A Thin Layer Imaging with the

Total Internal Reflection Fluorescence Microscopy

Neda Roostaie[1], Elham Sheykhi[1], Fariba Japelaghi[1], Mohammad Amin Bassam[2], Sharareh Tavaddod[3], Batool Sajad[*1]

[1] Department of physics, Alzahra University, Tehran, Iran

[2] Department of physics, Malek-Ashtar University of Technology, Tehran, Iran

[3] Department of physics, University of Edinburgh, Scotland

Abstract: Total internal reflection fluorescence microscopy (TIRFM) is an optical technique that allows imaging of a thin layer of the sample with a thickness of about 100-200 nm. It is used in science of cell biology to study cellular processes, especially near the membranes of living cells. This method is based on the total internal reflection phenomenon, where the evanescent wave is generated in the less dense medium. In fact, the evanescent wave is used to illuminate the sample. Consequently, the possibility of observing a superficial (instead of bulk) part of fluorophore labeled sample is opened up. In this work, a total internal reflection fluorescence microscope based on the light guide has been designed and assembled by means of the inverted microscope to image a thin layer from the surface of the sample. Operated experimental arrangement has been employed for the total internal reflection fluorescence imaging of cadmium selenide (CdSe) quantum dots.

Key words: Evanescent wave, fluorescence, microscopy, total internal reflection.

1. INTRODUCTION

Total internal reflection fluorescence microscopy was first introduced by Daniel Axelrod in 1980 [1]. This method uses the total internal reflection between the lower surface of the sample and the substrate to observe a thin layer of the sample. Total internal reflection occurs when the light beam propagating through a medium with a higher refractive index enters to a medium with a lower refractive index, for incidence angles greater than the critical angle.

From the wave optics view, when the total internal reflection occurs, a part of

the incident electromagnetic wave, which is called evanescent wave, partially penetrates into the medium with the lower refractive index. This wave illuminates the specific part of the sample which is in contact with the substrate surface.

According to Fig. 1, in the total internal reflection fluorescence microscopy, the sample is labeled with the fluorophore molecules, and then it is illuminated under the incidence angle larger than the critical angle. The generated evanescent waves selectively excite the fluorophore molecules near the interface coverslip-sample. Finally, the emitted radiation from the fluorophore molecules collected and provides the possibility of observing the specific sections of the sample.
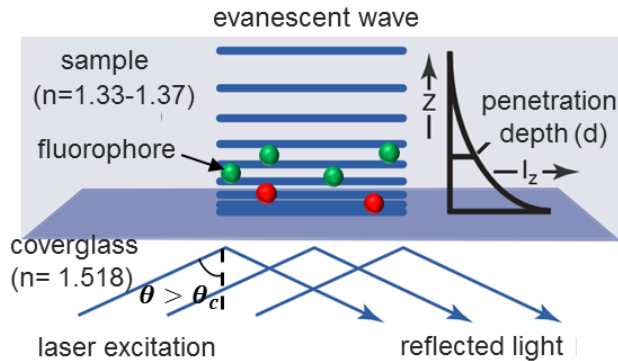

Fig. 1. Schematic diagram of the total internal reflection fluorescence (TIRF) phenomenon [2].

The intensity (I) and the penetration depth (d) of the evanescent wave are obtained by (1) and (2), respectively [3]:

$$I(z) = I_0 e^{-\frac{z}{d}} \tag{1}$$

$$d = \frac{\lambda_0}{4\pi\sqrt{(n_1 \sin\theta)^2 - n_2^2}} \tag{2}$$

Where n_1 and n_2 are the refractive indices of the first and second media respectively, I_0 is the intensity of light at the interface (at z=0), λ_0 is the wavelength of the incident light in vacuum and θ is the incidence angle. According to (1), the intensity of the evanescent wave decays exponentially with increasing the distance z from the interface. Thus, only a thin layer of the sample with a thickness of about 100 to 200 nm is illuminated to study some superficial properties of the sample such as adhesion. When viewing of the radiation only from a depth of below 200 nm is considered, the radiation from the depths of the specimen above 200 nm is referred as the background radiation

and is called the background noise. Therefore, images obtained by the total internal reflection fluorescence microscopy have a very low background noise.

According to (2), the penetration depth of the evanescent wave decreases with increasing the incidence angle. For supercritical angle, the penetration depth is generally in the order of λ_0 or smaller [3].

In the early 1980s, Axelrod described the experimental arrangements of the total internal reflection fluorescence microscope for inverted and upright microscopes [1, 4]. These arrangements are different in the illumination methods. In fact, a prism or waveguide or objective lens with high numerical aperture can be used to guide the beam to the sample and providing suitable conditions for the total internal reflection.

In the prism-based total internal reflection fluorescence microscope, the laser beam is guided to the coverslip-sample interface through a prism at the supercritical angle to occur total internal reflection phenomenon [5]. While in the objective-based total internal reflection fluorescence microscope, the laser beam passes through an objective lens before illuminating the sample [5]. This lens is used both for illuminating the interface and observing the emitted fluorescence. In the case of guide-based ones, the light rays are guided by the optical fiber from the light source into the optical waveguide which can be a rectangular or circular coverslip. After the light entering into the optical waveguide, the multiple reflections occur at the upper and the lower surfaces of waveguide and the vast area of the evanescent wave is generated.

The prism-based arrangement provides the best signal to noise ratio, but it is difficult to implement with open perfusion chamber on an inverted microscope. Objective-based arrangement is compatible with open perfusion chamber and there is access to the sample, but this arrangement needs the objective lenses with high numerical aperture (expensive) [6].

A light guide-based arrangement yields exceptional flexibility and it can be used by the objective lens with low numerical aperture and dry, water-, and oil-immersion objectives, while provides the well signal to noise ratio and moreover, setting up of the arrangement is not very expensive.

Total internal reflection fluorescence (TIRF) microscopy can be used in a wide range of cell biological applications, and is particularly well suited to analysis of the localization and dynamics of molecules and events near the plasma membrane [7]. Also, this method can be used to explore quantitatively the adhesion of living cells and compute the topography of cells with a nanometric axial resolution, typically 10 to 20 nm [8, 9, 10].

The advantages of using TIRF include the ability to obtain high-contrast images of fluorophores near the plasma membrane, very low background from the bulk of the cell as well as the reduced cellular photo damage and rapid exposure times.

In this study, the arrangement of the light guide-based total internal reflection fluorescence microscope has been set up by means of the inverted microscope to employ for the imaging of cadmium selenide (CdSe) quantum dots adhered to the surface of the typical sample. Quantum dots are the nanoparticles with unique optical properties such as higher light stability than the conventional fluorophore molecules, the narrow and separate of the excitation and emission spectrum, small size (2 to 8 nanometers) and being luminous. Their optoelectronic properties change as a function of both size and shape. Larger quantum dots (radius of 5 to 6 nm, for example) emit longer wavelengths resulting in emission colors such as orange or red. Smaller quantum dots (radius of 2 to 3 nm, for example) emit shorter wavelengths resulting in colors like blue and green, although the specific colors and sizes vary depending on the exact composition of the quantum dot [11]. Diversity of the emission wavelengths of these nanoparticles has provided the possibility of the use of simultaneous several markers in components of living cells and observation the intracellular processes.

2. EXPERIMENTAL ARRANGEMENT

The schematic diagram of the experimental arrangement of the total internal reflection fluorescence microscope based on the light guide at an inverted microscope is shown in Fig. 2. A diode laser with a wavelength of 405 nm and the nominal power of 150 mW is employed to illuminate the interface. The optical array includes an objective lens 25X (with NA=0.4), an eyepiece 10X and a trapezoid-shaped slide as a waveguide. The sample is placed on the slide and the laser beam is guided into the optical waveguide through the optical fiber. A MT-2024E CCD camera is connected to the microscope eyepiece for imaging.

The light guides to the slide-sample interface under supercritical angle in order to occur the total internal reflection into the waveguide. Therefore, a wedge-like piece is employed to adjust the irradiation angle. After the laser beam is guided to the interface of the slide-sample under the supercritical angle, the multiple total internal reflections occur inside the waveguide. Then the vast area of evanescent wave generates to excite the fluorophores. Finally, the radiation emitted from the sample passes through the objective lens, and is reflected by the flat mirror and then will be received through the eyepiece. The sample image is seen from the eyepiece.
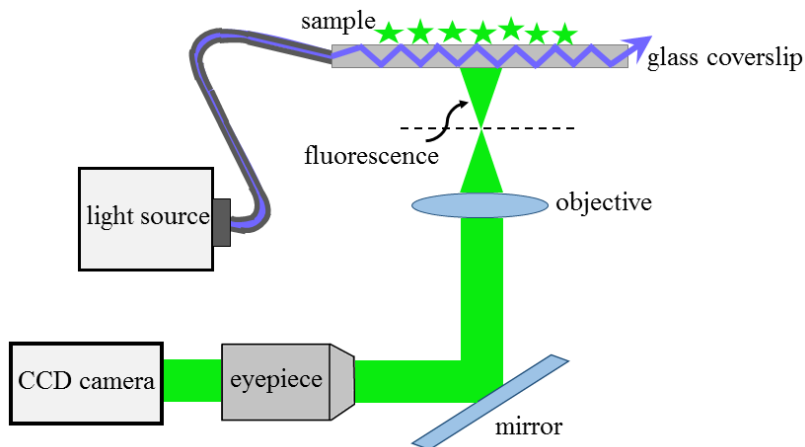
Fig. 2. The experimental layout of the total internal reflection fluorescence microscope based on the light guide.

The absorption and emission spectrum of a solution containing quantum dots with a concentration 4 gr/lit (water solvent) respectively is taken by the UV-VIS and PL spectrometers.

3. RESULTS

The experimental arrangement of the total internal reflection fluorescence microscope based on the light guide is employed for imaging of CdSe quantum dots. The absorption spectrum of the used CdSe quantum dots in the wavelength range 200 to 800 nm is shown in Fig. 3. It was seen that the solution containing CdSe quantum dots has a maximum absorbance at 400 nm wavelength region to determine the excitation wavelength. Fig. 4 shows the emission spectrum of the quantum dots due to the excitation wavelength 400 nm. The emission wavelength of the quantum dots is in the range of green.

In the total internal reflection fluorescence microscopy only the lowest surface of the sample is imaged which is in contact with the substrate. So the objective lens has been focused on the surface of the slide. Then a few drops of the solution containing CdSe quantum dots, placed on the trapezoidal slide to prepare the samples for imaging. The monochromatic laser light is guided into the waveguide to provide the condition of the imaging of quantum dots as recorded and shown in Fig. 5.
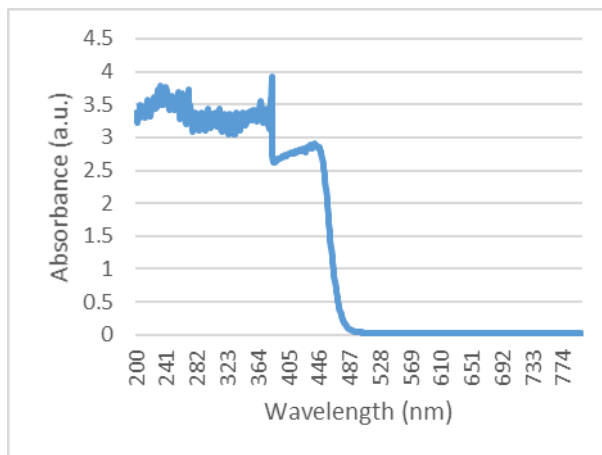
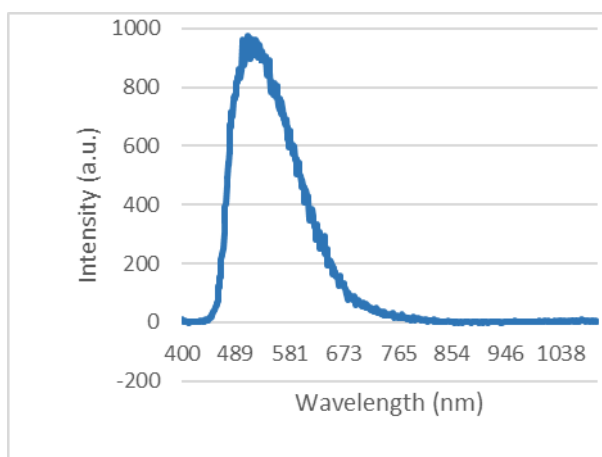Fig. 3. Absorption spectrum of the CdSe quantum dots in the wavelength range 200-800 nm.

Fig. 4. Emission spectrum of the CdSe quantum dots; excitation wavelength 400 nm has been considered.

As it is seen in Fig. 5(a), CdSe quantum dots adhered to the surface of the slide as well are seen. Since the objective lens is focused on the surface of the slide, it has been ensured that quantum dots are on the surface of the slide. The aggregation of the quantum dots which are sticking to the slide surface is observed in Fig. 5(b). Dark background of the recorded images indicates that the phenomenon of total internal reflection is occurred and the emission fluorescence from the quantum dots is caused by the evanescent wave.
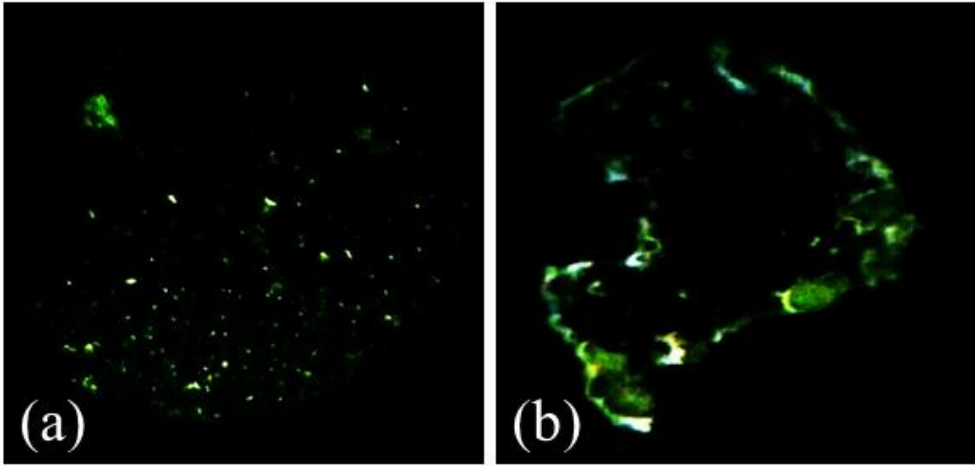
Fig. 5. Recorded images of the CdSe quantum dots which have been excited with a wavelength of 405 nm, (a) quantum dots adhered to the surface of the substrate, (b) aggregation of the quantum dots which are stuck to the substrate surface can be seen.

4. CONCLUSION

Total internal reflection fluorescence microscopy (TIRFM) is an optical technique that allows imaging of the thin layer of the sample with a thickness of about 100 to 200 nm. In this work, a total internal reflection fluorescence microscope based on the light guide has been designed and assembled by means of the inverted microscope to image a thin layer of the quantum dots adhering to the substrate surface.

Total internal reflection fluorescence microscopy technique based on the light guide arrangement is selected, because it can be used with the objective lens with low numerical aperture and it is an affordable way to view a thin layer of the sample. Moreover, it is very adjustable and provides easy access to the sample with a good signal to noise ratio.

Since only a thin layer with a thickness of about 100 nm from the sample is illuminated, this method provides the possibility of the study of the cell membrane and the cell adhesion to the substrate surface in the biological structures.

REFERENCES

[1] D. Axelrod, N.L. Thompson, and T.P. Burghardt. *Total internal reflection fluorescent microscopy*. Journal of microscopy 129 (1) (1983, Jan.) 19-28.
[2] Fish. K.N. *Total internal reflection fluorescence (TIRF) microscopy*. Current protocols in cytometry (2009, Oct.) 12-18.
[3] D. Axelrod, *Evanescent excitation and emission in fluorescence microscopy*. Biophysical journal 104 (7) (2013, Apr.) 1401-1409.

[4] D. Axelrod, T.P. Burghardt. and N.L. Thompson. *Total internal reflection fluorescence*. Annual review of biophysics and bioengineering 13 (1) (1984, Jun.) 247-268.

[5] Yildiz, Ahmet, and Ronald D. Vale. *Tracking movements of the microtubule motors kinesin and dynein using total internal reflection fluorescence microscopy.* Cold Spring Harbor Protocols 2015 (9) (2015, Sep.) pdb-prot086355.

[6] Török, Peter, and Fu-Jen Kao, Total internal reflection fluorescence microscopy, Optical Imaging and Microscopy: Techniques and Advanced Systems, vol. 87, 2007, 195-236.

[7] Mattheyses, Alexa L., Sanford M. Simon, and Joshua Z. Rappoport. *Imaging with total internal reflection fluorescence microscopy for the cell biologist.* J Cell Sci 123 (21) (2010, Nov.) 3621-3628.

[8] Dos Santos, Marcelina Cardoso, et al. *Topography of Cells Revealed by Variable-Angle Total Internal Reflection Fluorescence Microscopy.* Biophysical Journal 111 (6) (2016, Sep.) 1316-1327.

[9] D. Axelrod, *Cell-substrate contacts illuminated by total internal reflection fluorescence.* The Journal of cell biology 89 (1) (1981, Apr.) 141-145.

[10] Truskey, George A., et al. *Total internal reflection fluorescence microscopy (TIRFM). II. Topographical mapping of relative cell/substratum separation distances.* Journal of cell science 103 (2) (1992, Oct.) 491-499.

[11] Murray, Christopher B., C. R. Kagan, and M. G. Bawendi. *Synthesis and characterization of monodisperse nanocrystals and close-packed nanocrystal assemblies.* Annual Review of Materials Science 30 (1) (2000, Aug.) 545-610.