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Review

Quantitation of gold labelling and antigens in immunolabelled ultrathin sections

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INTRODUCTION

Immunocytochemistry employs immunological techniques to localise molecular components in biological material. The most commonly used approach is to label protein antigens with antibodies whose location can be revealed with a microscopic marker. At the ultrastructural level the technique is known as immunoelectron microscopy.

One of the major challenges in immunoelectron microscopy has been to find ways of exposing the antigens in the specimen to labelling reagents. Currently the method of choice is to make ultrathin 50–100 nm sections. These are then incubated with specific antibodies whose location is revealed using second step reagents such as protein-A complexed to colloidal gold. The colloidal gold particles are used because they have sufficient electron density to be distinguished easily from contrasted cellular structures and subsequently counted.

This review focuses exclusively on the quantitative aspects of gold labelling of antigens in cellular structures on ultrathin sections. It first addresses the problem of quantifying gold labelling itself and then focuses on methods which use gold labelling for absolute quantitation of antigens. Many of the approaches that are described rely heavily on principles used in stereology, a science that has been revolutionised recently by key breakthroughs (Gundersen et al. 1988*a*, *b*; Cruz-Orive & Weibel, 1990; Mayhew, 1992).

THE SECTIONS USED IN IMMUNOELECTRON MICROSCOPY

The thin slices used in immunoelectron microscopy are prepared after embedding the specimen in a medium solid enough for sectioning using glass or diamond knives. Two approaches are currently employed (see Fig. 1). One uses embedding in frozen water and is commonly referred to as ultrathin frozen sectioning, and the other relies on embedding in plastic resins.

Ultrathin frozen sections (Tokuyasu, 1973, 1978; Griffiths et al. 1984*a*) are prepared from chemically fixed specimens which have been infused with cryoprotectants such as sucrose and then frozen in liquid nitrogen. The ice is in a vitreous state and consequently lacks detectable ice crystals that could damage biological structures. After freezing, the sections are cut at between −90° and −120 °C. They are then thawed, mounted on plastic-coated EM grids and labelled. One disadvantage for quantitative purposes is that the sections are prone to folding and are difficult to prepare in series.

The most familiar resin technique uses aldehyde/osmium tetroxide fixation followed by embedding in epoxy resins. This has been used successfully for protein antigen localisation but, recently, specialised low temperature techniques have been developed and these improve the chances of obtaining gold labelling of protein antigens. In the most commonly employed low temperature method, the specimens are aldehyde-fixed and then dehydrated in progressively increasing solvent concentrations at decreasing temperatures (Progressive Lowering of Temperature; P.L.T.; Roth et al. 1981; Carlemalm et al. 1982). The temperatures are selected so that the solvent remains fluid throughout the procedure, the final steps of infiltration and embedding in resin being done at around −40 °C.

Another less well known low temperature method is cryosubstitution. This involves freezing the specimen and substituting the frozen water in the specimen, first by solvent and then by resin (Humbel & Schwarz, 1989). Polymerisation of the resin may be achieved at low (−40° to −80 °C) or at room temperatures. If the specimens are chemically fixed and cryoprotected,

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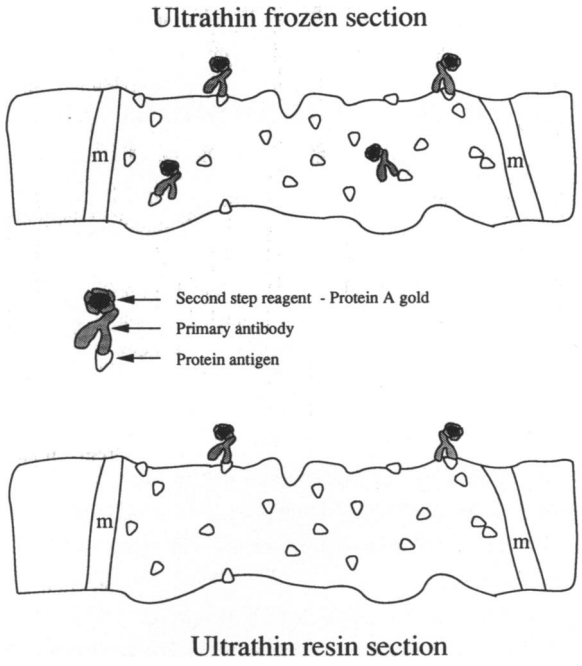

Fig. 1. Diagrammatic representation of ultrathin sections used for immunogold labelling. The sections are up to 100 nm thick. The protein antigens in this instance are soluble and are contained within a membrane (m)-bound compartment. Antibody molecules bound to antigen are located using the 2nd step reagent protein A-gold. The surface of ultrathin sections that is presented to the labelling reagents during labelling is complex. It is known that labelling reagents can enter some regions of ultrathin frozen sections (Stierhof & Schwarz, 1989), but this probably does not occur in resin sections (Bendayan, 1984). Nevertheless sections of some resins show a surface roughness with membranes protruding a few nanometers from the surface (Kellenberger et al. 1987).

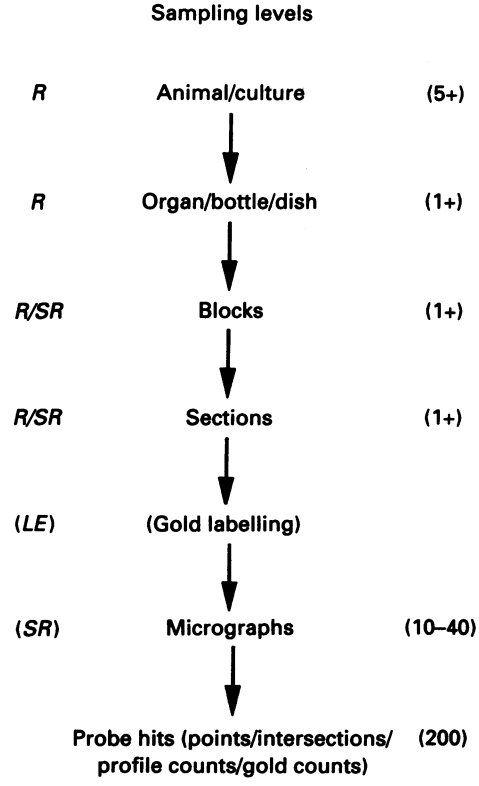

Fig. 2. Sampling of biological specimens for quantitation of immunolabelling on ultrathin sections. Numbers in brackets are the suggested number of sampling units needed to obtain precise estimates. Actual numbers will be determined by particular circumstances. *R* and *SR* refer to simple random and systematic random respectively. Gold labelling is itself a sampling step in which the labelling efficiency value (*LE*) describes the number of gold particles associated with the average antigen.

relatively large specimens (e.g. blocks of 0.5 mm side) can be processed (Van Genderen et al. 1991). However, if they are rapidly frozen, only thin ($\sim$ 10 μm thick) surface layers show good morphology. A general advantage of resin embedding in quantitation is that serial, unfolded sections can be prepared at room temperature.

SAMPLING

Ultrathin sections are so small compared with the specimen that each section usually represents much less than 1/1000th of the block and an even smaller proportion of the whole specimen! It is therefore absolutely essential that sections are representative samples. If they are not, the estimates of gold labelling may be biased which means they will consistently over or underestimate the true value for the specimen. As an example, bias would occur if sections were repeatedly taken from a region where an antigen is concentrated. The solution to the problem is to use random sampling which gives all antigenic sites the same chance of being in the section.

Even if estimates are unbiased, another concern is the variability of the results obtained from the section. In statistical terms very variable results are said to have a low precision and estimates with higher precision can usually be obtained by increasing the number of items sampled.

Precision is also related to efficiency which is defined by the amount of work needed to obtain a certain precision. Generally, efficiency can be improved by modifying the sampling design. For example, dramatic increases in efficiency can be obtained by spreading measurements, not in a simple random fashion, but at intervals throughout the specimen—a strategy called systematic sampling. This type of sampling is more efficient than simple random sampling (Gundersen & Jensen, 1987) because the variation present in the specimen is better represented in the sample and simple random samples may, for example, cluster together and overrepresent one region. Systematic samples are unbiased if the position of the first sample is itself random within the interval between the systematic samples (so called 'systematic random' sampling).

Sampling hierarchy

Experiments in immunoelectron microscopy are generally organised so that *animals* or *cultures* yield pieces of tissues or pellets of cells that are embedded as *blocks* from which *sections* are cut. The latter are *gold labelled* and quantitation is then carried out on a series of microscopic *fields*. At each of these sampling levels it must be decided which sampling method to use and how much to sample (see Fig. 2).

Animals/cell cultures. Experience has shown that the contribution of the biological units (animals/cultures) to the overall variation is relatively high compared with that at lower sampling levels and this means that it is generally better to examine more animals or cultures (optimally 5 or more) than concentrating many measurements on one alone (Gundersen & Østerby, 1981; Gupta et al. 1983; Cruz-Orive & Weibel, 1990).

Blocks and sections. In general, only a few blocks and sections are required. If the structure of interest is known to be distributed homogeneously, a single random selected block and section will generally allow precise estimates of gold labelling (Lucocq, 1992). Systematic sampling of blocks or sections is usually not required but might be needed if strong regional variation occurs. This is easily performed for blocks but for sections a complete section series must be prepared—a time-consuming procedure with resin sections and an extremely difficult one with ultrathin frozen sections.

Gold labelling. The number of gold particles almost never corresponds to the number of antigens in the section (see Fig. 1) and in most labelling experiments there are less gold particles on the section than there are antigens in it. Gold labelling is therefore a special sampling step for antigens in which the number of gold particles ($N(g)$) can be related to the number of antigens ($N(a)$) in a relationship called labelling efficiency ($LE = N(g)/N(a)$). The value of this parameter cannot be predicted a priori because it is determined by many physical, chemical and biochemical factors involved in section preparation and labelling (see sections on Absolute Antigen Quantitation below).

Fields. Microscopic fields contain the gold labelled structures that are either analysed directly at the electron microscope or indirectly by taking micrographs. The sampling method of choice is systematic sampling of fields with a random start. This is more efficient than simple random sampling, first because the distribution of structures and gold labelling can be quite inhomogeneous, and secondly because it is less complex and time consuming to perform—the fields can be positioned systematically using EM support grid bars as a guide (imagine trying to plot the location of fields at the electron microscope using random numbers!) It is often not necessary to examine more than 20 fields.

Gold and point counts. As we shall see, gold particle number can be related to structure parameters by applying simple stereological probes such as point, lines or quadrats (frames) to a set of fields. It has become clear that if sufficient sampling is done at higher levels, no more than about 200 of these events (gold particles, point hits, line intersections) need be counted for each biological unit sampled (culture/animal; Gundersen et al. 1988*b*). It is therefore simply not worthwhile to invest in automated measuring devices. These are no more efficient (Gundersen et al. 1981; Mathieu et al. 1981) and are much more expensive!

Section orientation. In stereology the orientation of probes and therefore sections is important when surface and length are estimated. The reason is that these parameters can have preferred orientations in space (anisotropy) so that unbiased estimates can be obtained only when the directions of the stereological probes are equally likely (isotropic) and their position in space is also uniform random (isotropic uniform random or IUR).

The effect of orientation on gold labelling of protein antigens has yet to be investigated but may be important when proteins form part of oriented filaments or membranes. Epitopes which lie on the surface of these proteins will display anisotropy and this could have a marked influence on gold labelling, especially when epitopes tend to face downwards or upwards in the section. In view of these theoretical worries it may be wise to use isotropic sections in quantitative studies where anisotropy of the antigen is suspected (see Mattfeldt et al. 1990, and Nyengaard & Gundersen, 1992, for methods of generating isotropic sections).

GOLD LABELLING RELATED TO STRUCTURAL PARAMETERS

Absolute amounts of antigen cannot be measured in immunoelectron microscopy without calibration of gold labelling (see below). Although in most experiments this is not done, biologically meaningful information about the antigens can still be obtained by relating the quantity of gold particles to structural parameters. As discussed below, it can be used to detect sites of antigen concentration and also, using

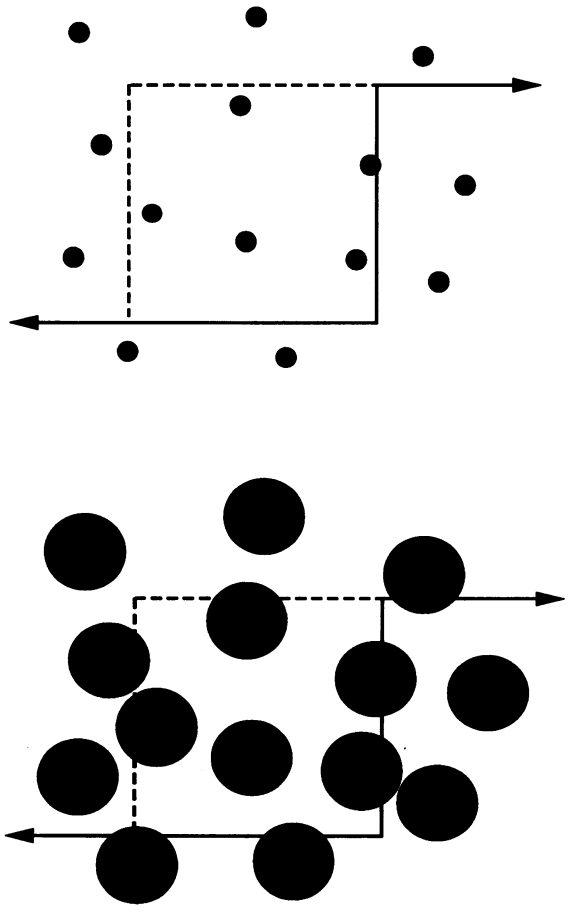

Fig. 3. Counting gold particles which lie completely within a rectangular quadrat is biased because the gold particles have a certain size. Increasing their size increases the bias (compare upper and lower figure). This bias can be avoided by using unbiased counting rules. The forbidden line rule excludes all particles from the continuous 'forbidden line' and its extensions. All other particles whether they fall partially or completely in the frame are counted. The number counted in each frame is 4.

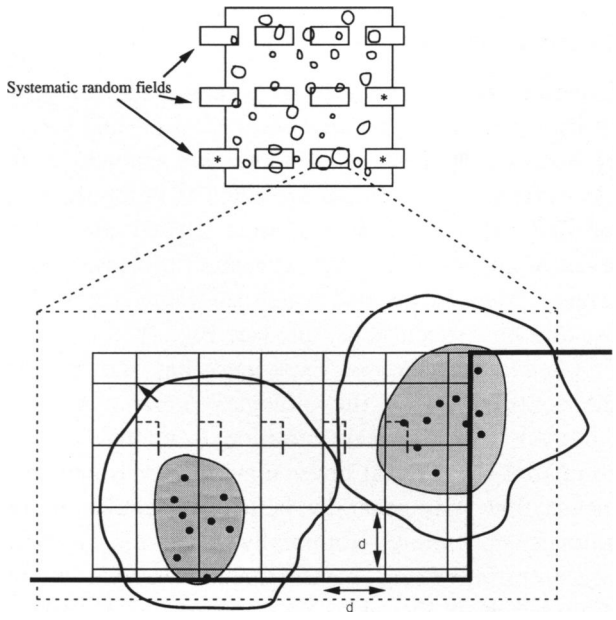

Fig. 4. Systematic sampling and estimation of numerical density of gold particles over nucleoplasm area. Micrographs of the pellet profile are taken at systematic positions with a random start. The magnification is the smallest at which both structure (nucleoplasm) and gold can be seen easily. Fields not containing the structure of interest (marked by an asterisk) are ignored. A rectangular counting frame is placed over the micrographs and the number of gold particles labelling the nucleoplasm is counted using 2-dimensional unbiased counting rules (14 particles). A lattice grid, in this case square, with known spacing d is placed at a random position over the counting frame. Points on the grid are defined by the upper left corner of each square, and 5 of them fall on the nucleoplasm. The area associated with *each* point is $5 \times d^2/\text{magnification}^2$. If the number of gold particles is very large then smaller counting frames may be superimposed on the basic lattice (for reasons of clarity only a few are shown here).

certain assumptions, to assess the relative amounts of antigen (for an excellent in depth discussion see Griffiths, 1993). Efficient methods for relating the amount of gold labelling to structure size or number are also discussed in this section.

Antigen detection. When polyclonal antibodies and protein A-gold are used, the measured labelling efficiency for a range of antigens lies somewhere between 1 and 20% (Hortsch et al. 1985; Griffiths & Hoppeler, 1986; Howell et al. 1987; Posthuma et al. 1987, 1988; Chang et al. 1988). This means that the number of gold particles represents between 1 and 20% of the number of antigens or that a single gold particle on average is associated with between 5 and 100 antigens. If structures lacking antigen were completely free of gold labelling there would be no theoretical lower limit to the amount of antigen that is detectable, even if the labelling efficiency were very low. However nonspecific sticking of labelling reagents does occur—an effect that is commonly referred to as 'background'. It is this background labelling which forces the detection limit for antigen upwards.

Taking a soluble protein as an example, nonspecific background staining over structures that do not contain the antigen is usually less than 1 particle per μm^2. This represents 10 particles/μm^3 if the sections are 100 nm thick. If antigen labelling over a structure containing the antigen were to produce 3 times this value (30 particles/μm^3, the specific signal would be 20 particles/μm^3 and this, at a labelling efficiency value of 2%, would correspond to 1000 antigens/μm^3 or approximately 2 μM protein (see Kellenberger et al. 1987, Stierhof & Schwartz, 1989, and Griffiths, 1993, for further discussion of detection limits). Relating counts of gold labelling to structural quantities is therefore clearly a valid way of deciding in which structures an antigen is concentrated (similar arguments can be applied to labelling of antigens that are associated with membranes; see Griffiths, 1993).

A word of caution, however: variation in back-

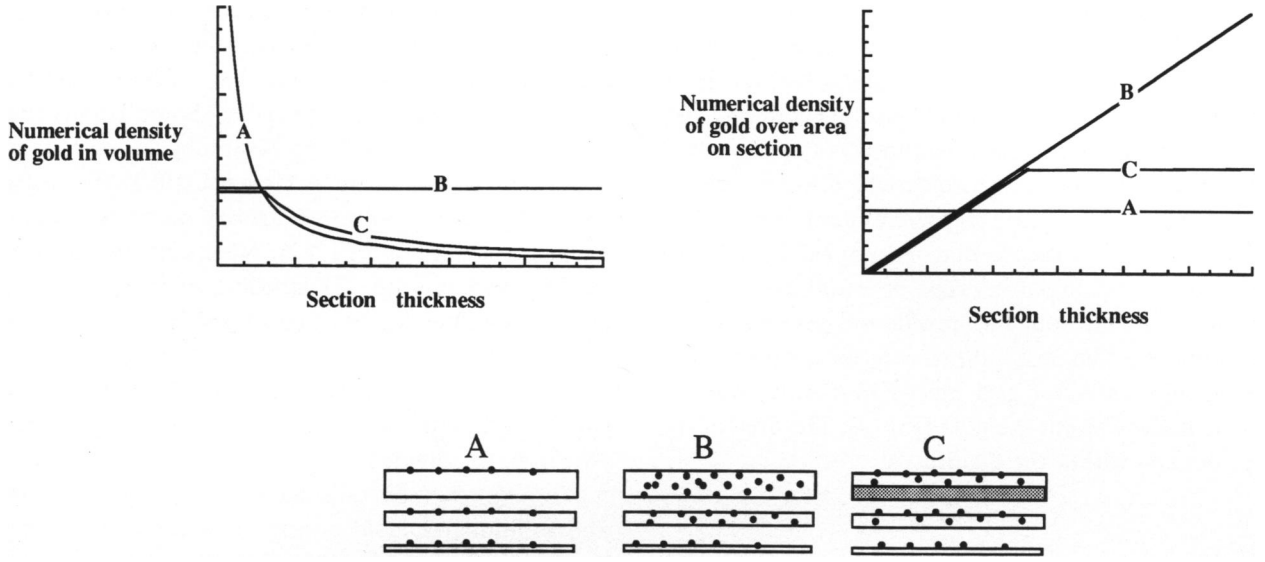

Fig. 5. The effect of section thickness on density of labelling in 2D and 3D. When there is no penetration (A) increases in section thickness cause a fall in the numerical density of gold in volume while numerical density over section area remains constant. Full penetration (B) on the other hand shows a constant density in volume but a linearly increasing density over section area. Theoretically it should be possible to detect and quantify partial penetration (C) because of the kink in the curves as section thickness is varied.

ground labelling can occur and give the impression of a weak gold signal over an antigen free structure. In such cases it is absolutely essential that controls for the specificity of the gold labelling are performed (see Griffiths, 1993, for discussion of different types of control).

Relative amounts of antigen. The major variation in labelling efficiency seems to occur between different cellular compartments or structures. However, within a single type of cellular structure the local conditions for labelling are likely to remain constant enough to produce constant labelling efficiency (Posthuma et al. 1984). This means that, if the specimen processing, sectioning and labelling conditions are kept constant, the relative change in labelling amount should then equal the relative change in antigen amount (providing the amount of gold labelling is above the detection limit and other factors, such as steric hindrance, do not cause fluctuations in the labelling efficiency). Put simply, an n-fold increase in gold label will represent an n-fold increase in antigen amount.

Unfortunately the measured labelling efficiency over *different* structures may differ by 2-fold (Griffiths & Hoppeler, 1986) or even much more (Chang et al. 1988) and consequently it is difficult to make conclusions about relative amounts of antigen in different structures. Fortunately a major cause of this variation has been traced to variable penetration of the labelling reagents which can be prevented using appropriate embedding, thereby equalising labelling efficiency (see section on Absolute Antigen Quantitation below).

Gold related to 2-dimensional (2D) parameters

Gold labelling of protein antigens can be related either to quantities displayed on the section (such as profile area or length or number), or alternatively to quantities in 3D space (such as structure volume, surface, length or number).

Areas. A systematic set of fields is photographed at the *lowest* magnification at which *both* gold particles and structure are visible. This includes the maximum of each in the sample and reduces field to field variation. All fields containing a structure profile, or parts thereof, are photographed. Quadrats placed over the microscopic fields (micrographs) are used to count gold particles and estimate structure profile area (Figs 3, 4).

Gold particles are not simply points, they have a certain size (those used for immunocytochemistry are between 4 and 15 nm). If gold particles lying exclusively within the confines of a rectangular frame of known area were counted, the density would be underestimated because particles hitting the edges of the frame are neglected. Increasing the gold size increases the error because larger profiles have less chance of lying completely within the rectangular frame (Fig. 3). This type of bias can be avoided by applying *unbiased counting rules* to the frame. One such rule uses a 'forbidden line' (Gundersen, 1977; Fig. 3). Any particle that crosses this line or its extension is excluded, the rest, including those which cross the other lines, are counted. (For the sceptic, a simple proof of the unbiasedness of this rule is to lay

a square lattice grid over a set of particle profiles and count all particles in individual unbiased counting frames formed by the squares—none is left out, none is counted twice.) Another unbiased 2D counting rule is to locate the centre point (centroid) of each particle and count only those centroids which fall inside the frame used for counting (Jensen & Sundeberg, 1986).

The area of structure that is sampled by a frame used to count gold particles can be estimated using the points that fall on the profile of interest. Most conveniently the test points can be arranged systematically as a test grid placed randomly over the frame used to count the gold (Fig. 4). The area of the profiles (A) within the quadrat is:

$$\frac{Pd^2}{M^2}$$

where d^2 is the area associated with one test point on the lattice and M, the linear magnification.

Notice that if the number of gold or test points counted is very large (i.e. much greater than 200) then the grid structure can be modified. Gold counts can be reduced by using small frames of known area placed within the register of the grid used for point counting (Fig. 4), while if there are too many points a coarser grid can be used.

Boundary lengths. When cell and organelle membranes are cut by ultrathin sections they display linear profiles. The length of such profiles can be estimated using intercepts between these features and a system of test lines. Unbiased estimates are obtained when the test lines and boundary interact isotropically and the lines have a random position (Fig. 4). The length B is given by $(\pi/4)I \times d$ where I is the sum of intersections between the boundary and the sets test lines that run perpendicular to each other and d is the real test line spacing on the specimen. Isotropy of the grid lines can be assured by selecting an angle between 1° and 180° using random numbers.

Profile number. In ultrathin sections 3D objects display profiles whose number can be related to the number of gold particles. This can be done by using the same type of 2D unbiased counting frame used for gold counting (see Fig. 4).

Gold related to sizes in 3 dimensions

In 3 dimensions gold labelling on ultrathin sections decorates structures with gold particles at a defined number and density. The labelling is mostly restricted to the surface of the ultrathin sections and so for any given structure the numerical density of labelling in 3D will depend on section thickness – thinner sections generate more sections and therefore more labelling. Estimation of 3D densities demands knowledge of section thickness and a number of methods for measuring this have been described. Some are suitable for use with resin sections (Gunning & Hardham, 1977; Bedi, 1987; Evans & Howard, 1989). Some are also suitable for ultrathin frozen sections (Small, 1968; Eusemann et al. 1982). Note that the effect of section thickness on 3D labelling density will vary according to the degree of penetration of the reagents (see Fig. 5).

The numerical density of gold particles in volume can be estimated from the gold density per area ($N(g)/A(s)$) divided by the section thickness, t. $N(g)/A(s)$ can be estimated as already described above. Similarly, gold density over boundary membrane length (estimated using both sets of perpendicular lines in Fig. 4) may be converted into density over membrane surface by applying the equation

$$\frac{N(g)}{B(s) \times 4/\pi \times t} = \frac{N(g)}{Idt}$$

as long as the sections are isotropic (see Mattfeldt et al. 1990, and Nyengaard & Gundersen, 1992).

The formula for numerical density of gold labelling over the membrane surface may also be derived by relating the gold to surface via volume (Griffiths & Hoppeler, 1986). The volume of structure over which the counts are made is Pd^2t where d is the point spacing on a square lattice grid. Using the same grid, the density of structure membrane in volume ($S_v(s,s)$) is given by $2I/L$, a well known stereological estimator, in which I is the number of intersections of test lines with the structure surface and L is an estimate of line length applied to the structure. (In the case of the grid used in Fig. 4, $L = P2d$.) The amount of surface examined for gold counting is then:

$$S_v(s,s) \times V(s) = \frac{2IPd^2t}{P2d}$$

and the gold density over surface is

$$\frac{N(g)}{2Idt}.$$

Gold related to individual structures

Gold densities in 2D or 3D are ratios and in microscopy problems arise when the denominator changes because of experimental or preparation effects. Volume changes are well known to occur during embedding in resins (King, 1991). Even more

serious are volume changes that are linked to experimental or other conditions (see Braendgaard & Gundersen, 1986). Such volume changes will alter the density of gold labelling per unit area and volume but are unlikely to change the total amount of gold label over a structure. It may therefore be important, and it is always safer, to relate the number of gold particles to an individual structure or to some other stable reference item such as the cell. Estimation of the number of gold particles per structure or per cell can be done using either indirect or direct methods.

Indirect estimation. Evaluating the number of gold particles per structure, or cell, requires stereological estimation of structural parameters. However, stereology is difficult to do on frozen sections and an indirect approach is adopted in which estimates of structure size (volume or surface) are first obtained on independent epoxy resin embedded specimens. These sizes of structures or cells can then be combined with the numerical density of gold particles within the same size parameter (volume or surface) to obtain an estimate of the number of gold particles per structure or cell. The methods are unbiased if the structure sizes are the same after the different processing methods (Griffiths & Hoppeler, 1986).

The number of gold particles associated with a structure ($N(g,s)$) can be estimated since $N(g,s) = N(g)/V(s) \times mean\ V(s)$ where $N(g)/V(s)$ is the numerical density of gold particles in volume and $V(s)$ the mean volume of that structure. Gold density is estimated on the immunolabelled section using methods described above, and mean structure volume can be estimated using one of the new stereological principles, the disector (Gundersen, 1986), selector (Cruz-Orive, 1987) or the nucleator (Gundersen, 1988). All of these methods first sample the structures unbiasedly in 3 dimensions and then estimate the volume. The sampling is always done using the disector which is 3 dimensional probe for counting or sampling the 3D objects which lie between 2 planes (see Sterio, 1984). One other method for estimating the volume of an individual structure deserves special mention, because of its generality. This is called Cavalieri estimation and uses a systematic sample of a complete set of sections through a structure with the first section positioned at random within the sampling interval. The volume is given by $Patj$; where P is the test point total, a is the area associated with each test point on a grid lattice, t the section thickness, and j the number of sections in the interval. Again, individual structures should be selected unbiasedly using the disector. The selector and nucleator estimates of volume demand isotropy of probes (or specimen) while Cavalieri estimation and the disector require only randomness of section location.

The number of gold particles associated with the total amount of a structure inside a cell (or other reference space; $N(g,s,cell)$) is given by $N(g)/V(s) \times V(s,cell)$ where $N(g)/V(s)$ is the numerical density of gold particles in structure volume and $V(s,cell)$ the volume of the structure in an average cell. The volume of the structure in an average cell ($V(s,cell)$) can be estimated from: $V(s,cell) \times V(cell)$ where $V_v(s,cell)$ is the volume fraction of the structure in the cell and $V(cell)$ is the mean cell volume. The volume fraction is estimated in systematic random fields by $\Sigma P(s)/\Sigma P(cell)$ where $\Sigma P(s)$ is the number of points falling over the structure and $\Sigma P(cell)$ is the number of points falling over the cell space. A variety of methods for estimating mean cell volume have been described, including the disector (Gundersen, 1986), selector (Cruz-Orive, 1987), the nucleator (Gundersen, 1988) and others specially adapted for use with cultured cells (Griffiths et al. 1984*b*). Finally by combining with the gold density in volume, the number of gold particles per aggregate structure in the cell, $N(g,s,cell)$ is obtained from $N(g)/A(s)t \times V_v(s,cell) \times V(cell)$. Notice that other sizes such as surface may be used in place of volume in this relationship and also that the structure volume may be replaced by the volume of a reference space such as the cell space. Thus using the formula $N(g)/A(cell)t \times V(cell)$ avoids the volume density estimation step.

Direct estimation. Unfolded sections in series can be prepared from resin-embedded specimens and the number of gold particles associated with a cellular structure or cell can be obtained using the same blocks that are used for immunoelectron microscopy. Two methods are described here.

(1) *An application of the double disector* (*Gundersen, 1986*). In this method the number of gold particles is related to the number of structures (e.g. cells) lying within a reference space that is included within a stack of thin sections. The first and last sections of the stack are mounted on slot grids and the reference structures are counted if their profiles are selected on one of the sections by a 2 dimensional unbiased counting frame but have disappeared in the other 'look-up' section (the disector principle; Sterio, 1984; Gundersen, 1986). The counts (Q^-) are related to the volume of a reference space in the disector by point counting (P_1) using a lattice grid in which the area per point is $a1$.

$$\text{Density of structure in reference space} = \frac{Q^-}{P(ref)\,a_1 t(j-1)}$$

where t is section thickness and $j-1$ is the number of sections in the disector (j is the number of sections in the whole stack).

One of the intervening sections is selected at random and is mounted on a mesh grid in the usual way and gold labelled. Gold particles are then counted in unbiased counting frames laid over systematically positioned micrographs and the volume of the same reference space in the sections estimated by point counting (points, *p(ref)*; area per point, *a2*).

The density of gold in reference space volume is

$$\frac{N(g)}{p(ref)a_2t}$$

and the ratio of gold density to reference structure density in the reference space is an estimate of the number of gold particles per reference structure,

$$N(g,s) = \frac{N(g)}{p(ref)a_2t} \times \frac{P(ref)a_1t(j-1)}{Q^-}.$$

Conveniently, the section thickness term t cancels. This technique has been used to compare the number of antigens with the number of gold particles to obtain labelling efficiency (Lucocq, 1992). In this case the counted reference structure and reference space was the cell itself but it can be any selected structure. Notice that although this approach is basically an adaptation of the double disector method described by Gundersen (1986), it differs in that the gold particles are not sampled from 3D space because they are added after slicing. However, the epitopes in the section (some of which are labelled) *are* sampled from 3D space. They are also small enough to be present in one section but absent from the next, so that if they could be seen they would all be counted using the disector principle. In fact, only a subset of these antigens is revealed by labelling and if this occurs in a random way then the above procedure could be considered as a double disector for gold sampled epitopes.

An advantage of this method is that structure volume and section thickness estimation are not required (although they appear in the equations). In addition the exact limits of the structure do not always need to be defined which may be difficult in tangential sections. It is only necessary to know when counting the gold particles that they do indeed belong to the structure.

(2) *An application of the fractionator* (*Gundersen, 1986*). If a structure is completely sectioned and a known fraction (f) of these sections is labelled then an estimate of the total gold particles labelling the structure ($N(g,s)$) is given by $I/f \times N(g)$ where ($N(g)$ is the total gold particles labelling the structure on the sampled sections. Systematic sampling provides efficiency and is unbiased when the position of the first section is random within the selected interval. The structures themselves may be selected unbiasedly in a disector whose size need not be known. This approach has yet to be used in immunoelectron microscopy but its advantages are that the number of gold particles per individual structure is obtained directly and there is no requirement for section thickness estimation. One disadvantage is the need to mount and label sections on EM grids that lack grid bars (e.g. slot grids) in order to visualise each selected profile completely. From experience gained from Cavalieri estimations of volume it is likely that, if the gold labelling is relatively homogeneous, as few as 5 sections per structure will yield precise estimates of $N(g,s)$ (Gundersen & Jensen, 1987).

ABSOLUTE ANTIGEN QUANTITATION

Factors affecting labelling efficiency (LE)

Absolute antigen quantitation depends on some knowledge of the relationship between the number of gold particles and the number of antigens—the labelling efficiency (LE). Before outlining methods for absolute antigen quantitation it is therefore important to discuss the factors which determine the LE. Broadly speaking these factors can be grouped into those related to labelling reagents, section prepared methods and, finally, antigen structure.

Labelling reagents. The affinity, concentration, time and temperature of application of antibodies and 2nd step reagents may all influence the final quantity of gold that binds to the antigens. The general type of reagent also has a marked effect. For example, polyclonal antibodies will recognise multiple epitopes on antigens and may therefore have a higher chance of producing gold labelling than monoclonals. Polyclonal antibodies are also used as 2nd step reagents complexed to gold particles. They will recognise multiple epitopes on the primary antibody and produce clusters of gold particles around the antigen and very much higher gold signals than protein *A*-gold (the latter produces mainly single gold particles in the vicinity of each labelled antigen). However, it is important to realise that despite the higher labelling efficiency (number of gold particles per antigen), each cluster of gold particles still represents only a single antigenic site so that the *fraction* of antigens labelled with antibody gold complexes is not much higher than that obtained with protein A-gold (Slot et al. 1988,

1989*b*). In other words the 2 reagents detect rather similar amounts of antigen.

Finally, labelling reagent activity can vary from day to day during storage leading to a variation in labelling efficiency. Such variability can largely be prevented if reagents are stored frozen as small aliquots at liquid nitrogen temperatures.

Specimen preparation. Aldehyde fixation produces extensive cross linking of protein in biological specimens to form a matrix that is stable in aqueous solution. A major effect of this matrix is to hinder the penetration of labelling reagents into the section and reduce labelling efficiency. Penetration varies according to the density of the fixed matrix and labelling is likely to be higher when the matrix density is low. Such variation has been demonstrated experimentally using glutaraldehyde-fixed gelatin: 5% gelatin allows penetration but 10% gelatin will not (Posthuma et al. 1987) and the labelling efficiency is therefore higher over the former. Interestingly the matrix density also has an effect on labelling efficiency in resins, even though resins are so highly crosslinked that labelling is restricted to the surface (Bendayan, 1984; see Fig. 1). The group of Slot and coworkers has looked for embedding methods which eliminate the effect of matrix density. One such method is polyacrylamide embedding which is suitable for use with ultrathin cryosections (Posthuma et al. 1987). Another is embedding in the resin HM20 (A. Oprins, H. J. Gueze, J. W. Slot, unpublished) after cryosubstitution. This group considers elimination of matrix effects in reference gels to be a strong indication that labelling efficiency is also equalised in different cellular structures, an important requirement for successful antigen quantitation (see below).

Finally, we should mention possible direct effects of aldehyde fixation on the antigen structure and also the possible modification, disruption, removal (Humbel & Schwarz, 1989) or even translocation of antigen during sectioning, all of which might further reduce labelling efficiency.

The antigen. As antigen concentration increases, antibody molecules and gold complexes will become crowded on the section surface and at some concentration the density of gold labelling will become maximal. This effect is commonly termed steric hindrance and will cause a decrease in labelling efficiency. Considering that 5 nm colloidal gold particle/protein A complexes associated with primary antibody may have a average diameter of between 10 and 30 nm the maximum number of particles per μm^2 of section could be between 1000 and 10000. In fact *LE* has been documented to remain remarkably stable at labelling densities up to the lower end of this range indicating that steric hindrance is not a problem in most labelling experiments (see Posthuma et al. 1988, and Ottersen, 1989*b*).

The majority of antigens localised in immunoelectron microscopy are proteins and these undergo postranslational modifications during their lifetime. These include glycosylation, phosphorylation, sulphation and proteolytic cleavage and there is clear evidence that such modifications can modify the binding of antibodies and therefore the amounts of gold labelling (e.g. Burke et al. 1983). Some knowledge of the antigen structure and the effects of any structural variation on antibody binding should therefore be obtained before quantitative studies using labelling efficiency are performed.

METHODS FOR ANTIGEN QUANTITATION

Immunolabelling must be calibrated if gold particles are used to count antigens. Calibration can be undertaken by counting the number of antigens in the structure using biochemical techniques and relating this to the number of gold particle which label it—the 'in situ' method. Alternatively the number of gold particles labelling the cell or structure can be compared to a distinct 'reference' specimen that contains known amounts of the antigen—the 'reference' method.

'*In situ*' *method*

The number of antigens ($N(a)$) is first counted using biochemical techniques (see Quinn et al. 1984, and Howell et al. 1987, for examples). $N(a)$ is then related to an immunocytochemical estimate for the number of gold particles ($N(g)$) labelling the antigens ($LE = N(g)/N(a)$). The problem is that the 2 quantities are estimated on different specimens and must be related through a bridge of reference. A convenient bridge is the cell number because this can be obtained in the 'wet' biochemical specimen and is unlikely to be affected during processing of the specimen used for immunocytochemistry.

$$LE = \frac{N(g)}{N(cell)} \times \frac{N(cell)}{N(a)}.$$

A number of strategies have been used to estimate the number of gold particles labelling a compartment in an average cell ($N(g)/N(cell)$). Some of these have already been described in detail in the section on labelling related to structures and have been divided into indirect or direct methods.

In the indirect method the labelled section is used to obtain 3D gold densities in volume or surface. The total size of the compartment in the cell is then obtained from stereology on conventionally processed material and gold particle labelling per cell can be estimated. Using volume as an example, the volume of a structure ($V(s)$) is given by $N(g)/V(s) \times V_v(s, cell) \times V(cell)$. In a pioneering and elegant study, Griffiths and coworkers (Griffiths & Hoppeler 1986; Griffiths et al. 1984*b*; Quinn et al. 1984) first estimated the number of newly synthesised viral spike proteins of Semliki Forest virus in endoplasmic reticulum and Golgi apparatus ($N(a)/N(cell)$). Then using stereological estimates of the total surface of each of these organelles in an average cell ($S(o)/N(cell)$) and the gold density over organelle surfaces ($N(g)/S(o)$) in ultrathin frozen sections, labelling efficiency could be obtained.

$$LE = N(g)/S(o) \times S(o)/N(cell) \times N(cell)/N(a).$$

Similar approaches to this problem have been published (Howell et al. 1987). As already stated the estimate of gold per cell requires both section thickness and cell volume estimations.

In the direct method the number of gold particles labelling a structure is related directly to the number of cells using the same blocks as used for immunoelectron microscopy (see above). The number of gold particles per cell is then related to the biochemical estimate of the number of antigens per cell to obtain the labelling efficiency. Using this approach an adaptation of the double disector method has been used to estimate the labelling efficiency for an antigen on the plasma membrane (Lucocq, 1992), giving reproducible estimates without requiring section thickness or cell volume estimation.

The use of LE values. Once known, an estimate of *LE* can be used as a tool for quantitating antigens, but only if certain conditions apply. The first condition is that the embedding, sectioning and labelling must be reproducible enough to allow *LE* values to be repeated in different experiments. The second is that, for a given antigen, the experimenter must know to which cellular locations and which antigen concentrations a particular *LE* value applies.

The prospects for controlling embedding, sectioning and labelling conditions seem good and reproducible estimates of $N(g)/N(cell)$ have already been obtained (Lucocq, 1992). One possibility for controlling labelling conditions, not so far explored in this approach, would be to label a standard specimen along with the test specimen, corrections being made for any variation in labelling reagent activity.

Concerning the antigen location, there are now strong indications that the use of polyacrylamide embedding for cryosectioning (Chang et al. 1988) and possibly HM20 embedding after cryosubstitution (A. Oprins et al. unpublished) will eliminate variation in *LE* caused by differing matrix density in different cellular structures. In addition, *LE* values seem to be quite stable over a large range of antigen concentrations (Posthuma et al. 1988; Ottersen, 1989*a*, *b*). It therefore seems that it will be possible, with the proviso that the antigen is biochemically homogeneous, to use a single *LE* value to quantitate antigens in a wide range of locations and concentrations.

'Reference' method

In this approach a reference specimen that contains known amounts of antigen is used to calibrate the labelling over the specimen. For proteins, the method has been worked out in the laboratory of Slot and coworkers (Posthuma et al. 1987, 1988; Slot et al. 1989*a*, *b*) and similar methods have also been used to quantitate amino acids (Ottersen, 1987, 1989*a*; see Figs 6, 7).

In principle the ultrathin sections containing the structure of interest are labelled along with a reference specimen that contains a known concentration of antigen in volume, ($N_v(a, ref)$). By comparing the density of labelling per area over a reference ($N(g, ref)/A(ref)$) with the density per area over the structure ($N(g, s)/A(s)$), the concentration of antigen in volume of the structure ($N_v(a, s)$) can be found.

For this approach to be valid 2 assumptions must apply: (1) *LE* must be equal over the reference and the structure and (2), the dimensional changes during processing must be the same in reference and specimen. If *LE*s are equal over reference and structure then

$$\frac{N(g, ref)}{N(a, ref)} = \frac{N(g, s)}{N(a, s)}$$

and if dimensions change equally during processing, concentration terms (numerical densities in volume) for antigen and gold can be introduced into this equation.

$$\frac{N_v(g, ref)}{N_v(a, ref)} = \frac{N_v(g, s)}{N_v(a, s)}$$

and since, $N_v(g, ref) = N(g, ref)/A(ref)t$ and $N_v(g, s) = N(g, s)/A(s)t$

$$\frac{N(g, ref)/A(ref)t}{N_v(a, ref)} = \frac{N(g, s)/A(s)t}{N_v(a, s)}$$

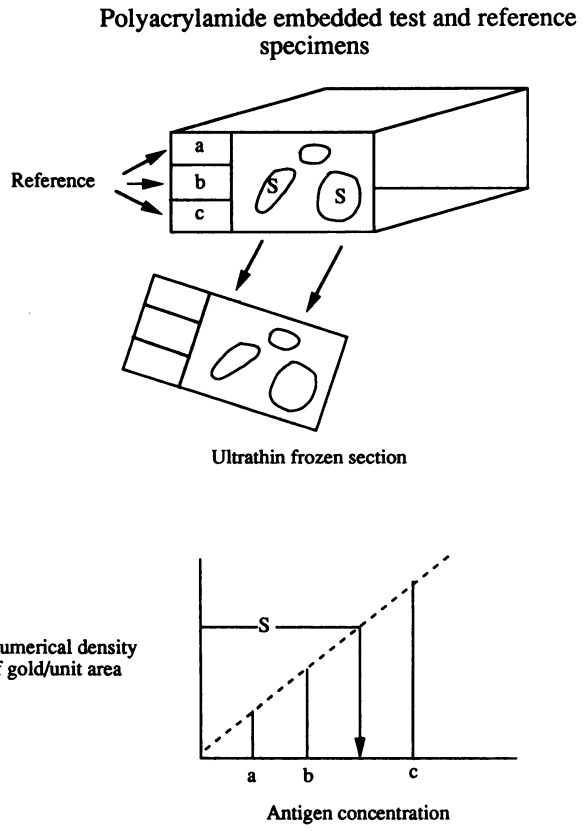

Fig. 6. The reference method developed by Slot and coworkers for quantitation of fixed protein antigens (see Posthuma et al. 1987, 1988, and Slot et al. 1989*a*, *b*, for details). The reference specimen contains known amounts of antigen mixed with gelatin and fixed with aldehyde. The test specimen contains the aldehyde-fixed structure of interest. These are embedded together in polyacrylamide, infiltrated with sucrose cryoprotectant, frozen in liquid nitrogen, sectioned and gold labelled for the antigen. The density of gold over area on the different concentration of antigen in the reference is used to calibrate the system and convert the density of the labelling over 2D profiles in the test specimen. S, labelled structure in the test specimen.

The section thickness t cancels and,

antigen density in volume,

$$N_v(a,s) = \frac{N(g,s)/A(s) \times N_v(a,ref)}{N(g,ref)/A(ref)}$$

$$= \frac{\textit{Gold labelling density per area over structure}}{\textit{Gold labelling density per area over reference}} \times \textit{Antigen concentration in reference volume}$$

These methods therefore use profile area as a bridge of reference between the reference specimen and the structure.

Slot and coworkers have used the reference method successfully employing ultrathin frozen sections to estimate the intracellular concentration of proteins on the exocytotic route (Posthuma et al. 1988) and in the cytosol (Chang et al. 1988) (see Fig. 6). They introduced polyacrylamide embedding of specimens

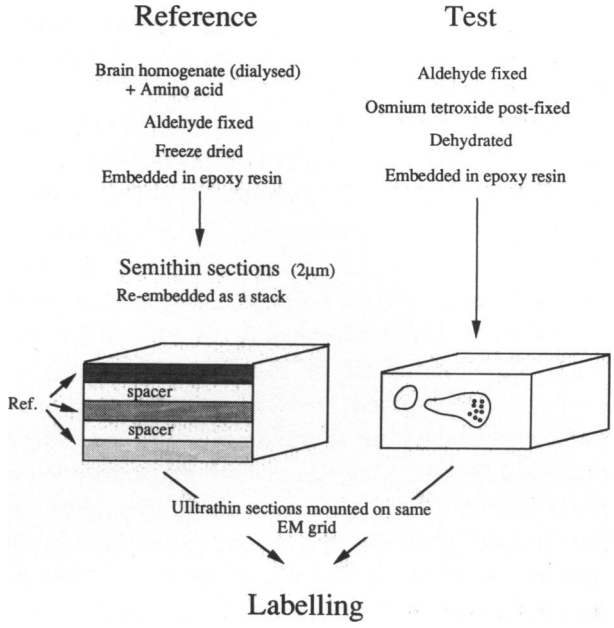

Fig. 7. The reference method developed by Ottersen and coworkers for quantitation of fixed amino acids (see Ottersen, 1987, 1989*a*, for details). The reference is prepared as shown and embedded in epoxy resin. Semithin sections of the reference are stacked, re-embedded and cut perpendicular to the original cutting direction. In this way a number of reference specimens containing different concentrations of amino acids can be sectioned together. The reference section is mounted on the same EM grid as the test specimen and they are then labelled together. Density of gold labelling over the reference is used to calibrate the immunolabelling.

which eliminates the effect of matrix density on labelling in the reference specimen and probably also equalises labelling efficiency in the structures especially if variable penetration of reagents into the section occurs. In one instance they have been able to confirm that the labelling efficiency and volume changes are similar over structure and reference specimen (Chang et al. 1988). Their method is well suited to any soluble protein that can be included in the reference gel at a known concentration. It has the additional advantage that the labelling conditions are controlled since the gel and structure are labelled simultaneously. Disadvantages are that the polyacrylamide embedding for cryosectioning is rather laborious and that the system has not yet been shown to work with membrane antigens. As mentioned above this group has investigated other embedding methods and found that embedding with Lowicryl resin HM20 eliminates the effect of matrix density on labelling (A. Oprins et al. unpublished). In this approach, aldehyde fixed specimens are first cryoprotected with sucrose and then frozen in liquid nitrogen. Subsequently they are freeze-substituted in methanol (−90 °C) and then infiltrated and embedded in HM20 (−40 °C). The ease with which unfolded

section series can be prepared using this resin makes it an ideal candidate for future quantitative studies of protein antigens.

Ottersen and coworkers have also developed a reference method which is used for the quantitation of amino acids in brain sections (for the method see Ottersen, 1987, 1989*a*, and for its application, Ottersen, 1989*b*; Zhang et al. 1990; Ji et al. 1991; Ottersen et al. 1991; Torp et al., 1991; Broman & Ottersen, 1992; Shupliakov et al. 1992). The principle is illustrated in Figure 7. The reference specimen is prepared from dialysed brain homogenates to which pure amino acids are added. This is then fixed in aldehydes, freeze dried and embedded in epoxy resin. Sections of the standards are labelled on the same drops of labelling reagents as sections of the specimen and the numerical density of gold labelling per area compared. Using this technique these workers have been able to assess the relative and local concentrations of different amino acids in neurons.

CONCLUDING REMARKS

By using sampling designs and principles developed in the field of stereology it is possible to obtain efficient and unbiased estimates of the amount of gold labelling associated with various structural parameters in 2D and 3D. Methods for absolute quantitation of antigens using immunogold labelling are now available and will, when combined with stereological techniques, allow detailed quantitative descriptions of important cellular processes at the ultrastructural level.

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