Proceedings of the 2005 IEEE
International Conference on Mechatronics
July 10-12, 2005, Taipei, Taiwan

# The Application of an Innovative MEMS Protein Chip in Real-time Total Internal Reflection Fluorescence Microscopy

Y. K. Yen, J. Y. Lee, M.C. Kuo and L. S. Huang*
*Institute of Applied Mechanics*
*National Taiwan University*
*No.1, Sec. 4, Roosevelt Rd., Taipei, Taiwan, ROC*
**lshuang@mems.iam.ntu.edu.tw*

***Abstract*** — **Single biomolecular detection and real-time motion tracking of an anti-IgG molecule in a microchannel was successfully demonstrated by using total internal reflection flurorescence (TIRF) microscopy. Fluorescence-labeled biomolecules were excited at the transparent near-wall region by the evanescent wave which occurred at the optically index-mismatch interface. The MEMS-based microchannels biochip was also well designed and fabricated to exploit to monitor the motion of a single biomolecule in the near-wall flow layer. The motion of a singe anti-IgG molecule has been tracked and analyzed under the speed limit 6 mm/s of image capturing system. The 3D positions of a molecule were also plotted to illustrate the biomolecular trajectory.**

## I. Introduction

The study of protein-protien association, dissociation and motion of a single molecule can provide insights into molecular genetics, biosensor design, drug design and development of targeted drug delivery systems. "Ref.[1]"It is well known that both electrostatic and hydrophobic interactions govern association at liquid-solid interfaces.The binding kinetics of protein on the surface of biosensor is limited by the boundary layer of flow has been studied [2]. TIRF microscopy is a well-suited technique for real-time imaging of the random motion of single protein molecules in nearwall free solution, since it may view labeled biomolecules with evanescent wave and also provides high signal-to-background ratio which can eliminate all the background of images [3]. Recent advances in charge coupled device (CCD) camera speed and continuous pixel miniaturization have enabled temporal resolution of 0.033 ms and spatial resolution of 0.3 μm.

In this study, the TIRFM and MEMS-based biochip was integrated to monitor the interactions of protein-protein in the pumping flow. The near-wall motion and association/dissociation behaviors of proteins at the glass and water interface were investigated as a function of flow rate and antigen concentration. The velocity of a single biomolecule in a real-time imaging experiment was conducted to quantify the biomolecular trajectory spatially as well as its associated velocity both in measurement and theoretical calculation.

## II. Theory

When light is incident from a medium of high refractive index $n_1$ into a second medium of low reflective index $n_2$ beyond the critical angle, total internal reflection occurs. The light beam is reflected back into the higher refractive index medium at which an evanescent field penetrates into the lower refractive index medium (e.g., water). Meanwhile, the associated electric field intensity of evanescent wave is exponentially decayed. The exponential decay constant $d$ of the evanescent filed intensity I(z) is typically 30-300 nm, which depends on material refractive indices, incidence angle and wavelength of illuminating light.

$$I(z) = I(0)\exp(-z/d), \tag{1}$$

where the penetration depth is

$$d = \lambda_0 / 4\pi\sqrt{n_1^2 \sin^2\theta_i - n_2^2} \tag{2}$$

$n_1$ and $n_2$ are the refractive indices; $\theta_i$ stands for the incidence angle; $\lambda_0$ is incident wavelength. The intensity at the interface, I(0) is determined with coherent superposition of the incident and reflected light by solving Maxwell's equations and appropriate boundary conditions [4]. For s-polarized light, it expressed as

$$I_s(0) = |A_s|^2 4\cos^2\theta_i / \left(1 - n_2^2/n_1^2\right), \tag{3}$$

and for p-polarized light

$$I_p(0) = |A_p|^2 \frac{4\cos^2\theta_i\left(2\sin^2\theta_i - n_2^2/n_1^2\right)}{(n_2/n_1)^4\cos^2\theta_i + \sin^2\theta_i - n_2^2/n_1^2} \tag{4}$$

## III. Experimental

### *A. Objective lens-coupled TIRFM*

The TIR illumination requires a high numerical aperture (NA) objective lens of greater than 1.4 that is capable of collecting more light onto specimens. Figure 1 shows the optical system based on the Olympus inverted microscope (IX71; Olympus, Japan). The light was illuminated from the solid state blue laser (475 nm, 10 mW, B&W Tek), ex-

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panded by lens in three-fold to the original beam size. A lens with a focal length 500 mm was used to focus the light to the back focal plane of objective (PlanApo 60X 1.4 Oil Objective, Olympus, Japan). Through the objective the light was struck onto the interface between the coverglass and aqueous samples conjugated with fluorochrome, which were excited by the laser beam and emitted in longer wavelength light (e.g., 520 nm). The emitted light was collected by objective through a fluorescence filter, blocking the exciting laser light. Finally, the emitted light was detected by the imaging system installed with the cooled CCD. A proprietary digital image process program (MetaMorph Imaging System, Universal Imaging Corporation) was used to control the camera and capture data.

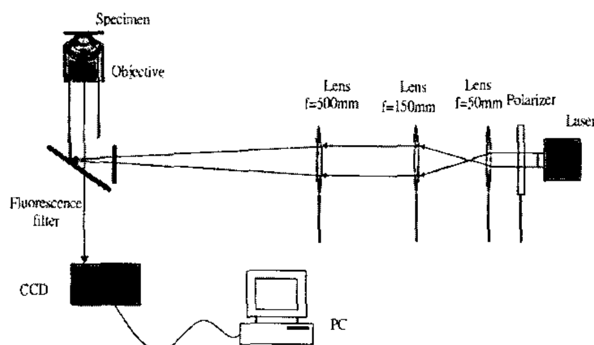


Fig. 1 Optical scheme of the objective lens-coupled TIRFM setup

*B. MEMS-based Microchannel Biochip*

The transparent MEMS-based biochip was developed, disposed on top of the inverted microscope. The biomolecular motion and interaction of proteins can be visualized with the biochip. The planar glass is essential as a viable substrate for TIRFM utilizing evanescent wave fluorescence excitation and fluorescence image-based detection (Figure 2). The preformed PDMS-based microchannel bound to the glass substrate was commonly used to exploit bio-related applications because of the enabling technology and mature fabrication processes [5]. The major purpose for the channel is to flow anti-IgG proteins above the coverglass for the immunoassay.

The process flow in manufacturing PDMS-based microchannels is illustrated in Figure 3. The structure of channels was patterned with the lithographic process. After this process, PDMS was prepared to transfer the structure of channels, the PDMS-based microchannels was readily made, followed by oxidation plasma for surface activation to bind the channels on a cover glass.

*C. Biochemical Modification of the Chip Surface*

To prepare the chip surface for protein immobilization, a coverglass was first soaked in 5% ethanol for 1~2 min, and treated in 5% 3-APTES (3-aminopropyltriethoxysilane, Sigma) to 30 min for surface silanization. After rinsing the surface with 5% ethanol and dry in air for 30 min, then immobilization for linker was carried out by being soaked in the 2.5% glutaraldehyde for 1h. After the linker immobilization the surface rinsed with phosphate buffer saline (PBS, pH 7.4) and exposed to 100 μg/ml of IgG overnight at 4 ℃ for covalent binding. Finally, Anti-IgG proteins conjugated with FITC which the concentration 100 μg/ml were prepared for immunoassay.

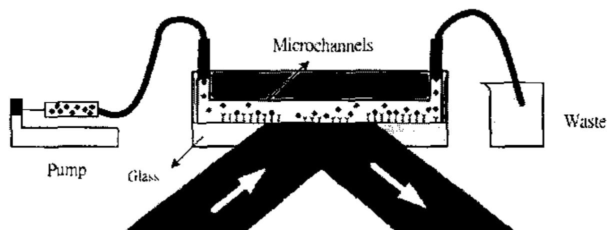


Fig. 2 The setup of combination of ITO-coated electrode and PDMS-based flow channel for TIRFM real-time monitoring and protein electrical manipulation.

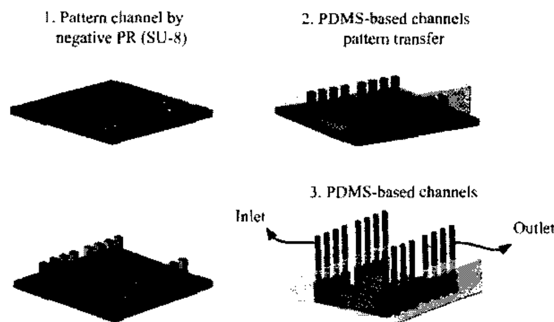


Fig. 3 The procedure of manufacturing PDMS-based microchannels. Channels were patterned with photoresist SU-8 100, then following by pattern transferred with PDMS

## IV. Results and Disscussuion

To monitor the motion and the associating process of a single anti-IgG molecule in the pumping flow, the PDMS-based microcannels biochip was made to conduct the experiment. Figure 4 shows the real biochip made by the MEMS fabrication technology, a sharp injection needle was inserted into the PDMS-based microchannels in order to transport the flow with bio materials into testing areas. The dimensions of flow channels were 16 mm long × 1 mm wide × 100 μm deep, and the flow rate of a peristaltic pump was set to be 10 μl/min. With such an experiment, the flow field in microchannel was fully developed. Therefore, in a steady state, incompressible flow field, the velocity profile is thus derived as

$$u(y) = (dp/dx)(1/2\mu)(y^2 - ay), \quad (5)$$

The flow rate is $1.67\text{x}10^{-4}\ \text{cm}^3/\text{s}$, the cross-section area of a channel is $9.36\text{x}10^{-4}\ \text{cm}^2$, and the viscosity coefficient is $9.03\text{x}10^{-4}\ \text{N.s/m}^2$. To input the parameters above, the results are obtained in comparison to the measured in Figure 7.

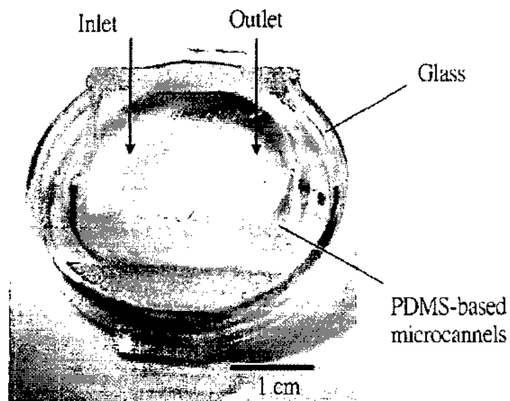

Fig. 4 The picture of the PDMS-based microchannels biochip

As for the TIRF testing, the fluorescent beads (Latex beads, carboxyl modified, Sigma) with accurate dimensions, are an appropriate sample. As mentioned before, the TIRFM allows to capture images with high signal-to-background ratio (SBR). Comparing to the background noise level in Epi-illumination mode, the TIRF configuration presents significant visual image within 300 nm from the interface by evanescent wave. Moreover, the lateral resolution measurement is fundamentally limited by the optical system and the resolution of image detection device. The fluorescent beads with diameter of 1.1 μm were immobilized on the coverglass, in turn taken into the assessment for further investigation. The beads were measured to be 1.125 μm in SEM picture, comparing to the measured size of 1.146 μm in TIRFM image. In this measurement, the TIRFM was verified, comparable to its size in SEM.

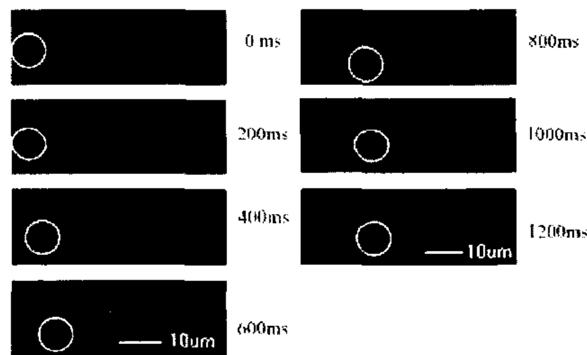

Fig. 5 The images of Real-time monitoring for motion of a single anti-IgG molecule in time interval

The association of a single protein molecule was first successfully visualized and monitored by using TIRF Microscopic system, as shown in Figure 5. The images were taken form the CCD in a speed of five frames per second, which was able to demonstrate different positions of the anti-IgG molecule in time interval. According to the speed limit of such a CCD system that captures the dynamic motion of a molecule to be 6 mm/s, the corresponding flow velocity is measured under this system limitation. In addition, the motion of an anti-IgG molecule was found to be tracked and simultaneously analyzed by the image processing program. Figure 6 shows the spatial positions of the biomolecule. The in-plane x-y positions was determined through the pixel and its associated magnified ratio of the optical system; the out-of-plane position in depth was determined by measuring the intensity of fluorescence light. In this setup, the penetration depth was found to be approximately 300 nm. It is clearly visualized that the anti-IgG biomolecule was motioned, approaching down to the surface in microchannels at which the IgG molecules were immobilized. The motion of anti-IgG molecules was governed by the flow field of the boundary layer [2]. In comparison of the measured and the calcuated results, Figure 7 (b) shows that the error was 70.3 % dramatically. This result may provoke the question, does the behavior of fluidics in the near-wall nanolayer still comply the typical laminar flow? This question remains unanswered and requires future experiments.

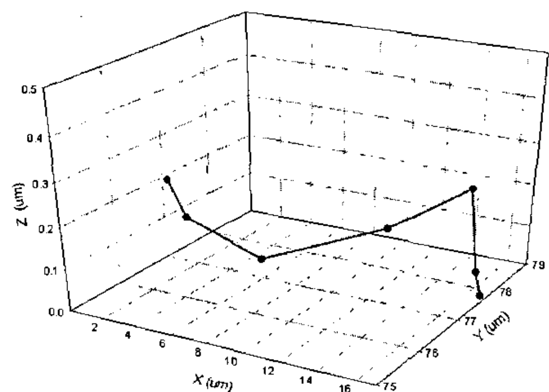

Fig. 6 The 3D plot of dynamic position of a single anti-IgG molecule during the association process

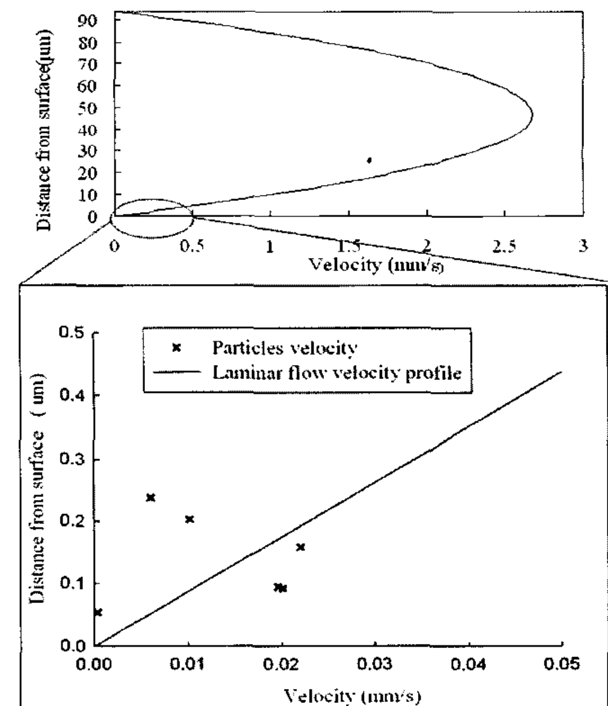

Fig. 7(a) The velocity distribution curve in the channel 7(b) The velocity distribution curve near the surface of biochip in 0.5μm

## V. Conclusions

In this study, we have successfully demonstrated a single biomolecular detection and real-time tracking of anti-IgG in a microchannel using the total internal reflection flurorescence (TIRF) microscopy for illustration of protein adsorption and recognition. The TIRF microscopy is a well-suited technique for real-time imaging and monitoring of a single protein molecule in nano-layer fluidics due to its unique evanescent wave at the optically index-mismatch interface that may excites fluorescences at the transparent near-wall region. Recent advances in CCD camera detection efficiency and speed have enabled the microscopy of temporal and spatial resolutions to be far-reaching 0.033 ms and 0.3 μm, respectively. The modified inverted TIRF microscopy was newly established, which allowed a directed laser beam underneath through the inverted microscopy to be incident in a critical angle into the surface inside the microchannel. In addition, the TIRFM successfully integrates a MEMS-based microchannels biochip to monitor and track down the motion of a single antigen molecule in which the real-time position and velocity of each frames were tracked and measured. In this near-wall region, the motion of the biomolecule was dominated by the hydrodynamic boundary layer. However, with the new TIRF technique introduced into the nano-scale measurement and visualization, the nanolayer of non-classical fludics was found to remain unanswered and open many opportunities for exploitation.

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