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Original Article

Potential application of $CuSO_4$ for the reduction of lipofuscin autofluorescence in formalin-fixed paraffin-embedded tissue

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ABSTRACT: The fluorescent pigment lipofuscin accumulates within the cytoplasm of cells of most of the organs. Because of its broad excitation and emission spectra, the presence of lipofuscin autofluorescence complicates the use of fluorescence microscopy. In this study, we examined chemical treatments of tissue sections with two concentration of $CuSO_4$ for their ability to reduce or eliminate lipofuscin-like autofluorescence without adversely affecting other fluorescent labels. We found that 4mM CuSO4 in 50 mM ammonium acetate buffer mildly eliminated lipofuscin autofluorescence in sections of human tonsil or mouse synovial tissue, whereas, at higher concentration (10mM) the autofluorescence was almost completely abolished. We found that this treatment had no effect on the fluorescence intensity of target tissue. We conclude that treatment of tissue with CuSO4 provides a reasonable compromise between reduction of lipofuscin-like fluorescence and maintenance of specific fluorescent labels.

KEYWORDS: Copper sulfate, autofluorescence, lipofuscin

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INTRODUCTION

Biological autofluorescence (AF), arising from endogenous fluorophores, is an intrinsic property of cells and tissues. AF properties of specific tissue constituents may be of diagnostic value[1]. This applies for lipofuscin granules and collagen fibers[2]. On the other hand, autofluorescence, either intrinsic or induced by fixation media and tissue processsing may either mask specific fluorescent signals or be mistaken for fluorescent labels[3,1]. There are many causes for autofluorescence of tissue. Some exist only in mammalian tissue, such as the fluorescent pigment lipofuscin, which accumulates with age in the cytoplasm of cells. The presence of lipofuscin can complicate the use of fluorescence microscopy in the brain as well as other tissues because of its broad excitation and emission spectra[4]. The spectra of lipofuscin overlap those of all commonly used fluorophores, making distinctions between specific labeling and nonspecific autofluorescence difficult or impossible[5]. Others are specific to plants or are independent of the species and find their origin in the embedding material. These causes of autofluorescence have one thing in common: they complicate the use of fluorescence microscopy. AF blocking efficacy varies depending on the tissue and the specimen-processing techniques, and to date there is no general formula for AF reduction.

Various histochemical techniques for the removal of autofluorescence have evolved. Ammonia–ethanol has been applied to remove some of the formaldehyde derived artifacts, sodium borohydride (borohydride) was used to control autofluorescence of glutaraldehyde-related condensates[6], and various dyes, including Sudan Black B (sudan), have been used to block autofluorescence in nerve tissue. However, a generally applicable recipe is still missing, particularly for routine paraffin sections of formaldehyde-fixed tissue.

Here we assessed the effects of $CuSO_4$ on autofluorescence in paraffin sections of archival formaldehyde-fixed human tonsil before fluorescence labeling. Thus, the aim of our study was to minimize autofluorescence to enable fluorescence microscopy in formaldehyde fixed archival tissue.

MATERIALS AND METHODS

Chemicals and antibodies: Monoclonal anti-human CD38 was purchased from Novocastra (Newcastle, UK), rat anti-mouse CD31 was from BD Bioscience (CA, USA), Vectashield from Vector Laboratories (Burlingame, CA, USA), $CuSO_4$ from Fisher Scientific (Pittsburg, PA, USA)

Tissue collection and preparation:

Human tonsils were obtained from patient underwent for tonsillectomy. Tissues were fixed in 10% phosphate buffered formalin for 24 hours, dehydrated and embedded in paraffin. Murine synovial tissue sample was collected after sacrificing the mice. These were washed in PBS, dehydrated and embedded in paraffin.

Immunofluorescence Labeling and copper sulphate treatment

From paraffin embedded tissue, three micron sections were cut from the paraffin blocks, dewaxed, and rehydrated. the sections were removed from the PBS wash, dipped briefly in distilled H_2O, and treated with CuSO4 in ammonium acetate buffer (pH 5.0) for 10 min, dipped briefly in distilled H_2O, and returned to PBS. Here we used 4mM and 10mM of $CuSO_4$ for this purpose.

Antigen retrieval was performed using Tris-EDTA buffer (pH 9.0) at 60 ℃, overnight followed by three wash with PBS. Non-specific binding sites were blocked with 5 % BSA (in PBS) for 20 minutes before applying anti-human rat monoclonal antibody (1:100, R&D system, Minneapolis, MN) and rat anti-mouse CD31 monoclonal antibody (1:50, BD Bioscience, USA). This was followed by the incubation of slide with appropriate secondary antibody conjugated with FITC. Finally, nuclear staining was performed with DAPI for three minutes, before mounting.

Microscope and Filter Systems

The sections were examined with Zeiss fluorescence microscope (Carl Zeiss, Jena, Germany) equipped for reflected fluorescence illumination and digital imaging. Filter bandpasses used in this study is given in table 1

Table 1: Microscopy conditions for fluorescence imaging using fluorescence microscope

Acquisition channel	Fluorophore used in this study	Excitation laser	Emission	Image display color
DAPI	NA	405 nm	425–525	Blue
FITC	FITC	473 nm	485–585	Green

RESULTS

General Observations

Lipofuscin-like autofluorescence was found throughout the tonsil (Figure 1). There appeared to be lipofuscin-like pigments in cells of all sizes, although not all cells emitted autofluorescence. For example, the majority of lipofuscin-like autofluorescence was observed surrounding cortical area, whereas less was observed in medulla. Furthermore, little or no autofluorescence was observed in cells comprising the molecular and granular layers of the tonsil.

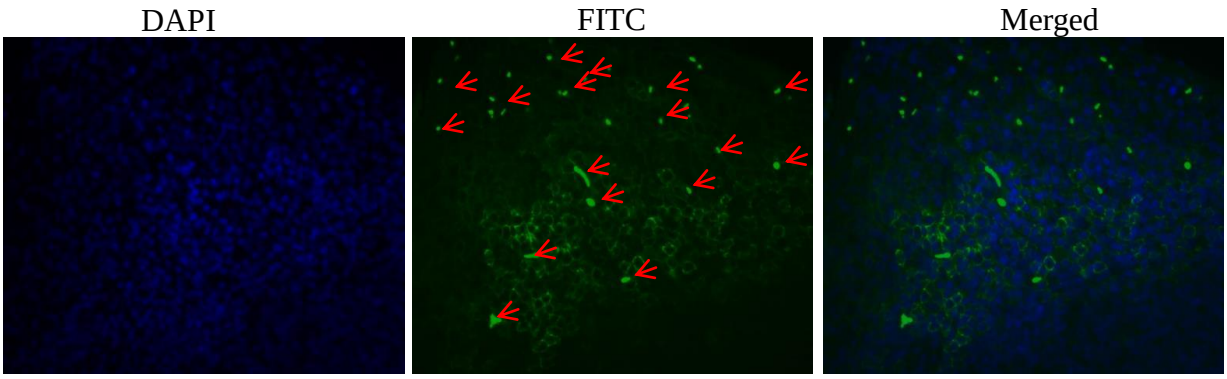

Figure 1. Autofluorescence from lipofuscin throughout the section (arrowhead). Magnification 200x for each pictures.

The excitation and emission characteristics of the lipofuscin-like autofluorescence were sufficiently broad that they could complicate the use of fluorescence techniques (Figure 2). Lipofuscin-like autofluorescent pigments were visible under fluorescent filters for fluorescein. Using other filters, it appeared like red (using the filters for rhodamine), the intensity of the lipofuscin-like compound could be as strong as that of the strongest immunofluorescence (data not shown). Thus, lipofuscin-like autofluorescence could mimic the appearance of immunofluorescent labeling.

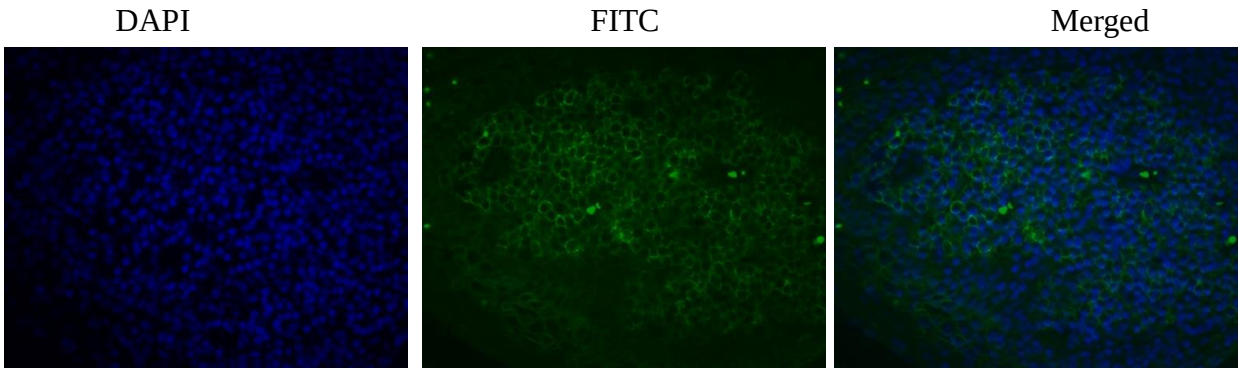

Figure 2: At 4mM concentration, CuSO4 largely reduces lipofuscin autofluorescence. Magnification 200x for each pictures.

Effect of CuSO4 Treatment on Lipofuscin-like Autofluorescence

At higher concentrations (10 mM CuSO4), almost all of the autofluorescent material was eliminated from tissue section (Figure 3), although some yellow fluorescent material remained throughout the structure.

We also employed murine synovial tissue to evaluate the efficacy of CuSO4 in eliminating lipofuscin autofluorescence. We found that there is massive lipofuscin pigment in this tissue also when mouse synovial tissue was stained with CD31 without the treatment of $CuSO_4$ (Fig. 4).

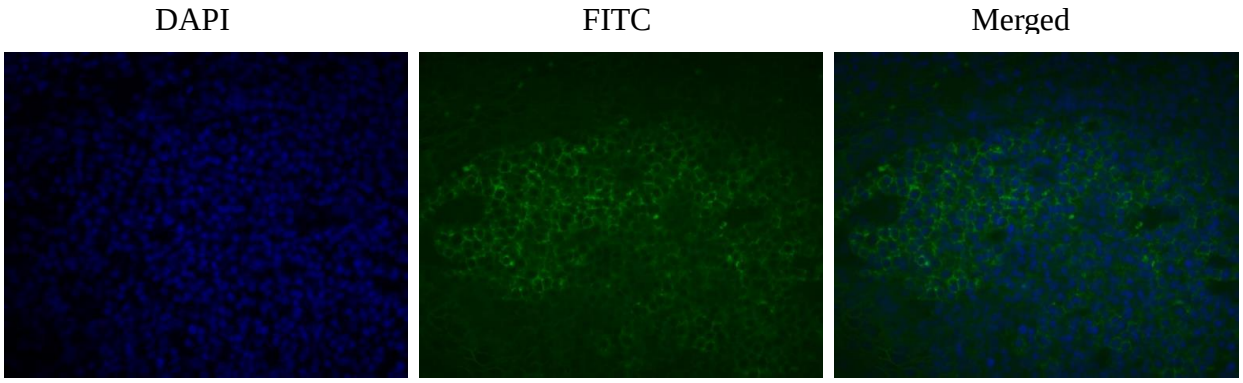

Figure 3. At 10mM concentration, $CuSO_4$ reduces lipofuscin autofluorescence is almost disappeared. Magnification 200x for each pictures.

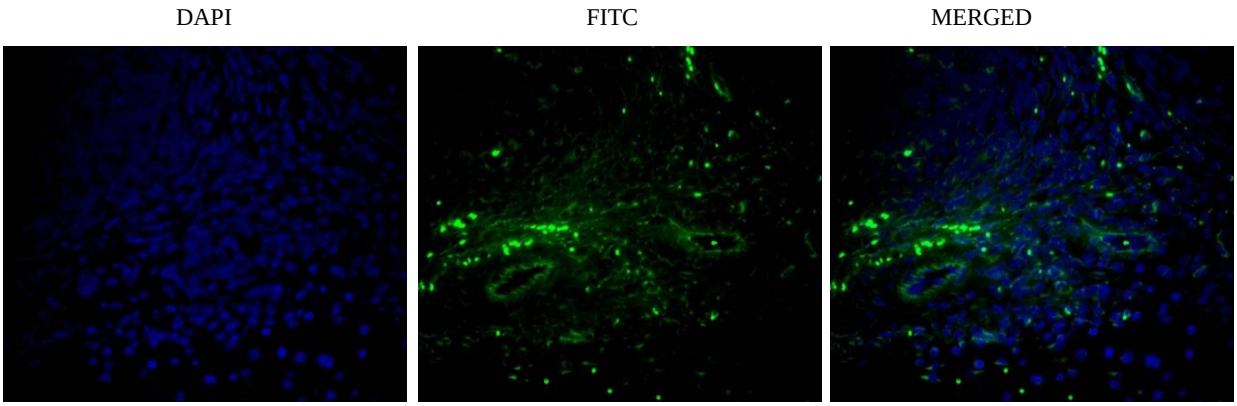

Figure 4. Profuse lipofuscinautofluorescent pigment in mouse synovial tissue. Magnification 200x for each pictures.

To determine the effect of $CuSO_4$ on the reduction of lipofuscin autofluorescene, we employed similar concentration of this chemicals (4 and 10mM) and incubated the slides for 10 minutes. We found mild reduction of autofluorescence signal when samples were treated with $CuSO_4$ at lower concentration (Figure 5). Higher concentration massively eliminated the signals. (Figure 6).

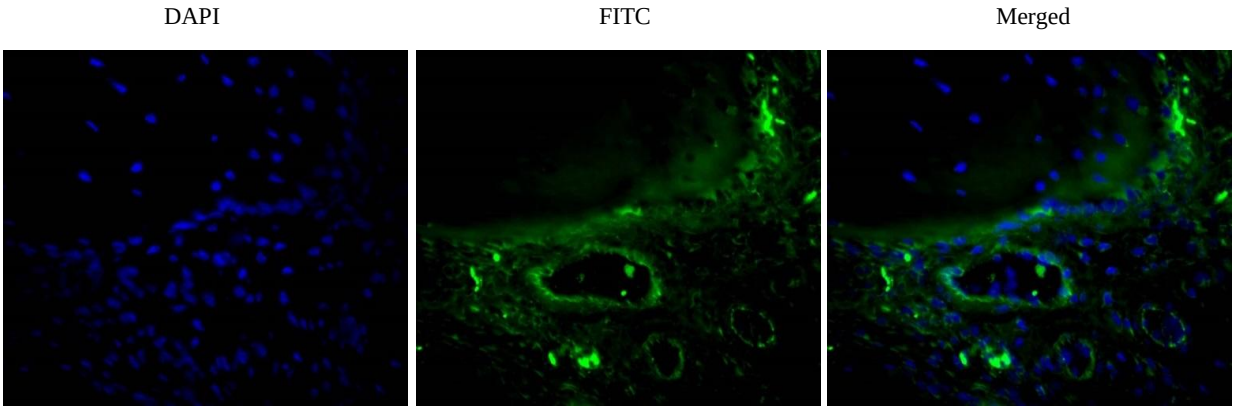

Figure 5. At 4mM concentration, CuSO4 reduces lipofuscin largely autofluorescence but still some persist. Magnification 200x for each pictures.

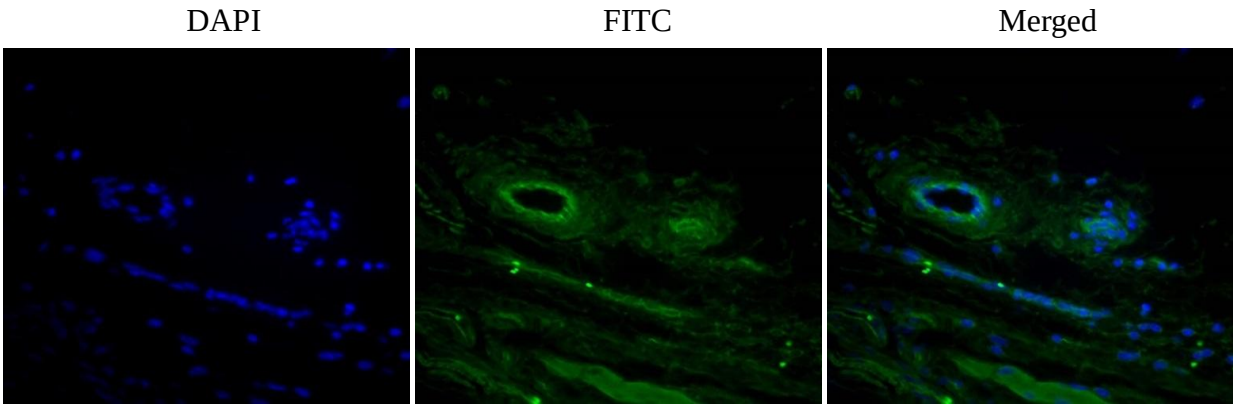

Figure 6. At 10mM concentration, CuSO4 greatly abolished lipofuscin autofluorescence. Magnification 200x for each pictures.

DISCUSSION

The interpretation of fluorescence microscopy of tissue from species in general, and from has been made difficult by the presence of the autofluorescent pigment lipofuscin, which accumulates in the cytoplasm of cells. It has been reported that the presence of lipofuscin can complicate the use of fluorescent immunocytochemical techniques because it fluoresces intensely using a variety of microscope filter systems[7, 5]. Therefore, it is difficult to distinguish specific labeling from that of lipofuscin.

Because of the difficulties presented by lipofuscin like autofluorescence, we were interested in finding a protocol that eliminated or reduced it and that was compatible with our fluorescent immunocytochemical and retrograde tract tracing procedures. However, because lipofuscin is visible under all common fluorescence filters, it may be possible to determine whether fluorescence is not due to lipofuscin. For example, if a cell fluoresces using the fluorescein filters but not using the rhodamine filters, one can reasonably conclude that the fluorescence is not due to lipofuscin[8]. However, this approach is of no help when the cell of interest contains lipofuscin and is of only limited use for multicolor fluorescence microscopy.

Previous studies have reported that lipofuscin could be extracted with a mixture of chloroform and methanol[9]. The results of these studies indicated that a blue fluorescent compound, possibly consisting of lipids, was the source of lipofuscin autofluorescence in tissues. However, it has also been shown that blue fluorescence can be induced artifactually through manipulations of the organic extract (e.g., light irradiation and chromatographic processes)[10]. Recently, a series of reports has demonstrated that a lipofuscin-like compound is extractable by aqueous but not by organic solvents[11]. Furthermore, this aqueous phase retains several of the same properties of lipofuscin-like autofluorescence found in situ (e.g., broad excitation and emission spectra and yellow fluorescence under UV illumination). Therefore, it appears that these recent studies may have identified the bona fide component responsible for lipofuscin-like autofluorescence.

It was found that treatment of the aqueous extract with certain metallic salts decreased or eliminated the yellow fluorescence, depending on the pH of the solution used[11]. On the basis of these results, we similarly treated sections of tissues to determine the ability of to reduce lipofuscin-like autofluorescence. In these experiments, we found that treatment of tissue sections with cupric sulfate reduced or eliminated lipofuscin-like autofluorescence without excessively reducing immunocytochemical labeling. We are uncertain about the chemical mechanism by which cupric sulfate (or cupric chloride) acts to reduce lipofuscin fluorescence. Fluorescence requires a photon of light to be absorbed by a molecule, causing an electron to be raised from its ground state to its excited state[12]. Fluorescence then occurs when that electron returns to its ground state, releasing energy in the form of another photon[12]. Quenching of fluorescence (i.e., reduction of its intensity) occurs when something interferes with this process. It would be reasonable to hypothesize that either collisional quenching, static quenching, or a combination of the two types of quenching occurs. It has been reported that Cu21 is an excellent "electron scavenger," suggesting that electrons could be transferred to it by collisional contact between it and a fluorescent molecule[13]. Transfer of electrons from the excited state of lipofuscin to Cu21 would circumvent the emission of its fluorescence. Regardless of the mechanism, we are convinced that Cu21 is important for reduction of lipofuscin-like fluorescence. Ferric or ferrous salts appeared to have little or no effect on lipofuscin-like fluorescence, in contrast to previous reports[11].

The little that is known regarding the chemical nature of lipofuscin has been reviewed by Pearse (1985)[14]. First, several chemical "bleaching" protocols (e.g., potassium permanganate, sodium borohydride, hydrogen peroxide) to which conjugated double bonds would be susceptible but aromatic double bonds would not, had no effect on its fluorescence. It, therefore, appears that any carbon–carbon double bonds necessary for fluorescence in lipofuscin must be aromatic in character. Second, lipofuscin is unlikely to undergo free radical formation, because illumination of lipofuscin for extended periods did not cause appreciable photobleaching as would be expected if free radical reactions were altering the compound. Third, its very broad excitation and emission characteristics suggest that lipofuscin might be composed of a mixture of different, although probably related, fluorescent molecules. Finally, our data are consistent with the existence of different types of lipofuscin because, $CuSO_4$ only reduced the intensity of lipofuscin autofluorescence in the inferior olivary nucleus rather than eliminating it. Furthermore, lipofuscin-like

labeling in vascular endothelium was less affected by $CuSO_4$ than was tonsil lipofuscin.

In summary, the experiments described here demonstrated that $CuSO_4$ that reduced the intensity of lipofuscin-like fluorophores were compatible with common fluorescent immunocytochemical and retrograde tract tracing techniques. Substantial reductions of lipofuscin-like autofluorescence in tissue sections were obtained with CuSO4. Furthermore, this substance were shown to be compatible with a wide range of fluorophores commonly used for immunocytochemical labeling. Although CuSO4 also reduced the intensity of immunocytochemical labeling, this reduction did not appear to decrease our ability to visualize specific labeling. In fact, the reduction of immunocytochemical labeling could be largely overcome by using longer exposures while imaging.

CONCLUSION

We conclude that treatment of paraffin embedded tissue with CuSO4 strikes an acceptable balance between reduction of lipofuscin-like autofluorescence and retention of immunocytochemical labeling. We also conclude that the cupric sulfate protocol may be of more general utility for the reduction of lipofuscin-like autofluorescence than other methods like Sudan black or ammonium nitrate.

REFERENCES

1. Noonberg, S.B., Weiss, T.L., Garovoy, M.R. and Hunt, C.A. 1992. Characterization and minimization of cellular autofluorescence in the study of oligonucleotide uptake using confocal microscopy. Antisense Res. Dev. 2, 303–333.
2. Banerjee, B., Miedema, B.E. and Chandrasekhar, H.R. 1999. Role of basement membrane collagen and elastin in the autofluorescence spectra of the colon. J Invest. Med. 47, 326–331.
3. Del Castillo, P., Llorente, A.R. and Stockert, J.C. 1989. Influence of fixation, exciting light and section thickness on the primary fluorescence of samples for microfluorometric analysis. Bas. Appl. Histochem. 33, 251–257.
4. Dowson, J.H. 1982. The evaluation of autofluorescence emission spectra derived from neuronal lipopigment. J. Microsc. 128, 261–270.
5. Kalyuzhny, A.E. and Wessendorf, M.W. 1998. Relationship of mu- and delta-opioid receptors to GABAergic neurons in the central nervous system, including antinociceptive brainstem circuits. J. Comp. Neurol. 392, 528–547.
6. Southern, L.J., Hughes, H., Lawford, P.V., Clench, M.R. and Maning, N.J. 2000. Glutaraldehyde-induced crosslinks: a study of model compounds and commercial bioprosthetic valves. J Heart Valve Dis. 9, 241–248.
7. Helen, P. 1983. Fine-structural and degenerative features in adult and aged human sympathetic ganglion cells. Mech. Ageing Dev. 23: 161–175.
8. Kalyuzhny, A.E., Arvidsson, U., Wu, W. and Wessendorf, M.W. 1996. mu-Opioid and delta-opioid receptors are expressed in brainstem antinociceptive circuits: studies using immunocytochemistry and retrograde tract-tracing. J Neurosci. 16, 6490–6503.
9. Katz, M.L., Gao, C.L. and Rice, L.M. 1996. Formation of lipofuscin-like fluorophores by reaction of retinal with photoreceptor outer segments and liposomes. Mech. Ageing Dev. 92, 159–174.
10. Kikugawa, K., Kato, T., Yamaki, S. and Kasai, H. 1994. Examination of the extraction methods and re-evaluation of blue fluorescence generated in rat tissues in situ. Biol. Pharmacol. Bull. 17, 9–15.
11. Kikugawa. K, Beppu, M., Sato, A., and Kasai, H. 1997.Separation of multiple yellow fluorescent lipofuscin components in rat kidney and their characterization. Mech. Ageing Dev. 97, 93–107.
12. Schnell, S.A., Staines, W.A. and Wessendorf, W.1999. Reduction of Lipofuscin-like autofluorescence in fluorescently labeled tissue.J Histochem.Cytochem. 47, 719–730.
13. Steiner, R.F. and Kirby, E.P. 1969. The interaction of the ground and excited states of indole derivatives with electron scavengers. J Phys. Chem. 73, 4130–4135.
14. Pearse, A.G.E. 1985. Histochemistry, Theoretical and Applied. Edinburgh, New York, Churchill Livingstone.