MORPHOLOGY AND PATHOMORPHOLOGY

Study of Optical Parameters of the Nucleoli under the Effect of Transcription Inhibitors by Coherent Phase Microscopy

V. P. Tychinskii, A. V. Kretushev, I. V. Klemyashov, T. V. Vyshenskaya, N. A. Filippova*, N. T. Raikhlin*, and A. A. Shtil'*

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 142, No. 10, pp. 465-470, October, 2006
Original article submitted March 6, 2006

The use of coherent phase microscopy for online quantitative registration of nucleolar reaction to transcription inhibition is validated. Reduction of phase thickness of the nucleoli was detected during the first minutes of the experiment; 30 min after addition of the drug rarefaction zones predominated and areas of condensation were seen. These changes reflect the dynamics of disorders in the nucleolar ultrastructure during transcription inhibition.

Key Words: *coherent phase microscopy; electron microscopy; transcription; actinomycin D*

Life-time imaging of biological objects is a most important requirement to evaluation of their structure and physiological status. We developed a method of coherent phase microscopy (CPM) and demonstrated the possibility of using it for studies of metabolism in chloroplasts, spores, and isolated mitochondria [5,6]. Analysis of optically dense organelles in live cells is a new application of CPM [1]. The nucleoli are intranuclear compartments, in which ribosome biogenesis is realized, specifically, transcription of genes encoding ribosomal proteins; these organelles are dynamic formations. Structural and functional changes in the nucleoli are an important component of cell reaction to stress and cause their adaptation to external exposure or death [3]. Hence, online monitoring of the nucleoli will help to analyze cell response to homeostasis disorders.

We carried out a quantitative analysis of the dynamics of changes in optical parameters of the nucleoli in response to transcription disorders. These disorders can be caused by direct inhibitors of transcription or result from modification of other intracellular targets, causing secondary morphofunctional changes in the transcription system. We studied changes in optical characteristics of the nucleoli under the effect of DNA-binding preparations actinomycin D and olivomycin (direct inhibitors of gene transcription). Electron microscopy data served as the criteria of impairment of nucleolar structure under the effect of these agents.

MATERIALS AND METHODS

Transformed human HCT 116 (rectal cancer), MCF-7 (breast cancer) strains, and immortalized mouse fibroblasts (NIH 3T3 strain) were used in the study.

Moscow State Institute of Radioengineering, Electronics, and Automatic Machines; *N. N. Blokhin Cancer Research Center, Russian Academy of Medical Sciences, Moscow. ***Address for correspondence:*** vtych@yandex.ru. V. P. Tychinsky

0007-4888/06/1424-0481

The cells were cultured in DMEM with 5% FCS (BioWhittaker), 2 mM L-glutamine, 100 Units/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO_2 in a humid atmosphere. Cells in the logarithmic growth phase were used; the cells were inoculated onto slides so that the inoculation density on the day of the experiment was about 50%. Actinomycin D (Sigma) and olivomycin (kind gift from M. N. Preobrazhenskaya, G. F. Gauze Institute of New Antibiotics Research, Russian Academy of Medical Sciences) in a final concentration of 1 μM were added to the cultures for 5-60 min, the slides were removed, and directly examined in a coherent phase microscope. The effects of transcription inhibitors on breast cancer cells obtained during tumor biopsy were studied in a series of experiments (the material was collected by I. V. Reshetov, P. A. Hertzen Moscow Oncological Institute). Biopsy specimens were carefully crushed and suspended in physiological saline. Cell suspension was placed into microscopy chamber and subjected to CPM, after which the studied substances were added into the chamber and optical parameters of the nucleoli were analyzed.

Some cells were observed in the CPM optical channel, after which their topograms and phase thickness profiles were measured. Up to 20 cells selected at random in different visual fields were analyzed in each sample. The measurements were carried out by Eiriscan coherent phase microscope with He-Ne laser (λ=633 nm) as the source, LI-620 dissector for registration of interference signal, and electron block for computer presentation of phase images (image input velocity 1 msec/pixel, for Olympus objective 20×/0.4 image field 12 μ). Transverse size of the nucleolus was evaluated at the level of half-height with an accuracy of up to 100 nm, phase thickness (Δh) with an accuracy of up to 3 nm.

For studies of the nucleolar ultrastructure, the cell monolayer was washed in physiological saline, separated from the substrate with Versene, precipitated by short-term centrifugation, and fixed in 2.5% glutaraldehyde solution. Transmission electron microscopy was carried out by the standard method. The preparations were examined under a JEM-1200 EX-II microscope.

RESULTS

The CPM philosophy (Fig. 1, *a*) consists in measurement of local optical path-length difference (optical thickness) $h(x,y)$, related to the refraction parameters of the object $n(x,y,z)$ and environment n_0:

$$h(x,y)=\int[n(x,y,z)-n_0]dz.$$

For interpretation of the results, the phase thickness profile parameters $h(x)$ were used: its maximum Δh value and transverse size or d diameter of the structural element. Physical model is presented (Fig. 1, *a*), where $n(x,y,z)$ is the object refraction coefficient, H physical (geometrical) thickness, and n_0 environment refraction coefficient. Optical path-length difference $h(x,y)$ in the object image and the maximum path-length difference value depend on the physical thickness and difference of the object and environment refraction coefficients $\Delta h= H[<n(x,y,z)>-n_0]$. Optically more dense nucleolus in the phase image of the cell (topogram; Fig. 1, *b*) was identified by local enlargement of the seeming (phase) thickness of the nucleus. The nucleolus refraction dn, determining its contrast in the phase image, depended on the difference of the refraction means $<n(x,y,z)>$ and the nucleus $<n_N>$ $\Delta n=\Delta h/H= <n(x,y,z)>-<n_N>\approx\Delta h/d$ in the approximation of the nucleolus spherical shape ($H\approx d$).

Three-dimensional images of the nucleolus and adjacent nuclear areas, obtained in analysis of individual cells by the CPM method, are presented (Fig. 1, *b*, *d*). The nucleolus clearly discernible in control (intact) HCT 116 cells as optically dense intranuclear formation (Fig. 1, *b*) becomes flat and optically less compact as early as just 5 min after addition of the transcription inhibitor (actinomycin D; Fig. 1, *c*). Thirty minutes after addition of actinomycin D the nucleolus had, along with loosened areas, local condensation areas, detected as sites with higher optical thickness (Fig. 1, *d*). Changes in optical thickness of the nucleolus in a cell depending on the duration of actinomycin D treatment are graphically presented (Fig. 2). Optical thickness of the nucleolus decreased during the first minutes of treatment, while 10 min after addition of the agent in addition to the loosened areas, whose total diameter was more than in intact cells, optically dense areas with lesser optical diameter appeared (Table 1). According to cytological data, the fibrillar component (FC) is the most electron dense in the nucleolus. The decrease in FC can be due to segregation of compact FC (Fig. 3). Hence, CPM shows not only the physical characteristics of the nucleolus in general, but detects the fine changes in these characteristics inside this small formation.

Studies of cell ultrastructure showed that the nucleoli in intact HCT 116 cells were mainly round and located in the center of the nucleus or near the nuclear membrane. Fibrillar centers, compact fibrillar component near the fibrillar centers, and less dense granular component were clearly seen in the nucleoli (Fig. 3, *a*). Ten minutes after addition of actinomycin D, the nucleoli acquired a peculiar

shape, fibrillar centers shrank, but their number increased. The granular component became less compact (Fig. 3, *b*). These signs indicate the beginning segregation of the nucleolus components, a phenomenon characterizing actinomycin D effect [5]. By the 30th min of exposure dissociation of the structures inside the nucleoli progresses: the number and size of disorderly scattered dense areas

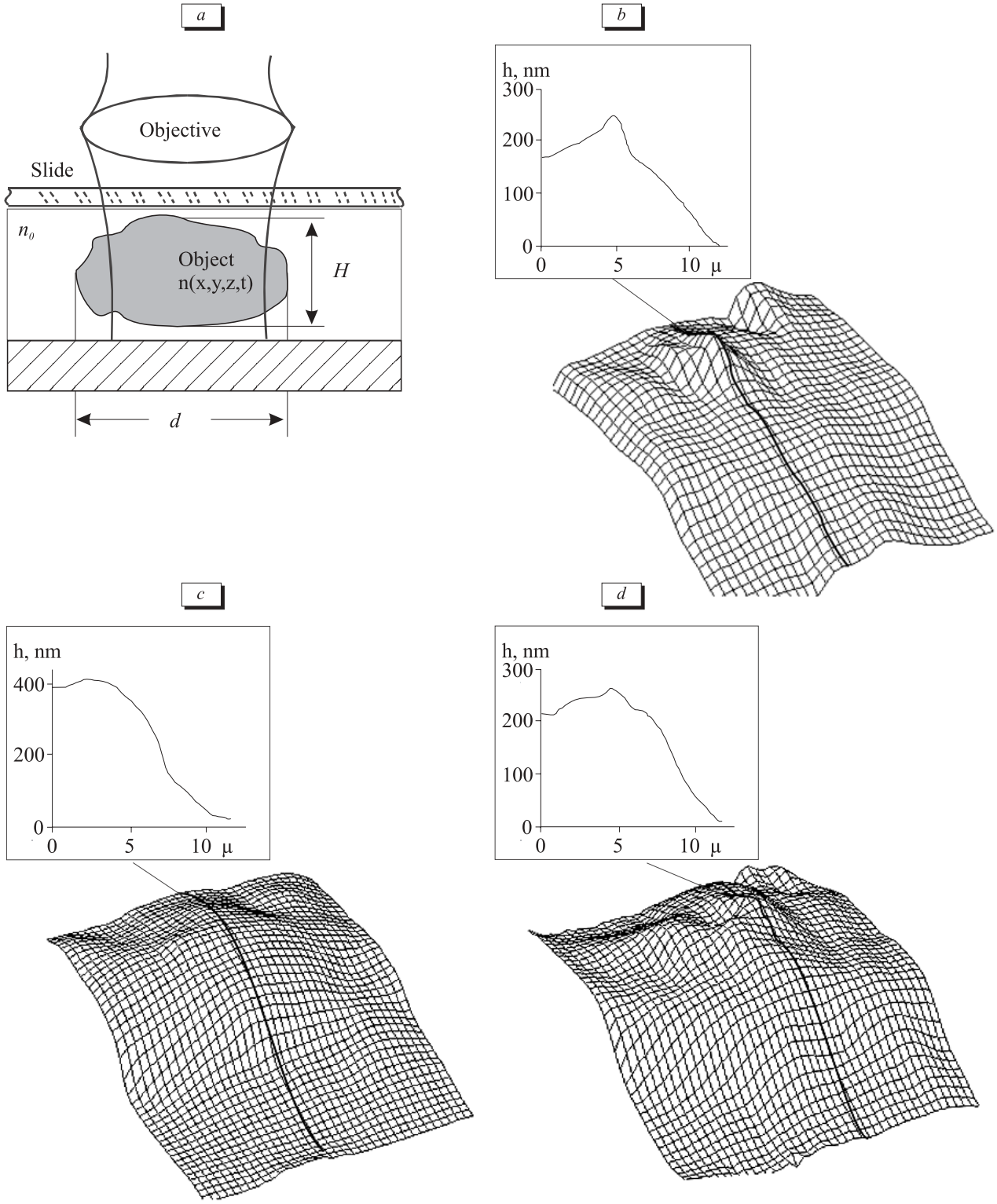

Fig. 1. Study of nucleolar structure by CPM. *a*) physical philosophy of the method; *b-d*) three-dimensional topograms of phase images of the nucleolus and adjacent nucleoplasm in HCT 116 cells: intact (*b*), 5 (*c*) and 30 min (*d*) after addition of 1 μM actinomycin D.

increases (compact FC), alternating with rarefaction zones (Fig. 3, *c*). Hence, CPM data on optical heterogeneity of the nucleolus as a reaction of this organelle to transcription inhibitor are in line with the picture of segregation of the nucleolar components, shown by electron microscopy.

Experiments demonstrate the possibility of analyzing individual cells by the CPM method. Statistical processing of these data provides quantitative expression of cell population response to transcription inhibitor by changes in the nucleolar optical parameters. Mean values of optical thickness and diameter and the nucleolar refraction values in NCT 116 cells before and after actinomycin D treatment (1 μM, 30 min) are presented (Table 1). The drug effect led to redistribution of the nucleoli by optical thickness: a statistically significant increase in the number of nucleoli with low optical thickness (number of optically rarefied nucleoli). Optical diameter (*d*) and refraction also decreased (Table 1).

Two groups of nucleoli are distinguished in intact cells: with phase thickness of 20-40 and 60-85 nm. By this parameter group 1 corresponds to nonfunctioning ("inert") nucleoli, group 2 to "active" ones [2]. Phase thickness of 20-35 nm was detected in the nucleoli after actinomycin D treatment (Table 1) and after cell incubation in medium with very low content of the serum. This means that phase thickness decreased under the effect of actinomycin D in the "active" nucleoli. Hence, phase thickness of the nucleolus is an indicator of its physiological activity: low values of the nucleolar phase thickness indicate a reduction (or suppression) of its function.

Olivomycin is an antibiotic of the aureolic acid group suppressing transcription mediated by RNA polymerases I and II, which caused a reduction of the nucleolar optical parameters similar to that under the effect of actinomycin D. Olivomycin aglycon possessing no antitranscription effect did not modify the nucleolar optical characteristics. In addition, the effects of transcription inhibitors were detected on several models: HCT 116 (Figs. 1, 2),

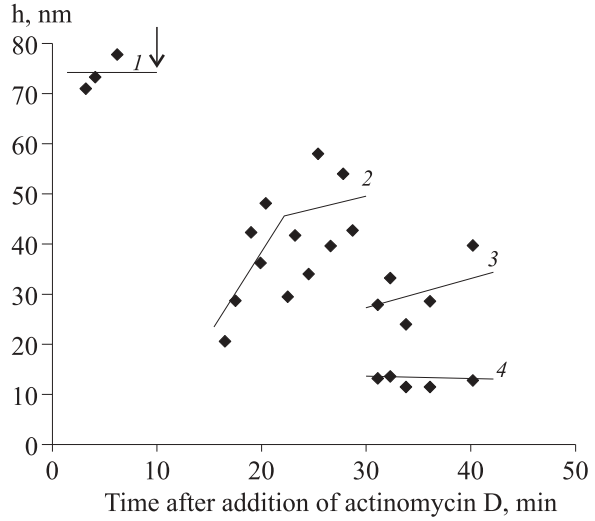

Fig. 2. Optical and morphological heterogeneity of the nucleoli under the effect of actinomycin D. Changes in nucleolar optical thickness depend on time elapsed after addition of 1 μM actinomycin D (arrow). *1*) before addition of actinomycin D; *2*) initial stage of sharp decrease (before 5 min) of phase height of the nucleolus and its subsequent increase; *3*, *4*) changes in phase height of different components of the nucleolus.

NIH 3T3, and MCF-7 strains. The decrease in the nucleolar optical thickness under the effect of actinomycin D was detected not only in cultured cells, but also in the cells from biopsy specimens from patients with breast cancer. This prompts the use of CPM in clinical practice for evaluating the cytotoxic effect of anticancer compounds and, maybe, prediction of tumor response to this or that drug.

The common pattern of changes in the nucleolar optical characteristics under the effect of transcription inhibitor consists in the decrease of their optical density. Considering the data on optical heterogeneity of the nucleoli in the course of response to actinomycin D (Fig. 2), this trend indicates the predominance of loosening over condensation. The mechanism of changes in optical parameters of the nucleoli during transcription disorders remains not studied. It is not clear which components of the nucleolus are responsible for its optical density. Presumably, the decrease in the nucleolar optical

TABLE 1. Time Course of Optical Parameters of "Active" Nucleoli after Addition of Actinomycin D

Parameter	HCT 116		NIH 3T3	
	control	actinomycin D*	control	actinomycin D*
Phase thickness (Δ*h*), nm	95	20	75	25
Optical diameter (*d*), μ	2.7	1.7**	2.8	1.8**
Refraction (Δ*n*)	0.040	0.015	0.045	0.016

Note. *Mean values of measurements in 10 series, each for 20 intact cells and 20 with 30-min exposure with actinomycin D. **Diameter values under the effect of actinomycin D are shown for the optically most dense area.

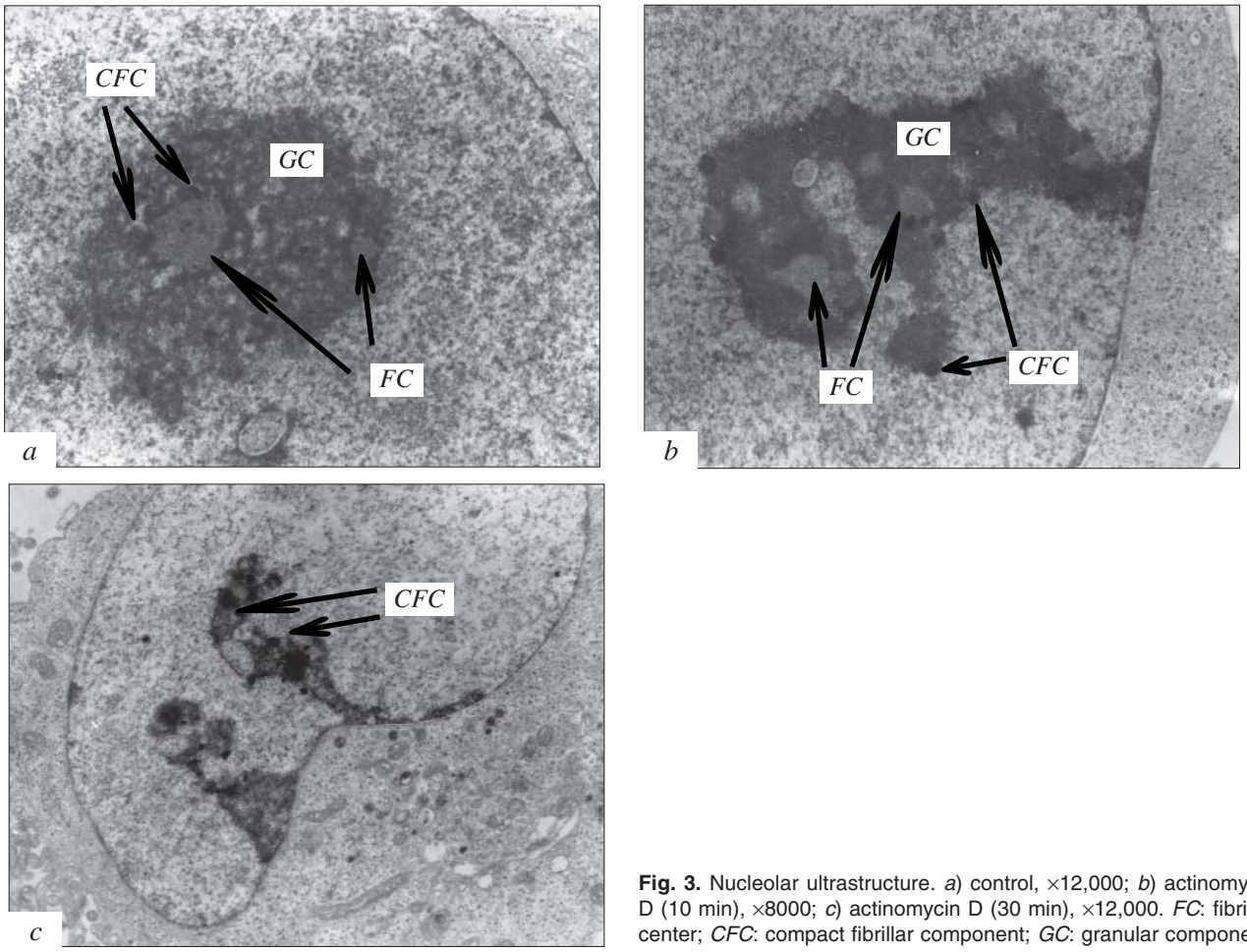

Fig. 3. Nucleolar ultrastructure. *a*) control, ×12,000; *b*) actinomycin D (10 min), ×8000; *c*) actinomycin D (30 min), ×12,000. *FC*: fibrillar center; *CFC*: compact fibrillar component; *GC*: granular component.

parameters is caused by active transport of proteins into the nucleoplasm, predominating over retrograde movement. This hypothesis is in line with dynamic changes in optical parameters: combination of rarefaction and condensation areas, in other words, formation of local optically heterogeneous objects inside the nucleoli. The decrease in the nucleolar phase thickness and their dysfunction can be caused by compactization because of shrinkage of the granular component. Comparison of CPM findings with electron microscopy data indicate that changes in optical parameters reflect the redistribution (segregation) of nucleolar structures during transcription inhibition [4].

Hence, the data of CPM, a new method for quantitative analysis of dynamic changes in the nucleolar structure under the effects of direct inhibitors of transcription, were verified by electron microscopy data. CPM detects early changes in optical parameters of individual nucleoli in the real time and evaluates the cell population response, requiring no fixation, staining, or long processing of the preparations. These advantages recommend this method for rapid diagnosis of disorders in the nucleolar structure during transcription suppression and for screening of new chemical compounds, potential cytotoxic (specifically, antitumor) drugs.

The study was supported by the Russian Foundation for Basic Research (grant No. 04-04-49132).

REFERENCES

1. V. P. Tychinskii, A. V. Kretushev, I. V. Klemyashov, *et al.*, *Dokl. Rossiisk. Akad. Nauk*, No. 4, 1-5 (2005).
2. Yu. S. Chentsov, *Introduction to Cellular Biology* [in Russian], Moscow (2004), pp. 152-185.
3. C. Mayer and I. Grummt, *Cell Cycle*, **4**, 1036-1038 (2005).
4. Y. Shav-Tal, J. Blechman, X. Darzacq, *et al.*, *Mol. Biol. Cell*, **16**, 2395-2413 (2005).
5. V. P. Tychinsky, A. V. Kretushev, T. V. Vyshenskaya, and A. N. Tikhonov, *Biochim. Biophys. Acta*, **1665**, 57-64 (2004).
6. V. P. Tychinsky, A. V. Kretushev, T. V. Vyshenskaya, and A. N. Tikhonov, *Ibid.*, **1708**, 362-366 (2005).