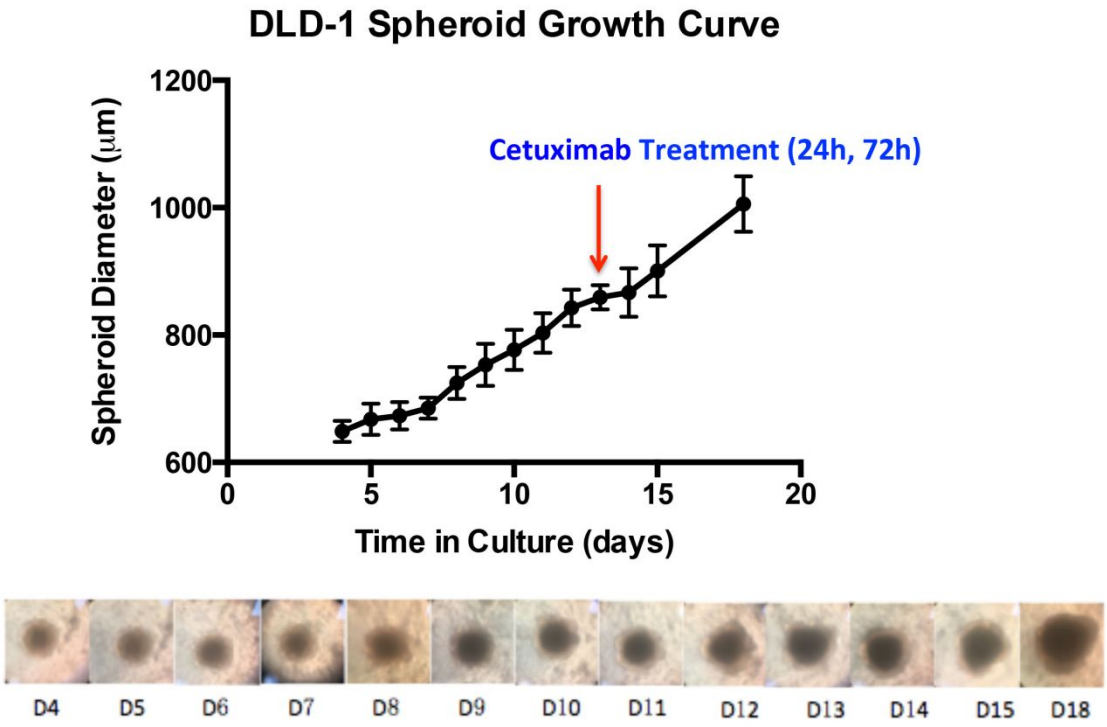
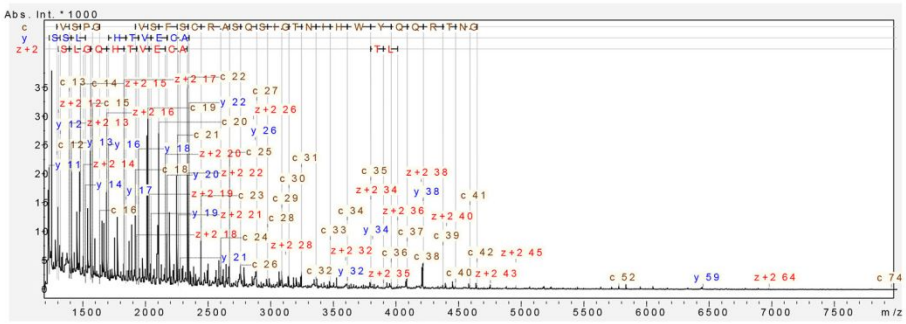
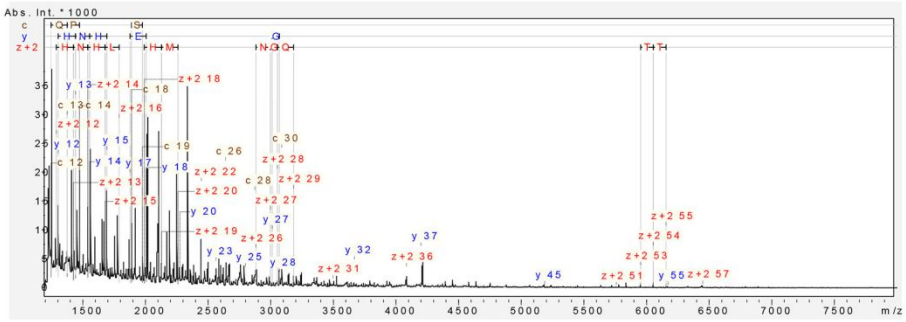
Supplemental Figure 1. Reproducibility of DLD-1 spheroid diameter. DLD-1 cells were seeded at 7000 cells/well. Photographs were taken of 16 spheroids daily and measured for spheroid diameter.



Supplemental Figure 2. MALDI-ISD spectrum of cetuximab in DAN matrix. The N-terminal c-ions and the C-terminal y- and z+2 ions are assigned in the spectra using BioTools software supported with de novo sequencing. Mass errors of fragments are <0.1 Da up to 8 kDa.

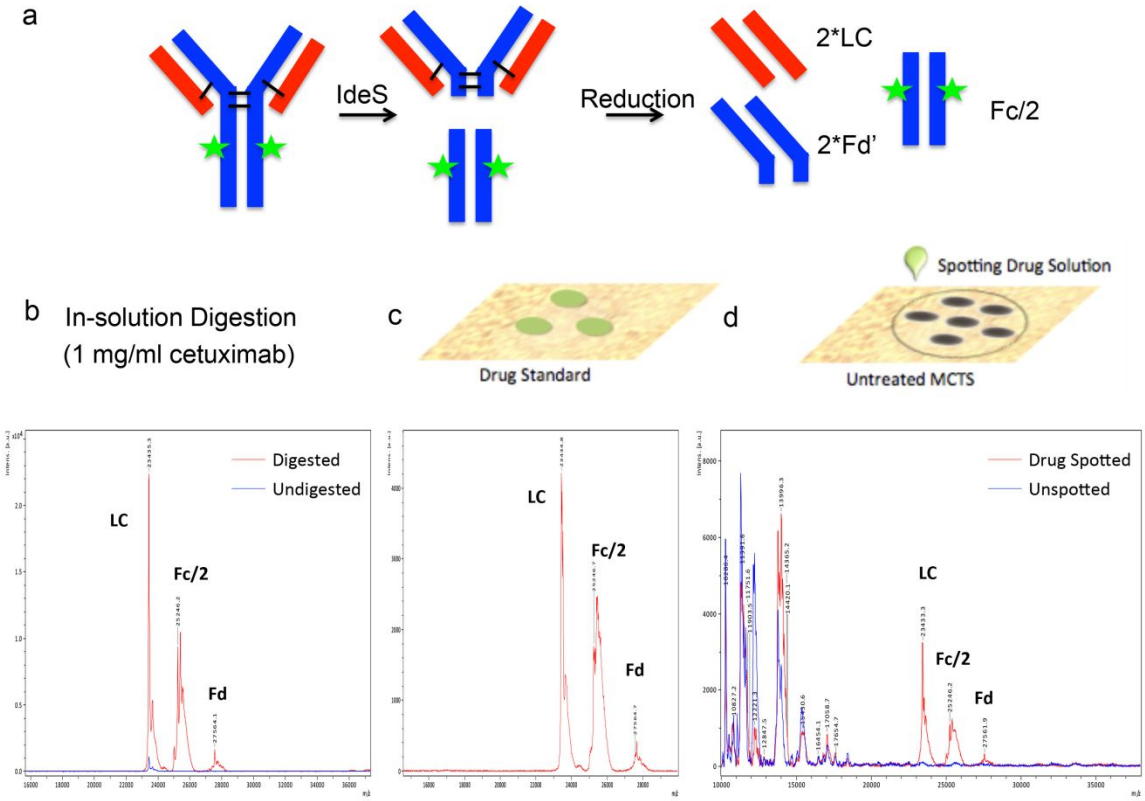


Light Chain of Cetuximab

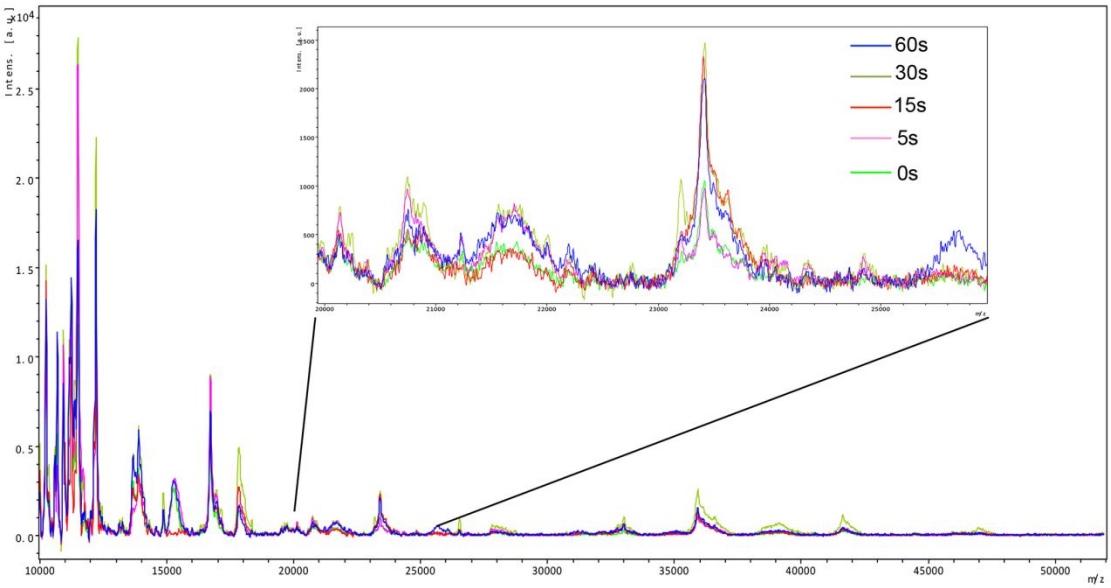


Heavy Chain of Cetuximab

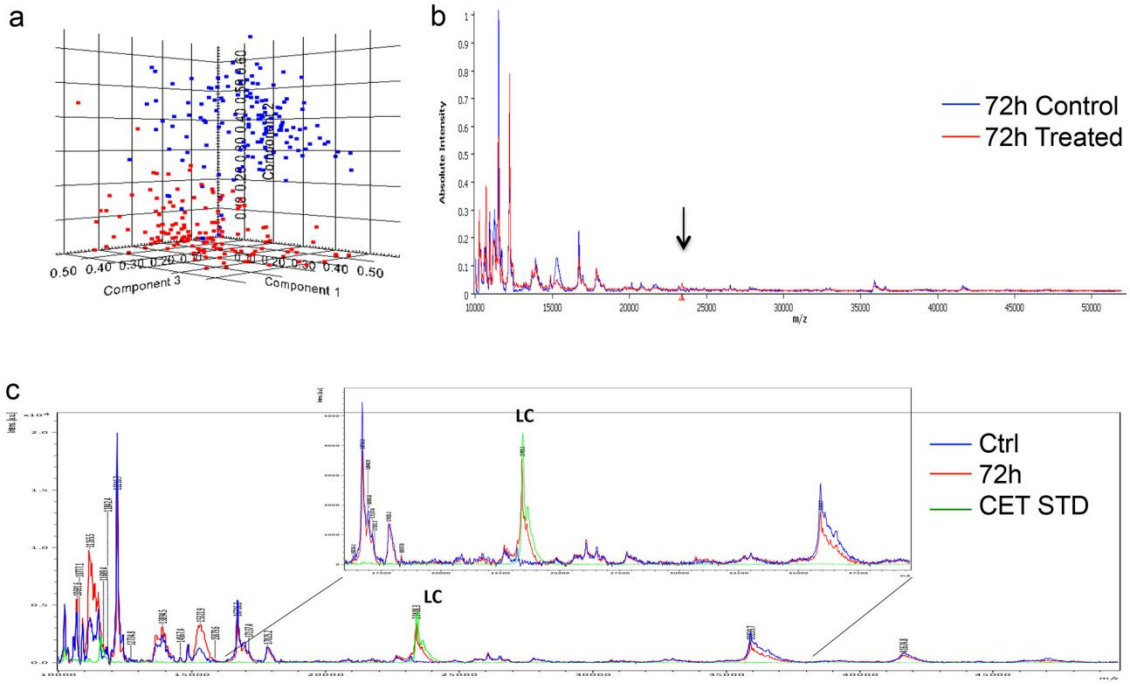
Supplemental Figure 3. ”Middle up” MALDI analysis of cetuximab using IdeS enzyme digestion followed by reduction. Illustration of the workflow is shown in (a). MALDI spectra were collected from samples of in-solution digestion of cetuximab (b), on glass slide digestion of spotted cetuximab (c), and on spheroid section digestion of spotted cetuximab (d).



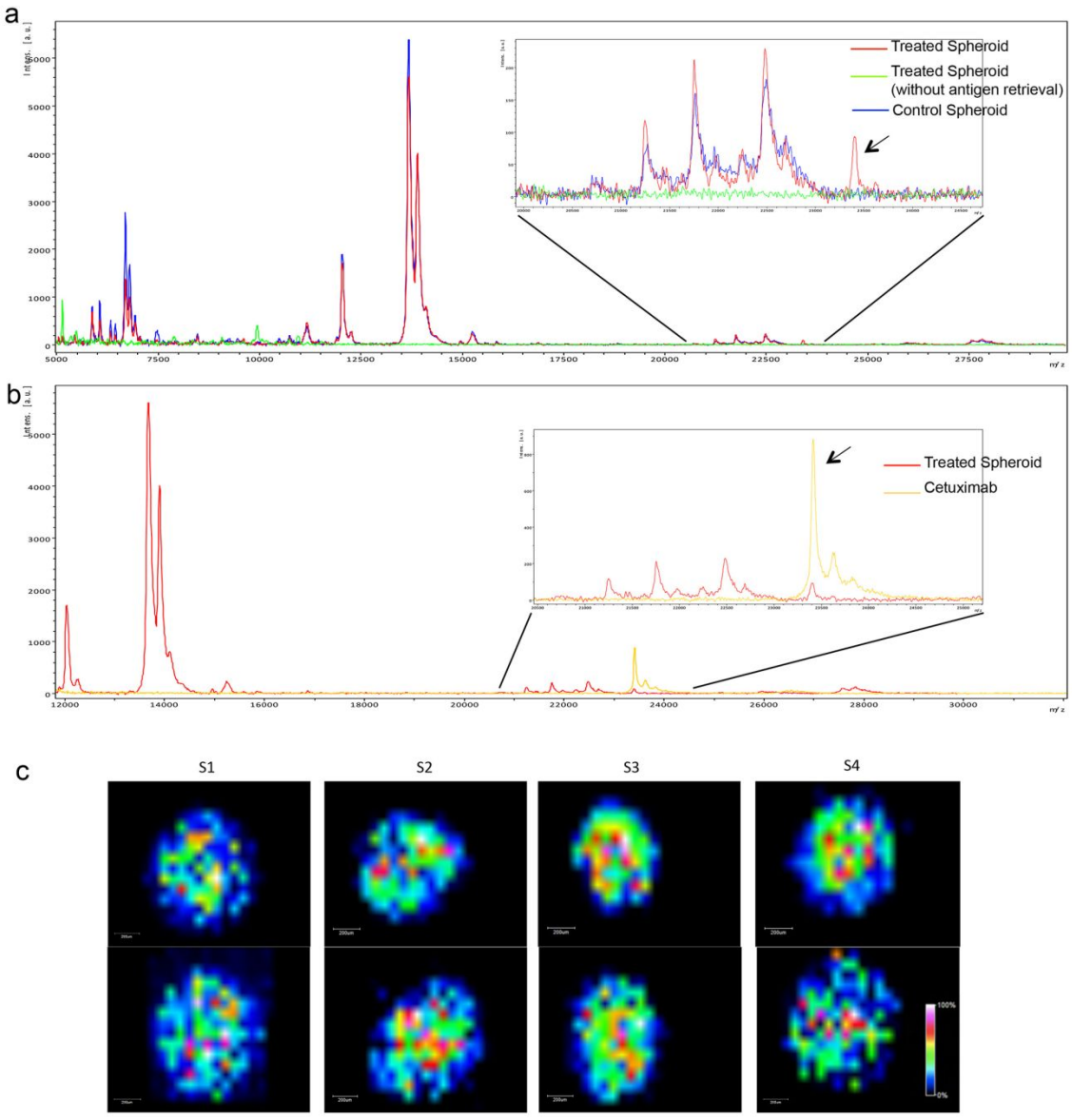
Supplemental Figure 4. MALDI MS protein profiles directly acquired from serial 72 h cetuximab treated spheroid sections not washed or washed with different washing conditions. All spectra were the sum of 3000 individual laser shots.



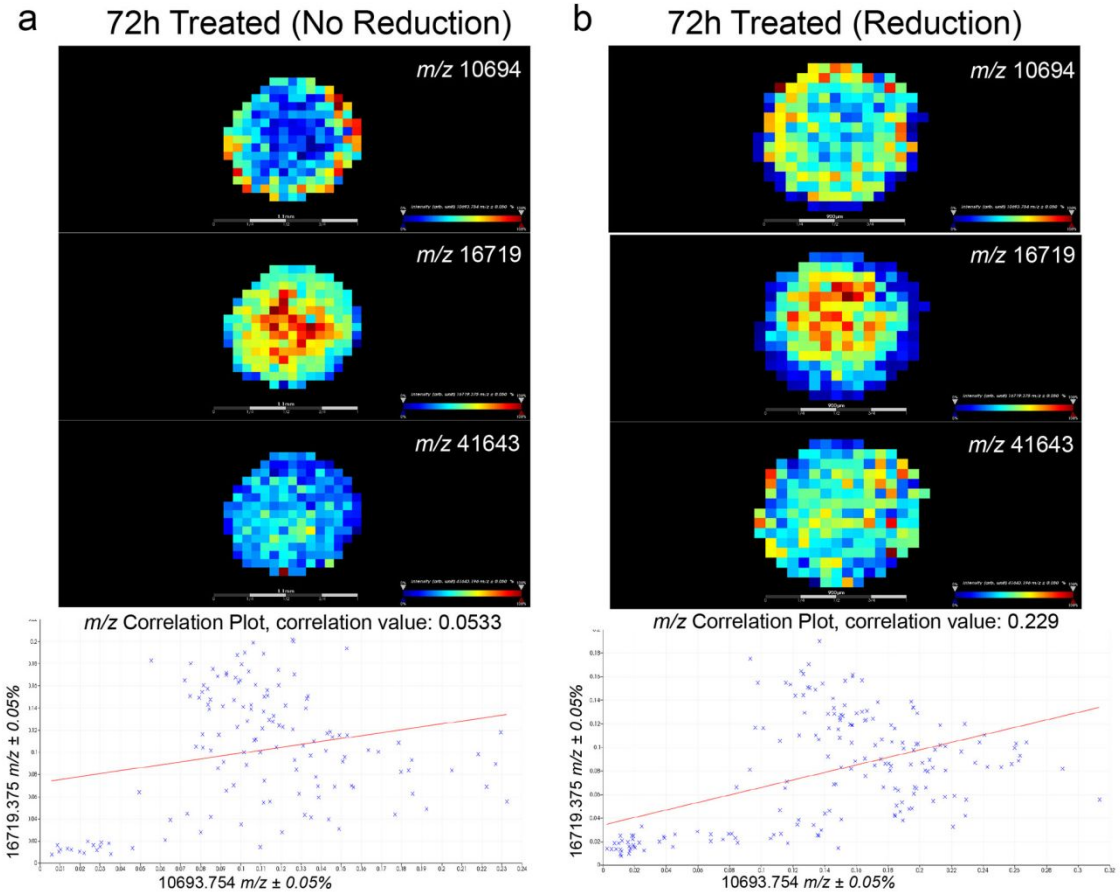
Supplemental Figure 5. pLSA was able to separate spectra from cetuximab treated and untreated spheroids as shown in the Score Plot (a). Summed spectra are shown in (b). MALDI spectra collected from drug treated spheroids were compared to MALDI results from analyzing reduced cetuximab standard solution (c), indicating that the peak detected in drug treated spheroid sample is from LC of cetuximab.



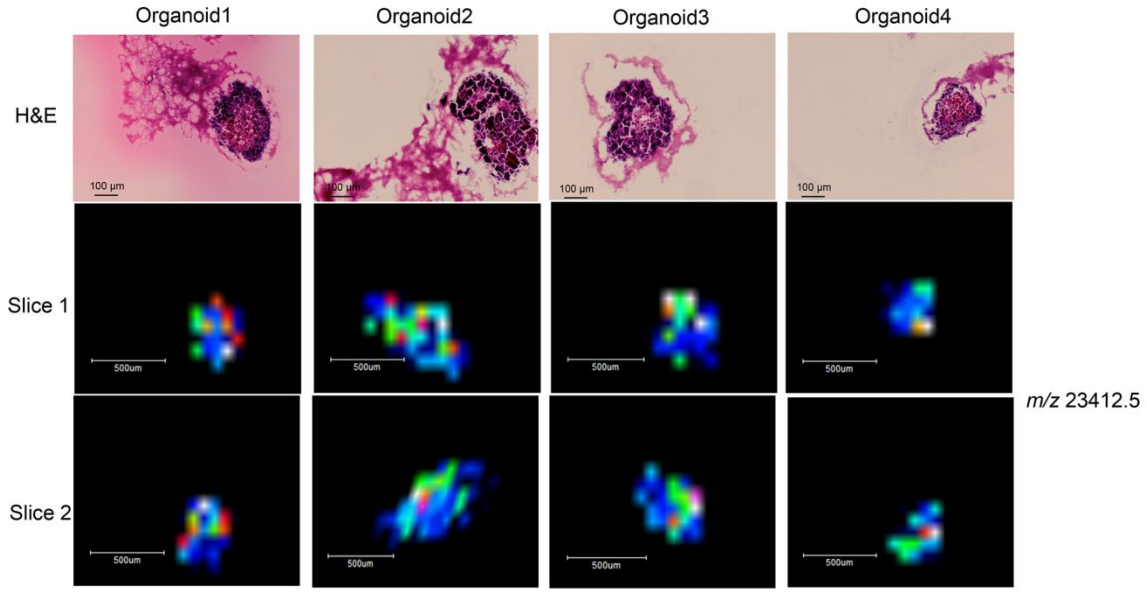
Supplemental Figure 6. MALDI MS protein profiles of formalin fixed HT-29 spheroids (a). MALDI spectra collected from drug treated spheroids were compared to MALDI results from analyzing cetuximab standard solution (0.1 mg/mL, 0.5 µl) spotted on slide followed by formalin fixation (b), indicating that the peak of *m/z* 23412.5 detected in drug treated spheroid sample is from cetuximab. (c) shows MALDI-MSI ion images with distribution of *m/z* 23412.5.



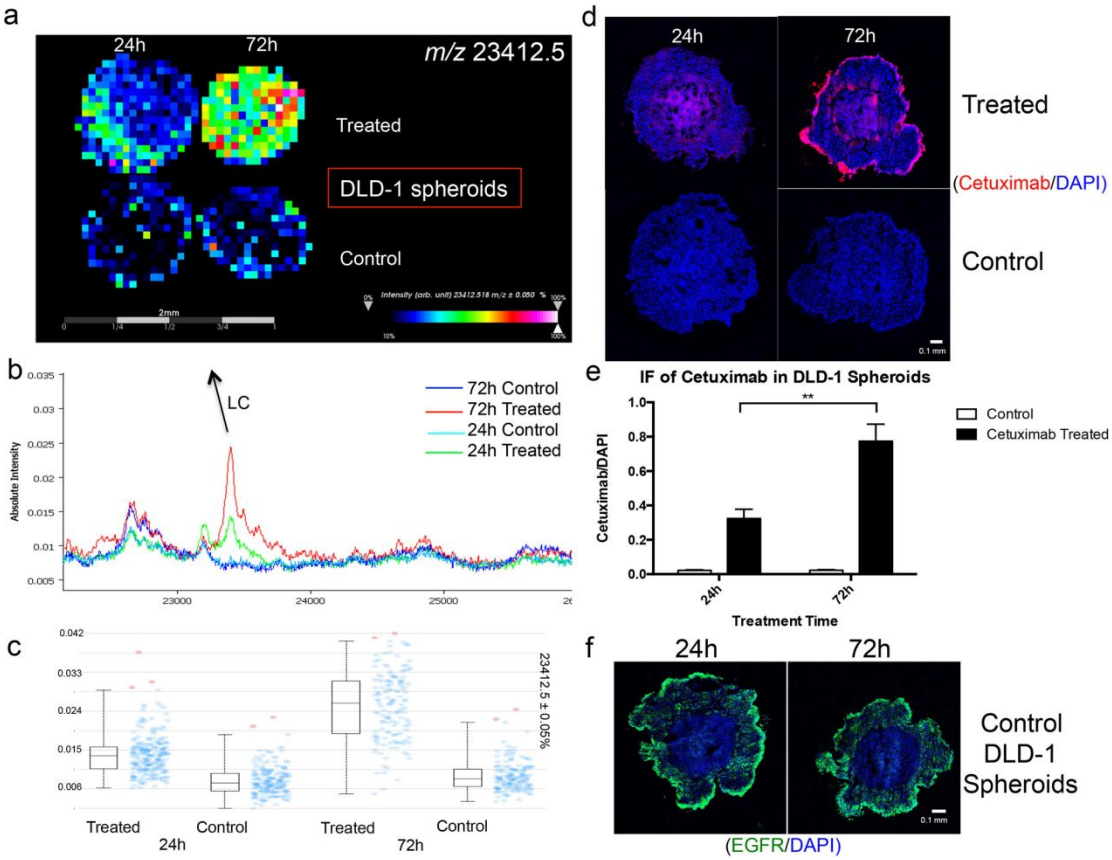
Supplemental Figure 7. Delocalization study of HT-29 spheroid sample with (b) and without (a) performing on-tissue reduction and alkylation. Ion density maps of three analytes (*m/z* 10694, *m/z* 16719, and *m/z* 41643) are shown, with the correlation analysis to evaluate co-localization of *m/z* 10694 and *m/z* 16719.



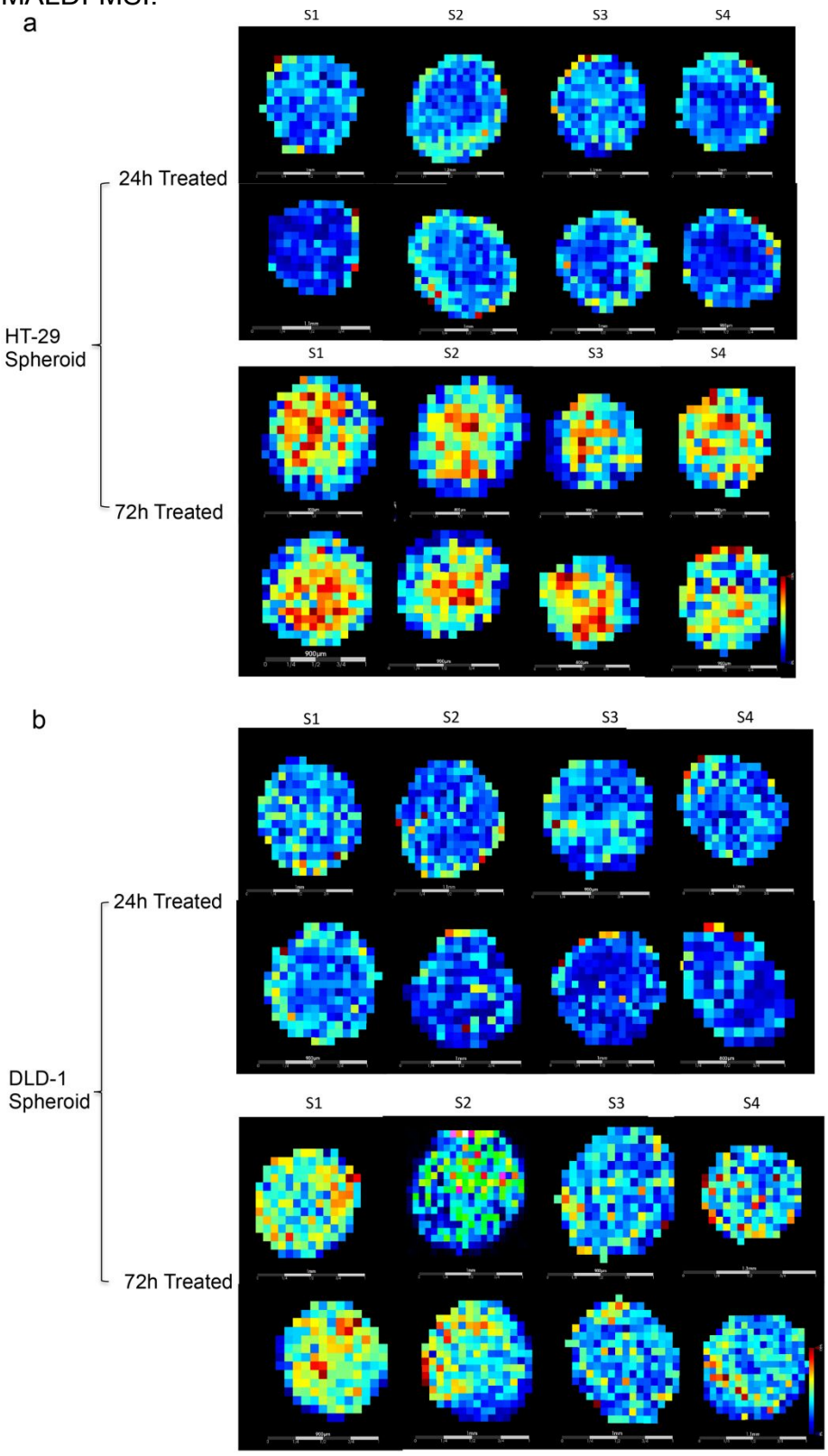
Supplemental Figure 8. Distribution of cetuximab in treated organoids analyzed using on-tissue reduction and alkylation combined with MALDI-MSI. H&E staining was performed on consecutive slices of organoids.



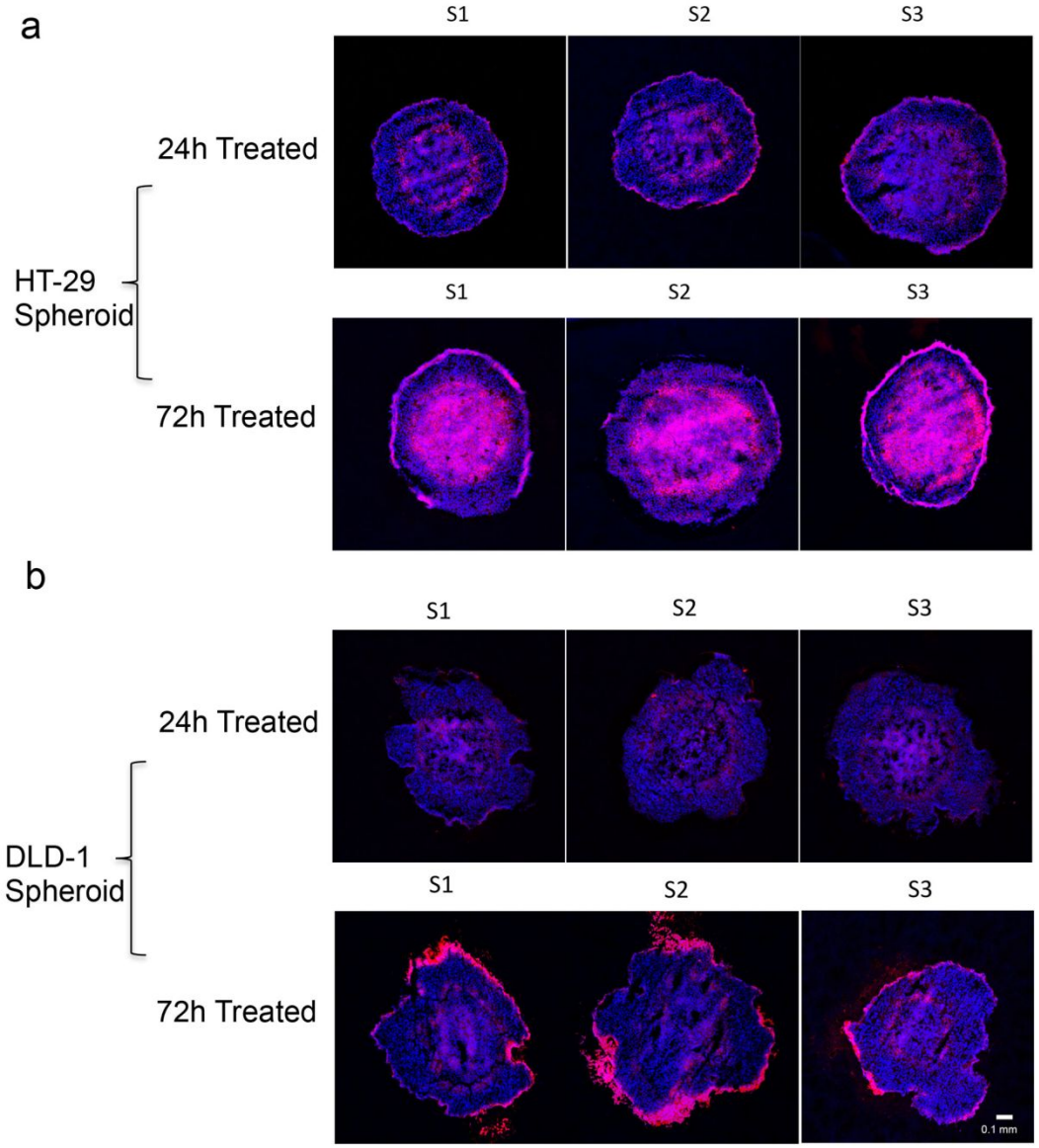
Supplemental Figure 9. MALDI-MSI ion intensity maps (a), summed mass spectra (b), and intensity box plots (c) of DLD-1 spheroids treated with 1 mg/mL cetuximab for 24 or 72 h. Immunofluorescence (IF) study of cetuximab localization is shown in (d). Data were normalized to DAPI intensity with statistical significance tested using Student *t*-test (e). n=6, * $p < 0.05$. (f) IF analysis of EGFR expression in DLD-1 control spheroids.



Supplemental Figure 10. Localization of cetuximab in treated HT-29 (a) or DLD-1 (b) spheroids analyzed using on-tissue reduction and alkylation combined with MALDI-MSI.



Supplemental Figure 11. Localization of cetuximab in treated HT-29 (a) or DLD-1 (b) spheroids analyzed using IF followed by fluorescence microscopy.



Supplemental Figure 12. IF study of cell proliferation (marker Ki-67) in DLD-1 spheroids with and without cetuximab treatment. (a) Typical IF images showing distribution of Ki-67 (green), and DAPI (blue) in 24h or 72 h treated and untreated spheroids (1 mg/mL). (b) Statistical relative quantification of Ki-67 changes in spheroids following drug treatment. Data were normalized to DAPI intensity. Student *t*-test was used to test the statistical significance. n=6, $*p < 0.05$