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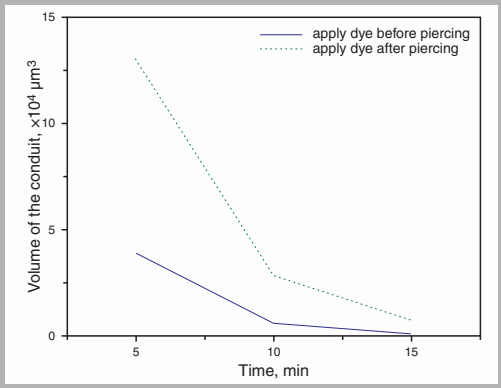
Abstract: Solid microneedles enhance the penetration of drugs into the viable skin but little is known about the geometry of the conduits *in vivo*. Therefore, laser scanning microscopy was used to visualize the conduits of a microneedle system with needles at a length of 300 μm in 6 healthy subjects over a period of time. The model drug, a fluorescent dye was applied before and after piercing. Laser scanning microscopy was evaluated as being an excellent method to monitor the geometry and closure of the conduits over time. The used microneedle system was evaluated as suitable to enhance the transport of model drugs into the viable epidermis without bleeding and a short closure time of the conduits at the skin surface.



Time dependent behavior of the mean values of the calculated conduit volumes when the dye was applied either before or after piercing

In vivo visualization of microneedle conduits in human skin using laser scanning microscopy

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1. Introduction

Dermal delivery of macromolecules is a challenge. A major hurdle is the skin barrier, which is located in the outermost layer of the skin, the stratum corneum. Although penetration enhancers and liposomes can be used to increase the penetration [1–4], these approaches are often only successful for low molecular weight drugs. When focusing on macromolecules, other approaches are required. One of the possibilities is the use of microneedles. Solid microneedles enhance the penetration of substances into the viable skin [5–8] by the generated conduits, which offer a route to transport the substances through the barrier. One field of application of these needles is dermal vaccination. Studies showed increased immune responses against diphtheria toxoid, influenza [9], and ovalbumin [10,11]. J.A. Mikszta et al. observed effective DNA vaccination after microneedle pre-treatment [12].

Although these results are promising, the differences between the microneedle arrays used is substantial. Not only do the needles differ in material, length, diameter and shape, but the different arrays in development are solid, hollow and sometimes pre-coated. Consequently, little is known about the geometry of the conduit formed, its behavior over a period of time and the diffusion of substances along these conduits *in vivo* in man. Furthermore, the ideal

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method of application remains unknown. One question to be asked is whether the application of the dye before piercing results in a more efficient penetration compared to dye application after piercing.

In the present study, the conduits of a microneedle system with needles at a length of 300 μm were visualized in 6 healthy subjects over a period of time. The model drug, a fluorescent dye, was applied before and after piercing.

2. Material and methods

2.1. Materials

The used microneedle array was the commercially available DermaStamp®. The DermaStamp® was kindly provided by H. Liebl (Dermaroller Deutschland S.a.r.l., Wolfenbüttel, Germany). The DermaStamp® has a central microneedle with 5 radially arranged needles. The needles were made out of stainless steel and have a very sharp needle tip. These microneedles have a length of 300 μm, a diameter of 120 μm and a needle distance of 2 mm. A fluorescent dye with a 0.2% solution of sodium fluorescein was used from Alcon Pharma GmbH (Freiburg, Germany).

2.2. Laser scanning microscopy

In vivo confocal laser scanning microscopy (CLSM) (Stratum® System, OptiScan, Melbourne, Australia) was used for visualization of the fluorescent dye within the skin and conduits. The system consists of a basic station containing an Argon laser (488 nm), a spectrometer and a control unit [13–15]. The basic station is connected to a hand-held device by optical fibers. The optical system and the focus tuning unit are integrated into the hand-held device [16,17]. This hand-held device can be used to investigate all body sites. The measuring area is 235×235 μm^2 and the penetration depth of the Argon laser radiation was ±200 μm [18]. A detailed description of the CLSM can be found in [14].

2.3. Volunteers

Six healthy volunteers aged between 20 and 58 years (mean age: 33 years, male/female: 17%/83%) were recruited. The volunteers had been informed about the aim and risks of the study and had given their written informed consent. The study had been approved by the University Hospital Charité Ethics Committee, Berlin, Germany in accordance with the Rules of Helsinki.

2.4. Study protocol

Piercing with the microneedle systems was performed in triplicate on the forearm of each volunteer in a randomized order. For each volunteer, one sterile microneedle was used and the skin area was disinfected before performing the examination. The sodium fluorescein dye was applied in two different methods: before and after piercing:

1. Dye application before piercing: application of dye was immediately followed by piercing. After piercing the dye was removed using soft tissues.
2. Dye application after piercing: piercing was immediately followed by an application of dye. The dye was removed using soft tissues after 1 minute of dye application.

For both conditions, one drop of sodium fluorescein was applied onto the site of CLSM evaluation to visualize the skin surface structures and conduits.

The DermaStamp® was applied three times in both conditions on the same area turning the stamp by about 45°. After applying the microneedle arrays and dye, the CLSM was used to localize a conduit. Representative images of the conduit were captured by varying the imaging depth at time points 5, 10, and 15 minutes after starting the CLSM evaluation. A series of pictures were taken over 1 minute but at least 5 pictures were taken to monitor the situation at the surface, the lateral and vertical distribution of the dye in the conduit and the maximal penetration depth of the dye into the viable tissue. Between the time points, the laser was set out of focus to avoid bleaching. The intensity of the laser varied between 450 and 520 μW. The application of a drop of sodium fluorescein on untreated skin for 1 minute served as a control. At a representative skin site, images were captured at different depths at time points of 5, 10, and 15 minutes after starting the CLSM evaluation.

2.5. Analysis

The pixel (fluorescence) intensity and area of selected pixel intensity was quantified using the public domain software ImageJ. The pixel intensities were categorized into three classes, referred to as fluorescent dye, diffusion and no signal. To set the threshold of these three classes of intensities in ImageJ, 31 random images with different depths were taken from two volunteers. The pixel intensity was calculated 10 times for each signal type in these 31 pictures. With an error rate of below 10%, the threshold values were set at pixel values 14 and 230 a.u. resulting in three categories. Above 230 a.u. the signal was referred to as "dye". Pixel intensity values between 14 and 230 a.u. were referred to as "dye diffusion", as the pixel intensity is weakened due to the diffusion process and values below 14 were not considered to be a signal. The autofluorescence of the skin was always below 14 a.u.

After automatically processing the images by ImageJ, the signal from other skin structures such as furrows was removed manually.

The following parameters were evaluated:

Time, min	Application of the dye	Dye at the surface, μm^2	Volume of the conduit, $\times 10^3$ μm^3	Maximum depth of dye diffusion, μm
5	Before	2200 ± 510	39 ± 9.2	130 ± 9.1
	After	4200 ± 1000	130 ± 40	170 ± 13
10	Before	450 ± 210	6.6 ± 3.0	93 ± 8.4
	After	1100 ± 310	2.9 ± 9.5	120 ± 12
15	Before	180 ± 140	1.1 ± 0.8	68 ± 4.6
	After	310 ± 120	7.0 ± 2.8	100 ± 9.2

Table 1 Mean values with standard errors of the area of dye at the surface, maximum depth of dye diffusion, and the volume of the conduit

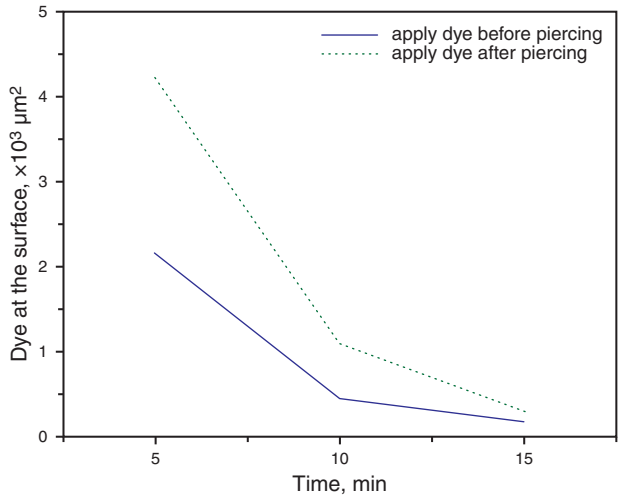

Figure 1 (online color at www.lphys.org) Time dependent behavior of the mean values of the dye area at the surface when the dye was applied either before or after piercing

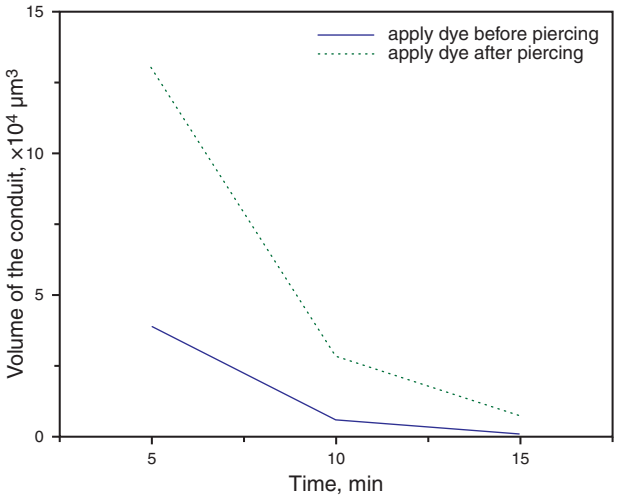

Figure 3 (online color at www.lphys.org) Time dependent behavior of the mean values of the calculated conduit volumes when the dye was applied either before or after piercing

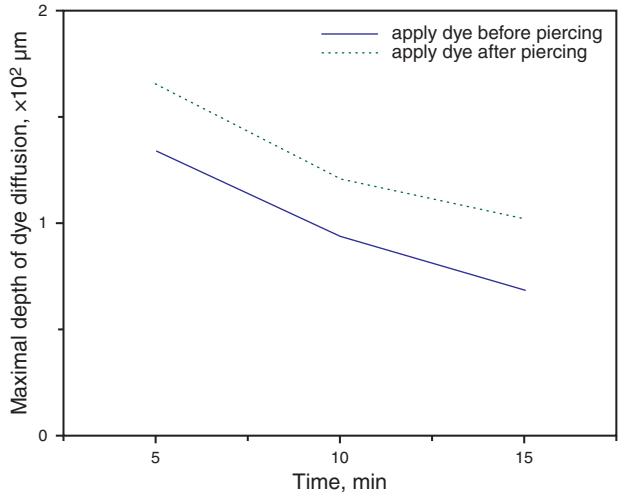

Figure 2 (online color at www.lphys.org) Time dependent behavior of the mean maximum depth of dye diffusion when the dye was applied either before or after piercing

- **Dye at the surface.** The fluorescence intensity of the dye at the surface was evaluated to investigate the closing of the conduit over a period of time. For this parameter the area of the dye at the surface at each time point was calculated from the CLSM image using ImageJ with a lower threshold of 230 for the pixel intensity.
- **Maximum depth of dye diffusion.** The maximal depth of dye diffusion was evaluated as the last depth where some fluorescence signal could still be detected, which has a fluorescent intensity over 14 a.u., but below 230.
- **Volume of the conduit.** The volume of the conduit was estimated assuming that the shape of the conduit is comparable to a cone. The base area of the cone was derived from the picture representing the maximum area of dye. This could be at the surface or inside the conduit (as it appears after 10 or 15 min). The length of the cone was calculated using the maximal depth of the dye minus the depth of maximum area of the dye. The calculation of the volume using the formula of a cone is an approximation especially at later time points.

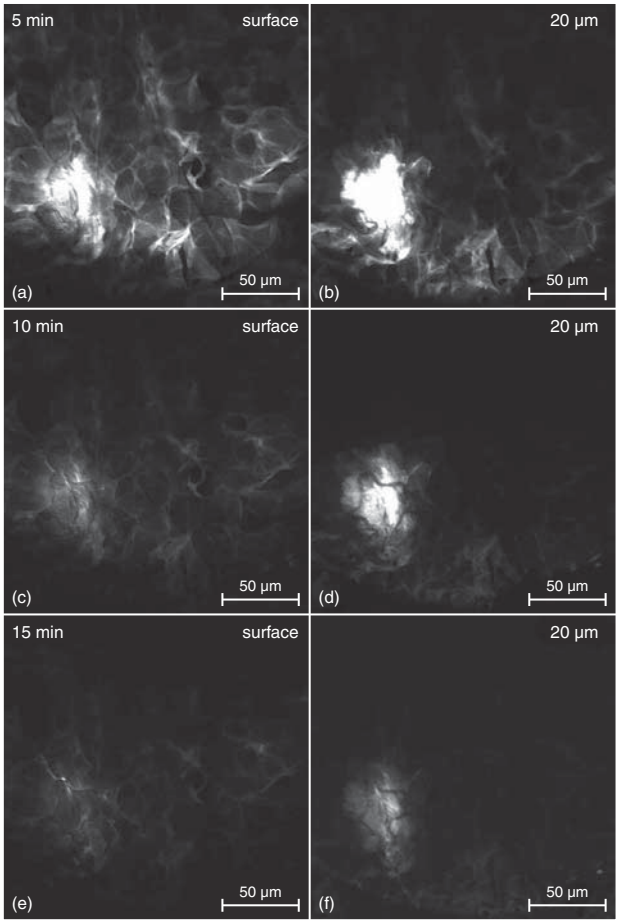

Figure 4 Example of CLSM images of a representative volunteer at different time points after piercing, when the dye was applied before piercing. The left images are at the surface and the images on the right show the described depth at different time points

	Dye at the surface	Volume of the conduit	Maximum depth of dye diffusion
5 min	(∗)	∗	∗
10 min	ns	∗∗	(∗)
15 min	ns	∗∗	∗

∗∗ $p < 0.010$ highly significant
∗ $p < 0.050$ significant
(∗) $p < 0.1$ trend
ns $p > 0.1$ non-significant

Table 2 Wilcoxon test of significance between the method of applying the dye, either before or after the application of microneedle systems

2.6. Statistics

Statistical analysis was performed with SPSS 16.0 for Windows (SPSS Inc., Chicago, Illinois). The Wilcoxon test was used, since the measurements in a volunteer are not independent. Factors such as time, before and after, and the data are not normally distributed.

3. Results

In all volunteers, no spots indicating bleeding were observed and the use of the microneedles was not perceived as painful. The changes over a period of time of the evaluated parameters are presented in Fig. 1 – Fig. 3 and Table 1. All values decrease over a period of time. The results are given for two different application methods: applying the dye before piercing, and application of the microneedle array after piercing. As shown in the figures, the application after piercing results in significantly higher values for the volume of the dye and the depth of the conduit (Table 2). Although the area of dye at the surface is also higher when applying the dye after piercing, this difference is not significant.

In Fig. 4, representative CLSM images are shown for applying the dye before piercing. Fig. 4a and Fig. 4b show the situation at 5 min after piercing and Fig. 4c, Fig. 4d and Fig. 4e, Fig. 4f at 10 and 15 min, respectively, after piercing. The left images present the surface conditions, the images on the right show the conditions at different depths. The results show that already after 5 min, the fluorescent intensity in the conduit at the surface is weaker compared to the fluorescent intensity at a depth of 20 μm in the skin. 10 min after piercing, almost no fluorescent intensity is visible at the surface. At 15 min after piercing, only a diffusion signal of the dye is visible in the tissue, which indicates that the conduit has been closed. When applying the dye for 1 min after piercing, more dye is present in the conduit and on average the dye is visible for a longer time compared to the images shown in Fig 4.

4. Discussion

For the first time, images of conduits induced by microneedles were visualized in human subjects. The CLSM offers the unique opportunity to obtain information on geometric parameters of the conduits and to monitor the behavior of a conduit and the dye over time. The strong decrease of the dye at the skin surface already after 10 min and in most cases after 15 min, indicates a fast closure of the conduits. This is an important effect as infections can be avoided due to open spots. The optical penetration depth of the used wavelength of 488 nm is between 150 and 200 μm depending on the amount of fluorescent dye and the optical properties of the investigated tissue. Therefore, in the case of dye application before microneedle treatment, the maximum depth of dye diffusion after 5 min could even be located at a greater depth. However,

because of the absence of bleeding the penetration depth should be in the range of the measured values. The measured depth of about 150 μm of the used microneedles is deep enough for macromolecules to reach the viable epidermis and probably even the dermis.

Different results of the parameter maximum depths of dye diffusion and the volume of the conduit between to the application of the dye before or after the piercing can be explained by the different time frame of dye penetration. Longer penetration times allow more diffusion of the dye into the conduits. The closure of the surface indicated by the dye at the surface does not differ significantly, when the dye is applied either before or after piercing. Therefore, both methods can be applied.

5. Conclusion

Laser scanning microscopy is an excellent method to measure the size of conduits produced by microneedles *in vivo* and to monitor the closure of the conduits over a period of time. The used microneedle system was evaluated as being suitable to enhance the transport of model drugs into the viable epidermis without bleeding and a short closure time of the conduits at the skin surface.

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