Master's Degree Thesis

Study on an application of bioengineering by layer TIRF measurement

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Abstract

Study on an application of bioengineering by layer TIRF measurement

The nature inside bacterial cell has traditionally been viewed as an environment where intermolecular interactions are governed by isotropic diffusion. However, in latest studies, it is reported that the interactions are sub-diffusive. In order to have better understanding on this phenomenon, it is necessary to conduct a 3-dimensional measurement of biological substance's high-speed motion at nano-scale.

In recent years, measurement of biological substance has significantly advanced since the introduction of TIRFM (Total Internal Reflection Fluorescence Microscopy). TIRFM uses evanescent light to illuminate and capture images from samples located a few hundred nano-meters from the microscope glass plate. Originally, TIRFM can only be used to measure samples in 2-dimensional. However, a method to image samples in pseudo 3-dimensional, called layer TIRF has been developed. In this method, it is possible to calculate the pseudo z-coordinate by matching the decaying ratio of evanescent light to the particle's z-axis location. Up until now, only low-speed measurement has been made.

In this work, a high speed measurement was achieved. First, calibration of static particles were conducted. Magnesium Fluoride (MgF_2) patterns with a width of 10[μm] and thickness of 100~200 [nm] were fabricated onto a glass plate. Bio-nanoparticles were placed onto the plate and z-axis coordinate were calibrated. Then, 3-dimensional Brownian motion of the bio-nanoparticles near the glass wall were captured using a high speed EMCCD camera. A low pass filter algorithm was used to reduce noise in captured images. The diffusion coefficient of bio-nanoparticles were calculated and the result obtained was close to the theoretical values.

Thesis structure

This thesis is constructed into the following contents;

Chapter 1: Introduction

First, the summary of this research will be briefly explained. Then, in details, background and latest discoveries on bioengineering field will be discussed. After that, the objective and of this research will be clarified.

Chapter 2: Concept and principle

In this chapter, the main concept and principle that was applied in this research will be discussed. The evanescent wave phenomenon, TIRFM optical system and layer TIRF method will be explained. Moreover, algorithms that were used in the image processing and vector calculations will be demonstrated. Lastly, the derivation of diffusion coefficient from Brownian motion will be explained.

Chapter 3: Experiment 1

This chapter focus on the method to calibrate the particle Z-axis using layer TIRF. Steps and optical system used in this experiments will be described. Then, the method to fabricate layer TIRF calibration plate will be explained. A few problems encountered during this phase will also be addressed. Finally, a method used to calculate the particle intensity from achieved results will be demonstrated.

Chapter 4: Experiment 2

In this chapter, the main content of this research, which is the calculation of bio-nanoparticle's diffusion will be explained. Experiment setup and method used in this experiment will be described. In addition, a brief explanation on the experiment results will be stated.

Chapter 5: Discussion and conclusion

This final chapter will conclude everything that I have done in this research. Moreover, a few ideas and suggestions to improve this research in the future will be stated.

Chapter 1: Introduction

1. 1. Research Background

Previously, bacterial cell has traditionally been thought as a "concentrated soup" where intermolecular interactions inside the cell are governed by diffusion. In other words, the conditions inside non-nucleated cell are often assumed to be close to those in a test tube. However, in a recent study [1], it is reported that the motion of macromolecules inside prokaryotic cells are actually sub-diffusive. In similar field, recently it is suggested that the weird behavior of Myosin V, a type of ATP-dependent motor proteins inside cells that translocates (walks along a straight path) might be caused by Brownian motions [2].

Little is actually known about movement of macromolecules inside cells. To have better understanding on how and what caused this phenomenon to occur, a 3-dimensional and high-speed measurement on motion of biological specimen at nano-scale is needed.

With the emerging of fluorescence imaging system, measurement of biological substance has significantly advanced. One of the most prominent tools in this type of system is TIRFM (Total Internal Reflection Fluorescence Microscopy)[3~5]. TIRFM exploit the unique properties of evanescent light to illuminate samples located a few hundred nano-meters from the microscope glass plate. Plus, the possibility to miniaturize the optical setup into a portable device making it suitable for in-situ cell analysis [6]. Originally, TIRFM can only be used to capture sample's information in 2-dimensional. However, a method to measure samples in pseudo 3-dimensional, called layer TIRF has been developed [7~8]. By applying this technique, it is possible to calculate the pseudo z-coordinate by matching the decaying ratio of evanescent light to the particle's z-axis location.

1. 2. Research Objective

In order to have better understanding regarding the diffusion phenomenon of biological substances at nano-scale, measurement of 3-dimensional diffusion coefficient is required. Therefore, in this research, layer-TIRF method is applied to calibrate z-coordinates of static bio-nanoparticles. The calibration result will be used to calculate the particle distance between glass surface when in motion. Finally, diffusion coefficient of the particles in 3-dimensional will be examined. The result will be evaluated whether it is possible to apply the layer TIRF method into the cell or bioengineering measurement.

Chapter 2: Concept and Principle

2.1. TIRFM

TIRFM is an abbreviation of "Total Internal Reflection Fluorescence Microscopy". This type of microscope use laser beam as a light source to illuminate samples, usually placed onto a glass plate. The incidence angle of laser beam is adjusted until it exceeds critical angle and total-internally reflected. Then, evanescent light wave will emerge at the glass surface boundary.

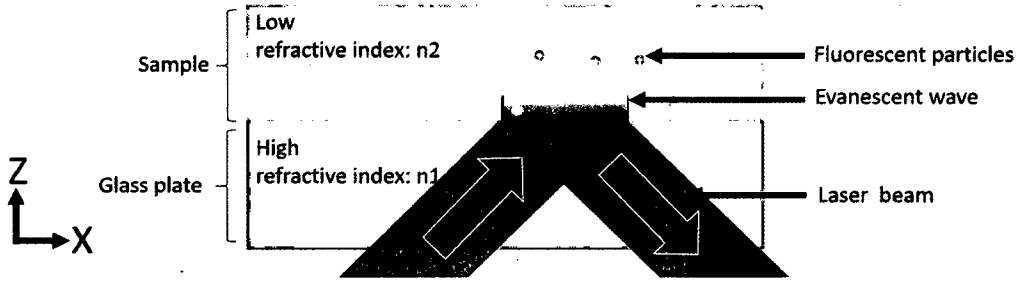

Fig.1 Total Internal Reflection Fluorescence Microscope

Evanescent light wave is near-field wave with an intensity that decays exponentially from the boundary at which the wave was formed. This tiny amount of light will penetrate through the glass towards the sample that is located a few hundreds nanometers from the glass wall. Fluorescent particles within that region will be excited by the evanescent light and emit fluorescent light. The emitted light is then transferred via the microscope optical system and captured into high S/N ratio images using CCD camera.

Mainly, there are two types of TIRF microscope; the objective lens and the prism type. The prism type TIRFM has advantages such as larger illumination range and the background light noise can be easily removed. However this type usually limits the sample thickness.

On the other hand, objective lens type usually combined with inverted microscope, which means it is commercially available and easier to operate. For this reason, this research use the objective lens type of TIRF microscope (Fig.) as the main optical system.

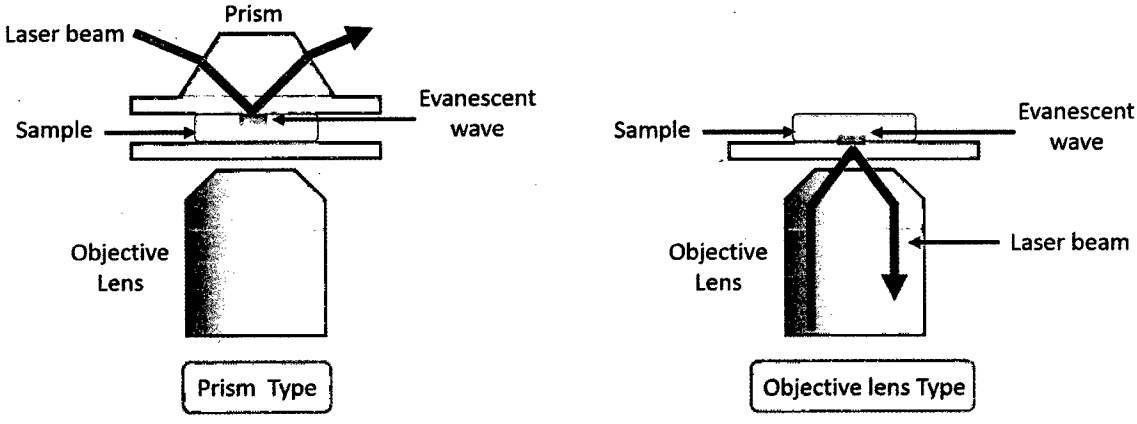

Fig.2 Two different types of TIRF optical system

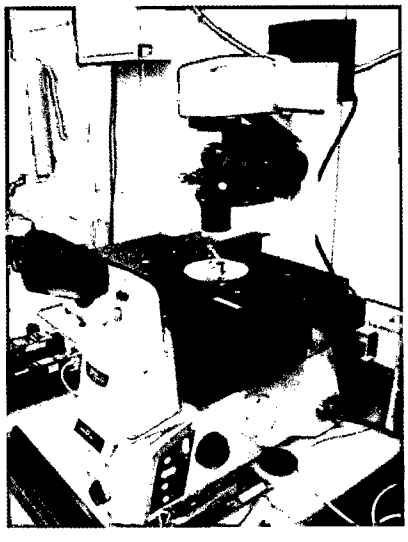

Fig.3 TIRF microscope (Nikon Eclipse Ti-E)

2.2. Evanescent Wave

The evanescent wave intensity decrease exponentially as it penetrate through the z-axis. The intensity decay can be expressed such that:

$$I(z) = I_0 e^{-\frac{z}{z_p}}$$

$$z_p = \frac{\lambda_0}{4\pi}(n_1^2 \sin^2\theta - n_2^2)^{-\frac{1}{2}}$$

Here, z is the distance from surface plane. λ_0 is the wavelength of light source (laser beam) and θ is the incidence angle. n_1 and n_2 are the refraction index of glass and sample(liquid). The term I_0 is the intensity at $z = 0$. Fig 4 shows the penetration depth of evanescent wave at different incidence angle. The parameter used for this calculation is showed in table 1.

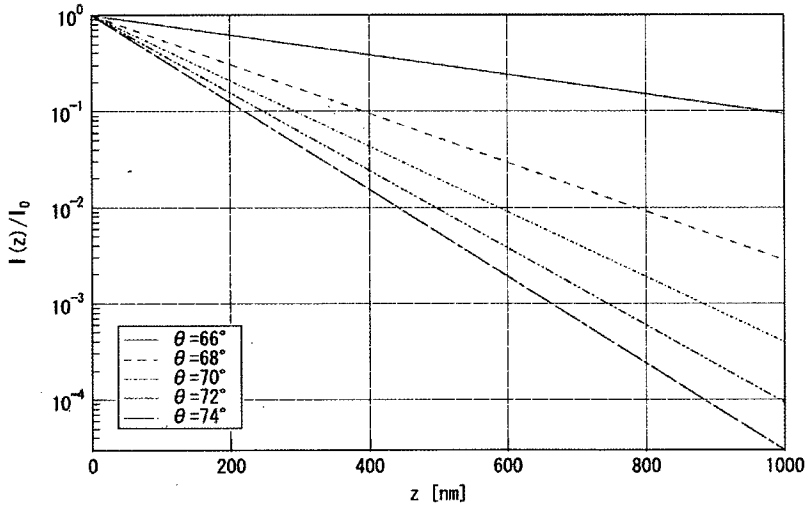

Fig.4 Penetration depth of evanescent wave

Parameter	Value
n_1	1.52
n_2	1.38
λ_0	561 [nm]

Table.1 Calculation parameter

2.3. Layer TIRF

Layer TIRF is a pseudo-3 dimensional measurement technique using TIRFM, developed by Yoda et al. The main concept of layer TIRF is discussed below;

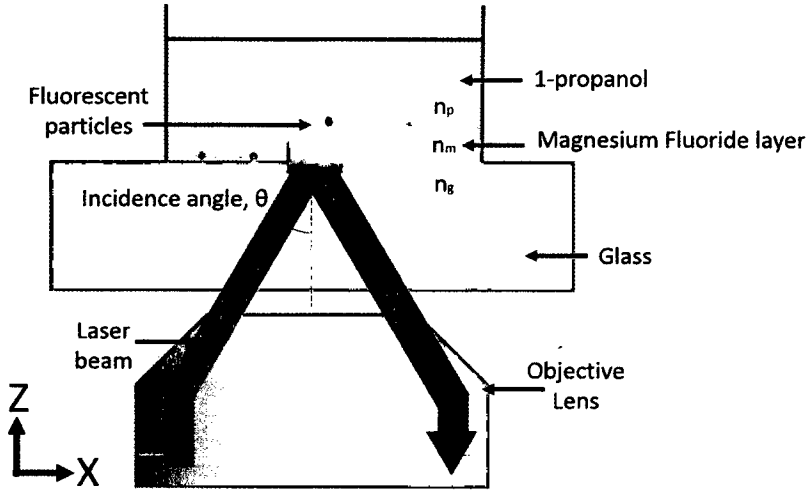

Fig.5 Main concept of Layer TIRF

First, as shown in figure 5, a thin layer of Magnesium Fluoride (MgF_2) with thickness of few hundreds nanometers is fabricated onto the microscope glass plate. Next, fluorescent particles are mixed into 1-propanol. The mixture is then poured onto the microscope glass plate. The plate is then viewed using TIRF microscope.

Due to refractive index matching between 1-propanol ($n_p = 1.38$) and MgF_2($n_m = 1.37$), diffraction effect can be ignored and the particles look as they are floated above the microscope glass surface. Then, the microscope glass plate is illuminated using laser beam (evanescent wave). The fluorescent particles attached onto the MgF_2 layer will produce lower fluorescent light emission compared to the ones on the glass surface. This is expected from the fact that evanescent wave decays as it penetrate through the z-axis, as mentioned in chapter 2.2.

By measuring the decaying intensity ratio between particles attached onto MgF_2 layer and microscope glass surface, the particle z-axis calibration can be made.

2.4. Atomic Force Microscopy

Atomic force microscopy (AFM) is a very high-resolution type of scanning probe microscopy, with demonstrated Z-resolution on the angstrom level (10–10 m). The high sensitivity of AFM making it one of the primary tools for imaging and measuring object's surface at the nano-scale.

The key principle of AFM is, the informations are collected by mechanically scanning the object's surface using a cantilever with a sharp tip (probe). The cantilever is usually made of silicon or silicon nitride with a tip radius of curvature on the order of nanometers. When the tip is brought close to the object's surface, forces between the tip and the sample lead to a deflection of the cantilever. The deflection is then measured using a laser spot reflected from the top surface of the cantilever into an array of photodiodes. Finally, the surface structure is calculated using computer based on the signal taken from the photodiodes.

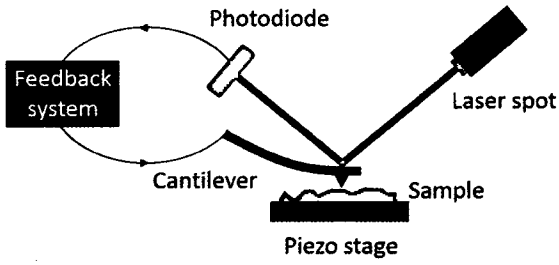

Fig.6 Main principle of atomic force microscopy

In this research, the AFM is used to measure the thickness of Magnesium Fluoride (MgF_2) that had been fabricated onto the microscope glass plate.

2.5. Noise filtering

To decrease noise in the captured images, a digital filter need to be applied. In this research, the noise inside the images are mainly high frequency random noise. A simple low pass filter can be applied to decrease this type of noise.

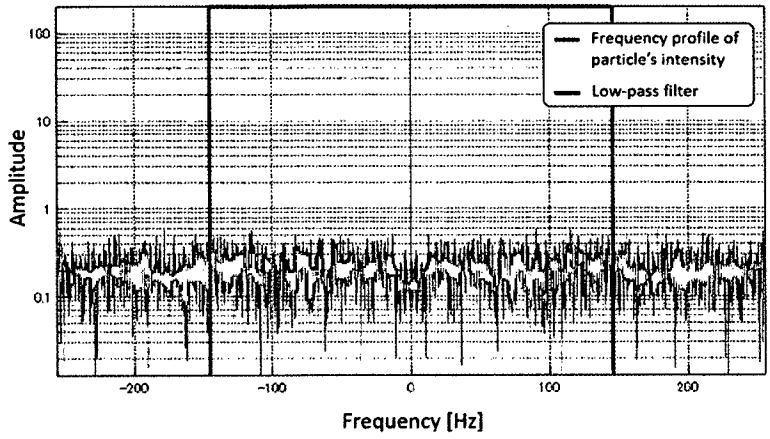

Fig.7 Low pass filter

In this approach, first the intensity change over time at point XY on the image is calculated. Then, Fourier transform is used on the time-domain data series to get the frequency distribution. Next, a low pass filter is applied to remove spectral higher than 128 Hz. Inverse Fourier transform is then applied to convert the signal back to time-domain. The process is then repeated for the whole pixels on the captured images. The advantage of using this method is, the random noise inside captured images can be reduced with low effect on spatial frequency distribution. The images before and after the low-pass filter is shown on Fig.8.

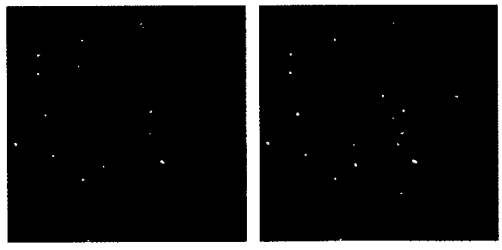

Fig.8 Images before and after low-pass filter

2.6. Calculation of particle's coordinate on XY plane

To calculate particle displacement, first, the coordinate for each particle is measured from captured images. Generally, the particle location is determined by the location of the pixel with highest intensity (peak). This approach however, will only give location as an integer. To get coordinate with higher precision, COG (Centre of gravity) algorithm is used.

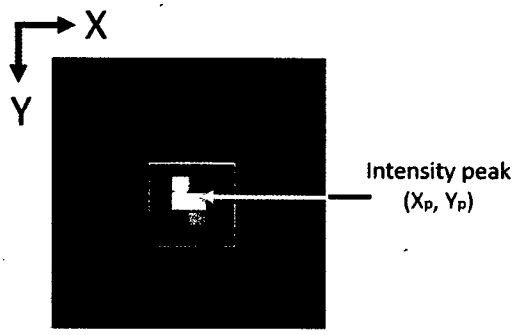

Fig.9 Intensity peak of fluorescence particle

In this algorithm, firstly, the location of particle's intensity peak is determine from the image. Then, as shown in Fig .111 a calculation window with the size of 3x3 pixels is created, setting the peak location as center. Next, the center of gravity from correspond window is calculated by taking the image's intensity as weight. The equation used in this algorithm is expressed in Eq.1. Gx and Gy is the particle's X and Y coordinate.

$$G_s = \sum_{x=X_p-2}^{X_p+2} \sum_{y=Y_p-2}^{Y_p+2} I(x,y)$$

$$G_x = \frac{1}{G_s} \sum_{x=X_p-2}^{X_p+2} \sum_{y=Y_p-2}^{Y_p+2} I(x,y) \cdot x$$

$$G_y = \frac{1}{G_s} \sum_{x=X_p-2}^{X_p+2} \sum_{y=Y_p-2}^{Y_p+2} I(x,y) \cdot y$$

Eq. 1 Centre of gravity

2.7. Particle tracking

Particle tracking is the process of matching particles within two consequent images to determine the particle's displacement and velocity vector. The algorithm used in this research is explained as follow:

First every particle location,$P(x.y,z)$ in image at time $= t$ is calculated. Then, for each P, particle location at time$= t + \Delta t$ is predicted by adding theoretical velocity. Since the particle only moves randomly, it is sufficient to create a circle searching region with radius $r = v_t \Delta t$ from point P. If any particle P'in image at time $= t + \Delta t$ is within the radius, then the distance between P and P' is concluded as the particle's displacement vector. In case there exist two or more P' within the search radius, no vector will be added and the calculation is proceeded to the next P. The velocity vector can be calculated by dividing displacement vector by Δt. Fig. shows the illustration of the tracking algorithm.

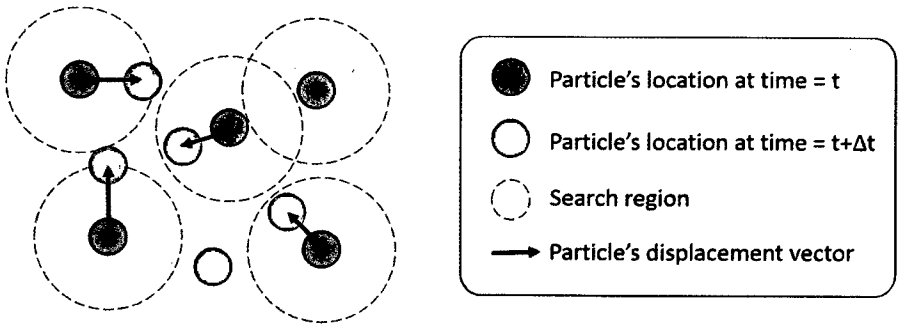

Fig.10 Particle tracking

2.8. Brownian motion

Brownian motion is the random motion of particles suspended in a fluid (a liquid or a gas) resulting from their collision with the surrounding moving molecules or atoms. Assuming that particles are moving in 3-dimensional and not affected by any external forces, the mean-square displacement,$\langle r^2 \rangle$ can be calculated using the following equation:

$$\langle r^2 \rangle = \frac{1}{n}\sum_{i=1}^{n} r_i^2 = \frac{1}{n}\sum_{i=1}^{n}\left(\Delta x_i^2 + \Delta y_i^2 + \Delta z_i^2\right)$$

Where n is the total vectors and Δx Δy Δz are changes in coordinates. By applying the Einstein equation[9], the following relation can be obtained. D is diffusion coefficient and Δt is the time interval.

$$\sqrt{\langle r^2 \rangle} = \sqrt{6D\Delta t}$$

Thus, D can be expressed as:

$$\mathrm{D} = \frac{\langle r^2 \rangle}{6\Delta t}$$

Here, D can be theoretically calculated using the following equation:

$$\mathrm{D_{theory}} = \frac{\kappa T}{3\pi\mu d_p}$$

Where κ is the Boltzmann constant, T is the fluid temperature, μ is the fluid viscosity and $d_p$is the particle's diameter. In this research, the evaluation of particle diffusion will be made based on value D obtained from experiments and comparing it to the theoretical value.

Chapter 3: Experiment 1, Layer TIRF calibration

3.1. Experiment setup

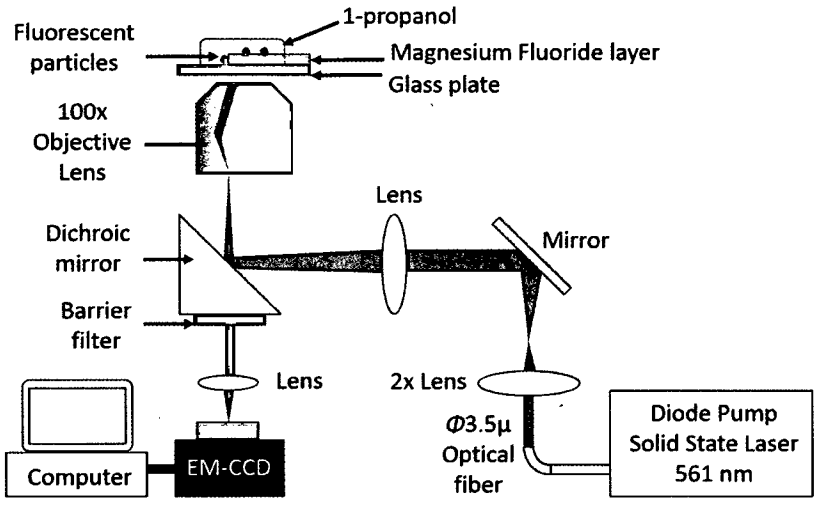

Fig.11 Optical system when using green laser

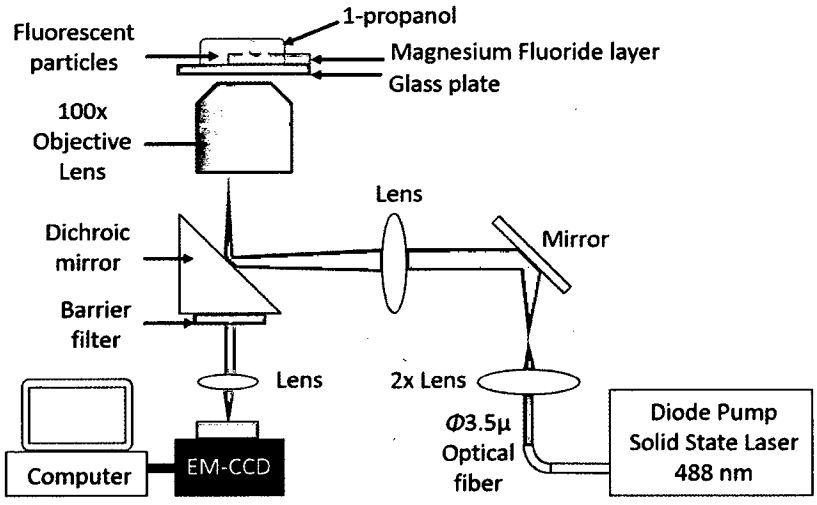

Fig.12 Optical system when using blue laser

Figure 11 & 12 shows experiment setup for layer TIRF calibration experiments. The experiment setup consist of TIRF microscope, laser, high speed CCD camera and fabricated calibration glass plate. Bio-nanoparticle and non-bio-nanoparticles were used. The process of calibration plate fabrication will be further explained in section 3.2.

The experiment method is explained as follow. First, the calibration plate is placed on the TIRFM stage and observed using halogen illumination. Next, the location of the MgF_2 pattern is searched. Then, fluorescent particles diluted at 1000x using 1-propanol is dripped onto the calibration plate. An interval of a minute is taken for the fluid to uniformly disperse. Afterwards, the calibration plate is observed using TIRF mode, in other words, laser illumination. The stage's Z axis is moved until the focus point is set to the glass region of the calibration plate. Images of the fluorescent particles are captured using EM-CCD camera, controlled using a computer. To avoid photo-bleaching, the acquisition time is shorten as much as possible. A reference background image is taken by screening the florescent light with a dichromatic filter. Finally, the plate is moved through XY plane and previous steps are repeated. Acquisition frequency is set to 512.82 Hz and exposure time is 1.93 [ms]. After images at different locations are taken, the decaying ratio is calculated using algorithm explained in section 3.3. Table. Shows the details on equipment and parameters used in this experiment.

Item	Model/Maker	Specification	
TIRFM	Nikon Eclipse Ti-E	Oil immersion objective lens	100x NA 1.49
CCD Camera	Andor DU-860	Resolution	128[px] x 128 [px]
		Pixel size	24 [μm] x 24 [μm]
		Acquisition interval	1.93 [ms]
Green Laser	Mellse Griot	Wavelength	561 [nm]
		Incidence angle	66.75°
Blue Laser	Coherent	Wavelength	488 [nm]
		Incidence angle	66.49°
Fluorescent particle	Invitrogen F8810, F8811, Thermo Sci. R200	Diameter	200 [nm]
		Density	0.00%
Glass plate	Matsunami glass	Refractive index	1.52
1-propanol	Showa Chemical	Refractive index	1.38
Magnesium fluoride		Refractive index	1.37

Table.2 Experiment equipment and parameter

3.2. Fabrication of calibration plate

As introduced in chapter 2.3, creating calibration plate for layer-TIRF involves a process of fabricating a thin layer of Magnesium Fluoride onto the microscope glass plate. During the early development of this research, an approach to cover roughly half of microscope glass plate's surface with MgF_2 layer was taken.

This approach took less time to create the calibration plate. However, it is very difficult to search for the boundary between glass and MgF_2 using TIRF microscope during the calibration phase. Moreover, the standard deviation of coating thickness near the boundary region was very high, increasing the calibration uncertainty due to large measurement errors.

To overcome this problem, instead of a whole layer of MgF_2, tiny patterns of MgF_2 are fabricated onto the microscope glass plate. The MgF_2 patterns are shaped into a square and aligned to form a line with a width of nearly 1 mm so the location can be easily located, even with naked eyes. The fabrication method is explained as below:

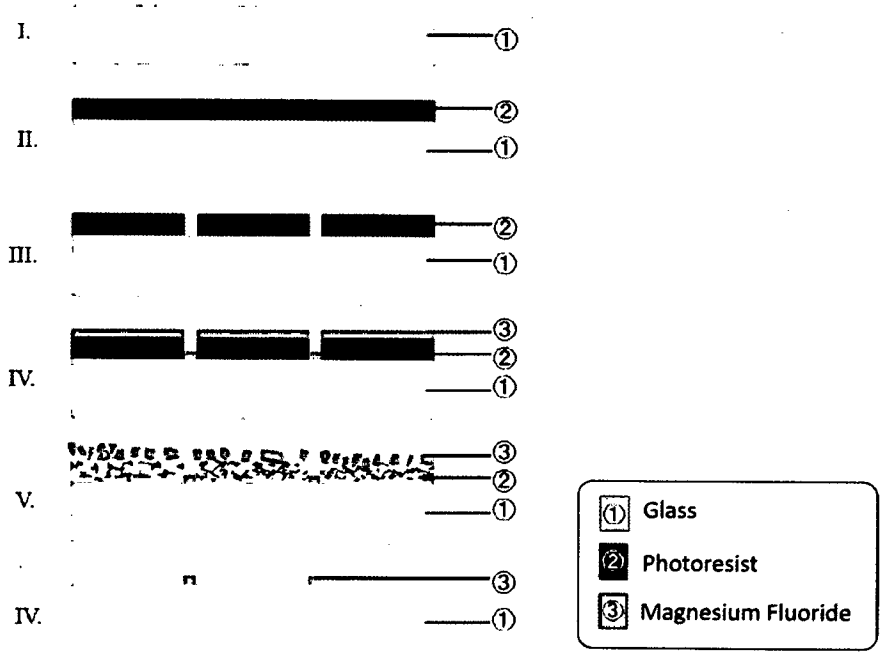

Fig.13 Steps for making layer TIRF calibration plate

In the fabricating process, a glass with thickness of 0.13~0.17mm was chosen as the microscope slide glass. First the glass plate is cleaned by sonication with purified water and left till dried. Next, as shown in Fig.13.II a layer of photoresist was coated on the plate using spin coating method. Afterwards, a pattern as illustrated in Fig.13.III was made onto the plate using electron beam. By using chemical vapor deposition method, a layer of MgF_2 was coated onto the plate. As manifested in Fig.4, MgF_2 crystal was heated inside a vacuum chamber($150[°C], 2 \times 10^{-3}[Pa]$)until it vaporized and condensate when come to contact with the glass plate.

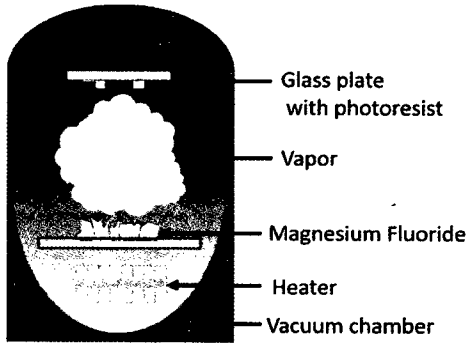

Fig.14 Chemical vapor deposition

Finally, the photoresist was removed, leaving behind MgF_2 pattern, as shown is Fig.13.IV. In theory, the coating thickness depends simply on the amount of MgF_2 used during the vapor deposition process. The thickness of MgF_2 layer is measured using AFM (Atomic Forced Microscopy). If the layer thickness has high standard deviation, or too thick for the evanescent light wave to penetrate (higher than 300nm), then it will not be used in calibration experiment.

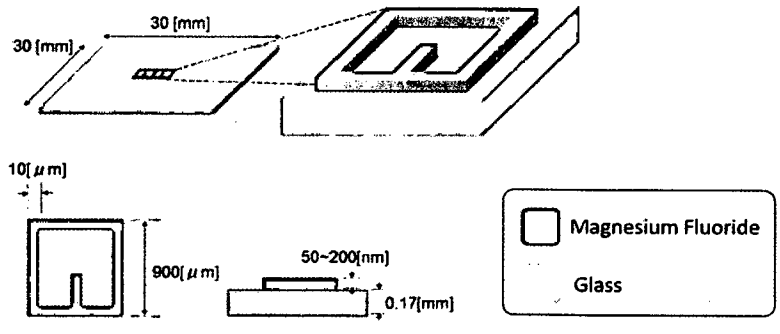

Fig.15 Calibration plate for Layer TIRF

3.3 Calculation of particle's intensity

The algorithm to calculate the decay ratio of fluorescent particle's intensity is described as follow: First, a number of particles are arbitrary chosen from captured images. Next, by using the background image as reference, glass and MgF_2 regions are identified. Refer Fig.16 for explanation. Then the particles located within the same region are grouped together. Then, total intensity inside 5x5 windows for each particles is calculated. Afterwards, the average intensity for particles within glass (I_g) and MgF_2 (I_m) regions, are calculated respectively.

The decaying ratio of particle's intensity is derived by dividing I_m over I_g. Finally, the intensity ratio is plotted against the MgF_2 thickness.

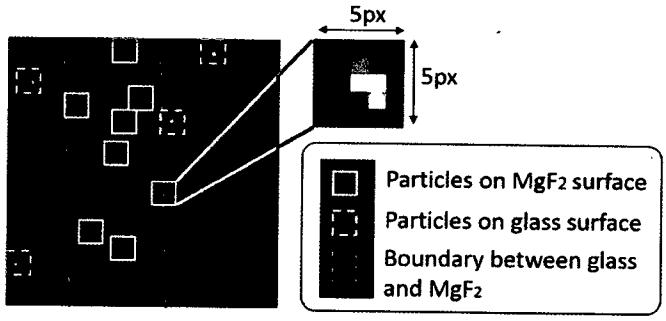

Fig.16 Particle intensity calculation