Raman Detection of Macular Carotenoid Pigments in Intact Human Retina

Paul S. Bernstein,[1] *Mihoko D. Yoshida,*[2] *Nikita B. Katz,*[1] *Robert W. McClane,*[2] *and Werner Gellermann*[2]

Purpose. To develop and test a novel noninvasive optical technique suitable for the objective measurement of macular carotenoid levels in human retina.

Methods. A resonance Raman scattering apparatus was constructed to measure carotenoid levels in flat-mounted human retinas and eyecups and in experimental animal eyes. Light from an argon laser was used to resonantly excite the electronic absorption of the carotenoid pigments, and scattered light was collected and analyzed by a Raman spectrometer. After carotenoid Raman measurements were completed on the retinal samples, macular carotenoid levels were determined by high-performance liquid chromatography (HPLC).

Results. Carotenoid resonance Raman scattering proved to be a highly sensitive and specific method for the noninvasive measurement of macular pigments in the human retina. Signal strength scaled linearly with actual macular carotenoid content as measured by HPLC. Our apparatus was also used to record resonance Raman signals from xanthophyll carotenoids stored in the retinal pigment epithelium of intact frog eyes.

Conclusions. This new noninvasive optical method will facilitate studies of ocular carotenoid distributions and their role in degenerative diseases of the eye and may allow for the rapid screening of carotenoid levels in large populations at risk for vision loss from age-related macular degeneration, the leading cause of blindness in the elderly in the United States. A prototype clinical instrument is under development. (*Invest Ophthalmol Vis Sci.* 1998;39:2003-2011)

Of the 10 to 15 carotenoid pigments usually found in normal human serum, only lutein and zeaxanthin are concentrated in high amounts in the cells of the macula lutea, the 5- to 6-mm diameter central area of the retina in which the visual acuity is highest.[1-5] These carotenoids give a characteristic yellow coloration to the macula, where they can act as a filter to attenuate photochemical damage and/or image degradation from short wavelength visible light.[6,7] In addition, they are thought to act as free-radical scavenging antioxidants.[6,7] Studies have shown that there is an inverse correlation between high dietary intakes and blood levels of lutein and zeaxanthin and risk of age-related macular degeneration (AMD), the leading cause of blindness in the elderly in the United States.[8,9] It has been demonstrated that macular carotenoid levels can be altered through dietary manipulation, and it has been reported that carotenoid levels are lower in autopsy eyes from patients with AMD.[10,11] Despite a lack of prospective randomized clinical trials, lutein supplements are already widely promoted by the health-food industry to those at risk for AMD. Although blood carotenoid measurements are relatively easy to perform, they are at best an indirect measure of ocular carotenoid status. Currently, the most commonly used noninvasive method for measuring human macular carotenoid levels is a subjective psychophysical flicker photometric test involving color intensity matching of a light beam aimed at the fovea and another aimed at the perifoveal area.[10-12] This method is time intensive and requires an alert, cooperative subject with good visual acuity. Thus, the usefulness of this method for assessing macular pigment levels in the elderly population most at risk for visual loss from AMD is severely limited. It is clear that a rapid objective and specific technique to noninvasively quantify macular pigment levels in human subjects would be a major advance in the study and the possible prevention of macular degeneration diseases.

The laser spectroscopic technique of resonance Raman scattering was investigated as a novel method for noninvasive optical measurement of the retinal carotenoid pigments. Blue/green argon laser lines were used to resonantly excite the electronic absorption of the pigments, and the Raman signals were recorded with a medium-resolution grating spectrometer using rapid detection with a cooled silicon charge-coupled detector (CCD) array. Carotenoids are known to have strong Raman signals when excitation is in the 450- to 550-nm range because they exhibit a resonance enhancement of 10^4 to 10^6 over the ordinarily weak Raman signals of carotenoids and

From the [1]Department of Ophthalmology, Moran Eye Center, University of Utah School of Medicine, Salt Lake City; and the [2]Department of Physics and Dixon Laser Institute, University of Utah, Salt Lake City.

Presented in part at the annual meeting of the Association for Research in Vision and Ophthalmology, May 1997, Fort Lauderdale, Florida, and at the annual meeting of the Society of Photograph-Optical Instrumentation Engineers, January 1998, San Jose, California.

Supported by National Institutes of Health, Bethesda, Maryland, Grant EY-11600; by grants from Research to Prevent Blindness, Inc., New York, New York; and by internal funds from the Dixon Laser Institute, University of Utah.

Submitted for publication January 20, 1998; revised June 4, 1998; accepted June 16, 1998.

Proprietary interest category: P.

Reprint requests: Paul S. Bernstein, Department of Ophthalmology, Moran Eye Center, University of Utah School of Medicine, 50 North Medical Drive, Salt Lake City, UT 84132.

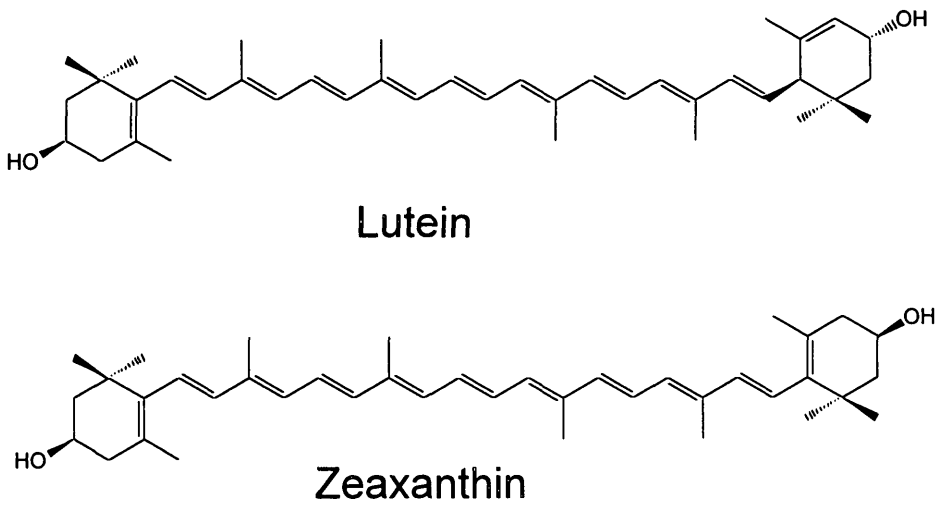

FIGURE 1. Chemical structures of lutein and zeaxanthin.

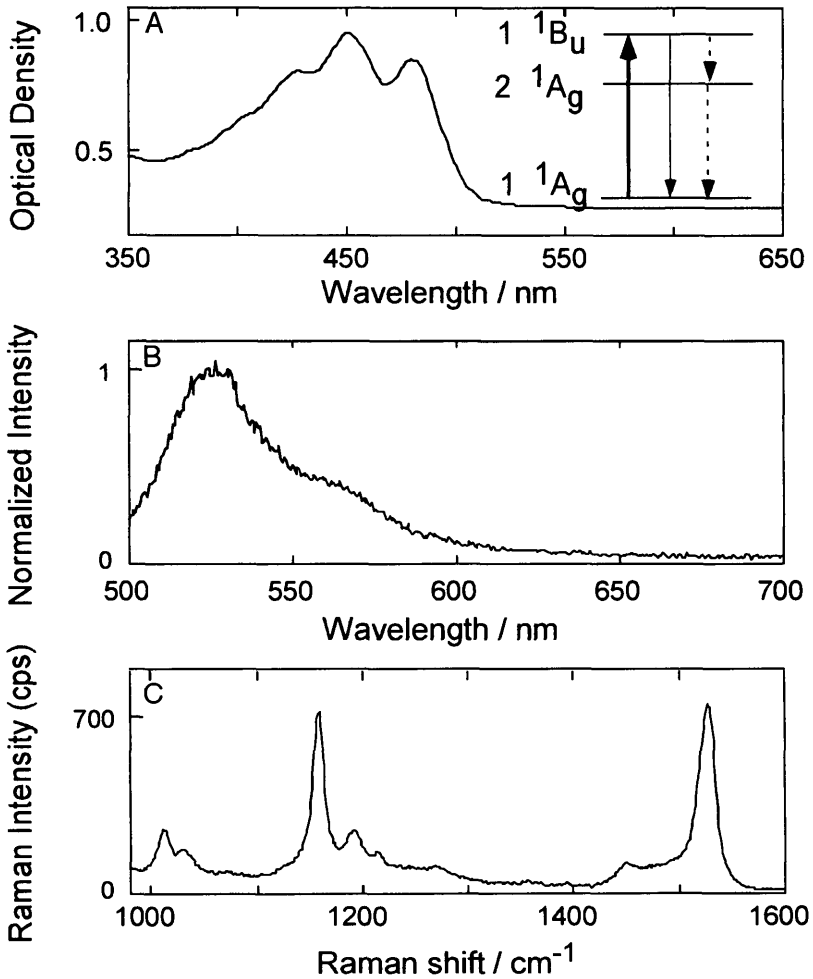

FIGURE 2. Optical properties of a 7 μM solution of lutein in tetrahydrofuran (THF). **(A)** Absorption spectrum measured through a 1-cm path length quartz cuvette with a spectrometer (Lambda-9; Perkin-Elmer, Norwalk, CT), the inset shows the electronic energy level diagram and corresponding radiative (*solid arrows*) and nonradiative (*dashed arrows*) transitions of the molecule (for details see text). **(B)** Luminescence spectrum measured under 488-nm excitation with a Spex fluorometer. **(C)** Raman spectrum (unpolarized) measured with a Spex Triplemate spectrometer under 514.5-nm excitation and 90° scattering geometry. The peak at 1030 cm^{-1} is an artifact and corresponds to THF. Resolution is approximately 4 cm^{-1}.

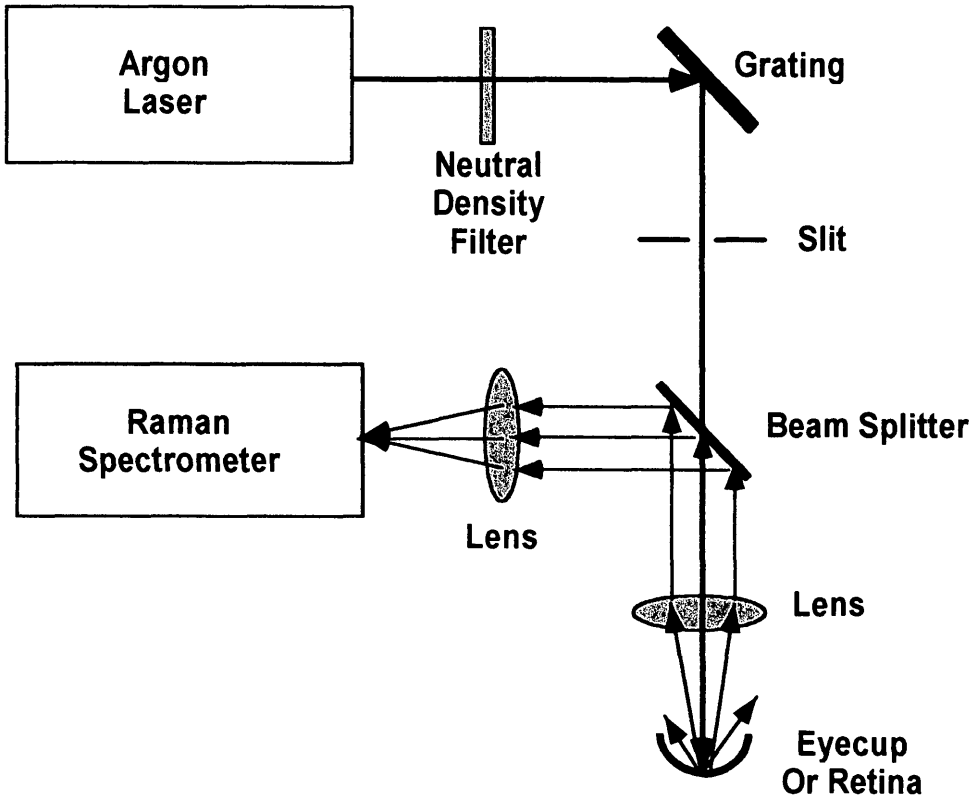

FIGURE 3. Apparatus for Raman detection of carotenoids in eyecup and flat-mounted retina samples. See the Materials and Methods section for device specifications.

other compounds encountered under nonresonant conditions.[13] Resonance Raman techniques have been used to measure carotenoid levels in atherosclerotic plaques,[14] breast biopsy specimens,[15] and the lens of the deep-sea hatchetfish,[16] but these methods have never before been applied to retinal tissue.

MATERIALS AND METHODS

Human Retina and Eyecup Preparation

Postmortem human eyes whose corneas had already been removed for transplantation were obtained from the Utah Lions' Eye Bank within 24 hours after death. Procurement and processing were performed with institutional approval and was in accordance with the tenets of the Declaration of Helsinki. Eyecups were prepared by removing the lens and iris. Eyes with discernible macular pathology were excluded. A 5-mm trephine was then used to cut out tissue centered on the fovea. The 5-mm macular punch was carefully dissected free from the vitreous and retinal pigment epithelium (RPE) and mounted flat between two glass slides separated by a plastic spacer to prevent crushing of the tissue. Flat-mounted macular tissue was stored at −20°C until used.

Experimental Apparatus for Raman Detection of Carotenoids in Eyecup and Flat-Mounted Retina Samples

As excitation sources, the vertically polarized 488- or 514.5-nm lines of an argon laser were used. The laser power was reduced to several milliwatts with a neutral density filter, and the laser plasma lines were eliminated with a combination of a 600 lines/mm grating and a slit. The laser was directed through a beam splitter and weakly focused onto the sample with a 10-cm focal length lens. The backscattered light was imaged onto the entrance slit of a Raman spectrometer with the beam splitter and an additional lens. The Spex Triplemate Raman spectrometer, (ISA, Edison, NJ) used two stray light rejection gratings with 300 lines/mm, a dispersion grating with 1200 lines/mm, and a liquid-nitrogen-cooled silicon CCD array with 25 μm pixel width. Raman signal intensity was recorded as counts per second (cps).

High-Performance Liquid Chromatography Analysis of Macular Carotenoids

After Raman measurements had been completed, the macular tissue was homogenized by brief sonication in 0.5 ml 50% methanol and then extracted into 0.4 ml hexane containing 0.5% butylated hydroxytoluene under dim light. Two-tenths of a milliliter of the hexane extract was injected onto a Waters high-performance liquid chromatography (HPLC) system fitted with a Microsorb cyano 100 Å, 5 μm, 250 × 4.6-mm column (Rainin, Woburn, MA) eluted with 80% hexane, 19.4% dichloromethane, 0.5% methanol, and 0.1% *N,N'*-diisopropylethylamine at 1.0 ml/min and detection at 450 nm. The integrated peak areas of the lutein and zeaxanthin peaks were converted to nanograms by use of a standard curve generated by diluting standard solutions of pure lutein (Kemin Industries, Des Moines, IA) and zeaxanthin (Hoffmann-La Roche, Basel, Switzerland).

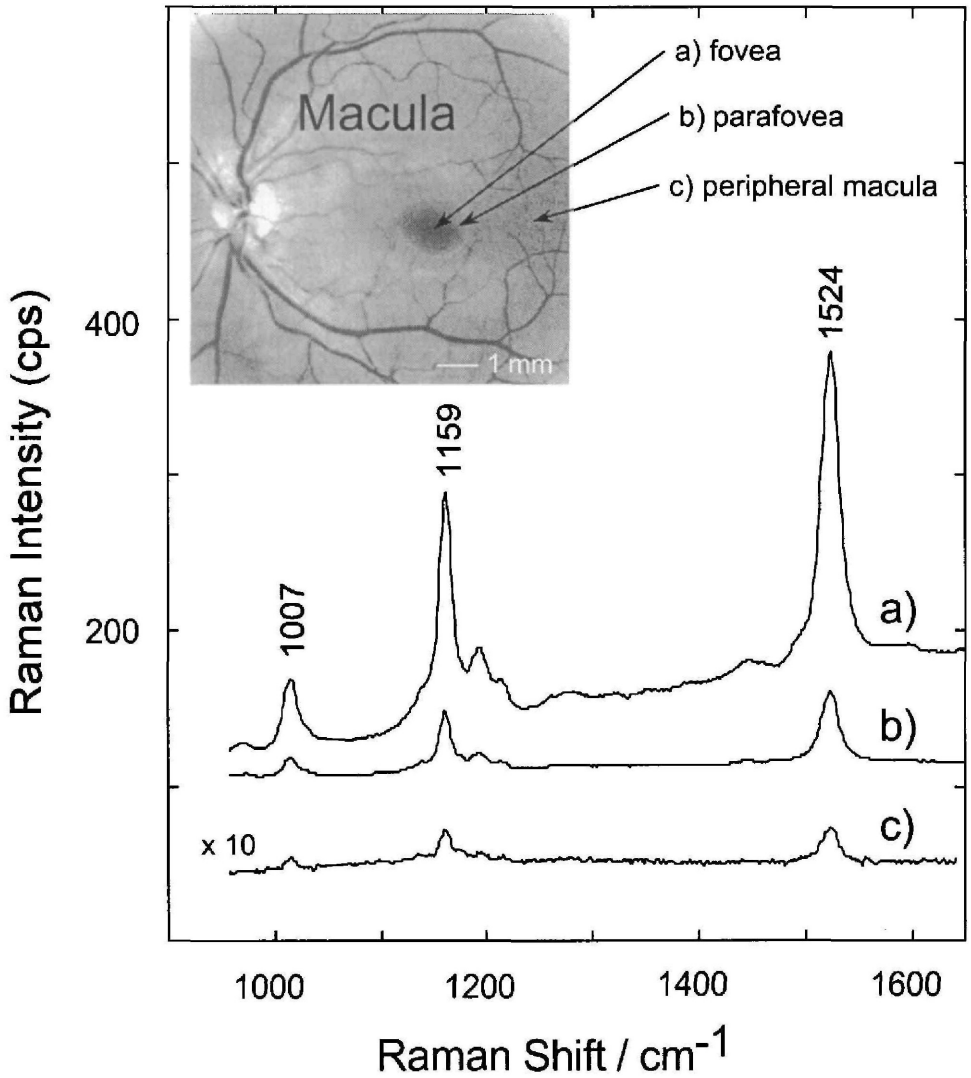

FIGURE 4. Resonance Raman spectra from a flat-mounted human retina. Spectra were obtained under excitation with 488-nm light in the center (*trace a*), 0.5 mm away from the center (*trace b*), and at the periphery (*trace c*) of the macula. The intensity scale of *trace c* is expanded by a factor of 10 for clarity. Illumination conditions: 488 nm, 40 mW, 300-μm spot size, 9 seconds.

Raman Measurement of Carotenoid Levels in Intact Frog Eyes

Rana pipiens frogs (Sullivan's Amphibians, Nashville, TN) were dark-adapted overnight and then immobilized by chilling to +4°C. Pupils were dilated with 1% tropicamide and 2.5% neosynephrine (Alcon, Fort Worth, TX). The Raman apparatus was as described above. Animal care and handling was in accordance with institutional guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

HPLC Analysis of Carotenoids in Frog Eyes

Dark-adapted *Rana pipiens* frogs were killed, and the eyes were enucleated. The combined retina, retinal pigment epithelium (RPE), and choroid from each eye were placed in separate 1.5-ml microcentrifuge tubes and homogenized with brief sonication into 0.5 ml 50% methanol. After homogenization, the carotenoids were extracted into 0.2 ml chloroform and evaporated to dryness. The extract from one eye underwent alkaline hydrolysis of carotenoid esters as described by Khachik at al.[17] Briefly, this entailed dissolving the material in 0.5 ml 5% KOH in methanol and heating to 85°C for 5 minutes. Adding three volumes of cold water stopped hydrolysis. Carotenoids were extracted into 0.5 ml HPLC running solvent that was washed with water until alkali-free and then evaporated. The saponified extract and the unsaponified extract from the fellow eye were redissolved into 200 μl HPLC running solvent. Ten-microliter portions of the samples were analyzed on the HPLC system as described above for human retinal tissue. Detection was performed at multiple wavelengths with a diode-array detector (UV6000LP; Thermo Separations, San Jose, CA).

RESULTS AND DISCUSSION

Lutein and zeaxanthin (Fig. 1) by virtue of their long conjugated isoprenoid backbones are ideally suited for detection through the use of resonance Raman spectroscopy. In Figure

2, we show the main optical properties (i.e., absorption, luminescence, and resonance Raman spectra) for a solution of lutein dissolved in tetrahydrofuran (THF). The electronic absorption is strong, occurs in a broad band (~100-nm width) centered at approximately 450 nm, and shows clearly resolved substructure with a spacing of approximately 1400 cm^{-1} (trace a). This behavior is due to electric-dipole-allowed vibronic transitions of the molecule's conjugated π-electron from the 1 ^1A_g singlet ground state to the 1 ^1B_u singlet excited state (see inset of Fig. 2A). Optical excitation within the absorption band leads to a weak luminescence with small Stokes shift (width ~50 nm, peak at ~530 nm), which again shows vibronic substructure (trace b). To detect sufficiently high luminescence signals in these measurements, a sensitive photomultiplier had to be used in combination with a photon counter. By analogy to the well-studied β-carotene molecule, the extremely low quantum efficiency of the lutein luminescence is most likely caused by the existence of a second excited singlet state, a 2 ^1A_g state, that lies below the 1 ^1B_u state (see inset of Fig. 2A). After excitation of the 1 ^1B_u state, the energy then relaxes very rapidly (within ~200 fs to 250 fs for β-carotene[18]) via nonradiative transitions to the lower 2 ^1A_g state, from which electronic emission to the ground state is symmetry-forbidden. The low 1 $^1B_u \rightarrow 1\ ^1A_g$ luminescence efficiency (10^{-5} to 10^{-4} for β-carotene[18]) and the absence of 2 $^1A_g \rightarrow 1\ ^1A_g$ fluorescence of lutein allow us to explore the resonant Raman scattering response of the molecular vibrations while avoiding potentially masking fluorescence signals. Indeed, we observed strong and clearly resolved Raman signals superimposed on a weak fluorescence background (trace c) under resonant laser excitation. The Raman response of lutein is characterized by two prominent Stokes lines at 1158 cm and 1528 cm^{-1} (corresponding to wavelength shifts of 29.2 and 39.3 nm, respectively, for 488-nm excitation), with nearly identical relative intensities. These lines originate, respectively, from carbon-carbon single-bond and double-bond stretch vibrations of the conjugated backbone.[19] In addition, several weaker but clearly distinguishable Stokes signals appear at 1008, 1195, 1220, and 1450 cm^{-1}. The 1008-cm^{-1} line is attributed to rocking motions of the molecule's methyl components.[19] Other carotenoids such as zeaxanthin, β-carotene, canthaxanthin, and astaxanthin had virtually identical resonance Raman spectra within the resolution limit (~4 cm^{-1}) of our spectrometer. This was expected because they all share a common conjugated polyene backbone structure.

An experimental apparatus suitable for Raman measurements on human retinal eyecup preparations and flat-mounted retinas was constructed as shown in Figure 3. Usable signals were obtained with either the 488-nm or 514.5-nm argon laser lines. Ordinarily, the 488-nm line is preferred over the 514.5-nm line because carotenoid Raman signal strength is three times higher at 488 nm, and background luminescence is lower. A typical result is shown in Figure 4 for a flat-mounted human retina. The Raman spectrum obtained for the macula is remarkably similar to the spectrum of the lutein solution shown in trace c of Figure 2. The peaks at approximately 1159 and 1524 cm^{-1} are obtained with a good signal-to-noise ratio when the beam is aimed at the foveal and parafoveal areas (traces a and b), and

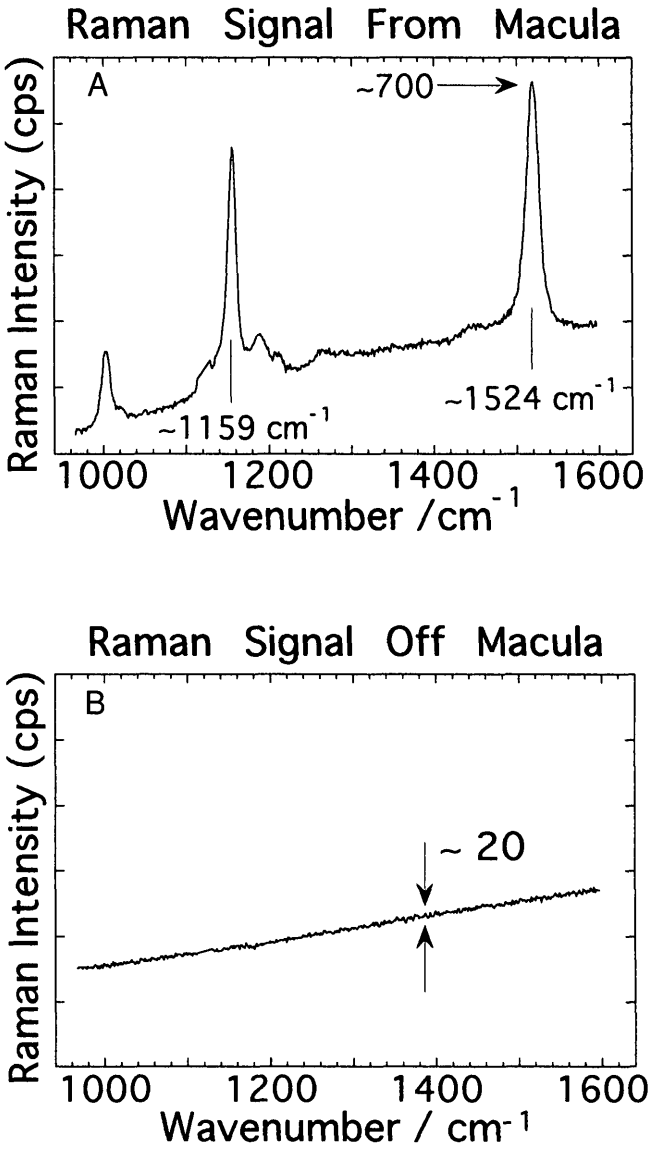

FIGURE 5. Carotenoid resonance Raman spectra from a human eyecup preparation. In the first experiment, a Raman measurement was obtained from the macula of a human eyecup preparation (**A**). Illumination conditions were 514.5 nm, 5 mW, and 1-mm spot size, 10 seconds. The same experiment was then repeated with the laser aimed at the peripheral retina (**B**). *y*-axis scales are the same in both experiments. As noted, the macular 1524 cm^{-1} peak height was approximately 700 cps. Using the same illumination conditions, no carotenoid Raman peaks could be identified in the peripheral retina above background noise (20 cps).

they decrease by a factor of 100 as the beam is moved toward the peripheral retina (trace c). This behavior correlates well with the known distribution of carotenoids in the human retina as determined by HPLC or psychophysically.[4,12] Other human ocular structures such as the cornea, lens, vitreous, RPE, choroid, and sclera do not generate any detectable or interfering Raman signals under comparable conditions, and carotenoid resonance Raman spectra was successfully recorded from a human eyecup preparation (Fig. 5). A linear correlation was also demonstrated between

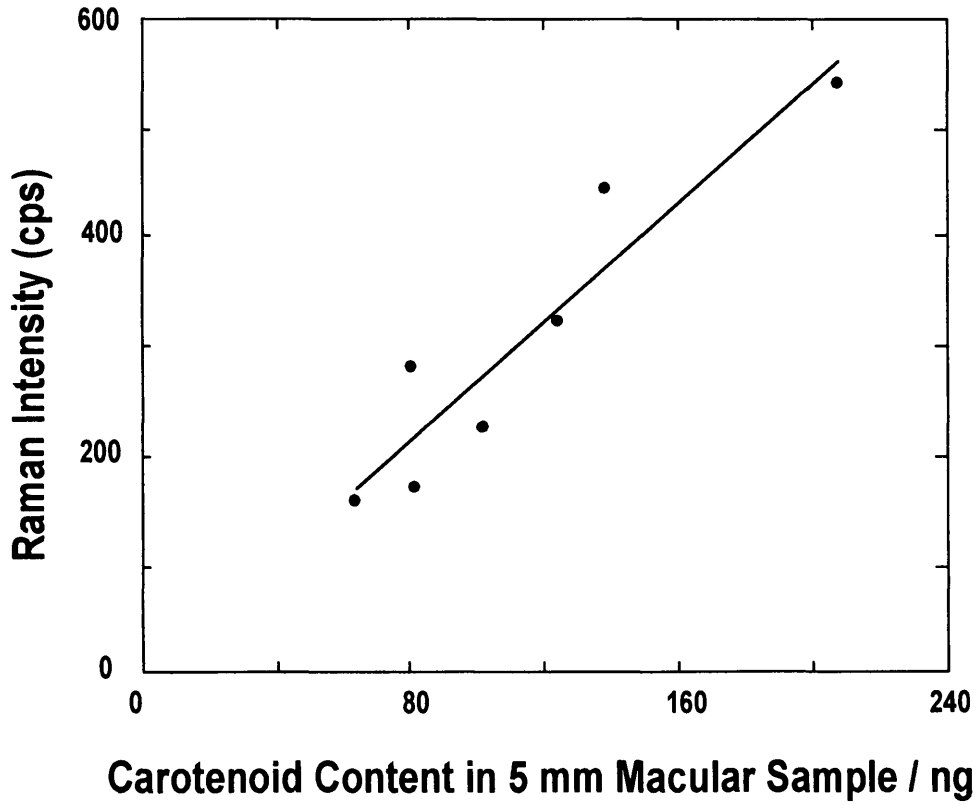

FIGURE 6. Integrated intensity of carotenoid 1524 cm^{-1} Stokes Raman line versus carotenoid content. Resonance Raman measurements and high-performance liquid chromatography (HPLC) analyses on seven human maculae were obtained as described in the Materials and Methods section. Illumination conditions were 488 nm, 2 mW, 4-mm spot size, 10 seconds. Plot demonstrates linear correlation between the two methods (R = 0.94). Single Raman measurements were made on each sample to minimize the possibility of pigment bleaching. Typical reproducibility of Raman intensity measurements and of carotenoid HPLC analysis is better than ±5%.

Raman signal strength and actual macular carotenoid levels as determined by HPLC (Fig. 6).

For the development of a Raman detection system useful for living humans, the light exposure of the retina has to be limited to a maximum permissible dose, which according to American National Standards Institute (ANSI) standard Z136.1 is 2.7 J/cm^2 (equivalent to a laser power density at the retina of 2.7 mW/mm^2 for 10 seconds).[20,21] The experiment described in Figure 6 in which the laser power density was 0.16 mW/mm^2 for 10 seconds shows that this is possible with a wide margin of safety. Repeated measurements on a single flat-mounted retina using light levels comparable to the ANSI standard showed that the Raman signal strength drops no more than 1 to 2% per measurement. HPLC analysis of this same retina showed no detectable generation of carotenoid photo-isomerization or photo-oxidation products. As future versions of our apparatus are developed, it is likely that the required light exposure to the retina could be reduced substantially through the use of holographic filters or monochromators with less dispersive optics and with higher light collection efficiencies. Moreover, for a clinical instrument it may not be necessary to scan such a large portion of the visible spectrum because photon counting at a few selected wavelengths may be all that is necessary to determine macular carotenoid levels with precision and accuracy.

After it had been established that strong Raman signals can be obtained from a postmortem human retina, a series of experiments were undertaken using living animal eyes as a model. Unfortunately, non-primate mammals accumulate only minuscule amounts of carotenoids in their retinas. The frog *Rana pipiens*, on the other hand, has been reported to accumulate large amounts of xanthophyll carotenoid esters in the RPE,[22] but their identities and amounts had never been established. Carotenoids were extracted from the frog retina/RPE/choroid and detected as a large peak at the chromatogram injection front that appeared to represent these xanthophyll esters (Fig. 7A). Saponification and HPLC analysis of the extract from the fellow eye (Fig. 7B) demonstrated a decrease of the peak at the injection front and the appearance of major peaks representing lutein (330 ng/eye) and zeaxanthin (240 ng/eye) (confirmed by diode-array spectra and by retention times identical with standards). In separate experiments, we found that more than 95% of the frog's carotenoid is in the RPE/choroid and that less than 5% is in the neural retina.

Carotenoid resonance Raman measurements were then performed on an intact eye of an immobilized and anesthetized dark-adapted frog *Rana pipiens*. A strong Raman signal specific for carotenoids was obtained, thus demonstrating that Raman measurements can be performed on whole eyes

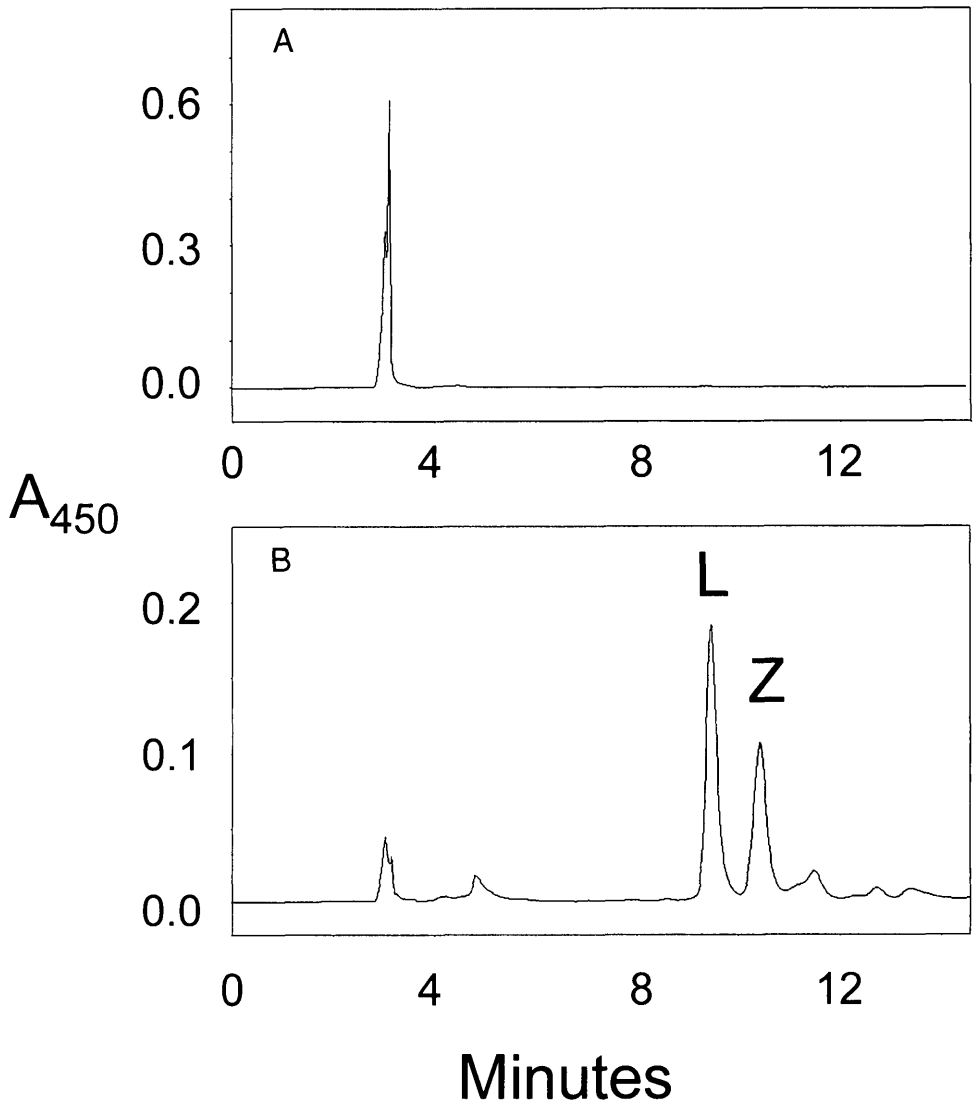

FIGURE 7. High-performance liquid chromatography (HPLC) analysis of carotenoids in frog retina/choroid/retinal pigment epithelium. Unsaponified (**A**) and saponified (**B**) extracts of *Rana pipiens* frog retina/RPE/choroid were prepared as described in the Materials and Methods section. The first HPLC trace is remarkable for a prominent peak at the injection front corresponding to the expected elution time for xanthophyll esters. Saponification of the extract from the fellow eye demonstrates a decrease in the size of the injection front peak in the second trace, and the appearance of two major peaks whose retention times and diode-array spectra correspond to lutein (L) and zeaxanthin (Z).

(Fig. 8). A weaker Raman signal was also observed close to the 1524-cm^{-1} carotenoid line, which disappeared after the eye was illuminated with a bright light for 60 seconds. The spectrally shifted position of this additional peak and the fact that it disappeared after the eye was adapted to light suggest that we may have measured a Raman signal from retinal rhodopsin or one of its bleaching intermediates, many of which are known to have strong resonance Raman peaks between 1545 cm^{-1} and 1569 cm^{-1} when stimulated with argon laser light.[23] Under light-adapted conditions, bleached opsin and unbound retinoids would be off-resonance to argon laser light, and their Raman signals would therefore be expected to be of very low intensity. It will be interesting to determine whether these findings can be replicated in living humans under light-adapted and dark-adapted conditions.

In conclusion, we showed that Raman scattering has the potential to be an outstanding new tool for the noninvasive measurement of macular carotenoids and other Raman active compounds in the intact human retina. It is remarkable that strong Raman signals with good signal-to-noise ratio can be obtained from the most light-sensitive area of the human retina at surprisingly low laser intensities that fall within safety standards. Because there is accumulating evidence that increased macular carotenoid levels may be protective against visual loss from AMD, this technique may prove to be important in assessing risk of visual loss in patients prone to develop AMD. Those patients

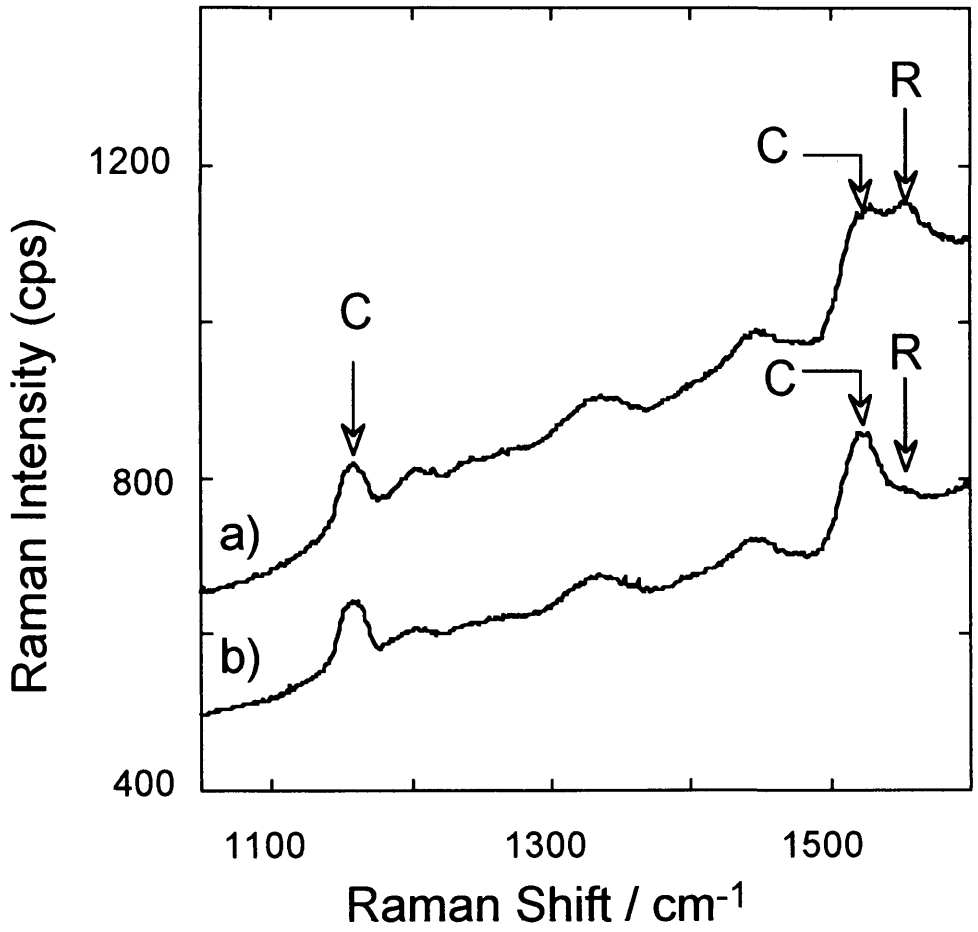

FIGURE 8. Resonance Raman spectra obtained from an intact frog eye. Resonance Raman spectra were obtained from the eye of a *Rana pipiens* frog dark-adapted overnight (*trace a*) and after exposure of the same eye to 60 seconds of bright light (*trace b*). Laser illumination conditions for carotenoid resonance Raman spectroscopy were 514.5 nm, 10 mW, 3-mm spot size, 60 seconds. C, carotenoid Raman peak; R, probable rhodopsin Raman peak. Note that R was no longer present after the eye had been illuminated by bright light.

found at risk could attempt to raise their macular carotenoid levels through dietary modification or nutritional supplementation, and their response to intervention could be monitored using the same device. A prototype Raman apparatus for use on living human eyes is currently under development.

Acknowledgments

The authors thank the Utah Lions Eye Bank for supplying postmortem human eyes and Olga A. Katz and Garth Muir for technical assistance. Lutein was a gift from Kemin Industries, Des Moines, IA, and zeaxanthin was a gift from Hoffmann-La Roche, Basel, Switzerland.

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