

Indocyanine green and protoporphyrin IX fluorescence imaging of inflammation, hypoxia, and necrosis of burns

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Burn injury affects nearly half a million people in the U.S. annually, resulting in over \$1 billion in direct annual treatment costs [1]. Burn injuries leave survivors with permanent scarring and adverse long-term sequelae. A main goal in modern burn management is reducing scarring for long-term wellbeing and quality of life, which relies on accurately assessing tissue damage. Furthermore, burns can progressively deepen and widen in the first few days after injury, resulting from excessive inflammation, compromised perfusion, hypoxia, or necrosis burden [2]. There are no objective and reliable methods clinically to assess these early pathophysiological processes and tissue damage early after burn injury to aid burn diagnosis and surgical decision making.

Fluorescence imaging has emerged as one of the most popular imaging modalities in preclinical research and clinical practice given its minimal invasiveness, real-time capability, and easy integration into clinical flow [3]. Two widely used Food and Drug Administration (FDA) approved human contrast agents in diagnostic fluorescence imaging guided surgery are indocyanine green (ICG) and protoporphyrin IX (PpIX). ICG angiography (ICGA) and second-window ICG (SWIG), which involves delayed imaging (usually 24 hours) after high dose (up to 5 mg/kg) ICG administration, have been studied in animal models and burn patients to predict burn depth and assist in surgical planning by measuring compromised perfusion or necrotic burn tissue [4, 5]. In contrast, PpIX is intrinsically produced in most tissues through the heme biosynthesis pathway. The selective accumulation of PpIX (prompt fluorescence, PF) and later quenching by tissue O₂ (delayed fluorescence, DF) have been used to demarcate glioma tumors and to identify tissue hypoxia [6, 7]. Our goal is to evaluate whether a combined ICG and PpIX fluorescence imaging can comprehensively characterize early burn pathophysiological processes including necrosis, compromised perfusion, vascular

permeability, and tissue hypoxia in rodent and human burn wounds.

The animal study was conducted in accordance with protocols approved by the University of Wisconsin Institutional Animal Care and Use Committee. Nude mice subjected to full thickness burns had fluorescence imaging at the burn site and its contralateral non-burn regions daily over 4 days post-burn. The mice were grouped depending on the time of ICG administration after burn, with a sample size of four to six mice per group.

The human subject study ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study?term=NCT05593523): NCT05593523) was approved by the University of Wisconsin Institutional Review Board. Two patients who admitted with burns were recruited for fluorescence imaging on the second day post-burn or the day prior to surgery, and 24 hours later.

Burn tissue biopsies were obtained and processed for histologic analysis to evaluate microscopic distribution of ICG and PpIX, tissue viability and presence of bacteria. Statistical differences at a significant level of 0.05 were conducted in Prism 8.0 (GraphPad Software, Inc., CA). Detailed materials, methods, and imaging devices are provided in Supplemental Appendix.

ICG was administered intravenously at 0, 24, or 48 hours post burn (hpb) to evaluate vascular response to burn over time. ICGA images showed that the central burn region had reduced fluorescence with a peripheral enhancing zone of hyperemia due to vascular dilation and permeability in response to injury (Figure S1b). Signal-background ratio (SBR) was quantified in burns at the peak of ICGA and showed hyperperfusion (SBR > 1) immediately after burn, hypoperfusion (SBR < 1) 24 hours later, and then normalized perfusion (SBR ~ 1) 48 hours later (Figure S1c). Vascular permeability increased in the area surrounding the

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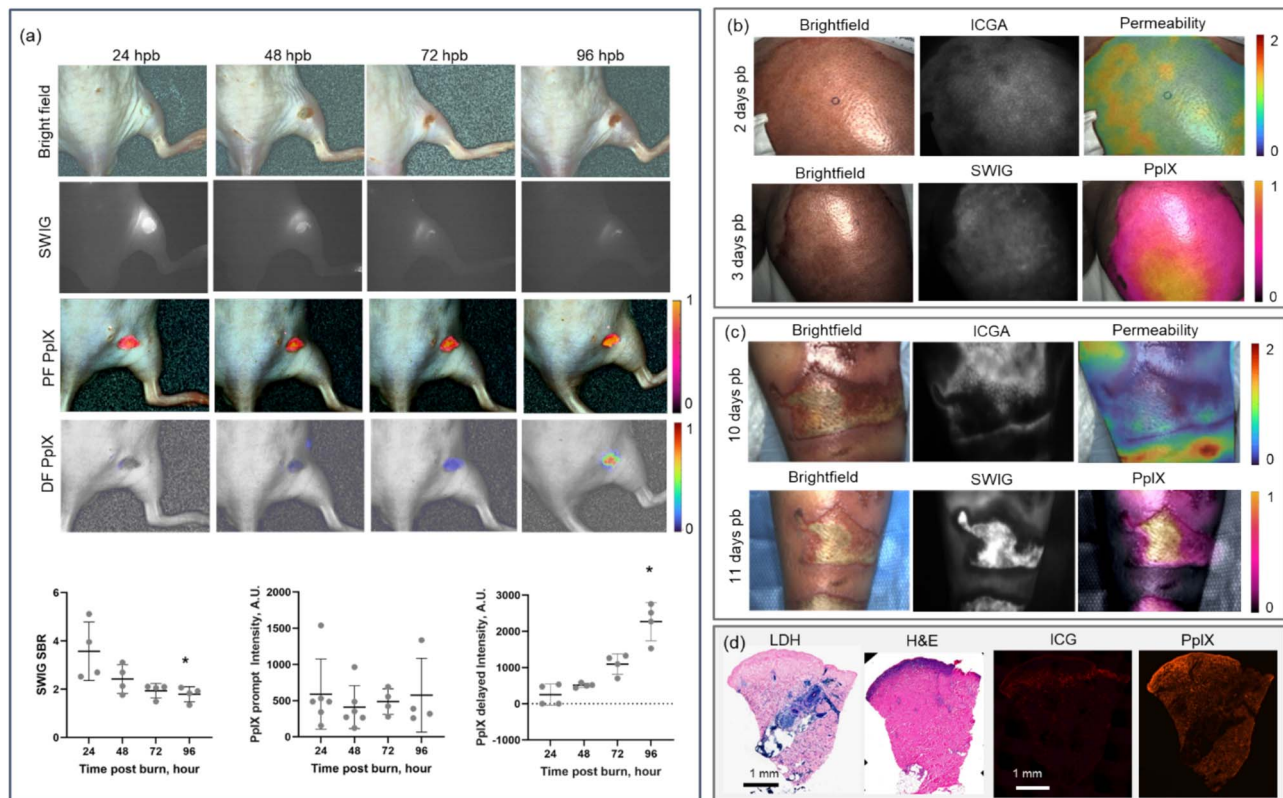


Figure 1. ICG and intrinsic PpIX signals detected in mouse and human burn wounds. **(a)** Longitudinal changes in SWIG, PpIX prompt (PF) and PpIX delayed fluorescence (DF) signals in burn wounds over 4 days in mice that had ICG administered at 0-hour post burn (hpb). Burn wounds in **(b)** patient 1 with a partial thickness burn and **(c)** patient 2 with a full thickness burn emitted endogenous PpIX signals during ICGA and SWIG imaging. **(d)** ICG and PpIX signal detected in a tissue biopsy from patient 1 and co-localized in necrotic tissue as shown in lactate dehydrogenase stain (LDH with blue stain = viable cells). H&E, hematoxylin and eosin. Blue circle in (b) corresponds to area of tissue biopsy for (d). *, $P < 0.05$ compared to 24 hpb

burn immediately after injury but diminished 24 hours later (Figure S1d, Video S1).

To examine the change in SWIG in burn wounds after a single injection of ICG administered at 0 hpb, SWIG images were taken daily in mice for four days (Figure 1a). As expected, SWIG SBR was highest 24-hours post-injection and reduced over time. The SWIG intensity and SBR were also dependent on the time when ICG was administered after burn (Figure S2). SWIG SBR was highest when ICG was administered immediately after burn, while the signal decreased significantly when ICG was administered at 24 hpb and 48 hpb.

Intrinsic PpIX PF was shown to selectively accumulate in burn wounds at 24 hours and persisted 4 days post-burn (Figure 1a). Similarly, the intensity of PpIX DF increased in burn wounds from 24 to 96 hpb, indicating that these burns became progressively hypoxic over time. At 96 hpb, the intensity of PpIX DF was significantly higher than 24 hpb, and the center of the burn appears to have the highest intensity of PpIX DF.

To confirm that intrinsic PpIX can be detected in human burns, PpIX images of burns in patients were obtained immediately before ICGA and SWIG imaging in two patients (Figure 1). Patient 1 presented with a partial thickness burn on the upper leg (Figure 1b). ICGA displayed higher fluorescence in the burn region compared to the non-burn region potentially due to hyperperfusion in the underlying

non-injured tissue. Higher vascular permeability was observed at in regions with darker ICGA. After 24 hours, burn regions demonstrated weak SWIG signals consistent with the partial thickness nature of the burn. On both days, PpIX signals were detected in the burn region. Patient 2 presented with a full thickness burn on the lower leg (Figure 1c). As expected, the full thickness burn had no ICGA signal, consistent with lack of perfusion whereas the peripheral region around the burn had hyper-fluorescence and high vascular permeability. SWIG and PpIX signals were higher in the burn wounds than the peripheral region. Patient 2 had lower ICGA and higher SWIG fluorescence in the full thickness burn wounds compared to those in the partial thickness burn in Patient 1. Vascular permeability observed in the full thickness burn area in Patient 2 (despite high permeability at the periphery) was lower than that observed in the partial thickness burn of Patient 1 presumably due to complete destruction of vasculature.

At the microscopic level, PpIX and ICG signals were compared to cell viability (LDH), skin architecture (H&E) and bacteria colonization (Gram stain) (Figure 1c, Figure S3 and Figure S4). The high levels of PpIX and ICG were found co-localized with non-viable regions in burn wounds, which were infiltrated with a mixture of numerous inflammatory cells and gram-positive or gram-negative bacteria based on the color, size and morphology in gram stain.

Burn wounds elicit an excessive and prolonged inflammatory response and one hallmark of inflammation is vasodilation and microvasculature structural changes. The high affinity of ICG to plasma proteins enables ICG to visualize tissue perfusion in intact vessels and to quantify vascular permeability in leaky vessels when the protein-ICG complex extravasates [8]. Here, we used ICGA to quantify tissue perfusion and vascular permeability in burns over time. Our ICGA data revealed that these inflammation-induced vascular responses were strongest immediately after the burn and decreased to much lower levels at later time points.

Since chronic or excessive hypoxia hinders healing and may lead to burn wound progression, the ability to monitor tissue hypoxia would be beneficial for earlier treatments to prevent progression of injury. This study demonstrated that PpIX DF mapped the spatial and time course of tissue hypoxic response to a full thickness burn wound in rodents *in vivo*. The increase of PpIX DF in burn wounds over time indicated that the burn wounds became progressively more hypoxic, possibly due to impaired perfusion and hypermetabolic activities that further reduce tissue oxygen levels.

This study also supports the utility of both SWIG and PpIX PF in burn demarcation at different times post burn. SWIG and PpIX PF were found selectively accumulated and co-localized with necrotic tissue in both mouse and human burns. SWIG accumulation displayed dependency on perfusion and vascular permeability (degree of inflammation) supporting the enhanced permeability and retention effect as a mechanism. SWIG signals were highest at early after burn and reduced over time when initial inflammation decreases in intensity after thermal injury. In contrast, intrinsic PpIX fluorescence increased in burn wounds over 4 days. Necrotic avidity of ICG has been proposed as the dominant mechanism of ICG retention in burn and is supported by this study [5]. The mechanisms by which intrinsic PpIX accumulates in burn wounds remain unclear but likely involve cell (inflammatory cells and bacteria that produce PpIX [9, 10]) migration into the burn tissue, since non-viable cells have no capacity to synthesize PpIX. In this study, we confirm the presence of inflammatory infiltrates and/or bacteria in the burned tissue in animals and patients. However, this does not explain the accumulation of PpIX in its entirety and warrants further study.

To our knowledge, this study is the first to explore combined ICG and PpIX fluorescence imaging to comprehensively characterize early burn pathophysiological processes, including necrosis, altered perfusion, vascular permeability, and tissue hypoxia in a rodent model of burn. The clinical relevance of this technique is also explored in a human pilot study, demonstrating that some of the features seen in the mice could be found in the human burn tissues, increasing the likelihood of future clinical translation. Further investigation into origins of the signals may lead to the development of image-based biomarkers for diagnostic or surgical guidance to identify irreversibly damaged tissue.

Abbreviations

ANOVA: Analysis of Variance; DF: Delayed Fluorescence; hpb: Hours Post Burn; ICG: Indocyanine Green; ICGA: Indocyanine Green Angiography; PpIX: Protoporphyrin IX; PF: Prompt Fluorescence; ROI: Region of Interest; SBR: Signal-to-background Ratio; SSIM: Structural

Similarity Index Matrix; SWIG: Second Window Indocyanine Green; 5-ALA: 5-Aminolevulinic Acid.

Author contributions

A.L., M.O. A.G., and B.P. conceived the experiment(s), A.L., M.O, and M.R. conducted the experiment(s), A.L. performed ICG imaging and data analysis, M.O performed PpIX imaging and analysis as well as vascular permeability data analysis for ICGA, M.O and B.P developed PpIX instrumentation, A.L. and M.O adapted microscopy setup, J.P performed histology, A.G and M.J acquired patient data, A.L and M.O wrote the manuscript. All authors reviewed and edited the manuscript.

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Supplementary data

Supplementary data is available at *Burns & Trauma Journal* online.

Ethic approval and consent to participate

The animal study was conducted in accordance with protocols approved by the University of Wisconsin Institutional Animal Care and Use Committee (IACUC No. M006719). The human subject study (ClinicalTrials.gov: NCT05593523) was approved by the University of Wisconsin IRB (IRB No.2022–1070). Written informed consent was obtained from the patients recruited in this study.

Consent for publication

Not applicable.

Conflict of interests

The authors declare that they have no conflict of interest.

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Data availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request. The MATLAB codes that analyzed vascular permeability in this study are available from the corresponding authors upon reasonable request.

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