



# Fluorescence calibration method for single-particle aerosol fluorescence instruments

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**Abstract.** Real-time, single particle fluorescence instruments used to detect atmospheric bioaerosol particles are increasingly common, yet no standard fluorescence calibration method exists for this technique. This limits the utility of these instruments as quantitative tools and complicates comparisons between different measurement campaigns. To address this need we have developed a

- 5 method to produce size-selected particles with a known mass of fluorophore, which we use to calibrate the fluorescence detection of a Wide-band Integrated Bioaerosol Sensor (WIBS-4A). We use mixed tryptophan-ammonium sulfate particles to calibrate one detector (FL1; excitation = 280nm; emission = 310-400nm), and pure quinine particles to calibrate the other (FL2; excitation = 280nm; emission = 420-650 nm). This procedure allows users to set the detector gains to achieve a known
- 10 absolute response, calculate the limits of detection for a given instrument, improve repeatability of instrumental set-up, and facilitate intercomparisons between different instruments. We recommend calibration of single-particle fluorescence instruments using these methods.

## 1 Introduction

Primary biological aerosol particles (PBAP) are of wide interest due to their potential impacts on
air quality (e.g. Prussin II et al. 2015), ecology (e.g. Morris et al. 2013), and Earth's climate (e.g. Creamean et al. 2013). PBAP comprise a broad class of atmospheric particles ranging from the small (viruses, as small as ~20 nm diameter) to the large (pollen grains, 5-100µm diameter) with bacteria, fungal and plant spores, and plant, insect, and animal fragments in between (Després et al., 2012). Despite the ubiquity of PBAP, many important questions remain regarding their atmospheric

20 impacts.





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The measurement of atmospheric PBAP has historically involved off-line techniques, such as culture-based methods and manual cell-counting by optical fluorescence microscopy. These methods require long air sampling periods, significant post-collection labor, and provide poor temporal resolution. In response to these shortcomings, a new generation of on-line, automated instruments for the measurement of PBAP, such as aerosol mass spectrometers (Tobias et al., 2005) and fluores-

cent particle spectrometers (Pan et al., 2003; Kaye et al., 2005), have recently been developed.

Measurements of single particle fluorescence has been used for rapid detection of PBAP in the fields of atmospheric science (Pöschl et al., 2010), public health (Bhangar et al., 2015), and biological warfare research (Greenwood et al., 2009). Many biological compounds, including certain amino

30 acids (e.g. tryptophan, tyrosine), metabolic small molecules (e.g. the reduced form of nicotinamide adenine dinucleotide, or NADH), and some proteins (e.g. Green Fluorescent Protein), are intrinsically fluorescent (Chudakov et al., 2010). In single-particle fluorescence instruments, fluorescence in such compounds is induced using ultraviolet excitation energy, and the resulting fluorescence is detected either in relatively broad emission bands using filters or with spectral resolution using a spectrometer. Fluorescent particle loadings are then used as a proxy for PBAP.

Despite the proliferation of single-particle fluorescence instruments (see Pan et al. 2003 and Kaye et al. 2005 for early prototype examples, and the Ultra-Violet Aerosol Particle Sizer (UV-APS; TSI, inc.) and Wideband Integrated Bioaerosol Sensor (WIBS; DMT, Inc.) for commercially-available examples), there is no standard method used to calibrate the magnitude of their fluorescence

40 signals. Fluorescently-dyed polystyrene latex spheres (FPSLs) are commonly used to assess detector performance, instrument alignment and excitation pulse timing. FPSLs, however, have significant batch-to-batch variability and suffer from poor shelf-life; thus they do not provide a repeatable, absolute calibration for fluorescence intensity. The lack of a standard limits our ability to compare observations made with different instruments, to track long-term instrument stability, and to assess 45 the fundamental limit of detection of the technique.

The amount of fluorescently emitted light that is measured is also a potentially useful metric for fluorescent particle attribution in single-particle fluorescence instruments. The use of these fluorescence magnitudes, however, varies widely in the published literature. Several recent studies have employed a binary yes-no classification of fluorescence above a threshold (e.g. Gabey et al. 2010,

- 50 Perring et al. 2015), essentially ignoring the fluorescence magnitude beyond the threshold. Fluorescence magnitudes have been used as input variables in automated particle-clustering analyses (Robinson et al., 2013; Crawford et al., 2015b) and to manually sort sampled particles into groupings (Wright et al., 2014). Particles emitting so much fluorescent light as to saturate fluorescence detectors are sometimes excluded from analysis (e.g. Toprak and Schnaiter 2013) and relatively
- 55 weak fluorescence has been proposed as a possible discriminator of interfering non-biological particles (Hill et al., 1999; Crawford et al., 2014, 2015a; Yu et al., 2016). The utility of fluorescence magnitudes will increase greatly with the development of an absolute fluorescence calibration strat-





egy applicable to any single-particle fluorescence measurement technology.

Here we present a reliable calibration strategy for fluorescence intensities measured by single-

60 particle fluorescence instruments. Methods for solution preparation, particle generation, and data analysis are presented.

#### 2 Materials & Methods

We evaluated the response of a wideband integrated bioaerosol sensor (WIBS-4A; Droplet Measurement Technologies; Boulder, CO, USA) to monodisperse aerosol particles containing a known
mass of fluorescent material. These experiments were conducted using fluorophores emitting in one or more of each of the fluorescent detectors and for different detector gains in the WIBS-4A. The following criteria guided our selection of fluorescent material:

- Fluorescent properties: fluorophores were chosen to match one or more of the excitation wavelengths (280, 370 nm) and emission bands (310-400, 420-650 nm) of the WIBS-4A.
- Stability: chemically inert fluorophores were chosen such that the signal from particles of a given size were constant over the course of a calibration.
  - Repeatability: the relationship (calibration curve) between fluorescence signal and fluorophore mass needed to be repeatable across multiple experiments with different batches of prepared solutions.
- 4. Availability & ease of preparation: all fluorophores used are inexpensive and easy to acquire. Importantly, each fluorophore chosen was soluble either in water or isopropanol for atomization.
  - 5. Safety: the materials used are all relatively safe to handle and prepare, though proper personal protective equipment was worn and exposure to exhausted particles was avoided.
- 80 Tryptophan and quinine fulfilled these requirements. NADH and naphthalene were also tested but each failed to meet one or more of the above requirements. Results from all materials tested are presented in Section 3. Below we present our detailed strategy for calibrating the fluorescence signals from the WIBS-4A with these materials.

# 2.1 Wideband Integrated Bioaerosol Sensor (WIBS-4A) operation

85 We validated the procedure using a commercially-available WIBS-4A, first described by Kaye et al. (2005) and later in significant detail by e.g. Gabey et al. (2010) and Perring et al. (2015). We will briefly describe its operating principles and the instrument settings used in this study.

The WIBS counts and sizes all incoming particles using elastic scattering from a continuouswave laser (635 nm, 12 mW). This scattering signal triggers the sequential flashing of two Xenon





- 90 lamps (5W L9455 modules, Hamamatsu Photonics K.K., Japan), one of which is filtered to emit light at 280 and the other at 370 nm. Any resulting fluorescent light is collected by two photomultiplier tubes (PMTs, H10720-110, Hamamatsu Photonics K.K., Japan) filtered to detect only specific wave bands: the FL1 detector detects 310-400 nm emission, and the FL2 detector detects 420-650 nm emission, though the peak sensitivity for each detector is in the 350-450 nm range. A
- 95 reference voltage input controls the gain on each PMT, which is changed manually with a variable potentiometer. We refer to this as the gain voltage throughout the rest of the paper. The FL2 detector also detects the scattering signal used for optical sizing. At reasonable particle sample rates, it can do so without interfering with the fluorescence measurement since the scattering event and the two flash lamp pulses are separated in time. Three fluorescence signals are therefore recorded for
- 100 a given particle: fluorescence between 310 and 400 nm following 280 nm excitation (referred to here as Channel A) and fluorescence between 420 and 650 nm with either 280 or 370 nm excitation (referred to as Channels B and C, respectively).

Both before and after the fluorescence calibration, the WIBS was run in forced trigger (FT) mode. In FT mode, the two xenon flashlamps are triggered automatically (as opposed to being triggered

- 105 by the presence of a particle) at ~2 Hz, to assess the background light detected in each PMT in the absence of particles. The FT background in each detector is a function of the flash lamp intensity, the flash lamp alignment, the efficiency of the filter at rejecting the excitation wavelength, the detector gain setting, and fluorescence from any material deposited within the instrument cavity (Toprak and Schnaiter, 2013). In general WIBS data analysis, FT data are used to determine a signal threshold
- 110 for each channel above which a particle is considered fluorescent. In ambient measurements, where a majority of particles are non-fluorescent, the fluorescent threshold can be assessed without taking the instrument off-line to run in FT mode, as there generally exists a dominant population of nonfluorescent particles that have a distribution of fluorescence magnitudes identical to the background data collected in FT mode (Perring et al., 2015). Here, sample particles were fluorescent by design
- 115 and we use 2-5 minutes of FT mode data to determine fluorescence thresholds for each channel. Gaussian functions were fit to FT signal peak intensity and fluorescence thresholds were defined as three standard deviations above the center of the fitted gaussian function (FT +  $3\sigma$ ).

All experiments presented here use a WIBS sample flow rate of 0.3 liter/min, and a sheath flow rate of 2.1 liter/min, close to typical factory settings. Laboratory tests reveal an inverse relation-

120 ship between optical size and particle velocity, as shown in Figure 1. This relationship likely is attributable to insufficient signal processing speed to fully resolve the scattering peak magnitude, a problem exacerbated at higher particle velocities. Due to this flow sensitivity, as well as flow dependence for the flash lamp timing, we note that instrument calibrations should be performed at the same flow rate that will be used in ambient or lab measurements.





#### 125 2.2 Particle generation and sampling

The experimental setup used for WIBS-4A calibration consisted of three general components: particle generation, particle conditioning, and measurement. This is shown schematically in Figure 2.

Fluorescent particles were generated by nebulizing a solution containing a fluorophore dissolved
in either isopropanol (99.9% purity, HPLC-grade, Pharmco) or deionized water, depending on the solubility of the fluorescent material. Additional non-fluorescent material was added to the nebulized solution, as needed, to adjust the per-particle mass of fluorophore. The desired range of fluorophore masses was determined empirically based on typical factory gain settings and previous observations of fluorescent magnitudes of known biological materials. For example, pure quinine (>98%, Sigma-

- 135 Aldrich) particles within the size range of the WIBS (>0.8 um) exhibited fluorescence intensities that were within the dynamic range of the detector at typical gain settings. For tryptophan (L-tryptophan, >98%, Sigma-Aldrich) on the other hand, pure particles saturated the detector at typical gain settings and produced much higher fluoresence signals than biological materials of comparable size. That the instrument is more sensitive to tryptophan than quinine on a mass basis is likely due to
- 140 the peak sensitivity of the WIBS-4A PMTs overlapping significantly with the tryptophan emission spectrum, and less so with that of quinine (Pant et al., 1990; Goldberg et al., 2012). Therefore tryptophan-based calibration particles were an internal mixture of tryptophan and ammonium sulfate "filler." Ammonium sulfate (≥99%, Sigma-Aldrich) was chosen because it is very soluble in water and has previously been shown not to fluoresce in the WIBS (Toprak and Schnaiter, 2013). In this
- 145 case the nebulized solution was prepared by mixing appropriate volumes of each stock solution (e.g. tryptophan in water and ammonium sulfate in water) with care taken to ensure the solution was well-mixed before nebulization. The composition of the particles is assumed to match that of the non-volatile components of the bulk solution. A full list of the gravimetric solutions used is presented in Table 1.
- 150 A nitrogen tank or HEPA-filtered room air was used to supply pressure to the nebulizer and a sealed dilution volume with inlet and outlet ports. The flows to the nebulizer and the dilution chamber were both controlled with rotameters tuned to provide adequate flow to the nebulizer while sufficiently diluting the output aerosol to maintain a manageable particle sample rate in the WIBS (limited by the recharge time of the flash lamps). The minimum flow rate required for the medical
- 155 nebulizers used here (B&F AeroMist Nebulizer; Allied Healthcare Products, Inc.; St. Louis, MO, USA) was determined to be ~1 liter/min, though this depended slightly on the solution. Typical dilution flow rates were 3-5 liter/min, yielding a total output flow of 4-6 liter/min. A bypassing port (a simple T-union with one end open to ambient) was installed downstream of the dilution chamber and upstream of a diffusion drier containing Drierite (anhydrous CaSO<sub>4</sub>), which reduced the RH
- 160 of the aerosol stream to <1%. The output from the drier fed into a differential mobility analyzer (DMA; custom-built at NOAA, Boulder, CO USA).





The DMA was used to select a narrow size-range of the incoming poly-disperse aerosol for sampling by the WIBS. We calculated the per-particle mass of fluorophore based on the selected particle mobility diameter and the mass fraction of nebulized solution assuming dry spherical particles. This
165 mass provides the basis for our calibration scheme, as it associates the fluorescence signals from the

WIBS with an absolute fluorescent mass. This experimental setup is shown in Figure 2a.

The flow rate of the aerosol stream through the diffusion drier and the DMA is controlled by the WIBS flowrate (0.3 liter/min) and a mass flow controller (MFC) downstream of the DMA. A sample flow through the DMA of 0.1 liter/min was optimal to select particles in the size range of interest

- 170 (650 nm 3  $\mu$ m). We used a DMA sheath flow of 1 liter/min (10:1 sheath to sample ratio). A make-up flow of 0.2 liter/min of HEPA-filtered lab air was controlled by the MFC after the DMA. It should be noted that this make-up flow was not dried. This 2:1 dilution from the make-up flow did not result in detectable evaporation or uptake of the size-selected particles, as determined by comparing measured particle sizes with and without this dilution, nor did it affect their fluorescence
- 175 signals. However, drying this make-up flow would remove any potential for water uptake, and could potentially be important in more humid environments. An RH probe (INTERCAP HMO60; Vaisala, Helsinki, FIN) installed in-line between the drier and the DMA showed that the measured humidity of the stream was between RH=1-2%, which indicated that particles were thoroughly dried prior to sizing. We estimate the residence time in the drier to be roughly 1.7 minutes.
- 180 In this configuration, the WIBS-4A sampled a stream of mono-disperse aerosol particles with a known mass of fluorescent molecules and the resulting fluorescence signal magnitudes were analyzed. We performed these experiments at several gain voltage settings and report the full results in Section 3. The experiment was conducted within a fume hood to contain all exhaust particles. In the absence of a fume hood, particulate filters on the exhaust lines are recommended to minimize any potential exposure to calibration particles.

#### 2.3 Calibration procedure

The starting point for most calibrations is preparation of the gravimetric fluorescent standard solution. The solutions used in these calibrations are listed in Table 1. We have verified that the signals from quinine and tryptophan do not significantly degrade over the course of two days under the

- 190 preparation and storage procedures used here. We have not assessed the stability of these solutions for longer terms and recommend solution preparation for semi-immediate use. Following solutions preparation, 12-15 mL was transferred to a clean medical nebulizer. This volume of solution was sufficient to last for 1.5 hours, the maximum duration of a typical calibration experiment. Unused solutions were stored in a dark refrigerator.
- 195 Prior to sampling fluorescent particles, the WIBS was run in FT mode for ~5 minutes. Following FT data collection, pressurized nitrogen was provided to the system, starting fluorescent particle generation as described above. The rotameter controlling the dilution flow upstream of the DMA





was adjusted to provide a particle count rate in the WIBS of <100 counts/s, below the maximum duty cycle of the flashlamps (125 counts/s), which allows for detection of all particles. Typical particle

- 200 concentrations in these experiments produed 10-100 counts/s, dependent on solute concentration in the nebulizer, selected mobility diameter, and dilution flow. For each selected mobility diameter, approximately 5 minutes of data were collected, providing enough particles to build high-fidelity histograms for determination of the central value and variability of fluorescence using gaussian fits. After a sufficient amount of data was collected, the voltage on the DMA was changed to select for
- another particle size, and this process was repeated until all data from all target sizes was collected. Data shown in the example experiment depicted in Figure 3 span the particle size range of 650 nm to 2.5  $\mu$ m. Following data collection from size-selected fluorescent particles, FT mode data was collected again for ~5 minutes.

# 2.4 Data analysis

210 A time series of single-particle data from a typical calibration of the FL2 detector using pure quinine particles is shown in Figure 3a. Black circles show individual fluorescence values and the pink line shows the 30-second average fluorescence intensity for the singly-charged ("Q1") population. Mobility diameters selected by the DMA are labeled with colored bands above the plot. Data are discarded during transitions from one DMA voltage to another (typically requiring ~30 seconds), 215 indicated by small gaps between the colored bands.

We construct fluorescence intensity histograms for each size (shown in Figure 3b) and fit a gaussian function to each singly charged mode, the center of which represents the modal fluorescence intensity for that size. In Figure 3b, each histogram is normalized to its maximum value and colored according to the colored bands in Figure 3a. We calculate the mass of fluorescent material for a

220 given size by assuming a spherical particle shape and complete removal of solvent. In the case of the mixed tryptophan-ammonium sulfate particles, we assume that the mass fraction in the dry particles is the same as in the atomizer solution. Fluorescence intensities as a function of fluorophore mass are then used to construct the calibration curves shown in Figure 4, which are discussed further in the Results section.

#### 225 2.5 Polydisperse fluorescent aerosol calibrations

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We also performed fluorescence calibrations using a polydisperse stream of fluorescent aerosol particles. Fluorescent particles were prepared in a similar manner as above with the exception that no DMA was used to size-select from the aerosol stream. This modified experimental set-up is shown in Figure 2b. Typical flowrates are shown in green in Figure 2b, but sometimes much higher dilution flow rates were used to ensure a particle count rate in the WIBS below 125 s<sup>-1</sup>.

Data were analyzed similarly to above, except WIBS scattering signals were used to provide particle size. A smoothed mie curve (so as to be monotonically increasing) based on our instrument's





geometry was used as a sizing calibration for the scattering signals. This sizing calibration curve was derived for each aerosol type used (quinine, ammonium sulfate-tryptophan) and for each gain

- 235 setting based on size-selected data. Each sizing calibration curve was then applied to scattering signals from the polydisperse aerosol data to provide particle diameter, which was converted to mass assuming complete drying and spherical particle shape. It should be noted that the application of a monotonically-increasing size calibration curve necessarily does not capture all of the mie scattering behavior. Thus, due to sizing errors, the polydisperse calibration slopes are expected to be less robust
- 240 than those generated using a DMA. Individual fluorescence signals were binned by size, and gaussian functions were fit to determine the modal fluorescence signal for a given bin. Only data from bins with >500 measured particles are displayed in Figure 4.

# 2.6 Xenon flash lamp intensity tests

- The relationship between fluorescence intensity and excitation power in fluorescence measurements can be complex. For instance, with too-high excitation power there can be saturation effects where fluorescent molecules are photobleached (Faris et al., 1997) or their excited states are depopulated through stimulated emission (Georges et al., 1996). With too-low excitation energy, fluorophores may not exhibit fluorescence at all (Kaye et al., 2005). Thus, the power of the excitation radiation can potentially have a large impact on the magnitude of fluorescent light measured.
- 250 The WIBS-4A provides a measurement of the relative power output of each xenon flash lamp pulse with a fiber optic sensor, which was designed to provide a measure of flash power over the lifetime of the lamp. These sensors, consisting of a silicon PIN-photodiode, which are placed near the arc lamp in the lens tube, provide a current that scales relative to the amount of light measured from each flash. However, they only provide a relative measure of light, and not an absolute measure of the excitation
- 255 energy experienced by each particle detected by the WIBS-4A. Moreover, this measurement is highly sensitive to the placement of the fiber optic sensor within the lens tube of the flash lamp, and may not provide a repeatable measurement when the fiber optic sensor is moved, either through instrument vibrations or when removing the flash lamp module for maintenance. In order to assess the response of our calibration particles to changes in lamp power, we performed fluorescence calibration tests
- 260 using neutral density filters with varying optical densities inserted within the lens tube of the flash lamps. Additionally, we performed our calibrations both with the original flash lamp, which has been installed in our instrument since its purchase ( $\sim$ 3 years ago), and a new flash lamp, in order to assess any possible degradation in power due to prolonged use.





## 3 Results and Discussion

# 265 3.1 Calibration results

Compiled results from the tryptophan and quinine calibrations are shown in Figures 4a and 4b, respectively, constructed as described above from single-particle fluorescence intensities measured for various fluorophore masses. Different symbols correspond to individual calibration experiments and error bars represent the width of the gaussian fits to observed fluorescence intensities for a given

- 270 mass. For both particle types we also tested different PMT detector gain voltages. All gravimetric standard solutions were prepared within two days of a given experiment. We collected FT mode data prior to each experiment, which is also shown on these graphs. The linear fits are constrained such that the y-intercept is equal to the average fluorescence signal from FT mode (second column in Tables 2 and 3.
- The detector gain clearly has a significant impact on the detector response for a given mass of fluorescent material. For instance, for the two FL1 gain settings shown in Figure 4a, the high gain (0.747 V) slope  $(25.0 \pm 1.7 \text{ counts/fg tryptophan})$  is ~4 times higher than for the low gain (0.632 V) slope  $(5.89 \pm 0.32 \text{ counts/fg tryptophan})$ . Thus, the mass of tryptophan that saturates the FL1 detector at the high gain setting is ~4 times lower than the low gain setting. As mentioned earlier,
- 280 two WIBS clustering studies (Robinson et al., 2013; Crawford et al., 2015b) excluded saturating particles from their analysis, and so understanding the range of measurable fluorophore mass is potentially important.

In Figure 4 we show an average linear fit to all data, but the slope of the reported calibration curves in Table 1 is the average slope across all of the individual experiments. Slopes for individual

- 285 calibration experiments, for a given FL channel and gain, were all within 15% of each other, and between 3 and 5 experiments were performed at each gain setting. The stability of these gain curves was assessed over a period of 4 months. For both quinine and mixed tryptophan/ammonium sulfate particles, the relationship between fluorescence intensity and fluorophore mass was linear. We take this as evidence that there is not significant shielding or quenching of fluorescence over the fluo-
- 290 rophore concentrations and particle sizes studied here. While there is noise in the data points shown in Figure 4, there are no systematic biases or individual experiments that are outliers, as the data from all experiments are distributed around the linear fit.

The variability in fluorescence signals in these calibration experiments are represented by error bars in Figure 4. These error bars are the width of the gaussian fits to the fluorescence intensities

295 at a given particle size. They represent the standard deviation of single-particle fluorescence values observed for a given mobility diameter selected by the DMA and are typically  $\pm$  20%. Because we are atomizing a well-mixed solution, we assume that all particles generated are uniform in composition, and so the variability in fluorescence can be attributed either to inherent noise in the WIBS, the transfer function of the DMA, or some combination of both. The variability in fluorescence inten-





- 300 sity is equivalent to a ~20% variability in fluorophore mass, which is roughly the the width of DMA transfer function on a mass basis. Thus, these error bars do not depict the fundamental precision of the WIBS itself, but rather the precision of this calibration technique using a DMA to select a narrow particle size range.
- Also shown in Figure 4 are results from the polydisperse fluorescent aerosol calibrations (dotted 305 colored lines). The fluorescence signal determined from the polydisperse aerosol data are within the error bars of the size-selected data points for almost all masses for both tryptophan and quinine. These data indicate that a polydisperse stream of fluorescent particles can provide an important qualitative (if not quantitative) check for fluorescence detector performance for the WIBS-4A. The ability to roughly determine a fluorescence calibration curve that does not necessitate the use of a
- 310 DMA makes this test a quicker, cheaper, and more field-deployable option for WIBS fluorescence calibration. However, due to the variability in optical size determination in the WIBS, this polydisperse fluorescence calibration method is not suggested as a substitute to using a DMA, but rather a supplemental technique that can be more easily and regularly applied.

#### 3.2 Effect of mass fraction tryptophan on fluorescence in mixed particles

- 315 For mixed particles containing tryptophan and ammonium sulfate, there was a dependence on the mass fraction of tryptophan in the atomizer solution for the measured fluorescence in the FL1 detector. Figure 5 shows calibration curves for particles containing different calculated mass fractions of tryptophan, the slopes of which increase with increasing mass fraction. For the range of mass fractions explored here (1-4% tryptophan in the mixed particles), we see an increase of 50% in the
- 320 calibration slope for the particles with the most tryptophan (mass fraction = 0.0409, purple) compared to the particles with the least tryptophan (mass fraction = 0.0110, red) The mass fractions displayed in the legend correspond to the calculated dry mass fractions in the particles, and are comparable to the last column in Table 1.

We were not able to determine the mechanism that explains this dependence on tryptophan mass 325 fraction. Nonetheless, the trend was repeatable across many experiments. Additionally, we found similar results for mixed-particles of tryptophan and dioctyl sebacate, which implies that the effect can not be attributed specifically to the presence of ammonium sulfate. Therefore, our calibration for the FL1 detector using the mixed particles is only "absolute" for the narrow concentration range (2.35 - 2.99% tryptophan) for the specific chemicals used here. However, given the repeatability of

- 330 our experiments, other users should be able to replicate our results using solutions prepared at the same nominal mass fraction. It is worth noting that while the difference in measured fluorescence intensity between the different solutions was significant, as determined by comparing the slopes of the fitted calibration curves, this difference is far less than the difference between the average slopes for the two gain settings tested and displayed in Figure 4.
- 335 Quinine, on the other hand, does not suffer this limitation as a calibrant, as pure quinine particles





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(as opposed to mixed-particles) exhibit fluorescence intensities within the range of values needed to calibrate these detectors. Thus, there may exist a more suitable calibrant than tryptophan for the FL1 detector in the WIBS. This calibrant would exhibit fluorescence intensity within the desired range for the FL1 detectors for pure particles of sizes in the WIBS detection range,. This substance could be sampled in the form of pure particles, thus not requiring "dilution" on a per-particle basis, as in

this case with tryptophan. Of course, an alternate calibration material for FL1 would still need to meet the requirements listed in Section 2.

## 3.3 Other materials

- We performed calibrations for several other fluorescent materials and found them to be inadequate as
  calibration standards for instruments like the WIBS-4A. For example, naphthalene particles, formed by nebulizing a solution of naphthalene (>99%, Sigma-Aldrich) dissolved in isopropanol (see Table 1), registered signal in our FL1 detector at a gain setting of 0.632 V, but were at the threshold of detection. As shown in Figure 6, only 22.5% or 7.5% of 1600 nm naphtalene particles registered above the FL1 threshold at a gain setting of 0.747 V, for FT+3σ (29 counts) or FT+4σ (35 counts)
- 350 thresholds, respectively. We experimented with more concentrated naphthelene solutions (to shift the polydisperse output from the nebulizer to larger sizes, making available more particles to be selected at higher DMA voltages), but these concentrations were so high that they clogged our nebulizers. However, it is worth noting that pure 1600 nm naphthelene particles (~2500 fg) had very low fluorescence.
- 355 Reduced nicotinamide adenine dinucleotide (NADH) was also explored as a calibrant for the FL2 detector (MP Biomedicals, LLC). It showed strong signal in both channels imaged by the FL2 detector (channels B and C), however signals from a given particle diameter showed systematic drift over long (~hour) timescales. As shown in Figure 7, the average signal observed in Channel B for 700 nm NADH particles (1-minute bins) steadily increased over the course of one hour, from 550 to 675
- 360 counts. These data are for a FL2 detector gain setting of 0.568 V. In other experiments (not shown), the average signal from NADH particles would oscillate on similar timescales. For comparison, neither quinine nor tryptophan exhibited this drift in fluorescence. This behavior is presumably due to ongoing chemistry leading to variable conversion in the atomizer volume between different oxidation states of NADH, only one of which is fluorescent. While NADH-fluorescence is widely used
- 365 to study enzymatic reactions (Lawkowicz et al., 1992), it is known to be highly unstable in solution (Rover, 1998) and the oxidized form (NAD+) is not fluorescent (Rost, 1992). So while NADH is an appealing potential calibrant for the WIBS, since it is a common metabolic molecule that exhibits fluorescence in the FL2 detector following both excitation wavelengths, stability issues prevented us from fully assessing it as we have done for quinine and tryptophan. However, we can say that the
- 370 fluorescence signal from NADH and quinine particles is on the same order of magnitude: NADH as we have prepared it here is roughly twice as fluorescent as quinine particles of the same fluo-





rophore mass in the FL2 detector following 280 nm excitation. So while quinine is not a biological molecule we expect to exist in any abundance in the atmosphere (unlike NADH), its stability and similar emission properties to NADH make it our calibrant of choice for the FL2 detector.

# 375 3.4 Detection limits

The development of an absolute calibration method allows us to examine limits of detection for both detectors as a function of gain voltage. As discussed above, we used the FT mode to assess the background noise in each channel, taking three standard deviations above the mean to be the threshold for designating a particle as fluorescent. This is a relatively conservative threshold for

- 380 which only 0.3% of FT observations would be falsely classified as fluorescent. The lower limit of detection (LOD), defined as the lowest mass that can be reliably distinguished from background (Armbruster and Pry, 2008), is then, in this case, the mass of a given fluorophore that would result in signal above this threshold at a particular gain setting. The upper LOD we define as the largest mass that gives signal without saturating the detector. Due to practical limitations, we did not measure
- either LOD mass directly for a given fluorophore. For example, in the case of the pure quinine particles, the only way to measure masses at the lower LOD is to select a smaller diameter using the DMA where detection efficiency decreases. Instead, we use the intersection of the calibration fit line with the fluorescence threshold (FT+3*σ*), and the saturation limit of the detector. The former gives the lower LOD (for which 50% of particles would be identified as fluorescent) and the latter gives 390 the upper LOD (for which 50% of particles would saturate the detector).

Figure 9 shows "detection bands" for each flourophore as a function of detector gain voltage. The left axis shows the mass at the lower LOD (bottom of band) and the upper LOD (top of band) as a function of gain for both tryptophan (Figure 9a) and quinine (Figure 9b). For both channels, increasing the gain voltage may marginally increase the lower LOD. For example, the lower LOD

- 395 of tryptophan in FL1 is 2.18 fg at a gain setting of 0.632 V and 2.17 fg at a gain setting of 0.747 V. At the same time, however, there is a dramatic shift in the saturating mass with 335 fg and 79.9 fg tryptophan saturating at 0.632 V and 0.747 V, respectively. Similarly the FL2 detector, the lowest gain setting (0.508 V) had the highest lower LOD compared to the medium and high gain settings, but also the highest upper LOD. Dotted lines on Figure 9 are visual guides that illustrate
- 400 the boundaries on the detectable range of fluorophore mass. The left axis is split because of the difference in the change of magnitude between the upper and lower LOD with gain voltage.

If the goal is to maximize detection of small amounts of fluorescent material, a higher gain setting may be beneficial with the understanding that fluorescence intensity information is lost for many strongly fluorescent particles. Using a binary above-below threshold classification scheme where the

405 fluorescence signals are not considered beyond the determination of whether a particle "fluoresces," this may not matter to the user. On the other hand, as in the clustering study performed by Robinson et al. (2013) in which saturating particles had to be excluded from analysis, a lower gain setting is





likely optimal in most cases, as the dynamic range of measurable fluorophore mass is much larger. The calibration slopes and LOD values for tryptophan and quinine at the instrumental settings used

410 here can be found in Tables 2 and 3, respectively.

# 3.5 Effect of flash lamp intensity on fluorescence signals

For both of our calibration materials, quinine and tryptophan, as well as fluorescent PSLs, fluorescence magnitudes decreased linearly with increasing optical density (OD) values for the filters used. Optical density is defined in terms of transmission (T), according to  $OD = \log_{10} T$ . Figure 8 shows

- 415 the fluorescence signals from 1.0 μm blue fluorescent PSLs (Fluoro-Max<sup>TM</sup>B0100; Thermo Scientific; Fremont, CA, USA) as a function of percent transmission of the native Xe flash lamp output. Each data point is the center of a gaussian function fit to a histogram of FL2 data from the FPSLs following the insertion of a neutral density filter in the lens tube between the lamp and the lens that focuses the lamp light on the particle beam. Experiments with both quinine particles and mixed am-
- 420 monium sulfate-tryptophan particles also showed this linear relationship in fluorescence magnitudes with transmission. We saw no difference between our original (3 year old) flash lamp and a brand new flash lamp in the magnitudes of fluorescence signals for any of the materials tested, indicating that they are producing the same amount of excitation power.
- Clearly, the fluorescence intensity measured in the WIBS-4A is highly dependent on the output power from the flash lamp. Due to the highly sensitive nature of the emitted fluorescent light on the input excitation energy, it would be desirable to have a direct measurement of the flash power, or at least a more robust relative measure of flash power that is not subject to very minor changes in e.g. alignment, as is the fiber-optic sensor in the WIBS-4A. We recommend this as an area of improvement for future models of the instrument. Nonetheless, the fluorescent particles described
- 430 here are a useful diagnostic tool, as any decrease in flash lamp power with time should be identifiable by decreases in signal from these fluorescent standards.

Using these two materials we have calibrated the detection sensitivity of a single-particle fluorescence instrument (WIBS-4A) to a given mass of fluoroescent material. Importantly, the overall sensitivity derived through these calibration experiments does not decouple the detector performance

- 435 from the excitation energy input. But, the method allows users to determine how their instrument responds to particles containing a known amount of quinine or tryptophan, and the resulting calibration curves can then be used to quantify, in absolute terms, the fluorescence of e.g. ambient fluorescent particles. Therefore, we propose reporting fluorescence intensities in units of fluorophore equivalent mass (e.g. "quinine equivalent mass" or "Q-units"). If WIBS-4A users report these "T-units" or "Q-units"
- 440 units" after properly assessing the sensitivity of their instruments, measurements of this fluorescence intensity should be directly comparable between instruments. As an example, we quantify the absolute fluorescence of the 1  $\mu$ m Blue FPSLs shown in Figure 8 being equivalent to the fluorescence of 3400 fg quinine (or 3400 "Q-units").





# 4 Conclusions

- 445 We have presented a simple fluorescence calibration technique for single-particle fluorescence spectrometers, and demonstrated its utility with a WIBS-4A instrument. We have shown different aerosolized solutions to be fluorescent in the WIBS-4A, stable with time, repeatable across different solution preparations and experiments, and safe to produce, thus meeting our requirements for use as calibration standards. Use of a DMA to select narrow particle size ranges allows for construction of
- 450 a fluorescence calibration curve that relates counts in the PMTs used to collect fluorescently emitted light to fluorophore mass. Reporting the calibration curves used in these instruments in sampling studies should improve the ability to directly compare results between instruments, and allow users to set the gains on their detectors in a more informed way. Instituting an absolute fluorescence scale should also lead to utilizing fluorescence intensities in ambient fluorescent aerosol studies and to
- 455 better comparisons between different instruments. We also highlight a need for the WIBS community to better monitor flash lamp power, as the fluorescence signals are in a linear response regime with respect to flash power.

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#### 460 References

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Armbruster, D. A. and Pry, T.: Limit of blank, limit of detection and limit of quantitation., The Clinical Biochemist Reviews, 29, S49–S52, 2008.

Bhangar, S., Adams, R. I., Pasut, W., Huffman, J. A., Arens, E. A., Taylor, J. W., Bruns, T. D., and Nazaroff,
W. W.: Chamber bioaerosol study: human emissions of size-resolved fluorescent biological aerosol particles,
Indoor Air, 26, 193–206, 2015.

Chudakov, D. M., Matz, M. V., Lukyanov, S., and Lukyanov, K. A.: Fluorescent Proteins and Their Applications in Imaging Living Cells and Tissues, Physiological Reviews, 90, 1103–1163, 2010.

Crawford, I., Robinson, N. H., Flynn, M. J., Foot, V. E., Gallagher, M. W., Huffman, J. A., Stanley, W. R., and Kaye, P. H.: Characterisation of bioaerosol emissions from a Colorado pine forest: results from the BEACHON-RoMBAS experiment, Atmospheric Chemistry and Physics, 14, 8559–8578, 2014.

- Crawford, I., Lloyd, G., Bower, K. N., Connolly, P. J., Flynn, M. J., Kaye, P. H., Choularton, T. W., and Gallagher, M. W.: Observations of fluorescent aerosol–cloud interactions in the free troposphere at the Sphinx high Alpine research station, Jungfraujoch, Atmospheric Chemistry and Physics Discussions, 15, 26067– 26088, 2015a.
- 475 Crawford, I., Ruske, S., Topping, D. O., and Gallagher, M. W.: Evaluation of hierarchical agglomerative cluster analysis methods for discrimination of primary biological aerosol, Atmospheric Measurement Techniques, 8, 4979–4991, 2015b.

Creamean, J. M., Suski, K. J., Rosenfeld, D., Cazorla, A., DeMott, P. J., Sullivan, R. C., White, A. B., Ralph, F. M., Minnis, P., Comstock, J. M., Tomlinson, J. M., and Prather, K. A.: Dust and Biological Aerosols from the Sahara and Asia Influence Precipitation in the Western U.S., Science, 339, 1572–1578, 2013.

- Després, V. R., Alex Huffman, J., Burrows, S. M., Hoose, C., Safatov, A. S., Buryak, G., Fröhlich-Nowoisky, J., Elbert, W., Andreae, M. O., Pöschl, U., and Jaenicke, R.: Primary biological aerosol particles in the atmosphere: a review, Tellus B, 64, 296, 2012.
- Faris, G. W., Copeland, R. A., Mortelmans, K., and Bronk, BV: Spectrally resolved absolute fluorescence cross
  sections for Bacillus spores, Applied Optics, 36, 958–967, 1997.

Gabey, A. M., Gallagher, M. W., Whitehead, J., Dorsey, J. R., Kaye, P. H., and Stanley, W. R.: Measurements and comparison of primary biological aerosol above and below a tropical forest canopy using a dual channel fluorescence spectrometer, Atmospheric Chemistry and Physics, 10, 4453–4466, 2010.

- Georges, J., Arnaud, N., and Parise, L.: Limitations arising from optical saturation in fluorescence and thermal lens spectrometries using pulsed laser excitation: Application to the determination of the fluorescence quantum yield of rhodamine 6G, Applied Spectroscopy, 50, 1505–1511, 1996.
  - Goldberg, J. M., Wissner, R. F., Klein, A. M., and Petersson, E. J.: Thioamide quenching of intrinsic protein fluorescence, Chemical Communications, 48, 1550–1552, 2012.
- Greenwood, D. P., Jeys, T. H., Johnson, B., Richardson, J. M., and Shatz, M. P.: Optical Techniques for
  Detecting and Identifying Biological-Warfare Agents, Proceedings of the IEEE, 97, 971–989, 2009.

Hill, S. C., Pinnick, R. G., Niles, S., Pan, Y. L., Holler, S., Chang, R. K., Bottiger, J., Chen, B. T., Orr, C. S., and Feather, G.: Real-time measurement of fluorescence spectra from single airborne biological particles, Field Analytical Chemistry and Technology, 3, 221–239, 1999.

Kaye, P., Stanley, W. R., Hirst, E., Foot, E. V., Baxter, K. L., and Barrington, S. J.: Single particle multichannel





bio-aerosol fluorescence sensor., Optics express, 13, 3583–3593, 2005.
 Lawkowicz, J. R., Szmacinski, H., Nowaczyk, K., and Johnson, M. L.: Fluorescence Lifetime Imaging of Free and Protein-Bound Nadh, Proceedings of the National Academy of Sciences of the United States of America, 89, 1271–1275, 1992.

- Morris, C. E., Conen, F., Alex Huffman, J., Phillips, V., Pöschl, U., and Sands, D. C.: Bioprecipitation: a
- feedback cycle linking Earth history, ecosystem dynamics and land use through biological ice nucleators in the atmosphere, Global Change Biology, 20, 341–351, 2013.
  - Pan, Y.-L., Hartings, J., Pinnick, R. G., Hill, S. C., Halverson, J., and Chang, R. K.: Single-Particle Fluorescence Spectrometer for Ambient Aerosols, Aerosol Science and Technology, 37, 628–639, 2003.
- Pant, D., Tripathi, U. C., Joshi, G. C., Tripathi, H. B., and Pant, D. D.: Photophysics of Doubly-Charged
  Quinine Steady-State and Time-Dependent Fluorescence, Journal of Photochemistry and Photobiology a-Chemistry, 51, 313–325, 1990.
  - Perring, A. E., Schwarz, J. P., Baumgardner, D., Hernandez, M. T., Spracklen, D. V., Heald, C. L., Gao, R. S., Kok, G., McMeeking, G. R., McQuaid, J. B., and Fahey, D. W.: Airborne observations of regional variation in fluorescent aerosol across the United States, Journal of Geophysical Research: Atmospheres, 120, 1153– 1170, 2015

515 1170, 2015.

530

- Pöschl, U., Martin, S. T., Sinha, B., Chen, Q., and Gunthe, S. S.: Rainforest aerosols as biogenic nuclei of clouds and precipitation in the Amazon, Science, 329, 1513–1516, 2010.
- Prussin II, A. J., Garcia, E. B., and Marr, L. C.: Total Concentrations of Virus and Bacteria in Indoor and Outdoor Air, Environmental Science & Technology Letters, 2, 84–88, 2015.
- 520 Robinson, N. H., Allan, J. D., Huffman, J. A., Kaye, P. H., Foot, V. E., and Gallagher, M.: Cluster analysis of WIBS single-particle bioaerosol data, Atmospheric Measurement Techniques, 6, 337–347, 2013.
  - Rost, F. W. D.: Fluorescence Microscopy, vol. 2, Cambridge University Press, Cambridge, UK, 1992.
  - Rover, L. e. a.: Study of NADH Stability Using Ultraviolet–Visible Spectrophotometric Analysis and Factorial Design, Analytical Biochemistry, 260, 50?55, 1998.
- 525 Tobias, H. J., Schafer, M. P., Pitesky, M., Fergenson, D. P., Horn, J., Frank, M., and Gard, E. E.: Bioaerosol Mass Spectrometry for Rapid Detection of Individual Airborne Mycobacterium tuberculosis H37Ra Particles, Applied and Environmental Microbiology, 71, 6086–6095, 2005.
  - Toprak, E. and Schnaiter, M.: Fluorescent biological aerosol particles measured with the Waveband Integrated Bioaerosol Sensor WIBS-4: laboratory tests combined with a one year field study, Atmospheric Chemistry and Physics, 13, 225–243, 2013.
  - Wright, T. P., Hader, J. D., McMeeking, G. R., and Petters, M. D.: High Relative Humidity as a Trigger for Widespread Release of Ice Nuclei, Aerosol Science and Technology, 48, i–v, 2014.
  - Yu, X., Wang, Z., Zhang, M., Kuhn, U., Xie, Z., Cheng, Y., Pöschl, U., and Su, H.: Ambient measurement of fluorescent aerosol particles with a WIBS in the Yangtze River Delta of China: potential impacts of
- 535 combustion-generated aerosol particles, Atmospheric Chemistry and Physics Discussions, pp. 1–31, 2016.







Fig. 1: Scattering signal dependence on total WIBS flow rate (sample + sheath) in liters/min (lpm). Data collected at a given sample flow rate are colored similarly. All data shown are for 1100 nm dioctyle sebacate particles.

	Mass %	Mass %	FL solution	non-FL solution	Mass % fluorophore
Expt #	FL solution	non-FL solution	[mL]	[mL]	(in dry particles)
1	0.648 Trypt. (Iso.)	0.090 AS (H <sub>2</sub> O)	3	9	2.35
2	0.832 Trypt. (Iso.)	$0.090 \text{ AS} (H_2O)$	3	9	2.99
3	0.648 Trypt. (Iso.)	$0.090 \text{ AS } (\mathrm{H_2O})$	3	9	2.35
4	0.648 Trypt. (Iso.)	$0.090 \text{ AS} (H_2O)$	3	9	2.35
5	0.959 Quin. (Iso.)	-	15	-	100.00
6	0.778 Quin. (Iso.)	-	15	-	100.00
7	0.514 Quin. (Iso.)	-	15	-	100.00
8	1.341 Quin. (Iso.)	-	15	-	100.00
9	0.535 Quin. (Iso.)	-	15	-	100.00
10	0.514 Quin. (Iso.)	-	15	-	100.00
11	6.332 Naph. (Iso.)	-	15	-	100.00
12	0.050 NADH (0.01 M NaOH)	_	15	-	57.29

Table 1: List of gravimetric solutions used for calibration experiments.







Fig. 2: Experimental set-up for fluorescent particle calibrations. Green numbers indicate flow rates in liters/min. (a) Size-selected calibration experiments. (b) Polydisperse calibration experiments.

Table 2: Calibration responses and limits of detection for tryptophan for different gain voltage settings.

Gain	FT	$FT + 3\sigma$	Slope $\pm$ s.dev.	Lower LOD	Upper LOD
[V]	[counts]	[counts]	[counts/fg]	[fg tryptophan]	[fg tryptophan]
0.747	50.0	105	$25.0\pm1.7$	2.17	79.9
0.632	11.1	24.3	$5.89\pm0.32$	2.18	335

Table 3: Calibration responses and limits of detection for quinine for different gain voltage settings.

Gain	FT	$FT + 3\sigma$	Slope $\pm$ s.dev.	Lower LOD	Upper LOD
[V]	[counts]	[counts]	[counts/fg]	[fg quinine]	[fg quinine]
0.634	64.8	132	$0.68\pm0.01$	98.4	2.90e3
0.568	23.7	49.7	$0.31\pm0.03$	83.9	6.55e3
0.508	10.1	21.5	$0.09\pm0.004$	122	2.17e4







Fig. 3: Data analysis scheme for calibration experiments illustrated by showing (a) FL2 WIBS data points for different sizes of mobility-selected particles, and (b) histograms of singly-charged fluorescent calibration particles. Colored bands at the top of (a) correspond to the selected particle sizes shown in legend of (b). This example illustrates the typical timeline of a calibration experiment for quinine, and how we analyze the data to construct calibration curves (shown in Figure 4).







Fig. 4: Fluorescence calibration curves for FL1 (**a**) and FL2 (**b**) detectors for different PMT detector gains. FL1 and FL2 detectors are calibrated with mixed tryptophan-ammonium sulfate and pure quinine particles, respectively. Each data point represents the central value of a gaussian function fit to fluorescence signals at a given particle mass, while the error bars are the standard deviation in fluorescence signals for that mass. Each symbol-shape refers to data from an individual calibration experiment. Dotted lines show results from polydisperse fluorescent aerosol calibration experiments. Black lines for both plots are linear fits to the mono-disperse data, all of which have R<sup>2</sup> values above 0.96.







Fig. 5: Fluorescence response to size-selected mixed-particles containing tryptophan and ammonium sulfate at different ratios. These calibration curves were taken at a lower gain setting than either of the curves in Figure 4a, and thus should not be directly compared.







Fig. 6: Fluorescence intensity histograms from FL1 detector of size-selected naphthalene particles and forced trigger (FT) background for a gain setting of 0.632 V. Gray lines are the raw data, while colored lines are smoothed for visualization purposes for each size and for FT. Only the tails of the naphthalene distributions are above the fluorescent threshold ( $3\sigma$  and  $4\sigma$  thresholds for this gain setting are indicated by vertical black lines).



Fig. 7: Time series from an example NADH calibration experiment, illustrating the lack of stability in the NADH fluorescent signal over long timescales. Signal from FL2 detector is plotted on y-axis for individual particles (black data points), and 1-minute averages (pink line with error bars representing  $\pm$  s.dev.).







Fig. 8: Fluorescence signal magnitudes vs transmission of Xenon flash lamp light. Fluorescence signal magnitudes are the central values of fitted gaussian functions to histograms of FL2 data from 1  $\mu$ m blue fluorescent PSLs. Nominal transmission values are calculated according to  $T = 10^{-\text{OD}}$ , where OD is the nominal manufacturer-quoted optical density.







Fig. 9: Detection "bands" for both FL1 and Fl2 channels showing the lower limit of detection and the mass that saturates the detector for tryptophan and quinine, respectively, as a function of detector gain voltage. Dotted lines are visual guides showing the range of fluorophore mass that is outside of the detection window.