Towards the Development of a Novel DNA Binding Fluorescent Cell Stain;

An Analysis of Common DNA Dyes and Their Applications

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Abstract

Fluorescent deoxyribonucleic acid (DNA) stains that permeate cells are used to observe

cellular processes *in vivo*, making them valuable tools. For the proposal of a new stain,

ethidium bromide, cyanine dyes, Hoechst stains, macarpine, DAPI and DRAQ5 are first

examined. Features contributing to DNA binding mechanism, toxicity, and cell

membrane transport are analyzed for these compounds. The mechanism of DNA binding

contributes to the toxicity of the compound. For dyes with well-understood transport

mechanisms, multi-drug protein transporters are vital players, though this remains an area

for ongoing research as many mechanisms are not well studied. A novel anthraquinone-

based compound is proposed and the DNA binding mechanism, cell permeability and

fluorescence are predicted based on the traits of existing dyes.

Towards the Development of a Novel DNA Binding Fluorescent Cell Stain

An Analysis of Common DNA Dyes and Their Applications

Background

Fluorescent molecules (fluorophores) are widely used in the biological sciences to visualize cells and subcellular structures. Fluorescent microscopy is a technique in which fluorescent stains are applied to a sample, which is viewed under a microscope. The stain is excited by a light source and emits colored light that can be easily visualized against a dark background. Fluorescent dyes can also be used to generate images through laser scanning confocal microscopy, which produces sharper and higher resolution images than traditional light microscopy and can be used to generate three-dimensional images of cells and their structures. Fluorescence recovery after photobleaching (FRAP) is a technique used to study the rate of movement inside a cell. A cell is first stained with a fluorescent molecule, and then a small portion of the cell undergoes photobleaching. Photobleaching refers to the permanent loss of color or fluorescence when a molecule is repeatedly struck by photons. The rate at which a fluorescent signal returns to this area of the cell provides information about the movement of cytoplasm and organelles within the cell, depending on which specific structures are stained, since new, unbleached particles must move into this area in order for the signal to return. Stains specific to DNA have been used to study chromosome movement during mitosis (Ring, 1982). They are also used to identify individual cells in cell sorting procedures such as flow cytometry (Basiji, Ortyn, Liang, Venkatachalam, & Morrissey, 2007). Because of the wide variety of applications for these stains, the synthesis of fluorescent biomarkers is a valuable area of

research. As novel dyes are developed, more specific niches can be filled, strengthening

the analytical power of bioimaging techniques and improving research capabilities for

biologists in a variety of fields.

The purpose of this research is to study the development of a new DNA binding

fluorescent stain and to test its effectiveness. Specifically, this study seeks to propose a

cell-permeating dye that can be used to stain DNA in live cells. In order to understand the

important features of this type of stain, some commonly used dyes are first examined and

their molecular structures are analyzed and compared. The common features of these

molecules are likely the necessary components of a cell-permeating fluorescent DNA

dye, and these will be incorporated into a novel stain. As will be discussed further below,

the most important traits to be considered for a stain of this kind are: DNA binding ability

and mechanism, toxicity, and cell membrane transport. This novel stain will bear some

similarity to DRAQ5 (an existing DNA dye which will be discussed in detail in a later

section) in basic structure, but the molecular structure will be altered so that the emission

wavelength is different, making the fluorescent signal a different color. This will be

especially helpful when simultaneously using several stains to identify multiple

subcellular structures in the same sample.

Fluorescence

Fluorescence is a phenomenon in which a molecule absorbs light and then emits it

at a longer wavelength. When a photon strikes a fluorescent compound, the photon is

absorbed and an electron is excited into a higher energetic state. This excited state is very

short-lived in fluorescent molecules, lasting only 10^{-10}-10^{-7}seconds before the electron

falls back into its ground (unexcited) state, and the energy is released in the form of light (Valeur, 2001). A fluorescent signal is sustained as different electrons in a sample are individually excited and fall back to the ground state, emitting energy. This emission energy is lower than that of the photon that excited the electron because some of the energy is consumed by the molecule. This energy difference (and corresponding wavelength difference) between the maximum wavelength of absorption and the maximum wavelength of emission is referred to as *Stokes shift.* Stokes shifts can vary for each molecule, so the emission wavelength is not necessarily directly related to the excitation wavelength. However, the emission energy is always lower than the excitation energy.

Not every molecule is fluorescent. In order for a molecule to fluoresce, electrons must be delocalized over multiple bonds. This occurs in a conjugated system, where there is an alternating sequence of single and double bonds, because there is a continuous path of π orbitals in which electrons can freely travel. The greater the extent of this conjugated system, the lower the energy of transition for an electron to go from the highest occupied molecular orbital to the lowest unoccupied molecular orbital and back (Valeur, 2001). In order for electrons to flow between π orbitals, the molecule must be planar. As will be shown in this discussion, most fluorescent molecules are systems of joined aromatic rings. These compounds are necessarily planar and have many conjugated bonds, which lowers transition energy and increases the likelihood of fluorescent electronic transitions. Conjugation can also be extended from these systems by the addition of some

substituents. This may improve the fluorescent signal so long as the molecule remains

planar, and the excitation and emission wavelengths are changed as a result.

Light falls into the larger category of electromagnetic radiation. It is important to

note here that light and energy are related. This relationship is expressed by the equation

$$E=h\nu \tag{1}$$

where E is the energy of a photon in Joules (J), h is Planck's constant ($6.626*10^{-34}$ J*s),

and ν is the frequency of a photon in Hertz (Hz) (Planck, 1901). The frequency of a wave

refers to the number of waves that pass by a point in space in one second, and is related to

wavelength by the equation

$$c=\lambda\nu \tag{2}$$

where c is the speed of light in a vacuum ($3.00*10^{8}$m/s), and λ is the wavelength in

meters (m). Thus the energy of a photon and the wavelength of electromagnetic radiation

is related by the equation

$$E=h(c/\lambda) \tag{3}$$

This makes it clear that when the electronic transition energies change in a conjugated

molecule, the excitation wavelength is affected. Since the emission energy is always

lower than the excitation energy, a decrease in excitation energy should cause a similar

decrease in emission energy, and a corresponding increase in emission wavelength. When

the electromagnetic radiation falls within the visible spectrum (approximately 400-700

nm), the wavelength of light determines the color. Shorter wavelengths (380-450 nm) are

violet-blue, while longer wavelengths (650-740 nm) are orange-red. All other colors fall

in this range as a continuous spectrum. This is important for the development of useful

fluorescent molecules. If the emission wavelength is outside this range, the light will not

be visible to the human eye, and the molecule will not be useful as a fluorescent dye.

Within this range, it is useful to have a variety of colors available so that a cell can be

stained with multiple dyes, and each dye be visually detected. Thus, excitation and

emission energies are important in the development of a fluorescent dye, and the

modification of these values by the addition of conjugated substituents is a valuable area

for experimentation.

Cell Membrane Permeability

The cell membrane is designed to separate the cell from its environment, and is

selectively permeable. The cell membrane is composed of a phospholipid bilayer,

interspersed with proteins and is associated with peripheral proteins and carbohydrates.

The phospholipid bilayer is hydrophilic on either side, since the polar head of the

phospholipids are exposed. The interior of the bilayer is hydrophobic, and is made up on

the non-polar fatty acid tails of the phospholipids. This hydrophobic interior makes it

difficult for charged molecules to get through the membrane, because hydrophobic

molecules repel particles that carry a charge or polarity. Nonetheless, some small, neutral

molecules can still diffuse freely across the membrane, driven by their concentration

gradient. Most notably among these are small, non-polar gases such as O_2, CO_2, and N_2

(Yang & Hinner, 2015).

Larger molecules and charged particles cannot passively diffuse across the

membrane, and these require transporters. Transport proteins shield charged molecules

from interactions with the hydrophobic portion of the cell membrane and can provide a

large enough channel for entry of large molecules. These transport proteins function by

several mechanisms. Channel proteins permit transport of small polar molecules like

water and may also transport ions such as Ca^{2+}, Na^+ or K^+. These channels may remain

open, or they may be ligand or voltage gated, depending on the molecule they are

designed to transport. These proteins do not require the input of energy to function, and

will only allow molecules to flow in and out of the cell with their concentration gradient.

If a molecule must be transported against a concentration gradient, a protein pump is

required. These pumps consume energy, usually in the form of adenosine tri-phosphate

(ATP), every time they pump a molecule through the membrane. These pumps are very

specific for the molecules they transport, and are often referred to as *active transporters*

because they require the input of energy.

Since protein transporters are very specific for what they allow through the

membrane, it is a challenge to introduce new compounds into the cell, such as stains or

intracellular drugs. This challenge has caused biologists to study other membrane

transport mechanisms that could be exploited. For example, some cell toxins such as

diphtheria and anthrax form their own pore complexes (Yang & Hinner, 2015). Evidently

this method is highly toxic to cells, but conjugating a fluorescent dye to one of these

toxins might be used to observe a fluorescent molecule within a cell for a period of time

before the toxin takes its effect. Viruses can also enter the cell, either directly through the

membrane, or by gaining access through endocytosis (Yang & Hinner, 2015). Viruses

that are taken up by endocytosis have the second challenge of escaping the endocytic

vesicle to enter into the cytoplasm. These therefore must employ mechanisms similar to the cellular toxins in order to be truly effective. Fluorescent molecules conjugated to viruses have been used to stain cells in organisms such as mice, in order to study cell proliferation (Grewe et al., 2017), demonstrating that there may be some future in this technique. However, as with the cell toxins, there are risks involved with intentionally introducing a virus into an organism, and this method may not be suitable for use in long-term live cell cultures or larger organisms.

One final possibility for transporting synthetic molecules across the membrane is to synthesize molecules that are chemically similar to molecules that are taken up naturally by the cell. The principle would be that these molecules would be similar enough to biologically relevant molecules that they would be able to use the same transport mechanisms to gain access to the cytoplasm. Difficulties with this exist because of the specificity of protein transporters and the fact that synthetic molecules must have certain structural and chemical properties in order to accomplish their intended purpose, whether therapeutic or analytical. However, some natural transport proteins have been used for intracellular drug-delivery (Borst & Elferink, 2002; Koepsell, 2004), so there may be a future in using this strategy to transport analytical dyes into the cell.

Detecting and Measuring Cell Permeation.

It should be simple to determine whether a fluorescent molecule has traversed the cell membrane, compared to non-fluorescent molecules, because the fluorescence of the intracellular space can be determined using confocal microscopy (Ensenat-Waser et al., 2002). However, this technique does not allow for quantitative data, it merely confirms

that the molecule has crossed the membrane and is present within the cell. Therefore it cannot be used to determine the permeability coefficient. The permeability coefficient is a quantitative measure (cm/s) of how fast a given molecule can cross a membrane (Yang & Hinner, 2015). Various methods exist to determine whether a particular molecule has successfully penetrated the cell membrane, including: selective isolation of the cytosol, digitonin-mediated permeabilization of the plasma membrane, and immunoprecipitation, which confirms that protein-protein interactions have been disrupted. Some more common methods include fluorescence microscopy, biological assays, and measuring the biological effect of the molecule intended to permeate the membrane (Yang & Hinner, 2015). These assays can be used to determine the efficiency of cell permeable fluorescent dyes; that is, whether they do in fact cross the membrane in relevant amounts. Once the dye traverses the membrane, which is a major barrier in the development of biological dyes, the next step is to synthesize a dye that will stain the DNA structure within the cell.

DNA Intercalation

Intercalation refers to the reversible introduction of a guest molecule into a compound with a layered structure, in which the structure of the host compound remains intact (Whittingha, 2012). DNA has a layered structure, with the nitrogenous bases stacked between two "backbones" of alternating deoxyribose and phosphate, twisted to form a double helix with a major groove and a minor groove. In order for a molecule to intercalate into this structure, it must be planar (Lyles & Cameron, 2002). Numerous important planar molecules have been shown to intercalate into the structure of DNA,
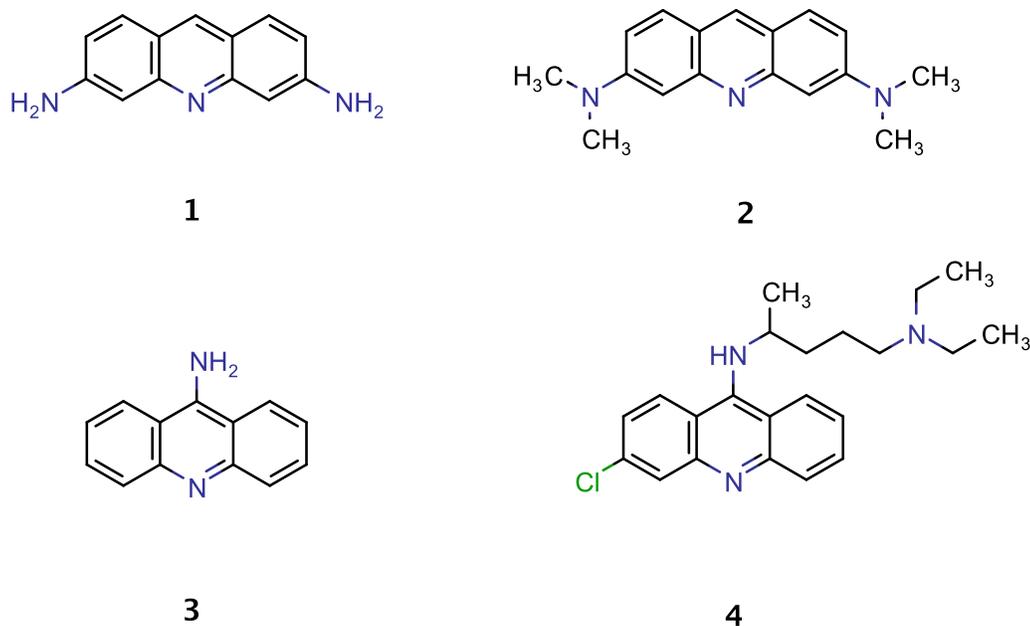
Figure 1. Pharmacologically significant intercalating compounds: proflavine (**1**), acridine

orange (**2**), 9-aminoacridine (**3**), and quinacrine (**4**)

including proflavine (**1**), acridine orange (**2**), 9-aminoacridine (**3**) and quinacrine (**4**)

(Whittingha, 2012). As seen in Figure 1, each of these compounds has a basic aromatic

core structure with varied substituents. Tanious et al. (1992) have shown that substituent

position can affect the way in which an intercalating compound such as anthraquinone

will intercalate into DNA structure. Anthraquinones substituted at the 1,4 and 1,8

positions intercalate with both substituent in either the major or minor groove, while 1,5

and 2,6-substituted compound will typically intercalate with one substituent in each

groove. Additionally, the substituents themselves have an effect on the intercalation in

DNA. Cations intercalate better than anions or neutral molecules because their positive

charge is electrostatically attracted to the anionic phosphate groups of the DNA

backbone. Similarly, polar molecules are preferable to non polar molecules because the bond to DNA can be strengthened by hydrogen bonding (Nafisi, Saboury, Keramat, Neault, & Tajmir-Riahi, 2007).

While the DNA structure is left intact after intercalation, it is necessarily distorted. This interferes with the normal functions of DNA including transcription and replication, and can lead to mutagenesis, carcinogenesis and cell death (Lyles & Cameron, 2002). For this reason, it is unlikely that any cell stained with a DNA intercalating dye would be able to proliferate normally, ultimately resulting in cell death. However, it may be possible to observe the DNA stained within a live cell during the period before the interrupted transcription causes the cell to become apoptotic. In this way, a live cell can be observed using fluorescent intercalating dyes, though not indefinitely.

In addition to intercalation, some DNA binding dyes bind to a strand of DNA by other mechanisms. Some molecules insert themselves into either the major or minor groove, stabilized by non-covalent interactions. These dyes behave similarly to intercalating dyes, however they do not insert themselves between the stacked nucleotide bases. This is less likely to disrupt the structure of the DNA, however these dyes could interfere with replication by preventing necessary proteins from binding to the strand of DNA. Similarly, some dyes interact non-covalently with the outer structure. Many compounds will interact with the negatively charged DNA backbone without interfering with the structure itself. These tend to be the least mutagenic, although they still may interfere with transcription machinery.

Considerations in the Development of Cell-Staining Dyes

As discussed above, the chemical structure of a fluorescent molecule is an important consideration in the development of a fluorescent dye because of its impact on excitation and emission wavelengths and the corresponding color. However, there are more traits that must be taken into account when preparing a dye for biological applications. These include: charge, cytotoxicity and mutagenicity, phototoxicity and background fluorescence. Each of these aspects varies in its importance depending on the target application of the dye. This study focuses on fluorescent dyes used in DNA staining.

The charge of a molecule is an important chemical property. It is especially important when considering molecules for biological application because the charge affects how the compound interacts with biological molecules. Many charged and partially charged molecules exist in the cell, and these interact in specific ways. For example, there are several partially charged amino acids. In proteins, the electrostatic interactions between the amino acids stabilize the structure, and a disruption in these interactions often results in a non-functional or partially functional protein, as is the case in many mutations. The electrochemical gradient on either side of the cell membrane is formed by charged molecules and ions, and drives the transport of signals between cells. Introducing a novel charged molecule into the cell will almost certainly alter these systems to some degree, and this must be considered.

The charge of a molecule will affect its effectiveness as a cell permeable DNA binding dye. A positively charged molecule will be electrostatically attracted to the

negative charges on the phosphate molecules in the DNA backbone, whereas a negatively

charged molecule will be repelled. However, it is difficult for charged molecules to

penetrate the cell membrane unassisted. These traits need to be balanced in order to

develop an effective dye.

Ideally, a cell staining dye should not kill the cell that is being studied. The degree

to which a substance causes damage to a living cell is referred to as cytotoxicity.

Cytotoxic compounds can destroy a cell by a variety of mechanisms. If a compound

compromises a cell's membrane integrity, the cell will most likely become necrotic or

apoptotic. Necrosis occurs when a cell's membrane rapidly deteriorates and the contents

of the cell are released into the environment in an uncontrolled manner. In multicellular

organisms this is often characterized by pain, redness, and swelling, and the process can

compromise surrounding cells, thereby aggravating the problem. A less extreme effect of

a cytotoxic compound is apoptosis. Apoptosis refers to controlled cell death, and also

occurs in multicellular organisms. When a cell has incurred enough damage that it can

not be repaired, apoptotic pathways are activated within the cell, causing it to condense

the chromosomes and break apart into multiple vesicles, which can be taken up by

phagocytes. The phagocytes then help to recycle the cellular material. This is a tightly

regulated pathway that can occur for multiple reasons. However, in the case of

introduction of cytotoxic material, apoptosis is a mechanism to destroy damaged cells for

the protection of the larger organism.

Sometimes, a substance will not directly kill the cell to which it is introduced, but

will prevent it from continuing in the cell cycle. This is known as cell cycle arrest, and a

compound that causes this is said to be cytostatic. Cell replication (mitosis) is another

tightly regulated biological process. A cell needs to receive multiple cytokine signals in

order to progress through mitosis. This occurs at two different checkpoints: G_1 and G_2-M.

If the cell fails to receive the necessary signals at the G_1 checkpoint, replication is halted

and the cell enters G_0 phase. If the cell is not correctly activated by cytokines at the G_2-M

checkpoint, the cycle is likewise halted. Failure to receive these progressive signals often

occurs as the result of DNA damage. This is a protective mechanism for the organism.

Failure of this cell cycle arrest system results in cancer, as genetically damaged cells are

able to replicate unchecked.

Similar to cytotoxicity, another important trait for a cell staining dye is its

phototoxicity. Light can cause harmful mutations in a cell, which can cause an otherwise

harmless molecule to become severely damaging. This happens most commonly with UV

light, which is subdivided into three categories: UVA (320-400 nm), UVB (280-320 nm)

and UVC (200-280 nm) (Pfeifer, You, & Besaratinia, 2005). Although all three of these

types of radiation can be harmful, the short-wave (higher energy) radiation (UVB, UVC),

is most directly damaging to cells (Kielbassa, Roza, & Epe, 1997). One of the most

common mutations induced at these wavelengths is dimerization, which usually occurs

between pyrimidines (Pfeifer et al., 2005). These dimers have been shown to contribute

to the development of cancer and a variety of other genetic disorders (Pfeifer et al.,

2005), making them toxic to the cell. Phototoxicity is an important concept in the

development of a fluorescent dye, because many fluorescent compounds are excited by

UV light, and some have emissions in this spectrum as well. Even if the structure of a

molecule does not directly cause damage to the cell, its use as a fluorescent dye may be harmful if it has excitation and emission peaks in this range. Therefore, it is preferable to have a molecule that is excited at longer wavelengths (600-700 nm), and emits in the far-red visible spectrum.

The final trait for consideration in the development of a fluorescent dye is background fluorescence. Ideally, if the dye is to be used for visualizing DNA, then it should fluoresce significantly more when bound to DNA. If the emission does not change upon binding, then it is difficult, if not impossible, to discern where the DNA is, thereby rendering the dye useless. The fluorescence of unbound dye is known as background fluorescence. For most DNA binding dyes, background fluorescence is minimal, because the structure of the molecule changes upon binding to DNA, forcing the fluorophore to assume a more planar conformation and reducing vibrational freedom. This trait should be present in any novel DNA binding dyes as well. In other cases, the chemical environment is an important factor, since fluorescence can be quenched through interactions with surrounding solvent molecules, which are prevented as the molecule binds to DNA and is shielded from interacting with surrounding molecules. This quenching by a solvent is convenient for reducing the background fluorescence caused by molecules not bound to DNA. Another possibility to avoid the problem of background fluorescence is that the molecule could emit at a different wavelength when bound to DNA. The DNA could then be distinguished in the cell according to color. However, this is only a useful feature if a single fluorescent dye is used at a time. If multiple dyes are

used, distinguishing between the colors and their meanings can quickly become

confusing.

Common Fluorescent Cell Staining Dyes

In order to better understand fluorescent DNA staining dyes, and to analyze the

physical and chemical properties that contribute to a molecule's toxicity, permeation, and

DNA binding capabilities, some of the most common stains are examined here. A wide

variety of fluorescent stains exist, each with their own advantages and difficulties. The

variety of unique stains allows biologists to study cells under different conditions. A

novel dye should bear some structural similarity to these existing stains, in order to

increase the likelihood that the structure will function in a similar way concerning DNA

binding mechanisms and cell membrane transport. However, it is important that a novel

stain also has enough unique features to justify its development; it must either address

difficulties encountered in the application of existing dyes, or else it must have a new

property that allows it to fill a niche where existing dyes are insufficient. Therefore, a

working understanding of the most common cell permeable DNA stains is important for

both understanding the necessary features for the development of a novel dye, and for

ensuring that the dye developed is truly novel.

Ethidium Bromide

Ethidium bromide (**5**) (EtBr) is an aromatic compound (Figure 2) that absorbs UV

light between 210 and 285 nm. It emits light at a wavelength of 605 nm, producing an

orange signal (Sabnis, 2010). The fluorescence of unbound EtBr is relatively weak, and
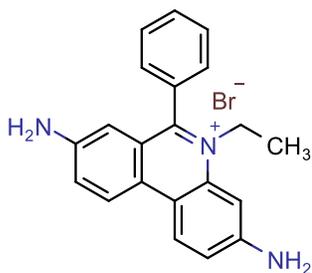
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Figure 2. Structure of ethidium bromide (**5**)

is markedly increased when bound to nucleic acids (Burns, 1972; Heller & Greenstock, 1994). The fluorescence lifetimes show a similar trend, with unbound EtBr having a lifetime of approximately 1.8 ns, which increases to about 23.1 ns upon intercalation (Heller & Greenstock, 1994). Additionally, the absorption and emission spectra are shifted when the dye is complexed with nucleic acids (Duhamel, Kanyo, Dinter-Gottlieb, & Lu, 1996). Thus EtBr is helpful for fluorescence microscopy because free EtBr can be easily distinguished from excess, unbound dye. The increase in fluorescence upon binding is due to the hydrophobic environment provided by the structure of the DNA molecule. The fluorescence of free EtBr is rapidly quenched by surrounding solvent, but the DNA-EtBr complex sterically hinders the solvent from quenching the fluorescence, thus extending the lifetime of the signal (Heller & Greenstock, 1994).

EtBr is a strong DNA binder that has a partial positive charge and is electrostatically attracted to the phosphate groups in the DNA duplex. Upon intercalation, it exhibits energetically favorable π-stacking interactions with nucleobase pairs, stabilizing the complex (Burns, 1972; Nafisi et al., 2007). This binding is reversible, and

occurs with all nucleic acids, DNA and RNA, irrespective of base composition (Stockert, 1974; Waring, 1965). EtBr is widely used to probe DNA structure and to examine changes caused by drug application or protein interactions (Nafisi et al., 2007). Unfortunately, it is difficult to use EtBr to stain nuclear DNA in living cells, possibly because of the presence of proteins on the surface of DNA, which may limit the access of EtBr to the appropriate nucleotide binding sites (Burns, 1969; Stockert, 1974). It is more commonly used to stain free DNA *in vitro*, or to stain RNA molecules present in the cytoplasm of a cell.

Cyanine Dyes

Cyanine dyes are symmetrical or unsymmetrical structures consisting of two nitrogen-containing cyclic structures connected by more than one methylene unit (Uno et al., 2017; Yarmoluk, Kovalska, & Losytskyy, 2008). They are a more effective alternative to traditional staining methods, particularly for the staining of live cells, and are to date the most pervasively used DNA probes (Fei & Gu, 2009; Uno et al., 2017). Similar to EtBr, cyanine dyes have a low fluorescence when free, but are highly fluorescent (increased up to 1000-fold) when bound non-covalently to nucleic acids, a common phenomenon among fluorescent dyes caused by the restriction of movement once the dye is bound to DNA, keeping it in an excited state for a longer period of time (Bruijns, Tiggelaar, & Gardeniers, 2016; Haugland, Yue, Millard, & Roth, 1995; Yarmoluk et al., 2008). These molecules bind to DNA by both groove binding and intercalation, with the primary binding mechanism depending on the DNA sequence and the structure of the dye (Yarmoluk et al., 2008).

One advantage of this class of stains is the variability of absorbance and emission wavelengths that can be obtained by altering the number of methylene units or the structure of the side chains (Uno et al., 2017). Uno et al. (2017) showed that one important component of an unsymmetrical cyanine dye is a dialkylamino group on the methylene bridge. This group increased the fluorescence lifetime and both the absorption and emission wavelengths as compared to the parent structure lacking the dialkylamino group (Uno et al., 2017).

Haugland et al. (1995) showed that when substituted with various cyclic structures containing O, N or S, cyanine dyes readily crossed the cell membranes of most prokaryotic and eukaryotic cells, and were shown to cross the nuclear membrane as well. Rather than intercalation, this family of stains bonded non-covalently to the DNA structure. Notably, these dyes had a very low toxicity in live cells, were highly sensitive, and displayed an unusual level of photostability (Bruijns et al., 2016), making this class of dyes one of the most promising for live-cell fluorescent staining. In 2015, Kadigamuwa et al. showed that two simple cyanines, 2,2'-cyanine (**6**) and 4,4'-cyanine (**7**) (Figure 3), accumulated in the cell through an active process that is not mediated by proteins. The precise mechanism is not clear, however it suggests that cell-permeating cyanine dyes may be able to enter the cell membrane without the help of protein transporters.
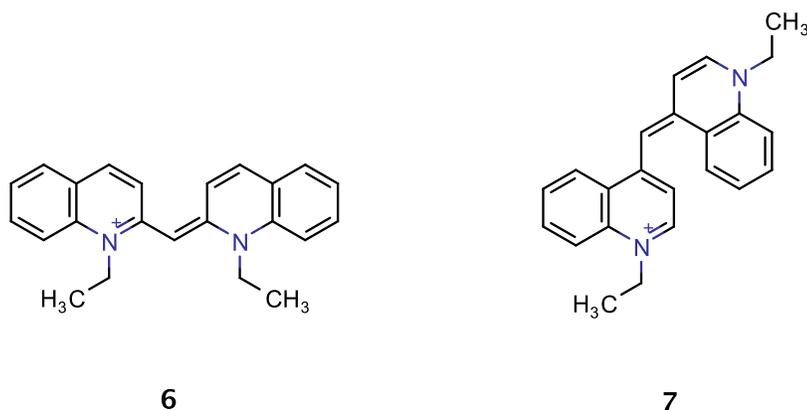
Figure 3. Cell permeating cyanines: 2,2'-cyanine (**6**) and 4,4'-cyanine (**7**)

A common example of a cyanine dye is Thiazole Orange (**8**) (Kienast & Schmitz,

1990; Lee, Chen, & Chiu, 1986) (Figure 4). This stain increases its fluorescence when

bound to DNA, likely because the interaction with the DNA bases forces the molecule

into a more planar conformation where electrons can freely flow between π orbitals (Lee,

Chen, & Chiu, 1986). Thiazole Orange has been demonstrated to intercalate into the

structure of DNA (Nygren, Svanvik, & Kubista, 1998), most likely due to the flat

structure of the molecule, which fits nicely between the bases of DNA. As an intercalator,

this stain shows a preference for GC-containing sequences (Yarmoluk, Kovalska, &

Losytskyy, 2008). Holzhauser et al. (2010) have used Thiazole Orange and its derivatives

to create modified oligonucleotides, which are incorporated into the DNA sequence as

artificial bases. These modified bases have a characteristic absorption range between 450

and 550 nm and emit between 500 and 700 nm, depending on the structure. This method

is useful for detecting specific DNA sequences, and the bases are potentially cell

permeable, but their incorporation into the DNA sequence renders them mutagenic and

therefore cytotoxic, in contrast with other cyanine dyes that bind non-covalently to the DNA structure. Some other cyanine dyes include: SBYR Green (**9**), PicoGreen (**10**), EvaGreen (**11**), and YOYO-1 (**12**), which are all commercially available (Biebricher et al., 2015; Bruijns et al., 2016; Chiaraviglio & Kirby, 2014; Dragan et al., 2010; Fei Mao, Leung, & Xin, 2007; Johnson & Spence, 2011; Ohta, Tokishita, & Yamagata, 2001; Shoute & Loppnow, 2018; Singer, Jones, Yue, & Haugland, 1997; Yoshinaga, Akitaya, & Yoshikawa, 2001). Of these dyes, only SBYR Green and PicoGreen permeate the cell membrane. The structures of these dyes are shown in Figure 4.

 Hoechst Stains

Hoechst stains (**13**) (Figure 5) are a family of minor groove binders commonly used to stain DNA for flow cytometry and cell sorting (Parrilla et al., 2004; Smith et al., 2013). DNA binding can be confirmed by the significant increase in fluorescence upon DNA binding (Chazotte, 2011). Parilla et al showed that these stains do not cause significant mutations in offspring produced by artificial insemination, meaning that Hoechst stains are not mutagenic and do not interfere with DNA replication in gametes (Parrilla et al., 2004). However, these same dyes have been shown to be cytotoxic in somatic cells at high concentrations (>20μM), and disruptive to mitosis and DNA synthesis even at low concentrations (Durand & Olive, 1982). Therefore their usefulness in live cell staining remains somewhat limited.
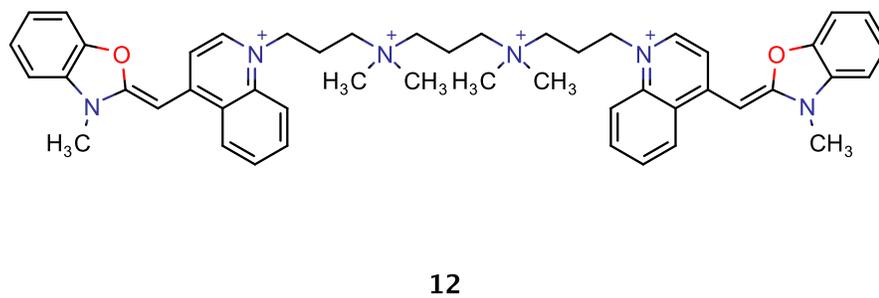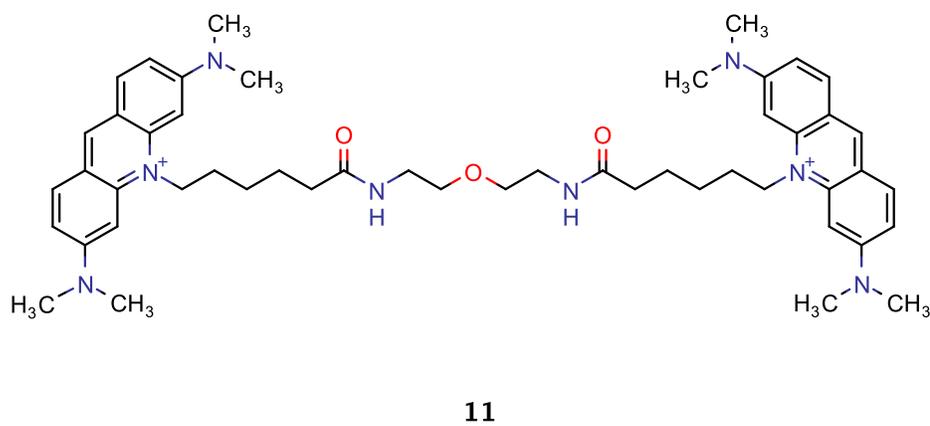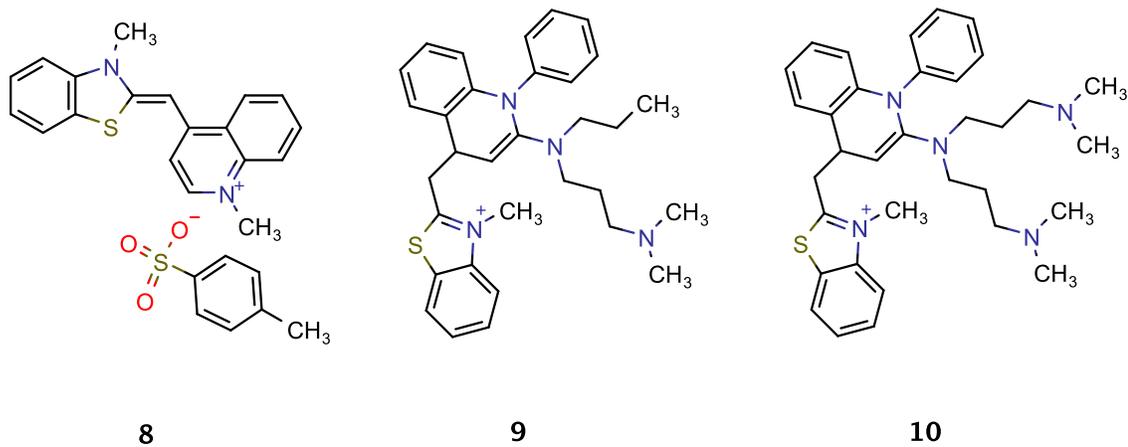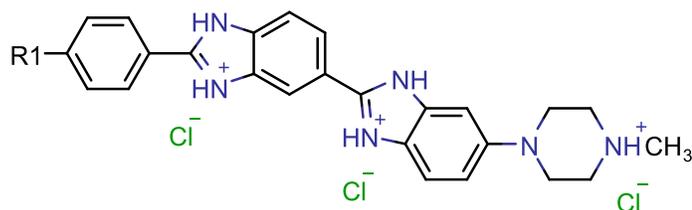
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Figure 4. Structures of select cyanine dyes: Thiazole Orange (**8**), SBYR Green (**9**), PicoGreen (**10**), EvaGreen (**11**), and YOYO-1 (**12**)

13

Figure 5. General structure of Hoechst stains (**13**). Common stains include Hoechst 33258 (R1= OH), Hoechst 33342 (R1=CH$_2$CH$_3$), and Hoechst 34580 (R1=N(CH$_2$)$_2$)

Hoechst stains are membrane permeating. It was demonstrated in 2007 that Hoechst 33342 is transported into the cell by LmrA-MD, a mutant ATP-binding cassette (ABC) transporter homolog from *Lactococcus lactis* that lacks a nucleotide-binding domain (Venter, Velamakanni, Balakrishnan, & van Veen, 2008). This suggests that the ABC transporters most likely play an important role in promoting intake of Hoechst stains into human cells, and that similar proteins may accomplish the same task in prokaryotes. ABC transporters have been implicated in the distribution and elimination of many drugs and are able to bind and transport several substrates, making them a pharmacologically important family of proteins (Borst & Elferink, 2002).

The most common Hoechst stains fluoresce under blue or UV light, which can be mutagenic and photototoxic, hindering their use for live cell staining. Lukinavicius et al. (2015) developed a novel Hoechst stain, SiR-Hoechst (**14**) (Figure 6), which is excited by far-red light and did not interfere with cellular reproduction within 24 hours of application, making it a safer option for live cell staining. SiR-Hoechst can be bound to

ligands that interact with DNA and with the cell membrane, providing both access to the

nucleus and specific DNA binding capability (Lukinavicius et al., 2015). This

development demonstrates the variability of Hoechst stains, and the broad range of

fluorescent properties that can be achieved by modifying the side chains of the dye.

Another use for Hoechst stains includes estimating DNA concentration.

Fluorometry is significantly more sensitive than spectrophotometry, and Hoechst stains

can be used to detect nanogram quantities of DNA in a sample (Green & Sambrook,

2017). These dyes have also been used to distinguish apoptotic cells from normal or

necrotic cells by analyzing DNA condensation in the nucleus (Crowley, Marfell, &

Waterhouse, 2016). However, it is important to note that most DNA binding dyes could

be used for these same methods.

14
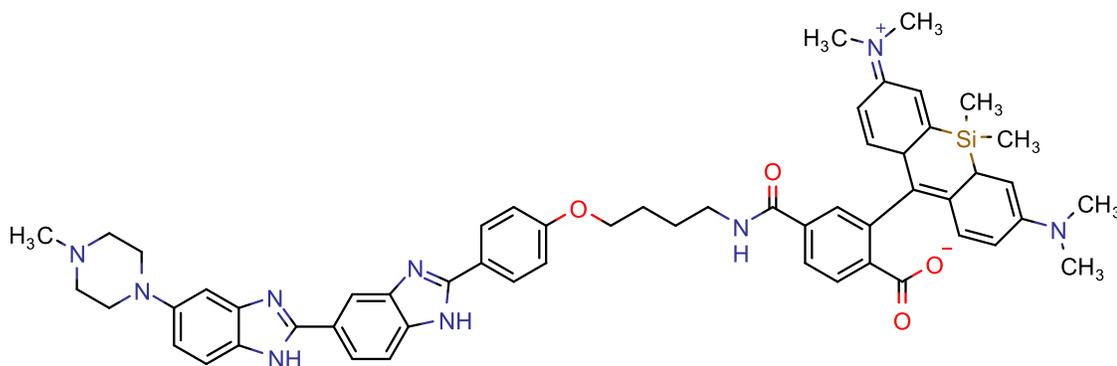
Figure 6. Stucture of SiR-Hoechst (**14**)

Macarpine

Macarpine (**15**) (Figure 7) is a recently developed DNA binding dye. It is a

quaternary benzophenanthridine alkaloid (QBA) isolated from *Stylophorum lasiocarpum,*

a perennial herb found in China (Sebrlova et al., 2015). It has also been artificially

synthesized by several procedures (Ishikawa, Saito, & Ishii, 1995; Sebrlova et al., 2015;

Slaninova et al., 2016). In addition to its fluorescent properties, it has been noted for its

antibiotic, antitumor and anti-inflammatory qualities, making it a compound of interest in

several branches of biological sciences (Ishikawa et al., 1995; Slaninova et al., 2016).

Macarpine is excited by light with wavelengths of 300-550 nm, but is maximally excited

at 390 nm and emits bright red light with the emission peak at 602 nm (Sebrlova et al.,

2015; Urbanová, Lubal, Slaninová, Táborská, & Táborský, 2009). Similar to other DNA

binding dyes discussed above, it shows a significant increase (18 fold) in fluorescence

when bound to DNA, and also shows an increase in fluorescence lifetime (Slaninova et

al., 2016; Rajecky, Sebrlova, Mravec, & Taborsky, 2015). This dye shows potential for

quantitative DNA studies, due to the fact that it binds to DNA stoichiometrically
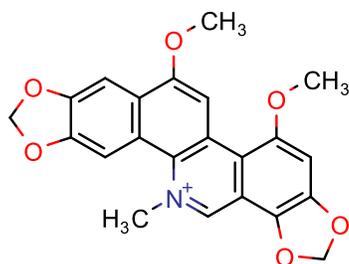
15

Figure 7. Structure of Macarpine (**15**)

(Slaninova, Slanina, & Taborska, 2007). The specific binding mechanism of macarpine to DNA has not been definitively determined. However, sanguinarine, a similar QBA, has been demonstrated to have an intercalating mechanism of binding. Based on the structural similarities of QBAs, it is reasonable to presume that macarpine binds to DNA by a similar mechanism (Rajecky, Sebrlova, Mravec, & Taborsky, 2015).

Slaninova et al. (2016) produced promising results regarding live-cell staining. They found that the nuclei of A-375 cells fluoresced strongly when stained with 0.1μg/mL macarpine, showing mitotic chromosomes. The cells were still viable 48 hours after staining and showed no appreciable DNA damage, suggesting that this concentration of dye is not cytotoxic. There was some phototoxicity detected when cells stained with macarpine were visualized using 488 nm light (Slaninova et al., 2016), indicating that excitation by longer wavelengths of light (550 nm) is preferable for viewing live cells.
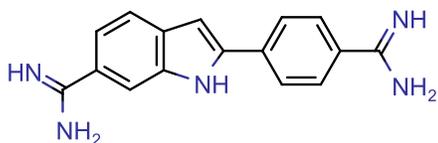
DAPI

DAPI (4', 6-diamidino-2-phenylindole) (**16**) (Figure 8) is a cell-permeable dye used extensively in fluorescent microscopy to detect AT-rich regions of DNA (Kapuscinski, 1995). Similar to Hoechst stains, the excitation wavelength for DAPI is near the UV spectrum (364 nm), leading to the toxicity problems discussed above. The emission wavelength is 454 nm (Żurek-Biesiada, Kędracka-Krok, & Dobrucki, 2013), which shows up as blue light. When excited by high energy UV light (340 nm), DAPI has been shown to undergo photoconversion and transition from a blue-emitting dye to a

green-emitting dye, which can cause confusion if more than one fluorescent dye is being

used in the same test (Żurek-Biesiada et al., 2013).

DAPI preferentially binds DNA in the minor groove, especially in AT-rich

regions, and has been shown to intercalate between DNA bases in CG-rich areas (Beccia

et al., 2012; Reis & Rocha, 2017). These interactions, particularly the intercalation,

happen largely because of the hydrophobic interactions between the nucleotide bases and

the dye structure (Beccia et al., 2012). When DAPI is dissolved in water, its light

emission dramatically increases upon binding with DNA, which is a useful indicator of

penetration. However, free DAPI fluoresces in ethanol, so the same effect is not observed

(Beccia et al., 2012).

It has been shown that DAPI can act as a substrate for the human organic cation

transporter (OCT)1/SLC22A1, which is found in the liver (Yasujima, Ohta, Inoue, &

Yuasa, 2011). This allows the dye to gain access into the cytoplasm, and provides insight

into the mechanisms by which fluorescent dyes can achieve permeation. Organic cation

transporters are polyspecific, so they can be used to transport multiple cationic

compounds into the cell (Koepsell, 2004). Though DAPI is not a cation, resonance allows

the electrons to flow between the two nitrogens on either end of the structure, giving the

16

*Figure 8.* Structure of DAPI (**16**)

nitrogen atoms a partial positive charge, which appears to be enough to allow the molecule to be treated as a cation. Since DAPI is taken up by an enzyme, this uptake conforms to Michaelis-Menton kinetics, with a K_m of 8.94μM, which indicates that the cation transporter has a much higher relative affinity for DAPI compared to its more well-known substrate, tetraethylammonium (TEA) (Yasujima et al., 2011). Interestingly, membrane potential appears to have no effect on the efficiency of DAPI transport, which is unusual for cation transporters, however it appears that Na^+ and Cl^- are critical for the optimum functioning of the transporter (Yasujima et al., 2011). This would seem to indicate that DAPI is imported by either a symport (process by which an integral protein transports different molecules across the membrane at the same time and in the same direction) or an antiport (transports molecules across the membrane at the same time and in opposite directions) mechanism.

DRAQ5 and Anthraquinone Compounds

DRAQ5 (**17**) (Figure 9) is a synthetic anthracene-based dye that readily crosses both the plasma and the nuclear membranes to stain DNA in a concentration-dependent manner (Wojcik & Dobrucki, 2008), making it an effective choice for visualizing DNA in live cells. This stain is a DNA intercalator that binds to double stranded DNA with high affinity and provides high resolution. It is maximally excited at 647 nm (although it can be excited by wavelengths as low as 488 nm) and fluoresces between 665-780 nm (P. Smith & Patterson, 2006; P. J. Smith, Wiltshire, Davies, Patterson, & Hoy, 1999). These wavelengths are red-shifted as the DNA-DRAQ5 ratio increases (P. Smith & Patterson, 2006; Smith et al., 2000). The excitation and emission wavelengths are useful as they

avoid phototoxic UV rays, as required for macarpine or Hoechst stains. The far-red

emission also does not interfere with other fluorescent cell staining dyes (Mari et al.,

2010), so DRAQ5 can be used alongside other stains in order to visualize multiple cell

components simultaneously. The dye showed little photobleaching, meaning that it can

be used for continuous study as long as the cells are viable (P. Smith & Patterson, 2006).

DRAQ5 is useful for biological applications because it is soluble in biologically

compatible solvents (P. J. Smith et al., 1999), a feature which contributes to the high cell

permeability.

The effective intercalation of DRAQ5 is likely due to the structure of the

compound. The conjugated aromatic rings form a planar structure that allows the

compound to slide in between the DNA bases. The binding is stabilized by the side

chains on either side of the core (P. Smith & Patterson, 2006), most likely with one chain

binding in each groove of the DNA strand (Nafisi et al., 2007). The compound is polar,

which may allow it to bind more effectively with the polar DNA strand.
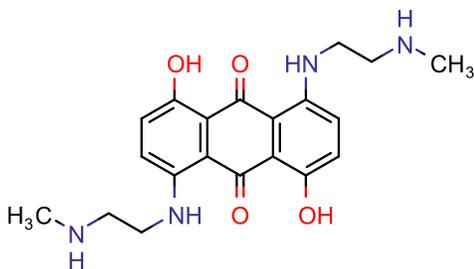
17

Figure 9. Structure of DRAQ5 (**17**)

While DRAQ5 is considered ideal because of its excellent cell permeable qualities, it can also cause damage in live cells. When the dye intercalates into the DNA, it separates the bases and unwinds the strand of DNA. This prevents H1 histones from associating with the DNA, which alters chromatin aggregation. This effect is notable even at low levels (Mari et al., 2010; Richard et al., 2011; Wojcik & Dobrucki, 2008) and proves to be cytotoxic.

Since DRAQ5 can effectively traverse both the plasma and nuclear membranes, and since it can effectively intercalate into the structure of DNA, it is logical that compounds of similar structure would have similar cell staining properties. DRAQ5 has an anthraquinone core (**18**) (Figure 10), and other substituted anthraquinone molecules have been used as cell staining dyes and have been shown to stain cells effectively (Li, Tan, Wang, Xiao, & Li, 2017; F. Mao, Leung, Cheung, & Roberts, 2015; P. Smith & Patterson, 2006). Anthraquinone is a well-known DNA intercalator (Bouquin, Malinovskii, & Häner, 2008), so any dye synthesized from this core should theoretically be a useful DNA staining dye. The experimental portion of this research focuses on the
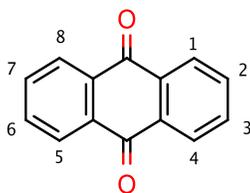
18

Figure 10. Structure of anthraquinone (**18**)

modification of anthraquinone for the purpose of developing a novel fluorescent dye with the same permeability qualities as DRAQ5.

Anthraquinone is an electron acceptor that is used in many biological and chemical applications, such as medicine, anion sensing, energy storage and food processing (Gerhardt et al., 2017; Li et al., 2017; Quevedo et al., 2010). It is a secondary metabolite that can be isolated from several plants in the *Rubiaceae* family (Quevedo et al., 2010). Two different pathways in these plants are used to synthesize anthraquinone. The first is the metabolism of glyceraldehyde-3-phosphate and pyruvate, occurring in the chloroplast, and the second is the metabolism of phosphoenol pyruvate and erythrose 4-phosphate (Quevedo et al., 2010). Interestingly, anthraquinone itself is a nonfluorescent molecule that is known to quench fluorescence significantly (Bouquin et al., 2008). Therefore, the side chains of anthraquinone dyes such as DRAQ5 must have a significant influence on the fluorescence of the molecule. This presents a suitable synthetic approach to the development of fluorescent anthraquinone-based dyes.

Analysis of Cell Permeable Fluorescent Dyes

This overview of cell staining dyes, as summarized in Table 1, shows the common traits of these stains and provides insight into to development of new cell-permeating molecules. It is clear that all cell-permeating DNA binding dyes are mutagenic and cytotoxic, although the degree of toxicity varies according to the DNA binding mechanism. Intercalators appear to be the most cytotoxic, because they insert directly into the layered bases of DNA, changing the overall structure. This change in structure interferes with the transcription of genes and replication, which usually results

Table 1. *Comparison of Cell Staining Fluorescent Dyes*

Compound	Excitation Wavelength (nm)	Emission Wavelength (nm)	DNA Binding Mechanism	Cell Permeation Mechanism	Signal Change When Bound to DNA
Ethidium bromide (**5**)	210-285	605	Intercalation	Does not permeate	Increased intensity
Thiazole Orange (**8**)	450-550	500-700	Intercalation	Undocumented	Increased intensity
SBYR Green (**9**)	497	520	Intercalation/minor groove binding*	Likely not protein mediated, mechanism uncertain	Increased intensity
PicoGreen (**10**)	500	523	Intercalation/minor groove binding	Likely not protein mediated, mechanism uncertain	Increased intensity
EvaGreen (**11**)	470-495	520-530	Intercalation/ external binding**	Does not permeate	Increased intensity, red-shifted excitation/ emission
YOYO-1 (**12**)	491	509	Intercalation	Does not permeate	Increased intensity
Hoechst 33528 (**13**)	352	451	Minor groove binding	Likely protein transporters	Increased intensity
Hoechst 33342 (**13**)	361	497	Minor groove binding	ABC transporter homologs	Increased intensity
Hoechst 34580 (**14**)	392	440	Minor groove binding	Likely protein transporters	Increased intensity
SiR-Hoechst (**15**)	652	672	Carboxylation and ligand complexation	Carboxylation and ligand complexation	Increased intensity
Macarpine (**15**)	390	602	Likely intercalation	Undocumented	Increased intensity and fluorescence lifetime
DAPI (**16**)	364	454	Minor groove binding, some intercalation	Organic cation transporter (OCT)1/SLC22A1	Increased intensity (in H_2O, effect not observed in EtOH)
DRAQ5 (**17**)	647	665-780	Intercalation, stabilized by major and minor groove binding	Undocumented	Red shift with increased DNA: DRAQ5 ratio

*SBYR Green binding mechanism depends on the base pair:dye ratio. At a higher pair:dye ratio, intercalation predominates. At lower ratios, minor groove binding is most common (Zipper, Brunner, Bernhagen, & Vitzthum, 2004).

**EvaGreen intercalates into DNA at low to moderate concentrations, but the binding mechanism switches to external binding at high concentrations (Shoute & Loppnow, 2018).

in cell-cycle arrest or apoptosis. If the cell does not die, it is likely to become cancerous, which is also damaging to the organism. For this reason, DNA intercalating molecules are hazardous and must be handled with care. It is probable that a novel stain that functioned by intercalation would be just as cytotoxic, based on these observations. This would limit its use in live organisms, but these types of stains can still be used in samples and cell cultures.

All of the molecules discussed here are capable of DNA binding. Some of these compounds intercalate, some are groove binding, and some bind by several mechanisms, depending on the DNA sequence and concentration of the dye. One important trait that they share is one or more positively charged nitrogen atoms. The positive charge (or partial positive charge in some molecules such as DAPI) is electrostatically attracted to the negatively charged phosphates in the DNA backbone. This electrostatic attraction allows the dye to bind tightly to a strand of DNA. In the case of long molecules such as DRAQ5, these positively charged portions can wrap around the double helix, forming multiple binding sites and further stabilizing the interaction. It is interesting that all of the molecules contain nitrogen as the positively charged atom, because nitrogen is an important component in the DNA bases, which also contain aromatic rings. It is likely that the structural similarity between the DNA bases and the DNA binding dyes encourages the interaction between the molecules.

Evidently one of the greatest challenges in developing a novel DNA binding dye is permeation of the cell membrane. An analysis of the transport of the dyes discussed above reveals a variety of transport mechanisms. Cyanine dyes do not appear to require

protein mediation to permeate the cell membrane, although the exact mechanism of their

penetration is uncertain. It is interesting that, of the specific cyanine dyes discussed in

this research, only the smaller molecules were cell-permeable. This would suggest that

the size of the molecule is a significant factor, which is a reasonable conclusion

considering that generally large molecules are excluded by the cell membrane in the

absence of a transporter. The importance of protein transporters is clearly demonstrated

by the permeation of SiR-Hoechst. This stain is a very large molecule, and therefore it

would not likely penetrate the cell membrane naturally. However, since Hoechst stain

transport is mediated by proteins, this stain is very cell-permeable. The transport of both

Hoechst stains and DAPI is achieved by polyspecific transporters that are often used in

pharmaceutical development. It would be reasonable to suggest that DRAQ5 and

macarpine dyes are transported by similar proteins, and this remains an area of potential

research. The investigation of these polyspecific enzymes, particularly the organic cation

transporters, which occur naturally in the human body, may lead to breakthroughs in the

synthesis of fluorescent molecules for DNA staining in live cells.

Experimental Cell Stain Development

The dye experimentally synthesized is an anthraquinone-based 1,4-disubstituted

molecule with extended conjugation as a result of the addition of a side group. It is

synthesized originally from 1,4-dichloroanthraquinone (**19**) (Figure 11). This compound

is an isomer of 1,5-dichloroanthraquinone (**20**), the starting material for DRAQ5

synthesis (P. Smith & Patterson, 2006). Based on the existing dyes analyzed above, this

molecule is expected to intercalate into the DNA structure so that the anthraquinone core

is inserted between the stacked bases, and the two substituent chains should both rest in

either the major or minor groove, though the side group may protrude from the groove

based on steric hindrance. This DNA binding method means that the dye will most likely

be highly mutagenic, since the structure of the DNA strand is necessarily interrupted by

the insertion of the dye. Transcription machinery will also likely be inhibited due to the

combined effects of the intercalation-induced structural change and the protruding side

group, which may block proteins from binding to the DNA strand.

It is more difficult to predict whether or not this new compound will be able to

permeate the cell membrane. Based on the dyes analyzed above, the compound has the

necessary features to allow this to take place. It is relatively small when compared to

large impermeable stains such as EvaGreen or YOYO-1. In fact, it is much smaller than

the Hoechst stains, dyes shown to be cell-permeating. Therefore, this molecule should not

be excluded based solely on size. The stain will contain a positively charged atom in one

of the substituent chains, but not in the core. With the exception of DRAQ5, all of the

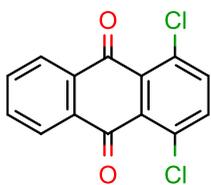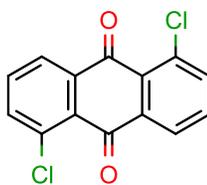stains analyzed in this study contain a positively charged nitrogen atom. Therefore,

19   **20**

Figure 11. Anthraquinone starting compounds: 1,4-dichloroanthraquinone (**19**), and 1,5-

dichloroanthraquinone (**20**)

although it is usually more difficult for charged particles to make it through the cell membrane, this charged portion should not interfere with permeation. If the dye is transported into the cell via cation transporters, as with DAPI, then the charged portion should improve the likelihood that the stain will be taken up. If the dye permeates the membrane independently, the effect of this cationic portion is difficult to predict.

The structure of the novel dye is highly conjugated and is predicted to be planar, so it should fluoresce provided it is not forced out of plane by steric hindrance. DRAQ5 emits between 665 and 780 nm, and it appears that an increase in conjugation changes excitation and emission wavelengths to lower energies. Therefore this dye will most likely fluoresce in the far-red spectrum. The emission wavelength will also likely be in this spectrum, meaning that phototoxicity should be reduced for this molecule. One possible risk is that the emission wavelength could be so long that it lies outside the visible spectrum. However, even if the wavelength of maximal emission is not visible to the human eye, some emission light should still be able to be seen, considering the large emission ranges of the other dyes examined in this study. Additionally, light that falls outside the visible spectrum can be detected using other methods, such as an infra-red camera.

Conclusion

The study of cell-permeating fluorophores does much to inform the development of a novel dye. This study reveals the structural factors contributing to a molecule's fluorescent excitation and emission, demonstrating that increasing aromaticity generally decreases excitation energy, thus decreasing phototoxicity. However, this increase in

conjugation must be balanced with the observation that small molecules generally pass through the membrane more readily than molecules with large, nonpolar regions. Though cellular transport is not well understood for all the dyes covered, it is clear that transport proteins play a significant role, and that an increased understanding of multi-drug transporters and their functions will almost certainly provide new methods of cellular penetration for live-cell staining. A brief overview of DNA binding methods highlighted the difficulty of developing a molecule that is non-toxic, due to the delicate nature of DNA replication and the tightly regulated processes that can be interrupted by a foreign molecule. Nonetheless, an intercalating dye such as DRAQ5 is highly effective for laboratory analysis, and a derivative of this structure has potential to expand the tools that cytologists and other analytical biologists can employ to better understand the cell.

The field of cell permeating dyes remains an area for active research. Though many molecules are shown to permeate the membrane, these mechanisms are not clear in most cases. Future studies should include searching for more transport proteins, and understanding the interactions between synthetic molecules and the cell membrane. A deeper understanding of these mechanics will greatly inform not only the development of new dyes, but also the efficient transport of intracellular drugs. Future studies of the experimental dye proposed here include analysis of DNA binding, including any potential signal or intensity shift. Studies will also include analyzing the interactions with live cells in order to study toxicity and membrane permeation, to see whether experimental results coincide with the predictions based on this study.

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