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Single-molecule analysis determines isozymes of human alkaline phosphatase in serum

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Abstract

Alkaline phosphatase (ALP) is an important biomarker, as high levels of ALP in blood can indicate liver disease or bone disorders. However, current clinical blood tests only measure the total concentration of ALP but are unable to distinguish enzyme isotypes. Here, we demonstrate a novel and rapid approach to profile various ALP isozymes in blood via a single-molecule-analysis platform. The microarray platform provides enzyme kinetics of hundreds of individual molecules at high throughput. Using these single molecule kinetics, we characterize the different activity profiles of ALP isotypes. By analyzing both healthy and disease samples, we found the single molecule activity distribution of ALP in serum reflects the health status of patients. This result demonstrates the potential utility of the method for improving the conventional ALP test, as well as for analyzing other enzymatic biomarkers, including enzyme isotypes.

Introduction

Alkaline phosphatase (ALP) is an important enzyme produced in multiple tissues that supports a wide range of processes, including the regulation of cell growth, death and migration, the transport of nutrients in the liver, the formation and growth of bones, and more[1]. Alterations in ALP functioning result in serious nutrition-related diseases, such as hypophosphatasia, which seriously affects the growth of teeth and bones.[2] Elevated ALP levels usually indicate damage to specific organs, such as the liver, kidney or bones. Therefore, ALP assays are included as routine blood tests for diagnostic screening of patients who may have these diseases.[3] Currently, routine ALP testing is performed by measuring the total phosphatase activity in the blood, which ranges from 20 U/L to 140 U/L in healthy individuals. However, this test is based on the assumption that ALP activity is directly related to the total amount of ALP present. Therefore, tests based on activity usually ignore the diversity of ALP enzymes and the heterogeneities between individual enzyme molecules. In fact, except for ALP from liver, bone, and kidney (also called tissue nonspecific ALP, or TNSALP), which usually contributes over 90% of the total ALP activity in blood, there are at least three more types of ALP in the blood: ALP from intestine (ALPI), ALP from placenta (ALPP) and ALPP-like (Regan) isozyme.[4] The levels of these isozymes is related to the health of the corresponding organs, but since they appear in such small amounts, there is no fast and easy way to distinguish these isotypes.[5] In recent years, with the development of new biomarker screening techniques, researchers have found the expression of ALP in cancer cells is helpful to understand carcinogenesis due to its key role in cell growth and death.[6] In particular, the Regan isotype was found to be related to pancreatic cancer cell growth and can be potentially used as a biomarker for early diagnosis.[4, 7] Therefore, developing an accurate method for ALP isotypes analysis would provide better patient stratification and potentially lead to better outcomes.

There are several different strategies to analyze ALP isotypes in blood or cell,[5] including (1) separation based on molecular weight, such as electrophoresis; (2) multiplexed detection with specific recognition, such as immunoassay with antibodies that are specific to each isozyme.[8] However, these methods require either a large sample input or multiple operation steps, making them less ideal for a fast blood test. Here, we aim to address this issue by proposing a new strategy to study ALP isozymes, based on measuring the activities of thousands of individual ALP molecules in blood simultaneously.

This strategy is inspired by our previous work on single molecule arrays, where a microwell-array-based digital ELISA platform was developed for the detection of protein biomarkers and achieved ultra-high sensitivity for many diagnostic applications.[9] In other related work, the array has been used to perform single molecule enzyme kinetics analysis. We demonstrated the ability to monitor as many as 6,000 enzyme molecules simultaneously, which allows for the study of heterogeneity in large enzyme populations.[10]

In this work, we have exploited this capability to observe many individual enzyme molecules simultaneously and use it to distinguish ALP isotypes in serum samples. ALP is present in high concentrations in serum. We diluted serum samples containing a mixture of ALP isotypes, added an ALP fluorogenic substrate, and sealed the resulting solution in a microwell array. The entire procedure was completely solution-based and avoided any solid-phase based separation. The experiments were designed in a way that the total number of microwells are at least 10 times higher than the total number of ALP molecules. Therefore, the majority of ALP molecules will be confined and isolated to a single microwell that follows Poisson statistics. The entire array was then imaged and monitored to acquire the individual kinetic information for thousands of ALP molecules contained in the serum sample. By analyzing the kinetics of each enzyme molecule, an activity distribution of a large number of ALP molecules in blood is collected. We therefore created activity histograms for individual patients so as to establish ALP activity profiles. We can determine both absolute and relative amounts of different ALP isotypes in a biological sample and we can also characterize how the histogram changes in patients with different disease conditions (Figure 1).

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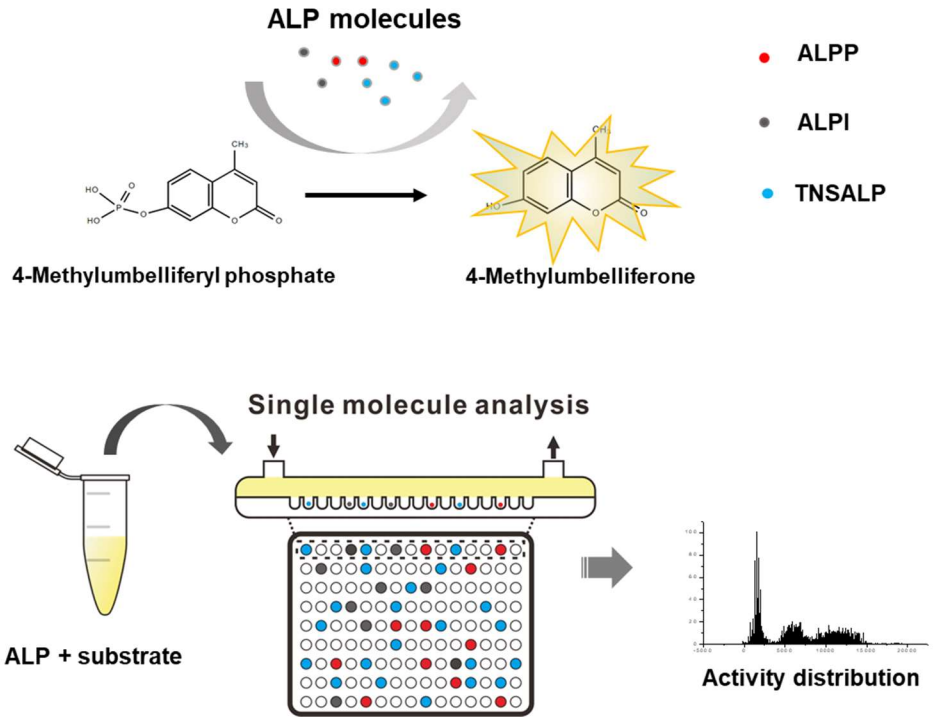

Figure 1. Single molecule analysis of ALP isotypes. Different ALP isotypes are able to react with the same substrate even though they have different kinetics. By taking advantage of the microarray platform, we are able to analyze the activity of individual molecules and collect an activity distribution histogram of the ALP population. The different colored dots stand for three different isotypes of ALP: ALPP (Alkaline phosphatase from placenta), ALPI (Alkaline phosphatase from Intestine), TNSALP (Tissue non-specific Alkaline phosphatase)

Results and Discussion

Microarray setup and the activity distribution of ALP standards

Single molecule activities of ALP in individual blood samples were obtained with a 24-channel microwell array disk. The microarray set-up was based on our previous reports with several changes as indicated in the Experimental section.[10d] One major change is that the microwell array disk was pre-treated before use by photo-bleaching to lower the background fluorescence. As shown in Figure S2a, a two-min photo-bleaching was able to lower background by 75% and minimize the background fluorescence when imaged. The auto-fluorescence intensity of the disk did not change in the dark or under daylight. The raw fluorescence intensity acquired from each image (as shown in RFU) was calibrated with a series of concentrations of the fluorescent product 4-MU (Figure S2b) and RFU changes were converted to enzymatic reaction turnover rates (s^{-1}) with the conversion equation 1 RFU/min = 3 s^{-1}. In order to simplify data processing, in the figures we used the fluorescence change in 20 min as a surrogate for the activity of individual enzyme molecules.

To be able to measure single enzyme molecule activities, the ALP concentration needs to be low enough that there are more wells than molecules, which can be determined by using the Poisson distribution. In our experimental set-up, we diluted pure ALP standard in such a way that the number of ALP molecules is less than 5% of the total number of microwells, which is equivalent to an ALP concentration of approximately 1.8 pM. Under this condition, 98% of the occupied wells contain a single enzyme molecule, according to Poisson statistics. For serum samples or cell lysates with unknown ALP concentrations, an initial dilution factor of 1000 × was used and the final "on" wells in each image were checked. According to the Poisson distribution, when the ratio of "on" wells to "off" wells is greater than 1:10, then the number of wells containing multiple enzyme molecules will be greater than 5% (Table S2 for a detailed calculation), which may lead to significant inaccuracy in the analysis. Therefore, data sets with enzyme counts higher than 10% of the microwells are excluded and/or the sample was further diluted before analysis.

The three standards used in this work were native ALPP protein, recombinant ALPI and primary hepatocyte cells (TNSALP) (native ALPI and TNSALP protein were not commercially available). To study ALP standards, we first studied their enzyme kinetics in bulk solutions. Figure S3a and Figure S4a show the curves of steady-state catalytic rate vs. substrate concentration for both ALPP and TNSALP. The Km values were calculated to be 63.9 μM for ALPP and 529.4 μM for TNSALP. Accordingly, we also studied the single molecule activities at different substrate concentrations, as shown in Figure S3b and Figure S4b. These figures show that the activities of ALP molecules increase with increasing substrate concentrations and reaches a plateau with 125 μM and 1 mM 4-MUP for ALPP and TNSALP, respectively. Based on these results, a substrate concentration of 1 mM was used for serum sample analysis. Figure 2 shows the single molecule activity distributions of ALPP, ALPI, and TNSALP. The x axis represents enzyme activity. The y axis presents the number of the molecules at each level of activity. From the distribution, it is clear that different isotypes can be distinguished based on their activities.

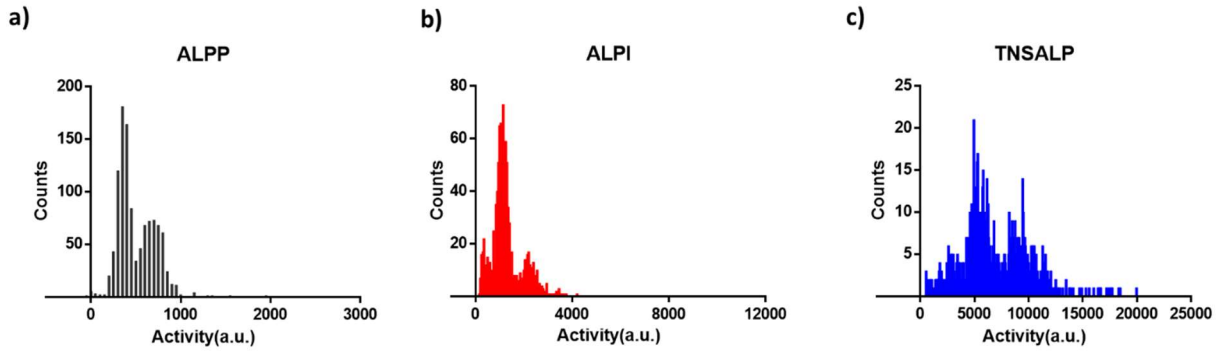

Figure 2. The activity distribution of the pure ALP standards: a) activity distribution of ALPP standards; b) activity distribution of ALPI standards; c) activity distribution of TNSALP from hepatocyte cell lysate. The

three standards are indicated by different colors. Each standard sample contains approximately 1000 ALP molecules. The fluorescence intensity change was used here to represent the enzyme activity.

The median turnover rates of ALP standards were calculated from the Gaussian-curve fitting of the single enzyme activity distribution. We found that (1) ALPP was comprised of one major population with a median catalytic rate of 85 ± 12 s^{-1} (about 572 a.u. in Figure 2); (2) ALPI contains two activity peaks. One peak comprises 80% of the population and has a median catalytic rate of 162 ± 6 s^{-1} (about 1080 a.u. in Figure 2), and the other takes up 20% of the population and has a median catalytic rate of 324 ± 6 s^{-1} (about 2160 a.u. in Figure 2); (3) TNSALP contains two broader populations at 873 ± 60 s^{-1} (about 5820 a.u. in Figure 2) and 1542 ± 174 s^{-1} (about 10280 a.u. in Figure 2). Based on our previous studies, the broad distribution of single-molecule activity is due to the static heterogeneity of the enzyme, which is caused by the existence of several stable protein conformations.[10d, 10e, 11] The two distinguishable sub-populations in ALPI and TNSALP may be due to the existence of two different oligomeric states, according to previous studies.[12] It is worth noting that TNSALP samples come directly from hepatocyte lysates, and we have ruled out the possibility of matrix effects by conducting dilution linearity experiments (Figure S5). We were able to reproducibly detect as few as 2 cells/mL (back-calculated concentration before lysis) of the hepatocytes in the 50 μL injected solution, which also demonstrate the high sensitivity of our platform.

Based on these results, the populations of ALPP and ALPI exhibit similar activities and overlap at 1000 a.u. in the distribution. In contrast, TNSALP shows a distinguishable and markedly higher activity. Therefore, we expected that a mixture of ALP isotypes in human samples would show similar distributions with one peak below an activity of 2000 a.u. for ALPP and ALPI, with two peaks at approximately 6000 a.u. and 10000 a.u. for TNSALP.

Single molecule analysis of healthy human serum samples

For serum ALP analysis, we obtained 18 serum samples from healthy donors. The serum samples were directly diluted 1000 fold with reaction buffer containing 1 mM 4-MUP substrate and loaded into the microwell array platform. Figures 3a-e show the real-time images of one typical serum sample. Each growing bright spot indicates the conversion of 4-MUP to 4-MU by a single ALP enzyme molecule. These spots are called active wells. The dark background indicates the microwells that contain no enzymes (called inactive wells). In Figure 3, several active wells from different ALP enzyme molecules are highlighted to represent the heterogeneity of their activities. The three circled active wells show distinct brightness changes. We suspect that these three enzyme molecules represent different isotypes. Next, we plotted the kinetic curves of all active wells as shown in Figure 3f. We found that the fluorescence intensity increased linearly, which suggests that the reactions reached steady-state during the experiment. We categorized the molecules into

three populations based on their activities (named as H, M, and L peaks). The median activities of these three populations were around 2500 a.u., 6000 a.u. and 11000 a.u..

We also studied the reproducibility of our platform. As shown in Figure S6, we implemented triplicate analysis of two serum samples and the results showed minimal variation between runs, which indicates the robustness of our platform.

When comparing the healthy samples to Figure 2, the L-peak was located in a region that is slightly higher than the characteristic region of ALPI, while the H and M peaks were aligned well with the two TNSALP peaks. We attributed the minor difference in activities to (1) the ALPI standard we used here was recombinant protein from E. coli, which might be slightly different from native ALPI in serum; (2) except for the three main types of ALP, there may be also other ALP isozymes or mutant ALPs in serum, which may affect the position of L peaks.

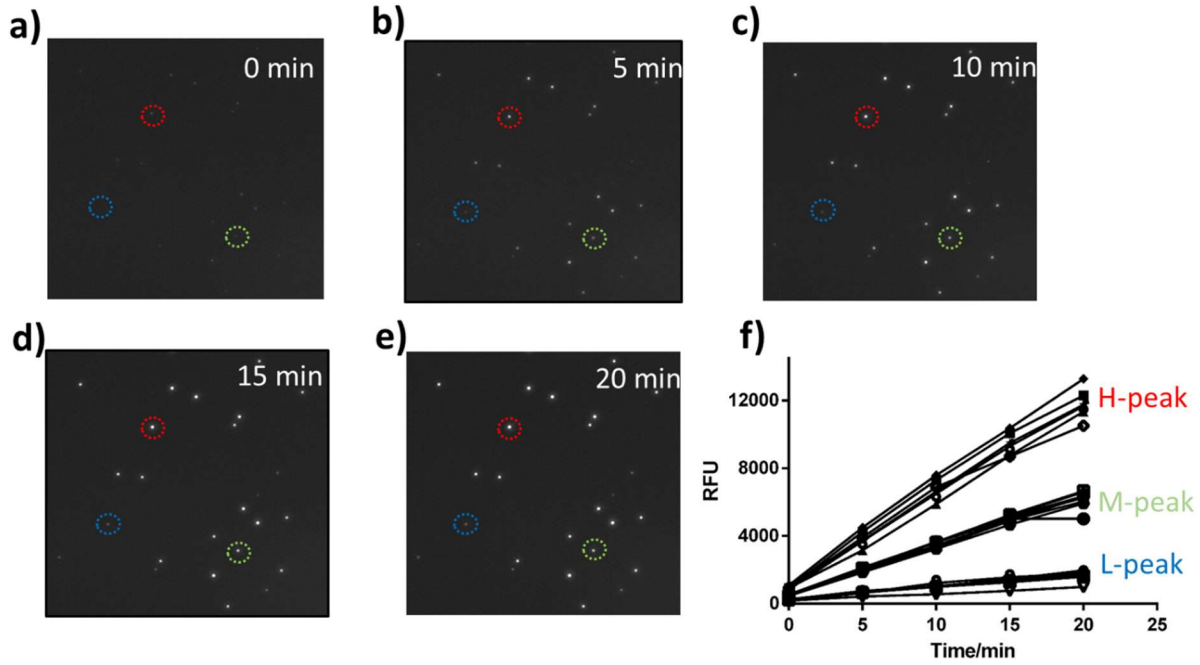

Figure 3. Single molecule activity measurements of one serum sample in real-time mode. a) to e) are fluorescence images at different times under the microscope. f) The average fluorescence intensity changes of the bright wells in a) to e) as a function of time. The colored circles are representative wells from H, M, and L peak molecules, respectively (color matched).

In order to further identify each peak, ALPP or TNSALP standards were spiked into three serum samples and the spike and recovery was calculated. Table S1 shows the spike and recovery result, which was based on the number of bright wells counted in the microarray. As shown Table S1, the spike and recovery of native ALPP protein ranged from 84% to 110% and the spike and recovery of hepatocyte lysates ranged from 80% to 130%. Figure

S7 shows the distribution changes after the standards were spiked in. Compared to the distribution in Figure 2, spiked ALPP and TNSALP standards only affect their own specific regions of the population, which means the serum didn't result in any matrix effects. We also noticed that spiked ALPP generated a separate population from the L peak, which means there is no or very little ALPP in serum. This result is consistent with the claim in the literature that ALPP concentration in blood is extremely low.[4, 13] Based on the spike and recovery results, we think the H peak and M peaks belong to TNSALP, the L peak belongs to ALPI, while the ALPP amount was too low to be distinguished in healthy donors' samples.

Single molecule analysis of patient serum samples

After analyzing all 18 samples (blue curves in Figure 4, and Figure S8a), we found all healthy samples consistently exhibit three enzyme populations, while the heights of each peak were different between individuals (error bars in Figures 4a and 4b show the variations among individuals, the raw distribution for each serum sample is shown in Figure S8).

In order to compare the activity distributions of different health conditions, serum samples from pregnant women and patients with liver disease were also analyzed. It has been reported that for pregnant women, ALPP in blood usually increases dramatically.[14] This elevation will cause confusion in the regular ALP blood test, and results in false-positive diagnosis of liver disease.[15] We analyzed four pregnant women's serum samples collected at 7 or 8 months' pregnancy from the Partner's Biobank.

From Figure 4a, it can be seen that in pregnant women, the height of the new "L-peak" (in red) was 200 to 1000 fold higher than the healthy samples' L-peak, and this characteristic peak can be clearly distinguished from the healthy control. This new "L-peak" matched the activity distribution of the ALPP standard, which has a lower activity distribution than the L-peak of healthy donors, and is consistent with our spike and recovery results. On the other hand, the heights of the other two peaks fall in the range of the healthy controls. For the ten serum samples from patients with liver disease, all the donors had elevated ALP levels (>200 U/L). We found all of these samples had a dramatic increase in their M-peaks (about 5-20 fold higher than healthy donors) and H-peaks (3-10 fold higher than healthy donors), while the L-peak remained in the same range as the healthy controls, as shown in Figure 4b.

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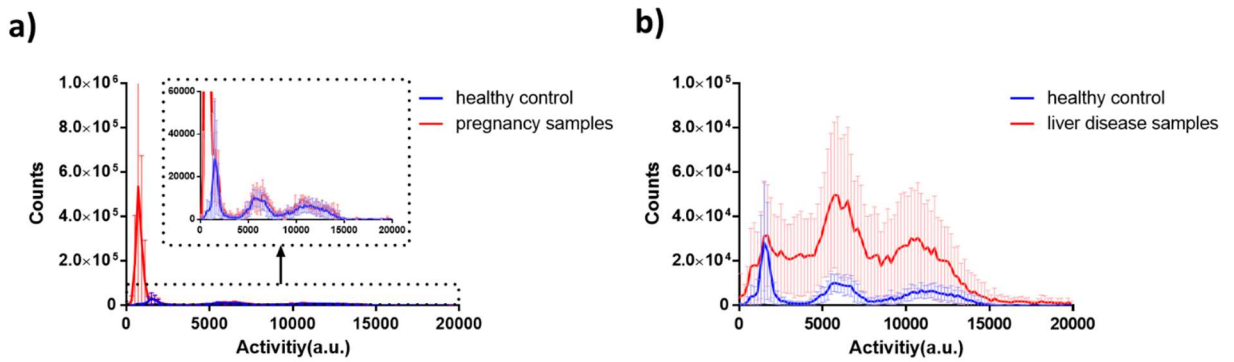

Figure 4. ALP distributions of different health conditions. a) Comparison of healthy donors' samples and pregnant women's samples. The error bars show the variations between individual samples. b) Comparison of healthy donors' samples and the samples from patients with liver disease. The error bars show the variations between individual samples. The counts on the y-axis account for dilution and are calculated based on the level of the undiluted serum sample.

Hypophosphatasia is a rare genetic disease due to mutations on patients' TNSALP genes.[2a] Patients with hypophosphatasia are unable to produce fully functional ALP for normal cell regulation. Our platform previously has shown the ability to analyze malfunctioning enzymes with better resolution than bulk measurements.[10d] Therefore, we predicted it would also be able to analyze malfunctioned ALP in hypophosphatasia patients' serum samples. We obtained seven serum samples from serious hypophosphatasia patients stored in the Biobank and measured their ALP activity distributions. Due to the low concentration of active ALP (lower than 20 U/L) in those serum samples, we had to use a 300 × dilution factor instead of 1000 × to obtain enough molecules to perform the analysis. As shown in Figure 5, there is a loss of the TNSALP peak in every serum sample, which was located in the H-peak region for healthy controls. Except for a change in the distribution pattern, the number of M-peak enzyme molecules on average was also much lower than the healthy controls. These results demonstrate that single molecule ALP measurements can be used as a personal enzyme function map for hypophosphatasia patients. Furthermore, the platform may be able to monitor hypophosphatasia related gene therapies since it can spontaneously monitor the enzyme expression levels and evaluate the function of each expressed enzyme molecule. We compared the number of ALP molecules (N_{ALP}) that have specific activities in each serum sample, and calculated the p-values based on the difference of N_{ALP} between disease samples and healthy samples (Figure S9). The p-values were in general < 0.0002 in each group comparison.

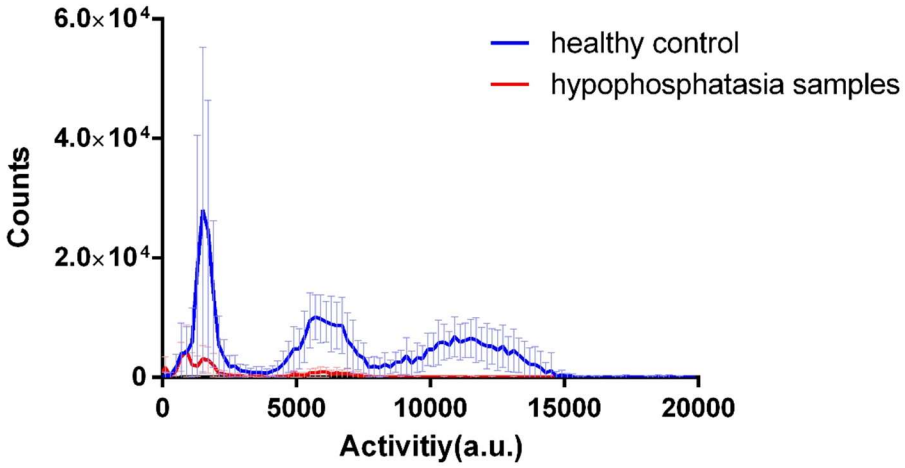

Figure 5. ALP activity distribution of seven serum samples from patients with hypophosphatasia. The counts on the y-axis account for dilution and are calculated based on the level of the undiluted serum sample.

Conclusion

In this work, we demonstrate how single-molecule activity analysis of ALP can distinguish between patients with various health conditions. By analyzing individual ALP molecules and plotting their activities, distinct populations comprising different ALP activities can be observed. The method offers promise for both diagnostics and therapeutics development. The platform provides a way to rapidly and easily distinguish different enzyme isotypes. In addition to enzymatic activities, other properties such as thermal stability and specific-resistance to inhibitors are also distinct among ALP isotypes[1b, 13]. These characteristics could potentially be used to improve the assay specificity when integrated with our platform. We also show that we can characterize malfunctioned enzyme molecules, which may lead to better methods for diagnosing and monitoring therapy. In addition, the linearity assay on hepatocyte lysates shows the platform can reproducibly detect TNSALP from as little as two cells in 50 μL buffer. This result shows the potential of our platform for single-cell analysis in the future.

Another advantage of the platform is that it can determine the quantity and function of targeted enzymes with a one-step set-up. Traditional ELISA or Mass Spec methods are able to quantify a target protein, but for enzyme biomarkers, characterization of activity and isozyme populations are important. We believe the single-molecule analysis reported here provides a more efficient and accurate approach to measuring enzyme activities than traditional methods.

Acknowledgments

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Keywords: Single molecule studies • Microarrays • Alkaline phosphatase • Enzymatic activity profiling

References

[1] a)R. B. McComb, G. N. Bowers Jr, S. Posen, *Alkaline phosphatase*, Springer Science & Business Media, **2013**; b)M. M. Kaplan, *Gastroenterology* **1972**, *62*, 452-468.

[2] a)M. P. Whyte, *Endocrine reviews* **1994**, *15*, 439-461; b)M. J. Weiss, D. Cole, K. Ray, M. P. Whyte, M. A. Lafferty, R. A. Mulivor, H. Harris, *Proceedings of the National Academy of Sciences* **1988**, *85*, 7666-7669; cM. P. Whyte, *Annals of the New York Academy of Sciences* **2010**, *1192*, 190-200.

[3] a)P. Wolf, *Archives of pathology & laboratory medicine* **1978**, *102*, 497-501; b)W. Nargis, B. Ahamed, M. Hossain, S. Biswas, M. Ibrahim, *Pulse* **2013**, *6*, 20-26.

[4] E. Mössner, G. Pfleiderer, K. Dittel, *Clinical Chemistry and Laboratory Medicine* **1984**, *22*, 467-472.

[5] a)H. Woitge, M. Seibel, R. Ziegler, *Clinical chemistry* **1996**, *42*, 1796-1804; b)C. P. Price, *Annals of Clinical Biochemistry* **1993**, *30*, 355-372.

[6] L. C. Tsai, M. W. Hung, Y. H. Chen, W. C. Su, G. G. Chang, T. C. Chang, *European journal of biochemistry* **2000**, *267*, 1330-1339.

[7] a)S. Liu, Q. Mao, W. Xue, X. Zhang, Y. Qi, Y. Wang, P. Chen, Q. Zhou, *Human pathology* **2019**, *86*, 49-56; b)R. Jemmerson, N. Shah, M. Takeya, W. H. Fishman, *Cancer research* **1985**, *45*, 282-287.

[8] S. T. Stinghen, J. F. Moura, P. Zancanella, G. A. Rodrigues, M. A. Pianovski, E. Lalli, D. L. Arnold, J. C. Minozzo, L. G. Callefe, R. C. Ribeiro, *BioMed Research International* **2006**, *2006*.

[9] D. H. Wilson, D. M. Rissin, C. W. Kan, D. R. Fournier, T. Piech, T. G. Campbell, R. E. Meyer, M. W. Fishburn, C. Cabrera, P. P. Patel, *Journal of laboratory automation* **2016**, *21*, 533-547.

[10] a)H. H. Gorris, T. M. Blicharz, D. R. Walt, *FEBS J.* **2007**, *274*, 5462-5470; b)D. M. Rissin, H. H. Gorris, D. R. Walt, *J. Am. Chem. Soc.* **2008**, *130*, 5349-5353; c)R. B. Liebherr, M. Renner, H. H. Gorris, *J. Am. Chem. Soc.* **2014**, *136*, 5949-5955; d)Y. Jiang, X. Li, B. R. Morrow, A. Pothukuchy, J. Gollihar, R. Novak, C. B. Reilly, A. D. Ellington, D. R. Walt, *ACS central science* **2019**, *5*, 1691-1698; e)X. Li, Y. Jiang, S. Chong, D. R. Walt, *Proceedings of the National Academy of Sciences* **2018**, *115*, 8346-8351.

[11] M. J. Rojek, D. R. Walt, *PLoS One* **2014**, *9*, e86224.

[12] a)D. B. Craig, E. A. Arriaga, J. C. Wong, H. Lu, N. J. Dovichi, *Journal of the American Chemical Society* **1996**, *118*, 5245-5253; b)K. V. Tabata, Y. Minagawa, Y. Kawaguchi, M. Ono, Y. Moriizumi, S. Yamayoshi, Y. Fujioka, Y. Ohba, Y. Kawaoka, H. Noji, *Scientific reports* **2019**, *9*, 1-13; c)S. Sakamoto, T. Komatsu, R. Watanabe, Y. Zhang, T. Inoue, M. Kawaguchi, H. Nakagawa, T. Ueno, T. Okusaka, K. Honda, *Science Advances* **2020**, *6*, eaay0888.

[13] U. Sharma, D. Pal, R. Prasad, *Indian Journal of Clinical Biochemistry* **2014**, *29*, 269-278.

[14] M. Salek, M. Hashemipour, A. Aminorroaya, A. Gheiratmand, R. Kelishadi, P. Ardestani, H. Nejadnik, M. Amini, B. Zolfaghari, *Experimental and clinical endocrinology & diabetes* **2008**, *116*, 352-356.

[15] Y. Bacq, O. Zarka, J. Brechot, N. Mariotte, S. Vol, J. Tichet, J. Weill, *Hepatology* **1996**, *23*, 1030-1034.

10.1002/anie.202007477

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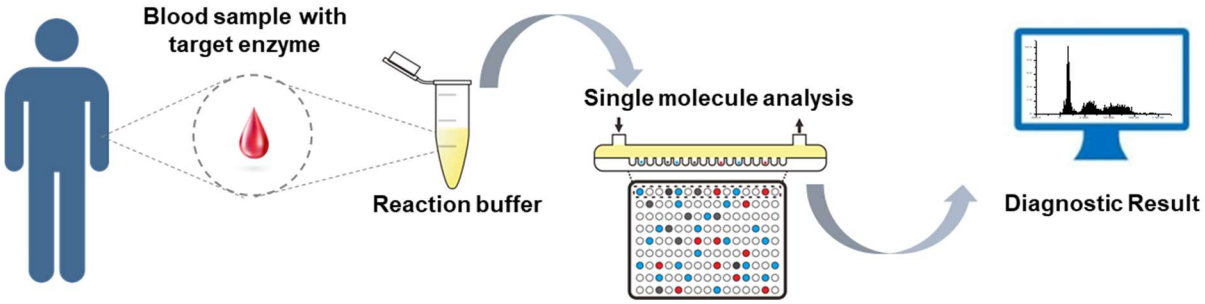

Single molecule analysis of isotypes of enzymatic biomarker