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TITLE Length-based Separation of DNA in Microfluidic Chips Using Oxazole Dyes

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ACCEPTED BY THE DEPARTMENT OF BIOMEDICAL ENGINEERING

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Length-based Separation of DNA in Microfluidic Chips using Oxazole Dyes

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in

Biomedical Engineering

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Abstract:

In this thesis, we present the use of oxazole dyes in order to fluoresce DNA in sequence-specific, length-dependent separation of DNA in capillary and microfluidic electrophoresis. Using alkane-tagged DNA strands tagged with alkane tails, electrophoretic separation is performed in a solution with micelles. DNA tagged with BODIPY-FL is intense enough to be used in capillary electrophoresis (CE) with laser-induced fluorescence (LIF). However, BODIPY-FL is not intense enough to be used in microfluidic chips due to its equimolar ratio of DNA:dye.

In microfluidic chips when using an ultraviolet source, BODIPY-FL is not an intense enough fluorophore to be used. As a result, intercalating dyes with significantly higher intensity due to the quantity of dye was investigated. In this work, the use of YOYO-1as a potential dye for use in ELFSE in microfluidic chips was investigated. Experimental evidence will demonstrate the use of YOYO-1 in free-solution electrophoresis, and its intensity in comparison to BODIPY-FL.

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Chapter 1: Introduction, Background, and Objectives

1.1 Motivation

Current standards of DNA separation are often slow, expose the user to potentially toxic chemicals (tetramethylethylenediamine (TEMED), ethidium bromide), and have poor resolution. DNA separation in free solution is an excellent alternative that is quick due to the low friction in a low viscosity solution, and requires few toxic chemicals. The newest methods of DNA separation that this paper will investigate are forms of capillary electrophoresis. However, in order to improve upon traditional methods of capillary electrophoresis, the use of microfluidic devices would allow for miniaturization, improved speed of separation (3-8 minutes instead of hours), and an increase in the throughput of DNA separation in a capillary-like system.

1.2 Length based detection and isolation of DNA

Detection, isolation and separation of DNA based on its size or length is important for numerous biological and engineering applications. Some of these applications include sequencing strands of DNA, separating DNA fragments for vector or plasmid cloning and the removal of genomic components from a PCR product. However, the current standards for DNA separation (i.e. gel electrophoresis) do this slowly and with low throughput. For example, using gel electrophoresis can separate 400 base-pair long strands of DNA in 1 hour (1), while novel technologies, such as End-Labeled Free-Solution Electrophoresis (ELFSE) could dramatically decrease the amount of time necessary for DNA separation, and potentially be applied to the separation of large DNA strands in one hour or less.

1.3 Introduction to ELFSE

ELFSE is a technique for separating DNA based on its length. Because DNA has a charge-to-friction ratio that is independent of its length, attempting to electrophoretically

separate DNA in a free buffer solution will not lead to separation, but will instead lead to co-migration of DNA strands in solution due to the hydrodynamic interactions with the surrounding fluid. As a result, modifying the charge-to-friction ratio for DNA is necessary for length-based separations. One way to modify this ratio is to add a constant-friction tag to the end of the DNA (2), thereby increasing the friction. Although adding a charged tag is not ideal, it will still result in modification of the charge-to-friction ratio. This additional friction is represented in mathematical modeling of the system as α, the theoretical friction of the tag converted to an equivalent number of DNA base pairs (3). This friction decreases the apparent electrophoretic mobility of the conjugate molecules by increasing α as shown in the following equation.

$$\mu_{EP} = \mu_0 \left(\frac{L}{L + \alpha} \right) \tag{1.1}$$

where μ_{EP} is the apparent electrophoretic mobility, μ_0 is the free-solution electrophoretic mobility, and L is the number of DNA base pairs. In addition to this formula, two other major formulas are needed for any electrophoretic mobility calculation:

$$\mu_{app} = \frac{L_d / t}{V / L_t} = \frac{L_d * L_t}{V * t} \tag{1.2}$$

$$\mu_{app} = \mu_{EOF} + \mu_{EP} \tag{1.3}$$

In these equations, μ_{app} is the apparent mobility of the DNA being separated, L_d is the length to the detector, L_t is the total length of the capillary, V is the separation voltage, t is the time of separation and μ_{EOF} is the free solution mobility under electro-osmotic flow (EOF). μ is generally reported in units of $cm^2/V*s$.

In electrophoresis, particularly in capillaries or microfluidic chips, EOF can be used to enhance separation protocols. EOF is the motion of a liquid in solution caused by an applied potential. In large pipes, this is not an issue, as the Debye layer is very small in comparison to the

radius of the pipe. However, in a microfluidic channel, the Debye layer is large enough relative to the radius of the channel that there is an additional flow relative to the electrophoretic direction caused by the layer of mobile ions in the Debye layer. This flow can be either in addition to or against the electrophoretic flow, however, for all systems described here, it will be assumed to be a counter-flow. As a result, this counter-flow becomes dominant when the surface is not passivated, i.e. the mobile ions in the Debye layer are not eliminated. EOF is eliminated by coating the wall of the capillary with a polymer such as poly-vinyl alcohol (PVA) or Performance Optimized Polymer-6™ (POP-6).

In a perfect system, each molecule attached to the DNA as a drag-tag would be perfectly monodisperse, i.e., the α of one drag tag would be exactly the same as an α of another drag tag, when attached to a DNA strand. Initial experiments with ELFSE chemically attached a synthetic streptavidin protein (2). However, the protein was not monodisperse enough for its intended application, despite gel purification leading to reasonable monodispersity. With respect to monodispersity, the drag tags may have the same polypeptide chain, but the folding and interactions between the different amino acids can have slightly different folding properties, leading to a variety of charged isomers with different amounts of friction. Polydisperse drag tags with a polydispersity index of 1.01 still led to a dramatic increase in the number of electropherogram peaks, as well as significant broadening of those peaks. In addition, because streptavidin is such a small protein, with α=24.15 bases, streptavidin would not be useful for separating DNA strands greater than 25-40 nucleic acids. In order to experimentally show good resolution, α=300 is required in order to separate 625 bases (2). Due to the fact that friction caused by a drag-tag increases linearly with respect to radius, and that a folded protein radius increases by molecular weight^(1/3), α=300 would require a 30 MDa protein, an impossible size

for a protein, especially one which is monodisperse (2). As a result, synthetically-synthesized polypeptides should not be used for this application, as they cannot be produced in perfect monodispersity.

1.4 Micelle ELFSE

As a result of monodisperse proteins not being suitable for strands longer than 40-50 base pairs, other techniques were investigated, including the use of micelle ELFSE. In micelle ELFSE, an alkane tail is attached to the end of one strand of single-stranded DNA (ssDNA). Depending on the technique used, the alkane tail can be labeled with a fluorophore. Illumination and fluorophores will be discussed in greater detail in section 1.5. Because of its hydrophobicity, the alkane tail interacts with the center of a micelle, creating a size-tunable micelle that acts as a drag-tag on the DNA. Larger micelles mean a larger α, leading to increased drag on the DNA molecule. Using micelle ELFSE provides unique operating conditions variables like capillary length, micelle size, and electric field strength, all of which necessary for large increases in throughput.

However, at any given instant, micelles are polydisperse, leading to tiny deviations in DNA velocity. This polydispersity is mitigated by the rapid size fluctuations in the size of the micelle, as shown in Figure 1. The rapidity of this fluctuation in micelle size is known as the trading frequency and must be very fast in order to lead to overall effective monodispersity.

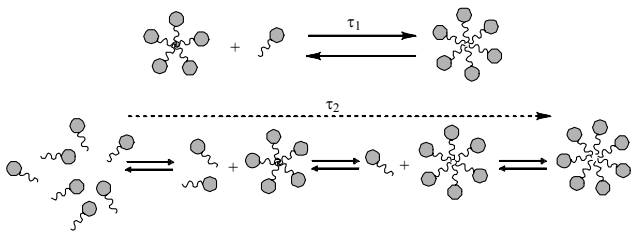


Figure 1: Micelle Trading

In order for micelle ELFSE to function correctly, a certain amount of time is required for the longest strands of DNA to resolve prior to the detector. This time is called the gap time.

Without this time, in addition to not leaving the DNA strands enough time to separate prior to detection, the separation would be considered under-resolved, because the micelles have not had enough time to equilibrate in size by trading monomers, and the longer strands have not had enough time to separate themselves into different sized peaks based on differences in velocity. This is generally an issue for the longest strands of DNA, as they move significantly faster in solution due to the small effect the micelles have on their mobility.

On the other end, slow molecules have significant problems with over-resolution – because the DNA molecules move so slowly, the separation distance between each size is magnified dramatically, meaning that the slowest molecules take a very long time to be detected, especially when using a separation medium that has a large α. This also means that slow molecules take a very long time to be seen during a separation. As a result, it is important to find a surfactant that produces micelles with an α that is appropriate for the separation – If the DNA molecules being separated are larger, the use of a larger α is required, but if the DNA molecules being separated are smaller, the use of a smaller α is more appropriate.

This is where the use of snapshot vs. finish-line detection becomes important. In snapshot detection, an image of the entire capillary is taken at a set time, but in finish-line detection, everything is sensed at a fixed distance from injection. Because the smallest, slowest strands resolve so quickly and easily, separating the smallest bands from the largest bands, and then detecting them with snapshot is a much more time-effective manner of separation, rather than waiting for them to resolve. See section 2.2 for a more in depth discussion of different detection methods.

Drag-tags can theoretically be added to both single-stranded DNA (ssDNA) strands of complementary double stranded DNA (dsDNA). The use of double-tagged DNA strands was

investigated theoretically by McCormick and Slater in 2005 and experimentally by Meagher and McCormick in 2006 (3). In these papers, Meagher presents equation 1.1 using different variables, describing α as "the total friction provided by the drag-tag, in terms of the number of additional uncharged monomers of DNA that would add equivalent friction" (3). However, this formula cannot explain why having two molecules of streptavidin attached to the DNA strand more than doubled the total amount of friction. They suggest

$$\mu = \frac{\mu_0}{N} \int_{\alpha}^{L+\alpha} (-0.65 + 0.62(\frac{n}{N})^{-\frac{1}{4}} + 0.62(1-\frac{n}{N})^{-\frac{1}{4}}) * (\frac{n}{N}) dn, \qquad (1.4)$$

where n represents the position of a charged monomer unit in the chain, and N is the total number of effective monomers (N=L+α for a drag-tag attached to one end, N=L+2α for a drag tag attached to both ends). A point of note for this formula is that if there is only a drag-tag at one end, then the α from the indices of integration disappear. In all cases, the addition of two drag tags to the ends of dsDNA and ssDNA adds more than double the expected drag due to end effects. It is suspected that this is caused by some form of bending of the DNA strand.

1.5 Capillary electrophoresis

For many applications of capillary electrophoresis, UV-fluorescence is appropriate (this is discussed in significantly more detail in section 3.2). However, for many applications of DNA in capillary electrophoresis, the use of a laser-induced fluorophore is necessary in order to observe the DNA in electropherogram form. A fluorophore is any molecule that releases a photon in response to absorption of the energy from a photon at a slightly higher energy level. For example, BODIPY-FL and YOYO-1, two fluorophores appropriate for ELFSE, are illuminated at 488 nm, and release a photon at 520 nm. This excitation wavelength is chosen because it is near, but not at the maximum of the fluorophore's quantum yield. Because the wavelengths of the input and output light are known, all other wavelengths can be filtered out

through the use of proper, light-filtering devices. This technique is also commonly used with fluorescence microscopy, described in significant detail in section 1.6. In capillary electrophoresis, lasers illuminate the sample from one side of the capillary, and a condenser and photomultiplier tube (PMT) are used to direct and sense the emitted light, and are built into the apparatus on the other side of the capillary. This means that only one filter is needed, as the excitation light is contained within a fiber optic, which then interfaces with the capillary in a closed very small environment, as shown in Figure 2. The bolded circle is the interface point between the laser and the capillary.

For the purposes of this discussion, normal polarity is considered (+)→(-), and reverse polarity is considered (-)→(+).

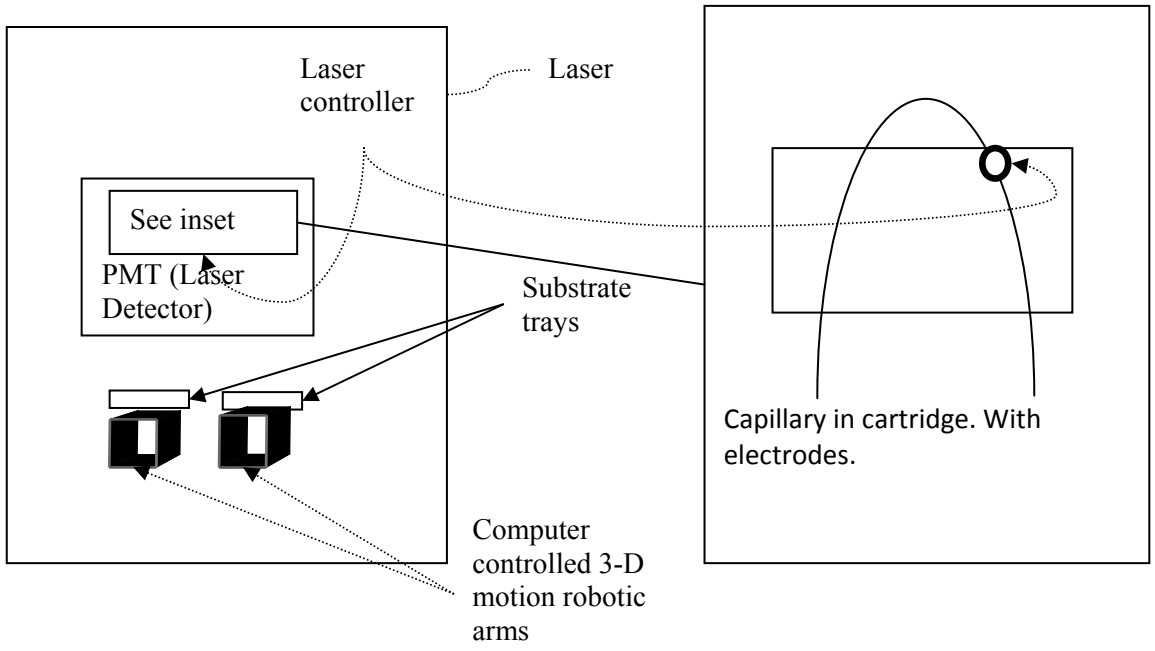


Figure 2: Schematic diagram of capillary electrophoresis machine

1.6 Fluorescence Microscopy

Fluorescence microscopes irradiate a specimen with a specific, desired band of wavelengths, called the excitation wavelength. Fluorophores are molecules that respond to these wavelengths and emit light at a higher wavelength, the emission wavelength, and lower amplitudes. Proper fluorescence microscopes use filters to eliminate wavelengths of light that are not the excitation wavelength on the way in, and the reflected emission wavelength in the light path to the camera or eyepiece. This allows for high contrast images with a very dark or black background. In order to do this, the excitation and ambient light must be eliminated in the light path between the sample and the eyepiece or camera, as the emitted fluorescence is several orders of magnitude weaker than the excitation light. This affects the limits of detection, as the contrast must be very high in order to see poorly illuminated structures (4).

Common fluorescence techniques illuminate the sample with either a laser or an ultraviolet source. This excitation light passes through a band-pass wavelength filter (excitation filter) and then reflects off a dichroic mirror onto the sample, illuminating the sample with intense light. If the wavelength is correct and the energy is high enough, then the molecule emits light at a slightly higher wavelength (emission wavelength). The emitted light is then passed back through the dichroic mirror and filtered by another band-pass filter (emission filter) at the desired wavelength. Good fluorophores have very little, if any, overlap between their excitation and emission spectra. Good

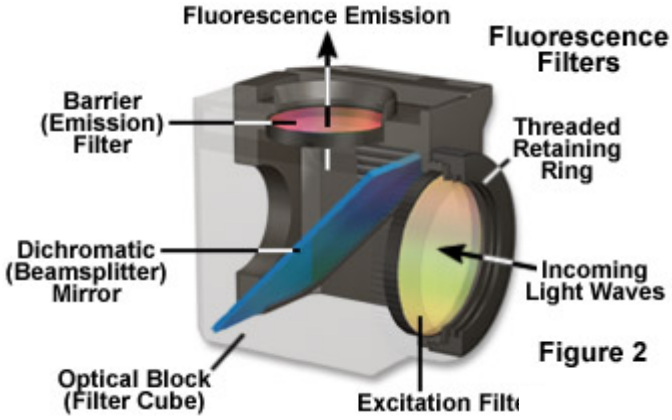


Figure 3: Filter Cube (4)

filters block out all of the excitation light; as a result, the only visible light is the emitted light. Unfortunately, the molecule radiates light in all directions, so only a fraction of the emitted light is seen. More often than not, these filters and mirrors are organized into a cube, as shown in Figure 3 (4). This setup is commonly used with microscopes and cameras, as the cubes fit into a rotating turret in the light path.

Chapter 2: Length-based separation of DNA in microfluidic chips based on current capillary electrophoresis technologies

2.1 Introduction

Capillary electrophoresis has been shown to separate DNA based on size when a monodisperse drag tag is attached to the DNA polymer chain. Microfluidic chips would use the same premise of capillary electrophoresis, but would take place in a microfluidic chip under a microscope and camera instead of in an automated, robotic machine using a laser and PMT. For this to work, significant equipment is necessary. See section 1.5 for a description of the apparatus.

Microfluidic research is particularly useful due to the potential to reduce DNA separation times to a matter of a few seconds with excellent resolution, in comparison to capillary electrophoresis or gels, which both require significant investments in time. In addition, the use of gels exposes the user to chemicals with carcinogenic (acrylamide and TEMED) and teratogenic (ethidium bromide) properties, and offers poor resolution for small molecules. Both capillary electrophoresis and microfluidic electrophoretic separations significantly reduce the amount of toxic chemicals needed, with the most dangerous chemicals regularly used being NaOH, HCl and methanol.

2.2 Advantages of microfluidic devices

As mentioned previously, capillary electrophoresis has been shown to work. Work in the Schneider lab has investigated a subset of capillary electrophoresis known as Micelle ELFSE. To this day, much of the research in this lab occurs in capillaries. However, numerous advantages to microfluidic chips exist that would be useful in furthering this research.

The biggest advantage is the ability to use snapshot detection as opposed to finish-line detection. Finish line detection is like runners running a race: whatever crosses the line of the detector is seen on an electropherogram as peaks comparing signal intensity to time. This means that a significant amount of time is spent waiting on the slowest DNA fragments to flow through the entire separation channel; in fact, some strands have so much drag that they are actually pushed backwards in the capillary. In comparison, snapshot detection takes a single image of the entire separation channel, then converts that into an electropherogram by looking at the light intensities with respect to distance down the channel. In equation(1.2), capillary electrophoresis varies t, whereas microfluidic systems vary L_d. Though microfluidic devices require finish line detection in addition to snapshot, they only use it to stop the separation when the leading DNA strand has maximized the separation distance. Once this is done, separation is considered complete, and a snapshot image is taken and converted to an electropherogram as described previously. Both the finish-line detection and snapshot detection can correlate either time or distance traveled with length of the DNA. For finish-line detection, the length to the detector is constant and time varies, whereas in snapshot detection, time is constant and the length to the detector is varied.

The next advantage is the theoretical minute quantities of DNA needed for separation in a microfluidic chip. In a capillary based system, 200 μL of 25 nM DNA is considered the standard injection volume needed, even if significantly less volume is actually injected. In comparison, using microfluidic chips and the correct setup, requires 0.3 μL of DNA diluted in distilled water and a small amount of buffer (5) to a total volume of 3 μL. Unlike capillary electrophoresis, this means that separation can be done in minute quantities and at low concentrations.

2.3 Drawbacks

As a result of these advantages, it was determined that an investigation of the mobility of DNA in microfluidic chips was warranted. The major question was what dye concentration would be required under UV fluorescence to be seen by a camera, or if a laser was necessary for detection. As dye concentrations correlate roughly to DNA concentrations depending on what preparation methods are used, this would also allow for quantification of DNA as well as determining the length of DNA in a similar mathematical manner to capillary electrophoresis.

One major drawback of microfluidic chips is that a device to use microfluidic chips would have to be designed from scratch. The chip used was a standard cross-T design, as shown in Figure 4. A cross-T chip was used to in order minimize the injection width; by injecting in the north-south channel first, then separating along the east-west channel with slight positive charges at both the north and south electrodes, the total injection width is the width of the north-south channel, an acceptable amount of variation with respect to the separation length of the east-west channel. Adapters to use this chip were needed in order to interface liquids (buffers, samples, waste, cleaning, etc.) with the inside of the chip. Four different design approaches were tested to interface with the chip. These will be explained in section 3.4.

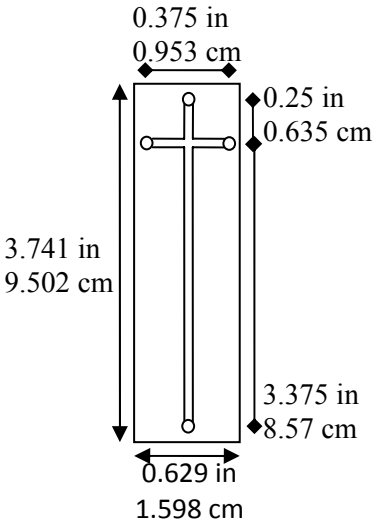


Figure 4: Chip dimensions. Thickness = 0.085 in, Inner Diameter of microfluidic capillary: 20 μm. Standard Cross-T chip design by Micralyne, Inc, Edmonton, Alberta, CA

Another major drawback is the cost of the chip. Though this chip is reasonably priced, the enormous cost of a custom-made chip in comparison to the easily replaced capillaries is

significantly higher. Thus, finding ways to make them reusable for repeated experiments is the only way to make them financially viable.

2.4 Apparatus and interfaces with microfluidic chips

Significant hardware was necessary for these experiments, as described in the following sections.

2.4.1 Data Collection with the microfluidic chip

Many attempts were made in designing an interface between the microfluidic chip and the outside world. However, a single hybrid device was designed that collected the advantages of all of the attempts prior. Each previous design used slightly different parameters which worked, but prevented the design from maximizing the benefits provided by microfluidic chips. Upchurch fittings and nanoports did not seal well to the chip, using either the epoxy rings or pressure, and also required larger volumes of buffers and samples than was desired. Pipette tips cut to size also required large volumes and had significant potential to leak, thus creating a salt bridge between wells. However, the use of a system with cut-end pipette tips with was ideal for cleanup at the end of the run.

The plates previously designed to hold pipette tips in place were used in three capacities. For filling of the channels with buffer solutions, the pipette tips were fitted into the wells, filled with whatever the desired buffers were and allowed to flow into the channels using head pressure. Upon completion of this, the pipette tips were removed, any spills were cleaned using a vacuum, and the wells were refilled using a micropipette or syringe. At this point, the plates were restacked into place, the tips of the electrodes were placed into the solutions in the wells, and were held in place by the holes in the pipette tips. The plates supported pipette tips that had been cut specifically to fit the holes in the top plate, without reaching the chip. How the plates and

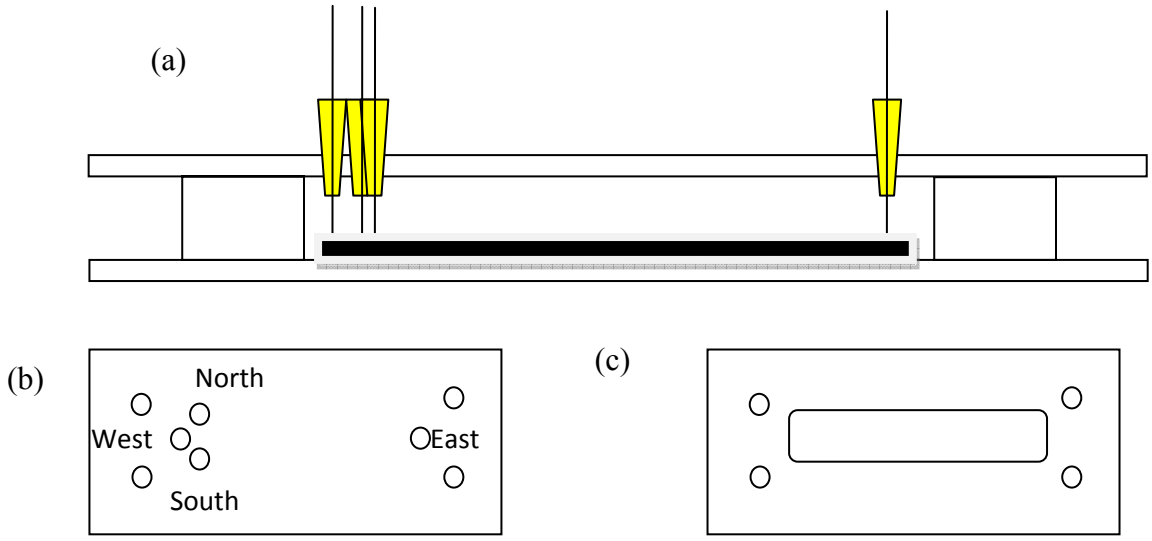


Figure 5: (a) Schematic of plates holding electrodes and chip in place. (b) Top plate. (c) Bottom plate.

accessories fit together, as well as not-to-scale schematics of the top and bottom plates are shown in Figure 5a, 5b, and 5c respectively. Finally, when cleaning the chips, 3 pipette tips were setup and filled up with running buffer, which was then sucked through the chip with a vacuum attached to the fourth well. This was found to be the best setup, and was thus chosen as the setup to use.

When setting up an apparatus like this, there are pitfalls to be wary of. First, it is important to clean up any leaks immediately; as these leaks dry, they can leave salt bridges, leading to current leakage and automatic shutoff of the power supply. Second, the use of nanoports on a standard cross-T chip is problematic – three nanoports need to be clamped in simultaneously on one end, which leads to misalignment and poor interfacing with the outside world. Third, it is extremely important that everything remain clean from dust, as this can clog a chip and make it unusable. In order to minimize contamination, vacuuming the top of the chip prior to use is important to clear any dust off the chip. Fourth, Kimwipes™ leave too much lint for this application and can scratch the chip and using lens paper is recommended instead.

2.5 Materials and Methods

2.5.1 Experimental reagents

Sodium hydroxide and sodium borate (borax) were purchased from Sigma-Aldrich Chemical Company. Ethanol was purchased from Fisher Scientific, Pittsburgh, PA. 10X Tris/Borate/EDTA (TBE) and all PCR reagents were purchased from Promega, Madison, WI. Nitrogen gas was purchased from Valley Gases, Wheeling, WV. BODIPY-FL was purchased from Invitrogen, Carlsbad, CA.

2.5.2 Hardware

In order to utilize microfluidic chips for DNA separation, significant equipment is necessary: a UV lamp (EXFO X-CITE 120, Lumen Dynamics, Mississauga, ON, Canada) to illuminate the dye molecule attached to the DNA; a high voltage power supply (μTk Microfluidic Tool Kit, Micralyne Inc, Edmonton, AB, Canada) to separate the DNA in free solution; a microscope (Eclipse TE-2000U, Nikon, Melville, NY, USA) and camera (Cascade 512B, Photometrics, Tucson, AZ USA) to observe the illumination of the dye passing through the channel, and computers to control the high voltage power supply and camera.

In addition, a standard cross-T chip produced by Micralyne Inc, Edmonton, Alberta, Canada was purchased and used for all microchip experiments. This chip was made of borofloat glass, a chemically resistant borosilicate glass.

2.5.3 UV test protocols

BODIPY-FL was serially diluted in H_2O. 25 μL of each sample was then placed in a 96 well plate in pairs. The plate was then tested in the Bio-Doc-it, a broad-spectrum UV illumeter.

Following the concentration profile from that, a similar experiment was run using the same concentration sweep, but on a microscope. One drop was placed on a microscope slide, a

cover slip was placed over it, and was then illuminated under UV light through a FITC cube. Note was made of whether or not it was visible via the microscope, the naked eye, and/or the camera with a live exposure.

2.5.4 Microchip Protocols

These protocols are adapted from protocols from Dr. Ketan Bhatt of CFD Research Corporation in Huntsville, AL. The first and most important step was to prepare the microfluidic chip by preparing the inner walls of the chip by soaking the chip in 10 mM sodium borate ($Na_2B_4O_7$*10 H_2O) overnight. This primed the capillary wall by eliminating any stray charges that may have accumulated on the walls during transport. It is important to vacuum the surface of the sodium borate solution prior to removal from the solution in order to remove any accumulated dust on the top of the solution.

Prior to running a DNA separation, it was important to prepare the channel walls for separations by activating the walls. Notation for channels is North channel/South channel/East channel/West channel, as noted in Figure 5. In order to this, the chip was filled with 10 mM NaOH, and then run under a current for 5 minutes at 300V/300V/300V/GND, with the GND varying to all 4 ports. This was run at the beginning of use for every chip, and after every third experiment on the chip. The protocol as previously described will be referred to as the 'start-up sequence,' though the buffers will change throughout the protocol.

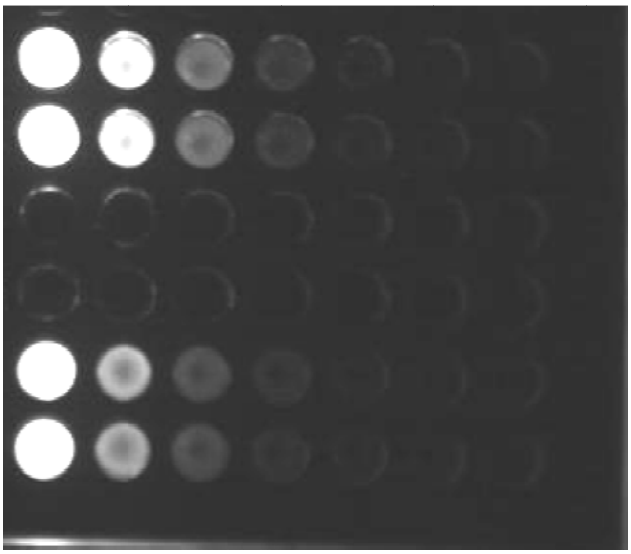
Following the sodium hydroxide start-up sequence, the chip was flushed with filtered nitrogen, and then refilled with a 1X TBE buffer. Once refilled with no air bubbles, the startup sequence was run again, but at 150V instead of 300V, as over-current shutdowns (a system error that stems from salt bridging and leakage) were common; operating the system at 150V eliminated most over-current shutdowns when salt bridges were prevented and eliminated.

At this point, the chip was ready for use. Though each method was slightly different, the separation protocols were fairly constant. The south well was vacuumed dry without pulling liquid out of the channel. The port was then filled with the experimental solution, and injected from the north to south channel using 1250/GND/GND/GND, and then separated down the E-W channel using 500/500/GND/2500. Variations of this protocol were used to try and discover what the minimum visible dye concentration using UV-fluorescence and a camera would be. Serial dilutions of BODIPY-FL were injected and separated from low concentrations to high concentrations, with concentrations ranging from 28-28000 nM. Because the ratio of BODIPY-FL to DNA is 1:1, and the intensity of BODIPY-FL is unrelated to whether or not it is bound to DNA, experiments were run using BODIPY-FL molecules that were not bound to any sort of DNA in order to find the minimum concentration of DNA required in order to use this system.

In order to visualize the BODIPY-FL in solution, light from a UV bulb was shown through a FITC cube, and a 10x objective was used. After focusing the objective on the cross of the microfluidic channels, the microscope was set to redirect all incoming light into the camera. At this point, the experiment was set to run by the protocol previously described. As it became harder and harder to visualize the sample with live imaging, various settings on the camera were adjusted, including contrast and exposure time. The exposure times tested were 0.1, 0.5, 1, 2, 5, 10, 20, and 30 seconds, and for ambient light testing under the blackout cage, 60 and 180 seconds.

This experiment was repeated under a variety of conditions and observation settings. This experiment was run with light-blocking curtains drawn and lights off and with a blackout cage with lights off and light-blocking curtains drawn. In addition, camera settings were adjusted as needed to try to enhance contrast and visibility of the dye.

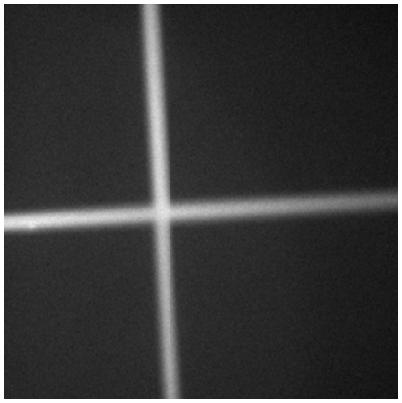
2.6 Results



L

Looking at the intensities of the wells on the Bio-Doc-it, it was found that the wells were clearly visible at concentrations as low as 28.8 μM BODIPY-FL. Using computer adjustments of contrast and brightness, it was found that the wells were visible at concentrations as low as 3.6 μM BODIPY-FL, as shown in Figure 6.

When using the microscope technique, it was found that the microscope and camera were significantly less sensitive on live settings than the naked eye. Using the microscope, the lowest concentration was 7.2 μM BODIPY-FL. Using the camera on a live setting, the BODIPY-FL was not visible at any concentration tested up to 28.8 μM BODIPY-FL. However, by the naked eye, the BOPIPY-FL was slightly visible at a concentration of 0.9 μM BODIPY-FL.



Numerous variables were tested in this setup, all involving the BODIPY-FL fluorophore. It was found that the minimum concentration of DNA that would be needed for this setup was on the order of 100 nM with a 10s exposure and the UV lamp set at maximum. However, similar testing was also done in microfluidic chips. As can be seen in Figure 7, in high concentrations, the BODIPY fluoresces well in the channels. In this image, the concentration is 1 μM in the channels.

2.7 Discussion

Because many DNA separations occur at DNA concentrations on the order of 10-20 nM, the 1:1 ratio of BODIPY: DNA suggests that BODIPY is not an intense enough fluorophore to be visualized with a camera and microscope in this application. Though the numbers seemingly match up, only femto- or atto- moles of DNA are flowing past the detector at any given time, so the dye needs to be visible at these minute concentrations. Additional major issues with how this experiment would have to be performed using BODIPY as a fluorophore include those discussed below.

The first problem is the low intensity of the BODIPY relative to the concentration of DNA. As previously mentioned, BODIPY-FL would need to be visible in significantly lower concentrations in order to make this setup functional. The use of special lenses or lasers would be able to make this concentration of BODIPY visible, but it is not feasible to do this while using the ultraviolet lamp setup. Nikon offers a 10x lens specific to fluorescence, but the benefit of this lens seems minimal in comparison to what is already on the microscope.

The second major issue is photobleaching. In past experiments, high concentration BODIPY was found to have lost significant intensity at 20 seconds when the UV excitation intensity was set to max. Because of the long-exposure times and exposure in areas that are not being visualized the microscope or camera, the use of UV leads to significantly wider exposure than desired. This is particularly problematic, since the concentration of DNA being used is so minute.

From here, there were a handful of different options that were considered. As previously stated, different optics or the use of a laser-based apparatus could have potentially been used to solve this problem. However, a better solution might be the investigation of a different dye with

either a significantly higher intensity (Alexa Fluor 488, Cy5, or Cy3), or a dye molecule that attaches to a DNA molecule numerous times, such as an intercalating dye (YOYO-1, TOTO-1, YO-PRO-1, TO-PRO-1). For experimental convenience, YOYO-1 became the next testable option.

Chapter 3: Length-based separation of DNA using intercalating oxazole dyes

3.1 Introduction

Because the intensity of BODIPY was too low for use in microfluidic chips with a microscope and camera setup, the next major question became what could be done to increase the intensity. Because of the collaborations available via my colleagues, it was decided that the use of a different dye would be the best option. Current protocols for ELFSE in the literature suggest that the most common method of making DNA fluoresce involves chemically binding a dye molecule to the DNA molecule. This is time consuming and can have poor yields when synthesized, and then separated by high-pressure liquid chromatography (HPLC). Better options must exist. Courtesy of Drs. Bruce Armitage and Danith Ly, an intercalating dye that had significant potential became my next research question. YOYO-1, an azole dye that intercalates between base pairs (Figure 8), can be used in free solution to dye DNA before, during, and after separation (herein referred to as pre-column, on-column and post-column labeling).

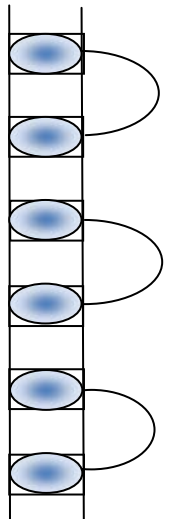


Figure 8: Intercalated DNA

In addition, research done by one of my colleagues suggests that YOYO-1, when intercalating pre-column, was leading to cleavage of DNA in solution, and as a result, a significant amount of previously unseen DNA was showing up at the tail end of the electropherogram. Because the DNA had been run through HPLC, it was assumed that it was something that was occurring during post-processing. As a result, one question in my new project was to try to figure out what that additional peak was, or to find a way to eliminate or reduce the peak at the tail end of the electropherogram.

3.2 Ultraviolet absorption vs. Laser-induced Fluorescence

When using capillary electrophoresis, two different illumination sources are available, both with advantages and disadvantages. Ultraviolet (UV) absorption does not require a dye to do DNA studies. However, in comparison to the other illumination standard, laser induced fluorescence (LIF), UV absorption requires significant concentrations of DNA. The signal from UV absorption's signal also is lower and tends to have broader peaks in comparison to LIF. LIF is a better standard for numerous reasons. For starters, the signals can be significantly more intense than those of UV. In addition, the concentrations of DNA, depending on the dye used, can be miniscule in comparison to UV, on the order of single nanomole concentrations. Using LIF, it is possible to see yoctomoles as they pass the detector, when illuminated with the correct laser and dye (6). The major disadvantage of using LIF is the additional time spent adding a fluorophore to the DNA molecules. However, considering that in order to use micelle ELFSE, an alkane tail is required to be added to the DNA molecule anyways, the addition of a fluorophore to the end of that alkane tail before binding the tail to DNA makes it easy enough to use an additional fluorophore.

3.3 Advantages and drawbacks of capillary electrophoresis

Capillary electrophoresis (CE) has many advantages over other technologies. The biggest is the option for high throughput testing. Numerous runs can be programmed to run through the same capillary before it needs to be replaced. This allows for more testing to be done in one programming session. In addition, because a capillary is easy to clean out or replace, should anything happen to the capillary, it can easily be thrown out and replaced by a new capillary in ~10 minutes. For capillary gel electrophoresis (CGE), high throughput means the ability to perform multiple separations on the same gel, as it is very easy to push everything out of the

current capillary using pressure, re-form a new gel, and separate all over again, assuming that the gel is not usable a second time.

Additionally, the small volumes necessary for one run of capillary electrophoresis means that the same samples can be reused numerous times. Though the concentrations are on the nanomolar scale, a good CE machine using LIF and a good detector should be able to sense zepto- or yocto- moles when passing the detector, as previously mentioned.

When used properly, the system should be able to resolve differences as small as a single base pair. Because the detector is sensing individual dye molecules that fluoresce very intensely, it does not require significant quantities of DNA in order to detect something.

There are also a number of drawbacks to using capillary electrophoresis. The first major drawback is the need to covalently bind a fluorophore of some variety to the DNA molecule. This means that there is a significant loss of DNA concentration due to both the chemical processing in the addition of the fluorophore (and tail for micelle ELFSE), as well as post-processing separations including HPLC, dehydration and re-suspension. This can be rectified through the use of intercalating dyes that interact with the DNA chain instead of needing to be covalently attached to the end of a DNA chain. In turn, this leads to the issue of poor correlation between signal intensity and concentration.

3.4 Chip loading

Sieben and Backhouse (2005) investigated various forms of labeling DNA for separation. There are three different potential operating regimes. The first is pre-column DNA intercalation, in which DNA is intercalated prior to any separation. The second is on-column labeling, in which the intercalating dye is mixed into the buffer or sieving matrix. The final is post-column separation, in which the separation occurs first, and then the DNA is labeled shortly before the

separation is complete. It must be noted that this paper is done entirely in sieving microfluidic chips, not free solution or gels; the end goal of this investigation is to move this research back to microfluidic chips (5).

The paper shows the effects of all three types of intercalation as being successful with various signal intensities. However, in order to make post-column separation viable, significantly higher concentrations of dye were used. In addition, the dye interacted significantly with the sieve. As a result, the dye never flows past a certain point in the microchannel. The results suggest that post-column intercalation leads to the best reproducibility of the time of a peak, but also gave the widest variation in signal. In addition, post-column labeling will lead to a decrease in overall signal as the number of runs per dye injection increases; the DNA will slowly diminish the amount of dye remaining until not enough remains to intercalate the entire concentration of DNA. This is partially due to nonuniform labeling of the DNA as it passes through the labeling zone and depletion of the labeling zone as the longer strands pass through. In addition, the use of these techniques suggests that the lower limit of detection is 9 fg/μL, though the setup as they used had a lower limit of 66 pg/μL.

Additionally, Sieben did some research involving adsorption of the dye molecules to the wall. It was found that with passivation (PVA coating), the YOYO molecule flowed down the entire channel in a plug, and labeled the DNA in free solution, similar to on-column labeling. However, because the PVA did not remain bound to the walls between runs, it was found to be more convenient to adsorb the dye molecule to the wall of the capillary and create a ‘labeling zone’ as found in post-column labeling, thus eliminating one advantage of on-column labeling over post-column labeling. In order to make the dye last for more than 2-3 runs, it is important to keep the DNA concentrations below 50 ng/μL (5).

There are a few other key points of note from this paper. CGE-based separations with on-column labeling take 25 minutes, significantly longer than either ELFSE or microchip CE. The reproducibility of post-column labeling is comparable to commercial systems, and better than on-column labeling. However, the peak area is significantly less reproducible than other techniques. They suggest that this is due to minor fluctuations that average out over the longer time span of separation in CE, but that microchip CE does not have enough time or separation distance to allow for this to happen.

3.5 YOYO-1

YOYO-1 is a dimeric intercalating dye that binds each fluorescent monomer between alternating pairs of DNA. This molecule's emission spectrum is shown in Figure 10 (7), and its structure is shown in Figure 9 (8). YOYO-1 can intercalate throughout the length of the DNA, leading to significant quantities of the dye throughout the DNA strand, and a significantly increased signal. In addition, YOYO-1 only fluoresces when bound to DNA – the dye does not fluoresce under either ultraviolet or laser-induced fluorescence when in free solution (9).

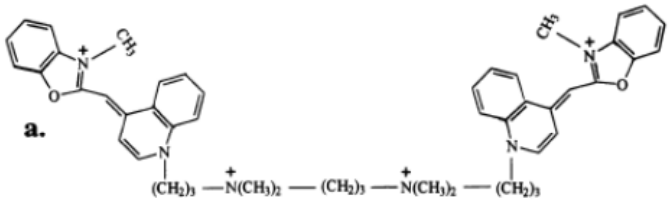


Figure 9: YOYO-1 Structure

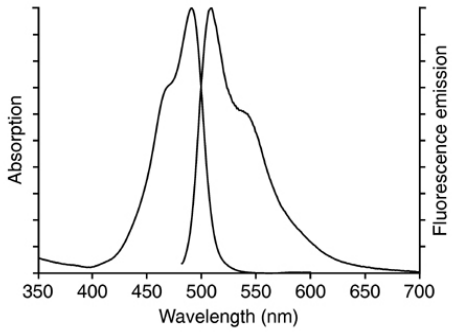


Figure 10: YOYO-1 Absorption and Emission Spectrum

As shown in Figure 8, the two ends of the dye molecule intercalate in between base pairs, stretching the DNA slightly from a normal separation of 3.4 Å between base pairs to ~3.8 Å (10)

between base pairs. This makes the DNA strand more susceptible to fracture, and led to an investigation of the different intercalation paradigms described in chapter 3.4.

YOYO-1 has also been well characterized in the literature. Literature suggests that bis-intercalating dyes such as YOYO-1 give band-splitting and band-broadening. In turn, this would lead to poor resolution and sensitivity (7). This makes sense, but this is also very easily rectified by using the correct ratio of dye to base pairs. According to Figeys et al. the ratio with the highest intensity is 10 bp: 1 dye molecule (11). In comparison, Reuter and Dryden suggest that saturation is achieved at a ratio of 3 bp: 1 dye (10). This phenomenon also suggests that post-column labeling would be the most effective form of labeling, even if the signal is dramatically reduced, due to its ability to limit any electrophoretic differences due to different binding ratios. Carlsson also suggests allowing very long times or elevating the temperature as ways to equilibrate the DNA and dye, but again, this problem is eliminated by post-column labeling (7).

A few other papers suggest using mono-intercalators like YO-PRO-1 or TO-PRO-1 as a way to eliminate double bands, even though there is a significant loss of intensity using the mono-intercalator. However, the equilibrium constant of binding (K_b) is 3 orders of magnitude lower than that of YOYO-1 or other bis-intercalators (9). Thus, mono-intercalators are not as good as their respective bis-intercalators. Reuter experimentally shows concentration-based time constants (τ) for intercalation. Extrapolating using an exponential fit of 2627.4*concentration^(-0.963) yields a time constant of 3.39 seconds, and a first order rate constant of 0.29 s^{-1} (10). Reuter goes one step further, claiming that at 40 nM, it is possible to achieve almost complete DNA saturation in ~100 seconds, something that conflicts with Carlsson's suggestions of saturation and equilibrium.

Finally, it appears that saturating the molecules dramatically increases the binding stability of the molecules YOYO-1 to the DNA strand, something that is unexplained by Carlsson (7). This could be caused by cooperativity, or by unforeseen interactions between YOYO-1, DNA, and the non-crosslinked polymer used in Carlsson's testing protocols.

3.6 Apparatus and setup

As shown in Figure 2, the P/ACE MDQ uses robotic arms to change the reagents as necessary. Programming is done in proprietary Karat32 software. Each arm has a 6x6 tray for various reagents and samples. Capillary lengths for all tests were 20 cm to the detector (L_d), and 30 cm total length (L_t). Reagents were placed in capped, 1.5 mL glass vials, and samples were placed in 200 μL plastic sample vials.

3.7 Materials and Methods

All capillary electrophoresis experiments were performed in a Beckman Coulter P/ACE MDQ Glycoprotein system.

3.7.1 DNA synthesis and preparatory reagents

All PCR reagents were purchased from Promega, Madison, WI. Dimethylaminopyridine (DMAP) and triethylamine (TEA) were purchased from Sigma-Aldrich, St. Louis, MO. Triamine pyrophosphate (TPP) and dipropyl disulfide (DPDS) were purchased from Fluka, a division of Sigma-Aldrich. Dimethylsulfoxide (DMSO) was purchased from Alfa Aesar, Ward Hill, MA. Glacial acetic acid and acetone were purchased from Fisher Scientific, Pittsburgh, PA. The storage container for cetyltrimethylammonium bromide (CTAB) was not found as of the writing of this paper.

3.7.2 Experimental reagents and supplies

Sodium hydroxide, C16 tails, deoxyribonucleic acid from calf thymus, and hydrochloric acid were purchased from Sigma-Aldrich Chemical Company. POP-6 was purchased from Applied Biosystems, Foster City, CA. Poly-N,N-dimethylmethacrylate (PDMA) was purchased from Scientific Polymer Products, Ontario, NY. Glycerol was purchased from MP Biomedicals, Solon, OH. Ethanol and methanol were purchased from Fisher Scientific, Pittsburgh, PA. 10X TBE and all PCR reagents were purchased from Promega, Madison, WI. All DNA oligomers were purchased from IDT Technologies, Coralville, IA. YOYO-1 and BODIPY-FL were purchased from Invitrogen, Carlsbad, CA. Benzyl alcohol was purchased from Acros Organics, Geel, Belgium. 50 μm ID tubing was purchased from Polymicro Technology, Phoenix, AZ.

3.7.3 DNA preparation

Oligomers of DNA were received from IDT technologies. As needed, PCR products were produced using the protocol as shown in appendix A.1, making sure to add the TAQ last, and keeping everything cold prior to injection into the PCR vials. The products were then separated using high-pressure liquid chromatography (HPLC). The desired products were saved and stored, and the waste was discarded.

One compliment to the DNA needs to have a tail attached in order to use micelle ELFSE. In order to do this, the protocol shown in appendix 2 was followed.

In order to make dsDNA, 2 complimentary DNA strands were mixed to a concentration of 25 nM of the dsDNA. One compliment was labeled with an alkane tail, or an alkane tail attached to a fluorophore (BODIPY and Cy-5).

3.7.4 Capillary preparation protocols

There are two different protocols depending on whether the system is running with or without surface passivation. With surface passivation (EOF suppressed), 10% w/v POP-6 is

hydrodynamically driven down a capillary precleaned with NaOH, with a 10 minute wait, then driven out of the capillary into a waste vial. With EOF suppressed, the system ran with reverse polarity.

Without surface passivation (EOF active), the inner surface of the capillary must be activated. In order to do this, the inner surface of the capillary was washed at 20 psi with the protocol shown in Table 1, with all volumes moving into a waste vial. With EOF active, the system operated with a normal polarity.

Table 1: EOF active preparation protocol	
Chemical	Time (min)
1M HCl	20
Millipore Water	5
50% Methanol, 50% Water	20
Millipore Water	5
1M NaOH	20
Millipore Water	5

In addition, between runs without surface passivation, a protocol of 1 minute each of H_2O, NaOH, H_2O was run, followed by 5 minutes of buffer. If the system ran with surface passivation, then the protocol was to flush 1X TBE with 0.1% POP-6, the running buffer, through the capillary at 20 psi for 5 minutes. At this point, the various injection methods occurred, as described in section 3.7.5-7.

3.7.5 Pre-column labeling

In pre-column labeling, dsDNA was mixed with YOYO-1 at a ratio of 4 BP DNA to 1 molecule of YOYO-1. For example, for a 40 base pair polymer strand (40mer) of dsDNA, that meant a ratio of 10:1 YOYO-1: DNA. Once mixed, the solution was allowed to sit for a minimum of 30 minutes. However, due to the separation protocol, the DNA often sat for 2 hours or longer prior to separation. This mixture was then injected into the capillary.

3.7.6 On-column labeling

In on-column labeling, dsDNA was prepared in one sample vial and 1 μM YOYO-1 was prepared in a different sample vial. These were then placed in trays on opposite sides of the

capillary. During the separation protocol, DNA was injected into the capillary and separated to a known point in the capillary. At first, a single YOYO-1 injection was performed, followed by separation in the normal buffer. A different protocol eliminated the injection of a plug of YOYO-1. Instead, at this point in the separation, the end of the capillary that had not had DNA-injected was moved into a vial containing 1 μM YOYO-1, and the remainder of the separation continued with the YOYO-1 flowing in the opposite direction to DNA due to their opposite charges.

3.7.7 Post-column labeling

With post-column labeling, YOYO-1 is hydrodynamically injected for 11.1 seconds at 20 psi to leave a labeling zone that is ~1 cm long beyond the detector. This number was calculated using the Hagen-Poiseuille equation as the time needed to be injected into a capillary to 11 cm. By performing the injection in this manner, the YOYO-1 is essentially smeared onto the wall. The capillary is then allowed to sit for 10 minutes in order for the YOYO-1 to adsorb to the wall. After the 10 minutes, the YOYO-1 is pushed out of the solution by buffer at 3 psi for 99 seconds, more than enough to push out the YOYO-1 in solution but, hypothetically, leave significant amounts of YOYO-1 adsorbed to the wall.

Once done, the DNA was injected like pre-column intercalated DNA was injected.

3.7.8 Injection protocols and types

There are two types of injection protocols. The first is hydrodynamic injection, in which the injected material, be it YOYO-1, buffer solution, or any other is injected with pressure into the capillary. This is done at 0.5 psi for 30 seconds, and can be used regardless whether EOF is active or suppressed.

The other option is electrokinetic (EK) injection. In this case, an electrophoretic motion moves the sample into the capillary. Without surface passivation, however, EOF is dominant,

and significant amounts of the desired sample cannot be injected into the capillary. This is typically performed at 4.0 kV for 30 seconds.

The biggest advantage to EK injection is the very flat profile seen at the end of the injected sample. With hydrodynamic injection, one will see a parabola-shaped plug. Examples of both can be seen in Figure 11. With electrokinetic injection, the interaction with the YOYO for on-column labeling was supposed to have a perfect plug-to-plug interface, leading to minimal mixing zones, just two plugs that pass one another and cause minimal broadening.

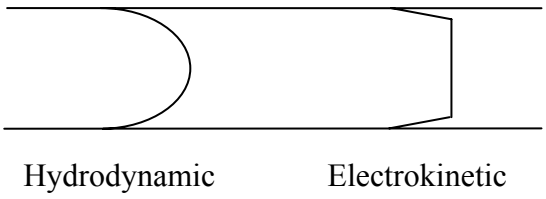


Figure 11: Injection shapes

3.7.9 Separation protocols

At the beginning of each run, the capillary was prepared as described in section 3.7.4. At this point, pre-column labeled DNA (3.7.5) would be injected, followed by the separation protocol. For on-column labeling (3.7.6) of DNA, unlabeled DNA was injected at this point for the initial separation distance, marking the beginning of the on-column labeling protocol. For post-column labeling (3.7.7) of DNA, the YOYO-1 was injected first as described in the protocol.

Once both DNA and YOYO-1 are both in the capillary for each respective form of labeling, the program is set to run for 10 minutes, separating with reverse polarities for passivated surfaces, and normal polarities for unpassivated surfaces.

3.7.10 Calf Thymus capillary cleanup protocol

Once the separation protocol was complete, it was necessary to clean the capillary from any YOYO-1 that had adsorbed to the wall, as it would no longer be a clean coating on the wall,

which could lead to minor diffusion and mixing effects. In order to do this, a high concentration of 'junk' DNA was flowed through the capillary at moderate pressure and for a long enough time to eliminate all of the YOYO-1 that had adsorbed to the wall. Though not necessary between every run, cleaning the capillary followed by placing a new layer of YOYO-1 allowed for EOF drift to be minimized and placement of a new, uniform layer of YOYO-1 on the capillary wall.

3.8 Results

Much research at first was done with on-column labeling. At first, it was investigated whether or not a plug of YOYO-1 would be sufficient to label the DNA flowing in the column. The first variable tested was the effect on the initial position of DNA. Though YOYO-1 was flowing down the capillary, the intensities never increased enough to suggest that there was significant intercalation. The signal of YOYO-1with pre-column intercalation is significantly more intense than BODIPY-FL. When using BODIPY at 25 nM, the expected signal intensity is on the order of 0.25 RFU, whereas the use of YOYO-1 is on the order of 1 or greater. In Figure 12, the DNA (t=0) refers to the position when the YOYO-1 was injected in section 3.7.7. As can be seen, there are three peaks. It is suspected that the slowest elution peak is the free-solution DNA, the middle elution peak is the double-stranded DNA, and the fastest elution peak is the modified single-stranded DNA.

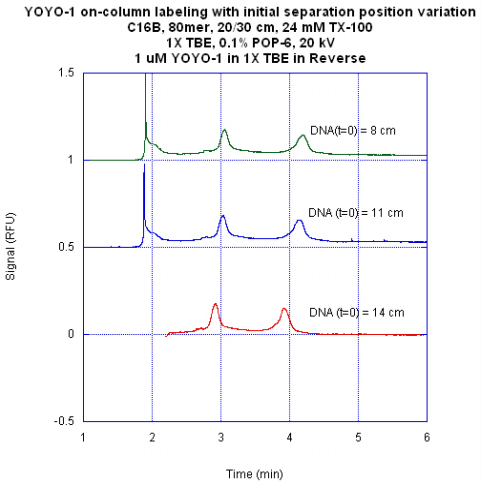


Figure 12: On column labeling with various DNA start points

The next investigation of on-column labeling used a single 24mer double-stranded DNA modified with a C16 tail with YOYO-1 in the buffer solution at the (+) electrode, which allowed it to flow electrophoretically down the column. As shown in Figure 14, the intensity is

significantly higher than any of the peaks shown in Figure 12. However, there is a significant tail and a second peak in the data.

The next step taken was to investigate the use of post-column labeling. It was found almost immediately that with EOF suppression, YOYO-1 did not adsorb to the walls of the capillary. As a result, the remainder of the experiments were performed with EOF active.

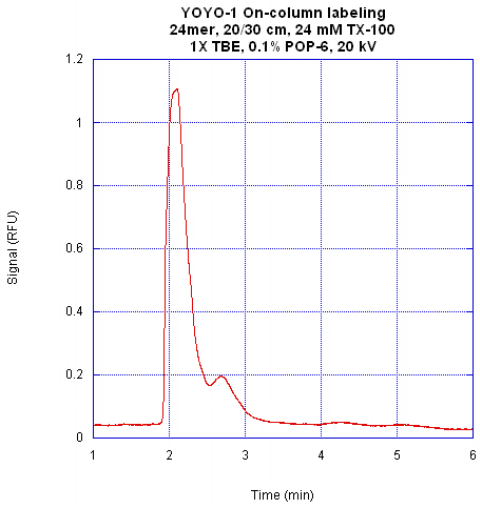


Figure 14: On-column labeling with YOYO-1 in reverse buffer

The next investigation was the use of post-column labeling in an EOF active system. There were a few important questions to answer before this system could be tested. The first was what sort of timeframe was necessary for YOYO-1 to adsorb to the wall. In order to do this, the post-column labeling procedure was run with wait-times of 0, 1, 3, and 10 minutes with YOYO-1 in the capillary, followed by a standard separation protocol, then looking to see which timeframe had the highest signal intensity. It was found that the 10 minute wait led to dramatically increased wall adsorption and, as a result, a higher signal.

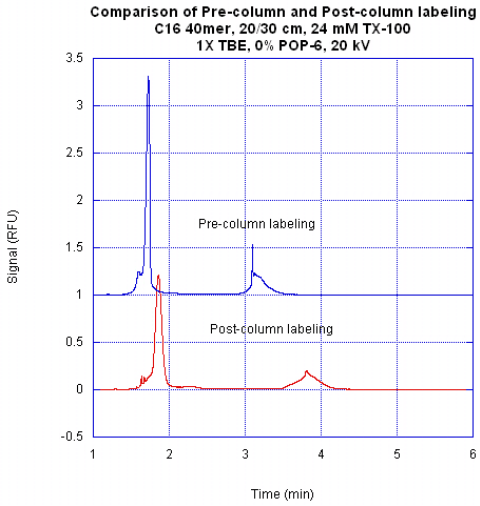


Figure 13: Post-column labeling

Once this 10 minute wait time was verified, experimentation began with on-column labeling. Experimentation with 40 bp and 80 bp was the standard mix. In order to verify the validity of post-column labeling, a simple run was performed in order to compare pre- and post-column labeling, as shown in Figure 13. Courtesy of my colleague, John Goldman, the top line

of Figure 15 has 24, 40, and 80 bp. It must also be noted that John's samples are run at 30 kV, whereas the rest are run at 20 kV. Table 2 compares the areas of each peak, corresponding roughly to the signal strength; it shows that the post-column signal is a dramatic decrease from the pre-column due to the lack of dye molecules that intercalate in the small labeling zone.

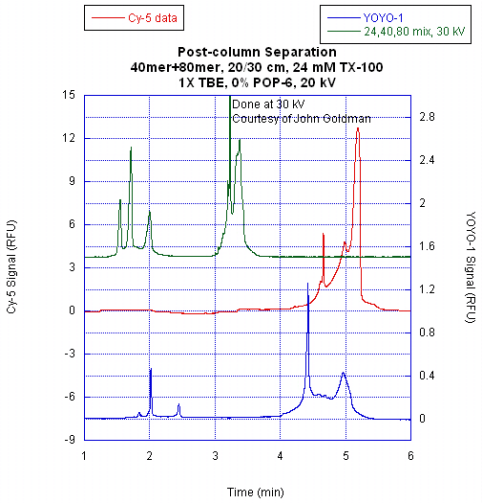


Figure 15: Comparison of Pre-column and Post-column labeling

Another question was what effect, if any, micelles may have on YOYO-1 signal intensity. It has been hypothesized that the use of surfactant micelles would strip the YOYO-1 off of the wall due to both having significant hydrophobic character. However, as Figure 16 shows, the signal intensity is approximately the same. In fact, the total areas under all of the peaks (0.165 for 0 mM TX-100, 0.194 for 24 mM TX-100) are higher for the micellated separation.

Table 2: Integrated areas under curves for figure 15

	Pre-column	Post-column
40mer	0.051	0.012
80mer	0.036	0.0057

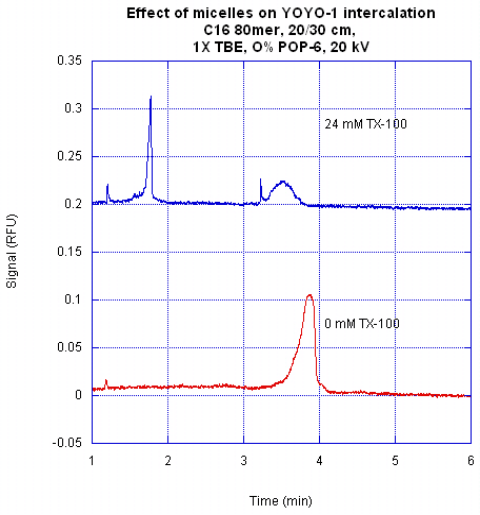


Figure 16: Effect of micelles

3.9 Discussion

When looking at Figure 14, one sees very wide bands in comparison to those shown in Figure 13. There are a number of reasons why. The first is shown in Figure 17. Because the YOYO-1 is labeling the flowing DNA in free solution, the dye is not intercalating instantly because intercalation

depends as much on orientation and position as concentration of dye. When a molecule of YOYO-1 intercalates with the DNA, it might be the only dye molecule attached; each successive addition of a molecule increases drag slightly. As a result, the DNA peak broadens. Without instantaneous labeling or a distinct labeling zone, it will eventually reach equilibrium, but by that point, the bands will have already broadened, which dramatically reduces the possible resolution.

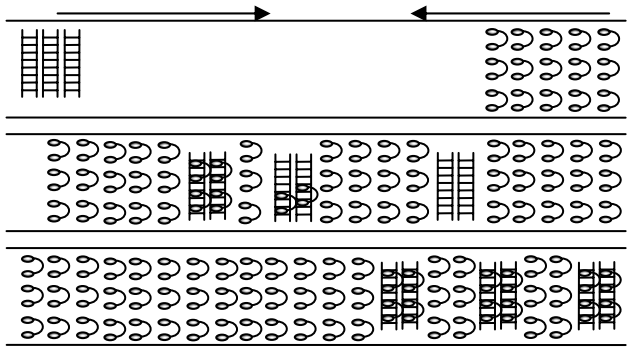


Figure 17: On-column labeling

In looking at the results as shown in Figure 14, there is a significant tail and a very small peak before the concentration of the DNA reaches zero. There are a handful of potential reasons for this, many of which are related to band broadening effects from both micelles and YOYO-1. As diagrammed in Figure 17 and previously discussed, YOYO-1 does not instantly intercalate DNA, and takes a significant amount of time to reach equilibrium. With pre-column intercalation, the standard intercalation time is 30 minutes, whereas with on-column, the total amount of time for DNA and YOYO-1 interaction is often less than 1-2 minutes before it crosses the detector. Yes, significant quantities of YOYO-1 will intercalate the DNA, but there will also be YOYO-1 with one of the dimers hanging off, and imperfect ratios to the different DNA strands. In addition, there will be some, albeit minimal interactions with the wall, even though the walls are passivated. When EOF is active, there can be significant wall effects and interactions with the Debye layer. Because POP-6 reduces the effect of the Debye layer, YOYO-1 and DNA are likely to have less interaction with the wall. Finally, the micelles also can lead to

significant sweeping effects that sweep out the back side of the peak, leading to significant tailing.

Based on the literature, post-column labeling should theoretically be the best way to eliminate any band broadening caused by imperfect intercalation by YOYO-1, but the data suggests that it is a poor alternative. Post-column labeled DNA should theoretically move faster due to the significant charge on DNA (reduced by YOYO-1).However, due to the very short labeling zone in the post-column system, the signal intensity is dramatically reduced, and there is little decrease in the width of the band due to the need for hydrodynamic injection. In the literature, much of the labeling is done in either gel electrophoresis or with capillary gel electrophoresis, which suggests that there might be an effect from the gel.

In addition, it is important to question the additional peaks at 4-5 minutes. What are these peaks? In Figure 13, the unmodified Cy-5 labeled peak appears at a time similar to that of the large peak, which suggests that this large peak is DNA that is moving at the free-solution mobility, with no interaction with micelles. However, considering how the oligomers are processed, this seems unlikely. After the alkane tails are added to the end of the DNA, the system is run through HPLC, which suggests that any off-products should be eliminated from the mixture. However, this is seemingly not the case.

Figure 16 also suggests that micelles may actually have some sort of enhancing effect on the YOYO-1. Considering that the micellated version of this experiment was performed after the micelle-free version, suggesting a lower YOYO-1 concentration in the injection vial, there is only one good reason for this. DNA flowing in this manner has minimal radial diffusion due to the plug flow. When DNA flows as a plug in free solution with no micelles, and with only diffusion and wall effects affecting the mobility and variance of the DNA, all of the DNA flows

through the labeling zone at once with only the edges of the DNA zone interacting with the wall (and the YOYO-1 layer), leading to decreased availability of DNA sites for the YOYO-1, as the DNA would need to pull the YOYO-1 off of the wall. In contrast, when micellated, there is length-based separation, and as a result, a lower concentration of DNA flowing past the detector at any one given point. This means that a greater percentage of intercalation sites are exposed on the plug of DNA as it passes through the labeling zone, leading to increased intercalation and a brighter signal in comparison to micelle-free systems.

Chapter 4: Summary, Conclusions, and Future Work

4.1 Summary

The major issue thus far has been that for free-solution DNA, it seems as if pre-column labeling with YOYO-1 is the most likely solution to moving this system back to microfluidic chips, the ultimate end goal of the project. The ability for YOYO-1 to enhance the brightness of DNA significantly under pre-column labeling means that any brightness issues could be fixed by YOYO-1. In addition, Nikon's MicroscopyU suggests that YOYO-1 will illuminate under a B-1E (FITC) filter-cube, exactly like BODIPY (12).

4.2 Conclusions and Future work

In chapter 2, theoretical advantages of microfluidic chips in comparison to capillary electrophoresis were found to include a shorter separation time due to the use of snapshot detection, higher resolution and minute quantities of DNA. However, due to BODIPY-FL not being intense enough, an investigation of other dyes was necessary. The use of capillary electrophoresis was chosen as a testing standard due its easy availability.

Significant research still must be performed in order to find out if YOYO-1 will work well as a good marker for DNA separation. Because YOYO-1 and other similar dyes are as good or better than fluorescein and rhodamine in CGE (5), but do not seem to work as well in free solution, there might be a similar transient interaction between DNA and YOYO-1 like micelles and DNA in micelle ELFSE. The major difference in this case is that the cross-linked gel in CGE would restrict the movements of the molecules and prevents significant radial diffusion, keeping the YOYO-1 and the DNA close enough together that the transient interaction would become a semi-permanent interaction. As a result, the DNA would be able to pick up the YOYO-1 in free solution at any point in the separation without a significant effect on the electropherogram peak

width during CGE; however, in ELFSE, there is no restrictive bound for the YOYO-1 and DNA complex. This would lead to radial diffusion between the two molecules, in which some YOYO-1 molecules might separate away from the DNA. Investigating this question is important in order to find out whether or not YOYO-1 is appropriate for use in free solution.

In addition, the signal for pre-column intercalation of DNA is high enough to suggest that, though there may be some stripping of the YOYO-1 off the DNA strand during separation, the bond strength and/or hydrophobic interactions of YOYO-1 with DNA is high enough that there is negligible stripping. An investigation of interactions between YOYO-1 and DNA is another important question to answer with respect to this system.

In addition, there is a question of the interactions between micelles and YOYO-1.As previously mentioned in chapter 3.8, data suggests that YOYO-1 binds to the walls of a capillary in an EOF active system. Due to the very low signal strength, there is a strong possibility that the YOYO-1 molecules are then stripped off the walls of the capillary by micelles in solution. The hydrophobic interactions between the inside of the micelle and the YOYO-1 molecule, which also has significant hydrophobic character, suggest that not only are the micelles preventing interactions between the YOYO-1 and DNA, but in fact are holding unbound YOYO-1 molecules on the inside of the micelles. As a result of this, there is significantly less YOYO-1 available to interact with the DNA, thus leading to a dramatically reduced signal when the YOYO-1 is intercalated on-column or post-column at the same YOYO-1 concentrations as pre-column intercalation. Whether or not there is significant interaction between YOYO-1 and micelles is a potential key to the use of YOYO-1 or any other hydrophobic dye molecules.

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Appendices

A.1 PCR Protocol

Stock Concentrations				Date:	12/5/2011
[primer ex]	5	mM		Type:	symmetric
[primer lim]	5	mM		F primer:	a-m13
[MgCl2]	25	mM		R primer:	fp5
[dNTPs]	10	mM		length:	259
[TAQ]	0.5	U/mL		T_a:	55
[SYBR]	0.001			# cycles	30
[template]	2.5	ng/mL			
Desired Concentrations (constant)					
[template]	0.1	ng/mL	0.04		
[primer 1]	500	nM			
[primer 2]	500	nM			
[nucleotides]	0.2	mM			
[$MgCl_2$]	2.5	mM			
[TAQ]	0.025	U/mL	0.04		
[SYBR]	0	X			
[salt]	0.05	M			
Volume Calculations					
V_T	25	mL			
****constant components****			100	% of V_T	
Order		Total:	25	mL	
1	Buffer		5	mL	
2	MgCl2		2.5	mL	
3	H_2O		9.75	mL	
4	primer rev		2.5	mL	
5	primer fw		2.5	mL	
6	template		1	mL	
7	dNTPs		0.5	mL	
8	TAQ		1.25	mL	

A.2 Tail Labeling

This protocol will enable labeling of amine terminated DNA with an aliphatic tail, with or without a fluorophore.

Reagents

Unless reagents are known to be fresh, remake each solution in 1.5mL centrifuge tube before continuing. Ensure that DMSO has been dried over molecular sieves and filtered through a hydrophobic syringe filter before mixing reagents.

1. 2mM of amine terminated DNA in DNA water
2. 5mM of aliphatic tail in DMSO
3. 0.15M CTAB (**C**etyl**T**rimethyl**A**mmonium **B**romide) in DNA water
    a. MW = 364.45 g/mol, add 54.7mg to 1mL DNA water
4. 1.0M DMAP (4-**D**i**M**ethyl**A**mino**P**yridine) in DMSO
    a. MW = 122.17 g/mol, add 122.17mg to 1mL DMSO
5. 0.5M TPP (**T**hiamine **P**yro**P**hosphate) in DMSO
    a. MW = 425.31 g/mol, add 212.655mg to 1mL DMSO
6. 0.5M DPDS (**D**i**P**ropyl **D**i**S**ulfide) in DMSO
    a. MW = 150.24g/mol, add 75.12mg to 1mL DMSO
7. Pure TEA (**T**ri**E**thyl**A**mine)
8. 0.1M TEAA (**T**ri**E**thyl**A**mmonim **A**cetate) in Millipore Water
9. 2.0 w/v% $LiClO_4$ (Lithium Perchlorate) in Acetone
10. HPLC grade Acetonitrile

Procedure

DNA Solution

1) In a 1.5mL centrifuge tube, mix 5µL of 2mM amine terminated DNA with 5µL of 0.15M CTAB solution
    i. CTAB is a cationic surfactant that will bind to the negatively charged aDNA and cause them to precipitate into a larger pellet after drying under low salt conditions
2) Vortex solution briefly and vacuum centrifuge for 30 minutes until dry

Reaction Solution

1) Place CTAB, DMAP, TPP, and DPDS in 80°C heat block to ensure reagents are in solution. TPP is usually cloudy and may take several minutes at temperature to dissolve into solution
2) In a new 1.5mL centrifuge tube add the following volumes in this order. Perform all mixing in a fume hood.
    i. 20µL of 5mM aliphatic tail
    ii. 1.0µL of pure TEA
    iii. 5.0µL of 1.0M DMAP
    iv. 5.0µL of 0.5M TPP
    v. 5.0µL of 0.5M DPDS
        i. NEED TO LOOK INTO WHAT THE MECHANISM IS EXACTLY. TEA ACTIVATES THE COOH GROUP, DMAP AND TPP ACT AS LEAVING GROUPS WHILE DPDS STABLIZES THE REACTION. CHECK PAPERS AND CHEMISTRY

3) Vortex solution and rest for 20 minutes at room temperature. The mixture needs to activate before being added to the dried aDNA.

Creating the labeled DNA

1) Once the aDNA has been dried and reaction solution activated, add the 36µL of reaction solution to the dry aDNA centrifuge tube.
2) Vortex briefly and shake for at least 6 hours (best if overnight)
3) Add 1mL of 2.0 w/v% $LiClO_4$ in acetone to shaken solution
4) Centrifuge for 20 minutes at 12,000 rpm in microcentrifuge
5) Pipette off supernatant and triple rinse with Acetone, ensuring not to disturb the pellet throughout
6) Add a small volume of Acetone to the tube (0.5mL) and resuspend the pellet with 15 seconds of sonication and adequate vortexing
7) Centrifuge for 20 minutes at 12,000 rpm in microcentrifuge
8) Pipette off the supernatant
9) Vacuum centrifuge off any remaining Acetone
10) Add 100µL of 0.1M TEAA to dry tube and resuspend with 15 seconds of sonication and adequate vortexing. May use 60°C heat block to encourage mixing if necessary but if performed correctly the pellet should easily enter solution

Purification with HPLC

1) Check buffer levels, 0.1TEAA and 99.99% Acetonitrile, making sure that they are well above the inlet tubes. Turn the helium gas tank and set the pressure between 20-40 psi.
2) Turn both switches on HPLC to "ON" position, one for the UV and one for the system
3) Open Millennium software and click "Run Sample" once logged in
    a. The HPLC must be on in order for the Millennium software to open properly
4) Select "Acetonitrile Wash" under methods and hit monitor. Wash for at least 15 minutes and look for the pressure to flat line at 0 psi for a few minutes
5) Hit "Abort" button on software to stop Acetonitrile wash
6) Select "TEAA Wash" under methods and hit monitor. Wash for at least 15 minutes and look for the pressure to oscillate around 3,000 psi for a few minutes
7) While washing with TEAA, rinse injection port on HPLC with TEAA to clean out any leftover runs.
    a. Use larger volume syringe with black plunger. Other tips with the wrong gauge needle will break the inlet.
    b. Wash a few times with 50-100µL in both "Load" position and in "Injection" position. When washing in "Injection" position, place syringe into inlet while in "Load" position, turn to "Injection" position, push plunger of syringe in to evacuate contents, turn back to "Load" position.
    c. Will be able to see liquid flowing out of outlet tubes next to injection site
8) Hit "Abort" button on software to stop TEAA wash
    a. If pressure never stabilizes around 3,000 psi, run steps 4-6 again
9) Select "TEAA 0 to 100% Acetonitrile" under methods and select set up
10) Check the fractional collector for empty tubes and making sure its flush against the back.
11) Name sample and ensure that the correct method is selected as well as injection volume of 50µL and run time of 60 minutes.
12) Once set up, the software will allow selection of "Injection" button under run conditions

13) Wait for software to prompt “Single Inject Waiting”
14) Slowly draw 50µL of labeled DNA in the syringe with the black plunger making sure there are no significant bubbles
15) Place tip into injection port while in the “Load” position. As quickly as possible, inject the sample, turn the position to “Inject”, and start the fractional collector by hitting “Start” on the top.
    a. Since the fractional collector takes 30 seconds samples, a long delay will cause the sample peak to be offset from the actual collection site
16) Hold the syringe in the injection site for 2 minutes since back pressure may cause the sample to leak out. Once removed, switch back to ”Load” position
17) Keep an eye on the fractional collector as sometimes the tubing gets tangled and needs to be moved out of the way
18) The Millennium software will update the absorbance and pressure plots in real time. Look for two sharp peaks during the run.
    a. The first peak will occur around 8 minutes. This peak represents any unmodified DNA
    b. The second peak will occur from 18-24 minutes depending on the tail length/type modification. Longer tails elute after longer periods of time.
19) Once both peaks are observed, click “Abort” on Millennium software and “End” on the fractional collector.
20) Look for what time the modified DNA eluted and collect 5 tubes around that time remembering that each fraction is a 30 seconds collection
21) Dispose the rest of the collection into the sink while running water
22) Rinse or replace any tubes near the 18-24 minutes mark (3^{rd}, 4^{th}, or 5^{th} rows) for the next run
23) Select “Acetonitrile Wash” under methods and hit monitor. Wash for at least 15 minutes and look for the pressure to flat line at 0 psi for a few minutes
24) Hit “Abort” button on software to stop Acetonitrile wash
25) Close Millennium software and turn the machine off
26) Take the 5 collection tubes and aliquot them into 1.5mL centrifuge tubes
27) Dry each tube in the vacuum centrifuge
28) Resuspend each dried DNA tube with DNA water to a combined volume of 1mL.
29) Once individually suspended, combine all the fractions into one 1.5mL centrifuge tube
30) Perform UV concentration test at 254nm wavelength. Calculate concentration using Beer’s law
31) Dry DNA in vacuum centrifuge and resuspend to 5mM in DNA water
32) Label the centrifuge tube and store in the freezer until needed