Analyzing Polyubiquitin Chains upon Ubiquitin Activating Enzyme Inhibition from Cell Culture & Tumor Lysates Using the Quanterix's Single Molecule Array (SiMoA) Technology

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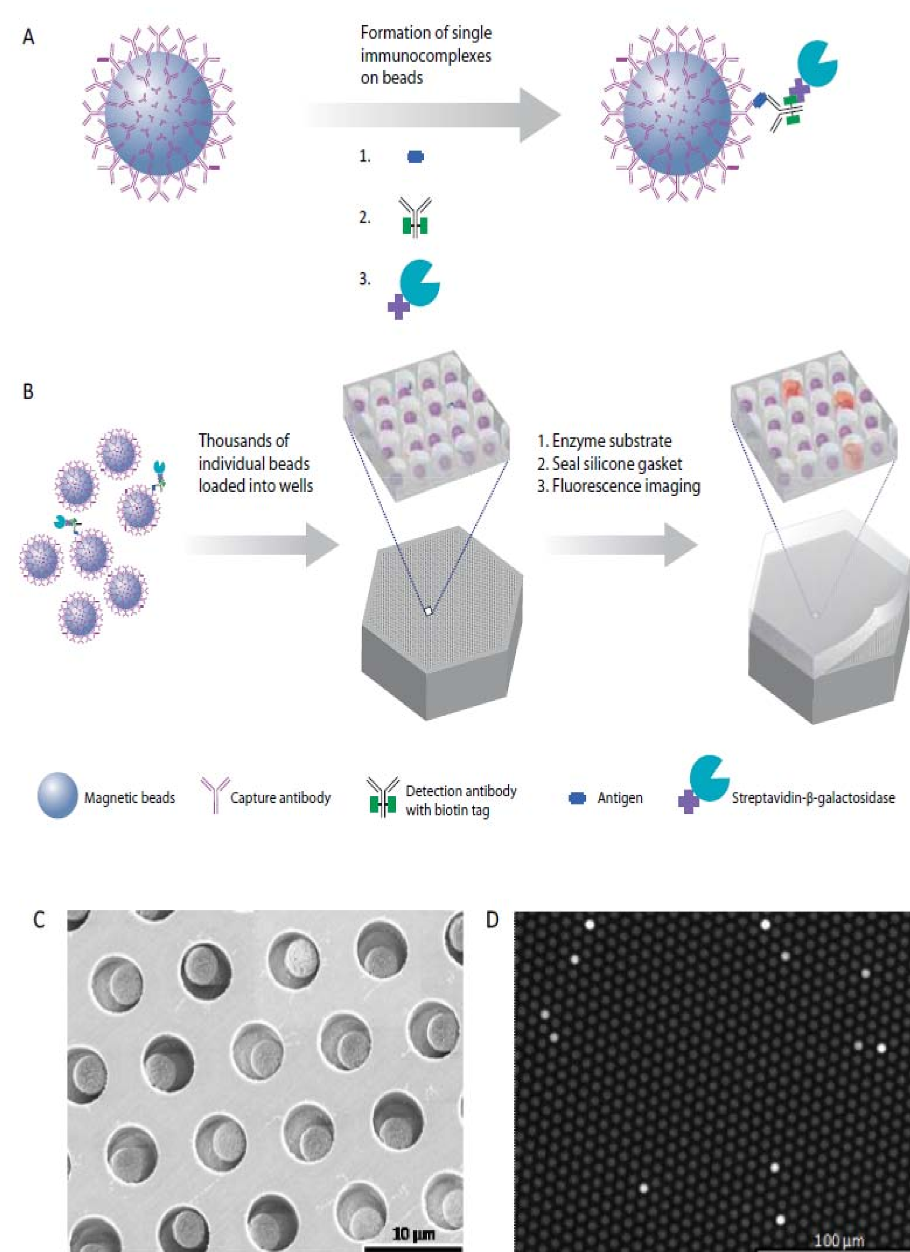
Abstract

Ubiquitination is a post-translational modification process which involves the attachment of ubiquitin(Ub) to lysine residues on either substrate proteins or itself resulting in protein monoubiquitination or polyubiquitination. This modification controls cellular activity by either protein degradation of the polyubiquinated target protein via the proteasome or alterations of signaling processes. Ubiquitination is mediated by the sequential action of three enzymes: the E1 (ubiquitin-activating enzyme(UAE)), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase) proteins. Targeting this process has been our focus to develop an anti-cancer drug. Monitoring polyubiquitin expression as a biomarker or pharmacodynamic (PD) marker in cancer cell culture or xenograft models treated with inhibitors of UAE is, therefore, a useful tool to identify a potential therapeutic molecule.

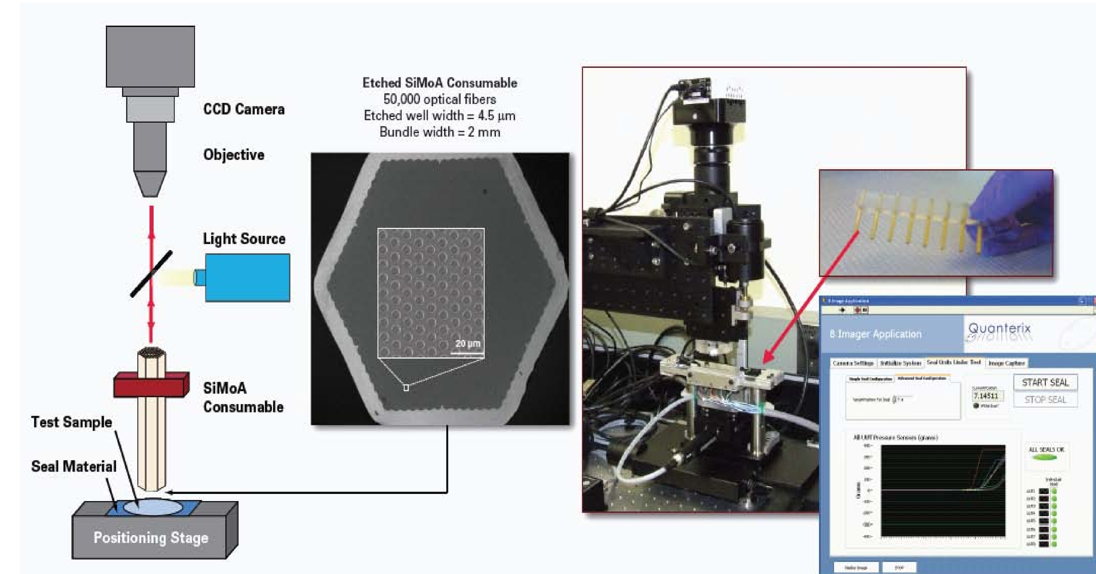
We employed a new technology from Quanterix called SiMoA (Single Molecule Array). This technology is capable of ultrasensitive protein measurements for monitoring the inhibition of polyubiquitination using femtoliter microwells to trap antibody captured molecules. Using both tissue culture and mouse xenograft samples and a complementary pair of commercially available antibodies against both linkage specific K48-polyUb and pan-polyUb, we first developed a protocol to establish a standard curve with recombinant K48-polyubiquitinated dihydrofolate reductase (polyUb$_5$-DHFR). We then compared samples treated or untreated with UAE inhibitors from HCT116 and WSU-DLCL2 cell culture as well as the corresponding xenograft tumor lysates to measure changes in poly-K48Ub levels. Our results show that the SiMoA technology is significantly more sensitive than other commercially available lysate-based platforms we have evaluated.

Technology Overview



AccuPSA based on arrays of femtoliter wells. (A) Capturing and labeling single protein molecules on beads using standard ELISA reagents. (B) Loading of beads into femtoliter well arrays for isolation and detection of single molecules. (C) SEM image of a small section of a femtoliter well array after bead loading. 2.7-μm-diam. beads were loaded into an array of wells with diameters of 4.5 μm and depths of 3.25 μm. (D) Fluorescence image of a small section of the femtoliter well array after signals from single enzymes are generated.

Instrumentation



Prototype SiMoA Instrumentation. Prototype SiMoA instrumentation was developed for assay validation and to support pharmaceutical and diagnostic collaborations. The SiMoA consumable is manufactured by etching tens of thousands of reaction vessels into the end of an optical fiber bundle. Strips of 8 fiber bundles are sized to sample one column of a microtiter plate, enabling convenient processing of up to 96-samples. The current bench-top instrument automatically seals the reaction vessels and concurrently reads the array of about 50,000 single molecule fluorescent assays. Sample preparation is done on a separate automated fluid handling workstation capable of processing hundreds of samples per shift.

Results

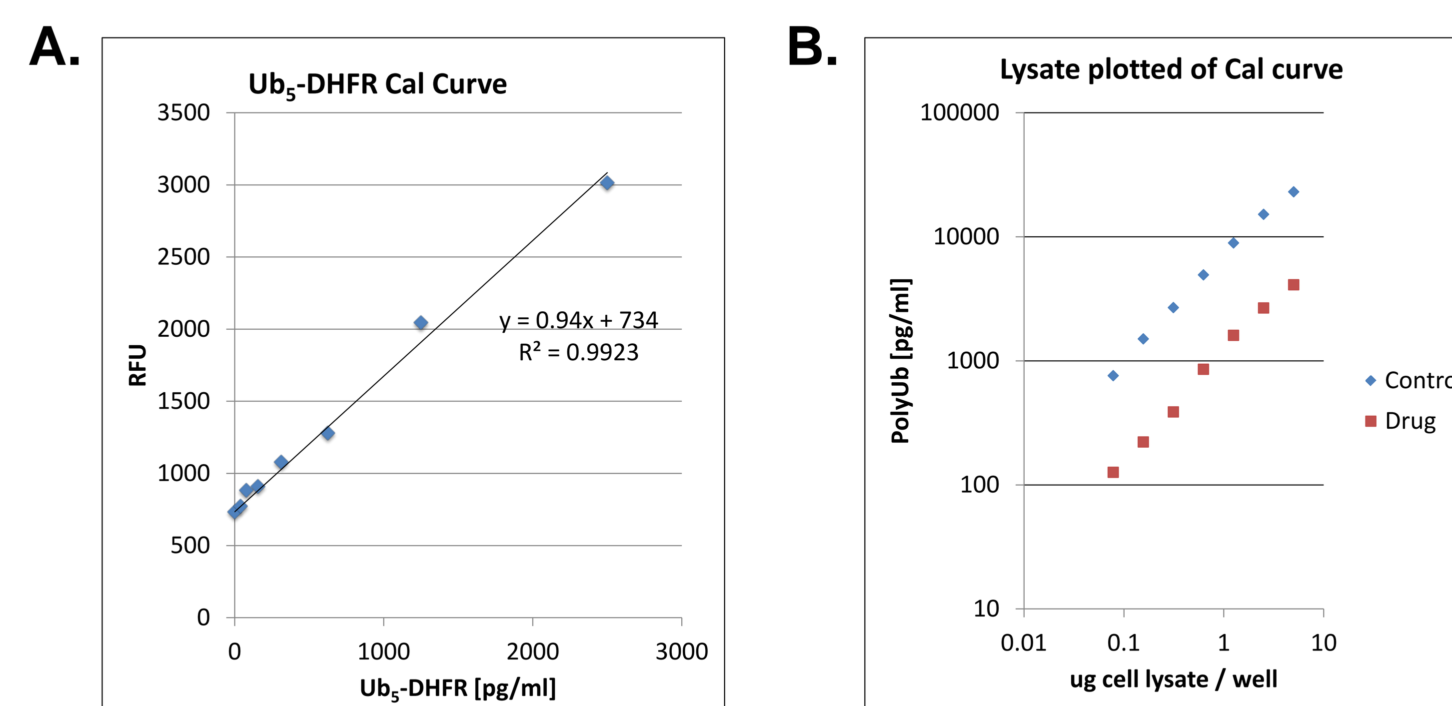


Fig 1. Establishing standard curve of K48 polyUb with QELISA (Panel A) to quantify cell culture lysates (Panel B). QELISA assay is a bead-based in ELISA format for high definition assay characterization and with less sensitivity as compared to SiMoA. Recombinant polyUb$_5$DHFR was synthesized in vitro and used to establish a standard curve (Panel A) titrated over an eight point two-fold dilution series in buffer. Complementary pairs of anti-pan polyUb (FK2) (Enzo) and anti-K48 linked polyUb (K48) antibodies (Millipore) were either labeled with biotin or conjugated directly to commercially available magnetic beads. Titration of HCT116 cell culture lysates derived from control or UAE inhibitor treated samples were then extrapolated from the polyUb$_5$ DHFR standard curve with detection of the analyte within 0.1 ug lysate protein per well (Panel B)

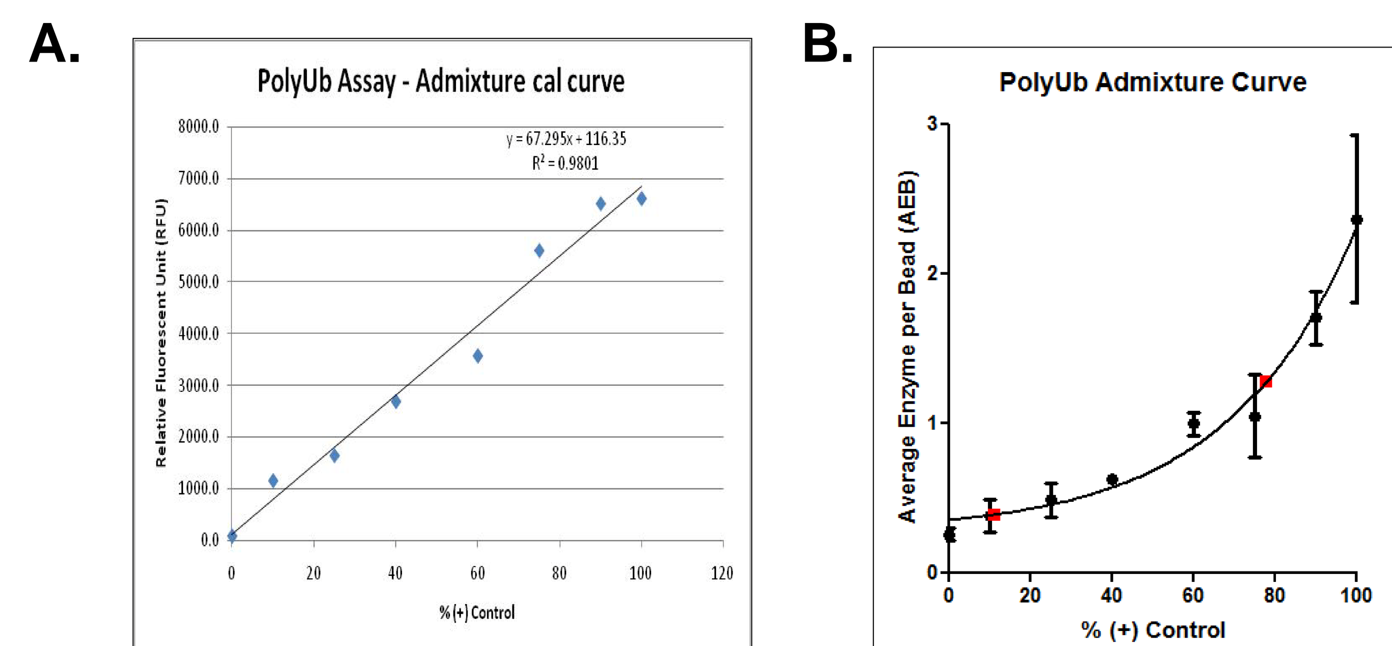


Fig 2. Establishing standard curve of admixture cell culture lysate in SiMoA. HCT116 cell culture lysates of DMSO control and UAE inhibitor treated (to knock down the polyUb expression to background) were mixed at differential ratios of control lysate in eight points series. Calibration curves were plotted in relative fluorescent unit (Panel A) or average enzyme per bead (AEB) (Panel B) using admixtures in SiMoA assay. HCT116 xenograft tumor lysates treated with vehicle or UAE inhibiting drug were plotted against the admixture curve (red square dots for 77.7 and 10.9 respectively).

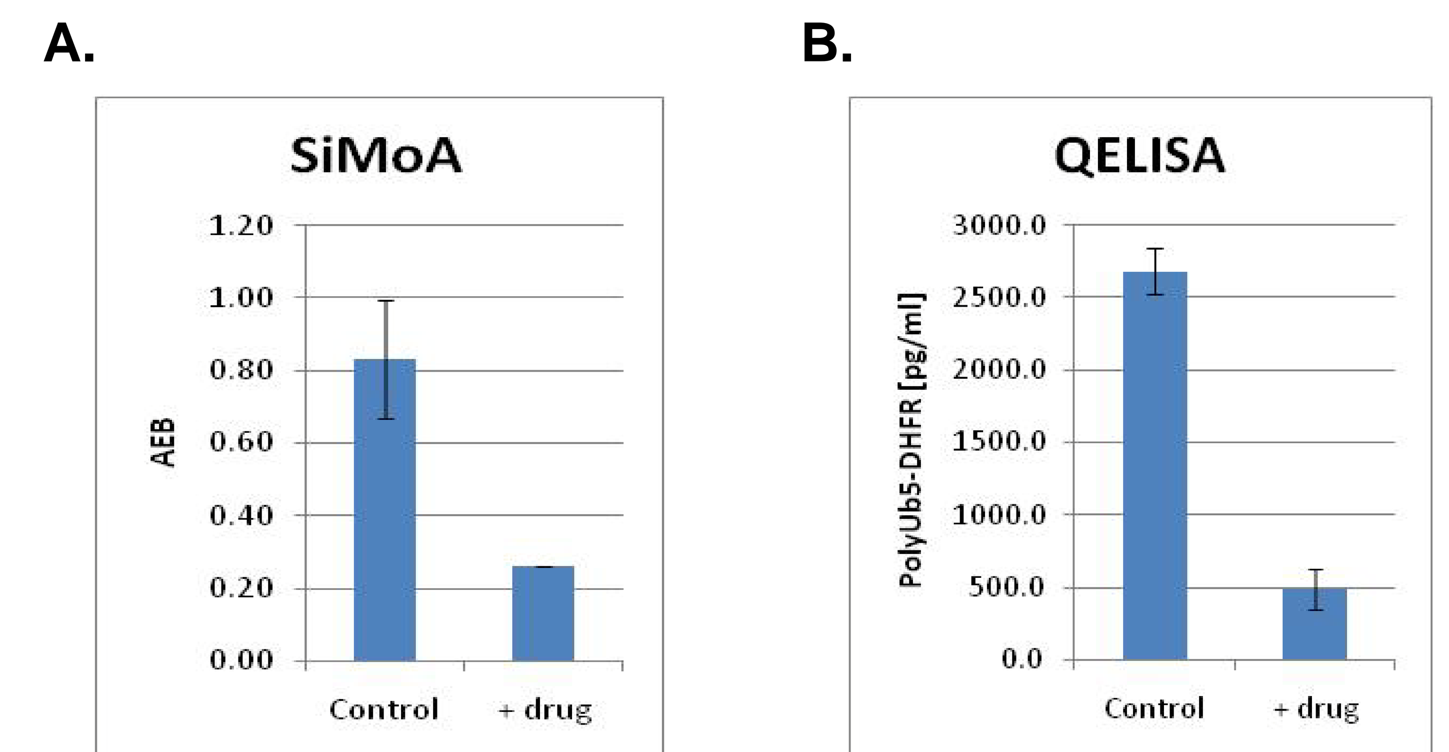


Fig 3. **Xenograft tumor samples were analyzed by SiMoA (Panel A) and QELISA (Panel B).** HCT116 xenograft tumor lysates -/+ drug treatment were analyzed in both SiMoA and QELISA. Results show that both methods generate comparable results.

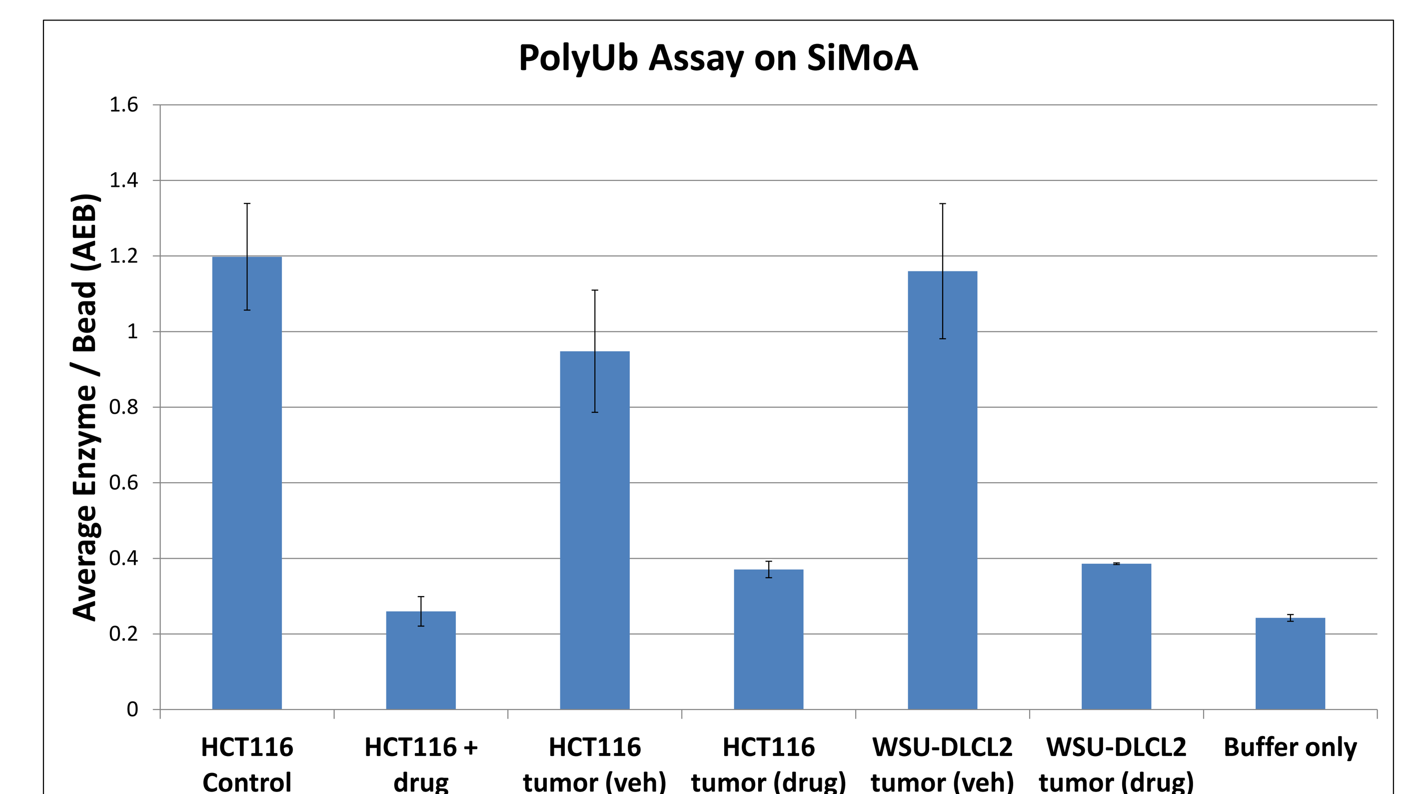


Fig 4. Cell culture or xenograft tumor lysates analysis with SiMoA. HCT116 cell culture or HCT116 and WSU-DLCL2 xenograft tumor bearing mice were treated with vehicle or an UAE inhibitor, samples lysed in mPER buffer(Pierce/Thermo) and analyzed using the same complementary pairs of pan polyUb/K48 linked polyUb antibodies as described in Fig. 1. Results showed clear discrimination between each control(DMSO) or drug treated lysates employing a total of 0.5ug protein/well of each sample. It would required more than 20ug lysate to get the similar sensitivity for quantification analysis on other commercial platforms we have evaluated (data not shown).

Conclusions

- Excellent assay sensitivity
- K48 polyUb can be measured from both cell culture and tumor lysates using Quanterix's SiMoA technology.
- Can use admixtures of control and drug treated cell culture lysate to create calibration curves in SiMoA.

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