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STUDYING CELLS WITH DIFFERENT METHODS IN SERIAL SECTIONS: PRINCIPLES OF MATCHING.
With potential applications in combined light and electron microscopy, and various types of histochemistry.

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ABSTRACT

Serial sections of cells can be used to combining results achieved with various investigation methods. Example: combination of light microscopic staining methods or autoradiography with electron microscopy. Histochemistry with its numerous applications benefits from the use of serial sections. Interpretation of results, however, is dependent on stereological principles outlined in this paper. It is possible to estimate the probability of a spherical cell being present in one serial section if it is seen in the other. Estimates can also be given on the number of cells in common in two serial sections, adjacent or separated by a known distance. Under certain conditions perfect matching of cells is possible if cell profiles with nuclei and corresponding location in both sections are matched. For perfect matching the distance of nuclei from each other in tissues is the most critical parameter. Matching of densely packed cells in serial sections is not always possible without modification of the traditional histological techniques. Psychological aspects of recognition are generally less restricting than the stereological conditions for perfect matching. Formulae to estimate the reliability of matching are outlined. Examples of applications are given.

Key words: serial sectioning, geometrical probability, light microcopy, electron microscopy, histochemistry, autoradiography

INTRODUCTION

Sectioning tissues in serial sections allows the investigator to solve stereological problems in histology and histopathology. It is probable that stereology in biological sciences

started with the use of serial sections, and serial sectioning is still the standard approach to 3-dimensional reconstruction (Gaunt 1971). Hans Elias used serial sections in his studies on the microanatomy of the liver and the kidney (Elias 1957) and approached the methods of mathematical stereology in biological context (e.g. Elias 1951, Elias 1954, Elias et al. 1955, Elias et al. 1961).

Serial sections have not given answers to all relevant questions and this has led research to quantitative methods and stereological models. Partly this development was due to the fact that serial section methodology was very laborious. It was also noted that serial sections of paraffin embedded tissue did not resolve well the stereological relations of small structures even though the structures could well be seen in individual sections. Embedding in plastic allowed thin sections for light microscopy and ultrathin sections for electron microscopy, and resulted in better resolution. The development of mathematical stereology helped especially in quantitation of tissue structures, leaving serial sectioning for special purposes. However, serial sectioning is still in many ways a unique methodology, in many contexts a kind of final proof for research done by applying stereological models on single sections of tissue. In fact there are many questions which cannot be solved without serial sectioning. The author of this paper has been involved with a couple of such studies: the structure of the lymphatic inlet valves (Collan and Kalima 1974) and regional abnormalities in respiratory cilia (Rautiainen et al. 1984). In addition to its standard use in studies on embryology (Gaunt 1971) serial sectioning, in combination with electron microscopy, has been applied to resolving the stereology of complicated membranous structures in cells (Andersson-Cedergren 1959, Stempak 1967).

The author's interest in serial sectioning started with the research on the structure of immunologically active cells in the epithelium of the small intestine. This subject was originally suggested to me by my teacher and boss, professor Harald Teir, M.D., as the title of my thesis which was published in 1972 (Collan 1972). Prof. Teir was interested in "physiological inflammation" (Teir 1966) - the inflammatory reaction present in the gut, skin and bronchi - which he daily met while microscopying histological samples. He also suggested that I should use electron microscopy in my studies. I soon noted that there were many researchers who had shown interest in studies of plastic embedded tissue with light microscopy (e.g. Bencosme et al. 1959, Thoenes 1960, Trump et al. 1961, Munger 1961, Marinozzi 1963, Cardno and Steiner 1965, Lynn et al. 1966, Laschi and Baccarani 1967, Collan 1969, Snodgress et al. 1972, Kaufmann and Stark 1972, Snipes 1977) and in combining electron and light microscopic information from serial sections. None of

the reports, however, had taken up the point of how to match the cells seen in such serial sections.

The immunologically active cells in the gut epithelium had special staining characteristics in plastic sections stained for light microscopy. This led me to compare the light and electron microscopic findings on individual cells. I soon realised, however, that the same cells could not always be found both in light and electron microscopy. This led the research to geometrical probability and estimates on the occurrence of the same cells in two serial sections. In connection with the study on immunologically active cells in gut epithelium serial sectioning was also used in another way: the volume variation of a number of these cells was estimated by using serial plastic sections (Collan 1972).

In the following I will try to review the principles involved in matching cells in serial sections. There are also examples to show how these principles can be applied in practice.

STEREOLOGICAL PRINCIPLES: WHOLE CELLS

The probability that a cell (or a tissue structure in general; diameter 2r perpendicular to the plane of sectioning = caliper diameter) will be included in two serial sections (thicknesses s_1 and s_2, cut at a distance of t from each other) if it is seen present in one of them, can be estimated with the formula (Collan and Collan 1970):

$$p = \frac{2r - t}{2r + s_1 + s_2 + t} \qquad (1)$$

The formula gives a way to estimate the probability of finding a cell, familiar to us from one section, in the corresponding location in the other serial section. In a population of cells, the caliper diameter of which can be considered equal, the formula tells us the fraction of cells that are common to both sections under investigation. The following example will further clarify the use of the formula.

Example 1.

Electron microscopy and light microscopy of spherical bone marrow blasts embedded in plastic were compared. First 1 µm thick plastic section was cut for light microscopy, thereafter 5 ultrathin sections were cut in series. The thickness of the ultrathin sections was 0.06 µm. The researcher wanted to compare the light microscope findings with the ultrastructure of 50 bone marrow blasts. The comparison could be made from the

light microscope section and the third EM section cut after the light microscope section. The diameter of the blasts was 10 μm. What fraction of the 50 blasts selected randomly from the light microscope section can be expected to be present in the ultrathin section ?

Solution:
The above formula gives:

$$p = \frac{10 - 0.12}{10 + 1 + 0.06 + 0.12} = 0.88$$

which is the fraction of cells that can be expected to be present in the ultrathin section if the cells are selected randomly. Because selection usually is not random, this value is worse than can be expected if only those cells are selected which show a large profile diameter in the light microscope section. I return to this point later.

CELL POPULATIONS WITH VARIABLE DIAMETERS

When the caliper diameter varies, the above formula can be applied in different fashion. If the mean caliper diameter $\overline{H}$ can be estimated, then $\overline{H}$ can be used instead of 2r in the above formula. Because the cell populations usually have a near lognormal size distribution, the mean p can also be estimated with the help of lognormal distribution. Guidelines have been given in an earlier study (Collan and Selkäinaho 1981).

Example 2.

Cells in tissue culture were studied. The sizes of the cultured cells were first estimated in a flow cytometer. The maximum diameter of these cells in flow cytometer was 16.9 μm. The size distribution is shown below:

Size class	Fraction of cells
15.0 - 16.9 μm	0. 10
13.0 - 14.9 μm	0. 15
11.0 - 12.9 μm	0. 05
9.0 - 10.9 μm	0. 30
7.0 - 8.9 μm	0. 40

The same cells were fixed and embedded in plastic. The largest diameter measured on the sections cut of the cells was 16.9 μms suggesting that no shrinkage had occurred. Under conditions that no shrinkage had occurred, calculate the mean caliper diameter of the cell population.

Solution:
A good estimate for the mean caliper diameter will be calculated as follows:

$\overline{H}$ = 16 x 0.10 + 14 x 0.15 + 12 x 0.05 + 10 x 0.30 + 8 x 0.40 = 10.5 (μm)

The mean caliper diameter can be used in formula (1). In case the distribution of cell sizes is lognormal or near lognormal, the mean p and its variance can be estimated by applying the characteristics of that distribution (Collan and Selkäinaho 1981).

STEREOLOGICAL PRINCIPLES: CELLS AND THEIR NUCLEI

In the practical situation one would rather like to be sure of that one is dealing with the same cell in the same place in two sections. If we consider whole cells the best we can get is the fraction of correctly matched cells. However, the investigator would be better off if only sections cut through the center of cells would be considered. Because the nucleus is situated at the center of the cell, the nucleus could tell us when we have a section through the center or near the center of the cell.

How this principle could be applied has been elaborated in an earlier study (Collan 1973). The study gives a solution to a situation with spherical cells of the same size. Because the biggest problems will occur with cells which touch each other, the solution is based on two cells with their membranes touching each other. These cells can be cut at any angle. To proceed we have to find criteria which allow us to make a distinction between the cells in the section. First, one should determine the cutting (caliper) distance over which two cells can be present in the same section so that they can be seen. In cells of similar size this distance varies between the diameter of the cells and zero. When (Fig. 1)

$$2r\ (\ 1 - \sin a\ ) > t \qquad (2)$$

(2r = diameter of the cells, a = cutting angle in relation to the line between the centers of the cells, t = distance between the sections) mismatching of the two cells is impossible. Under the above conditions, if one cell is seen in one section, the

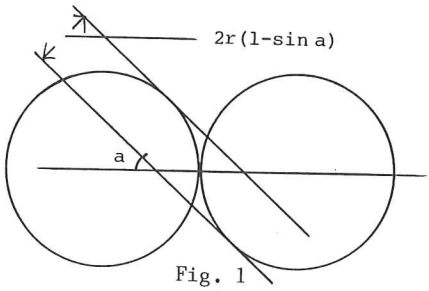

Fig. 1

same cell or both cells are seen in the other. When both cells are seen in both sections, there will be no problems in distinguishing between cells, either.

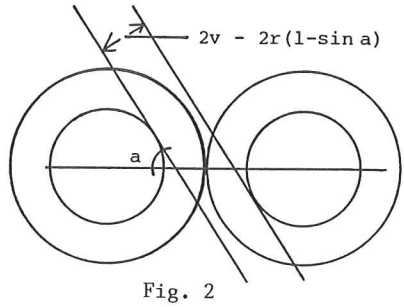

Fig. 2

On the other hand, because we would like to select only sections with nuclei for matching, the distance between the nuclei of two adjacent cells becomes important. Again the two adjacent cells can be cut at different angles and what now becomes important is the distance of the nuclei as measured from the cutting plane. When the sum of distances from the plane is larger than the sum of the thicknesses of the sections and the distance between the sections, a "nuclear" cell profile in a defined location in one section must be the same nuclear cell which is present in the other section in corresponding location. If the nucleus is not present in the other section, one cannot be sure of perfect matching. This gives us the formula (Fig. 2):

$$2v - 2r\,(1 - \sin a) > s + s + t \qquad (3)$$

in which v is the distance between the cell surface and the nucleus.

Because matching can be considered reliable when one or both of the inequalities hold, we can now combine them by summing both sides as follows:

$$\begin{array}{l} \quad 2r\,(1 - \sin a) > t \\ 2v - 2r\,(1 - \sin a) > s + s + t \\ \hline 2v \qquad\qquad\qquad > s + s + 2t \end{array} \qquad (4)$$

This gives a rule for matching. The distance between cell surface and the nuclear surface is decisive. If there is no intercellular material 2v corresponds to the distance between nuclei. In systems in which this distance is large enough it is possible to do perfect matching. Of course, perfect matching is also dependent on the thicknesses of the sections involved.

In a later study (Collan 1975) various systems of Epon embedded tissue were analysed in terms of their suitability for perfect matching. The study showed clearly that conditions for matching may be very strict. This applies especially to lymphocytes. The distance of nuclei in two adjacent lymphocytes in mouse spleen

may be very short, about 0.5 μm. This means that reliable matching is possible between light and electron microscopy if sections about 0.4 and 0.06 μms thick are used. Matching between light microscope sections is problematic because sections as thin as 0.2 μm are not always suitable for light microscopy.

Other systems are not as critical. In the mouse, the minimum distance between nuclei of spermatogonia, and kidney tubular cells was 1.7 μm. This allows reliable matching between light and electron microscopy. In fact 1 μm thick light microscope section can be used and several EM sections taken. Also three sections 0.5 μm thick can be used to allow perfect matching between light microscope sections. Paraffin embedded tissue cannot be sectioned as thin as this and it makes paraffin embedded tissue more or less unsuitable for perfect matching in these tissue systems.

Liver parenchymal cells in mouse had a distance of 8.5 μm or more between adjacent nuclei. This allows matching also in paraffin embedded tissue, but 3 μm sections or thinner should be recommended. Brain tissue generally has much intercellular material and matching will be easy and reliable in most instances when brain tissue is under study.

Example 3.

Brain glial cells are studied. In the area studied the cells are far apart and the minimum distance between cell nuclei is 25 μm. Nuclei are 8 μm in diameter and the cytoplasm around the nucleus is 5 μm thick. Is perfect matching possible in light microscopic immunohistochemistry by which the investigators would like to study the expression of 8 different protein antigens in the cytoplasm of these cells? What proportion of the cells can be perfectly matched?

Solution:
Let us first answer the last question. The solution is based on geometrical probability (Collan and Selkäinaho 1981, Collan et al. 1983, see also Weibel 1979). We consider only the case of adjacent serial sections - two sections following each other. We suppose that the thickness of the sections is 5 μm. First we imagine a cut with the microtome knife through the tissue, and suppose that the number of cut nuclei per area on cut tissue surface is N_A . Wicksell's formula can be applied here:

$$N_V = \frac{N_A}{8\ \mu m}$$

Thereafter the formula of Ebbeson and Tang can be applied:

$$\frac{N_A}{8\ \mu m} = (N_{A5} - N_A)/\ 5\ \mu m$$

in which N_{A5} is the number of nuclei seen in the 5 μm thick section.

From the above the ratio of N_A and N_{A5} can be calculated. The result is: 8/13 = 62%. This means that if 5 μm thick sections are used about 62% of cells seen in the section can be matched perfectly. One should not try to match the other cells, of course. The thinner the sections, the higher will be the fraction of cells which can be matched perfectly. The reader should realise that the distance between the nuclei has no relevance at this point.

The first question was partly answered above. Perfect matching is possible in two adjacent sections. The fraction of cells that can be perfectly matched can be estimated. But is it possible to cut 8 sections in series, stain each of these for different protein antigen and then match the sections ? This is not advisable because in that case the sections should be very thin, less than 1 μm thick. The nuclei are so small that only thin sections allow the same nuclei be present in all eight sections. If the section thickness will be within limits of 3 - 5 μms it is advisable to use pairs of adjacent sections. The first section of each pair would be stained for the same antigen and the second section would be stained for one of the seven other antigens. If the number of pairs of sections is big enough, it will be possible to find out what protein antigens are expressed simultaneously in the cells in question.

PSYCHOLOGICAL ASPECTS

The presence of parts of the same cells in the matched sections is the necessary prerequisite for reliable matching. However, this is not the only point to be considered. The other is correct recognition of the cells to be matched in the sections. An experiment on this aspect was made by using herring eggs embedded in paraffin (Collan and Collan 1970). Sections were cut of the paraffin blocks at 1200, 400, 200, 100, 40, 20, 12 and 4 μms apart from each other. Micrographs were taken of the sections and the micrographs were given as pairs to 15 observers. They also got pairs of micrographs taken from two different tissue blocks. The observers (many of which were specialist grade pathologists) were asked in which of the micrograph pairs they could locate same cells and find the corresponding locations. Distances 4 and 12 μm gave no problems. Thereafter up to 100 μm occasional picture pairs were considered too far apart to be matched by at least one of the

observers. However, sections above 200 µm apart were constantly too far apart to be recognized sufficiently well for matching. The mean diameter of the herring eggs was 0.77 mm. The ratio between the intersectional distance with still reasonably good matching (in this experiment 100 µm) and the mean diameter of the herring eggs was called the psychological safety limit. It was suggested that matching should not be done in systems composed of roughly spherical particles in which the distance between the upper surfaces of the sections divided by the diameter of the particles is above 0.13 - the psychological safety limit in the experiment.

The above result suggests that for perfect matching in microscopy 0.13 times the diameter of the nucleus is the greatest distance that can be allowed between the upper surfaces of the sections to be matched. This especially applies to systems in which the cells are densely grouped (spleen, lymph node).

Example 4.

1 µm thick sections are cut of spleen cells embedded in plastic. The diameter of lymphocyte nuclei in this system is 6 µm. Will the psychological safety limit be exceeded in this system if two adjacent cells are matched ?

Solution:
The distance between upper surfaces of the sections is 1 µm and nuclear diameter 6 µms. The ratio is 1/6 = 0.167. This means that recognition of corresponding places on the sections can be difficult. In addition one should realise that because the distances between nuclei in spleen can be very short, for geometrical reasons matching may also be imperfect under the conditions of the example.

DISCUSSION

Combination of light and electron microscopy of serial sections has certain special advantages. Such approach could be especially valuable in autoradiography, because light microscopic autoradiography is much more sensitive than EM autoradiography (Collan 1973). If light microscope autoradiography is done on sections cut in series with EM sections it will be possible to make the comparison without the need of re-embedding the light microscope section, as in the method suggested by Henken and Chernenko (1986). In a corresponding way one could apply the tissue block immunohistochemistry method, suggested by Eneström (1986) and find the immununohistochemically positive cells or structures by studying the ultrathin sections. Serial sections on individual cells can

also be used in light microscopy alone. Today it is not by any means unusual to hear on immunohistochemistry done on serial sections. The aim is to get an idea about the different types of antigens present in the same cells. The investigator can be expected to draw relevant conclusions from his findings if he or she is aware about the geometrical, stereological, and psychological principles involved in such experiment.

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