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# On contrast of biological X-ray nanomicroscopy

I.A. Artyukov, A.V. Vinogradov, N.L. Popov

*Abstract.* **We analyse the absorption contrast of histological and cytological preparations, which can be achieved in nanomicroscopic studies using monochromatic radiation in the spectral range of 90 – 600 eV (14 – 2 nm). Two types of unstained biological objects are considered: untreated and fixed in paraffin, and optimum wavelengths are determined for the study of samples with a thickness of 0.5 – 10 μm with a spatial resolution of 100 – 20 nm. Taking into account the efficiency of X-ray optics, the number of source photons required to produce a single image is estimated. It is shown that the greatest interest for the study of fixed objects represents the spectral region of 7 – 14 nm, for which, on the basis of rapidly developing compact sources of incoherent and coherent radiation and effective optics, microscopes for scientific and clinical research can be designed.**

***Keywords:*** *nanomicroscopy, spectral range of 2 – 14 nm, biological object, exposure dose.*

## 1. Introduction

Over the past two decades, X-ray microscopy has demonstrated impressive progress owing to the development of reflective and diffractive X-ray optical elements. The spatial resolution has increased by at least 100 times and constitutes a value of the order of 10 nm in the entire range from 100 eV to 10 keV [1]. These record results are obtained on the most advanced tunable sources of X-ray radiation – third-generation synchrotrons, the total number of which does not reach two dozen [2]. At the same time, commercially available X-ray microscopes have appeared that make it possible to study nanomaterials with a spatial resolution down to 50 nm [3, 4]; however, they can operate at a small number of wavelengths, limited by the capabilities of X-ray tubes. In this regard, one of the most urgent tasks, as before, is the development of compact, preferably tunable radiation sources available to small laboratories and scientific groups, which will offer an opportunity to use the achievements of X-ray optics, as well as the microscopy methods developed and tested for third-generation synchrotrons [5, 6]. This task is one of the practical goals of improving laboratory X-ray lasers, high harmonics generators of IR lasers, incoherent laser-plasma X-ray sources and Thomson laser-electronic generators [7].

In this work, we consider the spectral range of 90 – 600 eV, in which a number of compact X-ray sources are already available and used. Moreover, this is the region of resonances in the photoabsorption cross section of living matter. In particular, this region contains the K-edges of photoabsorption of the O, C, N and S elements composing the main amino acids and other biological materials. The spectral range of the water window, i.e. the spectral interval between the K-edges of photoabsorption of oxygen and carbon (284 – 543 eV), is ideally suited for microscopy in this spectrum region. This interval provides the greatest difference in the photoabsorption of these elements, i.e., the greatest image contrast of biological molecules containing mainly carbon, against the background of water that forms a basis of living tissue [8, 9]. Thus, in contrast to other modern methods of histological analysis, X-ray microscopy does not need any additional staining or colouring of samples.

To study dehydrated samples fixed in paraffin, Artyukov et al. [10] proposed to use a carbon window adjacent to the water window from the side of low energies. Despite a decrease in the contrast of biological molecules in this case, the use of a spectral range softer than the water window is of interest because of the above-mentioned rapid development of monochromatic coherent and incoherent sources of monochromatic radiation. In this case, two circumstances should be kept in mind. On the one hand, the number of wavelengths mastered by compact monochromatic sources (laboratory lasers, harmonic generators, laser plasma) with a quantum energy up to 100 eV is expanding. On the other hand, further advance of them towards shorter wavelengths encounters considerable difficulties.

In the present paper, in the range of 90 – 600 eV, we study the contrast of biological objects which are fixed with the aim of preserving their spatial structure. Fixation is achieved by dehydration and water replacement by paraffin [11]. The aim of the study is to determine the most favourable wavelengths of X-ray sources for microscopy of biological objects fixed in paraffin and to estimate the number of X-ray photons of a source, which is required to obtain a single image.

## 2. Model for determination of spatial resolution and exposure dose

Let a biological object with a characteristic size $\delta$ be enclosed in a layer of fixing material of thickness $L$ (Fig. 1). The image quality obtained in a monochromatic beam is characterised by the magnitude of the CNR contrast ratio. It is equal to the

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ratio of the average difference in the number of photons in the beams that passed through the layer, bypassing the object and through it, to the square root of the variance of this difference. Assuming that the number of photons incident on the sample obeys the Poisson statistics, we find:

$$\mathrm{CNR} = \frac{N_\delta \exp(-\mu L) - N_\delta \exp[-\mu L - (\kappa - \mu)\delta]}{\sqrt{N_\delta \exp(-\mu L) + N_\delta \exp[-\mu L - (\kappa - \mu)\delta]}} =$$

$$\sqrt{N_\delta \exp(-\mu L)}\,\frac{1 - \exp[-(\kappa - \mu)\delta]}{\sqrt{1 + \exp[-(\kappa - \mu)\delta]}}, \qquad (1)$$

where $N_\delta$ is the number of photons incident on the area $\delta$ of the sample surface; and $\kappa(\lambda)$ and $\mu(\lambda)$ are the absorption coefficients of the dehydrated object and fixing material, respectively, which depend on the source wavelength $\lambda$. For them, we shall use the values given in [12, 13].

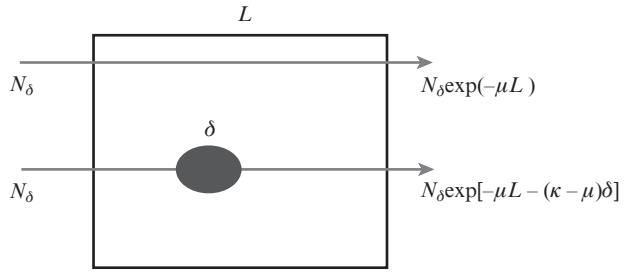


**Figure 1.** Bio-object with the characteristic size $\delta$ in a layer of fixing material with a thickness of $L$ (see other notations in the text).

Following Eqn (1) one can find the requirement to the total number of photons $N = M^2 N_\delta$ being necessary to obtain a single image containing $M^2$ pixels:

$$N \geqslant [(\mathrm{CNR})^2 M^2 \exp(\mu L)\frac{1 + \exp[-(\kappa - \mu)\delta]}{\{1 - \exp[-(\kappa - \mu)\delta]\}^2}. \qquad (2)$$

Strictly speaking, Eqn (1) is only valid for an ideal detector; therefore, in the transition from (1) to (2), there appears the sign $\geqslant$.

Relation (2) expresses the condition for obtaining images in microscopy based on the absorption contrast. It relates the total number of photons to the spatial resolution and has a simple meaning. The first factor gives the lower estimate for $N$ and corresponds to the total absorption in the object under study, $(\kappa - \mu)\delta \gg 1$. This optimistic estimate is only determined by the fixing material transmission and the number of pixels. The second factor shows in what degree the required number of photons $N$ increases due to incomplete absorption in the object. Relation (2) also explains the meaning of the value $(\mathrm{CNR})^2$, i.e. the number of photons required to record a single ($M = 1$) element $\delta$ in the case of total absorption [$(\kappa - \mu)\delta \gg 1$] and in the absence of background ($\mu L \ll 1$).

To analyse the condition for obtaining an image (2) at various wavelengths, it is convenient to represent it in the form $N = Gg$, where

$$G = (\mathrm{CNR})^2 M^2,$$
$$g(\kappa, \mu, L, \delta) = \exp(\mu L)\frac{1 + \exp[-(\kappa - \mu)\delta]}{\{1 - \exp[-(\kappa - \mu)\delta]\}^2}. \qquad (3)$$

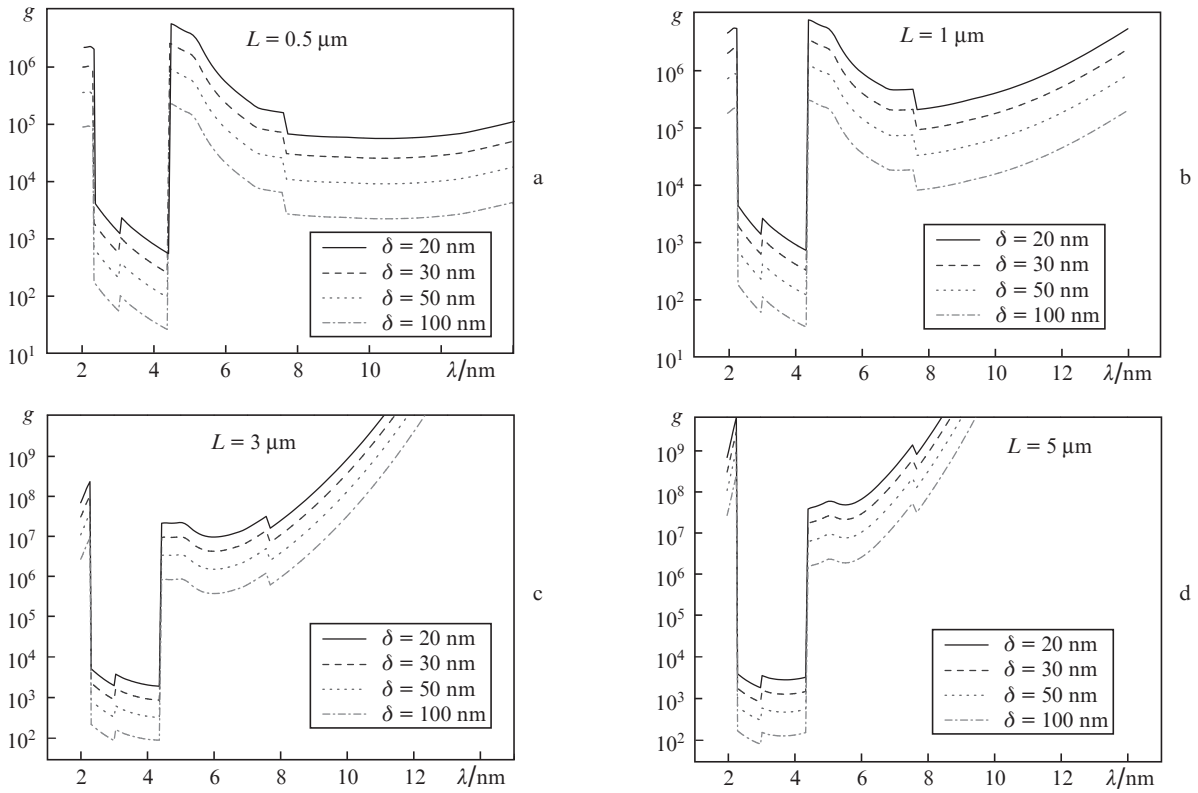


**Figure 2.** Dependences of the value of $g$ on the wavelength for protein structures in water at a slice thickness $L$ = (a) 0.5, (b) 1, (c) 3 and (d) 5 μm and various spatial resolutions.

The value of $g(\kappa, \mu, L, \delta)$ determines the dependence of $N$ on both the optical properties and sample geometry.

Figure 2 shows the values of $g(\kappa, \mu, L, \delta)$ for protein structures fixed in $H_2O$ at a spatial resolution of $\delta = 20$, 30, 50 and 100 nm and sample thicknesses $L = 0.5$, 1, 3 and 5 μm. The protein absorption coefficient was determined from the average density of 1.35 g cm$^{-3}$ [14] with weight fractions of the elements H, C, N, O and S given in [12] (p. 371). The corresponding curves for $\mu(\lambda)$ and $\kappa(\lambda)$ for water and protein are shown in Fig. 3. Figures 4 and 5 demonstrate similar data for the same protein structures fixed in paraffin $C_{20}H_{42}$ with a density of 0.9 g cm$^{-3}$.

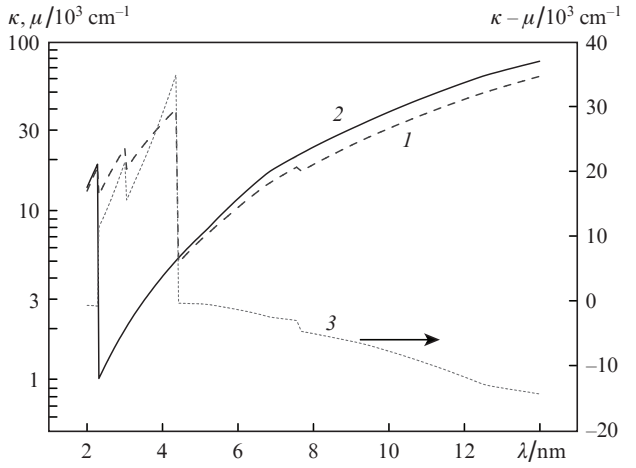


**Figure 3.** Spectral dependences of absorption coefficients of (*1*) protein and (*2*) water, as well as of (*3*) their differences on the wavelength.

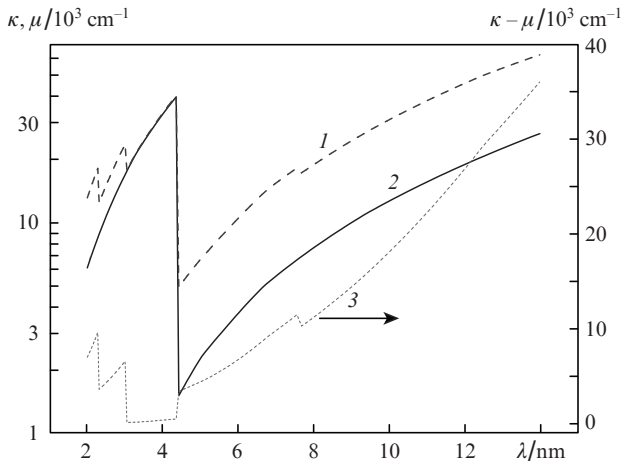


**Figure 5.** Spectral dependences of absorption coefficients of (*1*) protein and (*2*) paraffin, as well as of (*3*) their differences on the wavelength.

## 3. Discussion of calculation results

The results given in Figs 2 and 4 allow us to establish the requirements for monochromatic radiation sources in the wavelength range of 2–14 nm for imaging of biological structures fixed in paraffin, with a spatial resolution of 100–20 nm using a slice thickness of 10–0.5 μm. Note that this combination of the parameters cannot be achieved by means of optical or electron microscopy.

Table 1 demonstrates an example of using the data from Fig. 4 for a biological object fixed in paraffin. The Table gives

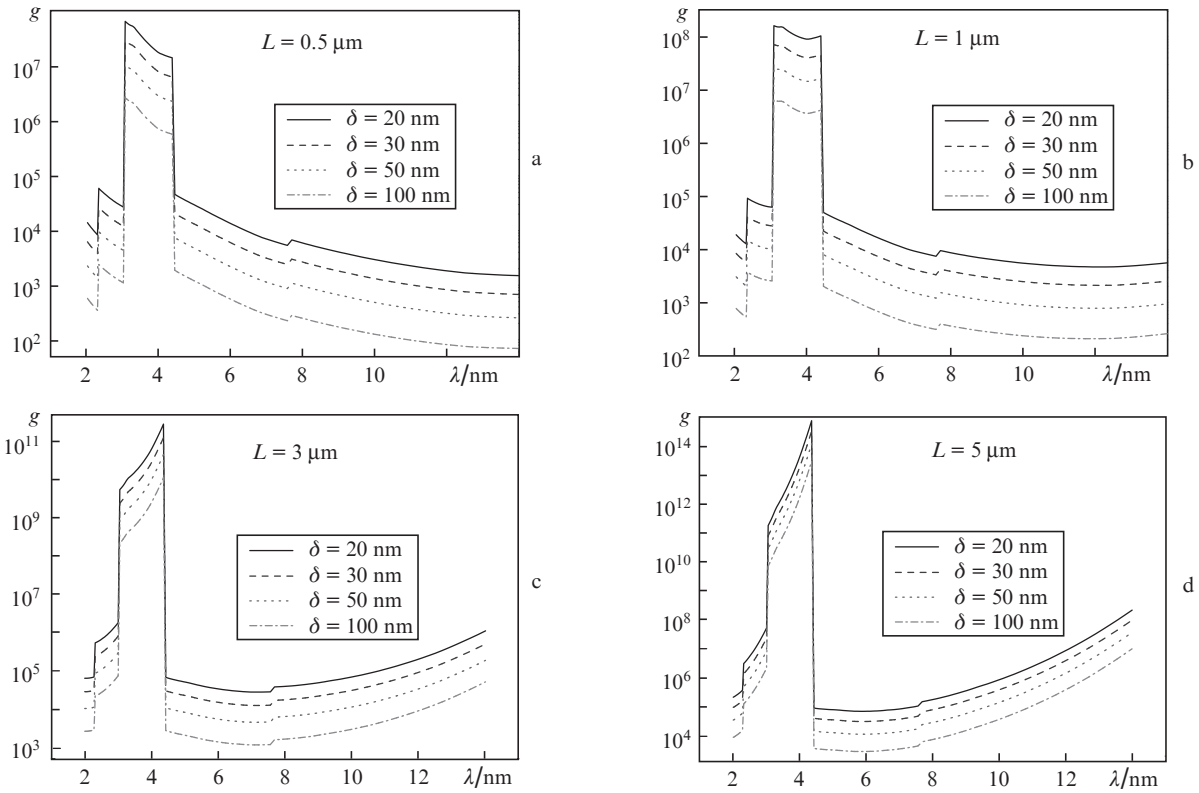


**Figure 4.** Dependences of the value of $g$ on the wavelength for protein structures in paraffin at a slice thickness $L$ = (a) 0.5, (b) 1, (c) 3 and (d) 5 μm and various spatial resolutions.

**Table 1.** Optimal wavelength ranges $\lambda$ and the number of photons $N$ required to obtain a single image of a biological object fixed in paraffin with a spatial resolution $\delta = 50$ nm [see (3) and Figs 2, 4].

| Slice thickness $L$/μm | Parameter | | | |
|---|---|---|---|---|
| | $\lambda$/nm | $G$ | $g$ | $N$ |
| 0.5 | 7–14 | $3.6\times10^7$ | $10^3-3\times10^2$ | $3.6\times10^{10}-10^{10}$ |
| 1 | 7–14 | $3.6\times10^7$ | $2\times10^3-10^3$ | $7.2\times10^{10}-3.6\times10^{10}$ |
| 3 | 5–10 | $3.6\times10^7$ | $4\times10^3-10^4$ | $1.5\times10^{11}-3.6\times10^{11}$ |
| 5 | 4.4–10 | $3.6\times10^7$ | $10^4-10^5$ | $3.6\times10^{11}-3.6\times10^{12}$ |

the optimal wavelength ranges $\lambda$ for microscopy, in which the number of photons $N$ required to obtain a single image with a spatial resolution of $\delta$= 50 nm is minimal. When calculating $G$ according to (3), the number of pixels was assumed to be equal to $1.6\times10^5$ with a contrast ratio CNR = 15.

Until now, we have not taken into account the presence of optics to magnify the image and to make consistent the transverse resolution, which we also assume equal to $\delta$, and the pixel size. Given fact that the efficiency of the zone plates is about 10% (see [15]), this gives an additional factor which is no more than $10^2$. Thus, to obtain a single image, the total energy of the source radiation on the surface of samples of various thicknesses (Table 1) should lie in the range 0.020–10 mJ.

## 4. Conclusions

1. To obtain images of 0.5–3-μm-thick biological slices fixed in paraffin with a resolution of 50 nm, monochromatic radiation sources in the wavelength range of 7–14 nm can be used. The number of photons required to obtain a single image does not exceed the value of $3.6\times10^{12}-3.6\times10^{13}$. At a photon energy of 100 eV, this corresponds to the total energy from 60 to 600 μJ on the sample, which can be provided both by laser plasma and laboratory X-ray lasers [16]. Similar parameters – spatial resolution and object thickness – cannot be attained by means of optical or electron microscopy.

2. The results of the calculations conducted in the work confirm the usefulness of developing compact and accessible X-ray sources in the spectral range starting from the carbon K-edge (4.4–14 nm) for the microscopy of biological objects fixed in paraffin. It should be noted that at present the paraffin based fixation technology is common for both clinical and research studies in biology and medicine but it cannot be used in the spectral region of the water window due to low contrast.

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