Holographic methods for phase microscopic objects study

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This chapter contains a short review of classical and holographic methods of microscopy for phase microscopic objects visualization, and their possibility for 3D imaging. The phase-, interference-, and polarization contrast methods are considered. The description of the digital holographic interference microscope (DHIM) and the experimental results of its application for phase microobjects study are presented.

Keywords: phase microobject; holographic microscopy; 3D imaging; digital holographic interference microscope; phase contrast; interference contrast; polarization contrast.

1. Introduction

The most of microobjects are phase microobjects. Phase microobjects do not change the intensity of the radiation transmitted through them, they insert only phase increments, and are inaccessible to direct observation by optical microscope. They are cells and tissues of human organism, crystals, thin films, etc. To observe such phase microobjects, phase increments, inserted by them into the transmitted wave, must be converted into intensity changes. It is necessary to use special methods for phase microobjects visualization. The two main methods were proposed: the phase-contrast and interference contrast methods. For the first time the problem of realizing phase-contrast was solved by F. Zernike in 1934 [1]. For the proposed phase-contrast method and the phase-contrast microscope he won Nobel Prize in Physics in 1953. The first interference-contrast microscope was described by M. Sagnas in 1911 [2]. Since there a lot of interference microscopes were proposed. They mainly differ by the method of beam splitting and combining.

Phase-contrast microscopes are widely used for phase microobjects visualization. But they allow only 2D imaging of the microobjects, measurement of their thicknesses is impossible. Interference microscopes give the possibility of quantitative measurements, but they are mainly used only for thin films thickness measurements. The problem of 3D visualization of phase micro objects has not been set and solved in classical microscopy. Till recently, electron microscopy was the only method for the 3D imaging of microobjects. However, this high-resolution method is «destructive». Long preliminary treatment of the sample is needed; this renders study of native cells impossible. In addition, such a treatment affects cells, and the reliability of the results of electron microscopy can be debated.

Improving resolution of electron microscope, D. Gabor proposed the method of holography (Nobel Prize in Physics in 1971) [3]. Though Gabor’s idea has not realized in microscopy, the development of holography as the method for the record and reconstruction of the phase and the amplitude of a wave resulted in the appearance of holographic analogs of the classical methods. They are holographic phase-contrast method, the method of holographic microinterferometry, and polarization contrast method [4–10]. The first holographic interference microscope, which realizes the methods, was created at the Laboratory of Holography, Kharkov National University, Ukraine. For the first time holographic addition and subtraction of waves in an interference fringe for obtaining contrast images of phase microobjects was realized. The contrast images of blood erythrocytes in a bright and dark interference fringes and their interferograms were obtained using the microscope.

Application of the holographic methods has opened quantitatively new possibility in microscopy of phase microobjects. Now it was made possible not only to visualize phase microobjects, but also to measure their geometrical parameters using their interferograms.

The development of computers and the methods for the digital data processing has led to a new stage in microscopy. The problem of 3D visualization of phase microobjects has been solved by combining the holographic methods with the methods for digital image processing. The first digital holographic interference microscope (DHIM) which allows the real-time 3D imaging of phase microscopic objects and measurements of their geometrical parameters has been created on the base of the holographic interference microscope [11, 12]. The instrument integrates holographic micro-interferometer with digital processing of interferograms. For the first time it has become possible to obtain 3D images of native cells. The first 3D images of the native human blood erythrocytes were obtained using the DHIM in 1998 [13]. Since there the DHIM is successfully used for study of different medical and technical microobjects [19–32].

In this chapter the review of classical and holographic methods, which are used for non-invasive visualization of phase microobjects, is given; and the layout of the digital holographic interference microscope is described. Experimental results of the DHIM application for 3D imaging of phase microobjects are presented.
2. Classical methods of phase microobjects visualization

2.1 Phase microobjects

When a transparent object, which is situated in the plane \( (x, y) \), is illuminated by the plane monochromatic wave, then the transmittance function \( F(x, y) \) of the object is defined by the relation

\[
F(x, y) = \frac{V(x, y)}{V_0(x, y)},
\]

where \( V_0(x, y) \) is the wave in the absence of the object; \( V(x, y) \) is the wave in the present of the object. In general, this function is complex, because the object can insert amplitude and phase changes into the light wave, transmitted through it. When the object changes only the amplitude of the wave, i.e. \( \arg F = 0 \), one can speak about the «amplitude» object. If the object change only the phase of the wave transmitted through it, i.e. \( |F| = 1 \), then we speak about «phase» object.

So, the transmittance function of the phase object has the form

\[
F(x, y) = e^{i\phi(x,y)}. \tag{2}
\]

where \( \phi(x,y) \) is the phase increment inserted by the phase object into the wave transmitted through it. If the phase microobject is observed through a microscope objective, then the intensity in the image plane of a microscope is not modulated by the phase relief of the object, and the phase microobject is invisible.

To visualize phase microscopic object, the phase increments, inserted by it into the transmitted light wave, must be converted into intensity changes. Interference is the phenomenon that the phase difference of two coherent interacting waves converts into changes of the resulting intensity. Two main methods based on the interference phenomenon are used in classical microscopy for the phase microscopic objects visualization. They are F. Zernike phase-contrast method and interference contrast method [2].

2.2 F. Zernike’s phase-contrast method

The first satisfactory wave theory of image formation by microscope was formulated by E. Abbe in 1873 [14, 15]. The theory is based on the idea that the microscopic image is the result of interference of the diffracted by the microobject wave and the zeroth-order wave (the direct, non-diffracted wave). These waves are spatially separated in the back focal plane of microobjective. The diffracted wave is shifted in phase by \( \frac{\pi}{2} \) relative to the phase of the zeroth-order wave, and has low intensity by comparison with the intensity of the zeroth-order wave.

Using E. Abbe’s theory as a basis, F. Zernike proposed a method of phase microobject visualization that became known as phase-contrast method [1]. The essence of the method is that it is possible to influence on the diffracted and direct waves independently, changing their phase relationship. So, a special phase plate, that retards or advances the phase of the zeroth-order wave by \( \frac{\pi}{2} \), is placed in zeroth order of diffraction in the microscope. The effect of the phase plate is described by the transmission function:

\[
A = a \cdot e^{i\frac{\pi}{2}}; a \leq 1, \tag{3}
\]

where \( a \) is the transmission coefficient of the phase plate., \( \frac{\pi}{2} \) is the phase increment inserted by this plate.

As a result, a phase difference equal 0 or \( \pi \) is created between the zeroth-order wave and the diffracted wave. The waves in the former case are in phase (addition of waves), and this strengthens the intensity of the image by comparison with the background. The waves in the latter case are anti-phased (subtraction of waves), and this weakens the intensity of the image by comparison with the background. If the phase increment \( \phi(x,y) \) inserted by the microobject into the transmitted light wave is small, the intensity in the image plane of microscope has the form [15]:

\[
I(x',y') = |c|^2 \cdot \left( a^2 \pm 2a\phi(x,y) \right), \tag{4}
\]

where \( |c|^2 \) is the intensity of the zeroth-order wave (non-diffracted wave).

Thus, in Zernike’s phase-contrast method, the phase increments inserted by the microobject into the wave transmitted through it are converted into intensity changes in its image. Waves of different intensities interact in the phase-contrast method. Therefore, adding or subtracting the waves does not provide high image contrast, and consequently the
method does not achieve high sensitivity. Absorbing phase plate \((\alpha < 0)\) are used to increase the image contrast, increasing the sensitivity of the method while reducing the overall illumination.

The phase-contrast method is based on the possibility of separately acting on the direct light without changing the light diffracted by the microobject. Thus, the phase-contrast method operates the more successfully, the smaller phase microobjects in optical thickness and dimensions.

Phase-contrast microscopes are widely used for phase microobject study in biology and medicine.

2.3 Interference contrast method

This method is also based on the phenomenon of interference of two waves. But in this case phase microobjects become visible due to the interference of two coherent waves of equal intensity, one of which passes through the microobject under study. Figure 1 shows the principle layout of the classical two-beam interferometer.

![Fig. 1](image)

The two compared waves are formed simultaneously, but propagate in different ways, and then they are combined and interfere. The interference contrast method can be realized in two main variants: interferometry in an infinitely wide fringe and interferometry in fringes of finite width [7].

The interferometry in an infinitely wide fringe solves the same problem as the phase-contrast microscopy. The intensity in the image of a microobject is modulated by the phase increments inserted by the microobject into the transmitted wave.

In the method of interferometry in fringes of finite width the pattern of interference fringes is modulated by the phase increments.

However, the phase-contrast and interference contrast methods are based on the interference of waves transmitted through a phase microobject and bypassing it, they have the difference that waves of equal intensity interact in the interference-contrast method. This makes it possible to maximize the image contrast and consequently the sensitivity of the method. Theoretically, the interference–contrast method is more efficient for phase microobject study than the method of phase–contrast. However, the advantages of the interference-contrast method are reduced by the complexity of the apparatus and difficulty of adjusting the interferometer. An especially complex problem in classical interferometry is the problem of obtaining two identical wave fronts.

There are several forms of classical interference microscopes [2]. They differ mainly in the method of separating and combining the light beams. In this case, the implementation of the various methods of interferometry requires separate devices.

The classical microinterferometers are mainly used only for study of thin films and measurements of their thicknesses.

2.4 Polarization contrast method

All materials can be divided into two classes: isotropic and anisotropic. Isotropic materials, which include liquids, gases, glasses and cubic crystals, demonstrate the same optical properties in all directions. They have only one refractive index, the state of the polarization of the light passing through them is not changed. Anisotropic materials, which include the majority of all solid substances (for example, crystals, soft tissues, etc.) have different optical and mechanical properties in different directions. Anisotropic materials change the polarization of light passing through them. This makes it possible to visualize such phase microscopic objects using the polarization contrast method. Figure 2 shows the layout of the classical polarization microscope.

![Fig. 2](image)

The microscope must be equipped by two polarizers. The first polarizer is positioned in the light path somewhere before the specimen. The second polarizer (which is called analyzer) is placed in the optical pathway between the microscope objective and observation camera. The contrast of the image arises from the interaction of the linear polarized light entering from the polarizer with the optically anisotropic specimen. Optical anisotropy manifests itself basically in the form of birefringence (double refraction). Birefringent specimens act as beam splitters and divide a ray...
of plane-polarized light into two individual rays. One of the rays follows the ordinary refraction laws, and so it is called the ordinary ray. The second one does not obey the law and is called the extraordinary ray. They are polarized in mutually perpendicular planes. The velocities of these rays are different and vary with propagation direction through the specimen. The birefringence can be formalized by assigning two different refractive indices to the material for different polarizations. The birefringence magnitude is then defined by

\[ \Delta n = n_e - n_o, \]

where \( n_o \) and \( n_e \) are, correspondently, the refractive indices for polarizations which are perpendicular (ordinary) and parallel (extraordinary) to the axis of anisotropy. The two waves with mutually orthogonal polarizations propagating in one direction inside the crystal gain the phase difference due to the difference of the refractive indices. The phase difference \( \Delta \phi \) is equal:

\[ \Delta \phi = \frac{2\pi}{\lambda} t \Delta n, \]

where \( \lambda \) is the wavelength of the light being used, \( t \) is the thickness of the microobject. So, the phase difference of the waves depends also on the thickness of the specimen. These waves can not interfere because they have orthogonal polarizations. Though, behind the linear analyzer, which transmits only vibrations, which is parallel to its transmittance plane, interference of the ordinary and extraordinary waves occurs. When the polarizer and the analyzer are crossed, no light is transmitted through the optical system in the absence of the specimen. When the anisotropic specimen is put in front of the microscope objective, its bright image, which is the result of interference of the ordinary and extraordinary waves, can be observed on the dark background. The intensity of the image depends on the phase difference \( \Delta \phi \). If the thickness of the specimen is nonuniform, then the intensity distribution in its image depends on the thickness distribution. Thus, the method of polarization contrast can be used for visualization of anisotropic microobjects. The polarization contrast method improves the quality of images of anisotropic specimens when compared with other techniques such as phase-contrast, interference contrast etc. The method allows obtaining the information about the structure of materials which can not be available with any other optical microscopy technique. Polarization microscopes are widely used in practice for anisotropic material investigation.

The classical methods of phase microobjects visualization (phase-, interference- and polarization-contrast methods) are based on the interference phenomenon. The phase-contrast method is optimal for investigation of small and thin microobjects. Interference-contrast method has a few advantages in comparison with the phase-contrast method. It allows investigating the broader class of microobjects as to their sizes and refractive indices. Moreover, the interference-contrast method is more sensitive because it allows obtaining the maximal contrast of images of phase microobjects. But these advantages are neutralized by structural and operation complexity of the classical interferometers. The method of polarization contrast allows obtaining high quality images with maximal contrast when compared with other techniques, but it can be applied only for anisotropic phase microobject. These classical microscopy methods solve only the problem of phase microobjects 2D visualization. The problem of 3D imaging of phase microobjects has not even been set within the framework of classical microscopy.

### 3. Holographic methods for phase microobjects 3D imaging

Development of holography as the method which makes it possible to record and reconstruct the phase and the amplitude of an object wave has resulted in the possibility of using the method of interferometry in microscopy; and the holographic analogues of the classical microscopy methods for phase microobject visualization have appeared. They are holographic phase-contrast method [4, 5], and the method of holographic microinterferometry [6, 7], and the holographic polarization-contrast method [8–10]. Combining the holographic methods with the methods of digital image processing has opened qualitatively new possibilities in microscopy of phase microobjects. And so, the problem of 3D imaging of phase microobjects has been solved.

#### 3.1 Holographic phase-contrast method (the method of holographic subtraction and addition in an interference fringe)

As it was mentioned above, the interference-contrast method in an infinitely wide fringe is more efficient for visualization of phase microobjects than the Zernike's phase-contrast method. If the advantages of the method are neutralized in the classical microscopy by the difficulties of obtaining two identical waves and adjustment of the interferometer, the use of holography removes these problems. Two identical waves transmitted along the same path but in different points of time interfere in the holographic interferometer. One of these waves is recorded and reconstructed from a hologram. Because the wave recorded and reconstructed from the hologram is a copy of the initial object wave, the problem of obtaining the two identical object waves, which is the main problem of classical interferometry, is completely removed.
Let us consider the result of interference of two identical coherent waves, one of which is transmitted through the phase microobject. The intensity $I_{im}$ in the image of the microobject, and the intensity of background $I_{b}$ are determined by the main interference equation [15]:

$$
I_{im}(x', y') = 2I_0[1 + \cos(\Delta \phi + \varphi(x, y))],
$$

$$
I_{b}(x', y') = 2I_0[1 + \cos \Delta \phi].
$$

Here $I_0$ is the intensity of each of the interacting waves; $\Delta \phi$ is the phase difference of the waves; $\varphi(x, y)$ is the phase increment inserted by the microobject into the wave transmitted through it. Equations (7) show that the image intensity differs from the background intensity, and is modulated by the phase increment inserted by the microobject.

If the phase difference of the waves $\Delta \phi = 0$, the interfering waves are in phase (addition of waves). Then

$$
I_{im}(x', y') = 2I_0[1 + \cos \varphi(x, y)],
$$

$$
I_{b}(x', y') = 4I_0.
$$

So, in the case of addition of the waves the phase microobject is observed as dark on a bright background.

If the phase difference of the waves $\Delta \phi = \pi$; the interfering waves are anti-phased (subtraction of waves). Then

$$
I_{im}(x', y') = 2I_0[1 - \cos \varphi(x, y)],
$$

$$
I_{b}(x', y') = 0.
$$

So, in the case of wave subtraction the phase microobject is observed as bright on a dark background.

The minimal intensity in the image of the microobject in the case of the wave addition corresponds to the maximal intensity in the image in the case of the wave subtraction, and vice versa, so the contrast of the images is inverted.

As it follows from Eqs. (8) and (9), the phase increment is equal:

$$
\varphi(x, y) = \arccos[\pm(1 - \frac{I_{im}(x', y')}{2I_0})].
$$

In accordance with the Eq. (8), $I_0 = \frac{I_{bs}}{4}$. Then

$$
\varphi(x, y) = \arccos[\pm(1 - \frac{2I_{im}(x', y')}{I_{bs}})],
$$

where $I_{im}(x', y')$ is the intensity in the image of the microobject on its interferograms; $I_{bs}$ is the intensity of the background in the case of addition of waves; the «+» sign corresponds to the case of wave subtraction; the «–» sign corresponds to the case of wave addition. Thus, measurements of the intensities in the interferograms of the microobject and the intensity of the background make it possible to reconstruct the phase relief of the microobject under study.

The intensity variations on the interferogram of the phase microobject are caused by the phase increment to the transmitted wave through the entire thickness of the microobject:

$$
\varphi(x, y) = \frac{2\pi}{\lambda} \int_{z_1}^{z_2} \Delta n(x, y, z)dz,
$$

where $\Delta n(x, y, z)$ is the difference of the refractive indices of the microobject and the ambient medium. If the microobject has a homogeneous refractive-index distribution, i.e. $\Delta n(x, y, z) = const$, we get

$$
\varphi(x, y) = \frac{2\pi}{\lambda} t(x, y)\Delta n,
$$

where $t(x, y) = z_2 - z_1$ is the thickness of the microobject in the point $(x, y)$. From this we obtain:

$$
t(x, y) = \varphi(x, y) \frac{\lambda}{2\pi\Delta n}.
$$

Taking into consideration Eq. (11), we get the equation for the thickness of the phase microobject in the point $(x, y)$:

$$
t(x, y) = \frac{\lambda}{2\pi\Delta n} \arccos[\pm(1 - \frac{2I_{im}(x', y')}{I_{bs}})],
$$
where $\lambda$ is the wavelength of the radiation being used, $\Delta n$ is the difference of the refractive indices of the microobject and ambient medium, $I_{mc}(x', y')$ is the intensity in the corresponding point on the interferogram of the microobject, $I_{bc}$ is the background intensity when the waves are added. The $\leftrightarrow$ sign corresponds to the case of subtraction of waves, and the $\leftrightarrow$ sign corresponds to addition of waves.

Thus, the method of holographic addition and subtraction of waves makes it possible to determine physical thickness of the phase microobjects in every point and reconstruct its 3D image.

The algorithm for computer reconstruction of the 3D image of a phase microobject by its interferogram regards the array of values $t(x, y)$ as an array of values of the third coordinate (the thickness).

However, because of certain difficulties, the holographic phase-contrast has not been used for obtaining images of phase microobjects. The difficulty consisted of providing the holographic addition and subtraction of the waves. This problem is easy to solve theoretically. The possibility of subtracting waves for obtaining a difference image was already predicted in the early papers of D. Gabor. The phase of the wave recorded and reconstructed from an amplitude hologram (the negative of the initial interference pattern), is shifted by $\pi$ relative to the phase of the initial wave. Therefore, the object wave and its anti-phased copy reconstructed from the hologram must quench each other when they are observed simultaneously (subtraction of waves). If a phase microobject is placed in one of the waves, then its bright image can be observed on the dark background. Such holographic subtraction can be realized in the real-time interferometry. On the other hand, when the unperturbed object wave and the object wave perturbed by a microobject are recorded on a single hologram, then the addition of waves is observed, because the phases of the both waves will be shifted by $\pi$ relative to the phase of the initial wave. And a dark image of the microobject can be observed on the bright background. So, the holographic addition of waves can be realized by the method of two-exposure interferometry. However, because of phase shifts that arise when the holographic emulsion is processed, and the very high sensitivity of the method to vibrations, it was not possible to obtain addition and subtraction of the waves in such an experiment. And the problem of obtaining in-phase and anti-phase waves remained open, like the problem of accomplishing phase contrast in holographic microscopy.

The problem of realizing phase contrast was solved in 1985 [4, 5], when the method of holographic addition and subtraction of waves in an interference fringe was proposed. The proposed method was called the holographic phase-contrast method. In this method the phase difference of waves, required for realizing subtraction or addition of the waves in an interference fringe is obtained by creating a small angle between the waves, so that the period of the resulting interference pattern significantly exceeds the size of the image of the microobject. When this is done, the conditions for waves to be anti-phase (subtraction of the waves) are automatically created within a dark interference fringe, and bright images of the phase microobjects can be observed on a dark background; while the conditions for the wave to be in phase (addition of the waves) are created within a bright interference fringe, and dark images of the microobjects can be observed on a bright background. Figures 3 show the first phase-contrast images of phase microobjects (native human blood erythrocytes), and their 3D images obtained by the holographic phase-contrast method using the DHIM [4, 5, 16].

![Fig. 3 Holographic phase-contrast images of native human blood erythrocytes in the cases of subtraction (a) and addition of waves (b), and their 3D images (c) obtained using the DHIM.](image-url)
3.2 The method of holographic interferometry in fringes of finite width

In the method of holographic addition and subtraction of waves the phase increments, inserted by the microobject into the wave transmitted through it, modulate the intensity in the microobject image, and it becomes visible (Fig.3). In the method of interferometry in fringes of finite width the system of interference fringes is modulated by the phase increments inserted by the microobject, and this manifests itself in deviations of the interference fringes. The method involves the interference of two waves with a certain angle $\theta$ between them. In this case instead of homogeneous field, a line field is obtained, with uniform alternation of intensity maxima and minima. The period of the interference pattern $T$ is equal:

$$T = \frac{\lambda}{\sin(\theta/2)},$$  \hspace{1cm} (16)

where $\lambda$ is the wavelength of the light being used.

When a phase microobject is situated in one of the waves, the intensity distributions $I_{\text{m}}$ in the interferogram of the microobject and in the interferogram of the background $I_{\text{b}}$ have the form [7]:

$$I_{\text{m}}(x',y') = 2I_0\left[1 + \cos\left(\Delta \phi + \phi(x,y) - \frac{2\pi x}{T}\right)\right],$$

$$I_{\text{b}}(x',y') = 2I_0\left[1 + \cos\left(\Delta \phi - \frac{2\pi x}{T}\right)\right].$$  \hspace{1cm} (17)

where $I_0$ is the intensity of each of the interacting waves, $\Delta \phi$ is the phase difference of the interacting waves, $\phi(x,y)$ is the phase increment inserted by the microobject into the transmitted wave, $x$ is the coordinate axis. As a result of the phase increments inserted by the microobject the interference picture is changed. That is manifested in deviations of the interference fringes in the image of the microobject. As it follows from equations (17), the deviation $h(x',y')$ of the interference fringe in some point $(x', y')$ in the interferogram of the microobject is proportional to the phase increment $\phi(x,y)$ inserted by the microobject in the corresponding point $(x, y)$:

$$h(x',y') = \frac{T\phi(x,y)}{2\pi}. \hspace{1cm} (18)$$

These deviations of the interference fringes show the phase silhouette of the microobject. So, measuring the deviations of the interference fringes $h(x',y')$ in the interferogram of the microobject, and the period $T$ of the background interference fringes, it is possible to determine the phase increments:

$$\phi(x,y) = \frac{2\pi h(x',y')}{T}. \hspace{1cm} (19)$$

Thus, the method of interferometry in fringes of finite width gives the possibility to visualize the phase microobject, and to measure its phase increments into the transmitted light wave.

If the microobject has a homogeneous refractive-index distribution, then it follows from Eq. (13) that the thickness $t(x,y)$ of the microobject at a point $(x, y)$ is equal:

$$t(x,y) = \frac{\lambda h(x', y')}{T\Delta n}, \hspace{1cm} (20)$$

where $h(x', y')$ is the deviation of the interference fringe in the corresponding point of the interferogram of the microobject, $T$ is the period of the system of reference interference fringes, $\lambda$ is the wavelength of the radiation being used, $\Delta n$ is the difference of refractive indices of the microobject and the ambient medium. In this case the method interferometry in fringes of finite widths allows one to determine the thickness of the microobject in every point, and reconstruct its 3D image.

The algorithm for the mathematical processing of the interferogram consists of interpreting the deviations of the interference fringes as the phase increments in accordance with the equation (19) for each point of the image, and an array is formed corresponding to the phase relief of the wave transmitted through the microobject. If the microobject has a homogeneous refractive-index distribution, and the phase increment is consequently a linear function of the thickness of the microobject, the algorithm for constructing a 3D image regards the array of values of the deviations $h(x', y')$ as an array of values of the thickness of the microobject in accordance with equation (20), followed by 3D visualization of the microobject, as well as the determination of its geometrical parameters. The problem of this type
has no exact mathematical solution. However, an approximation algorithm may be used, provided that the error that it introduces does not exceed the error introduced into the image by the fundamental deficiencies of the physical experiment (coherent noise, speckle structure, etc.). Moreover, the problem of reducing the influence of such noise on the image quality can also be solved by computer-processing the interferograms.

For the first time 3D images of phase microobjects were obtained by the interference contrast method when investigating the blood erythrocytes of patients suffering from hemolytic anemia in 1998 [13].

Figure 4 shows interferograms and reconstructed 3D images of the individual erythrocytes (a), and 3D image of the segment of the human blood smear (b) obtained using the DHIM.

The results make it possible to conclude that the method of holographic addition and subtraction of waves in an interference fringe (Fig.3) and the method of interferometry in fringes of finite width (Fig.4) can be effectively used for 3D visualization of phase microobjects. 3D images of phase microobjects not only give clear qualitative information concerning the shape of these objects, but they also make it possible to carry out various quantitative measurements of such geometrical parameters of these microobjects as linear size, the surface area, and the volume. When such microobjects as red blood cells are studied, the morphological parameters are important for medical diagnostics.

Each method has its own advantages and disadvantages for solving the problem of 3D visualization of phase microobjects.

When the method of holographic addition and subtraction in an interference fringe is used, fairly simple exact processing algorithms can be used for computer processing of the interferograms. However, since absolute values of intensities are used in this method, it is necessary for the transfer function of the image-recording unit to be linear in order to obtain adequate 3D imaging. At the stage of recording an interferogram this method imposes very high requirements on vibrational stability of the interferometer.

Though the problem of 3D imaging of phase microobjects when the method of holographic interferometry if fringes of finite width is used has no exact mathematical solution, there are no high requirements on the vibrational stability of the interferometer.

3.3 Holographic polarization contrast method

For visualization and 3D imaging of microobject that possess the anisotropy property the polarization contrast method can be used. Polarization contrast images can be obtained using a polarizer in front of the microobject and an analyzer (another polarizer) behind the microobjective in the microscope. The maximal contrast is obtained when the transmittance planes of the linear polarizers are orthogonal. The state of polarization of the reference beam can play the role of the analyzer in the case of hologram recording [8, 9].

As it follows from Eq. (6), the polarization-contrast image is modulated by the thickness of the microobject. This allows one to reconstruct its 3D image. Figure 5 shows the holographic polarization-contrast image of the biological microobject and its 3D image, and 3D image of the salt microcrystal reconstructed from the polarization-contrast holographic image, obtained using the DHIM. It is seen that polarization-contrast method improves quality of images in comparison with the phase and interference contrast methods due to removing coherent noise and filtering all others microobjects. Contrast images of anisotropic microobjects can be obtained with the maximal resolution.
4. Digital holographic interference microscope

The main disadvantage of the classical methods of phase microobjects visualization consists in the fact that realization of each method require a separate device. The advantage of holographic methods of phase microobjects study is that all the methods can be implemented in the same apparatus. The first digital holographic interference microscope (DHIM), which allows the real time 3D imaging of phase microobject and the quantitative measurements of their parameters, has been created in the Laboratory of Holography, Kharkov National University [11–13]. The DHIM is an easy-to-operate device that allows one to realize all holographic methods of phase microobjects investigation: the method of holographic subtraction and addition of waves, the method of interferometry in fringes of finite width, and the method of polarization contrast. The simple and one-axis construction of the DHIM makes it possible to use it as a usual optical microscope, a holographic microscope and a polarization microscope. The DHIM consists of three main units: holographic interference microscope (holographic microinterferometer), digital camera and computer. The interferograms and images of the microobjects under study obtained using the holographic microinterferometer are recorded by the digital camera. The digital interferograms and images are computer processed using the mathematical algorithms that makes it possible to reconstruct the 3D images of microobjects and to measure their geometrical parameters.

The optical layout of DHIM is shown in Figure 6.

A He-Ne laser that emits at 0.63 μm is used as a coherent radiation source. The radiation of laser 1 is divided into two beams by semitransparent mirror 5: the object beam and the reference beam. The object beam passes through microscope objective 11 and is directed to holographic plate (hologram) 12. The reference beam passes through collimator 9 and also is directed to hologram 12. Mirrors 3 and 4 are introduced into the system to rotate the rays. The reference beam plays an auxiliary role in the system. It is needed for recording and reconstructing the object wave from the hologram. A hologram of the «empty» object wave is recorded on holographic plate 12 in the absence of the specimen. The developed and fixed hologram is returned to its original position and serves as a permanent optical element of the microscope. The holographic microscope with the hologram becomes a holographic microinterferometer.

Because the wave reconstructed from the hologram is a replica of the real object wave, the problem of obtaining two identical wave fronts is completely removed. When the wave reconstructed from the hologram by means of the reference beam and the real object wave propagate simultaneously, their interference pattern can be observed by means of eyepiece 13.

Handling the interferometer is rather simple. It is implemented by small transversal shifting the hologram 12 from its initial position using the micrometer screws of the special holder. As a result, a certain angle $\theta$ is created between the interfering waves. This angle determines the period of interference fringes:

$$T = \frac{\lambda}{2\sin(\theta/2)},$$

where $\lambda$ is the wavelength of the laser radiation being used. If $\theta \approx 0$, then the holographic microinterferometer is adjusted on the infinitely broad fringes; and the holographic phase-contrast method can be used. If $\theta > 0$, then the holographic interferometer is adjusted on fringes of finite width, and the interference-contrast method can be realized. Changing the angle $\theta$, it is possible to control the period of the interference fringes. This allows obtaining the optimal quantity of interference fringes on images of microobjects of different sizes. Hologram 12 is placed between objective...
Fig. 6 Optical layout of a digital holographic interference microscope. 1 – laser; 2, 3 and 4 – mirrors; 5 – semitransparent mirror; 6, 7 and 8 – polarizers; 9 – collimator; 10 – test specimen; 11 – objective; 12 – hologram; 13 – eyepiece; 14 – image-recording unit.

11 and eyepiece 13. Since an unfocused image is recorded on the hologram, such a placement of the hologram makes it possible to increase the field of view of the microscope because of the possibility of displacing the eyepiece in a plane orthogonal to the observation direction, and to carry out additional focusing over the depth of the observed scene. When the test specimen 10 is placed in front of the microscope objective 11, its interferogram is observed.

If we work with different objectives, then a set of hologram for every objective is needed. The holographic interference microscope also can operate as usual optical microscope with laser illumination, and as a usual holographic microscope. The main shortcoming of holographic microscopy is the presence of coherent noise originating from different sources such as dust particles, scratches, and defects on and in optical elements and stray radiation. The coherent noise the more essential, the less the microobject under study. To obtain high quality of images, the quality of interferograms must be maximal. The quality of holograms and interferograms are determined by such a value as contrast. The contrast of the interference pattern is maximal and equal to 1, when the intensities and polarizations of the interfering waves are equal. To meet the conditions, polarizes 6, 7 and 8 are introduced into the system to equalize the intensities and polarizations of the interacting waves. The use of such polarization filtering in the holographic interference microscope makes it possible to reduce the influence of the coherent noise and to obtain interferograms of the microobjects of sizes equal to a few micrometers with the quantity of interference fringes on their images that is optimal for their 3D imaging. Moreover, with the polarizers, the microscope can operate as polarization microscope, and the method of polarization contrast can be used for anisotropic microobjects contrast imaging. Interferograms and images of the microobjects being investigated are recorded by digital camera 14.

A 40x0.65 objective and 10x eyepiece was used in the microscope. The holograms were recorded on PFG-03 plates. Developer GP-2 was used for processing the holograms.

Fig. 7 shows the images of native blood cells obtained using the DHIM. The DHIM was successfully used for investigation of blood erythrocytes 3D morphology in different diseases and under influence of different chemical and physical factors (ozone-therapy, radiation therapy), and thin films [19–32].

5. Conclusions

Combining the holographic methods with the methods for digital image processing has solved the problem of 3D visualization of phase microscopic objects. Creation of the digital holographic interference microscope (DHIM) allowing 3D real time imaging of phase microscopic objects and measurement of their geometrical parameters has led to a new stage in light microscopy. For the first time 3D imaging of untreated biological microscopic objects has become possible. Our pioneer results of DHIM application for investigation of medical and technical phase microobjects obviously testify that 3D imaging allows obtaining new and very important information on the microobjects under study. So, the DHIM can find application in medicine, biology and technique.
Fig. 7 3D image of the fragment of the human blood smear with a leukocyte and two erythrocytes (a). The fragment of the rabbit blood smear with pathological erythrocytes (b). The images are obtained using the DHIM.

References


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