CALIBRATION OF MICROSCOPES FOR MAGNIFICATION AND RESOLUTION

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With the rapidly increasing emphasis on quantitative results, it is very important to know what the magnification of a microscope really is. The various available calibrations are described, together with their limitations. It is furthermore important to ensure that a microscope is performing to specification, or at least to an adequate level for the experiment in hand. The appropriate tests for resolution and instrument performance are reviewed.

Keywords: Microscope, Calibration, Magnification, Resolution

Whenever we use any kind of microscope, be it a research light microscope, a scanning electron microscope or a TEM, we need to know the magnification. The manufacturers helpfully provide this information in approximate form, and for many applications this is all we need to know. However, as soon as any work involving the dimensions of the specimen under examination is undertaken the instrument needs more exact calibration.

Calibrating the light microscope

Measurements are normally made by using a graticule within the eyepiece, which lies in the focal plane of the eye lens. However, this graticule is only a comparative measure as it assumes the magnification of the objective lens and also the tube length of the microscope which in some instruments can be varied. It is necessary therefore to place a calibrated scale in the object position, and to project the magnified image on to the plane of the eyepiece graticule, so that the latter may be calibrated. Stage micrometers may be either transparent and covered with a cover glass or reflective and without a cover glass (for incident light instruments) (fig. 1). The calibration is only valid for the particular objective and eye lens being used and for the particular tube length at that time (one assumes that the objective lens is fully screwed into its mount, and that the eye lens is properly seated in the eyepiece tube).

Once this calibration has been carried out for a given pair of lenses, it will remain valid provided that there is no change in any of the mechanical parameters.

A recently developed test specimen is a silicon chip which has been electron beam written with a square mesh of lines of approximately 10 micrometres, and with thicker lines every 500 micrometers was primarily designed for the SEM but is found very useful also for incident light microscopes, as the contrast is good (fig. 3).

Figure 1

Calibrating the SEM

The electron lenses project a demagnified image of the electron source on to the specimen under examination, in a scanning raster normally controlled by electromagnetic deflector coils and driven in synchronism with the display tube raster. The magnification is determined by the size of the scanned raster on the display tube divided by the size of the raster on the specimen.

It is clear that the magnification therefore depends on the excitation of the scanning coils, modified by any remanent magnetism or accidental capacitance. It is quite possible that the two scan coils will not yield exactly the same magnification, so giving rise to orthogonal distortion. The scan on the display tube is also subject to distortion and non-linearity. Even after the system is calibrated, there will be no guarantee that the calibration will not change with time.

The magnification is also rather sensitively dependent on the working distance (objective lens to specimen) which is best determined in terms of the objective lens current. The calibration must take this factor into account.

Since the magnification range of a scanning electron microscope starts within the range of the light microscope but goes up to very high values, different calibration specimens are needed to cover the range.

As a matter of principle, in any regular object used for calibration, one should measure a number of intervals so as to average out any small variations in individual dimensions.

The calibration specimen frequently used for the lowest magnification is a piece of fine copper mesh with 1000 bars per inch (about 40/mm), a piece of which can be mounted directly on to a suitable stub (fig. 2). In the same size range would be the

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replica of a coarse grating of 19.7 lines per mm, which is one of the standards originally specified by SIRA as part of a calibration kit. A square mesh of fine lines etched on a silicon chip, with a repeat spacing of 10 μm is another useful specimen (fig. 3). At higher magnifications a much finer grating of 2160 lines per mm is suitable (fig. 4). This grating is made by forming a resin cast of a master diffraction grating and then replicating it in a metal so that there is a conducting specimen to be examined. In a scanning microscope it is particularly important to use orthogonally ruled gratings so that any distortion in the scan or raster can be readily perceived.

Calibrating the TEM

The image on the viewing screen of a transmission electron microscope is formed by successively magnified images in the objective lens, intermediate lenses and projector lens. The strengths of these lenses are altered by the excitation currents in the windings and the calibration given by the manufacturers assumes that the resultant magnetic fields which form the lenses are in constant relationship to the exciting currents. In practice there is a considerable hysteresis in the iron circuits and it is very important to approach the required value of excitation from a previously known direction of current (for example always working from maximum excitation down to the required value). It is also important that the current flow in each lens is always in one set direction (normally chosen by the manufacturer) as otherwise the calibration will be invalidated. In some instruments provision is made for cycling the lens current automatically when it is required to obtain reproducible magnifications in the system. The calibration is also sensitively dependent on the plane of the specimen in the objective lens.

In view of these qualifications, it is unrealistic to expect that a magnification calibration can be reproducibly obtained even to an accuracy of 1%, and the calibrating procedures used need only aim at this figure as a limiting performance.

At low magnifications the specimen support grid itself will normally provide an adequate magnification standard provided that the spacing of the grid bars is averaged over at least 20 bar spacings. The next range of magnification is satisfied by shadow cast carbon replicas formed from an original plastic impression of a diffraction grating (fig. 5). The common sizes are 1200 lines per mm and 2160 lines per mm. These gratings can be used into the middle range of magnification of a transmission electron microscope. Their accuracy depends however on the care with which they have been prepared, as the intermediate plastic replica may stretch if handled incorrectly. While such replicas should normally be good for 2% accuracy, it would be rash to guarantee an accuracy better than 5%.

At the top of the magnification range one can examine crystal lattices of known orientation which have accurately determined spacings. The finest of these in common use is the gold lattice in a single crystal layer in which spacings of 0.204nm, 0.143nm and 0.102nm can be detected (fig. 6). A series of crystals with slightly larger spacings are also available. Graphitised carbon black, with plane spacing 0.34nm, is commonly used.

Rather larger spacings of 1.75nm and 1.3nm, Murata et al (1) can be obtained from chlorocopper phthalocyanine crystals, but these are the largest commonly available as calibration specimens. Considerably larger spacings have been imaged by investigators studying more complex chemical compounds, but the procedures necessary to obtain the images may involve computer simulations, and these are not convenient for the average microscope user.

We are left with a gap in the middle of the magnification range which is not very satisfactorily served by any readily available calibration specimens. One could use the lattice spacings of catalase crystals (8.75nm and 6.85nm) (fig. 7) but there is considerable diversity in the published figures (Wrigley, (2) 1968) for this lattice and it cannot be regarded as a very accurate basis for calibration. The normal way of covering this region of the magnification range is to extrapolate from a diffraction grating replica and measure the distance between two features on a diffraction line whose spacing has been determined at a lower magnification. There is a danger here of multiplying errors involved in the earlier calibration.

Calibration by standard spheres

Mention may be made here of an earlier type of magnification standard which originated with the discovery of a batch of polystyrene latex spheres with a remarkably uniform diameter of 0.254 μm. This later led to the production of uniform latex particles by the Dow Chemical Company in sizes ranging from 0.1 μm up to 1.1 μm approximately. Particles of this type are very valuable in that they can be mixed with the preparation under examination and the comparison with the particles of a known size can be made directly. If the latex particles in known concentration are added to the preparation under examination which is to be prepared by a spray droplet method, it is even possible to calculate the accurate size/weight distribution in the unknown material.

A few cautionary observations should be made. There is no guarantee that any particular particle is accurately of the size stated as the mean diameter. For example, for a mean diameter of 0.22 μm the standard deviation is 0.0026 μm which appears to be remarkably small. This mathematic truth conceals the fact that there are still recognisable numbers of particles which are visibly larger or smaller than the mean. It is essential, therefore, that any preparation containing such latex particles should contain sufficient numbers for a statistically significant count to be made.

More recently, standard particles of larger dimensions have become available. Perhaps the most spectacular has been the particles prepared in space to obtain great accuracy in sphericity and size and certified by the National Bureau of Standards (fig. 8). These particles are of 9.89 μm mean size and so fill a very useful gap in the calibration range for light microscopes and scanning microscopes. However, even this uniform preparation with a very narrow gaussian distribution of size, still contains some larger particles (less than 1% of the total) which have to be excluded from consideration. Here again, therefore, it is essential to count a statistically significant number of particles. We decided that these exotic particles justified the title of 'Celestrial Spheres', rather than the more prosaic 'space balls'.

More recently still have become available a range of latex particles (Dynospheres) in a size range 0.5 μm to 20 μm with a very narrow size dispersion and which are widely used in light microscopy (fig. 9). The size accuracy is ±3% and the coefficient of variance is ±2%.

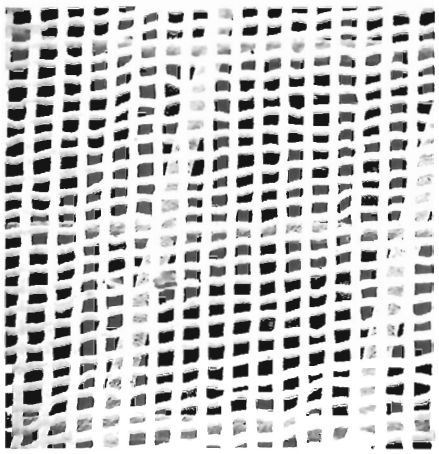
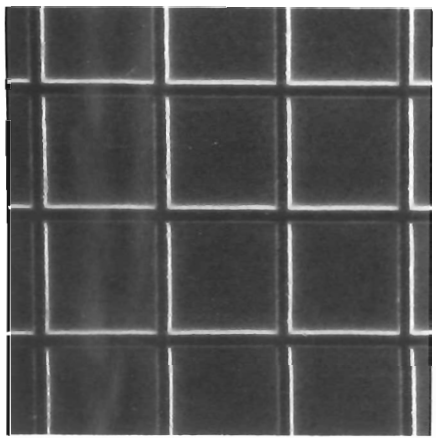


Figure 2



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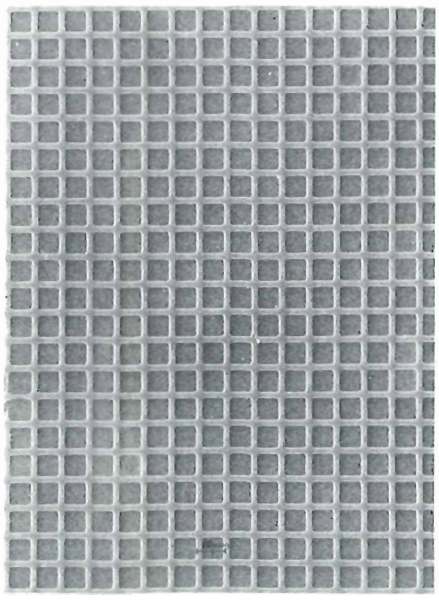

Figure 3

Measurement of resolution of an instrument

The resolution obtainable from any microscope is not needed to be known for interpretation of an image, but it is desirable to measure the performance occasionally in order to ensure that the instrument is operating optimally. There are very few specimens which can simulate the theoretical basis for assessing resolution and it is usual to

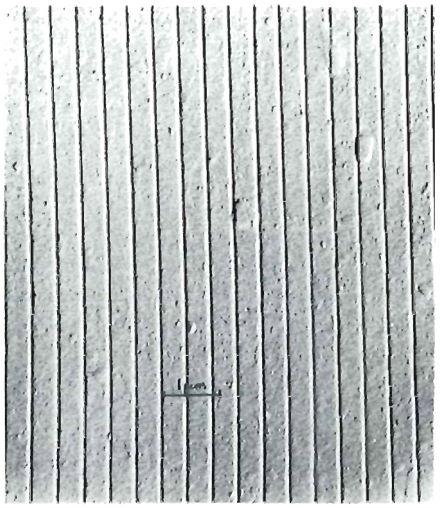

Figure 5

Figure 6

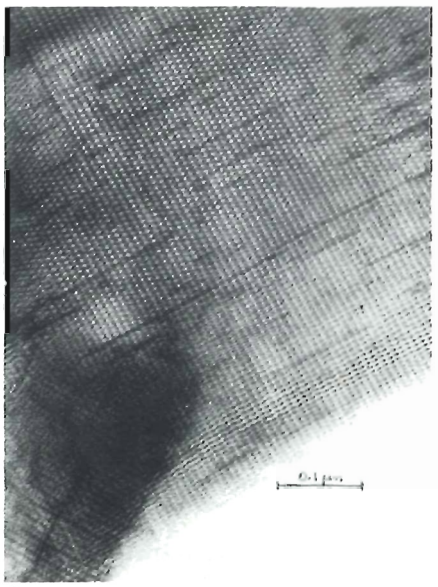

Figure 7

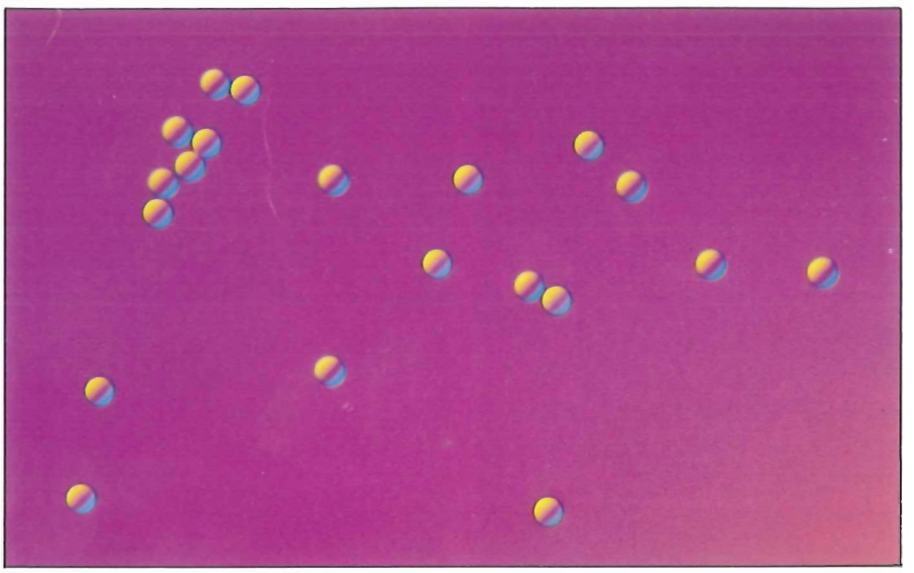

Figure 8

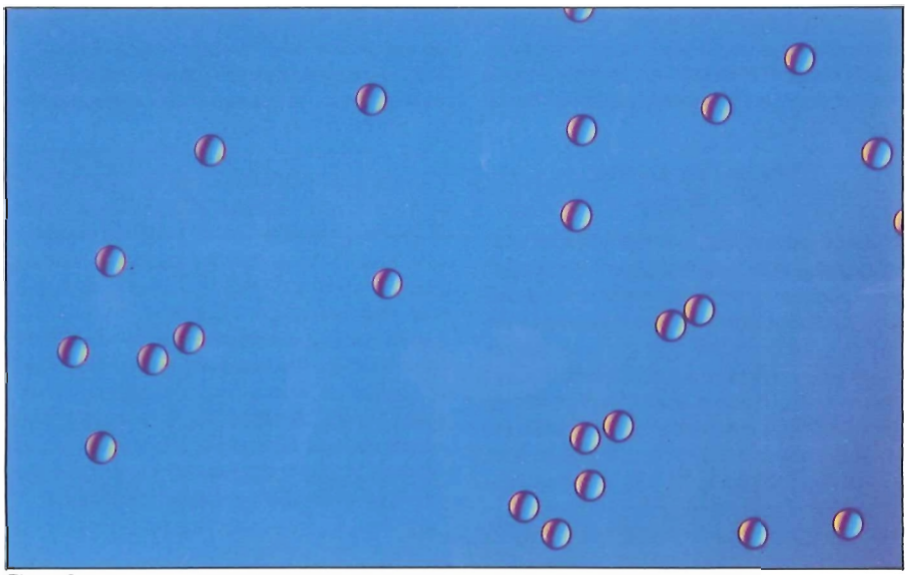

Figure 9

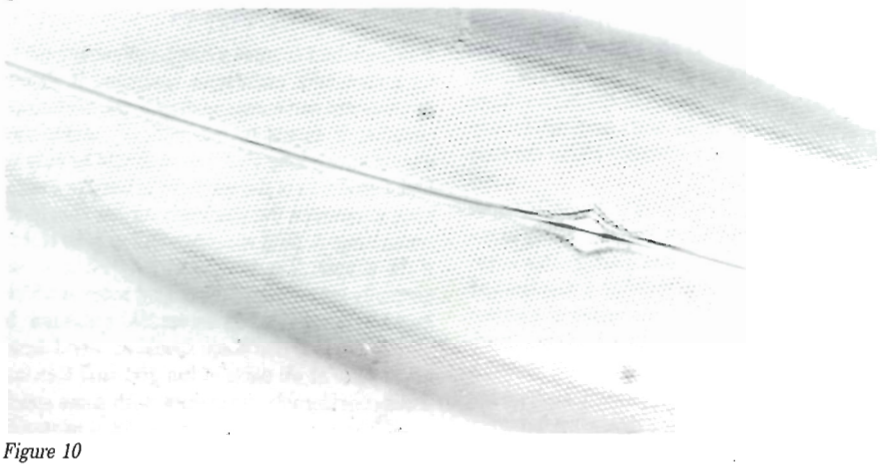

Figure 10

examine instead the appearance of known objects in order to assess the intrument performance.

Light Microscopy: The test of ultimate performance is quite difficult because the instrument is operating near the theoretical limit determined by the wavelength of the illumination. The depth of field is extremely small and diffraction effects can be troublesome. The test object has historically been one of the Diatoms which have very fine pores in a silicaceous structure which happen to lie near the resolution limit of a good microscope (fig. 10). The resolution is then judged by the detectability of these pores in an in-focus portion of the image. This is a highly subjective test, and of recent years, it has often been preferred to examine some of the Dow polystyrene spheres which are fairly well characterised as to size and which therefore make any judgement more quantitative. It is gratifying to know that the National Physical Laboratory is now investigating an objective method of measuring the instrument performance.

Scanning microscope

In the period when the performance of scanning microscopes was modest it was usual to examine small spikes of silver in silver mesh grids as a measure of the ultimate performance. One would now normally examine gold particles on a carbon background and look for two small adjacent particles which could be resolved as separate (fig. 11). The minimum space resolved between their edges, divided by the magnification, gives the resolution. The variation in sizes of the evaporated gold par-

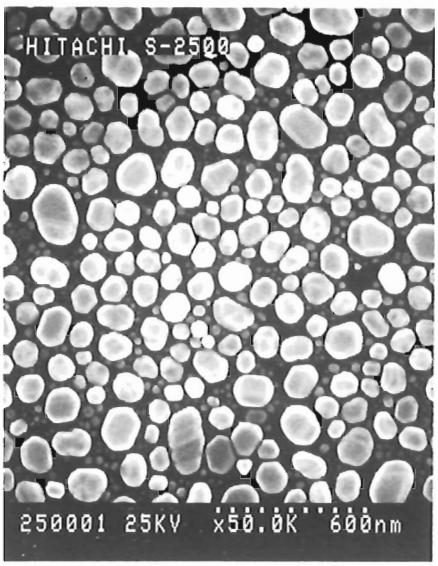

Figure 11

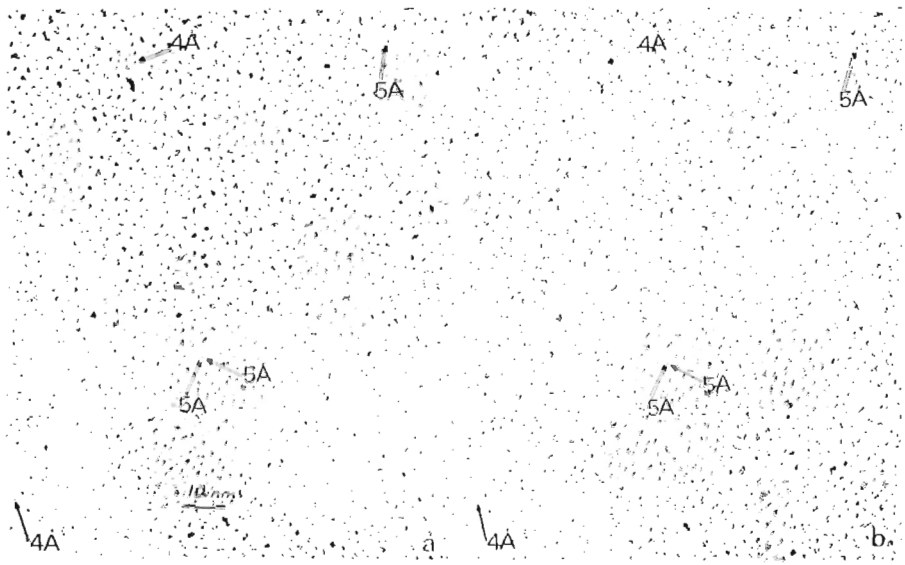

Figure 12

ticles and the spaces between them provides a convenient range of measurement to accommodate a considerable performance range from the different microscopes.

Transmission electron microscopes

For many years the test of resolution was the separation of small evaporated particles of platinum-iridium alloy on carbon support films. One sought to find pairs of particles separated by the minimum distance which were distinguishable in two successive micrographs, to ensure that there were no undue electron noise effects in the image (fig. 12). This test worked very well while the performance was in the range of 0.5nm to 1nm. However, an analysis by Dowell (3) showed that the number of corresponding pairs of particles in successive micrographs decreased very sharply for spacings less than 0.5nm, and this became explicable under the contrast transfer function theory when it was realised that the visibility of fine structure depended critically on the state of focus of the microscope and that at this level of resolution very small variations in the high voltage or current supplies could cause particles to vary between visibility and invisibility within the time scale of an observation.

Another popular way of testing the performance of a high resolution microscope is its ability to image lattice planes of very fine spacing. This is certainly an excellent test of stability of supplies and of the specimen stage, but the image of a lattice can be formed from just one diffracted spot in addition to the primary beam and the full aperture of the lens is not utilised in forming such an image. While the lattice plane image performance is therefore a sensitive test of stability, it has to be distinguished from a statement of the ultimate resolution of the microscope.

As was stated earlier, the appearance of an image of regular structure can change radically with the state of focus of the objective lens and for a very high performance microscope it is probably fruitless to look for a particular micrograph to prove its performance. A more useful test is an optical diffractogram prepared from a micrograph taken under optimum conditions. This will display the maximum spatial frequencies which have been transmitted through the imaging system in an unequivocal way, uncomplicated by considerations of electron noise or of image contrast. The same pattern also shows whether any astigmatism resides in the image.

Not everyone wishes to test the microscope in this way and a valuable check for factors affecting performance is provided by the examination of a perforated carbon film in a slightly over-focus condition. This test was first proposed by Haine and Mulvey (4) and while it does not serve as a true resolution test, it will indicate the source of any disturbance in the system which would limit the resolution. One can easily observe the effects of astigmatism, lens or high voltage instabilities, specimen drift or vibration. It is a quick and simple test and most of the information can be obtained by observation of the image under the telescope while the microscope is operating.

Requirements for a calibration specimen

It is important to meet a number of requirements for a successful calibration specimen. It should be clean and non-contaminating to the microscope vacuum, it should be mechanically stable and should have a long shelf life. It should be easy to find a good area of the specimen to use. For a scanning microscope the specimen surface should be conducting. A TEM specimen will only be stable if the support film is substantially intact — we normally set a limit of 5% of grid squares which may be damaged in an acceptable specimen. It is also desirable that there should be useful areas to examine at all parts of the grid, and this imposes considerable difficulties with some specimens. The requirements for a long life is relatively easy except with some of the larger lattice plane specimens which are often susceptible to beam damage.

Traceable standards

There is an increasing call for calibration specimens to be traceable to some national standard. This is readily achieved with stage graticules which can be calibrated at the National Physical Laboratory or could be compared against such standards with reasonably simple equipment. The coarser gratings used for calibration of scanning microscopes could be calibrated to an accuracy of about 1% at the limit of performance of existing interferometric methods. At the moment, there is no nationally recognised standard calibration available for the 2160 lines per mm gratings, although there are hopes that such calibrations will become available in the not too distant future. There is no early prospect of being able to check the validity of the replicas of diffraction gratings prepared for transmission electron microscopy.

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Legends to Figures

Figure 1. Stage micrometer (inset, magnified view of scale) (Courtesy Graticules Ltd.)
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Figure 3. Silicon chip with orthogonal ruled grating 10 μm spacing.
Figure 4. Diffration grating replica 2160 lines/mm. SEM micrograph.
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Figure 10. Diatom: Pleurosigma Spp., probably p. angulatum (Courtesy Dr S. Bradbury).
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Figure 12. Platinum-iridium particles evaporated onto a carbon substrate.