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***In vivo* characterization of minipig skin as a model for dermatological research using multiphoton microscopy**

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Abstract

Minipig skin is one of the most widely used non-rodent animal skin models for dermatological research. A thorough characterization of minipig skin is essential for gaining deeper understanding of its structural and functional similarities with human skin. In this study, three-dimensional (3-D) *in vivo* images of minipig skin was obtained non-invasively using a multimodal optical imaging system capable of acquiring two-photon excited fluorescence (TPEF) and fluorescence lifetime imaging microscopy (FLIM) images simultaneously. The images of the structural features of different layers of the minipig skin were qualitatively and quantitatively compared with those of human skin. Label-free imaging of skin was possible due to the endogenous fluorescence and optical properties of various components in the skin such as keratin, nicotinamide adenine dinucleotide phosphate (NAD(P)H), melanin, elastin, and collagen. This study demonstrates the capability of optical biopsy techniques, such as TPEF and FLIM, for *in vivo* non-invasive characterization of cellular and functional features of minipig skin, and the optical image-based similarities of this commonly utilized model of human skin. These optical imaging techniques have the potential to become promising tools in dermatological research for developing a better understanding of animal skin models, and for aiding in translational pre-clinical to clinical studies.

Graphical Abstract

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Author Contributions Statement

A.A. acquired the images, processed the data, and wrote the manuscript; E.C., D.S., P.H., J.L. and J.C. took care of the animals and anesthesia procedures during the study; M.Z. performed the data analysis; A.A., D.S., M.M., and S.F. contributed to the study design; Z.A. and S.B. designed and directed the study. All authors reviewed and revised the manuscript prior to publication.

Conflict of Interest Statement

S.B. received grant support from GlaxoSmithKline related to the research described here, and reports receiving consultation fees from and owning an equity interest in PhotoniCare Inc., Diagnostic Photonics, Inc., and LiveBx, LLC. A.A. and S.F. are employees and shareholders of GlaxoSmithKline.

3-D *in vivo* images of minipig skin was obtained non-invasively using a multimodal optical imaging system. Label free optical biopsy of minipig skin using multiphoton microscopy techniques enable *in vivo* non-invasive characterization of cellular and functional features of this commonly utilized model of human skin.

Keywords

Minipig skin; Optical biopsy; Fluorescence lifetime imaging microscopy; Animal model; *In vivo* imaging

1. Introduction

The complex structure of human skin and its physico-chemical characteristics helps to maintain homeostasis of the human body.¹⁻³ A better understanding of the skin structure, function and disease can have tremendous impact in research areas such as development of better treatment strategies for skin conditions, drug discovery, optimization of topical and/or transdermal drug delivery techniques, and testing of cosmetic products. Currently, researchers rely on engineered human skin equivalents or animal models for cutaneous disease characterization, drug permeability tests, and toxicity screening.^{4,5} Although there has been significant progress in developing 3D human skin equivalents *in vitro*, these engineered models do not necessarily recreate the complexity of various physiological processes and pathological mechanisms.⁶⁻⁸ Animal models are, therefore, at present essential to elucidate complex interactions underlying human skin physiology, function, or disease. Due to inter-species differences in structural, biochemical, and functional properties of skin, one of the main challenges for cutaneous studies using animal models are the difficulties inherent in extrapolating data from animals to humans.⁵ To ensure successful pre-clinical to clinical translation, anatomical and functional features of the animal models

used in skin studies need to be well-characterized, and their differences with respect to the human skin need to be taken in account.

Skin from several animal species including mice, rats, pigs, and monkeys are typically used for pre-clinical studies in dermatology.⁹ Among different animal models of skin, the pig or minipig is often the species of choice in dermal studies based on the anatomical, physiological, and immunological similarities with that of human skin.^{10–13} Similar to human skin, porcine skin has well developed rete ridges and dermal papilla, similar dermal-epidermal thickness ratio, epidermal turnover time, vascular anatomy, and collagen structure.^{14–16} Additionally, porcine skin is tightly attached to subcutaneous connective tissue as is human skin.¹⁶ However, porcine skin does not contain eccrine sweat glands, and unlike humans, apocrine glands are distributed throughout the skin surface.¹⁰ Porcine skin has been used as a human skin model for studies on wound healing^{16,17}, burns¹⁸, transdermal penetration and delivery¹⁹, and radiation impact²⁰, as well as in stem cell research.²¹ These features of the porcine skin model are valid for minipig skin as well.^{22,23} Furthermore, the minipig is the only non-rodent toxicology model where transgenic animals can be readily generated, and where close sequence homology exists between pigs and humans.²² These factors make the minipig an interesting model for safety and efficacy studies of various topical candidate drugs, and testing of biotechnology products.

Several techniques such as histology,¹⁴ confocal Raman microspectroscopy,²⁴ and infrared spectroscopy¹⁵ have been utilized previously to visualize and characterize the porcine skin model. These studies investigated various morphological, spectral, and biochemical similarities observed between porcine and human skin. However, all these comparative studies were performed *ex vivo*, and often on fixed or frozen (versus fresh) skin samples. The capability to visualize micro-morphological and biochemical features of minipig skin *in vivo* can be extremely valuable for improving our understanding of this widely- and well-established animal skin model. Endogenous fluorophores in skin such as nicotinamide adenine dinucleotide (phosphate) (NAD(P)H), flavins, porphyrins, elastin, keratin, and melanin allow label-free visualization of cellular and sub-cellular features of various skin layers.²⁵ Multiphoton microscopy (MPM) is a non-linear optical technique capable of acquiring 3-D images of skin with sub-micron resolution up to a depth of ~ 200 μm below the skin surface.^{26,27} In fluorescence lifetime imaging microscopy (FLIM), the fluorescence decay function of a fluorophore is measured with picosecond temporal resolution, which provides insight into the molecular interactions of the fluorophore with its biological microenvironment.²⁸ Previous studies have demonstrated the capability of FLIM to determine the biochemical composition of various skin layers.^{29,30} However, there are no prior reports of characterization of minipig skin model performed *in vivo*. In this study, a multimodal optical imaging system capable of simultaneously acquiring two-photon excited fluorescence (TPEF) and FLIM images was utilized to characterize microstructural and biochemical features of minipig skin *in vivo* in a non-invasive label-free manner. Additionally, the observed structural features were qualitatively and quantitatively compared with those of human skin, acquired with the same imaging technology and instrument.

2. Methods

2.1 Animals

Male minipigs (Strain: Göttingen, age = 2–3 months, n = 2) obtained from Marshall BioResources (North Rose, NY, United States) were used in this study. This breed of minipig was used because it is genetically controlled, making it an advantageous and reliable animal skin model. All animal procedures were conducted in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals, and were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois at Urbana-Champaign. All animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-International accredited facility in accordance with the *Guide for the Care and Use of Laboratory Animals* and all federal regulations. Sedation and biopsies were performed by professionally trained veterinary staff.

The minipigs were acclimated to a Panepinto sling device prior to study initiation. They were sedated intranasally with midazolam (0.1–0.5 mg/kg) and placed in the sling for imaging sessions. If additional sedation was needed, ketamine (20 mg/kg intramuscularly) was used in addition to the midazolam. These sedation/anesthesia procedures allowed for the acquisition of multimodal optical images with minimal motion artifacts. In order to ensure good coupling between the optical imaging system and the minipig skin, targeted areas were washed with water and hair was removed using clippers approximately 24 hours prior to imaging.

2.2 Multimodal optical imaging of minipig skin

Multimodal optical images were acquired using a CE-marked (certified) multimodal optical imaging system (MPTflex™ CARS, JenLab GmbH, Germany). The articulated imaging arm from the system was positioned at each imaging site and coupled to the skin (dorsal region) using a magnetic coupling ring (Figure 1k). A glass cover slip was placed on the coupling ring and attached to the skin using double-sided tape. For generating FLIM images, the excitation wavelength of the femtosecond laser was set to 725 nm and the incident laser power was set to 30 mW. Volumetric FLIM data sets were acquired from a volumetric region corresponding to 200 μm $\times$ 200 μm $\times$ 200 μm . FLIM images were taken in 5 μm steps from the skin surface down to a depth of 200 μm with spatial resolutions of < 0.5 μm horizontally and < 2 μm vertically. The time required to acquire each volumetric FLIM dataset was approximately 8 minutes.

2.3 Clinical imaging of human skin

In vivo images of human skin were obtained from the volar forearm region of healthy human subjects (Fitzpatrick skin type II, age range = 25 – 70 years, n = 5) using the same MPTflex™ CARS multimodal optical imaging system. The study protocol, the informed consent, and other documents that required pre-approval were reviewed and approved by the Institutional Review Board at Carle Foundation Hospital, Urbana, Illinois (Carle IRB no. 14072). Written informed consent was obtained from each subject prior to the performance of any study-specific procedure. The imaging procedure and system settings used for the clinical imaging were the same as those used for obtaining minipig skin images.

2.5 Image analysis

FLIM images were processed offline to determine the fluorescence decay parameters using the commercial software, SPCImage (Becker and Hickl GmbH, Berlin, Germany). Cell and nuclei sizes were measured using the lasso tool in ImageJ (National Institutes of Health, Bethesda, United States). During measurement, for each image, five representative cells were manually selected and measured. Python 3.7 with Matplotlib and Seaborn libraries were used for quantitative data analysis and visualization.

3. Results

3.1 Optical biopsy of minipig skin using multiphoton microscopy

MPM is capable of obtaining label-free *in vivo* images of skin non-invasively with sub-cellular resolution from the skin surface down to a depth of ~200 μm . This is possible due to the numerous endogenous fluorophores present in skin.²⁵ In this study, three-dimensional (3-D) multiphoton images of skin were obtained *in vivo* from different regions of the minipig that was maintained under sedation/anesthesia (Figure 1k). The stratified structure of the epidermis and the fibrous appearance of the dermis are clearly visible in the images [Figure 1 (a–e)]. In addition to the TPEF images, FLIM images were acquired simultaneously using this multimodal optical imaging system. Using FLIM, various endogenous fluorophores in skin can be differentiated based on their fluorescence lifetime, which gives insight into the biochemical composition of a target site. Figure 1 (f–j) shows FLIM images obtained from different layers of the minipig skin, false-colored within the fluorescence lifetime range of 100 ps (orange) to 3500 ps (blue). The mean fluorescence lifetime values showed a decreasing trend with depth, which is indicated by a shift in color towards yellow-orange. This shift in fluorescence lifetime is attributed to the increase in the number of melanocytes or melanin-containing keratinocytes closer to the basal layer of the epidermis.^{31,32} Figure 1l shows a representative 3-D rendered image of the minipig skin reconstructed from an image stack of 40 FLIM images obtained from different depths at a spacing of 5 μm . These data demonstrate the capability of multiphoton imaging techniques to visualize cellular/sub-cellular morphological and biochemical features of different layers of minipig skin *in vivo*.

3.2 Qualitative and quantitative comparison of minipig and human skin *in situ*

Numerous *ex vivo* studies have been conducted on porcine skin samples to characterize the thickness of different skin layers, and have compared the structural features with those of human skin.^{11,14,24} However, the structural and functional characteristics of a tissue start to degrade as soon as it is removed from an organism. As the preferred animal model for dermatological research, it is extremely valuable to compare the structural and functional features of minipig skin with respect to human skin *in situ*. During this study, 3-D multimodal optical images of minipig and human skin were obtained *in vivo* using the same imaging system with identical measurement parameters.

As shown in Figure 2, remarkable similarities in structural features were observed between minipig and human skin. The top-most layer, the stratum corneum (SC), was characterized by the presence of anucleate cornified dead cells in both minipig and human skin. The SC

layer of minipig skin appears to have some long fluorescence lifetime components (Figure 2a), which are absent in the SC of the human skin (Figure 2g). Further studies are needed to determine the source of this component. The next two layers within the viable epidermis, the stratum granulosum (SG) and the stratum spinosum (SS), are characterized by the presence of polyhedral living keratinocytes in both minipig and human skin. In both cases, cells become smaller towards the bottom layer of the epidermis, the stratum basale (SB), which is distinguished by the presence of melanocytes or melanin-containing keratinocytes, and the undulating rete ridge structure at the dermal-epidermal junction (DEJ). As expected, minipig skin (Figure 2d) exhibited a significantly lower amount of melanin pigmentation compared to human skin (Figure 2j) at the DEJ. The dermis was characterized by the fibrous appearance of the extracellular matrix tissue, such as elastin and collagen, in both minipig and human skin. These data, all of which were obtained *in vivo*, confirm observations from previous *ex vivo* studies^{14,15,24}, and qualitatively validate minipig skin as a well-suited pre-clinical model for dermatological studies.

Cells and their nuclear dimensions were similar across SG, SS and SB skin layers for both human and minipig skin (Figure 3(a–c)). Quantitative analysis of skin morphological structures reveals that the cell cross-sectional area (at the central section of a cell) in each of the optical slices are very similar between humans and minipigs, starting at around 1000 μm^2 at the SG, then getting progressively smaller at the SS and reaching a cross-sectional area of 100–200 μm^2 at the SB. Similarly, the cross-sectional area of nuclei was found to be 50–70 μm^2 at the SG and then decreased to 20 – 40 μm^2 at the SB in both species. The ratio of cytoplasm to cell cross-sectional areas followed a similar trend in all skin layers, with the exception of the SG, in both humans and minipigs (80–90% in SS and 70–75% in SB). A two-component exponential decay model was used for estimating the fluorescence lifetime parameters from FLIM images.²⁸ In terms of the two dominant fluorescence lifetime parameters, both minipig (n=2) and human (n=5) skin showed a similar trend with mean values for t_1 ~150 – 300 ps and t_2 ~1500 – 2300 ps, as shown in Figure 3(d–f). In minipig skin, the mean values of t_1 , t_2 and t_1/t_2 ratio was higher in the layers SG and SS, and the standard deviation of these parameters, especially t_2 , was lower compared to that of human skin. However, the fluorescence lifetime parameters in the SB was similar in both humans and minipigs.

4. Discussion

The minipig is considered an animal model of choice for skin studies, as this model shares many common features with human skin, including low hair density, epidermal morphology, and thickness.^{13,23} The morphological, biochemical, and biomechanical features of porcine skin that mimic human skin have been established in previous studies via *in vitro* methods.^{15,33–36} However, due to lack of tools capable of imaging skin *in vivo*, it has not been possible yet to confirm whether the results obtained *in vitro* are reflective of the *in vivo* tissue architecture and microenvironment. With the advances in laser technology, computational hardware, and beam delivery options, several non-invasive imaging technologies capable of investigating skin microstructure *in vivo* have been developed. Non-invasive 3-D imaging modalities such as optical coherence tomography (OCT)^{35,37}, high-frequency ultrasound (HFUS)³⁸, photoacoustic imaging (PAI)³⁹, MPM^{26,27} and reflectance

confocal microscopy (RCM)⁴⁰ have demonstrated their capability for acquiring skin images *in vivo*. In a recent study, images of a melanoma lesion and nearby healthy skin on a swine model were obtained *in vivo* using three different modalities, HFUS, OCT, and PAI.⁴¹ Although these techniques are capable of visualizing various morphological features in skin, one of their main limitation is insufficient resolution for cellular imaging. Among various non-invasive *in vivo* imaging modalities, RCM and MPM are both capable of providing images of skin with cellular/sub-cellular resolutions. Due to the lower scattering of light at longer wavelength regions, MPM offers deeper penetration (~200 μm) of light into skin than RCM. As shown in Figures 1 and 2, MPM allows for visualization of microstructural features associated with various skin layers in a label-free manner via nonlinear optical mechanisms such as TPEF and second harmonic generation (SHG). To our knowledge, this is the first study to report label-free (non-perturbative) MPM imaging of minipig skin with sub-cellular resolution non-invasively (non-destructively) and *in vivo* (under physiological conditions). Moreover, the micro-morphological features of the minipig and human skin were obtained using the same optical imaging system *in vivo* to bridge the gap between pre-clinical and clinical studies in dermatology.

One of the main research areas utilizing animal skin models is topical drug development and optimization of drug delivery techniques. During topical drug development, various pharmacological properties of a formulation, including its skin penetration, biodistribution, pharmacokinetics, and pharmacodynamics need to be characterized and optimized through *in vitro* and *in vivo* studies.⁴² Currently, most of these drug properties are evaluated on excised animal or human skin samples *in vitro* using Franz-type diffusion cells or using tape stripping methods.²³ Having the capability to perform these mechanistic studies on animal skin models *in vivo* will enable better prediction of the future performance of the topical treatments in humans. Optimization of the transdermal absorption of the active pharmaceutical ingredient across the skin barrier, assessment of its bioavailability at the site of action, and evaluation of its residence time at the target site are key parameters in topical formulation development.

Although *in vitro* drug permeation studies provide important information for screening topical drug delivery systems, these studies have limitations including compromised skin barrier function, absence of physiological response, and sample degradation with time. On the other hand, *in vivo* permeation studies on animal models may lack correlation with human skin. Hence, it is important to choose an animal model that is structurally and functionally most similar to human skin, and to have tools that are capable of evaluating these pharmacological parameters non-invasively, longitudinally, and *in vivo*. In a previous study, Gottingen minipig *in vitro* skin samples were used to study permeation through the skin.³⁴ This previous study compared the rate of permeation of three different drugs (nicotine, salicylic acid, and testosterone) through both human and minipig *ex vivo* skin samples and concluded that the fluxes of drug substances through minipig skin were similar to the fluxes through human skin. Furthermore, they concluded that the inter- and intra-variation in skin from the minipig was lower compared to human skin. In another study, Gottingen minipig skin reproduced the human pharmacokinetic profile for seven topical drugs, although the *in vivo* skin absorption in minipigs underestimated the absorption in the human skin.²³ Nonetheless, due to the structural and functional similarities with humans,

minipig skin is a promising translatable model for investigating and optimizing various pharmacological properties of topical formulations during the pre-clinical development stage.⁵

In this study, we demonstrated the suitability of the minipig as a model for dermatological human skin studies by confirming that the morphological and biochemical properties are very similar. There are several breeds of minipigs available for research. The Göttingen minipig, the experimental breed used in this study, is the most widely used breed due to their stable genetics and phenotype.⁴³ They are also one of the smallest purpose-bred minipigs with a light skin color available for research. Although the Göttingen minipig is not an albino,²² one of the limitations of this breed as a pre-clinical model in experimental dermatology is their non-pigmented skin. Other breeds such as Sinclair or Yucatan minipigs are better suited for dermatological research studies involving melanin assessment. Moreover, we have also identified some differences that may be important and require further investigation. Specifically, we measured some long fluorescence lifetime components in the SC layer of minipig skin, which were absent in the SC of human skin. These differences may be explained by environmental factors, but further studies are necessary to confirm this hypothesis. Also, the ratio between cytoplasm and whole cell cross-sectional area was significantly different in humans and minipigs at the SG layer.

A few challenges for conducting *in vivo* pre-clinical imaging studies using this state-of-the-art commercial optical imaging system were identified during this study. One such challenge was our ability to acquire high quality 3-D FLIM images *in situ* from multiple skin regions within a limited amount of time. Image acquisition required ~8 min to capture one 3-D FLIM dataset from one location on the minipig skin. It will be important to reduce image acquisition time if multiple datasets need to be taken from different regions while the animal remains under sedation/anesthesia. Furthermore, it was necessary to optimize the coupling procedure of the imaging probe to the skin. As this optical microscope was designed to acquire images of target regions with high spatial resolution, the acquisition process was extremely sensitive to motion artifacts. Even small movements of the target region due to cardiovascular or respiratory motions introduced artifacts in the acquired image data. As shown in Figure 1k, the dorsal region of a minipig, adjacent to its body midline, appeared to be the optimal location for capturing images with minimal motion artifacts. In order to obtain high quality, reliable, and reproducible data *in vivo*, it will be imperative to have both a stable probe coupling and fast imaging.

In conclusion, this study demonstrated the capability of multimodal multiphoton optical imaging techniques such as FLIM to visualize and characterize the micro-morphological features of minipig skin *in vivo*. As the most appropriate animal skin model for pre-clinical research, the 3-D structural and fluorescence lifetime characteristics of minipig skin were qualitatively and quantitatively compared with those of *in vivo* human skin, using the same imaging instrument and imaging parameters. The capability of optical imaging techniques such as FLIM to yield depth-resolved sub-cellular and cellular-level information of different skin layers *in situ* without any exogenous contrast agents offers researchers unprecedented information regarding skin structure and physiology in its unperturbed native environment. The data obtained using these non-invasive optical imaging techniques can play a major role

in developing a better understanding of animal skin models and can facilitate translation of pre-clinical study results into clinical medicine.

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Figure 1: Optical biopsy of in situ minipig skin.

(a-e) TPEF and (f-j) corresponding FLIM images of minipig skin obtained from different depths below the skin surface, which are denoted on the images. (k) Photograph of a sedated minipig coupled to the probe head on the articulated arm of the multimodal optical imaging system during imaging. (l) 3-D rendering of minipig skin reconstructed from 40 FLIM images obtained from skin surface down to a depth of 200 μm in steps of 5 μm . SC – stratum corneum, SG – stratum granulosum, SS – stratum spinosum, SB – stratum basale and D – dermis. Scale bars are 50 μm and are the same for all images. Color scale bar shows the range of mean fluorescence lifetimes.

Figure 2: Qualitative comparison of human and minipig skin.

FLIM images of (a-f) minipig and (g-l) human skin obtained from different skin layers below the skin surface. Depths are denoted on the images. The images are color-coded based on their mean fluorescence lifetime. The red dotted circles in (a) indicate regions with high fluorescence lifetime ($\tau_m > 2500$ ps) observed on the surface of minipig skin. SC – stratum corneum, SG – stratum granulosum, SS – stratum spinosum, SB – stratum basale and D – dermis. Scale bar is 50 μm and is the same for all images. Color scale bar shows the range of mean fluorescence lifetimes.

Figure 3: Quantitative comparison of human and minipig skin.

Two-photon FLIM images were quantified to compare morphological parameters: (a) cell size, (b) nucleus size, (c) Cytoplasm to cell area ratio in individual cells expressed as a percentage, and (d-f) fluorescence lifetime parameters. Vertical axis separates skin layers: 1 - stratum granulosum, 2-4 - stratum spinosum and 5 - stratum basale. For (a-c), five representative cells were selected and measured in each of the five skin layers. The data was obtained from the skin of two minipigs (dorsal region) and five humans volunteers (volar forearm).