RESEARCH ARTICLE

A multiplex assay to assess the transaminase activity toward chemically diverse amine donors

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Abstract: The development of methods to engineer and immobilize Amine transaminases (ATAs) improving their functionality and operational stability is gaining momentum. Nowadays, the quest for robust, fast, and easy-to-use methods to screen the activity of large collections of transaminases, is essential. This work presents a novel and multiplex fluorescence-based kinetic assay to assess ATA activity using 4-dimethylamino-1-naphthaldehyde as an amine acceptor. The developed assay allows us to screen a battery of amine donors using free and immobilized ATAs from different microbial sources as biocatalysts. As a result, using chromatographic methods, 4-hydroxybenzylamine was identified as the best amine donor for the amination of hydroxy methyl furfural. Finally, we adapt this method to determine the apparent Michaelis-Menten parameters of a model immobilized ATA at the microscopic (single-particle) level. Our studies promote the use of this multiplex, multidimensional assay to screen ATAs for further improvement.

Introduction

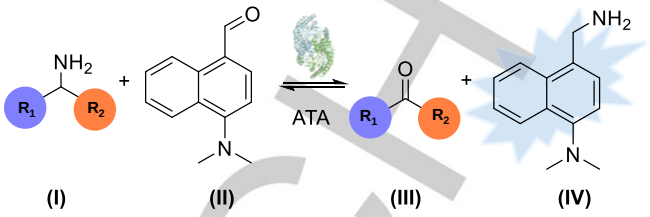
In the last decades, pharmaceutical and chemical industries are seeking for more efficient and sustainable methodologies to prepare chiral amines to be used as functional building blocks in their manufacturing pipelines.[1] Herein, biocatalysis emerges as a competitive and sustainable technology to transform available raw materials (i.e., aldehydes and ketones) into enantiopure high-added value amines under mild conditions and with exquisite selectivity.[2] Although amine dehydrogenases, reductive aminases, and imine reductases have bloomed into the biocatalysis landscape since the beginning of the XXI century, amine transaminases (ATAs) dominate the amination reactions in industry.[3] The activity of this enzyme family is aided by pyridoxal 5'-phosphate (PLP), which is a cofactor that allows the reversible transfer of an amino group from a donor (i.e., an amine) to an acceptor (i.e., aldehyde and ketones). [4] Unlike the amine oxidoreductases mentioned above, which depend on NAD(P)H redox cofactors that need to be recycled *in situ* by ancillary recycling systems, ATAs bind and self-recycle PLP within their active sites requiring a non-external recycling system. Hence, ATAs offer a sustainable route toward amines using a variety of aldehydes and ketones as amine acceptors, mainly using three major amine donors: L-alanine, isopropylamine, and 1-phenylethylamine (1-PEA). In particular, the amination of bio-based 5-(hydroxymethyl)furfural (HMF) is nowadays gaining importance in the context of biorefineries since the resulting 5-(hydroxymethyl)furfural amine (HMFA) is a valuable intermediate for the synthesis of antihypertensive drugs, preservatives, and other curing agents.[5] Unfortunately, only a few processes have been reported for the bioamination of HMF using transaminases, likely due to the lack of efficient enzymes able to perform such reactions through robust and efficient configurations.[6]

To further increase the potential of transaminases in applied biocatalysis, the scientific community has improved them through protein engineering to maximize their catalytic potential as well as their operational stability. This task can be achieved by discovering new ATAs from metagenomics, but also by engineering well-known ATAs (i.e., ATA from *Chromobacterium violaceum* (*Cv*ATA), from *Halomonas elongata* (*He*ATA), and from *Pseudomonas fluorescence* (*Pf*ATA)). Several seminal works demonstrate the power of directed evolution to reshape the active sites of ATA to perform outstanding biotransformations such as the marketed drug sitagliptin.[7] A major limitation in engineering and employing novel ATAs is the lack of fast and sensitive screening methods that allow the identification of novel

variants with superior activity and selectivity for challenging transamination reactions. The most common kinetic activity assay for ATAs is named the “acetophenone assay”. This method uses 1-PEA as an amine donor, which upon ATA action and in presence of an amine acceptor (i.e., pyruvate), is irreversibly transformed into acetophenone that is spectrophotometrically detected by UV-Vis. Unfortunately, this method is limited to 1-PEA as amine donor and requires a detection wavelength of 245 nm; a spectral area where signal interferences from other organic species are notorious. In an effort to overcome some of the challenges associated with the ‘acetophenone assay’, other UV-Vis activity assays have been developed either using quencher reactions enabling the detection of the products at higher wavelengths,[8] or coupling tandem reactions using auxiliary enzymes.[9] However, these methods are mostly end-point assays, thus the monitoring of the reaction in real-time is not functional and is time-consuming[8]. Remarkably, smart amine donors have been exploited to develop colour *in situ* due to the formation of a coloured precipitate upon the ATA action. These methods are excellent to shift the reaction equilibrium toward the amination reaction, enabling the amination of poor amine acceptors like acetophenone, but they fail to accurately measure enzyme kinetics.[10] All these enzyme assays are based on UV-Vis methods, however, a fluorescent-based method is demanded to increase the sensitivity and the throughput of the screening using cutting-edge microfluidic techniques.[11] As far as we know, there is only one screening method that assesses ATA activity using a fluorescent readout, but it is limited to screening only amine acceptors.[12] Therefore, none of the above activity assays covers all the needs for a versatile and selective ATA assay for high-throughput screening of this type of enzymes, with special focus on assaying amine donor panels.

To develop a new fluorescent-based assay for the assessment of the kinetics of transaminases, we investigated new molecules that upon ATA action turned out fluorescent. We identified aromatic aldehydes as promising substrates for amination as they are known to be weakly fluorescent or non-fluorescent in an aqueous solution. In contrast, their corresponding carboxylates are fluorescent.[13] Like the carboxylates, we hypothesize that the resulting amines upon ATA activity will be fluorescent, and thus monitored to assess enzyme kinetics in real time. A promising molecule for this end is the 4-dimethylamino-1-naphthaldehyde (4-DANAl, Scheme 1), which was employed as a substrate surrogate to detect and measure aldehyde dehydrogenase and oxidase activities.[13] In this work, the authors showed that the naphthaldehyde derivative displays moderate fluorescence over the visible spectral range, but after enzymatic reaction, a strong fluorescence of the product can be observed around 355 nm.

Based on the potential of 4-DANAl as an ATA substrate, we developed a multiplex enzymatic assay to evaluate the transamination activity of ATAs using different amine donors (Scheme 1). We synthesized and characterized the 4-(aminomethyl)-N,N-dimethylnaphthalen-1-amine (4-DANAm) to study its optical and fluorescent properties. When the amine acceptor (4-DANAl) was incubated with different transaminases, we could monitor the enzyme activity through both UV-Vis absorbance and fluorescence measurements and determine the transamination rates using different amine donors. Finally, this assay was used to quantify the Michaelis-Menten kinetic parameters of ATAs immobilized on solid carriers at the single-particle level through time-lapse fluorescence microscopy according to the image processing and analytics previously developed by our group for immobilized enzymes.[14]



Scheme 1. Reaction scheme of the multiplex ATA assay to screen amine donors. (I) Amine donor. (II) 4-(dimethylamino)-1-naphthaldehyde (4-DANAl) as an amine acceptor. (III) Carbonyl (aldehyde or ketone) as co-product and (IV) 4-(aminomethyl)-N,N-dimethylnaphthalen-1-amine (4-DANAm) as the fluorescent product. R1 and R2 are different aryl and alkyl substituents.

Results and Discussion

Fluorometric characterization of substrates and products. The aldehyde 4-DANAl is commercially available, or alternatively can be synthesized through $TiCl_4$-catalyzed arylation of trimetrylorthoformate with *N*,*N*-dimethyl-1-naphthylamine as previously described by Periasamy and coworkers[16]. On the other hand, the amine 4-DANAm was synthesized through reductive amination of 4-DANAl. Both, 4-DANAl and 4-DANAm were identified and characterized by NMR studies (see Experimental section and Figures S3-S5) to later investigate their photophysical properties. Figure 1 shows the UV-Vis absorbance, excitation, and emission spectra of both pure 4-DANAm and 4-DANAl. The aldehyde's maximum absorbance wavelength (λ_{max}) was found at 368 nm in agreement with the data previously reported,[13] unlike the amine which presents a λ_{max} of 290 nm. Regarding the fluorescence, both molecules presented their maximum emission wavelengths (λ_{em}) at 450 nm and their maximum excitation wavelengths (λ_{ex}) at 290 nm. After the spectral characterization of the compounds, calibration curves were executed to determine the molar extinction coefficient and the molar fluorescence intensity for each molecule (Figure S6 and Table S1). As a result, 4-DANAl exhibited a molar extinction coefficient at 370 nm (ε_{370}) of 6.53 mM^{-1} cm^{-1}, while 4-DANAm presented a ε_{290} of 3.81 mM^{-1} cm^{-1}, which was 1.7 times lower than the ε_{370} determined for the aldehyde. Regarding the fluorescence, the 4-DANAm emitted a fluorescence intensity per mol at 450 nm 11.5 times higher than 4-DANAl when they were excited at 290 nm. Hence, 4-DANAl is poorly fluorescent in aqueous conditions, whereas the amination of its position 1 seems to enhance the fluorescence properties of this molecule. Furthermore, the amination of this molecule has also consequences on the absorptivity of the molecule, since the aminated compound absorbs much less UV-Vis light than the corresponding aldehyde. Similar behavior was reported for other aromatic aldehydes when the conjugation between the aromatic ring and the carbonyl group was broken by introducing a carboxylic group. Quantitative differences in the UV-Vis and fluorescence spectra between 4-DANAl and 4-DANAm may allow real-time monitoring of the ATA-driven transformation of this aldehyde into the corresponding amine. A similar assay was reported for aromatic aldehydes like naphthaldehyde, which was oxidized to its corresponding carboxylate by aldehyde dehydrogenases and oxidases.[13] However, in that study 4-

DANAl was discarded due to its insufficient fluorescence yield for the enzyme assay.

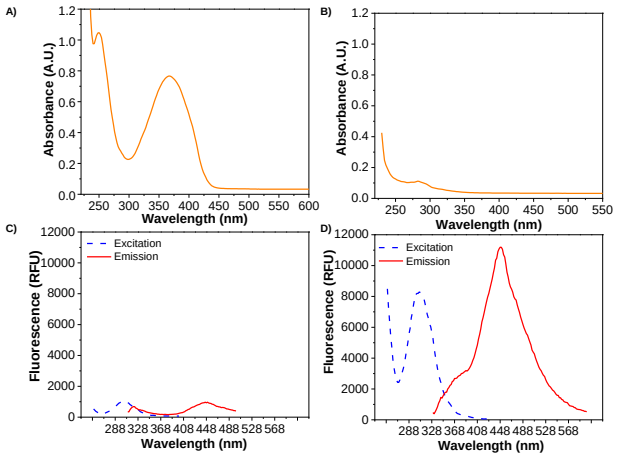


Figure 1. UV-Vis absorbance and fluorescence (emission and excitation) spectra of 4-DANAl (A and C) and 4-DANAm (B and D): **A)** 4-DANAl absorbance spectrum. The highest absorbance peak is 370 nm; **B)** 4-DANAm absorbance spectrum. The highest absorbance peak is 290 nm; **C)** Aldehyde fluorescence spectra; **D)** Amine fluorescence spectra. The excitation and emission peaks were 290 nm and 450 nm, for both the aldehyde and the amine, respectively.

Validation of the spectrophotometric and fluorometric measurements for a model transaminase assay

Encouraged by the UV-Vis and fluorescence properties of 4-DANAm, we set a dual UV-Vis/fluorometric assay to measure the activity of ATAs toward 4-DANAl in presence of a model amine donor (1-PEA). According to the spectra shown in Figure 1, we could measure the reaction course of the model transaminase *Pf*ATA by recording the absorbance at 370 nm (λ_{max} 4-DANAl) and 290 nm (λ_{max} 4-DANAm). Similarly, we could monitor the fluorescence by 4-DANAm at 450 nm (λ_{em}) upon excitation at 290 nm (λ_{ex}) (Figure 2). Therefore, the activity of ATAs can be measured by UV-Vis at two different wavelengths where we can monitor the consumption of the aldehyde (λ= 370 nm) and the production of the amine (λ= 290 nm). Nonetheless, absorbance at 370 nm provides a ΔAbs x time^{-1} roughly 3 times higher than the absorbance at 290 nm (Figure 2A and B). Remarkably, this colorimetric assay allows measurement of ATA activity at higher wavelengths in the UV-Vis spectrum than the conventional "acetophenone-assay" that forces us to measure at 245 nm. In this latter assay, the reaction mixture (including the enzyme) may also absorb at that low wavelength, interfering with the activity quantification. Simultaneously, the activity of ATA can also be determined by monitoring the fluorescence underlying 4-DANAm formation (Figure 2C). In this case, we can see how the shape of the curves resulting from the absorbance at 290 nm and fluorescence (λ_{ex} = 290 nm, λ_{em} = 450 nm) perfectly match, demonstrating that both measurements are accounting for the appearance of 4-DANAm upon the ATA-driven amination. One of the limitations of the fluorescent readout was its incompatibility with the use of exogenous PLP in the reaction mixture. Generally, 1-0.1 mM PLP is added to the reaction mix to boost the ATA activity,[4] however, we found that free PLP was incompatible with the fluorescence measurement. This cofactor gives a strong signal under the excitation/emission (290/450) conditions set for the reported assay (Figure S7). This interference is explained by the similarity between the excitation/emission fluorescence spectra of 4-DANAm and PLP 470 (λ_{ex} = 355 nm, λ_{em} = 470 nm).[22] To address the interference issues underlying the use of PLP, we performed the ATA assay in the absence of PLP. Under these conditions, the endogenous PLP bound to the active center of ATAs was enough to measure their activity without interfering with the fluorescence readout of the product. Hence, we could simultaneously monitor the consumption of 4-DANAl through absorbance, and the generation of 4-DANAm through both absorbance and fluorescence. Figure 2D shows significant differences between the different readouts. Using three ATAs from *Chromobacterium violaceum* (*Cv*ATA), *Halomonas elongata* (*He*ATA), and *Pseudomonas fluorescence* (*Pf*ATA), which are widely used in applied biocatalysis,[15] we found that the outcome activity was higher using the time courses recorded by fluorescence than those recorded by absorbance, indicating a higher sensitivity for the fluorescence readout. Despite this discrepancy, the activity trend between the three tested ATAs is kept and therefore the three readouts can be used for ATA screening and comparison. In all cases, the specific activity of *He*ATA was 5 and 17 times higher than the specific activities of *Pf*ATA and *Cv*ATA, respectively, towards the amination of 4-DANAl using 1-PEA as amine donor. Hence, this method is unique as no other transaminase activity assay can provide different readouts simultaneously in the same reaction chamber.

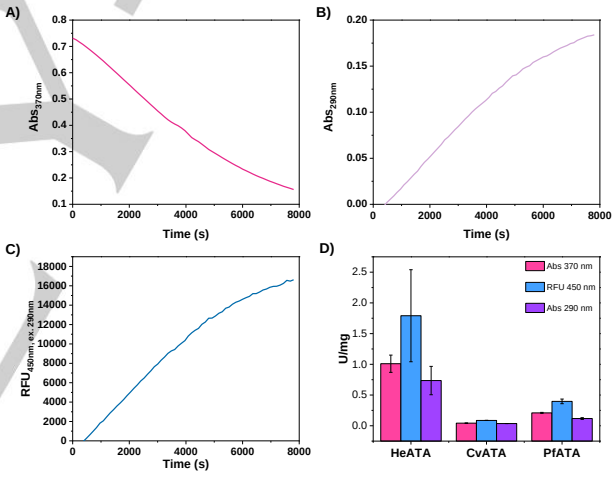


Figure 2. Multiplex transaminase assay: **A)** Reaction course at 370 nm monitoring the consumption of 4-DANAl; **B)** Reaction course at 290 nm following the production of 4-DANAm; **C)** Reaction course monitored through fluorescence at 450 nm upon excitation at 290 nm; **D)** ATA specific activity calculated from the slope of the time courses shown in panels A-C, using the corresponding calibration curves.

After measuring the enzyme kinetics using this novel spectrophotometric/fluorometric assay, we confirmed the consumption of the substrates and the formation of the products by HPLC coupled to a UV-Vis detector and using *Pf*ATA as model enzyme (Figure S8). In the absence of the enzyme (blank sample), the HPLC chromatogram shows a peak with an absorbance maximum of 370 nm corresponding to the aldehyde, meanwhile, no peak was detected corresponding to the amine in the chromatogram of 295 nm. When we analyzed the reaction crude incubated with the enzyme for 24 h, we identified a peak corresponding to acetophenone with an absorbance maximum at 245 nm as result of the deamination of 1-PEA. Moreover, we detected an unknown peak whose absorbance maximum was registered at 295 nm (Figure S8B). We suggest that such a new peak is 4-DANAm formed by the *Pf*ATA-driven transamination of

1-PEA to 4-DANAl. To confirm that such a new peak is 4-DANAm, we performed semipreparative HPLC to purify it. The purified sample was reanalyzed by HPLC-MS detecting the mass pattern that corresponds to 4-DANAm (Figure S9). Moreover, the enzymatically synthesized 4-DANAm shows the same UV-Vis spectra, fluorescence signal, and retention time as the standard 4-DANAm that we chemically synthesized. Hence, chromatography and different spectroscopy analysis demonstrate unequivocally that the *Pf*ATA catalyzes the conversion of 4-DANAl into 4-DANAm using 1-PEA as amine donor, and the activity of the enzyme can be monitored through a multiplex colorimetric/fluorometric assay.

Screening different amine donors for both soluble and immobilized transaminases and their capacity to catalyze the amination of HMF.

Having in hand a robust assay for the measurement of transaminase's activity, we next validated a screening method to test the activity of three model ATAs (*Cv*ATA. *He*ATA and *Pf*ATA) in solution toward a battery of amine donors. We selected a variety of amino acids, amines, and amino alcohols as amine donors. Table 1 shows the relative specific activity of the three enzymes toward different amine donors using the activity toward 1-PEA as a reference. The three enzymes presented different scopes for the amine donor, yet we can identify a common trend in the activity with the following preference order: aromatic amines > L-alanine > aliphatic amines > amino alcohols > other α-amino acids. Specifically, *Cv*ATA and *He*ATA exhibit their highest specific activity toward *p*-hydroxy-benzylamine, meanwhile, *Pf*ATA accepts 1-PEA as the best amine donor. These scopes match reasonably well with the scopes reported for these three enzymes using chromatographic methods.[16]

Moreover, this multiplex assay also served us to confirm the enantiopreference of the three enzymes. In all cases, the ATAs prefer the L-enantiomer of alanine over the D-enantiomer. This result also agrees with the S-specificity reported for the three ATAs herein studied.[15a, 16c] Remarkably, this method allows us to identify amino alcohols as potential amine donors for ATAs; a family of molecules never explored for that purpose. This enzymatic assay is not only relevant to screen different amine donors for newly discovered or engineered ATAs, but also to evaluate their performance on aromatic aldehydes like benzaldehyde, furfural, or 5-hydroxymethyl furfural, since 4-DANAl can be considered as a surrogate for those.

The multiplex method was not only limited to measuring the activity of soluble enzymes but also was useful to measure the activity of immobilized ones. Table 2 shows the specific activity of His-tagged *He*ATA immobilized on agarose microbeads and functionalized with cobalt-chelates (AG-Co^{+2}) for the amination of 4-DANAm using L-alanine, 4-hydroxybenzylamine, and 3-aminopropan-1-ol as amine donors. AG-Co^{2+} is a hydrophilic porous carrier widely used in enzyme immobilization of His-tagged enzymes as it allows their immobilization via a site-directed manner.[17] Regardless of the amine donor, the immobilization of *He*ATA led to a significant reduction of its activity, recovering only 5-8% of the activity measured for the free enzyme before the immobilization. This activity recovery falls in the same range as the recovered activity of other ATAs immobilized on similar carriers.[15, 18]

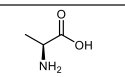
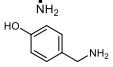
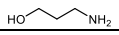
Table 1. Relative specific activity of *Chromobacterium violaceum, Pseudomonas fluorescens,* and *Halomonas elongata* transaminases against different amine donors.

		Relative Specific Activity (%)		
	Amine donor	***Cv*ATA**	***He*ATA**	***Pf*ATA**
Aminoalcohols	3-amino-1-propanol	22	30	2
	4-amino-2-butanol	10	21	2
	Serinol	0	0	0
Amines	1-phenylethylamine	**100**	**100**	**100**
	Isopropylamine	21	9	10
	Isopenthylamine	45	35	51
	4-hydroxybenzylamine	170	332	74
Amino acids	D-alanine	2	0	2
	L-alanine	84	97	53
	D-serine	0	0	0
	L-serine	0	0	3
	glycine	0	0	0
	D-glutamine	0	0	0
	L-glutamine	0	0	0

The 1-PEA was selected as the amine donor of reference assigning to it the value of 100%. The specific activity extracted from the fluorescence readout was 0.109 U mg^{-1}, 0.366 U mg^{-1}, and 1.824 U mg^{-1} for *Cv*ATA, *Pf*ATA, and *He*ATA, respectively using 4-DANAl and 1-PEA as substrates. All the reactions were carried out in 96-well plates where free enzymes were placed and mixed at a final volume of 220 µL with a reaction mixture consisting of 100 mM of HEPES at pH 8 with 4-(dimethylamino)-1-naphthaldehyde 0.1 mM and amine donor 2 mM. Reactions were monitored following the increase in fluorescence (λ_{ex} = 290 nm, λ_{em} = 450 nm).

As the valorization of renewable HMF into products like 5-(hydroxymethyl)furfuryl amine (HMFA) is gaining momentum in the landscape of biorefineries,[19] we assessed the accuracy of the herein developed ATA activity assay to screen for amine donors in the bioamination of aromatic aldehydes such as HMF (Figure 3A). To that aim, we studied the performance of *Pf*ATA and *Cv*ATA immobilized on AG-Co^{2+} for the transamination of HMF using one of the best (4-hydroxybenzylamine) and one of the worst (L-serine) amine donors according to our multiplex ATA assay herein reported (see Table 1). Figure 3B shows how both immobilized enzymes produced significantly more HMFA (quantified by HPLC, Figure S10) when using 4-hydroxybenzylamine than using L-serine.

Table 2. Specific activity (U mg^{-1}) from *Halomonas elongata* free and immobilized on AG-Co^{+2} beads using different amine donors.

Amine donor		U mg^{-1} free enzyme	U mg^{-1} immobilized enzyme
	L-alanine	1.774	0.099
	4-hydroxybenzylamine	6.064	0.502
	3-aminopropan-1-ol	0.556	0.048

All reactions were carried out in 96 well plates where immobilized enzyme on AG-Co^{+2} was placed and mixed at a final volume of 220 µL with a reaction mixture consisting of 100 mM of HEPES at pH 8 with 0.1 mM 4-(dimethylamino)-1-naphthaldehyde and 2 mM amine donor. Reactions were monitored following the fluorescence increase (λ_{ex} = 290 nm, λ_{em} = 450 nm).

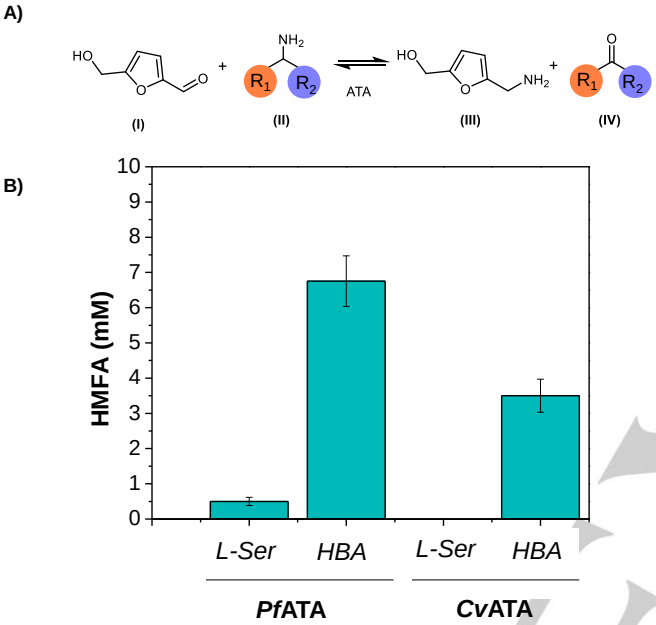


Figure 3. Bioamination of HMF with immobilized ATAs **A)** Reaction scheme where HBA (R1:4-hydroxyphenyl, R2:H) and L-serine (L-Ser) (R1: OH-CH_2-, R2:COOH) were tested as amine donors (II) to aminate HMF (I) giving rise to 5-hydroxymethyl furfurylamine (HMFA)(III) and the corresponding carbonyl group (IV); 4-hydroxybezaldehyde (R1:4-hydroxyphenyl, R2:H) or 3-hydroxy-2-oxo-propionate (R1: OH-CH_2-, R2:COOH). **B)** HMF bioamination yield catalyzed by *Pf*ATA and *Cv*ATA immobilized on AG-Co^{2+} using 4-hydroxybenzylamine (BA) and L-serine as amine donors. HMFA titers were determined by HPLC upon 24 h reaction with 10 mM HMF and 20 mM amine donor at pH 8 and 25 ºC.

When using 4-hydroxybenzylamine as an amine donor, immobilized *Pf*ATA reached 70% HMFA yield; 2-fold higher than the yield obtained when using the immobilized *Cv*ATA and the same amine donor. Contrariwise, we did not detect HMFA when using the immobilized *Cv*ATA and L-serine as an amine donor, indicating that such amino acid is not accepted by this enzyme to transfer the amine group to HMF. However, the immobilized *Pf*ATA was able to perform the amination of HMF although with a product yield lower than 5%. Hence, these results validate the new ATA multiplex assay for screening amine donors in the bioamination of aromatic aldehydes.

Single-particle analysis

Recently, our group has developed an analytic routine to unveil the kinetic parameters of immobilized enzymes through time-lapse microscopy under operando conditions at the single-particle level.[14] This methodology is highly useful to understand the intraparticle functional variability of immobilized enzymes. As the ATA activity assay herein developed allows us to measure the transamination activity through fluorescence, we exploited this assay in a single-particle experiment to reveal the sample heterogeneity of *Pf*ATA immobilized on AG-Co^{2+}. Moreover, the same experiment was performed with the free *Pf*ATA under the same settings to compare the behavior of the two enzyme formulations. This method allows us to follow the fluorescence increment resulting from the formation of 4-DANAm under the microscope in real-time and with a spatial resolution of 0.6 pixels μm^{-1} (Figure 4A). After processing the fluorescence time courses obtained for each microbead, we transformed the fluorescence into amine concentrations using calibration curves with the pure amine and the pure RhB-labelled enzyme to ultimately determine the single-particle kinetics of the immobilized *Pf*ATA (Figure 4B and Figure S2 and S11). We only observed an increase in the intraparticle fluorescence when the immobilized enzyme was incubated with 4-DANAl and 1-PEA. Negative controls without the enzyme or the substrates experienced negligible fluorescence changes over time (Figure S12). From the time courses of the immobilized and free *Pf*ATA (Figure 4B), we calculated their apparent Michaelis-Menten parameters by employing a local search method based on the Hooke & Jeeves algorithm using the software COPASI [20](Table S2). Figure 4C-D shows that the immobilized enzyme presents a 4.8-fold higher apparent K_M (52.59 µM) and 3.4-fold lower k_{cat} (0.023 s^{-1}) than its free counterpart. Single-particle experiments thus confirm that the immobilization of *Pf*ATA provoked a significant reduction in its catalytic efficiency as we observed for immobilized *He*ATA (Table 2). This result matches the data we previously reported elsewhere for the *Pf*ATA immobilized on agarose microbeads activated with cobalt chelates, and further exploited for the biosynthesis of amino alcohols[18a] and benzylamine.[18b]

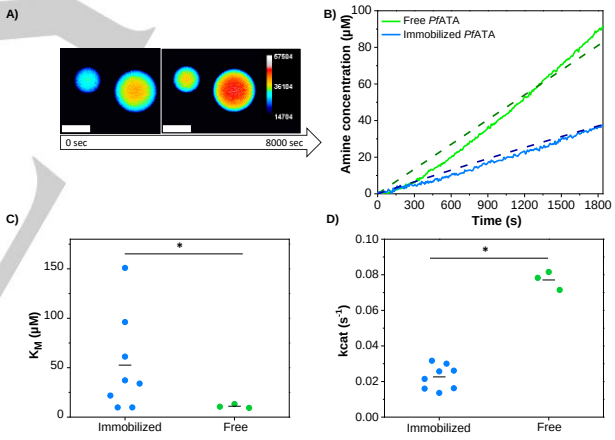


Figure 4. Single-particle assay to determine the apparent Michaelis-Menten parameters for immobilized *Pf*ATA: A) Fluorescence images of the reaction course of one single bead at the initial and the final time of reaction. Scale bar 100 µm; B) Reaction time courses of *Pf*ATA immobilized on one microbead (blue line) and its free counterpart (green line) measured through time-lapse imaging under the same microscope settings. The modeled time courses using COPASI are plotted with a dashed line in the corresponding color; C) Apparent K_M and (D) k_{cat} values of different microbeads (n = 8) with their corresponding mean values (black line). * p< 0.05

Strikingly, the immobilized sample exhibits a high functional heterogeneity between different microbeads with a radius range of 30-75 µm. This heterogeneity was more noticeable for the K_M (Figure 4C) than for the k_{cat} (Figure 4D). When we plotted these two different kinetic parameters as a function of the bead radius and the intraparticle enzyme concentration, we found no trend that may explain the origins of such functional heterogeneity in the microbead population (Figure S13). We suggest that the source of the functional variability found between microbeads may stem from the different diffusion rates of 4-DANAl across the different microbeads regardless of their size. As the set-up for

single-particle measurements is static, substrate gradients may arise within the well, explaining the different functionality of the microbeads depending on their position regarding the substrate injection point. Similar results were observed with CvATA immobilized on AG-Co^{2+}, where the initial rate of the enzyme varied from particle to particle (Figure S14).

Conclusion

In this work, we present a new colorimetric/fluorogenic assay to determine the activity of amino transaminases (ATAs) toward different amine donors employing 4-dimethylamine-1-naphthaldehyde (4-DANAl) as amine acceptor. The amination of this aromatic aldehyde yields an aminated molecule whose maximum absorbance differs from the one registered for the aldehyde. Furthermore, the fluorescence of the amine is higher than the fluorescence of the aldehyde under the same setting conditions. The photophysical properties of such aminated molecules allow us to develop a multiplex ATA assay where the enzyme activity can be monitored over time following three different readouts. This feature is unique to this novel assay as the conventional ATA assay is based on recording only one readout (normally the absorbance a 245 nm). This novel colorimetric/fluorogenic assay was exploited to characterize the amine donor scope of three different ATAs, from *Chromobacterium violaceum* (*Cv*ATA), *Halomonas elongate* (*He*ATA), and *Pseudomonas fluorescence* (*Pf*ATA), widely utilized in applied biocatalysis.

Furthermore, we used 4-DANAl as a surrogate for other aromatic aldehydes to find the best amine donor to aminate them. The best and worst amine donors resulting from this new ATA assay are also the best and worst ones for the bioamination of HMF using the corresponding ATA. Finally, the fluorescence features of this assay can be integrated into time-lapse microscopic analysis to perform single-particle kinetic studies of immobilized ATA. This application has been proven useful to unveil the functional heterogeneity of immobilized biocatalysts. We foresee this assay as a valuable analytical tool to screen new variants of engineered ATAs (free and immobilized) for the bioamination of aromatic aldehydes.

Experimental Section

Materials

Agarose-cobalt microbeads (50−150 µm diameter) were purchased from Agarose Bead Technologies (Madrid, Spain). 4-(dimethylamino)-1-naphthaldehyde (4-DANAl), 5-(hydroxymethyl) furfural (≥99%), acetonitrile, albumin bovine serum standard (BSA), D-alanine (98%), D-glutamine (98%), D-serine (98%), glycine, HEPES, isopentylamine (99%), kanamycin, L-glutamine (100%), L-serine (99%), NaCl, pyruvate, rhodamine B isothiocyanate mixed isomers (RhB) and serinol (98%) were purchased from Sigma-Aldrich (St. Louis, IL, USA). 4-amino-2-butanol (98%), 4-hydroxybenzylamine hydrate (97%), ampicillin, DL-1-phenylethylamine, imidazole (99%), isopropylamine (99%), isopropyl-β-D-thiogalactopyranoside (IPTG, 100%), L-alanine (99%), lysogeny broth (LB) and Tris-HCl were purchased from Fisher Bioreagents (Madrid, Spain). 3-amino-1-propanol (99%) was purchased from Cymit quimica S.L. (Barcelona, Spain). The Bradford protein assay dye reagent was purchased from BIORAD (Biorad. Hercules, CA, USA). Clear bottom black and white microplates (96- well) were purchased from Avantor (2021 VWR International, LLC). Dimethyl sulfoxide (DMSO) was purchased from Scharlab S.L. (Barcelona, Spain). NZY Auto-Induction LB medium (ZYM) was purchased from NZYtech (Lisbon, Portugal).

Enzyme expression and purification

Transaminase from *Chromobacterium violaceum* (*Cv*ATA), *Pseudomonas fluorescens* (*Pf*ATA), and *Halomonas elongata* (*He*ATA) with a 6-His tag at the N-terminus were expressed as described elsewhere.[21] *Cv*ATa and *He*ATA contained in a pMP89b plasmid were transformed into *E. coli* BL21 (DE3) chemical competent cells and cultivated under gently shaking at 37 °C in 50 mL of LB autoinducible medium (ZYM) supplemented with 30 µg/mL of ampicillin overnight. The cells were harvested by centrifugation at 4000 rpm for 30 min at 4 ºC and the pellet was resuspended in 5 mL of 0.1 mM PLP in 50 mM phosphate buffer (pH 8). The resulting suspensions were sonicated and centrifuged and the supernatant containing the enzyme was purified by immobilized metal affinity chromatography (IMAC). The enzymes were eluted with 0.1 mM PLP containing 500 mM imidazole in a 50 mM phosphate buffer (pH 8). *Pf*ATA contained in a pET28b plasmid was transformed into *E. coli* BL21 (DE3) chemical competent cells and cultivated under gently shaking at 37 ºC in 50 mL of LB medium supplemented with 30 µg mL^{-1} of kanamycin until the OD_{600nm} reached 0.4-0.6. At that point, the culture was induced with 10 µM IPTG and the cells were grown at 21 ºC for 16 h. Finally, cells were harvested by centrifugation at 4000 rpm for 30 min at 4 ºC. Then, the cell pellet was resuspended in 50 mM Tris−HCl buffer at pH 8 containing 100 mM NaCl, 30 mM imidazole, and 0.1 mM PLP. The resulting suspensions were sonicated and centrifuged, the supernatant containing the enzyme being purified by IMAC. The enzyme was eluted with 100 mM NaCl, 500 mM imidazole, and 0.1 mM PLP in 50 mM Tris−HCl buffer (pH 8).

Protein concentration determination

The protein concentration was determined by the Bradford assay using bovine serum albumin (BSA) as standard. Briefly, 5 µL of each standard or unknown sample were pipetted into a 96-well microplate. Then, 200 µL of the protein assay dye reagent was added to each well and incubated for 5 min at room temperature. Then the absorbance at 595 nm was measured using a microplate reader (Cytation 5 Cell Imaging Reader, BioTek Instruments).

Acetophenone assay

The activity of each enzyme was measured at 30 °C using a standard photometric assay for monitoring the increase of acetophenone concentration at 245 nm in a microplate reader (Cytation 5 Cell Imaging Reader, BioTek Instruments). The assay mixture contained 2 mM *rac*-1-PEA in DMSO, 2 mM pyruvate, and 0.1 mM PLP in 100 mM HEPES buffer (pH 8). All the measurements were performed in triplicates using 5 µL of enzyme and 200 µL of reaction mix in a 96 UV-well plate.

Chemical synthesis of 4-(dimethylamino)-1-naphthaldehyde (4-DANAl)

The chemical synthesis of the aldehyde was performed following an adapted protocol to the one described by Periasamy and co-workers.[22] A solution of $TiCl_4$ (1 M in CH_2Cl_2, 9.4 mL, 9.4 mmol)

was mixed with CH_2Cl_2 (10 mL), and added dropwise under an argon atmosphere over a cooled solution (0 ºC) of *N*,*N*-dimethyl-1-naphthylamine (805 mg, 4.7 mmol) and trimethyl orthoformate (771 mL, 7.1 mmol) in anhydrous CH_2Cl_2 (9.4 mL). The reaction mixture was stirred at 0 ºC for 30 min and then left warm at room temperature, stirring the reaction for an additional 5 h at this temperature. The reaction was quenched by the addition of a K_2CO_3 saturated aqueous solution and stirred for an additional 30 min. Then, the reaction mixture was filtered, and the organic layer was separated. The aqueous layer was extracted with CH_2Cl_2 (3 x 20 mL), and the combined organic phases were washed with brine (20 mL) and dried over anhydrous Na_2SO_4. The resulting organic phase was filtered, and the solvent was evaporated under reduced pressure. The reaction crude was purified by column chromatography on silica gel (5% EtOAc/hexane), yielding 4-DANAl as a brown solid (605 mg, 65% yield). The NMR data was in accordance with the ones already reported in the literature: ^{1}H-NMR (300.13 MHz, $CDCl_3$): δ 10.14 (s, 1H), 9.40–9.33 (m, 1H), 8.15 (dd, *J* = 9.0, 1.3 Hz, 1H), 7.76 (d, *J* = 7.9 Hz, 1H), 7.62 (ddd, *J* = 8.5, 6.9, 1.4 Hz, 1H), 7.50 (ddd, *J* = 8.3, 6.8, 1.4 Hz, 1H), 6.94 (d, *J* = 7.9 Hz, 1H) and 2.98 (s, 6H) ppm.

Chemical synthesis of 4-(aminomethyl)-*N*,*N*-dimethylnaphthalen-1-amine (4-DANAm)

4-DANAm was synthesized through the protocol of Bhattacharyya and co-workers described for the synthesis of other primary and secondary amines.[22] A mixture of 4-DANAl (145 mg, 0.7 mmol), $Ti(^iPrO)_4$ (432 mL, 1.5 mmol), and NH_3 in EtOH (2 M, 5.5 mL, 10.9 mmol) was stirred for 16 h in a sealed glass tube at room temperature and under argon atmosphere. Then, $NaBH_4$ (42 mg, 1.1 mmol) was carefully added to the reaction mixture, and the mixture was stirred for an additional 3 h. The reaction was quenched by pouring it into an aqueous ammonium hydroxide solution (2 M, 4 mL). The resulting inorganic precipitate was filtered off and washed with EtOAc (2 x 10 mL). The organic layer was separated, and the remaining aqueous layer was extracted with EtOAc (2 x 10 mL). The combined organic phases were extracted with HCl 1 M (20 mL), recovering the acidic aqueous extract that was washed with EtOAc (20 mL). The resulting aqueous solution was basified with an aqueous NaOH solution (2 M) until pH 10-12 and extracted with EtOAc (3 x 20 mL). The combined organic phases were washed with brine (10 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The reaction crude was purified by column chromatography on silica gel (5% NH_3/MeOH), yielding 4-DANAm as a brown oil (7.8 mg, 6% yield). ^{1}H-NMR (300.13 MHz, $CDCl_3$): δ 8.30 (dd, J = 8.6, 1.5 Hz, 1H), 8.08 – 8.01 (m, 1H), 7.46 (ddd, J = 13.8, 7.9, 3.2 Hz, 3H), 7.04 (d, *J* = 7.6 Hz, 1H), 4.85 (s, 2H), 4.30 (s, 2H) and 2.90 (s, 6H) ppm. ^{13}C-NMR (75.5 MHz, $CDCl_3$): δ 150.81 (C), 133.17 (C), 130.32 (C), 129.36 (C), 126.71 (CH), 126.03 (CH), 125.03 (CH), 124.88 (CH), 124.41 (CH), 113.48 (CH), 51.31 (CH_2) and 45.44 (CH_3) ppm.

4-DANAm enzymatic synthesis

Two samples were prepared to contain the reaction mix either with buffer (blank) or the enzyme. The reaction mix was composed of 0.1 mM 4-DANAl and 2 mM DL-1-PEA in 100 mM HEPES buffer (pH 8). Approximately 0.1 U mL^{-1} of enzyme in 50 mM phosphate buffer and 0.1 mM PLP (blank) were added to the reaction mix and the reaction was operating for 24 h at room temperature under orbital agitation (45 rpm). After this time, the samples were filtered to get rid of the enzyme and lyophilized ON. Both samples were resuspended in water and first analyzed by High-Performance Liquid Chromatography (HPLC) system Delta 600 (Waters) using acetonitrile (A) as mobile phase and water with 0.1% formic acid (B) following a gradient of 90:10. The 1-PEA of the amine was recollected using a preparative column and lyophilized ON to finally resuspend it in water.

Chromatographic methods

Ultra-high-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC/ESI-TOF MS) (Waters) was used to characterize the amine synthesized via enzymatic and chemical methods. An acquity UPLC BEH C18 17 µm (2.1 mm x 100 mm) column was used with a constant flow of 0.75 mL min^{-1} using a mobile phase composed of acetonitrile (A) and 0.1% formic acid in water (B) with a gradient of 60:40 for 15 min. Retention times were acetophenone (5.38 min), 4-DANAm (2.26 min), and 4-DANAl (8.58 min).

Fluorescence and absorbance characterization of 4-DANAl and 4-DANAm

The absorbance and fluorescence characterization were performed in 96-well UV plates (Thermo Scientific) with 200 µL of 4-DANAm or 4-DANAl diluted in acetonitrile up to a final concentration of 0.1 mM and 5 µL of a blank (50 mM phosphate buffer and 0.1 mM PLP) in a spectrophotometer Cytation 5 Cell Imaging Reader (BioTek Instruments). In both cases, the absorbance spectrum was performed in a range of 230 to 600 nm. The fluorescent excitation wavelength was scanned from 250 to 410 nm and the emission wavelength from 320 to 600 nm and 310 to 500 nm for the amine and aldehyde, respectively.

Multiplex readout ATA assay

The assay was performed in UV 96-well plates using a Cytation 5 Cell Imaging Reader (BioTek Instruments) with a wavelength of 290 nm for excitation and 450 nm for emission. The absorbance was also followed at 290 and 370 nm. The reaction was initiated by adding 20 µL of the enzymatic solution to 200 µL of the reaction mixture (4-DANAl 0.1 mM and DL-1-PEA 2 mM in 100 mM HEPES buffer, pH 8) and left to react for 30 min. As blank samples, the reaction mix was incubated under the same conditions and without the enzyme during the same analysis time (Figure S15). One unit of ATA activity was defined as the amount of enzyme required to produce one µmol of 4-DANAm per min at the assessed conditions.

Enzyme immobilization

Immobilized enzymes were prepared by mixing 10 volumes of crude cell extract containing the His-tagged protein with 1 volume of AG-Co^{2+} microbeads and incubated under orbital shaking for 2 h at 4 ºC. Then, the suspension was removed and the microbeads containing the enzyme were washed with 3 volumes of 50 mM phosphate buffer or 50 mM Tris-HCl buffer at pH 8 containing 0.1 mM PLP.

Different substrates as amino donors

The activity for the different amine donors was determined by fluorescence emission with a maximum peak at 450 nm using an excitation wavelength of 290 nm. The reaction was initiated by adding 20 µL of the enzymatic solution or suspension to 200 µL

of the reaction mixture 4-DANAl 0.1 mM and the corresponding amine donor 2 mM in 100 mM buffer HEPES pH 8. Some of the substrates were diluted in DMSO and others in water, depending on their solubility. The following substrates were tested as amine donors: 3-amino-1-propanol, 4-amino-2-butanol, and serinol as amino alcohols; isopropylamine, isopentylamine, 4-hydroxybenzylamine hydrate, and DL-1-PEA as amines; and D-alanine, L-alanine, D-serine, L-serine, glycine, D-glutamine, and L-glutamine as amino acids.

HMF amination

The amination of HMF using a biocatalyst was made by mixing 100 mg of biocatalyst with HMF (10 mM), amine donor (20 mM), and PLP (1 mM) in 100 mM HEPES buffer at pH 8. 4-hydroxybenzylamine and L-serine were the amine donors used for the amination of HMF. The reaction was followed by HPLC (Agilent Infinity 1260 II). Samples were detected in a range of 210 to 280 nm and were eluted at a 0.9 mL min^{-1} flow rate employing two mobile phases: phase A composed of acetonitrile, and phase B composed of trifluoroacetic acid (TFA) 0.01 % in water. The method followed consisted of a gradient of 5:95 for 1 min, 10:90 for 1 min, 55:45 for 13 min, 65:35 for 5 min, 75:25 for 5 min, and 5:95 for 15 min for a total of 40 min assay. The flow was constant at 0.9 mL min^{-1}. Retention times were L-serine 1.137 min, 5-hydroxymethylfurfurylamine (HMFA) 1.560 min, 4-hydroxybenzylamine 1.883 min, and HMF 3.517 min.

Enzyme fluorescent labeling.

Fluorescent labeling of ATAs with RhB was performed by mixing the enzyme in 100 mM sodium bicarbonate buffered solution at pH 8.5 and 0.1 mM PLP with RhB solution in DMSO (1:10 molar ratio) and incubated for 1 h under stirring at room temperature. The remaining RhB was removed by filtering the enzyme solution using a centrifugal filter unit (Amicon, Sigma-Aldrich, St. Louis, IL, USA) with 50 mM sodium phosphate buffer and 0.1 mM PLP solution at pH 8 until no fluorescence was detected in the filtered solution. Immobilization of the labeled enzyme was completed as previously described for the non-fluorescently labeled enzyme.

Single-particle assay

The assay was performed on a clear bottom, black 96-well microplate using a Cytation5 Cell Imaging Reader (BioTek Instruments). To observe the sample a phase objective Plan Fluorite 4X with a numerical aperture of 0.13 and coupled to an apotome grid WD with a working distance of 17 mm was employed. The LED/filter pairs were used for fluorescence imaging of fluorescent 4-DANAm and RhB labeled enzymes, including the blue channel (DAPI filter, λ_{ex} = 365 nm/λ_{em} = 447/90 nm) and red channel (RFP filter, λ_{ex} = 531 nm/λ_{em} = 593/40 nm), respectively. Moreover, to follow changes in the position of the microbeads during the experiment, the brightfield channel was also recorded. The time-lapse experiments were performed with 5 µL of a suspension 1:10 (w/v) of AG-Co^{2+} beads with the immobilized enzyme in 25 mM Tris−HCl 0.1 mM PLP at pH 8 in a well. Then, 191 µL of a solution containing 0.1 mM of 4-DANAl in HEPES buffer 100 mM pH 8 were added to the well, and initial fluorescence microscopy imaging in the blue channel was measured to follow fluorescence changes of 4-DANAm. Finally, 4 µL of 1-PEA 2 mM solution in DMSO were added to the solution to reach a total final volume of 200 µL, and the reaction was recorded every 12 s through time-lapse fluorescence microscopy for 1 hour. As negative controls we incubated the same suspension containing beads without immobilized enzyme, beads with the immobilized enzyme in HEPES buffer 100 mM pH 8, and beads without enzyme in the mix of reaction without 1-PEA or 4-DANAl to verify that the reaction was not occurring under those conditions.

The image processing and analysis were performed with a custom plugin for FIJI[23] recently developed in our group.[14, 24] After processing the images obtained, we obtained ROIs from 10 different beads and quantified the relative fluorescence units (RFUs) of each ROI at each time point. The RFUs were converted to amine concentration through data normalization and using a calibration curve of the pure amine (4-DANAm) under the microscope setup (see supporting information, Figure S1-S2). We also determined the enzyme concentration by measuring the fluorescence of the RhB-labeled enzyme immobilized on single particles using a calibration curve for the labeled enzyme under the same microscope set up (Figure S2). The data analysis is explained in full detail in the supporting information.

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Abbreviations

1-PEA - 1- phenylethylamine
4-DANAl - 4-dimethylamine-1-naphthaldehyde
4-DANAm - 4-(aminomethyl)-N,N-dimethylnaphthalen-1-amine
ATA – Amine transaminase (E.C. 2.6.1.X)
BA - 4-hydroxybenzylamine
CvATA - Amine transaminase from *Chromobacterium violaceum*
HeATA - Amine transaminase from *Halomonas elongata*
HMF - 5-(hydroxymethyl)furfural
HMFA- 5-(hydroxymethyl)furfural amine
PfATA - Amine transaminase from *Pseudomonas flourescens*
PLP - pyroxidal 5'-phosphate.
Rhb- Rhodamine B

Note

The authors declare no competing financial interests.

Keywords: Biocatalysis • Protein immobilization •Transaminases kinetics • Amine donor screening • Single-particle assay

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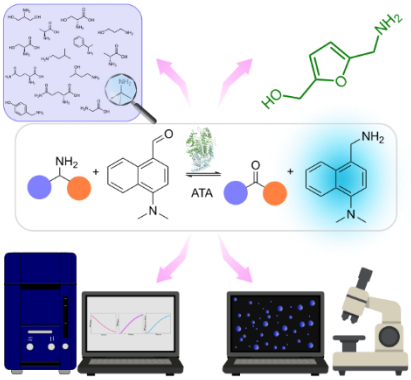

A multidimensional and multiplex fluorescence-based kinetic assay assesses transaminase activity using an aromatic aldehyde that is a surrogate of the 5-(hydroxymethyl)furfural (HMF). The assay also allows screening a battery of amine donors using free and immobilized ATAs as biocatalysts as well as study the biocatalyst at the microscopic level.

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