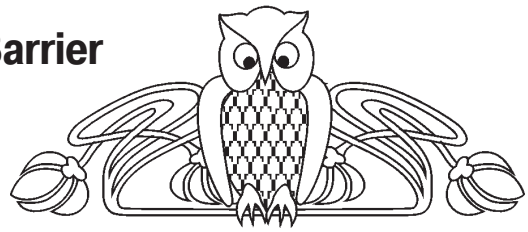
Non-invasive Methods for *in vivo* Determination of the Skin Barrier Function – Advantages of Confocal Raman Microspectroscopy



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The physical barrier of the stratum corneum (SC) is provided by corneocytes and the lateral organization of intercellular lipids, which necessarily includes the orthorhombic organization phase. A review of methods used for *in vivo* non-invasive measurement of skin barrier function has been carried out and it has been shown that all currently used methods are indirect. The most popular method is the measurement of the transepidermal water loss (TEWL), which does not provide information on biophysical parameters responsible for the barrier function of the SC. It has been shown that confocal Raman microspectroscopy is the most suitable non-invasive method to determine the depth profile of the lateral organization of intercellular lipids, i.e. to study the skin barrier function *in vivo*.

Keywords: intercellular lipids, lateral organization, orthorhombic organization, hexagonal organization, lipid lamella, stratum corneum, Raman spectroscopy, TEWL.

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1. Introduction

One of the main functions of the skin is the barrier function, i.e. protection against external physical and chemical influences, as well as against the penetration of pathogens into the body. The stratum corneum (SC), the uppermost continuously renewing layer of the epidermis, which represents a multifunctional system and serves as an effective physical barrier between the body and the environment, effectively protects against the penetration of pathogens (viruses, bacteria, allergens, microorganisms) into the "living" epidermis, and participates in the regulation of the water balance of the whole body.

The main components of the SC are keratin, lipids, water, natural moisturising factor molecules and antioxidants, whose concentrations are non-homogeneously distributed in the SC depth [1] and represent a morpho-functional unity, participating directly and indirectly in the formation and continuous maintenance of the skin barrier function. The physical barrier of the SC is provided by corneocytes and the lateral organization of intercellular lipids (ICL) [2], which necessarily include the orthorhombic organization phase, a strictly ordered and most densely packed structure [3]. Lipids are strictly structured inside lamellas in form of membrane and are oriented mainly perpendicular to the skin surface, which directly determines their lateral organization [3]. In the SC of healthy human individuals, where the lipid composition is balanced, the orthorhombic phase of the lateral organization dominates, in which lipids are most tightly packed, highly ordered and stay predominantly in a *trans*-conformation. The ICL matrix is also called the lipid barrier or the SC/skin barrier. The thickness of the SC on the human forearm is ≈19 μm [4] and varies depending on the skin area, being maximal on the palms and soles of the foot [5].

Methods for determining the lateral organization of ICL, which determines the skin barrier function [6], include wide angle and small-angle X-ray scattering, electron diffraction, electron paramagnetic resonance spectroscopy, infrared spectroscopy and confocal Raman microspectroscopy (CRM). All these methods are suitable for measuring skin biopsies *ex vivo*, and only CRM has an advantage of measuring the lateral organization of ICL *in vivo* [7, 8].

2. Barrier function of the SC is non-homogeneous in depth

The question of how the orthorhombic and hexagonal phases of the lateral organization of ICL coexist and how they are distributed in the

SC has remained unanswered for a long time. The first attempts to answer this question were made by Pilgram *et al*. [6] in an *in vitro* study of unfrozen human forearm SC tapes, obtained by applying tape stripping using the electron diffusion method to distinguish both phases of the lateral organization of ICL. The results showed that the hexagonal phase of the ICL organization prevails near the surface of the SC. The orthorhombic phase is characterized by a clearly defined maximum inside the SC at a depth corresponding to the 10-th tape application [6]. Yagi *et al*. [9], using the electron paramagnetic resonance spectroscopy method, confirmed in an *in vitro* experiment that the structural organization of the ICL matrix of the human SC is most dense at the intermediate SC depths. Later, using the combination of small-angle and wide-angle X-ray scattering methods, Doucet *et al*. [10] determined for the first time the distribution of the orthorhombic and hexagonal phases of the lateral ICL organization co-existing in the ICL lamellas of the human abdominal SC *ex vivo*. The results gave a complete picture for the entire SC thickness and showed that the orthorhombic phase of the lateral ICL organization prevails at intermediate SC depths, and a clear maximum is observed at a depth of ≈8–10 μm (≈50% of the SC thickness). The authors have shown in an *ex vivo* experiment that the hexagonal phase of the lateral ICL organization prevails at the surface and at the boundary of the SC and stratum granulosum, which coincides with the data obtained earlier in an *in vitro* experiment [6]. Thus, the results of *in vitro* and *ex vivo* measurements obtained by different groups showed a non-homogeneous distribution of the lateral organization of ICL in the SC with predominance of the orthorhombic phase at a depth corresponding to ≈35–50% of the SC thickness [6, 10]. Noninvasive *in vivo* measurements of the lateral organization of ICL in the SC became possible using CRM after the development of an algorithm for processing of Raman spectra [8], which is presented below.

3. An overview of non-invasive methods for *in vivo* assessment of the skin barrier function

In clinical practice, a dermatologist often only conducts a visual assessment of the state of the skin barrier function by indirect signs, based on practical recommendations to determine the severity of skin diseases. These can include those associated with impaired skin barrier function, such as psoriasis (PASI: Psoriasis Area and Severity Index) and atopic dermatitis (SCORAD: Scoring Atopic Dermatitis Index) to determine a number of clinical parameters such as erythema, dryness and swelling of the skin. This approach is completely non-invasive, however is subjective, requires the experience of a physician, and can only be used to make a primary diagnosis and the assessment of the skin barrier function. Obviously, an objective study of the skin barrier function requires the use of accurate measurement methods [11].

A generally accepted non-invasive method, often used *in vivo* in dermatology and cosmetology to assess the skin barrier function, is the measurement of the transepidermal water loss (TEWL) [12]. This method measures a vapor pressure gradient near the skin surface by sensors located inside an open or closed chamber [13]. This method is indirect, but the correlation between the content of the orthorhombic phase of the ICL organization in the SC, which directly characterizes the skin barrier function, with the TEWL [14] confirmed the validity of this method. Thus, an increased TEWL characterizes the impaired skin barrier function. Presently, there is no alternative to the TEWL measurements in determining the skin barrier function *in vivo*, because the methods of direct analysis of the lateral ICL organization require the extraction of a biopsy and, thus, are invasive. Study of the SC by tape stripping is not a useful alternative, because tape stripping itself is a minimally invasive *in vitro* method that does not provide information in the entire SC. Despite the popularity of the TEWL measurement method, it has a number of serious practical shortcomings. These include the complexity of the measurement procedure and preparation for the measurement (the requirement of long-term acclimatization of a volunteer, the need for stable temperature and humidity inside the room, etc.), the large deviation of measurement results, the impossibility to measure TEWL after topical skin treatment with cosmetic or medical products (due to the presence of water in the formulation and due to the occlusion of the SC), as well as the impact of perspiration. It should also be kept in mind that water vapors contained in the exhaled air of people in the vicinity of the measurement device may influence the TEWL results.

The skin barrier function can be determined *in vivo* by the measurement of the superficial pH: an increase of pH in the skin surface is associated with a weakening of the SC barrier [15]. Usually, the pH value on the skin surface is measured with a pH-sensitive glass electrode. However, pH microscopy using pH-sensitive fluorescent dyes and the subsequent measurement of fluorescence intensity and

fluorescence lifetime decay are also possible [16]. The pH measurements on the skin surface are not time-consuming, well reproducible and often used in dermatology [15], however, the pH measurement is also an indirect method for measuring the skin barrier function.

An indirect indicator of the skin barrier function is the thickness of the SC, which can be determined non-invasively and *in vivo* using imaging techniques such as, for instance, reflection confocal laser scanning microscopy [17], two-photon tomography [18] and optical coherence tomography [19]. These methods provide the large scanning area and the ability to visually identify inhomogeneity in the SC thickness. However, these devices are unable to perform structural analysis of the SC components, responsible for maintaining a skin barrier function.

Optical non-invasive methods based on Fourier-transform infrared spectroscopy [20] may serve for *in vivo* studies of the ICL organization. However, this method has one major limitation, strong absorption of radiation by water and as a result, the possibility to measure only the superficial SC depths [7], or the need to combine it with a minimally invasive procedure, removal of a part of the SC with tape stripping.

Recently, it has been proposed to use CRM to determine the skin barrier function to measure the counteraction of sodium lauryl sulfate penetration through the SC [21]. Despite the fact that the proposed method is also indirect and minimally invasive, the authors have shown a correlation with TEWL measurements. Kikuchi *et al*. [7] found a correlation between the lateral ICL organization of the human SC, measured *in vivo* and *ex vivo* by Raman spectroscopy, with the ratio of the content of orthorhombic and hexagonal phases of the lateral ICL organization, measured *ex vivo* on the skin biopsy by using wide angle X-ray scattering. Thus, it is once again confirmed that the orthorhombic phase of the lateral ICL organization in the SC directly determines the barrier function of the skin and can be studied noninvasively and *in vivo* by CRM.

4. Confocal Raman microspectroscopy for non-invasive *in vivo* determination of the skin barrier function

In the range of 2820–3030 cm^{-1} the ratio of lipid-related Raman band intensities at 2880 cm^{-1} (anti-symmetric stretching mode of CH_2 groups) and at 2850 cm^{-1} (symmetric stretching mode of CH_2 groups) I_{2880}/I_{2850} describes the lateral organization of lipids. In skin research, the main difficulty lies in the strong superposition of lipid- and keratin-related Raman bands in this spectral range. To solve this problem, an algorithm has been developed for separating the lipid and keratin contributions of the SC into the Raman band intensity. The algorithm uses known Raman spectra of model lipids and keratin and directly measured Raman spectra for the calculation of the parameters (Figure 1, *a*). The following formula for calculating the lateral organization of ICL in the SC was proposed [8]:

$$I_{2880}/I_{2850} = GE/KI = ((K_{2880} - P_K^{2880})/(1 - K_{2880}\,P_L^{2880})) \cdot ((1 - K_{2850}\,P_L^{2850})/(K_{2850} - P_K^{2850})),$$

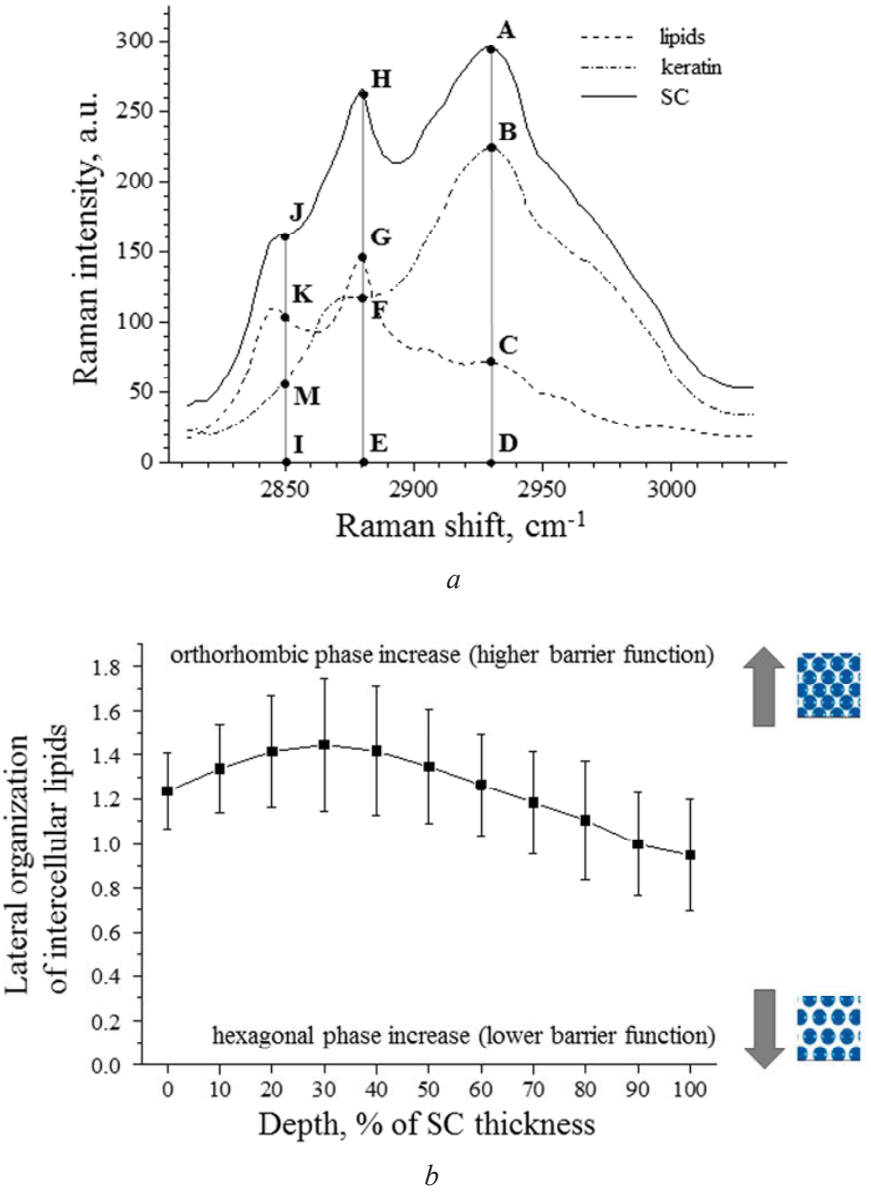
where $K_{2850} = JI/AD$; $K_{2880} = HE/AD$ – directly measured from the Raman spectra;

$$P_K^{2850} = MI/BD = 0.150 \pm 0.025;\ P_L^{2850} = KI/CD = 0.480 \pm 0.031,$$

$$P_K^{2880} = FE/BD = 0.440 \pm 0.024;\ P_L^{2880} = GE/CD = 0.320 \pm 0.026.$$

Further, using the developed algorithm, the distribution of lateral ICL organization in the human SC *in vivo* was determined for the first time (Figure 1, *b*). A higher I_{2880}/I_{2850} value characterizes a greater number of lipid chains in trans-conformation, and thus a denser orthorhombic packing of lipids [8]. As can be seen from Figure 1, *b*, on the surface of the SC, the ratio of I_{2880}/I_{2850} is ≈1.2. Then, it increases with increasing SC depth and reaches a maximal value of ≈1.45 (the highest content of the orthorhombic phase of the lateral ICL organization, the most dense and least permeable ICL package) at a depth of 20–40% of the SC thickness. Further, the I_{2880}/I_{2850} ratio decreases and reaches a minimum value of ≈0.95 (the highest content of the hexagonal phase of ICL organization, less dense and more permeable ICL packing) in the bottom of the SC.

In order to validate the CRM method, the lateral ICL organization has been measured in vivo in the skin of volunteers of two age groups (Figure 2, *a*) [22] and in comparison to the porcine ear skin ex vivo [23], which is known for lower skin barrier function [24] (Figure 2, *b*). The results show that differences are obvious not in the entire SC, but at certain SC depths: “older” skin is characterized with higher skin barrier function (more orthorhombic ICL phase) in comparison to “younger” skin at



a

b

Fig. 1. Raman spectrum of human SC (depth 4 μm) *in vivo* (solid line), lipids (dashed line) and keratin (dash-dotted line) (*a*) and the distribution of lateral ICL organization in the SC, measured as I_{2880}/I_{2850} (*GE*/*KI* ratio in (*a*)) obtained *in vivo* in the skin of 6 volunteers (mean±SD) (*b*). The SC thickness is normalized to 100%. Figures adopted from [8].
/ – more or less ordered lateral structure of ICL in the SC

20–30% SC depth (Figure 2, *a*); porcine ear skin has lower skin barrier function (more hexagonal ICL phase) in comparison to human forearm skin at 10–50% SC depth (Figure 2, *b*). This method can also be effectively applied to the treated skin in vivo, i.e. to analyze the influence of cosmetic and medical formulations on the barrier function related parameters of the SC [25]. The main limitation of the CRM is its costs and relatively long measurement and analysis time.

5. Conclusions

The lateral organization of lipids in the human SC measured *in vivo* is non-homogeneous and is characterized by a maximal content of the orthorhombic phase of the lateral ICL organization (the most dense and least permeable ICL package) at a depth of 20–40% of the SC thickness. In the superficial SC depths and in the bottom of the SC, the content of the hexagonal phase of the lateral ICL organization (less dense and more permeable ICL

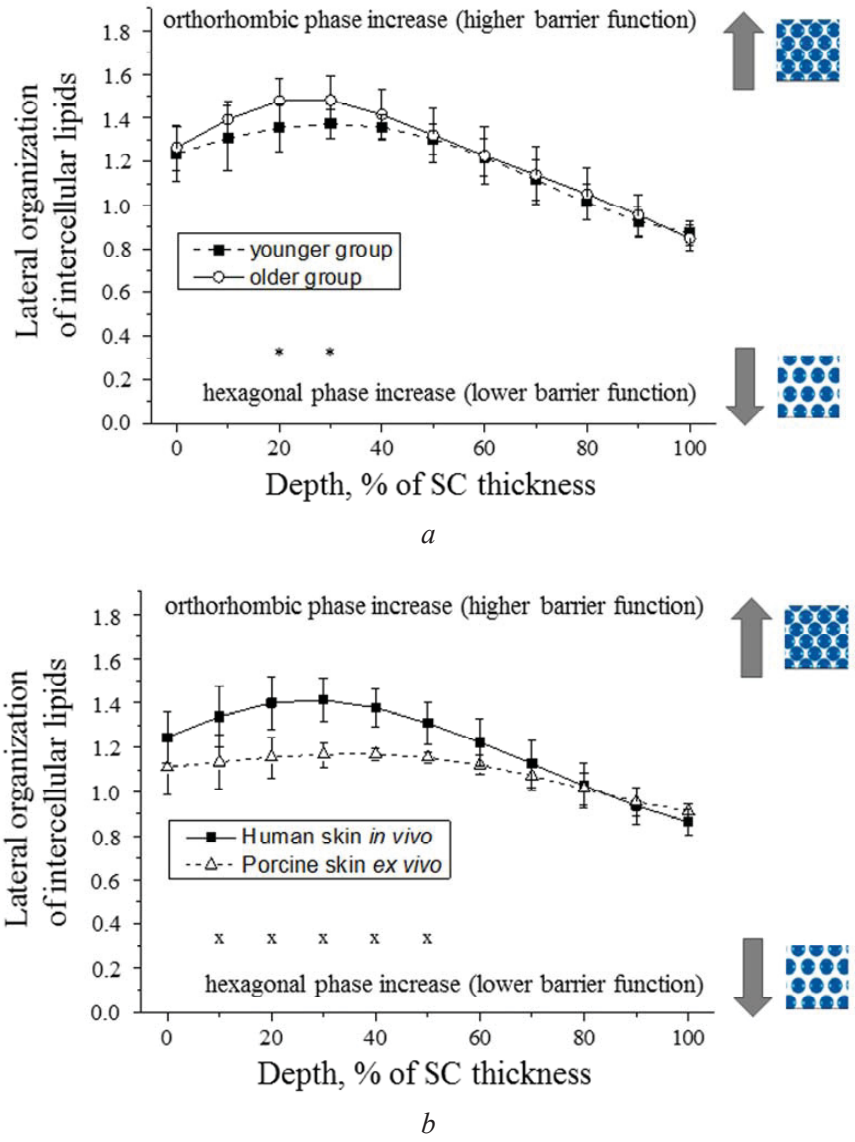


Fig. 2. The distribution of the lateral ICL organization in the SC obtained *in vivo* in the human skin of different age groups ("younger group": mean 29 y.o., 7 volunteers and "older group": mean 50 y.o., 4 volunteers) (*a*) and comparison to porcine ear skin *ex vivo* (*b*). The SC thickness is normalized to 100%. "*" and "x" represents modest ($p<0.1$) and strong ($p<0.01$) significant differences between the groups. Figures adopted from [22, 23].
– more / less ordered lateral structure of ICL in the SC

package) is maximal. Changes of skin barrier function were observed at the certain depths of the SC. Confocal Raman microspectroscopy is an optimal non-invasive method for *in vivo* study of the skin barrier function provided by the SC.

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Неинвазивные методы определения барьерной функции кожи *in vivo* – преимущества конфокальной микроспектроскопии комбинационного рассеяния света

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Физический барьер рогового слоя кожи обеспечивается корнеоцитами и латеральной организацией внеклеточных липидов, которая обязательно включает в себя орторомбическую фазу организации. Обзор методов, используемых для неинвазивного *in vivo* измерения барьерной функции кожи показал, что все используемые в настоящее время методы являются косвенными. Наиболее популярным методом является измерение трансэпидермальной потери воды, который не дает информации о биофизических параметрах, определяющих барьерную функцию рогового слоя кожи. Показано, что метод конфокальной микроспектроскопии комбинационного рассеяния света является наиболее подходящим неинвазивным методом для определения распределения степени латеральной организации внеклеточных липидов по глубине рогового слоя, т.е. для изучения барьерной функции кожи в *in vivo* эксперименте.

Ключевые слова: внеклеточные липиды, степень латеральной организации, орторомбическая организация, гексагональная организация, липидная ламелла, роговой слой, спектроскопия комбинационного рассеяния света, трансэпидермальная потеря воды.

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