Microscopy: Depth Of Focus, Optical Sectioning And Integrating Eyepiece Measurement

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INTRODUCTION

Often in histological and pathological work the specific volume or specific surface of a given phase with respect to the surround must be determined. For example, increasing emphasis is being made upon relation of tissue measurements to a unit volume of tissue rather than to less precise relationships such as mitoses per field. Desirable as unit volume relationships may be, few biologically oriented investigators using the microscope as one of their basic working tools know that methods have been worked out for determination of specific volume and specific surface. These methods are simple, effective and require a minimum of equipment. With their aid and an understanding of the reasoning behind their design, it is possible to measure microscopically and readily apparently impossible things such as the total alveolar surface area in the lung, the total capillary surface area in the kidney, the total surface area of bone in the intact and living skeleton, the volume ratio of acidophils to basophils and to entire gland in the pituitary, or the volume ratio of proximal to distal convoluted tubule in the kidney.

Often information of this type, in association with information gained by other means, leads to a complete synthesis of physiological events. It is the purpose of this paper to present the methods, the reasoning and describe the apparatus necessary for such measurements, partly for the interest of others and partly as a documentation of methods used in the Henry Ford Hospital Orthopaedic Research Laboratory in measuring the specific surfaces and volumes of bone and in measuring bone formation rates by several methods. It can be readily appreciated that the methods are too lengthy to include in a paper whose main topic is, first, unrelated to the stochastic theory involved and, second, itself lengthy.

The methods to be described depend upon the technique of optical sectioning which in turn is related to the depth of focus of microscope objectives.

The material will be divided and presented in the following categories: Depth of Focus; Optical Sectioning; Specific Volume Measurement; Specific Surface Measurement.
Diagram of a microscope and the relationship of its optical axis to the (X) (Y) (Z) axes of polar coordinate space. The optical axis coincides with the (Z) axis. The (X) and (Y) axes correspond to East-West and North-South movements, respectively, of the mechanical stage. The resolution of objectives along the (Z) axis is poorer than along the (X) and (Y) axes. The (Z) or vertical resolution is familiar to the camera enthusiast as the depth of focus.

**DEPTH OF FOCUS**

While vertical resolution can be calculated from theory outlined in texts such as those of Hardy and Perrin,\(^{12}\) Jenkins and White,\(^{15}\) or Valasek,\(^{24}\) in practical microscopy theory is only a baseline. The resolutions and contrasts actually achieved in a real situation may vary greatly from those predicted from theory. Recently published attention has been devoted by Foster,\(^{8}\) among others, to the fact that (X) (Y) resolutions with the light microscope may exceed those predicted by theory by from 20% to around 60% (Figure 1).
This is not a contradiction of physical constants; rather it is a reflection of some of the inadequacies of optical theory which has never been considered perfect by those who developed it or those who are conversant with it.

Some of the formulas used for calculation of objective resolution are given in figure 2. They are taken from Needham's excellent book. They are not intended to be precise and they illustrate the variation in basic assumptions made by different theorists to permit mathematical formulation. Actually complete theory is complex and, mathematically speaking, advanced.

A host of factors may affect resolution favorably or adversely, some of them being: whether the object is totally absorbing, self luminous, partially or wholly transmitting; the dominant wave length of the light source, the distribution of energy in the spectrum of the source and the relationship of these factors to color, reflectance and absorption by the object; phase effects within the object and between and mountant; phase effects, figure, surface perfection, reflectance and absorptance of the optical elements above and below the object; polarization effects in the object and optics; corrections of the objective, eyepiece, intermediate optical elements, coverslip, slide and homogeneity of mountant; numerical aperture; tube length, magnification; optical aberrations of the observer's eye.

### RESOLVING POWER

\[
\text{Abbe} = \frac{\lambda}{\text{N.A.}} \quad \text{(Narrow, central illuminating cone)}
\]

\[
d = \frac{\lambda}{2 \times \text{N.A.}} \quad \text{(Oblique illumination)}
\]

\[
d = \frac{2 \times \lambda}{\text{N.A}_{\text{obj}} + \text{N.A}_{\text{cond}}} \quad \text{(Dark Field)}
\]

Berek: \[
d = K \frac{\lambda}{\text{N.A.}} \quad \text{(Full cone; } K = 0.3 - 0.4)\]

Rayleigh: \[
d = \frac{\lambda}{2 \times \text{N.A.}}
\]

\[
d = \frac{1.22 \times \lambda}{\text{N.A.} \times K}
\]

\(d\) = minimum separation resolved, microns

\(\lambda\) = wavelength of light used, microns

\(K\) = a constant

\(\text{N.A.}\) = Numerical aperture

**Figure 2**

Some formulae for the (X) (Y) resolving power of microscope objectives. It can be seen that increasing N.A. and decreasing the dominant wavelength of the illumination improve (X) (Y) resolution. It can also be seen that different assumptions are made in formulating the physical systems involved by different theorists. Note that nothing is specified in these formulations about phase, polarization, absorptance, emission or refractive index aspects of the object. These omissions, consciously made by the theorists, have been overlooked by less competent individuals who have erroneously assumed these formulas are absolute and immutable. The theorists who derived them never intended this to be.
There are others but the point is made: the observed result of a given set-up is the summation of a large series of independent variables and is complex.

In the present study the depth of focus of a series of microscope objectives has been measured visually. The purpose was to obtain realistic values of the depth of focus of various objectives that would permit correction factors to be applied to measurements that depended on optical sectioning for validity. Since these measurements are done visually, the judgment of the microscopist is one of the important factors and must be measured with the optics. It is possible to measure the depth of focus photographically with much more precision and several such studies have been done in the past.

**Methods**

The ball-bearing fine motion of a Zeiss "WL" stand was used for all measurements. The adjustment was run in only one direction during each measurement to eliminate lost motion. The adjustment has no backlash. Seven or more separate measurements were taken.

![Graph](https://via.placeholder.com/150)

**Figure 3**

A plot or logarithmic scale of depth of focus of objective against the product of N.A. and objective magnification. The plot is nearly a straight line. The solid line represents the average plot of fluorite objectives. Apochromats of the same N.A. and magnification give improved resolution and are represented by the dotted line below the solid line. The achromats give slightly inferior resolution and are represented by the dotted line above the solid line. These differences are less evident at low N.A. and low magnification. An unknown objective can be interpolated along the plot provided it is adequately corrected. Poorly corrected objectives will produce values of focal depth above the plot. Objectives should not be criticized by the focal depth criterion unless used with the eyepieces designed for them and unless air-glass surfaces are clean.
done with each objective and optical set-up listed in Tables I and II and averaged. All measurements were done by one observer, said observer possessing normal accommodation and 20/20 visual acuity with less than 0.25 diopter astigmatism.

Numerical apertures were checked against a Metz apertometer made by Leitz. The degree of illumination of the objectives was checked by inspection through a phase telescope placed in the left hand tube of a binocular head. The measurements were done through the right tube. Kohler illumination was used unless specified, with the modification that a ground glass diffuser rather than the lamp filament was focused on the back focal plane of the objective and on the front focal plane of the substage condenser. This arrangement has become popular with the manufacturers of built-in light sources because it makes the manufacturing tolerances of the lamp less critical in ensuring proper centration and even illumination.

The object was the same for all of the studies and consisted of specks of dirt on the surface of a slide covered with a coverslip 0.15 mm thick, using H.S.R. (xyloc) as a mountant. The specks were selected because they were totally absorbing and so small that their finite dimensions in \((X) (Y) \text{ coordinate space were less than the resolving power of the objective being used. The field was a homogeneously radiant one. This set-up permits the maximum resolution compared to setups in which the object is radiant in a dark field and so on.}

The light source was a 15 watt low voltage tungsten filament lamp made by Zeiss for the illuminator of the “WL” stand. An achromatic, aplanatic condenser was used unless otherwise specified in Tables I and II.

**Measurements**

By vertical resolution is meant the distance along the \((Z)\) axis within which the object appears to be in sharp focus. (See Figure 1) Obviously, the experience of the microscopist and his judgment will affect any such measurements — and it is this effect that is needed to determine correction factors. The accommodative ability of the eye is more important as a source of variability in low N.A. objectives than with high N.A.

In Table I note that:

Increasing N.A. decreases depth of focus.

Increasing correction of the objective decreases the depth of focus. Achromats are the workhorse objectives corrected for two colors for definition and for one color with respect to spherical aberration and coma. Fluorites are considerably better corrected than achromats. Apochromats are the most highly corrected — and expensive — optical elements available in a microscope. They are corrected for definition with respect to three points in the spectrum and for spherical aberration, coma and chromatic difference of magnification with respect to two colors. Some of this correction is built into the compensating eyepieces which should be used with apochromats to obtain the full benefit of their corrections.

In Table II note that:

Considerable change in condenser N.A. with respect to the N.A. of the objective produces minor changes in vertical resolution. Resolution is increased by increasing the condenser N.A. See columns 2, 4, 5, 13, 14, 15.

Improvement in vertical resolution by using darkfield illumination was less than the probable error of the measurements (20 per cent). Using bluer light by selecting the proper Wratten filters significantly improves vertical resolution. See columns 6-9, 12, 13, 16.
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**Table I** DEPTH OF FOCUS ACCORDING TO PRIMARY MAGNIFICATION AND OBJECTIVE TYPE

All objectives listed are different; the table therefore lists tests on 14 objectives. All air-glass surfaces were cleaned with Kodak lens cleaner before the tests. Air glass surfaces between objective and eye were antireflection coated.

Zeiss (a): Western Zone, post WWII “K” eyepiece: Zeiss Compensating. Zeiss (b): Jena, pre WWII Zeiss (c): Jena, Post WWII

**Table II** DEPTH OF FOCUS ACCORDING TO ILLUMINATION AND EYEPICE

The same objective was used in columns 2-5; the same objective was used in columns 6-11, the same objective was used in columns 12-16.

In column 11 the K8 Quad. eyepiece is an adjustable Zeiss quadratic diaphragm eyepiece whose aperture in the focal plane was restricted to 300 μ in this test. The term IR in filter columns indicates an infra-red removing filter.

In column 2 a dry darkfield condenser of 0.8 N.A. illuminated the specimen.
Day to day variations in the microscopist significantly affect the reproducibility of the measurements, the range of variation found being on the order of 25 per cent of the values determined. See columns 6, 10 in Table I and columns 2, 13 in Table II.

Increasing the eyepiece magnification improves vertical resolution, but only to a certain point. Any gain in vertical resolution achieved in practice by this means is at the expense of the amount of the specimen visible at any one time.

In figure 3 the vertical resolution is plotted against the N.A. and magnification. The writer was surprised to find the vertical resolution of the N.A. 1.32 fluorite objective as large as 0.46 micron. Most texts which mention this problem imply that the vertical resolution of oil immersion, large N.A. objectives is vanishingly small; none of the references listed in the bibliography give concrete examples of depth of focus measurements on large N.A. objectives.

The focal depth-correction nomogram in figure 4 reveals the importance of the values for vertical resolution given in Tables I and II and graphed in figure 3.

**OPTICAL SECTIONING**

*The Importance of Ratio of Thicknesses*

Consider a 100 μ thick section cut through cortical bone. Observe a small area, say 1000 square microns, of this section. A number of osteocyte lacunae are seen lying within the area; assume them to be 20 in number. These lacunae will appear to occupy about 70 per cent of the total area of the 1000 square microns being considered. Already it is intuitively evident that the apparent occlusion of 70 per cent of the area by 20 osteocytes is not a true picture of the frequency of occurrence of osteocytes within bone, nor is it a true estimate of the proportion of the total volume of the bone that is occupied by osteocytes.

Assume now that the thickness of the section has been reduced to 1 micron by suitable grinding and the 1000 square micron area previously inspected is again inspected. Only one lacuna is seen where before there were 20. Because lacunar dimensions are about 5 x 15 x 20 microns, further reduction in thickness of the section will lead to nearly negligible further reduction in the apparent proportion of the 1000 square microns that is occupied by lacunae.

The 100 micron thick section was too thick to yield valid measurements of lacune, but the 1 micron thick section is satisfactory. Somewhat thinner sections would be slightly better. It becomes evident that the ratio of thickness of section to vertical dimension of the phase being measured is important in establishing the validity of all measurement done on a section. By a phase is meant any aspect of a tissue being measured. The phase might be the total volume of capillary lumen, the total volume of nuclei of osteoclasts or the total extracellular volume. When section thickness is an insignificant part of the vertical dimensions of the phase being measured, valid measurements may be made.

If the proportional area of a phase is measured on an infinitely thin section, the resulting measurement is also the measurement of the proportional volume of the phase in the solid. In the case of an infinitely thin section the vertical
dimension of the measured phase is immaterial. It is assumed for the moment that certain sampling and homogeneity requirements are fulfilled. The method of making the actual measurements will be dealt with later; for the moment it may also be assumed that, given a section, the area of any phase whatsoever may be measured.

Figure 4

Focal depth — correction factor nomogram. If focal depth \( f_D \) and average vertical dimension of measured phase \( D \) are known, the correction factor \( F_C \) which must be divided into the working measurements can be found. Lay a ruler on the value for \( f_D \) left hand vertical bar; holding this intersection point, slant the ruler until the edge intersects with the value of \( D \), middle vertical bar. Where the ruler intersects the \( F_C \) vertical bar, right, is the value of \( F_C \). The measurement of specific volume must be divided by \( F_C \) to obtain the value that would have been measured on an infinitely thin section.
MICROSCOPY

There are no physically real, infinitely thin sections. As a result, there is always some finite ratio between thickness of section and vertical dimension of the measured phase. This introduces a certain amount of error into measurements. This error becomes vanishingly small as the ratio between section thickness and vertical dimension of the measured phase becomes vanishingly small. But this means that if a real section thickness that is an insignificant fraction of the vertical dimension of the measured phase can be obtained, measurements may be made upon it that are valid. Furthermore, it should be possible to determine correction factors for the ratio of the thicknesses to correct measurements to zero error when the ratio of thicknesses is less than optimum.

Some Physical Solutions to Ratio of Thicknesses Problem

A polished surface of an opaque substance is an excellent real representative of an infinitely thin section and for most purposes may be considered infinitely thin, although in reality it is not. Such surfaces must be examined by reflected light. Replicas of solid surfaces, transparent or opaque, may be made for measurement and for most purposes are excellent. Replicas may be made of frozen tissue surfaces etched with various solvents.

Figure 5
Cross section, undecalcified, femur, 50 year old man. There are numerous large vascular channels termed resorption spaces. There are far more but smaller channels not resolved in this illustration.
FROST

To convert the preceding thought to physical feasibility (no sane person will drop a pin 10,000 times on a photograph), a device is needed which will cast arbitrarily spaced points over the image formed by the objective; which will permit the points to be seen in focus with the image from the objective and which will permit enough points to be seen in any one field so that an adequate number of total possible hits can be compiled in a reasonable time.

Point Counting Practice

Any micrometer eyepiece will permit the installation of a reticle which may be seen in a sharp focus at the same time as the image formed by the objective.1,2,10,11,12,14,17,22

A net micrometer installed in such an eyepiece will serve to provide the necessary points. Where the orthogonal lines of the reticle intersect, there is a point or possible hit. Such a reticle is illustrated in figure 6. The Zeiss Integrating Eyepiece I (Figure 7), is an example of such equipment.23 It was designed specifically for the present purpose, the number of possible hits and the angular widths of the reticle lines being adapted for average corrected vision. The actual eyepiece contains 25 possible hits, not the 10 illustrated.

In figure 8 a higher power view of the femoral cross section is illustrated with the Zeiss reticle superimposed. Two hand tallies are used in the measuring procedure. One records the total number of fields measured; this number, multiplied by 25, gives the total number of possible hits in these fields. The second tally records the actual hits which, when divided by the total possible hits, yields the area of the measured phase with respect to its surround in terms of the percent of the total area.

It can be seen that by rotating the eyepiece after each measurement, a new

![Figure 6](image-url)

![Figure 7](image-url)

In (a) the appearance of one style of net micrometer is seen as it would appear on looking through the microscope. For the purposes of the text, the points of intersection of the lines would be considered possible “hits”. There are 16 possible hits in this particular reticule. There are other styles containing 100 possible hits but the best number to use is between 20 and 30.

(b) refers to the text dealing with specific surface. The same net micrometer reticule may be used but in this case one ignores the vertical lines and observes only the intersections of the horizontal lines with the surface of the phase being measured with the microscope.

The appearance of the reticule in the Zeiss integrating eyepiece I is diagrammed. The real reticule has 25 possible hits instead of the 10 illustrated. A hit lies at the intersection of the short vertical bars with the longer horizontal ones.
distribution of the measuring points is obtained and a new field may be measured. Similarly, when making measurement with N.A. over 1.0, a change in the setting of the fine motion adjustment brings a new vertically displaced field into view and permits a new measurement. These steps increase local precision but are permissible only if all areas measured are treated alike.

The only remaining problem is that of sampling the tissue so that a representative portion of it is measured. If sampling is not adequate, very precise measurements on poorly chosen fields may lead to erroneous, and possibly embarrassing, figures.

**Sampling**

In figure 5 a femoral cross section is illustrated. Assume the vascular area is desired. Because of the longitudinal grain, and because of the thinness of the section, optical sectioning is not required and the N.A. of the objective is immaterial in that respect. Enough resolution is needed to give good definition of the walls of the spaces and to permit the observer to differentiate between Volkmann's canals.

**Figure 8**

An enlargement from the section illustrated in figure (5). Several Haversian canals may be seen in cross section. The numerous, small, oval features are osteocyte lacunae and the nucleus of the osteocyte may be seen in many of the lacunae. Superimposed on the illustration is part of the reticule of the Zeiss eyepiece, drawn in rather than photographed however. The short, vertical bars intersect with the continuous, horizontal ones. Each intersection is a possible hit. If the whole reticule has 25 possible hits, then each field also has 25 possible hits. One may therefore keep track of the number of fields and the number of hits, not bothering to count all of the possible but not actual hits with each field. This shortens the procedure appreciably.

There are 9 possible hits in the field. One of them, the farthest right on the middle row, is tangent on the wall of a vascular channel. There are no clear hits on vascular channels in the field.
FROST

cut into by the upper section surface (measured) and those not (not measured). A 10X objective, N.A. 0.32 is used. This objective has a rather small field so that complete scanning of the section in increments of exactly one field diameter and in strips exactly one field diameter apart would take an unnecessarily long time (Figure 9). Accordingly every second strip should be skipped. Under some circumstances one may choose instead to skip every second and third strip, or alternatively to skip every other field but not skip any strips. In this manner the section is sampled and no significant bias is introduced into the sample. To ensure orthogonality of scanning, a mechanical stage with orthogonal motions is essential.

Figure 9

A diagrammatic method of scanning a large section to obtain adequate sampling of it. Begin at the upper left, work along towards the right in steps exactly one field wide, and when the right edge of the section is reached, move down one field diameter and move the other way. Continue in this manner until the section is scanned to the bottom. Obviously when the endosteal or periosteal edge enters the field, that field is not counted but the next horizontal row is begun. With small sections and thin cortices large magnification will be needed and, usually, an increasing number of separate sections must be measured and averaged to obtain valid results.

Again consider figure 5. This time we wish to measure the area of osteocyte lacunae throughout the whole section. Because of their small dimensions, N.A. of over 1.2 is necessary to resolve them adequately and to produce the necessary thinness of the optical sections on which the measurements will be made.

Now consider figure 10. This is a cross section of an osteoporotic rib with very thin cortices. To measure the vascular space in this section high initial magnification is needed so that the thickness of the cortex fills the reticle of the micrometer eyepiece. Even when this is done, it is apparent that such a small sample of bone is on the slide that the results derived are not too valid for the entire bone; there is too much scatter. To measure the entire bone, therefore, it is necessary to measure a number of cross sections taken at regular intervals along the rib and average them to obtain an overall figure. From experience we know that reproducibility requires 5 or more sections to be averaged, and preferably over 7, for ribs.

Now consider figure 11. This is a photomicrograph of a sarcoma in bone. The longitudinal grain characterizing normal human cortex is absent. If we wish to know the volume of this tumor that is composed of osteoclasts, or of fibrous bone, a
Figure 10
A highly magnified portion of a cross section of a human rib which was osteoporotic. The average thickness of the cortex is about 300 microns. Sampling this cortex can only be done by measuring numerous sections and averaging them. The smaller the area of bone on a cross section the more sections must be averaged to obtain meaningful figures.

Figure 11
A decalcified, H & E stained section of an osteogenic sarcoma prepared by Villanueva's method (Am. J. Clin. Path, 36, 54, 1951). There is no longitudinal grain in this tumor. If one wished to know the specific surface of giant cells in this tumor, numerous sections cut at equal intervals through the substance of the lesion would have to be measured. Lamellar bone conveniently has a longitudinal grain which simplifies the sampling problem in dealing with lamellar bone.
series of complete sections cut at arbitrary but regular intervals must be cut through the tumor and the measurements of all sections averaged to a final result. If the sections are of differing sizes, the proportional relationships of the sections must be determined with respect to one of them so that weights may be assigned to the measurements of each and incorporated into the overall average.

As the reader begins to see, the process of doing specific volume and surface measurements is not difficult, but the process of designing and correcting the measurements to some valid value may entail a great deal of work and thought which must be omitted from publications to save space.

*Conversion of Area to Specific Volume*

Provided sampling is correct, any phalal area measurement based on the infinitely thin section principle may, when expressed as a percent of the total area, be directly converted to phalal percent of the total volume or specific volume. If, for example on corrected optical section-based area measurements it has been determined that osteocyte lacunae average 1.5 percent of the section area, then we know the specific volume of osteocyte lacunae in solid bone is 1.5 percent of the total solid.

![Diagram](image)

**Figure 12**

The top figure is the reticule of the Zeiss integrating eyepiece II in diagram. The total horizontal length of all 5 of these lines is measured with a stage micrometer, using the objective which will be used in measuring the tissue. This length is recorded as (L) for later use as described in the text. The bottom figure is part of the scale of a stage micrometer as seen looking through the microscope. Imagine the integrating eyepiece or net micrometer scale superimposed on the stage micrometer and the method of calibration may be understood.
It is as simple as that.

If the areas are initially expressed in finite units such as mm\(^2\) per mm\(^2\), convert them to percentage of area and the resulting percentage figure is also the percentage of the total volume of surround occupied by the measured phase.

**Specific Surface Measurement**

The term "specific surface" means the surface area of all the pores per unit volume of solid. Like "specific volume" the term had its origin in the field of the physics of porous media\(^{21}\). The total area of the lacunar walls per mm\(^2\) of cortical bone would be an example of specific surface. It might seem that such a measurement by microscopic means would be impossible and some gas adsorption technique would be preferable.

Again, Chalkley, Cornwall and Park\(^6\), Henning\(^{13}\) and Uspensky\(^{22}\) have developed the mathematical basis for a simple means of measuring the specific surface microscopically. The Zeiss firm has designed an integrating eyepiece specifically for this purpose; they term it Integrating Eyepiece II\(^{23}\). The essence of the technique is a reticle with several parallel, equidistant lines. The total length of these lines

![Figure 13](image)

Cross section of a tibia of a 9 year old boy. There are numerous large resorption spaces, some of them scalloped with Hwship’s lacunae.

Assume that the total area of bone surfaces containing Howship’s lacunae is wanted. Where the horizontal lines cut across such surfaces a hit is counted, and there are 4 such in this figure, all identified by an oblique line drawn in over the figure. There are other intersections between the walls of vascular channels and the reticule and if the vascular surface area were desired, it would be a simple matter to tally it as described in the text.
is measured with a stage micrometer with the objective that will be used for the measurements. This length is recorded for later use (Figure 12).

In figure 13 another cross section of cortical bone is illustrated. The lines of the Zeiss integrating eyepiece II are drawn over the photo. Any net micrometer will do however, the horizontal lines being measured as described while the vertical lines in the net are ignored as in figure 6B.

Where the horizontal lines cut across the interface between one phase and another, this interface being the surface that is to be measured, a hit is tallied. A separate talley counts the total number of fields measured. Where tangency of the reticle lines to the interface occurs, the even instances are counted as hits and the odd instances as misses; this strategem should also apply in the measurement of specific volume.

Sampling procedure, optical sectioning, and relationship of depth of the real or optical section to the depth of the phase being measured must be attended to as previously described.

At the conclusion of the measuring procedure, the average number of hits per field is calculated. The values of the average hits per field and the length of the reticle lines are substituted in the formula in figure 14.

Focal Depth Correction Factor:

\[ F_c = 1 + 1.5 \frac{f_o}{D} \]

\( F_c \) = Correction Factor  
\( f_o \) = Depth of Focus of Objective  
\( D \) = Vertical Depth Measured Phase

Specific Volume:

\[ V_s = \frac{H}{H + M} \]

\( V_s \) = Specific Volume  
\( H \) = Hits  
\( M \) = Misses

Specific Surface:

\[ A_{ss} = 2 \frac{H}{L} \]

\( A_{ss} \) = Specific Surface  
\( H \) = Hits  
\( L \) = Length of Reticle Lines

Figure 14

\[ A_{ss} = \frac{2H}{L} \]

Where \( A_{ss} \) is the specific surface area \( H \) is the average hits per field \( L \) is the total length of the lines in the reticle as calibrated from the stage micrometer.

The purely statistical significance of this measuring technique may be found from Table III under the 50 per cent column, 3rd from the bottom row. For example, if the total number of hits were 1000, the 95 per cent confidence limit would be plus or minus 3 per cent.

**SUMMARY**

The technique and theory of optical sectioning have been outlined. The visual depth of focus of a series of objectives has been determined.
MICROSCOPY

Simple, stochastic methods of measuring specific volumes and specific surfaces with the microscope, based on optical sectioning reasoning, are presented with some consideration of sampling technique.

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REFERENCES


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