Stony Brook University

Polarization and angle dependence of fluorescence from dye-labeled DNA molecules adsorbed and aligned on surfaces

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We, the thesis committee for the above candidate for the

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Abstract of the Thesis

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The absorption and emission of fluorescence radiation from oriented molecules are polarization and angular dependent, determined by the adsorption and emission dipole axes' ($\vec{\mu}_a$ and $\vec{\mu}_e$) alignment relative to the incident excitation direction and to the emission direction.

In this study, DNA molecules are aligned on polymethylmethacrylate (PMMA)-coated silicon surfaces and their fluorescence behavior is studied for different dye molecules (SyBr Gold, Acridine Orange and YOYO)for both double-stranded and single-stranded DNA(dsDNA and ssDNA). The polarization dependence for different angles of incidence and orientations of the DNA molecules was measured and used to infer the binding modes of the dye molecules. The dsDNA molecules, as expected, were found to have the dye molecules intercalated between the bases. However, surprisingly, in the case of ssDNA, the dye molecules were also found to bind with their absorption axes' parallel to the bases protruding from the single strand backbone. This is in contrast to conjectured binding modes of dyes aligned along the backbone of ssDNA.

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Introduction

Fluorescent dyes are widely used to visualize DNA molecules in microscopy and in sequencing experiments [1-8]. The binding of the fairly large dye molecules is known to affect the structure and dynamics of the DNA, changing its stiffness, base spacing and electrophoretic mobility, for example. A key element to understand the dye-DNA interaction is to determine the binding mode of the dye. The three main modes of binding are: 1) base intercalation, 2) minor groove binding and 3) major groove binding. An effective experimental technique used to probe the binding is the measurement of the polarization dependence [9-13] of absorption and emission of light from oriented DNA molecules. This report studies the polarization effects of three dye- labeled systems, DNA stained with SyBr Gold [14], YOYO [15] and Acridine orange dyes [16] (all provided by Molecular Probes). The polarization dependence of fluorescence absorption was measured for stretched and oriented DNA molecules deposited onto PMMA-coated silicon substrates. The emission intensities were measured as a function of polarization direction for different angles of incidence to the substrate surface and for different orientations of the DNA axes relative to the incident light direction. The emission intensities were measured both for native double-stranded DNA (dsDNA), as well as for heat-denatured single-stranded DNA (dsDNA). Somewhat surprisingly, the dyes studied were found to bind to ssDNA in a manner similar to that of the dsDNA, attached parallel to the bases and perpendicular to the DNA orientation axis. We also report on bleaching effects on the dye-DNA complex and determine the maximum time of beam exposure which may be used under our experimental conditions.

Experiment Section

Preparation of PMMA-coated Si substrates.

Si wafers are cut to 1×1 cm size, and then put into ultrasonic cleaner in ethanol for 15 minutes. Next, 1:1:3 ratio of Hydrogen Peroxide, ammonium hydroxide and de-ionized Water are mixed in a beaker with Si wafer. Heat them at boil ins for 15minutes to remove organic contamination on Si wafer, then change the solution into 1:1:3 ratio of Hydrogen Peroxide , Sulfuric acid and de-ionized Water and heat at 130℃ for 10 minutes to remove ionic/metallic impurities. The cleaned Si wafers are put to a spin caster one by one and a PMMA/ toluene solution is pipetted onto the sample. The sample is spun 30 seconds at 2500rpm. Finally, the thickness of the film on Si wafer is about 600 Å, as measured by ellipsometry.

Annealing the PMMA/ Si substrate in vacuum oven.

The PMMA/ Si is placed in a high vacuum oven at 130℃ for several hours to anneal the PMMA and to drive off surface contaminants adsorbed from the ambient.

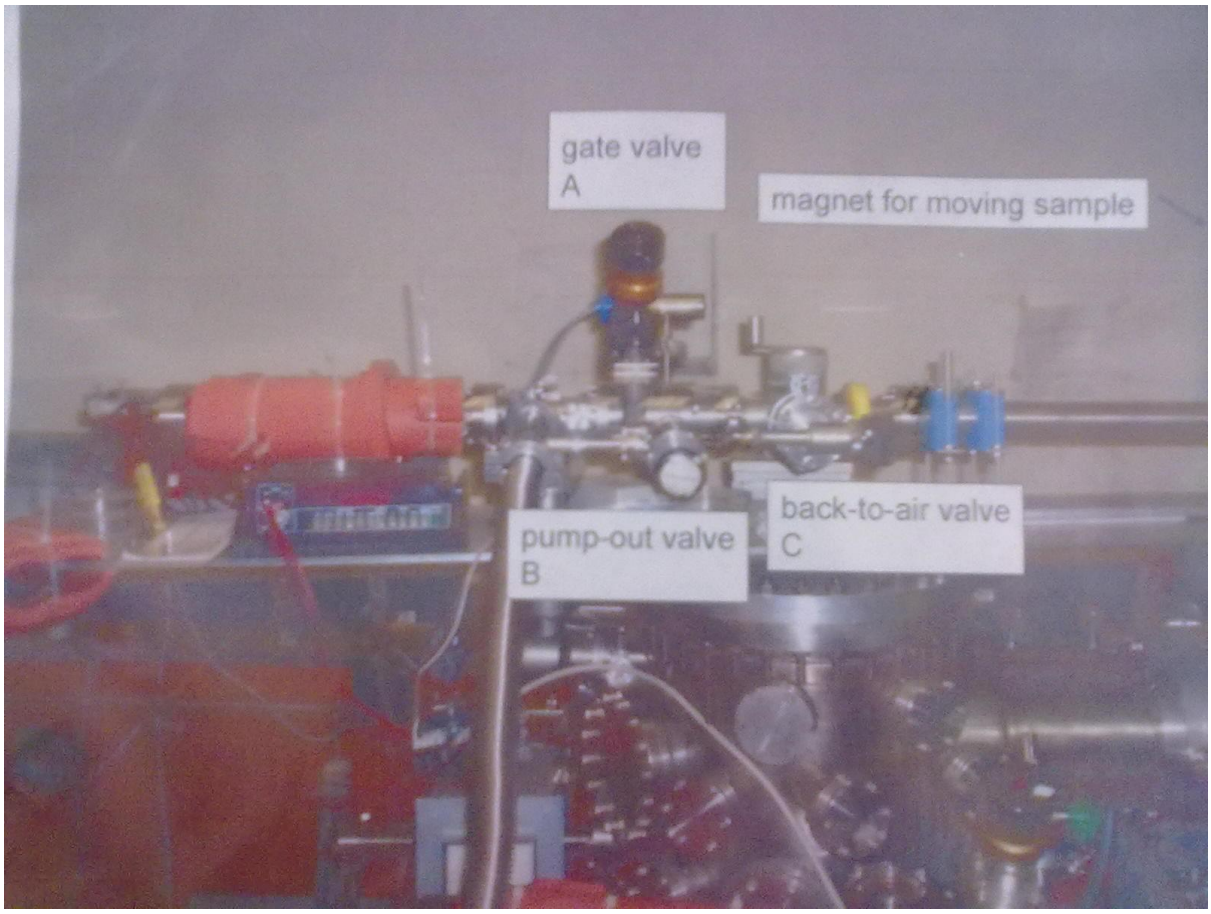


Figure 1. Hgh vacuum oven for annealing PMMA/Si samples.

Preparation of stock solutions of DNA and dye.

Double-stranded Lambda phage DNA (48503 base pairs) was diluted in a 6:50 buffer solution ratio of 0.1M NaOH and 0.02M MES. For different dye, the effect of fluorescence will change, and the concentration of DNA must change. If the concentration is too high, DNA molecules will be overlapped with each other and we cannot tell analyze single molecules and it's also hard to distinguish the background. And excessively low concentration will lead to few DNA strands in one microscope image.

Adsorption of DNA onto surfaces out of solution.

Figure 2 shows the deposition set-up. The top of the pipette with the tip adjusted to the appropriate height. We can adjust it using the microscope on the side (Figure 4). The pipette is filled with 2μl of DNA solution. The PMMA/ Si substrate is placed on Petri dish and placed under the pipette. The Petri dish to fit the position between the Si substrate and the top of the tip (Figure 3) is used. The screw A is rotated to squeeze out solution till there is a drop exiting the tip. The screw B is used to lower the pipette to the surface, tracking through the microscope. When its top contacts the Si substrate, the drop will be drawn down and the work is done. Usually we repeat this step 3 times so we have more choices when looking for DNA strands under microscope. Sometimes the drop does not contract inward so much and leave

little DNA strands on the surface because the drop is too small. And sometimes when we are waiting for the drops drying, the Petri dish tilts a little and leads to a graph shows accumulation of DNA strands on one side of the drop. There will be too few DNA strands if the DNA concentration was in the range of 0.5 µl/ml to 0.05 µl/ml. Dye concentration were typically 4 µl/ml.

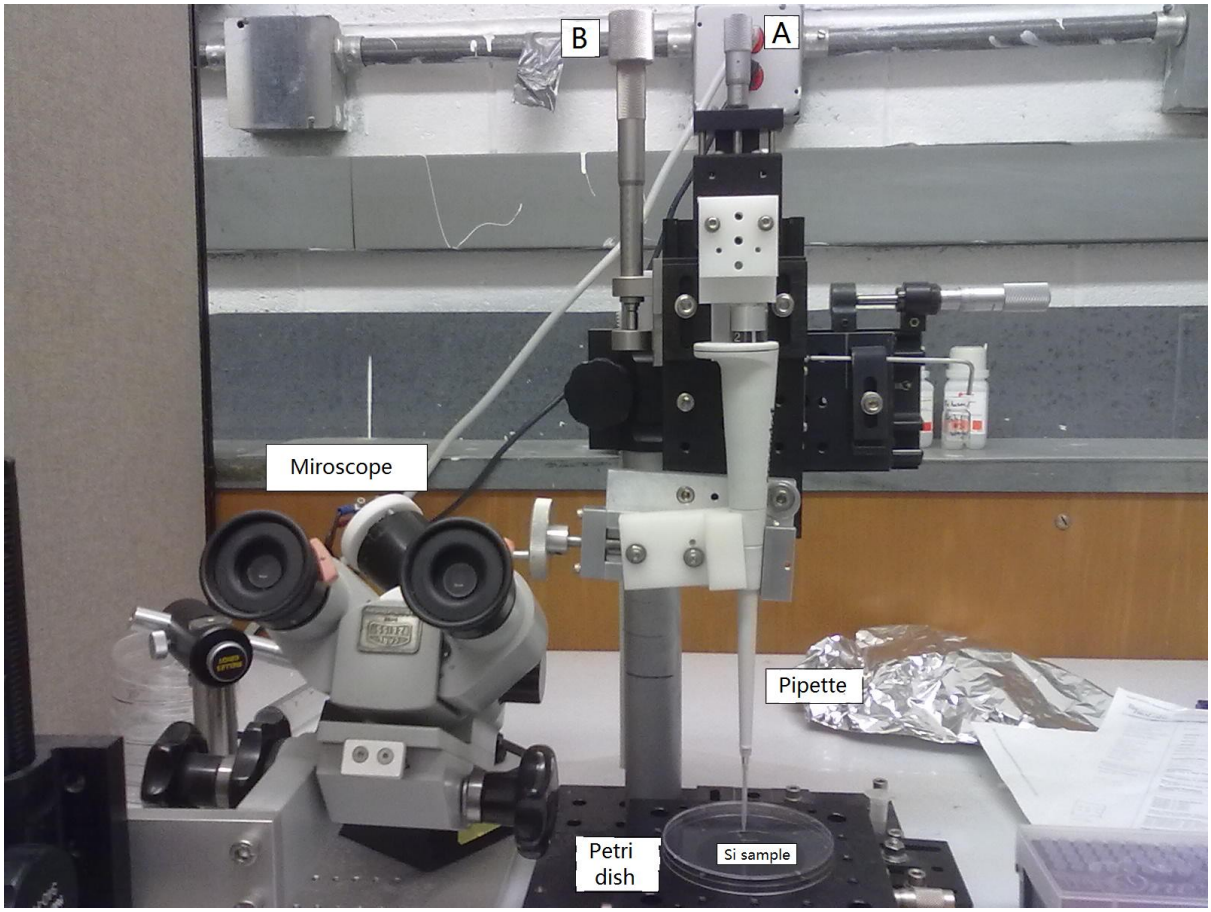


Figure 2. Setup for controlled deposition of drops of DNA solution onto PMMA/Si substrate. Pipette (2 µl capacity), mounted on XY stage, is at center. Viewing microscope is at left.

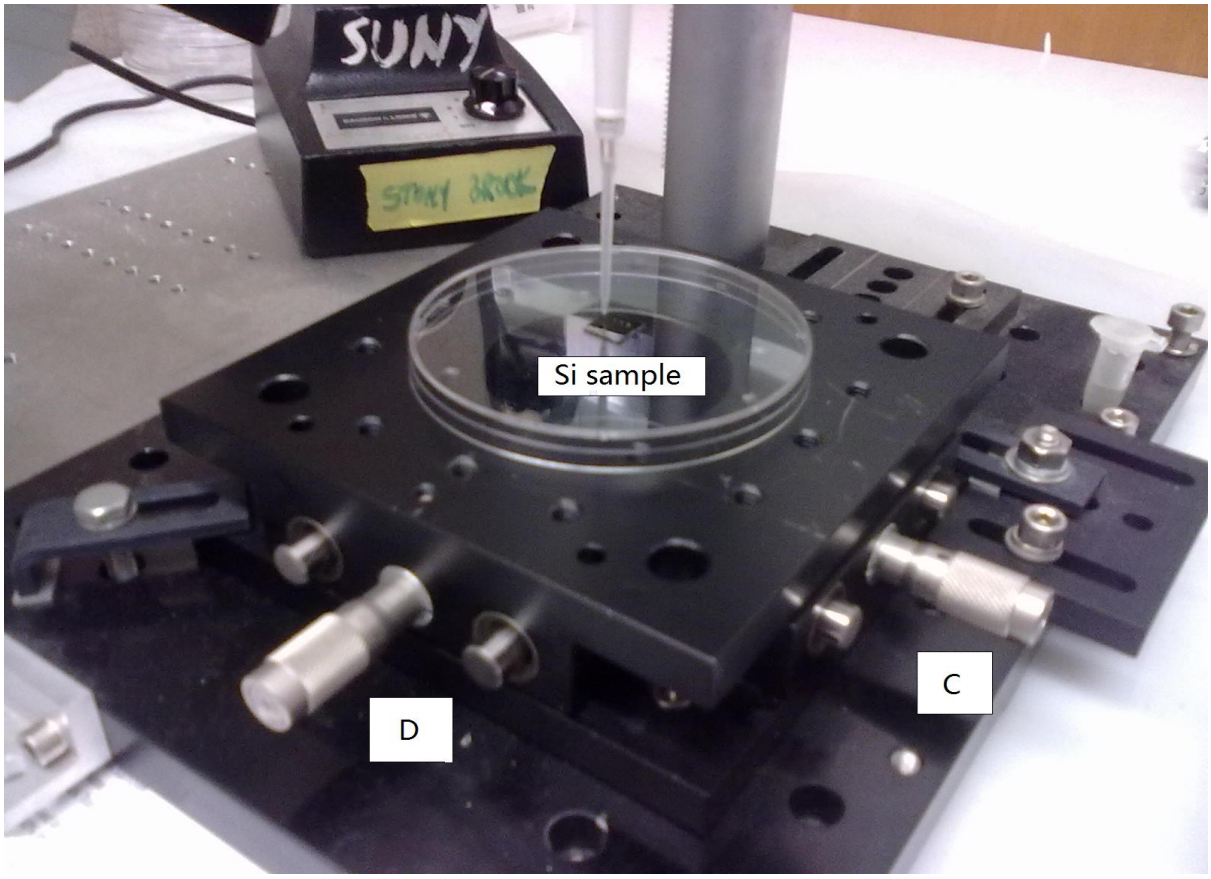


Figure 3. Pipette tip nearly in contact with PMMA/Si substrate placed on XY stage. Rotation of the screws C and D move the PMMA/Si substrate.


Figure 4. View through microscope of drop being deposited onto surface. Three drops on Si sustrate and one falling drop can be observed through the microscope.

Application of dye to DNA by PDMS soft lithography.

Most experiments of DNA adsorbed to the surface use dyes added in solution, prior to deposition. However, it is sometimes convenient (especially when using single-stranded DNA) to dye the NA after adsorption. This avoids possible dye effects on the adsorption process. The procedure is as follows: An elastomeric stamp (either flat of with grating) is prepared using a PDMS/ Crosslinker solution. A piece of PDMS stamp that is a little larger than the Si substrate is cut and immersed into a dye solution for 30 seconds (Figure 5a). As the space is quite small for the PDMS sheet, we don't need to fill it up at first and usually 500 µL is enough. The side with grating (if present) is placed face down on the PMMA/Si substrate (Figure 5b). We wait for 10 minutes to 1 hour to let the dye absorb on DNA strands with a weight pressing down on the stamp (Figure 5c).

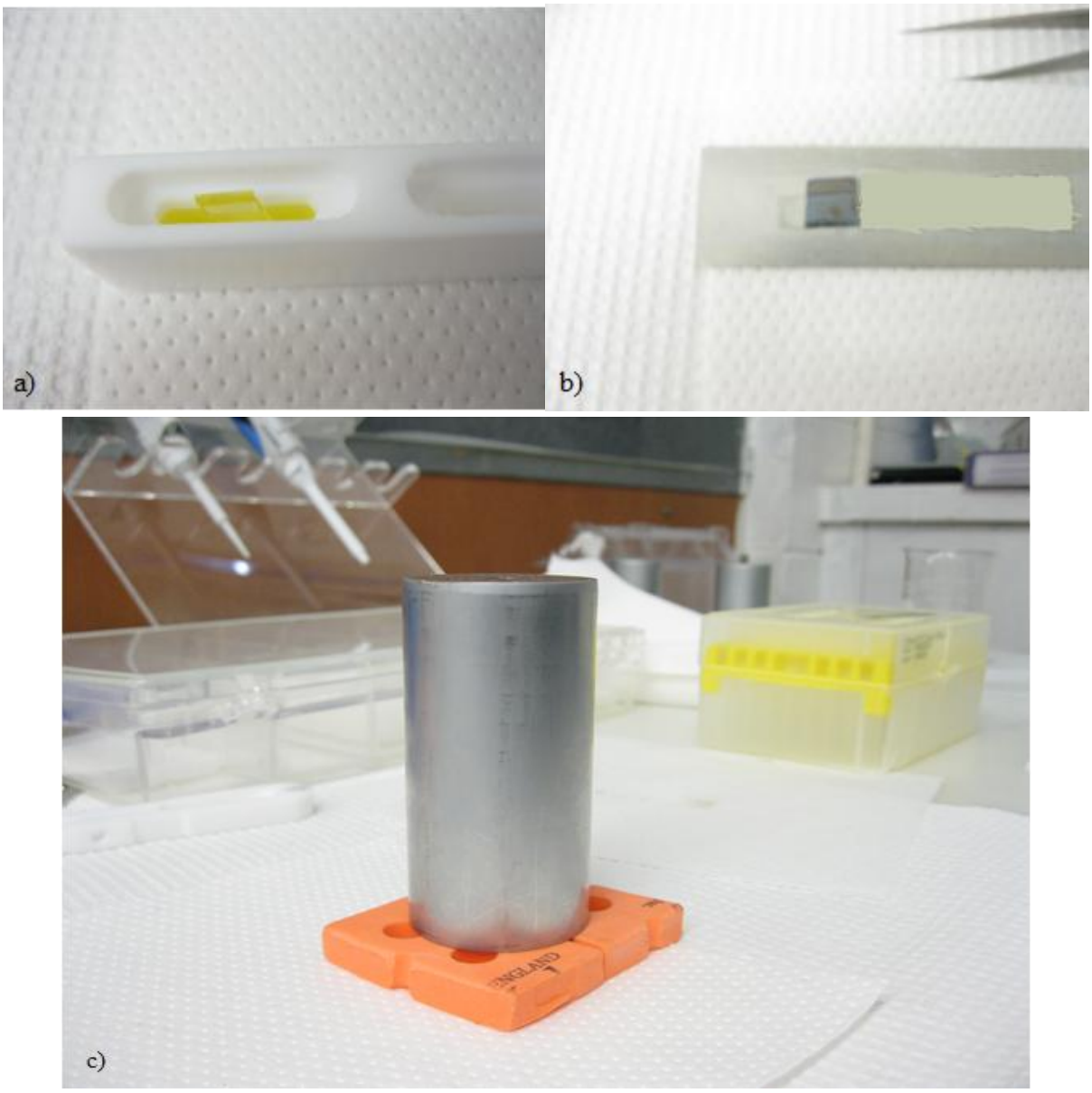


Figure 5. a) PDMS stamp placed in Teflon cell containing dye solution. b) Stamp is placed onto PMMA/Si substrate with DNA adsorbed. c) A weight is used to press onto stamp/substrate assembly.

Imaging using fluorescence microscopy.

There two kinds of light source used in this experiment, mercury light (50W, high pressure acr lamp) normally incident on the sample in epifluorescence mode and a laser (488nm blue laser diode source) at oblique incidence from the side of the sample (see Figure 6). The polarization of the mercury light is controlled by a rotatable polarizer insert in the microscope. The laser polarization is produced using a polarizer followed by a half-wave plate (Figure 7). In this way, we obtain polarized light with different angles to the surface of the sample by rotating the half-wave plate, which rotates the light polarization by double the angle of half-wave plate rotation.

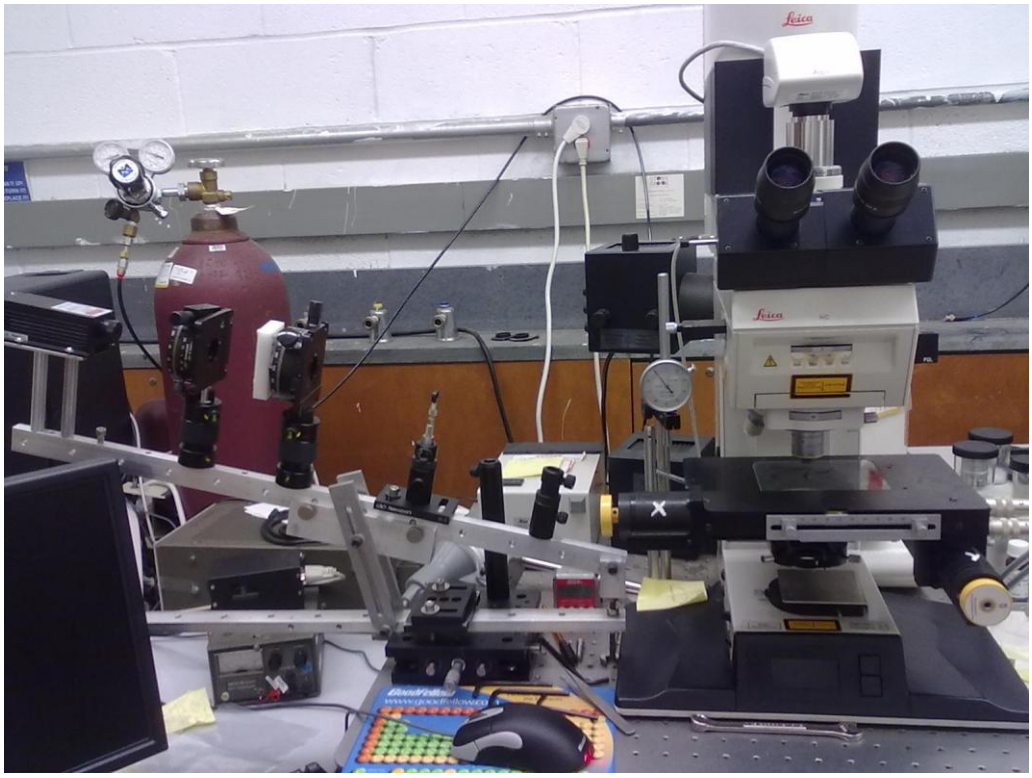


Figure 6. Optical rail setup for oblique angle of incidence laser excitation. Mounted onto the rail, left to right, are a blue laser(λ=488nm), polarizer, half-wave plate and beam defining apperture (see Figure 7 for schematic). The fluoresence microscope, housing a 50W Hg lamp in the epi-fluorescence mode, is shown at right.

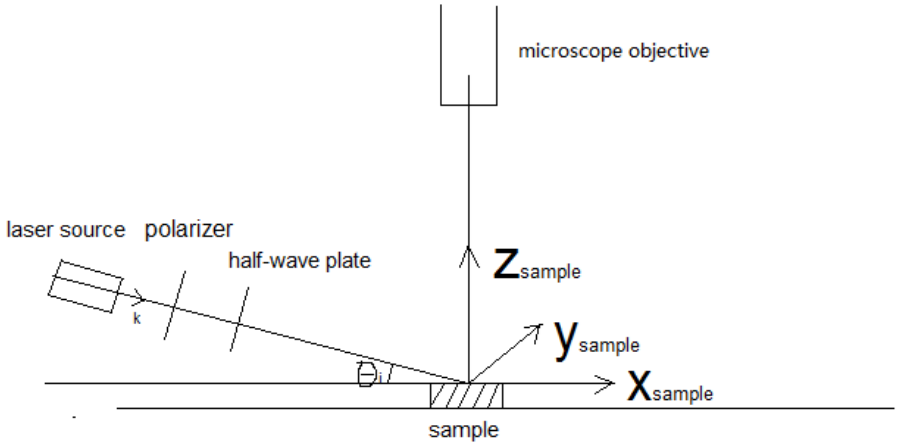


Figure 7. Schematic of optical setup in figure 6.

Here, θ_i is the incident angle of laser light on sample and is usually 18.1°, $\hat{z}_{sample}$ is the normal to the sample surface. $\vec{k}$ is the direction of the incident laser beam. $\hat{x}_{sample}$ is directed along the projection of $\vec{k}$ onto the sample plane. The plane of incidence is defined as the plane containing $\vec{k}$, $\hat{z}_{sample}$ and $\hat{x}_{sample}$ and $\hat{y}_{sample} = \hat{z}_{sample} \times \hat{x}_{sample}$.

Alignment of laser.

To analyze the polarization-dependence of fluorescence, we must calibrate the polarization angle with respect to the sample axes. The laser is partially polarized and we wanted to maximize the intensity passing through the first polarizer. We put laser source, polarizer and the detector in a line, rotated the polarizer and recorded the output of the detector every 5°. We plot (Figure 8) the output of the detector as a function of goniometer angle of the polarizer, and fit a $\cos^2(\theta_{lp})$ function to the data (where θ_{lp} is the angle between the laser polarization direction and the linear polarizer axis).

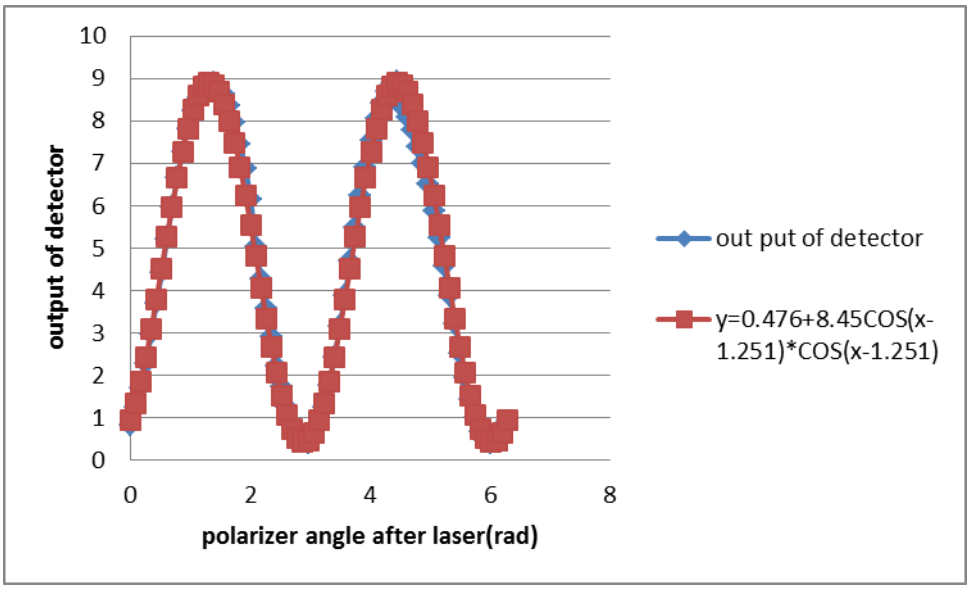


Figure 8. Output of detector when rotating the polarizer every 5 °.

When θ=1.251rad=71.67°, there is a maximum value of intensity, we choose this setting and lock it on the polarizer goniometer (Figure 9).

Figure 9. Rotate the polarizer to make the 71.67 °scale line aligned with the zero-scale line.

Next, we need to determine the relation between the polarization angle and the angle of half-wave plate. We place a linear polarization after the half-wave plate, aaligned with the sample $\hat{y}_{sample}$ direction. We call this "horizontal" polarization. Light polarized 90 ° with

respect to this will be called “vertical” polarization.

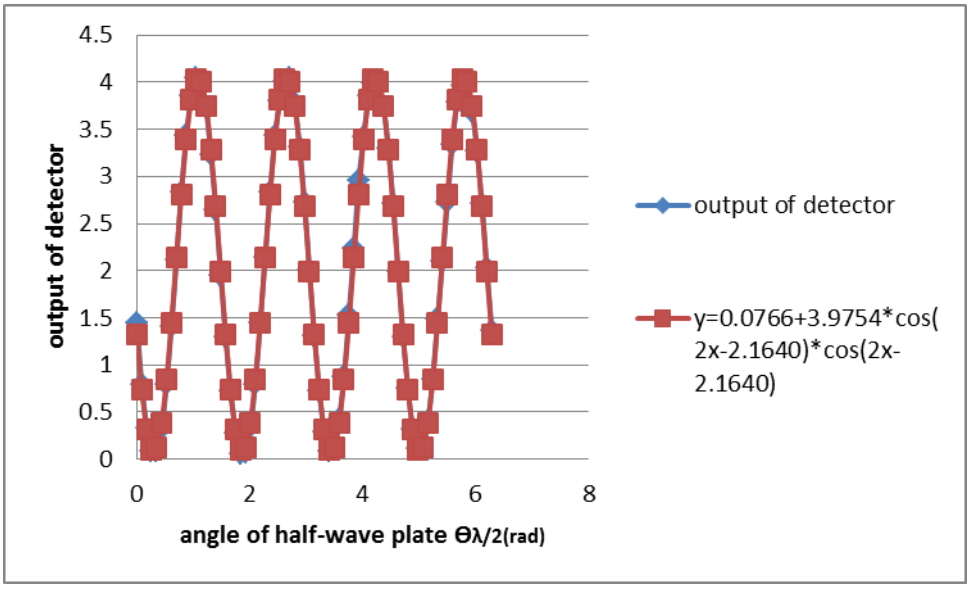


Figure 10. Output of detector after the laser goes through the polarizer, half-wave plate, and polarizer aligned with the sample $\hat{y}_{sample}$ direction (see Figure 7).

We again fit the data to the standard $\cos^2$ form. The fit gives $\theta = 2\theta_{\lambda/2} - 213.9°$, where we take θ=0° to be “vertical” polarization.

There are two expected modes that dye molecule may bind to DNA strands, one is parallel to the strands, and the other is perpendicular to the strands. We suppose that the dye molecule is perpendicular to the strands first (corresponding to dyes intercalating in the bases), and these are the calculation results.

For the mercury lamp data, we consider two cases:

Case 1: DNA strand is horizontal (along $\hat{x}_{sample}$). Then the intensity will be of the form:

$$I(\theta) = A \sin^2 \theta + B$$

θ is the angle of polarizer relative to $\hat{x}_{sample}$ axis, when θ=0°, the direction of polarization is along $\hat{x}_{sample}$, when θ=+90 °, the direction of polarization is along $\hat{y}_{sample}$. B takes into account background, In general

$$I = K_1 \cos^2(\hat{\mu}_{abs} \cdot \hat{E}_\theta) + K_2$$

Where $\hat{\mu}_{abs}$ =direction of absorption dipole, $\hat{E}_\theta$ =direction for electric polarization and K_1, K_2 are constants.

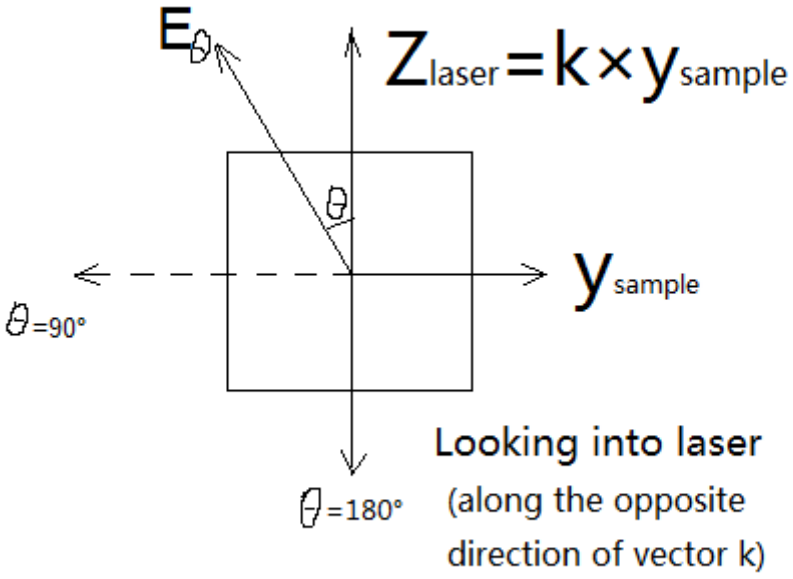


Figure 11. Diagram showing definition of the light polarization angle θ.

Case 2: DNA strand is vertical (along $\hat{y}_{sample}$). The intensity will be of form:

$$I(\theta) = A\cos^2\theta + B$$

A, B and θ have the same meaning as in case 1.

For the laser data, the intensity expression is similar and the only one thing is changed, that is the polarization angle θ here is as defined in Figure 11. It's the angle of polarizer with respect to z_{laser}, and $\hat{z}_{laser} = \hat{k} \times \hat{y}_{sample}$. We can see from the picture above that $\hat{k}$ is the direction of light incident on sample. Here (as found above) :

$$\theta = 2\theta_{\lambda/2} - 213.9°$$

We vary the half wave plate to change the orientation of the light polarization hitting the sample.

There are also two cases need to be considered.

Case 1: DNA strand is horizontal (along $\hat{x}_{sample}$). The intensity is of the form:

$$I(\theta) = A\sin^2(2\theta_{\lambda/2} - 213.9°) + B$$

Case 2: DNA strand is vertical (along $\hat{y}_{sample}$). The intensity is of the form:

$$I(\theta) = A\cos^2(2\theta_{\lambda/2} - 213.9°) + B$$

These are the relationship between intensity and polarization angle assuming that the dye dipole axis is perpendicular to the DNA axis.

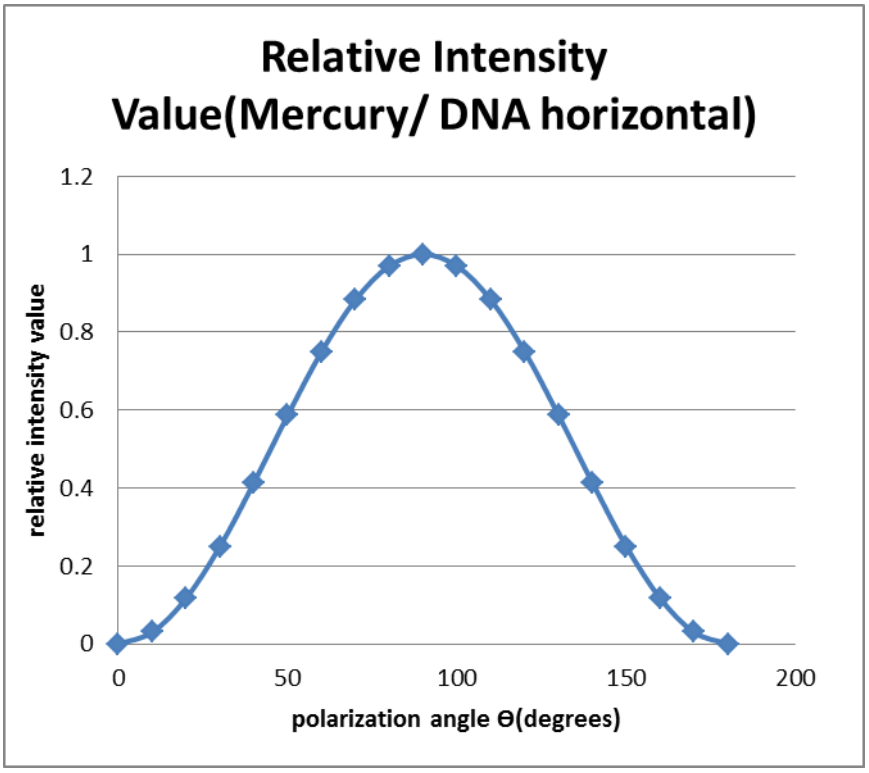


Figure 12. Calculated polarization dependence of emission for DNA axis along $\hat{x}_{sample}$, assuming dye absorption axis is normal to DNA axis (for intercalation of dye).

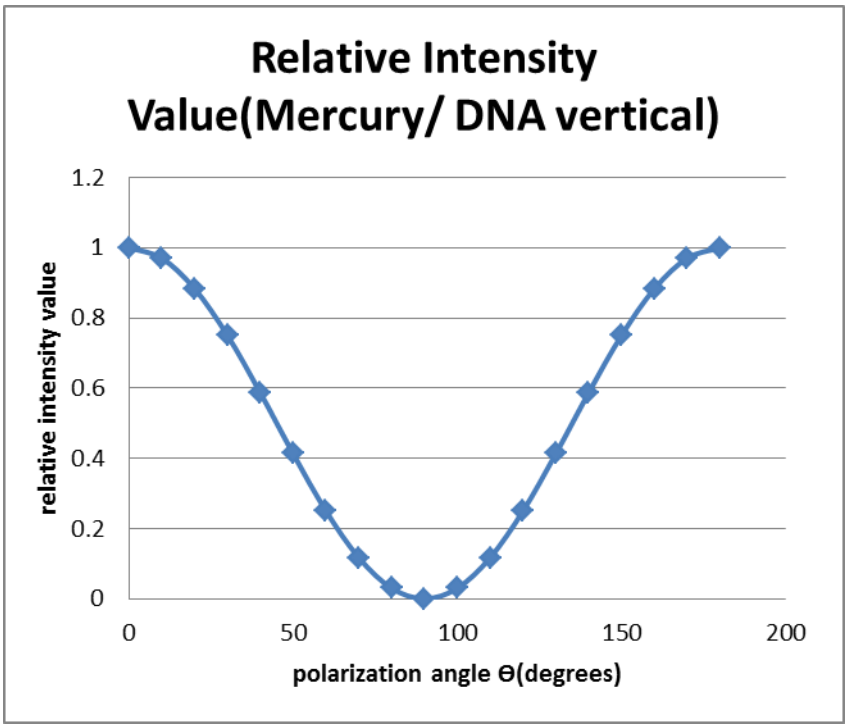


Figure 13. As in figure 12 but for DNA axis along $\hat{y}_{sample}$.

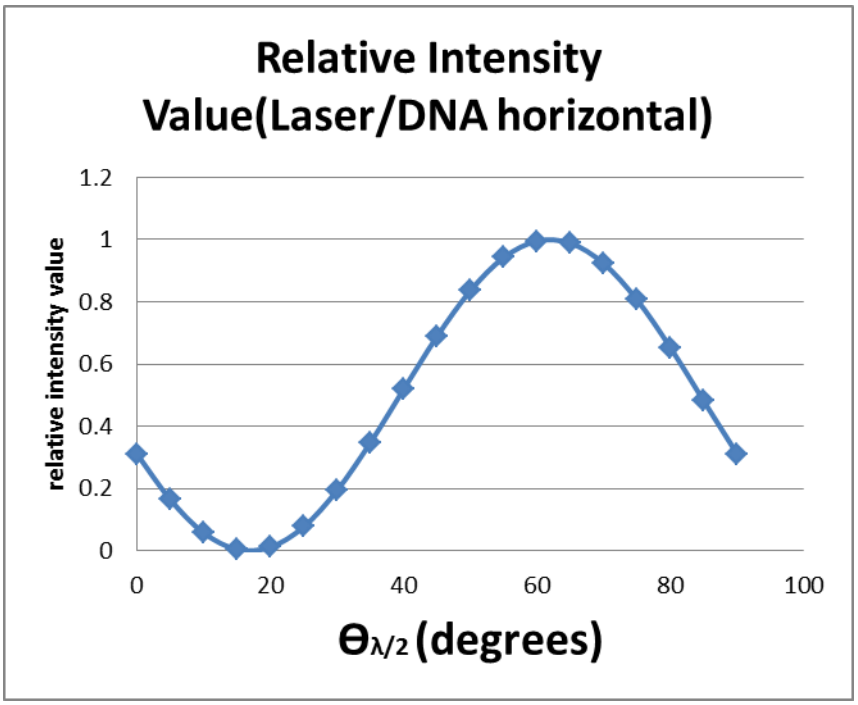


Figure 14. As in figure 12 but for laser light incident at 18.1 ℃ to surface.

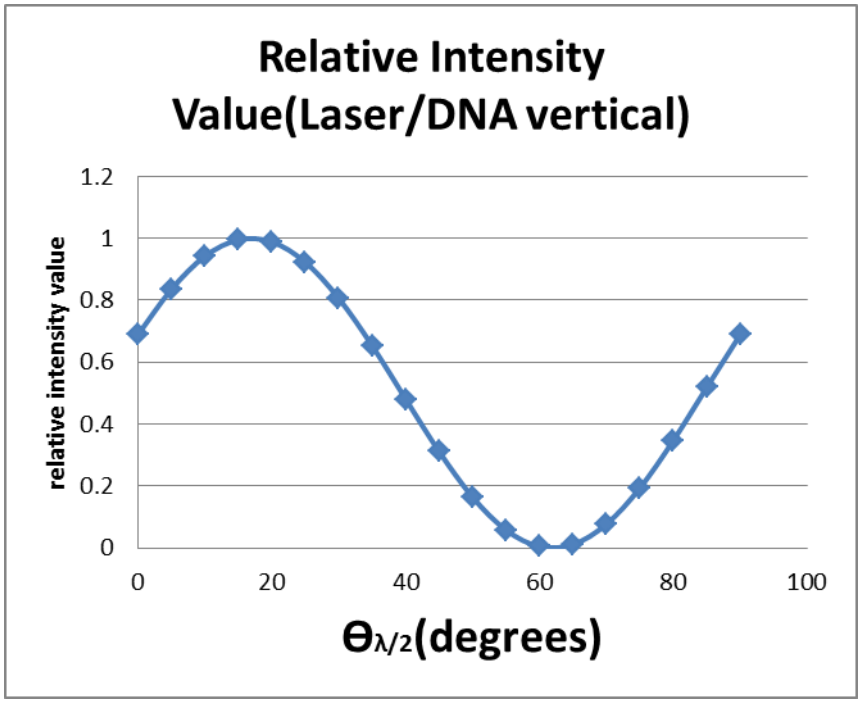


Figure 15. As in figure 14, but for DNA axis along $\hat{y}_{sample}$.

Note: If the dye is assumed to absorb along the DNA axis, the horizontal and vertical calculations would be reversed.

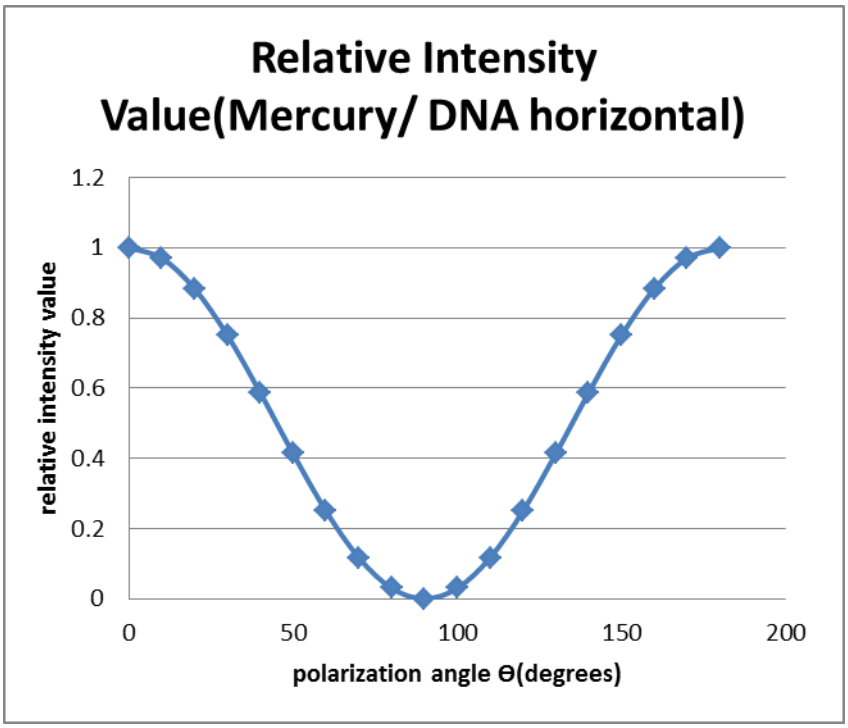


Figure 16. As in figure 12 but the dye is assumed to absorb along the DNA axis.

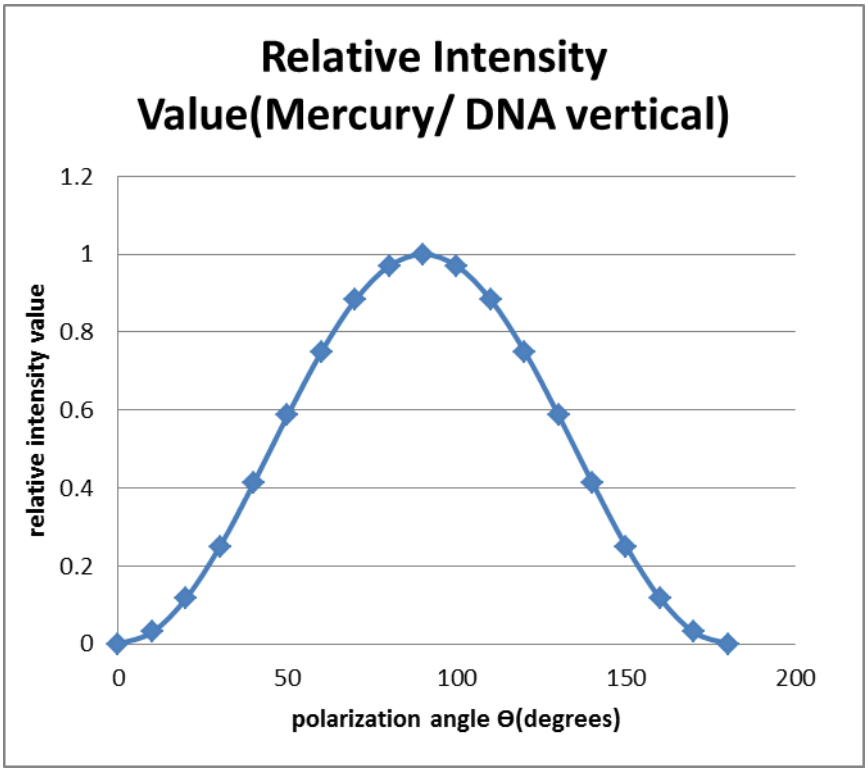


Figure 17. As in figure 13 but the dye is assumed to absorb along the DNA axis.

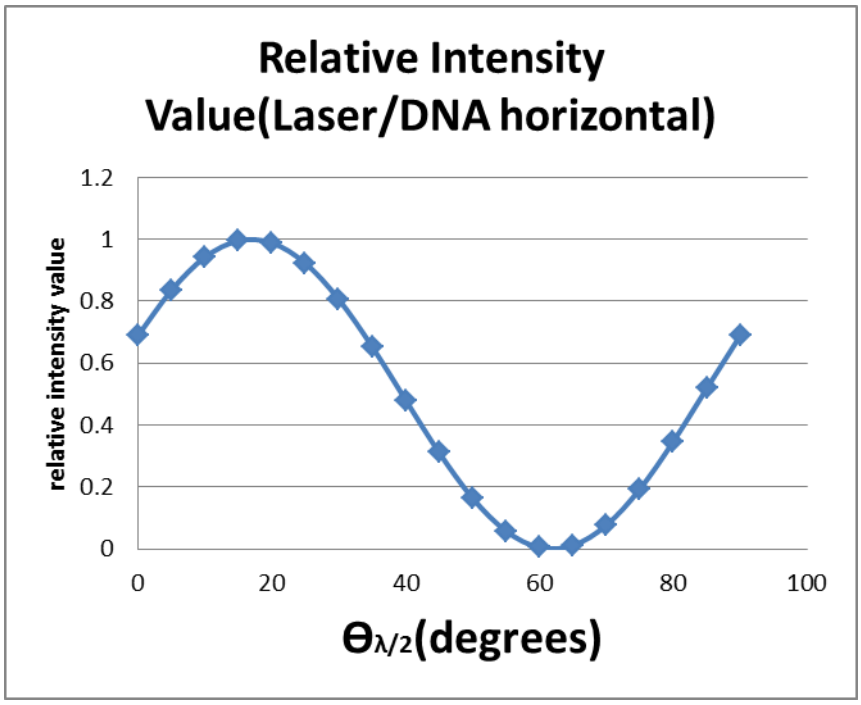


Figure 18. As in figure 14 but the dye is assumed to absorb along the DNA axis.

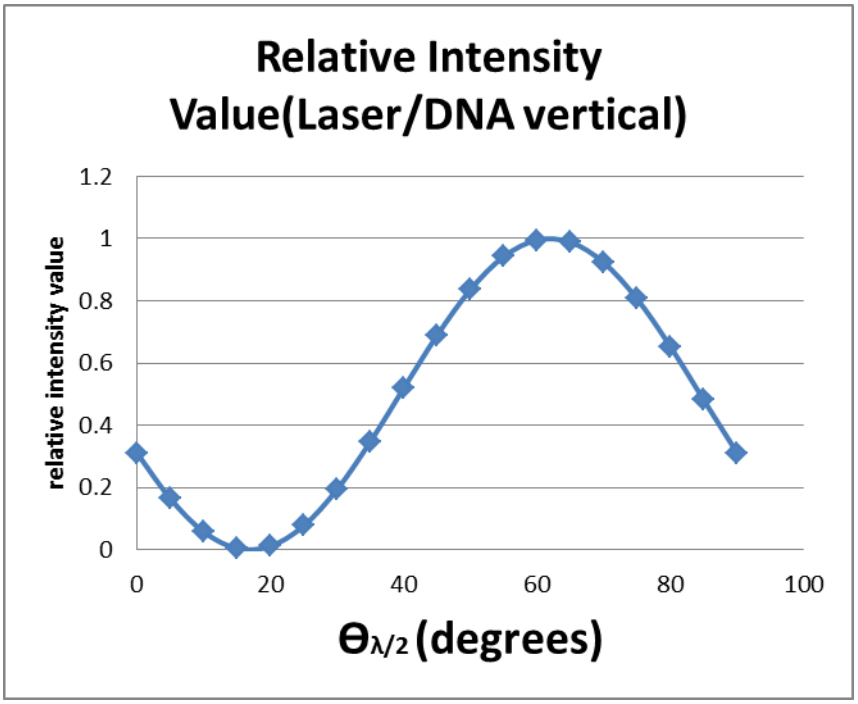


Figure 19. As in figure 15 but the dye is assumed to absorb along the DNA axis.

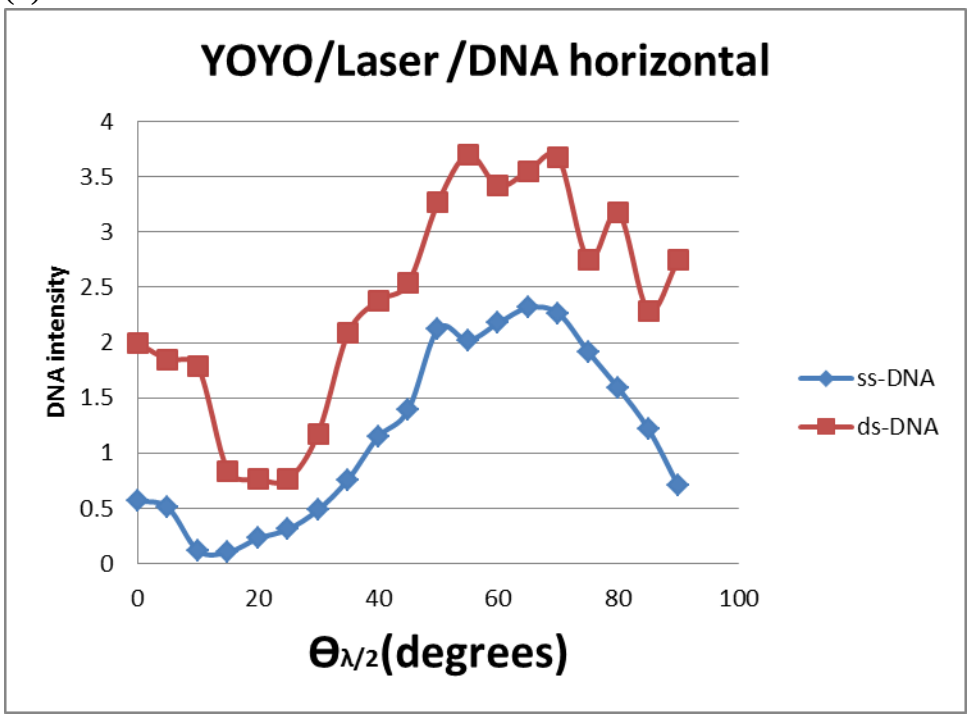
Results and Discussion.

Samples with aligned DNA molecules were prepared, as described above. Fluorescent emission intensities were measures as a function of incident polarization direction. Emission spectra were recorded using YOYO, SyBr Gold and AO (Acridine Orange) dyes for both

dsDNA and ssDNA. The light is incident at 90 °to the surface for Hg lamp data and 18.1 °for laser data. The laser wavelength is 488nm (blue) and a blue excitation. Filter was used with the Hg lamp.

There are two expected binding modes (dye axes either perpendicular or parallel to DNA axes), and we compare them with the calculation results. Here we just put the one that is compatible with the experiment data, the perpendicular orientation, indicating intercalation.

(1)



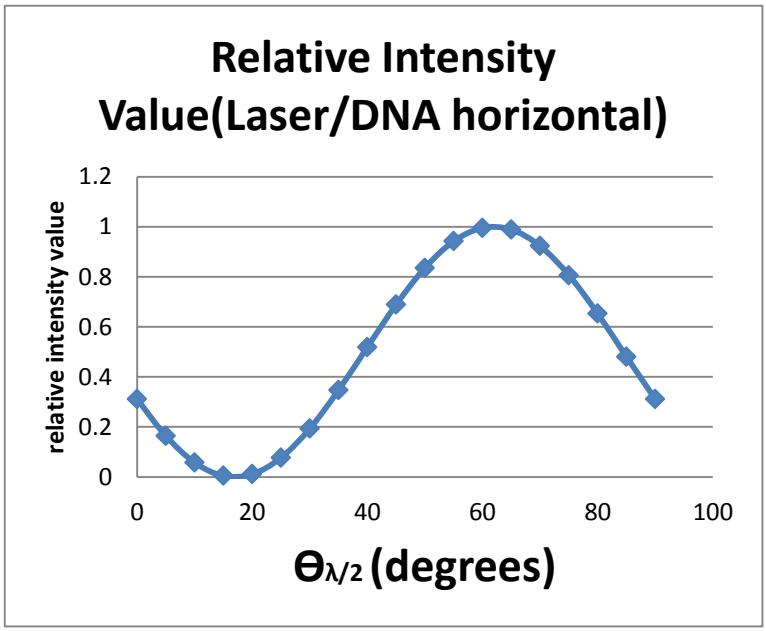
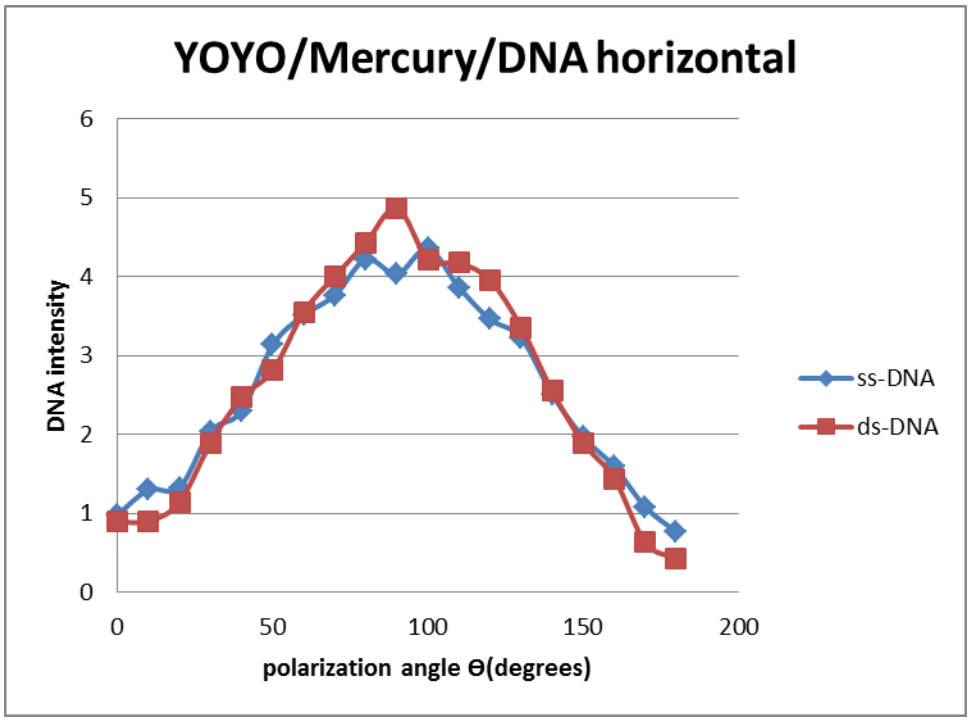


Figure 20. Polarized laser light and YOYO are used. The DNA strands are horizontal (along $\hat{x}_{sample}$). The comparison of experimental data with the corresponding calculation one is shown.

(2)



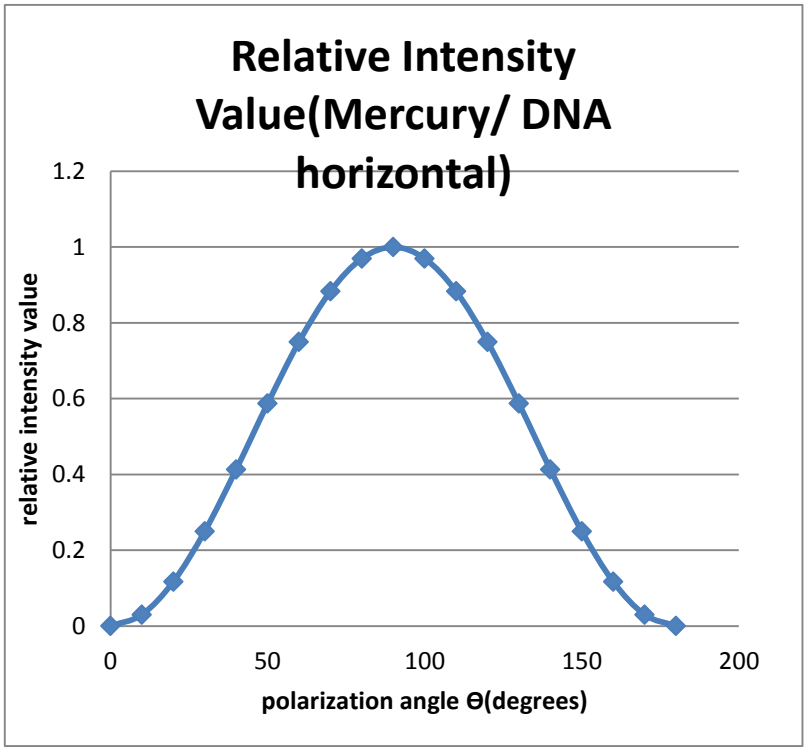
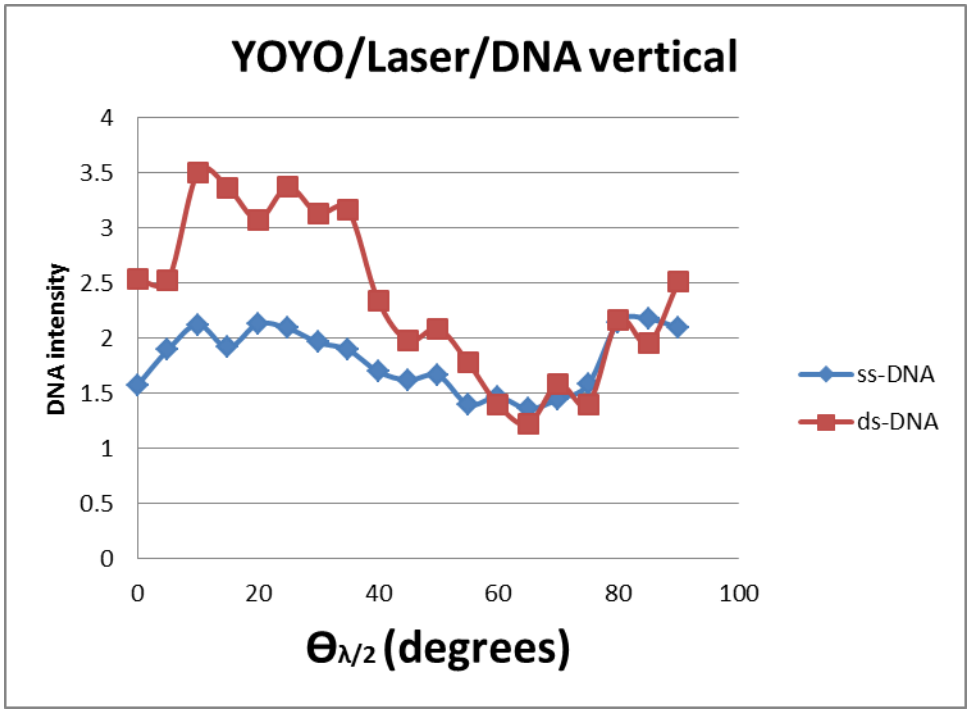


Figure 21. As in figure 20, but the polarized light is at normal incidence, using the Hg lamp.

(3)



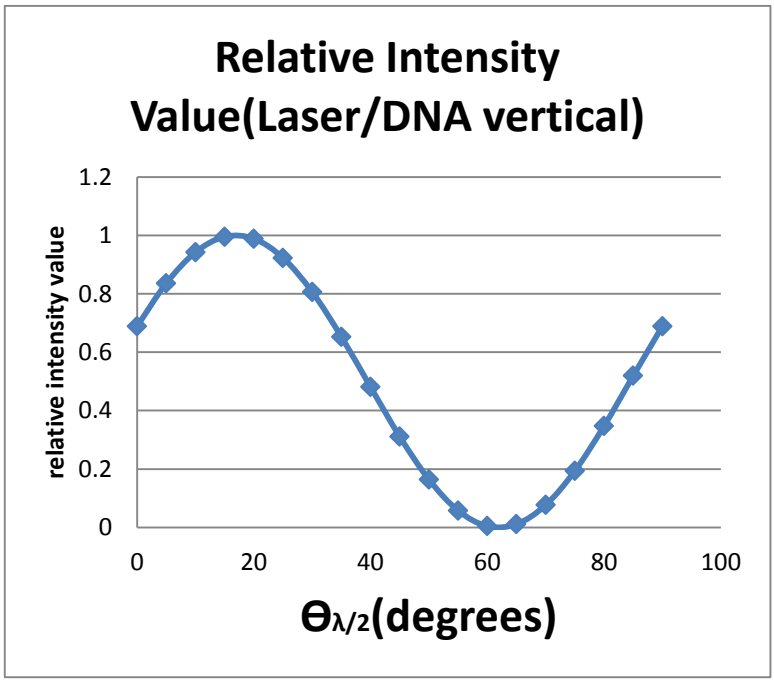
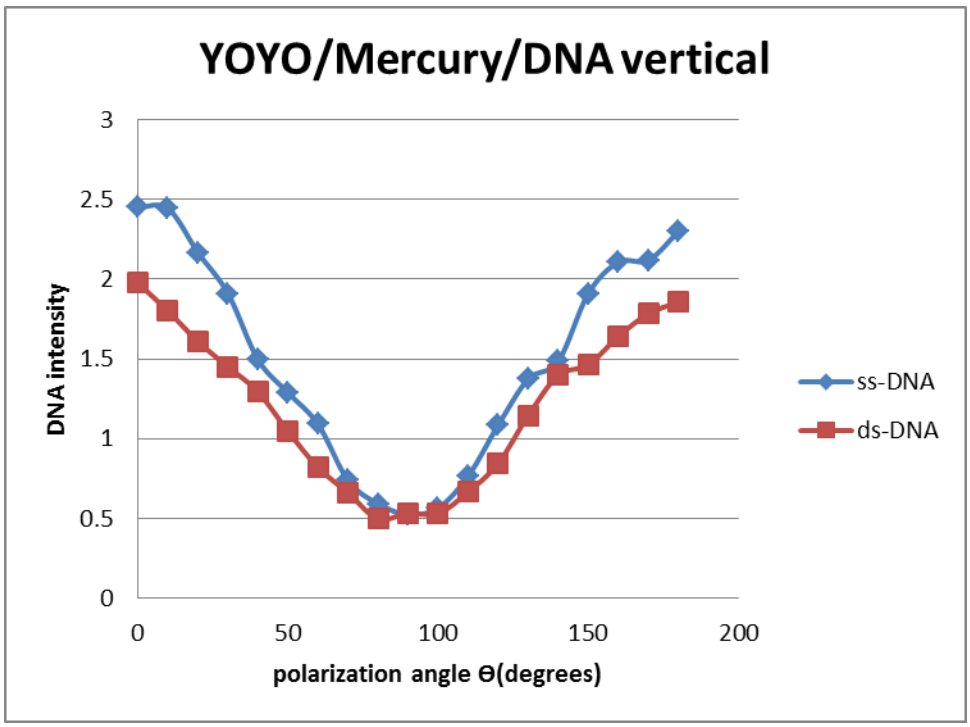


Figure 22. As in figure 20 but the DNA strands are vertical (along $\hat{y}_{sample}$).

(4)



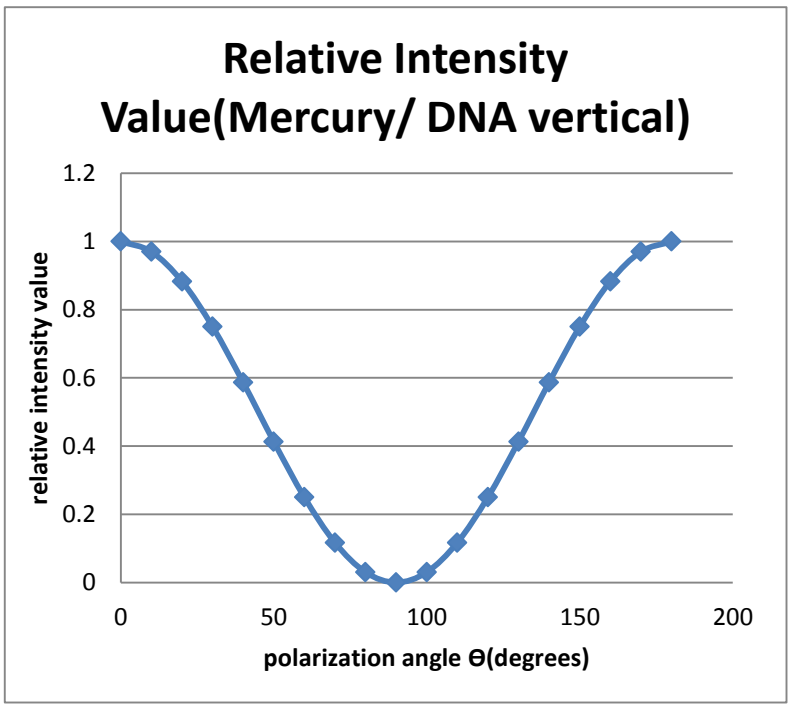
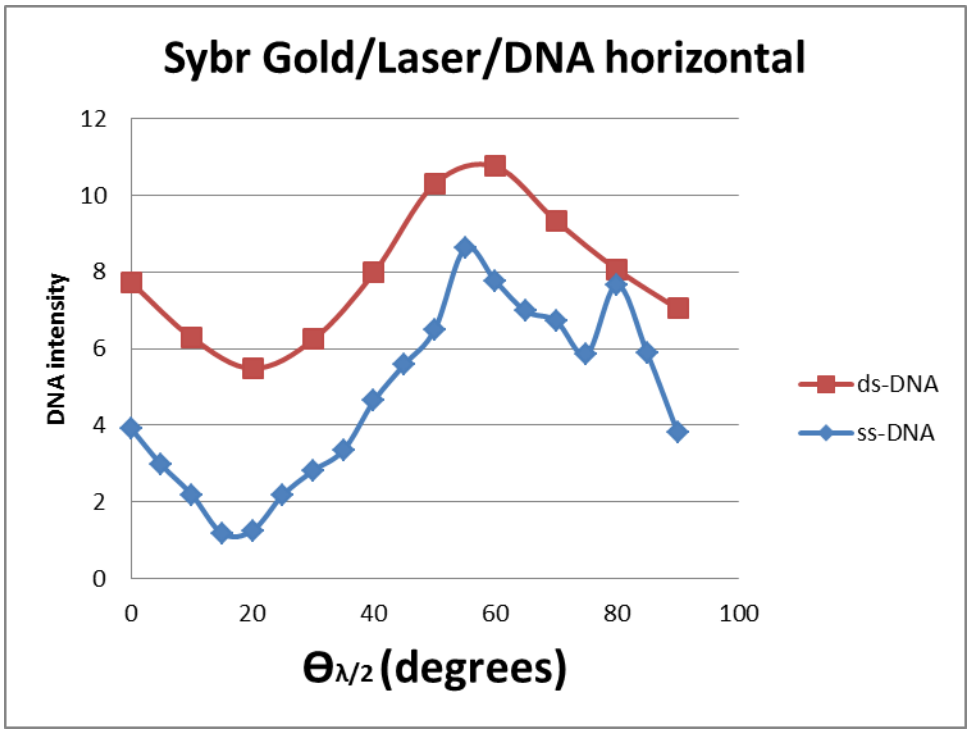


Figure 23. As in figure 22 but the polarized light is at normal incidence using Hg lamp.

In the next four figures, Sybr Gold, a recently developed dye (Molecular Probes) was used. Although Sybr Gold is somewhat less sensitive than YOYO, it still enables imaging of single DNA molecules and the toxicity is reported to be very low.
(5)



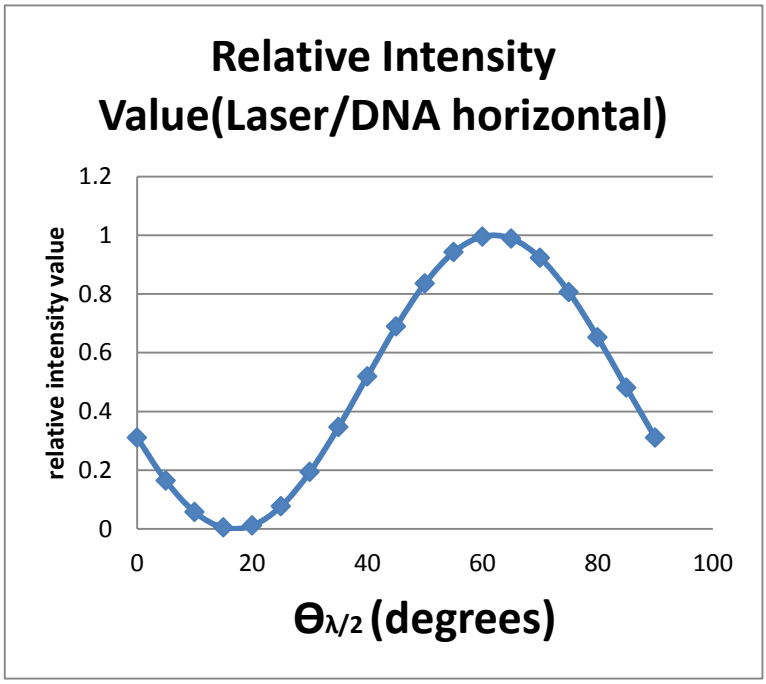
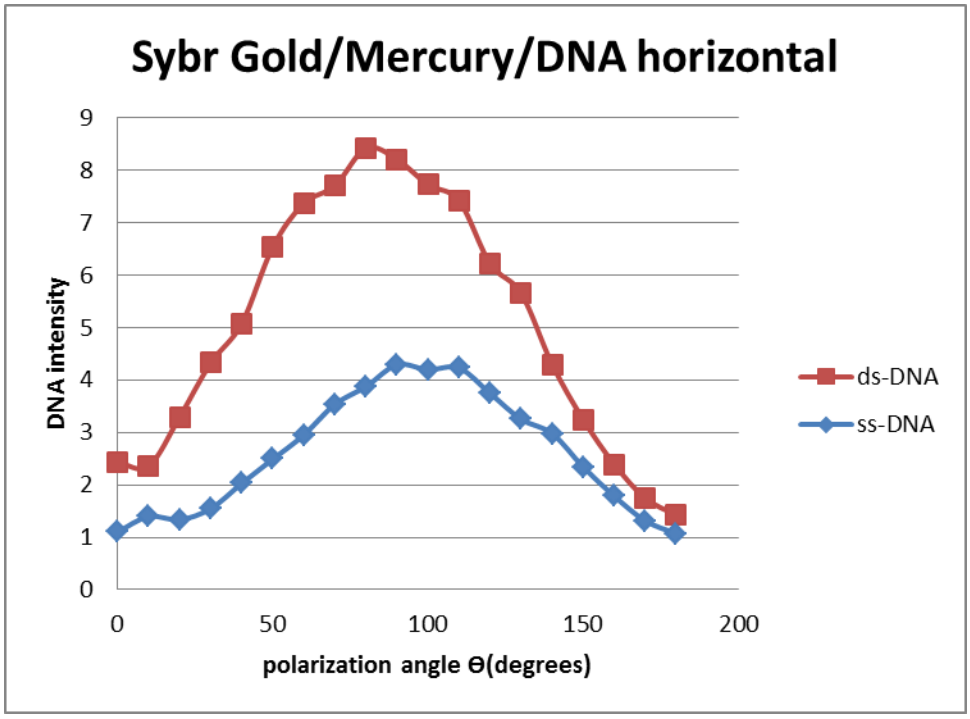


Figure 24. Polarized laser light and Sybr Gold are used. The DNA strands are horizontal (along $\hat{x}_{sample}$). The comparison of experimental data with the calculation one is shown.

(6)



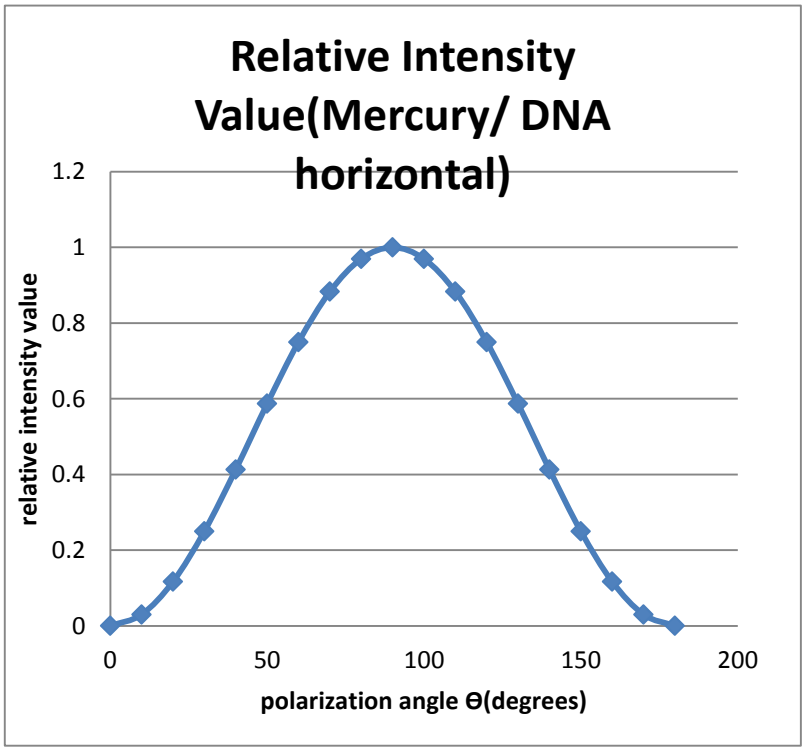
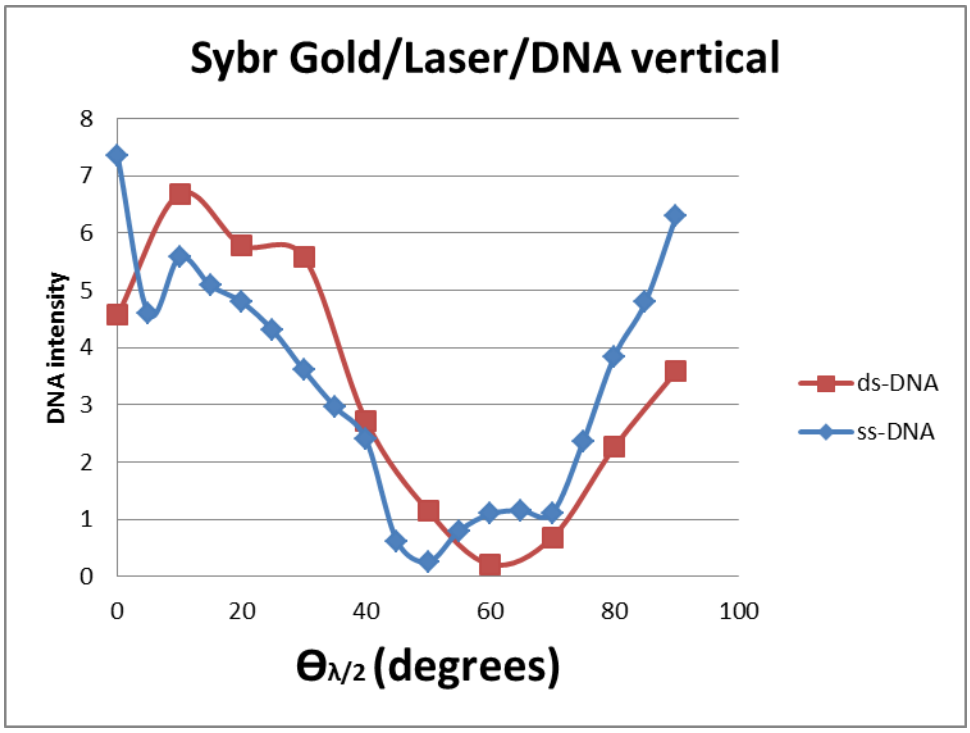


Figure 25. As in figure 24, but the polarized light is at normal incidence, using the Hg lamp. The lower panel is the corresponding calculation, assuming dye absorption moment perpendicular to DNA axis.

(7)



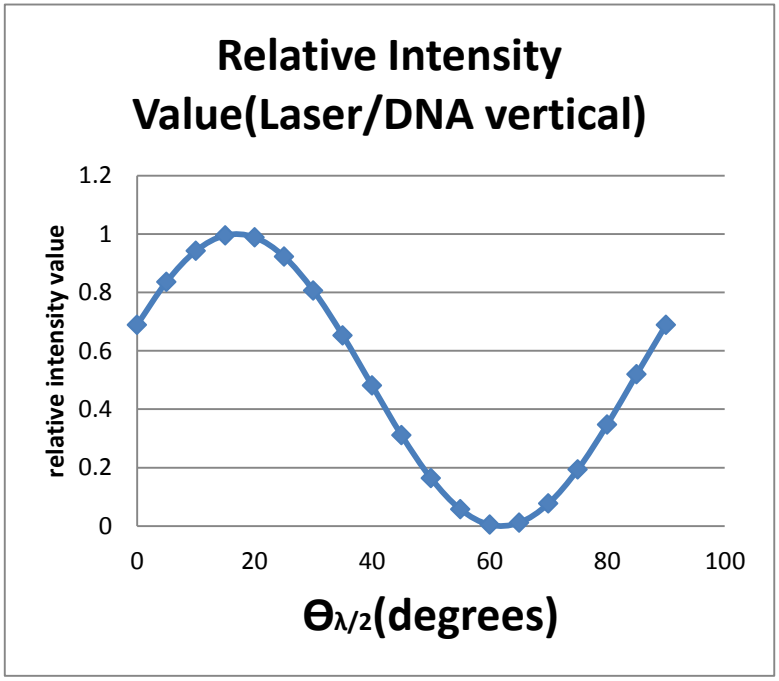
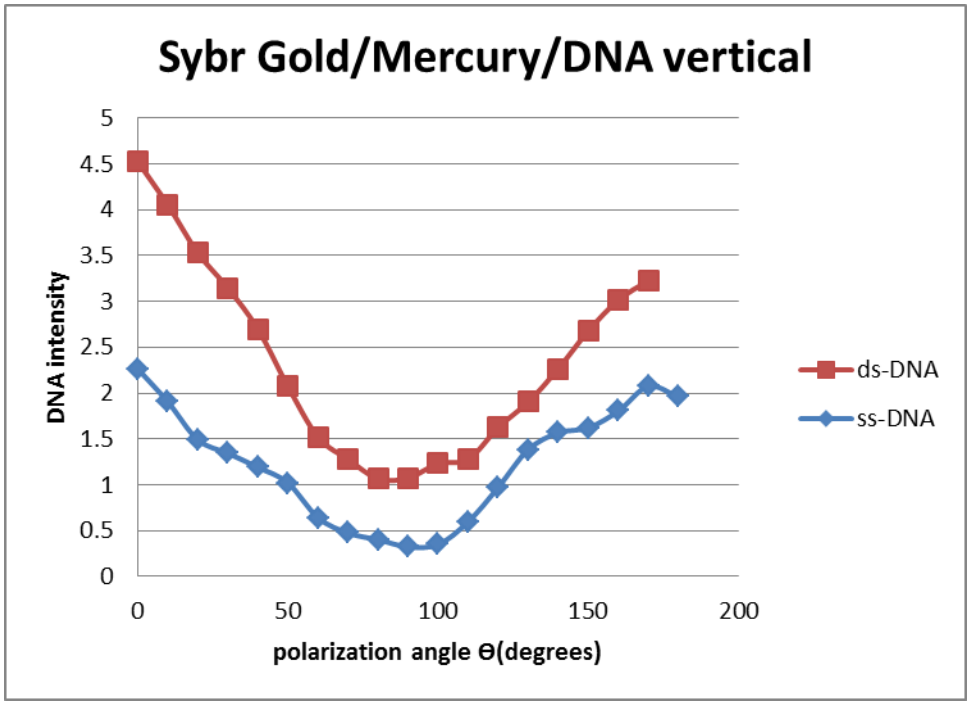


Figure 26. As in figure 24 but the DNA strand are vertical (along $\hat{y}_{sample}$).

(8)



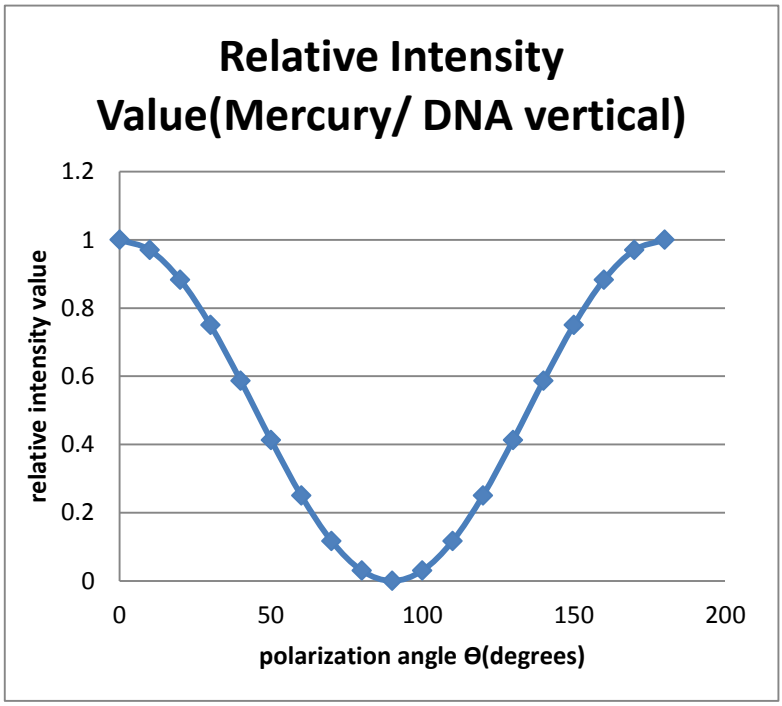


Figure 27. As in figure 26 but the polarized light is at normal incidence, using Hg lamp.

For the above figures, it can be seen clearly that the emission spectra are similar for dsDNA and ssDNA and that the binding mode is with dye dipole axis perpendicular to the DNA axis. In the case of dsDNA, the results are not surprising since many dyes are known to intercalate in the bases. However, for ssDNA it would appear to be less likely. One explanation could be that the ssDNA (prepared by heat denaturing) might actually reanneal in dsDNA during

depositon. In order to test this possibility, we use a third dye called Acridine Orange. When it binds to single-strand DNA, the emission light is red and when it binds to double-strand DNA, the emission light is green. With AO, we were able to prove that the way we used to produce single-strand DNA was effective.

As AO is a weaker dye than both YOYO and SyBr Gold, we needed to test its bleaching time first, so we can know how long to limit the exposure time when taking spectra to ensure the dye won't be overbleached before we finish all the tests. We kept the mercury light on continuously and recorded the intensity of the DNA every 30 seconds. Below we show the bleaching time of both single-stranded DNA and double-stranded DNA.

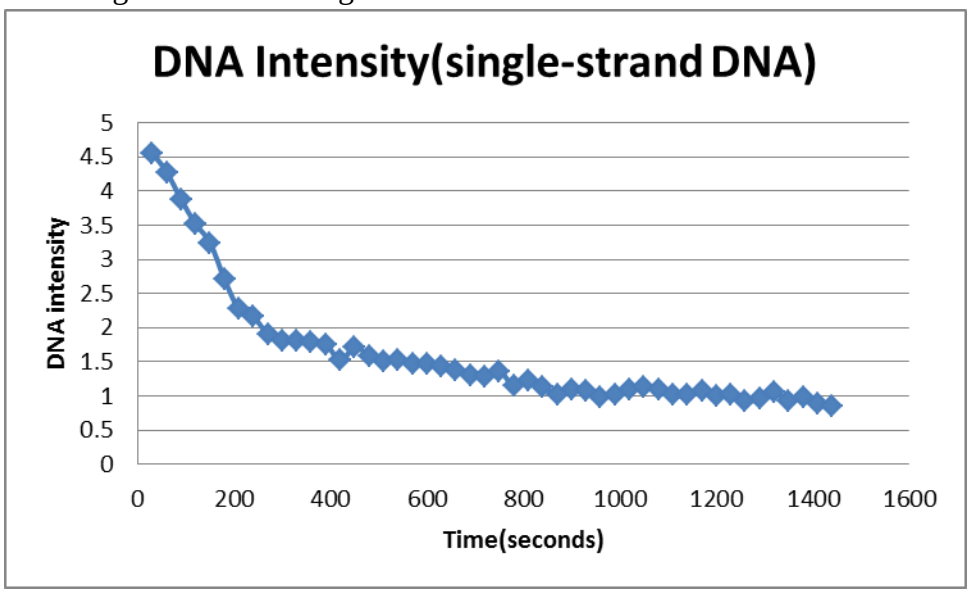


Figure 28. The mercury light is used. The intensity of ssDNA emission after every 30 seconds is shown.

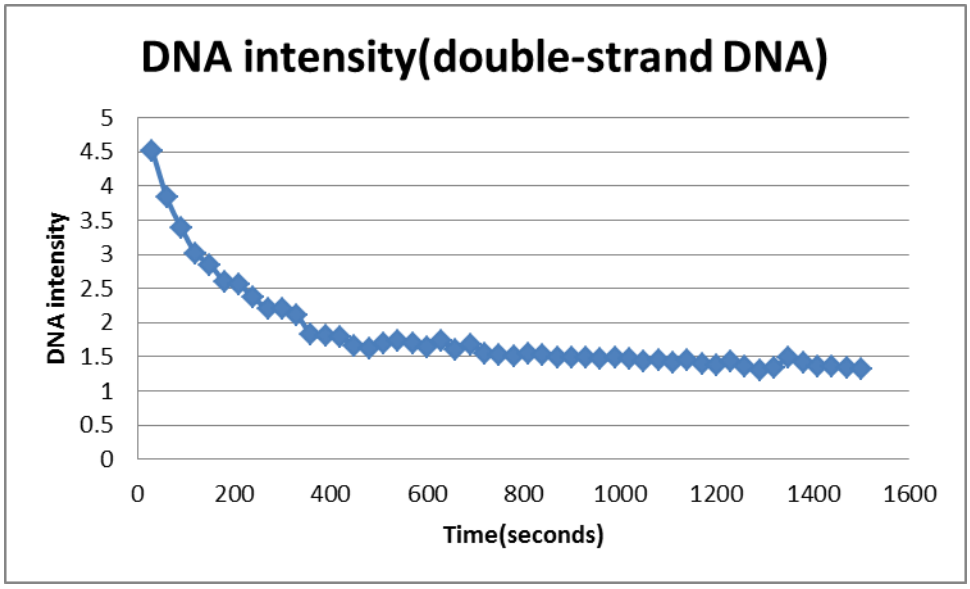
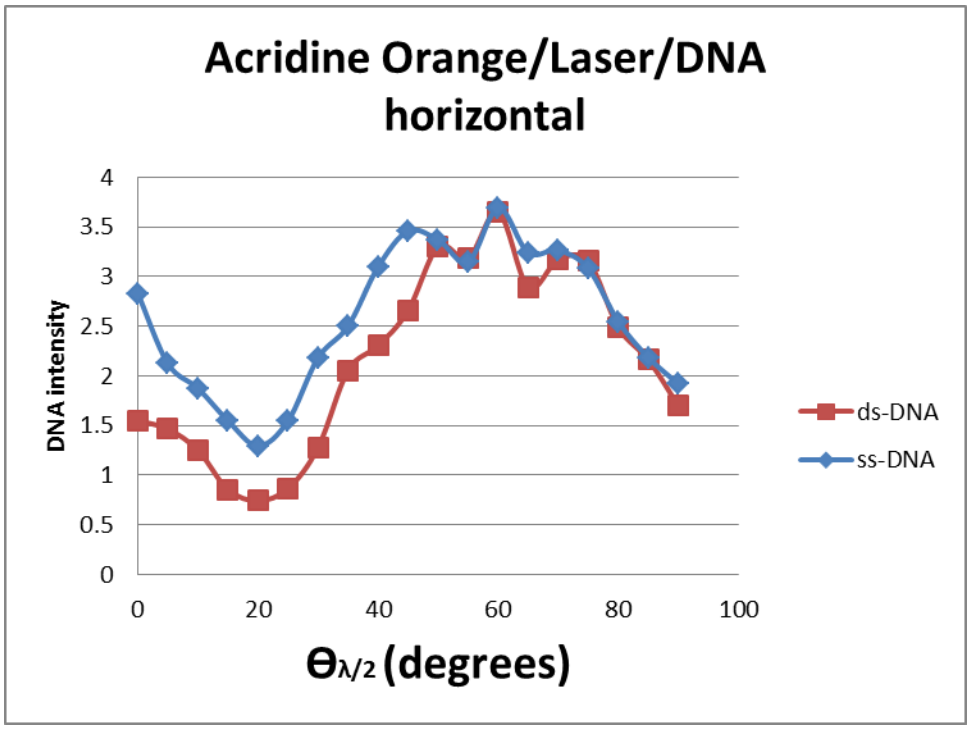


Figure 28. The mercury light is used. The intensity of dsDNA emission after every 30 seconds is shown.

We can see from the figures that the intensity of DNA goes down quickly at the beginning

and after about five minutes, it becomes steady, reduced by a factor of 3-4. It is clear that a total exposure of 100 seconds or less is desirable.
Now we start to use AO as the dye in the next tests.
(9)



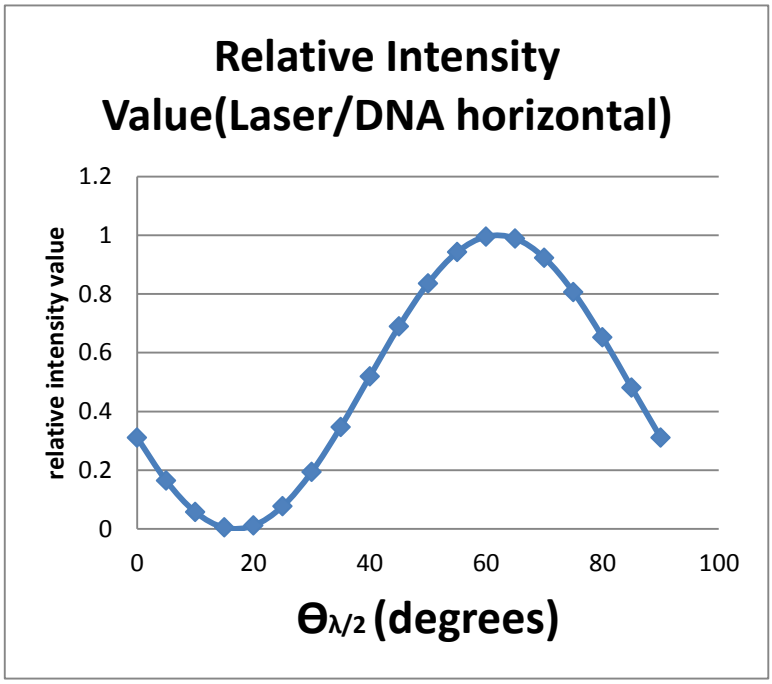
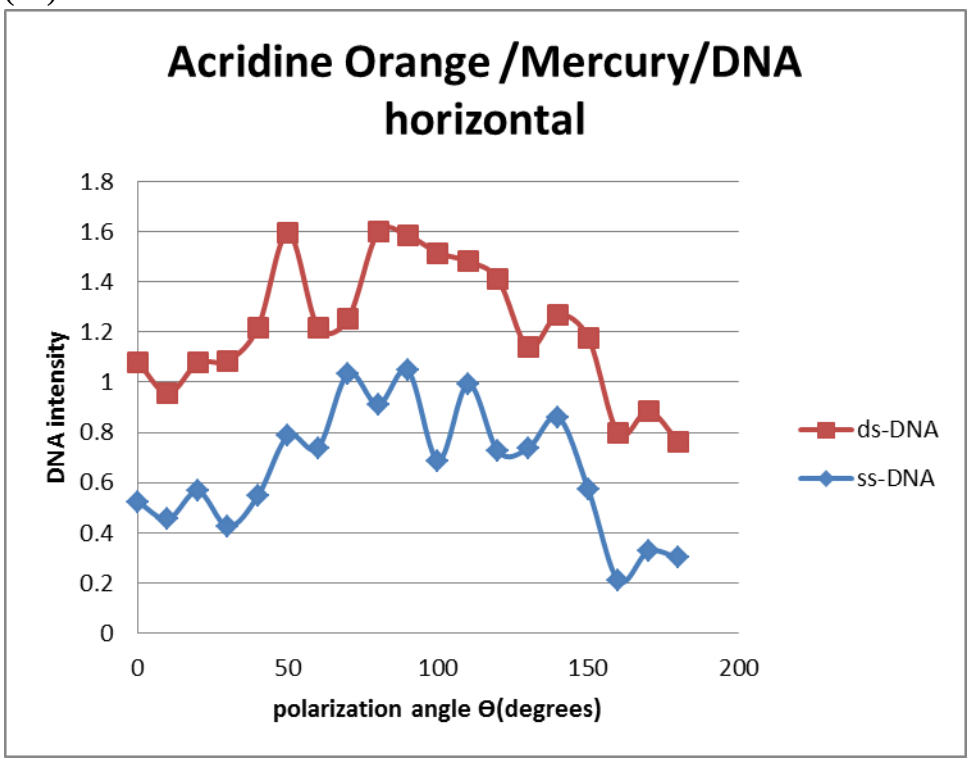


Figure 30. Polarized laser light and Acridine Orange are used. The DNA strands are horizontal (along $\hat{x}_{sample}$). The comparison of experimental data with the calculation is shown.

(10)



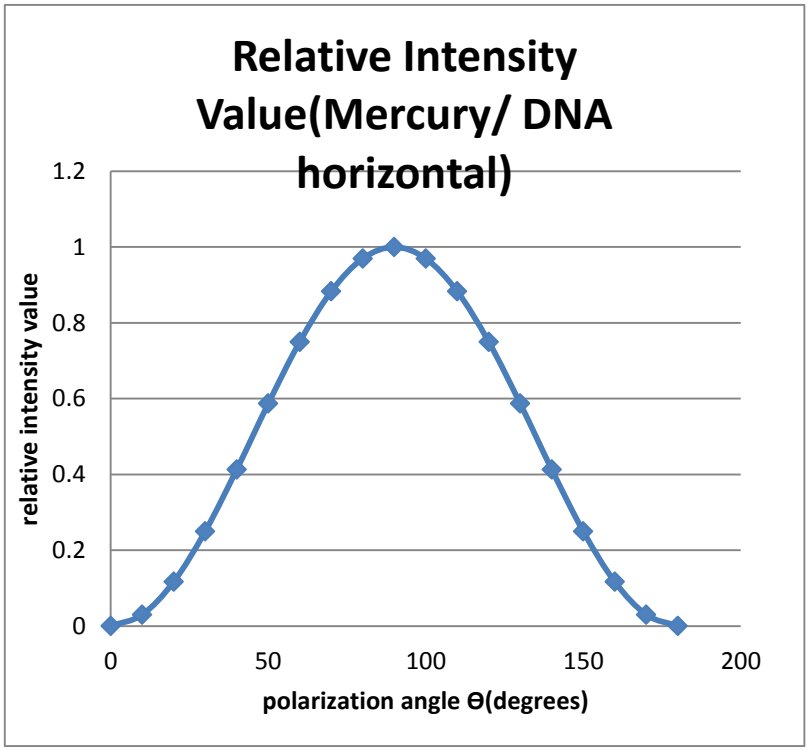
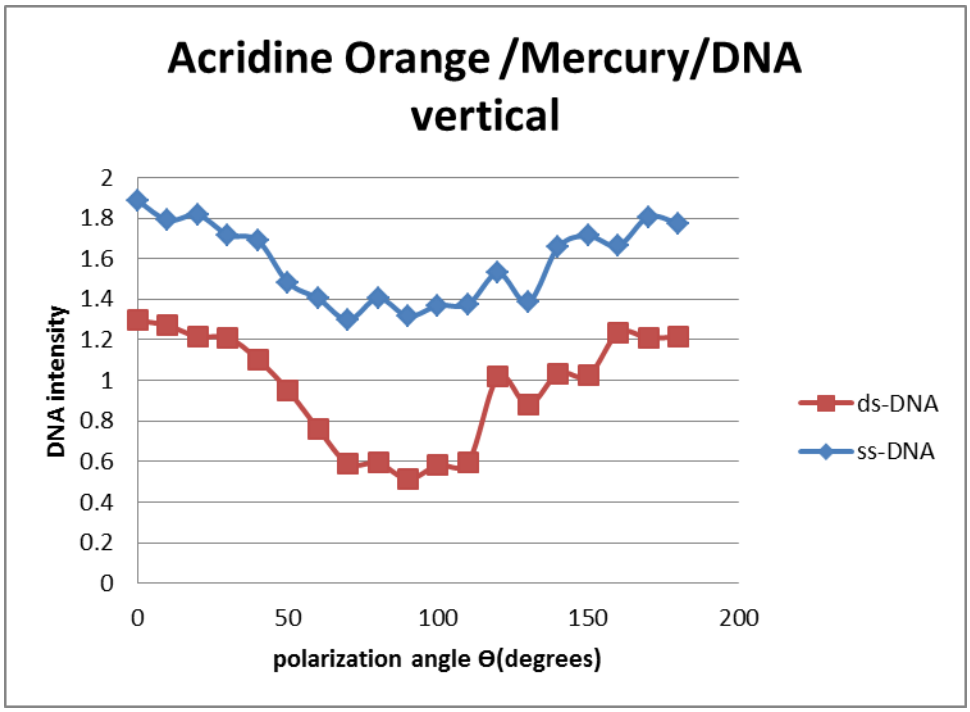


Figure 31. As in figure 30, but the polarized light is at normal incidence, using the Hg lamp.

(11)



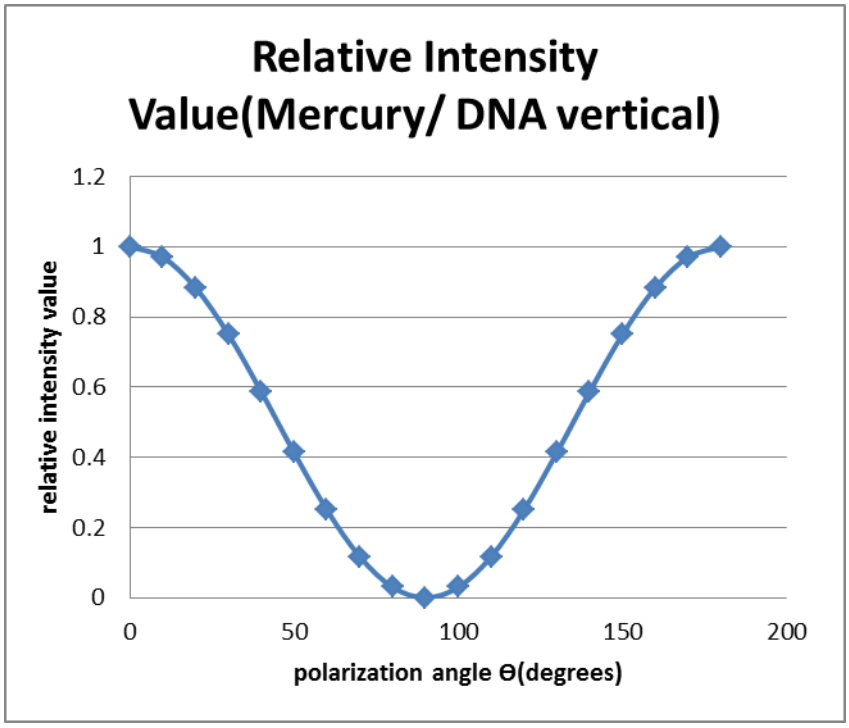


Figure 32. As in figure 31, but the DNA strands are vertical (along $\hat{y}_{sample}$).

Now it's clear that both in single-strand and double-strand DNA, dye molecules are binding perpendicular to the DNA strands.

Finally, we used both red and green filter to test whether red or green light takes the most part of the reflected light after exposure under mercury light.

The upper group is taken under 0 degree polarized angle, and the lower one is taken under 90 degree polarized angle. In each group, the upper one is the one with the red filter, and the

lower one is the one with the green filter.
(1)

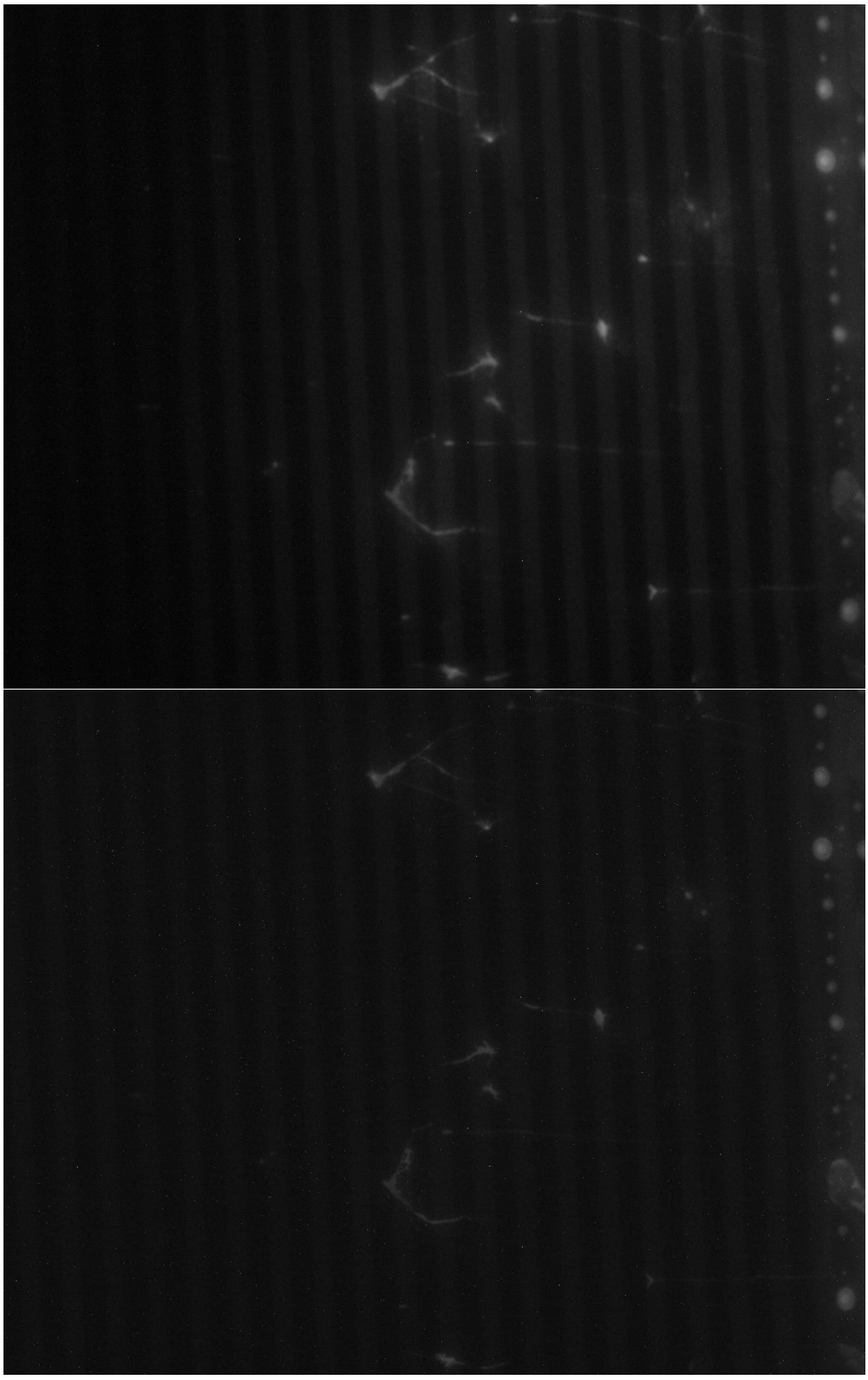


Figure 33. Mercury light and Acridine Orange were used. Comparison of the effect of red and green filter at 0° polarized angle on single-strand DNA is showed. Here, 40×lens was used and the size of the image area is 320μm*320μm.

(2)

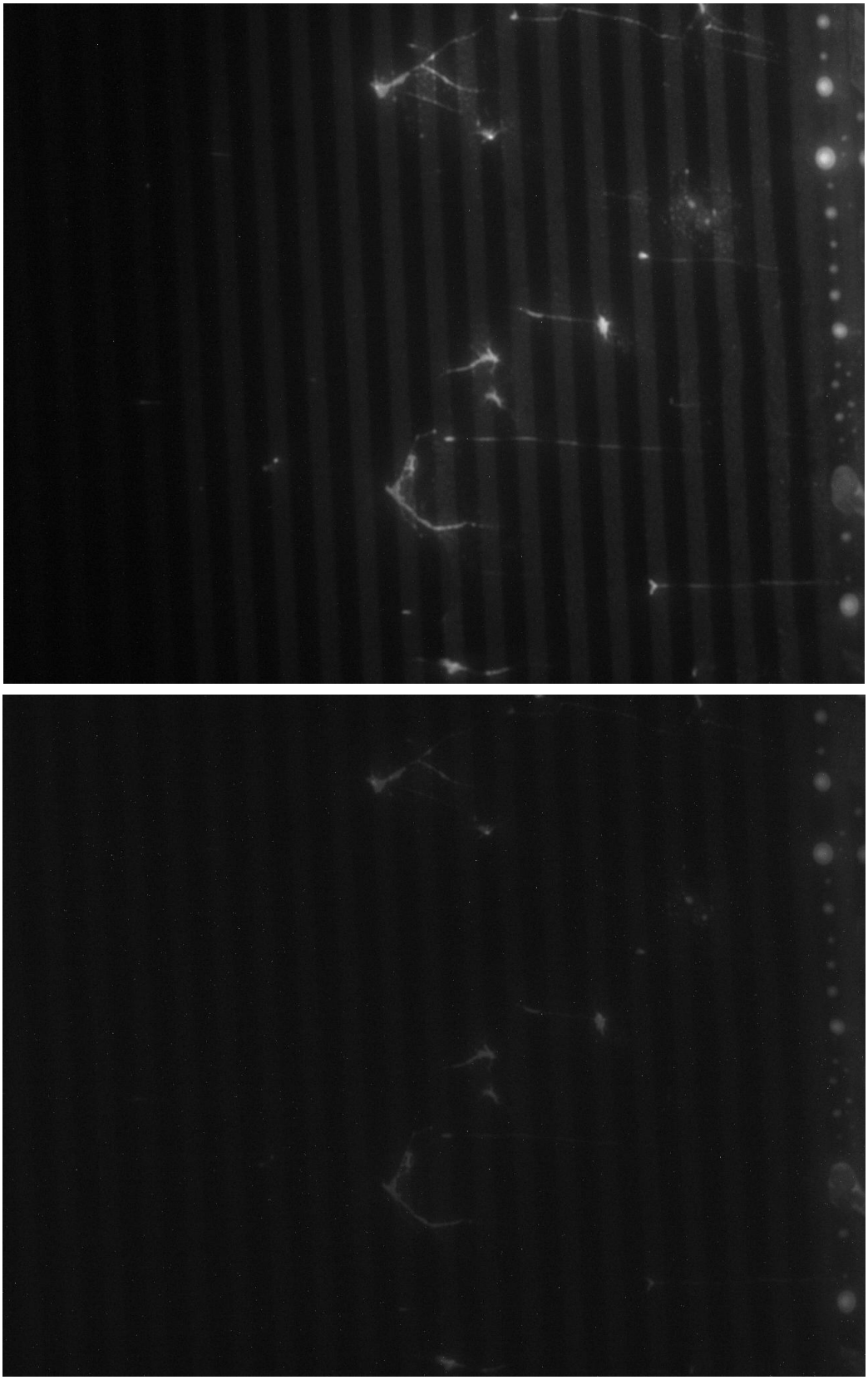


Figure 34. Mercury light and Acridine Orange were used. Comparison of the effect of red and green filter at 90 ° polarized angle on single-strand DNA is showed. Here, 40 ×lens was used and the size of the image area is 320 μm*320 μm.

In each group of pictures, it can be seen that the red one is brighter than the green one. And when we compare the picture taken at different polarized angle but with the same filter, we find out that the 90 degree polarized angle leads to brighter picture than the 0 degree polarized angle, which proves that the dye molecule is perpendicular to the DNA single-strand again.

But we still have two problems, one is that it's not so clear to distinguish by the naked eye directly through the picture above, we'd better to quantify the intensity; The other is that we only test once and the results maybe a coincidence and in order to clarify this question, we have to do the test repeatedly under the same condition.

Here we do three new tests. In the first two tests, we fixed the type of filter (red or green) , then we change the polarized angle repeatedly between 0 degree and 90 degree, In the last test, we do not use the polarizer and then change the filter again and again to check which filter leads to higher DNA intensity.

(1)

Figure 35. Mercury light and Acridine Orange were used. Change the polarized angle repeatedly at 0 ° and 90 ° with red filter. This is one of the photos. The ssDNA strand here is horizontal. Here, 40 ×lens was used and the size of the image area is 320 μm*320 μm.

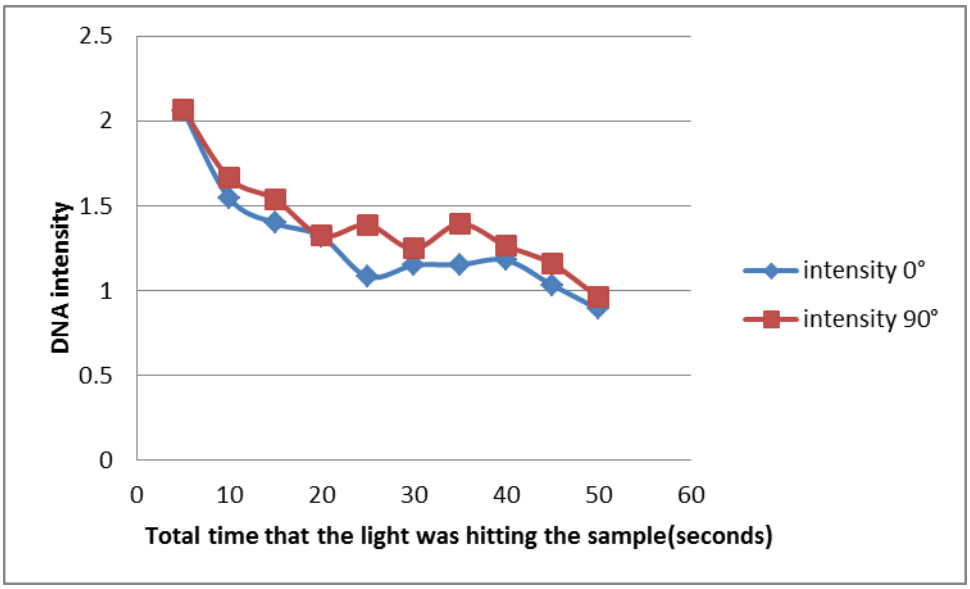


Figure 36. The intensity of DNA strands at 0 °and 90 °with red filter, respectively.

(2)

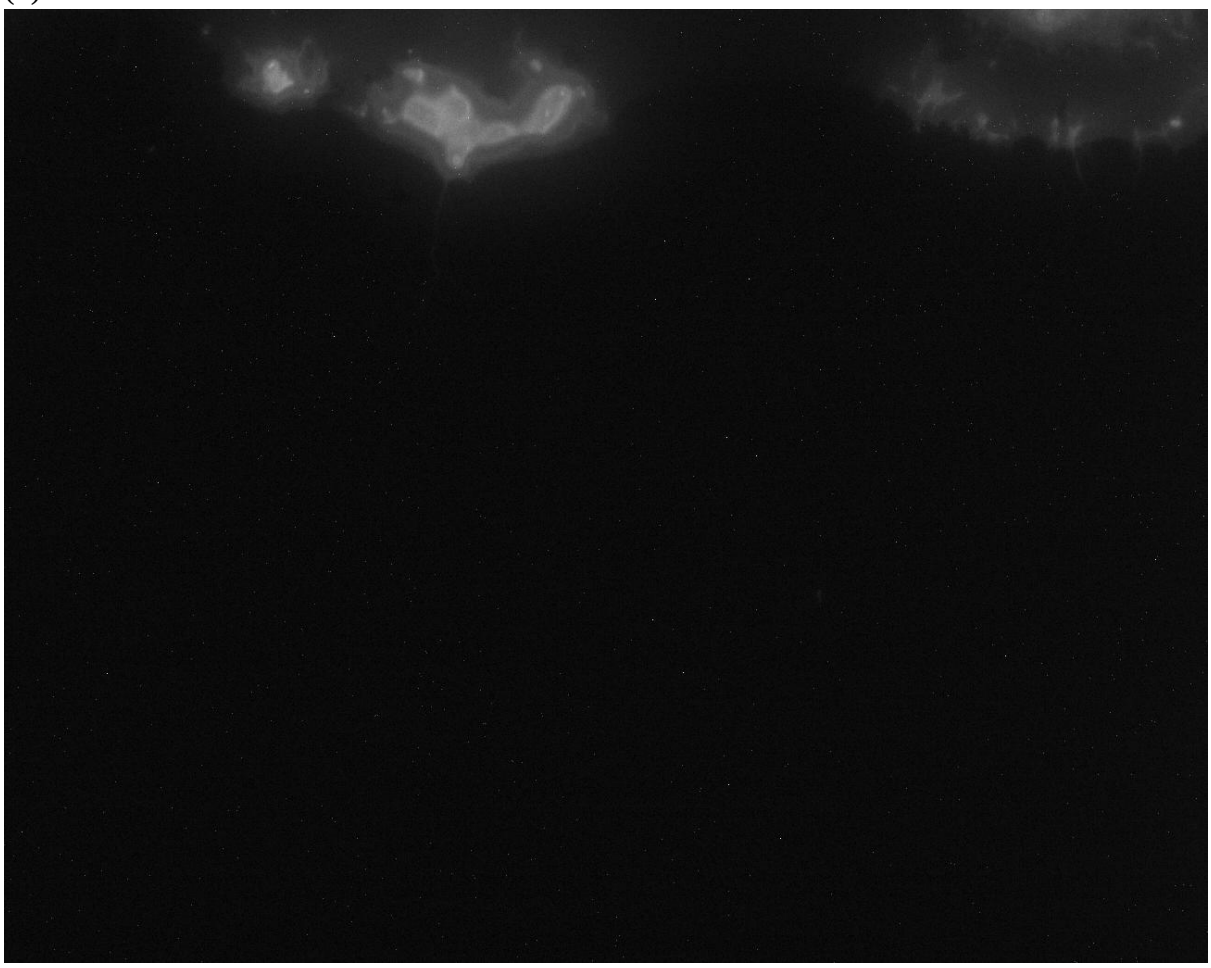


Figure 37. Mercury light and Acridine Orange were used. Change the polarized angle repeatedly at 0 °and 90 °with green filter. This is one of the photos. The ssDNA strand here is vertical. Here, 40 ×lens was used and the size of the image area is 320 μm*320 μm.

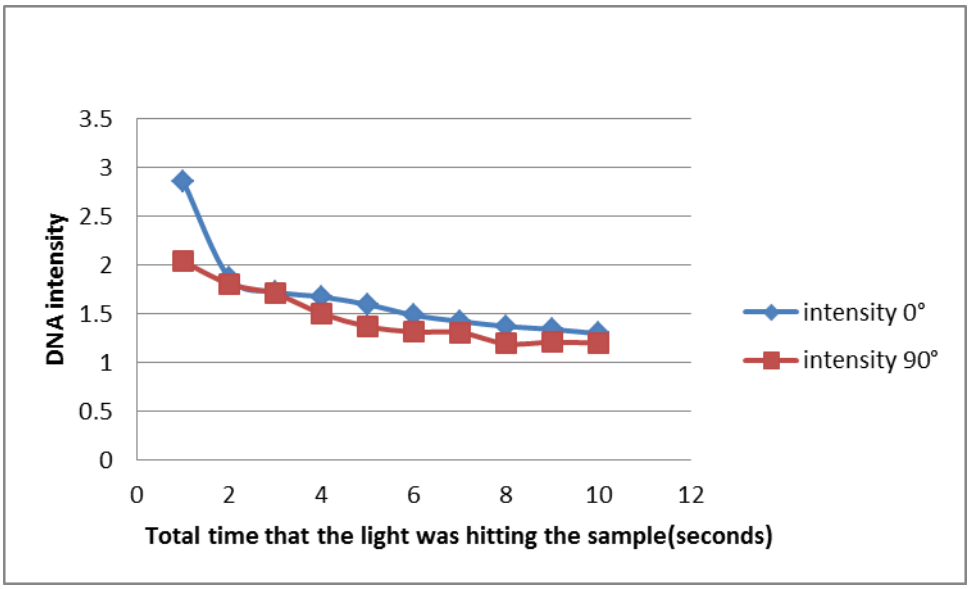


Figure 38. The intensity of DNA strands at 0 °and 90 °with green filter, respectively.

(3)

Figure 39. Mercury light and Acridine Orange were used. Change the filter repeatedly at the red one and the green one. This is one of the photos. The ssDNA strand here is horizontal. Here, 40 ×lens was used and the size of the image area is 320 μm*320 μm.

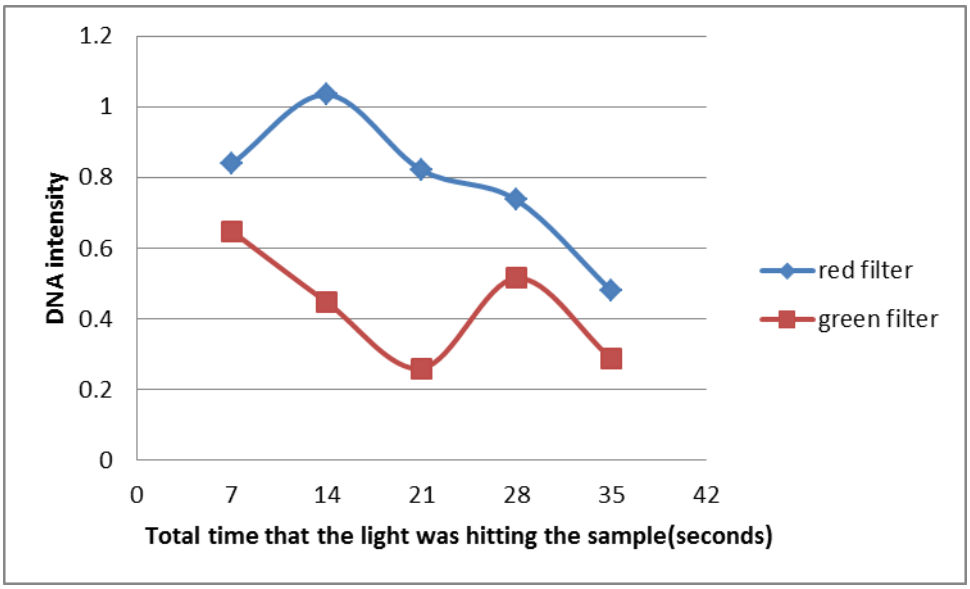


Figure 40. The intensity of DNA with red filter and green filter without the polarizer, respectively.

Conclusion.

The polarization dependence of fluorescence was successfully measured for three dye-DNA complexes using oriented, immobilized DNA molecules on PMMA- coated silicon substrates. The dyes used were SyBr Gold, YOYO and Acridine orange and all three dyes were found to intercalate into the bases for double-stranded DNA. Interestingly, it was found that the dyes also bound to single-stranded DNA with their dipole moment absorption axes perpendicular to the DNA axis.

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