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Application of TIRFM in Biomolecule Research and Clinical Medicine

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Abstract

With the increasing demand for imaging quality, many scientists constantly explore new imaging instruments to meet the requirements. The total internal reflection fluorescence microscopy has some incomparable superiorities, so it has become one of the research hotspots in recent years. It can show great performance in single-molecule imaging because it has unique imaging principles. This apparatus is used mainly in two fields, biomolecule research and clinical medicine. To know this instrument's function, the summary of applications in these two parts was given in this article. Now, scientists who have been focusing on this apparatus try to make this microscope combined with other late-model precise instruments that probe some unknown interaction mechanism of action. The TIRFM will show extraordinary talents in many aspects, and it will become a powerful tool for people to explore the mysteries of life.

Keywords

TIRFM, Single-Molecule Detection, Dynamic Imaging, Fluorescence Sensor

1. Introduction

The Total Internal Reflection Fluorescence Microscopy (TIRFM) is a fluorescence microscope based on the total internal reflection fluorescence theory. This fluorescence is achieved by employing an evanescent wave for excitation. As the energy of an evanescent field decreases with distance to the interface, only fluorophores in a certain proximity to the coverslip are excited, and fluorophores in the rest of the cell are hardly excited in the meantime. So, the interference of background fluorescence is greatly reduced. In other words, the secondary fluorescence emission has been limited in a very thin region, so we can get a higher signal-to-noise ratio [1] [2]. **Figure 1** shows the imaging theory of TIRFM. De-

pending on the different illumination pathways, the TIRFM can be divided into two systems, the prism-type TIRFM system, and the objective-type TIRFM system. Figure 2 shows pathways of both TIRFM types for an inverted microscope.

As an emerging apparatus, TIRFM has been greatly facilitated in recent years. With the development of TIRF technology, people coming from different fields pay more attention to this device. Compared with other fluorescence technology of biological single-molecule, TIRFM has incomparable advantages, especially in dynamic scanning and high-resolution imaging. TIRFM shows the superiorities in real-time imaging in Figure 3. TIRFM can show the real-time dynamic process

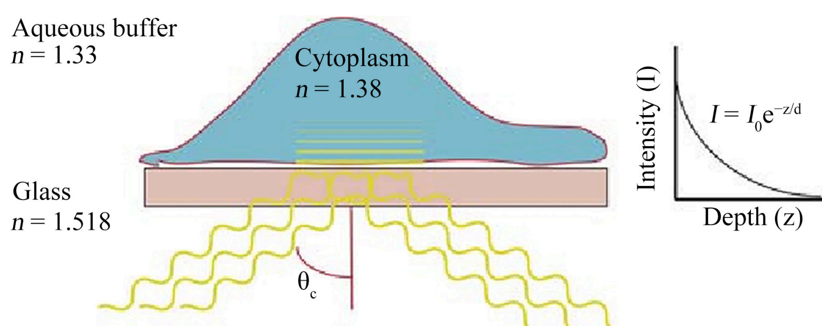

Figure 1. Total internal reflection fluorescence. TIRFM depends on the refraction and reflection of light from a planar surface. The incident beam is reflected from the glass surface rather than passing through the water. The reflected beam generates an evanescent field in the aqueous medium that decays exponentially into the sample. This picture was derived from reference [3].

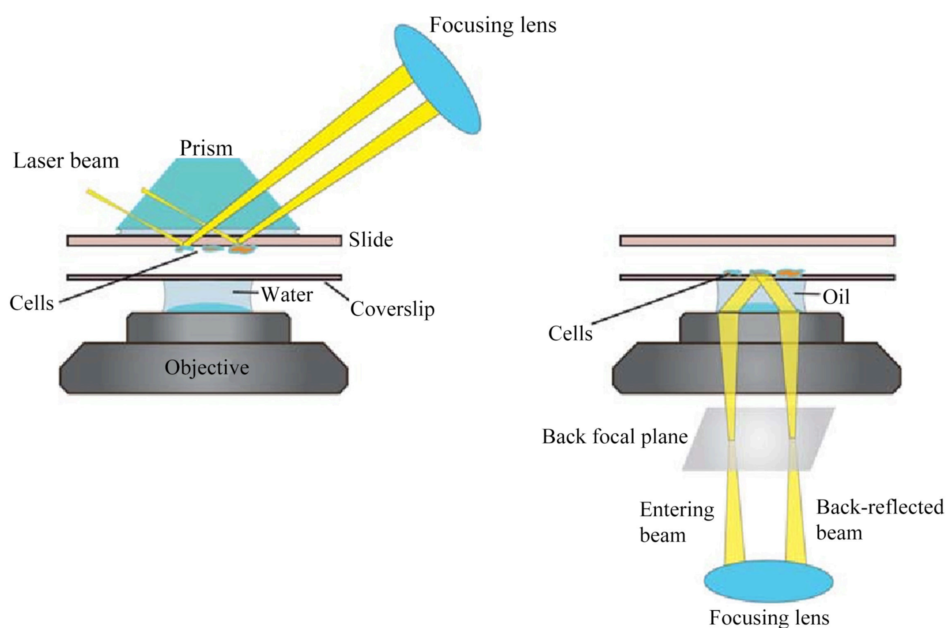

Figure 2. Two different illumination pathways of TIRFM. In a prism-type TIRFM system, the laser beam is guided onto a surface through a prism to achieve the correct angle. In an objective-type TIRFM system, the laser beam travels through the objective before illuminating the sample. This picture was derived from reference [3].

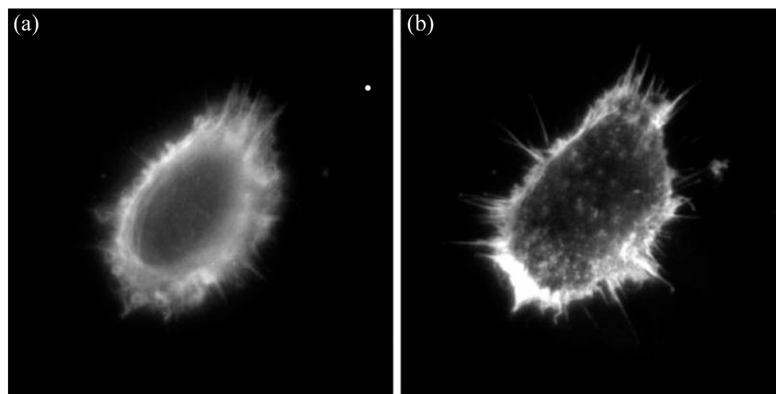

Figure 3. Information revealed by standard epifluorescence and TIRFM. (a) Standard epifluorescence; (b) TIRFM [2].

and high-resolution imaging. It has a high signal-to-noise ratio of images. It lowers phototoxicity, which can greatly decrease background fluorescence and let the researchers obtain high-quality imaging quality and reliable observation data. There is no doubt that this instrument has made notable achievements in biomolecule research and clinical medicine.

Many scientists have devoted themselves to explore and improve this technique in recent years, and they find this apparatus can be used in many studies of an object through constant research. Therefore, it's necessary to do an article to summarize the application of TIRFM. In this article, the application was divided into two categories, biomolecule research, and clinical medicine. In biomolecule research, the readers can learn about single-molecule detection like DNA and protein and biomolecular Interactions, including protein-protein and protein-DNA, and gain knowledge about immunization like innate immunity and acquire immunity as well as other mechanisms, such as nerve conduction and gynecological disease in clinical medicine.

2. Biomolecular Research

With the development of science and technology, scientists pay more attention to biomolecule research because the study of vital movement is based on the biomolecule study. Since early times, scientists try to find ways to observe and detect biomolecules, such as DNA, protein, RNA. As the research moves along, the scientists believe that the fluorescent technique has a significant effect in this field. TIRFM, like one of the applications of fluorescence technology, is a powerful tool for single-molecule study [4] [5]. This apparatus allows us to directly observe the dynamic process for the incomparable advantages, like super-resolution and higher signal-to-noise ratio [2]. It can be used in single-molecule detection and the study of biomolecular interactions.

2.1. Single-Molecule Detection

2.1.1. DNA

There is no doubt that TIRFM plays an unbeatable role in single-molecule re-

search since only a thin hierarchy of about 200 nanometers is excited by the evanescent wave, resulting in high sensitivity of detection and high signal-to-noise ratio of images. Much genetic information is contained in the DNA, and DNA can interact with other molecules, such as protein and RNA, achieving some life activities. The researchers carried out two techniques in the experiments. One is molecular combing, of which DNA molecules can be easily stretched, and the other is TIRFM. YOYO-1 is a fluorescence probe that can combine with DNA and form the DNA-YOYO-1 complex. It is noteworthy that photocleavage and photobleaching of the DNA-YOYO-1 complex were inevitable. And the two phenomena will limit the ability to image DNA molecules in real-time over long periods. Because imaging principle of TIRFM can effectively avoid photodegradation, it was used for high-definition imaging of the stretched DNA. It is easy to obtain high resolution image, and it provided the theory foundation for the following research. TIRF setup for real-time imaging is shown in **Figure 4**. This research has laid the foundation for visually studying the dynamic processes of interactions between DNA and proteins [4].

2.1.2. Protein

Proteins also play an important role in genetic inheritance. To be honest, protein is the direct performer of vital movement. Scientists never stop step from exploring protein structure and movement mechanisms.

It is technically unfeasible to make quantitative imaging of Intermediate Filaments (IFs) during the advanced phase of the assembly process since the structures are several μm long. And they exceed the field of view of many electron or atomic force microscopy techniques, so AFM can't realize the quantitative study. The researchers prepared fluorescently labeled vimentin for visualization by TIRFM. Recording the contour of filaments by TIRFM allows rapid determination

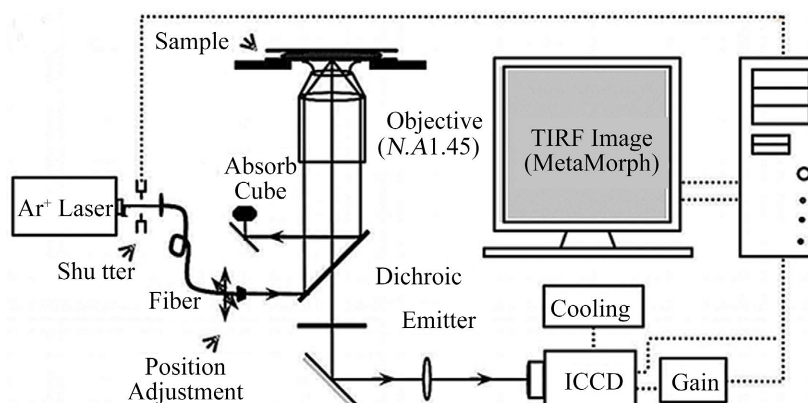

Figure 4. TIRFM setup for real-time imaging. Researchers used TE2000 inverted microscope and TIRF specialized oil-immersed objective for TIRF imaging and used Argon ion laser as the excitation light source. The laser was introduced into the microscope along the edge of the objective lens through the optical fiber, causing total internal reflection at the sample interface to generate hidden waves and induce fluorescence. Fluorescence signals were captured and imitated in re-al-time by an Intensified Charge-Coupled Device (ICCD) [4].

of their persistence length, so they can follow filament growth and the deposition process of IFs in real-time by TIRFM, which profits from its specific superiority, the high imaging rate. In this study, the experimenters have optimized the generation of fluorescently labeled vimentin for the unperturbed in vitro assembly of filaments [6]. At the same time, this research proved that this technique is quite efficient in observing the dynamic interaction of IFs with associated proteins [6].

Tropomyosin, an actin-binding protein, can combine with most actin filaments within a cell to form copolymers. Many scientists have used TIRFM to observe the molecular assembly of actin/tropomyosin filaments in vitro. TIRFM has proven to be a useful tool in observing actin dynamics and analysis of the interaction with actin-binding proteins (ABPs) [7]-[12].

2.2. Biomolecular Interactions

2.2.1. Protein-Protein

Various actin, including monomeric actin, G-actin, F-actin, as a consequential component of cellular structures, can control many processes, such as vesicle trafficking and cell motility. Tropomyosins (Tpms) are a family of ABPs, which play an important role in regulating the plethora of cellular functions in both muscle and non-muscle cells [13]. The scientists were based on actin polymerization dynamics to directly observe actin assembly and protein dynamic by using TIRFM. They can detect the image of the polymerization of monomeric actin and palladin (an actin-binding protein) in real-time with TIRFM. They expect to know how palladin accelerates actin-based cell motility in metastatic cells, which will positively impact developing a new therapy in cancer progression [14].

Anillin, as an actin-binding protein, can bundle actin filaments and link to the plasma membrane in the ring-like actin network. To illuminate the latent intermolecular interaction between the actin filaments and actin-binding domain (ABD) that is a component of anillin structure, the researchers showed real-time imaging of actin filament crosslinking dynamic by TIRFM [15]. The researchers observed fluorescent images of single Anillin-FL-GFP (full-length anillin fused to green fluorescent protein) and Anillin-ABD-GFP molecules on actin filaments adhered to glass coverslips using TIRFM and performed Actin Cross-linking Protein (ACP) affinity assays based on single-molecule fluorescence imaging with TIRFM in the study [16].

People have been aware of the importance of cell-cell contact-based information exchanges in recent years. There is a mass of interaction between receptor and ligands. The scientists confuse about how these interactions mediate the formation and stability of cell-to-cell adhesion focal planes. With the help of advanced state-of-the-art live-cell imaging techniques, such as TIRFM, they have a profound comprehension of a cell-to-cell interaction [17]. In TIRFM imaging experiments, an antigen presentation system supported by fluid Planar Lipid Bilayer (PLB) membranes has been widely used to mimic the cell-cell contact in-

terface [18]. It has shown an enormous result in exploring the action mechanism until cooperating with the advanced TIRFM imaging technique. They can do quantitative analysis and examine some specific processes, like the formation of B-Cell Receptor (BCR) microclusters, basing on the TIRFM visually imaging in real time [19]. The experiment uses a new method to do the studies on the complicated receptor and ligand interactions during cell-cell crosstalk [20].

2.2.2. Protein-DNA

To better understand archaeal chromatin proteins Cren7 and Sul7d from Sulfolobus architectural roles in chromosomal DNA organization, the scientists with total single-molecule internal reflection fluorescence microscopy (SM-TIRFM) and Atomic Force Microscopy (AFM). Researchers presume that the two proteins form protein-DNA filaments in different patterns [21]. For the first time, they prove that the two proteins enabled DNA bridging, and both DNA binding and bridging were involved in DNA compaction. In the study, researchers visualized the influence of binding by the two proteins on the conformation of DNA at the single-molecule level. They found the assembled protein-DNA complexes were maintained for a long term without decomposition, and learn more about the structural basis of DNA bridging by Cren7 and Sis7d with TIRFM [22].

3. Clinical Applications

All vital movement is dependent on biomolecular movement. The experiments mentioned above about biomolecular interaction establish a basis for research. Therefore, people can use the former research to further study the clinical application, especially in some pathogenesis of serious human disease, like various cancers, immunological diseases, nerve conduction, and gynecological disease.

Scientists devote themselves to study clinical applications. It is the key point of the study that they must know the mechanism. The best solution is to understand how cells interact, meaning people need to see the real-time dynamic process and high-resolution imaging. Compared with other traditional fluorescence technology of biological single-molecule, TIRFM has an unbeatable advantage. In addition, if it is connected with some advanced detector, it will play a bigger role in the study.

3.1. Immunization

3.1.1. Innate Immunity

Arabidopsis Hypersensitive Induced Reaction (AtHIR) proteins play a role in plant innate immunity. The latent mechanism of AtHIR in plant innate is ambiguous. Using VA-TIRFM and Forster Resonance Energy Transfer (FRET), the researchers contribute to the mechanism research in the experiment. They used VA-TIRFM to monitor the dynamics of individual AtHIR1 (a part of HIR family genes) particles at the Plasma Membrane (PM) with high resolution in living leaf epidermal cells [23] [24]. It is coming into increasing use for rapid and sensitive detection of fluorescent probes with background rejection, which has been used

to obtain high-quality images in the plant cell cortex. VA-TIRFM provides a powerful method to analyze or character the details of complex dynamics at the single-molecule level in living plant cells, and other traditional methods needed larger fields of view and greater out of focus light rejection, therefore VA-TIRFM can be outfitted with devices to perform targeted photobleaching and photoactivation of probes, thus combining sensitive detection and extended fluorescence life with the ability to perform photomanipulation experiments [25]. Some specific information, like the change of the diffusion coefficients and the motion range of AtHIR1-EGFP (Enhanced Green Fluorescent Protein) fusion protein, can be used for characterization analysis by using VA-TIRFM to observe. Protein complex formation is a critical aspect of plant innate immunity [26]. AtHIR1 may form a compound with other proteins, so it is significant to monitor and analyze it [27].

3.1.2. Acquired Immunity

Compared with innate immunity, the researchers focus more on the application of TIRFM in acquired immunity. This technique has been greatly facilitated in this field to explore the virus's pathogenic mechanism, which is due to its incomparable advantages.

The leading reason for respiratory infection in children is human Metapneumovirus (hMPV). However, the virus characterization and infection mechanism are still poorly understood. In this study, TIRFM was used to investigate early events of hMPV infection, and researchers used TIRFM to observe GFP-hMPV contiguously and analyze virus characterization in every frame. Scientists usually use the technique to analyze exocytosis, endocytosis, and protein dynamics in prior studies [28] [29]. The researchers daringly selected TIRFM to make continuous imaging of tracing of the virus. Compared with the traditional fluorescence microscopic imaging technology, this technique can avoid fluorescence interference of other sites for its special fluorescence achieved by employing an evanescent wave for excitation. It had been proven that TIRFM, as a highly efficient technique, can be used to discuss the virus movement [30].

Extracellular Vesicles (EVs) are lipid bilayer-delimited particles released from cells. EVs have drawn extensive attention because they have been recognized as efficient platforms of cell-cell communication and intercellular transfer of materials, especially in immune response [31] [32]. The thorny issue is that it is difficult to accurately characterize and analyze them for EVs' small size [33]. The researchers select TIRFM as a tool to confirm EV content. They can obtain a super-resolution type of microscope by augmenting Z resolution [34]. The study describes herein several methods to isolate and analyze extracellular vesicles by TIRFM, and they offer a complete solution from human peripheral blood lymphocyte purification to EV isolation and TIRF imaging [35].

Human Immunodeficiency Virus (HIV) must first fuse its lipid envelope with the host cell plasma membrane to enter a cell and establish infection. The dynamic process of the fusion can be tracked by TIRFM [36]. In this study, the re-

searchers used a combination of Cryo Electron Tomography (CryoET) and TIRFM in various research experiments. They detect the single-molecule fusion behavior with TIRFM and establish a 3D configuration of proteins and lipids at intermediate steps. Because of this, the researchers think this system is deadly suitable for imaging timed snapshots of 3D volumes [37]. This technical innovation has shown the enormous possibility for future study.

It follows that TIRFM not only plays a crucial part in acquired immunity by itself but also can combine with other advanced instruments to play a greater role in this level.

3.2. Other Applications

3.2.1. Nerve Conduction

It has been proved that large Fields of View (FOVs) in TIRFM via waveguides are extraordinarily effective for visually imaging robust cell types in a short time [38] [39]. But this technology is not applied to sensitive cell types like delicate primary neurons.

The TIRFM is usually used for imaging, detection, and analysis for its excellent properties. Unfortunately, on the one hand, scientists expect magnification of the TIRFM as high as possible to detect dynamic processes and analyze molecules. On the other hand, this request will limit the accomplishable FOVs, and imaging throughput. What's worse, qualitative and quantitative studies also were affected [40]. And the conventional TIRFM can't be used for measuring the cellular response under a drug treatment or other stimulus over a mass number of cells. Because of that, the researchers put forward a new apparatus based on the waveguide-based TIRFM for live-cell imaging of demanding samples, named chip-Scope. Compared with the traditional TIRFM, this new TIRF imaging platform allows for capturing a large amount of data for statistical study relying on the correlation in both space and time, which facilitates the obtaining of statistically significant results also for studies where the time dimension is of the essence. The TIRFM plays a crucial part in the spatially confined illumination and the high contrast and essentially decreases the phototoxicity [41] [42] [43]. The photonic waveguides provide the exceptionally large excitation areas and superior illumination homogeneity in the new apparatus. The combination has an enormous and bright prospect in nerve study.

In the study, the researchers succeeded in culturing and imaging fibroblasts, primary rat hippocampal neurons, and axons of Xenopus retinal ganglion cells (RGCs) by using this apparatus.

The experiment results found clear support for the improved TIRFM can be used for live-cell neuroimaging application. TIRFM will provide many new imaging applications through constant technological improvement.

3.2.2. Gynecological Disease

Ovarian cancer is the leading cause of patients' death among all gynecological cancers. The fatality of ovarian cancer remained stubbornly high is that this cancer

is difficult to be detected at an early diagnosis and is easy to develop drug resistance [44] [45] [46] [47]. Platinum- and taxane-based drugs usually were used in Epithelial Ovarian Cancer (EOC), while this cancer cell produces strong drug resistance to platinum- and taxane-based drugs. Therefore, preventing or solving chemoresistance is a thorny issue in ovarian cancer therapy [48]. Cancer metastasis is based on coordinated and dynamic regulation, but the mechanism of action is unknown [49].

Some studies showed that the SOCE (Store-operated Ca^{2+} entry) blocker is effective in combination with chemotherapies to treat refractory tumors, while the effect of SOCE for chemoresistance is ill-informed [50] [51]. Because of that, the researchers explored the influences of SOCE on focal adhesion dynamics and migration in ovarian cancer cells' chemoresistance in the experiment. It is an indisputable fact that TIRFM has high resolution and higher signal-to-noise ratio and can show efficient imaging in real-time, which has played a prominent part in this study. The experimenters assess the turnover of focal adhesions with EGFP-tagged focal adhesion molecules by using TIRFM to imaging at 30-s intervals per image for 1 h [52]. The excellent characteristics of TIRFM lay a foundation for the accuracy and reliability of the experiment.

4. Conclusions

It is believed that TIRFM plays a significant role in biomolecule research and clinical medicine. TIRFM can be used to analyze the single-molecule structure and state of motion, explore the mechanism of molecular interaction in biomolecule research, detect pathogenicity mechanisms and investigate the mechanism of drug therapy in clinical medicine.

The newest imaging instrument has a high-resolution and higher signal-to-noise ratio, which is the greatest strength compared with other fluorescence technology of biological single-molecule. Because of its excellent trait and low cost, this apparatus has a high potential for commercialization. It is a research hotspot in the future.

However, the drawbacks should not be ignored. Firstly, it's hard to make eligible products, like some high-precision instruments. Secondly, TIRFM is limited to imaging at the interface of two different media having suitable refractive indices. It's not a major defect because a great number of applications are ideally suited to the technique. Thirdly, the occurrence of fringing artifacts is inevitable in conventional laser-TIRF. Thirdly, the high magnification of lenses required for TIRFM limits the accomplishable FOVs and imaging throughput. Finally, it has low signal strength because it is difficult to detect. This issue can be effectively solved by cooperating with some high-sensitivity detectors for analysis. And combining with other facilitates is the future direction of development.

Now some people raise new ideas and methods to improve and ameliorate its characters. Alexander Kogel and his colleague put forward a new system that is

the Light-Emitting Diode (LED)-based implementation of objective-type TIRF (LED-TIRF), which can overcome fringing artifacts. Traditional MA-TIRFM (Multi-Angle Total Internal Reflection Fluorescence Microscopy) has a problem in the lateral super-resolution and doesn't relay well to perform depth imaging in densely distributed regions. The trouble is how to raise lateral super-resolution while keeping the high temporal resolution, so Zheng and his partner presented a new method to overcome this issue, termed P-TIRF (Polarized TIRF), which shows optimal three-dimensional imaging and enables the illustration of three-dimensional dynamic change of live cells.

In the future, TIRFM should make continuous technical innovation and continue to be merged with other complementary techniques, like AFM (the Atomic Force Microscope). And considering the limitation of two-dimension imaging, scientists need to seek a breakthrough in imaging theory. There is no doubt that this device will be used in various fields in the foreseeable future.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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