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Assignment of the Number of Equivalent Reference Fluorophores to Dyed Microspheres

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A procedure will be described to assign to each dyed microsphere a number called the Equivalent number of Reference Fluorophores (ERF). The ERF unit gives the number of reference fluorophores in solution which produce the same fluorescence signal as a single dyed microsphere. In the first step, fluorescence measurements were carried out on serial dilutions of a solution of reference fluorophores. The resulting fluorescence intensities and the corresponding concentrations were used to calibrate the response of the fluorometer. The calibration consisted of establishing a linear relation between the intensities and concentrations. In the second step, the fluorescence intensity from a suspension of microspheres was measured in order to determine the equivalent concentration of reference fluorophores which gave the same fluorescence intensity as the suspension of microspheres. This was performed by utilizing the calibration line obtained in the first step. In the third step, a flow cytometer and a light obscuration apparatus were used to measure the total concentration of microspheres in the suspensions used for the fluorescence measurements. In addition to the total microsphere concentration, the flow cytometer also enabled the measurement of the concentration of a sub population of microspheres which are used to calibrate the fluorescence scale of a flow cytometer. The fourth step utilized the data collected in steps one, two, and three to assign a value of ERF to individual microspheres. The set of microspheres with assigned ERF values will be used to establish a linear fluorescence scale in each channel of a flow cytometer. The discussion will emphasize the estimate of uncertainties in each step of the assignment process.

Key words: ERF; flow cytometer; calibration; microspheres; concentration; fluorescence.

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1. Introduction

Microspheres with assigned number of equivalent reference fluorophores (ERF) are used to calibrate the fluorescence response of flow cytometers used for clinical diagnostics [1]. To insure comparability, it is important that the manufacturers of microspheres assign ERF values using a well-defined protocol. This manuscript describes a protocol which has been vetted by the flow cytometry community and has been implemented at National Institute of Standards and Technology (NIST). The assignment is performed using a fluorometer whose fluorescence response was calibrated by measuring the fluorescence intensity from a set of serial dilutions of a solution of reference fluorophores with a known concentration. The calibrated fluorometer was used to convert the fluorescence intensity from a suspension with microsphere concentration $N_{microspheres}$ (1/mL), to an equivalent concentration of reference fluorophores, $C_{equivalent}$ (mol/L), which yielded the same fluorescence intensity as the suspension. The ratio of these two measured values yielded the value of the equivalent number of reference fluorophores for a single microsphere (ERF) as shown in Eq. (1).

$$ERF = \frac{6.0221*10^{23}}{1000}\frac{C_{equivalent}}{N_{microspheres}} \quad (1)$$

The total uncertainty in ERF is due to uncertainties in the values of $C_{equivalent}$ and $N_{microspheres}$ both of which will be discussed in the following.

2. Measurement of the Fluorescence Intensity and its Uncertainty

Fluorescence emission spectra from the reference solutions were measured over a wide range of fluorophore concentrations. Fluorescence intensity was defined as the sum of the signals over all of the wavelengths in the spectrum. A typical spectrum contained over 4000 different wavelengths separated by about 0.07 nm. The maximum signal at each wavelength was about 60,000 DU (digital units). To cover the wide range of fluorophore concentrations, and concomitant signals greater than the dynamic range of the instrument response, it was necessary to vary the laser intensity (with neutral density filter) and CCD acquisition time (mechanical shutter). At lower fluorophore concentrations, it was necessary to subtract the solvent Raman signal. Finally, it was necessary to perform a correction for the relative spectral response of the CCD detector. The steps needed to acquire a fluorescence spectrum and obtain the fluorescence intensity are discussed below with emphasis on the estimate of uncertainty.

2.1 Subtraction of Buffer Signal

The fluorescence intensity in a given pixel, FI_s, was found by subtracting the intensity from the pure buffer, BI, from the measured intensity, FI, of the solution containing the fluorophore. The subtraction was carried out according to Eq. (2).

$$FI_s = FI*\frac{10^{ND}}{T} - BI*\frac{10^{ND_b}}{T_b} \quad (2)$$

The symbols ND and T give respectively the optical density of the neutral density filter and the collection time of the CCD detector used in the acquisition of the emission spectrum. The symbols ND_b and T_b are the analogous quantities used in the acquisition of the buffer spectrum. Usually ND_b was 0 since the full laser power was needed to obtain a good response from the buffer. The values of T and T_b ranged between 0.5 s to 2 s. Division by the collection time normalized all data to a common collection time of 1 s. Multiplication by 10^{ND} adjusted the data to that obtained with full laser intensity. The measurements of the fluorescence intensity from dilutions of the reference fluorophore solutions were always performed from the low to high concentrations. The procedure summarized in Eq. (2) permitted the acquisition of fluorescence intensity over a dynamical range of 10^4 while keeping the CCD detected signal between 5,000 to 60,000 digital units (DU). (The instrument dynamic range was 1000 DU to 64000 DU). The quantity FI_s was obtained from two different measurements. Assuming that each measurement was a Poisson process, the uncertainty in the value of FI_s at each pixel is given by Eq. (3).

$$\sigma_{FI_s} = \sqrt{\left(\sqrt{FI}*\frac{10^{ND}}{T}\right)^2 + \left(\sqrt{BI}*\frac{10^{ND_b}}{T_b}\right)^2} \quad (3)$$

It was assumed in Eq. (3) that the values of the optical density of the neutral density filter and the collection time of the CCD detector were exact. Any deviation in the value of these quantities from the true value will be considered as a systematic uncertainty.

2.2 Relative Spectral Response Correction

Subsequent to the subtraction of the buffer signal, the digital number obtained from a pixel of the CCD detector will be called the measured fluorescence intensity FI_s. The wavelength dependence of the measured fluorescence intensity may be different from the wavelength dependence of the intensity of fluorescence, *F*, emitted by the fluorophores in the illuminated solution. The relation between the two fluorescence intensities is given in Eq. (4).

$$FI_s = G\Gamma F \tag{4}$$

The symbol G is the detection gain of the CCD pixel associated with the wavelength λ. The symbol Γ represents the monochromator throughput that can be understood as follows [2]. For a properly designed system, the collection lens situated in front of the entrance slit of the monochromator, images the monochromator entrance slit inside the sample cuvette. Any photon originating from a fluorophore located in the slit image, and emitted into the angle subtended by the collection lens will arrive at the CCD detector. There will be a wavelength dependence of the throughput but it will be negligible. In practice, the throughput is usually considered to be a constant. It is difficult to measure the absolute values of the throughput and the CCD gain. A practical approach utilizes a calibrated reference lamp to determine a relative detection gain. The aperture of the reference lamp unit was placed at the location of the sample. The relation between the measured lamp intensity LI and the portion of the lamp calibrated radiance L entering the monochromator is given by a relation similar to Eq. (4) and written below as Eq. (5).

$$LI = G\Gamma L \tag{5}$$

Clearly the product of gain and throughput can be estimated by the ratio of lamp intensities as written in Eq. (6),

$$\frac{LI}{L} = G\Gamma. \tag{6}$$

It is difficult to estimate L since the reference lamp has a variable aperture to control its intensity and the diameter of the aperture is not calibrated. A practical approach is to divide both sides of Eq. (6) by respective values at some chosen wavelength, for example 490 nm. Then it is possible to form the relative response correction factor, called c, given in Eq. (7) [3].

$$\frac{LI_{490}}{L_{490}}\frac{L}{LI} = \frac{G_{490}\Gamma_{490}}{G\Gamma} = c \tag{7}$$

The uncertainty in *c* arises mainly from the measured lamp intensity. The calibrated radiance values are usually given by a parameterized function while the uncertainty in ratio at 490 nm will be assumed to be negligible. With these assumptions, the uncertainty in c can be written as $\sigma_c = c/\sqrt{LI}$. Multiplying Eq. (4) by c gives a measured fluorescence intensity corrected for relative spectral response as shown in Eq. (8).

$$FI_{cor} \equiv c * FI_s = G_{490}\Gamma_{490}F \tag{8}$$

Equation (8) states that the corrected fluorescence intensity, FI_{cor} is proportionate to the true fluorescence intensity emitted by the fluorophore solution. In practice the normalization wavelength is chosen at the peak of the emission spectrum so that the relative spectral response correction mainly changes only those values at wavelengths at the sides of the peak. The same normalization wavelength was used for the spectrometer calibration and the microsphere measurement. This ensured that the microsphere fluorescence intensity was represented correctly in terms of the concentration of reference fluorophores.

2.3 Calculation of the Uncertainties of the Total Fluorescence Intensity

The solid trace and the dashed trace in Figure 1a show the collected spectrum from a Coumarin 30 (C30) reference solution and the calibrated reference lamp (X10) respectively. In both cases, the extended spectrum from 390 nm to 700 nm was obtained by joining five partial spectra collected at five different settings of the monochromator grating angle. Each partial spectrum consisted of 1024 pixels spanning a wavelength range of approximately 70 nm resulting in about 10 nm overlap between adjacent partial spectra. The joining of the partial spectra was performed by an algorithm contained in the data acquisition software that came with the spectrometer. The calibrated lamp spectrum was used to correct for joining artifacts and for the relative spectral response of the detector. The Coumarin30 spectrum shown in Fig. 1a was multiplied by the correction factor resulting in the final spectrum shown in Fig. 1b. The value of the normalization wavelength was chosen near the maximum of the emission spectrum. The sum over a specified range of wavelengths in Fig. 1b was defined as the total fluorescence intensity.

Let FI_{cor} and FI_s represent the corrected and measured fluorescence intensities respectively related by the equation $FI_{cor} = FI_s \times c$ where c is the correction factor given by Eq. (7). The final fluorescence intensity and the uncertainty in the final fluorescence intensity are given by Eqs. (9a) and (9b), respectively.

$$FI_{cor} = \sum_i FI_{cor,i} \tag{9a}$$

$$\sigma_{FI_{cor}} = \sqrt{\sum_i FI_{cor,i}^2 * \left(\frac{1}{LI_i} + \frac{\sigma_{FI_{s,i}}^2}{FI_{s,i}^{\ 2}} \right)} \tag{9b}$$

Here the index i runs over all pixels included in the calculation of the fluorescence intensity. The number of photons arriving at a given pixel is a Poisson process. However, there are several corrections that have to be made to translate the number of photons into a fluorescence intensity. Therefore, we do not consider the sum of pixel intensities as a compound Poisson process with the uncertainty given simply by the square root of the value of the sum. Rather, as shown in Eq. (9b), the uncertainty in the sum of intensities from all pixels was obtained from the sum of uncertainties associated with each pixel. This procedure resulted in a significantly larger uncertainty than the uncertainty obtained from the square root of the sum in Eq. (9a).

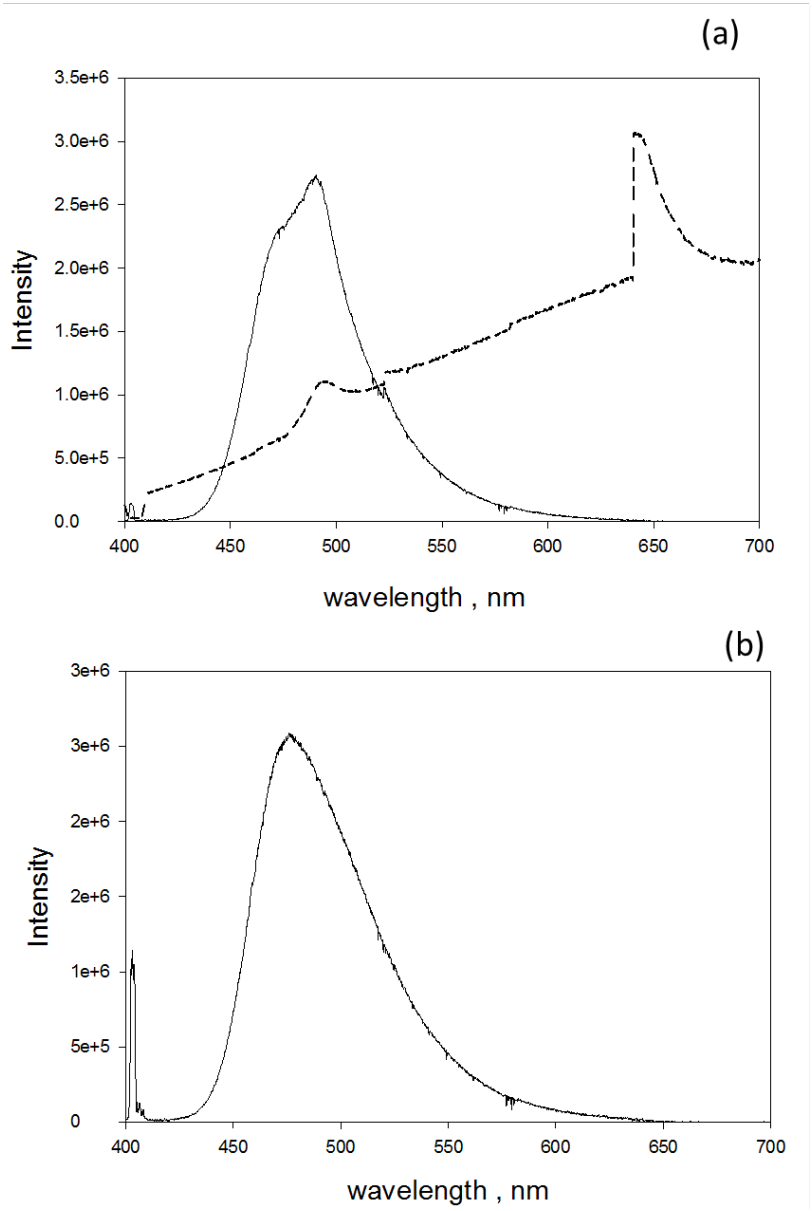

Fig. 1. (a) The solid trace shows the fluorescence emission spectrum from a solution of Coumarin 30 (C30) in isopropanol. The excitation wavelength was 405 nm. The spectrum was prepared according to Sec. 2 in the text. The dashed trace shows the spectrum of the reference lamp scaled to fit on the plot. The lamp spectrum was used in Eq. (7) to form the relative spectral correction, c. (b) The solid trace shows the C30 spectrum after it was multiplied by the correction factor c. The sharp feature at 405 nm is due to leakage of the excitation light.

3. Calibration of Fluorometer

The equivalent concentration of reference fluorophores, which will be called simply C, was determined from the calibration curve given by Eq. (10).

$$\log(FI_{ref}) = a + b * \log\left(C_{ref}\right) \tag{10}$$

FI_{ref} and C_{ref} are the fluorescence intensity and concentration of the reference fluorophore solutions used in drawing the calibration line. The fluorescence intensity of the fluorophore solutions was obtained using the procedure leading up to Eq. (9a). Figure 2 shows the calibration points for serial dilutions of Coumarin 30 in isopropanol. The uncertainties in the calibration line parameters *a* and *b* were determined from Excel® fitting algorithm LINEST[1] [4]. Appendix A discusses the dependence of the fit parameters *a* and *b* on measurement conditions. Clearly, it is very important to monitor the laser light intensity. A good quality power meter is essential to keep track of the laser power. The optical density of the neutral density filters needs to be measured accurately. The polarization of the laser light should be kept constant.

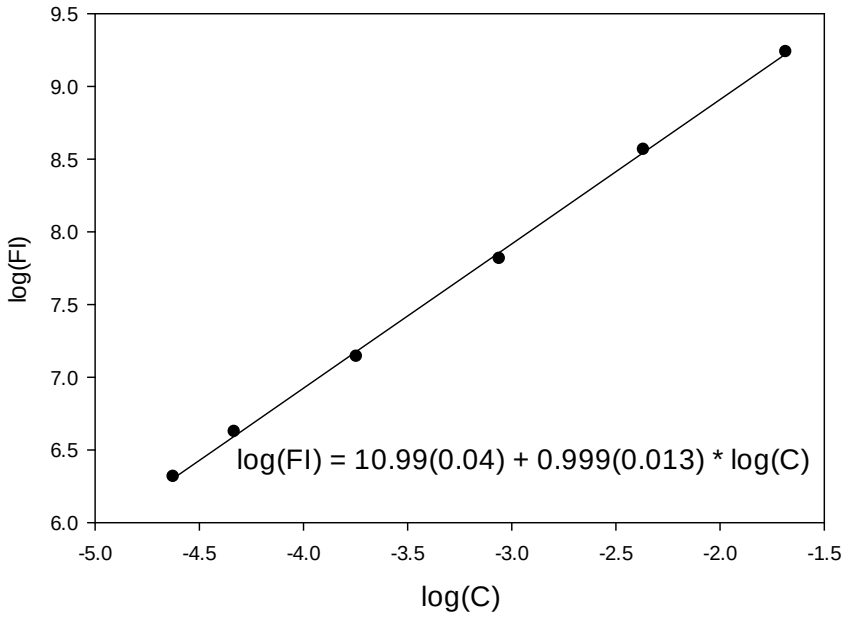

Fig. 2. The solid circles show a plot of the log of the measured fluorescence intensities of dilutions of reference C30 solution versus the log of the concentration of C30 in the respective solutions. The straight line shows the best linear fit to the solid circles. The slope of 0.999±0.013 indicates that the fluorescence intensity is proportionate to the concentration of C30.

4. Measurement of Microsphere Fluorescence Intensity

In the following, the different microsphere populations will be designated by the letters URB (SPHERO™ Ultra Rainbow Beads) followed by an integer. The value of the integer in the designation refers to the dye loading of that microsphere population. Thus URB1 refers to the microsphere population with no extrinsic dye (also called "blank"), and URB2 and URB5 refer to populations with lowest and highest dye loading respectively. In the flow cytometry literature, the URB microspheres are sometimes designated as URB peak #2, URB peak #5, etc. This later designation originates from the fact that when the URB microspheres are passed through a flow cytometer the different URB populations produce distinct peaks in the fluorescence intensity distribution plot. Increased dye loading of the microsphere results in a corresponding higher peak position.

Figure 3a shows the spectra of blank microspheres, bottom trace, and the URB2 microspheres, top trace. The water Raman band at 470 nm was the major background. The two spectra were adjusted for excitation power and

[1] Certain commercial equipment, instruments, or materials are identified in this paper in order to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.

integration time and the difference spectrum is shown in Fig. 3b. The water Raman line is subtracted out. Corrections were made for relative spectral response and splicing artifacts in the same way as described for the reference solutions. The spectrum in Fig. 3b was summed over a specified wavelength range, and the result was the microsphere fluorescence intensity. This integrated microsphere fluorescence was inserted into the calibration plot shown in Fig. 2 to obtain the equivalent concentration of reference C30 fluorophores which gave the same *FI* as the microspheres. The wavelength range which defines the fluorescence intensity of the microspheres can be changed to accommodate the filters used to define specific fluorescence channels in a flow cytometer. Of special interest are the start and stop index values in Eq. (9a), and normalization wavelength in Eq. (7). The value of the normalization wavelength has significant effect on the magnitude of the fluorescence intensity value and should be the same for the reference fluorophore and microsphere measurements. The start and stop indexes can be different for the reference solutions and the microsphere suspension.

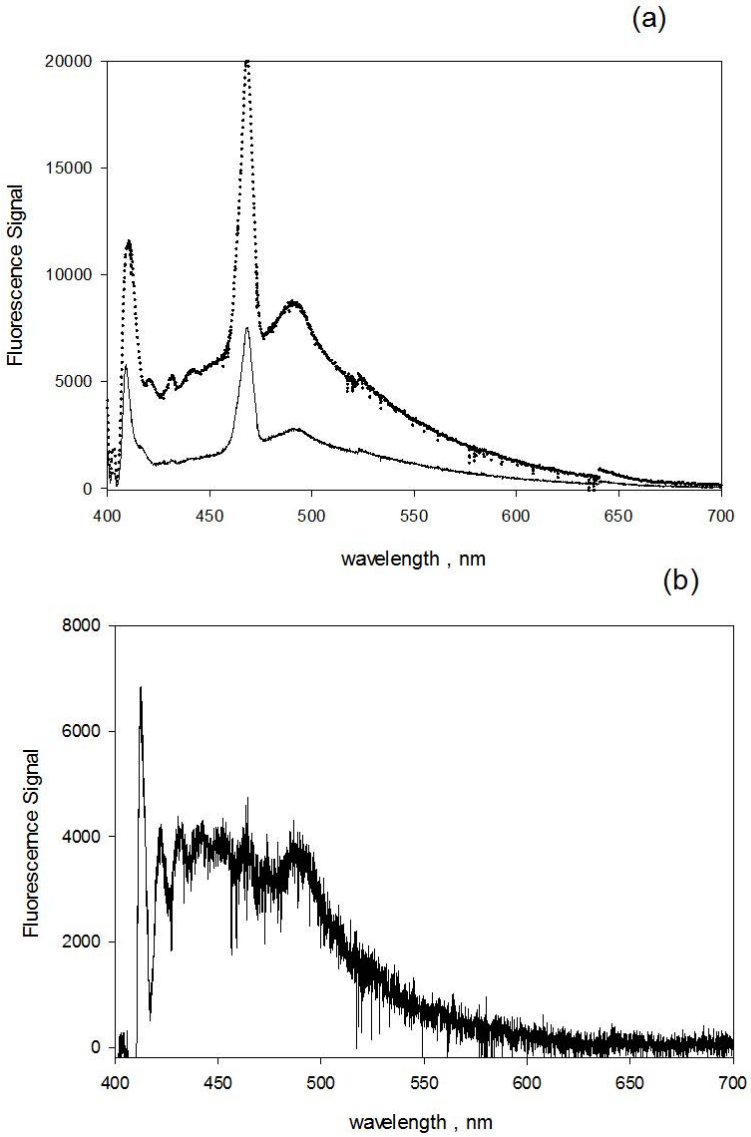

Fig. 3. (a) The bottom trace shows the emission spectrum from blank SPHERO™ Ultra Rainbow URB1. The top trace shows the emission from the dimmest microspheres, SPHERO™ Ultra Rainbow URB2. The large feature at 470 nm is the water Raman band. The collection time was 0.2 s for URB1 and 0.5 s for URB2. (b) The fluorescence emission spectrum of URB2 after subtraction of the "blank" spectrum and correction for relative spectral response.

5. Determining Equivalent Concentration and its Uncertainty

Given the calibration line, the log of the equivalent concentration, *C*, can be found from the measured microsphere fluorescence intensity, *F*, by inverting Eq. (10) as shown in Eq. (11).

$$\log(C) = \frac{\log(F) - a}{b} \tag{11}$$

Therefore, the equivalent concentration is given by $C = 10^{\log(C)} * 10^{-6}\ , mol / L$. The factor of 10^{-6} arises because all of the concentrations in the dilution calculations were in units of µmol/L. The uncertainties in the calibration fit parameters, *siga* and *sigb*, were obtained from the fitting algorithm. With the uncertainties in *a*, *b*, and *F* we can get the uncertainty in C as shown in Eq. (12) [5]

$$sigC = 2.303 * 10^{\log(C)} * siglogC * 10^{-6} \tag{12}$$

where

$$siglogC = \left|\log(C)\right| * \left(\frac{siglogF^2 + siga^2}{\left(\log(F) - a\right)^2} + \frac{sigb^2}{b^2} \right)^{1/2}$$

$$siglogF = 0.434 * \frac{sigF}{F}.$$

6. Measurement of Microsphere Concentration

The microsphere concentration was measured using two different methods, flow cytometry, described in Sec. 6.1, and light obscuration (LO), described in Sec. 6.2. The measurement of microsphere concentration using a flow cytometer was described previously [6]. This method yields an estimate of the total microsphere concentration as well as the concentration of the major microsphere population, which is identified by a scattering pattern in the FSC-SSC (forward scattering channel-side scattering channel) dot plot, and characteristic signal pattern in the relevant fluorescence channels. The LO technique detects the reduction of signal caused by the passage of a microsphere between the light source and a detector. The LO technique is complementary to the technique which detects light scattering. In the following discussion, each technique is described in the context of measurements meant to validate the technique. In the case of ERF assignments, a critical condition was that both techniques give the same concentration for the same microsphere suspension. The concentration from flow cytometer measurement was used in the assignment of ERF; the LO measurement served the critical role of verifying the flow cytometer result.

6.1 Measurement of Microsphere Concentration with a Flow cytometer

The flow cytometer measures the microsphere concentration by detecting scattered light and fluorescence emission from microspheres passing through a laser beam. Figure 4 shows the worksheet produced by the Attune flow cytometer software for a typical run of a mixture of Trucount™ (TC) and SPHERO™ Ultra Rainbow microsphere URB4 in PBS+BSA buffer. The sample was made by putting in 0.0997 g of URB4 stock suspension and 1.9713 g of buffer directly into the TC tube that contained the lyophilized reference

microspheres. The gates in Fig. 4 were designed to emphasize fluorescence and obtain the total concentration of all fluorescing microspheres. The total concentration was used in comparison with fluorometer measurements (where all fluorescence is included), as well as comparison with concentration measurements with light obscuration particle counter, which counted all microspheres. The table in Fig. 4 is a compilation of all events contained in various gates. The names of the gates are given in the leftmost column of the table and arranged in hierarchical order. For example, the row labeled R1 shows the events that occurred in gate R1 in the FSC-SSC dot plot with the further condition that the events were also contained in gate R4 in the VL2-VL3 dot plot. Gate R4 included all TC microspheres. Similarly, the row labeled R6 shows the events that occurred in gate R6 in the FSC-SSC dot plot with the further condition that the events were also contained in gate R5 in the VL2-VL3 dot plot. Gate R5 included all URB4 microspheres that fluoresced. Choosing gates R2 and R3 in the BL1-BL2 dot plot with blue laser excitation instead of R4 and R5 (violet laser excitation) gave almost identical results. The ratio of events in R5 and R4 or the ratio in R3 and R2 or the ratio in R6 and R1 could be used to calculate the concentration of URB4 microspheres and associated uncertainty of the measured concentration of URB4. The concentration of TC in the tube was obtained from the known number of TC microspheres and the volume of URB4 suspension and PBS+BSA buffer added. The uncertainty of the microsphere concentration was obtained from the standard deviation of 3 or more repeated measurements. The concentration obtained using the data in Fig. 4 compared favorably with the concentrations determined with the Light Obscuration method.

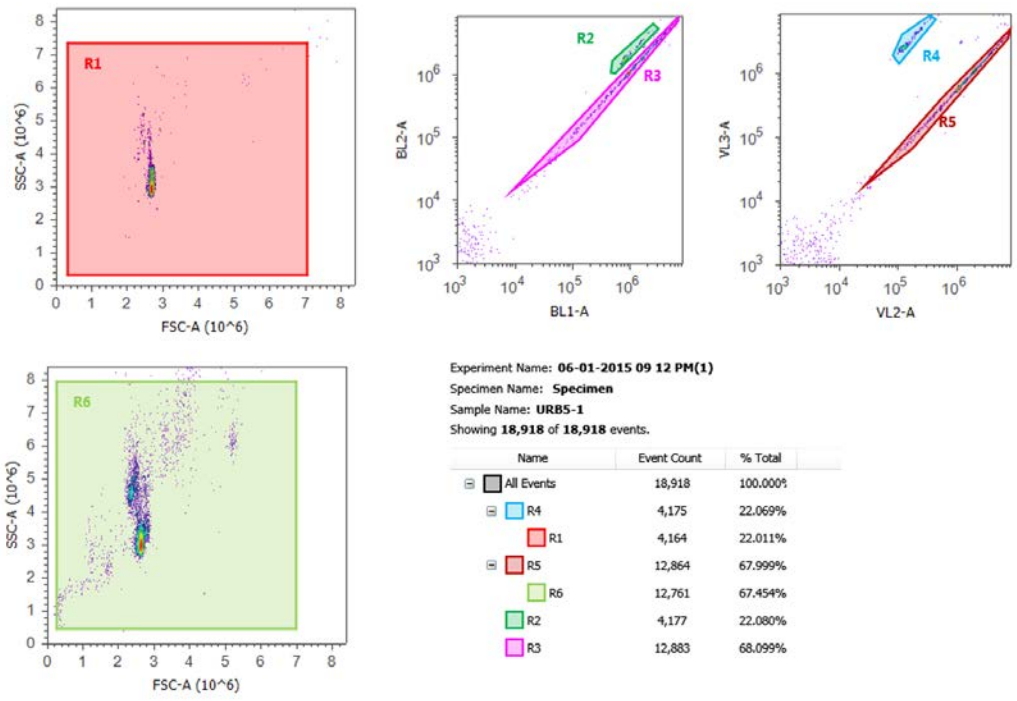

Fig. 4. Worksheet produced by the Attune™ flow cytometer software. Gates R2 and R4 show the population of Trucount™ microspheres identified by fluorescence emission excited by blue (488 nm) and violet (405 nm) lasers. Gates R3 and R5 show the population of URB5 microspheres identified by fluorescence emission excited by blue and violet lasers. The number of events in these gates, shown in the Table at the bottom, was used to determine the concentration of URB5 microspheres relative to the known concentration of TC microspheres. The upper left scattering plot shows scattering events which were also in the R2 gate. Similarly, the lower left plot shows the scattering events which were also in the R3 gate. The characteristic distribution of events in the two scattering plots was used to confirm the occurrence of TC or URB5 events.

6.2 Measurement of Microsphere Concentration with Light Obscuration Instrument

The Light Obscuration (LO) instrument was a PAMAS model SVSS-C with a HCB-LD-25/25 sensor head, S/N U32757. Deionized, UV-light sterilized and filtered water was used as a blank to measure the background of the instrument. A background of less than 50 mL^{-1} measured over the instrument's size range (1.0 μm to 200 μm) was achieved by flowing the buffer prior to the measurement of the microsphere samples. The performance of the instrument was verified by measuring the size and concentration of Thermo Count-Cal 5 μm microspheres. The measurement of a narrow distribution of sizes (4.6 μm to 5.8 μm) centered at 5 μm, and a concentration within 10% of the manufacturer's specification of 3000 mL^{-1} indicated that the instrument was operating properly. The diameter of the Count-Cal beads is NIST traceable, but the concentration is not. Each sample was shaken or vortexed for 10 seconds, then sonicated for 15 seconds, and then gently stirred by tipping the sealed container just before a set of nine measurements were performed. Instrument parameters that did not change for all runs are pre-run volume = 0.6 mL, measuring volume = 0.4 mL, rinse volume = 0.6 mL, fill rate = 10 mL/minute, empty rate = 200 mL/minute, and rinse rate = 10 mL/minute.

A suspension of TruCount™ microspheres was prepared by adding 3.5 g of ISOTON II® gravimetrically to the TruCount™ test tube, which contained 50,650 microspheres as certified by the manufacturer. This number has been confirmed by measurements taken at PTB on their quantitative flow cytometer, which has been qualified for high accuracy concentration measurements. The test tube was then vortexed for 15 seconds and immediately transferred to a 30 mL PETG (polyethylene terephthalate copolyester, glycol modified) Nalgene bottle and diluted gravimetrically with ISOTON II® to the 10 mL graduation on the bottle. This procedure produced a concentration of approximately 5000 microspheres / mL, which is in the linear concentration range for this instrument.

Suspensions of URB1, URB2 and URB5 were prepared by adding 10 mL of PBS with 0.02% Tween™20 to a 30 mL PETG Nalgene bottle and then adding 40 μL of the corresponding URB stock solution. Dilution factors were calculated gravimetrically by measuring the mass of the bottle before and after each addition. This procedure produced concentrations of approximately 4000 microspheres / mL, which is in the linear concentration range for this instrument.

The LO instrument uses a 1mL syringe with a piston pump to measure and deliver the amount of sample into the system. A "rinse volume" of 0.6 mL was flushed through the system before three consecutive sample volumes of 0.4 mL each were measured. This data collection procedure was repeated three more time for each sample, giving a total of 12 data points for each sample. The average concentration and standard deviation were calculated and multiplied by the dilution factor to give the concentration and statistical uncertainty for the corresponding stock solution. The number of microspheres per mL was measured for a size range from 2.3 μm to 6.5 μm. Note that under optimum conditions, the concentrations of background "particles" in this size range observed for deionized (DI) water and PBS solvent are < 5 mL^{-1} and < 50 mL^{-1}, respectively.

Validation of LO determination of microsphere concentration included gravimetric calibration of the delivered volume, dependence on microsphere concentration to determine linear range (or correct for coincidence), sampling error due to microsphere adsorption, and pump volume dependence to determine timing error. Calibration of the delivered volume by the piston pump was done by measuring the mass of a 25 mL container of DI water before and after nominal 0.4 mL aliquots of the water were removed from the container by the pump. Note that 0.01 % sodium dodecyl sulfate (SDS) was added to the water to reduce surface tension and 1 mL of vacuum pump oil was added to cover the surface of the water to inhibit water evaporation.

The linear concentration range of the instrument was determined for microspheres in aqueous suspension. Suspensions with concentrations less than 6000 mL^{-1} were found to behave linearly with concentration. Negative bias was observed at concentration of 8000 mL^{-1} or greater, probably due to coincidence error, i.e., multiple microspheres passing through the detection region simultaneously. Note that this effect was diminished by using ISOTON II® as a solvent and this effect will be explored more in

the future. The optimal concentration range for aqueous suspensions was determined to be 2000 mL^{-1} to 5000 mL^{-1}. The measured microsphere concentration was found to be independent of the measured sample volume in the range from 0.2 mL to 1.0 mL.

7. Assignment of ERF

The values of ERF were obtained from Eq. (1). The uncertainty in ERF, called *sigERF*, was obtained by applying error propagation rules to Eq. (1). The result is shown in Eq. (13) below.

$$sigERF = ERF * \left(\frac{sigC^2}{C^2} + \frac{sigN^2}{N^2} \right)^{1/2} \tag{13}$$

The uncertainty sigC was obtained from Eq. (12). Section 8 below presents an example of the ERF assignment procedure for two populations of microspheres. The results are shown in Table 4.

8. Assignment of ERF Value to Ultra Rainbow Microspheres, URB2 and URB5

In the following, the procedures described above will be applied to assign a value of ERF to Ultra Rainbow microsphere URB2 and URB5 in terms of Coumarin 30 reference fluorophores. The URB microspheres were suspended in phosphate buffer saline (PBS), pH 7.4 with 0.02% (w/w) Tween™20, while the C30 reference solution was diluted in isopropanol. The reference solution of C30 in acetonitrile (ACN) is certified to have a concentration of 130.5 ± 1.7 µmol/kg. However, fluorometers measure fluorescence emitted from a fixed sensing volume, and the fluorescence intensity is proportionate to the total number of fluorophores given by the product of the sensing volume and the fluorophore concentration in units of µmol/L. Therefore, the certified concentration has to be multiplied by the density of the solvent to convert the concentration to units of µmol/L. The density of ACN is 0.7801±0.0003 kg/L at 22 °C as given in NIST/TRC Web Thermo Tables [7]. Multiplying the certified concentration by the ACN density yields a C30 concentration of 101.8 ±1.3 µmol/L. The fluorometer was calibrated using a number of serial dilutions of the reference C30 solution. The diluent was isopropanol instead of ACN. It was found that at low concentrations the fluorescence intensity of C30 depended non-linearly on the concentration of C30 when the diluent was ACN. The density of isopropanol is 0.7838 kg/L at 22 °C. This value differs from the density of ACN by less the 0.5%, therefore we did not consider the small amount of ACN included in the first dilution of the reference solution. Table 1 gives the results of the dilutions obtained by weighing the reference solutions and the diluent isopropanol. The mass shown in the second column refers to the mass of solution in the row above. The uncertainties in the mass were obtained from the standard deviation of five consecutive readings of the scale.

Table 1. Serial dilutions of C30 reference solution.

Identifier	Solution mass, g	Error g	Isopropanol mass, g	Error g	Concentration µmol/L	Error µmol/L
SRM 1934					101.8	1.3
1	0.03763	0.00001	3.77835	0.00001	1.004	0.012
2	0.10101	0	4.70682	0.00001	0.02109	0.00027
3	0.78881	0.00001	3.01438	0.00001	4.374e-03	0.056e-03
4	0.76759	0	3.01045	0.00001	88.87e-05	1.13e-05
5	0.78207	0.00001	3.01883	0	18.29e-05	0.23e-05
6	1.18410	0.00001	3.38672	0.00001	4.737e-05	0.061e-05
7	1.56967	0	1.50504	0.00001	2.418e-05	0.031e-05

The details of the dilution measurements are shown in the Excel® file "ERF Assignment" in the Supplemental Materials folder.

Next, the fluorescence intensity of the diluted reference solutions was measured following the steps outlined in Sec. 2. Table 2 gives the compilation of the results. Solution 1 was an intermediate dilution and its fluorescence intensity was not measured. The solution concentrations shown in column 2 and 3 in Table 2 are reproduced from Table 1. The normalization wavelength in Eq. (8) was set to 465 nm and the start and stop wavelengths were 440 nm and 650 nm respectively.

Table 2. Measured fluorescence intensity of reference solutions.

Identifier	Concentration µmol/L	Uncertainty µmol/L	Fluorescence Intensity DU	Uncertainty DU
2	0.02109	0.00027	2.103e09	8.65e05
3	4.374e-03	0.056e-03	4.483e08	1.55e05
4	88.87e-05	1.13e-05	7.993e07	2.54e04
5	18.29e-05	0.23e-05	1.640e07	6.96e03
6	4.737e-05	0.061e-05	4.966e06	4.23e03
7	2.418e-05	0.031e-05	2.404e06	3.73e03

The straight line was fitted to the values of $\log(FI_{ref})$ versus the values of $\log(C_{ref})$. The resulting straight line fit is shown in Fig 2. The numbers in parentheses in the fitting equation are the uncertainties of the fit parameters. The slope of the line is within error of the value 1, the expected result if the fluorescence intensity is proportionate to the concentration. Using Eq. (11), the linear relationship was used to find the concentration of reference fluorophores that gave the same FI as the microsphere suspension.

Next we measured the fluorescence intensity and concentration of URB microsphere suspensions. The microsphere vials shipped by the manufacturer have a nominal microsphere concentration of about 10^7/mL. Using a pipette, a dilution of 10 was performed by putting 1 mL of the stock microsphere suspension into 9 mL of PBS, pH 7.4 with 0.02 % w/w Tween™20. The diluted solution was appropriate for the measurement of fluorescence intensity. Typical fluorescence emission spectrum of URB2 excited with 405 nm laser light is shown in Fig. 3.

In order to measure the microsphere concentration with a flow cytometer, the diluted suspension used for the fluorescence intensity measurement was further diluted by a factor of 20. The second dilution was performed gravimetrically in order to get an accurate dilution factor. The concentrations of the suspensions used for fluorescence measurements are shown in the second column of Table 3. They were obtained from flow cytometer worksheets such as shown in Fig. 4 and the second dilution factor. The details of the concentration measurements are shown in the Excel® file "URB concentrations" in the Supplemental Materials folder. The fluorescence intensities were measured under the identical fluorometer conditions used for the diluted reference solution measurements. The fluorescence emission spectrum of URB5 was similar in shape to that shown in Fig. 3 but had less noise. The concentrations of the URB2 and URB5 microsphere suspensions and the corresponding fluorescence intensities are shown in the second and fourth column of Table 3, respectively.

Table 3. Concentration and fluorescence intensity of URB microsphere suspensions.

Identifier	Concentration #/mL	Uncertainty #/mL	Fluorescence Intensity, DU	Uncertainty DU
URB2	1.034e06	0.015e06	4.420e06	1.2e04
URB5	1.046e06	0.011e06	2.279e08	8.1e04

The data presented in Tables 2 and 3 were used in Eq. (1) to calculate the values of ERF for URB2 and URB5 microspheres. The detailed procedure for the calculation was given in Sec. 7, and the details of the

calculations are given in the Excel® file "ERF Assignment" in the Supplemental Materials folder. The final results are shown in Table 4.

Table 4. Assigned values of ERF.

Identifier	ERF Value	Uncertainty in ERF
URB2	2.64e04	4.3e03
URB5	1.35e06	1.7e05

The uncertainties in the ERF values are about 15%. The largest contribution to the uncertainties comes from the uncertainties of the calibration line parameters *a* and *b* as given in Eq. (12). If the uncertainties of the calibration parameters are set to 0, the ERF errors are reduced to about 2 %.

9. Calibration of a Flow Cytometer using URB Microspheres

The URB microspheres, with assigned values of ERF, will be used to construct a linear fluorescence intensity scale for all fluorescence channels of a flow cytometer. The currently available URB microspheres are polydisperse. As shown in the lower left FSC-SSC dot plot in Fig. 4, there is a major population with a reasonably well defined size, and a minor population with a more diffuse size distribution. The linear scale is based on the major population of microspheres as determined by placing an appropriate gate in the FSC-SSC dot plot. On the other hand, the measurements of fluorescence intensity used to assign ERF values were performed in fluorometers using suspensions of URB microspheres which included all populations of microspheres. It is therefore necessary to make a small adjustment to the values of ERF so that they reflect the true ERF values of the microspheres in the major population used to construct the linear scale. In the supporting information of a previous publication [8] it was stated that the ERF value of the major population could be written as a product of the assigned ERF value and a ratio of the mean fluorescence intensities (MFI) of the major and total populations respectively, as shown in Eq. (14).

$$ERF_{major} = ERF_{assigned} * MFI_{major} / MFI_{total} \quad (14)$$

Equation (14) was justified because the "ratio of ERF values describes the relative fluorescence response of different microsphere populations and is therefore identical to the ratio of the mean fluorescence intensities measured in the flow cytometer" [8]. The values of ERF_{major} obtained from Eq. (14) are appropriate for developing the linear fluorescence scale for a flow cytometer.

9.1 Experimental Validation of Equation (14)

To estimate the accuracy of Eq. (14), a vial containing about 3 mL of URB4 microspheres was spun down and the microspheres were resuspended in a PBS buffer with 0.02% Tween™20. The resuspended microspheres were sorted on the FACSAria flow cytometer with the requirement that the scattering signal from the sorted microspheres fell inside the region characterizing the major microsphere population. The sorted URB4 sample, with a 99% purity, had a volume of about 2.5 mL with a nominal microsphere concentration of $1*10^6$ /mL. The sorted microspheres and another vial of unsorted URB4 microspheres (called mixed in the following discussion) were used to prepare 20-fold dilutions of sorted and mixed populations of URB4 microspheres. In addition, a 20-fold dilution of "blank" microspheres (URB1) was also prepared. The "blank" microspheres are identical to the URB4 microspheres except that they are not loaded with dye. The Attune flow cytometer was used to measure the concentration of the three samples

relative to the Trucount™ standard. The Fluorolog® 3 fluorometer with laser excitation was used to measure the fluorescence spectra of the sorted and mixed suspensions.

Figures 5 and 6 show the Attune worksheets for the determination of the concentration of mixed and sorted samples of URB4 microspheres respectively. The R1(1) gate is much "cleaner" in Fig. 6 as expected for a sorted sample. The concentration of the "blank" sample was determined in a similar manner except that instead of the dye fluorescence, the microsphere auto fluorescence was used as the additional identifier of the microspheres. The fluorescence spectra of the mixed and sorted microsphere samples were taken with a Fluorolog® 3 spectrofluorometer equipped with 405 nm and 638 nm laser excitation. In both cases, the "blank" microsphere sample was used to subtract the background auto fluorescence and the water Raman line. The detector arm of the fluorometer was equipped with a notch filter to suppress scattered light. Additional scattered light was eliminated during the background subtraction using the "blank" microsphere spectra. The "ERF" values were estimated by dividing the integrated fluorescence intensity by the respective microsphere concentrations. The quotation marks around ERF indicate that the fluorescence intensity units are arbitrary and not related to a concentration of reference fluorophores. Equation (14) was rewritten as Eq. (15) to reflect the relation between the mixed and sorted results. The left side of Eq. (15) gives the "ERF" value of the major population for the sorted sample. The right hand of Eq. (15) gives the corrected "ERF" value for the mixed sample.

$$\frac{FI_{sorted}}{C_{sorted}} = \frac{MFI_{major}}{MFI_{mixed}} * \frac{FI_{mixed}}{C_{mixed}} \tag{15}$$

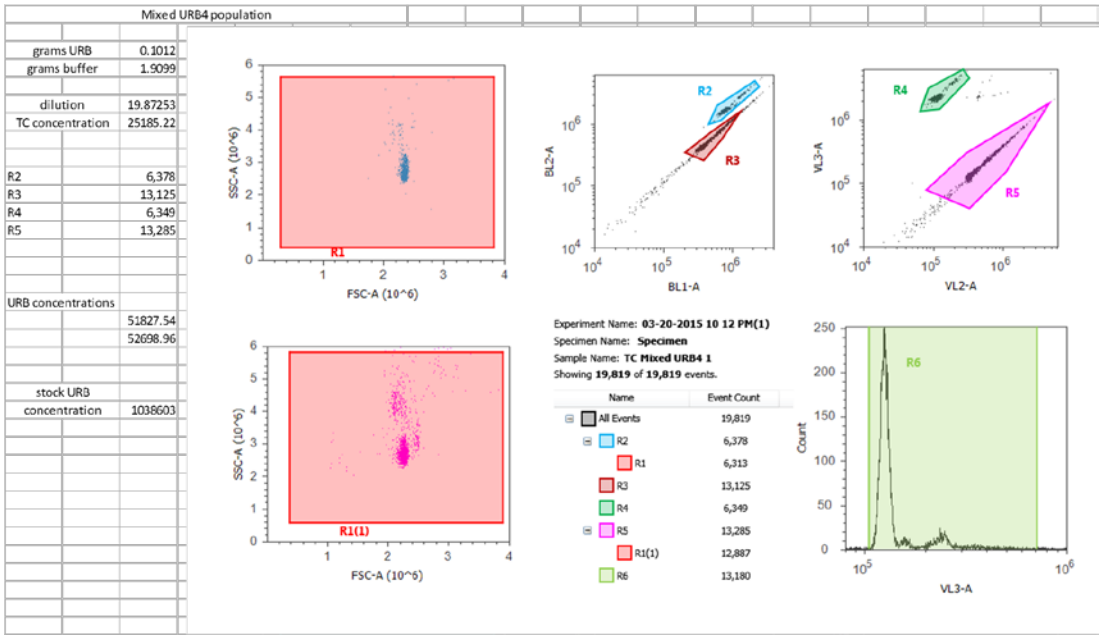

Fig 5. The Attune worksheet for a sample of URB4 microspheres taken directly from the vial of URB4 microspheres send by the manufacturer. The Gate R1(1) shows the pattern of scattering associated with the URB4 microspheres which consists of a tightly packed set of events which we call the major population, and a more diffuse set of events which define the minor population. The fluorescence intensity from the mixed suspension is due to the combined major and minor populations.

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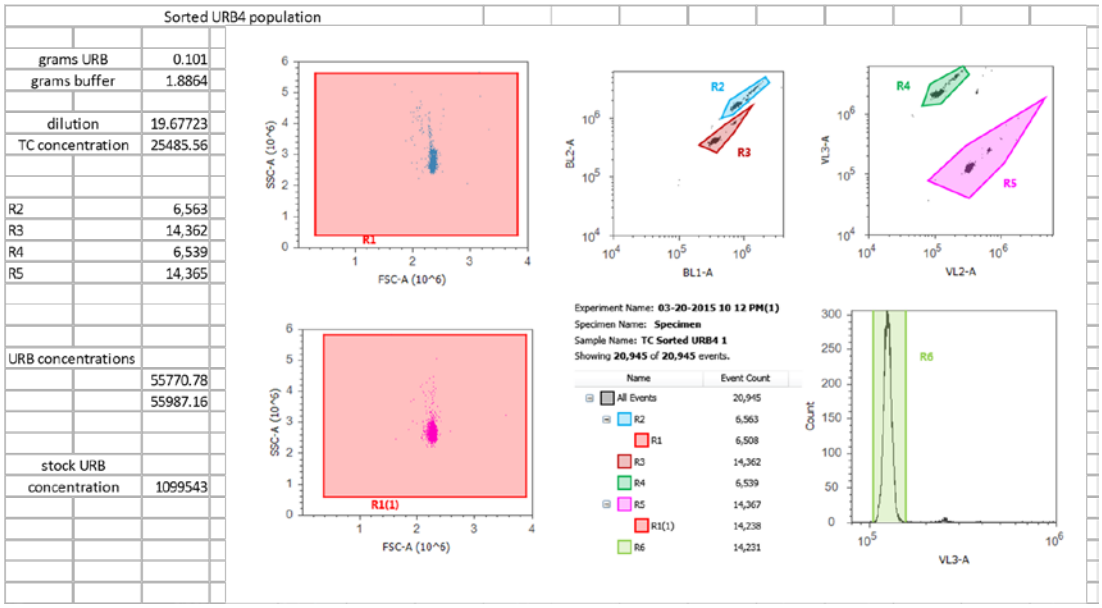

Fig 6. The Attune worksheet for a sample of "sorted" URB4 microspheres. The sorting was performed using an Aria sorting flow cytometer. During sorting, only URB4 microspheres whose scattering signal fell in the gate associated with the major population were retained, all other microspheres were discarded. The scattering pattern in Gate R1(1) shows a tightly packed set of events, and none of the diffused events evident in Gate R1(1) in the previous Fig. 5. The data in Fig. 6 allows the measurement of the concentration of the microspheres in the major population. The fluorescence intensity from the sorted suspension will be due to only the major population.

The ratios of the fluorescence intensity and microsphere concentration provide an estimate of the "ERF" values. The mean fluorescence intensity, MFI_{mixed}, was obtained from Fig. 5 by calculating the mean fluorescence intensity of gated region R6 with result of 13180. The gated region R6 was then narrowed to include only the main peak in the histogram giving the value of 11110 for MFI_{major}. Using the data in Figs. 5 and 6, and the measured intensities in the fluorometer, the left and right sides of Eq. (15) were evaluated yielding 44.5(0.2)=0.843*54.42(0.2) for 405 nm excitation, and 21.1(0.2)=0.843*26.5(0.2) for 638 nm excitation. The "ERF" values on the left and right sides of Eq. (15) differ by about 18%. Multiplying the right side "ERF" by the ratio of the MFI (0.843) brings the value of "ERF" to within 4% of the value on the left side of Eq. (15). The close correspondence between the calculated values of the right and left sides of Eq. (15), supports the validity of the proposed adjustment (Eq. (14)) in the assigned ERF values.

9.2 ERF Values of the Major Microsphere Population

The relation shown in Eq. (14) was used to evaluate the ERF_{major} from the assigned values shown in Table 4. The ratio of the mean fluorescence intensities (MFI) of the major and total populations was obtained from flow cytometer measurements on the same suspensions used in measuring the fluorescence spectra. The major population was defined as the number of events that had a signal in the VL3 channel and a scattering signal appropriate for the major population. The total population was defined as events that had VL3 signals but were not constrained by the scattering signals. The ratios were 0.824(0.014) for the URB2 suspension and 0.852(0.026) for the URB5 suspension. Multiplying the assigned values of ERF shown in Table 4 by the ratios yielded the ERF_{major} values of the major populations. The results are shown in Table 5.

Table 5. Values of ERF_{major}.

Identifier	ERF_{major}	Uncertainty	ERF_{total}	Uncertainty
URB2	2.22e04	3.6e03	2.64e04	4.3e03
URB5	1.15e06	1.5e05	1.35e06	1.7e05

Table 5 is the final result of the process of assignment of ERF values to microspheres. The ERF_{total} values are the result of fluorometer measurement of the microsphere fluorescence intensity and flow cytometer measurement of microsphere concentration. The ERF_{major} values are based on fluorescence measurements with the flow cytometer to identify the major microsphere population and make the appropriate adjustment in the assigned ERF_{total} value.

10. Summary

The assignment of ERF values requires a series of measurements starting with the measurement of the fluorescence intensity from serial dilutions of a solution of reference fluorophores with a known concentration. The data is used to calibrate the response of a fluorometer which is then used to measure the fluorescence intensity of a suspension of microspheres. The calibrated fluorometer allows the expression of the microsphere fluorescence intensity in terms of an equivalent concentration of reference fluorophores. The equivalent concentration is divided by the concentration of microspheres to yield the equivalent number of reference fluorophores (ERF) per single microsphere. The fluorometer calibration contributes a large portion of the uncertainty in ERF values. An examination of the calibration process suggests that the ERF uncertainty could be minimized by an accurate measurement of the laser intensity incident on the suspension (see Appendix A). This involves a measurement of laser power as well as accurate determination of the optical density of the ND filters, and finally an accurate determination of the integration time used in accumulating CCD spectra. A procedure was described for adjusting the assigned ERF values to those appropriate for the major population of the calibration microspheres. The major population is used to calibrate the fluorescence channels in the flow cytometer.

11. Appendix A

Model of the Fluorescence Response

Start with Eq. (8) in the text. This equation gives the corrected fluorescence intensity in digital units in terms of the fluorescence intensity emitted by the fluorophores in the solution. The factor F in Eq. (8) can be expanded in terms of the relevant fluorophore and solution properties as shown in Eq. (A1) by the term in the parenthesis.

$$FI_{cor,\lambda} = G_n \Gamma_n \left(I_{ex} \sigma_{ex} l f_\lambda PC \right) \tag{A1}$$

The subscripts n, ex, and λ in Eq. (A1) refer to the normalization wavelength, the excitation wavelength, and the emission wavelength respectively. I_{ex} gives the intensity of the excitation light entering the suspension, σ_{ex} is the absorption cross section at the excitation wavelength, l is the path length, and f_λ stands for the probability that after excitation a fluorescence photon will be emitted with wavelength λ. The

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quantity *P* refers to the effect of polarization on the intensity of the emitted fluorescence. The polarized laser light selectively excites fluorophores whose electric dipole moments are aligned with the polarization vector. The emission of fluorescence is maximum in a direction perpendicular to the electric dipole moment. During the lifetime of the excited state, the fluorophores undergo rotational diffusion so that the initial optimal alignment of dipole moment is lost resulting in a decrease in detected intensity. The rotational diffusion is expected to be different for reference fluorophores and fluorophores inside the microspheres. Next, Eq. (A1) is summed over the specified range of emission wavelengths to obtain the total fluorescence intensity. The result is rewritten in Eq. (A2) specifically for the case of reference fluorophores.

$$FI_{cor,r} = \sum_{\lambda} FI_{cor,\lambda} = G_n \Gamma_n \left(I_{ex} \sigma_{ex,r} Q_r l P C_r \right) \tag{A2}$$

The subscript *r* identifies the properties of the reference solution. The symbol Q_r stands for the quantum yield of the reference fluorophore, it originates from the summation of the probabilities of emission over the entire fluorescence emission spectrum shown in Fig. 1b. Equation (A2) shows that the fluorescence intensity is proportionate to the concentration of fluorophores. Taking the log of both sides of Eq. (A2) and separating the concentration from the other terms, gives a representation of the parameter *a* in Eq. (10) in the text. Explicitly, $a = G_n \Gamma_n I_{ex} \sigma_{ex,r} Q_r l P$. The parameter *b* in Eq. (10) is equal to 1. Since the parameter *a* depends on the intensity of the laser, it is important to monitor the laser intensity incident on the sample and normalize the measured fluorescence intensity for different dilutions of the reference solution to a common value. It is also important to insure that the polarization of the laser light and the path length remain constant. In practice, the fluorescence emission is collected for a preset time interval. This is equivalent to the excitation laser lasting for the duration of the interval. Clearly the collection time interval has to be known accurately.

Supplemental Materials

- Excel® file “ERF Assignment”: details of the procedure for assigning ERF values
- Excel® file “URB concentrations”: details of the concentration measurements

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