Supplementary Information for

In situ drug-receptor binding kinetics in single cells: a quantitative label-free study of anti-tumor drug resistance

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1. Statistical analysis on the kinetic constants of individual cells

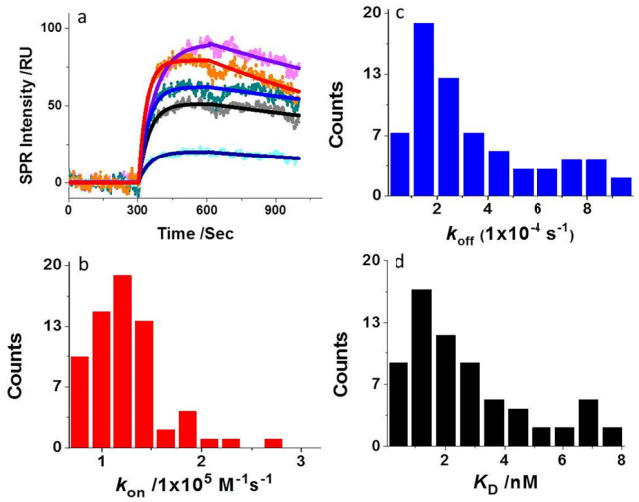

Fig. S1. (a) In order to quantitatively study the variability of the Herceptin-Her2 interaction on different single cells, SPR sensorgrams of 5 typical individual cells are displayed. The Langmuir binding model was subsequently applied to fit all the 60 sensorgrams obtained in two parallel experiments. (b-d) The corresponding histograms of (b) association rate constant (k_{on}), (c) dissociation rate constant (k_{off}) and (d) equilibrium binding constant (K_D), respectively.

2. Receptor regeneration in native cell membranes

Receptor surface regeneration is needed in order to perform repeated kinetic measurements on the same sample at different concentrations of ligand molecules. Many different reagents, such as NaOH, Glycine-HCl, have been used to regenerate receptor surfaces in the typical purified protein-based surface plasmon resonance (SPR) studies. In the present work, NaOH was found to effectively regenerate Her2 receptors in cell membranes. Immunofluorescence and SPR measurements were utilized to evaluate the performance of NaOH regeneration.

Fig. S2a shows the negative staining of Her2 in SK-BR3 cells without incubation with Herceptin (primary antibody), showing that non-specific adsorption of the secondary antibody to the cell membrane is negligible. After Herceptin (primary antibody) binding, the cells were exposed to 100 mM NaOH solution for 30 seconds and followed by incubation with the secondary antibody. The immunofluorescence image (Fig. S2b) shows little intensity, indicating effective release of the bound Herceptin molecules from the membrane in the NaOH regeneration step. To ensure that the regeneration procedure did not damage Her2, immunofluorescence staining was performed on the same cells by incubating them with Herceptin and secondary antibody. The recovery of the strong immunofluorescence emission was found (Fig. S2c), suggesting that NaOH treatment did not damage the Her2 receptors in the cell membrane. Additionally, the SPR measurements were carried out on the same cells before and after NaOH regeneration, and the results (Fig. S2d) show that both the maximum

SPR intensity and the binding kinetics did not change significantly, further indicating that the NaOH regeneration did not affect the binding of Herceptin to the Her2 receptors.

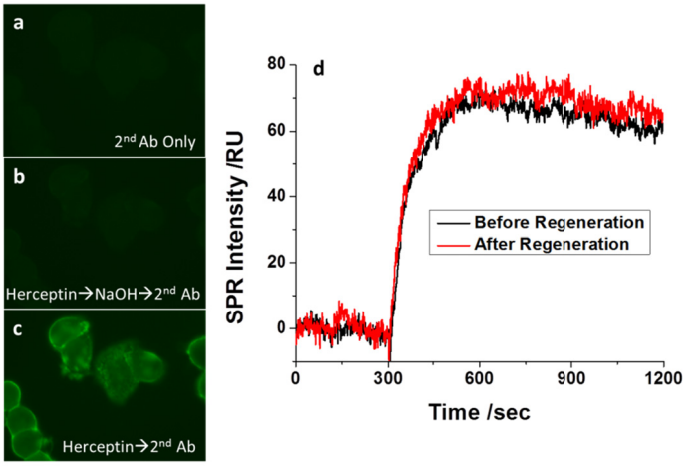

Fig. S2. Regeneration of Her2 binding sites by NaOH. Fluorescence images of cells incubated with: (a) secondary antibody; (b) Herceptin, NaOH and then secondary antibody, sequentially; and (c) Herceptin and then secondary antibody, sequentially. (d) SPR sensorgram of Herceptin binding to a cell before (black curve) and after the NaOH regeneration procedure (red curve).

3. Her2 Expression levels studied by immunofluorescence

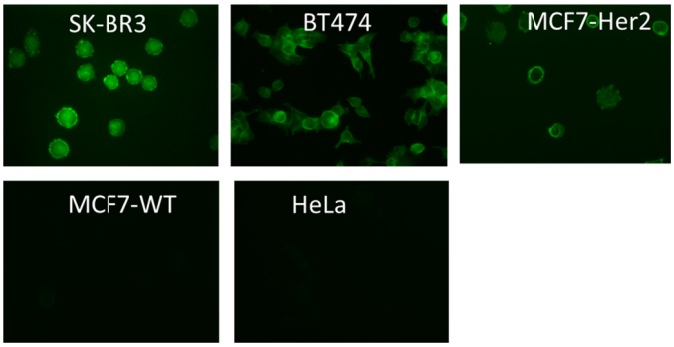

Fig. S3. Her2 Expression levels in five different cell lines used in the present work were studied by immunofluorescence (see Experimental section for details).

4. Herceptin-Her2 binding in human breast tumor primary cells

To examine the relevance of the above findings for the cell lines to the cells isolated from real tissues, the binding of Herceptin to human breast tumor primary cells was studied. The primary cells were extracted and purified from a patient who was diagnosed with early stage Her2 positive breast tumor. The cells were provided by CHI scientific Inc. (Jiangying, Jiangsu, China) as frozen vials. Upon receiving, the cells were cultured by following the manufacturer instructions and the 3rd to 5th passages of the primary cells were used in the SPRi experiments (See more details in Handbook of Primary Cell Culture: A Practical Manual to the Labtoratory Standard, http://www.chiscientific.com/show.aspx?id=63). In brief, primary cells were cultured in

freshly-prepared collagen I-coated flask (Corning, NY) to enhance the adhesion. The basal culture medium (CHI scientific Inc.) was used and the culture medium was changed twice every week. 0.5% Dispase I (Sigma, w/v) was used to harvest the primary cells from the flask. The cells were also fixed prior to the experiments.

Fig. S4a displays the SPR image of the 3rd passage of human breast tumor primary cells adhered on a SPR sensor chip. Note that the cells represent a mixed population of breast tumor and normal primary cells, as well as other supporting cells, such as fibroblast, due to the imperfect isolation and purification of breast tumor primary cells from biopsy tissues. The binding behaviors of Herceptin onto these cells are completely different, which is best shown in the Her2 distribution map in Fig. S4b. Significant increase in the SPR intensity was observed in only a few locations marked by white arrows. For the cells that exhibit SPR intensity increase, the magnitude of the increase varies from cell to cell, indicating different expression of Her2 in these cells. This variability in Her2 abundance, as well as binding kinetics, is more clearly shown by the sensorgrams from different locations (Fig. S4c).

The study presented above shows that SPR imaging may be used to evaluate the performance of anti-tumor drugs at single cell level in mixed population of cells. Important information includes 1) quantification of the amount and local distribution of Her2 receptors on membrane of individual cells; 2) analysis of Herceptin-Her2 binding kinetic constants, which are directly relevant to local drug residence time.

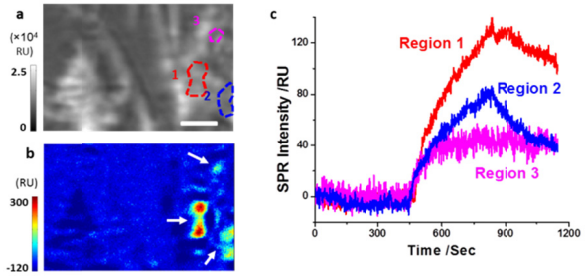

Fig. S4. Herceptin interaction with human breast tumor primary cells. (a) SPR image of adhered primary cells isolated from patient biopsy. Three regions of interest were indicated by color shapes. (b) Differential SPR image after injection of 4.2 μg/mL Herceptin solution. White arrows indicate the locations of regions 1, 2 and 3, respectively. (c) SPR sensorgrams in regions 1, 2 and 3 as shown in (a). Scale bar, 50 μm.

5. Cell proliferation of Herceptin sensitive and resistant subclones.

SKBR3 herceptin resistant (C5 and H6) sub clones keep growing in the presence of Herceptin, while sensitive (C11 and E8) cells not, as demonstrated by cell proliferation assay and observed on the micrographs (Fig S5). We confirmed that these subclones were all derived from the parental SKBR3, after performing a 24 SNP's analysis where 100% of concordance among all SKBR3 cells was found; in contrast less than 5% of the SNP's matched with other cell lines (MCF7, HeLa and 293T).

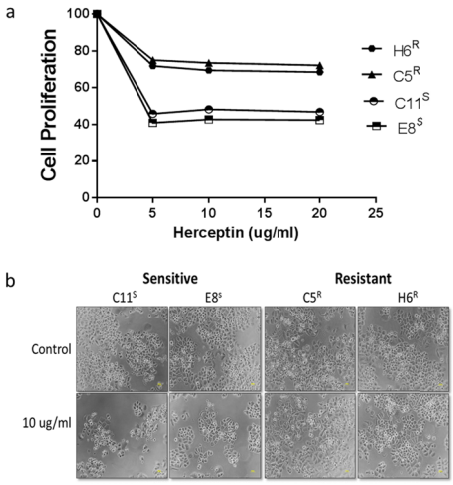

Fig. S5. (a) Cell proliferation of Herceptin sensitive (C11 and E8) and resistant (C5 and H6) subclones measured by resazurin, showing that resistant subclones continue to proliferate in presence of Herceptin after 9d of treatment. (b) Microscopy images showing more cells after herceptin treatment in resistant population compare to sensitive one.

6. Calculation of Herceptin Resistance Index (RI)

The HRI is defined as the percentage of ineffective population among the total Her2 population, which can be calculated from a single cell SPR sensorgram by using the following equation:

$$RI = 1 - \frac{\text{SPR intensity at the end of dissociation } (I_{end})}{\text{Maximal SPR intensity at the end of association}(I_{max})}$$

Briefly, if all the Her2 population exhibits ineffective Herceptin binding, the weakly bound Herceptin will be rapidly washed off and the SPR intensity at the end of dissociation equals to zero, leading to a HRI of one. Such cells are considered to be most resistant to Herceptin. Oppositely, if all the Her2 population shows effective Herceptin binding, majority of the Herceptin would remain on the cells at the end of dissociation, resulting in a HRI tending to zero. In this case, HRI has similar meaning as the drug residence time, in which slower k_{off} also results in better drug performance.

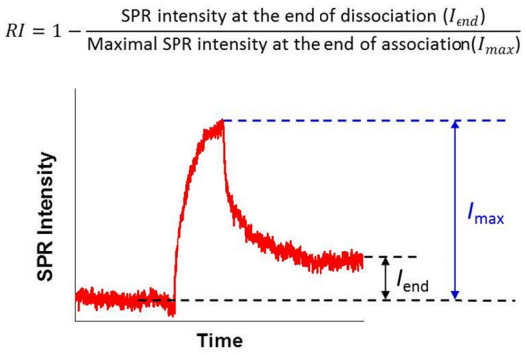

Fig. S6. The illustration of calculation of resistance index (RI).

7. Immunofluorescence Staining of MUC4 in Herceptin-sensitive clones

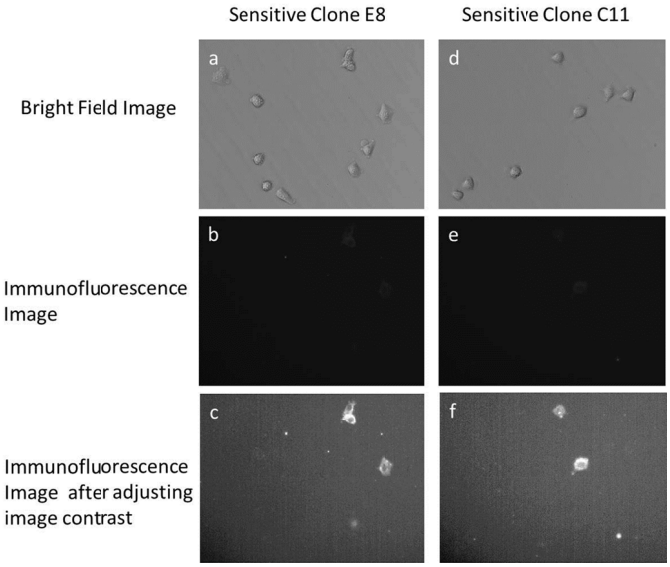

Fig. S7. The bright-field images (a,d), immuno-fluorescence images with the same capture conditions as those described in Fig. 5A (b,e) and MUC4 immuno-fluorescence images after adjusting the contrast for better visualization (c,f) for sensitive clones E8 (a-c) and C11 (d-f), respectively. While some of the sensitive cells indeed show minor amount of MUC4 expression, the expression levels are significantly lower than those in resistant clones (Fig. 5A).

8. Overall Her2 expression levels when using a different anti-Her2 antibody other than Her2.

Because it was found that Herceptin-Her2 interaction was at least partially affected in Herceptin-resistant cells, a different anti-Her2 polyclonal antibody (Cell Signaling Technology, Catalog No. #2242) was used to study the overall Her2 expression level in Herceptin-sensitive (C11 and E8) and Herceptin-resistant (C5 and H6) clones. The results below (Fig. S8) indicate that the amount of Her2 expression does not significantly change in Herceptin-resistant cells. These results are consistent with the fact that maximum SPR intensity in Herceptin-resistant cells was comparable with that in Herceptin-sensitive cells (Fig. 4A).

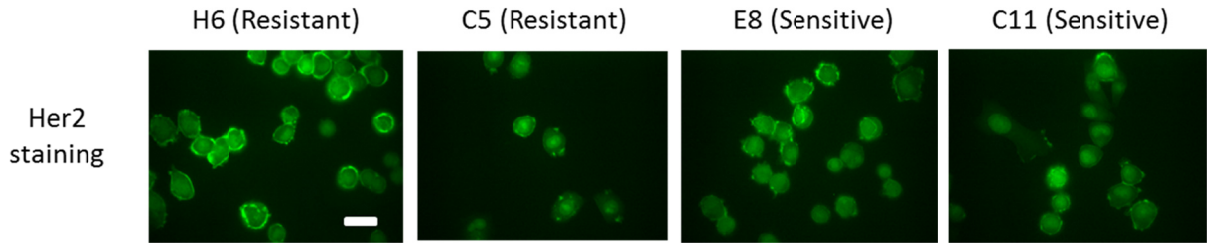

Fig. S8. Her2 staining results when using a different anti-Her2 primary antibody other than Herceptin.

9. Cross validation with dual staining of Her2 and MUC4

The cross validation of two pairs of primary and secondary antibodies were performed to show that there was no cross reaction during the dual staining of Her2 and MUC4 of cells on the same chip surface. From the results shown below (Fig. S9), it was found that the cross reaction between these two pairs of antibodies are minimal and the staining images shown in Fig. 5b represented the appropriate distribution of Her2 and MUC4, respectively.

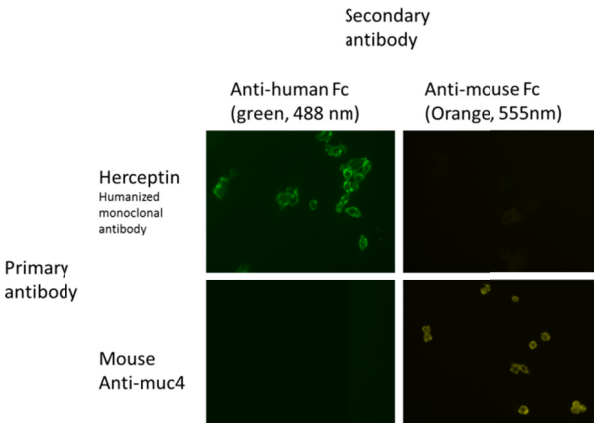

Fig. S9. Cross validation of two pairs of primary and secondary antibodies for Her2 and MUC4.