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Abstract. A tip nanobiosensor for monitoring DNA replication was presented. The effects of excitation power and polarization on tip-enhanced fluorescence (TEF) were assessed with the tip immersed in fluorescein isothiocyanate solution first. The photon count rose on average fivefold with radially polarized illumination at 50 mW. We then used polymerase-functionalized tips for monitoring loop-mediated isothermal amplification on Hepatitis C virus cDNA. The amplicon-SYBR® Green I complex was detected and compared to real-time loop-mediated isothermal amplification. The signals of the reaction using 4 and 0.004 ng/μl templates were detected 10 and 30 min earlier, respectively. The results showed the potential of TEF in developing a nanobiosensor for real-time DNA amplification. [DOI: 10.1117/1.JBO.20.2.027005]

Keywords: tip-enhanced fluorescence; loop-mediated isothermal amplification; Hepatitis C virus; radial polarized illumination.

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1 Introduction

Metal-enhanced fluorescence (MEF) is a convenient method for improving signal sensitivity.[1] It has been applied to observe molecular interactions and sense biomolecules.[2] Two processes contribute to MEF. The first is local electric field enhancement by the lightning rod effect near the sharp edge of the metallic structure. This lightening rod effect can be further enhanced by local-mode surface plasmon polariton, when the excitation light is at or close to the resonance frequency of the nanostructures.[3] The second process is the purcell effect with the fluorophore near the metal surface. The purcell effect causes the increase of both radiative and nonradiative decay rates and leads to changes in fluorescence lifetime, spectral shape, and quantum yield.[4] However, when the distance between the metal surface and the fluorophore is narrowed, the ohmic loss by nonradiative decay seriously quenches the fluorophore despite the strong enhancement of the electric field.[5] An appropriate distance is considered to be around 2 to 5 nm to balance the enhancement effect and the ohmic loss.[6,7]

MEF has been applied to endpoint biosensors for the improvement of sensitivity. For example, in 2005, Hong and Kang increased an immunoassay signal as much as 10 fold with nanogold particles;[8] in 2007, Sabanayagam and Lakowicz reported a 28-fold signal enhancement for DNA hybridization arrays with silver nanoparticles.[9] Besides nanoparticles, the tip structure was also used to enhance fluorescence. In 1999, Sánchez et al. introduced tip-enhanced fluorescence (TEF) to a scanning near-field two-photon microscopy.[10] The electric field at the apex effectively decreased the excitation volume to overcome the diffraction limit of optical imaging.[11] Similar near-field optical microscopes have been used for imaging the cell membrane, DNA, and other biological molecules.[12]

Although numerous endpoint sensors and imaging technologies using MEF have been developed, the application of an MEF sensor to DNA replication is less explored. DNA replication techniques are important in clinical molecular diagnostics. For example, the polymerase chain reaction has been broadly used for genetic disease diagnosis, oncogene screening, pharmacogenomics, and infection disease detection. In addition, DNA replication is a basic reaction in most of the DNA sequencing technologies. Loop-mediated isothermal amplification (LAMP) in particular has become a popular rapid detection method owing to its high sensitivity and high specificity as in nested-PCR.[13] Also, helicase or precise thermal cycling is not required in LAMP since the polymerase used has optimal replication and displacement activities around 60°C to 65°C.[14,15]

We propose that TEF can increase the sensitivity of time series LAMP detection (tip-LAMP). The effects of excitation power and polarization on TEF were assessed to figure out appropriate excitation conditions. A properly polarized illumination on a tip could reduce the excitation volume and maximize the enhancement effect. Our results also revealed the trend of TEF with the increment in excitation power. Tip-LAMP was then demonstrated at three different Hepatitis C virus (HCV) cDNA template concentrations. With signal enhancement, a laser beam of minimum intensity was used to minimize sample damages in monitoring tip-LAMP. To the best of our knowledge, this is the first time that TEF has been applied to sense time series isothermal amplification in nanoscale. According to our results, TEF was a promising technology for developing a miniaturized biosensor with high sensitivity and quick response for monitoring real-time DNA amplification.

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2 Methods and Materials

2.1 *Optical Setup*

The schematic diagram of our optical setup is illustrated in Fig. 1. An ultrafast laser (Tsunami) was used with a wavelength of 780 nm. To study the effect of laser polarization on TEF, a polarization converter (Arcoptix S.A) was set to switch between radial polarization (RP) and azimuthal polarization (AP). The intensity of the laser was controlled by a half-wave plate and a linear polarizer. The illumination power was measured by a power meter in front of the polarization converter. To collect the emission, a 60× water-immersion condenser was used as an objective. The emitted fluorescence passed through a band-pass filter of 520 ± 10 nm before being detected by the photomultiplier. The focal spot images of polarized beam were displayed in Fig. 1, which showed that the RP beam produced a sharper focus than linear polarization (LP) and AP. A three-dimensional scan was performed before each experiment to verify the apex position. All images were at a 49 nm/pixel scale with an integration time of 0.1 ms/pixel.

2.2 *Loop-Mediated Isothermal Amplification Hepatitis C Virus cDNA*

The amplification process of LAMP consisted of two steps: first, a dumbbell-like new template was formed with the help of forward/backward inner primers (FIP/BIP) and forward/backward primers (FP/BP) (Fig. 2). Second, the loop structures in the new template could be the primer of the template itself for elongating a new strand. The newly elongated amplicon then formed a flower-like DNA by self-annealing. The added forward/backward loop primers (LF/LB) were used to accelerate the elongation process. The primer sequences for HCV LAMP were listed in Table 1.

The mixture of reaction solutions was prepared for general LAMP and tip-LAMP. The premixture included 0.4 mM dNTPs, 0.4 M Mg_2SO_4, 1 × Bst buffer (New England Biolabs Inc.), 1 M betaine (Sigma-Aldrich Co.), and the primer mixture. Mg ion was the cofactor for polymerases. The betaine could reduce the required energy for separating DNA double strands. The primer mixture mentioned here contained 10 pmol of FP/BP, 20 pmol of FIP/BIP, and 5 pmol of LF/LB. Additionally, the asymmetrical cyanine dye (SYBR® Green I) green dye was added as a reporter on newly forming double-stranded DNA. All materials above were dissolved in ultrapure water. For general LAMP, target DNA templates and 8 U of large fragment Bst polymerase (76 kDa, New England Biolabs Inc.) were added to the mixture and heated to 65°C for 1 h. The HCV cDNA templates used here were prepared with the pGEM-T easy vector system (PROMEGA). The subsequent processes of tip-LAMP are explained in Sec. 2.3.

2.3 *Preparation of Nano Tips for Loop-Mediated Isothermal Amplification (Tip-LAMP)*

The apex size of Pt/Ir coated tips (NanoInk Inc.) was inspected via VHX-2000 microscopy (Keyence Co.) and scanning electron microscopy (SEM) before and after gold film deposition. The Au film was deposited onto the tips by sputtering [Fig. 3(a)-1] and verified with an ellipsometer (EP3, nanofilm) to confirm the thickness. Because the electric field distribution and excitation volume at the apex were affected by the thickness of the Au film and the sharpness of the apex,[16,17] all tips used in subsequent experiments were with a 7-nm-thick Au film, which corresponded to 55 to 57 nm in apex diameter. The gold covered tips were then immersed into 1 mM 16-mercaptohexadecanoic acid (MHA) in acetonitrile for 30 min to form a MHA self-assembled monolayer. MHA is a long carbon chain structure with sulfhydryl and carboxyl groups at its two ends,

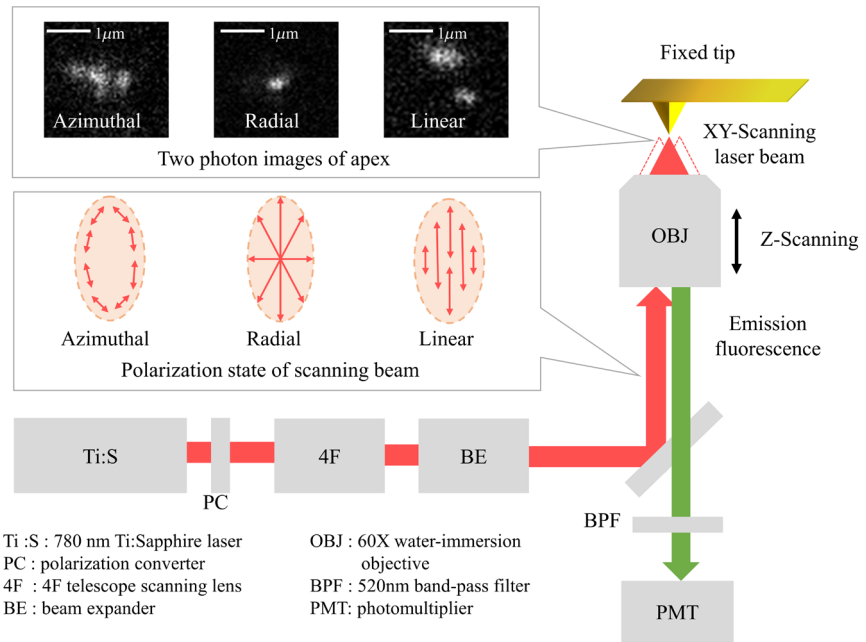

Fig. 1 Illustration of our two-photon microscopy. The samples were scanned with three differently polarized laser beams, respectively. The upper-left image indicates the sharper focus with radially polarized illumination than the other two polarized states. The profile of the images was also affected by the shape of apexes.

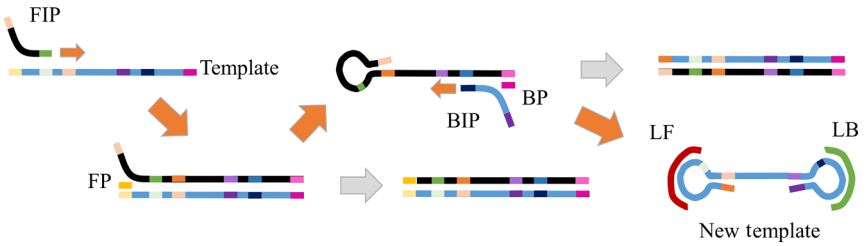

Fig. 2 Dumbbell-like new template was formed in the first step of loop-mediated isothermal amplification (LAMP). (FIP: forward inner primer, BIP: backward inner primer, FP: forward primer, BP: backward primer, LF: forward loop primer, LB: backward loop primer).

respectively. The sulfhydryl group could react with the Au film surface by thiol-metal bond (Au-S bond). After being washed with ethanol and ultrapure water, the MHA coated tips were soaked into 400 mM ethyldimethylaminopropyl carbodiimide and 100 mM N-hydroxysuccinimide (NHS) in ultrapure water for 5 min to activate the carboxyl group on MHA [Fig. 3(a)-2]. For polymerase coating, Bst polymerase was dropped onto a cellulose membrane. The chemically modified tips were dried by N_2 and brought into contact with the cellulose membrane by the approaching process of dip-pen nanolithography platform (NanoInk) [Fig. 3(a)-3]. The carboxyl group of MHA would covalently bind to the amine group on polymerase. Finally, the polymerase functionalized tips were mixed with the reagent premixture mentioned in Sec. 2.2 and sealed inside a reaction chamber for LAMP reaction [Fig. 3(a)-4].

Serious quenching occurs when the fluorophore is too close to the metal surface. Therefore, we estimated the distance between the tip and the fluorophore. By assuming that the shape of the polymerase was spherical, the diameter of Bst polymerase was calculated to be 5.6 nm.[18] Since the length of the MHA linker was around 1.5 nm, the Au-fluorophore distance was estimated to be 6.6 to 7.1 nm [Fig. 3(b)], which was sufficient to seriously avoid quenching through ohmic loss. The SEM images of an unmodified and modified tip are shown in Fig. 3(c).

3 Results

In Sec. 3.1, we briefly report the effect of background signal, excitation power, and polarization on TEF. A gold film covered tip was immersed into a fluorescein isothiocyanate (FITC) solution and scanned with a polarization-switchable laser beam for imaging. In Sec. 3.2, we demonstrate the application of TEF to monitor the LAMP reaction at the tip apex.

Table 1 The primer sequences for loop-mediated isothermal amplification (LAMP) on Hepatitis C virus (HCV).

Primer	Sequence (5′-3′)
Forward inner primer	TATGGCTCTCCCGGGAGGGG TTGCCATGGCGTTAGTATGAGT
Backward inner primer	TGAGTACACCGGAATTGCCAGG TTGGGGGCACGCCCAAAT
Forward primer	CTTCACGCAGAAAGCGTCTA
Backward primer	CCTTTCGCGACCCAACACTA
Forward loop primer	TCCTGGAGGCTGCACGA
Backward loop primer	ACGACCGGGTCCTTTCTTG

3.1 *Excitation Power and Excitation Polarization Effect on Tip-Enhanced Fluorescence*

We first inspected the background signals from our system, tip, and fluorescence solution. The system background was measured in air without a tip. The result in Fig. 4(a) indicated that system background was not relevant to the excitation power. Meanwhile, the photon count, either of the tip apex in water or of the focal spot in FITC solution, was proportional to the excitation power [the upper-left plot in Fig. 4(a)]. Even though some of the background signals slightly increased with power, the TEF photon count in FTIC solution was much greater than the background signals. In addition, the photon count at apex was found to be higher than the photon count on a flat Au surface. In view of Fig. 4, we confirmed that a metallic tip provided the strongest enhancement, and the interference from the background signals could be neglected in our experiments.

Polarized illumination affects the intensity distribution of the electrical field at the tip. According to the computer simulation, RP illumination was thought to provide sharp longitudinal field distribution at the focal point whose size was approximately 0.4λ (full width at half maximum).[19] The longitudinal electric field could excite a strong and highly concentrated optical field in tip-based near-field optics.[20] We experimentally inspected the TEF intensity under RP and AP illuminations. Figure 4(b) indicates that the enhancement was proportional to the excitation power. In addition, an RP excitation beam provided stronger enhancement than AP in our system. Apart from this, the enhancement with RP illumination reduced the diameter of the excitation spot and the minimum requirement of the excitation intensity. Figure 4(c) displays the diameter differences of excitation spots under RP or LP illumination. We took images of the apex in FITC solution such as the upper-left two photon images shown in Fig. 1. The RP illumination reduced around 100 nm in spot diameter compared to LP. Furthermore, Fig. 4(c) shows that RP illumination was of benefit to the apex imaging with an excitation power lower than 50 mW.

The enhancement ratio (ER) of the tip in Fig. 4(a) was calculated and plotted in Fig. 5. The ER here is defined as the value of the brightest pixel in the apex image divided by the average count of a random acquired 30-pixel-square area out of the apex in the solution [Fig. 5(a)]. The ER of the tip apex in the FITC solution with RP illumination was shown in Fig 5(b). The ER of TEF was proportional to the incident laser intensity. At an excitation power of 50 mW, the ER could be five on average; at an excitation power of 95 mW, it could be around 30 on average. This rise in ER was inferred to be a result of the increase of

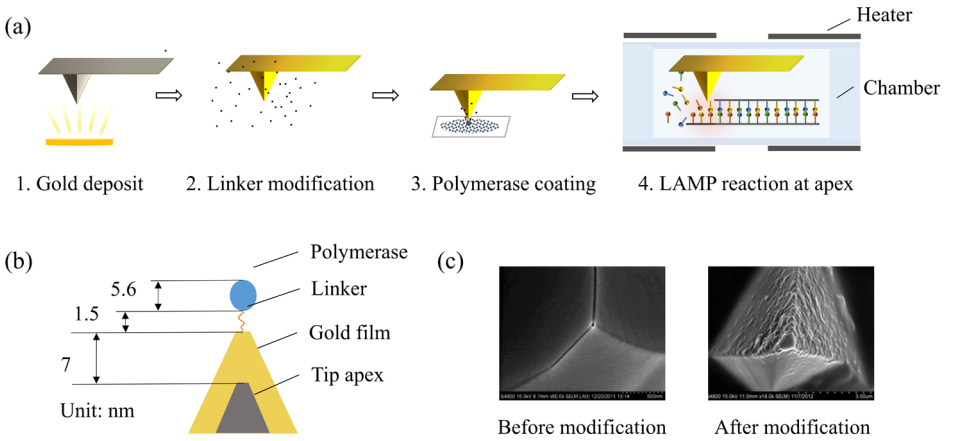

Fig. 3 Tip preparation. (a) Undamaged tips were functionalized with MHA/EDC/NHS and polymerase. Heating plates with a temperature sensor and feedback control was covered in both sides of the chamber. (b) The distance of Au-fluorophore was estimation to be 6.6 to 7.1 nm (d = thickness). (c) Scanning electron microscopy (SEM) image of the unmodified/modified tip.

excitation volume,[21] and the curve should reach a plateau at a higher incident power.[10] We chose a power of 50 mW for excitation in Sec. 3.2. It should be noted that the measured power of 50 mW before the polarization converter corresponded to an after-objective power of 160 μW in our optical system.

3.2 *Tip Enhanced Fluorescence for Monitoring Loop-Mediated Isothermal Amplification*

To perform the tip-LAMP, a premixture solution, polymerase functionalized tip, and 4 ng/μl HCV cDNA templates were sealed in the reaction chamber. We heated the reaction chamber to 65°C for LAMP reaction. The two photon images of the apex were continuously captured during the reaction. The results in Fig. 6(a) indicate that the brightness of the apex images increased with the reaction time, which corresponded to the accumulation of amplicon-SYBR® Green I complex. We created histograms of the photon counts at different time points from Fig. 6(a) and divided the photon count values into three different intensity groups. The histograms shifted to the right with time [Fig. 6(b)]: before the start of the reaction, more than 90% of the pixels had an intensity of less than 1000 counts; after 70 min reaction, around 30% of the pixels had a count more than 1000, and about 25% of the pixels had a count more than 10,000.

To figure out the performance of tip-LAMP in quantification, tip-LAMP reactions at three different template concentrations were carried out: 4, 0.4, and 0.004 ng/μl. The premixture solution was separated into two parts as mentioned in Sec. 2.2 for

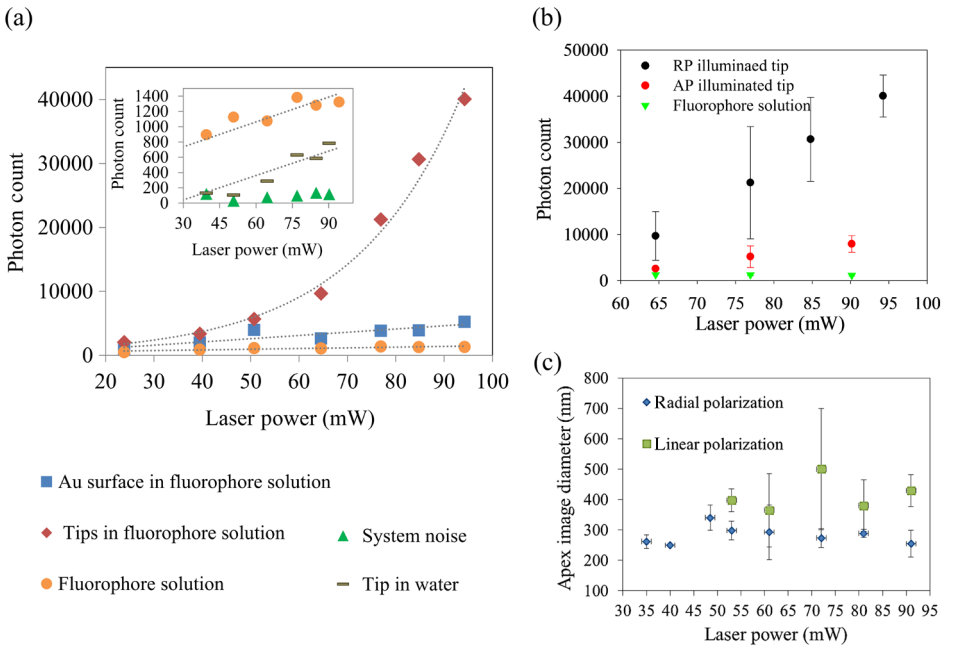

Fig. 4 Tip strongly enhances the fluorescence. (a) Tip is with stronger emission than flat Au. Upper-left plot shows the weak background signal, which can be ignored in our experiments. (b) Effect of polarization on tip-enhanced fluorescence (TEF) signal intensity. (n = 5). (c) The focal spot diameter is around 100 smaller with RP illumination than LP. TEF with RP illumination increased the emission intensity and made the emission spot clear to be seen.

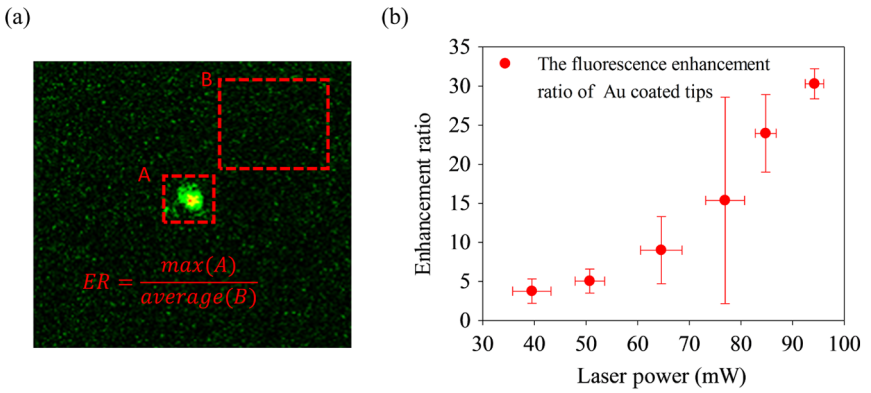

Fig. 5 (a) Two-photon image of the tip apex under radially polarized illumination. The enhancement ration was calculated followed the formula with red color. B is a 30-pixel square and A is a random selected rectangular region including the apex image. (b) The enhancement ratio (ER) of tip apex in fluorescein isothiocyanate (FITC) solution with radial polarized illumination.

general real-time LAMP and tip-LAMP. The general real-time LAMP was performed with a BIO-RAD CFX real-time PCR detection system. The results of general LAMP were shown in Fig. 7(a). In the meantime, the tip-LAMP was performed with the protocol mentioned earlier in this section. The apex images with the size of 10×10 pixels were continuously acquired within the reaction time. The photon counting values in each apex image were averaged and plotted in Fig. 7(b) for all the time points. Compared to the calibration curve of general LAMP, the increasing phases of the tip-LAMP were about 10, 20, and 30 min earlier for the reactions with 4, 0.4, and 0.004 ng/μl templates, respectively.

4 Discussion

To develop a TEF nanobiosensor with high sensitivity, we investigated the effects of the excitation polarizations and intensities on TEF. With an excitation power lower than 50 mW, the emission excited by AP or LP beam was as weak as the background signals, which made the emission hard to detect. However, RP illumination provided the strongest field enhancement, which made the fluorescence recording feasible under low excitation power. Besides the effect of polarization, our results revealed the nonlinear proportional relationship between ER and excitation power. According to previous research in metal enhanced fluorescence, the rise of ER with excitation power finally saturated and reached a plateau. Instead of showing the increasing or plateau phase, Fig. 5 demonstrated the changing of ER with a low excitation intensity. The minimum excitation power with an obvious enhancement was 50 mW. Thus, a 50 mW RP laser beam was used to excite the amplicon-SYBR® Green I complex fluorescence in the tip-LAMP. The 50 mW laser will be attenuated to 160 μW after passing through the other optical components and the objective in our optical setup. The low power illumination and fast image scanning could avoid damages in biological samples during a long-time observation. According to our results, the Bst polymerase was still active under the illumination of the chosen power.

Because of the signal enhancement in TEF, tip-LAMP could be a highly sensitive DNA nanobiosensor. Two mechanisms in our design magnified the signals. First, the quantity of HCV cDNA was increased by the LAMP reaction. Second, the fluorescence was amplified with TEF. However, since there was only one reaction spot in the tip-LAMP, mass transportation under low template concentrations could be a limitation for real-world applications. A long and changeable time will be required for molecular collisions with low template concentrations. A tip array to increase the reaction points is one of the options that could be used to overcome this problem.

The signal variations in our results could be attributed to the orientation, diffusion, and Brownian motion of fluorophore particles. The individual apex size deviations in different tips should also be considered. Moreover, the fluctuation of the fluorescence signal during the biosynthesis process in Fig. 7(b) could also be caused by stochastic bioreactions and the movement of DNA strands in solution. Furthermore, the randomized

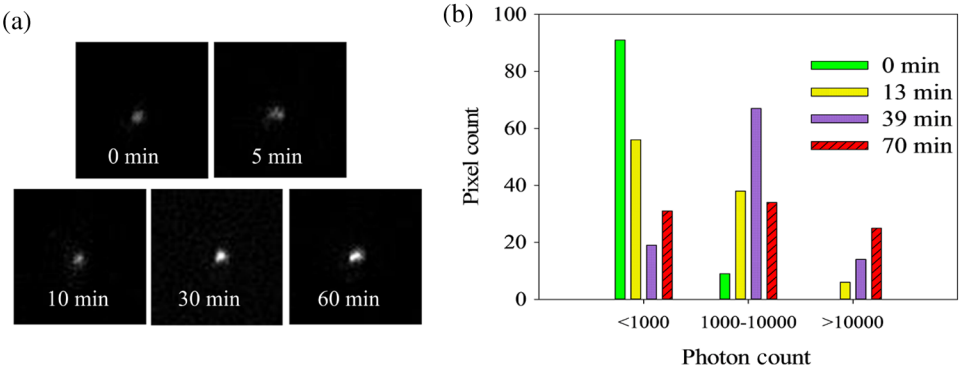

Fig. 6 (a) Two photon images of this tip-LAMP in selected time points. (b) Grouped pixel intensity histogram of apex fluorescence images at different times during the tip-LAMP with 4 ng/μl HCV cDNA.

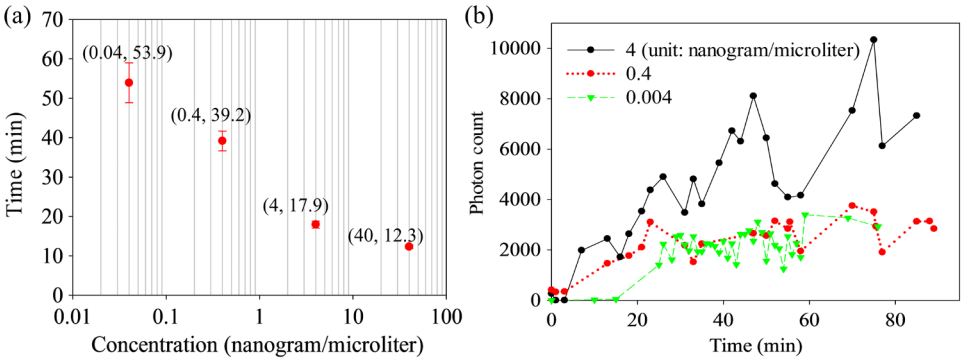

Fig. 7 (a) The calibration curve of general real-time LAMP performed by BIO-RAD CFX real-time PCR detection system. (b) Results of the tip-LAMP reaction with three different template concentrations.

molecule collision and individual differences in polymerase processing could lead to different initiation times and subsequent ability in replication. In other words, amplicons of variant lengths would be produced. In addition, the amplicon-SYBR® Green I complex complex might also leave, drift, or continue to the next replication cycle owing to thermodynamic effects, repulsion of negatively charged DNA, and optical gradient force on the apex. Despite the existence of these uncertainties in the tip-LAMP, the overall result showed the high sensitivity and quantitative ability of the tip nanobiosensor.

5 Conclusion

In conclusion, radially polarized illumination produced a strong and well-confined optical field at a tip, which was used to enhance the emission of FITC and amplicon-SYBR® Green I complex. With an illumination intensity of 50 mW, FITC emission was increased five times on average by TEF. Moreover, we monitored the LAMP on Hepatitis C virus cDNA by TEF. The increment of amplicon-SYBR® Green I complex at three different template concentrations was found 10 to 30 min earlier than general LAMP. These results revealed the potential use of TEF as a miniaturized real-time biosensor with high sensitivity. In the future, we expect the development of a tip sensor array for monitoring single biomolecular reactions or detecting bioreactions in extremely low concentrations.

Acknowledgments

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