Microarray Technology

# Enzyme Microarrays: On-Chip Determination of Inhibition Constants Based on Affinity-Label Detection of Enzymatic Activity**

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The completion of the human genome's deciphering is opening previously unforeseeable opportunities. This major step towards the understanding and control of living bodies lays a fertile ground for proteomic science and, in particular, for the identification of novel enzymatic therapeutic targets. The understanding of the biochemical complexity is paralleled, in the chemical sciences, by an ever-increasing ability to generate large numbers of vastly diverse chemical structures.[1] For the evaluation of the activity of these compounds as enzyme-activity modulators, a plethora of high throughput screening (HTS) assays are currently explored. In particular, protein chips have been proposed to bring high throughput assays into the picoscale range.[2] It was thought that just as DNA chips are a pivotal contributor to genomic science, protein chips would become a major tool for proteomics, including HTS. However, protein-chip technology until recently has failed to provide information about the most crucial property of microarrayed enzymes:[3,4] the state of their catalytic activity. Herein we report a kinetic approach to on-chip enzyme-activity determination and its detailed demonstration for papain, a member of the cystein protease family. This method, due to the data quality that it provides, its automation potential and its demonstrated robustness should soon find applications in large scale HTS.

The developed method uses, as its key step, the intrinsic property of tagged affinity labels[5,6] to react with enzymes in an activity-dependent manner. The methodology (Figure 1) involves the covalent immobilization of the enzyme of interest on a functionalized glass slide. The remaining,

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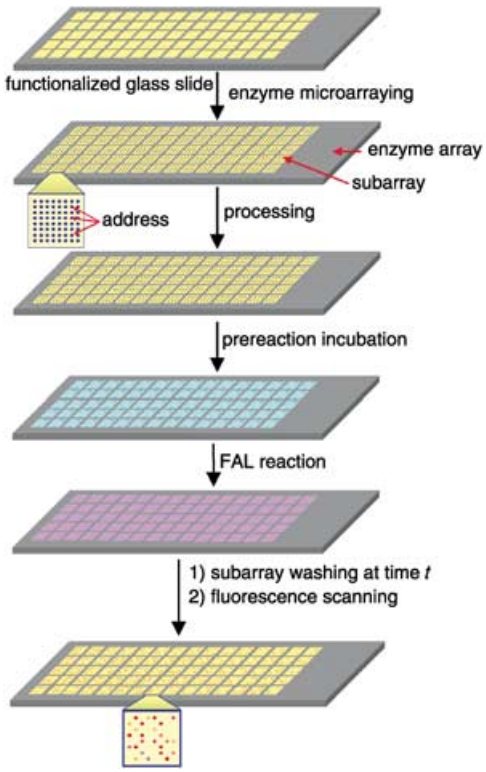
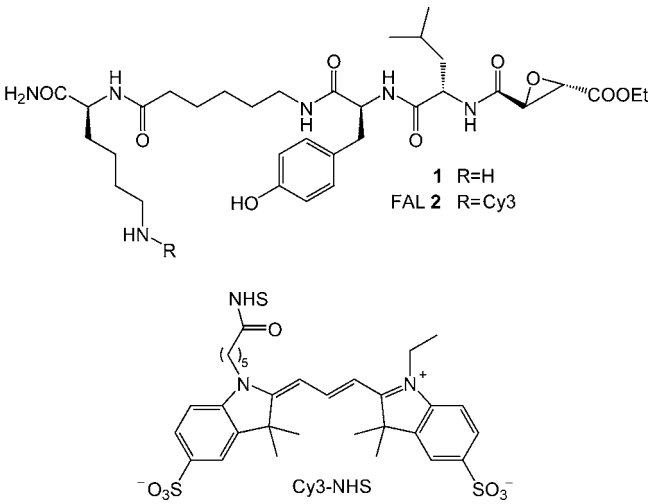



***Figure 1.*** General methodology for the affinity label on-chip enzyme inhibitor screening (ALCEIS). At the final stage, the fluorescence of each address correlates with the amount of reacted enzyme at time *t*.

unconverted surface functionalities are blocked with a surface-reactive compound. After a series of washings, an appropriate fluorescently tagged affinity label (FAL) is incubated on the top of the enzyme-immobilized area for the desired time (in absence or presence of a potential inhibitor). The amount of reacted enzyme microarrayed at a given address can then be correlated with the fluorescence measured at that address. Based on this correlation, kinetic parameters of an immobilized enzyme can be readily obtained.

The successful realization of such a system requires, as the key components, a surface-immobilization chemistry that maintains the activity of the studied enzyme and a FAL that efficiently and specifically reacts with the active enzyme(s) of interest and has very low reactivity towards other surface-immobilized molecules. To prove the principle of such a system, we synthesized a cystein protease FAL derived from the work of Barett et al.[7] and Bogyo and co-workers,[5] by the conjugation of N-hydroxysuccinimide functionalized Cy3 dye to the amino terminal group of compound **1** (see Scheme 1).[8] The obtained compound was purified by reverse phase (RP) HPLC and characterized by ESMS. Its capacity to efficiently and specifically label papain but not trypsin, bovine serum albumin or E-64 inhibited papain was demonstrated by gel-electrophoresis.

Furthermore, we evaluated the on-chip reactivity of FAL **2** towards papain that was microarrayed on a hydrogel–NHS-functionalized glass slide that contained squares of $3 \times 3\,mm^2$ each (subarrays), separated by a 20 μm-thick teflon coating.




***Scheme 1.*** Structure of the FAL **2** used in this study.

Such an array design allows each subarray to be addressed both individually and collectively.

Incubation of the subarrays for different periods of time with different concentrations of **2** followed by washing with hot SDS buffer reveals that significant fluorescence is associated with papain-containing areas (spots) within each subarray (Figure 2a) and that the level of fluorescence is time-dependent, with a saturation time dependent on the concentration of **2** used. The washing conditions employed prove that **2** is covalently bound to the microarrayed enzyme. Indeed, when identical washing conditions were used for an array on which biotinilated albumin was immobilized and subsequently detected by a noncovalent biotin-streptavidin interaction with streptavidin-phycoerythrin (SAPE), no significant signal was observed. After the array had been washed, the background fluorescence level was very low (signal:noise ratio $>50$), thus demonstrating the specific reaction of **2** with papain. Moreover, the reaction in the absence of dithiothreitol (DTT) or with microarrayed heat-denatured papain or other, non-cystein protease enzymes (such as trypsin or chymotrypsin) resulted in no significant fluorescence signal. The quality of the data obtained with this method is very high: the typical standard deviation between spots within a $4 \times 4$ array is less than 7% (Figure 2d, e) and less than 10% between different, identically treated subarrays. These data all strongly support the fact that **2** is an excellent candidate for the on-chip determination of microarrayed cystein proteases.

To obtain a quantitative model for the kinetics of the reaction between the immobilized cystein protease and **2**, the fluorescence data was quantified by comparing these data with a calibration microarray and fitted to several models.[9] The best fit was obtained (Figure 2b) for a reaction sequence derived from a Michaelis–Menten kinetic model. A slow side reaction ($k_{side} = 0.53(4)\,M^{-1}s^{-1}$) had to be taken into account, which presumably can be attributed to the attack by free thiol groups from inactive, immobilized protein on the epoxide moiety of **2**. Correspondingly, analysis of the initial velocities $v_0$ versus the concentration of **2** (Figure 2c) obeys to what is

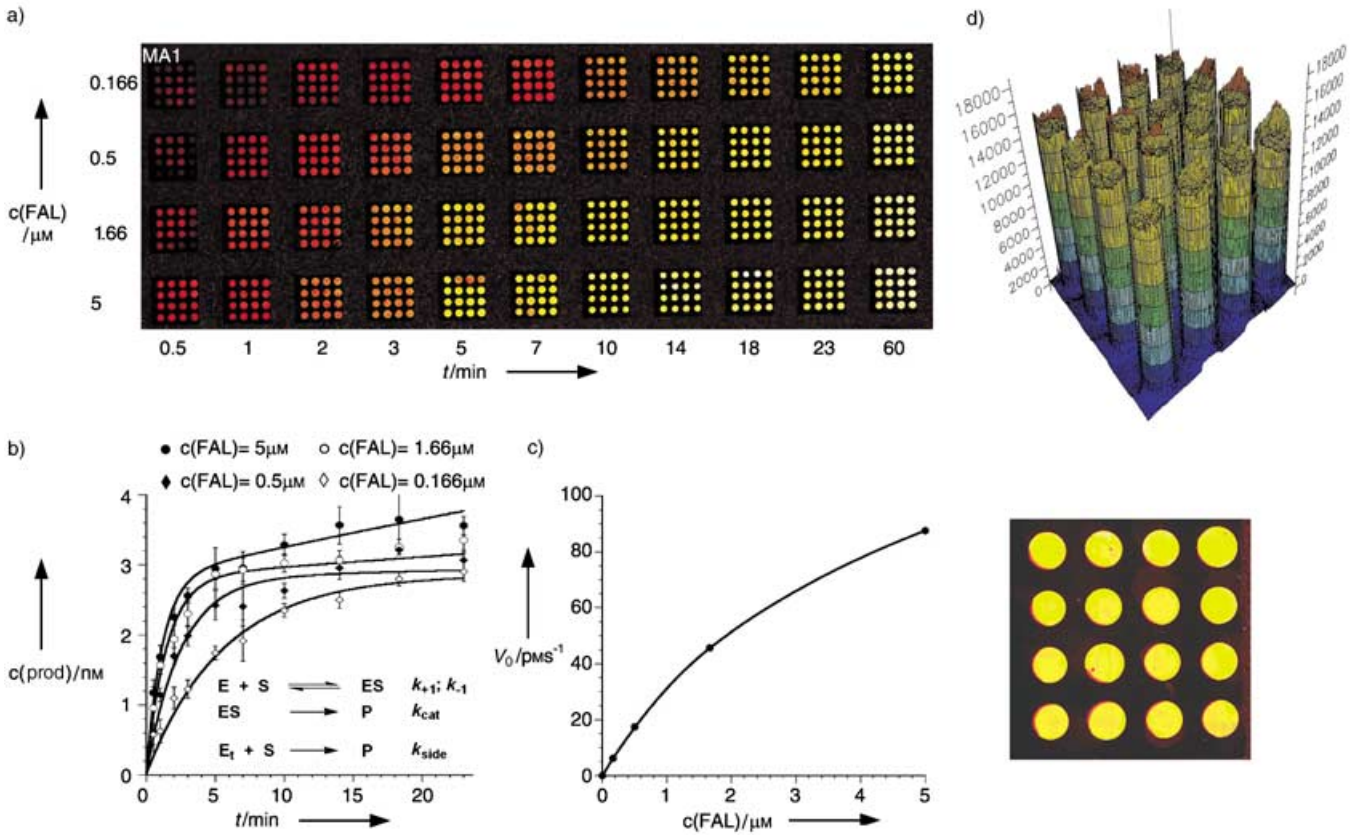



***Figure 2.*** a) Image of a microarray (MA1) composed of 44 subarrays of 4×4 papain spots each, treated for 11 different reaction times with four different concentrations of **2**. b) Quantified data corresponding to the microarray MA1 and best-fit reaction model, derived from a Michaelis–Menten kinetic. c) Plot of initial velocities ($v_0$) versus concentration of FAL **2**, $c$(FAL). d) Spot-to-spot reproducibility within a subarray for the presented method. e) Typical fluorescence image of a sub array.

expected for an enzyme-catalyzed reaction with $K_M^{app} = 3.9(5)$ μM and $v_{max} = 0.152(11)$ nM s$^{-1}$ ($c_{papain} = 2.8(3)$ nM), though no turn over is completed (see Supporting Information for detailed discussion). Simplification of the model did not lead to a good agreement of the theoretical progress curve with the experimental data, while including diffusion or further pre-equillibria did not improve the goodness of fit. However, comparison of on-chip kinetics with the corresponding rate constants, that were established by an independent solution experiment, revealed a tenfold decreased rate constant for the binding of **2** to papain ($k_{+1}$) on the surface. This result supports the argument in favor of a diffusion limitation of the fast binding processes. Therefore, for all models that involve the binding of a small molecule, a pre-equilibrium was implemented to account for this diffusion limitation. Consequently, the difference of the pseudosecond-order rate constants $k_{2nd}$ determined for the reaction of **2** with papain on chip and in solution falls below the error range of the constants (Table 1).

To demonstrate that this method can be used for the discovery and characterization of inhibitors—both reversible and irreversible—we preincubated microarrays with four different concentrations of leupeptin (competitive papain inhibitor; MA2) or E-64 (irreversible inhibitor for cystein proteases; MA3), before **2** was added and incubated for various times. The obtained time curves (Figure 3b, d) clearly indicate that addition of leupeptin slows down the reaction between **2** and the active enzyme in a concentration-dependent manner, but does not influence the saturation level reached after a long reaction time. Typically for reversible inhibition, the saturation level corresponds to the concentration of the active microarrayed papain (Figure 3b). However when E-64 is used as an inhibitor, the saturation level

***Table 1:*** Characteristic kinetic constants of tested inhibitors

| Array | $k_{2nd}$ (**2**) [μM$^{-1}$ s$^{-1}$] | $k_{2nd}$ (E-64) [μM$^{-1}$ s$^{-1}$] | $K_i$ (Leu) [nM] |
|---|---|---|---|
| MA1 | 0.14(3) | – | – |
| MA2 | 0.14(3)[a] | – | 120(12) |
| MA3 | 0.14(3)[a] | 0.32 (18) | – |
| MA4[b] | 0.14(3)[a] | 0.53 (9) | 150(5) |
| MA4[c] | – | 0.12 (16) | 100(21) |
| solution | 0.16 (3)[d] | 0.37–0.64[e] | 160(49)[f] |

[a] Determined on MA1 and not refined for this experiment. [b] Preincubation with inhibitor for 1 h before addition of **2**. [c] Simultaneous addition of inhibitor and **2**. [d] Quantification of solution experiment analogous to [8]. [e] Literature values.[2] [f] Quantification of solution experiment analogous to reference [8]. The value determined at pH 5.8 with a fluorogenic substrate is 2.5.[11]

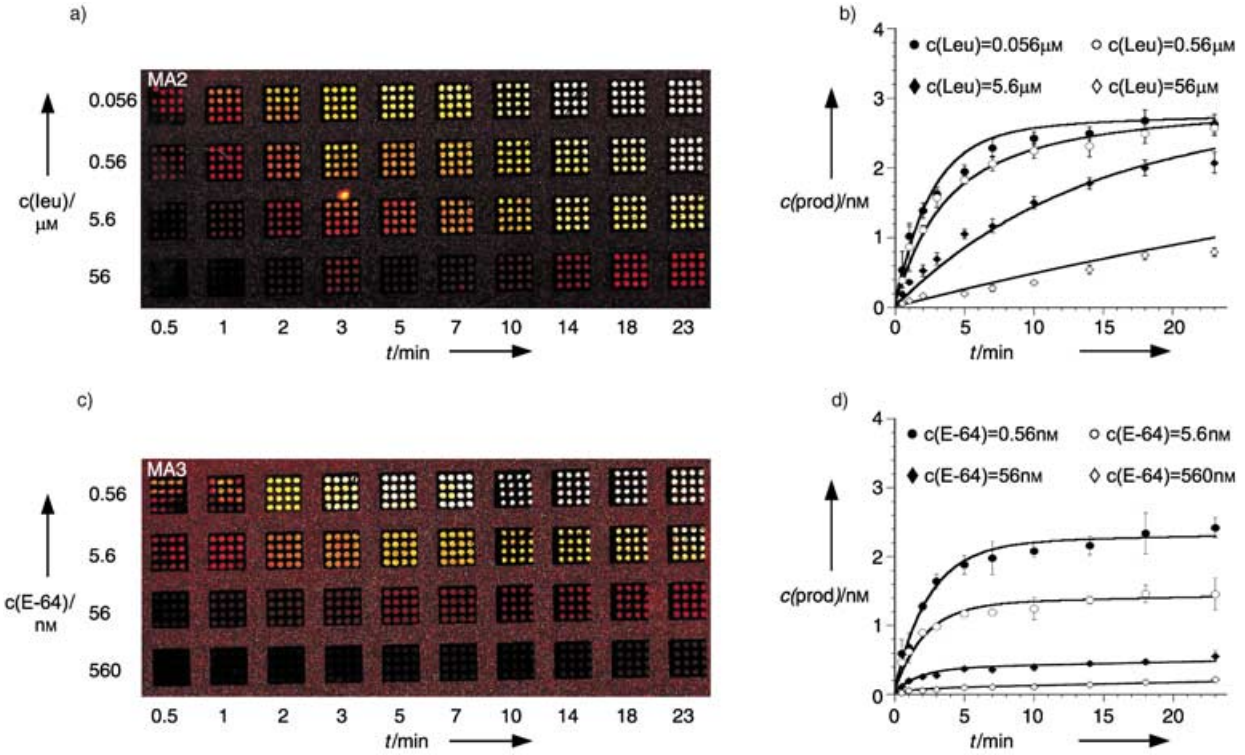



***Figure 3.*** a, c) Image of two microarrays (MA2 and MA3) composed of 40 subarrays each preincubated for 1 h with four different concentrations of leupeptin (respectively E-64) before reaction with **2** at 0.5 μM for ten different times. b) Quantified data corresponding to the microarray MA2. The data corresponding to one sub-array (3 min, $c(\text{Leu}) = 56$ μM) of MA2 was excluded from the analysis due to experimental error. d) Quantified data corresponding to the microarray MA3.

reached is correlated with the E-64 concentration, as it is expected for irreversible inhibition (Figure 3d).

These facts show that the presented method allows one to distinguish between reversible and irreversible inhibition, provided that a minimum of two time points are measured at relevant times. Based on the model and the kinetic constants derived from MA1, we could determine characteristic on-chip inhibition constants for leupeptin and E-64 (Table 1). While for E-64 the pseudosecond-order rate constant of $k_{2nd} = 0.32(18)\ \mu M^{-1} s^{-1}$ corresponds well to the known value,[10] the reversible inhibition constant for leupeptin of $K_i = 115(12)$ nM is about 50 times higher than previously reported values.[11] This difference can partly be attributed to the influence of DTT (present at much higher concentrations on the chip) on the complex equilibria that take place in an aqueous solution of leupeptin.[11] A determination of $K_i$ in solution under similar conditions (see Supporting Information) as they were applied on the chip resulted in a value of 150(5) nM.

After having established the measurement of on-chip kinetic constants, we set out to demonstrate that our method is well suited to derive quantitative inhibition constants from only one time point, an important asset for the use of this method in HTS. Thus, two experimental setups were envisioned: in the first case, 11 subarrays were preincubated for 1 hour with decreasing concentrations of inhibitor. Subsequently each subarray was treated with **2** for 2 minutes (Figure 4a, MA4, bottom 2 rows). Alternatively, in a direct competition experiment, each subarray was incubated with a DTT activation buffer, followed by 2 minutes treatment with a mixture of **2** and decreasing concentrations of inhibitor (Figure 4a, MA4, top 2 rows).

Extraction of the inhibition constants is straightforward from the preincubation experiment. $K_i$ values for leupeptin can be obtained from the ratios of the initial velocities $v_i/v_0$ at a given inhibitor concentration,[12] since the measurement takes place prior to a substantial inactivation of the enzyme by the FAL and the FAL is applied in a concentration below its reversible binding constant. The $K_i$ values, derived from any of the points satisfying the condition $0.1 < v_i/v_0 < 0.9$, were within the same order of magnitude, which is suitable for high throughput screening. To retrieve $k_{2nd}$ values for E-64, the $v_i/v_0$ ratio can be used to calculate the amount of active enzyme after a certain preincubation time, which is directly correlated to the pseudosecond order velocity of reaction of **2** with papain.

In the co-incubation situation, the inhibition constants can only be derived with a full understanding of the underlying kinetic model and the kinetic constants from MA1. Thus, this method is based on a faster experiment, but needs a predetermination of the kinetic parameters of the process. The kinetic parameters derived are listed in Table 1. Overall, they correspond well with the constants determined in solution. Whereas for irreversible inhibition, the preincubation methodology provides superior results, for competitive inhibition both methods are acceptable.

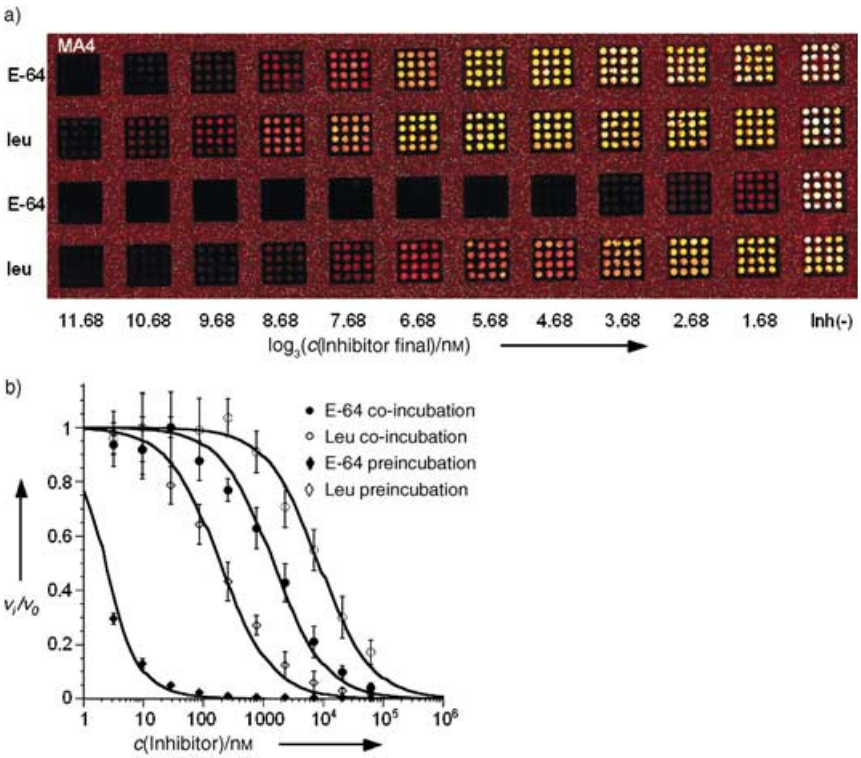



***Figure 4.*** a) Image of MA4 composed of 48 subarrays. Top two rows were co-incubated with **2** and different concentrations of E-64 (leupeptin) for 2 minutes. Bottom rows were preincubated with different concentrations of E-64 (respectively leupeptin) for 1 h before **2** was added for 2 min. b) Evolution of the ratio between the initial velocities ($v_i/v_0$) as a function of the inhibitor concentration ($c$(Inhibitor)) derived from the quantified data of MA4.

Placed in the context of other on-chip methods that use mainly binding events for detection,[2] this method has several noticeable advantages: first, it clearly differentiates between the capacity of an arrayed enzyme to catalyze a chemical reaction rather than only bind a chemical partner. The second important advantage is the independence of the obtained result on the postprocessing washing step. Indeed, it usually is difficult to ensure consistent washing conditions for two different batches for example, whereas, as this method involves covalent binding of the activity-reporter molecule, very drastic washing conditions can be used with little variation in the observed fluorescence of the active enzymes. This adds to the enzyme chip technology an additional robustness, which is an important fact for their reliable exploitation. The practical possibilities to reliably use ALCEIS with many classes of enzymes require optimization of the immobilization chemistry as well as a full kinetic characterization of each of the immobilized enzyme. Since this latter aspect, including reaction modeling, must be determined only once for a given set of enzymes, the method described can be used in a high-throughput system. By using such an assay, as well as appropriate automation it appears realistic to run more than a million enzymatic tests a day with a single working station and a significantly reduced amount of material consumption with respect to current techniques. The method presented herein complements well the recently demonstrated use of FALs for the determination of inhibitor's specificity profile in tissue proteomes.[13,14] Different in nature, our method requires at this stage purified enzymes, yet has the advantage of a higher throughput. Considering the recent spectacular progress made in this field,[15] one can expect a rapid broadening of the method presented herein.

In conclusion, we have proven the possibility of quantitatively and reliably measuring on a chip the activity level of reversible and irreversible inhibitors by taking advantage of the specific reaction between a class of enzymes and fluorescently tagged affinity labels. We do provide both the theoretical and practical basis for further developments in the promising field of enzyme(s) microarrays.

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**Keywords:** enzymes · fluorescent probes · high-throughput screening · inhibitors · microarrays · on-chip kinetics

[1] see for example M. D. Burke, S. L. Schreiber, *Angew. Chem.* **2004**, *116*, 48; *Angew. Chem. Int. Ed.* **2004**, *43*, 46, and references therein.
[2] For reviews see: G. McBeath, *Nat. Genet.* **2002**, *32*, 526; the state of the art in this field is comprehensively reviewed in the recent issue dedicated to protein microarrays of *Proteomics* **2003**, *3*, 2077–2270.
[3] This technique effectively solves the on-chip diffusion problem; however it does not measure the activity of immobilized enzymes and has inherent advantages as well as disadvantages over the method presented herein. D. N. Gosalia, S. L. Diamond, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 8721.
[4] Chen et al. have shown that affinity labels can be used to label microarrayed enzymes. G. Y. J. Chen, M. Uttamchandani, Q. Zhu, G. Wang, S. Q. Yao, *ChemBioChem* **2003**, *4*, 336.
[5] D. C. Greenbaum, W. D. Arnold, F. Lu, L. Hayrapetian, A. Baruch, J. Krumrine, S. Toba, K. Chehade, D. Brömme, I. D. Kuntz, M. Bogyo, *Chem. Biol.* **2002**, *9*, 1085.
[6] G. C. Adam, E. J. Sorensen, B. F. Cravatt, *Nat. Biotechnol.* **2002**, *20*, 805.
[7] A. J. Barett, A. A. Kembhavi, M. A. Brown, H. Kirschke, C. G. Knight, M. Tamai, K. Handa, *Biochem. J.* **1982**, *201*, 189.
[8] D. Greenbaum, K. F. Medzihradszky, A. Burlingame, M. Bogyo, *Chem. Biol.* **2000**, *7*, 569.
[9] Kinetic Parameters were extracted by best fit analysis by using the Program DYNAFIT: P. Kuzmic, *Anal. Biochem.* **1996**, *237*, 260–273.
[10] H.-H. Otto, T. Schirrmeister, *Chem. Rev.* **1997**, *97*, 133.
[11] R. M. Schultz, P. Varma-Nelson, R. Oritz, K. A. Kozlowski, A. T. Orawski, P. Pagast, A. Frankfater, *J. Biol. Chem.* **1989**, *264*, 1497.
[12] P. Kuzmic, S. Sideris, L. M. Cregar, K. C. Elrod, K. D. Rice, J. W. Janc, *Anal. Biochem.* **2000**, *281*, 62.
[13] M. P. Patricelli, D. K. Giang, L. M. Stamp, J. J. Burbaum, *Proteomics* **2001**, *1*, 1067–1071.
[14] D. Leung, C. Hardouin, D. L. Boger, B. F. Cravatt, *Nat. Biotechnol.* **2003**, *21*, 687.
[15] D. A. Jeffery, M. Bogyo, *Curr. Opin. Biotechnol.* **2003**, *14*, 87.

  