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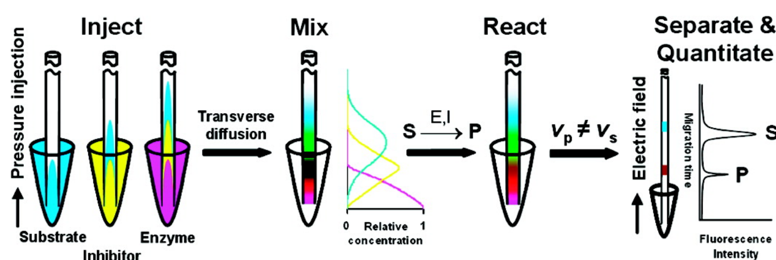

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"Inject-Mix-React-Separate-and-Quantitate" (IMReSQ) Method for Screening Enzyme Inhibitors

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Many enzymes involved in regulatory cellular processes are considered attractive therapeutic targets and their inhibitors are potential drug candidates.[1] Screening of combinatorial libraries for enzyme inhibitors is pivotal to identifying hit compounds for the development of enzyme-targeting drugs. Here we introduce the first method for screening enzyme inhibitors which is applicable to regulatory enzymes and consumes only nanoliter volumes of the reactant solutions. We name the method inject-mix-react-separate-and-quantitate (IMReSQ). The concept of the method is shown in Figure 1. First, nanoliter volumes of substrate, candidate inhibitor, and enzyme solutions are injected separately (from microliter volumes in cupped vials) by pressure into a capillary as separate plugs without the need of nanoliter-scale liquid handlers. Second, the plugs are mixed inside this capillary microreactor by transverse diffusion of laminar flow profiles (TDLFP).[2] Third, the reaction mixture is incubated to form the enzymatic product. Fourth, the product is separated from the substrate inside the capillary by electrophoresis. Fifth, the amounts of the product and substrate are quantitated. In this proof-of-principle work, we applied the method to study inhibition of recently cloned protein farnesyltransferase (FT) from parasite *Entamoeba histolytica* (*Eh*); this enzyme is a potential therapeutic target for antiparasitic drugs.[3,4] We identified three previously unknown inhibitors of *Eh*FT and proved that IMReSQ could be used for accurately ranking the potencies of inhibitors.

Methods for screening enzyme inhibitors can be divided into two broad categories: homogeneous, which monitor product formation without its physical separation from the substrate, and separation-based, which separate the product from the substrate by means of chromatography or electrophoresis prior to its quantitation. Recent advances in printing chemical libraries made it possible to transfer homogeneous methods from microtiter plates to microarrays, which require only nanoliter volumes of reagents.[5] Microarrays, however, require substrates that do not fluoresce before being converted into fluorescent products. Such fluorogenic substrates are not available for the majority of regulatory enzymes, for example, prenyltransferases, glycosyltransferases, and kinases. In separation-based methods, simple fluorescently labeled substrates can be used instead of fluorogenic substrates. Fluorescently labeled substrates are available for many regulatory enzymes.[6] If separation is carried out in a narrow-bore capillary, only nanoliter volumes of reaction mixtures are consumed. Because of the lack of a generic way of mixing solutions inside the capillary, however, the reaction mixture must be prepared in a vial outside the capillary with a volume of at least several microliters. This work was inspired by the insight that TDLFP can be used to mix nanoliter volumes of enzyme, substrate, and candidate inhibitor, injected into the capillary as separate plugs. Thus far, TDLFP had been used to mix two reactants only.[2] Here, we demonstrate the TDLFP-based mixing of four reactants—enzyme, two substrates, and inhibitor—followed by enzymatic product formation, separation of the product from the substrate, and quantitation of the formed product and remaining substrate.

The *Eh*FT enzyme was a recombinant protein produced as described elsewhere.[4] The enzyme transfers the farnesyl group from farnesyl pyrophosphate (substrate 1, S1) to a fluorescently labeled pentapeptide (substrate 2, S2), which mimics Ras protein, its native substrate. The farnesylated pentapeptide (product, P) was separated from S2 by capillary electrophoresis and fluorescent detection was used to quantitate the amounts of P and S2. Farnesyltransferase inhibitors (FTIs) tested in this work were commercial FTI-276 and FTI-277, previously shown to inhibit *Eh*FT[4] and human FT,[7] respectively, as well as new compounds, FTI-343, FTI-391, FTI-651, and FTI-656, recently proven to inhibit mammalian FT[8] but never tested for *Eh*FT. Fluorescence detection was used in this work; however, other detection approaches, such as light absorption and mass-spectrometry, can also be used in IMReSQ.

First, we studied TDLFP mixing of the four reaction components using computer simulation (see the Supporting Information). An algorithm for the optimization of the plug order in TDLFP mixing has not been developed yet; therefore, strictly speaking, we could not optimize the plug order. We could, however, numerically simulate the concentration profiles of the mixed reaction components along the capillary for any given order of plugs (Figure 2A). Using such a simulation we tested several plug orders that seemed reasonable on the basis of two simple criteria: the number of plugs had to be small while the spatial overlap of the components after mixing had to be significant. The plug order chosen for further work was: S1, S2, enzyme (E), inhibitor (I), and S1 again (Figure 2A). Plugs of an enzymatic buffer were injected before and after injecting the reaction components to (i) isolate the reaction mixture from an electrophoresis buffer containing a surfactant and (ii) improve the quality of mixing. The simulated after-mixing concentration profiles of the four components did not overlap perfectly; however, they revealed a significant reaction zone with all four components present (Figure 2A). There was also a reaction zone with S1, S2, and E, but without I, suggesting that complete inhibition with this mixing scenario was not achievable.

In TDLFP, the required mixing time is defined by the time of transverse diffusion of the largest molecule, *Eh*FT. Our computer simulation showed that for the experimental conditions used, the sufficient mixing time was less than 1 min. The reaction time was longer than the mixing time, which suggested that only a negligible amount of the product was formed during mixing.

Second, we experimentally demonstrated that TDLFP indeed mixed the four reaction components and that the product formation could be observed along with the inhibition. The absence of either of the components meant that the concentration of this component in the injected plug was zero (an enzyme buffer was injected). In

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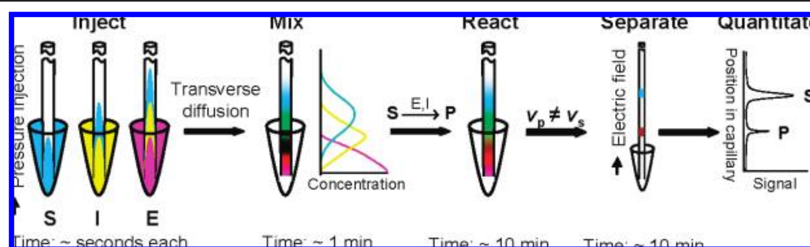

Figure 1. The concept of IMReSQ. The reaction components (substrate, S; inhibitor, I; and enzyme, E) are injected into the capillary as separate plugs, mixed by TDLFP, reacted to form the product (P), and separated. S and P are quantitated after separation.

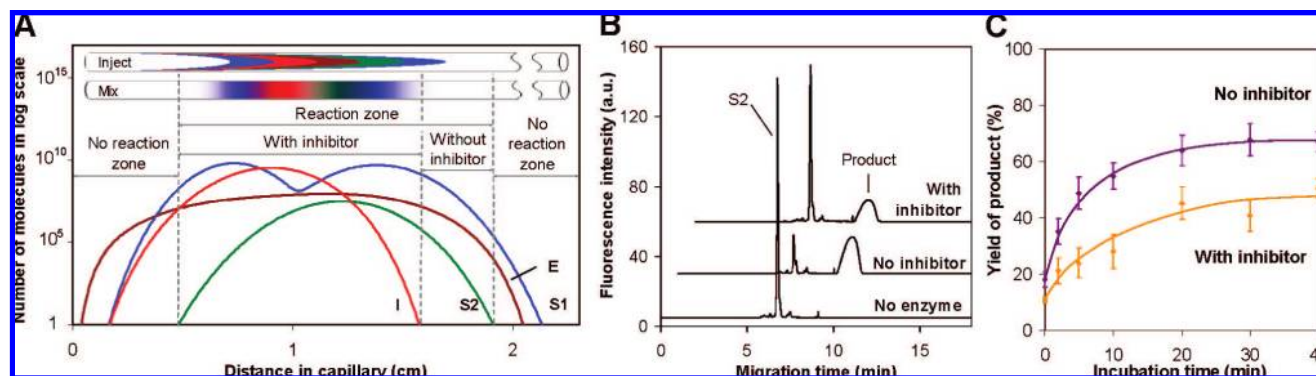

Figure 2. IMReSQ for inhibition of *Eh*FT enzyme. (A) Simulated distribution of reaction components in the capillary after injection (top) and after mixing (bottom) for the following plug order: enzymatic buffer (white), FPP (S1, blue), fluorescent peptapeptide substrate (S2, green), *Eh*FT enzyme (E, brown), inhibitor (I, red), FPP again, and enzymatic buffer again. (B) Experimental result of IMReSQ analysis of *Eh*FT inhibition by FTI-276 after 40-min incubation following injection and mixing of reaction components depicted in panel A. The preinjection concentrations were 75 μM for S1, 0.2 μM for S2, 3 μM for E, and 50 μM for I. (C) Kinetics of product formation with and without inhibitor (50 μM FTI-276).

this part of our study, we used the FTI-276 inhibitor that had been proven to inhibit *Eh*FT.[4] When the concentration of E was zero, no P was formed and a single peak of S2 was detected (Figure 2B, lower trace). In the presence of E but without I, S2 was converted in to P and, accordingly, two peaks were observed after P was separated from the remaining S2 (Figure 2B, middle trace). Finally, in the presence of I, the reaction rate was lower and the amount of P formed during the same incubation time was smaller. The peak of P was smaller while the peak of the remaining S2 was higher (Figure 2B, upper trace) than those in the absence of I. When the incubation time varied, the reaction kinetics could be measured. The kinetics in the absence and presence of I were classical Michaelis kinetics (Figure 2C). Thus, all four components were mixed to the level at which the rate of P formation depended on I.

Third, we tested if the IMReSQ method could be used to quantitatively rank the potencies of inhibitors. Conventionally, IC_{50} values (inhibitor concentrations that cause 50% reduction of the reaction rate) are used to rank inhibitors. IC_{50} depends on enzyme and substrate concentrations and, thus, inapplicable to IMReSQ, in which the solutions are not ideally mixed. To quantitatively rank inhibitors by IMReSQ, we suggest PIC_{50}, which is a preinjection inhibitor concentration that causes 50% reduction in the reaction rate. We compared ranking of several potential inhibitors of *Eh*FT by a "traditional" method using IC_{50} and by the IMReSQ method using PIC_{50}. In the traditional method, microliter volumes of S1, S2, I, and E were mixed in a vial and incubated to form P. A nanoliter volume of the reaction mixture was injected into the capillary and P was separated from remaining S2 and their amounts were quantitated. The PIC_{50} to IC_{50} ratio was identical within the error limits (Table 1). The constant ratio suggests that IMReSQ can be used for quantitatively ranking potencies of the inhibitors. Four inhibitors of mammalian farnesyltransferase (FTI-276, FTI-277, FTI-651, and FTI-656) were found to be potent for *Eh*FT and can, therefore, be used as hit compounds for drug development. To conclude, IMReSQ is the first method that consumes nanoliter volumes of the reaction components per analysis and is applicable to regulatory enzymes. IMReSQ can be used with commercially available single-capillary instrumentation for screening small libraries. Screening large libraries with IMReSQ will require specialized multicapillary instrumentation.

Table 1. IC_{50} and PIC_{50} for Inhibition of *Eh*FT Determined by Traditional (in-Vial Reaction) and IMReSQ Methods, Respectively

candidate inhibitor	traditional IC_{50} (μM)	IMReSQ PIC_{50} (μM)	PIC_{50}/IC_{50}
FTI-276	1.1 ± 0.3	11.2 ± 3.2	10.2 ± 2.9
FTI-277	2.1 ± 0.4	18.9 ± 3.8	9.0 ± 1.8
FTI-343	not measurable	not measurable	
FTI-591	not measurable	not measurable	
FTI-651	71 ± 17	800 ± 70	11.3 ± 2.7
FTI-656	61 ± 18	620 ± 90	10.2 ± 3.1

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Supporting Information Available: Supporting materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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