Electron Microscope Autoradiography with Special Reference to the Problem of Resolution*

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In 1956 LIQUER-MILWARD first tried to apply the autoradiographic technique to electron microscopy. In her experiments the nuclei of tumor cells, which were labelled with $^{60}$Co, were examined autoradiographically using the Ilford G-5 emulsion and the tracks of $\beta$-particles were demonstrated. Although her observations have little value in the light of present knowledge, her challenge to electron microscope autoradiography has established an eternal monument as it has provoked present day progress in this field of science. Following her and other technical pioneers such as O'BRIEN and GEORGE (1959) and HARFORD and HAMLIN (1961), many technical difficulties were overcome by CARO (1961 a, b, 1964), SALPETER (1967) and other investigators. Thereafter excellent works applying these results have been published. Besides technical improvements, theoretical considerations laying emphasis on the problem of resolution have also been published (CARO, 1962 a, b; PELC, 1963; GRANBOULAN, 1963; SALPETER and BACHMANN, 1964; BACHMANN and SALPETER, 1965; SALPETER et al., 1969; et al.). Summarizing these workers' opinions, the limit of resolution has been determined as about 1,000 Å when the nuclide in $^3$H, the section thickness is 500–1,000 Å, and the diameter of silver halide grains in emulsion is less than 1,000 Å. They, however, agreed that the limit of resolution could be theoretically reduced by obtaining thinner sections and by reducing the diameter of silver halide crystals.

The authors made an attempt to couple autoradiography with electron microscopy for the first time in Japan in 1964. Since then the authors have employed nuclear research emulsion manufactured in Japan. At first improvement in the emulsion was required, and this was achieved with help of Dr. A. HIRATA of the Konishiroku Institute. Another elaboration was a new developing procedure for the purpose of improvement in resolution. This technique was provisionally named extremely fine grain development. And up to now, several considerations of resolution of electron microscope autoradiography were studied (MIZUHIRA, 1965; MIZUHIRA and UCHIDA, 1966, 1967, 1968 a, b; UCHIDA, 1965, 1969).

When an autoradiographic preparation is treated with ordinary developers (for example, D-19, Conidol-X, etc., following prescribed developing procedures), the diameter of the developed silver grains is 3,000–4,000 Å, i.e. 3–4 times larger than the original silver halide crystals. Since this size is in the optical microscopic order, the authors cannot help feeling pessimistic over the reliability of electron microscopic observations of such gigantic grains. In fact, the filamentous silver grains obtained

* This paper is dedicated to Prof. Toshio Ito upon his retirement.
by ordinary development (Fig. 11) do not reveal the initial point of development (i.e. the radiation source). In other words, does development begin on the mitochondrial membrane or in the matrix; or do the silver grains observed in the E.R. originate inside the E.R., or do they originate in the surrounding cytoplasm? These questions cannot be answered by ordinary development.

If the localization of an incorporated substance cannot be determined, it is nonsense to use the electron microscope for the merit of electron microscope autoradiography depends on its excellent resolution for locating the distribution of a certain substance at the macromolecular level. The authors have attempted to determine the limit of resolution of electron microscopic autoradiography with the newly devised technique of extremely fine grain development.

**Method of Electron Microscope Autoradiography**

**A. Preparation of grid meshes**

Untreated grid meshes will cause contamination by reacting with emulsion and chemicals during autoradiographic procedures. To avoid these undesirable effects, grid meshes are dipped in a 2-3% collodion solution and allowed to dry on a filter paper side by side. This procedure not only prevents the copper surface from reacting with chemicals, but also aids in attaching sections firmly. This technique is also useful in ordinary electron microscopic sectioning for protecting the section from being detached or contaminated.

**B. Treatment of sections**

About 300-500 Å thick ultrathin sections are electronic-stained with saturated uranyl acetate solution for about 1 hr. The radioactivity of the uranium is estimated to be extremely small, and does not affect the emulsion coating enough to cause background fog. After the electronic staining, direct contact between the section and the emulsion may cause background fog by gradually reacting with chemicals during a long exposure time. A carbon coating of about 50-100 Å will prevent these effects.

**C. Characteristics of Japan-made nuclear research emulsions and their treatment**

1. **Characteristics**

Of the emulsions listed in Table 1, Sakura NR-H1 and NR-H2 are characterized by fine grain, simple treatment, high sensitivity and excellent stability.

<table>
<thead>
<tr>
<th>Emulsions</th>
<th>Grain size (in µ)</th>
<th>Exposure period (in weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sakura NR-M1</td>
<td>0.14</td>
<td>6-8</td>
</tr>
<tr>
<td>NR-H1</td>
<td>0.07</td>
<td>6-8</td>
</tr>
<tr>
<td>NR-H2 (NR-H1-T)</td>
<td>0.07</td>
<td>3-4</td>
</tr>
<tr>
<td>Fuji ER-29-M</td>
<td>0.07</td>
<td>2-3</td>
</tr>
</tbody>
</table>

2. **Coating emulsion**

Filmy emulsions coated onto ultrathin sections are provided to be “a close but even
monolayer.” The methods of making such filmy emulsions are classified as follows: touching (O’BREIN, 1959; CARO and TUBERGEN, 1962), pipetting (SALPETER and BACHMANN, 1964), bubbling (CARO, 1961 a), dipping (CARO and PALADE, 1964; SALPETER and BACHMANN, 1964), wire-looping (HAY and REVEL, 1963 a, b; MEEK and MOSES, 1963), silver evaporating (SILK et al., 1961) and centrifugation (KOELER et al., 1963). The touching method seems to be the most handy and provides consistent results even when one is treating many grid meshes at the same time. The original emulsion of Sakura NR-H1 or NR-H2 is diluted 12-13 times with distilled water, and the diluted emulsion is coated onto ultrathin sections while maintaining a temperature of 40-45°C throughout the procedure. The dilution is experimentally determined so as to make the silver halide grains “close but even monolayered.”

3. Exposure

Emulsion-coated and thoroughly dried sections are kept in a cold dark box (4-6°C) with a desiccative for a suitable period. The long period necessary for exposure may make the emulsions deteriorate in air so the dark box should be evacuated or filled with inert gases such as nitrogen.

The exposure period is listed in Table 1. The exposure period must be as short as possible to decrease the “background fog” as well as to minimize the size of the developed silver grains for the purpose of obtaining high resolution, because the grain size depends not only on the developing conditions but also on the exposure period.

The difference in the silver grain size between ³H and other isotopes such as ³⁵S, ¹⁴C and ⁴⁵Ca, whose energies are about ten times stronger than ³H, is observed by using the same exposure period (generally at least 3 weeks for ³H) and the same developing conditions. Despite its lower energy the grain size of ³H is greater than the others.

D. Developing procedure

Ordinary developers are generally as chemical developers, e.g. D-19, Dektol, Microdol-X, Loveland, etc.; and physical developers, e.g. paraphenylenediamine method. After many trials, the combination of the gold-latensification and the Elon-ascorbic acid method has been found to be the best of all, because each latent image formed by the impact of a β-particle appears as an arrangement of dots or short strands in accordance with the macromolecular distribution of the applied labelled compound. An overgrowth of silver grains tends to produce coiled filaments. Therefore the developing method is at fault if such a figure appears.

When treating several grid meshes simultaneously, the slow development method should be used because the longer the developing time, the smaller is the difference between each mesh.

The authors’ extremely fine grain development: Previous to development with Elon-ascorbic acid, incremental treatment is given by dipping into gold chloride solution for about 10 sec. Washing with water is necessary between the two processes. The relation between the growth of the developed silver grains and the developing time as well as the temperature of the developer was examined, and the best condition was at 17°C for 14 min. With this method the diameter of the developed silver grains was between 100–400 Å. Fixation was with Kodak F–5 for 5 min, followed
by rinsing. All treatments were carried on by changing the solutions in a 10cc beaker. Strict control of time and temperature is required using a timer and a water bath provided with a thermostat. Contact between meshes or excessive agitation of the solution must be avoided.

E. Removal of gelatin and electronic staining

After development, gelatin in the filmy emulsion has to be removed and electronic staining applied. By dipping the developed section into a 50:1 MIZUHIRA-KUROTAKI’s solution (MIZUHIRA and KUROTAKI, 1964) for about 30 min, the gelatin is dissolved in an alkali and at the same time electronic staining with lead takes place. Washing with distilled water should be most carefully done lest the developed silver grains on the section become detached or dislocated.

F. Application dose of isotope

It seems that the larger the dose of radioisotope the better, but radiation disorders should be considered if experiments continue for a long time after incorporation. The minimum useful dose is 10–20 μC/g of body weight. The mean particle number decayed during 60 days after applying of isotope per unit area was theoretically calculated under the conditions stated in Table 2, with the resultant value of 10^{-1}/μ^2. Judging from this value, the dose of 10 μC/g may seem insufficient; however, the

| Table 2. The theoretical number of isotopes decayed in the ultrathin section (Emulsion NR-Hi) |
|---|---|---|
| Supposition 1 | Cell diameter | 20 μ |
| | Cell volume | 4/3π × (10^{-3})^3 = 4 × 10^{-9} cm^3 |
| Supposition 2 | S.G. of the cell | 1 g/cm³ |
| | Cell weight | 4 × 10^{-3}/g |
| | Number of cells per unit weight (1g) of tissue | 1/4 × 10^{-3} = 2.5 × 10^{9}/g |
| | Application dose of isotope | 10 μC/g (animal weight) |
| | Number of β-particles decayed during 60 day’s exposure | 10 × 3.7 × 10^{4} × 60 × 60 × 24 × 60 ÷ 2 × 10^{12} |
| | Number of decayed particles in a single cell | 2 × 10^{12} ÷ (2.5 × 10^{9}) = 8 × 10^{3} |
| Supposition 3 | Thickness of an ultrathin section | 500 Å |
| | Number of decayed particles within 1.μm² section | 500 × 5 × 10^{-4} × 8 × 10^{-3} = 10^{-1}/μ² |

isotope is not evenly distributed throughout the tissue but is incorporated specifically by a particular structure, the target structure. Therefore, this dose is effective enough in practice for the present purpose. The isotope is applied intraperitoneally, subcutaneously, intramuscularly or intravascularly, and if necessary can be incorporated into any organ with a canula. The intravascular injection, though occasionally difficult, gives excellent results with a relatively small amount of isotope.

G. Problem of isotope fixation within the tissue

During the procedures of specimen preparation, such as fixation, dehydration
and embedding, the labelled compound must remain in situ without becoming dissolved or dislocated. If the labelled compound is either a protein itself or a substance which is readily incorporated into protein, the process of preparation offers no problem, since the labelled compound will certainly be fixed with the routine fixatives such as osmium tetroxide, glutaraldehyde, etc. The commonly used compounds, on the other hand, are in most cases either water-soluble or lipid-soluble, and are not easily fixed in the tissue. If a soluble compound is made insoluble by adding certain reagents to the fixative, then the precipitates can be viewed. Although there are many compounds which cannot be made insoluble by any means, hypertonic fixative adjusted with sucrose will more or less inhibit the dissolution of the labelled compound. In this case, the radioactivity of the discarded solution should be examined at each step of the process to check the loss of isotope. Fishman and Gershon (1964) also reported that the water-soluble compounds were made almost insoluble by hypertonic fixatives, that is, sucrose added to the formalin or osmium tetroxide.

If a large amount of radioactivity is found in the discarded solution, this does not always indicate a failure, because spurious labelled compounds other than the fraction which incorporated into the living organism as the tissue components must be washed away during the process. Careful re-examination must be made in this case using biochemical methods.

Several compounds which are soluble either in water or lipid can be fixed by the following methods.

1. In order to fix the soluble isotope $^{22}\text{Na}$, a fixative containing potassium pyroantimonate ($\text{K}_2\text{Sb}_2\text{O}_7$) that is usually applied for the detection of Na-ions in electron microscopic histochemistry, gives good results by forming $\text{Na}_2\text{Sb}_2\text{O}_7$ as a fine insoluble precipitate. In this case the pH of the fixative was adjusted by measuring the potassium pyroantimonate without the buffer.

2. For the fixation of $^{45}\text{Ca}$, sodium dihydroxytartrate osazone or potassium oxalate in the fixative makes an insoluble precipitate with Ca-ions.

<table>
<thead>
<tr>
<th>Salts</th>
<th>Water pH 4.0</th>
<th>Water pH 7.0</th>
<th>Alcohol 50%</th>
<th>Alcohol 80%</th>
<th>Alcohol 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{B}_1\text{-NDS}$</td>
<td>2.12</td>
<td>20.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{B}_1\text{-HSCN}$</td>
<td>24.9</td>
<td>6.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{B}_1\text{-}\text{(BiI)}_2$</td>
<td>3.67</td>
<td>3.97</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{B}_1\text{-DMDPDS}$</td>
<td>0.40</td>
<td>4.85</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{B}<em>1\text{-II}</em>{\text{PtCl}}_4$</td>
<td>0.095</td>
<td></td>
<td>0.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{SBT}-\text{II}_{\text{PtCl}}_4$</td>
<td>0.15</td>
<td></td>
<td>0.34</td>
<td>0.27</td>
<td>0.05</td>
</tr>
<tr>
<td>$\text{B}_1\text{-HCl}$</td>
<td></td>
<td></td>
<td>1000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NDS: Naphthalene-1, 8-disulfonate
DMDPDS: 2, 2-dimethy-1, 3-diphenylpropane-4, 4'-disulfonate
SBT: S-benzoyl thiamine monophosphate

3. Various reagents were tested for precipitating soluble thiamine and its derivatives. In cooperation with Dr. H. Shindo of the Central Research Laboratory, Sankyo Co. Ltd., chloroplatinic acid ($\text{H}_2\text{PtCl}_6$) was found to be the best; it makes a highly
insoluble metallic compound of thiamine (Table 3). Specimens were prepared in the fixative and a series of dehydration mediums which were supersaturated with chloroplatinic acid and chloroplatinic salt of thiamine (thiamine-PtCl₆). The precipitate of thiamine-PtCl₆ is shown in Figures 22 and 23. A detailed description is given below.

4. For the fixation of lipid-soluble cholesterol, the digitonide method was applied. By adding digitonin to the process from fixation to dehydration a good amount of cholesterol was retained in the tissue (Fig. 1). The residual quantity of cholesterol varied according to the organ (Fig. 2).

5. After many attempts to fix steroids, a prolonged fixation method with both glutaraldehyde and osmium tetroxide was found to be the best way to demonstrate developed silver grains at the target structure (Fig. 12–15). Although a considerable amount of lipid-soluble steroids was found in the discarded solution, the main part taken up by a cell and combined with cell components seems to have been retained in situ by this method.
Generally speaking, the freezing and drying method is an excellent procedure for keeping the incorporated material from being dissolved away or dislocated, but this method is apt to destroy the ultrastructure of the cell. Since the object of electron microscope autoradiography is to investigate the localization of a certain substance at the macromolecular level, one of the above mentioned methods of fixation is preferable for observing cellular structures in a good state of preservation.

Resolution of Electron Microscope Autoradiography

Theories on resolution in electron microscope autoradiography were published by Caro (1962 a, b), Caro and Schnöss (1965), PELC (1963), Granboulan (1963), Salpeter and Bachmann (1964), Bachmann and Salpeter (1965), Salpeter et al. (1969), etc. The minimal resolution was estimated by them to be about 1,000Å for $^3$H and about 3,000Å for $^{32}$P. Bachmann and Salpeter (1965) distinguished two types of error: the photographic process (Ep) and geometric factors (Eg). The error Ep is determined by two factors; the diameter of the silver halide crystals ($a$) and the size of the developed silver grain ($b$). The mean photographic error is expressed as $\sqrt{\frac{a^2}{5} + \frac{b^2}{12}}$.

The geometric error, $Eg$, in terms of the resolution limiting thickness, is presented as $\frac{t_e}{3} + \frac{t_s}{2} + t_i$, where $t_e$ is the emulsion thickness, $t_s$ is the section thickness, and $t_i$ is the thickness of the layer between the emulsion and the section. The thicknesses $t_e$ and $t_s$ are more influential than $t_i$. The total error is expressed as $\sqrt{Ep^2 + Eg^2}$. As a device for reducing $Ep$, the Eastman Kodak NTE emulsion (the diameter of the
silver halide grains is about 0.03–0.05 µ) is sufficient to obtain a resolution of 700–900 Å, which, moreover, can be improved to about 300 Å under optimum conditions.

Prescinding from these opinions and theories, the authors reconsidered the problem of resolution on the basis of histological observations.

A. Histological observations

Several histological photographs of electron microscopic autoradiography are presented for the discussion of the problem of resolution.

The placental labyrinth, 30 min after the intravenous administration of cholesterol-1, 2-3H, was fixed by the prolonged fixation method with both glutaraldehyde and osmium tetroxide, and then autoradiography was applied (Fig. 12). Cholesterol becomes incorporated into the membranous structure in the cell, and this was shown by the large number of developed silver grains adherent to the membrane. Of the 30 plates observed, the number of developed silver grains attached to the membranous structure were 87% of the total. Although it is uncertain from what point the silver dots or rods began to develop, the center of the dot may be supposed to be the initial point of development. The distance from the center of the developed silver grains to the membrane was measured as 85.6 Å on the average of about 200 silver dots (Fig. 3). This value is 1/10 of the reported limit of resolution of electron microscope autoradiography, which was previously assumed to be 1,000–1,500 Å. In order to discover the reason why such a high resolution could be obtained, an experiment was tried to see what part of the latent images, which appeared by impact of β-particles in a silver halide crystal, remained as the extremely fine dots without washing away.

The section was developed as carefully as possible to obtain extremely fine grains at 17°C for 14 min in Elon-ascorbic acid developer following pre-treatment with gold chloride solution, and observed under the electron microscope without removing the gelatin. The developed silver halide grains appeared as small conglomerates which looked cat footprints. The authors called this a “cat’s paw pattern” (Fig. 17). The tissue of a green rice leaf hopper (Nephotettix cincticeps), a carrier of rice dwarf virus, also showed the cat’s paw pattern by treatment with uridine-5-3H (Fig. 18). The average diameter of about 200 patterns was measured as 853 Å, which coincides with the diameter of silver halide grains in the emulsion, namely 700–900 Å (Fig. 4). At a higher magnification, the conglomerate which composes the cat’s paw pattern is formed by dot-like images. The dot-like image seems to be formed in a silver halide crystal by the impact of one or more β-particles, and is supposed to be ionized silver
atoms freed from the crystal lattice to aggregate on the crystal surface.

![Graph showing distribution of developed grain size](image)

Fig. 4. Distribution of the developed grain size. See the text on resolution.

The latent images of developed silver halide grains are rather dispersed in the case of $\beta$-particles, while those in the case of light are fairly large and concentrated. It is true that the latent image becomes manifest when it reaches a certain size as a result of the aggregation of silver atoms toward the sensitivity nucleus, but, if developed slowly with Elon-ascorbic acid at a relatively low temperature ($17^\circ$C, 14 min) after gold impregnation, the latent image is assumed to be manifest in its original size and location. The authors will term this image a "silver speck." The mean number of silver specks in a cat's paw pattern was 10.5 in about 200 samples (Fig. 5). This value is reasonable for the number of latent images which are formed by the impact of $\beta$-particles on the surface of a silver halide crystal.

It is important to note that fine-developed silver grains remained after gelatin removal within 100 Å from the membranous structure, while the cat's paw pattern was seen if the procedure of gelatin removal was skipped. If a fine-developed silver grain is one of the silver specks of a cat's paw pattern, it should be considered whether any particular one of the silver specks is destined to remain after the gelatin removal.

![Graph showing distribution of counts of developed silver grains silver specks](image)

Fig. 5. Distribution of the counts of developed silver grains silver specks. See the text on resolution.
If an arbitrary one of the latent images, i.e. silver specks, should remain on the section surface on the assumption that the silver specks are evenly distributed inside a silver halide grain, then fine-developed silver grains are dispersed at the distance of twice the diameter of an undeveloped silver halide grain (Fig. 6). The range would therefore be $1,500 \sim 2,000 \AA$.

![Fig. 6. Latent images in a silver halide grain (SG) hit by $\beta$-particles have varied probability of remaining on the section surface ($S$). See the text on resolution.](image)

This is contradictory to the authors' observation because fine-developed silver grains were confined within $100 \AA$ from the membranous structure. It should also be pointed out that the theories of photography hitherto advanced do not sufficiently explain the present observations, and that a revised theory on resolution is required.

Similar results were obtained by the administration of estradiol and testosterone. Figure 13 shows a uterine epithelial cell of a mouse which was fixed 1 hr after the intravenous administration of estradiol-17$\beta$-6, 7-3H. Figures 14-16 also show a capsular cell of the tubuli seminiferi of the mouse testis which was fixed 1 hr after the intravenous injection of testosterone-1, 2-3H. Extremely-fine-developed silver grains are observed in contact with membranous structures, probably target structures, by the same fixation method as in Figure 12.

**B. Theoretical considerations**

Up to now, the resolution of electron microscope autoradiography has been considered to be about one diameter of a silver halide grain on the basis that a silver halide grain is the minimal unit of the detector of $\beta$-particles. According to this concept, the silver halide grain size of 0.07–0.1 $\mu$ of the Sakura emulsion NR-H1 and NR-H2 is the limit of resolution. Our observations, however, indicated that the resolution might be as small as $100 \AA$ as described above. For the interpretation of our observations, together with Dr. K. Konishi of the Department of Radiology, the Tokyo Medical...
and Dental University, the following are to be considered.

A certain $\beta$-emitter is supposed to be present in the specimen. The probability that a $\beta$-particle may hit a silver halide grain of the monolayered emulsion covering the $\beta$-emitter, is to be estimated geometrically (Fig. 7); a silver halide spherical grain with a radius $a$ may be hit by a $\beta$-particle whose radiation source is located at the distance $x$ from the grain. The depth of the radiation source from the specimen surface is $b$, and the $\beta$-radiation is assumed to be straight (the $\beta$-radiation is considered to be straight by primary approximation, because the degree of resolution is within a few tenths of the mean $\beta$-particle range in the authors’ experiments with $^3$H). Then the probability $p(x)$ is proportional to $1 - \sqrt{1 - a^2/(x^2+(a+b)^2)}$. The relation between $P(x)$ and $X=x/a$ in the section between $b=0$ and $b=a$ draws such a curve that $P(x)$ rapidly increases as the silver halide grain approaches the radiation source so far as $b$ remains extremely small, while the curve inclines gently as $b$ becomes larger (Fig. 8). It is evident that the resolution improves as the section thickness becomes thinner even without considering the grain size.

From a practical standpoint the radius of a silver grain $a$ is regarded as the apparent radius $r$, which equals the radius of a tangent circle of the effective solid angle. The $r$ is expressed as follows:

$$r = \frac{a \sqrt{x^2+b^2+2ab}}{\sqrt{x^2+(a+b)^2}}$$

where, $x^2+b^2+2ab < x^2+(a+b)^2$

therefore, $r < a$.

Moreover, the energy of $\beta$-rays is in inverse proportion to $x^2$, so that remote grains receive far less energy than adjacent grains. The value of $a$, or $r$, is negligibly small if the value of $x$ is large.

Concerning the continuous spectrum of $\beta$-rays, the low-energy series has a large stopping power with a short range, and therefore probably forms stable latent images only in the immediately proximate silver halide grains. Taking these suppositions into consideration, the probability curve will be steeper, $P(x)$ will rapidly increase in
the smaller \( x \) region, and the resolution will be higher than the value estimated by the above calculations (Fig. 9).

The resolution of autoradiography has hitherto been believed practically to be the diametral size of a single emulsified silver grain; in other words, a single silver halide grain has been considered the minimal unit of detection. Apart from these preconceptions, the authors demonstrated excellent resolution from the figure in which the silver dots were distributed within an aver-

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**Fig. 8.** Cross-section of silver halide grains at various distance from the source. See the text on resolution.

**Fig. 9.** Frequency distribution of distance from the radiation source to the silver halide grain. See the text on resolution.
age of 100 Å from the target membrane structures after developing by the extremely fine grain method (Fig. 3). The following formula is introduced on the assumption that the bottom plane of the silver halide crystal was the only detector of the radiation.

$$1 - \frac{x}{\sqrt{x + \left(\frac{b}{a}\right)^2}}$$

The value obtained by this hypothetical formula is shown as a dotted line in Figure 9, and this curve is quite similar to the empirical curve (solid line). This is explained by the following supposition.

If a β-particle hits a silver grain, whether the radiation source is directly below the contact print between the silver halide grain and the section surface as at β₁, or whether it is not as at β₂ and β₃, specks A......G... are formed in the silver halide grain (Fig. 6). Specks which are not on the contact point will be washed away by development and gelatin removal, or will be distributed at random within about 1,500 Å from the contact point.

Even when the radiation source is not directly below the contact point, the impact of β-particles on the contact point from the side of a silver halide grain is possible. Nevertheless, from the standpoint of the geometrical relations between the radiation source and the detector as depicted in Figure 7, the longer the distance, the smaller is the effective solid angle. Therefore there is every probability that most detectors are hit from directly below. If the detector were frequently hit by particles from a farther radiation source, the developed silver grains would be more widely distributed from the target membrane structure.

Another problem is the correlation between the point where the β-rays penetrate into a silver halide grain, and the point where the latent image appears. According to the photographic theory, which is now generally accepted, the two points do not always coincide, but the point where the latent image is formed is one of the pre-existing sensitivity centers of the silver halide grain. The authors' present observations, however, suggest the following considerations.

The authors' idea stands on the assumption that β-particles carry many electrons to the incident point of a silver halide grain, where many metallic silver atoms are attracted from the inside of the grain, and the probability of forming the latent image is the highest at the incident point. Then the latent image is probably formed at the surface of the impact area.

If the radiation source lies directly below the contact point between the silver grain and the section surface as at β₁ in Figure 6, the silver halide grain right above it will in all probability be hit by β-particles in very thin sections. At the impact point an aggregate of metallic silver atoms is formed to make a latent image. The aggregate, which touches the section surface, remains there after development as the developed silver grain. Other metallic silver aggregates that are formed at places other than the contact point and are not in contact with the section surface will be washed away during gelatin removal. Supposing that the remaining developed silver specks are observed as silver grains under the electron microscope, then a high resolution as obtained in the present experiments is reasonable.

Autoradiography with the isotopes such as ³⁵S and ⁴⁵Ca, which have an energy ten times higher than ³H and a lower energy loss against silver bromide, also demonstrat-
ed distinct localization as in the case of $^3$H (Fig. 19). In this experiment the grain size was smaller than in case of $^3$H with the same exposure period and the same development procedure, and each silver grain consisted of much finer grains (Fig. 20). The autoradiogram of $^{35}$S-treated articular cartilage was similar. These isotopes have much lower energy loss than $^3$H, or, in other words, have less probability of making latent images in silver halide grains whose diameters are under 0.1 µ. Nevertheless, silver specks are detected at the target structure by the same experimental method as in the case of $^3$H, though each grain containing subunits is smaller than with $^3$H.

These observations are explained as follows. Even very faint latent images, or aggregations of metallic silver atoms, which seem undetectable by ordinary development procedures may be recognized as developed silver grains by the gold impregnation method followed by Elon-ascorbic acid development. The latent image at the surface of the incident point, which is identical to the contact area between the silver grain and the section surface, is a looser aggregate of metallic silver atoms than that formed by $^3$H. Therefore the subunit structure in the developed silver grain remains at the section after development procedures. The subunit structure indicates that metallic silver atoms in this case are incapable of forming a solid mass as in the case of $^3$H, because the high-energy substance are so small in energy loss that the silver atoms at the site of impact are few in number and loose in arrangement. Although the subunit structure is observed by routine procedures, a prolonged exposure tends to form the same silver specks as in the case of $^3$H.

The hypothesis that the contact point between a silver halide grain and the section surface is hit by β-rays from directly below (from the direction of the highest geometrical efficiency), making a latent image at that point which then remains in situ after development and gelatin removal, seems to be valid for the explanation of such an extraordinary high resolution as demonstrated in the figures. It is true that this is merely a hypothesis, but most photographic theories accepted at present are also composed of assumptions. Several observations, however, which may be considered as practical evidence, are described in the following section.

G. Further improvement in resolution (Experiments with thiamine)

The limit of resolution has been discussed so far on the basis of electron-optical images obtained by extremely fine grain development as well as on the basis of theoretical evidence deduced from the authors’ experiments. A silver grain, which lies directly below the incident point of β-particles, makes the maximum latent image which becomes apparent through the development procedures while other silver grains which are not in contact with the section surface are washed away at the stage of gelatin removal in the course of procedures. This explanation seems to be more than a mere hypothesis because the authors believe to produced very convincing pictures.

To follow the resorption process of $^3$H-labelled thiamine in the intestinal canal, thiamine must first be precipitated as an insoluble metallic compound in order to observe its distribution under the electron microscope. Table 2 shows several insoluble compounds of thiamine produced in cooperation with Dr. H. Sando. Since the chloroplatinic salt of thiamine was the least soluble, the fixative containing chloroplatinic acid ($\text{H}_2\text{PtCl}_6$) was the best for demonstrating the distribution of precipitates
A labelled thiamine compound—S-benzoyl thiamine monophosphate (BTMP)—was incorporated into the ligated intestinal canal. After 1–30 min, pieces of the intestinal wall were fixed with 2.5% glutaraldehyde and later with 2% osmium tetroxide. The fixative as well as a graded series of alcohol for dehydration were supersaturated with chloroplatinic acid and at the same time with chloroplatinic salt of thiamine (B1-PtCl₆) to keep the precipitates from being washed away during the process. Embedding in Epon 812 and ultrathin sectioning were as usual.

One molecule out of 500,000 BTMP molecules is estimated to be labelled with ³H. Developed silver grains must therefore be far fewer than thiamine-precipitates.

At several places in Figures 25–30, B1-PtCl₆ precipitates of light contrast are overlapped by strikingly contrasted developed silver grains of 150–400Å in diameter. In some places the silver grain overlaps the edge of the precipitate while in others the grain is completely superimposed on the precipitate. These figures indicate a high degree of resolution.

Another experiment was performed in order to examine the interrelationship between B1-PtCl₆ precipitates and developed silver grains. To one of two test tubes containing collodion solution ³H-thiamine was added and to the other test tube H₂PtCl₆. Then the two solutions were mixed. The mixture was a slightly turbid collodion solution which was coated on the grid mesh to make a membrane. The electron microscopic observation of this collodion-coated grid revealed fine precipitates (Fig. 32) which were later identified as chloroplatinic acid by selected area electron diffraction (Fig. 33). Another collodioned grid was coated by the emulsion NR-H2, exposed for 4 weeks, developed with extremely fine grain development, and observed

Fig. 10. Schematic representation of high resolution electron microscope autoradiography. See the text on resolution.
electron-microscopically after gelatin removal. Developed silver grains with diameters of 100–300 Å were completely superimposed on the precipitates (Fig. 31). The precipitates were not always mounted by the silver grains while the silver grains were never found outside the precipitates.

The precipitates, whether they may be visible or not in a ultrastructure level, are identical with the labelled compound which was made insoluble with precipitant and are therefore the radiation source. The coincidence of the developed silver grain and the radiation source indicates that the developed silver grain mounts the radiation source, and consequently seems to have proved the authors' theory on extremely high resolution.

Experiments with thiamine labelled with 35S and 14C produced the same result, and therefore the resolution is not hardly affected by the range and the energy of isotopes so far as extremely fine grain development is concerned. Figure 10 summarizes the discussions that the resolution is controlled by spatial relationship between the silver halide grain and the section surface. In other words, that the contact point of the two (Fig. 10 c), or the contact surface of polyhedral silver grain (Fig. 10 d, e), is the only detector of radiation (Fig. 9, 10). Although the possibility of forming a few latent images originating from oblique radiation by neighbouring β-particles (Fig. 10 a) is not denied, most of silver grains are considered to be those that are hit from directly
below. The reliability of extremely high resolution is thus evident from the present observations obtained by extremely fine grain development (Fig. 10 b, c).

**Summary**

1. A method of electron microscope autoradiography was established using nuclear research emulsions manufactured in Japan: Sakura NR-H1 and NR-H2. Several biological applications were performed, and simultaneously the improvement in resolution was studied.

2. The aim of electron microscope autoradiography is to reveal the exact localization of certain substances at the macromolecular level. In order to attain this object the establishment of an extremely fine grain development method is indispensable.

3. Although the limit of resolution in electron microscope autoradiography is supposed to be the diameter of silver halide grains in emulsion, it may be improved by considering the fact that the contact area between the silver grain and the section surface is the minimum unit of resolution.

4. Thiamine, labelled with 3H, was made insoluble and precipitated with chloroplatinic acid at fixation. Developed silver grains completely overlap the B1-PtCl₆ precipitates.

5. The minimum resolution of the electron microscope autoradiography was determined histologically to be within 100 Å. The following hypothesis is proposed to explain this high resolution although the introduction of extremely fine grain development is also considered one of the factors.

Some of latent images are formed at the contact point (surface) between the polyhedral silver halide grain and the section surface. The latent image seems to be formed by the impact of β-particles from directly below, and since it is in contact with the section it remains in place even after development and gelatin removal. This latent image finally becomes a developed silver grain in the electron microscope autoradiogram.

電子顕微鏡的オートラジオグラフィー、とくに分解能の検討（内容自抄）

1. 原子核研究用乳剤 さくら NR-H1 および NR-H2 を用いて電子顕微鏡的オートラジオグラフィの手技を確立し、その生物学的応用をここに、主として組織切片写真上の所見をもととして電子オートラジオグラフィの分解能について検討した。

2. 電顕オートラジオグラフィの目的とする物質の局在を高分子のレベルでとらえるためには超微粒子現像を行うことが不可欠である。

3. 分解能を検討する目的で種々の実験を行い得られた所見により、超微粒子現像を行う限り、切片と切片上に一層に並ぶ臭化銀粒子との接点（接面）のところに、直下からβ線でヒットされた時にできたその接点（接面）の部分の潜像のみが、現像と脱ゼラチン処理ののちに、そのままの位置で最後に切片上に現像銀としてみとめられる確率が非常に高い。

4. 電顕オートラジオグラフィの分解能の向上が、従来乳剤臭化銀粒子の直径程度までは可能であるとされていた。しかし、われわれの検討の結果、分解能はさらにかなりよいもので、臭化銀粒子の切片との接点（接面）のみを detector として考え得る可能性
Resolution in Electron Microscope Autoradiography

References


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