

Toward Quantitative Comparison of Fluorescent Protein Expression Levels via Fluorescent Beads

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1. MOTIVATION

Establishing an effective engineering discipline always requires standardized and comparable units of measurement. Such measurements serve as a means of communication between the people and machines interacting with a project, ensure compatibility between components, and allow prediction of the results of design decisions. Regulating gene expression is foundational for organism engineering, and flow cytometry is an excellent means of quantifying large numbers of single cell gene expression measurements. At present, however, flow cytometry data is still often acquired in arbitrary or relative units, without standardizing the measurement by comparison to an independent reference material (i.e., one enabling precise calibration of measurements). Some have proposed standardizing to a biological cultured reference material (e.g., [3]), but fluorescence from such materials varies strongly, unpredictably, and often not proportional to the samples it is intended to be a reference for, thus resulting in a large degree of uncertainty in measurement.

In contrast, stable reference materials have been developed, in the form of beads with a defined fluorescence quantified in terms of molecules of equivalent reference fluorophores (ERF; alternately MEF or ME[fluorophore]) [5]. These reference materials have been primarily employed in medical applications of flow cytometry, which typically use a small number of standard dyes rather than a wide range of fluorescent protein variants, and where the goals of measurement are typically focused on the “digital” goal of classifying cells into distinct populations, rather than the more “analog” goal of precisely quantifying levels of gene expression.

Fluorescent beads have already been used as a reference material for engineering gene expression in a number of studies, including making high-precision circuit predictions (e.g., [2]), engineering novel biological sensors (e.g., [4]), and debugging circuit design problems (e.g., [1]). We now aim to validate these methods through interlaboratory studies and to develop supporting methods and recommended practices that will simplify widespread adoption of well-defined units in flow cytometry, thus accelerating scientific development and simplifying the engineering of biological organisms.

2. USAGE SCENARIOS

We have identified four key usage scenarios for bead-based

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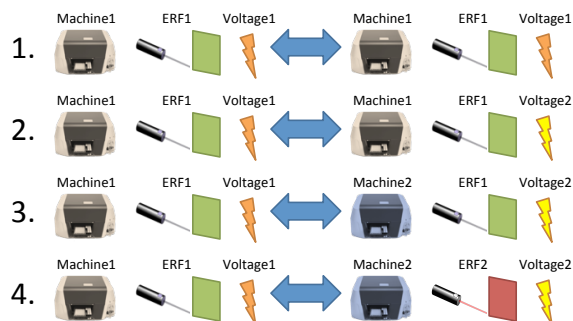


Figure 1: Scenarios for use of ERF-calibrated beads in comparing flow cytometry data.

comparison of fluorescence measurements, along with key target applications motivating their development. In these scenarios, an “ERF-quantified laser/filter combination” means that a set of beads has been assigned ERF values for their intensity when excited with a particular laser frequency and observed through a particular optical filter (e.g., via the process in [5]). If a significantly different laser or filter are used, then the quantification does not apply. The four scenarios (also illustrated in Figure 1) are, in increasing levels of complexity:

1. Comparison of samples with the same ERF-quantified laser/filter combination, on the same machine, with the same voltage settings. *Target application: fusion of data sets.*
2. Comparison of samples with the same ERF-quantified laser/filter combination, on the same machine, but different voltage settings. *Target applications: fusion of data sets, extension of data range.*
3. Comparison of samples with the same ERF-quantified laser/filter combination, but different machines (making voltage comparison moot). *Target applications: fusion of data sets, validation of material or method transfer.*
4. Comparison of samples with different ERF-quantified laser/filter combinations (making machine and voltage comparison moot). *Target applications: fusion of data sets, validation of material or method transfer, comparison of multiple signals.*

Note that in no case are the target applications focused on comparison of a cell sample to ERF-quantified beads, per se. Rather, the goals are focused on comparison of cell samples, as enabled by comparing each sample to a set of beads.

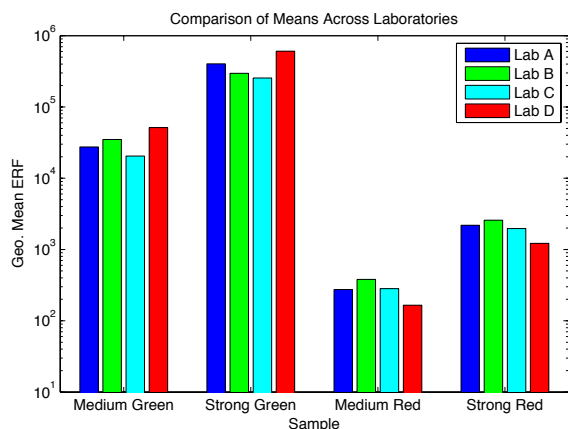


Figure 2: Pilot study measurements show a 1.43-fold geometric standard deviation of mean ERF measurements across laboratories (laboratories indicated by color; red is the laboratory with unmatched channels).

Thus, validity of measurements depends primarily on the relationship between cell samples and beads remaining stable across space and time, rather than the actual relationship between cellular fluorescent protein and ERF, which is much more difficult to assure.

3. PILOT STUDY RESULTS

As an initial test of using ERF-quantified beads to establish common units for Scenarios 1-3, we conducted a pilot interlaboratory study of bead-calibrated measurements of *E. coli* expressing GFP and mCherry across four flow cytometers, each in a different laboratory. Three of the flow cytometers had closely matching filters for GFP and mCherry, while the fourth had significantly different filters for both. Five sets of samples of *E. coli* were prepared at one of the four laboratories: empty vector, medium GFP expression, strong GFP expression, medium mCherry expression, and strong mCherry expression. Each sample was then split into aliquots and shipped frozen, to be measured at each laboratory in three independently prepared replicates of each sample at three channel voltages, chosen to spread measurements across the range of each instrument.

Figure 2 and Figure 3 present statistical results from these samples computed by gating for cell events using a gate based on the empty vector sample, calibrating to ERF units using the provided ERF values for SpheroTech RCP-30-5A beads of the appropriate batch (MEFL for green, MEPTR for red), then computing geometric mean and standard deviation (geometric statistics are used throughout due to the fact that the distribution of each sample is roughly lognormal, as is often observed with flow cytometry).¹ Figure 2 compares the geometric mean of ERF values for each laboratory. Note that the geometric means are all fairly close to one another: their standard deviation is 1.43-fold across all laboratories and 1.23-fold for the three laboratories with matched channels. Examining the geometric mean

¹The strong GFP samples, however, had a very low density of events, and so for those samples we report the mode of the upper distribution component instead.

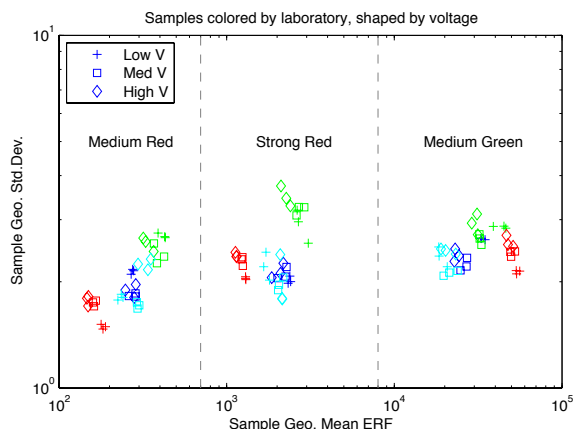


Figure 3: Geometric mean and standard deviation for individual samples are clustered tightly for each laboratory and for each voltage within a laboratory.

and standard deviation of the events in each individual sample (Figure 3), we find that laboratory-to-laboratory variation amongst laboratories with matched channels is on the same order as replicate-to-replicate variation (standard deviation 1.05-fold) and voltage-to-voltage variation (standard deviation 1.11-fold).

4. CONTRIBUTIONS AND FUTURE WORK

As expected, these preliminary results indicate that fluorescent beads can be used for precise quantitative comparison of fluorescent protein expression, even despite some degree of variation in instrument configuration. We are now in the process of scaling up to a larger and more comprehensive interlaboratory study, with which we hope to definitively establish the efficacy of commercially available ERF-quantified calibration beads for quantification of fluorescent protein expression. Further goals include validating methods for comparison of the expression levels of different fluorescent proteins, improving analytical software to make these methods readily accessible, and quantifying the sources of variation.

5. REFERENCES

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