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The Role of Laser Speckle Imaging in Port-Wine Stain Research: Recent Advances and Opportunities

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(Invited Paper)

***Abstract*—Here, we review our current knowledge on the etiology and treatment of port-wine stain (PWS) birthmarks. Current treatment options have significant limitations in terms of efficacy. With the combination of 1) a suitable preclinical microvascular model, 2) laser speckle imaging (LSI) to evaluate blood-flow dynamics, and 3) a longitudinal experimental design, rapid preclinical assessment of new phototherapies can be translated from the lab to the clinic. The combination of photodynamic therapy (PDT) and pulsed-dye laser (PDL) irradiation achieves a synergistic effect that reduces the required radiant exposures of the individual phototherapies to achieve persistent vascular shutdown. PDL combined with antiangiogenic agents is a promising strategy to achieve persistent vascular shutdown by preventing reformation and reperfusion of photocoagulated blood vessels. Integration of LSI into the clinical workflow may lead to surgical image guidance that maximizes acute photocoagulation, which is expected to improve PWS therapeutic outcome. Continued integration of noninvasive optical imaging technologies and biochemical analysis collectively are expected to lead to more robust treatment strategies.**

***Index Terms*—Antiangiogenic, biomedical optical imaging, biophotonics, dorsal window chamber, image-guided surgery, intravital imaging, surgery.**

I. Introduction

In an estimated three children an per 1000 live births (~400 000 per year) [1], hypervascular skin lesions known as port-wine stain (PWS) birthmarks develop. They are due purportedly to irregularities in neural development and genetic mutations. Since the seminal publication by Anderson and Parrish on selective photothermolysis [2], photothermal therapy of PWS birthmarks has become a classic example of how judicious use of wavelength and pulse duration can result in selective photo-induced damage to subsurface targets. Here, we review our current understanding of PWS, including their etiology and microscopic architecture; optical methods used currently in the clinic for treatment; and the critical role that laser speckle imaging (LSI) can play both in rapid evaluation of new phototherapy protocols and intraoperative monitoring and guidance during laser surgery. We conclude with recommendations for future research directions.

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II. PWS Etiology

The cause and origin of PWS remain incompletely understood. Researchers have proposed two central hypotheses to describe the pathogenesis of PWS birthmarks. One hypothesis is that axonal denervation may contribute to the development of PWS birthmarks. Supporting evidence includes: 1) PWS typically occur in regions that normally are innervated by certain axonal branches (i.e., trigeminal nerve [3]; and 2) nerve fiber density is significantly decreased in PWS as compared to normal skin [4].

A second hypothesis proposes that genetic mutations may contribute to formation of PWS. Shirley *et al.* [5] discovered that sporadic somatic guanine nucleotide-binding protein, G alpha subunit q (GNAQ) mutation (R183Q), was found in PWS with an average mutation frequency lower than 5%. However, the specific cell-type distributions of this mutation and its pathogenic roles in PWS development remain unknown. Mutation(s) in a single gene alone may be insufficient to cause PWS, but may contribute its pathogenesis in combination with other genetic alterations.

Many signaling pathways are aberrantly activated in PWS. We have identified an activation profile of various kinases during different stages of PWS progression, including (1) c-Jun N-terminal kinases and extracellular signal regulated kinases in infantile to nodular PWS, which may contribute to both the pathogenesis and progressive development of PWS; (2) AKT and phosphatidylinositol 3-kinases, which may be involved in the progressive dilation of PWS blood vessels; and (3) phosphoinositide phospholipase C γ subunit, which may lead to the formation of nodules [6]. Furthermore, PWS have elevated

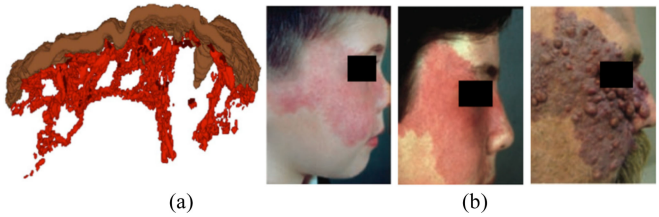

Fig. 1. (a) Representative (a) 3-D image of PWS skin. The epidermis (brown) and general dermal structure (red) are similar for normal and PWS skin. The image is a digital representation of 70 adjacent histological sections. Adapted with permission from REF. [9]. (b) Example of progression of a PWS over four decades of life. PWS birthmarks tend to darken in color and become hypertrophic, as evidenced in this case by nodule formation. Adapted with permission from REF. [11].

expression of both vascular endothelial growth factor (VEGF) and VEGF receptor subtype 2 (VEGFR-2) [7].

In summary, aberrant activation of signaling pathways in PWS may be a result of mutations of GNAQ, or VEGFR-2 activation, or some combination thereof.

III. Appearance and Microscopic Architecture of a PWS

PWS involves enlargement and an increased density of capillaries and post-capillary venules. Histopathological studies [see Fig. 1(a)] of PWS show an abnormal plexus of dilated blood vessels located in the dermis [8]. The skin contains clusters of blood vessels with diameters ranging primarily between 10 to 50 μm [see Fig. 1(a)] [9], although larger (200 μm) diameter vessels are observed in histological sections [8].

PWS are well demarcated and grow proportionately in surface area with age. In infants and young children, PWS are flat red to pink macules [see Fig. 1(b)]. However, PWS tend to darken progressively to deep red or purple [see Fig. 1(b)] and, by adult age, often become raised as a result of the development of vascular papules or nodules on the skin surface, which can often bleed spontaneously with incidental trauma [10], [11]. These changes in color and contour are attributed to progressive dilatation of the abnormal dermal vascular plexus.

IV. Standard Treatment: Photothermal Therapy

A. Selective Photothermolysis

In 1983, Anderson and Parrish [2] made the seminal observation that judicious selection of laser parameters can result in precise targeting of subsurface structures, such as hemoglobin in blood. As a direct result, the treatment of PWS became one of the earliest successful applications of laser therapy. For PWS laser treatment, the goal is selective photocoagulation of the subsurface vessels, with subsequent replacement of the damaged vessels with normal microvasculature [see Fig. 2(a)]. Laser light targets the optical absorbers oxyhemoglobin and deoxyhemoglobin in the blood vessels. The absorbers convert the optical energy to heat, resulting ideally in thermal damage and complete thrombosis in the targeted vessels [12], [13]. To take advantage of the selective photothermolysis principles outlined by Anderson and Parrish, medical laser technology has evolved toward

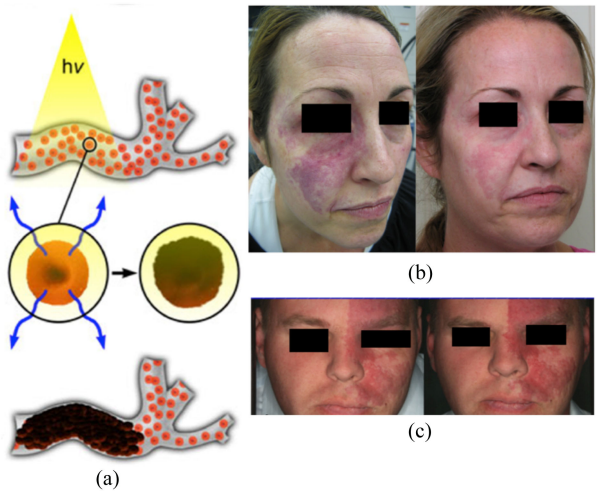

Fig. 2. (a) Selective photothermolysis is the governing principle clinicians to use to treat PWS birthmarks. Selective absorption of the optical energy in the superficial PWS vasculature results in generation of heat energy, leading to local temperature elevation and, with sufficiently high temperatures, photocoagulation of blood constituents and/or the walls of the microvasculature. Complete and selective acute photocoagulation of the vessel is considered to be a prerequisite of subsequent vessel removal during wound healing. Adapted with permission from REF. [13]. (b) Example of a good treatment outcome. Photographs were taken (left) before PDL treatment and (right) after six laser treatments. Based on visible reflectance spectroscopy measurements, the PWS blanching was 80%. (c) Example of a poor treatment outcome. Photographs were taken (left) before PDL treatment and (right) after three laser treatments. Quantitative analysis demonstrates that the PWS birthmark actually darkened in color.

use of longer wavelengths, from 577 nm [14], [15] to 585 nm [16] and to 595 nm [17]; and longer pulse durations from 0.5 to 1.5 ms [18].

The approach of pulsed-dye laser (PDL) therapy is based on the premise that acute, selective photocoagulation of PWS vessels results in vascular remodeling that replaces photocoagulated vessels with normal-sized capillaries [see Fig. 2(a)]. The maximum permissible radiant exposure is limited due to the competitive absorption by epidermal melanin. Photocoagulation by PDL therapy is limited primarily by the optical scattering of skin to a maximum depth of $\sim$2 mm. Recent improvements in PDL technology have included incorporation of cryogen spray cooling in an effort to reduce epidermal damage and pain and permit the use of higher radiant exposures [19].

Since the 1980s, clinicians have used PDLs emitting yellow light (577–595 nm) devices. Alternate light sources include the frequency-doubled Nd:YAG laser (532 nm) and broadband intense pulsed light sources. To achieve deeper penetration of light to treat hypertrophic or nodular PWS birthmarks, the alexandrite laser (755 nm) can be used [20].

Consistent removal of PWS in children is expected to eliminate the psychosocial damage these lesions inflict, and will significantly and positively impact the life of affected individuals and their families. Previous studies have demonstrated that early treatment of PWS generally achieves better outcomes (i.e. greater lightening in a shorter period of time [21], [22] than treatment at adult ages. Improved results in infants are likely due to the presence of PWS vessel diameters that are more amenable to photocoagulation and thinner overall lesions.

B. Current State of PDL Therapy

The PDL is the standard of care for the treatment of PWS throughout the world [see Fig. 2(b)]. However, current treatment options have significant limitations in terms of efficacy and risk [23], [24]. When treated by PDL, PWS often become lighter in color, but patients typically require multiple treatments (15+ to obtain the optimal therapeutic result in terms of lesion fading [25]). When general anesthesia is used, treatments can cost upwards of $2000 per session.

Even with numerous treatment sessions, complete removal often is not achieved. Patients and physicians are often frustrated as some lesions do not lighten with treatment [see Fig. 2(c)] and PWS recurrence is common. A large population of patients with PWS responds poorly to PDL treatment, with a range of 12 to 85% of patients achieving less than 50% clearance, regardless of the treatment modality [26]–[29]. Huikeshoven *et al.* [30] published a 10-year follow up on 51 patients who had undergone PDL treatment for PWS. Using objective skin color measurements, they reported significant re-darkening of PWS following an initial course of PDL therapy (although the PWS remained significantly lighter than before treatment). Furthermore, only 59% of patients were satisfied with the overall treatment result.

Several factors that play a primary role in limiting PDL treatment efficacy have been identified. First, competitive absorption of therapeutic laser light by epidermal melanin reduces the light dosage reaching the targeted subsurface vessels. This is particularly a problem in patients with darker skin types. Second, PDL therapy is capable of inducing acute photocoagulation within intermediate-sized vessels greater than 20 μm in diameter, but small superficial vessels remain difficult to photocoagulate due to the rapid heat diffusion from these vessels into the perivascular tissue [31]. Finally, revascularization may occur as a result of neovascularization and reperfusion of partially damaged vessels [13], [27], [32]. Treatment response may depend on the degree of innervation and microvascular density [4]. We and other research groups [33]–[43] have reported on potential optimization of treatment parameter selection (i.e., wavelength, spot size, pulse duration, radiant exposure) on an individual treatment basis. However, due to the considerable heterogeneity of PWS vascular architecture and lack of current knowledge on how extracted skin characteristics can be used to guide treatment parameter selection, this premise remains difficult to test and impractical to perform in the clinic.

V. Development of New Treatment Approaches

During the last 20 years, PWS treatment outcomes have remained largely unchanged [44]. To reduce the financial burden and potential risks of repeated treatments under general anesthesia, there is a need for innovative methods to maximize the reduction in PWS redness per treatment session. Without addressing this need, the efficacy of PWS laser therapy will remain variable, because protocols will remain based primarily on the impression and overall experience of the treating physician. To enhance PWS therapeutic outcome, we focus our research efforts along three themes:

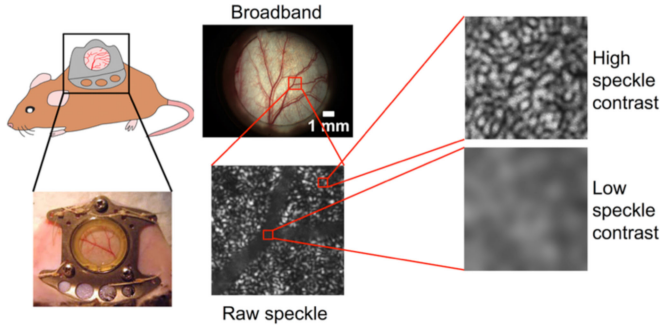

Fig. 3. (a) Rodent dorsal window chamber model used for preclinical evaluation of phototherapies. (b) (Left) Rodent dorsal window chamber under (top) broadband illumination and (bottom) 633-nm HeNe laser illumination. (Right) In regions with blood flow, the speckle pattern has low contrast, whereas in surrounding regions the pattern has high contrast.

1) Develop new strategies to increase prevalence of acute photocoagulation
2) Modulate biological response to phototherapy. Reformation and reperfusion of PWS blood vessels following therapy is a barrier that must be overcome to achieve improved therapeutic outcome after laser treatment [12], [32], [45], [46]
3) Develop imaging approaches to provide intraoperative feedback during phototherapy

A. Animal Model

Extensive evaluation and optimization of new approaches to therapy, requires use of an animal model. Currently, a model bearing a lesion identical to a PWS, does not exist. Previous studies used the rooster comb [47]–[49] and chick chorioallantoic membrane [18], [50]–[52] models as a surrogate for PWS vasculature. For our recent studies, we selected the rodent dorsal window chamber model [see Fig. 3(a)] based on extensive evaluation and use of which revealed the following important criteria: 1) The size of blood vessels in the dorsal window chamber is similar to that observed in PWS skin, 2) the dorsal window chamber model is an established method for microvascular evaluation, and 3) studies utilizing this method for PWS research have demonstrated correlation with studies on *in-vivo* PWS skin. Detailed protocols [53], [54] describe the materials and surgical procedures involved with the window chamber.

The size and depth of blood vessels in the dorsal window chamber are similar to that observed in PWS skin. Barsky *et al.* [8] analyzed biopsies from 100 PWS patients and determined the mean vessel depth to be 460 μm. With further analysis of this data, we determined that mean PWS vessel diameters range between 50 and 75 μm and the blood volume fraction varied from 2 to 8% [41]. In comparison, the depth ($\sim$500 μm) of the subdermal vascular network of the hamster dorsal window chamber is within $\sim$10% of the mean PWS vessel depth. Diameters of vessels in the window chamber range typically between 10 to 120 μm, and the blood fraction of analyzed window chambers is between 3 to 6%. Collectively, these physical characteristics

of window chamber microvasculature compare favorably with those found in PWS skin

B. LSI of Blood-Flow Dynamics

To characterize longitudinally the microcirculation of the dorsal window chamber, intravital microscopy is typically used [55]–[58]. This method provides invaluable information on the microvasculature, but its use is limited largely to study only a small subregion of the window chamber. Here, we describe use of LSI, a flexible, wide-field optical imaging approach that we have used extensively to image both the dorsal window chamber and subjects with PWS.

Several excellent descriptions of LSI exist in the literature [59]–[62]. Here, we summarize only the most salient points. With illumination of an object with coherent light and imaging of the remitted light with a camera, a speckle pattern is observed [see Fig. 3(b)]. If the camera exposure is sufficiently short, the velocity distribution in the field will be mapped on the photograph as variations in speckle contrast [see Fig. 3(b)]. In their seminal paper on LSI, Fercher and Briers [63] derived a speckle contrast imaging equation, which since has been corrected to [64]:

$$\frac{\sigma_s}{I} = \left[\beta \frac{\exp\left(-2T/\tau_c\right) - 1 + 2T/\tau_c}{2\left(T/\tau_c\right)^2}\right]^{1/2} \tag{1}$$

where $\sigma_s(T)$ and $\langle I \rangle$ are the local standard deviation and mean, respectively, of the speckle pattern collected over exposure time $T[s]$, β is a term that accounts for correlation loss due to spatial sampling of the speckle pattern and polarization, and τ_c is the speckle correlation time [s]. Thompson *et al.* [65] presented modeling and experimental data that suggests the importance of β for LSI analysis.

Various methods can be used to estimate τ_c from measurements of K, including use of look-up tables. Ramirez-San-Juan *et al.* [66] and Cheng and Duong [67] derived a simplified speckle imaging equation for contrast values between 0 and 0.5:

$$\tau_c = 2TK^2. \tag{2}$$

The simplicity of (2) facilitates rapid calculation of τ_c from knowledge of T and measurement of K.

C. Need for Long-Term Monitoring of the Microcirculation

With selective photothermolysis, the target short-term response is acute photocoagulation and substantial reduction in blood flow. The desired long-term response is substantial vascular remodeling within the region. A poor response is associated either with removal of the coagulum, leading to restoration of blood flow, or integration of the coagulum into the restructured vessel lumen [12], [13]. To study the preclinical efficacy of new therapeutic approaches, the dorsal window chamber is an effective model. With use of common exposure times (1–10 ms), our *in-vitro* blood flow phantom data [68] demonstrate a linear response range spanning low-flow conditions (i.e., capillaries) and flow in arterioles and venules, which are the primary blood vessels of interest in the rodent dorsal window chamber model.

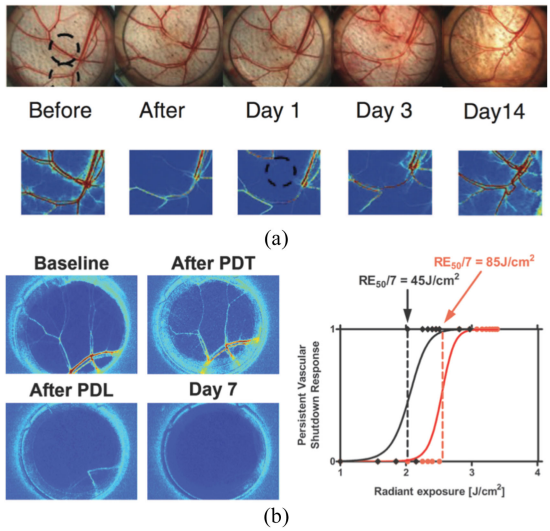

Fig. 4. (a) Long-term monitoring of irradiated blood vessels is essential for assessment of the degree of photoinjury to targeted vessels. Wide-field color reflectance images (top row) and corresponding speckle flow index (SFI) images (bottom row) were acquired after two arteriole-venule pairs (dashed circles in "Before" image) were irradiated with simultaneous 532 and 1064 nm laser pulses. In the absence of complete vascular shutdown, restoration of blood flow is observed, in a strikingly similar architecture to that observed in the "Before" image. Color reflectance image dimensions (H × V): 13 × 10 mm^2, SFI image dimensions: 9 × 7 mm^2. Adapted with permission from REF. [32]. (b) Combined NPe6-mediated PDT and PDL irradiation induces persistent vascular shutdown at reduced individual radiant exposures. (Top) SFI images collected before phototherapy, after PDT, after ensuing PDL irradiation, and at seven days post-irradiation. (Bottom) Combined with a fixed PDL radiant exposure of 6 J/cm^2, the required characteristic PDT radiant exposure to achieve persistent vascular shutdown, decreased from 85 J/cm^2 to 45 J/cm^2. Reprinted with permission from REF. 100.

We have demonstrated that the short-term (<24 h) microvascular response to light-based therapeutic intervention differs considerably from the long-term response [see Fig. 4(a)] [32]. In the absence of complete acute photocoagulation of the irradiated vessels with PDL, the region of interest remains perfused. This is in agreement with seminal clinical observations [24], [69]–[72].

With the window chamber, we set out to develop new phototherapies to achieve persistent vascular shutdown. Persistent vascular shutdown is defined as a lack of blood flow in the targeted blood vessel or in the entire window chamber, at a specified time point after phototherapy. To this end, we have studied the ability of two new approaches to achieve this level of shutdown: 1) combined photodynamic and photothermal therapies; and 2) photochemotherapy.

D. Approach #1: Combined Phototherapies

Photodynamic therapy (PDT) involves use of a CW light source to activate an otherwise harmless photosensitizer [73]. Since the low optical powers (typically mW) associated with PDT typically are insufficient to cause photothermal injury to the epidermis, patients with all skin types can be treated, although darker skin types may require longer light exposure times to achieve the desired therapeutic effect [74]. PDL therapy tends to spare small (7 to 20 μm diameter) blood vessels

[31], while PDT can photocoagulate vessels of all sizes. With PDT, vascular injury can accumulate at progressively deeper regions as exposure time is increased.

Nearly all published data involving PDT of PWS are from studies conducted in China using either photocarcinorin (PSD-007) or hemoporfin as the photosensitizer [75]–[85]. Data from large-scale clinical studies suggest that PDT can achieve good treatment outcomes [79], [86]. Zhang *et al.* [80] report that PDT treats pink PWS birthmarks better than PDL therapy, although their comparison involves use of PDL treatment without cryogen spray cooling. However, this clinical outcome was associated with undesirable aspects of the treatment protocol, including the reported photosensitivity period of four to eight weeks; treatment sessions greater than one hour in duration; and adverse side effects in pediatric patients including scarring and skin necrosis [75], [80], [87]. Furthermore, similar to PDL therapy, complete clearance of PWS birthmarks is difficult to achieve, even with multiple PDT sessions [75], [77]. Hence, further study of PDT as an alternate treatment to PDL therapy is warranted.

We first studied the efficacy of Benzoporphryin derivative monoacid ring A (BPD) as a photosensitizer. The U.S. Food and Drug Administration approved by BPD for the treatment of wet age-related macular degeneration, skin carcinoma, and brain tumors. With a series of preclinical studies [88]–[91], we demonstrated promising results with the combination of BPD-mediated PDT combined with PDL irradiation. We then initiated clinical translation of the protocol in a Phase I FDA approved trial of PDT + PDL for treatment of PWS [92] and demonstrated that PDT + PDL is more effective for selective removal of cutaneous microvasculature, as compared to PDL alone.

Based on promising preclinical data [93], we evaluated talaporfin sodium (NPe6) as a candidate photosensitizer in treatment of PWS. NPe6 has proven selective vascular effects in preclinical studies, an acceptable photosensitivity period of five to seven days, and a positive safety profile [94]–[97]. We selected a light source to target a secondary absorption peak of 664 nm [98].

We first studied NPe6-mediated PDT. With dose-response analysis, we quantified a characteristic radiant exposure ($RE_{50/7}$) capable of achieving persistent vascular shutdown at day 7 after phototherapy [99]. We then combined NPe6-mediated PDT with PDL therapy, with either PDT or PDL performed at radiant exposures below their respective $RE_{50/7}$ values of 85 and 7.1 J/cm^2 [100]. We determined that $RE_{50/7}$ for NPe6-mediated PDT decreased substantially when combined with PDL [see Fig. 4(b)]. Initial analysis of the data suggests that PDT and PDL act in a synergistic manner, although the mechanism of action remains unknown and is a topic of further study.

Collectively, our preclinical and clinical observations on PDT + PDL suggest that complete, persistent vascular shutdown can be achieved with reduced light doses, which is an important first step toward enhancing PWS therapeutic outcome. Achieving this outcome was accelerated with longitudinal optical imaging of a suitable preclinical model. A Phase I clinical study evaluating this approach is underway.

E. Approach #2: Photochemotherapy

Angiogenesis associated with the normal wound healing response after laser exposure can cause regeneration of coagulated blood vessels [32], [45], [46], [101]. PDL treatment of PWS causes acute hypoxia due to intense damage to blood vessels. Local hypoxia leads to upregulation of hypoxia-inducible factor 1-alpha (HIF-1α) and subsequent transcription of numerous pro-angiogenic genes, including VEGF [102], [103]. VEGF is the predominant growth factor that regulates angiogenesis pathways by signaling via VEGFR-2 [104]. We previously studied the use of Imiquimod, an antiangiogenic agent approved by the U.S. Food and Drug Administration for treatment of external genital warts, superficial basal cell carcinoma, and actinic keratosis, in combination with PDL therapy [105], [106]. The preliminary data suggest that the combined PDL + imiquimod protocol leads to a higher degree of blanching than PDL alone, although some regression in color change was observed. Further clinical trials are warranted to assess safety and efficacy of this approach.

Activation of VEGFR-2 also leads to activation of the mammalian target of rapamycin (mTOR) signaling pathway [102], [107]–[109]. mTOR can phosphorylate 4E-binding protein 1 (4E-BP1) [110] and S6 kinase [111], [112], which in turn mediates efficient cap-dependent translation initiation and subsequent regeneration and reperfusion of injured blood vessels.

We hypothesize that the combination of PDL therapy and rapamycin-mediated inhibition of mTOR signaling may enhance PWS therapeutic outcome using photochemotherapy. Recent studies demonstrate the potential of FDA-approved rapamycin [113], [114] as an anti-angiogenic agent. Rapamycin 1) decreases VEGF production, 2) mitigates the response of vascular endothelial cells to stimulation by VEGF [115], [116], 3) inhibits upstream Akt-induced signaling in endothelial cells [117], and 4) reduces the angiogenic effects of hypoxia [118], [119].

Based on these findings, we performed preclinical evaluation of photochemotherapy on the dorsal window chamber model and used LSI to monitor the treatment response [101]. When blood vessels were exposed to laser irradiation combined with daily 1% topical rapamycin for 14 days, we observed minimal regeneration of blood vessels [see Fig. 5(a)]. Even after rapamycin application was discontinued, we did not observe any revascularization during the one-month monitoring period.

Based on the preclinical results, we initiated a Phase I clinical study to determine the safety and efficacy of photochemotherapy [45]. With combined PDL and rapamycin therapy, we observed a persistent blanching response as long as 13 months after therapy [see Fig. 5(b)]. In contrast, considerable re-vascularization of the PDL-only sites was observed. Furthermore, in a clinical study involving 23 subjects with Sturge-Weber syndrome and PWS, Marqués *et al.* [120] reported that the combination of PDL and topical rapamycin led to a higher degree of blanching than PDL alone.

In summary, the integration of LSI as a longitudinal monitoring tool facilitated rapid discovery of new phototherapy protocols that now are in Phase I clinical trials.

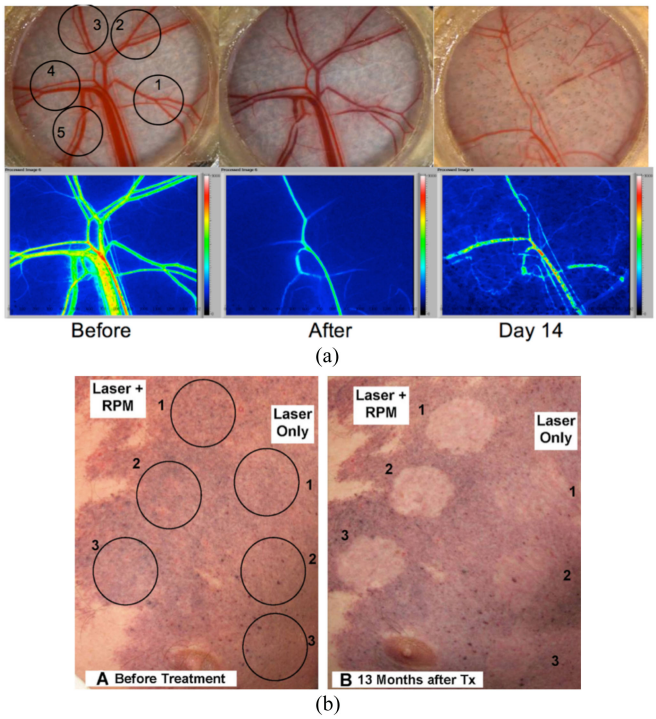

Fig. 5. Combined PDL therapy with Rapamycin induces persistent vascular shutdown. (a) Preclinical evaluation of window chamber treated with targeted PDL pulses in conjunction with 1% topical rapamycin application. Acute shutdown of the targeted vessels was observed and persisted through Day 14. Adapted with permission from REF. [101]). (b) First clinical demonstration of photochemotherapy for treatment of PWS birthmarks. At test sites treated with PDL alone, near-complete re-perfusion was observed 13 months after treatment. When PDL was used in conjunction with oral rapamycin administration, persistent blanching was observed at 13 months after treatment. Adapted with permission from REF. [45].

VI. Real-Time, Clinical LSI During PDL Therapy

Since the goal of laser therapy is acute photocoagulation of the blood vessels, we hypothesize that treatment outcome correlates with intraoperative measures of blood-flow reduction. With LSI measurements collected before and after laser therapy, we identified that regions of persistent perfusion oftentimes existed after treatment [121], [122]. We postulated that immediate re-treatment of these regions would lead to an improved treatment response, which then would result in a decrease in the number of required treatment sessions to achieve complete PWS blanching.

We then integrated LSI into the clinical workflow within the operating room. To achieve this goal, it was imperative to enable real-time processing and visualization of the raw speckle images. With the fast 'roll' algorithm described by Tom *et al.* [123], we achieved real-time blood-flow imaging at 10 frames per sec with use of the processing power of a NVIDIA graphics processing unit (GPU) [124]. We also changed the hardware to a tripod-based setup, as a first step toward flexible positioning during laser surgery [13], [125].

We utilized the real-time LSI system to measure PWS perfusion in the operating room during PDL treatment (Fig. 6, Video 1) [125]. In a study of 24 subjects, we determined that treatment outcome correlated with the magnitude of blood-flow reduction measured during laser therapy.

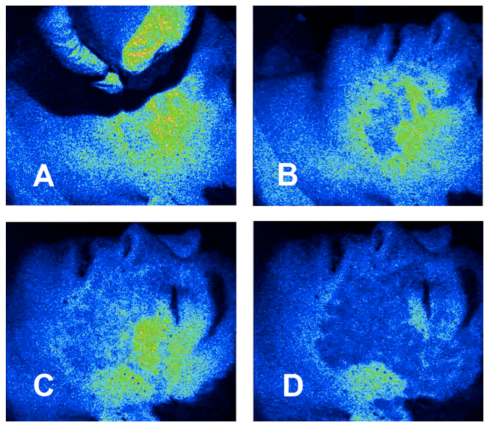

Fig. 6. Images captured from a live video display of SFI during PDL surgery of a patient with a PWS birthmark on the face. (A) Before treatment, (B,C) two time points during treatment, (D) immediately after the first pass is complete. Note the considerable reduction in SFI achieved with the laser. Multimedia file Video 1 shows the unaltered SFI video during the first laser pass.

Other research groups have reported complementary findings. Qiu *et al.* [126] used LSI to measure perfusion dynamics during PDT on seven subjects with PWS. They found that PWS perfusion increased for several minutes during the course of treatment, followed by a sustained decreased in perfusion during the remaining treatment time. Perfusion levels at the end of treatment did not uniformly drop below initial values, leading the authors to suggest that additional treatment time may be required to achieve maximal birthmark blanching. Ren *et al.* [127] used LSI to quantify PWS perfusion in 40 subjects before PDT and at a three-to-six month follow-up. They found that the reduction in PWS perfusion during PDT was correlated to lesion blanching.

Collectively, these clinical studies indicate that intraoperative LSI can be used to monitor treatment progress and ultimately be used as real-time feedback to guide the clinician regarding the potential need for immediate additional treatment to regions exhibiting persistent perfusion. Larger-scale studies coupled with additional mechanistic insight will be necessary to assess the potential of LSI to provide a real-time measure of phototherapy dosimetry in the clinic.

VII. Summary of Results

With the combination of 1) a suitable microvascular model [see Fig. 3(a)], 2) LSI to evaluate blood-flow dynamics [see Fig. 3(b)], and 3) a longitudinal experimental design [see Fig. 4(a)], rapid preclinical assessment of new phototherapies can be achieved. The combination of PDT and PDL irradiation achieves a synergistic effect that reduces the required radiant exposures of the individual phototherapies to achieve persistent vascular shutdown [see Fig. 4(b)], which may lead to a reduction in the complications associated with PDT and PDL therapy. The combination of PDL therapy or PDT with anti-angiogenic agents is a promising strategy to achieve persistent vascular

shutdown (Fig. 5). Integration of LSI into the clinical workflow may lead to surgical image guidance that maximizes acute photocoagulation, which is expected to improve PWS therapeutic outcome (Fig. 6).

VIII. Future Research Directions

Open issues remain related to our basic understanding of the etiology of PWS, the activated signaling pathways following phototherapy, and integration of optical imaging technologies into the operating room to inform clinicians during phototherapy of PWS.

Chemical modulation of PWS. With immunohistochemical analysis of biopsies from PWS, we concluded that different protein kinases may be activated during different stages of PWS development [6]. Our data suggest that use of protein kinase inhibitors may serve as a potential therapeutic protocol for PWS.

Preclinical assessment of blood flow during photochemotherapy. Continued assessment of the dosing, scheduling, and safety of anti-angiogenic agents in combination with phototherapy, is an area of intense activity. In addition to rapamycin, we recently reported on use of topical axitinib, which can modulate multiple signaling pathways associated with angiogenesis, in conjunction with PDL irradiation [128]. Further preclinical assessment of photochemotherapy is expected to yield candidate drug/light combinations for evaluation in clinical trials. LSI will continue to play a critical role in evaluation and translation of photochemotherapy to the clinic.

Intraoperative assessment of blood flow during PDL therapy. Based on our recent intraoperative LSI data (Fig. 6, Video 1) [125], we propose that development of a comprehensive image-guided treatment approach for individualized therapy. This approach is expected to increase the efficacy of each session (regardless of treatment methodology), enhancing PWS removal while reducing the frequency and duration of the treatment course and associated medical care burden. Future work should build upon our initial clinical study, to 1) determine the correlation between acute measurements of blood-flow changes and treatment response, 2) determine why some patients who experience major acute reductions in blood flow have poor treatment responses, and 3) determine how patient demographics (age, PWS anatomic location, gender, etc.) affect the acute reduction in blood flow and treatment response.

Clinical assessment of blood flow during photochemotherapy. Future work should investigate how blood flow changes during photochemotherapy. The observed reduction in blood flow immediately after PDL therapy (Fig. 6, Video 1) [125] may induce local hypoxia and stimulate activation of pro-angiogenic factors. Continued monitoring of blood flow may enable a personalized approach to the administration of anti-angiogenic agents.

Integration of complementary optical imaging technologies into the preclinical and clinical workflow. Future work also should focus on evaluation of refinements to LSI and alternate optical imaging technologies, especially as devices become more user friendly in the clinic.

Doppler Optical Coherence Tomography (OCT). We have demonstrated the potential of Doppler OCT to image changes in the microcirculation associated with PDL irradiation [see Fig. 7(a)] [43], [129]–[131]. Doppler OCT enables detailed, depth-resolved visualization of the perfused microcirculation, with the potential for real-time assessment. With the development of new light sources and continued refinement of algorithms, the speed and the resolution of Doppler OCT has improved, enabling more accurate *in vivo* characterization of PWS skin. For example, improved stability of components can further enhance the signal-to-noise ratio in Doppler flow images. In addition, a faster swept source is expected to increase the imaging speed, and hence extend the imaging area. Finally, integration of GPU-based processing is expected to enable faster calculation of blood flow and real-time 3-D display of the microvasculature in real time.

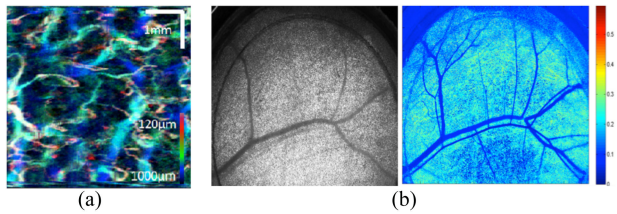

Fig. 7. (a) Maximum intensity projection of Doppler optical coherence tomogram taken from a PWS located on the upper extremity. Adapted with permission from REF. [131]. (b) (Left) Raw speckle and (Right) speckle contrast image of a dorsal window chamber. We collected the raw speckle image with a converted monochrome Canon 7D dSLR camera (LDP LLC, Carlstadt, NJ, USA).

Photoacoustics. First described in biophotonics by Oraevsky *et al.* [132], photoacoustics is a promising approach for noninvasive imaging and characterization of biological tissues. Wang and Gao [133] published a comprehensive review of the technology. Few publications exist on use of photoacoustics to characterize PWS. Viator *et al.* [134] first applied photoacoustics for depth profiling of PWS, and showed reasonable agreement with the depth of vasculature derived from Doppler OCT images. Kolkman *et al.* [135] published images from three PWS and, although a plexus of vasculature was visible on each subject individual vessels were difficult to identify. Seminal developments in photoacoustic technology have enabled impressive imaging of the microcirculation in multiple tissue types, including brain [136], breast [137], and skin [148]. Furthermore, with development of clinic-friendly photoacoustic devices [138] and improvements in imaging speed [138], we believe that photoacoustic technologies should be evaluated for assessment of PWS and that it has potential for surgical image guidance.

Spatial Frequency Domain Imaging (SFDI). With calibration data and model-based fitting of the image set collected at multiple spatial frequencies, SFDI has the unique capability of enabling quantification of spatially resolved optical absorption and scattering parameters, allowing wide-field quantitative mapping of tissue optical properties [139]–[142]. By decoupling the multi-spectral absorption and scattering optical properties, SFDI removes the crosstalk in reflectivity changes resulting from physically distinct contrast mechanisms. With spectral unmixing of the absorption maps, it is possible to achieve quantitative assessment of the oxyhemoglobin, deoxyhemoglobin and total hemoglobin contents, and hemoglobin oxygen saturation.

Early preliminary data suggest the potential of SFDI to study PWS hemodynamics associated with phototherapy [141]. Further development of clinic-friendly SFDI devices [143] are expected to facilitate integration of the technology into the clinical workflow.

Refinements to LSI. We previously reported on the use of a color dSLR camera to perform LSI [144]. Such cameras already are integrated into the general clinical workflow, but the presence of the Bayer filter used to enable color photography, leads to a marked reduction in speckle contrast and hence measurement dynamic range. With use of a monochrome dSLR camera, the advantages of the form factor are maintained, but with the added benefit of full pixel sampling of the speckle pattern due to the absence of the Bayer filter [see Fig. 7(b)]. Also, speckle contrast values are affected not only by changes in blood flow but also by the local optical properties [145]. Novel approaches that account for optical property dynamics, such as coherent SFDI [146] or multiple-exposure LSI [147], can improve on the accuracy of LSI measurements, but currently at the penalty of increased acquisition and processing times. Continued refinement of these methods is expected to improve the quantitative accuracy of LSI for monitoring of phototherapy of PWS, especially in longitudinal studies.

IX. Conclusion

We have reviewed our current knowledge on the etiology and treatment of PWS. Preclinical and clinical experimental studies demonstrate the critical role that optical imaging can play in development of new treatment strategies. Acute photocoagulation and persistent vascular shutdown may be key factors that lead to improved PWS therapeutic outcome, and the associated hemodynamics and biological response warrant further study. Continued integration of noninvasive optical imaging technologies and biochemical analysis collectively are expected to lead to more robust treatment strategies.

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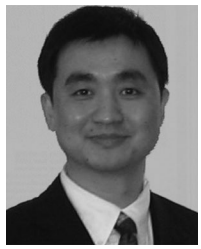

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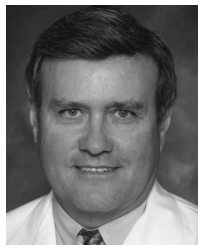

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