Electronic Supplementary Material (ESI) for Lab on a Chip

Supplementary Information:

Scaling Advantages and Constraints in Miniaturized Capture Assays for Single Cell Protein Analysis

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Keywords: single cell proteomics; dip pen nanolithography; immunoassay; miniaturization; microfluidics

Supplementary Information

Cell Culture & Preparation

The BE colon carcinoma cell line was used as previously described in addition to the breast cancer cell line MCF7.[1] The cells were cultured using Dulbecco's Modified Eagles Medium (DMEM; Invitrogen, UK) supplemented with 10% (v/v) foetal bovine serum (FBS; Sigma, UK). Cells that were used in experiments were detached from culture flasks using accutase (Invitrogen, UK), which was inactivated by suspension in L-15 medium (Sigma, UK) supplemented with 10% FBS. L-15 medium allows cells to remain viable outside the incubator for a longer period of time. A suspension of single cells was created by gentle agitation with a pipette before sieving through a nylon mesh of 40μm pitch (BD Falcon, UK) to remove any cell clumps.

Surface Derivistisation

Coverslips were surface derivatised as in [2]. Briefly, microscope coverslips were cleaned by sequential sonication in 1M KOH, acetone and 1M KOH with subsequent rinse and sonication in ultrapure water after each step. Coverslips are rinsed in H_2O, dried by nitrogen then immersed in APTES in 5% acetic acid in methanol for 30 min. Coverslips are rinsed in methanol followed by H_2O and dried by nitrogen. PEGylation solution is prepared by mixing mPEG and mPEGbiotin in 100 mM sodium bicarbonate buffer. Silanated coverslip pairs are sandwiched with 80 μL of PEGylation solution and incubated in a sealed humid chamber for 3 hours. Coverslips are disassembled, washed copiously with H_2O, dried and stored under nitrogen. Immediately prior to printing, PEGylated coverslip pairs are sandwiched with 80 μL of 0.1mg/mL neutravidin in MQ H_2O for 30 mins. The background when using this functionalisation is less than 0.003 molecules/μm^2 (less than 50 molecules per 137 μm × 137 μm field of view).

DPN Printing

DPN probes (**figure S1**) are fragile and susceptible to damage if excessively overdriven into the substrate surface. Prior to printing the z-plane of the coverslip surface is determined by manually driving the probes until contact is observed. This may be performed for each of the 25 locations where antibody spots will be printed per chip. However, a z-plane requires 3 enclosing coordinates and can be excessively time consuming to complete for all spot locations. Instead, z-planes were completed per 5 spot locations which spans approximately 5 mm.

Given a particular substrate chemistry, spotting solution and probe contact force, the printed spot size is determined by the probe dwell time on the surface and number of 'overprints' i.e. number of repeated prints at the same spot coordinate. It is observed that spot size increases with increased dwell time and more print repeats whereas spot variation decreases with more print repeats.

Microfluidic Device Fabrication

The single cell analysis MAC chip was fabricated using SU-8-based soft-lithography techniques for PDMS. PDMS was mixed at a ratio of 10:1 precursor to curing agent and poured over the SU-8 mould before being degassed in a desiccator chamber and set

to cure at 60 °C for 2+ hours. The Si-wafer surface was passivated by vapour-deposition of (tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane (Sigma-Aldrich, UK) under vacuum to enable easy lift-off of cured PDMS. The cured PDMS was removed from the mould and 300 μm access holes were drilled (Diama, UK). The PDMS is exposed to air plasma for 1 minute before the microchannels are sealed by a coverslip (thickness #1.5; Corning, UK) upon which antibody spot have been printed in defined locations using the Omnigrid or NLP2000 DPN arrayer. The antibody microspots are aligned using a home built translation stage. Plasma treatment destroys surface functionalisation and the antibodies so only the PDMS surface is plasma treated to enhance bonding. The bonded device is incubated at 4 °C in a sealed chamber overnight before use.

Single Molecule Counting

The number of proteins captured within one chamber are counted using the methods previously described.[1] Single molecules are detected by identifying 4-9 clustered pixels, with circularity greater than 0.5 and with pixel intensities at least 3 times the background standard deviation plus its mean value. When protein copy number is high, bound proteins become congested and the single molecule images overlap and are individually indistinguishable. In this regime, the number of proteins captured can be estimated from dividing the image intensity by the known average intensity of a single molecule.

Capture Assay Model

By modelling the behaviour of the sandwich immunoassay used in a MAC chip it is possible to determine how performance scales as a function of spot radius and chamber volume. The performance of an immunoassay may be characterised by the parameters of sensitivity, dynamic range and signal to noise ratio. With reference to **figure S2**, the model immunoassay comprises a primary antibody (Ab_1), which binds its antigen (Ag) with affinity K_{D1}, which in turn binds a detection antibody (Ab_2) with an affinity K_{D2}. A single cell will contain many non-antigenic species (X) that may bind Ab_1 and/or Ab_2 with an average affinity K_{D1X} and K_{D2X}, respectively, and contributes to the immunoassay as background.

All of the measurements are made at equilibrium and therefore the calculations presented here all reflect the levels of binding expected at equilibrium. It is, however, convenient to evoke these equilibria as ratios of separable rate constants. The differential equation for the formation of the trimolecular primary antibody-antigen-detection antibody species is

$$\frac{d}{dt}\left[Ab_1AgAb_2\right] = k_{2,on}\left[Ab_1Ag\right]\left[Ab_2\right] + k_{1,on}\left[Ab_1\right]\left[AgAb_2\right] - \left[Ab_1AgAb_2\right]\left(k_{1,off} + k_{2,off}\right) \quad \text{(S1)}$$

where Ab_1AgAb_2 is the concentration of the trimolecular primary antibody-antigen-detection antibody species; Ab_1Ag and $AgAb_2$ are the concentrations of the bimolecular species of antigen bound to the primary and detection antibodies, respectively; and $k_{1,on}$ (10^6 $M^{-1}s^{-1}$), $k_{1,off}$ (10^{-3} s^{-1}), $k_{2,on}$ (10^6 $M^{-1}s^{-1}$) and $k_{2,off}$ (10^{-3} s^{-1}) are the on and off rates for the primary and detection antibodies to the antigen. The rate constants are typical of those for a high on-rate antibody having a K_D of 10^{-9} M and all of our modelling is done for antibodies with nanomolar K_Ds.[3,4] Nevertheless, it is easy to extrapolate the results of the model for antibodies of differing affinity.

Similarly, differential equations may be constructed for the intermediate bimolecular species Ab_1Ag and $AgAb_2$. The model also allows the formation of non-antigenic bimolecular (Ab_1X and XAb_2) and trimolecular (Ab_1XAb_2) species as a simple assessment of non-specific background binding. The hypothetical average on and off rates for the primary and detection antibodies to the non-antigens are $k_{1X,on}$ (10^2 $M^{-1}s^{-1}$), $k_{1X,off}$ (10^{-2} s^{-1}), $k_{2X,on}$ (10^2 $M^{-1}s^{-1}$) and $k_{2X,off}$ (10^{-2} s^{-1}). An additional background contribution is made by antibody cross-talk (Ab_1Ab_2) with the rate constants $k_{12C,on}$ (10^3 $M^{-1}s^{-1}$), $k_{12C,off}$ (10^{-2} s^{-1}), which is estimated from experiment.

For the calculations which follow we take the following parameters. The typical protein content of a cell is approximately 10 % (w/w).[5] A typical mammalian cell has a radius of 5-7.5 μm and a volume of 0.5-1.8 pL, which, assuming an average molecular mass of 30 kDa, suggests a total protein content on the order of 10^9 proteins.[6] The level at which individual proteins are expressed in a cell can vary between 1 and 10^5 for low and high abundance proteins, respectively.[7] The number of primary antibodies is the product of the antibody density and the immunospot area while the number of detection antibodies is chosen to such that there is a minimum of 10^6/chamber and are in excess of the number of captured molecules. The model assumes the reaction is not diffusion limited and that equilibrium has been reached in the chamber.

The signal to background noise ratio (SNR) is the ratio of target proteins bound to non-target proteins bound due to the finite specificity of the antibodies. In fact one of the key reasons for using a sandwich assay is that it greatly improves this signal to noise since the overall specificity becomes the product of the specificities of both antibodies used. The SNR can therefore be calculated by

$$SNR = \frac{N\left(Ab_1AgAb_2\right)}{3\sqrt{N\left(Ab_1XAb_2\right)+N\left(Ab_1Ab_2\right)}} \qquad \text{(S2)}$$

where N(x) denotes the number of species x present in the chamber volume. The minimum detectable signal is the sensitivity of the immunoassay and is determined to be when the SNR > 1. The factor 3 in the denominator is to reflect the need for the signal to be three standard deviations above background to provide a confidence of 99.7%. In order to determine the absolute number of protein species within a single cell, it is beneficial for the immunoassay to operate within the mass sensing regime, where the vast majority of the analyte is bound, as opposed to the ambient analyte regime.[8] The fraction of antigen bound at equilibrium is given by

$$B_{Ag} = \frac{N\left(Ab_1AgAb_2\right)}{N\left(Ag\right)_T} \qquad \text{(S3)}$$

where $N(Ag)_T$ is the total number of antigens present in the chamber volume. Unless otherwise stated, when discussing B_{Ag} the total number of antigens in the chamber is 10^4.

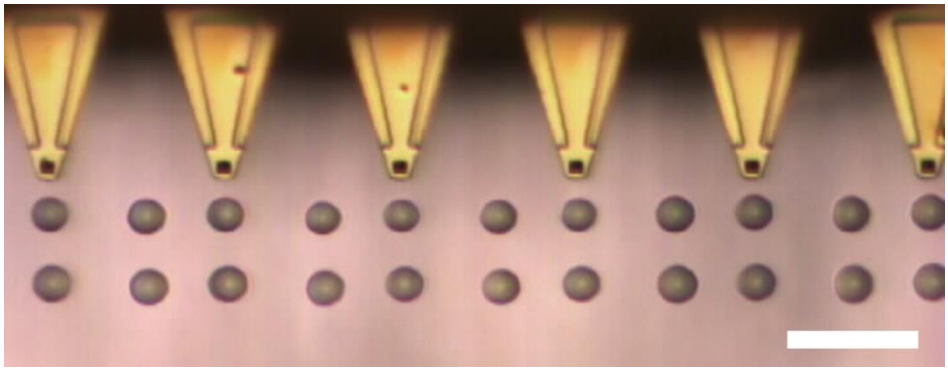

Fig. S1 Image showing a few DPN cantilevers from a 12-probe array and the deposited antibody spots (scale bar 50 μm).

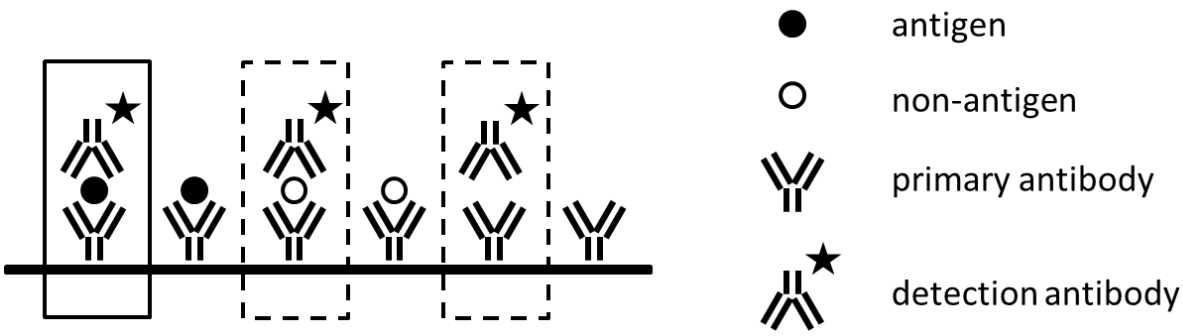

Fig. S2 Schematic of trimolecular antibody binding model with noise showing the permutations of primary antibody trimolecular species. Additionally, antigen, non-antigen and detection antibody may remain free in solution. The species enclosed by the solid and dashed-line rectangles contribute to the signal and background, respectively.

References

(1) Salehi-Reyhani, A.; Kaplinsky, J.; Burgin, E.; Novakova, M.; DeMello, A. J.; Templer, R. H.; Parker, P.; Neil, M. A. A.; Ces, O.; French, P.; Willison, K. R.; Klug, D. *Lab on a Chip* **2011**, *11*, 1256–61.

(2) Roy, R.; Hohng, S.; Ha, T. *Nature methods* **2008**, *5*, 507–16.

(3) Zhuang, G.; Katakura, Y.; Omasa, T.; Kishimoto, M.; Suga, K.-I. *Journal of Bioscience and Bioengineering* **2001**, *92*, 330–336.

(4) Lin, S.; Shih-Yuan Lee, A.; Lin, C.-C.; Lee, C.-K. *Current Proteomics* **2006**, *3*, 12.

(5) Hellmich, W.; Pelargus, C.; Leffhalm, K.; Ros, A.; Anselmetti, D. *Electrophoresis* **2005**, *26*, 3689–96.

(6) Dovichi, N. J.; Hu, S.; Michels, D.; Mao, D.; Dambrowitz, A. In *Single Cell Analysis: Technologies and Applications (D. Anselmetti)*; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2009.

(7) Sims, C. E.; Allbritton, N. L. *Lab on a chip* **2007**, *7*, 423–40.

(8) Ekins, R. P.; Chu, F. W. *Clinical chemistry* **1991**, *37*, 1955–67.