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A simple and effective calibration method to determine the accuracy of liquid-handling nano-dispenser devices

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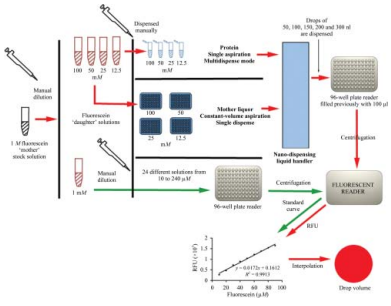
The accurate delivery of small volumes is a critical factor in the crystallization of macromolecules as it influences the reproducibility of the screening experiments. Crystallographic screening technologies have made it possible to perform experiments using volumes as low as 50 nl. The accuracy of the dispenser has usually been calibrated by weight measurements. In this work, a simple and inexpensive fluorescence-based calibration method that is sensitive and that can be used to monitor the precision and accuracy of any liquid-handling nano-dispenser device is presented. The results suggest that the protocol described here can be useful to determine volumes ranging from 50 to 300 nl with precision. Therefore, the pipetting of volumes as low as 50 nl can be calibrated periodically to ensure that precision and accuracy are maintained. The suggested calibration protocol can be executed in 6 h per instrument, including the calibration curve, which is the most time-consuming step; the rest can be completed in approximately 2 h.

1. Introduction

The availability of instruments delivering very small volumes reproducibly has had a tremendous impact in biotechnology and drug discovery. In particular, their use in the screening of protein crystallization conditions has increased widely in academic and industrial laboratories (Rose, 1999). With the development of structural genomics efforts, the efficient use of robots to automate protein crystallization became critical (DeLucas *et al.*, 2003; Stock *et al.*, 2005; Tickle *et al.*, 2004). The purpose of liquid-handling nano-dispensing technologies is to permit the rapid delivery of small-volume (∼50 nl) drops repeatedly and reproducibly, expediting the screening of a large number of crystallization conditions using a limited volume of highly purified protein.

Liquid-handling nano-dispensing devices can be generally classified as either contact or noncontact depending on their delivery mechanism. The two instruments that have been used and calibrated in our laboratory use both techniques. The Phoenix/RE (Art Robbins/Rigaku, USA) uses self-contained air pressure and a vacuum pump to aspirate and dispense the liquid. It has 96 syringes that contact the surface to transfer the liquid and it additionally has an on-the-fly noncontact protein nano-dispenser (Deerac-type). The other instrument, the Cartesian Dispensing System (Genomic Solutions, USA), uses the noncontact dispensing method, specifically syringe–solenoid technology with eight tips.

Independent of the delivery mechanism, one of the most important parameters is the amount of time that the valve remains open. The open time is approximately the time required for the fluid to move through the valve. Thus, the open time can be changed as a function of the viscosity and the drop size. The open time is a parameter that can be adjusted directly in the Cartesian. In the Phoenix, specifying a viscosity constant for each liquid class can change the open time. Therefore, the instruments allow different volumes and viscosities to be worked with. Each instrument has a specific sensitivity, but the basic principle is the same: the open time that the valve remains open.



In order to perform adequately, liquid-handling nano-dispensing devices should be calibrated consistently and periodically to ensure reproducibility. Usually, these types of devices are in a multi-user facility and the operators need to be confident of the correct performance of the instruments. In addition, the personnel in charge of the service need to answer questions from the users about their experiment by performing a routine calibration of the robots. Ideally, routine calibration protocols should be fast and inexpensive, and should provide robust data for the precision and accuracy of the different liquid-handling nano-dispensing devices in any laboratory.

Precision (also referred to as reproducibility) relates to the extent to which a group of measurements differ from one another. An accepted parameter to assess the precision of a group of measurements is the relative standard deviation (RSD) from the mean, also given as the coefficient of variability (CV) in percentage units. CV is the absolute value of the RSD (see the legend of Table 2 for definitions). For drug-discovery applications CV must be below 10% and preferably within 5% for the most stringent applications (Rose, 1999). Accuracy is a measurement of the variation of the actual drop volume with respect to the expected drop volume (or target volume) and it is calculated as the standard error (SE) of the expected volume {SE = 100[(mean − target)/target]} (Table 2). Standard errors of less than 5% are acceptable for most applications (Rose, 1999). A robust correlation between the actual and the predicted dispensed volume is essential and the figures of merit used to evaluate such instrumentation can be defined as precision and accuracy, using the size of the deviations to establish the relative ranking.

Calibration methods typically fall into two different classes: gravimetric and fluorescence-based. Standard gravimetric measurements are unreliable for testing the accuracy of liquid-handling nano-dispensing devices (Rhode *et al.*, 2004) and require special conditions to complete. Fluorescence-based methods rely on a concentration measurement of a fluorophore and are often used for the calibration of volumes. Fluorescence probes are typically expensive; for example, Oregon Green 488 (Invitrogen, USA) costs 64 euros per 10 mg. The cost of routine calibration using these compounds would be unreasonably high for small academic laboratories. For this reason, fluorescein sodium salt (Sigma–Aldrich, USA) has been used in this calibration protocol. On one hand, fluorescein is inexpensive (0.4 euros per gram) and it has been already validated as a useful fluorophore for testing the precision of nanolitre dispensing (Harris & Mutz, 2006). On the other hand, the fluorescein signal is highly dependent on the fluorescence reader; therefore, the correct

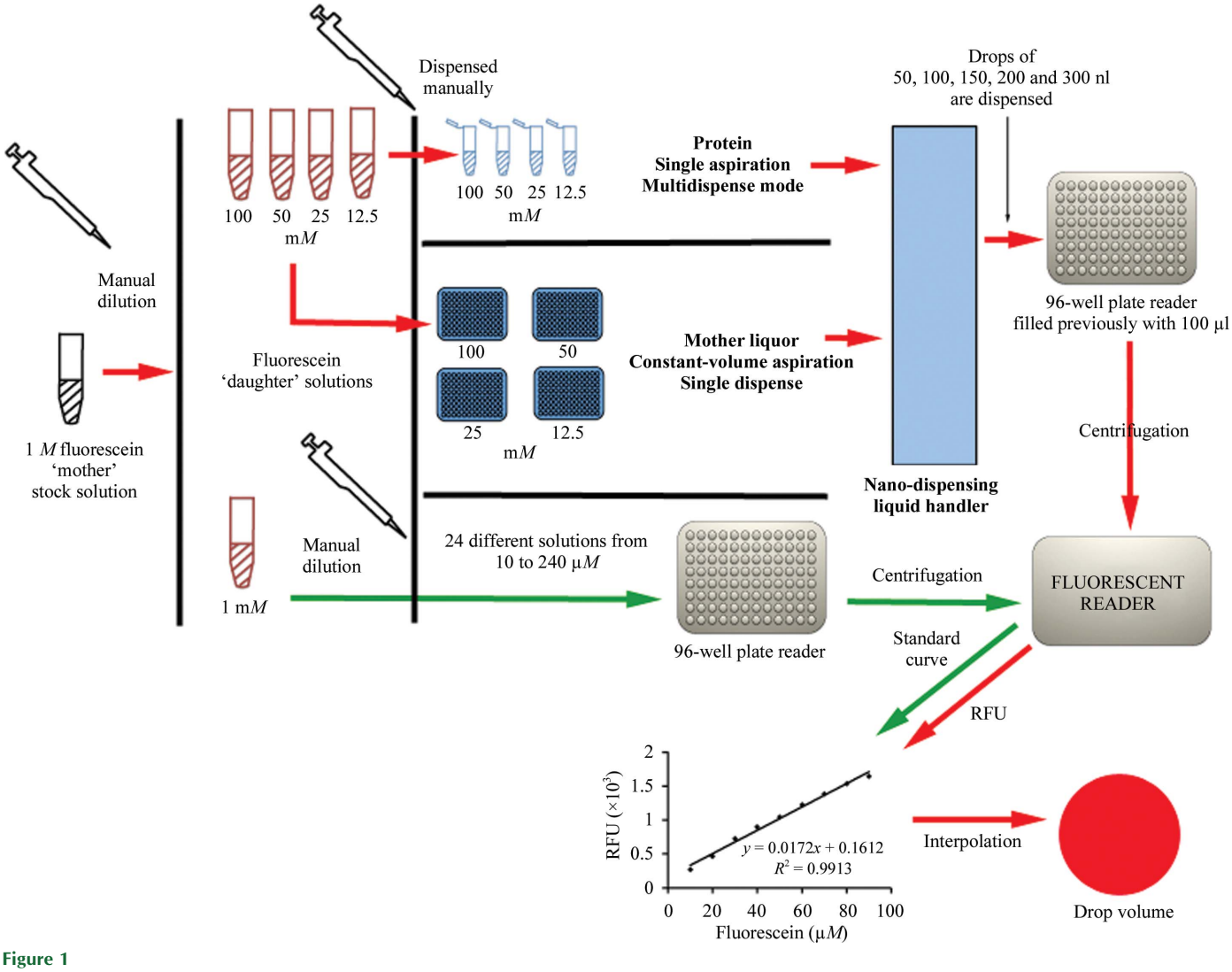


Figure 1
Diagram showing a schematic representation of the workflow of the calibration protocol.

Table 1
Summary of the solutions (volumes and concentrations) needed for the proposed calibration protocol.

(*a*) No glycerol.

	Stock solutions		Solutions for the dilutions	
	1 *M* fluorescein, 0% glycerol	1 *M* fluorescein, 10% glycerol	0.1 *M* Tris pH 8, 0% glycerol	0.1 *M* Tris pH 8, 10% glycerol
Fluorescein (g)	18.8	18.8	—	—
85% glycerol (ml)	—	5.9	—	29.4
0.1 *M* Tris pH 8† (ml)	50	50	250	250

(*b*) 10% glycerol.

1 *M* fluorescein, 0% glycerol (*x* ml)	0.1 *M* Tris pH 8, 0% glycerol (*y* ml)				
1 *M* fluorescein, 10% glycerol (*x* ml)	0.1 *M* Tris pH 8, 10% glycerol (*y* ml)	Initial concentration (m*M*)	Volume dispensed by the robot (nl)	Final volume into the plate (µl)	Final concentration (µ*M*)
5	50	100	50	100	50
2.5	50	50	100		50
2.5	50	50	150		75
1.25	50	25	200		50
0.625	50	12.5	300		37.5

† The final volume of solution which was reached using 0.1 *M* Tris pH 8.

concentration range of the probe must be found before performing the calibration.

In this communication, we present a rapid, effective and inexpensive fluorescence-based calibration protocol that can easily be adapted to various liquid-handling nano-dispensing devices for routine and periodic calibration. We propose that this method should be used routinely (at least twice a year) to calibrate liquid-handling nano-dispensing devices in protein crystallographic laboratories in order to ensure more reproducible and consistent crystallization results and to document the reliability of the equipment to different users. This protocol should also be used when problems with the liquid dispensing are observed or even suspected.

2. Materials and instrumentation

Two fluorescein 'mother' solutions (1 *M*) with different viscosities were prepared as follows. Solution 1 containing 0% glycerol was prepared by adding 18.8 g fluorescein (sodium salt; Sigma–Aldrich, USA) to 50 ml 0.1 *M* Tris pH 8 (Sigma–Aldrich, USA).

Solution 2 containing 10% glycerol was prepared by adding 18.8 g fluorescein (sodium salt) and 5.9 ml 85% glycerol (Sigma–Aldrich, USA) to 0.1 *M* Tris pH 8 to give a final volume of 50 ml.

Fluorescein 'daughter' solutions were prepared manually (using a Pipetman, Gilson, USA) by diluting *x* ml of the 'mother' solution (with 0 and 10% glycerol) into *y* ml of 0.1 *M* Tris pH 8 (with 0 and 10% glycerol) to give fluorescein concentrations of 100, 50, 25, 12.5 and 1 m*M*. The protocols are summarized in Fig. 1 and Table 1.

All the plates were centrifuged for consistency (1000 rev min^{-1}, 2 min), as centrifugation improves the fluorescence CV values (Petersen & Nguyen, 2005), and read at optimal sensitivity based on the best signal-to-noise ratio obtained pre-reading. 96-well Corning plates (CLS3651; Sigma–Aldrich, USA) were used and read in a FL6000 fluorescence microplate reader (BioTek, USA).

3. Methods

3.1. Preparation of a standard curve

In order to prepare a calibration standard curve, it is critical to find a stable fluorescence signal. Therefore, a wide range of concentrations (100–28 m*M*) of the fluorophore were tested, with the aim of finding the most suitable linear intervals. Although a nanomolar range has been used for testing instrument performance in other laboratories with confocal fluorescence readers (Taylor *et al.*, 2002), our fluorimeter did not respond reliably in this concentration range and we instead used a higher concentration.

The fluorescence signal increased from 10 to 600 µ*M* and decreased from this concentration to 28 m*M*. The deviation of the curve from linearity can be explained by a self-quenching effect (Imasaka *et al.*, 1977). Therefore, the range 10–240 µ*M* was selected

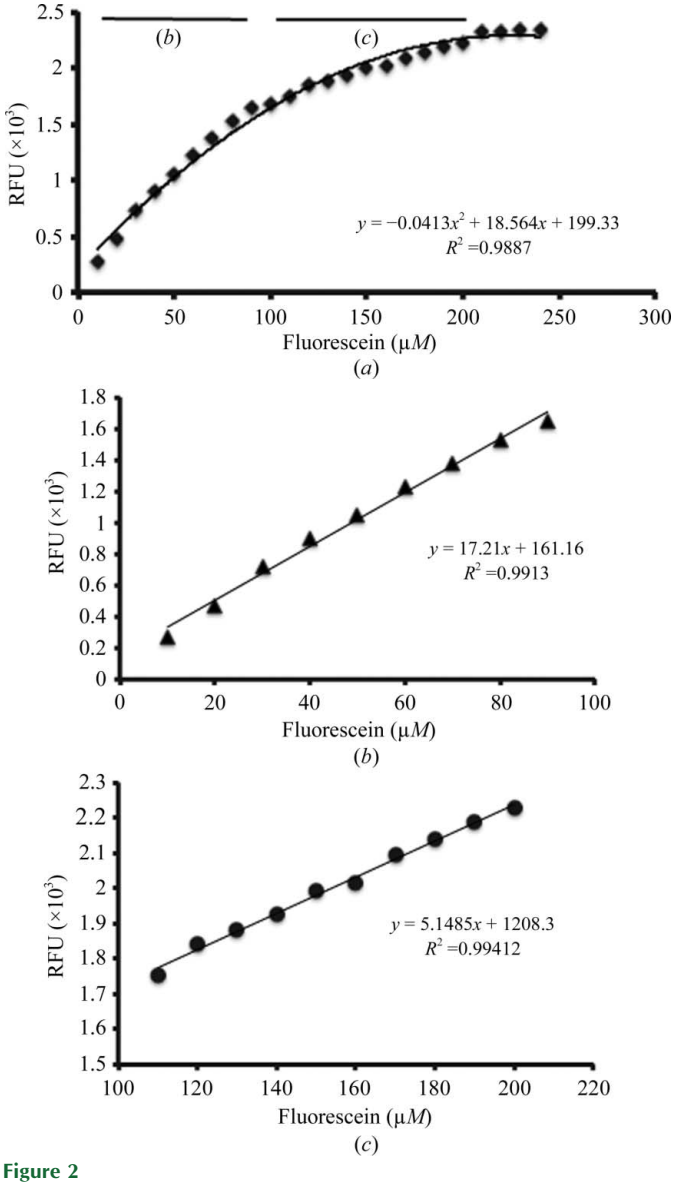


Figure 2
Standard curve of fluorescence response *versus* fluorescein concentration. (*a*) Relative fluorescence units (RFU) *versus* fluorescein concentration in the range 10–240 µ*M*. The overall regression curve for concentrations in the range 10–240 µ*M* is shown; two distinct linear ranges have been noted and are shown in (*b*) and (*c*). (*b*) Regression line for the concentration range 10–90 µ*M*. (*c*) Regression line for the concentration range 110–210 µ*M*.

Table 2
Calibration results for liquid-handling nano-devices.

(*a*) Protein-dispensing method: tip dispensers 4, 8 and overall mean of the two tips for the Cartesian instrument, nano-tip (Deerac-type) for the Phoenix. The mean for the Cartesian device corresponds to 48 measurements for tip 4 and 48 measurements for tip 8, giving a total of 96 measurements for both tips for comparison with the 96 measurements for the Phoenix tip. 'Mean' does not correspond to the mean value of the two tips averaged separately.

		50 nl			100 nl			150 nl			200 nl			300 nl		
	Tip	Dispensed volume (nl)	CV† (%)	SE† (%)	Dispensed volume (nl)	CV (%)	SE (%)	Dispensed volume (nl)	CV (%)	SE (%)	Dispensed volume (nl)	CV (%)	SE (%)	Dispensed volume (nl)	CV (%)	SE (%)
0% glycerol	Tip 4	43.9	9.8	12.3	104.6	6.9	4.6	140.2	2.9	6.5	201.9	6.2	1.0	308.7	9.0	2.9
	Tip 8	48.3	14.8	3.4	98.8	6.3	1.2	145.8	4.0	2.8	204.4	6.9	2.2	291.4	6.8	2.9
	Mean	46.4	13.9	7.2	101.7	7.2	1.7	143.1	4.0	4.6	203.2	6.5	1.6	300.6	8.5	0.2
	Nano tip	50.0	6.2	0.1	94.4	3.9	5.6	146.9	4.5	2.1	194.7	4.5	2.6	303.5	6.8	1.2
10% glycerol	Tip 4	43.6	9.9	12.8	104.1	3.0	4.1	145.6	4.0	2.9	198.6	5.7	0.7	308.5	6.6	2.8
	Tip 8	48.8	15.2	2.5	100.9	2.4	0.9	142.4	5.4	5.0	198.1	6.3	1.0	288.4	5.3	3.9
	Mean	45.9	13.9	8.2	102.5	4.2	2.5	144.0	4.8	4.0	197.9	6.0	1.1	298.1	6.8	0.6
	Nano tip	50.2	4.2	0.3	100.1	4.5	0.1	148.4	4.8	1.1	190.5	3.7	4.7	303.6	4.9	1.2

(*b*) Mother-liquor dispensing method. The values for the eight Cartesian tips and the 96 Phoenix tips are shown.

		50 nl			100 nl			150 nl			200 nl			300 nl		
		Dispensed volume (nl)	CV (%)	SE (%)	Dispensed volume (nl)	CV (%)	SE (%)	Dispensed volume (nl)	CV (%)	SE (%)	Dispensed volume (nl)	CV (%)	SE (%)	Dispensed volume (nl)	CV (%)	SE (%)
0% glycerol	8 tips, Cartesian	46.1	5.6	7.8	95.5	5.0	4.5	152.2	3.8	1.5	194.6	5.6	2.7	292.4	5.7	2.5
	96 tips, Phoenix	45.5	3.5	9.0	96.2	4.0	3.8	147.9	2.4	1.4	194.1	2.7	2.9	295.7	3.8	1.4
10% glycerol	8 tips, Cartesian	47.0	5.1	6.1	99.6	5.8	0.4	145.6	4.3	3.0	201.3	3.9	0.7	291.9	5.3	2.7
	96 tips, Phoenix	46.6	3.2	6.7	93.3	3.7	6.7	144.9	2.3	3.4	191.0	3.5	4.5	290.4	3.4	3.2

† The precision is expressed as the coefficient of variability (CV) in percentage units. The accuracy is calculated as the standard error (SE) of the coefficient between the 'mean dispensed volume' and the expected volume. CV (%) = 100 ($\sigma/\langle x\rangle$), where σ and $\langle x\rangle$ are the standard deviation and the mean value, respectively. SE (%) = 100 [(actual − target)/target].

to further analyze the fluorescein solutions as follows. The 1 m*M* fluorescein 'daughter' solution was diluted into 0.1 *M* Tris pH 8 manually to prepare 24 different solutions with concentrations ranging from 10 to 240 µ*M* in a final volume of 100 µl (Fig. 1). This volume is the minimum that is needed to ensure proper functionality of the fluorescence reader. The probe was excited at 460 nm and the emission was read at 530 nm. The solutions were prepared in a 96-well plate and fluorescence readings were measured to obtain an extended calibration curve that was fitted to a quadratic polynomial function (Fig. 2*a*).

The fluorescence signal increased linearly from 10 to 90 µ*M*. Above 100 µ*M* the response was still robust (110–210 µ*M*), but the slope decreased, maintaining linearity with a regression coefficient (R^2 = 0.9941) similar to the lower (10–90 µ*M*) range (R^2 = 0.9913). Consequently, the concentration ranges 10–90 and 110–210 µ*M* can be selected and fitted by a regression line in order to determine the accuracy and the precision of both liquid-handling nano-dispenser devices (Figs. 2*b* and 2*c*). In this work, the range 10–90 µ*M* was used.

3.2. Calibration of the protein and mother-liquor dispensing methods

For protein delivery, the sample is dispensed using the single-aspiration multiple-dispense mode; this involves filling the tip with the sample, which is then dispensed in multiple drops onto the 96-well plate.

For mother-liquor dispensing, a constant volume is aspirated from a reservoir and is individually dispensed into a destination well. The calibration protocol (shown in Fig. 1) was adapted depending on the instrument and the dispensing method.

3.2.1. Protein dispensing. For the Phoenix, the nano-tip aspirated a predetermined volume from the 'daughter' solutions at concentrations of *x* m*M* (where *x* = 12.5, 25, 50 and 100 m*M*). The nano-tip then dispensed different volumes (*y* = 50, 100, 150, 200 and 300 nl) using the appropriate 'daughter' solution shown in Table 1. The well plate was previously filled manually with 100 µl 0.1 *M* Tris pH 8 in order to prevent any evaporation from the nanodrops.

Since the viscosity of the sample can affect protein dispensing, 'mother' and 'daughter' solutions containing 0 and 10% glycerol were used.

The protocol was adapted for the Cartesian since this equipment has two protein nano-tip dispensers (tips 4 and 8). Each nano-tip aspirated a constant volume (5, 6.5, 9, 11.5 or 16.5 µl) as a function of the *y* volume value. It dispensed one single drop of the corresponding volume (50, 100, 150, 200 or 300 nl) in each well. In order to obtain sound statistics, 48 fluorescence values were obtained for each *y* volume and each tip. This device was programmed to use the same open time for samples containing 0 and 10% glycerol (Table 2*a*).

The Phoenix nano-tip was calibrated for two different sample viscosities with 0 and 10% glycerol by changing the viscosity constant, which is directly related to the open time. The nano-tip then aspirated a constant volume (10, 15, 20, 25 or 35 µl) as a function of the *y* value and dispensed one drop of the corresponding volume in each well of a 96-well plate. 96 fluorescence values were obtained for each *y* volume for statistical calculations.

3.2.2. Mother-liquor dispensing. The operation mode is a constant-volume aspiration and single dispense. All of the tips of the devices aspirate a constant volume of the 'daughter' solution from a reservoir of a 96-well plate and then single-dispense each *y* volume in a second plate.

A calibration was performed in duplicate for solutions with 0 and 10% glycerol in order to assure that the mother-liquor dispensers accurately dispense different viscosities with the same open-time.

The eight nano-tips of the Cartesian robot aspirated a constant volume (10 µl) from the first plate and dispensed one drop of each *y* volume in a second 96-well plate. For statistical calculations, 12 fluorescence values were obtained for each tip of a predeterminated volume.

The 96 nano-tips of the Phoenix aspirated a constant volume (10 µl) from a first plate and dispensed a drop in each well of a 96-well plate. Three plates were set up for each volume in order to obtain an average for statistical calculations. The drop volume for both dispensing methods was calculated by interpolation of the RFU value in the standard calibration curve (Table 2*b*).

4. Results and discussion

The purpose of this study was to develop a rapid and effective calibration method for evaluating the accuracy and precision of liquid-handling nano-dispenser devices. The proposed protocol is based on fluorescence measurements using fluorescein as an inexpensive fluorophore. It consists of three parts: (i) preparing the stock solutions, 'mother' and corresponding 'daughter' solutions, (ii) obtaining the calibration curve of fluorescein concentration *versus* fluorescence and (iii) calibrating the instruments based on the previous step. The most time-consuming part of the procedure is to adjust the fluorescein concentration of the 'daughter' solutions to the sensitivity of the local fluorimeter so that the response of the calibration curve is in the linear range and provides robust statistics. Once this range has been found, the calibration protocol is effective and fast. The 'mother' and 'daughter' solutions can easily be prepared and the nano-dispenser devices can be calibrated rapidly. The whole protocol can be performed in approximately 6 h per instrument, including the calibration curve. Once the curve has been calculated, the calibration measurements and statistical analysis can be completed in about 2 h. Unless the mechanical or optical configuration of the fluorimeter (or fluorimeter reader) has changed, the first part of the protocol does not need to be repeated.

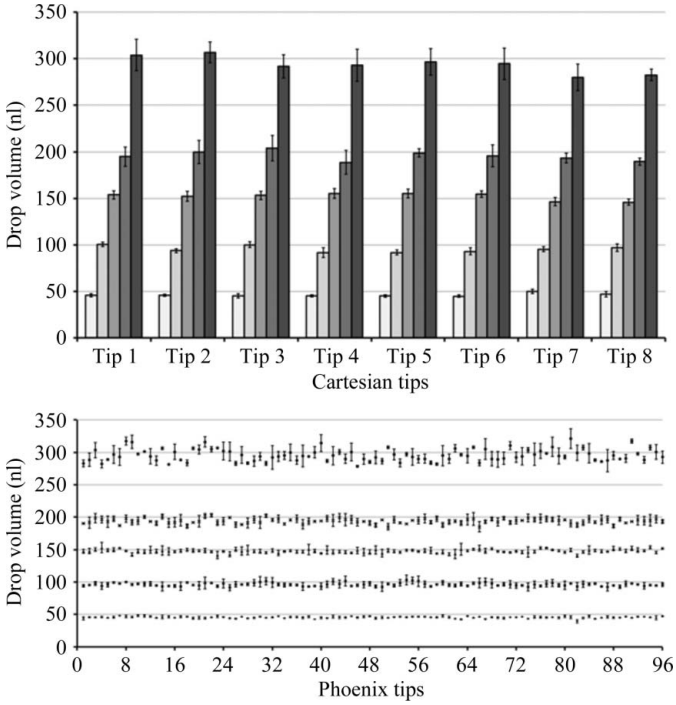


Figure 3
Diagrams of the mother-liquor-dispensing calibration results at 0% glycerol. The eight different tips for the Cartesian and the 96 tips for the Phoenix are shown on the abscissa. Error bars correspond to the different measurements for each tip and the number of observations were as follows. N = 12 measurements for the Cartesian; each tip dispenses a predetermined volume 12 times (one row) in a well of a plate. For the Phoenix device, the 96 tips dispense a predetermined volume in a well of a plate; N = 3 is for three different plates.

The most effective way to select the concentration range for the fluorescein 'daughter' solutions is as follows. From a 1 *M* 'mother' solution, series of dilutions are performed so that the final concentration for the calibration curve is in the range 1–250 µ*M*. This may depend on the specific fluorescence reader and is a critical step, since it has been reported that self-quenching occurs in solutions of free fluorescein at concentrations higher than 1.8×10^{-6} *M* (Imasaka *et al.*, 1977). For our instrument, the response was linear in the 10–90 and 110–210 µ*M* ranges, but the slope changed. For practical reasons, the concentration range selected for calibration of our instruments was 10–90 µ*M* (Fig. 2*b*). Details of the dilutions selected are presented in Table 1.

Following the above-described protocol, the results of the calibration of our Phoenix and Cartesian robots are shown in Table 2(*a*) for protein dispensing and in Table 2(*b*) and Fig. 3 for mother-liquor dispensing. In the protein dispensing, the CV is consistently below 10% in both sets of equipment for solutions with 0 and 10% glycerol. However, for the 50 nl volume Cartesian dispensing the CV is 13.9 for both viscosities. These results indicate that the instrument could not dispense this volume accurately using the single-aspiration multi-dispense method. The volumes of 100, 150, 200 and 300 nl are all dispensed with a CV of less than 10% and an SE of less than 5% (except for tip 4, 150 nl without glycerol). Whether the discrepancy with the lower volume is within the tolerance and specification of the instrument should be addressed for each particular robot.

In our laboratory, we have tried this calibration protocol using 40 mg ml^{-1} lysozyme with and without glycerol and did not observe any significant differences in the CV and SE statistical parameters in the presence or absence of protein. For this reason, we used only glycerol and not a mixture of protein and glycerol for calibration. We inferred that proteins of average viscosity and at concentrations of up to 40 mg ml^{-1} can be dispensed precisely and accurately using our instruments.

The CV for mother-liquor dispensing is lower than that for protein dispensing in the majority of cases (Table 2*b*). The critical volume, 50 nl, is dispensed with a lower CV but with an SE greater than 5%. Therefore, the aspirate/dispense mode dispenses smaller volumes more precisely, but the accuracy is not always acceptable. A graphical representation of these values is shown in Fig. 3, in which the error bars correspond to the number of times that the calibration has been performed, as indicated in §3.2.2.

Additionally, volumes of 100, 150, 200 and 300 nl are dispensed more precisely and accurately than those in the protein-dispensing mode. The presence of 10% glycerol does not affect the CV or SE parameters in the dispensing of the mother-liquor solutions. This reflects the capacity of these instruments to dispense mother liquor containing reagents of medium-to-low viscosity. A pictorial summary of the numerical values included in Table 2(*b*) is presented in Fig. 3.

5. Conclusions

A simple, inexpensive and effective calibration protocol has been described and tested in our laboratory that permits an assessment of the performance of liquid-handling nano-dispensing devices. For our instruments, the precision for protein dispensing (CV < 10%) shows that the protocol described here can be useful in determining volumes ranging from 100 to 300 nl for solutions with 0 and 10% glycerol. In the mother-liquor dispensing mode volumes of 100–300 nl are dispensed with better values of the CV and SE than those in the protein-dispensing mode. In both dispensing methods the CV and SE

parameters for the critical volume of 50 nl are barely within the acceptable range (slightly greater than 10%).

The calibration curve is the most time-consuming step (∼4 h) of the proposed protocol; the rest can be completed in approximately 2 h. It is suggested that this protocol should be used routinely (twice a year) to ensure the performance of liquid-handling nano-dispensing instruments in academic or industrial laboratories. Alternatively, it could be used when malfunction is suspected to provide reliable data to the repair personnel.

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