nature nanotechnology

REVIEW ARTICLE

PUBLISHED ONLINE: 3 DECEMBER 2014 | DOI: 10.1038/NNANO.2014.261

Advancing the speed, sensitivity and accuracy of biomolecular detection using multi-length-scale engineering

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Rapid progress in identifying disease biomarkers has increased the importance of creating high-performance detection technologies. Over the last decade, the design of many detection platforms has focused on either the nano or micro length scale. Here, we review recent strategies that combine nano- and microscale materials and devices to produce large improvements in detection sensitivity, speed and accuracy, allowing previously undetectable biomarkers to be identified in clinical samples. Microsensors that incorporate nanoscale features can now rapidly detect disease-related nucleic acids expressed in patient samples. New microdevices that separate large clinical samples into nanocompartments allow precise quantitation of analytes, and microfluidic systems that utilize nanoscale binding events can detect rare cancer cells in the bloodstream more accurately than before. These advances will lead to faster and more reliable clinical diagnostic devices.

Increasingly, advances in medicine rely on understanding the multimolecular causes, effects and signatures of diseases. Personalized therapies targeted to highly specific disease sub-states now leverage insights into the molecular origins and signatures of these illnesses[1,2]. As therapeutic regimes become increasingly sophisticated and targeted, many diseases are now more treatable than before.

However, many approaches to diagnostic testing remain decades — and in some cases centuries — old[3]. One of the major impediments to effective diagnosis is the slow turnaround of most diagnostic techniques. Using culture to diagnose fast-spreading infectious diseases, for example, can take days to weeks[4]. Furthermore, poor sensitivity in many approaches means that for some diseases detection is possible only at the advanced stages, where they are difficult to treat[5]. These issues weigh on already-strained health care systems and hinder the delivery of optimal patient outcomes. The economic burden associated with the limitations of present-day diagnostic paradigms contributes to the unsustainable health care systems of many countries in the developed world, and also limits adoption of new treatments in the developing world[6,7]. Many of the global health crises that burden the developing world will benefit substantially from implementation of user-friendly, inexpensive diagnostic tools to deliver effective care to areas where sophisticated lab facilities are not available.

The last decade has yielded breakthroughs that are poised to transform disease diagnosis based on molecular signatures. In particular, the latest generation of devices addresses three distinct and physically important length scales that impact the detection process: the nanoscale dimensions of biological analytes; the micrometre length scale of biomolecular diffusion; and the macroscopic scale of clinical samples that contain millilitres of fluid to be processed. In this Review, we summarize the challenges related to achieving clinically relevant levels of performance with diagnostic technologies, and we discuss recent progress in the development of advanced detection systems through the lens of multi-length-scale integration. We highlight several new high-performance systems that have achieved record-breaking levels of speed, sensitivity and accuracy. We also discuss new frontiers in this field and identify challenges that remain.

Critical length scales for biomolecular detection

The detection of biological molecules involves phenomena occurring across different length scales (Fig. 1). For example, the regime of biomolecular recognition lies between 1 nm and 10 nm. The binding regions of nucleic acids having sequence-specific roles in biology are in the range of 20 base pairs and above, corresponding to 6 nm and greater. Protein–protein interactions also occur on the nanometre length scale.

Between 10 μm and 100 μm corresponds to the regime of molecular diffusion in solution. The size and shape of the biomolecular analytes of interest, combined with physiological temperatures, dictate that in the minutes-to-hours timescale appropriate for rapid biomolecular detection, typical large biological analyte molecules can diffuse 10–100 μm.

The regime of clinical sample size is millimetres to centimetres (or microlitres to millilitres). For many applications where the analyte of interest is rare, it is important to sample large volumes. The detection of bacterial pathogens to diagnose bloodstream infections and the analysis of circulating tumour cells (CTCs) are two examples of such applications where only a few cells may be present in a millilitre of blood, necessitating that several millilitres must be sampled.

Over the last two decades, the increasing availability of bottom-up and top-down strategies for generating nanomaterials[8,9], combined

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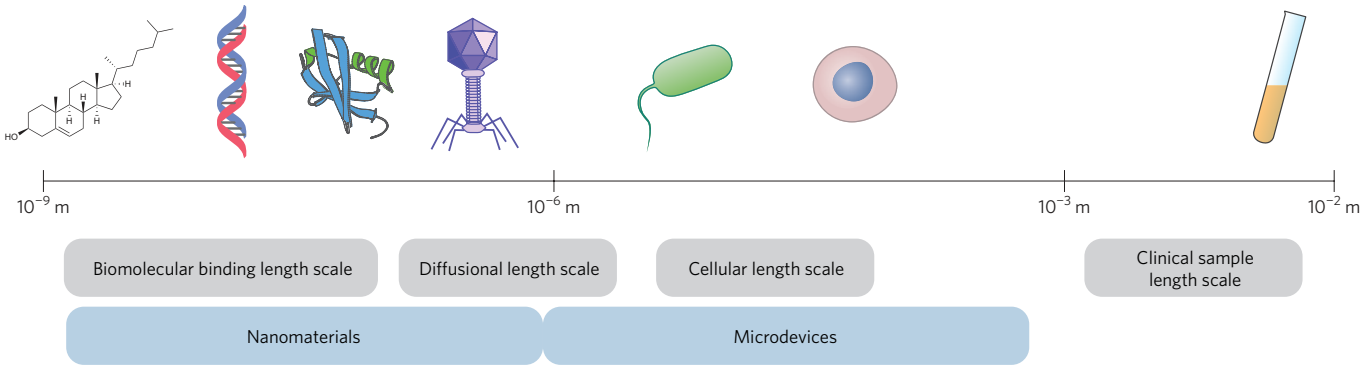

Figure 1 | Length scales of interest for biomolecular detection. Bioanalytical detection approaches monitor binding events between analytes of interest and specific receptors that occur on the nanoscale. Nanomaterials are ideal for biomolecular display because their dimensions approach the molecular scale, which promotes binding behaviour similar to that observed in solution-based processes. For reference, a molecule of cholesterol is shown on the left; this molecule is nanometre-sized. A DNA molecule (red/blue) and a protein molecule (green/blue), both structures that have sizes on the order of tens of nanometres are also depicted. A bacteriophage (purple), bacterial cell (green) and human cell (pink/blue) span the hundreds of nanometres to micrometre length scale. The microscale length regime is also important for biomolecular detection because most large molecules can only diffuse micrometres while detection is in progress. Devices can straightforwardly be engineered on this length scale, which facilitates the analysis of cells and processing of macroscale clinical samples (depicted as a test tube).

with expanded methodologies that allowed conjugation of biological receptors, have prompted a significant focus on miniaturized detectors[10]. Furthermore, microfluidic devices have become increasingly sophisticated[11]. These efforts have produced many examples of high-performance sensing systems, and several microfluidic platforms have been commercialized for research applications.

Early achievements in this area also highlighted the limitations of these approaches when used in isolation. While the ability of nanoscale detectors to detect biological molecules with excellent sensitivity became well documented, it was recognized that sensors with very small surface areas could not efficiently and rapidly capture and detect large, slow-moving analyte molecules within clinical samples[12,13]. Microfluidic devices that automate the steps required for biomolecular detection using methods such as the polymerase chain reaction or immunoassays served to complement, rather than supplant, diagnostic technologies that offer complete solutions in themselves.

Recently, combining microscale systems with nanomaterial-based strategies has permitted new types of biomolecular analysis to be developed and high levels of sensitivity to be validated with clinical specimens. Furthermore, using microscale devices to divide large samples into nanoscale aliquots has provided new ways to quantitate biomolecules. Moreover, microscale devices can leverage nanoscale binding events to sense rare analytes and cells at parts per million levels. In this Review, we explore these concepts and their impact on advances in protein biomarker analysis, nucleic acid detection and rare-cell analysis.

Performance challenges

To be effective at analysing clinical samples, biomolecular detection systems must achieve high levels of sensitivity, equivalent to low limits of detection. Stringent specificity is also required to ensure accurate discrimination among the abundance of biomolecules found in a sample. These levels of performance must be maintained in heterogeneous samples such as the complex biological matrices of blood, urine and saliva, a requirement that adds a significant challenge in light of the need to overcome nonspecific binding. Furthermore, to enable routine use of diagnostic test results, rapid turnaround on the timescale of a physician office visit (~20 minutes) is highly desirable.

The enzyme-linked immunosorbent assay (ELISA) is the laboratory workhorse technology for protein biomarker detection[14]. This technique, which uses an antibody sandwich assay format, can achieve clinically relevant levels of specificity and accurately report on levels of a target protein analyte. However, because of the technique's moderate (picomolar) sensitivity, it fails to detect even lower, but potentially clinically relevant levels of biomarkers often found in many samples of interest (for example, femtomolar to attomolar concentrations). Indeed, many potential markers of cancer, cardiovascular disease and neurodegenerative disease are present at these low levels in affected patients. A grand challenge for the protein detection field has thus been to improve sensitivity dramatically relative to the picomolar sensitivity of ELISA. Assays carried out on solid surfaces or in a laminar flow format concentrate the signal required for readout into a specific area; however, the target molecules in a sample must come into direct contact for a binding event to take place. This type of two-dimensional approach becomes limited by the slow diffusion of biomolecules.

The polymerase chain reaction (PCR), along with other enzymatic amplification methods that copy target molecules until they can be detected by fluorescence or other macroscale techniques, is the present-day gold standard for nucleic acid analysis[15]. The sensitivity and specificity of PCR allow detection at single-digit copy numbers and specificity at the single-nucleotide level. The level of automation for PCR-based assays achieved in several commercial platforms has enabled moderate-complexity labs to conduct sophisticated molecular diagnostic testing. However, the multiple-hours turnaround times and the need for lab skills to obtain accurate answers with PCR assays have not allowed them to be used in point-of-care settings.

For molecular diagnostics to extend their reach into the clinic, complete, sample-to-answer test automation will be required. This penetration to the clinic will demand short turnaround times compatible with physician office workflow. Achieving these capabilities is particularly important for indications such as infectious disease where the spread and severity of an infection can be limited with a rapid diagnosis. Therefore, any new nucleic acid biosensing technologies must be simple, fast, highly sensitive and specific.

Another key challenge to bioanalysis relates to implementing protein and nucleic acid analysis when rare cells must be isolated from clinical samples. For example, the analysis of CTCs may allow for non-invasive sampling of markers that define a tumour's phenotype and metastatic potential. However, these cells are present at concentrations as low as 5 cells per millilitre, and are therefore outnumbered by billions of normal cells in one millilitre of blood. Commercially available platforms for CTC immunocapture have,

despite the known limitations that cause many types of CTC to evade detection, brought CTC enumeration into mainstream cancer research. Advanced platforms with improved sensitivities and expanded versatility (for example, capture based on new markers, or even marker-free capture) are required to bring CTC analysis into routine clinical cancer management.

Advances in ultrasensitive protein detection

Achieving a clinically relevant combination of sensitivity, specificity and speed in protein detection has recently seen rapid progress through the use of materials of different length scales. Nanomaterials (for example, nanoparticles) have high surface-to-volume ratios and their surfaces are therefore well suited for the binding of reporter groups for biomolecular detection assays; however, microscale materials with lower surface-to-volume ratios that possess a larger core of active material are better choices for manipulations such as magnetic capture. Hence, combining these materials to detect bioanalytes brings together features that promote efficient binding of target analytes while also facilitating the collection of captured molecules.

A means of bringing together engineered micro- and nanoparticles for high-performance biomarker detection is illustrated by a class of assays that use DNA molecules as bio-barcodes[16] (Fig. 2a). Because their sequences are connected with specific analytes when the assay is designed, the DNA molecules serve as barcodes that report on what analyte has been detected. The first component of the capture is a microparticle with a magnetic core, and the second is a spherical nucleic acid (SNA)[17]. SNAs are gold nanoparticles functionalized with DNA molecules that can also be decorated with different functionalities, in this case a monoclonal antibody. Even at low concentrations, the complex can — once the target protein is sandwiched between the magnetic microparticle and DNA-coated gold nanoparticle — rapidly be isolated using a magnetic field. Subsequently, the gold nanoparticle conjugate that is part of the sandwich complex is rapidly dissolved, thereby releasing the DNA barcode strands. Mapping a specific DNA sequence onto each protein target of interest enables multiplexing, wherein the sequence is then detected using established microarray methods. The efficient capture of targets, enabled by the ability of the SNAs to interrogate the sample in three dimensions and combined with protein-to-DNA amplification, allows for femtomolar, and in some cases even attomolar, limits of detection.

This type of assay has been used for the detection of the HIV-1 p24 Gag protein, and was shown to have excellent analytical (Fig. 2b) and clinical (Fig. 2c) sensitivity[18]. Another example highlighting the sensitivity of this approach involved a clinical study that sought to correlate levels of the prostate-specific antigen (PSA) with prostate cancer recurrence in men post-prostatectomy[19]. Because the assay has a limit of detection 300 times lower than the most sensitive ELISA assay available commercially, it was possible to obtain accurate PSA measurements on patients who could then be informed that their low PSA levels were stable and that it was likely that their cancer would not recur. This was true for more than half of the patient pool; with the other half, the enhanced sensitivity allowed detection of increasing levels of PSA, which are linked to recurrence. The study also suggested that the assay could be used to monitor patient response to adjuvant or salvage therapies.

The bio-barcode strategy has also been used to track markers of Alzheimer disease in cerebral spinal fluid, a sample matrix in which the protein marker concentration is too low (<1 pM) to be detected using conventional ELISA and immunoassay technologies[20]. The assay was used to analyse the presence of amyloid-β-derived diffusible ligands (ADDLs) in cerebral spinal fluid from patients who had been definitively diagnosed with the disease post-mortem. ADDLs are potential soluble pathogenic Alzheimer disease markers. A 30-person study showed that all subjects, both with and without Alzheimer disease, had measurable levels of ADDL, and that all but two of the patients with Alzheimer disease had statistically elevated levels of ADDL compared with samples from healthy age-matched controls. The ability to track levels of ADDL in cerebral spinal fluid, and potentially also in blood, offers a path to using precise diagnostic tools to track the responses of patients who have Alzheimer disease to new therapies.

Using nanoscale reporter groups combined with microscale capture agents has produced significant advances in the sensitivity that can be achieved for the detection of clinically relevant protein biomarkers. This capability will be useful in a clinical context to monitor disease recurrence and to utilize biomarkers that are present at very low levels. An interesting and important question remains open and of interest to this field: what is the lowest concentration at which clinically relevant biomarkers can be present in patient samples? Since most biomarker discovery efforts are performed with very small samples, it remains possible that rare, yet highly specific biomarkers, could be missed in light of today's sensitivity limits. As discovery methods turn up rarer molecules, diagnostic technologies will be required to continue to analyse ever larger samples and further improve detection limits.

Nucleic acid detection with record-breaking speed

Protein biomarkers are important tools for the non-invasive screening and diagnosis of disease, but in many cases it is also essential to analyse nucleic acid sequences. Analysing genetic mutations and alterations that are implicated in cancer subtyping, bacterial antibiotic resistance and adverse reactions to many drugs are all examples of diagnostic applications where nucleic acid sequences must be delineated.

Today, the analysis of panels of nucleic acids is achieved predominantly in centralized laboratories. Most commercial assays rely on the enzymatic amplification of a target sequence, followed by readout of the amplified sequence using an optical method. A major unmet challenge for the field is the rapid detection of nucleic acids (that is, in under 30 minutes) with sample-to-answer automation — an achievement that would enable analysis outside of sophisticated labs. Realization of this goal would provide a powerful solution for the diagnosis of infectious diseases and for other indications where treatment decisions are time sensitive.

As an alternative to nucleic acid detection approaches based on enzymatic amplification, strategies based on amplification-free direct detection methods have received significant attention as they may represent a better solution for testing outside of the laboratory environment. Eliminating amplification may provide a way to speed the delivery of test results, and also simplify assay workflow to enable straightforward automation. For example, several integrated circuit-based strategies have sought to use electronic transduction schemes for sensitive direct nucleic acid analysis. By functionalizing silicon nanowires with nucleic acid probes[21], field-effect transistors can be made that have conductivities that depend on the presence of a complementary target sequence. Target hybridization can be monitored quantitatively using this approach as a function of time[22], and small changes in sequence can also be detected[23]. These devices combine high levels of performance with simple electronic devices that can be mass manufactured using the same infrastructure leveraged by the consumer electronics industry.

Direct electrochemical readout of specific nucleic acid sequences represents another solution to the rapid molecular analysis challenge. Electrochemical readout differs from electronic readout because it uses the flow of current at the surface of a sensor to determine the presence or absence of an analyte, rather than detecting a change in the properties of an electronic material. In general, electrochemical detection schemes involve the recruitment of redox-active reporter groups to the surface of a sensor in response to the binding of a specific analyte. Several different approaches to

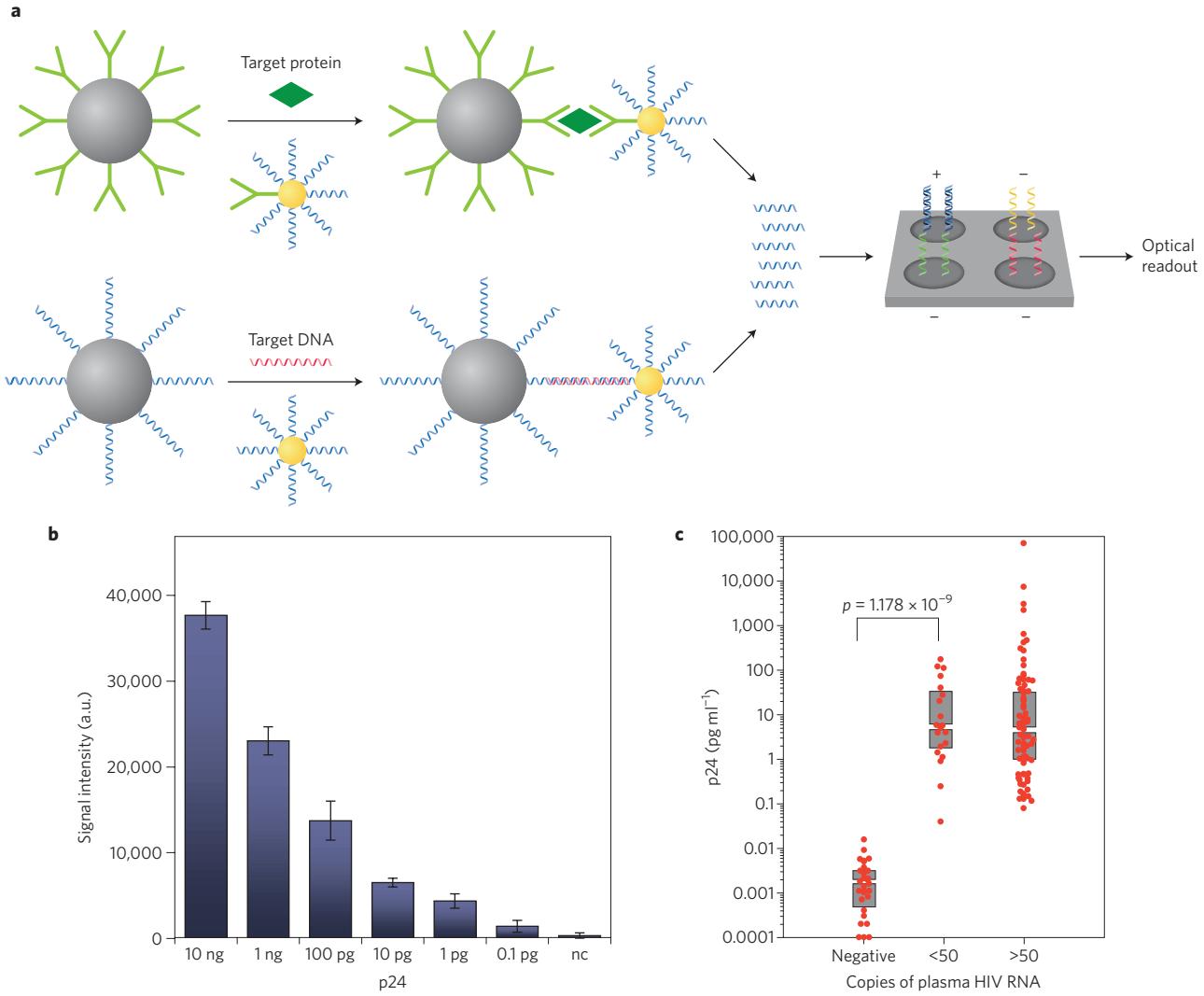

Figure 2 | The bio-barcode assay combines micro- and nanoparticles for biomolecular detection. a, A protein target (green diamond) is bound by a magnetic microparticle (grey) functionalized with a specific antibody (green Y-shape). The microparticles are probed with gold nanoparticles (yellow) that are functionalized with antibodies (green) and barcode sequences (blue). After isolation, the barcodes are released and detected on an array. The pattern of barcode binding on the array displaying a variety of barcode-binding sequences reports on the presence or absence of particular analytes. The same approach can be used to detect specific DNA sequences (bottom). **b**, Data showing HIV p24 Gag protein is detected at picogram levels using the bio-barcode assay described in **a**. nc, noncomplementary control. Error bars represent standard deviations. **c**, Clinical data showing sensitivity and specificity of the bio-barcode assay as compared to the gold standard (PCR). The *x*-axis values correspond to levels of HIV RNA in blood samples drawn from 146 patients measured by PCR. The *y* axis displays p24 protein concentrations in these same specimens measured by the new barcode assay. Each red dot represents a data point collected from a patient sample, and grey bars indicate the median and interquartile range. This data set shows excellent correspondence between the gold-standard PCR method used to detect HIV RNA and the new barcode assay that measures p24 protein, and there is strong statistical significance of the data for positive versus negative patients. Panels **b**,**c** reproduced with permission from ref. 18, © 2008 Future Medicine Ltd.

electrochemical nucleic acid detection have been pursued, including methods that use sandwich-type assays to deliver reporter groups[24,25], enzymatic labels to produce amplified currents[26,27], molecular beacon-like E-DNA assays[28,29], bio-barcode assays[30] and non-covalent reporter systems[31,32].

In designing electrode-based detectors for electrochemical sensing, it is particularly important to consider the implications of the nano-to-micro length scale. In contrast to the particle-based approach mentioned above, electrode sensors are tethered to a measurement system during target binding and therefore must employ an alternative strategy to thoroughly interrogate a three-dimensional sample. It is advantageous to miniaturize electrodes to minimize signal-to-noise, but detectors that are too small will not experience enough collisions with slow-moving analytes to attain high sensitivity.

Indeed, rationally engineering the structures of electrochemical sensing elements both at the nanometre and micrometre scales has recently been shown to enable improvements in both the sensitivity and speed[33,34] of nucleic acid detection, permitting detection of clinically relevant levels (fM) of nucleic acids in 30 minutes. Patterned microelectrodes on the surface of a glass (Fig. 3a) or silicon chip are generated by metal electrodeposition (Fig. 3b), and then a finely nanostructured surface is created using a second electrodeposition step (Fig. 3c). By creating a 100-μm sensor footprint, the latest approaches promote collisions with slow-moving messenger RNA (mRNA) molecules in solution, thereby removing the diffusional limitations of other chip-based sensors that suffer from low collisional frequencies[35]. The nanoscale roughness implemented in the coating of these large sensors promotes the display of probes in conformations that promote efficient binding of target nucleic acids[35].

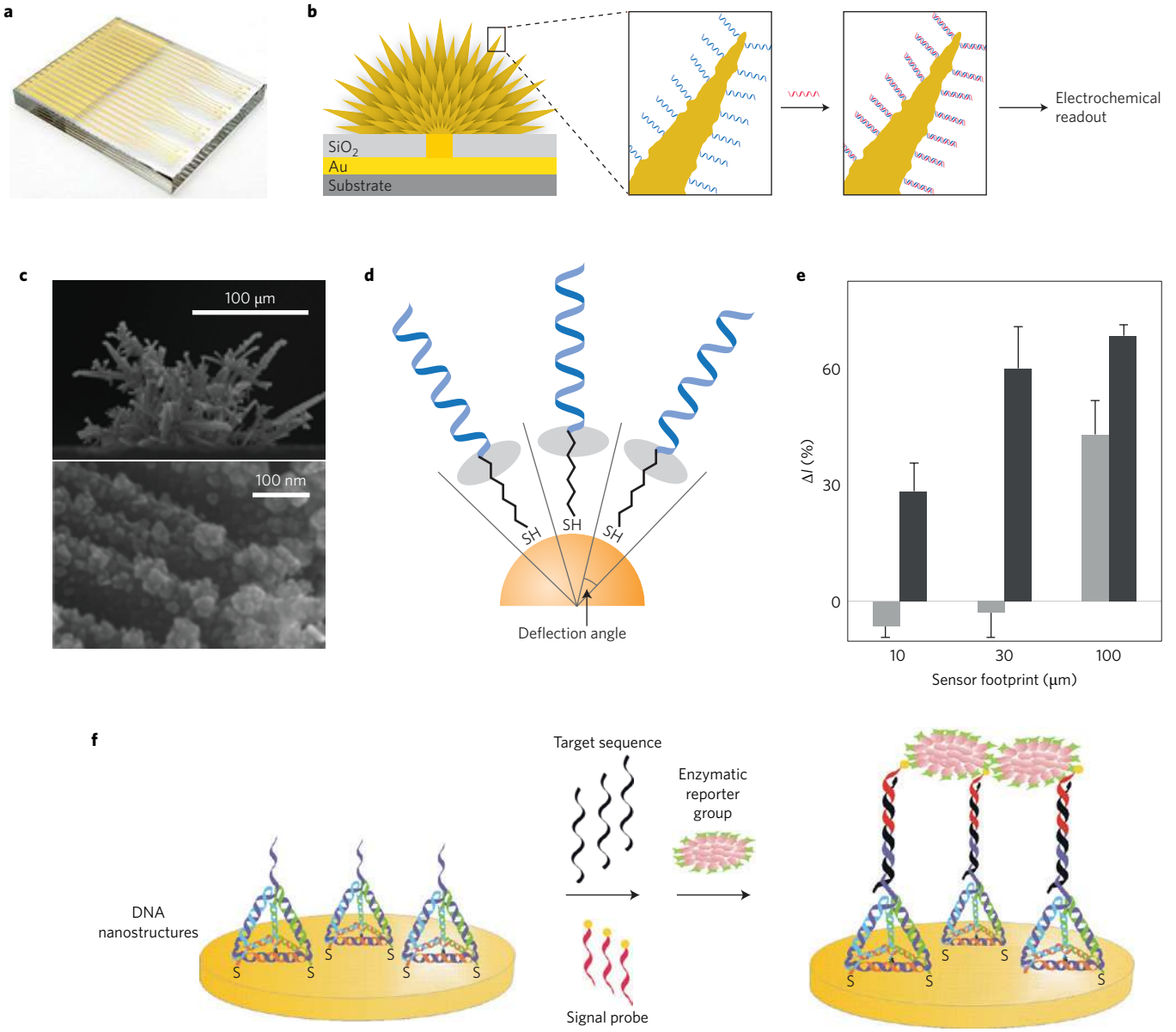

Figure 3 | Advances in rapid nucleic acid detection based on micro-to-nanoscale engineering of electrode properties. a, A lithographically patterned chip that serves as a substrate for the patterning of nanostructured microelectrodes (NMEs). **b**, NMEs combine the advantage of a large surface area with a finely nanostructured surface. Gold is electroplated on the surface of the chip through apertures etched in a passivation layer. Probe molecules (blue lines) are attached to the electrodes, and after a target-containing sample (red lines) is introduced, reporter groups are incubated with the sensor to give an electrochemical readout. **c**, Electron micrographs showing NMEs have 100-µm footprints (top), but feature nanoscale roughness on the order of 10 nm (bottom). **d**, Nanoscale roughness promotes the creation of a deflection angle that decreases steric interference between probe molecules (blue) immobilized with a thiolated tether (black) on a highly nanostructured surface (orange). The grey ovals represent the zone within which the probe strand has additional space to interact with a target sequence because of the deflection angle. **e**, Achievement of rapid nucleic acid detection. Data collected when different concentrations of bacterial lysates were incubated with NMEs with 10, 30 and 100 µm footprint for 30 minutes. While all the sensors showed measureable levels of current change (ΔI) when lysates containing a high (150 cells per microlitre; black bars) level of bacteria were introduced, only the largest 100 µm sensors could detect the lower (1.5 cells per microlitre; grey bars) concentration of bacteria, illustrating the importance of the microscale footprint of the sensor. Error bars represent standard error values. **f**, DNA nanostructures enhance the electrochemical detection sensitivity of nucleic acids. DNA tetrahedral display capture probes with well-defined nanoscale spacing, and target sequences are read-out using an enzymatic label. Figures adapted with permission from: **b**,**d**, ref. 33, © 2011 American Chemical Society; **c**, ref. 37, © 2009 American Chemical Society; **e**,**f**, ref. 48, 2012 Nature Publishing Group.

Fundamental investigations have shown that nucleic acid probe molecules immobilized on nanostructured microelectrode (NME) surfaces exhibit an angle of deflection[36] (Fig. 3d) that limits steric interactions between probes[35,37], thereby enhancing the rate and efficiency of hybridization. The NMEs have been coupled with an electrocatalytic reporter strategy[32] for the readout of target sequences; electrocatalysis is triggered when a negatively charged sequence binds to the sensor surface that in turn attracts a positively charged redox reporter. The electrochemical reduction of this reporter is made catalytic by the inclusion of a second electron acceptor that regenerates the cationic reporter.

The electrocatalytic approach permits direct analysis of mRNAs that serve as markers of pathogenic bacteria and associated antibiotic resistance down to concentrations as low as 1 cell per microlitre

(refs 33,38; Fig. 3e) within 30 minutes, a level of sensitivity not achieved previously with such fast turnaround. This strategy does not require sample clean-up, another feature that speeds the delivery of results[36]. Methods employing enzymatic amplification or active concentrating of samples lag behind this sample-to-answer turnaround time, with the commercial real-time PCR system[39] possessing the highest level of automation typically delivering results in 2–3 hours.

Electrochemical nucleic acid analysis carried out using NMEs has also been applied to the quantitation of a range of analytes including small molecules and proteins[40], and biomarkers including cancer-related gene fusions[41,42] and microRNAs[43]. In view of its low limit of detection, the strategy also shows promise in the analysis of gene expression in rare CTCs isolated from the bloodstreams of patients with cancer[44].

DNA-based nanostructures[45,46] represent another means to enhance the sensitivity of electrochemical nucleic acid detection. Tetrahedral structures made of DNA oligonucleotides display probes with well-defined distances to promote accessibility and overall detection sensitivity (Fig. 3f). Attomolar concentrations of microRNAs were detected using this approach[47,48], indicating that the display of probe sequences on both organic and inorganic nanostructures produce high levels of sensitivity. Nanomolar levels of small-molecule and protein[49] analytes can also be detected using sensors based on DNA nanostructures[50].

While advances in multi-length-scale engineering have improved the sensitivity and speed of nucleic acid detection, key challenges that remain include the demonstration of the robustness of direct nucleic acid detection strategies in a clinical setting and to evaluate whether the diverse range of biological backgrounds encountered in clinical samples will interfere with approaches where trace levels of specific sequences are recruited to electrochemical sensors. While eliminating front-end sample processing of biological samples simplifies the instrumentation required significantly, it renders the requirement of excellent specificity and sensitivity even more stringent.

High-accuracy digital biomolecular quantitation

While the methods discussed thus far are fast, sensitive and highly specific, there are limits to their capacity to quantitate biological analytes accurately over a large dynamic range of concentration. Several technologies have been developed recently that implement digital[51,52] methods to address the quantitation challenge. In the digital approach, macroscopic patient samples, typically possessing millilitre to microlitre volumes, are subdivided into many much smaller subvolumes, typically in the nanolitre to picolitre range[11,53,54]. Dividing samples in this manner ensures that, on average, only a small integer number of target molecules exist within each compartment[52]. As a result, the occupancy of compartments may be described using Poisson statistics, ideally in such a way that each compartment contains either zero or one molecule of the target analyte (Fig. 4a). Nucleic acid amplification of the contents of these subvolumes can be used to provide either a binary outcome (hence the digital nomenclature), and the concentration of the target analyte can be calculated from this measured distribution. In principle, digital approaches provide a wide dynamic range in the determination of target concentrations compared with traditional bulk ensemble measurements. The size of the compartments can be varied across the micro- to millilitre length scales to provide an expanded dynamic range.

Microfluidic implementation of this approach has enabled quantitative biomolecular analysis for a variety of applications. Genetic alterations known as copy-number variations can be monitored to understand how individual patients respond to cancer drugs[55], viral nucleic acids can be quantitated for infectious disease monitoring[56,57], and single-cell gene expression profiling can be carried out to facilitate the identification of biological heterogeneity[58]. It has also been utilized to quantify unequal expression of the two copies of each gene found in the diploid human genome[52] and profile low levels of residual disease biomarkers that may remain after cancer treatment[55,59]. In prenatal diagnostics, it can provide non-invasive detection of genetic defects[60,61], an approach complementary to advanced sequencing strategies[62].

Digital amplification may also provide an effective solution for the quantification of nucleic acid targets in limited-resource settings — such as a remote clinic, in the field, home or any other location where the sophisticated equipment required for PCR is unavailable. Making reliable, quantitative measurements using cost-effective devices will require merging simple microfluidic approaches for sample preparation with amplification strategies that are straightforward to implement. Equipment-free compartmentalization of molecules[63–66] is a microfluidic strategy particularly appropriate for limited-resource settings, as it eliminates the need for pumps. Furthermore, isothermal amplification chemistry also simplifies the workflow needed to detect nucleic targets by removing the requirement for thermocycling and is a good candidate for lab-free testing. Technologies combining these approaches have been validated in initial studies, wherein viral nucleic acids of HIV and the hepatitis C virus were partitioned in microcompartments, quantified and equivalence was shown between PCR and isothermal amplification chemistries[64,65] (Fig. 4b). These studies were performed using a 'SlipChip', a glass substrate patterned with microcompartments of varying sizes that can be filled with sample and amplification reagents via relative rotation of a similarly patterned substrate.

Robustness, that is, consistent performance despite variation in external conditions, is a crucial requirement in low-resource settings and one of the key advantages of digital single-molecule counting approaches[67]. Because of its reliance on binary outputs, the digital format gives isothermal amplification chemistries an unexpected high degree of robustness with respect to perturbations in temperature[64], reaction time and imaging quality. Digital isothermal assays have been read using a mobile phone camera that captures an image of a chip and transmits the pattern of the positive and negative compartments wirelessly to a cloud server for analysis[67].

A recently reported approach that uses microwells, rather than microfluidics, also applies the same digital logic to detect proteins[68–70]. Here, micrometre-sized magnetic beads with a capture antibody bound to their surface are added to a patient sample (Fig. 5a,b). Because the number of beads added exceeds the number of molecules in the sample by nearly an order of magnitude, the molecules bind to the beads as described by a Poisson distribution[69]. Most beads do not capture any molecules while about 10% will capture only a single molecule[70]. As in ELISA, a sandwich is formed in which the protein bridges the capture and detection antibodies, and thus any bead that has captured the target of interest carries an enzyme. The beads are then distributed into 40-femtolitre microwells, with each microwell sized to fit a single bead. A fluorogenic substrate is added to the bead-loaded microwell array and fluorescent product accumulates in wells containing an enzyme. Quantitation of the number of the original target protein molecules is thus obtained by counting the number of fluorescent wells. Femtomolar concentrations of an analyte within a sample can be detected, compared with low picomolar (pM) detection limits for the best commercial protein assays using the same reagents.

This single-molecule-array strategy can also be used to detect synthetic nucleic acids present at femtomolar concentrations without the need for target amplification[71], and was also adapted to detect bacterial DNA at attomolar levels[72]. The approach has also been used to detect tau protein in samples from patients with Alzheimer disease[73] and brain injury[74]. The resulting data, when combined with measurements of Abeta42 levels, can predict cognitive outcome after brain hypoxia due to myocardial infarction[75,76]. The strategy

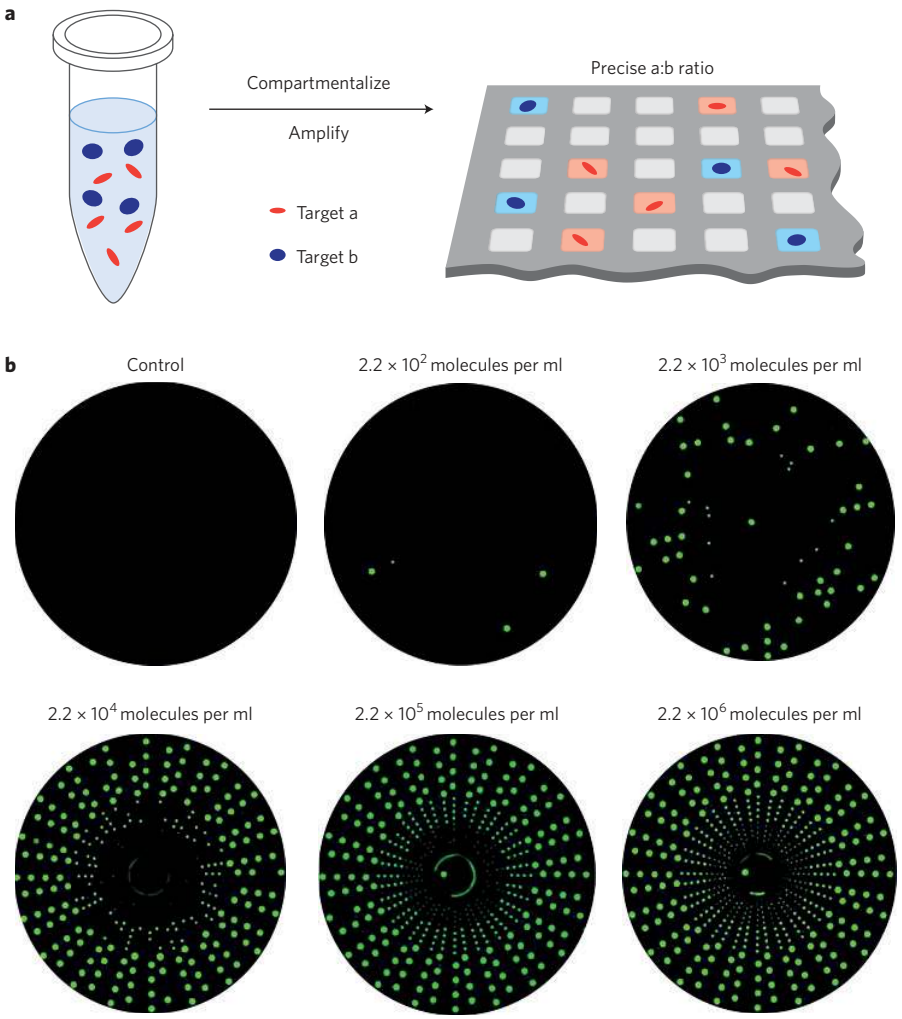

Figure 4 | Dividing samples into nano- to femtolitre volumes allows highly accurate quantitation of clinically relevant targets. a, In digital single-molecule amplification, target molecules (blue and red) are compartmentalized into smaller volumes. Nucleic acid amplification of the targets in a much smaller volume than during bulk amplification gives a signal that rises above the background level and above the signal arising from non-specific amplification. The quantitation of positive and negative wells allows a highly precise ratio of two analytes to be determined. This approach provides a wider dynamic range than traditional bulk ensemble measurements when determining the concentrations of target molecules. **b**, Quantitation of viral RNA within a SlipChip microfluidic device containing wells of different sizes via reverse transcription and PCR. A SlipChip is a piece of plastic or glass patterned with an array of microcompartments that can be filled with sample, and then using a similarly patterned top plate, amplification reagents can be introduced into each well by slipping the position of the two substrates. After the amplification is complete, fluorescent products (green) can be detected visually, and the number of positive wells reports on the concentration of a target analyte. At very low concentrations of RNA, the large wells have a higher probability of capturing and amplifying a molecule of RNA (larger green spots). At high concentrations of RNA, small wells capture and amplify RNA (small green spots). Quantification is performed over the range of concentrations from where several large wells turn green up to where several small wells remain dark. Panel **b** is reproduced with permission from ref. 64, © 2011 American Chemical Society.

has also been employed to detect toxins[77], to detect early HIV infection[78], to predict recurrence of prostate cancer[76,79], and to measure inflammatory markers in patients with Crohn disease[80]. The digital ELISA method has also been used to measure the low (pg ml^{-1}) levels of PSA in patients with prostate cancer who have undergone radical prostatectomies — these levels cannot be measured with a conventional ELISA[79] (Fig. 5c).

The quantitative nature of digital approaches brings new precision to the measurement of disease-related biomarkers, and enriches the precision of information content that can be obtained with clinical samples. It will enable biomolecular markers to be detected and monitored, thereby allowing response to therapy to be followed, and disease progression to be tracked accurately. However, clinical interpretation of the results will be more complicated and greater clinical validation may be required for the implementation of new quantitative tests that are currently performed on a qualitative basis.

Highly efficient capture and analysis of rare cancer cells

While molecular biomarker detection is integral to effective clinical diagnostic technologies, another important set of challenges relates to the capture and analysis of specific types of intact cell. The concentration of CTCs in the bloodstream of patients who have cancer as well as phenotypic status offers important information that may provide valuable diagnostic and prognostic insights. For this application, attaining high levels of sensitivity and specificity is a challenge. CTCs can be present at very low levels in blood (1–10 cells per millilitre), and are overwhelmingly outnumbered by normal blood cells a billion-fold.

Early technologies developed for CTC isolation and enumeration relied on tagging cells with magnetic nanoparticles displaying an antibody specific for the epithelial cell adhesion molecule (EpCAM), which is a marker present on the surfaces of many cancer cells[81]. CellSearch, an instrument approved by the Food and

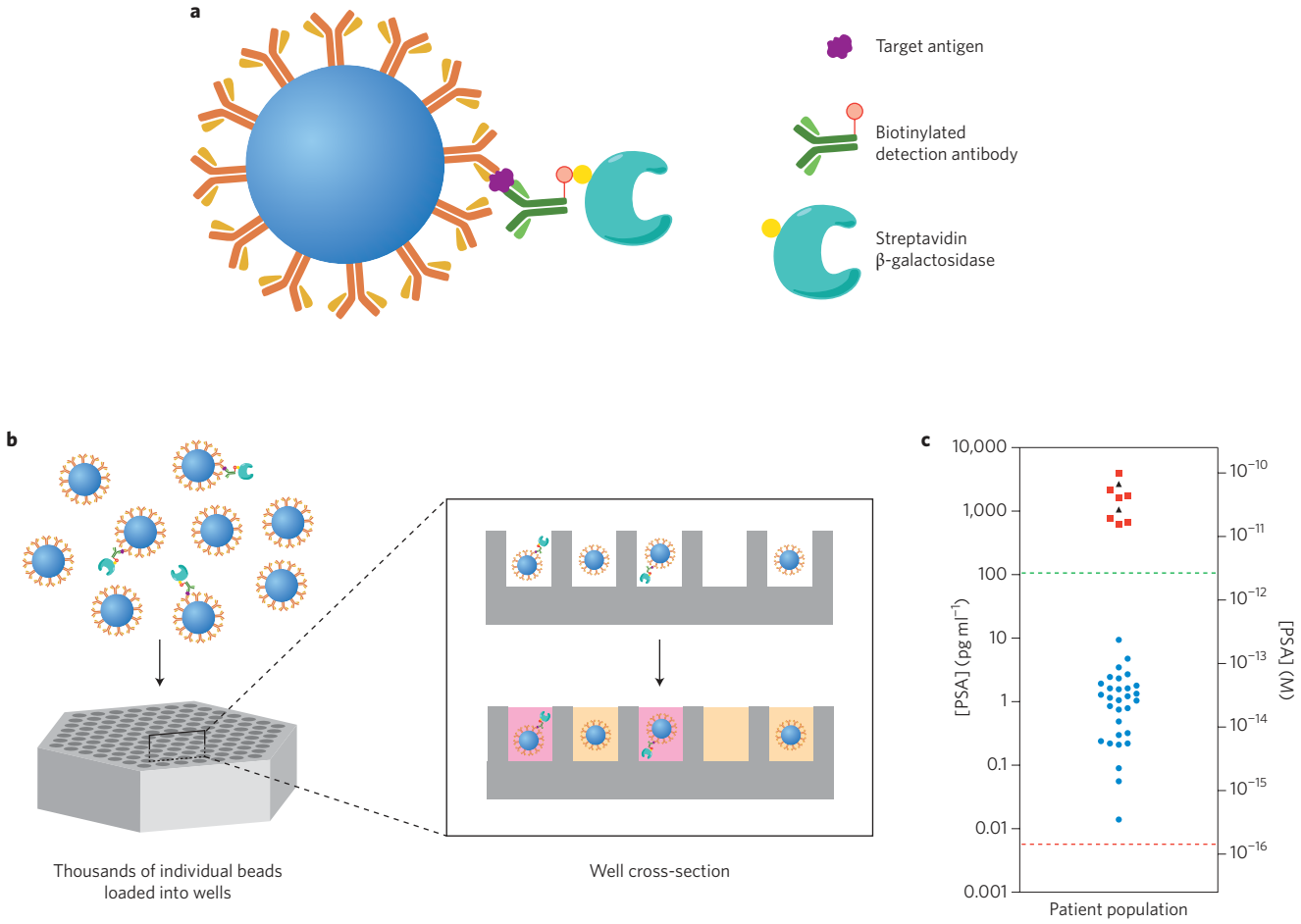

Figure 5 | Microwells separate analytes into femtolitre compartments for quantitative protein detection. a, A target antigen incubated with magnetic beads (blue circle) binds to capture antibodies (orange Y shape) on the bead surface. Biotinylated detection antibodies and an enzymatic reporter (β-galactosidase in this case) form a sandwich complex. **b**, Once deposited in an array of microwells, a fluorescent substrate is added to the sample, and wells are quantitated to analyse levels of protein biomarkers. **c**, Analysis of clinical samples for PSA levels. Concentrations of PSA were measured in serum samples from patients who had undergone a radical prostatectomy (blue circles), healthy control samples (red squares) and Bio-Rad PSA control samples (black triangles) determined using a digital ELISA. All samples yielded levels above the detection limit of the digital method (red line). In contrast, the detection limit of a commercially available ELISA test (green line) is too high to provide comparable data. Panel **c** is reproduced from ref. 68, 2010 Nature Publishing Group.

Drug Administration for monitoring treatment efficacy using this approach, is considered by many to be the present-day gold standard for CTC counting. While the analytical performance of CellSearch as assessed using cell lines is reasonable (with a reported analytical sensitivity of 1 CTC in a 7.5-ml sample), it typically identifies CTCs in the bloodstream for less than 50% of patients with cancer with metastatic disease[82]. As a result, it is primarily used only as a prognostic tool.

A first step in advancing next-generation CTC systems was to improve the efficiency of capture in patient samples. Sophisticated microfluidic devices that feature EpCAM-coated surfaces have proven to be effective for non-destructive CTC capture[83,84]. By displaying a nanoscale capture agent, for example an antibody to EpCAM, on a microfluidic device, capture efficiencies increased significantly and led to CTC detection sensitivities in patient samples that were improved by a factor of 2–3 times relative to CellSearch. The utility of these microfluidic devices has been shown for monitoring prostate cancer[84,85] and the identification of potential drug targets based on expression profiling of CTCs in pancreatic cancer[86]. Microfluidics-enabled strategies that rely on differences in deformability[87], size[88] or density[89] of CTCs relative to other cells in the bloodstream have also been developed for the sensitive isolation of rare CTCs. Affinity-based methods for isolating and separating cells remain powerful tools in light of the heterogeneity of CTCs and the possibility that only certain subpopulations may be clinically relevant[90,91]. The use of antibody cocktails to capture both the epithelial and mesenchymal phenotypes of CTCs showed the first demonstration of the epithelial-to-mesenchymal transition in patients with breast cancer[92].

Work on advanced CTC capture systems has increasingly focused on expanding the types of surface marker that are used for cell selection. Recently, several CTC capture systems have been reported that can use markers other than EpCAM for capture and/or analysis[93], or that rely on negative selection so that *a priori* knowledge of the surface expression characteristics of CTCs is not required[94]. For example a microfluidic inertial focusing platform — the iCTC chip (Fig. 6a,b) — combines several types of separation[95]. It depletes smaller red blood cells and platelets in the first microfluidic stage using a mechanical filter. The system then allows CTCs and leukocytes to be sorted using either positive or negative selection via the attachment of magnetic microparticles to the remaining cells, and a magnetic field directs the cells to different isolation chambers. For positive selection of CTCs, a marker such as EpCAM can be used to bind magnetic beads to CTCs for isolation, while for negative

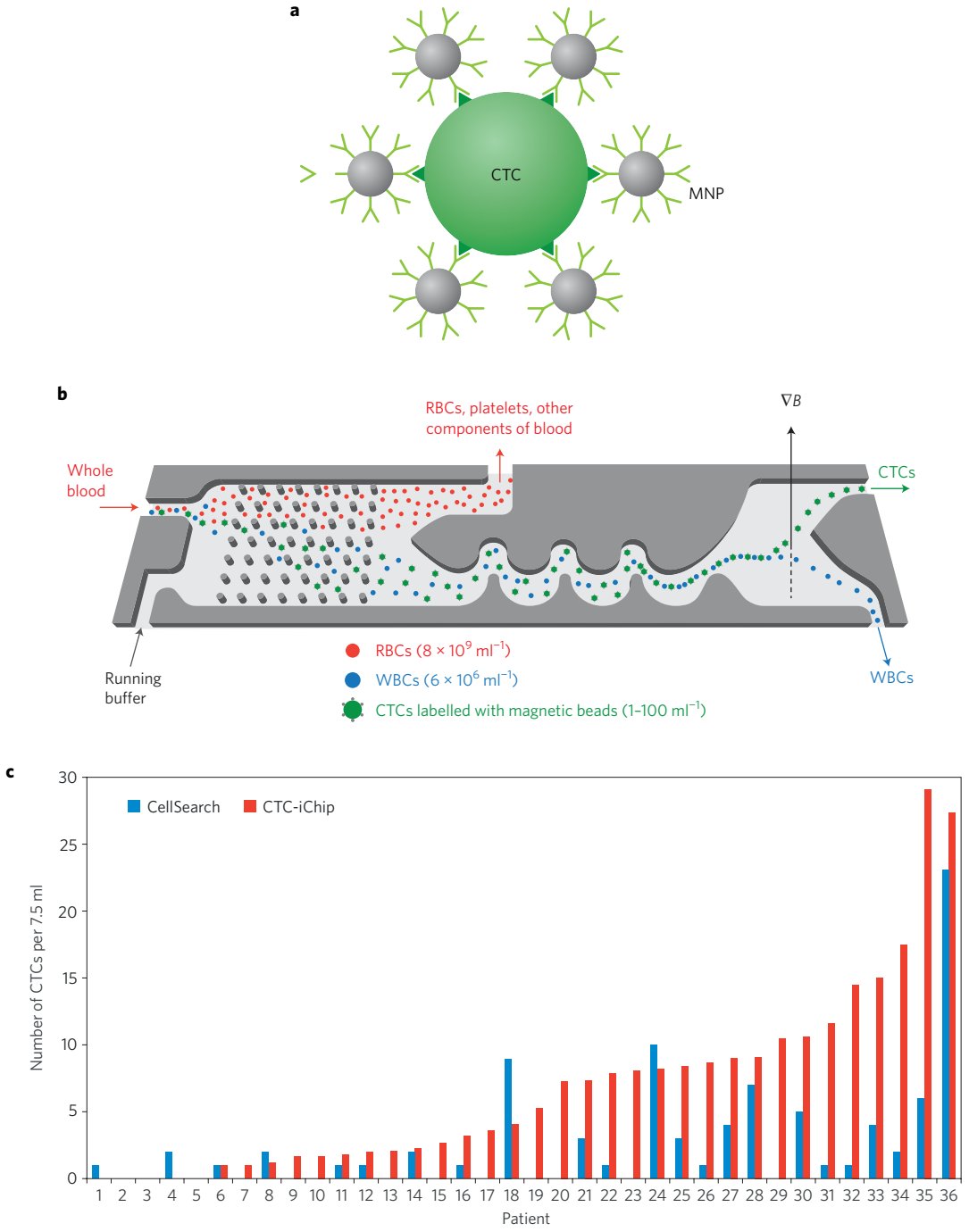

Figure 6 | Advanced microdevices for circulating tumour cell isolation. a, CTCs (green) are recognized by antibody-functionalized magnetic particles (MNPs; grey). **b**, A microengineered CTC isolation chip allows negative and positive selection for CTCs by manipulating the flow of microparticle-tagged cells. Whole blood is injected into the device, and passed through an array of microposts that separate the cells based on size. This allows white blood cells (WBCs) and CTCs to be separated from abundant red blood cells (RBCs). Either the WBCs or CTCs can be labelled with magnetic particles, and the application of a magnetic force (*B*) then forces the different cells to exit the chip via different outlets where they can be isolated as an enriched population. **c**, CTC levels isolated from blood samples collected from patients with metastatic breast, prostate, pancreatic, colorectal, or lung cancer. The samples were analysed with the iChip or the gold-standard CellSearch method. Significantly higher levels of CTCs were observed when the iChip was used, and many samples that appear negative for CTCs when CellSearch was used for analysis were found to have significant levels of cancer cells with the iChip. This indicates that CTC counting is much more accurate with this new approach and enables CTC monitoring even in patients with low counts. Panels **b**,**c** adapted from ref. 95, © 2013 American Association for the Advancement of Science.

selection, leukocytes and granulocytes can be labelled with a cocktail of antibodies to separate them from the CTCs. This approach was shown to be effective with patient samples having low CTC loads. For samples where <30 CTCs were detected in a 7.5-ml sample using the iChip, CellSearch often did not report any CTCs (Fig. 6c).

An additional recent study documented the use of a micro-Hall detector for the simultaneous analysis of several different markers[96]. This approach does not capture CTCs selectively, but instead identifies cells with a particular marker profile in an unpurified sample, thereby avoiding the loss of cells, which is often incurred

in purification. Magnetic nanoparticles with varying magnetization properties are functionalized with different antibodies to surface antigens, and the presence of specific particles is analysed with a micro-Hall detector. This approach increased CTC detection frequency in patient samples by 25% over what could be achieved with CellSearch. Another recent study documented a highly versatile ensemble-decision aliquot ranking approach in which any antibody of choice can be employed to analyse the marker profile of a CTC[97]. Multiple antibodies are labelled with different flurophores, incubated with CTC-containing blood, and the sample is pumped through a microfluidic channel, where fluorescent cells are counted. This system also outperformed CellSearch and detected CTCs in 82 out of 90 patients with stage-IV metastatic breast cancer (91%), while CellSearch only detected cells in 44% of the patients.

Significant progress has been made in the isolation and analysis of CTCs and promising advances that combine microscale engineering with nanoscale binding events are on the horizon. Advanced methods for intracellular RNA expression may play an important role in CTC characterization. Engineered nanoparticles coated with oligonucleotide probes, for example, can enter cells, bind to an mRNA sequence of interest and concomitantly release a fluorophore-labelled sequence[98]. This has the potential to search for CTCs based on unique genetic signatures.

Future frontiers for biomolecular detection technologies

Progress towards sensitive, specific, rapid, automated diagnostics has been brisk and impressive; nevertheless, significant opportunities to address more fully the goals of this field still exist, and multi-length-scale engineering is likely to remain important. The degree of automation varies from platform to platform, and typically is less complete in more complex assays. Full automation of biomolecular detection platforms will broaden the uptake of new detection capabilities and promote efficiency in resource-constrained laboratories. Better sample processing interfaces that introduce samples into analysis platforms with a high level of automation will facilitate the generation of accurate results even in the absence of highly trained operators. The platforms discussed above often focus on the automation of key elements of the sample processing chain (for example, automated sample processing or readout), but ultimately retain manual steps to connect them. An automated workflow with no human intervention between the introduction of a sample and data presentation is the goal for most assays so that they can be used outside of laboratory facilities.

Furthermore, although much progress has been made in multiplexing, with the technologies reviewed here finding successful application in the detection and even quantitation of multiple biomarkers, further advances are needed. It is projected that research into genetic fingerprints of disease will produce a demand for tests employing hundreds of biomarkers. In addition, tools for pre-clinical research that seek to enable the discovery and winnowing down of new gene expression fingerprints will benefit from rapid, automated and low-cost analysis of thousands of markers from libraries of prospective targets. Technologies that scale to this order of biomarker multiplexing will be in demand, and given the importance of miniaturized diagnostic devices, combinations of micro- and nanofabrication will be an important tool.

Conclusions

Knowledge of the molecular basis of disease is advancing rapidly, with molecular targets emerging in many disease states that represent promising targets for earlier detection and targeted treatment. As summarized in this Review, technologies that can detect and quantitate protein and nucleic acid biomarkers with the desired level of accuracy, cost and automation will be critical for the integration of these markers into routine clinical practice. The advances highlighted herein all perform at high levels because they leverage the advantages of working on several length scales to achieve high levels of sensitivity and specificity. Uniting features of both nano- and microscale materials brings together compelling capabilities for analyte capture and analysis, and combining microdevices with materials that allow for tagging of molecules and cells at the nanoscale has yielded devices with superior performance. These advances provide new means of searching for the markers of cancer, and neurodegenerative and infectious diseases.

Received 9 March 2014; accepted 13 October 2014; published online 3 December 2014

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Acknowledgements

S.O.K. and E.H.S. acknowledge Genome Canada, the Canadian Institute of Health Research, the Natural Sciences and Engineering Research Council, and the Ontario Research Fund for support of their work. M.T. acknowledges the National Institute of Health (NIH) P41 Resource Center, NIH National Institute of Biomedical Imaging and Bioengineering Quantum Grant. R.F.I acknowledges NIH grant R01EB012946 and the Defense Advanced Research Projects Agency (DARPA) Cooperative Agreement HR0011-11-2-0006 for support. D.R.W. acknowledges generous support from DARPA (HR0011-12-2,0001: SUB #5-55065) and a Department of Defense Innovator Award BC100510(W81XWH-11-1-0814). C.A.M. acknowledges support from the Center for Cancer Nanotechnology Excellence (CCNE) initiative of the National Institutes of Health (NIH), the Nanoscale Science and Engineering Centers (NSEC) initiative of the National Science Foundation, the Prostate Cancer Foundation, National Institute of Arthritis and Musculosketal and Skin Diseases/NIH, and DARPA.

Additional information

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Competing financial interests

R.F.I. is a scientific founder, a Director, and has equity in SlipChip. D.R.W. is a scientific founder, a Director, and has equity in Quanterix. S.O.K. is a founder, a Director, and has equity in Xagenic. E.H.S. holds equity in Xagenic.