Handbook of Tissue Optical Clearing
New Prospects in Optical Imaging
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The role of optical clearing to enhance the applications of in vivo OCT and photodynamic therapy: Towards PDT of pigmented melanomas and beyond

Publication details
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Published online on: 09 Feb 2022

How to cite :- Layla Pires, Michelle Barreto Requena, Valentin Demidov, Ana Gabriela Salvio, I. Alex Vitkin, Brian C. Wilson, Cristina Kurachi. 09 Feb 2022, The role of optical clearing to enhance the applications of in vivo OCT and photodynamic therapy: Towards PDT of pigmented melanomas and

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The role of optical clearing to enhance the applications of in vivo OCT and photodynamic therapy: Towards PDT of pigmented melanomas and beyond

Layla Pires, Michelle Barreto Requena, Valentin Demidov, Ana Gabriela Salvio, I. Alex Vitkin, Brian C. Wilson, and Cristina Kurachi

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Introduction
This chapter presents an overview of the use of optical clearing of tumors, considering the effects in vivo on both optical coherence tomography (OCT) imaging and photodynamic therapy (PDT), particularly in melanoma.

Tumor tissue and light scattering. Given the limited penetration of visible/near-infrared light in tissues [1], high-resolution studies of the tumor microenvironment, including the microvasculature, have required the use of several ex vivo analytic optical techniques, such as frozen-section histopathology or optical sectioning through confocal laser-scanning microscopy. However, the need for real-time in vivo diagnostics and the use of light-based therapies such as PDT have motivated the development of techniques to overcome the limited light penetration in tissue.

Light attenuation in tissue is caused by absorption and (elastic) scattering, with the latter contributing particularly in non-pigmented lesions. Optical clearing agents (OCA) have been successfully used to decrease the light scattering in tissue and to improve imaging quality in-depth, mostly in normal skin or benign cutaneous lesions [2]. Its use to enhance the efficacy of both photodiagnostics and phototherapeutics in pigmented or thicker malignant lesions is of interest, given the potential clinical applications.

Optical clearing for in vivo tumor spectroscopy/imaging
Photonics-based approaches to assessing tumors and their microenvironment are actively being explored for cancer staging and prognosis, both in preclinical research and in the clinic [3–5]. An non-exhaustive list of noninvasive optical modalities includes diffuse reflectance spectroscopy, bioluminescence imaging, photoacoustic imaging, steady-state or time-resolved fluorescence spectroscopy/imaging [6], confocal and/or multi-photon approaches in white-light reflectance or fluorescence mode, and optical coherence tomography (OCT).

OCT is arguably one of the most explored techniques for tissue structural analysis and microvasculature visualization. Using the principles of electromagnetic interference and coherence gating to enable depth-resolved imaging of tissue morphology and architecture on the micron scale, OCT has emerged as viable and robust contrast-agent-free “in-vivo microscope.” In addition to its impressive volumetric visualization of fine tissue microstructures (often down to the cellular level), a variety of additional OCT contrast mechanisms have been developed to measure other important functional characteristics of tissues, for example blood and lymphatic microvasculature (e.g., speckle-based OCT methods) and tissue biomechanics (OCT elastography). Its various scientific underpinnings, technological advances, preclinical studies, and clinical uses have been summarized in recent reviews [7–10]. However, while generating cross-sectional or volumetric images of high spatial resolution (typically ~10 μm), the imaging depth of OCT is only ~1–2 mm, due to rapid attenuation of the coherently backscattered light. For tumor imaging, this has prevented imaging of the whole lesion, often necessitating biopsy or resection, followed by ex vivo analysis.

Using both OCT and diffuse reflectance spectroscopy (DRS), we have recently demonstrated that optical clearing agents can approximately double the effective depth of light penetration in a mouse model of pigmented cutaneous
melanoma [11]. Tape stripping to remove the stratum corneum was used prior to topical application of the OCA (PEG 400 and 1.2 propanediol mix, 19:1) to enhance its penetration. White-light DRS demonstrated diffusion of the OCA through the skin layers over time: for short wavelengths (~500 nm) the signal decreased within the first few minutes, while for longer wavelengths (>~600 nm) up to 45 min was required to achieve the DRS-measured maximum clearing effect. Further, OCT imaging was performed using a swept-source system centered at 1300 nm; here, the maximum clearing effect was observed considerably later than DRS (at 4 h after the OCA application), and enabled the visualization of tumor microvasculature up to a depth of 750 μm, or about double that without clearing, in this challenging pigmented tumor model [11]. These initial results show the potential of optical clearing agents to improve the utility of optical spectroscopy and imaging techniques to study tumor microenvironment, even in the highly absorbing milieu of pigmented melanoma. If translated to the clinic, such OCA approaches could enhance the utility of optical diagnostics, particularly in the context of tumor staging and treatment response assessment (Figure 31.1).

**Photodynamic therapy of tumors**

Photodynamic therapy (PDT) induces (tumor) cell death due to cytotoxic photoproducts such as singlet-state oxygen generated by light-activated photosensitizers [12]. In most non-dermatological applications, the PDT procedure is initiated by systemic administration of the photosensitizer. After a suitable time interval (minutes to days), depending on the photosensitizer uptake and clearance kinetics [13], this is followed by local irradiation using laser or LED light. In order to achieve the desired photodynamic effect, a minimum light fluence
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at the target tumor depth is required and typically shows a threshold dose response. The spatial distribution of both the photosensitizer and the light throughout the full depth and volume of the tumor target is critical to a successful outcome [12].

The effective treatment depth (or radius in the case of interstitial fiberoptic light delivery) depends on the photosensitizer concentration, optical extinction coefficient, and quantum yield of the cytotoxic photoproduct(s), as well as on the intrinsic photodynamic sensitivity of the treated tissue to the specific photosensitizer. Considering the light distribution, the treatment depth depends exponentially on the effective light penetration depth, $d_{\text{eff}}$, which is the reciprocal of the effective attenuation coefficient, $\mu_{\text{eff}}$.

$$\mu_{\text{eff}} = \sqrt{\frac{1}{\mu_a} + \frac{1}{\mu'_a}}$$

where $\mu_a$ and $\mu'_a$ are the optical absorption and transport scattering coefficients of the tissue at the treatment wavelength. This approximation works when the scattering is significantly greater than absorption, which is true in most tissues at longer visible/near-infrared wavelengths; this may or may not be valid in the context of pigmented melanomas, as we discuss below. However, regardless of the light propagation regime, the penetration depth and effective treatment depth depend on both absorption and scattering properties of tissue.

Topical PDT is approved in several countries for basal cell carcinoma and non-oncological conditions [14, 15]. It is recommended for actinic keratosis (pre-malignant lesions), Bowen’s disease (squamous cell carcinoma in situ), superficial basal cell carcinoma (BCC), and nodular BCC [16]. Currently, three commercial prodrugs are approved for topical use, all based on 5-aminolevulinic acid (ALA) formulated as an emulsion (Levulan®, DUSA Pharmaceuticals, Inc., USA), a nanoemulsion (nc-ALA: Ameluz® Biofrontera Inc., USA) [17], or a more lipid-soluble metyle derivative (Metvix®, Galderma, USA). When thus supplied in excess, ALA leads to increased production of the fluorescent photosensitizer protoporphyrin IX (PpIX) through the heme biosynthesis pathway. This treatment has low systemic toxicity, can be repeated without resistance or hypersensitivity, and preserves normal tissue structure and nerve function [17]. Additionally, PDT (whether ALA-based or with other photosensitizers) can have significant antitumor effects through immune stimulation, reducing or preventing tumor metastasis, and potentially altering tumor progression and reducing tumor recurrence [18–20]. These immune effects are particularly relevant in tumors where surgery is the primary treatment, since there is increasing evidence that surgery-related stress can reduce immune system function, leading to increased metastatic spread. This has been demonstrated preclinically in melanoma and breast and lung tumors [21–23], while immune dysfunction has been implicated in postsurgical metastatic spread of prostate [18] and pancreatic [23] cancers in patients. For highly metastatic cancers such as melanoma, these positive PDT-induced immune responses make it, in principle, an attractive alternative or adjuvant to surgery.

Topical application of ALA-based photosensitizers is recommended for superficial BCC, particularly in sites that show poor healing after surgery, or are important for good cosmesis, or involve treatment of multiple or large superficial lesions. It is also considered as an option for nodular BCC lesions <= 2 mm thick [17]. Cure rates of >90% can be achieved in superficial BCC [24]. However, for nodular lesions, the response rate is only around 50% [25]. PDT is also not currently recommended for pigmented tumors such as pigmented BCC or melanoma.

Preclinical studies of melanoma have explored the use of infrared light and more potent photosensitizers to improve response rates, but in most cases have not achieved full tumor eradication [26]. While there may also be differences in the intrinsic photodynamic sensitivity of melanoma cells, these poor results are likely the result of limited penetration of the photosensitizer into the deeper tumor layers and/or the high light attenuation due to melanin absorption.

To address the former, several chemical and physical pre-treatment procedures have been explored, including different drug formulations, the use of agents to enhance PpIX biosynthesis, and physical manipulations such as the use of microneedles, iontophoresis and temperature modulation [27]. Deep curettage to debulk lesions before topically applying the photosensitizer or the ALA prodrug has improved outcomes [28, 29]. Bay et al. compared physical methods to improve methyl aminolevulinate (MAL) delivery, including the use of laser ablation of the tissue surface [30]. To overcome the photosensitizer depth penetration limitation, systemic PDT can be an option for nonmelanoma skin cancer, but general skin photosensitivity must be minimized [31, 32]. Further, light penetration remains an intrinsic limitation for pigmented lesions.

### Optical clearing in PDT treatment of melanoma

Melanoma, a highly pigmented skin cancer, continues to present significant challenges for light-based techniques and other treatments. The strong light absorption of melanin and scattering by the melanosome granules present in high concentration within the tumor both limit light penetration, preventing full lesion imaging assessment and treatment using optical techniques. Globally, more than 55,000 patients die from melanoma every year [33]. The prognosis for primary cutaneous melanoma depends on tumor thickness [34]. Importantly, melanoma metastasizes in 30% of patients following primary tumor excision, with consequent high mortality rates [35] even in thin (<1 mm) lesions (5%–15%) [36, 37]. Local recurrence rates after resection range from 3%–5% (Stage 0) to >13% (Stage 4). Hence, there are significant unmet clinical needs, both to treat the primary tumor more effectively and to reduce the risk of life-threatening tumor progression and metastatic spread.

Two main factors may be advantageous in the use of optical clearing to improve the effective treatment depth in cutaneous melanoma, namely improved optical coupling at the lesion surface and reduced attenuation through refractive index matching within the tissue. The first mechanism is well known, for example through its use in high-resolution microscopy utilizing oil-immersion lenses. In topical application at the tumor surface, OCA gel fills micro-irregularities to enhance light coupling into the tissue. For the second mechanism, light scattering within the tissue is reduced through the reduction of
the refractive index contrast, based on OCAs with a refractive index of ~1.4. The overall result is deeper light penetration with depth and more homogeneous light distribution, both of which can improve the PDT efficacy.

We have recently reported [38] the use of optical clearing combined with PDT in intradermal pigmented melanoma in a mouse model using the clinical photosensitzers Visudyne (vascular-targeted) and Photodithazine (tumor cell-targeted), either alone or in combination. The photosensitzers were administered systemically to circumvent the limited penetrance of topical applications (see below). Tumors grown from amelanotic (nonpigmented) cells that were otherwise essentially identical to the pigmented cells were used as controls to isolate the role of melanin in the treatment response in vivo.

Optical clearing had no significant effect on the PDT response of the amelanotic tumors in any treatment group. This was expected, since the tumor thickness (<1 mm) and lack of significant absorption allowed adequate (i.e., above threshold) light dose throughout the full lesion depth. Encouragingly, in the pigmented tumors, optical clearing significantly improved the PDT response in all treatment groups. Most importantly, complete tumor eradication was achieved when optical clearing was combined with dual-photosensitizer PDT (Figure 31.2). This demonstrated the critical role that melanin plays in attenuating the light and affecting the treatment outcome [38]. It should be emphasized that optical clearing did not reduce the high light absorption of melanin per se but did likely reduce the contribution of the melanin granules to light scattering, which impacts the overall light attenuation. It is possible that further improvements could be achieved by combining optical clearing and optical whitening agents, such as kojic acid, that destroy the melanin granules and potentially would reduce the absorption of the PDT treatment light [40].

We have also carried out additional studies with sub-therapeutic PDT doses combined with optical clearing to determine the maximum response depth in the melanoma tumor [39]. Immediately after PDT with and without optical clearing of pigmented tumors, ex vivo Raman spectra were taken using a confocal Raman microscope (Alpha 300 RAS microscope, WITec, Ulm, Germany), with 785 nm excitation and 100–3200 cm⁻¹ wavenumber range detection. The images and spectra were collected at 20× and 50× magnifications, and spectra were taken at different depths in the PDT-treated tumor (from 25 μm to 2 mm in 100 μm increments). With the addition of optical clearing, PDT-induced spectral changes were observed up to a depth of 725 μm (Figure 31.2, bottom panel) and the spectra were similar to those at superficial depths of 25–125 μm where the light attenuation was low [39]. In contrast, the PDT group without optical clearing showed marked heterogeneity in the Raman spectra even at intermediate depths of 225–325 μm. That is, it appears that the use of optical clearing promoted a more homogenous and deeper PDT response, in agreement with the direct histopathological evaluation (Figure 31.2).

Complementing these ongoing preclinical studies, we are currently carrying out the first clinical trial at Amaral Carvalho Hospital, Brazil, applying optical clearing in patients receiving MAL-PDT treatment of thin (<2 mm) BCC lesions [41]. The preliminary results in 12 patients demonstrate that the technique is safe, with no side effects observed and no negative impact on PDT treatment. The next step will be to use optical clearing in thicker lesions that have poor PDT response [41].

Despite the initial indications of improvement in the PDT outcomes, optical clearing still requires topical application in most cases, so that methods to improve OCA diffusion into the tumor tissue (e.g., increase the amount and reduce the time) are needed to translate the concept into clinical practice. Hence, we are currently investigating the use of polymer microneedle patches that perforate the superficial layers of the tissue to improve the diffusion of both OCA and photosensitizer into the tumor tissue [42]. Emerging OCT-based tumor delineation methods [43–46] may substantially improve tumor boundary detection and its longitudinal response monitoring post-treatment. Our recently proposed fully automatic volumetric tumor delineation method based on quantitative speckle analysis of OCT images has been demonstrated in vivo and validated in two different biological models of human cancers grown in experimental mice, including pigmented melanoma (Figure 31.3). The same microstructural OCT datasets were also used to simultaneously obtain volumetric images of tissue microvasculature, furnishing a more complete functional tumor picture. The method was shown to (1) robustly delineate tumor from normal tissue, enabling a variety of biomedical applications (e.g., early disease detection/diagnosis or therapy response quantification), and (2) reflect some of the tumor changes following ionizing irradiation. We are currently investigating the capability of these novel techniques for detection of early signs of melanoma response to PDT and possible effects of optical clearing, which will be reported in separate publications.

Other potential optical clearing applications for PDT beyond the skin include endoscopic and interstitial scenarios [49, 50]. In the former, optical fibers placed through an endoscope instrument channel allow either surface or interstitial light delivery in hollow organs such as the esophagus and bronchus [51]. Nontoxic optical clearing agents could also be delivered directly to the tumor surface via the instrument channel and reapplied if necessary during the light irradiation; the smooth mucosal surface and absence of a significant physical barrier (e.g., stratum corneum as in skin) may enable easier penetration of OCAs.

In interstitial PDT, including endoscopic approaches, optical fibers are inserted using needles or catheters directly into tissues, typically for treating thicker and larger-volume lesions; often the use of cylindrical diffusing-tip fibers improves the homogeneity of light distribution throughout the entire lesion volume. This approach involves more complex dosimetry with image-based treatment planning [52], but has shown promising results for the treatment of cancers in prostate [52, 53], pancreas [54], head and neck [55, 56], and brain [57, 58]. In this scenario, the optical clearing agent could be injected intratumorally prior to the irradiation to increase the effective treatment volume per fiber and improve the light homogeneity.

Optical clearing agents could be also used in other phototherapeutic modalities, such as photothermal therapy, in which the absorbed light leads to tissue heating above ~55°C to destroy tumors by coagulative necrosis [59]. Again, the use
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of OCAs could improve the effective treatment volume and homogeneity, especially in larger lesions.

**Conclusions**

In summary, optical clearing is a safe and inexpensive method to modify tissue optical properties, not only increasing the light penetration depth but also improving the light distribution throughout the target lesion, with potential to enable improved PDT response. OCA may expand the use of light-based diagnostic and treatment techniques to different tumors that are commonly considered unsuitable for biophotonic approaches. The case in point demonstrated here is pigmented melanoma, a dangerous and difficult-to-treat malignancy. This chapter highlighted initial in vivo preclinical and clinical studies using OCAs for optical imaging and PDT treatments and has discussed the overall prospects of OCA-enabled PDT in refractory lesions and beyond.
FIGURE 31.3 Recently developed OCT volumetric tumor delineation method[47] applied in vivo for detection of pigmented melanoma grown in a nude mouse skin. (a) Microphotograph of melanoma. Scale bar is 1 mm; (b) OCT-scanned skin surface of the tumor shown in (a) 90 min after OCA application. Yellow arrows indicate the OCA residuals on the skin surface; (c) Depth-encoded tumor microvasculature obtained with conventional speckle variance method [48] from the same microstructural OCT dataset (depth scale in mm: green = top tissue layers, black = deepest tissues); (d) OCT-derived 3D parametric image of the tumor and surrounding nonmalignant tissues. The sharp tumor-skin “interface” at specific delineation parameter range allows for accurate tumor delineation through parametric thresholding for further analysis.

Acknowledgments

The authors wish to thank the Princes Margaret Foundation (Invest in Research program) and the Cancer Research Society, Canada, and FAPESP and CNPq, Brazil, for financial support of this work.

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