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Abstract. The paper presents a method for recording fluorescent images of single molecules using the total internal reflection fluorescence microscopy. This method allows determining the location of a molecule with subdiffraction accuracy and a high signal-to-noise ratio of the detected radiation. Methods for computer processing of fluorescence images are considered. The obtained experimental data are presented and the possible areas of application of the obtained results are discussed.

1. Introduction

Total internal reflection fluorescence microscopy (TIRFM) is widely used in biomedical research to visualize single molecules, for example, to study gene expression and the dynamics of the interaction of proteins with the cell membrane [1]. One of the main goals in cell biology is to determine in vivo the local distribution of cellular components and its dynamic changes. Such visualization of individual membrane proteins in living cells opens new perspectives for studying cellular organization and processes at the molecular level. Using fluorescent molecules as probes in a biological sample under study, it is possible to obtain information about the local nanometer environment of the molecules [2]. TIRFM is limited within one focal plane. A relatively short exposure time (many snapshots per second) is used in obtaining images. It makes the method suitable for visualizing dynamic processes. One of the advantages of this method is the ability to display several fluorophores simultaneously or in very rapid succession. This method is optimal for displaying very fast events occurring at the microscopic level. Computer image analysis is performed for information about the behavior of single molecules. With the help of image processing, it is possible to obtain information in a form convenient for visual perception that allows you to select and measure the objects under study, without disturbing the accuracy of the images obtained. [3, 4].

This paper presents the results of image processing obtained from developed total internal reflection fluorescence microscopy experimental setup.
2. Total internal reflection fluorescence microscopy
TIRFM is based on the phenomenon of total internal reflection. At a certain angle, called critical, the incident light beam totally internally reflects at the interface between two mediums. An electromagnetic field called the evanescent wave penetrates a small distance into the liquid medium with refractive index $n_2$. Evanescent wave propagates parallel to the surface in the plane of incidence. However, part of the electromagnetic field penetrates into the medium with refractive index $n_2$ to a depth of about half the wavelength of the incident radiation [5-7]. Penetrated radiation is able to selectively excite fluorophores located near the interface [8].

The developed optical setup consists of a fluorescence excitation source, which uses a semiconductor laser with a wavelength of 532 nm, a prism of total internal reflection on which the object under study is located, a set of filters (excitation and emission) and a low-noise camera (62 dB), high efficiency (95%), high spectral (380-650 nm) and dynamic (50 dB) ranges. A detailed description of the experimental setup is presented in [9]. R6G is chosen as the object of study.

3. Image processing
A set of image analysis processes is required to obtain the particle's dynamic information from the optical image. It includes subtraction of the background noise, localization of the particle position [10], connection and reconstruction of the trajectory, as well as classification of the diffusion behavior.

Trajectory analysis is an important step for understanding the dynamic behavior of a biological object of interest [11]. Time dependence of mean-square displacement of the single molecules is the main method for determining the motion of it. This makes it possible to additionally obtain several important parameters (the diffusion coefficient and the velocity of the particle) [12].

Analyzing the time dependence of mean-square displacement the trajectory of an individual particle can be basically divided into the following four regimes [13-15]:
- direct diffusion (movement of particles along the cytoskeleton),
- normal diffusion (movement of particles in Brownian motion),
- anomalous diffusion (movement of particles when they collide with obstacles or interact with other particles),
- limited diffusion (the movement of particles within a particular region).

4. Results
Processing cluster images obtained in the experiment consists of the following steps:
- segmentation of regions of interest in the characteristic features;
- selection of regions;
- evaluation parameters of the areas.

In the first step, the division of the image by color is introduced. Distinctive features of the selected image object, namely single molecules, is their color (orange range radiation). The processing of the image on this step consists of:
- indexing images – image conversion to the color space CIELAB partitioning the colors of an image on indices.
- classification of image fragments on the principle of nearest neighbor – assigns the picture elements of the truth, in case they belong to the class of orange when using the color space CIELAB.

The result of the work at this stage is shown in figure 1. This step of processing allows us to subtraction of the background noise.
The next step is the selection of the desired areas. This procedure was carried out by morphological image processing. This allowed us to determine the individual objects that fit the description from the point of view of color. The result was allocated in the following areas, shown in figure 2.

After the procedure of selection was determined by the following parameters [16-19]:
- change in equivalent background intensity of radiation of molecules;
- fluctuation of the position of molecules over time;
- change in equivalent intensity of a single molecule.

All parameters changed over time in 0.1-second steps. The background intensity was determined as a mathematical expectation for pixel brightness in regions of interest. Depending on the time, the following function graph was obtained, shown in figure 3.
Figure 3. Dependence of relative intensity radiation on time.

Fluctuation of the position of the molecule was determined as a mathematical expectation of the Euclidean distance between the two largest molecules (figure 4).

Figure 4. Dependence of relative movement (fluctuation) the provisions of molecule on time.
Figure 5. Change in the equivalent intensity of a single molecule.

Figure 5 shows the emission intensity of the maximum size of molecules among the found. From the obtained results, the relative intensity and movement of the selected regions with molecules changes over time. Consequently, the studied molecules of rhodamine 6 G perform a Brownian motion. In the future, it is planned to detect the movement for a longer time to track the trajectory of movement of the isolated molecular clusters. Also, the applied algorithms are suitable for analyzing images from developed experimental setup of total internal reflection fluorescence microscopy.

5. Conclusions
In this work the analysis and processing of the obtained fluorescence images was presented. Samples of R6G in low molecular concentration were selected for experimental studies. The main stages for image processing of single molecule were considered. At this stage of processing, the following actions were carried out: segmentation of areas of interest by characteristic features, selection of regions and assessment of area parameters. Obtained results confirm using experimental setup of TIRFM to visualize single molecules. This method is optimal for displaying very fast events occurring at the microscopic level that important for cell biological applications

In the future, such an analysis will help to study the nature of the movement of molecules in a system, their interactions will allow to analyze in detail the dynamic properties of various molecular systems and to study the influence of the dynamics of molecules on the properties of a substance [20].

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