Image Anal Stereol 2009;28:1-10
Review Paper

STEREOLOGY AND SOME STRUCTURAL CORRELATES OF RETINAL AND PHOTORECEPTOR CELL FUNCTION

TERRY M MAYHEW
Centre for Integrated Systems Biology and Medicine, School of Biomedical Sciences, University of Nottingham, Queen's Medical Centre, Nottingham NG7 2UH, UK
e-mail: terry.mayhew@nottingham.ac.uk
(Accepted March 3, 2009)

ABSTRACT

The retina is the part of the eye which detects light, transduces it into nerve impulses and plays a significant role in visual perception. Sensitivity to light is multi-factorial and depends on the properties of photopigment molecules, their synthesis and incorporation into photoreceptor membranes and the neural circuitry between photoreceptor cells, bipolar neurons and ganglion neurons. In addition, it depends on structural factors such as the absolute and relative numbers of different types of photoreceptor neurons, their subcellular morphology, their distribution across the retina and the physical dimensions (especially surface areas) and spatial arrangements of their photoreceptor membranes. At the molecular level, these membranes harbour photosensitive pigment molecules comprising transmembrane glycoproteins (opsins, which vary between photoreceptor cells) and a non-protein chromophore. Phototransduction involves a conformational change in the chromophore and activation of an opsin. A transducer G protein, transducin, lowers levels of cGMP and triggers changes in membrane ion permeability including the closure of Na^+ channels. This causes the plasmalemma to become less depolarized and the relative hyperpolarization stimulates ganglion cells whose axons form the optic nerve. Phosducin is a light-regulated phosphoprotein located in inner and outer segments of rod photoreceptor cells. It modulates phototransduction by binding to beta and gamma subunits of transducin. This review briefly illustrates ways in which stereology can contribute to our understanding of these processes by providing quantitative data on photoreceptor number, disk membrane surface area and the subcellular immunolocalisation of key molecules.

Keywords: immunogold quantification, membrane surface area, number, photoreceptor neurons, retina, stereology.

INTRODUCTION

In adult humans, the eye has a diameter of about 20 mm and about 70% of its internal surface is occupied by the retina. It has been estimated that there are about 120 million photoreceptor neurons (114 million rods and 6 million cones) within the retina and about 1.1 million fibres within the optic nerve (Kupfer *et al*., 1967; Williams *et al*., 1989). This represents an overall photoreceptor:fibre convergence ratio of 110:1 but the ratio varies across the retina from centrally to peripherally and between rods and cones. The fovea centralis contains only cones and displays the greatest visual acuity and the cone:fibre convergence ratio is 1:1. By contrast, the periphery is rod-dominated, there is greater sensitivity and reduced acuity and many rods converge on each bipolar neuron and many bipolar neurons converge on a single ganglion cell.

In the vertebrate neural retina, light sensitivity is multi-factorial and depends on the properties of photo-pigment molecules, their synthesis and incorporation into photoreceptor membranes and the neuronal circuitry between photoreceptor, bipolar and ganglion neurons. In turn, pigment incorporation and light-responsiveness are influenced by structural factors, *e.g.* the number and subcellular morphology of photoreceptor cells (PRCs), their spatial distribution and the physical dimensions and arrangements of their photoreceptor membranes.

PRCs are arranged radially through several layers of the retina and show differences in morphology, function and behaviour which distinguish them as rods or cones. Despite the differences, each PRC possesses well-defined subcellular domains concerned with particular functions including phototransduction, aerobic metabolism, gene expression and transmitter release. Phototransduction, the conversion of incident light into nerve impulses (Wassle and Boycott, 1991), occurs in the outer segment (OS) domain in which photoreceptor membranes are arranged as stacks of

disks, the sizes and numbers of which vary according to species and PRC type and position. Photoreceptor membranes are renewed in a circadian rhythm following shedding at the OS tips and subsequent endocytosis by cells of the retinal pigment epithelium, RPE (Goldman *et al*., 1980; Lavail, 1980; Cahill and Besharse, 1995). Rods and cones differ not only in terms of the shape of the OS domain but also in the mechanisms of renewal of photoreceptor membranes (Steinberg *et al*., 1980; Eckmiller, 1987; 1990). PRCs also vary in their responses to lighting conditions by changes in length (Burnside and Dearry, 1986; Rey and Burnside, 1999).

Photoreceptor disk membranes harbour photosensitive pigment molecules comprising one of a number of transmembrane glycoproteins (opsins) and a non-protein chromophore (11-cis-retinal). Opsins vary between PRCs and between cones. In darkness, the rod cell plasmalemma is depolarized and the rods secrete neurotransmitters. Channels are kept open by cyclic GMP (cGMP). Phototransduction involves a conformational change in the chromophore and activation of an opsin. Phosducin, a light-regulated phosphoprotein, binds to subunits of transducin, a transducer G protein (Lee *et al*., 1987; Savage *et al*., 2000), and this lowers levels of cGMP and triggers changes in ionic permeability (including the closure of Na^+ channels). The PRC plasmalemma becomes less depolarized and the relative hyperpolarization ultimately leads to ganglion cell stimulation (Grusser, 1983; Liebman *et al*., 1987).

While phototransduction occurs in the OS domain of PRCs, aerobic metabolism occurs in the ellipsoid domain which is rich in mitochondria (55-85% of ellipsoid volume in macaques, Hoang *et al*., 2002). Gene expression and protein synthesis occur in the cell body of PRCs. Another domain, the myoid, lies between the OS and cell body and it is in this domain that PRC elongation/contraction occur in response to changes in light conditions (Rey and Burnside, 1999). These movements help to optimise the position of OS segments: rods elongate in bright light whereas cones elongate in dim light or darkness. Finally, transmitter release by exocytosis takes place in the synaptic terminal. From the latter site, impulses are transmitted to bipolar neurons and, from them, to ganglion neurons whose axons form the optic nerve.

Here, we illustrate some ways in which stereology can contribute to our understanding of these processes, in particular visual phototransduction. This is illustrated by taking examples from studies on the numbers of PRCs and their disk membrane surface areas and on the subcellular distribution of immunolabelled phosducin within PRC domains.

EXAMPLE 1: THE RETINA AND ITS PHOTORECEPTORS

This example draws on a stereological study of rat retina (Mayhew and Astle, 1997) designed in order to characterise PRCs at whole organ and average cell levels. To this end, eyeballs were removed from inbred rats, immersed in cold fixative solution (2% glutaraldehyde, 1% paraformaldehyde, 3 mM calcium chloride and 1% sucrose in 0.1 M cacodylate Millonig's buffer, pH 7.4) and, after fixation, trimmed of excess fat. Subsequently, they were postfixed with 1% osmium tetroxide in Millonig's buffer, washed in cacodylate buffer plus 6% sucrose (pH 7.2) and block-stained in 2% uranyl acetate in distilled water. Following dehydration, pieces of tissue from each eye were embedded in Transmit resin at a standard orientation.

Sampling

The aim of sampling was to accord all regions (and all PRCs) an equal chance of being selected. The sampling was multistage (Mayhew and Astle, 1997) and, consequently, it was important to aim for consistency of definition of tissue compartments when moving from one stage to another (Cruz-Orive and Weibel, 1981). The design provided three primary outcome measures:

[a] total retinal volume, V_{ret};

[b] total number of PRCs per retina, N_{prc}, and

[c] total surface area of disk membranes per retina, S_{dm}.

Stage 1 sampling. The aim here was to obtain macroscopic estimates of retinal surface area, S_{ret}. Under a binocular dissecting microscope, two mutually perpendicular lengths across the eyeball (the distances between tangents to its outer surface) were measured using the parallel lines of an eyepiece graticule. This was superimposed on the projected image of each eyeball so as to be random in position and orientation. After measurement, the eyeball was rotated and the exercise repeated. The average length was then taken to correspond to eyeball mean projected height and used to obtain rough estimates, assuming sphericity, of eyeball volume and outer surface area.

To calculate S_{ret}, each eyeball was cut along its visual axis. After removing the lens and cornea, the remaining tissue was cut into several pieces and flattened under a glass coverslip by gentle pressure.

Stage 2 sampling. The aim here was to derive light microscopic (LM) estimates of mean retinal

thickness, T_{ret}, and the volumes of the retina and its outer nuclear and outer segment layers (V_{ret}, V_{onl} and V_{osl} respectively). Pieces of retina were positioned and oriented in resin blocks so that full-depth (vertical) slices could be cut. Blocks selected for microtomy by the lottery method were rotated about their long axis in order to satisfy sampling conditions for estimation of surface areas from vertical sections (Baddeley *et al*., 1986). Sections approximately 1 μm thick were mounted on glass slides and stained with toluidine blue.

Sectional images were projected onto white paper at a final magnification of x 1550 which was calibrated using a stage micrometer scale as an external standard. By means of two step-motors, LM fields of view were selected in a systematic uniform random fashion (Gundersen and Jensen, 1987; Mayhew, 2007). Tracings were made of the boundaries of the entire retina (extending from the outer segment/RPE interface to the internal limiting membrane), the outer segment layer and outer nuclear layer.

Stages 3 and 4 sampling. Stage 3 provided low-power transmission electron microscopic (TEM) estimates of the volume-weighted mean volume, v_{Vnuc}, of PRC nuclei within the outer nuclear layer, and the volume density of nuclei in the layer, V_{nuc}/V_{onl}. At stage 4, high-power TEM estimates of the packing density of PRC disk membrane surface area, S_{dm}, within the volume of the OS layer (S_{dm}/V_{osl}) were derived.

Ultrathin sections (thickness ca. 70 nm) were cut from the same blocks, mounted on copper support grids, and stained with lead citrate. TEM fields were recorded and printed at x 2216 (outer nuclear layer) or x 26860 (outer segment layer) with the aid of grating replicas as external calibration standards. Montages were made so that the vertical direction could be monitored within each layer.

Stereological estimations

Stage 1 estimates. Retinal surface area was estimated by test point counting using the relation

$$\text{est } S_{ret} = \Sigma P_{ret} \text{ x } a(p),$$

where a(p) is the area associated with one test point. Retinal area, divided by the outer area for the eyeball, gave an estimate of the fractional surface occupied by retina.

Stage 2 estimates. Projected images of full-depth vertical slices were used to obtain estimates of the arithmetic mean thickness of the retina, T_{ret}, which was taken to be the mean distance from the inner aspect of the RPE to the internal limiting membrane. The volume of the retina was estimated as

$$\text{est } V_{ret} = S_{ret} \text{ x } T_{ret}.$$

In addition, the thicknesses of the outer nuclear and OS layers were recorded and the relative thicknesses used as estimators of relative volumes.

Stage 3 estimates. Micrographs of the outer nuclear layer provided estimates of the volume-weighted mean volumes of PRC nuclei and the fractional volume of the layer occupied by those nuclei. Estimates of v_{Vnuc} were obtained by measuring point-sampled intercept lengths (Gundersen and Jensen, 1985) using test lines superimposed at sine-weighted angles with respect to the vertical axis on the sections (Baddeley *et al*., 1986; Cruz-Orive and Hunziker, 1986). A sine-weighted angle between 0^o and 97^o was chosen at random for the first montage and subsequent montages from the same retina were analysed by rotating the lines systematically at an angle of 37^o. When a test point fell on a nuclear profile, the length of line passing through the point in the specified direction and intercepting the nuclear profile was measured.

The estimate of v_{Vnuc} was calculated from the mean of the cubed intercept lengths (l^3) using the equation

$$\text{est } v_{Vnuc} = (\pi/3) \text{ x } (l^3)/M^3,$$

where M is the final linear magnification. This volume overestimates the number-weighted mean volume, v_{Nnuc}, of nuclei and the relative bias was estimated using the selector (Cruz-Orive, 1987). The coefficient of variation (CV) of the distribution of nuclear volumes within a retina averaged 57%. Therefore, the relative bias was about 33% and nuclear volume and number estimates were corrected for this degree of bias:

$$\text{est } N_{prc} = V_{onl} \text{ x } (V_{nuc}/V_{onl}) \text{ x } 1/v_{Nnuc},$$

where v_{Vnuc} represents the number-weighted mean volume of PRC nuclei.

Stage 4 estimates. Micrographs of the outer segment layer were sampled in order to estimate the surface density of disk membranes in PRC outer segments and the fractional volume of the layer occupied by those segments. Again, the latter was estimated by test point counting. Disk membrane surface densities within outer segments were estimated by counting intersections using cycloid test arc lattices (Baddeley *et al*., 1986) and the formula

$$\text{est } S_{dm}/V_{os} = 2 \text{ x } \Sigma I_{dm}/\Sigma P_{os} \text{ x } (l/M),$$

where ΣP_{os} x (l/M) is the total length of cycloid arcs on the specimen scale. Micrographs were sampled only where OS membrane images were clear so as to avoid errors due to image loss by oblique sectioning (Mayhew and Reith, 1988).

The surface density of disk membranes in the OS was multiplied by the fractional volumes of outer segments in order to calculate the membrane surface density in the complete layer, S_{dm}/V_{osl}, and then the total membrane surface per retina was estimated as

$$\text{est } S_{dm} = V_{osl} \text{ x } (S_{dm}/V_{osl}).$$

Findings

Key outcome measures are summarised in Table 1. The retina possessed a volume of roughly 16 mm^3 and a total surface area of 80 mm^2. The latter represents about 56% of the external eyeball surface but this slightly underestimates the fraction of the internal surface occupied by retina because it does not take into account the thickness of the eyeball. The retina also contained 30 million PRCs each possessing, on average, about 700 disks per stack and 2600 μm^2 of disk membrane surface area. Collectively this equates to a global surface area of approximately 770 cm^2 per retina.

EXAMPLE 2: THE IMMUNOLOCALIZATION OF PHOSDUCIN IN RODS

This example illustrates an application of recently-developed immunogold counting methods for high-resolution subcellular localization of interesting molecules (Mayhew, 2007; Mayhew and Lucocq, 2008a;b). In particular, their practical utility is illustrated by analysis of gold particles labelling the phosphoprotein, phosducin, in subcellular domains of rod photoreceptors in the retinas of light- and dark-adapted groups of rats (Chen *et al*., 2005). Each domain represents a volume-occupying compartment.

Four rats per group were examined. Following enucleation, eyeballs were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer at room temperature. After a buffer wash, retinas were cut into smaller fragments, dehydrated in increasing concentrations of ethanol and block-embedded in LR White embedding medium. From a random selection of blocks, ultrathin sections (50-100 nm thickness) running vertically through the retina were cut and collected on copper support grids for TEM analysis.

Post-embedding immunogold labelling was undertaken (Chen *et al*., 2005). Sections were incubated in buffer containing 10% donkey serum followed sequentially by glycine, buffer wash and overnight incubation with phosducin primary antibody diluted 1:1000 in buffer containing bovine serum albumin. Grids were subsequently washed again prior to incubation with the secondary antibody (donkey anti-rabbit IgG) conjugated with 12-nm colloidal gold particles. Following rinses in deionized water, randomly-chosen sections were contrasted with 1% uranyl acetate and randomly-chosen fields of view were recorded for purposes of gold counting.

Stereological estimations

Gold particles were counted in rod PRCs and assigned to one of five compartments, each of which constituted a subcellular domain. The selected domains were the OS, ellipsoid, myoid, nucleus and synaptic terminal. In the original study (Chen *et al*., 2005), percentage frequencies of gold particle distributions were presented for each domain together with labelling densities expressed as gold particles per μm^2. In practice, a simpler and more efficient estimator of labelling density is gold particles per test point where points are randomly superimposed on TEM images (Mayhew *et al*., 2003). For present purposes, the original data were used to estimate gold and point counts and these were normalised to roughly 200 per group to represent a reasonable workload in terms of overall precision of estimation (Lucocq *et al*., 2004).

Table 1. *Stereological estimates for the entire retina and average PRC. Values are means (CVs) for n=6 rats (data based on Mayhew and Astle, 1997).*

Variable	Quantity per eyeball
Volume of retina, mm^3	15.9 (8%)
Volume of outer segment layer, mm^3	1.6 (14%)
Volume of outer segments, mm^3	1.1 (14%)
Surface area of eyeball exterior, mm^2	144 (3%)
Surface area of retina, mm^2	80.4 (4%)
Surface area of disk membranes, mm^2	76700 (24%)
Number of PRCs (rods and cones), millions	30 (18%)
Disk membrane area per PRC, μm^2	2610 (27%)
Number of disks per stack	704 (25%)

The observed gold particles falling on different domains in light- and dark-adapted retinas were used to test the null hypothesis of no difference in phosducin labelling distributions between the two study groups. For within-group comparisons, the observed gold particles and predicted distributions (obtained from point counts) were used to test the null hypothesis of no difference in labelling between compartments.

The between-group comparisons of labelling patterns were undertaken using the raw gold counts on each subcellular domain followed by a c x r contingency table analysis with c = 2 columns (= photo-adaptation groups) and r = 5 rows (= subcellular domains). The total chi-squared, χ^2, for 4 degrees of freedom (2-1 columns x 5-1 rows) was used to decide whether the group labelling distributions were different.

For within-group comparisons of labelling distributions, a two-sample χ^2 analysis was applied with c = 2 columns (= observed and expected distributions) and r = 5 rows (= subcellular domains). From observed (N_o) and expected (N_e) gold counts, relative labelling indices (RLI = N_o/N_e) were calculated for each PRC domain. Each RLI value indicates the degree to which a subcellular domain is labelled in comparison to random labelling. The distribution of expected ('randomly-distributed') gold gold particles is simulated simply by randomly superimposing lattices of test points (Mayhew *et al*., 2002). If a domain is preferentially labelled, RLI > 1 but it is necessary to confirm this by monitoring the corresponding partial χ^2 values for each domain. The total χ^2 for 4 degrees of freedom (2-1 columns x 5-1 rows) indicates whether any of the domains are preferentially labelled. This required that two criteria were met: (i) the domain RLI had to be > 1 and (ii) its partial χ^2 value had to account for 10% or more of total χ^2 (Mayhew, 2007; Mayhew and Lucocq, 2008b).

Findings

Results of TEM analysis of gold particle probes localising phosducin in the selected domains of light- and dark-adapted groups are summarised in Tables 2-4. For the between-group comparison (Table 2), total χ^2 amounted to 4.74 and, with 4 degrees of freedom (df), this corresponded to a probability level of P = 0.315. Consequently, the null hypothesis of no difference between groups was accepted. The distribution of phosducin labelling did not shift between compartments as a result of photo-adaptation.

Two within-group comparisons were drawn, the one for domains in light-adapted eyes and the other for those in dark-adapted eyes. In the light-adapted group, χ^2 analysis yielded a total value of 226.6 and, for df = 4, this corresponded to P < 0.001 (Table 3). The null hypothesis was rejected and we conclude that the distribution of phosducin is not random. Analysis of RLI values indicated three cell domains with RLI > 1. However, only the myoid and synapse domains showed partial χ^2 values which made substantial contributions to the total. These amounted to roughly 27% (myoid) and 49% (synaptic terminal) of total χ^2. We conclude that the synapse domain showed almost 4-fold higher labelling, and the myoid domain almost 3-fold higher labelling, than might be predicted on the basis of random labelling. Similar conclusions were drawn when domain labelling patterns were compared in the dark-adapted group (Table 4, P < 0.001). In this case, myoid and synapse domains had RLI values of 2.10 and 3.88 and partial χ^2 values accounting for 14% and 68% respectively of total χ^2.

Table 2. *Subcellular localization of gold-labelled phosducin in light- and dark-adapted groups of rod cells. Values are observed (expected) numbers of gold particles in each domain (based on data in Chen et al., 2005). Contingency table analysis reveals that labelling distributions in light- and dark-adapted groups are not significantly different (total χ^2 = 4.74, degrees of freedom = 4, P = 0.315).*

Rod Cell Domains	Light-adapted Group	Dark-adapted Group	Row Totals	χ^2 values
Outer segment	30 (33.5)	34 (30.5)	64	0.37, 0.41
Ellipsoid	55 (51.3)	43 (46.7)	98	0.26, 0.29
Myoid	52 (46.1)	36 (41.9)	88	0.76, 0.83
Nucleus	32 (37.7)	40 (34.3)	72	0.87, 0.95
Synaptic terminal	51 (51.3)	47 (46.7)	98	0.00, 0.00
Column Totals	220 (220)	200 (200)	420	4.74

DISCUSSION

EXAMPLE 1: THE RETINA AND ITS PHOTORECEPTORS

This example was based on a study prompted by the desire to establish stereological baseline data on the number of PRCs and the surfaces of their disk membranes (Mayhew and Astle, 1997). These structural quantities are important correlates of retinal function. The findings revealed the substantial extent of disk membrane area (770 cm^2) created by densely packing 30 million PRCs on a retinal surface of only 80 mm^2.

Volume-weighted mean volumes (Gundersen and Jensen, 1985; Baddeley *et al*., 1986) were converted to number-weighted volumes using correction factors obtained using the selector (Cruz-Orive, 1987). These correction factors cannot be transferred uncritically to other species, or even to retinas in other experimental groups from the same species, because the distribution of nuclear volumes may vary with experimental manipulation. Instead, it would be preferable to adopt a direct approach to estimating PRC number (and separate numbers for rods and cones) using some variant of the disector or fractionator principles (Sterio, 1984; Gundersen, 1986; 2002). Resolving rods and cones would need to be undertaken using morphological or other criteria. Because of the dense packing of PRC nuclei within the outer nuclear layer, such estimates are probably best obtained using the optical rather than the physical disector. In the original study (Mayhew and Astle, 1997), this would have been more technically demanding since combined LM and TEM was required in order to estimate disk membrane areas.

A practical benefit of using volume-weighted mean is that it can be estimated without knowing section thickness or making assumptions about nuclear shape. The bias arising from assuming that each PRC has one nucleus is likely to be negligible and biases arising from the presence of cone nuclei (which appeared to be bigger) are also likely to be trivial since there is a consensus that cones account for a tiny proportion (< 4%) of all PRCs (Lavail, 1976; Carter-Dawson and Lavail, 1979; Mayhew and Astle, 1997).

Previous studies on primates have indicated that there are 700-1300 disks per OS (Young, 1967; Williams *et al*., 1989). The figure for rat retina, 700

Table 3. *Subcellular localization of gold-labelled phosducin in light-adapted rod cells (based on data in Chen et al., 2005). Two-sample chi-squared analysis reveals that the labelling distribution is not random (total χ^2 = 226.6, degrees of freedom = 4, P < 0.001). Three domains appear to be preferentially labelled on the basis of their relative labelling index (RLI > 1) but only the myoid and synapse domains can be regarded as preferentially labelled because their partial χ^2 values make substantial contributions to total χ^2 (accounting for about 27% and 49% respectively).*

Rod Cell Domains	Observed Golds, N_o	Test Points, P	Expected Golds, N_e	RLI = N_o/N_e	χ^2 values
Outer segment	30	65	70.10	0.43	22.9
Ellipsoid	55	39	42.06	1.31	4.0
Myoid	52	17	18.33	2.84	61.8
Nucleus	32	71	76.57	0.42	25.9
Synaptic terminal	51	12	12.94	3.94	111.9
Column Totals	220	204	220	1.00	226.6

Table 4. *Subcellular localization of gold-labelled phosducin in dark-adapted rod cells (based on data in Chen et al., 2005). Chi-squared analysis reveals that the labelling distribution is not random (total χ^2 = 147.4, degrees of freedom = 4, P < 0.001). Three domains appear to be preferentially labelled (RLI > 1) but only the myoid and synapse domains can be regarded as preferentially labelled because their partial χ^2 values make substantial contributions to total χ^2 (accounting for about 14% and 68% respectively).*

Rod Cell Domains	Observed Golds, N_o	Test Points, P	Expected Golds, N_e	RLI = N_o/N_e	χ^2 values
Outer segment	34	61	61.62	0.55	12.4
Ellipsoid	43	38	38.38	1.12	0.6
Myoid	36	17	17.17	2.10	20.7
Nucleus	40	70	70.70	0.57	13.3
Synaptic terminal	47	12	12.12	3.88	100.4
Column Totals	200	198	200	1.00	147.4

disks per OS, is at the lower end of this range. For the mouse retina, samples taken from areas of high disk packing density suggest values of 500 (cones) to 980 (rods) disks per cell (Carter-Dawson and Lavail, 1979). These comparisons imply that the membrane amplification factor within PCRs (roughly equal to twice the disk number per cell) is smaller in rats than primates. Since OS diameters (1-2 μm in primates and 1.4 μm in rats) and disk thicknesses (ca. 20 nm) seem to be similar between species, the figure of 120 million PRCs for the human retina suggests a total disk membrane area of 6000 cm^2 and this area is roughly 8 times more extensive than in the rat retina.

Whilst the observed coefficient of variation between rats was relatively low (2-14%) for several variables, it was greater (18-27%) for the PRCs and their disk membranes. The higher CVs for the latter variables probably reflect the fact that the numbers and sizes of PRCs vary across the retina and that disk membrane loss and renewal vary through the day (Steinberg *et al*., 1973; Goldman *et al*., 1980; Lavail, 1980; Cahill and Besharse, 1995).

The disk membrane surface area (ca. 770 cm2) represents a substantial substrate on which to incorporate photopigment molecules. This area is associated with a stack of 700 disks and, if the phagocytosis of disk membranes by RPE cells operates at a comparable rate in the rat to that in humans (turnover every 10 days, *see* Young, 1967), each PRC would have to renew 70 disks every day. At the cellular level, this is equivalent to about 260 μm^2 of photoreceptor membrane but, for the retina as a whole, 77 cm^2 of membrane per day. It is worth noting that the latter is almost 54-fold greater than the surface area of the eyeball exterior.

Morphometric studies on the rat optic nerve have established its fibre content at 100,000 to 140,000 (Treff *et al*., 1972; Mayhew, 1990; Fukui *et al*., 1991). With the present estimate of 30 million PRCs, this equates to a convergence ratio of roughly 260:1. This is higher than the value of 110:1 suggested for human retina (Kupfer *et al*., 1967; Williams *et al*., 1989) and is consistent with the greater sensitivity of the scotopic retina of the rat.

EXAMPLE 2: THE IMMUNOLOCALIZATION OF PHOSDUCIN IN RODS

Based on data presented in Chen *et al*. (2005), the findings illustrate the value of a portfolio of new methods for comparing the immunogold labelling distributions of different compartments within a cell group and of the same set of compartments in different groups of cells (Mayhew and Lucocq, 2008b). Provided properly-randomised sampling is conducted at each stage of the multistage selection process, the methods provide unbiased estimates of the numbers of gold particles associated with interesting subcellular domains. Another advantage of the between-group method is its ability to permit analysis of sets of compartments which are mixtures of organelles, membranes and filaments rather than compartments belonging solely to a single class. A further attraction is the possibility to alter the final magnification between experimental groups of cells provided that this does not compromise the ability to recognise or resolve compartments. The method is well-suited to between-group comparisons but comparing labelling patterns between compartments within a group of cells requires an alternative approach (Mayhew *et al*., 2002; 2003). Although operating under more constraints, the latter does provide information related to the intensity of compartment labelling and these quantities will assist in interpreting effects, interactions and translocation pathways within cells.

Quantifying gold labelling distributions in volume-occupying compartments requires a sampling scheme which randomises only the location of items, particularly the spatial encounters between section planes and compartments. By contrast, quantifying distributions involving surface-occupying and linear compartments requires a scheme that also randomises spatial orientation. It follows that the latter is required when the study aim is to quantify mixtures of organelles, membranes and filaments. Systematic uniform random sampling schemes for meeting these demands are described elsewhere (Baddeley *et al*. 1986; Mattfeldt *et al*. 1990; Nyengaard and Gundersen, 1992; Gundersen *et al*., 1999; Howard and Reed, 2005; Mayhew, 2008).

Here, the immunogold quantification methods were applied to examine the distributions of phosducin in retinas from light- and dark-adapted eyes. They confirmed the main findings of the earlier study (Chen *et al*., 2005) although present sample sizes were created artificially and standardised to approximately 200 gold particles and 200 test points per group. The reason for decreasing the gold particle counts was to reduce workloads to acceptable levels: counts of roughly 200 golds on each of two sections have been found to yield reasonably precise estimates (Mayhew *et al*., 2002; Lucocq *et al*., 2004). Another consideration is that, for statistical testing by contingency table analysis, it is recommended that no expected number (of gold particles) should be smaller than five. Observed

numbers of gold particles may be less than this. Clearly, this recommendation may influence the choice of compartments. For example, it may be possible to achieve the target minimum of expected gold particles by the simple expedient of creating composite or portmanteau compartments.

Phosducin is a light-regulated phosphoprotein which binds to subunits of transducin (Lee *et al*., 1987; Savage *et al*., 2000). Phosducin expression in the retina appears to be confined to PRCs. In an earlier study (Chen *et al*., 2005), high immunogold labelling densities were found in the synaptic terminal, lower densities in the ellipsoid and myoid domains and minimal labelling in the rod OS and nucleus. These patterns were seen in both light- and dark-adapted groups. The present study has confirmed these findings and shown that, in terms of numbers of gold particles, the synaptic terminal contains roughly 4 times the amount of phosducin than might be predicted on the basis of a random distribution of molecules throughout the rod cell. Expressed in the same way, the myoid domain exhibits 2-3 times the amount whilst the OS and nucleus have less phosducin than might be predicted on the same basis. The high concentration of phosducin in the synaptic terminal is consistent with the notion of a regulatory role in synaptic functioning whilst that in the nucleus might suggest a role in regulating transcription (Yusim *et al*., 2000; Zhu and Craft, 2000; Nakano *et al*., 2001).

CONCLUDING REMARKS

It is anticipated that these simple and efficient sampling and stereological estimation procedures will contribute to future studies involving quantitative structural analysis and immunoelectron microscopy of the retina to complement those undertaken on other regions of the nervous system. The methods should complement biochemical, physiological and other studies to facilitate our understanding of retinal function. From a molecular perspective, the recently-introduced immunolabelling methods offer convenient and effective ways of comparing labelling distributions between groups and of making mechanistic interpretations of observed shifts in labelling patterns. These complement other approaches for detecting interesting molecules, *e.g.* proteomic analysis, but with the added benefit of localization rather than mere detection or visualization. Though the within-group comparisons here were illustrated using subcellular volume domains, comparable principles may be used to study labelling in surface-occupying compartments, *i.e.*, membranes (Mayhew *et al*., 2002) or in mixtures of volume- and surface-occupying compartments (Mayhew and Lucocq, 2008a).

ACKNOWLEDGEMENTS

TMM is grateful to the BBSRC and MRC for current research funding.

REFERENCES

Baddeley AJ, Gundersen HJG, Cruz-Orive LM (1986). Estimation of surface area from vertical sections. J Microsc 142:259-76.

Burnside B, Dearry A (1986). Cell motility and the retina. In: Adler R, Farber D, eds. The Retina, pp. 151-206. Orlando: Academic Press.

Cahill GM, Besharse JC (1995). Circadian rhythmicity in vertebrate retinas: regulation by a photoreceptor oscillator. Prog Retinal Eye Res 14:267-91.

Carter-Dawson LD, Lavail MM (1979). Rods and cones in the mouse retina. Structural analysis using light and electron microscopy. J Comp Neurol 188:245-62.

Chen J, Yoshida T, Nakano K, Bitensky MW (2005). Subcellular localization of phosducin in rod photo-receptors. Vis Neurosci 22:19-25.

Cruz-Orive LM (1987). Particle number can be estimated using a disector of unknown thickness: the selector. J Microsc 145:121-42.

Cruz-Orive LM, Hunziker EB (1986). Stereology for anisotropic cells: application to growth cartilage. J Microsc 143:47-80.

Cruz-Orive LM, Weibel ER (1981). Sampling designs for stereology. J Microsc 122:235-57.

Eckmiller MS (1987). Cone outer segment morphogenesis: taper change and distal invaginations. J Cell Biol 105: 2267-77.

Eckmiller MS (1990). Distal invaginations and the renewal of cone outer segments in anuran and monkey retinas. Cell Tissue Res 260:19-28.

Fukui Y, Hayasaka S, Bedi KS, Ozaki HS, Takeuchi Y (1991). Quantitative study of the development of the optic nerve in rats reared in the dark during early postnatal life. J Anat 174: 37-47.

Goldman AI, Teirstein PS, O'Brien PJ (1980). The role of ambient lighting in circadian disc shedding in the rod outer segment of the rat retina. Invest Ophthalmol Vis Sci 19:1257-67.

Grusser O-J (1983). Vision and eye movements. In: Schmidt RF, Thews G, eds. Human Physiology, pp. 234-73. Berlin: Springer.

Gundersen HJG (1986). Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson. J Microsc 143:3-45.

Gundersen HJG (2002). The smooth fractionator. J Microsc 207:191-210.

Gundersen HJG, Jensen EB (1985). Stereological estimation of the volume-weighted mean volume of arbitrary particles observed on random sections. J Microsc 138: 127-42.

Gundersen HJG, Jensen EB (1987). The efficiency of systematic sampling in stereology and its prediction. J Microsc 147:229-63.

Gundersen HJG, Jensen EBV, Kiêu K, Nielsen J (1999). The efficiency of systematic sampling in stereology - reconsidered. J Microsc 193:199-211.

Hoang QV, Linsenmeier RA, Chung CK, Curcio CA (2002). Photoreceptor inner segments in monkey and human retina: mitochondrial density, optics, and regional variation. Vis Neurosci 19:395-407.

Howard CV, Reed MG (2005). Unbiased stereology. Three-dimensional measurement in microscopy. Second Edition. Abingdon: Garland Science/BIOS Scientific Publishers.

Kupfer C, Chumbley L, Downer J de C (1967). Quantitative histology of optic nerve, optic tract and lateral geniculate nucleus of man. J Anat 101:393-401.

Lavail MM (1976). Survival of some photoreceptor cells in albino rats following long-term exposure to continuous light. Invest Ophthalmol 15:64-70.

Lavail MM (1980). Circadian nature of rat outer segment disc shedding in the rat. Invest Ophthalmol Vis Sci 19:407-11.

Lee RH, Lieberman BS, Lolley RN (1987). A novel complex from bovine visual cells of a 33,000-dalton phosphoprotein with beta- and gamma-transducin: purification and subunit structure. Biochemistry 26: 3983-90.

Liebman PA, Parker KR, Dratz EA (1987). The molecular mechanism of visual excitation and its relation to the structure and composition of the rod outer segment. Ann Rev Physiol 49:765-91.

Lucocq JM, Habermann A, Watt S, Backer JM, Mayhew TM, Griffiths G (2004). A rapid method for assessing the distribution of gold labelling on thin sections. J Histochem Cytochem 52:991-1000.

Mattfeldt T, Mall G, Gharehbaghi H, Möller P (1990) Estimation of surface area and length with the orientator. J Microsc 159:301-17.

Mayhew TM (1990). Efficient and unbiased sampling of nerve fibers for estimating fiber number and size. In: Conn PM, ed. Methods in Neuroscience, Volume 3, Quantitative and Qualitative Microscopy, pp. 172-87. London: Academic Press.

Mayhew TM (1992). A review of recent advances in stereology for quantifying neural structure. J Neurocytol 21:313-28.

Mayhew TM (2006). Stereology and the placenta: where's the point? – a review. Placenta 27, suppl A:S17-S25.

Mayhew TM (2007) Quantitative immunoelectron microscopy: alternative ways of assessing subcellular patterns of gold labelling. In: Kuo J, ed. Methods in Molecular Biology Series, Volume 369, Electron Microscopy: Methods and Protocols, 2nd Edition, pp. 309-329. Totowa: Humana Press Inc.

Mayhew TM (2008). Taking tissue samples from the placenta: an illustration of principles and strategies. Placenta 28:1-14.

Mayhew TM, Astle D (1997). Photoreceptor number and outer segment disk membrane surface area in the retina of the rat: stereological data for whole organ and average photoreceptor cell. J Neurocytol 26:53-61.

Mayhew T, Griffiths G, Habermann A, Lucocq J, Emre N, Webster P (2003). A simpler way of comparing the labelling densities of cellular compartments illustrated using data from VPARP and LAMP-1 immunogold labelling experiments. Histochem Cell Biol 119:333-41.

Mayhew TM, Lucocq JM (2008a). Quantifying immunogold labelling patterns of cellular compartments when they comprise mixtures of membranes (surface-occupying) and organelles (volume-occupying). Histochem Cell Biol 129:367-78.

Mayhew TM, Lucocq JM (2008b). Developments in cell biology for quantitative immunoelectron microscopy based on thin sections: a review. Histochem Cell Biol 130:299-313.

Mayhew TM, Lucocq JM and Griffiths G (2002) Relative labelling index: a novel stereological approach to test for non-random immunogold labelling of organelles and membranes on transmission electron microscopy thin sections. J Microsc, 205, 153-164.

Mayhew TM, Reith A (1988). Practical ways to correct cyto-membrane surface densities for the loss of membrane images that results from oblique sectioning. In: Reith A, Mayhew TM, eds. Stereology and Morphometry in Electron Microscopy. Problems and Solutions, pp. 99-110. New York: Hemisphere Publishing Corporation.

Nakano K, Chen J, Tarr GE, Yoshida T, Flynn JM, Bitensky MW (2001). Rethinking the role of phosducin: light-regulated binding of phosducin to 14-3-3 in rod inner segments. Proc Nat Acad Sci USA 98:4693-8.

Nyengaard JR, Gundersen HJG (1992). The isector: a simple and direct method for generating isotropic, uniform random sections from small specimens. J Microsc 165:427-31.

Rey HL, Burnside B (1999). Adenosine stimulates cone photoreceptor myoid elongation via an adenosine A2-like receptor. J Neurochem 72:2345-55.

Savage JR, McLaughlin JN, Skiba NP, Hamm HE, Willardson BM (2000). Functional roles of the two domains of phosducin and phosducin-like protein. J Biol Chem 275:30399-407.

Steinberg RH, Fisher SK, Anderson DH (1980). Disc morphogenesis in vertebrate photoreceptors. J Comp Neurol 190:501-18.

Steinberg RH, Reid M, Lacy PL (1973). The distribution of rods and cones in the retina of the cat (Felis domesticus). J Comp Neurol 148:229-48.

Sterio DC (1984). The unbiased estimation of number and sizes of arbitrary particles using the disector. J Microsc 134:127-36.

Treff WM, Meyer-Konig E, Schlote W (1972). Morphometric analysis of a fibre system in the central nervous system. J Microsc 95:337-43.

Wassle H, Boycott BB (1991). Functional architecture of the mammalian retina. Physiol Rev 71:447-80.

Williams PL, Warwick R, Dyson M, Bannister LH (1989). Gray's Anatomy, 37th edition. Edinburgh: Churchill Livingstone.

Young RW (1967). The renewal of photoreceptor cell outer segments. J Cell Biol 33:61-72.

Yusim K, Parnas H, Segel LA (2000). Theory for the feedback inhibition of fast release of neurotransmitter. Bull Math Biol 62:717-57.

Zhu X, Craft CM (2000). Modulation of CRX transactivation activity by phosducin isoforms. Mol Cell Biol 20: 5216-26.