Fluorescent detection and analysis of single molecules

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Abstract. Total internal reflection fluorescence microscopy for detection and analysis of single molecules is considered. The experimental setup for the detection of single molecules by total internal reflection fluorescence microscopy was developed. Water solution of Rhodamine 6G was investigated.

1. Introduction
There are several methods of single molecule detection. Nowadays, researchers are mostly interested in the fluorescence microscopy method as the non-invasive technique for molecule detection [1]. In comparison with classical light microscopy, fluorescence microscopy has a number of advantages. For example, possibility to determine the distance between molecules, sufficient for interaction between them [2]. In addition, the use of sets of fluorophores has allowed researchers to identify the microscopic cellular components and specific cells among substances which are not fluorescent.

One of the most common types of fluorescence microscopy is the so-called Total internal reflection fluorescence (TIRF) microscopy. TIRF microscopy produces a thin excitation light field (the evanescent field) that nominally decays exponentially. Selective excitement of fluorophores near the sample interface is seen by this field. This method is widely used in biomedicine: researching exocytosis and endocytosis of neurotransmitters in the synapse, researching the dynamics of proteins interactions on the cell membrane [3]. The method we consider allows us to obtain images of single molecules. Thus we will be able to obtain information about the local nanometer environment of molecules. This information will allow us to study the structure and dynamics of single molecules at microscopic level.

One of the advantages of TIRFM in comparison with other methods is its ability to obtain an optical layer of better quality at large depths of thick samples. In addition, the scattering of both stimulating and fluorescent light influences the signal reception in this method not so much as in confocal microscopy. The TIRF method is less toxic and has a high signal-to-noise ratio. TIRFM is also more capable of collecting data on high-throughput interactions within and between single molecules given low background noise. TIRFM tends to be more reliable in terms of single-molecular tracking, which becomes important when considering the drifting effects of microscope equipment.

2. Techniques
The considered method is based on the phenomenon of full internal reflection, which is described by the Snellius law [5]. According to which certain conditions must be fulfilled:

- the angle of incidence of radiation should exceed the value of the critical angle (1)
- the refractive index of the medium ($n_1$) from which the light falls to the surface shall be greater than the refractive index of the environment ($n_2$).
\[ \theta_c = \sin^{-1} \frac{n_2}{n_1} \]  

In our case, the angle value is \( \theta_c = \arcsin(1.14) = 48.7^\circ \).

\[ d(\theta) = \frac{\lambda/n_1}{4\pi(s\sin^2\theta - (n_2/n_1))^{-1/2}} \]  

The depth of penetration of electromagnetic radiation into a medium with a smaller refractive index for our object of investigation \( d(\theta) = 219 \text{ nm} \) (2).

3. Experimental setup

The scheme of the experimental setup is shown in Fig.1. The light flux passes through the prism of total reflection, is reflected at the interface and passes through the opposite face of the prism, while a part of the electromagnetic radiation penetrates into a less dense medium, thereby exciting fluorescence in the object under investigation. The reflected light of the incident beam generates an electromagnetic field in a medium with a lower index of refraction.

![Figure 1. Experimental setup. 1 — laser, 2 — prism of total internal reflection, 3 — sample, 4 — emission, 5 — microscope lens, 6 — filter, 7 — CCD camera, 8 — computer.](image)

Fluorophores located closest to the surface of the glass are selectively excited when interacting with this field, and the secondary fluorescence from these radiators (fluorophores) is collected by the optics of the microscope. The principle of this process is shown in Fig.2. Then the signal passes through a filter installed in a microscope, and is detected by a CCD camera. In this work, a camera with a low noise level (62 dB), high efficiency and high dynamic (50 dB) and spectral (380 – 650 nm) ranges was chosen. The received signal is then displayed on the computer as an image in Altami Studio.

The light that hits the matrix elements is converted into an electric charge, a charge picture is formed, which is proportional to the illumination in each cell. The matrix can accumulate charges for a certain period of time. The total charge accumulated in the cell is equal to the product of charges for the exposure time. To obtain a color image, the light beam passes through a set of special filters of green,
blue and red color. According to the received pictures, we can, for example, monitor the trajectories of rhodamine molecules.

![Image](image1.png)

**Figure 2.** Total internal reflection fluorescence. 1 — laser, 2 — emission, 3 — prism, 4 — evanescent field, 5 — sample, 6 — objective.

4. **Samples**

As a test sample, a water solution of rhodamine 6G with fluorescence excitation length within 526 nm was used. In accordance with this, a laser with a wavelength of 532 nm was selected. Refractive indices of rhodamine (n2=1,332) and glass (n_1=1,518) [3,4]. Rhodamine dyes are used in biotechnology in such methods as fluorescence microscopy, flow cytometry and enzyme immunoassay. The dye has surprisingly high photostability, high quantum yield of fluorescence (0.95 [6]), and its generation is close to the absorption maximum (approximately 530 nm). The dye forming range is from 570 to 660 nm with a maximum at 590 nm.

5. **Results and discussion**

The first results of the experiment confirmed the potential of the chosen method in the detection of rhodamine molecules 6G. The application of total internal reflection fluorescence microscopy on prism allowed us to increase the sensitivity in comparison with standard methods.
In the Fig. 3 fluorescent molecules in different concentration are presented. We can determine location of the fluorescent centers in studied samples on the basis of these pictures. The research of single molecules by the fluorescence method in the mode of total internal reflection will help to understand how the biotic processes in the cell proceed. It will also be possible to introduce single molecules as probes, which will allow us to study the structure and dynamics of materials in which molecules will be introduced at such a microscopic level [8].

Nowadays, TIRF microscopy has a solid theoretical basis, and the practical application of this technique has been greatly simplified due to recent technological advances. Not surprisingly, it is increasingly being used in biomolecular research and research in cell biology. The configuration of TIRF systems based on direct or inverted microscopes is relatively simple when using a laser light source, but can also be performed using a conventional arc lamp, provided that the light passing through the central part of the lens is blocked. Now, complete modular microscopic systems assembled for TIRF microscopy in combination with other optical techniques are now available, and some manufacturers produce high numerical aperture lenses designed specifically for applications that use internal reflection. The TIRF method is compatible with various lighting modes, including light-field, darkfield, phase-contrast, differential interference contrast and traditional epifluorescence. A special advantage of systems with illumination through the lens is their compatibility with various mechanisms of manipulation with biomolecules, such as atomic force microscopy. Probably, the tendency of combining TIRF microscopy with other additional techniques will continue. Also, TIRFM has potential advantages in any application where visualization of small structures or single molecules is required in samples with a large number of fluorophores located outside the optical plane of interest, such as molecules in solutions with Brownian motion, vesicles undergoing endocytosis or exocytosis, or transfer of individual proteins in cells [6,7].

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