Tomographic Interference Microscopy of Living Cells

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BIOGRAPHY
Gennady Vishnyakov received his engineering physics degree from Moscow Engineering Physics Institute in 1978. Since that time he has worked in the Russian Research Institute For Optical and Physical Measurements (VNIIOFI). Gennady received his PhD in 1985 for the thesis 'Tomographic methods in holographic interferometry' and his DSc in 2000 for the thesis 'Optical tomography of multidimensional objects'. His interests include optical tomography and 3D microscopy, optical data and image processing, holography, optical profilometry and interferometry. He is the author of more than 60 scientific papers and the book "Optical Tomography" (1989), in Russian.

INTRODUCTION
Research on the internal structure of living cells gives important information about morphology, spatial distribution of proteins and concentration of chemical drugs inside the cell. In microscopy three types of samples are usually investigated: fluorescent or emissive samples; stained or amplitude samples; and transparent or phase samples. For each type of sample a different method of image acquisition is required.

There are various approaches to microscopy of 3D fluorescent samples. In the first approach widefield microscopy and digital image processing are used [1-3]. The second approach is confocal scanning microscopy. The next approach was suggested in [4] in which the optical microscope is used as tomographic device for projection acquisition of fluorescent samples. Earlier in [5] we have offered a tomographic approach to the description 3D imaging properties of optical systems (see [5]).

Another wide class of samples in microscopy is phase and absorbing samples. The living cell is a phase object because it is transparent to optical radiation. For 3D absorbing or phase samples it is necessary to use the methods of computed tomography (CT). In this case the microscope is an optical setup for projection acquisition at various probing angles. The authors of [6] were one of the first who proposed to connect CT and microscopy. They used cone-beam microtomography for the reconstruction of absorption coefficient distribution of stratified mediums.

For the first time in [7] a microscope with oblique illumination for tomography of phase samples was suggested. Phase contrast was used for visualization of projections. Therefore this method is suitable to study phase objects with small gradients of refractive index.

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probing beam. Scanning the sample through the stationary or rotation or in tomography can be realized in two evaluation of the projection data. Angular probing (method) and interference contrast. DIC is a method of shearing interferometry. As mentioned above, an optically transparent sample is described by a 3D spatial distribution of refraction index. Therefore such a sample basically causes a phase shift of a probing light wave. In microscopy phase contrast microscopy the number of projections and the angle of view are limited. Possible techniques of angular probing in microscopy are shown in Fig. 1. The numerical apertures of objectives 3 and 5 restrict the maximal viewing angle.

The principle of angular probing shown in Fig 1a has been realized in our papers [8], and then in [9]. The technique in Fig 1b has been suggested in [10]. A scanner based on a digital micromirror device (DMD) placed in aperture diaphragm plane of a microscope is used. This technique is suitable only for small samples. The size of sample must be smaller than the diameter of the beam waist near the focal plane of the objective.

For large objects it is necessary to use the technique represented in Fig 1e. The original procedure of the collected data processing, patented by us [11], allows us to obtain parallel projections.

As mentioned above, an optically transparent sample is described by a 3D spatial distribution of refraction index. Therefore such a sample basically causes a phase shift of a probing light wave. In microscopy phase contrast and interference methods are used for its visualization. Phase contrast (proposed by Zernike) does not give the quantitative information about the phase of the optical wave. There are two interference methods: DIC (differential interference contrast or Nomarski method) and interference contrast.

DIC is a method of shearing interferometry. It allows the measuring of a wave phase gradient in a direction of shift. However interference fringes code the information about the phase gradient and it requires an operation of decoding. It is more preferable to use the interference method. Special objectives are available for the Michelson and Mirau methods. However these objectives have a small numerical aperture and a small angle of view. The Linnik microinterferometer has the largest numerical aperture. Therefore in the present work we have used it with the technique of angular probing as shown in Fig 1a.

Projection acquisition

For projection acquisition we used a Linnik interference microscope (Fig. 2). Tilted illumination of the sample was achieved by displacement of a point light source [12]. For automatic interferogram decoding a method of 4th phase steps (Carre algorithm) was used. In the Linnik microscope the sample was placed on a mirror, and therefore the probing light beam passes through the sample under different angles twice (Fig 3a). To reduce this problem to usual transmission tomography it is necessary to sum the sample and its own reflected image (Fig 3b).

After decoding an interference pattern, one obtains the system of equations:

$$\Delta N_i = \frac{2\pi}{\lambda} (n(s) - n_0) ds$$

where \(\Delta N_i\) is the phase difference for \(i\)th ray; \(B_i\) and \(C_i\) are points of its input and output in the sample; \(n(s)\) is the refractive index of sample along this ray; \(n_0\) is the refractive index of the environment medium, which is assumed to be constant; and \(\lambda\) is the wavelength of a light.

The set of these integrals along the parallel rays at a fixed angle is called a two-dimensional parallel projection. The 2D projection is characterized by polar angle \(\theta\) and the azimuthally angle \(\phi\) which determine the probing vector direction in 3D space.

Tomogram reconstruction algorithms and projection preprocessing

After discretization the tomographic problem is reduced to the system of linear algebraic equations [14]:

$$A g = f$$

Here \(g\) is a required vector, it represents the difference between the refraction indexes of sample and medium. \(J\) is the number of voxels (elementary volumes) where refraction index is reconstructed; \(f\) is a vector of the projection data. \(I\) is the number of integrals measured according to (1); and \(A\) is a matrix of dimension \(I \times J\) (projecting matrix). The element of a projecting matrix \(A_{ij}\) is usually defined as length of crossing ith ray with jth voxel.

For solving the system of equations (2) in [15] the combined algebraic algorithm cART with the a-priori information has been used:

$$g^{(n)} = \hat{f}^{(-1)} H_c^{(-1)} FA^{(-1)} g^{(n-1)}$$

where \(A\) is the realization the iteration of ART algorithm, \(H_c\) is an operator in Fourier space, which change the 3D Fourier transform to the central slice theorem [14], \(\hat{f}\) and \(\hat{g}\) are operators for a-priori information in spatial and frequency domain; and \(F\) is an operator for 3D Fourier transform.

In our case the operator \(\hat{g}\) has been replaced to 1 (i.e. the a-priori information in frequency domain was not used). In the structure of the operator \(\hat{f}\), in various combinations could be included: (a) averaging with window \(3 \times 3 \times 3\); (b) median filtering with the same window; (c) zeroing outside of two spheres; and (d) procedure of mirror reflection.

The first problem of projection preprocessing was the alignment of projection contours with the contours of the cell. This alignment has been carried out using the relationship between the first geometrical moment of 3D object and the first moments of its 2D pro-
plane \( Z = 0 \). After each iteration, negative values of reconstructed function were replaced by zero. The area of reconstructed function was two spheres of radius 0.5. The centers of spheres were located on axis \( Z \), a distance 0.5 from a mirror. Outside these spheres the reconstructed functions were replaced by zero. In Fig 5 a 3D reconstruction of lymphocyte internal structure as some density surfaces is demonstrated. The 3D tomogram resolution was 128 x 128 x 128 voxels.

**RESULTS**

Living lymphocytes were chosen as the phase samples. Cells were placed between a thin cover glass and a flat mirror in a physiological solution with refractive index 1.334. According to the conclusions of work [13] the ‘square’-scanning trajectory was used for projection acquisition. In this case the geometrical place of points of a probing vector angular coordinates on rectangular plane \((\varphi, \theta)\) coincides with points of a square grid. The angular range of probing for a 100x, 1.25 NA oil immersion objective was 90 degrees. The total number of 2D projections was 43. The projection resolution was 256 x 256 pixels and the real size of the projections was 23 x 23 \(\mu m\). All projections are shown in Fig 4.

For tomogram reconstruction the combined iterative algorithm cART was used. The reconstruction volume was a cube with length of the side equal to 2. All distances are given in dimensionless units. The mirror was placed in a plane \( Z = 0 \),..