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Determination of Subcellular Localization of Flavonol in Cultured Cells by Laser Scanning

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1. Introduction

Flavonoids are widely distributed in the plant kingdom including in edible plants such as vegetables and fruits. They are polyphenolic compounds comprising of fifteen carbons, with two aromatic rings connected by a three-carbon bridge (C₆-C₃-C₆). Flavonoids are divided into seven subclasses; the flavones, flavonols, flavanones, catechins, anthocyanins, isoflavones and the chalcones, according to their basic skeletal structure (Fig. 1). They are known to have various beneficial pharmacological effects such as anti-cancer, anti-obesity, anti-inflammatory and anti-oxidative activities (Ahn, et al. 2008; Boots, et al. 2008; Chu, et al. 2007; Hsu and Yen 2006; Kuzuhara, et al. 2008; Murakami, et al. 2008). To investigate the beneficial effects of trace food components, it is important to understand their metabolism, absorption, tissue distribution, and subcellular localization. Recently, the metabolism, including absorption and tissue distribution, of trace food components has been investigated using cultured cells and experimental animals (de Boer, et al. 2005; Urpi-Sarda, et al. 2008; Wang, et al. 2003). To determine the physiological concentrations and chemical structures of flavonoid metabolites, high-performance liquid chromatography (HPLC) or HPLC combined with mass spectrometry (MS) is conventionally used (de Boer, et al. 2005; Mullen, et al. 2006; Urpi-Sarda, et al. 2008; Wang, et al. 2003; Yanez, et al. 2008). The subcellular localization of the flavonoids is currently not fully understood, although the preparation of subcellular fractions using centrifugation, followed by HPLC analysis to determine the cellular localization of trace food components, including flavonoids, has been described (Gagne, et al. 2006). Radioactive isotope labeling has also been used to estimate tissue localization of trace food components in animals (Hirosawa and Yamada 1981), and this method could be applied to estimating subcellular localization in cultured cells. However, cross contamination between fractions during the preparation of subcellular fractions can limit the accuracy of such techniques. In this chapter, we demonstrate the value of confocal laser scanning fluorescence microscopy in the subcellular localization of flavanoids, by the detection of autofluorescence in intact culture cells. During this study, we focused on the flavonol subclass, as this subclass features stronger autofluorescence properties than other flavonoid subclasses.

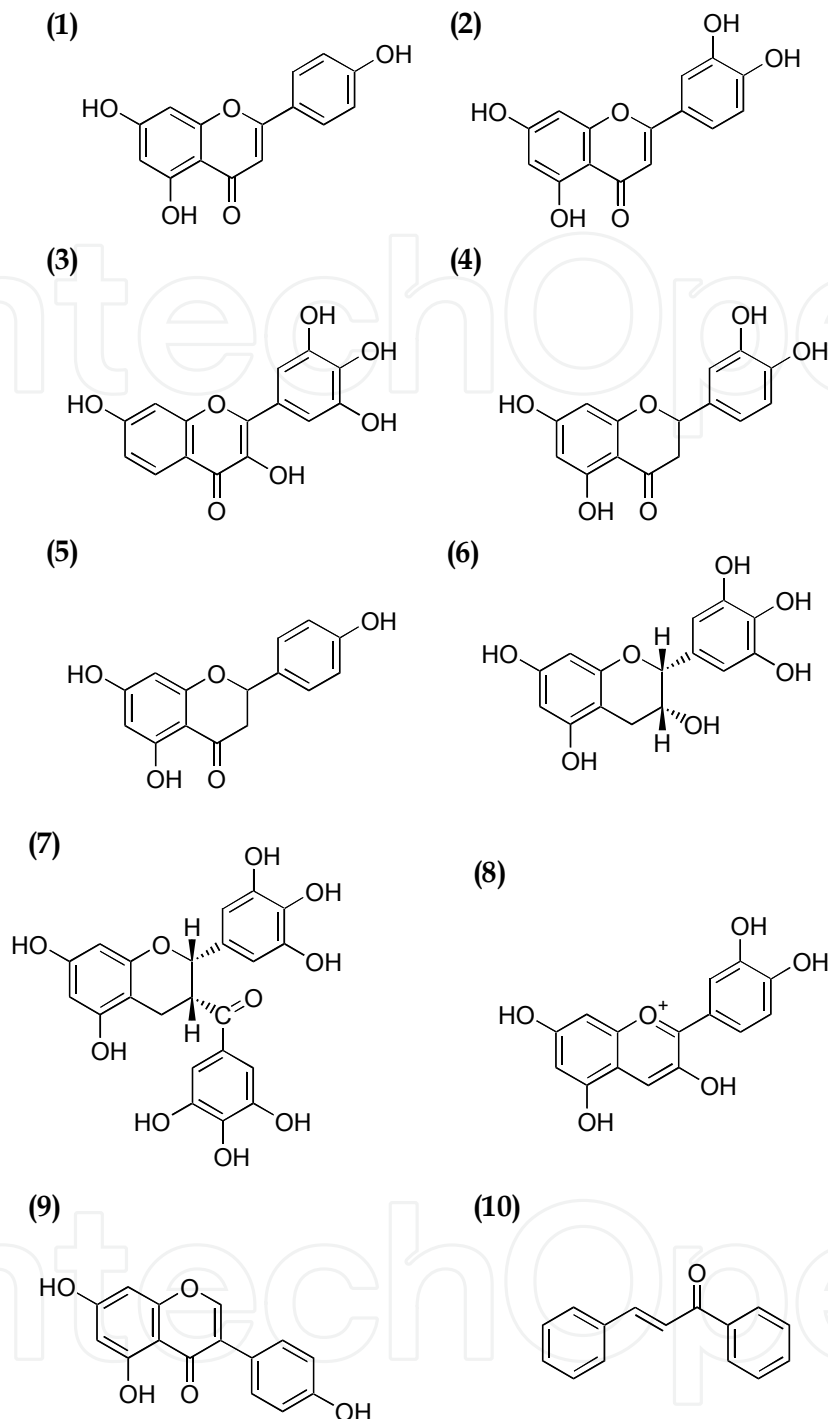


Fig. 1. Representative compounds from each flavonoid subclass

Apigenin (1) and luteolin (2) belong to the flavone subclass and robinetin (3) to the flavonol subclass. Eriodictiol (4) and naringenin (5) belong to the flavanone subclass while (-)-epigallocatechin (6) and (-)-epigallocatechin gallate (7) belong to the catechin subclass. Cyanidin (8), genistein (9) and chalcone (10) belong to the anthocyan, isoflavone and chalcone subclasses, respectively.

The flavonol subclass feature a hydroxyl group in the 3-position of the flavonoid structure (Figure 2). Flavonols are found in onions (*Allium cepa* L.), broccoli (*Brassica oleracea* var.

botrytis L.), and apples (*Malus pumila* Mill.) (Sakakibara, et al. 2003). It has been reported that flavonols are found in the liver, kidney, muscle, heart, lung, brain, testes, spleen, thymus, bone, brown fat, and white fat of rats, and in the liver, lung, white fat, muscle, brain, kidney, heart, and spleen of pigs after consumption of flavonol containing food stuffs (Bieger, et al. 2008; de Boer, et al. 2005).

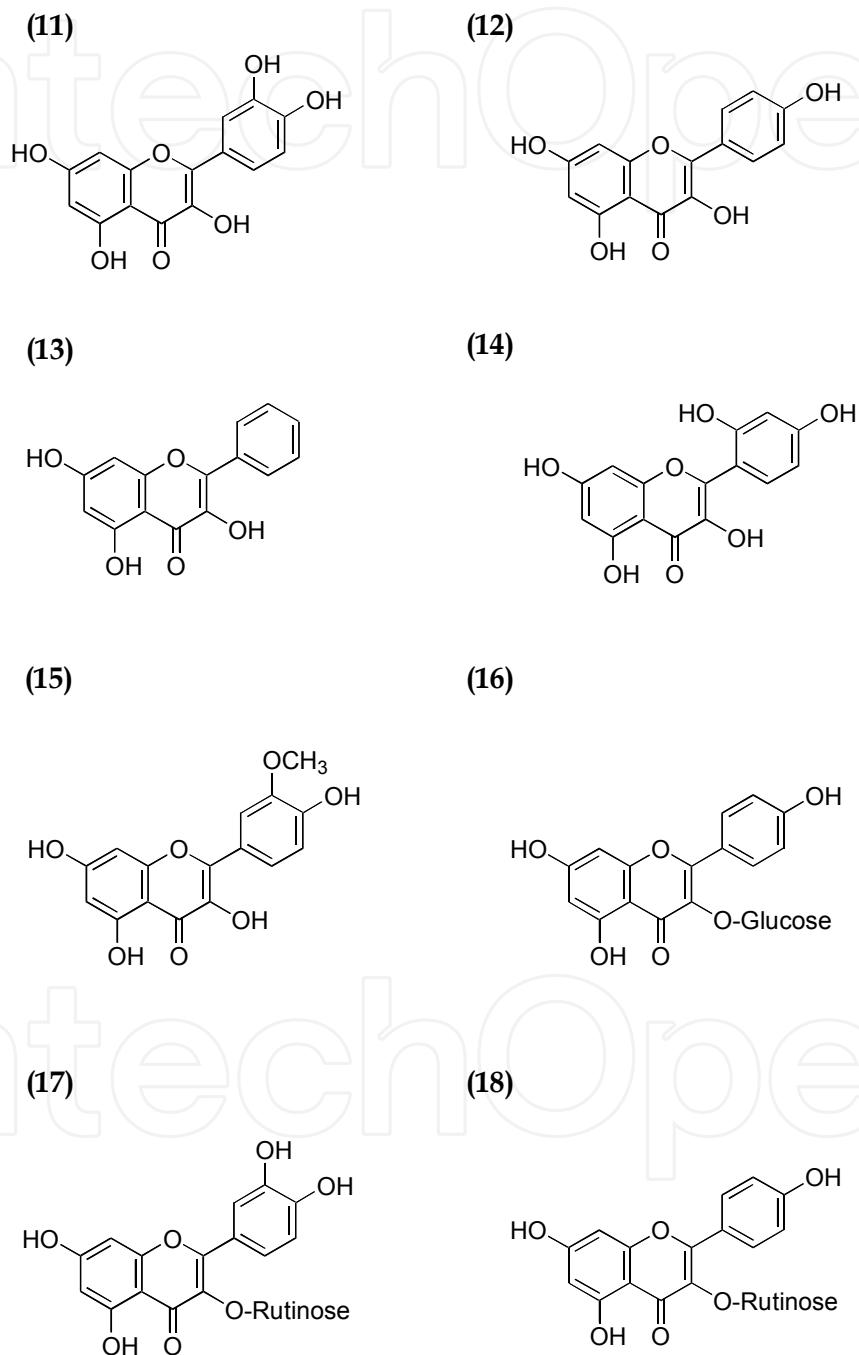


Fig. 2. Examples of the flavonol subclass. Structures of quercetin (11), kaempferol (12), galangin (13), morin (14), isorhamnetin (15), kaempferol-3-glucoside (16), quercetin-3-rutinoside (17), and kaempferol-3-rutinoside (18).

Thus, after dietary intake, flavonol becomes widely distributed in various tissues and is known to exert beneficial effects in many tissue types. In mouse liver, flavonol has been reported to reduce the toxicological effects of dioxin by suppressing the dioxin-induced activation of the aryl hydrocarbon receptor (Mukai, et al. 2009a). Flavonol has been shown to reduce lipid peroxidation in the aorta tissue of rabbits fed a high cholesterol diet (Kamada, et al. 2005). In the research that demonstrates the effect of flavonol on the brain, the nerve cell is generally used. In PC12, the nerve-like cells, flavonol glucuronides suppressed production of reactive oxygen species (Shirai, et al. 2006). The cellular absorption and metabolism of flavonoids in the intestines, human colonic adenocarcinoma Caco-2 cells have been used as a model. It was reported that flavonoids are incorporated into Caco-2 cells by passive diffusion (Walgren, et al. 1998) and/or the active sodium-dependent glucose transporter 1 (Walgren, et al. 2000b), and are excreted through an efflux transporter such as multidrug resistance-associated protein-2 (Walgren, et al. 2000a). Previously, it has also been reported that flavonol emits fluorescence in Caco-2 cells (Walgren, et al. 2000b) and platelet cells from healthy volunteers (Wright, et al. 2010) detectable by fluorescence microscopy. However, these reports did not address the subcellular localization of the flavonoids. To date, the fluorescence properties of flavonoids have not been taken advantage of in studies of flavonoid subcellular localization. Here, we present data showing the subcellular localization of flavonol aglycones in intact cultured cells using confocal laser scanning fluorescence microscopy.

2. Detection of flavonoid in cultured cell by fluorescence microscopy

Since liver tissue is known to accumulate flavonols due to its role in their metabolism, the mouse hepatoma cell line, Hepa-1c1c7 was used as a model cell-line in these studies. Hepa-1c1c7 cells were grown and maintained at 37 °C in α -minimum essential medium (α -MEM) containing 10% fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml of streptomycin under a humidified atmosphere containing 5% CO₂. Hepa-1c1c7 cells were seeded onto a glass-bottomed culture dish and were grown until 80% confluent. Cells were incubated with fresh α -MEM supplemented with 5% fetal bovine serum for 24 h prior to treatment. The cells were treated with each flavonoid by addition of the solubilized flavonoid (in α -MEM containing 0.1% dimethyl sulfoxide) to the medium, and the cells were incubated for 70 min. It is noteworthy that the culture medium itself regularly showed fluorescence, hindering cellular visualization. To avoid this problem, common physiological buffers such as Krebs-Ringer HEPES buffer (50 mM HEPES, pH 7.4, 137 mM NaCl, 4.8 mM KCl, 1.85 mM CaCl₂, and 1.3 mM MgSO₄) were also applied to the cells during the incubation period. The fluorescence images obtained were shown in Fig. 3. Washing the cells with ice-cold phosphate buffered saline (PBS, pH 7.4) was important to obtain clear images (Fig. 4). After washing, the cells were fixed with 4% paraformaldehyde and 0.2% picric acid in 100 mM sodium phosphate buffer (pH 7.2) overnight at 4 °C in the absence of light. Then, autofluorescence from the flavonoid was observed under a confocal laser scanning fluorescence microscope (LSM 510 invert, Carl Zeiss, Jena, Germany) with excitation by an argon laser at 488 nm and a 515–535 nm band pass filter. A low energy laser was used during observation, but a full energy laser was used for generating images. The uptake of flavonoids (listed in Table 1) into Hepa-1c1c7 cells was examined under a confocal microscope. When the cells were treated with five flavonol aglycones, namely kaempferol, galangin, isorhamnetin, morin and quercetin, green autofluorescence of the

cells was observed (Fig. 5). In contrast, cells treated with either flavonol glycosides (kaempferol-3-glucoside, kaempferol-3-rutinoside and quercetin-3-rutinoside), or flavonoids belonging to other subclasses (flavone, apigenin, luteolin, naringenin, eriodictyol, (-)-epigallocatechin and (-)-epigallocatechin gallate), did not emit any autofluorescence (data not shown). The uptake of flavonol aglycones in Hepa-1c1c7 cells was observed with a confocal microscope. However, flavonol glycosides and compounds belonging to other subclasses could not be visualized.

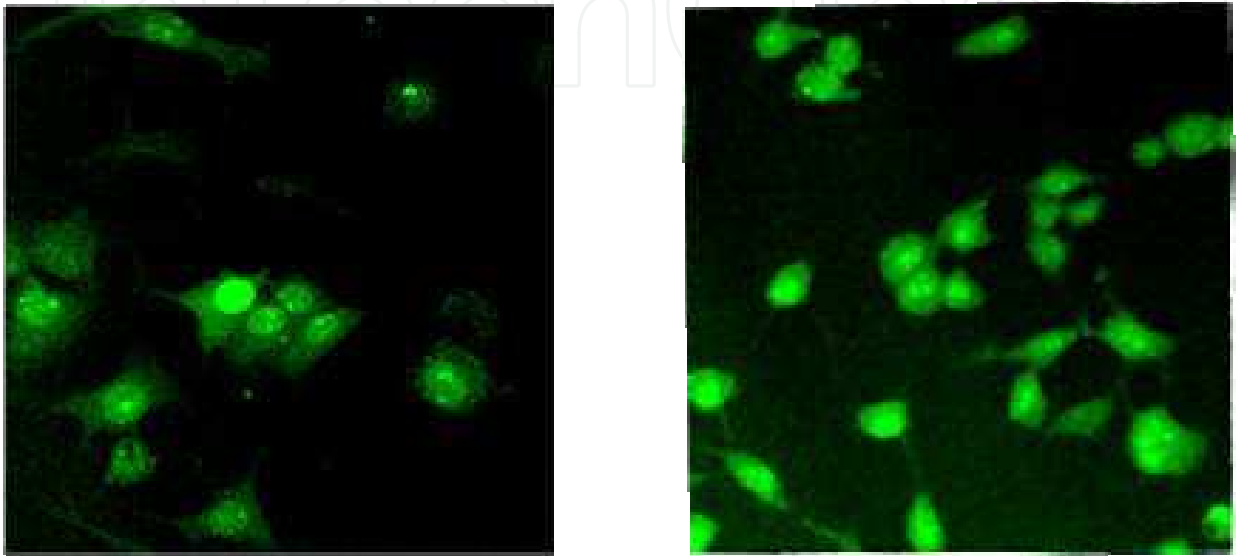


Fig. 3. Both medium and physiological buffer could be used during incubation in uptake experiments. Here, cells treated with the flavonol kaempferol at 50 μ M in either α -MEM culture medium (left) or Krebs-Ringer HEPES buffer (right) for 70 min are shown.

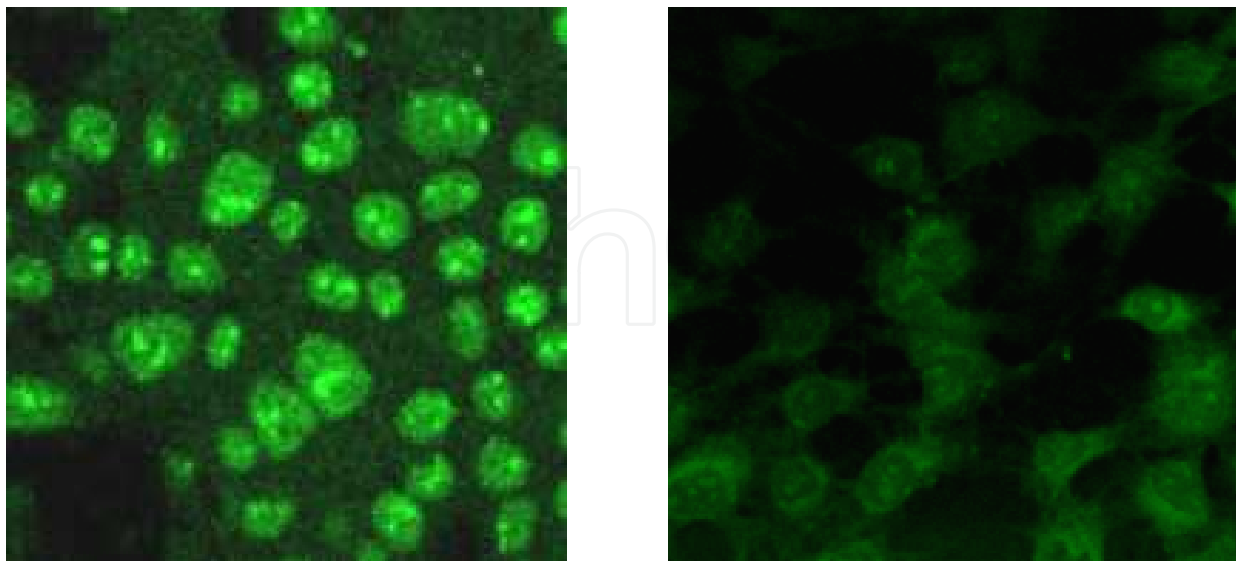


Fig. 4. Images to indicate the importance of cell washing for successful imaging . Here, cells treated with the flavonol kaempferol at 50 μ M, in α -MEM culture medium for 70 min, and either washed with PBS prior to imaging (left) or directly imaged (right) are shown.

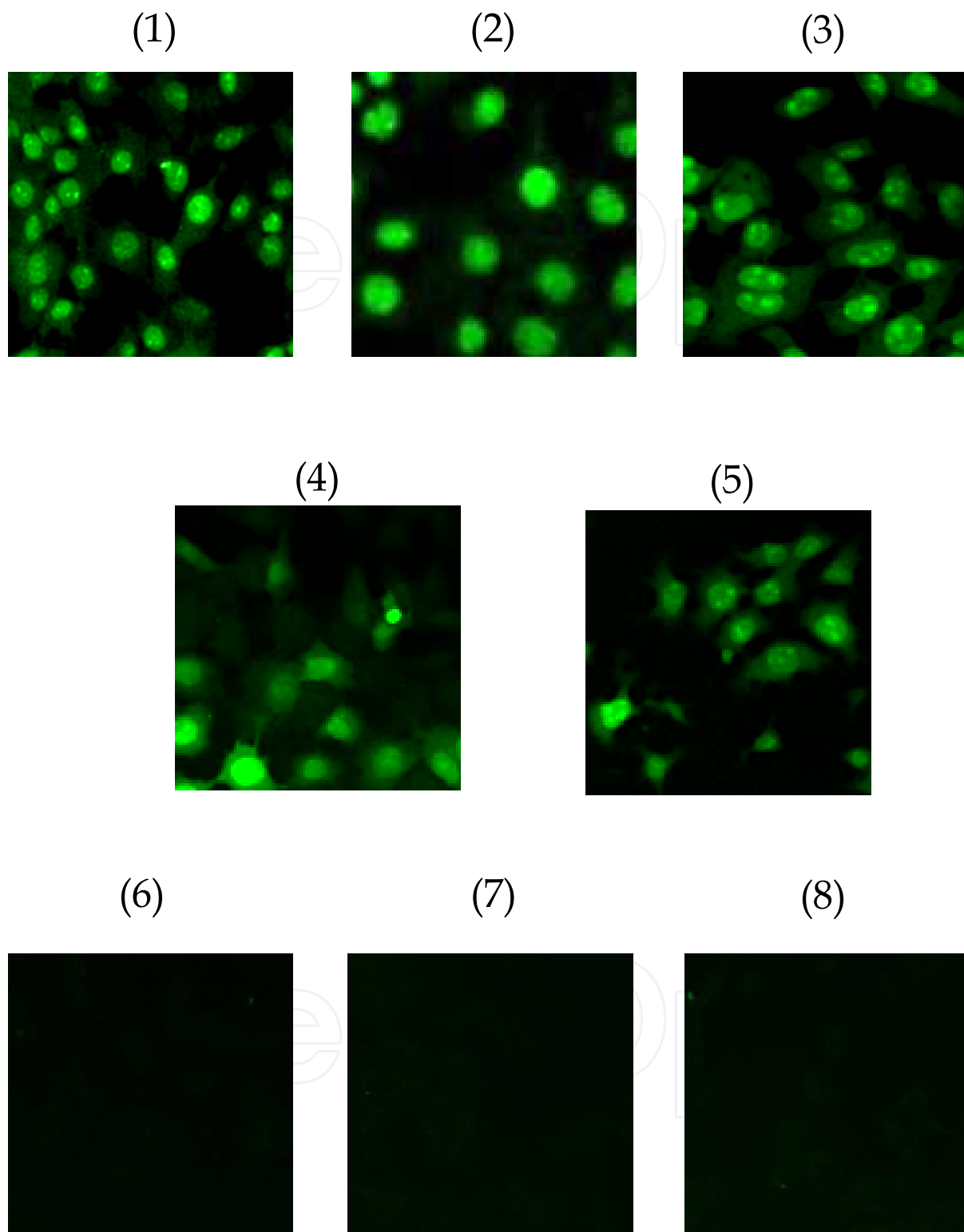


Fig. 5. Cellular uptake of various flavonoids as visualized by fluorescence microscopy. Hepa-1c1c7 cells were seeded onto glass bottom dishes and treated with (1) kaempferol, (2) galangin, (3) isorhamnetin, (4) morin, (5) quercetin, (6) kaempferol-3-glucoside, (7) kaempferol-3-rutioside and (8) quercetin-3-rutinoside at 50 μ M for 70 min. Green fluorescence was measured under a confocal microscope. (Mukai, et al. 2009b)

Subclass	Name of Flavonoid
Flavonol	kaempferogalangin
	isorhamnetin
	morin
	quercetin
	kaempferol-3-glucoside
	kaempferol-3-rhamnoside
	quercetin-3-rhamnoside
Flavone	flavone
	apigenin
	luteolin
Flavanone	naringenin
	eriodictyol
Catechin	(-)-epigallocatechin
	(-)-epigallocatechin gallate

Table 1. Flavonoids examined in this study and their subclass categorization

The excitation spectra of selected flavonoids were measured using a fluorescence spectrophotometer (F2500, Hitachi, Tokyo, Japan) with an excitation wavelength range between 460 and 490 nm and fluorescence emission detected at a wavelength of 520 nm.

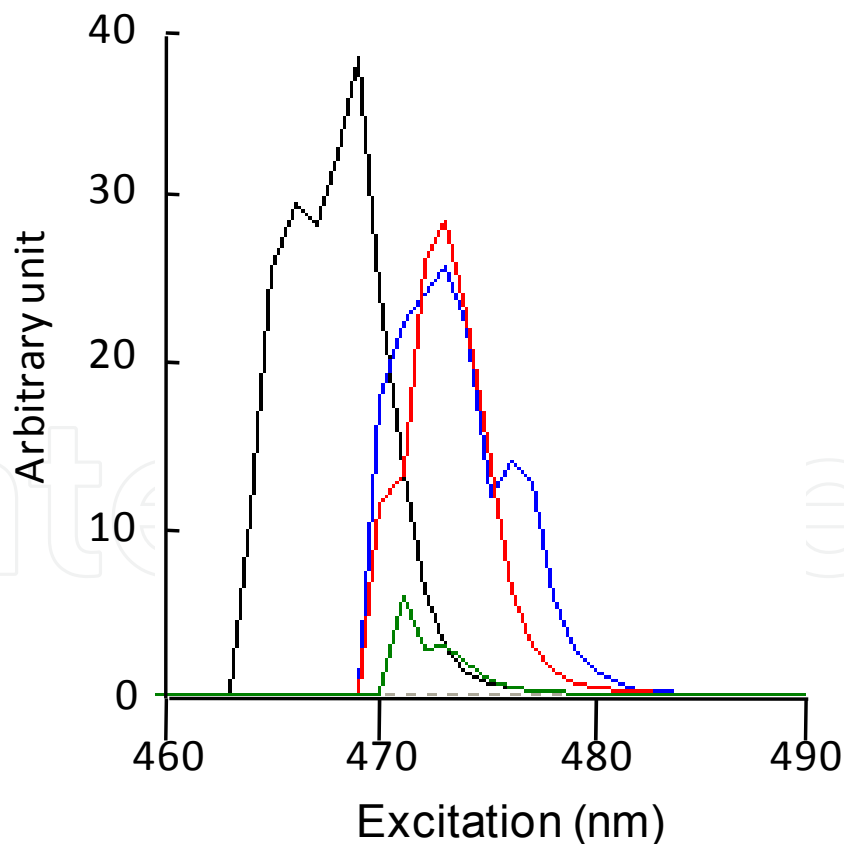


Fig. 6. Excitation spectrum of flavonoids. Excitation spectrum (ex. 460-490 nm) of kaempferol (black), quercetin (blue), quercetin-3-rutinoside (green), and naringenin (red) as measured using a fluorescence spectrophotometer. The fluorescence emission was detected at 520 nm.

These spectra indicate that kaempferol, quercetin, quercetin-3-rutinoside, and naringenin emits fluorescence with an excitation wavelength of 470-480 nm (Fig. 6), whereas apigenin, luteolin, and (-)-epigallocatechin do not (data not shown). These results indicate that only flavonols emit fluorescence and other flavonoid subclasses do not (with the exception of naringenin). Interestingly, not only do the flavonol aglycones, but also the glycosides, emit fluorescence.

3. Application of fluorescence microscopy to the estimation of flavonol cellular uptake

The viability of fluorescence microscopy as a means of estimating of the cellular uptake of flavonols, was tested. The detection limit and dose-dependency were determined and compared with a biochemical method using a radioactive compound. Fluorescent microscopic analysis was carried out using Hepa-1c1c7 cells treated with varying concentrations of kaempferol as a model compound (0, 10, 50 and 100 μM for 70 min).

The cells were treated with [^3H]-kaempferol at 1, 10 and 20 μM for 70 min. The cell lysate was transferred to vials with a scintillation cocktail, and the radioactivity incorporated into the cells was measured using a liquid scintillation counter. Data are represented as the mean \pm SE (n=3). (Mukai, et al. 2009b)

Conditions for the fluorescent microscopic analysis are the same as those described in section 1. The uptake of radioactive flavonol by hepatocytes was tested as follows. Hepa-1c1c7 cells, seeded onto a 24-well culture plate at a density of 2×10^5 cells/well and incubated for 24 h, were treated with 1, 10 and 20 μM [^3H]-kaempferol (Moravek Biochemicals Inc.) in Krebs-Ringer HEPES buffer for 70 min. The cells were washed three times with ice-cold PBS and lysed by addition of 250 μL of 0.05 M NaOH and incubation for 4h. The lysate was transferred to a vial with a scintillation cocktail, and the radioactivity incorporated into the cells was measured using a liquid scintillation counter.

Monitoring the autofluorescence of the Hepa-1c1c7 cells after treatment with various concentrations of kaempferol using the confocal scanning microscope indicated that autofluorescence was too weak for imaging in cells treated with 10 μM kaempferol (Fig. 7) but green autofluorescence could be detected in cells treated with 50 μM or more. On the other hand, measurement of the incorporated radioactivity, reflecting kaempferol levels in the cells, increased in a dose-dependent manner and could be readily observed with cell treatment with only 10 μM kaempferol (Fig. 8). Under our experimental conditions, incorporation levels (ratio of the amount of uptake to the amount of treatment) were almost the same; about 7.1 to 7.6% of kaempferol was incorporated into Hepa-1c1c7 cells.

From these results, the sensitivity of the confocal microscopy method is lower than that of the biochemical method using radioactive compound. This may be because the former method only allows detection of incorporated flavonol aglycone, where the latter method allows detection of the aglycone as well as its metabolites. This indicates that the sensitivity of the confocal microscope method is likely to be dependent on the cell type analyzed, because the metabolism of flavonoids differs between different cell types (see section 3).

The time-dependence of kaempferol uptake was tested by monitoring the autofluorescence of cells treated with 50 μM kaempferol for incubation periods of 0, 1, 5, 10, 15, 30, 45, and 70 min, and the intensity of fluorescence was observed to increase in a time dependent manner (Fig. 9). After kaempferol treatment for 15 min, fluorescence was observed in the cells.

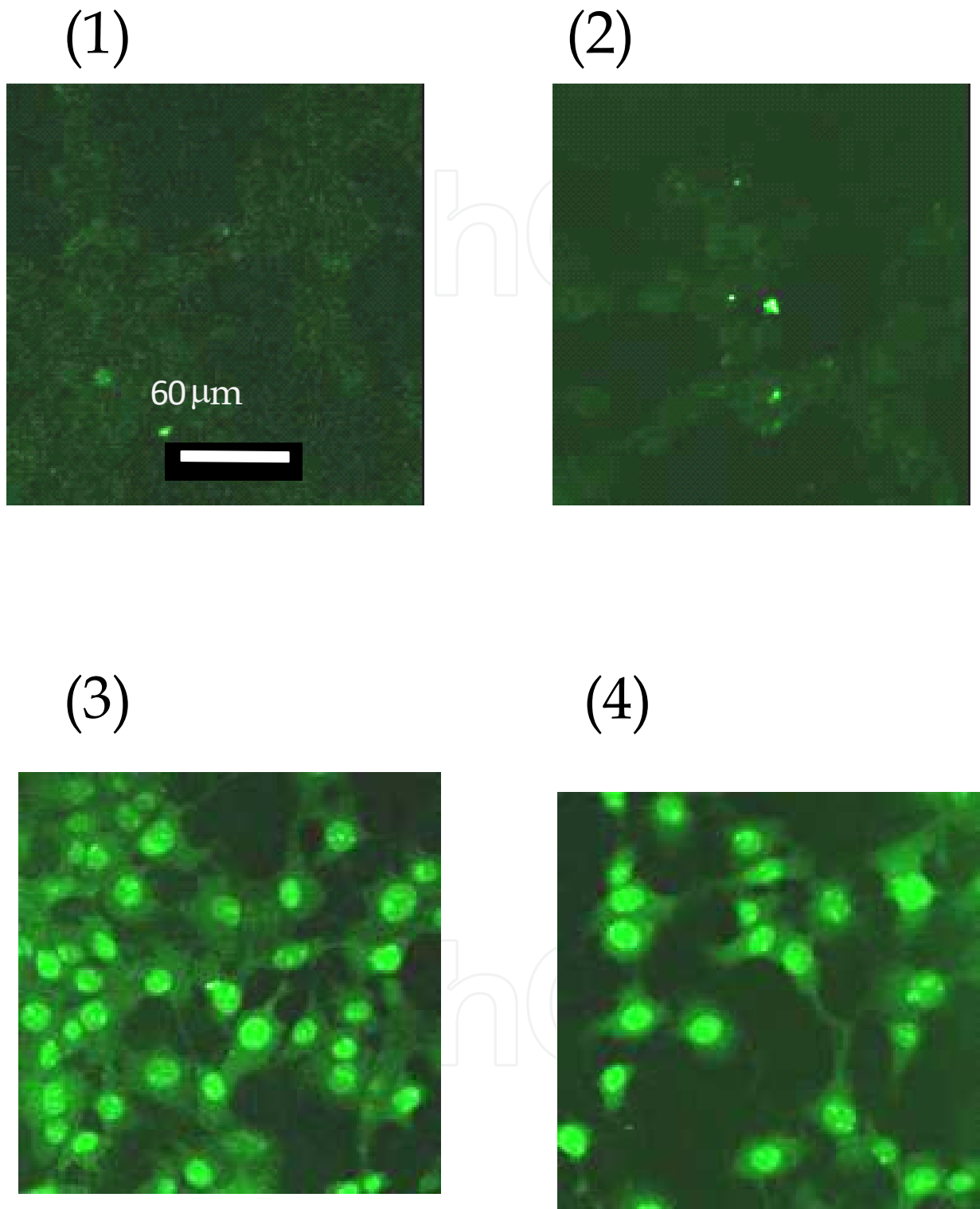


Fig. 7. Cellular uptake of kaempferol was visualized by fluorescence microscopy in Hepa-1c17 cells. The cells were seeded onto glass bottom dishes and treated with kaempferol at (1) 0 μM , (2) 10 μM , (3) 50 μM and (4) 100 μM for 70 min. Green fluorescence was observed under a confocal microscope (Ex. 488 nm- Em. 515-535 nm). (Mukai, et al. 2009b)

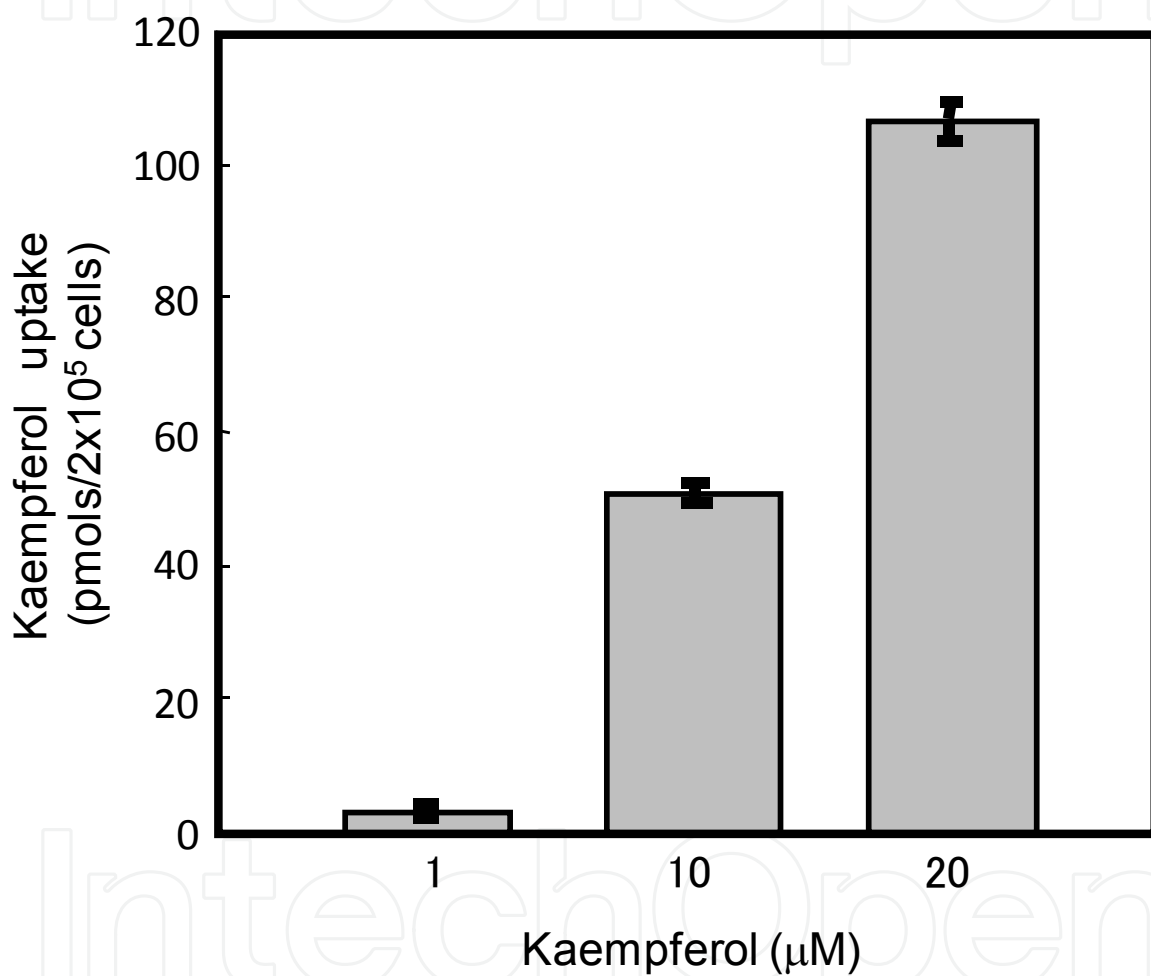


Fig. 8. The cells were treated with [³H]-kaempferol at 1, 10 and 20 μM for 70 min. The cell lysate was transferred to vials with a scintillation cocktail, and the radioactivity incorporated into the cells was measured using a liquid scintillation counter. Data are represented as the mean ± SE (n=3). (Mukai, et al. 2009b)

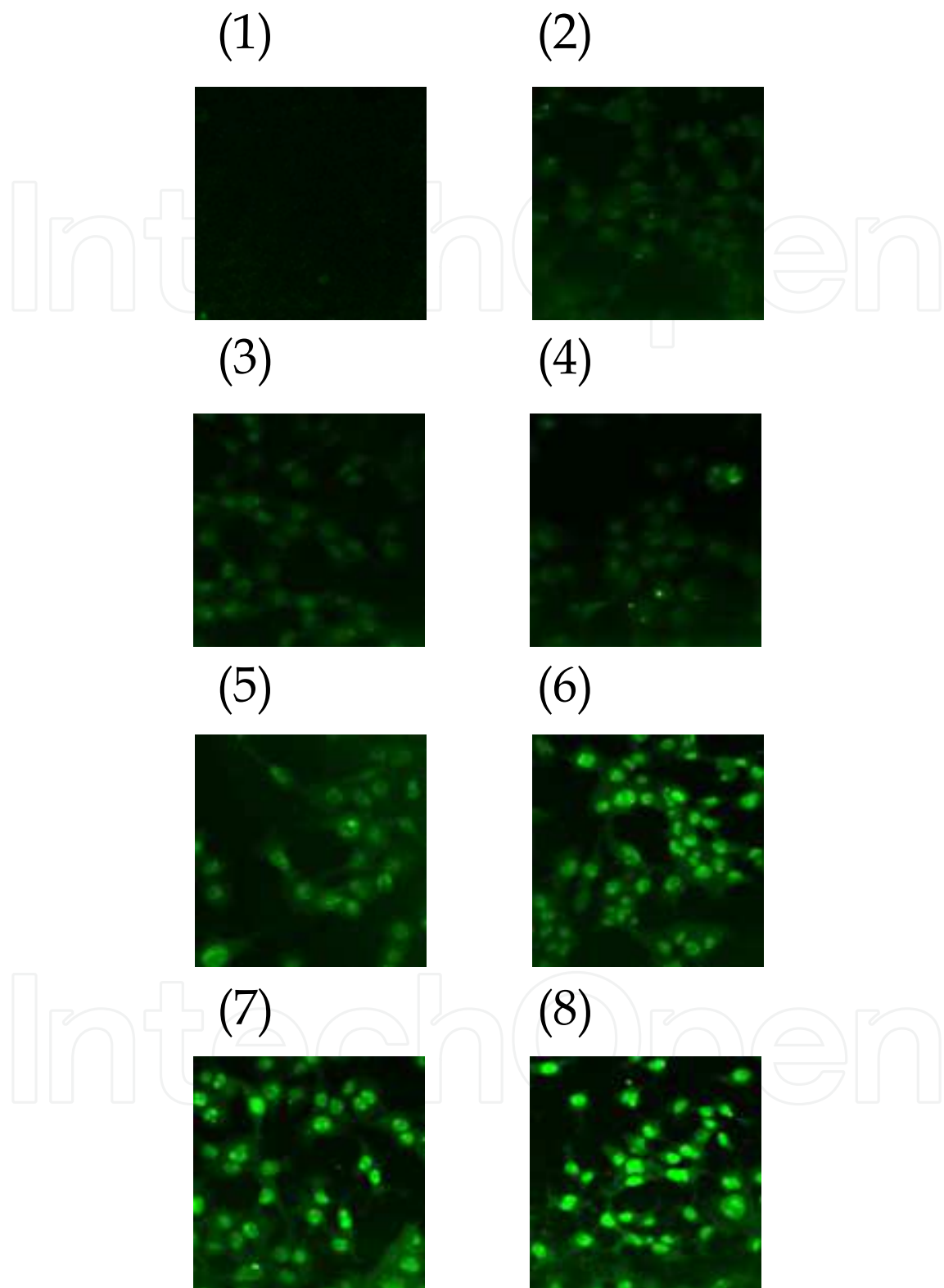


Fig. 9. Time-dependent cellular uptake of kaempferol in Hepa-1c1c7 cells. The cells on glass bottom dishes were treated with kaempferol at 50 μ M for (1) 0, (2) 1, (3) 5, (4) 10, (5) 15, (6) 30, (7) 45, and (8) 70 min. Green fluorescence was observed under a confocal microscope. (Mukai, et al. 2009b)

4. Determination of subcellular localization of flavonol in cultured cells

Culture conditions of Hepa-1c1c7 cells are described in sections 1 and 2. Human umbilical vein endothelial cells (HUVEC) were grown and maintained in a medium specially designed for endothelial cells obtained from Cell Applications Inc. (San Diego, CA). Neuro2A neuroblastoma cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% BSA and penicillin-streptomycin at 100 U/ml and 100 µg/ml respectively, and after incubation for 24h, the cells were maintained in serum free DMEM containing penicillin-streptomycin. Caco-2 human colonic adenocarcinoma, were maintained in DMEM containing 15% BSA, 0.1% non-essential amino acids, 100 U/ml and 100 µg/ml streptomycin. Cells were maintained at 37 °C under a humidified atmosphere containing 5% CO₂.

All cells were seeded onto glass-bottomed culture dishes (MatTek Corp., Ashland, MA) and were grown until confluent. The cells were incubated with fresh culture medium for 24 h prior to treatment, and then treated with the test flavonols for 70 min. Kaempferol, galangin and quercetin were used as the model flavonols and were added as a solution at 100 µM in culture medium containing 0.1% dimethyl sulfoxide. The cells were washed with ice-cold PBS (pH 7.4) and then fixed with 4% paraformaldehyde and 0.2% picric acid in 100 mM sodium phosphate buffer (pH 7.2) overnight, at 4 °C and in the absence of light. To determine the subcellular localization of flavonols in intact mouse hepatoma Hepa-1c1c7 cells, HUVEC, mouse neuroblastoma Neuro2A cells and human colonic carcinoma Caco-2 cells, the nucleus was counter-stained with propidium iodide (PI, Fig. 10 and 11). The cells were washed with PBS, and incubated in PBS containing 0.3% tritonX-100 for 20 min at room temperature. The cells were incubated in 2X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) containing 100 µg/ml RNase for 30 min at 37 °C. The cells were rinsed 3 times in 2X SSC for 1 minute each time. The cells were incubated with 500 nM PI in 2X SSC for 30 min at room temperature and rinsed again as above. These cells were stored at 4 °C and used for measurement of autofluorescence within 24 h. Autofluorescence from the flavonol was observed using confocal laser scanning fluorescence microscopy with excitation by an argon laser at 488 nm and a 515–535 nm band pass filter. PI fluorescence was monitored at 543 nm (HeNe excitation) with a 560–615 nm band pass filter. Low energy laser excitation was used during observation, but maximum energy laser excitation was used for imaging the autofluorescence of the flavonols.

In hepa-1c1c7 cells and HUVEC (Figs. 10 and 11), the red fluorescence (PI) overlapped with the green fluorescence from the flavonols and yellow fluorescence was observed in merged images. (Mukai, et al. 2009b) Therefore, flavonols incorporated into Hepa-1c1c7 cells or HUVEC accumulated in the nucleus. In neuro2A (Fig. 11), the red fluorescence (PI) was separate from the green fluorescence of flavonol. The merged image therefore showed both green and red fluorescence, indicating that the flavonols incorporated into neuron 2A cells accumulated in the cytoplasm. In the case of Caco-2 cells (Fig. 11), the green fluorescence from flavonol completely covered the cells. In the merged image, orange fluorescence was observed, and flavonol fluorescence did not overlap with the PI. Thus, most of the flavonol was associated with the cellular membrane of Caco-2 cells, and did not penetrate into the cells.

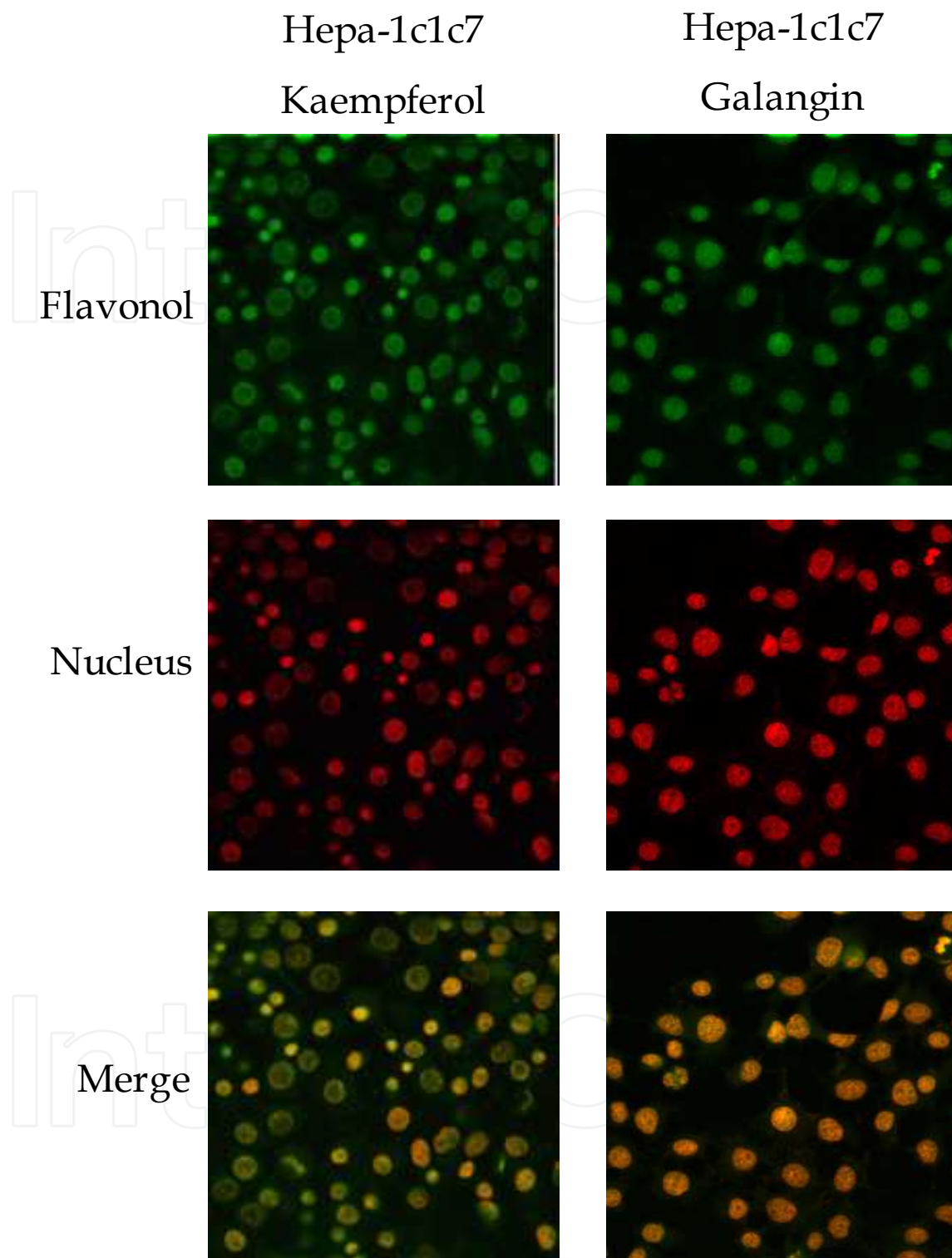


Fig. 10. Subcellular localization of kaempferol and galangin in Hepa-1c1c7 cells. The cells were seeded onto glass bottom dishes, treated with kaempferol (left) or galangin (right) at 50 μ M for 70min, and stained with PI. Green fluorescence (Ex. 488 nm- Em. 515-535 nm) from flavonol (top) and red fluorescence (Ex. 543 nm-Em. 560-615) from PI (middle) were monitored under a confocal microscope, and the images were merged (bottom). (Mukai, et al. 2009b)

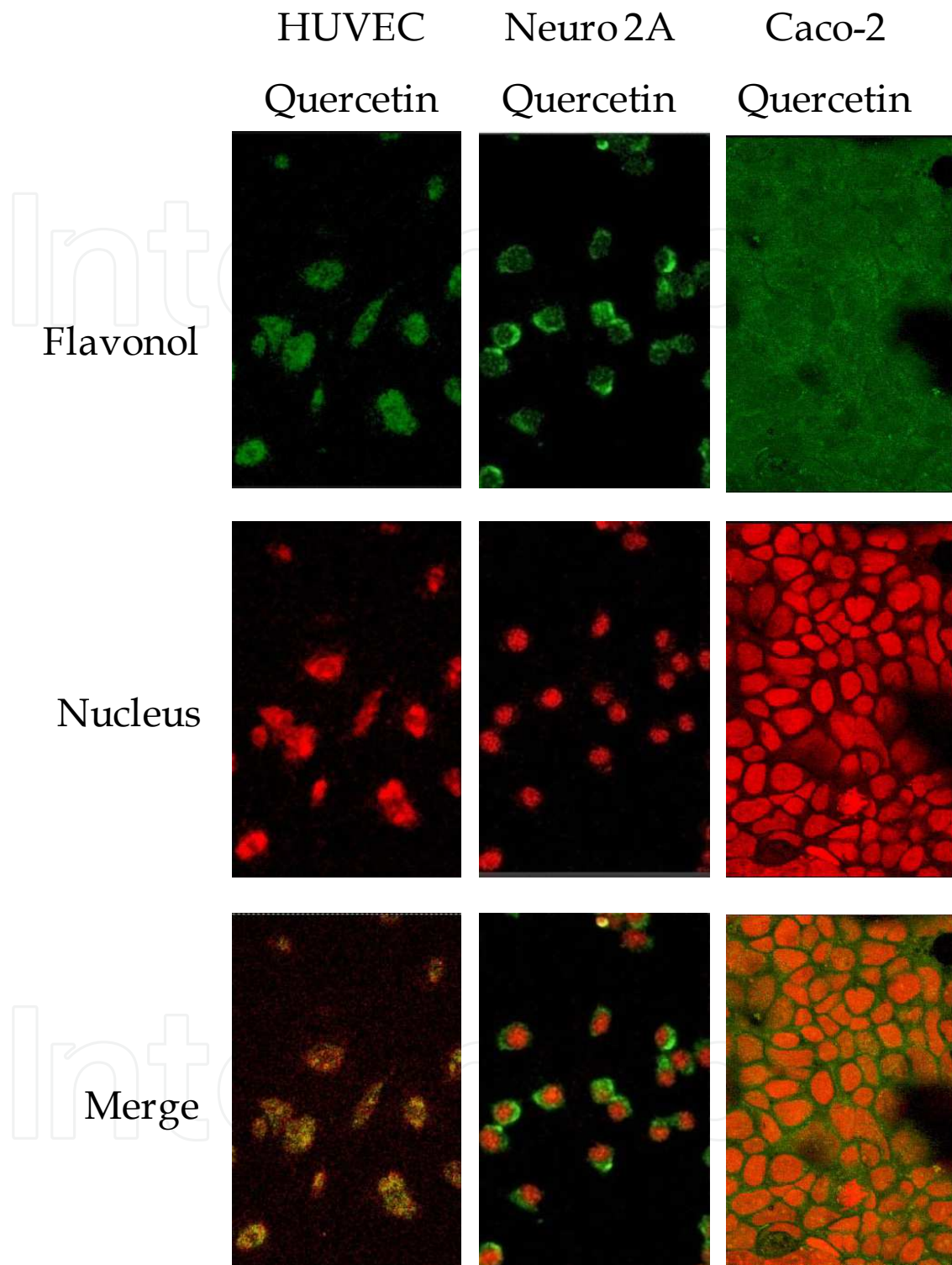


Fig. 11. Subcellular localization of quercetin in HUVEC, neuron 2A, and Caco-2 cells. HUVEC (left), neuron 2A (center), and Caco-2 cells (left) were seeded onto glass bottom dishes, treated with quercetin at 50 μ M for 70min and stained with PI. Green fluorescence (Ex. 488 nm- Em. 515-535 nm) from flavonol (top) and red fluorescence (Ex. 543 nm-Em. 560-615) from PI (middle) were monitored under a confocal microscope, and the images were merged (bottom).

5. Discussion and conclusion

We describe here the use of laser scanning microscopy as an effective tool for the estimation of subcellular localization of flavonols in intact cultured cells. The results demonstrate that the subcellular localization of flavonol aglycones is different in different cell types: flavonol aglycones accumulate into the nucleus of Hepa-1c1c7 cells (Mukai, et al. 2009b) and HUVEC, into the cytoplasm of neuro 2A, and are associated with the cellular membrane of Caco-2 cells (Figs. 10 and 11). This method for subcellular localization is superior to HPLC and LC/MS methods, as it does not require pretreatments such as fractionation of the cells and extraction of the compound from the cells. Moreover, this method allows analysis of subcellular localization more rapidly than pretreatment methods. This method therefore allows estimation of flavonol aglycone localization in intact cells with non-fluorescent medium, however, it does require relatively high concentrations of the target compound to be used, and does not allow cellular concentrations to be quantified. When fluorescence-labeled compounds are used, it is necessary to consider whether the fluorescent label influences subcellular localization of the target compound. A further advantage of this method is therefore that changes in localization as a result of fluorescent labeling do not need to be considered, as the flavonol is used in its native form. As with all flavonol, observation should be carried out as quickly as possible and the sample should be kept in darkness, to avoid quenching of the autofluorescence, because flavonol itself easily undergoes decomposition.

A correlation between chemical structure and autofluorescence is observed, with the hydroxyl group at the C3-position in the flavonol skeleton being important to fluorescence, and no fluorescence observed in cells treated with other flavonoids. Even flavonols did not show autofluorescence after treatment of Hepa-1c1c7 cells if they were in the form of glycosides at the C3-position (Fig 4), although rutin (quercetin-3-rutinoside) showed fluorescence between ex. 470 - 480 nm without the presence of cultured cells (Fig.6). This difference may be due to a lack of uptake of the intact flavonol glycoside by hepatic cells. It has been reported that during the absorption process into the body, quercetin glycosides are hydrolyzed to the aglycone form by epithelial hydrolytic enzymes or enterobacteria in the intestinal tract (Murota and Terao 2003; Terao 2009). During absorption and metabolism, the flavonol aglycone is converted to a glucuronide or sulfate conjugate (Murota and Terao 2003). Therefore, it has been suggested that flavonol glycosides are unable to cross cellular and nuclear membranes without hydrolytic cleavage or glucuronic acid/sulfuric acid conjugation at the C3-position. The flavanone naringenin emitted fluorescence in the absence of cultured cells (Fig. 6), but not in the presence of cells (data not shown). It has been reported that the cellular uptake of flavanone naringenin is lower than that of flavonol (Tourniaire, et al. 2005), suggesting that the cellular uptake of naringenin is insufficient for observation by the fluorescent microscopy. To elucidate these points, further study is needed to clarify the structure-fluorescence relationship and absorption mechanism in cultured cells.

Cellular efflux is also an important event in flavonol metabolism, and flavonols are known to be excreted from cells through a cell membrane efflux transporter (Brand, et al. 2008; Ofer, et al. 2005). Treatment of cells with an inhibitor of the efflux pump such as P-glycoprotein increases the cellular concentration of flavonol and enhances its beneficial effects in cultured cells and experimental animals (Mukai, et al. 2009a; Wang, et al. 2005). It is also known that flavonols can have pharmacological effects in both the cytoplasm and

nucleus of cultured cells. For example, flavonol affects the function of aryl hydrocarbon receptors in cytoplasm (Mukai, et al. 2010). In the nuclei of cancer cells, flavonols induce the oxidative cleavage of cellular DNA in the presence of copper ions and may therefore slow the progression of cancer growth (Hadi, et al. 2007). In this chapter, we have shown that subcellular localization of flavonols varies between cell lines (Fig. 10 and 11) although what factors mediate these differences in flavonol localization are not known. Further study is therefore needed to clarify these results and to investigate the relationship between the function and subcellular localization of flavonols in various cell lines. Flavonols have been detected in the liver, lung, white fat, muscle, brain, kidney, heart, and spleen of experimental animals by HPLC and LC/MS (de Boer, et al. 2005; Urpi-Sarda, et al. 2008; Wang, et al. 2003). It would therefore be of interest to investigate the subcellular localization of flavonols using fluorescence microscopy in other cell types in addition to those investigated in this chapter. It could be powerful tool for confirming the detailed subcellular localization by stains for cellular organelles and cytoskeletons such as cellular membrane, mitochondria, tubulins, and actins. In addition, cells have polarity for example enterocyte, which has apical side and basolateral one. The polarity of cell is involved in the intercellular and/or intracellular transport. To determine the relationships between cell-polarity and flavonol-transport, three-dimensional observation using confocal fluorescent microscopy would be an excellent method for detecting localization.

This method is expected to be applicable for the study of subcellular localization for other fluorescent trace food components, drugs, and Chinese medicine components in various types of cells. It is hoped that this method will contribute to clarifying the relationship between subcellular localization of target chemicals and their beneficial and/or toxicological functions in various cell types in future.

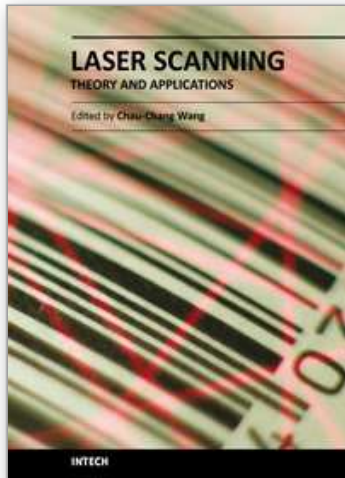
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Ever since the invention of laser by Schawlow and Townes in 1958, various innovative ideas of laser-based applications emerge very year. At the same time, scientists and engineers keep on improving laser's power density, size, and cost which patch up the gap between theories and implementations. More importantly, our everyday life is changed and influenced by lasers even though we may not be fully aware of its existence. For example, it is there in cross-continent phone calls, price tag scanning in supermarkets, pointers in the classrooms, printers in the offices, accurate metal cutting in machine shops, etc. In this volume, we focus the recent developments related to laser scanning, a very powerful technique used in features detection and measurement. We invited researchers who do fundamental works in laser scanning theories or apply the principles of laser scanning to tackle problems encountered in medicine, geodesic survey, biology and archaeology. Twenty-eight chapters contributed by authors around the world to constitute this comprehensive book.

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