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**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

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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 (>10⁴ 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 >10⁴ CFU/g, and CSS missed detection of 85% of these wounds. FL significantly increased detection of bacteria (>10⁵ 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 lifetime\(^1\) and the incidence of wounds continues to rise as the population ages and co-morbidities mount\(^2\). As a result, management of chronic wounds accounts for >5% of total health care expenditures in the US and UK\(^3\)-\(^6\).

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 healing\(^9\)-\(^11\) and increases the risk of wound complications, including infection, sepsis and amputation\(^12\)-\(^14\). Guidelines advise that early diagnosis of high bacterial burden is essential to prevent the wound from progression to local or systemic infection\(^15\). 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 CSS\(^18\). 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 years\(^20\), 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)\textsuperscript{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 \textit{Staphylococcus}, \textit{Proteus}, \textit{Klebsiella} and \textit{Pseudomonas} genera\textsuperscript{22,23} endogenously emit red or cyan fluorescent signatures\textsuperscript{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\textsuperscript{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)\textsuperscript{30} in chronic wounds and burns\textsuperscript{27,28,31-35}, however these studies lacked rigour and statistical power. The \textbf{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.

\textbf{Materials & Methods}

\textbf{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 checklist. 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 guidelines^{15}, 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 mm^{36}. 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 porphyrins^{37}, it uniquely produces a cyan fluorescent signal due to endogenous pyoverdine, a virulence factor^{26}. 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 bacteria^{36}. 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.

**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 diluted 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 35ºC 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 described. 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 Pepe. 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 reported\textsuperscript{42}.

In 82% (287/350) of wounds, bacterial loads >10\textsuperscript{4} CFU/g were observed, confirmed by microbiological analysis (Figure 3). Median (range) total bacterial load of all wounds was 1.8 x 10\textsuperscript{6} CFU/g (0.0 to 7.7 x 10\textsuperscript{9} CFU/g). A higher proportion of males (69.7%) than females (30.3%) had microbiology positive wounds (>10\textsuperscript{4} CFU/g). Of the microbiology positive wounds, 19.5% were on systemic antibiotics, and bacterial load of these wounds averaged (SD) 1.4 x10\textsuperscript{7} CFU/g (3.1 x10\textsuperscript{7} CFU/g); over 50% of microbiology negative wounds (<10\textsuperscript{4} CFU/g) were on systemic antibiotics. Bacterial loads >10\textsuperscript{6} 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\textsuperscript{6} CFU/g, which some consider to be indicative of infection\textsuperscript{17}; in 16.9% (59/350) of wounds, bacterial loads >10\textsuperscript{8} CFU/g were observed, while 18% (63/350) of wounds had bacterial loads ≤10\textsuperscript{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\textsuperscript{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 practices\(^\text{15}\). 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 \(1.8 \times 10^6\) 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\textsuperscript{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 infection\textsuperscript{18}. 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)\textsuperscript{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\textsuperscript{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”\textsuperscript{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\textsuperscript{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 wounds \textsuperscript{47}. 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\textsuperscript{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\textsuperscript{3,4,49}, and contributes to some of the 196 daily DFU-related amputations in the US\textsuperscript{50}. Under- and over-treatment can lead to sub-optimal wound care, inflated costs, and antibiotic misuse\textsuperscript{51}. The robust performance characteristics of FL reported here demonstrate the applicability of this diagnostic procedure to facilitate earlier detection of detrimental wound bacterial burden\textsuperscript{15}.

According to guidelines\textsuperscript{15}, intervention is mandated in wounds when bacterial colonisation turns into local infection (≥10\textsuperscript{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\textsuperscript{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:

\textbf{(1) Guided wound bed preparation in ≥ 90% of wounds in this and other studies\textsuperscript{35,52}.} Information on location of bacterial burden at point-of-care has been shown to be highly impactful for debridement\textsuperscript{52,53}, selection of appropriate cleanser\textsuperscript{30}, and general wound bed preparation prior to application of advanced therapies\textsuperscript{30}. Advanced therapies such as cellular and tissue-based products and skin grafts often fail when high bacterial loads are present\textsuperscript{54-56}.

\textbf{(2) Alerted clinicians to unexpected location of bacterial loads\textsuperscript{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 region\textsuperscript{57} and sampling is rarely performed outside of the wound bed\textsuperscript{58-60}. The FL information in this study provided objective evidence on location of bacteria to facilitate targeted eradication.

\textbf{(3) Provided information on efficacy of antibiotics and guided stewardship decisions without delay\textsuperscript{35}.} 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 stewardship$^{51}$ 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 heterogenous 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 insufficiency$^{61}$ and protease activity$^{62}$ 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\textsuperscript{21,28,29,63}. As with other diagnostic imaging modalities\textsuperscript{64-66}, we anticipate that the performance measures reported should be improved with increased experience\textsuperscript{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\textsuperscript{32,53}. Due to the limited (1.5 mm) depth of excitation\textsuperscript{36} and inability to detect non-porphyrin producing bacteria, including species from the \textit{Streptococcus}, \textit{Enterococcus} and \textit{Finegoldia} genera (which account for an estimated 12% of the most prevalent wound pathogens\textsuperscript{23} and rarely occur monomicrobially\textsuperscript{69}), it is recommended that FL be used in combination with CSS.

\textbf{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\textsuperscript{6} CFU/g, that some suggest are indicative of infection\textsuperscript{18}. 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\textsuperscript{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)\textsuperscript{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.

\textbf{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 (>10⁴ 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 (>10⁴ 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 (>10⁴ 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%).

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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


7. Cho S. Development of a Model to Predict Healing of Chronic Wounds Within 12 Weeks. *Advances in Wound Care*. 2020;0(0).


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).


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.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All patients (n=350)</th>
<th>Microbiology positive (n=287)</th>
<th>Microbiology negative (n=63)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age mean (SD)</td>
<td>60.19 (12.44)</td>
<td>59.95 (12.11)</td>
<td>61.27 (13.87)</td>
<td>0.45</td>
</tr>
<tr>
<td>Female</td>
<td>125.00 (35.71)</td>
<td>87 (30.31)</td>
<td>38 (60.32)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systemic antibiotic use (Yes)</td>
<td>90 (25.71)</td>
<td>56 (19.51)</td>
<td>34 (53.97)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Delayed healing present</td>
<td>185 (52.86)</td>
<td>158 (55.05)</td>
<td>27 (42.86)</td>
<td>0.094</td>
</tr>
<tr>
<td>Fitzpatrick score</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light (I or II)</td>
<td>224 (64.00)</td>
<td>179 (62.37)</td>
<td>45 (71.43)</td>
<td>0.50</td>
</tr>
<tr>
<td>Medium (III or IV)</td>
<td>83 (23.71)</td>
<td>74 (25.78)</td>
<td>9 (14.29)</td>
<td></td>
</tr>
<tr>
<td>Dark (V or VI)</td>
<td>43 (12.29)</td>
<td>34 (11.85)</td>
<td>9 (14.29)</td>
<td></td>
</tr>
<tr>
<td>Wound type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DFU</td>
<td>138 (39.43)</td>
<td>123 (42.86)</td>
<td>15 (23.81)</td>
<td></td>
</tr>
<tr>
<td>PU</td>
<td>22 (6.29)</td>
<td>20 (6.97)</td>
<td>2 (3.17)</td>
<td>0.009</td>
</tr>
<tr>
<td>SSI</td>
<td>60 (17.14)</td>
<td>44 (15.33)</td>
<td>16 (25.40)</td>
<td></td>
</tr>
<tr>
<td>VLU</td>
<td>106 (30.29)</td>
<td>79 (27.53)</td>
<td>27 (42.86)</td>
<td></td>
</tr>
<tr>
<td>Wound duration</td>
<td>&lt;3 months</td>
<td>3-12 months</td>
<td>&gt; 12 months</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>-----------</td>
<td>-------------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>24 (6.86)</td>
<td>21 (7.32)</td>
<td>3 (4.76)</td>
<td></td>
</tr>
<tr>
<td>Median (range) total bacterial load</td>
<td>1.80 x 10^6 (0.00 – 7.70 x 10^9)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Estimates of positive predictive value, negative predictive value and accuracy for detection of bacterial loads $>10^4$ 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.

<table>
<thead>
<tr>
<th></th>
<th>CSS</th>
<th>CSS+FL</th>
<th>FL</th>
<th>CSS vs CSS+FL</th>
<th>CSS vs FL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Positive Predictive Value (PPV)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% [95% CI]</td>
<td>91.67 [83.85, 99.49]</td>
<td>94.59 [91.34, 97.85]</td>
<td>96.00 [93.10, 98.90]</td>
<td>0.19</td>
<td>0.14</td>
</tr>
<tr>
<td><strong>Negative Predictive Value (NPV)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% [95% CI]</td>
<td>19.54 [15.06, 24.01]</td>
<td>32.12 [25.00, 39.25]</td>
<td>32.00 [25.09, 38.91]</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Accuracy</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% [95% CI]</td>
<td>29.43 [24.90, 34.41]</td>
<td>65.14 [60.01, 69.95]</td>
<td>64.00 [58.84, 68.85]</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Impact on diagnosis and patient care</th>
<th>FL+</th>
<th>FL-</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No./Total (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Improved patient care</td>
<td>169/315 (53.65)</td>
<td>146/315 (46.35)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Changed diagnosis of bacterial burden</td>
<td>141/183 (77.05)</td>
<td>42/183 (22.95)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Changed clinic treatment plan</td>
<td>148/241 (61.41)</td>
<td>93/241 (38.59)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Increased clinician confidence (if no change to wound assessment)</td>
<td>41/134 (30.60)</td>
<td>93/134 (69.40)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Aspects of wound bed preparation influenced by fluorescence imaging

<table>
<thead>
<tr>
<th>Any aspect of wound bed preparation</th>
<th>FL+</th>
<th>FL-</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No./Total (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue management</td>
<td>131/236 (55.51)</td>
<td>105/236 (44.49)</td>
<td>0.004</td>
</tr>
<tr>
<td>Infection or inflammation</td>
<td>158/267 (59.42)</td>
<td>109/267 (40.58)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Advances in Wound Care

Diagnostic Accuracy of Point-of-Care Fluorescence Imaging for the Detection of Bacterial Burden in Wounds: Results from the 350-Patient FLAAG Trial (DOI: 10.1089/wound.2020.1272)

This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

| 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 (>10⁴ 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 (>10^6 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 criteria\textsuperscript{14}), 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\textsuperscript{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.