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SHORT COMMUNICATION

"Triple Observation Method (TOM)" to Discriminate Optically Autofluorescence from Porphyrins Versus that from Copper-Metallothioneins

Kenji Nakayama · Yoshinobu Katoh · Mamoru Tamura

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Abstract We propose a conclusive difference observed between the excitation conditions required to observe porphyrins and copper-metallothioneins in cells and/or tissues using an ordinary fluorescence microscope. We have emphasized the importance of examining the spectral properties of the emissions to avoid any serious mistakes such as confusing porphyrins with copper-metallothioneins in the liver and kidneys. However, microspectrophotometry is not a conventional method for either histochemical, cytochemical, or pathological studies because microspectrophotometers are both expensive and difficult to operate. Therefore, we demonstrate a simple comparative method using ordinary excitation filter arrangements. When using our technique, it becomes possible to optically discriminate more accurately between the autofluorescence properties arising from porphyrins and those arising from copper-metallothioneins. We would like to name our simple technique "Triple Observation Method (TOM)".

K. Nakayama (✉)
Department of Health and Environmental Science,
Hokkaido Institute of Public Health,
North 19 West 12, Kita-ku,
Sapporo 060-0819 Hokkaido, Japan
e-mail: ken_naka@iph.pref.hokkaido.jp

Y. Katoh
Department of Biological Science,
Hokkaido Institute of Public Health,
Sapporo 060-0819 Hokkaido, Japan
e-mail: kyoshi@iph.pref.hokkaido.jp

M. Tamura
Rm C111, School of Medicine, Tsinghua University,
Haidian District, Beijing 100084, China
e-mail: mtamura@mail.sci.hokudai.ac.jp

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Introduction

Analytical techniques based on autofluorescence monitoring can be utilized in order to obtain information concerning the morphological and physiological conditions of cells and/or tissues. Moreover, autofluorescence analyses can be performed in real time because they do not require any treatment with fixing or staining of the specimens. In the last decade, fluorescence spectroscopy and imaging techniques have been developed to be utilized both in basic research and clinical diagnoses. Therefore, the useful and convenient signals emitted by endogenous fluorophores have been revealed by spectroscopy and imaging. These molecules are often related to fundamental biological processes in cells and/or tissues. They are significant parameters for tracing the states in cells and/or tissues. Consequently, analytical parameters based on autofluorescence monitoring have great potentiality both in research and diagnostics, and the interest in applying these new analytical tools both in vitro and in vivo is growing [1].

The detection of autofluorescence in the liver is more important than in other organs. This is because the many colors of autofluorescence have been reported in the liver. The endogenous fluorophores in hepatocytes include lipopigments, matrix proteins such as collagen and elastin, reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavins, bile constituents, and vitamin A. Among them, the red-orange light arising from porphyrins is well known. There are many reports concerning the red-

orange autofluorescence in the hepatocytes with liver disease, such as hepatitis, liver cirrhosis, porphyria cutanea tarda and especially hepatocellular carcinoma. However, unfortunately, we cannot now see the precious color photographs of the red-orange autofluorescence from porphyrins in those livers because most reports were published from the 1950s to the 1980s [2–8].

Another important endogenous fluorophore is yellow-orange light arising from copper-metallothioneins (Cu-MTs) in the liver and kidneys. MTs are low-molecular-weight proteins, which bind unusually high amounts of heavy metal ions such as Cu, zinc, cadmium and mercury [9, 10]. Cu-MTs, in which minutely cuprous [Cu(I) or Cu^+] ions are chelated by thiolate groups, emit visible yellow-orange light at around 600 nm when illuminated with UV light, around the 300 nm region at room temperature [11–13]. It is generally found that Cu(I)-MTs isolated from mammals, fungus and yeast are autofluorescent [13]. Using this unique property of Cu(I)-MTs, the localizations of these proteins were visualized in both the livers [14] and the male kidneys [15] of Long-Evans Cinnamon (LEC) rats. The LEC rat is an animal model for Wilson's disease (WND) because these animals exhibit many clinical, biochemical and molecular-biological features similar to those occurring in the human disorder [16]. The two articles cited earlier concerning the autofluorescence of Cu(I)-MTs were the first reports of the direct observations of the lights from Cu(I)-MTs in the hepatocytes and the male renal cells, respectively [14, 15]. In the LEC rats, the yellow-orange emission in the livers and the red-orange emission in the male kidneys were identified as specific autofluorescence from Cu(I)-MTs by several quenching tests, as well as histochemical, biochemical and immunocytochemical confirmations. From those examinations, both emissions of the LEC rat were believed to be the autofluorescence arising from Cu(I)-MTs.

However, in our report on fluorescence microspectrophotometric analyses of these emissions in the male renal sections [17], we confirmed that the endogenous fluorophores emitting red-orange light in the male LEC rat kidneys are porphyrins, not Cu(I)-MTs. The male renal red-orange emission suggested an abnormal accumulation of highly carboxylated porphyrins. This evidence indicated that the quenching tests, as well as histochemical, biochemical and immunocytochemical confirmations for the emission from Cu(I)-MTs, could not differentiate between autofluorescence from Cu(I)-MTs and porphyrins which were abnormally accumulated at the same time. Therefore, we demonstrated the necessity of using microspectrophotometry for the differentiation of autofluorescence from porphyrins and Cu(I)-MTs to examine the spectral properties of these emissions [17–19].

Recently, Quaglia et al. published a report concerning the autofluorescence of Cu-MT in human hepatocytes [20]. They detected the bright red-orange autofluorescence in the granules in human liver tissues from characterized patients with WND or chronic cholestasis and suggested the autofluorescence arose from Cu-MTs. However, their filter arrangement using a 420±30 nm excitation filter of a fluorescence microscope was suitable for the observation in the autofluorescence arising from porphyrins rather than Cu-MTs. This is because the excitation maxima of porphyrins and Cu-MTs are located at around 405 nm [3–5, 7, 21, 22] and around 300 nm [11–13], respectively. Therefore, they discovered an abnormal accumulation of porphyrins in those human liver tissues. We have concluded that their report was a typical mistake in confusing porphyrins with Cu(I)-MTs. Therefore, we demonstrated the necessity of using microspectrophotometry for the differentiation of autofluorescence from porphyrins and Cu(I)-MTs to examine the spectral properties of the emissions [17–19].

Unfortunately, microspectrophotometry is not a conventional method for either histochemical, cytochemical, or pathological studies because microspectrophotometers are both expensive and difficult to operate. Therefore, a simple differentiation method using an ordinary fluorescence microscope is needed in order to discriminate optically between autofluorescence from porphyrins and that from Cu(I)-MTs.

In this short communication, we propose a simple method for differentiation of autofluorescence from porphyrins and Cu(I)-MTs, by which an appropriate combination of excitation and emission filters is selected using an ordinary fluorescence microscope.

Materials and Methods

The preparation of sections was performed as described in our previous reports [17–19]. All animal procedures were approved by the Animal Care and Use Committees of Hokkaido Institute of Public Health. All procedures using rats were carried out according to the regulations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. In this study, the hepatic and renal sections of 30-week-old male Long-Evans Cinnamon (LEC) rats were utilized for the observations of autofluorescence from Cu(I)-MTs and porphyrins, respectively. The bright red-orange autofluorescence is barely observable in the kidneys of the 30-week-old female LEC rats because the levels of porphyrins in the female kidneys are reduced markedly compared with those of the male kidneys [18]. The tissues were cut in a cryostat microtome (Model CM-41; Sakura Seiki Inc., Tokyo, Japan) at −20°C. The frozen sections (8 μm) were mounted on slides, thawed at room temperature, and immersed in acetone for 5 min to

dehydrate the tissues. The specimens were embedded rapidly. We chose an Entellan new (Merck, Darmstadt, Germany) as a rapid embedding medium to avoid autofluorescence quenching, since the medium is characterized by low absorbance in the 300–400 nm region, faint emission in the visible region, high chemical stability under UV excitation, and because it enables the use of easily recyclable quartz cover slips and slides. The conditions required for the observation of the hepatic and renal emissions were compared with the aid of an Olympus BX50-FLA microscope (Olympus, Tokyo, Japan) equipped for epifluorescence using a 100-W mercury lamp. The embedded specimens were photographed serially under three conditions as follows: using a U-MWU filter cube (a 330–385 nm excitation filter, a 400 nm dichroic mirror and a 420 nm barrier filter); a modified U-MWU filter cube (a 330–385 nm excitation filter, a 400 nm dichroic mirror and a 515 nm barrier filter); and a modified U-MWB filter cube (a 400–440 nm excitation filter, a 500 nm dichroic mirror and a 515 nm barrier filter). All of the observations were performed within 6 h after the embedding.

For fluorescence microspectrophotometric observations, the preparation of sections was performed as described in our previous report [17]. A microscope photometer (MPM800; Carl Zeiss, Oberkochem, Germany) was used both for the fluorescence microscopic observations and the spectroscopic measurement. It was equipped with a 100-W mercury-xenon high-pressure lamp. By selecting the appropriate filter units (an excitation filter of 300±30 nm for spectrum scanning, or of 340±30 nm for photographic recording, a 400 nm dichroic mirror and a 420 nm barrier filter), the specimens were viewed, and the emission spectra of the autofluorescence were recorded in the visible region from 450 to 750 nm at room temperature. All procedures were also performed within 6 h after the embedding. The spectrum shown in this study was corrected using the blank spectrum of the embedding medium.

Results and Discussion

"Triple Observation Method (TOM)" Using Ordinary Excitation Filter Arrangements

All of the observations in the hepatic and renal sections of 30-week-old male LEC rats (Fig. 1) were exactly the same as those described in previous reports [14, 15, 17, 18], and all of the emissions also satisfied the quenching tests for Cu (I)-MTs (data not shown). For observations of the hepatic yellow-orange emission, a 330–385 nm excitation filter (Fig. 1a and b) was the most efficient. A 420 nm barrier filter (Fig. 1a) was more specific but less sensitive than a 515 nm barrier filter (Fig. 1b). We concluded that there was little difference between them, since the difference was subjective, depending upon the observer. Using a 400–440 nm excitation filter, the yellow-orange emission could hardly be detected (Fig. 1c). These excitation properties were in agreement with those of Cu(I)-MTs because the excitation maxima of the proteins are located at around 300 nm [11–13]. In the observation of the renal red-orange emission, the brilliant lights were detected under all three conditions (Fig. 1d–f). In contrast to the conditions required

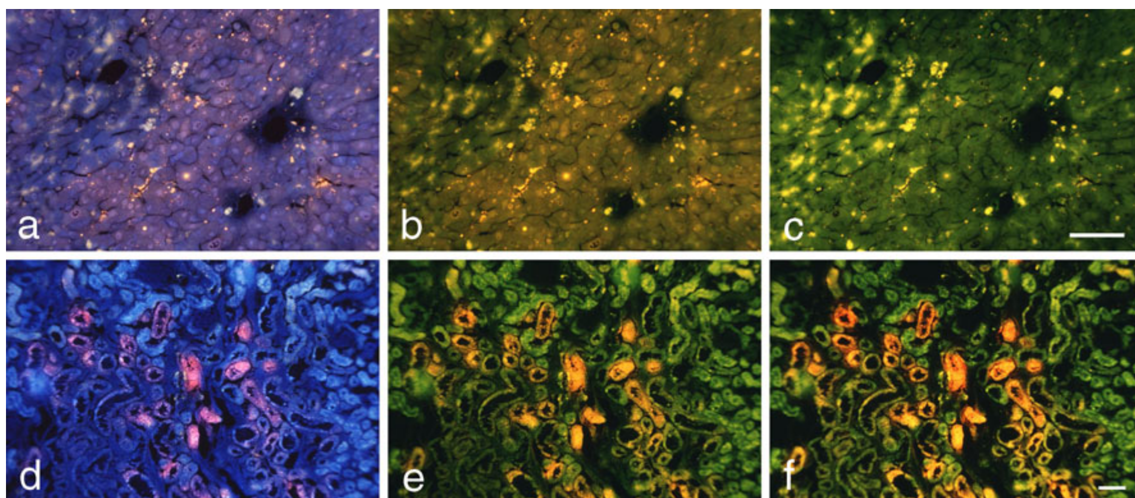

Fig. 1 Triple Observation Method (TOM): Comparison of the conditions required for the observation of autofluorescence in hepatic (**a**–**c**) and renal (**d**–**f**) sections of 30-week-old male LEC rats, performed with the aid of an ordinary fluorescence microscope. The specimens were photographed serially under the three following conditions: with a U-MWU filter cube (a 330–385 nm excitation filter, a 400 nm dichroic mirror and a 420 nm barrier filter; **a** and **d**); a modified U-MWU filter cube (a 330–385 nm excitation filter, a 400 nm dichroic mirror and a 515 nm barrier filter; **b** and **e**); and a modified U-MWB filter cube (a 400–440 nm excitation filter, a 500 nm dichroic mirror and a 515 nm barrier filter; **c** and **f**). Original magnifications, x 50 (**a**–**c**), x 25 (**d**–**f**). Bar=100 μm

for the observations of emissions from Cu(I)-MTs, the renal biomolecules under the 400–440 nm excitation filter emitted light equal to or more intense than those under the 330–385 nm excitation filter. These excitation properties were in accord with those of porphyrins whose excitation maxima are located at around 405 nm [3–5, 7, 21, 22]

The proposed method is very simple. However, it is possible to discriminate optically between autofluorescence from porphyrins and that from Cu(I)-MTs by which an appropriate combination of excitation and emission filters is selected using an ordinary fluorescence microscope.

Confirmation of Yellow-Orange Autofluorescence in the Liver of a WND Patient by Using Microspectrophotometry

The results of a clinical application of fluorescence microspectrophotometric observations are shown in Fig. 2. The following filter sets were selected: an excitation filter of 340±30 nm; a 420 nm dichroic mirror; and a 400 nm barrier filter. This filter set was appropriate for photographing yellow-orange autofluorescence arising from Cu(I)-MTs in the liver using an ordinary fluorescence microscope.

A portion of a fresh frozen hepatic tissue was kindly gifted from Dr. M. Kubota (Department of Pediatrics, Hokkaido University Graduate School of Medicine) after a portion of the liver was voluntarily transplanted from the informed patient's mother to the patient. The patient was a Japanese boy at the age of 12 years. Both hepatitis B and C viral infections were negative. He was diagnosed as a WND patient at the hepatitis stage on the basis of the following clinical data [23]: the abnormal reduction of serum ceruloplasmin level; the abnormal accumulation of hepatic Cu; the detection of Kayser-Fleischer rings; and the detection of one WND-causing mutation, 2871 del. C [24, 25] on one chromosome. Hepatic cirrhosis was observed in the tissue (the data of hematoxylin and eosin staining were not shown).

Bright yellow-orange autofluorescence was observed in hepatocytes in the regenerative nodules surrounded by fibrotic bands (Fig. 2a). Almost all of the emissions were detected in granules in the hepatocytes (Fig. 2b). The emission spectrum of the region indicated by a white spot shown in Fig. 2b is shown in Fig. 2c. The maximum of the yellow-orange autofluorescence in the granule was detected at around 615 nm and its spectral shape was broad. These analyses were identical with those of in vitro Cu(I)-MTs purified from the livers of the LEC rats [17]. Therefore, these results clearly prove that the endogenous fluorophores emitting yellow-orange light in the granules in the WND hepatocytes are Cu(I)-MTs. No red-orange autofluorescence, which was characterized as arising from porphyrins, was observed in the hepatocytes of this patient.

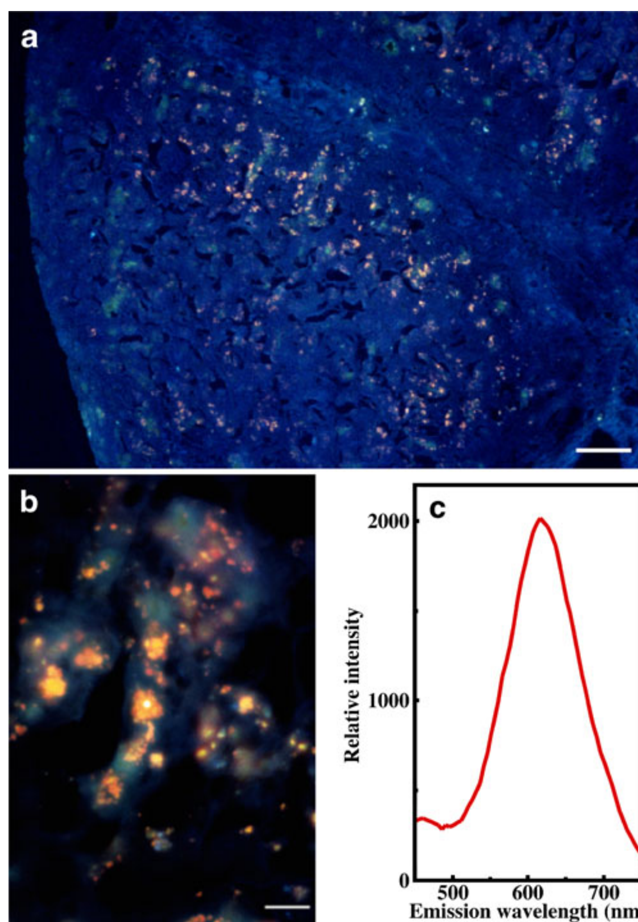

Fig. 2 Microspectrophotometric observations of bright yellow-orange autofluorescence from CU(I)–MTs in hepatic sections of a patient with WND. **a** Fluorescence microscopic photograph. Original magnification, x 25. Bar=50 μm. Bright yellow-orange autofluorescence was observed in hepatocytes in the regenerative nodules surrounded by fibrotic bands. **b** Fluorescence microscopic photograph. Original magnification, x 250. Bar=5 μm. Almost all of the emissions were detected in granules in the hepatocytes. A white spot in the photograph indicates a measurement point (a diameter of 0.16 μm) for the emission spectrum analysis shown in Fig. 2c. **c** Emission spectrum of the region indicated by the white spot shown in Fig. 2b. The maximum of the yellow-orange autofluorescence in the granule was detected at around 615 nm and its spectral shape was broad. These analyses were identical with those of in vitro Cu(I)-MTs purified from the livers of the LEC rats [17]. Therefore, these results prove that the endogenous fluorophores emitting yellow-orange light in the granules in the WND hepatocytes are Cu(I)-MTs

Conclusions

In this short communication, we propose a simple method for differentiation of autofluorescence from porphyrins and Cu(I)-MTs, by which an appropriate combination of excitation and emission filters is selected using an ordinary fluorescence microscope. When using our technique, it becomes possible to optically discriminate more accurately between the autofluorescence properties arising from porphyrins and those arising from Cu(I)-MTs. Therefore,

we wish to emphasize the importance of examining the spectral properties of the emissions to identify the endogenous fluorophores in the cells and/or the tissues. Our observations were practical attempts at this. The microspectrophotometric analysis of the WND patient supported our simple technique: the conclusive differences observed between the excitation conditions required to observe porphyrins and Cu(I)-MTs are effectual for an optical discrimination between them using an ordinary fluorescence microscope. We would like to name our simple technique “Triple Observation Method (TOM)”.

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Conflict of interest The authors declare no conflict of interest.

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