



Quantitative Molecular Characterization of Bovine Vitreous and Lens with Non-invasive Dynamic Light Scattering

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The non-invasive technique of dynamic light scattering (DLS) was used to quantitatively characterize vitreous and lens structure on a molecular level by measuring the sizes of the predominant particles and mapping the three-dimensional topographic distribution of these structural macromolecules in three spatial dimensions. The results of DLS measurements in five fresh adult bovine eyes were compared to DLS measurements in model solutions of hyaluronan (HA) and collagen (Coll). In the bovine eyes DLS measurements were obtained from excised samples of gel and liquid vitreous and compared to the model solutions. Measurements in whole vitreous were obtained at multiple points posterior to the lens to generate a three-dimensional 'map' of molecular structure. The macromolecule distribution in bovine lens was similarly characterized.

In each bovine vitreous (Bo Vit) specimen, DLS predominantly detected two distinct particles, which differed in diffusion properties and hence size. Comparisons with model vitreous solutions demonstrated that these most likely corresponded to the Coll and HA components of vitreous. Three-dimensional mapping of Bo Vit found heterogeneity throughout the vitreous body, with different particle size distributions for Coll and HA at different loci. In contrast, the three-dimensional distribution of lens macromolecules was more homogeneous. Thus, the non-invasive DLS technique can quantitate the average sizes of vitreous and lens macromolecules and map their three-dimensional distribution. This method to assess quantitatively the macromolecular structure of vitreous and lens should be useful for clinical as well as experimental applications in health and disease. © 2001 Academic Press

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1. Introduction

The intricate three-dimensional organization of vitreous macromolecules results in a visco-elastic gel that maintains transparency in the center of the eye while physically protecting and physiologically supporting surrounding tissues. The lens requires a similarly complex arrangement of macromolecules to achieve unhindered transmission and accurate refraction of light within the eye. Advances in understanding vitreous and lens structure in health and disease have long been hampered by the fact that these tissues are *designed* to be invisible (Sebag, 1997). To date, there has been a lack of investigational techniques that characterize vitreous and lens structure at the molecular level without disrupting the inherent molecular organization or inducing confounding artifacts. Specialized preset lens biomicroscopy (Takahashi, Trempe and Schepens, 1980) and NMR spectroscopy (Aguayo et al., 1985) of vitreous have been employed in some centers, but are not presently used routinely. While the LOCS clinical classification system of lens opacification (Chylack et al., 1989) has provided a more organized and systematic approach

with a higher degree of reproducibility and inter-observer congruity, this system involves subjective evaluations that limit its value as a quantitative assessment. Perhaps as a consequence, the LOCS classification has to date not achieved widespread clinical use.

Current methods of examining and evaluating vitreous and lens are not sufficiently quantitative and reproducible for accurate clinical assessment or investigation. This feature has limited the utility of preset lens biomicroscopy (Takahashi et al., 1980) and ultrasonography (Green and Byrne, 1989) for research in vitreous, in spite of their utility in clinical practice. Scheimpflug photography of the lens has been developed as a means to quantitate lens opacification, but this technique may not be sensitive enough to detect early changes (Ansari et al., 1998; Datile, 1999). Thus, new non-invasive methodologies are needed to characterize the molecular effects of systemic disorders on vitreous and lens as well as the senescent changes in molecular structure and organization that lead to posterior vitreous detachment and cataracts, important causes of diseases affecting vision.

Because vitreous and lens are essentially suspensions of macromolecules in a fluid medium, this study evaluated the use of the non-invasive technique of dynamic light scattering (DLS) (Ansari and Suh,

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1998) to measure the sizes of vitreous and lens macromolecules and to determine their three-dimensional distribution.

2. Materials and Methods

DLS

DLS is an established laboratory technique to measure the average size or size distribution of microscopic particles (3 nm–3 μm in diameter) suspended in a fluid medium where they undergo random Brownian motion. Light scattered by a laser beam passing through such a dispersion will have intensity fluctuations in proportion to the Brownian motion of the particles. Since the size of the particles influences their Brownian motion, analysis of the scattered light intensity yields a distribution of the size(s) of the suspended particles.

In these experiments, visible light from a laser diode (power 50 μW) is focused into a small scattering volume inside the sample. The detected signal is processed via a digital correlator to yield a time autocorrelation function (TCF). For dilute dispersions of spherical particles the slope of the TCF provides a quick and accurate determination of the particle's translational diffusion coefficient, which can be related to its size via a Stokes–Einstein equation, provided the viscosity of the suspending fluid, its temperature, and its refractive index are known. The self-beating experiments reported here measure a normalized intensity–intensity temporal autocorrelation function $g^2(\tau)$, which is related to the normalized electric field temporal autocorrelation function of the fluctuations in the scattered light amplitude $g^1(\tau)$ via the Siegert relation,

$$g^2(\tau) = A[1 + \beta|g^1(\tau)|^2] \quad (1)$$

where $A = \langle I \rangle^2$ is the average D.C. photocurrent or the baseline of the autocorrelation function, and β ($0 < \beta < 1$) is an empirical experimental constant and is a measure of the spatial coherence of the scattering geometry of the collection optics which can be related to signal-to-noise (S/N) and hence considered a measure of the efficiency of a DLS spectrometer. Earlier studies of protein diffusivity in eye lenses by Tanaka and Benedek (1976) have established the presence of two decay constants. Equation (1) can be re-written in terms of a fast and a slowly decaying component, due to alpha crystalline diffusion and its larger aggregates, respectively, as

$$g^1(\tau) = I_\alpha \exp(-D_\alpha q^2 \tau) + I_{\alpha A} \exp(-D_{\alpha A} q^2 \tau) \quad (2)$$

where I_α and $I_{\alpha A}$, D_α and $D_{\alpha A}$ are the scattering strengths, and corresponding translational diffusion coefficients due to small α crystallins and their larger aggregates in an eye lens, respectively, and $q = [(4\pi n/\lambda)\sin(\theta/2)]$ is the magnitude of the scattering vector.

The Stokes–Einstein relation [$D = KT/6\pi\eta R$] together with the knowledge of the scattering strengths from each size species leads to a particle size distribution. For the lens and vitreous the viscosity ($\eta = 0.8904$ centipoise) and a refractive index (n) of 1.333 at room temperature of approximately 25°C was used to extract macromolecular sizes. The autocorrelation functions were analyzed using the commercial data inversion routines supplied by the Brookhaven Instruments Corporation (Holtsville, NY, U.S.A.). These include a double exponential and an exponential sampling program. The details are described in greater detail elsewhere (Stock and Ray, 1985).

New generation DLS instrumentation (Ansari et al., 1996; Ansari and Suh, 1997), developed at NASA to conduct fluid physics experiments on-board the space shuttle and space station orbiters, is employed in this study (Fig. 1). The input beam from a semi-conductor laser (670 nm wavelength) at 50 μW power was projected into the specimens (see below) and the scattered signal was collected by the DLS probe for a duration of 10 sec. The signal was then detected by an avalanche photodiode detector system (EG&G Canada, Model PCS2). A TCF was constructed using a digital correlator card (Brookhaven Instrument, BI9000). The slope of the TCF provides a measure of the particle sizes (Stock and Ray, 1985) in the selected measurement sites (volume = 50 μm^3) within the vitreous and lens.

In specimens where the DLS probe was used to obtain measurements from the entire vitreous body and lens for three-dimensional analysis, scanning was performed in conjunction with a micropositioning assembly, as shown in Fig. 1. The translation stages (Newport, Model 423) with motorized actuators (Newport, Model 850B) control the motion in the X, Y, and Z directions. A motion controller (Newport, Model MM1000DC) receives a series of commands from the Pentium processor-based computer/correlator (EPS, 7600 series) via RS232 cable and drives the actuators for precise positioning of the probe, and in turn the measurement site. The correlator software (Brookhaven Instrument, BI9000) is modified to achieve on-line control of the probe position without leaving the program environment. This setup enabled semi-automated measurements from a sufficient number of sites within the vitreous body to enable the creation of a three-dimensional map of the distribution of the average particle sizes of Bo Vit macromolecules.

Study Specimens

Model vitreous solutions. HA was prepared as a 0.1 mg ml⁻¹ solution in isotonic saline and filtered through a 0.22 μm filter (Millipore, Bedford, MA, U.S.A.) to remove impurities. Coll was similarly prepared in isotonic saline at a concentration of 0.1 mg ml⁻¹ and filtered. These concentrations were

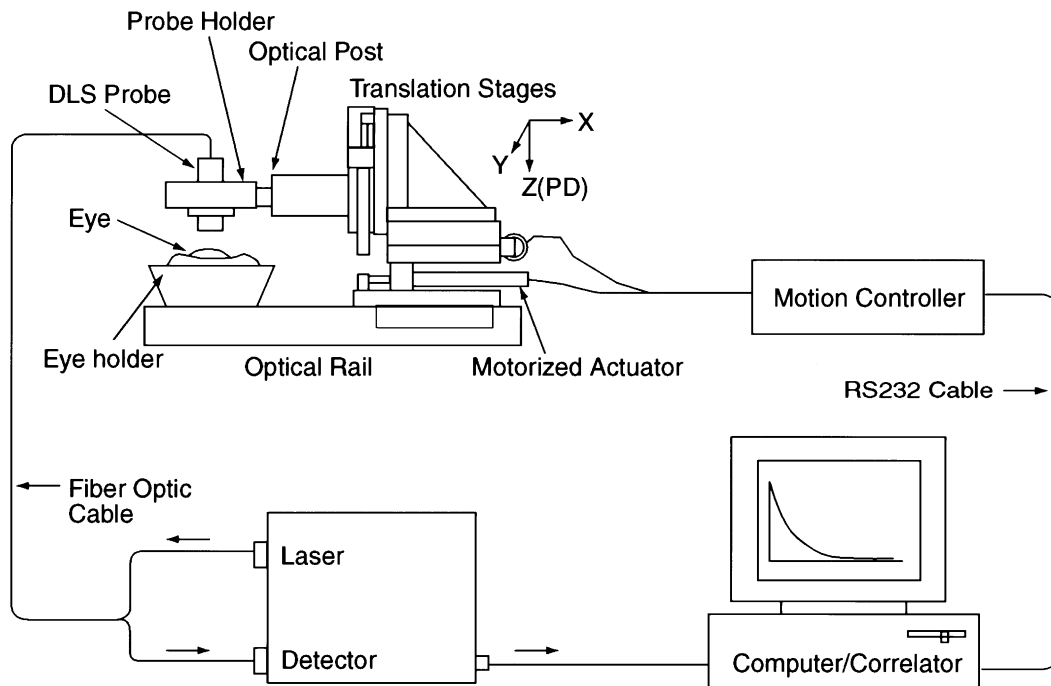


FIG. 1. Schematic diagram of DLS instrumentation for data capture and analysis.

selected as they are close to human vitreous concentrations in situ:

$[HA]_{\text{gel vitreous}} = 0.15 \text{ mg ml}^{-1}$ until the age of 20 years, 0.18 mg ml^{-1} thereafter;

$[HA]_{\text{liquid vitreous}} = 0.1$ to 0.15 mg ml^{-1} until the age of 70 years;

$[Coll] = 0.05 \text{ mg ml}^{-1}$ in youth, increasing slightly to 0.1 mg ml^{-1} in later years

(d'après Denlinger and Balazs, 1982).

Bo Vit specimens. Five adult bovine eyes were obtained fresh from the local abattoir. In two eyes the globe was transected at the equator. The vitreous body from one eye was removed from the globe and placed in an open container for DLS measurements from the open end of the container. A second eye was transected in this manner and the entire vitreous was removed from the eyecup and placed into a container. Whole Bo Vit was centrifuged at $10\,000 g$ and the supernatant liquid vitreous was decanted into an optical cuvette for DLS measurements.

The remaining three bovine eyes were prepared by dissecting away the cornea, exposing the lens and the vitreous body in normal anatomic positions within the eye. The DLS probe was positioned over the specimen, which was positioned with the anterior segment pointing upward and the posterior segment resting on the surface of the dish. Serial measurements were obtained from multiple sites within the vitreous body using the automated micro-positioning assembly, as described above.

Bovine lens specimens. Three intact bovine eyes were obtained from a local abattoir within 6 hr after the

animals were killed. DLS measurements were performed in the intact eye via the cornea, which was kept moist by regularly applying isotonic saline solution.

3. Results

Vitreous

Fig. 2 shows the DLS measurements, in terms of a TCF, in whole vitreous explanted from a Bo Vit and model solutions of Coll and HA. The Bo Vit TCF shows a major ('slow') component (the long tail of the TCF on the right side of the graph), and a minor ('fast') component on the left side of the graph. The TCFs from model solutions suggest that the particle in vitreous that is most likely to be represented by the slow component of the whole vitreous TCF is Coll, while the fast component most likely reflects HA (Fig. 2).

Particle size distributions were determined from the DLS measurements that are presented in Fig. 2 using the CONTIN method (Stock and Ray, 1985) and the results are displayed in Fig. 3. It can be seen from these results that there is a definite, albeit minor, contribution to the whole vitreous TCF by the HA molecules. The reason for the limited contribution to the whole vitreous TCF is that in gel vitreous the HA molecules are bound in a 'rigid' molecular framework, and are therefore less mobile and have limited diffusive motion, and thus contribute less to the TCF.

DLS measurements in the liquid component isolated from the Bo Vit showed a very rapidly decaying TCF (Fig. 4). This suggests that the sample contains one predominant particle, which contributes a fast component to the TCF. As it is known that liquid vitreous

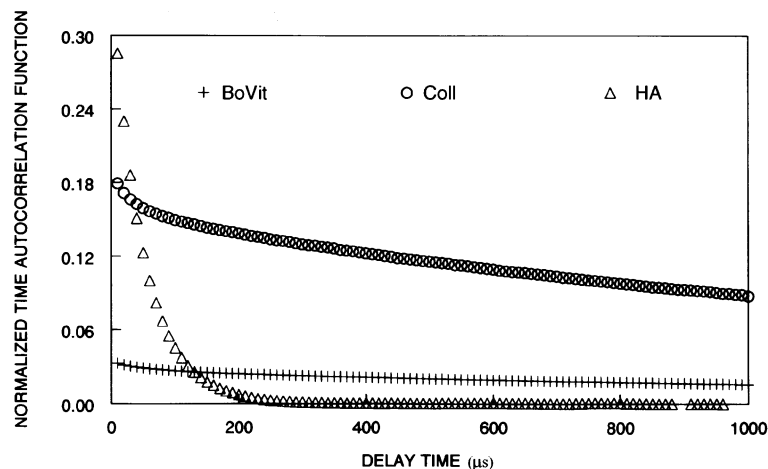


FIG. 2. TCF curve obtained from DLS measurements of explanted Bo Vit, and model vitreous solutions of HA and Coll placed in optical cuvettes. The abscissa is the delay time, which represents the diffusive motion of constituent particles in the sample. The ordinate is derived from a correlation function of the intensity of scattered light. As described in the text, the pattern of exponential decay shown for Bo Vit most likely results from the two major structural molecules present in gel vitreous. The fast component (that portion of the curve on the left side of the plot) likely reflects the contribution of HA, while the slow component (that portion on the right side of the curve) most likely reflects the contribution of Coll molecules.

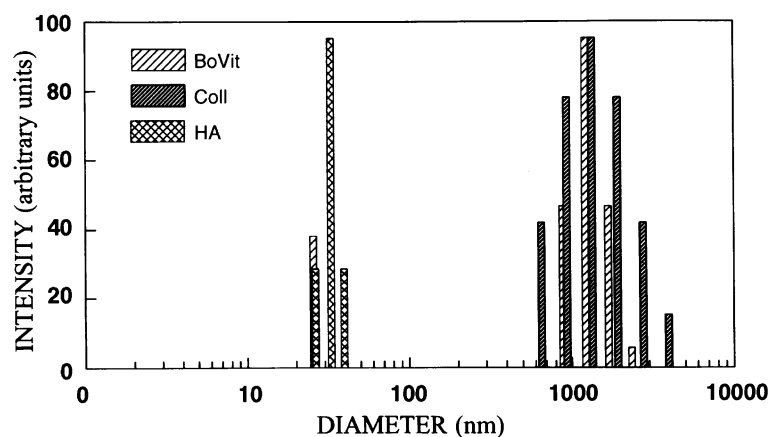


FIG. 3. Particle size distributions for whole Bo Vit and model vitreous solutions of HA and Coll determined from the DLS TCFs shown in Fig. 2.

consists primarily of HA with little or no Coll (Sebag, 1998), these results support the aforementioned postulate that the fast component of the TCF curve obtained from whole vitreous is primarily due to HA, while the slow component likely results from vitreous Coll.

DLS measurements from multiple points in whole Bo Vit (whole eyes with cornea excised) revealed considerable variation in the distribution of particle sizes throughout the entire vitreous body. Fig. 5 displays the three-dimensional map generated from these DLS measurements. The X axis is the horizontal distance from the central (optical) axis of the eye. The Y axis is the distance along the sagittal (front to back) plane from the central starting point. From this starting point, whose location is represented as the Z co-ordinate defined as mm posterior to the lens along the optical axis in the central vitreous, measurements were obtained at 0.5 mm steps in both the X and Y directions. Along the Z axis (optical axis or sagittal

plane), measurements were obtained every 2 mm, from 14 mm behind the back surface of the lens to 24 mm back. The considerable topographic heterogeneity in particle sizes of macromolecules throughout the vitreous body can be appreciated in these plots, with the height of the lines representing the average particle size for all molecular constituents at the measurement site.

Lens

Fig. 6 shows the composite of TCF results obtained from DLS measurements at various points along the antero-posterior axis of the bovine lens. The X axis is the horizontal distance from the central (optical) axis of the eye. The Y axis is the distance along the sagittal (front to back) plane from the central starting point. From this starting point, whose location is represented as the Z co-ordinate defined as mm posterior to the anterior capsule of the lens

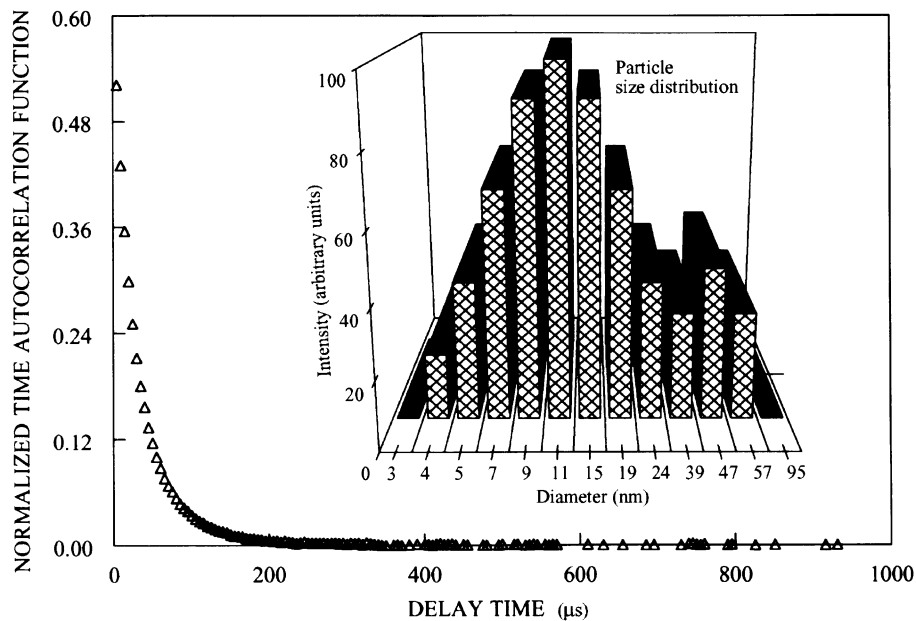


FIG. 4. DLS TCF of liquid compartment of Bo Vit. The results correlate closely with those obtained from the HA solution (Figs 2 and 3) and are consistent with the concept that liquid vitreous is primarily composed of HA and water.

along the optical axis, measurements were obtained at 0.5 mm steps in both the X and Y directions. Along the Z axis (optical axis in the sagittal plane) measurements were obtained every 2 mm, from 2 mm posterior to the anterior lens capsule up to 10 mm into the lens. In contrast to the results seen in vitreous, there is much greater homogeneity in the distribution of lens macromolecules, with the height of the lines representing the average particle size for all molecular constituents present at the measurement site.

4. Discussion

In recent years there has been a growing appreciation for the fact that changes in vitreous structure with aging and disease are the direct consequence of alterations on a molecular level, particularly with respect to the interaction of Coll, HA, and other extracellular matrix molecules. Similarly, it has long been appreciated that lens opacification results from changes in the macromolecular organization of the lens. Yet, there has been a dearth of methods by which these tissues can be precisely and reproducibly studied quantitatively in the laboratory and adequately evaluated to detect early molecular changes clinically. The advent of methodologies that introduce no artifacts and yield information about the molecular composition and organization of vitreous and lens can greatly improve the understanding of physiology in health and pathology in disease. Preliminary studies (Rovati et al., 1998; Sebag et al., 1999) found interesting results using DLS to evaluate diabetes effects upon vitreous.

This study demonstrates that by measuring the particle sizes present in vitreous and lens, the non-invasive methodology of DLS can evaluate vitreous and lens structure on a molecular level. Comparisons of measurements in whole vitreous with measurements in liquid vitreous, gel vitreous, and model solutions of vitreous showed that DLS provides useful information about the two major structural components of vitreous, i.e. Coll and HA. By not employing any extraneous substances, this non-invasive technique avoids introducing any artifact, making it useful for laboratory investigation of vitreous structure and physiology. Furthermore, the non-invasive nature and low energy level of this laser based methodology make it potentially very useful for clinical applications. Indeed, it may soon be feasible to improve the ability to evaluate vitreous clinically, for example, by quantitating the degree of liquefaction or presence of a posterior vitreous detachment with greater precision and reproducibility. Although the lens differs from vitreous insofar as it is a much more highly concentrated protein solution, DLS was able to assess the molecular morphology quantitatively. It is also possible that the protein concentration is slightly higher in the nuclear region, possibly lowering the diffusion coefficient artifactually and erroneously increasing the observed particle sizes displayed in Fig. 2. If this is a factor at all, it is of a minimal degree. Thus, the non-invasive and highly reproducible DLS methodology will likely be useful to detect the earliest molecular events in cataractogenesis and monitor the response to therapy intended to either retard or reverse cataract formation.

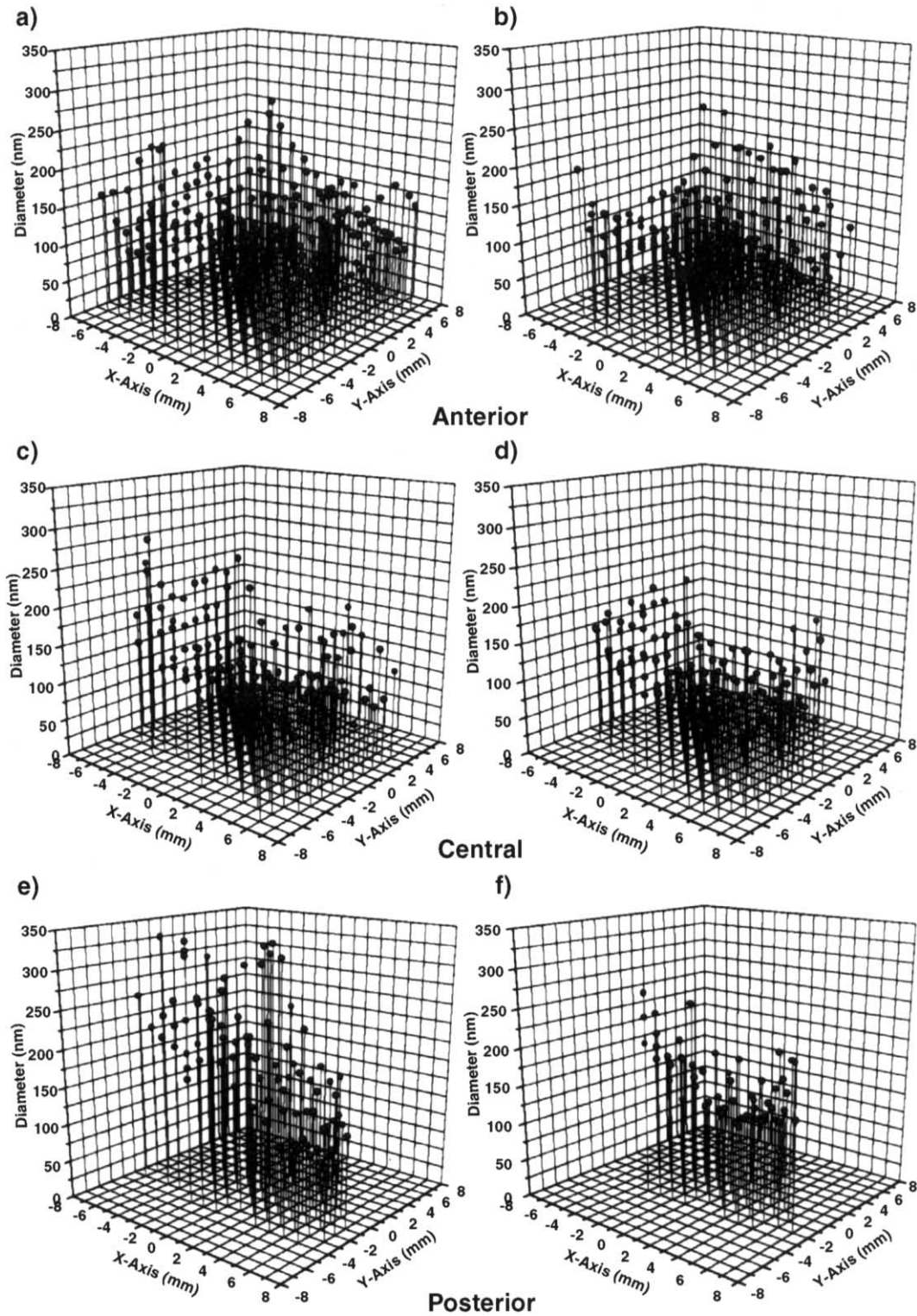


FIG. 5. Three-dimensional map of DLS measurements in the Bo Vit body, in situ. The X axis is the horizontal distance from a central (near-optical) axis of the eye. The Y axis is the distance along the sagittal (front to back) plane from the central starting point of measurements on the optical axis of the eye. From this starting point, whose location is represented as the Z coordinate and is defined as mm posterior to the lens along the optical axis in the central vitreous, measurements were obtained at 0.5 mm steps in both the X and Y directions. Along the Z axis (in the sagittal plane), measurements were obtained every 2 mm, from 14 mm behind the back surface of the lens to 24 mm posterior to this point. The heterogeneous distribution of macromolecules throughout the vitreous body can be appreciated in these plots with the height of the lines representing the average particle size for all molecular constituents (cumulant fit of both fast HA and slow Coll) at the measurement site.

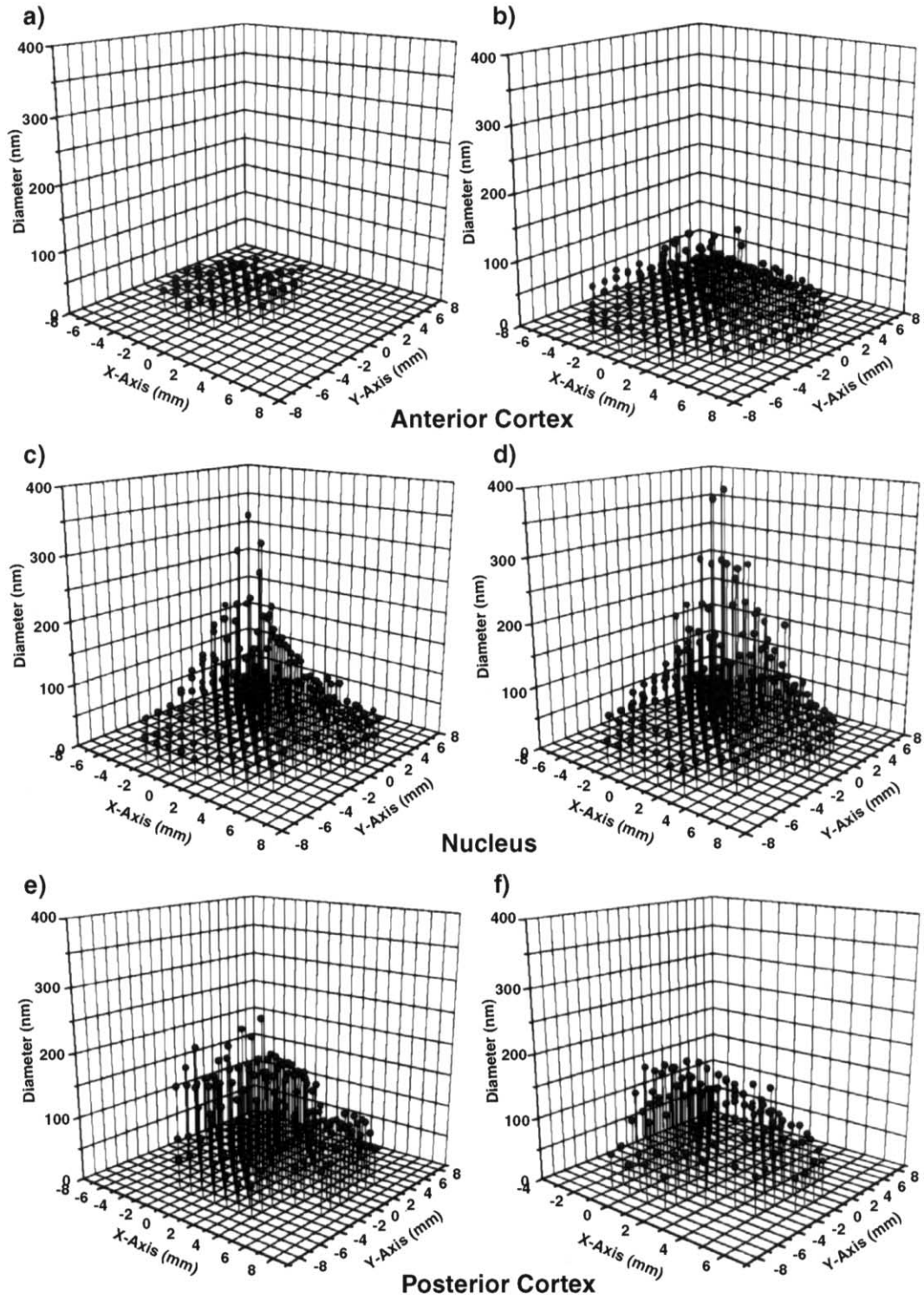


FIG. 6. Three-dimensional map of DLS measurements in the bovine lens. The X axis is the horizontal distance from a central (near-optical) axis of the eye. The Y axis is the distance along the sagittal (front to back) plane from the central starting point. From this starting point, whose location is represented as the Z co-ordinate (defined as mm posterior to the anterior capsule of the lens along the optical axis), measurements were obtained at 0.5 mm steps in both the X and Y directions. Along the Z axis (optical axis in the sagittal plane), measurements were obtained every 2 mm, from 2 mm posterior to the anterior lens capsule up to 10 mm into the lens. In contrast to the results seen in the vitreous (Fig. 5), there is much greater homogeneity in the distribution of lens crystallins, with the height of the lines representing the cumulant fit of the average particle size of the crystallins at the measurement site.

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