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# Revisiting Optical Clearing with Dimethyl Sulfoxide (DMSO): *In Vitro* and *In Vivo* Studies

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## ABSTRACT

Functional optical characterization of disease progression and response to therapy suffers from loss of spatial resolution and imaging depth due to scattering, impacting the ability of researchers to localize and quantify molecular processes. Here we report on the ability of dimethyl sulfoxide (DMSO) to reduce temporarily the optical scattering of skin. Data collected from *in vitro* phantom images and *in vivo* fluorescence images demonstrate the potential of this simple method to mitigate the blurring effects of scattering with topical application, which we expect will improve the accuracy and localization of *in vivo* molecular imaging studies.

## INTRODUCTION

Light-based therapeutic and diagnostic techniques currently employed in the biomedical field suffer from a loss of spatial resolution and imaging depth due to the scattering and absorption events intrinsic to biological tissues. At the visible and near-infrared wavelengths critical to many optical imaging modalities, scattering dominates over absorption and is thus the primary contributor to the reduction of light penetration into biological tissues. If the optical scattering events of biological tissues at these wavelengths of light could be reduced or minimized, it is reasonable to expect that the potential applications of imaging modalities would expand in scope. In fact, many studies have already demonstrated that an overall reduction in the number of scattering events within a biological tissue can lead to increased penetration depth and contrast in a number of optical imaging techniques [1–8].

Optical clearing is a method for inducing a transient reduction in the scattering properties of a tissue using an optical clearing agent (OCA) [1]. Although it has been concluded that a myriad of chemical agents possess appreciable

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optical clearing potential (OCP), many of these agents possess hydrophilic properties which prevent them from penetrating the lipid-rich stratum corneum. Without bypassing this hydrophobic region of the tissue, most known OCA are incapable of inducing optical clearing unless the stratum corneum is bypassed. An OCA which cannot be applied topically is severely limited in its potential applications to most imaging modalities. Thus, the focus of our research has been to induce optical clearing using an OCA which can be applied topically yet still achieve a meaningful reduction in the degree of tissue scattering.

Due to the known ability of DMSO to penetrate intact skin and its known OCP with dermal application (4, 11), we set out to study the potential of using DMSO as a simple and effective OCA. Collectively, our recently published *in vitro* and *in vivo* results provide compelling evidence that DMSO *by itself* is an effective topical OCA and is capable of inducing a three-fold reduction in skin optical scattering as well as improved visualization of subsurface blood vessels (25). The focus of the preliminary data presented herein summarizes our recent efforts to expand upon these results. Specifically, we have focused on experiments designed to demonstrate the ability of DMSO to improve resolution and imaging depth in imagining modalities used routinely by other research scientists. To achieve this objective, a silicone tissue phantom with mock vasculature was employed to study the expected improvements in imaging depth and resolution following DMSO application to *in vitro* human skin. Furthermore, we present an *in-vivo* demonstration of improved fluorescence signal strength in a mouse model which has undergone DMSO-mediated optical clearing.

## MATERIALS AND METHODS

### *In Vitro Flow Phantom Experiments:*

***Skin Preparation:*** Cryo-preserved, dermatomed human skin (Science Care, Phoenix, AZ) was thawed to room temperature (~26°C). With a single-edged razor blade, the skin was cut into ~2.5 cm x 2.5 cm samples. The thickness of each sample was measured by placing it between two glass slides, clamping the preparation with binder clips to provide consistent compression and uniform thickness, and measuring the preparation thickness with a micrometer (Mitutoyo, City of Industry, CA). Sample thickness ranged between 1.2 and 1.5 mm. To minimize systematic error and maximize repeatability of the measurement method, we recorded the preparation thickness after one click of the fine adjustment screw on the micrometer. By calculating the difference between the thickness of the preparation and the slides, we determined thickness of each sample.

***Silicone Phantom:*** A silicone phantom was synthesized with dimensions 9cm x 9cm x 1.5cm in a Petri dish. A 1:10 ratio of P-4 Curing Agent (Eager Plastics)/P-4 translucent silicone rubber (Eager Plastics) was used during phantom preparation. To achieve a reduced scattering coefficient of 4 $mm^{-1}$, titanium oxide ($SiO_2$, TI602, Atlantic Equipment Engineers) was mixed by multiplying the total volume of the silicone rubber and curing agent by 0.002656 g/ml. Prior to phantom solidification, four flexible plastic tubes (Tygon® formulation S-54HL) with an inner diameter of 250 μm were embedded within the phantom in a staggered conformation, separated by 5 mm and embedded at depths of 1, 2, 3, and 5 mm. We focused on absorption contrast in this set of experiments, using nigrosin as a biological marker at a concentration of 300 mg/mL.

***Experimental Protocol:*** For each experiment, a freshly thawed skin samples was placed on top of the phantom to insure the tubes were covered. To collect reflectance images, a 12-bit thermoelectrically-cooled CCD camera (QImaging 2000R) equipped with a macro lens was used. A low-coherence 785-nm laser diode (B&W TEK) was used as an excitation source. The skin samples were treated either with 14M DMSO (experimental condition) or isotonic saline (negative control). A total of six experiments was performed. Images were taken at 0, 20, 40, 60 min after agent application, in addition to baseline images of the phantom alone to serve as measures of the actual tube width and reflectance of nigrosin.

***Image Analysis:*** Prior to agent application, we identified regions from which pixel-intensity line profiles were extracted. Due to the rigid positioning of both the camera and phantom, the same regions were analyzed in all subsequent images.

**In-Vivo *Fluorescence Imaging:***

For these experiments, adult (~25 g) male C3H mice were used. Each animal was anesthetized with isoflurane gas followed by injection of a ketamine/xylazine (4:3 ratio, 0.4 mL/100 g mass) cocktail. The animal was placed on a custom stage with the dorsum facing upwards. A modified Hilltop chamber with a 7-mm-diameter hole removed from its center was placed over two adjacent regions on the mouse dorsum to hold the solutions of interest (see below). A multispectral imaging camera (Nuance, CRI, Woburn, MA) was used to collect fluorescence images with the liquid

crystal tunable filter set to transmit 530 nm radiation. The animal dorsum was excited with a broadband light source equipped with a 488-nm line filter.

Each animal was injected i.p. with 100 μL of fluorescein sodium. To minimize the influence of potential DMSO-induced vasoconstriction on fluorescein diffusion and hence on interpretation of the time-resolved images, we waited 30 min after fluorescein administration for a steady-state fluorescein distribution to be reached. DMSO (14M) was applied topically within one of the Hilltop chambers and removed at specific time points (0, 10, 20, 30, 40, 50, and 60 min after initial DMSO application) to enable collection of fluorescence emission directly from the skin surface. After image collection, fresh DMSO was reapplied immediately. A similar procedure was followed for the adjacent Hilltop chamber site, but with isotonic saline applied in lieu of DMSO, as a control site.

## RESULTS

### In-Vitro *Silicone Phantom Imaging:*

With DMSO application, we observed a progressive decrease in 785-nm reflectance, towards the baseline reflectance values of the phantom itself (Figure 1). This observation is consistent with our expectation of improved spatial resolution and nigrosin quantitation. For all experiments, only the tubes at depths of 1 and 2 mm were perceivable in the images. Our inability to observe tubes at deeper depths was due in part to a nonuniform illumination pattern; future experiments will address this issue.

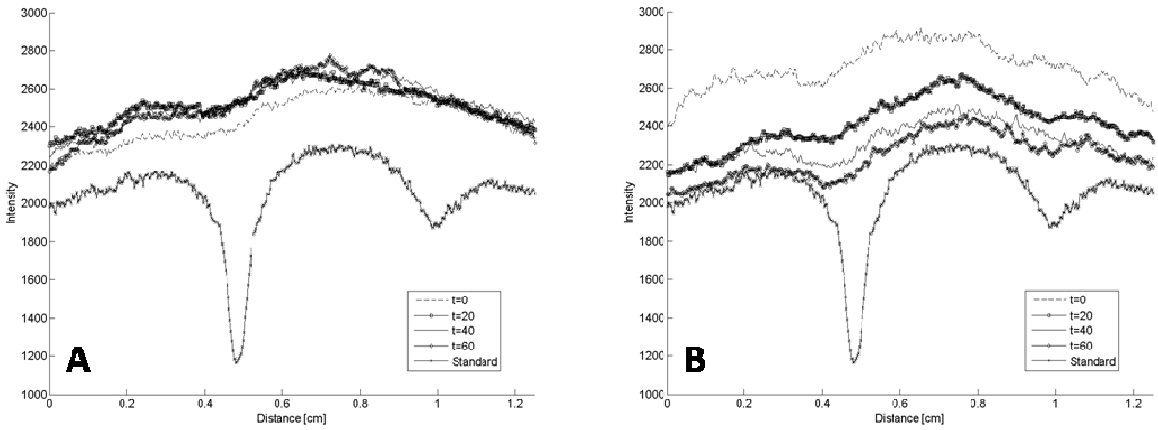


**Figure 1. DMSO-mediated optical clearing enables enhanced quantitative characterization of subsurface structures. Either (A) isotonic saline or (B) 14M DMSO was applied topically to *in vitro* human skin samples. Each sample was placed on top of a silicone phantom with embedded tubes filled with nigrosin as a simulated absorber. Two distinct troughs (at x positions of**

**~0.45 and 1.00 cm) represented the decrease in diffuse reflectance due to absorption at nigrosin at depths of 1 and 2 mm, respectively. After placing the skin sample above the phantom, an increase in reflectance was observed. With application of saline, no appreciable change in sample reflectance was observed. With application of DMSO, a marked decrease in diffuse reflectance was observed, as observed with the downward trend of the laterally-resolved reflectance curves.**

### In-vivo *Fluorescence Microscopy:*

We observed a ~70% average increase in 530-nm fluorescence intensity from baseline (i.e., 0 min) to 10 min after DMSO application, compared to a ~10% increase with saline application (Figure 2). For both DMSO and saline sites, this increase was sustained for ~50 min, after which a decrease in fluorescence intensity was observed at both sites.

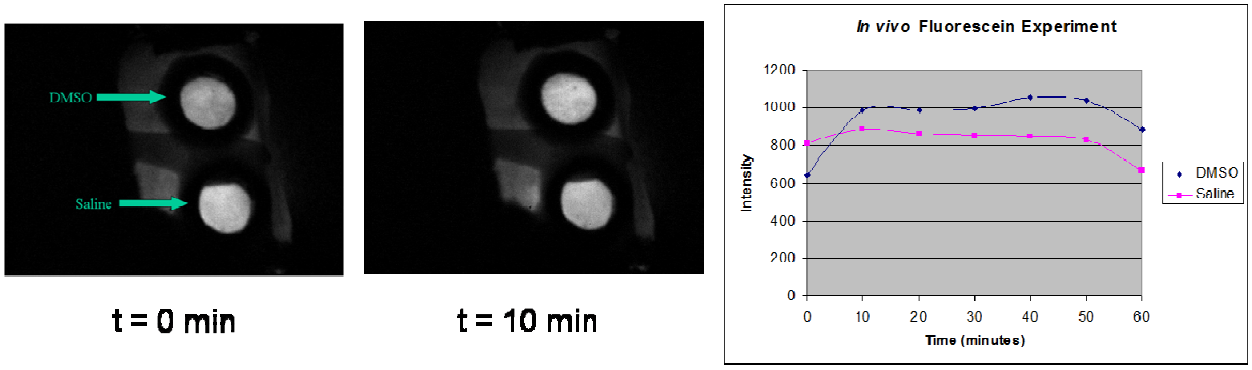


**Figure 2. DMSO-mediated optical clearing increases the quantity of fluorescence emission *in vivo*.**

## DISCUSSION

Studies involving optical imaging and microscopy focus typically on use of molecular absorption and fluorescence characteristics as a source of contrast. Optical scattering has been viewed as an unavoidable restriction limiting the ultimate potential of optical-based methods. The use of optical clearing to improve characterization of subsurface molecular and cellular processes uniquely addresses the restrictions imposed by optical scattering on accurate localization and quantification of the optical signals of interest. Our *in vitro* and *in vivo* data presented herein and in the literature provide compelling evidence that DMSO *by itself* is an effective topical optical clearing agent (25). Although not conclusive, the preliminary data presented in this paper serve to highlight some of our previous findings by illustrating improvements to near "real world" experimental designs resulting from implementation of a DMSO-mediated optical clearing protocol.

Previous studies on the topic of optical clearing have used *in vitro* preparations to demonstrate enhanced optical spectroscopy (11-13), fluorescence imaging (14), bioluminescence and chemiluminescence imaging (15, 16).

Furthermore, widely accepted OCAs such as glycerol, propylene glycol, and glucose, have been shown to improve the contrast and imaging depth for confocal as well as multi-photon microscopy (17-19). Lastly, improvements in other imaging modalities such as optical coherence tomography have also been witnessed following an optical clearing protocol (10, 20, 21). The potential benefits of optical clearing to laser therapy of subsurface targets such as tattoo pigments and microvasculature have also been demonstrated by a number of researchers (22-24). From the evidence presented, we conclude that an optical clearing protocol should be developed to enhance the functionality of these imaging modalities.

If a standardized optical clearing method is to be implemented with optical imaging protocols, topical application is a highly desirable feature of the chosen OCA. To the best of our knowledge, DMSO is the only individual agent which possesses an appreciable OCP (a three-fold reduction in reduced optical scattering, according to our recent data with topical application (25). Furthermore, the preliminary data presented herein (Figures 1 and 2) and in our recent publication indicate that DMSO can induce a reduction in optical scattering within 15 min after topical application (25). Collectively, our data provide compelling evidence which warrants further study of DMSO as an OCA for *in vivo* applications.

Finally, if DMSO proves to be a viable OCA for the variety of imaging techniques mentioned, it is imperative to discuss the issue of acute and systemic toxicity. We believe that the stigma derived from claims of the purported systemic toxicity of DMSO have overshadowed its potential clinical applications. Interestingly, a comprehensive literature review to date has confirmed only localized erythema as a ubiquitous side effect of DMSO application (25). Studies investigating claims of serious systemic effects are contradictory. Such side effects are associated with chronic DMSO application, and hence should not be cited as conclusive evidence against the occasional use of DMSO for therapeutic and diagnostic biophotonic applications.

The application of optical clearing is expected to enable clinicians and scientists employing optical methods to interrogate and target previously unattainable biological tissue features in situ, extending the range of suitable applications for biophotonics. The short time frame needed to induce DMSO mediated optical clearing, combined with DMSO's ability to be applied topically to the skin are two very persuasive reasons why DMSO should be strongly considered as a potential OCA for extending research in biophotonics. We postulate that the expected benefits of using

DMSO as an OCA to studies employing molecular sensing of subsurface events outweigh the current concerns of toxicity and thus future research into DMSO mediated optical clearing is warranted.

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