The Investigation of Multi-reflection Images From Small Crystallites using Dark-Field Electron Microscopy

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An investigation of multi-reflection dark-field imaging with the conventional transmission microscope shows that diffraction data can be obtained from small crystalline particles less than 100 Å in diameter. Particles down to a few unit cells in diameter reveal their crystal habit giving the planar spacing and symmetry for a given orientation. In the case of Au, single crystallites as small as 18 Å in diameter gave good data. This method uses magnifications intermediate between diffraction patterns and high-resolution images, so large fields of particles are imaged with low beam intensities and hence minimized radiation dose and specimen damage. The multi-reflection technique also yields much lower background signals from the support films than selected-area diffraction and allows even faint diffraction images of individual crystallites to be observed. Au crystallites on amorphous carbon films were employed to demonstrate several features of the method. Lattice spacings as small as 2 and 1.4 Å were measured by observing the shift of the images at two defocus values with known difference. Several individual particles produced multiple diffraction images with high degrees of symmetry (100, 110 and 111 orientations). Useful information is obtained from the shifts in individual diffraction images that are functions of spherical aberration and defocus and the fine structure in the images similar to bend contours caused by beam divergence. The multi-reflection technique was used to study individual iron cores of ferritin molecules. For single-crystal cores, six and fourfold crystal symmetries were observed with lattice spacings consistent with spacings of 2.55 and 2 Å reported by X-ray and electron diffraction. Many iron cores were found to be either polycrystalline or amorphous structures, with single-crystal cores being rather rare, implying that the core is a poorly crystallized structure.

I. Introduction

With the transmission electron microscope the observation of diffraction patterns from small crystalline particles less than a few hundred Ångstroms in size, in addition to bright-field and dark-field images, is of prime importance in determining their lattice constants and orientation. However, the recording of diffraction patterns of individual particles can only be accomplished with great difficulty in the conventional transmission microscope and in this paper we examine another technique for obtaining electron diffraction data; multi-reflection dark-field imaging.

Briefly, the multi-reflection method can be described in terms of the types of images created in the microscope. For each crystalline particle in a field, a normal bright-field image is produced as well as several dark-field images corresponding to particular Bragg reflections. It has been shown by Hall (1948, 1949) that the dark-field images are displaced from the bright-field shadow image by amounts depending on the spherical aberration and defocus of the objective lens. If a central beam stop similar to the type described by DuPouy (1967) is used, the bright-field image is eliminated and the dark-field images with enhanced contrast are obtained which reveal the particle's crystallographic habit in a direct manner. Essentially, the method involves separating the multiple diffraction images in a dark-field micrograph. This technique has been employed by Poppa, Heinemann & Elliot (1971) in the study of epitaxic growth of gold on mica and Heinemann & Poppa (1972) in an investigation of gold crystallites grown on PbS substrates (also see Heinemann, 1971).

In this paper, several properties of multiple dark-field images which have not yet been reported are demonstrated with Au particles. The second part of this investigation is concerned with the structure of the iron core of ferritin molecules.

II. Experimental procedures

II(a) Construction of the central beam stop

Our central beam stops consist of a thin platinum wire, 5 μm in diameter, supported across standard platinum–iridium apertures with hole diameters ranging from 50 to 100 μm. The wire is commercially available in the form of Wollaston wire in which the 5 μm platinum wire is surrounded by a 50 μm diameter silver wire. The silver is removed with HNO₃ and treated with a 25% solution of NH₄OH to remove any residual silver left by the HNO₃. The bare platinum wire is mounted on the aperture under a 50-power microscope with the aid of a micro-manipulator and is fixed in place with epoxy. The wire and aperture are

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coated with gold by vacuum vapor deposition to cover any contamination. The beam stop is then ready for use in the microscope.

II(b) Electron microscopy

The electron microscope used for all the central beam stop experiments was a Siemens Elmsikop 101 operated at 100 kV with an objective lens focal length $f = 2.35$ mm, and spherical aberration coefficient $C_s = 1.35$ mm. A 200 $\mu$m diameter aperture was used in the second condenser lens and the illuminating system was adjusted to produce a direct beam spot of about 3 $\mu$m diameter in the back-focal plane of the objective lens when the incident radiation was focused onto the specimen plane. The angular beam divergence at the specimen was $\sim 7 \times 10^{-4}$ rad and all micrographs were taken at or near this condition. The electron optical magnifications were between 20 000 and 100 000 $\times$ and exposure times were between 3 and 8 s, depending on the particular experiment. A liquid-nitrogen cooled anticontamination device surrounded both the specimen and the central beam stop.

The correct analysis of an individual particle's multi-reflection images and their relation to the particle's crystallography required calibration of the central beam stop apertures. Here, a single-crystal gold film a few hundred Angstroms thick along the [001] direction was used. Selected area diffraction patterns of this gold film together with the superimposed images of contrast stop apertures with diameters of 60 and 80 $\mu$m respectively were obtained. Four strong 200 Bragg reflections are transmitted with the 60 $\mu$m apertures. Thus, if one were looking at randomly oriented Au crystallites, multiple images of planes of the type 111 and 200 would be transmitted within the 60 $\mu$m aperture. With the 80 $\mu$m aperture additional Bragg reflections of the 220 type are transmitted.

III. Experimental results and analysis of gold clusters

The general features of image formation using a central beam stop and defocusing have been given by Poppa et al. (1971) and will not be repeated here. In this

Fig. 1. Central stop dark-field image of a large field of Au crystallites. $\Delta f \sim 14000$ Å, $C_s = 1.35$ mm, $\lambda = 0.037$ Å, electron optical magnification $\sim 50 000 \times$. 
section, several examples of multi-reflection dark-field imaging of gold crystallites demonstrate the types of images obtained and their characteristics.

Table 1. Number of reciprocal lattice reflections in Au corresponding to given lattice orientations, or crystal directions parallel to the optic axis of the microscope, for aperture size 60 and 80 μm

<table>
<thead>
<tr>
<th>Number of images</th>
<th>Lattice orientation</th>
</tr>
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<tbody>
<tr>
<td>60 μm* 80 μm†</td>
<td></td>
</tr>
<tr>
<td>6 8</td>
<td>[110]</td>
</tr>
<tr>
<td>4 8</td>
<td>[100]</td>
</tr>
<tr>
<td>0 6</td>
<td>[111]</td>
</tr>
<tr>
<td>2 4</td>
<td>[211]</td>
</tr>
<tr>
<td>2 2</td>
<td>[310]; [123]§</td>
</tr>
</tbody>
</table>

* Includes 111 and 200 reflections.
† Includes 111, 200 and 220 reflections.
‡ Two 200 reflections.
§ Two 111 reflections.

III(a) Multi-reflection images from a large field of crystallites

In a selected area diffraction (SAD) pattern from a region of a carbon film specimen which contains several crystalline gold nuclei in the field, it is virtually impossible to determine which reflections were produced by a given crystallite. The same specimen region taken under central beam stop dark-field conditions with a central beam stop aperture of 60 μm diameter is shown in Fig. 1. Here the image was defocused by 14 000 Å and the gold crystallites produce multiple images. The beam stop in this case was mounted on an aperture that passed only the Bragg reflections of the 111 and 200 type planes. Thus, from the known geometry of the reciprocal lattice for gold crystals, multiple images are expected with 6, 4, 2, 0 images for each crystallite, depending on the orientation of the crystallite with respect to the incident beam. The

![Multi-reflection images of an Au crystallite with its [001] direction parallel to the optic axis of the microscope. (a)-(e) focal series with 200 and 220 type Bragg reflections. (f)-(h) focal series with 220 type Bragg reflections. Electron optical magnification 100 000 x. (See text for focal steps.)](image)
number of reciprocal lattice reflections that correspond to given lattice orientations when this \( 60 \ \mu m \) diameter aperture is used are presented in the first column of Table 1. The electron micrograph in Fig. 1 contains a high percentage of crystallites with six reflections which correspond to \([110]\) oriented particles, \(i.e.\) particles with a \([110]\) direction parallel to the optic axis of the microscope and the incident electron beam.

III(b) Individual particle orientation

A demonstration of multiple dark-field imaging from different orientations of an individual gold crystallite on a carbon film perpendicular to the microscope axis is shown in Fig. 2. The crystallite is oriented with its \([100]\) direction along the optic axis and the multiple dark-field images were taken with both \(60\) and \(80 \ \mu m\) diameter beam stops. Table 1 summarizes the number of multiple images obtained for these apertures and different crystal orientations.

An analysis of Fig. 2 shows a crystallite that has the same orientation as the continuous film used to obtain the diffraction patterns to calibrate the beam stops. Fig. 2(a)-(e) is a through-focus series of images obtained with an \(80 \ \mu m\) aperture where eight strong Bragg reflections of type \(200\) and \(220\) are transmitted. The figures’ defocus values are approximately \(+28000, -21000, 0, -21000\) and \(-28000 \ \AA\) in going from (a) to (e). Here, zero defocus represents the best focus image, \(i.e.\) where all the images are in closest juxtaposition, so that the values listed are relative to this defocus value. In going from (a) to (e), there is a change in the image from a barrel to a pin-cushion distortion of the image pattern, because the image shift due to spherical aberration is nonlinear. The combined effects of spherical aberration and defocus are additive when overfocused but are opposing at underfocus. Since the \(220\) reflections are more severely affected by spherical aberration than the \(200\) spots the total image [see Fig. 2(a)-(e)] appears distorted from the ideal diffraction pattern.

Fig. 2(f)-(h) shows a focal series on the same particle with the \(60 \ \mu m\) aperture where only the four \(200\) Bragg reflections are transmitted. The defocus steps between pictures are approximately \(7000 \ \AA\).

III(c) Minimum particle size

The question arises as to how small a gold crystallite will form multiple images of adequate contrast in the central beam stop dark-field method. To determine...
this, small gold particles were nucleated in an atmosphere containing a partial pressure of argon and small isolated gold particles down to 18 Å in size were obtained. A multiple dark-field image and the corresponding bright-field image are shown in Fig. 3. The size range of these particles is similar to other observations with a contrast stop aperture (Poppa et al., 1971), but here crystal symmetries are readily observed. Particle sizes below 18 Å were not found in our investigation. Normal bright-field images of particles in this size range had high contrast with respect to the carbon support film and we conclude that smaller particles do not exist in this specimen, since they are not visible in our electron micrographs. If stable nuclei could be formed on very thin films, crystal shape effects would be significant in the multi-reflection images and structural information about the earliest stages of nucleation growth should be attainable. Some indications of shape effects have been observed by the streaking of individual diffraction images, though the effect was weak and did not produce high contrast micrographs.

In other regions small polycrystalline particles were found and contained numerous multi-reflection dark-field image spots oriented randomly in rings. Fig. 4 shows a high-magnification dark-field micrograph of such a gold particle where its crystallographic projection is observable to the 2 Å planar resolution level.

III(d) Convergent-beam diffraction effects

For our microscope illuminating conditions, in which the primary beam divergence is on the order of $10^{-3}$ rad, the SAD pattern obtained of a single-crystal film will consist of a number of diffracted beams, each spread into a circular disc. The intensity variation across each diffracted spot disc gives the variation of diffracted beam intensity as a function of the incident beam direction with respect to the crystal. These intensity variations have also been used in the determination of scattering potentials (extinction distances) by matching observed and calculated profiles (Goodman & Lehmpfuhl, 1967).

We have observed the same intensity variations using the multi-reflection technique. Fig. 5 is an ex-

Fig. 4. Multi-reflection image of a polycrystalline Au particle.
ample showing multiple images from three Au crystallites with [110] directions parallel to the optic axis of the microscope. Each particle has several diffraction images and each image contains several band-like structures. For different images of a given crystal, both the intensity and spacings of the bands are different, as is to be expected from n-beam dynamical calculations of rocking curves (bend contours) for Au crystals (Krakow & Hines, 1970). The spacings are sensitive to small changes in crystal orientation so that none of the three crystallites shown are at exactly the same orientation.

A further complication enters, however, since the thickness also varies as a function of position across each particle’s image. Therefore, the bands that are seen cannot be directly compared to rocking curve calculations at a constant thickness, but some type of extinction contour that is a function of both beam tilt and thickness would have to be generated.

IV. Analysis of the iron core structure of ferritin
IV(a) Background information

Farrant’s (1964) early study of the morphology of the iron core of ferritin (ferric-hydroxide-phosphate complex) led him to the conclusion that it exists as micelles of about 55 Å in diameter consisting of sub-units of about 27 Å in diameter and that the micelles are located inside a protein shell. Publications by Haggis (1965, 1966) support the concept of micelles, but he called the subunits of the iron cores the micelles. He was able to show with different bright-field micrographs and electron diffraction experiments that the iron core of ferritin consists of crystallites of varying size and number in different molecules. The results Haggis (1965) obtained by electron diffraction experiments have been substantiated by other authors (Towe & Bradley, 1967; Harrison, Fischbach, Hoy & Haggis, 1967) using both X-ray and electron diffraction.

Recent investigations by others using lattice imaging have further substantiated the X-ray and electron diffraction work regarding the crystallinity of the iron core of ferritin (Matsuo & Ono, 1973; Massover & Cowley, 1973a, b; Massover, 1972). The work of Massover & Cowley has shown that the crystal lattice plane spacings in ferritin are observable using tilted-beam dark-field lattice imaging. They found that a diversity of conditions exists in the core’s ultrastructural appearance due to crystallite orientation, size, variable iron content and occupancy. Their results are consistent with the hexagonal model of Towe & Bradley who constructed a model related to hematite.

Fig. 5. Central beam stop images of Au crystallites with their [110] directions parallel to the optic axis of the microscope. The images have band-like structures in each Bragg reflection image produced by convergent beam illumination. Beam divergence \( \sim 7 \times 10^{-4} \) rad, electron optical magnification \( \sim 50000 \times \).
We have employed the multi-reflection technique as an alternative to the investigations mentioned above to obtain additional information on the iron core of ferritin.

IV(b) Experimental procedures and results

In these experiments, Pentex horse spleen ferritin, which was six times crystallized with cadmium removed, was used and supported on thin carbon films 50–100 Å in thickness. The microscope was operated at 100 kV for all the ferritin experiments with magnifications between 20 000 and 100 000 ×. A 5 μm central beam stop mounted across a 60 μm diameter objective aperture was used for all the dark-field imaging.

Dark-field micrographs of a dispersion of ferritin molecules using the central beam stop are shown in Fig. 6. They were taken at an electron optical magnification of 80 000 ×. Fig. 6(a) is the in-focus image in which the objective lens is focused so the image is considered sharpest. Fig. 6(b) is defocused by 8000 Å. Arrows indicate the same molecules in each micrograph. It is apparent from the molecules that are diffracting strongly that there is a considerable size variation of the micelles while, in some instances, no diffraction images are present.

Individual ferritin molecules that have single-crystal iron cores can diffract strongly at several Bragg angles simultaneously, corresponding to different reciprocal lattice points. Fig. 7 shows a focal series of central beam stop dark-field micrographs of a ferritin molecule that has a hexagonal diffraction pattern (the defocus steps were −8000 Å between micrographs). Fig. 7(c) shows the decomposition into six images corresponding to the six different Bragg reflections. The measured lattice spacing of the iron core giving this hexagonal arrangement was ~2.7 Å. This parameter was determined by measuring the image shift as a function of focus and was found to be consistent with the spacing of 2.55 Å reported by others using X-rays (Harrison et al., 1967), electron diffraction (Haggis, 1965) and lattice imaging (Massover & Cowley, 1973a, b). Fig. 7(d) shows the corresponding bright-field image of the molecule taken at a large underfocus value. There appear to be four subunits present in the bright-field micrograph but this structure does not correspond to the six dark-field images or their symmetry. Other subunit arrangements can be found at different objective lens defocus values in bright-field images. Since the size of the multiple diffraction images in Fig. 7(b) are quite close to the size of the bright-field image, the iron core must be essentially a single crystal and the four subunits observed in Fig. 7(d) are undoubtedly an imaging artifact produced by the phase contrast from the supporting carbon film.

Another example of a crystalline iron core of ferritin is shown in Fig. 8. Here the image decomposes into four diffraction images (Fig. 8a–d) and the measured displacement with defocus give planar spacings of 2.2 Å. This spacing has also been obtained in the other previously mentioned investigations. An interesting feature of Fig. 8(d) is the dark line image at the center of the pattern corresponding to the position of the central beam stop. The image is visible because there is an appreciable amount of diffuse scattering present in this region. The sizes of the individual multiple images in this example are considerably smaller than their bright-field images, thus the whole iron core cannot be a single crystal. The rest of the iron core either contributes to the diffuse scattering background observed or is not oriented to give a strong Bragg reflection. In the investigation of multi-reflection images of well ordered gold crystallites, this diffuse scattering was not found.

Fig. 6. Multi-reflection dark-field images of a large field of ferritin molecules (a) in-focus, (b) overfocus 7000 Å. Electron optical magnification ~80 000 ×.
Examples of ferritin cores which are polycrystalline are shown in Fig. 9. From Fig. 9(b), it can be seen that some cores must have several micelles since the patterns exhibit rings with several Bragg reflection spots, both strong and weak, arranged in a random manner around the ring. Other molecules exhibit a diffuse halo, characteristic of an amorphous iron core or a poorly oriented particle.

It is worth mentioning that both structural models of ferritin have a c axis dimension of 9.4 Å (Towe & Bradley, 1967; Harrison et al., 1967), but only Massover & Cowley observed this spacing by using direct lattice imaging. In our case, the weak diffuse halo of the ferritin did correspond to a region of reciprocal space with spacings larger than 4 Å and covering spacings on the order of the 9.4 Å unit-cell dimensions. However,
we could not distinguish whether this broad region in reciprocal space corresponded to the crystal structure of the iron core or whether its contribution is due to the apoферритin acting as an amorphous scatterer since the diffraction images from the iron cores were not significantly above the background noise level in this spatial region.

V. Discussion

V(a) Amorphous substrate intensity

In these experiments amorphous carbon films were used to support the crystalline particles. We have found experimentally that the background scattering contribution of the support film is much less in multi-reflection dark-field imaging than in SAD. The reason for this advantage is clear if the amplitudes of the scattering background from an amorphous film are compared for each of the two cases.

In selected-area diffraction all the \( N \) atoms in the area of the amorphous substrate defined by the field-limiting aperture scatter over a range of angles. The amplitude distribution at a particular angle, \( A_{\text{SAD}}(\alpha) \), from the substrate is proportional to \( N f(\alpha) \); where \( f(\alpha) \) is the atomic scattering factor and the phase relations between scatters are neglected.

In the multi-reflection dark-field (MRDF) technique, reciprocal space refers to the position in the image of a particular Bragg reflection for a fixed value of defocus; and each object point of the substrate is spread out into a disc in the image whose area is defined by the spherical aberration and defocus of the objective lens. The maximum size of this disc is defined by the radius of the dark-field objective aperture and every object point within it contributes background intensity to the center of the disc. In the MRDF method the amplitude contribution by the \( N' \) atoms of the substrate imaged in a particular image area is proportional to the integrated scattering from these \( N' \) atoms over the whole objective aperture (back-focal plane) divided by the solid angle subtended by this aperture. Thus, if the phase relations between scatters are neglected, the amplitude at the center of the disc due to the substrate film is proportional to:

\[
N' \int_0^{2\pi} \int_0^{\alpha_{\text{max}}} f(\alpha)\alpha d\alpha d\phi / \int_0^{2\pi} \int_0^{\alpha_{\text{max}}} \alpha d\alpha d\phi
\]

where \( \alpha_{\text{max}} \) corresponds to the largest scattering angle where there is transmission by the central beam stop aperture. The ratio of the amplitude distributions in the MRDF and SAD techniques can be used to compare the background intensity levels

\[
\frac{I_{\text{MRDF}}}{I_{\text{SAD}}} = \left| \frac{A_{\text{MRDF}}}{A_{\text{SAD}}} \right|^2 = \left| \frac{N' \int_0^{2\pi} \int_0^{\alpha_{\text{max}}} f(\alpha)\alpha d\alpha d\phi / \int_0^{2\pi} \int_0^{\alpha_{\text{max}}} \alpha d\alpha d\phi}{N f(\alpha) \int_0^{2\pi} \int_0^{\alpha_{\text{max}}} \alpha d\alpha d\phi} \right|^2
\]

The advantage of the multi-reflection technique is realized when \( N' \ll N \). For SAD, the smallest effective area which produces interpretable diffraction patterns is of the order of several thousand Ångströms in diameter, while the multi-reflection techniques can be used to image object areas comparable in size to particles from which crystallographic information is desired. As an example, for a given specimen area the ratio of amplitudes is approximately proportional to the ratio of the number of atoms, so taking the ratio between an area 0.5 \( \mu \)m in diameter in the field stop aperture for SAD and comparing it to an area 500 Å in diameter from which multi-reflections are imaged, the ratio of scattering amplitudes will be approximately \( A_{\text{MRDF}} / A_{\text{SAD}} = 10^{-1} \) and have an intensity ratio of \( 10^{-2} \). Under these conditions, the multi-reflection technique gives a much lower background signal than SAD and allows even faint diffraction images of individual crystallites to be observed (e.g. 18 Å crystal of Fig. 4).
Multi-reflection dark-field microscopy uses a magnification that is intermediate between a diffraction pattern and a high-resolution image. By using this relatively low magnification, the illuminating beam intensity can be low and hence the radiation dose and specimen damage can be minimized. The MRDF method only requires a level of resolution and magnification necessary to image the dark-field images corresponding to the Bragg reflections of each particle. For example, 20 Å particles with 2 Å planar spacings would require a magnification of at least 200,000 x for dark-field lattice fringes to be observed on a photographic plate (0.04 mm/spacing). The 20 Å particle's multi-reflection dark-field images would be visible at a magnification only 1/5 to 1/10 that required for the lattice imaging mode. If the same photographic conditions are used to record the image on the photographic plate for both the dark-field lattice images and multi-reflection dark-field images, the total beam exposure per unit area at the specimen will be reduced for the MRFD method by 1/25 or even by 1/100, a considerable reduction in the primary beam dose.

The MRDF method can provide more crystallographic information on individual particles than high-resolution lattice images. For multi-reflection dark-field imaging, examples of Au particles with eight simultaneous reflections with planar spacings of 2 Å and 2 Å were imaged. Even in the case of ferritin, six reciprocal lattice reflections with 2 Å planar spacings were visualized simultaneously. At best, lattice imaging would allow fewer reflections to be visualized simultaneously, 2 or 3 at this level of resolution. Exacting conditions of microscope defocus, astigmatism and beam tilt are required to form high-resolution lattice images in the phase-contrast imaging mode. The important point is that the multi-reflection dark-field method does not require this precision to extract planar spacings and crystal symmetries. The lattice imaging mode will allow more accurate measurements of planar spacings on a limited field of view, since it is limited only by the accuracy of magnification calibration or an internal standard. The multi-reflection method on the other hand can be used to sample a large field of particles in a single micrograph.

In our investigations, amorphous carbon films were used as support for small crystallites. The films' surfaces were perpendicular to the optic axis of the microscope since we employed a standard stage in the objective lens of the microscope. It should be pointed out that a single multi-reflection micrograph of a given particle may not have all the multiple images with the full symmetry that the exact reciprocal lattice plane will give owing to the curvature of the Ewald sphere and the exact orientation of the particle with respect to the substrate support. If our microscope had been equipped with a tilt stage, we could have readily brought the important crystal reflections of a given particle into a strong Bragg condition rather than searching for properly oriented crystals. Furthermore, several different orientations of a given particle could have been investigated to give full crystallographic information of each particle rather than only obtaining data from one reciprocal lattice plane. MRDF is particularly advantageous in this respect when compared to lattice imaging and SAD, since it is a straightforward method of observing a given field of view and the diffraction images over a wide range of orientations. In lattice imaging, the specimen has to be shifted at high magnification to obtain the correct image field. In SAD, the optics of the electron microscope must be switched between the image and diffraction modes to assure that the same specimen area is being sampled and is limited to relatively large crystalline areas with a high substrate background 'noise'. MRDF provides a straightforward method of observing a given field of view with several crystallites and their diffraction images over a wide range of orientations with relatively non-critical instrumental adjustments.

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