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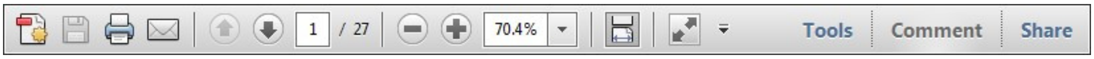
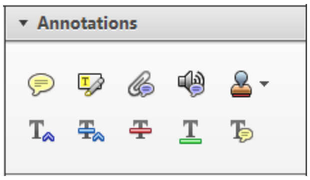
Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

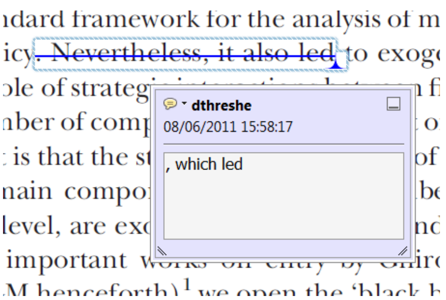


1. Replace (Ins) Tool – for replacing text.

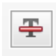
Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.



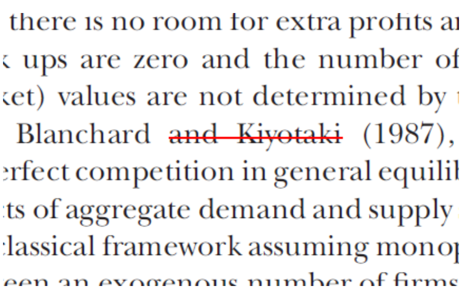
2. Strikethrough (Del) Tool – for deleting text.



Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

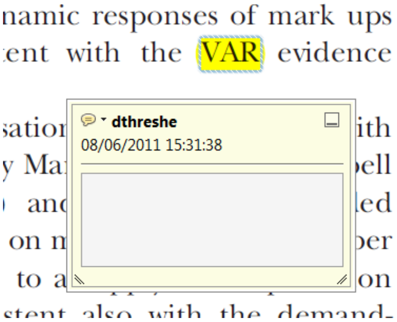


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

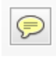
Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.



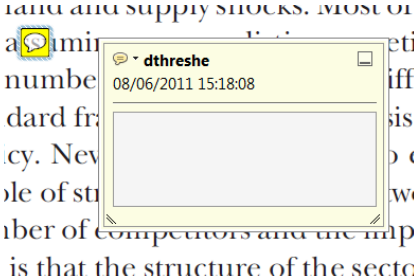
4. Add sticky note Tool – for making notes at specific points in the text.



Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.

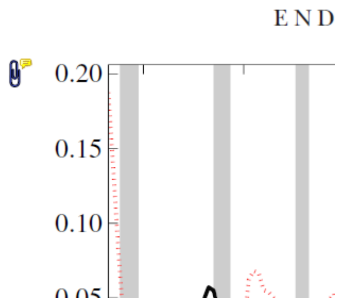


5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate place in the text.

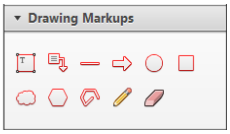
How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.



6. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks.



How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
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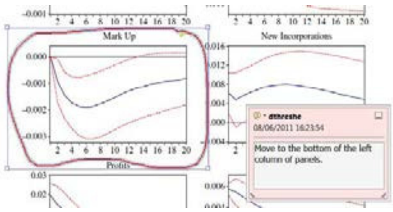
- Double click on the shape and type any text in the red box that appears.



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Observation of *In vivo* Morphologic Changes after Carbon Dioxide Ablative Fractional Laser in a Mouse Model Using Noninvasive Imaging Modalities and Comparison with Histologic Examination

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ABSTRACT

Ablative fractional carbon dioxide (CO_2) lasers have been widely used for several types of cosmetic dermatosis. A number of previous studies have evaluated this technique in animals or human beings by observing morphologic changes using an invasive modality such as skin biopsy. In this study, we assessed *in vivo* skin changes after CO_2 ablative fractional laser treatment in a mouse model using noninvasive imaging modalities (Folliscope® and Visioscan 98®), and each results was compared with data from histologic examination. An ablative fractional CO_2 laser was applied with different pulse energy between 7 to 35 mJ/microspot. As results of above methods, we also confirmed that the CO_2 ablative fractional laser generated injuries with increasing width and depth with increasing pulse energy. Although numerous papers have described application of this laser *in vivo* skin specimens, our study evaluated the feasibility of using relative noninvasive imaging modalities for assessing the outcome of laser ablation. Based on our data, we suggest that these technologies may be useful alternative modalities for assessing laser ablation that are easier to perform and less invasive than skin biopsy.

INTRODUCTION

The fractional laser combines the benefits of ablative and nonablative resurfacing lasers. Local resurfacing with a 1550-nm nonablative laser using an array of microscopic thermal wounds was found to be effective, with minimal patient downtime and morbidity. However, the procedure required multiple sessions and the results often varied (1,2). A novel CO_2 ablative fractional laser has been used in a technique similar to that of the traditional fractional laser. By depositing a pixilated pattern of microscopic ablative wounds surrounded by healthy tissue, the CO_2 ablative fractional laser increases the efficacy of ablative techniques and reduces the downtime associated with treatment (3,4).

The ability to observe skin changes *in vivo* is important when evaluating the efficacy of laser treatment. To date, several authors have evaluated series of skin biopsies by histopathologic examinations at different times following laser treatment in initial *in vivo* studies, and such studies have demonstrated the histologic and clinical effects of CO_2 ablative fractional laser (5–9). However, these previous studies investigated morphologic changes using only skin biopsy, which is invasive and requires a relatively long time for evaluation (10,11).

Therefore, in this study we assessed *in vivo* skin changes after CO_2 ablative fractional laser treatment in a mouse model using noninvasive imaging modalities in combination with microscopic histologic evaluation to determine whether increasing pulse energy affected the width and depth of injury created.

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The first two authors contributed equally to this work.

MATERIALS AND METHODS

Subjects. Five-week-old female BALB/c-nu mice (SLC Shizuoka, Japan) were used in all of the experiments in this study. Mice were housed and bred under conventional conditions (temperature: 23 ± 3°C, relative humidity: 55 ± 15%) at the R&D Center of the College of Medicine in Chung-Ang University, Korea. Animal care was performed according to ethical guidelines and the experimental protocol was approved by the Institutional Review Board of Chung-Ang University. After an acclimation period of 7 days, mice with healthy appearance were randomly allocated into six groups with six mice per group as follows: Group 1: untreated control, Group 2: 7 mJ pulse energy, Group 3: 14 mJ pulse energy, Group 4: 21 mJ pulse energy, Group 5: 28 mJ pulse energy and Group 6: 35 mJ pulse energy. Two mice in each group were sacrificed at 0, 3 and 7 days after the procedure, and their tissues were collected and fixed.

Laser treatment. The mice were treated with a CO_2 ablative fractional laser (Fraxis®, Ilooda Inc., Korea). The day before the experiment, the hair was removed and the back of the mouse was marked with four dots to make a square along the long axis. This region was then irradiated with one pass in a pattern of a rectangle sized 9 × 13 mm (a total of 140 dots), with a spot size of 100 μm and various pulse energy of 7 to 35 mJ/microspot. The processing methods are summarized in Fig. 1.

Evaluation criteria. To investigate the width of lesions in the laser application area, we used a magnified imaging device (Folliscope®, LeadM Corp., Korea). It is a small simple USB-based apparatus that is easily operated using a computer screen as an interface. It has been used in several studies to analyze hair and skin. In this study, we obtained photographs of the skin surface and measured the width of the microholes.

The depth of lesions was assessed by data provided by an optical imaging device (Visioscan 98®, C+K Electronic GmbH., Germany). It is a unique UVA light video camera with high resolution that allows direct study of the skin surface. The images produced by this camera show the skin structure and level. Furthermore, with combination of complementary software (Skin-Visiometer®), it can show the micro changes of the

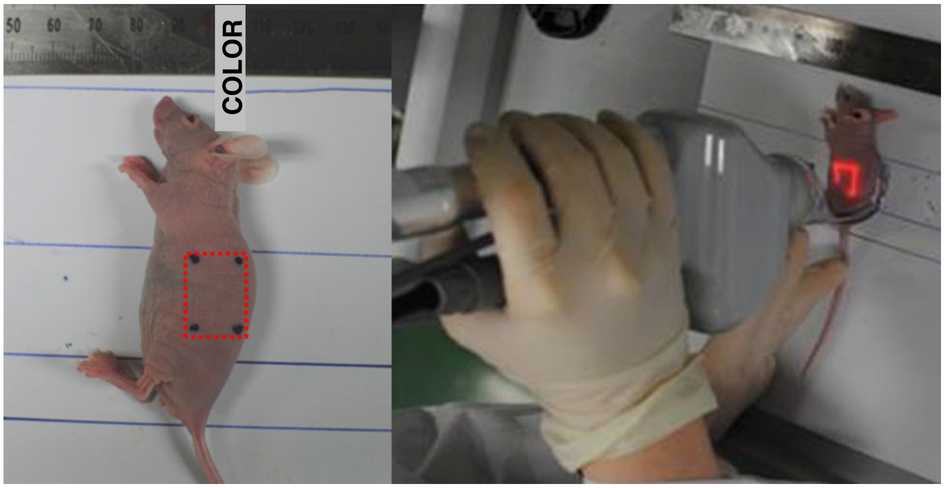

Figure 1. Method for applying laser treatment.

skin by transmitting light (3D negative image of the skin). Thus, the 3D coordinates for each pixel of the digitalized image are easily identified as different gradations of the color bars, although not providing an absolute numerical value.

To compare the width and depth of injury generated, samples were fixed in 10% formaldehyde, embedded in paraffin and stained with standard hematoxylin & eosin (H&E). The width and depth of the lesions were measured using a computer program that quantified the injury based upon a standard micrometer measurement. A camera (DP 70®, Olympus BIOSCOPS, Central Valley, PA) coupled to microscope (BX51®) was used to take the histologic images, and computerized digital imaging micrometer software was used (Olympus Stream Modular Imaging Software®).

Statistical analysis. The wilcoxon signed rank test was used to compare if there was any significant difference in width measured by Folliscope® and H&E. A P-value less than 0.05 was considered significant.

RESULTS

An increase in width with increasing pulse energy was observed by Folliscope®. The mean ablation width was 0.130 ± 0.009 mm for 7 mJ, 0.241 ± 0.0011 mm for 14 mJ, 0.305 ± 0.019 mm for 21 mJ, 0.322 ± 0.027 mm for 28 mJ and 0.343 ± 0.023 mm for 35 mJ (Table 1). The microhole size was measurable in the images until 3 days after the laser application, but most of the damage had healed after the 7th day. Although some marks remained, they appeared to be keratinized and could not be analyzed in all groups (Fig. 2). Similar pattern changes were obtained in H&E-stained samples. The mean value was 0.103 ± 0.008 mm for 7 mJ, 0.218 ± 0.0027 mm for 14 mJ, 0.245 ± 0.036 mm for 21 mJ, 0.274 ± 0.018 mm for 28 mJ and 0.267 ± 0.025 mm for 35 mJ (Table 1). Wilcoxon signed rank test revealed that the difference between values obtained by folliscope and H&E was not statistically significant.

In evaluation of depth changes, 3D images taken by Visioscan 98® showed higher and brighter green bars with increasing pulse energy, clearly confirming that microholes with greater depth were generated by increasing pulse energy (Fig. 3). After 7 days, the wound healing process was generally complete and most lesions had a normal appearance. Thus, the exact length of time to heal could not be calculated and interpreted in all groups (data not shown). A similar pattern of change was confirmed in H&E. The mean ablation depth at 0 days after treatment was 140.0 ± 20.0 μm for pulse duration of 7 mJ, 164.7 ± 21.5 μm for 14 mJ, 170.0 ± 13.1 μm for 21 mJ, 184.3 ± 9.0 μm for 28 mJ and 203.0 ± 7.0 μm for 35 mJ (Table 1).

DISCUSSION

The efficacy of the fractional laser depends on the depth of the injury; deeper zones of thermal damage result in greater clinical efficacy (15). Several initial *in vivo* studies have demonstrated the histologic and clinical effects of CO_2 ablative fractional laser treatment by examining a series of skin biopsies and the histopathologic state at different times following treatment (16). However, skin biopsy is an invasive procedure and histologic evaluation requires a fairly long time (10,11). To overcome this problem, in this study we analyzed the width and depth of microholes following CO_2 ablative fractional laser using noninvasive imaging modalities in addition to histologic evaluation and compared the resulting data.

The Folliscope® has gained recent popularity in specialized dermatologic fields and is increasingly being used for diagnosis and follow-up of several skin conditions (12). It has the advantages of clear visualization of the subject and instant and automatic measurement of different parameters such as skin surface components. The values of different numerical parameters can also be compared with previous measurements for the same subject. Consequently, it is often used as a valuable objective method for assessing the course of disease and the response to therapy. In this study, we used it for evaluating the depth of microholes compared with H&E staining.

Techniques for direct measurement of skin topography using the Visioscan 98® have been developed especially to study the skin surface. The camera features a high-resolution black and white video sensor and a ring-shaped UVA light source (that is completely harmless to the skin) for uniform illumination of the skin. With its special light, it produces a very sharp and nonglossy image. Furthermore, the camera can be connected to the computer directly via a port so that a live image is always visible. The distribution of the image is used to evaluate micro changes in the skin within 1 s. In addition to the image processing function, special software allows the calculation of a variety of skin surface parameters. The four clinical parameters used to quantitatively and qualitatively describe the skin surface as an

Table 1. Changes in mean ablation width and depth at different pulse energy. G1: untreated control, G2: 7 mJ pulse energies, G3: 14 mJ, G4: 21 mJ, G5: 28 mJ and G6: 35 mJ.

	Methods	Day	G1	G2	G3	G4	G5	G6
Width (mm)	Folliscope	0	0	0.130 ± 0.009	0.241 ± 0.0011	0.305 ± 0.019	0.322 ± 0.027	0.343 ± 0.023
	H&E		0	0.103 ± 0.008	0.218 ± 0.0027	0.245 ± 0.036	0.274 ± 0.018	0.267 ± 0.025
	Folliscope	3	0	0	0.225 ± 0.024	0.275 ± 0.037	0.254 ± 0.020	0.295 ± 0.023
	H&E		0	0	0.183 ± 0.004	0.217 ± 0.005	0.215 ± 0.003	0.245 ± 0.004
Depth (μm)	H&E	0	0	140.0 ± 20.0	164.7 ± 21.5	170.0 ± 13.1	184.3 ± 9.0	203.0 ± 7.0
		3	0	55.0 ± 11.5	59.0 ± 14.4	73.7 ± 12.5	112.3 ± 17.6	98.0 ± 4.0

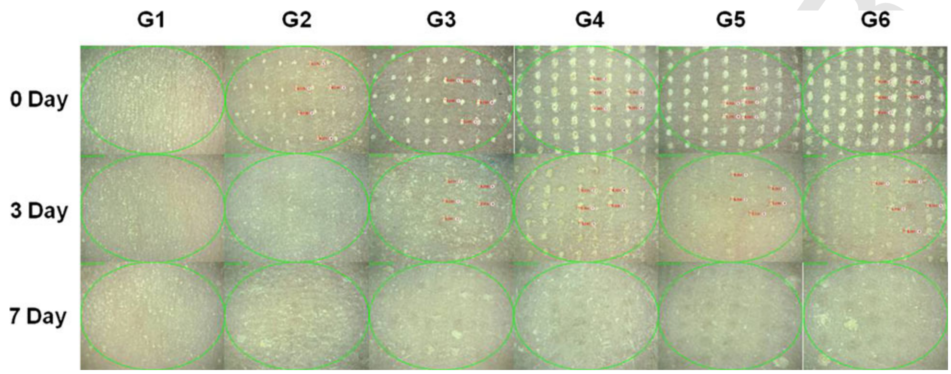

Figure 2. To determine changes in lesion width, images were taken using a folliscope and then measured. A proportional increase in width for each increase in pulse duration was found. The microhole size was measurable until 3 days after the laser application but most of the damage had healed after the 7th day posttreatment, leaving some marks that appeared to be keratinized. ~~G1: untreated control, G2: 200 μs pulse duration, G3: 400 μs, G4: 600 μs, G5: 800 μs, G6: 1000 μs.~~

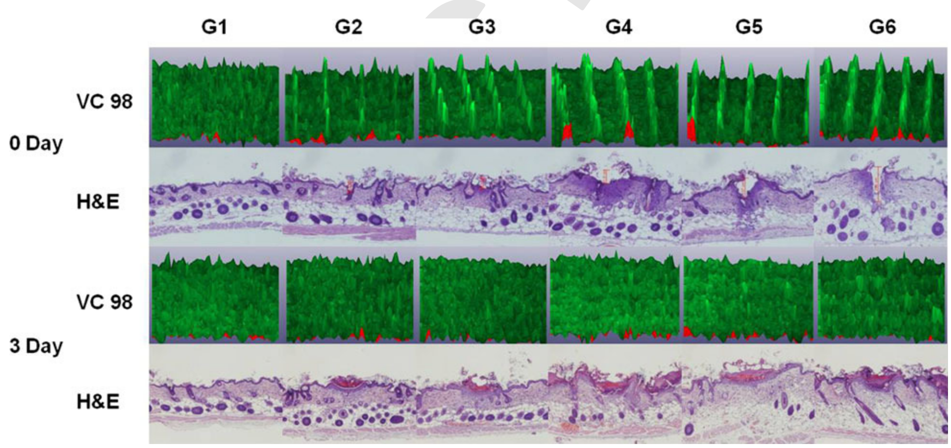

Figure 3. Histologic results (H&E, ×40) and VC 98 images of CO_2 fractional laser for determining changes in depth in mouse skin samples. Day 0: in all treatment groups, the epidermis showed the presence of stratum corneum disruption and microholes. The depth of injury was greater when the pulse duration was longer and increased depending on the pulse duration. Day 3: the wound healing process was evident at this point. ~~G1: untreated control, G2: 200 μs pulse duration, G3: 400 μs, G4: 600 μs, G5: 800 μs and G6: 1000 μs.~~

index are skin smoothness, roughness, scaliness and wrinkles. Thus, before-and-after treatment comparisons of the same skin site enable evaluation of trends in skin conditions. The software provided with it offers many additional interesting functions. For example, hair measurements, such as hair length after shaving, can easily be performed. The application of this flexible tool is without limitation. Consequently, it has been applied in numerous fields, such as testing cosmetics, pharmaceuticals and detergents and for objective clinical diagnoses in dermatology (~~13–15~~).

It can also be used together with other combination instrument (Skin-Visiometer®) as complementary technology, to analyze the micro changes of the skin by transmitting light (3D negative of the skin). Using this method, the 3D coordinates for each pixel of the digitalized image are known and profiles on the images can indirectly be predicted and compared with real depth by H&E staining. A colored 3D image can also be displayed quickly. All results can be stored or printed out together with the images. Measurements obtained using it generally provide images within a short period of time (less than 1 minute) (~~13~~).

In this study, 3D images taken by it showed higher and brighter green bars with increasing pulse energy. When compared with data from H&E staining, we were able to easily confirm that microholes with greater depth were generated with increasing pulse energy. Consequently, the imaging method used in this study provide comparative data that may allow assessment of the average depth of the microholes without invasive biopsy.

The present study has one important limitation with regard to differences between human and mouse skin. Although the skin of pigs is most similar to that of humans, mice are used for most animal studies because of their wide availability, small size and tractable nature. However, mouse skin is loose and thin. In addition, skin re-epithelialization is faster in mice than in humans because of the high hair density (17). Therefore, longer treatment intervals are necessary for CO_2 ablative fractional laser in humans.

We are entering an era of development of diverse laser modalities for use in the treatment of cosmetic conditions. The efficacy of laser treatment is generally assessed through clinical scores to evaluate visible changes. However, for microscopic analysis of structural skin changes, histologic examination of a skin biopsy has traditionally been the best option. In the current study, we demonstrated the feasibility of analyzing skin changes using imaging modalities, that are easier to use and less invasive than biopsy.

CONCLUSIONS

This study was conducted to investigate the efficacy of the CO_2 ablative fractional laser through using noninvasive imaging modalities and tissue analysis. We documented the correlation between increasing pulse energy and ablation width and depth using all of these approaches. Although numerous previous reports have described a similar function of this laser in *in vivo* skin specimens, in this study we analyzed treatment effectiveness by noninvasive imaging modalities (Folliscope® and Visioscan 98®). Based on our data, we suggest that these technologies may be useful modalities for confirming laser efficacy with greater ease of use and less invasiveness than biopsy.

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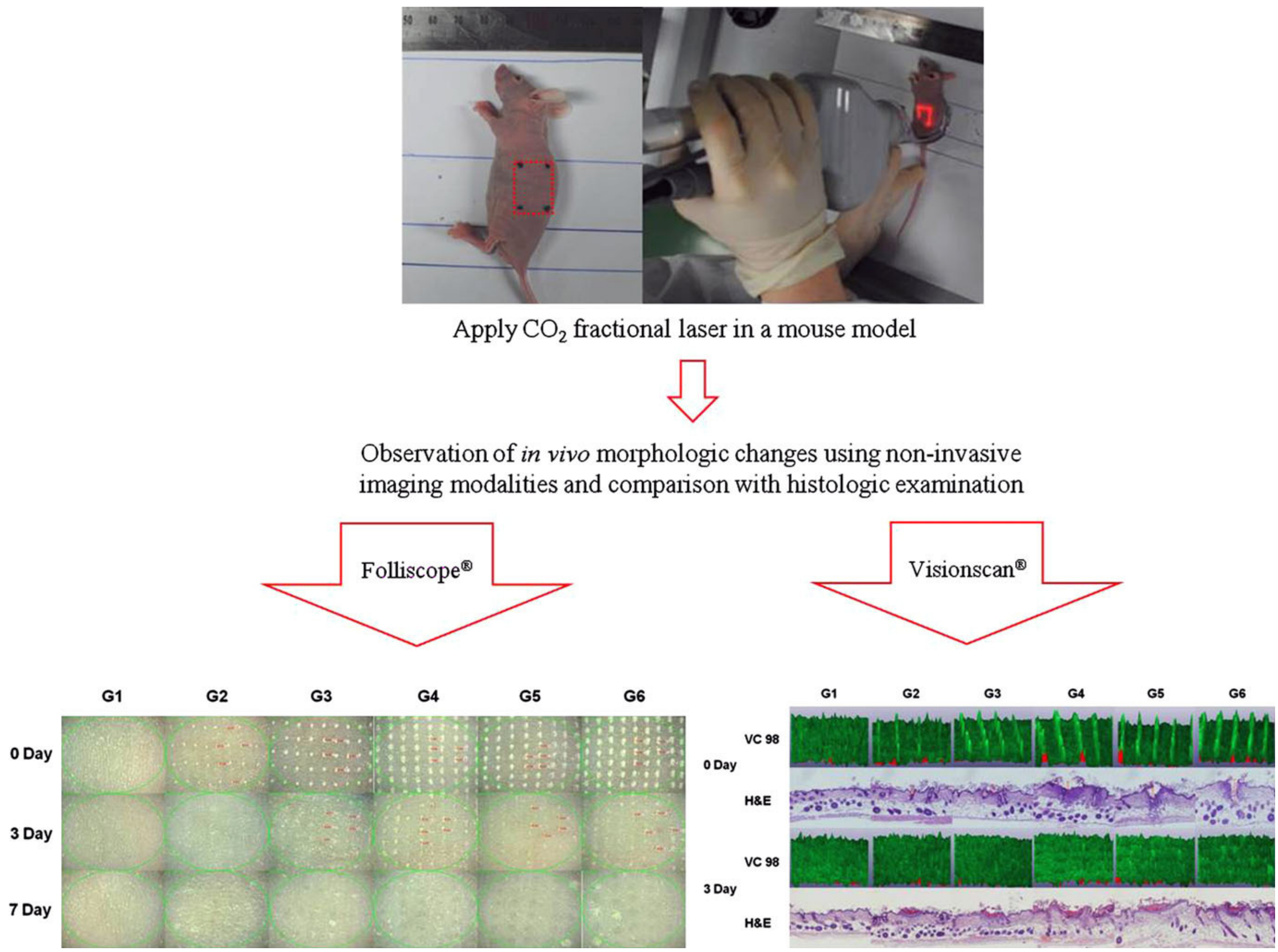

The ability to observe skin changes *in vivo* is important when evaluating the efficacy of laser treatment. This study was conducted to investigate the efficacy of the CO_2 ablative fractional laser through using noninvasive imaging modalities (Folliscope® and Visioscan 98®) and tissue analysis. Although numerous previous reports have described a similar function of this laser in *in vivo* skin specimens, in this study we analyzed treatment effectiveness by noninvasive imaging modalities. Based on our data, we suggest that these technol-
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Insert hyphen	=	=
Start new paragraph	¶	¶
Transpose	⊔⊓	⊔⊓
Close up	linking ⁐ characters	⁐
Insert or substitute space between characters or words	#	#
Reduce space between characters or words	⁀‿	⁀‿