# Simultaneous measurement of one- and two-photon excited fluorescence from a single sample: a detection method for oligonucleotides

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A new method has been developed that is based on the use of a single-excitation wavelength from a cw laser to excite simultaneously one-photon and two-photon fluorescence (TPF). Fluorescence bands of a sample containing two oligonucleotides, one labeled with a one-photon fluorescence dye and the other with a TPF dye, can be measured simultaneously. The two fluorescence bands are well separated, because the one-photon excited fluorescence band is redshifted, whereas the TPF band is blueshifted from the excitation wavelength. The spectral separation was found to be as large as 200 nm when ADS 840NCS was used to label one oligonucleotide for one-photon fluorescence and Rhodamine Red-X dye was used for TPF. Spectral overlapping problem that plagues current DNA sequencing techniques can be eliminated effectively with this method. © 2002 Optical Society of America

*OCIS codes:* 120.0120, 140.0140, 300.0300, 000.1570, 170.6280, 230.1040.

## 1. Introduction

Fluorescence technique has been widely used as the detection method for DNA sequencing.[1–6] This popularity stems from the fact that the technique has low detection limits, high sensitivity, ease of automation, on-line detection, and multiplexing capability.[1–6] Often, more than one fluorescence dye is used to label oligonucleotides in order to increase the throughput and eliminate the problem of lane-to-lane misalignment. Four different fluorescence dyes usually are used for multiplexing. Because four-color sequencing relies on spectral separation of the fluorescence spectra of the dyes, any overlapping among their spectra may result in cross talk between detection channels.[1–4] As a consequence, considerable efforts have been made in the last few years to ameliorate this problem. Limited progress has been made, including the development of novel set of four dyes that have relatively narrower fluorescence spectral bandwidth.[7,8] Unfortunately, because fluorescence spectra are inherently broad, overlap still persists among the spectra of these dyes.[7,8] It is therefore of particular importance that a novel fluorescence detection method be developed that can be used for DNA sequencing with higher accuracy and resolution.

As described above, because fluorescence spectra have inherently broad spectral bandwidths, it would be very difficult to avoid overlap among spectra of two or more fluorescence dyes when only one wavelength is used for excitation. However, if the fluorescence spectra are produced by different absorption processes, namely conventional one-photon absorption and simultaneous two-photon absorption, it is possible to obtain well-resolved (well separated) fluorescence spectra of two or more fluorescence dyes with a single-excitation wavelength, Take, for example, a sample containing two fluorescence label dyes that are excited simultaneously by a single-excitation wavelength. Fluorescence of the first dye is produced by the one-photon absorption process. Accordingly, it is redshifted compared with the excitation wavelength. The second dye absorbs excitation light through a simultaneous two-photon absorption process. Its fluorescence spectrum is therefore blueshifted. Consequently, there is no possible overlap between the two fluorescence spectra because they are well separated; i.e., they are on either side of the excitation wavelength. While this possibility has been known for some time, it has not been actually used in multiplexing fluorescence detection. This is because the probability of the instantaneous absorp-

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Received 27 July 2001; revised manuscript received 5 November 2001.

0003-6935/02/122285-07$15.00/0

tion of two photons is rather low. As a consequence, pulsed lasers are often used for excitation, and elaborate and sophisticated detection methods, including photon counting, time discrimination, and second-harmonic detection, are often needed to extract the small two-photon excited fluorescence (TPF) signals in the presence of a large background.[9–13] However, such procedures are not necessary, as we were able to demonstrate recently that TPF signal can be generated by exciting with a cw laser and detecting with a simple detection system such as those used for the one-photon fluorescence technique.[14] Our success was based on the use of an acousto-optic tunable filter (AOTF) for spectral dispersion.[14] The electronically driven, spectrally tunable AOTF is known to have higher throughput (>90% diffraction efficiency) and better background discrimination (based on the difference in the polarization of the diffracted and transmitted light) than conventional monochromators.[15–17] Additionally, because the light diffracted from the AOTF can be readily amplitude modulated (by modulating the corresponding rf signal applied to the AOTF), a phase-detection technique can be used to enhance further the signal and to reduce the background noise.[15–17] As a result, it was possible for us to measure TPF with cw laser excitation.

The information presented is indeed provocative and indicates clearly that it is possible to obtain well-resolved fluorescence spectra from a sample with only one excitation wavelength. Such considerations prompted us to initiate this study that aims to demonstrate this possibility, namely the use of a single-excitation wavelength (from a cw laser) to produce simultaneously one-photon and TPF spectra from a single sample. The sample contains two oligonucleotides, one labeled with one-photon excited fluorescence dye and the other with TPF dye. Preliminary results are presented in this paper.

## 2. Experiment

Figure 1 shows a schematic diagram of the AOTF-based fluorimetric detector that was used in this study. As illustrated, a tunable Ti:sapphire laser (maximum output of 1.2 W at 780 nm) pumped by the multiline output of a 6.5-W Argon-ion laser was used as the excitation source. A long-pass filter was placed before the sample to block any light leaking from the Argon pump laser from reaching the sample. To increase the photon flux, the Ti:sapphire excitation beam was focused tightly into the sample. Light emitted from the sample as a result of the two-photon-excited fluorescence was collected at 90° and collimated onto a noncollinear $TeO_2$ visible AOTF (Crystal Technologies, 1040 E. Meadow Circle, Palo Alto, Calif. 94303). Light diffracted by the AOTF was focused into and detected by a red sensitive Hamamatsu photomultiplier tube (PMT) and cooled by a two-stage Peltier cooler (Model R6060–02, Hamamatsu Corp., Hamamatsu City, Japan). This AOTF was placed between two cross-axis Glan-Thompson polarizers (Karl Lambrecht Corp., 4204 N. Lincoln Ave., Chicago, Ill. 60618) to block the transmitted beam and pass the diffracted beam. The sample also emitted one-photon near-infrared (NIR) fluorescence, and this fluorescence was collimated onto a second noncollinear $TeO_2$ AOTF (labeled as NIR AOTF in the figure). This NIR AOTF was placed at a right angle to the excitation beam and on the other side of the sample. A second red sensitive Hamamatsu PMT was used to detect the diffracted NIR fluorescence. Both PMT signals, which were amplitude modulated at the same frequency as the AOTFs (11 kHz), were demodulated and amplified by lock-in amplifiers (Model SR810, Stanford Research Systems, 1290 Reamwood Ave., Sunnyvale, Calif. 94089). The lock-in amplifier outputs were connected to a microcomputer through a 16-bit interface board. Modifications were made to the previously used C++ program to allow each AOTF to be scanned independently.

The visible fluorescent label, Rhodamine Red-X, was purchased from Molecular Probes (Eugene, Ore.) as an amino reactive coupling kit. Each kit contained the reactive label, a labeling buffer, a column-loading buffer, a wash buffer, an elution buffer, dimethyl sulfoxide, and solid-phase extraction spin columns. The fluorophore with the reactive amino group was supplied with a six-carbon spacer to minimize any possible spectral changes when the dye is coupled to the oligonucleotide. The NIR fluorescent label, ADS 840NCS, was custom synthesized by American Dye Source (Quebec, Canada).

Two different 20-mer oligonucleotides were custom synthesized by Integrated DNA Technologies (Coralville, Iowa). The first oligonucleotide, T1, which had the sequence 5′-TCA GTT CAC GTC AAG CTA TT-3′, was coupled with the Rhodamine Red-X visible label. The second oligonucleotide, T2, which had the sequence 5′-CGT TAA CGC TAC GCC AGC GG-3′, was coupled with the NIR fluorescent label. The oligonucleotides were modified with amino groups at their 5′ ends to facilitate the attachment of the fluorescent label. Both oligonucleotides were received in 1-μmol quantities. The oligonucleotides were coupled with the reactive amino group of the dye in three steps: 1) purification of oligonucleotide, 2) coupling with fluorescent label dye, and 3) purification of labeled oligonucleotide. The labeling procedure optimized for 0.1–1.0-mg quantities of 20-mer oligonucleotides is shown in Table 1.

### A. Oligonucleotide Purification Procedure

The oligonucleotide was dissolved in a minimum volume of distilled deionized water. The solution was then extracted three times with chloroform to remove organic impurities. Following extraction, the addition of 0.1 volume of aqueous 3.0 M NaCl and 2.5 volumes of absolute ethanol to the solution led to the precipitation of the oligonucleotide when the mixture was stored at −20° C for 30 min. The cooled mixture was then centrifuged at 12,000 g for 30 min to facilitate the collection of the precipitated oligonucleotide. Subsequently, the supernatant

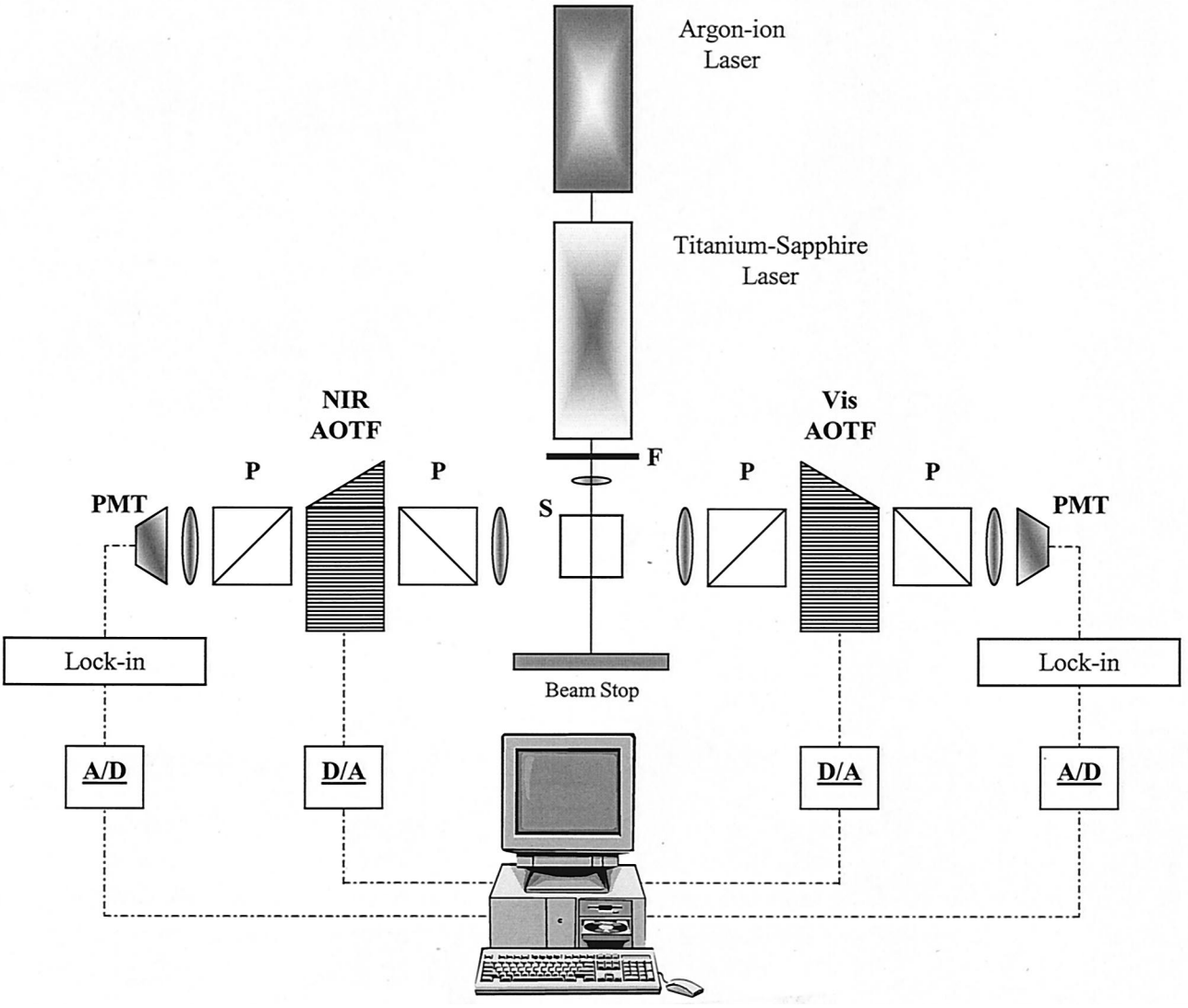


Fig. 1. Schematic diagram of the AOTF-based fluorimeter: AOTF, acousto-optic tunable filter; lock-in, lock-in amplifier; PMT, photomultiplier tube; A/D, analog to digital converter; D/A, digital to analog converter.

was removed and the pellet was washed twice with cold 70% ethanol. After drying under vacuum the pellet was dissolved in distilled deionized water to give the final oligonucleotide a concentration of approximately 25 μg/μl. The resultant oligonucleotide stock solution was archived at −20° C for later use.

### B. Coupling Procedure

The dye with the reactive amino group was dissolved in dimethyl sulfoxide to give a fluorescent label concentration of approximately $1.7 \times 10^{-3}$ M. 14.0 μl of this dye solution was used during the coupling reaction. 75.0 μl of the labeling buffer, 7.0 μl of distilled deionized water, and 4.0 μl of the oligonucleotide stock solution was added to the reactive label solution. The reaction mixture was protected from light and allowed to react at room temperature for six hours with gentle agitation.

### C. Purification of Labeled Oligonucleotide

Following coupling, the conjugated and any unconjugated oligonucleotides were precipitated by adding 0.1 volume of 3.0 M aqueous NaCl and 2.5 volumes of absolute ethanol and stored at −20° C for 30 min. Following cooling, the precipitated oligonucleotides were collected by centrifuging the mixture at 12,000g for 30 min. Subsequent to centrifugation, the supernatant was removed and the pellet was washed twice with cold 70% ethanol and dried under vacuum. The dried pellet was then dissolved in 750 μl of the column-loading buffer. Prior to the application of the oligonucleotide mixture, the solid-phase extraction spin column was equilibrated by washing with 1.0 ml of the equilibration buffer four times at 1280 g for 30 s each. We applied the oligonucleotides to the equilibrated spin column by pipetting the solution onto the column and centrifuging at 1280 g for 30 s. Unconjugated oligonucleotides that adhered to the spin column were eluted through centrifugation six

Table 1. Oligonucleotide Labeling Procedure

| Process | Step | Procedure |
|---|---|---|
| Oligonucleotide Purification | 1 | Dissolve oligonucleotide in minimum volume of $H_2O$ and extract with equal volume of Chloroform (3X) |
| | 2 | Precipitate Oligonucleotide by adding 0.1 volume 3.0 M NaCl, 2.5 volume absolute ethanol and storage at −20°C for 30 min |
| | 3 | Following cooling, centrifuge mixture at 12,000 g for 30 min |
| | 4 | Remove supernatant and rinse pellet with cold 70% ethanol (2X) and dry under vacuum |
| | 5 | Dissolve pellet in $H_2O$ at a concentration of ~25 μg/μL and store solution at 20°C for further use |
| Coupling | 6 | Dissolve the amino-reactive label in ~14 μL dimethyl sulfoxide |
| | 7 | Add 75 μL labeling buffer, 7 μL ddH2O, and 4 μL of purified oligonucleotide solution (25 μL/μL) to the fluorescent label solution. |
| | 8 | Protect the reaction mixture from light and allow the coupling reaction to proceed for 6 h at room temperature |
| Labeled Oligonucleotide Purification | 9 | Precipitate labeled and unreacted oligonucleotide by adding 0.1 volume 3.0 M NaCl, 2.5 volume absolute ethanol and storage at −20°C for 30 min |
| | 10 | Following cooling, centrifuge mixture at 12,000 g for 30 min |
| | 11 | Remove supernatant and rinse pellet with cold 70% ethanol (2X) and dry under vacuum |
| | 12 | Dissolve pellet in 750 μL of column-loading buffer |
| | 13 | Equilibrate solid phase extraction spin column by pipetting 1 mL of equilibration buffer onto column and centrifuge at 1280 g for 30 s (4X) |
| | 14 | Pipette oligonucleotide in loading buffer onto column and centrifuge at 1280 g for 30 s |
| | 15 | Pipette 1 mL of wash buffer onto column and centrifuge at 1280 g for 30 s to elute unconjugated oligonucleotides (6X) |
| | 16 | Elute labeled oligonucleotide by pipetting 1 mL of elution buffer onto column, followed by centrifugation at 1280 g for 30 s (2X) |
| | 17 | Pool labeled oligonucleotide fractions, dry under vacuum, and store in dark at −20°C for further use |

times with a 1.0-ml wash buffer at 1280 g for 30 s each. Subsequently, the conjugated dye-oligonucleotide adduct was eluted through centrifugation twice with a 1.0-ml elution buffer at 1280 g for 30 s each time. Fractions containing conjugated oligonucleotide were pooled, dried under vacuum, and stored in the dark at −20 °C for further use.

### 3. Results and Discussion

The NIR fluorescent dye, ADS 840NCS, is sparingly soluble in water. However, it can be dissolved in a surfactant solution such as sodium dodecyl sulfate (SDS). According to the manufacturer, ADS 840NCS has a molar absorptivity ($\epsilon$) of $2.21 \times 10^5$ L $cm^{-1}$ $mol^{-1}$ at 835 nm in methanol. It was found, however, that in $6.25 \times 10^{-2}$ M SDS solution, the maximum absorption ($\lambda_{max}$) of the dye was shifted slightly to 846 nm. Careful examination of the absorption spectra of uncoupled ADS 840NCS and ADS 840NCS coupled to an oligonucleotide (840NCS-ONT) reveals that the $\lambda_{max}$ of ADS 840NCS undergoes slight changes following a coupling to an oligonucleotide. Specifically, subsequent to coupling, the $\lambda_{max}$ was shifted from 846 nm to approximately 836 nm concomitant with the appearance of an additional band at 769 nm. The changes in the spectra may be due to the influence of the attached oligonucleotide because, contrary to the Rhodamine Red-X, ADS 840NCS is not separated from the oligonucleotide by a hydrocarbon spacer.

The ADS 840NCS dye can be excited by light in the NIR region. Figure 2 shows the one-photon-excited fluorescence spectrum of ADS 840NCS. The sample that contained $2.85 \times 10^{-6}$ M ADS 840NCS in SDS solution was excited with 700 mW of 845-nm light. As illustrated, the maximum fluorescence intensity occurs at about 865 nm.

It was found that in $6.25\times 10^{-2}$ M SDS solution, Rhodamine Red-X has a $\lambda_{max}$ of 573 nm. Figure 3 shows the one-photon-excited fluorescence spectrum of Rhodamine Red-X in $6.25 \times 10^{-2}$ M SDS (obtained with 520-nm excitation light). As illustrated, the peak of the emission spectrum is at 579 nm, and its FWHM is 36 nm.

It is expected that through the simultaneous absorption of two NIR photons it may be possible to

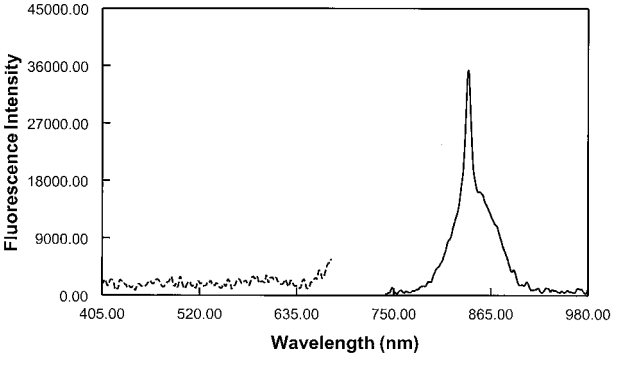


Fig. 2. One-photon fluorescence spectrum of $2.85 \times 10^{-6}$ M ADS 840NCS uncoupled dye in $6.25 \times 10^{-2}$ M SDS (excitation wavelength, 845 nm).

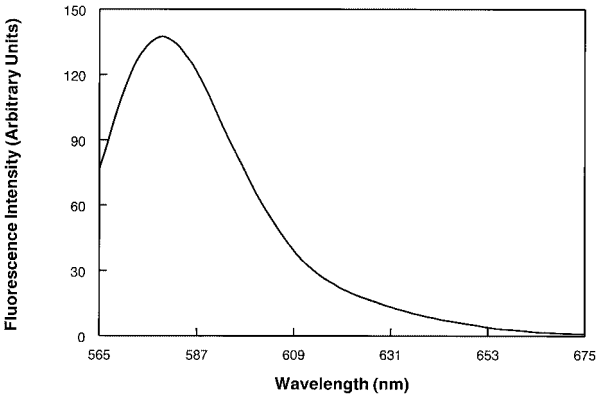


Fig. 3. One-photon-excited fluorescence spectrum of Rhodamine Red-X in $6.25 \times 10^{-2}$ M, obtained with 520-nm excitation light.

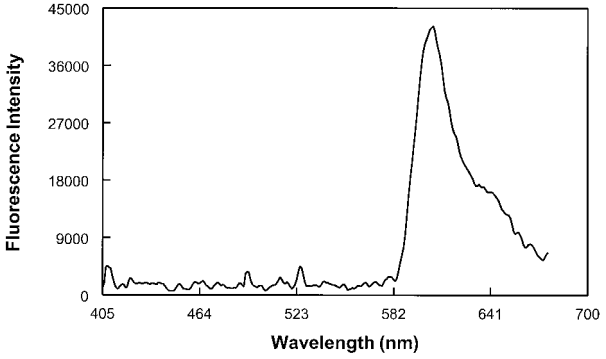


Fig. 5. Two-photon-excited fluorescence spectrum of $1.0 \times 10^{-5}$ M Rhodamine Red-X in $6.25 \times 10^{-2}$ M SDS, excited at 845 nm.

excite the visible fluorescence dye Rhodamine Red-X with NIR light. This was indeed the case, and the results are shown in Fig. 4. Specifically, the figure shows the corrected two-photon excitation spectrum of the Rhodamine Red-X in SDS solution. The spectrum was measured with the AOTF-based fluorimeter (shown in Fig. 1) and obtained by scanning sequentially the Ti:sapphire laser from 790 to 865 nm with the detecting wavelength set at 606 nm (i.e., at the maximum emission of the Rhodamine Red-X). We then corrected the two-photon excitation spectrum by taking the ratio of the fluorescence intensity and the laser emission profile. As illustrated in the figure, the two-photon absorption spectrum of the Rhodamine Red-X remains close to zero until about 800 nm. From 800 nm to 865 nm, significant increase in two-photon absorption is observed. Most notably, in this selected range, the most efficient two-photon absorption occurs at 865 nm.

Figure 5 shows the fluorescence spectrum of $1.0 \times 10^{-5}$ M Rhodamine Red-X excited with a cw laser beam in the NIR region at 845 nm with a power of 700 mW. As illustrated, the dye was excited by the simultaneous absorption of two NIR photons. The excited dye emitted fluorescence with a maximum at about 606 nm. The bandwidth of this TPF spectrum is about 30 nm, which is relatively narrower than that of the one-photon-excited fluorescence spectrum (i.e., 36 nm). This is hardly surprising, as it was reported previously that TPF spectra generally have narrower bandwidth than the corresponding one-photon-excited spectra. The TPF spectrum also shifted toward a longer wavelength (maximum at about 606 nm), compared with the one-photon spectrum (maximum at about 579 nm, Fig. 3). A variety of reasons might be account for this observation, but the most likely one might be the reabsorption of the emitted photons by the dye following two-photon excitation with NIR light. This possibility stems from the fact that compared with the one-photon-excited fluorescence, the TPF spectrum is unsymmetric and shifted toward the longer wavelength region.

The intensity of the TPF spectra at 606 nm versus the excitation laser power for a sample containing $1.00 \times 10^{-5}$ M Rhodamine Red-X in SDS solution is plotted in Fig. 6. This log–log plot shows that the fluorescence intensity is proportional to the laser excitation power; however, the slope of this plot is not at the expected value of 2.0 but rather at 1.36. This may be due to the defocusing of the excitation laser beam by the SDS surfactant solution. In fact, surfactant solutions are known to produce a defocusing effect on strong laser beams through the nonlinear optical effects such as electrostriction. The yield for the two-

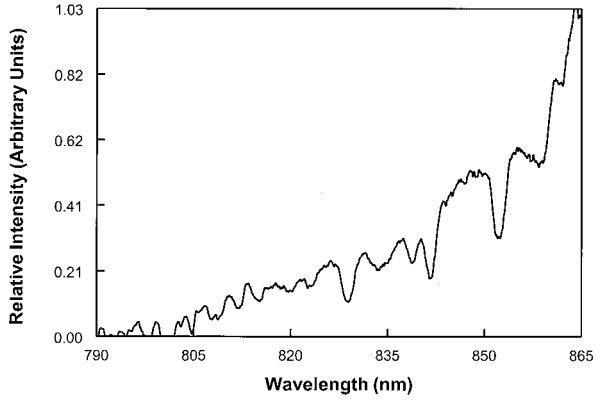


Fig. 4. Two-photon excitation spectrum of Rhodamine Red-X in $6.25 \times 10^{-2}$M (monitored at 606 nm).

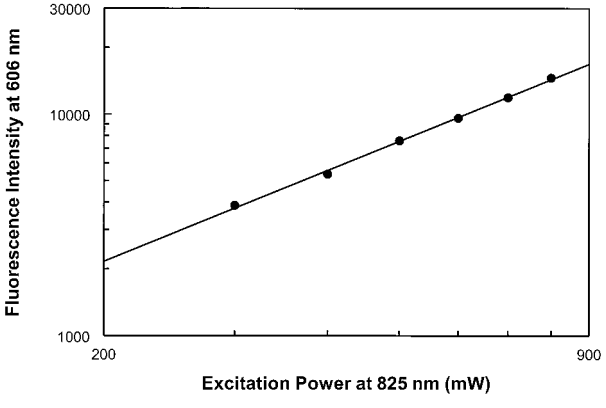


Fig. 6. Plot of intensity of two-photon-excited fluorescence versus excitation laser power for $1.0 \times 10^{-5}$ M Rhodamine Red-X.

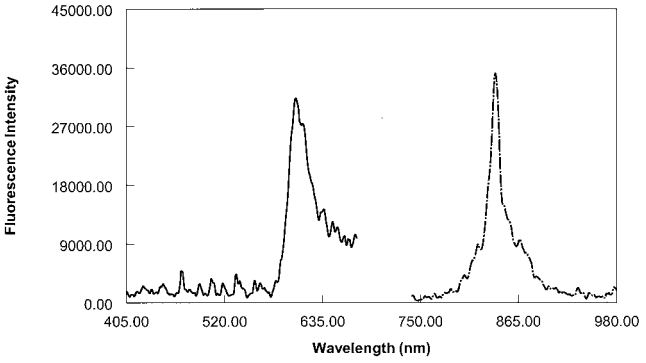


Fig. 7. One- and two-photon-excited fluorescence spectra of a sample containing ADS 840NCS dye coupled to an oligonucleotide, and Rhodamine Red-X coupled to an oligonucleotide. The spectra were obtained through an excitation of the sample with a single excitation wavelength of 845 nm; the dashed line is the one-photon fluorescence spectrum of the ADS 840NCS coupled oligonucleotide and the solid line is the two-photon excited fluorescence spectrum of Rhodamine Red-X coupled oligonucleotide.

photon-excited fluorescence was decreased as a consequence of this defocusing effect.

Subsequent experiments were designed to demonstrate that with a single-excitation wavelength, two different types of fluorescence, namely the one-photon-excited fluorescence and TPF, can be measured simultaneously from a sample that contains an oligonucleotide labeled with the NIR fluorescence dye ADS 840NCS and an oligonucleotide labeled with the Rhodamine Red-X. The wavelength must be chosen that allows excitation of both labeled oligonucleotides. 865 nm would provide strong a TPF for the Rhodamine Red-X sample but is not suitable for the sample labeled with the ADS 840NCS NIR dye. This is because 865-nm light is outside the absorption band of the ADS 840NCS in SDS solution. Both the ADS 840NCS and the Rhodamine Red-X samples can be excited by cw laser light in the region from 650 to approximately 850 nm. It should be noted that excitation in this region is less efficient for Rhodamine Red-X than with 865-nm excitation. A compromised wavelength of 845 nm was selected to facilitate excitation of both labeled oligonucleotides. The fluorescence of a sample containing $1.00 \times 10^{-5}$ M Rhodamine Red-X labeled nucleotide and $2.85 \times 10^{-6}$ M ADS 840NCS labeled nucleotide in SDS solution was measured with an 845-nm excitation wavelength (700 mW). The spectra obtained are shown in Fig. 7. As illustrated, the fluorescence bands of both labeled oligos are well separated. The visible fluorescence band from approximately 577 to 680 nm is similar to the spectrum shown in Fig. 5. The band therefore can be attributed to the TPF of the Rhodamine Red-X labeled oligonucleotide compound. The fluorescence band in the NIR region (from 750 to 910 nm) is similar to the spectrum shown in Fig. 2. It can therefore be assigned to the one-photon-excited fluorescence spectrum of the ADS 840NCS labeled oligonucleotide compound. Taken together, the results presented clearly demonstrate that it is possible to measure simultaneously both one- and two-photon-excited fluorescence spectra from a single sample through simple use of a single-excitation wavelength from a cw laser. The two fluorescence bands are well separated (more than 200 nm) because the one-photon-excited fluorescence band is redshifted, whereas the TPF band is blueshifted from the excitation wavelength.

## 4. Conclusion

It has been demonstrated that high diffraction efficiency and better background discrimination of the AOTF make it possible to measure TPF from a labeled oligonucleotide when the sample is excited by light from a cw laser. This discovery leads to the development of a novel method in which, by simple use of a single excitation wavelength from a cw laser, one-photon fluorescence and TPF can be measured simultaneously from a single sample. There are two oligonucleotides in the sample, one labeled with a one photon fluorescence (NIR) dye and the other labeled with a TPF dye. The one-photon and TPF spectra are on either sides of the excitation wavelength and hence are well separated (more than 200 nm). As a result, the developed method is superior to other methods currently used in the multiplexed fluorescence DNA sequencing because it can effectively eliminate the spectral overlapping problem.

In this study, two AOTFs and two PMTs were used to separately disperse and detect the one-photon and two-photon excited fluorescence. This arrangement was made because of the limited spectral tuning ranges of the AOTFs that we had available in the laboratory when this study was performed. Specifically, the visible AOTF can only be tuned spectrally from 400 nm to 700 nm, whereas the tuning range for the NIR AOTF is from 700 nm to 1000 nm. As a consequence, it was not possible to use only one of them for the simultaneous dispersion of both one- and TPF light. It is important to note that through use of an array (or two arrays) of piezoelectric transducers to provide acoustic waves with a relatively wider frequency range, it is possible to construct an AOTF with a spectral tuning range that includes both the visible and the NIR region (e.g., from 400 nm to 1000 nm). In fact such an AOTF has been theoretically reported and constructed. When such an AOTF is used, it would be possible to perform the present study with only one AOTF and one PMT. Furthermore, rather than sequentially scanning the AOTF from the visible range (for the TPF) to the near infrared range (for one-photon fluorescence), it is possible to use the AOTF to disperse both ranges simultaneously. This can be accomplished by using the same procedure as we did in our earlier study, namely the use of the AOTF in the multiplexing configuration.[18–20] Specifically, light diffracted from the AOTF does not need to be a monochromatic light. Multiwavelength light can be diffracted from the AOTF when several rf signals are applied simultaneously to the filter. Accordingly, two rf signals corresponding to the visible and the NIR range can be applied simultaneously to the AOTF.

Through individual and sinusoidal modulatation of the visible and the NIR rf signal at the desired frequency, the corresponding visible and NIR component in the diffracted beam will be amplitude modulated at that frequency.[18–20] Both the visible and the NIR therefore can be detected simultaneously by a single detector through the phase-lock detection method. It is therefore evident that it is possible to use one AOTF and one PMT to measure simultaneously one- and two-photon-excited fluorescence from a single sample containing oligonucleotides labeled with both one- and TPF dyes. The complexity and cost of the instrument will therefore be substantially reduced. Such possibility is the subject of our intense investigation.

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