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Abstract
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Keywords
Fluorescence intensities, Neurological disorders, Noninvasive examination, Spectroscopic signatures, Transmissible spongiform encephalopathies, bovine spongiform encephalopathy, pathology, pathophysiology, retina, encephalopathy, scrapie

Disciplines
Chemistry | Veterinary Pathology and Pathobiology | Veterinary Physiology

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The feasibility of exploiting fluorescence spectra of the eye for diagnosis of transmissible spongiform encephalopathies (TSEs) was examined. Retinas from scrapie-positive sheep were compared with scrapie-negative sheep using fluorescence spectroscopy, and distinct differences in the fluorescence intensity and spectroscopic signatures were observed. The characteristic fluorescent signatures are thought to be the result of an accumulation of lipofuscin in the retina. It appears that the eye, in particular the retina, is a useful tissue for noninvasive examination of some neurological pathologies such as scrapie. The development of procedures based on examinations of the eye that permit the detection of neurological disorders in animals holds great promise.

Neurological disorders of the central and peripheral nervous system are caused by a disease, trauma, or injury to the nervous system. They can be difficult to treat and often are debilitating. Transmissible spongiform encephalopathies (TSEs) are one of many other well-known neurological disorders such as Parkinson's disease, Alzheimer's disease, Huntington's disease, systematic amyloidosis, and maturity-onset diabetes that involve accumulation of extracellular aggregates leading to tissue damage and disease.[1−4] TSEs are slowly progressive, insidiously degenerative diseases that affect the central nervous system (CNS) of both humans and animals and are usually accompanied by the production of "spongiform" changes in the brain. TSEs are believed to be transmitted by abnormal proteins, which are resistant to enzymatic degradation, called prions and in the case of scrapie, designated as PrP^{sc}.[5]

The TSE of most concern for the food supply is bovine spongiform encephalopathy (BSE), a fatal neurodegenerative transmissible disease in cattle. It is thought to be associated with variant Creutzfeldt−Jakob disease (vCJD) in humans.[6] The oral route of infection is considered to be the most probable path for transmission of BSE to humans.[7,8] In order to reduce the risk of human exposure, specified risk material (SRM, e.g., brain and spinal cord) from cattle is removed during slaughter and processing. Prohibition of SRM in the human food chain is considered of critical importance for protection of consumers from BSE.[9,10] Regulations regarding SRM have been promulgated by the European Commission (Annex V Commission Regulation (EC) No. 999/2001) and for the United States by the Food Safety and Inspection Service (99 CFR 310.22). Many other countries have also banned bovine CNS tissues from meat products, and its presence is cause for import rejection and international trade disputes. Developing technology for monitoring CNS tissue in meat and other food products as well as for diagnosing animals for TSEs will be increasingly important in securing the safety of the world's food supply.

Fluorescence spectroscopy has been commonly used in a variety of biological applications, and its feasibility for the detection of fecal contamination by exploiting fluorescent chlorophyll metabolites on meat during slaughter has been previously reported.[11] Instruments developed for this application are currently being used to screen carcasses in real time at beef processing plants in the United States and France. A similar approach can be applied to CNS detection by exploiting lipofuscin, a highly fluorescent, heterogeneous, high-molecular weight material that has been shown to accumulate in high concentrations in neural

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(1) Kelly, J. W. *Structure* **1997**, *5*, 595–600.

(2) Tan, S. Y.; Pepys, M. B.; Hawkins, P. N. *Am. J. Kidney Dis.* **1995**, *26*, 267–285.

(3) Sunde, M.; Serpell, L. C.; Bartlam, M.; Fraser, P. E.; Pepys, M. B.; Blake, C. C. F. *J. Mol. Biol.* **1997**, *273*, 729–739.

(4) Conway, K. A.; Harper, J. D.; Lansbury, P. T., Jr. *Biochemistry* **2000**, *39*, 2552–2563.

(5) Prusiner, S. B. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 13363–13383.

(6) Ironside, J. W. *Brain Pathol.* **1996**, *6*, 379–388.

(7) Cousens, S.; Smith, P. G.; Ward, H.; Everington, D.; Knight, R. S.; Zeidler, M.; Stewart, G.; Smith-Bathgate, E. A.; Macleod, M. A.; Mackenzie, J.; Will, R. G. *Lancet* **2001**, *357*, 1002–1007.

(8) Comer, P. J.; Huntly, P. J. *Stat. Methods Med. Res.* **2003**, *12*, 279–291.

(9) E.F.S.A. *EFSA J.* **2005**, *307*, 1−135.

(10) Harvard Center of Risk Analysis, and Harvard School of Public Health, and Center of Computational Epidemiology, College of Veterinary Medicine, Tuskegee University. Access at http://www.fsis.usda.gov/Science/Risk_Assessments/.

(11) Ashby, K. D.; Wen, J.; Chowdhury, P.; Casey, T. A.; Rasmussen, M. A.; Petrich, J. W. *J. Agric. Food Chem.* **2003**, *51*, 3502–3507.

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tissues.[12−17] Our previous work has shown that brain and spinal cord samples display highly conserved spectral characteristics, and these characteristics have potential for use in meat processing applications.[18−20] We have investigated the spectra and spectral intensities of the two most important SRM tissues (brain and spinal cord) and compared the data to non-CNS tissues. On the basis of a study of the spectral signatures of CNS tissue, we have previously suggested the development of devices for detecting CNS tissue in meat products by the rapid monitoring of fluorescence spectra.[19] It is known that the amount of fluorescent lipofuscin in CNS tissue increases as a result of spontaneous and experimentally induced Creutzfeldt−Jakob's disease (CJD). Boellaard et al. have demonstrated a relationship between lipofuscin production, a decline in autophagocytosis activity, and the experimental induction of CJD in mice.[13] Furthermore, there is substantial documentation linking neurological disease produced by TSEs to eye damage and the accumulation of lipofuscin.[13,21−28]

In this work, we investigate the use of fluorescence spectroscopy of the retina to identify scrapie, which we use as a model for TSEs. Scrapie is the most widespread TSE affecting sheep and goats worldwide. It is characterized by a gradual onset; and advanced cases show typical disease characteristics including unthriftiness, compulsive itching, balance and ambulatory abnormalities, convulsions, and eventual death. The disease has been observed for centuries. At present, it is incurable and the most common form of control is quarantine, euthanasia, and proper disposal of the carcass. Scrapie is not considered to be infectious to humans.

While several research groups are actively involved in developing spectral examinations of the eye to assess the extent of macular degeneration and other abnormalities,[27−35] to our knowledge, few have reported on the use of the retinal scans for this particular pathological application.[25,26,36,37] The objective of this work is to find spectral signatures of intrinsic fluorescent markers of eye that can provide a nonlethal and noninvasive means of determining if a living animal is infected with a TSE. Our experiments were designed to address the following questions: (1) Can spectra obtained from the eye be used for identification of neurological disease? (2) Can the effects of neurological disease observed in the eye be distinguished from those associated with normal aging? (3) Can images of the eye be obtained that report on neurological disease? With these questions in mind we have now extended previous work and investigated fluorescence spectroscopy of the retina for potential diagnosis of neurological disease. Our investigations, which have focused on scrapie-positive and scrapie-negative sheep, suggest that the retina is a most promising part of the eye for revealing spectroscopic signatures indicative of neurological disease.

MATERIALS AND METHODS

Materials. DAKO Target Retrieval Solution and biotinylated antimouse IgG (made in horse) were obtained from DAKO Corp., Carpinteria, CA, and Vector Laboratories, Burlingame, CA, respectively. A Basic Alkaline Phosphatase Red Detection Kit and NexES IHC modules were acquired from Ventana Medical Systems, Inc., Tucson, AZ. All other chemicals used for pathological procedures were obtained from Sigma Aldrich.

Animal Tissue Samples. Tissue samples from both scrapie-positive and scrapie-negative sheep were obtained from several sources. Samples from 73 sheep were collected for the study for a total of 140 eyeballs. In some cases only one eye was collected from the animals sampled. Both positive and negative tissue samples were harvested according to a standardized necropsy procedures from sheep identified through the National Scrapie Surveillance Plan administered by the Animal and Plant Health Inspection Service (APHIS), United States Department of Agriculture (USDA).[38] Samples were also obtained from the Caine Veterinary Teaching Center, University of Idaho, Caldwell, ID. The identity of individual producers if known has been withheld. Scrapie-negative tissue samples were also obtained locally from sheep at the National Animal Disease Center (NADC), Ames, IA. All procedures relating to the care and experimental use of live sheep used on site at NADC were approved by the NADC Institutional Animal Care and Use Committee. Information regarding breed, age, and sex was also obtained for each tissue sample used in this study (see Table S-1 in the Supporting Information).

Disease Diagnosis. All animals were diagnosed for scrapie infection status using conventional pathological methods. To

(12) Yin, D. *Free Radical Biol. Med.* **1996**, *21*, 871–888.
(13) Boellaard, J. W.; Schlote, W.; Tateishi, J. *Acta Neuropathol.* **1989**, *78*, 410–418.
(14) Terman, A.; Brunk, U. T. *Acta Pathol., Microbiol., Immunol.* **1998**, *106*, 265–276.
(15) Tsuchida, M.; Miura, T.; Aibara, K. *Chem. Phys. Lipids* **1987**, *44*, 297–325.
(16) Curtis, H. J. *Biological Mechanisms of Aging*; Charles C. Thomas: Springfield, IL, 1966.
(17) Strehler, B. L. *Time, Cells, and Aging*; Academic Press: New York, 1977.
(18) Chowdhury, P. K.; Halder, M.; Choudhury, P. K.; Kraus, G. A.; Desai, M. J.; Armstrong, D. W.; Casey, T. A.; Rasmussen, M. A.; Petrich, J. W. *Photochem. Photobiol.* **2004**, *79*, 21–25.
(19) Schönenbrücher, H.; Adhikary, R.; Mukherjee, P.; Casey, T. A.; Rasmussen, M. A.; Maistrovich, F. D.; Hamir, A. N.; Kehrli, M. J.; Richt, J.; Petrich, J. W. *J. Agric. Food Chem.* **2008**, *56*, 6220–6226.
(20) Adhikary, R.; Schönenbrücher, H.; Rasmussen, M. A.; Casey, T. A.; Hamir, A. N.; Kehrli, M. E.; Richt, J. A.; Petrich, J. W. *Photochem. Photobiol.* **2009**, *85*, 1322–1326.
(21) Foncin, J. F.; Gaches, J.; Le Beau, J. *Rev. Neurol. (Paris)* **1964**, *111*, 507–515.
(22) Jacobson, S.; Koenig, H.; Ross, E. *J. Neuropathol. Exp. Neurol.* **1967**, *22*, 152–153.
(23) Kirschbaum, W. R. *Jakob-Creutzfeldt Disease*; Elsevier: New York, 1968.
(24) Miyashita, M.; Stierstorfer, B.; Schmahl, W. *J. Vet. Med. B* **2004**, *51*, 209–215.
(25) Smith, J. D.; Greenlee, J. J.; Hamir, A. N.; Richt, J. A.; Greenlee, M. H. W. *Vet. Pathol.* **2009**, *46*, 810–818.
(26) Smith, J. D.; Greenlee, J. J.; Hamir, A. N.; Greenlee, M. H. W. *Vet. Rec.* **2009**, *165*, 179–181.
(27) Hortells, P.; Monzon, M.; Monleon, E.; Acin, C.; Vargas, A.; Bolea, R.; Lujan, L.; Badiola, J. J. *Brain Res.* **2006**, *1108*, 188–194.
(28) Rubenstein, R.; Gray, P. C.; Wehlburg, C. M.; Wagner, J. S.; Tisone, G. C. *Biochem. Biophys. Res. Commun.* **1998**, *246*, 100–106.
(29) Holz, F. G.; Bellman, C.; Staudt, S.; Schutt, F.; Volcker, H. E. *Invest. Ophthalmol. Visual Sci.* **2001**, *42*, 1051–1056.
(30) Smith, R. T.; Nagasaki, T.; Sparrow, J. R.; Barbazetto, I.; Koniarek, J. P.; Bickmann, L. J. *J. Biomed. Opt.* **2004**, *9*, 162–172.
(31) Delori, F. C. *Arch. Biochem. Biophys.* **2004**, *430*, 156–162.
(32) Schweitzer, D.; Hammer, M.; Schweitzer, F.; Anders, R.; Doebbecke, T.; Schenke, S.; Gaillard, E. R. *J. Biomed. Opt.* **2004**, *9*, 1214–1222.
(33) Schmitz-Valckenberg, S.; Holz, F. G.; Bird, A. C.; Spaide, R. F. *Retina* **2008**, *28*, 385–409.
(34) Surguchev, A.; Surguchov, A. *Brain Res. Bull.* **2010**, *81*, 12–14.
(35) Smith, R. T.; Gomes Nuno, L.; Barile, G.; Busuioc, M.; Lee, N.; Laine, A. *Invest. Ophthalmol. Visual Sci.* **2009**, *50*, 3907–3914.
(36) Smith, J. D.; Greenlee, J. J.; Hamir, A. N.; West Greenlee, M. H. *J. Comp. Pathol.* **2008**, *138*, 12–22.
(37) Greenlee, J. J.; Hamir, A. N.; Greenlee, M. H. W. *Vet. Pathol.* **2006**, *43*, 733–739.
(38) National Scrapie Surveillance Plan, APHIS, USDA. 2008, access at http://www.aphis.usda.gov/vs/nahss/sheep/national_scrapie_surveillance_plan_08192008.pdf.

obtain brain tissue, the disarticulated heads of sheep were split near the sagittal midline with a bandsaw. Each half of the brain was carefully removed from the calvarium and one of the halves was immersion fixed in 10% neutral buffered formalin for 2−3 weeks. The formalin-fixed brain was cut into 2−4 mm wide coronal sections at four levels: (1) brainstem at the obex, (2) cerebellum at midlevel lateral lobe, (3) midbrain at the rostral colliculus, and (4) hippocampus and adjacent cerebral cortex. The tissue blocks were placed in histopathology cassettes, dehydrated, and embedded in paraffin wax. Paraffin-embedded tissues were sectioned to 5 μm thickness, affixed to charged glass slides, and stained with hematoxylin and eosin (HE) for examination by light microscopy or left unstained for immunohistochemistry (IHC) processing.

An automated IHC method for detection of infectious prion protein (PrP^{sc}) was used as described previously.[39] In brief detail, following deparaffinization in xylene and rehydration through gradations of alcohol to water, tissue sections were autoclaved (121 °C, 1.38×10^5 Pa) for 30 min in an antigen retrieval solution and labeled with an indirect avidin−biotin system designed for an automated immunostainer. The primary antibody was a cocktail of two monoclonal antibodies, F89/160.1.5[40] and F99/97.6.1,[41] each used at a concentration of 5 μg/mL, and incubation was carried out at 37 °C for 32 min. The secondary antibody was biotinylated antimouse, diluted 1:200. The preparation was incubated for 8 min at 37 °C.

Sample Preparation. The eyeballs collected were stored frozen until use. After thawing, the eyeballs were dissected; and the retina or other eye tissue was removed and placed on a plain glass microscope slide (25 mm × 75 mm × 1 mm). The retina, in particular, was oriented as a thin layer on the microscope slide enabling the entire area of the sample to be exposed to the excitation light.

Steady-State Measurements. Steady-state fluorescence spectra were obtained on a SPEX Fluoromax-2 (ISA Jobin-Yvon/SPEX, Edison, NJ) with a 5 nm band-pass unless otherwise specified and corrected for lamp spectral intensity and detector response. Fluorescence spectra were collected in the front-faced orientation. For the experiments reported in this study, the samples were excited at $\lambda_{ex} = 470$ nm with an interference filter on the excitation side, and emission was collected at $\lambda_{em} \geq 505$ nm using a cutoff filter before the detector to eliminate scattered light. Polarized fluorescence spectra were obtained using two polarizers, with one on the excitation side and another on the emission side with the appropriate excitation interference and cutoff emission filters. Fluorescence spectra were obtained using horizontal−horizontal (HH) and horizontal−vertical (HV) orientations of the polarizers.

Hyperspectral Fluorescence Imaging Microscopy. The hyperspectral images were captured on a system located in the Roy J. Carver Laboratory for Ultrahigh Resolution Biological Microscopy, Iowa Sate University. This system was based on a NIKON ECLIPSE TE 2000-E microscope. The illumination source was an X-Cite 120 PC from EXFO. Samples were excited at 470 nm using an interference filter, and fluorescence was collected at ≥500 nm with a long-pass filter and a Nikon Plan Fluor 10×/0.30 PH1-DL objective. The collected fluorescence was dispersed using a Spectral-DV spectrometer from Optical Insights and captured by a Photometrics Cascade 512 B CCD camera (Roper Scientific). The software used for image capture was Mélange V3.7.

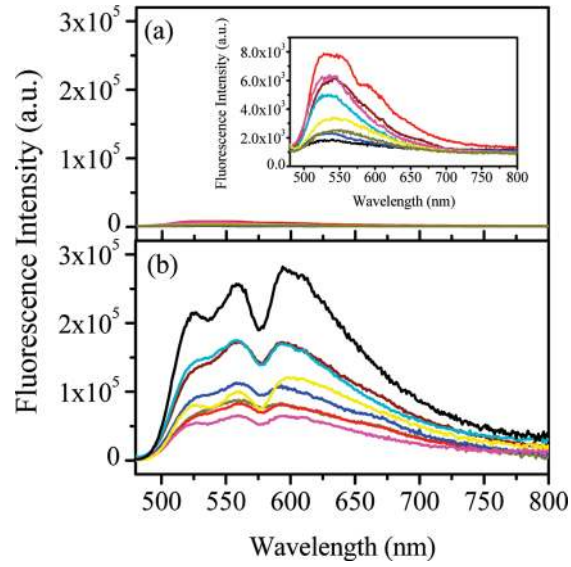

Figure 1. Fluorescence spectra of the retina from scrapie-negative and scrapie-positive sheep. Comparison of (a) scrapie-negative and (b) scrapie-positive sheep retinas at $\lambda_{ex} = 470$ nm. Representative, different, front-faced fluorescence spectra from eight individual retinas are shown for each scrapie-negative and scrapie-positive animal. Note that the intensity values are in the same range for both data sets on the primary graphs. The inset graph for the scrapie-negative data uses an expanded ordinate. Significant differences of fluorescent intensity exist between the two data sets.

RESULTS AND DISCUSSION

We studied a total of 140 sheep eyeballs from 73 animals obtained from several sources. A total of 35 of the animals were scrapie-positive and 38 of the animals were scrapie-negative. In the sheep identified as scrapie-negative, there was no evidence of spongiform change or other significant lesions, as assessed by microscopic examination of hemotoxylin and eosin stained brain sections. Likewise, there was no labeling indicative of PrP^{sc} seen in brain tissue sections examined following IHC (immunohistochemical) processing. Conversely, in sheep diagnosed as scrapie-positive, the observation of both lesions and IHC labeling confirmed a positive diagnosis for scrapie.

Fluorescence spectra from various parts of the sheep eye (cornea, iris, lens layer, lens gel, lens, vitreous humor, retina, tapetum, optic nerve, and sclera) were obtained at different excitation wavelengths using a fluorometer in a front-faced geometry. The retinas (and the sclera, see Figure S-1 in the Supporting Information) show large differences in spectral features when comparisons are made between scrapie-negative and scrapie-positive samples. The sclera is the protective tissue that covers the entire eyeball except the cornea. The retina is the preferred part of eye for noninvasive diagnosis of TSE affected animal because it can be directly accessed by light to excite the pigments, in contrast with the sclera. Front-faced fluorescence spectra of solid retina samples from scrapie-negative and scrapie-positive

(39) Hamir, A. N.; Kunkle, R. A.; Richt, J. A.; Miller, J. M.; Cutlip, R. C.; Jenny, A. L. *J. Vet. Diagn. Invest.* **2005**, *17*, 3–9.
(40) O'Rourke, K. I.; Baszler, T. V.; Miller, J. M.; Spraker, T. R.; Sadler-Riggleman, I.; Knowles, D. P. *J. Clin. Microbiol.* **1998**, *36*, 1750–1755.
(41) O'Rourke, K. I.; Baszler, T. V.; Besser, T. E.; Miller, J. M.; Cutlip, R. C.; Wells, G. A. H.; Ryder, S. J.; Parish, S. M.; Hamir, A. N.; Cockett, N. E.; Jenny, A.; Knowles, D. P. *J. Clin. Microbiol.* **2000**, *38*, 3254–3259.

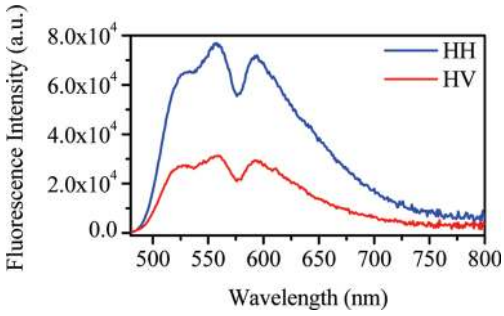

Figure 2. Polarized fluorescence spectra from a scrapie-positive sheep. Spectra were collected in a front-faced orientation. Samples were excited at 470 nm with an interference filter in the excitation light path, and fluorescence was collected after 505 nm with a long pass filter. In order to determine whether the observed structure is due to scattered light, the experiments were conducted with two polarizers in either of two positions: HH (horizontal excitation−horizontal emission) or HV (horizontal excitation−vertical emission). The data indicate that the spectral structure observed is authentic and not an artifact.

sheep excited at 470 nm are presented in Figure 1. The data indicate that fluorescence spectra from eyes (especially the retina) are very rich and vary considerably with excitation wavelength (λ_{ex}). When $\lambda_{ex} = 470$ nm, the fluorescence intensity of the scrapie-positive retinas is significantly greater than that of scrapie-negative sheep. The spectra from scrapie-positive samples were also more structured and displayed two intense peaks at ~560 and ~600 nm with a shoulder at ~525 nm. The scrapie-positive samples also displayed a minimum at ~575 nm. The literature indicates that TSE is caused by an accumulation of protease-resistant prions in central nervous system tissues such as brain, spinal cord, and eye.[5] It is also known that lipofuscin accumulation occurs in the diseased eye (e.g., in retinal pigment epithelium).[42−48] The spectral differences in scrapie-positive retinas compared to scrapie-negative retinas can be attributed to the result of altered or increased lipofuscin owing to prion infection.

Given the pronounced structural differences observed in the spectra of the scrapie-positive tissue, additional control experiments were undertaken to ensure that these data were not an experimental artifact, since spectra were collected from solid samples, which may have contributed to excessive light scattering. Although spectral structure was not pronounced in the scrapie-negative samples, it was necessary to determine that the results were independent of the polarization of the excitation radiation. The data in Figure 2 demonstrate that the spectra observed were,

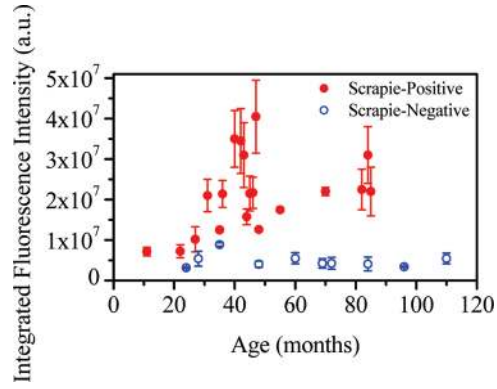

Figure 3. Plot of the integrated fluorescence intensity from scrapie-positive and scrapie-negative sheep retinas as a function of age. All points represent multiple spectroscopic determinations with standard error bars. Those with visible error bars are an average of 4−12 experimental replications. The remaining data points with error bars smaller than the symbol are with $N = 2$. Fluorescence spectra were collected as described in a front-faced orientation. For all experiments, samples were excited at 470 nm with an interference filter in the excitation light path, and fluorescence was collected at wavelengths greater than 505 nm to eliminate scattering. Note that the differences in total fluorescence due to disease status greatly exceed any differences that result from age.

indeed, independent of polarization. We suggest that these spectral differences are the result of neurological disease or in this case, scrapie.

It has been reported that the intracellular fluorescent pigment lipofuscin accumulates in the retina with increasing age.[49−51] It is important to distinguish the spectral differences arising from scrapie with respect to those arising from normal aging. A plot of integrated fluorescence intensity obtained from both scrapie-positive and scrapie-negative sheep retinas as a function of age is shown in Figure 3. Samples were obtained from sheep up to 9 years of age (see Table S-1 in the Supporting Information). The data indicate clear differences, with the scrapie-positive retinas being more fluorescent than those from the scrapie-negative retinas. The differences in total fluorescence due to disease status greatly exceed any differences that result from other factors, such as age.

In addition, hyperspectral fluorescence microscopy was used to compare images of retinas with respect to disease status. Representative images from different regions of sex- and age-matched retinas for scrapie-negative and scrapie-positive sheep are displayed in Figure 4. The scrapie-positive retina shows significantly higher fluorescence intensity as opposed to the scrapie-negative retina, as indicated by the grayscale intensity gradient of the images. This result is also consistent with the data presented in Figures 1 and 3 indicating that the scrapie-positive retinas are more fluorescent than those of scrapie-negative retinas. The scrapie-positive images also contain aggregates of fluorescent material, as indicated by the arrows in Figure 4 and are more heterogeneously fluorescent. In contrast, the scrapie-negative

(42) Gaillard, E. R.; Atherton, S. J.; Eldred, G.; Dillon, J. *Photochem. Photobiol.* **1995**, *61*, 448–453.
(43) Docchio, F.; Boulton, M.; Cubeddu, R.; Ramponi, R.; Barker, P. D. *Photochem. Photobiol.* **1991**, *54*, 247–253.
(44) Dillon, J.; Zheng, L.; Merriam, J. C.; Gaillard, E. R. *Exp. Eye Res.* **2004**, *79*, 753–759.
(45) Mata, N. L.; Weng, J.; Travis, G. H. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 7154–7159.
(46) Boulton, M.; Docchio, F.; Dayhaw-Barker, P.; Ramponi, R.; Cubeddu, R. *Vision Res.* **1990**, *30*, 1291–1303.
(47) Gaillard, E. R.; Zheng, L.; Merriam, J. C.; Dillon, J. *Invest. Ophthalmol. Visual Sci.* **2000**, *41*, 1454–1459.
(48) Glickman, R. D. In *Progress in Brain Research*; Ripps, K. H. H., Wu, S., Eds., 2001; pp 699−712.
(49) Wing, G. L.; Blanchard, G. C.; Weiter, J. J. *Invest. Ophthalmol. Visual Sci.* **1978**, *17*, 601–607.
(50) Delori, F. C.; Staurenghi, G.; Arend, O.; Dorey, C. K.; Goger, D. G.; Weiter, J. J. *Invest. Ophthalmol. Visual Sci.* **1995**, *36*, 2327–2331.
(51) Mukherjee, P.; Bose, S.; Hurd, A. A.; Adhikary, R.; Schonenbrucher, H.; Hamir, A. N.; Richt, J. A.; Casey, T. A.; Rasmussen, M. A.; Petrich, J. W. *Photochem. Photobiol.* **2009**, *85*, 234–238.

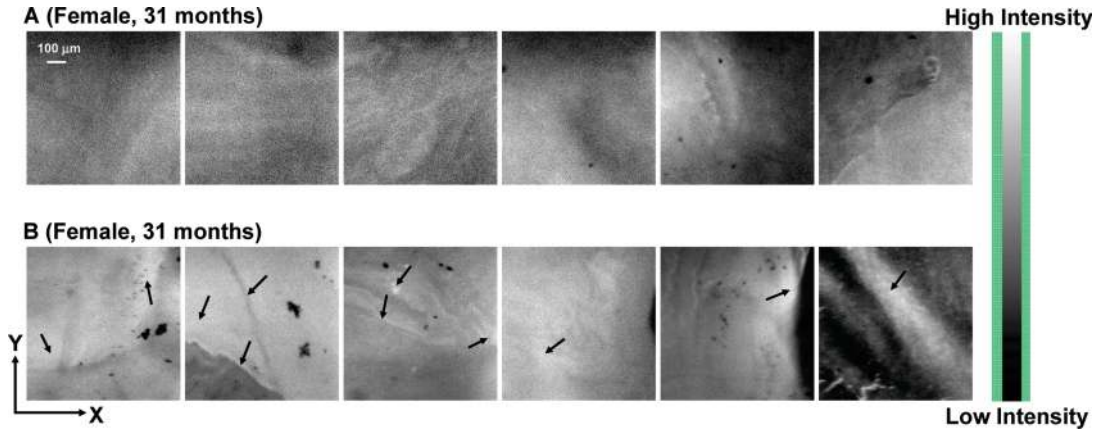

Figure 4. Hyperspectral fluorescence images of the retina of sheep. Representative images from different regions of sex- and age-matched (31 months) (A) scrapie-negative and (B) scrapie-positive sheep retinas. The samples were excited at 470 nm using an interference filter, and fluorescence was collected at wavelengths greater than 500 nm using a long-pass filter. The images shown are 800 μm by 800 μm. All images were captured under identical conditions. High intensity is represented by white; low intensity by black on a grayscale gradient. Note the more intense fluorescence and aggregation of fluorescent material in the scrapie-positive images.

tissue is more homogeneous and is weakly fluorescent. This further supports our observations from front-faced fluorescence spectra that scrapie-positive retinas show higher fluorescence with prominent structure. From this comparison of age-matched retinas, we conclude that the scrapie-positive retinas contain very bright regions of highly fluorescent material, which is suggestive of neurological damage as a result of disease.

Others have reported observations that support this current research. Recently Smith et al. have shown that the function and morphology of retinas are altered in TSE-infected cattle and sheep infected with scrapie.[25,26] Hortells et al. concluded that PrP^{sc} in the retina was highly correlated with the occurrence of scrapie[27] whereas Rubenstein et al. used ultraviolet fluorescent spectroscopy to detect different forms of PrP^{sc} in the eye.[28] Fundus autofluorescence has also been investigated for possible diagnostic use in a large number of retinal diseases[33,35] including the "conformational diseases" caused by accumulation of proteinaceous aggregates.[34] Autofluorescence has also been used to map lipofuscin distribution in the retina of humans.[33] In this study we have not determined the specific structures that are responsible for the fluorescence but others have suggested that lipofuscin and other macromolecular aggregates are responsible.[27,34]

CONCLUSIONS

Given our results, we suggest that the distinct differences in the spectral signatures of the retina are diagnostic of animals naturally infected with scrapie. We further suggest that the eye, and in particular the retina, will be a useful tissue for noninvasive determination of neurological pathologies such as scrapie.

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SUPPORTING INFORMATION AVAILABLE

Fluorescence spectra of sclera and additional information mentioned in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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