Elemental analysis of tissue pellets for the differentiation of epidermal lesion and normal skin by laser-induced breakdown spectroscopy

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Abstract: By laser induced breakdown spectroscopy (LIBS) analysis of epidermal lesion and dermis tissue pellets of hairless mouse, it is shown that Ca intensity in the epidermal lesion is higher than that in dermis, whereas Na and K intensities have an opposite tendency. It is demonstrated that epidermal lesion and normal dermis can be differentiated with high selectivity either by univariate or multivariate analysis of LIBS spectra with an intensity ratio difference by factor of 8 or classification accuracy over 0.995, respectively.

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References and links

1. M.-Q. Man, Y. Hatano, S. H. Lee, M. Man, S. Chang, K. R. Feingold, D. Y. M. Leung, W. Holleran, Y. Uchida, and P. M. Elias, “Characterization of a hapten-induced, murine model with multiple features of atopic dermatitis: structural, immunologic, and biochemical changes following single versus multiple oxazolone challenges,” J. Invest. Dermatol. 128(1), 79–86 (2008).

2. Y. Matsunaga, Y. Ogura, R. Ehama, S. Amano, T. Nishiyama, and H. Tagami, “Establishment of a mouse skin model of the lichenification in human chronic eczematous dermatitis,” Br. J. Dermatol. 156(5), 884–891 (2007).

3. Mosby, Mosby’s Medical Dictionary, 8th Edition (Elsevier, 2009)

4. J. A. Rothnagel, A. M. Dominey, L. D. Dempsey, M. A. Longley, D. A. Grenchalgh, T. A. Gagne, M. Huber, E. Frenk, D. Hohl, and D. R. Roop, “Mutations in the rod domains of keratins 1 and 10 in epidermolytic hyperkeratosis,” Science 257(5073), 1128–1130 (1992).

5. G. Klinda, P. Urban, C. M. Philipp, and H.-P. Berlien, “532 nm laser treatment of molluscum contagiosum,” Med. Laser Appl. 26(4), 172–175 (2011).

6. C. Hafer and T. Vogt, “Seborrhoeic keratosis,” J. Dtsch. Dermatol. Ges. 6(8), 664–677 (2008).

7. B. R. Duus, T. Philipsen, J. D. Christensen, F. Lundvall, and J. Sondergaard, “Refractory condylomata acuminata: a controlled clinical trial of carbon dioxide laser versus conventional surgical treatment,” Genitourin. Med. 61(1), 59–61 (1985).

8. M. Lapidoth, H. Israeli, D. Ben Amitai, and S. Halachmi, “Treatment of verrucous epidermal nevus: experience with 71 cases,” Dermatology (Basel) 226(4), 342–346 (2013).

9. Y. K. Kim, D.-Y. Kim, S. J. Lee, W. S. Chung, and S. B. Cho, “Therapeutic efficacy of long-pulsed 755-nm alexandrite laser for seborrhoeic keratoses,” J. Eur. Acad. Dermatol. Venereol. 28(8), 1007–1011 (2014).

10. E. Papadavid and A. Katsambas, “Lasers for facial rejuvenation: a review,” Int. J. Dermatol. 42(6), 480–487 (2003).

11. M. R. Alexiades-Armenakas, J. S. Dover, and K. A. Arndt, “The spectrum of laser skin resurfacing: nonablative, fractional, and ablative laser resurfacing,” J. Am. Acad. Dermatol. 58(5), 719–740 (2008).

12. C. A. Nanni and T. S. Alster, “Complications of carbon dioxide laser resurfacing. An evaluation of 500 patients,” Dermatol. Surg. 24(3), 315–320 (1998).

13. D. A. Cremers and L. J. Radziemski, Handbook of laser-induced breakdown spectroscopy (John Wiley & Sons Ltd, 2013).

14. V. K. Singh and A. K. Rai, “Potential of laser-induced breakdown spectroscopy for the rapid identification of carious teeth,” Lasers Med. Sci. 26(3), 307–315 (2011).
15. A. Kumar, F.-Y. Yueh, J. P. Singh, and S. Burgess, “Characterization of malignant tissue cells by laser-induced breakdown spectroscopy,” Appl. Opt. 43(28), 5399–5403 (2004).

16. A. El-Hussein, A. K. Kassem, H. Ismail, and M. A. Harith, “Exploiting LIBS as a spectrochemical analytical technique in diagnosis of some types of human malignancies,” Talanta 82(2), 495–501 (2010).

17. R. Kanawade, F. Mehrabi, C. Knipfer, M. Rohde, K. Tangermann-Gerk, M. Schmidt, and F. Stelzlé, “Pilot study of laser induced breakdown spectroscopy for tissue differentiation by monitoring the plume created during laser surgery — An approach on a feedback Laser control mechanism,” Spectrochim. Acta Part B At. Spectrosc. 87(1), 175–181 (2013).

18. R. Kanawade, F. Mehrabi, F. Klämpfl, M. Rohde, C. Knipfer, K. Tangermann-Gerk, W. Adler, M. Schmidt, and F. Stelzlé, “Qualitative tissue differentiation by analysing the intensity ratios of atomic emission lines using laser induced breakdown spectroscopy (LIBS): prospects for a feedback mechanism for surgical laser systems,” J. Biophotonics 8(1–2), 153–161 (2015).

19. J. H. Han, Y. Moon, J. J. Lee, S. Choi, Y.-C. Kim, and S. Jeong, “Differentiation of cutaneous melanoma from surrounding skin using laser-induced breakdown spectroscopy,” Biomed. Opt. Express 7(1), 57–66 (2016).

20. F. Mehrabi, M. Rohde, C. Knipfer, R. Kanawade, F. Klämpfl, W. Adler, F. Stelzlé, and M. Schmidt, “Laser induced breakdown spectroscopy for bone and cartilage differentiation - ex vivo study as a prospect for a laser surgery feedback mechanism,” Biomed. Opt. Express 5(11), 4013–4023 (2014).

21. F. Benavides, T. M. Oberyszyn, A. M. VanBuskirk, V. E. Reeve, and D. F. Kusewitt, “The hairless mouse in skin research,” J. Dermatol. Sci. 53(1), 10–18 (2009).

22. H. Jin, R. He, M. Oyoshi, and R. S. Geha, “Animal models of atopic dermatitis,” J. Invest. Dermatol. 129(1), 31–40 (2009).

23. C. S. Potten, R. Saffhill, and H. I. Maibach, “Measurement of the transit time for cells through the epidermis and stratum corneum of the mouse and guinea-pig,” Cell Tissue Kinet. 20(5), 461–472 (1987).

24. L. A. Nylander-French, “A tape-stripping method for measuring dermal exposure to multifunctional acrylates,” Ann. Occup. Hyg. 44(8), 645–651 (2000).

25. A. L. Stinchcomb, F. Pirot, G. D. Touraille, A. L. Bunge, and R. H. Guy, “Chemical uptake into human stratum corneum in vivo from volatile and non-volatile solvents,” Pharm. Res. 16(8), 1288–1293 (1999).

26. R. Kanmer, H. Tinnerberg, and K. Eriksson, “Evaluation of a tape-stripping technique for measuring dermal exposure to pyrene and benzo(a)pyrene,” J. Environ. Monit. 13(8), 2165–2171 (2011).

27. F.-Y. Yueh, H. Zheng, J. P. Singh, and S. Burgess, “Preliminary evaluation of laser-induced breakdown spectroscopy for tissue classification,” Spectrochim. Acta Part B At. Spectrosc. 64(10), 1059–1067 (2009).

28. Y. Moon, J. H. Han, J. J. Lee, and S. Jeong, “Influence of water content on the laser-induced breakdown spectroscopy analysis of human cell pellet,” Spectrochim. Acta Part B At. Spectrosc. 114, 27–33 (2015).

29. N. B. Zorov, A. A. Gorbatenko, T. A. Labutin, and A. M. Popov, “A review of normalization techniques in analytical atomic spectrometry with laser sampling: From single to multivariate correction,” Spectrochim. Acta Part B At. Spectrosc. 65(8), 642–657 (2010).

30. I. J. Berridge, M. D. Bootman, and M. L. Roderick, “Calcium signalling: dynamics, homeostasis and remodelling,” Nat. Rev. Mol. Cell Biol. 4(7), 517–529 (2003).

31. D. E. Clapham, “Calcium Signaling,” Cell 131(6), 1047–1058 (2007).

32. D. D. Bikle, A. Ratnam, T. Mauro, J. Harris, and S. Pillai, “Changes in calcium responsiveness and handling during keratinocyte differentiation. Potential role of the calcium receptor,” J. Clin. Invest. 97(4), 1085–1093 (1996).

33. P. Elias, S. Ahn, B. Brown, D. Crumrine, and K. R. Feingold, “Origin of the epidermal calcium gradient: regulation by barrier status and role of active vs passive mechanisms,” J. Invest. Dermatol. 119(6), 1269–1274 (2002).

34. M. Denda, S. Fuziwara, and K. Inoue, “Influx of calcium and chloride ions into epidermal keratinocytes regulates exocytosis of epidermal lamellar bodies and skin permeability barrier homeostasis,” J. Invest. Dermatol. 121(2), 362–367 (2003).

35. M. F. Kulesz-Martín, D. Fabian, and J. S. Bertram, “Differential calcium requirements for growth of mouse skin epithelial and fibroblast cells,” Cell Tissue Kinet. 17(5), 525–533 (1984).

36. J. A. McGrath, R. J. Eady, and F. M. Pope, “Anatomy and organization of human skin,” in Rook’s Textbook of Dermatology (Blackwell Publishing, Inc., 2004), pp. 45–128.

37. G. J. Tortora and B. H. Derrickson, Principles of anatomy and physiology, 13th edition (Wiley, 2011).

38. E. D. Desouza, I. A. Atiya, A. El-Ebraheem, B. C. Wainman, D. E. B. Fleming, F. E. McNeill, and M. J. Farquharson, “Characterization of the depth distribution of Ca, Fe and Zn in skin samples, using synchrotron micro-x-ray fluorescence (SuXRF) to help quantify in-vivo measurements of elements in the skin,” Appl. Radiat. Isot. 77, 68–75 (2013).

39. T. von Zglinicki, M. Lindberg, G. M. Roomans, and B. Forslind, “Water and ion distribution profiles in human skin,” Acta Derm. Venereol. 73(5), 340–343 (1993).

40. Y. Werner-Linde, J. Pallon, and B. Forslind, “Physiologically important trace elements of paraleusal psoriatic skin,” Scanning Microsc. 12(4), 599–608 (1998).

1. Introduction

Chronic inflammation of skin tends to induce morphologic changes such as epidermal hyperplasia or hyperkeratosis, the increase of tissue amount due to abnormal cell proliferation or the thickening of corneal layer which is the outermost layer of skin,
respectively [1–3]. Hyperkeratotic epidermal lesion may develop as a result of repetitive mechanical injury of skin, for example, by scratching, as observed in prurigo nodularis and lichen simplex chronicus [2]. In dermatology, hyperkeratotic epidermal lesion has been observed in various pathological states such as epidermolytic hyperkeratosis [4], molluscum contagiosum [5], seborrheic keratosis [6], condyloma acuminata [7], and verrucous epidermal nevus [8]. For the treatment of focal hyperkeratotic epidermal lesions, lasers have been widely used for selective destruction of abnormal skin lesion [9]. However, ablative laser modalities are occasionally associated with adverse effects like post-inflammatory hyperpigmentation, hypopigmentation or scar due to unwanted damage in the mid-dermis [10, 11]. Since epidermis is in contact with dermis, laser ablation treatment of epidermal lesion can easily produce a dermal injury unless the ablation depth is precisely controlled. Especially, a dermal injury in the facial area such as the perioral, periorbital, chin and neck regions was known to be particularly prone to scar formation [12]. Thus, to avoid the unwanted damage of dermis during laser ablation treatment of epidermal lesion, it is desired to be able to accurately locate the depth or identify the layer being ablated in situ so that only the hyperkeratotic epidermal lesion can be removed with minimal damage of dermis.

For this purpose, laser induced breakdown spectroscopy (LIBS) can provide an effective solution. LIBS is a technique to measure the spectral intensity of plasma emission generated by pulsed laser irradiation of a target. Since the spectral intensity of LIBS plasma depends on the elemental composition and/or concentration of the target [13], the change of ablation target, for example, from epidermal lesion to dermis, can be identified by monitoring LIBS signals provided that epidermal lesion and dermis bear significant enough differences in elemental composition or concentration. LIBS analysis needs no or little sample preparation, can be completed in a short time (seconds), and can be performed in air with good depth resolution. Thus, the application of LIBS for real time monitoring or as the feedback mechanism during laser treatment has been investigated in various ways such as the identification of carious teeth [14], classification of different soft tissues [15–19], or differentiation of bone and cartilage [20].

In this work, the results for LIBS analysis of hyperkeratotic epidermal lesion and dermis using tissue pellet samples are reported. LIBS analysis results are then compared with the elemental compositions determined by other analytical methods, inductively coupled plasma optical emission spectrometer (ICP-OES) and atomic absorption spectrometer (AAS). It is demonstrated that hyperkeratotic epidermal lesion and dermis can be clearly distinguished by LIBS when proper signals are selected as bio-markers.

2. Experiment

2.1 Sample preparation

Female SKH-1 hairless mice of 6-8 weeks old (Orient Bio Inc., Gyeonggi-do, Korea) were used as the sample. These mice are immunocompetent, have normal immunologic response to the allergen and irritant, and are widely used for the study of skin inflammation process, wound healing and carcinogenesis due to their similarity to human skin [21]. Sample preparation was carried out under the approval of the animal care committee of Gwangju Institute of Science and Technology (GIST2015-05) as follows. Mice were acclimatized at least 1 week prior to experiments and housed in standard conditions of 12h light/12h dark cycle, 24 ± 2°C temperature, and 50 ± 10% relative humidity. Figure 1 shows the clinical images of a mouse sample before (Fig. 1(a)) and after (Fig. 1(b)) hyperkeratotic lesion induction. To develop hyperkeratotic epidermal lesion in the mouse skin, allergic inflammation was induced using oxazolone (Sigma-Aldrich, St. Louis, MO, USA) in acetone and olive oil mixture (3:1). Oxazolone is a well-known hapten molecule that binds protein and induces allergic inflammation [22], and oxazolone induced animal models are widely used in biology research, especially in the fields of allergic inflammation and atopic dermatitis. A repetitive application of oxazolone in acetone and olive oil mixture on the dorsal skin causes barrier disruption, irritation, inflammation and itch, which results in the
formation of epidermal hyperplasia and hyperkeratosis [2]. Specifically, the dorsal skin of a mouse was first sensitized with 2% oxazolone solution. Then, after 1 week, 0.5% solution was applied three times a week for 8–12 weeks depending on individual mouse, which was followed by 2-4 weeks of washout period. Note that the turnover time of normal epidermis of a hairless mouse was reported to be about 8–9.5 days, shorter in disease condition [23]. Thus, the skin samples collected after the washout period was considered to be free from the possible oxazolone residues. Hyperkeratotic epidermal lesion tissues were then collected using forceps under anesthesia by ketamine (70 mg/kg, Yuhan Ketamine 50, Yuhan Co., Ltd, Seoul, Korea) and xylazine (7 mg/kg, Bayer Korea, Gyeonggi-do, Korea). Serum crusts and oozing material in epidermis were excluded from collection.

The mice samples from which epidermal lesion tissue was collected were also used for the collection of dermis tissue. To remove the remnant epidermis existed on a mouse skin after epidermal lesion collection, an adhesive tape (Scotch Transparent Tape 600, 3M, St. Paul, MN, USA) was pressed onto the skin and stripped with constant force under anesthesia [24–26] and the tape-stripping was repeated ten times. Figure 1(c) shows the mouse after tape-stripping from which dermis tissue was collected by excising dorsal skin. The collected epidermal lesion tissue and dorsal skin were then freeze-dried for 24 h at −120°C (FDCF-12006, Operon Co., Korea). For a complete removal of the locally remaining epidermal lesion shown in Fig. 1(c) and of possibly unstripped epidermis layer, a thin layer of the freeze-dried dermis was removed by gently grinding with a grinder (Dremel 4000, Bosch Leinfelden, GER). Thirty female SKH-1 hairless mice were consumed to collect epidermal lesion tissue sufficient to produce a pellet.

To make the epidermal lesion pellet, the freeze-dried epidermal lesion tissue was ground to powder and pressed into a mold for 1 min using a digital hydraulic press (PIKE 181-1110, PIKE Technologies, USA) at the pressure of 6 ton. The same procedure was taken to prepare the dermis pellet. The accordingly prepared pellet samples (diameter = 13 mm, height = ~1 mm) are shown in Figs. 1(d) and 1(e).

Fig. 1. Photographs of a mouse with different skin conditions and pellet samples.

2.2 LIBS measurement

A commercial LIBS system (RT250EC, Applied Spectra Inc., USA) of which a schematic diagram is shown in Fig. 2 was used for the analysis. The epidermal lesion or dermis pellet was irradiated by a second harmonic Nd:YAG laser (\(\lambda = 532\) nm, \(\tau = 5\) ns, top-hat profile) with a laser spot diameter of approximately 200 \(\mu\)m at the sample surface. The pellet sample was placed inside a chamber covered with a quartz window in order to prevent the focusing and collection lenses from possible contamination by ablation particles. The laser pulse energy in experiments was 8.84 mJ behind the quartz window (corresponding laser fluence =
28.2 J/cm²). The emission from laser plasma was captured by a collection lens and delivered to the CCD spectrometer via an optical fiber bundle. The gate delay of the CCD spectrometer was set to 0.2 μs. The gate delay and laser pulse energy in experiments were determined after test measurement for the values at which the emission signals of the major elements (C, Mg, Ca, CN, Na, H, O, N, K, Cl) were clearly detected. The gate width of the CCD spectrometer was fixed by the system to 1.05 ms.

LIBS spectra were collected from 30 different spots on each pellet sample and each of the 30 measurement spots was irradiated by 15 consecutive laser shots at the repetition rate of 5 Hz. The LIBS signal from the first shot on each measurement spot was eliminated from the analysis in order to avoid the possible effects by contamination during handling, and the spectra from the 2nd to 15th shot were accumulated. Figure 3(a) shows the ablation craters produced on the epidermal lesion pellet after 15 laser shots, which shows little changes in color and morphology. The shot-to-shot variation of LIBS signal intensity of, for example, Ca lines in Fig. 3(b) also remained within the range of standard deviation. Based on these data, it is considered that there was little change in pellet chemistry during laser shots. The accumulated spectra from 30 measurement spots were then averaged to obtain the LIBS spectra of each pellet.

For cross check, the pellet samples completed of LIBS measurements were also analyzed with ICP-OES (Varian-720US, Agilent, USA) and AAS (ICE 3500 AA system, Theme, USA). ICP-OES was used for Ca and Mg concentrations (argon flow rate = 15L/min, Ca(II) 315.887 nm and Mg(I) 285.213 nm lines), whereas AAS was carried out for K and Na concentrations (air-acetylene flow rate = 0.9L/min, K(I) 769.896 nm and Na(I) 589.592 nm lines).

![Fig. 2. Schematic diagram of the LIBS system.](image-url)
3. Results and discussion

3.1 LIBS signal intensity

The averaged LIBS spectra of the epidermal lesion and dermis pellets are shown in Fig. 4. First, Fig. 4 shows that the same emission lines are detected from both pellets with difference in intensity of some spectra. Next, the spectra reveal that the elements being detected from the pellets include C, Mg, Ca, CN, Na, H, N, K, O, and Cl which are the elements frequently detected in biological samples and selectively used as the bio-markers in earlier LIBS studies [15–19, 27].

The mean intensity values of the emission lines of the epidermal lesion and dermis pellets are presented in Table 1. For the calculation of spectral intensity of an isolated emission line, the area under the peak was integrated after background subtraction [28]. The intensities of overlapped peaks of the same element with similar spectral properties, such as Mg(II) 279.553 nm and 280.270 nm, Na(I) 588.995 nm and 589.592 nm, and N(I) 742.364 nm, 744.229 nm, and 746.831 nm, were added together and represented by a single value. These overlapped lines of each element have the same upper and lower level energies as shown in Table 1 and thus their intensities are expected to change with similar tendency under the local thermodynamic equilibrium (LTE) condition [29]. In fact, the two Ca lines of similar spectral properties in Fig. 3(b) showed nearly identical changes in shot-to-shot intensity, which was understood to imply that LTE condition was satisfied for the plasma.
The results in Table 1 show that the intensity of Ca signals of epidermal lesion is significantly higher than that of dermis (2.2–4.9 times), whereas the intensities of Na and K of epidermal lesion are much lower than those of dermis (0.4–0.5 times). Also, the peak value of Cl(I) 819.442 nm line of epidermal lesion is 0.45 times smaller than that of dermis; note that the peak value instead of the integrated intensity was used for the Cl(I) 819.442 nm line because this line was largely overlapped with the neighboring Na(I) 818.326 nm line. On the other hand, the intensities of C, Mg, CN, H, N, and O lines of both samples were similar.

Figure 5 shows the LIBS signal intensities of four elements (Mg, Ca, K, and Na) of epidermal lesion and dermis in comparison with the concentrations measured by ICP-OES and AAS. Since the LIBS signal was not calibrated by concentration, the LIBS signal intensity ratio does not necessarily show the same proportionality with the ICP-OES or AAS measured concentrations. Nonetheless, it is clear in Fig. 5 that the ICP-OES and AAS results show the same tendency as the LIBS signal intensities, that is, a significantly higher Ca concentration but much lower Na and K concentrations in epidermal lesion than dermis. Mg concentrations of both samples were nearly the same as the LIBS signal intensities, confirming that the elemental concentrations of epidermal lesion and dermis samples determined by ICP-OES and AAS are in reasonable agreement with the LIBS data.
Table 1. Spectral properties and measured LIBS signal intensities of selected emission lines (laser fluence = 28.2 J/cm², 30 points average)

| Elements | Wavelength (nm) | Lower level energy (eV) | Upper level energy (eV) | Mean intensity (RSD %) | Intensity ratio |
|----------|----------------|-------------------------|-------------------------|------------------------|-----------------|
|          |                | Epidermal lesion         | Dermis                  |                        |                 |
| C(I)     | 247.856        | 2.68                    | 7.68                    | 3117.58 (7.67)         | 2830.40 (7.49)  | 1.10            |
| Mg(II)   | 279.553        | 0.00                    | 4.43                    | 4091.58 (24.71)        | 3654.15 (19.68) | 1.12            |
| Mg(II)   | 280.270        | 0.00                    | 4.42                    | 985.91 (46.32)         | 216.44 (42.05)  | 4.56            |
| Ca(II)   | 315.887        | 3.12                    | 7.05                    | 2850.37 (46.26)        | 577.34 (47.90)  | 4.94            |
| Ca(II)   | 317.933        | 3.15                    | 7.05                    | 22096.34 (6.99)        | 23180.75 (8.80) | 0.95            |
| CN       | 388.340        | (Molecular band)        |                         |                        |                 |
| Ca(II)   | 393.366        | 0.00                    | 3.15                    | 22088.09 (24.98)       | 7374.82 (26.38) | 3.00            |
| Ca(II)   | 396.847        | 0.00                    | 3.12                    | 13938.11 (26.18)       | 4348.98 (28.01) | 3.20            |
| Ca(I)    | 422.673        | 0.00                    | 2.93                    | 2929.92 (23.76)        | 1341.76 (17.75) | 2.18            |
| Na(I)    | 588.995        | 0.00                    | 2.10                    | 28951.38 (14.16)       | 53778.23 (13.63) | 0.54            |
| Na(I)    | 589.592        | 0.00                    | 2.10                    | 13938.11 (26.18)       | 4348.98 (28.01) | 3.20            |
| Ca(I)    | 612.222        | 1.89                    | 3.91                    | 724.76 (36.92)         | 247.33 (24.83)  | 2.93            |
| Ca(I)    | 616.129        | 2.52                    | 4.53                    | 908.88 (36.01)         | 297.19 (23.74)  | 3.06            |
| Hα       | 656.277        | 10.20                   | 12.09                   | 24432.90 (15.07)       | 20029.87 (11.25) | 1.22            |
| N(I)     | 742.364        | 10.33                   | 12.00                   | 11577.96 (16.85)       | 9378.88 (12.01) | 1.23            |
| N(I)     | 744.229        | 10.33                   | 12.00                   | 6858.89 (18.37)        | 16797.79 (14.97) | 0.41            |
| N(I)     | 746.831        | 10.34                   | 12.00                   | 6858.89 (18.37)        | 16797.79 (14.97) | 0.41            |
| K(I)     | 766.490        | 0.00                    | 1.62                    | 13396.91 (15.32)       | 11075.89 (11.69) | 1.21            |
| K(I)     | 769.896        | 0.00                    | 1.61                    | 13396.91 (15.32)       | 11075.89 (11.69) | 1.21            |
| O(I)     | 777.194        | 9.15                    | 10.74                   | 1799.67 (Peak value)   | 4101.47 (Peak value) | 0.44          |
| Na(I)    | 818.326        | 2.10                    | 3.62                    | 2735.2 (Peak value)    | 6067.23 (Peak value) | 0.45          |
| Cl(I)    | 819.442        | 8.99                    | 10.50                   | 2735.2 (Peak value)    | 6067.23 (Peak value) | 0.45          |

The observed high Ca level in epidermal lesion may be understood in the following points of view. Epidermal Ca is located in the intracellular organelles, extracellular fluid and intracellular fluid [30] among which intracellular organelles and extracellular fluid cover the largest portion of Ca profile in epidermis [31]. Epidermal Ca is known as an essential regulator of keratinocyte differentiation, the process by which epidermis cell gradually changes from the basal layer to spinous and granular layers and finally to the corneal layer [32]. As a result, normal mammalian epidermis develops a distinct Ca gradient characterized by a low Ca level in the lower epidermis layers (basal and spinous layers), an increase toward the outer epidermis layer (up to the granular layer), and again a decrease in the outermost corneal layer. Especially, high polar ions such as Ca are hard to be dissolved in the corneal layer, because the corneal layer has large amount of extracellular lipids with relatively less water [33]. However, this original Ca gradient in normal epidermis is to be lost when epidermal barrier is disrupted during the development of epidermal lesion [34]. Furthermore, repetitive and chronic barrier disruption induces epidermal hyperplasia. The resulting high cellularity in epidermis causes an increase of intracellular organelles such as endoplasmic.
reticulum which stores Ca. The high Ca level of the epidermal lesion sample in this study is understood to agree with the expected increase of organelle density.

![Graph showing LIBS signal intensities of Mg, Ca, K, and Na and their concentrations determined by ICP-OES and AAS of the epidermal lesion and dermis pellets.](image)

Unlike epidermis, Ca content in dermis is not fully elucidated yet [35]. Whereas almost 95% of epidermis is composed of keratinocytes [36], dermis consists of various constituents: various types of cells (fibroblasts, macrophages and adipocytes) and matrix components such as collagen, elastin, hyaluronan, proteoglycans, glycoproteins and other substance [37]. Thus, Ca level in dermis depends on the actual constituent components. However, it was reported in a recent depth profiling study of skin using synchrotron micro-x-ray fluorescence (μXRF) that Ca content in epidermis was higher than that in upper dermis [38], which also supports the higher Ca level in epidermal lesion than in dermis measured by LIBS.

On the other hand, earlier studies for the contents of Na, K, and Cl in skin showed little consistency. For example, based on x-ray microanalysis, Von Zglinicki et al. [39] reported that Na, K, and Cl ions were in equilibrium between epidermis and dermis under normal condition, whereas Werner-Linde et al. [40] reported that K increased weakly in the basal and spinous layers but decreased in the corneal layer and dermis. Cl content was reported to be low in the corneal layer but gradually increased toward dermis [40].

### 3.2 Classification by univariate analysis

The observed difference in spectral intensity between epidermal lesion and dermis of the emission lines in Table 1 suggests that the classification of epidermal lesion and dermis may become possible by monitoring the change of LIBS signal intensities of properly selected emission lines. While the intensity of an emission line varies widely as the measurement parameters such as laser energy and detector delay change, the relative intensity between two spectral lines remains much more consistent with respect to experimental conditions. Accordingly, intensity ratio is frequently adopted in LIBS analysis for the classification of biological tissues [15, 16, 18].

For the selection of appropriate spectral lines for classification, the lines whose intensity ratio varied most significantly upon target change from epidermal lesion to dermis are first considered. Table 1 shows that the LIBS signal intensity ratio of epidermal lesion to dermis is highest (4.94) for Ca(II) 317.933 nm line but lowest (0.41) for K(I) 766.490 nm and 769.896 nm lines, implying that $I_{Ca(II)}^{317.933 \text{ nm}} / I_{K(I)}^{766.490 \text{ nm}}$ will show the most conspicuous change. However, the high relative standard deviation (RSD) of Ca(II) 317.933 nm line intensity (46.3~47.9%) implies that this signal fluctuates over a wide range. Thus, Ca(II) 396.847 nm line which has a high intensity ratio (3.20) as well as relatively low RSD (26.2~28.0%) is selected as the Ca analytic line. When these lines were used, the intensity...
ratio $I_{\text{Ca(II) 396.847 nm}} / I_{\text{K(I) 766.490 nm}}$ became 2.02 for the epidermal lesion but dropped to 0.26 for the dermis (see Table 2), almost a factor of 8 difference upon sample change. Besides the K(I) 766.490 nm and 769.896 nm lines, Na(I) 588.995 nm and 589.592 nm lines also have a low ratio of epidermal lesion signal to dermis signal (0.54). When Na(I) 588.995 nm line was used, the intensity ratio $I_{\text{Ca(II) 396.847 nm}} / I_{\text{Na(I) 588.995 nm}}$ changed by a factor of 6 as the target was changed from epidermal lesion to dermis, implying that Ca(II) 396.847 nm and Na(I) 588.995 nm is also a good combination of spectral lines for the classification.

Alternatively, carbon signal is often chosen as the internal standard because carbon is one of the major elements composing human tissue and known to be independent of malignancy status of a tissue [16]. When C(I) 247.856 nm was used, the normalized intensity of Ca(II) 396.847 nm line ($I_{\text{Ca(II) 396.847 nm}} / I_{\text{C(I) 247.856 nm}}$) showed a moderate factor of 3 difference upon the sample change from epidermal lesion to dermis. Similarly, when one of the elements whose intensity was nearly invariant between epidermal lesion and dermis was used, for example, Mg(II) 279.553 and 280.270 nm, a similar level of change in intensity ratio (2.9) was obtained as shown in Table 2.

These results by univariate analysis demonstrate that the change of target tissue can be identified with high certainty by monitoring LIBS signal intensity ratio as the ablation front advances from epidermal lesion to dermis during laser treatment.

| Spectral line (nm) | Intensity Ratio       |
|-------------------|----------------------|
|                   | Dermis | Epidermal lesion | Epidermal lesion / Dermis |
| Ca(II) 396.847 / C(I) 247.856 | 1.54   | 4.47             | 2.90                    |
| Ca(II) 396.847 / Mg(II) 279.553 & 280.270 | 1.19   | 3.41             | 2.87                    |
| Ca(II) 396.847 / Na(I) 588.995 & 589.592 | 0.08   | 0.48             | 6                      |
| Ca(II) 396.847 / K(I) 766.490 | 0.26   | 2.02             | 7.77                    |

### 3.3 Multivariate analysis

The classification by univariate analysis above is based on the accumulated LIBS spectra from 30 measurement points. However, since precise depth control is critical during laser treatment, the capability to identify tissue change between pulses is more desired. Thus, a multivariate analysis was carried out using the Matlab statistical and bioinformatics tool box (Mathworks Inc., USA) to differentiate epidermal lesion and dermis from single-shot LIBS spectra. For this purpose, five spectral regimes of 240 nm ~ 281 nm, 310 nm ~ 325 nm, 390 nm ~ 425 nm, 586 nm ~ 593 nm and 762 nm ~ 773 nm which included the above discussed emission lines of interest of C, Mg, Ca, Na, and K were selected as the data for multivariate analysis. Also, the entire spectra from total 840 shots (2 pellet samples × 30 points × 14 shots) were included in the multivariate analysis as follows.

First, principal component analysis (PCA) was applied to the data in order to reduce dimension, which showed that the data can be almost completely expressed by 10 principal components up to 98.2% of variance. Figure 6 shows a scatter plot of PCA scores for the first two derived variables of epidermal lesion and dermis: the first (PC1) and the second (PC2) principal components covered 71.5% and 20.1%, respectively, of the total variance. Based on the PCA results, linear discriminant analysis was carried out to classify the epidermal lesion and dermis using the first ten principal components, and the classification results were verified by 10-fold cross-validation. Table 3 shows the confusion matrix obtained as a result of linear discriminant analysis, which demonstrates that high classification performance can
be achieved for epidermal lesion and dermis with single shot LIBS plasma. Only 2 cases out of 420 dermis spectra were misidentified as epidermal lesion while the epidermal lesion was perfectly predicted. The classification accuracy of epidermal lesion and dermis became 1 and 0.995, respectively.

4. Conclusion

Based on LIBS analysis of pellet samples, it was shown that the concentrations of common elements in epidermal lesion and dermis differ significantly, which was also confirmed by ICP-OES and AAS results. The intensity ratio of properly selected emission lines showed a difference as high as factor of 8 between epidermal lesion and dermis. When multivariate analysis was applied, a classification accuracy of 1 and 0.995 was achieved for epidermal lesion and dermis, respectively, with single shot LIBS spectra. Combined with the intrinsic capability of shot-to-shot depth control of laser ablation, these results suggest that LIBS can provide a reliable method to identify the target tissue during laser treatment of skin lesion so that unnecessary damage of dermis can be minimized. It should be noted, however, that the possible matrix effects of real skin on LIBS signal due to blood and water or the inhomogeneity of real skin need to be further investigated in order to apply LIBS for in vivo classification of epidermal lesion and dermis.

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