On-Line Diffusion Profile of a Lipophilic Model Dye in Different Depths of a Hair Follicle in Human Scalp Skin

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In skin and hair research, drug targeting to the hair follicle is of great interest in the treatment of skin diseases. The aim of this study is to visualize on-line the diffusion processes of a model fluorophore into the hair follicle at different depths using fresh human scalp skin and confocal laser scanning microscopy. Up to a depth of 500 μm in the skin, a fast increase of fluorescence is observed in the gap followed by accumulation of the dye in the hair cuticle. Penetration was also observed via the stratum corneum and the epidermis. Little label reached depths greater than 2000 μm. Fat cells accumulated the label fastest, followed by the cuticular area and the outer root sheath of the hair follicle. Sweat glands revealed very low staining, whereas the bulb at a depth of 4000 μm was visualized only by autofluorescence. From this study, we conclude that on-line visualization is a promising technique to access diffusion processes in deep skin layers even on a cellular level. Furthermore, we conclude that the gap and the cuticle play an important role in the initial diffusion period with the label in the cuticle originating from the gap.

Key words: confocal laser scanning microscopy/on-line/hair follicle/transport/human skin
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Investigations of transport and delivery processes in skin aim at systemic or local delivery of drugs in order to improve their efficacy. Follicular delivery is one main area of interest when treating skin diseases originating in the pilosebaceous unit (hair follicle and sebaceous gland). Therefore, it is crucial to measure increases and decreases of a drug in the target area.

Reviews of the hair follicle as a route for drug delivery have been published (Lauer *et al*, 1995; Weiner, 1998; Agarwal *et al*, 2000) and summarize the status of pilosebaceous drug delivery. Numerous studies have been performed studying the role of follicular contribution to the transport of which only a selection is cited here (Kao *et al*, 1988; Illel *et al*, 1991; Hueber *et al*, 1994). These studies focus on the comparison of diffusion profiles over time between hairless or follicle-free skin and normal skin.

Visualization of follicular penetration in the upper hair follicle has also been examined. This has been achieved by fluorescence or laser scanning microscopy (Schaefer *et al*, 1989; Rolland *et al*, 1993; Lauer *et al*, 1995; Allec *et al*, 1997; Lademann *et al*, 1999), autoradiography (Fabin and Touitou, 1991; Chu *et al*, 1996), scanning electron microscopy (Schaefer *et al*, 1989), microphotography in combination with UV light (Foreman *et al*, 1979), and light microscopy (Meidan *et al*, 1998). Penetration into deeper hair follicle regions has been reported by only three authors (Serizawa *et al*, 1995; Lieb *et al*, 1997; Genina *et al*, 2002). Lieb *et al* reported transport of oligomers (24 and 25 mer) into the hair bulb. Fluorophore penetration in non-fixed skin containing the subcutaneous fat was studied previously using an on-line visualization technique. In these studies, we focused on either permeation in the epidermis (Grams *et al*, 2004a) or the hair follicle (Grams *et al*, 2004b). The first aim of this study is to extend the previous studies and demonstrate that visualization of diffusion processes can be extended up to a depth of at least 4000 μm. An additional aim is to visualize on-line the penetration of one model substance into the hair follicle, with special focus on deep follicular regions in the dermis and the subcutaneous fat. An attempt has been made to visualize the diffusion into the hair bulb. On-line visualization has not been achieved previously without chemical fixation or fixation by freezing.

Results

Several time series (on-line) of the diffusion of BFL (Bodipy FLCs) focusing on the hair follicle at various depths have been recorded. Figure 1 displays a schematic drawing of a hair follicle in human scalp skin and the approximate location of the various time series from the skin surface up to the subcutaneous fat.

In the initial diffusion period the fluorescence of the dye is primarily located along the gap and the cuticle At the skin surface, a diffusion pattern (Grams *et al*, 2004b) is obtained as reported previously. Ten minutes after application of the donor phase, no dye was detected in the hair follicle and in the skin. Within the next hour, diffusion commenced via the hair follicle and the stratum corneum. The images (Fig 2*A*, 1 h) show that in the initial time period, the gap and the cuticle of the hair follicle are stained very bright and that

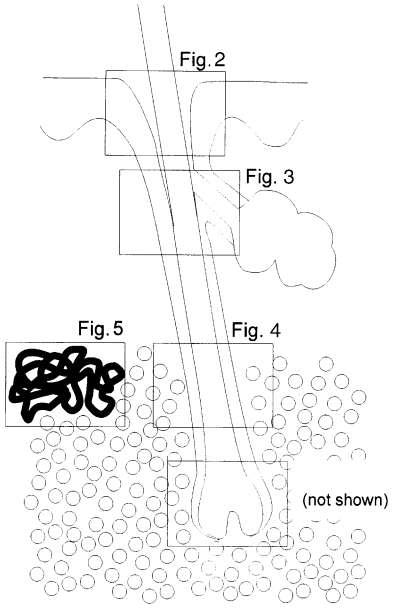

Figure 1
Cross-sectional overview of the hair follicle in human scalp skin. The deeper regions of the hair follicle are located in the subcutaneous fat. Marked areas indicate the position of the various time series selected in this study. Each time series requires a new hair follicle cross-section.

the fluorescence reaches deeper as compared to the staining in the non-follicular route. This strongly indicates permeation of the dye via the gap and cuticle. Up to 3 h after application, the fluorescence intensity of the follicular gap increases. Subsequently, a decrease in fluorescence intensity in the gap is observed. This fluorescence decrease is not detected in the cuticle. The epidermis is weakly labelled after 1 h, whereas fluorescence enters the dermis after 2 h. The fluorescence intensity of these non-follicular regions, however, remains lower than the intensities in the gap and in the cuticle. After termination of the on-line experiment, an image with higher magnification of the gap area (Fig 2*B*) was taken.

An intensity gradient is observed from the cuticle (high intensity) to the outer root sheet (low intensity) At a skin depth of 800 μm (Fig 3*A*), an intensity gradient is observed between the cuticle (high intensity) and the outer root sheet (low intensity), which remains during the entire diffusion period of 16 h. Additionally, the onset of the appearance of the fluorescence is delayed compared with the superficial layers (Fig 2*A*). Only after 2 h, fluorescence appears in the area of the gap and cuticle. After 3 h (Fig 3*A*, image 4) fluorescence is also detected in the outer root sheath simultaneously with the dermis; however, the labelling is less bright than that in the gap and cuticle. The more intense staining of the gap and the cuticle compared with the surrounding tissue remains even after 16 h. Furthermore, the intensity gradient in the gap and cuticle is much higher than in the surrounding tissue. This implies that in deeper areas of the hair follicle, the observed difference in fluorescence intensity between the cuticle and the outer root sheath is decreased. A higher magnification image at the end of the diffusion experiment (Fig 3*B*) confirms that the cuticle is still stained stronger than the outer root sheath, with a higher difference in intensity closer to the skin surface. The hair shaft, the dermis, and a region where the inner root sheath is disintegrating is hardly labelled at all.

Deep in the skin (2100 μm) the fluorescent intensity remains low (Fig 4) Five hours after starting the diffusion, the first staining occurs in the fat cells. Due to the low staining at this depth, the contrast and the brightness of the images had to be adjusted to obtain information about changes in label distribution. After 7 h, the cuticle of the hair follicle is stained and increases in fluorescence intensity over time. Furthermore, the outer root sheath is weakly stained after 9 h and the staining increases with time. As has already been observed in the previous images, the inner root sheath and the hair shaft are hardly stained at this depth.

Only a very low staining is observed in the sweat glands at a depth of 2030 μm (Fig 5*A*). Since only very little fluorescence was detected, image adjustments (contrast and brightness) were required. Immediately after starting the experiment, typical dot-like autofluorescence in the sweat glands were visible, which did not change over time. The first staining was observed after 4–5 h in the fat cells surrounding the sweat gland. As the diffusion proceeded, the autofluorescence-free area of the sweat glands also retained the dye, with black non-stained areas remaining. In Fig 5*B* a cross-section is depicted to illustrate the location of the sweat gland.

Proceeding even into deeper regions of the skin (4000 μm), only autofluorescence was observed in the hair bulb during the entire experiment (not shown). Not even the surrounding fat cells showed an increase in fluorescence intensity.

Discussion

The technique of on-line visualization has already been proven to be effective for time-dependent visualization of diffusion processes (i) in the stratum corneum, epidermis, and dermis (Grams *et al*, 2004a) and (ii) in the hair follicle at the skin surface (Grams *et al*, 2004b). Our studies indicate that the technique is also suitable for "in depth visualization" of diffusion processes over time; however, care has to be taken in the positioning of the skin piece to visualize an appropriate hair follicle (Fig 6). The donor phase has to be in direct contact with the hair follicle opening. Furthermore, the follicle should be located close to the skin surface, but not too close to the cutting plane, which would result in damaged follicular structures. Therefore a parallel positioning of the hair follicle to the cutting plane is crucial. A divergence from the parallel orientation can result in a very large distance from the cutting plane (Fig 6*B*), and therefore insufficient fluorescence intensity for image detection in the area of the hair bulb. Figure 6*C* shows a non-parallel orientation of the hair follicle. The bulb is close enough to the cutting surface; however, the hair follicle does not reach the skin surface, making it unusable. The situation in Fig 6*D* allows visualization of the intact follicle only in the upper half of the skin. Although preparations have to be judged critically, this technique provides access to diffusion processes

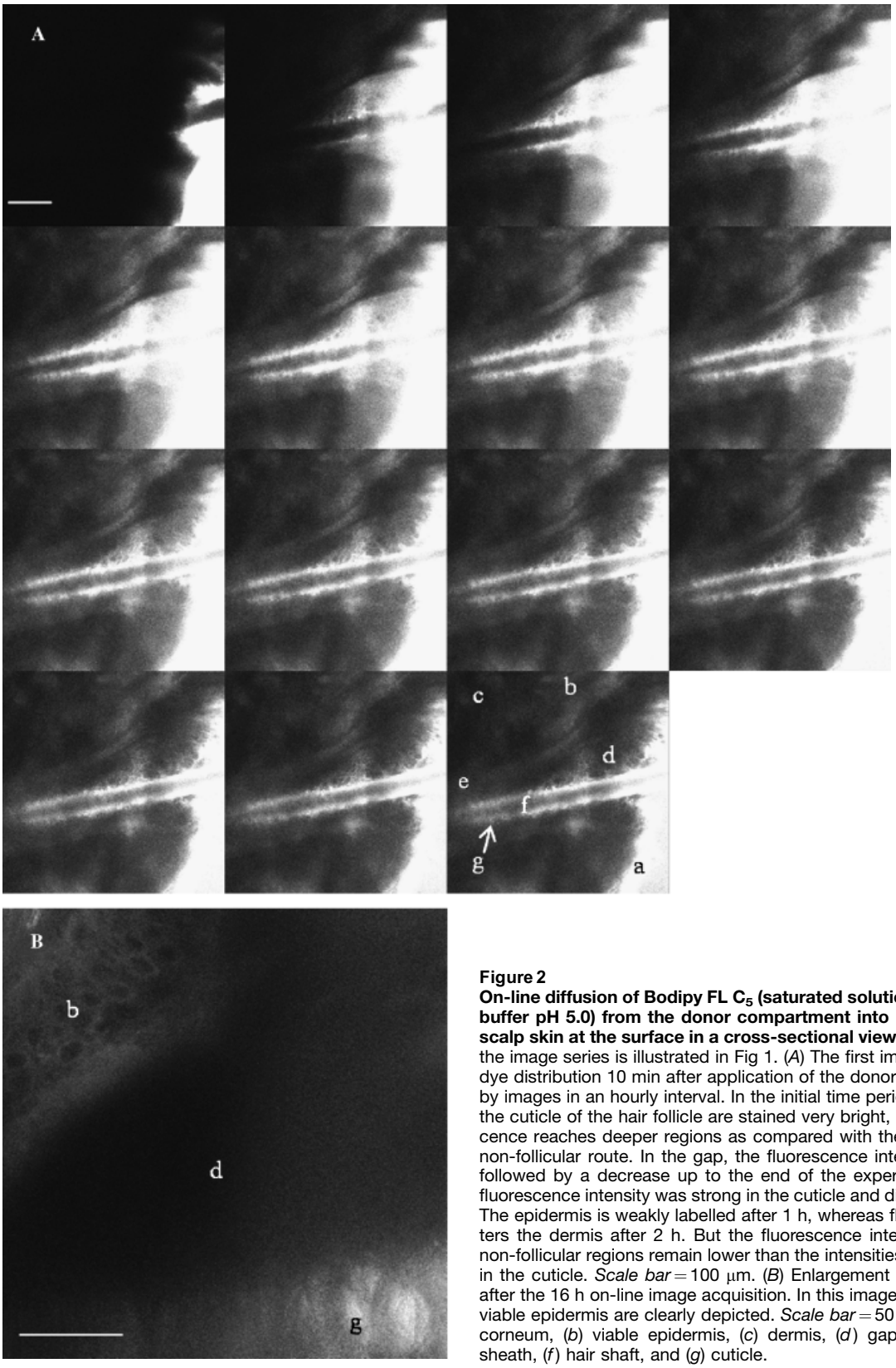

Figure 2
On-line diffusion of Bodipy FL C_5 (saturated solution in citric acid buffer pH 5.0) from the donor compartment into unfixed human scalp skin at the surface in a cross-sectional view. The location of the image series is illustrated in Fig 1. (*A*) The first image depicts the dye distribution 10 min after application of the donor phase followed by images in an hourly interval. In the initial time period, the gap and the cuticle of the hair follicle are stained very bright, and the fluorescence reaches deeper regions as compared with the staining in the non-follicular route. In the gap, the fluorescence intensity increases followed by a decrease up to the end of the experiment while the fluorescence intensity was strong in the cuticle and did not decrease. The epidermis is weakly labelled after 1 h, whereas fluorescence enters the dermis after 2 h. But the fluorescence intensities of these non-follicular regions remain lower than the intensities in the gap and in the cuticle. *Scale bar* = 100 μm. (*B*) Enlargement of the gap area after the 16 h on-line image acquisition. In this image, the cells in the viable epidermis are clearly depicted. *Scale bar* = 50 μm. (*a*) Stratum corneum, (*b*) viable epidermis, (*c*) dermis, (*d*) gap, (*e*) outer root sheath, (*f*) hair shaft, and (*g*) cuticle.

in deeper follicular structures of non-fixed human skin including the subcutaneous fat.

Scheuplein (1967) has already predicted the importance of the follicular pathway in the initial diffusion processes. This diffusion process, however, has not been visualized on-line to date. In the on-line time series at the skin surface and at a depth of 800 μm, the gap of the hair follicle exhibits very early strong staining followed by the staining of the cuticle when using this lipophilic model substance. These images strongly indicate that the labelling of the cuticle originates from the dye present in the gap. Since the epidermis is weakly stained shortly after the gap and the cuticle, BFL

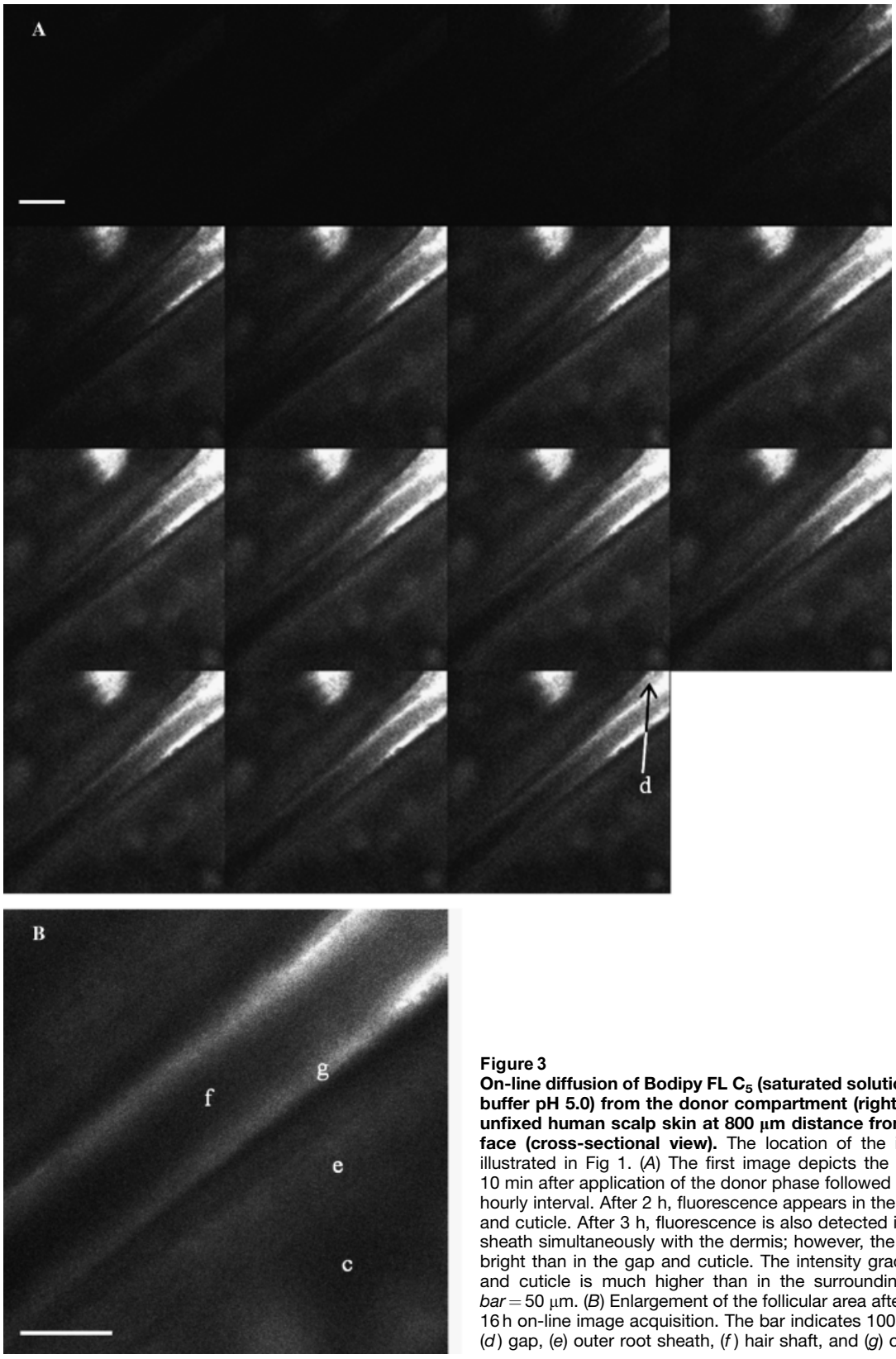

Figure 3
On-line diffusion of Bodipy FL C_5 (saturated solution in citric acid buffer pH 5.0) from the donor compartment (right of image) into unfixed human scalp skin at 800 μm distance from the skin surface (cross-sectional view). The location of the image series is illustrated in Fig 1. (*A*) The first image depicts the dye distribution 10 min after application of the donor phase followed by images in an hourly interval. After 2 h, fluorescence appears in the area of the gap and cuticle. After 3 h, fluorescence is also detected in the outer root sheath simultaneously with the dermis; however, the labelling is less bright than in the gap and cuticle. The intensity gradient in the gap and cuticle is much higher than in the surrounding tissue. *Scale bar* = 50 μm. (*B*) Enlargement of the follicular area after the end of the 16 h on-line image acquisition. The bar indicates 100 μm. (*c*) Dermis, (*d*) gap, (*e*) outer root sheath, (*f*) hair shaft, and (*g*) cuticle.

also penetrates through the stratum corneum at early time points. This is in agreement with the general opinion that small (BFL: 300 g per mol) and medium lipophilic compounds (BFL: $\log P = 2.5$) penetrate relatively fast through the stratum corneum (Flynn, 1990; Guy, 1996; Hadgraft and Pugh, 1998; Barry, 2001). Keeping in mind that the gap and the cuticle are stained strongly, whereas the epidermis is stained moderately in the beginning of the diffusion process, the origin of the label in the outer root sheath is not clear. The direct connection from the epidermis to the outer root sheath and their similarities in cell structures make this a possible route of penetration. On the other hand, the label

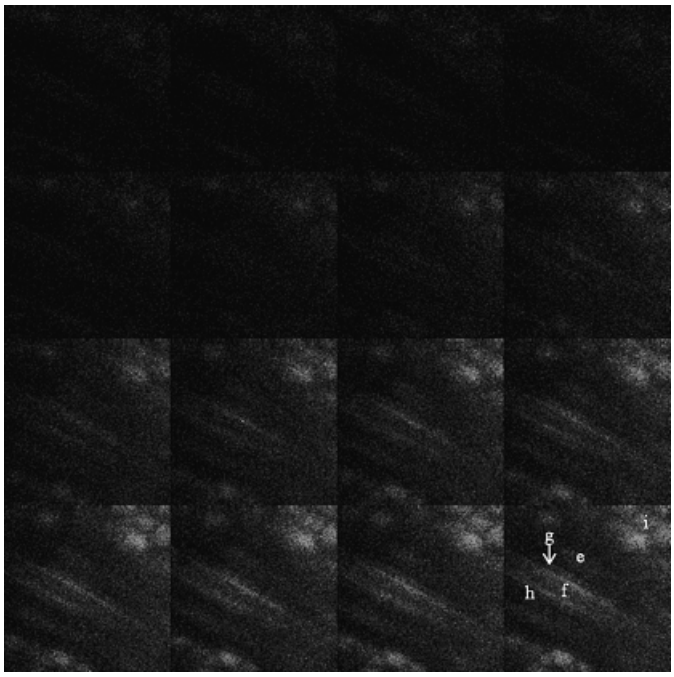

Figure 4
On-line diffusion of Bodipy FL C_5 (saturated solution in citric acid buffer pH 5.0) from the donor compartment (right of image) into unfixed human scalp skin at 2100 μm distance from the skin surface (cross-sectional view). The location of the image series is illustrated in Figure 1. Contrast and brightness of the image series were optimized due to weak labelling. The first image depicts the dye distribution 10 min after application of the donor phase followed by images in an hourly interval. Despite low fluorescence intensity, the first staining of skin structures, namely the subcutaneous fat cells, is visible 5 h after the start of the experiment. After 7 and 9 h, the cuticle and the outer root sheath of the hair follicle are stained and increase in fluorescence intensity over time. The inner root sheath and the hair shaft are hardly stained. (*e*) Outer root sheath, (*f*) hair shaft, (*g*) cuticle, (*h*) inner root sheath, and (*i*) subcutaneous fat cells.

can penetrate from the gap through the thinner stratum corneum of the infundibulum into the outer root sheath. The inner root sheath is not yet present at the level of the infundibulum and starts to appear below the sebaceous gland (isthmus). Since the lipophilic dye penetrates the stratum corneum relatively fast and is detected in a high amount in the follicular gap, none of the two routes can be excluded. Since the dermis is labelled after the outer root sheath, however, the observations indicate strongly that the dye in the outer root sheath originates from the gap or the epidermis and not from the dermal tissue. Deeper in the hair follicle, where the gap disappears, the fluorescent gradient in the cuticle is much stronger than the gradient in the surrounding tissue (Fig 3*B*). This strongly indicates that at this depth, in the hair follicle, label is diffusing from the cuticle to the surrounding tissue, subsequently being the inner, outer root sheet, and the dermis. Particulate structures such as titanium dioxide microparticles have also been reported to accumulate in the follicular opening (Lademann *et al*, 1999; Toll *et al*, 2004). Due to their prolonged presence and higher concentration in the follicular orifice, particulate carriers therefore have the potential to increase the penetration via the follicular route.

In even deeper skin regions, little label was observed. This implies that even *in vitro* in the absence of active blood vessels only a small amount of applied drug reaches skin structures at a depth of 2000 μm or more. Although in the upper part of the skin staining was always first observed in the gap and the cuticle, in the deeper skin regions the fat cells of the subcutaneous tissue were labelled first. Since this is observed in regions surrounding the hair follicle as well as in regions surrounding the sweat gland, it is very likely that the fat cells retrieve their label from the dermis. Structures of the appendages (cuticle and outer root sheath of the hair follicle and sweat gland cells) can be stained by label from appendageal structures closer to the surface or via the dermis. Since the inner root sheath is hardly stained throughout time and depth, we suggest that the cuticular structures of the hair follicle obtain their label from the cuticle further up in the skin. The bulb of the hair follicle provides a target for drugs and nutrients since the hair follicle starts developing in this region. The images show that insufficient label molecules reach a skin depth of 4000 μm for the confocal laser scanning microscopy (CLSM) detection. Not even fat cells are stained, suggesting that the majority of the label is captured and retained by fat cells at a depth of 2000 μm. This subcutaneous fat might function as a depot for the lipophilic label. In order to increase the transport along the hair follicle, different application techniques have to be considered. In the literature, it has been reported that an electric current increases the presence of a model fluorophore in the hair follicle (Turner and Guy, 1998) in addition to opening the follicular orifice (Schaefer and Lademann, 2001). Furthermore, liposomes and viral vectors are reported to have the potential of follicular delivery of small and large molecules as well as DNA molecules (Hoffman, 1998; Gupta *et al*, 2001; Ciotti and Weiner, 2002; Hoffman, 2004).

The on-line visualization presented in this paper uses skin *in vitro* and therefore lacks blood flow at depths of 200 μm, around the sweat gland, and around the hair follicle. If blood flow were to be present, clearance of the drug by the bloodstream in the dermis and the hair follicle close to the epidermal–dermal junction would likely occur. This would reduce the amount reaching the fat cells and deeper layers of the hair follicle. Simultaneously, however the blood vessels might also provide a fast transport system to the highly vascularized hair bulb and redistribute the previously gathered drug molecules to the hair bulb. Although the basic staining pattern of the hair follicle between BFL, a less lipophilic, and a more lipophilic substance is comparable (Grams and Bouwstra, 2002; Grams *et al*, 2003), extrapolation of the results described in this work to other substances has to be investigated in the near future.

The decrease of fluorescence over time in the gap is due to a depletion of the dye in the donor phase. In this early on-line diffusion cell design, a static setup was chosen to prove the general usage. In future experiments, a flow-through diffusion system should be established to maintain a constant donor phase concentration and circumvent the depletion of the donor phase. Pilot studies have shown the technical feasibility of a flow-through on-line setup. This setup would create the appropriate conditions to study steady-state diffusion, which might facilitate the calculation of diffusion coefficients of different penetrants in the various skin regions.

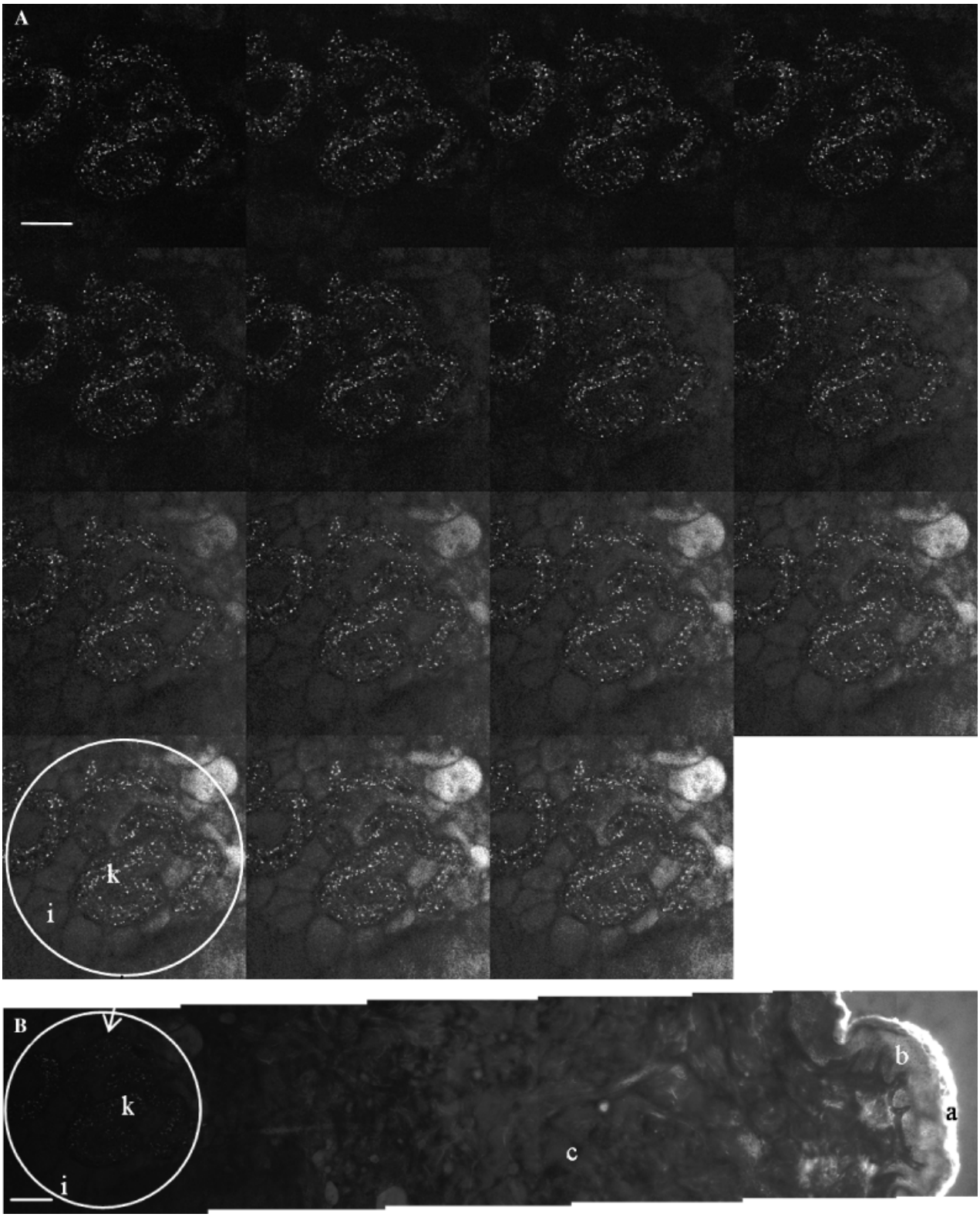

Figure 5
On-line diffusion of Bodipy FL C_5 (saturated solution in citric acid buffer pH 5.0) from the donor compartment (right of image) into the sweat gland of unfixed human scalp skin at 2040 μm distance from the skin surface (cross-sectional view). The location of the image series is illustrated in Fig 1. (*A*) The first image depicts the dye distribution 10 min after application of the donor phase followed by images in an hourly interval. Contrast and brightness of the image series were optimized due to little labelling. Dot-like autofluorescence is visible in the sweat glands. The first fluorescence staining is observed after 4–5 h in the fat cells surrounding the sweat gland. As the diffusion proceeds, the autofluorescence-free area of the sweat glands also retains the dye, with black non-stained areas remaining. (*B*) After 16 h of on-line diffusion, a cross-section of the skin was taken to determine the depth of the on-line time series indicated by a white circle. Contrast and brightness were not optimized due to the strong labelling of the stratum corneum. *Scale bars* = 100 μm (*A*, *B*). (*a*) Stratum corneum, (*b*) viable epidermis, (*c*) dermis, (*i*) subcutaneous fat cells, and (*k*) sweat gland.

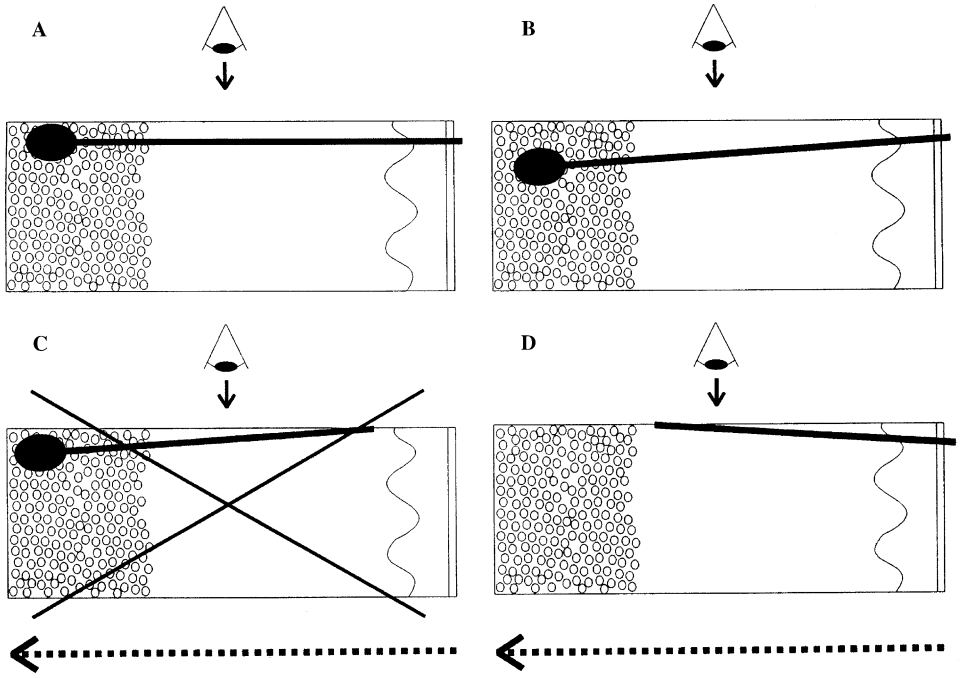

Figure 6
Positioning of the hair follicle in the skin to enable visualization using the on-line cutting device. The eye indicates the direction of visualization while the dashed arrow indicates the direction of diffusion after application in the donor compartment. (*A*) Optimal positioning of the hair follicle, namely with the opening in the donor compartment, parallel and close to the cross-section. (*B*) Sub-optimal positioning where the fluorescence of the deeper follicle section will be substantially decreased due to scattering and absorption processes. This positioning is only suitable for visualization in the middle and eventually the upper section. (*C*) A follicle positioned without an opening into the donor compartment results in erroneous diffusion profiles. (*D*) Sub-optimal positioning where the hair bulb is removed during the cutting procedure. This positioning is only suitale for visualization in the upper section where the hair follicle is still intact.

Materials and Methods

The study was approved by the LACDR Scientific board. Bodipy FL C_5 was obtained from Molecular Probes, Leiden, the Netherlands. Fresh human scalp skin from cosmetic face-lift surgery was transported on a filter paper soaked with phosphate-buffered saline (PBS), pH 7.4 (139 mM NaCl, 2.5 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 25 mg per L streptomycin, and 25,000 U per L penicillin) and stored at 4°C until use. Within 4 h after face-lift surgery, the experiment was started, thereby preventing alteration of the skin due to extended storage and freezing. The dental clay Impregum F (Espe) for sealing of the on-line diffusion cell was purchased from Dental Union BV, Nieuwegein, the Netherlands.

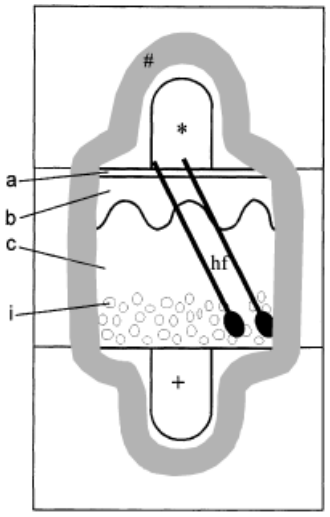

Figure 7
Cross-Sectional cutting surface. View of the cross-sectional cutting surface displaying the stratum corneum (*a*), the viable epidermis (*b*), the dermis (*c*), the subcutaneous fat tissue (*i*) and the hair follicle (hf) in the combined cross-section/on-line device. The cross-section is sealed by dental clay (#) to create closed donor (*) and acceptor compartments (+). After sealing, hair follicle parts are imaged by confocal laser scanning microscopy.

Preparation for the diffusion experiment The acceptor phase consisted of PBS, pH 7.4. The donor phase consisted of a saturated solution (0.1 mg per mL) of Bodipy FL C_5 (excitation/emission = 505/511 nm) in 50 mM citric acid buffer (CAB) at a pH of 5.0, equivalent to that of the skin surface.

Upon arrival in our laboratory, the surface of the scalp skin was gently wiped with PBS, followed by 70% ethanol to remove any contaminants of the subcutaneous fat from the skin surface. The hair was cut with surgical scissors to a length of 2 mm above the surface. Subsequently, a skin square of 8 × 8 mm with the hair follicles parallel to two sides of the square was cut with a stanza. The skin square included the epidermis (viable and non-viable), dermis, and subcutaneous fat.

Preparation of the on-line diffusion cell The preparation of the on-line diffusion cell has been described previously (Grams *et al*, 2004a). Basically, the on-line diffusion cell is part of a combined cutting device/on-line diffusion cell. The skin block is mounted into the cutting device, with the stratum corneum being supported by a silicone square to prevent artifact formation during sectioning. After mechanical fixation, the skin is cut from the dermis side to the stratum corneum side, resulting in a flat cross-sectional cutting plane with the acceptor compartment at the dermis side. Subsequently, the donor compartment is screwed onto the mechanically fixed skin with the acceptor compartment. In order to seal donor and acceptor compartments and to prevent any leakage along the cutting plane, the on-line diffusion cell is completed by fixing a pioloform-coated (0.5% (wt/vol) pioloform in chloroform) cover glass with dental clay (Impregum F) to the diffusion compartments (Fig 7). The dental clay seal allows injection of the acceptor and donor phase at the start of the experiment.

Image acquisition using CLSM The confocal microscope (Leica DM IRBE Leica, Rijswijk, the Netherlands) was equipped with an argon laser of an excitation wavelength of 488 nm and a Leica HC PL APO 20 × objective. Fixed microscopic settings of the CLSM were used and determined in pilot experiments. At these fixed settings, autofluorescence did not interfere with our measurements. But autofluorescence was visible at the most sensitive settings, which is an essential tool for the location of the hair follicle prior to the application of the donor phase. A hair follicle, which can be analyzed, has to emerge from the skin a few micrometers below

the cutting plane, but, at a sufficient distance to prevent visualization of damaged cells due to the mechanical cutting procedure.

Images were collected every 60 min starting 10 min after application of the donor phase for a period of 15 h. Time series at different depths have been obtained from different on-line cell preparations and different donors. A new cross-section with another hair follicle is required for each time series.

Conclusion

This technique allows the visualization of the diffusion process of a fluorescent dye in deeper layers of the skin up to a depth of the subcutaneous fat. This is crucial for monitoring the penetration pathway of this dye in follicular delivery since the terminal hair follicle extends into the subcutaneous fat tissue. We conclude that for BFL, the initial diffusion occurs via the gap and the cuticle of the hair follicle. From the cuticle, the label most probably diffuses into the surrounding tissue rather than from the surrounding tissue into the cuticle. Diffusion also occurs via the stratum corneum and the epidermis. The label source of the outer root sheath is not clear since it can originate from the gap via the thinner stratum corneum of the infundibulum or from the direct connection of the epidermis with the outer root sheath. Furthermore, we conclude that the label in the subcutaneous fat does not originate from the appendages but more likely from the label in the dermis. Additionally, the subcutaneous fat has the potential to function as a depot for lipophilic compounds with the possibility of constant release over time. Using saturated BFL buffer solution, no detectable amount of our label reached the hair bulb in the *in vitro* conditions. For the extrapolation of these results to penetrants with different physicochemical properties and different formulations, additional experiments are necessary.

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