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TITLE: AN APPROACH TO SINGLE-MOLECULE DETECTION BY LASER - INDUCED FLUORESCENCE

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An approach to single-molecule detection by laser-induced fluorescence

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Abstract

A sheath flow cuvette was evaluated in laser-induced fluorescence determination of aqueous rhodamine 6G. A detection limit of 18 attograms was obtained within a one-second signal integration time. The concentration detection limit was 8.9×10^{-14} mole per liter. An average of one-half rhodamine 6G molecule was present within the 11 pL excitation volume. However, during the signal integration time a total of 22,000 analyte molecules passed through the excitation region in a 0.42 microliter volume.

The biomedical technique of flow cytometry has been used to study the fluorescence and light scatter properties of biological cells and cellular components.[1] The hydrodynamic focusing property of the sheath flow cuvette employed in flow cytometry provides a well designed flow chamber for laser-induced fluorescence analysis of small volume samples. The sheath flow cuvette has been applied as a laser-induced fluorescence detector in high performance liquid chromatography and flow injection analysis.[2-4] A tightly focused laser beam was used in those experiments to define an excitation volume of several nanoliters.

In the present report, the performance of the sheath flow cuvette is considered for fluorescence analysis in excitation volumes of several picoliters.[5] The sample cell is shown in Figure 1.

SAMPLE TUBE

SHEATH STREAM

SAMPLE STREAM

QUARTZ WINDOW
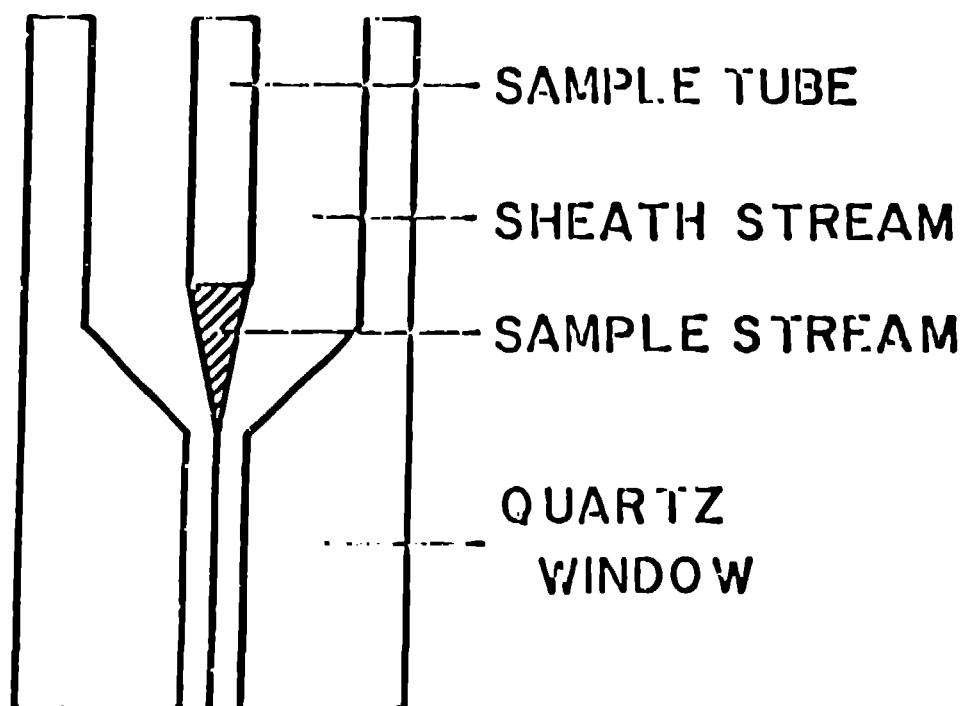
Figure 1. In the sheath flow cuvette, a liquid sample stream is injected into the center of a flowing liquid sheath stream. At the low flow rates employed, a stable laminar profile is produced within the cuvette. The sample injection tube is about 0.3 mm in diameter and the sheath stream is about 2.0 mm in diameter at the injection point. Hydrodynamic focusing occurs as the flow chamber necks down to a square probe region, 0.25 mm on a side and 1.6 cm long. The sample stream radius was measured to be about 15 μm.

. The sample and sheath streams were pumped by regulated air pressure. Polyethylene tubing was used throughout the apparatus. Samples were held in disposable test tubes. A sample flow rate of 25 μL/min was measured by weighing the sample test tube before and after flowing for a fixed time interval. The linear velocity of the sample stream was 0.6 m/s. Due to the narrow sample stream diameter, the velocity variation across the sample stream diameter due to a laminar flow profile was negligible.

Fresh solutions of aqueous rhodamine 6G were prepared within 48 hours of the measurement from a 3.5×10^{-7}M stock solution by serial dilution. Low impurity water was used both to prepare the samples and as the sheath fluid. Background measurements were made by flowing only sheath fluid. Samples were prepared in 100 mL volumetric flasks. The low surface-to-volume ratio of these flasks minimized sample loss due to adsorption on flask walls. The experimental diagram is shown in Figure 2.
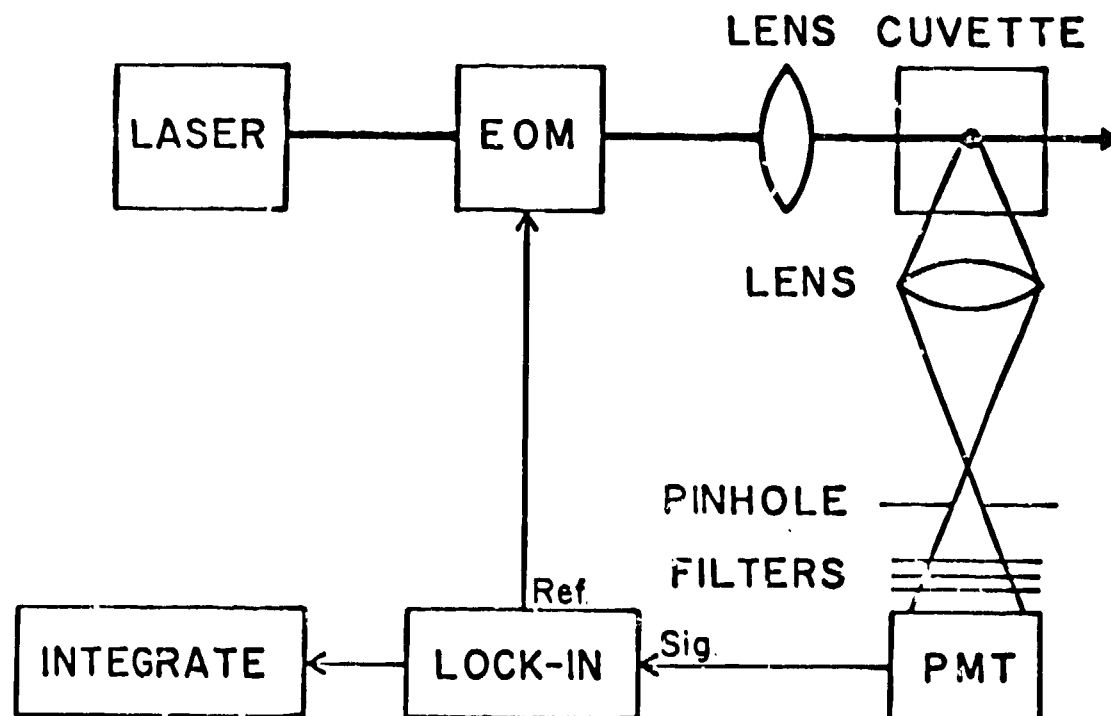

Figure 2. An argon ion laser, Spectra Physics 165-05, was used in the light-regulated mode as the excitation source. To achieve near shot noise limited performance, the laser output was modulated in a 50% duty cycle square wave at 10 kHz with an electro-optic modulator (EOM), Coherent Associates Model 50. The measured noise of the laser beam at the modulation frequency within a one-hertz bandwidth was measured to be less than 0.3%. The modulated beam was focused to a 11-μm radius waist located in the sample stream. An average laser power of 250 mW was measured. Due to the 50% duty cycle, the laser power during the "on" portion of the square wave was 500 mW. The corresponding intensity, 1.3×10^6 W/cm² was nearly an order of magnitude less than the measured saturation parameter for rhodamine 6G.[6]

The high optical quality quartz windows of the sheath flow cuvette facilitates effective spatial masking of laser light scattered from the cuvette windows and sample stream. An industrial microscope objective (N.A. = 0.60), Leitz 569-109, produced an image of the illuminated sample stream at 32X magnification on a 0.75-mm diameter pinhole. The objective is designed for use with a 1.8-mm thick quartz window, similar to that provided by the sheath flow cuvette. The sample stream image slightly overfilled the pinhole, so that light scattered from either the sheath stream or the cuvette windows is not transmitted.

Fluorescence was collected with 6% efficiency. A sequence of three filters blocked scattered light while passing much of the fluorescence. A 40 nm full width at half-maximum bandpass interference filter centered at 550 nm was used first. Two long wavelength pass filters were also used with a 50% cut-on wavelength of 530 nm, one an interference filter and the second a colored glass filter. Light which was transmitted by the filter train was detected with a photomultiplier tube, EMI 9789A, with a S-20 photocathode. The photomultiplier tube was selected for high quantum yield and low noise.

. The photomultiplier signal was preamplified and sent to a PAR Model 124 lock-in amplifier, phase referenced to the electro-optic modulator. The lock-in output was integrated for a one-s   d interval with a voltage-to-frequency converter and a fast counter. A sequence of 5   10 voltage readings was manually recorded and the mean and standard deviation were calcu ted.

A calibration curve was constructed for rhodamine 6G samples ranging in concentration from 3.5×10^{-8} to 3.5×10^{-11} M. The calibration curve was linear across the concentration range studied, r>0.999. The detection limit, two standard deviations above the blank signal, was 18 ag (1.8×10^{-14} g) in mass units and 8.9×10^{-14} M in concentration units. One may define the excitation volume as the product of the laser beam spot size with the sample stream area. With this definition, an average of one half of an analyte molecule would be present within the 11-pL excitation volume at the detection limit. This detection limit does not represent true single-molecule detection because 22,000 analyte molecules passed through the excitation volume during the one-second integration time. It should be noted that there is nothing fundamental about this integration time. The value was chosen only as a convenient rate to manually record the data.

The low mass detection limit, nearly two orders of magnitude superior to previously reported values,[7] was a result of several experimental design choices. The use of a modulated laser beam and phase-sensitive detection electronics resulted in nearly shot-noise limited detection of fluorescence. Careful spatial masking of the sample image greatly reduced the background signal from light scatter at the cuvette window and the sheath stream. Since the sheath and sample streams have the same refractive index, there is no light scatter at their interface. The spectral filters used in this experiment minimized the collection of Rayleigh or Raman light scatter from the sample stream while transmitting the fluorescence wavelengths at high efficiency. Finally, the small sample volume analyzed over the one-second integration time, 0.45 µL, is important in achieving low mass detection limits.

It is interesting to consider improvements in this fluorescence instrument. Most importantly, the signal integration time may be reduced to 37 µs, the transit time of an analyte molecule across the laser beam. For uncorrelated noise, such as shot noise, there is a square root dependence of detection limit upon integration time. If a 37-µs time constant were chosen, a detection limit of 67 fluorescent tags on a single species is predicted. The ultimate detection limit of a single analyte molecule should result if several additional improvements are considered: pulsed laser excitation and time filtered detection,[8] smaller excitation volume, improved collection efficiency, optimized spectral and spatial filtering, higher quantum yield of detection, and electronic correlation filtering.[9]

Single molecule counting would represent a significant advance in chemical analysis. The advance is due to the binary nature of single molecule counting. An analogy may be drawn with photon counting[10] In both cases, individual quantized events are detected and counted. The detection process requires only the discrimination between two possible signal levels corresponding to the presence or absence of an event. In comparison, analog processes require the estimation of a quantity by considering a large number of possible signal levels. Binary processes inherently provide greater noise immunity than analog processes.[11] Additional advantages of single molecule counting may be anticipated from the demonstrated advantages of single photon counting: direct digital processing of quantized information, decreased background signal, improved signal-to-noise ratio, low detection limit, accurate long-term signal integration, and reduced sensitivity to environmental fluctuations (1/f noise).

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