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Application of Photoacoustic Microscopy to Analysis of Biological Components in Tissue Sections

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Photoacoustic microscopy was applied to the determination of dye (alcian blue 8GS) spotted on a mucin layer. The determination range in a 40 μm diameter area was 40—1200 pg. The total dye quantity in the spot (*ca.* 2 mm diameter) was determined by integrating the quantities in the 40 μm diameter area within 15% error, irrespective of the uniformity of the distribution. This method was applied to the analysis of acidic mucopoly-saccharide in the rat rectum and in rat eyeball sections stained with alcian blue 8GS. The dye quantity was estimated to be 40—640 pg in the microregion of 40 μm diameter area and 1.5—1.8 μg in the whole region in *ca.* 8 μm thick sections. These quantities corresponded to 1.6—26 ng and 60—72 μg of mucin, respectively, based on the weight binding ratio of 40 of mucin to the dye in solution.

Keywords——photoacoustic microscopy; solid surface analysis; alcian blue 8GS; micro-distribution; acidic mucopolysaccharide; tissue; stained rat rectum; stained rat eyeball

The photoacoustic method[1] detects acoustic (thermal) waves generated by intermittent light absorption and thermal relaxation. Since the photoacoustic method measures the acoustic (thermal) wave intensity, it is advantageous to apply the method to the analysis of highly light-scattering solid samples. Hitherto, qualitative analyses have been performed not only on inorganic solid materials such as ceramics[2] and metal[3] but also on biological samples (blood smear,[4] human tissue,[5] animal tissue,[6] bacterial membrane[7] and plant samples[5a,8]), and quantitative analyses have been performed on some biological materials in solution.[9]

In the photoacoustic method, by using a sharply focused light beam for irradiation and X–Y scanning of the sample stage, a photoacoustic microscopic (PAM) image[10] which represents the microdistribution of light absorbing substances is obtained. In our work[11] the PAM method was applied to the determination of dye in microregions of a solid biopolymer layer. The PAM method was applied to the analysis of acidic mucopolysaccharide in stained sections of rat tissues. The applicability of the PAM method to the quantitative analysis of a biological component in the tissue is presented in this paper.

Experimental

Materials——Rectum and eyeball were excised from freshly killed rat (adult male Wistar rats weighing *ca.* 250 g) and prepared as follows. Samples were fixed with 10% formaldehyde (for rectum) or Carnoy solution (for eyeball), embedded in paraffin, and then sectioned with a microtome. After staining a deparaffinized section by immersing it in 1% alcian blue 8GS solution containing 3% acetic acid for 60 min, the section was dried and placed in the photoacoustic cell. The thickness of the stained section was *ca.* 8 μm as determined by microscopic measurement.

Alcian blue 8GS was obtained from Chroma-Gesellschaft, GFR, and mucin (Type I from bovine submaxillary glands) was from Sigma Chemical Co., U.S.A.

All other chemicals were of guaranteed grade and were used as received.

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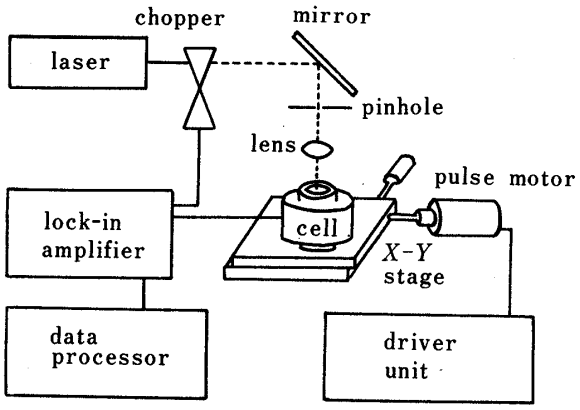

Fig. 1. Schematic Diagram of the Photoacoustic Microscopic Apparatus

Apparatus——A single beam photoacoustic apparatus was assembled in our laboratory as shown schematically in Fig. 1. A He–Ne laser (25 mW, 633 nm, GLS-5700, NEC, Japan) was used as the light source. The light beam was modulated at 43 Hz by means of a mechanical chopper and focused to a 40 μm diameter (ϕ) spot by using a pinhole and a lens. The focused light beam was used to irradiate a sample in the photoacoustic cell. The cell, with a volume of 0.3 cm^3, was tightly sealed with two quartz windows and O-rings. Acoustic waves generated in the cell were detected by using an electret-type condenser microphone and amplified by a lock-in amplifier (model 126, Princeton Applied Research Co., U.S.A.) at the phase angle of 102°, which gave the maximum signal intensity. The cell was set on the *X–Y* stage, which was driven by a pulse motor (Oriental Motor Co., Japan). The photoacoustic signal was analyzed by a data processor, Chromatopac, C-R3A (Shimadzu Co., Japan).

Binding Ratio of Mucin to Alcian Blue 8GS——The binding ratio of mucin to alcian blue 8GS in solution was estimated in the following way. Mucin solution (1 mg/ml) (300 μl) was mixed with 1 ml of alcian blue 8GS solution at various concentrations and allowed to stand for 30 min at ambient temperature. Then, 2 ml of a mixture of ethanol, acetic acid and distilled water (7 : 1 : 2) was added in order to accelerate the precipitation of mucin and dye complex. The solution was filtered to trap the complex on an acetyl cellulose membrane filter previously saturated with dye. The absorbance of the filtrate containing the free dye was measured at 633 nm with a spectrophotometer. The binding ratio was estimated to be in the range of 37—41 (mean 40).

Results and Discussion

Calibration by Using Standard Dye Spots

The calibration curve of dye was obtained by using standard dye spots on the solid biopolymer. A standard dye spot was prepared as follows. Mucin solution (5 mg/ml) of (60 μl) was spotted on a cover glass and dried on a hot plate (*ca.* 100 °C). Then, 0.5 μl of alcian blue 8GS solution at various concentrations was spotted on the mucin layer and dried slowly. Typical PAM images of standard dye spots (*ca.* 2 mmϕ) are shown in Fig. 2. Almost uniform distribution of dye in the spot was achieved by careful spotting and slow drying. Different procedures of spotting and drying gave various distributions of dye. The thickness of the spot on the mucin layer was determined to be *ca.* 8 μm by microscopic measurement. All the dye present in a spot seemed to give a photoacoustic signal because the laser beam could pass through the section. The calibration curve of dye in the area of the laser beam bundle (40 μmϕ) was obtained by PAM measurement of uniform dye spots as shown in Fig. 3. The data showed a non-linear dependence on the dye quantity. The least-squares analysis of the relation between the logarithm of the signal intensity in the 40 μmϕ area and that of the dye quantity in the same area gave Eq. 1.

$$\log[\text{signal intensity}] = 5.29 + 0.816 \times \log[\text{dye quantity}] \quad (1)$$

This equation can be rewritten as follows;

$$[\text{dye quantity}] = 3.30 \times 10^{-7} \times [\text{signal intensity}]^{1.23} \quad (2)$$

The factors in the equations depend on the optical and thermal characteristics of samples. The

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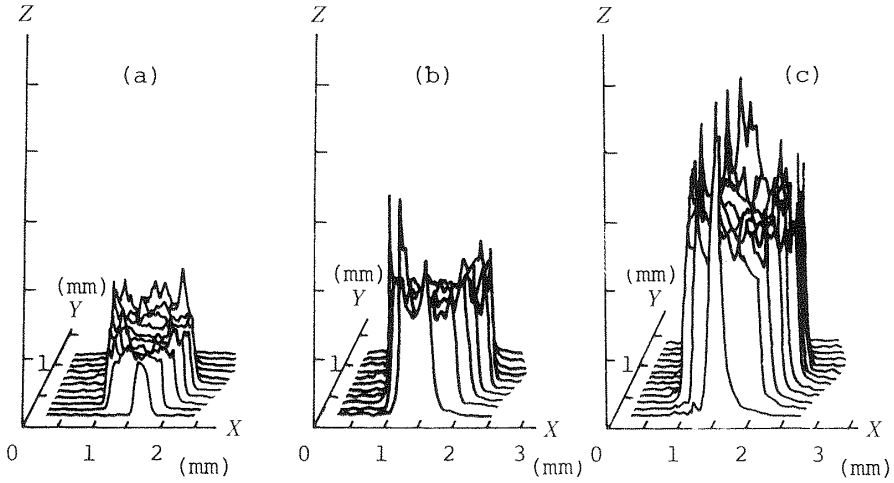

Fig. 2. PAM Images of Uniform Alcian Blue 8GS Spots on the Mucin Layer
Spotting quantity; (a) 250 ng, (b) 500 ng, (c) 1000 ng.

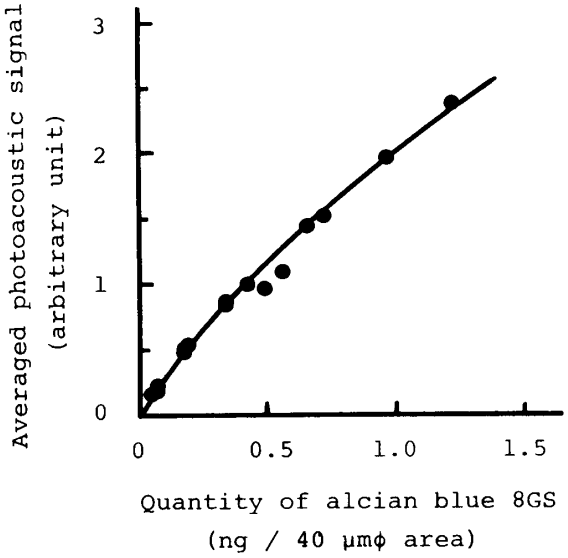

Fig. 3. Calibration Curve of Alcian Blue 8GS on the Mucin Layer

deviations of these factors obtained from almost uniform spots of the same thickness were within 20%. The determination range in the microregion of 40 μmϕ was 40—1200 pg. The upper limit of 1200 pg was due to the difficulty in preparing a more concentrated uniform dye spot.

Determination of Total Dye Quantity

The dye quantity in a whole dye spot was obtained by integrating the dye quantity in the microregion. Equation 2 was programed in a Chromatopac C-R3A data processor and the PAM signal was integrated to determine the total quantity of the dye.

The determination by this method was applied to spots having a non-uniform distribution as shown in Fig. 4. The scanning was carried out by changing the mode (scanning interval and direction). As shown in Table I, a scanning interval of less than 80 μm was necessary in order to determine the dye quantity with an error of less than 2%. The deviation caused by changing the scanning axis by 90° was less than 5%.

The results for non-uniform spots are summarized in Table II. The overall error in the determination of spots with various quantities of dye was 15% at the maximum. The difference of local thickness of the dye spot might be partly responsible for the error.

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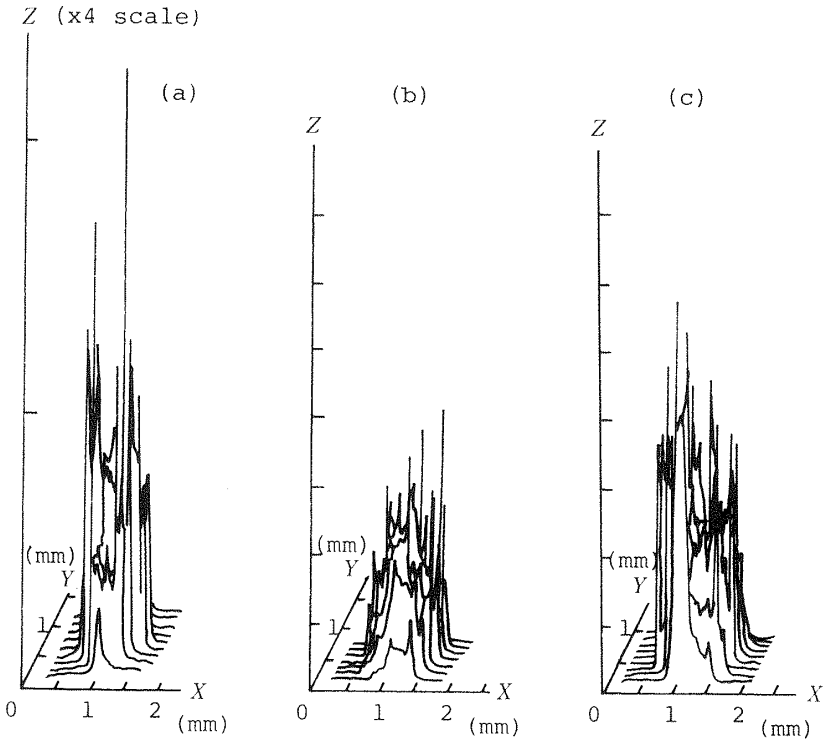

Fig. 4. PAM Images of Non-uniform Alcian Blue 8GS Spots on the Mucin Layer
Spotting quantity; (a) 125 ng, (b) 250 ng, (c) 500 ng.

TABLE I. Variation of Analytical Data with Scanning Mode

Scanning interval (μm)	Alcian blue 8GS quantity spotted (ng)			
	125	250	500	750
	Quantity found (ng)			
80	118 ± 2.8 (1.5%)	—	517 ± 7.1 (1.0%)	—
160	± 8.1 (5.4%)	279 ± 11.3 (2.9%)	± 30.9 (4.8%)	737 ± 46.7 (4.1%)
320	± 11.4 (8.0%)	± 21.0 (5.6%)	± 34.3 (5.7%)	± 103.2 (10.7%)
Deviation with 90° change of scanning axis	2.1%	4.9%	2.4%	4.1%

Determination of Dye in Tissue Sections

The PAM method mentioned above was applied to the determination of alcian blue 8GS in stained rat tissue sections. This dye is well known in histochemistry to bind with acidic mucopolysaccharide (mucin) with high selectivity. The calibration curve shown in Fig. 3 was used in these cases. Both the thickness (*ca.* 8 μm) and the phase lag (102° ± 10°) of the stained sections were almost the same as those of the standard dye spots on the mucin layer (thickness of *ca.* 8 μm and phase lag of 102° ± 10°). These results suggested good similarity of the optical and thermal characteristics of the stained section and the dye-spotted mucin layer. The PAM images and photographs of rat rectum and eyeball are shown in Figs. 5 and 6. The dye quantity in each 40 μmϕ area of Figs. 5 and 6 was within the determination range.

The PAM image of the rectum showed that the dye was distributed mainly in the

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Table II. Total Dye Quantity

Dye quantity		Error (%)
Spotted (ng)	Found (ng)	
125	140	+12.0
	120	−4.0
	139	+11.2
500	479	−4.2
	572	+14.4
	461	−7.8
750	708	−5.6
	674	−10.1
	737	−1.7

Determined at a scanning interval of 80 μm.

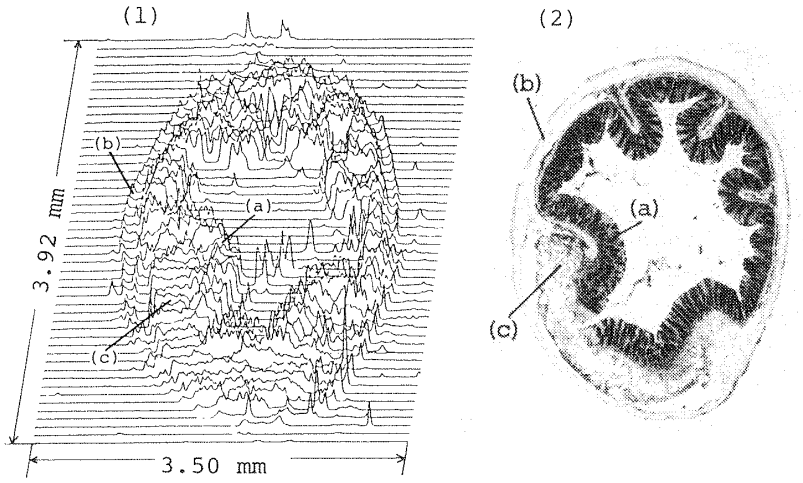

Fig. 5. PAM Image (1) and Photograph (2) of Stained Rat Rectum Section
(a) Mucosal layer, (b) serosa and (c) submucosa.

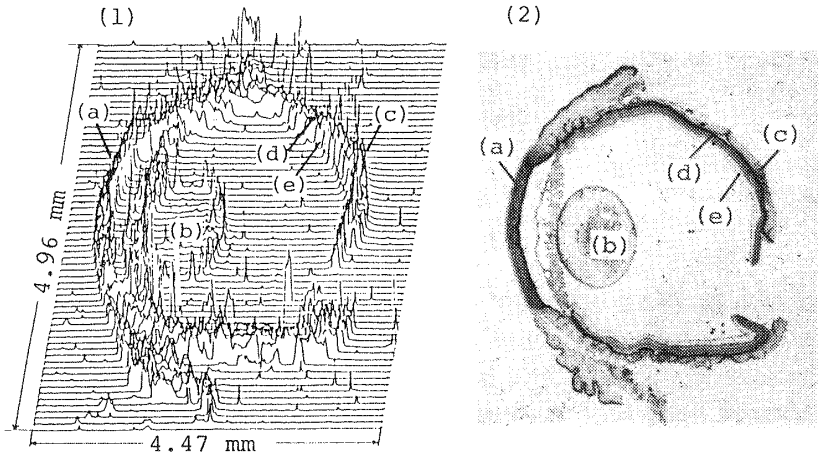

Fig. 6. PAM Image (1) and Photograph (2) of Stained Rat Eyeball Section
(a) Cornea, (b) lens, (c) sclera, (d) choroid and (e) retina.

mucosal layer, and the pattern was in good agreement with the histochemical pattern as shown by the photograph. Alcian blue 8GS in the rectum (oval shape of 2.8 × 3.5 mm with *ca.* 8 μm thickness), shown in Fig. 5, was 1.8 μg in total, corresponding to 72 μg of mucin from the

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binding ratio, with the microdistribution of *ca.* 500 pg (20 ng of mucin) at the highest in the mucosal layer and less than 250 pg in other parts (submucosa, muscularis, serosa and so on) in each 40 $\mu m\phi$ area.

In the PAM image of the eyeball, the signal intensity in the cornea and choroid was higher than that in other parts such as the lens and retina. The result is also in good agreement with the result of the photograph. Alcian blue 8GS in the eyeball (round shape of *ca.* 3.7 mm diameter with *ca.* 8 μm thickness) shown in Fig. 6 was 1.5 μg in total (60 μg of mucin) with the microdistribution of *ca.* 600 pg (24 ng of mucin) at the highest in the cornea and in choroid and *ca.* 300 pg (12 ng of mucin) at the edge of the lens and in the retina in a 40 $\mu m\phi$ area.

Since alcian blue 8GS did not show metachromasia in this study, the dye could be determined by using a single-wavelength laser. However, the staining selectivity of the dye should be checked carefully because there is no chemical evidence for selective interaction.

The determination limit was 40 pg in the 40 $\mu m\phi$ area. The present PAM apparatus was at least 5 times more sensitive than the reflectant absorption method with a thin layer chromatogram scanner (CS-930, Shimadzu Co., Japan) though it is very difficult to compare the sensitivity under identical conditions. The laser beam may be focused to *ca.* 1 μm by improving the lens system, and this would result in still higher sensitivity and higher resolution of the PAM method. Studies to achieve this are in progress in our laboratory.

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