# Analysis of Nitrocellulose-Based Solid-State Calibration Standards for Point-of-Care (POC) Fluorometers

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***Abstract*— This study presents an analysis of nitrocellulose-based solid-state calibration standards for point-of-care (POC) fluorometers. Our findings show that the manual assembly process introduces significant variations in the alignment/offset of printed lines with respect to the optical aperture. A Claremont BioSolutions and BioDot automated dispenser were compared to determine efficacy in producing high-quality calibration standards. Qualitative analysis of line morphology shows less pronounced irregularities in lines printed by the BioDot. The BioDot also demonstrated significantly lower variation in the width of printed lines, with a CV of 6.4% compared to the Claremont's 27.0%. However, fluorescence intensity measurements using a POC fluorometer revealed a mean inter-sample CV of 15.0% and 14.2% for the Claremont and BioDot, respectively. Direct and indirect fluorophore immobilization methods were compared. The results indicated that the direct method is a viable approach, but the indirect method presents challenges with increased background and fluorophore depletion, rendering it unsuitable for calibration standard production.**

***Clinical relevance*— This work investigates the efficacy of nitrocellulose-based solid-state standards for calibrating POC fluorometers, which are essential for ensuring accurate diagnosis and screening of patient samples.**

## I. INTRODUCTION

Fluorometers play an important role in diagnostics and biomedical research due to their high sensitivity and specificity [1]. In point-of-care (POC) applications, fluorometers enable rapid, on-site analysis, which is essential for early detection and effective interventions [2]. Accurate and reliable calibration of POC fluorometers is vital for ensuring consistent performance and reproducible results. Calibration involves using standard references to ensure consistent instrument response across channels, thereby compensating for hardware-specific variations in the device [3]. This process is critical for maintaining the precision and accuracy of POC fluorometers, ensuring they provide reliable data for diagnostic and monitoring purposes.

Traditional solution-based calibration standards are commonly used for accurate calibration of fluorometers; however, they pose challenges related to compatibility, photostability, and handling [4]. Solid-state calibration standards have emerged as promising alternatives due to their ease of use, enhanced photostability, and reusability [5]. These standards typically involve immobilizing fluorophore onto solid substrates [6]. The quality and reproducibility of the immobilization process can significantly influence the performance of calibration standards.

In this work, we present a comprehensive analysis of nitrocellulose-based solid-state calibration standards for POC fluorometers. We examine the reproducibility of line alignment, morphology, and width, focusing on their impact on the quality of the printed lines. We evaluate the performance of two automated dispensers to determine their efficacy in producing high-quality calibration standards. Additionally, we compare two methods of fluorophore immobilization: the direct method, which involves printing the fluorophore directly onto the nitrocellulose, and the indirect method, which involves printing immunoglobulin G (IgG) onto the nitrocellulose followed by capture of the fluorophore-conjugated secondary. We aim to identify the most effective strategies for producing reliable and accurate calibration standards for POC fluorometers and identifying potential causes of significant inter-sample variation.

## II. MATERIALS AND METHODS

### A. Calibration Standard Assembly

The solid-state calibration standards employed in this study consist of two lateral flow test strips enclosed within a 3D-printed cartridge (Fig. 1c). Each lateral flow strip (70 mm × 5 mm) is composed of a sample pad, adhesive-backed nitrocellulose membrane, absorbent pad, and four analysis sites (Fig. 1a). The cartridge was fabricated in acrylonitrile butadiene styrene (ABS) using a fused deposition modeling (FDM) printer. The top and bottom components feature 3.8 mm × 3.8 mm apertures for light transmission (Fig. 1b). The strips are secured within the cartridge utilizing an interference fit.

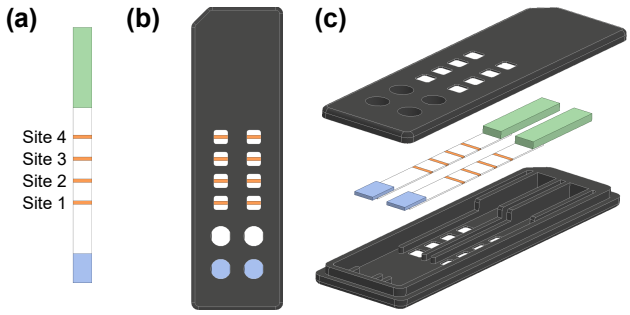


Fig. 1. (a) Schematic of the lateral flow strip with the sample pad (blue), nitrocellulose (white), absorbent pad (green), and analysis sites indicated. (b) Top and (c) exploded views of the strip/cartridge assembly.

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### B. Automated Dispensing Systems

In this study, we utilized a Claremont BioSolutions Automated Lateral Flow Reagent Dispenser (ALFRD) and BioDot XYZ3060 Dispense System to fabricate nitrocellulose-based calibration standards. The Claremont dispenser is an economical ($4,750), compact device designed for precision reagent dispensing onto lateral flow membranes [7]. In contrast, the BioDot XYZ3060 is a high-end system ($55,499) with advanced precision and versatility in dispensing a wide range of fluids with high accuracy [8]. This study aims to compare the performance of these two dispensing systems to assess whether the substantial increase in cost with the BioDot XYZ3060 results in enhanced fabrication of nitrocellulose-based calibration standards.

### C. Fluorophore Immobilization Techniques

Two techniques were evaluated for immobilizing fluorophores onto nitrocellulose membranes. For both methods, Cy3 was conjugated to anti-human IgG secondary antibodies and diluted in phosphate-buffered saline (PBS) to ratios of 1:25, 1:50, 1:100, and 1:200. The direct method involved printing the fluorophore-conjugated secondary antibody directly onto the nitrocellulose membrane. The indirect technique involved first dispensing IgG onto the nitrocellulose. Cy3-conjugated secondary was subsequently applied to the sample pad, migrating through the strip via capillary action.

### D. Fluorescence Instrumentation and Image Analysis

The POC fluorometer used in this work is an updated iteration of the device developed by Obahiagbon et al. [9]. This device features eight analysis sites operating in a transillumination configuration. The excitation side contains 520 nm LEDs mounted on a printed circuit board (PCB) equipped with individual excitation filters. The emission side is equipped with PIN photodiodes operating in photovoltaic mode fitted with individual emission filters. A charge integration circuit and microcontroller are employed for signal readout.

A benchtop fluorescence scanner (Lionheart FX, BioTek) and image analysis software (Gen5, BioTek) were employed for image acquisition and processing. The fluorescence scanner was used to capture high-resolution images of the printed lines and the image analysis software was used for object measurements and fluorescence quantification. The platform offered precise quantification and detailed visualization of the data, serving as a reference for comparing results obtained with the POC fluorometer.

### E. Consideration of Photobleaching Effects

Photobleaching is a concern in fluorescence measurements because it reduces signal intensity over time, resulting in potentially inaccurate and/or biased data [10]. Photobleaching is the irreversible degradation of a fluorophore caused by light exposure [11]. The degree of photobleaching experienced by a fluorophore is significantly impacted by excitation intensity and exposure time [12]. For example, Cy3 experiences an approximate 15% reduction in fluorescence intensity after 30 seconds of continuous illumination with a 100 W mercury arc lamp [13]. Although Cy3 is generally considered a bright and photostable fluorophore, measures were implemented in the experimental approach to minimize photobleaching effects.

Low-power (96 mW peak) 520 nm LEDs were used in the POC reader for excitation. A constant current LED driver was implemented to enable pulse width modulation (PWM) for adjusting perceived LED brightness. A sequential activation pattern provided continuous illumination at a 10% duty cycle for a maximum of 20 seconds per channel. Preliminary data demonstrated negligible crosstalk between channels, indicating a low probability of photobleaching contributions from adjacent channels. In the benchtop fluorescence scanner, a 523 nm LED module coupled with a red fluorescent protein (RFP) filter cube was used for excitation. The LED intensity was set to 10%; camera gain and integration time were adjusted to enhance image brightness without increasing sample illumination. We employed pulsed illumination (100 ms) during image acquisition to further mitigate photobleaching effects.

## III. RESULTS AND DISCUSSION

### A. Alignment Reproducibility

The fabrication process of the calibration standards is highly manual, potentially introducing variations in measured fluorescence intensity unrelated to printing quality and immobilization technique. In this experiment, we assessed reproducibility of line placement by measuring the offset of each line relative to the center of the cartridge aperture (Fig. 2a). The resulting histogram revealed a slightly left-skewed distribution with a mean offset of 554 µm (Fig. 2b). The minimum and maximum offsets were 27 µm and 1039 µm, respectively, with a coefficient of variation (CV) of 43.5%. These findings indicate significant variation and low reproducibility in the alignment of the printed lines with respect to the center of the cartridge aperture. This variation likely yields inconsistent fluorescence signals and has a negative impact on calibration accuracy in the POC fluorometers.

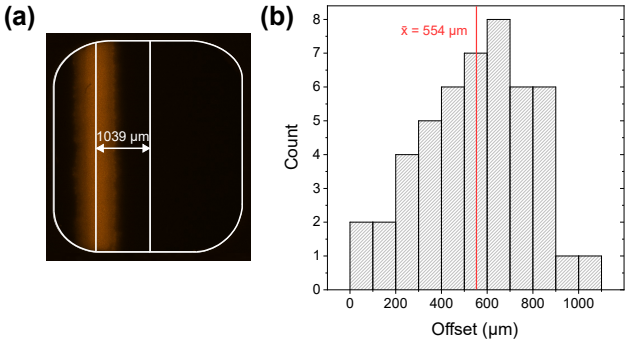


Fig. 2. Example of the line with the greatest offset with respect to the center line of the aperture.

### B. Qualitative Analysis of Line Morphology

This experiment aimed to qualitatively analyze and compare the morphology of lines printed by the Claremont and BioDot dispensers due to observed irregularities in the printed lines. The results showed that both dispensers produced lines with anomalous morphology (Fig. 3). However, the Claremont dispenser produced lines with more pronounced irregularities. The BioDot dispenser displayed less deviation between normal and anomalous lines. Visual inspection of the Claremont tubing indicated that air bubbles were likely responsible for the irregular line shapes. These findings suggest that the sophisticated design of the BioDot system may effectively mitigate printing issues, resulting in improved line morphology.

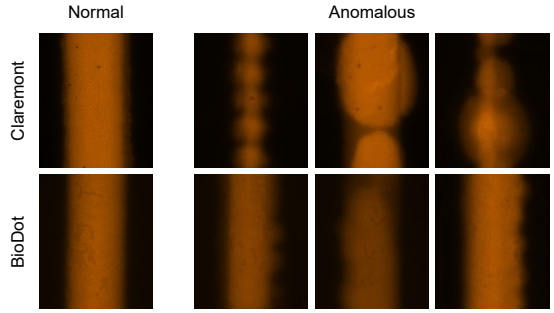


Fig. 3. Normal and anomalous lines produced by the Claremont and BioDot printers.

### C. Line Width Reproducibility

We observed notable variation in the width of the printed lines, prompting a comparison of the reproducibility of line widths produced by the Claremont and BioDot dispensers. The results indicated a greater range of line width measurements for the Claremont dispenser compared to the BioDot (Fig. 4). The Claremont printed lines had a minimum, maximum, and mean width of 570 µm, 1640 µm, and 1018 µm, respectively. The BioDot printed lines with a minimum, maximum, and mean width of 1245 µm, 1704 µm, and 1383 µm, respectively. The BioDot printed lines demonstrated significantly less variation, with a coefficient of variation (CV) of 6.4%, compared to 27.0% for the Claremont. These results indicate substantially higher reproducibility of line width for the BioDot system.

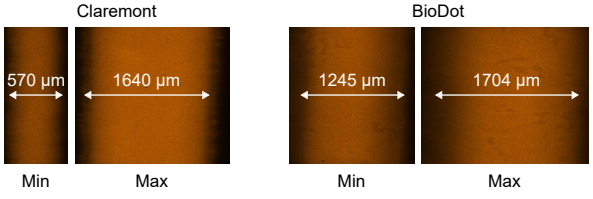


Fig. 4. Minimum and maximum line widths produced by the Claremont and BioDot dispensers.

### D. Fluorescence Intensity Variability

We examined whether improved repeatability in line width printing would reduce variations in fluorescence intensity. Although the BioDot system produced lines with more consistent widths, our results showed that lines of nearly identical width can still exhibit significantly different fluorescence intensities. A line with only a 0.4% greater width exhibited a 34.6% greater intensity in fluorescence emission (Fig. 5a). Fluorescence intensity measurements from the POC fluorometer yielded a CV for the Claremont of 13.3%, 13.3%, 13.2%, and 20.3% at 1:200, 1:100, 1:50, and 1:25 dilutions, respectively (Fig. 5b). CV for the BioDot at the same dilution factors were 12.3%, 15.2%, 11.2%, and 18.1%. The average CV across all dilutions were 15.0% for the Claremont and 14.2% for the BioDot. These findings suggest that greater printing precision with the BioDot does not necessarily translate to significantly reduced variation in fluorescence intensity measurements in the POC reader.

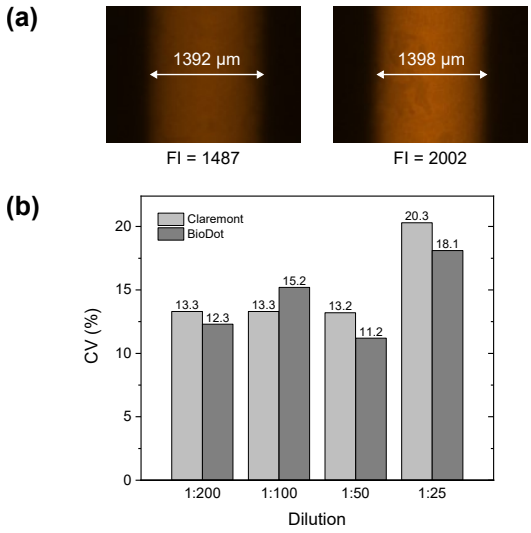


Fig. 5. (a) Example of two lines with nearly identical widths but significantly different fluorescence intensities (FI). (b) CV for lines printed with the Claremont and BioDot at various dilution ratios.

### E. Background Signal Intensity

We evaluated the indirect method of fluorophore immobilization to compare its performance with the direct method, focusing on its impact on background signal. Four strips were tested by flowing the Cy3-conjugated secondary antibody through the strip. Background signal intensity was quantified using a line profile of the exposed nitrocellulose between the sample and absorbent pads (Fig. 6a). The fluorescence intensity line profiles revealed significant differences in background signal across strips (Fig. 6b). Background signal was inconsistent across sites within a strip, with two of the four strips displaying peaks due to fluorophore accumulation. Additionally, baseline background signal varied between strips, with some strips exhibiting a 2- to 3-fold higher baseline signal than others. These results indicate that the indirect immobilization method introduces additional variability in

fluorescence intensities, having a potentially negative effect on calibration.

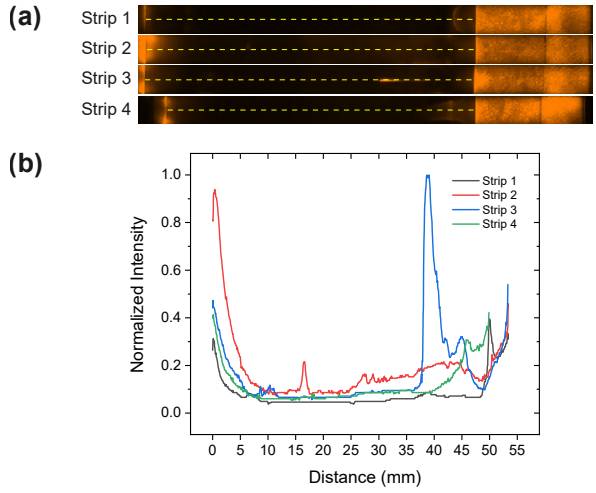


Fig. 6. (a) Visualization of background signal intensity with yellow dashed lines indicating line profiles used for quantification. (b) Background intensity measurements generated from the line profiles for each strip.

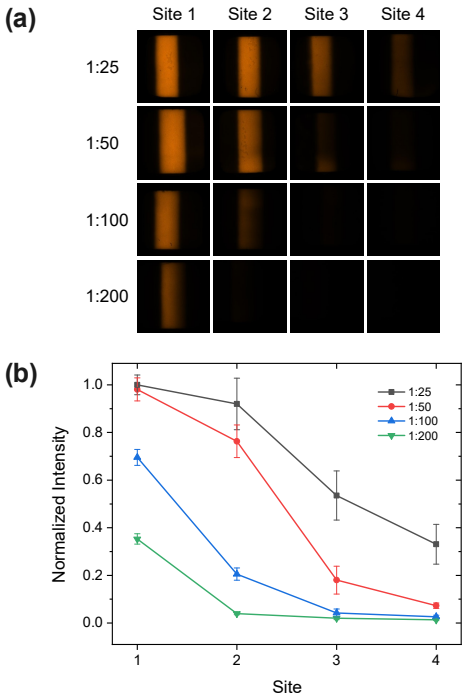


Fig. 7. (a) Visualization and (b) quantification of fluorophore depletion across analysis sites for various dilutions.

### *F. Fluorophore Depletion*

We assessed the inter-line variability of strips prepared using the indirect immobilization technique to compare it with that of strips prepared using the direct method. The results showed that fluorophore depletion occurred as the solutions flowed across the nitrocellulose (Fig. 7a). Even at the highest concentration tested, a 67% reduction in fluorescence intensity was measured from the first to the last line (Fig. 7b). This depletion resulted in a CV of 45.5%, 88.4%, 129.0%, and 154.7% for the 1:25, 1:50, 1:100, and 1:200 dilutions, respectively. These findings demonstrate that depletion significantly impacts inter-line variability and calibrator performance, rendering the indirect method unsuitable for producing consistent fluorescence intensities across sites.

## IV. CONCLUSION

This study highlights the feasibility and limitations of nitrocellulose-based solid-state calibration standards for POC fluorometers. Although direct immobilization of fluorophores onto nitrocellulose using automated dispensers provides acceptable performance, the moderate reproducibility underscores the need for higher quality calibration standards. The BioDot dispenser's enhanced precision demonstrates some benefits, but it does not fully address the issues of inter-sample variation. Our findings emphasize the necessity for more advanced and consistent calibration methods to ensure reliable and accurate diagnostics in POC applications.

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