

Chapter 3

An Introduction to Stereological Analysis: Morphometric Techniques for Beginning Biologists

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Introduction

A major goal of biology is to integrate structural and functional studies in order to better understand living things. A correlation between structural and functional data has, in the past, been difficult because while functional studies (e.g., physiology and biochemistry) have tended to produce quantitative data, structural studies (e.g., morphology and anatomy) have tended to be qualitative. In the last few decades morphometric techniques have been developed which allow structural data to be quantified and thus facilitate comparison with functional studies. Stereology, the study of three-dimensional objects through the interpretation of two-dimensional images, is one such technique. Not only does stereology provide a means of correlating structure and function, but it does so in a way that (1) removes the bias of the investigator, (2) allows the detection of small changes which may otherwise go unnoticed, and (3) provides a reliable means of comparing data, which can then be analyzed statistically.

The purpose of this chapter is to describe several student exercises which may be used to introduce students to the techniques of stereological analysis. In addition, each exercise adds a new dimension to the particular topic under investigation, thus augmenting the traditional approaches used to teach the topic.

The exercises presented are intended for introductory-level college students. They would be equally applicable, however, both for upper-division collegians and advanced secondary students. As Weibel (1973:291) notes: "Many biologists shy away from using stereological methods because of the apparent need for advanced mathematical knowledge... In fact, the only mathematics needed for applying stereology is an elementary knowledge of statistics." In reality, the only mathematical knowledge necessary to generate data is the ability to count; it is only the level of sophistication of data analysis that is enhanced by a knowledge of statistics and this can be adjusted to the level of the students.

Furthermore, once students have been introduced to various stereological techniques through laboratory exercises, such as are described here, they may utilize appropriate techniques to enhance independent research projects at virtually any level (precollegiate through graduate).

Each exercise is designed to be one component of a laboratory examining a particular topic, in this way supplementing traditional laboratory material. Thirty to 40 minutes should be sufficient time to introduce the technique, explain the procedure, and generate and analyze data. Subsequently, as similar procedures are used in other exercises, less time would be required.

The time required in preparation will vary with the resources available to the instructor. Ideally each student would be supplied with several (different) photomicrographs of the study material and a sample probe. In such a case time and materials would have to be expended to prepare the

photomicrographs and test probes. These are reusable, however, so when the exercise is repeated in the future everything would be ready immediately. An alternative, which I originally used, was to allow students to use their individual prepared microscope slides on a demonstration microscope with a video camera and a television monitor. A single grid probe was taped to the television screen so that individual students, or groups of students, could analyze their own slides. A third alternative, which would also allow students to analyze their own slides, would be to equip student microscopes with Weibel discs or other similar eyepiece reticules. Depending on the material to be examined this may either be the quickest and most efficient method (for relatively small counts) or next to impossible (for larger counts where already scored sample points cannot be “crossed off”). A final alternative would be to utilize published photomicrographs such as in a textbook. In this case all that would be required is a sample probe. It would not be possible to generate statistically significant data using this alternative because all counts would be made off a single sample, nevertheless this approach would be suitable for teaching the technique and would involve little cost and virtually no set-up time.

Student Outline

Stereology is the study of three-dimensional objects through the interpretation of two-dimensional images. This is useful not only because it allows us to study the structure of entire cells and tissues based on thin sections or photomicrographs of sections, but also because it allows us to study this structural quantitatively. For example, the physiology of the tissue (e.g., rate of photosynthesis) could be directly compared with the anatomy of the same tissue (e.g., volume of photosynthetic cells). If you take a moment to think about it you will recognize that there will be some basic problems in trying to interpret the three-dimensional structure of tissues given only thin sections. One of the most serious is that the plane of section will often determine the profile of the sectioned object. For instance, a sphere, ellipsoid, cone, or cylinder may all yield a circular profile when cut in thin section (Figure 3.1). Experience derived from studying many sections of tissue is necessary to overcome this problem. You will not be asked to interpret the shape of unknown structures in any of the following exercises, but you must keep this problem in mind if you plan to utilize stereological techniques in independent research projects.

A second problem is easily dealt with by stereological techniques themselves. The size of an object is an important parameter of any quantitative tissue study, yet the size of a profile will again depend upon the plane of section (Figure 3.1A,C,D). It is our good fortune that in cutting an object, such as the sphere in Figure 3.1A, the probability of obtaining each possible circular profile is not the same. In fact, the closer the profile diameter is to the true diameter of the sphere, the greater is its probability of being encountered in a section. It can be shown, using a rigorous statistical approach, that the probability of obtaining a profile greater than or equal to $1/2$ of the actual diameter is about 87% (Weibel, 1973, 1980). A careful examination of Figure 3.2 should convince you of this. The probabilistic relationship between the size of a randomly obtained profile and the true diameter of the object from which the profile was obtained allows the latter to be inferred from the former. One must simply sample, in random fashion, a large number of profiles of the sample.

Stereological techniques are not limited to the biological sciences. Actually, they were first devised as a method of analyzing the mineral composition of various rocks. Much of their practical application today is in the field of metallurgy and modifications of the techniques are used in ecology, geography, and earth sciences. Most of the application in biology has been by electron microscopists but many important discoveries have been, and continue to be, made using stereology and the light microscope (see Aherne and Dunnill, 1982; Steer, 1981; Weibel, 1979). The following exercises have been designed in order to acquaint you with commonly used stereological techniques.

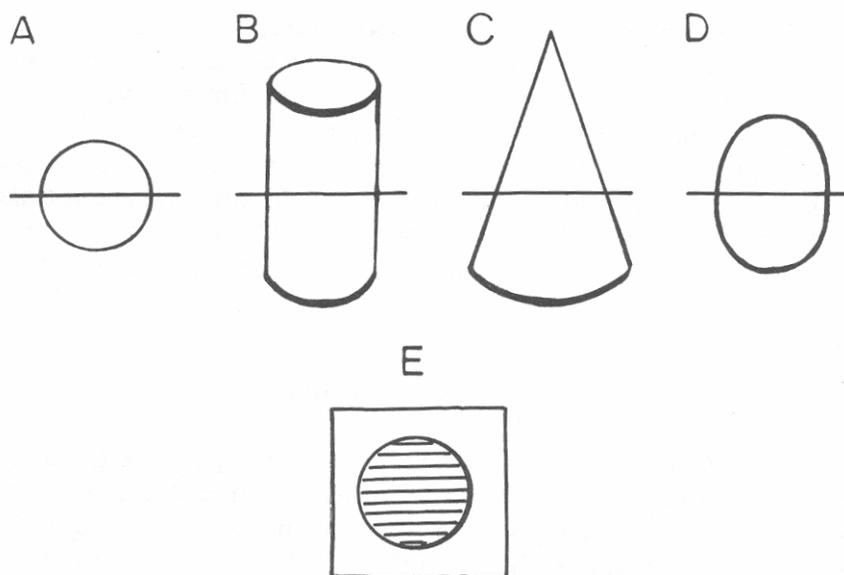


Figure 3.1. A series of three-dimensional objects (A, sphere; B, cylinder; C, cone; D, ellipsoid) which will produce a circle of identical area, E, if cut in the plane indicated by the horizontal lines

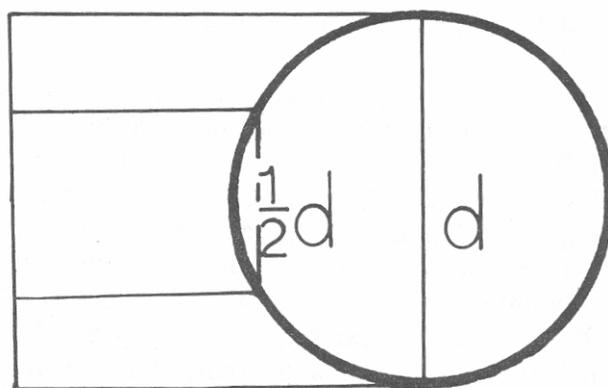


Figure 3.2. AA diagram of a circle cut in two of the possible planes of section: along its diameter and along a line one half its diameter. The probability of cutting at a position $> 1/2d$ is much larger than the probability of cutting $< 1/2d$ (86.6% versus 13.4%).

Although stereology is a powerful quantitative tool you should not be intimidated into believing that complex mathematics are involved. An understanding of statistics is necessary to fully appreciate why stereology works but the only prerequisite to applying the techniques to many basic problems is the ability to count.

The fundamental relationship of stereology is volume density. Volume density (VV) is defined as the volume of a component relative to the containing volume = V. component/V. total. This relationship may be illustrated with the following example. An irregularly shaped cell is contained within a cube of tissue of unit dimensions (Figure 3.3). If the block of tissue is serially sectioned, the area of tissue in each section will be constant, A. Furthermore, the area of the cell can be measured on each section, a. The volumes of both the tissue and cell may then be calculated by adding together the serial areas and multiplying by the thickness of each section (t). Thus, volume density is calculated as:

$$V_v = \frac{\Sigma(a)t}{\Sigma(A)t} = \frac{\Sigma a}{\Sigma A}$$

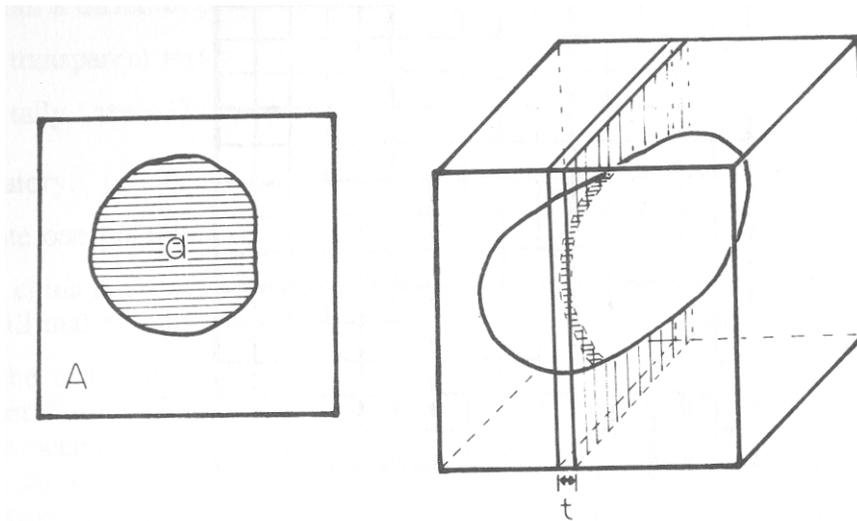


Figure 3.3. Diagram of a hypothetical cell in a cube of tissue. For the section illustrated the area of the cell (a) is shaded, while the area of the tissue (A) is unshaded. The volumes of tissue and cell in that section are the corresponding areas times the section thickness (t). The summations of volumes for all possible sections would be the actual tissue and cell volumes of the specimen.

It is impractical to serially cut tissue and measure every cell of interest, but, if a relatively large number of measurements are made on randomly selected cells, you will be able to infer the actual volumes of the cells. The question then becomes how to measure the areas of randomly selected sections. One of the traditional methods is to cut and weigh pieces of photomicrographs or line drawings corresponding to the cells of interest. The weight of the pieces relative to the photomicrograph as a whole will be proportional to the area of the cells relative to the tissue as a whole. Of course, the pieces must be cut accurately and carefully weighed and the procedure is time consuming and laborious. Another traditional method is to use a planimeter to carefully outline the profiles of the cells of interest in a photomicrograph. Again, this procedure is somewhat time consuming and laborious and you must have access to a planimeter. A third method, point counting, is a stereological technique which will be utilized in the following exercises.

Point counting may be done either directly through the microscope or on photomicrographs. In either case a test probe must be superimposed over the image of the specimen. The test probe consists of a set of points, either randomly arranged or frequently in a regular lattice, which are superimposed randomly over the image of the specimen. A convenient probe consists of a square lattice (Figure 3.4) in which each line intersection serves as a sample point. Ideally, the spacing of points on the test probe should correspond to the size of the object to be analyzed. The square lattice probe may be used on objects of various sizes by using the corners of individual squares as test points (for small objects) or various multiples of squares to correspond with the size of the object to be analyzed. For example, in the lower left corner of Figure 3.4 test probes of dimensions 12, 22, 32, 42, and 52 units are in heavy outline. To use point counting in determining volume density, you simply orient the test probe randomly over the specimen image and tally the points lying over the object of interest and the total number of points over the tissue as a whole.

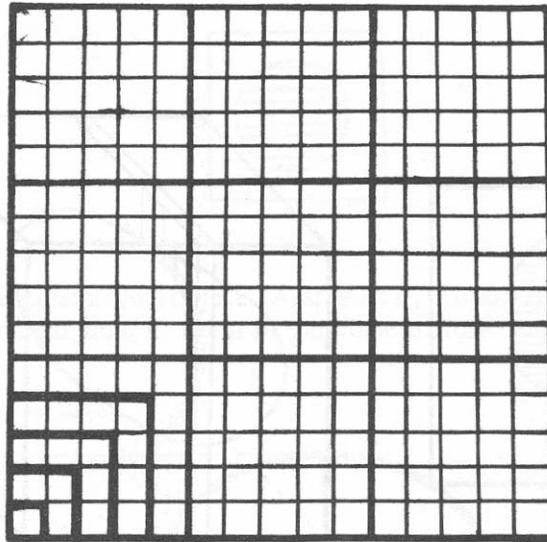


Figure 3.4. Illustration of a sample grid probe. Line intersections indicate sample points. This regular lattice pattern may be used on samples of various sizes by adjusting the number of grids used to designate a sample point as illustrated in the lower left hand corner. [From Sundberg (1984). Reprinted with permission.]

Notes for the Instructor

Exercise 1: A Quantitative Comparison of Sun and Shade Leaves (Sundberg, 1984)

Most introductory botany textbooks qualitatively describe the internal anatomy of sun and shade leaves as an example of an ecological modification of leaf structure. Sun leaves, those grown in full sunlight, are described as thick in cross-section with a relatively thick and tightly compacted layer of palisade mesophyll and a thick layer of spongy mesophyll with relatively small amounts of intercellular space. Shade leaves, those from within the canopy or otherwise shaded, as described as being thinner in cross-section with less well developed mesophyll layers and more extensive intercellular spaces. Commercially-prepared microscope slides of sun and shade leaves, available from biological supply houses, may be used to demonstrate these anatomical differences quantitatively.

Prior to the laboratory:

1. Prepare photomicrography sets of sun and shade leaves (Figure 3.5; see Appendix A). Ideally each student should have a unique pair of photomicrographs with each micrograph preferably taken from a different prepared slide.
2. Prepare transparent test probe overlays.
3. Prepare tally sheets (Table 3.1)

In the laboratory:

1. Distribute one photomicrograph set, test probe, tally sheet, and china marker to each student.
2. Using a china marker, outline the profile of each tissue region on the paired photomicrographs. (This will make it easier for students to tally test points in the subsequent step.)
3. Orient the test probe randomly over the photomicrograph of the sun leaf (Figure 3.6). It is important that the test probe is randomly oriented with respect to the photomicrograph. Stereological procedures are based on probability theory and require that either a probe with a random array of sample points is used or, if a regular test probe is used, the probe is randomly oriented to the tissue. This is especially important if there is a regular pattern in the tissue being examined.
4. Count and record the number of sample points overlying each tissue type. (Each intersection of grid lines on the test probe represents a sample point).
5. Tally the total number of points sampled on the sun leaf.
6. Repeat steps 3–5 for the shade leaf.
7. Combine individual student data to obtain class totals.
8. Calculate the volume density for each tissue type in both the sun and shade leaves (VV).

$$V_V = \frac{\text{points tallied for tissue type}}{\text{total points tallied for leaf}}$$

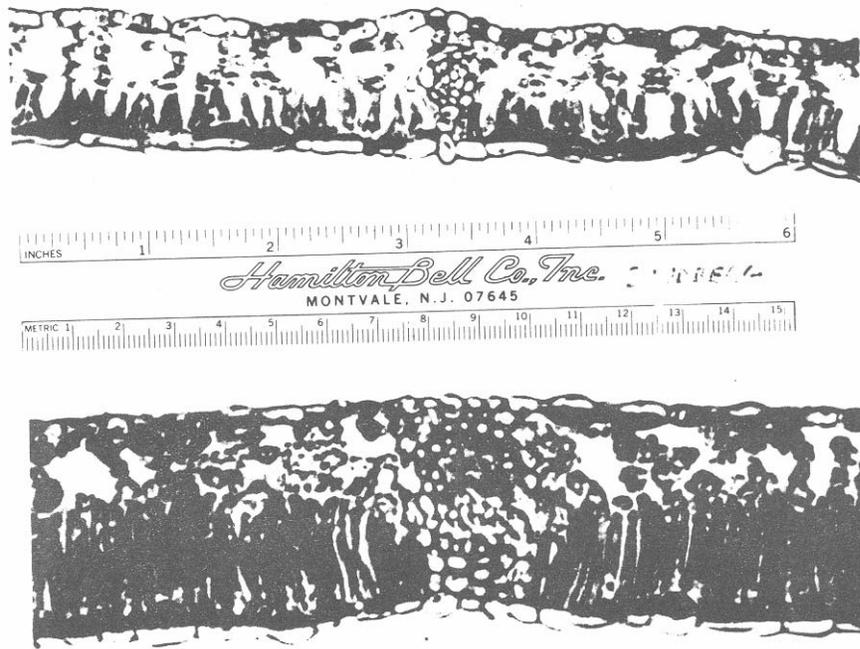


Figure 3.5. Sample student photomicrograph set with photographs of sun (bottom) and shade (top) leaves, at identical magnification, mounted together on a card. [From Sundberg (1984). Reprinted with permission.]

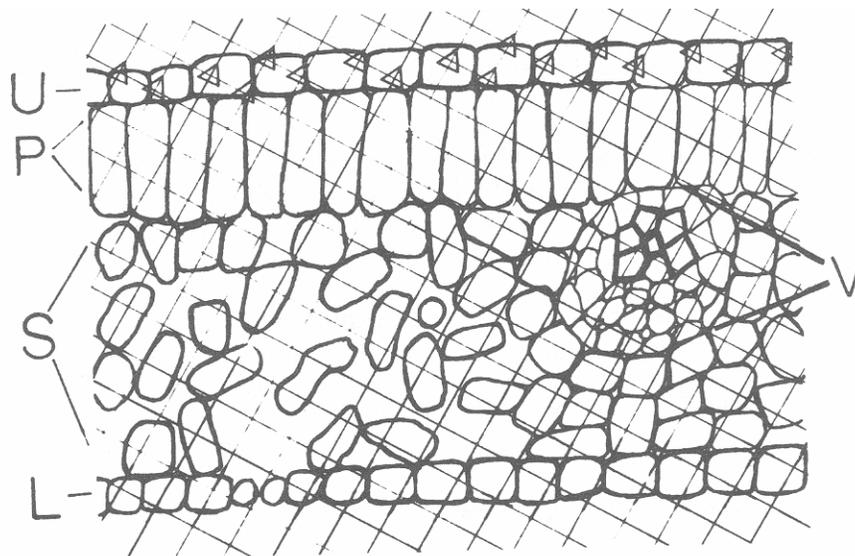


Figure 3.6. Sketch of a hypothetical sun-leaf photomicrograph with a superimposed test grid. The various tissue regions are indicated: U, upper epidermis; P, palisade mesophyll; S, spongy mesophyll; L, lower epidermis; V, vascular bundle. Test probe points overlying the upper epidermis are indicated by triangles.

Table 3.1. Sample student data tally sheet for sun and shade leaf exercise.

Sun leaf			
Tissue type	Point count	Volume density	% Leaf volume
Upper epidermis			
Palisade mesophyll			
Vascular tissue			
Spongy mesophyll			
Intercellular space			
Lower epidermis			
Total			

Shade leaf			
Tissue type	Point count	Volume density	% Leaf volume
Upper epidermis			
Palisade mesophyll			
Vascular tissue			
Spongy mesophyll			
Intercellular space			
Lower epidermis			
Total			

Table 3.2 illustrates the data obtained from the hypothetical leaf diagrammed in Figure 3.6 and is typical of student data generated from a single photomicrograph. Table 3.3 illustrates a typical student's data for the complete exercise. Table 3.4 presents a set of combined class data. These data quantitatively verify what was qualitatively described in the textbook. A total of 5600 points were tallied for the shade leaf while 8658 were scored for the sun leaf. Thus, the shade leaf is thinner, having only 65% of the volume of the corresponding sun leaf. Both the palisade and spongy mesophylls are more extensively developed in the sun leaf, having volume density of 0.396 and 0.187, respectively, than in the shade leaf, whose corresponding volume densities are 0.261 and 0.162. Intercellular spaces occupy relatively more volume in the shade leaf (20.5%) than in the sun leaf (10.0%).

Volume density is a probabilistic function whose accuracy is based on the sample size. In general, the larger the sample size the more likely the sample values obtained will approach the true values of the parameters being examined. In fact, we can choose to make the sample as accurate as we want by adjusting the sample size. Suppose you determine from your initial count that the volume density of spongy mesophyll in the sun leaf is 0.201 (Table 3.3). How many points must be counted in order to have a reliable estimate of the volume density of spongy mesophyll? This can be determined using the formula:

$$P_T = (0.453) \frac{(I - V)}{V_V \times E^2}$$

where VV is the preliminary volume density, E is the desired level of accuracy (the error you are willing to accept), and PT is the total number of points which must be counted to attain that level of accuracy. For example, if you are willing to accept a 10% chance of error for sun leaf spongy mesophyll, the number of points necessary is:

$$P_T = (0.453) \frac{(1 - 0.201)}{(0.201)(0.10)^2} = 180 \text{ points}$$

On the other hand, if you want to be 95% confident in your sample,

$$P_T = (0.453) \frac{(1 - 0.201)}{(0.201)(0.05)^2} = 720 \text{ points}$$

Table 3.5 demonstrates that counts of less than 200 are sufficient to obtain 95% confidence for volume densities greater than or equal to 0.50. Counts of this magnitude are often obtainable from a single slide (Table 3.3). By combining individual results to obtain class data sufficient counts may be obtained to achieve significant values for all parameters (Table 3.4).

Table 3.2. Hypothetical data based on Figure 3.6.

Tissue type	Point count	Volume density (VV)	% Leaf volume
Upper epidermis	18	0.098	9.8
Palisade mesophyll	49	0.266	26.6
Vascular tissue	14	0.076	7.6
Spongy mesophyll	56	0.304	30.4
Intercellular space	32	0.174	17.4
Lower epidermis	15	0.082	8.2
Total	184	1.000	100.0

Exercise 2: Correlation of Cell Type to Specific Gravity in Wood

The physical properties of various woods are due to their cellular composition and arrangement. Stereological techniques may be used to quantify cell composition of woods which can in turn be compared with certain physical characteristics. In this exercise the volume density of vessels, tracheids, fibers, ray parenchyma and wood parenchyma are calculated for several different woods. The volume density of each cell type is then plotted against specific gravity to determine if a correlation exists.

Commercially-prepared microscope slides of various woods will be examined in cross-section. Students must first be able to identify the different cell types. Vessels, tracheids, and fibers are all dead cells at maturity, therefore they will lack cytoplasmic contents in prepared slides. Vessels have the largest diameter and will frequently appear round in cross section. Fibers are very small in cross section and their uniformly thick walls often nearly occlude the cell lumen. Tracheids are intermediate in size. Both parenchyma types will be recognized by their cell contents. The ray parenchyma forms linear files of cells in cross section while the wood parenchyma usually occurs in patches often surrounding vessel members. Tables of specific gravity and other wood properties may be found in various wood products texts (Table 3.6).

Table 3.3. Representative student data.

Leaf type	Tissue type	Point count	Volume density	% Leaf volume
Sun	Upper epidermis	28	0.108	10.8
	Palisade mesophyll	105	0.405	40.5
	Vascular tissue	30	0.116	11.6
	Spongy mesophyll	52	0.201	20.1
	Intercellular space	25	0.097	9.7
	Lower epidermis	19	0.073	7.3
	Total	259	1.000	100.0
Shade	Upper epidermis	22	0.122	12.2
	Palisade mesophyll	42	0.232	23.2
	Vascular tissue	28	0.155	15.5
	Spongy mesophyll	29	0.160	16.0
	Intercellular space	38	0.210	21.0
	Lower epidermis	22	0.122	12.2
	Total	181	1.001	100.1

Table 3.4. Representative class data.

Leaf type	Tissue type	Point count	Volume density	% Leaf volume
Sun	Upper epidermis	1069	0.123	12.3
	Palisade mesophyll	3430	0.396	39.6
	Vascular tissue	870	0.100	10.0
	Spongy mesophyll	1619	0.187	18.7
	Intercellular space	870	0.100	10.0
	Lower epidermis	800	0.092	9.8
	Total	8658	0.998	99.8
Shade	Upper epidermis	838	0.150	15.0
	Palisade mesophyll	1459	0.261	26.1
	Vascular tissue	422	0.075	7.5
	Spongy mesophyll	909	0.162	16.2
	Intercellular space	1146	0.205	20.5
	Lower epidermis	826	0.148	14.8
	Total	5600	1.001	100.1

Table 3.5. Points required for a given level of accuracy.

Volume density (VV)	Test points required (PT)	
	$E = 0.10$	$E = 0.05$
0.95	2	10
0.75	15	60
0.50	45	181
0.25	136	544
0.10	408	1631
0.05	861	3443
0.01	4485	17,938
0.005	9015	36,058

Table 3.6. Some properties of various woods (after Newell, 1927).

Species	Specific gravity	
	Green	Oven-dry
<i>Tilia americana</i>	0.33	0.40
<i>Ulmus americana</i>	0.44	0.34
<i>Larix occidentalis</i>	0.48	0.59
<i>Quercus alba</i>	0.60	0.71
<i>Robinia pseudoacacia</i>	0.66	0.71

Prior to the laboratory:

1. Prepare photomicrographs of various woods in cross section (Figure 3.7; see Appendix A). Five or more individual micrographs should be prepared for each wood.
2. Prepare transparent test probe overlays.
3. Prepare tally sheets (Table 3.7).

In the laboratory:

1. Distribute photomicrographs, test probes, tally sheet, and china markers to students. Students should be divided into groups with each group given the responsibility for one species (i.e., each student in a group should have a different photomicrograph of the same species).
2. Orient the test probe randomly over the photomicrograph.
3. Using the china marker, outline the profiles of the less conspicuous groups of cells (e.g., tracheids, fibers, and wood parenchyma).
4. Count and record the number of sample points overlying each cell type.
5. Tally the total number of points sampled.

6. Combine the individual student data within each group to obtain summary data for each species.
7. Calculate the volume density for each cell type of each species.
8. For each cell type plot the calculated volume density against the corresponding specific gravity.
9. Calculate the equation and correlation for each graph produced (Appendix B).

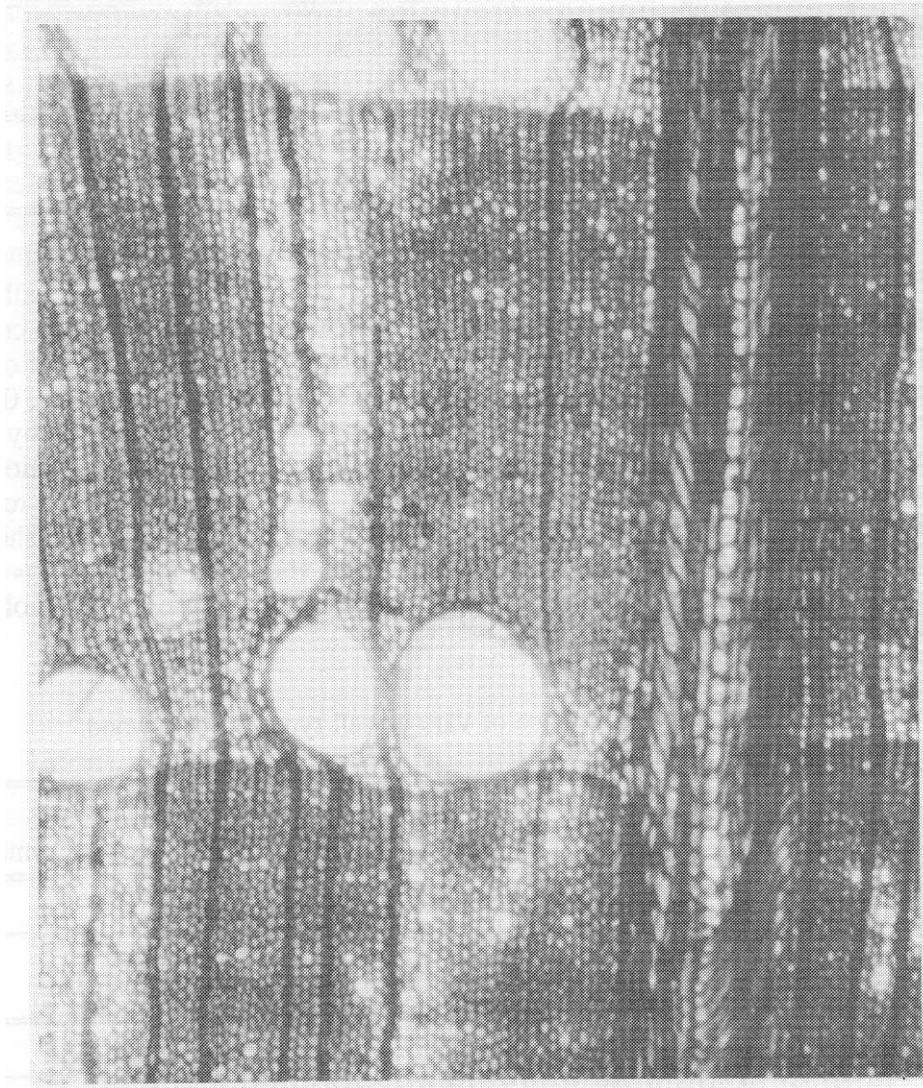


Figure 3.7. Photomicrograph of Quercus wood in cross-section.

Table 3.7. Sample student data tally sheet for wood structure exercise.

Species examined:						
	Vessels	Tracheids	Fibers	Ray parenchyma	Wood parenchyma	Total
Your data						
Group data						
Volume density						

Table 3.8 illustrates typical student data for one micrograph of *Quercus* as well as the combined group data of students for that species. In Figure 3.8 the volume density of each cell type is plotted against the specific gravity for each species. For example, the specific gravity of oven dry *Quercus* is 0.71 while the volume densities of each cell type are: vessels, 0.05; tracheids, 0.19; fibers, 0.47; ray parenchyma, 0.14; and wood parenchyma, 0.14. These values are indicated by a triangle on the appropriate graphs. It can be seen from the graphs that a good positive correlation exists between volume density of fibers and specific gravity ($r = 0.95$). In other words, as the relative volume of fibers in a wood increases, the specific gravity also increases. The graphs also show that there are reasonable negative correlations between specific gravity and volume of tracheids ($r = 0.79$) and volume of vessels ($r = 0.83$). In both cases the specific gravity decreases as the relative volume of tracheids or vessels increases.

Table 3.8. Occurrence of various cell types on Oak wood.

	Vessels	Tracheids	Fibers	Ray parenchyma	Wood parenchyma	Total points
Student 1	15	178	124	28	43	388
Group total (10 students)	197	694	1681	517	497	3586
Volume density	0.06	0.19	0.47	0.14	0.14	
% of wood volume	6	19	47	14	14	

Both of the exercises outlined above demonstrate how the relative volumes of various components may be estimated by calculating volume density. These relative values can easily be converted into absolute values if one of the parameters can be measured. An example of such a conversion is a recent study of stomatal distributions in various xerophytes (Strobel and Sundberg, 1984).

Stomates are the openings in the surface of aerial plant parts, especially leaves, through which gases are exchanged and water vapour is lost. It seems reasonable to expect that plants growing in arid environments would have fewer stomates per unit area so as to reduce water loss. Indeed, this is a xeromorphic adaptation described by plant anatomists into the second half of this century (Eames and MacDaniels, 1947). More recently it has been shown that many xerophytic plants actually have an increase in the number of stomates per unit surface area (Cutter, 1971; Esau, 1965; Fahn, 1974). We proposed to test the hypothesis that succulent and non-succulent

xerophytes evolved different strategies to deal with water stress, specifically that succulents tend to have a reduced stomatal density while the stomatal density in non-succulents is increased.

Stomatal densities could easily be determined in most cases by making epidermal peels, however, in many xerophytes the stomates are sunken below the epidermis (e.g., *Ficus*) or arranged in complex sunken crypts (e.g., *Ammophila* and *Nerium*) (Figure 3.9). In the latter two instances it is impossible to determine stomatal densities from peels, therefore stereological techniques were used to determine the volume density of stomates (guard cells) in the epidermis. *Ficus* served as a control because in this case individual stomates are sunken beneath the epidermis. Although the stomates themselves are not visible in a peel of *Ficus*, their density can easily be estimated from the number of “holes” in the peel. A single stomate is associated with each “hole.”

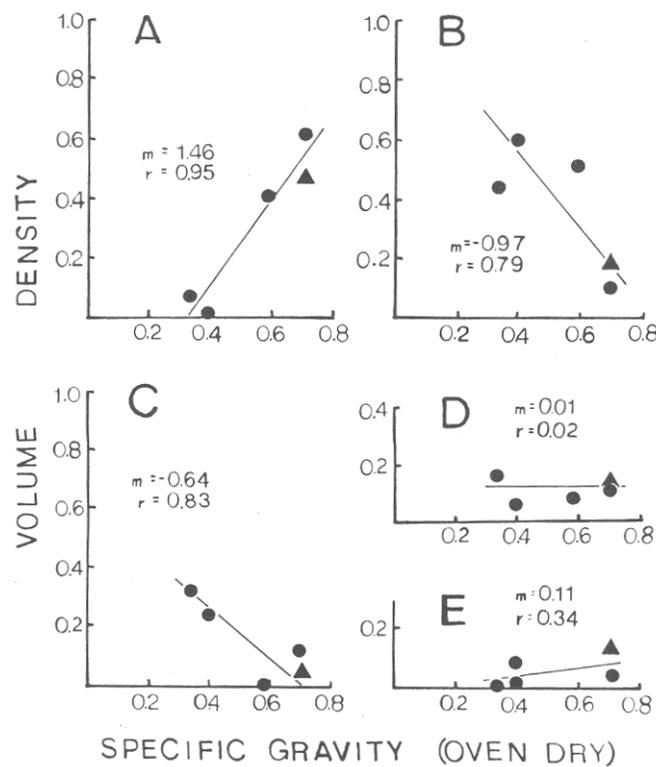


Figure 3.8. Sample plots of volume density of cell types versus specific gravity of wood for Basswood, Elm, Larch, Oak, and Black Locust based on actual class data. Data for Oak (Table 3.6) is illustrated by triangles. A, fibers; B, tracheids; C, vessels; D, ray parenchyma; E, wood parenchyma; m = slope; r = correlation coefficient.

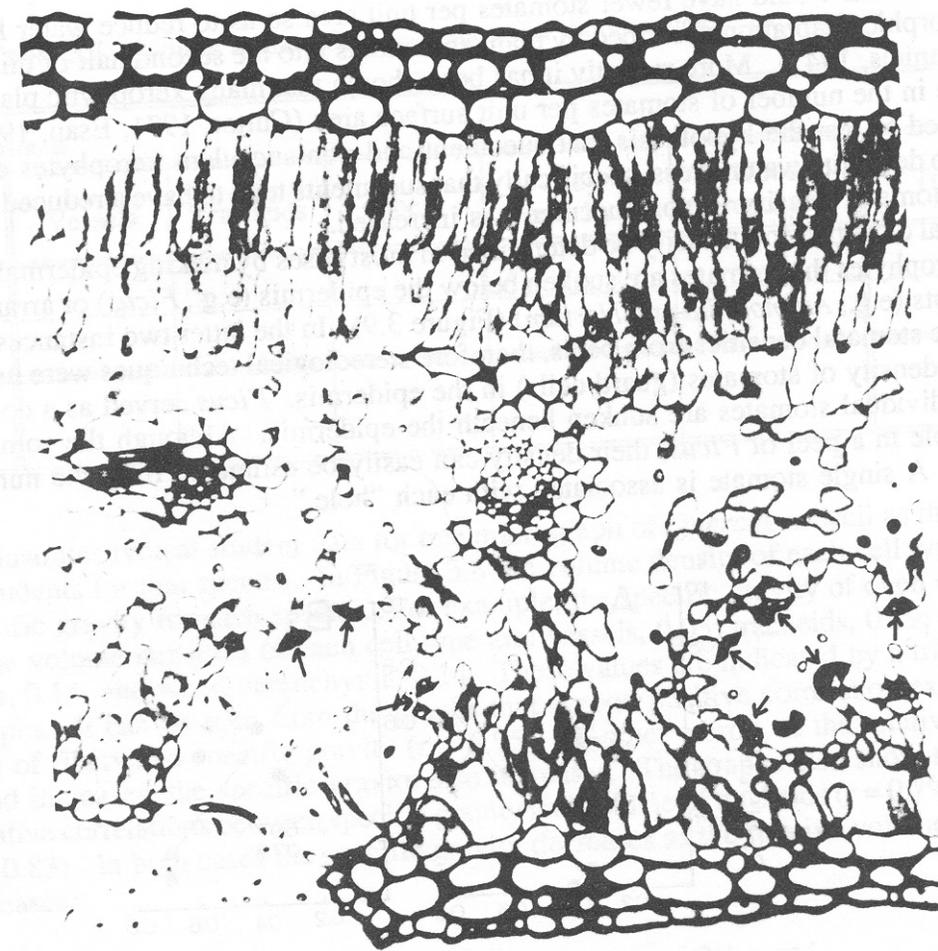


Figure 3.9. Photomicrograph of leaf cross-sections of *Nerium oleander*. Stomates are indicated by arrows.

In order to determine the stomatal density on leaves with encrypted stomates, slides of leaf cross-sections were used. An ocular grid test probe was superimposed over randomly selected fields and counts of epidermal cells and guard cells were tallied. After computing the volume density of guard cells to total epidermis the average epidermal cell area was determined using a calibrated ocular micrometer. Volume densities were then transformed to absolute densities using the formula $D = 1/a(VV)$, where D is the stomatal density, a is the average epidermal cell area, and VV is the volume density of stomates.

The data are summarized in Figure 3.10. Data for *Crassula* and the three species of *Kalanchoe*, all succulents, were obtained directly from peels and demonstrate a reduced stomatal density compared to average mesophyte leaves, although stomates occur on both surfaces of the leaves. In the remaining three species stomates occur only on the lower surface. In *Ficus* the density determined stereologically ($146/\text{mm}^2$) was nearly identical to that determined directly from peels ($150.5/\text{mm}^2$). The densities determined for *Ammophila* and *Nerium* are among the highest recorded for any plant (cf. Popham, 1966).

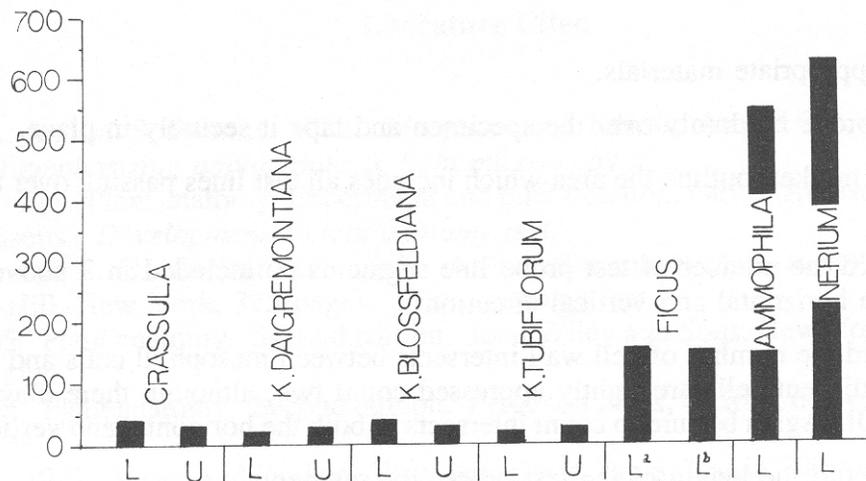


Figure 3.10. Average stomatal density on leaves of seven xerophytes. Crassula and Kalanchoe are succulents with stomates present on both the upper (U) and lower (L) leaf surfaces. Ficus, Ammophila, and Nerium are all non-succulents with stomates occurring only on their lower leaf surfaces. Data obtained directly from epidermal peels (L_a) and stereology (L_b) are presented for Ficus. [From Strobel and Sundberg (1984). Reprinted with permission.]

Exercise 3: Estimation of the Surface Area to Volume Ratio of Mesophyll Cells

in Sun and Shade Leaves

A second important stereological relationship is surface density (SV). This relationship defines the surface area to volume ratio and is calculated by the formula $SV = 2(I/L)$, where L is the total length of test lines used and I is the total number of intersects of the test line with the image boundary (Toth, 1982; Weibel, 1973).

In Exercise 1 we determined that there are significant differences in the relative volumes occupied by various tissues of sun and shade leaves. We also know that these leaves differ in size and shape: shade leaves are generally broader but thinner. It is reasonable, therefore, to suspect that the sizes of individual cells in a particular tissue might also vary with the environment.

Since surface to volume ratio is affected by size (area increases by the square while volume increases by the cube), and this ratio is important in transport processes across the cell membrane, this morphological relationship is of great interest for many physiological processes.

Prior to the laboratory:

Prepare photomicrographs, test probe overlays, and tally sheets. The same micrographs and probes prepared for exercise one may be used although it may be easier for the student to identify individual cells, especially in the palisade mesophyll, on micrographs of higher magnification.

In the laboratory:

1. Distribute the appropriate materials.
2. Orient the test probe randomly over the specimen and tape it securely in place.
3. Using the china marker, outline the area which includes all test lines passing over the mesophyll tissue.
4. Count and record the number of test probe line segments (l) included in 3 above. Be sure to include both the horizontal and vertical directions.
5. Count and record the number of cell wall intersects between mesophyll cells and the test probe lines. Where adjacent cells are tightly appressed count two, although there may appear to be only a single wall. Again be sure to count intersects in both the horizontal and vertical directions.
6. Measure and record the length of the test probe line segments.
7. Calculate and record the total length of line segments ($LT = \text{number of segments, } l, \times \text{segment length}$) and surface density ($2I/LT$).

Typical data for a group of four students are presented in Table 3.9. The average surface density of mesophyll cells in the shade leaf is $0.73 \text{ mm}^2/\text{mm}^3$ while in the corresponding sun leaf it is $0.37 \text{ mm}^2/\text{mm}^3$. In other words, there is twice the surface area per unit volume in shade-leaf mesophyll cells as in sun-leaf mesophyll—an indication that the former cells may be smaller and that their diffusion rates may be greater.

For research purposes the values obtained for SV must be corrected for magnification. The formula for this, as well as for the calculation of the sample size necessary for a given level of error, may be found in Weibel (1979).

Table 3.9. Surface density of mesophyll in sun and shade leaves.

Type	Counts (I)	Segment (l)	Segment length (mm)	2I	LT	2I/LT (mm^2/mm^3)
Shade	320	234	5	640	1170	0.55
	324	219	5	648	1095	0.59
	538	216	5	1076	1080	1.00
	434	276	5	710	880	0.81
Total				3074	4225	0.73
Sun	368	366	5	734	1830	0.40
	336	455	5	791	2275	0.35
	396	438	5	834	2190	0.38
	376	482	5	858	2410	0.36
Total				3217	8705	0.37

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APPENDIX A
Materials

Photomicrographs of sun and shade leaves:

I have used commercially-prepared slides of Acer sun and shade leaves. Suitable magnification is obtained by enlarging 35-mm negatives, exposed at 100X magnification, to produce full frame, ca. 5" × 7", prints. Each photomicrograph set is prepared by mounting glossy prints of one sun leaf and one shade leaf on a piece of artist's board. The mounted prints are then laminated in plastic so that students may mark directly on the micrographs with china markers or water soluble pens when tallying data. Transparent test probe overlays may be made by making photocopies of appropriately-scaled graph paper onto clear plastic sheets. Alternatively, a regular grid may be ruled onto clear plastic sheets using permanent ink. The spacing of points on the test probe should be approximately the size of the object to be analyzed; in this exercise the spacing should be approximately the width of a mesophyll cell on the photomicrograph.

Photomicrographs of woods:

I have used commercially-prepared cross-sections of the following woods: Larix, Quercus, Robinia, Tilia, and Ulmus. Random fields of each slide were photographed at 100X using a 35-mm photomicroscope set-up. The negatives were enlarged to print four 8" × 10" prints from each frame. At this magnification the test probes from the above exercise may be used.

Materials per class of 36 students:

Photomicrographic set-up

Black and white film (e.g., Kodak plus-X or panatomic-X) (2 rolls, 36 exposure)

Enlarging paper (e.g., Kodabromide F-3, 8" × 10") (36 sheets)

Transparent grid sheets (Thermofax or equivalent photocopies of standard 0.5-cm graph paper on 8" × 10" transparent sheets are adequate) (36)

China markers or water-soluble pens to aid in scoring (36)

Tally sheets

Prepared slides of sun and shade leaves and wood cross-sections:

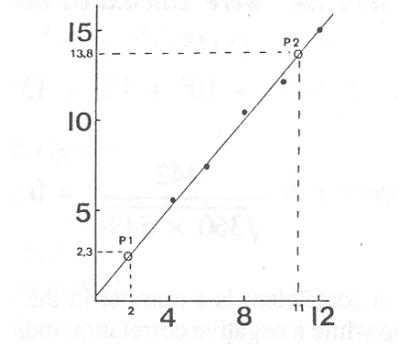
Carolina Biology Supply
2700 York Road
Burlington, NC 27216

Triarch Prepared Microscope Slides
P.O. Box 98
Ripon, WI 54971

APPENDIX B
Calculations of Slope and Correlation Coefficient

The slope of a line may be determined graphically by carefully plotting the data and “eyeballing” the best-fitting line. For instance, the following data may be plotted as below:

X	Y
4	5
6	7
8	10
10	12
12	15



The slope of this line can be estimated by computing the “rise over run.” Choose two parts on the line (e.g., P1, P2) and drop vertical lines from these points to the x-axis and horizontal lines to the y-axis as illustrated above. In this case the coordinates of P1 and P2 are (2, 2.3) and (11, 13.8), respectively. Rise over run = $(y_2 - y_1)/(x_2 - x_1) = 12.5/10 = 1.25$. Because the sign of the slope is positive, y increased with x. If the sign were negative, y would decrease as x increased.

A more accurate way of estimating the slope (m) is to use the method of “least squares.” The slope is calculated by the equation: $m = SP/SS_x$. Using the data above the calculations would be as follows:

$$SP = \sum XY - (\sum X \sum Y)/n$$

$$\sum XY = (4 \times 5) + (6 \times 7) + (8 \times 10) + (10 \times 12) + (12 \times 15) = 442$$

$$\sum X = 4 + 6 + 8 + 10 + 12 = 40$$

$$\sum Y = 5 + 7 + 10 + 12 + 15 = 49$$

$$n = 5$$

$$SP = 442 - (40)(49)/5 = 50$$

$$SS_x = \sum X^2 - (\sum X)^2/n$$

$$\sum X^2 = 4^2 + 6^2 + 8^2 + 10^2 + 12^2 = 360$$

$$(\sum X)^2 = 40^2 = 1600$$

$$SS_x = 360 - 1600/5 = 40$$

therefore, the slope (m) = $SP/SS_x = 50/40 = 1.25$

The correlation coefficient (r) is calculated by the equation:

$$r = \frac{\Sigma XY}{\sqrt{(\Sigma X^2)(\Sigma Y^2)}}$$

ΣXY and ΣX^2 were calculated above and are 442 and 360, respectively.

$$\Sigma Y^2 = 5^2 + 7^2 + 10^2 + 12^2 + 15^2 = 543$$

$$\text{therefore } r = \frac{442}{\sqrt{360 \times 543}} = 0.99$$

The correlation coefficient is a number in the range from -1.0 to +1.0. A positive correlation indicates a direct relationship while a negative correlation indicates an inverse relationship. If $r = 0$ there is no correlation, therefore, the closer r is to $|1|$, the stronger the relationship.