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Invited review article

Multiphoton microscopy in dermatological imaging

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ABSTRACT

A minimally invasive imaging modality that provides both cellular and extracellular structural information with subcellular resolution is helpful for clinical diagnosis as well as basic laboratory research in dermatology. Multiphoton microscopy (MPM), using femtosecond laser as the light source, is efficient in non-linear excitation of endogenous fluorophores and induction of second harmonic generation signals from non-centrosymmetric biomolecules such as collagen. This imaging modality is minimally invasive in the sense that much of the traditional histological procedures can be bypassed en route to obtain morphological and structural information of high scattering skin tissues. This unique feature has allowed clinical dermatological diagnosis, both *ex vivo* and *in vivo*. In addition to discussing the basic principles of multiphoton microscopy, this review is aimed at emphasizing its specific applications to dermatological imaging, including characterizing stratum corneum structures, visualizing and quantifying transcutaneous drug delivery, detecting skin cancers, exploring collagen structural transitions, and monitoring laser–skin interactions.

Contents

1. Introduction

Traditionally, both basic researchers and clinicians of dermatology rely heavily on histology for characterization and quantification of biological or pathological features in skin. However, the invasive nature of biopsy does not allow dynamic analysis of the subtle cutaneous changes in the targeted area. In addition to the biopsy procedure, further processing needed for histological examination including fixation, section and staining also makes it impossible to promptly monitor the physiological or pathological alterations. Development of a non-invasive or minimally invasive imaging modality that allows real-time observation of skin at high resolution is highly valuable both in laboratory research and clinical dermatological practice.

Due to the advent in laser technology and image processing technology, multiphoton microscopy (MPM) has become a popular minimally invasive biomedical imaging modality [1–5].

Abbreviations: MPM, multiphoton microscopy.

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In conventional fluorescence microscopy or confocal fluorescence microscroscopy, fluorophores are excited by absorption of one photon of relative high energy in the visible or ultraviolet spectrum. In MPM, excitation of the fluorophores relies on the simultaneous absorption of two or more photons of lower energy in longer wavelengths, usually in the near-infrared spectrum. To achieve efficient non-linear excitation of fluorophores, extremely short duration laser sources with high peak power and pulse durations in the range of tens to hundreds of femtoseconds are used. Microscopic imaging with the non-linear optical phenomena induced by the multiphoton transition offers an important advantage in that only a very tiny volume of fluorophores in the focal point is excited. This feature gives MPM an optical sectioning ability with subcellular resolution without the need for a confocal pinhole and therefore results in higher light collection efficiency from highly scattering tissues. The longer wavelength of the infrared light source is also advantageous in enhancing achievable tissue imaging depths and reduced phototoxicity.

In addition to multiphoton fluorescence excitation, the ultrashort femtosecond laser is also efficient in producing the nonlinear polarization effect of second harmonic generation (SHG) from its interaction with non-centrosymmetric biological structures, such as collagen and myosin [6,7]. Since SHG does not involve absorption of the incident light and no energy is lost in the process. The interaction of incident light with non-centrosymmetric structures generates photons of exactly half the wavelength of the incident photons. Because the wavelength of photons from SHG is shorter than that of fluorescence from multiphoton excitation, SHG signals can be separated from fluorescence and provide contrast during tissue imaging. The structural sensitivity of SHG can also be applied to the detection of structural transitions of collagen and myosin in normal and pathological conditions with higher sensitivity than that achievable in conventional histology [6,8–10].

In addition to multiphoton imaging, reflectance confocal microscopy (RCM) has also gained popularity as a clinical, noninvasive imaging tool in dermatology by providing cellular morphological information [11,12]. Compared with MPM, the ability of RCM to resolve the dermal extracellular matrix is limited. Since the endogenous fluorophores including keratin, NAD(P)H, elastic fibers, etc., can be efficiently excited by MPM [5,7], the combination of autofluorescence and SHG in MPM imaging can provide morphological, structural and even chemical information of both cells and extracellular matrix of skin without the need of extrinsic labeling [13,14]. With fluorescence labeling, the vascular networks can also be clearly visualized [15].

The availability of commercial multiphoton diagnostic instrumentation for human skin imaging *in vivo* demonstrates the applicability of multiphoton imaging in the clinics. In the following sections, we will discuss the different dermatological topics that this unique imaging modality can address.

2. Transcutaneous drug delivery and stratum corneum structural alterations induced by penetration enhancers

The unique structure of stratum corneum provides most of the barrier function by insulating the body from the external environment and also hinders transcutaneous delivery of drugs. The stratum corneum barrier is made up of protein-rich corneocytes embedded in intercellular lipid, a brick-and-mortar model [16,17]. Both the regular packing of intercellular lipid lamellae and the intracellular keratin networks are believed to be responsible for the barrier function as various physical and chemical penetration enhancers have been developed to alter this barrier [18,19]. Conventionally, the structure of intercellular lipid in stratum corneum can only be analyzed by transmission electron microscopy or X-ray diffraction [20–22]. In addition, the effect of penetration enhancers on the transcutaneous delivery of drugs is investigated by diffusion chamber or determination of tissue drug levels *in vitro* or by determining the biological effect and/or blood level of the drug under transcutaneous delivery *in vivo* [23–25]. However, the detailed dynamics of drug penetration across skin and the associated structural alterations of stratum corneum cannot be effectively evaluated using these approaches.

Due to the availability of keratin autofluorescence, the corenocytes can be imaged by MPM without extrinsic labels (Fig. 1a, normal corneocytes). Recently, we demonstrated the disruption of the intracellular keratin networks and intercellular cohesion of corneocytes by the penetration enhancer of a depilatory cream by MPM visualization (Fig. 1b, c and g) [26]. We found that the pretreatment of depilatory creams can detach corneocytes from stratum corneum and also generates intracellular pores in the remaining corenocytes by disrupting the intracellular keratin, providing shunts for drug penetration.

In addition to intracellular keratin, the introduction of model fluorescent drugs that can be spectrally separated from keratin autofluorescence enables the visualization of the dynamics of drug penetration in stratum corneum (Fig. 1d, e and f) [26–28]. Without the need for removing stratum corneum for the determination of drug concentrations, MPM can directly quantify the spatial drug concentration distribution, calculate the vehicle to skin partition coefficient of drugs and visualize the preferential drug penetration routes. Hence, the selective penetration through either transcellular route or intercellular route under penetration enhancer can be visualized and quantified (Fig. 1e and f) [26,28].

MPM is also powerful in visualizing the penetration and diffusion of nanoparticles in skin [29,30]. Through the SHG generated from collagen matrix as a contrast, the diffusion dynamics of fluorescent nanoparticles of quantum dots in dermis can be analyzed by multiphoton imaging [30]. In addition, the effect of penetration enhancers on the microtransport of nanoparticles can also be directly investigated by analyzing the SHG signals from zinc oxide nanoparticles [31].

In addition to the morphological changes of corneocytes, MPM can also analyze the changes in intercellular lipids of stratum corneum. By the use of Nile red staining, the distribution of intercellular lipid can be visualized (Fig. 1h and i). This technique characterizes the disruption of intercellular lipid by the penetration enhancer and the result is well correlated with the result in transmission electron microscopy [26]. In addition to characterizing lipid distribution at the cellular level, MPM is also powerful in characterizing the packing, preferential orientation and structural alterations of lipid at the molecular level [32]. The preferential alignment of intercellular lipid lamellae can be determined by varying the excitation and emission polarizations of Laurdan labeled stratum corneum [33]. For example, with the treatment of penetration enhancer of oleic acid, the regular packing of intercellular lipid molecules is disrupted and becomes increasingly randomized [34].

Conventionally, the investigation of the transdermal process is focused on the stratum corneum. The dynamics of the permeation of drugs in the viable keratinocytes and dermis have been rarely investigated. Due to the ability of MPM in delineating cells, extracellular matrix and blood vessels, the detailed process of drug absorption across the viable epidermis to the dermis and even to the blood vessels can be investigated in the future. We expect that this technique can be helpful in characterizing the effects of various transdermal penetration enhancers and shedding more light on the dynamic penetration of drugs across skin under the effect of various penetration-enhancing strategies. In addition to *in vitro* study, this technique can also be applied to the study of human transcutaneous drug delivery *in vivo* [3].