Multimodal combinational holographic and fluorescence fluctuation microscopy to obtain spatial super-resolution

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Abstract. Ways of combination of holographic and super-resolution fluorescent techniques in the same optical scheme are described. The key parameters influencing achievement of maximum possible resolution are considered. The possibility to choose different fluorescence technic for different types of fluorophores without any optical scheme changes is presented. As a result in case of visualization of the samples, which transparent in optical band, three-dimensional super resolution is received that significantly expands possibilities of the noninvasive analysis of biological samples.

1. Introduction

Fluorescence microscopy allows three-dimensional investigation of living cell cultures and tissues. But the resolution in such sort of systems is limited by optical Abbe’s diffraction limit. Other techniques, like atomic force microscopy, electron microscopy and scanning tunnelling microscopy achieve molecular-level resolution, but are not fit for the live cells imaging. Last decades, the optical diffraction limit has been overcome by using a several new methods, pioneered by stimulated emission depletion (STED) [1], structured illumination microscopy (SIM) [2,3], image interference microscopy[4] and ground-state depletion[5], photo-activated localization microscopy (PALM)[6] and (fPALM) [7], stochastic optical reconstruction microscopy (STORM) [8], and variants thereof [9,10] also improved stochastic techniques using photoswitchable probes. PALM and STORM techniques reach nanometre resolution, but with slow registration speed (minutes to hours). STED has got video frame rate but the method is extremely exacting of dyes and labelling procedures [11]. Recently, superresolution microscopy at 11 Hz has been exposed by using SIM, achieving a 2-fold increased longitudinal resolution[12]. All this approaches are able achieving three-dimensional super-resolution, but with the serious technical modifications to the microscope optical scheme[13-15].

All of these methods require special sample preparation techniques making these methods more elaborate and complicated than traditional fluorescence wide-field microscopy. One of the another superresolution techniques is bleaching/blinking assisted localization microscopy (BaLM). It relies on the intrinsic bleaching and blinking behaviours characteristic of commonly used fluorescent probes [16]. The main idea of this approach is that all fluorophores blink: at some instant of time they are exited and begin to shine and early or late they are bleached. And they do it independently of one another. This fact is the base of BaLM image series acquisition and data analysis (gSHRImP [17], 3-B [18]). This technique is suitable for all commonly used synthetic fluorescent dyes and genetically
expressed fluorescent proteins. But in the result of reconstruction researcher have only the most probable location of fluorophore with artificial brightness. Other methods based on fact, that the signal from object space is convolved with the point spread function (PSF) of the imaging apparatus. Digital image recording leads to a projection of the convolved signal onto a discrete set of points in space (pixels) and intensities. Thus the microscopy images are a vector of real data of finite length. Regularization techniques have been applied by Facciolo et al [19]. The power of the new incomplete Dykstra’s algorithm (ICD) is demonstrated by using it as preprocessor for the super-resolution optical fluctuation imaging (SOFI) method by Dertinger et al [20]. The negative side of this approach is, that the number of frames for SOFI applications has to be large enough to resolve the statistical properties of the fluorescence signal [21,22] and crucial for the feasibility of applying it as preprocessor. On the other hand, SOFI is not exacting to level of signal to noise and is suitable for endogen fluorophores.

Though BaLM and SOFI need a sufficient amount of images to resolve the statistical behaviour of the temporal fluctuations of the observed signal, its strength is that any of them can be combined with a variety of microscopy techniques without any modifying the experimental setup, but using proper methodologies and next data processing. That’s why, holographic scheme is suitable for use of these methods too. Application of holographic techniques for creation of the three-dimensional structure of biological samples is free of these limitations so it is the perspective direction in study of biological objects with achievement of ultrahigh three-dimensional and necessary time resolution.

We show optical scheme, which consist of off-axis digital holography and super resolution fluorescent setup. Such combination allows to research live biological objects dynamic changes. As a result of application of the holographic recording method and further reconstructing of phase and amplitude information of object, there was an opportunity to carry out visualization of the dynamic changes in live biological samples. The developmental results on the example of neuronal plasticity research were obtained. Application of holographic approach allows receiving ultrahigh sensitivity to longitudinal changes in optical length. But, transversal resolution is the same as in diffraction limited microscopy. For resolve of this restriction it is possible to combine holographic method with one of approaches to improve transversal resolution, which meets certain requirements. Depend of biological object we able choose data analysis method to correct reconstruction of sequence of fluorescent images.

2. Methods to be combined

2.1 Holography
Holography makes it possible to reconstruct variations of the light intensity and the phase increment during the passage through the transparent or semi-transparent object. Holographic reconstruction allows obtaining information on the three-dimensional structure of an object using only one hologram in the transverse plane. Holography is divided into digital and analog ones by the recording media and reconstruction approach. The main drawback of digital holography as compared with the analog one is a lower information capacity [23,24]. The main advantage of digital holography is high rate of registration and numerical processing of the results[23,24]. So, the digital recording type was chosen for combined scheme. In this case it is possible to record holographic video (a set of sequential holograms) with minimum possible delays between frames and then reconstruct them for the phase dynamics visualization. The recording rate is determined by the using digital matrix. The available time resolution is inversely proportional to a chosen active area of these digital detectors.

2.2 Bleaching/bleaking assisted localization microscopy
BaLM fluorescence analysis consists in excitation of fluorescence and registration and analysis of fluorophore blinking. Then consecutive frames are subtracted the each other. As a result of this operation, signals only from blinking or bleaching molecules stay on the difference images. So, there are images of separate molecules in the form of their point spread function (PSF). All difference elements that are less, than the estimated PSF size, aren't considered. It makes possible to determine
the location of individual fluorophore molecules with the diffraction limit. Processing consists in
dilation of the difference image in order to eliminate intensity dips [25]. For the pixels which do not
undergo dilation, i.e., retain their initial intensity, the mean intensity within a circle of several pixels
should be calculated. The obtained mean value is compared with a certain threshold value determined
by the noise of the initial data. If the mean value exceeds a specified threshold, then this diffraction
spot is approximated by a two-dimensional Gaussian distribution using the Levenberg–Marquardt
algorithm [26]. The coordinates of the centre of this function are assumed to be the desired position of
the molecule. Using the Gaussian filter, the coordinates of the found centre are converted into a new
image with resolution, that is already beyond the diffraction limit[27]. The filter width corresponding
to the resolution of the new image is mainly determined by noise. This algorithm appears to be
resistant to additive noise with an amplitude of up to 40% of the maximum signal amplitude. Under
these conditions, pixelization (efficient resolution) of the new image is performed with density
exceeding the initial one by 10 times.

2.3 Super-resolution optical fluctuation imaging
A different approach is SOFI (super-resolution optical fluctuation imaging) [20,28], which also
improve resolution due to the temporal behaviour of the imaged object signal. SOFI does not require
controlled or synchronized photoactivation, and relies on the independent stochastic fluctuations of the
emitters. It works without the need for sophisticated electronics or special acquisition setup. The main
aspect of SOFI is that the fluorescent label has to exhibit at least two different emission states.
Different emitters have to switch between states over and over again and independently from each
other in a stochastic manner. A SOFI image pixel value (of the order n) is received from the n-th
order cumulant of the original pixel time series. Using standard imaging applications the signal in a
pixel is represented by the superposition of the fluorescence coming from different, closely emitters.
The n-th order cumulant is quantity linked to the n-th-order correlation function. It's filtering the signal
on the base of its fluctuations and only highly correlated fluctuations are left over.

3. Combination of the holographic and superresolution fluorescent microscopy in the same
optical setup
We show optical scheme consist of off-axis digital holography and super resolution fluorescent setup
on Figure 1. This combination allows to research live biological objects dynamic changes. Optical
scheme consist of several basic elements: 1- a laser, 2 and 3- a collimator lenses, 4, 4'- beam splitters,
5 and 5'- mirrors, 6- Petri dish with cells, 7 – objective, 8- beam splitter cube, 9 – filter, 10 and 10'-
digital array matrixes for holographic and fluorescent channels.
In this case, reference beam, which is coherent with the object beam, is introduced at the optimum convergence angle from the outside[24]. For combination we have to upgrade standard off-axis holography scheme to divide object beam on two parts by beam splitter 4’. First of resulting wave is an object beam and interfering with reference beam on one digital detector 10. It should be mentioned, that the intensity of object and reference beam should not differ significantly. “VEC-545” digital cameras based on the complementary metal–oxide–semiconductor structure (CMOS camera) with a matrix diagonal of 0.4 inch, 2592×1944 pixels, and a 2.2μm pixel dimension were used. Second beam after filtering excitation wavelength is registering by another digital detector 10’ - Flea-3 8,8 megapixel CMOS camera with 1.55 um pixel size. The coherent laser beam was used both for recording of holograms and for fluorescence excitation. He–Ne laser 1 with a wavelength of 633 nm was used, which could be replaced by a laser operated at any other wavelength, depending on the tasks assigned. As for a holography, illumination power influences only to exposure time. But for the super resolution fluorescent microscopy is extremely important to select the source of light type and parameters. And it’s necessary to select optimum excitation wavelength, depending on an absorption spectrum characteristic of a specific fluorophore.

4 Features of using

4.1 Excitation parameters in case of Bleaching/blinking assisted localization microscopy

The most important for BaLm method to estimate the excitation light power correctly[29]. Record of a frame series with blinking is necessary for the localization of separate fluorophore molecules accurately, i.e. it is necessary to detect acts of separate molecules light emission on all field of view in order to define their centres. Therefore parameters of a fluorophore, exciting light, and also registering digital matrix shall be factored into in calculation of optimum excitation power. First of all it is necessary to select correct excitation wavelength $\lambda$. It has to be guided by excitation spectrum of
visualizing fluorophore. Fluorescence from the molecules which are in "the diffraction volume" $V_d$, depending of $\lambda$ and numerical aperture of a lens $NA$, will get to a diffraction spot from which all information registers. Knowing concentration $n$ of a fluorophore in a sample, it is possible to calculate quantity of molecules of $N$ in volume of excitation. From all photons of excitation which propagate through the volume only part of them will be absorbed by molecules of a fluorophore. It is proportional to the fluorophore absorption section $\sigma$. Also it is necessary, that only one molecule of a fluorophore would be excited in a diffraction volume. Power of exciting light will be equal to sum energy of photons radiated during time $t$ between two subsequent frames of record with $f$ frequency, divided on this time. It is able to calculate the number of photons, flying from a source through the focal diffraction volume during time $t$, respectively. Also we should keep in mind, that another fluorophores shouldn’t be excited around a distance equal to PSF diameter near the PSF of blinked one. Then the optimal excitation power is

$$P = \frac{3fhcSN\lambda^4}{16\theta_F^4\pi^2}, \quad (1)$$

where $\theta_F$ is the quantum yield of a fluorophore. So, optimal excitation wavelength power depends of several different parameters. Concentration of $n$ is controlled by dye loading. Parameters $\theta_F, \lambda$ and $\sigma$ depend on the selected fluorophore. Value of $NA$ depends on the selected lens. And value of a beam area of $S$ is defined by the chosen collimator. Digital camera influence on frame rate frequency $f$. Thus, for receiving cross ultrahigh resolution, possible to calculate the necessary laser line power for correct operation of the BaLM fluorescent analysis. Using a formula (1) it’s able to select optimum excitation light power in each experiment.

4.2 Reconstruction

For holography, the image of object is reconstructed numerically by double Fourier transformation algorithm with filtering in the frequency area [24]. As a result of application of the holographic recording method and further reconstructing of phase and amplitude information of object, there is an opportunity to carry out visualization of the dynamic changes in live biological samples. For fluorescent channel is able to use BaLM or SOFI method. It depends of fluorophores (endogenous or exogenous), necessary speed of reconstruction, period of object changes and type of reconstructed data.

5. Discussion

In contrast to STORM (PALM) method SOFI approach allows to develop overlapping fluorophores and not requires single photon sensitivity. So, unlike above we used not scientific CMOS camera, but choose with the smallest pixel size one. The most important aspect of BaLM is that the fluorophore molecule images can overlap due to diffraction blur, but lighting up and extinguish events during the inter-frame time gap should be located at a distance exceeding the diffraction limit. The number of blinking molecules is determined by the excitation intensity, absorption cross section, the quantum efficiency and the amount of marker per volume unit. SOFI is devoid of this disadvantages, but in case of fluorophores with difference brightness able to lost the faintest signal during increasing cumulant order. So, we are able to choose different approach for different situation without any changes in optical setup, excitation or recording parameters. Therefore, by combining these advantages with the holographic microscopy method, one can achieve very high sensitivity to optical path difference along Z-axis with superresolution in XY plane due to fluorescence fluctuation analysis algorithms. Moreover since different approaches are used in the combination they not only improve resolution but reveal different sample properties making possible to get complementary information, e.g. structural and functional imaging simultaneously with high sensitivity and space-temporal resolution.
Acknowledgments
This work has been financially supported by Russian Foundation for Basic Research (grant No. 15-42-02658).

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