

**Electronic Supplementary Information:**

**High-throughput Protease Activity Cytometry Reveals Dose-dependent Heterogeneity in PMA-mediated ADAM17 Activation**

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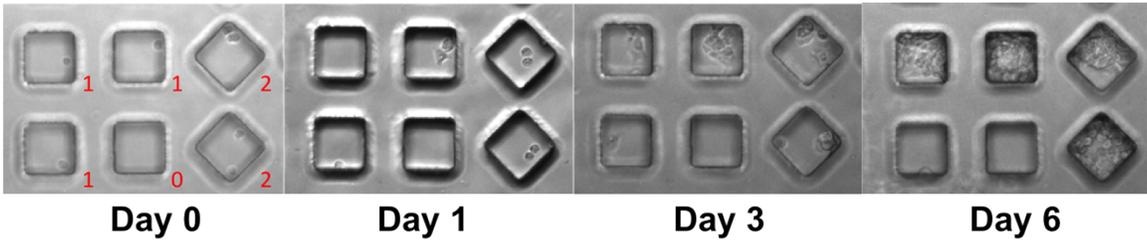
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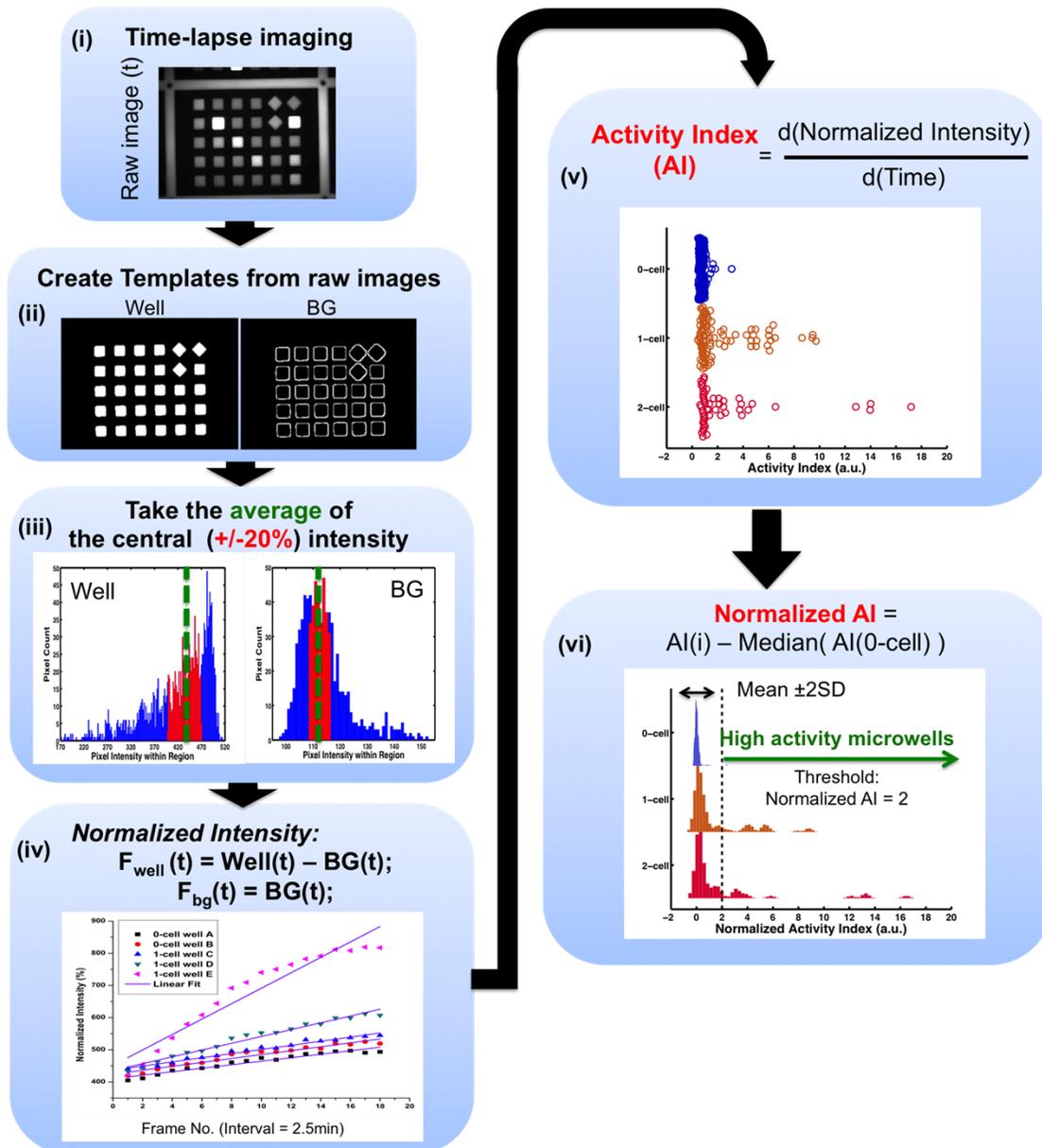
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**Supplementary Figures**



**Fig. S1.** Representative images for HepG2 cells cultured on collagen-coated microwell array. The red digits indicate the number of cells in each microwell immediately after seeding on Day 0. Cell spreading and proliferation was observed over time during complete medium culture. And the images were taken using a phase contrast microscope.



**Fig. S2.** Schematic for signal acquisition, processing and normalization. (i) The time-lapse raw images for the substrate cleavage were captured by fluorescence inverted microscope and then stabilized using Image Stabilizer plugin of Image J® software. Given the non-uniform illumination within observation window and the well-to-well interference due to light scattering, a normalization method was developed for signal processing. (ii) Briefly, two templates were generated based on the raw images with the aid of MATLAB and defined the regions within each microwells (Well) and the background regions around the wells (BG), respectively. (iii) The pixel intensity within each region was further sorted in ascending order and the extreme values at both ends would be discarded. The average value (green dash line) of the central  $\pm 20\%$  pixel intensity (red histogram) was then considered as the intensity indicator for that region. In this manner, one can reduce the variance introduced by inaccurate microwell border identification and the presence of bright objects within microwell. (iv) For background regions, the

average value became the normalized intensity. But for microwell regions, the average value of each well region was further subtracted by the normalized intensity of its surrounding background region. Then, one can obtain time-lapse profile of normalized intensity for individual microwells. Example profiles of microwells with 0 or 1 cell are shown here. (v) We modeled the protease-mediated substrate cleavage as the classical Michaelis-Menten model, where the initial rate of cleavage is proportional to the concentration of active enzyme in the system with excessive substrate of nearly constant concentration. Therefore, we defined the protease activity index (AI) as the increasing rate (i.e. slope) of normalized fluorescence intensity and extracted the AI value for each region via robust linear least-squares fitting of the time-lapse normalized intensity profile. A representative “bee swarm” plot is shown for the microwell AI values of 0-cell, 1-cell and 2-cell wells using data derived from a cell-loaded microwell array challenged with 0.8  $\mu\text{M}$  PMA. (vi) For array-to-array comparison among different cell-loaded microwell arrays, we used the median AI value of empty microwells (negative control) from each array as the reference value to account for the spontaneous substrate cleavage occurred in the absent of cells within that particular array. We further calculated the normalized AI value by subtracting each microwell AI value with the reference value of the same array and used the resultant normalized AI value to evaluate the protease response of cells within different microwell array. A representative histogram is shown for the normalized AI values of 0-cell, 1-cell and 2-cell wells, and the data is derived from the same assay shown in Fig. S2(v). Notice that all the histograms shown in this manuscript have been smoothed using MATLAB function *ksdensity().m* where the density estimation was based on a normal kernel function and the locations of kernel smoothing windows were robustly estimated via function *histogram().m*. Based on the normalized AI values of 0-cell wells, we also defined a threshold for high activity microwells to be normalized AI = 2, which is beyond 2 standard deviations away from the mean of 0-cell wells’ normalized AI values. Percentage of high activity microwells derived from a given protease assay then provides an indicator to quantify the overall protease response of all the single cells measured during that particular assay.