Supplementary Information

Validation of alamarBlue viability assay

In order to confirm the viability results achieved by the alamarBlue assay in this paper, we compared the results to the viabilities obtained by fluorescence microscopy imaging using live/dead stain. We stained the A431.H9 spheroids (initially 1500 cells/spheroid) treated by 5-FU with various concentrations for 48 hrs using LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (L3224, Invitrogen). The kit measures the cell viability based on the integrity of cell membranes. The live cells are stained by Calcein AM, which emit green fluorescence light (517 nm) when excited by blue light (494 nm); while the dead cells are stained by Ethidium homodimer-1, which emit red fluorescence light (617 nm) when excited by green light (528 nm).

To estimate the viabilities from the fluorescence images of 3D spheroids, we captured the fluorescence images at 10 different focal planes with 20 μm apart. The images were then reconstructed by stacking focused parts of each figure into a single image using an image processing and analysis software ImageJ developed by NIH with a plug-in, Stack Focuser, which is developed by Michael Umorin (http://rsbweb.nih.gov/ij/plugins/stack-focuser.html). Figure S1A shows the comparison between two methods, and Fig. S1B shows the reconstructed fluorescence images. For the four concentrations tested in the experiments, the viabilities obtained from the two methods have discrepancies less than 10%, except for the 1 μM 5-FU case. Such viability discrepancy may be caused by the different principles for estimating the cell viability in these two methods. In addition, due to the 3D structures of the spheroids, it is usually challenging to image an intact spheroid. Also, fluorescence dye may not be able to freely diffuse into an intact spheroid.

Moreover, we tested the linearity of the alamarBlue assay for 3D spheroids viability measurements. We transferred uniformly sized 1500-cell A431.H9 spheroids into 96-well plates at various number of spheroids per well, and performed alamarBlue assay (6 hr-incubation) for the wells with different spheroid numbers. Figure S1C shows the measurement results, which suggests the excellent linearity of the assay. In summary, precisely estimating cell viability for 3D cultures in a high throughput manner is still a challenging task, and alamar Blue assay demonstrated in this paper provides a reasonable solution.

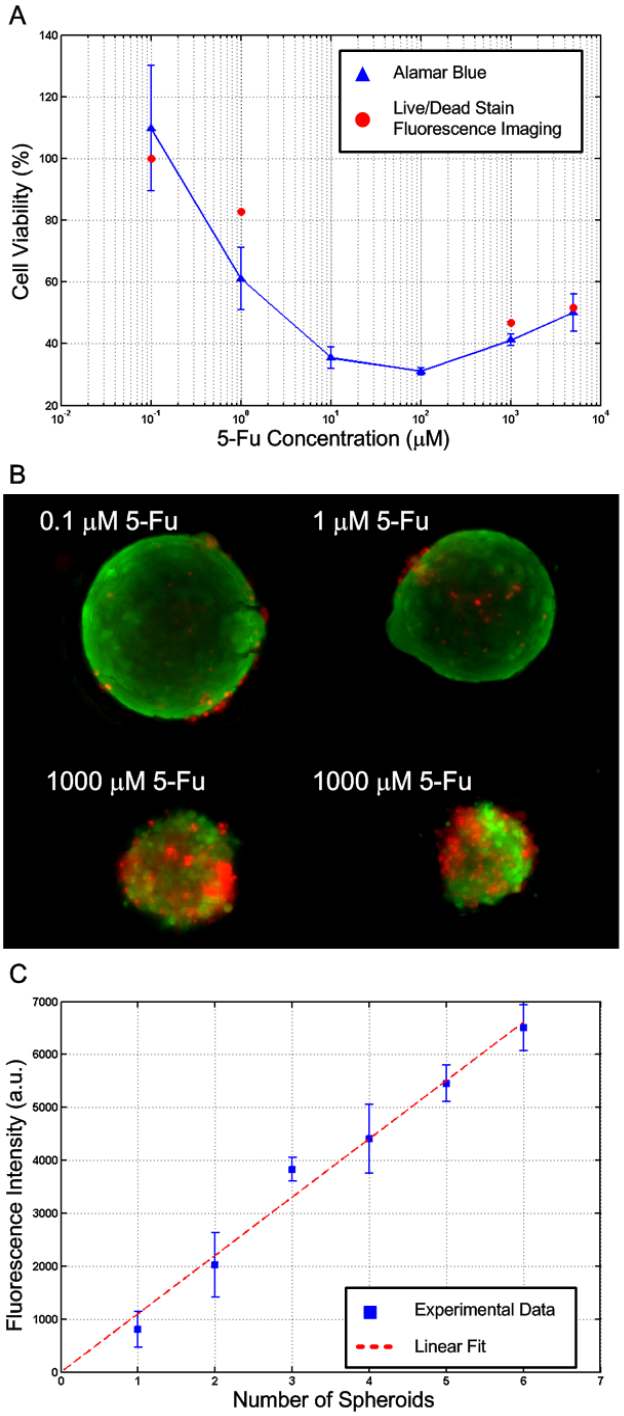

Fig. S1 (a) Comparison of cell viability of A431.H9 spheroids based on alamarBlue assay and fluorescence microscopy imaging with live/dead stain. (b) Fluorescence images of the A431.H9 spheroids treated with different concentrations of 5-Fu. (c) Alamar Blue assay linearity characterization: fluorescence intensity measurements in the Alamar Blue assay for different numbers of A431.H9 spheroids. n = 5 for each 5-FU concentration in (a), and n = 5 for each spheroid number group in (c). Data are expressed as the mean ± standard deviation.