Fluorescence Lifetime to image epidermal ionic concentrations

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ABSTRACT

Measurements of ionic concentrations in skin have traditionally been performed with an array of methods which either did not reveal detailed localization information, or only provided qualitative, not quantitative information. FLIM combines a number of advantages into a method ideally suited to visualize concentrations of ions such as H^+ in intact, unperturbed epidermis and stratum corneum (SC). Fluorescence lifetime is dye concentration-independent, the method requires only low light intensities and is therefore not prone to photobleaching or phototoxic artifacts, and because multiphoton lasers of IR wavelength are used, light penetrates deep into intact tissue. The standard method to measure SC pH is the flat pH electrode, which provides reliable information only about surface pH changes, without further vertical or subcellular spatial resolution; i.e., specific microdomains such as the corneocyte interstices are not resolved, and the deeper SC is inaccessible without resorting to inherently disruptive stripping methods. Furthermore, the concept of a gradient of pH through the SC stems from such stripping experiments, but other confirmation for this concept is lacking. Our investigations into the SC pH distribution so far have revealed the crucial role of the Sodium/Hydrogen Antiporter NHE1 in generation of SC acidity, the colocalization of enzymatic lipid processing activity in the SC with acidic domains of the SC, and the timing and localization of emerging acidity in the SC of newborns. Together, these results have led to an improved understanding of the SC pH, its distribution, origin, and regulation. Future uses for this method include measurements of other ions important for epidermal processes, such as Ca^{2+}, and a quantitative approach to topical drug penetration.

Keywords: Epidermis, Fluorescence Lifetime Imaging, pH, Calcium

I. INTRODUCTION

The fundamental importance of ions, and their cellular transporters for cell and organ function, is being increasingly appreciated, as shown by the recent award of the Nobel Prize for Chemistry to Peter Agre and Roderick MacKinnon for "discoveries concerning channels in cell membranes". In epidermis, modulation of Ca^{2+} concentration and/or flux regulates numerous critical functions, including cell adhesion, growth, differentiation, apoptosis, and lamellar body (LB) secretion, and thereby permeability barrier function[3,4,7,20,19,29,31]. Moreover, defective ion transport has been shown to play a role in the pathogenesis of a wide range of extracutaneous and skin diseases[15]. Prominent recent examples in the field of Dermatology are the pathogenesis of Hailey-Hailey and Darier's diseases, wherein intracellular calcium-ATP'ases are defective[14,35,37].

Epidermis displays two unique and characteristic electrolytic gradients: First, a calcium (Ca^{2+}) gradient, which regulates numerous epidermal functions via cell-physiologic processes and effects on differentiation. The Ca^{2+} gradient ascends steeply towards the stratum granulosum (SG), and declines again in the stratum corneum (SC)[25]. Second, the acidic pH of the SC also forms a functional endpoint late in epidermal differentiation, which regulates epidermal functions such as extracellular lipid processing and desquamation[1,8,34]. Alterations of both the epidermal Ca^{2+} gradient and in surface pH have been reported for dermatoses, e.g. atopic dermatitis, and psoriasis.

Using Fluorescence Lifetime Imaging Microscopy (FLIM), both pH and Ca^{2+} concentration gradients and subcellular localization within the epidermis can be investigated concurrently[1,2].

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An acidic pH begins to form just above the SG, and becomes progressively more acidic across the SC. The acidic pH is also required for normal permeability barrier function[19,8], and several other key SC functions. For example, the activity of acidic, lysosomal type, lipid processing enzymes is required to fully establish the permeability barrier[13,12,16,36], and an acidic pH controls the activity of proteases necessary for SC integrity and desquamation[9,38,33,17]. We recently demonstrated that the Sodium-Hydrogen-Exchanger 1 (NHE1) plays a key role in establishing this critical acidic SC milieu[2]. Our studies also revealed the functional importance of progressive SC acidification during the immediate post-partum development period[1].

Adhesive tape-stripping to remove sequential layers of SC from the skin's surface is, despite its limitations[41], an established technique in epidermal research, often likened to the epidermal treadmill exam[10,32]. This technique has been used for a wide array of questions in epidermal biology, and is therefore well characterized. As follow-up assays are used electron microscopy to assess structural changes, and assays to establish expression and distribution of cellular components, e.g. (confocal and/or immuno) fluorescence microscopy, biochemical methods (Western Immunoblots), and molecular methods (PCR, rt-PCR). Nevertheless, the notion of the existence of a pH gradient across the upper epidermis, i.e., the SC, was generated through experiments combining tape-stripping with pH measurements.

To date no other method aside from FLIM appears useful to confirm or disprove on a microscopic scale this and other concepts surrounding ionic gradients in epidermal biology. Here, we demonstrate FLIM to measure and localize pH domains in epidermis and how results obtained with this method compare to conventionally used method(s), possibly opening avenues for other/different biological explanations and a more detailed view and understanding of biology.

II. METHODOLOGY

Animals: Male hairless mice (SKH1 hr/hr, Charles River Laboratories, Wilmington, MA) were fed Purina mouse diet and water ad libitum. Animals were 8-12 weeks old at time of experiments.

Materials: All dyes were purchased from Molecular Probes (Eugene, OR).

Fluorescence Lifetime Imaging Microscopy: pH was determined using the lifetime-sensitive fluorescent pH indicator BCECF (Molecular Probes, 100 mM applied in pure ethanol), as reported previously[9,2,1]. Animals were kept at ambient temperature and humidity for the duration of the dye incubation. A biopsy was taken approximately 15 minutes following the last dye application, mounted for microscopy, and directly visualized.

In brief, two-photon fluorescence lifetime imaging microscopy (FLIM)[24,39,40] to determine pH was performed by using a Millenia-pumped Tsunami titanium:sapphire laser system (Spectra-Physics) as the two-photon excitation source. Excitation of the sample was achieved by coupling the 820 nm output of the laser through the epifluorescence port of a Zeiss Axiovert microscope. The fluorescence was collected using a Hamamatsu (R3996) photomultiplier placed at the bottom port of the microscope. Scanning mirrors and a 40x infinity corrected oil objective (Zeiss F Fluar, 1.3 N.A.) were used to image areas of 107 μm^2. Z-slices (1.7 μm per slice) were obtained by adjusting the objective focus with a motorized driver (ASI Multi-Scan 4). Lifetime data were acquired using the frequency-domain method (80 MHz). Fluorescein was used as the reference lifetime standard (τ_f = 4.05 ns, pH 9.5). Data-evaluation and visualization were performed directly with the in-house software SIM-FCS. Fluorescence-intensity images were adjusted to enhance structural features and to visualize dye distribution and penetration.

The resulting pH-maps are displayed on the same color-scale to facilitate comparisons. The pH-value distribution within these images is depicted in the corresponding histograms. Individual images were combined using Adobe Illustrator (Adobe Systems Incorporated, San Jose, CA), but no further image processing was performed. Background fluorescence was measured in samples of unstained tissue, treated otherwise identically. Intensity counts in such images were always below 10/50μsec, or below 5% of low intensity images[9].

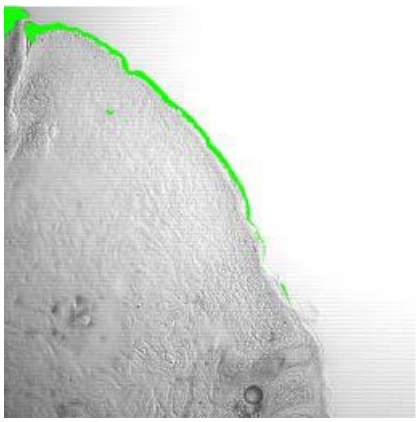
Conventional surface pH measurements were performed using a flat glass surface electrode (Mettler-Toledo, Giessen, Germany) with a pH meter (Skin pH Meter PH 900; Courage & Khazaka, Cologne, Germany). Adhesive tape to assess changes over depth as described earlier (D-squame disks, Acaderm, Menlo Park, CA).

III. RESULTS

Dye distribution in epidermis

10-100 mM BCECF applied in pure ethanol was used to label the SC, four times over the course of an hour, and reapplied only after remaining ethanol had evaporated. A biopsy was taken approximately 15 minutes following the last dye application, mounted for microscopy, and directly visualized. Under these conditions, epidermal baseline functions remain undisturbed (as discussed earlier,[28,27,23,18]), and the dye distributes throughout the SC (Fig.1).

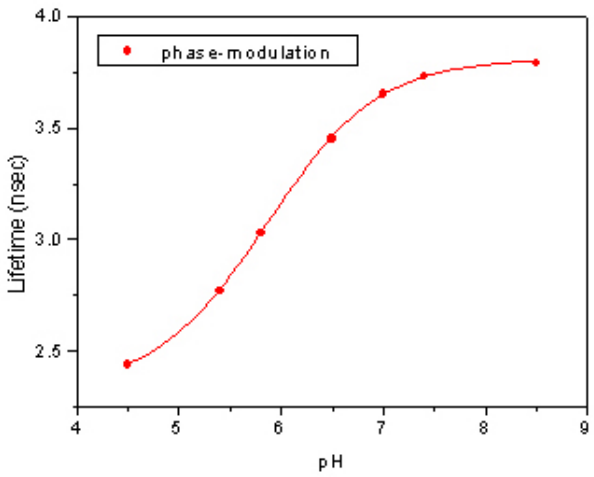
Figure 1: Hairless mouse skin labeled with BCECF as outlined above, and visualized on a Leica TCS-SP confocal microscope. Note the equal distribution of the pH sensitive dye throughout the epidermis, without further penetration into deeper skin layers.



Dye calibration in buffer

Lifetime-values were converted to pH-values, based on a calibration of BCECF in a series of buffers of different pH, using the Henderson-Hasselbalch equation. Control experiments to assess the influence of the solvent and lipids present in the tissue revealed that a saturated solution of Cholesterol in buffer did not affect lifetimes, when corrected for its index of refraction. Similarly, water/ethanol mixtures with up to 5% Ethanol also did not change BCECF lifetime, even without a correction for refraction. Nevertheless, the questions of lipid/protein/pH interactions (and the modeling systems used) are a whole field of research unto itself (e.g., [6,11,30]), which cannot be addressed in this study.

Figure 2: Calibration graph for BCECF in a series of pH-adjusted buffers. Typical graph as used for the conversion of raw lifetime values obtained from tissue samples to tissue pH-values.



Surface pH changes by flat electrode measurements

The typical experiment to assess pH changes over depth uses commercially available adhesive tape, or the D-Squame disks that are less-disruptive and allow for better control over the amount of material removed per strip. Here the results of a group of hairless mice, stripped to a similar SC-damage as controlled by transepidermal water-loss measurements (TEWL; approximately 7-9g/m^2/hr), and repeatedly pH measured over 24 hrs (n=18 to 24 mice). No significant change in post-tape strip pH can be observed over the 24 hr follow-up period (columns in Fig.3), while the standard assay for barrier repair and recovery, TEWL, shows close to completely re-established epidermal homeostasis (graph in Fig. 3).

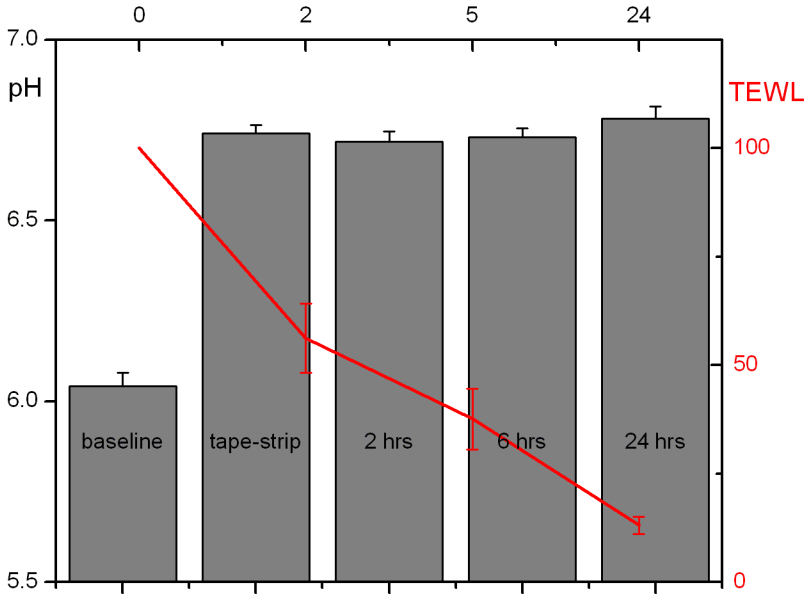


Figure 3: Surface pH on hairless mice before, at time 0 (= tape strip), and at 2, 5, and 24 hrs post tape-strip (gray columns). Concomitant TEWL measurements (red graph), showing reduction of the initial damage induced through tape stripping (100% at 0 hrs), decreasing over time. Note the clear absence of pH changes over time post strip, while TEWL clearly demonstrates barrier repair.

pH changes assessed by FLIM

By comparison, FLIM clearly distinguishes a series of changes in pH microdomain distribution: on the surface, both with and without tape-stripping, the typical two-compartment distribution of a dominant, approximately pH 6 domain and a lesser pronounced approximately pH 7 domain are present (histograms in top panels, Figs. 4 and 5). Following tape-stripping, the pH at the SC/SG interface is clearly more acidic than in undisturbed tissue, where at this depth neutral values predominate (histograms in bottom panels, Figs. 4 and 5).

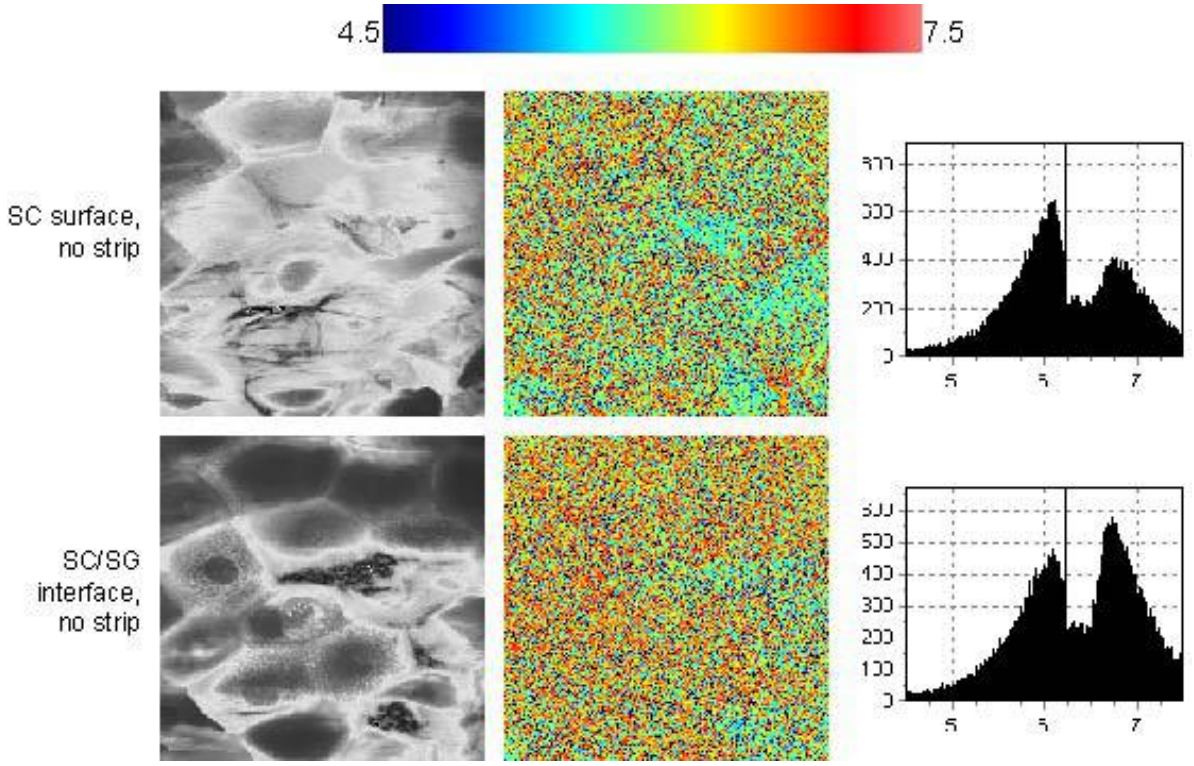


Figure 4: (previous page) Fluorescence Lifetime images from hairless mouse skin not subjected to tape stripping. The images show en face optical sections from the SC surface (0 μm, top panel), and the SC/SG interface (approx. 6 μm into the epidermis, bottom panel) of mouse skin labeled with BCECF, but otherwise untreated. Fluorescence-intensity images (for structural information, left), compared to fluorescence lifetime, which was converted to pH maps (the actual pH distribution of this optical section, middle images), and a histogram for this pH distribution (right); pH color-scale at top.

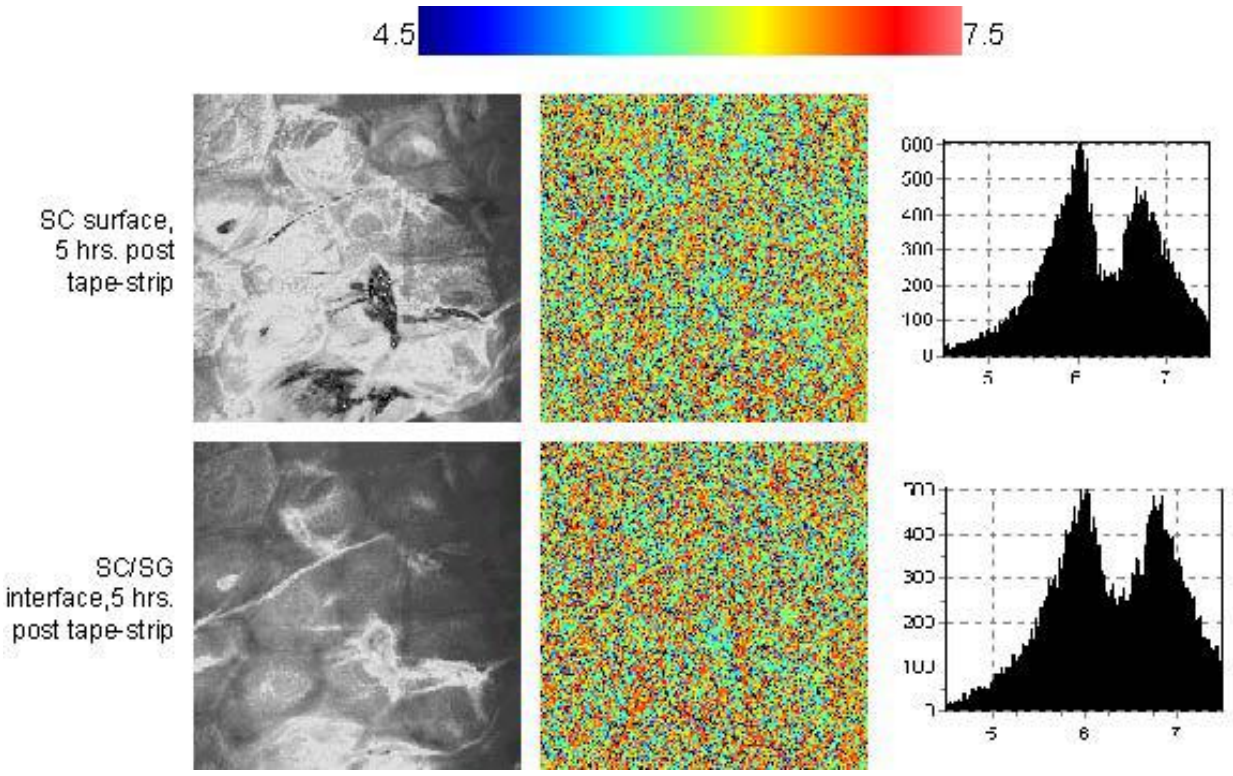


Figure 5: Fluorescence Lifetime images from hairless mouse skin, 5 hrs following tape stripping. The images show analog to Fig.4 en face optical sections from the SC surface (0 μm, top panel), and the SC/SG interface (approx. 6 μm into the epidermis, bottom panel) of tape-stripped mouse skin labeled with BCECF. Fluorescence-intensity images (for structural information, left), compared to fluorescence lifetime, which was converted to pH maps (the actual pH distribution of this optical section, middle images), and a histogram for this pH distribution (right); pH color-scale at top.

IV. CONCLUSIONS

Epidermal pH is a well-known characteristic of epidermal homeostasis, and changes of pH accompany a range of physiologic responses as well as skin diseases[34]. Nevertheless, until the application of FLIM to the observation of this phenomenon, opportunities for insight into underlying mechanisms were limited. FLIM led to a new understanding of pH distribution on a microscopic level, which now allows the testing of hypotheses about the origin, function and role of epidermal pH.

Intracellular/intraorganellar pH cannot be distinguished through FLIM, as the point-spread function of the microscopic system –and any comparable optical system would differ only slightly– imposes a limit of resolution in Z-direction. Here, the limit lies at approximately 1.7 μm, such that specifically organellar pH cannot be measured. Nevertheless, intracellular pH is best studied in cell-culture systems, where pharmacologic manipulations are easily achieved.

The en-face view that the system used here generates appears to acquire rather homogeneous SC domains, based on the columnar stacking of corneocytes, where the vertical inter-corneocyte spaces are rather big. By comparison, the

horizontal inter-corneocyte spaces are small, also compared to the corneocyte volume[5,22,21,42]; the overall error through "mixing" of domains should therefore be small.
Previously, we demonstrated that the Sodium-Hydrogen-Exchanger 1 (NHE1) plays a key role in establishing the critical acidic SC milieu[2], and how generating this milieu is of key functional importance in the post-natal adaptation and maturation period of mammalian epidermis[1]. The data presented here again demonstrate the inhomogeneous distribution of pH across the epidermis. Yet, we also demonstrate here how FLIM can be used to resolve mechanistic questions: The re-acidification of SC following tape-stripping is necessary in light of the acidic pH dependence of a range of lipid- and protein-processing enzymes, e.g., β-Glucocerebrosidase, and others[13,16,36], normally present and active in SC, and required for barrier repair following such an experimental insult. Still, the established method of surface pH measurements cannot demonstrate that such a change actually occurs, and therefore one would postulate that pH does not play a role in such processes. Nevertheless, experimentally altering the pH environment on the surface delays barrier recovery; this experiment proved the functional necessity for an acidic SC milieu[26].
Using FLIM we here demonstrate the selectively enhanced acidification at the SC/SG interface following tape-stripping, which indicates that an acidic pH is actively generated and present where enzymatic activity is required, i.e., at the SC/SG interface, subsequent to release of precursor lipids from lamellar bodies, specific acidic pH dependent enzymes are active/can be active due to the appropriate pH environment to convert the lamellar body derived lipids to mature extracellular bilayers, necessary for epidermal permeability barrier function.

V. OUTLOOK

We have successfully established FLIM to measure and localize pH in undisturbed samples of skin. This method so far has lead to a range of insights into longstanding questions of epidermal physiology, which could not be resolved with earlier methodology. Still, as mentioned above, the other and physiologically more important epidermal electrolytic gradient, at the same time a key regulator for numerous cell-biological events, is the epidermal calcium (Ca^{2+}) gradient. We recently made progress in establishing calcium-FLIM in epidermis. Still, the pitfalls for this application are numerous, and a discussion or presentation of our preliminary results in this field is beyond the scope of this paper.

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