

Article type: Technology Advances**Title:** Diagnostic Accuracy of Point-of-Care Fluorescence Imaging for the Detection of Bacterial Burden in Wounds: Results from the 350-Patient FLAAG Trial**Running title:** Fluorescence Imaging of Bacteria in Wounds**Authors:** Lam Le MD¹, Marc Baer DPM², Patrick Briggs DPM³, Neal Bullock, DPM⁴, Windy Cole DPM⁵, Daniel DiMarco D.O⁶, Rachel Hamil MD⁷, Khristina Harrell RN⁸, Maria Kasper DPM, FACFAS⁹, Weili Li PhD¹⁰, Keyur Patel D.O¹¹, Matthew Sabo DPM FACFAS¹², Kerry Thibodeaux MD FACS CWSP¹³, Thomas E. Serena MD⁸**Author affiliations:** ¹The Heal Clinic, Tulsa, OK, ²Foot & Ankle Center, Bryn Mawr, PA, ³HCA-Houston Healthcare Gulf Coast Foot and Ankle Specialists, Webster, TX, ⁴Royal Research Corp, Pembroke Pines, FL, ⁵Kent State University College of Podiatric Medicine, Kent, OH, ⁶St. Vincent Wound & Hyperbaric Centre, Erie, PA, ⁷St. Mary's Center for Wound Healing, Athens, GA, ⁸SerenaGroup Research Foundation, Cambridge, MA, USA, ⁹ Martin Foot and Ankle, York, PA, ¹⁰Li & Li Statistical Consulting, Toronto, ON, Canada, ¹¹ARMSTRONG County Memorial Hospital, Kittanning, PA, ¹²The Foot and Ankle Wellness Center of Western PA, Butler, PA, USA, ¹³The Wound Treatment Center at Opelousas General Health System, Opelousas, LA,**Corresponding Author Information**

Dr. Thomas E. Serena

SerenaGroup Research Foundation

125 Cambridge Park Drive, Ste 301

serena@serenagroups.com

+1 (814) 688-4000

word count: 4407

Key words: diagnostic accuracy, fluorescence imaging, wound assessment, wound infection

Abstract

Objective High bacterial load contributes to chronicity of wounds and is diagnosed based on assessment of clinical signs and symptoms (CSS) of infection, but these characteristics are poor predictors of bacterial burden. Point-of-care fluorescence imaging (FL) can improve identification of wounds with high bacterial burden ($>10^4$ CFU/g). FL detects bacteria, whether planktonic or in biofilm, but does not distinguish between the two. In this study, diagnostic accuracy of FL was compared to CSS during routine wound assessment. Post-assessment, clinicians were surveyed to assess impact of FL on treatment plan.

Approach: A prospective multi-center controlled study was conducted by 20 study clinicians from 14 outpatient advanced wound care centers across the US. Wounds underwent assessment for CSS followed by FL. Biopsies were collected to confirm total bacterial load. 350 patients completed the study (138 diabetic foot ulcers, 106 venous leg ulcers, 60 surgical sites, 22 pressure ulcers, and 24 others).

Results: 287/350 wounds (82%) had bacterial loads $>10^4$ CFU/g, and CSS missed detection of 85% of these wounds. FL significantly increased detection of bacteria ($>10^4$ CFU/g) by 4-fold, and this was consistent across wound types ($p<0.001$). Specificity of CSS+FL remained comparably high to CSS ($p=1.0$). FL information modified treatment plans (69% of wounds), influenced wound bed preparation (85%), and improved overall patient care (90%) as reported by study clinicians.

Innovation: This novel non-contact, handheld fluorescence imaging device provides immediate, objective information on presence, location, and load of bacteria at point-of-care.

Conclusion: Use of FL facilitates adherence to clinical guidelines recommending prompt detection and removal of bacterial burden to reduce wound infection and facilitate healing.

Introduction

An estimated 1-2% of the population in developed countries will experience a chronic wound in their lifetime¹ and the incidence of wounds continues to rise as the population ages and co-morbidities mount². As a result, management of chronic wounds accounts for >5% of total health care expenditures in the US and UK³⁻⁶.

Chronic wounds fail to progress through a timely sequence of repair. It is known that increased microbial load is a key predictor of non-healing wounds^{7,8}. Proliferation of bacteria resulting in moderate-to-heavy loads ($>10^4$ CFU/g) delays healing⁹⁻¹¹ and increases the risk of wound complications, including infection, sepsis and amputation¹²⁻¹⁴. Guidelines advise that early diagnosis of high bacterial burden is essential to prevent the wound from progression to local or systemic infection¹⁵. To reduce bacterial burden, clinicians choose from an armamentarium of antiseptic wound cleansers, debridement techniques, and antimicrobial options. This is done without objective information on bacteria at point-of-care and without information on treatment efficacy.

Clinical Problem Addressed

Treatment selection at point-of-care is largely based on evaluation of clinical signs and symptoms (CSS) of infection or high bacterial loads. However, numerous studies have reported that patients with high bacterial burden are frequently asymptomatic^{11,16,17}. Furthermore, comorbidities in wound patients (e.g. diabetes, autoimmune disease) can blunt immune responses and exacerbate patient-to-patient variability of CSS¹⁸. Together, this results in poor sensitivity of CSS for detection of infection^{16,17,19}; hindering immediate identification of wounds with high bacterial burden. Quantitative tissue cultures of wound biopsies are the reference standard to quantify bacterial load, but prolonged turnaround time between biopsy and microbiological results limits the rapid decision making needed to effectively manage bacterial burden in wounds. The relative inconsistency of CSS and delays in results from microbiological culture and PCR analysis may explain why 12-week wound healing rates are below 60%⁷ and have remained stagnant over the past 40 years²⁰, despite tremendous advances in wound treatments.

To address the pervasive problem of bacteria-related delayed healing and facilitate a more proactive approach to treatment planning, objective diagnostic information on bacterial burden in wounds is needed. Point-of-care diagnosis of bacterial burden in wounds is achieved using a handheld fluorescence imaging device (MolecuLight *i:X*, MolecuLight Inc., Toronto, Canada) that detects endogenous fluorescence from bacteria (at loads $>10^4$ CFU/g)²¹. Macroscopic imaging of bacteria is not possible as bacteria themselves are microscopic. However, when bacteria accumulate at high loads ($>10^4$ CFU/g), the fluorophores they collectively emit are detectable via fluorescence imaging. Under safe violet light illumination, common wound pathogens including bacteria from the *Staphylococcus*, *Proteus*, *Klebsiella* and *Pseudomonas* genera^{22,23} endogenously emit red or cyan fluorescent signatures^{23-25,26}. By detecting these fluorescent signals, fluorescence imaging provides immediate information on bacterial location, without use of contrast agents (Figure 1). Multiple clinical studies have consistently reported positive predictive values (PPV) of these fluorescent signals, averaging 95.6% (range 87.5%-100%) to detect moderate-to-heavy loads of bacteria, confirmed by microbiological analysis^{21,27-29}. Recent evidence indicates that the fluorescence imaging (FL) procedure facilitates more appropriate treatment selection and timing of advanced therapies (e.g. grafts and skin substitutes)³⁰ in chronic wounds and burns^{27,28,31-35}, however these studies lacked rigour and statistical power. The **Fluorescence imaging Assessment and Guidance (FLAAG)** study, a large, multi-center prospective controlled clinical trial targeting wounds of various type and duration, was established to evaluate: (1) whether FL improves detection of wounds with high ($>10^4$ CFU/g) bacterial loads, and (2) how point-of-care information on bacterial presence and location impacts treatment planning.

Materials & Methods

Study Population & Design

This prospective, single-blind, multi-center cross-sectional study (clinicaltrials.gov #NCT03540004) had two independent co-primary endpoints: i) superiority in sensitivity of CSS and fluorescence imaging (CSS + FL) vs. CSS alone, to identify wounds with moderate or heavy ($>10^4$ CFU/g bacterial load); and ii) non-inferiority of specificity of CSS + FL vs. CSS

alone with region of indifference of 10% to identify wounds with moderate-to-heavy bacterial load. These co-primary endpoints were independent of each other. A sample size of 160 patients, consisting of 100 positive cases to demonstrate superiority in sensitivity and 60 negative cases to demonstrate non-inferiority of specificity, was chosen to achieve >80% power for both primary endpoints. The study included adult (>18 years) patients presenting with wounds: 138 diabetic foot ulcers (DFUs), 106 venous leg ulcers (VLUs), 22 pressure ulcers (PUs), 60 surgical sites (SS) and 24 others of unknown infection status (Supplemental Figure 1). To ensure adequate representation of wound variety, a minimum of 20 participants were recruited with each wound type (e.g. DFU, VLU, PU, SSI). Due to the high prevalence of patients with bacterial loads $>10^4$ CFU/g, rolling recruitment was performed until a sufficient number of microbiologically negative wounds ($<10^4$ CFU/g) to achieve statistical power was met, at enrollment of 371 patients. An independent third-party (Ironstone PD, Toronto, ON) was used to control for bias and ensure appropriate blinding. Patients were recruited from 14 U.S. outpatient advanced wound care centers by 20 clinicians (12 podiatrists, 4 surgeons, 1 ER physician, 1 wound care physician, and 2 nurse practitioners). Patients were excluded if they had been treated with an investigational drug within the last month, had recently (<30 days) had a wound biopsy, were not able to consent, had any contraindications to routine wound care and/or monitoring, or if their wounds could not be imaged due to anatomical location. Only one wound per patient was eligible for inclusion. Before beginning the study, clinicians were provided with on-site and online training on use of the device, image interpretation, good clinical practice, and trial procedures. Clinicians were required to pass (>80%) a color blindness and image interpretation test prior to enrolling participants. The study was conducted in accordance with Health Insurance Portability and Accountability Act guidelines, adhered to tenets of the International Conference on Harmonisation E6 Good Clinical Practice (ICH GCP) and the Declaration of Helsinki, and received ethics approval by an external institutional review board (Veritas IRB, Montreal, QC).

Assessment of clinical signs and symptoms of infection and fluorescence imaging

Clinicians reviewed patient history and visually inspected wounds for CSS using the International Wound Infection Institute (IWII) Wound Infection checklist¹⁵. Assessment of

infection was based on clinician judgement; wounds with ≥ 3 criteria present were considered positive for moderate-to-heavy ($>10^4$ CFU/g) bacterial loads, per guidelines¹⁵, but if one overwhelming sign or symptom was present, clinicians had the discretion to deem the wound positive for CSS. A 4-week treatment plan was created based on assessment of CSS. Immediately following CSS assessment, standard and fluorescence images were captured with the fluorescence imaging device. To ensure uniform fluorescence imaging, the device is held at a 90-degree angle to the wound. The device's LEDs emit safe 405 nm violet light to excite fluorophores in the wound up to a penetration depth of 1.5 mm³⁶. This excitation wavelength causes most bacterial species in wounds to emit a red fluorescent signal due to endogenous porphyrins in the heme pathway^{23,25}. While *Pseudomonas aeruginosa* also produces porphyrins³⁷, it uniquely produces a cyan fluorescent signal due to endogenous pyoverdine, a virulence factor²⁶. These fluorescent signals from bacteria that accumulate in a region of the wound at loads $>10^4$ CFU/g are detectable by the device^{21,29}. Specialized optical filters on the device allow transmission of only relevant fluorescence from tissue and bacteria³⁶. Connective tissues (e.g. collagen) produce green fluorescent signals^{38,23,25,26} and flaky skin appears a brighter green with white edges. Images where red or cyan fluorescence was observed by clinicians were considered positive for moderate-to-heavy bacterial loads ($>10^4$ CFU/g)²¹ (Figure 2). A new treatment plan was documented incorporating information about bacterial fluorescence. Clinicians then completed a survey indicating how FL influenced diagnosis of bacterial burden in the wound, guided procedure, and treatment selection (i.e. frequency of treatment including cleaning, debridement, and use of topical antimicrobials and antibiotics) or influenced patient care.

Microbiological analysis of total bacterial load

Punch biopsies from wounds were collected to quantify total bacterial load. Up to three biopsies (6 mm diameter) were obtained under local anesthetic: a biopsy from the wound center, or if applicable, a biopsy outside of the wound center from a region of the wound positive for bacterial fluorescence, or region positive for CSS. In wounds where bacterial fluorescence was observed, clinicians were directed to collect a biopsy from the region of the wound that was brightest for bacterial fluorescence. Biopsy samples were cut

to a depth of 2 mm (to restrict bacterial contents to the penetration depth of imaging device) and transported in Remel ACT-II transport media to a central lab (Eurofins Central Laboratory, Lancaster PA) for microbiological culture analysis of load and species. Fluorescence can only be detected from bacteria that are alive, thus necessitating the use of quantitative culture analysis to confirm the total bacterial loads detected by fluorescence imaging. This method may not fully capture the microbiological diversity in the wound, including some fastidious bacterial species, therefore every effort was made to provide optimal conditions for bacteria that are challenging to culture. To prepare for analysis, a small portion of the tissue was prepared for gram staining on a sterile slide. The remaining biopsy sample was homogenized and serially diluted³⁹ for quantitative microbiological analysis (range of detection from 0 to 10^9 CFU/g). Diluted biopsy homogenates were cultured on BAP/Chocolate agar (nonselective growth), Columbia CAN agar (select gram positive), MacConkey agar (selective gram negative) or Brucella agar (anaerobes) and incubated at 35°C in the appropriate atmosphere. Aerobe cultures were assessed for growth after 24 hours of incubation and incubated up to 48 hours; anaerobes were assessed after 48 hours of incubation, and then reviewed every 24 hours up to 7 days. A wound was considered microbiologically positive if the total bacterial load (the sum of all bacteria from any biopsy) was $>10^4$ CFU/g. MALDI-TOF mass spectrometry (Bruker Daltonics, Germany) was used to identify bacterial species, as previously described⁴⁰. Microbiologists were blinded to the results of the CSS assessment and FL.

Statistical Analysis

One-sided exact McNemar tests were used for comparisons of sensitivity, specificity and accuracy of detecting bacterial loads $>10^4$ CFU/g. Comparisons of predictive values (PPV and NPV) were performed using an asymptotic method as described by Moskowitz and Pepe⁴¹. Sample proportions and 95% confidence intervals were used to estimate the diagnostic accuracy characteristics. Fisher's exact test was performed to assess association between fluorescence diagnosis (FL+ or FL-) and reported survey outcomes; statistical significance was set at $p=0.05$. All analyses were performed using R version 3.6.2.

Results

Between May 2018 and April 2019, 371 patients with various wound types (DFUs, VLUS, PUs, SS, and others) were screened. Of the 371 patients screened, only 4 (1.1%) were excluded from the study and microbiology data was completed for 350. Basic demographic information along with antibiotic use, wound type, wound duration, and total bacterial load are reported in Table 1. Mean (SD) age of participants was 60.2 (12.4) and 35.7% were female. Wound duration exceeded 3 months in 69.7% of wounds and delayed healing was observed in 52.9%. No serious adverse events resulting from use of the device were reported⁴².

In 82% (287/350) of wounds, bacterial loads $>10^4$ CFU/g were observed, confirmed by microbiological analysis (Figure 3). Median (range) total bacterial load of all wounds was 1.8×10^6 CFU/g (0.0 to 7.7×10^9 CFU/g). A higher proportion of males (69.7%) than females (30.3%) had microbiology positive wounds ($>10^4$ CFU/g). Of the microbiology positive wounds, 19.5% were on systemic antibiotics, and bacterial load of these wounds averaged (SD) 1.4×10^7 CFU/g (3.1×10^7 CFU/g); over 50% of microbiology negative wounds ($<10^4$ CFU/g) were on systemic antibiotics. Bacterial loads $>10^4$ CFU/g were most prevalent in diabetic foot ulcers and wounds of ≥ 12 months duration. Of the 350 wounds in the study, 183 (52.3%) had bacterial loads $>10^6$ CFU/g, which some consider to be indicative of infection¹⁷; in 16.9% (59/350) of wounds, bacterial loads $>10^8$ CFU/g were observed, while 18% (63/350) of wounds had bacterial loads $\leq 10^3$ CFU/g. One hundred and six different bacterial species (51 genera) were detected from 1053 isolates; species detected included: 68 gram positive, 38 gram negative, 78 aerobes and 28 anaerobes. In 85.7% (246/287) of microbiology positive wounds (loads $>10^4$ CFU/g), mixed bacterial colonization was present. *Staphylococcus aureus* was the most prevalent species observed, present in 71.1% of microbiology positive wounds. *Pseudomonas aeruginosa* was prevalent in 13.9% (40/287) of microbiology positive wounds and was associated with presence of cyan fluorescence, as expected. Supplemental Table 1 lists bacterial species detected from all study wounds. An average of 2.8 bacterial species were detected per biopsy collected from the center of the wound. In most wounds, the center of the wound was also the brightest region of fluorescence. However, in 78 wounds, an additional FL-guided biopsy was

collected outside of the wound center. From these FL-guided biopsies taken outside of the wound center, an average of 3.1 bacterial species were detected. This was significantly higher than the average number of bacterial species detected in biopsies collected from the center of the same wound (2.2; $p < 0.001$). The inclusion of 98.9% (367/371) of the population screened suggests that these findings are representative of bacterial loads in typical wound populations.

Diagnostic accuracy of FL was assessed on its own and in combination with information provided by CSS assessment (CSS+FL). Clinicians diagnosed 302/350 wounds as negative for CSS. Addition of FL to CSS improved sensitivity (61.0% [95% CI, 55.3%-66.6%]) to detect wounds with bacterial loads $>10^4$ CFU/g by 4-fold compared to CSS alone (15.33% [95% CI, 11.16, 19.50]; $P < 0.001$, Figure 4A), consistent across wound types (Figure 4D). Sensitivity of FL was comparable to CSS+FL. Detection of false positives using CSS and FL was rare, resulting in specificity of 84.1% (95% CI, 75.1%-93.2%; Figure 4B) of CSS+FL that was comparable to CSS. Specificity of FL remained similarly high relative to CSS across all wound types (Figure 4E). DOR of CSS+FL was 8.3 (95% CI, 4.1-17.0), and was 3.1-fold higher than CSS (2.7 [95% CI 0.9-7.7]; Figure 4C). PPV of FL (either alone or in combination with CSS) was comparably high (96.0, 95% CI [93.1-98.9] and 94.6, 95% CI [91.3-97.9], respectively) to CSS alone (91.7, 95% CI [83.9-99.5]), but NPV and accuracy of CSS+FL were significantly increased by 64.4% and 2.2-fold respectively, compared to CSS (Table 2; $p < 0.001$). CSS alone had poor discriminative power to predict wounds with high bacterial loads (Figure 5); FL drove improvements in discriminative power to identify wounds with bacterial burden $>10^4$ CFU/g at point-of-care. With FL, high bacterial burden was identified in 131 wounds otherwise missed by CSS. FL provided additional benefits at time of diagnosis by locating bacterial burden outside of the wound bed in 128/302 (42.4%) wounds negative for CSS. The enhanced sensitivity, accuracy and discriminative power of FL compared to CSS resulted in identification of a larger proportion of wounds with bacterial loads $>10^4$ CFU/g.

The impact of FL information on care planning was evaluated using a clinician survey. The survey asked clinicians to report which aspects of wound care were most impacted by FL. Clinicians reported that FL resulted in improvements to patient care

(which includes wound bed preparation, treatment planning, patient engagement and monitoring treatment efficacy) in 90.0% of study wounds. FL information also resulted in changes to diagnosis of bacterial burden in 52.3% of wounds (Figure 6). The objective, diagnostic information provided by FL changed clinical treatment plans in 68.9% of wounds (Figure 6A). FL-information guided wound bed preparation in 84.6% of wounds; and had the greatest impact on primarily tissue management (67.4%) and infection control (76.3%; Figure 6B). Wound care decision making stems from assessment, thus not surprisingly, assessment was heavily influenced by FL-information (78.6%). Downstream aspects of care including sampling location (44.6% of wounds), cleaning (42.9%), debridement (48.0%), treatment selection (55.4%), and wound documentation (45.1%) were also influenced (Figure 6C). Table 3 summarizes the aspects of care that were impacted by fluorescence information and compares impact of that information in wounds deemed fluorescence (bacteria) positive vs fluorescence negative. As expected, changes to care plan, (with the exception of wound assessment, moisture imbalance and edge advance), were more prevalent among wounds positive for bacterial fluorescence compared to those negative for bacterial fluorescence ($p < 0.001$), indicating that the enhanced detection of bacteria provided by fluorescence information significantly influenced clinicians' care planning.

Discussion

Bacterial load in wounds is underestimated and the incidence of infection in the wound care population is underreported^{17,18}, and therefore undertreated. The presence and severity of bacterial loads in wounds is typically inferred from CSS^{43,44}. However, CSS is inherently subjective and can miss detection of wounds with moderate-to-heavy bacterial loads^{16,17}. More accurate methods to identify wounds with clinically significant loads of bacteria can facilitate better management of wounds according to standard of care practices¹⁵. In this study, fluorescence imaging of bacteria to detect bacterial loads $>10^4$ CFU/g was used in combination with standard of care assessment of CSS to determine if detection of wound with high bacterial loads ($>10^4$ CFU/g) could be improved. Microbiological analysis of wound biopsies revealed median bacterial load of 1.8×10^6 CFU/g, with 36.6% of study wounds having bacterial loads $>10^7$ CFU/g. At bacterial loads of 10^4 CFU/g, clinical signs of infection may not manifest but delayed wound healing is

observed^{9,10}. CSS assessment failed to detect 84.7% (155/183) of wounds with bacterial loads $>10^6$ CFU/g, a threshold that some consider indicative of infection¹⁸. CSS (individual and combined criteria) had poor discriminatory power in identifying wounds with bacterial loads $>10^4$ CFU/g. Delayed healing, which had high sensitivity, was the clear exception, but had poor specificity, likely due to presence of physical characteristics that may delay healing (e.g. presence of biofilm, vascular insufficiency, poor offloading)^{15,45}. Four signs of infection (purulent discharge, inflammation, hypergranulation, and erythema) fell below the line of chance and were ineffective at predicting bacterial loads $>10^4$ CFU/g, consistent with previous reports^{16,17}. The poor discriminatory power of CSS would have resulted in 84.7% (243/287) of patients with bacterial loads $>10^4$ CFU/g receiving inappropriate treatment to address bacteria at time of assessment. Indeed, a recent meta analysis of CSS effectiveness concludes “the apparent lack of utility of a combination of findings identified by infectious disease experts (Infectious Diseases Society of America criteria) as useful for diabetic foot infection is both surprising and disappointing but highlights the difficulty in making the diagnosis”¹⁷. To overcome stagnant wound healing trends, improved methods of identifying and treating bacterial load needs to be prioritized.

Detection of bacteria in wounds using fluorescence imaging has been previously validated through *in vitro* and *in vivo* studies that elegantly demonstrated the correlation between intensity of fluorescent signal (from bacterial porphyrins) and bacterial load and showed that FL can detect both planktonic and biofilm encased bacteria^{23,46}, though it cannot distinguish between these two states of bacteria. Biofilm detection and eradication is of tremendous importance in wound care, with biofilm prevalence estimated in up to 90% of chronic wounds⁴⁷. Even without distinguishing between planktonic and biofilm encased bacteria, the ability of FL to detect bacteria in biofilm and target treatment to regions that potentially contain biofilm is a significant advancement for the field.

In vitro results lack the tissue in which wound bacteria are dispersed and other factors present in the wound that may influence capacity to detect high bacterial loads in wounds. This makes clinical studies critical to assess the true performance of this device to detect bacteria above 10^4 CFU/g. Consistent with prior clinical studies^{33,35,48}, use of the FL diagnostic procedure to detect bacterial loads $>10^4$ CFU/g resulted in higher sensitivity (4-

fold) and accuracy (2.2-fold), enhanced detection of high bacterial burden in wounds otherwise missed by CSS, and immediately impacted treatment plans. Inaccurate or late diagnosis of bacteria and infection plagues chronic wounds at great costs to the patient and healthcare systems^{3,4,49}, and contributes to some of the 196 daily DFU-related amputations in the US⁵⁰. Under- and over-treatment can lead to sub-optimal wound care, inflated costs, and antibiotic misuse⁵¹. The robust performance characteristics of FL reported here demonstrate the applicability of this diagnostic procedure to facilitate earlier detection of detrimental wound bacterial burden¹⁵.

According to guidelines¹⁵, intervention is mandated in wounds when bacterial colonisation turns into local infection ($\geq 10^6$ CFU/g). Intervention at this critical point prevents further escalation up the infection continuum and damage to host tissue. In this study, FL provided real-time evidence of high ($>10^4$ CFU/g) bacterial loads in 131 wounds negative for CSS, prompting intervention in the form of bacterial-targeted therapies (e.g., cleansing, debridement or use of antimicrobials). The inclusion of FL as part of routine wound assessment provided information on bacterial burden that led to additional improvements in care:

(1) Guided wound bed preparation in $\geq 90\%$ of wounds in this and other studies^{35,52}.

Information on location of bacterial burden at point-of-care has been shown to be highly impactful for debridement^{52,53}, selection of appropriate cleanser³⁰, and general wound bed preparation prior to application of advanced therapies³⁰.

Advanced therapies such as cellular and tissue-based products and skin grafts often fail when high bacterial loads are present⁵⁴⁻⁵⁶.

(2) Alerted clinicians to unexpected location of bacterial loads^{27,52}. In this study more than 80% of wounds (150/185) positive for fluorescence from bacteria had bacterial burden outside of the wound bed. Treatments to minimize bacterial load (e.g. debridement) are not typically targeted to this region⁵⁷ and sampling is rarely performed outside of the wound bed⁵⁸⁻⁶⁰. The FL information in this study provided objective evidence on location of bacteria to facilitate targeted eradication.

(3) Provided information on efficacy of antibiotics and guided stewardship decisions without delay³⁵. In this study 56 microbiology positive wounds were on systemic

antibiotics at time of enrollment. Fluorescence imaging revealed the presence of red or cyan fluorescence, indicative of bacterial loads $>10^4$ CFU/g in 39.3% (22/56) of these wounds. Biopsy analysis later confirmed the presence of bacteria at loads $>10^4$ CFU/g in these wounds. Together, these findings suggest inadequacy of the antibiotic treatment.

A recent international position paper on antimicrobial stewardship⁵¹ highlighted diagnostic uncertainty in wounds as a key factor contributing to antimicrobial misuse, and recommends the use of rapid, diagnostic testing to ensure judicious use of antimicrobials. Here, we show evidence that supports this recommendation; FL resulted in more appropriate diagnosis of 46% of wounds with bacterial loads $>10^4$ CFU/g compared to CSS and impacted antimicrobial stewardship decisions in 53.1% of wounds. Diagnostic imaging provides actionable information to better implement gold standard wound care.

Strengths and Limitations

This study of 350 patients included a heterogeneous sample of wounds, across multiple clinical sites. The minimal participant exclusion criteria and diverse wound types included in the study increases the generalisability of results to the overall chronic wound population. Furthermore, the use of wound biopsy and culture analysis to confirm bacteria loads enhanced confidence in the diagnostic accuracy measures reported. However, there were limitations to these methodologies. First, due to the imprecision of soft tissue biopsy trimming, the biopsies were cut to a greater depth than the 1.5 mm excitation limit of the imaging device, thus it is possible that the biopsy may have detected more anaerobic bacteria than the device was able to. Second, the conditions of culture analysis are not favorable for fastidious bacteria and may have resulted in underreporting the diversity of bacteria species present in the wound. This study focused primarily on high bacterial loads as a contributor to delayed wound healing but additional systemic factors that were not reported here, including vascular insufficiency⁶¹ and protease activity⁶² must also be considered. Clinicians had limited experience using FL in a clinical context prior to the study, which may have contributed to lower sensitivity to detect bacteria at loads $>10^4$ CFU/g than previously observed. In prior FL studies, sensitivity estimates ranging from 72-

100% were reported, likely due to longer clinician experience using the device^{21,28,29,63}. As with other diagnostic imaging modalities⁶⁴⁻⁶⁶, we anticipate that the performance measures reported should be improved with increased experience^{67,68}. This single time point study meant that effectiveness of changes in treatment plan based on FL could not be measured. Longitudinal randomized controlled trials assessing wound healing may further elucidate the impact of point-of-care diagnostic imaging of bacteria. Evidence from small longitudinal observational studies demonstrate accelerated wound area reduction with use of FL^{32,53}. Due to the limited (1.5 mm) depth of excitation³⁶ and inability to detect non-porphyrin producing bacteria, including species from the *Streptococcus*, *Enterococcus* and *Fingoldia* genera (which account for an estimated 12% of the most prevalent wound pathogens²³ and rarely occur monomicrobially⁶⁹), it is recommended that FL be used in combination with CSS.

Conclusion

The severity of bacterial burden in wounds is grossly underappreciated. Our results from 350 wounds reveal failure of current standard of care assessment to detect 84.7% of wounds with bacterial loads $>10^6$ CFU/g, that some suggest are indicative of infection¹⁸. Incorporation of the non-invasive FL diagnostic procedure to wound assessment greatly improved detection of high bacterial burden across a variety of wound types and provided information on bacterial location at point-of-care. This represents a paradigm shift in wound assessment, in which clinicians now have immediate information on bacterial burden to guide treatment selection and inform the frequency of re-assessment to determine the efficacy of selected treatments at point-of-care^{34,53}. The point-of-care information provided by FL facilitates a rapid switch to a more effective bacterial-targeting agent (e.g. cleanser, bandage)^{34,70}. Study results, collected across 14 study sites from 20 clinicians of varying skill levels, indicate the widespread utility of FL to inform wound assessment, wound bed preparation, and overall treatment planning.

Innovation

Despite advances in wound therapies, wound healing rates in the last 40 years have remained stagnant as clinicians continue to work blindly to address bacterial burden in

wounds. In this study, fluorescence imaging increased detection of high loads ($>10^4$ CFU/g) of bacteria by 4-fold and informed the location and extent of bacteria in wounds. This actionable information enabled early detection of bacteria, especially in highly prevalent asymptomatic wounds, and allowed clinicians to treat bacterial burden without delays. Information provided by this non-contact point-of-care imaging device can be used to inform treatment planning and evaluate the efficacy of selected treatments.

Key Findings

- 82% of study wounds (287/350) had clinically significant bacterial loads ($>10^4$ CFU/g) that were missed by standard of care assessment of clinical signs and symptoms of infection (CSS).
- Incorporation of MolecuLight *i:X* fluorescence imaging device with standard of care assessment of CSS increased point-of-care detection of wounds with high bacterial loads ($>10^4$ CFU/g) by 4-fold compared to CSS alone.
- Use of this non-contact point-of-care bacterial imaging device significantly impacted downstream aspects of patient care including sampling location (44.6% of wounds), cleaning (42.9%) and debridement (48%) and selection of antimicrobials (53.1%) and other treatments (55.4%).

Acknowledgements and Funding Sources

Funding for the study was provided by MolecuLight Inc. Ironstone Product Development contributed to the study design and conducted data auditing. All authors had access to relevant data, approved the final version, and were responsible for the decision to submit the manuscript for publication.

Author Disclosure and Ghostwriting

SerenaGroup research foundation received funding from MolecuLight Inc. to cover conduct of the study. No competing financial interest exist for other authors. The authors thank MolecuLight Inc. for assistance with preparation of the schematic in Figure 1.

About the Authors

Lam Le MD is the medical director of the St. John Wound Center and a certified wound specialist.

Marc Baer DPM is a podiatrist with a special interest in wound care and limb salvage and practices at the

Bryn Mawr Hospital Wound Care Center.

Patrick Briggs DPM is a podiatrist current part of Texas Gulf Coast Medica Group.

Neal Bullock, DPM is a podiatric surgery specialist currently practicing in Pembroke Pines, Florida.

Windy Cole DPM is an Adjunct Professor and Director of Wound Care Research at Kent State University College of Podiatric Medicine.

Daniel DiMarco D.O is a family medicine specialist in Fairview, Pennsylvania.

Rachel Hamil MD is a wound care and emergency medicine specialist in Athens, GA. She currently practices and is the medical director at St Mary's Center for Wound Healing and is affiliated with Saint Mary's Hospital.

Khristina Harrell RN is a licensed practical nurse and Chief Nursing Officer of the SerenaGroup research foundation. Her primary responsibilities include consulting and conducting clinical trials.

Maria Kasper DPM, FACFAS is a practicing podiatrist in York, PA. She currently practices and is the medical directory at Martin Foot and Ankle and is affiliated with Wellspan/York Hospital and UPMC Pinnacle Memorial Hospital.

Weili Li PhD is cofounder of Li & Li Statistical Consulting. She holds a PhD in Biostatistics and has more than 10 years of experience in theoretical and applied statistics.

Keyur Patel D.O currently serves as Medical Co-Director of the Wound & Hyperbaric Center and attending Emergency Physician at Armstrong County Memorial Hospital outside of Pittsburgh, PA.

Matthew Sabo DPM FACFAS is a Clinical Associate Professor at Temple University School of Podiatric Medicine and currently serves as the associate director of research at The Snyder Institute of Armstrong County Memorial Hospital.

Kerry Thibodeaux MD FACS CWSP is a general surgeon in Opelousas, Louisiana and is affiliated with Opelousas General Health System.

Thomas E. Serena MD is Founder and Medical director of the SerenaGroup, a family of wound, hyperbaric and research companies and is board certified in Surgery with practice in wound healing.

List of Abbreviations

CFU	Colony forming units
CSS	Clinical signs and symptoms of infection
DFU	Diabetic foot ulcer
DOR	Diagnostic Odds Ratio
FL	Fluorescence imaging
FLAAG	Fluorescence Imaging Assessment and Guidance
IWII	International Wound Infection Institute
NPV	Negative Predictive Value
PPV	Positive Predictive Value
VLU	Venous Leg Ulcer

References

1. Gottrup F. A specialized wound-healing center concept: importance of a multidisciplinary department structure and surgical treatment facilities in the treatment of chronic wounds. *Am J Surg.* May 2004;187(5A):38S-43S.
2. Sen CK, Gordillo GM, Roy S, et al. Human skin wounds: a major and snowballing threat to public health and the economy. *Wound Repair Regen.* Nov-Dec 2009;17(6):763-771.
3. Nussbaum SR, Carter MJ, Fife CE, et al. An Economic Evaluation of the Impact, Cost, and Medicare Policy Implications of Chronic Nonhealing Wounds. *Value Health.* Jan 2018;21(1):27-32.
4. Guest JF, Ayoub N, McIlwraith T, et al. Health economic burden that different wound types impose on the UK's National Health Service. *Int Wound J.* Apr 2017;14(2):322-330.
5. Phillips CJ, Humphreys I, Fletcher J, Harding K, Chamberlain G, Macey S. Estimating the costs associated with the management of patients with chronic wounds using linked routine data. *Int Wound J.* Dec 2016;13(6):1193-1197.
6. Brem H, Stojadinovic O, Diegelmann RF, et al. Molecular markers in patients with chronic wounds to guide surgical debridement. *Mol Med.* Jan-Feb 2007;13(1-2):30-39.
7. Cho S. Development of a Model to Predict Healing of Chronic Wounds Within 12 Weeks. *Advances in Wound Care.* 2020;0(0).
8. Tuttle MS. Association Between Microbial Bioburden and Healing Outcomes in Venous Leg Ulcers: A Review of the Evidence. *Adv Wound Care (New Rochelle).* Jan 1 2015;4(1):1-11.
9. Caldwell MD. Bacteria and Antibiotics in Wound Healing. *Surg Clin North Am.* Aug 2020;100(4):757-776.
10. Xu L, McLennan SV, Lo L, et al. Bacterial load predicts healing rate in neuropathic diabetic foot ulcers. *Diabetes Care.* Feb 2007;30(2):378-380.

11. Browne AC, Vearncombe M, Sibbald RG. High bacterial load in asymptomatic diabetic patients with neurotrophic ulcers retards wound healing after application of Dermagraft. *Ostomy Wound Manage*. Oct 2001;47(10):44-49.
12. Turtiainen J, Hakala T, Hakkarainen T, Karhukorpi J. The impact of surgical wound bacterial colonization on the incidence of surgical site infection after lower limb vascular surgery: a prospective observational study. *Eur J Vasc Endovasc Surg*. Apr 2014;47(4):411-417.
13. Gardner SE, Frantz RA. Wound bioburden and infection-related complications in diabetic foot ulcers. *Biol Res Nurs*. Jul 2008;10(1):44-53.
14. Misic AM, Gardner SE, Grice EA. The Wound Microbiome: Modern Approaches to Examining the Role of Microorganisms in Impaired Chronic Wound Healing. *Adv Wound Care (New Rochelle)*. Jul 1 2014;3(7):502-510.
15. IWII. Wound infection in clinical practice (International Wound Infection Institute). *Wounds International*. 2016.
16. Gardner SE, Frantz RA, Doebbeling BN. The validity of the clinical signs and symptoms used to identify localized chronic wound infection. *Wound Repair Regen*. May-Jun 2001;9(3):178-186.
17. Reddy M, Gill SS, Wu W, Kalkar SR, Rochon PA. Does this patient have an infection of a chronic wound? *JAMA*. Feb 8 2012;307(6):605-611.
18. Gardner SE, Hillis SL, Frantz RA. Clinical signs of infection in diabetic foot ulcers with high microbial load. *Biol Res Nurs*. Oct 2009;11(2):119-128.
19. Serena TE, Hanft JR, Snyder R. The lack of reliability of clinical examination in the diagnosis of wound infection: preliminary communication. *Int J Low Extrem Wounds*. Mar 2008;7(1):32-35.
20. Fife CE, Eckert KA, Carter MJ. Publicly Reported Wound Healing Rates: The Fantasy and the Reality. *Adv Wound Care (New Rochelle)*. Mar 1 2018;7(3):77-94.
21. Rennie MY, Lindvere-Teene L, Tapang K, Linden R. Point-of-care fluorescence imaging predicts the presence of pathogenic bacteria in wounds: a clinical study. *J Wound Care*. Aug 2 2017;26(8):452-460.
22. Cavallaro G, Decaria L, Rosato A. Genome-based analysis of heme biosynthesis and uptake in prokaryotic systems. *J Proteome Res*. Nov 2008;7(11):4946-4954.

23. Jones LM, Dunham D, Rennie MY, et al. In vitro detection of porphyrin-producing wound bacteria with real-time fluorescence imaging. *Future Microbiol.* Feb 26 2020.
24. Nitzan YK, M. Endogenous Porphyrin Production in Bacteria by δ -Aminolaevulinic Acid and Subsequent Bacterial Photoeradication. *Lasers in Medical Science.* 1999;14(4):8.
25. Philipp-Dormston WK, Doss M. Comparison of porphyrin and heme biosynthesis in various heterotrophic bacteria. *Enzyme.* 1973;16(1):57-64.
26. Meyer JMA, M.A. The Fluorescent Pigment of *Pseudomonas fluorescens*: Biosynthesis, Purification and Physicochemical Properties. *Microbiology.* 1978;107:9.
27. Farhan NJ, S.L.A. Utility of MolecuLight for managing bacterial burden in pediatric burns. *J Burn Care Res.* 2019.
28. Hurley CM, McClusky P, Sugrue RM, Clover JA, Kelly JE. Efficacy of a bacterial fluorescence imaging device in an outpatient wound care clinic: a pilot study. *J Wound Care.* Jul 2 2019;28(7):438-443.
29. Serena TE. Evaluation of MolecuLight i:X as an Adjunctive Fluorescence Imaging Tool to Clinical Signs and Symptoms for the Identification of Bacteria-containing Wounds. *Clinicaltrials.gov #NCT03540004* 2019.
30. Aung B. Can Fluorescence Imaging Predict the Success of CTPs for Wound Closure and Save Costs? *Today's Wound Clinic.* Vol 13 2019:22-25.
31. Blumenthal E, Jeffery SLA. The Use of the MolecuLight i:X in Managing Burns: A Pilot Study. *J Burn Care Res.* Jan 1 2018;39(1):154-161.
32. DaCosta RS, Kulbatski I, Lindvere-Teene L, et al. Point-of-care autofluorescence imaging for real-time sampling and treatment guidance of bioburden in chronic wounds: first-in-human results. *PLoS One.* 2015;10(3):e0116623.
33. Hill R, Rennie MY, Douglas J. Using Bacterial Fluorescence Imaging and Antimicrobial Stewardship to Guide Wound Management Practices: A Case Series. *Ostomy Wound Manage.* Aug 2018;64(8):18-28.
34. Raizman R. Fluorescence imaging guided dressing change frequency during negative pressure wound therapy: a case series. *J Wound Care.* Sep 1 2019;28(Sup9):S28-S37.

35. Serena TE, Harrell K, Serena L, Yaakov RA. Real-time bacterial fluorescence imaging accurately identifies wounds with moderate-to-heavy bacterial burden. *J Wound Care*. Jun 2 2019;28(6):346-357.
36. Rennie MY, Dunham D, Lindvere-Teene L, Raizman R, Hill R, Linden R. Understanding Real-Time Fluorescence Signals from Bacteria and Wound Tissues Observed with the MolecuLight i:X(TM). *Diagnostics (Basel)*. Feb 26 2019;9(1).
37. Amin RM, Bhayana B, Hamblin MR, Dai T. Antimicrobial blue light inactivation of *Pseudomonas aeruginosa* by photo-excitation of endogenous porphyrins: In vitro and in vivo studies. *Lasers Surg Med*. 2016;48(5):562-568.
38. Zhao HL, Zhang CP, Zhu H, Jiang YF, Fu XB. Autofluorescence of collagen fibres in scar. *Skin Res Technol*. Nov 2017;23(4):588-592.
39. Buchanan K, Heimbach DM, Minshew BH, Coyle MB. Comparison of quantitative and semiquantitative culture techniques for burn biopsy. *J Clin Microbiol*. Feb 1986;23(2):258-261.
40. Sauget M, Valot B, Bertrand X, Hocquet D. Can MALDI-TOF Mass Spectrometry Reasonably Type Bacteria? *Trends in Microbiology*. 2017;25(6):447-455.
41. Moskowitz CS, Pepe MS. Comparing the predictive values of diagnostic tests: sample size and analysis for paired study designs. *Clin Trials*. 2006;3(3):272-279.
42. Serena TE, Cole W, Coe S, et al. The safety of punch biopsies on hard-to-heal wounds: a large multicentre clinical trial. *Journal of Wound Care*. 2020;29(Sup2c):S4-S7.
43. Bowler PG. Wound pathophysiology, infection and therapeutic options. *Ann Med*. 2002;34(6):419-427.
44. Cutting KF, White RJ. Criteria for identifying wound infection--revisited. *Ostomy Wound Manage*. Jan 2005;51(1):28-34.
45. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science*. May 21 1999;284(5418):1318-1322.
46. Lopez AJ. In vivo detection of bacteria within a biofilm using a point-of-care fluorescence imaging device (Abstract). Paper presented at: Symposium on Advanced Wound Care 2020; virtual.

47. Attinger C, Wolcott R. Clinically Addressing Biofilm in Chronic Wounds. *Adv Wound Care (New Rochelle)*. Jun 2012;1(3):127-132.
48. Blackshaw EL, Jeffery SLA. Efficacy of an imaging device at identifying the presence of bacteria in wounds at a plastic surgery outpatients clinic. *J Wound Care*. Jan 2 2018;27(1):20-26.
49. Olsson M, Jarbrink K, Divakar U, et al. The humanistic and economic burden of chronic wounds: A systematic review. *Wound Repair Regen*. Jan 2019;27(1):114-125.
50. Fakorede FA. Increasing awareness about peripheral artery disease can save limbs and lives. *Am J Manag Care*. Dec 2018;24(14 Spec No.):SP609.
51. Lipsky BA, Dryden M, Gottrup F, Nathwani D, Seaton RA, Stryja J. Antimicrobial stewardship in wound care: a Position Paper from the British Society for Antimicrobial Chemotherapy and European Wound Management Association. *J Antimicrob Chemother*. Nov 2016;71(11):3026-3035.
52. Raizman R, Dunham D, Lindvere-Teene L, et al. Use of a bacterial fluorescence imaging device: wound measurement, bacterial detection and targeted debridement. *J Wound Care*. Dec 2 2019;28(12):824-834.
53. Cole W, Coe S. Use of a bacterial fluorescence imaging system to target wound debridement and accelerate healing: a pilot study. *J Wound Care*. Jul 1 2020;29(Sup7):S44-s52.
54. Hogsberg T, Bjarnsholt T, Thomsen JS, Kirketerp-Moller K. Success rate of split-thickness skin grafting of chronic venous leg ulcers depends on the presence of *Pseudomonas aeruginosa*: a retrospective study. *PLoS One*. 2011;6(5):e20492.
55. Xu Z, Hsia HC. The Impact of Microbial Communities on Wound Healing: A Review. *Ann Plast Surg*. Jul 2018;81(1):113-123.
56. Zekri A KW. Success of skin grafting on a contaminated recipient surface. *Eur J Plast Surg*. 1995;18:40-42.
57. Moelleken M, Jockenhöfer F, Benson S, Dissemond J. Prospective clinical study on the efficacy of bacterial removal with mechanical debridement in and around chronic leg ulcers assessed with fluorescence imaging. *International Wound Journal*. n/a(n/a).

58. Copeland-Halperin LR, Kaminsky AJ, Bluefeld N, Miraliakbari R. Sample procurement for cultures of infected wounds: a systematic review. *J Wound Care*. Apr 2016;25(4):S4-6, S8-10.
59. Huang Y, Cao Y, Zou M, et al. A Comparison of Tissue versus Swab Culturing of Infected Diabetic Foot Wounds. *Int J Endocrinol*. 2016;2016:8198714.
60. Tedeschi S, Negosanti L, Sgarzani R, et al. Superficial swab versus deep-tissue biopsy for the microbiological diagnosis of local infection in advanced-stage pressure ulcers of spinal-cord-injured patients: a prospective study. *Clin Microbiol Infect*. Dec 2017;23(12):943-947.
61. Thomas Hess C. Checklist for Factors Affecting Wound Healing. *Advances in Skin & Wound Care*. 2011;24(4):192.
62. McCarty SM PS. Proteases and Delayed Wound Healing. *Advances in Wound Care*. 2013;2(8):438-447.
63. Jeffery S. The utility of MolecuLight bacterial sensing in the management of burns and traumatic wounds. Paper presented at: SPIE Photonic Diagnosis and Treatment of Infections and Inflammatory Diseases; March 7, 2019, 2019.
64. Cooper L, Gale A, Darker I, Toms A, Saada J. *Radiology image perception and observer performance: How does expertise and clinical information alter interpretation? Stroke detection explored through eye-tracking*. Vol 7263: SPIE; 2009.
65. Wood G, Knapp KM, Rock B, Cousens C, Roobottom C, Wilson MR. Visual expertise in detecting and diagnosing skeletal fractures. *Skeletal Radiol*. Feb 2013;42(2):165-172.
66. Nakashima R, Kobayashi K, Maeda E, Yoshikawa T, Yokosawa K. Visual Search of Experts in Medical Image Reading: The Effect of Training, Target Prevalence, and Expert Knowledge. *Frontiers in Psychology*. 2013-April-05 2013;4(166).
67. Esserman L, Cowley H, Eberle C, et al. Improving the accuracy of mammography: volume and outcome relationships. *J Natl Cancer Inst*. Mar 6 2002;94(5):369-375.
68. Brealey S, Scally A, Hahn S, Thomas N, Godfrey C, Coomarasamy A. Accuracy of radiographer plain radiograph reporting in clinical practice: a meta-analysis. *Clin Radiol*. Feb 2005;60(2):232-241.

69. Wolcott RD, Hanson JD, Rees EJ, et al. Analysis of the chronic wound microbiota of 2,963 patients by 16S rDNA pyrosequencing. *Wound Repair Regen.* Jan-Feb 2016;24(1):163-174.
70. Hill R. How effective is your wound cleanser?: An evaluation using bacterial fluorescence imaging. Paper presented at: SAWC Spring 20192019.

Table 1: Baseline characteristics of study participants. Wounds that were ‘microbiology positive’ had bacterial loads $>10^4$ CFU/g. Fischer’s exact test was used to compare microbiology positive and microbiology negative subsets of each characteristic described. Statistical significance was set at $p=0.05$; bold values indicate significance.

Characteristic	All patients (n=350)	Microbiology positive (n=287)	Microbiology negative (n=63)	P value
Age mean (SD)	60.19 (12.44)	59.95 (12.11)	61.27 (13.87)	0.45
Female	125.00 (35.71)	87 (30.31)	38 (60.32)	<0.001
Systemic antibiotic use (Yes)	90 (25.71)	56 (19.51)	34 (53.97)	<0.001
Delayed healing present	185 (52.86)	158 (55.05)	27 (42.86)	0.094
Fitzpatrick score				
Light (I or II)	224 (64.00)	179 (62.37)	45 (71.43)	0.50
Medium (III or IV)	83 (23.71)	74 (25.78)	9 (14.29)	
Dark (V or VI)	43 (12.29)	34 (11.85)	9 (14.29)	
Wound type				
DFU	138 (39.43)	123 (42.86)	15 (23.81)	0.009
PU	22 (6.29)	20 (6.97)	2 (3.17)	
SSI	60 (17.14)	44 (15.33)	16 (25.40)	
VLU	106 (30.29)	79 (27.53)	27 (42.86)	

Other	24 (6.86)	21 (7.32)	3 (4.76)	28
Wound duration				
<3 months	106 (30.29)	79 (27.53)	27 (42.86)	0.008
3-12 months	118 (33.71)	93 (32.40)	25 (39.68)	
> 12 months	126 (36.00)	115 (40.07)	11 (17.46)	
Median (range) total bacterial load		1.80 x 10 ⁶ (0.00 – 7.70 x 10 ⁹)		

Table 2: Estimates of positive predictive value, negative predictive value and accuracy for detection of bacterial loads >10⁴ CFU/g. PPV, NPV and accuracy were estimated for CSS, CSS+FL and FL using microbiological analysis of total bacteria load to serve as ground truth. Clinical signs and symptoms of infection (CSS) combined with fluorescence imaging (FL) were compared with CSS and FL alone at the participant level. All p-values were derived from one-sided tests.

	CSS	CSS+FL	FL	CSS vs CSS+FL	CSS vs FL
	% [95% CI]			P-value	P-value
Positive Predictive Value (PPV)	91.67 [83.85, 99.49]	94.59 [91.34, 97.85]	96.00 [93.10, 98.90]	0.19	0.14
Negative Predictive Value (NPV)	19.54 [15.06, 24.01]	32.12 [25.00, 39.25]	32.00 [25.09, 38.91]	<0.001	<0.001
Accuracy	29.43 [24.90, 34.41]	65.14 [60.01, 69.95]	64.00 [58.84, 68.85]	<0.001	<0.001

Table 3: Impact of fluorescence imaging (FL) on care plan. Clinicians completed a survey on utility of fluorescence information after capturing images. The total number of participants where fluorescence information influenced care plan is listed in column 1. For each survey item, a Fischer's exact test was performed to assess differences between wounds deemed positive (FL+) or negative (FL-) for bacterial fluorescence. Statistical significance was set at $p=0.05$; values in bold indicate significance.

	No./Total (%)	FL+	FL-	P value
Impact on diagnosis and patient care				
Improved patient care	315/350 (90.00)	169/315 (53.65)	146/315 (46.35)	<0.001
Changed diagnosis of bacterial burden	183/350 (52.29)	141/183 (77.05)	42/183 (22.95)	<0.001
Changed clinic treatment plan	241/350 (68.86)	148/241 (61.41)	93/241 (38.59)	<0.001
Increased clinician confidence (if no change to wound assessment)	134/350 (38.29)	41/134 (30.60)	93/134 (69.40)	<0.001
Aspects of wound bed preparation influenced by fluorescence imaging				
Any aspect of wound bed preparation	296/350 (84.57)	160/296 (54.05)	136/296 (45.95)	<0.001
Tissue management	236/350 (67.43)	131/236 (55.51)	105/236 (44.49)	0.004
Infection or inflammation	267/350 (76.29)	158/267	109/267	<0.001

		(59.18)	(40.82)	
Moisture imbalance	57/350 (16.29)	27/57 (47.37)	30/57 (52.63)	0.77
Edge Advance	65/350 (18.57)	32/65 (49.23)	33/65 (50.77)	>0.99
Aspects of wound care influenced by fluorescence imaging:				
Wound assessment	275/350 (78.57)	142/275 (51.64)	133/275 (48.36)	0.30
Cleansing	150/350 (42.86)	95/150 (63.33)	55/150 (36.67)	<0.001
Debridement	168/350 (48.00)	105/168 (62.50)	63/168 (37.50)	<0.001
Sampling location	156/350 (44.57)	121/350 (77.56)	35/156 (22.44)	<0.001
Treatment selection	194/350 (55.43)	116/194 (59.79)	78/194 (40.21)	<0.001
Antimicrobial stewardship	186/350 (53.14)	120/186 (64.52)	66/186 (35.48)	<0.001
Wound documentation	158/350 (45.14)	97/158 (61.39)	61/158 (38.61)	<0.001

Figure Legends

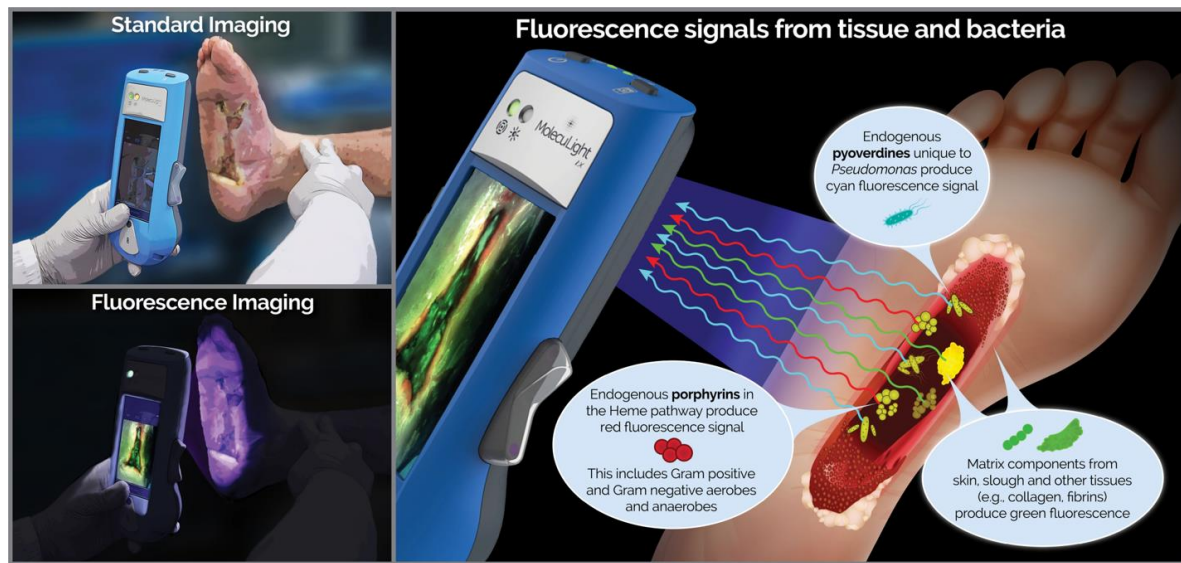


Figure 1: (A) Standard and (B) fluorescence imaging using the MolecuLight *i:X*. The green range finder LED indicates that the device is within optimal range (8-12 cm) and correctly positioned for imaging. Darkness is required (achieved by turning off room lights turned or using a DarkDrape) to capture fluorescence images. (C) When a wound is illuminated by the safe, violet (405 nm) light, components in the wound are excited up to a depth of 1.5 mm. Porphyrin-producing bacteria within the wound emit red fluorescence signals, *Pseudomonas aeruginosa* emits cyan fluorescence signals and tissue components (e.g., collagen, fibrins) emit green fluorescence signals. An optical filter on the device captures these relevant signals and prevents reflected violet light from contaminating the image without any digital processing.

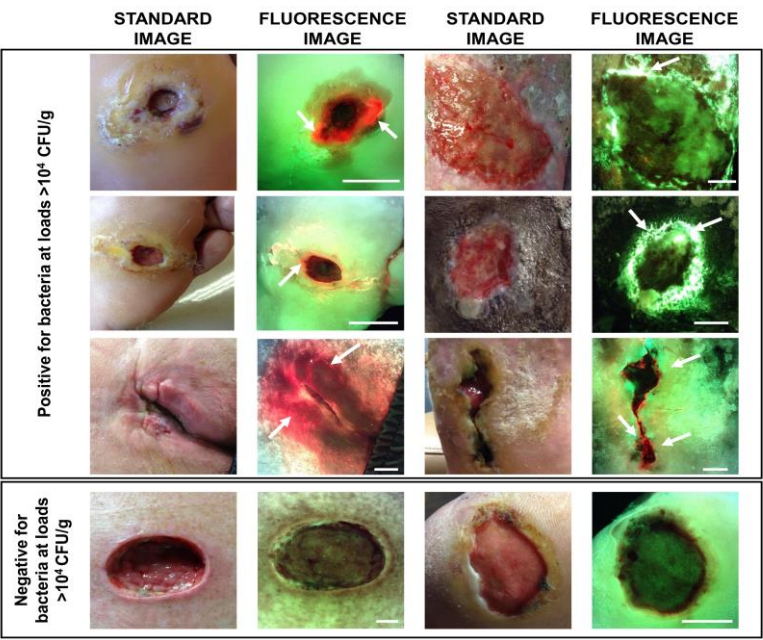


Figure 2: Representative fluorescence images of wounds that were positive or negative for moderate-to-heavy loads of bacteria ($>10^4$ CFU/g) in and around the wound bed. White arrows indicate regions of red or cyan fluorescence from bacteria; scale bars represent 1 cm.

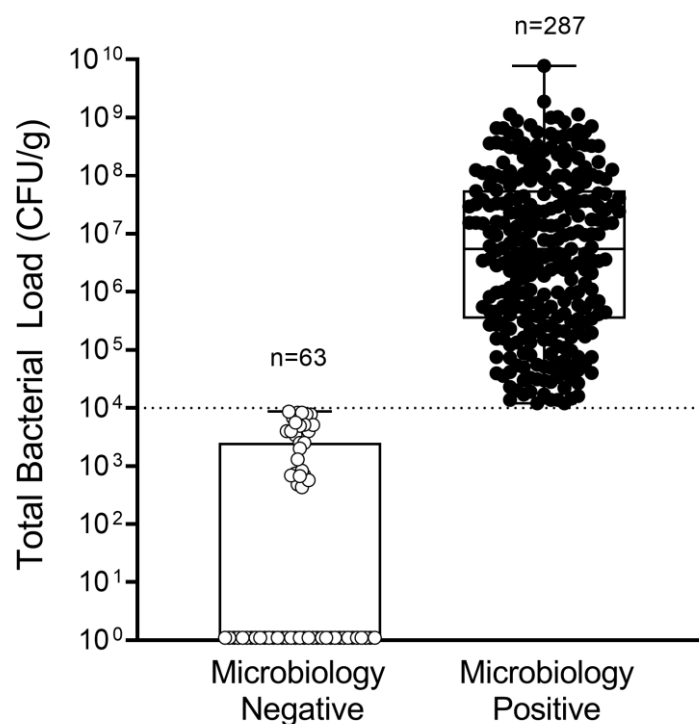


Figure 3: Box plot shows the distribution of total bacterial load (CFU/g) of each wound biopsied (n=350 wounds total) based on whether wounds were microbiologically negative (bacterial load $<10^4$ CFU/g; n=63) or positive ($>10^4$ CFU/g; n=287). Boxes contain the 25th to 75th percentiles of data set while center line indicates median bacterial load of all wounds (10^6 CFU/g). Black whiskers represent minimum and maximum values. Dashed line indicates lowest threshold (10^4 CFU/g) at which bacteria can be detected using fluorescence imaging. Of the microbiology negative wound biopsies, 36 had total bacterial load of 0.

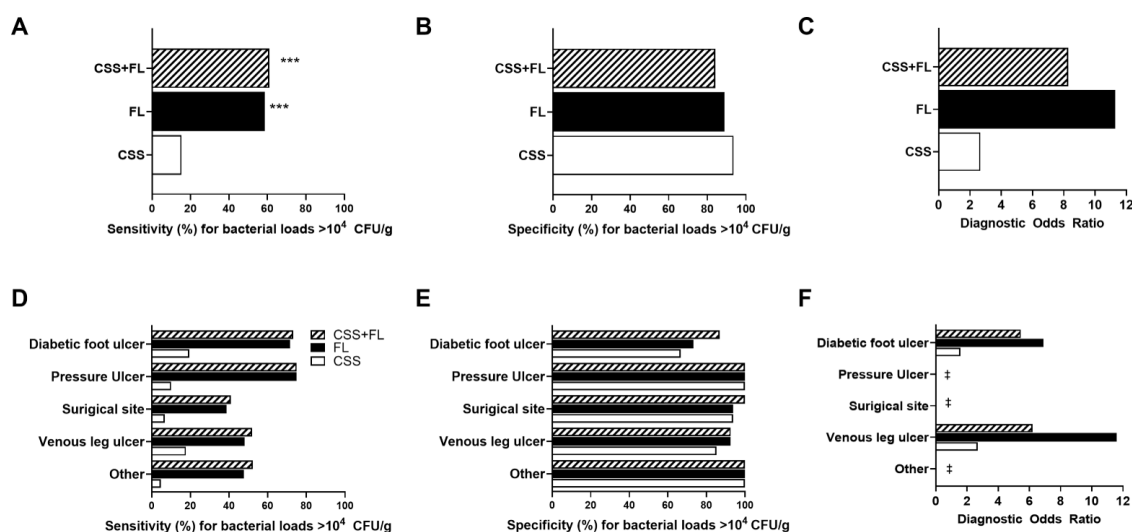


Figure 4: Clinical signs and symptoms of infection (CSS) combined with fluorescence imaging (FL) were compared with CSS and FL alone at the participant level for sensitivity (A), specificity (B) and diagnostic odds ratio (C) (n=350). Comparisons were also made between CSS, FL, and CSS+FL for each wound type (D-F). ***p<0.001 derived from a one-sided McNemar exact test. ‡When specificity was 100%, a DOR could not be calculated and compared between groups.

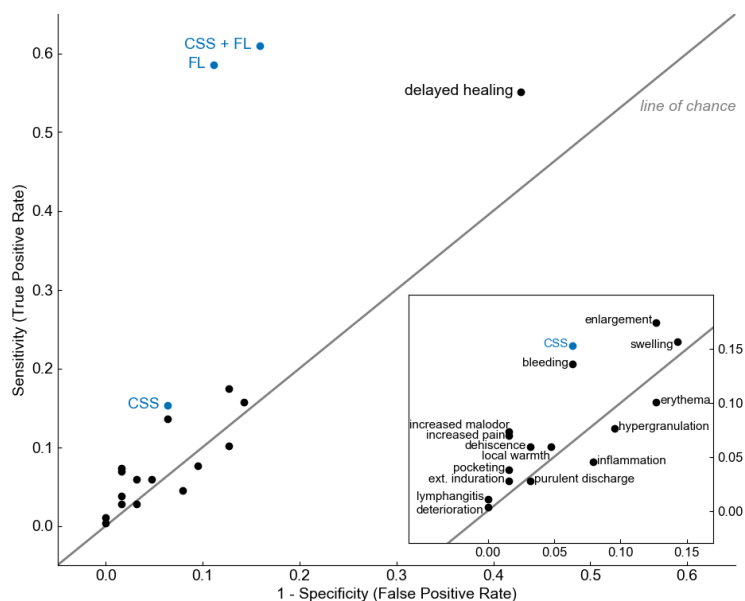


Figure 5: Scatter plot (pairs of sensitivity, 1-specificity) comparing discriminative power of clinical signs and symptoms of infection (CSS, based on IWII criteria¹⁴), individual signs of infection, fluorescence imaging (FL), and CSS+FL. Values in the top left corner indicate high discriminative power. Erythema, hypergranulation, inflammation and purulent discharge all fell below the line of chance indicating they were no better than ‘flipping a coin’ at predicting bacterial loads $>10^4$ CFU/g in wounds.

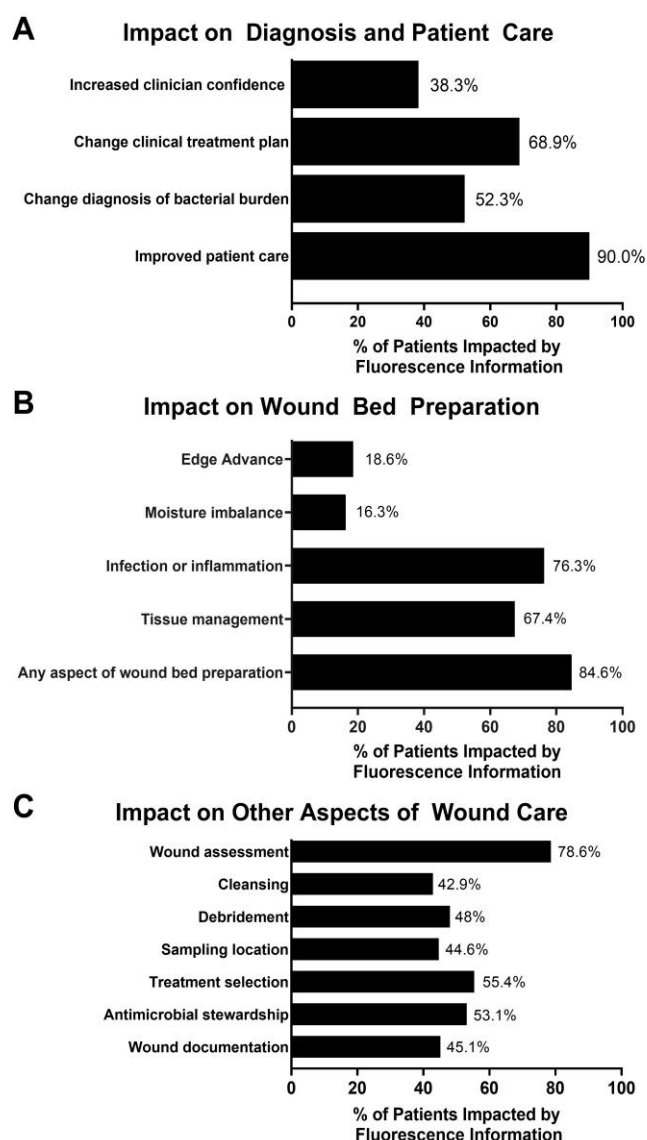


Figure 6: Impact of fluorescence imaging (FL) on care plan. Clinicians completed a survey on utility of fluorescence information after capturing images. Clinicians reported on how FL information impacted diagnosis and patient care (A), wound bed preparation (B), and other aspects of wound care (C). Values indicate the percent of wounds impacted by FL information.