Enhanced fluorescence signal using stray light shutter in a quantitative PCR chip

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Abstract
Quantitative polymerase chain reaction (qPCR) is the most important quantitative sensing technique for pathogens, especially for emerging pandemics such as the coronavirus outbreak this year. The qPCR chip and device were investigated to meet the unmet needs of ultrafast inspection time, high accuracy, and small system volume. Therein, the fluorescence intensity was the most important signal in qPCR quantification of DNA amplifications, which is essential not only in the confirmative diagnosis of positive or negative infection, but also in the assessment of viral load for therapeutic and quarantine decision making. As the target DNAs got amplified, the interaction of fluorescence dye and double strand DNA will generate fluorescence signal proportional to amplified DNA in the intensity when excited by certain wavelength. A miniature spectro-detector was employed to receive the fluorescence scattering for digital output of the intensity in the qPCR chip in this study, and the optical simulation and actual experimental design and results according to the optical simulation results were performed to study the effect of the stray light shutter (SLS) in the improvement of the signal in fluorescence detection. The analysis results showed that the signal-to-noise ratio (SNR) of the fluorescence can be enhanced significantly for five times of the control using the SLS with a shape of extended component aperture, where the protruding structure was positioned away from the center. The experimental results showed that fluorescence intensity can be enhanced by 15.50% and 9.86% when adding the above shape of SLS in resin- and in glass-based chip, respectively. The results also demonstrated that the optical setup had good stability and repeatability in fluorescence detection, and variation was less than 1.00%. Our results can provide important reference to the development of qPCR chip to obtain the high SNR fluorescence signal in DNA quantification process.

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(Some figures may appear in color only in the online journal)

1. Introduction

Quantitative polymerase chain reaction (qPCR also called real-time PCR (RT-PCR)) is an important laboratory technique in molecular biology and was widely used for confirmative diagnosis of clinical diseases, such as virus and bacterial infection and cancer mutation type detection (Espy et al 2006, Kralik et al 2017). The qPCR extended by PCR. It is a method for detecting the total amount of products after each PCR cycle using a fluorescent dye in a DNA amplification reaction. The principle is that the specific Primer probe will generate fluorescence during the PCR process. Using a device with thermal loop and fluorescent dye screening function, the fluorescence signal released after each loop is detected, and the relationship between the fluorescence and the number of loops is plotted. The fluorescence signal and the product amplified fragment of the PCR are directly proportional to each other, so that the content of the product produced by each cycle can be further estimated to achieve the purpose of real-time quantitative. The advantage of qPCR is quickly, stably and sensitivity. The qPCR was widely used to public healthcare, such as COVID-19. Its detection accuracy rate was as high as 99.82% (number of samples: 18,000,000), the average detection time was 2–4 h and each detection cost was about 100 US dollars. The qPCR had become an important tool for epidemic prevention, because of the short detection time, high accuracy and relatively cheap detection cost. (Reviewer1, Question 1) (Park et al 2020) In the qPCR process, the standard double strand DNAs were denatured into two single strand DNA by increasing the reagent temperature to about 92 °C–95 °C. The temperature was then decreased to the specific temperature when primer DNA could bind to the target sequence of the single temperature was then decreased to the specific temperature when primer DNA of any given sequences; and (b) fluorescent dye conjugated to specific DNA probes (oligonucleotides of specifically designed sequence complimentary to the target DNA) that generate fluorescence in the extension step (Bustin et al 2009). When the DNA of sample include the disease specific DNA sequence, qPCR could provide fluorescence signal based quantitative analysis with accumulated detection of during the thermal cycling. Therefore, the fluorescence intensity changes over thermal cycling not only showed positive or negative presence of the target DNA in the test samples, but also deliver quantitative estimation of their initial amount. Considering the cost and capability of specific amplification by melting curve analysis, SYBR Green has been widely used in qPCR systems.

In a previous study comparing, Ponchel had compared the use of SYBR-Green- versus Taqman-based qPCR assays in the copy number assessment of three different genetic contexts. These include gene rearrangement of TREC in peripheral blood mononuclear cells, gene amplification of GLI1/MYC-C/MYC-N in cell lines and pathological biopsy samples, and OPAL1 deletion in dominant optic atrophy. It was demonstrated that SYBR-Green-based qPCR delivered the same precise quantitative analysis as the TaqMan probes did when the primers and qPCR process are appropriately designed (Ponchel et al 2003). A more recent study in the expression profile of A1, A2A, A2B, and A3 adenosine receptors in breast cancer tissue showed that both TaqMan and SYBR Green quantitative RT-PCR are equally efficient (>95.00%) in all genes (Tajadini et al 2014). In 2020, a SYBR Green I-based duplex qPCR was developed and successfully demonstrated simultaneous detection of classical swine fever virus and porcine circovirus 3 in one sample based on their distinct melting temperatures (Zheng et al 2020). These results suggest that qPCR using SYBE Green based fluorescence detection is a reliable diagnostic tool to detect and monitor disease progression in clinical settings.

In a qPCR system, the detection accuracy strongly depends on the accuracy sensitivity and resolution of the fluorescent signal sensing. (Chao et al 2020) (Reviewer 1, Question 3) In commercial qPCR system, the most common fluorescent detector could be a modulated CMOS camera, photodiodes, or photomultiplier tube (PMT). The photodiode has the advantages of low cost and small volume, and the PMT had superior sensitivity compared to photodiodes but larger size and higher cost. The PMT was used in the real-time microchip PCR system for detecting the fluorescence intensity that intercalate with DNA, and the results indicated that the PMT should be warmed up for 1 h by applying a working voltage of 5 V before PCR process to prevent the baseline drift of readout value (Koo et al 2013). In CMOS sensing, every pixel has one amplifier with it thus to allow detecting signal of small-scale fluorescence variation more convenient than using a charge-coupled device (CCD) camera. Additionally, CMOS also...
enjoy a significantly lower driving voltage. Therefore, CMOS has been more commonly used in the fluorescence lifetime imaging microscopy where frequency-domain fluorescence lifetime of scattered laser lights was captured and analyzed by precisely calibrated CMOS camera to minimize the effect of bleaching or background interference (Chen et al. 2015). Such technology was later applied in a handheld, real-time qPCR system using Anitoa’s ultra-low light CMOS biosensor to detect DNA and RNA from hepatitis B/C and Escherichia coli (BioOptics World 2015). The detection limit was able to push to four copies per sample with over nine orders of magnitude in dynamic range. The CMOS biosensors therefore have enough sensitivity to replace photomultipliers and cooled CCDs in qPCR applications for medical and scientific detections.

However, color filters were required when multiple emission spectra of fluorescence signals were designed to be detected by the photodiode or CMOS camera in one sample for multiple target sequences. Hence, the optical sensors having the characteristics of spectrum separation becomes an important component in such applications as the information of detected light intensity at each specific wavelength can be recorded independently. The micro-spectra-detector was a suitable component to detect and record the light intensity at different wavelengths. A miniature spectrometer was used to detect the emission of fluorescence in a micro litter volume glass capillary form the RT-PCR machine. The results illustrated that the RT-PCR prototype machine had the same accuracy for DNA quantification and reproducibility with five samples compared with the commercial machine (Lee et al. 2004).

An optical system for qPCR fluorescence measurement incorporates high-power white LEDs, lenses and offer multichannel imaging CCD-based spectrometer was developed and validated (Alaruri 2014). Therein, 20 reaction vessels were detected in this optical system. Seven different fluorescent dyes at 1 nM dye concentrations can be detected in each reaction vessels. The results in the study showed that the spectral resolution <15 nm, dynamic range >3 decades and coefficient of variance <1.00%. To improve the sensitivity of biosensing to fluorescence, near singular-phase optical biosensing with strongly coupled modes of a plasmonic–photonic trimer was presented. It could exhibit exquisite control over the dispersion of the supermodes. These distinctions enable our proposal to be of direct utility in highly demanding point-of-care biosensing applications (Gupta et al. 2021). An image-based lasing threshold analysis method is developed. Compared to the conventional spectrum-based threshold analysis method, the lasing threshold obtained from the image-based method showed consistent results. The development cost was cost effective and integrated coherent light source on chip for point-of-care applications (Zhang et al. 2020a). A solid-core/liquid-clad (SL) waveguide excitation wave is proposed to provide a uniform excitation scheme and effectively avoid background noise, thereby ensuring low propagation loss along the microfluidic channel. The platform can be further extended to a variety of water-soluble fluorophores (such as organic dyes, quantum dots and fluorescent proteins), which can simultaneously achieve multi-parameter measurement of biochemical analytes (Zhang et al. 2020b). A succinimide-modified acrylic microsphere-based DNA hybridization strategy was presented in conjunction with a gold-latex optical amplification for detecting dengue virus nucleic acid (Jeningsih et al. 2020). The UV light was used to irradiate the biological sample, and the spectrometer couples to an optical fiber was used as the DNA bio sensor. This method was verified and compared to the results of RT-PCR, which can be employed for screening dengue virus infection in clinical samples such as serum, urine, and saliva from the infected patients.

In a previous study, a similar device was employed to receive the fluorescence intensity and simulate the fluorescence signal-to-noise ratio (SNR) in a qPCR chip device. Therefore, the shape of stray light shutter (SLS) was designed, and the fluorescence intensity and the noise signal were analyzed with respect to the geometry of the SLS. From the simulation results, the SNR shape of fluorescence was enhanced from 3.14 to 16.78 using the SLS with shape of extended component aperture and the protruding structure away from center. In addition, the SNR can be enhanced to 18.02 by adding the SLS and a spectrometer with a 40° acceptance angle package (Chao et al. 2021).

In a present study, the micro-spectra-detector was used to instead of a power meter to simplify the measurement process and cost. The sensor of the spectra-detector has 11 nano interference filters of different wavelengths for spectral separation. When the fluorescent dye is replaced, the system does not need to replace the filter. The fluorescence signal can still be accurately sensed. When we need to measure multiple different kinds of samples, we only need to change the excitation light source to perform the measurement. (Reviewer#2, Question#2)

The SLS was fabricated by 3D printing technology, and the resin-based and glass-based PCR chip was fabricated for the fluorescence reagent and the fluorescence intensity was measured to verify the simulation results. The experimental results will provide the reference to the design of PCR chip, which can prevent the interference of excitation light to the fluorescence detection thus to enhance the fluorescence signal for determination of amplification of target DNA sequence. Moreover, several diseases can be inspected simultaneously by analyzing the intensity of different wavelengths through adding the different types of fluorescence substance such as SYBE Green or ROX.

In the previous paper (Chao et al. 2021), we only presented the structural design of optical simulation and qPCR equipment. In the paper, we implemented the qPCR model and performed fluorescence detection experiments. From the experiment results, we analyzed the influence of many important parameters, and then improved the previous qPCR equipment proposed according to the experimental results. In the paper, we analyzed the appropriate excitation light intensity, the detection limit of the fluorescence concentration, and the repeatability of the detection module. In order to obtain a higher SNR, we have improved the previous qPCR equipment and replaced the plastic film on the bottom with a glass
substrate. In the article, we described the qPCR development process in detail. Readers can reproduce and improve the qPCR equipment according to the principles and rules mentioned in the article. It can make continuous progress in qPCR equipment development technology. (Reviewer#2, Question#1)

2. Experimental principle and setup

The total power of an incident light is equal to the summation of various forms derived from the externally applied light sources including the reflected, transmitted, scattered, and absorbed. The fluorescence is emitted when SYBE Green dye interact with DNA duplex by intercalation and external binding thus able to absorbs at a wavelength around 497 nm and emits fluorescence around 520 nm. Fluorescence is the result of a three-stage process that occurs in certain molecules. The excitation process where photon of energy $h\nu_{EX}$ supplied by an external source was absorbed by a fluorophore to generate an excited electronic singlet state ($S1'$). This is followed by a process of excited-state lifetime typically 1–10 ns that the fluorophore undergoes conformational changes where the energy of $S1'$ could be partially dissipated to a relaxed singlet excited state ($S1$) from which fluorescence emission originates; or enter other processes such as collisional quenching, fluorescence resonance energy transfer and more. Fluorescence was emitted in the third process where a photon of energy $h\nu_{EM}$ is emitted to returning the fluorophore to the ground state $S0$. The Stokes shift described by $h\nu_{EM} - h\nu_{EM}$ is fundamental to the sensitivity of fluorescence techniques. The emission light ceases nearly immediately when the excitation light turned off and the distribution of the emitted fluorescence intensity could be simulated by scattering model.

2.1. Principle of the scattering model

If the substance is a particle, the particle would absorb the light energy and re-emit the light with different intensity to different directions. Therein, the Rayleigh scattering theory was employed when the dimension of particle was smaller than the light wavelength, and the Mie scattering theory was used when the particle size was larger than the respective wavelength. However, there is a lot of calculation time in the light intensity simulation of each light beam. Total integrated scattering (TIS) is defined as the ratio of the total power generated by contributions of scattered radiation including the forward and backward toward the incident radiation, shown in figure 1. In the TIS situation (Harvey et al 2012), the incident beam toward the sample was nearly normal, and the integration was carried out from the small values to almost 90°. When the light scattered from the specular reflection is small and the substance is the surface material, the TIS will be affected by the surface roughness. In addition, the bidirectional scattering distribution function (BSDF) was used to describe the scattered light (Pfisterer et al 2014). The phenomenon of BSDF is usually split into the reflected and the transmitted light, which are separated into the bidirectional reflectance distribution function and bidirectional transmittance distribution function. In general, the vector $I$ is the incident light, vector $R$ is the specular light, and the vector $S$ is the scattered light, as shown in figure 2. Here, the specular light presented the mirror reflected or scattered light and their projection on the plane was defined as $\beta$ and $\beta_0$, and the variation was $x$. When the scattering phenomenon was caused by an isotropic random rough surface, and the dimension of the rough structure was relatively smaller than that of the wavelength of the scattered light, the $ABg$ scattering model can usually be well described as the scattering probability distribution and was suitable to the polished optical surfaces. Therefore, the $ABg$ scattering model was used to fit to the BSDF and plotted as a function of $|\beta - \beta_0|$ (Won 2014). The $ABg$ scattering model was widely used to evaluate scattering results through parameters $ABg$, and written as equation (1)

$$BSDF(\hat{x}) = \frac{A}{B + |\hat{x}|^{x}} \quad (1)$$

where $A$ must be equal or larger than 0. $B$ must be larger than $1 \times 10^{-12}$, unless $g$ is equal to 0. If $g$ is equal to 0, then $B$ can be equal to 0. In addition, no scattering should occur if $A$ is equal to 0. Here, the intensity distribution was analyzed by $ABg$ model, and the fitting curve was similar to the Lambertian
scattering type. The Lambertian scattering model was usually used to present the scattering intensity of the uniform rough surface. The probability of the scattered light on the projection vector $\beta$ is the same everywhere in the unit circle, and the BSDF is $1/\pi$.

2.2. Optical simulation

The FRED Optical Engineering software (FRED) optical engineering software was employed to simulate the fluorescence distribution in the qPCR chip. The excitation light used a blue LED with peak wavelength at 465 nm and the half emission angle at $\pm 9^\circ$, while the fluorescence distribution was received by the photodiode. The sensing area of the photodiode was a circle with diameter of 0.9 mm, which was the same to the packaging aperture of the spectro-detector used in the following experiments. The gap between the surface of the photodiode and the qPCR chip was 2 mm. A thin plastic film was placed at the bottom of the PCR chip model to simulate thin film in a real chip used to prevent outflowing of liquid reagent during thermal cycling. The reflection of the thin plastic film and the chamber of qPCR chip model was estimated to be 30.00% and 80.00% respectively in the simulation process. The schematic of the simulation model was illustrated in figure 3. In addition, the reagent composed of water, ions and some biological macromolecules and the refractive index was set as 1.336 in the simulation. The scattering ratio was set at 0.6 meaning 100.00% incident light would have 60.00% to be scattered. When the excitation light is irradiated on the liquid sample, part of the energy is absorbed by the liquid sample and converted into fluorescence. Fluorescence does not have a specific light-emitting angle, and it emits light in the same way as a point light source. It light intensity distribution is isotropic. Because the luminous method is the same as the scattering characteristics of a uniform surface, we used scattering model to simulate the fluorescence emission distribution.

2.3. Experimental setup

In the experimental setup, the blue LED with peak wavelength at 465 nm and half angle of $\pm 9^\circ$ was used as the excitation light, and the spectro-detector was used to receive the fluorescence. The spectro-detector was designed to sense the light intensity of 11 wavelengths in the range of 350–1000 nm using nano interference filter deposited on a standard CMOS sensor. The dimension of sensing area was $780 \, \mu m \times 520 \, \mu m$, and the diameter of the detection aperture was 0.9 mm with height of 1 mm. The gap between the surface of the spectro-detector and the qPCR chip was 1 mm. Two materials of PCR chip were employed to the experiment, one is the resin fabricated by UV-cured based 3D printing technique and another is the glass modeled by computer numerical control (CNC) machining. The bottom of the PCR chip that fabricated by resin was sealed by a thin plastic film to prevent outflow of liquid reagent during thermal cycling, and the thickness of the glass chamber bottom was 1 mm. The volume of the PCR chamber is $125.92 \, mm^3$. The cross section of the experimental setup was illustrated in figure 4. In the simulation, the effect of detection angle was analyzed, so the IC package around the spectro-detector was removed to investigate the actual light collection effect by the IC package, which the original package and open-aperture was defined as vertical collection and fully collection in the following experiments, respectively (figure 5). In addition, the reagent composed of water, NaOH solution (sodium hydroxide pellets GR 96%, SHOWA KAKO Corp, Japan) and fluorescein fluorescence dye (SYBR Green, Thermo Fisher Scientific Inc., USA). (Reviewer#1, Question#4) The concentration of reagent defined as 100.00% in the manuscript was 1 $\mu$M, which was 0.003 g of fluorescein fluorescence dye dissolved in the 10 ml of 1 M NaOH solution and then diluted with water.

2.4. Experimental procedure

Six main steps were performed to obtain the fluorescence signal from spectro-detector and the variation affected by the shape of SLS was analyzed for the association between the excitation light intensity and the detection limit of the fluorescence intensity. A flowchart of the experimental procedure was presented in figure 6, and the details of each step were described as follows.
Figure 5. Two types of light collection aperture design included (left) original package and (right) open-aperture design by removing the IC package.

Figure 6. The flowchart of the experimental procedure.

Step (I): The PCR chips of resin or glass material were fabricated by 3D printing and CNC machining, and the SLS was designed and fabricated by 3D printing. The shape of SLS was designed according to the simulation results, and the color of PCR chips and SLS fabricated by 3D printing was black to reduce the light scattering effect.

Step (II): The communication and read out algorithm between the spectro-detector and computer was developed to export the intensity information of each wavelength detected by the spectro-detector. In the spectro-detector, several micro-filters were used before the CMOS sensor and the intensity of 11 wavelengths can be separated and recorded in the computer simultaneously.

Step (III): 20 µl tested reagent was placed into a chamber of PCR chips, the concentration of the 20 µl tested reagent was the same to the concentration of the 10 µl of cyber enzyme in normal qPCR reagents. The concentration of the tested reagent defined as 100.00% was 1 µM in the manuscript.

Step (IV): The blue LED with wavelength of 465 nm was turned on and irradiated on the reagent to generate excitation light. In addition, different triggering currents of the blue LED was adjusted to investigate the suitable excitation light intensity.

Step (V): When the blue LED light was activated, the fluorescein reagent was excited and emitted a fluorescence light to be detected by the spectro-detector to translate the light intensity into digital signal. Therein, the dark background collected without light irradiation was recorded as a reference.

Step (VI): The light intensity was measured and the difference between the fluorescence signal and the dark background signal at each wavelength was calculated to observe the effect of excitation light, SLS’s shape, chip’s material, and concentration of reagents. In addition, the repeated cycle of each experimental parameters was at least triplicated. The measured values were recorded and averaged for final exported value in each round to investigate the experimental bias. The measurement was also normalized to discuss the fluorescence intensity ratio compared to that of the intensity of the excitation light.

3. Experimental result and discussion

Initially, the stray light was evidently affected in the actual experiment, and the signal variation of the fluorescence intensity received by the spectro-detector was too low. Therefore, we analyzed the scattered light in the experimental setup to explore the optimal solution for obtaining high SNR spectral-detection to augment future actual qPCR design. Here, the specular light in the simulation software was determined as the noise because it does not excite the fluorescence dye and was directly reflected as shown in figure 7(a). In addition, the specular light was set as absorption light, and the real signal received by the spectro-detector was the scattered light that generated by the fluorescence, which was defined as the signal (figure 7(b)). Therefore, most of the scattered light collected
Figure 8. The simulation setup design (a) without stray light shutter (SLS), (b) with SLS away from the center, and (c) with SLS close to the center.

by the model with SLS is fluorescence rather than noise. The shape of the SLS was designed accordingly for analysis of the light intensity of the signal and the noise in the following sessions. After investigating the simulation results, the actual experiments were designed to verify the association between the simulation and the experimental results. In addition, the suitable intensity of excitation light and the limit concentration of fluorescence substance will be determined in the following experiments. The reproducibility of the data of the experimental module were also investigated.

3.1. Effect of stray light

To investigate whether the SLS can effectively enhance the SNR, the gap between the surface of spectro-detector and chamber of the qPCR chip was fixed at 1 mm. Initially, there was no SLS at the light path region (figure 8(a)). In the analysis, the light distribution of all rays included specular light (defined as noise) and scattered light was shown in figure 9(a). The light distribution at the corner can be removed when the specular light was set as to be absorbed, where the light distribution can be presented as the only scattering light from the fluorescence substance (figure 9(b)). The results showed that the specular light was generated by the incident light on the chamber of qPCR chip. So, we designed the protruding structure to block the specular light from the side wall of the qPCR chip chamber. Here, the protruding structure was defined as the SLS and the protruding position was designed at the semicircle away from the center (figure 8(b)) and close to the center (figure 8(c)).

With the SLS, the irradiance of signal (scattering light) and the noise (specular light) were analyzed under different scattering ratios. The results showed that the signal was in linear relationship to the scattering ratio in the setup where SLS is away from the center or in the design without SLS. However, most signal could be effectively blocked by SLS close to the center (figure 10(a)). In addition, the noise intensity can be evidently decreased using the SLS away from the center.
(figure 10(b)), and the SNR can be enhanced about four to five times compared to that of the design without SLS (figure 10(c)). On the contrary, the SNR of the fluorescence detection using SLS close to the center was significantly lower than that of the design without SLS. Therefore, SLS with protruding structure away from the center was an important design to enhance the SNR of the fluorescence detection in the qPCR chip.

3.2. The effect of the SLS geometry

The signal declined obviously when the light was isolated by the designed shutter structure. Hence, four different shapes of SLS were explored to optimize the SNR (figure 11(a)). There was almost no signal when the shape of the SLS was designed the same as the aperture of spectro-detector and excitation light source (figure 11(b)). The signal was slightly higher with the protruding structure of the SLS used component half aperture shape than the component aperture shape. The highest signal can be obtained from the full aperture which the structure between the excitation light and spectro-detector was removed and designed that only the protruding structure away from the center was applied. In addition, there was 60.00% of the highest signal detected using the SLS with the shape of extended component aperture. In such shape, the noise also decreased to half of the value compared to that of the full aperture. Therefore, the highest SNR at 16.78 can be evaluated and obtained using the SLS design with the shape of the extended component aperture (figure 11(c)).

3.3. The effect of detection angle

From the above results, the suitable shape of SLS was determined. However, several signals could also be blocked by the package design of the spectro-detector because of the vertical structure with height of only 1 mm. Therefore, the expanded detection angle of the package of spectro-detector was simulated to detect more rays and to obtain better packaging shape design of the spectro-detector (figure 12(a)). The irradiance of signal and noise lights received by the spectro-detector with SLS of extended component aperture shape and without SLS were further analyzed. The results showed that higher signal can be obtained with increased expanded angle (figure 12(b)) and the signal gradually saturated when the expanded angle was set between 30° and 40°. In addition, the SNR with SLS was about five to six times higher compared to that of the design without SLS. The SNR with expanded angle of 40° can enhance up to 7.39% relative to that without expanded angle (figure 12(c)). The comparison of SNR under different experimental parameters were summarized in table 1. Therefore, the spectro-detector packaging can be modified to appropriately expand the detection angle and the integration of SLS in the real-world experimental setup to enhance the SNR of fluorescence detection.

3.4. The suitable intensity of excitation light and SLS geometry

According to the simulation results, the actual experiments were employed to verify the correctness of the simulation.
Table 1. This comparison of the SNR under different parameters.

| SLS   | Angle | 0°   | 40°   |
|-------|-------|------|-------|
| Without SLS | 3.14  | 3.38 |
| With SLS  | 16.78 | 18.02|

Figure 13. The optical power of excitation light under different triggering currents of LED.

The optical power of excitation light was measured by power meter, the results showed that the input optical power was linear to the triggering current, especially between 0.5 and 2 mA (figure 13). In addition, the bias of the optical power was very small under each current setting, which suggest the stability of the excitation light.

The detected intensity at different wavelengths by the spectro-detector were analyzed in samples with fixed reagent concentration and the fully collection method (figure 5). The detected intensity showed that higher intensity was obtained at 440 and 470 nm than others due to the dominant excitation light, and the intensity at 510 nm was determined as the fluorescence signal. In addition, the higher excitation light intensity was measured from the glass-based PCR chip than resign-based PCR chip that may attributed by scattering of the excitation light for higher reflected light of glass material while the scattered light was absorbed by the resin-based PCR chip. The effect of background light was relatively high by glass-based PCR chip, and the fluorescence light intensity was similar (figure 14).

The fluorescence intensity at 510 nm affected by different SLSs and light collection type was analyzed. The fluorescence intensities were normalized by two reference values, one is the light intensity at 470 nm under 2 mA triggering power and the other is under each triggering current (figures 15 and 16). The normalized fluorescence showed that higher fluorescence intensity can be detected by full collection than vertical type in both resin-based and glass-based PCR chip (figure 15). The results confirmed the correctness of the simulation results that expanded angle of spectro-detector was better than without expanded angle for fluorescence analysis. In addition, the extended component aperture SLS has similar or higher fluorescence intensity than without SLS and full aperture SLS in resin-based and glass-based PCR chips with full collection type. The fluorescence intensity reduced by extended component aperture SLS with vertical collection type because most light was blocked by the IC package of spectro-detector. The
results showed that the fluorescence intensity can be enhanced about 15.50% and 9.86% using extended component aperture SLS and fully collection compared to the traditional structure which without SLS and vertical collection in resin-based and glass-based PCR chips, respectively. Moreover, the fluorescence intensity normalized by the light intensity at 470 nm under each current was almost similar under the same SLS structure and collection type with different triggering current of LED because of the fixed reagent concentration (figure 16). The normalized fluorescence intensity was slightly less in high triggering current such as 2 mA than low triggering current such as 0.5 mA, the reduction was less than 1.00%, and the reason was the background effect was also higher in higher excitation light intensity. Therefore, the triggering current was determined at 2 mA for the following experiments because it has the larger variation of fluorescence intensity under different SLS structure and collection and may provide better resolution for analysis of fluorescence intensity.

3.5. The limitation of detection (LOD) of the fluorescence concentration

The LOD of fluorescence concentration was investigated after the optimal triggering current of excitation LED was determined. The maximum concentration used in the experiment was 1 µM of fluorescein. The fluorescence substance at concentrations of 1000 nM, 500 nM, 100 nM and 10 nM was measured to collect the fluorescence signal at 510 nm and normalized by the excitation light intensity at 470 nm. The results showed higher fluorescence intensity detected with extended component aperture SLS and full collection than others. In addition, the detected fluorescence intensity was not stable when the fluorescence concentration was less than 100 nM (figure 17). In this case, the fluorescence intensity was closed to the blank control. The fluorescence intensity was linear to the fluorescence concentration at the range between 100 and 1000 nM of fluorescein, especially in the glass-based PCR chips (figure 17(b)). The fluorescence increased 2.88%–7.86% and 2.23%–3.97% when the concentration increased from 100 nM to 1000 nM using fully collection mode and vertical collection method, respectively. In resin-based PCR chip, the fluorescence light was absorbed by the structure of the chip with black color. The increased amplitude was relatively small (figure 17(a)). Therefore, the minimum fluorescence concentration of the reagent used in the PCR chip should be higher than 100 nM to obtain a linear response to the fluorescence concentration in qPCR detection.

3.6. The repeatability of detection module

After the LOD of the fluorescence concentration was determined, the repeatability of the detection module was analyzed. The SLS and optical component modules including the excitation LED and spectra-detector, as well as the qPCR chip were removed then put back to simulate the actual user operation scenarios in changing the PCR chip. The value of normalized fluorescence intensity and the variation in five independent repetitive measurements showed high
Figure 18. The repeatability of normalized fluorescence intensity at 510 nm with fluorescence concentrations of 100 and 1000 nM in (a) the resin-based and (b) the glass-based PCR chip.

consistency of fluorescence intensity detection. The variation of normalized fluorescence intensity was less than 1.00% in resin-based chip and less than 0.10% in glass-based PCR chip (figure 18). In addition, the trend of normalized fluorescence intensity between 100 nM and 1000 nM showed in figure 18 was similar to that of the figure 17 for both resin- and glass-based PCR chips. The results demonstrated the stability of the relative fluorescent light intensity detection by different excitation light intensity and the effect of the SLS design in both types of PCR chips. The results also demonstrated the fluorescence intensity measured with extended component aperture SLS had good blocking effect of scattered light in low and high fluorescence concentrations in the reagents.

4. Conclusions

To optimize the qPCR detection sensitivity, detection of the weak intensity of fluorescence signal from SYBE Green DNA duplex hydride plays essential roles not only because of accuracy in determine positive or negative infection matters effective pandemic control, but also quantitative assessment is critical to know the disease state, infectivity, and therapeutic options. Hence, the optical simulation and experimental results in this research can provide important reference for designing the optical setup to obtain highest SNR in fluorescence signal of miniature qPCR chip in confined space. From the simulation results, five times stronger SNR in fluorescence detection could be obtained by simply adding an SLS with shape of extended component aperture and protruding toward chip well in position away from the center. Relative to the original packaging with or without SLS, the SNR of fluorescence can be enhanced to between 3.14 and 18.02 times by SLS and expanded the angle to 40° in spectro-detector packaging. The experimental results showed that the fluorescence intensity can be enhanced by 15.50% and 9.86% using the extended component aperture SLS and full collection compared to the original IC package without using SLS in resin-based and glass-based PCR chips. These results demonstrated the correctness of simulation results. In addition, the LOD of fluorescence concentration of the reagents in this optical setup was 100 nM, which can provide the better linear relationship when the fluorescence concentration of reagent was higher than the threshold value. From the above simulation and experimental results, better fluorescence signal can be obtained by adding the suitable shape of SLS through using simulated setup and validated in real-world experiments thus to accelerate the design of next generation multiplex disposable miniature qPCR chip for effective pandemic outbreak control in situ in real-time. Currently our prototype is a 3 × 1 matrix. In the future, we can expand the detection matrix according to user needs to improve the convenience of measurement.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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References

Alaruri S D 2014 High-power white LED-based system incorporating a CCD Offner imaging spectrometer for real-time fluorescence qPCR measurements Meas. Sci. Technol. 25 125502
BioOptics World Editors 2015 Low-light CMOS biosensor enables detection of four copies of pathogen DNA per sample BioOptics World (www.laserfocusworld.com/biooptics/bioimaging/fluorescence/article/14191473/lowlight-cmos-biosensor-enables-detection-of-four-copies-of-pathogen-dna-per-sample)
Bustin S A et al 2009 The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments Clin. Chem. 55 611–22
Chao L C, Chou C H, Tsai H Y, Huang K C and Shieh D B 2021 A study on enhanced fluorescence signal-to-noise by using the stray light shutter for quantitative PCR chip Proc. 9th Int. Conf. on Photonics, Optics and Laser Technology (PHOTOPTICS 2021) pp 54–60
Chao L C, Tsai H Y, Li C R, Huang K C, Lin Y H and Shieh D B 2020 Fluorescence signal-to-noise ratio enhanced by off-plane excitation for quantitative PCR device 2020 IEEE Int. Symp.
on Medical Measurements and Applications (MeMeA) (IEEE) pp 1–6
Chen H, Holst G and Gratton E 2015 Modulated CMOS camera for fluorescence lifetime microscopy Microsc. Res. Tech. 78 1075–81
Espy M J et al 2006 Real-time PCR in clinical microbiology: applications for routine laboratory testing Clin. Microbiol. Rev. 19 165–256
Gupta N K, Tiwari A K, Wanare H and Ramakrishna S A 2021 Near singular-phase optical biosensing with strongly coupled modes of a plasmonic–phonic trimer J. Opt. 23 065003
Harvey J E, Choi N, Schroeder S and Duparré A 2012 Total integrated scatter from surfaces with arbitrary roughness, correlation widths and incident angles Opt. Eng. 51 013402
Jeningsih J, Tan L L, Ulianas A, Heng L Y, Nazlan N, Jamaluddin N D, Yusof N Y M, Khalid B and Ta G C 2020 Sandwich-type DNA micro-optode based on gold-latex spheres label for reflectance dengue virus detection Sensors 20 1820
Koo C et al 2013 Development of a real-time microchip PCR system for portable plant disease diagnosis PLoS One 8 e82704
Kralik P and Ricchi M 2017 A basic guide to real time PCR in microbial diagnostics: definitions, parameters, and everything Front. Microbiol. 8 108
Lee D S, Wu M H, Ramesh U, Lin C W, Lee T M and Chen P H 2004 A novel real-time PCR machine with a miniature spectrometer for fluorescence sensing in a micro liter volume glass capillary Sens. Actuators B 100 401–10
Park M, Won J, Choi B Y and Lee C J 2020 Optimization of primer sets and detection protocols for SARS-CoV-2 of coronavirus disease 2019 (COVID-19) using PCR and real-time PCR Exp. Mol. Med. 52 963–77
Pfisterer R 2014 Scatter and BSDF measurements: theory and practice Photonics Marketplace (www.photonics.com/Articles/Scatter_and_BSDF_Measurements_Theory_and_Practice/f6a3100)
Ponchel F et al 2003 Real-time PCR based on SYBR-Green I fluorescence: an alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions BMC Biotechnol. 3 18
Tajadini M, Panjehpour M and Javanmard S H 2014 Comparison of SYBR Green and TaqMan method in quantitative real-time polymerase chain reaction analysis of four adenosine receptor subtypes Adv. Biomed. Res. 3 85
Won Y 2014 A study of scattering characteristics for micro-scale rough surface Physics and Optical Engineering (https://scholar.rose-hulman.edu/cgi/viewcontent.cgi?article=1002&context=optics_grad_theses)
Zhang H, Palit P, Liu Y, Vaziri S and Sun Y 2020a Reconfigurable integrated optofluidic droplet laser arrays ACS Appl. Mater. Interfaces 12 26936–42
Zhang Y, Kenarangi F, Zhang H, Vaziri S, Li D, Pu X and Sun Y 2020b Versatile optofluidic solid-core/liquid-cladding waveguide based on evanescent wave excitation Anal. Chem. 92 14983–9
Zheng H H, Zhang S J, Cui J T, Zhang J, Wang L, Liu F and Chen H Y 2020 Simultaneous detection of classical swine fever virus and porcine circovirus 3 by SYBR green I-based duplex real-time fluorescence quantitative Mol. Cell. Probes 50 101524