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Spatial mapping by Imaging Mass Spectrometry offers advancements for rapid definition of human skin proteomic signatures

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Abstract

Investigations of the human skin proteome by classical analytical procedures have not addressed spatial molecular distributions in whole skin biopsies. The aim of this study was to develop methods for detection of protein signatures and their spatial disposition in human skin using advanced molecular imaging technology based on mass spectrometry technologies. This technology allows for the generation of protein images at specific molecular weight values without the use of antibody while maintaining tissue architecture. Two experimental approaches were employed: MALDI-MS profiling, where mass spectra were taken from discrete locations based on histology, and MALDI-IMS imaging, where complete molecular images were obtained at various MW values. In addition, proteins were identified by *in situ* tryptic digestion, sequence analysis of the fragment peptides, and protein database searching. We have detected patterns of protein differences that exist between epidermis and dermis as well as subtle regional differences between the papillary and reticular dermis. Furthermore, we were able to detect proteins that are constitutive features of human skin as well as those associated with unique markers of individual variability.

Keywords

MALDI-IMS; proteomics; human skin; Thymosin β -4; dermis

Introduction

Mass spectrometry (MS) has become an indispensable tool to detect and identify proteins in health and disease (1–5). Proteomic approaches have proven useful for homogeneous *in vitro* studies with dermal fibroblasts or keratinocytes, but such techniques are not optimal for capturing global signatures within *in vivo* settings (6, 7). In 2006, Huang *et al.* reported

an *in vivo* mass spectrometry detection technique coupled with capillary ultrafiltration probes used to identify secreted proteins during murine wound healing (8). Other reports applied 2D-DIGE technology to homogenized samples of scleroderma skin (9, 10). At present, what is known of protein localization to specific cells of interest in skin is limited to indirect evidence from immunohistochemical staining within biopsies.

MS technologies such as matrix-assisted laser desorption ionization (MALDI) imaging MS has high throughput potential (11, 12) and can generate many hundreds of protein-specific ion density maps correlated with tissue architecture (13–18). MALDI-IMS permits imaging of the tissue distribution for low molecular weight compounds such as metabolites (19–22). Sugiura and Setou recently reported that MALDI-IMS uniquely provides for simultaneously visualizing a parent drug, its metabolites, as well as endogenous metabolites within targeted organs (23). Application of spatially retentive technologies allows for study of complex interaction between cells and their microenvironment at the molecular level, a type of systematic analysis particularly attractive for examination of the complex architecture in cutaneous samples (16, 24–31). Additionally, MALDI MS offers the potential for detection of molecular species present in a single tissue section regardless of whether a given protein perturbation has been previously implicated or whether a specific antibody has been developed for its immunodetection. In combination with rapid advances in sample preparation (14, 16, 27, 32–34) and data processing (35, 36), IMS now offers a precise means of analyzing protein signatures within complex microenvironments that develop during pathophysiologic or pharmacologic modifications in skin diseases (37, 38).

The present study was designed to optimize MALDI techniques for the detection and definition of proteomic signatures in normal human epidermis and dermis. Two experimental approaches were employed: MALDI-MS profiling, where mass spectra were taken from discrete locations based on histology, and MALDI-IMS imaging, where complete molecular images were obtained at various MW values. In addition, proteins were identified by *in situ* tryptic digestion, sequence analysis of the fragment peptides, and protein database searching.

Materials and Methods

Tissue specimen collection and processing

Following institutional review board approval, skin samples were obtained from the trunk region of normal health patients ages ranging from 36 to 66 (mean = 51.8 years) undergoing elective surgical procedures (N=10). Samples were snap frozen in liquid nitrogen and stored at -80°C until ready for processing. Companion pieces were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained for immunohistochemical confirmation of proteins discovered during MS analysis.

Frozen Tissue Preparation

Human skin samples were sectioned at $12\text{ }\mu\text{m}$ using a cryostat (CM 3050 S, Leica Microsystems GmbH, Wetzlar, Germany) at a setting of -20°C . Serial sections were collected on MALDI gold plates (Applied Biosystem Inc, Foster City, CA, USA) for MS analysis. After thaw mounting, gold plates were placed in a desiccator for 10 min to allow tissue adherence and equilibration to room temperature. Serial sections collected on microscope slides were stained with Hematoxylin and Eosin used to determine matrix placement for MALDI MS studies.

Tissue Fixation and Contaminant Removal

Before matrix deposition, each plate was rinsed at room temperature with solvent (39). Solvents tested included 70% isopropanol followed 95% isopropanol, 70% methanol followed by 95% methanol, 70% ethanol followed by 95% ethanol, dual rinses in 100% hexane, and dual rinses in 100% toluene. Each rinsing procedure lasted for 30 sec. Protein spectra were evaluated for TIC (total ion current), background noise and the number of peaks recorded. The TIC proved highest for isopropanol and lowest for toluene. Ethanol produced comparable data, but for IMS, more ions were localized in epidermis layer after isopropanol washing. In the cases of hexane and toluene, only a few signals in the low mass range were detected. Overall, isopropanol was determined to show the highest relative signaling intensities and the highest number of peaks observed. Our final procedure of choice consisted of 70% isopropanol followed by 95 % isopropanol for 30 sec each and 30 min of vacuum desiccation. The aim of the described procedure was to remove interfering species, such as salts and lipids, that can promote adduct formation, ion suppression and poor matrix crystallization (32).

Tissue preparation for Profiling

A robotic acoustic droplet ejection system was used for matrix deposition (Portrait 630 reagent multi-spotter, Labcyte, Sunnyvale, CA) (14). Two different areas of interest, epidermis and dermis, were targeted for repeated deposition of matrix. Sinapinic acid (20 mg/mL) in 1:1 ACN/0.1% TFA (aq.) was deposited over a series of 6 iterations at 10 droplets per iteration. After completion of matrix deposition, gold plates were immediately returned to vacuum desiccation at room temperature until MS analysis the same day.

Tissue preparation for Imaging

MALDI matrix was spotted by the robotic spotter (Labcyte Portrait 930). Sinapinic acid (20 mg/mL) in 1:1 ACN/0.1% TFA (aq.) was spotted *on tissue* into an array incorporating 200 μm (center-to-center) spacing between individual spots, each with a diameter of 150 μm . The robotic spotter deposited over a series of 20 iterations at 1 drop per iteration. Profiling and imaging analysis were carried out by Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) operating in positive polarity and linear mode.

Tissue Preparation for Tryptic Digestion

Trypsin solution was prepared in 50 mM acetic acid (40, 41) and activated by adding 500 μL of 100 mM ammonium bicarbonate, reaching pH ~8.0 with a final concentration of 0.038 $\mu\text{g}/\mu\text{L}$. Enzyme solution was automatically spotted onto frozen sections using the robotic spotter in a 200 μm array, over a series of 30 iterations at three droplets per iteration and 120 sec time intervals per iteration to allow for drying. Trypsin digestion proceeded at room temperature (~22° C) for ~3 h. Following digestion, a matrix solution containing 10 mg/ml of α -cyano-4-hydroxy-cinnamic acid (CHCA) in 1:1 ACN/0.5% TFA (aq.) was spotted. Peptide spectra were acquired using Ultraflextreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA). The mass spectrometer was operated with positive polarity *reflectron* mode and spectra acquired in the range of m/z 400–4700.

MS/MS sequence analysis of tryptic peptides and protein identification

Digested peptides were isolated as *precursor ions* (*parent ions*) and fragmented to generate MS/MS spectra. Latter were converted into a single MASCOT generic format data file and run against the Swiss Prot database to match tryptic peptide sequences to their respective intact proteins. MS/MS spectrum searches were performed with a mass tolerance of 0.3 Da and fragment ion tolerance of ± 0.1 Da. Search criteria also included up to three missed

cleavages and variable modifications, such as methionine (M), histidine (H) and tryptophan (W) oxidation and also N-terminal (N-term) acetylation.

Immunohistochemical Staining

Immunohistochemical staining was performed using commercial antibodies specifically directed against thymosin β -4 (Abcam, Cambridge, MA) and lumican (R&D Systems, Minneapolis, MN). Formalin-fixed paraffin embedded normal skins were sectioned at 5 μ m, placed on slides and warmed overnight at 60°C. Slides were deparaffinized and rehydrated with graded alcohols ending in tris buffered saline (TBS-T, LabVision, Fremont, CA). For thymosin β -4 staining, pepsin (ready-to-use or RTU, Invitrogen, Camarillo, CA) was applied for 20 mins at 37°C. Endogenous peroxidases and non-specific background were blocked by subsequent incubations in peroxidase block (RTU, DAKO, Carpinteria, CA) and serum-free protein block (RTU, DAKO). Primary antibody to thymosin β -4 was used at 1:1000 for 1 hour, followed by incubation in EnVision+ HRP labelled polymer (RTU, DAKO). For lumican staining, proteinase K (RTU, DAKO) was applied for 5 mins. Endogenous peroxidases were blocked as before. Non-specific background, secondary, and tertiary labelling of target was accomplished by use of Vector's ABC Elite Goat IgG kit (Vector Laboratories, Burlingame, CA). Primary antibody to lumican was used at 1:300 for 1 hour. Slides were rinsed with TBS-T between each reagent treatments. All steps were carried out at room temperature unless otherwise noted. Visualization of both antibodies was achieved with Nova Red chromogen (Vector Laboratories). Slides were counterstained with Mayer's hematoxylin, dehydrated through a series of alcohols and xylenes, and then coverslipped with Acrytol Mounting Media (Surgipath, Richmond, IL). Microscopic examination and imaging was performed with an Olympus BH-2 microscope with a Polaroid digital microscope camera 2 (Melville, NY).

Statistical Analysis

Multiple spectra per region of interest (the upper and lower dermis) were selected from the MALDI-IMS data. In particular, 150 spectra per area of interest were exported using the ion density maps of specific molecular features as coordinates to distinguish the two dermal areas. Comparisons of these regions of interest were conducted using principal component analysis (PCA). PCA analyses were performed since this is a statistical method commonly used to reduce the dimensionality of a multivariate data set by displaying and ranking its variance within a data set. This statistical tool was used to generate classification models based on protein profile patterns. These data were used to confirm the existence of two disparate sub-regions within the dermis in normal subjects.

Results

Skin Protein Profiling/Imaging

A typical analysis of proteins directly from tissue follows two main experimental approaches: profiling and imaging. Profiling involves analysis of discrete areas of the tissue sections to enable comparisons between distinct areas on tissue sections, such as normal healthy area versus a diseased area, or between two different specimens (Figures 1a–c). High-resolution imaging of a tissue section requires that the entire tissue section be analyzed from an ordered array of laser ablated spots in which spectra are acquired from those spots at intervals that define the image resolution (Figures 1d–f). Imaging software generates two-dimensional ion-maps, by plotting the intensity of signals obtained as a function of xy coordinates. This procedure allows for rapid assessment of protein localization and the visualization of the molecular differences between and among samples.

To optimize skin-specific detection parameters, frozen sections were prepared from trunk skin from 3 male and 7 female adult patients who were undergoing elective surgery. Initial experiments were performed by MALDI MS in order to profile different areas of the tissue. Hematoxylin and eosin stained sections were used to determine histological coordinates for matrix application over the epidermis and dermis (Fig 1a). Visualization prior to spotting helped avoid matrix deposition over epidermal appendages such as pilosebaceous units or eccrine sweat glands that are dispersed throughout the dermis. Figures 1b & 1c show the same section before and after matrix deposition. We deposited 20 matrix spots per area in order to establish the protein pattern in these focal areas of interest. E-Figure 1a presents the protein profiles in the mass range from 2000 to 16,000 Da related to the epidermis (e-Fig 1a) and dermis (e-Fig 1b). The data expressed in e-Figs 1a & b are expressed as averages. Prior to averaging we examined the spot-to-spot variability among m/z and their relative ion intensities. The displays of individual spectra from each matrix are shown in the epidermis from two representative patients in e-Figure 1c & d. The remarkable consistency in the relative intensity of peaks from spot-to-spot suggests uniformity in ionization. These data from two patients show considerable consistency in the m/z ions although there are some patient to patient variabilities. This suggests to our team that MALDI MS as well as IMS may be a tool that will enable cutaneous researchers to tease out individual differences in an age when the next frontier of medicine will be to apply individualized treatment plans based on proteomic data.

As expected, differences were observed comparing the profiles obtained from the epidermis and dermis reflecting the expected expression differences between a stratified squamous keratinizing epithelium (e-Fig 1a) and the dense irregular connective tissue of the dermis (e-Fig 1b). For example, signals at m/z 10178, 11051, 11308 and 11607 were only observed in the epidermis (e-Fig 1a), whereas the signals at m/z 3369, 3440 and 5170 were only detected in the dermis (e-Fig 1b). Moreover, other peaks such as m/z 2936, 7767 and 8567 were found in both epidermis and dermis although with different expression levels. Dermal spectra showed fewer peaks than those from epidermis, especially in the high mass range (e-Fig 1b). Many dermal peaks were observed in the mass range 3–5 kDa, such as the signal at m/z 4965, the base peak of every spectrum acquired. The dermis expressed many other characteristic peaks such as m/z 6432 and 6633 and also m/z 8772 and 8845. The comparison between protein profiles of the two main skin layers was carried out using averaged and normalized mass spectra in order to minimize spectrum-to-spectrum differences. Numerous protein peaks were consistently detected in both regions while others showed an apparent pattern of individuality. We attribute differences among inter-spot intensity to the sensitivity of MALDI MS that captures slightly variable tissue morphology from one dermal spot to another, i.e., one spot may be centered over an area with a high population of resident cells whereas another spot may center over a capillary endothelium and yet another spot over extracellular matrix molecules.

Figures 1 e & f present optical images of a 12 μ m skin section mounted on a gold MALDI plate prior to and after matrix deposition for IMS analysis. We conducted preliminary matrix deposition tests and selected 200 μ m (center-to-center) spacing. Figure 2 presents a series of ion density maps or images from proteins, with different intensities, that were localized in different areas of the section. Figure 2a shows three representative ion density maps over the epidermis related to the ions at m/z 4285, 8224 and 11,656, while Fig 2b depicts several ions with unique dermal distributions. Figure 2c–e shows ion density maps for m/z 4965, corresponding to thymosin β -4 (TYB-4), distributed throughout the dermis in all the samples analyzed, as well as the ions at m/z 5056 and 6444. Further, IMS localized some signals, for example at m/z 10,096, 10,220 and 13,790, that were largely restricted to the upper papillary dermis (Fig 2f). Figure 2g depicts ions selectively concentrated in the reticular dermis at m/z 4938, 4965 and 5171. All 10 patients did not show this distinctive sub-localization pattern

within the dermis (compare Fig 2g for m/z 4965 with Fig 2c–e). The reason of this patient-to-patient variability is not yet clear. On the basis of our IMS optimization and analysis of 10 normal patients, we found that the signal at m/z 4965 is a predominant protein in dermal regions while those at 8215, 8565 and 11,656 are uniquely expressed proteins in normal human epidermis.

Protein Identification on Tissue Sections Using *in situ* Tryptic Micro Digestion

We utilized an *in situ* tryptic micro digestion protocol to generate peptides directly on discrete areas of tissue for sequence analysis (40, 42). Signal intensities of tryptic peptides are mediated by several factors, e.g. protein concentration differences, variation in enzymatic digestion efficiency and differences in desorption and ionization efficiencies. The protease hydrolysis step is essential to generate peptide fragments and enable identification directly from their specific location in the tissue. Peptides were sequenced using a MALDI TOF/TOF instrument and were subsequently correlated to the respective intact proteins for epidermis or dermis through a protein database search. Currently we have identified selected proteins directly from human skin using tandem MS, carried out to achieve mass accuracies of approximately 0.1 Da. Thymosine β -4 was sequenced intact off the tissue by MS/MS, whereas other proteins were identified using a *bottom-up* approach: enzymatic digestion followed by peptide sequencing. Five keratin proteins found in the epidermis are listed in the Supplemental Table 1. As expected, keratin 14 is restricted to the undifferentiated epidermal stratum while keratins 1 and 10, were highly characteristic of the outer more differentiated epidermal strata. Table 1 features the peptide at m/z 2872, part of K1C10-human for which ions at m/z 1090, 1364, 1492, 1706 were also detected. Some areas of the epidermis revealed low signals for the ion at m/z 1667.851. This signal was detected in higher relative abundance in the dermis and corresponds to the partial protein sequence SLEYLDLSFNQIAR (Fig 3), characteristic of lumican, a prototypic leucine-rich proteoglycan with keratin sulfate side chains that binds to type I collagen fibrils and plays a role in collagen fibril assembly (43, 44). Three more peptides were sequenced from the dermal region from signals for ions at m/z of 1753, 1225 and 1024 (Supplemental Table 1). Our digestion protocol also detected collagen molecules in the dermis. These included collagen alpha-3 (VI) chain for which a total of 12 tryptic peptides from CO6A3-human were detected. Decorin, a proteoglycan from the small leucine-rich proteoglycan (SLRP) family, was also identified. Dermis also was found to have actin protein (ACTA-human) and beta-actin-like protein 2 (ACTBL-human) which, after digestion, a total of 8 and 7 peptides were detected, respectively (Supplemental Table 1). All skin samples contain a rich vascular network so the presence of three different peptides with AA sequences for hemoglobin (HBB-human) served as an internal positive control. Finally, glypican-2 (GPC2-human), a protein from the glypican family of heparan sulfate proteoglycans, was identified. This protein, known to modify cell signaling pathways and contribute to cellular proliferation and tissue growth, was spatially restricted to the dermal region as were hemoglobin, decorin, actin and actin-like proteins.

Immunohistochemical Confirmation

Positive immunoreactivity for TYB-4 was present within a dermal population (presumed resident fibroblasts and immunocytes) (Figs 3a & b). These images show intense staining of capillary endothelium for TYB-4 while this secreted protein reveals a diffuse distribution over the extracellular matrix. Lumican, an extracellular matrix molecule that is incompletely characterized in the skin, was quite evident by *in situ* tryptic digestion coupled with MALDI MS (e-Figure 2). Figure 3c–e show variable epidermal displays for lumican revealing intense immunostaining over capillaries. Lumican shows a variable distribution in the dermis with a sub-epidermal concentration that was particularly strong in one patient (Fig 3e) but displays a broad dispersal pattern throughout the reticular dermis in another patient

(Fig 3f). Many keratinocytes comprising the secretory segments of the apocrine (Fig 3g) and eccrine sweat glands (Fig 3h) show intense staining for lumican.

Principal Component Analysis

A supervised principal component analysis was carried out on multiple spectra classes acquired from a whole tissue section. Here, PCA was applied as a tool to define spectral clustering from MALDI-IMS for examination of the composite proteome within the upper (papillary) versus lower (reticular) dermal regions (Fig 4). By analysis of total significant protein components in the sample, the software was able to cluster the m/z from the lower dermal area left of the zero mark on the x-axis based on 29% of the variances. Thus, proteins within the dermis as a whole were distinctively separated into two clusters. Stated differently, 71% of the protein features are comparatively homogenous with no distinction between upper or lower dermis. Not surprisingly, the variances that contribute to the subtle differences that define upper and lower dermal composition are low at 29%.

Discussion

The protein profiles obtained in this study showed distinct differences between focal regions of the skin (epidermis versus dermis) due to their different cellular compositions. In addition MALDI IMS was able to detect unique differences between the papillary and reticular dermis. We consistently detected constitutive markers of normal skin regions as well as unique markers indicative of individual variability. This latter finding suggests that these advanced MS techniques may find increasing applicability in the realm of individualized medicine. Since skin is a prime example of an organ with clear spatial patterns and niches that reflect morphologic and functional specialization (45), the potential of this highly sensitive and spatially precise analysis is particularly attractive for the study of cutaneous lesions. For example, it is known that skin grafts maintain unique donor site specificities. Only recently investigators have begun to uncover fibroblast gene expression mechanisms that are responsible for this phenomenon of anatomic diversity and positional variation in human skin (45–47). Other investigators have studied the role of stem cells in cutaneous processes, such as wound healing (48). With the MS techniques we have developed for skin analysis, we can map such site-specific/cell specific cutaneous nuances by virtue of their ability to provide a more comprehensive proteomic signature in register with unique tissue architecture. We believe the field is poised to move beyond the data that can be averaged during homogenization.

While protein identification in skin has been a standard approach for many decades, such discoveries have been laborious and using techniques such as Western Blotting, ELISA, and immunohistochemistry, only known proteins can be studied. Another modern proteomic approach, 2D-DIGE/MS, is limited to homogenization of whole skin biopsies and detects high-abundance proteins at a MW between 20–200 kDa, but loses spatial information at the cellular level. All proteomic techniques, including MALDI-IMS, have limitations (49)(23). Because this is true, multiple proteomic techniques can be utilized to provide complementary information.

Rapid advancements in MALDI-IMS techniques have made it possible to study complex disease states. MALDI-IMS has been utilized to study prognostic markers in non-small cell lung cancers (50), and to demarcate tumor margins in renal carcinoma samples (51). Schwamborn and Caprioli's review of MALDI-IMS discusses several instances where this technique has been used to study clinical disease states (17).

Technologies utilized in the present paper produced complementary data to other techniques by detecting proteins in the 2–20 kDa range with highest sensitivity. This mass range

included TYB-4 that has already been implicated in events occurring during wound repair (52), mast cell exocytosis (53), activation of hair follicle stem cells (54), angiogenesis (54, 55), matrix metalloproteinase expression (52) and anti-inflammatory events (56). TYB-4 regulates dynamics of the actin cytoskeleton in cell types for which cell migration is essential (57). We also confirmed the presence of this protein in normal human skin using immunohistochemical techniques (58). In sum, the presence of TYB-4 in each dermal spectrum suggests that an ample supply of this protein is held in readiness during skin homeostasis awaiting a response to stimuli.

Although IMS technology detected a number of interesting proteins, in this report we restricted our focus to lumican, a protein that is less abundant and has been poorly characterized in skin (59). Bhattacharjee *et al* depicted the presence of lumican in the subepidermal region (59). We confirmed a similar moderate distribution in this location. Since this proteoglycan is known to interact with collagen by limiting growth of fibril diameter (43), its distribution in this area may explain the visibly smaller caliber of the collagen fibrils in this microniche within an otherwise dense irregular connective tissue. The presence of this protein led us to examine additional locations of lumican within the skin. We have found that lumican is also present in certain differentiated keratinocytes in the outer strata of the surface epidermis as well as in secretory keratinocytes of sweat glands. The diffuse presence within the extracellular matrix of the reticular dermis where collagen fibrils are not constrained in diameter, leads us to suspect several more unexplored roles for lumican in both dermis as well as keratinocytes. Our data suggest that proteomic signatures resulting from newer, more sensitive MALDI IMS techniques can be equally useful in generating novel hypotheses to explain cutaneous structure and function.

In summary, IMS offers new opportunities for the proteomic investigation of tissues such as human skin permitting a systems biology approach for monitoring cellular modifications occurring in skin diseases. Advances using MALDI-IMS have provided scientists with a unique discovery tool for visualizing multiple elements in complex environments (60, 61). Though this technology has not yet been applied to give a comprehensive proteomic approach for studying skin, the purpose of this study was to optimize and assess the utility of mass spectrometry to analyze proteins from normal human skin using IMS to observe the distribution and localization within different areas of human skin. We highlight the potential value of high resolution images that allow for the evaluation of cutaneous proteomic details unreachable using other lower resolution investigative techniques.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

a–c) illustrate a typical MALDI MS (profiling) experiment. a) shows hematoxylin and eosin staining of a normal human skin section b) shows the same tissue section mounted on a gold MALDI plate before and c) after matrix robotic deposition with 20 spots arrayed over the epidermis and 20 spots arrayed over the dermis. d–f) illustrated a typical MALDI IMS sample preparation d) shows hematoxylin and eosin staining of a different human skin section. e) shows a tissue section on a gold MALDI plate before and f) after a 200 μm spacing matrix was arrayed for IMS analysis. Scale bar = 5000 μm .

Figure 2.

Illustrates a series of ion density maps from MALDI IMS. a) reveals 3 representative ions with a distribution of m/z consistent with an epidermal spatial distribution and b) reveals ions with a generalized spatial distribution throughout the dermis. c–e) show the ion density maps from 3 different patients illustrating the distribution for thymosin β -4 at m/z of 4965. f) shows ion density maps from a single patient depicting 3 m/z ions with selective spatial distribution restricted to the papillary dermis and g) ion density maps from the same patient showing site-specific distribution in the reticular dermis. Scale bar = 2000 μ m

Figure 3.

Immunohistochemical confirmation and distribution for thymosin β -4 and lumican a) shows thymosine β -4 in many cells within the dermis b) shows a diffuse dispersal for this secreted protein over the extracellular matrix in the reticular dermal region c–e) shows the highest distribution for lumican in association with capillaries. e) shows a modest staining for lumican in the uppermost papillary dermis at the dermal-epidermal junction f) shows a wide spatial dispersal pattern throughout the reticular dermis g) shows intense staining in numerous keratinocytes in the apocrine sweat gland h) shows intense staining in many keratinocytes within the secretory segment of the eccrine sweat gland. Scale bar (e & f) = 200 μ m. Scale bar (all others) = 100 μ m

Figure 4.

Display of the principal component analysis of the differing protein features between the upper and lower dermis. Each green dot depicts the significant protein features from the lower reticular dermal region and each red dot depicts the protein features from the upper dermal region.