

# FLUORESCENCE SPECTROSCOPY OF DERMAL WOUNDS IN RATS

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(Paper JBO-088 received Apr. 26, 1996; revised manuscript received Sep. 9, 1996; accepted for publication Oct. 23, 1996.)

## ABSTRACT

Fluorescence spectra of dermal lesions on 48 rats were investigated using excitation wavelengths of 275, 300, and 340 nm. Emissions at 340 and 460 nm were measured in both the forequarter and hindquarter lesions as function of time after incision. Unlike the 460-nm emission, intensity at 340 nm increased with time and then saturated. Control studies on intact skin and lesions in dead rats failed to demonstrate any time-dependent changes at these wavelengths. It appears that the increase in the 340-nm intensity is due to changes in the tryptophan level, and may reflect accumulation of fibrinogen in the early wound healing process. © 1997 Society of Photo-Optical Instrumentation Engineers.

**Keywords** optical spectroscopy; fluorescence dermal wound; healing.

## 1 INTRODUCTION

The use of optical spectroscopy to observe and influence biological processes is of growing interest for many medical applications. Recently, optical spectroscopy has been used to detect physical and chemical changes in cells and tissues and distinguish cancerous tissues from healthy ones in various organs<sup>1-10</sup> and in normal and atherosclerotic aorta.<sup>11-14</sup> Various investigators have also reported that laser irradiation at low energies might accelerate wound healing in both humans and animal experimental models.<sup>15-19</sup>

In the work reported here, fluorescence spectroscopy was used to investigate initial changes in dermal wound healing. It is hoped that this methodology will lead to the development of noninvasive tools and procedures in the real time for clinical treatment and evaluation of wounds and burns.

## 2 EXPERIMENTAL METHOD AND MATERIALS

Spectroscopic measurements were performed using a Mediscience Technology Corp. CD Scan. This instrument is based on a Perkin-Elmer LS50 spectrophotometer with key software. A schematic optical layout is shown in Figure 1. The light source is a xenon flash lamp. The power of the excitation light source incident on the sample is about  $1/2 \mu\text{W}$ . There are two monochrometers, one in the excitation beam path and the other in the emission beam

path. The excitation wavelengths are selected by adjusting the angle of the grating, which is controlled by a stepping motor, and an adjustable slit. The selected excitation beam is focused by a mirror into a bifurcated optical fiber bundle, with a small portion of the excitation beam reflected by a beam splitter into a reference photomultiplier. The output signal of the reference photomultiplier is used to correct the emission signal for variation in excitation intensity. The emission light is collected by the fiber bundle and is passed to the entrance slit of the emission monochromator. The emission wavelength is selected by adjusting the angle of its grating, which is also controlled by a stepping motor. Only the emission light at the selected wavelength is passed into the signal photomultiplier for detection. The probe consisted of a UV transmitting quartz fiber bundle. The sample end of the probe is a 6-cm diameter circular aperture, recessed 2 mm from the end of its ferrule. The individual fibers in the probe are randomly split into two separate bundles, with  $2 \times 6$ -cm rectangular apertures to match the spectrometer optics. During measurements, the probe was positioned normal to the scanned region. Special software was used for data collection, processing, and plotting.

The fluorescence spectra from dermal lesions were obtained using 275, 300, and 340 nm as excitation wavelengths. The excitation intensity at 300 nm (340 nm) was approximately 25% (80%) greater than the excitation intensity at 275 nm; however, the spectrophotometer normalized the signal by the

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FLUORESCENCE AND EXCITATION INSTRUMENT

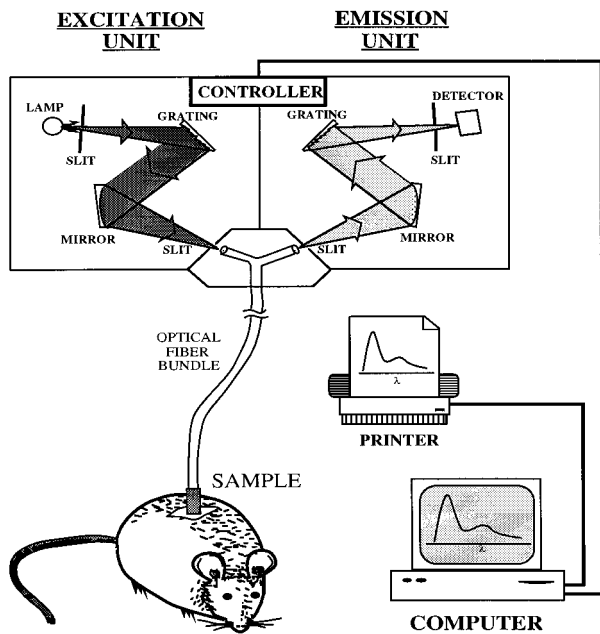


Fig. 1 Schematic diagram of fiber-based fluorescence measurement system.

excitation intensity. Two methods were employed to examine changes in the spectral response. (1) The whole spectrum was examined at 5-min intervals over a 2-h period. (2) The intensity of nine emission wavelengths was measured at 1-min intervals up to 150 min after incision. In addition the *in vitro* responses of separated blood plasma and various photoresponsive bioactive molecules in 0.9% sodium chloride solution were measured and the measured wavelengths are displayed in Table 1.

Table 1 Measured excitation and emission wavelengths from selected biological molecules.

Excitation (nm)	Emission (nm)	Origin
275	310	Collagen III
275	340	Tryptophan
275	395	Collagen II
300	340	Tryptophan
300	375	Pyridoxal 5'-phosphate
300	435	Pyridoxic acid
340	390	Collagen III
340	410	Elastin
340	460	NADH

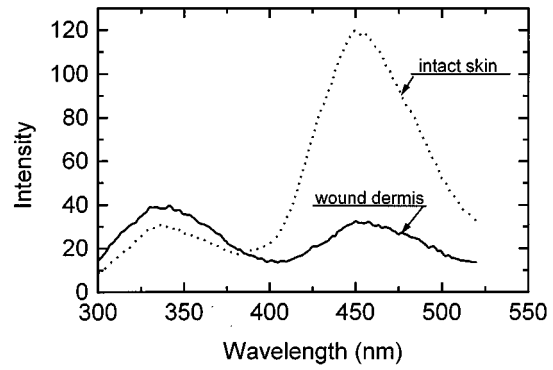


Fig. 2 Typical emission spectra of wound site and intact skin excited by 275 nm.

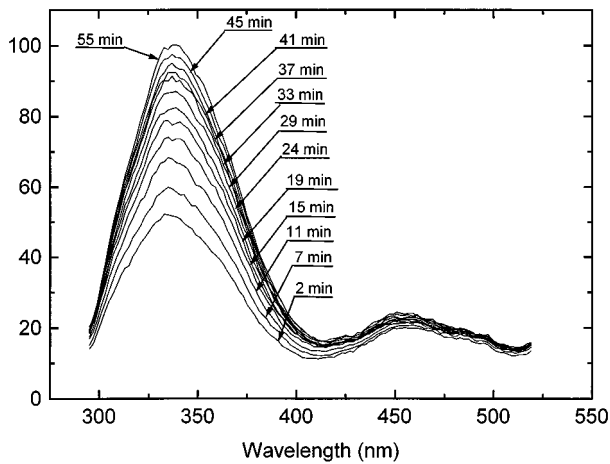
Forty-eight 250 to 400-g male Wistar rats were employed in the *in vivo* study. This study consisted of six experimental groups. The first group had a single lesion placed in either the right or left forequarter. Following the collection of data from the first lesion, the second group had an additional lesion placed on the opposite forequarter. The third group had a single lesion placed in either hindquarter. The fourth had two lesions, one in the forequarter and one in the hindquarter. The fifth group were controls consisting of shaved rats without lesions. The sixth group were controls consisting of shaved rats with lesions that died during the course of the study.

To prevent movement during measurements, all animals were anesthetized with a 40-mg/kg body weight dose of sodium pentobarbital administered intraperitoneally. The area of the wound sites was first shaved and cleaned with sterile alcohol swabs and air dried. Then, taking advantage of the loose pelt of this species, 1-cm long lesions made with sterile scalpel blades were extended through the dermis about 0.5 mm deep but no further. During the course of data collection the anesthetized rat was placed in a prostrate position and the optical fiber bundle was placed proximal to the lesion or to the shaved intact skin. The time course was recorded after  $t=0$  when the lesion was made.

For *in vitro* examinations, 5 ml of femoral blood was extracted from each of 6 anesthetized rats. To prevent clotting, an equal volume of heparinized saline (50 units/0.9% NaCl) was added to each sample. A tabletop clinical centrifuge spinning at 3000 rpm for 20 min was then used to separate plasma from hematocrit. Four-milliliter quartz cuvettes were then filled with the separated plasma and the emission spectra determined in the standard manner.

3 RESULTS

Figure 2 shows a typical fluorescence spectrum for intact shaved skin and a wound. In the shaved intact skin, fluorescence spectra for excitation wave-

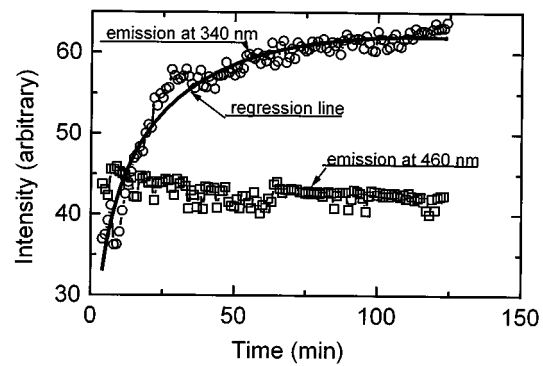


**Fig. 3** The temporal behavior of the spectra of the wound site area after incision, excited by 275 nm.

lengths 275 or 300 nm are similar. For these wavelengths, there are two emission intensity peaks; one is near 340 nm and the other is near 460 nm. The amplitude of these two peaks varies with excitation wavelength. The dermal wound site also exhibited two peaks at 340 and 460 nm. When a wound is compared with intact skin, the amplitudes of these peaks are quite different. The peak emission near 460 nm is markedly decreased and the peak emission at 340 nm is enhanced. When excited at either 300 or 275 nm, both forequarter and hindquarter lesions exhibited emissions at 340 and near 460 nm.

The most important observation was that in the wound site, the intensity of the 340 nm emission markedly increased with time after incision before finally reaching a plateau while that at 460 nm did not change in time (Figure 3). This observation at 340 nm was not dependent on the measuring methods (whether the whole spectrum was measured every 5 min or only the amplitude of the fluorescence spectrum was measured at 1-min intervals). The emission kinetics at 340 nm was in sharp contrast to the emission at near 460 nm, which was unaffected by the passage of time (Figure 4) from the time after incision of the wound.

The analysis of the average rise time of 340-nm emissions for the first wound (Figure 5) was calculated using 15 rats with one forequarter lesion. The average rise time of a 340-nm emission for the second wound was calculated using 9 rats with forequarter lesions (Figure 5). Emission intensities were monitored at 1-min intervals. Rise-time curves were then constructed for each rat. The curves were then normalized and their combined means and standard deviations calculated. The average rise time of 90% maximum is  $52.6 \pm 37$  min for forequarter and  $44 \pm 31$  min for hindquarter. It was found that the second wound's pattern of spectral emissions did not statistically differ from that of the first. As in the first wound, the intensity of emission at 340 nm

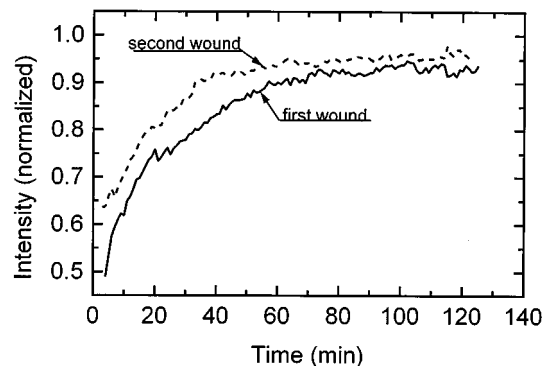


**Fig. 4** The peak intensity from a wound area at 340 nm and near 460 nm as a function of time after incision. The sample is excited by 275 and 340 nm, respectively.

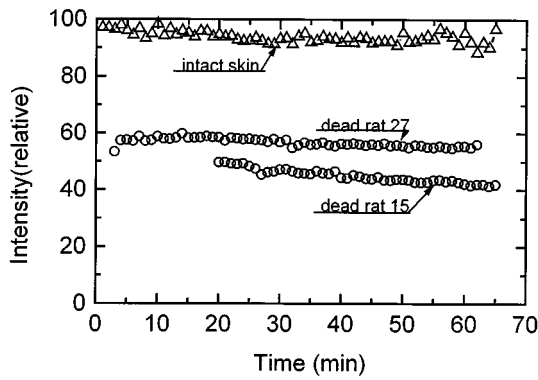
increases with time while the intensity near 460 nm is unaltered.

To determine whether the observed emissions were due to the inadvertent selection of a unique portion of the skin, single lesions were made on the contralateral side in 6 rats and on the hindquarter in another group of 6 rats. Both contralateral and hindquarter lesions exhibited emissions at 340 and 460 nm. The two curves appear to be similar and neither the order of the lesion nor its location altered the wound's rise time. In the control groups, emissions at 340 nm from intact skin and emissions from wounds in dead animals showed no alterations in intensities with time, as shown in Figure 6 for either 340- or 460-nm wavelengths.

The fluorescence peak at 340 nm excited by 275 nm, and the peak at 460 nm excited by 340 nm is associated with tryptophan and reduced nicotinamide adenine dinucleotide (NADH) (Table 1), respectively. It is possible that the presence of tryptophan and NADH in dermal wounds is due to proteins leaking from the blood. Since whole blood does not respond to optical excitation, testing the above hypothesis required the separation of plasma from hematocrit. The emission spectra shown in



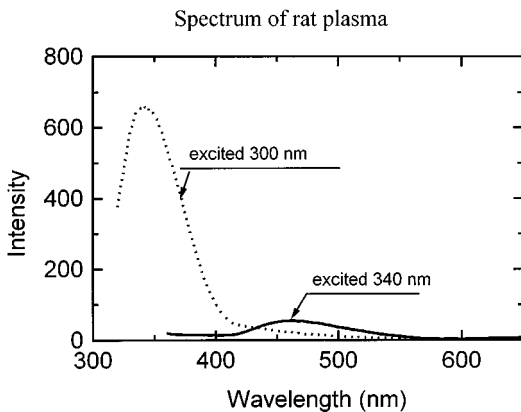
**Fig. 5** Average intensity at 340 nm at first wound site area as a function of time ( $n=15$  rats) and average intensity of second wound area at 340 nm as a function of time ( $n=9$  rats).



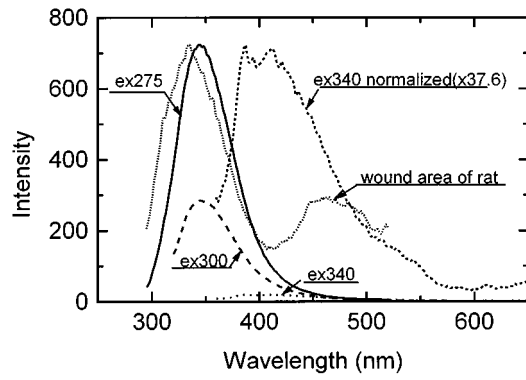
**Fig. 6** Average intensity at 340-nm emission, excited by 275 nm, for intact skin in live rats and lesions in dead rats, as a function of time.

Figure 7 confirm that tryptophan and NADH were found in the plasma samples. This suggests that the leakage of tryptophan into the wound increased while the NADH was unaltered with time. The hypothesis of leakage from the blood is not supported. Blood does not produce this level of signal.

It is also possible that the presence of tryptophan in the wound is due to the selective leaking of fibrin and fibrinogen from blood vessels. To test this concept, the *in vitro* spectrum of fibrin and fibrinogen in the solid state and dissolved in saline solution were measured. The spectra were nearly same for the two situations. Normalized spectra of fibrin and fibrinogen in saline and the wound area of rats are shown in Figures 8 and 9. When excited with 275 nm, fibrin and fibrinogen emit with a peak near 340 nm. The peak shift for fibrin is small. When excited with 340 nm, the spectrum of fibrin emits a broader peak from 400 to 450 nm. When excited with 275 nm, however, fibrinogen emits a peak near 340 nm and a very small intensity at 460 nm. The change in the spectrum emitted from fibrinogen would be closer to the emission spectrum of the wound area than that of fibrin.



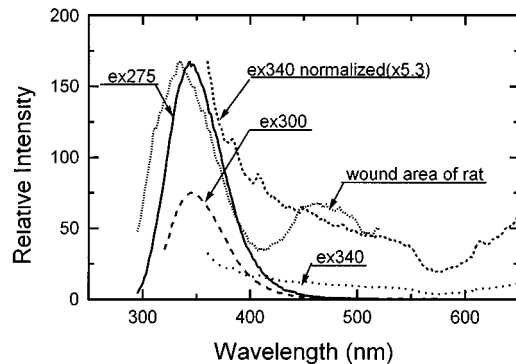
**Fig. 7** Emission spectra of rat plasma excited by 300 and 340 nm.



**Fig. 8** Emission spectrum of the fibrin in saline excited by 275, 300, and 340 nm, and the spectrum of the wound area excited by 275 nm as reference. The normalized factor excited by 340 nm is 37.6.

#### 4 DISCUSSION

The significant observation was that when optically excited at 300 and 340 nm, rat skin induces peak emissions at 340 and 460 nm, respectively. The intensity from dermal wounds at 340 nm emissions grew, reaching 90% of its plateau value in 52.6 min, which the 460 nm did not change after incision of a wound. Several questions arise from these observations. What produced the 340-nm emission? Would the rising intensity of 340-nm emissions occur in lesions on other areas of skin? Were the 340- and 460-nm emissions due to simple bleeding? Was the 340-nm emission due to the lesion stimulating the release of a blood-borne hormonelike factor? Or was the 340-nm emission a sign of local, perhaps wound-healing processes? The initial observations were made on right forequarter lesions. The intensity of the 340-nm emission from the wounds increased with time. This is consistent with an accumulation of a tryptophan-like factor. To determine if this accumulation of a tryptophan-like factor was unique to right forequarter wounds, one and two



**Fig. 9** Emission spectrum of the fibrinogen in saline excited by 275, 300, and 340 nm, and the spectrum of wound area of a rat excited by 275 nm as reference. The normalized factor excited by 340 nm is 5.3.

transdermal lesions were made on other dorsal areas. All lesions emitted a similar spectrum.

It is possible that the tryptophanlike factor in dermal wounds is due to the stimulated release of a hormonelike blood-borne factor containing tryptophan. If such were the case, having already been stimulated, the tryptophanlike spectrum in a second wound would be expected to be more intense or at least to plateau earlier. To examine this hypothesis, the emission spectra of the second wounds were compared with those of the first. The second wound's pattern of spectral emissions did not differ from the first. The intensity of emission at 340 nm rose at the same apparent rate and intensity as in the first wound, while emission intensity at 460 nm was unaltered. These results suggest that whatever the origin of the 340-nm emission, it is most likely not due to hormonal action. It is likely that the 340-nm emission is due to extravasation from unchanging blood stores or from dermal tissue.

A common observation was the accumulation of clear fluid in the wound. These observations suggested that the 340-nm emissions might be due to diapedesis from blood plasma. Since whole blood is spectrally inert, this hypothesis was examined by investigating the induced emission of blood plasma. When optically stimulated, plasma separated by centrifugation was found to generate emissions at 340 and 460 nm. Furthermore, these emissions were unaltered with time. Since only the 340-nm emission factor and not the 460-nm emission factor accumulated in the wound, the above observation supports the proposal that the source of wound tryptophanlike factor is not due to the unfiltered accumulation of blood plasma. Since neither shaved dermis nor dermal wounds in dead rats accumulated this tryptophanlike factor, it appears that the factor was not released from dermal stores. Since fibrin, when excited at 340 nm wavelength, emits a peak near 460 nm, and fibrinogen does not, it appears most likely that the apparent accumulation of tryptophan is derived from fibrinogen. It appears that fibrinogen is a reasonable molecule for a built-up signal at 340 nm in a wound. When the dermis is damaged, the blood vessels in the region become selectively more permeable to fibrinogen. Our hypothesis that dermal wounds lead to a selective increase in fibrinogen permeability in the local vascular bed requires further study.

#### Acknowledgment

We thank Dr. H. Savage of the Sloan Kettering Cancer Center for help with the wound plasma work, Dr. A. Katz of the Mediphotonics Laboratory at The City College of New York for helpful suggestions, and New York State Technology Foundation and Mediscience Technology Corp. for support.

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