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Note: A method to isolate and detect a large number of single molecules by microdroplet fluorescence spectroscopy

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A laser induced fluorescence system, in combination with a glass-frit nebulizer and a photo-voltaic cell detector, is described for single molecule detection. The glass-frit nebulizer continuously generates a large number of droplets with an average droplet size of three micrometers in diameter. Rhodamine 6G molecules were detected at the 10^{-12} M level. Concentrations 10^{-12} – 10^{-10} M would provide mostly single molecules (0, 1, 2, 3, ...) in the individual droplets, as determined by Poisson distribution. © 2012 American Institute of Physics. [<http://dx.doi.org/10.1063/1.3697723>]

Chemical detection has reached the sensitivity for single molecules. Laser-induced fluorescence (LIF) is one of the powerful techniques used in single molecule detection (SMD).^{1,2} Conditions required for SMD include a small probe volume for minimizing background signals and a highly diluted solution for allowing far-apart separations of single molecules.^{1,2} The small volume of a microdroplet is ideal for LIF-SMD. Spherical microdroplets provide enhancement effects for photon-energy through cavity resonance, internal reflection and refraction, and focusing.³ Molecules in a droplet will experience enhanced excitation, resulting in highly intense emission.^{3,4} Molecular lasing in a microdroplet has been reported.^{3,5} By using electrodynamic levitation of a single glycerol droplet, Ng *et al.*⁶ and Barnes *et al.*⁷ have detected and counted single molecules. Barnes *et al.* in an attempt to increase the sampling rate, used the approach of falling-droplet-stream in an electrodynamic-focused quadrupole and detected single molecules.^{8,9} The droplets in these works were produced with a piezoelectric ink-jet type droplet generator.^{6–9} Other droplet generators available include electrosprays,¹⁰ pneumatic nebulizers,^{11,12} ultra-sonic nebulizers,¹³ and glass-frit nebulizers.^{14–18} Each nebulizer type has its own set of operation requirements, performances, and equipment costs. The glass-frit nebulizer generates very small droplets, with an average size of three micrometers in diameter and has the lowest equipment cost. The number of molecules in a droplet is calculated with Poisson statistics, which predicts that the smallest droplet will contain the fewest number of molecules.⁶

The use of glass frits as a droplet generator was introduced by Apel *et al.*^{14,15} Fritted glass is manufactured from particles of glass which are fused, or sintered into a solid, but porous body; it can be sealed into glass tubing as a filter made entirely of glass. The droplet generation involves delivering a sample solution to the bottom surface of the fritted disk, passing a pressurized gas from the top surface of the fritted disk, and removing the produced droplets from the bottom surface of the disk by the same pressurized gas above.

This note describes the use of a glass-frit nebulizer for LIF-SMD. Aqueous solutions of Rhodamine 6G (R6G) dye were used. The analytical performances obtained with the microdroplet fluorescence spectrometer are compared with those of a commercial cuvette fluorescence spectrometer.

The glass frit nebulizer used is shown in Figure 1. The nebulizer is made from a fritted tube (Fisher Scientific); the frit-disk is 2 cm in diameter and is fine-porosity grade. A 1-mm inside diameter capillary Teflon tubing, cut with $\sim 45^\circ$ opening, was oriented to touch the bottom of the glass disk. A peristaltic pump was used to deliver the sample solution through a Tygon tubing, with a 2 ml/min solution uptake rate. The argon gas was operated at 20 psi. The aerosols exit through a 1-mm opening of a pipet tip. Figure 2 shows the schematic diagram of the microdroplet spectrometer. The concave elliptical mirror was 89 mm in diameter and used with an object (focal) distance of 17.0 mm and an image distance of 140 mm. The air-cooled argon laser (MWK Industries) was operated at ~ 30 mW (all lines and beam diameter of ~ 1 mm) and the detector (Oriental model #71904, Photo-voltaic silicon detector) was thermoelectrically cooled (Oriental model #70708 temperature controller power supply) to -40°C . The pre-amplifier used was model 70710 from Oriental. The bandpass spectral filter used was 560 ± 10 nm. The lock-in amplifier was EG&G model 5210. The emission intensity was observed using a linear strip-chart recorder and a voltmeter (Bel Merit Model 3050).

The commercial fluorescence spectrometer employed in the comparison study was Model LS50 Spectrofluorometer from Perkin Elmer (PE). This instrument utilized a xenon arc lamp for excitation, with a grating-monochromator for selecting excitation radiations and another grating-monochromator for isolating the emission. A diode array UV-Vis spectrometer (Hewlett Packard) was used to obtain the absorption wavelengths for the R6G solution. The spectro-fluorometer was set for the maximum absorption (excitation wavelength of 526 nm) and for the highest emission wavelength (signal) of 565 nm.

Several operation parameters were optimized for highest signal and lowest noise levels. It was found that the higher the argon pressure the higher the signal-to-noise ratio; high

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FIG. 1. The glass frit nebulizer.

gas pressure, however, could risk damaging the glass frit. Therefore, 20 psi argon pressure was chosen. The chopper frequency was optimized at 750 Hz after varying the frequency from 500 to 1000 Hz. The tip length and its orifice size for the 4-cm aerosol pipet-tube were also optimized; it was found that a short (~ 4 mm) tip with a 1.0 mm orifice worked best. The best excitation and observation distance from the tip orifice was 10 mm. Note that the solution uptake rate of 2 ml/min is dependent on the availability of a relatively large volume of sample solution. The effective solution delivery rate (as droplets) is $\sim 1\%$ of the solution uptake rate.¹⁶ It is possible to restrict the solution uptake rate while sacrificing some of the detection sensitivity. The glass-frit also showed a significant signal carry-over from solutions with concentrations greater than 10^{-9} M. For our interest in single molecule detection, solutions of lower than 10^{-9} M were utilized. Running a water-blank for 1–2 min was sufficient in removing a residual signal to the baseline level. It was also possible to speed up the peristaltic pump in the washing step so that the frit could be cleaned in less than 1 min. Both water and ethanol were evaluated as the solvent; water worked better, presumably its higher surface tension gave better quality and/or higher quantity droplets. It should be noted that solvent evaporation will

FIG. 3. Log-log plot of emission intensity versus concentration of rhodamine aqueous solution: (a) the developed microdroplet fluorescence spectroscopy system and (b) the commercial cuvette fluorescence spectrometer.

reduce the droplet volume and that the efficiency for photon coupling is droplet size-dependent.³

The emission signals (in arbitrary units) obtained with the microdroplet spectrometer (MS) are compared with those obtained with the commercial spectrometer (CS) and are shown in the log-log plot in Figure 3. The MS signals at the low concentration range are linear but those of the CS show virtually noise. The MS signals at the high concentrations correspondingly increase but those of the CS eventually saturated. These results indicate the MS is superior in both the detection limit and the linear dynamic range. The PE spectrophotometer is a relatively an old unit. Newer models could produce better analytical results. Generally, fluorescence spectrometers provide a detection limit of 10^{-11} M.¹⁹

The lowest detection level for the microdroplet spectrometer is pico (10^{-12}) molar. The droplets generated with the glass frit nebulizer have been studied for size distribution; the droplet diameter ranged from 0.5 to 6.5 μm with the highest number droplet distribution around 3 μm .¹² At detection levels of 10^{-12} – 10^{-10} M, the probabilities of having molecules in the droplets, calculated with the Poisson Statistics, are highest for zero and one molecule in a droplet.⁶

The microdroplet fluorescence spectrometer has been evaluated to be capable of isolating and identifying a large number of single molecules. Other systems use ultra-sensitive detectors, signal correlation electronics, and software for identifying bursts of photons emanated from a single molecule in the probe volume; this system probes a large number of droplets continuously and therefore the total number of single molecules provides a bunch of signals that can be detected with a standard cooled detector. Some potential applications may include droplet screening, single target-molecule identification, separation, collection, and study.

FIG. 2. The microdroplet fluorescence spectroscopy system.

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