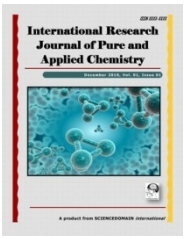

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Application of Newton's Zero Order Caustic for Analysis and Measurement: Part II Fluorescence

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AAG and LN designed the study and wrote the first draft of the manuscript. Authors CH and AAG measured and analyzed the results. Authors LN and VM reviewed the optical ray tracing and managed the literature searches. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

An oblate spheroid-shaped liquid solution acts as a lens through the phenomenon of Newton's zero order caustic resulting in the focusing of a low power source into high intensity light within the sample to detect and measure fluorescence. Microwatt LED sources can be used to measure fluorescence in two ways, depending on the properties of the solute. For a weakly fluorescing solute, emitted light can be detected as a beam emanating from the spheroid sample along the path of illumination. For strongly fluorescing solutes, emitted light can be detected radiating from the surface orthogonal to the excitation source. Three fluorescent molecules (tartrazine, SYBR Green I, and BBT anion) were studied using photodiode excitation sources of 430 and 470 nm maximum wavelengths respectively. Image capture for strongly fluorescent molecules was performed with a digital phone camera, followed by image analysis in order to calibrate light detected as a function of fluorescent molecule or complex concentration.

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ABBREVIATIONS

x_s = distance between a point source and the center of the sample being illuminated; b = major axis of a spheroid; a = minor axis of a spheroid; SYBR Green I = DNA intercalating dye used to measure dsDNA by fluorescence; BBT = a substrate for alkaline phosphatase that fluoresces in its anionic form; ALP = alkaline phosphatase; PTFE = polytetrafluoroethylene; PBT = Polybutylene terephthalate; Cartesian coordinates = (x,y,z) Radius of spheroid =R; light intensity= I; OpAmp = operational amplifier.

1. INTRODUCTION

Fluorescence measurement is a versatile and widely used method in diagnostics, molecular and cellular biology research, environmental studies, and chemical analyses. Advances in nanomaterials, highly sensitive fluorescent detection strategies, as well as probes with a wide array of emission wavelengths, continue to expand its use, especially when specific detection and measurement is desired at very low concentrations – even down to a single molecule. In the first part of this 3 part series, Newton's zero order caustic was shown to be an optical system that can generate high intensity light within the sample being detected, without the use of high power light sources, lasers or lenses. In fluorescence, a relatively high intensity of light at the required excitation band is usually preferred, as long as photobleaching can be minimized, in order to maximize the detection range and sensitivity. Emission of fluorescent light at low fluorophore concentration is given by the equation:

$$I_f = 2.3\Phi_f I_o \varepsilon l C \tag{1}$$

where Φ_f is the quantum efficiency; I_o is the intensity of the excitation light; ε is the molar absorption coefficient of the fluorescent molecule; *l* is the path length, and C is the molar concentration of the fluorescent molecule (or limiting species if a probe fluoresces in the presence of a target molecule). For example, the DNA detection molecule SYBR Green I is considered to have a molar absorption coefficient of 50,000-70,000, quantum efficiency of 0.8, and at a dilution of 1:5,000 from the standard 10,000x stock, a concentration of 3.9 micromoles per liter. Generally, quantitative fluorimetry is done using instrument-specific factors rather than explicitly calculating quantum efficiency, absorptivity and path length. The emission spectrum is normally of longer wavelength than that of the excitation band, and if this difference, which is known as the Stokes shift, is sufficiently separated, detection of the emitted light can be performed, although optical filters are frequently employed as well.

Fluorescence detection can be performed using a variety of geometries, filters, and light collection methods, depending upon the particular application. One method that uses fluorescence from a spherical liquid sample in air is FACS (fluorescence-activated cell sorting) whereby cells suspending in small drops are irradiated with a laser, sorted and collected, typically based on the fluorescent characteristics of a cell labeled with fluorophore-conjugated antibodies. However, the authors are unaware of a method or device whereby excitation light is intensified within a liquid sample for detection and measurement of the fluorescing species concentration.

Fluorescence microscopy and spectroscopy rely on excitation sources in the range of 10-100 W, while a single, low cost LED is typically much less powerful using 50-100 milliwatts of

electrical energy and providing less than a milliwatt of light energy in a particular direction. Thus, being able to generate sufficient intensity of excitation light within a sample using low power LED sources would be a very useful way to lower power needs in fluorescence measurements.

In part I, it was shown that an oblate spheroid can generate a cusp of revolution and an axial spike caustic within the spheroid due to the refraction of light without any internal reflections. The description of light rays incident on a drop of water with refraction but without reflection is termed Newton's zero order caustic in the literature [1,2]. For oblate spheroids where incident light is aimed at the major axis, not only are light intensities in the forward scattering direction significantly higher than the incident light intensities, but for a wide range of aspect ratios the highest intensity light can be focused in the center of the sample [1]. Due to the high forward illumination intensity in this system, it would then be reasonable to expect that fluorescence could be generated within the sample, but it is unclear how the excitation light and sample shape can be best employed to simplify detection while maintaining high sensitivity and linear calibration at low fluorophore concentration.

This paper explores the use of Newton's zero order caustic using experimental results for fluorescent solutions shaped into spheroids and measured via a simple optical platform. Two-dimensional ray tracing is used to qualitatively illustrate where high intensity excitation light occurs using different spheroid shapes, which can be created by lowering the surface tension of the sample. Relevant scientific literature on light scattering and rainbows is also used to help link 2-D ray tracing with experimental results.

2. MATERIALS AND METHODS

2.1 Sample Stage

The sample stage consisted of a PTFE coated slide with 30 wells of 3 mm diameter (Tekdon Inc., Myakka City, FL). Two wells along the center line of the slide were employed to pin drops of 160 microliters of the liquid sample, forming a flattened spheroid shape without the need of a sample container. Each multi-well slide can measure 5 different samples and each position can be used for multiple tests of the same sample solution. At room temperature, evaporation is very slow since the aqueous drop is pinned and held in an oblate spheroid shape rather than allowed to spread into a thin film. Both the lower surface to volume ratio of the spheroid and the stagnant film of air on the lower half of the sample contributed to low evaporation rates such that the sample volume changed only by 7% after 15 minutes, which for the purposes of fluorescent measurements for highly fluorescent molecules, introduces negligible variations in the data. The oblate spheroid shape of the samples were verified using ImageJ (http://rsbweb.nih.gov/ij/) and found by edge analysis and measurement to have ratios of major to minor axis typically in the range of b/a = 1.5 – 2.0, depending upon the concentration of surfactant in the drop and the relative effects of pinning and surface tension that balance and keep the sample intact. Due to drop spreading via gravitational effects for a larger 160 microliter drop as compared to the 120 microliter drops used in Part I, the sample has a flat zone on the bottom of the drop. This shape deviation is not considered to negate the assumption of a oblate spheroid used in 2-D ray tracing because experimental results corroborate the model predictions. Also, for measurement of fluorescence from an orthogonal position using highly fluorescing molecules, the change in drop shape does not affect the ability to calibrate the system using the integrated density software tool in ImageJ.

Refraction, reflection, and light loss due to the material properties of a container is avoided in the experimental platform since the sample is a free standing drop.

2.2 Photodiode Device

Excitation light was generated using two different blue LED photodiodes (430 nm and 470 nm peak wavelengths) with internal micro-lens and a PBT housing (Industrial Fiber Optics, Tempe, Arizona). Each photodiode was powered by a standard, constant voltage, DC circuit based on the manufacturer's recommended value of 4.5 Volts at 35 mA using a 9 volt battery. The spectral bandwidth of the 430 nm photodiode is wider (65 nm) than that of the 470 nm photodioded (25 nm). Typical optical output power levels for these photodiodes, which are normally used in fiber optic communications, are 25 micro Watts for the 430 nm photodiode and 75 micro Watts for the 470 nm photodiode.

2.3 Fluorescence Measurements

The same optical holder for the 30 well slides was used in Part I of this 3 part series. However, only one photodiode was used and the acrylic body was painted flat black. Measurements were conducted in a dark room, with a low wattage desk spot lamp pointing away from the sample stage in order to apply the samples and record images or spectra. In order to conduct the measurements for SYBR Green I and BBT anion, an 80 microliter aliquot of the fluorescent molecule solution is first applied to the slide followed by a second 80 microliter aliquot of DNA or alkaline phosphatase. Mixing of drops using these 30 well slides has been observed to take place in less than 2 seconds, and thus images can be acquired shortly after the second drop is applied. Typically, the photodiode is left on during the experiments for several hours since the power usage is relatively low.

Detection of light when studying SYBR Green I and BBT anion samples was conducted using an HTC Incredible 2 Android phone camera to acquire images. The ImageJ software program was used after the photo image was recorded to apply a digital color filter (selecting the green filter image), and measure the integrated density in a section of the spheroid sample. Background fluorescence near the drop was also recorded and subtracted from the integrated density measurement. The camera was kept between 4-5 cm from the sample.

2.4 Spectrophotometry

For tartrazine, a fiber optic spectrometer (model USB2000 Miniature Fiber Optic Spectrometer, Ocean Optics) was used to detect the light exiting the spheroidal sample. This spectrophotometer accepts incident radiation transmitted through a single-strand optical fiber (400 μm fiber diameter, UV/VIS Laboratory-grade Patch Cord Optical Fiber Assembly, Ocean Optics) and disperses it via a fixed grating across a 2048-element linear CCD array detector. The operating software (SpectraSuite, v. 2.0.109, Ocean Optics) runs on a Mac Pro laptop (Apple, Cupertino, CA). An achromatic collimating lens (350-2000 nm, model 74-ACR, Ocean Optics) was used to collect the incident light, which is then transmitted through the optical fiber to the grating. Spectra were taken as described in the experimental section of Part I of the 3 part series.

2.5 Chemicals and Reagents

Tartrazine exhibits a maximum light absorbance at 427 nm and fluoresces when excited by a relatively broad band of UV light (280-380 nm), generating a strong fluorescent light at 565 nm [3]. SYBR Green I, N',N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine, (Invitrogen, Grand Island, NY) is an asymmetrical cyanine dye that when bound to dsDNA absorbs blue light at a wavelength maximum at 497 nm and emits green light at a wavelength maximum at 520 nm. In the absence of dsDNA, SYBR Green I has a relatively weak, but detectable, fluorescence. BBT, 2'-[2'-benzothiazoyl]-6'-hydroxybenzothiazole, (Promega, Madison Wisconsin) is a substrate for alkaline phosphatase and fluoresces in its anionic form after enzymatic reaction, producing a fluorescent species with a wavelength maximum for excitation at 435 nm and an emission wavelength maximum at 575 nm.

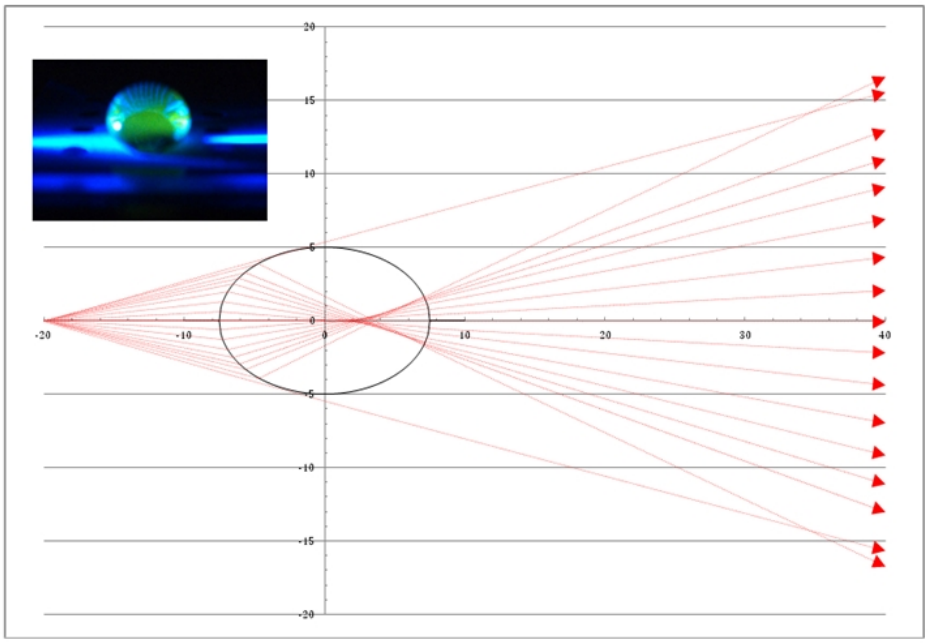
3. RESULTS AND DISCUSSION

3.1 2-D Ray Tracing

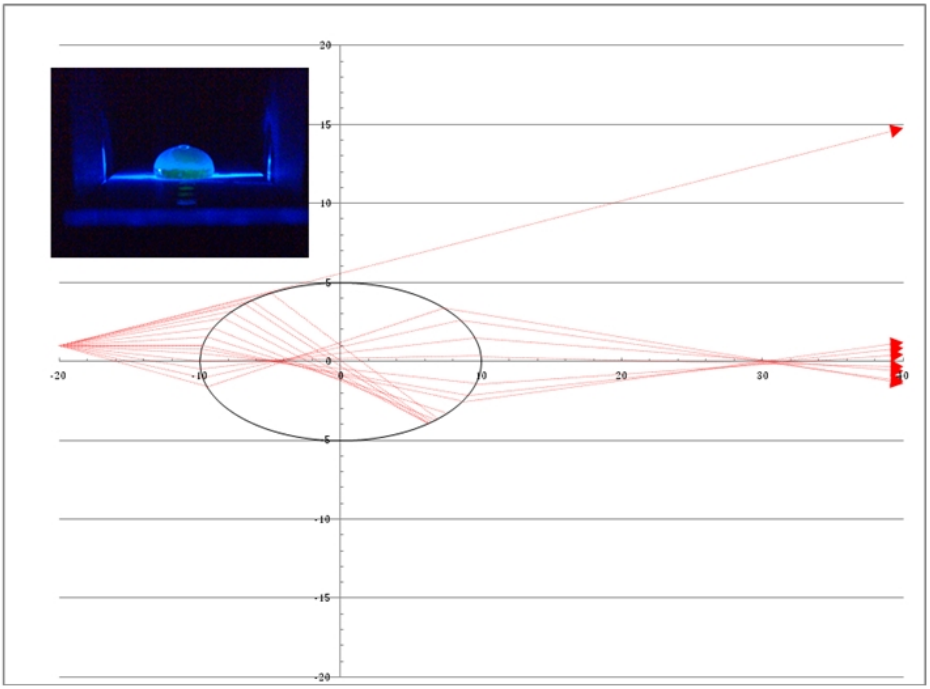
Light ray tracing analysis in the literature [1,4] indicate that light irradiating an oblate spheroid shaped sample edge-on will coalesce within its volume into Netwon's zero order caustic. Through observation of different fluorescent species and solutions with and without surfactants, it became apparent that the volume and aspect ratio of the spheroid affect the fluorescent light detected since the source light used is of very low intensity and requires focusing within the sample to generate an easily detectable fluorescence intensity. Also, the position and geometry of the excitation light source with respect to the center of the drop is crucial for understanding the way light refracts within the sample. Lock [1] and Lock and McCollum [2] derived equations for the ray tracing of light with no reflections for spheres, elliptical cylinders, and spheroids. The equations from these authors are used to compute the path of light rays in 2-D in order to better interpret images and observations of fluorescence. Computed and graphed results were compared to similar geometries and source types (point or uniform) given in these articles, as well as by Kofler [5], in order to verify the detailed calculations.

Fig. 1a illustrates rays for a point source placed at a distance from the center of the sample of four times the spheroid's minor axis ($x_s/a = 4$), with a ratio of major to minor axis (b/a) of 1.5. It should be noted that the bottom of the sample drop (see insert for SYBR Green I detection of dsDNA) is flatter than this idealized representation, but the difference is not significant for the purposes of this discussion since ray tracing is being used solely as a qualitative guide to indicate where the highest intensity light is found. Fig. 1b shows ray tracing for a spheroid with $b/a = 2.0$, where rays exiting the drop $y < -5$ are not shown to approximate the more pronounced difficulties in justifying using an oblate spheroid as the appropriate geometry since there is an appreciable flattening of the drop, for this b/a ratio. In this Figure, the caustic focus appears to be shifted to near the part of the spheroid where light enters, which is directly attributable to the higher value of b/a and the fixed position of the light source in the experimental system used. To illustrate the significance of this effect, the insert to Fig. 1b shows an experimental image for b/a=2 and the top half of the spheroid is seen to have a diffuse glow of blue light. Not shown is that in the room light the sample in the Fig. 1b insert appears essentially like water and similar in clarity to the insert of Fig. 1a. By comparison, in Fig. 1c, a 2-D ray tracing of a spherical sample shows that offsetting the point source for a sphere shifts the caustic focus slightly below the plane x= 0. This

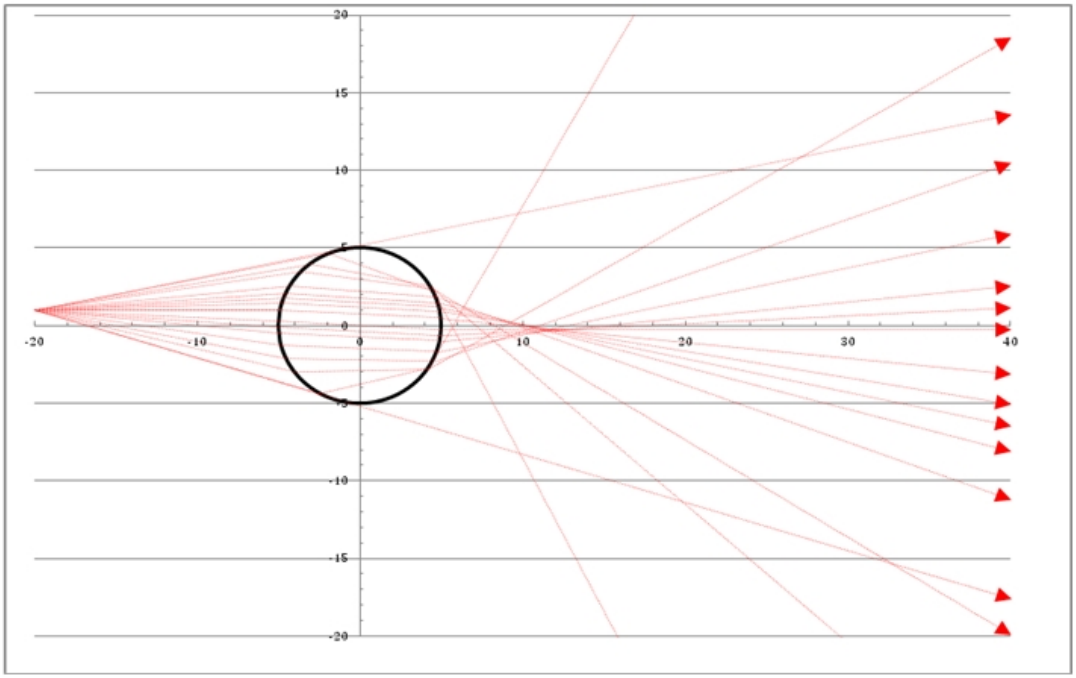
exploration of shape and point source position illustrates that 2-D ray tracing can help interpret deviations due to sample and source geometric changes. Fig. 1a and 1b also indicate that the spatial location within the sample most useful for quantitation is influenced by b/a.



(a)



(b)



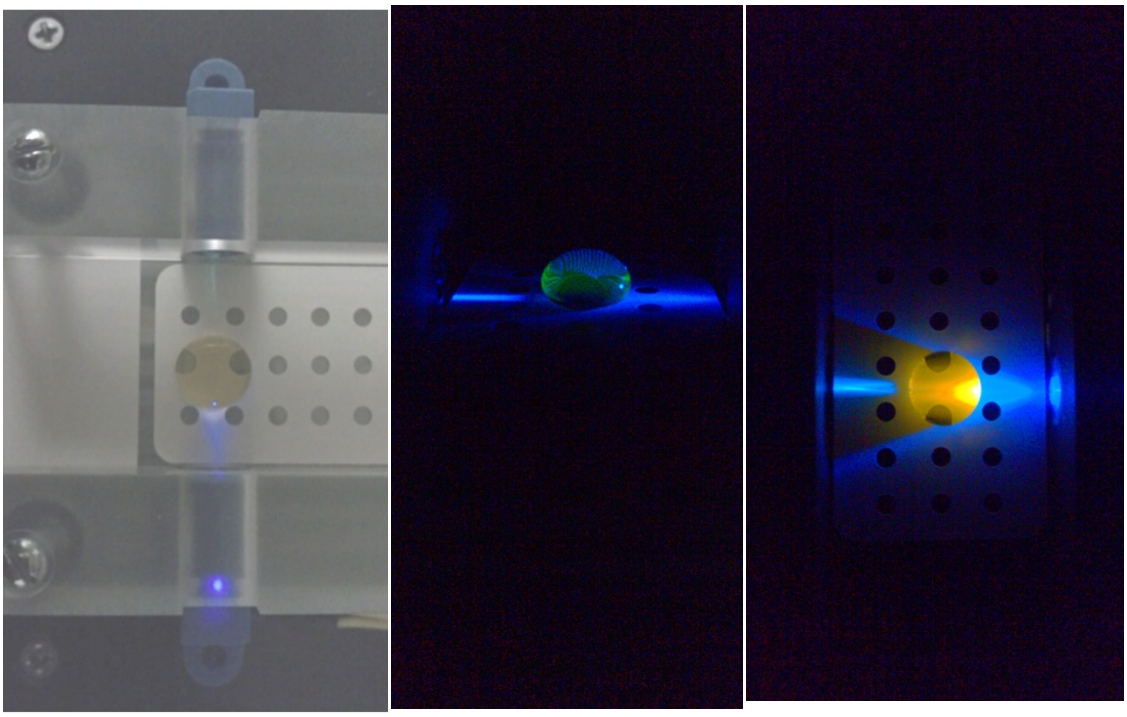
(c)

Fig. 1. 2-D Ray tracing for (a) a centered point source illuminating an oblate spheroid of b/a=1.5, (b) a point source offset from the centerline with respect to an oblate spheroid of b/a=2 with some bottom rays removed to represent effect of spreading of a liquid sample at high surfactant concentration, and (c) a point source offset from a sphere for comparison purposes. Figs. 1a insert is an image of SYBR Green I with calf thymus DNA with b/a=1.5. Fig. 1b insert is another image of SYBR Green I with calf thymus DNA with added surfactant in order to increase b/a to a value of 2

It should be noted that 2-D ray tracing of a point source illuminating a spheroid is geometrically problematic since spheroidal symmetry does not match the spherical symmetry of a point source, making it difficult to see how Figs. 1a and 1b could be directly interpreted in 3-D. Lock and McColum [2] correctly matched a point source to a cylinder of water with an elliptical cross section for their ray tracing, and Lock [1] limited his study of oblate spheroids to uniform sources. However, even in these cases, Ren and colleagues point out that interference effects of all orders of rays are not taken into account making it difficult, in the 2-D approach, to take into account scattering in all directions which can be done with a vectorial complex ray model instead [6]. Horvath at al [7] studied water drops on leaves, provided ray tracing calculations for sunlight at different angles and drops that took into account flattening at the bottom of the drop due to the leaf surface. Qualitatively, the 2-D results in Figs. 1a and 1b follow similar trends in the position of the caustic focus with b/a and incident light angle shown in all of these studies, so it is assumed that as long as the 2-D ray trace is not considered to represent a symmetrical 3-D result, the shape and coordinate mismatch should not limit their use in qualitatively interpreting the experimental images.

3.2 Comparison of Fluorescent Molecules

Figs. 2a-c compare the way fluorescence can be observed through the use of Newton's zero order caustic. In Part I, it was found, based on deviation of the absorbance calibration from the Beer Lambert law, that tartrazine fluoresces at higher solution concentrations. It was also shown that the fluorescence could be detected by analyzing the spectra of the light exiting the sample. By comparing Figs. 2a and 2c, which have high concentration (0.2 millimolar) of tartrazine and BBT anion respectively, beams of colored light are apparent in both images. This effect is more striking in Fig. 2c since the emitted light is yellow and, as predicted by ray tracing in Fig. 1a, there is a divergence of light exiting the sample. The more intense beam of blue light emanating from the sample in Fig. 2c is an interesting contrast to what is seen in Fig. 2a where the light corresponds to the emission spectra for tartrazine. The difference between Fig. 2a and 2c is attributed to both the higher molar absorbance of tartrazine for the excitation light, the respective bandwidths of the excitation sources, and the higher fluorescence of BBT anion. At 0.2 millimolar concentration, tartrazine absorbs much of the 430 nm blue light. But the excitation of tartrazine by light of less than 400 nm wavelength results in fluorescence that can be visually observed, even in room light. On the other hand, BBT anion is used at low concentration and much of the 470 nm light concentrated along the axis of the sample is not absorbed by the sample.



(a) (b) (c)

Fig. 2. Observations of fluorescence for tartrazine (a), SYBR Green I with DNA (b), and BBT anion with ALP (c), in order to compare and contrast how fluorescence is observed and to illustrate the use of 2-D ray tracing as a guide to interpret the features seen in (b) and (c)

The same light source used to generate the image in Fig. 2c was used to generate the image in Fig. 2b, which was created by combining SYBR Green I with 80 ng of calf thymus DNA. In a typical experiment, with diluted SYBR Green I and low surfactant concentration, there is a distinct green color within the oblate spheroid, however striped lines of blue are seen at the top and some green patterns on the sides. These striped patterns are believed to be reflections of light from the Teflon coated slide and glass wells at the bottom of the slides. Another important observation made through imaging hundreds of fluorescent images of SYBR Green I [8] and BBT anion solutions is that the bottom middle of the sample is relatively dark, which is not surprising since the 2-D ray trace suggests that the spheroid receives no refracted or transmitted light in that region. When viewing BBT anion solution from the side (Fig. 3), blue stripes also appear at the top of the spheroid until the concentration reaches a level where the fluorescent light is bright enough to mask the reflected excitation light.

3.3 SYBR Green I

Based on the calculations by Lock, for a spheroid where b/a = 1.5 and using a planar source of light, end on, there is a high degree of forward scattering in small oblate spheroid water drops with a peak increase in intensity to nearly $I/I_o = 1 \times 10^6$ at scattering angles between 0 - 2 degrees, and with I/I_o between 100-1,000 for scattering angles between 3-30 degrees. Since power is proportional to intensity, if area is kept constant, this calculation suggests a sizeable increase in light intensity is possible with this experimental optical platform. Kofler [5] used geometric and wave optics to predict a global intensity increase at the caustic focus of 1,000-fold. These predictions suggest that for the photodiodes used, which have output optical power when coupled with a plastic fiber of between 25-75 micro Watts, the caustic focus may yield an optical power equivalent to as much as several Watts, at low scattering angles. Measurement of the light intensity generated is beyond the scope of this paper, but instead the utility of the system for making useful molecular measurements was explored experimentally. Overall, it was observed that the limit of detection using SYBR Green I for DNA using a dilution of 1:5,000 was 1 ng/ml, which is comparable to the detection range found in commercial fluorimeters, but with much less instrumentation complexity. Similar performance when measuring alkaline phosphatase using BBT, suggests that the caustic focus using a spheroid sample leads to adequate light intensity for calibration. Also, because the most intense light is limited to a fraction of the sample volume, photobleaching of the available fluorophores is slowed, permitting steady-state fluorescence measurement over longer periods of time. It is important to note that depending the concentration of surface active agents in the sample, the area used to calculate fluorescence using integrated density needs to be guided by the ray tracing shown in Fig. 1. In general, as the concentration of surfactant increases, the area used to calculate fluorescence should be smaller and closer to the zone of highest light intensity in the droplet in order to have reproducible fluorescence measurements.

Fig. 3 shows typical results from the optical system when measuring calf thymus DNA using SYBR Green I, compared to published results by Rengarajan and colleagues [9]. The data shown is based on triplicate measurements, and a linear best fit accompanies the data. For the typical SYBR Green I dilution of 1:5,000 from an original solution dissolved in DMSO, the calibration is not linear at DNA concentration much above 1 microgram/ml. It was also useful to see whether the optical platform could yield reasonable detection sensitivities for a variety of cell phone cameras. First-year students at Arizona State University [8] tested the optical platform using their own cell phone cameras to record the images and calibrate the system.

In all cases, the images were taken using a side view and processed using ImageJ as described in the experimental section.

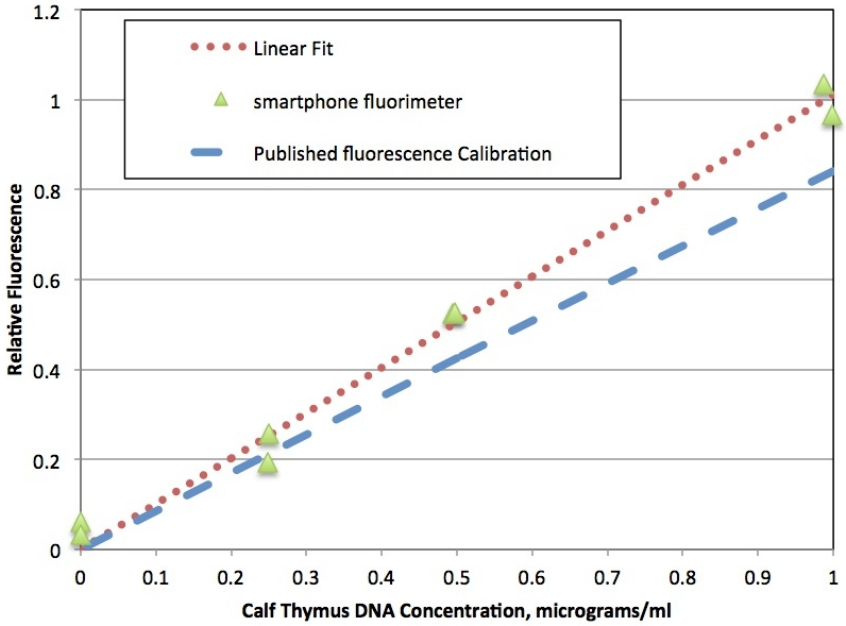


Fig. 3. Calibration curve for SYBR Green I detection of calf thymus DNA and comparison with published results using a standard fluorimeter [8] (blue dashed line). Each green triangle represents the average of triplicate measurements for a sample. The red dashed line is a linear fit of the data. More calibration data using a variety of smartphones can be found online [9]. For the concentration of SYBR Green I used, it is well known that relative fluorescence is not linear with DNA concentration above 1 microgram per ml, and thus higher concentrations are not shown in this figure

3.4 BBT

Fig. 4 shows a sequence of images over time to illustrate the evolution of a fluorescent species due to enzymatic action by alkaline phosphatase on BBT. As seen in Fig. 4a, before ALP reacts to produce BBT anion, the drop appears relatively dark in the interior with blue light illuminating from the right side of the image and seen exiting the left side. A gradual brightening of the drop is evident as the reaction proceeds. A series of measurements were taken after 7 minutes and compared to the manufacturer's published results for alkaline phosphatase measurement after 15 minutes. The slopes of the calibration lines were very similar (1290 RFU/minute versus 1280 RFU/minute) when the images were processed and referenced to the linear range below 0.8 milliunits.

3.5 Tartrazine

Tartrazine fluorescence was discussed briefly in Part I, but for comparison with SYBR Green I and BBT, other observations and measurements are instructive. Fig. 5a shows an image of a 0.2 mM tartrazine solution from a side view in a dark room, while Fig. 5b shows the same sample with the room lights turned on. Fig. 5a does not indicate the presence of

fluorescence, but in Fig. 5b it appears that the light leaving the sample is in its emission spectra. In fact, using a fiber optic spectrometer (Fig. 6) to detect the light exiting the solution in the forward direction indicates that the light is in the expected emission wavelength band. However, it does appears that the fluorescence intensity is much weaker than SYBR Green I or BBT anion fluorescence since measurement using cell phone imaging is not possible, because the sample appears to have an integrated density similar to the background, even after color filtering.

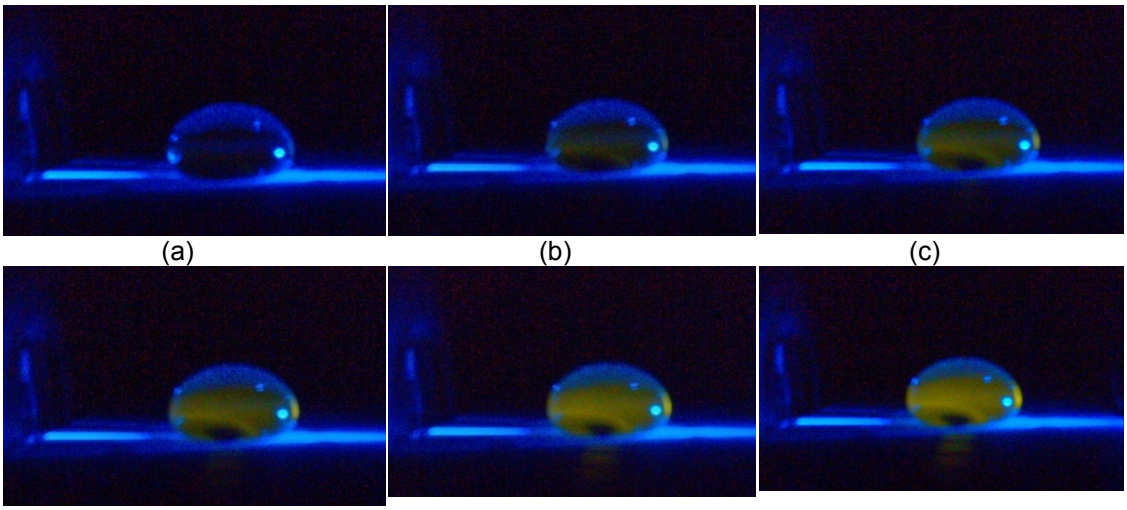


Fig. 4. Sequence of 6 images used in calibrating alkaline phosphatase (ALP) detection using BBT as a substrate. In these images, ALP concentration of 0.16 milliunits is being analyzed. The time sequence of images after combining the substrate with the enzyme are: (a) 0; (b) 1; (c) 2; (d) 3; (e) 5 and (f) 7 minutes. Data were taken at 27 C

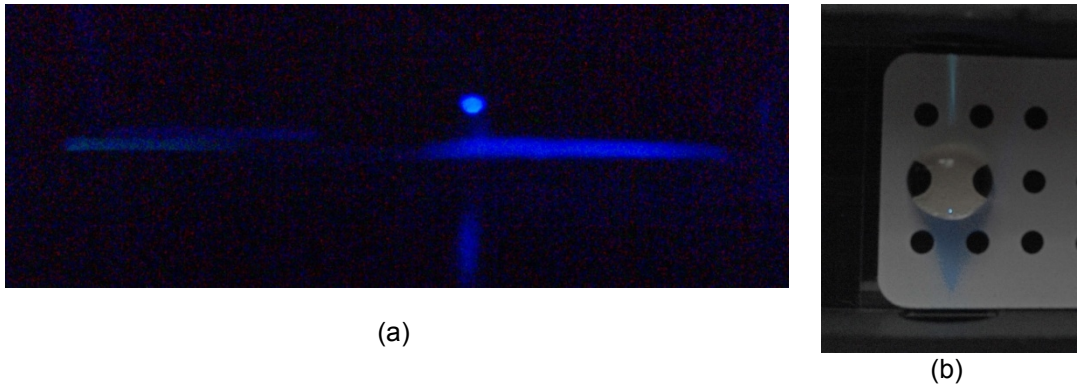


Fig. 5. Images of 0.2 millimoles per liter of tartrazine in water: (a) side view of the sample in the dark with excitation light illuminating the spheroid droplet from the right, (b) top view of the same sample with the lights turned on

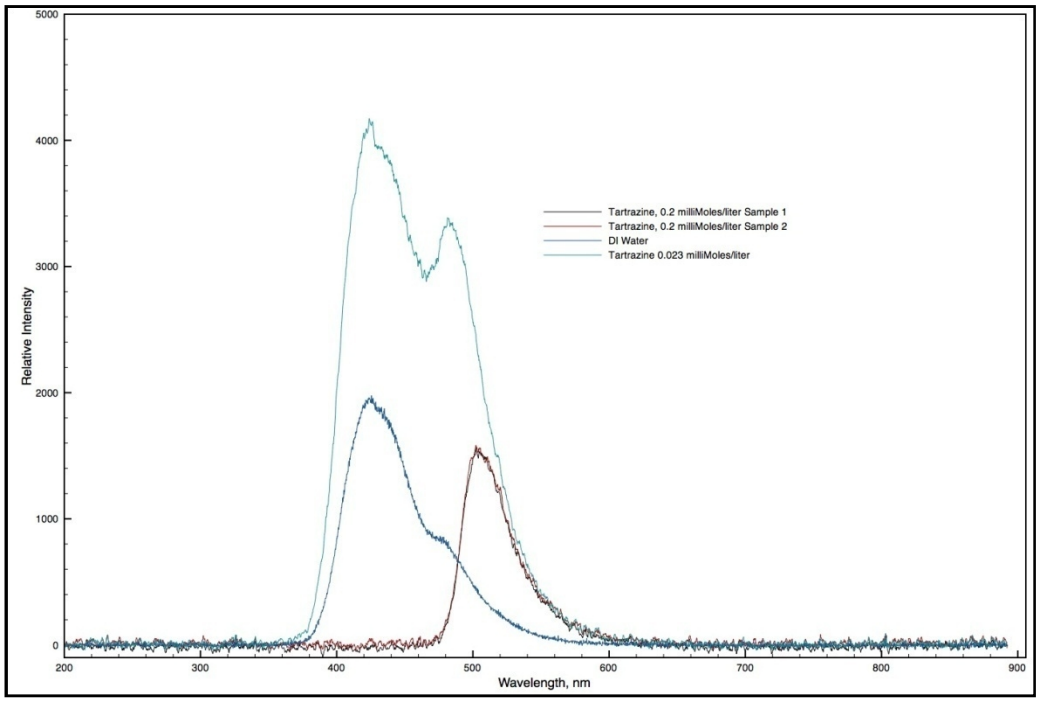


Fig. 6. Four spectra recorded by a fiber optic spectrometer for light from a blue LED at 430 nm maximum wavelength exiting an oblate spheroid containing either an aqueous solution of tartrazine or DI water. The green line is the spectra for 0.023 millimoles per ml tartrazine, the blue line is for DI water, and the red and black lines are for 2 separate measurements of 0.2 mM tartrazine

From Fig. 6, it is evident that for 0.2 mM tartrazine, the light exiting the sample is significant shifted from the spectra of the Blue LED being recorded leaving a droplet of DI water. For a tartrazine concentration of 0.023 mM, the spectra exiting the sample is seen to have mostly the excitation light, but there is a shoulder detectable that corresponds to the presence of some fluorescence. It should be noted that the intensity recordings vary in Fig. 6 since the caustic focus was intentionally not identical in each measurement and hence the spectral shift can be more clearly presented.

4. CONCLUSION

Newton's zero order caustic can be used to detect fluorescence in a liquid sample, using a low power source, by shaping the sample in order to refract light and create a zone of high intensity light within the sample. In this system, no optical lenses or filters need be employed, which significantly differs from what is commonly found in commercially available instruments. For weakly fluorescent molecules, the beam exiting the sample can be used to detect the emission spectra, especially when the concentration is high enough to also absorb much of the incident excitation light. For strongly fluorescing molecules such as those used to detect dsDNA or for molecules used biological probes, the light exiting the sample shaped into an oblate spheroid can be detected with a digital phone camera and analyzed using software developed for electrophoresis gel quantitation. Changing the major to minor axis in the sample can be used to shift the caustic focus, but reflection and zones of

low light intensity should be avoided since they can limit the area for analysis in quantitative measurements.

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COMPETING INTERESTS

Authors declare that no competing interests exist.

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