Synthesis of Various Classes of Cyanine Fluorophores and Their Application In In Vivo Tissue Imaging

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SYNTHESIS OF VARIOUS CLASSES OF CYANINE FLUOROPHORES AND THEIR APPLICATION IN IN VIVO TISSUE IMAGING

by

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Under the Direction of Maged M. Henary, Ph.D

ABSTRACT

A novel series of near-infrared fluorescent contrast agents was developed and characterized. Their physicochemical and optical properties were measured. By altering functional groups of cyanine fluorophores, the selective targeting of endocrine glands, exocrine glands, cartilage and bone using NIR fluorescence to visualize the targeted tissue has been reported. These agents have high specificity for tissue targeting inherent to the chemical structure of the fluorophore. After a single low-dose intravenous injection these agents have high specificity for tissue targeting inherent to the chemical structure of the fluorophore. The results lay the foundation for future improvements in optical imaging in endocrine surgery, tissue engineering, joint surgery, and cartilage-specific drug development.
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College of Arts and Sciences
Georgia State University
May 2017
DEDICATION

This dissertation is dedicated to my grandparents, Myrna and Dan Kranis, who I know have been waiting eagerly and patiently for this day to come. It is also dedicated to my “Grandpa Hats” who would have turned 90 on the day that I earned my doctorate and my Grandma Enid who passed away on the eve of me receiving my undergraduate degree.
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Most importantly, I thank my parents, Ellen and Jeff Levitz, for their steadfast support and my sister Hillary for always being around when needed. This still would not have been possible without you. Lastly, thank you to the rest of my large extended family for all their support through the entirety of my education.
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1 Introduction to Real-Time Biomedical Imaging

While medication for the treatment of human diseases continues to improve, surgery remains a central component for many complicated indications. The ability to fully resect tumorous tissue in cancer patients while avoiding vital tissues present in the surgical field greatly affects the patient’s long-term survival. The past decade has seen a rapid growth and technological advancement of imaging techniques. Computerized tomography (CT), MRI, ultrasonography and endoscopy all aid in the presurgical process to ensure the possibility of death from surgery is worth the risk for potential cure, but their utility during surgery is limited. In general, the surgeon must distinguish diseased tissue from normal tissue solely by their eyesight and physical analyses, even during complex resections. During surgery, the question contemplated by every patient and practitioner is “how do you know if you got it all?” Surgeons typically have to submit specimens for histological evaluation and wait for the results to determine if additional removal is necessary.

1.1 Imaging Modalities

New technologies are revolutionizing the way surgery is performed. The medical imaging community has pursued various avenues by translating spectral imaging techniques from existing preoperative methods to offer surgeons real-time visualization of the surgical field. Most of these modalities are tomographic imaging, including single photon emission computed tomography (SPECT) or positron emission tomography (PET). Of the two, SPECT is more widely used as providers have more access to machines and protocols for acquisition, processing, and reporting. PET offers further advantages over SPECT, such as improved image quality, greater interpretive certainty, higher diagnostic accuracy, lower exposure to radiation, and shorter imaging times through its use of positron-labeled molecules in smaller doses. While both of these have had some success, nonspecific uptake of radioactive tracers leads to a less distinguishable signal rendering the surgical field still challenging to interpret. In addition,
these tracers involve the use of deep penetrating radioactivity limiting the overall potential of their real-time translation due to their short lifespan which can be shorter than one hour. This can require the contrast agents to be injected multiple times during a surgery exposing the patient to higher amounts of damaging radiation. The use of these radioactive tracers can be the equivalent of anywhere from 2-250 x-rays.\textsuperscript{6} Due to the need for lead collimators to make SPECT imaging 3D, it has a very low detection efficiency (\(<10^{-4}\) times the number of emitted gamma rays).\textsuperscript{7} The subsequent use of reconstruction algorithms to visually generate the spatial distribution of signal sources allows for limited spatial resolution and poor temporal resolution.\textsuperscript{8}

![Absorbance of water, hemoglobin and oxyhemoglobin highlighting the NIR window from 650-900 nm](image)

**Figure 1.1. Absorbance of water, hemoglobin and oxyhemoglobin highlighting the NIR window from 650-900 nm**

Intraoperative fluorescence imaging or fluorescence-guided surgery (FGS), can provide high fidelity visualization for real-time localization, resection and margin confirmation in surgery\textsuperscript{9} without the use of ionizing radiation. FGS relies on the emission of light through fluorescence for illumination and an imaging system to assist surgeons in distinguishing different tissues through the injection of fluorescent detection agents. The disadvantages of FGS are the tissue penetration depth and autofluorescence. As a result, fluorescence imaging in the visible spectrum hinders resolution and contrast with increasing tissue depths. As shown in Figure 1.1, the NIR range from about 650-900 nm is useful due to low autofluorescence and
scattering from tissues. Tissue penetration by this light is enhanced in this range due to low tissue florescence and absorption from hemoglobin and can reach a penetration depth of almost 1 cm as shown in Figure 1.2. Based on tissue penetration depth, a high signal-to-background ratio (SBR) is required.

![Figure 1.2. Effective penetration depth of light](image)

### 1.2 Fluorescence Guided Surgery Technology

Many groups have invested in developing imaging systems for NIR FGS over the past few years$^{10-23}$ and now some of these systems have been applied clinically. These systems can be classified as portable, functional and endoscopic, and laparoscopic. Many of these systems have already been utilized in clinical diagnosis and treatment. These different types of systems offer distinct advantages such as operation convenience, improved image assessment and increasing detection depth. At least three of these systems have already been approved by the FDA.

Portable FGS systems satisfy the criteria for convenient operation. Hammamatsu’s Photodynamic Eye (PDE™) is an FDA approved handheld imaging system that emits NIR light and detects it through the *in vivo* uptake of fluorescent 2D images. Additionally, the FDA approved Artemis™ system includes intraoperative visible images to give an overlay providing excellent utility for nerve surgeries.$^{24,25}$ A third FDA approved portable system is the SPY system that has been applied to monitor skin perfusion in nipple-sparing mastectomies.$^{26}$
The Fluorescence Assisted Resection and Exploration (FLARE) system developed by the Frangioni lab at Harvard Medical School is characterized as a functional intraoperative system. It uses three cameras to simultaneously collect images from two NIR channels (700 and 800 nm) and one visible channel, exploiting the large NIR wavelength window and theoretically allowing for a disease-targeted fluorophore to be distinguished from another contrast targeting vital tissues. The FLARE system has successfully been applied to sentinel lymph node mapping in breast cancer, cervical cancer, and vulvar cancer patients. Although this system is not portable and has a large hardware footprint, a mini FLARE system has also been developed.

![Figure 1.3. Schematic of dual channel intraoperative imaging](image)

Recently, this technology has been applied to the surgical removal of liver metastases, breast cancer, ovarian cancer, melanoma, vulvar cancer, and cervical cancer, but an additional part of this is targeting healthy tissue that should be avoided during surgery. With the help of FGS technology, the prognosis of patients will undoubtedly improve. For example, Loja et al. used FGS with Alexa-647 labeled pH Low Insertion Peptide, pHLIP (a pH responsive peptide), to detect alterations in extracellular pH in head and neck squamous cell carcinoma in order to assess tumor margins during surgery, improving the prognosis of this cancer through better detection.
In recent years FGS has been coupled with endoscopic and laparoscopic technology to work around the issue of penetration depth. These systems have been applied to cancer surgery and have assisted in minimally invasive cancer therapy.\textsuperscript{31} Disadvantages to these systems are determining the optical path length to simultaneously view both visible and NIR fluorescent images. It is also difficult to alter the workflow procedure to integrate the FGS technology. Glatz \textit{et al.} developed a color and NIR fluorescence laparoscopy system for the identification of tumor margins.\textsuperscript{32} Another laparoscopic system, the Fluoscope developed by Harvard Medical School, is currently recruiting participants for clinical trials for sentinel lymph node mapping in lung cancer.

![Figure 1.4. High tech goggles help a surgeon visualize cancer cells. Image reprinted with permission Washington University School of Medicine in St. Louis/Siteman Cancer Center](image)

Most recently, a goggle system with metal-oxide-semiconductor and a see-through display has been developed by the Achilefu lab at Washington University, shown in Figure 1.4. This is the first application of a dual-modal see-through display in NIR fluorescence imaging. The goggles include light source, control, detector and display modules with an 830 nm emission filter and can detect a fluorescence signal as low as pM concentrations.\textsuperscript{23}

### 1.3 Contrast Agents

The most important components of FGS technology are the surgical navigation system and the imaging contrast agents. The surgical navigation system is required to activate these
fluorophores with NIR light and detect their fluorescence on a live video screen for accurate surgical guidance after image registration processing by a computer.11

Because fluorescence is only one pathway that an excited photon can return to lower energy, the chemical structure of contrast agents must be designed such that fluorescence is the dominant relaxation pathway resulting in a high quantum yield of photon emission. They should also have a high molar absorptivity; thereby giving the agents a higher molecular brightness. Agents should be specific and cleared quickly from non-target areas to give a lower background signal. There are three ways that a dye can target specific tissues; active targeting, activatable targeting, and passive targeting. Active targeting involves conjugation of a contrast agent to a targeting moiety.33 The success of approach is dependent on several engineering obstacles. Most importantly is the choice of and overall efficacy of the targeting ligand. Next a satisfactory contrast agent must be chosen with high molecular brightness and fast clearance (before ligand attachment).34 Lastly, and the often overlooked is the isolating linker. The linker must be flexible enough to allow the targeting ligand to bind and chemically inert so as to avoid interactions with the targeting ligand, contrast agent, or binding site. It is also important to utilize the linker to optimize the physicochemical properties of the of the entire molecule. While this rapid process offers the potential for high SBR, the introduction of a large dye into the structure of a targeting moiety often alters its properties causing it to no longer specifically target. An activatable contrast agent is one in which the contrast agent’s fluorescence is quenched until a particular stimulus (i.e., pH, enzyme, redox potential) alters the structure of the agent activating its fluorescence. Passive targeting, also referred to as structure inherent targeting relies on the biodistribution of the contrast agent to be selective. The biodistribution of a contrast agent can be due to the size, hydrophobicity (often measured as logD or logP), molecular recognition, and/or permeability of the tissue. This structure inherent targeting allows for the smallest agents and the avoidance of obstacles to active and activatable targeting.
1.3.1 FDA Approved Contrast Agents

![Chemical structures of FDA approved NIR contrast agents](image)

**Indocyanine Green**  
**Methylene Blue**

Figure 1.5. Chemical structure of FDA approved NIR contrast agents

There are currently two NIR contrast agents that have been approved by the Food and Drug Administration (FDA); the cyanine dye indocyanine green (ICG) and the phenothiazine methylene blue (MB), shown in Figure 1.5. These contrast agents have been used in intraoperative imaging for the treatment of sentinel lymph node mapping,\textsuperscript{10,28,35,36} hepatic micrometastases detection,\textsuperscript{37} and the visualization of the gastrointestinal tract, bile duct, and ureters.\textsuperscript{10,38-42} However, both of these agents display strong non-specific binding to proteins \textit{in vivo} and are not selective. The inability to selectively target specific tissues has limited their usefulness in application. In addition, the structures of ICG and MB are such that it cannot be covalently conjugated to targeting moieties without significant alteration of the core structure.\textsuperscript{43} Methylene blue has mediocre optical properties compared to other contrast agents. In serum MB displays a molar absorptivity of 71,200 M\textsuperscript{-1}cm\textsuperscript{-1} and a quantum yield of 3.8\% owing to a molecular brightness of 2,706 M\textsuperscript{-1}cm\textsuperscript{-1}. In comparison cyanine dyes such as ICG typically display molar absorptivities above 100,000 M\textsuperscript{-1}cm\textsuperscript{-1} (121,000 M\textsuperscript{-1}cm\textsuperscript{-1} for ICG in serum) and quantum yields closer to 10\% (9.3\% for ICG). This will be discussed in more detail in Chapter 3.

While the imaging community generally agrees that MB and ICG are not ideal fluorophores, they are presently the only NIR fluorophores that have been FDA approved.
Numerous imaging systems have been developed but there is clearly a need for the development of new and better fluorophores to provide the best clinical options for both patients and surgeons.
2 SYNTHESIS OF A LIBRARY

2.1 Cyanine Dyes

Significant advancements are being made to develop NIR-fluorescent compounds that target specific tissues and help surgeons to visualize them in real time without changing the overall look of the surgical field. A class of dyes that has shown outstanding promise in the area of NIR-fluorescence image-guided surgery are cyanine dyes. These dyes, first discovered in 1856, display a range of absorption and fluorescence covering the electronic spectrum from the ultraviolet to the infrared. Due to their extreme sensitivity to light, their inconceivable value was not discovered for another 20 years when they were used to increase sensitivity of the photographic plate. Since then cyanine dyes have been used in an incredible amount of applications including but not limited to laser printing, pH sensing, data storage, and medicine.

Cyanine dyes are distinguished from other dyes in that they possess two nitrogen containing heterocycles that are connected by an electron-deficient conjugated methine bridge as shown by the general structure in Figure 2.1. This causes the cation to be delocalized throughout the conjugation and is the cause of their high absorption and fluorescence wavelengths.

Figure 2.1. General Cyanine Dye Structure

The heterocycles act as both electron donors and acceptors creating an electron deficient system throughout the molecule that gives cyanines a wide range of absorption and fluorescence in the visible and infrared regions. They are also characterized as having narrow absorption bands and high extinction coefficients. Their names depend on the how many methine groups are found in the bridge connecting the two heterocycles. Dyes containing n =
0, 1, 2, and 3, are classified as mono-, tri-, penta-, and hepta-, respectively. There are multiple well described routes to the synthesis of monomethine and trimethine cyanines, but pentamethine and heptamethine cyanines have one general route. This route is through the condensation of methylsubstituted quaternized heterocyclic compounds with an $\alpha,\omega$ dialdehyde or its equivalent.

Their narrow absorption bands and high extinction coefficients are important properties that have allowed them to be used in various applications over the last 140 years. Monomethine and trimethine cyanines typically fluoresce in the 500-600 range of the electronic spectrum and the addition of each double bond to the methine chain causes a bathochromic shift of about 100 nm resulting in fluorescence around 700 nm for pentamethine and around 800 nm for heptamethine cyanines. These wavelengths are ideal for the 700 and 800 nm cameras of the FLARE imaging system. These compounds have appealing optical properties including their red-shifted fluorescence and elevated quantum yields.

In addition to excellent optical properties, cyanines are highly modifiable. As discussed previously, their absorption and fluorescence wavelengths can be tuned by modifying the length of the methine bridge. Besides the length of the methine bridge, the $N$-alkyl groups (Figure 2.2 blue circle) can be modified to add or reduce hydrophilicity. This position has little effect on optical properties, but lipophilicity can be introduced by adding large hydrophobic groups such as propyl phenyls or decyls while hydrophilicity can be introduced by the addition of charged groups such as quaternary ammoniums or sulfonates. A middle ground can be found in compounds including a quaternary pyridinium group that adds water solubility while keeping hydrophobic character. In addition functionality can be introduced in this position such as alcohols, carboxylic acids, esters, sulfonates, etc. The heterocycle (Figure 2.2 red) can be altered to introduce small wavelength changes of a few nm such as from a benzothiazole to a 3,3-dimethylindolenine or larger wavelength changes up to 100 nm as seen when a quinoline
or benz[c,d]indole is introduced. These flatter more conjugated heterocycles also have large influences on fluorescence which need to be taken into account as they oftentimes have lower quantum yield if they are even fluorescent at all. These heterocycles can also be substituted (Figure 2.2 yellow), often with electron donating or electron withdrawing substituents that have small effects on the optical properties of the compounds but can have large effects on binding. The meso-carbon atom can have substitutions on it as well. Most commonly a halogen is seen at this position which is useful for further addition to the cyanine scaffold, but using different intermediate salt linkers, alkyl or aromatic groups can be introduced directly to the methine bridge. Nucleophiles such as amines, alcohols and thiols can be attached by substitution reactions and organoboronic acids can be used to achieve carbon-carbon cross coupling. Lastly, a cycloalkene (Figure 2.2 purple) is often introduced on the methine bridge in heptamethine dyes for additional stability. This is generally done with a cyclohexene, which has little effect on optical properties, but has been done with cyclopentene and cycloheptene as well. The cyclopentene greatly alters the angle of the bonds in the methine bridges and causes a large redshift in absorption wavelength.

Figure 2.2. Modification sites on cyanine dyes

These properties along with low tissue autofluorescence and the ability to deeply penetrate tissue in the NIR window have allowed cyanine dyes to be promising for use in in vivo imaging. Fluorescence imaging techniques with near-infrared dyes are generally superior in terms of sensitivity and selectivity. Due to the rigidness of near-infrared dye fluorophores during binding, fluorescence increases significantly. Commonly, near-infrared emission agents are conjugated with ligands that are known to target specific tumors, but these
agents’ application have been limited due to problems with delivery and specificity. Employing near-infrared dyes as contrast agents in optical imaging, a non-radiative technique,\(^4\) is safer than current radiological techniques, however, the applications of cyanine dyes in cancer diagnosis and detection is yet to be fully explored.\(^5\)

While many groups are currently working on cancer specific contrast agents, it is the objective of this work to synthesize contrast agents that are tissue specific. This has dual purposes: first because the FLARE system uses two detection cameras, the tumor specific contrast agents can be used to label vital tissues that need to be avoided during surgery, but also can be used if the specific tissue is the surgical target.

Our lab has previously synthesized a series of pentamethine cyanines and their biodistribution was studied in vivo. This series could specifically target pituitary glands, pancreas, and lymph nodes with dependence on molecular characteristics.\(^57,58\) Through this series, it has been shown that targeting can be predicted by correlating physicochemical properties to where the dyes localize in mice. For instance, the optimal log\(D\) value for targeting the thyroid gland was determined to be between 3.5 and 6.5.\(^57\) Before synthesis of the library of heptamethine compounds, tables were designed comparing physicochemical properties, such as log\(D\), hydrogen bond donors and acceptors, molecular weight, and volume. Table 2.1 is an example of one of these tables presenting how altering the heptamethine linker can affect these properties. Shown in this table are proposed dyes (Figure 2.3) with very small modifications made to the structure either on the methine bridge or as a substituent on the heterocycle all with \(N\)-methyl alkyl chains. These small changes allow the molecular weight (mw) of the compounds to be varied from 409 to 641 with an average mw right around 500. They are hydrophobic compounds with log\(D\) ranging from 4.0 to 6.6. None of them have any hydrogen bond donors, but they all have between 1 and 4 hydrogen bond acceptors. This table
shows how widely the small changes in these compounds can affect their physicochemical properties and thereby could also affect their biodistribution.

Table 2.1 Physicochemical properties of select proposed dyes

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<tr>
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<td>452.09</td>
<td>3</td>
<td>29.03</td>
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<td>2</td>
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</tr>
<tr>
<td>28b</td>
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<td>5.60</td>
<td>488.97</td>
<td>3</td>
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<td>0</td>
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</tr>
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<td>474.58</td>
<td>3</td>
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<tr>
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<td>5.24</td>
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</tr>
<tr>
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<td>6.37</td>
<td>528.7</td>
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<td>0</td>
<td>1</td>
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</tr>
<tr>
<td>31a</td>
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<td>542.83</td>
<td>4</td>
<td>28.46</td>
<td>0</td>
<td>2</td>
<td>67.38</td>
</tr>
</tbody>
</table>

Figure 2.3 Select proposed dyes for physicochemical property calculations

2.2 Modification of Heterocycles

Toward creating a library of cyanine dyes for biodistribution screening, optimized routes for altering the structure of the cyanine pharmacophore have been developed. The synthesis of cyanine dyes begins with the Fischer indole cyclization reaction of various phenylhydrazines 1a-f with 3-methyl-2-butanone in acetic acid.59-61 This provides 2,3,3-
trimethylindolenines 2a-f in very good yields as shown in Equation 1. The yields range from 80-91% by conventional methods but our group has shown that 100% yield can be obtained using microwave. By altering the substituents on the phenylhydrazine the heterocyclic substituents of the final cyanines can be modified. The inclusion of sulfonate groups can make the dye water soluble, while a methoxy substituent will redshift the wavelength about 10 nm.

![Chemical structure](image)

Equation 1

1a: R¹ = H  
1b: R¹ = OMe  
1c: R¹ = Br  
1d: R¹ = Cl  
1e: R¹ = F  
1f: R¹ = SO₃⁻  
2a: R¹ = H  
2b: R¹ = OMe  
2c: R¹ = Br  
2d: R¹ = Cl  
2e: R¹ = F  
2f: R¹ = SO₃⁻

2.3 Quaternary Ammonium Salts

As shown in Equation 2, these 2,3,3-trimethylindolenines 2a-f as well as 1,1,2-trimethyl-1H-benz[e]indole are alkylated by refluxing various alkyl halides or sultones in acetonitrile to prepare quaternary ammonium salts 3a-t which are precipitated in ether. These compounds are synthesized in fair to excellent yields ranging from 50% to 91%. The yields are typically lower for compounds 3h-k with the additional quaternary ammonium ranging from 50% to 81%. These compounds use 3-bromopropyltrimethylammonium bromide as starting material which also precipitates in either and makes the purification more difficult. Compounds 3l-o also tend to have lower yields ranging from 56% to 80% due to reaction kinetics. However by conventional methods these reactions typically are not completed overnight. By using microwave excellent yields of 98% can be achieved in minutes.
Another heterocyclic modification is described in Scheme 2.1. Benz[c,d]indole quaternary salts are formed by first alkylating benz[c,d]indol-2(1H)-one 4 before thionation with phosphorous pentasulfide in boiling pyridine. The thio ketone 6 is then methylated to a thioether by stirring with iodo methane overnight at room temperature. The thioether 7 is then replaced by Meldrum’s acid by heating for 6 hours under basic conditions in ethanol. Last, the Meldrum’s acid is removed by dissolving compound 8 in acetic acid and refluxing for 20 minutes. Hydrochloric acid is dripped into the solution before cooling to room temperature forming the acidic methyl position on the benz[c,d]indole quaternary ammonium salt 9.63
2.4 Methine Linkers Modification

In parallel to quaternary salt formation, intermediate salt linkers are synthesized. To obtain the linker used for the development of pentamethine cyanines containing a meso-halogen mucochloric or mucobromic acid 10 was reacted with aniline in warm ethanol and upon cooling and dilution with diethyl ether, the intermediate salt linker precipitated as a yellow solid 11 (Equation 3).

Another pentamethine linker was synthesized through a Vilsmeier formulation on bromoacetic acid as shown in Equation 4. Phosphorous oxychloride (POCl₃) was added dropwise to a cooled solution of DMF at 0 °C which causes the solution to turn a pale orange color. After allowing the solution to warm to room temperature bromoacetic acid 12 was added in portions and heated overnight at 70 °C. The reaction was then quenched with water and the
pH increased to 9 with sodium carbonate before filtration of inorganic salts and extraction of triformaldehyde 13. This linker allows for the formation of pentamethine cyanines with an aldehyde at the *meso*-position.

\[
\text{Br} \quad \text{OH} \quad 1) \text{POCl}_3/\text{DMF} \quad 2) \text{Na}_2\text{CO}_3, \text{H}_2\text{O} \quad \text{O} \quad \text{O} \quad 13
\]

As shown in Equation 5, the heptamethine linkers are synthesized through a similar Vilsmeier reaction. Substituted or unsubstituted cyclohexanones 14 in dichloromethane were added dropwise to the POCl₃ mixture at 0 °C. The solution was then heated at 100 °C for 2 hours and cooled to room temperature. The dimethyliminium is trapped with aniline to increase long term stability before being precipitated in water with hydrochloric acid as Vilsmeier reagents 15.

\[
\text{O} \quad 1) \text{DMF/POCl}_3 \quad 2) \text{Aniline} \quad \text{Ph} \quad \text{N} \quad \text{Y} \quad \text{Z} \quad \text{15}
\]

14a: Y = Cl, Z = H  
14b: Y = Br, Z = H  
14c: Y = Cl, Z = Ph  
15a: Y = Cl, Z = H  
15b: Y = Br, Z = H  
15c: Y = Cl, Z = Ph

The same procedure can be used with cyclopentanones 16a or cycloheptanones 16b to change the size of the cyclohexene in the methine bridge as shown in Equation 6. The inclusion of a cyclopentanone redshifts the wavelength of the final dyes to about 820 nm due to the strain put on the methine bridge by the cycle.
When cyclic ketones are utilized for the formation of the Vilsmeier reagents \( \text{15} \) and \( \text{17} \) halogens are installed in the meso- position that allow for further conjugation of the dye to nucleophiles by substitution or carbon-carbon coupled through Suzuki coupling. By preparing Vilsmeier reagents \( \text{19a-b} \) with methyl and phenyl substituents in the meso- position from 1-substituted cyclohexenes, Suzuki coupling of the dye can be avoided later.

![Chemical structure](image)

\[
\begin{align*}
\text{16a: } n &= 0 \\
\text{16b: } n &= 2 \\
\text{17a: } n &= 0 \\
\text{17b: } n &= 2
\end{align*}
\]

### 2.5 Monomethine Cyanine Preparation

As described by our lab, the benz[\( c,d \)]indole heterocycle redshifts the wavelength about 100 nm.\(^{65,66}\) Although these dyes are usually not fluorescent, it has been shown that when they have restricted rotation such as in a viscous solvent or bound to a biomolecule they will fluoresce. While the fluorescence of monomethine cyanines is generally around 500 nm, monomethine cyanines including two of these benz[\( c,d \)]indole heterocycles fluoresce in the near infrared region. They are formed by heating quaternary ammonium salt \( \text{9b} \) (Scheme 2.1) with thioketone \( \text{7b} \) in acetonitrile in the presence of triethyl amine for 2 hours as shown in Equation 8. Dye \( \text{20} \) was synthesized in 72% yield after purification by column chromatography and would fluoresce at 700 nm.
2.6 Trimethine Cyanines

Trimethine dyes typically fluoresce in the visible region, but their wavelengths can correspondingly be shifted using the benz[c,d]indole heterocycle. As shown in Equation 9, symmetrical trimethine cyanines are formed by heating quaternary ammonium salts with triethyl orthoacetate in acetic anhydride for 2 hours. The dyes are precipitated out of solution and washed with ether. This synthesis pathway has been optimized to yield pure dyes with no further purification and dye 21 was synthesized in 76% yield. These symmetrical trimethine cyanine dyes containing two benz[c,d]indole heterocycles fluoresce around 780 nm and would be useful with the 800 nm camera of the FLARE system.

A trimethine cyanine dye containing only one benz[c,d]indole heterocycle would also fluoresce in the NIR region ~680 nm and would be useful with the 700 nm camera of the FLARE system. As shown in Scheme 2.2 the key step in the synthesis of unsymmetrical trimethine cyanines is the formation of an aldehyde 22 by Vilsmeier formulation on a quaternary ammonium salt. This aldehyde can then be reacted with another salt to form an
unsymmetrical trimethine cyanine. This synthesis pathway has also been optimized to yield pure dyes with no further purification. Dye 23a was synthesized in 55% yield while dye 23b was synthesized in 79% yield.

Scheme 2.2. Synthesis of unsymmetrical trimethine cyanines 23a-b fluorescing in the NIR region

2.7 Pentamethine Cyanines

The major synthetic route to pentamethine cyanines generally involves condensation of quaternary ammonium salts with malondialdehyde derivatives. This reaction generally takes place in acetic anhydride in the presence of sodium acetate as shown in Equation 10. Four new pentamethine cyanines with a meso-chlorine atom were synthesized and purified by column chromatography with yields ranging from 68%-82%.

The pentamethine cyanine with a meso-aldehyde was synthesized through the condensation of triformyl methane 13 with quaternary ammonium salt 3b. Sodium acetate was
not strong enough for this reaction and therefore pyridine was used in ethanol as shown in Equation 11. Dye 25 was purified by column chromatography in 65% yield.

![Chemical structure of 3b and 25](image)

2.8 **Heptamethine Cyanines With an Open Chain**

The use of heptamethine cyanines as fluorescent markers in biological systems has generated enormous interest. The general synthetic route is similar to that of pentamethine cyanines. Heptamethines are formed by heating individual quaternary ammonium salts 3 with intermediate linkers in acetic anhydride for 2 hours in the presence of sodium acetate as shown in Equation 12. These dyes range in yield from 15% to 90%. Tricationic dyes 26h-k were responsible for many much of the lower yields ranging from 15% to 62% as they are more difficult to purify and large amounts of dye are lost in recrystallization or during reverse phase column chromatography. The methoxy dyes also have lower yields potentially due to more rapid decomposition. After the pure dye eluted from the column, brown decomposition could be seen and these dyes were synthesized in yields ranging from 47% to 86%. The rest of the dyes with no substitutions or electron withdrawing substitutions were synthesized in yields of 60% to 90%.
The mechanism for heptamethine cyanines has been described as shown in Scheme 2.3.

The reaction is started with deprotonation of the quaternary ammonium salt by the acetate anion from sodium acetate. The mechanism is similar for pentamethine cyanines, but with two less carbons in the methine linker.
Scheme 2.3. Mechanism of heptamethine cyanine formation

Pentamethine and heptamethine cyanine synthesis often does not go to completion and therefore requires more rigorous purification. The optimal method for purification of these compounds is through a reprecipitation process which involves dissolving the compounds in a minimal amount of warm methanol before being precipitated in ether. Column chromatography is necessary for dyes that cannot be purified through these precipitation processes. Hydrophobic dyes usually elute in dichloromethane with 1-5% methanol. Dyes containing multiple charges such as 26h-k tend to be more difficult to purify. They are generally purified by reverse-phase column chromatography using commercially available C18 silica gel that can be costly. Crystallization of highly polar cyanines has previously seldom furnished pure
compounds. Our lab has shown that these compounds can easily be recrystallized in good yield after dissolving in water and precipitating with acetone (1:50) or from dimethyl sulfoxide with ethyl acetate (1:100).

2.9 Heptamethine Cyanines Containing a Cyclic Ring in the Methine Chain

Many heptamethine cyanines have been designed to contain a six-membered carbocyclic subsystem as part of the methine chain such as in compounds 27. This subsystem increases the rigidity of the molecule typically increasing the stability of these compounds and decreasing aggregation in solution. As shown in Equation 13, a set of eleven heptamethine compounds containing a cyclohexene ring and meso-chlorine atom were synthesized. These dyes are used for modifications described in Section 2.10. These dyes were synthesized in yields ranging from 66% to 84%.

\[
\begin{align*}
27a: & \ R^1 = \text{Me}, \ R^2 = R^3 = H \\
27b: & \ R^1 = -\text{CH}_2\text{CH}_2\text{CH}_2\text{SO}_3, \ R^2 = R^3 = (\text{CH}_2=\text{CH})_2 \\
27c: & \ R^1 = -\text{CH}_2\text{CH}_2\text{CH}_2\text{SO}_3, \ R^2 = \text{OMe}, \ R^3 = H \\
27d: & \ R^1 = \text{TMAB}, \ R^2 = \text{SO}_3, \ R^3 = H \\
27e: & \ R^1 = \text{Me}, \ R^2 = \text{OMe}, \ R^3 = H \\
27f: & \ R^1 = \text{Me}, \ R^2 = \text{Br}, \ R^3 = H \\
27g: & \ R^1 = -\text{CH}_2\text{CH}_2\text{CH}_2\text{Ph}, \ R^2 = R^3 = (\text{CH}_2=\text{CH})_2 \\
27h: & \ R^1 = -\text{CH}_2\text{CH}_2\text{CH}_2\text{Ph}, \ R^2 = \text{Cl}, \ R^3 = H \\
27i: & \ R^1 = \text{Et}, \ R^2 = F, \ R^3 = H \\
27j: & \ R^1 = -\text{CH}_2\text{CH}_2\text{CH}_2\text{Ph}, \ R^2 = F, \ R^3 = H \\
27k: & \ R^1 = \text{Et}, \ R^2 = R^3 = H \\
27l: & \ R^1 = -\text{CH}_2\text{CH}_2\text{CH}_2\text{Ph}, \ R^2 = \text{OMe}, \ R^3 = H
\end{align*}
\]

* X = I for \( R^1 = \text{Me}, \text{Et}, \text{Bu} \)
\[
X = \text{Br} \text{ for } R^1 = \text{TMAB}, \text{-CH}_2\text{CH}_2\text{CH}_2\text{Ph}
\]

Another class of heptamethine dyes with cyclopentane or cycloheptene in the methine bridge were synthesized. Alteration of a molecule to a similar but more planar and rigid
molecule often increases the quantum yield of fluorescence. A five-membered ring would further increase rigidity and increase quantum yield while a seven-membered ring would allow additional flexibility while still preventing rotation around the methine bridge in the fused positions. These two heptamethine cyanines are prepared following a similar reaction as shown in Equation 14. Because of the increased rigidity of the methine bridge caused by the five-membered ring, the reaction to 28a did not go to completion and was purified by column to a 53% yield. Dye 28b was also purified by column in a 79% yield.

Heptamethine cyanines bearing a bromine in the meso-position could undergo Suzuki coupling reactions with organoboronic acid reagents to give dyes substituted in this position. These heptamethines with a meso-bromine 29 are synthesized using Vilsmeier 15b as described in Equation 15 and were synthesized in 74% and 65% yield for 29a and 29b, respectively.

As mentioned previously, this Suzuki coupling step with organoboronic acids can potentially be avoided by using Vilsmeier reagents 19a-b as shown in Equation 16. Here heptamethine cyanines with meso-methyl and phenyl groups were synthesized by condensation of quaternary ammonium salts 3 with methyl and phenyl substituted Vilsmeier reagents 19a-
b. To do this using Suzuki coupling, methylboronic acid or phenylboronic acid would be reacted with the chloro- or bromo-substituted dye with an expensive palladium catalyst. In addition, this has generally only been done with water soluble dyes. The carbon-carbon coupled final dye must then be separated from the starting material dye by column chromatography. Dyes 30a-j, synthesized by reacting the corresponding quaternary ammonium salts 3 with Vilsmeier reagents 19a-b, are purified solely by washing with methanol and were synthesized in 63-85% yield.

Modification of the heterocycles has focused almost entirely on the meso-position of the methine bridge. There is a lack of literature modifying other sites of the cyclic ring on the bridge. An example of another point of modification is 5 position of the cyclohexene as shown in Equation 17. This position is not conjugated into the system and therefore allows for modification of the structure and hydrophobicity with less effects on optical properties. The dyes were substituted in very good yields above 80%.
2.10 Modifications to the Heptamethine Core

Because these broadly used fluorophores suffer significant drawbacks such as poor stability, likely due to nucleophilic exchange reactions at the meso-position, cyanines modified at this position are desirable. The chlorine atom at this position of heptamethine dyes allows for conjugation to various nucleophiles, such as phenols, through the $S_{RN}1$ displacement pathway. This differs from regular nucleophilic substitutions in that deactivating groups are not necessary. These substituted compounds should be more stable while maintaining excellent optical and physical properties, however they have rarely been synthesized. For oxygen attachments to the cyanine core, sodium hydride (NaH) is used to generate the phenoxide ion $\text{in situ}$ because the protonated oxygen exhibits limited nucleophilicity as shown in Equation 18. A small shift in maximum absorption wavelength from 776 nm to 762 nm was observed. Dye 32 was synthesized in 81% yield.
Aromatic amines are more difficult to couple via conventional amide-coupling strategies because of electron delocalization across the ring and therefore required microwave heating as described in Equation 19. The reaction would not proceed by conventional methods due to the instability of cyanine dyes and heat and time required for the reaction to take place. Optimal microwave conditions were determined by small scale reactions followed by UV-Vis spectroscopy as described in Table 2.2 before scaling up for purification. Optimization began by heating at 100 W and increasing the time. After 20 minutes, crude yield began decreasing. The temperature was then increased which also decreased the crude yield, but decreasing the temperature to 80 °C increased crude yield. Trial 6 was determined to be the optimal conditions achieving a 72% yield.

![Chemical structures](image)

**Table 2.2. Optimization of microwave conditions for nucleophilic addition of amines**

<table>
<thead>
<tr>
<th>Trial</th>
<th>Reaction Time (min)</th>
<th>Temp (°C)</th>
<th>Power (W)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>100</td>
<td>100</td>
<td>32</td>
</tr>
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<tr>
<td>8</td>
<td>20</td>
<td>80</td>
<td>80</td>
<td>55</td>
</tr>
</tbody>
</table>

In order to synthesize a dye containing a sulfonazide, acetylaminobenzene was first sulfonated using chlorosulfonic acid. The acid was then converted to a sulfonyl chloride using thionyl chloride. The sulfonazide was then formed through nucleophilic substitution of the chlorine with sodium azide, before deprotection of the amine using hydrochloric acid. The
aromatic sulfonazide was more difficult to couple via conventional amide-couple strategies and microwave heating was necessary. The optimized microwave method described in Table 2.2 was used. This dye was purified by column chromatography using 0.2% methanol in dichloromethane as the eluting solvent. Dye 39 was synthesized in 46% yield.

![Scheme 2.4. Synthesis of a heptamethine cyanine 39 with sulfonazide](image)

### 2.10.1 Smiles Rearrangement

To synthesize a highly charged heptamethine cyanine a Smiles rearrangement was utilized. Smiles rearrangements require both strong bases and elevated temperatures to drive the α,δ-transposition of heteroatoms to the thermodynamically preferred isomer. A distinct alternative selectively integrates an electrophile on the heteroatom originally attached to the unsaturated carbon providing otherwise unreachable products allowing for more simple access to meso-oxygen substituted heptamethine cyanines from more facile tertiary amino precursors. This method is able to counteract the disadvantages of the more conventional attachment of
alkoxides. The *meso*-aliphatic oxygen substitution provides the opportunity to develop agents with improved properties relative to those prepared through current strategies. Here, an additional positive charge is added to a hydrophobic heptamethine cyanine to make it more hydrophilic. As shown in Scheme 2.5, the secondary amine *N*-methylethanolamine was first attached to the dye by heating in a sealed tube. Characteristically this nitrogen addition to the methine chain causes blue-shifted absorption of about 100 nm, a broad spectrum, and a larger Stokes shift. Upon Smiles rearrangement to the oxygen substitution by microwave heating, a visible blue to green color transition occurs as the maximum absorption wavelength shifts to 762 nm along with a smaller Stokes shift and greater molar absorptivity. In order to synthesize a dicationic heptamethine cyanine, iodomethane was included in the Smiles rearrangement reaction to quaternize the amine. Dye 41 was synthesized in 70% yield.

![Scheme 2.5. Smiles rearrangement on heptamethine cyanine 40](image)

### 2.10.2 Suzuki Coupling

Another common method of preparing monofunctional cyanines is through the stepwise conjugation of two different heterocycles with one of these heterocycles containing monocarboxy functionality, but this approach is problematic due to difficult purification of from the undesirable symmetrical dyes that form. In addition, the ether, thioether and amide linkages are chemically labile rendering the fluorophores susceptible to cleavage. To avoid this problem, a direct carbon substitution is preferable. Organoboronic acids are convenient reagents for cross coupling reagents due to their thermal stability and lack of reactivity with water. Hydrophilic chloro-substituted heptamethine cyanines have been carbon-carbon
coupled rarely, and cross coupling has been seen even less with hydrophobic cyanines.\textsuperscript{71} The reaction of chloro dye 27a and an arylboronic derivative in a mixture of DMF and water in the presence of tetrakis(triphenylphosphine)palladium(0) (Pd(PPh\textsubscript{3})\textsubscript{4}) allowed the reaction to proceed as shown in Equation 20. The dye was synthesized in poor yield of 21\% due to the reaction not working well with water insoluble compounds. The absorption spectra of dye 42 shows characteristic broadening and a hypsochromic shift of ~20 nm indicating a direct interaction of the aryl group with the chromophore system similar to dyes 30f-j with a phenyl ring attached at the \textit{meso-} position.
3 TARGETING

Through a collaboration with the Choi lab at Harvard Medical school, an initial screen of the synthesized NIR fluorophore library injected IV into CD-1 mice identified three potential pharmacophores for endocrine uptake with emission wavelengths of 700 and 800 nm. The endocrine glands are tissues of considerable clinical importance, yet they are problematic to locate during surgery.

3.1 Adrenal Gland Targeting

The adrenals glands (AGs) are paired endocrine glands that produce hormones such as epinephrine, norepinephrine, androgens, estrogens, aldosterone, and cortisol. They are also the site of benign and malignant tumors that can lead to Cushing’s syndrome, Conn syndrome, virilization, and feminization. Radical adrenalectomy is traditionally performed by conventional open surgery. However, since its initial description in 1992, laparoscopic adrenalectomy has expanded significantly for the resection of functional adrenal tumors and incidentalomas smaller than 8-10 cm. The advantages of a laparoscopic approach include a better cosmetic result, shorter recovery time, and significantly less pain, while it may cause shorter survival, less complete removal of the tumor, and shorter time to and a greater chance of the tumor returning. In addition, laparoscopic instruments could rub against the tumor during surgery and allow tumor cells to spread to other parts of the abdomen. Real-time image guidance during the AG surgery can help guarantee a complete removal of the tumor while preserving vital tissues neighboring the glands, which is also key to shortening intraoperative time and effort.

It was reported by Obermeyer et al. that visualizing the AGs after a single intravenous injection of methylene blue (MB) shortened the operative time of laparoscopic adrenalectomy. However, the use of MB is not efficient because color visualization demands high dose injections, while retention time in the target is too short for surgery. Indeed, the
The effective dose of MB used for the aforementioned pig study was 7.5 mg/kg, which is 5-fold higher than the typical clinical dose (1.5 mg/kg). In addition, the duration of bluish color changes in the AG was less than 15 min.\textsuperscript{81}

### 3.1.1 Optical and Photophysical Properties

The two novel, high performance NIR fluorophores $24c$ and $26e$ that were identified from the library of ~100 compounds through screening that target the AGs, emit at 700 nm and 800 nm, respectively. Their structures can be seen in Figure 3.1. In this study, their performance was compared, quantitatively, to MB and ICG in both small and large animal models of AG surgery. After initial screening, $24c$ (700 nm NIR) and $26e$ (800 nm NIR) were identified as AG-targeted agents.

![Chemical structures of NIR fluorophores $24c$ and $26e$. Log$D$ was calculated using MarvinSketch (ChemAxon)](image)

Because these dyes are hydrophobic (log$D$ 6.49 and 6.78) and need to be dissolved in aqueous media to be useful for \textit{in vivo} studies, their solubility and optical properties were studied in different solvents. As shown in Table 3.1 both $24c$ and $26e$ are most soluble in dimethyl sulfoxide (DMSO) with solubility greater than 25 mg/mL and almost completely insoluble in PBS buffer. They are also both slightly soluble in fetal bovine serum (FBS).
Table 3.1. Solubility and optical properties of compounds 24c and 26e in PBS, FBS, MeOH, and DMSO

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Solvent</th>
<th>Solubility (mg/mL)</th>
<th>Extinction Coefficient (M⁻¹cm⁻¹)</th>
<th>Absorbance Maximum (nm)</th>
<th>Emission Maximum (nm)</th>
<th>Stokes Shift (nm)</th>
<th>Quantum Yield (%)</th>
</tr>
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<tbody>
<tr>
<td>24c</td>
<td>PBS</td>
<td>&lt; 0.01</td>
<td>63,000</td>
<td>706</td>
<td>720</td>
<td>14</td>
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<tr>
<td></td>
<td>FBS</td>
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<td>179,000</td>
<td>692</td>
<td>707</td>
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<td>16.0</td>
</tr>
<tr>
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<td>MeOH</td>
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<td>681</td>
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</tbody>
</table>

The absorbance spectra in these solvents can be seen in Figure 3.2. The dyes have the highest molar absorptivity in methanol followed by DMSO. The lowest molar absorptivity is seen in PBS due to their insolubility in the solvent. As shown in Figure 3.2B, with as little as 1% DMSO in saline almost no aggregation is seen and the dyes are completely dissolved. The continued increase in molar absorptivity with increased DMSO concentration is due to the higher molar absorptivity seen in DMSO compared to saline.

Figure 3.2. Absorbance spectra of compounds 24c and 26e in various solvents. (A) Absorbance spectra in PBS, FBS, DMSO and MeOH. (B) Solubility in saline-DMSO mixtures. 5 mM stock solutions were prepared in DMSO and added to saline containing 10% FBS
The chemical structures and optical properties in neutral buffered serum of all NIR fluorophores used in this study are shown in Figure 3.3. MB and 24c exhibited fluorescence properties compatible with the 700 nm NIR fluorescence channel of the FLARE imaging system while ICG and 26e exhibited fluorescence properties compatible with its 800 nm NIR fluorescence channel. In FBS, the synthesized fluorophores had higher molar absorptivity than the commercially available dyes with 179,000 M⁻¹cm⁻¹ for 24c compared to 71,200 M⁻¹cm⁻¹ for MB and 135,000 M⁻¹cm⁻¹ for 26e compared to M⁻¹cm⁻¹ 121,000 for ICG. The synthesized compounds had higher quantum yields as well. MB had the lowest extinction coefficient and QY of all, consistent with prior reports.³⁸ Molecular brightness is generally a better indicator of utility in imaging applications because it takes into account both the amount of photons absorbed and the ratio of photons emitted compared to those absorbed. The synthesized compounds had higher molecular brightness than their counterparts with 24c at 28,282 M⁻¹cm⁻¹ and 26e at 14,580 M⁻¹cm⁻¹. MB displays a significantly lower molecular brightness than all of the cyanine dyes at 2,706 M⁻¹cm⁻¹.

<table>
<thead>
<tr>
<th></th>
<th>MB</th>
<th>24c</th>
<th>ICG</th>
<th>26e</th>
</tr>
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<tbody>
<tr>
<td>Extinction Coefficient (ε, M⁻¹cm⁻¹)</td>
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<td>Molecular Brightness (ε × Φ, M⁻¹cm⁻¹)</td>
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<td>28,282</td>
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<td>14,580</td>
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</table>

Figure 3.3. Optical properties of NIR fluorophores in FBS, pH 7.4., 3D structures and representative absorbance (Abs) and fluorescence (FL) spectra of each fluorophore in 100% FBS supplemented with 50 mM HEPES, pH 7.4.
3.1.2 In Vivo Imaging

Despite the improved optical properties, both 24c and 26e have poor water solubility because of high logD. To improve the solubility, the dyes were dissolved in DMSO (5 mM), and diluted into saline containing 10% serum proteins to a final concentration of 200 µM, where both fluorophores were completely dissolved without any precipitates (Figure 3.1). As shown in Figure 3.4, 24c and 26e resulted in significant signal intensity in the AGs (SBR > 2.0 relative to muscle and SBR ≈ 1.5 relative to kidney), which was much higher and more sustained than either MB or ICG. However, in mice, background tissue uptake in the liver of all four NIR fluorophores was high, resulting in some cases of an SBR < 1.0. Based on the kinetics of AG uptake, 60 min post-injection was selected as the optimal time point in mice and used for dose ranging.

![Figure 3.4](image)

Figure 3.4. Kinetics of AG signal relative to surrounding tissues and organs. SBR of AG compared to surrounding major tissue/organs was measured after 10 nmol intravenous injection of each fluorophore
As shown in Figure 3.5, there was little dose dependency on either MB or ICG uptake in the AG. On the other hand, 24c and 26e showed a dose-dependent uptake in AGs, and 26e reached the maximum SBR (AG/Mu) > 4.0 when 50 nmol of each compound was injected intravenously in CD-1 mice. A significant difference in the SBR (AG/Mu) was found for injected doses 10 nmol for both 24c and 26e.

![Figure 3.5. Dose dependence of AG signal. 2-50 nmol of NIR fluorophores were injected intravenously 1 h prior to quantitation of SBR.](image)

Based on these kinetic and dose ranging studies, intraoperative imaging of AGs (Figure 3.6) was performed at 60 min post-injection of a dose of 10 nmol of contrast agent and resulted in distinct highlighting of the glands, and easy differentiation from surrounding muscle and fat, and even kidney in mice.
Figure 3.6. Intraoperative NIR imaging of AG and surrounding organs (10 nmol at 1 h). Bl, bladder; Ki, kidney; Li, liver; Sp, spleen. Scale bars = 0.5 cm. Red pseudo-color was used for 700 nm and lime green for 800 nm NIR in the merged image.

After resection of the AGs, NIR fluorescence microscopy revealed that both 24c and 26e showed significant uptake in adrenal cortex, particularly in the zona fasciculate (Figure 3.7). The fact that these molecules target the adrenal cortex specifically over the adrenal medulla suggests that they might share structural similarity to precursor molecules used in hormone production in the adrenal cortex, although this hypothesis remains unproven at present.
Figure 3.7 NIR fluorescence microscopy of adrenal glands resected from animals shown in Figure 3.5. Red pseudo-color was used for 700 nm and lime green for 800 nm NIR in the merged image. C, cortex; M, medulla; ZF, zona fasciculate. Scale bars = 50 µm.

Because the mouse adrenal gland is so thin (~2.5 mm) and the penetration of NIR light can be almost 1 cm, the mouse model actually shows a lower SBR than what we would expect to see in humans. To confirm NIR fluorophore kinetics in a large animal model system approaching the size of humans, the fluorophores were injected intravenously into 35 kg Yorkshire pigs and signal intensity of the AGs and surrounding tissues and organs was measured over time. MB was not able to generate a SBR > 1.0 at any time point. ICG generated a measurable SBR relative to muscle, but not relative to kidney or liver. As shown in Figure 3.8, both 24c and 26e, however, resulted in a significant SBR relative to all nearby tissues and for an extended period of time. In fact, 26e generated an SBR ≥ 3.0 for 4 hours post-injection. No changes in vital signs were observed over the 4 hours experiment.
Figure 3.8. Kinetics and intraoperative imaging with 2 µmol intravenous injection of each NIR fluorophore in pigs (n = 3): A) Kinetics of AG signal relative to surrounding tissues and organs. SBR of AG compared to surrounding major tissue/organs was measured at indicated time points up to 4 h. Red pseudo-color was used for 700 nm NIR and lime green for 800 nm NIR in the merged image. AG, adrenal gland; Ki, kidney; Li, liver. Scale bar = 3 cm.

Both 24c and 26e appear to perform well for identifying normal AGs across species. At first glance, performance of these molecules in mice appears to be lower than that in pig. However, the lower SBR in mice is likely the result of the thin diameter of the mouse AG. AGs in mice are so thin (~ 1 mm), and the penetration of NIR light so deep (> 5 mm) that thick organs like the kidney and liver appear much brighter than they are on a per gram basis. In pig and human, however, the AG signal is a true reflection of the relative difference in fluorophore uptake among the various tissues and organs. Because of rapid uptake of both fluorophores in AGs (within 5 min), injections can be repeated intraoperatively, if needed, to keep the signal strong throughout the surgical procedure. What is not known, though, is how they will perform in the setting of adrenal tumors. Tumors in the medulla are less likely to exhibit high uptake...
based on our histological data. Tumors of the adrenal cortex may exhibit high uptake, in which case these NIR fluorophores could be used to find local and distant metastases. Given the small sample size and complexity of mouse models in these diseases, and the fact that tissue path length (see above) will confound results, a clinical trial of patients with adrenocortical malignancies may be the most efficient way of evaluating the utility of these agents in tumor resection. Another concern is the potential toxicity. Although a wide range of doses (2-50 nmol) has been tested with a clinically compatible formulation (e.g., 10% serum-containing saline), neither adverse reactions nor in vivo toxicity were observed. This may be because both agents neither cross blood-brain barrier nor accumulate into the reproductive organs in mice and pigs (data not shown). It is also well-known that ICG containing the same polymethine backbone with 24c and 26e has shown only limited adverse reactions in the clinical use.

Finally, 24c and 26e were chosen for use with the two (700 nm and 800 nm) independent NIR fluorescence channels of the FLARE imaging system. This, in turn, enables mixing and matching of any number of NIR fluorophores for complex surgical procedures. For example, 26e (800 nm emission) could be used to highlight the AGs in conjunction with a 700 nm NIR fluorophore, such as bolus injection of MB, which highlights blood vessels. Thus, one channel of FLARE could be used to either find or resect (depending on intention) the AGs while the other channel is used to quickly find feeding arteries. Similarly, 24c (700 nm emission) could be used to find AGs while ICG (800 nm emission) is used to find feeding vessels. Similarly, 26e could be used in conjunction with T700-F (700 nm emission to readily image the pancreas and adrenal glands allowing the pancreas to be distinguished from the adrenal gland rather easily as seen in Figure 3.9.
Simultaneous Dual Channel Imaging of Pancreas and Adrenal Gland

2.5 µmol of T700-F was injected intravenously for pancreas imaging 4 h prior to adrenal gland imaging with 2 µmol intravenous injection of 26e 30 min prior to imaging (n = 3 pigs). Shown are color image, 700 nm NIR fluorescence, 800 nm NIR fluorescence, and a merged image of the three. For the merged image, FLARE™ channel #1 (700 nm) is pseudo-colored in red and channel #2 (800 nm) in green. Abbreviations used are: AG, adrenal gland; In, intestine; Pa, pancreas. Arrowheads indicate tiny branch from adrenal gland. Scale bars = 1 cm. All NIR fluorescence images have identical exposure times and normalizations.

3.2 Parathyroid Gland Targeting

An initial screen of a NIR fluorophore library containing ~100 novel compounds, injected IV into CD-1 mice identified a single potential pharmacophore 26a for parathyroid uptake at an emission of 800 nm. The parathyroid glands are four tiny glands, about the size of a grain of rice, located behind the thyroid gland which produce parathyroid hormones (PTH) that regulate the level of calcium in the blood. Calcium is the primary element that causes muscle contraction. PTH must be kept within a narrow window. If too much PTH is released hyperparathyroidism occurs. If too little PTH is released hypoparathyroidism occurs leading to hypocalcemia and increased levels of blood phosphorus (hyperphosphatemia). This is a rare condition and most commonly occurs due to damage or removal of parathyroid glands during surgery.

These glands are of considerable clinical importance, yet they are difficult to locate during surgery. Particularly, a partial or complete resection of the thyroid gland requires accurate identification and preservation of the parathyroid glands. In addition, complications can occur when all functional parathyroid glands are accidentally damaged or removed during thyroidectomies or when they are not completely removed during parathyroidectomies.
Using the naked eye, the visualization of normal parathyroid glands and accessory thyroid tissue is problematic in high-risk procedures such as those required for central neck surgery, thyroid cancer or Graves disease. Not only are the tissues tiny, but their location varies widely from person to person. Because of this microscopy is required to dependably tell the difference between parathyroid tissue, thyroid tissues and surrounding lymph nodes. Currently, the only agent known to target glands in the head and neck is MB, which exhibits uptake in a parathyroid tumor (adenoma), but not in normal parathyroid glands. MB fluoresces at 690 nm so there are no known fluorophores to target the parathyroid glands in the 800 nm region. Surgeons are currently required to rely on visual identification and of the different tissues and their experience which can be subjective and inconclusive. NIR fluorophores that exhibit structure-inherent specific uptake in the parathyroid glands could provide surgeons with unmistakable guidance during head and neck surgery after a simple IV injection.

### 3.2.1 Optical and Physicochemical Properties

Based on the pharmacophore of structure of 26a, the initial hit identified during screening, a series of fluorophores was designed and synthesized following the same procedure in Equation 12 to optimize parathyroid gland uptake. The NIR fluorophores, shown in Figure 3.10, emitting at 800 nm were composed of heptamethine cores and decorated with various side chains including hydrogen, methoxy, and fluoro groups. The physicochemical and optical properties of these fluorophores are shown in Table 3.2. In order to systematically alter hydrophilicity, hydrophobicity, polarity and electron resonance the heterocyclic substituents of the polymethine core were altered without changing the nonresonant side chains. All three

![Figure 3.10. Structures of fluorophores for parathyroid gland targeting](image-url)
fluorophores had molecular weights between 400 and 470 and logD between 3.7 and 4.4 making them relatively hydrophobic compounds. All targeted fluorophores exhibited maximum fluorescence in the NIR region and high molecular brightness which together minimize tissue autofluorescence and maximize fluorescence signal. Dye 26l had the lowest molecular brightness at 8,036 M$^{-1}$cm$^{-1}$ due to the electron donating nature of the methoxy group donating electrons back into the system. The fluorine substitution slightly raised the quantum yield to almost 19% giving it the highest molecular brightness at 20,790 M$^{-1}$cm$^{-1}$.

Table 3.2. Physicochemical and optical properties of compounds 26a, 26l, and T800-F in 100% serum, pH 7.4. In silico calculations of logD at pH 7.4 and total polar surface area were calculated using Marvin and JChem calculator plug-ins (ChemAxon)

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<th></th>
<th>26a</th>
<th>26l</th>
<th>T800-F</th>
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<tr>
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<tr>
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<td>Stokes shift (nm)</td>
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<tr>
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<tr>
<td>Molecular brightness (M$^{-1}$cm$^{-1}$)</td>
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<td>8,036</td>
<td>20,790</td>
</tr>
</tbody>
</table>

3.2.2  **In Vivo Imaging in Mice**

As a preliminary *in vivo* test for thyroid targeting, the NIR fluorophores were IV injected into CD-1 mice and imaged at 1 and 4 h. T800-F showed the highest signal-to-background ratio (SBR) values compared to other molecules. Signal intensity of the thyroid tissue in mice decreased only 17% between 1 and 4 hours post-injection. All three fluorophores had SBR greater than 1.5 after 1 h, but the SBR from T800-F was greater than 6 after 1 hour and sustained above 4 for over 4 hours (Figure 3.11).
Figure 3.11. Preliminary thyroid targeting in CD-1 mice. All NIR images have identical exposure time. SG, salivary glands; TG, thyroid glands (arrows).

Because T800-F showed the highest SBR, it was chosen for further studies. As shown in Figure 3.12, the optimal dose for parathyroid imaging was determined by IV injecting doses ranging from 25-100 nmol into mice and the SBR was measured over time. The optimal single IV injection dose for CD-1 mice was determined to be 50 nmol (1 mg/kg). T800-F displayed clearance through both the liver and kidneys 4 hours after injection, however the fluorescent absolute amounts of dye excreted into the urine and feces were difficult to detect.
3.2.3 In Vivo Parathyroid Imaging in Pigs

Pigs were used as a large animal model for parathyroid identification because they represent a worst-case scenario. Unlike in humans, pig parathyroid glands are located in the thorax, distant from the thyroid glands and without anatomical markers. Thus, it is more challenging to differentiate pig parathyroid glands with the naked eye because of their small size (a few mm) and location embedded within the surrounding thymus. T800-F was IV injected into Yorkshire pigs at a dose of 5 μmol and imaged over a period of 5 h. The parathyroid was able to be identified with high sensitivity and specificity with SBR greater than 6 after 1 hour and SBR sustained above 4 for over 4 hours post injection. Higher fluorescence was seen in the pig parathyroid than in the thyroid 1 hour post-injection and this signal was maintained for over 5 h. This can be seen in the parathyroid signal to thyroid signal ratio curve (PTR) which reaches a maximum at 3 hours post injection (Figure 3.13). Due to this high PTR, T800-F could be used for specific identification of the parathyroid glands.
3.2.4 Simultaneous in vivo NIR imaging of parathyroid and thyroid

The dual-NIR channel capability of the FLARE imaging system was exploited to provide surgeons with clear-cut markers during head and neck surgery. Rats were initially chosen as they possess a single pair of parathyroid glands located on the anterior and lateral aspects of the thyroid lobes. For dual-channel imaging of the glands, T800-F was injected concurrently with a dye previously synthesized by our lab that targets the thyroid gland and fluoresces at 700 nm, T700-F. T800-F was IV injected into a 250 g Sprague-Dawley (SD) rat 2 hours before imaging followed by T700-F injected into the same animal 6 hours before imaging. These are the optimal doses and timing determined during initial screening tests. Under these conditions T800-F allows for the parathyroid to be visualized unmistakably and T700-F simultaneously highlights both glands. The tissues were resected to confirm their identities by NIR fluorescence microscopy and consecutive H&E staining. As predicted, T700-F was observed in both thyroid and parathyroid glands, whereas T800-F remained only in the parathyroid gland. To confirm that these results are independent of species, the experiment was repeated in the pig model (Fig 3.14). T800-F was determined to target the chief and oxyphil cells of the pig parathyroid.
Figure 3.14. Dual channel NIR imaging in pigs using both T700-F and T800-F. T800-F was IV injected 4 h before imaging followed by T700-F 2 h later. La, Larynx; PG, parathyroid glands (arrowheads); TG, thyroid gland (arrows). Pseudocolored red and green colors were used for 700-nm and 800-nm channel images, respectively, in the color-NIR merged image. Scale bars, 300 μm. All NIR fluorescence images have identical exposure and normalizations.

The mechanism of uptake and retention of T800-F in specific cell types of the parathyroid glands is presently unknown. It seems that site-specific halogenation on the heterocyclic moiety of the cyanine dye is critical for targeting and it is possible that these molecules are being mistaken by iodine-processing cellular machinery (such as transporters and enzymes) as endogenous substrates. Future studies could focus on the identification of the intracellular targets of these contrast agents; however, it should be noted that contrast agent optimization can continue even when only the target cell type and preliminary pharmacophore are known.

The ability to identify the thyroid and parathyroid glands simultaneously after simple IV injection using the dual-NIR channel capability of the FLARE imaging system advances the potential for head and neck surgery to be performed with better precision and thus fewer surgical accidents and lower mortality. This study also provides design considerations for developing disease-specific contrast agents utilizing a structure inherent targeting strategy.

Discovering these agents is an arduous process. In the case of the adrenal and parathyroid glands, it required the synthesis and screening of a large NIR fluorophore library.
of ~300 compounds before optimization of the presumed pharmacophore. The strategy can be applied to almost any desired target and as the library continues to increase in size, pharmacophores for any particular target become more available.95,96

3.3 Cartilage Targeting

Sections 3.1 and 3.2 have shown that structure inherent targeting can be discovered through screening libraries of compounds, but it is important to note that structure inherent targeting can also be achieved by introducing targeting groups into the nonresonant structure of the fluorophore. For example, phosphonate derivatives have been widely used for targeting bone due to their high affinity for minerals and calcium salts found in bone surfaces.97 In this section the development of a cartilage specific fluorophore based on this type of rationale design is discussed.

Healthy cartilage is an essential component to a good quality of life. The functions of cartilage include cushioning joints and intervertebral spaces, giving the ears and nose form, and protection of the trachea. All three histological types of cartilage are produced by chondrocytes, or more precisely chondroblasts, until they are trapped within their own cellular matrix.98-100 The types of cartilage differ mainly in the relative amounts of collagen, proteoglycans, and elastin they contain.

Cartilage dysfunction leads to several acute and chronic indispositions. In degenerative joint disease (DJD, osteoarthritis), destruction of cartilage on the articular surfaces of joints leads to pain and debilitation. DJD affects 27 million patients in the US alone and results in huge economic losses.101 Unlike bone, cartilage does not have the ability to regenerate. Replacement therapies through tissue engineering and pharmacological treatment that prevent destruction are being intensely researched. However, even if these therapies were available currently, there would be no way to evaluate these treatments noninvasively or to guide surgery for neo-cartilage transplantation.
Despite its importance, cartilage is difficult to image. Most efforts to image and quantify cartilage have focused on magnetic resonance imaging (MRI)\textsuperscript{98,102} however, cartilage is a poor target for MRI due to its water-deficient composition.\textsuperscript{103} It is also a poor target for clinical computer tomography (CT) due to low X-ray absorption, and when using conventional microCT, it is essentially invisible.\textsuperscript{104} A SPECT radiotracer for cartilage was reported in 2001,\textsuperscript{105} but recent preclinical study literature shows poor image quality\textsuperscript{106} and clinical studies have not followed. To date, there is not a single optical contrast agent for cartilage and certainly not one in the NIR range.

In the 1970s improved isolation and chromatographic procedures allowed for the purification and analysis of tissue proteoglycans and glycosaminoglycans (GAGs). The separation of the large aggregating proteoglycans from cartilage left a complex proteoglycan, hyaluronan, and link protein. These GAGs consist of repeating disaccharide units composed of \( N \)-sulfated hexosamine. This high density of negative charges allow cartilage to retain a large amount of water and be surrounded by cations that form the gel in the extracellular matrix of cartilage;\textsuperscript{100} therefore, it was hypothesized that the introduction of cationic groups on a fluorophore should allow it to interact with anionic cartilage tissue. A proposed tricationic pharmacophore for NIR cartilage targeting can be seen in Figure 3.15.

\begin{center}
\includegraphics[width=0.5\textwidth]{figure3_15.png}
\end{center}

Figure 3.15. Proposed pharmacophore for high-specificity cartilage binding in vivo.

3.3.1 Physicochemical and Optical Properties

The physicochemical and optical properties of the proposed pharmacophore with modifications to the heterocycle are summarized in Table 3.3. The hydrophobicity, polarity,
and electron-resonance properties of the final NIR fluorophores could be systematically altered without affecting the emission wavelength by modifying the nonresonant substituents on the heterocycles as shown in the structures in Figure 3.16. The modifications included a methoxy group as an example of an electron donating substitution, an additional benzene on the heterocycle for increased hydrophobicity, and a sulfonate group making the net charge +1. All four compounds had similar optical properties with emission ranging from 788-818 nm and molar absorptivities from 101,500 M\(^{-1}\)cm\(^{-1}\) to 179,000 M\(^{-1}\)cm\(^{-1}\). The compound with the benz[e]indole heterocycle had the highest molecular brightness, followed by the methoxy and sulfate substituted compounds.

Table 3.3 Physicochemical and optical properties of NIR fluorophores 26h-k. Theoretical calculations of the molecular properties were calculated by using Marvin and JChem calculator plugins. All optical measurements were performed at 37 °C in 100% FBS buffered with 50 mm HEPES, pH 7.4.

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</table>

Figure 3.16 Structures of tricationic NIR fluorophores 26h-k

26h: R = H
26i: R = OMe
26j: R = SO\(_3\)⁻
3.3.2  *In vivo imaging*

As a preliminary test for cartilage targeting *in vivo*, the NIR fluorophores were intravenously injected into CD-1 mice. After 4 hours the mice were imaged and the fluorescent signals in the costal cartilage tissues were quantified (Figure 3.17).

![Figure 3.17. *In vivo* cartilage targeting with in mice. Each fluorophore was injected intravenously into CD-1 mice 4 h prior to imaging. The SBR (Ca/Mu) was calculated from the fluorescence intensity of costal cartilage tissues versus the signal intensity of neighboring muscle obtained over the imaging period. All NIR fluorescence images have identical exposure and normalization. Scale bars=1 cm.](image)

As expected, the sulfonated control compound 26k with a net charge of +1 showed no cartilage uptake. This compound had increased hydrophobicity compared to the others (logD at pH 7.4 of -1.67) and the negatively charged sulfonates could cause repulsion from the anionic GAGs. In addition, the polarity value for 26k is near 120 Å², which generally is outside the range of values where good cell membrane permeability would be expected. While the dye 26j showed cartilage uptake, its increased hydrophobicity and unbalanced 3D conformation, due to the benz[e]indole heterocycle, caused high nonspecific background signals. Interestingly, the methoxy substituted compound 26i exhibited the highest SBR (calculated from the difference in fluorescence intensity between costal cartilage and neighboring muscle) values compared to the other molecules, and 26h also showed high cartilage uptake. Although the synergistic effects of the methoxy groups on the cationic polymethine structure for efficient cartilage binding are not well understood, the addition of methoxy groups improved the hydrophilicity (LogD at pH 7.4 < -4.0) and increased the polar surface area (24.71 Å²).
Because **26i** showed the highest SBR for cartilage tissues among the different compounds tested, the methoxy-substituted NIR fluorophore was selected for further *in vivo* studies. All major cartilage tissues were imaged 4 hours post injection with **26i** in CD-1 mice to determine whether cartilage type (hyaline, elastic, or fibrocartilage) had an effect on uptake. After a single intravenous injection of **26i**, all cartilage tissues were clearly visualized, including ears/nose, knee joints, costal cartilages, and intervertebral discs.

![Figure 3.18. In vivo NIR imaging of cartilage tissues from ear, knee joint, and thoracic cavity in mice. Dye 26i was intravenously injected into 25 g CD-1 mice 4 h prior to imaging. The arrows in the knee joint indicate the growth plates. The white arrows labeled 1 and the white arrowheads labeled 2 in the chest cavity indicate hyaline and fibrocartilage, respectively. NIR images of resected cartilage tissues were obtained from the same animal. All NIR fluorescence images for each condition have identical exposure times and normalizations. Scale bars=1 cm.](image)

Because the composition of each cartilage tissue is different, histological evaluations with Alcian blue and hematoxylin and eosin (H&E) staining were performed. Alcian blue (dark blue staining) mainly stained acidic GAGs in cartilage, where strong NIR fluorescence was also observed. The histology data also confirm binding to all three types of cartilage. Since cartilage tissues are all produced by chondrocytes (or more precisely chondroblasts) and differ mainly in the relative amounts of collagen, proteoglycans, and elastin present, these NIR fluorophores are most likely binding either to the surface of chondrocytes, or more likely, to a secreted molecule with a high local concentration. Interestingly, the growth (epiphyseal) plate which is composed largely of hyaline cartilage was also targeted along with the fibrocartilage of the knee joint in the young mice (8 weeks) used for the study suggesting that these NIR
fluorophores could even bind cartilage in early developmental stages because the growth plate is generally only found in children and adolescents.

3.3.3 *Simultaneous in vivo NIR imaging of cartilage and bone*

Lastly, the dual-channel capability of the FLARE imaging system was utilized to highlight cartilage and bone tissues simultaneously in real time. The combination of cartilage targeting and bone targeting was tested by injecting 26i with a bone targeting agent previously synthesized by our group P700-SO3 together into mice and pigs. Cartilage and bone tissues were easily identifiable by different channels of the FLARE system as shown in Figure 3.19. This method could prove to be useful in future tissue engineering studies, in which transplanted neocartilage could be visualized in one NIR channel and either bone or blood vessels could be visualized in the other. During human surgery, these new imaging agents might prove useful for assessing cartilage thickness during arthroscopy, or for finding discreet damage to joints.

**Figure 3.19. Dual-channel *in vivo* fluorescence imaging of cartilage and bone tissues in the same animal.**
3.4 Conclusion

Along with pancreas-specific\textsuperscript{58} and bone-specific NIR fluorophores\textsuperscript{97} these novel specific NIR fluorophores described herein are examples of “structure inherent targeting”, in which tissue-/organ-specific targeting is engineered directly into the nonresonant structure of a NIR fluorophore. The incorporation of tissue-targeting moieties into the chemical structure of the contrast agent creates a bifunctional molecule eliminating the need for conjugation of targeting and fluorophore domains and thereby enabling subsequent optimization of in vivo properties including biodistribution, clearance, and elimination. As the number of NIR fluorophores developed with specific targets increases, the combinations that can be used together increase significantly, as do the amount of complex surgical procedures that can benefit from the technology. It is hoped that the present findings will lay the foundation for improved diagnosis and treatment of diseases.
4 STEM CELL TRACKING

4.1 Introduction

Longitudinal tracking of live cells in living organisms is crucial to understand the function, toxicity and therapeutic mechanism of systemically or implanted stem cells in clinical use.\textsuperscript{109} Currently, it is difficult to find the localized and differentiated cells after extracting the tissue construct from the body (i.e., histological analysis).\textsuperscript{110} The ability to monitor localization and behavior of administered cells for extended periods of time using high-resolution \textit{in vivo} optical imaging techniques would provide critical information in the treatment of diseases. To identify administered cells from the host tissue for real-time \textit{in vivo} analysis and \textit{ex vivo} post-analysis, the target cells must be labeled with a nontoxic and stable imaging probe that can retain its fluorescence in a biological environment long enough to be tracked.\textsuperscript{111} General strategies to track cells using contrast agents for optical imaging include endogenous biomolecule tracers\textsuperscript{112} and fluorescent proteins,\textsuperscript{113,114} however, these suffer from photobleaching, leakage of tracers, and engulfment by nearby cells resulting in false-positives.\textsuperscript{115,116} In order to overcome these limitations, exogenous functional molecules, especially NIR fluorophores, have been developed for staining cellular components.\textsuperscript{107,117} Optical fluorescence imaging has the potential to solve these problems, but two fundamental problems remain. Long-term \textit{in vivo} tracking is difficult due to the chemical and metabolic degradation of fluorophores\textsuperscript{118} and even agents retained in cells are washed out or destroyed during histopathological tissue processing.\textsuperscript{119}
Figure 4.1 Known agents for cell staining

To solve these fundamental problems, many subcellular components and organelles, such as DNA, lysosome, endoplasmic reticulum (ER), Golgi complex and mitochondria, have been targeted.\textsuperscript{120,121} Several functionalization strategies have been developed to design such imaging probes with high affinity to selective organelles. For example, hydrogen peroxide-targeted and pH-sensitive fluorophores, such as Cy-O-SeH (Figure 4.1) rapidly target lysosomes and mitochondria; however, due to their photo- and physicochemical instability long-term monitoring has not been achieved.\textsuperscript{122,123} Genetically encoded tagging to the membrane proteins and DNA with compounds such as firefly luciferase (FLuc, Figure 4.1) is generally more stable, but requires multiple replications and can be difficult to reach the fluorescence density required for imaging cells in a short period of time.\textsuperscript{124,125} Current focus has fixated on the development of optical probes targeting membrane organelles such as ER,
mitochondria (Mitotracker Orange, Figure 4.1) and lysosome (Lysotracker Red, Figure 4.1) to trap the probes inside the membrane by chemical alteration using membrane potential, proteins, and fixatives to eliminate efflux and washout after destruction. However, most of these agents are in the visible range (400-650 nm) and only a few are available in the NIR wavelength.¹²⁶

4.2 Rationale

The major challenges of fluorescence imaging for *in vivo* cell tracking are insufficient photostability and cytotoxicity, which limit imaging to fixed cells without continuous recording of the fate of cells in real time after administration of stained cells. An innovative design of fluorophores with two functional domains allows for avoidance of physical or biochemical interference of fluorophores to the cellular activities by inducing rapid passive diffusion into the cell followed by selective lysosomal sequestration and then trapping the fluorophores inside the cells. In addition, the fixable property of fluorophores allows for retaining in the tissue after resection and chemical treatment for histological analysis.

---

**Figure 4.2. Chemical structures of 45, 32 and IR786**
The two main functional domains the NIR probe was designed to contain were a lipophilic imaging core and an ionizable docking chain as shown in Figure 4.2. The imaging domain uses a heptamethine cyanine core, which emits at 790 nm with high photostability.\textsuperscript{73,127} Sufficient photostability is an essential property for prolonged live cell imaging. Additionally, this imaging core provides appropriate lipophilicity (LogD >1.0 at pH 7.4) allowing the fluorophore to penetrate inside the live cell membrane. As illustrated in Figure 4.3, the rationale is to introduce a primary amine that, once inside the acidic environment of the lysosome, will undergo a shift in equilibrium between charged and uncharged favoring the ionized form that could limit the diffusion of 45 back across the lysosomal membrane into the cytosolic space.\textsuperscript{128} As a result, the protonated 45 will be retained inside the cell, which is of importance to be an inert cell tracer with enhanced photo-activity.\textsuperscript{129}

![Figure 4.3 Lysosomal sequestration of 45](image)

How suitable an optical probe is for \textit{in vivo} live cell imaging is dependent on the different ionizable docking functional groups attached to the heptamethine cyanine imaging core. To compare selective cellular retention and further docking in the subcellular component, the \textit{meso}-carbon position of the heptamethine was functionalized with a primary amine for 45 and a carboxylic acid for 32. The commercially available heptamethine IR786 containing an unmodified chloride on the fixable group was used as a control.
Table 4.1 Physicochemical properties of lipophilic NIR fluorescent fluorophores 45, IR786 and 32

<table>
<thead>
<tr>
<th>Physicochemical property</th>
<th>45</th>
<th>IR786</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Formula</strong></td>
<td>C_{42}H_{50}N_{3}O</td>
<td>C_{32}H_{36}ClN_{2}</td>
<td>C_{41}H_{45}N_{2}O_{3}</td>
</tr>
<tr>
<td>Molecular weight (Da)</td>
<td>612.87</td>
<td>484.09</td>
<td>613.81</td>
</tr>
<tr>
<td>LogD at pH 7.4</td>
<td>3.5</td>
<td>4.79</td>
<td>6.54</td>
</tr>
<tr>
<td>Total charges at pH 7.4</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>pK_{a}, strongest basic</td>
<td>9.81</td>
<td>1.92</td>
<td>4.05</td>
</tr>
<tr>
<td>Topological polar surface area (Å²)</td>
<td>41.5</td>
<td>6.25</td>
<td>52.78</td>
</tr>
<tr>
<td>Hydrogen bond acceptor(s)</td>
<td>3</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Hydrogen bond donor(s)</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

The physicochemical properties of the NIR fluorophores used in this study are summarized in Table 4.1. All three probes are lipophilic cations (logD >3.0 at pH 7.4) which can readily diffuse across the cell membrane by passive diffusion, but only 45 becomes protonated (pK_{a}>6.0) in the acidic environment of lysosome (pH 4-5).^{130}

4.3 Results and Discussion

4.3.1 Synthesis

![Scheme 4.1 Synthesis of amine containing heptamethine cyanine](image)

As described in Chapter 2, chloro substituted dyes were synthesized through salt condensation with Vilsmeier-Haack reagent to build the heptamethine cyanine core. This dye was then functionalized by reaction of the meso chlorine atom of 27k with the appropriate nucleophile through the S_{RN1} displacement pathway as described in Scheme 4.1. Tyramine hydrochloride 43 was first Boc protected to form compound 44 and ensure selective reactivity.
at the oxygen atom. The Boc-protected product is then converted to the free amine by treatment with trifluoroacetic acid (TFA) in dichloromethane at 0 °C to yield dye 45 which was purified by flash column chromatography. The synthesis of dye 32 was described in Section 2.10.

4.4 Optical Properties

Comparative absorption and fluorescence emission spectra were recorded for three fluorophores and all three probes had similar emission at 790-800 nm as shown in Figure 4.4.

The optical properties of the NIR fluorophores used in this study are summarized in Table 4.2. Extinction coefficient and fluorescence quantum yield values for 45 and 32 in serum-containing media were similar with extinction coefficients \( \sim 150,000 \, \text{M}^{-1}\text{cm}^{-1} \) and quantum yields of 29% owing to molecular brightness of 44,660 \( \text{M}^{-1}\text{cm}^{-1} \) and 39,825 \( \text{M}^{-1}\text{cm}^{-1} \), respectively. These values are significantly higher than the commercially available IR786 which has an extinction coefficient of 117,500 \( \text{M}^{-1}\text{cm}^{-1} \) and quantum yield of 23% owing to its molecular brightness of 27,025 \( \text{M}^{-1}\text{cm}^{-1} \).

<table>
<thead>
<tr>
<th>Optical property in media</th>
<th>45</th>
<th>IR786</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance maximum (( \lambda_{\text{abs}} ), nm)</td>
<td>778</td>
<td>786</td>
<td>773</td>
</tr>
<tr>
<td>Emission maximum (( \lambda_{\text{em}, \text{nm}} ))</td>
<td>790</td>
<td>800</td>
<td>786</td>
</tr>
<tr>
<td>Stokes shift (nm)</td>
<td>12</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>Extinction coefficient (( \varepsilon ), ( \text{M}^{-1}\text{cm}^{-1} ))</td>
<td>154,000</td>
<td>117,500</td>
<td>137,500</td>
</tr>
<tr>
<td>Quantum yield at 770 nm (( \Phi ), %)</td>
<td>29.0</td>
<td>23.0</td>
<td>29.0</td>
</tr>
<tr>
<td>Molecular brightness (( \text{M}^{-1}\text{cm}^{-1} ))</td>
<td>44,660</td>
<td>27,025</td>
<td>39,825</td>
</tr>
</tbody>
</table>
4.4.1 Cell testing

To compare the cellular permeability and retention, all 3 NIR fluorophores were loaded into living PC3 cells individually, and their cellular behaviors were compared at various conditions. All 3 lipophilic cations achieved saturation of loading into cells after only 10 min with extracellular concentrations ranging from 0.5 to 10 µM. Even at these high concentrations, no evidence of toxicity was observed, i.e., there has been no observed change in cellular morphology, no decrease or increase in the rate of proliferation, and no change in cell plating efficiency. In addition, as shown in Figure 4.5, the strong signal strength of 45 was maintained in active proliferation of the cells for over 24 hours after incubation, while cells stained with IR786 and 32 had almost no signals after 24 hours due to the efflux of fluorophores.

![Figure 4.5 Long-term cellular stability of 45, IR786, and 32 in live PC3 cells](image)

To show that 45 is fixable, the cells were treated with 4% formaldehyde after staining and washed with 1% Tween 20 (Figure 4.6). Despite potential interaction of the chloride atom of IR786 with intracellular proteins, virtually no intracellular retention was observed after fixation and detergent washing. Dye 45, however, is fixed in place with no loss of signal after detergent washing. Dye 45 demonstrated high efficiency and rapid loading into live cells, as well as exhibited excellent partitioning behavior and retention.
4.4.2 Animal studies

To validate the cell studies for in vivo cell tracking, PC3 cells stained with either 45 or IR786 were suspended in the media solution and intravenously administered into syngeneic C57BL/6J mice. The mice were sacrificed at 5 minutes and 24 hours post-injection, and then the thoracic cavity was opened and imaged under real-time image guidance using the intraoperative optical imaging system (Figure 4.7). Initially, the cells with both 45 and IR786 showed similar signal intensity from the lung. However, after 24 hours, only the cells in the lung stained with 45 could be observed. The signal intensity of the lung was rather attenuated at 24 hours due to migration of the cells from the lung capillaries to the liver, which was traceable by an increased signal in the liver.
Figure 4.7. *In vivo* tracking of stained cells using the intraoperative optical imaging system. 45 and IR786-stained cells were trapped in the lung with strong fluorescent signal at 5 min post-intravenous injection. The cells stained with 45 were found in the liver as an evidence of cell migration from the lung capillaries at 24 h post-injection. All NIR fluorescence images are identically normalized. Abbreviations used are: He, heart; Li, liver; Lu, lung. Scale bars = 3 mm.

This *in vivo* cell tracking was emphasized by changing the injection method. As shown in Figure 4.8, cells orthotopically injected into the liver were found in the lung 6 hours post-injection, which implicates migration of the cells through hepatic vasculatures. NIR *in vivo* fluorescence imaging confirmed that prolonged lysosome sequestration of 45 is the key to real-time visualization of tracing administered cells. This mechanism differs from other lysosomal targeting and pH sensitive probes where their purpose is to monitor cellular trafficking process. In this case, probes are effluxed out of the targeted organelle in 1-2 hours. The fixable property of 45 is unique from all other subcellular-targeted probes. It has potential for clinical translation, especially for cell-based therapy, such as immune cancer therapy and cardiac stem cell regeneration which can be solved using 45, where the homing and behavior of cells after administration can be monitored and analyzed.
Figure 4.8. In vivo tracking of cells stained with 45 injected into the liver using the intraoperative optical imaging system. The cells were found in the lung after 6 h, implicating liver-to-lung trafficking of the cells. All NIR fluorescence images are identically normalized. Abbreviations used are: He, heart; In, intestine; Ki, kidney; Li, liver; Lu, lung. Scale bars = 3 mm.

Another major problem with the use of cell tracking fluorophores is the inability to fix contrast agents intracellularly using formalin or paraformaldehyde. Washout and/or destruction of such fluorophores during H&E histological staining is currently a common challenge. H&E is the gold standard for analyzing biopsy specimens from clinical trials, and cell tracking technology must be compatible to make a substantial impact in the field. However, H&E processing is extremely harsh, requiring exposure to organic solvents as strong as xylene, and heating to at least 65 °C. Currently there are no fixable NIR fluorophores that can withstand these severe conditions. Dye 45 has the ability retain high stability while fixed covalently within the cell using formalin or paraformaldehyde. To prove this, the same process used clinically on every human tissue biopsy, using the cell pellet and lung tissue was followed with 45 and IR786. The cell pellets underwent the same histological preparation to evaluate the fluorescence retention or attenuation. As shown in Figure 4.9a, while IR786 was largely washed away during histological processing, 45 was fixed in place and withstood organic solvent washing and H&E staining. The lung tissues were harvested after intravenous administration of the stained cells, and a cryosection was performed followed by H&E staining.
As expected, \textbf{45} was resistant to the harsh processing and allowed further visualization of the cells (Figure 4.9b). This makes it possible to combine NIR fluorescence and H&E staining of the same slide, and thus to locate a single cell on an H&E stained tissue slice while co-staining for differentiation markers.

![Figure 4.9. Stability of histological processes, including formaldehyde fixation and H&E staining. (a) Cell pellets mimicking the tissue structure and (b) the lung tissue sections harvested from mice after intravenous administration of the stained cells. All NIR fluorescence images have identical exposure and normalization. Scale bars = 25 µm.](image)

Finally, to confirm the lysosomal sequestration, PC3 cells were stained with \textbf{45} and a commercially available LysoTracker dye (Figure 4.10). They clearly co-registered each other, however, \textbf{45} displayed prolonged retention compared with the LysoTracker, although both showed similar stability in serum. This proves that \textbf{45} is fixable and ionizable inside the lysosome limiting efflux elimination and thereby enabling quantitation of cell survival and differentiation at the single cell level.

![Figure 4.10. Lysosomal sequestration of \textbf{45} (left), LysoTracker (middle), and merged image (right). Scale bars = 25 µm.](image)
4.5 Conclusion

In this study, a single NIR fluorophore was synthesized to monitor the fate of injected cells \textit{in vivo} and \textit{ex vivo} using high-resolution optical imaging techniques. Specifically, this amino-functionalized fluorophore 45 selectively targets lysosomes and exhibits outstanding physicochemical and optical properties in serum as well as \textit{in vivo}, which enabled longitudinal tracking of living cells in the body and even after extraction from the body. The novel cell tracking NIR fluorophore 45 survives all steps in H&E histological tissue processing while retaining excellent NIR fluorophore properties. The lipophilic cation structure allows for cellular membrane permeation, and the primary amine is used for rapid sequestration followed by efficient intracellular fixation by formalin through reductive amination. This amine structure also reduces the risk of efflux of NIR fluorophores from the cell membrane enabling tracking of single cells for determining the mechanism of various diseases in the body. Therefore, 45 has the potential to be useful in labeling various types of cells to study their \textit{in vivo} and \textit{ex vivo} fate at the single cell level.
“Turn on” fluorescence response of monomethine cyanines caused by noncovalent binding to ct-DNA

5.1 Introduction

Imaging of macromolecules such as DNA by staining with fluorescent compounds is of great interest; therefore, expanding the options of available probes is vital to several areas of research spanning from medical diagnostics to genomics. The synthesis of low cost, easy to manipulate systems for fast analysis is required. Fluorescent detection has rapidly become one of the most widely used techniques due to its sensitivity and noninvasiveness. Ethidium bromide has commonly been used for the detection of DNA, however it has mutagenic effects and poses other environmental concerns. On the other hand, cyanine dyes are sensitive, safe and highly modifiable.

As described previously by our group, monomethine cyanines possess a valuable characteristic of only displaying fluorescence when rotation around the methine bridge is restricted. These dyes are the best non-convalent binding nucleic acid labels due to their high binding constants, large molar absorptivities and quantum yields and high fluorescence signals upon binding. Two commercially available monomethine cyanines are among the most popular intercalating agents and have recently been used for DNA and chromatin imaging with super-resolution fluorescence microscopy. While there is still much to explore regarding what effect structure has on the photophysical properties, our lab has developed ways to redshift the monomethine scaffold by altering the terminal heterocycles. Understanding how to redshift the monomethine cyanine dyes allows for the enhancement of existing molecular probes minimizing background absorption and fluorescence. While it is well known that monomethine cyanines tend to bind DNA there is a lack of literature on what causes or prevents a cyanine from binding to DNA. Herein a series of monomethine cyanines that
differ only in one heterocycle were synthesized to investigate how these different heterocycles influence DNA binding.

5.2 Results and Discussion

5.2.1 Synthesis

Toward correlating the various heterocyclic moieties and their ability to bind DNA, we began to rationally design monomethine cyanines containing the benz[c,d]indole heterocycle on one half of the dye. The other side possessed different heterocycles including 2-methylbenzothiazole, 2-methylbenzoxazole, 2-methylquinoline, or 3,3-dimethylindole, heterocycles all with butyl side chains on the nitrogen. As shown in Table 5.1, the physicochemical properties of these four compounds are all very similar with molecular weight from 397-423 (without counterion) and polarizability from 76-80. They are all hydrophobic molecules with LogP between 7 and 10. With the exception of the benzoxazole containing dye they have similar topological polar surface area (TPSA). The oxygen atom typically raises the TPSA, but there is no way to keep it in line with the other compounds without altering the structure drastically. Due to the similarity of their physicochemical properties any change observed in binding to DNA should be based on the 3D structure of each dye.

Table 5.1. Physicochemical properties of monomethine cyanines 46

<table>
<thead>
<tr>
<th>Dye</th>
<th>MW (amu)</th>
<th>LogP</th>
<th>TPSA (Å²)</th>
<th>Polarizability</th>
</tr>
</thead>
<tbody>
<tr>
<td>OX</td>
<td>397.54</td>
<td>7.68</td>
<td>5.479</td>
<td>76.21</td>
</tr>
<tr>
<td>BTZ</td>
<td>413.60</td>
<td>8.25</td>
<td>1.674</td>
<td>77.39</td>
</tr>
<tr>
<td>Q</td>
<td>407.58</td>
<td>8.69</td>
<td>1.165</td>
<td>78.36</td>
</tr>
<tr>
<td>IN</td>
<td>423.62</td>
<td>9.23</td>
<td>1.752</td>
<td>80.37</td>
</tr>
</tbody>
</table>

The asymmetrical red-shifted monomethine cyanine dyes were synthesized as shown in Scheme 5.1. The key intermediate 7b which was used as one heterocycle for the monomethine cyanines was synthesized as described in Scheme 2.1. The second heterocycle included 2-methylbenzothiazole, 2-methylbenzoxazole, 2,3,3-trimethylindole, and 2-
methylquinoline were alkylated, respectively, with iodobutane various alkyl halides in acetonitrile to form quaternary ammonium salts 46. The two heterocycles 7b and 3 were then connected by a condensation reaction in acetonitrile with triethylamine to afford final dyes 46.

![Scheme 5.1. Synthesis of monomethine cyanines](image)

The monomethine reaction begins with the deprotonation of the methyl group at the 2 position of the heterocycle. This activated methylene group of the various heterocyclic salts 3 displaces the methyl sulfide moiety of 7b and results in the formation of the monomethine dyes. After isolation of the final dyes via precipitation from methanol with diethyl ether, the dyes were characterized by HRMS, 1H and 13C NMR and their optical properties were investigated.

### 5.2.2 Optical Properties

**Table 5.2. Energy values and optical properties of the monomethine cyanines 46**

<table>
<thead>
<tr>
<th>Dye</th>
<th>$\lambda_{\text{max}}$(exp.)</th>
<th>$\lambda_{\text{max}}$(calc.)</th>
<th>$\varepsilon$($M^{-1}cm^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OX</td>
<td>500</td>
<td>490</td>
<td>39,900</td>
</tr>
<tr>
<td>BTZ</td>
<td>560</td>
<td>505</td>
<td>30,700</td>
</tr>
<tr>
<td>Q</td>
<td>585</td>
<td>522</td>
<td>35,900</td>
</tr>
<tr>
<td>IN</td>
<td>559</td>
<td>530</td>
<td>25,200</td>
</tr>
</tbody>
</table>

The optical properties of the monomethine cyanines were measured in ethanol. As shown in Table 5.2 the absorption maxima range from 500 nm in the benzoxazole containing dye OX to 585 nm with the increased conjugation of the quinoline system in Q. Their molar
absorptivities range from 25,000 M\(^{-1}\)cm\(^{-1}\) in the dimethylindolenine compound IN to almost 40,000 M\(^{-1}\)cm\(^{-1}\) in the benzoxazole compound OX. Benzoxazole compounds are known to be brighter. In general, the molar absorptivities are low for monomethine cyanines due to the benz\([c,d]\)indole heterocycle present on the other end of the dye, but it is this heterocycle that causes the absorbance to be redshifted about 100 nm from where the dyes would generally absorb if they were symmetrical.\(^{69}\) Scientists have attempted to approximate wave functions and energies for atoms and ions since the late 1920s.\(^{142}\) Over the past 90 years there has been significant improvements for predicting behaviors of these systems and more complex ones. The ability to use theoretical calculations to predict optical properties of fluorophores, such as monomethine dyes is a useful tool for screening compounds which can eliminate the need to synthesize them first thus reducing harmful environmental impact. The ability to use theoretical calculations of optical properties for fluorophores, such as monomethine dyes could be useful for the development of viscosity detection fluorophores or bioimaging agents with desirable optical profiles.\(^{66}\) The dyes were modeled with a restricted dihedral angle to disallow rotation around the methine carbon. Their geometries were optimized using Hartree-Fock dynamic functional theory (HF-DFT) hybrid exchange-correlation functional and B3LYP/6-31G* basis set to predict the energy of the compounds at ground state.\(^{143}\) The experimental results mostly agree with the trend of the computationally predicted energy gaps. None of these compounds display significant fluorescence unaccompanied in ethanol.

### 5.2.3 DNA Binding

It was previously shown by our lab that only when the monomethine cyanine’s ability to rotate around the methine bridge is restricted do the dyes relax by fluorescence.\(^{65}\) The dyes generally do not fluoresce in free flowing solvent, but when the rotation around the methine bridge is restricted such as in glycerol or when bound, fluorescence can be measured. The binding of these dyes to ct-DNA restricts this rotation and allows for the unique “turn-on”
fluorescence of these compounds. This property was exploited to determine how strongly each heterocycle interacts with DNA compared to the others. Table 5.3 shows the binding constants and emission maxima of the monomethine cyanines.

<table>
<thead>
<tr>
<th>Dye</th>
<th>λ&lt;sub&gt;emission&lt;/sub&gt; (nm)</th>
<th>K&lt;sub&gt;b&lt;/sub&gt; (M&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OX</td>
<td>555</td>
<td>1.6 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>BTZ</td>
<td>583</td>
<td>1.1 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Q</td>
<td>680</td>
<td>1.8 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>IN</td>
<td>686</td>
<td>1.6 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>TO&lt;sup&gt;a&lt;/sup&gt;</td>
<td>525</td>
<td>1.7 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Published values λ<sub>emission</sub> and K<sub>b</sub>*

As shown in Figure 5.1, dye OX displayed the greatest increase in fluorescence when in the presence of ct-DNA with a 700-fold increase. The dye goes from non-fluorescent with no DNA present to almost 700 fluorescence units with 20 μM DNA. This is due to both the binding affinity of the dye (1.6 x 10<sup>4</sup>M<sup>-1</sup>) and the tendency of benzoxazole to decrease non-radiative deactivation due to fast intersystem crossing.<sup>146</sup> The benzothiazole containing dye BTZ shows a similar binding affinity (1.1 x 10<sup>4</sup>M<sup>-1</sup>), but less fluorescence with only about a 40-fold increase. The sulfur atom in benzothiazole can cause the opposite effect of the oxygen in benzoxazole due to a lower singlet-triplet energy gap raising the opportunity for non-radiative relaxation.<sup>69</sup> The change in binding affinity is not very significant, but is likely due to the larger size of the sulfur atom compared to oxygen. This dye is similar to the commercially available dye thiazole orange (TO) which has a binding affinity in the order of 10<sup>5</sup>M<sup>-1</sup>.<sup>145</sup> TO likely has higher affinity due to steric hindrance of the butyl groups in our dyes as well as the larger size of the benz[c,d]indolium heterocycle compared to the quinoline in TO. These values are also on par with similar monomethine cyanine dyes.<sup>133</sup>
Figure 5.1. Representative fluorescence spectrum of OX and IN at constant dye concentration of 10 μM with increasing ct-DNA concentrations from 0-20 μM

Unlike the BTZ and OX compounds which showed excellent binding, the IN compound containing a 3,3-dimethylindolenine heterocycle showed almost no binding with a binding affinity of $1.6 \times 10^{-3}$ M$^{-1}$. This compound was 14 orders of magnitude lower than the benzothiazole and benzoxazole compounds and any binding it may have shown is likely from the benz[c,d]indole heterocycle on the other side of the dye. Because all of the physicochemical properties of the dye have been kept similar and the only change is the dimethyl group, it can be concluded that the dimethyl groups are perpendicular to the plane of the heterocycle providing steric hindrance that prevents this binding. Our group has reported similar properties with the 3,3-dimethylindolenine preventing duplex DNA binding but still allowing for binding to G-quadruplex DNA in other cyanine classes. This can be seen in the 3D electrostatic potential maps shown in Figure 5.2.

Figure 5.2. Electrostatic potential maps of monomethine cyanines 46
This finding is of utmost importance as the dimethyl functionality can be exploited to prevent off site binding in the medicinal applications of these dyes. **OX** could be beneficial as a molecular probe due to its redshifted nature over current commercially available DNA probes (30 nm higher than TO). In addition, this dye should have higher fluorescence intensity than the commercially available probes due to the exclusion of quinoline from the structure. Quinolines are known to experience non-radiative return of decay. These compounds will continue to become more useful as the fluorescence approaches the optical window (650-900 nm) and gets further from the absorption of human tissues.

### 5.2.4 Conclusion

A series of monomethine cyanines were synthesized in good yield with red-shifted absorbance properties in comparison to previously synthesized monomethine cyanine dyes. Although the benz[c,d]indolium containing monomethine cyanine dyes in this report are non-fluorescent in free flowing solvent, their fluorescence is “turned on” through the restriction of the methine bridge caused by binding to DNA. Computational methods were shown to be useful as a predictive tool for determining their optical properties. In keeping everything the same except for a single heterocycle, binding affinity to ct-DNA of these heterocycles were able to be compared. **OX** stood out as the best candidate as a starting point for building a new molecular probe due to its strong binding affinity and 700-fold increase in fluorescence from binding. In addition, **IN** with a 3,3-dimethylindolenine heterocycle showed almost no binding due to the steric hindrance of the dimethyl group. This could be exploited to prevent offsite binding. Utilizing the described techniques these dyes could be further developed as potential biological probes. Future studies will investigate how different substitutions on the heterocycles could increase binding affinities and increase optical activity to various biological targets.
6 Conclusions

In conclusion, a series cyanine dyes were systematically designed and synthesized to be used as probes in biomedical applications. They were characterized by $^1$H and $^{13}$C NMR and HRMS. Near-infrared dyes underwent biodistribution testing in CD-1 mice and fluorophores with specific targets were further tested in large animal models. Through screening and rational design, specific targeting of the adrenal gland, parathyroid glands, and cartilage was achieved. One of these NIR fluorophores synthesized to monitor injected cells in vivo and ex vivo was able to penetrate the cell membrane where it was fixed inside the lysosome preventing efflux from the cell and could be useful in labeling cells for determining the mechanism of various diseases in the body. A few visible range dyes were tested as probes for DNA binding comparing. Small changes in the structures were made to determine what may cause or prevent binding. One of these dyes showed a large increase in fluorescence upon binding and could potentially be used as a DNA probe due to its redshifted properties compared to current probes. It is our hope that these findings will lay the foundation for improved diagnosis and treatment of diseases.
7 Experimental

7.1 Chemicals and Instruments

All chemicals and solvents were of American Chemical Society grade or HPLC purity and were used as received. All chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA), Sigma-Aldrich (Saint Louis, MO), and Acros Organics. The reactions were followed using silica gel 60 F254 thin layer chromatography plates or reversed-phase silica-gel thin-layer chromatography plates (Merck EMD Millipore, Darmstadt, Germany) with 5% methanol in water as the mobile phase. Open column chromatography was utilized for the purification of all hydrophobic final compounds using 60-200u, 60A classic column silica gel (Dynamic Adsorbents, Norcross, GA) and C18 reversed-phase silica gel (Dynamic Adsorbents, Norcross, GA) for highly charged products. The $^1$H NMR, $^{13}$C NMR, and $^{19}$F NMR spectra were obtained using high quality Kontes NMR tubes (Kimble Chase, Vineland, NJ) rated to 500 MHz and were recorded on a Bruker Avance (400 MHz) spectrometer. NMR abbreviations used throughout the experimental section are as follows: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, dd = doublet doublets, and bs = broad singlet. High resolution mass spectra (HRMS) were obtained at the GSU Mass Spectrometry Facility using a Waters Q-TOF micro (ESI-Q-TOF) mass spectrometer. The purity of each compound tested as determined by using LC/MS instrument possessing a Waters 2487 single wavelength absorption detector. The column used in LC was a Waters Delta-Pak 5 μM 100Å 3.9×150 mm reversed phase C18 column, with a flow rate of 1 mL/min employing a 5-100% Acetonitrile/water/0.1% formic acid gradient; a SEDEX 75 Evaporative light scattering detection (ELSD) was also utilized in tandem with liquid chromatography to confirm purity. LysoTracker™ was obtained from Molecular Probes (Eugene, OR), and IR786 was purchased from Sigma-Aldrich. Imaging experiments were performed by Harvard Medical School at Beth Israel Deaconess Medical Center.
7.2 Synthesis and Characterization

7.2.1 Heterocycles

5-methoxy-2,3,3-trimethyl-3H-indole, 2b: A mixture of 4-methoxyphenylhydrazine hydrochloride and 3-methyl-2-butanone was refluxed in glacial acetic acid for 24 hrs. Acetic acid was then removed by rotary evaporation and the solution was diluted with dichloromethane. Sodium bicarbonate was added to neutralize any remaining acid and the 5-methoxy-2,3,3-trimethyl-3H-indole 2b was extracted three times with 50 mL dichloromethane, dried with magnesium sulfate, and concentrated under vacuum to form a brown oil; 91% yield (lit 95% yield)\textsuperscript{148}.

5-bromo-2,3,3-trimethyl-3H-indole, 2c: was synthesized by a similar procedure to 72b; 91% yield (lit 98% yield)\textsuperscript{148}.

5-chloro-2,3,3-trimethyl-3H-indole, 2d: A mixture of 4-chlorophenylhydrazine hydrochloride, 3-methyl-2-butanone, deionized water, and sulfuric acid were added to a 10 mL microwave vessel. The reaction vessel was capped and heated under microwave irradiation for 10 min at 100 °C. The water was decanted and the reaction was neutralized with a saturated solution of sodium bicarbonate. The liquid was poured off and the resulting oil was dissolved in a dichloromethane, dried with magnesium sulfate, gravity filtered and concentrated; 100% yield.

2,3,3-trimethyl-3H-indole-5-sulfonate, 2f: was synthesized by our group as previously reported\textsuperscript{127}.

7.2.2 Quaternary Ammonium Salts

1-ethyl-2,3,3-trimethyl-3H-indol-1-ium iodide, 3b: A mixture of 2,3,3-trimethyl-3H-indole and iodobutane were refluxed in acetonitrile for 72 hrs. The reaction was cooled to room
temperature. The solution was then poured into ether and the precipitate was filtered; 82% yield; mp 217-221 °C (lit mp 165-166 °C).  

1-butyl-2,3,3-trimethyl-3H-indol-1-ium iodide, **3c**: was synthesized by a similar procedure to **3b**: 80% yield; mp 116-119 °C (lit mp 102 °C, 83% yield).  

2,3,3-trimethyl-1-(3-phenylpropyl)-3H-indol-1-ium bromide, **3d**: was synthesized by a similar procedure to **3b**: 80% yield; mp 156-158 °C; $^1$H NMR (400 MHz, DMSO-d$_6$): δ 7.98 (t, $J = 5.2$ Hz, 1 H), 7.84 (t, $J = 5.2$ Hz, 1 H), 7.46 (m, 2 H), 7.29 (m, 5 H), 4.51 (t, $J = 8.0$ Hz, 2 H), 2.81 (m, 5 H), 2.17 (m, 2 H), 1.53 (s, 6 H). $^{13}$C NMR (100 MHz, DMSO-d$_6$): δ 14.3, 22.0, 28.8, 31.7, 47.4, 54.1, 115.4, 123.5, 126.0, 128.2, 128.3, 128.8, 129.3, 140.6, 141.0, 141.8, 196.6. HRMS m/z: calc for C$_{20}$H$_{24}$N$^+2$ 278.1904, obsd 278.1915.  

3-butyl-1,1,2-trimethyl-1H-benzo[e]indol-3-ium iodide, **3f**: was synthesized by a similar procedure to **3b**: mp 142-145°C, 87% yield (lit mp 127-129°C, 90% yield).  

2,3,3-trimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium bromide, **3h**: was synthesized by our group as previously reported.$^{149}$  

5-Methoxy-2,3,3-trimethyl-1-(3-(trimethylammonio)propyl)-3H-indolium bromide, **3i**: Yield 88%; $^1$H NMR (400 MHz, DMSO-d$_6$): δ 1.55 (s, 6 H), 2.33 (m, 2 H), 2.87 (s, 3 H), 3.12 (s, 9 H), 3.63 (t, $J = 8.4$ Hz, 2 H), 3.87 (s, 3 H), 4.48 (t, $J = 8.4$ Hz, 2 H), 7.16 (d, $J = 8.8$ Hz, 1 H), 7.58 (s, 1 H), 8.03 (d, $J = 8.8$ Hz, 1 H); HRMS m/z: calc for C$_{17}$H$_{28}$N$_2$O$_2^{2+}$ 138.1096, obsd 138.1010.
1,1,2-Trimethyl-3-(3-(trimethylammonio)propyl)-1H-benzo[e]indolium bromide, 3j: Yield 52%; 1H NMR (400 MHz, DMSO-d$_6$): δ 1.79 (s, 6 H), 2.42-2.37 (m, 2 H), 3.03 (s, 3 H), 3.41 (s, 9 H), 3.65 (t, J = 8.0 Hz, 2 H), 4.64 (t, J = 8.0 Hz, 2 H), 7.82-7.75 (m, 2 H), 8.26 (t, J = 8.8 Hz, 2 H), 8.33 (d, J = 8.8 Hz, 1 H), 8.39 (d, J = 8.8 Hz, 1 H). 13C NMR (100 MHz, DMSO-d$_6$): δ 15.08, 21.98, 22.11, 45.34, 53.11, 56.13, 62.42, 113.93, 123.89, 127.70, 127.77, 128.93, 130.20, 131.18, 133.55, 137.33, 138.90, 198.11. HRMS m/z: calc for C$_{20}$H$_{28}$N$_2$ 2+ 148.1121, obsd 148.1148.

2,3,3-trimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-5-sulfonate bromide, 3k: was synthesized by our group as previously reported.

5-methoxy-1,2,3,3-tetramethyl-3H-indolium iodide, 3l: Yield 79%; 1H NMR (400 MHz, DMSO-d$_6$): δ 1.51 (s, 6 H), 2.71 (s, 3 H), 3.87 (s, 3 H), 3.94 (s, 3 H), 7.15 (d, J = 8.8 Hz, 1 H), 7.47 (s, 1 H), 7.81 (d, J = 8.8 Hz, 1 H).

1-ethyl-5-methoxy-2,3,3-tetramethyl-3H-indolium iodide, 3m: 1H NMR (400 MHz, DMSO-d$_6$): δ 1.43 (t, J = 8.8 Hz, 3 H), 1.52 (s, 6 H), 2.77 (s, 3 H), 3.87 (s, 3 H), 4.45 (q, J = 8.8 Hz, 2 H), 7.15 (dd, J = 8.8 Hz, 1 H), 7.49 (s, 1 H), 7.87 (d, J = 8.8 Hz, 1 H).

1-butyl-5-methoxy-2,3,3-trimethyl-3H-indol-1-ium iodide, 3n: was synthesized by a similar procedure to 3b; 56% yield; mp 145-147 °C; 1H NMR (400 MHz, DMSO-d$_6$): δ 0.93 (t, J = 7.2 Hz, 3 H), 1.43 (m, 2 H), 1.53 (s, 6 H), 1.80 (m, 2 H), 2.80 (s, 3 H), 3.86 (s, 3 H), 4.43 (t, J = 7.6 Hz, 2 H), 7.13 (dd, J = 10.8 Hz, 1 H), 7.50 (s, 1 H), 7.89 (d, J = 9.2 Hz, 1 H); 13C NMR (100 MHz, MeOD-d$_6$): δ 12.4, 19.1, 21.6, 29.0, 47.9, 53.8, 55.7, 108.4, 114.5, 116.0, 133.4, 143.5, 160.7, 192.3; HRMS m/z: calc for C$_{16}$H$_{24}$NO$^+$ 246.1852, obsd 246.1857.
1-ethyl-5-fluoro-2,3,3-trimethyl-3H-indol-1-ium iodide, \textbf{3r}: was synthesized by a similar procedure to \textbf{3b}: 84% yield; $^1$H NMR (400 MHz, DMSO-\textit{d}$_6$): $\delta$ 1.43 (t, $J =$ 7.2 Hz, 3 H), 1.54 (s, 6 H), 2.81 (s, 3 H), 4.48 (t, $J =$ 7.6 Hz, 2 H), 7.51 (t, $J =$ 8.8 Hz, 1 H), 7.85 (d, $J =$ 8.4 Hz, 1 H), 8.04 (d, $J =$ 8.8 Hz, 1 H).

1-butyl-2-methylbenzo[c,d]indol-1-ium iodide, \textbf{9b}: was synthesized by our group as previously reported.$^{63}$

### 7.2.3 Polymethine Linkers

\textit{N-((1Z,3E)-2-chloro-3-(phenylimino)prop-1-en-1-yl)aniline} hydrochloride, \textbf{11a}: $^1$H NMR (400 MHz, DMSO-\textit{d}$_6$): δ ppm 7.33 (t, $J =$ 7.2 Hz, 2 H), 7.51 (t, $J =$ 7.6 Hz, 4 H), 7.65 (d, $J =$ 8.0 Hz, 4 H), 9.43 (s, 1 H), 11.83 (s, 1 H).

\textit{N-((E)-(2-methyl-3-(E)-(phenylimino)methyl)cyclohex-2-en-1-ylidene)methyl}aniline hydrochloride, \textbf{19a}: Yield 75%; mp >260 °C; $^1$H NMR (400 MHz, DMSO-\textit{d}$_6$): δ ppm 1.80 (m, 2 H), 2.55 (m, 4 H), 2.57 (s, 3 H), 7.23 (t, $J =$ 7.6 Hz, 2 H), 7.44 (t, $J =$ 7.6 Hz, 4 H), 7.65 (t, $J =$ 7.6 Hz, 4 H), 8.44 (s, 2 H).

\textit{N-((E)-(6-((E)-(phenylimino)methyl)-4,5-dihydro-[1,1'-biphenyl]-2(3H)-ylidene)methyl}aniline hydrochloride, \textbf{19b}: Yield 75%; mp 201-203 °C; $^1$H NMR (400 MHz, DMSO-\textit{d}$_6$): δ ppm 1.95 (m, 2 H), 2.72 (t, $J =$ 6.0 Hz, 4 H), 7.08 (d, $J =$ 7.6 Hz, 4 H), 7.16 (t, $J =$ 7.6 Hz, 2 H), 7.26 (s, 2 H), 7.35 (t, $J =$ 7.6 Hz, 4 H), 7.41 (dd, $J =$ 7.6 Hz, 2 H), 7.57 (m, 3 H).

### 7.2.4 Monomethine Cyanine

1-butyl-2-((1-butylbenzo[c,d]indol-2(1H)-ylidene)methyl)benzo[c,d]indol-1-ium iodide, \textbf{20}: Yield 72%; mp >260 °C; $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 0.98 (t, $J =$ 7.2 Hz, 6 H), 1.58
(m, 4 H), 2.00 (m, 4 H), 4.73 (m, 4 H), 7.00 (s, 1 H), 7.28 (s, 2 H), 7.44 (d, J = 7.2 Hz, 2 H), 7.51 (t, J = 7.6 Hz, 2 H), 7.62 (t, J = 7.6 Hz, 2 H), 7.73 (d, J = 8.4 Hz, 2 H), 8.09 (d, J = 8.0 Hz, 2 H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 14.0, 20.3, 31.3, 46.7, 90.4, 112.0, 124.5, 125.6, 128.2, 128.9, 129.4, 129.6, 130.2, 133.2, 141.5, 159.0.

7.2.5 Trimethine Cyanines

1-Butyl-2-[3-(1-butylbenz[c,d]indol-2(1H)-ylidene)-1-propen-1-yl]- iodide, 21: was synthesized as previously published.$^{63}$ Yield 80% (lit yield 60%).

1,3,3-trimethyl-2-((1E,3E)-3-(1-methylbenzo[c,d]indol-2(1H)-ylidene)prop-1-en-1-yl)-3H-indol-1-ium bromide, 23a: 55% yield; mp 232-234 °C; $^1$H NMR (400 MHz, DMSO-$d_6$): δ ppm 1.76 (s, 6 H), 3.81 (s, 6 H), 6.86 (t, J = 7.6 Hz, 2 H), 7.39 (t, J = 7.6 Hz, 1 H), 7.54 (m, 3 H), 7.67 (t, J = 7.6 Hz, 1 H), 7.72 (d, J = 7.6 Hz, 1 H), 7.98 (t, J = 7.6 Hz, 1 H), 8.27 (d, J = 8.0 Hz, 1 H), 8.35 (d, J = 7.6 Hz, 1 H), 8.69 (t, J = 13.2 Hz, 1 H); $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ ppm 32.8, 37.4, 54.8, 112.3, 114.5, 117.6, 127.4, 127.7, 129.7, 131.3, 133.9, 134.8, 135.2, 136.2, 146.6, 147.7, 153.5, 180.7.

2-((1E,3E)-3-(1-Butylbenzo[c,d]indol-2(1H)-ylidene)prop-1-en-1-yl)-1,3,3-trimethyl-3H-indol-1-ium iodide, 23b: 79% yield; mp 128-130 °C; $^1$H NMR (400 MHz, MeOD-$d_4$): δ ppm 1.04 (t, J = 7.6 Hz, 3 H), 1.50-1.60 (m, 2 H), 1.85 (s, 6 H), 1.87-1.95 (m, 2 H), 3.84 (s, 3 H), 4.32 (t, J = 7.6 Hz, 2 H), 6.82 (d, J = 13.6 Hz, 1 H), 6.87 (d, J = 13.6 Hz, 1 H), 7.41 (t, J = 7.2 Hz, 1 H), 7.39-7.54 (m, 3 H), 7.64 (d, J = 7.6 Hz, 1 H), 7.68 (d, J = 7.6 Hz, 2 H), 7.77 (d, J = 8.4 Hz, 1 H), 7.95 (t, J = 7.6 Hz, 1 H), 8.23 (d, J = 8.4 Hz, 1 H), 8.38 (d, J = 7.2 Hz, 1 H), 8.91 (t, J = 13.6 Hz, 1 H); $^{13}$C NMR (100 MHz, MeOD-$d_4$): δ ppm 12.8, 14.0, 19.8, 27.3, 29.2, 31.0, 43.5, 65.4, 105.9, 106.8, 109.2, 111.5, 122.1, 122.5, 125.0, 126.0, 126.6, 128.6, 129.3, 129.5,
130.2, 131.1, 141.3, 141.4, 142.5, 149.2, 155.4, 175.9. HRMS m/z: calc. for C_{29}H_{31}N_{2}^+ 407.2482, obsd 407.2499.

1,3,3-Trimethyl-2-((1E,3E)-3-((3-(trimethylammonio)propyl)benzo[cd]indol-2(1H)-ylidene)prop-1-en-1-yl)-3H-indol-1-ium bromide, 23c: Yield 75%; mp 134 °C; \(^1\)H NMR (400 MHz, MeOD-\textit{d}_4): \(\delta\) ppm 1.77 (s, 6 H), 2.23-2.30 (m, 2 H), 3.10 (s, 9 H), 3.62-3.66 (m, 2 H), 4.33 (t, \(J = 6.4\) Hz, 2 H), 7.05 (d, \(J = 13.6\) Hz, 1 H), 7.12 (d, \(J = 12.8\) Hz, 1 H), 7.42 (t, \(J = 7.6\) Hz, 1 H), 7.54 (t, \(J = 7.6\) Hz, 1 H), 7.58 (d, \(J = 7.2\) Hz, 1 H), 7.64 (d, \(J = 8.0\) Hz, 1 H), 7.72 (t, \(J = 6.8\) Hz, 1 H), 7.74-7.79 (m, 2 H), 8.00 (t, \(J = 7.6\) Hz, 1 H), 8.28 (d, \(J = 8.4\) Hz, 1 H), 8.36 (d, \(J = 7.2\) Hz, 1 H), 8.73 (t, \(J = 13.6\) Hz, 1 H); \(^{13}\)C NMR (100 MHz, MeOD-\textit{d}_4): \(\delta\) ppm 22.5, 28.0, 32.9, 40.9, 50.3, 52.9, 63.0, 107.7, 108.4, 109.3, 113.1, 122.4, 123.0, 125.2, 126.9, 127.1, 129.2, 129.8, 130.0, 130.2, 130.6, 131.4, 141.4, 142.0, 142.9, 148.8, 154.2, 176.4; HRMS m/z: calc. for C_{31}H_{37}N_{3}^{2+} 225.6488, obsd 225.6170.

7.2.6 Pentamethine Cyanines

2-((1E,3Z)-3-chloro-5-((E)-5-methoxy-1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-5-methoxy-1,3,3-trimethyl-3H-indol-1-ium iodide, 24a: Yield 48%; mp 244-245 °C; \(^1\)H NMR (400 MHz, MeOD-\textit{d}_4): \(\delta\) ppm 1.73 (s, 12 H), 3.67 (s, 6 H), 3.85 (s, 6 H), 6.34 (d, \(J = 12.0\) Hz, 2 H), 6.99 (d, \(J = 8.0\) Hz, 2 H), 7.14 (s, 2 H), 7.29 (d, \(J = 8.0\) Hz, 2 H), 8.23 (d, \(J = 12\) Hz, 2 H); \(^{13}\)C NMR (100 MHz, DMSO-\textit{d}_6): \(\delta\) ppm 27.0, 31.9, 49.8, 56.3, 99.7, 109.3, 112.7, 114.0, 121.8, 136.5, 143.4, 146.1, 158.5, 173.7. HRMS m/z: calc. for C_{29}H_{34}ClN_{2}O_{2}^{+} 477.2303, obsd 477.2327.

2-((1E,3Z)-3-chloro-5-((E)-1-ethyl-5-methoxy-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1-ethyl-5-methoxy-3,3-dimethyl-3H-indol-1-ium iodide, 24b: Yield 72%; decom 223 °C; \(^1\)H NMR (400 MHz, MeOD-\textit{d}_4): \(\delta\) ppm 1.30 (t, \(J = 7.2\) Hz, 6 H), 1.71 (s, 12 H), 3.82 (s, 6
2-((1,3Z,5E)-3-chloro-5-(3-ethyl-1,1-dimethyl-1H-benzo[e]indol-2(3H)-ylidene)penta-1,3-dien-1-yl)-3-ethyl-1,1-dimethyl-1H-benzo[e]indol-3-ium iodide, **24c**: Yield 68%, mp 231-233 °C; $^1$H NMR (400 MHz DMSO-$d_6$) $\delta$: 1.39 (s, 6 H), 2.00 (s, 12 H), 4.37 (s, 4 H), 6.38 (d, $J$ = 16 Hz, 2 H), 7.55 (t, 2 H), 7.73 (t, 2 H), 7.84 (d, $J$ = 8.0 Hz, 2 H), 8.12 (m, 4 H), 8.27 (d, $J$ = 4.0 Hz, 2 H), 8.65 (d, $J$ = 16.0 Hz, 2 H); $^{13}$C NMR (100 MHz DMSO-$d_6$) $\delta$: 11.8, 25.8, 50.7, 98.5, 111.1, 121.6, 121.7, 124.6, 126.9, 127.3, 129.4, 130.0, 131.1, 133.4, 138.5, 146.1, 174.1; HRMS m/z: calc. for C$_{29}$H$_{34}$ClN$_2$O$_2^+$ 505.2616, obsd 505.2632.

$^2$-(1,3Z,5Z)-3-chloro-5-(3-methylphtho[2,1-d]thiazol-2(3H)-ylidene)penta-1,3-dien-1-yl)-3-methylphtho[2,1-d]thiazol-3-ium iodide, **24d**: Yield 82%, mp 243-245 °C; $^1$H NMR (400 MHz DMSO-$d_6$) $\delta$: 3.92 (s, 6 H), 6.24 (d, $J$ = 12.8 Hz, 2 H), 7.36 (t, $J$ = 7.2 Hz, 2 H), 7.57 (t, $J$ = 7.2 Hz, 2 H), 7.76 (d, $J$ = 7.6 Hz, 2 H), 7.91 (m, 6 H), 8.07 (d, $J$ = 9.2 Hz, 2 H); $^{13}$C NMR (100 MHz DMSO-$d_6$) $\delta$: 34.7, 97.9, 113.4, 119.4, 121.7, 123.2, 126.3, 128.9, 129.8, 130.5, 140.3, 144.2, 164.3.

1-ethyl-2-((1,3Z)-5-((E)-1-ethyl-3,3-dimethylindolin-2-ylidene)-3-formylpenta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium iodide, **25**: Yield 65%; $^1$H NMR (400 MHz MeOD-$d_4$) $\delta$: 1.52 (t, $J$ = 7.2 Hz, 6 H), 1.83 (s, 12 H), 4.40 (q, $J$ = 7.2 Hz, 4 H), 7.36 (m, 2 H), 7.42 (m, 2 H), 7.53 (d, $J$ = 7.2 Hz, 4 H), 8.32 (d, $J$ = 11.2 Hz, 2 H), 9.90 (s, 1 H).

### 7.2.7 Heptamethine Cyanines with an Open Chain

1,3,3-trimethyl-2-((1,3E,5E)-7-((E)-1,3,3-trimethylindolin-2-ylidene)hepta-1,3,5-trien-1-yl)-3H-indol-1-ium iodide, **26a**: Yield 53%; mp 159-161 °C; $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ ppm 1.63 (s, 12 H), 3.58 (s, 6 H), 6.33 (d, $J$ = 13.6 Hz, 2 H), 6.53 (t, $J$ = 12.4 Hz, 2 H), 7.22 (t, $J$ = 7.2 Hz, 2 H), 7.39 (m, 4 H), 7.57 (d, $J$ = 7.2 Hz, 1 H), 7.78 (t, $J$ = 12.4 Hz, 1 H), 7.84 (t, $J$ = 12.4 Hz, 2 H); $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ ppm 27.5, 31.6, 49.0, 104.3, 111.3,
122.7, 125.0, 128.8, 141.3, 143.3, 151.1, 172.1. HRMS m/z: calc. for C_{29}H_{33}N_{2}^{+} 409.2638, obsd 409.2655.

1-ethyl-2-((1E,3E,5E)-7-((E)-1-ethyl-3,3-dimethylindolin-2-ylidene)hepta-1,3,5-trien-1-yl)-3,3-dimethyl-3H-indol-1-iium iodide, 26b: Yield 90%; mp 142-144 °C; \(^{1}\text{H} \) NMR (400 MHz, DMSO-d\(_6\)) \(\delta\) ppm 1.27 (t, \(J = 7.2 \text{ Hz}, 6 \text{ H}\)), 1.63 (s, 12 H), 4.12, (q, \(J = 7.2 \text{ Hz}, 4 \text{ H}\)), 6.37 (d, \(J = 13.6 \text{ Hz}, 2 \text{ H}\)), 6.54 (t, \(J = 12.8 \text{ Hz}, 2 \text{ H}\)), 7.23 (t, \(J = 7.2 \text{ Hz}, 2 \text{ H}\)), 7.39 (m, 4 H), 7.58 (d, \(J = 7.2 \text{ Hz}, 2 \text{ H}\)), 7.77 (t, \(J = 12.8 \text{ Hz}, 1 \text{ H}\)), 7.88 (t, \(J = 12.8 \text{ Hz}, 2 \text{ H}\)). \(^{13}\text{C} \) NMR (100 MHz, DMSO-d\(_6\)) \(\delta\) ppm 12.6, 27.5, 49.1, 103.8, 111.2, 122.9, 125.0, 125.7, 128.9, 141.5, 142.1, 151.5, 171.2.

1-butyl-2-((1E,3E,5E)-7-((E)-1-butyl-3,3-dimethylindolin-2-ylidene)hepta-1,3,5-trien-1-yl)-3,3-dimethyl-3H-indol-1-iium iodide, 26c: Yield 69%; mp 174-176 °C; \(^{1}\text{H} \) NMR (400 MHz, MeOD-d\(_4\)): \(\delta\) ppm 1.03 (t, \(J = 7.2 \text{ Hz}, 6 \text{ H}\)), 1.50 (m, 4 H), 1.71 (s, 12 H), 1.79 (m, 4 H), 4.04 (t, \(J = 7.2 \text{ Hz}, 4 \text{ H}\)), 6.29 (d, \(J = 13.6 \text{ Hz}, 2 \text{ H}\)), 6.58 (t, \(J = 12.4 \text{ Hz}, 2 \text{ H}\)), 7.27 (m, 4 H), 7.41 (t, \(J = 7.6 \text{ Hz}, 2 \text{ H}\)), 7.49 (d, \(J = 7.6 \text{ Hz}, 4 \text{ H}\)), 7.63 (t, \(J = 12.4 \text{ Hz}, 1 \text{ H}\)), 7.95 (t, \(J = 12.8 \text{ Hz}, 2 \text{ H}\)). \(^{13}\text{C} \) NMR (100 MHz, MeOD-d\(_4\)): \(\delta\) ppm 12.7, 19.7, 26.6, 29.2, 43.3, 103.3, 110.4, 121.9, 124.6, 128.3, 141.0, 142.3, 151.6. HRMS m/z: calc. for C_{35}H_{45}N_{2}^{+} 493.3577, obsd 493.3567.

2-((1E,3E,5E)-7-((E)-3,3-dimethyl-1-(3-phenylpropyl)indolin-2-ylidene)hepta-1,3,5-trien-1-yl)-3,3-dimethyl-1-(3-phenylpropyl)-3H-indol-1-iium bromide, 26d: Yield 36%; mp 148-150 °C; \(^{1}\text{H} \) NMR (400 MHz, CDCl\(_3\)): \(\delta\) ppm 1.61 (s, 12 H), 2.00 (m, 4 H), 2.74 (t, \(J = 7.6 \text{ Hz}, 4 \text{ H}\)), 4.10 (t, \(J = 7.6 \text{ Hz}, 4 \text{ H}\)), 6.24 (d, \(J = 13.6 \text{ Hz}, 2 \text{ H}\)), 6.45 (t, \(J = 12.8 \text{ Hz}, 2 \text{ H}\)), 7.23 (m, 8 H), 7.31 (m, 6 H), 7.37 (t, \(J = 7.2 \text{ Hz}, 2 \text{ H}\)), 7.75 (t, \(J = 12.8 \text{ Hz}, 1 \text{ H}\)), 7.85 (t, \(J = 12.8 \text{ Hz}, 2 \text{ H}\) ). \(^{13}\text{C} \) NMR (100 MHz, DMSO-d\(_6\)): \(\delta\) ppm 27.6, 29.1, 32.5, 43.5, 49.1, 111.3, 122.8, 125.0,
126.5, 128.8, 128.8, 141.4, 141.5, 142.5, 171.6. HRMS m/z: calc. for C₄₅H₉₉N₂O₂⁺ 617.3890, obsd 617.3896.

3-ethyl-2-((1E,3E,5E,7E)-7-(3-ethyl-1,1-dimethyl-1,3-dihydro-2H-benzo[e]indol-2-ylidene)hepta-1,3,5-trien-1-yl)-1,1-dimethyl-1H-benzo[e]indol-3-i um iodide, 26e: Yield 64%; mp 191-193 °C; ¹H NMR (400 MHz, DMSO-d₆): δ ppm 1.33 (t, J = 7.2 Hz, 6 H), 1.91 (s, 12 H), 4.26 (s, 6 H), 6.45 (d, J = 13.2 Hz, 2 H), 6.59 (t, J = 11.6 Hz, 2 H), 7.50 (t, J = 7.2 Hz, 2 H), 7.65 (t, J = 7.6 Hz, 2 H), 7.73 (d, J = 8.8 Hz, 2 H), 7.82 (t, J = 13.2 Hz, 1 H), 8.02 (m, 4 H), 8.25 (d, J = 8.4 Hz, 2 H). ¹³C NMR (100 MHz, DMSO-d₆): δ ppm 13.0, 27.1, 50.9, 103.6, 111.8, 122.6, 125.1, 128.1, 130.3, 130.8, 131.7, 133.6, 139.8, 150.5, 172.4. HRMS m/z: calc. for C₃₉H₄₁N₂⁺ 537.3264, obsd 537.3255.

3-butyl-2-((1E,3E,5E,7E)-7-(3-butyl-1,1-dimethyl-1,3-dihydro-2H-benzo[e]indol-2-ylidene)hepta-1,3,5-trien-1-yl)-1,1-dimethyl-1H-benzo[e]indol-3-i um iodide, 26f: Yield 61%; mp 179-181 °C; ¹H NMR (400 MHz, DMSO-d₆): δ ppm 0.955 (t, J = 7.2 Hz, 6 H), 1.45 (m, 4 H), 1.73 (m, 4 H), 1.91 (s, 12 H), 4.21 (t, J = 7.2 Hz, 4 H), 6.44 (d, J = 13.6 Hz, 2 H), 6.60 (t, J = 12.8 Hz, 2 H), 7.50 (t, J = 7.6 Hz, 2 H), 7.65 (t, J = 7.6 Hz, 2 H), 7.72 (d, J = 8.8 Hz, 2 H), 7.81 (t, J = 12.8 Hz, 1 H), 8.04 (m, 6 H), 8.25 (d, J = 8.4 Hz, 2 H). ¹³C NMR (100 MHz, DMSO-d₆): δ 14.3, 19.9, 27.2, 29.9, 43.9, 50.8, 103.8, 112.0, 122.6, 125.1, 125.9, 128.0, 128.1, 130.3, 130.7, 131.6, 133.6, 140.3, 150.5, 172.8.

2-((1E,3E,5E,7E)-7-(1,1-dimethyl-3-(3-phenylpropyl)-1,3-dihydro-2H-benzo[e]indol-2-ylidene)hepta-1,3,5-trien-1-yl)-1,1-dimethyl-3-(3-phenylpropyl)-1H-benzo[e]indol-3-i um bromide, 26g: Yield 85%; mp 160-162 °C; ¹H NMR (400 MHz, DMSO-d₆): δ 1.88 (s, 12 H), 2.04 (m, 4 H), 2.75 (t, J = 7.6Hz, 4 H), 4.21 (t, J = 7.2 Hz, 4 H), 6.23 (d, J = 13.6 Hz, 2 H),
6.47 (t, \( J = 12.8 \) Hz, 2 H), 7.27 (m, 10 H), 7.49 (t, \( J = 7.6 \) Hz, 2 H), 7.63 (m, 5 H), 7.32 (m, 5H), 7.95 (t, \( J = 13.2 \) Hz, 2 H), 8.03 (d, \( J = 9.2 \) Hz, 4 H), 8.22 (d, \( J = 8.8 \) Hz, 2 H). \(^{13}\text{C} \) NMR (100 MHz, DMSO-\( d_6 \)) \( \delta \) 27.1, 29.4, 32.4, 43.6, 50.8, 103.7, 111.8, 122.6, 125.2, 126.5, 128.0, 128.1, 128.9, 128.8, 130.3, 130.7, 131.6, 133.6, 140.1, 141.4, 150.4, 172.8.

\( 2-((1E,3E,5E)-7-((E)-3,3\text{-dimethyl-1-(3-(trimethylammonio)propyl} \text{indolin-2-ylidene}) \text{hepta-1,3,5-trien-1-yl})-3,3\text{-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-i um}) \) tribromide, \( \text{26h: Yield 46%}; \) mp 191-193 °C \(^1\text{H} \) NMR (400 MHz, MeOD-\( d_4 \)): \( \delta \) ppm 1.69 (s 12 H), 2.32 (m, 4 H), 3.25 (s, 18 H), 3.85 (t, \( J = 8.0 \) Hz, 4 H), 4.32 (t, \( J = 7.6 \) Hz, 4 H), 6.62 (d, \( J = 13.2 \) Hz, 2 H), 6.77 (t, \( J = 12.0 \) Hz, 2 H), 7.23 (t, \( J = 7.2 \) Hz, 2 H), 7.40 (t, \( J = 7.6 \) Hz, 2 H), 7.49 (t, \( J = 8.0 \) Hz, 4 H), 7.61 (t, \( J = 12.0 \) Hz, 1 H), 7.91 (t, \( J = 11.6 \) Hz, 2 H). \(^{13}\text{C} \) NMR (100 MHz, MeOD-\( d_4 \)) \( \delta \) ppm 20.9, 26.7, 40.3, 48.9, 52.4, 52.5, 52.5, 63.1, 110.5, 122.0, 124.7, 126.6, 128.4, 141.0, 141.9, 152.2, 171.6. HRMS m/z: calc. for \( \text{C}_{39}\text{H}_{57}\text{N}_{4}^{3+} \) 193.8189, obsd 193.7919.

\( 5\text{-methoxy-2-}((1E,3E,5E)-7-((E)-5\text{-methoxy-3,3\text{-dimethyl-1-(3-(trimethylammonio)propyl} \text{indolin-2-ylidene}) \text{hepta-1,3,5-trien-1-yl})-3,3\text{-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-i um}) \) tribromide, \( \text{26i: Yield 15%}; \) mp 183-185 °C; \(^1\text{H} \) NMR (400 MHz, MeOD-\( d_4 \)): \( \delta \) ppm 1.65 (s 12 H), 2.30 (m, 4 H), 3.25 (s, 18 H), 3.78 (s, 6 H), 3.84 (t, \( J = 8.4 \) Hz, 4 H), 4.32 (t, \( J = 8.4 \) Hz, 4 H), 6.66 (t, \( J = 12.0 \) Hz, 2 H), 6.93 (d, \( J = 8.4 \) Hz, 2 H), 7.08 (s, 2 H), 7.43 (m, 3 H), 7.77 (d, \( J = 10.8 \) Hz, 2 H). \(^{13}\text{C} \) NMR (100 MHz, MeOD-\( d_4 \)) \( \delta \) ppm 20.9, 26.6, 40.5, 49.0, 52.5, 54.9, 63.1, 108.7, 111.2, 113.2, 125.9, 135.4, 142.7, 150.6, 158.2, 170.6. HRMS m/z: calc. for \( \text{C}_{41}\text{H}_{61}\text{N}_{4}\text{O}_{2}^{3+} \) 213.8260, obsd 213.7877.
(2-((1E,3E,5E,7E)-7-(1,1-dimethyl-3-(3-trimethylammonio)propyl)-1,3-dihydro-2H-benzo[e]indol-2-ylidene)hepta-1,3,5-trien-1-yl)-1,1-dimethyl-3-(3-(trimethylammonio)propyl)-1H-benzo[e]indol-3-ium tribromide, 26j: Yield 62%; mp 219-221 °C; $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 1.92 (s, 12 H), 2.20 (m, 4 H), 3.16 (s, 18 H), 3.76 (t, $J$ = Hz, 4 H), 4.13 (t, $J$ = Hz, 4 H), 6.74 (m, 4 H), 7.50 (t, $J$ = 7.2 Hz, 2 H), 7.65 (t, $J$ = 7.2 Hz, 2 H), 7.87 (m, 3 H), 8.07 (m, 6 H), 8.26 (d, $J$ = 8.4, 2 H). $^{13}$C NMR (100 MHz, DMSO-d$_6$) $\delta$ 21.61, 27.27, 50.89, 53.02, 62.81, 104.43, 112.03, 122.67, 122.67, 125.22, 126.66, 128.06, 128.17, 130.35, 130.77, 131.74, 133.52, 140.05, 172.74. HRMS m/z: calc. for C$_{41}$H$_{61}$N$_4$3+ 227.1627, obsd 227.1599.

2-((1E,3E,5E,7E)-7-(3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)propyl)indolin-2-ylidene)hepta-1,3,5-trien-1-yl)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3H-indol-1-ium-5- sulfonate bromide, 26k: Yield 53%; mp >260 °C; $^1$H NMR (400 MHz, DMSO-d$_6$): $\delta$ 1.68 (s, 12 H), 2.16 (m, 4 H), 3.10 (s, 18 H), 3.51 (t, $J$ = 7.6 Hz, 4 H), 4.14 (t, $J$ = 7.6 Hz, 4 H), 6.44 (d, $J$ = 13.6 Hz, 2 H), 6.58 (t, $J$ = 13.2 Hz, 2 H), 7.36 (d, $J$ = 8.0 Hz, 2 H), 7.68 (d, $J$ = 8.4 Hz, 2 H), 7.79 (s, 2 H), 8.91 (m, 3 H). $^{13}$C NMR (100 MHz, DMSO-d$_6$): $\delta$ 21.30, 27.27, 50.89, 53.02, 62.81, 104.43, 112.03, 122.67, 122.67, 125.22, 126.66, 128.06, 128.17, 130.35, 130.77, 131.74, 133.52, 140.05, 172.74. HRMS m/z: calc. for C$_{39}$H$_{55}$Na$_3$O$_6$S$_2$3+ 739.3558, obsd 739.3571.

5-methoxy-2-((1E,3E,5E)-7-((E)-5-methoxy-1,3,3-trimethylindolin-2-ylidene)hepta-1,3,5-trien-1-yl)-1,3,3-trimethyl-3H-indol-1-ium iodide, 26l: Yield 53%; mp 189-190 °C; $^1$H NMR (400 MHz, DMSO-d$_6$): $\delta$ ppm 1.62 (s, 12 H), 3.55 (s, 6 H), 3.80 (s, 6 H), 6.23 (d, $J$ = 13.6 Hz, 2 H), 6.45 (t, $J$ = 12.8 Hz, 2 H), 6.95 (dd, $J$ = 12.8 Hz, 2 H), 7.26 (m, 4 H), 7.67 (t, $J$ = 12.8 Hz, 1 H), 7.78 (t, $J$ = 13.2 Hz, 2 H). $^{13}$C NMR (100 MHz, DMSO-d$_6$): $\delta$ ppm 24.5, 27.5, 31.6, 49.2,
56.2, 109.5, 111.9, 113.7, 136.9, 143.0, 157.9, 171.2. HRMS m/z: calc. for C₃₁H₇₇N₂O₂⁺ 469.2850, obsd 469.2848.

1-ethyl-2-\(((E,3E,5E)\)-7-{(E)-1-ethyl-5-methoxy-3,3-dimethylindolin-2-ylidene}hepta-1,3,5-trien-1-yl\)-5-methoxy-3,3-dimethyl-3H-indol-1-iium iodide, \textbf{26m}: Yield 35%; mp 142-144 °C; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 1.25 (t, \(J = 7.2\) Hz, 6 H) 1.62 (s, 12 H), 3.80 (s, 6 H), 4.08 (q, \(J = 7.2\) Hz, 4 H), 6.27 (d, \(J = 13.6\) Hz, 2 H), 6.46 (t, \(J = 12.8\) Hz, 2 H), 6.95 (dd, \(J = 8.8\) Hz, 2 H), 7.27 (m, 4 H), 7.67 (t, \(J = 12.8\) Hz, 1 H), 7.79 (t, \(J = 13.2\) Hz, 2 H). \(^13\)C NMR (100 MHz, DMSO-\(d_6\)) \(\delta\) 12.7, 27.5, 39.1, 49.3, 56.2, 103.2, 109.7, 111.8, 124.9, 135.6, 143.3, 150.2, 157.9, 170.3.

1-butyl-2-\(((E,3E,5E)\)-7-{(E)-1-butyl-5-methoxy-3,3-dimethylindolin-2-ylidene}hepta-1,3,5-trien-1-yl\)-5-methoxy-3,3-dimethyl-3H-indol-1-iium iodide, \textbf{26n}: Yield 86%; mp 106-108 °C; \(^1\)H NMR (400 MHz, MeOD-\(d_4\)) \(\delta\) 1.02 (t, \(J = 7.2\) Hz, 6 H), 1.48 (m, 4 H), 1.69 (s, 12 H), 1.77 (m, 4 H), 3.85 (s, 3 H), 4.05 (m, 4 H), 6.20 (d, \(J = 13.2\) Hz, 2 H), 6.60 (t, \(J = 12.0\) Hz, 2 H), 6.96 (d, \(J = 8.0\) Hz, 2 H), 7.10 (s, 2 H), 7.18 (d, \(J = 8.4\) Hz, 2 H), 7.54 (t, \(J = 11.2\) Hz, 1 H), 7.86 (t, \(J = 12.8\) Hz, 2 H). \(^13\)C NMR (100 MHz, MeOD-\(d_4\)) \(\delta\) 12.8, 19.7, 26.6, 29.2, 43.5, 49.0, 55.0, 102.8, 108.6, 111.0, 113.2, 124.7, 135.8, 142.7, 150.2, 158.2, 170.8.

5-methoxy-2-\(((E,3E,5E)\)-7-{(E)-5-methoxy-3,3-dimethyl-1-(3-phenylpropyl)indolin-2-ylidene}hepta-1,3,5-trien-1-yl\)-3,3-dimethyl-1-(3-phenylpropyl)-3H-indol-1-iium bromide, \textbf{26o}: Yield 41%; mp 131-133 °C; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 1.61 (s, 12 H), 1.98 (m, 4 H), 2.72 (t, \(J = 7.2\) Hz, 4 H), 3.79 (s, 6 H), 4.06 (t, \(J = 7.2\) Hz, 4 H), 6.13 (d, \(J = 13.6\) Hz, 2 H), 6.37 (t, \(J = 12.8\) Hz, 2 H), 6.93 (dd, \(J = 8.8\) Hz, 2 H), 7.23 (m, 9 H), 7.32 (m, 5H), 7.64 (t, \(J = 8.8\) Hz, 1 H), 7.77 (t, \(J = 13.2\) Hz, 2 H). \(^13\)C NMR (100 MHz, DMSO-\(d_6\)) \(\delta\) 27.6, 29.1, 32.5,
1-butyl-2-((1E,3E,5E)-7-((E)-1-butyl-5-chloro-3,3-dimethylindolin-2-ylidene)hepta-1,3,5-trien-1-yl)-5-chloro-3,3-dimethyl-3H-indol-1-iium iodide, 26p: Yield 80%; mp 169-171 °C; \(^1\)H NMR (400 MHz, MeOD-\(d_4\)) \(\delta\) 1.02 (t, \(J = 7.2\) Hz, 6 H), 1.48 (m, 4 H), 1.71 (s, 12 H), 1.77 (m, 4 H), 4.07 (t, \(J = 7.2\) Hz, 4 H), 6.30 (d, \(J = 13.6\) Hz, 2 H), 6.60 (t, \(J = 12.4\) Hz, 2 H), 7.26 (d, \(J = 7.6\) Hz, 2 H), 7.41 (dd, \(J = 8.4\) Hz, 2 H), 7.55 (s, 2 H), 7.65 (t, \(J = 12.4\) Hz, 1 H), 7.96 (t, \(J = 7.6\) Hz, 2 H). \(^{13}\)C NMR (100 MHz, MeOD-\(d_4\)) \(\delta\) 12.7, 19.7, 26.4, 29.1, 43.6, 49.3, 103.7, 111.9, 122.5, 126.2, 128.3, 130.0, 141.1, 142.9, 151.8, 170.7.

5-chloro-2-((1E,3E,5E)-7-((E)-5-chloro-3,3-dimethyl-1-(3-phenylpropyl)indolin-2-ylidene)hepta-1,3,5-trien-1-yl)-3,3-dimethyl-1-(3-phenylpropyl)-3H-indol-1-iium bromide, 26q: Yield 78%; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 1.63 (s, 12 H), 1.98 (m, 4 H), 2.72 (t, \(J = 7.6\) Hz, 4 H), 4.09 (t, \(J = 7.2\) Hz, 4 H), 6.23 (d, \(J = 13.6\) Hz, 2 H), 6.47 (t, \(J = 12.8\) Hz, 2 H), 7.29 (m, 13 H), 7.44 (dd, \(J = 8.4\) Hz, 2 H), 7.74 (t, \(J = 12.8\) Hz, 2 H), 7.86 (t, \(J = 12.8\) Hz, 2 H). \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)) \(\delta\) 27.4, 29.0, 32.4, 43.8, 49.3, 104.5, 112.7, 123.3, 126.5, 128.7, 128.8, 129.3, 141.4, 141.5, 143.5, 151.7, 171.5.

1-ethyl-2-((1E,3E,5E)-7-((E)-1-ethyl-5-fluoro-3,3-dimethylindolin-2-ylidene)hepta-1,3,5-trien-1-yl)-5-fluoro-3,3-dimethyl-3H-indol-1-iium iodide, 26r: Yield 56%; mp 165-167 °C; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 1.26 (t, \(J = 7.2\) Hz, 6 H), 1.64 (s, 12 H), 4.10 (t, \(J = 7.2\) Hz, 4 H), 6.35 (d, \(J = 14.0\) Hz, 2 H), 6.53 (t, \(J = 12.8\) Hz, 2 H), 7.24 (m, 2 H), 7.39 (m, 2 H), 7.58 (m, 2 H), 7.75 (t, \(J = 12.8\) Hz, 1 H), 7.86 (t, \(J = 12.8\) Hz, 2 H). \(^{19}\)F NMR (115 MHz, DMSO-\(d_6\)) \(\delta\) -119.8.
1-butyl-2-((1E,3E,5E)-7-((E)-1-butyl-5-fluoro-3,3-dimethylindolin-2-ylidene)hepta-1,3,5-
trien-1-yl)-5-fluoro-3,3-dimethyl-3H-indol-1-ium iodide, 26s: Yield 81%; mp 225-227 °C; 1H
NMR (400 MHz, DMSO-d$_6$) δ 0.93 (t, J = 7.2 Hz, 6 H), 1.38 (m, 4 H), 1.64 (s, 12 H), 1.67 (m, 4 H), 4.06 (t, J = 7.2 Hz, 4 H), 6.35 (d, J = 14.0 Hz, 2 H), 6.53 (t, J = 12.4 Hz, 2 H), 7.23 (m, 2 H), 7.39 (m, 2 H), 7.58 (m, 2 H), 7.75 (t, J = 12.4 Hz, 1 H), 7.86 (t, J = 12.8 Hz, 2 H). 19F
NMR (115 MHz, DMSO-d$_6$) δ -120.0.

5-fluoro-2-((1E,3E,5E)-7-((E)-5-fluoro-3,3-dimethyl-1-(3-phenylpropyl)indolin-2-
ylidene)hepta-1,3,5-trien-1-yl)-3,3-dimethyl-1-(3-phenylpropyl)-3H-indol-1-ium bromide,
26t: Yield 71%; mp 135-137 °C; 1H NMR (400 MHz, DMSO-d$_6$) δ 1.62 (s, 12 H), 1.98 (m, 4 H), 2.73 (t, J = 7.6 Hz, 4 H), 4.08 (t, J = 7.6 Hz, 4 H), 6.20 (d, J = 13.6 Hz, 2 H), 6.44 (t, J = 12.4 Hz, 2 H), 7.23 (m, 8 H), 7.34 (m, 6 H), 7.57 (dd, J = 12.4 Hz, 2 H), 7.71 (t, J = 12.4 Hz, 1 H), 7.83 (t, J = 12.8 Hz, 2 H). 19F NMR (115 MHz, DMSO-d$_6$) δ -120.0.

7.2.8 Heptamethine Cyanines Containing a Cyclic Ring in the Methine Chain

2-((E)-2-((E)-2-chloro-3-((E)-1,3,3-trimethylindolin-2-ylidene)ethylidene)cyclohex-1-en-
1-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide, 27a: was synthesized as previously
described.$^{150}$ 1H NMR (400 MHz, DMSO-d$_6$) δ 1.67 (s, 12 H), 1.85 (m, 2 H), 2.72 (t, J = 6.0 Hz, 4 H), 3.68 (s, 6 H), 6.30 (t, J = 14.0 Hz, 2 H), 7.28 (m, 2 H), 7.44 (m, 4 H), 7.62 (d, J = 7.2 Hz, 2 H), 8.25 (d, J = 14.4 Hz, 2 H).

3-((E)-2-((E)-2-chloro-3-((E)-2-(1,1-dimethyl-3-(3-sulfonatopropyl)-1,3-dihydro-2H-
benzo[e]indol-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1,1-dimethyl-1H-
benzo[e]indol-3-ium-3-yl)propane-1-sulfonate, 27b: Yield 71%; 1H NMR (400 MHz, DMSO-
d$_6$) δ 1.25 (t, J = 7.2 Hz, 6 H), 1.64 (s, 12 H), 4.10, (q, J = 7.2 Hz, 4 H), 6.37 (d, J = 13.6 Hz, 2
H), 6.56 (t, J = 12.8 Hz, 2 H), 7.39 (d, J = 8.4 Hz, 2 H), 7.45 (dd, J = 6.8 Hz, 2 H), 7.76 (m, 3 H), 7.89 (t, J = 13.2 Hz, 2 H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 12.6, 27.3, 49.3, 104.2, 112.6, 123.4, 126.3, 128.8, 129.3, 141.1, 143.6, 151.8, 171.0.

2-((E)-2-((E)-2-chloro-3-((E)-5-methoxy-1,3,3-trimethylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-5-methoxy-1,3,3-trimethyl-3H-indol-1-ium iodide, 27e: Yield 71%; mp 238-240 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 1.66 (s, 12 H), 1.84 (m, 2 H), 2.68 (t, J = 7.6 Hz, 4 H), 3.65 (s, 6 H), 3.82 (s, 6 H), 6.21 (d, J = 14.4 Hz, 2 H), 6.99 (dd, J = 8.8 Hz, 2 H), 7.30 (s, 2 H), 7.36 (d, J = 8.4 Hz, 2 H), 8.17 (d, J = 14.0 Hz, 2 H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 20.9, 26.3, 27.7, 32.0, 49.4, 56.3, 101.8, 109.5, 112.5, 125.6, 136.8, 141.9, 143.2, 147.0, 158.3, 172.0.

5-bromo-2-((E)-2-((E)-3-((E)-5-bromo-1,3,3-trimethylindolin-2-ylidene)ethylidene)-2-chlorocyclohex-1-en-1-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide, 27f: Yield 76%; mp 247-249 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 1.68 (s, 12 H), 1.86 (t, J = 6.0 Hz, 2 H), 2.72 (m, 4 H), 3.67 (s, 6 H), 6.29 (d, J = 14.4 Hz, 2 H), 7.39 (d, J = 8.4 Hz, 2 H), 7.62 (dd, J = 8.4 Hz, 2 H), 7.89 (s, 2 H), 8.24 (d, J = 14.0 Hz, 2 H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 20.8, 26.3, 27.5, 32.1, 49.5, 102.7, 113.7, 117.9, 126.1, 127.2, 131.7, 142.7, 143.2, 143.7, 148.5, 172.7.

2-((E)-2-((E)-2-chloro-3-((E)-2-(1,1-dimethyl-3-(3-phenylpropyl)-1,3-dihydro-2H-benzo[e]indol-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1,1-dimethyl-3-(3-phenylpropyl)-1H-benzo[e]indol-3-ium bromide, 27g: Yield 72%; mp 211-213 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 1.88 (m, 2 H), 1.94 (s, 12 H), 2.09 (m, 4 H), 2.56 (m, 4 H), 2.80 (t, J = 7.6 Hz, 4 H), 4.33 (t, J = 7.6 Hz, 4 H), 6.15 (d, J = 13.6 Hz, 2 H), 7.29 (m, 10 H), 7.53 (t, J
= 7.6 Hz, 2 H), 7.66 (t, J = 7.6 Hz, 2 H), 7.76 (d, J = 8.4 Hz, 2 H), 8.09 (t, J = 8.8 Hz, 4 H),
8.31 (t, J = 13.2 Hz, 4 H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 26.3, 27.4, 29.2, 32.4, 43., 51.2,
101.4, 112.1, 122.7, 125.5, 126.5, 127.9, 128.3, 128.8, 128.9, 128.9, 130.4, 130.9, 131.9, 134.1, 140.1,
141.3, 142.3, 147.8, 173.7.
5-chloro-2-((E)-2-((E)-2-chloro-3-((E)-5-chloro-3,3-dimethyl-1-(3-phenylpropyl)indolin-2-ylidene)ethyldiene)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-1-(3-phenylpropyl)-3H-indol-1-
im bromide, 27h: Yield 84%; mp 206-208 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 1.65 (s, 12 H), 1.82 (m, 2 H), 2.02 (m, 4 H), 2.75 (t, J = 7.2 Hz, 4 H), 4.19 (t, J = 6.8 Hz, 4 H), 6.10 (d, $J$
= 14.4 Hz, 2 H), 7.26 (m, 10 H), 7.48 (m, 4 H), 7.80 (s, 2 H), 8.20 (d, $J$ = 14.0 Hz, 2 H) $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 26.2, 27.7, 28.8, 32.4, 49.6, 102.1, 113.3, 123.5, 126.5, 127.2, 128.7, 128.9, 129.9, 141.2, 141.4, 143.5, 143.5, 148.8, 172.4.
2-((E)-2-((E)-2-chloro-3-((E)-1-ethyl-5-fluoro-3,3-dimethylindolin-2-
ylidene)ethyldiene)cyclohex-1-en-1-yl)vinyl)-1-ethyl-5-fluoro-3,3-dimethyl-3H-indol-1-
ium iodide, 27i: Yield 71%; mp >260 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 1.29 (t, $J$ = 7.2 Hz, 6 H), 1.67 (s, 12 H), 1.85 (m, 4 H), 2.71 (t, $J$ = 6.0 Hz, 4 H), 4.25 (q, $J$ = 7.2 Hz, 4 H), 6.29 (d, $J$
= 14.0 Hz, 2 H), 7.30 (t, $J$ = 8.8 Hz, 2 H), 7.48 (d, $J$ = 8.8 Hz, 2 H), 7.64 (d, $J$ = 8.4 Hz, 2 H)
8.24 (d, $J$ = 14.4 Hz, 2 H). $^{19}$F NMR (115 MHz, DMSO-$d_6$) $\delta$ -119.21.
2-((E)-2-((E)-2-chloro-3-((E)-5-fluoro-3,3-dimethyl-1-(3-phenylpropyl)indolin-2-
ylidene)ethyldiene)cyclohex-1-en-1-yl)vinyl)-5-fluoro-3,3-dimethyl-1-(3-phenylpropyl)-3H-
indol-1-i um bromide, 27j: Yield 66%; mp 143-145 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 1.65 (s, 12 H), 1.84 (m, 4 H), 2.03 (m, 4 H), 2.75 (t, $J$ = 7.2 Hz, 4 H), 4.19 (t, $J$ = 7.2 Hz, 4 H), 6.07
(d, J = 14.4 Hz, 2 H), 7.28 (m, 12 H), 7.46 (m, 2 H), 7.63 (m, 2 H), 8.19 (d, J = 14.0 Hz, 2 H).

$^1$H NMR (115 MHz, DMSO-$d_6$) $\delta$ -119.17.

2-((E)-2-((E)-2-chloro-3-(2-((E)-5-methoxy-3,3-dimethyl-1-(3-phenylpropyl)indolin-2-ylidene)ethyldiene)cyclohex-1-en-1-yl)vinyl)-5-methoxy-3,3-dimethyl-1-(3-phenylpropyl)-3H-indol-1-ium bromide, 27l: Yield 78%; mp 135-137 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$

1.64 (s, 12 H), 1.81 (t, J = 7.6 Hz, 2 H), 2.02 (m, 4 H), 2.74 (t, J = 7.6 Hz, 4 H), 3.37 (m, 4 H), 3.81 (s, 6 H), 4.16 (t, J = 7.6 Hz, 4 H), 6.00 (d, J = 14.4 Hz, 2 H), 6.98 (dd, J = 8.4 Hz, 2 H), 7.25 (m, 6 H), 7.33 (m, 8 H), 8.14 (d, J = 14.4 Hz, 2 H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$

20.7, 26.2, 27.8, 28.9, 32.4, 43.8, 49.6, 53.3, 101.3, 109.6, 112.6, 114.1, 125.6, 126.5, 128.8, 128.9, 135.9, 141.3, 142.1, 143.3, 147.3, 158.3, 171.5.

2-((E)-2-((E)-2-chloro-3-(2-((E)-1,3,3-trimethylindolin-2-ylidene)ethyldiene)cyclopent-1-en-1-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide, 28a: Yield 53%; mp >260 °C; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$

1.73 (s, 12 H), 3.10 (m, 4 H), 3.77 (s, 6 H), 6.13 (d, J = 14.0 Hz, 2 H), 7.16 (d, J = 8.0 Hz, 2 H), 7.24 (t, J = 8.0 Hz, 2 H), 7.40 (m, 4 H), 7.83 (d, J = 14.0 Hz, 2 H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$

27.1, 28.0, 32.6, 49.0, 102.9, 110.6, 122.0, 125.1, 128.8, 136.8, 138.3, 140.9 142.9, 151.8, 171.4. HRMS m/z: calc for C$_{31}$H$_{34}$N$_2$Cl$^+$ 469.2405, obsd 469.2387.

2-((E)-2-((E)-2-chloro-3-(2-((E)-1,3,3-trimethylindolin-2-ylidene)ethyldiene)cyclohept-1-en-1-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide, 28b: Yield 79%; mp 139-141 °C; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$

1.74 (s, 12 H), 1.91 (m, 4 H), 2.83 (m, 4 H), 3.79 (s, 6 H), 6.31 (d, J = 14.4 Hz, 2 H), 7.19 (d, J = 8.00 Hz, 2 H), 7.26 (t, J = 7.2 Hz, 2 H), 7.38 (d, J = 7.2 Hz, 2 H), 7.44 (t, J = 7.2 Hz, 2 H), 8.37 (d, J = 14.0 Hz, 2 H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$

23.9, 26.9,
28.0, 32.8, 49.2, 101.4, 110.7, 122.0, 125.2, 128.8, 131.2, 142.8, 147.2, 154.7, 173.1. HRMS m/z: calc for C_{33}H_{38}N_{2}Cl^+ 497.2718, obsd 497.2698.

2-((E)-2-((E)-2-bromo-3-((E)-1,3,3-trimethylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide, **29a**: Yield 74%; mp >260 °C; \(^1^H\) NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 1.69 (s, 12 H), 1.85 (m, 2 H), 3.91 (m, 4 H), 3.69 (s, 6 H), 6.23 (d, \(J = 14.4\) Hz, 2 H), 7.29 (m, 2 H), 7.44 (m, 4 H), 7.63 (d, \(J = 7.2\) Hz, 2 H), 8.26 (d, \(J = 14.0, 2\) H).

\(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)) \(\delta\) 27.5, 31.9, 49.3, 102.6, 111.9, 122.8, 125.6, 128.4, 129.0, 141.4, 143.3, 144.9, 146.7, 173.1. HRMS m/z: calc for C_{32}H_{36}N_{2}Br^+ 527.2056, obsd 527.2040.

5-bromo-2-((E)-2-((E)-2-bromo-3-((E)-5-bromo-1-butyl-3,3-dimethylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1-butyl-3,3-dimethyl-3H-indol-1-ium iodide, **29b**: Yield 65%; mp 195-197 °C; \(^1^H\) NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.02 (t, \(J = 7.2\) Hz, 6 H), 1.51 (m, 4 H), 1.60 (s, 12 H), 1.82 (m, 4 H), 2.00 (m, 2 H), 2.80 (t, \(J = 6.0\) Hz, 4 H), 4.25 (t, \(J = 7.2\) Hz, 2 H), 7.08 (d, \(J = 8.4\) Hz, 2 H), 7.48 (s, 2 H), 7.53 (d, \(J = 8.4\) Hz, 2 H), 8.33 (d, \(J = 14.0, 2\) H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 13.9, 20.3, 27.9, 28.0, 29.5, 38.1, 45.2, 49.3, 59.5, 102.5, 112.4, 118.3, 125.6, 128.5, 130.3, 134.4, 141.4, 143.0, 144.1, 147.1, 153.9, 171.6.

1,3,3-trimethyl-2-((E)-2-((E)-2-methyl-3-((E)-1,3,3-trimethylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3H-indol-1-ium iodide, **30a**: Yield 71%; mp 217-219 °C; \(^1^H\) NMR (400 MHz, CDCl\(_3\)) \(\delta\) ppm 1.73 (s, 12 H), 1.92 (t, \(J = 5.6\) Hz, 2 H), 2.43 (s, 3 H), 2.62 (t, \(J = 5.6\) Hz, 4 H), 3.72 (s, 6 H), 6.16 (d, \(J = 13.6\) Hz, 2 H), 7.16 (d, \(J = 8.0\) Hz, 2 H), 7.23 (d, \(J = 7.6\) Hz, 2 H), 7.38 (m, 4 H), 8.05 (d, \(J = 13.2\) Hz, 2 H). \(^{13}\)C NMR (100 MHz,
CDCl₃) δ ppm 15.1, 20.9, 25.7, 28.3, 32.2, 100.6, 110.3, 122.1, 124.8, 128.7, 132.1, 140.5, 142.4, 156.0, 171.5. HRMS m/z: calc. for C₃₃H₃₉N₂⁺ 463.3108, obsd 463.3091.

5-methoxy-2-((E)-2-((E)-3-((E)-5-methoxy-1,3,3-trimethylindolin-2-ylidene)ethylidene)2-methylcyclohex-1-en-1-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide, 30b: Yield 62%; mp 122-124 °C; ¹H NMR (400 MHz, CDCl₃) δ 1.72 (s, 12 H), 1.91 (m, 2 H), 2.40 (s, 3 H), 2.58 (t, J = 6.4 Hz, 4 H), 3.67 (s, 6 H), 3.88 (s, 6 H), 6.06 (d, J = 13.6 Hz, 2 H), 6.91 (dd, J = 8.4 Hz, 2 H), 6.95 (s, 2 H), 7.09 (d, J = 8.4 Hz, 2 H), 7.99 (d, J = 13.6 Hz, 2 H). ¹³C NMR (100 MHz, CDCl₃) δ 15.0, 21.0, 25.6, 28.3, 32.3, 48.9, 56.1, 100.1, 109.3, 110.9, 113.1, 131.2, 136.6, 141.2, 142.2, 157.9, 170.5.

5-bromo-2-((E)-2-((E)-3-((E)-5-bromo-1,3,3-trimethylindolin-2-ylidene)ethylidene)2-methylcyclohex-1-en-1-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide, 30c: Yield 73%; mp 233-235 °C; ¹H NMR (400 MHz, CDCl₃) δ 1.72 (s, 12 H), 1.91 (t, J = 6.0 Hz, 2 H), 2.42 (s, 3 H), 2.62 (t, J = 6.0 Hz, 4 H), 3.72 (s, 6 H), 6.17 (d, J = 13.6 Hz, 2 H), 7.05 (d, J = 8.4 Hz, 2 H), 7.45 (s, 2 H), 7.51 (d, J = 8.8 Hz, 2 H), 8.02 (d, J = 13.6 Hz, 2 H). ¹³C NMR (100 MHz, CDCl₃) δ 15.2, 20.8, 25.7, 28.3, 32.6, 48.8, 101.1, 111.8, 117.7, 125.4, 131.7, 132.9, 142.1, 142.3, 156.4, 170.8.

5-chloro-2-((E)-2-((E)-3-((E)-5-chloro-1,3,3-trimethylindolin-2-ylidene)ethylidene)2-methylcyclohex-1-en-1-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide, 30d: Yield 68%; mp 228-230 °C; ¹H NMR (400 MHz, CDCl₃) δ 1.72 (s, 12 H), 1.91 (m, 2 H), 2.42 (s, 3 H), 2.62 (t, J = 6.0 Hz, 4 H), 3.72 (s, 6 H), 6.17 (d, J = 13.6 Hz, 2 H), 7.09 (d, J = 8.4 Hz, 2 H), 7.31 (s, 2 H), 7.36 (dd, J = 8.8 Hz, 2 H), 8.03 (d, J = 14.0 Hz, 2 H). ¹³C NMR (100 MHz, CDCl₃) δ 15.2,
2.8, 25.7, 28.2, 32.6, 48.8, 101.2, 111.3, 122.6, 128.8, 130.3, 132.9, 141.7, 142.0, 142.3, 156.2, 170.9.

2-((E)-2-((E)-3-((E)-2-(1,1-dimethyl-3-(3-phenylpropyl)-1,3-dihydro-2H-benzo[e]indol-2-ylidene)ethylidene)-2-methylcyclohex-1-en-1-yl)vinyl)-1,1-dimethyl-3-(3-phenylpropyl)-1H-benzo[e]indol-3-ium bromide, 30e: Yield 65%; mp 148-150 °C; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 1.25 (t, \(J = 5.6\) Hz, 2 H), 1.94 (s, 12 H), 2.09 (m, 4 H), 2.37 (m, 4 H), 2.45 (s, 3 H), 2.80 (t, \(J = 7.2\) Hz, 4 H), 4.27 (t, \(J = 7.2\) Hz, 4 H), 6.03 (d, \(J = 14.0\) Hz, 2 H), 7.29 (m, 10 H), 7.50 (t, \(J = 7.2\) Hz, 2 H), 7.65 (t, \(J = 7.2\) Hz, 2 H), 7.70 (d, \(J = 9.2\) Hz, 2 H), 8.07 (m, 6 H), 8.25 (d, \(J = 8.8\) Hz, 2 H). \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)) \(\delta\) 15.0, 25.3, 27.6, 29.0, 32.5, 43.5, 50.8, 100.0, 111.9, 122.6, 125.1, 126.5, 128.0, 128.1, 128.8, 128.9, 130.3, 130.8, 131.7, 133.4, 140.3, 141.3, 154.7, 172.4.

1,3,3-trimethyl-2-((E)-2-((E)-6-(2-(((E)-1,3,3-trimethylindolin-2-ylidene)ethylidene)-3,4,5,6-tetrahydro-[1,1'-biphenyl]-2-yl)vinyl)-3H-indol-1-ium iodide, 30f: Yield 77%; mp >260 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.11 (s, 12 H), 1.95 (m, 2 H), 2.68 (t, \(J = 6.0\) Hz, 4 H), 3.58 (s, 6 H), 6.17 (d, \(J = 14.0\) Hz, 4 H), 7.16 (m, 4 H), 7.25 (d, \(J = 8.4\) Hz, 2 H), 7.34 (m, 4 H), 7.46 (d, \(J = 7.6\) Hz, 2 H), 7.62 (m, 3 H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 21.3, 24.5, 27.3, 31.5, 48.5, 100.7, 111.3, 122.7, 124.9, 128.5, 128.8, 129.0, 129.6, 131.0, 139.1, 140.9, 143.3, 147.3, 161.5, 172.1. HRMS m/z: calc. for C\(_{38}\)H\(_{41}\)N\(_2\)\(^+\) 525.3264, obsd 525.3241.

5-methoxy-2-((E)-2-((E)-6-(2-(((E)-5-methoxy-1,3,3-trimethylindolin-2-ylidene)ethylidene)-3,4,5,6-tetrahydro-[1,1'-biphenyl]-2-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide, 30g: Yield 73%; mp 238-240 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.17 (s, 12 H), 2.06 (m, 2 H), 2.70 (t, \(J = 6.4\) Hz, 4 H), 3.61 (s, 6 H), 3.82 (s, 6 H), 6.03 (d, \(J = 14.0\) Hz, 2 H), 6.76 (s, 2 H), 6.86...
5-bromo-2-(((E)-2-((E)-6-(2-((E)-5-bromo-1,3,3-trimethylindolin-2-ylidene)ethylidene)-3,4,5,6-tetrahydro-[1,1'-biphenyl]-2-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide, 30h: Yield 75%; mp > 260 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 1.12 (s, 12 H), 2.08 (m, 2 H), 2.76 (t, $J = 6.4$ Hz, 4 H), 3.66 (s, 6 H), 6.15 (d, $J = 14.0$ Hz, 2 H), 6.99 (d, $J = 8.4$ Hz, 2 H), 7.15 (d, $J = 14.0$ Hz, 2 H), 7.21 (dd, $J = 7.6$ Hz, 2 H), 7.46 (dd, $J = 8.4$ Hz, 2 H), 7.56 (m, 3 H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 21.0, 25.0, 27.5, 32.5, 48.4, 100.9, 111.7, 117.7, 125.3, 128.2, 128.5, 129.4, 131.5, 133.2, 138.7, 142.0, 142.4, 148.1, 162.9, 171.2.

5-chloro-2-(((E)-2-((E)-6-(2-((E)-5-chloro-1,3,3-trimethylindolin-2-ylidene)ethylidene)-3,4,5,6-tetrahydro-[1,1'-biphenyl]-2-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide, 30i: Yield 78%; mp > 260 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 1.50 (s, 12 H), 2.11 (m, 2 H), 2.79 (t, $J = 6.4$ Hz, 4 H), 3.65 (s, 6 H), 6.12 (d, $J = 14.0$ Hz, 2 H), 7.05 (d, $J = 8.4$ Hz, 2 H), 7.17 (m, 6 H), 7.30 (t, $J = 8.4$ Hz, 2 H), 7.56 (m, 3 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 21.1, 25.0, 27.5, 32.5, 48.5, 100.8, 111.3, 122.5, 128.2, 128.5, 128.7, 129.4, 130.2, 133.0, 138.7, 141.5, 142.1, 148.2, 163.0, 171.4.

1,1,3-trimethyl-2-(((E)-2-((E)-6-(1,1,3-trimethyl-1,3-dihydro-2H-benzo[e]indol-2-ylidene)ethylidene)-3,4,5,6-tetrahydro-[1,1'-biphenyl]-2-yl)vinyl)-1H-benzo[e]indol-3-ium iodide, 30j: Yield 63%; mp > 260 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 1.50 (s, 12 H), 2.11 (m, 2 H), 2.79 (t, $J = 6.4$ Hz, 4 H), 3.77 (s, 6 H), 6.16 (d, $J = 14.0$ Hz, 2 H), 7.30 (m, 2 H), 7.44 (m, 4 H), 7.55 (t, $J = 7.6$ Hz, 2 H), 7.66 (m, 3 H), 7.92 (m, 6 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ
2-((E)-2-((E)-4-chloro-5-(2-((E)-1,3,3-trimethylindolin-2-ylidene)ethylidene)-1,2,5,6-tetrahydro-[1,1′-biphenyl]-3-yl)vinyl)-1,3,3-trimethyl-3\textit{H}-indol-1-ium iodide, 31\text{a}: Yield 72%; mp 171-173 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 1.68 (s, 12 H), 2.70 (m, 2 H), 3.04 (m, 1 H), 3.15 (m, 2 H), 3.65 (s, 6 H), 6.32 (d, $J = 14.0$ Hz, 2 H), 7.29 (m, 3 H), 7.41 (m, 6 H), 7.48 (d, $J = 7.2$ Hz, 2 H), 7.63 (d, $J = 7.6$ Hz, 2 H), 8.31 (d, $J = 14.0$ Hz, 2 H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 27.8, 32.0, 33.6, 38.7, 49.3, 102.4, 111.9, 122.8, 125.6, 126.3, 127.1, 127.9, 129.0, 141.5, 143.3, 145.4, 147.7, 173.3; HRMS m/z: calc. for C$_{38}$H$_{40}$N$_2$Cl$^+$ 559.2875, obsd 559.2855.

2-((E)-2-((E)-4-chloro-5-((E)-2-((1,1′-trimethyl-1,3-dihydro-2\textit{H}-benzo[\textit{e}]indol-2-ylidene)ethylidene)ethylidene)-1,2,5,6-tetrahydro-[1,1′-biphenyl]-3-yl)vinyl)-1,1,3-trimethyl-1\textit{H}-benzo[\textit{e}]indol-3-ium iodide, 31\text{b}: Yield 82%; mp 182-184 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 1.97 (s, 12 H), 2.74 (t, $J = 12.8$ Hz, 2 H), 3.08 (m, 1 H), 3.20 (m, 2 H), 3.78 (s, 6 H), 6.37 (d, $J = 14.4$ Hz, 2 H), 7.31 (t, $J = 7.2$ Hz, 1 H), 6.42 (t, $J = 7.2$ Hz, 2 H), 7.52 (m, 4 H), 7.67 (t, $J = 7.2$ Hz, 2 H), 7.77 (d, $J = 8.8$ Hz, 2 H), 8.09 (t, $J = 9.2$ Hz, 4 H), 8.31 (d, $J = 8.4$ Hz, 2 H), 8.43 (d, $J = 14.4$ Hz, 2 H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 27.3, 32.4, 33.7, 51.1, 102.0, 112.2, 122.7, 125.4, 126.3, 127.1, 127.8, 127.9, 128.2, 129.0, 130.4, 130.8, 131.9, 134.0, 140.9, 142.3, 145.4, 147.2, 174.5.

2-((E)-2-((E)-4-(2-carboxyethyl)phenoxy)-3-((E)-1,3,3-trimethylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1,3,3-trimethyl-3\textit{H}-indol-1-ium iodide, 32: 81% yield; mp 161-163 °C; $^1$H NMR (400 MHz, MeOD-$d_4$): δ 1.35 (s, 12 H), 2.05 (m, 2 H), 2.52 (t, $J = 7.2$ Hz, 2 H), 2.75 (t, $J = 6.0$ Hz, 4 H), 2.86 (t, $J = 7.2$ Hz, 2 H), 3.60 (s, 6 H), 6.13 (d, $J = 14.4$ Hz, 2 H), 7.03 (d, $J = 8.8$ Hz, 2 H), 7.25 (m, 4 H), 7.38 (m, 4 H), 8.00 (d, $J = 14.4$ Hz,
2-((E)-2-((E)-2-((4-(2-carboxyethyl)phenyl)amino)-3-(2-((E)-1,3,3-trimethylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide, 33: Yield 72%; 1H NMR (400 MHz, CDCl3): δ 1.77 (s, 12 H), 1.98 (m, 2 H), 2.66 (t, J = 5.6 Hz, 4 H), 2.88 (t, J = 5.6 Hz, 4 H), 3.37 (s, 6 H), 5.63 (d, J = 13.6 Hz, 2 H), 6.87 (d, J = 8.0 Hz, 2 H), 7.07 (t, J = 7.6 Hz, 2 H), 7.32 (t, J = 8.0 Hz, 2 H), 7.38 (t, J = 7.6 Hz, 2 H), 7.45 (d, J = 7.2 Hz, 2 H), 7.52 (t, J = 7.2 Hz, 2 H), 7.89 (d, J = 12.8 Hz, 2 H); 13C NMR (100 MHz, CDCl3): δ ppm 22.4, 22.6, 23.8, 28.3, 29.7, 29.9, 94.2, 107.9, 112.3, 121.9, 122.2, 126.7, 128.3, 129.4, 132.1, 139.3, 140.0, 142.9, 143.8, 165.9.

2-((E)-2-((E)-2-((4-(azidosulfonyl)phenyl)amino)-3-(2-((E)-1,3,3-trimethylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide, 39: Yield 46%; 1H NMR (400 MHz, CDCl3): δ 0.89 (m, 4 H), 1.27 (s, 12 H), 2.19 (t, J = 6.0 Hz, 2 H), 3.37 (s, 6 H), 6.59 (m, 2 H), 6.67 (m, 2 H), 6.92 (d, J = 8.8 Hz, 4 H), 7.33 (d, J = 8.8 Hz, 4 H), 8.31 (m, 2 H), 8.45 (d, J = 11.6 Hz, 2 H).

2-((E)-2-((E)-2-((2-hydroxyethyl)amino)-3-(2-((E)-1,3,3-trimethylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide, 40: Yield 76%; mp 160-162 °C; 1H NMR (400 MHz, CDCl3) δ 1.66 (s, 12 H), 1.86 (m, 2 H), 2.52 (t, J = 6.8 Hz, 4 H), 3.45 (s, 6 H), 3.60 (s, 3 H), 4.03 (d, J = 7.6 Hz, 2 H), 4.11 (d, J = 7.6 Hz, 2 H), 5.64 (d, J = 13.2 Hz, 2 H), 6.93 (d, J = 8.0 Hz, 2 H), 7.10 (t, J = 7.6 Hz, 2 H), 7.31 (m, 4 H), 7.70 (d, J = 13.2 Hz, 2 H); 13C NMR (100 MHz, CDCl3) δ 21.7, 25.0, 29.2, 45.2, 47.9, 59.2, 59.9, 94.7, 108.4, 122.1, 123.0, 123.6, 128.1, 140.2, 141.8, 143.5, 143.5.

1,3,3-trimethyl-2-((E)-2-((E)-2-((trimethylammonio)ethoxy)-3-(2-((E)-1,3,3-trimethylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3H-indol-1-ium diiodide, 41: Yield 70%; mp 108-110 °C; 1H NMR (400 MHz, CDCl3) δ 1.76 (s, 12 H), 1.97 (m, 2 H), 2.62 (t, J = 6.0 Hz, 4 H), 3.69 (s, 6 H), 3.72 (s, 9 H), 4.62 (d, J = 5.6 Hz, 2 H), 4.67 (d, J = 5.2 Hz, 2 H), 5.95 (d, J = 14.0 Hz, 2 H), 7.12 (d, J = 8.0 Hz, 2 H), 7.25 (t, J = 7.6 Hz, 2 H), 7.39.
(t, J = 7.6 Hz, 2 H), 7.44 (d, J = 7.6 Hz, 2 H), 8.00 (d, J = 14.0 Hz, 2 H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 20.8, 24.9, 28.4, 32.3, 49.4, 55.3, 64.2, 70.1, 99.9, 110.2, 122.6, 123.2, 125.2, 128.6, 140.1, 140.5, 142.8, 169.3, 172.3.

2-((E)-2-((E)-4′-(2-carboxyethyl)-6-(2-((E)-1,3,3-trimethylindolin-2-ylidene)ethylidene)-3,4,5,6-tetrahydro-[1,1′-biphenyl]-2-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide, \(^{42}\):
Yield 21%; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta\) 0.39 (s, 12 H), 1.25 (m, 2 H), 1.95 (m, 6 H), 2.30 (t, J = 7.2 Hz, 4 H), 2.75 (s, 6 H), 5.30 (d, J = 14.0 Hz, 2 H), 6.36 (m, 6 H), 6.54 (m, 6 H), 6.72 (d, J = 8.4 Hz, 2 H); \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)): \(\delta\) 21.1, 24.2, 26.5, 29.9, 30.3, 35.8, 99.3, 110.0, 121.8, 124.4, 128.2, 128.6, 129.3, 136.7, 140.7, 141.7, 142.9, 148.3, 172.5, 174.9.

2-((E)-2-((E)-2-(4-(2-aminoethyl)phenoxy)-3-(2-((E)-1-ethyl-3,3-dimethylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1-ethyl-3,3-dimethyl-3H-indol-1-ium iodide, \(^{45}\):
Yield 92%, mp 218-221 °C; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta\) 1.12-1.30 (m, 18 H), 1.90-1.99 (m, 2 H), 2.70-2.75 (m, 4 H), 2.77-2.97 (m, 4 H), 4.20 (q, J = 6.5 Hz, 4 H), 6.20 (d, J = 14.1 Hz, 2 H), 7.10-7.16 (m, 1 H), 7.19-7.25 (m, 1 H), 7.28 (d, J = 8.3 Hz, 1 H), 7.37-7.41 (m, 4 H), 7.48-7.53 (d, J = 7.5 Hz, 2 H), 7.82 (d, J = 14.1 Hz, 2 H), 7.99 (s, 2 H). \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)): \(\delta\) 11.5, 20.1, 23.2, 27.6, 34.0, 41.3, 48.0, 99.2, 110.4, 133.7, 120.7, 121.9, 124.2, 127.9, 129.8, 122.7, 140.4, 141.1, 145.3, 157.5, 162.3, 170.5. HRMS m/z: calc. for C\(_{42}\)H\(_{50}\)N\(_3\)O\(_2\)+ 612.3949, obsd 612.3951; calc. for C\(_{42}\)H\(_{51}\)N\(_3\)O\(_2\)+ 306.7011, obsd 306.7009.

### 7.3 Optical and Physicochemical Properties

All optical measurements were performed in phosphate-buffered saline (PBS), pH 7.4, 100% FBS supplemented with 50 mM HEPES, pH 7.4, methanol (MeOH), ethanol (EtOH) and DMSO after preparing 3 mM stock solutions in DMSO. Samples were also diluted in saline containing 10% FBS to confirm aggregation and precipitation in solvent mixtures. UV-
Vis/NIR absorption spectra were recorded on a Cary 50 spectrophotometer (Varian, Palo Alto, CA, USA) interfaced with Cary WinUV Scan Application v3.00 using VWR disposable polystyrene cuvettes with a 1 cm pathlength. Laser Induced Fluorescence (LIF) emission spectra were acquired using Shimadzu RF-5301 Spectrofluorophotometer (Shimadzu Corporation Analytical Instruments Division, Duisburg, Germany) interfaced to a PC with RF-5301PC software using Sigma-Aldrich disposable polystyrene fluorimeter cuvettes with a 1 cm pathlength. All spectral measurements were recorded at room temperature. The data analysis and calculations were carried out using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). For fluorescence quantum yield (QY) measurements, oxazine 725 in ethylene glycol (QY = 19%) and ICG in DMSO (QY = 13%) were used as calibration standards, under conditions of matched absorbance at 655 and 765 nm. In silico calculations of physicochemical properties such as molecular weight, charge, distribution coefficient (logD at pH 7.4), pKa, refractivity, topological polar surface area (TPSA), H-bond donors/acceptors, and rotatable bonds were calculated using JChem calculator plugins (ChemAxon, Budapest, Hungary). Data plotting was performed using Prism version 4.0a software (GraphPad, San Diego, CA) and Microsoft Excel (Redmond, WA).

7.4 Animal Models

Animals were housed in an AAALAC-certified facility and were studied under the supervision of BIDMC IACUC in accordance with the approved institutional protocol (#101-2011 for rodents and #046-2010 for pigs. Male CD-1 mice weighing ~25 g and male Sprague-Dawley (SD) rats weighing ~250 g (Charles River Laboratories) were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine intraperitoneally (Webster Veterinary, Fort Devens, MA). Female Yorkshire pigs (E.M. Parsons and Sons) averaging 35 kg were induced with 4.4 mg/kg intramuscular Telazol (For Dodge Labs) intubated and maintained with 2% isoflurane
(Baxter Healthcare). After anesthesia, EKG, heart rate, pulse oximetry and body temperature were monitored throughout surgery.

7.5 **NIR Fluorescence Imaging System**

The dual-NIR channel FLARE imaging system has been described in detail. In this study, 4 mW/cm² of 670 nm excitation and 11 mW/cm² of 760 nm excitation were used with white light (400–650 nm) at 40,000 lx. Color and NIR fluorescence images were acquired simultaneously with custom software at rates up to 15 Hz over a 15 cm diameter field of view. In the color-NIR merged images, 800-nm fluorescence was pseudocolored green and 700-nm fluorescence was pseudocolored red. The imaging system was positioned at a distance of 18 inches from the surgical field. For each experiment, camera exposure times and image normalization were held constant.

7.6 **Quantitative Analysis**

At each time point, the fluorescence and background intensity of a region of interest over each tissue was quantified using custom FLARE software. The signal-to-background ratio (SBR) was calculated as SBR=fluorescence/background, where background is the signal intensity of neighboring tissues, such as muscle or skin, measured over the imaging period. All NIR fluorescence images for each particular fluorophore were normalized identically in all conditions of a given experiment. At least three animals were analyzed at each time point. Results were presented as mean +/- sd and curve fitting was performed using Prism version 4.0a software (GraphPad). Statistical analyses were carried out using one-way analysis of variance followed by Tukey's multiple comparisons test. Statistical analysis was carried out by using the unpaired Student’s t-test or one-way analysis of variance (ANOVA). Results were presented as mean +/- SD and curve fitting was performed using Prism version 4.0a software (GraphPad, San Diego, CA). One-way ANOVA was used for statistical evaluation. P values
less than 0.05 were considered significant: *P < 0.05, **P < 0.01 and ***P < 0.001. The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment.

7.7 **Histology and NIR Fluorescence Microscopy**

Tissues from rats and pigs were preserved for hematoxylin and eosin (H&E) staining and NIR fluorescence microscopic assessment. Tissues extracted from the animals after intraoperative imaging were placed in 2% paraformaldehyde in PBS for 30 min before mounting in Tissue-Tek OCT compound (Fisher Scientific, Pittsburgh, PA) and flash-freezing in liquid nitrogen. Frozen samples were cryosectioned (50 μm per slice for parathyroid and 10 mm per slice for cartilage), observed by fluorescence microscopy and then stained with H&E.

NIR fluorescence microscopy for resected tissues was performed on a Nikon Eclipse TE300 microscope system with four filter sets as previously described. The microscope was equipped with a 100-W mercury light source (Chiu Technical Corporation), NIR-compatible optics, a NIR-compatible 10× Plan Fluor objective lens and a 100× Plan Apo oil immersion objective lens (Nikon). Images were acquired on an Orca-AG (Hamamatsu). Image acquisition and analysis was performed using iVision software (BioVision Technologies). Two custom filter sets (Chroma Technology Corporation, Brattleboro, VT) composed of 650 +/- 22 nm and 750 +/- 25 nm excitation filters, 675 nm and 785 nm dichroic mirrors, and 710 +/- 25 nm and 810 +/- 20 nm emission filters were used to detect signals in the frozen tissue samples. A custom filter set (Chroma Technology Corporation) composed of 750 +/- 25 nm excitation filters, 785 nm dichroic mirrors, and 810 +/- 20 nm emission filters were used to detect T800-F signals in the frozen tissue samples.
7.8 Live cell labeling and in vitro imaging

Human prostate cancer cells (PC3 cells) were seeded into 24-well plates (5×10^4 cells per well), and incubated at 37 °C in humidified 5% CO₂ incubator in DMEM containing 10% FBS and 1% penicillin streptomycin for 2 days. After washing twice with media solution, 45, IR786, and 32 were added to each well at a concentration of 2 µM and incubated for 30 min at 37 °C. In order to improve image contrast, cells were washed three times with media solution prior to imaging. The images were acquired either right after washing or additional washing with 1% Tween 20 to compare fluorescent signal reduction among the contrast agents. The live cell imaging was performed using Nikon TE2000 epifluorescence microscope equipped with a 75W Xenon light source and an Orca-ER (Hamamatsu, Bridgewater, NJ) camera. The filter set (Chroma Technology Corporation, Brattleboro, VT) composed of 710 +/- 25 nm excitation filter, 785 dichroic mirror, and 810 +/- 20 nm emission filter was used to detect all NIR fluorophores. These cells were observed again after 2 days to compare long-term stability.

7.9 In vivo cell tracking and histological analysis

Animals were housed in an AAALAC-certified facility and were studied under the supervision of BIDMC IACUC in accordance with the approved institutional protocol (#057-2014). 6 weeks old C57BL/6 mice (male; 20-25 g) were purchased from Charles River Laboratories (Wilmington, MA). Animals were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine intraperitoneally (Webster Veterinary, Fort Devens, MA). B16F10 cells were stained with 2 µM of CTNF126 or IR786 for 30 min at 37 °C, and detached with cell scraper. 1×10^6 cells per mouse were injected intravenously into the mice, respectively. Animals were sacrificed 5 min and 24 hours post-injection of the cells, respectively. The lung cavity was opened to observe injected cells in the lung using our custom-built intraoperative NIR imaging system, which equipped 760 nm excitation light (3.6 mW/cm²) and white light (400–650 nm) at 5500 lux. Color and NIR fluorescence images were acquired simultaneously with custom
software at rates of up to 15 Hz over a field of view with a diameter of 15 cm. The lungs were resected and embedded in Tissue-Tek O.C.T. compound (Sakura Finetec, Torrance, CA). The tissues were then cryosectioned at 10 µm interval and stained with hematoxyline and eosin (H&E) for further visualization with TE2000 NIR fluorescence microscope. For validation of fluorescent signal retention during histological analysis, we preceded a standard method using cell pellets. PC3 were seeded onto 100 x 15 mm petri dishes and incubated at 37 °C in humidified 5% CO₂ incubator in the media solution until the cell confluency reached 90%. 1×10⁷ cells (~ 5 petri dishes) were stained with 45 or IR786 at a concentration of 2 µM and incubated for 30 min at 37 °C. After washing with media solution, the cells were collected using cell scraper and centrifuged at 1200 rpm for 10 min. After the same histological preparation was conducted, the sectioned slides were imaged using the microscope using 20X objective.

7.10 DNA Binding Studies

A stock solution of each dye (1x10⁻⁴ M) and ct-DNA type 1 (7.5x10⁻³ M) were prepared in EtOH and Tris-HCl buffer solution, respectively. Fluorescence titration with ct-DNA concentrations (0-200 mM) were made by mixing 35 µL dye solution with Tris-HCl buffer solution with and without ct-DNA to a total volume of 3500 µL in a fluorescence cuvette to make working solutions of 10 µM. Fluorescence spectra were measured in duplicate with excitation at 530 nm and slit widths of 5 nm for both excitation and emission.

8 References


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APPENDICES

The following appendices contain the supplemental information including NMR and MS.
100% MeOH+0.1% HCOOH

ANDY_AL_27_HENARY_082511_POS 89 (1.655) Cm (80.89)

TOF MS ES+ 2.44e3

18:18:43 25-Aug-2011

26c
50% MeOH+0.1% HCOOH
100% MeOH+0.1% HCOOH

ANDY_AL_25_HENARY_071811 66 (1.229) Cm (66.73)

537.4

182.1

305.2

26e

16:59:45 18-Jul-2011
TOF MS ES+
4.76e3
100% MeOH+0.1% HCOOH

ANDY_AL_31_HENARY_083111 37 (0.689) Cm (37:42)


TOF MS ES+ 7.24e3
100% MeOH+0.1% HCOOH

ANDY_AL_22_HENARY_071511 53 (0.988) Cm (49:53)

17:24:24 15-Jul-2011

TOF MS ES+

7.28e3