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Quantitative study of mammalian cells by scanning transmission soft X-ray microscopy

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Abstract. Molecular distribution in mammalian cells was studied by soft X-ray scanning transmission microscopy with respect to the quantitative aspect of analysis. NEXAFS profiles at the C, N and O K-absorption edges were combined and used for the analysis. For the estimation of quantity for nucleic acids and proteins, NEXAFS profiles of DNA and bovine serum albumin (BSA) at the N K-absorption edge were applied assuming that those were their representatives. The method has a potential to explore the other molecular components than nucleic acids and proteins.

1. Introduction

Scanning transmission X-ray microscope (STXM) has been established and used in a wide area of science. In a biological field, distribution of DNA in a bean chromosome [1] and a bull sperm [2] has been imaged by the spectromicroscopy. In addition, quantitative approach has been developed for the analysis of molecules and elements in biological specimens such as microbial biofilms [3], quinoa [4] and bacterial endospores [5] using STXM. For the quantitative analysis of molecular distribution of DNA and proteins, near edge X-ray absorption fine structure (NEXAFS) at carbon K-absorption edge has been applied. We have proposed that NEXAFS at the N K-absorption edge has a merit of clear separation between DNA and proteins in addition to the exclusion of biomolecules containing no N [6].

2. Materials and methods

Cultured CHO cells at interphase were collected, fixed with ethanol-acetic acid (3:1) solution or glutaraldehyde (2.5% in 0.075M KCl) and dropped on the commercially available formvar membrane (#10-1008, Okenshoji. Japan). The cells and molecules such as DNA and bovine serum albumin (BSA; protein) were observed with scanning transmission X-ray microscope (STXM) installed at BL4U, UVSOR-III, Okazaki, Japan. The results were analyzed using the aXis2000 program. For quantitative analysis, we use NEXAFS spectra of DNA and BSA at the N K-absorption edge, since nucleic acids and proteins are major components of cells containing nitrogen and were clearly separated at 1s-π^* transition.

Calculation has been made as follows: At the first step, each spectrum of image, DNA or BSA at the C, N and O K-absorption edges was combined to form one absorption spectrum covering the

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energy range from the C K-absorption edge to O K-absorption edge. Combined image spectrum was named as spect1. DNA spectrum was adjusted to spect1 so that it fitted the optical density (OD) difference between ODs at 399.6 eV and 398 eV (the peak and the valley in DNA NEXAFS) of spect1 and named as DNAspect. DNAspect was subtracted from spect1 resulting in a spectrum named as spect2. BSA spectrum was adjusted to spect2 so that it fited the OD difference between ODs at 401.4 eV and 398 eV (the peak and the valley in BSA NEXAFS) of spect2 and named as Prspect. Prspect was subtracted from spect2 resulting in a spectrum named as spect3. ODs in DNAspect and Prspect were converted to mass absorption coefficient. For comparison of the profile of each spectrum, it was rescaled so that the difference of maximum level and minimum level was adjusted to be the same OD difference and set to be the same at the left end of the energy.

3. Results and Discussion

3.1. NEXAFS of DNA and BSA

Figure 1 shows the NEXAFS profiles of DNA and BSA at the C, N and O K-absorption edges. Peaks were referred to the papers for DNA [7] and proteins [8]. NEXAFS of DNA at the N K-absorption edge shows a peak of 1s to π* transition of N=C bond at 399.6 eV, which is clearly separated from a peak of 1s to π* transition of amide at 401.4 eV in BSA. It should be noted that DNA and RNA include the same type of N=C bond, hence the present analysis of DNA corresponds to nucleic acids. In addition, amide is common in proteins, so that the analysis of BSA is for proteins.

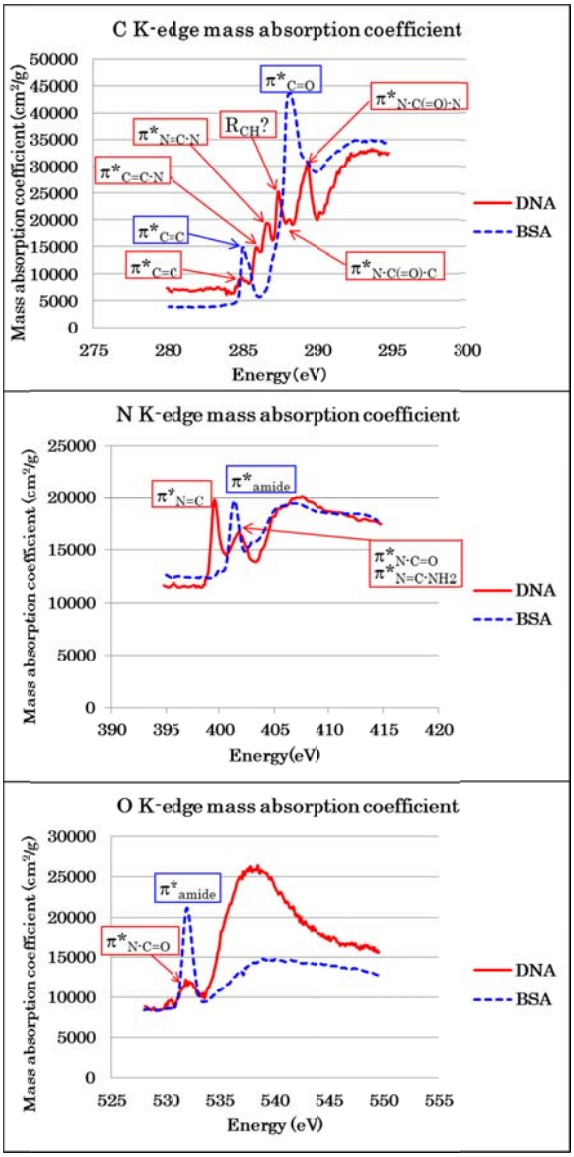


Figure 1. Mass absorption coefficient of DNA (solid line) and BSA (dotted line) at the C, N and O K-absorption edges.

3.2. Images of CHO cells

Figure 2 shows the optical density images at 285 eV and RGB maps for interphase cells fixed with either ethanol-acetic acid or glutaraldehyde obtained with STXM. The spect3 was estimated from the average absorption spectrum (spect1) of a whole imaging area by the method shown in section 2. RGB maps

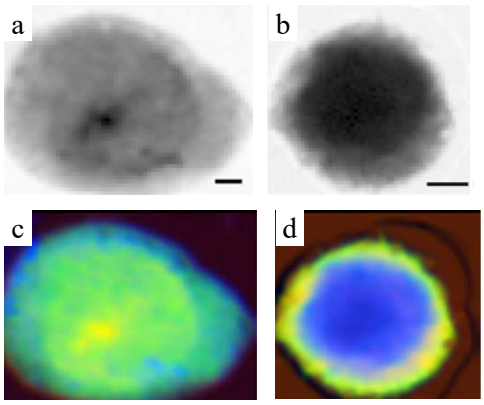


Figure 2. Optical density images (a,b) and their RGB maps (c,d) of CHO cells fixed with ethanol-acetic acid (a,c) and glutaraldehyde (b,d). In RGB maps (c,d), red, green and blue are for nucleic acid, protein, and spect3, respectively. Bar, 2 μm.

were obtained by the aXis2000 program with the mass absorption coefficient spectra of DNA and BSA, and OD of spect3.

The results in figure 2 show different RGB maps due to the difference in the fixatives of the cells, ethanol-acetic acid and glutaraldehyde. In the center of the cells (nucleus) fixed with glutaraldehyde (figure 2d), color is blue meaning that the main component was spect3 (residual component other than nucleic acids and proteins). The result is not reasonable to our knowledge that nucleus mainly consists of DNA and proteins. In contrast, the result for the cells fixed with ethanol-acetic acid shows the reasonable feature. The reason for this discrepancy is attributable to the glutaraldehyde. The observed data for the cells fixed with glutaraldehyde may not be sensitive enough to separate the spectra of DNA and proteins at the present condition. The results suggested that fixatives of the cells should be selected for the purpose of the analysis, though tomographic sliced-image will clear the problem.

3.3. NEXAFS of local areas in CHO cells

The NEXAFS profiles for the local areas in CHO cells were studied as the next step. The areas were chosen for nucleus (1, green), nucleus containing nucleolus (2, blue) and cytoplasm (3, pink) as shown in figure 3. It should be noted that the areas for region 1 and 2 in the cells fixed with glutaraldehyde was not adopted because of the reason mentioned above. Figure 4 shows the spect1 of each local area

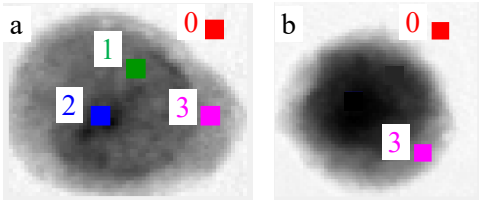


Figure 3. Local areas in CHO cells fixed with ethanol-acetic acid (a), and glutaraldehyde (b) for the analysis. Parts of 0 (red) for outside the cells (background), 1 (green) for nucleus, 2 (blue) for nucleus including mainly nucleolus, and 3 (pink) for cytoplasm. The sizes of the areas were a, 2.25 μm^2 each and b, 0.73μm^2.

for comparison. In figure 4, each part in figure 3 corresponds to the same colour and number, i.e., part1 for green (solid line), part2 for blue (broken line), part3 for pink (broken at long distance) of the cells fixed with ethanol-acetic acid (figure 3a), and g-part3 for purple (dotted line) of the cells fixed with glutaraldehyde (figure 3b).

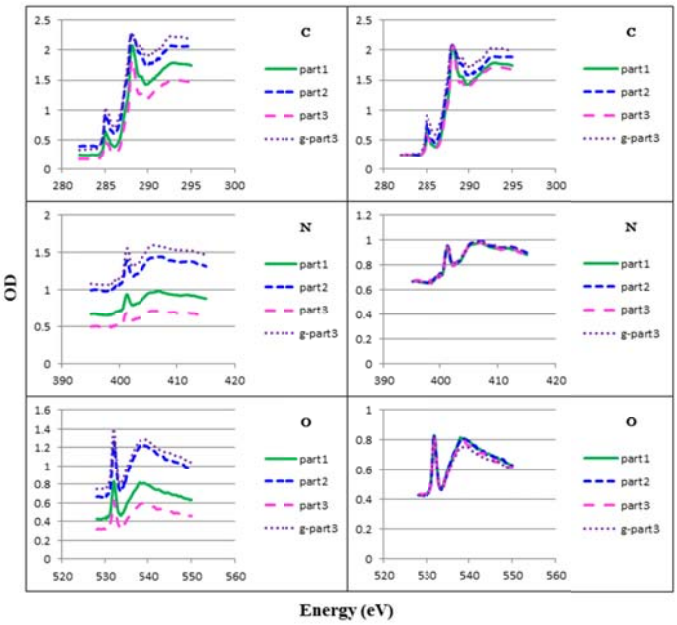


Figure 4. Spect1 of three local areas (figure 3a parts1-3) in a CHO cell fixed with ethanol-acetic acid and one area (figure 3b part3; g-part3) in a CHO cell fixed with glutaraldehyde for the spectra at the C (top), N (middle) and O (bottom) K-absorption edges. Left half shows direct comparison of observed spect1 and right half is for the rescaled spectra.

It is interesting to note that the profiles of each area are quite similar at the N and O K-absorption edges (right half in figure 4). However, the profiles of the spectra from different position are not the same at the C K-absorption edge which may be applicable to identify the molecules other than nucleic acids and proteins, or even among the nucleic acids and proteins. Left half in figure 4 reflects the comparison of the density difference of the molecules. The density is low at cytoplasm and high at nucleus for the cells fixed with ethanol-acetic acid. In contrast, the density in cytoplasm (figure3b, 3) for the cells fixed with glutaraldehyde was much higher compared to the cells fixed with ethanol-acetic acid.

3.4. NEXAFS of spect3 for local areas in CHO cells

Figure 5 shows the spect3 of each local area for comparison. The spect3 indicated the spectrum for the molecules other than nucleic acids and proteins. Therefore, it should be useful to find the molecular components in the cells. Figure 5 suggests that there is not much difference at all the local areas in cells for the distribution of molecules containing nitrogen and oxygen, but carbon, at which significant difference in the NEXAFS was observed. A sharp decrease in the spectra may be caused by either a small shift of the peak for proteins or the presence of component(s) which have a peak close to the C=O amide absorption peak.

With the present results for DNAspect and Prspect, the amount of nucleic acids and proteins at the local areas were estimated (Table1). The ratio of nucleic acids/proteins were almost the same regardless of the local areas in the cells fixed with ethanol-acetic acid in addition to the cytoplasm of the cells fixed with glutaraldehyde, though molecular species should be different, i.e., mainly DNA for nucleus and RNA for cytoplasm.

Table1. Ratio of nucleic acids per proteins estimated from DNAspect and Prspect for local areas in CHO cells shown in figure 3.

part	nucleic acids(N)	Proteins(P)	N/P
	μg/cm^2		
1	6.71	33.1	0.20
2	10.3	48.4	0.21
3	5.5	23.5	0.23
g-part3	11.1	57.7	0.19

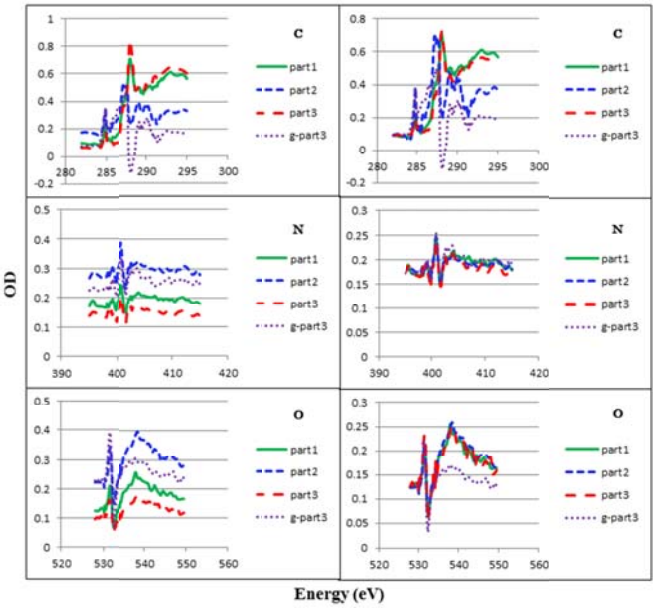


Figure 5. Spect3 of three local areas (figure 3a parts1-3) in a CHO cell fixed with ethanol-acetic acid and one area (figure 3b part3; g-part3) in a CHO cell fixed with glutaraldehyde for the spectra at the C (top), N (middle) and O (bottom) K-absorption edges. Left half shows direct comparison of the spectra and right half is for the rescaled spectra.

In conclusion, quantitative analysis using combined NEXAFS profiles at C, N, and O K-absorption edges showed that the ratios of nucleic acids per proteins were the same level in all the cell regions.

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