

APPLICATIONS OF STEREOLOGY IN PATHOLOGY: A BRIEF GUIDE

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ABSTRACT

The value of using stereological techniques to investigate pathological tissues is enormous, but its full potential has yet to be realised. In the present report, a brief guide to some practical considerations is provided, and the problems of sampling pathological material are discussed. By applying the methods, much valuable information can be obtained from the analysis of histological material which exists in abundance in all pathology departments.

INTRODUCTION

Stereology has been defined as a body of mathematical methods relating three dimensional parameters defining a structure to two dimensional measurements obtainable on sections of the structure (Weibel, 1979). In the biological sciences, stereological methods have been applied to investigate the histology and ultrastructure of a variety of normal and experimentally treated tissues. The pioneering work in this field began just over twenty years ago and there now exists a wealth of information characterising many normal tissues, such as the lung (Weibel, 1963), exocrine pancreas (Bolender, 1974), stratified squamous epithelia (Landay and Schroeder, 1977; White et al., 1980), skeletal muscle (Eisenberg et al., 1974), the central nervous system (Haug et al., 1976) the reticulo endothelial system (Mayhew and Williams, 1974 a, b) and the liver (Loud, 1968; Weibel et al., 1969).

It is axiomatic that before we can understand the plethora of changes which result from disease processes, we have first to understand the structure and function of normal tissues. Whilst in many areas the subject of histology is being rewritten in quantitative terms, our understanding of normal tissue structure is by no means complete but should improve enormously over the next decade. Many workers in the disciplines of experimental biology and pathology are now applying stereological methods to investigate a number of aspects of normal and abnormal tissue structure. The single most important aspect of quantitative morphology as provided by stereology is that it provides greater objectivity of description of biological tissues. In pathology departments, a large proportion of time is spent making diagnoses from the qualitative interpretation of tissue architecture. This interpretation is essentially subjective and can vary considerably (Saxen, 1979). Ultimately it will depend upon the skill, training and experience of the individual pathologist. Therefore greater objectivity through the use of quantitative techniques can only be a valuable asset.

Unfortunately there is still a certain mystique regarding morphometric and stereological methods which often seems to deter an individual with a problem which can be readily solved by their application. There is indeed a very well established and often complicated mathematical basis for all stereological procedures, but for most of us these are not important. You do not need to be a motor mechanic in order to drive a car. The application of stereological methods can be an extremely simple process, and can nevertheless provide considerable quantities of valuable objective information. There are however certain guidelines which need to be followed.

SOME ASPECTS OF SAMPLING

One of the fundamental principles of stereology is that it is a science of averages. We use stereology to generate information from biological tissues by the analysis of random tissue sections. It is essential that as large a number of random samples of the particular tissue under investigation as possible is analysed in order to provide data which is representative of that tissue. A random sample may be defined as one which is free from selection bias, and therefore we should obtain a

sample of micrographs which is selected without regard to their quality or content, preferably from blocks of tissue selected in like fashion, for example by lottery.

All measurements are liable to uncertainties. In biological studies, unlike other natural sciences, an irreducible uncertainty is present known as biological variation. This is the variation which occurs naturally between individuals. In biological experimental design, we try to minimise this variation by selecting matched groups of animals or individuals; this matching may be for strain, age, sex or body weight. Further, we normally attempt to minimise the contribution of biological variation to the variance of a group mean by acquiring as large a number of individuals as possible. There are several steps involved in acquiring tissues for stereological analysis. A typical sampling scheme, which has been variously described as nested, cascade or multi-level, is illustrated in Fig. 1. From each of the steps in the scheme we attempt to obtain representative random samples. These steps introduce further sources of uncertainty into the experimental design.

From a matched sample of randomly selected animals we obtain random samples of organs or tissues, which we then process for light or electron microscopical examination. This provides us with a sample of tissue blocks. From these further selection is made at random, and these blocks are then sectioned and may be either examined directly or photographed. Microscopical fields should be selected without regard to their quality or content. Finally the sampling terminates with the confrontation of the microscopical field, or its representation, the micrograph, with a test system from which the quantitative data are generated. General problems of sampling have been dealt with by Weibel (1970, 1979) and Stuart (1976).

Two aspects of sampling which are pertinent to the novice wishing to undertake studies on pathological tissues will now be considered. These are sample quality and sample size.

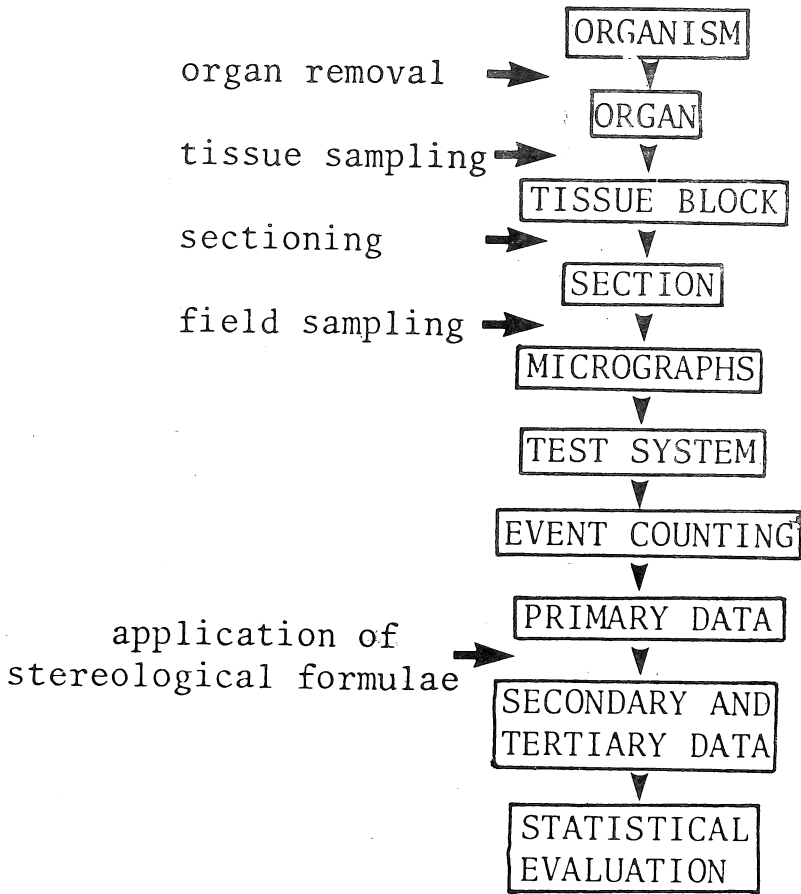


Fig. 1. A typical cascade sampling design for stereology

a. Sample Quality

The quality of the sample is important in that measurements should be made from microscopical fields which are representative. However random samples may not be representative and it may be necessary to deviate from the ideal sampling scheme in order to satisfy the requirements for a particular investigation. For example, if we are interested in evaluating cytological changes which occur in successive strata during the differentiation of stratified squamous epithelium, a random sample of sections will generate many fields which are parallel to the epithelial surface in which it is impossible to establish the level of sectioning.

Sections cut perpendicular to the epithelial surface will provide far more useful information since they provide an accurate overall picture of the individual cells from all layers, which can then be appropriately sampled. In this case, we have biased our sample of sections from a random one to one in which we select only those cut perpendicular to the surface. This sampling procedure has been used by a number of investigators (Schroeder and Münzel-Pedrazzoli 1970; White et al. 1980; White and Gohari 1981a).

When undertaking studies on pathological material, it is common to find that the diseased tissue differs markedly in its architecture from the normal tissue of origin. Usually, we seek to compare quantitative data from experimentally treated or pathological tissue with that from an appropriate control, but strict comparability may be difficult to achieve where the disease process has produced severe disorganisation. In this review, it is obviously impossible to discuss the nature and extent of all possible pathological changes, but the investigator must seek to ensure comparability and this may necessitate modification of the sampling design or the selection of a more appropriate stereological parameter.

b. Sample Size

The second aspect of sampling which requires some clarification is that of sample size. At each of the different sampling levels in Fig. 1., how large should the sample be in order to acquire accurate, representative data? Unfortunately, there are no hard and fast rules, and optimal sample size varies with the stereological parameter and with the particular experimental system. Sample size is influenced by the variability present in the sampling design. In pathological studies, diseased tissues may show a much greater heterogeneity than normal tissues and therefore a larger sample may be required to obtain representative data. Sample size is also affected by the magnitude of the changes we wish to detect: smaller samples may be required for studies in which particular components are quantitatively very different. The precision with which we wish to detect changes is also an important consideration and maximal precision can be achieved by measuring maximal numbers. This has to be balanced practically against the final cost of achieving it and this cost may be measured in terms of

time, effort and money. It has been shown that above a certain size, the improvement in precision is negligible in relation to the extra effort involved (Shay 1975; Nicolson, 1978).

There are several ways of determining minimal sample size. Minimal sample size can be defined as that required from each animal to obtain an acceptable level of precision. One of the simplest is the cumulative mean approach (Chalkley 1943; Schroeder and Münzel-Pedrazzoli 1970), which involves measuring an initially large sample of micrographs. The primary data are then used to compute progressive mean estimates for increasing numbers of micrographs (Fig. 2). Other methods are available such as the relative standard error method which can deal with volume density (Hally 1964) and surface density (Weibel 1976) estimates. These have been dealt with in depth by Weibel (1979).

Precision of measurement on an individual micrograph is not essential. Indeed evaluations of efficiencies of some commercially available automatic or semi-automatic analysers have indicated that it can take longer to obtain data with these expensive machines than by using a simple transparent test system (Mayhew 1981). Further, precision of measurement of an individual field is usually of minor importance when compared with the differences between fields, blocks and animals (Gundersen and Østerby 1981). In a variety of stereological experiments (Mayhew et al. 1982; Gupta et al. 1982), it has been estimated that the largest source of variation is that present between animals. Further Mayhew et al. (1982) have described considerable differences in the contribution to the overall variance in similar estimates obtained from similar tissues undergoing a gradual pathological change. Whilst biological variation is thus a significant factor, inter-block and inter-animal variation can be reduced by using more blocks and more animals, and inter-micrograph variation can be minimised by recording them at the lowest possible magnification which permits resolution of the feature being measured. In general, it is better to spend a given amount of time analysing micrographs from many blocks from many animals than to spend the time analysing more micrographs from fewer blocks from fewer animals. There is also nothing to be gained from quantifying individual micrographs with great precision. Mayhew et al. (1982) have illustrated how stereological sampling schemes may be optimised in pathological studies, and the principle

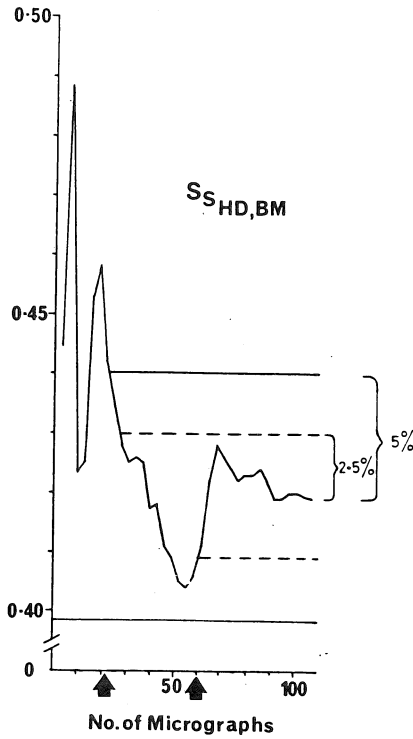


Fig. 2. An example of the cumulative mean plot curve. Raw data from one animal for the parameter $S_{SHD,BM}$, the relative surface of basal plasma membrane occupied by hemidesmosomes is collected in groups of 3. The mean of the first group of three is calculated and plotted; subsequently the means of 6, 9, 12, etc. sets of data are calculated and plotted to provide a curve which initially fluctuates widely but which subsequently stabilizes. A 5% (± 2.5) or a 10% (± 5) level of precision from the sample can be obtained by analysing 60 or 22 micrographs respectively.

advocated by Gundersen and Østerby (1981) of "Do more less well" is an extremely valuable one.

In pathological studies, adequate sampling may be problematical. In studies designed to investigate uncommon lesions, for example, it may prove impossible to acquire an adequate number of specimens from different individuals. Difficulty may also arise in instances where it is possible to obtain only very small specimens from which the production of several tissue blocks may not be feasible. In cases such as these, the principle of "Do more, less well" still applies, even though it may not be possible to obtain large numbers of animals/patients or blocks respectively.

DATA GENERATION

The sequence of events leading up to the acquisition of a representative micrograph sample has been briefly described and it is now pertinent to describe how to proceed in order to obtain measurements from this sample. The simplest and cheapest way of obtaining information is to superimpose a transparent test system over each micrograph. The test system may comprise a series of points, lines or areas depending upon the type of information sought. The primary data is obtained by event counting, that is, by evaluating the chance encounters between the feature or features of interest and the test probes. These primary data are converted to secondary parameters by substitution into appropriate stereological formulae. There are numerous reviews which describe the practical steps in generating stereological data, and the reader is directed to some of them (Weibel and Bolender 1973, Weibel 1974, 1979; Mayhew 1979; White et al. 1982).

The most basic and widely applied stereological parameters are those used to quantify volume, length, surface area and number, and it is these which should provide useful starting points for quantitative research in pathology (Table 1). These parameters are density estimates and for example, the volume density, V_V , relates the volume of a component of interest to a reference volume. Thus we can determine the volume of mitochondria present in unit volume of cell cytoplasm, or the volume of fibroblasts present in a unit volume of tendon. The reference unit is not always necessarily a volume, and in some instances it

Table 1

Some useful stereological parameters

Volume Density	V_V
Length Density	L_V
Surface Density	S_V, S_S
Numerical Density	N_V, N_S

Each relates the volume, length, surface or number of a specified component to a unit containing (reference) volume or surface.

may be more relevant biologically to relate a feature to a surface than to a volume (Mayhew 1979; White et al. 1982). Thus a parameter such as a surface density of a structure which is present on a surface, such as a desmosome which is differentiated on a plasma membrane, may be defined as S_V , the surface density of desmosomes related to unit volume of cells upon which they are present. It may be more appropriate to define surface density as S_S , and to relate the surface density of desmosomes to the surface upon which they are situated i.e. the plasma membrane (see McNutt, 1976; White and Gohari 1981c). Thus before starting a quantitative analysis, both the feature of interest and the compartment within which it is found must be clearly recognisable. In some cases, it may be necessary to identify components by the use of special staining techniques or histo- or cytochemistry.

CONCLUSIONS

In the majority of studies on pathological tissues, stereological methods are readily applicable. The use of these relatively simple techniques can be used to provide a wealth of information which can be used to gain insights into the pathogenesis of disease. Stereology can provide information on the extent of a particular change which might be implicated in a disease process and it is often possible that unsuspected changes may be simultaneously elucidated (Haug 1980). In particular, morphometry and stereology are being applied to studies of tumour pathology (Wiernik et al. 1973; Lawrence et al. 1980; McNutt 1976; Pauli et al. 1978; Franklin and Smith, 1980; Baak et al.

1981; White and Gohari 1981 a,b,c) where problems of diagnosis are of direct importance. Saxen (1979) has clearly shown that there are significant discrepancies in the diagnosis of thyroid malignancies by experienced pathologists and it would thus appear that an approach incorporating more reliability and objectivity, such as from the use of quantitative morphological methods, would indeed be a valuable aid. Before this can be achieved, a wide range of stereological parameters from an even wider range of pathological lesions will require evaluation before these techniques can be used routinely in clinical diagnosis.

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