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Metallic Tip Enhanced Fluorescence for DNA Replication Monitoring*

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***Abstract*—We have successfully performed localized loop-mediated isothermal reactions of hepatitis B virus (HBV) and hepatitis C virus (HCV) on the apex (50~100 nm) of metallic tips coated with Bst polymerases. The SYBR green molecules binding to the new formed HBV DNA inside the optical near fields were excited by two-photon fluorescence microscopy, and directly imaged in far field. Another reporter primer is used for HCV replication detection. Preliminary results are presented in this manuscript.**

I. Introduction

Biomolecule interactions in nano-scale are broadly interested to scientists for years. Comparing to macroscopic experiments, the interactions under microcosmic view could provide more direct information to reveal molecules behavior. For example, the DNA replication process is revealed by optical tweezers and the helicase efficiency is measured by fluorescence correlation spectroscopy. To observe the molecule reactions in nano-scale volume, the enhancement of signal should become indispensable.

Metal-enhanced fluorescence (MEF) is a phenomenon describing the enhancement of fluorescence intensity while the excited fluorophores are in the proximity of the metal nanostructures or surfaces in a few angstroms to nanometers. The phenomenon is generally thinking to be contributed by two processes. The first one is the near-field enhancement of the incident lights and emission fluorescence on metal structures. The second one is the excited-state fluorophores induced plasmonic effect which altering the quantum yield and fluorescence lifetime by changing the radiative decay rate [1].

Due to the MEF effect, the fluorescence signals of fluorophores with low quantum yields can be typically increased by 2 to 10 folds [2, 3]. The enhanced effect has been applied to many research fields, like biomolecule interactions, immunoassays, and clinical sensors for higher sensitivity and detection limitations [4-6]. In 2007, Jian Zhang revealed the possibility of single molecule Cy3 and Cy 5 detection by MEF, and the other report about single green fluorescent protein detection by MEF then was published by Yi Fu in 2008. Both of them use silver nanoparticles to enhance the fluorescence signal. Silver, however, are unstable and easy to interact with other molecules. Instead, gold with higher biology compatibility may be a more promising material for biological purpose. In our previous work [7], we utilized the Au coated tip apex for detect the DNA replication process named loop-isothermal amplification (LAMP) [8]. Due to the isothermal and time-saving characteristics, LAMP reaction has been a popular method in many gene sensing biochips [9-11].

In this manuscript, we demonstrate a SYBR Green based method and a new fluorescence reporter design for the localized HBV and HCV LAMP reaction, which reveal the possibility of MEF in submicron scale biosensor application and molecular reaction observation.

II. Methods

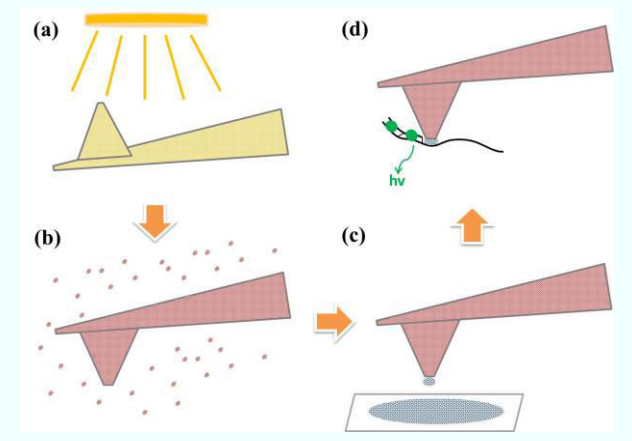

Figure 1. The experimental processes are showed in this scheme. (a) The Au film is deposit to the tip. (b) The gold covered tip is immersed to the MHA solution and then activated with EDC/NHS solution. (c) The modified tip is then approach to the cellulose membrane containing the Bst polymerase. (d) The functionalized tip is used to perform a localized DNA replication.

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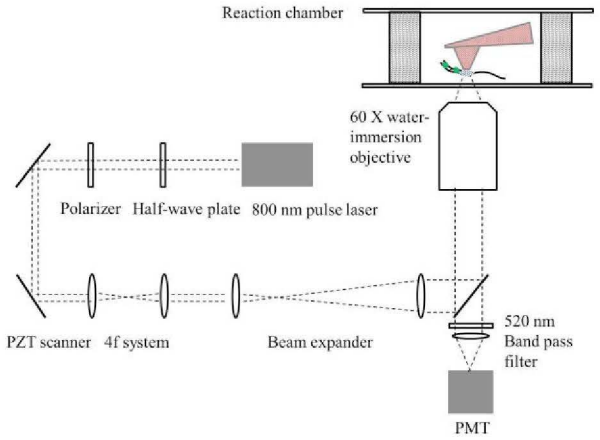

Figure 2. The construction drawing is the two photon microscope and the PDMS reaction chamber used in our experiment. The SYBR green molecules are excited by 800 nm pulsing laser, and the emission fluorescence is detected by PMT with a 520 nm band pass filter.

The experiment scheme is illustrated in Fig.1. Silica Pt/Ir coating tips were deposited with Au film by sputters under 35 Watt and 10^{-5} torr for 150 second. After Au sputtering, the apex size was around 57 nm; the size of apex was checked by scanning electric microscope.

The Au deposited tips were then immersed into mercaptohexadecanoic acid (MHA) solution in acetone nitrite for 30 minute. Self-assembled monolayer will be left on the Au film after ethanol and ultra-pure water washing. The carboxyl group of the MHA linker was then activated by 400 mM ethyldimethylaminopropyl carbodiimide (EDC) and 100 mM N-Hydroxysuccinimide (NHS) solution dissolved in water. The tips were washed with ethanol solution and dried before use.

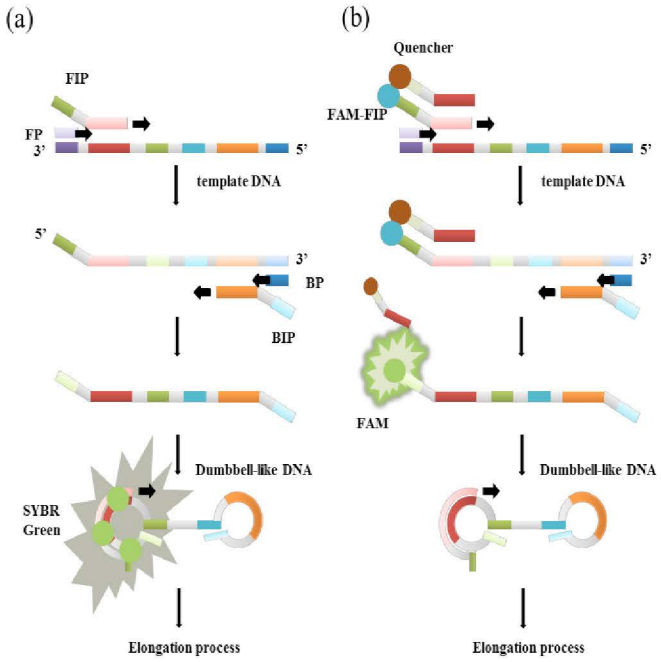

Figure 3. The reaction process of (a) the SYBR green-based LAMP reaction and (b) the new primer design for LAMP reaction. In reaction (a), the SYBR emti fluorescence while chelated to double strand DNA. In (b), the FAM-labled primer will emit fluorescence when the quencher strand are displaced by new forming BIP leading strand.

To immobilize the protein to apex, the functionalized tips were approached to a cellulose membrane pre-immersed to Bst polymerase solution by mechanical motor stage and stay for 5 min. The tip body was blocked with bovine serum albumin.

The coated tip products preserved in PBS were used immediately to avoid the decrease of polymerase activity. The reagent mixture which contained LAMP pre-mixture, designed primer pairs, HBV or HCV DNA and SYBR dye was prepared previously. The mixture and functionalized tips were then sealed between two cover glasses. To achieve the Bst polymerase working temperature, a flexible heating plate with temperature sensor was settled on the microscope sample stage to control the temperature around 63 to 65°C. The temperature was maintained for two hours for the LAMP reaction monitoring.

A custom-built two-photon excitation fluorescence microscope with 800nm laser in 100 picosecond pulse duration and a water-immersion 60X objective was used to collected fluorescence photon counts at a wavelength of 520±10 nm (Fig. 2). The background noise can be reject and both enhancement factor and image contrast will increase by combining the metallic tip enhancement with the two-photon fluorescence excitation microscopy [12].

The SYBR dye chelated to new formed double strand HBV DNA product, like Fig. 3 (a) showing, will emit fluorescence signal and detect by our system. For the HCV LAMP reaction observing, a FAM-NAD labeled primer pair design in figure 3 (b) was used, which fluorescence was quenched before reaction and emitted after the replications started.

III. Result and Discussion

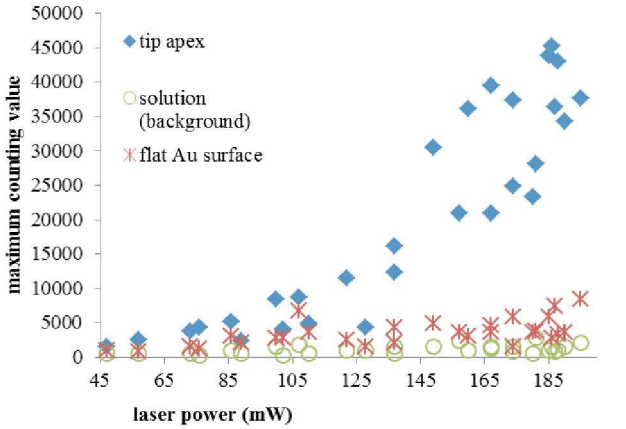

Figure 4. The metallic tip structure provides stronger optical signal enhancement than metal surface and bare fluorophore. The enhancement will dramatically increase with the increase of excitation laser intensity. The laser power is measured in the laser output.

The enhancement of fluorescence signal effectively decrease the power needed to excite the fluorescence and increase the quantum yield of the fluorophores.

Figure 4 shows the dramatically increase of photon counts by the assist of metallic tips. While the metal surfaces provide around two fold of signal intensity in our experiment, the metallic tip structure can enhance the optical signal about ten to twelve folds than the bare fluorophores. The results from five tips are presented in this figure. The photon counting variation may cause by the slightly differences of the tip apex.

In addition to the enhancement, the molecules fixing process also plays an important role in increasing the signal to noise ratio in localized bioreaction. The modification of protein functional method by the mechanical approaching and BSA blocking process successfully prevents the uncontrollable contaminant of Bst polymerase spreading to the cantilever beam when withdrawing the tip from the cellulose membrane surface and insure the positioning reaction.

The metallic tip enhanced fluorescence results of the localized HBV LAMP reaction are showed in Fig. 5. The increasing of the fluorescence intensity means the increasing of double strand DNA. The signals were taken in a five to ten minute interval. A slightly increase of photon counts will happens after a few minute heating, and then the value start to fluctuate. The point 1 in figure shows the signal fluctuation which may infer to the stochastic diffusion of free form SYBR Green, SYBR-chelated primer-DNA complex or short replicating DNA segment. The photon counts of point 2 in Fig. 5 indicate that the new strands HBV DNA start to be numerous produced an hour after the heating start.

Because of the increase of quantum yield, the nonspecific fluorescence from the free form SYBR and the SYBR chelated to primer annealed template DNA may cause the

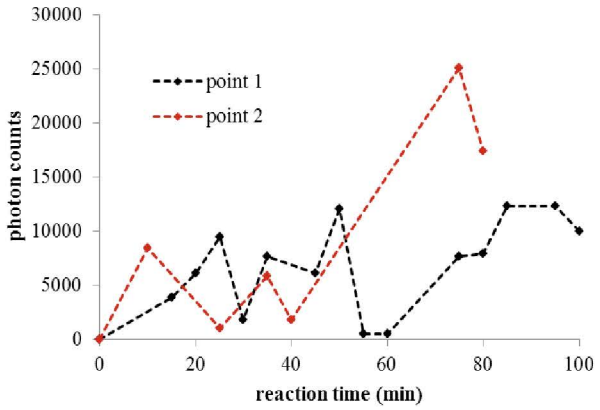

Figure 5. (point1) The backgound noise come from the free form SYBR or the SYBR chelated to short double strand DNA of primer and template. (point2) The localized HBV LAMP reaction on tip apex. The results infer that the reaction start after heating an hour.

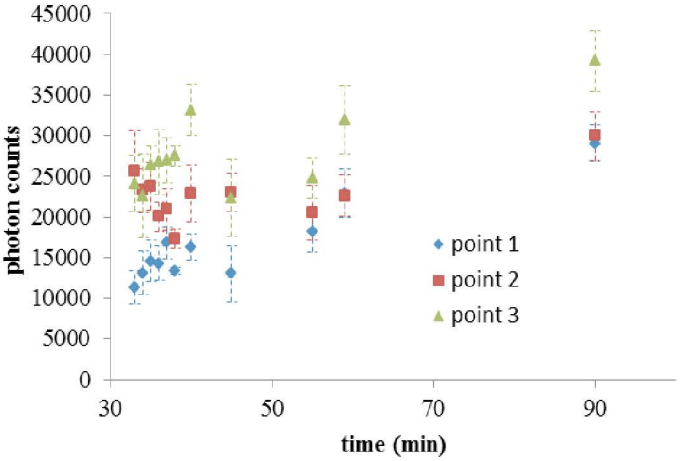

Figure 6. The locsalized HCV LAMP reaction on the metallic tip apex. The photon counts of the reaction slightly increase at 40 to 45 minute and stronly elevate around 55 to 60 minute.

increase of background noise. Hence, we design another fluorophore labeled method to avoid this problem by using a fluorophore-quencher primer pair for the HCV LAMP reaction.

Instead of reporting the newly synthesized double strands DNA, the optical signals emit in case the quencher leave apart from the fluorophore molecules. The quencher-labeled strand will be kick out by Bst polymerase in the last step of dumbbell-like structure forming stage in LAMP reaction, like Fig. 3 (b) shows.

Fig. 6 is the real-time monitoring plot of HCV LAMP reaction. The elevations of photon counts show up after fifty-five minutes, which may indicate the massively start of the looping process of LAMP reaction and the increasing quantity of DNA product. The fluctuation of signal appears before fifty minutes which may infer to the variation quantity of primer-DNA complex or the discontinued LAMP reactions.

IV. Discussion and Conclusion

We have successfully performed the metallic tip enhanced fluorescence method for monitoring the localized Hepatitis B virus and Hepatitis C virus loop-mediated isothermal amplification. Due to the reasons of the signal fluctuation are complex and hard to be analyzed, to successfully perform this reaction, the background light noise and protein coating process should be carefully considered. Primer-SYBR complex, FAM-conjugated primers and short DNA segment replication are possible causes of noise in our inference. Nevertheless, the dramatically increase of fluorescence should be undoubtedly noted as the massive DNA replication.

Although there are still uncertain parameters in this experiment, the geometrical space limitation on tip apex and the fluorescence enhancement on metallic nanostructure of

this method have shown the potential for nano-scale sensing or single molecule interaction observation with a well experiment skill in the future.

ACKNOWLEDGMENT

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