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Section II: Morphology and Development

Past, present and future methods of quantification in anatomical and ultrastructural studies of lichens

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SUMMARY

This paper provides a general view of the state of the art in quantification methods for light and electron microscopy, with especial reference to botany and lichenology. The need of quantification in anatomical and ultrastructural studies of lichens is contrasted with the wide range of topics already tackled by means of numbers. The applicability and reliability of stereological methods are based on a consistent theoretical combination of statistics and geometry. The modern tools of stereology ensure a wide range of unbiased and efficient estimators of the main quantities (volume, surface, length and number). The most useful methods and their problems and requirements are mentioned with brief comments and associated key references. As a result of our studies, some proposals for lichenological works dealing with quantification are given. Of great interest are the concepts of sampling design, effort investment, variability and its sources, and anisotropy. Some final comments on the future of quantitative microscopy are included.

The state of the art in quantification methods

By definition, the product of microscopy is an image, and the job of the microscopists is to obtain images, to study them and to extract the biologically significant information and convey this in an intelligable form to the scientific community. Image quantification has always been one of the aims of botanical microscopists, as an efficient way of dealing with these tasks. Until the end of the 1970's, the few anatomical or ultrastructural studies within the field of botany that included quantifications were usually based on morphometric parameters as surface area, diameter, perimeter, number of objects per section or micrograph. Morphometrical data are simple two-dimensional estimators of the so-called classical quantities (Volume, Surface, Length and Number) and, despite their limited applications, are still present in many botanical papers.

The first tentative references to the third dimension were poorly regarded because of the use of very unreliable methods for measuring the number and size of particles (30). The procedures were collectivelly known as "model-based", since they held in common the requirement of very strict and unrealistic model assumptions about the geometrical shape of the objects of study, the most common assumption being that all the objects had to be spherical. These methods have been reviewed by Cruz-Orive (9). A combination of theoretical mathematics, statistics and geometry has led to the creation of the stereology, described in detail for the first time by Weibel (43). This branch of the geometric probability theory is based on the statement that the two-dimensional images of a section (such as a micrograph) contain quantitative information about three-dimensional structures but only in a statistical sense. The strenght of stereology is that it reports data for three-dimensional structures in terms of three-dimensional

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quantities, and this is done directly from the product of microscopy, the two-dimensional image. The two concepts referred to the methods that the stereological literature of the 80's has especially stressed are, "unbiased", that is, without systematic deviation from the true value, and "efficient", meaning with a low variability after spending a moderate amount of time. Modern stereology and the arise of several new stereological tools have been reviewed with accuracy by their principal creators and promoters, Gundersen (17, 19, 20) and Cruz-Orive (10, 11).

Interest of quantification of structures in lichenology

In modern histology and cell biology, physiological and biochemical studies traditionally rely on quantitative methods, and one would therefore expect the associated microscopical study to be quantitative as well. Quantification provides objective information for studies concerning 1) description of structures, 2) comparisons among structures, 3) changes in structures, and 4) relationship between structure and function. If measurements of thickness and diameters (i. e. thallus layers, cell wall, spores, etc.) are not considered, there is no study, as far as we know, describing lichen structures quantitatively. However, quantification has been carried out in many cases with the aim of reporting changes in structures and their possible implications for lichen physiology. Most of them are ultrastructural studies, where the response variable is the relative abundance of a certain cellular structure or group of structures. And most of them, unfortunately, are limited to two dimensions, despite the fact that in several cases the leap in the third dimension could be very simple. Let's see a brief enumeration of them.

Cellular damage caused by pollution was traditionally tackled by estimating the percentage of the total algal cells that showed collapsed contents (23, 25, 26, 27, 28, 29). The works of Eversman and Sigal (12, 13) dealing with pollutants and structural damage of lichens represented a significant methodological advance with respect to the previous papers, but their measurement methods include aspects which still need reexamination. Ultrastructural changes due to seasons (15, 24) or to transplantation (40) were reported with special mention of the amount or size of the lipidic storage bodies of the photobiont. The effects of hydration and desiccation on photobiont fine structure have been studied by means of quantification of the particles of the plasmalemma (35), the storage bodies (1, 37) and the pyrenoid and pyrenoglobuli (6). Similar works have been carried out assessing the effects of light and dark (7) and lipoxygenase enzyme (38) on the organelles of the photobiont, and the influence of prefixation in the study of both symbionts of *Lobaria* (2).

In the work of Collins and Farrar (8), possibly the first lichenological study in which stereology was used, the quantitative data concerning both symbionts are related to measurements of photosynthesis rate and ribitol transfer. But the works of Ascaso and Valladares (3, 42) are the first ones to apply the modern unbiased sampling methods to study symbiont fine structure and also the first to reveal the great potential of the simple stereological methods applied to lichens. Taking into account the wide variety of lichenological topics where quantification has proven to be a crucial aspect, the need for simple but reliable methods for quantifying in anatomical and ultrastructural studies seems to be clear.

The methods and their problems

According to Briarty (5), in attempting to measure cellular structure from sectioned material, the investigator is faced with having to take one of two contrasting approaches: either to study in detail one or a few particles (e. g. cells) or to sample a large particle population and to estimate average values for parameters which may not actually be the case for any of the individual particles making up the population. In the first case, serial sectioning followed by three-dimensional reconstruction is involved, the explanation of which exceeds the space available (in case of interest see 45, 46). The second approach, the statistical one, constitutes the main body of stereology and their more relevant aspects will be briefly expounded below.

When three-dimensional quantities are estimated from two-dimensional images one has to admit at least four sources of error (Fig. 1): 1) one single object can generate sections of different shape, 2) one single object can produce more than one disconnected profiles when sectioned, 3) the sections of a membrane will generate on average a larger thickness than the real thickness of the membrane, and 4) the sections of a spherical structure will provide on average a smaller diameter than the real one. Many stereological parameters tend to compensate these error source, and, for instance, it is recommended that the measurements of diameters should be multiplied by $4/\pi$ (43). However, other sources of bias are pendant of practical solutions, as for example, the resolution used to observe the section image, since the better the resolution with which a membrane is studied the higher the estimate of the membrane surface area (34). This effect depends on some geometric characteristics of the membrane that can be assessed by estimating its "fractal dimension", a fascinating concept introduced by Mandelbrot (31, 32).

With the aid of stereology, two different groups of quantitative parameters can be estimated: absolute values or proportional values (ratios). In general, the more simple and widespread stereological parameters are ratios, that is the value of a certain quantity (volume, area, etc.) in relation to a certain space of reference. In Table 1, the most common stereological parameters together with their abbreviations, method of calculation and units are shown. The approach to them is simple and their basic principles are intuitively plausible. Once the reference space is well specified, by measuring the area of our structure and the reference area we are estimating the volume density (Vv), and subsequently the surface density (length or perimeter

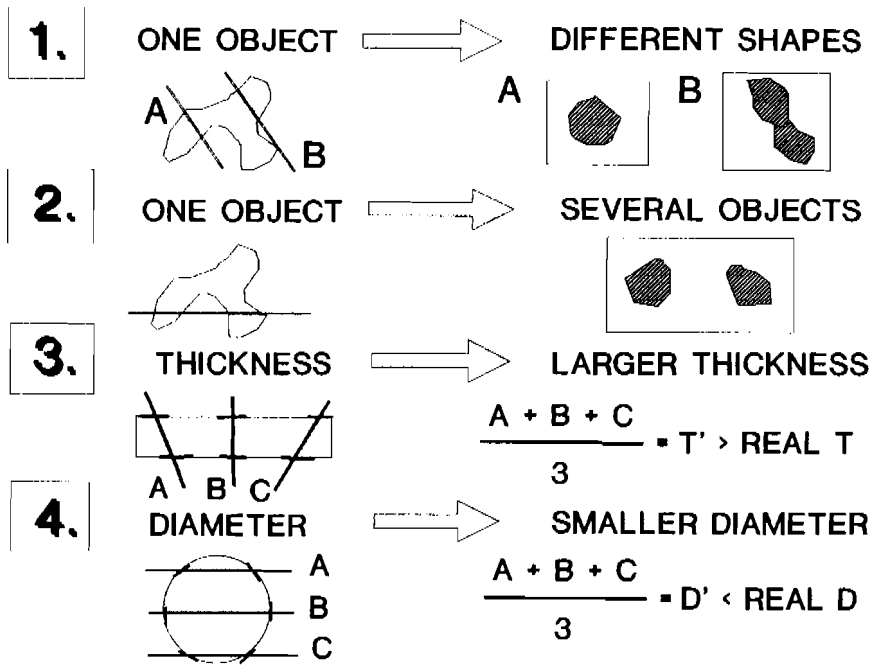

Fig. 1. Four sources of error when measuring 3-D objects in 2-D images (e.g. micrographs of transversal sections of lichen thallus, hyphae, algal cells).

Table 1. Abreviation, units and way of calculation for the four most common and widespread stereological parameters. For the volume and surface densities, two different formula are shown. U represents arbitrary units.

Parameter	Abreviation	Units	Calculation
Volume density	Vv	U^3/U^3 or %	i) structure area / REFERENCE AREA ii) points hitting structure / P. HIT. REFERENCE
Surface density	Sv	U^2/U^3 or U^{-1}	i) $(4/\pi)$*(structure perimeter/REF. AREA) ii) 2* (structure intersections/REF. LENGTH)
Length density	Lv	U/U^3 or U^{-2}	2* number of structure profiles/REF. AREA
Numeric density	Nv	Num./U^3 or U^{-3}	number of structure profiles/DISECTOR VOLUME

to reference area ratio, Sv), the length density (number of profiles to reference area ratio, Lv) and numeric density (number of countables profiles to disector volume ratio, Nv, see below for further explanations).

When the process of measuring begins, one has to choose between a computer or a piece of paper and a ruler. The computerized image analysers are very attractive but for an example with a few exceptions (14), they are not able to distinguish between a mitochondrion and a storage body. The grid test systems are of simple use and provide unbiased data, just by counting points and interceptions over a micrograph (Fig. 2). These methods, when assisted by computerized data acquisition systems, are very efficient and can only be surpassed by fully automatic and expensive image analyzers, if these are applicable to the problem, which they are too often not (44). A real advantage of the grids is that the user can control the effort to be invested, since a compromise between the precision and the effort can be reached by adjusting the size of the test system. In other words, the effort invested on measuring with a grid depends on the researcher "ambition coefficient" but not on the stereological parameters themself.

An appropriate experimental design is essential and the optimal use of stereology requires the most efficient distribution of effort between the steps in the practical sampling procedure. Gundersen and Osterby (18) demonstrated that variations at the lower levels of sampling (e.g. number of sections) are of minor importance, while the "biological variation" between specimens plays an all-important role. In this sense, the measuring precision is of little importance and on this relies their advice of "Do more less well".

Some proposals for lichen studies

The extremely wide variability of the lichen anatomy and ultrastructure makes the sampling and experimental design a point of vital importance. First of all, we recom-

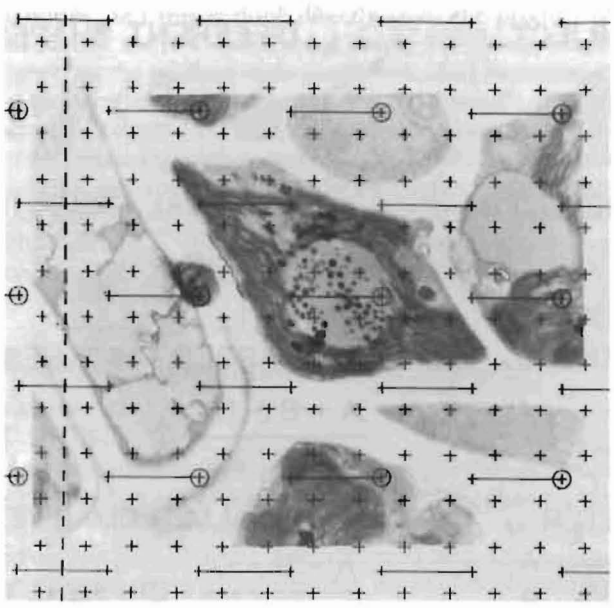

Fig. 2. TEM micrograph of the algal layer of *Lasallia hispanica* covered with a test system grid used to count points and interceptions in order to estimate Vv (volume density) and Sv (surface density) values of the organelles of both symbionts.

mend the usage of a number of thalli as large as possible (at least five or more). If we want to reduce the error by 30% we have to double the effort, but, do we use twice as many thalli, twice as many sections per thallus or twice as many micrographs per section? The better way to solve this is a preliminary nested anova designed to check the main sources of variation. In order to determine the minimum number of thalli needed, the crucial factor is the variance of the parameter under study. In the case of comparing two groups of thalli, if the square of the difference between the mean of each group is equal or larger than the variance of the parameter, then a small number of thalli (5 to 10) will be enough. If it is not the case, the nested anova will tell at which level of sampling more effort is needed, usually it will require an increase in the number of thalli examined.

Our studies have been based mainly on the stereological parameter volume density (Vv). This gives a broad idea of "amount" of a certain structure under study and is rather simple to obtain (Fig. 3). Some volume densities of interest in lichenology are those of the photobiont and mycobiont cells in relation to the algal layer or to the thallus volume, and those of the organelles and cellular structures of both symbionts in relation to the cell volume. Another interesting parameter is the surface density (Sv), that gives an idea of the surface area of a structure contained in a certain volume. However, this parameter is very sensitive to the *anisotropy*. And the lichens are undoubtely very anisotropic. In Fig. 4, an example of what anisotropy means is given. In brief, when a structure of interest is not distributed at random in the containing space but instead shows a certain spatial arrangement, it is anisotropic. While the area of the structure to the reference area ratio (Vv) is the same in two perpendicular sections of an anisotropic structure, the perimeters of the same structure differ from one section to another, and subsequently the perimeter-reference area ratio will be different. Fortunately, in lichens it is easy to obtain a "vertical section", that is, a section perpendicular to a certain well-defined plane, and by applying a cycloid test system on a vertical section (Fig. 5) the question of anisotropy can be easily solved (4, 11).

In lichenology much work has gone into counting particles, especially algal cells (e.g. 15, 16, 22, 36) or organel-

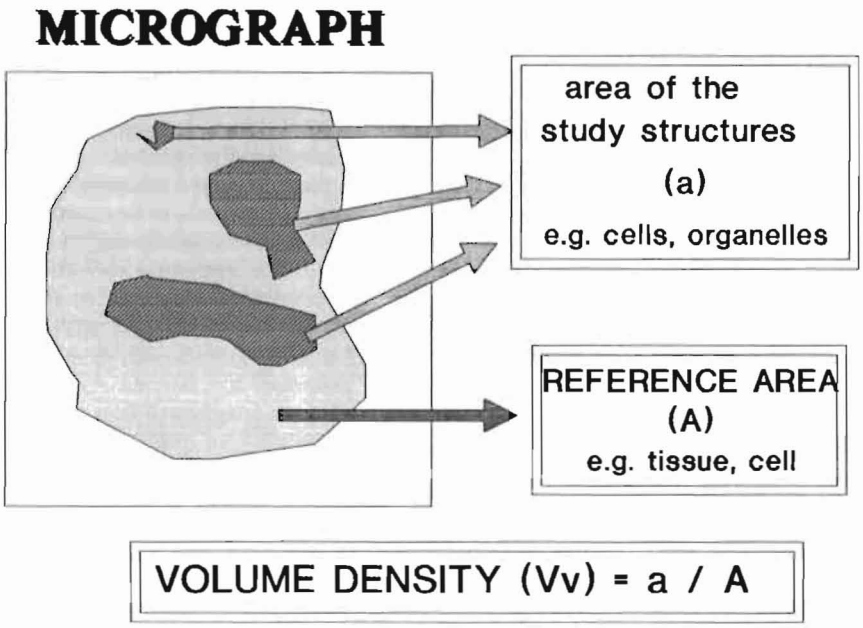

Fig. 3. Sketch of the procedure to obtain the volume density (Vv) from a micrograph.

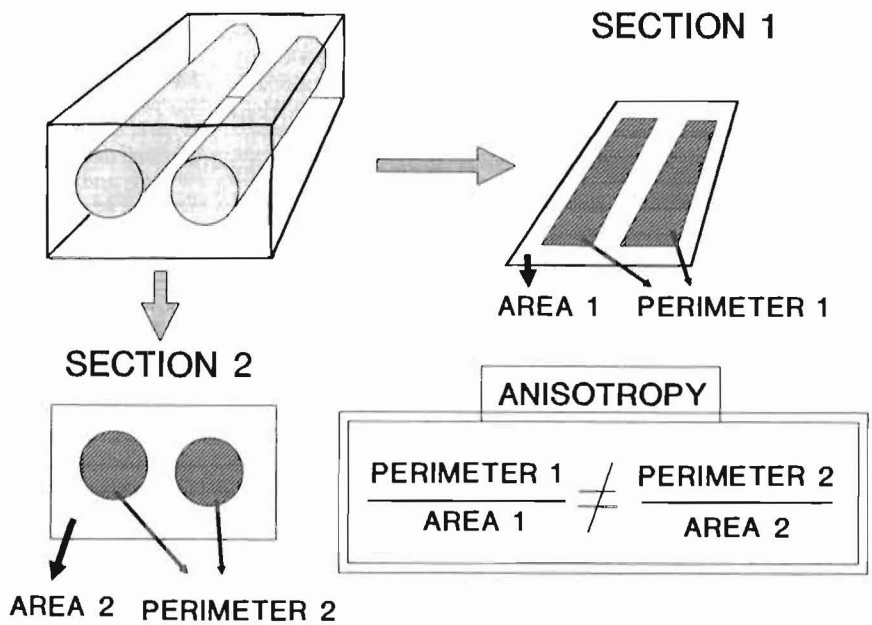

Fig. 4. Illustration of what anisotropy means. In a structure with a certain spatial order, the orientation of the section introduces bias in the measurements of some parameters (e.g. surface density, which is related to the perimeter).

les within the cells (e.g. 7, 40), but a reliable method has never been applied. The "disector" (41, 20) has been proposed as an unbiased method for counting particles. The disector consists of two parallel planes, a known distance apart. They can be serial sections. Then, in accordance with the method of counting profiles in 2-D (19), the number of particles that are in one section but not in the next is considered and related to the disector volume: disector area (reference) multiplied by the distance between sections (the disector thickness). The result of doing this in up to 50 pairs of sections is a very realistic estimation of number of particles contained in a certain volume.

Finally, a commentary about the plasticity of the stereological parameters that can be transformed and adapted to others. For instance, in foliose or crustose lichens, the amount of photobiont cells under a square centimeter of the upper surface can be of great potential in comparative studies. This value (Vs) can be simply obtained by multiplying the volume density (Vv) by the average thallus thickness (in centimeters, if we want the result in these units). The logic underlying this example is easy to understand and can be used in many different ways.

Final considerations

Besides the classical quantities mentioned throughout this paper, there exists the so called "truly 3-D quantities" or second-order properties, that include the connectivity, the size distribution, the real feature shape and the spatial distribution. Despite the existence of several works dealing with them (for spatial distribution see 21, 33, 39) they are far from being satisfactorily tackled by stereology. The confocal laser and light scanning microscopy are starting to be used in the estimation of those properties, but in the study of cell organelles serial sections are still necessary. In

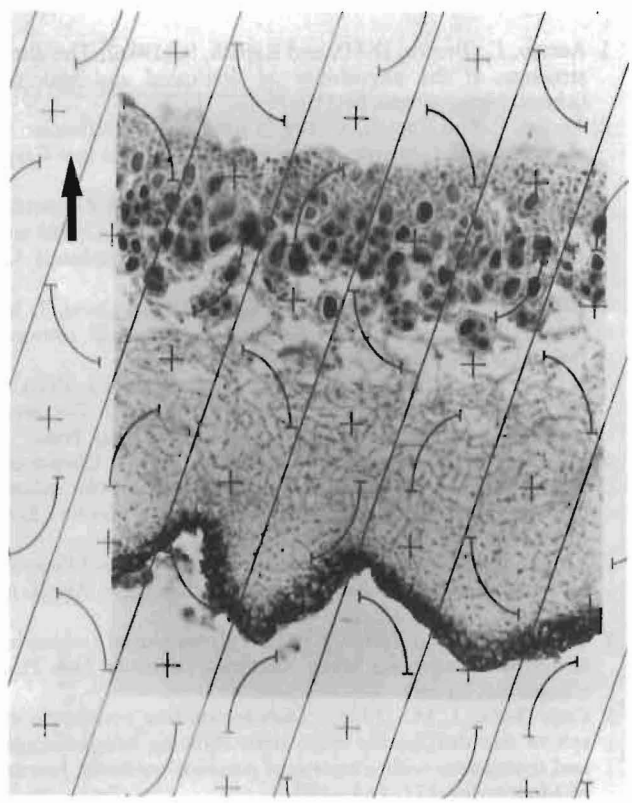

Fig. 5. Light micrograph "vertical section" of a thallus of *Umbilicaria ruebeliana* covered by a cycloid test system, used to estimate the surface density (Sv) of the cells correcting the problems generated by anisotropy. The arrow indicates the "vertical" axis.

the future, an evolution of the stereological tools together with a development of light, laser and electron microscopy techniques will make possible a more convincing approach to those and other structural properties not well worked out at present.

As Weibel commented already in the 80's, when the question is "where are we?", with respect to stereology in cell biology the answer is "not as far as we could be". And this is especially true not only in lichenology but in the general field of botany.

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