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# Yoctomole detection limit by laser-induced fluorescence in capillary electrophoresis

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**Abstract**

Laser-induced fluorescence detection in a sheath flow cuvette is the most sensitive method of detecting fluorescent molecules for capillary electrophoresis. This manuscript demonstrates the detection of 50 yoctomoles (1 yoctomole = 1 ymol = $1 \times 10^{-24}$ mol) of rhodamine 6G. A 1-mW He–Ne laser ($\lambda = 543.5$ nm) was used as the excitation source. This detection limit is a 10 times improvement compared to the previous state-of-the-art detection limit in separation science.

## 1. Introduction

The small sample volume used in capillary electrophoresis requires high-sensitivity detectors. Different kinds of detectors can be employed for this purpose. Mass spectrometry [1,2], electrochemistry [3–5], UV–visible absorbance [6,7], radioactive isotopes [8], laser induced thermo-optical absorbance techniques [9–12] and laser-induced fluorescence [13–15] are among the most often used detection methods.

Laser-induced fluorescence is particularly interesting because the detection sensitivity extends to single molecule level in neat solutions [16–20] and in levitated microdroplets [21,22]. The detection of single molecules of rhodamine 6G, Texas Red, phycoerythrin, and some near-infrared dyes has been reported. Unfortunately, it is difficult to use the detection scheme in neat solutions as a tool to identify real samples; fluorescence is a relatively information-poor technique. However, combination of a high-sensitivity detector with a high-efficiency separation system allows the identification of a large number of analyte at extremely low levels; combination of high-sensitivity detection with high-selectivity separation results in an analytical tool with unrivaled performance. Previous work from this group has shown detection limits of zeptomoles ($10^{-21}$ mol) of amino acids labeled with fluorescein isothiocyanate, separated by capillary electrophoresis [14], and 300-molecule detection limits of tetramethyl-rhodamine-isothiocyanate [23]. With the improvement in laser technology and computer-controlled data acquisition system, we have constructed an inexpensive higher-sensitivity detection system. This manuscript demonstrates the combination of an extremely sensitive detector, the sheath flow cuvette laser induced fluorescence detector, and a very efficient

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separation system, the capillary electrophoresis system. Detection limits for this system are 30 molecules or 50 yoctomoles (1 ymol = $10^{-24}$ mol) of highly fluorescent analyte. We are approaching analysis at the single-molecule level.

## 2. Experimental

### 2.1. The detection system

The detection system was constructed on an optical bread board (Melles Griot, Nepean, Canada) (Fig. 1). A 1.0-mW helium–neon laser (GreNe, Melles Griot) with a wavelength of 543.5 nm was used as the excitation source. The beam was focused with a 25 mm focal length 5 × microscope objective (Melles Griot) into the cuvette. A sheath flow cuvette is used as the fluorescence detector. The cuvette has 1-mm thick walls, a 200-$\mu$m square inner chamber, and a length of 1 cm. Fluorescence was imaged by a 60 × 0.7 numerical aperture (NA) microscope objective (Universe Kogaku Model 60X-LWD, Oyster Bay, NY, USA) onto an iris which was adjusted so that the fluorescent light is allowed to go through, while the scattering light is blocked. The transmitted light was filtered with a 570–610-nm band pass interference filter (Omega Optical, Brattleboro, VT, USA) and was detected with a R1477 photomultiplier tube (Hamamatsu, Bridgewater, NJ, USA). The output signal of the photomultiplier tube was collected by a photon counter (C1230, Hamamatsu), and digitized by a Macintosh IIsi computer. The program used to collect data is written in LabView (National Instruments, Austin, TX, USA). All chemicals used were of the highest available purity (analytical-reagent grade).

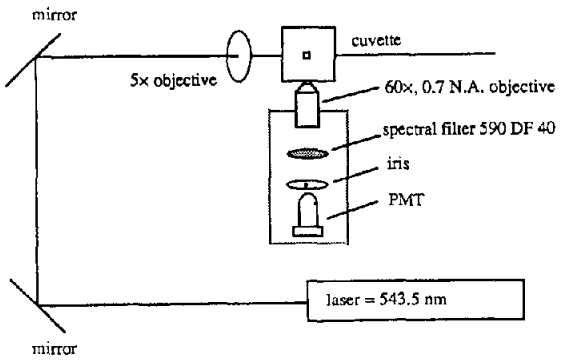


Fig. 1. Optical arrangement for the laser-induced fluorescence detector. PMT = Photomultiplier tube.

### 2.2. Electrophoresis system

The electrophoresis is driven by a Spellman CZE1000R high-voltage power supply (Spellman, Plainview, NY, USA). The capillary (Polymicro, Phoenix, AZ, USA) was 40 cm × 10 $\mu$m I.D. × 140 $\mu$m O.D. Rhodamine 6G bulk solution was prepared in ethanol with a concentration of $4.13 \cdot 10^{-3}$ *M*, then serial dilution was used to generate a $4.13 \cdot 10^{-11}$ *M* solution in 5 m*M* borate buffer with 10 m*M* sodium dodecyl sulfate, pH 9.2. As a simple source of sheath fluid, a 250-ml wash bottle was filled with buffer solution, and the buffer level was set to be a few centimeters higher than the buffer level in the waste container. Because of the siphon effect, the sheath buffer flows through the cuvette by gravity; this flow is very smooth, with no pulses to disturb the fluorescence signal. The sheath flow-rate can be controlled by adjusting the buffer level in the wash bottle while keeping the buffer levels in the injection vial and waste cell constant. The level difference was set at 7 cm in this case.

## 3. Results and discussion

Typically, the sample was injected at 500 V for 5 s, the electrophoresis was performed at 30 000 V, and the retention time of Rhodamine 6G was about 3 min. Fig. 2 shows electropherograms of repeated injections of about 360 molecules of rhodamine 6G into a capillary (the first electropherogram is a blank). The full width at half height of the peaks is about 0.5 s, corresponding to *ca.* 700 000 theoretical plates. This plate count is slightly lower than that expected for this size dye molecule [15] and probably reflects difficulty

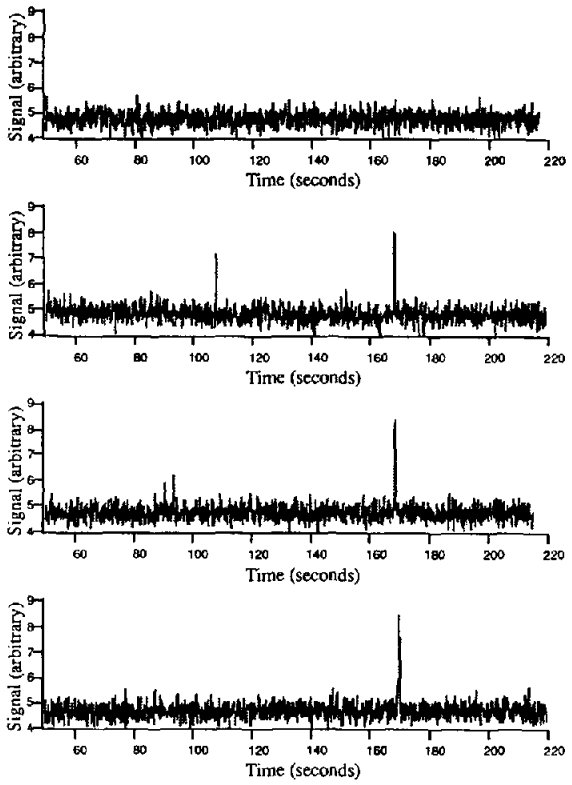


Fig. 2. Electropherograms of repeated injections of 300 molecules of rhodamine 6G into a capillary.

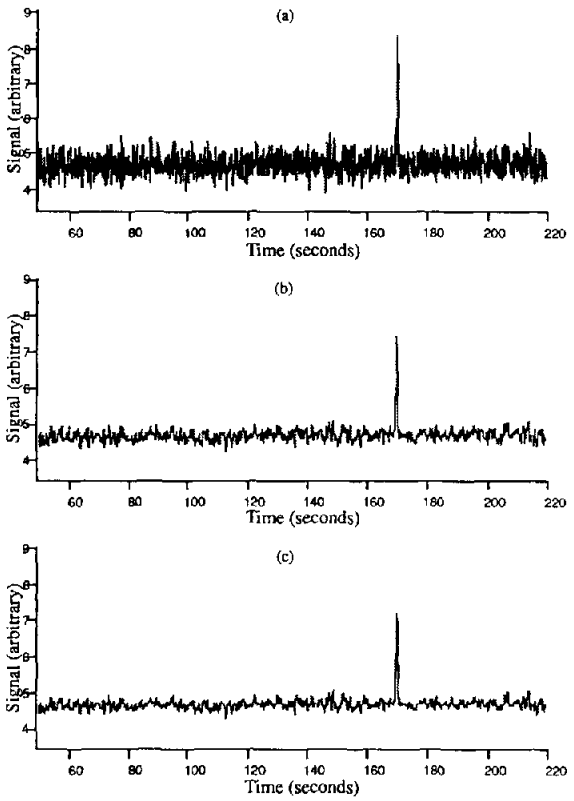


Fig. 3. (a) One of the electropherograms in Fig. 2. (b) The results of 3 times binomial smoothing. (c) The results of 5 times binomial smoothing.

in estimating the peak width of these very sharp peaks.

The detection limits are calculated by the method of Knoll [24]. Data were collected for more than 220 s, so the $K_{LOD}$ of 0.6536 for $N = 100$ is used, where $N$ is the length of the electropherogram (220 s) divided by the full width at half maximum (FWHM) (0.5). The capillary volume is 33 nl and the injection volume is about 15 pl. Assuming 360 molecules were injected (the actual amount will vary by *ca.* 5% because of sampling error), the detection limit is less than 60 molecules.

Because the data were collected in the digital form by a computer, digital signal processing like smoothing, Fourier transform low pass filtering, or time domain filtering can be easily performed. Fig. 3 shows one of the electropherograms from fig. 2, and the results of binomial smoothing operations. Fig. 3a shows the raw data, the detection limit is 59 molecules, Fig. 3b is the result of 3 times smoothing, the detection limit is 42 molecules, and Fig. 3c is the result of 5 times smoothing, the detection limit is 40 molecules.

With the help of Fourier transform, it is possible to transform a time domain data to frequency domain. Fig. 4 shows the real part of a fast Fourier transform of the same electropherogram. From the Fourier transform of the time domain data shown in Fig. 4b, the noise characteristics can be determined. In this case, the signal peak mainly contributes to the lower frequency part of the transform result. At frequencies above 1 Hz, there appears to be some noise added to the signal. When the frequency is higher than 2 Hz, the components are mainly noise. With the help of Fourier transform, appropriate digital filters can be designed for different data so that most noise can be rejected in the frequency domain while reserving as much signal

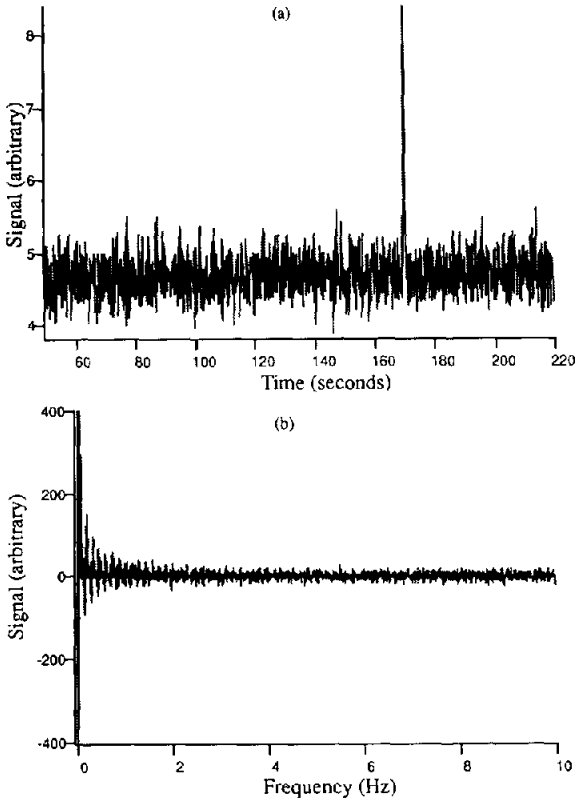


Fig. 4. (a) Raw data, (b) real part of a fast Fourier transform of the same electropherogram as Fig. 3a.

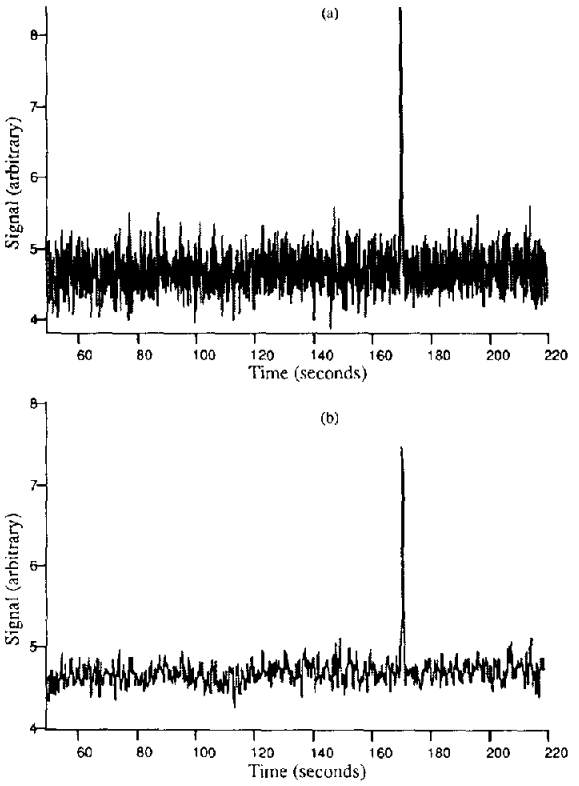


Fig. 5. The effect of Fourier transform low-pass filtering. (a) Raw data, (b) filtered data.

component as possible. Because the signal components are mainly at low frequency, a second order digital filter was used with a 1-Hz cut off frequency.

Fig. 5 shows the effects of Fourier transform low-pass filtering. Figure 5a shows the same electropherogram as Fig. 4a, the detection limit is 59 molecules; Fig. 5(b) shows the filtered electropherogram, the cut off frequency of the filter equals 1 Hz, and the order equals 2, the detection limit for the filtered data is 40 molecules.

Another way of digital signal processing is Savitzky–Golay type time domain filtering. The correlation of two signals is equivalent to multiplication of their Fourier transforms. Therefore, correlating the electropherogram to a Gaussian shaped function gives a time domain filtering, which is equivalent to a perfectly designed filter in the frequency domain of the Fourier transform provided the Gaussian function has the same standard deviation as the signal peaks. Another electropherogram is used as an example in Fig. 6: Fig. 6a is the raw data, the detection limit is 53 molecules; Fig. 6b is the resultant electropherogram from time domain filtering by a Gaussian shaped filter, the detection limit is 30 molecules. The experimental conditions were the same as other injections except a newly conditioned capillary was used. With this system, both rhodamine 6G and tetramethylrhodamine isothiocyanate were tested, the detection limits were around 30 molecules of injected sample for both analyte.

These detection limits represent the state-of-the art for capillary electrophoresis. With the sheath flow cuvette, better microscope objective, more powerful laser and digital signal processing, the detection limits have been improved by

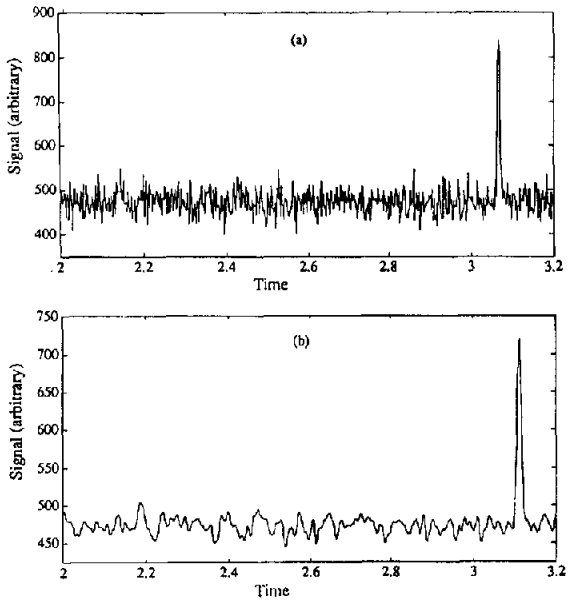


Fig. 6. Comparison of the original electropherogram and the resultant electropherogram after a Gaussian shaped time domain filter. (a) Raw data, the detection limit is 53 molecules; (b) resulting electropherogram from time domain filtering by a Gaussian shaped filter, the detection limit is 30 molecules. The horizontal axis is time in min, and the vertical axis is number of photons.

a factor of 10 compares to the previous results. We anticipate that improved optics and data processing will soon drop detection limits to the single molecule level. By combining the ultimate in quantitative analysis (single-molecule detection) with the exquisite separation efficiency of capillary electrophoresis, we will have constructed an extremely powerful tool for analysis of biological samples.

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