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Fabrication of a Single Molecule Detection System and Its Application: Connection between Ensemble and Single Molecule Measurements

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A laser scanning fluorescence microscope system has been fabricated for single molecule detection (SMD). Problems associated with the system set-up have been discussed along with proper suggestions. Based on the SMD results obtained by using the apparatus, a statistical method has been suggested to determine the minimum number of required molecules to form a group of uniform average in a selected error range.

key words: single molecule detection, fluorescence, nile red, Cy3, uniform average

INTRODUCTION

Since the observation of fluorescence from an individual dye molecule at room temperature [1], single molecule detection (SMD) technique has emerged as a useful tool to attain detailed molecular information in physical, chemical and biological researches [2-6]. SMD technique has proven to be effectively applicable to the elucidation of photophysical properties of various molecular systems such as light emitting polymers [7-9], photonic wires [10], and biomolecules labeled with fluorophores [11-12]. Although the results from ensemble measurements are essential for the investigation of molecular properties, they often preclude detailed information on single molecular behaviors by averaging and mixing all individual signals. On the other hand, SMD provides new information on individual molecular properties such as spectral shift [13], fluctuation of fluorescence intensity due to local environment [14-15], and temperature dependent line broadening [16] of single molecules that could not be seen in ensemble measurement.

Among various single molecule detection apparatus, laser scanning fluorescence microscope has been widely used owing to its high sensitivity and low excitation energy in fluorescence detection [17-18]. By using an oil-immersion objective lens with high numerical aperture (NA), the spatial resolution as high as optical diffraction limit could be attained. This resolution is enough for distinguishing the location of single fluorescent molecules spread on a transparent polymer film. In this work, we explain the details in fabricating an SMD apparatus based on the laser scanning fluorescence microscope system. From the single molecule intensity data, a statistical method has been suggested to determine the minimum number of molecules required to form a group of uniform average.

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EXPERIMENTAL SECTION

An inverted microscope (Axiovert-25CFL, Carl Zeiss) was used for the main body of the SMD apparatus. The sample scanning was achieved by using an *x-y* scanner consist of two electrostrictive actuators (AD-100, Newport) and a two-axis linear aluminum ball bearing stage (Newport). The excitation laser beam of 543.5 nm He-Ne laser (25LGR193-230, Melles Griot) was delivered to the input port of the microscope through a single-mode optical fiber (P3-3224-FC, Thorlabs Inc.). The laser beam from the fiber was filtered by a narrow-band interference filter (F10-546.1-4-1.00, CVI Laser Corp.), and then reflected up to the microscope objective lens (Plan-neofluar 100 × NA=1.3, Carl Zeiss) by using a dichroic beam splitter (565DCXR, Chroma). The laser beam was expanded to 6 mm in diameter before it impinged the excitation filter to get higher optical resolution. The relationship between the beam diameter and the focus size will be discussed later in detail. Fluorescence signal from the sample was collected by the same microscope objective lens and focused through a 150 mm-focal length lens onto a single photon counting module (SPCM-AQR-13-FC, PerkinElmer Optoelectronics). Scattered light around the excitation wavelength was removed by inserting a holographic notch filter (543.5 nm SuperNotch-Plus™, Kaiser Optics) between the objective lens and the focusing lens. The electrical pulses from the photodetector (SPCM) were counted by using a computer plug-in counter board (PCI 6602, National Instruments). Sample stage scann-ing and data acquisition were controlled by using a visual basic program running on a Pentium PC.

Nile Red (NR) (Molecular Probe) and Cy3 (Amersham Biosciences) were used without further purification. The NR solution was prepared in methanol with successive dilution. Then NR was finally diluted in poly(methylmethacrylate) (PMMA) (Aldrich)/toluene solution. The concentration of the NR was in the range of 10^{-9}~10^{-10} M. About 150 μl of this solution was spin coated at 2,000 rpm onto a rigorously cleaned coverglass (180 μm thick, Fisher Scientific). For observation of single Cy3 fluorescence, aqueous solution of Cy3 (10^{-8} M) was spin coated on a PMMA film.

RESULTS AND DISCUSSION

Calibration and stabilization of electrostrictive scanner

In our apparatus, fluorescence image was obtained by scanning the sample stage in *x-y* direction with linear motion. Since the moving step for image scanning was very small (~100 nm), electrostrictive actuator (AD 100, Newport) with power supply (ESA-C controller, Newport) was used for the fine displacement. The displacement was monitored and calibrated by using optical interferometric method. Scattered light image of ruled grating (700 nm) also provided a medium resolution calibrator.

For optical interferometric measurement, Twyman-Green type interferometer [19] was used in which the position of one reflecting mirror was controlled by the actuator (Fig. 1). Since one period of the interferogram corresponds to the half of laser wavelength ($\lambda/2$ = 271.75 nm), the number of waves in the interferogram gives the expanded length of actuator by applied electric field change. The optical interferometric method provides highly accurate length measurement depending on the detection stability. The relative intensity fluctuation was measured to be 1.5% in our interferogram, and the expansion length of the actuator could be measured with *ca.* 4 nm resolution. Much higher resolution down to sub-Å can be achieved through more careful detection of interferogram with the use of energy stabilized laser source, vibration isolation and temperature control.

Measured expansion length of an actuator by using interferometric method is shown in Fig. 1b. As shown in Figure 1b, the expansion length did not linearly depend on the applied electric field. Moreover the expansion length was significantly different from the data supplied by the manufacturer due to the intrinsic property of electrostrictive actuator materials [20]. The electrostrictive actuator needs to be calibrated periodically for accurate operation by using a proper method such as interferometric measurement.

While the optical interferometric calibration provides a standard technique for the expansion length measurement, it gives a result for one dimensional length change. Two dimensional calibration of the sample stage could be done by the scattered light imaging of a ruled grating for comparison with the known value of grating interval.

Although electrostrictive actuators conveniently provide movement with high resolution in response to the applied electric field, several characteristics such as hysteresis, temperature effect, creep and nonlinear response should be considered for a proper use. We tried to minimize the problems associated with the use of electrostrictive actuators as follows;

1) Hysteresis is a directional property by which the expansion length differs depending on the direction of change (stretching or shrinking) [20]. To prevent the hysteresis effect, sample scanning was done under unidirectional electric field change. The grating image showed that the hysteresis effect was not pronounced within the image resolution of 230 nm by unidirectional scan.

2) The displacement of electrostrictive actuator is highly

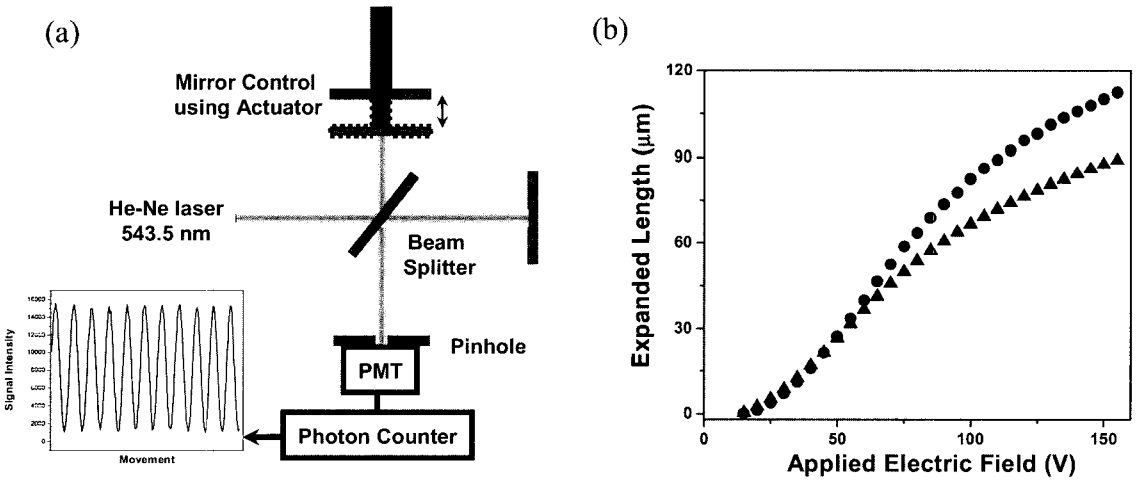

Figure 1. (a) A schematic diagram of a Twyman-Green interferometer used for calibration of electrostrictive actuator, and (b) the expansion length of actuator by the applied electric field. (°„) represents the data supplied by manufacturer, and (°‹) represents the results obtained by using interferometric measurement. (The expansion length can vary after long-term absence of use due to the physical property of electrostrictive materials)

dependent on the ambient temperature [21]. When the expansion length was measured with repetitive scanning, it became shorter as the number of runs increased. This result was due to the temperature rise of the actuator body under repetitive scan. The effect could be minimized by maintaining the temperature of actuator body slightly lower than the ambient temperature. Silicon flexible tube of 4 mm in diameter was used to circulate chilled water around the actuator to keep the body temperature at 18°C. The displacement was stabilized within the image resolution after the temperature control.

3) Nonlinear response of the electrostrictive actuator could not be avoided. The deviation from the linearity was negligible for expanded length up to 20 μm. Image distortion was negligible when the scanned area was less than 20×20 μm. We used the electrostrictive actuator without any correction for nonlinearity. In case of single molecule measurement, repositioning stability is more important than the length accuracy.

Measurement and reduction of the focused beam diameter

When 543.5 nm laser beam is focused by using an objective lens with 1.3 NA, the size of focus can be as small as 209 nm according to the diffraction theory of light as in Eqn. (1).

$$\Phi = \lambda/(2\,NA) \quad (1)$$

Φ : focused beam diameter
λ: wavelength of the laser light
NA : numerical aperture of the lens

It is possible to measure the focused beam diameter by observing the single molecule fluorescence intensity with scanning as shown in Fig. 2. Since the size of molecule corresponds to 1-2 nm, single fluorescent molecule can be considered as a point light source compared with the optical resolution scale.

When a collimated laser beam is focused through a lens, the boundary of an Airy disk can be obtained by Eqn. (2) [22].

$$\Phi = 1.22\, f\lambda/D \quad (2)$$

Φ : Radius of Airy disk
f : focal length of the objective lens
D : collimated beam diameter

The collimated laser beam diameter from the optical fiber output was *ca.* 1.5 mm. When the beam was focused through an objective lens (NA = 1.3), the beam diameter was measured to be 900 nm at the focus. This is almost the same as the calculated value of 880 nm by Eqn. (2). Since the focal length of the objective lens and the wavelength of the light were fixed, only the beam diameter could be changed. According to Eqn. (2), the focused laser beam size becomes smaller as the beam diameter is increased. Thus we expanded the laser beam size as large as 6 mm by using a beam expander (× 4).

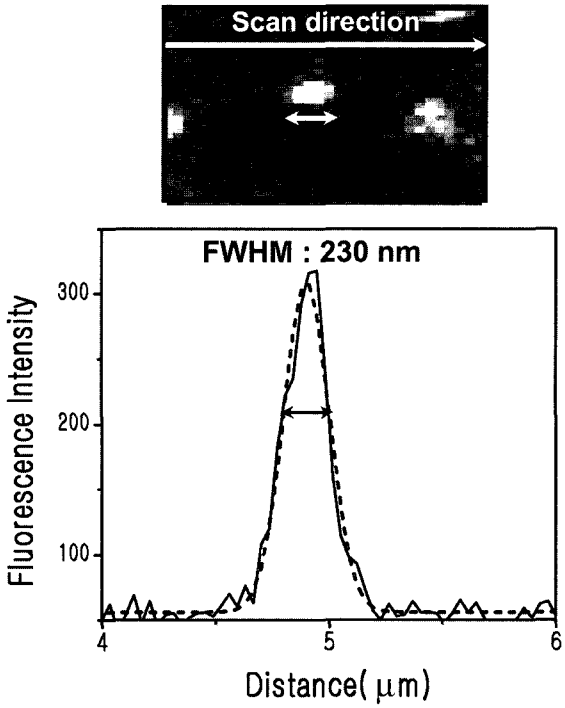

Figure 2. An example of the laser beam size measurement at the focus by using SMD of fluorescence. The spot size could be estimated from the FWHM of the Gaussian fit for the fluorescence intensity as a function of distance.

The enlarged collimated beam resulted in the focus size as small as 230 nm, which was obtained by fitting the scanned fluorescence intensity of a single molecule with Gaussian function (Fig. 2).

Effect of ground vibration

When we observe the signal from the focal point of an objective lens, the mechanical vibration can easily influence the signal intensity. Since the detection area is confined to a very small region (4.2×10^{-10} cm^2 for 230 nm focal spot), a slight movement of the laser beam or target sample results in a large signal change.

We used a pneumatic vibration isolation workstation (Model VH3648W-OPT, Newport) to minimize the noise from the ground vibration. All the optical detection components including microscope were fixed on this workstation. By using the pneumatic isolation, the noise due to mechanical vibration from the ground could be attenuated down to 1/30 when we monitored the signal intensity fluctuation at a fixed position.

Laser light was delivered from the laser to the input port of the microscope through a single-mode optical fiber. This geometry is better than direct coupling of laser light and microscope since the distance between the laser source and the microscope objective lens can be minimized. By using a pneumatic vibration isolator and optical fiber delivery system, noise signal due to the mechanical vibration could be

effectively minimized.

Sample preparation and confirmation of SMD

The fluorescence image of NR molecules embedded in a PMMA thin film was observed. Figure 3 shows 10×10 μm fluorescence images for NR molecules spin-coated at various concentrations (10^{-9}~10^{-10} M). As the concentration of the dye solution was decreased, the number of detected fluorescent spots was decreased as shown in Fig. 3a-e. The *log-log* plot of this correlation exhibited a linear relationship over the concentration range of 10^{-9}~10^{-10} M with the slope of 1.0. This value indicated that the spots consisted of single molecules otherwise the slope would be higher than unity. For example, the slope would be 2 if the spots were fluorophore dimers.

The photobleaching of each spot occurred as a single step with a discrete intensity drop when we observed the fluorescence intensity transient (Fig. 3f). This behavior is a strong indication of SMD because the photobleaching of a bunch of molecules might occur in multiple steps or non-discrete pattern.

Fluorescence image of single spots was also observed with linearly polarized light excitation. Single fluorescent molecules are expected to have a well-defined absorption and emission dipole orientation [23]. Thus, the fluorescence intensity of single molecule depends on the relative orientation of the molecular transition dipole to the excitation polarization. If the molecular transition dipole is parallel to the excitation polarization, the fluorescence intensity of the spot would be high. On the other hand, the spot might not emit fluorescence if the dipole orientation is perpendicular to the excitation polarization. Therefore an alternative appearance of the fluorescence spots is expected by the change of the excitation light polarization. The fluorescence intensities of some spots were dramatically changed when the excitation light polarization was modulated perpendicularly. This is another evidence of SMD since a bunch of molecules would not show polarization behavior due to averaged random orientation of constituent molecules. Not all the single molecules would show polarization behavior in a fixed geometry. Single molecules having dipole orientation of 45° to the excitation polarization would not show any polarization behavior since the molecules can interact with both perpendicular components of linear polarization.

Finally, the detection rate of photons from a single spot could be estimated. The maximum rate of detected photons can be estimated by Eqn. (3).

$$N=k\phi_F\sigma I \tag{3}$$

N : The number of observed photons per second
κ : Instrumental constant of detection efficiency
ϕ_F : Fluorescence quantum yield of NR
σ :Absorption cross section of NR
I : Excitation power

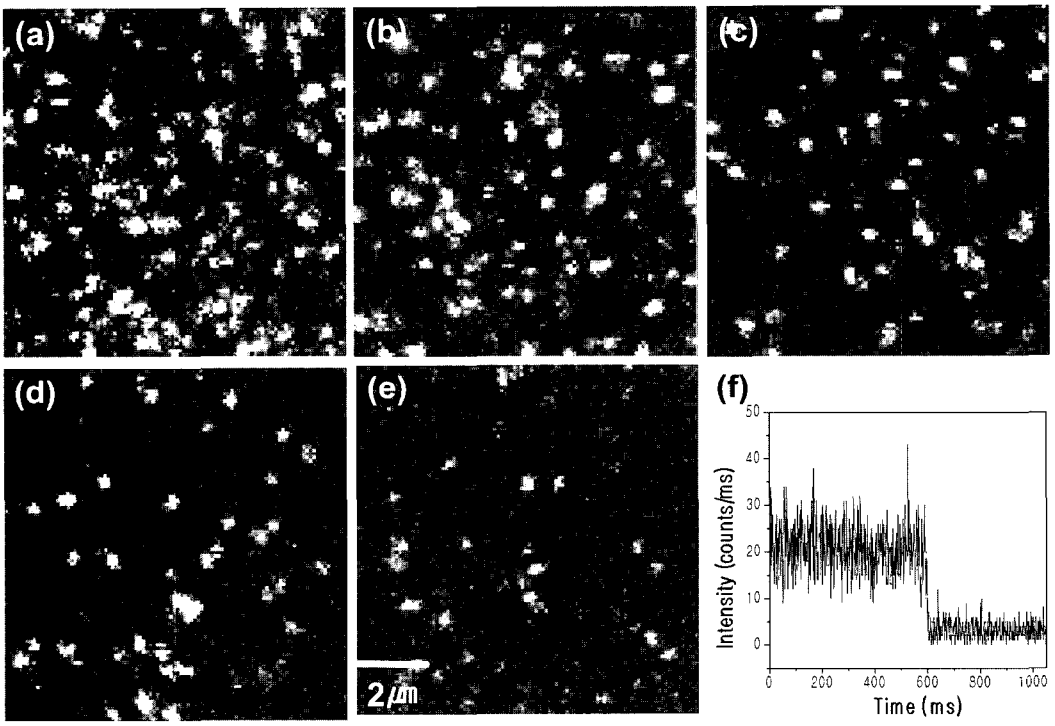

Figure 3. Fluorescence image of single NR molecules embedded in a thin polymer film (PMMA). The bright spots correspond to the individual NR molecules. The concentration of NR solutions before spin coating were (a) 1.4×10^{-9} (b) 1.1×10^{-9} (c) 7.0×10^{-10} (d) 4.7×10^{-10} (e) 3.5×10^{-10}, respectively. (f) The discrete jump in the fluorescence intensity transient of a spot indicating that the spot consisted of a single molecule.

The maximum detection rate was calculated to be 10 kHz when we used 1 μW excitation at 543.5 nm. The fluorescence quantum yield of NR was assumed to be 0.5. The κ value was estimated to be 0.05 by considering the transmittance and numerical aperture of the objective lens, reflectivity and transmittance of optics, and the quantum efficiency of the photodetector. When we observed the fluorescence from single NR spots with 1 μW excitation for 10 ms period, the detected number of photons did not exceed 100 counts. Since the count rate did not exceed the maximum value obtainable from the single NR molecule, most of the observed spots were believed to be single fluorophores.

Connection between SMD and ensemble measurements

It can be expected that the result by SMD could be a unit of molecular optical property such as fluorescence intensity. If every single molecule results in the same fluorescence intensity, it is possible to estimate the number of fluorophores in the observation volume by measuring the fluorescence intensity and divide it with the unit intensity of each fluorophore. We have measured the fluorescence intensities of single molecules to test if single molecule can provide a primary unit of fluorescence intensity.

When we measured the single Cy3 fluorescence with unpolarized laser light excitation, a wide distribution of intensity histogram was observed as shown in Fig. 4. The intensity histogram obtained from 1849 Cy3 molecules on PMMA matrix could be fitted by a Gaussian distribution function with the mean value and standard deviation of 115 and 57, respectively. This result indicates that the signal intensity varies from 50 to 150% within m ± σ range, where m is the mean value and σ is the standard deviation. Such a large fluctuation in single molecule fluorescence intensities might come from the variation of absorption and emission

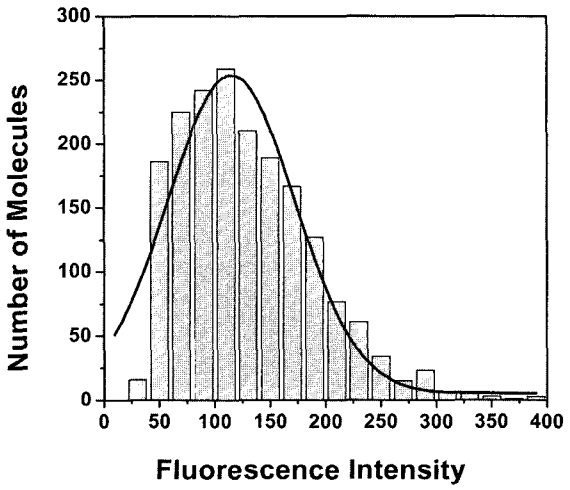

Figure 4. Fluorescence intensity histogram of 1849 single Cy3 molecules. The histogram could be fitted by using a Gaussian function with mean value and standard deviation of 115 and 57, respectively.

efficiencies of individual molecules.

The orientation of absorption dipole affects the transition probability of a molecule from its ground state to excited states. A molecule which has transition dipole orientation parallel to the light propagation would not absorb photons since the transition dipole and the electric field of light is orthogonal to each other. On the other hand, a molecule that has transition dipole moment perpendicular to the light propagation would absorb unpolarized light very efficiently. Thus the difference in the molecular orientation relative to the light propagation results in a large difference of molecular absorption and fluorescence intensity. Since Cy3 molecules on PMMA film are not easily diffusing or rotating, the random orientation of molecular transition dipole would be represented as the difference in fluorescence intensity.

It is also known that the absorption spectrum and fluorescence quantum yield of molecule depend on its environment [24]. Since we excite molecules with a fixed wavelength of laser light, the change in the absorption spectrum of each molecule due to its nanoenvironment influences the degree of light absorption. As a result, the fluorescence intensities of single molecules would largely differ depending on their molecular orientation and surrounding environment. The fluorescence intensities of single Cy3 molecules vary from 3.2 to 200% relative to the mean value within the range of 95% confidence interval. Accordingly, it is difficult to consider SMD result as a unit of molecular optical property due to large diversity in case that the molecules are not in free rotation or diffusion.

The fluorescence intensities of single Cy3 molecules were read from spot image in the direction of left to right and top to bottom. Since the reading procedure has no correlation with the fluorescence intensity, we could assume that the sequence of data is random. We tried to deduce a minimum number of required molecules that should be involved to show a uniform average intensity. When we average the intensities of a number of samples, the mean values would become uniform as the number of samples increase. The mean and standard deviation of sample means from a population is given by,

$$E(\bar{X}) = m$$
$$\sigma(\bar{X}) = \sigma/\sqrt{n} \quad (4).$$

Here, X, $E(\bar{X})$ and m are the sample mean, mean of the sample means and the population mean, respectively. $\sigma(\bar{X})$ and σ are the standard deviations of sample means and population, respectively. n is the number of samples extracted from the population. Based on Eqn. (4), we estimated the minimum number of samples averaged that are required for the sample mean to be within ±10% of the population mean. When we measured the intensity values of 1849 samples, σ/m was calculated to be 0.5. Since the intensity histogram could be well fitted by Gaussian function, it might be considered to follow the normal distribution function. In case of normal distribution, the σ/m value should be less than 0.051 for the

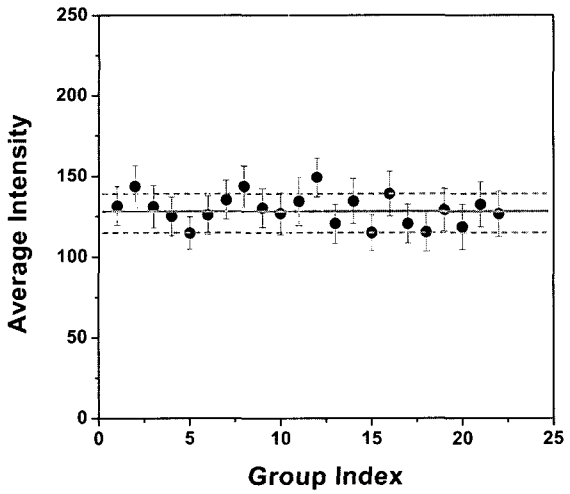

Figure 5. The plot of sample means for 22 different groups with 83 samples. The error bars represent one standard deviation of the sample values in each group. The solid line represents the total mean of 1849 molecules, and the dashed lines represent ±10% error range from the total mean.

sample means to be within the range of $m \pm 0.1m$ with 95% confidence.

From the above consideration and the σ/m value of 0.5, the minimum number of samples required was deduced to be 83. The average value of fluorescence intensities of 83 spots falls within ±10% of the total mean. Figure 5 shows the plot of sample means for 22 different groups with 83 samples. As shown in Fig. 5, the sample means were confined within 10% of the total mean with 95% confidence. The simple statistical method could be used to determine the minimum number of single molecules required to form a group of uniform average within a selected error range. This method can be used to determine the minimum required sample number in observing optical properties of molecules such as emission spectra, fluorescence quantum yield, excited state lifetime, and the total number of photons emitted before the molecule is bleached.

CONCLUSION

We have fabricated a laser scanning fluorescence microscope system that has sensitivity down to single fluorescent molecule detection. Adequate solutions were suggested for several problems associated with SMD system such as nanoscale positioning with sample stage, ground vibration, focused beam diameter reduction, and sample preparation for single molecule detection and confirmation. We also deduced a statistical method to estimate the minimum number of single molecules required to form a group of uniform average in a selected error range. The SMD results would converge to the result of ensemble measurement as the number of molecules considered increases. The above statistical method provides a connection between ensemble and SMD results by determining the number of minimum required single molecules to be a representative group.

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