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UV- and visible-excited fluorescence of nucleic acids separated by capillary electrophoresis

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Abstract

UV- and visible-excited fluorescence detection strategies were compared for nucleic acids separated by capillary electrophoresis (CE). A dual-polymer sieving matrix consisting of hydroxypropylmethylcellulose and poly(vinylpyrrolidone) was used to separate DNA fragments from a 100-base pair ladder and RNA from individual cells. Two nucleic acid dyes, SYBR Gold and SYBR Green I, were evaluated for their performance at both UV (275 nm) and visible (488 nm) excitation wavelengths. While SYBR Gold-bound RNA from single cells yielded a substantially reduced UV-excited signal compared to that with visible excitation (as expected), the sensitivity of SYBR Gold-bound double-stranded DNA was comparable for UV and Vis excitation wavelengths. This study reveals the first demonstration of using SYBR Gold dyes for DNA detection following separation with CE and also the first example of SYBR-based detection of RNA sampled and separated from individual cells.

Keywords: Laser-induced fluorescence detection; Detection, electrophoresis; Nucleic acids; DNA; RNA

1. Introduction

The use of laser-induced fluorescence (LIF) to detect nucleic acids following separation by slab-gel electrophoresis or capillary electrophoresis (CE) is becoming common for high-sensitivity measurement of these analytes. This, in turn, has precipitated a substantial increase in the development of fluorescent probes that bind to nucleic acids to cause enhanced fluorescent properties of the dye:nucleic acid complex. Although nucleic acids do exhibit native (i.e., intrinsic) fluorescence after UV excitation [1,2], the inherently low fluorescence quantum yield may make detection of ultra-low amounts of nucleic acids difficult, such as the levels present in single mammalian cells. Ethidium bromide (EtBr) is a common fluorescent dye used for detection of nucleic acids following separation in both gels and in CE, as cited in these representative examples [3–6]. Recently, we have demonstrated the direct sampling and separation of RNA in individual cells using CE with EtBr detection [7]. However, newer dyes with higher fluorescence quantum yields, lower background fluorescence, and higher nucleic acid binding affinities, especially for single-stranded DNA (ssDNA) and RNA, are becoming increasingly popular [4].

Two examples of such dyes are SYBR Green I and SYBR Gold, which are unsymmetrical cyanine dyes primarily used for staining nucleic acid bands following slab gel electrophoresis [8]. SYBR Green I

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exhibits higher affinity for double-stranded DNA (dsDNA) than EtBr, as well as moderate affinity for ssDNA and RNA [4,9,10]. SYBR Gold has many of the same characteristics of SYBR Green I, but shows higher sensitivity for both ssDNA and RNA [4]. The spectral properties of SYBR Green I and SYBR Gold present an additional advantage over EtBr, in that these dyes can be excited in both the UV and visible regions of the spectrum [9,10], increasing their versatility. The excitation maxima for SYBR Green I are at 290, 380 and 497 nm [9] while those for SYBR Gold are at 300 and 495 nm [4,10]. These wavelengths allow the use of argon-ion lasers in both the UV and visible regions as a means for LIF detection of nucleic acids.

In this paper we examine the sensitivity of SYBR Green I and SYBR Gold for LIF detection of dsDNA [100-base pairs (bp) standard sample] and single-cell (non-extracted) RNA following separation by CE. Both UV (275 nm) and visible (488 nm) excitation wavelengths are used to detect the DNA standard and the single-cell RNA with each dye. SYBR Green I has been used extensively for the detection of dsDNA in CE [11–15] and gel electrophoresis [4,16–19]. However, limited use of SYBR Gold has been reported for the detection of dsDNA and RNA standards following gel electrophoresis [4,20–22]. We present here the first example of the detection of non-extracted, single-cell RNA using both of these dyes and the first use of SYBR Gold for LIF detection of DNA following CE. Furthermore, this is the first demonstration, to our knowledge, of the use of these dyes with a UV excitation source for LIF detection of dsDNA and RNA separated by CE.

2. Experimental

2.1. Chemicals and reagents

Tris(hydroxymethyl)aminomethane (Tris), mannitol, and sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO, USA). Hydroxypropylmethylcellulose (HPMC-5), molecular mass 10 000, was obtained from Aldrich (Milwaukee, WI, USA). Ethylenediamine tetraacetic acid disodium salt (EDTA) and 0.25% trypsin-EDTA were from Life Technologies (Gaithersburg, MD, USA). Poly(vinylpyrrolidone) (PVP), molecular mass 1 000 000, was purchased from Polysciences (Warrington, PA, USA). The DNA ladder (Promega, Madison, WI, USA) contained 11 double-stranded fragments of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, and 1500 bp. As supplied, the concentration of the 500-bp fragment is threefold higher than the other fragments so that it can serve as a marker. SYBR Gold and SYBR Green I, which are nucleic acid dyes, were from Molecular Probes (Eugene, OR, USA). Deionized water (resistance $\geq$18 MΩ) was obtained from a Milli-Q system (Bedford, MA, USA). All other chemicals were obtained from Fisher Scientific (Fairlawn, NJ, USA).

2.2. Cell preparation

The Chinese hamster ovary (CHO-K1) cell line, obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), was used in all single-cell experiments. The cells were cultured in F-12K medium (ATCC) containing 2 m*M* L-glutamine and 1.5 g/l sodium hydrogencarbonate. The medium was supplemented with 10% fetal bovine serum, 0.18 μg/ml streptomycin, and 0.18 IU/ml penicillin (Life Technologies). The cells were grown in an incubator set at 37°C with a 5% CO_2 level. Cells were isolated for experiments 2–4 days after passage under sterile conditions. Following removal of the medium from the culture flask, the cells were suspended in 3 ml of 0.25% trypsin-EDTA and incubated at 37°C for 5 min. The cells were then centrifuged at 1000 *g* for 2 min and the trypsin-EDTA was removed. The remaining cell pellet was then washed four times with phosphate-buffered saline (PBS, containing 9 g/l sodium chloride, 5 m*M* D-glucose, 1 m*M* sodium phosphate monobasic, and 3 m*M* sodium phosphate dibasic; pH was adjusted to 7.4 with 2 *M* sodium hydroxide). Following the washing procedure, the cell pellet was resuspended in PBS.

2.3. Buffer preparation

The sieving matrix used for separation has been described previously [7] and contained 1% HPMC, 0.5% PVP, and 6% mannitol dissolved in a solution of 89 m*M* Tris, 89 m*M* boric acid, and 1 m*M* EDTA (TBE). The polymers and the mannitol were com-

bined until a relatively homogeneous dry mixture was achieved; prior mixing of the polymers aids dissolution. The TBE was then added and the mixture was stirred using a magnetic stirrer for at least 30 min. The pH of the sieving buffer was adjusted to 7.5 with 2 *M* sodium hydroxide. Following pH adjustment, the nucleic acid dye was added (either SYBR Gold or SYBR Green I) such that a 10 000-fold dilution of the original dye was achieved (absolute concentration was not provided by the manufacturer).

2.4. CE–LIF systems

The experiments were performed on two laboratory-built CE–LIF systems, one utilizing 488-nm excitation and the other utilizing 275-nm excitation. Separations were performed in both systems using 40-cm (25-cm effective length) bare fused-silica capillaries (50 μm I.D.×360 μm O.D.; Polymicro Technologies, Phoenix, AZ, USA). The polyimide coating was burned off with a lighter to create a ~1-cm detection window. The coating was also removed from the inlet end of the capillary to facilitate cell injection. The high-voltage power supply was obtained from Glassman High Voltage (Whitehouse Station, NJ, USA). Positive voltage (+6 kV) was applied at the outlet end (anode) to drive the electrophoresis; the inlet (cathode) was grounded. A DT 2804 data acquisition board was used to digitize the data and electropherograms were generated using ChromPerfect software (Justice Innovations, Mountain View, CA, USA). The original data files were converted to ASCII and the electropherograms were replotted using Excel.

2.4.1. Visible excitation

The 488-nm line of a reconditioned (air-cooled) argon-ion laser was isolated with a prism and used for excitation. This laser was focused onto the detection window using a 1-cm focal length lens. Fluorescence was collected through a 10× microscope objective (Edmund Scientific, Barrington, NJ, USA) 90° from the incident light and was imaged onto a photomultiplier tube (PMT, R928; Hamamatsu, Bridgewater, NJ, USA). A spatial filter and an emission filter (or filters) were mounted to the front of the PMT to isolate fluorescence emission and to reduce noise caused by stray light. Different long-pass filters were used, including an OG-515, an OG-570, and an OG-570/RG-610 combination (Schott Scientific Glass, Parkersburg, WV, USA).

2.4.2. UV excitation

The 275-nm line of an argon-ion laser (Coherent, Innova Sabre TSM, Santa Clara, CA, USA) was isolated with a fused-silica prism (Edmund Scientific) and used for excitation. This laser was focused onto the detection window using a fused-silica lens (1-cm focal length). Fluorescence was collected through a 10× microscope objective (Melles-Griot, Irvine, CA, USA) 90° from the incident light, passed through a spatial filter and an OG-515 filter (Schott Scientific Glass), and focused onto a photomultiplier tube (R105; Hamamatsu).

2.5. SYBR Gold fluorescence emission spectra

Fluorescence emission spectra (488 and 275 nm excitation) were obtained for unbound SYBR Gold and dsDNA-bound SYBR Gold using a Spex Fluorolog τ3 fluorimeter (Instrument S.A., Edison, NJ, USA). The unbound SYBR Gold sample was prepared by diluting SYBR Gold 10 000-fold in the separation sieving matrix. The dsDNA-bound sample was prepared by adding 20 μg/ml dsDNA (from the 100-bp ladder) to a sample of the SYBR Gold sieving matrix. The sample was mixed and then allowed to incubate for 1 h. A quartz microcuvette was used for all analyses. Spectra were collected at 2-nm increments with 1-s integration using DataMax for Windows software (Instruments S.A.). During 275-nm excitation, emission spectra were collected from 290 to 700 nm, and a high-pass filter was used to eliminate interference resulting from second order excitation light. During 488-nm excitation, spectra were collected (without a filter) from 490 to 700 nm.

2.6. Single-cell injection

A Micromaster III microscope (Fisher Scientific) with a 10× objective (100× total magnification) was used for cell injection. A capillary mount was constructed to hold the inlet of the capillary stationary. The capillary outlet was connected to a syringe (containing running buffer) with a short piece of

plastic tubing (0.010 in. I.D.; 1 in.=2.54 cm). Once the inner channel of the inlet end of the capillary was in focus on a microscope slide, a 20-μl droplet of the cell suspension was placed on the tip of the capillary. By applying suction with the syringe, a single cell was drawn into the capillary, using the microscope to monitor the process. After the cell was pulled into the capillary, the microscope slide was moved to a clean location (while the capillary was held fixed). A 20-μl droplet of 0.2% SDS solution (prepared in TBE) was then placed over the tip of the capillary. A small plug of the solution was slowly brought into the capillary to lyse the cell. After lysis, the ends of the capillary were placed in the respective buffer reservoirs and electrophoresis was begun.

3. Results and discussion

3.1. DNA analysis

A comparison of 488-nm and 275-nm detection of DNA using SYBR Gold was first performed using a 100-bp dsDNA ladder. Representative electropherograms are shown in Fig. 1A and B. Although the excitation maximum in the UV region is much smaller than in the visible region [4], both the 275-nm and 488-nm modes provide sensitive detection of the dsDNA standard with comparable limits of detection (LODs). Using 488-nm excitation an LOD of 0.1 ng/ml (S/N~2 for the 400-bp peak) was achieved, while the LOD for that same peak using 275-nm detection was 0.2 ng/ml (S/N~3). SYBR Green I, another nucleic acid dye that is excitable at both 488 nm and 275 nm was also used for DNA detection and the results were consistent with the SYBR Gold results (data not shown). The similar DNA detection limits for UV and visible excitation are peculiar, considering that the excitation spectra for DNA-bound SYBR Gold and SYBR Green I are much more intense in the visible region than in the UV region [4]. Hence, better (lower) LODs are expected with 488-nm excitation. A detailed explanation of this phenomenon is provided below in the section on SYBR Gold Emission (Section 3.3).

Clearly, under our experimental conditions either mode of detection is suitable. However, a linear dependence of the signal on DNA concentration is not observed. The signal drops off exponentially after a concentration threshold is reached. For example, in our 488-nm detection of the DNA ladder, the 0.1 ng/ml sample gave a S/N~2 for the 400-bp peak. However, the 400-bp peak of the 0.2 ng/ml sample had a S/N~15. Similar results are seen using 275-nm detection, in which the 400-bp peak of a 0.4 ng/ml sample had a S/N~10 while that of a 0.2 ng/ml sample had a S/N~3. Thus, the non-linearity is not wavelength specific. We have also observed this phenomenon when detecting DNA using ethidium bromide, indicating that it is also not specific to one particular dye. The non-linearity in DNA detection is believed to be owing to our procedures for sample

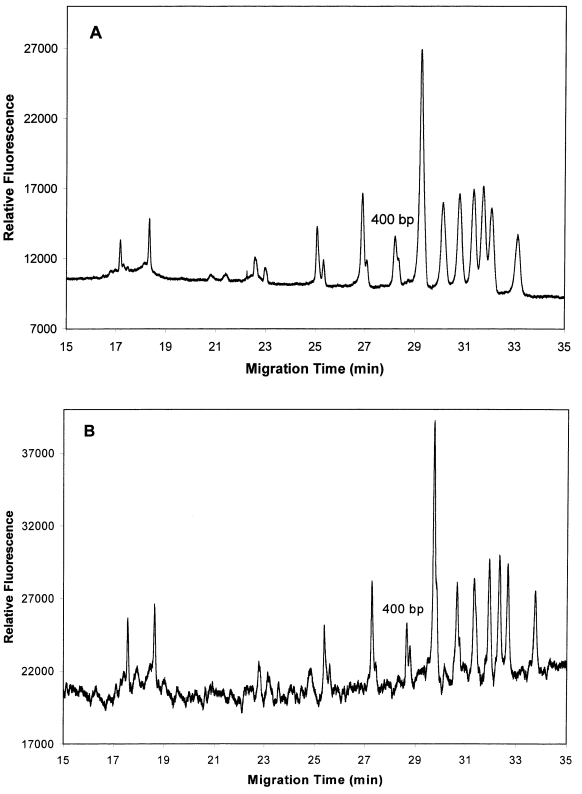

Fig. 1. Electropherograms of 100-bp DNA standard using SYBR Gold dye and LIF detection. Sieving buffer: 1% HPMC-5, 0.5% PVP, and 6% mannitol, in TBE buffer (pH 7.5) containing a 10 000-fold dilution of SYBR Gold; capillary: 40 cm (25 cm effective length) ×50 μm I.D.; injection: 5 s at −6 kV; electrophoresis voltage: −6 kV. (A) 2.0 ng/ml DNA standard using 488-nm excitation and OG-570/RG-610 filters. (B) 2.0 ng/ml DNA standard using 275-nm excitation and an OG-515 filter.

dilution and the resulting competition of ions during injection. All of our DNA samples are prepared in water rather than in buffer, to prevent preferential electrokinetic injection of buffer salts that have higher charge-to-mass ratios than the DNA [23]. At these very low concentrations of DNA, the concentration of hydroxyl ions in the water (0.32 μ*M* for pH 7.5) becomes significant and exceeds the DNA concentration by several orders of magnitude. For example, 0.1 ng/ml DNA corresponds to a concentration of ~0.3 p*M* for the 400-bp fragment. The much higher mobility (and greater concentration) of OH^- results in competition with DNA as the charge carrier, which has been shown to cause lower injected amounts than expected [24]. Depending on how narrow the threshold window is for these competing processes, it appears that a very sharp drop in signal can result from a small decrease in DNA concentration.

3.2. Single-cell RNA analysis

The detection of RNA (using SYBR Gold and SYBR Green I) from single CHO-K1 cells was also compared using both 488-nm and 275-nm excitation. While both dyes have been used to detect DNA standards [4,11–22] and to a lesser extent, RNA standards [4], this is to our knowledge the first example of RNA detection from single cells using either of these dyes. Fig. 2A and B show representative single-cell electropherograms using SYBR Gold with 488-nm and 275-nm excitation, respectively. The first group of peaks in Fig. 2 (~15–17 min) corresponds to the low-molecular-mass (LMM) fractions, consisting of smaller ribosomal RNA (e.g., 5S, 5.8S rRNA) and transfer RNA (tRNA) fragments [7]. The last two peaks (>22 min) are tentatively identified as the larger rRNA fractions (18S and 28S, respectively). The large spikes, seen prominently in the 488-nm excited electropherogram, are probably due to the presence of small air bubbles or cell debris in the capillary. No genomic DNA is detected in any of the single-cell runs, as confirmed in earlier experiments [7]. Our experimental conditions are sufficiently mild to prevent physical degradation of the DNA into fragments small enough to migrate.

Unlike the DNA detection limits, which were similar for SYBR Gold at both wavelengths, the ability to detect RNA differed significantly between the two wavelengths. As seen in Fig. 2A, 488-nm excitation results in much larger signals for the RNA peaks than those shown in Fig. 2B for 275-nm excitation. This difference, which is not observed with DNA, is actually expected. As previously stated, the excitation spectrum for SYBR Gold bound to dsDNA is much lower in intensity in the UV region than it is in the visible region [4]. The lower absorption in the UV should logically give rise to a less intense signal when excited at 275 nm compared to 488 nm as indicated in Fig. 2.

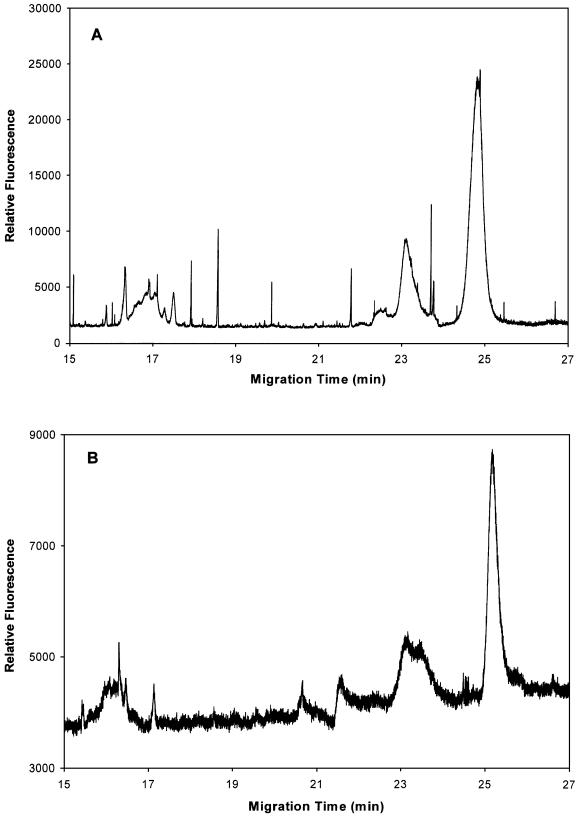

Fig. 2. Electropherograms of RNA from single CHO-K1 cells using SYBR Gold dye and LIF detection. Cell lysis was performed with 0.2% SDS solution. Other conditions as in Fig. 1. (A) 488-nm excitation. (B) 275-nm excitation.

Fig. 3A and B show single-cell electropherograms using SYBR Green I with 488-nm and 275-nm excitation, respectively. The difference between visible and UV excitation is even more striking with this dye than with SYBR Gold. While excitation at 488

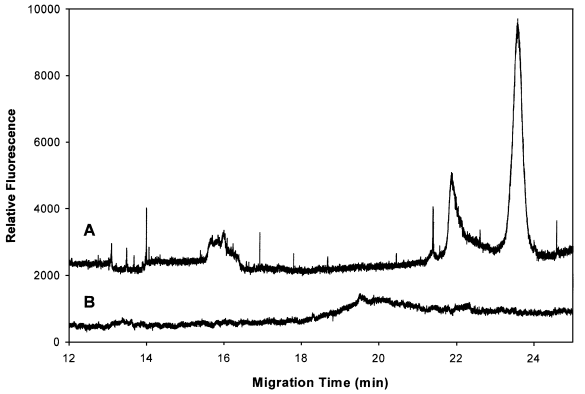

Fig. 3. Electropherograms of RNA from single CHO-K1 cells using SYBR Green I dye (10 000-fold dilution) and LIF detection. Cell lysis was performed with 0.2% SDS solution. Other conditions as in Fig. 1. (A) 488-nm excitation. (B) 275-nm excitation.

nm results in the typical electropherogram seen for single-cell RNA, 275-nm excitation results in no signal. As with SYBR Gold, the fluorescence excitation spectrum for dsDNA-bound SYBR Green I has a much lower intensity in the UV than it does in the visible region [9]. In addition, SYBR Green I is much less sensitive for detecting RNA than is SYBR Gold [8]. These intrinsic factors combined with the low levels of RNA contained within a single cell justify the lack of signal at 275 nm.

3.3. SYBR Gold emission

Emission spectra for dsDNA-bound SYBR Gold with 488-nm and 275-nm excitation are shown in Fig. 4A and B, respectively. It is clear from Fig. 4 that the two excitation wavelengths result in different fluorescence spectra. The 488-nm excited emission (Fig. 4A) is typical of what has been reported for dsDNA-bound SYBR Gold [4]. The emission ranges from ~500 to 680 nm, with a peak intensity at 534 nm. However, the 275-nm excited emission (Fig. 4B) is quite different. The emission response ranges from ~500 to 640 nm, with a much sharper cut-off than the 488-nm spectrum. In addition, the peak emission is shifted such that there is a slight plateau at 534 nm, with the emission maximum occurring at 550 nm. The spectra of the unbound dye also differ between the two wavelengths (Fig. 4A and B, insets). For 488 nm excitation, the peak emission is

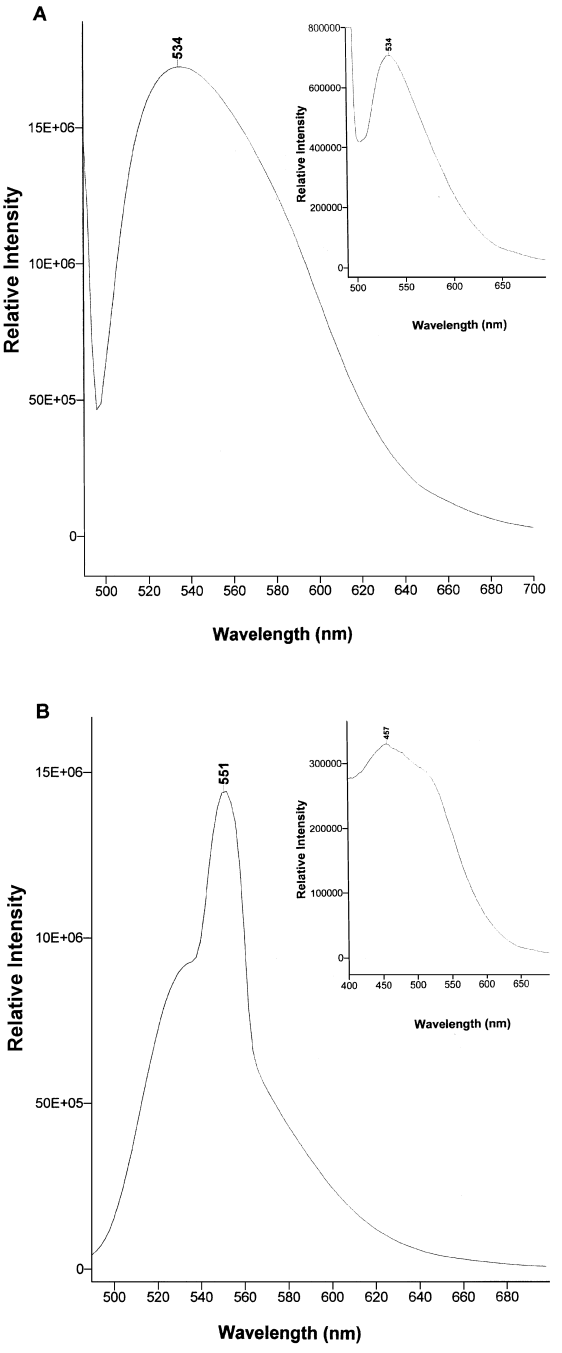

Fig. 4. Fluorescence emission spectra for dsDNA-bound SYBR Gold. Insets show emission spectra for unbound SYBR Gold (10 000-fold dilution in sieving buffer) at the respective excitation wavelengths. (A) 488-nm excitation. Emission was collected from 490 to 700 nm. (B) 275-nm excitation. Emission was collected from 290 to 700 nm.

at 534 nm, while for 275-nm excitation the peak emission is blue-shifted to 457 nm. In addition, 488-nm excitation causes a more intense emission than does 275-nm excitation. This blue-shift, coupled with the lower background intensity, results in emission properties that coincide with different emission filters for the two excitation wavelengths.

In order to optimize the sensitivity of 488-nm excitation, a set of experiments was devised to determine which optical filter combination would collect maximum nucleic acid fluorescence while minimizing the background signal. Three high-pass filter combinations were tested: an OG-515 filter, an OG-570 filter, and an OG-570/RG-610 filter combination; the spectral properties of the filters are listed in Table 1. The results of these filters for the detection of both DNA and single-cell RNA are shown in Table 2. While the fluorescence characteristics of nucleic acids with 275-nm excitation warrant the use of an OG-515 filter, this filter is unsuitable for use with 488 nm. Although OG-515 should spectrally allow maximal fluorescence collection, it actually gives poor results for both DNA and RNA detection, as expected. The LOD for DNA using OG-515, 1.3 ng/ml (S/N~3), is 10-fold worse than for the other filter combinations. In addition, OG-515 results in a very high background fluorescence caused by the presence of unbound dye in the sieving buffer. Surprisingly, the OG-570/RG-610 combination, which cuts almost all of the nucleic acid fluorescence emission, provides the best detection limits for DNA. Optimal sensitivity for single-cell RNA determinations results from the OG-570 filter, for which the S/N is approximately two-times higher than with the OG-570/RG-610 combination and four-times higher than with the OG-515 filter.

The previous discussion on nucleic acid emission also answers the question of why the DNA detection limits were about the same with both UV and visible excitation. Using the OG-515 filter with 275-nm excitation allows collection the majority of the fluorescence emission to be collected (partially owing to the observed spectral shift). However, it was necessary to use a filter with a higher cut-on wavelength for 488-nm excited detection to eliminate background fluorescence of unbound SYBR Gold. The OG-570/RG-610 filter combination, which gave the best DNA detection limits, actually permits only a small fraction of the total fluorescence to be detected. Thus, despite the lower intensity from 275-nm excitation, the fact that most of the visible-excited emission is being discarded while most of the UV-excited emission is being collected leads to similar detection limits at both wavelengths. We suspect that the spectral shifts present in dsDNA-bound SYBR Gold, which are partially responsible for this phenomenon, do not exist for RNA-bound SYBR Gold. However, it would be difficult to duplicate the complex cellular environment from which the RNA was sampled in order to obtain meaningful spectra for RNA-bound SYBR Gold.

While these initial experiments show no distinct advantages of using UV excitation for the detection of nucleic acids, they do take the first step toward characterizing the UV-excited fluorescence of nucleic acids bound to SYBR Green I and SYBR Gold,

Table 2
DNA detection limits for various filter combinations[a]

Filter	LOD (ng/ml)	S/N (400-bp peak)
OG-515	~1.3	~3
OG-570	~0.3	~3
OG-570/RG-610	~0.1	~2

[a] DNA (100-bp ladder) was injected for 5 s at −6 kV. Electrophoresis voltage: −6 kV. Sieving buffer: 1% HPMC-5, 0.5% PVP, and 6% mannitol in TBE containing a 10 000-fold dilution of SYBR Gold. LIF detection: argon-ion laser excitation at 488 nm. Fluorescence passed through the designated filter prior to collection by the PMT.

Table 1
High-pass filter specifications[a]

Filter	Cut-on (nm)	50% Max. transmission (nm)	Max. transmission (nm)
OG-515	490	515±6	≥570
OG-570	550	570±6	≥630
RG-610	560	610±6	≥660

[a] Spectral information obtained from the Filter Glass Catalog (Schott Technologies; www.schottglasstech.com).

beyond the traditional scope of gel electrophoresis. Further, more comprehensive studies may lead to the development of UV-excitable dyes with higher sensitivity than currently available visible-excited dyes. This may lead to increased use of UV excitation for the detection of nucleic acids in conventional studies, as well as to the development of assays in which UV excitation is necessary.

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