paper and note on methodology

New approach to assess the cholesterol distribution in the eye lens: confocal Raman microspectroscopy and filipin cytochemistry

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Abstract Confocal Raman microspectroscopy (CRM) is a non-invasive, non-destructive, and sensitive analytical tool for the study of some aspects of the molecular organization of cells and tissues with high spatial resolution. Filipin, a polyene antibiotic, specifically binds to cholesterol, and its molecular structure predicts it to be Raman-active. The aim of the present study was to assess the potentialities of a combined CRM-filipin approach to study the distribution of cholesterol in the human eye lens. Paraformaldehyde-fixed human lenses were sliced (0.7 mm), incubated with filipin, and analyzed by CRM. Filipin proved to give a specific Raman signal at 1586 cm^{-1}, hardly interfering with signals from lens proteins. The CRM-filipin approach proved to be extremely sensitive, allowing detection of cholesterol in the femtogram range. It has an excellent spatial resolution (0.2–0.5 μm^3) when using point measurements. Due to the intrinsic anisotropy of membranes in the eye lens and therefore of the cholesterol distribution, a line-scan approach has to be adopted when fiber-to-fiber changes in cholesterol are of interest. The distribution of filipin along the optical axis of four human eye lenses was compared with data from the literature. The combined CRM-filipin approach is a highly specific and sensitive method for the study of cholesterol within cells and tissues. The spatial resolution is high and can be adapted to the desired discriminative power. The gross distribution of filipin along the optical axis obtained in this study is similar to that found in biochemical studies.—**Duindam, H. J., G. F. J. M. Vrensen, C. Otto, G. J. Puppels, and J. Greve.** New approach to assess the cholesterol distribution in the eye lens: confocal Raman microspectroscopy and filipin cytochemistry. *J. Lipid Res.* 1995. **36:** 1139–1146.

Supplementary key words membranes • cholesterol • eye lens • Raman microspectroscopy • filipin

During the lifelong growth of the eye lens, the differentiation of the postmitotic equatorial epithelial cells into lens fibers involves an elongation process, increasing the plasma membrane about 1000-fold (1, 2). Several studies revealing differences in membrane architecture, protein composition, permeability, and capacitance of superficial versus deep cortical and nuclear fibers (3–6) support a two-compartment model of the eye lens as proposed by Garner (7) and Smeets et al. (8): a superficial compartment (about 30% of its volume), biochemically and physiologically active, with "normal" plasma membranes, and a nuclear compartment (about 70%) biochemically inert with impermeable, uncoupled, rigid, and non-leaky membranes. The cholesterol to phospholipid (C/PL) ratios reported for the eye lens range from 0.8 up to 6 and are the highest reported for any animal or human tissue (4, 5, 9–13). The significance of cholesterol for the eye lens is furthermore emphasized by the observation that cholesterol biosynthesis-inhibiting drugs cause cataract formation in the rat, dog, and human (9, 13–16). All these effects indicate that cholesterol in the lens plays an important physiological role, which also pertains to maintaining lens transparency.

The distribution of cholesterol inside human eye lenses has been investigated with biochemical and freeze-fracture techniques in which the accuracy of localization is dependent on the manner of dissection i.e., fraction size or fracture plane (5, 9, 17).

The polyene antibiotic filipin complexes with unesterified 3-β-OH-sterols with an apolar chain at C17 in a 1:1 sterol to filipin ratio (18–21). The only sterol in the eye lens qualifying for complex formation with filipin is cholesterol (5, 22). Filipin, therefore, can be considered to be a specific cholesterol probe inside the eye lens. The determination of the reporter molecule filipin can be done using confocal Raman microspectroscopy. CRM using a 660 nm incident laser beam is a non-destructive technique suited to study the macromolecular composition of biological material with a high spatial resolution (23, 24).

Abbreviation: CRM, confocal Raman microspectroscopy.

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Filipin is expected to be strongly Raman-active, on account of its five double carbon bonds (25) (**Fig. 1**).

Combining confocal Raman microspectroscopy and filipin cytochemistry could thus create a new sensitive method to determine the distribution of cholesterol with high spatial resolution inside intact lenses or lens slices.

The aim of this study was to test this novel approach using filipin as a cholesterol-specific and Raman-active probe.

MATERIALS AND METHODS

Human donor eye lenses obtained from the Corneabank Amsterdam, extracted within 24 h post mortem, were fixed in 0.08 M cacodylate-buffered 1% paraformaldehyde, pH 7.3. The fixed lenses were sliced by cutting along the optical axis in posterior direction with three razor blades, obtaining two axial (24) slices of approximately 0.7 mm thickness. One of the slices was incubated, under continuous agitation, for 4 h at 38°C in the dark in a 0.08 M cacodylate-buffered filipin (type III Sigma lot 69F4019) solution (0.1 mg/ml, pH 7.3). After incubation the slice was washed thoroughly with 0.08 M cacodylate buffer, pH 7.3 (preparation procedure according to Van Marle et al. (17)). The other slice was stored in the same buffer and used for control measurements.

Raman spectra were recorded with a confocal Raman microspectrometer (23). The excitation light with a wavelength of 660 nm was emitted by an Argon-ion laser (Coherent) pumped DCM dye laser (Spectra Physics, model 375B).

A water immersion objective (Zeiss Plan Neofluar, 63x, NA = 1.2) was used to focus the laser beam in the lens slice and to collect backscattered light. The high numerical aperture of 1.2 of this objective contributes to the small dimensions of the measuring volume (± 0.2 μm^3), the form of which can be approximated by an ellipsoid with axial and lateral radii of ± 0.65 μm and ± 0.225 μm, respectively. Raman spectra were recorded using a data collection time of 2 min from different points or from 25-μm lines along the optical or equatorial axis of the slice at a constant depth below the surface of the slice. Constant depth was achieved by focusing on the surface of the lens slice at every position and then moving the object exactly 30 μm towards the objective. For line scanning the slice was moved to and from 25 μm along its optical or equatorial axis while recording the Raman spectrum. Given the 2-min data collection time and a scanning speed of 100 μm per min, one line-scan was the integration of eight measurements. Exact positioning and scanning of the lens slice was done using two computer-controlled, hysteresis-corrected stepmotors capable of making steps of 0.1 μm.

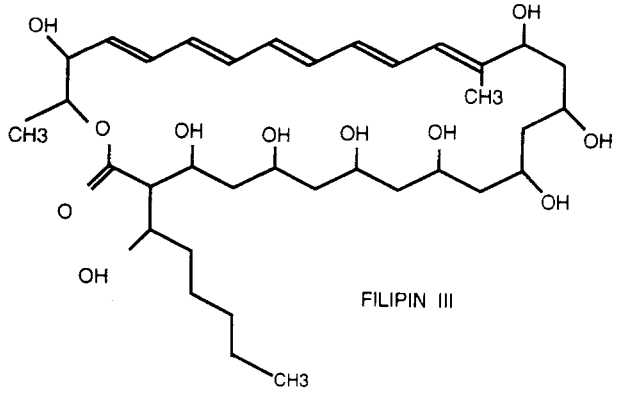

Fig. 1. The conjugated carbon double bonds of filipin III are expected to account for the Raman activity at 1586 cm^{-1}.

The Raman spectra were corrected for wavelength-dependent transmission of the spectrometer and variation of pixel sensitivity of the CCD camera. Wavenumber calibration was performed with an indene spectrum recorded at the same setting of the instrument as used in the lens slice measurements. The calibrated and corrected Raman spectra were analyzed with a spectrum analysis program RAMPAC (26).

As filipin is known to penetrate up to maximally 70 μm into the slice, the Raman spectra from positions 100 μm below the surface do not show a filipin peak. Therefore, at every position in the lens, the intensity of the 1586 cm^{-1} filipin peak was determined by subtracting the spectrum taken at 100 μm below the surface of the slice from the spectrum taken at 30 μm below the surface. The subtraction was done after normalization for protein content using the 1375–1500 cm^{-1} C-H-deformation band, which is generally accepted as an internal protein standard (8, 27). The total integrated intensity of the 1586 cm^{-1} filipin peak was used as a measure of relative local filipin content in the lens slice. In order to allow comparison among human lenses of different age and size and to compare the present results with previous biochemical studies, the positions on the optical or equatorial axis are given as distances from the anterior or equatorial edge related to the actual optical or equatorial axial sizes.

RESULTS

Raman spectra recorded from pure filipin in (DMSO) (A), fixed human eye lens slices with (B), and without (C) filipin treatment, and the difference spectrum (D: B minus C) are shown in **Fig. 2.**

The difference-spectrum is similar to the Raman spectrum from pure filipin indicating that the signal contribution of eye lens material to the intensity of the 1586 cm^{-1} filipin peak (Fig. 2: arrow) of the difference spectra is

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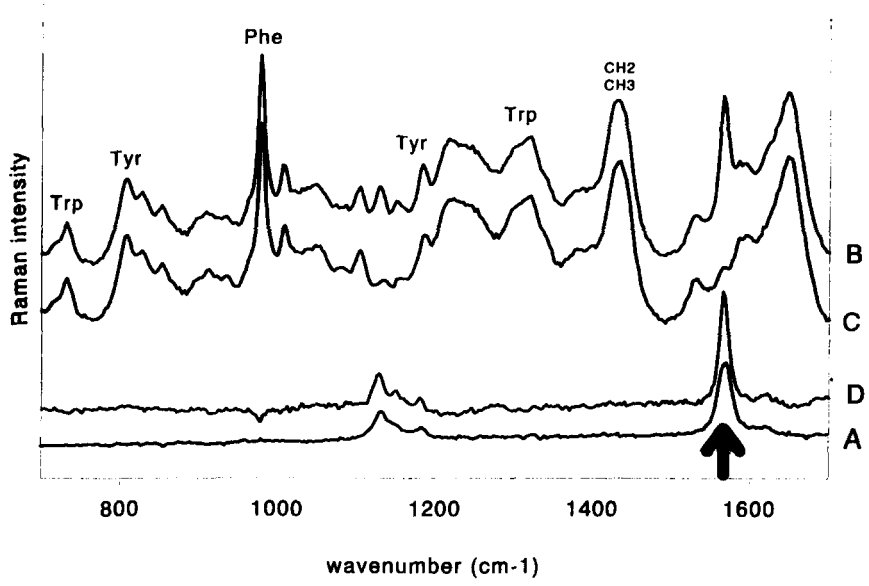

Fig. 2. In this figure the 700–1700 cm^{-1} Raman spectral region from filipin in DMSO (A), a 31-year-old human lens with (B) and without (C) filipin and the difference spectrum (D: B minus C) are shown (arrow: 1586 cm^{-1}; Trp: tryptophan, Try: tyrosine, Phe: phenylalanine, CH2 CH3: protein).

negligible. No differences were found among the Raman spectra recorded from pure solid filipin, filipin dissolved in dimethylsulfoxide, or filipin dissolved in 0.08 M cacodylate buffer, pH 7.3 (data not shown).

To evaluate the spatial resolution of the CRM-filipin approach we estimated for several lens slices the integrated 1586 cm^{-1} signal from different points 1 μm apart along the optical axis of the slice at a specified (20 μm)

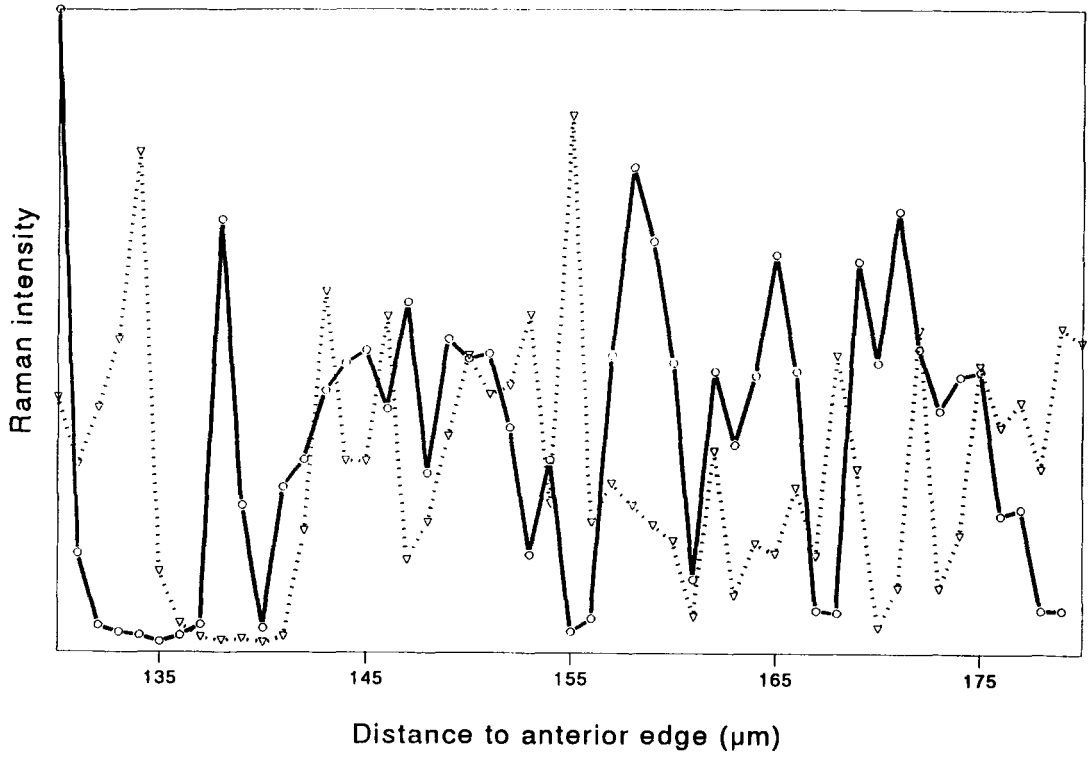

Fig. 3. Filipin signal from points 1 μm apart at 20 μm (circles and solid line) and 25 μm (triangles and dotted line) below the surface of the lens slice.

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depth below the surface. An example of such a measurement is given in **Fig. 3** (uninterrupted line). Repeated measurements at exactly the same position in the lens slice give nearly identical Raman spectra (Pearson correlation coefficient, 0.9997). Within regions known to have a constant protein content (28), the C–H deformation peak (1375–1500 cm^{-1}) remains constant, whereas the filipin peak varies depending on the position in the slice, indicating that the protein peak is not affected by the filipin reaction. The extreme variation in filipin content from one point to the other was most disappointing. If this is due to the method used, it would make the approach useless for assessment of the cholesterol distribution within the lens. To get more insight into the reasons behind these extreme variations we measured exactly the same trajectory at a slightly (5 μm) deeper level in the slice (Fig. 3, dotted line). At many axial positions the filipin signal is high at the 20-μm level and low at the 25-μm level. This strongly suggests that the variations in filipin signal are due to the anisotropic organization of membranes inside the lens, i.e., depends on whether the small focused laser probe (0.2–0.5 μm^3) comprises a membrane or only includes fiber cytoplasm as schematically indicated in **Fig. 4.**

As point measurements would render the approach unsuitable for measuring the changes in cholesterol content throughout the lens, we adopted a line-scan approach, i.e., Raman signals were constantly recorded while scanning the laser probe over a distance of 25 μm at a constant depth (8 times to and fro during 120 sec). The results for five human lenses are given in **Fig. 5** and **Fig. 6.**

The overall picture of the filipin distribution in the human eye lens emerging from these observations is that the filipin signal gradually increases reaching maximal values at distances below the posterior and anterior sur-

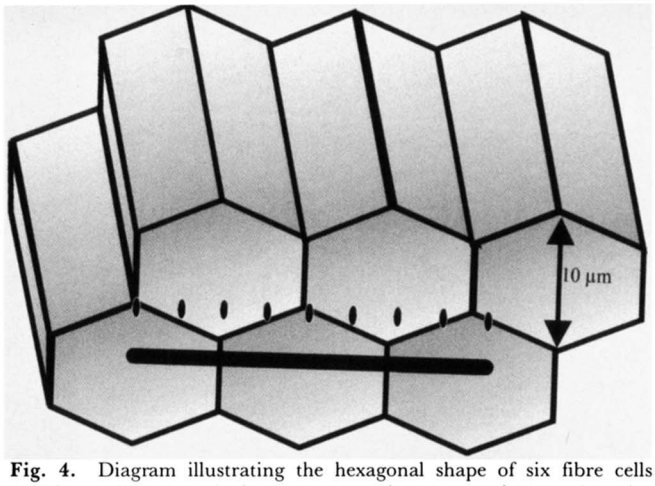

Fig. 4. Diagram illustrating the hexagonal shape of six fibre cells (width: ±10 μm) and the two measuring approaches used: point measurements (an ellipse approximates the measuring volume of the laser probe) and line scanning (the line approximates the measuring volume of the laser probe). This figure also depicts that point measurements at two positions can give high or low filipin signals due to the position of the measuring probe (black ellipses) exclusively in the cytoplasm or in cytoplasm including membranes.

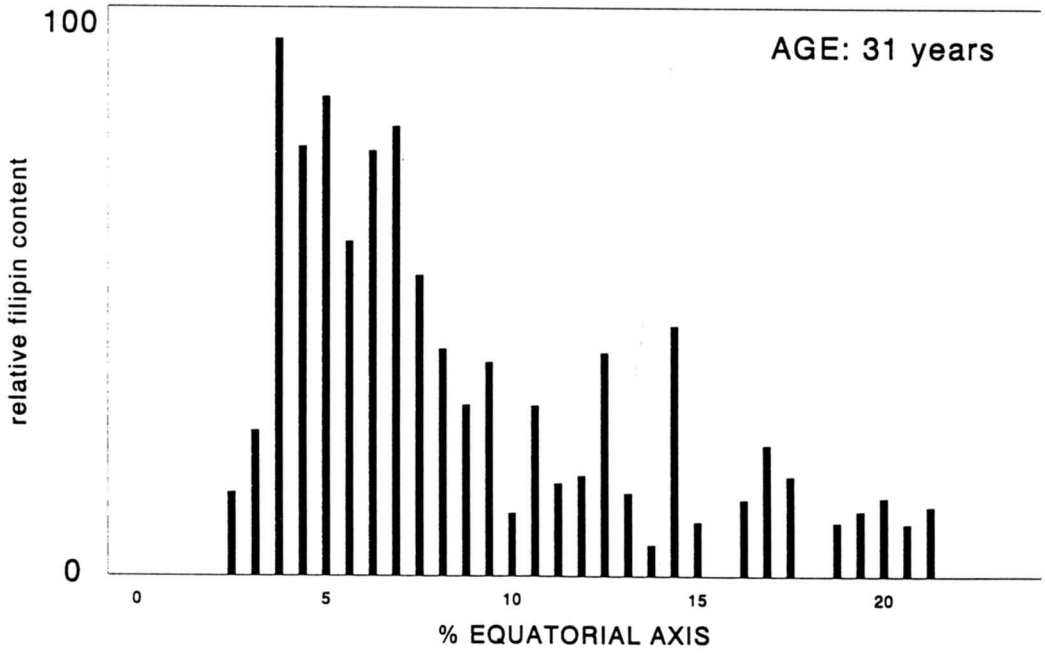

Fig. 5. Intensity of filipin Raman signals along a part of the equatorial axis of a 31-year-old human eye lens. The position along the axis is normalized and given in distance to the equatorial edge relative to the total equatorial axis of the lens.

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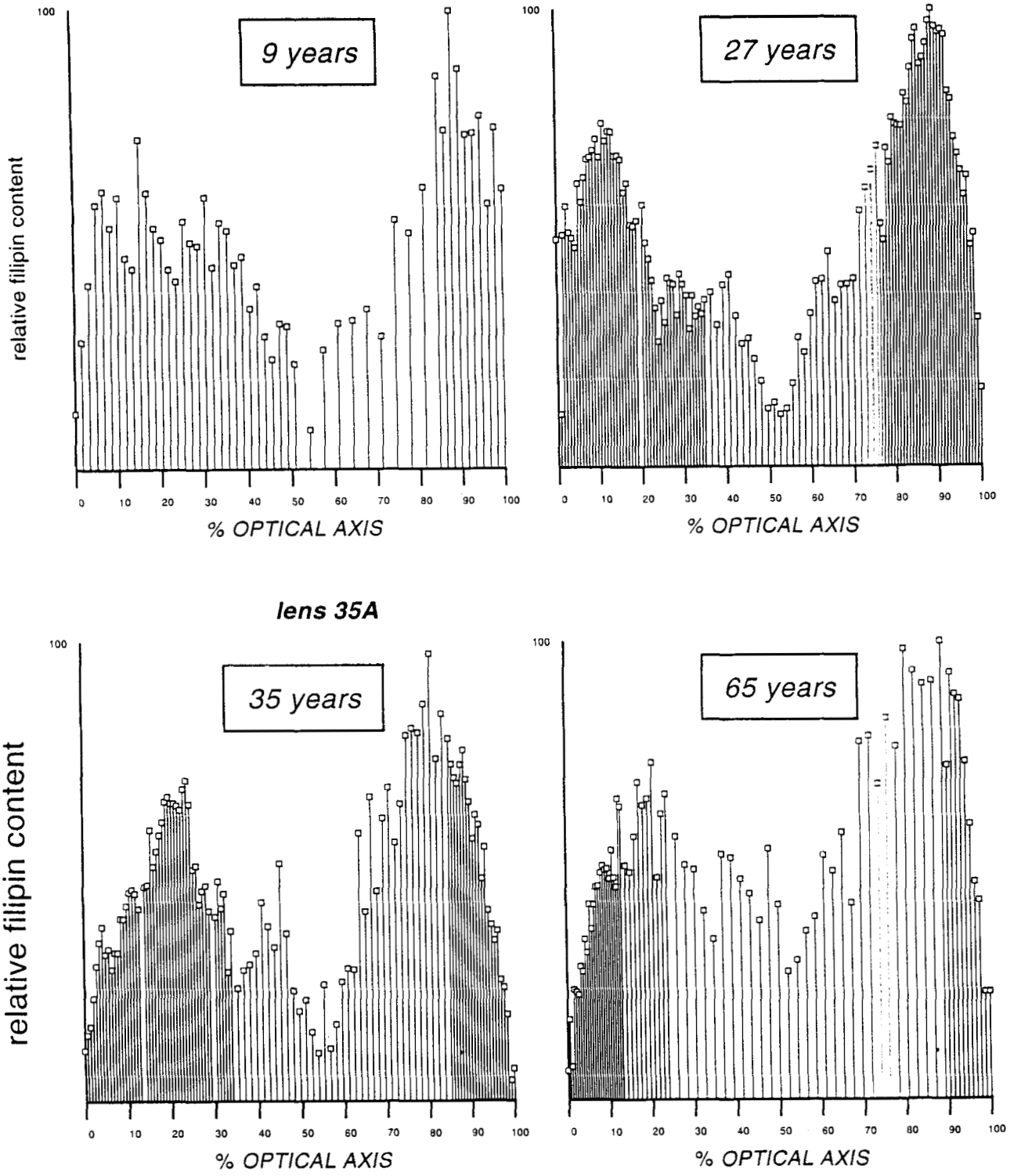

Fig. 6. Filipin distribution along the optical axis of four human lenses of different ages (9, 27, 35, and 65 years). The position along the axis is normalized and given in distance to the anterior edge relative to the total optical axis of the lens.

face varying between 15 and 20% of the optical or equatorial diameter. Towards the nucleus the filipin signals decrease and have a minimum around 50% of the lens diameter, i.e., in the right center of the lens.

To compare our results with a previous biochemical study (5), the filipin signals in the anterior and posterior parts were grouped and averaged corresponding to the (volume) fractions as given by Li, So, and Spector (5), i.e.,

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0–3.4%, 3.4–7.6%, 7.6–19.4%, 19.4–31.2%, 31.2–37.1%, 37.1–43%, 43–57.7%, and 57.7–100%. The results of this regrouping and averaging for the four human lenses of Fig. 6 are given in **Fig. 7A.** The filipin distribution that was regrouped (Fig. 7A) according to Li et al. (5) shows the same profile as the cholesterol distribution presented in their study (Fig. 7B).

DISCUSSION

The present study aimed to evaluate the specificity and sensitivity of a combined CRM-filipin approach to assess the cholesterol distribution within the eye lens with high spatial resolution. As summarized in Fig. 2, filipin bound to the cholesterol in lens membranes gives a specific Raman signal at 1586 cm^{-1} with negligible interference with signals from lens proteins or lipids, indicating that Raman microspectroscopic detection of filipin and therefore of cholesterol within lens tissue is specific.

In this study a 63× objective with NA 1.2 was used to collect the Raman scattered light. Using this objective, Puppels et al. (23) have shown that an axial and lateral spatial resolution of 1.3 μm and 0.45 μm, respectively, can be obtained. This high spatial resolution allows the study of variations in filipin content between locations with a lateral distance of at least 1 μm (Fig. 3). An obvious disadvantage of this high resolution is given in Figs. 3 and 4. The lens consists of close-packed, hexagonal fiber cells, interdigitating at their lateral and apical edges with complex protrusions and has minimal extracellular space (2–3%) (29, 30). The absence of cell organelles in the vast majority of fibers (31) implies that the lens has an intrinsic anisotropy of membranous elements and thus of cholesterol. Due to this anisotropic distribution of cholesterol in the lens, differences in cholesterol on a fiber-to-fiber base are difficult to assess using point measurements. In this study a line-scan approach was adopted to circumvent this problem. This line-scan approach gave the results illustrated in Figs. 5 and 6 which, on the whole, resemble the cholesterol distribution observed in biochemical studies (5, 9, 10) and follow the distribution of the filipin-cholesterol pits observed in freeze-fracture studies (17). The resemblance of the cholesterol distribution obtained by the present study (Fig. 7A) and of the biochemical data (5) (Fig. 7B) is striking. Considering the specificity and stoichiometry of the formation of the filipin-sterol complex (18–21) and the similarity of the measured filipin distribution with the cholesterol distribution from other studies (5, 9, 10), we assume that the relative filipin content determined by the Raman measurements reflects the relative content of unesterified cholesterol along the optical and equatorial axis of the eye lens. As outlined by Miller (32) filipin incubation will affect the topographical localization of cholesterol and leads to a lateral redistribution of cholesterol from one site to another within one membrane. This means that filipin probing is not a reliable technique to discriminate between the cholesterol levels within one membrane. This can partly be avoided by changing the incubation time. However, the same author (32) also states that filipin can reliably be used to localize cholesterol on a continuous sheet of membrane (topological studies). Considering the dimensions of the laser probe (**Fig. 8A and 8B** and the last paragraph of the discussion) we are measuring membrane areas of 0.16–0.46 μm^2 in the present study. Given the dimensions of a filipin-cholesterol pit between 20–25 nm the surface areas of these pits are approximately 0.0004 μm^2. This means that redistribution of cholesterol by filipin incubation will not or minimally affect our results. Moreover, a random distribution of filipin-cholesterol pits was observed in a previous freeze-fracture study (17), indicating that redistribution of cholesterol does not occur on a topological

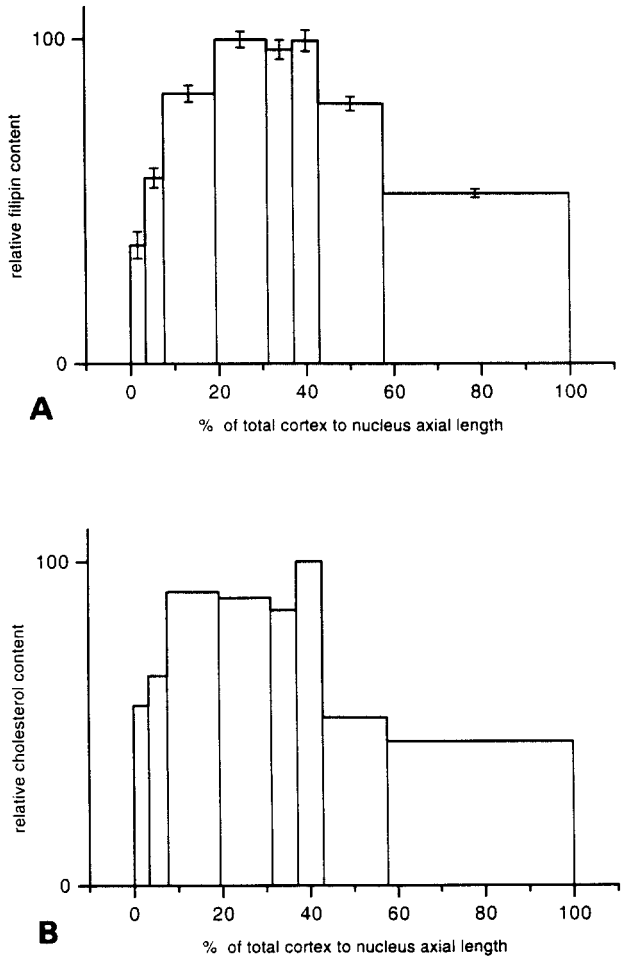

Fig. 7. A: Mean filipin signal of four human lenses regrouped on the optical axis according to the concentric fractions used by Li et al. (5). A detailed description is given in the Materials and Methods section. B: Cholesterol content of a 63-year-old human left lens (from Li et al., Fig. 2, upper graph (5)).

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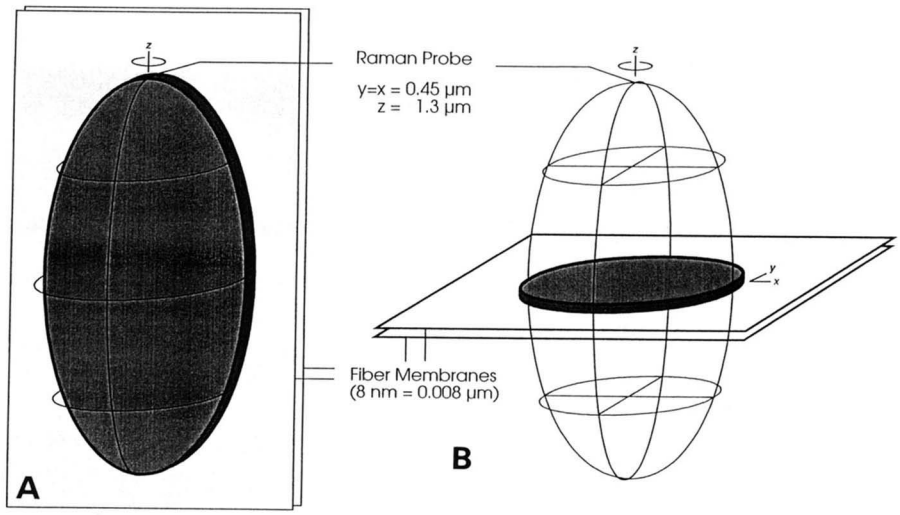

Fig. 8. A: Diagram illustrating the intersection of the laser probe with a membrane oriented parallel to the optical axis of the laser probe (long axis). B: Diagram illustrating the intersection of the laser probe with a membrane oriented perpendicular to the optical axis of the laser probe (small axis).

scale in the lens. Finally, the resemblance to previous biochemical data (5, 9, 10) supports the reliability of the present topological estimation of cholesterol in the human eye lens using this CRM-filipin approach.

To evaluate the sensitivity of the CRM-filipin approach we calculated the quantity of cholesterol detected by the volume of the laser probe (point measurements). The measuring volume of the Raman probe using a 63× objective with NA 1.2 is approximately 0.2–0.5 μm^3 depending on the boundaries accepted, 50% and 25%, respectively. The axial and lateral resolutions are 1.3 and 0.45 μm, respectively (20). The form of the probe (Fig. 8) can be approximated by an ellipsoid. The lens fibers have transsectional diameters of 5–10 μm depending on their position in the lens. This means that the measuring volume will maximally encompass only one opposing pair of fiber limiting membranes of two adjacent fibers, which are both 8 nm (0.008 μm) thick and have minimal intercellular separation (29). This means that the measuring volume encompasses $(\pi \times 0.65 \times 0.225) \times (2 \times 0.008) = 0.0073\ \mu m^3$ membrane material when the membranes are positioned in the direction of the long axis of the laser probe (Fig. 8A). When positioned perpendicular to the direction of the long axis, the measuring volume contains $(\pi \times 0.225^2) \times (2 \times 0.008) = 0.0025\ \mu m^3$ membrane material (Fig. 8B). Assuming that the membranes are pure phospholipid bilayers with an approximated relative density of 0.95 g/ml and an approximated cholesterol to phospholipid molar ratio of 0.8 (true for superficial lens fibers (5) and a molecular weight of cholesterol of 386 and an averaged molecular weight of phospholipids of 700, the cholesterol content per cm^3 is given by $0.95 \times (8 \times 386)/((8 \times 386) + (10 \times 700)) = 0.29$ g or 1 μm^3 contains 0.29×10^{-12} g cholesterol. This means that the cholesterol content encompassed in the measuring volume is $0.0073 \times 0.29 \times 10^{-12} = 2.1 \times 10^{-15}$ g or 2.1 femtogram for the membranes in an ideal axial position (Fig. 8A) and $0.0025 \times 0.29 \times 10^{-12} = 0.7$ femtogram in an ideal lateral position (Fig. 8B). Because we know that natural membranes also contain intrinsic membrane proteins, the amount of cholesterol within the measuring volume will be even lower. As the Raman spectra show a good signal-to-noise ratio, the CRM-filipin approach is extremely sensitive in addition to being highly specific.

CONCLUSION

The combination of confocal Raman microspectroscopy and filipin cytochemistry is a highly specific and extremely sensitive method for the study of the local distribution of cholesterol in cells and tissues.

The preliminary results presented for human lenses are perfectly in line with biochemical and filipin freeze-fracture studies on cholesterol distribution in the human eye lens. Furthermore, it must be emphasized that the high spatial resolution, (1.3 μm axial and 0.45 μm lateral) allows the detection of strict local changes due, for example, to cataractous insults in lenses, and theoretically even allows distinction between the cholesterol content of the membranes of distinct cellular compartments such as the nucleus, the reticular meshwork, etc.

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