Clinical Chemistry 58:4
000–000 (2012)

Editorials

Colorful Molecular Diagnostics

Achillefs N. Kapanidis[1*] and Robert Crawford[1]

Most diagnostic assays used daily in clinical laboratories are based on identifying and quantifying the presence of specific molecules that serve as markers of pathologic conditions and physiological status; however, these molecules are neither visualized nor counted directly. Instead, amplified signals generated through various strategies [e.g., real-time PCR, enzyme-linked assays (ELISAs), or simply blood culture] quantify the molecules of interest in an indirect fashion. These bioassays provide easy and robust detection on affordable instrumentation, but their amplification procedures also are usually associated with additional costs, additional time to results, increased labor, and a certain error rate. Furthermore, most of these methods monitor a single marker in a single sample.

Consequently, methods that detect molecular markers directly are highly desirable. In most cases, this quest involves fluorescence spectroscopy and imaging, owing to its flexibility, low cost, and sensitivity in visualizing and characterizing single molecules in very small sample volumes *(1)*. Until recently, single-molecule fluorescence detection was not a trivial task, because the signals generated from an individual molecule (for example, a fluorophore attached to a protein or DNA) were extremely weak. Only custom-built microscopes featuring the best combination of illumination sources, fluorescent probes, and ultrasensitive detectors could provide the sensitivity required to "see" and count single fluorescent molecules. Improvements introduced to facilitate studies of biological mechanisms in vitro and in vivo have made single-molecule microscopy instruments more sensitive, streamlined, and affordable. In fact, such instruments are leading the race for third-generation DNA sequencing *(2, 3)*.

In this issue of the Journal, Kim et al. *(4)* introduce a different application of single-molecule fluorophore detection, this time to the field of diagnostics. The new technique, which is based on single-molecule fluorescence detection in solution, is an extension of a family of methods known as alternating-laser excitation (ALEX)[2] spectroscopy of single molecules *(5)*. ALEX methods sort molecules on the basis of fluorophore stoichiometry (a fluorescence ratio called *S*) and fluorophore proximity [expressed as another fluorescence ratio, fluorescence resonance energy transfer (FRET) efficiency, which is sensitive to distances of 2–10 nm]. In its original incarnation *(5)*, ALEX used laser light that alternated between a wavelength that excited the FRET donor directly (and could probe the presence of FRET) and a wavelength that excited the FRET acceptor directly (and could probe the donor–acceptor stoichiometry). The major contribution of ALEX methods rested in their ability to sort molecules *(6)* according to their desired donor–acceptor stoichiometry and then analyze donor–acceptor distances in the molecular groups of interest. In this way, sorting single molecules with ALEX achieves for molecules what fluorescence-activated cell sorting and flow cytometry achieve for single cells. In fluorescence-activated cell sorting, a single cell flows through a detection zone, is illuminated with several lasers, and produces fluorescence signals that allow its identification and physical sorting. During an ALEX experiment, the transit through the detection zone occurs simply by diffusion; the sorting is only analytical but is sufficient for the direct analysis of the molecules present in solution.

Kim et al. *(4)* move from established 2- and 3-color formats *(7)* to introduce an impressive and novel 4-color ALEX format that uses 4 lasers and 4 spectral regions. This increase in complexity also means that the methods can now "decode" more information. This capability is key for the present technology, because it offers the considerable advantage of being able to check for the presence of multiple molecular subpopulations by using 2 kinds of codes: a "color" code (corresponding to the stoichiometry ratio) and a "distance" code (corresponding to the FRET efficiency ratio). For example, molecules "painted" blue–green can be differentiated from molecules painted blue–green–infrared on the basis of stoichiometry. Once the subpopulations with the same color combination are selected, then the distance between fluorophores (e.g., a blue–green pair

[1] Department of Physics and Biological Physics Research Group, Clarendon Laboratory, University of Oxford, Oxford, UK.
* Address correspondence to this author at: Department of Physics, Clarendon Laboratory, University of Oxford, Parks Rd., Oxford OX1 3PU, UK. Fax 0044-1865-272-400; e-mail a.kapanidis1@physics.ox.ac.uk.
Received January 16, 2012; accepted January 18, 2012.
Previously published online at DOI: 10.1373/clinchem.2011.180802

[2] Nonstandard abbreviations: ALEX, alternating-laser excitation; FRET, fluorescence resonance energy transfer.

with a 4-nm distance vs a blue–green pair with a 10-nm distance) acts as the second "code." Using this method, the authors claim that 4-color ALEX can generate and detect up to 100 codes in the same solution, an extremely impressive number that opens many opportunities for exciting diagnostics applications.

The ability to code is obviously important for multiplexed assays, which can test for panels of multiple sequences (or, in general, analytes) in a single measurement, greatly aiding the diagnostic potential of any assay. But, how is the coding being used to monitor different molecular targets? The answer lies in the fact that the coding fluorophores are attached to biomolecules that bind specifically to the targets of interest. For example, specific DNA sequences are detected through hybridization to complementary strands that carry the coding information (different fluorophore labels), whereas specific proteins are detected by using antibodies that target different epitopes on the surface of the protein target.

To showcase their multiplexing power of 4-color ALEX, Kim et al. *(4)* first demonstrate successful sorting of 25 different synthetic double-stranded DNA fragments (labeled with 1 to 4 fluorophores) in a single measurement. The authors next demonstrate the great diagnostic potential of the assay by detecting 8 PCR-amplified bacterial genetic markers (including 3 antibiotic-resistance determinants found in staphylococci and enterococci known to cause septicemia) with a sensitivity of $\leq$10 genomic equivalents and a quantification range of greater than 2 orders of magnitude. Furthermore, the authors demonstrate the simultaneous detection of all 8 markers amplified in a single well via multiplex PCR and subsequent hybridization to the full range of fluorophore-labeled DNA probes.

The assay of Kim et al. *(4)* also performed well with protein-based tumor markers. They selected 6 tumor markers for analysis with antibody pairs labeled with combinations of different-color fluorophores and detected the markers on the basis of differences in stoichiometries, an observation only available with ALEX. Simultaneous detection of all 6 markers spiked into serum was achieved by molecular sorting in "*S* space" in a "mix-and-read" format. Although sensitivities for the markers varied, depending on antibody affinities and dye properties, 3 markers were detectable at or below their clinically relevant threshold concentrations. A linear interval for detection, as well as quantification over a range of 2 orders of magnitude, was demonstrated for each marker.

This study is an impressive demonstration of the potential of single-molecule fluorescence methods for diagnostics. It would be interesting to see whether DNA sequences can be detected without amplification and what the sensitivity would be in such a case. That would reduce the time to results, an important consideration that increases the value of the assay. Additional targets (especially nucleic acids) can be easily included, and the preparation of the probes is straightforward. The protein assay, however, depends on the availability of high-affinity antibodies for the target of interest. The development of methods for sample preparation and handling and their integration with the instrument, as well as the development of a robust and affordable microscope, will be important for the wide adoption of the diagnostic assay. Combination with other bioassays compatible with ALEX, such as protein biosensors that use ALEX-based DNA coincidence *(8)*, can also improve the versatility of single-molecule diagnostic assays.

It is too early to say whether these molecular assays will be able to compete effectively with existing diagnostic platforms, but given the reduction in the complexity and costs of single-molecule instrumentation and the ability of sensitive multiplexed detection with minute samples, it is reasonable to expect that they will have an increasing share in the clinical diagnostics laboratory. The future of single-molecule diagnostics seems colorful and quite bright.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *No authors declared any potential conflicts of interest.*

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