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# Hyperintensity of Integrin-Targeted Fluorescence Agent IntegriSense750™ Accurately Predicts Flap Necrosis Compared to Indocyanine Green

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## Abstract

**BACKGROUND—**Flap necrosis is a feared complication of reconstructive surgery. Current methods of prediction using indocyanine green (ICG) lack specificity. IntegriSense750™ is a fluorescence agent that binds sites of vascular remodeling. We hypothesized that IntegriSense750™ better predicts flap compromise compared to ICG.

**METHODS—**Fifteen mice underwent lateral thoracic artery axial flap harvest. Mice received an injection of ICG (n=7) or IntegriSense750™ (n=8) daily from post-operative days (POD) 0–3 and were imaged daily. Mean signal-to-background ratios quantified the change in fluorescence as necrosis progressed, then compared.

**RESULTS—**Mean signal-to-background ratio was significantly higher for IntegriSense750 ™ compared to ICG on POD 0 [1.47 ± 0.17 vs. 0.86 ± 0.21, p=0.01] and daily through POD 3 (2.12 ± 0.70 vs. 0.96 ± 0.29, p<0.001).

**CONCLUSIONS—**IntegriSense750™ demonstrates increased signal-to-background ratio at areas of flap distress compared to ICG which may increase identification of flap necrosis and improve patient outcomes.

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**Keywords**

Axial pattern flap; Murine flap model; Flap necrosis; Indocyanine green; Integrisense750

## INTRODUCTION

Pedicled axial flaps are an important reconstructive option following oncologic resection especially in a salvage surgery or in those patients with medical comorbidities precluding the use of microvascular free flaps.[1] Flap failure for axial flaps is a rare but major complication that threatens the integrity of the reconstruction and can cause significant morbidity. Failure of axial flaps is usually secondary to insufficient perfusion of the rotated tissue, which leads to distal necrosis in the flap. This is the result of either poor flap design, vascular pedicle compromise, or poor wound healing.[2,3] To prevent tissue necrosis, an adequate perfusion of the skin flap is essential with sufficient blood flow from arteries through capillaries to draining venules.[4]

Close monitoring of the pedicled flap is critical to its success, as early signs of flap failure could alter the clinical management course of patient care. Perfusion assessment of a pedicled flap is usually based on subjective clinical findings such as skin turgor, color, capillary refill, and dermal bleeding.[5] Areas of borderline perfusion and watershed areas that cross adjacent angiosomes are more difficult to assess with these subjective clinical strategies.

Indocyanine green (ICG) fluorescence angiography is an established and objective tool to assess flap perfusion. ICG has gained popularity for its minimal invasiveness and good sensitivity (95–100%) and specificity (98.8–100%) in assessing flap perfusion.[5,6] ICG is a water-soluble, tricarbocyanine dye that absorbs light in the near-infrared spectral range and fluoresces maximally at 835nm.[7] It demonstrates strict occupancy to the intravascular space and has a short half-life of 2.5–4 minutes.[7] ICG is most commonly used in designing fasciocutaneous pedicled flaps within the head and neck such as supraclavicular artery island, submental, and paramedian forehead flaps.[8,9,10]

ICG is widely distributed in the intravascular space and its activity is not specific to watershed areas at risk of necrosis. Because of this, ICG does not adequately delineate between healthy and non-viable tissue due to vascular compromise. Therefore, there is a need for improved imaging agents to more specifically assess necrosis. A targeted fluorescent agent to directly evaluate vasculature provides a method to better predict areas of threatened necrosis. IntegriSense750™ (VisEn Medical, Bedford, MA) is a fluorescence imaging agent consisting of a near-infrared fluorochrome coupled to a non-peptide small molecule integrin $\alpha_v\beta_3$ antagonist.[11] Integrins are transmembrane cell surface receptors that mediate signal transduction, cell-to-cell interactions, and cell-to-extracellular matrix adhesion, and are thought to congregate in areas of vascular remodeling. We hypothesized that IntegriSense750 would predict flap compromise earlier and more accurately than ICG in a murine axial flap model because of its binding specificity. If Integrisense750 can better predict areas of flap compromise before clinical signs, it has the potential to be an objective assessment tool in flap monitoring.

# MATERIALS AND METHODS

## Animal Models

All animal procedures were approved by the International Animal Care and Use Committee (IACUC) of the University of Alabama at Birmingham. SKH-1 nude, immunocompetent mice (Charles River Laboratories, Hartford, CT, USA), aged four-six months, were used for this study. Mice were housed in a pathogen-free environment, allowed free access to food and water, and were kept on a standard cycle of 12 hours of light and 12 hours of darkness.

## Flap Harvest

The lateral thoracic artery supplies the majority of the mouse dorsum on each side. The lateral thoracic artery-based flap can be used to study distal flap necrosis by designing its length to cross into another angiosome, resulting in inadequate vascularization of the distal portion of the flap. Fifteen mice were anesthetized with isoflurane (VetOne®, Boise Idaho; Distributed by MWI Animal Health®), and the dorsum was disinfected with 70% isopropyl alcohol wipes. Mice were positioned on a sterile operating board under a direct heating lamp to ensure body temperature was maintained throughout surgery. A marking pen was used to draw a 1×4 cm flap overlying the right side of the dorsum based on the lateral thoracic artery. A length of 4 cm was chosen to ensure the distal end of the flap crossed into the second angiosome supplied by the deep circumflex iliac artery. A width of 1 cm was chosen in order to surgically divide contributions from the intercostal perforators. These flap dimensions were based on a prior study demonstrating that a lateral thoracic artery flap with dimensions 1.5×3.5 cm avoids distal ischemic necrosis.[12] Boundaries of the flap were the midline of the dorsum (medial), right axillary line (lateral), infrascapular line (superior), and the iliac crest (inferior). Using sterile technique, flaps were raised in a caudal to cranial direction and consisted of skin, subcutaneous tissue, and a thin layer of panniculus carnosus muscle. Care was taken to preserve the pedicle as it entered the skin flap from a superolateral direction. Hemostasis was achieved with direct pressure. After the flap was elevated, it was sutured down in its original position with simple interrupted 5–0 polypropylene suture. After the procedure, mice were given a subcutaneous injection of carprofen/buprenorphine (manufactured by Vericore Limited Dundee, United Kingdom and distributed by Pfizer Animal Health, Pfizer, New York, NY, USA) for post-procedural pain control. The duration of each procedure was approximately 45 minutes. Post-procedure, flaps were monitored by visual inspection.

## Imaging agent administration

Immediately following surgery, mice received a single tail vein injection of either ICG (PerkinElmer, Waltham, MA USA) (n=7) or IntegriSense750 (PerkinElmer, Waltham, MA USA) (n=8). ICG was given at a dose of 0.8 micrograms per mouse. IntegriSense750 was given at a dose of 2 nmol per mouse per manufacturer recommendations. Mice were injected daily from post-operative days (POD) 0–3.

### Imaging analysis of flap necrosis

After each injection, flaps were imaged using the Luna Imaging System (Novadaq Technologies Inc., Toronto, ON Canada). Flaps were imaged immediately following surgery, 3 hours post-surgery, and then daily for 5 days. Quantitative analysis was performed using the Novadaq onboard system software (SPY-Q). Regions of interest were drawn based on the area of necrosis seen on POD 5 as determined by a reconstructive head and neck surgeon. This region of interest represented the area of ultimate necrosis. Background values were acquired from areas superior to the necrotic area that were deemed viable tissue. Pixel intensity from regions of interest is provided by the SPY-Q software as Relative Fluorescent Units. Signal-to-background ratio was calculated from the Relative Fluorescent Units of POD 5-based region of interest divided by the Relative Fluorescent Units from the background region of interest. This ratio was then used to quantify the change in fluorescence of the flap and compared with progression of necrosis. Qualitative analysis was performed using OsiriX DICOM viewer.

### Clinical analysis of flap necrosis

Daily high-resolution photographs were taken of each mouse flap for documentation of flap necrosis. Photographs were taken using magnification at a distance of 20cm from the mouse. The end point was defined as POD 5. Photos were uniformly cropped to highlight the flap on the mouse dorsum. For clinical analysis, cropped flap photos were presented to three blinded reconstructive head and neck surgeons (CMT, JCF, ELR) in a random order, and each surgeon determined presence of necrosis (yes/no) and percentage of necrosed flap based on clinical assessment.

### Automated analysis of flap necrosis

Following image acquisition, a custom MATLAB script was compiled to detect and quantify the necrotic region within each image. Segmentation of necrotic regions was completed with k-means clustering, an unsupervised machine learning technique, which permitted the ability to threshold heterogeneity within the color hue of interest. After conversion to the CIELAB color space, the sutures were removed from the images by thresholding to exclude pixels with a negative b* value. A cluster number of four was used to separate the background, light skin tones, darker skin tones, and the necrotic region. The clusters were sorted and assigned based on the nonzero mean of the red layer pixel intensity for consistencies and automation. The code-generated values were the raw necrotic cluster of the entire image (area necrosis/total image pixels)*100. To normalize for slight variations with image acquisition height, mouse size and flap size, a correction factor (blinded) was added to determine the area of the necrotic tissue, calculated as a percentage of the total flap.

### Statistical Analysis

Statistical analysis was performed using two-tailed, unpaired t-tests with SAS Statistical Analytics Software. $p<0.05$ was considered significant. Error bars represent the cohort's standard deviation.

# RESULTS

## Near-infrared fluorescent imaging

Flaps treated with IntegriSense750 demonstrated a significantly higher signal to background ratio in the area of flap compromise compared with ICG. As shown in Figure 1a, this effect was sustained throughout POD 0–3. Mean signal-to-background ratio was significantly higher for IntegriSense750 (1.47+/−0.17) compared to ICG (0.86+/−0.21) at 3 hours after flap harvest (p=0.01) (Figure 1b). This trend continued on POD 1 with an IntegriSense750 signal-to-background ratio of 1.92+/−0.55 versus ICG signal-to-background ratio of 0.85+/−0.31 (p<0.001) (Figure 1c). The IntegriSense750 intensity was also significantly higher on POD 2 (p<0.01) and POD 3 (p<0.01). For all observations, IntegriSense750 signal-to-background ratio did not significantly deviate from 1.75 (p=0.04) in necrosed areas as determined on POD 5. No hyperintensity of ICG was noted, as the mean signal-to-background ratio did not significantly deviate from 1 throughout the post-operative period (0.96+/−0.28, p=0.6). IntegriSense750 identified areas of flap compromise before a change in the clinical appearance of the flap was recognized, as shown qualitatively in Figure 2.

## Fluorescence signal heterogeneity

IntegriSense750 imaging demonstrated a greater homogeneity in signal compared to ICG in areas of flap necrosis. The standard deviation of IntegriSense750 mean fluorescence intensity (2.41+/−0.54) was significantly lower (p=0.008) than ICG (8.9 +/−5.92) on POD 1 imaging (Figure 3). Representative images of IntegriSense750 and ICG flaps are also shown for comparison.

## Automated quantification of percent necrosis using custom software

To ensure there was consistent flap necrosis between the IntegriSense750 and ICG groups, a custom MATLAB script was compiled to identify and quantify necrotic areas throughout the study. Using this algorithm, a percentage of the total flap area (pixels) was calculated from brightfield images at 3hr, 3-day, and 5-day post-surgery. Representative images from the IntegriSense750 group are shown in Figure 4a with inset algorithm output displaying the threshold-corrected image. There was no significant difference in percent flap necrosis between the IntegriSense750 (2.03%+/−0.53) and ICG (2.29%+/−1.23) groups at 3hr post (p>0.05) and POD 5 (57.6%+/−22.7 versus 31.2%+/−30.5, respectively) (p=0.09) as shown in Figure 4b. However, there was a significant difference in percent flap necrosis between timepoints 3hr post and POD 5 for both IntegriSense750 (p<0.01) and ICG (p<0.05) groups (Figure 4b).

## Clinical grading assessment of flap necrosis

A total of 93 uniformly cropped flap images were presented to three blinded reconstructive surgeons for yes/no grading on presence of necrosis as well as percent necrosis. For the clinical assessment (Figure 4c), there was no significant difference in percent flap necrosis between the IntegriSense750 (0%) and ICG (0.47%+/−1.25) groups at 3hr post (p>0.05) and POD 5 (50.5%+/−25.0 versus 29.9%+/−32.2, respectively) (p=0.2). Similar to the automated analysis, there was a significant difference in flap necrosis between timepoints 3hr post

and POD 5 for both IntegriSense750 ($p<0.01$) and ICG ($p<0.05$) groups (Figure 4c). The subjectivity of clinical flap assessment is demonstrated in Table 1, which shows the number of flaps with necrosis based on the quantitative assessment and the percent discordance between the 3 blinded clinical assessments. There was unanimous consensus regarding the absence of necrosis in all flaps on POD 0 and the presence of necrosis in all flaps on POD 5. There were discordant opinions regarding the presence of necrosis throughout the rest of the postoperative period. The degree of discordant opinions ranged from 14.3–37.5%.

## DISCUSSION

Flap necrosis is a feared complication of reconstructive surgery. Clinical judgement remains the most common method of assessing flap viability, which is rooted in subjectivity and has been shown to have a relatively low specificity in predicting flap failure.[13,14] In this study, we also demonstrate a discordance of opinions on the presence/absence of necrosis between three reconstructive surgeons. Fluorescence angiography is an objective measure of flap perfusion and has been studied both *in vitro* and *in vivo*. ICG is the most well-recognized near-infrared fluorophore used for assessing vascular perfusion, but few studies have assessed its use in murine models. Most of the small animal model studies using ICG as a tool for assessing flap viability have taken place in rats.[15–18] Although the results have been promising in these select studies, it is important to appreciate the different mechanisms by which these studies quantify necrosis.

Han *et al.* compared ICG angiography with thermal imaging and near-infrared spectroscopy in a rat axial flap model. They found that ICG demonstrated the most accurate discrimination of necrotic tissue.[15] Mucke *et al.* also demonstrated a significant difference in ICG intensity in vital tissue compared to necrotic tissue in a rat epigastric axial flap model.[16] This particular study used the integrated mathematical software tool FLOW® 800 to evaluate microvascular blood flow. Jones *et al.* compared ICG to multispectral near-infrared reflectance imaging to assess tissue perfusion in an axial flap model in rats.[17] This study used the Kent KD203 Snapshot near-infrared system, which images the tissue hemoglobin oxygen saturation of the flap, and therefore provides information regarding tissue perfusion. In these mentioned studies, ICG angiography has been used to assess tissue perfusion through its ability to demonstrate hyperintensity in areas of vascular flow. A lack of signal, therefore, indicates an area of insufficient vascular flow with risk of necrosis. Here we present the first study that uses SBR of ICG angiography to measure flap necrosis in a mouse model. SBR is a measure of contrast, which quantitatively represents what is observed visually, making it the optimal metric for imaging analysis. Our findings contradict those of the aforementioned studies in that we did not demonstrate ICG angiography to be capable of predicting flap necrosis using SBR.

In this proof of principle study, we were interested in evaluating a different fluorescence imaging modality to assess flap perfusion for several reasons. First, ICG is relatively non-specific in that it circulates freely within the intravascular space. Second, the ability of ICG angiography to detect areas of concerning vascular perfusion relies on interpreting a lack of signal. Our goal was to explore another near-infrared fluorophore agent that would have an increased signal in areas of concerning vascular flow to aid in visualization of

these areas. IntegriSense750 is a selective ligand of $\alpha_v\beta_3$ integrin used in the detection of angiogenesis during vascular remodeling.[19] Integrins play a key role in cellular processes such as adhesion, differentiation, shape, migration, signaling, invasion and proliferation.[20] Specific to angiogenesis, $\alpha_v\beta_3$ integrin mediates the process of capillary sprouting which requires an interaction between the endothelial cell growth and the surrounding supporting tissue or extracellular matrix.[11] Our hypothesis was that IntegriSense750 would show a better specificity than ICG to areas of threatened vascularity due to the role of integrins in vascular remodeling. We hypothesized that integrins are upregulated in areas of vascular compromise in an attempt to proceed with angiogenesis and neovascularization.

There are very few studies that have used IntegriSense in mouse models, and most of the studies have used IntegriSense primarily for tumor detection[21] and as molecular imaging biomarkers for atherosclerosis. [22] Tamplenizza *et al.* used IntegriSense680 to detect angiogenesis in a mouse model of scaffold implantation. They showed the ability of IntegriSense680 to concentrate in areas of angiogenesis through quantifying signal intensity in a defined region of interest.[23] Tekabe *et al.* showed a higher fluorescence signal of IntegriSense750 in an ischemic hindlimb of a mouse when compared to the non-ischemic side.[11] This was the first study to show the use of IntegriSense750 in imaging the angiogenic response to an ischemic condition. Their results showed the promising potential of using near-infrared imaging in quantifying angiogenesis during vascular compromise.

Our results show that IntegriSense750 concentrates in areas of threatened vascularity by exhibiting a hyperintensity and thus a significant signal-to-background ratio. We show that this hyperintensity is visible even before clinical changes concerning for necrosis. We asked three reconstructive surgeons to evaluate each flap starting from 3-hours post-harvest through POD 5 to determine the presence or absence of necrosis using the clinical signs that are the standard of flap failure assessment. We found a surprising level of discordant opinions among the clinical interpretations. Out of the 12 times points assessed, there were discordant opinions at 6 (50%), and discordance ranged from 14.3% to 37.5%. Figure 5 shows an example of an IntegriSense750 flap that had discordant clinical opinions on POD 1 and POD 2, yet all surgeons agreed on the presence of necrosis by POD 5. On POD 1, although the clinical signs of necrosis are debatable, there is already clear hyperintensity in the distal portion of the flap. This is also seen on POD 2. This is an example of the value of using an objective imaging modality to reveal threatened tissue prior to a definitive clinical picture of necrosis. This could allow for earlier interventions with the potential to salvage flaps and improve flap survival.

During the study, a pattern of signal heterogeneity in the ICG groups was observed, specifically in the areas of future necrosis. This was not observed in the IntegriSense750 group as the standard deviation of the flap fluorescence intensity was significantly lower than the ICG group, indicating a greater uniformity of fluorescence signal. This was an important observation considering that lesser intra-flap heterogeneity yields a lower quantitative dynamic range among the pixel values, making the quantitative data more uniform. This is beneficial as it allows for more sensitive imaging and the high intensity areas are more easily distinguishable, especially when using a hyperintensive imaging agent.

Our study also shows that ICG was not able to predict flap necrosis, as it did not exhibit an average signal-to-background ratio that deviated from 1 throughout the post-operative period. This finding strays from findings of previously mentioned studies in murine animal models and should be considered in the context of the methodology used for quantification of the ICG signal. In our study, we used a signal-to-background noise ratio to quantify signal intensity. Other studies have used a variety of mechanisms for signal interpretation and quantification and could therefore lead to differing interpretations.

## CONCLUSION

Here we present the first study to show the utility of using IntegriSense750 as an imaging modality to predict flap necrosis in a murine axial flap model. Furthermore, we show that IntegriSense750 produces a hyperintensity within the failing region of the flap, and this hyperintensity is appreciable as early as post-operative day zero. This finding is important, as it reveals the ability of InegriSense750 to highlight areas of potential flap failure before the onset of clinical signs, potentially allowing for earlier intervention for flap salvage.

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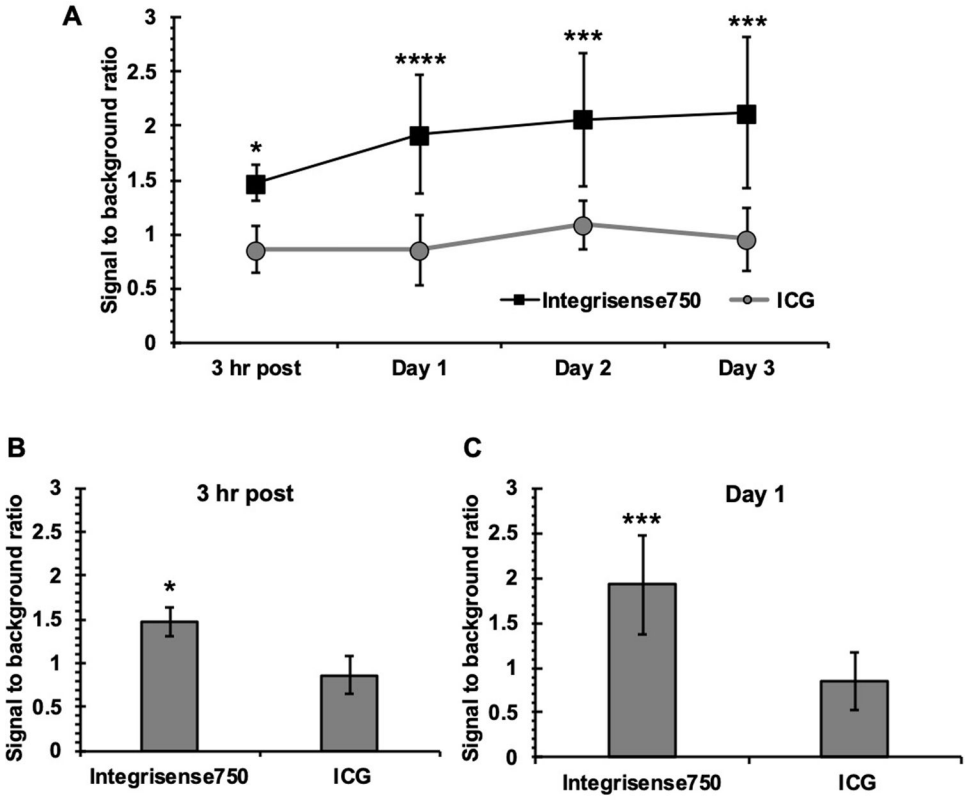


**FIGURE 1: Signal-to-background ratio demonstrates hyperintensity of IntegriSense750 vs Indocyanine green**

A. IntegriSense750 flaps demonstrated a significant signal-to-background ratio starting at 3-hours post flap harvest which was sustained until POD 3. ICG flaps did not demonstrate a significant signal-to-background ratio, as it did not deviate significantly from 1.0 throughout the postoperative period.

B. Mean signal-to-background ratio was significantly higher for IntegriSense750 (1.47+/−0.17) compared to ICG (0.86+/−0.21) at 3 hours after flap harvest ($p=0.01$).

C. Mean signal-to-background ratio was significantly higher for IntegriSense750 (1.92+/−0.55) compared to ICG (0.85+/−0.31) at POD 1 ($p<0.001$).

Abbreviations: Indocyanine green (ICG); Post-operative day (POD).

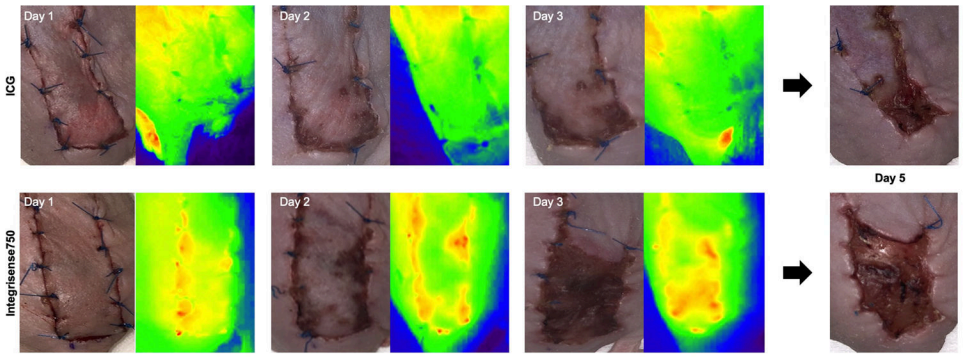


**FIGURE 2: IntegriSense750 demonstrates hyperintensity of threatened areas while Indocyanine green does not**

Top: ICG demonstrates a lack of signal that is difficult to interpret and does not easily correspond to clinical necrosis.

Bottom: IntegriSense750 shows hyperintensity in areas of impending flap necrosis that intensifies as the necrosis progresses.

Abbreviations: Indocyanine green (ICG)

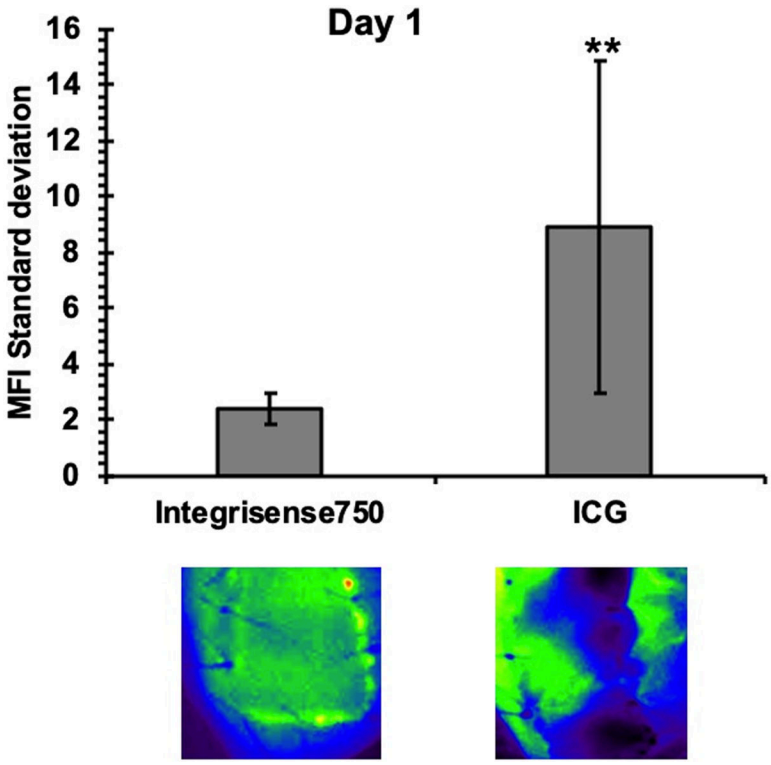


**FIGURE 3: Intra-flap homogeneity is high with IntegriSense750 compared to Indocyanine green**

IntegriSense750 imaging demonstrated a greater homogeneity in signal compared to ICG in areas of flap necrosis. The mean standard deviation of IntegriSense750 mean fluorescence intensity (2.41+/−0.54) was significantly lower (p=0.008) than ICG (8.9 +/−5.92) on POD 1. Abbreviations: Indocyanine green (ICG); Post-operative day (POD).

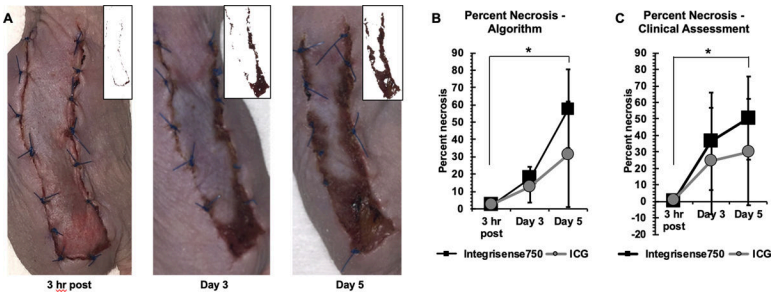


**FIGURE 4: Quantitative and qualitative assessment of necrosis shows no difference in necrosis between IntegriSense750 and Indocyanine flaps**

A) Representative images from the IntegriSense750 group with inset algorithm output displaying the threshold-corrected image for quantifying necrosis.

B) There was no significant difference in percent flap necrosis between the IntegriSense750 and ICG groups at 3hr post (2.03%+/−0.53 versus 2.29%+/−1.23; $p>0.05$) and POD 5 (57.6%+/−22.7 versus 31.2%+/−30.5; $p=0.09$), respectively. There was a significant difference in percent flap necrosis between timepoints 3hr post and POD 5 for both IntegriSense750 ($p<0.01$) and ICG ($p<0.05$) groups.

C) For the qualitative clinical assessment, there was no significant difference in percent flap necrosis between the IntegriSense750 and ICG groups at 3hr post (0% versus 0.47%+/−1.25; $p>0.05$) and POD 5 (50.5%+/−25.0 versus 29.9%+/−32.2; $p=0.2$), respectively. There was a significant difference in flap necrosis between timepoints 3hr post and POD 5 for both IntegriSense750 ($p<0.01$) and ICG ($p<0.05$) groups.

Abbreviations: Indocyanine green (ICG); Post-operative day (POD).

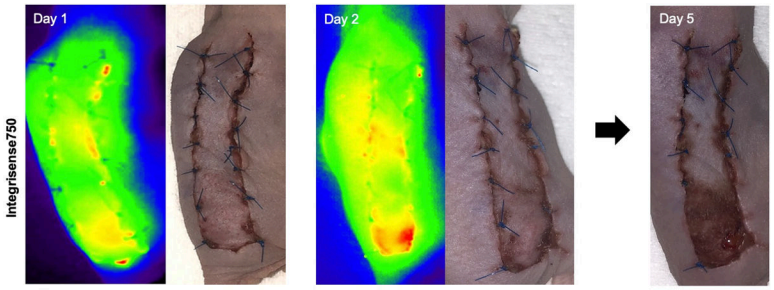


**FIGURE 5: Example of an axial flap with discordant clinical interpretation of necrosis**

An IntegriSense750-treated flap shows discrete hyperintensity in the distal portion of the flap on both POD 1 and POD 2 that corresponds to eventual clinical necrosis by POD 5. There were discordant opinions among the 3 reconstructive surgeons regarding the clinical appearance of necrosis on POD 1 (1/3 surgeons reported necrosis) and POD 2 (1/3 surgeons reported necrosis).

Abbreviations: Post-operative day (POD).

**TABLE 1:**

**Clinical assessment of axial flap necrosis.**

Three blinded reconstructive surgeons were asked to assess all flaps (n=93) for the presence of necrosis across all time points. If one of the surgeons rated yes for the presence of necrosis, that was counted as a flap with necrosis. If all 3 surgeons did not provide the same response for a flap, that was labeled as discordant clinical interpretation. Hr = hour, ICG = Indocyanine Green, POD = postoperative day.

| | Integrisense$^{750}$ POD 0 | ICG POD 0 | Integrisense$^{750}$ POD 1 | ICG POD 1 | Integrisense$^{750}$ POD 2 | ICG POD 2 | Integrisense$^{750}$ POD 3 | ICG POD 3 |
|---|---|---|---|---|---|---|---|---|
| **Flaps with necrosis** | 0% | 0% | 37.5% | 43.0% | 100% | 100% | 100% | 100% |
| **Necrosis grading discordance** | 0% | 0% | 37.5% | 0% | 25% | 28.6% | 25% | 14.3% |