Visualization of Cutaneous Distribution of Minocycline of a Topical Gel in Human Facial Skin with Two-Photon Excited Fluorescence Lifetime Imaging Microscopy (FLIM) and Phasor Analysis

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Introduction

To limit systemic exposure to antibiotic, and to achieve localized delivery into the skin, a topical minocycline (BPX-01) is being developed clinically to address acne vulgaris by targeting the lesions directly. Knowing the efficiency of topical delivery could translate to a better understanding of clinically effective dose. We have previously demonstrated transepidermal and transfollicular penetration of minocycline with conventional fluorescence microscopy. However, because of poor signal-to-noise (SNR) due to high endogenous tissue fluorescence, this technique required 'infinite' dosing. Recently, we have demonstrated the use of two-photon excitation fluorescence (2PEF) microscopy in identifying minocycline with the equivalent of about 2.5 daily doses.

In the current study, we introduced a novel method of visualization and quantification of minocycline within human skin tissue by utilizing a phasor approach to fluorescence lifetime microscopy (FLIM) to further enhance the SNR. Fresh frozen human facial skin specimens undergoing only a single freeze-thaw cycle were used in the study. Skin specimens were dosed with 2.5 mg/cm^2, equivalent to a single daily dose, at 1% and 2% BPX-01 for 24 hours, and then cut to 30-μm sections. Femtosecond laser pulses from an optical parametric oscillator (OPO) tuned to 780 nm were used to generate two-photon excited fluorescence signals from the tissue sections. Signals were acquired with photomultiplier tubes (PMT) to reconstruct FLIM images. In phasor analysis of FLIM, the fluorescence decay trace from each pixel in the FLIM image is plotted as a single point in the phasor plot. Since every fluorophore has a specific decay trace, we can identify a specific molecule by its position in the phasor plot.

The unique signature of minocycline in FLIM phasor analysis was successfully differentiated from the endogenous fluorescence of human tissue. Based on our preliminary analysis, we believe that the visualization and quantification method using a phasor approach to FLIM can play an important role in future pharmacokinetics (PK) and pharmacodynamics (PD) analyses.

Methods

Ex vivo human facial skin specimens were obtained and stored at -80 °C frozen condition. Prior to experimentation, the specimens were let to thaw. Each donor specimen was divided into blank (negative) control, vehicle control, 1% BPX-01 and 2% BPX-01 treatment arms. For the vehicle, 1% and 2% BPX-01 arms, the specimens were treated with 2.5 mg/cm^2 of the topical gel. All four arms were maintained at 32 °C for the dosing period of 24 hours. Post treatment the tissues were embedded in OCT, cut to 30-μm thick sections, and mounted onto microscope slides.

The samples were then placed onto a FLIM setup as shown in Figure 1. Femtosecond laser pulses at 780 nm were delivered to each sample for two-photon excitation, and time-resolved fluorescence signals were acquired by a photomultiplier tube (PMT) along with the excitation source reference signal.

The fluorescence signals were time-correlated to the FLIM reference, and in Phasor analysis the time-domain fluorescence signals were converted to frequency-domain using Fourier transform. The resulting amplitude and phase information from this operation allowed the dataset to be rearranged in a scatter plot (Phasor plot) in polar coordinate as shown in Figure 2.[1,2]

Such approach accentuated the signals originating from an exogenous fluorophore such as minocycline because of its distinct fluorescence lifetime from that of endogenous tissue fluorophores, which was generally absent in the blank and vehicle groups.

False colors were then assigned to differentiate minocycline (Red color in right column of Figure 3) from endogenous fluorescence and re-mapped to produce the FLIM images.

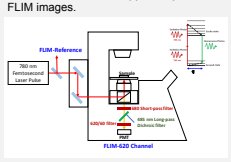

Figure 1. Experimental Setup for Fluorescence Lifetime Imaging Microscopy (FLIM). 780-nm femtosecond laser pulses were delivered to the sample; two-photon excitation fluorescence was filtered with a 680-nm short-pass and a 620-nm bandpass filters, with the time-resolved signal captured by a photomultiplier tube and time-correlated with the FLIM reference. From the two signals the fluorescence lifetime, τ_1 (inset) specific for minocycline or endogenous fluorophores may be determined.

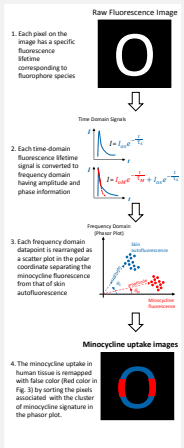

Figure 2. FLIM analysis with Phasor plot and image overlay

Results: Pharmacokinetic Tomography of a Single Daily Dose of BPX-01

The FLIM experimental setup enabled co-registration of a series of images acquired in the bright field and 2PEF, as shown in Figure 3. Previously, our team have shown that the 2PEF approach alone was adequate in demonstrating minocycline uptake and distribution in an *ex vivo* human facial skin model with approximately 2.5x daily dose or higher of BPX-01 at 4 hrs and 24 hrs.[3]

In the current study, only a single daily dose of BPX-01 was applied. As a result, we found it difficult to determine among the 2PEF images if minocycline fluorescence was present when compared to the blanks (negative controls) and the vehicle controls, as endogenous tissue fluorescence appeared to be overwhelming any fluorescence from the drug substance.

However, with the Phasor approach to time-correlated single photon counting fluorescence lifetime microscopy (FLIM), we revealed the presence of minocycline distribution within all anatomical structures of interest in the skin – the epidermis, hair follicle and sebaceous gland – in both the 1% and 2% BPX-01 treatment groups. We observed a general trend among the FLIM images in which minocycline distribution was most concentrated in the epidermis, followed by the hair follicle, and with noticeable trace amount in the sebaceous gland, in a single daily dose treatment after 24 hrs of incubation time. These observations were supported by the amount of identifiable data points associated with minocycline fluorescence among the corresponding Phasor plots from different areas of the skin. The Phasor approach also enabled quantification of local concentrations of minocycline as shown in the far right column in Figure 3.

In conclusion,

With FLIM analysis, tissue samples treated with a single daily dose of both 1% and 2% BPX-01 exhibited substantial uptake in the epidermis and the hair follicle. Trace amount of minocycline was also found in the sebaceous gland where it would have otherwise been difficult to identify with 2PEF microscopy.

Quantitative estimation of minocycline local concentrations appeared viable with FLIM-Phasor analysis. This is the first such study designed to demonstrate the potential of FLIM-Phasor analysis in understanding the absorption and distribution of a drug substance, minocycline, as part of a translational research program.

References

1. M. A. Digman, V. R. Caiolfa, M. Zamai, E. Gratton, "The Phasor Approach to Fluorescence Lifetime Imaging Analysis," BioPhys J: Biophys Lett., L14-16, 2007
2. Lakner, P.H., Monaghan, M.G., Moller, Y., Olayioye, M.A. & Schenke-Layland, K. "Applying phasor approach analysis of multiphoton FLIM measurements to probe the metabolic activity of three-dimensional in vitro cell culture models", Sci. Rep. 7, 42730 (2017).
3. M. Hermsmeier, T. Sawant, D. Lac, A. Yamamoto, X. Chen, U. Nagavarapu, C. L. Evans; K. F. Chan, "Visualizing and Quantifying Drug Distribution in Tissue," Proc. SPIE Vol:10046, 100460A (2017); doi: 10.1117/12.2256621

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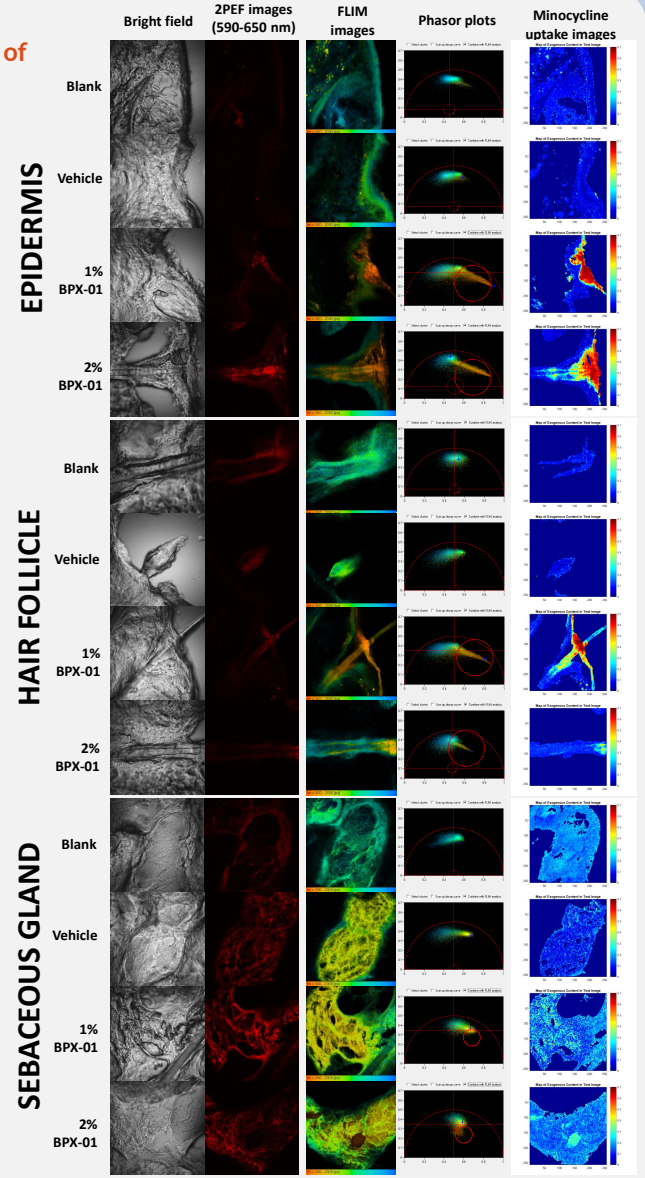

Figure 3. FLIM analysis starting (from left to right column) bright field images, two-photon excitation fluorescence (2PEF) images, FLIM images, Phasor plots corresponding to FLIM images, and quantitative analysis of local minocycline concentration, in the epidermis, hair follicle and sebaceous gland by phasor analysis.