A New Method to Determine the Distribution of a Fluorophore in Scalp Skin with Focus on Hair Follicles

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INTRODUCTION

The contribution of the follicular pathway for passive transdermal drug penetration is still unclear. In literature it has been discussed that the characteristic of the formulation and/or of the drug itself is crucial for the delivery and absorption process (1–8). In developing new dermal delivery systems especially focused on local targeting in the skin, quantification of substances in the various compartments of the skin is important. Recently skin imaging techniques (9) and quantification procedures used in skin research (10) has been summarized.

Quantitative access to deposition of substances in the follicular region is a challenging task, as the hair follicle extends down into the subcutaneous fat. The distribution profiles of compounds were mostly determined by tape stripping in combination with biopsies (11), immunohistochemical analysis (5,12) or isolation and dissolution of skin parts (12). However, these techniques limit the analysis of label distribution to the skin surface and bear the danger of delocalisation of the label due to fixation procedures. Non-invasive methods of quantification such as depth-resolved near-IR spectroscopy have unfortunately still an insufficient resolution (31 μm) for the detailed visualization of the hair follicle (13). Turner and Guy (14) have published relative quantification data in the follicular region of cryo-fixed hairless mouse skin using Confocal Laser Scanning Microscopy (CLSM). However, details of the fluorophore distribution in the various parts of the hair follicle were not reported which is essential for the understanding of the transport route of a substance into the skin and the hair follicle.

The advantage of CLSM is its high resolution and the possibility to circumvent tissue fixation, thereby avoiding label delocalisation during the preparation method. Furthermore the label distribution can be visualized in deeper layers of the skin using optical cross-sections. The objective of this paper is to introduce a new method of relative quantification with CLSM. This method enables the determination of the degree of accumulation in the stratum corneum, epidermis, dermis, outer root sheath, inner root sheath, cuticular area and hair shaft as a first quantitative step in non-fixed fresh human scalp skin.

MATERIALS AND METHODS

Materials

Bodipy® 564/570 C_5 (B564) was purchased from Molecular Probes, The Netherlands. Fresh human scalp skin from patients aged 45 to 66 years was obtained within 3 h after cosmetic surgery. The pieces were temporarily stored on a filter paper soaked with phosphate buffered saline (PBS) at pH 7.4 (139 mM NaCl, 2.5 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 25 mg/L Streptomycin and 25000 U/l Penicillin). Flow-through diffusion cells prepared from Kel-F (Chlorotrifluoroethylene) were used made by the Fine Mechanical Department at the Gorlaeus Laboratories in Leiden.

Methods

In vitro delivery experiments

On arrival in our laboratory the skin was directly dermatomed to a thickness of 1100 μm +/– 150 μm, equivalent to the total thickness of epidermis and dermis. The skin surface was cleaned and the skin mounted in the diffusion cell with a supporting membrane (dialysis membrane, MW cut off 50000, SpectraPor). The donor phase consisted of a saturated B564 solution (5.5 μg/ml) in 50 mM citric acid buffer pH 5.0 (CAB) containing 30% (v/v) ethanol. The spectrum of our selected model penetrant is insensitive to pH and solvent polarity according to the manufacturer. The acceptor phase consisted of PBS at pH 7.4. It was pumped at a flow rate of 2.0 +/– 0.2 ml/h. After 18 h of diffusion, the label was removed from the donor compartment and the skin was washed with an excess of purified water. To determine autofluorescence a skin piece without label was analyzed from each donor. In a pilot experiment CAB containing 30% (v/v) ethanol without dye served as control for permeation and visualization.

Visualization by Confocal Laser Scanning Microscopy (CLSM)

The confocal microscope consisted of a Bio-Rad MRC 600 unit, equipped with a HeNe laser (543 nm). The microscopic unit was an inverted Zeiss IM-35. Fixed microscopic settings of the CLSM were used, whereas the laser intensity varied between 3 and 30% of its total intensity. At the fixed settings autofluorescence did not interfere with our measurements.

After completion of the diffusion experiments the skin was directly transferred to the confocal microscope. The skin was visualized in the x/y-direction parallel to the skin surface and at the bottom edge (dermis side of the skin). To obtain a cross-sectional view, the unfixed skin was manually cut perpendicular to the surface of the skin using a device modified from Meuwissen *et al.* (15). This cross sectional view allows the visualization of the compound with increasing depth and parallel to the hair shaft circumventing the scattering and absorption drawback. Follicular images were obtained either

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ABBREVIATIONS: B564, Bodipy® 564/570 C_5; CLSM, Confocal Laser Scanning Microscopy; Kel-F, Chlorotrifluoroethylene; PBS, Phosphate buffered saline; CAB, Citric acid buffer; I_{av}, Intensity per pixel; n_{pix}, Number of pixels; IPI, Integrated pixel intensity; ID, Intensity density; F_{rel}, Relative fluorescence; V_{rel}, Relative volume; f_{acc}, Relative accumulation factor.

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from the bottom or the cross sectional perspective at a depth of 800–1100 μm. Investigated skin areas are the stratum corneum, epidermis, dermis, outer and inner root sheath, cuticle and the hair shaft.

Relative quantification: Calculation of the relative accumulation factor (f_{acc}). For the final calculations at least 3 different donors have been analyzed. The general calculation procedure follows the scheme depicted in Fig. 1. Capital letters (A-F) in the figure correspond to the equivalent calculation step described below in this section.

A. Calculation of the Intensity Density (ID_i) of every area in each picture. The CoMOS software (Bio-Rad) of the CLSM is based on gray scaling and provides the average intensity per pixel (I_{av}), the area (A) in μm^2, the number of pixels (n_{pix}) and the total pixel value of a selected area. The total pixel value provided by the CoMOS software is the integrated pixel intensity (IPI) of the selected area, which is calculated according to equation 1:

$$IPI = I_{av} * n_{pix} \quad (1)$$

The intensity density (ID) of the selected area normalizes the Intensity to μm^2 and can directly be calculated from the provided information (equation 2):

$$ID_i = IPI / A \quad (2)$$

As the intensity per pixel is in fact the integral intensity over a certain depth in the z-direction perpendicular to the optical cross section, which is the resolution in z-direction (1-2 μm), the intensity density is not per unit area but per unit volume. In our calculation we assume that this "band width" of the confocal image in z-direction is the same for the different parts in the tissue when pinhole and objective are kept constant.

B. Combination of the surface pictures with the follicle pictures of one donor. From the two types of images (follicle, surface) the relative intensity densities ($ID_{rel\ i}$) have to be calculated. Since the dermis is present in both pictures, the ID_i of the dermis has been used as scaling instrument. In doing so, we assume that the distribution within the dermis is independent of depths and that the ID's are independent from the image direction accepting minimal underestimation of accumulation in the top layers.

C. Calculation of the relative intensity density ($ID_{rel\ i}$) for part i of the skin in percent. After averaging the ID_i's for each area, the sum of all ID_i's can be calculated ($ID_{i\ tot}$). The relative intensity density ($ID_{rel\ i}$) per area of interest is then calculated as follows (equation 3):

$$ID_{reli} = ID_i / ID_{toti} * 100\% \quad (3)$$

In fact these values provide the relative intensity distribution in the various parts of the skin assuming that all areas occupy the same volume.

D. Calculation of the relative fluorescence ($F_{rel\ i}$) for part i of the skin. For calculating the relative fluorescence for part i of the skin, the relative volumes occupied by each area of interest have to be estimated. For this purpose we assumed a

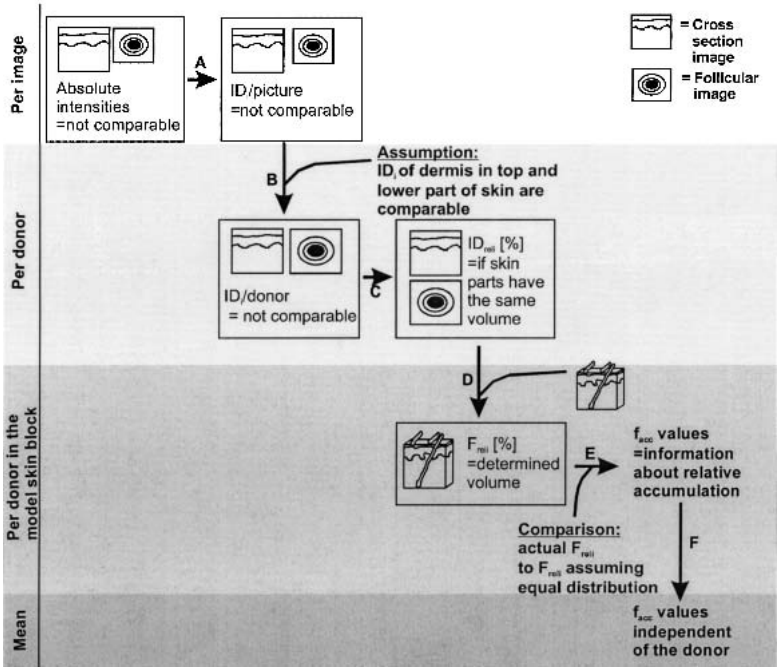

Fig. 1. Scheme of the relative quantification of the label distribution in human scalp skin. Average fluorescence intensities are obtained from CLSM images (cross section and follicular image). (A) The ID_i is calculated for every skin part and (B) scaled for every donor (C) followed by the determination of the relative ID in percent. (D) For every donor the F_{rel} is determined by incorporating specific volumes of an assumed skin block (1.1*1.1*1.1 mm^3). (E) The f_{acc} value is obtained for every donor by comparing the actual F_{rel} with the F_{rel} at equal distribution ($= V_{rel}$). (F) The f_{acc} values are averaged over different donors. ID_i = intensity density in part i of the skin, ID_{rel} = relative ID, F_{rel} = relative fluorescence, V_{rel} = relative volume.

skin block of a standard size (1.1 mm * 1.1 mm * 1.1 mm) with n follicles. To decompose this volume in the various regions the size of each area was determined from confocal pictures (Table I), whereas the number of follicles was counted (160/cm^2). The volumes of the various follicular areas have been approached by assuming a cylindrical shape perpendicular to the skin surface. Microscopically the cuticle of the follicle and of the hair could not be distinguished. Therefore both cuticles will be referred to as cuticle.

To be able to determine the integral relative fluorescence $F_{rel\ i}$ of each skin part, the total volume and the volume of each part of the skin were calculated (Table I). The calculated relative intensity density per μm^3 of each part of the skin has to be multiplied by its calculated volume to obtain the total relative intensity in that area. Since we are interested in the contribution of each part to the total relative intensity in the skin, we divide the total relative intensity of each part ($ID_{rel\ i}*V_i$) by the total relative intensity of the tissue ($(\Sigma ID_{rel\ i}*V_i)$) (equation 4). The sum of the relative fluorescence of all the skin parts is 1 (equation 5) and can be expressed as percentage (equation 6). V_i is the volume of part i of the skin whereas $F_{rel\ i}$ is the relative fluorescence of part i of the skin:

$$F_{rel\,i} = \frac{ID_{rel\,i} * V_i}{\Sigma(ID_{rel\,i} * V_i)} \quad (4)$$

$$\Sigma F_{rel\,i} = 1 \quad (5)$$

$$\Sigma F_{rel\,i} * 100 = 100\% \quad (6)$$

The percentage $F_{rel\ i}$ value gives only information about the integral relative amount of intensity in the volume of a certain area compared to the total relative amount of the intensity in the assumed skin block. That means, it includes the relation between the volume of a certain region to the total volume.

E. Calculation of the accumulation factor ($f_{acc\ i}$) for part i of the skin. To obtain information about the degree of accumulation in each skin part, we have to go one step further. The actual relative distribution values ($F_{rel\ i}$) have to be compared to the relative distribution assuming no accumulation in any part of the skin. In the latter situation, the distribution value ($F_{rel\ i}$) should be the same as the percentage of the respective volume compared to the total volume ($V_{rel\ i}$). Dividing the relative fluorescence ($F_{rel\ i}$) by the relative volume ($V_{rel\ i}$) in percent leads to an accumulation factor (f_{acc}) for each examined part of the skin:

$$f_{acc\,i} = \frac{F_{rel\,i}\,(in\ \%)}{V_{rel\,i}\,(in\ \%)} \quad (7)$$

If the compound is equally distributed in all skin parts, the accumulation factor ($f_{acc\ i}$) for all parts would be 1. If the relative distribution value ($F_{rel\ i}$) is higher than the equal distribution value (expressed in $V_{rel\ i}$ [%]), i.e. a value above 1, label accumulation in the selected part is present.

F. Averaging the f_{acc}'s from all donors. At this point, the accumulation factors of the different donors can be averaged and the standard deviation can be calculated. This enables as well the statistical analysis. It is important to realize that from the accumulation factor no conclusions can be drawn about the absolute amounts of the penetrant in the skin.

RESULTS

The lipophilic B564 exhibited very bright staining of the epidermis, whereas the stratum corneum and the dermis showed a lower content of dye (Fig. 2A). The hair follicle, which reaches down into the dermis reveals a characteristic staining. Whereas the outer root sheath and the cuticular area exhibit strong staining, only a low amount of label was detected in the inner root sheath and hardly any label was seen in the hair shaft itself (Fig. 2B).

When calculating the relative fluorescence intensity ($F_{rel\ i}$) according to equation 4, 77% of the label is located in the dermis (Table I) although in the image only low fluorescence intensity was observed in this region. 10% of the total relative fluorescence was estimated in the epidermis and less than 1% in the stratum corneum of the model skin block. The hair follicle including the hair shaft contains 12% of the total relative fluorescence. Therefore the relative fluorescence intensity values give access to the relative content of a target area. However, they do not represent a sensitive means to investigate the changes in accumulation of the label in certain skin areas, since due to the limited follicular volume, the total amount of fluorescence in the follicular area is limited.

Focusing on the relative accumulation, f_{acc} values of 2.3 for the epidermis, 2.2 for the outer root sheath, 1.5 for the inner root sheath and 1.8 for the cuticular area were determined (Fig. 3). These values which are above 1.0 indicate accumulation of the dye in these areas. The stratum corneum and the hair shaft exhibited values, which were not different from 1.0, while a relative accumulation value below 1.0 has been detected for the dermis indicating no accumulation.

DISCUSSION

In stratum corneum B564 (log $P_{oct/wat}$ value of 4 at pH 5.0) penetrates mainly paracellular in the lipid regions. Since

Table I. Measured Thickness or Radius and Calculated Volumes of the Different Parts of the Skin from 20 CLSM Images for the Determination of the Relative Fluorescence Intensity Values ($F_{rel\ i}$). $V_{rel\ i}$ is the Relative Volume of part i of the Skin. Determined $F_{rel\ i}$ Values of B564 in Various Skin Parts after 18 h Penetration of 0.025% Dye from CAB Containing 30% (v/v) Ethanol Are Shown as Well.

	Stratum corneum	Epidermis	Dermis	Outer root sheath	Inner root sheath	Cuticle	Shaft
Thickness [μm]	8.4	50.9	1040.7	50.4	28.0	4.0	
Radius [μm]							31.9
Volume [10^6 μm^3]	9.5	57.6	1178.4	59.0	18.3	1.8	6.7
$V_{rel\ i}$ [%]	0.7	4.3	88.5	4.4	1.4	0.1	0.5
$F_{rel\ i}$ [%]	0.8	10.1	76.7	9.6	2.1	0.2	0.5

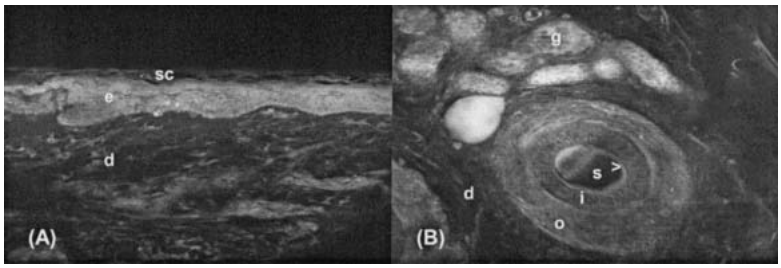

Fig. 2. Distribution of B564 in fresh human scalp skin. In the cross sectional view (A) the stratum corneum (sc), the epidermis (e) and the dermis (d) are visualized. In the parallel view from the dermis side of the skin (B) a hair follicle with its characteristic areas, outer root sheath (o), inner root sheath (i), cuticular area (>) and the hair shaft (s) as well as a sweat gland (g) and the dermis (d) is depicted.

no label was detected inside the corneocytes only a small relative accumulation value in this skin barrier was determined. The adjacent viable epidermis is much brighter stained than the stratum corneum. In the viable epidermis, the cornified envelope has not been developed yet, which allows the label to permeate into the cells (Fig. 2A). The deeper layer of the skin (dermis) shows only slight labeling (Fig. 2A and 2B) and no accumulation of the dye (Fig. 3). However parts of the hair follicle, which are situated in this part of the dermis exhibit increased accumulation values, namely the outer root sheath and the cuticular area. These results lead to the discussion whether the dermis has a low affinity for the fluorophore or whether the hair follicle represents a favored penetration route for the lipophilic B564. Since it is known from literature, that various small substances (14,16,17), charged lower molecular weight dextrans (5) and adapalene-loaded microspheres with a diameter of 5 μm (6) showed increased penetration via the follicular route, it might also be the case for B564.

To obtain a tool for the elucidation of the follicular distribution, the qualitative visualization via Confocal Laser Scanning Microscopy was extended by quantifying the relative dye distribution in the hair follicle. In CLSM an absolute quantification of label concentration within a certain skin area is impossible (18).

During relative quantification the bleaching of the fluorophores has to be minimised in CLSM. Bleaching of our dye is minimal, since even after 150 scans the reduction in fluorescence intensity was difficult to detect. In our studies 5 scans (Kalman) were used. Additionally short one-time exposures were chosen and z-scans were avoided. The thickness of the optical slice is also a crucial parameter in relative quantification. It depends on the objective used (magnification and numerical aperture) and the confocal aperture (pinhole). Therefore the same objective and pinhole are used during the visualization studies.

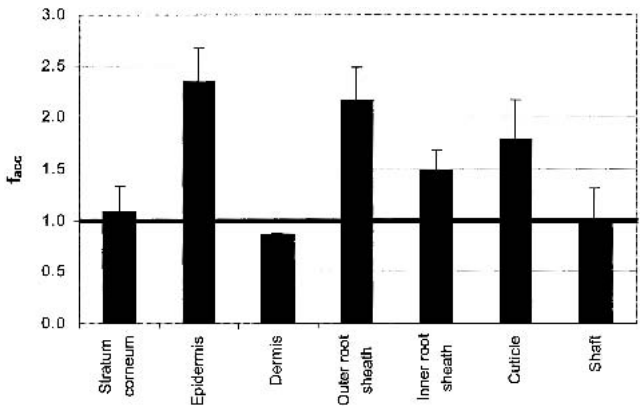

Fig. 3. Relative accumulation values (f_{acc}) of B564 from citric acid buffer pH 5.0 containing 30% (v/v) ethanol (EtOH) are depicted after application of 0.025% (w/v) label on 4 different donors.

In the course of the calculation procedure the CoMOS software determines the intensity of the marked pixels and the size of the marked area. Since the intensity density is expressed per μm^2, this value is independent of the pixel density and thus independent of the zoom used for the image. The assumed total volume of the skin block and the relative volumes of each skin part do not have any influence on the intensity density values. When evaluating the relative fluorescence (F_{rel}) data the more voluminous parts of the skin i.e. dermis and epidermis exhibit the highest relative fluorescence values. When focusing on targeting the hair follicle one aims at an increased accumulation of the investigated drug in the target area. Therefore the relative accumulation value (f_{acc}) gives more information about a certain skin and follicle region. However, it has to be noted that the F_{rel} and the f_{acc} are dependent on the relative volume of each skin region but independent of the assumed total volume of the skin block.

However, the relative quantification has certain limits. First, in case of small areas, the selection of this region on the computer screen might introduce some inaccuracy. This can result in an underestimation of the amount of label deposited in that specific area, e.g., cuticular region, stratum corneum. Secondly, relative accumulation values give no information regarding the absolute concentration in certain areas of interest. This means, that although the accumulation value for a certain region increases, the total amount of label in the skin might be decreased at the same time. Therefore, the relative accumulation values determined in this publication do not correlate with the total amount of label in the skin.

Turner and Guy (14) were the first to semi-quantitatively determine the amount of calcein in the follicular area after iontophoresis using CLSM. Values obtained from one type of image were used for the determination of the different transport fractions. Images taken from the top were analyzed semi-quantitatively whereas the cross section images (manual or optical) were used for qualitative evaluation of the diffusion pathway. The resolution was limited to a non-follicular area, a follicular area and the hair shaft itself. With the analysis

presented in this paper, follicular layers deep in the skin can be analyzed thereby giving detailed information about the distribution and accumulation within the hair follicle.

The main advantage of this relative quantification method is that it enables numerical access to accumulation changes in various skin parts in deeper skin layers. Unfixed skin can be used minimising delocalisation of the dye and artifact formation in skin. Indirect comparison of two images from one donor is also possible. However this method requires fluorophores and one post-experimental cut of unfixed tissue, which limits this method to *in vitro* and *ex vivo* studies. At this moment studies are in progress in our laboratory to examine the accumulation of various compounds and vehicles in the hair follicle. Different compounds and/or delivery vehicles will be compared to optimise a formulation. Hence, this analysis procedure can be of great advantage not only in the research of follicular targeting but also in local skin targeting.

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REFERENCES

1. R. Agarwal, O. P. Katare, and S. P. Vyas. The pilosebaceous unit: A pivotal route for topical drug delivery. *Methods Find. Exp. Clin. Pharmacol.* **22**:129–133 (2000).
2. B. Illel. Formulation for transfollicular drug administration: Some recent advances. *Crit. Rev. Ther. Drug Carrier Syst.* **14**:207–219 (1997).
3. A. C. Lauer, L. M. Lieb, C. Ramachandran, G. L. Flynn, and N. D. Weiner. Transfollicular drug delivery. *Pharm. Res.* **10**:179–186 (1995).
4. A. C. Lauer, C. Ramachandran, L. M. Lieb, S. Niemiec, and N. D. Weiner. Targeted delivery to the pilosebaceous unit via liposomes. *Adv. Drug Deliv. Rev.* **18**:311–324 (1996).
5. L. M. Lieb, A. P. Liimatta, R. N. Bryan, B. D. Brown, and G. G. Krueger. Description of the intrafollicular delivery of large molecular weight molecules to follicles of human scalp skin *in vitro*. *J. Pharm. Sci.* **86**:1022–1029 (1997).
6. A. Rolland, N. Wagner, A. Chatelus, B. Shroot, and H. Schaefer. Site-specific drug delivery to pilosebaceous structures using polymeric microspheres. *Pharm. Res.* **10**:1738–1744 (1993).
7. C. C. Sumian, F. B. Pitre, B. E. Gauthier, M. Bouclier, and S. R. Mordon. A new method to improve penetration depth of dyes into the follicular duct: Potential application for laser hair removal. *J. Am. Acad. Dermatol.* **41**:172–175 (1999).
8. N. Weiner. Targeted follicular delivery of macromolecules via liposomes. *Int. J. Pharm.* **162**:29–38 (1998).
9. P. Corcuff and G. E. Pierard. Skin imaging: State of the art at the dawn of the year 2000. *Skin Bioeng.* **26**:1–11 (1998).
10. E. Touitou, V. M. Meidan, and E. Horwitz. Methods for quantitative determination of drug localized in the skin. *J. Control. Release* **56**:7–21 (1998).
11. B. Kammerau, A. Zesch, and H. Schaefer. Absolute concentrations of dithranol and triacetyl-dithranol in the skin layers after local treatment: *in vivo* investigations with four different types of pharmaceutical vehicles. *J. Invest. Dermatol.* **64**:145–149 (1975).
12. W. G. Reifenrath, G. S. Hawkins, and M. S. Kurtz. Percutaneous penetration and skin retention of topically applied compounds: an *in vitro-in vivo* study. *J. Pharm. Sci.* **80**:526–532 (1991).
13. N. G. Nerella and J. K. Drennen. Depth-resolved near-infrared spectroscopy. *Appl. Spectrosc.* **50**:285–291 (1996).
14. N. G. Turner and R. H. Guy. Visualization and Quantitation of Iontophoretic Pathways Using Confocal Microscopy. *J. Invest. Dermatol. Symp. Proc.* **3**:136–142 (1998).
15. M. E. M. J. Meuwissen, J. Janssen, C. Cullander, H. E. Junginger, and J. A. Bouwstra. A cross-section device to improve visualization of fluorescent probe penetration into the skin by Confocal Laser Scanning Microscopy. *Pharm. Res.* **15**:352–356 (1998).
16. F. Hueber, H. Schaefer, and J. Wepierre. Role of transepidermal and transfollicular routes in percutaneous absorption of steroids: *in vitro* studies on human skin. *Skin Pharmacol.* **7**:237–244 (1994).
17. B. Illel, H. Schaefer, J. Wepierre, and O. Doucet. Follicles play an important role in percutaneous absorption. *J. Pharm. Sci.* **80**:424–427 (1991).
18. M. Laurent, G. Johannin, N. Gilbert, L. Lucas, D. Cassio, P. X. Petit, and A. Fleury. Power and limits of laser scanning confocal microscopy. *Biol. Cell* **80**:229–240 (1994).