated together before irradiation. If the biotin, avidin, and lipid concentrations for this assay are optimized that the intensity difference between pores of type A (Figure 6.8) and all other pores can be improved to give a more definitive yes or no answer to a molecular recognition event than the one shown.
Figure 6.8. (A-D) contain C6 (10^{-5} M) and DT-BP adduct (30 mM) in 1,4-DCB. To pores (A) and (C) was added biotin capped lipid (9.5x10^{-5} M) while pores (B) and (D) contained POPS (9.5x10^{-5} M). (A) and (B) were incubated with 0.01 M PBS pH 7.5 containing (Av2) (10^{-5} M) while (C) and (D) were incubated with 0.01 M PBS pH 7.5 for 4 hours before irradiation. Pores (A) were the only ones which contained both biotin and avidin. (FL) indicates only C6 (10^{-5} M) was present while (Empty) indicates the pore held 1,4-DCB and buffer. Irradiation time is noted on each image.

While POPS appeared to prevent avidin from “sticking” to the solvent it did not prevent some insertion of the sensitizer to initiate photocleavage. In addition the assay needs to be developed so that biotin capped lipid (the ligand) can be printed onto the surface of a pore. This means that the solution used in all pores should remain the same (POPS must be a component) with the exception of the biotin capped lipid which is the ligand to be printed. To determine the effect of POPS on biotin-avidin molecular recognition the experiment in Figure 6.7 was repeated in the presence of different ratios
of POPS:Biotin. The results (Figure 6.9) show that the presence of POPS does decrease or eliminate the ability for detection of biotin-avidin recognition depending on the ratio of POPS:biotin when the concentrations of biotin and avidin remain the same as in previous experiments. The best results obtained, in the presence of POPS, were for (C) and (D) at 1.5 and 2 POPS: 1 biotin, giving a difference in the normalized fluorescence intensity for [biotin – no biotin] of 0.16 and 0.35 after about 100 minutes of irradiation respectively.

A photoinduced amplified fluorescence quenching methodology for a fluorescence “turn-off” assay for the detection of biological targets and ligands has been developed and tested by the molecular recognition pair of biotin and avidin. In experiments, both in fluorescence cells and on a chip, we could distinguish between samples where both biotin and avidin were present and those where one or neither was available, if POPS was used to prevent avidin from “sticking” to the solvent layer. While an attempt was made to optimize the POPS concentration, to improve the detection limit, further work needs to be done to finalize these optimizations, to optimize the concentration of avidin, and to determine its detection limit. Following the completion of these optimizations an utilizable photoamplified fluorescence “turn-off” assay for the detection of biological targets and ligands will be completed.
Figure 6.9. (A-H) contain C6 (10^{-5} M) and DT-BP adduct (30 mM) in 1,4-DCB. In addition each cell contained the following: (A) biotin capped lipid (9.5\times10^{-5} M), (B) POPS (9.5\times10^{-5} M) (C) biotin capped lipid (9.5\times10^{-5} M) + POPS (9.5\times10^{-5} M), (D) POPS (9.5\times10^{-5} M), (E) biotin capped lipid (9.5\times10^{-5} M) + POPS (1.43\times10^{-4} M), (F) POPS (1.43\times10^{-4} M), (G) biotin capped lipid (9.5\times10^{-5} M) + POPS (1.9\times10^{-4} M), and (H) POPS (1.9\times10^{-4} M). All samples were incubated with 0.01 M PBS pH 7.5 containing (Av2) (10^{-5} M) for 4 hours before irradiation.
C. Experimental

C.1. General

Common solvents were purchased from AAper Alcohol and used as is, except for hexanes. Hexanes was distilled over calcium hydride before use. All reagents for which a synthesis is not referenced in the text or described here were purchased from Alfa Aesar, TCI America, Fisher Scientific, AK Scientific, ChemImpex, or Aldrich. All reagents were used without purification unless otherwise noted. All NMR spectra were recorded at 25 °C on either a Varian Mercury 400 MHz instrument, or a Bruker Biospin 500 MHZ instrument, in CDCl$_3$ with TMS as an internal standard (unless noted otherwise). Column chromatography was performed on silica gel, 32-63μ mesh and the eluent is noted in the procedure.

C.2. General information regarding bulk photoreactions

Photoreactions in bulk solvent were carried out using an in house built 365 nm five LED carousel reactor outfitted with a 300-400 nm long pass filter. The concentrations of each reagent in the photoreactions are stated in the text where the reaction is presented.

C.3. General information regarding reactions on the CCD imager w/ “mini” reactor

Photoreactions carried out in capillary chips, described in Chapter 5, were irradiated using two 365 nm LED’s from Nichia Corporation and imaged on the CCD imager with a single 400 nm LED excitation source, a mini carousel reactor on top, and
the fluorescence filter block was optimized for C6, all described in Chapter 5. The capillaries used to prepare the chips were purchased from AM-Systems Inc. OD 1.5 mm x ID 0.86 mm x 4 in.

C.3.1. General information regarding the fluorescence analysis of images obtained on the CCD imaging device.

All images from the CCD imager in this chapter were analyzed visually. The results were confirmed by opening each image in Adobe Photoshop and using the marquee tool a circle was drawn around the image of the first pore. The mean pixel intensity value (from 0 to 255) was determined by the histogram tool for all pixels inside the marquee tool circle. The mean pixel intensity was recorded in Excel where the fluorescence was normalized as $I_{\text{norm}} = (I/I_{\text{std}})/I_0$, where $I$ is the pixel intensity of the pore being normalized, $I_{\text{std}}$ is the intensity of the pore containing fluorophore only in a given image and $I_0$ is $(I/I_{\text{std}})$ for the same pore from the image at $t=0$. Following normalization the intensity difference between samples could be determined.

C.4. Preparation of dithiane adducts of benzophenone

All dithiane adducts of benzophenone used in this chapter were previously described in either Chapter 3 or Chapter 4.

C.5. Preparation of compounds 1 – 7

2-bromo-9H-xanthen-9-one (1): Sodium metal (~2.4 g) was dissolved in methanol (60 mL). 4-bromophenol (10 g, 58 mmol) and 2-chlorobenzoic acid (9.2 g, 59 mmol) were
added and methanol was removed under vacuum to give a cake like solid. Copper powder (cat.) was added, the reaction was heated with an open flame (bunsen burner) applied until dense white smoke spread over the entire solid. The reaction was cooled for 30 min. Concentrated sulfuric acid (100 mL) was added and the mixture was heated for 4 hours at 90 °C, cooled to room temperature, poured over ice, extracted with dichloromethane (2 x 150 mL), and washed with 5% aq. NaOH solution. The organic layer was collected, dried over anhydrous sodium sulfate, and removed under vacuum. The resulting solid was purified by gel filtration (eluted by DCM) and the solvent was removed to give the product (1.4 g, 9% yield). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.46-8.47 (d, 1H, \(J = 2.5\) Hz), 8.32-8.35 (dd, 1H, \(J = 1.7, 8.0\) Hz), 7.77-7.82 (ddd, 1H, \(J = 0.6, 2.5, 8.9\) Hz), 7.73-7.76 (m, 1H), 7.48-7.51 (d, 1H, \(J = 8.5\) Hz), 7.39-7.43 (m, 2H) (NMR tpg27159).

**methyl 11-(9-oxo-9H-xanthen-2-yl)undecanoate (3):** To a pressure vessel containing methyl-10-undecenoate (2.45 mL, 10 mmol) was added 9-BBN (0.5 M in THF, 22 mL, 10 mmol). The reaction mixture was heated to 85-90 °C with stirring for 18 hrs to provide (2). After cooling to room temperature water was added (0.15 mL) to quench unreacted 9-BBN, and this mixture was combined with (1) (1.4 g, 5.1 mmol), Pd(PPh\(_3\))\(_4\) (0.6 g, 0.5 mmol), and potassium phosphate tribasic (7 g) in a round bottom flask. The reaction was heated at reflux for 18h, filtered, and the solvent was removed under vacuum. The residue was dissolved in ethyl acetate and purified via gel filtration (eluted with hexane:ethyl acetate (20:1)) to give the purified white solid (0.877 g, 41 % yield).
**1H NMR (500 MHz, CDCl3) δ 8.34-8.36 (dd, 1H, J = 1.7, 8.0 Hz), 8.13 (s, 1H), 7.70-7.74 (ddd, 1H, J = 1.8, 7.1, 8.7 Hz), 7.54-7.57 (dd, 1H, J = 2.3, 8.6 Hz), 7.48-7.50 (d, 1H, J = 8.6 Hz), 7.41-7.43 (d, 1H, J = 8.6 Hz), 7.36-7.41 (ddd, 1H, J = 1.0, 7.1, 8.0 Hz), 3.66 (s, 3H), 2.71-2.75 (m, 2H), 2.28-2.31 (t, 2H, J = 7.6 Hz), 1.55-1.67 (qd, 4H, J = 7.6, 15.1 Hz), 1.25-1.35 (m, 12H). (NMR tpg27160A or tpg27160B)**

**11-(9-oxo-9H-xanthen-2-yl)undecanoic acid (4):** (3) (0.877 g, 2.2 mmol) was suspended in MeOH (50 mL) with stirring. NaOH (2 g) dissolved in H2O (10 mL) was added to the suspension. This mixture was refluxed for 4 h, poured over ice, and made acidic using conc. HCl. The solid was collected via filtration to give the product (0.714 g, 86% yield). **1H NMR (500 MHz, CDCl3) δ 8.34-8.36 (dd, 1H, J = 1.6, 8.0 Hz), 8.14 (d, 1H, J = 2.2 Hz), 7.70-7.74 (ddd, 1H, J = 1.7, 7.2, 8.7 Hz), 7.54-7.57 (dd, 1H, J = 2.3, 8.6 Hz), 7.48-7.50 (d, 1H, J = 8.0 Hz), 7.41-7.43 (d, 1H, J = 8.5 Hz), 7.35-7.39 (t, 1H, J = 8.0 Hz), 2.71-2.75 (m, 2H), 2.33-2.37 (t, 2H, J = 7.5 Hz), 1.59-1.69 (m, 4H), 1.23-1.37 (m, 12H). (NMR tpg27161_pure)**

**11-(9-oxo-9H-xanthen-2-yl)undecanoic acid N-hydroxysuccinamide ester (5):** (4) (0.75 g, 1.97 mmol), DIPEA (0.1 mL), DMAP (cat.), N-hydroxysuccinimide (0.45 g, 3.9 mmol), and EDC (1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride, (0.60 g, 3.15 mmol) were dissolved in THF:CH2Cl2 (2:1, 75 mL) and stirred for 24 hours. The solution was washed with water (60 mL) and saturated aq. sodium bicarbonate (60 mL), followed by NaCl (60 mL). The organic layer was dried over anhydrous sodium sulfate
and removed under vacuum to give the product (0.94 g, 99% yield).  

\[^1\text{H NMR (500 MHz, CDCl}_3\})\ \delta \ 8.34-8.36 \ (dd, \ 1H, \ J = 1.7, \ 8.0 \ Hz), \ 8.13 \ (d, \ 1H, \ J = 2.2 \ Hz), \ 7.70-7.74 \ (ddd, \ 1H, \ J = 1.7, \ 7.1, \ 8.6 \ Hz), \ 7.55-7.57 \ (dd, \ 1H, \ J = 2.2, \ 8.6 \ Hz), \ 7.48-7.50 \ (d, \ 1H, \ J = 8.5 \ Hz), \ 7.42-7.43 \ (d, \ 1H, \ J = 8.6 \ Hz), \ 7.36-7.39 \ (m, \ 1H), \ 2.85 \ (m, \ 4H) \ 2.71-2.74 \ (m, \ 2H), \ 2.58-2.61 \ (t, \ 2H, \ J = 7.5 \ Hz), \ 1.64-1.76 \ (m, \ 4H), \ 1.25-1.43 \ (m, \ 12H). \ (\text{NMR tpg27486})

11-(11-(9-oxo-9H-xanthen-2-yl)undecanamido)undecanoic acid (6):  (5) (1.31 g, 5.8 mmol) was dissolved in DMF (30 mL), 11-aminoundecanoic acid (2.36 g, 11.7 mmol), NEt\textsubscript{3} (0.2 mL), and DMAP (cat.) were added and the reaction was heated at 100 °C overnight with stirring. The reaction was poured over ice and the product was collected by filtration and purified by gel filtration (eluted with 7% MeOH in DCM) to give the product (0.62 g, 20 % yield).  

\[^1\text{H NMR (500 MHz, CDCl}_3\})\ \delta \ 8.25-8.27 \ (d, \ 1H, \ J = 8.0 \ Hz), \ 8.04 \ (s, \ 1H), \ 7.67-7.70 \ (t, \ 1H, \ J = 7.8 \ Hz), \ 7.51-7.53 \ (dd, \ 1H, \ J = 1.9, \ 8.6 \ Hz), \ 7.44-7.46 \ (d, \ 1H, \ J = 8.5 \ Hz), \ 7.38-7.39 \ (d, \ 1H, \ J = 8.6 \ Hz), \ 7.31-7.34 \ (t, \ 1H, \ J = 7.5 \ Hz), \ 3.11-3.14 \ (t, \ 2H, \ J = 7.2 \ Hz), \ 2.65-2.68 \ (m, \ 2H), \ 2.20-2.23 \ (t, \ 2H, \ J = 7.5 \ Hz), \ 2.07-2.10 \ (t, \ 2H, \ J = 7.6 \ Hz), \ 1.51-1.62 \ (m, \ 8H),1.35-1.43 \ (m, \ 4H) \ 1.10-1.25 \ (m, \ 20H). \ (\text{NMR tpg27511, 31})

11-(11-(9-oxo-9H-xanthen-2-yl)undecanamido)undecanoic acid N-hydroxysuccinamide ester (7):  A mixture of (6) (0.5 g, 0.89 mmol), NEt\textsubscript{3} (0.05 mL), DMAP (cat.), N-hydroxysuccinimide (0.154 g, 1.3 mmol), and EDC (1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride, (0.204 g, 1.1 mmol) was
dissolved in DMF (15 mL) and stirred for 24 hours. The solvent was removed under vacuum. The product was dissolved in DCM and purified by gel filtration eluted with DCM (50 mL) and then 10% MeOD in DCM (100 mL) to give pure product (0.135 g, 23% Yield). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.34-8.36 (d, 1H, \(J = 1.7, 8.0\) Hz), 8.14 (d, 1H, \(J = 2.2\) Hz), 7.70-7.74 (ddd, 1H, \(J = 1.7, 7.1, 8.6\) Hz), 7.55-7.57 (dd, 1H, \(J = 2.2, 8.5\) Hz), 7.49-7.50 (d, 1H, \(J = 8.4\) Hz), 7.42-7.44 (d, 1H, \(J = 8.5\) Hz), 7.36-7.39 (t, 1H, \(J = 7.5\) Hz), 3.21-3.25 (dd, 2H, \(J = 7.0, 13.1\) Hz), 2.71-2.74 (m, 2H), 2.58-2.61 (t, 2H, \(J = 2.5, 7.5\) Hz), 2.13-2.16 (m, 2H), 1.70-1.76 (dt, 2H, \(J = 7.5, 15.2\)), 1.60-1.68 (m, 6H), 1.46-1.50 (m, 2H) 1.22-1.43 (m, 22H). (NMR tpg27513_pure)

**C.6. General procedure for avidin-xanthone conjugation:**

Avidin (5 mg, 0.000074 mmol), purchased from Invitrogen or Pierce Biotechnology (now ThermoScientific) was dissolved in PBS (0.01 M, pH 7.5, 1 mL). The appropriate xanthone-NHS ester was dissolved in DMSO (5 mg/1 mL) and 0.1 mL of this solution was added to the buffer containing Avidin. The resulting mixture was shaken gently overnight at room temperature and purified on a Sephadex G-25 column (to 2.5 g of Sephadex G-25, purchased from Aldrich, was added a 0.01 M PBS to make a slurry, this slurry was poured into the appropriate column, the mixture was added and eluted with the phosphate buffer). 16 fractions (1 mL each) were collected and tested by UV/Vis. The conjugated avidin is generally found between fractions 3 and 8 containing the product with an average of 3 to 5 xanthone molecules per avidin.
The Solver in Excel was used to carry out a least squares analysis between a model system (a fit the sum of OD’s for free ketone and protein, at known concentrations) and the actual UV/Vis spectrum of a fraction of the conjugated protein. The solver determined the relative concentrations of ketone and avidin present in the conjugate, from which the ratio could be taken to determine the number of coupled xanthone molecules per avidin. Each avidin generally carried between 3 and 5 xanthones, depending on the experiment and the fraction. Using the absorbance of xanthone at 365 nm the actual concentration of xanthone ($\varepsilon_{365} = 635$) was determined and from this the approximate concentration of avidin was calculated for each fraction. Avidin for each binding experiment was then diluted from these fractions.

**Analysis of avidin-(5) conjugation (Av1):** Each avidin tetramer carries approximately 2 to 3 xanthone molecules. The concentration of xanthone was calculated to be $1.4 \times 10^{-4}$ M, from this it was determined the approximate concentration of avidin was $8 \times 10^{-5}$ M. This fraction was diluted to the appropriate concentrations as indicated in the text before use. Below is the fitting of OD’s for free avidin and free xanthone at $10^{-4}$ M to the actual avidin-xanthone conjugate spectrum. (tpg27480)
Analysis of avidin-(7) conjugation (Av2): Each avidin tetramer carries approximately 5 xanthone molecules. The concentration of xanthone was calculated to be $1.4 \times 10^{-4}$ M, from this it was determined the approximate concentration of avidin was $8 \times 10^{-5}$ M. This fraction was diluted to the appropriate concentrations as indicated in the text before use. Below is the fitting of OD’s for free avidin and free xanthone at $10^{-4}$ M to the actual avidin-xanthone conjugate spectrum. (tpg27518)
C.7. General information regarding biotin-avidin binding experiments

The avidin-fluorescein conjugate used in the fluorescent binding studies was purchased from Invitrogen and contained 4 moles dye/mole avidin. All reactions for photoamplified fluorescence quenching by molecular recognition of biotin-avidin were prepared at the concentrations described in the text. Biotin solutions were prepared from a stock solution purchased from Avanti Polar Lipids at a concentration of 10 mg/mL in CHCl₃. POPS solutions were prepared from a stock solution purchased from Avanti Polar Lipids at a concentration of 20 mg/mL in CHCl₃.

All experiments in which biotin-avidin molecular recognition took place were left at room temperature to allow for binding. In the fluorescence binding studies the times are noted in the text. All other experiments were left for 4 hours unless otherwise noted in the text.
Chapter 7: Conclusion

The objective of my research was to develop a novel method which couples the molecular recognition-triggered photoamplification chain in diaryl ketone adducts of dithiane with a "turn-off" and/or “turn-on” fluorescence-based assay for the detection of biological targets and ligands, regardless of their nature, through a molecular recognition event. Throughout this dissertation several key steps to the development of such an assay have been studied and optimized. The most significant of these steps include: (1) optimization of photochemistry as it applies to the development of an ultra-sensitive photoamplified fluorescence turn-off assay, (2) implementation of the method in the design and fabrication of chips for ultra-sensitive screening of microarrays, and (3) integration of this microchip assay into a fluorescence based signal transducer for ultra-sensitive detection of molecular recognition events. By addressing these key steps I have shown that photoamplification of dithiane adducts of BP can be utilized in an ultra-sensitive fluorescence assay for the detection of molecular recognition events.

A. Dithiane Adducts of Benzophenone

At the beginning of this project a better understanding of the photoamplification of dithiane adducts of BP was obtained by studying the mechanism of fragmentation in adducts of 2-alkyl substituted dithianes with BP. From this study it was determined that
the quantum efficiency of cleavage steadily increases for adducts of dithianes substituted with longer alkyl chains (Table 2.1).

The activation enthalpies ($\Delta H^\neq$) were determined to be 4.7 kcal/mol for the MDT-BP adduct and 1.5 kcal/mol for the EDT-BP adduct, which is in accord with the activation energies obtained by Whitten for the thioindigo sensitized fragmentation in vicinal amino alcohols.[59] Based upon methyldithianyl radical being more stable than that of tert-butyl, which in turn is more stable than the unsubstituted dithiane-2-yl radical[53], it is not unreasonable to assume that the apparent $\Delta H^\neq$ of 4.7 and 1.5 kcal/mol correspond to fragmentation of the oxygen-centered radicals, releasing methyldithianyl and ethyldithianyl radicals respectively. Therefore, we believe that the reaction takes place via the O-centered radical mechanism and not via the original ‘Grob-like’ mechanism in the case of adducts of benzophenone.

To support these findings computational and NMR studies of dithiane BP adducts were undertaken. Computations show the lowest energy conformations for both methyl and ethyl derivatives, the eMgO/eEgO conformers (Figure 2.3), had the smaller alkyl substituent in the equatorial position, whereas the bulky benzhydryl group was axial, with oxy-radical being gauche to methyl/ethyl (that is, anti to one of sulfur atoms). These conformers have the lowest energies at both the minima and the transition states. Low temperature NMR experiments confirm these results. In the methyl and ethyl derivatives of the adduct the multiplets for the axial and equatorial protons $H_2C^{(3)}$ and $H_2C^{(5)}$ split into two sets. If the rotation of the benzhydrol group has been stopped at this temperature this would indicate that the most stable isomer is unsymmetrical.
While the findings in Chapter 2 did not rule out the ‘Grobe-like’ mechanism for photofragmentation, they do provide further evidence that supports the alternative oxygen-centered radical mechanism. Both the experimental and computational studies show apparent enthalpies of activation for photoinduced fragmentation which are in agreement, and indicate that the most stable conformation of the ethyl and methyl adducts of BP are unsymmetrical.

B. Fluorophores for a “Turn On” or “Turn Off” Assay

The search for fluorophores to be used in both a “turn-on” and “turn-off” fluorescence assay was undertaken. Three approaches to the design of a fluorescence “turn-on” assay (Figure 3.1) were explored: (1) A fluorophore masked with dithiane which recovers fluorescence upon cleavage; (2) A fluorophore conjugated to an adduct carrying a quencher, which upon cleavage releases the quencher allowing for fluorescence recovery; and (3) An adduct carrying self quenching fluorophores, that upon release, recovers fluorescence. In the end none of these approaches was successful and the focus of the project was directed toward identifying a fluorophore for and developing a “turn-off” assay.

Because our adduct cleaves into two parts upon photofragmentation, BP and dithiane, one or both of these parts must be capable of quenching the fluorophore. In the case of the developed assay BP plays this role. Two fluorophores were identified which meet the requirements for use in this assay (Chapter 2, B). The first, p-QPh is quenched via collisional quenching with BP, leading to an effective turn-off assay. The problem
with this assay is that the emission of p-OPh is in the UV region \( \lambda_{\text{em max}} = 376 \text{ nm} \), and most biological assays utilize fluorophores which emit in the visible spectrum.

7-Diethylaminocoumarin-3-carboxylic acid and coumarin 6 (C6) were later identified as reliable fluorophores for the turn-off assay, both of which are quenched via a de-ethylation reaction catalyzed by BP. After verifying that C6 does not interfere with photoamplification we decided to pursue its use in the photoamplified fluorescence turn-off assay.

C. Optimizing the “Turn-off” Assay

The masked sensitizer – dithiane adduct of BP – and the solvent were optimized for the turn-off assay. The solvent was optimized for 7 key parameters: (1) hydrophobicity; (2) solubility of the masked sensitizer; (3) method of purification for the adduct; (4) quantum yields of fragmentation; (5) lack of interference with photochemistry and fluorophore emission; (6) miscibility with aqueous physiological buffers; and (7) diffusion of benzophenone – for which the diffusion coefficient was experimentally determined in many solvents (Table 4.1).

Various adducts were prepared and modified with alkyl chains at several different positions (Figure 4.1). The \( \Phi_{\text{sens}} \) was experimentally determined to range from 0.12 to 0.18 depending on where the adducts are substituted. In addition, the solubility of each adduct was tested in high boiling alkanes to determine their suitability if such a solvent should be needed. Purification of the adducts was also undertaken, but as there is currently no method to differentiate between a minute impurity of BP and very small
amounts of direct (unsensitized) cleavage of the adduct, we cannot determine whether the BP present is truly an impurity or if it is a product of cleavage and therefore will always determine our detection limit. Therefore, the method by which purification takes place has a large effect on the detection limit of the assay.

In the case of DCM (Figure 4.2) $10^{-7}$ M BP (18 ppb) was reproducibly detected. This translates into a 100,000 fold amplification of the original sensitizer. This signal amplification affords scientists the ability to detect a wide range of molecular binding events. It not only improved the signal strength from those events which are already detectable at reasonable limits in other such assays, but also affords the ability to identify binding events which, without this amplification, would be difficult or even impossible to detect.

A thorough study of solvents for this assay was then undertaken to try and identify a solvent “type” which would allow for the use of a recrystallizable adduct. Unfortunately, these solvents did not provide results anywhere close to those achieved in DCM and their use was abandoned. Finally, ionic liquids were tested due to their unique properties. Six IL’s were prepared (Figure 4.5) and tested for this assay only to conclude that while IL’s may help facilitate the photocleavage reaction they cannot be used because they quench the fluorophore and they slow down diffusion of BP, lowering the detection limit of the assay.

DCM became our gold standard in regards to the detection limit of the “turn-off” assay, thus we looked to chlorinated alkanes to try and solve our solvent problem. 1,4-DCB was identified as a preferable solvent for this assay, for which the detection limit of
BP in bulk solution is $10^{-6}$ M (0.1 μM, 180 ppb). This is considered to be a biologically relevant concentration.

D. Designing a Chip and Imager

A variety of chips were designed and prepared which allowed for free 3-dimensional collisional quenching in solution and provided a structural element suitable for a pixilated spatially addressable array. Some of these chips utilized organogels to provide the structural element while other were pre-formed and provided to us. As a result we decided to pursue the use of a sealed end capillary chip.

Several imagers were designed and tested, but in the end we have developed an inexpensive imaging device and method based upon a FL microscope, and a “mini” reactor chip in which to carry out our biotin-avidin molecular recognition studies. The imager developed easily allows for the visible detection of seeded $10^{-5}$ M BP (1.80 ppm).

We determined that increasing the path length up to 5 mm does improve the fluorescence intensity of the image, but beyond this intensity is not affected. It has also been shown that the imager can visibly distinguish between solutions seeded with $10^{-4}$ M BP, $10^{-5}$ M BP, and no BP, giving a visual detection limit of $10^{-5}$M BP. Further analysis of the intensities of the pores gives a detectable difference in normalized fluorescence intensity between a pore containing $10^{-4}$ M, $10^{-5}$ M, or $10^{-6}$ M BP and one without BP, after 25 minutes of irradiation, of 80%, 65%, and 25% respectively. Thus, we now have the chip and imager necessary to develop an on-chip method for photoamplified fluorescence quenching initiated by a molecular recognition event.
E. Detection of a Molecular Recognition Event

The final task was to implement this methodology for the detection of a molecular recognition event – biotin-avidin binding. First, the minimum starting concentrations for binding to occur were determined, using avidin-fluorescein conjugate and a biotin-capped lipid in a capillary tube which could be imaged on our in-house built CCD imager. These results showed that after 2 to 4 hours $3 \times 10^{-9}$ M avidin binds to biotin, in solution at a concentration of $9.5 \times 10^{-5}$ M, at an easily detectable limit by fluorescence imaging. Because of the solubility of the biotin capped lipid using lower concentrations was not possible.

Utilizing this information, photoamplified fluorescence quenching initiated by molecular recognition of the biotin-avidin pair was studied. In experiments, both in fluorescence cells and on a chip, we can distinguish the fluorescence intensity of samples where both biotin and avidin are present from those where only one or neither is present on the condition that POPS was used to prevent avidin from “sticking” to the solvent layer. An attempt was made to optimize the POPS concentration to improve the detection limit, showing that the concentration of POPS does affect molecular recognition of biotin and avidin. Overall, these experiments showed promise that this assay does work, and that molecular recognition of the biotin-avidin pair does initiate photoamplified fluorescence quenching, which can be imaged on a chip by an inexpensive CCD imager.
F. Final Results and the Future

A novel method which couples the molecular recognition-triggered photoamplification chain in diaryl ketone adducts of dithiane with a "turn-off" fluorescence-based assay for the detection of biological targets and ligands, regardless of their nature, through a molecular recognition event has been developed. Each component of this new photoinduced amplified fluorescence quenching methodology – the fluorophore, solvent, and masked sensitizer – has been optimized. We have shown that the assay can achieve a detection limit of $10^{-7}$ M (18 ppb) of sensitizer in bulk solution, a 100,000 fold amplification. A proof of concept has been carried out that shows molecular recognition of the biotin-avidin pair does lead to photoamplified fluorescence quenching.

Work which remains to be completed includes optimization of the aspects related to biotin-avidin binding and prevention of non-specific photoinitiation due to “sticking” of avidin to the organic solvent or lipid layers. This work should include further optimization of the POPS concentration in relation to biotin in the lipid layer, and optimization of the avidin concentration and determination of its detection limit. Following the completion of these optimizations an utilizable photoamplified fluorescence “turn-off” assay will be completed. This assay, carried out on a chip, will be able to provide a yes or no result showing that a molecular recognition event between a biological target and its ligand (known or unknown) has taken place.
References


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