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Authors: Mochizuki, Yasuhiro, Park, Min Kyun, Mori, Takao, and Kawashima, Seiichiro

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The Difference in Autofluorescence Features of Lipofuscin between Brain and Adrenal

YASUHIRO MOCHIZUKI, MIN KYUN PARK, TAKAO MORI and SEIICHIRO KAWASHIMA

Zoological Institute, Graduate School of Science, University of Tokyo, Tokyo 113, Japan

ABSTRACT—Lipofuscin is the autofluorescent material, which accumulates with aging in the cells of various tissues. However, its autofluorescence characteristics are different among tissues. In the present study, the autofluorescence features of lipofuscin in the brain and adrenal were compared. In 18–21-month-old rats, the brain lipofuscin was granular and its autofluorescence was bright whitish-yellow to bright orange. On the contrary, the adrenal lipofuscin was not demarkated as granules, and its autofluorescence was subdued orange. The emission maximum of the bright whitish-yellow brain lipofuscin was 540 nm to 570 nm and that of the adrenal lipofuscin was 640 nm to 660 nm, when excited at 330 nm to 380 nm. When the spectra were drawn after correcting the wavelength-dependent bias of microspectrofluorometer, the autofluorescence spectra were consistent with microscopically observable tint. To conclude, the present results showed that the autofluorescence features of the bright whitish-yellow brain lipofuscin and the adrenal lipofuscin were quite different.

INTRODUCTION

The striking and common phenomenon in animal aging is the accumulation of lipofuscin in the cells. Lipofuscin is positive to neutral-lipid and acid-phosphatase stainings, and is thought to be derived from secondary lysosomes. The relation of lipofuscin with aging [3, 11, 14, 15, 28], drugs [16–18, 22, 27] and diets [4, 12, 19, 21, 29, 31] has been well documented. The most characteristic feature of lipofuscin is its autofluorescence. The chemical nature and structure of lipofuscin have not yet been well understood, but the autofluorescence has been regarded as the most reliable qualitative and quantitative marker of lipofuscin. Siakotos *et al.* [24] first partially isolated lipofuscin by extraction with chloroform : methanol (2 : 1) solution and measured autofluorescence spectrum by a spectrofluorophotometer. In their study, the excitation maximum was 360 nm and the emission spectrum peaked at 435 nm. Their extraction method and autofluorescence measurement were used by a number of investigators [1, 5, 6, 10, 26, 30]. From its autofluorescence characteristics, it was suggested that lipofuscin contained conjugated Schiff-base product in its structure [2].

However, there exists discrepancy that extracts of lipofuscin-loaded cells emit blue light, whereas under a fluorescence microscopy, lipofuscin emit yellow light. This discrepancy has not yet been elucidated. Therefore, Sohal and Brunk [25] suggested that the blue-emitting fluorescent material should not be termed "lipofuscin or lipofuscin-related or lipofuscin-derived". For this discrepancy, Eldred *et al.* [7] stated that the blue emission maximum might be resulted from instrumental bias and that in measuring over 500 nm with a spectrofluorophotometer, some special spectral corrections were needed. They reported that the corrected emission maximum of retinal pigment epithelium (RPE) lipofuscin showed a broad peak at 540 nm to 580 nm and was consistent with the autofluorescence of lipofuscin granules under a fluorescence microscope.

We observed several tissues under a fluorescence microscope, and realized that the autofluorescence of materials termed lipofuscin was different among tissues. Therefore, we attempted to obtain autofluorescnce spectra of lipofuscin without incorporating any discrepancy and verify the observable difference among tissues on the basis of corrected autofluorescence measurement.

The present paper briefly deals with the corrected autofluorescence spectra of lipofuscin in young and old rats, and the difference of autofluorescence characteristics of lipofuscin between the brain and adrenal will be discussed.

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MATERIALS AND METHODS

Young (4 months of age) and old (18–21 months of age) male rats of the Wistar/Tw strain maintained in this laboratory were used. They were housed in a temperature-controlled room under daily photoperiods of 12 hr-light and 12 hr-dark cycle, and were fed with laboratory chow (CA-1 : CE-7 = 1 : 3; Japan Clea Inc., Tokyo) and tap water ad libitum.

In each age group, three animals were killed by decapitation, and the brain and adrenals were quickly removed and fixed in 10% formaldehyde for a few days. The fixed tissues were embedded in paraffin. Sections were cut at 10-μm thick, deparaffinized in xylene (100%) and mounted using Entellan^{R} (E. Merck, Darmstadt)

Fluorescence microscopy and corrected microspectrofluorometric analysis of tissue lipofuscin were performed using a

Nikon fluorometric microscope system P102 (excitation filter; EX330-380, dichroic mirror; DM400, light source; USH-102DH 100W high pressure marcury lamp, pinhole-diaphragm; 0.2mm, object glass; x40) and monochromator G-70. All spectra were corrected using a program FSMS (version 4.00, Nikon corporation, Tokyo). Based on a calibrated tungsten lamp, FSMS is programed to correct the wavelength-dependent bias of instrument. Thus, the measured emission intensities were proportional to the amount of light quantum indepedent of the emission wavelength. In all cases, the pinhole-diaphragm was significantly smaller than the area of aimed autofluorescence source, and therefore no background autofluorescence was subtracted from the measured autofluorescence spectra. Arbitrary unit was a relative value but adjusted to be able to compare the intensity of each autofluorescence spectrum.

RESULTS

Fluorescence microscopy

Age-related accumulation of autofluorescent materials was evident in both the brain and adrenal. In the present study we designated the materials as lipofuscin in both the brain and adrenal.

In the brain of 4-month-old rats, some neurons contained a few lipofuscin granules but most neurons did not contain any. In 18–21-month-old rat brain, a huge number of lipofuscin granules were found in many neurons. Two autofluorescence types of lipofuscin were found in the brain; bright whitish-yellow type and bright orange type. For example, in the pons of old rats large neurons markedly accumulated bright whitish-yellow lipofuscin in the cytosol, whereas few lipofuscin granules were encountered in the cytosol of neurons of young rats (Fig. 1A, B), and in the cerebellum some Purkinje cells contained bright whitish-yellow lipofuscin and some contained bright orange one. However, in some brain regions such as the cerebrum and midbrain, it was very difficult to distinguish the neurons with bright whitish-yellow type from the bright orange one, indicating that there was no substantial difference between the two autofluorescence types. Even at old age, the neurons bearing no lipofuscin granules were encountered in all brain regions. The brain lipofuscin generally appeared granular.

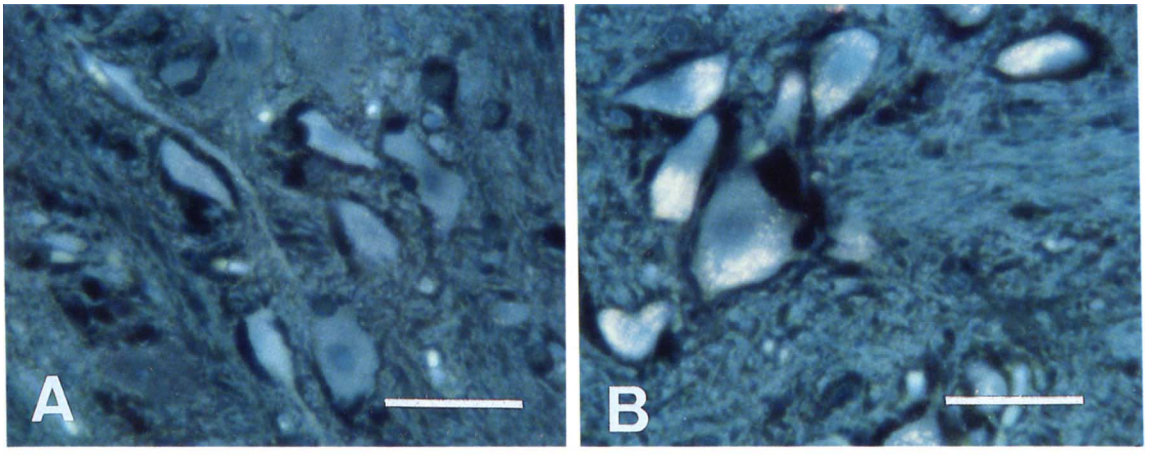

FIG. 1. Fluorescence photomicrographs of a part of the pons. A) 4-month-old rat. B) 18-month-old rat. The autofluorescence of lipofuscin in the neurons of the pons was bright whitish-yellow, but some lipofuscin granules appeared bright orange. Bar: 50 μm.

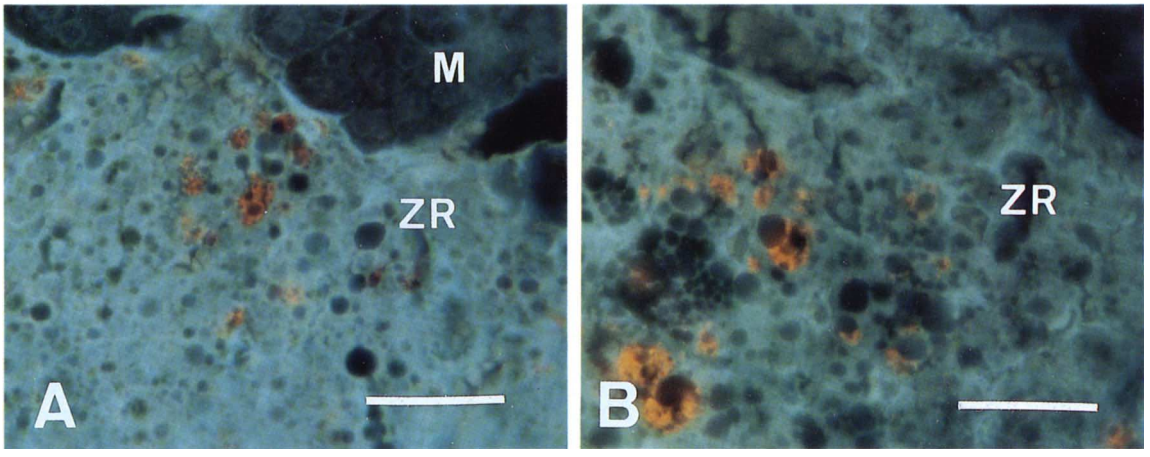

FIG. 2. Fluorescence photomicrographs of the zona reticularis of adrenal cortex. A) 4-month-old rat. B) 18-month-old rat. The autofluorescence of the adrenal lipofuscin was subdued orange. M: medulla, ZR: zona reticularis. Bar: 50 μm

In the adrenal of 4-month-old rats, some lipofuscin was present in the zona reticularis of the cortex, but no lipofuscin depositions were observed in other adrenal cortical zones and the medulla (Fig. 2A). In the adrenal of 18-21-month-old rats, the distribution of lipofuscin did not show any difference from that of young rats, and the size, rather than the number, of the orange cluster significantly increased with aging (Fig. 2B). In either age group, the adrenal lipofuscin was not granular in shape, unlike the brain lipofuscin and its autofluorescence was always subdued orange.

Corrected microspectrofluorometric analysis

In measuring the fluorescence, in order to preclude other fluorescence than lipofuscin, it is desirable that the pinhole-diaphragm is small enough and lipofuscin is dense and abundant. In the fluorescence microscopy, it was observed that the pons neurons of old rats markedly accumulated the bright whitish-yellow lipofuscin (Fig. 1B). Therefore, the pons was choosen as the sample for microspectrofluorometric analysis of the bright whitish-yellow lipofuscin of the brain. In the pons of 18–21-month-old rats, there were no neurons which vigorously accumulated bright orange lipofuscin enough to cover the whole scope of the pinhole-diaphragm. Thus, no microspectrofluorometric data were obtained on the bright orange brain lipofuscin. Typical corrected autofluorescence spectrum of the bright whitish-yellow brain lipofuscin is shown in Figure 3A. It was a broad and gently-sloping spectrum with no sharp peaks. The emission maximum was found at 540 nm to 570 nm, and shoulders were present at 480 nm to 520 nm and 620 nm to 660 nm, when excited at 330 nm to 380 nm (Fig. 3A and Table 1). The typical corrected autofluorescence spectrum of the cytosol of neurons in 4-month-old rats is shown in Figure 3B. The low fluorescence intensity in 4-month-old rats accorded well with the result of few lipofuscin granules in the cytosol shown in Figure 1A. The comparison of the two autofluorescence spectra made it clear that the autofluorescence spectrum between 480 nm to 660 nm covered the autofluorescence of the bright whitish-yellow brain lipofuscin.

Typical corrected autofluorescence spectrum of the adrenal lipofuscin in 18–21-month-old rats is shown in Figure 4A. It was also a broad and gently-sloping spectrum, but its

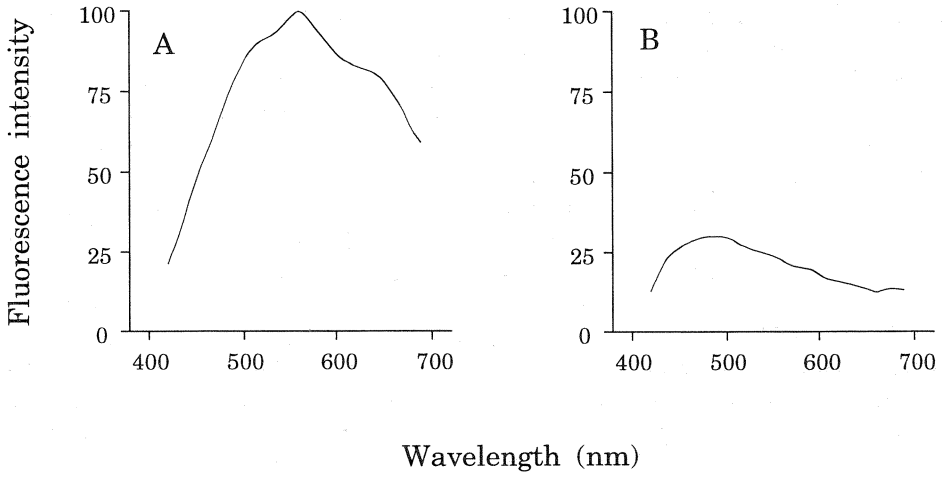

FIG. 3. Typical corrected autofluorescence spectra of the neurons of the pons. A) Brain lipofuscin in a 21-month-old rat. B) Cytosol of a neuron devoid of lipofuscin in a 4-month-old rat. These spectrum profiles were similar in other neurons. All autofluorescence spectra were measured using a Nikon fluorometric microscope system P102 and monochromater G-70, and corrected using the FSMS program. Excitation wavelength at 330 nm to 380 nm.

TABLE 1. Fluorescence characteristics of the brain and adrenal lipofuscin

	Age (month)	Excitation wavelength (nm)	Fluorescence	Emission maximum wavelength (nm)	Shoulders (nm)
Brain lipofuscin	18–21	330–380	bright whitish-yellow	540–570	480–520 620–660
			bright orange	ND	ND
Adrenal lipofuscin	4	330–380	subdued orange	640–660	530–580
	18–21			640–660	530–580

No data were presented on brain lipofuscin in 4-month-old rat, because in 4-month-old rat brain, no lipofuscin granules were found in the cytosol (Fig. 1A).

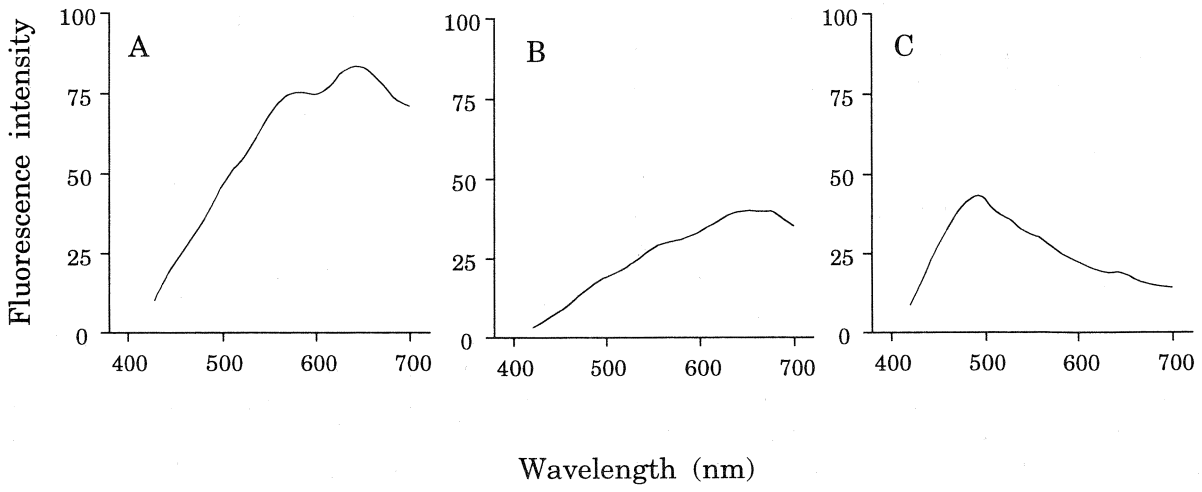

FIG. 4. Typical corrected autofluorescence spectra of the cells of zona reticularis of adrenal. A) Adrenal lipofuscin in an 18-month-old rat. B) Adrenal lipofuscin in a 4-month-old rat. C) Cytosol of adrenal cells devoid of lipofuscin in a 4-month-old rat. These spectrum profiles were similar in other cells. All autofluorescence spectra were measured using a Nikon fluorometric microscope system P102 and monochromater G-70, and corrected using the FSMS program. Excitation wavelength at 330 nm to 380 nm.

emission maximum was 640 nm to 660 nm, and a shoulder was present at 530 nm to 580 nm, when excited at 330 nm to 380 nm (Fig. 4A and Table 1). In the adrenal of 4-month-old rats, enough lipofuscin to measure was present (Fig. 2A), and the autofluorescence spectrum was measured (Fig. 4B). The spectrum pattern of the adrenal lipofuscin in 4-month-old rats was very similar to that in 18–21-month-old rats (Fig. 4A, B). The emission maximum was 640 nm to 660 nm, when excited at 330 nm to 380 nm, and a small shoulder was present at 530 nm to 580 nm (Table 1). However, the fluorescence intensity of the 4-month-old rats adrenal lipofuscin was weaker, and the peak and shoulder were more gently-sloping than those of the 18–21-month-old one. The typical corrected autofluorescence spectrum of the cytosol devoid of lipofuscin in the zona reticularis of 4-month-old rats is shown in Figure. 4C. The spectrum pattern was apparently different from those of the adrenal cells loaded with lipofuscin (Fig. 4A, B). Therefore, the autofluorescence spectrum between 530 nm to 660 nm was regarded as specific for the autofluorescence of the adrenal lipofuscin.

DISCUSSION

In the fluorescence microscopy, the brain lipofuscin and the adrenal lipofuscin appeared quite different. The brain lipofuscin was present in granular-shape and its autofluorescence was bright whitish-yellow to bright orange when excited with ultraviolet light, whereas the adrenal lipofuscin was not granular-shape and emitted only subdued orange. However, in electron microscopic observation, it was reported that adrenal cytosolic lipofuscin was present as granules [13, 20], suggesting that the subdued orange cluster may be composed of fine granules. The autofluorescence spectra of the bright whitish-yellow brain lipofuscin and the adrenal lipofuscin were quite different and neither of these autofluorescence characteristics was identical to those for RPE lipofuscin so far reported [7]. In the present study, the analysis of autofluorescence spectra of the bright whitish-yellow brain lipofuscin and the adrenal lipofuscin after correcting the wavelength-dependent bias of instrument showed that there were three major autofluorescence groups which feature the autofluorescence of these lipofuscin; 480 nm to 520 nm emitters (blue to green range), 540 nm to 570 nm emitters (yellow range) and 640 nm to 660 nm emitters (red range) (Table 1). The bright whitish-yellow brain lipofuscin contained all the three major groups. However, 540 nm to 570 nm emitters were dominant which yields autofluorescence tint of bright whitish-yellow. On the contrary, the autofluorescence of the adrenal lipofuscin was composed of 540 nm to 570 nm and 640 nm to 660 nm emitters, but the proportion of 640 nm to 660 nm emitters were dominant and 540 nm to 570 nm emitters were minor, yielding autofluorescence tint of subdued orange. These results indicate that the difference in the ratio of three major autofluorescence groups causes the difference between bright whitish-yellow for the brain lipofuscin and subdued orange for the adrenal lipofuscin.

Recently, Eldred and Katz [8] analyzed RPE lipofuscin with thin layer chromatography and corrected spectrofluorometry, and reported that RPE lipofuscin was composed of four color emitters; green emitters, yellow-green emitters, golden yellow emitters and orange-red emitters. By comparing the autofluorescence characteristics among the bright whitish-yellow brain lipofuscin, the adrenal lipofuscin and the RPE lipofuscin, it may be suggested that 480 nm to 520 nm emitters are identical with green emitters, 540 nm to 570 nm emitters are identical with yellow-green emitters, and 640 nm to 660 nm emitters are close to orange-red emitters. Based on the comparison, it may further be concluded that lipofuscin is composed of a few major autofluorescence groups and the autofluorescence features of lipofuscin is the

outcome of different ratio in the content of various autofluorescence groups.

The chemical composition of lipofuscin has not yet been defined. Some authors reported that the autofluorescence around 520 nm was not derived from lipofuscin *per se* but flavin [2, 8, 11]. In the present measurement on the autofluorescence of the part of cytosol where lipofuscin granules were packed, we observed autofluorescence around 520 nm. Therefore, it is probable that lipofuscin granules contain flavin as their component.

There were some reports on uncorrected autofluorescence spectra of lipofuscin using a microspectrofluorometer [21, 23]. Its emission maximum was around 500 nm and there were no peaks over 500 nm, and this was not consistent with the autofluorescence tint observed under the fluorescence microscope. In the present study, the corrected autofluorescence spectra were consistent with the autofluorescence under the fluorescence microscope.

Taking all these findings into consideration, it is suggested that instrumental bias must be corrected in order to obtain true autofluorescence spectra of lipofuscin. The true autofluorescence characteristics of the brain and adrenal lipofuscin were quite different.

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