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Design of Portable Time-Resolved Fluorometer

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Abstract: A miniaturization time-resolved fluorescence analyzer was designed based on immunofluorescence and embedded technology, this instrument can be utilized to detect the immunochromatographic strip to achieve quantitative inspection and analysis of analytes in human blood or body fluid. By mechanical scanning means point-by-point assay, using UV-LED irradiation the fluorescence labeled immunocomplex on the immunochromatographic strip, and the fluorescence was emitted after being excited. Fluorescent light passes through narrow-band optical filters and converted to electrical signal by Silicon photodiode (Si PIN). After amplification and analog-digital conversion, the signal is sent to the micro-controller STM32F103 for processing. The fluorescence intensity distribution of the reagent strip was obtained after the scan detection finished, and the concentration of the substance can be calculated based on the known standard curve. After testing, the instrument has a wide dynamic range of measurement. It not only has a reliable repeatability (CV<0.2%) performance in the detection of concentration larger than 100μg/mL, but also hasdemonstrate stable validate features (CV=2.6%) when the concentration only at 1μg/mL. It has commendably application prospects in point-of-care test detection (POCT).

Keywords: Time-Resolved, Fluorescence Immunoassay, TRFIA, Quantitative Detection, Si PIN, POCT

1. Introduction

Fluoroimmunoassay (FIA) is mature methodology in the immunoassay with advantages of high specificity and can detect a variety of antibodies [1-2]. And The time-resolved fluoroimmunoassay (TRFIA) technology is based on the fluoroimmunoassay using lanthanide rare earth metals chelation as a fluorescent tracer. The lanthanide performs a significant fluorescence characteristics, such as europium (Eu) absorption bands 300~500nm and emission spectrum with 615±10nm, stokes shift from 250nm to 350nm, fluorescence lifetime 60~900 μs [3], which are conducive to the realization of time-resolved detection to improve detection accuracy [4-6]. The TRFIA technique can be utilized for the determination of endocrine hormones, proteins, peptides, nucleic acids, neurotransmitters, receptors, cytokines, cell surface antigen, tumor markers, blood drug concentration of various bioactive substances [7], can be facilitated to the diagnosis of infectious diseases, endocrine, cancer, drug testing, immunology, blood type identification a variety of diagnostic categories [8-10], has become one of common of analysis means in biological medical research and clinical super trace biochemical test [11].

Although there are several existing clinical practices have automated immunofluorescence test equipments put into utilize, however such equipments are always company of various defects such as huge volume [12], significant costs, and most of them cannot be utilized for Point of Care Testing [13].

To remedy these deficiencies, we proposed a novice miniaturization, full automatization and rapid TRFIA based on embedded technology and electro-optical sensor technology. This instrument proposed to utilize for chromatography detection which is based on the double-antibody sandwich Elisa method, with full use of time-resolved detection technology in the implementation, which improves fluorescence detection accuracy and sensitivity, and can be utilized for Point of Care Testing.

2. Theory of Immunochromatographic Testing

The TRFIA analyzer apparatus for detecting immunochromatographic strips which bases on double

antibody sandwich format. The immunochromatographic is an assembly of multi-plain porous carriers impregnated with immunoreagents, the fibrous layer material is as the stationary phase, and the test liquid is as the carriers. The liquid sample flows along the carriers, and the immunocomplex formed in a certain zones in the test strip.

This kind of immunity strip set the binding zone, the test line and the control line on the fiber material layer [14]. Antigen and first antibody combined with specific immune response in the binding zone. The test line is fixed with second antibody 1, the control line fixed with the second antibody 2. The Second antibody 1 and Antigen-antibody conjugates specific immune reaction occurs, and then the immunoconjugate generated which concentration is related with the antigen concentration. The Second antibody 2 was able to react with the first antibody, however the degree of the reaction was negatively correlated with the concentration of the antigen [15]. The concentration of the two conjugates can be detected by immunofluorescence, and the quantitative detection needs to measure of the antigen.

Detection system to detect the type of chromatography reagents principle is shown in Figure 1, processes are as follows:

(1) Placing sample in sample zone of chromatography strip, waiting for immunological reaction process complete;
(2) Utilizing UV-LED stimulates the fluorescence labeled immunocomplex on the immunochromatographic strip, leads fluorescence emitted after being excited.
(3) After passes through narrow-band optical filters, fluorescent light is converted to electrical signal by photosensor. After amplification, analog-digital conversion, the signal is sent to the CPU for processing.
(4) A small movement controlled by the mechanical part, repeat steps (2), step (3), until all the windows could be detected and obtain a fluorescence intensity distribution spectral;
(5) The concentration of the substance can be calculated with the known standard curve by the CPU.

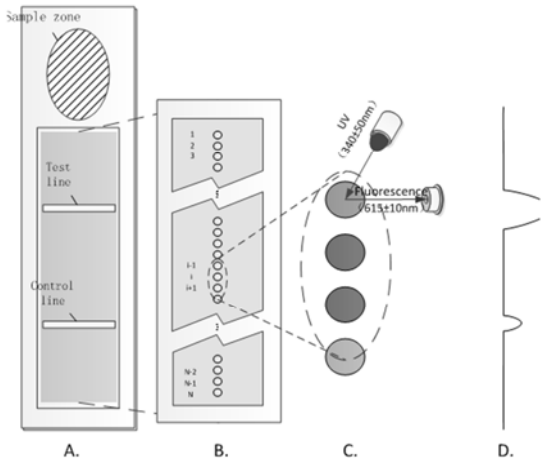

Figure 1. *Immunochromatographic Strip structure diagram. A. Immunochromatographic Strip; B. The Test window; C. Point-by-Point Detection; D. Immune fluorescence spectrum.*

3. System Implementation

The TRFIA analyzer mainly consists of four parts: the hardware integration, the optical module, the mechanical structure and the system software. The hardware integration and software design are the dual pill of the whole system, which can drive the light source, opto-electrical converter, A/D converter, stepper motor and other peripheral equipment to implementing the scan detection, and calculating and displaying the test results. The optical module includes a fluorescence light path and a collection optical path, and plays a key role in the front of the fluorescence detection. The mechanical structure provides the power, and the auxiliary realizes the scanning process.

3.1. Hardware Integration

As shown in Figure 2, the STMicroelectronics micro controller STM32F103 is employed as the main controller, this micro-controller based on ARM core Cortex-M3, frequency is 72MHz, 128KB FLASH, integrated USB bus interface, motor controller as well as other various peripherals, with high performance, low power consumption and so on. The STM32F103 can meet the design for lighting, mechanical, A/D and other peripheral control needs, as well as the complete calculation process of the test results.

The UV LED driving circuit drives the UV LED to emit an ultraviolet light which is stable and intensity controllable. The photoelectric sensor converts the fluorescence intensity in the receiving plane to weak electrical signal which is positively correlated. In order to effectively collect and measure the intensity of fluorescence, the electrical signal is amplified by the signal conditioning circuit, converted to digital signal by the AD977A and sent to the microcontroller. Through the motor driver, the system provides power for stepper motor and flexibly controls the direction and speed which drivers the strip stage horizontal movement during the scanning process. The light gate is utilized for detecting the position of the stage, in case of stage movement derailed. The hardware circuit also includes display, printer, IC card interface, PC communication interface, USB interface and other peripheral interfaces, which are mainly utilized for man-machine interface.

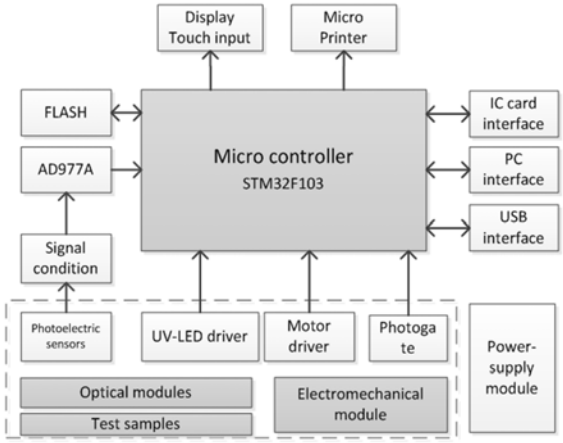

Figure 2. *System hardware block diagram.*

3.2. Optical Platform Design

Optical system is the core part of the immune fluorescence analyzer consisting of light source, optical path and the photoelectric sensor. In order to reduce the size of the system, the UV LED was utilized as the light source to produce the light which spectrum is matches with the absorption spectrum of fluorescent label. For that the absorption wavelength of La series rare earth metal elements is 340nm, which means the peak of emission spectrum of UV light source should be in the vicinity of 340nm. According to the requirements, this paper selects the KED365UH as the source of UV and which produced by Japanese Nichia Semiconductor Company. Because of the wavelength of KED365UH is narrow, and it's luminescence spectrum near the absorption spectrum of the complex of La. Compared with halogen lamps, UV LED has the advantages with portable drive briefness, as well as fast switching function under time-resolved immunofluorescence detection.

Photon detector take advantage of current intensity produced by photoelectric effect to detect the number of photons, there are various types of detectors such as silicon photo diodes, avalanche photodiode and photomultiplier tube etc. Because the weak signal of fluorescence, we need to choose the photoelectric converter device with accurate sensitivity and high S/N ratio. Consider about the portable design, miniature size of the sensor should be chosen. To sum up, the selection of photoelectric devices need to have a high sensitivity approach the 615nm. Based on the above requirements, the silicon photo diodes (SiPIN) S1226-5BK of Hamamatsu Corporation (HPK.) is our final selection. Due to the addition of high impedance material, SiPIN has the characteristics of wide depletion layer and low terminal capacitance, thus the response speed is faster than the PN type photodiode [16]. Figure 3 shows the profile and spectral characteristics of the S1226-5BK.

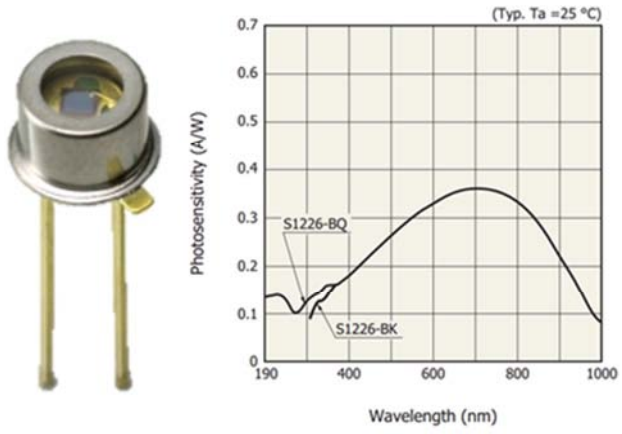

Figure 3. *The appearance and response characteristic of silicon photodiode S1226-5BK.*

S1226-5BK's spectral response range is 320~1000nm, which displays an accurate sensitivity, specific parameters are shown in Table 1.

Table 1. *Parameters of Silicon photodiode S1226-5BK.*

Parameter Name	Value
Photosensitive area size	2.4 × 2.4 mm
Spectral response range (λ)	320 to 1000 nm
Peak sensitivity wavelength (λp)	720 nm
Photosensitivity (S)	0.36 A/W
Dark current (I_D)	5 pA
Terminal capacitance (Ct)	160 pF

The light path is divided into two parts: the excitation optical path and collection optical path. Collection optical path plays the role of focusing and aberration correction. As shown in figure 4, the first lens is a biconvex, second parallel plates is a filter，the filter can be moved freely in the working distance. Excitation optical path adopts lens which same as the collection optical path to focus light, that save cost and increase energy of lighting spot.

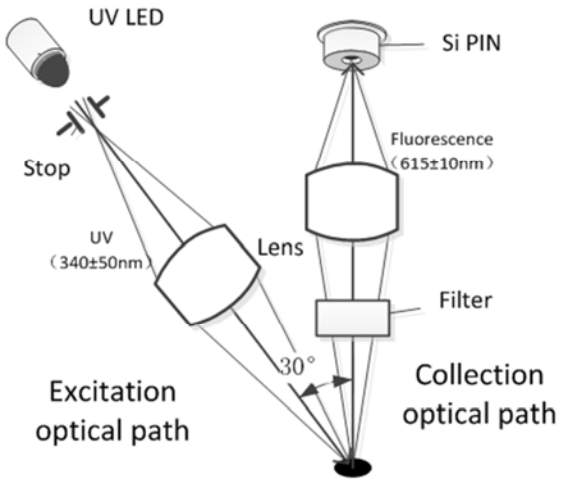

Figure 4. *Schematic diagram of optical path.*

3.3. Mechanical Module

The scanning mechanism comprises a stepping motor, a guide rail, a objective table and a connectors. In the process of detecting the reagent strip, the photoelectric diode is utilized as a photoelectric conversion device to detect one single point during each conversion procedure. In order to detect the whole immunochromatographic strip and set scanning mechanism to drive the immunochromatographic strip, the fluorescence distribution pattern was obtained after all points were detected by scanning method, which consequently affects the scanning results accuracy bye mechanical structure.

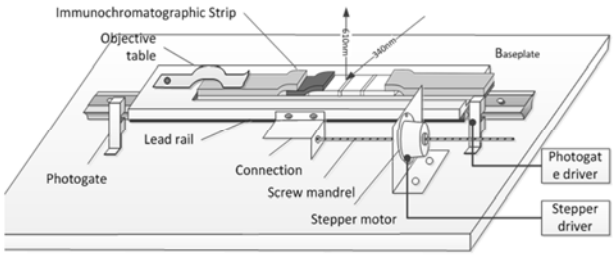

Figure 5. *Schematic diagram of mechanical mechanism.*

3.4. Time-Resolved Acquisition

The autofluorescence is primary aspect leads the decrease of detection accuracy in TRFIA, nevertheless the autofluorescence's lifetime is shorter then fluorescence of the Lanthanum Series. According to the lifetime differ, detection after sort life fluorescence (autofluorescence) cancellation is a excellent method to reduce the interference of autofluorescence and improve the accuracy of test results. In our study, the time resolved acquisition process is achieved, and the detection process is shown in Figure 6.

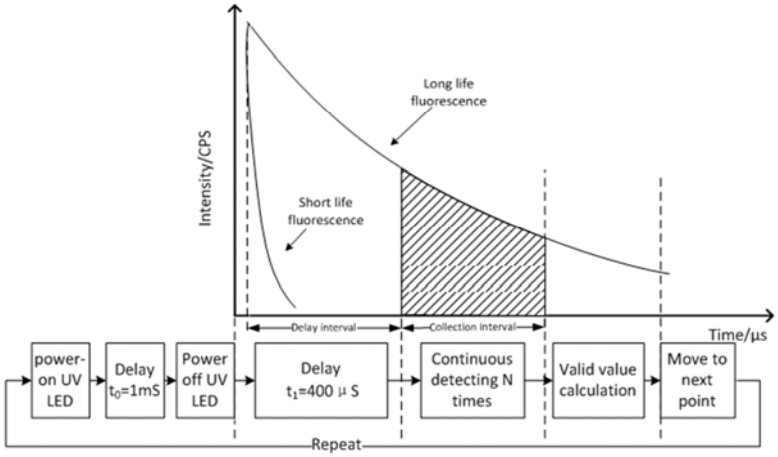

Figure 6. *Time-resolved measurements.*

As shown in Figure 6, the detection procedures for a single point includes:

(1) Power on the UV LED duration time is T_0, stimulates the fluorescent substance on the immunochromatographic strip emiting fluorescence;

(2) Power off the UV LED, delaying for time T_1, until the autofluorescence is quenched;

(3) Repeated detection *n* times, and calculate the effective value with algorithm to represent the intensity of this point;

(4) Step to the next point, repeat (1) (2) (3) steps.

According to the life characteristics of La, the delay time *T1* is about 400μs. In the step (3), a point of continuous *n* acquisition, we will finally work the value based on the *n* captured value to eliminate random interference. Due to the limited sampling rate of ADC, the number of acquisition times cannot be so large, the value *n* is set according to ADC acquisition speed and fluorescence quenching rate.

3.5. Data Processing

System sequentially scans every single detection point on the strip. In the process of scanning, position located through two photogates. The total collection point in the scanning process is set to *LENGTH*. These collection points generate form a fluorescence intensity spectral distribution as shown in Figure 7.

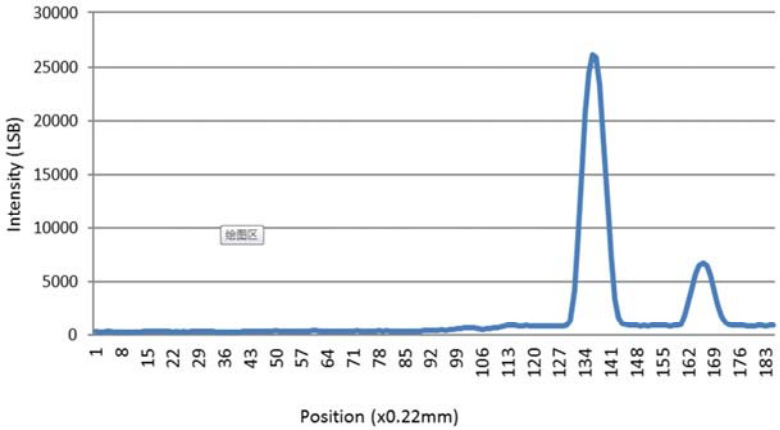

Figure 7. *Fluorescence intensity spectra (LENGTH=190).*

After scanning the spectral distribution, the MCU automatically searches for the position and value of the test line and control line in the spectrum of the fluorescence intensity. The value on the test line and control named as T and C, which utilized for calculation of substance concentration combine with the standard curve, the standard curve describing the relationship between the substance concentration of strip testing and T/C, and the standard curve is provided by strip manufacturers. There are two ways to describe the standard curve: Point–to-Point and Cubic Curve.

Point–to-Point saves some scattered of T/C and the corresponding concentration. The program read point-to-point data from the RF card and matching the measurement data with it during the measurement, which leads to calculate the concentration of the substance to be measured. In the cubic curve mode, the equation of the relationship between the concentration and the T/C value was recorded in the RF card. By implement the fitting equation, the program can calculate the concentration of the measured value according to the measured T/C value. Assume the measured value is a=T/C, and the substance concentration is y, we can obtain:

$$y = F_{stand}(a) = C_0 + C_1 \cdot a^1 + C_2 \cdot a^2 + C_3 \cdot a^3 \quad (1)$$

4. Result and Analysis

The system was validated by the standard of the strip, and the performance of the system was evaluated in both measurement repeatability and dynamic range.

4.1. Repeatability

In our study, the coefficient of variation (CV) is introduced to evaluate the repeatability index of our system, the coefficient of variation is obtained by the standard deviation (SD) as well as the mean (M) of samples.

SD reflects the degree of deviation between the measured values and the overall M. Assuming that the n (n, 1,... N) sampling results for the strip is y_n, the composition of the sample named *Y*. The average value of N times sample is μ, as a result:

$$\mu_Y = \frac{1}{N}\sum_{n=1}^{N} y_n \quad (2)$$

Standard deviation SD values is σ, and:

$$\sigma_Y = \sqrt{\frac{1}{N}\sum_{n=1}^{N}\left(y_n - \mu_Y\right)} \quad (3)$$

Then the value of the coefficient of variation is CV_Y, and then:

$$CV_Y = \sigma_Y / \mu_Y \times \%100 \quad (4)$$

SD is facilitated in comparison to the standard deviation of the average level of similar samples, which reflects the real stability of the measurement. Whereas SD cannot be utilized to compare multiple samples with average values is in various levels. In this case, we introduce CV to compare the differences [17].

During our experiments, it is need to repeat the measurement several times on the same strip with at certain intervals. CV is employed to the result analysis. Specific parameters of our test environment are: reagent concentration: 50μg/mL; environment: night, indoors, daylight lamp lighting; test interval: 30s, the results are shown in Table 2.

Table 2. *Repeatability of measurement results (T/C's).*

Test No	Contrast instrument	Our instruments
1	0.1373	0.157297
2	0.1373	0.154922
3	0.1368	0.155431
4	0.1358	0.155292
5	0.1354	0.157735
6	0.1348	0.155611
7	0.1352	0.155935
8	0.1354	0.154761
SD	0.00099	0.00108504
M	0.1360	0.155873
CV	0.727904%	0.696105%

Test results demonstrate: (1)The mean value after 8 times measurement is 0.15587, which is close to the contrast instrument; (2) The CV value is little lower to than the results of clinical armarium, which means the achieve even better repeatability index.

4.2. Dynamic Ranges

Dynamic range represents the interval scale of the instrument in the range of accuracy. Dynamic range and error measuring data of our instrument are shown in Table 3. Figure 8 shows the stability measurements according different concentrations, which demonstrates a better measurement stability (CV = 0.2%) performance when the concentration of the analyte concentration is greater than 100μg/mL; When the measured substance concentration is less than 100μg/mL, the stability rete starts to decline, yet the coefficient of variation can be restricted under 2.6%; When the concentration is less than 1μg/mL, the stability rate of the measurement decreases rapidly.

Table 3. *Test results and errors at different concentrations.*

Concentration (μg/mL)	0	1	10	50	100	200	400
1	0.028509	0.028851	0.053145	0.179586	0.24778	0.426448	0.738307
2	0.028217	0.028842	0.051171	0.177742	0.247855	0.423139	0.737425
3	0.029248	0.029544	0.053537	0.178899	0.24689	0.425992	0.740499
4	0.028523	0.029814	0.054599	0.178542	0.248549	0.426015	0.73704
5	0.029022	0.029756	0.054481	0.175831	0.246024	0.425725	0.739618
6	0.028126	0.028207	0.053935	0.181784	0.246934	0.425324	0.740076
7	0.029586	0.030671	0.05432	0.177411	0.24771	0.424805	0.735237
8	0.028904	0.028864	0.052882	0.180708	0.24626	0.423377	0.739214
M	0.0287669	0.0293187	0.0535088	0.1788129	0.247250	0.4251031	0.738427
CV	1.77516%	2.64346%	2.11492%	1.05901%	0.3506%	0.29209%	0.241%

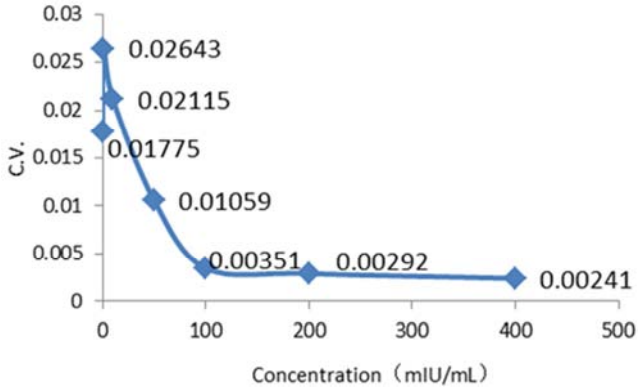

Figure 8. *Stability of measurement results at different concentrations.*

5. Conclusion and Discussion

In this paper, we conducted a system integration scheme of a time-resolved fluorescence Analyzer, conbining bio-technology, chemical technology and electronic technology together, this instrument is a sophisticated product of multi-disciplinary knowledge integration.

This analyzer includes the following modules: light sources, optical path design, electro-optical sensors, signal conditioning and conversion, signal processor, displayer and interaction, mechanical systems, all these components were selected small-packaged structures, size of complete appliance is only 26cmx20cmx13cm.

The detection process is employed a maximum utilize of time-resolved fluorescence immunoassay theory, which generated a satisfied repeatability (CV<0.2%) in measuring the. concentration ≥ 100μg/mL immunochromatographic strip, furthermore it also behaved reliable performances (CV=2.6%) when the concentration rate is as low as only 1μg/mL, consequently has an extensive application in the prospect of POCT.

The deficiency of our research at this stage is validation strip is limited utilizing the standard strip instead of actual testing strip, nevertheless the curve detected in actual usage scenarios demonstrated a low satisfaction level, which means higher demandingness of this instrument still needs to be upgraded in our future studies.

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