Noninvasive imaging of *Staphylococcus aureus* infections with a nuclease-activated probe

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Technologies that enable the rapid detection and localization of bacterial infections in living animals could address an unmet need for infectious disease diagnostics. We describe a molecular imaging approach for the specific, noninvasive detection of *S. aureus* based on the activity of the *S. aureus* secreted nuclease, micrococcal nuclease (MN). Several short synthetic oligonucleotides, rendered resistant to mammalian serum nucleases by various chemical modifications and flanked with a fluorophore and quencher, were activated upon degradation by purified MN and in *S. aureus* culture supernatants. A probe consisting of a pair of deoxythymidines flanked by several 2′-O-methyl–modified nucleotides was activated in culture supernatants of *S. aureus* but not in culture supernatants of several other pathogenic bacteria. Systemic administration of this probe to mice bearing *S. aureus* muscle infections resulted in probe activation at the infection sites in an MN-dependent manner. This new bacterial imaging approach has potential clinical applicability for infections with *S. aureus* and several other medically important pathogens.

Diagnosis of focal bacterial infections, such as osteomyelitis, septic arthritis and pyomyositis, initially entails the evaluation of several nonspecific symptoms, including pain, swelling and fever¹–³. Definitive evidence of infection and identification of the causative bacterial species can only be obtained via tissue biopsy and culture. Whereas focal bacterial infections can be life-threatening situations in which time is of the essence, such diagnostic procedures typically consume many hours to days. Moreover, current diagnostic approaches are prone to false negative results⁴. Rapid, sensitive and noninvasive molecular imaging assays for bacterial infections could thus address an unmet need for infectious disease diagnostics.

Preclinical development of molecular imaging approaches for bacterial infections offers hope for the future availability of such technologies⁵–¹¹. Although promising, none of these approaches provides a platform for the development of species-selective imaging probes for multiple bacterial species. Bacterial imaging probes that provide species-specific information are desirable because they could facilitate the rapid administration of appropriate therapy (for example, antibiotic treatment tailored for the species) in clinical settings. Nucleases, which have widespread expression and diversity, are one category of proteins that could be used to specifically identify a variety of bacterial species¹². As degrading enzymes, nucleases can be detected with activatable probes¹³. Activatable probes release their signal (usually fluorescence) only upon encountering their target molecule, which greatly increases the sensitivity of target detection and thus provides an important advantage over nonactivatable probes¹⁴. We recently reported that several as-yet-unidentified nucleases produced by a species of mycoplasma (*Mycoplasma fermentans*) can efficiently digest chemically modified oligonucleotides that are resistant to degradation by mammalian nucleases¹⁵. This finding suggested the use of chemically modified oligonucleotides as the basis of activatable probes for the detection of *Mycoplasma fermentans*.

We began this study by asking whether bacterial pathogens that are responsible for substantial morbidity and mortality might also express nucleases capable of degrading chemically modified oligonucleotides. Such nucleases could potentially be used as the probe-activating component of an imaging approach for pathogenic bacteria. We focused our efforts on *S. aureus*, the most common cause of many types of focal infections in humans¹–³. *S. aureus* secretes a nuclease known as MN, a very well-studied enzyme that is among the first proteins secreted by bacteria upon invasion of a mammalian host. MN exhibits robust DNase and RNase activities and is active on both single- and double-stranded DNA substrates, and its nuclease activity has been used to classify bacterial isolates for decades¹⁷,¹⁹. Here we report the noninvasive detection of *S. aureus* infections with a quenched fluorescent oligonucleotide probe that we tailored to be specifically activated by MN. The probe is expected to be both nontoxic and inexpensive, and it enables infection localization in less than an hour when administered at low doses. These elements could facilitate the translation of this infection imaging approach into clinical practice.

**RESULTS**

We sought a short oligonucleotide substrate that is both sensitive to MN and resistant to serum nucleases. Such an oligonucleotide

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Received 1 February 2013; accepted 26 April 2013; published online 2 February 2014; doi:10.1038/nm.3460
could form the basis of a quenched fluorescent imaging probe that is specifically activated (fluorescence is unquenched) upon digestion by MN\textsuperscript{13}. Because the susceptibility of chemically modified oligonucleotide substrates to MN digestion is poorly understood, we tested the ability of MN to degrade oligonucleotide substrates with chemical modifications that are known to promote resistance to degradation by mammalian serum nucleases\textsuperscript{20}. To facilitate the subsequent development of imaging probes, we tested the various oligonucleotide compositions in a quenched fluorescent probe format: short (10- to 12-mer) oligonucleotides flanked with a 5` fluorophore (fluorescein amide (FAM)) and 3` quenchers (ZEN and Iowa Black RQ)\textsuperscript{13,15}.

One such probe, made with an oligonucleotide composed exclusively of locked nucleic acid–modified nucleotides, was not digested by MN (F.J.H., unpublished observation), whereas oligonucleotides composed exclusively of 2'-fluoro- or 2'-O-methyl–modified nucleotides were relatively weak substrates (data not shown)\textsuperscript{20,21}. Next, we compared the MN and serum nuclease susceptibility of RNA oligonucleotides composed of 2'-fluoro- or 2'-O-methyl–modified pyrimidines and unmodified purines with that of a DNA oligonucleotide, as DNA is the preferred substrate for MN among unmodified nucleic acids (see Table 1 for probe sequences and modifications)\textsuperscript{17}. A 1 U µl\textsuperscript{-1} MN concentration yielded complete or near-complete digestion of these oligonucleotides after short incubations and was thus used as a normalization control for the assays. More dilute MN (0.1 U µl\textsuperscript{-1}) provided an intermediate degree of digestion after 15 or 60 min, thus enabling assessment of the relative degree of digestion of the substrates. The DNA probe was digested by MN (in PBS supplemented with CaCl\textsubscript{2} and MgCl\textsubscript{2}) more efficiently than either the 2'-fluoro- or 2'-O-methyl–modified pyrimidine RNA oligonucleotides, but was, as expected, also substantially digested in serum (Fig. 1a). In contrast, the 2'-fluoro- and 2'-O-methyl–modified pyrimidine RNA oligonucleotides were more stable in serum but less efficiently digested by MN. A second-generation probe, composed of a pair of deoxothymidines flanked by several 2'-O-methyl–modified nucleotides, was designed to maximize sensitivity to MN, which is known to efficiently digest oligonucleotides with deoxythymidine nucleotides while also resisting serum nuclease degradation\textsuperscript{22}. This 'TT probe' was substantially more sensitive to MN digestion than the other chemically modified oligonucleotides tested, and it also exhibited robust serum stability (Fig. 1a). Probes in which AT or AA were substituted for the central TT exhibited reduced sensitivity for MN digestion and were not pursued with further experiments (Supplementary Fig. 1). Measurement of TT probe activation by MN (0.05 U µl\textsuperscript{-1}) as a function of time revealed rapid activation kinetics, with an activation half-time of 7.7 min (Supplementary Fig. 2). The TT probe exhibited comparable activation by MN in the

| Table 1 Nucleic acid probe sequences and modifications |
|------------------------------------------------------|
| 2'-F Pyr | FAM-Fu-Fu-Fu-Gu-Fu-Fu-Gu-Fu-Fu-ZEN-RQ |
| 2'-Ome Pyr | FAM-mC-mC-Gu-mC-Gu-mC-Gu-mC-Gu-mC-ZEN-RQ |
| 2'-A | FAM-Fu-Fu-Fu-Fu-A-A-Gu-Fu-Fu-Fu-Fu-ZEN-RQ |
| 2'-Ome A | FAM-mC-mC-Gu-mC-Gu-mC-Gu-mC-Gu-mC-ZEN-RQ |

Figure 1 Nucleic acid probe activation by MN and serum nucleases. (a,b) Activation of various nucleic acid probes (see Table 1 for probe details) by MN and mouse and human serum (a) and MN-expressing and MN-negative (Newman and UAMS-1 strains) S. aureus culture supernatants (b). Each of the indicated probes was incubated at 37 °C with 1 U µl\textsuperscript{-1} (positive control) or 0.1 U µl\textsuperscript{-1} MN in Dulbecco's PBS (includes physiological levels of calcium and magnesium), or with 90% mouse or human serum (a) or with 90% of culture supernatants of the indicated S. aureus strains (prepared as described in the Online Methods). Mean fluorescence values of all reactions (measured in triplicate) with a given probe were normalized to the mean fluorescence measured with digestion of the probe with 1 U µl\textsuperscript{-1} MN. Data represent mean ± s.d. of the triplicate fluorescence measurements.
context of mouse or human serum and buffer (PBS), suggesting its in vivo utility (Supplementary Fig. 3).

To evaluate the activation of these probes in the context of the complex mixture of S. aureus–secreted factors, we incubated the probes with culture supernatants of the Newman and UAMS-1 strains of S. aureus (Fig. 1b, also see Supplementary Table 1 for bacterial strains used in this study). The TT probe was completely digested after a 60-min incubation in either supernatant (Fig. 1b). This was not unexpected because we previously measured extracellular MN levels of 0.45–0.6 U µl−1 in cultures of these strains23. The digestion observed here was primarily mediated by MN, as incubation of the TT probe in supernatants of MN-negative versions of each strain yielded minimal probe activation (Fig. 1b)32,34. In summary, among the serum nuclease–resistant oligonucleotides tested, the TT probe clearly exhibited the greatest sensitivity to digestion by MN, both in purified form and in culture supernatants.

The utility of visible-light fluorophores, such as fluorescein (excitation/emission (ex/em), 494/521 nm), for in vivo imaging is severely limited by tissue autofluorescence and scattering of visible light25. In contrast, tissue penetration of light with substantially longer wavelengths, especially within the near-infrared (NIR) range, is much greater, and tissue autofluorescence with NIR is minimal25. Indeed, fluorescence imaging with NIR light is estimated to be feasible at tissue depths of 7–14 cm (ref. 25). To prepare an MN-detecting imaging probe based on the TT probe that would be more suitable for in vivo imaging, we substituted Cy5.5 (ex/em: 685/706 nm), a fluorophore with emission in the NIR range, for the FAM moiety used in the initial TT probe version. The fluorescence of this intact probe was weak, but after digestion with MN, its fluorescence was comparable to that of a TT probe version. The weak TT probe activation observed upon incubation with MN-negative S. aureus cell suspensions (data not shown) is consistent with this conclusion. There were no overt differences in the extent of bacterial colonization, necrosis or inflammation between the wild-type and MN-negative infections in histological sections or in levels of circulating neutrophils in mice bearing these infections (Supplementary Figs. 8 and 9). Thus, the probe activation seen in the wild-type S. aureus–infected animals does not seem to be related to the inflammatory response to the infections. Quantification of probe fluorescence levels at the infection sites and in the uninfected legs of TT probe–injected mice yielded an approximately fourfold target-to-background ratio at 45 min after probe injection (Supplementary Fig. 10).

Although these results indicate that MN activates the TT probe at S. aureus infection sites in vivo, we observed that much of the probe fluorescence extended beyond the boundary of the bioluminescence signal. A simple and plausible explanation is that the intravenously administered probe may have limited access to the infection site in the setting of our pyomysitis infection model. Indeed, when bacterial infections progress to the stage of tissue necrosis, liquefaction or abscess formation, penetration of intravenously administered agents (including antibiotics) is limited and access becomes problematic30. To explore this possibility, we injected (via tail vein) the unquenched TT probe into mice with S. aureus–infected thigh muscles. The mice were subsequently euthanized and dissected to provide a clearer picture of the infection sites. The unquenched probe appeared to have very limited access to the infection site (Fig. 2f). We also observed activation of the TT probe adjacent to the infection site after killing and dissection (Fig. 2g). Moreover, histological examination of S. aureus–infected mouse thigh muscles (Supplementary Fig. 11) revealed lesions with substantial necrosis, an observation consistent with the notion that the infection sites have reduced blood perfusion and, consequently, reduced probe access. These results suggest that the probe activation seen in infected animals (Fig. 2d,g) may have resulted from the probe encountering MN that had leaked out of the primary infection site. The penetration rate that MN (a ~17 kDa protein) would need to exhibit in the muscle tissue to account for the probe activation observed (~4 mm in 48 h, or 0.08 mm h−1) is well within the limits found by others who have studied macromolecular penetration of muscle tissue (0.39–0.6 mm h−1 for 20-kDa dextran)33,34. In summary, the TT probe detected the presence of S. aureus, despite having only limited access to the region where the bacteria, and presumably MN, were most concentrated.

The clinical diagnostic value of assays that noninvasively detect bacterial infections such as pyomysitis, septic arthritis, etc., will depend, in part, on their ability to simultaneously identify the type of bacteria present. We thus sought to determine whether the TT probe, or any of the others we have tested, might also be activated by nucleases produced by other bacterial pathogens that cause some of the same types of infections as S. aureus. Of the culture supernatants of seven such bacterial species tested (Supplementary Table 2 lists colony-forming unit (CFU) ml−1 values), none substantially digested the TT probe, whereas Staphylococcus lugdunensis and Streptococcus agalactiae (group B Streptococcus) supernatants both digested the probes that included 2′-fluoro–modified nucleotides (Fig. 3a). The lack of activation of the DNA probe by culture supernatants of bacteria known to secrete DNases (for example, S. agalactiae), possibly reflects an incompatibility of the particular sequence used in this probe with the secreted DNases. Of the bacterial cell suspensions of the cultures tested, only that of S. lugdunensis (an emerging pathogen of the same genus as S. aureus) produced any appreciable digestion of the TT probe (~25%)
in a 1-h incubation (Fig. 3b). Bacterial cell suspensions of *S. agalactiae* and *Streptococcus pneumoniae* both digested the probes that included 2′-fluoro–modified nucleotides (Fig. 3b), possibly resulting from the activity of surface-localized nucleases. Together, these results demonstrate a high degree of nuclease specificity for the TT probe.

**DISCUSSION**

The imaging tools that are currently available for diagnosing bacterial infections cannot directly detect bacteria or material derived from them; they are used to measure downstream consequences of infections, such as tissue destruction or inflammatory responses. Such assays provide no direct evidence of either the type or the presence of bacteria at sites of potential infections. As a result of these limitations, physicians currently depend heavily on a variety of nonspecific criteria together with biopsy and culture (which is time consuming) to diagnose infections. In this proof-of-concept study, we developed an activatable probe that enables the noninvasive imaging of *S. aureus* infections in mice in less than an hour. To the best of our knowledge, the present study is the first to demonstrate the noninvasive imaging of a disease condition in animals with a nuclease-activated probe.

**Figure 2** Activation of the Cy5.5-TT probe by MN in vitro and in *S. aureus*-infected mice. (a–e) For in vitro evaluation (a), the indicated amounts of probe were incubated with (+MN) or without (−MN) MN at 37 °C for 1 h. Controls include buffer only (DPBS) and the unquenched TT probe. Fluorescence signal strengths are indicated with the vertical color bar on the right. To evaluate probe activation in mice with *S. aureus*-derived pyomyositis, uninfected mice (b, n = 4 mice; c, n = 3 mice), and mice infected with lux+ MN-expressing *S. aureus* (d, n = 5 mice) or with lux− MN-negative *S. aureus* (e, n = 4 mice) in their right thighs were imaged with Cy5.5-channel fluorescence before (Bkgd) and after tail vein administration of Cy5.5-TT probe (b,d,e) or unquenched TT probe (e). Luminescence images acquired before probe injections (panels on left) indicate location of infections (in d and e). Vertical color bars indicate the relationship between pseudocolors and signal strength. Fluorescence display levels are adjusted to show light levels that exceed tissue autofluorescence, unactivated TT probe fluorescence and the low levels of luminescent light bleedthrough and activation by serum nucleases. Times listed above fluorescence images indicate the time elapsed after probe administration. For imaging after killing and dissection, mice with thigh-MN-expressing *S. aureus* pyomyositis and injected with unquenched TT probe (f, n = 4 mice) or TT probe (g, n = 4 mice) were euthanized 45 min after probe injection; dissected muscle tissue was imaged with luminescence (green) and the Cy5.5 fluorescence channel (red). Scale bar, 1 cm.

*This text is a direct transcription of the content from the provided image, without any additions or modifications.*
Our exploration of the substrate specificity of MN provided a framework for the design of a probe that would be efficiently activated by MN without being susceptible to serum nucleases. The incorporation of unmodified deoxynucleotides into the TT probe was intended to make it highly sensitive to MN digestion, and in vitro probe activation assays with purified MN indicated that MN does, in fact, efficiently activate the probe. Culture supernatants of two common strains of \textit{S. aureus} also activate the probe. The activation of the TT probe by \textit{S. aureus} (supernatants or in infected animals) can be attributed to MN activity because we saw minimal probe activation in experiments with an \textit{S. aureus} mutant that does not express MN. Notably, the TT probe is resistant to degradation by mouse and human serum nucleases, probably owing to the 2′-O-methyl–modified nucleotides that flank the central deoxynucleotides, as this modification is known to provide a high degree of resistance to serum nucleases. The nucleases present in the culture supernatants of our panel of additional pathogenic bacterial species failed to digest any of the oligonucleotides with 2′-O-methyl–modified nucleotides. The inclusion of the flanking 2′-O-methyl–modified nucleotides in the TT probe may thus be responsible for its resistance to these other bacterial nucleases, as well. In any case, the fact that this probe is preferentially activated by MN suggests that the imaging approach described here has the potential not only to detect pathogens, but also to indicate the type of bacteria that may be present.

Several preclinical bacterial imaging approaches can detect bacteria nonspecifically.\textsuperscript{6–8,10,11} Our approach and the prothrombin-based approach of Panizzi et al.\textsuperscript{9} provide a means of specifically detecting \textit{S. aureus}. Whereas nonspecific bacterial detection has the advantage of detecting a wide variety of infections, species-specific detection can potentially guide physicians in antibiotic selection at an early stage in the diagnostic process and thus provides complementary, and highly valuable, information. In contrast to the prothrombin-based approach, the nuclease-activated approach provides a blueprint for the species-specific detection of a variety of medically important bacterial pathogens. The present study therefore represents a proof-of-concept study that may lead to a new category of species-specific, activatable imaging probes for bacterial infections. The broad diversity of nuclease structures and enzymatic mechanisms supports the notion that specific probes can be engineered for many additional target nucleases.\textsuperscript{12} Indeed, the distinct substrate specificities of the other (non-\textit{S. aureus}) bacterial nucleases tested and the fact that some of these can digest oligonucleotides that are resistant to serum nucleases suggests the broader applicability of the approach. Although this approach is applicable only to pathogens that produce targetable nucleases, there are, nonetheless, several prominent pathogens besides \textit{S. aureus} that are known to produce robust nuclease activity.\textsuperscript{32–34} The wide variety of existing synthetic nucleotide modifications is also likely to facilitate the engineering of additional specific probes.\textsuperscript{20}

The bacterial detection approach we describe here seems well suited for clinical translation. The probes are expected to be nontoxic because the oligonucleotide portion is composed of naturally occurring modified (2′-O-methyl) and DNA nucleotides. A recent preclinical toxicity study of IRDye 800CW, an NIR fluorophore that would be an attractive alternative for Cy5.5 for clinical translation of the approach (it is excited and emits at longer wavelengths, which yield better tissue penetration), found that this fluorophore has low toxicity in rats.\textsuperscript{35} TT probe activation was detected at infection sites after administration of low doses (~1 mg per kg body weight), further increasing the likelihood that toxic effects can be avoided. Current nucleic acid synthesis and conjugation technologies are sufficiently advanced to make the large-scale good manufacturing practice synthesis of such probes both feasible and economical. Although deep-tissue infections (for example, kidney abscesses) are beyond the reach of current noninvasive optical imaging approaches, there remains a wide variety of bacterial infections that are shallow enough to be detectable with NIR fluorescence imaging (for example, many joint and bone infections). Ongoing efforts by several groups to translate NIR imaging approaches to the clinic may make NIR clinical imaging instrumentation commonplace in clinical settings in the near future, thus providing an infrastructure for the accommodation of NIR-based bacterial imaging diagnostic approaches.\textsuperscript{36–39} For these reasons and because this approach would address an important unmet clinical need, we anticipate its clinical translation in the coming years.
ACKNOWLEDGMENTS

Research reported in this publication was supported by the National Institute of Allergy and Infectious Diseases of the US National Institutes of Health under awarded numbers AI083211 (A.R.H.) and AI101391 (J.O.M. II). The content is solely the responsibility of the authors and does not necessarily represent the official views of the US National Institutes of Health. F.J.H. was supported by a postdoctoral fellowship from the American Heart Association. The authors thank J. Kavanaugh for technical assistance with bacterial cultures and R. Schmidt and J. Widness for sharing a Sysmex XT-200i Automated Hematology Analyzer.

AUTHOR CONTRIBUTIONS

F.J.H. developed the experimental methodology, designed experiments, carried out experiments and analyzed data. L.H., M.E.O. and K.M.P. generated new reagents and provided crucial technical input. M.E.O. also measured the CFUs of bacterial cultures. L.H. measured blood counts of mice. D.K.M. carried out the histological staining and data analysis. D.K.T. carried out the magnetic resonance imaging and analysis. M.A.B. provided crucial input for probe design and generation, oversaw probe generation and contributed to the conceptual development of the project. J.O.M. II, A.R.H. and F.J.H. conceived of and developed the underlying concepts of the project. A.R.H. oversaw the generation of genetically modified bacteria and the preparation of bacterial cultures. J.O.M. II designed experiments, analyzed data, coordinated the various aspects of the project and wrote the manuscript with help from all of the authors.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Oligonucleotide probe synthesis and purification. Oligonucleotide probes were synthesized and purified at Integrated DNA Technologies (IDT). Briefly, all the FAM-labeled probes were synthesized using standard solid-phase phosphoramidite chemistry, followed by high-performance liquid chromatography (HPLC) purification. For the Cy5.5-labeled probes, the sequences were first synthesized with ZEN and Iowa Black quenchers or inverted dT on the 3′ ends and amine on the 5′ ends using the standard solid-phase phosphoramidite chemistry and purified with HPLC. We set these purified sequences to react with Cy5.5 NHS ester (GE Healthcare) (to chemically conjugate the Cy5.5 label to the sequences) and then isolated the Cy5.5-labeled probes with a second HPLC purification. We confirmed all probe identities with electron spray ionization mass spectrometry (ESI-MS) using an Oligo HTCS system (Novatia LLC). The measured molecular weights are within 1.5 Da of the expected molecular weights. The purity of the probes, as assessed with HPLC analysis, is typically greater than 90%. Quantitation of the probes was achieved by calculating from their ultraviolet absorption data and their nearest-neighbor–model–based extinction coefficients at 260 nm. Extinction coefficients of 2′-O-methyl–modified nucleotides and 2′-fluoro–modified nucleotides are assumed to be the same as that of RNA.

Fluorescence plate reader nuclease assays. Fluorescence plate reader assays were carried out as described15. Briefly, for each reaction, we combined 1 µl of a stock solution of each probe (50 µM concentration) with 9 µl of each sample (buffer, buffer plus purified nuclease, serum, culture supernatant, culture broth or washed bacteria) and incubated at 37 °C for the time periods indicated in the figures. We then added 290 µl of PBS supplemented with 10 mM EDTA and 10 mM EGTA to each and then loaded 95 µl of each diluted reaction per well into a 96-well plate (96F nontreated black microwell plate (NUNC)). We measured fluorescence levels with an Analyst HT fluorescence plate reader (LIJ Biosystems). We measured background fluorescence levels of probes incubated in buffer or broth and autofluorescence levels of the various preparations and subtracted them from the probe-activation reaction values prior to normalizations. Except where stated otherwise, we carried out these subtractions as follows. We subtracted the fluorescence of each of the probes incubated in DPBS from the corresponding reactions consisting of purified MN diluted in DPBS. We subtracted the fluorescence of each of the probes incubated in DPBS plus the autofluorescence of each serum (mouse or human) from the serum-containing reactions. We subtracted the fluorescence of each of the probes incubated in the appropriate unconditioned culture broth from the corresponding culture supernatant reactions. We subtracted the fluorescence of each of the probes incubated in DPBS plus the autofluorescence of each appropriate bacterial suspension from each bacterial suspension reaction. For assessment of probe activation in cultures of various pathogenic bacterial species, the data shown were collected in several distinct experiments, each of which included the positive control (for normalization) and background measurements (for subtractions). We obtained purified MN from Worthington Biochemical Corporation, Dulbecco’s phosphate-buffered saline (DPBS), containing physiological levels of calcium and magnesium, from Invitrogen, human serum from Sigma-Aldrich and mouse serum (C57BL/6) from Valley Biomedical.

In vitro evaluation of the Cy5.5-TT probe. We incubated 10, 40, 160 or 640 pmol of the Cy5.5-TT probe in 100 µl DPBS or DPBS plus 1 µM MN at 37 °C for 1 h. We measured fluorescence in 96-well plates in a Xenogen IVIS 200 imaging system. Controls included buffer only (DPBS) and equivalent amounts of the unquenched TT probe.

Bacterial cultures and growth conditions. We maintained bacteria in tryptic soy broth (TSB), Luria Bertani (LB) or Todd Hewitt + yeast (THY) broth as defined (Supplementary Table 1) for each strain. To prepare cultures for assays, we subcultured overnight cultures 1:500 into 5 ml fresh broth and grew for 24 h at 37 °C with shaking. The only exceptions were S. pneumoniae and S. agalactiae (group B Streptococcus), which were grown under static conditions in a 37 °C incubator supplemented with 5.0% CO2. To prepare spent media for nuclease assays, we centrifuged 1 ml of each culture at 6,000g for 10 min and saved the supernatant. To prepare bacterial suspensions for nuclease assays, we washed pelleted bacteria with 1 ml DPBS and resuspended in 100 µl of DPBS.

Genetic manipulation of S. aureus. Bacteriophage 11 was used to transduce the P. luminescens luxABCDE genes from strain AH1362 into strains Newman and Newman nuc−/LbrB as previously described16. We selected transductants carrying the lux genes on tryptic soy agar (TSA) with kanamycin supplemented at 50 µg ml−1. We confirmed bioluminescence production (lux+) in the resulting strains using a Tecan Infinite M200 plate reader (see Supplementary Table 1).

S. aureus pyomositis model. We prepared S. aureus cultures for infection into mice as follows. First, we inoculated 5 ml of TSB supplemented with kanamycin (50 µg ml−1) with frozen stocks of MN-expressing or MN-negative lux+ S. aureus of the strain Newman genetic background (Supplementary Table 1). We grew the cultures overnight at 37 °C with shaking at 200 rpm and then subcultured each strain 1:100 into 5 ml of fresh medium and grew for another 12 h at 37 °C with shaking. We washed the bacteria once with PBS and then resuspended in PBS to an approximate cell density of ~2 × 10^6 CFU ml−1 for injection into mice. We serially diluted the bacteria, plated on TSA and incubated at 37 °C to determine bacterial concentration.

For animal infections, we injected 50 µl of 2 × 10^6 CFU ml−1 (1 × 10^7 CFU total) intramuscularly (thigh muscle) in 6- to 8-week-old C57BL/6 female mice under isoflurane anesthesia. Mice were shaved before injections. We evaluated injection sites with bioluminescence imaging immediately thereafter. Mice were imaged or euthanized for imaging, blood harvest or histology 48 h after injections. All animal experiments were approved by the University of Iowa Institutional Animal Care and Use Committee.

In vivo evaluation of nuclease-activated probes. We carried out bioluminescence and epifluorescence imaging with a Xenogen IVIS 200 imaging system (Caliper). First, we anesthetized mice with 2% isoflurane gas anesthesia and placed them on the imaging platform in the optical system for dorsal imaging. We recorded luminescence images with a 1-min exposure time and an open emission filter. We recorded epifluorescence images with a 1-s exposure time and excitation and emission filters appropriate for the Cy5.5 dye. To avoid saturation, we reduced the exposure time for the acquisition of epifluorescence images of the mice injected with the unquenched TT probe to 0.5 s. We acquired bioluminescence and fluorescence images before probe injections and then acquired additional fluorescence images following tail vein injections (time points are indicated in figures) of the probes. For probe administration, we injected 3 nmol of each probe diluted in 120 µl PBS via tail vein. We used IVIS 4.2 software to perform acquisition, image processing (including soft binning and smoothing) and analysis and preparation of pseudocolored overlays of luminescence, fluorescence and grayscale images. We carried out fluorescence and luminescence imaging of tissues after killing and dissection as described for the live animal imaging, but with field of view adjusted for image acquisitions.

Histological analysis of infected and uninfected tissue. For histological analyses, we euthanized mice via carbon dioxide intoxication. We photographed the gross lesions of the mice with pyomositis with a digital camera before and after removal of the skin. Then we carefully dissected the soft tissues of the S. aureus–infected (right) leg. For comparison with uninfected tissues, we also dissected the corresponding portion of the uninfected (left) leg. We then fixed all tissues in 10% neutral buffered formalin for 48 h at room temperature. We gross-sectioned the fixed tissues and then routinely processed them in a series of alcohol and xylene baths, paraffin–embedded them and stained 4-µm sections with HE and/or Gram stain as previously described17. Slides were examined by a veterinary pathologist (D.K.M.) for histopathologic interpretation. We acquired high-resolution digital images with a DP71 camera (Olympus) mounted on a BX51 microscope (Olympus) with MicroSuite Pathology Edition Software (Olympus).

Target-to-background ratio measurements. We used IVIS 4.2 software to measure fluorescence intensity within regions of interest (ROIs) of mouse.
images acquired 45 min after TT probe administration. The following three groups of mice were included in the analysis: uninfected mice, *S. aureus*–infected mice and MN-negative *S. aureus*–infected mice. We determined target-to-background ratios as follows. For infected animals, the fluorescence measured within an ROI placed over the probe-activation site of the infected leg was divided by fluorescence measured within an ROI placed over the contralateral uninfected leg. For each uninfected mouse, we calculated the ratio of fluorescence within ROIs of uninfected versus contralateral uninfected leg. We subtracted the tissue autofluorescence level (measured within each ROI in the background fluorescence image (taken before probe administration)) from each of the corresponding ROI fluorescence values measured at the 45-min time point before the ratio calculations.

**Magnetic resonance imaging of infected mice.** We carried out magnetic resonance imaging (MRI) with a 4.7-Tesla Varian Unity/INova small-bore scanner with a 25-mm diameter transmit/receive coil. First, we anesthetized the mice with isoflurane (3% induction, 1.5% maintenance) and transferred them to the scanner. Following localizer scans, we acquired three sets of T2-weighted fast spin-echo scans (one each in axial, coronal and sagittal orientations). Pulse sequence parameters were TR/TE = 2,000/60 ms, echo train length = 8, slice thickness of 0.8 mm, and in-plane resolution of 0.16 mm over a 256 × 256 acquisition matrix with six signal averages for a scan time of about 8 min per orientation. Overall scan time was about 30 min for each mouse.

**Blood counts.** For blood collection, we anesthetized mice with ketamine/xylazine and collected at least 150 µl blood from each mouse via submandibular venous puncture into an EDTA tripotassium salt multivette (Multivette 600 K3E; Sarstedt). We measured complete blood counts (CBCs) from undiluted blood samples within 30 min of collection using a Sysmex XT-200i Automated Hematology Analyzer (Sysmex America).

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