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REVIEW

Computerized fluorescence microscopy of microbial cells

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Abstract
The upgrading of fluorescence microscopy by the introduction of computer technologies has led to the creation of a new methodology, computerized fluorescence microscopy (CFM). CFM improves subjective visualization and combines it with objective quantitative analysis of the microscopic data. CFM has opened up two fundamentally new opportunities for studying microorganisms. The first is the quantitative measurement of the fluorescence parameters of the targeted fluorophores in association with certain structures of individual cells. The second is the expansion of the boundaries of visualization/resolution of intracellular components beyond the "diffraction limit" of light microscopy into the nanometer range. This enables to obtain unique information about the localization and dynamics of intracellular processes at the molecular level. The purpose of this review is to demonstrate the potential of CFM in the study of fundamental aspects of the structural and functional organization of microbial cells. The basics of computer processing and analysis of digital images are briefly described. The fluorescent molecules used in CFM with an emphasis on fluorescent proteins are characterized. The main methods of super-resolution microscopy (nanoscopy) are presented. The capabilities of various CFM methods for exploring microbial cells at the subcellular level are illustrated by the examples of various studies on yeast and bacteria.

Keywords Microscopy · Fluorescence · Computer image processing and analysis · Super-resolution microscopy · Nanoscopy · Microorganisms · Bacteria · Yeast

Abbreviations

CFM	Computerized fluorescence microscopy,
FP	Fluorescent protein
CIPA	Computer image processing and analysis
GFP	Green fluorescent protein
fPALM/PALM	Fluorescence photoactivation localisation microscopy
STORM	Stochastic optical reconstruction microscopy
SIM	Structured illumination microscopy
STED	Stimulated emission depletion microscopy
SMLM	Single molecule localization microscopy
smFISH	Single molecule fluorescence in situ hybridization
QCM	Quantitative confocal microscopy

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Introduction

Methods for the study of microorganisms based on the phenomenon of fluorescence can be conditionally divided into two categories. The methods of the first category provide visualization of individual cells using fluorescence microscopes. With their help, it is possible to visually detect fluorescence of single cells and determine its spatial distribution. The methods of the second category are based on the quantitative measurement of the main parameters of fluorescence, such as intensity, fluorescence and excitation spectra, the lifetime of the excited state, and polarization. These parameters make it possible to study the physicochemical properties of cells or their components at the molecular level with special devices, (spectro)fluorimeters of various configurations. For technical reasons related to the sensitivity of the devices, measurements are carried out on bulk sample preparations containing many cells. As a result, the data obtained are averaged, and the properties of individual cells are leveled (Lakowicz 2006).

Over the past twenty years, the research of microbial cells has moved to a qualitatively new level due to the development of *computerized fluorescence microscopy* (CFM).

This methodology improves *subjective* visualization and combines it with *objective quantitative* analysis of the microscopic data. CFM appeared due to a number of innovations.

Firstly, fluorescence microscopes have been significantly improved due to the use of lasers, highly sensitive photomultipliers, digital photo/video cameras and powerful computers. This made it possible to detect ultra-weak light fluxes from microscopic objects in the form of images in *digital format* (Sanderson et al. 2014).

Secondly, *computers and software* for processing and analyzing digital images with high speed were created. On the one hand, they provided the opportunity to improve the visual perception of microscopic objects; on the other hand, they made it possible to quantitatively characterize both the optical parameters and the geometry of the objects (Puchkov 2016a).

Thirdly, an extensive set of fluorescing molecules with various properties for introduction into microbial cells has been produced (Johnson and Spence 2010; Spahn et al. 2018). In addition, special methods based on the use of fluorescent proteins (FP) have been developed (Tsien 1998; Snapp 2005). They made it possible to obtain images of intracellular structures with a resolution exceeding the capabilities of conventional light microscopy and even to track the localization and movement of individual molecules. The combination of these methods is called *super-resolution microscopy* (Coltharp and Xiao 2012; Yao and Carballido-López 2014) or *nanoscopy* (Hell 2007; Requejo-Isidro 2013).

Figure 1 shows the main steps of CFM.

The purpose of this review is to demonstrate the unique research potential of CFM in studying the fundamental aspects of the structural and functional organization of microbial cells. For this purpose, the basics of computer processing and analysis of digital images are briefly described. Then, the fluorescent molecules used in CFM with an emphasis on fluorescent proteins are characterized. Next, the main methods of super-resolution microscopy (nanoscopy) are presented. Finally, the capabilities of various CFM methods for exploring microbial cells at the subcellular level

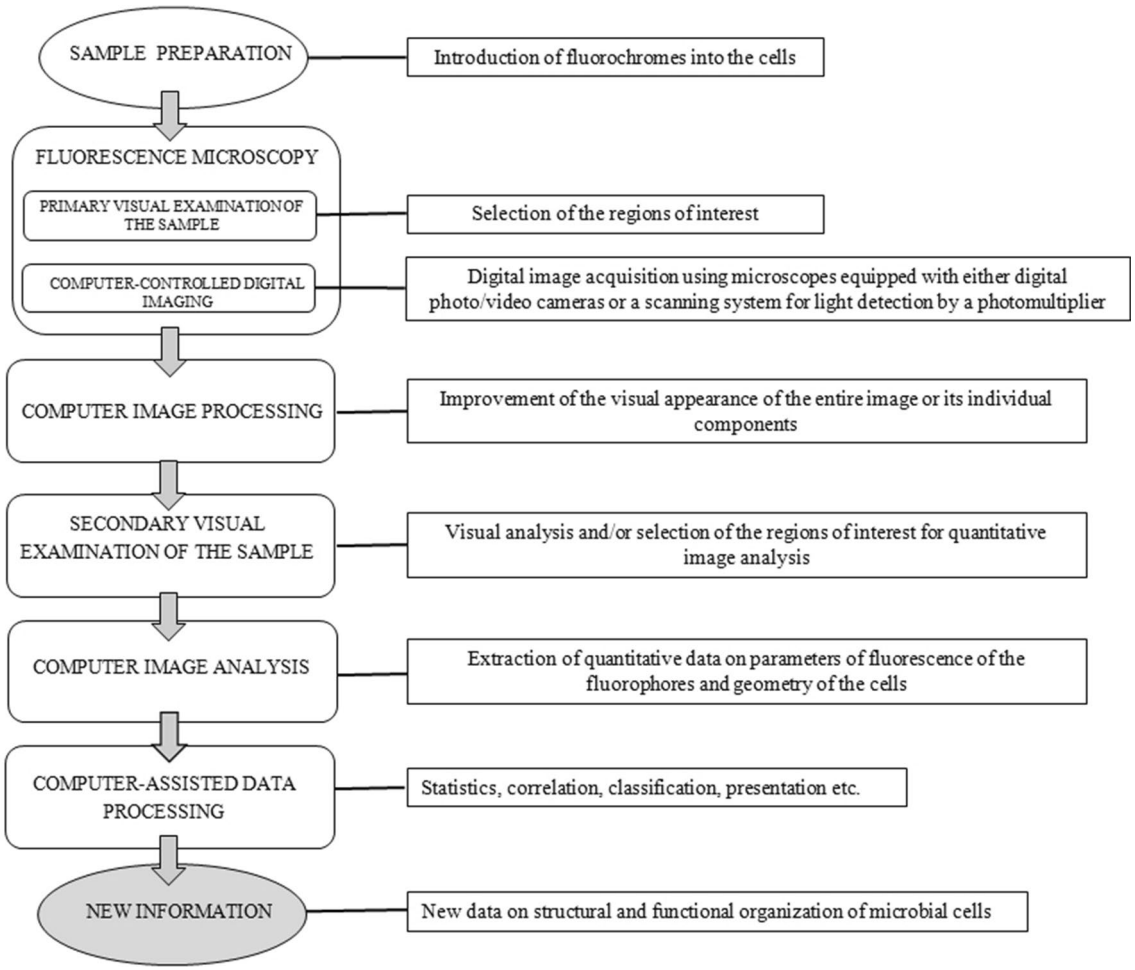

Fig. 1 A flowchart diagram of the computerized fluorescence microscopy of microbial cells

are illustrated by the examples of various studies on yeast and bacteria.

The paper is intended to provide *a bird's-eye view* of the CFM. Principles of fluorescence and numerous fluorescence-based techniques including microscopy have been addressed in depth in (Lakowicz 2006; Sanderson et al. 2014; and http://www.microscopyu.com). Many specific aspects of CFM and its particular applications in the studies of microorganisms have been reviewed by others and will be cited accordingly.

Computer image processing and analysis

The basics of computer digital image processing and analysis (CIPA) were developed in the middle of the twentieth century. However, this methodology has found wide application in many fields of science and practice to date only with the advent of affordable computer equipment of the necessary power. The general principles of CIPA are described in sufficient detail in a number of publications (Pratt 2001; Gonzalez and Woods 2002; Sonka et al. 2007; Solomon and Breckon 2010; Kan 2017), including those focused on applications in microscopy (Wilkinson and Schut 1998; Sbalzarini 2016; Nketia et al. 2017; Wallace et al. 2018). The principles on how CIPA works in microscopy, including fluorescence microscopy, can be briefly presented as follows (Puchkov 2016a).

CIPA deals with images, for example, photos in a digital format. This means that they are a set of elements called pixels if the image was obtained in two-dimensional (2D) space, or voxels if the image was obtained in three-dimensional (3D) space. Each pixel (voxel) contains digitally encoded information about its X–Y–(Z) position in Cartesian coordinates, as well as about the optical properties at this point in space. The nature of the optical information depends on which optical system was used when the image was captured. If this information was obtained using monochrome digital photo/video cameras or a photomultiplier, it represents data on the light intensity in units of the gray scale. This scale in the 8-bit representation consists of 28 variants or 256 levels from black (0) to white (256) color. If the images were obtained using color photo/video cameras or systems based on light filters, such as multispectral or hyperspectral systems (Hagen and Kudenov 2003; Gao and Smith 2015), they are a matrix of pixels with information encoded according to one of the models of so-called "color spaces", namely, RGB, HSV, CIE-Lab, and YCrCb. RGB is the most popular model used in CFM, which is based on a 24-bit combination of Red, Green and Blue color intensities with 8 bits for each color. The initial data in digital format is processed by a computer as parameters of a special mathematical model constructed from various algorithms. Processing the raw data with this model allows them to be manipulated in such a way as to improve the visual appearance of the entire image or its individual components. This procedure is called *image deconvolution.* An example of using image deconvolution in CFM of *Saccharomyces cerevisiae* cells is shown in Fig. 2. In addition, the data encoded in the digital image can be used for their quantitative analysis. This approach is called *computer image analysis*. With the help of computer image analysis, the optical properties of objects are determined in conjunction with their spatial characteristics. Two examples of using image analysis in computerized fluorescence microscopy of *S. cerevisiae* cells are shown in Figs. 3 and 4.

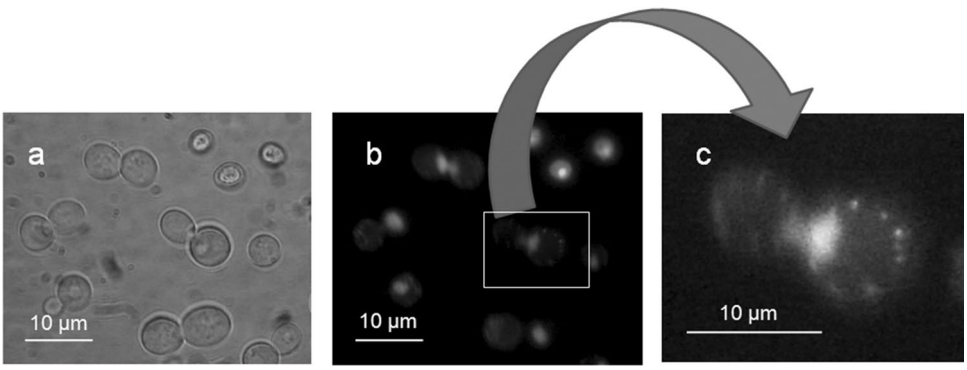

Fig. 2 Transmitted (**a**) and fluorescence (**b**) microscopy images of live *Saccharomyces cerevisiae* cells stained with nucleic acid dye 4′,6-diamidino-2-phenylindoleand (DAPI). **c** The rectangular fragment of the image in **b** after the deconvolution (ImageJ and Adobe Photoshop Cs6 software). The deconvolution made it possible to visualize intracellular localization of the nucleus at the stage of division (in the middle) and the mitochondria (small dots in the periphery) fluorescing blue light. Experimental conditions were as in (Puchkov 2016b). (Unpublished results)

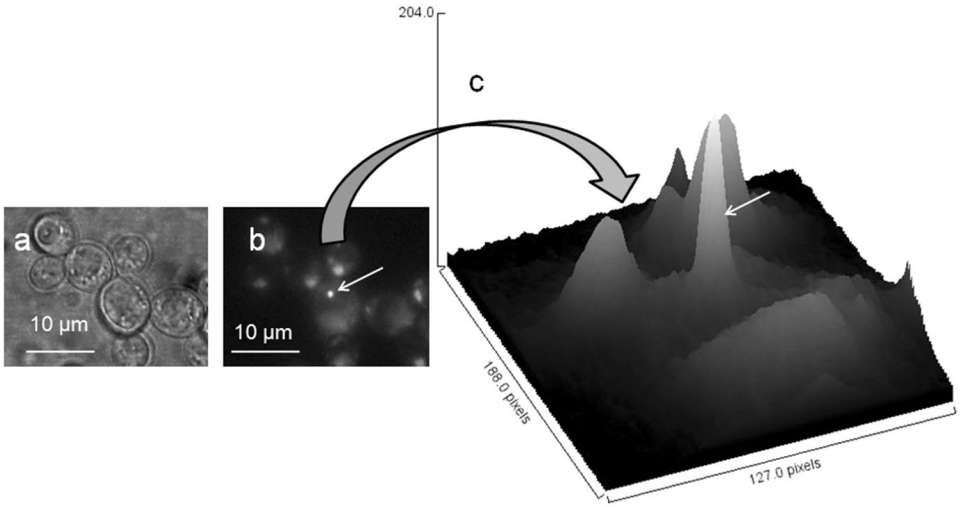

Fig. 3 Transmitted (**a**) and fluorescence (**b**) microscopy images of live *Saccharomyces cerevisiae* cells stained with nucleic acid dye 4′,6-diamidino-2-phenylindoleand (DAPI). **c** The surface profile of fluorescence intensity of two cells (ImageJ and Adobe Photoshop Cs6 software). White arrows in **b** and **c** indicate a particle of inorganic polyphosphate complex in the vacuole of one cell, which had bright yellow fluorescence. The data allow quantitative fluorescence intensity measurements in relation to certain intracellular structures. Experimental conditions were as in (Puchkov 2016b). (Unpublished results)

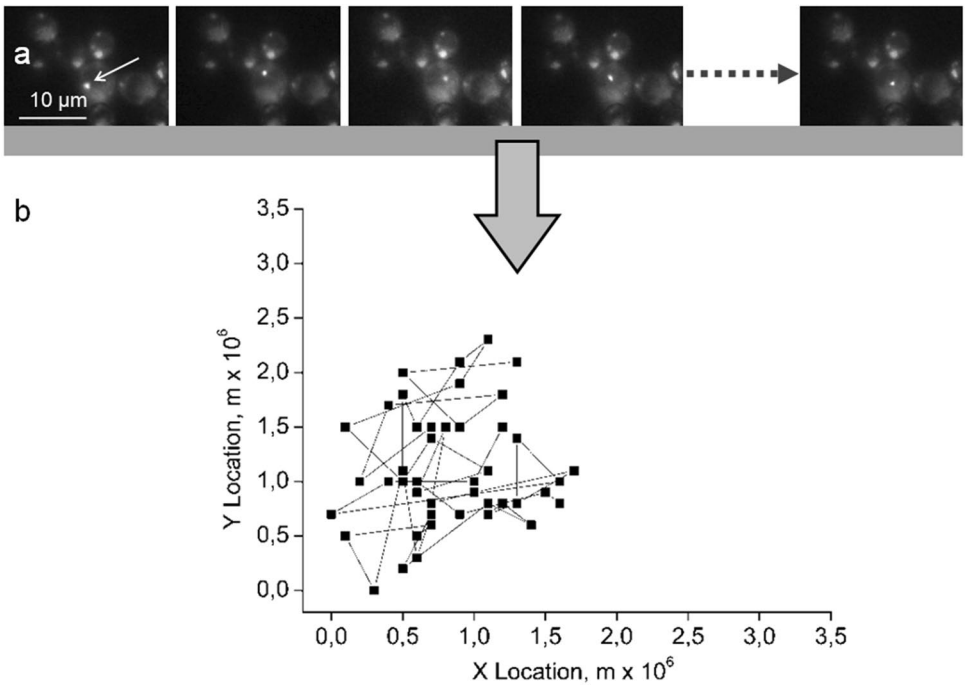

Fig. 4 **a** A series of fluorescence microscopy images of live *Saccharomyces cerevisiae* cells stained with nucleic acid dye 4′, 6-diamidino-2-phenylindoleand (DAPI) taken at 5 s intervals. White arrow indicates a particle of inorganic polyphosphate complex in the vacuole of one cell, which had bright yellow fluorescence and visually was in constant movement. **b** Positions of the particle of inorganic polyphosphate complex in the vacuole derived from the series of images (**a**) (ImageJ and OriginPro 7.5 software). The data indicate that the particle movement is chaotic or Brownian motion. Further studies of this phenomenon made it possible to assess viscosity in the vacuoles of the cells (Puchkov 2016b). Experimental conditions were as in (Puchkov 2016b). (Unpublished results)

Fluorescent molecules

In most microbial cells, there are few natural fluorescent molecules. In addition, the spectral properties of their fluorescence are such that it is almost impossible to use them for microscopy. Therefore, specially synthesized molecules are used. In conventional fluorescence microscopy, they are called *fluorescent dyes* or *stains*, since their main role is to visualize the entire cell as a whole or its individual components. In fluorimetry of bulk samples, fluorescing molecules are called *fluorophores* or *fluorochromes* (the latter term is used less often). The term fluorophore has more general meaning regardless of the fluorescent method used (Lakowicz 2006). So it is suitable to use it in CFM.

Fluorophores are conventionally divided into two categories, *probes* and *tags (labels)*. Fluorescent probes are fluorophores that are capable of *non-covalent physical* interaction with cell components. This interaction is of a certain degree of specificity and causes changes in some parameters of fluorescence. For example, fluorescent dye of nucleic acids 4′,6-diamidino-2-phenylindoleand (DAPI) can be used as a probe, since its non-covalent interaction with nucleic acids gives rise to its blue fluorescence, and interaction with inorganic polyphosphates causes yellow fluorescence. These fluorescence changes can be both visually observed and quantitatively measures in CFM (Puchkov 2016b) (Figs. 3 and 4). Quinacrine is another example. In CFM, it may serve both as a dye that stains vacuoles and as a probe for assessing intravacuolar viscosity in single yeast cells by fluorescence polarization measurements (Puchkov 2016b). There are numerous fluorescent probes for the studies of membranes, nucleic acids, intracellular organelles, pH of the cytoplasm, the content of inorganic ions, and some others (Johnson and Spence 2010).

Fluorescent tags specifically bind to certain macromolecules by *covalently bonds* (proteins, lipids, carbohydrates, and others), thereby making them fluorescent. Fluorescent tags are very popular in labeling antibodies. Fluorescently labeled proteins can be generated in microorganisms by molecular biology techniques (see below).

The use of many fluorescent probes and tags is difficult or even impossible for intracellular studies of live cells. This is due to the selective permeability of the cytoplasmic membrane, through which many hydrophilic fluorophores cannot enter the cell. One of the possible ways to overcome this hindrance is the creation of hydrophobic derivatives of the hydrophilic fluorophores. Upon penetration into the cells, the hydrophobic "tail" is cleaved off by intracellular enzymes (for example, esterases) (Johnson and Spence 2010).

New research capabilities of CFM were opened up due to the development of special techniques based on the generation and use of FPs.

The first technology of the generation of FPs was invented due to the finding of a natural protein of the jellyfish *Aequorea victoria*, which fluoresces in the green region of the spectrum and therefore is known as *green fluorescent protein* (GFP) (Tsien 1998). It was found that the cloned GFP gene is self-sufficient for the biosynthesis of a full-fledged fluorescent protein in various cells. This served as a prerequisite for the development of molecular biology methods by which GFP can be linked (fused) to other proteins as a fluorescent tag. Later, natural and artificial GFP homologues were discovered and synthesized, which were able to fluoresce in the longer wavelength region of the spectrum. They also can be used to create hybrid FPs. The history of development and the principles of the technology for creating FPs based on GFP homologues are well described by Snapp (2005) and Campbell (2008).

The principle of the second technology is based on the creation of a hybrid of an intracellular target protein with a special protein tag, which has the ability to covalently attach low-molecular mass fluorophores. Due to this ability such proteins are called "self-labeling proteins". They are enzymes that covalently bind fluorescent fragments attached to their substrates. The technology includes two stages. First, a hybrid of the target protein with the corresponding tag is created in the studied cells using molecular biology methods. Then, a substrate that contains a fluorophore is added. As a result, upon enzymatic reaction, the entire structure including the target protein and the tag is labeled with an exogenous fluorophore (Fernández-Suárez and Ting 2008; Hinner and Johnsson 2010).

One of the popular proteins that can "label themselves" with fluorophores was generated on the basis of human O^6-alkyl guanine-DNA alkyltransferase. It was found that the fusion of this enzyme with target proteins in the cells of bacteria, yeast and CHO culture and the subsequent addition of fluorescent derivatives of O^6-benzyl guanine, the substrate of this enzyme, led to fluorescent labeling of hybrid proteins (Keppler et al. 2003). Following these studies, a genetically engineered product of the O^6-alkyl guanine-DNA alkyltransferase gene, SNAP-tag, was obtained, which is currently commercially available as part of various expression vectors. Its substrates are fluorescent derivatives of O^6-benzyl guanine (Juillerat et al. 2003). There are also other systems for labeling intracellular proteins with exogenous fluorophores using this technology, for example, CLIP-tag and Halo-tag. Their substrates are fluorescent derivatives of O^6-benzyl cytosine (Gautier et al. 2008) and primary alkylgalides (Los et al. 2008), respectively. The combined use of SNAP-tag and CLIP-tag for the introduction of two different fluorophores into cells was described (Macias-Contreras et al 2020).

The technology of creating FPs using protein tags that can "label themselves" with fluorophores has several advantages

compared to the technology based on GFP homologues. The FPs obtained in this way have a large quantum yield and increased stability when excited by high-intensity light sources (Fernández-Suárez and Ting 2008; Hinner and Johnsson 2010). In addition, the possibility of using an extensive set of commercially available low molecular mass fluorophores (Johnson and Spence 2010) with this technology opens up new areas of CFM application.

Interesting experimental possibilities appeared after discovery of so-called photoswitchable FPs. Some of such FPs acquire the ability to fluoresce only after photoactivation with light of certain wavelengths. This transition can be either reversible (Dronpa protein is an exapmle) or irreversible (photoactivated GFP is an example). Other photoswitchable FPs can irreversibly change their spectral properties under appropriate illumination. For example, EosFP that fluoresces green, after being illuminated with blue light, begins to fluoresce red. The properties and possibilities of using such FPs in CFM are discussed in detail in the reviews of Chozinski et al. (2014) as well as in Minoshima and Kikuchi (2017).

It should be noted that the use of the FP technologies on microorganisms may have problems. First of all, there may be difficulties in the study of live cells due to the impermeability of the membranes and/or cell walls of bacteria and yeast for most hydrophilic fluorescent substrates. This difficulty can be circumvented, for example, by using hydrophobic derivatives of fluorophores (Keppler et al. 2003) or electroporation (Stagge et al. 2013). It should also be kept in mind that both technologies for creating FPs imply modification of target proteins, which must be considered when interpreting the data obtained with their help.

One of the most important results of the combined application of CFM and the technologies for the generation of intracellular FPs was the development of the methods of *super-resolution microscopy* or *nanoscopy* (Hell 2007).

Super-resolution microscopy (nanoscopy)

The resolution of standard optical microscopy is limited due to the diffraction properties of light. This so-called diffraction limit of spatial resolution is approximately equal to half the light wavelength of the illumination used. In practice, this means that it is impossible to distinguish two objects that are located closer than half the wavelength of the light used (in most cases, it is several hundred nanometers) with a conventional optical microscope (Sanderson et al. 2014). Super-resolution microscopy methods made it possible to overcome this limit in various ways. This possibility was demonstrated for the first time by the so-called *near-field* microscopy. It was based on the recording of light fluxes by a special optical probe, which was located at a distance from the sample surface less than the wavelength of the light used. However, methods based on conventional *far-field* microscopy turned out to be more practical for studying biological objects. For their implementation, microscopes of a design with a special optical probe are not required, and they provide the opportunity to explore not only the surface, but also deeper areas of the sample. To emphasize the possibility of studying objects below the "diffraction limit" with a resolution in the nanometer range, the term *nanoscopy* was introduced (Betzig and Chichester 1993).

Various methods of the nanoscopy of biological objects were described in a number of reviews focused, in particular, on the general principles of nanoscopy (Hell 2007; Requejo-Isidro 2013; Han et al. 2013; Lakadamyali 2014; Nienhaus and Nienhaus 2016), special issues related to the use of photoswitchable fluorophores (Chozinski et al. 2014; Minoshima and Kikuchi 2017), the study of the localization of single molecules (MacDonald et al.2015; Sydor et al. 2015; Lelek et al. 2021), as well as the specifics of application for the study of microorganisms (Coltharp and Xiao 2012; Sanderson et al. 2014; Yao and Carballido-López 2014; Gahlmann and Moerner 2014; Tuson and Biteen 2015; Kocaoglu and Carlson 2016; Tuson et al. 2016; Endesfelder 2019; Ho and Tirrell 2019; Cambré and Aertsen 2020; Singh and Kenney 2021).

Conventionally, nanoscopy methods can be divided into two categories.

1. *Methods of single molecule localization microscopy* (SMLM) (Lelek et al. 2021), such as fluorescence photoactivation localization microscopy (fPALM/PALM) (Betzig et al. 2006; Hess et al. 2006) and stochastic optical reconstruction microscopy (STORM) (Rust et al. 2006; Bates et al. 2007). The general principle that underlies this category of methods is as follows. The fluorescent molecules in the test sample are excited in such a way that only a fraction of them can fluoresce at the same time. This procedure is carried out repeatedly with the imaging. The images are superimposed on each other. The resulting image includes all the fluorescent molecules, including those that are located at such a close distance from each other that they would be indistinguishable with conventional microscopy. To implement this principle in the fPALM/PALM methods, special photoswitchable FPs are used which can be "turned on/off" by certain combinations of excitation illumination (Dickson et al.1997; Lippincott-Schwartz and Patterson 2009; Heilemann et al. 2009). In STORM methods, specially selected pairs of fluorophores are use. One of this pair acts as an "activator" and the other as an "acceptor" of excitation (Rust et al. 2006; Bates et al. 2007). The resolution of PALM/STORM methods in the lateral and axial directions is about 10–20 nm and

20–100 nm, respectively. Some other methods of this category can be found in (Lelek et al. 2021).

2. *Methods based on special illumination of samples*, such as structured illumination microscopy (SIM) (Gustafsson 2005) and stimulated emission depletion microscopy (STED) (Hell and Wichmann 1994; Klar et al. 2000; Hell 2007). To implement this category of methods, an ultra-high sensitivity for recording weak fluorescence of individual molecules is not required. In the microscopes for SIM, the excitation light flux passes through a special optical lattice. This leads to the formation of spatially structured illumination in the focal plane of the sample due to the interference (Moire) effect. After processing a series of fluorescent images of the sample obtained in this way by special computer programs, the resolution can be improved to approximately 100 nm. In STED microscopy, excitation with two laser pulses is used. The first pulse excites the photoswithchable fluorophores to a fluorescent state. The second pulse is used to suppress fluorescence in a fraction of the fluorophores in the area surrounding the narrow focus area of the objective lens. The sample is scanned under this excitation mode, and a fluorescent image of the entire sample is obtained. The resolution of this method in the lateral direction is of 30–60 nm.

A wide range of capabilities of various CFM methods for the exploration of microorganisms at *subcellular level* are illustrated below by the examples of studies performed on yeast and bacteria.

Subcellular level studies of yeast and bacteria with CFM

Yeast

CFM can be performed on a standard fluorescence microscope with a digital photo/video camera and ImageJ image analysis software available on the Internet. The possibility of *subcellular microfluorimetry* of single cells of the yeast *S. cerevisiae* with such a relatively simple set of experimental tools was demonstrated. This approach was used to assess viscosity in the vacuoles by the measurements of the Brownian motion of inorganic polyphosphate complexes and the polarization of quinacrine fluorescence. In addition, a so-called "pseudospectral" analysis was developed, which made it possible to localize the intracellular distribution of the anti-cancer compound doxorubicin in yeast cells, used as a model of animal and human cells (Puchkov 2016b).

Computer software CalMorph was created for *morphometry* based on quantitative processing of fluorescence microscopy data on the intracellular localization of nuclei and actin labeled with the corresponding fluorophores along with parameters of the geometry of *S. cerevisiae* cells (Ohtani et al. 2004). A database of morphological data of haploid cells with non-lethal mutations was developed using this software (Ohya et al. 2005). It was used to study the relationship of the functions of certain genes with morphological features (Nogami et al. 2007; Ghanegolmohammadi et al. 2017). Later, this approach was upgraded by supplementing the fluorescence microscopy data on the localization of mitochondria, vacuoles, endoplasmic reticulum, Golgi apparatus, endosomes, veretin and septin labeled with various fluorophores and FPs (Negishi et al. 2009). A morphometric software F-CalMorph similar to CalMorph was developed for the yeast *Schizosaccharomyces pombe* (Suzuki et al. 2006). The possibility of using morphometric indicators of the yeast form of fungi and artificial intelligence methods for the development of antifungal drugs was demonstrated (Gebre et al. 2015).

Methods of visualization and quantification of single RNA molecules have been developed (Femino et al. 1998), which were used for the *single molecule fluorescence* in situ *hybridization* (smFISH) on *S. cerevisiae* yeast. It was shown with smFISH that two isoforms of mRNA of one of the kinetochore proteins were synthesized at different stages of the cell cycle and exported from the nucleus to the cytoplasm (Chen et al. 2018). The kinetics of expression and intracellular distribution of mRNA of the STL1 and CTT1 genes in *S. cerevisiae* cells under osmotic shock were studied (Li and Neuert 2019).

High speed single-molecule tracking of functional Mig1 protein (Zn-containing transcription factor) was used to study the *dynamics of its binding* to the DNA of live *S. cerevisiae* cells. The obtained data made it possible to characterize the 3D distribution of the protein binding sites on DNA. Subsequent computer simulation allowed for the prediction of the 3D architecture of the genome (Wollman et al.2020).

Quantitative confocal microscopy (QCM) (Jonkman et al. 2014) and nanoscopy were used to study the *localization* of macromolecules, as well as the *dynamics* of intracellular processes in *S. pombe*. For example, QCM was used to measure the distances between the cluster of Chr1 genes, the cytoplasmic membrane and the polar body of the spindle depending on the growth conditions of the cells (Bjerling et al. 2012). The QCM and fPALM methods were used to study the structure and dynamics of assembly/dissociation of interphase nodules, precursors of the cytokinetic contractile ring (Laplante et al. 2016; Akamatsu et al. 2017). The mechanism of polymerization of actin filaments during clathrin-induced endocytosis were studied with PALM (Arasada et al. 2018). SIM enabled to identify the spatial interaction of the protein components of the polar spindle bodies during their duplication (Burns et al. 2015; Bestul et al. 2017).

Bacteria

A number of reviews on the use of various CFM methods in the studies of bacteria at the subcellular level have been published. They were focused, in particular, on the structure of the cytoskeleton and nucleoid (Yao and Carballido-López 2014); the dynamics of transcription (Stracy and Kapanidis 2017) and translation (Gahlmann and Moerner 2014); the dynamics of protein-DNA interaction (Uphoff 2016); DNA replication and repair (Li et al. 2018), the organization of secretion systems and intracellular compartmentalization (Schneider and Basler 2016); intracellular signaling and gene expression dynamics (Kentner and Sourjik 2010); spatial organization and life-style of pathogenic bacteria (Singh and Kenney 2021); mechanisms of action of antimicrobial compounds (Choi et al. 2016); as well as technical aspects (Haas et al. 2014) and prospects for using CFM in the study of intracellular biochemistry at the level of single molecules (Endesfelder 2019).

The development of new CFM methods for the studies of bacteria at subcellular level has many directions. The research is conducted to find new fluorophores in combination with various techniques of nanoscopy for specific tasks and bacterial species. The examples below illustrate this research.

To implement the STORM methodology on live *E. coli* cells, an original technique has been developed based on the creation of hybrid with "self labeling" proteins using the eukaryotic enzyme N-myristoyl transferase. Spirolactam derivatives of rhodamine, which can be "turned on/off" under the light of specific wavelength, were linked to the Tar and CheA chemotoxis proteins and the FtsZ and FtsA division proteins as fluorescent tags. This made it possible to localize these proteins in the cells using STORM with an accuracy of 15 and 30 nm in the radial and axial directions, respectively (Ho and Tirrell 2019).

The application of the method of labeling target proteins of live *Caulobacter crescentus* cells by attaching the so-called *fluorogen-activating peptides* was described (Szent-Gyorgyi et al. 2008). The dye malachite green was used as a fluorogen, and the peptide dL5 was used as a fluorogen-activating peptide (Szent-Gyorgyi et al. 2013). The complex of this peptide and dye had a well-pronounced fluorescence in the red region of the spectrum, photostability and high affinity of the components. Fluorescent images of three proteins labeled in this way in bacterial cells were obtained using STED. It was shown that the developed technique allows for a four-fold improvement in resolution compared to standard microscopy. In addition, it was noted that the addition of dL5 to one of the proteins (CreS) significantly less affected its structure compared to FPs (Saurabh et al. 2016).

The possibility of improving the brightness and stability of FP fluorescence in *Vibrio cholerae* cells using *plasmon resonance* phenomenon was investigated. For this purpose, a special substrate of gold nanoparticles was created, on which the cells with the TcpP virulence regulator protein labeled with mCherry FP were immobilized. The excitation of the fluorescence of hybrid proteins in such a system made it possible to increase the number of recorded fluorescence photons before the onset of fluorescence decay. The conclusion was made about the possibility of using plasmon-enhanced fluorescence for localization of individual protein molecules in live bacterial cells in general and in *V. cholerae*, in particular (Flynn et al. 2016).

Another CFM technique based on fPALM was developed to study the mutual disposition of proteins of the polar complex associated with chemotaxis and flagella in *V. cholerae* cells. It allowed for more precise localization of target proteins labeled with FPs. This was achieved by simultaneous recording of the fluorescent image of both the cell contours and target proteins, due to the labeling of the cytoplasmic membrane, periplasm and polar complex proteins with specific photoswitchable FPs. Special software have been created that enabled processing fPALM data simultaneously on the structure of at least 100 cells and on more than 10,000 molecules of target proteins. It was found with this approach, in particular, that one of the proteins of the polar complex tends to be strictly localized in the site close to the internal bend of the cytoplasmic membrane (Altinoglu et al. 2019).

Spatiotemporal *image correlation spectroscopy* (Hebert et al. 2005) was used to characterize the movement of protein molecules in live *E. coli* cells. This technique made it possible to overcome the problem of blurring of the fluorescent image caused by the difference in the speeds of image recording and the movement of molecules. The approach was based on the exact determination of the width of the correlation function by direct calculation of the variance of the correlation function instead of the commonly used Gaussian fitting procedure. The effectiveness of the technique was confirmed by assessing the diffusion parameters of the photoswitchable mMaple3 fluorescent protein in live *E. coli* cells (Rowland et al. 2016).

The potentials of CFM for studying yeasts and bacteria at the subcellular level are illustrated but not restricted by the examples presented in the Table 1.

Conclusions and future perspectives

CFM has opened up two fundamentally new opportunities for studying microorganisms. *The first* is the quantitative measurement of the fluorescence parameters of the targeted fluorescent probes and tags in association with certain structures of individual cells. *The second* is the expansion of the boundaries of visualization/resolution of intracellular components beyond the" diffraction limit" of light microscopy into the nanometer

Table 1 Selected examples of the CFM use in the studies of single microbial cells at the subcellular level

Studied microorganisms		Technique	Analyzed features	References
Yeasts	*Saccharomyces cerevisiae*	Wide-field fluorescence microscopy with ImageJ software image analysis	Viscosity in the vacuoles Intracellular localization of an anti-cancer compound	Puchkov (2016b)
		Wide-field fluorescence microscopy with CalMorph software image analysis	Cell morphology with localization of intracellular organelles	Ohtani et al. (2004), Negishi et al. (2009)
		Single molecule fluorescence in situ hybridization (smFISH)	mRNA synthesis and localization	Chen et al. (2018) and Li and Neuert (2019)
		Astigmatism microscopy imaging and STORM with custom software image analysis	Dynamics of protein binding to DNA	Wollman et al. (2020)
		Single-molecule total internal reflection fluorescence microscopy with Micro-Manager software image analysis; wide-field microscopy with custom Python software image analysis	Dynamics of protein binding to DNA	Donovan et al. (2019)
	Schizosaccharomyces pombe	Wide-field fluorescence microscopy with F-CalMorph software image analysis	Cell morphology with localization of intracellular organelles	Suzuki et al. (2006)
		QCM	Distances between the cluster of Chr1 genes, the cytoplasmic membrane and the polar body of the spindle	Bjerling et al. (2012)
		QCM and fPALM	Structure and dynamics of assembly/dissociation of interphase nodules	Laplante et al. (2016) and Akamatsu et al. (2017)
		fPALM	Polymerization of actin filaments	Arasada et al. (2018)
		SIM	Spatial interaction of the protein components of the polar spindle bodies	Burns et al. (2015) and Bestul et al. (2017)
		fPALM	Nanoscale architecture of the contractile ring	McDonald et al. (2017)
Bacteria	*Bacillus subtilis*	fPALM	Visualization of actin-like proteins	Yao and Carballido-López (2014)
	Escherichia coli	fPALM	Organization of the nucleoid-associated proteins	Wang et al. (2011)
		STORM	Supramolecular protein assembly	Jessop et al. (2021)
		STORM	Intracellular protin localization	Ho and Tirrell (2019)
	Caulobacter crescentus	fPALM	Dynamics, localization and superstructure of proteins	Biteen et al. (2011)
	Salmonella enterica	fPALM	DNA–protein interaction	Liew et al. (2019)
	Vibrio cholerae	QCM and 3D STORM	Molecular architecture of biofilm	Berk et al. (2012)
		fPALM	Single-molecule tracking of proteins	Haas et al. (2015)
		fPALM	Protein localization	Altinoglu et al.2019
	Streptococcus pneumoniae	3D SIM	Visualization of septum formation with high resolution	Wheeler et al. (2011)

Table 1 (continued)

Studied microorganisms		Technique	Analyzed features	References
Archaea	*Haloferax volcanii*	Wide-field fluorescence microscopy with ImageJ image analysis and QCM	Localization and dynamics of DNA-binding proteins	Lestini et al. (2013)
		Wide-field fluorescence microscopy with Fiji software and SIM with softWoRx software image analysis	Localization and dynamics of DNA- binding proteins	Delpech et al. (2018)
		STORM	Single-molecule localization of proteins	Turkowyd et al. (2020)
	Sulfolobus islandicus	Wide-field fluorescence microscopy with Volocity 6.3 software image analysis	Localization and dynamics of DNA- binding proteins	Martínez-Alvarez et al. (2017)

range. This enables to obtain unique information about the localization and dynamics of intracellular processes at the molecular level.

CFM makes it possible to combine quantitative fluorescence measurements with visual analysis, as well as to relate the parameters of fluorescence with the structural elements of cells. It is also important that many CFM techniques can be implemented on live cells. To date, many CFM-based methods have been developed to solve a wide range of problems related to the structural and functional organization of microbial cells.

All this determines a special place of CFM in the modern *analytical toolbox for single cell studies* of microorganisms (Puchkov 2021). This methodology is in continuous upgrading due to the development of new software, improvement of microscopes, as well as methods of fluorescent probing specific targets in the cells. The most promising ways of application of CFM in future will be those that combine this methodology with other methods for studying single cells of microorganisms, as well as genetics and molecular biology. Future perspectives of CFM are also associated with implementation of numerous analytical tools based on the phenomenon of fluorescence (Lakowicz 2006) for studies of single cells of microorganisms at the *subcellular level*.

Declarations

Conflict of interest The author declares that there is no conflict of interests.

Research involving human participants and animals The article does not contain data obtained in the course of animal and human studies.

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