Supporting Information

The impact of dissociation constant on the detection sensitivity of polymerization-based signal amplification reactions

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Experimental Details

Materials

Poly(ethylene glycol) diacrylate (PEGDA) (M_n=575), triethanolamine (TEA), 1-vinyl-2-pyrrolidinone (VP), eosin Y disodium salt, sodium (meta) periodate, HABA, 10x phosphate buffered saline (PBS), 4'-hydroxyazobenzene-2-carboxylic acid (HABA), Triton® X-100, and Tween® 20 were purchased from Sigma Aldrich and used without further purification. Eosin 5-isothiocyanate was obtained from Marker Gene Technology. Streptavidin was purchased from Rockland Immunochemicals Inc. 10x bovine serum albumin blocker solution, EZ-Link NHS-LC biotin, and a BCA Protein Assay Kit with bovine serum albumin standards were purchased from Pierce/Thermo Scientific. 100x Denhardt's solution and Coomassie Brilliant Blue were obtained from Bioexpress. Glass slides (75x25x1 mm) were purchased from VWR. Seakem LE Agarose was purchased from Lonza. Imidazole (99%) was obtained from Alfa Aesar and cOmplete Mini Protease Inhibitor Cocktail was purchased from Roche. Rosetta™ host strains were purchased from Novagen. Sodium chloride, sodium phosphate monobasic, and sodium phosphate dibasic were purchased from Mallinckrodt Chemicals. Bacto agar and Difco LB broth were purchased from Becton, Dickinson, and Company. Chloramphenicol and kanamycin sulphate were obtained from Calbiochem. Isopropyl β-D-1 thiogalactopyranoside (IPTG) was purchased from Omega Bio-Tek. HisTrap™ FF crude 1 mL columns were purchased from GE Healthcare. Silicone isolators (9 mm diameter, 1 mm well depth) were obtained from Electron Microscopy Sciences. Cy3 NHS ester was purchased from Lumiprobe. UltraCruz™ Micro G-25 Spin Columns were purchased from Santa Cruz Biotechnology, Inc. 4-15% Mini-PROTEAN® TGX™ Precast Gels were purchased from Bio-Rad Laboratories, Inc.

Protein Expression and Biotinylation

Plasmids (pEThK-Fn3 clones B, D, and E)[30] were obtained from the K.D. Wittrup Lab. To express each clone, plasmids were transformed into Rosetta (DE3) E. coli, which were then grown on LB agar plates (with 30 mg/mL kanamycin and 25 mg/mL chloramphenicol) at 37°C. Starter cultures were prepared by transferring single colonies to 5 mL of LB media (with 30 mg/L kanamycin and 25 mg/mL chloramphenicol). These cultures were grown at 37°C at 250 rpm for ~16 hours and then added to 100 mL of LB media (with 30 mg/L kanamycin and 25 mg/mL chloramphenicol) in a 250 mL flask to be grown at 37°C and 250 rpm. Once the absorbance at 600 nm had reached ~1, expression was induced by adding IPTG to a final concentration of 0.5 mM. The cells were then incubated for a further 24 hours at 37°C and 250 rpm. At this point, the cells were pelleted at 15,000xg for 15 minutes (4°C). The supernatant was decanted and the cells were resuspended in 25 mL wash buffer (300 mM NaCl, 50 mM sodium phosphate, 10 mM imidazole, pH 7.4). A cOmplete Mini protease inhibitor cocktail tablet was added to the cell suspension. The sample tube containing the cell mixture was placed in a container packed with ice and sonicated 3 times for 60 seconds each (Branson sonifier 250, output control set to 5, 50% duty cycle). The sample was centrifuged at 15,000xg for 15 minutes (4°C) and the supernatant was filter sterilized with a 0.2 μm filter in preparation for metal affinity purification. The proteins were purified using an ÄKTAFPLC and HisTrap FF Crude columns. The elution buffer consisted of 300 mM NaCl, 50 mM sodium phosphate, 300 mM imidazole (pH 7.6). (Supplementary Figure 1)

Following purification, the proteins were biotinylated with the EZ-Link NHS-LC-Biotin and subsequently purified using UltraCruz™ Micro G-25 Spin Columns. Total protein was quantified using a BCA assay with BSA standards.

HABA Assay for determining relative degree of biotinylation

A standard curve for determining the degree of biotinylation was generated by adding varying concentrations of biotin to HABA-streptavidin solutions. The standards were prepared in a 96-well microplate format; 20 µL of various concentrations of biotin stock solutions in pH 6, 0.05 M sodium phosphate, 0.15 M NaCl buffer were added to 180 µL HABA-streptavidin (175.6 µL 0.5 mg/mL streptavidin in pH 6, 0.05 M sodium phosphate, 0.15 M NaCl buffer and 4.4 µL 2.42 mg/mL HABA in 10 mM NaOH). The samples were prepared by mixing 20 µL of each biotinylated protein with 180 µL of the HABA-streptavidin solution. The samples were mixed for 5 minutes prior to reading the absorbance at 500 nm using a Plate Reader. The standard curve was constructed by plotting the change in absorbance for the biotin dilutions (relative to a sample to which 20 µL of buffer had been added) as a function of biotin concentration. (Supplementary Figure 2)

Densitometry

Because the BCA assay determines the total amount of protein, the fraction of the total corresponding to each of the fibronectin clones was estimated using densitometry. Duplicate samples of each of the fibronectin clone protein purifications were run at a total protein concentration of 1.5 µg on an SDS-PAGE gel along with BSA standards (2 µg, 1.5 µg, and 1 µg). Following Coomassie staining, the gel was imaged and analyzed using ImageJ. The image was

inverted and the integrated intensities of equal areas fully encompassing each of the protein bands as well as an area removed from the bands (to be used as the background intensity) were measured. The integrated intensities were then background corrected and the quantity of each of the fibronectin clones was determined through normalization by the integrated intensity of the BSA standard bands. (Supplementary Figure 3)

EGFR/Fc purification

An EGFR (extracellular domain)-Fc receptor fusion was isolated from a mixture of EGFR-Fc fusion and Fc receptor using size exclusion chromatography (Superdex 75 10/300 GL gel filtration column). The resulting fractions were collected and run on an SDS-PAGE gel to identify the fraction containing the fusion. Fraction 7 contained the fusion without any contaminating Fc. (Supplementary Figure 4)

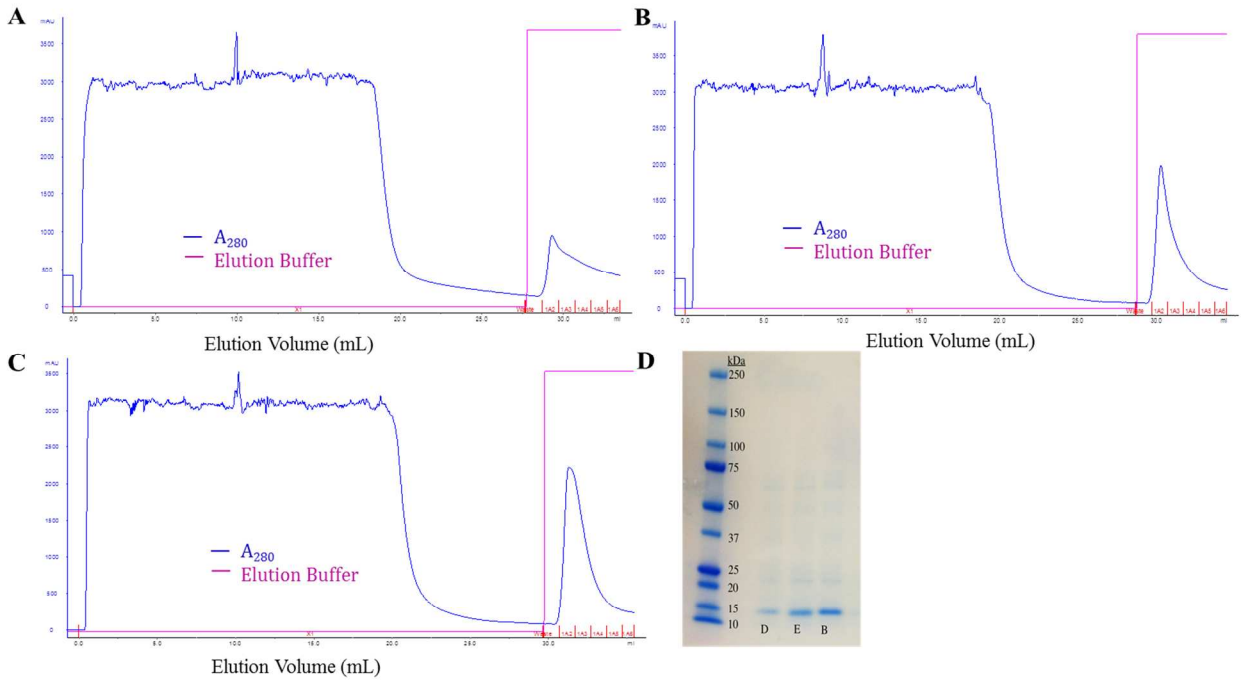
Details of Aldehyde Functionalized Agarose Surface Preparation

2 mL of a solution of 0.2 wt% agarose in distilled water (briefly heated in the microwave) were pipetted onto a glass slide and dehydrated overnight under ambient conditions. Activation of the agarose was achieved by immersing the slides in 20 mM sodium (meta) periodate (in distilled water) for 30 minutes. Following activation, the surfaces were rinsed with distilled water and dried under ambient conditions for 2 hours prior to protein printing.

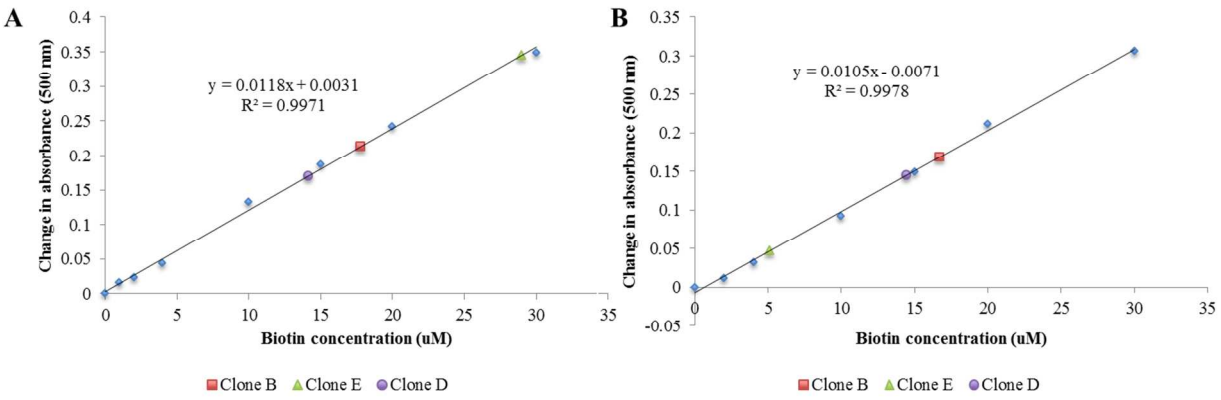
Antigen Density Determination

We quantified the number of binding-accessible EGFR molecules per square micron in each spot using a streptavidin-Cy3 conjugate and fluorescence analysis (Agilent microarray scanner)

against a Cy3 calibration array (Full Moon Biosystems). The surfaces were developed according to the standard protocol; in brief, the surfaces were blocked for 10 minutes with 1% BSA in 1x PBS and rinsed with 1x PBS, 40 μL of each of the biotinylated fibronectin clones (10 μg/mL total protein) was added to separate isolators and the binding interaction was given 30 minutes to reach completion before unbound protein was rinsed away using 1x PBS. At this point, the test surfaces were contacted with 0.1 μM streptavidin-Cy3 in 0.75% BSA in 1.5x PBS, 5x Denhardts for five minutes in a humid chamber. Sequential rinses with PBST (1x PBS, 0.1% Tween 20), 1x PBS, and ddH_2O were used to remove unbound streptavidin-Cy3. The background fluorescence was determined by preparing surfaces with the corresponding unbiotinylated fibronectin clones in place of the biotinylated binders. The fluorescence intensity of the EGFR spots was then quantified and subtracted from the fluorescence intensity of features contacted with the biotinylated binders. These background-corrected fluorescence signals were compared with a standard curve generated using the Full Moon Biosystems calibration array where features containing only spotting buffer were used to calculate background signal. The arrays were scanned at 100% PMT with an excitation wavelength of 532 nm (20 mW) and emission wavelengths between 550 and 610 nm.



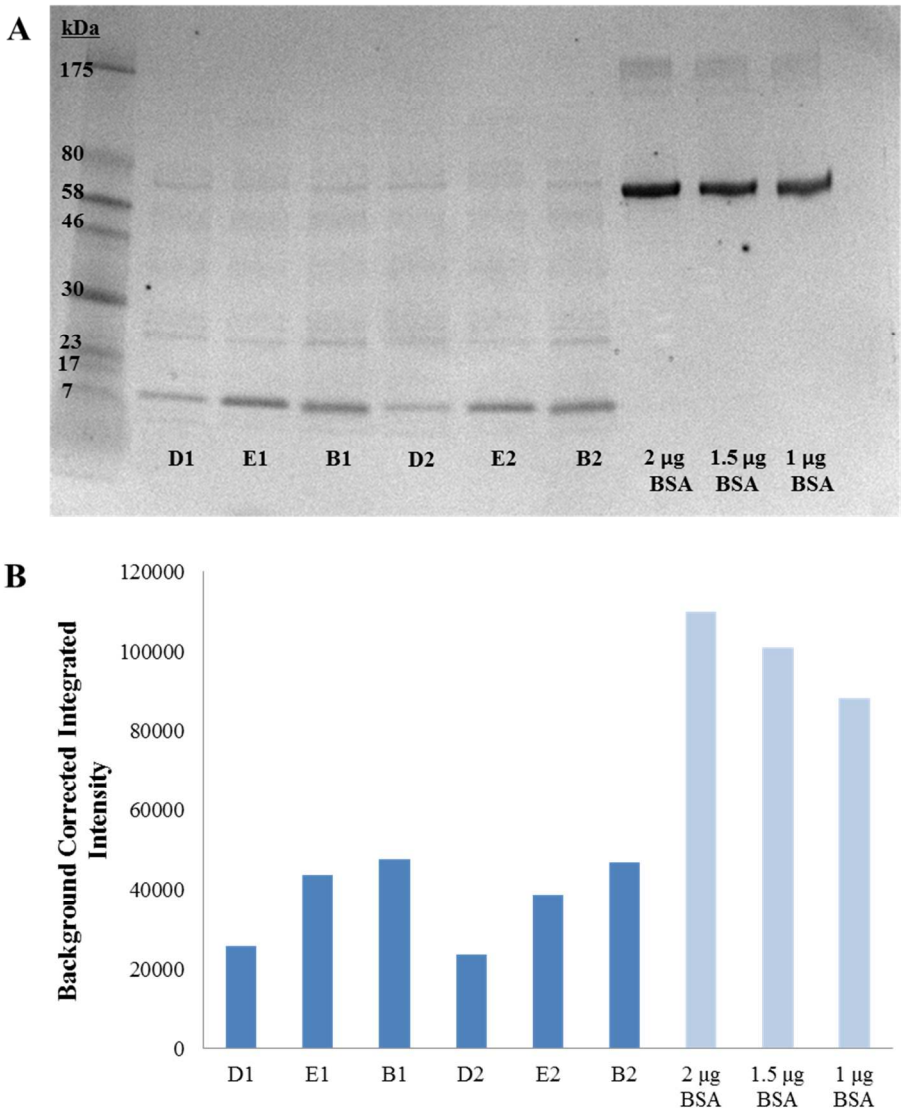
Supplementary Figure 1. Fibronectin clone purifications. (A) Chromatogram for metal affinity purification of clone B showing the absorbance at 280 nm and the introduction of elution buffer. The fractions eluted (in red) were pooled and the amount of protein was quantified using a BCA assay. (B) Chromatogram for metal affinity purification of clone D showing the absorbance at 280 nm and the introduction of elution buffer. The fractions eluted (in red) were pooled and the amount of protein was quantified using a BCA assay. (C) Chromatogram for metal affinity purification of clone E showing the absorbance at 280 nm and the introduction of elution buffer. The fractions eluted (in red) were pooled and the amount of protein was quantified using a BCA assay. (D) SDS-PAGE gel. Based on total protein quantification (BCA assay), 1.5 µg of each of the protein purifications was loaded onto an SDS-PAGE gel, which was then run for 30 minutes at 150 V.



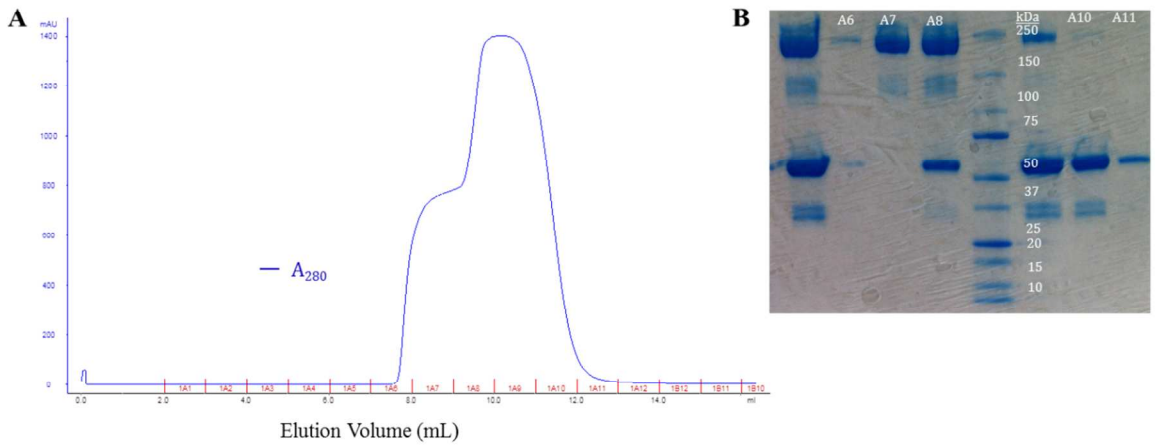
C

Fibronectin clone	Biotin/protein (standard curve A)	Biotin/protein (standard curve B)
D	10	10
E	8	7
B	12	11

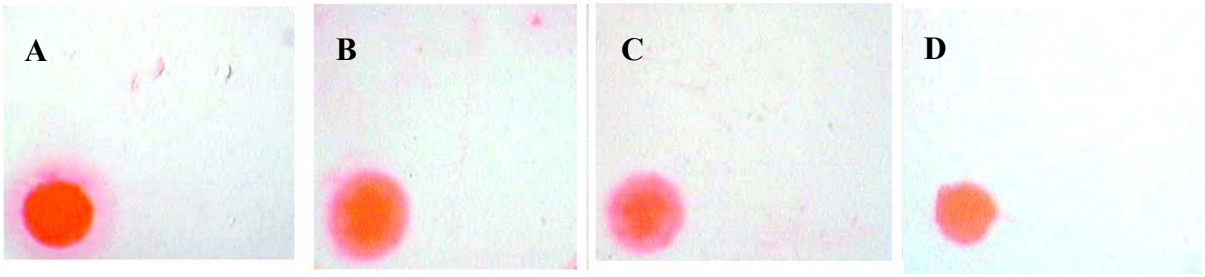
Supplementary Figure 2. HABA assay on biotinylated fibronectin clones. (A) Standard curve for determining the degree of biotinylation generated by adding varying concentrations of biotin to HABA-streptavidin solutions. The change in absorbance for the biotin dilutions (relative to a sample to which no biotin was added) is plotted as a function of biotin concentration. The change in absorbance for each of the clones is shown on the plot. (B) Replicate HABA assay performed on a different day. (C) Table summarizing the number of biotin per protein determined using the two separate standard curves. The number of biotin per protein is defined as the moles of biotin (as indicated by the assay) divided by the moles of protein (based on a BCA assay and assuming that there are not any contaminating proteins present). Because the proteins differ only with respect to a small subset of amino acids, it is reasonable to assume that they are similarly reactive; therefore, we hypothesize that the differences in the number of biotin per protein are attributable to differences in the relative purities of the protein preparations.



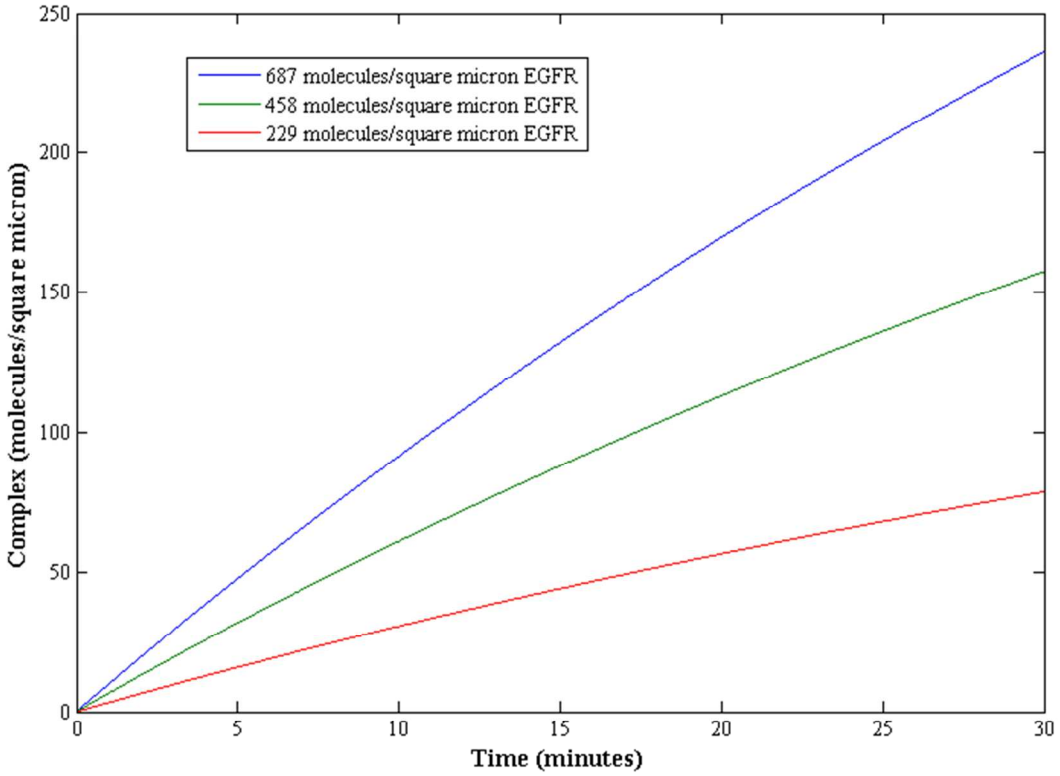
Supplementary Figure 3. Densitometric analysis. (A) SDS-PAGE gel. 1.5 µg total protein (based on a BCA assay) of each fibronectin clone protein preparation was loaded in duplicate onto an SDS-PAGE gel along with a protein ladder and BSA standards at the indicated quantities. The gel was imaged and densitometric analysis was performed in order to determine the amount of each fibronectin clone in the respective protein preparations. (B) Quantification of the SDS-PAGE gel presented in A. Using ImageJ, the image was inverted and the integrated intensities of equal areas fully encompassing the bands as well as an area removed from the bands (to be used as the background intensity) were measured. The integrated intensities were then background corrected (shown above) and the quantity of each of the fibronectin clones was determined through normalization by the integrated intensity of the BSA standard bands.



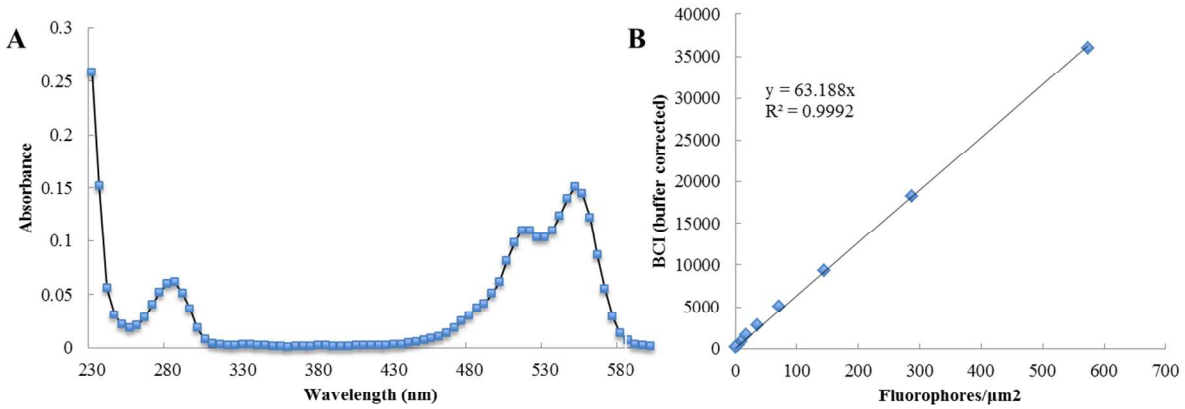
Supplementary Figure 4. EGFR-Fc fusion purification. (A) Chromatogram for size exclusion purification of the EGFR-Fc fusion showing the absorbance at 280 nm. (B) SDS-PAGE gel of fractions collected during the size exclusion purification. The fractions collected from the size exclusion purification of the EGFR-Fc fusion were diluted 1:1 with Laemmli buffer and loaded onto an SDS-PAGE gel (run at 150 V for 30 minutes). The fraction collected of the EGFR-FC fusion and used in this study is labeled A7.

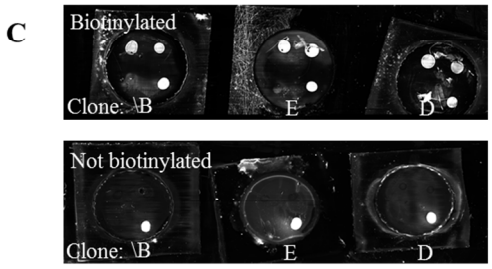


Supplementary Figure 5. Negative and positive controls. (A) The test surface was prepared omitting the incubation with a biotinylated fibronectin clone. This demonstrates that nonspecific binding of streptavidin-eosin to EGFR does not result in a false positive. (B) The test surface was prepared with 1 uM unbiotinylated clone B in place of its biotinylated counterpart. (C) The test surface was prepared with 1 uM unbiotinylated clone E in place of its biotinylated counterpart. (D) The test surface was prepared with 1 uM unbiotinylated clone D in place of its biotinylated counterpart. The latter three cases demonstrate that nonspecific binding of streptavidin-eosin to the fibronectin clones does not result in a false positive. In all cases, polymerization is observed in response to the binding of the streptavidin-eosin conjugate to the biotin covalently coupled to the surface as the positive control spot.



Supplementary Figure 6. Complex concentration increases as the concentration of immobilized EGFR molecules increases. Complex concentration as a function of time is shown for the EGFR surface density determined experimentally (229 molecules/μm^2) as well as double and triple this concentration. For the results generated above, the concentration of fibronectin clone D (K_d=0.25 nM) was set to 2.4 nM. The experimental data (Figure 3 in the text) shows that the initiator density achieved with a surface density of 229 molecules EGFR/μm^2 and 2.4 nM of clone D in solution is not sufficient after a 30-minute incubation to initiate polymerization. This simulation shows that one way to achieve an initiator density above the threshold required for polymerization while holding the solution concentration of the target biomolecule (clone D) constant would be to increase the surface density of EGFR.

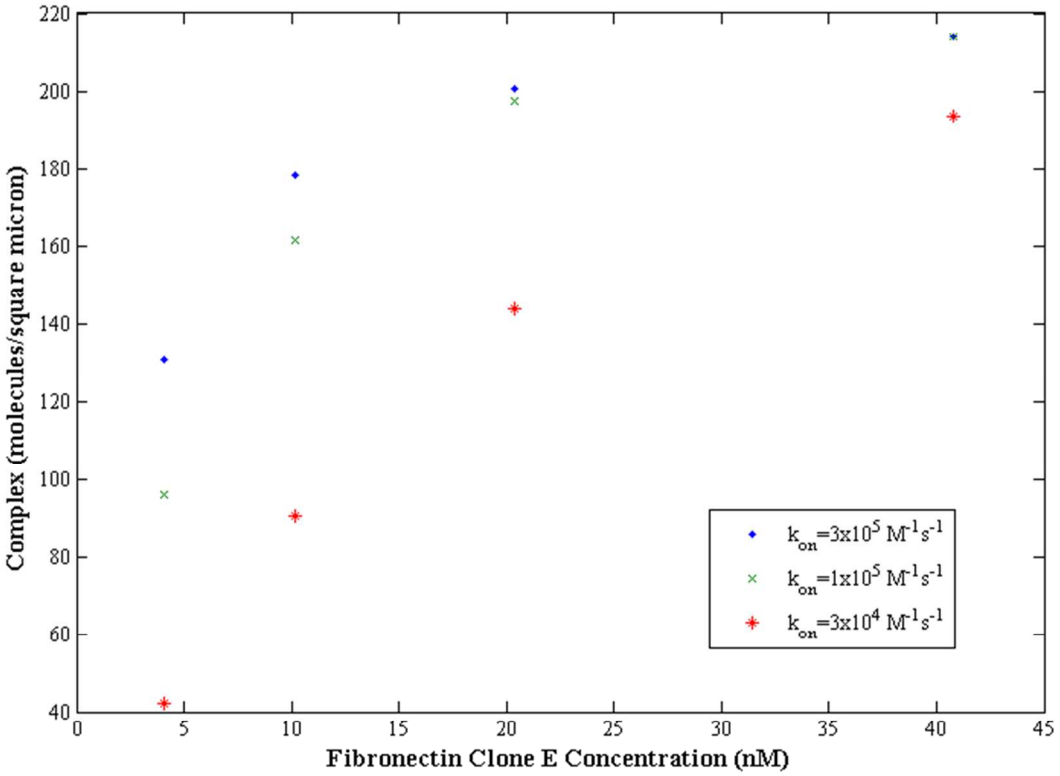




D **Streptavidin per square micron**

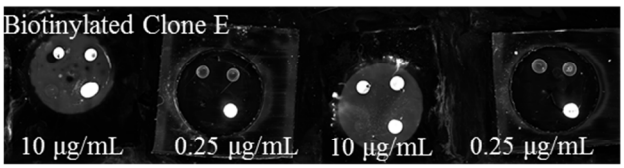
Fibronectin Clone	EGFR (1)	EGFR (2)	Positive Control	Negative Control
10 µg/mL B-b	220	230	270	60
10 µg/mL E-b	260	260	270	30
10 µg/mL D-b	230	180	240	70
Average	230		260	50

Supplementary Figure 7. Protein surface density quantification. (A) A streptavidin-Cy3 conjugate with 3.5 Cy3 molecules per streptavidin was prepared using the same method outlined above, though with a NHS ester-functional dye in the place of an isothiocyanate dye. Extinction coefficients used in the analysis: $\varepsilon_{cy3,552} = 150{,}000\ M^{-1}cm^{-1}$, $\varepsilon_{cy3,280} = 12{,}000\ M^{-1}cm^{-1}$ $\varepsilon_{SA,280} = 173{,}000\ M^{-1}cm^{-1}$. (B) Standard curve generated using a Full Moon Biosystems calibration array. The signal intensities were determined using ImageJ to compute average intensities within specified regions of constant area. BCI (background corrected intensity) is defined as the difference between the signal and the background (in the case of the calibration array, an array of buffer spots). The array was scanned at 100% PMT with an excitation wavelength of 532 nm (20 mW) and emission wavelengths between 550 and 610 nm. (C) (Top) A biochip test surface reacted with 10 µg/mL (total protein) of each of the biotinylated fibronectin clones (from left to right: clones B, E, and D) followed by 0.1 µM streptavidin-Cy3 conjugate as described in the text and imaged using an Agilent microarray scanner with an excitation wavelength of 532 nm (20 mW) and emission wavelengths between 550 nm and 610 nm. The PMT setting was 100%. The top two features in each array correspond to the EGFR-Fc fusion immobilized on the surface, while the bottom right feature is the positive control spot (biotinylated clone B). The rightmost array demonstrates how surface defects (arising through contact with the end of a pipet tip, for example) can result in false positives. (Bottom) A biochip test surface developed in the same way as the top image with the exception that the surface has been reacted with 10 µg/mL (total protein) of each of the unbiotinylated fibronectin clones (from left to right: clones B, E, and D). (D) Table summarizing the number of streptavidin bound per square micron for the indicated surface features based on average signal intensities determined using the images presented in (C). The bottom image in (C) was used as the background correction for the top image and the intensity values were converted to the number of streptavidin bound per square micron using the calibration array standard curve (B).

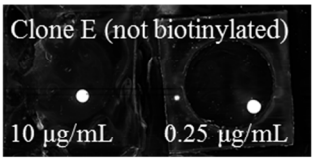


Supplementary Figure 8. Reducing the on-rate below 10^5 $M^{-1}s^{-1}$ results in a reduction in the complex concentration for lower concentrations of the fibronectin clone. For the results generated above, K_d=2.9 nM (fibronectin clone E) and the binding reaction time was set to 30 minutes. This simulation shows the extent to which slight changes in the on-rate (which may occur from clone to clone as clones were selected using equilibrium titrations rather than kinetic screens) result in changes in the surface concentration of the EGFR-Fn complex. For example, looking at the 10 nM points, a deviation of the on-rate from the standard order-of-magnitude assumption of 10^5 $M^{-1}s^{-1}$ could place the initiator density either well above or well below the observed threshold required for polymerization.

A



B



C **Streptavidin per square micron**

Fibronectin Clone	EGFR (average of 4)
10 µg/mL E-b	240
0.25 µg/mL E-b	70

Supplementary Figure 9. Protein surface density quantification for clone E. (A) A biochip test surface reacted with the indicated concentration (total protein) of biotinylated fibronectin clone E followed by 0.1 µM streptavidin-Cy3 conjugate as described in the text and imaged using an Agilent microarray scanner with an excitation wavelength of 532 nm (20 mW) and emission wavelengths between 550 nm and 610 nm. The PMT setting was 100%. The top two features in each array correspond to the EGFR-Fc fusion immobilized on the surface, while the bottom right feature is the positive control spot (biotinylated clone B). Based on densitometry, 10 µg/mL clone E corresponds to 410 nM and 0.25 µg/mL corresponds to 10 nM. (B) A biochip test surface developed as described for A, with the exception that unbiotinylated clone E was used in place of the biotinylated clone. (C) Table summarizing the number of streptavidin bound per square micron for the EGFR surface features based on average signal intensities determined using the images presented in A and B. The fluorescence intensities of the relevant features in the image in B were used as the background correction for the image in A and the intensity values were converted to the number of streptavidin bound per square micron using the calibration array standard curve shown in Supplementary Figure 7B.