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Accurate Prediction of Tissue Viability at Post-Operative Day 7 Using Only 2 Intraoperative Sub-Second Near-Infrared Fluorescence Images

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Abstract

Background—The ability to predict the future viability of tissue, while still in the operating room and able to intervene, would have a major impact on patient outcome. Although several objective methods to evaluate tissue perfusion have been reported, none to date has sufficient accuracy.

Method—In eight Sprague-Dawley rats, reverse McFarlane dorsal skin flaps were created. Continuous near-infrared (NIR) fluorescence angiography using indocyanine green (ICG) was performed immediately after surgery, for a total of 30 min. These dynamic measurements were

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Conflict of Interest

John V. Frangioni, M.D., Ph.D.: Dr. Frangioni is founder, owner, and CEO of Curadel, LLC, which has licensed FLARE imaging systems and contrast agents from the Beth Israel Deaconess Medical Center.

Author's Contributions

HW, BTL, and JVF designed the experiments. HW, CV, JA, MAP, OH and BTL performed the experiments. HW, CV, JA, BTL and JVF reviewed, analyzed, and interpreted the data. HW, BTL and JVF wrote the paper. All authors discussed the results and commented on the manuscript.

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used to quantify ICG biodistribution and clearance, and to develop a simple metric that accurately predicted tissue viability at post-operative day (POD) 7. The new metric was compared to previously described metrics.

Results—Reproducible patterns of ICG biodistribution and clearance from the flap permitted quantitative metrics to be developed for predicting flap viability at POD 7. Previously described metrics, which set the boundary between healthy and necrotic tissue as either 17% or 25% of peak NIR fluorescence at 2 min post-ICG injection underestimated the area of necrosis by 75% and 48%, respectively. Our data suggest that both the shape and area of clinical necrosis occurring at POD 7 can be predicted intraoperatively, with the boundary defined as NIR fluorescence intensities of 40-55% of peak fluorescence measured at 5 min.

Conclusion—Two 750 msec intraoperative NIR fluorescence images obtained at times 0 and 5 min post-injection of ICG accurately predicted skin flap viability 7 days after surgery.

INTRODUCTION

Necrosis is a debilitating morbidity associated with many different types of surgery, including vascularized composite allografts¹, mastectomy skin flaps^{2,3}, local and free tissue transfer, as well as many other clinical situations where tissue is at risk for decreased perfusion. Although incidence is gradually decreasing as surgical techniques improve, it remains rather high at 4.9-16%⁴⁻⁷. In addition to cosmetic concerns, necrosis and tissue loss can lead to infection, longer hospital stays, increased cost of care, and the delay of oncologic treatment⁸. Therefore, the development of early and accurate predictors of skin and tissue viability is desperately needed.

The gold standard today for the assessment of tissue perfusion is comprised of several qualitative clinical indicators, such as skin color, hardness, temperature, capillary refill, and dermal bleeding. However, because these assessments are subjective, their interpretations are variable, and their reliability questionable^{9,10}. For this reason, many objective methods for assessing tissue perfusion have been developed such as fluorescein angiography, laser Doppler imaging, thermography, and tissue oxygen saturation measurements¹¹⁻¹⁴. To date, though, none have achieved the necessary accuracy to enable intraoperative intervention.

We and the other investigators have previously reported that near-infrared (NIR) fluorescence angiography using indocyanine green (ICG), which is commercially available and FDA-approved for skin perfusion mapping, provides real-time intraoperative identification of perforator vessels and can help predict flap viability¹⁵⁻¹⁸. The metrics most widely used in the literature for this application include a threshold of from 17% to 25% of peak NIR fluorescence, measured within 2 min post-injection of indocyanine green (ICG), in order to define the area of skin at risk for failure¹⁹⁻²⁴, and therefore the boundary between future healthy and necrotic tissue. However, statistical analysis of these metrics, such as sensitivity and specificity, is lacking or is not satisfactory^{22,23}.

In this study, we employed dynamic ICG angiography over a period of 30 min post-injection to find patterns of ICG biodistribution and clearance that might better predict long-term flap outcome using simple, rapid, intraoperative NIR fluorescence images.

MATERIALS AND METHODS

Animal Models

Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-certified facility and were studied under the supervision of Beth Israel Deaconess Medical Center's Institutional Animal Care and Use Committee (IACUC) in accordance with approved institutional protocols #030-2013. Male Sprague-Dawley rats ($n = 8$) averaging 365 g (Charles River Laboratories, Wilmington, MA) were induced, and anesthesia was maintained, with 2-3% isoflurane. Meloxicam (Bimeda, Oakbrook Terrace, IL) was administered subcutaneously 20 min prior to the end of surgery and at 24 h for analgesia.

Surgical Procedures

The dorsum of anesthetized animals was shaved and swabbed with betadine. McFarlane and reverse McFarlane rat dorsal skin flaps have been described previously^{25,26}. As shown in Figure 1A, a caudally based 3×10 cm skin flap including the panniculus carnosus muscle was incised preserving the base of the flap. After the incision of the skin, the dissection of the subcutaneous connective tissues was performed bluntly from the level of scapulae to the level of iliac crests. The skin flaps were elevated vertically with the base over the bilateral iliac crests, while carefully confirming there was no active bleeding on the back of the flaps and on the surface of the muscle. The flaps were then sutured with 4-0 nylon suture back to their original location (Figure 1B).

NIR Fluorescence Angiography

The dual-NIR channel FLARE imaging system has been described in detail previously²⁷. In this study, the illuminance of white light (400-650 nm) and 760 nm NIR excitation light were 40,000 lx and 11.0 mW/cm², respectively. Color images (67 msec exposure) and >785 nm NIR fluorescence images (750 msec) were acquired over a 15 cm diameter field of view (FOV) and merged with custom software. The exposure time of 750 msec was chosen to ensure that all measured NIR fluorescence intensities were within the linear range of the 12-bit camera. The imaging system was positioned at a distance of 18 inches from the surgical field.

ICG was purchased from MP Biomedicals (Santa Ana, CA) and dissolved in distilled water at 2.5 mg/ml (3.2 mM) immediately prior to injection. 0.5 mg/kg of ICG was injected into the rats intravenously 15 min after completion of flap suturing. Images were recorded from the time of the injection to 30 min post injection, every 2 s for the first 2 min, every minute until 15 min, and every 5 min until 30 min. After 30 min observation, animals were allowed to recover from anesthesia in a separate cage and housed singly for 7 days, with color images of the skin flap taken at postoperative day (POD) 1, 2, 3, and 7. Clinical assessment including skin color, hardness, warmth, and capillary refill were also performed on these dates. Euthanasia was performed immediately after POD 7 imaging and assessment and some of the 5×5 mm of skin tissues was resected from each skin flap for the histological analysis after euthanasia. A group of 3 independent, experienced plastic surgeons, blinded to the results of intraoperative NIR fluorescence imaging, evaluated flap necrosis using the

color image at POD 7 and drew a boundary between what they believed was the viable and necrotic areas of the flap.

Quantitative Assessment and Statistical Analysis

At 2 and 5 min post-ICG time points, the fluorescence intensity (FI) of each pixel over the flap was quantified. A small region of interest (ROI) for background was drawn outside of the flap, in an area of maximally perfused skin, and its FI was also quantified. The signal-to-background ratio (SBR) was defined as $SBR = (FI \text{ of the pixel at 2 or 5 min} - FI \text{ of the same pixel at 0 min}) / (FI \text{ of BG at 2 or 5 min} - FI \text{ of BG at 0 min})$. Subtraction of the 0 min values eliminated autofluorescence as a confounding variable. Each threshold was analyzed using the SBR in each pixel on the flap using the 2 or 5 min images. The boundaries of necrosis drawn by three different plastic surgeons were translated to a graph composed of each pixel on the POD 7 images and consensus curves were drawn by calculating the mean and standard error of the mean (SEM) of those boundaries from the ground truth. The region of necrosis established by each boundary, the consensus curve on the color picture, and the region of each threshold on NIR images were outlined and the area of each region was measured using Image J (National Institutes of Health, Rockville Pike, MD). Quantitative assessments were performed using FLARE custom software and MATLAB (MathWorks, Natick, MA). A one-way ANOVA among each area was performed using GraphPad Prism Version 6.0 (GraphPad Software Inc., La Jolla, CA) and 2-D correlation coefficient was calculated among each region using MATLAB. A P value < 0.05 was considered significant.

RESULTS

Animal Model

We chose a reverse McFarlane dorsal skin flap in rat (Figure 1) as the model is well validated, reproducible, and readily available for confirmation by other groups^{25,26}. This model also produces a pattern of skin necrosis that progresses over 7 days (Figure 2A), as well as histological changes (Figure 2B), which are consistent with those seen clinically in failed flaps. An important point is that animals must be followed until POD 7 because skin necrosis continues to mature between POD 3 and POD 7 (Figure 2A). The necrotic area of the flap becomes either black and hardened or brown but not hardened. Histologically, blackened areas (Figure 2B, a) displayed full-thickness necrosis of the skin including epidermis, dermis, skin appendages and subcutaneous fat, and underlying muscle while brown-colored areas (Figure 2B, b) displayed necrosis of the epidermis, dermis, and appendages to the level of the mid dermis with inflammation. As expected, surviving skin had complete preservation of the epidermis, dermis, skin appendages, subcutaneous fat, and muscle (Figure 2B, c).

Intraoperative NIR Fluorescence ICG Angiography

The NIR fluorescence signal produced by ICG typically appeared within 5 s after bolus injection then quickly spread throughout the flap (Figure 3). The signal reached maximum FI within 30 s. Interestingly, although the absolute signal intensity decreased over time, the extent of ICG penetration into the flap expanded over the period between 2 min and 5 min then stabilized. As shown in Figure 3, the sharpest delineation between the skin having high

and low perfusion was seen at 5 min. Measurements at 5 min were therefore used in all subsequent experiments. Importantly, there was no perceptible change in the color image over this 30 min period, except for minor purpura in the area of grasping by forceps.

Defining an Intraoperative Metric that Predicts Future Tissue Viability

Several investigators have reported that the boundary defined from 17% to 25% of peak NIR fluorescence measured at 1 to 2 min post-ICG intraoperatively can predict flap outcome post-operatively^{19–24}. In order to quantify the precise boundary of flap necrosis at POD 7, we employed 3 experienced, board-certified plastic surgeons to review the color images from POD 7 and draw a boundary between healthy and dead skin (Figure 4A). All surgeons were blinded to the intraoperative NIR fluorescence results. In most animals, the consensus boundary differed minimally, but in some there was disagreement about what was healthy and what was dead, highlighting the subjective nature of clinical assessment. In most animals, all of the surgeons classified both black-colored and brown-colored areas of the flap as clinical necrosis.

The boundary drawn by the plastic surgeons was used as the defined territory for tissue viability at POD 7 and for quantitative comparison to intraoperative NIR fluorescence images. The areas of individual flap necrosis of all 8 rats are shown in Supplemental Digital Content 1, Table shows the area of individual flap necrosis for each rat at POD 7, INSERT LINK, and the comparison of 2-D correlation coefficients among the necrotic areas evaluated by different plastic surgeons is shown in Supplemental Digital Content 2, Figure shows the Comparison of 2-D correlation coefficients among the necrotic areas evaluated by three different plastic surgeons at POD 7, INSERT LINK. The boundaries drawn by all 3 evaluators were highly correlated in all 8 rats. As shown in Figure 4A, we compared the literature metrics of 17% or 25% peak fluorescence at 2 min post-injection of ICG to a range of peak NIR thresholds measured at 5 min. As shown graphically in Figure 4B and numerically in Table 1, in terms of the area, NIR peak thresholds of 17% or 25% at 2 min were statistically and significantly different from true necrosis at POD 7, underestimating the extent of necrosis by 75% and 48%, respectively. Measurements at 5 min, however, had a much stronger correlation with clinical outcome when using 40% to 55% of peak NIR fluorescence as the threshold. There was no statistical difference between the boundaries defined by 40% to 55% intensities. In addition, the shape of the region of 17% to 60% of peak NIR fluorescence was compared with clinical necrosis, with the comparison among each peak NIR fluorescence shown in Figure 4C. Again, the region defined by 40% to 55% of peak NIR fluorescence at 5 min showed high correlation with clinical outcome with $r > 0.7$, while the regions defined by 17% and 25% at 2 min were much less correlated.

DISCUSSION

A Holy Grail of surgery is the ability to predict future outcome based on intraoperative metrics. If provided this information, a surgeon would be able to intervene before morbidity could develop. The need for such a technology is profound, especially since over 90% of surgeons still rely on subjective clinical assessment to determine tissue perfusion²⁸.

Our study builds upon previous attempts in the area of intraoperative prediction of future clinical outcome, and provides a simple metric that is easy to measure with devices and drugs already approved for this indication. NIR fluorescence angiography using ICG has been compared directly to fluorescein angiography²² and laser Doppler imaging²⁹ and found to be superior. There is currently no standard, however, for quantitative metrics using ICG angiography, and most published studies focused on measurements obtained between 1-2 min after injection^{22,30,31}.

Our key finding is that ICG undergoes changes in its biodistribution and clearance within a skin flap over a time frame much longer than previously reported. By waiting for the extent of ICG penetration into the flap to peak and stabilize, one is able to measure a boundary intraoperatively that appears to perfectly correlate with the future viability of the flap at POD 7. Because only two sub-second measurements are required, one prior to ICG injection and one at 5 min post-injection, the technology does not impede clinical workflow. Both the area analysis and the shape analysis concluded that the 40% to 55% of peak NIR threshold at 5 min are the most accurate predictors of future clinical outcome. While a threshold of 40% of peak NIR fluorescence was closest to clinical necrosis from the standpoint of surface area, 45%-50% of peak NIR fluorescence had higher correlation with outcome at POD 7 in terms of the shape. We believe this discrepancy resulted from the obvious contraction and transformation of the necrotic skin at POD 7 relative to skin measured intraoperatively. We therefore believe that a threshold of 50% of peak NIR fluorescence measured at 5 min is the best predictor for future studies. Applying a flat field correction, and/or correction for NIR absorption of the skin could also further improve precision of the measurements.

A major unanswered question is the mechanism of the delayed biodistribution and clearance and why it correlates so well to future tissue viability. We believe that the combination of hypoxic and mechanical injury incurred when raising the flap triggers an irreversible sequence of events that has, as either a marker or primary pathogenesis, changes in local perfusion. These changes are missed when measurements are taken too early after injection because low peak thresholds and early times are mainly measuring arterial inflow. Yet there is an entire zone of altered perfusion, which is not destined to fail, and which permits ICG to flow in and out, but at a slower rate than in otherwise healthy tissue. This “intermediate” zone would not have to be repaired or resected intraoperatively because it appears to recover in all cases. Using previous metrics, a much larger area than necessary would be labeled a failure.

Our study is not without limitations. First, the present study had small sample size. The results need to be confirmed in the larger animal study first, then in patients undergoing skin flaps for various indications. Because the absolute incidence of necrosis is rather low, though, confirmatory studies will necessarily be large. Second, we used a dose of 0.5 mg/kg in rats. This dose would scale, by weight, to an injected dose of 35 mg in an adult human or, by body surface area, to a dose of 7 mg. Both doses are within the approved dose range for ICG, but defining the lowest dose required in humans remains to be determined. Finally, the timing of the ICG relative to suturing of the flap may differ in rat and human, as might the exact threshold of peak NIR fluorescence at 5 min that correlates with clinical outcome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Caudally-based reverse McFarlane dorsal skin flap in rats

A. Schematic drawing of the skin flap.

B. Preoperative markings (top row) and sutured skin flap immediately after surgery (bottom row).

Scale bars = 1 cm.

Figure 2. The development of skin flap necrosis during the post-operative period

A. Clinical necrosis on POD 3 and POD 7 were compared clinically for each rat, with 2 representative animals shown. Scale bars = 1 cm.

B. Histological analysis of skin flaps at POD 7 biopsies. Resected tissues included black-colored hardened areas (a), brown-colored areas (b), or normal-appearing areas (c). Each section was stained with H&E and observed using brightfield microscopy. Scale bars = 1 cm on the image of rat skin flap and 1 mm on the histological images.

Figure 3. Intraoperative NIR fluorescence angiography using ICG

0.5 mg/kg of ICG was injected intravenously and images acquired at time 0 (injection) and over the next 30 minutes in N = 8 Sprague-Dawley rats averaging 365 g total. Shown are a representative color image (67 msec exposure) and NIR fluorescence images (750 msec exposure) at times 0, 0.5, 2, 5, and 30 min post-injection. All NIR fluorescence images have identical exposure times and normalizations. Scale bars = 1 cm. Abbreviations used are: NIR, near-infrared; ICG, indocyanine green.

Figure 4. Quantitative comparison between intraoperative NIR images and clinical necrosis at POD 7

A. Intraoperative color and NIR fluorescence images (at 2 and 5 min post-injection of ICG) were acquired (750 msec exposure) and compared to the POD 7 color image. NIR thresholds of either 17% or 25% of peak fluorescence at 2 min post-injection, or 35% to 60% of peak fluorescence at 5 min were quantified and the area of necrosis defined by the dotted lines. The consensus curve for the POD 7 image was calculated using the mean $\pm$ SEM of boundaries drawn by 3 independent plastic surgeons blinded to NIR fluorescence results. All NIR fluorescence images have identical exposure times and normalizations. Scale bars = 1 cm.

B. The area predicted by intraoperative NIR fluorescence imaging at 2 and 5 min post-ICG injection compared to clinical necrosis at POD 7 for various NIR thresholds. * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$.

C. Comparison of 2-D correlation coefficients between the shape of the region defined by each peak NIR fluorescence and the region of clinical necrosis at POD 7.

Abbreviations used are: ICG, indocyanine green; NIR, near-infrared; POD, postoperative day; SBR, signal-to-background ratio; SEM, standard error of the mean.

Table 1
Measured Areas of Skin Necrosis using Different Methods

Time of Measurement	Measurement Type	Area of Necrosis (%)
2 min	17% of Peak NIR Threshold	9.9 ± 7.6
2 min	25% of Peak NIR Threshold	21.0 ± 10.4
5 min	35% of Peak NIR Threshold	31.0 ± 5.7
5 min	40% of Peak NIR Threshold	40.7 ± 6.4
5 min	45% of Peak NIR Threshold	44.8 ± 6.3
5 min	50% of Peak NIR Threshold	49.4 ± 8.3
5 min	55% of Peak NIR Threshold	50.6 ± 7.5
5 min	60% of Peak NIR Threshold	55.0 ± 6.2
POD 7	Clinical Necrosis	41.8 ± 3.5

Abbreviations used: NIR = near-infrared.