Research Techniques Made Simple: Two-Photon Intravital Imaging of the Skin

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Over the last few years, intravital two-photon microscopy has matured into a powerful technology helping basic and clinical researchers obtain quantifiable details of complex biological mechanisms in live and intact tissues. Two-photon microscopy provides high spatial and temporal resolution in vivo with little phototoxicity that is unattainable by other optical tools like confocal microscopy. Using ultrashort laser pulses, two-photon microscopy allows the visualization of molecules, cells, and extracellular structures up to depths of 1 mm within tissues. Consequently, real-time imaging of the individual skin layers under both physiological and pathological conditions has revolutionized our understanding of cutaneous homeostasis, immunity, and tumor biology. This review provides an overview to two-photon microscopy of the skin by covering the basic concepts and current applications in diverse preclinical and clinical settings.

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Description: This article, designed for dermatologists, residents, fellows, and related healthcare providers, seeks to reduce the growing divide between dermatology clinical practice and the basic science/current research methodologies on which many diagnostic and therapeutic advances are built.

Objectives: At the conclusion of this activity, learners should be better able to:

- Recognize the newest techniques in biomedical research.
- Describe how these techniques can be utilized and their limitations.
- Describe the potential impact of these techniques.

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INTRODUCTION

The theory underlying two-photon excitation was described initially by Maria Göppert-Mayer in 1931, and the first two-photon microscope was pioneered and patented by Winfried Denk and colleagues almost six decades later in 1990 (Weigert et al., 2010). Two-photon microscopy (TPM) enables examination of the deeper layers of live specimens, including the skin, and has many advantages over conventional microscopic imaging methods. Using long-wavelength, ultrashort-pulse laser sources, the excitation volume in TPM is confined to the focal plane, thus excluding out-of-focus background excitation, which is observed, for

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Abbreviations: SHG, second harmonic generation; THG, third harmonic generation; TPM, two-photon microscopy

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BENEFITS

- Depth of light penetration
- Reduced photobleaching and phototoxicity outside the focal plane
- Optical sectioning and label-free visualization of autofluorescent molecules and structures

LIMITATIONS

- Potential phototoxicity in the focal plane after long-term imaging
- Distorted z-resolution, in particular at higher depths levels
- Potential thermal damage due to high-laser power pulses
- High costs of instrument compared with confocal and conventional microscopy
- High level of expertise required

example, in confocal microscopy. This minimizes photobleaching and phototoxicity. Another useful feature of two-photon excitation relevant to biologic imaging is the capacity of TPM to take advantage of higher-order interactions between light and tissue components, for example, second (SHG) and third (THG) harmonic generation signals, which can provide architectural information of the investigated tissue (Yew et al., 2014).

Based on its optical features, intravital TPM offers an experimental and diagnostic method that can be used to uncover the homeostatic principles of normal skin and events resulting in skin diseases (Perry et al., 2012). The skin is a complex multilayer organ, which imparts optical challenges for imaging. For example, each layer exhibits different optical properties such as the refractive index (i.e., 1.51 in stratum corneum, 1.34 in epidermis, and 1.41 in the dermis). Other potential limitations include the high cost, reduced z-resolution (in particular at depths > 500 μm), and potential thermal tissue damage due to absorption of high-power laser light (Lo et al., 2005; Olivieri et al., 2013). TPM can be expanded by combining it with other optical methods like Forster resonance energy transfer (i.e., FRET) and fluorescence recovery after photobleaching (i.e., FRAP) (Broussard and Green, 2017; Erami et al., 2016).

THE BASIC PRINCIPLES OF TPM

TPM relies on nonlinear photoexcitation of molecules, whereby two low-energy photons are almost simultaneously (within 10^{-18} to 10^{-16} seconds) absorbed in the same focal point, resulting in fluorescence emission. Tunable short (femtosecond)-pulsed lasers facilitate such rare collisions. This principle also eliminates the need for a pinhole, which is used in confocal microscopy, because the excitation outside the focal plane is too weak to cause appreciable fluorescence. In addition, short-pulse lasers can keep the average power at the sample low and thereby reduce tissue damage, enabling long-term imaging (Weigert et al., 2010). Compared with confocal microscopy, which in the skin is limited to a depth of approximately 50–60 μm, light penetration in TPM goes beyond the epidermis and superficial dermis to about 300–600 μm, depending on site, excitation wavelength, and fluorophores, allowing visualization of endogenous and exogenous fluorophores and structures like collagen or elastin (Table 1) (Nwaneshiudu et al., 2012; Yew et al., 2014).

SHG signals add a unique advantage to TPM by allowing label-free visualization of non-centrosymmetric structural components, such as extracellular matrix proteins (Rehberg et al., 2011). The signal in TPM is generated when excited photons decay to their ground state and emit a photon with a frequency less than double of its original. SHG signal is generated when scattered incident photons recombine into a single photon without energy loss (Figure 1) (Olivieri et al., 2013). SHG is a useful feature of TPM when, for example, studying tissue architecture, for instance, the delineation of boundaries between normal and malignant tissue. Apart from SHG, other less common higher-order processes, THG and

Table 1. Skin layers and a selected list of endogenous components and their spectral positions in TPM

Skin Main Layer	Skin Sublayer	Cells in Each Layer	Endogenously Detectable Component	Spectral Positions (Excitation/Emission in nm)
Epidermis	Stratum corneum	5–6 layers of cornified dead cells	Keratin	(760–860/477–503)
	Stratum lucidum	Dendritic epidermal T cells Keratinocytes Dendritic cells Langerhans cells Melanocytes Merkel cells	NADPH (in living keratinocytes)	Free 460 (730–780/460–480)
	Stratum granulosum			Bound to protein 440 (730–780/460–480)
	Stratum spinosum		Melanin (eumelanin and pheomelanin)	440-420-475 (800/550)
	Basal cell layer			
Dermis		Dermal dendritic cells Dermal T cells Neutrophils Macrophages Other immune cells Stromal cells	Collagen fibers	(800–860/400–430)
			Elastin fibers	(730–760/460–480)

Abbreviation: TPM, two-photon microscopy.

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