Validation of Arrayed Imaging Reflectometry Biosensor Response for Protein-Antibody Interactions: Cross-Correlation of Theory, Experiment, and Complementary Techniques

SUPPORTING INFORMATION

Rashmi Sriram,[‡,†] *Amrita R. Yadav,*[§,†] *Charles R. Mace,*[#] *and Benjamin L. Miller* [‡,#,&,]

[‡]Department of Biomedical Engineering, [§]Physics and Astronomy, [#]Biophysics and Biochemistry, [&]Dermatology, University of Rochester, Rochester, NY, 14642

* To whom correspondence should be addressed. E-mail: Benjamin_miller@urmc.rochester.edu. Telephone: (585) 275-9805.

[†]These authors contributed equally towards this study.

Index

1. Abbreviations Used

MPBS (modified PBS):	Aqueous buffer containing 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 150 mM NaCl at pH 7.2
MPBS-ET:	MPBS buffer containing 3 mM EDTA and 0.005% Tween-20
MPBS-BET:	MPBS-ET supplemented with 1% (v/v) BSA
HBS:	Aqueous buffer containing 20 mM HEPES, 150 mM NaCl, at pH 7.2
ddH_2O:	Glass-distilled deionized water
VEGF:	Vascular endothelial growth factor
FGF-2:	Fibroblast growth factor-2
AIR	Arrayed imaging reflectometry
SPR:	Surface plasmon resonance

2. Materials. Six inch silicon wafers (p-type, <100>) diced into 1 cm x 1 cm chips with an average of 1460 Å of thermally grown silicon dioxide were obtained from the Infotonics Technology Center, Inc. (Canandaigua, NY). (3-aminopropyl)dimethylethoxysilane (APDMES) and glutaraldehyde (50% aqueous solution) were purchased from Gelest, Inc. and TCI America, respectively. Recombinant human VEGF, recombinant human FGF-2, purified anti-human IFNγ and anti-human VEGF (Clone 26503) were purchased from RnD Systems Inc.; purified anti-human FGF-2 (Clone JKFb-1) was purchased from Biolegend. Anti-fluorescein was purchased from Rockland Immunochemicals Inc. Bovine serum albumin (BSA) used for blocking was purchased from Sigma Aldrich, and sterile BSA used as carrier in protein dilutions was purchased from Equitech-Bio Inc. SPR experiments were carried out using Biacore X or Biacore T100 SPR instruments and CM5 SPR chips (GE Healthcare). Spectroscopic ellipsometry measurements were carried out on an Alpha SE instrument (J. A. Woollam). All simulations were carried out using MATLAB (Mathworks, Natick, MA).

3. AIR Chip Preparation

The 1 cm x 1 cm silicon chips from the wafer were incubated in 7:3 (v/v) of 10 M sodium hydroxide:ethanol for 30 minutes under mild shaking conditions to remove traces of a protective adhesive material remaining from the wafer dicing process. Then, the chips were rinsed repeatedly with glass-distilled deionized water (ddH_2O) and dried under a stream of nitrogen. Following this, the chips were etched using dilute hydrofluoric acid (HF) solution to a final oxide thickness, as measured by spectroscopic ellipsometry, of 1387.5 Å for the VEGF experiments and 1390 Å for FGF-2 experiments. This difference was adopted to compensate for the differential immobilization thicknesses of the two antibodies on the aldehyde substrate. The etched chips were washed in piranha solution (3:1 (v/v) conc. sulfuric acid to 30% hydrogen peroxide; CAUTION: piranha solution reacts vigorously with organic materials and must be used with care) for 30 minutes, followed by thorough rinsing with ddH_2O and drying under a stream of nitrogen. The chips were then incubated with a 1% (v/v) solution of (3-

aminopropyl)dimethylethoxysilane (APDMES) in anhydrous toluene for 20 minutes on an orbital shaker. The chips were then repeatedly washed with anhydrous toluene, dried under a stream of nitrogen and baked at 110 ^{o}C for 30 minutes. After the chips had cooled to room temperature (ca. 5 minutes), a solution of 1.25% (v/v) glutaraldehyde in modified PBS buffer (MPBS) was poured over them, and the chips were left in this solution on a shaker for 60 minutes. Afterwards, they were washed with MPBS and ddH_2O and dried under a nitrogen stream. The aldehyde-functionalized chips were then subjected to antibody immobilization via amine-aldehyde coupling chemistry (Schiff base formation).

Antibody immobilization was carried out using both macro and microarray formats. In the case of macrospots, antibodies of interest (anti-human VEGF or anti-human FGF-2) were immobilized in the form of one large spot (ca. 6 mm in diameter) per chip. 30 µL of the antibody, diluted to 400 µg/mL in MPBS, was deposited in the center of the chips and allowed to react for 60 minutes at ~75% relative humidity at room temperature. After antibody immobilization, the remaining aldehyde groups were blocked by incubating the chips in 0.2 mg/mL BSA solution in HBS buffer for 60 minutes. Post-blocking, the chips were washed with MPBS and placed in separate wells of a 24-well plate. Sets of chips were then exposed to 500 µL of their corresponding protein serially diluted 10-fold in MPBS-BET starting from 1 µg/mL and going down to 100 fg/mL for 16 hours under mild shaking conditions at room temperature. After protein incubation, chips were washed with ddH_2O and dried under a stream of nitrogen.

Microarrays were also prepared to test AIR reflectance for FGF-2 and VEGF, and were printed using a Virtek Chip Writer Pro arrayer (Virtek Vision Inc., Ontario, Canada) and an SMP2XB pin (Arrayit Corporation, ~1 nL spot volume, ~120 micron spot diameter). For FGF-2 microarrays, five replicate spots of 300, 400 and 500 µg/mL anti-human FGF-2, 300 µg/mL anti-IFNγ and 300 µg/mL anti-fluorescein were printed onto the chips and allowed to immobilize for 60 minutes at 75% relative

humidity. The anti-fluorescein and anti-IFNγ spots served as negative controls. In the case of VEGF, 400 and 500 μg/mL anti-human VEGF were used for the experiments and 300 μg/mL anti-IFNγ and 200 μg/mL anti-fluorescein were used as negative controls. The same blocking and protein incubation procedures as used for the macro spots were then used for the antibody microarrays.

4. Ellipsometric Measurements

The total thickness of the combined oxide and linking chemistry layers after aldehyde functionalization was determined using spectroscopic ellipsometry. Thickness of the antibody layer was calculated as the change in the thickness before antibody immobilization and after incubation of the antibody modified chips with the MPBS-BET buffer alone. Finally, thickness of the protein layer at a particular solution protein concentration was calculated as the change in the thickness of the chip from the antibody modified chip subjected to MPBS-BET alone. The maximum thickness, t_{max}, for a given protein was determined as the thickness change for saturating protein concentrations of 1 μg/mL and 10 μg/mL.

5. AIR Measurements

Both macro spot chips and microarrays were imaged on a prototype AIR imaging system (Adarza Biosystems, Inc.). The imaging setup consists of a linearly polarized 10 mW HeNe laser (632.8 nm) followed by a Glan-Thompson polarizer, which enforces a 100,000:1 s to p polarization intensity. The light from the polarizer is collimated and is then incident on the chip, which is mounted on a sample stage. The reflected light is then directed onto a camera via an Offner relay (used to create a 1:1 image of the chip on the camera sensor). The angle of incidence of the light can be finely adjusted using a micrometer. The images were acquired using LuCam capture software (Lumenera Corporation). The camera gain and integration time were adjusted to acquire a maximum intensity for the highest concentration chip (1 μg/mL) without saturating the camera response. This enabled reasonable

sensitivity at the lower concentrations while still retaining information at the higher concentrations used in the experiments. Chip images for both FGF-2 and VEGF are shown in Figures S5, S6 and S7.

The acquired AIR images were analyzed using ImageJ[1] software. For the macro spots, a circular region of interest with a diameter of 120 pixels was selected from each spot, and its mean intensity was taken as a measure of the reflectance of the spot. Antibody-functionalized chips treated with MPBS-BET alone acted as interchip negative controls. For microarrayed spots, the average intensity of the five replicates corresponding to the 500 μg/mL of antibody concentration was taken as the reflectance measurement. The difference in the intensities of chips subjected to the protein and the negative control chip was calculated for each solution protein concentration. An additional normalization using anti-fluorescein as the intrachip negative control was carried out for the microarrays, and all intensity changes were scaled to the maximum intensity change observed for the chip exposed to 1 μg/mL of target protein.

6. Determination of t_{max}

In principle, the maximum thickness of the target protein (t_{max}) can be calculated using equation 5, which is based on the number of antibody binding sites on the surface, the interaction stoichiometry, and the size of the protein using a relationship similar to that commonly used for SPR,

$$t_{\max} = t(antibody) \times n \times \frac{Mol.Wt.of\ protein}{Mol.Wt.of\ antibody}, \qquad \text{(Eq.S1)}$$

where *t(antibody)* is the thickness of the immobilized antibody and *n* is the stoichiometry of the interaction. However, this calculation is complicated by the fact that it is not possible to estimate the exact amount of active antibody available on the chip surface in the correct orientation to bind the protein, and the 100% surface coverage condition is ill defined in terms of the precise amount of protein present on the surface. Therefore, t_{max} was determined experimentally by measuring the thickness change

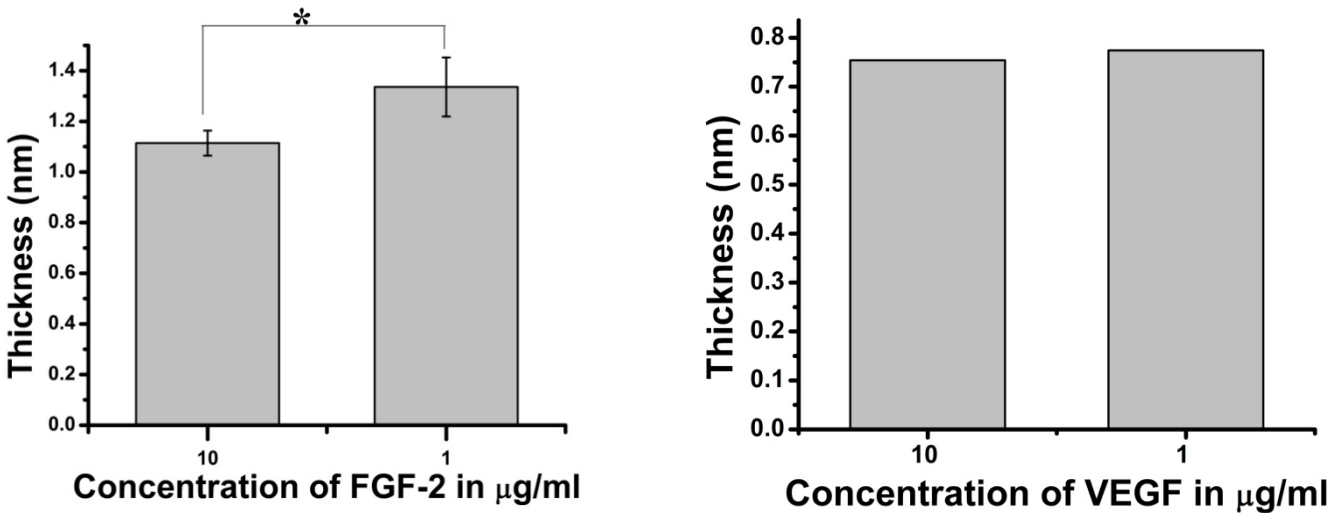
induced by protein binding at a high concentration of 10 μg/mL and 1 μg/mL (corresponding to molar concentrations of 575 nM and 57.5 nM for FGF-2 and 250 nM and 25 nM for VEGF) using spectroscopic ellipsometry. These concentrations were chosen because the preliminary simulations using the measured K_D values suggested that the protein-antibody interaction signal saturates around 1 μg/mL (i.e., 57.5 nM and 25 nM for FGF-2 and VEGF, respectively). The t_{max} for FGF-2 and VEGF was measured to be 1.3 nm and 0.8 nm respectively (*vide infra*). These values were then used in the AIR simulation.

7. Maximum Thickness Change (t_{max}) Measurements for FGF-2 and VEGF

To determine the maximum or saturating thicknesses (t_{max}) of FGF-2 and VEGF, spectroscopic ellipsometry (J. A. Woollam, Alpha-SE) was used to measure thicknesses on chips with one large spot of antibody. Oxide chips were functionalized with aminosilane and aldehyde, and the total thickness of the combined oxide and linking chemistry layers was measured. Then, 30 μL of 400 μg/mL of anti-FGF-2 or anti-VEGF was manually spotted (~6 mm in diameter) onto the aldehyde-modified chips. For FGF-2, two chips were treated with MPBS-BET which served as control, two other chips were incubated with 10 μg/mL of FGF-2 and the last two were treated with 1 μg/mL of FGF-2. For VEGF, one chip was used as control and each of the other two chips were subjected to 10 μg/mL and 1 μg/mL of VEGF protein respectively. Each chip was incubated with 500 μL of protein or buffer solution in separate wells of a 24 well plate at room temperature and left on an orbital shaker with mild shaking for ~16 hours. Afterwards, they were rinsed with ddH_2O and dried under a stream of nitrogen gas. Following this, the chips were mounted on the sample stage of spectroscopic ellipsometer such that the light beam fell entirely in the region defined by the immobilized antibody. Chips exposed to MPBS-BET alone served as reference chips for the antibody layer thickness, which was calculated as the change in

the thickness before the antibody immobilization and after the overnight (~16 hours) incubation with buffer alone. Once this was determined, the thickness of the protein layer at a particular concentration was calculated as the change in the thickness before and after protein treatment.

Figure S1 shows the thickness measurements obtained for FGF-2 and VEGF at two different concentrations. For FGF-2 thickness measurements, we carried out a two tailed t test at a 95% significance level to see if the difference in the thickness measurements at the two concentrations was significant or not. The test resulted in a value of $p > 0.05$, suggesting that there is no significant difference in the thickness of the FGF-2 layer at the two concentrations considered. Hence, the saturating thickness or t_{max} for FGF-2 is ~ 1.3 nm and we used this value for all our simulations. For VEGF, we were able to carry out only one thickness measurement for each concentration owing to the high cost (attributed to the amount of protein) of this assay. The saturating thickness for VEGF from these measurements is ~ 0.8 nm and this was used in the simulation.



(A) **(B)**

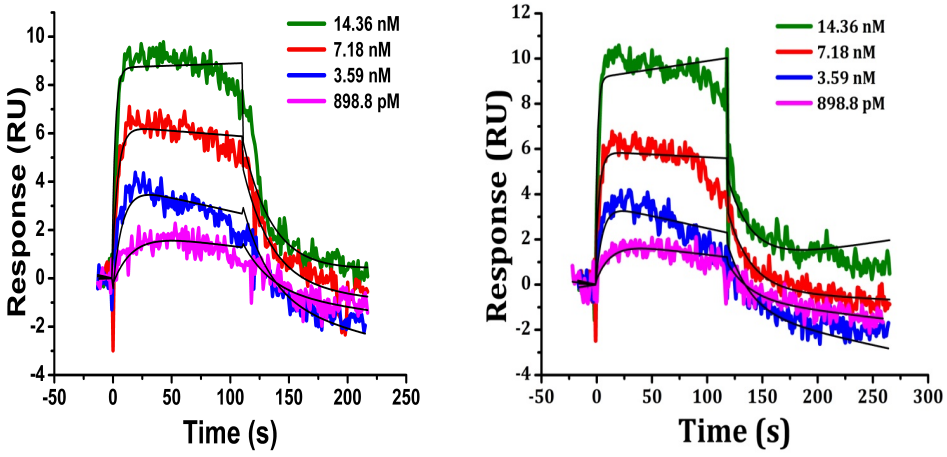
Figure S1. (A) Thickness measurements for FGF-2 at two different concentrations. Error bars correspond to standard deviation of the measurements from the mean value for n=2. (*) corresponds to a value of $p > 0.05$ obtained by carrying out a two-tailed t test. This suggested that there is no significant difference between the two measurements. **(B)** Thickness measurements for VEGF at 10 and 1 μg/mL concentration.

8. SPR Experiments

Kinetic and thermodynamic binding parameters for both protein-antibody pairs were determined using Biacore X or Biacore T100 SPR instruments equipped with research grade CM5 chips. The CM5 chips were activated using a 1:1 ratio of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 0.1 M N-hydroxysuccinimide (NHS), and subsequently functionalized with ~400 RU (response unit, 1 RU = 1 pg/mm^2) and ~3000 RU of anti-FGF-2 and anti-VEGF respectively. Antibodies were immobilized with different densities in the two cases because of the different assays used to determine binding affinities: in the case of FGF-2, the affinity was determined by monitoring binding kinetics, and

hence a low immobilization density was desirable to minimize mass transfer effects; however, in the solution affinity assay used for VEGF (*vide infra*), the SPR chip was used to determine the concentrations of free VEGF in different solutions, and a high immobilization density was desirable to ensure the maximum sensitivity at low concentrations.

For FGF-2, different concentrations of the protein (14.36 nM, 7.18 nM, 3.59 nM and 898.8 pM) were flowed over the antibody-functionalized chip at a rate of 50 µL/min and the binding kinetics were monitored. The resulting binding curves were globally fit to a 1:1 Langmuir binding model that accounts for the drifting baseline using BIA evaluation software (version 4, GE Healthcare), and the affinity constants for the interactions were extracted from the kinetic on and off rates.



(A) (B)

Figure S2. **(A)** and **(B)** represent a plot of SPR response units with time for different concentrations of FGF-2 flowed over an anti-FGF-2 chip in duplicates. Colored traces correspond to the real-time response for different concentrations of FGF-2 and the black traces represent the 1:1 Langmuir model which also accounts for the drifting baseline, fit to the data. The χ^2 for the obtained fits was 0.244 ± 0.001, which is well within the accepted value of $\chi^2 < 2$ for a good fit. The observed baseline drifts over the entire period of the sensorgram were within the noise level of the instrument (± 2 RU). [2]

For VEGF, an SPR-based solution affinity assay was used to determine the strength of interaction.[3,4] 500 pM VEGF was incubated with different concentrations of anti-VEGF prepared in serial two-fold dilutions ranging from 50 nM to ~6 pM. The solutions were allowed to equilibrate for 16 hours and subsequently introduced serially to the anti-VEGF CM5 chip. The concentration of free VEGF in each of these solutions was determined by using a separately established calibration curve with known concentrations of free VEGF. In brief, known concentrations of free VEGF were flowed over the anti-VEGF chip for a period of 120 s. The signal change at the end of the injection was plotted as a function of concentration, and the data were fit to a straight line. The slope and intercept of the line could then be used to determine the unknown free VEGF concentrations on the basis of the observed SPR signal change. The amount of bound VEGF could thus be calculated as a function of antibody concentration. This bound concentration was plotted against the total binding site concentration assuming two binding sites per antibody. The dissociation constant (K_D) was obtained by fitting the data to a ligand depletion model.

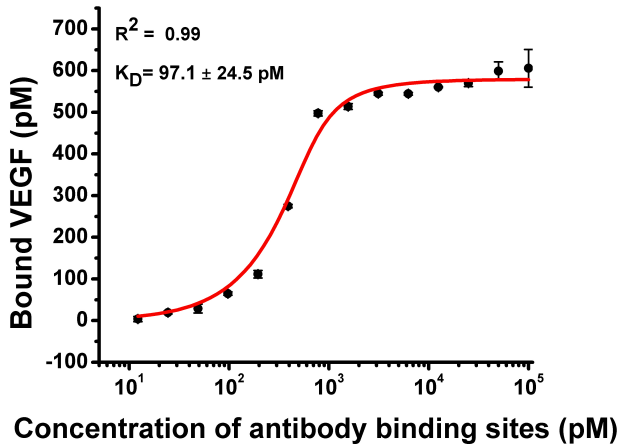


Figure S3. Plot of bound VEGF concentration against total concentration of anti-VEGF binding sites (two sites available per antibody). The red trace represents the ligand depletion model fit to the data. Error bars represent standard deviations for n = 2 replicates, and are typically illustrated by the size of the data point.

9. Ligand Depletion Model. In order to extract the K_D of interaction from the SPR based solution affinity assay for VEGF and anti-VEGF, a ligand depletion model was used. The amount of bound VEGF was plotted against the concentration of available binding sites (two times the antibody concentration), and the data were fit using the equation:

$$y = \frac{(x + K_D + B_{max}) - \sqrt{(x + K_D + B_{max})^2 - 4 \times x \times K_D}}{2} \qquad \text{(Eq.S2)}$$

where y is the bound VEGF concentration, x is the total binding sites concentration, K_D is the dissociation constant for the interaction and B_{max} is the maximum binding capacity and corresponds to the VEGF concentration used in this particular assay. Both B_{max} and K_D were allowed to vary during the fit. The K_D was found to be 100 ± 20 pM, the χ^2 and R^2 for the obtained fit were 653.5 and 0.99, respectively.

10. Theoretical Model Depicting the Variation of Raw Reflectance Values with the Concentration of

Protein.

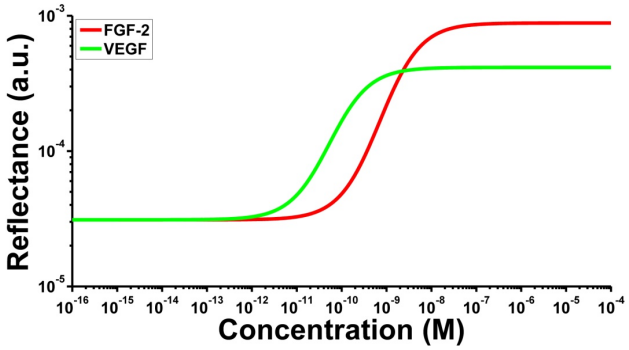


Figure S4. AIR response curves for FGF-2 and VEGF simulated by incorporating 1:1 one-site Langmuir binding model into the AIR thickness based model. The plot shows the variation of raw reflectance values with the protein concentration.

11. AIR Chip Images. (a) FGF-2 Macro spots

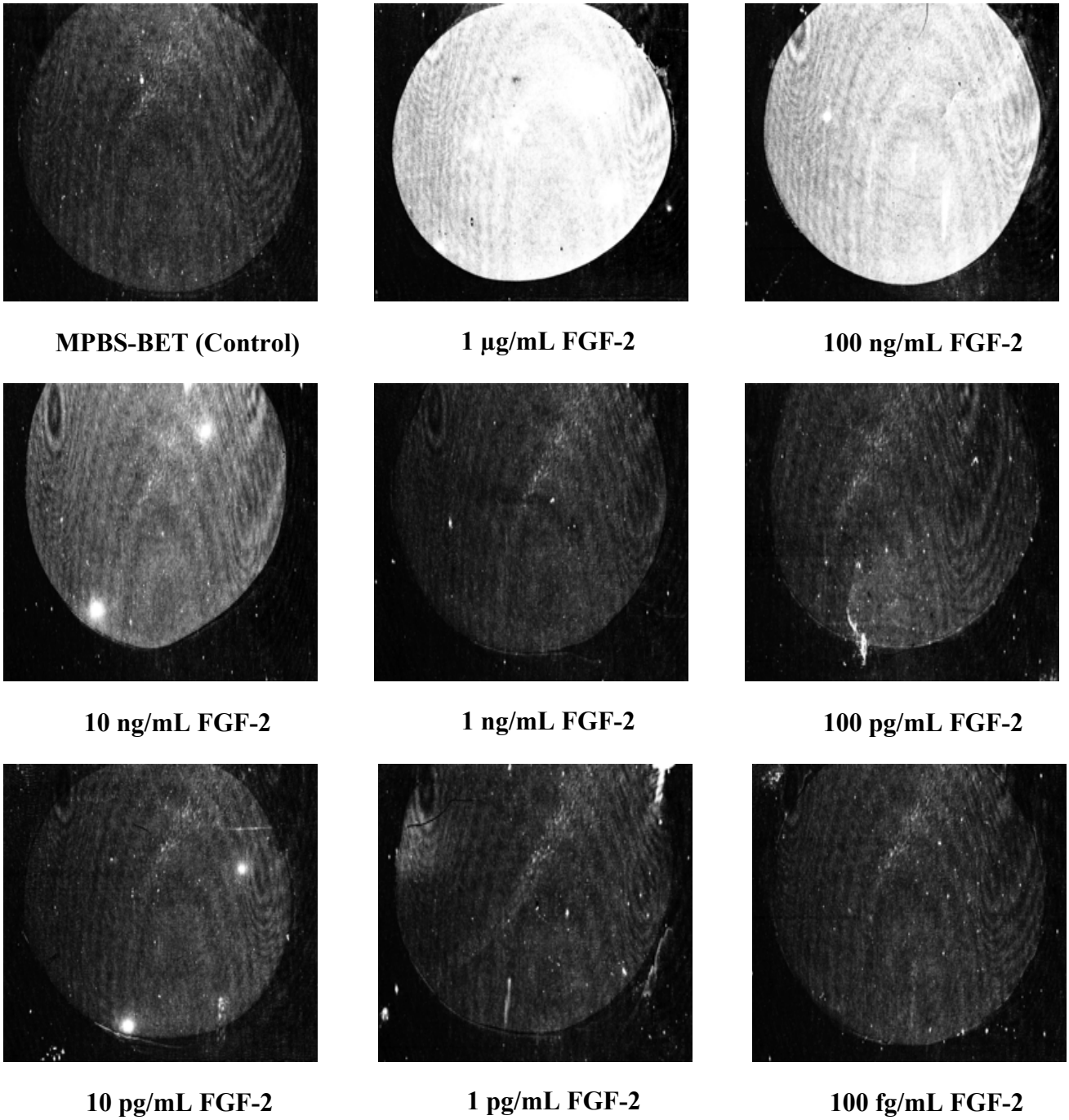


Figure S5. AIR images of one set of chips subjected to different concentrations of FGF-2 diluted in MPBS-BET. The CCD gain of 9 and exposure of 250 ms was used to image these chips on AIR optical setup. Note that these are presented for qualitative comparison only, and should not be evaluated for absolute intensity.

(b) FGF-2 Microarrays

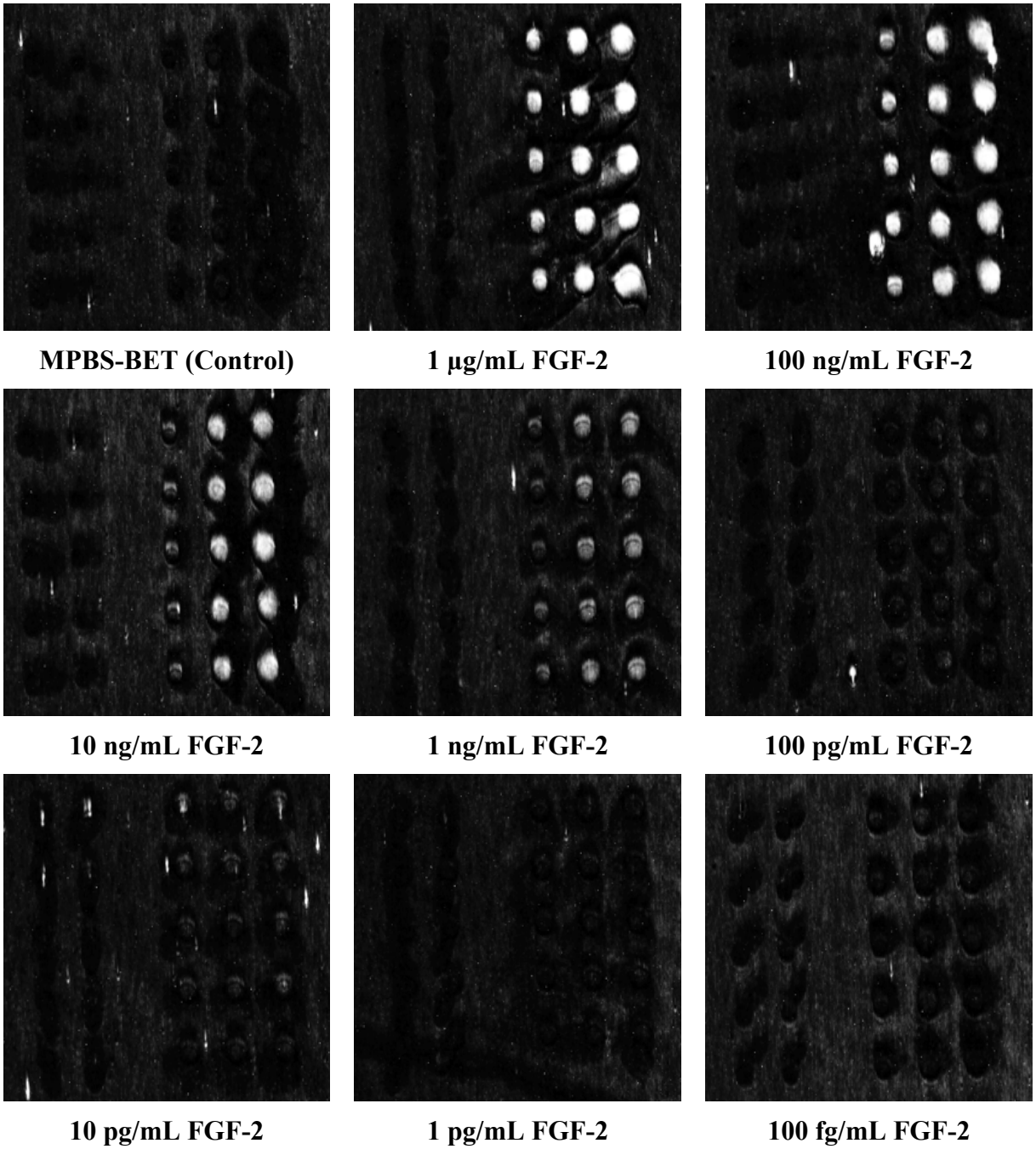


Figure S6. This panel shows the AIR images of one set of anti-FGF-2 microarrayed chips subjected to different concentrations of FGF-2 diluted in MPBS-BET. The CCD gain of 6.5 and exposure of 275 ms was used to image these chips on AIR optical setup. The 5 spots in a row are replicates, and the columns from left to right correspond to 300 µg/mL anti-fluorescein, 300 µg/mL anti-IFNγ, a blank column, 300, 400 and 500 µg/mL anti-FGF-2. Note that these are presented for qualitative comparison only, and should not be evaluated for absolute intensity.

(c) VEGF Microarrays

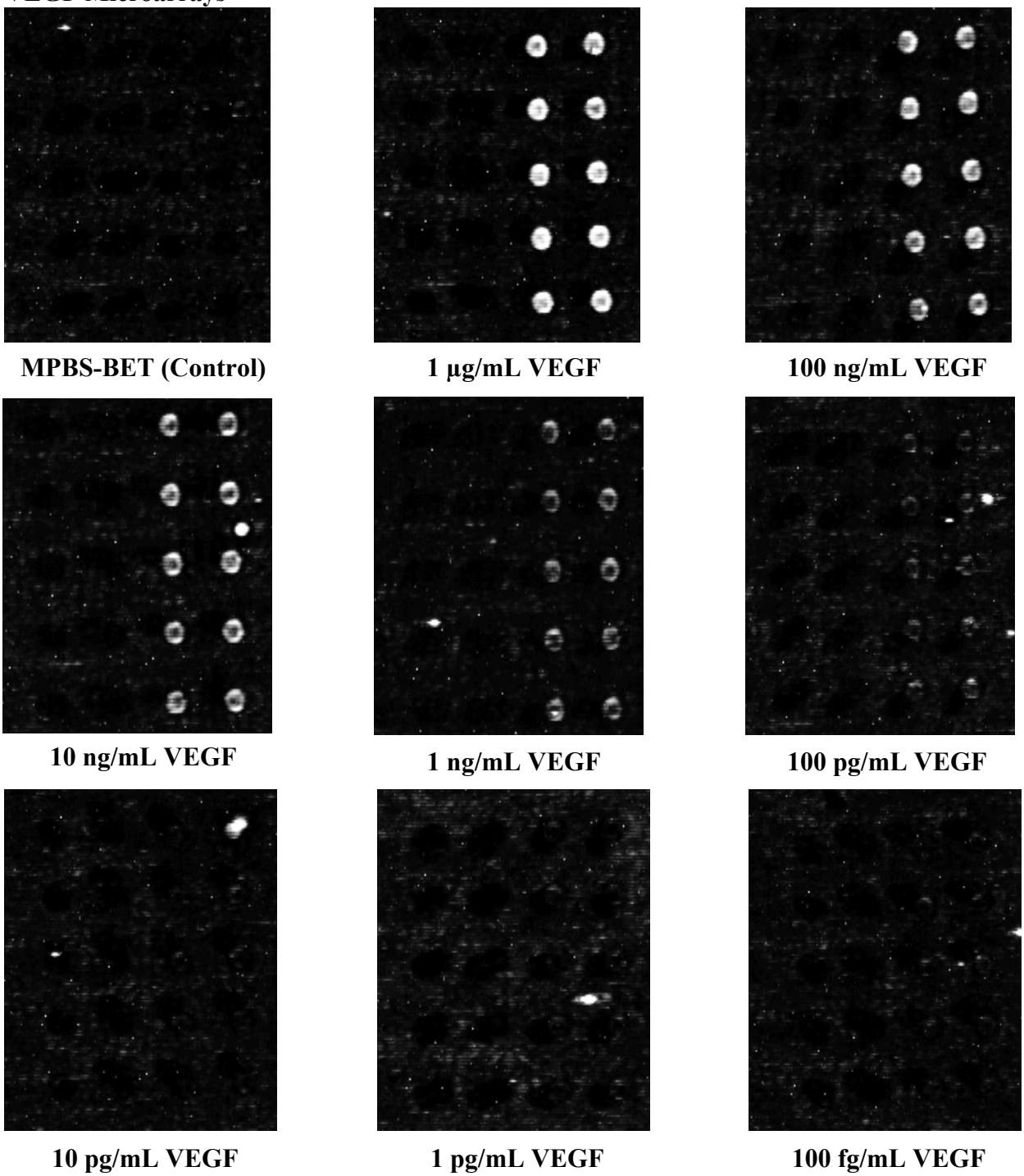
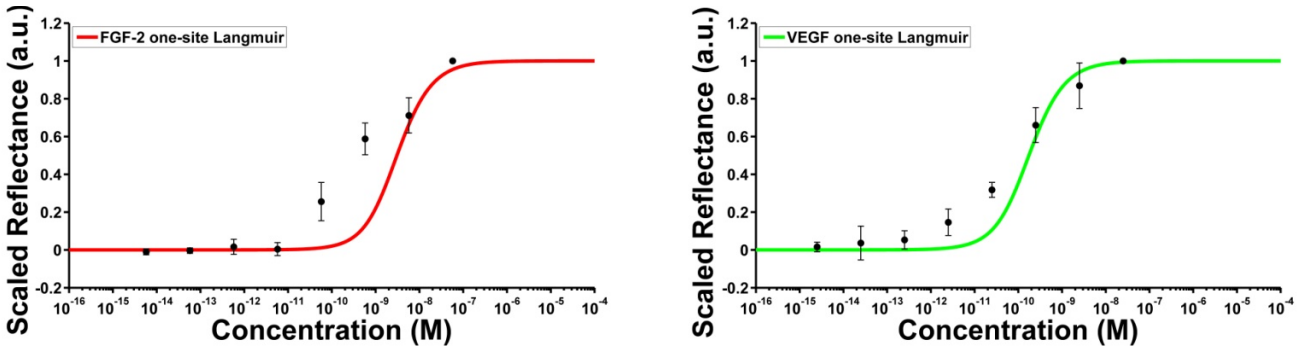


Figure S7. AIR images of one set of anti-VEGF microarrayed chips subjected to different concentrations of VEGF diluted in MPBS-BET. A CCD gain of 9 and exposure of 300 ms was used to image these chips on AIR optical setup. The 5 spots in a row are replicates, and the columns from left to right correspond to 200 µg/mL anti-fluorescein, 300 µg/mL anti-IFNγ, 400 µg/mL anti-VEGF and 500 µg/mL anti-VEGF. Note that these are presented for qualitative comparison only, and should not be evaluated for absolute intensity.

12. One-site Langmuir fits to FGF-2 and VEGF Microarrays



(A) **(B)**

Figure S8. One-site Langmuir model based AIR fits to FGF-2 **(A)** and VEGF **(B)** microarrays. Red and green traces correspond to the simulated curves and the black points represent the experimental scaled reflectance measurements. Error bars correspond to the standard deviation of the scaled reflectance values from the mean for three replicate experiments.

13. (a) Sips Isotherm to Describe the Protein-Antibody Binding Interactions on the Surface

A Sips isotherm, expressed in terms of fractional surface coverage as,

$$\Gamma = \frac{C^{a}}{C^{a} + (K_{Da})^{a}}, \qquad \text{(Eq.S3a)}$$

was also used to model the AIR response after converting it to an equation providing the protein layer thickness as,

$$t = t_{max} \frac{C^{a}}{C^{a} + (K_{Da})^{a}}. \qquad \text{(Eq.S3b)}$$

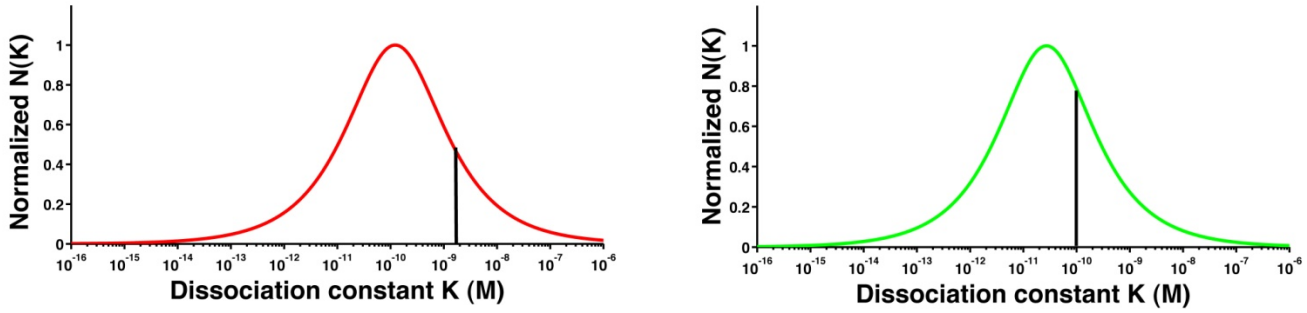
In the above equations, Γ is the fractional surface coverage at solution concentration C of the protein, a is the heterogeneity index describing the width of the affinity distribution about the average binding affinity of K_{Da}, t is the thickness of the protein layer at concentration C and t_{max} is the saturation thickness of the protein layer. The thickness of the protein layer obtained from Eq. (S3b) can be converted into the normalized AIR reflectance change as described in the methods section.

(b) Calculated Affinity Distributions for Obtained Sips Isotherm Parameters

The Sips isotherm models interactions for which the affinities of interaction assume a continuous range of values. The distribution of interaction affinities can be calculated by using the relation:[5]

$$N(K) \propto \frac{\left(\frac{K_{Da}}{K}\right)^{a} \cdot \sin(\pi \cdot a)}{1 + 2 \cdot \left(\frac{K_{Da}}{K}\right)^{a} \cdot \cos(\pi \cdot a) + \left(\frac{K_{Da}}{K}\right)^{2a}} \qquad \text{(Eq.S4)}$$

where $N(K)$ represents the number of binding sites with the affinity K, K_{Da} is the average affinity and a is the heterogeneity index. For a qualitative look at the distribution, the proportionality constant can be adjusted so as to assign a peak value of 1 to $N(K)$. The affinity distributions for the best-fit parameters obtained for our data are plotted in Figure S9(a) for FGF-2 and Figure S9(b) for VEGF. The vertical line represents the K_D for the particular antibody-protein pair as determined by SPR.



(A) **(B)**

Figure S9. Calculated normalized affinity distributions on the chip surface for **(A)** anti-FGF-2 and **(B)** anti-VEGF. The distributions are centred about 122.2 pM and 27.2 pM for FGF-2 and VEGF, respectively. The black vertical lines represent the K_D measured for the respective protein-antibody pair using SPR, namely 1.6 nM for FGF-2 and 100 pM for VEGF.

(c) FGF-2 Macro Spot data Fit to Sips Isotherm

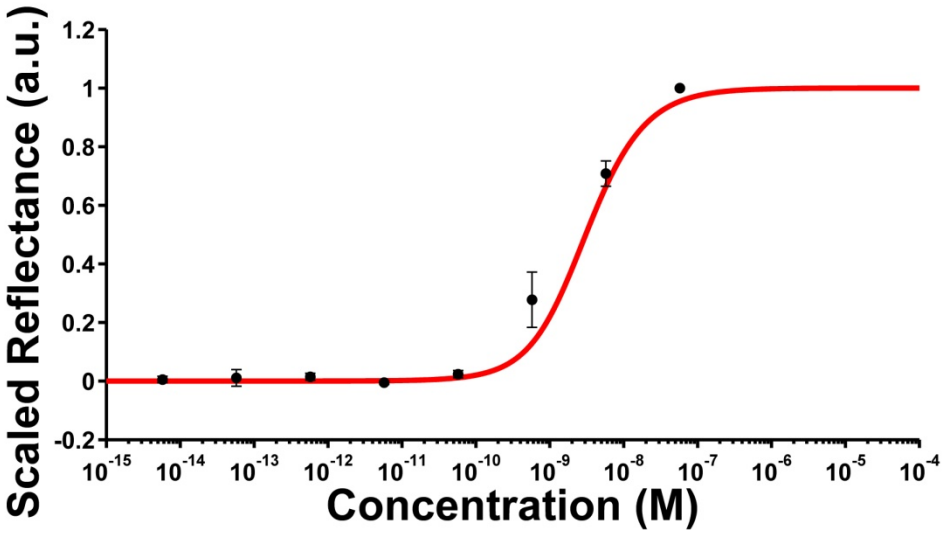


Figure S10. FGF-2 Macro spot data fit to the Sips-based AIR curve. For the fits a K_D of 1.6 nM (determined by SPR) and a t_{max} of 1.3 nm was used and this resulted in a width of distribution, a, to be 1.

References

(1) Abramoff, M. D.; Magelhaes, P.J.; Ram, S.J. *Biophotonics Int*. **2004**, 11, 36-42.

(2) Biacore X Intsrument Handbook; Biacore AB: Uppsala, Sweden, 2001.

(3) Karlsson, R. *Anal. Biochem*. **1994**, *221,* 142-151.

(4) Cochran, S.; Li, C.; Fairweather, J. K.; Kett, W. C.; Coombe, D. R.; Ferro, V. *J. Med. Chem*. **2003**, *46,* 4601-4608.

(5) Sips, R. J. Chem. Phys. 1948, 16, 490-495.