Development of a Quantifiable Optical Reader for Lateral Flow Immunoassay

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Abstract—**An optimized optical reader was developed for quantitative fluorescence detection of lateral flow immunoassay in point-of-care test (POCT). Different from existing readers for qualitative test, the developed reader is able to perform quantitative test with a line-shaped excitation beam to obtain the distribution of fluorescence signal intensity along the lateral flow strip. To reduce system complexity, a custom Y-shaped optical fiber, instead of one set of optical lenses, was adopted to couple the excitation and emission channels. A high power Light Emitting Diode (LED) with a specific wavelength interfaced with one of the two split ends of the optical fiber for excitation, while a highly sensitive photodiode with the other split end for detection. A linear stage with high resolution, which was driven by a step motor, was incorporated to facilitate strip scanning. Considering the inhomogeneous nature of lateral flow immunoassay, an optimized global searching algorithm was developed to identify test and control peaks and their boundaries, which further improves reader's sensitivity and specificity. Finally, a calibration model was built into the reader to report the concentration of the test sample. As an example, quantitative CRP (C - reactive protein) test was performed on this reader achieving a reasonable clinical diagnostics range from 1 μg/mL to 200 μg/mL. With its machine reading function, simplicity and relatively low cost, the optical reader could play an important role in POCT.**

Keywords- Optical Reader; Lateral Flow Immunoassay; Point-of-Care Test (POCT); Quantitative Detection; Global Searching Algorithm; CRP (C - reactive protein)

I. INTRODUCTION

In recent years, point-of-care test (POCT) has become increasingly popular in medical society [1-4]. It is a type of medical testing that is performed at or near the site of patient care with distinct characteristics of immediacy, easiness, convenience, portability and low-cost [2]. Immunoassays exploit the sensitivity and specificity of antibody–antigen interactions for the detection of analyte of interest [5]. Immunoassays play a prominent role in medical diagnostics for the detection of biomarkers of disease, metabolites, and other molecular targets. As a good example of POCT, immunochromatographic tests implemented as nitrocellulose lateral flow (LF) strips provide an elegantly simple realization of an immunoassay that is rapid, inexpensive, and easy to use [6, 7]. Such LF strips are widely used by at clinics, pregnancy and drugs of abuse tests. In addition, they are often employed to detect food and water for bacterial contamination [8].

In a common embodiment, the LF strip assay is comprised of a narrow (2-4 mm wide, 10-30 mm long) nitrocellulose membrane to which a porous sample loading pad containing dried reporters (e.g., gold or carbon particulate labels conjugated with anti-target antibodies) is affixed at one end, and an absorbent pad is attached to the opposing end [9]. A 'test line' zone to capture analyte is formed on the nitrocellulose membrane by immobilizing antigen or antibodies that bind the analyte, while a 'control line' zone is formed downstream the 'test line' for assay verification. To run a test, a liquid sample (e.g., blood, urine, saliva or food) is applied to the sample loading pad. Any analyte contained in the sample binds the reporter, and the resulting target-label complex flows along the porous nitrocellulose membrane due to capillary forces, induced in part by the absorbent pad at the downstream end of the strip. As the sample wicks down the strip, the labeled analyte binds at the test line. The accumulated gold- or carbon-labeled target complex at the test line provides a visible indicator for the presence of analyte.

Despite their substantial advantages, lateral flow strip immunoassays are generally limited to screening applications, particularly at the point of care, because they are not easily quantifiable, not sufficiently reproducible, and are not sensitive enough for certain applications when gold or carbon particulate labels are used [10]. Therefore, people attempted to replace traditional visible labels with others, such as fluorescence particle reporters to improve the sensitivity and accuracy, which when optically excited emit an optical signal of characteristic wavelength [11-12]. With fluorescence labels,

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the sensitivity and accuracy of the lateral flow test can be significantly improved because of wide dynamic range. For example, qualitative lateral flow test with an Uplink reader has been developed to improve detection sensitivity with significantly low background by using up-converting phosphor (UCP) particles [13]. However, for quantitative test, the lateral flow immunoassay needs to be further improved to decrease the effect of inhomogeneity. At the same time, parried optical readers for fluorescence signal reading need to be built [14]. For most of them, a laser and Photo Multiplier Tube (PMT) were used respectively for excitation and detection with sets of optical lens. For example, a 2-D optical scanner was developed for imaging and quantification of up-converting phosphor (UCP) labels on a strip within 8 min, whose optical module was mounted on a motorized dual-axis linear translation stage to perform 2-D scanning [15].

C-reactive protein (CRP) is a protein found in the blood, whose level rises in response to inflammation or infection [16, 17]. The detection of CRP in blood serum is of great value in the surveillance of the presence and intensity of inflammation and infection, as well as in distinguishing between bacterial and viral infections [18]. In addition, CRP can also be utilized in diagnostics for cancer, cardiovascular diseases, fibrosis and obstructive sleep apnea [19-22]. Existed semi-quantitative or quantitative CRP detection formats include immunoradiometric ELISA, latex-enhanced immunonephelometric method, automated immunoturbidimetric method, barcode-style lateral flow assay, and solid-phase sandwich fluorescence immunoassay [22-25]. Due to the complicated procedure with multiple steps and the heavy diagnostics devices, most of them are limited to central clinical labs. Therefore, for point-of-care test, simple, easy-to-use, rapid and accurate CRP diagnostics methods need to be developed.

Previously, for pouch-actuated, lateral flow immunoassay microfluidic cassettes, we have developed a portable analyzer which houses a compact, qualitative OEM type UCP detector to scan and read fluorescence signal on a strip [13, 26]. As a continued work, here, an optimized reader was developed to perform quantitative test. The compact reader relies on a LED and photodiode respectively for excitation and detection. Instead of using a pair of coupled optical lenses a custom Y-shaped optical fiber was designed and adopted to couple the excitation and emission channels with low complexity. Different from 2-D scanning [15], a fast quantitative test could be done by smoothly moving the strip on a motorized 1-D moving stage under a line-shaped excitation beam within 1 minute, which reduces the risk of non-specific binding in lateral flow immunoassay. Considering both the inhomogeneous nature of the lateral flow immunoassay and the potential signal noise, an optimized global searching algorithm was developed to analyze the signal reading of the strip, which further improves the reader's sensitivity and specificity. Finally, a simplified calibration model was built to figure out the concentration of the test sample based on the fluorescence signal.

As a proof of effectiveness, quantitative CRP test was performed on the developed reader with the reasonable clinical diagnostics range from 1μg/mL to 200μg/mL achieved successfully. With the characteristics of quantification, low cost, portability, and convenience, the developed POCT reader is expected to enhance accurate lateral flow test on traditional cassettes or even microfluidic chips.

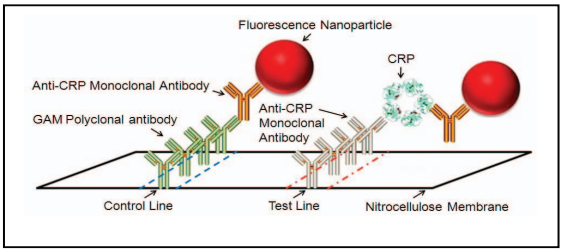

Figure 1. A schematic depiction of the sandwiched lateral flow immunoassay

II. MATERIALS AND METHODS

A. *Immunoassay*

The lateral flow immunoassay strip for CRP detection is comprised of a narrow (~4 mm wide, ~10 mm long) nitrocellulose membrane (HF135, EMD Millipore, Inc. Massachusetts, USA), of which a porous sample loading pad containing dried fluorescence reporters is attached to one end, and an absorbent pad is affixed to the opposing end. The antibodies conjugated with fluorescence particles are 10C5, which are affinity-purified mouse against human monoclonal antibodies made in house. The fluorescence particles (fluoro-max-fluorescent-carboxylate-modified-particles, Thermal Scientific Inc. Texas, USA) with the diameter of 0.322 μm were purchased from Thermal China. Its excitation wavelength is 365nm and emission wavelength 610 nm. A 'test line' zone to capture analyte is formed on the nitrocellulose membrane by immobilizing anti-CRP monoclonal antibodies that can bind to the CRP protein. Downstream the 'test line' a 'control line' zone formed by immobilizing polyclonal antibodies that can bind to anti-CRP monoclonal antibodies. The antibodies immobilized on the test line are 7D9, which are affinity-purified mouse against human monoclonal antibodies made in house. The antibodies on the control line are affinity-purified goat against mouse (GAM) polyclonal antibodies made in house. For quantitative detection, the lateral flow immunoassay protocol was optimized to improve its homogeneousness, stability and repeatability. Fig. 1 is a schematic depiction of the sandwiched immunoassay carried out on the LF strip.

The monoclonal antibodies (7D9) in buffer were spotted onto the test line area of the nitrocellulose membrane with the concentration of 2 mg/mL using the spotting machine (BioDot XYZ3050, BioDot, Inc. California, USA), and polyclonal antibodies in buffer with the concentration of 1 mg/mL onto the control line area. The fluorescence particles (volume concentration: 1%) were conjugated with monoclonal antibodies (10C5) with the reagent of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Thermal Scientific, Inc. Texas, USA) within 2 hour incubation at room temperature in house based on the standard protocol [27]. The conjugates were freeze-dried on the sample pad (8x4 mm) with the antibody concentration of 0.1μg/mL.

To run a CRP test, a liquid sample is blotted on the sample loading pad first. Any CRP protein contained in the sample binds to the fluorescence conjugates, and the combined CRP-fluorescence conjugate complex flow along the porous nitrocellulose membrane due to capillary forces, induced in part by the absorbent pad of the strip. When the sample wicks down the strip, the labeled CRP proteins are captured by the test line, while the free fluorescence conjugates by the control line. After incubated for a predefined time, such as ~15 minutes, the fluorescence signal of the test and control lines can be read out with an optical reader for CRP concentration calculation.

B. Quantitative Detection of CRP

It can be easily understood that the amount of CRP, or CRP-fluorescence conjugate complex captured on the test line is proportional to their corresponding fluorescence signal intensity. However, existed methods with a single excitation light point cannot obtain the fluorescence signal intensity distribution of the entire test line unless 2-D scanning is used as in ref. [15]. To simplify quantifiable detection, an upgraded 1-D scanning with a line-shaped excitation beam is developed and presented here to obtain the entire fluorescence signal intensity distribution of the detection window, which covers the area between the test and control line of the strip. A moving strip is continually excited by a line-shaped excitation beam whose length is close to that of the test line, and at the same time, the strip's fluorescence emission is collected by the detector, until the detection window is scanned completely. Fig. 2 depicts signal readings of strips with low and high positive CRP samples in mV units as functions of position along the strip. In Fig. 2, the total scanning length is 10mm, where totally 200 data points spread averagely. In Fig. 2, the inset is a schematic of strip scanning with a line-shaped excitation beam.

In Fig. 2, the letters T and C, denote respectively the test and control peaks. It is generally accepted that the area under the test peak is proportional to the amount of target analyte. The low positive sample yielded a low test peak and a high control peak. The high positive sample yielded a high test peak and a low control peak. In principle, the amplitude or the area of the test or control peak can be regarded as the signal reading. As what has been adopted by other existed lateral flow tests, the area value under the test line (T) is normally divided by that of the control line (C) for calibration before it is regarded as the final test result, as following:

$$\text{Signal Ratio} = T / C \tag{1}$$

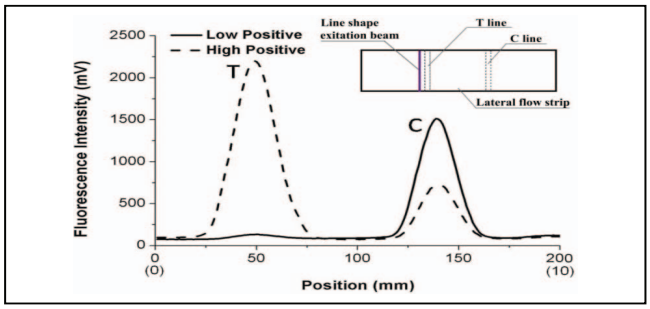

Figure 2. Scans of LF strips for detecting the presence of CRP in diluted samples. The read signal is depicted as a function of position along the LF strip for low positive (solid line), and high positive samples (dash line). The inlet is a schematic of strip scanning with a line-shaped excitation beam.

C. Response Curve Fitting Model

Instead of using existed four- or five- parameter logistic (4PL or 5PL) model to estimate the detection response [28], a simplified logarithmic model with only two parameters was developed and built into the optical reader, as following:

$$\lg(\text{Signal Ratio}) = a * \lg x + b \tag{2}$$

where x is the analyte concentration, a is the slope factor, and b is the bias. In the exponential log space, it is a simple linear model between the detection signal and the analyte concentration. Model parameters can be identified with the least square method which is able to identify the best fitting curve by minimizing the sum of squared errors (SE)[29], defined as

$$SE = \sum_{i=1}^{N} (y_i - y_i')^2 \tag{3}$$

where N is the number of analyte concentration, yi and yi' are respectively the detected result and fitted response at each analyte concentration.

D. Optical Reader

The optical reader consists of an optical module, a motorized scanning stage, a photoelectric conversion module, a LED module, and an electrical module for data acquisition and system control, as shown in Fig. 3.

Compared to the existed readers including separate excitation and detection modules [6], the complexity of the optical module of the developed reader has been significantly reduced with a custom Y-shaped optical fiber (Nanjing Hecho Technology Co., Ltd, China). The common end of the Y-shape optical fiber was divided into three zones, one for excitation, another two for detection, see B-B' view in Fig. 3. The line-shaped excitation beam (width: 0.5 mm), which consists of hundreds of thin optical fibers, transmits the excitation light

from an UV LED coupling with one of the split ends of the Y-shaped fiber to the strip. The two detection zones adjacent to the excitation line in the common end consists of hundreds of thin optical fibers to collect the fluorescence emission from the strip through two focusing lens, and finally hitting the photodiode through the optical fiber bundle.

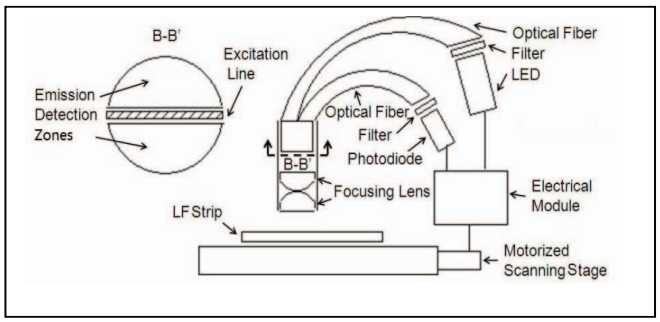

Figure 3. Schematic diagram of the optical reader

The central excitation and emission wavelengths of the fluorescence particles are 365 nm and 610 nm respectively. Therefore, a 365 nm UV LED (Sensor Electronic Technology Inc. USA) with a glass band-pass filter (320-390 nm, Beijing Bodian Optical Tech. Co. Ltd, China) was adopted for excitation, and a sensitive photodiode (S2386-5K, Hamamatsu, Japan) with a glass longpass edge filter (LP580, Beijing Bodian Optical Tech. Co. Ltd, China) was adopted for detection. As shown in Fig. 3, the UV LED and the photodiode with filters were attached respectively to two split ends of the custom Y-shape optical fiber.

The linear scanning stage was driven by a step motor (SST39C1010, Dong Guan Shinano Motor Co., Ltd., China). The electrical module guided the step motor to perform strip scanning with a step motor driver, and simultaneously collected the photoelectric conversion signal from the photodiode. A 32-bit microcontroller (STM32F407V, STMicroelectronics, Swiss) with the Cortex-M4 CPU was used to operate the reader. A constant current source with good stability was used to drive the UV LED with the flexibility of amplitude setting. For analog to digital signal conversion, a 16-bit A/D convertor (AD7707, Analog Devices, USA) was applied to ensure detection resolution and accuracy. After sampling and filtering, 400 data points, which spread averagely within 10mm scanning range, were used to analyze the fluorescence signal intensity distribution along the LF strip. One test can be finished within 1 minute after the strip is put into the reader. The optical reader can be easily operated with a touch-screen through by PIC controller (PIC24FJ256D, Microchip, USA), and the test data can be saved, printed out or transmitted to other place for further analysis.

E. Global Searching Algorithm

As discussed before, to figure out the analyte concentration, it is necessary to analyze the fluorescence signal along the lateral flow strip with reasonable accuracy. Witnessing a couple of facts including inhomogeneous nature of lateral flow assay, non-specific bonding and potential signal noise from the electrical system of the reader, an optimized global searching algorithm, which is able to identify the test and control peaks as well as their boundaries with good repeatability and accuracy, was developed to improve the reader's sensitivity and specificity. Fig. 4 is the flowchart of the developed algorithm.

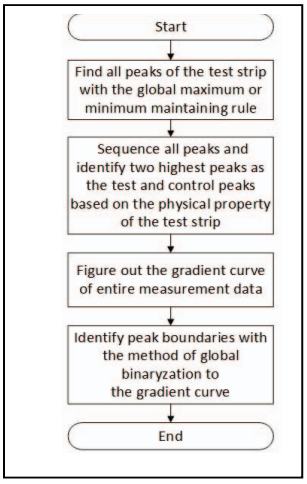

Figure 4. Flow chart of the developed global searching algorithm

First, all the measured data is processed with the maximum-minimum maintaining rule to find out all the peaks or troughs. At the first step, each data point is compared with the former one to find out the local maximal point until the amplitudes of the data points start to decrease. At the second step, with the local maximal data point as the starting point, each continuing data point is compared with the former one to identify the local minimal point until the amplitudes of the data points start to increase. To improve the algorithm's immunity to signal fluctuation caused by noise or inhomogeneous assay, each state (rising or descending) is recognized only when the difference amplitude between each two data points is larger than a pre-defined threshold. The state switching between rising and descending indicates a peak or trough. In this way, all the peaks and troughs of the scanning curve can be identified without loss. After that, all the peaks are sorted in order based on their amplitude values. The first and second highest peaks with physically reasonable distance are regarded as the test and control peaks.

The threshold value for state recognition (for example, rising or descending) is defined based on a self-adapting strategy. The initial value is pre-set as (maximum data reading)/1000. When the number of the identified peaks is less than two, the threshold value will be reduced to half of the old one until the number of peaks is not less than two.

Next, the peak's boundaries are identified with a global binaryzation method with good immunity to background fluctuation. At the first step, the gradient curve of measurement data is built by subtracting each data point with the continued one. At the second step, binaryzation with a predefined threshold value to the gradient curve is performed twice to locate the test and control peak's boundaries respectively. The

threshold value for test line is equal to the average value of a sampling region covering the test peak, and so does the threshold for the control line.

With the developed global searching algorithm, even the low test peak can be identified consistently, which significantly enhances the reader's sensitivity with specificity.

III. RESULTS

Six test samples with different concentration (1 μg/mL to 200 μg/mL), were prepared by diluting standard CRP sample with PB (PH: 7.3, 20 mM) buffer to build the calibration model. Each time, 70 μL of test sample was applied into the sample inlet of the strip cassette. And then, each strip was incubated for around 15 minutes before starting scan. The fluorescence signal intensity reading along the strip for six tests is shown Fig. 5. As estimated, the low positive sample (1 μg/mL, solid line) yielded a low test peak and a high control peak. When the concentration increased, the test peak became higher, and the control peak became lower.

As discussed previously, in principle, there have two potential ways to figure out the test result based on fluorescence signal reading. One is the peak ratio between test and control lines, and the other is the area ratio. To achieve better repeatability, here the area ratio (ST/SC) between the test and the control peaks is used. Fig. 6 is the dose-response curve of the calibration model.

The related coefficient of the calibration model is 0.995. More than ten tests were performed to evaluate the reproducibility of the dose-response curve of the calibration model. It was found that all the related coefficients were larger than 0.99, and their coefficient of variation (CV) value was less than 1.0%. The model parameters in (2) were identified with the least square method. The identified model for concentration calculation is described as following:

$$C = 69.343 * (S_T / S_C)^{1.285} \quad (4)$$

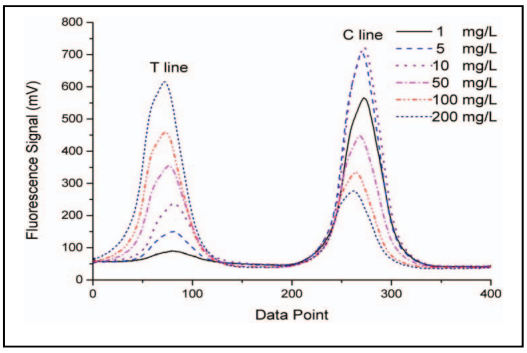

Figure 5. Scanning curves of six tests with different concentration

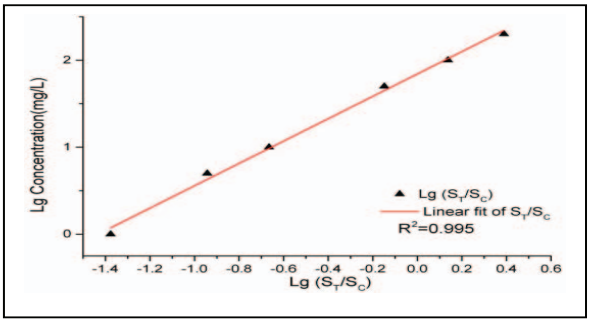

Figure 6. Dose-response curve between lg(concentration) and lg(ST/SC)

The accuracy and repeatability of the optical reader were evaluated with test samples of different concentrations. For clinical test, the quantifiable test range of the developed reader is found as 1-200μg/mL even though the test sample with lower concentration can be read out. When the test sample's concentration is out of the quantifiable range, the reader will just give a qualitative test result (negative or positive). Different samples with concentrations of 1μg/mL, 100μg/mL, and 200μg/mL were used to test the reader's reproducibility. Each sample was tested for 20 times, and the achieved CV (Coefficient of Variation) values were 1.52% for 1μg/mL, 0.71% for 100μg/mL, and 0.47% for 200μg/mL, which were all well below 2.5%, a typical level accepted generally.

IV. DISCUSSION

Parallel tests of CRP with real blood serum sample were done with both the developed reader and the traditional ELISA method. Compared to the developed reader, CRP test with a normal ELISA device (RT-6100, Rayto Inc. Shenzhen, China) needs more sample volume (for example, 100μL) and longer reaction time (more than one hour) with the detection limit of 0.625 ng/mL and 100% of specificity. The sensitivity of the developed reader reaches 1μg/mL with the specificity (for both negative and positive) of 100%. Because the ELISA reader with a specific kit (HuiJia Biotechbology Co. Ltd, China) was designed for ultra-highly sensitive CRP test, its sensitivity is significantly higher than that of the developed reader. Totally ten real blood serum samples were tested to build the correlation curve between the developed reader and the ELISA method, and the correlation coefficient is 0.993, which proofed the accuracy of the developed reader. For clinical application, therefore, the accuracy, sensitivity and specificity of the developed method is satisfied.

Compared to other readers without moving parts, adoption of a moving stage with a step motor slightly increases the cost of the developed reader. However, a photodiode and LED, instead of a photo multiplier tube (PMT) and laser, were used respectively for detection and excitation here to reduce the reader's cost and size. Because of the replaceable LED and photodiode with different wavelength, it is possible for the developed optical reader to be conveniently modified for diagnostics of other bio-markers in POCT.

V. CONCLUSION

For POCT, a portable reader for quantifiable lateral flow immunoassay has been developed and presented here. With a custom Y-shape optical fiber, the excitation channel and the emission detection channel have been coupled with low complexity and cost, which reduces the difficulty of optical alignment with high precision. With a line-shaped excitation beam from a Y-shaped optical fiber, the developed optical reader is able to achieve the distribution of the fluorescence signal intensity of the lateral flow strip efficiently. To reduce the reader's cost and size, a high power LED was adopted for excitation, and a highly sensitive photodiode was used for detection. Strip scanning was performed with a motorized linear stage. For good sensitivity and specificity, an optimized global searching algorithm was developed to identify the test and control peaks and their boundaries, which reduces the effect of the inhomogeneous nature of the strip. The reader's performance was evaluated by performing quantitative CRP test. The coefficient of variation (CV) of the optical reader for CRP test is less than 2.5% within the entire detection range between 1-200 μg/mL.

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