Review

CellPress

Digital detection of biomarkers assisted by nanoparticles: application to diagnostics

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Single-molecule detection and counting is the new frontier in biomarker analysis. Here, we report on recent techniques for the digital detection of biomolecules for clinical application. First we highlight methods based on the immunocapture of proteins onto microparticles, followed by isolation of individual particles in microenvironments so that a sufficient signal is acquired for each binding event to make a binary decision, thus dramatically enhancing the signal:noise ratio. Various approaches are categorized based on the method used for particle confinement in an isolated microenvironment. We go on to describe methods for the detection of individual biological nanoparticles as well as the digital detection of proteins by artificial nanoparticle labels. The discussion of the methods emphasizes the practical considerations and their clinical applicability.

Digital and analog detection of biomarkers

The clinical use of protein biomarkers to differentiate between healthy and disease states, and to monitor disease progression, requires selective measurement of low concentrations of proteins in complex samples. Current immunoassays typically measure proteins at concentrations above 10^{-12} M [1]. However, the serum concentrations of most proteins that are important in cancer [2], neurological disorders [3,4], and the early stages of infection [5] are in the range 10^{-16}–10^{-12} M.

Attempts to develop methods capable of measuring these minute concentrations of proteins have focused on the replication of nucleic acid labels on proteins [6,7] or on measuring the bulk ensemble properties of labeled protein molecules using a variety of transduction and amplification mechanisms, such as electrochemical [8,9], mechanical [10,11], or optical [12–14] techniques.

The new frontier in biomarker analysis is single-molecule counting or digital detection, an approach that provides resolution and sensitivity that are not obtainable with ensemble measurements. Digital detection is a disruptive technology potentially allowing most advanced disease diagnostic tools to become available at a low cost and at the point-of-need. This is similar to how digital audio on compact discs enabled access to the highest-quality music recordings without expensive Hi-Fi equipment. Before digital recording, the sound quality from a vinyl analog recording depended on the sophistication of the player accurately reproducing the precise analog signal. Starting with the digital recording of audio on compact discs, sound quality no longer depended on the reader because it was easier to measure the presence or absence of signal than to detect the absolute amount of signal. Similar to reading a binary recording (1 s and 0 s), detection of single particles, when possible, is easier than the precise measurement of the ensemble quantities.

The principle of single-molecule counting can be best illustrated with the case of fluorescence detection (Figure 1). Each fluorophore reporter emits many photons before photobleaching, leading to fluorescent signals proportional to the fluorescent species present (Figure 1A). This type of measurement can be seen as an analog signal that increases with the concentration of the species being measured. By contrast, digital detection allows for a binary decision for each detectable signal (ideally for each single molecule) enabling measurement in discrete counts rather than as an analog intensity (Figure 1B). In theory, any method that is sensitive enough to detect an event associated with a single molecule would enable digital detection by counting and offer significant advantages over ensemble measurements in terms of sensitivity. In fact, the ultimate limit to analytical sensitivity is the reliable detection of a single molecule.

Almost all methods for single-molecule detection and analysis that we review here exploit an indirect detection principle because they do not detect the biomolecule itself, but instead particles or labels that are associated with it.

Here, we also provide examples of single-molecule detection and their application in high-throughput biomarker profiling, as well as discussing the challenges associated with these efforts. Given that nanoscale and complex sensing elements used in single-particle detection techniques may limit clinical applicability, we focus only on

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Keywords: biomarkers; single-molecule; diagnostics; nanoparticle; virus; digital detection; diagnostics.
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0167-7799/
 http://dx.doi.org/10.1016/j.tibtech.2015.03.002

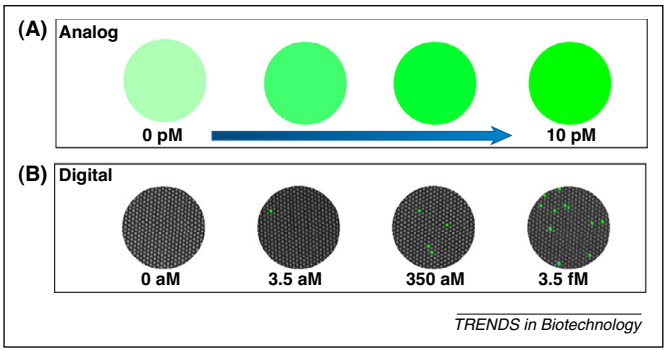

Figure 1. Analog and digital detection. **(A)** Analog measurements give increasing intensity as the concentration increases. **(B)** By contrast, digital measurements are independent of intensity and simply rely on a signal/no signal readout. Reproduced, with permission, from [12].

methods that are intrinsically robust and mature enough to be suitable for application in clinical diagnostics.

Digital molecule counting through single-molecule compartmentalization

It is a challenge to detect a signal from individual biomolecules or from labels attached to them, especially in a large volume. In traditional enzyme-linked immunosorbent assays (ELISAs), the fluorescent substrate is allowed to diffuse into a large volume, thus 'diluting' the signal. An effective way to overcome this problem is to concentrate the protein analytes by capturing them on micro- and/or nanoparticles using specific antibodies. By confining the particles in small compartments, the fluorescent substrate is forced to concentrate into a small interrogation volume, significantly increasing the local signal in comparison with standard bulk immunosorbent assays. The second challenge in achieving digital detection is the sparsity of the sample required to make a binary decision for each detection event (i.e., each molecule needs to be spatially separated to be detected as an individual particle). For very dilute samples requiring ultra-high sensitivity, sparsity is usually not a significant concern, but it can ultimately limit the sensor performance at high concentrations and, thus, the dynamic range.

The advances in optical methods enabling the detection of single molecules were reviewed recently [12,15]. The diverse microfabrication approaches (microwells, optical fibers, microfluidics compartments, and nanodroplets) allowing fractionation of the micro- and/or nanoparticles and the way these advancements lead to detection of subfemtomolar concentration in biological samples are discussed below.

Compartmentalization of single molecules bound to microparticles

Microfabricated microwells

The advent of microwells generated the opportunity to create arrays of nanovessels that can be used to confine single molecules in a small volume to create a locally high concentration. If a single molecule is trapped in a 1-femtoliter volume, the local concentration is approximately 2 nM and, thus, is relatively easy to detect [16] provided that one has captured the molecule of interest, knows where to detect it, and the transduction mechanism generates a signal well above the background. This observation was the basis for developing arrays of femtoliter-scale reaction wells called single-molecule arrays (Simoas), which are used to isolate and detect single enzyme molecules derived from enzyme-linked immunosorbent assays [17]. The test comprises a fluorescence sandwich immunoassay performed on the surface of an antibody-coated magnetic microbead. When analyzing samples with a very low concentration of the target protein, more capturing beads are added than the number of target molecules in the sample; therefore, the percentage of labeled beads, after the assay, will follow a Poisson distribution, with beads carrying either one single immunocomplex or none, thus providing a digital readout [18]. It would not be possible to detect such a low number of labeled molecules using standard ELISA plates and readers because the fluorophores generated by each enzyme would diffuse into the large assay volumes and fluorescence would result in a low signal above background. By contrast, if single microparticles are confined in microwells, the high local concentration of fluorophores will ensure a detectable signal. Specifically, the wells in this work, fabricated by optical fiber bundles, have a volume of 50 fL each and ensure the confinement of one single bead per well in such a way that, by acquiring fluorescence images of the array, it is possible to distinguish 'on' signals corresponding to labeled beads and 'off' signals corresponding to unlabeled beads. This digital ELISA platform was applied to the analysis of prostate-specific antigen (PSA) in serum samples of patients who had undergone radical prostatectomy, detecting PSA ranging from 14 to 9.4 fg/mL whereas the limit of detection of standard ELISA assays is in the 0.1 ng/mL range. Similar sensitivities were obtained by isolating magnetic nanobeads by arrays of femtoliter-sized wells fabricated in cyclic olefin polymer using injection molding based on DVD manufacturing [19]. Even if the specific diagnostic benefit of measuring PSA in those patients is still uncertain, this work clearly demonstrated that, by isolating and detecting single immunocomplexes in femtoliter-scale reactors, digital ELISA enables the measurement of biomarkers in complex samples at concentrations well beyond the sensitivity of analog ELISAs. A clinical evaluation of single molecule-level PSA measurement was later performed [20], demonstrating that, with a limit of detection of 3 pg/mL, this digital test can be a predictor of recurrence-free survival after radical prostatectomy with a sensitivity and specificity of 100% and 75%, respectively.

The Simoa approach was also used to detect p24 antigen for the detection of acute HIV infection with the same sensitivity provided by nucleic acid amplification testing and 7–10 days earlier than conventional immunoassays [21]. It was also used for the detection of inflammatory cytokines in plasma samples from patients with Crohn's disease [22].

The Simoa concept was then extended to multiplexed immunoassays demonstrating the simultaneous detection of a panel of four cytokines [23]. The multiplexing capability is enabled by the use of paramagnetic particles labeled with different fluorescent dyes to create optically distinct