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# Development of a high-content imaging assay for screening compound aggregation

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A B S T R A C T

Aggregated compounds can promiscuously and nonspecifically associate with proteins resulting in either false inhibition or activation of many different protein target classes. We developed a high-content imaging assay in a 384-well format using fluorescently labeled target proteins and an Operetta cell imager to screen for compound aggregates that interact with target proteins. The high-throughput assay can not only directly detect the interaction between compound aggregators and the target of interest, but also determine the critical aggregation concentration (CAC) of a given promiscuous small molecule.

An underappreciated challenge in qualifying hits identified in a high throughput screening (HTS) campaign is detecting and removing promiscuous inhibitors and occasionally activators which often manifest as “false positives”. A significant source of these “false positives” is aggregation. Aggregated compounds function through a non-specific mechanism of inhibition resulting from the interaction of compound aggregates (which are comprised of many compound molecules) with a given target protein, rather than the specific binding of individual molecules to the target [1–6]. These aggregates form submicron particles in aqueous media, and have been reported to sequester nearly 10,000 protein molecules per aggregate particle [1,7].

Assays to detect promiscuous aggregates have been developed that either measure the detergent-sensitivity of aggregation based inhibition [6,8,9] or use dynamic light scattering (DLS) to measure the particle size of the aggregate [1,10,11]. Limitations exist for both types of assays. The detergent-based assays require an enzyme activity based readout and optimization of the detergent, as aggregation is concentration and solution condition dependent [6]. Light scattering can result from other aggregative phenomena, such as precipitation, resulting in a false positive readout [8]. Furthermore, DLS data acquisition has limited throughput. It can take up to 3 h per 384-well plate due to the size heterogeneity of aggregates in buffers, resulting in an increased number of data points needed for DLS analysis [8]. In the same study, the authors showed that a number of compounds tested scattered light with an intermediate intensity, providing an ambiguous measurement of compound aggregation [8]. An underlying cause of these limitations is the inability of these assays to directly measure the interaction between the compound aggregates and the target protein. Here we describe an image based, high-throughput assay that can not only directly detect the interaction between compound aggregators and the target of interest, but also determine the critical aggregation concentration (CAC) of a given promiscuous small molecule.

Visual evidence for the direct interaction between proteins and compound aggregates has been reported in the literature [1]. First, transmission electron microscopy (TEM) was used to directly observe the interaction between the β-galactosidase enzyme and its aggregated inhibitor, I4PTH [4]. Next, confocal fluorescence microscopy enabled the visualization of the interaction between green fluorescent protein (GFP) and the I4PTH aggregates as discrete bright spots approximately 1 μm in diameter, whereas GFP alone only produced a diffuse field of fluorescence. Based on these observations, we designed a high content imaging assay to detect the discrete binding event formed when compound aggregators interact with a fluorescently labeled protein, then quantify the intensity and number of bright spots to determine the CAC for a given compound.

In our initial experiments, we tested the ability of three known promiscuous compounds (sulconazole, econazole, and I4PTH) to aggregate and form discrete bright spots in the presence of GFP [11].

*Abbreviations used:* CAC, critical aggregation concentration; HTS, high throughput screening; GFP, Green Fluorescent Protein; DLS, dynamic light scattering; I4PTH, tetraiodophenolphthalein; SirT1, human NAD-dependent protein deacetylase sirtuin-1; TEM, transmission electron microscopy; CLND, Chemiluminescent Nitrogen Detection

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Prednisone was selected as negative control (non-promiscuous compound) due to its aqueous solubility and known specificity for nuclear receptors [12]. GFP was expressed and purified as reported [13]. We titrated GFP protein concentrations for the assay with 250 μM sulconazole to get the best ratio of signal to background in terms of bright spot formation, which represented a binding event between sulconazole and GFP. This titration resulted in an optimal signal to background ratio when 20 μg/ml of GFP was used (data not shown). The assay was run in 384-well poly-D-lysine clear plates, with an assay volume of 20 μl per well. Next, 20 μg/ml of GFP was incubated with different concentrations of sulconazole in PBS buffer for 2 min at room temperature. After incubation, the assay plates were read by the Operetta imaging system using the 40 × objective. Four images of each well were collected in the green (Alexa488) channel. The raw data was then exported as events per image analyzed. An algorithm was created to count the number of bright spots in each well image using the Columbus® High Content Imaging Analysis Software. We used the "Find nuclei function "and method "M" to count the population with the following parameters: "Splitting coefficient" = 0.00 and "Common threshold" = 0.75. After calculating imaging intensity, the populations were filtered by "nuclear intensity" (Alexa488) > 1600 to reduce the background. The output of the algorithm was the number of aggregated compound particles (bright spots) per image analyzed. Our initial data analysis indicated a direct correlation between sulconazole concentration and the number of bright spots detected (Fig. 1A). The sample wells containing the highest concentration of aggregator compound resulted in the formation of the most and brightest spots, while the sample with lowest concentration of sulconazole yield the fewest number of spots. The summary of the algorithm used to quantify the spots formed by compound aggregation in the presence of GFP is shown in Fig. 1B. To further demonstrate the utility of this assay to determine the CAC, three known aggregators (sulconazole, econazole, I4PTH, and the negative control prednisone) were tested over a 2-fold serial dilution range from 1000 μM to 0.488 μM with 20 μg/ml GFP. The results indicated that the three known aggregating compounds induced spot formation in a concentration dependent manner. Dose-response curves were plotted for the three known aggregating compounds (Fig. 1C). The dose response curves for the known compound aggregators indicate that initial bright spot formation occurs at concentrations between 32 μM and 62.5 μM. These numbers are similar to the reported DLS determined CACs: sulconazole is 20 μM, econazole is35 μM, and I4PTH is 10 μM [4] [11]. This illustrates the utility of this imaging assay as an orthogonal readout or even a replacement for DLS to screen for compound aggregation. Moreover, the data obtained from this imaging assay provided an additional layer of information compared to DLS, as the assay directly measures the interaction of target protein and compound aggregation. When combined with biochemical assay data, this assay can provide a useful filter for false positives from high throughput screening campaigns.

We chose the human NAD-dependent protein deacetylase sirtuin-1 (SIRT1) as an example of an early discovery target to evaluate the broader applicability of this assay. SIRT1 utilizes NAD to catalyze the removal of an acetyl moiety from the ε-amino group of lysine residues within numerous protein substrates, yielding the deacetylated protein substrate, nicotinamide, and 2′-O-acetyl-ADP-ribose [13]. The broad substrate promiscuity of SIRT1, coupled with its ability to serve as a sensor of the cytosolic ratio of NAD+/NADH, renders SIRT1 as an important regulator of transcription, energy metabolism and nutrient sensing [14,15]. SIRT1 activation has been suggested to have beneficial effects in several disease processes, thus triggering significant drug discovery efforts to find sirtuin activating compounds (STACs) [14]. Current literature evidence combined with internal data suggested that SIRT1 activators may be prone to nuisance mechanisms, including protein mediated compound aggregation [16]. For these reasons, we

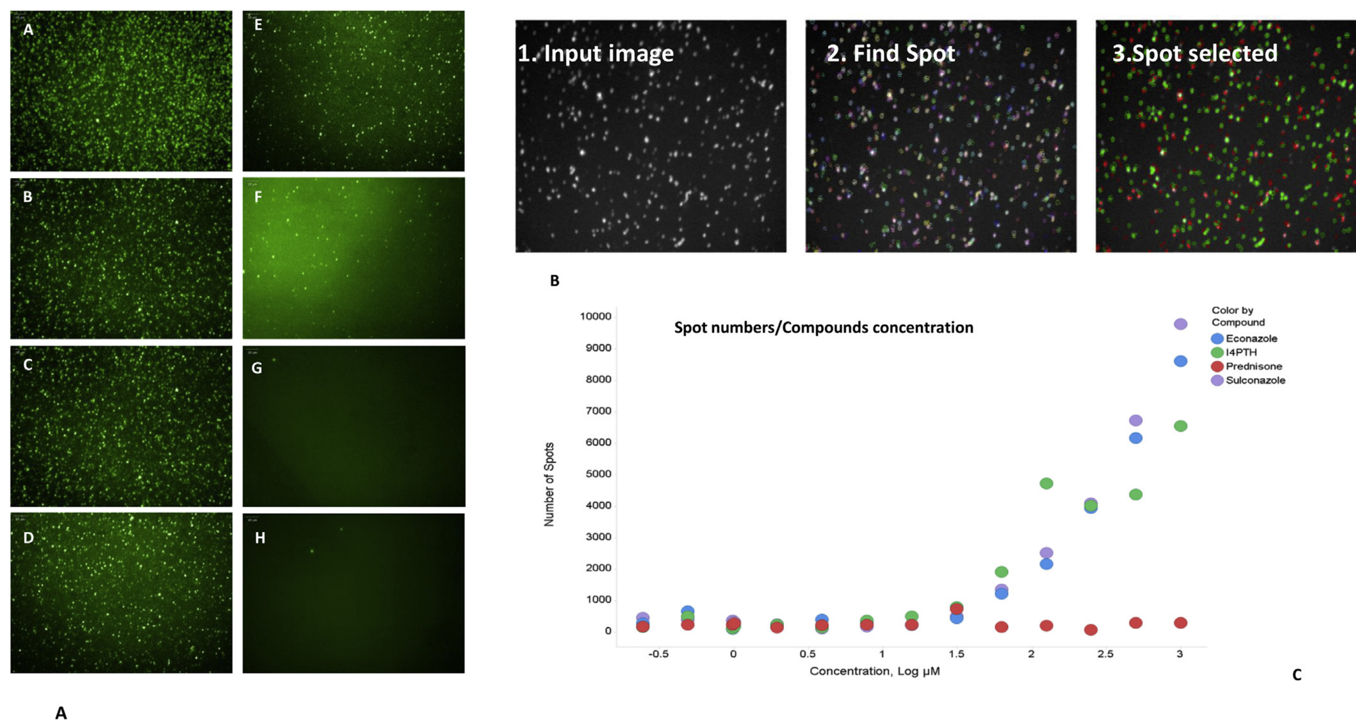


**Fig. 1.** A Visualization of sulconazole binding to GFP protein to form bright spots. The sample pictures from the wells with different sulconazole concentrations were acquired on a PerkinElmer Operetta. Protein concentration: 20 μg/ml for all wells, (A) GFP with 1000 μM sulconazole (B) GFP with 500 μM sulconazole (C) GFP with 250 μM sulconazole (D) GFP with 125 μM sulconazole (E) GFP with 62.5 μM sulconazole (F) GFP with 31.5 μM sulconazole (G) GFP with 15 μM sulconazole (H) GFP with 0 μM sulconazole B. Image analysis strategy with the Columbus® High Content Imaging Analysis Software. 1.) input images, 2.) find spots in the Alex 488 channel and calculate image properties, 3.) define the results (the final number of spots selected per spot area which represented the association of aggregated protein. C. Dose response for four control compounds in this assay (sulconazole, econazole, I4PTH and prednisone).

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