

# Enzymatic degradation of human skin dermis revealed by fluorescence and reflectance spectroscopy

Ye Yuan and Patricia Relue

Cellular Engineering Laboratory, The University of Toledo, Ohio 43606  
[prelue@eng.utoledo.edu](mailto:prelue@eng.utoledo.edu)

**Abstract:** Dermis is the major source of the fluorescence and light scattering of skin. Tumor-induced degradation of the dermis is expected to change the fluorescence and light scattering properties of skin. To investigate how these fluorescence and light scattering properties are changed, human skin dermis was degraded with enzymes to mimic tumor invasion. The enzymatic erosion process was investigated with fluorescence and reflectance spectroscopy. Dermis degradation by the enzymes resulted in a decrease in fluorescence emission and light scattering in the dermis. Fluorescence anisotropy, however, could not detect the change in the dermis induced by the enzyme treatments.

©2008 Optical Society of America

**OCIS codes:** (170.6510) Spectroscopy, tissue diagnostics; (170.6930) Tissue; (170.6280) Spectroscopy, fluorescence and luminescence; (170.4580) Optical diagnostics for medicine.

---

## References and links

1. N. Ramanujam, "Fluorescence spectroscopy of neoplastic and non-neoplastic tissues," *Neoplasia* **2**, 89-117 (2000).
2. T. J. Romer, M. Fitzmaurice, R. M. Cothren, R. Richards-Kortum, R. Petras, M. V. Sivak Jr. and J. R. Kramer Jr, "Laser-induced fluorescence microscopy of normal colon and dysplasia in colonic adenomas: implications for spectroscopic diagnosis," *Am. J. Gastroenterol.* **90**, 81-87 (1995).
3. B. Mayinger, M. Jordan, P. Horner, C. Gerlach, S. Muehldorfer, B. R. Bittorf, K. E. Matzel, W. Hohenberger, E. G. Hahn and K. Guenther, "Endoscopic light-induced autofluorescence spectroscopy for the diagnosis of colorectal cancer and adenoma," *J. Photochem. Photobiol. B* **70**, 13-20 (2003).
4. I. Georgakoudi, E. E. Sheets, M. G. Muller, V. Backman, C. P. Crum, K. Badizadegan, R. R. Dasari and M. S. Feld, "Trimodal spectroscopy for the detection and characterization of cervical precancers in vivo," *Am. J. Obstet. Gynecol.* **186**, 374-382 (2002).
5. I. Pavlova, K. Sokolov, R. Drezek, A. Malpica, M. Follen and R. Richards-Kortum, "Microanatomical and biochemical origins of normal and precancerous cervical autofluorescence using laser-scanning fluorescence confocal microscopy," *Photochem. Photobiol.* **77**, 550-555 (2003).
6. I. Georgakoudi, B. C. Jacobson, M. G. Muller, E. E. Sheets, K. Badizadegan, D. L. Carr-Locke, C. P. Crum, C. W. Boone, R. R. Dasari, J. Van Dam and M. S. Feld, "NAD(P)H and collagen as in vivo quantitative biomarkers of epithelial precancerous changes," *Cancer Res.* **62**, 682-687 (2002).
7. T. Vo-Dinh, M. Panjehpour, B. F. Overholt, C. E. Julius, S. Overholt and M. N. Phan, "Laser-induced fluorescence for the detection of esophageal and skin cancer," *Adv. Biomed. Clin. Diagn. Syst.* **4958**, 67-70 (2003).
8. U. Utzinger, N. D. Kirkpatrick, R. A. Drezek and M. A. Brewer, "Endogenous fluorescence emission of the ovary," in *Optical Diagnostics and Sensing V*, A. V. Priezzhev and G. L. Cote, eds., *Proc. SPIE* **5702**, 92-96 (2005).
9. Z. Huang, H. M. Cheah, T.-C. Chia and T. L. Ching, "Laser-induced microscopic fluorescence and images of skin tissues," in *Laser Microscopy*, K. Koenig, H. J. Tanke and H. Schneckenburger, eds., *Proc. SPIE* **4164**, 43-47 (2000).
10. R. Na, I. M. Stender and H. C. Wulf, "Can autofluorescence demarcate basal cell carcinoma from normal skin? A comparison with protoporphyrin IX fluorescence," *Acta Derm.-Venereol.* **81**, 246-249 (2001).
11. L. Brancaleon, A. J. Durkin, J. H. Tu, G. Menaker, J. D. Fallon and N. Kollias, "In vivo fluorescence spectroscopy of nonmelanoma skin cancer," *Photochem. Photobiol.* **73**, 178-183 (2001).
12. M. Panjehpour, C. E. Julius, M. N. Phan, T. Vo-Dinh and S. Overholt, "Laser-induced fluorescence spectroscopy for in vivo diagnosis of non-melanoma skin cancers," *Laser Surg. Med.* **31**, 367-373 (2002).
13. R. Richards-Kortum and E. Sevick-Muraca, "Quantitative optical spectroscopy for tissue diagnosis," *Annu. Rev. Phys. Chem.* **47**, 555-606 (1996).

14. I. Georgakoudi, B. C. Jacobson, J. Van Dam, V. Backman, M. B. Wallace, M. G. Muller, Q. Zhang, K. Badizadegan, D. Sun, G. A. Thomas, L. T. Perelman and M. S. Feld, "Fluorescence, reflectance, and light-scattering spectroscopy for evaluating dysplasia in patients with Barrett's esophagus," *Gastroenterology* **120**, 1620-1629 (2001).
15. G. K. Steigleder and R. Uerdingen, "Response of collagenous tissue to pigmented tumors," *Hautarzt* **27**, 153-154 (1976).
16. D. E. Woolley and C. A. Grafton, "Collagenase immunolocalization studies of cutaneous secondary melanomas," *Brit. J. Cancer* **42**, 260-265 (1980).
17. K. Sokolov, M. Follen and R. Richards-Kortum, "Optical spectroscopy for detection of neoplasia," *Curr. Opin. Chem. Biol.* **6**, 651-658 (2002).
18. Y. Yuan, "Investigation of skin and skin components using polarized fluorescence and polarized reflectance towards the detection of cutaneous melanoma," Ph.D. dissertation, The University of Toledo, Toledo, OH, 2006.
19. D. R. Ralston, C. Layton, A. J. Dalley, S. G. Boyce, E. Freedlander and S. Mac Neil, "The requirement for basement membrane antigens in the production of human epidermal/dermal composites in vitro," *Brit. J. Dermatol.* **140**, 605-615 (1999).
20. M. Regnier, M. Prunieras and D. Woodley, "Growth and differentiation of adult human epidermal cells on dermal substrates," *Front Matrix Biol.* **9**, 4-35 (1981).
21. N. Kollias, R. Gillies, M. Moran, I. E. Kochevar and R. R. Anderson, "Endogenous skin fluorescence includes bands that may serve as quantitative markers of aging and photoaging," *J. Invest. Dermatol.* **111**, 776-780 (1998).
22. B. Banerjee, B. E. Miedema and H. R. Chandrasekhar, "Role of basement membrane collagen and elastin in the autofluorescence spectra of the colon," *J. Invest. Med.* **47**, 326-332 (1999).
23. J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, 2nd ed., (Kluwer Academic/Plenum, New York, 1999).
24. X. Wang and L. V. Wang, "Propagation of polarized light in birefringent turbid media: A Monte Carlo study," *J. Biomed. Opt.* **7**, 279-290 (2002).
25. P. R. Odetti, A. Borgoglio and R. Rolandi, "Age-related increase of collagen fluorescence in human subcutaneous tissue," *Metabolism* **41**, 655-658 (1992).
26. N. Kollias, G. Zonios and G.N. Stamatias, "Fluorescence spectroscopy of skin," *Shedding New Light on Disease: Optical Diagnostics for the New Millennium* **28**, 17-23 (2002).
27. K. A. Piez and A. H. Reddi, *Extracellular Matrix Biochemistry* (Elsevier, New York, c1984).
28. K. S. Stenn, R. Link, G. Moellmann, J. Madri and E. Kuklinska, "Dispase, a neutral protease from *Bacillus polymyxa*, is a powerful fibronectinase and type IV collagenase," *J. Invest. Dermatol.* **93**, 287-290 (1989).

## 1. Introduction

Decreased tissue fluorescence has been observed to be associated with tissue malignancy in many tissue types [1]. The decrease of tissue fluorescence has been attributed to the loss of collagen fluorescence in colon [2, 3], cervix [4-6], esophagus [6, 7], ovary [8], and skin [7, 9-12], as the tissues progress towards malignancy. In addition, tissue scattering is also changed due to the structural changes of the connective tissue associated with tumor progression [5, 13, 14]. Studies of skin cancer have demonstrated that collagen matrix is reduced at the locations where tumor cells reside [15]. The residual collagen structures in skin cancer are associated with collagenase, suggesting a role of the collagenase in facilitating extracellular matrix breakdown and tumor invasion [16]. Therefore, as the collagen in the dermis surrounding the tumor is lost, the scattering should decrease and become lower than in the normal dermis.

Collagen cross-links are major fluorophores of the connective tissue. Decreased collagen fluorescence in the tumor tissue is ascribed to a reduced number of collagen cross-links in the tumor stroma, which is suggested to be the result of the degradation of collagen fibers by the collagenases secreted by the tumor and stromal cells [4-6]. The decrease of fluorescence by basal cell carcinomas (BCC) is greater than that by squamous cell carcinomas (SCC), suggesting a more extensive invasion of the dermis by BCC than by SCC [11].

The changes of collagen fluorescence and scattering properties can occur in the early stages of cancer development [14, 17]. Therefore, optical techniques, such as fluorescence and reflectance spectroscopy, can be useful in probing the events occurring in the early stages of cancer development. In this study, tumor invasion of the dermis was experimentally modeled using dermis prepared from cadaver skin. Dispase was used to break down the basement membrane on the dermis to mimic the earliest step of tumor invasion. To model later tumor

invasion into the dermis, dispase was used to completely remove the basement membrane [18], and Type I collagenase was applied to the dermis to partially erode the matrix structure of the dermis. Fluorescence spectroscopy with non-polarized illumination was used to investigate how the enzymatic degradation of the dermis changes the intensity of the fluorescence emission of the dermis. Reflectance spectroscopy with polarized illumination was used to investigate the enzyme-induced changes of the scattering property of the dermis, since the degree of linear polarization (DLP) calculated with the partially polarized backscattered light from the dermis provides information about multiple scattering within the dermis, where increased scattering tends to depolarize incident polarized light to a greater extent, leading to a lower DLP [18]. Since enzymatic erosion of the dermis may also change the physicochemical properties of the collagen fluorophores and their microenvironment, fluorescence anisotropy of the enzyme-treated dermis was also investigated using fluorescence spectroscopy with polarized illumination.

## 2. Materials and methods

### 2.1 Experimental setup

Figure 1 shows the fluorescence and reflectance spectroscopy set-up. An HBO 100W mercury arc lamp (Osram, Danvers, MA) was used as a light source. Excitation light was selected by three excitation bandpass filters ( $350\text{nm}\pm 20\text{nm}$ ,  $360\text{nm}\pm 20\text{nm}$ , and  $375\text{nm}\pm 20\text{nm}$ , Chroma Technology) mounted on a Lambda 10 filter wheel (Sutter Instruments, Novato, CA). A dichroic mirror ( $390\text{nm}$ ) was used to reflect the UV excitation light which was coupled into an illumination fiber. Two multimode silica-core optical fibers (Thorlabs, Newton, NJ) with the core diameter of 1mm and NA of 0.22 were used to deliver the excitation light and collect fluorescence emissions. The distance of the fiber tip to the sample surface was 1 cm for both the illumination and collection fibers. The excitation light beam was normal to the sample surface. The angle between the illumination and collection fibers was  $45^\circ$ . The first linear polarizer (polarizer) was a UV passing glass polarizer (Edmund Optics). The polarization axis of the polarizer could be set to be either parallel or perpendicular to the scattering plane which was defined by the excitation light beam and the light path of the backscattered light collected by the collection fiber. The second linear polarizer (analyzer) consisted of a pair of film polarizers (Edmund Optics) oriented orthogonally to one another. The polarization axis of one of the pair of film polarizers was set to be parallel to the scattering plane and the polarization axis of the other of the pair was set to be perpendicular to the scattering plane. The film polarizers passed light of wavelengths greater than  $380\text{nm}$ . A custom-designed holder was used for the fibers, the polarizer, and the analyzer to ensure reproducible positioning. The collection fiber was connected to a USB2000 fiber optic spectrometer (Ocean Optics, Dunedin, FL) as the detector. Both the filter wheel and the spectrometer were controlled by a PC. For reflectance spectroscopy, a LS-1 tungsten halogen light source (Ocean Optics, Dunedin, FL) with a 1.0 neutral density filter was used. A multimode optical fiber same as those described above was used to deliver the halogen light. Fluorescence and reflectance spectra collected by the spectrometer were displayed and stored on the PC. To collect non-polarized spectra, the linear polarizers were removed from the light path.

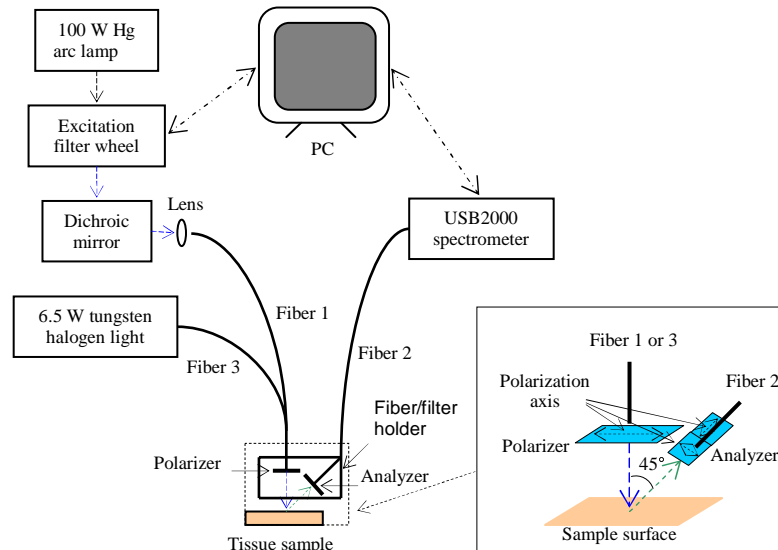


Fig. 1. Fluorescence and reflectance spectroscopy set-up. For fluorescence spectroscopy, fiber 1 was used for illumination. For reflectance spectroscopy, fiber 3 was used for illumination. Fiber 2 was used to deliver either fluorescence emission or reflectance from the sample to the spectrometer. The inset shows the details of the light paths near sample surface and the polarization orientation of the polarizer and the analyzer.

## 2.2 Enzymatic degradation of acellular dermis

Skin samples were collected from a local tissue bank. Acellular dermis (hereinafter referred as dermis) was made using methods described in literature [20]. The dermis of about 1mm thick was cut with a sterile scalpel into small pieces of about 10mm×20mm. For enzyme treatment, one piece of dermis was transferred to one well of a six-well tissue culture plate (Greiner Labortechnik) with the papillary dermis side facing up. A sterile stainless steel ring with an inner diameter of 8mm was placed on the dermis. A 0.5ml aliquot of 1.2U/ml dispase (Sigma-Aldrich) in acetate buffer (1mM sodium acetate, 5mM calcium acetate, pH 7.50) was placed into the ring, and the rest of the dermis was submerged with 3 ml of acetate buffer. To determine the time required for dispase removal of the basement membrane, three pieces of dermis were treated with dispase and incubated at 37°C in a water-saturated incubator (NAPCO). The dispase-treated dermis was removed at incubation times of 1, 2, and 3 hours, fixed in 10% formalin, and stained with periodic acid-Schiff staining (PAS staining) for the basement membrane [19].

To model the process of tumor-associated degradation of the dermis, eight pieces of dermis were placed in the wells of two six-well tissue culture plates. Two pieces of dermis were treated with acetate buffer only as a control (hereinafter referred as dermis control). The remaining 6 pieces of dermis were treated with dispase. All of the dermis samples were incubated at 37°C in the incubator. One piece of dermis was removed for spectroscopy after 1-hour incubation with dispase; all others were incubated for 2 hours (time determined in this study to remove the basement membrane). One piece of dispase-treated dermis and one piece of dermis control were removed for spectroscopy after 2 hours of incubation. The remaining 5 pieces of dermis were rinsed 3 times with Hank's balanced salt solution (HBSS) buffer (pH7.50). A 0.5ml aliquot 100U/ml Type I collagenase (Sigma-Aldrich) solution in HBSS (pH 7.50) was added to the dispase-treated area on each of the 4 pieces of dermis in the same way as for the dispase treatment. The rest of the dermis was submerged with 3 ml of HBSS. The dermis control was submerged with 3.5ml of HBSS buffer only as a control. The remaining dermis samples were again incubated at 37°C in the incubator. The collagenase-

treated dermis was removed for spectroscopy after incubation times of 2, 4, 8, and 16 hours. The dermis control was evaluated spectroscopically after 16 hours of incubation.

### 2.3 Fluorescence and reflectance spectroscopy of the dermis

Collagen fluorophores that contribute to the fluorescence of dermis are complicated and largely unknown. Enzymatic degradation may cause different changes to the collagen fluorophores. Since the collagen fluorophores can be excited by UVA light [21], three excitation wavelengths at 350, 360 and 375nm were used in this study to better characterize the complex nature of dermal fluorescence. These 3 excitation wavelengths are optimal for the excitation of Type I, Type III, Type IV collagen, and elastin [22], all of which are components of the dermis. The dermal fluorescence spectra excited at these 3 wavelengths showed a red-shift with increasing the excitation wavelength (data not shown).

The dermis samples were rinsed 3 times with sterile deionized water after enzyme treatment. Fluorescence and reflectance spectra were collected from 2 different sampling areas on each piece of dermis, one area being enzyme-treated and the other being an adjacent untreated control area. For the dermis control, one sampling area was on one half of the dermis and the other sampling area was on the other half. The dermis samples were kept moist during the measurement by adding drops of sterile deionized water to the edges of the samples. To investigate the changes of fluorescence intensity induced by enzymatic degradation, two non-polarized fluorescence spectra were collected at each excitation wavelength from each sampling area on the dermis. Sequential polarized fluorescence spectra were then collected with the polarization orientation between the polarizer and analyzer set as parallel, perpendicular, perpendicular, and parallel. The polarization orientation of the illumination light was set as parallel to the scattering plane. Polarized reflectance spectra were collected in the same manner immediately following the collection of the polarized fluorescence spectra. The fluorescence anisotropy (FA) and DLP were calculated as,

$$FA = \frac{I_{f//} - I_{f\perp}}{I_{f//} + 2I_{f\perp}} \quad (1)$$

$$DLP = \frac{I_{r//} - I_{r\perp}}{I_{r//} + I_{r\perp}} \quad (2)$$

where  $I$  is the intensity of a spectrum, and  $f$  and  $r$  refer to fluorescence and reflectance spectra, respectively, and  $//$  or  $\perp$  represent either parallel or perpendicular alignment of the polarizer and analyzer [23]. The two parallel and two perpendicular polarized spectra were averaged, respectively. FA and DLP were then calculated with the averaged spectra.

## 3. Results

### 3.1 Fluorescence emission of dispase and collagenase treated dermis

The dermis was treated with the enzymes for differing times and the fluorescence spectra for both the enzyme-treated area and its adjacent control area on the dermis were collected. As a reference, fluorescence spectra were also collected from two sampling areas on the dermis controls. The enzyme treatment did not lead to a noticeable decrease of the thickness of the dermis. The fluorescence measurements were repeated on 9 batches of dermis on different days. The fluorescence spectra were averaged over the two measurements taken on each sampling area; no photobleaching was observed when comparing the two measured fluorescence spectra from the same sampling areas. To compare enzyme-induced fluorescence changes, fluorescence spectra ratios were calculated by ratioing the treatment area spectra to the control area spectra. The fluorescence spectra ratios calculated for the 9 batches of dermis were averaged. The average fluorescence spectra ratios of the enzyme-treated dermis for 360 nm excitation are shown in Fig. 2. The fluorescence spectra ratios of the two dermis controls are very flat and equal to unity, showing the reproducibility of the spectra generated within a

sample. After a 1 hr treatment with dispase, the fluorescence spectra ratio is still flat but has decreased slightly. After treatment with dispase for 2 hrs, the fluorescence spectra ratio is no longer flat, but decreases considerably with increasing emission wavelength to a value near 0.85. For all subsequent treatments with collagenase, the fluorescence spectra ratios are basically unchanged regardless of the collagenase treatment time.

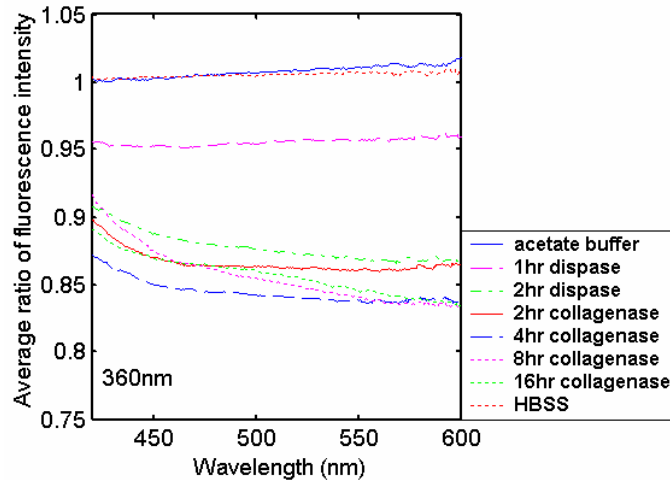


Fig. 2. Average fluorescence spectra ratios of the dermis treated with dispase and collagenase. The data of the two dermis controls were averaged over 8 batches of dermis. Since the behavior is relatively independent of the excitation wavelength, only data for 360nm is shown.

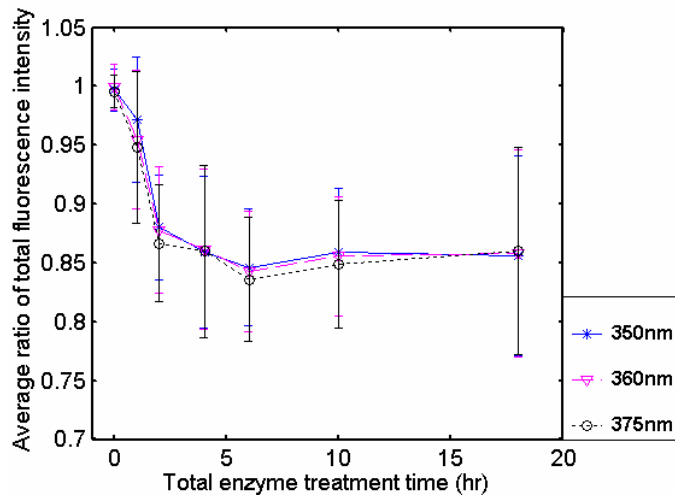


Fig. 3. The average ratio of total fluorescence is shown as a function of total enzyme treatment time. Data show the means and standard deviations for 8 batches of dermis. After 2hrs of treatment with dispase, the total fluorescence intensities at all 3 excitation wavelengths significantly decreased as compared to the control ( $p < 0.001$ ) and 1hr treatment with dispase ( $p < 0.05$ ). No significant decrease in the total fluorescence intensities was observed for 1hr treatment with dispase.

To quantify the changes in the fluorescence emissions, the total fluorescence for a sampling area was calculated from the fluorescence emission spectra by integrating over the

entire spectral range from 420nm to 600nm. The ratio of the total fluorescence in the treatment area to the control area on each piece of dermis was calculated. The average ratios of total fluorescence were calculated from 8 batches of dermis and the results are shown in Fig. 3. A sharp decrease of the fluorescence emission occurred during the first 2 hrs of treatment with dispase. The subsequent treatments with collagenase did not cause any additional decrease of the fluorescence emission. The enzyme-induced changes in the fluorescence emission are very similar for all 3 excitation wavelengths.

### 3.2 Fluorescence anisotropy of dispase and collagenase treated dermis

To investigate if enzymatic erosion changes the physicochemical properties of the collagen fluorophores and their microenvironments and to determine if enzymatic treatment results in excitation wavelength dependent changes in the fluorescence anisotropy, polarized fluorescence spectra were collected on the enzyme-treated dermis. The fluorescence anisotropy for each piece of dermis was calculated at each excitation wavelength. The fluorescence anisotropies were averaged over 9 batches of dermis. No excitation wavelength dependent difference in the fluorescence anisotropies were observed on the enzyme-treated dermis (Fig. 4). Considering that no difference is detected between the fluorescence anisotropies and between the decreases of the fluorescence emission from the 3 excitation wavelengths, the results suggest that the excitation wavelengths seem to target the same fluorophores, or the enzymes non-differentially digest all the fluorophores which are excited with these 3 excitation wavelengths.

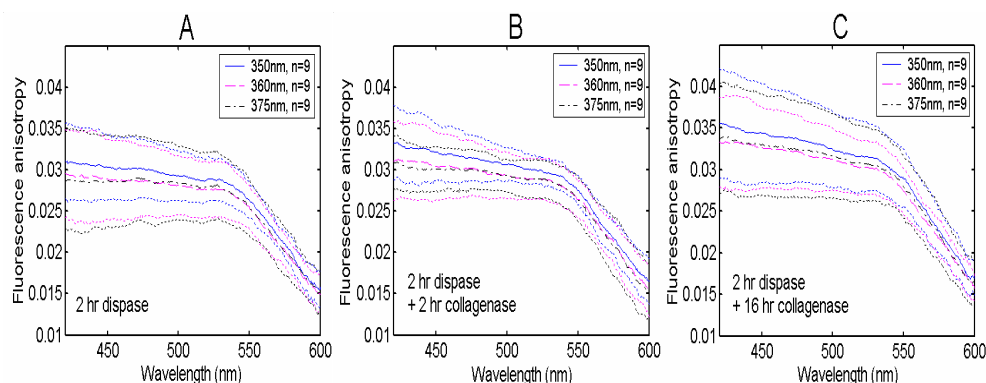


Fig. 4. Representative fluorescence anisotropies of the enzyme-treated dermis as a function of excitation wavelength. The dermis was treated with dispase for (A) 2 hrs, and further treated with collagenase for (B) 2 hrs and (C) 16 hrs. Dashed lines represent  $\pm$  one standard deviation for the data set.

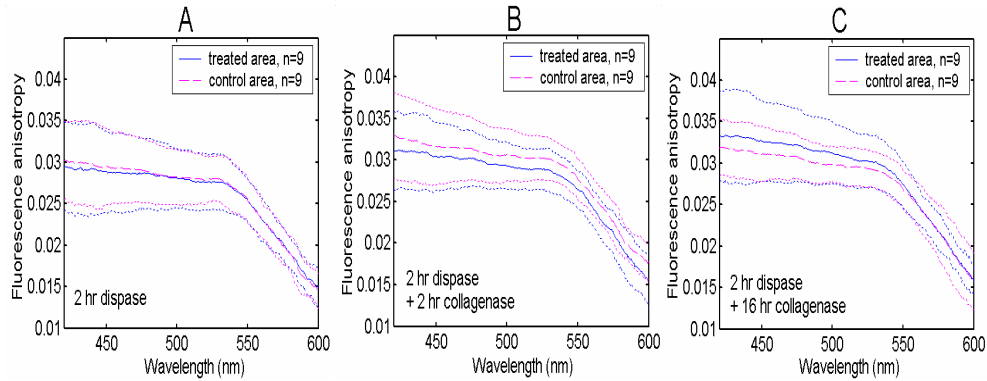


Fig. 5. Comparison of the fluorescence anisotropy of the enzyme-treated areas with that of the control areas. The dermis was treated with dispase for (A) 2 hrs, and further treated with collagenase for (B) 2 hrs and (C) 16 hrs. Dashed lines represent +/- one standard deviation for the data set.

The representative fluorescence anisotropies of the enzyme-treated and control areas are shown in Fig. 5. The fluorescence anisotropies were averaged over 9 batches of dermis; results are shown for 360nm excitation. For all the treatment conditions tested, no significant differences are observed between the enzyme-treated and control areas. Thus, it appears that the enzymatic degradation of the matrix did not change the rotation ability of the collagen fluorophores.

### 3.3 Dispace and collagenase treated dermis probed with polarized reflectance

To investigate whether enzymatic erosion changes the optical scattering properties of the dermis, polarized reflectance spectra were collected from the enzyme-treated dermis as previously described. The DLPs were calculated from the spectra collected on 9 batches of dermis and averaged. The average DLPs of the backscattered light from the enzyme-treated and control areas on the dermis are shown in Fig. 6. The average DLPs of the enzyme-treated and control areas are nearly identical for up to 4 hrs of total enzyme treatment. However, after 6hrs of enzyme treatment (2 hrs of dispase followed by 4 hrs of collagenase), the average DLP of the enzyme-treated areas increased appreciably relative to the control areas. The average DLP continued to increase with increased collagenase incubation time. The average DLPs of the treatment and control areas are replotted in Fig. 7. No change of the DLP is seen in any of the control areas. In addition, no change of the DLP is seen with the basement membrane degradation by dispase. However, The DLP increased with increasing the time of collagenase treatment, indicating a loss of scattering structures, most likely collagen fibers, in the dermis. It should be noted that collagen fibers are not only scattering structures but also birefringent structures. Linear birefringence in the dermis also contributes to the depolarization of the initially polarized incident light [24]. With the destruction of the collagen fibers by the enzymes, linear birefringence might decrease. Therefore, in addition to decreased scattering, a decrease in birefringence might also contribute to the increase of the DLP.

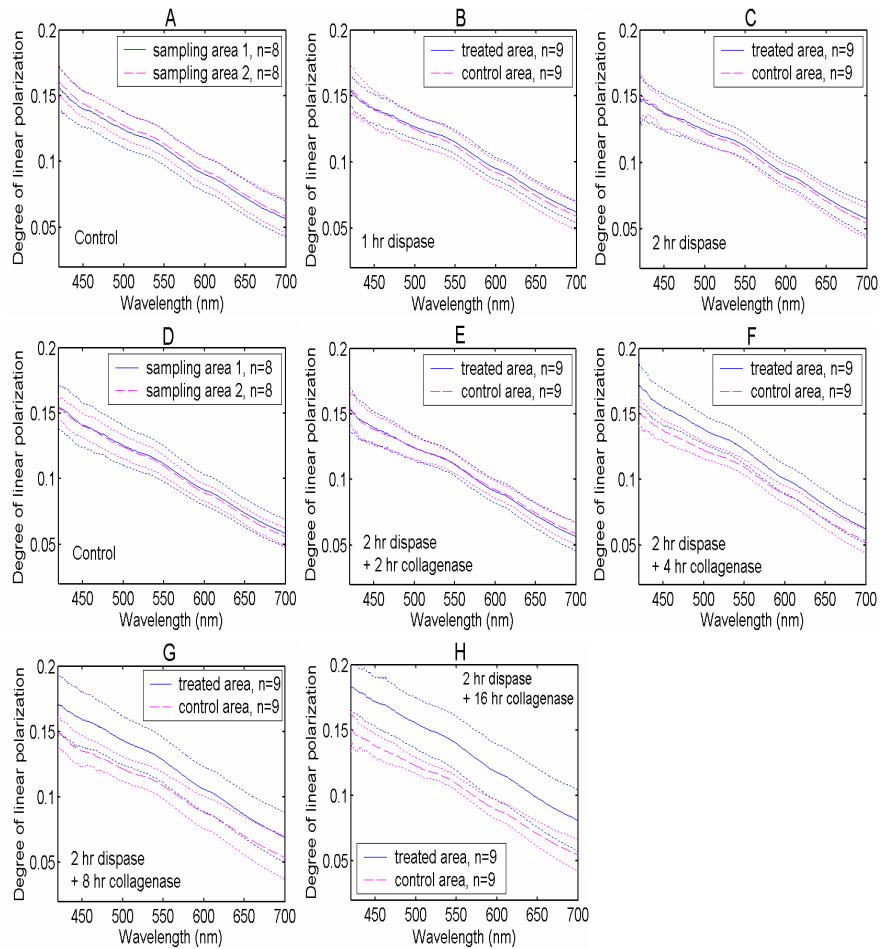


Fig. 6. Comparison of the DLP of the enzyme-treated areas with that of the control areas for the treatment with dispase for (B) 1hr and (C) 2 hrs; and further treatment with collagenase for (E) 2 hrs, (F) 4 hrs, (G) 8 hrs, and (H) 16 hrs. The DLP of the dermis controls is shown in (A) for the dermis controls incubated in the acetate buffer for 2 hrs, and (D) for the dermis controls incubated in the acetate buffer for 2 hrs, followed by incubation in HBSS for 16 hrs (See methods). Dashed lines represent +/- one standard deviation for the data set.

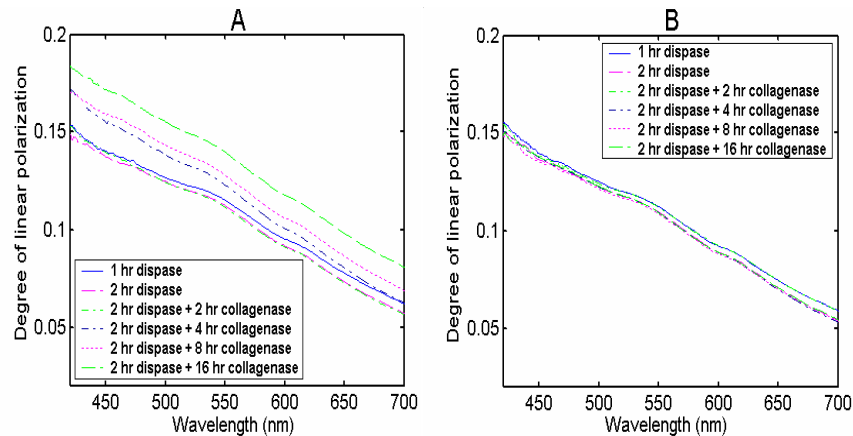


Fig. 7. Comparison of average DLP with treatment time for (A) the enzyme-treated areas and (B) the control areas. All averages were calculated from the DLPs calculated from sampling areas on 9 batches of dermis.

#### 4. Discussion

Previous studies have revealed that a decrease in fluorescence emission and light scattering is associated with tissue malignancy in various tissue types, including skin. Collagenase-induced dissolution of the extracellular matrix has been suggested to be the cause of the reduction of the fluorescence emission and light scattering of the malignant tissue. In this study, enzymatic erosion of the dermis was employed to mimic the breakdown of the dermis during tumor progression. Fluorescence and reflectance spectroscopy were used to detect the enzyme-induced changes of the dermis. Due to the complex nature of the fluorophores in the dermis, the excitation wavelengths of 350nm, 360nm, and 375nm were used in this study. However, enzymatic degradation of the dermis, either by the dispase to remove the basement membrane or by the Type I collagenase to break down the Type I and Type III collagen structures, did not show any excitation wavelength-dependent changes in the fluorescence emission. The 3 excitation wavelengths may target the same fluorophores, which could be the collagenase-digestible collagen cross-links [21, 25, 26]. Alternately, the actions of dispase and collagenase may have no specificity for any of the different types of fluorescent cross-links in the dermis. Collagenases specifically cleave some peptide bonds on the  $\alpha$  chains of the collagen molecule but not the cross-links of the collagen fibril [27]. The resulting collagen fragments spontaneously denature at temperatures greater than 33°C [27]. Therefore, the cleavage of the dermis by the collagenases may lead to a uniform degradation of all the various fluorophores in the dermis.

The fluorescence anisotropies of the dermis excited at the 3 excitation wavelengths did not show any excitation wavelength dependency. This, again, may be due to the same fluorophores being excited by all 3 excitation wavelengths, or to the different fluorophores having similar rotational diffusion rates. The fluorescence anisotropies of the dermis did not show any changes with the enzyme treatments at any of the excitation wavelengths. It appears that the enzyme treatments did not change the physicochemical properties of the remaining fluorophores and their microenvironment but simply destroyed the fluorophores. It can be concluded from these results that the fluorescence measurements of the enzyme-treated dermis with the excitations at 350nm, 360nm, and 375nm provide redundant information.

Enzymatic erosion of the dermis appreciably decreased the fluorescence emission relative to the untreated control in this study, especially during the first 2 hrs of incubation with the dispase. Extended treatment of the dermis with the Type I collagenase did not result in a further decrease in the fluorescence emission. Dispase specifically cleaves Type IV collagen

but only minimally degrades Type I collagen [28]. This study has determined that the basement membrane structure, namely Type IV collagen-rich lamina densa, was totally removed by 2 hr incubation with the dispase (data not shown). Thus, the sharp decrease in the fluorescence emission during the first 2 hrs of treatment with the dispase was most likely due to the degradation and destruction of the fluorophores within the lamina densa. Why did the subsequent treatment with the Type I collagenase not cause a further decrease in the fluorescence emission? Several explanations are possible. One explanation is that the Type I collagenase destroyed the fluorophores in the papillary dermis by gradually removing this part of the dermis through the prolonged incubation. Since the papillary dermis is considerably thinner (about 100 $\mu$ m) and has lower fluorescence emission than the reticular dermis as observed in this study (data not shown), the removal of the papillary dermis might lead to excitation of more fluorophores in the reticular dermis, and thus, stronger backscattered fluorescence emission which might counteract the reduction of fluorescence emission due to the erosion of the papillary dermis. Another explanation is that the collagenase cleaves the specific peptide bonds on the  $\alpha$  chains of the collagen molecule but not the cross-links of the collagen fibril. Thus, collagen monomers might be cleaved but the fragments would still be held in place by the cross-links. As a result, the fluorophores may survive the enzyme treatment. The first explanation seems more reasonable, since some pieces of the dermis incubated with the Type I collagenase for long times (i.e. 16 hours) showed that the enzyme-treated areas were eroded a very little bit.

The polarized reflectance is not as sensitive as the fluorescence emission in detecting the early changes of the dermis resulting from the enzyme treatment. The DLP of the enzyme-treated dermis only increased appreciably relative to the control after 2 hrs of dispase treatment followed by 4 hrs of collagenase treatment. The DLP of the enzyme-treated dermis increased further with the prolonged incubation with the Type I collagenase. Enzymatic erosion destroyed the scattering and birefringent structures in the dermis, namely the collagen fibers, and more collagen fibers were destroyed during the prolonged treatment with collagenase, thus leading to a higher DLP of the enzyme-treated dermis. The measured DLP is lower from the papillary dermis than the reticular dermis (data not shown). Thus, loss of the papillary dermis by the enzyme treatment may also contribute to the increase in DLP. However, the enzymatic degradation of the collagen fibers in the dermis might be the major cause, since the DLP of the enzyme-treated dermis increased further with the prolonged incubation with the Type I collagenase.

In summary, the structural changes of the dermis induced by the enzymes can be monitored with fluorescence emission spectroscopy and polarized reflectance spectroscopy. In this study, the fluorescence emission and light scattering as well as the birefringence of the dermis decreased with the enzyme treatment. These results support the literature findings with *in vivo* and *ex vivo* tissues that tissue malignancy leads to decreased fluorescence emission and light scattering in the tumor stroma. The results in this study also confirm that the reduction of the fluorescence emission and light scattering of the stroma are consistent with the enzymatic cleavage of the collagen fibers in the extracellular matrix. Under the conditions in this study, the fluorescence emission intensity was very sensitive to the early structural changes of the dermis as the basement membrane was being removed by the dispase. However, the dispase-treated dermis did not show any change in DLP. Conversely, the DLP increased as the Type I collagenase destroyed the underlying Type I and Type III collagens, but the fluorescence emission intensity did not change during this period of prolonged incubation. Fluorescence measurements of the dermis with multiple excitation wavelengths between 350nm and 375nm appear redundant, and the fluorescence anisotropy of the dermis did not change with the enzyme treatment. Therefore, the enzymatic cleavage of the dermal collagens seems to non-differentially destroy the various fluorophores in the dermis due to the action mechanism of the collagenase, and the enzymatic cleavage does not change the physicochemical properties of the remaining fluorophores and their microenvironment. Thus, combined fluorescence emission and polarized reflectance spectroscopy can be used to probe the enzyme-induced structural changes of the extracellular matrix. It should be noted that the

model developed in this study was used to solely mimic the breakdown of the extracellular matrix of stromal tissue by tumor progression. This model does not include tumor cells, stromal cells, blood vessels, and other structures which are typical components of in vivo tumor and its surrounding stromal tissue. Increased number and activities of tumor cells and enhanced vasculature are associated with tumor progression. Therefore, further efforts are needed to develop fluorescence and reflectance spectroscopy for in vivo tumor diagnosis.

### **Acknowledgments**

We acknowledge the Department of Pathology at the Medical University of Ohio for the histology of the dermis.