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Limitations of clinical and biological histology

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Summary Histology, including histochemistry, histopathology, electron microscopy and immunocytochemistry, can be considered as two disciplines – *clinical* and *biological*. The former is used to make a clinical diagnosis, based on empirical comparisons between, on the one hand, the clinical health of a normal subject with the histological appearances of the organs, and on the other hand, the clinical disease or syndrome of a patient, with the different histological appearances of the organs believed by the clinicians to be affected. It is intended that biological histology should be the most accurate description possible of the structure and chemistry of the living tissue in the intact healthy organism. © 2000 Harcourt Publishers Ltd

INTRODUCTION

The following stages of histological procedures are considered: killing the animal; fixation of the tissue; its dehydration; clearing, infiltration; embedding; sectioning; rehydration; staining; second dehydration, second infiltration; mounting. Each step of the procedure is examined to see how it may change the structure, and chemistry of the tissue, the cells and its subcellular organelles. When one examines the effects of the different reagents and agents on the tissues, many of the conclusions of biological histology are thrown into doubt. Two strategies are possible in this situation. *Either*, one can embark upon a long series of control experiences examining the effects of each of the reagents and agents used on the structure and chemistry of the cells, *or*, one should study living or near-living systems usually by low-energy non-disruptive procedures. Histologists and cytologists should no longer continue to ignore the effects of the powerful reagents they use on the biology of cells.

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LOGICAL NOTE

Conclusions, which have been arrived at, which are wittingly or unwittingly in disobedience of the laws of solid geometry, thermodynamics or physical chemistry, cannot be saved by any other evidence, apparently compatible or not incompatible, or not relevant, to those conclusions. No conclusion or theory should ignore fundamental doubts. The correct reaction to doubts on *any* grounds is to: (i) examine them carefully to see if, indeed, they *are* based on ignoring fundamental laws; (ii) ask if the procedure could be so modified that the experiments would then obey fundamental laws; (iii) carry out such calculations or control experiments to find out, if the disobedience to fundamental laws has so little effect on the final results of the experiment, that it can be regarded as not significant; (iv) carry out such control experiments to measure quantitatively the effects of the reagents and agents, and use this information to correct the results of the experiments.

DEFINITION

Histology here will be taken to include: all procedures of human and animal histology, irrespective of the technique of microscopy used; histochemistry; immunocytochemistry and electron microscopy – including

transmission, scanning and several freezing techniques. The review will not cover microscopy, studies of unfixed cells, subcellular fractionation, cellular enrichment, or historical aspects of histology.

There have been extensive theoretical studies on the *chemistry* of individual stages of histology (for example, (1–7), but remarkably few on the effects of histological *procedures* on microscopical appearances (8–11); none has examined the effects on the biochemistry of cells in living intact animals.

CLINICAL HISTOLOGY

Clinical histology, which originated as a branch of pathology, is used for diagnosis of disease. The clinician knows that living nuclei are not violet, nor cytoplasm pink; and that all stains are artefacts, because they colour, or deposit on parts of cells, which, in life, have no natural colour and few deposits. It is not important that the colours and deposits are artefacts, because the same artefacts have been imposed on the tissues originating from a healthy person and those from an ill person, and the histopathologist recognises the differences between the two in the light of previous clinical knowledge about the diagnosis. The health or illness of the tissue has been previously established empirically by comparing the clinical condition with the cellular appearance at biopsy or post-mortem of the tissue.

CYTOLOGICAL STUDIES USING HISTOLOGICAL TECHNIQUES

The cytologist aims to understand the structure and chemistry of the tissue as it was in the living intact animal. Any agent or manoeuvre, which changes these parameters significantly, for example, by fixing tissues (arresting metabolism), dehydrating them (shrinking them), rinsing them (extracting water-soluble substances) etc. alters their structures or chemistry, and make information obtained from them of less value than that derived from examining living tissues (12). This applies to all tissue from biopsies, post mortems, smears, spreads, tissue cultures, subcellular fractions, all cells containing water, etc., studied by histology, histochemistry, immunochemistry, and electron microscopy (13).

In general, histological procedures involve: dying of an animal and fixing the tissue, which effectively destroys the dynamic relationships present in the living organism; dehydration, which removes the water in which nearly all chemical reactions occur in the living tissue; and shrinkage, which distorts the dimensions and concentrations of substances in each subcellular compartment. Whereas such changes do not matter in clinical histology, they are very important, when one is trying to find out what happens in the cells in the living intact animal.

TISSUE CULTURE

Much photographic information about some kinds of cells is derived from tissue cultures, in which the tissue is definitely living. However, one must bear in mind that the tissue is different from that in the living intact animal in the following respects:

a. The cell structure often *looks* different from that in the presumed parent tissue;
b. its appearance changes with time in culture;
c. cultures are usually derived from embryonic or cancerous tissues, whose cytology and chemistry are known to be different from those of adult animals and human beings;
d. tissues obtain their nutrients from their culture media, while in the intact animal, they obtain them from the network of capillaries;
e. tissues in culture are not subjected to the hydrostatic pressure from the circulation, nor the lateral and vertical pressure from the surrounding tissue, as the organs are in the living intact animal;
f. the chemistry, the morphology and the growth in culture of the tissues is *totally* dependent on the media and the culture conditions in which they are grown;
g. tissues in culture have different cellular environments than they do in the intact animal;
h. tissues in culture have never been shown to stain similarly or have the same immunochemical properties, as they do in the intact parent tissue, although it is popularly and tacitly assumed that they do;
i. tissues de-differentiate in culture;
j. often tissues which have been grown in living cultures are, nevertheless, examined after fixation and full histological procedures;
k. a culture is only made of one or a few kinds of cellular components of a whole animal.

REAGENTS USED IN HISTOLOGY

The most obvious problems arise from the use of a large number of unnatural and powerful reagents and agents, and the untested but testable assumptions, that the latter themselves have little or no effects on the structure and chemistry of the cells. A list of the classes of reagents and agents used routinely is given (Table 1). The obvious question here is, 'What are the consequences to the structure and chemistry of living organisms of subjecting them to agents and reagents which are: (a) unnatural; (b) very powerful chemically; (c) toxic; (d) extract important natural ingredients?'

Table 1 Classes of reagents, agents and electromagnetic radiations, used in histology. In the doses used, only water, buffers, and carcinogenic and teratogenic substances, are compatible with the life of cells or organisms. In addition, few of the reagents are isotonic

Water
Strong acids, alkalis, aldehydes, oxidising and reducing agents
Dehydrating agents
Lipid extracting reagents, (may be same as latter)
Dangerous solvents and vehicles
Buffers
Poisonous salts of heavy metals
Plasticisers, accelerators and hardeners
Carcinogenic and teratogenic substances
High and low temperatures
Electron beams
X-rays and ultraviolet radiation

All reagents used have the following effects:

a. they dilute the chemical constituents of the tissue in which they are soluble. For example, in light microscopy a 100 nm to 10 μm thick section of 10 mm × 10 mm, weighs less than 1 mg. In electron microscopy, a section 40–120 nm thick of 2 mm × 2 mm weighs less than 0.5 μg. These specimens are immersed in 5–10 ml of reagents, which represents huge volumes relative to the tissue dimensions. There will be an exchange of soluble materials between the relatively large area of thin tissue and the large volume of reagent, due to diffusion and osmosis; these will be accelerated by any shaking, heating, and heat released by reactions between the tissue, earlier reagents and the new reagents. Water soluble materials include ions, carboxylic acids, fatty acids, amino acids, and proteins of low molecular weight. Diffusion only stops at a temperature of absolute zero, in totally dehydrated tissues, or if the tissue contents have been precipitated *and* bound to the tissue firmly;
b. freezing techniques include rapid freezing, freeze fracture, freeze-drying, freeze substitution and freeze etching. These cause large changes in the tissue, with respect to the osmotic pressure, rates of diffusion, solubility, pressure, hydration, entropy, viscosity, vapour pressure, and rates of reaction, in all the constituent chemicals in all the compartments and reagents used in the procedures. Freezing is carried out to slow down post mortem changes and to act as a fixing agent, but a chemical fixative is usually used after the freezing;
c. a basic tenet of histology is that each different phase in the tissue is of different chemical composition. Minimally, there are: the extracellular compartment; the cell membrane; the cytoplasm; the mitochondrial membrane; the mitochondrial matrix; the nuclear membrane; the nucleoplasm; the nucleolus. Thus, each of the reagents and agents in Table 1 would *change* each compartment differently and *affect* the metabolism and chemistry of each compartment differently.

OTHER PROBLEMS OF HISTOLOGICAL PROCEDURES

Even with such standard procedures as, haematoxylin and eosin, silver staining, or osmic acid staining, the titles of the procedures condition one to think that these important reagents are the main or only powerful chemicals employed in the named procedures. Usually, 10–20 reagents are used in each procedure.

All histological procedures are empirical, as are culinary recipes. Whereas the latter use the subjective judgement of pleasant taste, the former often use the criterion of aesthetic appearance to assess the usefulness of a procedure. This is because histologists feel that there are no *cytological* techniques available to indicate the original appearance or chemistry of the tissues in the intact living animals, or the histologists have been unwilling to use procedures, which might be able to yield such information (see Tables 2 & 3).

Microscopical slides have so much information in them due to the magnification, that a histologist has to select the slides, the fields and the micrographs, for demonstration

Table 2 Some non-invasive and minimally disruptive techniques which can be used in cytology intended to elucidate the properties of intact cells. Many of the references are old, because these techniques are out of favour, compared with highly energetic disruptive systems, involving such techniques as subcellular fractionation and electron microscopy

Technique	Comments
Phase contrast and dark ground microscopy (33,37,38)	Unfixed cells, can be used with time lapse
Intracellular pipettes (39–41)	Measuring transmembrane voltage and ion movements
Transplantation of nuclei (42–44)	Micromanipulation under the microscope
Addition of single reagents (45–47)	In unfixed tissue, to bathing media
Microinjection (46,47,48)	Into extracellular fluid or cytoplasm
Microspectrophotometry (49,50)	Non-invasive
Microchemistry (51,52)	Whole cells, nuclei, cytoplasm or extracellular fluids
Fluorescence (53,54)	Only using antibody in isotonic conditions, with no other reagents
Laser microbeam trap (55)	Can move nuclei or mitochondria
Microcentrifugation (56,57)	Under direct microscopic supervision
Supravital staining (20,58,59)	Assumes stains do not alter the reactions being observed
Other in vivo techniques (60)	Mostly blood vessels

Table 3 Preparations which can be studied with minimal disruption to the original tissue

Kinds of preparation	Cells or fluids	Comments
Normally single cells	Red cells	These can be examined in vitro in media simulating those in vivo
	Ova	
	Sperm	
	Lymphocytes	
	Protozoa	
Easily isolated cells	Buccal epithelium	Taken from cheeks
	Cells in urine	Deposit after mild centrifugation; have not been used hitherto
	Normal cells from vaginal smears	Have to be identified as healthy
	Hepatocytes	Liver is taken up into a syringe
	Neuron cell bodies	Microdissected by hand
	Myelinated axons	Must be teased under microscope
	Cells from biopsies	Must be teased under microscope
	Healthy tissue removed at operation	Must be teased under microscope
	Rods and cones	Separate easily from retinas
Extracellular fluids	Plasma	These fluids are in such large volumes relative to the size of cells that they can not reflect rapid changes in the cells which they bathe. However, local small samples can be drawn off
	Urine	
	Lymph	
	Salivary juice	
	Gastric juice	
	Duodenal fluid	
	Spermatic fluid	
	Tears	
	Sweat	
	Milk	
	Tubular fitrate	
	Microdialysis	
Microchemistry of single or few cells	DNA, RNA in neurons	Most of this work has been done in mammalian cell bodies
	proteins in neurons	
	enzymes in neurons	
	ions in neurons	
Microchemistry of cellular constituents	Tubular filtrates	
	Pipette specimens of extracellular fluid and cytoplasm	
Isolated parts of cells	Neuronal cell membrane	
	Microdissected nuclei	
	Myelinated nerve fibres	
	Unmyelinated nerve fibres	
	Isolated muscle fibres	

and publication. Such choice of evidence is unacceptable in any other branch of science, whereas it is required in histology. In an attempt to make the choice as objective as possible, the research workers show 'typical' views of the structure, which they are describing. Yet frequently, electron microscopists have reacted to criticism about micrographs they show by asserting vehemently that they deliberately *choose* the illustrations they use, and this makes it unfair to assess their appearances from published pictures. Obviously an image should be chosen to be typical of a structure, not a rare one which illustrates the particular point the author is making. Any jobbing histologist – especially an electron microscopist – knows that even with the 'best' histological technique, one can find a very large range of appearances, which one can choose to illustrate any structure which one wishes to show.

Clinical histologists do not feel that they need statistics, because the pathological lesions are overwhelmingly associated with the disease states of the patients. Probably, also, it is a recognition of the subjectiveness or experience they exercise in choosing particular examples of the wide variety of appearances in sections.

PROBLEMS OF CLINICAL HISTOLOGY

Firstly, the correlations between clinical disease and histopathology are not always absolute. Two examples are worth noting. In about a quarter of clear clinical cases of coronary thrombosis, post mortem examination of the arteries showed no relevant significant lesions, and many patients who died of other diseases were found to have had two or more blocked coronary arteries (14–16). A

significant percentage of appendices excised surgically, were found to appear quite normal (17–19).

Secondly, it is sometimes difficult to pinpoint the microscopic lesion by looking at the gross appearance, and so, occasionally, biopsies or post mortem specimens are taken from a healthy tissue, or from disrupted tissue between the lesion and the healthy tissue.

Thirdly, the choice of optimal staining procedure to show up a particular lesion must be somewhat subjective.

Fourthly, some lesions appear rather similar in different diseases.

Fifthly, different histologists do not always agree on such parameters as the extent of the lesion, the malignancy of a cancer, the nomenclature, etc; the latter is especially true of lesions of the brain.

However, the histopathologist usually knows the age, clinical history and signs and symptoms of the patient, and this knowledge influences the diagnosis. It represents evidence for the conclusion, and has a complex relationship to observer bias.

INDIVIDUAL STEPS OF HISTOLOGICAL PROCEDURES

Killing the animal and obtaining human specimens

Occasionally, the killing of animals is discussed briefly in textbooks (20,21), but not in its relation to possible effects on the final appearance of sections. Perhaps, the histologists believe that the method of killing the animal – or indeed, the procedure of taking a biopsy – have little or no influence on the structure or chemistry of tissues, or they consider that they can do nothing about it. The fact that butchers believe that the taste of meat depends, *inter alia*, upon the method of slaughter, means that there is a *prima facie* case for discussing the matter, bearing in mind the desire of biologists to find out about the chemistry of the cells in living intact organisms.

Tissue is usually taken from biopsies of patients in clinics, surgical operations or experimental animals. Patients may be under local or general anaesthesia, when biopsies are taken. The samples of tissue are immersed immediately in a fixative, or are rapidly frozen and then fixed. The sections are then prepared for diagnosis by the pathologist, who examines their cellular appearance. In so far as this is done for clinical purposes, the events occurring during dying and post mortem will not affect the decision about diagnosis; the same is also true of tissues taken from the bodies of dead patients for histological examination.

However, the experimental cytologist must ask how might killing the animal affect the appearance or biochemistry of the tissues. Animals are normally killed by: dislocation of the neck; stunning by striking the head followed by exsanguination; injection of excess anaesthetic; exsanguination under anaesthesia; guillotining; being dropped into liquid nitrogen; exposure to high concentrations of carbon dioxide; intravenous air embolus; microwaving the brain. When the tissues are obtained from slaughter houses, the animals will have been killed by electric stunning or captive bolt, followed by sticking and exsanguination, or by cutting the throat followed by exsanguination. All techniques of killing animals, except those carried out under anaesthesia, cause the animal some pain for seconds – although one cannot know its intensity, or precise duration. It is likely that they feel pain until fall of blood pressure, fainting, or anoxia to the sensory system in the brain, makes them unconscious.

Pain causes stress which activates the sympathetic nervous system. In addition to the stress on the living animal, the process of dying initiates a number of effects on the tissues, and these continue after death. All death, which is not caused by the destruction of tissues, occurs as a consequence of tissue hypoxia. The events during dying and the consequences to tissues are shown in Table 4.

Table 4 Effects of dying and death on tissues. Events above the horizontal mid-line cannot be prevented even by rapid freezing. Those below the line are diminished if the animal is killed quickly, and the tissues processed without delay

Events	Results
Pain due to killing procedure	Release of catecholamines and steroids
	Hyperventilation
	Lowered blood P_{02} and pH, raised PC_{02}
	Lowered adenosine triphosphate and phosphocreatine in muscles
Hypoxia during dying of animal, organ, tissue and cells	Cellular hypoxia
	Raised serum Na^+, lowered K^+ and raised phosphate
	Raised tissue Na^+
	Lowered tissue P_{02} and raised P_{co2}
Dying	Agonal bacteraemia
Fall of temperature	Slowing down of metabolism
Autolysis	Proteolysis and glycolysis
Loss of immunological response	Antibodies fall
Bacterial invasion	Tissue degradation and gas formation
Collapse of transmembrane gradients	Compartmentation of soluble components diminished
Rigor mortis	Muscles in spasm

Table 5 Effects of fixation on chemistry of tissues. Generally, fixatives denature proteins, inhibit enzymes, and change the volume of tissues

Type of fixative	Examples	Other effects
Aldehydes	Formaldehyde Glutaraldehyde	Shrinkage
Oxidising agents	Osmium tetroxide Potassium permanganate Potassium dichromate	Breakdown of proteins Enzyme inhibition
Early fixatives	Acetic acid Ethanol	Dehydration
Heat	Boiling Microwave	Release of gases Enzyme inhibition
Cold	Freezing to –200°C	Dehydration
Others	Mercuric chloride Picric acid Carbodiimides	Various

It might be thought that the latter effects would be reversed by incubating the tissue, but this has its own problems, of which the four most troublesome are: swelling of tissues during incubation; unknown loss of tissue components to the medium; unknown and often unknowable extent of recovery of metabolism during incubation; difficulty of knowing tissue weight (22).

It is thus clear that it is difficult to know the effects of dying and death on cells.

Fixation

There are about 2000 fixatives and fixative mixtures, as well as heating and freezing. The chemicals can be classified into various groups (Table 5), (2,10,23,24).

The aims of fixation are: (i) to precipate the macromolecules, preferably at their sites in the living intact animal, but if this is not possible, at their sites in the tissue from the dead animal; (ii) to prevent changes in shape or size of any part of the tissue; (iii) to hold its chemical reactions to their equilibria in life; (iv) to kill organisms which would cause its decay; (v) to preserve the tissue for a long time. Fixation cannot stop: (a) the effects of dying and death on the whole organism, the tissues or the cells, (b) osmosis of water within or between compartments; (c) diffusion of solutes, solvents or gases between cellular compartments; (d) and the heat exchanges consequent upon the addition of reagents to each other and the tissue.

Fixation may prevent relocation of molecules and particles which they precipitate, such as proteins and glycogen. The intention is that they should precipitate at the sites where they were in the living cell. Unfortunately, there is no way of knowing how much movement occurs during dying and fixation, nor whether the reagents precipate the particles and the macromolecules at their original sites, or whether they can move after precipitation. However, it is reasonable to believe that they remain in the same compartments as they were in life, at least until the tissue is sectioned.

Fixation generally causes shrinkage of whole cells, extracellular fluid, cytoplasm, mitochondrial matrix, nucleoplasm, and nucleolus, not always to the same extent (8,11,25). The cross-linkage of proteins, their denaturation, the loss of co-factors by dilution and the inhibition of enzymes, mean that fixatives should only be used in immunocytochemistry, when their effects on tissue constituents under study have been examined and corrected for. This is a very complex situation, since: firstly, the effects of the reagents on the naturation of proteins or enzyme activities have often only been measured in purified systems, whereas in living tissues, the natural chemicals occur in small concentrations often in the presence of other substances at higher concentrations; secondly, the fixative extracts water-soluble compounds from the tissue; thirdly, changes in volume alter the dimensions and concentrations of all the substances in the organelles; fourthly, all fixatives – including boiling and freezing – have osmotic effects, usually because they are not isotonic. It is unlikely that any fixative can be regarded as isotonic, since they change substantially the permeability of the cell membranes; fifthly, freezing procedures, however rapid, dehydrate the tissues when the temperature falls below the eutectic point of the extracellular fluid and cytoplasm.

Freezing

Since 60–80% of cells consist of water, freezing must produce dehydration, when the water crystallizes out. Indeed, publications on electron microscopical techniques indicate that the tissues must be fully dehydrated before they are put into the instrument, and bombarded

by electrons (*6*,*26*,*27*). Critical point drying – in which it is claimed that the water remains in the tissue – is a misnomer, because the critical point is the temperature and pressure at which the solid, liquid and vapour phases are in equilibrium. At this point drying will not occur. Only when the temperature is raised or the pressure is lowered from the critical point, can drying ensue.

Often when rapid freezing is used initially to make the tissue harden enough to section, fixatives are subsequently added.

It is widely believed that freezing can be done so rapidly that there is not enough time for fluid movements, tissue distortion or crystal formation. Of course, the more rapid the freezing, the smaller the crystals. However, the glasses, which have been shown experimentally, are normally in pure solutions – because impurities prevent glass formation – and extracellular fluid, cytoplasm, mitochondrioplasm and nucleoplasm are simply not pure solutions.

The tissue freezes when it is immersed in liquid nitrogen or isopentane at −190°C to −150°C; carbon dioxide freezes it to about −70°C. During the cooling, dissolved gases such as oxygen, carbon dioxide and ammonia, bubble out of solution because they are much less soluble at these temperatures than they are at 37°C.

The cooling liquids are on the outside of the tissues. The outside becomes more viscous, and it solidifies and contracts before the deeper part of the tissue. The large pressure gradient causes the tissue to crack, and one hears this clearly when tissue is dropped into liquid nitrogen or isopentane. The more rapid the cooling, the greater the cracking. It is widely believed that the more rapid the cooling, the less time the tissue has to be dehydrated and shrink, and that dehydration does not occur if the freezing is rapid enough. Unfortunately, the rapidity allows less time for the gases to escape. Although it is widely believed that rapid freezing prevents dehydration and shrinkage, there is no experimental evidence for this assertion.

Dehydration

Ethanol, acetone, dioxane and freezing, are used for dehydration. The former two extract lipids, so that it is very unlikely that any lipids would be left after the exposure of small pieces of tissue to such enormous volumes of reagents. Dehydration and infiltration at least twice each extract lipids, lipid-soluble constituents, water and water-soluble constituents. Yet electron microscopists claim, firstly that they can see the 'unit membranes', (28); secondly, that the unit membrane contains about 40% lipids (*29*); thirdly, that they can measure the thickness of the membrane.

Furthermore, since Singer and Nicholson in 1972 (30), it has generally been believed that cell membranes are 'fluid', and that the fluids are mainly water and lipids, so that dehydration would alter the membrane dynamics completely.

Each phase of the extracellular fluid and the cells contain different amounts of water, so that dehydration alters the relative concentrations of all the constituents in each of the phases.

A real difficulty here is that one cannot measure the total lipids in the living cell membranes (31). Nevertheless, those who believe that they can prepare a membrane fraction would be doing a major service to membranology to find out how much of the lipids – and other constituents – survive dehydration, and, subsequently any complete histological procedure.

Clearing

Sections are often cleared in light microscopy, using such reagents as xylene, toluene, benzene, trichlorethylene, chloroform, cedar oil and clove oil. Some of these reagents, such as the first three, are also used for infiltration. They increase the refractive index of the tissue and make it more transparent. All of these reagents except the last two – and also methanol and ethanol – are routinely used in biochemical procedures to extract lipids.

Anderson and Gordon (32) list the criteria for choosing a suitable clearing agent as: speedy removal of dehydrating agents; easy removal by molten paraffin wax; minimal tissue damage; flammability; minimum toxicity and cost. Presumably, extraction of lipids would be included in their category of 'tissue damage'.

Infiltration

Since the dehydrating agents are not miscible with the embedding agents, such as wax, acrylic or epoxy resins, intermediate substances such as xylene, 2-hydroxy methyl acrylate and polyethylene glycol, or propylene oxide respectively, are used to infiltrate the tissue before the embedding. In electron microscopy, highly chemically-active plasticizers, accelerators and hardeners are added; many of them are potentially toxic to the histologists.

Embedding

Tissue is embedded to make it hard enough for thin sections to be cut. During the infiltration and embedding, polymerisation occurs. Sections for electron microscopy are cured by heat or ultraviolet light, and they harden. The mixtures used and the times for curing are chosen empirically to enable the tissue to be cut at the thickness and hardness indicated in the literature as being optimal to show up particular structures. These arbitrary choices

make the appearances of the sections largely dependent on the intention of the histologists and electron microscopists.

Cutting sections of tissue

For light microscopy, sections are cut 1 μm to 150 μm in thickness. The thinnest sections are cut with glass knives, but most sections are cut 4–6 μm thick, using stainless steel knives. The thicker sections are used to show thin dendrites, axons and fibres, mainly in the nervous system. Electron microscopists cut sections 40–500 nm thick. The thicknesses between 40 and 120 nm are assessed by their colour. Most figures given for the thicknesses of sections are microtome settings, yet the tissue is compressed during section, and swells during subsequent processing, so that one does not know the real thickness of the section when examining the tissue under the microscope. Further considerable quantities of energy are liberated along the narrow cutting edge during shearing and cutting the section; this is probably translated into a local temperature rise. Cutting may also spread the content of some cells, especially of any granules or particles in them.

If one takes thin sections of an egg of the same thickness relative to the size of a whole nerve cell body, or simply, if one makes sections of a banana, it becomes quite obvious that *without serial sections*, one cannot tell the shape or size of the whole object. If one pursues the analogy of a nerve cell body, one cannot know the shape or size of the cell, its symmetry, the presence or number or position of its mitochondria, nucleus or nucleolus; nor could one know the presence or number of any processes arising from it.

From a single section, one could know only the number of cells which had been cut by that section. One could try to calculate the dimensions of a large identifiable group of cells, if one made assumptions about their shapes in three dimensions, and measured the section thickness. This is most easily done with spherical cells, such as spores, ova, fat droplets or nuclei. Nevertheless, the resultant shapes and sizes of the cells would be those of the fixed dehydrated embedded cells, not those of them living or unfixed.

One can take fresh unfixed cells in their normal environments, for example, ova, nuclei, and protozoa, and view them by phase contrast microscopy. One can then photograph them during each phase of a histological procedure and see how that stage affects their shapes and dimensions. One could then extrapolate back to the unfixed state (*9*,11,33). This procedure has its own problems. Firstly, taking the cells out of their normal environments may change them. Secondly, when one looks at their projected areas, one cannot necessarily know that the projected areas change in the same way in the coordinates at right angles to these areas; sometimes the weight of the cover glass or osmosis causes the cells to deflate. Thirdly, one can only measure the dimensions of, say, cell bodies, nuclei or processes by light microscopy, because they are within the resolution of the microscope, but one cannot measure smaller objects, beyond the resolution. Thus, there are limitations to extrapolating back from the histological appearance of tissues to their likely appearance unfixed, and even more so to those in the living intact animals.

A section of a tissue cuts through cells, nuclei and mitochondria, because the section is usually much thinner than their embedded diameters. One does not know whether the battery of reagents up to embedding, increases or decreases the permeability of the cell membranes to antibodies. However, any organelle cut by microtome must become completely permeable to any antibody. It should not then be necessary for immunocytochemists to add 'permeabilisers', such as detergents, to show up the cytoskeleton. It is much more likely that the permeabiliser precipitates cytoplasm itself.

Floating out and mounting sections

The thin sections are floated out and mounted on slides, sometimes with an adhesive. They stick to the slides sufficiently firmly that they do not come off, when they are subjected to the other reagents used subsequently. After the sections have been mounted, they do not usually shrink because they are 'anchored' to the slide, possibly by Van der Waals forces.

Rehydration

Most stains used in light microscopy are aqueous, so the sections have to be rehydrated before staining. This is done by adding xylene and going through from 100% ethanol to water. Once again any substances soluble in ethanol or water, still left in the sections, will be extracted from them.

Staining

There are about 2500 stains and staining procedures. They may be classified in many different ways, for example, whether they are acidophil or basophil, the colour, the types of cells they stain, the organelles or granules they show up, the tissues for which they are believed to be specific, or whether they are colour or deposit stains.

Many of the original colour stains were developed from the aniline dye industry (34). Colour stains, such as methylene blue, neutral red or malachite green, stain cytoplasm, nuclei, membranes and fibres; tissues may also be counter stained. Of the clinical procedures,

haematoxylin and eosin is the most widely used. The nuclei appear violet and the cytoplasm pink. Of course, the same structures can be seen by phase contrast microscopy, interference microscopy, or dark ground illumination, without staining. Colour stains enhance the visibility of the cells and make them look more pleasing.

Deposit stains, on the other hand, are precipitates of salts of heavy metals, such as osmium, silver, lead, manganese, mercury or tungsten, usually on membranes and nuclei. Some salts can form deposit or colour stains, such as silver or osmium. They may appear as colours, until the salt and the tissue exceeds the solubility product, and then they precipitate. One cannot know whether a precipitate is due to the stain reacting with a soluble or insoluble material in or around the cell, or is depositing on a structure present there. Nor does one have any way of knowing if the apparent structure has moved from the site in the living cell, unless that structure can be seen in living cell culture or in unfixed cells. If it is seen with a number of quite different staining procedures, this strengthens the possibility that it exists in the living cell.

Deposit stains are used in electron microscopy. The original tissue would be destroyed by the electron bombardment, the low pressure and the radiation, so that one has to deposit a heavy metal on it, and them bombard the heavy metal. Obviously, any structure or compound, which does not react with the heavy metal salts, will not be seen. However, when the electron microscopists show what they believe to be cell membranes, receptors, transmembrane molecules or deoxyribonucleic acid, they are actually looking at metal deposits, sited where they believe the structure or macromolecule to have been. Since the structures will have been dehydrated and subjected to very low pressures, the heavy metal salts coating them *must* be on the outside and therefore larger than the structures at this stage.

It is widely believed that the heavy metal salts indicate the sites of the membranes precisely, but this is not the case. The stains *deposit* on the outsides of the membranes. A line is a geometrical abstraction, because it has position but no thickness. Every real membrane has a finite thickness, so that any deposit will rest on *both* of its surfaces. Thus any real membrane must appear as two lines, if the microscope has the resolution to separate them – as has the high power electron microscope.

It is sometimes argued that the heavy metal stain replaces the 'unit membranes', so that one line of stain marks the site of a single layer of membrane. *Either*, as indicated here, the stains are deposits, *or* they dissolve the membranes and occupy their sites. However, if the membranes were, indeed, dissolved, the stains would not have a site on which to deposit.

The thickness of the cell membranes – even if they are much greater than the 7–10 nm seen under the electron microscope, may well be below the 200–250 nm resolution of the light microscope, but a discussion of this matter is beyond the scope of this paper. There is no doubt that the cells do have a cell and a nuclear membrane, but despite a huge amount of electron microscopy *compatible* with the Davson–Danielli and Singer Nicholson hypotheses (29,30), the suggested structure of the membranes and their chemistry in the intact organism is currently unknown and possibly unknowable; this is because one cannot isolate them from their environments without grossly insulting them chemically (12,31). The subcellular and electron microscopic evidence are both grossly inadequate.

Much has been written about the necessity to fix and stain tissues with buffered solutions. The pH of a solution is the concentration of hydrogen ions in water, and isotonic solutions, demonstrated in red cells, would not cause the tissue to swell or shrink. However, neither the hydrogen ion concentration nor tonicity can have any chemical meaning after dehydration. If buffering of any agent used during the procedure improves the appearance of the final section, this must represent a purely empirical finding not explicable theoretically, as buffering. In other terms, it is obviously optimal physiology to bathe a living tissue in a buffered solution, because extreme pHs are incompatible with life, but the pH at earlier stages of histological procedures cannot be considered relevant to the tissue behaviour after dehydration.

Second dehydration

This is done to enable the sections to be embedded in non-aqueous media, and will further extract any materials soluble in water or ethanol from the section, and any others which have become water or ethanol soluble due to the action of reagents used since the first dehydration before embedding.

Mounting

For light microscopy, tissues are mounted in non-aqueous resins, such as DPX, Canada Balsam or Euparal. Previously, aqueous media were used, such as glycerine, gelatine, chloral gum and polyethylene glycol. These mountants were difficult to seal properly, since they sometimes leaked when solvent evaporated. Sometimes they dried out or became contaminated by bacteria and fungi.

Sections for electron microscopy are usually mounted in epoxy resins, but they require immersion of the sections in mixtures of propylene oxide and the particular epoxy resin before being embedded in the concentrated resin. The tissue shrinks when the solvent evaporates. The mountant polymerises, often accelerated by the addition of accelerators and hardeners, so that the preparation becomes permanent.

Table 6 Types of information which can be derived using histological techniques. Some of the information is clinical*

The identity of an organ*
Particular regions in heterogeneous organs, such as brains, spinal cords, kidneys and adrenals*
The diagnosis of lesions of the organs, such as inflammation, cancer, dystrophy or degeneration*
The density of a particular stained cell in a part of a microscopical field, after correcting for the geometry
The presence of parasites, bacteria, fungi or viruses*
If a particular subcellular organelle or microbe is present or absent in a particular population of cells, but not its absolute incidence
Relations of cells and tracts to each other
Degeneration of regions or tracts
Whether there are membranes around cells
Whether regeneration has occurred in life, for example, of nerve fibres or liver

Some mountants for light microscopy cause the tissue to become pale, but bright light is the biggest threat to the colour of tissues.

Mountants are often classified by their refractive indices, but these refer to the bottles of rack reagents, and the refractive indices of the mounted sections change much as the solvents evaporate.

It is generally thought that the shrinkage of the tissue when it is dehydrated is reversed, when it is rehydrated. Unfortunately, this is not so. Single neuron cell bodies were visualised by phase contract microscopy, and the same cells were photographed as each reagent was added. The projected areas of the cell bodies shrunk to 20% of their original areas when stained with haematoxylin and eosin, or by the Palmgren procedure, and to 15% when stained with osmic acid, as for electron microscopy (11).

Of course, sections are mounted, so that they can be kept permanently for diagnosis by pathologists, and for teaching histology and histopathology to students of biology and medicine. Unfixed cells, smears and teased tissue do not last long before they become infected, so that their appearances can only be preserved by photography.

Information which can be derived from histology

Classes of information may be listed (Table 6). Examination of this table is instructive. The first two categories mainly relate to testing the knowledge of undergraduate medical students. The diagnosis of lesions and the detection of parasites are the preoccupation of histopathologists and haematologists. The measurement of the density of cells, and regeneration, are both clinical and experimental. Whether a particular subcellular organelle is *present* or *absent* can be known, because one can see many regions of the same type of cell in a succession of slides, but the incidence of subcellular organelles is difficult to know or calculate except in serial sections. Thus histology when used for biological purposes is most valuable in answering 'yes' or 'no' questions. Any counting or measurements have to be corrected in respect of their size, shape, thickness of section, and the shrinkage during preparation.

Table 7 Information about living cells in intact organisms which can*not* be derived using histological procedures

Volume of extracellular fluid
Chemistry of extracellular fluid
Thickness of cell membranes
Permeability of cell membranes
Presence of receptors on cell membranes
Presence of granules on cell membranes
Shapes of cells, nuclei or mitochondria
Number of connections with other cells
Chemistry of the cytoplasm, nucleoplasm or nuclei
Nature or direction of movements between or within compartments
Presence or rates of reaction of any constituents soluble in water or organic solvents

WHAT INFORMATION *CANNOT* BE DERIVED ABOUT LIVING INTACT TISSUE USING HISTOLOGICAL PROCEDURES

One may summarise further considerations in this paper by listing the information about living cells, which can*not* be derived using histological procedures. The main effects are on the dimensions, shapes and chemistry of the tissues (Table 7). The most satisfactory approach to these very burdensome problems is to use minimally disruptive techniques. The Second Law of Thermodynamics dictates that both putting energy into a system, and changing this entropy, result in a change of free energy, which drives all physico-chemical and chemical reactions. Therefore, one should avoid – as far as possible – all energetic and disruptive procedures, if one wishes to know about the state of the cells in the living intact animal. It is widely believed that no other approaches are possible. A list of minimal energetic and disruptive procedures, with key authors, is given (Table 2), and the kinds of preparations, upon which they may be employed, follows (Table 3). This illustrates the profusion of more law-abiding cytological techniques which are to be recommended.

Two other approaches are possible. One way is to examine the analysis in this paper, and show how it is wrong. I should be grateful to receive any such criticisms. Secondly, one can carry out many control experiments on current histological procedures. For example, one can control for the effect of haematoxylin and eosin procedure

on the shape and dimensions of such cells as sperm, ova, red cells or lymphocytes, which can be easily isolated in their natural milieux. Under the phase contrast microscope, one can photograph the shapes, dimensions and refractive indices of the cells at each step of any histological procedure. One can then extrapolate back to the dimensions of other cells prepared in a similar way. Similarly, one can measure the lipid, protein, carbohydrate, water and nucleotide content at each stage of the histological procedure. One can observe under the microscope the effects of each of the reagents used, on the intracellular movements, the shapes and chemistry of cells in tissue culture, on the assumption that such cells behave similarly to those in the living intact animal, bearing in mind the reservations listed earlier. Another way is to study the effects of each of the reagents in the concentrations used in the histological procedures on the parameters one is studying.

CONCLUSIONS

While clinical histology is a useful empirical tool, a vast repertoire of control experiments will have to be done, before one can conclude with certainly that histology has contributed significantly to the cytology of intact animals. It seems probable that the current belief in the value of biological histology developed historically from the usefulness of clinical histology. The latter discipline developed in the second half of the nineteenth century (35,34,36) before the modern concepts of control experiments, physical chemistry, artefacts, specificity, stereoscopy, electronics, and questioning received wisdom, had become common currency among light microscopists, histologists and electron microscopists.

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