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BACKGROUND: Single-molecule detection (SMD) technologies are well-suited for clinical diagnostic applications by offering the prospect of minimizing precious patient sample requirements while maximizing clinical information content. Not yet available, however, is a universal SMD-based platform technology that permits multiplexed detection of both nucleic acid and protein targets and that is suitable for automation and integration into the clinical laboratory work flow.

METHODS: We have used a sensitive, specific, quantitative, and cost-effective homogeneous SMD method that has high single-well multiplexing potential and uses alternating-laser excitation (ALEX) fluorescence-aided molecule sorting extended to 4 colors (4c-ALEX). Recognition molecules are tagged with different-color fluorescent dyes, and coincident confocal detection of ≥2 colors constitutes a positive target-detection event. The virtual exclusion of the majority of sources of background noise eliminates washing steps. Sorting molecules with multidimensional probe stoichiometries (S) and single-molecule fluorescence resonance energy transfer efficiencies (E) allows differentiation of numerous targets simultaneously.

RESULTS: We show detection, differentiation, and quantification—in a single well—of (a) 25 different fluorescently labeled DNAs; (b) 8 bacterial genetic markers, including 3 antibiotic drug–resistance determinants found in 11 septicemia-causing Staphylococcus and Enterococcus strains; and (c) 6 tumor markers present in blood.

CONCLUSIONS: The results demonstrate the assay’s utility for clinical molecular diagnostics applications by means of multiplexed detection of nucleic acids and proteins. Future integration of additional technology components to minimize preanalytical sample manipulation while maximizing throughput should allow development of a user-friendly (“sample in, answer out”) point-of-care platform for next-generation medical diagnostic tests that offer considerable savings in costs and patient sample.

Next-generation diagnostic tools need to be able to measure a broad range of biomarkers and combine their information content to reach clinically meaningful conclusions in a timely fashion. Given that patient samples are limited and that biomarker or pathogen concentrations are often extremely low, serial analysis is not practical. Thus, only exceptionally sensitive methodologies with deep single-well multiplexing capability will be able to query multiple targets simultaneously and boost differential diagnostic value substantially.

For maximizing clinical information content obtainable from a single small patient sample, single-molecule detection (SMD) technologies are the most promising for the development of next-generation medical diagnostics. While single-molecule sequencing methods have undergone recent important advances, the development of a universal SMD-based platform technology for rapid and cost-efficient detection of both nucleic acid and protein targets has lagged...
behind. We describe the successful extension of confocal microsecond-scale alternating-laser excitation (ALEX) single-molecule fluorescence spectroscopy to 4 colors, providing substantially enhanced single-well multiplexing capabilities, and we demonstrate its diagnostic utility for multiplexed detection of nucleic acid and protein targets.

ALEX was originally developed as a novel single-molecule method capable of detecting static and transient macromolecular interactions with ultrahigh sensitivity and specificity via 2-color and 3-color coincident detection of single molecules in a confocal detection volume (approximately 1 fL) (1–5). This method introduces additional laser excitation source(s) to probe the fluorescent acceptor (A) in a direct, fluorescence resonance energy transfer (FRET)-independent fashion, and it obtains both donor (D)-excitation–based and A-excitation–based observables for each single molecule. This scheme recovers distinct emission signatures for all species involved in molecular interactions by calculating 2 fluorescence ratios: the FRET efficiency $E$ (6–8) which reports on the $D$–$A$ distance, and a new, distance-independent ratio (stoichiometry-based ratio, or $S$) which reports on the $D$–$A$ stoichiometry of the various species. The combination of $E$ and $S$ on 2-dimensional histograms allows virtual sorting of single molecules, which is defined as “fluorescence-aided molecular sorting.” This analysis mode for single molecules in a complex solution is analogous to fluorescence-activated cell sorting for cells in complex solutions. The ALEX methodology is superior to conventional FRET, because ALEX allows determination of stoichiometries of interacting molecules in addition to accurate distance measurements. Furthermore, accurate quantification of the concentrations of multiple targets is possible by this molecule-by-molecule counting method.

Materials and Methods

**FOUR-COLOR ALEX SPECTROSCOPY**

Three-color ALEX single-molecule fluorescence spectroscopy, previously described by Lee et al. (5), was expanded to 4 colors (4c-ALEX) (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol58/issue4). Alteration of 3 excitation laser sources [blue (B), 488 nm; green (G), 543 nm; red (R), 635 nm] is controlled by a single acousto-optic modulator (Neos Technologies), and the fourth laser [infrared (I), 730 nm] is modulated directly. Four individual single-photon counting modules (SPCM-AQR(H)-14C; PerkinElmer Optoelectronics) were used for detection. Fluorescence signals arising from a femtoliter confocal volume are spatially filtered by a pinhole (100-μm diameter) in a custom-built confocal microscope and are subsequently divided into 4 separate channels [B, G, R, I] for SMD through time stamping of arrived photons modulated in time.

For data analysis, probe stoichiometry ratios ($S_{DA}$) and proximity ratios ($E_{DA}$) to construct multidimensional $ES$ histograms for deep multiplexing capability of 4c-ALEX have been calculated with the following formulas:

$$S_{DA} = \frac{F_{Dex}}{F_{Dex} + F_{Aex}}$$

and

$$E_{DA} = \frac{F_{Axem}}{F_{Dex} + F_{Aex}},$$

where $D$ (donor) is B, G, or R, and $A$ (acceptor) is G, R, or I. For $S_{DA}$, $F_{Dex}$ is the intensity of the fluorescent photon signal during the donor excitation period, and $F_{Aex}$ is the intensity of the fluorescent photon signal during the acceptor excitation period. For $E_{DA}$, $F_{Axem}$ is the intensity of the fluorescent photon signal in the donor-emission channel during the donor excitation period, and $F_{Dex}$ is the intensity of the fluorescent photon signal in the acceptor-emission channel during the donor excitation period. $E_{DA}$ is FRET without considering cross talk, direct excitation, and leakage. For accurate $E$ calculations, correction formulas (2, 5) may be implemented. Empirically determined $S$ ranges (see Table 1 in the online Data Supplement) were used to sort species first, and 2-dimensional $ES$ and 3-dimensional $EE$ histograms further sorted them according to their FRET differences.

**DETECTION OF FLUORESCENTLY LABELED DNA MOLECULES**

Twenty-five singly, doubly, triply, and quadruply labeled DNA species were prepared with amino linker–modified oligonucleotides labeled with reactive N-hydroxysuccinimide ester–activated dyes: Alexa488 (Invitrogen), Cy3B (GE Healthcare), Atto647N (ATTO-TEC), and Cy7 (GE Healthcare). Multiply labeled oligonucleotides were generated either by hybridizing complementary singly labeled oligonucleotides or by using the ligase chain reaction with singly labeled oligonucleotides as primers on respective oligonucleotide templates, followed by PAGE gel purification. The primer sequences are given in Table 2 in the online Data Supplement. The buffer conditions for the ligase chain reaction were 20 mmol/L Tris–HCl (pH 7.5), 20 mmol/L KCl, 10 mmol/L MgCl$_2$, 1 mL/L Igepal (Sigma-Aldrich), 0.01 mmol/L ATP, and 1 mmol/L dithiothreitol. The ligase chain reaction began with 2 min of predenaturation at 92 °C, followed by 30 cycles of a
15-s denaturation at 92 °C and a 2-min ligation at 68 °C.

DETECTION OF BACTERIAL GENES

Purified bacterial genomic DNAs and viable organisms were obtained from the ATCC or NARSA (Network on Antimicrobial Resistance in Staphylococcus aureus). The following bacteria strains were used: Staphylococcus aureus Mu50 [MRSA (mecillin-resistant S. aureus)], S. aureus Wood46 [MSSA (mecillin-susceptible S. aureus)], S. epidermidis RP62A [MRSE (mecillin-resistant S. epidermidis)], S. epidermidis PCI 1200 [MSSE (mecillin-susceptible S. epidermidis)], Enterococcus faecium MMC4 [VRE<sub>faecium</sub> (vancomycin-resistant E. faecium), a vanA<sup>6</sup> (d-alanine–d-lactate ligase) gene cluster–carrying strain], E. faecium NCTC 7171 [VSE<sub>faecium</sub> (vancomycin-susceptible E. faecium)], E. faecalis V583 [VRF<sub>faecalis</sub> (vancomycin-resistant E. faecalis), a vanB gene cluster–carrying strain], and E. faecalis ATCC 29212 [VSE<sub>faecalis</sub> (vancomycin-susceptible E. faecalis)]. The following strains were used as controls: Bacillus subtilis 168, Escherichia coli K-12, S. haemolyticus SM131, S. hominis R22, S. lugdunensis LRA 260, S. saprophyticus NCTC 7292, S. schleiferi N850274, Enterococcus avium AmMS112, E. casseliflavus 84–10–088. Purified bacterial genomic DNAs were quantified with Quant-iT™ Pi-coGreen® dsDNA Reagent and Kits (Invitrogen), and genomic equivalents (GE) were calculated before PCR amplification. Human genomic DNA, which was added as background to PCRs at 2.5 μg per reaction to mimic clinical samples after DNA extraction from blood, was purchased from Roche Applied Science.

Oligo 7 software (Molecular Biology Insights) was used to design both PCR primers and hybridization FRET probe pairs (see Table 3 in the online Data Supplement). A universal amplification primer (5′-GCGTACTAGCGTACCACGTGTCGACT-3′) and 30 nmol/L (multiplexed target detections) by a ramping step from 68 °C to 60 °C after a denaturation step for 10 min at 95 °C. Final FRET probe concentrations were 15 nmol/L (separate target detections) and 30 nmol/L (multiplexed target detections). Probes labeled with Alexa488 and Atto647N were synthesized by Biosynthesis. Probes labeled with Cy3B were synthesized in house.

DETECTION OF TUMOR MARKERS

Purified carcinoembryonic antigen (CEA), prostate-specific antigen (PSA), α-fetooprotein (AFP), neuron-specific enolase (NSE), human chorionic gonadotropin (hCG), and mucin 16 (MUC16) tumor marker antigens, as well as respective monoclonal antibody pairs that bind to 2 mutually exclusive epitopes on each marker, were purchased from Sigma-Aldrich, Abnova, and Abcam. Labeling and buffer-exchange kits were purchased from Thermo Fisher Scientific. Before monoclonal antibody labeling, storage-buffer solutions were exchanged with a sodium borate solution (50 mmol/L) via Zeba desalting columns (Thermo Fisher Scientific). Labeling reactions were carried out with DyLight labeling kits (Thermo Fisher Scientific) with DyLight488, DyLight549, DyLight649, and DyLight750 dyes. Before 4c-ALEX–based measurements, PBS (10 mmol/L phosphate buffer, pH 7.4; 150 mmol/L NaCl) with 1 mL/L Tween 20 was used as a dilution buffer for SMD. The antibodies labeled with the infrared dye did not suffer from photobleaching or blinking owing to the presence of multiple fluorophores per antibody.

Results

MULTIPLEXING POTENTIAL OF 4c-ALEX SPECTROSCOPY

4c-ALEX–based spectroscopy (see Fig. 1 in the online Data Supplement) greatly extends the ability to detect multiple biomolecules diffusing in solution by the use of probes labeled with up to 4 different fluorescent dyes, in conjunction with multicolor excitation and detection (Fig. 1). Results are analyzed in a multidimensional vector space consisting of mutually orthogonal
Fig. 1. 4c-ALEX allows highly multiplexed biomolecule detection, differentiation, and quantification subsequent to subpopulation analysis in multidimensional space.

(A), Schematic overview for clinical diagnostics applications. According to target type, a small patient sample is processed with standard clinical diagnostic laboratory procedures (e.g., serum preparation, DNA extraction). For low-copy nucleic acid targets (e.g., pathogens causing early-onset septicemia), multiplex PCR is used for initial target amplification. Target-recognition molecules (e.g., antibodies, DNA oligonucleotides) tagged with different-color fluorescence dyes are mixed with targets, incubated, and analyzed with 4c-ALEX single-molecule fluorescence spectroscopy. Coincident confocal detection of 2, 3, or 4 colors constitutes a positive target-detection event, allowing implementation of bar coding and identification of numerous targets simultaneously in a single well. (B), The 3-dimensional stoichiometry tetrahedron histogram illustrates the ability of the methodology to separate molecular species with different stoichiometry parameters. The colored circles represent B, G, R, and I fluorophores. Indicated are singly labeled species at the 4 vertices, doubly labeled species at the 6 sides, triply labeled species on the 4 faces, and the quadruply labeled species at the center of the tetrahedron. (C), Subsequent projections to 2-dimensional ES histograms sort the doubly labeled species [BR low, medium (med), and high (high)] via FRET for different DNA species with the same stoichiometry ratios but different dye distances. (D), Three triply labeled species with different FRET efficiencies (GR_B, BR_G, BG_R; the underscore indicates dye separation ≥10 nm—i.e., low E, otherwise high E) are sorted by projecting to a 3-dimensional E histogram involving B, G, and R fluorophores. (E), Two quadruply labeled species (RI_BG, GI_BR) are sorted in 2 consecutive 3-dimensional E histograms, because of the presence of 6 independent pairwise fluorophore distances involving 6 orthogonal E_j parameters. The first 3-dimensional E histogram selects the subpopulations of the quadruply labeled species with 1 set of 3 E_j values; the second 3-dimensional E histogram further sorts the different species by using the other set of 3 E_j values.
vectors with 6 $S_i$ and 6 $E_j$ parameters ($i = B, G, R$; and $j = G, R, I$, where $i \neq j$). Initial stoichiometry-based subpopulation selection in $S_i$ space is followed by molecule sorting and quantification through successive projections to multidimensional $E_j$ spaces. By placing reasonable detection and feasibility restrictions on these multidimensional spaces (see Supplemental Data in the online Data Supplement), we estimate that we can differentiate at least 10 doubly labeled species, 50 triply labeled species, and 100 quadruply labeled species.

MULTIPLEXED DETECTION AND DIFFERENTIATION OF 25 DNA SPECIES

For a proof of principle, we demonstrated that 4c-ALEX can sort and count molecules with different probe stoichiometries and FRET distances by analyzing a solution containing 25 singly, doubly, triply, and quadruply labeled DNA oligonucleotide species (10 pmol/L concentration each; see Table 2 in the online Data Supplement). The species with different stoichiometry ratios were sorted by using empirically determined S ranges along each $S_{BR}$ axis, as shown in the $S_{BR}$ vs $S_{GI}$ 2-dimensional histograms (see Fig. 2A in the online Data Supplement). FRET efficiencies that report on donor–acceptor proximities (without leakage, direct excitation, or background-correction terms) were used for further sorting of species through successive projections on 2-dimensional ES and 3-dimensional E histograms. Sorted species with different FRET efficiencies were clearly distinguishable in a single measurement (see Fig. 2B in the online Data Supplement). A representative example for molecule sorting based on stoichiometry and FRET is shown in Fig. 3 in the online Data Supplement. The measurement time needed for target detection, i.e., for obtaining sufficient fluorophore bursts above background to allow statistical analysis, is dependent on the concentration of the target fluorophore signal and dye stability. Measurement times ranged from 5 min to 1 h for individual and multiplexed target detection, respectively.

MULTIPLEXED DETECTION AND QUANTIFICATION OF BACTERIAL GENES

To show the suitability of ALEX-based spectroscopy for the development of diagnostic biodetection assays that can query multiple DNA-based targets simultaneously, we selected 8 bacterial genetic markers, including 3 antibiotic drug–resistance determinants found in septicemia-causing staphylococci and enterococci (10–12). The selected markers are resistance specific for methicillin [mecA (adapter protein MecA)], vancomycin A (vanA), and vancomycin B (vanB), as well as species specific for $S. aureus$ [nuc (thermonuclease precursor)], $S. epidermidis$ [nrdE (ribonucleotide-diphosphate reductase subunit α)], $E. faecium$ [$ddl_E_{faecium}$ (D-alanine–D-alanine ligase)], $E. faecalis$ [$ddl_E_{faecalis}$ (D-alanine–D-alanine ligase)], and $B. subtilis$ [sfp (4′-phosphopantetheinyl transferase)]. Gram-positive $B. subtilis$ was chosen to serve as a positive control for monitoring DNA extraction efficiency for future assays. To achieve maximal sensitivity, which is necessary to determine septicemia onset in small blood samples, we applied the PCR for initial target amplification. Subsequent ALEX-based detection, differentiation, and quantification used hybridized dye-labeled FRET probe pairs, which increased specificity and allowed implementation of bar coding. Primer and probe sequences, as well as assigned dye color combinations and FRET distances, are shown in Table 3 in the online Data Supplement.

Using various amounts of purified genomic DNA from MRSA, MRSE, VRE$_{faecium}$ carrying a vanA gene cluster, VRE$_{faecalis}$ carrying a vanB gene cluster, and $B. subtilis$, we first separately amplified, detected, and quantified the 8 genetic markers. We demonstrated a detection sensitivity of $\leq10$ GE and a quantification interval over $\geq2$ orders of magnitude for each target (see Fig. 4 in the online Data Supplement). We then amplified—in a single well—all 8 genes via a multiplex PCR in presence of a human genomic DNA background (2.5 μg—to mimic a clinical blood sample after DNA extraction), followed by hybridization with the full set of dye-labeled FRET probe pairs and ALEX-based spectroscopy (Fig. 2). Analysis of 3-dimensional $S_{BG}$–$S_{BR}$–$S_{GR}$ and individual ES histograms (see Fig. 5 in the online Data Supplement) confirmed the detection sensitivity and the quantification interval. Stoichiometry and FRET efficiency values corresponding to each target were comparable to those observed in separate target detections, confirming the absence of interfering probe–probe interactions or nonspecific hybridizations. Additional specificity analysis with a mixture of other closely related Staphylococcus species (S. hemolyticus, S. hominis, S. lugdunensis, S. saprophyticus, S. schleiferi) and Enterococcus species (E. avium, E. casseliflavus, E. gallinarum), together with $B. subtilis$, demonstrated an absence of assay interference.

MULTIPLEXED DETECTION AND QUANTIFICATION OF TUMOR MARKERS

To establish 4c-ALEX utility for diagnostic assays for detecting and measuring panels of clinically relevant protein biomarkers, we selected the following 6 tumor markers present in blood for multiplex analysis: CEA, PSA, AFP, NSE, hCG, and MUC16/CA-125. Increased marker concentrations above the established thresholds may be indicative of tumorigenesis (13), and panels of multiple tumor markers have proved more informative than individual markers (14–17).
Commercially available antibody pairs that bind each marker at 2 nonoverlapping epitopes, were labeled with different-color fluorophore combinations to exploit the multiplexing capability of 4c-ALEX for molecular sorting based on stoichiometry ratios only, without FRET involvement [generally, dye-labeled antibody pairs do not undergo FRET when bound to a target antigen, owing to a 10-nm separation between donor and acceptor fluorophores; however, barcoding may be retained by attaching multiple labels to antibodies via the use of dye–DNA conjugates (18)]. Using purified tumor markers spiked into serum at various concentrations, we demonstrated simultaneous detection and molecular sorting in the multidimensional $S_0$ space of all 6 markers in a simple “mix-and-read” assay format, without any additional sample-preparation steps (Fig. 3A; see Fig. 6 in the online Data Supplement). Detection sensitivities vary for each marker and mostly depend on antibody affinities, dye interference with target recognition, dye emission energies, and dye stabilities. Three of the 6 markers (CEA, PSA, and AFP) were detectable at or below their clinically relevant threshold concentrations of 2.5 μg/L (14 pmol/L), 4 μg/L (121 pmol/L), and 10 μg/L (145 pmol/L), respectively ($P < 0.05$). Separate detection and quantification established a linear interval of quantification over $\geq 2$ orders of magnitude for each marker, with obtained sensitivities of 14 pmol/L (CEA), 100 pmol/L (PSA), and 100 pmol/L (AFP) (see Fig. 7 in the online Data Supplement). To demonstrate the specificity and the absence of signal cross talk for the detection of these 3 markers, we reconfirmed the detection sensitivity of each marker at or below its clinically relevant threshold concentration in the presence of high concentrations of the other 2 markers and hCG (Fig. 3B).

Discussion

The results we have presented are the first description of ALEX use for clinical molecular diagnostic applications via multiplexed detection of nucleic acids and proteins on the same platform. Previously, the analytical power of ALEX was mostly used in 2-color and/or 3-color modes for biochemical studies and for analyzing the molecular dynamics of important complex biological processes (1, 4, 19–25). For example, transcription initiation by RNA polymerase has been shown to occur through a “DNA scrunching” mechanism (19). 4c-ALEX now allows rapid and simultaneous detection of numerous targets in a single well, especially when FRET is exploited to monitor distances between fluorescent donors and acceptors incorporated at specific sites on individual molecules, thereby...
Fig. 3. Multiplexed detection, identification, and quantification of 6 tumor markers directly in a human serum background in a “mix-and-read” assay format.

(A), Samples consisted of purified tumor markers spiked into 50% human serum at various concentrations. Addition of a mix of dye-labeled antibody pairs was followed by a 1-h incubation and 4c-ALEX–based burst count measurements (1 h) for stoichiometry-based quantification. Negative control: 50% human serum without spiked tumor markers. All low concentrations (clinically relevant threshold concentrations, except hCG): 14 pmol/L CEA, 100 pmol/L PSA, 100 pmol/L AFP, 77 pmol/L NSE, 54 pmol/L hCG, and 5.6 nmol/L MUC16; all high concentrations: 600 pmol/L CEA, 260 pmol/L PSA, 346 pmol/L AFP, 2.6 nmol/L NSE, 468 pmol/L hCG, and 52 nmol/L MUC16. P values were calculated for comparisons between threshold concentrations and the negative control (n = 3; data are expressed as the mean and SE). *P ≤ 0.05 for CEA, PSA, and AFP. Note that the negative control may contain endogenous tumor markers present at unknown increased levels, possibly influencing significance calculations for hCG, NSE, and MUC16. Corresponding 3-dimensional S histograms visualizing the different burst counts for each subpopulation of the 6 tumor markers are shown in Fig. 6 in the online Data Supplement. (B), Demonstration of detection specificity for CEA, PSA, and AFP. Each tumor marker was spiked individually into 50% human serum at or below its clinically relevant threshold concentration (CEA, 14 pmol/L; PSA and AFP, 100 pmol/L), together with high concentrations of the other 2 markers and hCG (1.4 nmol/L each), followed by dye-labeled antibody incubation for 1 h. Simultaneous 4c-ALEX–based detection and quantification (30-min burst count measurements) show an absence of signal cross talk and reconfirm detection sensitivities (n = 3; data are expressed as the mean and SE). *P ≤ 0.05.
allowing the implementation of bar coding. Counting the total number of different-color coincident fluorescence bursts arising from probe-bound target molecules passing through the confocal detection volume allows the derivation of the target concentration. The multicolor coincidence detection by laser alternation allows one to find “a needle in a haystack,” i.e., via virtual exclusion of the majority of (high-concentration) background sources, eliminating washing steps, and enabling superior sensitivity, specificity, and accuracy of target quantification. This improved methodology is well suited for diagnostics applications in which rapid simultaneous and accurate quantification of several DNA-, RNA-, or protein-based targets in limited amounts of complex clinical matrices is of vital importance but is not achieved cost-effectively with current technologies.

Compared with ALEX, other promising sensitive nucleic acid–detection methodologies put limits on multiplexing capabilities (e.g., real-time PCR is limited to 4 targets/well). Microarray-based screening techniques have drawbacks owing to the immobilization of detection molecules, which can hinder hybridization to target molecules, and owing to washing steps, which reduce sensitivity and limit accurate quantification. The widely used ELISA for protein detection has limited multiplexing capability, requires larger-sized patient samples, and cannot be performed in a simple mix-and-read fashion because of necessary washing steps. Emerging multiple analytic–detection systems, such as those offered by Luminex and eBioscience, are more complex and time-consuming bead-based assays for the flow cytometer that require washing steps and use ensemble fluorescence measurements. Nonfluorescence methods that use gold nanoparticles (Nanosphere) have gained recent visibility owing to the methods’ high sensitivity; however, these methods involve target capture, silver signal amplification, and multiple washing steps. For the detection of septicemia, traditional blood culture, which takes at least 24 h, is still the gold standard. False-positive results arising from contamination and false-negative results (due to, for example, antibiotic treatment at the time of sampling) are well known problems. Nucleic acid–based methods offer the potential to supplement traditional blood cultures.

The 4c-ALEX–based assay for multiplexed detection of bacterial genetic markers after PCR amplification has the capacity to simultaneously interrogate the presence of 11 bacterial strains that are major pathogens responsible for nosocomial and community-acquired infections (10–12). These strains include MSSA, MRSA, MSSE, MRSE, VSE<sub>faecalis</sub>, vanA VRE<sub>faecalis</sub> vanB VRE<sub>faecalis</sub>, VSE<sub>faecium</sub> vanA VRE<sub>faecium</sub>, vanB VRE<sub>faecium</sub> and VRSA. A dynamic interval over at least 2 orders of magnitude was demonstrated with 10, 100, and 1000 GE as target inputs (Fig. 2; see Fig. 4B in the online Data Supplement). Albeit at low resolution, the range of GE resembles different bacterial loads observed in patients and thus may allow guidance for antibacterial treatment dosing in the future. Linear regression analysis of results for the separate detection of targets (see Fig. 4B in the online Data Supplement) revealed variations in regression line slopes that may be caused by different amplification efficiencies. Thus, conditions for such targets as <i>add</i>/E. faecium require optimization by, for example, varying the primer and probe sequences. Furthermore, instead of the use of equimolar target input in multiplexed analysis, ratio variations resembling coinfections with different bacterial loads will require thorough examination and optimization.

After optimization, the current assay can be expanded to include other strains carrying specific mutations, other species and drug resistances, and virulence factors and toxins, as well as host-response biomarkers of exposure. This assay should be useful, in combination with standard DNA-extraction methods as performed in clinical diagnostics laboratories, for rapid etiologic diagnosis and early administration of adequate antimicrobial therapy soon after a patient’s arrival at the hospital, which are crucial for the successful management of sepsis and septic shock (26).

The 4c-ALEX–based assay for multiplexed tumor marker detection serves as a proof of principle for the development of diagnostic tests for detecting and measuring clinically relevant protein biomarker panels. Tumor markers may be used to help diagnose cancer, predict a patient’s response to particular therapies, check a patient’s response to treatment, or determine if cancer has returned; however, effective implementation of personalized cancer therapeutic regimens based on tumor marker panels depends on successful identification and clinical validation (27) of additional informative biomarkers. Yet, in combination with genomic and transcriptomic methods for patient stratification, tumor marker panels may offer the promise of additional insight into cancer disease states. In this context, 4c-ALEX could present a unique opportunity to improve existing tests [e.g., the popular PSA test, which lacks clinical specificity and leads to overdiagnosis and unnecessary biopsies (28)] by adding not only other informative protein-based but also microRNA-based biomarkers. Numerous microRNAs have now been shown to be deregulated in human cancers (29, 30), and we recently achieved amplification-free detection of a prostate cancer-specific microRNA (miR-141) at 1 fmol/L (data not shown), which is below its clinically relevant threshold concentration. Furthermore, the technology may be useful for the development of tests...
for personalized-medicine applications by simultaneously querying multiple single-nucleotide polymorphisms and copy number variants, in addition to the development of biomarker panels for health status assessments with fingerprick-size blood samples or other samples.

The ALEX methodology, however, still faces limitations and challenges. Absolute burst counts corresponding to target concentrations vary between targets, owing to differences in amplification and FRET efficiencies, dye emission energies, and dye stabilities. In particular, low populations of infrared-emitting species were observed because of photobleaching and blinking of the infrared dye. This property therefore requires the identification of dyes with improved stabilities. Photobleaching can also be greatly reduced by implementing microfluidics-based sample handling under a nitrogen environment (31) and the use of triplet quenchers such as a Trolox–cysteamine mix (32). Moreover, quantum dots may be considered instead of organic dyes to avoid photobleaching.

Considering protein targets, there is limited stoichiometry-based multiplexing potential, because the number of dyes per antibody reflects an average of the currently used random labeling process, which does not allow FRET-based differentiation. That could be improved, however, by introducing dually labeled antibody pairs or by conjugation of a chemical scaffold carrying multiple fluorophores that would allow implementation of FRET-based bar codes. Furthermore, the dependence of limits of detection on antibody affinities requires the identification of commercially available high-affinity antibodies or affinity maturation via, for example, molecular evolution using yeast display (33).

The current technology lacks fast-performance, high-throughput, and automated liquid-handling functionalities. To develop a “sample in, answer out” type of analytical instrument that can be used reliably without highly trained personnel will require integration of additional technology components. The feasibility of integrating microfluidics-based sample handling (34), including a continuous-flow system that uses digital droplet PCR (35–37), up-front target-enrichment procedures (38), and emerging multifoci photon-counting detectors for increased throughput (39), offers the exciting opportunity to develop a rapid, fully automated, ultrasensitive, and ultraspecific highly multiplexed clinical diagnostic platform based on 4c-ALEX single-molecule spectroscopy.

The appealing aspect of the single-molecule approach, particularly when combined with microfluidics-based sample handling (34), is the ability to use very small amounts of precious reagents and patient samples. The exquisite sensitivity associated with SMD to record subtle changes in target concentrations in ultrasmall volumes allows considerable savings in costs and patient sample. Because the ALEX methodology permits the detection of both protein- and nucleic acid–based targets with the same instrumentation, further cost savings are expected for end users.

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