Detectability of reflectance and fluorescent contrast agents for real-time *in vivo* confocal microscopy

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Abstract: Reflectance agents (liposomes, polystyrene microparticles, aluminum chloride salts, acetic acid) and fluorescent agents (polymer- and cosmetic actives-tagged fluorescein and rhodamine compounds, green fluorescent protein) enhance contrast of real-time confocal images of skin and microcirculation in vivo. Quantitative analysis of signal detectability versus contrast agent properties, and experimental images are presented. These results provide a basis for optimizing confocal microscope design and imaging parameters for real-time in vivo applications.

Introduction: Confocal microscopy is a non-invasive optical imaging modality with typical lateral resolution of 0.5-1.0 μm and axial resolution (section thickness) 2-5 μm within living tissues. This resolution allows histologic detail to be imaged and visualized in human and animal tissues in vivo. Nuclear and cellular detail, organelles, circulating blood cells, collagen, and connective tissues have been imaged to maximum depth of 200-500 μm in human skin [1-3] and human oral mucosa in vivo [4], and surgically exposed animal tissues in situ [5, 6]. However, the lack of structure-specific reflectance or fluorescent contrast limits the usefulness of confocal microscopy to morphologic investigations at the cellular- and nuclear-level. Morphologic and functional imaging at specific organelle- and ultrastructure-levels will be possible only if we develop contrast agents that may be used and detected in vivo.

We carried out a preliminary quantitative analysis and imaging experiments of the detectability of reflectance and fluorescent contrast agents for visualizing human skin and animal microcirculation in vivo. The goal was to understand signal detectability and contrast as a function of the optical properties of contrast agents and instrumentation parameters that would be necessary for real-time confocal imaging in skin in vivo.

Detectability of reflectance contrast agents: Based on Mie's optical scattering theory, we expect strong back-scatter and high contrast from organelles and microstructures that have size (d) on the order of the illumination wavelength (λ), and refractive index (n) greater than that of the surrounding epidermis (n=1.34) or dermis (n=1.38). When imaging at the infrared wavelength of 1064 nm, we detect typically 100-10000 photons/pixel (experimentally measured) from the epidermis to dermis, relative to a background of 100 photons, which then gives a signal-to-noise ratio of about 3-40 and signal-to-background of 1-100 [3].

Liposomes (n=1.41, d=0.7 μm) could thus be detected and they strongly enhance the contrast of microcirculation in the dermis of Sprague-Dawley rats. Topically applied aluminum chloride salts and polystyrene microspheres (diameter 0.1-0.5 μm) were found to enhance the contrast of sweat ducts and hair follicles in human skin; brighter and deeper imaging is possible with these agents. Topically applied 5% acetic acid causes the intra-nuclear chromatin (which normally exists as 30-

100 nm-thin diffuse filaments) to condense into convoluted 1-5 μm-thick strands. The condensed chromatin strongly back-scatters light, making the nuclei appear bright and easily detectable; this contrast-enhancing technique allowed detection of non-melanoma cancers in skin [7].

Detectability of fluorescent contrast agents: In fluorescence, detection of contrast agents in vivo and at video-rate (i. e., pixel integration time of 100 nsec) is much more challenging [8]. In general, our analysis predicts ~10^5 molecules in a high-resolution confocal probe volume of about $6x10^{-12}$ ml (measured lateral resolution 0.5 μm and section thickness 2 μm) when we use a 0.9 NA water immersion objective lens, a detection pinhole of diameter 10 resels and blue illumination at 488 nm. After accounting for losses due to scattering through tissue, collection efficiency of the objective lens and transmission through the confocal optics, 5000-10000 photons per pixel will typically be detected from the dermis, relative to a background of 500 (experimentally estimated). Thus the signal-to-noise ratio will be 50-80 and signal-to-background 10-20.

With non-toxic dosage of ~1 mg/kg, we could image polymeric particles labelled with either FITC or rhodamine B sulfonyl chloride in the microcirculation of Sprague-Dawley rats. In another pilot study, the penetration of topically-applied hydrophilic and hydrophobic compounds through human stratum corneum in vivo was investigated. The compounds were labelled with either fluorescein or octadecyl fluorescein ester. For the various compounds, we could visualize either the adsorption in the superficial corneocytes or the inter-cellular penetration to a depth of 10-20 μm in the stratum corneum. Topically applied fluorescein has also proven useful to characterize the distribution of corneocytes in healthy versus unhealthy skin. In another experiment, we have been able to image the expression of green fluorescent protein in mouse epidermis in vivo.

Summary: The analysis and experiments provide an understanding of confocal detectability of exogenous reflectance and fluorescent contrast agents within human and animal skin in vivo. Such information will provide a basis for developing optimum optical properties of contrast agents and optimum confocal microscope design and imaging parameters for real-time in vivo applications.

References

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