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

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.

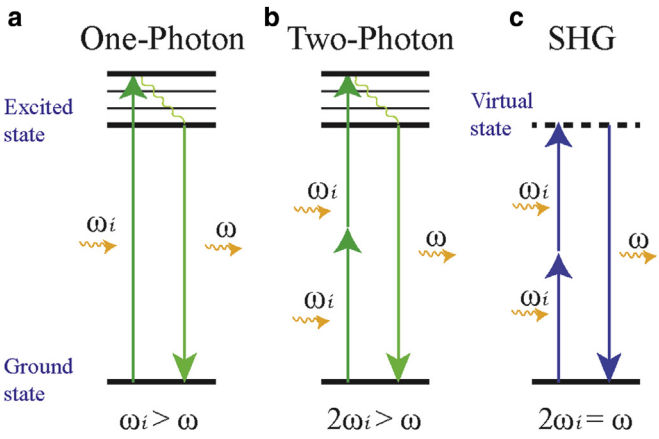

Figure 1. Perrin-Jablonski fluorescence diagram describing one-photon excited fluorescence versus two-photon and SHG signals. (**a**) In one-photon excitation, a higher energy source is required and absorbed to excite the photon from the ground state to the excited state. Visible light is emitted when this photon returns to its ground state, with the emitted photon having slightly lower radiation energy at a frequency of ω than the original light frequency ω*i*. **(b)** In two-photon excited fluorescence, this process is replicated with the simultaneous absorption of two lower-energy photons. Both processes involve real energy transition of electrons where emitted light energy is partially lost. **(c)** In SHG no energy is absorbed, and all the scattered incident photons are recombined into a single photon, without energy lost and at the same frequency as 2ω*i*. SHG, second harmonic generation.

fourth harmonic generation, also exist (Yew et al., 2014). THG is induced by changes in refraction index occurring at interfaces such as cell nuclei and cytoplasm or cytoplasm and interstitial fluid (Rehberg et al., 2011). Moreover, fourth harmonic generation signal is the sum of the frequency generation from THG and pump light (Karvonen et al., 2015). These signals can be used to obtain further information on structural tissue components.

OVERVIEW OF METHODOLOGY (SAMPLE PREPARATION AND FILTER SETUP)

In this review, we focus on the ear skin model to illustrate the experimental setup and use of TPM in intravital imaging. Mice are appropriately anesthetized, for example, by the intraperitoneal injection of ketamine/xylazine. Hair is then removed from the region of interest using depilatory cream, after which the animal is stably positioned on a custom-built, temperature-controlled mounting platform (Figure 2a). The ear is covered with a coverslip and a solution of glycerin-phosphate buffered saline. Intravenous injection of plasma markers such as Evans Blue dye, high-molecular FITC-dextran, or quantum dots can be used to delineate blood vessels (Li et al., 2012). A mode-locked titanium-sapphire laser at 920 nm wavelength can be used to excite eGFP (excitation/emission = 488/507 nm), Evans Blue (excitation/emission = 620/680 nm), and SHG (excitation/emission = 415/455 nm). Signals are detected with different photomultiplier tubes (filter setup is outlined in Figure 2b). For the imaging presented here, a ×20 water immersion objective was used. TPM imaging sessions commonly produce large-size datasets (giga- to terabyte range), the analysis of which requires powerful hardware and software instrumentation. Postacquisition processing of data can be achieved using commercial software packages such as Imaris (Bitplane, Zurich, Switzerland), Metamorph (Molecular Devices, Sunnyvale, CA), and Volocity (PerkinElmer, Waltham, MA), as well as shareware including ImageJ (National Institutes of Health, Bethesda, MD) (Figure 2c). Publicly accessible newer software like FocusStack and StimServer provide minimal memory footprint and are thus more cost effective. These packages have the capability of performing stack alignment, automated re-randomization of time-lapse data, and automated cell segmentation, with the additional option of direct incorporation into MATLAB (MathWorks, Natick, MA)-based analysis tools (Muir and Kampa, 2014). A variety of

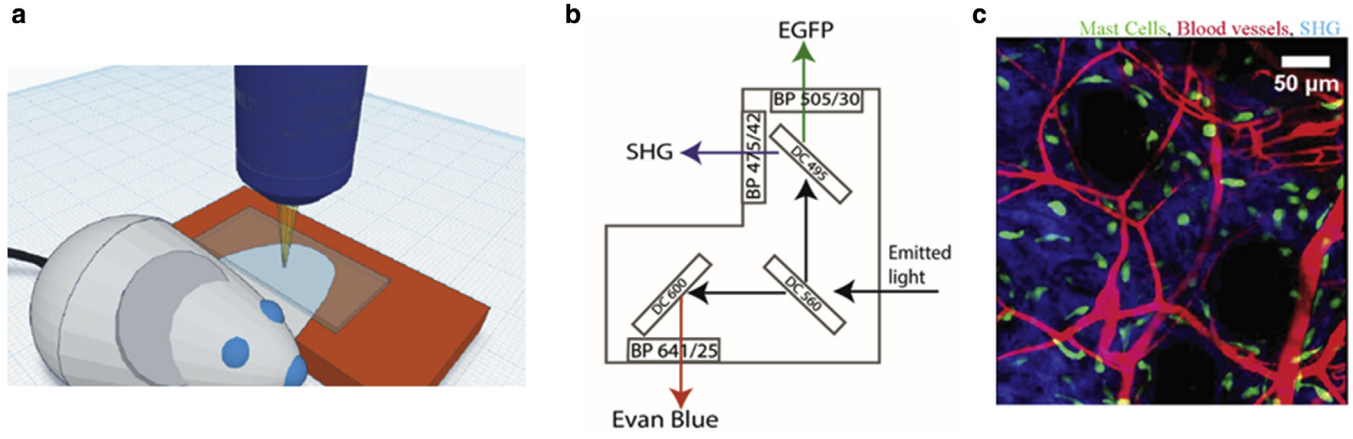

Figure 2. Visualization of mast cells in vivo using the mouse ear skin model. (**a**) Close-up schematic view of a mouse ear positioned on a temperature-controlled mounting platform designed using Thinkercad (Autodesk, San Rafael, CA). **(b)** For detection of fluorophores, ear skin was simultaneously exposed to a mode-locked titanium-sapphire laser at 920 nm wavelength for excitation of eGFP (excitation/emission = 488/507 nm), Evans Blue (excitation/emission = 620/680 nm), and SHG (excitation/emission = 415/455 nm). The fluorescence signals were detected using independent photomultiplier tubes after transmitting through or getting transmitted and reflected by dichroic mirrors. Band-pass filters were used to further restrict the wavelength detected to decrease background noise and spectral overlaps. **(c)** Skin of a c-kit−GFP transgenic mouse was imaged. Mast cells are green. The extracellular matrix in the dermis was detected by SHG signal (blue) and the blood vessels (red) (Evans Blue). The imaging was performed using a ×20 water-immersion objective. SHG, second harmonic generation.

parameters such as cell motion patterns, cellular localization, and interactions can be computed to describe the orchestration of immune responses in cutaneous biology (Germain et al., 2012; Li et al., 2012).

EXPERIMENTAL DERMATOLOGY RESEARCH AND TPM

Arguably, the immune system is where multiphoton microscopy has had the greatest impact in basic dermatological studies in both the steady state and during inflammation, where it is possible to study single cell behavior in real time and molecules within an intact living environment (Weninger et al., 2014). Currently, the most common sites for intravital multiphoton imaging of the skin in mice include the ear (Roediger et al., 2008), hind footpad (Graham et al., 2009), and dorsal skin (Amornphimoltham et al., 2011). The footpad is hairless, but hair removal is essential in the ear and back skin because the autofluorescence of hair shafts obscure image acquisition (Li et al., 2012; Roediger et al., 2008). In addition, ear and footpad skin are less affected by respiration, and it easier to produce a stable image. However, it is important that the mouse reaches a stable temperature before image recording, otherwise drift in the x-, y-, or z-axis can occur, resulting in an unstable time-lapse video (Li et al., 2012). Long-term time-lapse imaging can be achieved by exploiting various in vivo cell and tissue labeling techniques and fluorescent reporter mice.

The application of multiphoton microscopy can, therefore, permit quantitative measurements of spatial distribution, motility, interactions, and response dynamics of leukocytes under homeostatic and inflammatory conditions, as well as host-tumor responses, which otherwise would not be possible. For example, using CXCR6-GFP transgenic reporter mice, differences in $EGFP^{+}$ γδ T-cell morphology and their density in dermis compared with epidermis was investigated in ear skin (Sumaria et al., 2011). Using CD11c-EYFP mice, dendritic cell function and migratory behavior were evaluated in sterile skin injury in which the chemoattractants to the site of injury were proposed to rise from the resident or recruited inflammatory cells (Goh et al., 2015). TPM microscopy can also be enhanced by combining it with other optical techniques. Using a Cre-inducible E-cadherin—GFP transgenic mouse model, Erami et al. (2016) combined TPM and fluorescence recovery after photobleaching to assess alteration in cadherin-based cell-cell junction integrity in the setting of tumor progression. Although this is not a comprehensive review of all the advances made through multiphoton imaging of the skin, these examples serve to show that this technology has significantly advanced our understanding of the spatiotemporal interactions of immune cell subsets in lymphoid organs and peripheral tissues, including the skin (Germain et al., 2012; Jain and Weninger, 2013). Moreover, although animal models can be used to elucidate the basic cellular and molecular mechanisms to obtain real-time, quantifiable details of complex biological mechanisms in intact tissues, the ultimate goal is to translate our understanding into clinical applications.

CLINICAL DERMATOLOGY AND TPM

Multiphoton microscopy is now being considered as a potential noninvasive diagnostic tool in dermatology, because the skin is very accessible for imaging. Although currently it is only possible to derive structural information from autofluorescent signals within human tissue in vivo, TPM is able to provide near-histological grade images without the need for a skin biopsy or tissue processing. The use of autofluorescence as a source of natural contrast has been shown in reflectance confocal microscopy. However, the multiphoton microscope has multiple advantages, allowing "optical biopsies" of human skin in vivo (Luo et al., 2014). Nevertheless, there are limited human safety data for multiphoton imaging in the skin, and thus its use currently is restricted to the experimental setting. Intravital imaging studies using TPM in mouse skin have not shown signs of

MULTIPLE CHOICE QUESTIONS

1. Which of the following is true about the basic principles of two-photon microscopy (TPM)?
   A. A two-photon microscope uses a pinhole.
   B. TPM requires collision of two low-energy photons almost simultaneously.
   C. UV spectra are used in TPM.
   D. Generation of second harmonic generation (SHG) requires absorption of two low-energy photons.
2. Which of the following is not an advantage of TPM?
   A. The depth of light penetration
   B. Less photobleaching outside the confocal volume
   C. Excellent z-resolution
   D. None of the above
3. TPM has been used in which of the following applications?
   A. Aging studies
   B. Inflammatory dermatoses
   C. Skin cancer diagnosis
   D. All of the above
4. Endogenous autofluorescent signals in the skin can be generated from which of the following?
   A. Elastin and collagen
   B. NAD(P)H
   C. Melanin
   D. All of the above
5. Based on Figure 2b, using a mode-locked titanium-sapphire laser at 920 nm wavelength, which band-pass (BP) filter is most appropriate?
   A. Evans Blue with a BP filter of 505/30
   B. SHG with a BP filter of 641/25
   C. EGFP with a BP filter of 505/30
   D. None of the above

acute phototoxicity (Li et al., 2012; Roediger et al., 2008), and clinical studies indicate minimal erythema and cellular damage from two-photon excitation (Fischer et al., 2008).

Commercial multiphoton microscope systems are now available and have been used experimentally in the clinical setting to investigate the structural composition of human skin with the promise of future application in disease processes in vivo (Shirshin et al., 2017). These studies have mainly focused on the utility of this technology in skin aging studies (Koehler et al., 2009), inflammatory dermatoses (Koehler et al., 2012; Sugata et al., 2011), and role of skin cancer diagnosis (Balu et al., 2015). In addition, in combination with fluorescence lifetime imaging, TPM has been used to study the metabolic state of keratinocytes in normal and inflamed human epidermis (Huck et al., 2016). Moreover, similar to the murine laser injury model (Ng et al., 2011), it has been proposed that the same femtosecond laser used to excite and image endogenous fluorophores in human skin can be used as a dermal cutting tool. Because of the two-photon effect, the desired femtosecond laser ablation occurs at the focal point within the intact skin (Garvie-Cook et al., 2016). Although such approaches are purely experimental, it does highlight the potential significance of in vivo imaging, with possible application in the simultaneous treatment of human skin conditions.

FURTHER READING

Two-photon excitation has been extensively reviewed in many recent articles (e.g., Secklehner et al., 2017). In addition, the US National Institutes of Health Resource for Biophysical Imaging (http://www.drbio.cornell.edu/ and http://www.jenlab.de/) provides further information on fluorophores and equipment for multiphoton microscopy for the interested reader.

CONCLUSIONS

TPM is an advanced optical imaging technique that uses brief, intense, long-wavelength laser pulses with the capacity to penetrate into the deep layer of the skin. This technology has thus become popular in experimental and clinical dermatology research to investigate the mechanisms underlying skin pathologies. Commercial instruments are now available for real-time microscopy using endogenous autofluorescent components like melanin, elastin, collagen, and NAD(P)H. Among many advantages of TPM are penetration depth, minimal out-of-focus signals, minimal photobleaching, and reduced phototoxicity, as well as the ability to image structures label free. The increased availability of transgenic mice and fluorescent probes and instrumentational improvements like laser safety, together with enhanced analytical capability, make TPM an important part of the biomedical investigation.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

PO and PLT were involved in figure development and writing the manuscript. WW contributed to concept and design, writing the manuscript, and final approval.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to this paper. Teaching slides are available as supplementary material.

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