

**NIST Special Publication 260**  
**NIST SP 260-264**

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## **Abstract**

The latest protocols are described for the NIST assignment of fluorescence intensity values to calibration spheres or beads, which is primarily used for the accurate measurement of particle intensity values in quantitative flow cytometry. Procedures for measuring fluorescence intensity and bead concentration are described and the history of these assignments for the NIST Flow Cytometry Standards Consortium and its members are reviewed.

## **Keywords**

beads; calibration; cytometer; flow cytometry; fluorescence channels; intensity; microspheres; nanospheres; standards.

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## 1. Introduction

Fluorescence intensity values of cytometer calibration microspheres, or beads, in units of equivalent reference fluorophores (ERF) [1-4] are assigned by NIST for members of the Flow Cytometry Standards Consortium (FCSC). [5] A member sends NIST their calibration beads in suspension and the fluorescence intensity values and number concentrations of the samples are measured. The two measured values for a sample are then used to assign the mean fluorescence intensity value per bead. The ERF scale is an instrument-independent fluorescence intensity scale and assigned beads are referred to as ERF beads. ERF beads can be used to calibrate the fluorescence intensity scale for each fluorescence channel of a flow cytometer. Once calibrated, the flow cytometer can then be used to assign fluorescence intensities to cells, bioparticles and other particles. [6, 7] The ERF assignment process was described previously, [8] but what is reported here has been revised to include the latest reference fluorophores, laser wavelengths and protocols used by NIST, as well as a new, instrument-dependent fluorescence intensity value assignment, i.e., absolute reference intensity (ARI), which is proportional to the fluorescence intensity that reaches the instrument's detector.

## 2. Experiment

Reference fluorophore solutions of known concentrations are used to assign fluorescence intensities to the beads in units of ERF. The fluorescence intensity values of both the reference fluorophore solution and the bead suspension are measured using a fluorescence spectrometer, not a flow cytometer, under the same instrument conditions. The number concentration of the bead suspension is measured using a particle counting technique or combination of two nano-sized particle counting methods depending on the size range of the calibration particles.

### 2.1 Reference Fluorophores – Purity and Concentration Determinations

#### 2.1.1 Fluorescein (FL)

Fluorescein solution at a concentration of  $60 \mu\text{mol}\cdot\text{dm}^{-3}$  in  $0.1 \text{ mol}\cdot\text{dm}^{-3}$  borate buffer (boric acid and water) at pH 9.5 (pH adjusted with  $0.1 \text{ mol}\cdot\text{dm}^{-3}$  NaOH) was aliquoted (1.25 mL) into amber glass ampoules that were flame-sealed under argon. The fluorescein material in powder form was produced by Molecular Probes, Inc. (Eugene, OR) as Highly Purified Fluorescein MPR #71358, WO #18073 P2. The fluorescein (2-(6-hydroxy-3-oxo-3H-xanthen-9-yl) benzoic acid,  $\text{C}_{20}\text{H}_{12}\text{O}_5$ , relative molecular mass  $M_r = 332.311$ , CAS No. 2321-07-5) powder was custom-produced for NIST and is not a commercial product. Boric acid granules were obtained from Mallinckrodt, Lot 2549 KVTK,  $M_r = 61.83$ . Sodium hydroxide pellets were from Mallinckrodt, Catalog No. 7708, Lot 7708M484721,  $M_r = 40.00$ . All water used was NIST deionized water that was then passed through a deionizer (Millipore Mill-Q A10) to produce water having a resistivity  $\geq 18 \text{ M}\Omega\cdot\text{cm}$ .

The purity of the solid fluorescein material used to prepare the reference solution was determined by proton nuclear magnetic resonance spectroscopy and independently verified by analysis of impurities. The concentration of the reference solution was determined using mass measurements taken during gravimetric preparation of the solution and the certified purity of the dye.

This reference solution was first sold by NIST as SRM 1932 Fluorescein Solution in 2003. [9] Its stability has been confirmed every 5 years to the present using fluorescence and absorbance spectroscopy to compare the spectral shapes and peak intensities of fluorescein's spectra over time. No changes have been observed within the uncertainties of the measurements. This reference solution and its certified value and uncertainty for concentration have been used for ERF assignments from 2016 to the present.

### **2.1.2 Nile Red (NR) and Coumarin 30 (C30)**

Nile Red and Coumarin 30 solutions at concentrations of  $118.7 \mu\text{mol}\cdot\text{kg}^{-1}$  and  $130.5 \mu\text{mol}\cdot\text{kg}^{-1}$ , respectively, in acetonitrile were aliquoted (1.25 mL) into amber glass ampoules that were flame-sealed under argon. The NR was produced by Molecular Probes® (Life Technologies/Thermo Fisher Scientific) as Cat. # N1142, Lot # 1189983, and determined by the manufacturer to be 99% pure using HPLC with UV absorption detection at 254 nm. It was supplied in several vials, each containing 25 mg of NR. The C30, also known as Coumarin 515, was produced by Aldrich (Sigma-Aldrich) as Cat. # 546127, Lot # BCBH1078V, and determined by the manufacturer to be 99% pure with unspecified analysis techniques. It was supplied in several vials, each containing 100 mg of C30.

The purity of the solid NR and C30 materials used to prepare the reference solutions were determined by proton nuclear magnetic resonance spectroscopy that was quantified using an internal standard. The concentration of the reference solutions was determined using mass measurements taken during gravimetric preparation of the solutions and the certified purities of the dyes.

These reference solutions were sold by NIST as constituents of SRM 1934 Fluorescent Dyes for Quantitative Flow Cytometry from 2016 to 2024. [10] Their stability has been confirmed every 5 years to the present using fluorescence and absorbance spectroscopy to compare the spectral shapes and peak intensities of their spectra over time. No changes have been observed within the uncertainties of the measurements. SRM 1934 was discontinued for sale in 2025 but is still used by NIST internally. These reference solutions and their certified values and uncertainties for concentration have been used for ERF assignments from 2016 to the present.

### **2.1.3 Allophycocyanin (APC)**

The first lot of APC suspension used by NIST for ERF assignments from 2016 to 2024, referred to here as Lot 1, was supplied by Life Technologies in 2016 (Catalog #: A803, Lot #: 1575860, Product information: ~ 4 mg/mL, M.W. ~ 104 000, medium, 60 % saturated ammonium sulfate, 50 mmol/L potassium phosphate, pH 7.0). Lot 1 was aliquoted (105  $\mu\text{L}$ ) after gentle vortexing into 0.5 mL polypropylene Sarstedt tubes (#72.730.105) and sealed with a hand-tightened screw cap.

The purity of Lot 1 was estimated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion chromatography (SEC) for material qualification. [11, 12] The stability of Lot 1 was monitored by measuring its absorbance and the absorbance was found to be decreasing in 2024, implying degradation of the material. A new lot of APC suspension, referred to here as Lot 2, was supplied by Thermo Fisher Scientific in 2025 (Catalog #: not commercially available, Lot #: 1575860, Product information: ~ 4 mg/mL, M.W. ~ 104 000, medium, 60% saturated ammonium sulfate, 50 mmol/L potassium phosphate, pH 7.0).

Lot 1 was sold as a reference suspension by NIST as a constituent of SRM 1934 Fluorescent Dyes for Quantitative Flow Cytometry from 2016 to 2024. Lot 1 and its non-certified value and uncertainty for concentration were used for ERF assignments from 2016 to 2024 and Lot 2 from 2025 to the present.

#### **2.1.4 Pacific Orange (PO), Alexa Fluor 700 (AF700) and Alexa Fluor 750 (AF750)**

The Pacific Orange (PO) was supplied in 2019 by Molecular Probes® (Life Technologies/Thermo Fisher Scientific) as Pacific Orange triethylamine salt SKU # MT38404, Lot # EN0061-023-YH, and determined by the manufacturer to be about 99 % pure using HPLC with absorption detection at 398 nm. The Alexa Fluor 700 (AF700) was supplied in 2019 by Molecular Probes® (Life Technologies/Thermo Fisher Scientific) as Alexa Fluor 700 carboxylic acid, tris (triethylammonium salt) SKU # MT35335, Lot # 1967899, and determined by the manufacturer to be about 99 % pure using HPLC with absorption detection at 700 nm. The Alexa Fluor 750 (AF750) was supplied in 2019 by Molecular Probes® (Life Technologies/Thermo Fisher Scientific) as Alexa Fluor 750 carboxylic acid, tris (triethylammonium salt) SKU # MT35338, Lot # 1877862A, and determined by the manufacturer to be about 99 % pure using HPLC with absorption detection at 750 nm.

The three dyes were found to contain a significant amount of water, about 5 % by weight. The dyes absorbed this water from the air over a course of minutes, making weighing problematic. Therefore, it was necessary to develop a procedure for drying and then weighing the dyes under dry conditions, as described here, to determine accurate mass concentrations for the dye solutions. Note that this hygroscopic behavior was not observed for any of the other reference dyes, so this procedure was only used for the three dyes in this section.

A microbalance was put onto a stable, vibration-free table covered by a glove bag. A glass crucible with a volume of approximately 100  $\mu$ L was used to weigh the dye. The mass of the empty crucible was measured accurately three times using the microbalance. Dye was added to the crucible with a spatula, using the microbalance to measure the approximate amount of dye needed in the crucible. Each crucible with dye was put into a small glass dish (10 mL volume) without a cap to prevent spilling. The small glass dish with crucible and dye were transferred to a vacuum oven. Thermogravimetric analysis (TGA) was used earlier to estimate the amount of time (45 minutes to 60 minutes) and the temperature (about 100 °C) needed to drive off water and any other solvents that might be present without decomposing or melting the dye. After drying, the crucible(s) were immediately transferred to a desiccator containing silica desiccant. The desiccator was used for short-term storage. The desiccator was put next to the microbalance in the glove bag and the glove bag was sealed to the table using duct tape. The relative humidity

(RH) and temperature were monitored in the glove bag using a portable meter. 60 psi of dry nitrogen gas was flowed from a tank into the glove bag for about 2 hours to bring the RH in the glove bag down from 40 % RH to about 3 % RH. The latter value being approximately the lowest RH value attainable under these conditions. Once this low RH value was reached, the mass of the dye with crucible was measured in the glove bag by transferring the crucible with the dish out of the desiccator and transferring the crucible from the dish to the balance using tweezers. Each crucible was measured three times. The crucible was transferred into a glass vial that was then crimp-sealed with a silicone septum in the glove bag. The vial with dye was stored in a desiccator in a refrigerator until it was used to make the reference solution.

When making the reference solution, the crimping lid was removed from the glass vial. Dimethyl sulfoxide (DMSO) was added to the vial, filling about halfway using a glass pipette. Since DMSO is hygroscopic, a new bottle was opened and used to prepare the reference solutions for PO, AF700, AF750 and AF405. Alternatively, an open bottle of DMSO can be stored in a desiccator to prevent water contamination from the air. The dye solution was stirred carefully using the glass pipette. The solution was added to an amber glass bottle with cap. The glass vial was refilled with DMSO several times, which was then transferred to the amber glass bottle to ensure that all dye was transferred. About one liter of DMSO was then added to the amber bottle.

Dye solutions were dissolved in DMSO and concentrations were determined gravimetrically. The DMSO used was Sigma-Aldrich, anhydrous,  $\geq 99.9\%$ , Cat. # 276855-2L, Lot # SHBK7268 (used with PO) and SHBK9388 (used with AF700 and AF750). The microbalance used for dye mass measurements was a Mettler Toledo XPR2U (S/N B735599409) in 227/B153, which was internally calibrated. The balance used for solvent mass measurements was a Sauter RC4021 (S/N SV-03094). Both balances are also calibrated annually using external standard weights. All measurements were taken at temperature  $T = 22.0\text{ }^{\circ}\text{C} \pm 1.0\text{ }^{\circ}\text{C}$ , 30 % RH (outside glove bag) and 4 % RH (inside glove bag) and 1003 mbar atmospheric pressure. Quantitative NMR (qNMR) with an internal standard was used to determine the absolute purity of the three solid samples.

These reference solutions and their non-certified values and uncertainties for concentration have been used for ERF assignments from 2019 to the present. Their stability has been confirmed every 5 years to the present using fluorescence and absorbance spectroscopy to compare the spectral shapes and peak intensities of their spectra over time. No changes have been observed within the uncertainties of the measurements. These reference solutions have never been sold by NIST to date.

### **2.1.5 Alexa Fluor 405 (AF405)**

The Alexa Fluor 405 (AF405) was supplied in 2019 by Molecular Probes<sup>®</sup> (Life Technologies/ Thermo Fisher Scientific) as Alexa Fluor 405 carboxylic acid (triethylammonium salt) SKU # MT35750, Lot # 520821, and determined by the manufacturer to be about 99 % pure using HPLC with absorption detection at 254 nm. The purity of the solid AF405 material used to prepare the reference solution was determined by proton nuclear magnetic resonance spectroscopy that was quantified using an internal standard. The concentration of the reference solution in

DMSO was determined using mass measurements taken during the gravimetric preparation of the solution and the purity of the dye.

This reference solution and its certified value and uncertainty for concentration have been used for ERF assignments from 2024 to the present. Its stability will be confirmed every 5 years using fluorescence and absorbance spectroscopy to compare the spectral shapes and peak intensities of its spectra over time. This reference solution has never been sold by NIST to date.

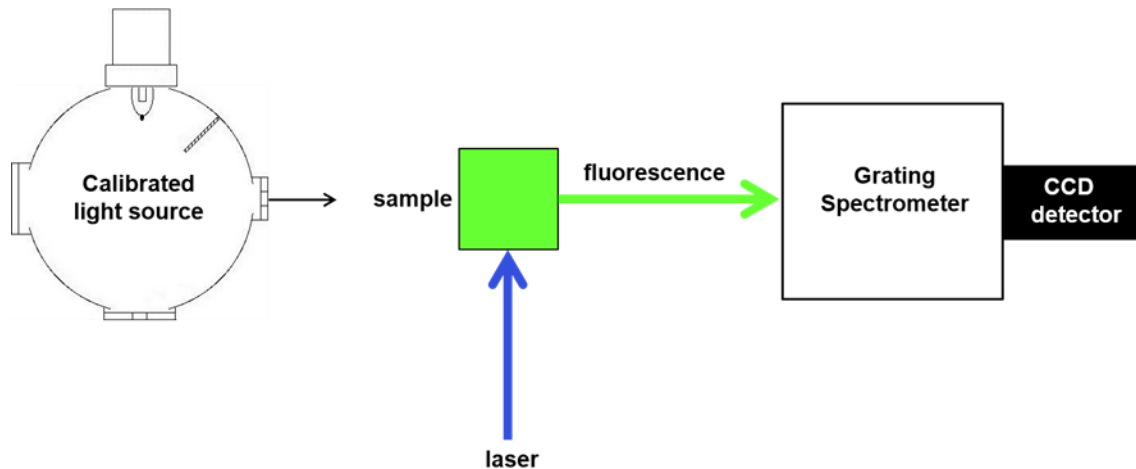
### **2.1.6 Quantitative NMR for Fluorophore Purity Determination**

Proton nuclear magnetic resonance spectroscopy quantified using an internal standard (<sup>1</sup>H-qNMR) was used to determine the purity of the solid dyes, except for fluorescein and APC. Dimethyl sulfone was used as the internal standard for the NR and C30 determinations, and 1,3,5-trimethoxybenzene (Sigma-Aldrich, TraceCERT®, Lot BCBW3670) was used as the internal standard for PO, AF700 and AF750 determinations. The purity of each dye was determined as a mass fraction, traceable to the International System of Units (SI) through NIST PS1 Primary Standard for qNMR (Benzoic Acid) [13-15].

The purity of fluorescein was also determined using qNMR; however, this determination was done before NIST had developed the internal standard method. In the earlier determination, NIST identified all fluorescein peaks and all impurity peaks in the NMR spectrum and integrated their areas. The ratio of the area of the fluorescein peaks to the summed areas of fluorescein and impurity peaks was used to express the purity as a mass fraction. Note that the identity of all impurities had to be determined to calculate the mass fraction, which is not the case for the internal standard method. The purity of APC was not determined using qNMR (see section 2.1.3).

## **2.2 Fluorescence Spectrometry for Fluorescence Intensity Determination**

Each fluorescence spectrum was measured using a fluorescence spectrometer with a charge-coupled device (CCD) detector and laser excitation (see Figure 1). The relative radiometric accuracy as a function of wavelength of the signal (emission) detection system was corrected using a calibrated light source [16], traceable to the NIST realization of the International System of Units (SI) [17-21]. All fluorescence measurements were taken at 21 °C ± 1.0 °C using a 90° transmitting geometry with the excitation beam incident on and normal to one of the polished surfaces of the sample cuvette. All emission spectra were corrected for the responsivity of the detection system and normalized to the mean laser intensity measured over the same time period as each spectrum was taken. As of 2025, the excitation laser wavelengths available on the fluorescence spectrometer are 375 nm, 405 nm, 488 nm, 561 nm, 638 nm and 808 nm. For initial assignments in 2017, only 405 nm, 488 nm and 633 nm lasers were available. In 2019, 375 nm and 561 nm, and in 2023, 808 nm lasers were added. In 2025, the individual lasers were replaced with a laser module with a colinear design, capable of higher laser power densities, tighter focus down to 10 microns on the sample, simultaneous multi-laser excitations, and a 638 nm laser replacing the 633 nm laser.



**Fig. 1. Schematic of the fluorescence spectrometer used for assignment of fluorescence intensities to calibration bead suspensions in ERF units. The spectrometer is spectrally corrected for relative intensity of emission using a calibrated light source.**

Fluorescence intensity was measured by comparing the integrated intensity of the fluorescence emission spectrum (fluorescence intensity versus emission wavelength) of a reference fluorophore solution with that of a calibration bead suspension. The reference fluorophore solution (e.g., APC in Figure 2) spectrum is integrated over the bandwidth of the main peak area down to about 10 % of the maximum intensity. This bandwidth is fixed for a particular reference fluorophore and the integrated fluorescence intensity acts as a standard light bulb, i.e., standard intensity, used to quantify the fluorescence intensity of an unknown, i.e., the bead suspension in this case. The concentration of the reference fluorophore solution is known and used to quantify the fluorescence intensity in fluorophore concentration units. The calibration bead suspension spectrum (see Figure 2 as an example) is integrated over the bandwidth of an emission (EM) filter, either the EM filter used in the fluorescence channel of the flow cytometer being calibrated or a standard EM filter defined by NIST. When the instrument's EM filter is used, the integrated standard intensity of the bead suspension is proportional to the fluorescence intensity that reaches the instrument's detector, expressed in absolute reference intensity (ARI) units, the magnitude of which depends on the EM filter used by the flow cytometer. When the standard filter is used, the integrated standard intensity of the bead suspension is instrument independent and expressed in equivalent reference fluorophores (ERF) units. The use of ERF units enables the standardization of biomarker expression analysis across different cytometer platforms.

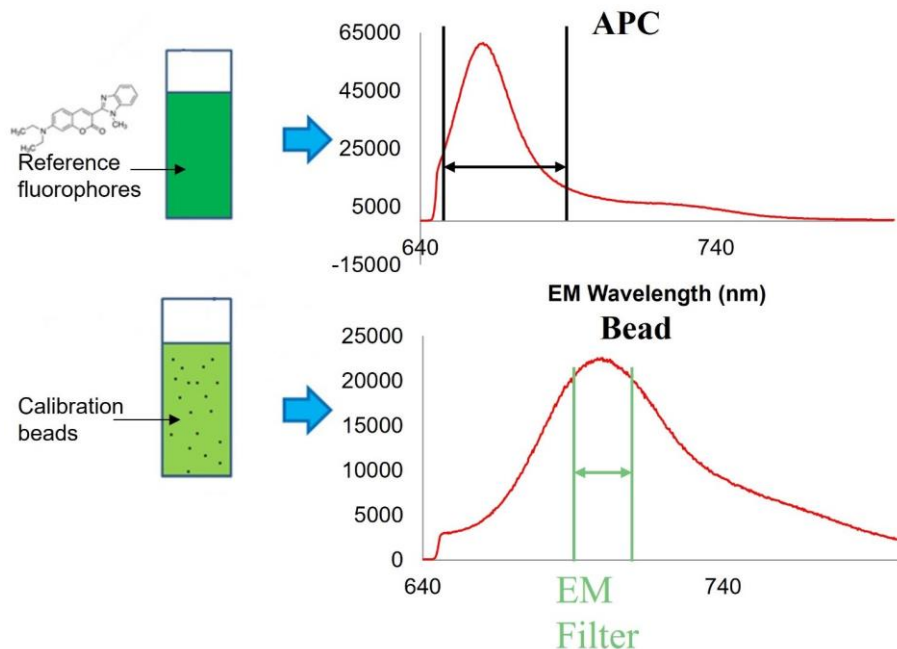


Fig. 2. Fluorescence spectra of a reference fluorophore (APC) and a calibration bead (Bead) are integrated over a fixed bandwidth and an emission (EM) filter bandwidth. The integrated intensities are compared to determine standard intensities in ARI and ERF units.

## 2.3 Bead Concentration Measurements

### 2.3.1 Micrometer-sized Particle Concentration Determination

A light obscuration (LO) based, liquid particle counter was used to determine the bead concentration of suspensions for beads with mean diameters greater than or equal to 2  $\mu\text{m}$  [22, 23]. The LO counter was a PAMAS model SVSS-C with an 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 20  $\text{mL}^{-1}$  was achieved before samples were measured. The daily performance of the instrument was also verified by measuring the size and concentration of Thermo Count-Cal 5  $\mu\text{m}$  beads. The measurement of a narrow distribution of sizes centered at 5  $\mu\text{m}$  and a concentration within 10 % of the manufacturer's specification of 3000  $\text{mL}^{-1}$  was recognized as a successful verification. The diameter of the Count-Cal beads is NIST traceable, but the concentration is not. Based on our estimates of accuracy and lot variation, a 10 % uncertainty in the specified concentration was assumed. Each sample was shaken or vortexed for 10 seconds, then sonicated for 10 seconds, and then gently stirred by tipping the sealed container just before a set of ten measurements were collected.

A stock suspension of calibration beads was prepared at a nominal number concentration of  $10^6$   $\text{mL}^{-1}$  in the appropriate solvent for the bead. A sample suspension for LO measurements was prepared by diluting the stock solution with the appropriate solvent to a bead concentration of approximately 5000  $\text{mL}^{-1}$ .

Particle concentration is obtained by dividing a particle count by the sample volume. Traceability to the SI is ensured by determining the confidence that all particles within the sample volume are counted and by determining the actual sample volume. Qualification of the particle counter for high accuracy measurements and determination of uncertainties includes 1) gravimetric calibration of volume, 2) pump volume dependence of particle counts to determine timing error, and 3) concentration dependence of particle counts to determine the linear range, correct for coincidence and determine sampling error due to bead adsorption to surfaces [22].

A flow cytometer, typically a CytoFlex LX (Beckman Coulter Life Sciences, Indianapolis, IN, USA), [24] was also used to confirm the LO-based bead concentration. This was done by using TruCount beads (BD Biosciences) as an internal standard in the calibration bead suspension. A sample suspension for flow cytometer measurements was prepared by adding 100  $\mu\text{L}$  of the stock solution to a TruCount tube and diluting with 400  $\mu\text{L}$  of the appropriate solvent. Each TruCount tube contains a specified number of TruCount beads with a nominal value of 50 000 beads. The variation in the number of TruCount beads from tube to tube has been estimated to be as much as 6 %. The LO measurement is used to calculate the ERF values, because the uncertainties in the LO measurement are more thoroughly understood, such that the resulting bead concentration is traceable to the SI and considered a certified value by NIST.

### **2.3.2 Submicrometer-sized Particle Concentration Determination**

Flow Cytometry (FCM), CytoFlex S or CytoFlex LX flow cytometers (Beckman Coulter Life Sciences, Indianapolis, IN, USA), was used as the primary technique for the determination of the number concentration of submicrometer particle suspensions. NIST has explored multiple techniques for number concentration determination and found flow cytometry to be one of the most accurate. [25] Violet side scatter (SSC) from a 405 nm laser served as a trigger for the detection of submicrometer particles and discrimination of the noise. To date, all measured suspensions displayed fluorescence signals following 488 nm laser excitation with four spectrally separated emission intensities collected from 505 nm to 545 nm, 564 nm to 606 nm, 665 nm to 715 nm and 750 nm to 810 nm. Two independent methods are used to determine particle concentration from the FCM data. The first uses an internal counting standard with a known particle concentration and the second uses a protocol to calibrate the flow rate of the flow cytometer. Studies have shown that comparable results are obtained using the two independent counting methods [26, 25].

TruCount (TC) microspheres (BD Biosciences, San Jose, CA, USA) were used in the first method as an internal counting standard for determining absolute particle counts. The number of TC microspheres per tube was calibrated by the manufacturer using the dry mass (DM) method to assure SI traceability and has demonstrated agreement with other primary methods in the micrometer range [27, 28]. The large dynamic range of FCM enables direct calibration of submicrometer sphere number concentration through the measurement of micrometer-sized TC spheres. Note that for determining the number concentration of monodisperse spheres, linearity in signals between the submicrometer and micrometer sizes is not necessary. The buffer used for the measurement was filtered with a 20 nm filter from GE Healthcare Life Sciences. The sample line was carefully cleaned between each sample measurement.

PBS buffer and Tween 20 were added to a TC tube by mass, such that the sample for measurement contained 0.1 % Tween 20, had a total volume of 0.5 mL and number concentrations of about  $1 \times 10^5 \text{ mL}^{-1}$  for TC microspheres and of between  $0.5 \times 10^6 \text{ mL}^{-1}$  and  $5 \times 10^6 \text{ mL}^{-1}$  for unknown bead samples. The number concentration of the TC spheres is known. The ratio of the number of sample counts to the number of TC counts and the number concentration of the TC microspheres were used to calculate the number concentration of the unknown samples.

In the second method, volumetric counting is implemented as an absolute number counting method using a flow cytometer equipped with a peristaltic pump-based fluidic system, capable of accurate volumetric calibration. A flow rate of 30  $\mu\text{L}/\text{min}$  was set in the software and used for measuring samples. Calibrating the flow rate using the manufacturer's protocol prior to acquisition was vital to ensure the accuracy of the results, where the flow rate and signal collection time were used to calculate sample volume. Flow rate is calibrated by collecting the sample that flows through the flow cytometer during a known time period and measuring its mass using an analytical balance (Mettler Toledo: Columbus, OH, USA) that was calibrated with SI-traceable reference masses.

Unknown bead samples were diluted as mentioned earlier, and the measured positive events per unit volume were used for calculation. The largest uncertainties in the volumetric flow cytometry technique are systematic biases that can cause some particles to not be counted. These biases include adsorption of particles on surfaces, non-uniformity in the sample distribution along the fluidic sampling system, particle aggregation and coincidence error. Based on our experience with other flow techniques such as light obscuration and discussions with the manufacturer, we estimated that these types of bias add an expanded uncertainty of 5% or less. Experimental determinations of these biases are planned for future study. The uncertainty in sample volume is estimated to be relatively small in comparison. Because all possible uncertainties have not been explored in the determination of number concentration using flow cytometry, the submicrometer particle concentrations are reported as non-certified values.

### 3. Results and Discussion

#### 3.1 Reference Fluorophores – Purity and Concentration Determinations

The certified purity and uncertainty of the solid fluorescein material were  $97.55 \% \pm 0.64 \%$ . The certified concentration and uncertainty of the fluorescein reference solution were  $60.97 \mu\text{mol}\cdot\text{kg}^{-1} \pm 0.40 \mu\text{mol}\cdot\text{kg}^{-1}$ . All uncertainties reported here are expanded uncertainties with an expansion coefficient of  $k=2$ .

The solid NR and C30 materials' certified purity and uncertainty were  $97.74 \% \pm 1.02 \%$  and  $97.35 \% \pm 0.46 \%$ , respectively. The certified concentration and uncertainty of the reference solutions were  $118.7 \mu\text{mol}\cdot\text{kg}^{-1} \pm 2.3 \mu\text{mol}\cdot\text{kg}^{-1}$  and  $130.5 \mu\text{mol}\cdot\text{kg}^{-1} \pm 1.7 \mu\text{mol}\cdot\text{kg}^{-1}$  for NR and C30, respectively. All uncertainties reported here are expanded uncertainties with an expansion coefficient of  $k=2$ .

The purity of APC Lot 1 was estimated, using SDS-PAGE and size exclusion chromatography (SEC), as the mass fraction to be greater than 95 %, with impurity bands at less than 5 % of the total intensity. The concentration of Lot 1 was determined to be  $30.0 \mu\text{mol}\cdot\text{L}^{-1} \pm 2.5 \mu\text{mol}\cdot\text{L}^{-1}$  using amino acid analysis [11, 12]. The molar extinction coefficient at  $\lambda = 652 \text{ nm}$  in PBS, pH 7.4 with 0.02% (w/w) Tween 20 was calculated to be  $8.81 \times 10^5 \text{ L}\cdot\text{cm}^{-1}\cdot\text{mol}^{-1} \pm 0.74 \times 10^5 \text{ L}\cdot\text{cm}^{-1}\cdot\text{mol}^{-1}$  using the absorbance value divided by the concentration and the pathlength. The absorbance of Lot 2 was compared to that of Lot 1 and found to be about 2% less. Assuming the extinction coefficient of Lots 1 and 2 are equal, the absorbance was used to calculate the concentration of Lot 2 as  $29.4 \mu\text{mol}\cdot\text{L}^{-1} \pm 2.9 \mu\text{mol}\cdot\text{L}^{-1}$ . Note that the values for the purity, concentration and extinction coefficient are non-certified values.

The solid PO, AF700 and AF750 materials' stoichiometry found by qNMR for each dye was not an integer for the counter ion. The stoichiometric ratio for the counter ion versus the dye was determined to be 1.05, 3.3 and 3.9 for PO, AF700 and AF750, respectively. This implies that the molecular weight of the dye with counter ion could not be determined accurately, therefore, the molecular weight of the dye without counter ion was used to determine the purity in grams of dye per gram of solid sample. These values of  $0.784 \text{ g/g} \pm 0.018 \text{ g/g}$ ,  $0.712 \text{ g/g} \pm 0.012 \text{ g/g}$  and  $0.605 \text{ g/g} \pm 0.016 \text{ g/g}$  were determined at a 95 % confidence interval ( $U_{95}$ ) for PO, AF700 and AF750, respectively. The reference value concentration of the dye solutions was corrected for the purity of the dyes by multiplying the dye solution concentration by the purity of the dye. The concentration and uncertainty for each reference dye solution are given below. They are given as non-certified values, even though the magnitude of the uncertainties for purity and gravimetry are within acceptable limits, because the nature of the unknown impurities was not identified.

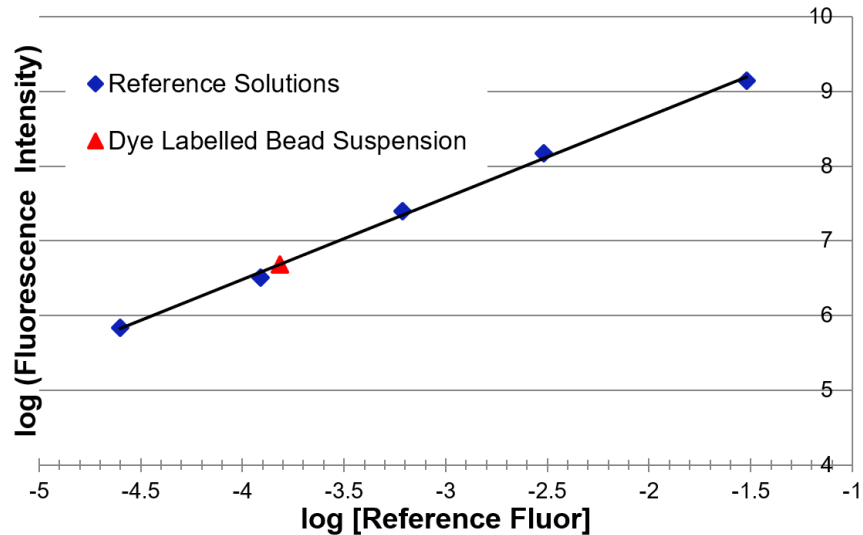
**Table 1: Concentration and Uncertainty for PO, AF700 and AF750**

<b>[PO]</b>	<b><math>U_{95}</math></b>	<b>[AF700]</b>	<b><math>U_{95}</math></b>	<b>[AF750]</b>	<b><math>U_{95}</math></b>
mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
20.0951	0.462	20.1497	0.340	15.9852	0.428
mol/kg	mol/kg	mol/kg	mol/kg	mol/kg	mol/kg
3.724E-05	8.6E-07	2.044E-05	3.4E-07	1.812E-05	4.9E-07
mol/L	mol/L	mol/L	mol/L	mol/L	mol/L
4.091E-05	9.4E-07	2.245E-05	3.8E-07	1.991E-05	5.3E-07

The solid AF405 material's certified purity and uncertainty were  $97.41 \% \pm 0.54 \%$ . The certified concentration and uncertainty of the reference solution was  $100 \mu\text{mol}\cdot\text{kg}^{-1} \pm 1 \mu\text{mol}\cdot\text{kg}^{-1}$ . All uncertainties reported here are expanded uncertainties with an expansion coefficient of  $k=2$ .

### 3.2 Fluorescence Spectrometry for Fluorescence Intensity Determination

The integrated fluorescence intensity of the particle suspensions was measured in terms of reference fluorophore concentration. This was achieved by first determining plots of integrated fluorescence intensity versus reference fluorophore concentration using serial dilutions of the appropriate reference solution (see Fig. 3). A straight line was fitted to the plot. This straight line defines the reference fluorophore intensity scale for both ARI and ERF units. The absorbance and emission spectra of all reference fluorophores and the excitation laser wavelengths



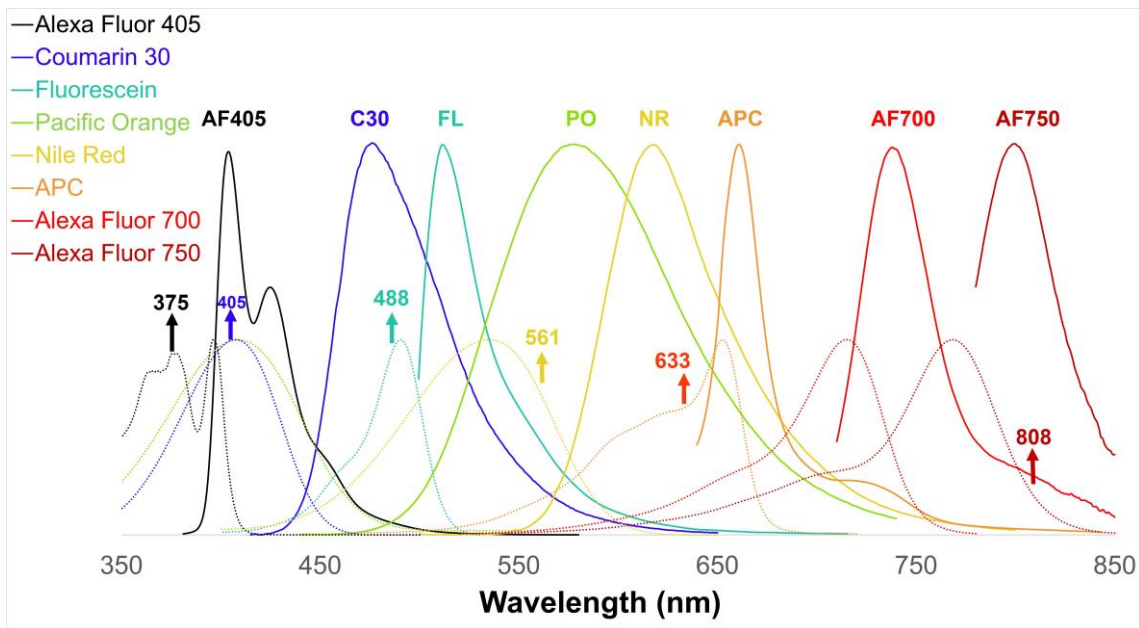
**Fig. 3. Calibration curve, using a log-log plot, for fluorescence intensity as a function of reference fluorophore concentration. A straight calibration line is used to determine the measured fluorescence intensity of the bead suspension shown by a red triangle symbol in terms of the reference fluorophore concentration.**

available to the fluorescence spectrometer are shown in Figure 4 and in Table 2. The absorbance spectrum determines which laser excitation (EX) wavelengths can be used to excite a reference fluorophore. The fluorescence emission (EM) spectrum defines the possible EM ranges that can be used with a reference fluorophore for ERF assignments.

The integrated fluorescence intensity of a bead suspension was then measured using the same fluorescence spectrometer settings as those for the reference fluorophore (see Figure 2). The location of the suspension's integrated fluorescence intensity on the fitted straight line was determined, giving the number of reference fluorophores needed to produce a fluorescence intensity equal to that of the bead suspension (see Figure 3).

**Table 2: EX Wavelength and EM Range for Reference Fluorophores**

Reference Fluor	EX Laser (nm)	EM Range (nm)
Alexa Fluor 405 (AF405)	375	390-480 (violet)
Coumarin 30 (C30)	375 405	390-550 420-550 (blue)
Pacific Orange (PO)	375 405	500-700 500-700 (yellow-green)
Fluorescein (FL)	488	500-580 (green)
Nile Red (NR)	488 561	570-700 580-700 (orange)
APC	633	640-740 (red)
Alexa Fluor 700 (AF700)	633	570-800 (red-near IR)
Alexa Fluor 750 (AF750)	633 808	750-850 (near IR)



**Fig. 4.** Fluorescence emission (solid lines) and absorbance (dotted lines) spectra of the reference fluorophores used to assign fluorescence intensities in ARI and ERF units. The upward arrows show the positions of the excitation lasers for the fluorescence spectrometer.

### 3.3 Bead Concentration Measurements

For micrometer-sized particles, the number concentrations of calibration beads with mean diameters of 2  $\mu\text{m}$ , 3.5  $\mu\text{m}$  and 10  $\mu\text{m}$  have been measured using LO, and the corresponding values measured using FCM with an internal standard were compared. The difference in the values between the two techniques was typically less than 5 %. LO only differentiates particles based on size, whereas FCM differentiates based on fluorescence intensity. Since calibration beads are not completely homogeneous, there is typically a main fluorescence population with fluorescence intensities clustered very close together, representing 85 % to 95 % percent of the total bead population, and a smaller population with fluorescence intensities that are more scattered. The main population is gated during FCM data analysis and is the bead population of interest during calibration of a flow cytometer. Therefore, a flow cytometer was used to determine the mean fluorescence intensity (MFI) of the main and total populations and the corresponding MFI ratio as well as serving as an orthogonal method for verifying the particle concentration measurement carried out by using LO.

For submicrometer-sized particles, the number concentrations of calibration beads were measured using both FCM with an internal counting standard and FCM with volumetric calibration. The values for the two methods were averaged and recorded as noncertified values for number concentration with their corresponding uncertainties.

### 3.4. ERF Assignments Per Bead

The mean ARI and ERF values for the integrated fluorescence intensity of a single calibration bead was determined by dividing the fluorophore concentration value for the bead suspension, as determined in Figure 3, by the number concentration of the bead suspension. The mean ARI and ERF values for the total bead population were then multiplied by the MFI ratio of the main and total populations to determine the mean values for the main population.

The EM range in flow cytometers is defined by the bandpass filter that the fluorescence passes through just before reaching the detector. Each fluorescence channel of a flow cytometer is defined by a single EX wavelength and a single EM range. The fluorescence channels that have been assigned by NIST were reported to customers, along with the reference fluorophore, EX wavelength ( $\lambda_{\text{EX}}$ ), center wavelength of the emission range ( $\lambda_{\text{Em}}$ ) and bandwidth of the fluorescence channel ( $\Delta\lambda$ ) used for the assignment. The total uncertainties in ARI and ERF value assignments have been determined at the 95 % confidence level (expansion coefficient  $k = 2$ ) and have ranged from 5 % to 13 % of the value per bead. These values and corresponding uncertainties were given to customers in an ERF Assignment Report.

#### 4. References

- [1] Wang L, Gaigalas AK, Marti GE, Abbasi F, Hoffman RA (2008) Toward Quantitative Fluorescence Measurements with Multicolor Flow Cytometry. *Cytometry* 73A, 279-288.
- [2] Wang L, Gaigalas AK (2011) Development of Multicolor Flow Cytometry Standards: Assignment of ERF Units. *J. Res. NIST* 116, 671-683.
- [3] Hoffman RA, Wang L, Bigos M, Nolan JP (2012) NIST/ISAC Standardization Study: Variability in Assignment of Intensity Values to Fluorescence Standard Beads and in Cross Calibration of Standard Beads to Hard Dyed Beads. *Cytometry Part A*, 81A, 785-796.
- [4] Wang L, DeRose PC, Gaigalas AK (2016) Assignment of the Number of Equivalent Reference Fluorophores to Dyed Microspheres. *J. Res. NIST* 121, 264-281.  
<https://www.nist.gov/programs-projects/quantitative-flow-cytometry-measurements>
- [5] NIST (2016) Flow Cytometry Quantitation Consortium. *Fed. Regist.* 81(136), 46054-46055 (July 15, 2016).  
<https://www.nist.gov/programs-projects/quantitative-flow-cytometry-measurements>
- [6] Wang L, Abbasi F, Gaigalas AK, Hoffman RA, Flagler D, Marti GE (2007) Discrepancy in Measuring CD4 Expression on T Lymphocytes Using Fluorescein Conjugates in Comparison with Unimolar CD4-Phycoerythrin Conjugates. *Cytometry Part B, Clinical Cytometry* 72B: 442-449.
- [7] Wang L, Stebbings R, Gaigalas AK, Sutherland J, Kammel M, John M, Roemer B, Kuhne M, Schneider RJ, Braun M, Leclere N, Dikshit D, Abbasi F, Marti GE, Porcedda P, Sassi M, Revel L, Kim SK, Marshall D, Whitby L, Jing W, Ost V, Vonski M, Neukammer J (2015) Quantification of Cells with Specific Phenotypes II: Determination of CD4 Expression Level on Lyophilized Human PBMC Surface Labeled with Anti-CD4 FITC Antibody. *Cytometry* 87A, 254-261.
- [8] DeRose P, Tian L, Elsheikh E, Urbas A, Zhang Y-Z, Wang L (2020) Expanding NIST Calibration of Fluorescent Microspheres for Flow Cytometry to More Fluorescence Channels and Smaller Particles, *Materials*, 13, 4111.  
doi:10.3390/ma13184111
- [9] NIST (2003) Certificate of Analysis, Standard Reference Material 1932, Fluorescein Solution.  
<https://tsapps.nist.gov/srmext/certificates/1932.pdf>
- [10] NIST (2016) Certificate of Analysis, Standard Reference Material 1934, Fluorescent Dyes for Quantitative Flow Cytometry (Visible Spectral Range).  
<https://tsapps.nist.gov/srmext/certificates/archives/1934.pdf>
- [11] Anders, JC (2002) Advances in Amino Acid Analysis, *BioPharm*, 32-67.
- [12] MacColl, R (2004) Allophycocyanin and Energy Transfer, *Biochimica et Biophysica Acta*, 1657: 73-81.
- [13] NIST (2018) Certificate of Analysis, NIST PS1, Primary Standard for quantitative NMR (Benzoic Acid).
- [14] Duewer, DL, Parris, RM; White, EW, May, WE, Elbaum, H (2004) Metrologically Sound Traceable Assessment of the Chemical Purity of Organic Reference Materials. *NIST Special Publication* 1012.

- [15] Nelson, MA, Waters, JF, Toman, B, Lang, BE, Rück, A, Breitruck, K, Obkircher, M, Windust, A, Lippa, KA (2018) A New Realization of SI for Organic Chemical Measurement: NIST PS1 Primary Standard for Quantitative NMR (Benzoic Acid). *Anal.Chem.* 90:10510.
- [16] DeRose PC, Early EA, Kramer GW (2007) Qualification of a Fluorescence Spectrometer for Measuring True Fluorescence Spectra, *Rev.Sci.Instru.*, 78:033107, 1.
- [17] Walker, JH, Saunders, RD, Hattenburg, AT (1987) Natl. Bur. Stand. (U.S.) *Special Publication* 250-1 (U.S. GPO, Washington, D.C.).
- [18] Yoon, HW, Gibson, CE (2011) NIST (U.S.) *Special Publication* 250-89 (U.S. GPO, Washington, D.C.).
- [19] Larason, TC, Houston, JM (2008) NIST (U.S.) *Special Publication* 250-41 (U.S. GPO, Washington, D.C.).
- [20] Larason, TC, Bruce, SS, Cromer, CL (1996) *J. Res. NIST*, 101:133.
- [21] P.Y. Barnes, PY, E.A. Early, EA, A.C. Parr, AC (1998) NIST (U.S.) *Special Publication* 250-48 (U.S. GPO, Washington, D.C.).
- [22] Ripple, D, DeRose, PC (2018) Primary Determination of Particle Number Concentration with Light Obscuration and Dynamic Imaging Particle Counters. *J.Res. NIST* 123: -002.
- [23] DeRose, PC, Wang, L (2018) NIST Fluorescence-based Measurement Services. *BioPharm Int.* 31:(12) 24.
- [24] Brittain GC, Chen YQ, Martinez E, Tang VA, Renner TM, Langlois M-A, Gulnik SA (2016) Novel Semiconductor-Based Flow Cytometer with Enhanced Light-Scatter Sensitivity for the Analysis of Biological Nanoparticles. *Sci.Rep.* 9, 16039 (13pp).
- [25] DeRose PC, Benkstein KD, Elsheikh EB, Gaigalas AK, Lehman SE, Ripple DC, Tian L, Vreeland WN, Welch EJ, York AW, Zhang Y-Z, Wang L (2022) Number Concentration Measurements of Polystyrene Submicrometer Particles. *Nanomaterials*, 12, 3118. <https://doi.org/10.3390/nano12183118>
- [26] Saraiva L, Wang L, Kammel M, Kummrow A, Atkinson E, Lee JY, Yalcinkaya B, Akgöz M, Höckner J, Ruf A, Engel A, Zhang Y-Z, O'Shea O, Sassi MP, Divieto C, Lekishvili T, Campbell JJ, Liu Y, Wang J, Stebbings R, Gaigalas AK, Rigsby P, Neukammer J, Vessillier S (2019) Comparison of Volumetric and Bead-based Counting of CD34 Cells by Single-platform Flow Cytometry. *Cytometry Part B*, 96B: 508-513.
- [27] Neukammer J, Kammel M, Höckner J, Kummrow A, Ruf A (2015) Reference Procedure for the Measurement of Stem Cell Concentrations in Apheresis Products. *PTB Mitt.* 125, 70–73.
- [28] Stebbings R, Wang L, Sutherland J, Kammel M, Gaigalas AK, John M, Roemer B, Neukammer J (2015) Determination of CD4+ Cell Count per  $\mu\text{L}$  in Reconstituted Lyophilized Human PBMC Pre-labelled with Anti-CD4 FITC Antibody. *Cytometry* 87A, 244–253.