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Review

Structural and dynamical aspects of skin studied by multiphoton excitation fluorescence microscopy-based methods

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This article is dedicated to the loving memory of Prof. Robert (Bob) Clegg

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ABSTRACT

This mini-review reports on applications of particular multiphoton excitation microscopy-based methodologies employed in our laboratory to study skin. These approaches allow in-depth optical sectioning of the tissue, providing spatially resolved information on specific fluorescence probes' parameters. Specifically, by applying these methods, spatially resolved maps of water dipolar relaxation (generalized polarization function using the 6-lauroyl-2-(N,N-dimethylamino)naphthale probe), activity of protons (fluorescence lifetime imaging using a proton sensitive fluorescence probe – 2,7-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein) and diffusion coefficients of distinct fluorescence probes (raster imaging correlation spectroscopy) can be obtained from different regions of the tissue. Comparative studies of different tissue strata, but also between equivalent regions of normal and abnormal excised skin, including applications of fluctuation correlation spectroscopy on transdermal penetration of liposomes are presented and discussed. The data from the different studies reported reveal the intrinsic heterogeneity of skin and also prove these strategies to be powerful noninvasive tools to explore structural and dynamical aspects of the tissue.

Contents

Abbreviations: ACBP, acyl-coenzyme a binding protein; ATTO-647N-PE, ATTO-647N 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; ATTO-647N-STREP, ATTO 647N Streptavidin; BCECF, 2,7-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; cc, cross correlation; FCS, fluorescence correlation spectroscopy; FLIM, fluorescence lifetime imaging microscopy; GP, generalized polarization; LAURDAN, 6-lauroyl-2-(N,N-dimethylamino)naphthale; RhB, rhodamine B ([9-(2-carboxyphenyl)-6-diethylamino-3-xanthenylidene]-diethylammonium chloride); RhB-PE, lissamine-rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine; RICS, raster imaging correlation spectroscopy; SC, stratum corneum; SHG, second harmonic generation; TMR-DEX, tetramethylrhodamine dextran 3000 MW.

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1. Introduction

Since their introduction in the early 90s, multi-photon excitation laser scanning microscopes have been a breakthrough in biomedical imaging allowing to collect structural and dynamical information of a broad range of processes in living specimens (Denk et al., 1990). Compared with regular single-photon excitation confocal fluorescence microscopy, multi-photon excitation offers important advantages to image thick specimens (Helmchen and Denk, 2005; Masters et al., 1997, 1998; Williams et al., 2001; Wustner et al., 2011). The multiphoton excitation process is nonlinear with respect to the intensity of the excitation light resulting in a confinement of the excitation process to a small volume, i.e. the focal volume. In other words, in contrast to single photon excitation confocal fluorescence microscopy the sectioning effect is inherent to the excitation process (does not require a pinhole to produce a confocal image). The ability to make optical sections without a pinhole enables the collection of scattered light from thick samples. This combined with the deeper penetration of the infrared excitation light enables imaging up to 1 mm into the sample, providing superior contrast at depths >150 μm, which is the limit for the excitation wavelengths used in regular single-photon excitation confocal fluorescence microscopy. Also, the confined excitation volume of multiphoton excitation reduces photodamage and photobleaching in thick biological specimens allowing imaging of live specimens for prolonged time periods.

The availability of fluorescence microscopes coupled with ultrafast pulsed lasers, special optics, advanced detection systems and electronics has allowed development of particular methods that can measure different fluorescence parameters from fluorescent probes. This provides important spatially resolved information of the specimen's inherent structure but also associated dynamics. Examples are fluorescence correlation spectroscopy (FCS (Berland et al., 1995; Elson, 2011)) and raster imaging correlation spectroscopy (RICS (Digman et al., 2005a,b)) that provide information on diffusion coefficients of fluorescent probes; fluorescence lifetime imaging microscopy (FLIM) that informs on the fluorescence lifetime of, for example, ion sensitive probes (Behne et al., 2003, 2002; Hanson et al., 2002), and 6-lauroyl-2-(N,N-dimethylamino)naphthalene generalized polarization (LAURDAN GP) imaging, that allows a measure of the content and dynamics of water in the local milieu of the probe (Bagatolli, 2006; Parasassi et al., 1997; Yu et al., 1996).

Multiphoton excitation fluorescence microscopy has become a very effective tool to study skin tissue. Skin is an ideal target because it is easily reachable with regular microscope objectives allowing potential in-depth structural and dynamical studies under excised and *in vivo* conditions. Different studies using extrinsic fluorescent probes, but also exploiting the autofluorescence signal of the tissue, have been reported using FLIM and multiphoton excitation fluorescence intensity imaging (Behne et al., 2011; Bloksgaard et al., 2012a,b; Breunig et al., 2012; Carrer et al., 2008; Celli et al., 2010, 2009; Hanson et al., 2002; Iwai et al., 2012; Konig et al., 2011, 2010; Seidenari et al., 2012; Sun et al., 2004; Zhao et al., 2012). For example, applying the latter technique structural studies of the tissue were performed in our group using skin autofluorescence (Nielsen et al., 2011). Specifically, the effect of different storage conditions on excised skin was evaluated by linking tissue autofluorescence imaging and measurements of transdermal penetration of caffeine. This study showed that not all storage conditions are suitable to optimally preserve the intrinsic structure of the tissue, influencing the transdermal penetration of caffeine; for details see (Nielsen et al., 2011). Furthermore, dynamical studies where performed in skin using fluctuation techniques such as image correlation spectroscopy (Tanner et al., 2009), RICS (Brewer et al., 2012) and FCS (Guldbrand et al., 2010). Combined strategies using the aforementioned methodologies are becoming a powerful tool to gain new basic knowledge about the structural and dynamical features of skin, with important applications in non-invasive clinical diagnostics (Seidenari et al., 2012; Zhao et al., 2012).

The purpose of this mini-review is to summarize some multiphoton excitation-based fluorescence microscopy methodologies currently employed in our laboratory to study structural and dynamical aspects of skin tissue. Examples from different studies of skin structure, hydration level, proton activity, and diffusion maps using particular fluorescent probes are presented.[3] Specifically, information of the hydration level of the stratum corneum (SC) of normal and abnormal tissue, i.e. normal retro auricular or ear canal human skin vs. Cholesteatoma (Bloksgaard et al., 2012b); wild type vs. Acyl-CoA binding protein -ACBP- knockout mice (Bloksgaard et al., 2012a), as well as changes in the activity of protons in wild type vs. ACBP knockout mice (Bloksgaard et al., 2012a). Similarly, we present examples of acquisition of diffusion maps of distinct fluorescent probes in skin tissue, as well as of their usefulness in transdermal drug delivery experiments using liposomes (Brewer et al., 2012). To conclude, we briefly provide an example of another nonlinear phenomenon, the so called second harmonic generation (SHG), to study collagen structure in heat treated skin (Andersen et al., 2011). This technique has been applied by other groups in previous skin related studies (Konig and Riemann, 2003; Lin et al., 2005).

2. LAURDAN generalized polarization (GP) imaging

2.1. Definition of the GP function

The favorable partitioning of the amphiphilic LAURDAN probe to membranes permits studying membranous regions in skin. As an example, LAURDAN has been used to characterize the packing of stratum corneum (SC) lipid membranes as well as cellular membranes existing in the lower epidermis (Carrer et al., 2008). The first extensive characterization of LAURDAN fluorescence properties was performed in membrane model systems (glycerophospholipid vesicles) by (Parasassi et al., 1986b). These authors reported on a pronounced red shift of the emission spectra of LAURDAN during the solid ordered (s_o, or gel) to liquid disordered (l_d) phase transition occurring in membranes composed of glycerophospholipids. This is characterized by emission of blue light when the membranes are in the s_o phase that gradually changes to green when the membrane is transitioning to a liquid disordered phase (440–490 nm respectively). In order to explain the particular response of the probe, the authors proposed the existence of an environment that can orient with the LAURDAN excited state dipole (Parasassi et al., 1986b). In other words, during the membrane phase transition the dynamics occurring in the local probe's milieu correspond with the fluorescence lifetime of the probe. This environmental influence has been correlated with the extent of water dipolar relaxation and water content (polarity) surrounding the fluorophore (Bagatolli and Gratton, 1999; Parasassi et al., 1997) and a two state model was used to describe the response of the probe (Parasassi et al., 1986a,b), providing the basis for the definition of the generalized polarization (GP) function (Parasassi et al., 1990, 1991). The GP function reflects thus, the response of the probe's emission spectrum to water content and dynamics, a phenomenon that is connected to the lateral packing of membranous systems. The GP function was originally defined as:

$$\mathrm{GP} = \frac{I_B - I_R}{I_B + I_R} \tag{1}$$

[3] The reader is encouraged to revise the original references if information regarding instrumentation or material and methods for the different experiments presented in this article are desired.