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Protein measurements in microwells

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Abstract

Protein measurements are essential to many fields ranging from fundamental biochemistry to clinical diagnostics. The ability to make measurements of proteins with ultra-sensitivity will enable early diagnosis of diseases by accessing a concentration regime below the detection limit of present protein assay methods. Furthermore, while single cell analysis is becoming an essential tool, most single cell analytical methods are aimed at measuring genetic targets. Single cell protein measurements will be critical to obtaining a complete picture of the cell. Microwells and microwell arrays are powerful platforms for making protein measurements. Confining molecules and cells to small volumes creates high local concentrations. This Insight discusses the present status of microwell arrays for making protein measurements and describes some of the fundamental challenges as well as opportunities for using microwells in the future.

Present Status of the Field

This Insight aims to convey the value of performing protein measurements in a microwell format. Today we are being inundated with new discoveries in genomics—nearly every day a new species is being sequenced, new disease genetic markers are being discovered, and fundamental information about new genetic control and regulation mechanisms are being reported. But genes code for proteins, many of which are enzymes that carry out catalytic reactions in metabolic pathways; other proteins are signaling molecules for important regulatory pathways such as immune function, and still other proteins provide structural roles for preserving the integrity of the organism. Differences in protein expression or protein modifications, resulting from mutations or changes in post-translational modification, can manifest as disease. Proteins are closer to the action than either the genes or the messenger RNAs that code for them. A good example of where protein measurements are important is in human health. Yet protein measurement techniques have not changed much in many decades. The basic format for the enzyme linked immunosorbent assay (ELISA) remains intact since it was first introduced other than changes in the transduction method (e.g. electrochemiluminescence). ELISAs are limited to measuring protein

concentrations above single digit picomolar (pM). Consequently, the ability to diagnose disease rests on the roughly 100 proteins of clinical utility in the blood^[1]. But many disease-relevant protein biomarkers are likely present at much lower concentrations or are only measurable when the disease has progressed to the point where intervention is either ineffective or useless.

So what is the path forward for protein measurements? Clearly, we need to access the concentration regime below pM. One way to perform such ultra-sensitive measurements is to employ microwells. For making protein measurements in microwells, there are two distinct steps that need to be considered. First, one must capture the protein of interest using a capture reagent. Such reagents can be antibodies, aptamers, or specific proteins that interact with the protein of interest. The second step is detecting the bound complex. A variety of methods can be used for this step including enzyme labels, fluorescent labels, and nucleic acids. For example, in ELISAs, the capture reagent is an antibody and the detection reagent is an enzyme linked antibody that recognizes a different epitope on the target protein. Such assays are typically carried out in microwells in a microtiter plate and have been so successful that they are the gold standard for protein assays in the clinical arena. But the term “microwells” is a misnomer by today’s standards. When microtiter plate formats were first introduced, they revolutionized high throughput screening because they replaced sampling from what was then a state of the art auto-analyzer, in which large sample cups were consecutively sampled by aspiration from a carousel. Microtiter plates advanced both sampling and measurement by enabling assays to be performed in a 96 well format, a 384 well format, and finally a 1536 well format. The working volumes of the wells in a 96, 384, and 1536 well plates are 75-200, 20-80, and 2-8 μL respectively. In this format, the “micro” in microwell refers to the microliter volumes. For readers who don’t typically think about how length relates to volume, a microliter is a volume defined by a cubic millimeter. In contrast, today the term “microwells” refers to wells with micrometer (micron) dimensions, analogous to the term “microarray” in which features with micron-sized dimensions are arrayed on a substrate. Since volume scales as the cube root, a cubic micron corresponds to a femtoliter: 1×10^{-15} liters. The chemistry and physics of femtoliter volumes is very different than microliter volumes and the practical aspects that devolve from the chemistry and physics create both opportunities and significant challenges. In this Insight, I will restrict the discussion of microwells to include chambers between 0.1-10 micron dimensions. Such volumes span the size regime of microfluidics and also bacterial and mammalian cells.

The earliest microwells were developed in the mid 1990’s. My laboratory reported the first high density

microwell array in which we acid etched the cores of an optical fiber bundle to create microwells with several thousand wells on the end of the optical fiber array^[2]. By melting and pulling the fiber followed by etching, we were able to prepare nanowells with significantly smaller dimensions and 3 attoliter (3×10^{-18} L) volumes (Figure 1). Whitesides and coworkers reported a scalable microwell array prepared from a silicon master using PDMS replicas^[3].

The advent of microwells generated an opportunity to create arrays of small vessels; these vessels could be used as miniature reaction chambers, or as containers to trap or confine molecules. A thought experiment—if one confines a single molecule in a 1 femtoliter volume, the local concentration is roughly 2nM. Confinement in small volumes creates a locally high concentration. If the confined molecule is a fluorescent dye, it should be relatively easy to detect it as long as one knows where to look. But confinement is insufficient for making analytical measurements. If one wanted to distribute just 1 microliter into 10 fL wells, it would require 10^8 wells to confine the entire solution. Such an array of wells (approximately $2\mu\text{m}$ diameter \times $2\mu\text{m}$ deep) would require a 3 cm \times 3 cm surface. Attempting to fill such an array would be an enormous challenge, with evaporation only one of the difficulties. Of course, most samples of biological or clinical interest are much larger than a few microliters. Consequently, it is important to pre-concentrate the protein molecules of interest. One way to concentrate is via affinity binding reactions.

There is a fundamental limit with binding reactions at low concentration when two species are brought together and one of the binding partners is attached to a surface. Calculations by Corn, based on first principles and some reasonable assumptions regarding on and off rate and surface coverage, indicate that a 10fM solution would take three years to equilibrate with a 1cm^2 surface—what Corn refers to as the “Tyranny of the Langmuir Isotherm”, which have been discussed in detail for affinity binding assays^[4]. In practice, binding times deviate significantly from the theoretical values but can be many hours or days for dilute solutions of analyte. Thus, there is a mismatch between the need for rapid assays to detect disease and the long incubation times required to capture the target protein of interest.

One alternative is to bring the capture surface to the analyte rather than trying to bring the analyte to the capture surface. Microspheres and nanoparticles are ideal surfaces to accomplish this goal. The first example of combining binding surfaces with microwells was the development of bead arrays by Walt and coworkers, which enabled localized delivery of binding reagents into the microwells^[5].

Today, microwells are used in multiple ways for protein detection. My laboratory extended the bead array idea to single molecule protein detection. In conjunction with colleagues at Quanterix Corporation, we developed the Single Molecule Array (Simoa) format for performing immunoassays using digital ELISAs^[6] (Figure 2). In this format antibody-coated microspheres are used to capture extremely low abundance target proteins from solution. The digital ELISA involves capturing single protein molecules on individual microspheres and then visualizing the bound single molecules by using an enzyme amplification scheme. To calibrate the reader, 100 μL of a 1 fM target protein solution contains 60000 molecules. In the Simoa digital ELISA format, we employ microspheres or beads, each decorated with hundreds of thousands of Ab molecules. 500000 antibody-coated microspheres are added to the solution to bring the affinity capture surface to the solution. The combination of the large number of beads combined with the high number of capture Abs per bead creates a locally high concentration of antibody and drives the reaction to the bound complex. Furthermore, the distance between microspheres is short relative to the diffusion path length of the target proteins. All of these factors significantly accelerate binding times compared to diffusion of a low concentration protein to a large area planar surface. Because there are many more microspheres than molecules added to solution, the target molecules bind such that there is either one or zero molecules per microsphere—calculable from the Poisson equation. Using a conventional ELISA format, after washing, the captured proteins are labeled with a biotinylated detection antibody followed by labeling with β -galactosidase conjugated to streptavidin. Beads are then loaded (either using gravity or a magnetic field) into microwell arrays containing between 50000-216000 microwells with dimensions of 4 μm in diameter and 3 μm deep and sealed along with a fluorogenic substrate for the β -gal. Beads containing a β -gal molecule, and therefore a target protein, generate a high local concentration of fluorescent product and can be imaged and counted. The ratio of fluorescent microwells to the total number of microsphere-containing microwells corresponds to the protein concentration. By using this approach for digital detection of proteins, limits of detection can be reduced between 100-1000 fold to the single digit femtomolar or even high attomolar range.

Ismagilov and coworkers have developed a SlipChip consisting of two plates—one plate contains microwells preloaded with reagents and a second plate contains ducts^[7] (Figure 3). When the two plates are moved relative to one another, reagents are transferred and allowed to mix without the need for pumps or other active fluidic delivery mechanisms. Several immunoassay formats for SlipChips have

been developed in which protein binding occurs either directly to the wells or to magnetic beads that are then delivered to the wells^[8].

In addition to the classical enzyme-labeled immunoassays, there are other methods that can be used for detecting target proteins. One class of assays employs nucleic acids as labels. The Proximity Extension Assay (PEA) developed by Landegren and coworkers and commercialized by OLink Biosciences is a homogeneous assay in which two DNA-labeled antibodies bind to a target protein in solution^[9]. When bound to the target protein, proximity of the DNA labels enables complementary binding, which primes a PCR reaction and creates real-time PCR amplicons. Another nucleic acid based protein detection system is immuno PCR^[10], in which a nucleic acid labeled detection antibody is used to detect a bound target protein. PCR amplification of the DNA label enables sensitive detection of the bound protein. The PCR reaction is somewhat more cumbersome than a fluorogenic or chromogenic enzyme amplification but the sensitivity can be superior.

Another area in which protein measurements in microwells is valuable is for following enzymatic reactions at the single molecule level. Although early work in the field of single molecule enzymology employed water in oil emulsions to create micro-reaction vessels, the use of microwells to confine single enzyme molecules is more recent. Single enzyme molecules can be trapped in microwells^[11] and the reactions can be observed by monitoring the kinetics of fluorogenic substrates being converted into fluorescent products. Microwells enable reactions to be monitored for long periods of time, which enables reaction mechanisms to be elucidated that cannot be observed with ensemble measurements.^[12] An advantage of microwell measurements compared to the popular Total Internal Reflection Fluorescence (TIRF) methods is that microwells enable the enzyme to remain in solution rather than being attached to a surface. When microwells are used for such measurements, they must be passivated with proteins such as BSA or with a polyethylene glycol (PEG) surface layer to avoid surface adsorption.

On a side note, several DNA sequencing methods have taken advantage of microwells—Webb's zero mode waveguides^[13], 454 Sequencing uses the beads in wells approach^[14], Ion Torrent^[15] uses microwells to confine protons generated when nucleotides are added by DNA polymerase to a growing DNA template within microwell arrays and the change in pH is measured via an ion selective field effect transistor. The ability to measure even a single proton underscores the value of confining chemical

species to small volumes. The confinement creates large local concentration/activity changes that can be readily measured using today's sensitive detectors.

Future scenarios

High throughput screening hasn't really benefitted from microwell arrays because there is no way to deliver different reagents (e.g. chemical libraries) to different wells. Delivery of small volume solutions with the flexibility to deliver specific samples and reagents to each of many microwells is important because it would enable extremely small amounts of precious materials to be used for screening.

There has been significant interest in single cell analysis with the goal of capturing or trapping cells to analyze their contents. Most research in this area is directed at genetic analysis because DNA and RNA can be amplified using PCR or other amplification methods. There isn't a PCR analog for proteins. The volumes of cells and microwells are perfectly matched such that microwells can be used to trap and/or confine cells^[16]. Cells have volumes in the tens of femtoliters to single picoliter range. The first microwell single cell array was published in 1999 by Taylor and Walt^[17]. Love and coworkers have used picoliter wells to measure protein release. They use captured cells in microengraved wells and have measured release of various proteins—primarily immunoregulatory markers^[18]. One could potentially use the wells to confine cells such that they can be lysed and their contents released in order to prevent dilution of the small number of proteins into a much larger volume.

Problems and issues that need to be overcome

The use of microwells for protein detection offers a significant advantage over performing bulk measurements. Improved sensitivity, even down to the single molecule level can be accomplished. As discussed above, there remain numerous challenges for making protein measurements in microwells.

One of the biggest issues with any affinity measurement is non-specific binding (NSB). For protein measurements using capture reagents, the biggest challenge is to reduce non-specific binding. Non-target proteins, as well as the detection reagents (often large enzymes), stick to surfaces and give false positive signals. Such responses are particularly challenging when trying to measure a presence/absence response; for example, the presence of a highly infectious viral or bacterial protein

that should not be present. In this case, any signal resulting from even a single reporter molecule binding non-specifically to the assay vessel surface could be interpreted as a positive signal. The analytical criterion of a true signal being 3x the standard deviation of the background provides more measurement confidence but any background creates a problem in that it increases the limit of detection. Ideally, there would be no binding at zero concentration of an analyte. NSB has been a nagging problem for protein measurements and will continue to pose a challenge in microwell formats. In this regard, proximity assays that involve two binding partners are advantageous because NSB is reduced since the reaction takes place in solution and does not require a binding surface. But localization of the bound complex in microwells becomes a challenge either because the solution is too dilute or because a capture reagent is required that causes NSB to become a problem (a Catch 22 situation). Good solutions to NSB are urgently needed, requiring new surface chemistries and architectures, new solution additives that prevent active surfaces from being occupied by non-target proteins, and new binding schemes that are intrinsically more selective. One solution to the NSB problem is to dilute the solutions—both sample and reagent solution—so the concentration of proteins is lower and the probability that they will adsorb to the surface is reduced.

Another issue is concentration. There is a huge mismatch between the relatively large sample volumes and the extremely small volumes of microwells. The need to go from large samples to small assay volumes remains the most vexing problem. As discussed above, the total volume of microwell arrays is small so there is a need to either pre-concentrate or else have a way to distribute a large volume into many wells. If there is a large volume of a highly dilute solution, then shot noise, due to Poisson sampling errors becomes a challenge whereby distributing aliquots of the sample into microwells may not adequately represent the true concentration in the solution being sampled. Clever microfluidics can play a role here.

Multiplexing is another challenge. For many clinical or basic biology measurements, it is insufficient to only measure a single protein. With limited sample volumes available for many of these applications, it is essential to measure multiple proteins in a single aliquot. Encoded capture beads can be used for multiplexing but the number of microwells required to carry out multiplexed assays scales with the number of assays required. Incompatibilities between sample volume and the large surface areas required for high density microwell arrays create a challenge for high levels of multiplexing.

Pre-concentration is both a challenge and an opportunity. Today, the challenges for proteins are to make measurements of both low concentrations and low absolute numbers of protein molecules in small volumes. A 1fM solution contains 6×10^{-5} molecules for every 100 fL. Therefore, at least 100000 wells are required to trap a single molecule. As described above, binding reactions are incredibly slow at low concentrations. While capture beads and nanoparticles serve to soak up low concentrations of target protein, they still take time and also suffer from NSB due to the high surface areas involved. Heller and coworkers solved this problem for DNA by employing an electrode array containing bound single stranded DNA sequences^[19]. By applying an electric field, the charged complementary strands in solution were rapidly attracted to the surface. Using the electric field, specific binding could be encouraged while minimizing NSB of non-target sequences. Perhaps there is an analog of this mechanism that can be used to pre-concentrate proteins.

Affinity binding reagents are another major need. Antibodies are large, sticky molecules derived from animals or cell culture. Batch-to-batch variability plagues the research and clinical communities as the reliability and reproducibility of assays drift over time. Alternative binding reagents include single chain antibodies, aptamers, and SOMAmers^[20]—all of which are homogeneous materials.

Finally, for making measurements of single enzyme molecules in microwells, there is a tremendous need for better fluorogenic substrates for more classes of enzymes. While there are many fluorogenic enzyme substrates available, most of them are either unstable and generate high backgrounds in the absence of the enzyme, or else are sufficiently perturbed in structure that the enzymes have slow turnover numbers, making them inappropriate for single molecule studies. Creative organic chemistry, informed by knowledge of both fluorescent molecule design and enzyme structure-activity relationships will be needed to address this issue.

Future outlook

There are a multitude of prospects for improving protein measurements in microwells. Microfluidics will clearly play a major role in delivering fluids to microwells, including integrating the microwells into a fluidic device as has been recently reported.^[21] The volumes that can be manipulated by microfluidics are perfectly matched to the volume regime of microwells. An integrated solution that addresses the

size of many different microwell arrays—individual well volumes as well as the total array areas that need to be filled—would advance the field significantly.

One major opportunity is in the area of single cell analysis. In the introduction of this Insight, I mentioned how proteins were closer to the action than nucleic acids. The marriage of single cells with microwells will enable tremendous knowledge to be gleaned about cell-to-cell variability. Such analyses will be essential for solving “needle-in-a-haystack” problems such as identifying a highly invasive metastatic cell in a large background of normal cells from a biopsy. Again, microfluidics will play a significant role. Delivering individual cells to microwells, lysing the cells and manipulating the resulting lysate for analysis will be essential tools.

Proteins serve as catalysts, signal messengers, and structural components. Making protein measurements in microwells offers the ultimate in sensitivity—molecular counting. There are many opportunities for solving challenging physical and chemical limitations as well as designing creative engineering solutions for fluidic partitioning and delivery.

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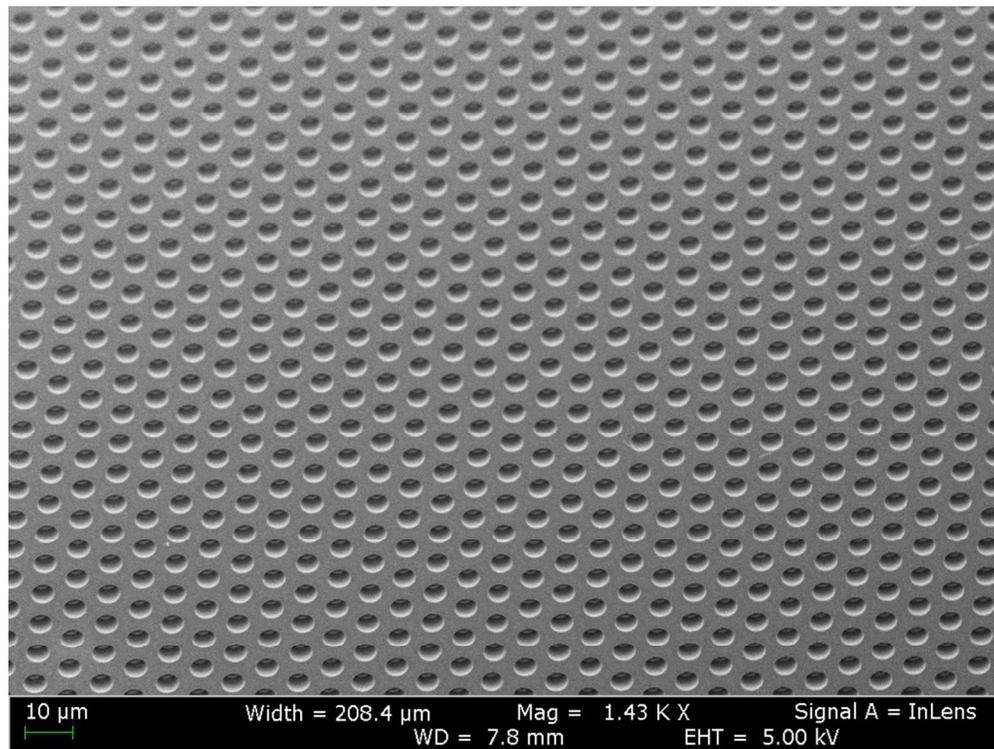


Figure 1—Scanning electron micrograph of an etched optical fiber array showing highly-ordered, high-density microwells.
26009x19507mm (1 x 1 DPI)

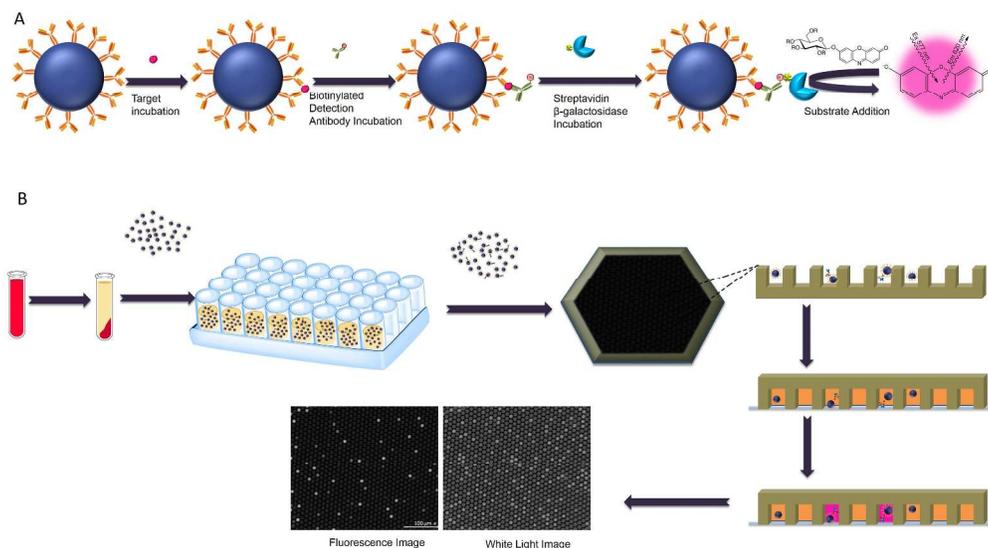


Figure 2—Schematic of Simoa Assay. A. Magnetic bead containing capture antibodies is first incubated with sample containing the target protein of interest. After washing, a biotinylated detection antibody is used to label the captured protein. Labeling of the biotin with streptavidin-labeled β -galactosidase results in a single enzyme-labeled complex on each bead containing a bound protein. Exposure to a substrate generates a fluorescent product. B. Protocol for performing digital ELISAs using Simoa. Beads are added to a blood sample containing the protein of interest. Many more beads are added than the number of target protein molecules in the sample such that most beads contain zero molecules while some beads contain only one bound protein molecule. The remaining steps are the same as in A. Beads containing a bound protein molecule carry a β -galactosidase label. The beads are then loaded onto an optical fiber microwell array and sealed with a substrate solution. Enzyme-labeled beads generate a detectable fluorescence signal while the beads containing no target protein remain colorless. Bottom left shows both white light and fluorescence images of the wells.

664x371mm (150 x 150 DPI)

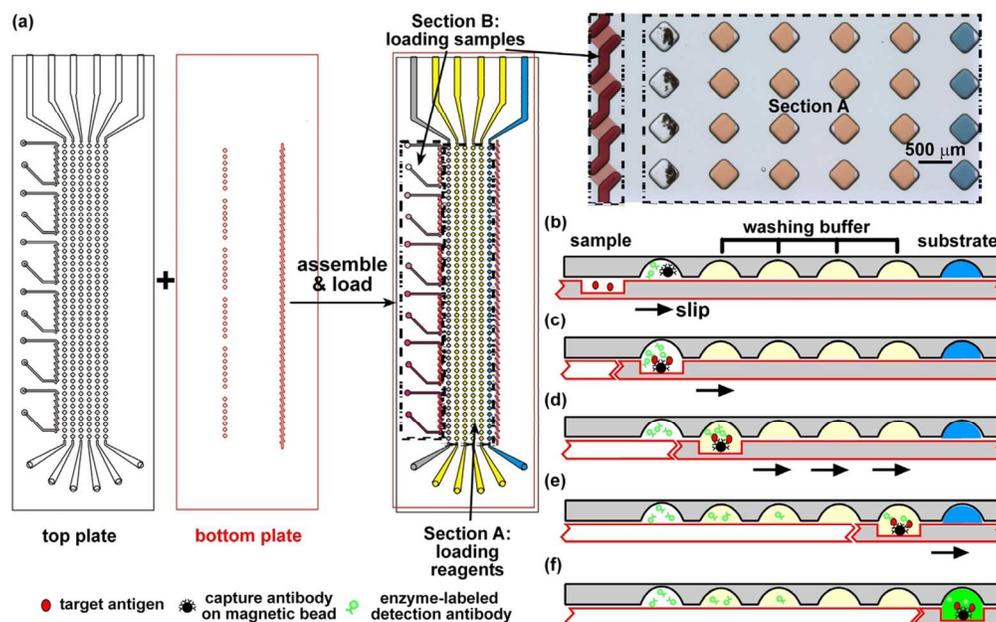


Figure 3— Performing heterogeneous immunoassays with multiple nanoliter samples in SlipChip. (a) A schematic of the SlipChip designed for calibration on the two plates of microfabricated glass. The top plate of this SlipChip (outlined in black) contained inlets, outlets, and wells for the various reagents (section A) and inlets, outlets, and ducts to load the samples: six standard solutions (section B). All wells and ducts were 80 μm deep. The bottom plate (outlined in red) of this SlipChip contained the 80 μm deep ducts to load the reagents (section A) and 10 μm deep wells for the sample (section B). The two plates were assembled to form the fluidic path for loading the reagents and samples. In section A, the wells were loaded with reagents. The gray wells of row 1 were loaded with the solution containing magnetic beads coupled with the capture antibody and an enzyme-labeled detection antibody. Wells in rows 2–5 (yellow) contained the washing buffer. Wells in row 6 (blue) contained the substrate. Section B was designed to load six samples into seven wells each. A microphotograph on the right shows the wells filled with different dye solutions (rows 2–6 of sections A, and section B) or a suspension of beads (row 1 of section A). (b–f) Schematics of step-by-step operation of the bead-based immunoassay in SlipChip. Reprinted with permission from the

American Chemical Society, Reference 8.

109x68mm (300 x 300 DPI)

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