Published in final edited form as:

Talanta. 2009 December 15; 80(2): 744–748. doi:10.1016/j.talanta.2009.07.060.

Six orders of magnitude dynamic range in capillary electrophoresis with ultrasensitive laser-induced fluorescence detection

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Abstract

An ultrasensitive laser-induced fluorescence detector was used with capillary electrophoresis for the study of 5-carboxy-tetramethylrhodamine. The raw signal from the detector provided roughly three orders of magnitude dynamic range. The signal saturated at high analyte concentrations due to the dead time associated with the single-photon counting avalanche photodiode employed in the detector. The signal can be corrected for the detector dead time, providing an additional order of magnitude dynamic range. To further increase dynamic range, two fiber-optic beam-splitters were cascaded to generate a primary signal and two attenuated signals, each monitored by a single-photon counting avalanche photodiode. The combined signals from the three photodiodes are reasonably linear from the concentration detection limit of 3 pM to 10 μ M, the maximum concentration investigated, a range of 3,000,000. Mass detection limits were 150 yoctomoles injected onto the capillary.

Keywords

capillary electrophoresis; dynamic range; linear range; laser induced fluorescence

Introduction

Capillary electrophoresis provides outstanding resolution and near-Gaussian peaks. As a result, trace components can be detected in the presence of interfering compounds present at many orders of magnitude higher concentration. The dynamic range of the detector limits the ability to determine simultaneously the amounts of both trace and abundant components.

In capillary electrophoresis, absorbance measurements are limited to two or three orders of magnitude in dynamic range, whereas fluorescence detectors are able to achieve perhaps four orders of magnitude dynamic range. The latter tend to employ photomultiplier tubes as photodetectors with 16-bit analog-to-digital converters (ADC). The ADCs are typically operated so that the noise in the measurement is larger than the least significant digit in the ADC. A single measurement will typically have a dynamic range of less than 10,000; averaging the signal from many measurements in the presence of uncorrelated noise can improve the

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dynamic range to a limited extent [1]. Higher dynamic range ADC converters are becoming available but are seldom employed in fluorescence detection.

In principle, photon-counting detectors can achieve an arbitrary dynamic range limited only by the size of the register used to accumulate counts. However, photon counters have a finite dead time during which they do not respond to a second photon [2]. In a paralyzable detector, the arrival of a second photon restarts the dead time. At very high incident rates, the detector will saturate and record no signal at all. For commercial avalanche photodiode photon counters, the dead time is on the order of 50 ns, and the detector will saturate as the incident photon rate approaches 20 MHz. The dead-time characteristic of detectors has received significant attention from the atomic and nuclear physics communities, and a number of models are available to correct for dead-time effects [3,4].

There are cases where very high dynamic range is desirable, for example when detecting minute amounts of enzymatic product in the presence of a very large excess of substrate [5,6]. We report a simple method to increase dynamic range without limit in fluorescence detection and employ this method to produce over six orders of magnitude dynamic range while retaining yoctomole detection limits. In this system, fluorescence generated in a sheath-flow cuvette is collected by a microscope objective and imaged onto a gradient index-coupled fiber optic. The light captured by the fiber is sent to a cascade of fiber-optic beam-splitters, repeatedly dividing the signal into successively lower intensity channels. These beam-splitters require no alignment, are robust, and are inexpensive.

Each channel is equipped with a dedicated avalanche photodiode photon counting module. The highest intensity signal is used to characterize components present at very low levels and the most attenuated signal is used to characterize the highly abundant components. In principle, an arbitrary number of splitters can be cascaded to generate an arbitrary dynamic range measurement; we demonstrate this approach with a cascade of two splitters and three photodiodes.

Material and methods

Reagents

Unless specified, reagents were from Sigma. Water was from a Barnstead Nanopure water supply. 5-carboxy-tetramethyl rhodamine (TAMRA) was from Invitrogen. Capillaries were from Polymicro Technologies.

Capillary electrophoresis

The capillary electrophoresis system was similar to others developed by this group [7-9]. Briefly, a 23-cm long, 20- μ m ID, and 150- μ m OD uncoated fused silica capillary was used for the separation. The separation was performed using a running buffer composed of 10 mM sodium tetraborate, 35 mM sodium deoxycholate, and 5 mM methyl- β -cyclodextrin. The running voltage was 18 kV. Injection was for 1 second at 1 kV. Counts were recorded at 50 Hz.

High-dynamic range, ultrasensitive laser-induced fluorescence detector

The instrument was equipped with a post-column sheath-flow cuvette for fluorescence detection [10,11]. The optical design of the high-dynamic range detector is shown in figure 1. The majority of the system was identical to that described earlier. Excitation was provided with a 10 mW diode-pumped solid-state (DPSS) laser at 532 nm (Coherent) that was focused in a sheath-flow cuvette. Fluorescence was collected by a 0.45 NA microscope objective, passed through a 580 DF40 bandpass filter, and imaged onto a GRIN-lens coupled fiber optic.

We employed fiber-optic beam-splitters to divide the fluorescence into two channels; one channel retained most of the original intensity and the second presented an attenuated version. The beam-splitters (Thorlabs) have a nominal 99:1 split ratio at 850 +/- 40 nm. However, the split ratio degraded at the 580 nm wavelength of the fluorescence. One splitter generated a split ratio of ~15:1 and the other generated a split ratio of ~4:1. The splitters were placed in series, where the intense output of the first splitter was directed to a single photon counting avalanche photodiode (APD1) and the attenuated output was directed to the second splitter. The outputs of that splitter were connected to two additional single-photon counting avalanche photodiodes (APD2 and APD3). PerkinElmer SPCM single photon counting modules were employed in the experiment; all three had nominal dead time of 55 ns.

The fiber-optic cables attached to these beam-splitters are relatively transparent in the visible portion of the spectrum. Room light striking the fibers will increase the dark count. We cover the fibers with an opaque plastic sheet to eliminate this source of background signal.

The outputs of the photodiodes were digitized at 50 Hz by counters (National Instruments) in a PC. The data were then processed on a Macintosh computer using Matlab. Data were treated with a three point median filter to remove spikes due to light scatter from particles, and then convoluted with a 44-point wide Gaussian function with 5 point (100 ms) standard deviation. The MatLab routine cftool was used to fit a Gaussian function to the TAMRA peak; peak area was estimated by multiplying the peak amplitude with the standard deviation.

Results and discussion

Single avalanche photodiode detector

We employed the most sensitive photodiode of figure 1 to construct a calibration curve for the injection of 45 pL of various concentrations of 5-carboxy-tetramethylrhodamine. The data were treated with a 3-point median filter to remove the occasional noise spike associated with the passage of a particle through the detector and then convoluted with a 100 ms wide Gaussian filter, which matched the width of the electrophoretic peak.

Determining the linearity of wide dynamic range signals is not straightforward. We measured the signal for samples prepared by ten-fold serial dilution. A simple plot of signal vs. concentration is virtually guaranteed to generate a high correlation coefficient in a least-squares plot. Instead, we inspect the slope of the least-squares fit of a straight line to the logarithm of concentration vs. the logarithm of signal; the slope should be close to 1.0 if the signal is linearly related to concentration.

Figure 2 presents a log-log plot of the calibration data. The signal indeed increased linearly with concentration from 10 pM to 10 nM; the log-log plot generated a slope of 0.92 ± 0.04 . The signal saturated at higher concentrations.

The concentration detection limit (3 s) was 3.4 pM and the mass detection limit was 150 ymol (100 copies) injected onto the capillary. The linear dynamic range from the detection limit to the start of the nonlinear portion of the calibration curve was 3,000. The peaks in the linear portion of the calibration curve generated $300,000 \pm 20,000$ theoretical plates.

Paralizable detector

The observed photon count rate, N_{obs} , from a paralizable detector is related to the true photon count rate, N_{true} , and the detector dead time, t_d [4].

$$N_{\text{obs}} = N_{\text{true}} e^{-N_{\text{true}} t_d} \quad (1)$$

A plot of the incident and observed count rates predicted from equation 1 is shown in figure 3 for a paralyzable photodetector with a 55-ns dead time, the nominal dead time for our photodiodes. The observed count rate is within 5% of the true count rate for signals less than 1 MHz, but becomes highly nonlinear at higher count rates. The observed signal reaches a maximum at an incident count rate equal to $1/t_d$ and then decreases at higher count rates. The maximum signal is $1/(t_d e)$.

Figure 4 presents the electropherogram generated by a 1 μM TAMRA solution. The lower amplitude impurity peaks at 81 and 86.5 seconds are Gaussian in shape, whereas the main peak is severely distorted, showing a truncated peak with a dip at the center. This dip occurs because N_{obs} is not monotonically related to N_{true} ; the true count rate near the dip exceeds $1/t_d$. The signal exceeds the maximum predicted for a 55-ns dead time detector; equation 1 is not accurate for very high signals.

An inverse function can be used to estimate N_{true} for $N_{\text{true}} < 1/t_d$. Several models have been presented for the inverse function. The most common correction function is [12]

$$N_{\text{true}} \approx \frac{N_{\text{obs}}}{1 - N_{\text{obs}} t_d} \quad (2)$$

A second order term can be included, which improves the accuracy of the inverse function [4].

$$N_{\text{true}} \approx \frac{N_{\text{obs}}}{1 - N_{\text{obs}} t_d - (N_{\text{obs}} t_d)^2} \quad (3)$$

The latter correction is accurate to within 2% for $N_{\text{true}} < 0.65 / t_d$ ($N_{\text{obs}} < 0.34 / t_d$).

We employed equation 3 with $t_d = 55$ ns to correct the nonlinear response of the photodiode. The resulting calibration curve was reasonably linear from the detection limit to 100 nM concentration (slope of the log-log plot = 0.91 ± 0.04 , $r = 0.9974$). The non-monotonic relationship between N_{true} and N_{obs} prevented an accurate correction of the 1 μM data. The detection limit was not affected by this procedure, and the linear dynamic range was increased to $\sim 30,000$.

Fiber-optic beam-splitter cascade

We employed a cascade of two fiber optic beam-splitters to divide the fluorescence intensity. The first beam-splitter divided the intensity in $\sim 15:1$ ratio; the second divides intensity by $\sim 4:1$. The splitting ratios were determined by first correcting the signal from each photodiode for detector dead time and then comparing the signal intensity of low concentration samples. The first photodiode received the vast majority of the fluorescence signal and was useful for the lowest amplitude signals; the third diode received only 2% of the fluorescence signal and was useful for higher amplitude signals.

The beam-splitters have some loss. We measured the baseline signal with the APD directly connected to the GRIN lens and the signal after going through the beam-splitters. The signal

from photodiode 1 was attenuated by about 35%. 5% of that loss was due to light directed to the other photodiodes and could be recovered by summing the photodiode signals. The detection limit of a shot-noise limited experiment scales inversely with the square-root of the signal. A 30% decrease in signal corresponds to 20% degradation in detection limit. That loss of performance is accompanied by a two order of magnitude increase in dynamic range and should be acceptable in most experiments.

Figure 5 presents the electropherogram generated by the 1 μ M sample as observed by the three photodiodes. The traces have been corrected for the detector dead time with equation 3 and scaled based on the measured fiber-optic beam-splitter ratio. Although the peak signal from photodiode 1 could not be corrected by equation 3, the lower amplitude signals were well within the linear range of the photodiode. The corrected signal from photodiode 3 was multiplied by 10,000 to highlight trace components, and the corrected and scaled signal from photodiode 2 was multiplied by 100 to highlight intermediate amplitude components. The trace component at 77 s was roughly four orders of magnitude less intense than the main TAMRA peak.

The high dynamic range allows a detailed characterization of peak shape. In particular, the main peak shows a modest amount of tailing in the photodiode 3 signal, which becomes much more prominent in the higher sensitivity signal of photodiode 1. That tailing is not the result of the convolution filter employed to treat the data. The convolution function is symmetrical and can not introduce tailing. The effect of the filter is shown in figure S1 of the supporting information for this paper.

Figure 6 presents the calibration curves generated by combining the scaled peak areas from the three diodes; photodiode 1 was used for corrected and scaled signals up to 1 MHz, photodiode 2 was used for corrected and scaled signals between 50 kHz and 100 MHz, and photodiode 3 was used for signals between 50 kHz and 1 GHz. The combined calibration curve generated a log-log slope of 0.93 ± 0.02 , $r = 0.9981$ ($n = 20$), ranging from the detection limit of 3×10^{-12} M to 1×10^{-5} M, a range of 3,000,000 in concentration. The slope is not within experimental error of unity; the origin of that discrepancy is not clear, but may be associated with the photophysical phenomena described below. Because the slope is not unity, it is necessary to construct a calibration curve using at least two concentrations of analyte to determine the slope. In practice, the splitter combination should be evaluated across a wide concentration range to characterize its performance.

Of course, additional beam-splitters and detectors can be added to the system to increase the dynamic range to higher concentrations. However, we are near the limits provided by the electrophoretic and spectroscopic properties of this dye. There are three phenomena that occur at higher analyte concentrations to generate nonlinear responses. First, dimer formation occurs at sub-millimolar concentrations of highly conjugated aromatic dye molecules [13]. These dimers have different molar absorptivity than the free dye and will lead to deviations from Beer's law. Antonov has reported a dimer dissociation constant of 3×10^{-4} M for rhodamine 6G, a similar dye to TAMRA. Second, capillary electrophoresis peak shapes become distorted as the ionic strength of the sample approaches the ionic strength of the buffer; in our system, analyte concentration can be increased to ~ 1 mM before electrophoretic phenomena distort peak shape. Finally, the fluorescence signal will become nonlinear when absorbance across the stream radius becomes significant, which again will become important for tetramethyl rhodamine concentration greater than a few hundred micromolar.

We also point out that Merz and Mort employed a liquid-crystal programmable attenuator in the emission channel of a fluorescence detector [14]. This device was used to keep the fluorescence intensity within the linear dynamic range of their photodetector and was

particularly useful at high analyte concentrations. Unfortunately, a calibration curve was not generated to evaluate the performance of this system.

Finally, the dynamic range of the instrument can be improved at lower concentrations. Most simply, the capillary inner diameter can be increased, allowing an increase in injection volume by at least one order of magnitude. As the ultimate improvement, a beam-splitter cascade can be incorporated into a single molecule detector [15], which would allow the widest possible dynamic range in fluorescence detection--nine or ten orders of magnitude should be possible, limited only by the photochemistry of the dye molecule.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (R01NS061767). CDW acknowledges an American Chemical Society –Division of Analytical Chemistry Graduate Fellowship. We thank one of the reviewers who brought reference 14 to our attention.

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Figure 1.

Optical diagram. Fluorescence is divided with a cascade of fiber-optic beam-splitters into three channels. APD 1 provides the highest sensitivity but saturates at relatively low signals. APD 3 provides the poorest sensitivity but saturates at relatively high signals.

Figure 2.

Log-log calibration curve for uncorrected peak area for diode 1. The straight line is the result of a linear regression analysis to the log-log data for concentrations of 10^{-11} , 10^{-10} , 10^{-9} , and 10^{-8} M; slope = 0.92 ± 0.04 , $r = 0.9980$. The dashed lines are to guide the eye.

Figure 3. Plot of equation 1 for a paralyzable detector with a dead time of 55 ns. The observed count rate maximizes near 6 MHz and decreases at more intense signals.

Figure 4.

Uncorrected electropherogram of a 1 μ M solution of TAMRA recorded with photodiode 1. Data were treated with a 3-point median filter and then convoluted with a Gaussian filter with a standard deviation of 100 ms. Note the dip at the center of the peak at 92 s due to detector saturation.

Figure 5.

Corrected electropherogram of a 1 μM solution of TAMRA and recorded with three photodiodes. The signals from the photodiodes have been filtered and corrected for detector dead time. The signals from photodiodes 2 and 3 have also been scaled to account for the attenuation introduced by the fiber optic beam-splitter cascade. Finally, the corrected signal from diode 1 has been multiplied by 10,000 and the corrected and scaled signal from diode 2 has been multiplied by 100 to highlight low and medium abundance components.

Figure 6.

Log-log calibration curve combining peak area for the three diodes. Red “ Δ ” is the corrected area for diode 1 (from 10^{-11} to 10^{-7} M), green “ $\circ$ ” is the corrected and scaled area for diode 2 (from 10^{-10} to 10^{-6} M), and black “+” is the corrected and scaled area for diode 3 (from 10^{-10} to 10^{-5} M). The line is a least-squares fit of a line to the log-log data; the slope is 0.93 ± 0.02 , $r = 0.9981$, $n = 20$.