**In situ bio-distribution and residency of a topical anti-inflammatory using fluorescence lifetime imaging microscopy**

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**Running Head:** Non-invasive detection of a topical anti-inflammatory using FLIM
What’s already known about this topic: Fluorescence lifetime imaging (FLIM) has been utilized to non-invasively image cellular features in human subjects. It has demonstrated the potential to monitor morphological and metabolic changes at the subcellular level that occur in subjects with skin cancer and atopic dermatitis. Its potential for wider utilization in dermal drug discovery or dermatologic disease monitoring has seen limited development.

What does this study add: This is a first of its kind clinical study in which FLIM was used to monitor drug distribution and residency. It is a first step in establishing the unique capabilities multi-photon imaging could provide to patients through non-invasive drug detection. More information was generated per subject while reducing the subject burden and population size using FLIM. Imaging endpoints can be utilized to improve patient outcomes through improved monitoring capabilities.

ABSTRACT

Background: In this Phase I clinical trial, the spatial bio-distribution and residency of GSK2894512 within the epidermis and dermis of healthy human participants was investigated non-invasively using fluorescence lifetime imaging microscopy (FLIM). Two topical drug formulations containing 1% GSK2894512 were applied to the right and left forearms of 6 participants for 7 consecutive days, followed by 7 days of observation for residency.

Objective: FLIM images were obtained daily throughout the study approximately every 24 hours. During the treatment phase of the study, images were collected from each participant pre-treatment, reflecting the residual dose from the previous day. Three punch
biopsies from each participant of one formulation was obtained from the treated region during the post-treatment follow-up period between days 8 and 14 for comparison with FLIM results.

**Results:** Cellular and sub-cellular features associated with different epidermal and dermal layers were visualized non-invasively, down to a depth of 200 µm. Results yielded 3D maps of GSK2894512 spatial distribution and residency over time. This fluorescence data provided a marker that was used as a monitor for day-to-day variance of drug presence and residency post-application.

**Conclusions:** These results suggest FLIM could be a viable alternative to skin biopsies without the usual patient discomfort and limitations, thereby enabling the direct measurement of skin distribution through longitudinal monitoring. These results are the first step in establishing the unique capabilities that multi-photon imaging could provide to patients through non-invasive drug detection.

**INTRODUCTION**

Understanding penetration characteristics of the active pharmaceutical ingredient (API) in topical formulations such as volumetric dispersion, penetration, and residency are necessary for successful treatment of dermatological conditions. To gain this understanding, a variety of different methods can be utilized to investigate penetration and bio-distribution within skin either *ex vivo* or *in vivo*. An overview of many of these methods can be found in the supplemental materials along with limitations of these techniques that could be addressed by advanced imaging methods. Due to these limitations, an imaging technique that can provide label-free mapping of spatial distributions, penetration pathways, and residency of
topical formulation on skin *in vivo* will be an extremely valuable tool for topical formulation development and monitoring disease outcomes.

The purpose of this paper is to report results of a Phase I clinical trial of a topically delivered investigational drug GSK2894512, a drug being developed for treatment of atopic dermatitis and psoriasis, in healthy participants utilizing interpreted results from fluorescence lifetime imaging microscopy (FLIM) as the primary endpoint. FLIM is a relatively new non-invasive multiphoton optical imaging technique that measures fluorescence lifetimes of fluorophores to generate image contrast, and can be used to monitor metabolic, chemical, and morphological changes *in vivo*. Although excitation and emission wavelengths of bound and unbound NAD(P)H are the same, fluorescence lifetimes of NAD(P)H in each state are significantly different, with unbound being ~0.4 ns and bound being ~2 ns. This differentiation can be utilized as a marker for metabolic activity. In a previous study investigating the transdermal kinetics of a drug formulation in human skin sections *ex vivo*, FLIM was utilized to monitor the drugs diffusion pathway by measuring the variation in bound NAD(P)H fluorescence lifetime before and 2 h post-drug application. In addition to NAD(P)H, a variety of additional endogenous fluorophores are also observed in skin. These characteristics makes FLIM a promising technique for label-free skin imaging.

In this clinical study of healthy participants, penetration, spatial distribution, and residency of two formulations of topical creams (A and B) of GSK2894512 were evaluated longitudinally using FLIM. This drug exhibits inherent fluorescence properties (lifetime ~3000 ps), which allows differentiation from endogenous fluorescent components in human skin. Utilizing fluorescent properties of GSK2894512, penetration kinetics of this drug was followed in six healthy participants for both formulations daily throughout a 14-day residency study. To our knowledge, this is a first of its kind clinical study in which FLIM was used to monitor drug distribution and residency in a clinical setting, and is the first step toward
establishing the unique capabilities that this type of imaging provides to non-invasively follow drug effects on patient outcomes in a clinical setting.

MATERIALS AND METHODS

Study participants and topical treatment

Seven healthy, white males (Fitzpatrick skin type I – IV) from 18 to 32 years of age were enrolled into this Phase I clinical trial during a 3-month period (July to September). Six participants completed the study; one participant withdrew consent on day 6. Each participant received 3 mg/cm² of 1% concentration of each study cream, A and B. Cream A was applied to one volar forearm and cream B was applied to the other volar forearm once daily for 7 days. To monitor background fluorescence in FLIM, vehicle creams were imaged on day 1 in a separate area of the forearm (at least 1.3 cm from where study drug was applied).

The human subject research component was approved by the Carle Foundation Hospital Institutional Review Board. Informed consent was obtained from healthy participants prior to any study procedures.

FLIM imaging

The study participants were comfortably seated on a recliner chair with armrests and the volar forearm of the subject was securely positioned on the armrest with cushion support, which helped to reduce the motion artifacts during the imaging session. Prior to imaging, using double sided tape, a glass cover slip was placed on a magnetic coupling ring which was then attached to the volar forearm imaging site. Subsequently, the articulated arm of the imaging system was attached to the magnetic coupling ring (Figure 1e). A CE certified
multimodal optical imaging system (MPTflex™ CARS, JenLab GmbH, Germany) was used for acquiring FLIM images in vivo. FLIM images were obtained from study participants from day 1 to day 15 including control images on day 1 (prior to initial drug application). For generating FLIM images, the excitation wavelength of the femtosecond laser was set to 725 nm and in situ laser power was set to 30 mW, which is well below ANSI Z136.1 (2014) safety limits. Two sets of volumetric FLIM data sets corresponding to 200 µm x 200 µm x 200 µm volumes were acquired from within 7 cm x 14 cm treatment region per study day. 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. Time required to acquire each volumetric FLIM dataset was approximately 8 minutes.

The details of detection part of this multimodal imaging system has been described previously. The imaging system comprised of four different detection channels where the autofluorescence signal was collected by the first detection channel (405 to 565nm) and the vast majority of drug fluorescence signal was detected by the second channel (370 to 405 nm). As most of the drug signal was detected in the second channel (Supplementary figures 1 and 2), only the signal collected in this channel was analyzed for determining the drug distribution and residency from the FLIM images.

**FLIM image analysis**

Fluorescence decay data from FLIM images were processed offline using commercial software, SPCImage (Becker and Hickl GmbH, Germany). Fluorescence decay curves obtained from each pixel of the FLIM dataset were fitted using a standard multi-parameter exponential decay, full details of which can be found in the supplementary material. From the fitting, three variables were used to discriminate endogenous fluorescence from the study
drug: mean lifetime ($\tau_m$), second lifetime component ($\tau_2$) from a 2-component fit, and absolute third fractional component ($a_3$) from a 3-component fit. Typically, endogenous fluorophores in healthy skin have $\tau_m<2200$ ps, $\tau_2<3500$ ps and no appreciable $a_3$ component. These variables were selected based on literature evidence, and experimentally confirmed by comparing FLIM images from control regions (day 1 and vehicle cream images) with those obtained from the treatment regions. Fluorescence signal attributed to GSK2894512 from each pixel was determined using an analysis algorithm detailed in Figure 2. Total drug fluorescence was quantified by integrating drug fluorescence measured from all pixels within a full volumetric dataset using a custom-written MATLAB (MathWorks Inc., United States) algorithm.

**Liquid chromatography / mass spectrometry / mass spectrometry (LC-MS/MS) analysis**

GSK2894512 was extracted from human skin tissue homogenate by protein precipitation, using acetonitrile containing [2H213C6]-GSK2894512 as an internal standard. Extracts were analyzed by HPLC-MS/MS using a TurboIonSpray™ interface with negative ion multiple reaction monitoring. This method was validated over the range 0.5-1000 ng/mL and the LLQ was 0.5 ng/mL using a 50 µL aliquot of human tissue homogenate. Concentrations of GSK2894512 was normalized to ng/mg of skin tissue.

**RESULTS**

**Optical biopsy of human skin**

FLIM allow non-invasive visualization of microstructural organization and biochemical composition of human skin *in situ*. Endogenous fluorophores in human skin
such as NAD(P)H, flavins, porphyrins, elastin, keratin, and melanin\textsuperscript{6-8} enable label-free visualization of cellular and sub-cellular details of different skin layers \textit{in vivo}. In comparison to confocal microscopy, TPEF provides superior spatial resolutions and deeper penetration, up to \(~200\,\mu\text{m}\) in scattering tissues. As shown in Figure 1a, different sub-layers of epidermis such as stratum corneum (SC), stratum granulosum (SG), stratum spinosum (SS) and stratum basale (SB), can be differentiated based on cellular features in those layers. As shown in previous studies\textsuperscript{3}, fluorescence lifetime depends on the chemical composition of that region and is an indicator of its metabolic state. Figure 1b shows FLIM images of different skin layers and Figure 1c shows histograms of mean fluorescence lifetime at corresponding depths. Since melanin has a lower fluorescence lifetime than keratin, melanocytes and keratinocytes can be easily distinguished from surrounding tissue in FLIM images. A 3D rendering of the imaged volume of human skin reconstructed from FLIM images (Figure 1d), demonstrates the ‘optical biopsy’ capabilities of these imaging modalities.

**Mapping and quantification of 3D bio-distribution of the topical creams \textit{in vivo}**

Fluorescence signal from the topical cream was discriminated from endogenous fluorescence based on its longer fluorescence lifetime. FLIM images were color-coded according to fluorescence lifetime, where regions with a mean fluorescence lifetime ($\tau_m$) longer than 3500 ps are presented in blue. Figure 3a shows penetration of the topical cream including penetration along a hair follicle. These FLIM images were obtained 24 hours post-application and showed that most of the topical cream remained in the SC layer and accumulated along ridges of the skin. A 3D rendering depicting penetration of the topical cream along the hair follicle is shown in Figure 3b. Depth of penetration of the topical cream
was determined from these depth-resolved FLIM images (Figure 3c). Using these approaches, it was also possible to detect both stronger signals from concentrated drug and weaker signals from dispersed drug (Supplementary Figures 3-5). In addition to the fluorescence decay fitting technique, the differentiation of the drug fluorescence signal from endogenous fluorescence was confirmed using phasor analysis, and drug bio-distribution estimated using both analysis approaches were similar (Supplementary Figure 6).

**Determining skin residency of a topical cream**

Two different formulations of the same API were applied on the volar forearm of participants for 7 consecutive days and FLIM images were obtained from treated regions 24 hours post-application (control images were obtained on day 1 prior to application). To determine the skin residency of the topical cream, FLIM images were taken daily throughout the study. Spatial distribution, depth of penetration, and residency of the topical formulation were determined based on these FLIM images. Figure 4 depicts FLIM images from one study participant demarcating drug signal (shown as blue regions) at different depths on different days of this longitudinal study. Optimal parameters for discriminating drug signal were determined experimentally by analyzing control images from pre-dose on day 1 and vehicle control images obtained 24 hours post-dose from day 1 application. During treatment days 2 through 7, most of the drug signal was detected on the skin surface [Figure 4 (c & d)]. Accumulation of the topical formulation along skin ridges is also visible in images obtained during days 2, 8 and 9 [Figure 4 (i, j, k, p)]. By day 10, there was no detectable fluorescence from formulation residing in the skin [Figure 4 (f, l and r)]. Figure 4 shows results from one participant with inter-participant variability large in terms of amounts of drug present on skin and its residency (Supplementary Figures 7 and 8).
**FLIM clinical readouts**

Drug fluorescence detected using FLIM from different skin layers for both creams were semi-quantified. Figure 5 (a & b) show drug fluorescence signal, depth of penetration, and residency in one study participant for cream A and B, respectively. In this participant, fluorescence signal from both formulations were detected above limits of detection on all treatment days, except on day 4 for cream A (Figure 5a). However, no appreciable amount of drug fluorescence was detected from both creams during the post-treatment period, i.e. after day 8.

Figure 6a shows average depth-integrated fluorescence signals from both creams on different days of the study (n=6). Large day-to-day variations were observed in fluorescence signal for both creams during the treatment period with maximum fluorescence signal observed on day 7 for cream A and day 6 for cream B. For both creams, an average drug fluorescence was not observable below 60 µm (Figure 6b). During the post-treatment period, fluorescence from drug was detected above the detection threshold 48 hours post-dose (day 9) for cream A and 24 hours post-dose (day 8) for cream B.

Punch biopsies (4 mm) were taken from the cream B-treated region from all participants on day 8. Participants were randomized to have two more biopsies/participant collected between day 9 and day 14. Biopsies from cream A were not taken as confidence in the ability to observe its behavior with FLIM was high based on the drug’s optical characteristics, allowing the trial to be reduced from 12 subjects to 6 thus reducing participant burden. Biopsies were analyzed using liquid chromatography - mass spectrometry / mass spectrometry (LC-MS/MS) to characterize pharmacokinetic properties of the drug. Bioanalytical results indicated that there was a mean ± SEM of 172.5 +/- 46.132 ng drug/g tissue present in the skin 24 hours after last application (day 8, n=6), and 20.9 +/- 20.937 ng/g
present 48 hours after last application (day 9, n=2), with lower limits of quantification (LLQ) being approximately 25 ng per gram of tissue (Figure 6c). LC-MS/MS results of cream B showed a similar trend to the results obtained using FLIM during this time period.

DISCUSSION

In this study, two formulations (A and B) of topical cream GSK2894512 were investigated to detect their penetration and residency in healthy participants. GSK2894512 has inherent fluorescent properties and a long fluorescence lifetime, which enabled its detection from endogenous fluorescence signal using FLIM *in situ*. The non-invasive nature of the method significantly increased the amount of information that could be generated per participant, while simultaneously reducing participant burden. Penetration and residency of both formulations of GSK2894512 were imaged in all six participants daily (in duplicate) through vehicle control, treatment, and post-application phases of the study, whereas biopsies were only collected from participants during the post-application phase of the study from one formulation. To achieve a similar evaluation of these formulations penetration and residency using only traditional methods would have required biopsies for each day from six participants for both tested formulations in all phases of the study. This would have required 180 physical biopsies to be collected. In this study, a maximum of three biopsies were allowed per participant, which would have required at least 60 participants to perform a similar study using biopsies. These calculations are based on a single data point per participant. Twice as many participants and biopsies would be have been needed to achieve comparable information as was acquired through FLIM as it was taken in duplicate. Utilizing FLIM, a significant reduction in clinical trial size is achievable, reducing overall costs of the trial, burden on participants, and likelihood of complications from biopsy.
There was significant variation in day-to-day observation of drug fluorescence from both formulations throughout the treatment phase of this study as shown by FLIM. This is an interesting finding as drug was applied on a daily basis by clinicians to ensure compliance with drug delivery. Additionally, participants were required to refrain from activities that would remove the drug, such as showering, strenuous activity for the first four hours post-dose, swimming, and hot tubs throughout the duration of the study. From a clinical perspective, this type of variation could have important implications on establishing a dosing schedule, because significant variation in drug presence on skin reduces the efficacy of the formulation, regardless of the drug. Because both formulations indicated this variation, it is likely that there are factors, outside controls within the protocol, influencing retention of the drug. For topical treatments, it is important to understand and limit, or compensate for these variations to ensure best patient outcomes.

The post-application phase of this study was followed by both FLIM and bioanalysis of biopsies from cream B. Bioanalytical results showed detectable drug on day 8, significantly less drug on day 9, and below limit of detection of drug on days 10 to 14. Although quantitative concentrations of drug were not measured with FLIM, bioanalytical results compare well with fluorescence signal monitored by FLIM. Due to the small number of participants and limitations in biopsy collection, results from FLIM cannot be completely validated by bioanalytical analysis from this study alone. Significant drug presence was still observable with FLIM 24-hours post last dose (day 8), and only minor quantities present at 48-hours after last dose (day 9). Since spatial information was also observed with FLIM, it was also possible to observe the spatial distribution of GSK2894512. The minor quantities of drug present were largely found in the furrows of the skin and not in its deeper layers. This type of spatial information would not have been visible using traditional bioanalytical methods.
It is worth noting that absence of fluorescent signal may not necessarily mean lack of significant picomolar quantities of drug presence, which may still be exerting effect in dermis. Due to the scattering of emitted fluorescent light in dense tissues such as skin, there can be limitations on the sensitivity of non-invasive optical imaging techniques such as FLIM to detect small quantities of drug in deeper regions. Although the lack of observation of drug in the skin by LC/MS/MS gives confidence that these smaller drug quantities are probably not present. Moreover, metabolism or ligand engagement of the drug could alter the fluorescent properties of the drug such that they are not detectable with this method. Another aspect to consider is the likelihood of photobleaching that may occur during the imaging duration, which should be accounted for when quantifying the drug fluorescence signal. With these limitations in mind, we can suggest that the majority of the FLIM signal for both formulations was dispersed in the SC, furrows of the skin, and in hair follicles. This important finding helps contribute to both our understanding of the importance of the SC/SG barrier, as well as to establish where within the skin the drug is dispersing. There were only limited differences in dispersion of the drug between the two formulations in this study.

In conclusion, this exploratory study demonstrated the capability of FLIM as a novel, non-invasive imaging method to follow in vivo the distribution and residency of topical medication, GSK2894512, in healthy skin. It was possible to localize fluorescence signal of the drug emerging from different layers of skin in a label-free manner, which enabled observation of 3D bio-distribution and depth of penetration of two topical formulations in situ. Results obtained show several advantages offered by FLIM over the current practices in topical drug development. In addition to allowing longitudinal monitoring of the same treatment region without any patient discomfort or sampling limitations, use of imaging endpoints can decrease the workload involved in tissue analysis, provide information of the safety and optimal dosing of test formulations, and thus improve the efficiency of the drug
development process. More importantly, imaging tools such as FLIM might provide biomarkers of a disease process and much earlier surrogate markers of treatment success. Besides cellular morphological details, FLIM also provides information related to biochemical compositions of the target site, which was not utilized in this study. The variations in fluorescence lifetime can be related to metabolic changes happening in drug-treated regions. Hence, besides quantifying drug fluorescence distribution and penetration, FLIM can also be utilized to monitor pharmacodynamic changes in different skin layers and cells following topical treatment. We have shown that FLIM is a promising imaging tool that can be used for studying pharmacokinetic properties of a topical cream in vivo, accelerating drug development processes, reducing costs, and developing more effective and appropriate methods to perform post-treatment monitoring.

CONFLICT OF INTEREST

S.A.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. and Diagnostic Photonics Inc. Z.A., A.A., S.F., H.A., and L.V.J. are all employees and shareholders of GlaxoSmithKline.

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REFERENCES


Figure 1: Optical imaging of human skin in vivo. a) TPEF images of different skin layers, b) FLIM images of different skin layers, c) Histogram of mean lifetime distribution at different skin layers, d) 3D rendering of skin from FLIM images, and e) Coupling tomograph head to skin for in vivo imaging (SC – stratum corneum, SG – stratum granulosum, SS – stratum spinosum, SB – stratum basale, DEJ – dermal-epidermal junction and UD – upper dermis).
Figure 2: Flow chart of FLIM data analysis. Raw FLIM data were analyzed using SPCImage (Becker and Hickl, Germany) software. Output parameters such $t_m$, $t_2$, $t_3$, $a_3(\text{abs})$, $a_3(\%)$, and pixel intensities are imported to MATLAB for further analysis. Each FLIM image comprised of 127 x 127 pixels with 40 frames per volumetric dataset. In MATLAB, images are analyzed pixel by pixel as shown in the flowchart to determine the spatial distribution and contribution of drug to the measured fluorescence signal.
Figure 3: Drug penetration into skin through a hair follicle. a) FLIM images acquired from a region treated with cream B 24-hours post-dose. These FLIM images are obtained from different skin layers, whose corresponding depths below the skin surface are denoted on the images. The blue regions are pixels with fluorescence lifetime > 3500 ps, which corresponds to the fluorescence signal from drug. The extent of drug penetration and penetration pathways are visible in these FLIM images. b) A 3D rendering of the volumetric FLIM dataset obtained from same region, depicting the drug penetration through the hair follicle (red arrow). c) Estimation of the drug penetration depth by quantifying the drug fluorescence at different depths. In this representative FLIM dataset, penetration depth was determined to be 60 µm (denoted by red dashed line).
Figure 4: Longitudinal FLIM images showing distribution of cream A in different skin layers such as (a-f) SC, (g-l) SG and (m-r) DEJ during different days of the study. All the blue regions indicate fluorescence signal from drug. Each row represents images taken from different depths (0 µm, 25 µm and 50 µm). SC – stratum corneum, SG – stratum granulosum and DEJ – dermal-epidermal junction.
Figure 5: Determining depth of penetration and skin residency of the topical formulations using FLIM. Representative plots showing the fluorescence signal quantified from different depth sections of a healthy participant on different days of the study for a) cream A and b) cream B. Day 1 C and day 2 V represent measurements from control images obtained before treatment on day 1 and from vehicle images on day 2, respectively. Days 1 - 7 are treatment days and FLIM images were obtained until day 14.
Figure 6: Combined readout of (a) skin residency, and (b) depth of penetration determined by analysis of FLIM images for creams A and B (n=6). c) Pharmacokinetic concentration-time plot for cream B obtained from biopsy samples using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). The lower limit of quantification (LLQ) of the assay was 25 ng/g. Day 1 C and day 2 V represent measurements from control images obtained before treatment on day 1 and from vehicle images on day 2, respectively. Days 1-7 are treatment days and FLIM images were obtained until day 14. Biopsies for LC-MS/MS measurements were taken during the post-treatment period (day 8 – day 14). The results in the sub-plots are presented as mean +/- SEM.