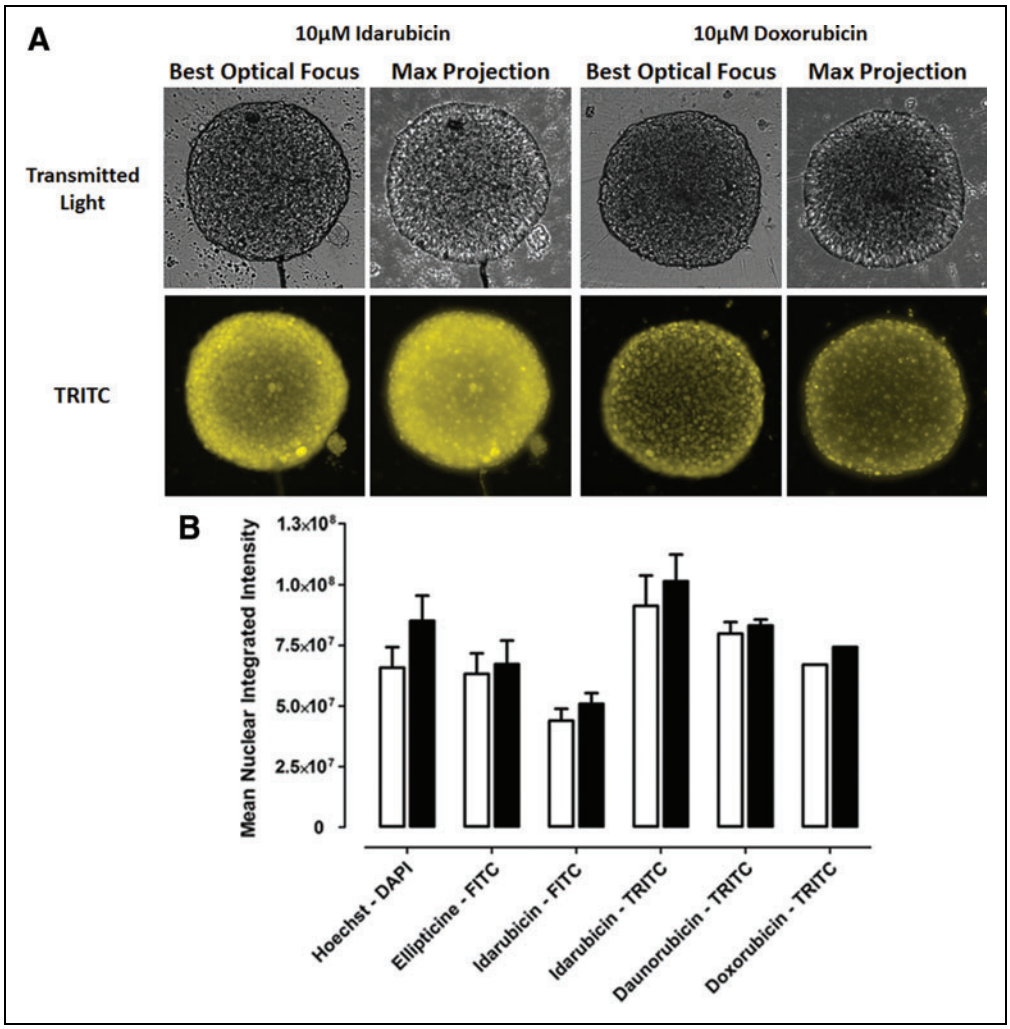

Supplementary Fig. S2. *Z*-stack image acquisition and 2D maximum projection versus single best focus image acquisition; **(A)** transmitted light and TRITC images of FaDu MCTSs exposed to idarubicin or doxorubicin. FaDu cells were seeded into 384-well ULA plates at 5,000 cells per well; after 24 h in culture, 10 μM of idarubicin or doxorubicin was added to the MCTSs, incubated for 1 h, fixed in formaldehyde, and washed with PBS. Transmitted light and TRITC images of MCTSs were then acquired on the IXM. *Z*-stacks of 10–20 images each separated by 20 μm were acquired and then collapsed by the MetaXpress software to generate a single 2D maximum projection image. To generate best focus images, we used the image-based autofocus method of the MetaXpress software in the DAPI channel to select the best focal plane and a single best focus image of each MCTS was then acquired for each channel. Representative transmitted light images and fluorescent images colored by channel (TRITC—*yellow*) are presented. **(B)** Fluorescent drug accumulation in FaDu MCTSs. The MWCS image analysis module was used to analyze the fluorescent drug accumulation in 2D maximum projection images (■), and single best focus images (□) of FaDu MCTSs were exposed to 2 μg/mL Hoechst or 10 μM ellipticine, idarubicin, daunorubicin, or doxorubicin for 1 h. The mean ± SD ($n = 6$) of the mean integrated fluorescent intensities from replicate MCTS wells for Hoechst-DAPI, ellipticine-FITC, idarubicin-FITC, idarubicin-TRITC, doxorubicin-TRITC, and daunorubicin-TRITC are presented. Representative data from one of three independent experiments are shown. 2D, two-dimensional; DAPI, 4,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; IXM, ImageXpress® Micro; MCTSs, multicellular tumor spheroids; MWCS, multi-wavelength cell scoring; TRITC, tetramethylrhodamine; ULA plates, ultra-low attachment microtiter plates.