33rd Annual International Conference of the IEEE EMBS
Boston, Massachusetts USA, August 30 - September 3, 2011

Detection of Tip-enhanced Fluorescence from Loop-mediated Isothermal Amplification of Hepatitis B Virus by Two-photon Microscopy

Shih-Chung Wei[a], Tsung-Liang Chuang[b], Hui-Hsin Lu[b], Chia-Chen Chang[b], Da-Shin Wang[b], Kung-Bin Sung[a], and Chii-Wann Lin[b], *Member, IEEE*

***Abstract*—Tip-enhanced fluorescence of localized DNA replication by loop-mediated isothermal amplification (LAMP) is a potential way to observe real-time biological reaction confined in nanometer scale. We successfully coated Bst polymerase on the apex (~100 nm) of an atomic force microscope (AFM) tip and performed localized LAMP reaction of hepatitis B virus (HBV). By using this tip-based reaction, the replicated HBV DNA can be directly imaged to be 400~500 nm spots by using two-photon excitation fluorescence microscopy.**

I. Introduction

Development of highly sensitive, inexpensive and fast DNA screening methods is of significant interest to researchers and clinicians. Loop-mediated isothermal amplification (LAMP), first published by *Notomi et al*. in 2000 [1], has been shown to have higher sensitivity and specificity than polymerase chain reaction (PCR) [2]. During a LAMP reaction occurring at a constant temperature, the target nucleic acids are replicated to many dumbbell strands at first stage and many of the replicas are then connected to form a stem-loop structure which is several times longer than original target. The isothermal character of LAMP makes it suitable to develop a simple and fast screening tool. However, the results of LAMP reactions are usually detected by using fluorescence labeling electrophoresis or turbidity, both of which need laboratory equipment and a large quantity of replicas to achieve sufficient signal-to-noise ratios. This inevitably increases the time of the reaction, especially for the detection of low concentration targets. Even many of miniaturized devices are developing; we believe the simplification of device including detection will limit the detection sensitivity. Enhancement of signal from the newly synthesized DNA strands has the potential to overcome the bottleneck of current DNA screening methods.

One promising technique to improve the low detection limit is using an AFM probe with metal coating which can confine photons in an extremely small volume to enhance the intensity of fluorescence emission of molecules near the metal nanostructure [3,4]. Many researchers have demonstrated the effects of tip-based enhancement for fluorescence emission or Raman scattering and have applied them to many fields [5, 6,7].

Manuscript received March 26, 2011. This work was supported in part by the National Science Council in Taiwan.

[a] Institute of Biomedical Electronics and Bioinformatics, National Taiwan University, Taiwan.
[b] Institute of Biomedical Engineering, National Taiwan University, Taiwan.
*Contacting author:
Chii-wann Lin, Medical MicroSensor and System Laboratory, Institute of Biomedical Electronics and Bioinformatics, National Taiwan University, No.1, Sec.4, Roosevelt Road, Taipei, 10617 Taiwan; Tel: +886-2-33665272, 33665271; Fax: +886-2-23620586; Email: xcwlin@gmail.com
Kung-Bin Sung, Biomedical Optical Spectroscopy and Imaging Laboratory, Institute of Biomedical Electronics and Bioinformatics, National Taiwan University, No.1, Sec.4, Roosevelt Road, Taipei, 10617 Taiwan; Tel: +886-2-33669675; Email: kbsung@cc.ee.ntu.edu.tw

II. Materials and Methods

A. AFM Tip Preparation

The tip coating and protein immobilization process is illustrated in Fig.1. Silica tips with Pt/Ir coating were coated with an Au layer under 30 Watt and 105 torr for 150 sec by a sputter. The size of the apex was around 100 nm after Au sputtering. The Au covered tips were immersed into supersaturated mercaptohexadecanoic acid (MHA) dissolved in ethanol for 30 min to form a self-assembled monolayer. After washing with ethanol once and ultra-pure water (type 1 water) twice, the carboxyl group of MHA was activated by 400mM ethyldimethylaminopropyl carbodiimide (EDC) and 100mM N-Hydroxysuccinimide (NHS) solution in ultra-pure water for 30 min. The tips were washed with ultra-pure water twice and dried by N_2.

For protein immobilization, the surface-modified tips were made in contact with a cellulose membrane containing Bst polymerase for 5 min by Dip Pen Nanolithography (DPN). The z-axis position of the approaching tip was monitored by a laser beam reflected from the back surface of the tip toward a quarter photo diode to prevent overloading. Non-reacted areas of the tips were blocked with bovine serum albumin (BSA) (Sigma) and washed with Phosphate buffered saline (PBS).The final products were preserved in PBS.

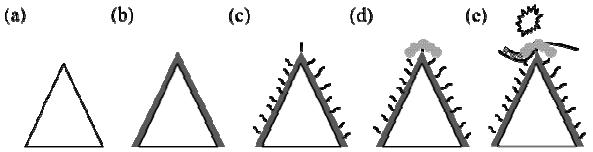

Fig. 1 The tip preparation process: (a) uncoated tip (b) Au film sputtered (c) surface modified with MHA (d) protein coated on the apex (e) DNA replication on apex.

FTTC-conjugated avidin was immobilized on AFM tips following the pro tocol and observed with a fluorescence microscope to verify the protein immobilization protocol.

A. *Preparation of HBV Sample*

HBV DNA was prepared by using QIAamp Viral DNA Mini Kit (QIAGEN) for isolation and pGEM-T Easy vector (PROMEGA) for amplification. Three pairs of primers as shown in Table 1 were designed following our previous work [9].

Table 1 Names and sequences of the three pair primers used in HBV LAMP reaction designed by S.Y. Lee (2007)

Primer	Sequence
FIP	5'-TGGAATTAGAGGACAAACGGG TGCTGCTATGCCTCATCTT-3'
BIP	5'-CTGCTCAAGGAACCTCTATGTTTCG ATGATGGGATGGGAATACA -3'
F3	5'-GGCGTTTTATCATCTTCCT -3'
B3	5'-AGGTTACTTGCGAAAGCC-3'
Loop F	5'-TACCTTGATAGTCCAGAAGAACC -3'
Loop B	5'-CTACGGACGGAAACTGAC -3'

B. *Loop-mediated Isothermal Amplification and Data Acquisition*

The LAMP reagent mixture contained the primers, betaine (Sigma), dNTPs, Mg_2SO_4, and Bst buffer (New England Biolabs) [9].

A conventional LAMP experiment was performed as a positive control. The reagent mixture plus 8U of Bst polymerase and the target DNA were heated to 65°C and left to react for 40 min. After the LAMP reaction was completed, the solutions were cooled to 4°C. Electrophoresis was performed in 1.5% agarose gel with 100 Volt and stained with ethidium bromide (EtBr).

In tip-enhanced LAMP experiment, the reagent mixture was mixed with 10^6 copies of HBV DNA as the target and one 1 of SYBR dye for fluorescence labeling of the newly formed DNA strands. A reaction chamber for tip-enhanced LAMP was made by Poly-methylmethacrylate (PMMA) on cover glass. LAMP reagent mixture and protein coated tips were inserted into the reaction chamber and the chamber was sealed. An Aluminum heating plate with temperature sensing and feedback control of the input voltage to a power supply was located on top of the chamber to maintain the temperature at 65°C. Heating the aluminum plate to 65°C took one hour and the temperature was kept constant for another hour (the actual temperature on the apex is hard to measure).

The SYBR dye was used as an indicator of double helix DNA production and detected by a custom-built two-photon excitation fluorescence microscope equipped with a pulsed laser at a wavelength of 800 nm (Fig. 2). Fluorescence emission at a wavelength of 520±10 nm was collected with a water-immersion 60X objective and detected by a PMT. Combining the metal tip enhancement with two-photon fluorescence excitation microscopy can reject most of the background signal and increase both enhancement factor and image contrast [10].

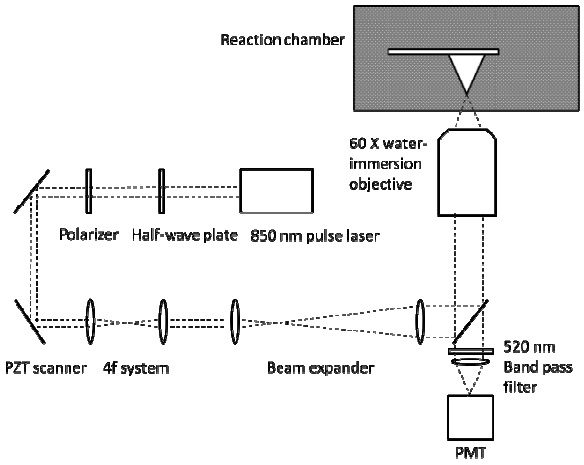

Fig. 2 Schematics of two photon microscopy setup and the reaction chamber

III. RESULTS

A. *Immobilizing Protein on Tip Apex*

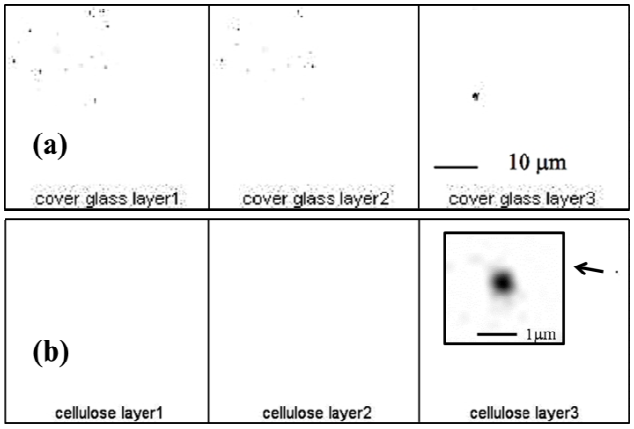

Fig. 3 Two photon excitation fluorescence images of FITC-avidin coated tips. (a) The tip approach to the cover glass substrate. Contamination of avidin can be seen in layer 1 and layer 2. (b) The tips approaching to the cellulose substrate. Layer 1 and layer 2 are very clean without any fluorescence protein. A magnified image of the coated spot in layer 3 is shown in the inset. The z distance between adjacent images was 2.5 μm and was controlled by a PZT.

Fig. 3 shows TPM images of tips coated with FTTC-conjugated avidin. Layer 1, 2 and 3 correspond to the cantilever, tip body and the apex of the tips, respectively. Two kinds of substrate including cover glass and cellulose membrane were used. The results of using cover glass as the substrate showed splashes of avidin over the whole tip as shown in Fig. 3 (a). Figure 3 (b) shows more successful coating by using the cellulose substrate. Only layer 3, top of the apex, showed a fluorescence spot about 550~600 nm in diameter. Based on the result, cellulose substrate was used to immobilize the Bst polymerase for LAMP reactions.

B. *LAMP Reaction*

Fig. 4 shows the electrophoresis results of the LAMP products resulting from different original HBV DNA concentrations. The first lane was the marker ladder. The second lane was control group without any target DNA. The concentration values of the other lanes were sequentially

$1x10^2$, $1x10^3$, $1x10^4$, and $1x10^5$. The result suggests that the low limit of detection was between 100 and 1000 copies.

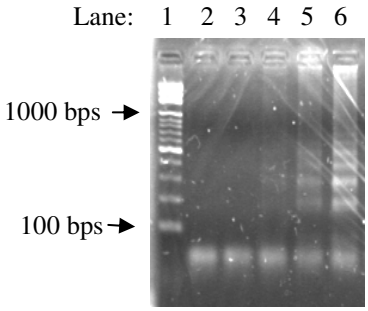

Fig. 4 Electrophoresis result of HBV DNA LAMP with various conecentrations. Lane1: marker ladder 100~1000 base pairs, Lane2: control, Lane3: 100 copies, Lane 4: 1000 copies, Lane 5: 10000 copies, Lane 6: 100000 copies)

C. Detecting Tip-enhanced Fluorescence of LAMP Reaction

Both the fluorescence intensity and covered area in the ROI increased with heating time, which suggests an increasing number of double helix DNA. The observed multipoint reactions around the apex could be caused by multiple polymerases coated at or near the apex.

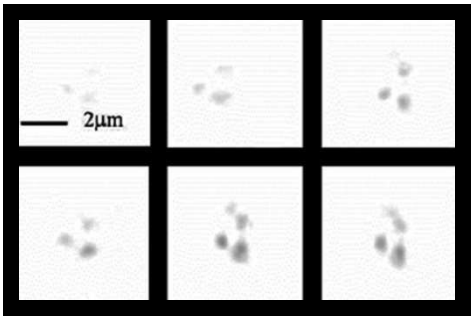

Fig. 5
Selected ROI of fluorescence images from the tip-enhanced experiment (from left to right, top row: 60 min, 70 min, 80 min; bottom row: 90 min, 100 min, 110 min)

To analyze the fluorescence intensity during the LAMP reaction, a region of interest (ROI) was selected (30x30 pixels) and the total photon counts measured by the PMT were calculated at a time interval of 10 minutes after the heater reached 65°C. The integrated photon counts in the ROI increased with time, indicating the progress of replication. The maximum photon counts appeared at 40 min. Afterwards, the reaction slowed down possibly due to steric hindrance caused by previous LAMP products..

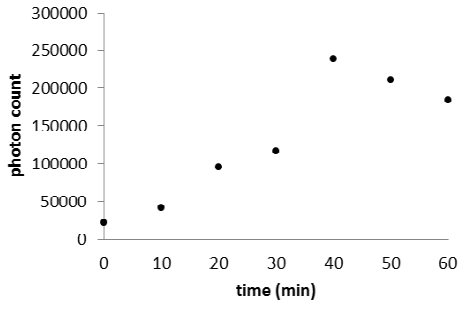

Fig. 6. Time-lapse measurement of fluorescence shows the integrate piexal values within an 30x30 pixels ROI.

IV. DISCUSSION

The immobilization of polymerase on the apex is an important step to ensure the inhibition of optical signal interference from the cantilever and increase the signal-to-noise ratio by avoiding multipoint reaction out of the apex at the same time. In our work, the tip coating process could be further optimized to concentrate the reaction to a single point.

Two-photon excitation fluorescence microscopy with PMT confines the excitation volume and minimizes noise from out-of-focus regions. It was used in this preliminary study to analyze the fluorescence intensity at different layers of the AFM tip. Other low cost optical detection techniques such as a photodiode can be used in future implementation once the coating process is optimized to only cover the apex. .

The increase in the area covered with fluorescence indicates increased volume occupied by newly synthesized DNA since the SYBR dye has stronger fluorescence after chelating to the newly synthesized double helix DNA. The decrease in photon counts after 40 min might suggest photobleaching or the release of synthesized DNA, and more experiments are needed to confirm the reason. The results of tip-enhanced fluorescence detection of HBV DNA LAMP reaction suggest that this method could be used to observe biological reactions confined in a nanometer-scaled volume in real time, which provides a potential way to reveal reaction mechanism or analyze the detailed structural information of bio-molecules such as in DNA sequencing applications.

V. CONCLUSION

We have successfully demonstrated the observation of DNA replication by tip-enhanced fluorescence. The immobilized LAMP reaction of HBV on an AFM tip provides an easy way to potentially achieve observation of reactions at tiny volume. The repeatability and quantification of this method, though, should be confirmed by more experiments. Tip-based biomolecular reaction may be a useful strategy for the developments of real-time detection of other biomolecular reactions and highly sensitive technique for reaction mechanism study.

VI. REFERENCES

[1] T. Notomi, H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino, T. Hase, Loop-mediated isothermal amplification of DNA, Nucleic Acids Res. vol.28, E63, 2000.

[2] Y. Mori, K. Nagamine, N. Tomita, T. Notomi, Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation, Biochem. Biophys. Res. Commun., vol.289, p.150–154, 2001.

[3] Lukas Novotny, Randy X. Bian, and X. Sunney Xie, Theory of Nanometric Optical Tweezers, Physical Review Letters, vol.79, p.645-648, 1997.

[4] Yves C. Martin, Hendrik F. Hamann, and H. Kumar Wickramasinghe, Strength of the electric field in apertureless near-field optical microscopy, J. Appl. Phys., vol.89, p.5774-5778,2001.

[5] S. Kawata, Y. Inouye, Scanning probe optical microscopy using a metallic probe tip, Ultramicroscopy, vol.57, p.313-317, 1995.

[6] Ziyang Ma, Jordan M. Gerton, Lawrence A. Wade, and Stephen R. Quake, Fluorescence Near-Field Microscopy of DNA at Sub-10nm Resolution, Physical Review Letters, vol.97, 260801-1 -4, 2006.

[7] Andrew Downes, Donald Salter, and Alistair Elfick, Finite Element Simulations of Tip-Enhanced Raman and Fluorescence Spectroscopy J. Phys. Chem. B, vol.110, p.6692-6698, 2006.
[8] B.J. McMahon, Epidemiology and natural history of hepatitis B, Semin. Liver Dis.25 (Suppl.1) p.3–8, 2005.
[9] S.Y. Lee, C.N.Lee, Holl Mark, Deirdre R. Meldrum, C. W. Lin, Efficient, specific, compact hepatitis B diagnostic device: Optical detection of the hepatitis B virus by isothermal amplification, Sensors and Actuator B, vol.127, p.598-605, 2007.
[10] Erik J. Sánchez, Lukas Novotny, and X. Sunney Xie, Near-Field Fluorescence Microscopy Based on Two-Photon Excitation with Metal Tips. Physical Review Letters, vol.82, p.4014-4017, 1999