Quantitative Detection of Protein Arrays

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Quantitative Detection of Protein Arrays

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We introduce a quantitative method that utilizes scanning electron microscopy for the analysis of protein chips (SEMPC). SEMPC is based upon counting target-coated gold particles interacting specifically with ligands or proteins arrayed on a derivative microscope glass slide by utilizing backscattering electron detection. As model systems, we quantified the interactions of biotin and streptavidin and of an antibody with its cognate hapten. Our method gives quantitative molecule-counting capabilities with an excellent signal-to-noise ratio and demonstrates a broad dynamic range while retaining easy sample preparation and realistic automation capability. Increased sensitivity and dynamic range are achieved in comparison to currently used array detection methods such as fluorescence, with no signal bleaching, affording high reproducibility and compatibility with miniaturization. Thus, our approach facilitates the determination of the absolute number of molecules bound to the chip rather than their relative amounts, as well as the use of smaller samples.

The DNA array field provides important insight into *relative* mRNA transcription levels between different cell states and growth conditions, yet absolute quantification of gene expression remains unanswered.[1,2] In addition, transcription levels provide only indirect information concerning the final output of the biological system, i.e. the proteins, whose levels are influenced by mRNA stability, posttranslational modifications, protein stability, etc.[3] Several recent reports have demonstrated the feasibility of protein microarrays for a variety of applications.[4−7] Such arrays are required to produce highly sensitive, quantitative, and reproducible measurements of protein levels.[8−10]

We herein propose a strategy for quantification of chip technology based upon the particulate nature of gold bead markers and on the ability of the scanning electron microscope (SEM) to accurately detect these particles (Figure 1). We demonstrate how the quantitative capabilities of our approach can be used to elevate the accuracy and readout sensitivity of protein chips. The generalization to DNA chips is straightforward, yet we believe that the automated, high-resolution detection capability of small quantities[2,8,11,12] will eventually have more impact on protein than on DNA chips. This is largely due to the fact that sample size is a crucial issue in protein analysis, in contrast to the advantage PCR offers DNA analysis.

In a protein chip, a solution of interest (e.g., cell lysates) is probed for a specific molecule M by placing its cognate partner P on the chip's surface. The conjugation process extracts M out of the fluid with an efficiency that can be measured by the fraction α of M that binds to its cognate, immobilized partner P. The P−M complex is detected with an efficiency β via further reactions with a labeled probe (Figure 1). Measurement of α and β are fundamental for a quantitative detection of the chip and arise from the basic steps of the SEMPC (Figure 1).

The immobilized molecules and the molecules in the bulk solution interact within an effective volume determined by the diffusion coefficient, the hybridization reaction time, and the area on the chip where the immobilized molecules are attached. Thus, for a given concentration of molecules in the bulk (M in Figure 1), there will be m_V molecules in the hybridization volume (see below) that can interact with the cognate partner molecule P immobilized on the spot of the chip. The number of P−M pairs that will form at the surface is $m_S = m_V\alpha$, as there are more molecules immobilized to the chip surface than molecules in the bulk so that saturation will not occur and we retain the quantitative capability. The efficiency α typically incorporates a number of intermediate factors ($\alpha = \alpha_1 \times \alpha_2 \times ... \times \alpha_\nu$). It can depend, for example, on the preparation of the slide and its coverage by reactive groups (α_1), on the efficiency of the further coverage of the substrate by the provided partner P (α_2), and on K_D.

The conjugated P−M pair is detected with efficiency β by attaching a gold-labeled probe to M. The attachment may be

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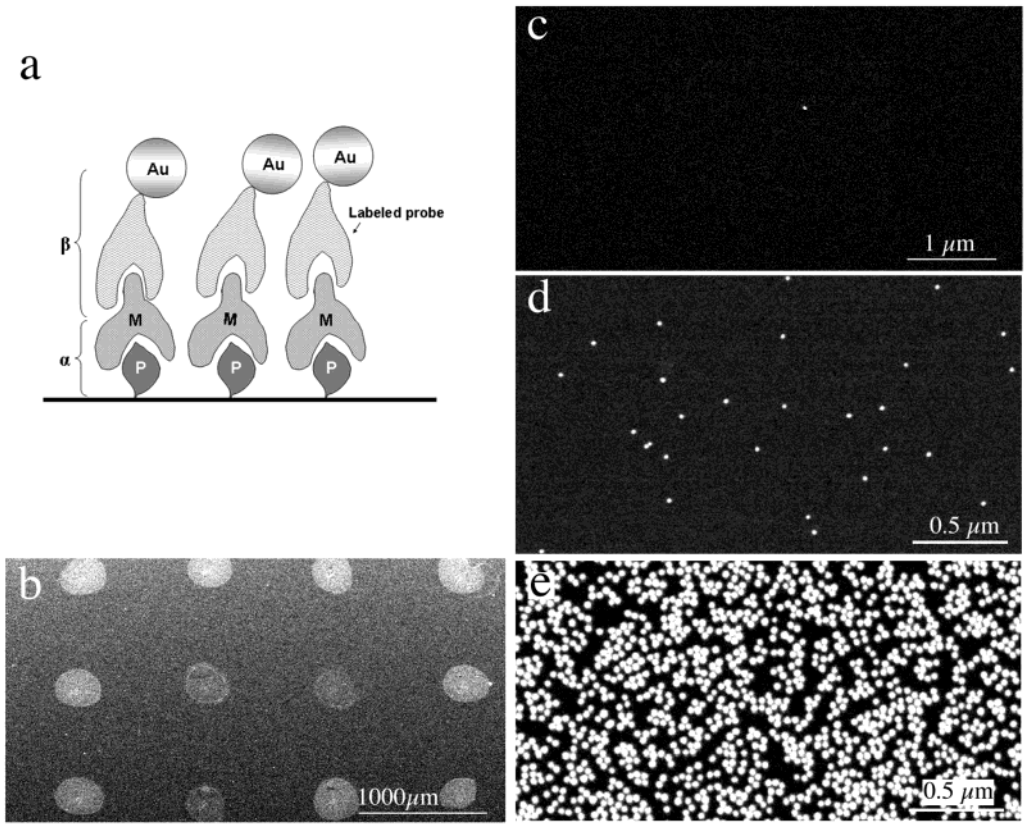

Figure 1. (a) Schematic illustration of the experimental system, depicting the two coefficients being measured. A specific molecule M is probed by placing its cognate partner P on the chip. M is attached to the surface with efficiency α. M is then detected by the gold probe with efficiency β (drawn out of scale for clarity). (b) Low-magnification SEM image of the spots in the b-BSA chip that was probed with 20-nm gold colloids conjugated to streptavidin.The bright spots were spotted with 15 μM b-BSA. The three fainter spots in the middle are the flag, which were spotted with 1.5 μM b-BSA. The flag spots remain visible also when the rest of the array is at low concentration and are therefore hard to discern at this magnification. Some of the inherent noise that arises from the Biorobotics TAS arrayer is apparent in the inhomogeneity of the spots. (c) High-magnification image of a background control spot where only BSA was spotted (no b-BSA). (d) High-magnification image of a spot where the ratio between b-BSA and BSA was 1:10000 (0.0015 μM b-BSA was spotted). (e) High-magnification image of a spot where the ratio between b-BSA and BSA was 1:10 (1.5 μM b-BSA was spotted).

achieved by a variety of interactions; in our case, we biotinylate M prior to exposing it to the chip and use streptavidin-coated gold colloids. In general, α, which is the more important process, will depend on the system used and on its biochemical details and will exhibit some noise inherent to the biological system. On the other hand, β is a more universal feature of the measuring technique that will depend primarily on the efficiency of the biotinylation process and on the interaction between the gold-conjugated protein and its ligand. The number of molecules detected is therefore $m_D = m_S\beta = m_V\alpha\beta$.

As the efficiency of the full process is determined by the product $\alpha\beta$, we present below two experiments, each designed to quantify a separate set of these coefficients for the SEMPC system, turning it into a fully quantitative assay. We first describe a simple procedure for measuring β, using a model system based on the streptavidin−biotin interaction that controls the number m_S of cognate pairs attached to the surface. These results are distinctly better than those we obtained using a commercial fluorescence tagging and scanning detector technique. We then demonstrate the high resolution and the low noise in a more realistic hapten−antibody system that enables us to measure the product $\alpha\beta$.

MATERIALS AND METHODS

Chemical Modifications. Biotin-BSA (b-BSA) was prepared as follows: biotinamidocaproic acid *N*-hydroxysuccinimide ester (Sigma; 1.9 μmol in DMF) was added to BSA (75 nmol in 0.1 M $NaHCO_3$) and the resultant mixture stirred 3 h at 4 °C followed by extensive dialysis in 20 mM phosphate buffer + 150 mM NaCl (PBS). A total of 12 ± 4 biotin molecules was conjugated per BSA molecule as assessed from a sandwich assay using streptavidin−peroxidase conjugate (Sigma) on a streptavidin-coated microtiter plate (Nunc) (data not shown). Purified monoclonal antibodies D2.3 (65% specific activity as measured by fluorescence quenching titration)[13] and anti c-Myc (clone 9E10 from Sigma) were conjugated to biotin (b-D2.3 and b-Myc, respectively) as above. *p*-Nitrobenzyl phosphonate *N*-glycylglutarate (h) synthesis and its conjugation to BSA (h-BSA) were described previously.[14]

Protein Array Preparation. A total of 256 spots was spotted on aldehyde slides (Telechem International, SuperAldehyde Substrates) using a Biorobotics TAS arrayer, arranged as 16 4 × 4 subarrays. Each spot contained ~15 nL of BSA, b-BSA, or h-BSA (40% glycerol in PBS at pH 7.5) and covered an area of $\sim 10^5$ μm^2.

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A triangle of three spots in each subarray, usually in the corner, was spotted with b-BSA (0.1 mg/mL), creating gold-rich "flags" for detection and alignment of the slide in the SEM, and one was spotted with only BSA (1 mg/mL) to serve as background control. b-BSA was mixed at varying ratios (1:10^6−1:1) with nonlabeled BSA and spotted while keeping the total BSA concentration at 15 μM. h-BSA was spotted from a 3 μM solution. The spotted areas on each slide were separated by drawing paraffin lines (PAP PEN, Daido Sangyo Co.). In the h-BSA slides, subareas were created within the spotted area by drawing additional paraffin lines between the subarrays. The spotted slides were incubated in a humid chamber for 2−3 h in room temperature. A 10 mg/mL BSA solution in PBS solution was used to rinse the slides and to block remaining aldehyde groups by immersion at ambient temperature with gentle agitation for 1 h. The slides were then briefly washed in PBS. The b-BSA-spotted slides were used without further manipulations to be probed by the streptavidin-coated gold colloids or the fluorescently labeled streptavidin. The h-BSA-spotted slides were submitted to a further step of hybridization with the cognate antibody or controls (see below) before being probed with the streptavidin-coated gold colloids.

Antibody Capture. Drops of antibody b-D2.3 (20 μL) in TRIS, pH 8.3, containing 20% glycerol and 1 mg/mL BSA (1.3−1.3 × 10^5 pM) were applied to the subareas separated by the paraffin lines in the h-BSA slide. Following 3-h incubation at ambient temperature in a moisture chamber, the slides were rinsed three times in PBS + 0.04% Tween 20. As controls, nonbiotinylated D2.3 and a nonrelevant biotinylated antibody (b-Myc) were used under the same conditions.

Probing Slides with Streptavidin-Coated Colloids. Streptavidin-coated gold colloids of 20- or 40-nm diameter (British Biocell International) were washed by repetitive cycles of centrifugation at 4 °C (20 min.; 15000*g*) in PBS supplemented with 0.04% Tween 20 and 1 mg/mL BSA (twice) and in the same buffer only replacing Tween 20 with 20% glycerol and 0.5 M NaCl. The beads (15 nM) were resuspended in the latter buffer and used for probing the arrayed slides.

A 40-μL aliquot of the above colloids was applied to each printed area for 4 h in a humid chamber at ambient temperature. Following the incubation, the slides were washed three times in PBS + 0.04% Tween 20 (3-min incubations). In some cases, the slides were also immersed in a fixation solution (3% paraformaldehyde, 2% glutaraldehyde in ddH_2O; 30 min). The slides were then washed in ddH_2O (10 min), spun (1000*g*; 5 min.) to remove access liquid, and dried in a vacuum chamber overnight. Finally, the slides were cut into 1-in.^{2} sections to fit on the microscope's mount using a diamond glasscutter and coated with a 200-μm carbon coat using Edward's carbon coater, and their edges were colored with conducting silver paste **(**Ted Pella Inc**.)** to prevent charging of the samples. The fixation, carbon coating, and cutting steps were omitted once we moved to work with a newly installed Philips ESEM.

The gold-probed slides were visualized with a SEM (JEOL 6400 or Philips ESEM XL30). Typically, five images of 6.25−48-μm^2 frames inside each spot were taken via a backscattered electrons detector. Gold colloids were counted for each image using NIH Scion Image processing software. The number of gold colloids per spot was calculated as the average number of gold colloids in a frame multiplied by the number of frames per spot of $r = 200$ μm.

Fluorescent Probing of Slides. The slides were processed according to McBeath and Schreiber.[4] Briefly, 40 μL of Cy3-conjugated streptavidin (Amersham; 17 nM in PBS containing 20% glycerol, 1 mg/mL BSA) was incubated at ambient temperature (4 h), followed by washing and centrifugation as described above for the gold colloid slides treatment. The Cy3-probed slides were scanned at 5-μm resolution using Packard ScanArray 4000. The intensity of the spots was determined in two ways. In the first, the standard Quantarray software was used. However, manual analysis of the intensity of the spots that corresponded to the spots analyzed in the gold-probed slides, using the NIH Scion Image processing software, resulted in better fluorescence signals and was therefore used for subsequent analysis.

Determining the Concentration of Surface-Bound Proteins. A radioactive assay[15] was conducted to evaluate the number of BSA molecules conjugated to the SuperAldehyde slide's surface, using trace [^{132}I]BSA (2.2 million cpm mL^{-1}) mixed with unlabeled BSA (0.03−1 mg/mL). Using the same binding and washing protocol as above, the number of BSA molecules per unit area was measured to be (1.5 ± 0.3) × 10^{12} molecules/cm^2 or 1.9 × 10^9 BSA molecules/spot ($r = 200$ μm) (average over three independent experiments using concentration of 0.2−1 mg/mL BSA; data not shown). The BSA coverage is ∼30% of the aldehydes' capacity of the glass slide (5 × 10^{12} reactive group/cm^2 as measured by the manufacturer).

Estimating the Number of Molecules in the Hybridization Volume. m_v, the number of molecules in the bulk solution available for binding the chip-immobilized molecules, was calculated using the diffusion equation for a disklike adsorber of radius r in a semi-infinite medium.[16] Given the diffusion current, $I = 4DrA[\mathrm{M}]$, where D is the diffusion coefficient, [M] is the molar concentration of molecules in the bulk,[16] and A is Avogadro's number (6.02 × 10^{23}), then taking t for the hybridization time yields

$$m_V = It = 4DrA[\mathrm{M}]t = V_{\mathrm{eff}}A[\mathrm{M}]$$

(V_{eff} is hybridization volume)

In our experimental setup, $D_{\mathrm{antibody}} = 3.8 \times 10^{-7}$ cm^2/s,[17,18] $r = 200$ μm, $t = 3$ h, and [M] is the overall antibody concentration in the droplet, resulting in

$$V_{\mathrm{eff}} = 330\ \mathrm{nL} \quad \text{and} \quad m_V = 1.98 \times 10^{17}[\mathrm{M}]$$

RESULTS

Biotin−Streptavidin System. (1) Effect of Colloid Size. In the first set of experiments, a gradient of biotin-BSA concentrations in constant overall concentration of BSA was coated on the glass surface (with repeats within each subarray). Tagging with the 20-nm streptavidin-coated gold colloids (STP20) exhibited ∼4-fold

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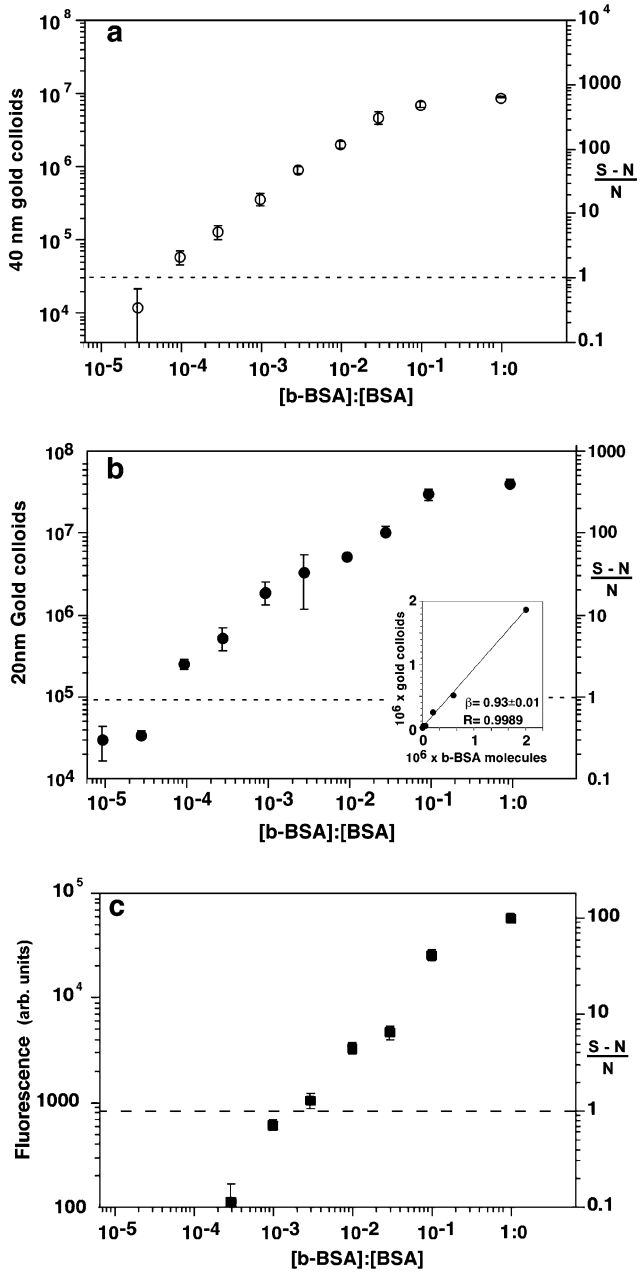

Figure 2. Streptavidin−biotin system**.** Detected signal as a function of the ratio between b-BSA and BSA in a spot. For gold-probed slides, the detected signal is m_D, the number of gold colloids detected in the spot, minus the number of colloids detected in the control spot (N). For the fluorescence slide, the signal is the average pixel intensity in the spot (I) minus the average pixel intensity in the BSA control spot (N). The signal-to-noise ratio is defined as $(S - N)/N$ with $S = m_D$ for gold and $S = I$ for fluorescence. Dashed line represents $(S - N)/N = 1$, a common criterion[30−32] of 2-fold change in signal as a confidence limit for detection. (a) Results for the 40-nm gold probes. (b) Results for the 20-nm gold probes. Inset in (b) presents m_D, the detected number of molecules as a function of m_S, the expected number of molecules (see Materials and Methods for calculations). The slope gives the efficiency of detection β. (c) Results for the fluorescence Cy3 probes.

improvement of the detection signal and dynamic range, as compared with the 40-nm colloids (STP40) (see Figure 2b vs 2a). Since the largest number of colloids able to pack closely in a given area limits the upper detection limit, we expect that decreasing the size of the colloids will further extend the dynamic range in its upper limit. Judging from the current literature,[19] smaller gold colloids may have better binding properties, suggesting the use of smaller colloids as a way to further improve the sensitivity and extend the lower limit of the dynamic range.

Moreover, by careful normalization, it will be possible to carry out concurrently relative measurements of a variety of protein samples on the same array spot using different sizes of colloids as well as combinations of different heavy metal colloids,[20,21] thus increasing the versatility and applicability of the proposed system.

(2) Gold versus Fluorescence Tagging. The difference between gold- and fluorescence-tagged probes is demonstrated in Figure 2. Using the gold probes (STP20) extended the dynamic range where signal scales linearly with protein concentration by a factor of 20 and the sensitivity of detection by ~50-fold relative to the fluorescent probes. A rough estimate for the reproducibility is the coefficient of variation (CV), the ratio of the second moment of the distribution of m_D to the first moment, measured over slides in all the different experiments we conducted. We obtained significantly better reproducibility with SEMPC (CV = 0.20) vis à vis fluorescence probing (CV = 0.47). Our results are comparable to CV values of DNA chip fluorescence, typically ranging between 0.2 and 0.7,[22,23] even though our protein chip involves an additional hybridization step.

A further important point is that decreasing the spot radius r will have no effect on our results, while it significantly decreases (essentially like r^2) the resolution of fluorescence-based measurements.

(3) Measuring *β*. The efficiency of detection β (see Figure 1) is the ratio between m_D, the number of molecules detected by the gold-tagged probe in the SEM, and m_S, the number of molecules actually attached to the surface. To control and vary m_S in a precise manner, we added a competing molecule that limits and controls the fraction of M that actually binds to the surface. Biotinylated BSA (b-BSA), representing M, was mixed with nonbiotinylated BSA to ensure that the total number of BSA molecules is kept constant and large, while the ratio of biotinylated to nonbiotinylated BSA is varied. When solutions with b-BSA + BSA were spotted on the aldehyde-coated glass slide, the excess of BSA over the active surface−aldehyde molecules, ensured saturation and a uniform coating of the surface. The relative amount of biotinylated BSA in each spot is determined only by the initial ratio in the bulk fluid. This renders the process independent of the BSA−aldehyde conjugation efficiency, of the coverage of the substrate by aldehydes, and of all other factors (notably the cognate M−P interaction mentioned above) that make up α. This procedure produces an array of spots that simulates a range of different α cases, each spot containing a different number m_S of b-BSA. These b-BSA molecules could now be probed with gold-tagged streptavidin and detected with a SEM to obtain m_D (Figure 2a and b). Since m_S is determined by the protocol and m_D is measured, the efficiency of detection β is the slope presented in the inset of Figure 2b.

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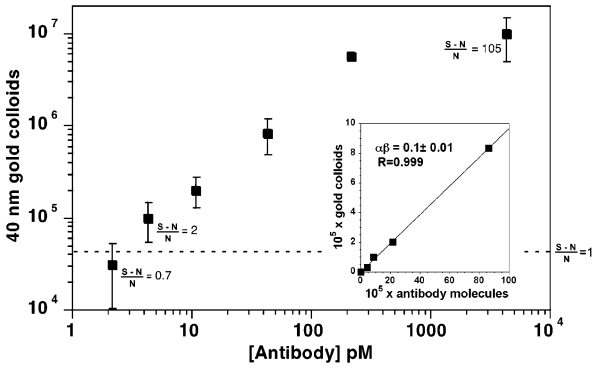

Figure 3. Hapten−antibody system. Detected signal in a spot as a function of antibody concentration. Detected signal is defined as m_D, the number of gold colloids detected in a spot minus the average number of gold colloids detected in the control spots (N). Controls were hybridized either with the nonbiotinylated antibody or with a nonrelevant biotinylated antibody (see Materials & Methods). Signal-to-noise ratio was defined as in Figure 2. Dashed line represents $(S - N)/N = 1$, or 2-fold change in signal. Inset presents the detected number of molecules m_D as a function of the expected number of molecules m_V in the hybridization volume (see Materials and Methods for details). The slope gives the efficiency of detection $\alpha\beta$.

Using the measured coverage of BSA molecules on the glass slide (see Materials and Methods), we obtain an efficiency $\beta \approx 1$ (full detection) for STP20 and $\beta \approx 0.2$ for STP40. It seems clear that we are reaching *quantitative* molecular counting detection within the limits of our linear dynamic range.

Hapten−Antibody System. To demonstrate the SEMPC's sensitivity to low analyte concentrations while determining $\alpha\beta$, we used a second model system, based on a "sandwich" assay. The analyte protein of interest M (a biotinylated antibody) is bound by both an immobilized capture ligand P (hapten-BSA, h-BSA) and a soluble, gold-labeled ligand (streptavidin). Advantageously, a saturated level of the universal labeling ligand (e.g., streptavidin) can be used in all systems, while the proteins of interest need only be biotinylated. In our model system, h-BSA was spotted on the glass slide and then hybridized with different concentrations of its cognate biotinylated D2.3 monoclonal antibody (M). The hapten−antibody complex was then probed with gold-tagged streptavidin (STP40). Results for the hapten−antibody system are shown in Figure 3. Antibody concentrations as low as 5 pM could be detected with a 2-fold higher signal relative to noise. On the basis of our streptavidin−biotin results (see also ref 19), we expected to obtain a significant advantage by using STP20, but in fact, the signal improvement was small while the signal-to-noise ratio remained somewhat better for STP40 (data not shown). It is possible that the advantage of STP20 is reduced because the antibody anchor/spacer facilitates access of STP40 to the biotin marker.

(1) Measuring $\alpha\beta$. To calculate $\alpha\beta$, we plotted the detected number of molecules m_D against the number of molecules in the bulk m_V (see Figure 3, inset). m_V is derived from the known concentration of the antibody, [M], and the hybridization volume as described in Materials and Methods. The slope of the linear regression yields $\alpha\beta = 0.1$. While we do not know β precisely for this system, we can approximate it using the results of the biotin−streptavidin system (Figure 2; $\beta \approx 0.2$ for STP40). Here the value may be comparable, resulting in an estimate of $\alpha \approx 0.5$. In principle, $\alpha \approx 1$ could be expected since the K_d is 4 nM and the concentration of the surface-bound h-BSA is 100 nM (calculated using the hybridization volume, molecular coverage of BSA on the surface, and ~10 hapten molecules/BSA). The departure from unity may be due to an overestimation of β or to a reduction in the binding efficiency caused by the heterogeneous conjugated surface, which may prevent some of the antibodies from binding the h-BSA.

DISCUSSION

The advantages of using the SEM as a detection method for the chip are mostly because of the quantification capabilities, but also because it enables elimination of the major components of the background noise signal. This becomes apparent when compared with other array detection methods such as fluorescence. The backscattered imaging mode provides the ability to distinguish between elements of high atomic number (e.g., gold, silver, and titanium) and elements of low atomic number (e.g., various salt deposits). Thus, two major sources of noise typical of biochips are eliminated. First, the substrate noise—autofluorescence in fluorescence techniques or surface roughness in the AFM[24]—has little to no impact on the SEM detection. Second, salts accumulating on the substrate from the different buffers do not disturb the SEM, while they do emit autofluorescence and are hard to distinguish from the gold particles in the AFM. Using the SEMPC, we are left to deal only with noise due to nonspecific binding of the gold-tagged probe to the chip. This can be addressed by further optimization of the chip's surface[25] (e.g., different 'blocking' reagents), the hybridization solutions, and the washing solutions.

SEMPC does not require a new protocol for the preparation of the chip. The use of a probe that is labeled with gold and not with fluorescence requires small changes in the standard protocol (see, for example, MacBeath et al.[4] and Haab et al.[5]) such as an increase of hybridization time and addition of salts to reduce ionic attractions. While the manual manipulation of the SEM, as carried out in this study, is more cumbersome than using the commercially available fluorescence array scanners, high throughput and automation of the SEM was implemented with a high degree of success by the semiconductor industry.[26] Similar advances could greatly enhance the productivity and efficiency of SEMPC, making it as easy to use as current array scanners. The preparation for the SEM is in fact independent of the type of buffers and materials used, and there is only a weak dependence on the "hands" that prepare the sample so that higher reproducibility can be obtained. Our results indicate that reproducibility for gold is more than a factor of 2 better than that of fluorescence.

Recently, labeling with gold has been extensively used.[12,21,24,27−29,32] The advantages of gold labeling are numerous, including quantification, localization, repeated reading, storage,

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and easy preparation. Since the area scanned by the SEM is typically 10−50 μm^2, our detection method is compatible with further miniaturization of the chip.[11] This is in stark contrast with fluorescence techniques, whose integrative measurement deteriorates linearly with the area.

Molecular counting capability is an outcome of the particulate nature of the gold colloids. If only one molecule were attached to each gold particle, we would be assured that each gold bead we detect in the SEM signals one molecule. Since that is usually not the case, we cannot be assured of a one-to-one correspondence between colloidal particles counted and molecules on the substrate unless the molecules are spaced on the substrate at distances that are larger than the colloid size. Indeed, we found that in the large range of lower concentrations where this criterion is met (more than 2 orders of magnitude) a linear ratio of detected-to-expected molecules exists. Experimental interest is often exactly in this lower concentration region. This range may be further extended by the use of smaller gold colloids.

Beyond the technical advantages of SEMPC, its higher resolution and enhanced dynamic range, the ability to fully quantify the results of chip analysis opens a fundamentally different set of questions that can be addressed. Currently used chip techniques yield only integrated light-intensity measurements and enable detection of only relative amounts of molecules in solution.[9] Using the SEMPC, it is possible to assess the absolute amounts, reducing the need for comparison assays. We have further shown how to separate the detection process of the chip into two steps and to quantify their efficiency experimentally. Beyond this, each of the intermediate steps that constitute these two steps may further be examined and quantified. In the future, questions regarding the number of copies of a given molecule that exist in a cell lysate may also be answered.

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