Biotechnology Advances 66 (2023) 108171

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

In-depth analysis of biocatalysts by microfluidics: An emerging source of data for machine learning

Michal Vasina [a,b], David Kovar [a,b], Jiri Damborsky [a,b], Yun Ding [c], Tianjin Yang [c,d], Andrew deMello [c], Stanislav Mazurenko [a,b,*], Stavros Stavrakis [c,**], Zbynek Prokop [a,b,*]

[a] Loschmidt Laboratories, Department of Experimental Biology and RECETOX, Faculty of Science, Masaryk University, 602 00 Brno, Czech Republic
[b] International Clinical Research Centre, St. Anne's University Hospital, 656 91 Brno, Czech Republic
[c] Institute for Chemical and Bioengineering, ETH Zürich, 8093 Zürich, Switzerland
[d] Department of Biochemistry, University of Zurich, 8057 Zurich, Switzerland

ARTICLE INFO

Keywords:
Enzyme
Biochemical characterization
Biotechnology
Catalytic activity
Thermostability
Steady-state kinetics
Protein crystallography
Big data
Protein engineering
Artificial intelligence

ABSTRACT

Nowadays, the vastly increasing demand for novel biotechnological products is supported by the continuous development of biocatalytic applications that provide sustainable green alternatives to chemical processes. The success of a biocatalytic application is critically dependent on how quickly we can identify and characterize enzyme variants fitting the conditions of industrial processes. While miniaturization and parallelization have dramatically increased the throughput of next-generation sequencing systems, the subsequent characterization of the obtained candidates is still a limiting process in identifying the desired biocatalysts. Only a few commercial microfluidic systems for enzyme analysis are currently available, and the transformation of numerous published prototypes into commercial platforms is still to be streamlined. This review presents the state-of-the-art, recent trends, and perspectives in applying microfluidic tools in the functional and structural analysis of biocatalysts. We discuss the advantages and disadvantages of available technologies, their reproducibility and robustness, and readiness for routine laboratory use. We also highlight the unexplored potential of microfluidics to leverage the power of machine learning for biocatalyst development.

1. Introduction

Biocatalysts offer environmental and economic advantages to accelerate industrially and pharmaceutically important biotechnological processes (Badenhorst and Bornscheuer, 2018). Unfortunately, enzymes sometimes exhibit low catalytic efficiency, insufficient stability under operating conditions, or poor stereo- and/or regioselectivity, which limits their wide use (Reetz, 2013). Thus there is a need to either find novel enzymes in the natural diversity by (meta)genomic approaches or to improve the known enzymes by exploring the artificial diversity generated by protein engineering techniques (Wahler and Reymond, 2001).

The process of novel biocatalysts discovery for biotechnologies, either from natural or artificial diversity, has been significantly

Abbreviations: 3D-MiXD, 3D-printed, X-ray-compatible microfluidic device; ADE, acoustic droplet ejection; Cryo-EM, cryogenic electron microscopy; DOME, data, optimization, model and evaluation; DOT, drop-on-tape technique; DMS, deep mutational scanning; DESI, desorption electrospray ionization; DSC, differential scanning calorimetry; DMF, digital microfluidic; DLS, dynamic light scattering; EPR, electron paramagnetic resonance; ESI, electrospray ionization; EC, Enzyme Commission; FACS, fluorescence-activated cell sorting; FRET, fluorescence resonance energy transfer; Gboost, gradient boosting; HLDs, haloalkane dehalogenases; HPLC, high-performance liquid chromatography; HT-MEK, High-Throughput Microfluidic Enzyme Kinetics; HTS, high-throughput screening; IMER, Immobilized microfluidic enzyme reactors; KNN, K-nearest neighbors; LASSO, least absolute shrinkage and selection operator; ML, machine learning; MS, mass spectrometry; MicroPEX, microfluidic profile explorer; MMSA, microfluidics-based mobility shift assay; NN, neural network; NGS, next-generation sequencing; NMR, nuclear magnetic resonance; PLS, partial least squares; PDMS, poly(dimethylsiloxane); PCA, principal component analysis; POC, proof-of-concept; SODA, sequential operation droplet array; SFX, serial femtosecond crystallography; SSX, serial synchrotron crystallography; SAXS, small-angle x-ray scattering; SVM, support vector machines; SERS, surface-enhanced Raman spectroscopy; SPR, surface plasmon resonance; XFEL, X-ray free-electron laser;.

* Corresponding authors at: Loschmidt Laboratories, Department of Experimental Biology and RECETOX, Faculty of Science, Masaryk University, 602 00 Brno, Czech Republic.
** Corresponding author.
E-mail addresses: mazurenko@mail.muni.cz (S. Mazurenko), stavros.stavrakis@chem.ethz.ch (S. Stavrakis), zbynek@chemi.muni.cz (Z. Prokop).

https://doi.org/10.1016/j.biotechadv.2023.108171
Received 24 January 2023; Received in revised form 4 May 2023; Accepted 4 May 2023
Available online 5 May 2023

improved thanks to next-generation sequencing (NGS) and high-throughput screening (HTS), which increasingly rely on microfluidics (Bunzel et al., 2018; van Dijk et al., 2014). However, the most promising biocatalysts identified within these campaigns must be biochemically characterized (Fig. 1). The biochemical characterization can easily become the most time- and sample-demanding step, especially when conventional low-throughput technologies are applied (Vasina et al., 2020). Miniaturization has been successfully utilized in modern NGS and HTS methods. The same opportunity arises in developing modern techniques for the in-depth experimental analysis of the ever-increasing number of identified promising biocatalysts.

This review presents the state-of-the-art and recent trends in the

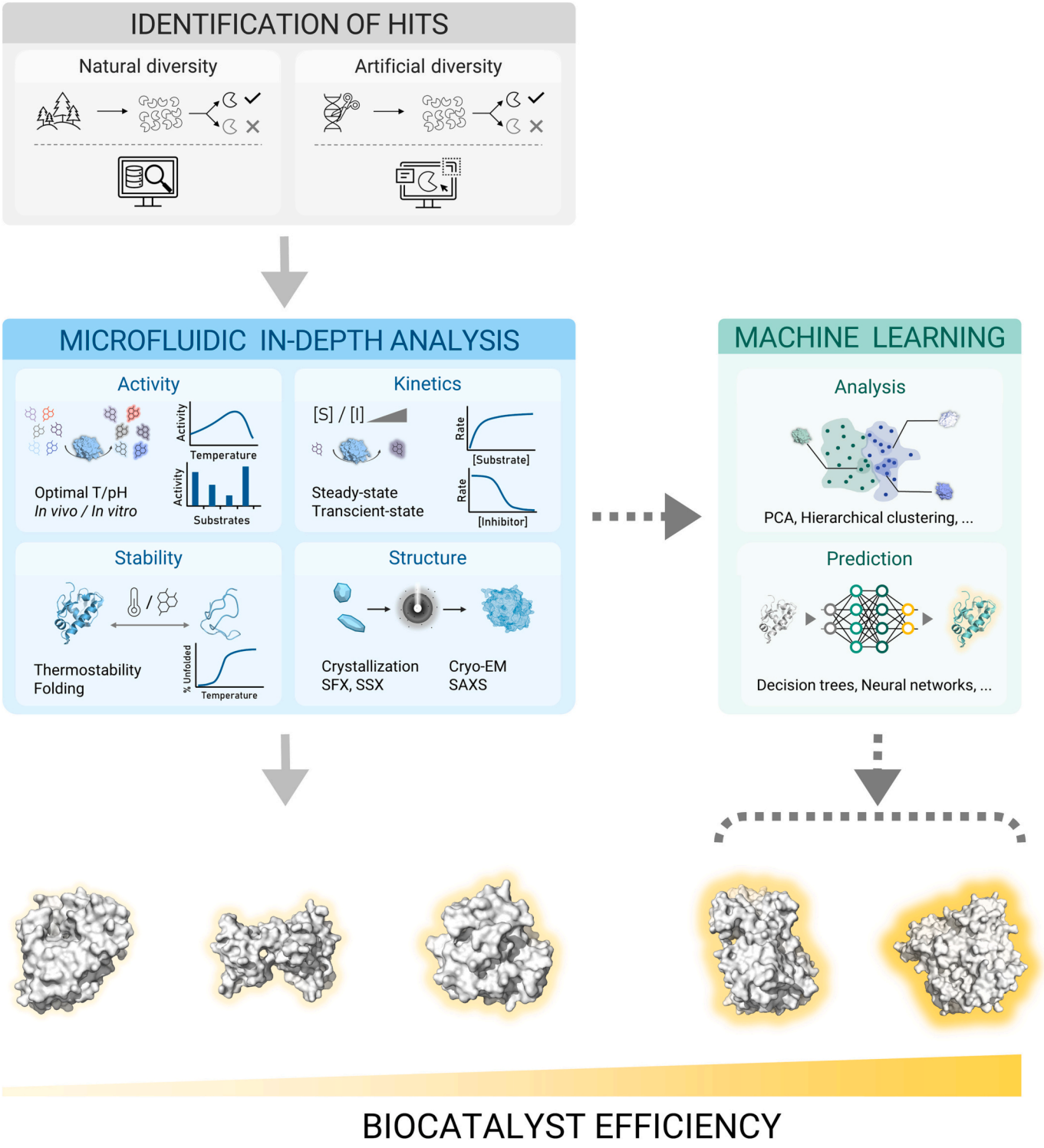

Fig. 1. The synergy of microfluidic characterization and machine learning for developing efficient biocatalysts. Interesting biocatalysts are usually identified by screening natural or artificial diversity (top, grey). Microfluidics (left, light blue) facilitates the in-depth experimental analysis of biocatalysts in terms of activity, kinetics, stability, and structure. Machine learning (right, light green) can work with data collected by microfluidics to analyze them and make predictions, which can yield more efficient biocatalysts (dashed arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

development, applications, and commercialization of microfluidic devices for the biochemical characterization of biocatalysts. Rather than focusing on microfluidic HTS methods, such as droplet sorting, we direct the readers to several excellent reviews on this topic (Neun et al., 2020; Sesen et al., 2017). In addition, this review does not cover deep mutational scanning (DMS), which combines HTS and NGS to assess the effects of individual mutations on the enzyme function (Fowler and Fields, 2014; Romero et al., 2015).

After a brief introduction to biocatalyst discovery (Section 2), we describe the microfluidic technology applicable to the experimental analysis of biocatalysts (Section 3). Section 4 provides an overview of currently available microfluidic techniques for analyzing key properties of enzymes, including the four major categories of activity, kinetics, stability, and structure. We evaluate the maturity, robustness, and readiness of the microfluidic technology for routine laboratory use. Finally, we highlight the advantages and opportunities of combining microfluidics with machine learning (Section 5). Not yet fully established, this synergy can result in the generation and leveraging of large datasets in enzyme design and prediction of enzymes with improved target properties (Fig. 1).

2. Discovery of novel biocatalysts

With the many challenges humanity faces in ecology, industry, and healthcare, there is a growing need for novel or improved biocatalysts to accelerate the desired biotechnological processes. Their discovery usually requires two main steps: (i) effective screening of the available diversity and (ii) subsequent in-depth experimental analysis of the identified variants regarding the key properties needed for successful applications in biotechnology. The available diversity comprises natural diversity – represented by exponentially growing (meta)genomic databases – and artificial diversity designed by protein engineering methods (Fig. 1). Both can be efficiently explored using well-established experimental HTS protocols or semi-rational approaches combined with *in silico* methods. Yet, with the ever-increasing number of promising candidates, it is necessary to find new sample-efficient high-performance techniques that will allow their analysis.

2.1. Natural diversity exploration

Microorganisms represent the largest reservoir of novel enzymes on Earth (Simon and Daniel, 2011) while also inhabiting the widest variety of ecosystems (Berini et al., 2017), including extreme habitats such as hot springs, acid mine waters at near zero pH, or Antarctic ices (Mirete et al., 2016). However, approximately 99% of microorganisms are uncultivable (Culligan et al., 2014; Handelsman, 2004). Metagenomics offers the culture-independent analysis of the microbial community in any environmental sample (Sleator et al., 2008). After collecting the environmental sample and DNA isolation, several ways are available to screen for interesting genes. The two most popular kinds of screening are (i) function-based (functional metagenomics) and (ii) sequence-based (genetic screening) (Berini et al., 2017).

Function-based screening for biocatalysts is based on the enzyme activity readout (Yang and Ding, 2014), and it is carried out mostly on agar plates (Ngara and Zhang, 2018). The acceleration of such screening campaigns is typically achieved using various HTS methods (Bunzel et al., 2018). These include microtiter plates (Lafferty and Dycaico, 2004), ultrahigh-throughput HTS platforms, such as fluorescence-activated cell sorting (FACS) (Bonner et al., 1972) or in-vitro compartmentalization (Tawfik and Griffiths, 1998), and more recently, microfluidic systems (Sesen et al., 2017), e.g., microcapillary arrays (Chen et al., 2016) or droplet sorting devices (Colin et al., 2015). The (ultra) high-throughput screening platforms often employ detection via fluorogenic or chromogenic substrates, which might differ from the desired reaction and thus will be less relevant (You and Arnold, 1996). Another limiting factor of these approaches is the potential biased and insufficient expression of proteins of interest in the library host, most commonly *Escherichia coli* (Uchiyama and Miyazaki, 2009). Such expression issues can be addressed by choosing an alternative expression host strain, e.g., *Bacillus subtilis, Pseudomonas putida* or *Pichia pastoris*, which can significantly increase the success rate of enzyme expression despite higher laboratory demands (Katzke et al., 2017).

In contrast, genetic screening methods are independent of gene expression. In this approach, the polymerase chain reaction (PCR) with primers specific for conserved regions of the genes (e.g., regions carrying catalytic residues for enzymes) is used (Coughlan et al., 2015). Either the clones in metagenomic libraries or directly the extracted environmental DNA can be screened in this manner (Berini et al., 2017). The main drawback of genetic screening is its bias toward the members of already known gene families (Culligan et al., 2014), whereas function-based screening enables the discovery of completely new classes of enzymes bearing low similarity to known sequences (Ufarté et al., 2015). The recent rapid advancement of NGS techniques, mainly the “shotgun” sequencing of isolated environmental DNA (Quince et al., 2017), has given birth to alternative *in silico* screening approaches (Berini et al., 2017). Various sequence- or structure-based bioinformatics analyses can subsequently help to identify and rationally select attractive targets for further experimental testing (Höhne et al., 2010; Vanacek et al., 2018), although the hits are usually sequentially homologous to the known enzymes.

2.2. Artificial diversity exploration

Apart from the discovery of enzymes from natural diversity, it is also possible to explore the diversity created artificially, e.g., by protein engineering, to improve the desired properties of the enzyme (Bornscheuer, 2016; Wahler and Reymond, 2001). As a dominant strategy, protein engineering uses directed evolution (Zeymer and Hilvert, 2018), where random mutagenesis, created, e.g., by DNA shuffling (Stemmer, 1994) or error-prone PCR (Cadwell and Joyce, 1992), is performed to introduce diversity and obtain a library of mutant variants. Many studies also harnessed the “small-but-smart” combinatorial libraries (Jochens and Bornscheuer, 2010), performing the simultaneous multiple site-saturation mutagenesis, which in many cases increased the number of obtained hits (Alejaldre et al., 2021). The subsequent procedure is practically identical to the function-based screening mentioned above. The alternative strategy is rational design, where site-directed mutagenesis (Hutchison et al., 1978) is applied to substitute a particular amino acid in the protein sequence with another one. This approach, however, usually requires information about the parental enzyme structure to guide the decision on the exact position and substitution (Yang and Ding, 2014).

With the increasing availability of enzymology data, researchers started combining both of these fundamental approaches in a semi-rational design (Lutz, 2010). Furthermore, modern enzyme engineering leverages a number of alternative strategies to improve enzymes (Chen and Arnold, 2020), with the increasing use of phylogenic analyses and machine learning (more in Section 5). For example, ancestral sequence reconstruction, a method inferring the evolutionary precursors of extant proteins, was used to increase the thermostability and enantioselectivity of a wide panel of industrial Ene reductases (Livada et al., 2023). Thermostable and catalytically efficient luciferases were constructed using deep learning-based *de novo* protein design called hallucination.(Yeh et al., 2023).

2.3. High-throughput characterization of biocatalysts

After identifying the hits in natural or artificial diversity, these enzymes must be produced and biochemically characterized. Researchers often need to deal with the issues associated with the expression and purification of soluble enzymes, yet these limitations are outside the scope of this article, and we refer the readers to several reviews on this

topic (Correa and Oppezzo, 2015; Ki and Pack, 2020). The subsequent biochemical characterization can be regarded as one of the bottlenecks in the whole process of enzyme discovery/improvement (Bornscheuer, 2016) due to the necessity of performing an in-depth experimental analysis of various important enzyme properties (Roodyn, 1970; Vasina et al., 2022b), including activity, kinetics, stability, and structure (Fig. 1). Basic enzyme characterization parameters, which often need to be determined, are summarized in Table 1.

Conventional biochemical characterization methods carried out in test tubes suffer from low throughput and high sample consumption, requiring large-scale protein production and purification of enzymes for in-depth biochemical characterization. Throughput and sample consumption have been improved by integrating microtiter plate-based technology (Rachinskiy et al., 2009) and robotic handling (Dörr et al., 2016). Such miniaturization of characterization techniques leads to increased throughput and requires much smaller amounts of proteins, which could be efficiently delivered by small-scale or cell-free expression and purification systems (Vasina et al., 2020). In this regard, the technology of microfluidics offers favorable capacities in terms of higher throughput and lower sample consumption (Bunzel et al., 2018; Gielen et al., 2015). We describe the benefits of microfluidics in Section 3, while in Section 4, we summarize the recent endeavors to use this technology for various biochemical analyses of biocatalysts.

3. Principles of microfluidic technology

This section describes the various microfluidic types used for the experimental analysis of biocatalysts, the practical aspects of microfluidics, and the most commonly used detection methods. Compared to conventional (macroscale) instrumentation, microfluidic technologies offer the seductive advantages of enhanced analytical performance, low unit cost, reduced sample consumption, process integration, system automation, operational flexibility, and small instrumental footprints. Microfluidic systems can be categorized according to the technique used to manipulate the contained fluid. Primary examples include continuous flow microfluidics, droplet microfluidics, programmable (valve-based) microfluidics, and digital microfluidics. (Fig. 2A).

Table 1
The definitions of selected enzyme characteristics.

Characteristics	Parameters	Definition and units
Activity	In vitro activity	[kat] = [mol]/[s], [IU] = [μmol]/[min]
	Specific enzyme activity	[kat]/[kg], [IU]/[mg]
	pH/temperature optimum	pH or temperature (°C) with the maximum observed activity
Kinetics	Maximum velocity V_{max}	V_{max} = [mol]/[s], or [μmol]/[min]
	Turnover number k_{cat}	$k_{cat} = V_{max}/[E]_0$
	Michaelis constant K_M	$K_M = (k_{-1} + k_{cat}) / k_1$
	Catalytic efficiency k_{cat}/K_M	$k_{cat}/K_M = k_{cat} \cdot k_1/(k_{-1} + k_{cat})$
	half-inhibitory concentration IC_{50}	inhibitor concentration, decreasing enzyme activity to half
	Inhibition constant K_i	$K_i = IC_{50}/(1 + [S]/K_M)$
	E-value (enantioselectivity)	$E = (k_{cat}/K_M)^R/(k_{cat}/K_M)^S$
Stability	Melting temperature T_m	temperature (°C) at which 50% of protein molecules are folded and 50% unfolded
	Half-life $\tau_{1/2}$	time until activity reduces to half of the original activity at a given temperature
Structure	CD ellipticity profile	ellipticity (°) dependence on wavelength
	Oligomeric states	Number of subunits of the polypeptide chain
	Resolution (X-ray/Cryo-EM)	a measure of the resolvability in the electron density map of a molecule, expressed in [Å]

The above-used abbreviations are kat = katal, IU = international unit, $[E]_0$ = total concentration of enzyme, k_1 and k_{-1} are rate constants of formation and reverse dissociation of Enzyme-substrate complex – ES, respectively.

3.1. Types of microfluidic systems

3.1.1. Continuous flow microfluidics

In continuous flows, streams of fluid pass through a channel, with mixing occurring through diffusion orthogonal to the flow direction. Fluid flow can be controlled by external pumps or other types of fluid handling based on electric, magnetic, or capillary forces. Flow-focusing schemes can be used for extremely fast mixing of reagents (on the order of a few microseconds), making them particularly useful for probing rapid reactions (Burke et al., 2013). Another type of continuous flow mixer uses special geometries (such as groves in the channel surface) to efficiently mix solutions under laminar flow conditions (Buchegger et al., 2012). Continuous flow microfluidic systems have been utilized for the biochemical characterization of enzymes since the turn of the 21st century (Hadd et al., 1999). Hadd et al. combined flow injection analysis and electrophoretic separation within a microfluidic device to assess the inhibition of acetylcholinesterase. Current designs of automated continuous-flow microfluidics deals with valve-based microfluidic technology that implements more complex and user-defined fluidic operations (Subsection 3.1.4). For example, coupling pneumatic valves with continuous flow microfluidics has led to "microfluidic large-scale integration" (Thorsen et al., 2002), which can be viewed as a microfluidic equivalent to a well plate with extra functionalities. Despite numerous applications in chemistry and biology, continuous flow systems are often compromised due to issues associated with Taylor dispersion (causing smear of the concentration distribution of the reaction mixture composition over time), cross-contamination, and solute-surface interactions (Shang et al., 2017).

3.1.2. Droplet microfluidics

Droplet microfluidics has gained significant popularity over the past decade due to the elimination of dispersion effects of continuous flow systems by localization of reagents in isolated reaction vessels in a controllable and configurable manner (Ding et al., 2020). Using water-in-oil droplets as biochemical vessels has engendered the miniaturization of assay volumes to sub-nanoliter (nL) scales and augmented analytical throughputs to above 10^6 assays per day (Sesen et al., 2017). The formation of droplets within a microfluidic device involves the dispersion of one fluid inside another immiscible or partially immiscible fluid. This process can be achieved using various techniques, and several recent reviews focus on the topic in great detail (Shang et al., 2017; Suea-Ngam et al., 2019; Zhu and Wang, 2016). One of the main limitations of droplet microfluidics is the leakage of hydrophobic reagents out of the aqueous phase (Chen et al., 2012). This issue can be addressed by selecting the right surfactant/oil combination or by chemical modification of the substrate – addition of charged groups to the molecule (Neun et al., 2020). Alternatively, the partitioning of small molecules between the used phases can be turned in our favor, e.g., by a continuous supply of the hydrophobic reagent from the oil phase (details in Subsection 4.1.3) (Buryska et al., 2019) or from organic phase (Xiang et al., 2021).

One of the early attempts to use droplet microfluidics to study enzymatic reaction was shown by Song et al. (Song and Ismagilov, 2003). They utilized a droplet-based microfluidic platform to perform single-turnover ribonuclease A kinetics with millisecond time resolution. Using exposure times in the range of seconds, they acquired images constructed from the average signal of hundreds of identical droplets. Droplet microfluidics has many applications in biomolecular kinetics due to rapid mixing, absence of dispersion, and immediate control of individual droplet payloads. Since then, droplet microfluidics has been applied to determine various biochemical characteristics of enzymes, as described in several excellent reviews (Ding et al., 2020; Hess et al., 2020; Shao et al., 2022) and the latest applications covered in Section 4.

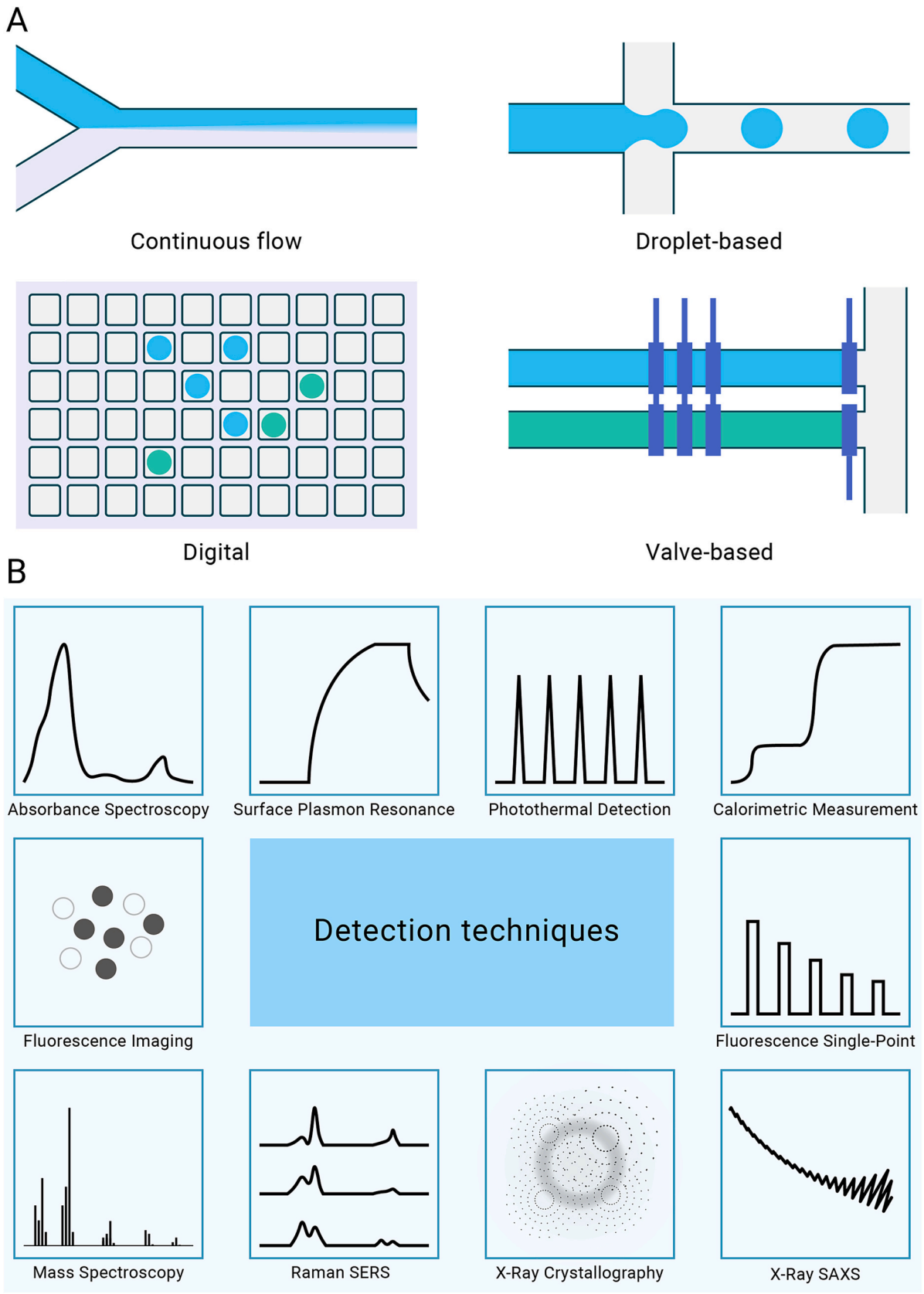

Fig. 2. Microfluidic technology. A) Four types of fluid manipulation approaches in microfluidic devices, including continuous flow (laminar flow of two aqueous phases), droplet-based (aqueous drops in an immiscible oil), digital microfluidic array, where droplet movements are controlled by electrodes, and valve-based devices incorporating an additional level of fluid control by integrating pneumatic valves. B) Summary of the most important detection methods for monitoring enzymatic reactions within microfluidic platforms with schemes of the obtained signal.

3.1.3. Digital microfluidics

A "digital microfluidic" (DMF) platform manipulates droplets on arrays of electrodes coated with a dielectric layer. Manipulating the electric field by varying the voltage applied to the electrodes modifies the wettability (Chatterjee et al., 2006) of the hydrophobic surface upon which the droplet moves. This allows its transport across multiple electrodes by sequentially applying a voltage to adjoining electrodes on the desired path. Digital microfluidic devices are inherently programmable since each electrode can be randomly addressed for actuation, enabling droplet movement along arbitrary paths (Hadwen et al., 2012). These systems also allow simultaneous control over the motion of multiple droplets, in theory providing a high level of parallelization (Eydelnant et al., 2014).

The portability of these platforms, when coupled with detection mechanisms such as fluorescence, was suitable for automating a fucosyltransferase inhibition assay (Leclerc et al., 2019). DMF platforms have also been successfully applied to study homogeneous enzymatic assays, such as the enzyme kinetics of alkaline phosphatase and the effects of its inhibition with inorganic phosphate, producing kinetic constants that are consistent with those reported in the literature (Miller and Wheeler, 2008). Heinemann et al. used an integrated DMF platform with mass spectrometry (MS) to quantify 20 enzyme assays in parallel, using 150 nL droplets (Heinemann et al., 2017). However, despite the potential for parallelization of these devices, the maximum number of parallel operations that could be achieved with a given number of electrodes was limited compared to microchannel-based droplet devices (Banerjee et al., 2015) due to control complexity or fabrication constraints. In general, DMF systems manipulate droplets that are typically on the order of 1 μL, approximately 1000 times larger than the volumes handled in microchannel-based devices, and thus may benefit less from the advantages of performing reactions in small volumes.

3.1.4. Valve-based microfluidics

Microscale valves employing pneumatic actuators can be integrated into microfluidic devices to achieve an additional level of control. The development of soft lithography (Xia and Whitesides, 1998) and the extension of this technique to the fabrication of integrated elastomeric microvalves by multilayer soft lithography (Unger et al., 2000) have enabled the robust production of monolithic devices composed of poly (dimethylsiloxane) (PDMS) with thousands of active microscale pneumatic valves able to rapidly and accurately control fluid flow. Valves control the timing of reagent flow through serially connected chambers for multistep reactions where reagent mixing between chambers for consecutive protocol steps is performed by diffusion (Fan et al., 2011). The valve-based devices can be programmable platforms since individually addressable valves can direct fluid flow through fluidic structures in a multilayer microfluidic device. PDMS valves in a hybrid PDMS-glass device were used to programmably manipulate nL volume scale sample flow through a square array to perform an enzymatic assay, mixing, and serial dilution (Jensen et al., 2010). Based on this concept, 114 individually addressable valves in a programmable microfluidic device were used to control flow for fluid metering and active mixing, surface immunoassays, and cell culture (Fidalgo and Maerkl, 2011). To achieve higher levels of functionality, sample compartmentalization in droplets has previously been exploited in valve-based devices (Raj et al., 2016; Wu et al., 2009; Zeng et al., 2009). This led to the development of programmable droplet platforms by combining droplet- and valve-based microfluidic concepts. A valve-based device incorporating a rotary mixer was used to mix multiple programmable proportions of aqueous solutions, and the resulting solution was emulsified for transport to one of the several addressable storage chambers (Urbanski et al., 2006). This concept was extended as a screening platform for protein crystallization, in which programmed mixtures of buffers and protein were formulated in droplets and transported into micron-sized channels for incubation and modulation of osmotic strength (Lau et al., 2007). Performing multiple operations within droplet-based microfluidic platforms often requires the integration of an addressable unit for droplet merging and storing, especially when droplets must be incubated for long periods (Lee et al., 2018). Such storage arrays allow stable entrapment and merging of generated droplets in confined spaces, creating various profiles of concentration gradients and enabling monitoring of droplet populations over extended periods. Droplets cannot be manipulated independently in these devices since the same valves control all storage chambers. To address this issue, the same group proposed a highly addressable static droplet array, using two control layers: one for valve manipulation and the other to achieve manipulation of individual droplets (Jeong et al., 2016).

3.2. Microfluidics for controlling mass and heat transport

Continuous and droplet-based microfluidic systems have proved to be of great utility in large-scale biological experimentation since they consume minimal sample, operate at high analytical throughput, are characterized by efficient mass and heat transfer, and offer high levels of integration and automation. Since the typical size of a microfluidic channel ranges between tens to hundreds of microns, the fluid is laminar. Under such conditions, the mixing of fluid streams is mediated solely by diffusion, with the extent of mixing being defined by channel dimensions, the mean diffusion coefficient, and the average residence time. The reduced diffusion distances associated with microchannels shorten the diffusive mixing times, and thus diffusion can be effective in allowing both rapid and controllable mixing (Kuo and Chiu, 2011). Accordingly, due to the high surface area-to-volume ratios of microfluidic channels, performing chemical reactions within these structures can be highly advantageous since the mass transfer can be significantly enhanced. Especially droplet microfluidic systems are highly useful for a range of enzyme-related experiments due to the enhancement of both mass and thermal transfer transport within droplets.

The experimental acquisition of kinetic and thermodynamic parameters for protein unfolding or aggregation at different temperatures is a powerful tool for understanding the molecular mechanisms underlying these important biochemical processes. Therefore, precise temperature control is crucial. Temperature can be controlled in different ways (Miralles et al., 2013), for example, by external heating modules or electromagnetic radiators. External heating approaches can be easily realized by directly placing the microfluidic device on a Peltier element. For instance, Yang et al. used two Peltier elements, one for heat conduction and the second for heat removal, arranged in an opposing geometry to generate a linear temperature gradient (between 40 and 70 °C) across a microfluidic channel (Yang et al., 2014). This arrangement probed protein aggregation by acquiring UV absorption spectra along the channel. By integrating an indium tin oxide-coated glass slide into a droplet-based platform, Yang et al. recently developed a droplet-based temperature-jump platform for assessing protein unfolding kinetics (Yang et al., 2022). The platform acquired UV–Vis time-resolved spectra over a wide range of temperatures and thus has broad utility in studying enzyme kinetics and protein stability at elevated temperatures.

Electromagnetic radiation can produce heat in a microfluidic device by illuminating it with IR radiation, thus generating a rapid temperature jump in microsecond or millisecond timescales. For instance, using a pulsed-laser source allows the formation of a defined temperature jump with a square-shaped temperature profile and a duration ranging from microseconds to seconds (Guo et al., 2012), depending on the power of laser input. Compared with a commercial temperature jump apparatus, which requires multiple heating steps to achieve the requested temperature, the radiation-induced temperature jump technique, combined with microfluidics, can provide temperature changes on much shorter timescales. This feature is especially important for studying fast biomolecular kinetics or conformational transitions on a microsecond scale, such as protein unfolding/refolding (Ebbinghaus et al., 2010; Guo et al., 2012). For example, Ebbinghaus et al. (Ebbinghaus et al., 2010) combined fluorescence microscopy and temperature jump to probe reaction

dynamics inside living cells with a high spatiotemporal resolution, allowing cooling in less than 50 ms. The fast unfolding/refolding kinetics and the thermodynamics of folding (between 23 °C and 49 °C) of a fluorescently labeled phosphoglycerate kinase were conveniently measured using fluorescence resonance energy transfer (FRET) techniques.

Moreover, Polinkovsky et al. used a gold-coated sapphire coverslip as the bottom layer of a microfluidic device, providing 20 times greater thermal conductivity than normal glass, while the gold layer enhanced IR light absorption by more than two orders of magnitude (Polinkovsky et al., 2014). This aspect, combined with a small channel depth (0.4 μm), allowed the temperature to increase from 25 to 80 °C within 1 μs. This device studied the rapid folding and unfolding dynamics of DNA hairpins at different ionic strengths, making this approach applicable to probing the folding of a wide variety of proteins under native conditions.

3.3. Microfluidic detection methods

Microfluidic devices are compatible with diverse analytical detection techniques (Fig. 2B) and are thoroughly described in specialized reviews (Ghazal et al., 2016; Zhu and Fang, 2013). The most important detection methods used in microfluidic platforms are optical methods (fluorescence and UV–vis spectrometry), while recent studies have leveraged the detection capability of MS (Ha et al., 2021) (Section 4). This section describes how fluorescence and label-free detection methods can acquire information-rich data.

3.3.1. Fluorescence detection

The popularity of fluorescence detection owes much to its facile integration with microfluidic-based formats (Măriuţa et al., 2020), its heightened sensitivity (down to the single-molecule level) (Jeffries et al., 2010; Rane et al., 2010), high information content, and ability to operate on ultra-short timescales (thus allowing access to rapidly occurring processes) (Srisa-Art et al., 2010). Unsurprisingly, a variety of fluorescence detectors have been used in microfluidic experiments. For instance, wide-field fluorescence imaging can record the fluorescent intensities of thousands of droplets simultaneously, providing valuable statistical information reflecting the heterogeneity of individual droplets (Hess et al., 2015). By imaging droplet arrays, single-cell enzyme activity can also be measured (Schmitz et al., 2009).

One limitation of fluorescence imaging for quantitative analysis is that the frame rate of a fluorescence charge-coupled device camera is typically lower than the droplet generation frequency. Thus, image-based fluorescence detection is limited in applications where particular droplets need to be analyzed individually on a very short time scale, such as droplet sorting. Single-point fluorescence detection schemes can solve the problem by providing information through a photodetector. Unlike fluorescence-based wide-field imaging of a homogeneous set of droplets in kinetic analysis, which provides average values by integrating images over many seconds, single-point detection schemes can probe reaction kinetics with single droplet resolution (Clausell-Tormos et al., 2010; Srisa-Art et al., 2008), providing quantitative and accurate information relating to droplet heterogeneity.

While fluorescence-based detection schemes provide excellent sensitivities, there are also some limitations and disadvantages, e.g., photobleaching, low quantum yield, intrusion by fluorescent labels, labeling efficiency, etc. In this respect, it is desirable to have alternative detection strategies that provide for rapid and sensitive detection of analyte molecules in a label-free manner.

3.3.2. Label-free detection

UV–visible absorption detection is one of the most commonly used label-free detection methods. However, traditional approaches for performing absorption detection within such nL-pL volumes are compromised by a number of factors, including short integration times (Richard M. P. Doornbos et al., 1997), scattering of light at droplet interfaces (Gielen et al., 2016), and, most importantly, reduced optical pathlengths (Nightingale et al., 2020). All these obstacles significantly impede the transfer of a range of established biological assays (routinely performed in cuvettes and microtiter plates) to droplet-based systems. Several solutions have been developed to overcome these limitations. Differential Detection Photothermal Interferometry, a quasi-pathlength independent approach, facilitates single-point absorbance detection in pL- and fL-volume droplets at frequencies exceeding 10 kHz (Maceiczyk et al., 2017). Unlike techniques based on light attenuation, photothermal signals are only weakly dependent on the optical path length, making them highly suitable for integration with microfluidic channels. Another approach to circumvent the reduced optical pathlength issue was reported by Yang et al., in which a droplet was squeezed through an extended channel section, and single-wavelength absorbance signals were recorded via two liquid-core waveguides embedded in close proximity to the channel (Yang et al., 2017). Such an approach enhanced absorbance sensitivity and reduced the limit of detection for fluorescein to 400 nM. The issue with scattering at droplet edges has been successfully reduced by matching the refractive index of the oil and aqueous phases using the addition of 1,3-bis(trifluoromethyl)-5-bromobenzene to the oil (Salmon et al., 2016).

Although the approaches mentioned above have achieved low detection limits, they lack the broadband character of conventional absorption spectroscopy. Accessing the absorbance broadband spectrum of reaction species yields much more information than those extracted from a single-point readout (Mao et al., 2015; Neil et al., 2011). Probst et al. presented a platform for the sensitive acquisition of broad-band absorption spectra from rapidly moving pL-volume droplets (Probst et al., 2021). Combining confocal illumination with an ultra-fast acquisition rate and a post-processing algorithm eliminates spectral contribution from the continuous phase and achieves a high signal-to-noise ratio.

Alongside the traditional optical methods, MS has become popular in recent years for its simplicity, availability, and ability to provide a label-free qualitative and quantitative analysis. MS combines ambient ionization techniques such as electrospray ionization (ESI) and the desorption approach (DESI). DESI-MS does not require sample preparation and is high-salt tolerant. Therefore, it is valuable for the screening and analysis of enzymatic reactions performed directly from complex reaction matrices (Ho et al., 2003; Hollerbach et al., 2017; Morato et al., 2020).

4. Accelerated data collection by microfluidics

The development of the microfluidic technologies covered in previous sections accelerates their adoption for biocatalyst characterization. Here, we summarize the recent developments and applications of microfluidic technologies for the in-depth experimental analysis of enzymes. We describe the criteria used within the literature search in Appendix. The selected key studies are described in Table 2 (the full results of our literature search can be found in the Full literature review, see the section Data availability). We collected additional information for each study (Appendix) to explore general trends within this research field. Next, we describe microfluidics-enabled enzyme characterization regarding four enzyme characteristics categories: activity, kinetics, stability, and structure. Within these subsections, we focus on studies using commercial microfluidic instruments and on the platforms which were systematically applied for analyzing biocatalysts. The last subsection describes the trends in this field regarding the collected information and bibliographic analysis, summarized in Figs. 3 and 4.

4.1. Enzyme activity

Catalytic activity is one of the initial pieces of information which scientists gather to biochemically characterize an enzyme. Yet, the catalytic activity highly depends on the experimental conditions

Table 2
Selected studies using microfluidics for the characterization of biocatalysts.

Characteristics	Specific characterization	Enzymes	Study	Enzyme variants/study
Activity	In-vitro activity testing	alkaline-phosphatase	(Jiang et al., 2020)	3
		β-galactosidase	(Li et al., 2018)	4
		β-glucuronidase	(Jiang et al., 2019)	5
	In-vivo activity testing	alkaline phosphatase	(Girault et al., 2021)	6
		β-galactosidase	(Lou et al., 2021)	3
	Temperature/pH profiling	alkaline-phosphatase	(Gilboa et al., 2022)	1
		haloalkane dehalogenase	(Vasina et al., 2022a)	24
	Substrate profiling	glycosyl transferase	(Xu et al., 2017)	3
		haloalkane dehalogenase	(Vasina et al., 2022a)	24
			(Jiang et al., 2020)	3
			(Markin et al., 2021)	~1000
Kinetics	Steady-state kinetics	alkaline phosphatase	(Grant et al., 2018)	1
		glutathione reductase	(Hess et al., 2021)	3
		haloalkane dehalogenase	(Vasina et al., 2022a)	6
		matrix metalloproteinase	(Guo et al., 2018)	1
		peptidylarginine deiminase	(Grant et al., 2022)	1
		kinase	(Fogarty et al., 2017)	1
		β-secretase	(Gajiwala et al., 2017)	2
			(Sawaguchi et al., 2021)	3
			(Liu et al., 2017)	1
	Transient-state kinetics	β-galactosidase & horseradish peroxidase	(Hess et al., 2021)	2
		lactate dehydrogenase	(Reddish et al., 2017)	1
Stability	Thermostability	lysozyme	(Mukhametzyanov et al., 2022)	1
			(Mitić et al., 2017)	1
	Refolding	cytochrome *c*	(Srour et al., 2018)	1
		lysozyme	(Fatkhutdinova et al., 2022)	1
			(Ferreira et al., 2020a)	1
Structure	Crystallization conditions	lysozyme	(Wang et al., 2019)	3
		lysozyme & trypsin	(Liang et al., 2017)	2
			(Monteiro et al., 2020)	2
	Serial crystallography	aspartate α-decarboxylase	(Olmos et al., 2018)	1
		β-lactamase	(Rabe et al., 2021)	1
		isopenicillin *N* synthase	(Beyerlein et al., 2017)	1
		lysozyme	(Holmes et al., 2022)	1
		methane monooxygenase	(Srinivas et al., 2020)	1
		ribonucleotide reductase	(Fuller et al., 2017)	1
	Cryo-EM structure	recombinase	(Mäeots et al., 2020)	1
	SAXS structure	dipeptidase	(Schewa et al., 2020)	2
		lysozyme	(Pham et al., 2017)	2
			(Rodríguez-Ruiz et al., 2017)	1

(Bisswanger, 2014). We have identified that microfluidics was predominantly used to test enzyme activity *in vitro* and *in vivo*. At the same time, fewer studies explored the operational conditions, such as pH or temperature profiling, or studied the preference of enzymes toward multiple substrates (substrate profiling) (Fig. 3).

4.1.1. *In vitro activity testing*

In vitro activity testing is a simple way of demonstrating a proof of concept (POC), often employing benchmark enzymes coupled with well-established assay and optical readout, mainly fluorescence. However, microchamber arrays that allow single-molecule/digital enzymology studies have demonstrated systematic usage and commercialization of the developed systems (Noji et al., 2022). As an example, Walt et al. made use of the microwell array disk from Quanterix to perform various activity and kinetic studies, e.g., understanding the subunit function of β-galactosidase (Li et al., 2018), studying the hysteretic behavior of mutant β-glucuronidase (Jiang et al., 2019), or characterizing various isozymes of human alkaline phosphatase in serum (Jiang et al., 2020).

4.1.2. *In vivo activity testing*

Microfluidics offers convenient *in vivo* activity testing at the single-cell level. Although this feature is predominantly used for HTS (Neun et al., 2019; Tesauro et al., 2015), several research groups have developed and systematically applied microfluidic systems also for quantitative analysis of *in vivo* enzyme activity. The Fang group introduced the Sequential Operation Droplet Array (SODA) in 2013 (Zhu et al., 2013), and since then, it has been applied for various purposes, including HTS, single molecule analysis, or single cell analysis (Dong and Fang, 2020). Recently, they have developed a simplified version of SODA, called Manual Droplet Operation System, which they applied to a single-cell enzyme assay of β-galactosidase (Lou et al., 2021).

Another systematic application of microfluidic *in vivo* activity testing was demonstrated by the Baret group. Their microfluidic chip enables the single-cell measurement of plankton alkaline phosphatase activity (Girault et al., 2018). They utilized this system to study the adaptive evolution of several phytoplankton species in the context of environmental changes, as the alkaline phosphatase activity correlates with plankton growth (Girault et al., 2021).

4.1.3. *Optimal activity conditions*

The heavy dependence of catalytic activity on experimental conditions drives scientists to explore different temperature ranges, pH, various buffers, or co-solvents (Bisswanger, 2014). Microfluidic systems are well suited for exploring optimal conditions, especially in their droplet or array formats, which enable parallelization. For example, the aforementioned microwell array disk from Quanterix was used to study temperature and pH effects on the heterogenous activity of alkaline phosphatase (Gilboa et al., 2022).

Our group systematically characterized optimal conditions several times in the studies of haloalkane dehalogenases (HLDs) converting hydrophobic haloalkanes. The delivery of such hydrophobic compounds is difficult due to the leakage into the carrier oil and represents one of the significant issues of droplet microfluidics (Chen et al., 2012). We have overcome this issue by introducing a capillary-based microfluidic platform called Microfluidic Profile Explorer (MicroPEX), where the controlled oil-water partitioning and microdialysis of hydrophobic

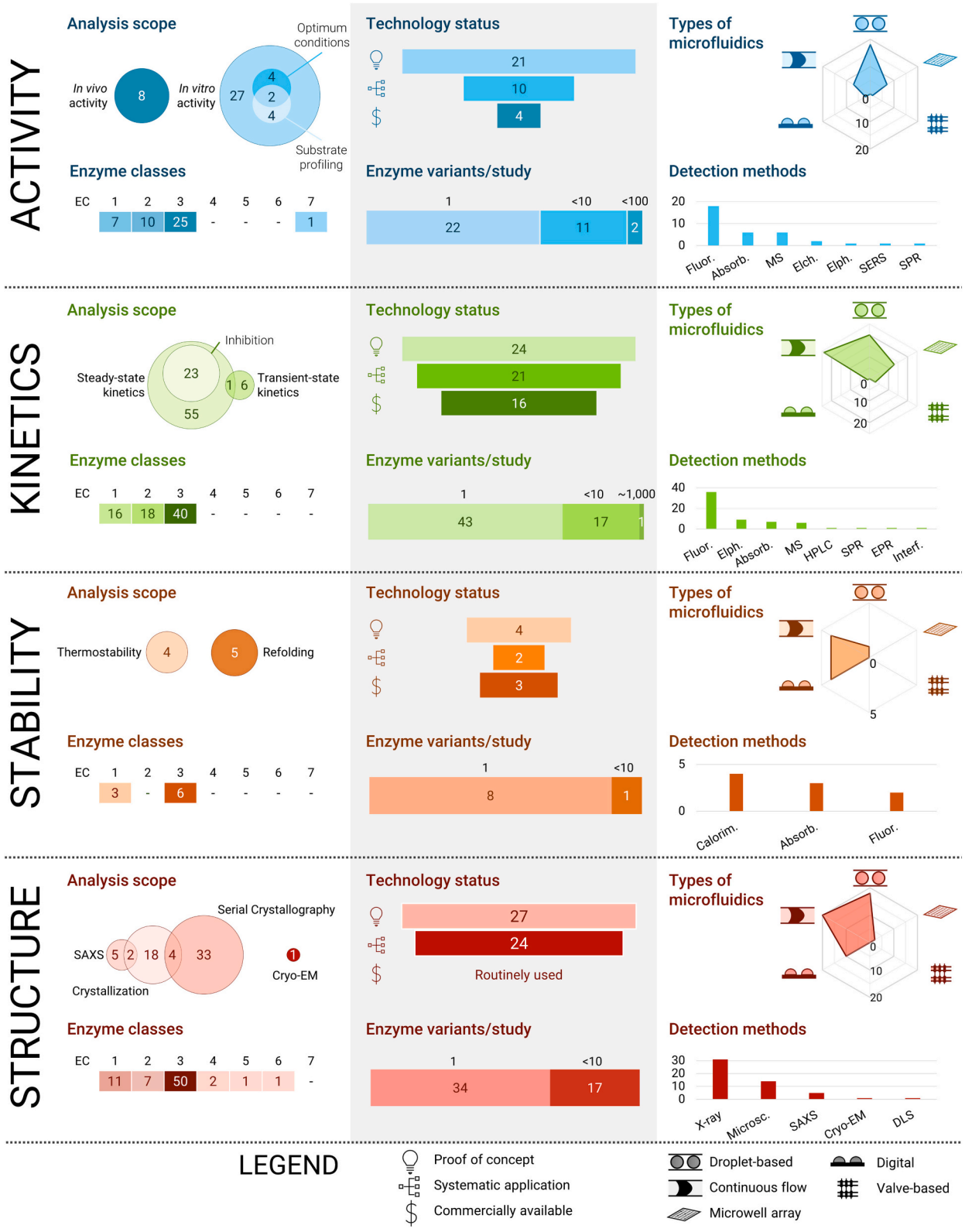

Fig. 3. Practical aspects of microfluidic biochemical characterization of enzymes. For each characteristic (activity, kinetics, stability, and structure), the figure shows the analysis of data from the literature search (the Full literature review, see Data availability) reflecting the analyzed enzymes (left column), the performance of the microfluidic technology (middle column in grey), and technical aspects of microfluidics (right column). Numbers in each type of graph indicate the number of publications. Pictograms are explained in the legend (bottom) respectively to each column. Several detection methods are abbreviated, including fluorescence (fluor.), absorbance (absorb.), electrochemical detection (elch.), electrophoresis (elph.), interferometry (interf.), calorimetry (calorim.) and microscopy (microsc.). Other standard abbreviations are defined in the list of abbreviations.

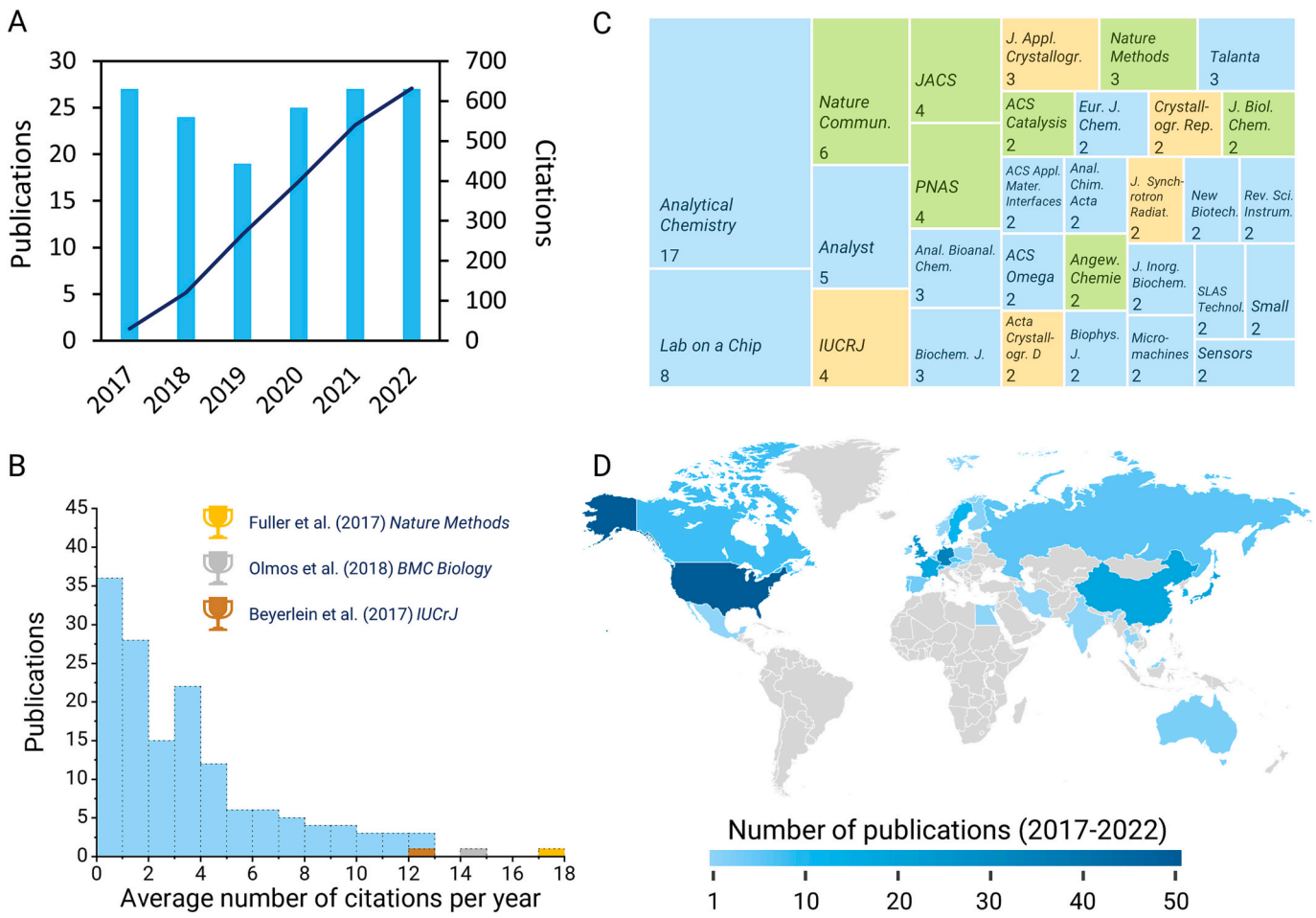

Fig. 4. Bibliographic analysis of the microfluidic characterization of enzymes published 2017–2022. A) The number of publications and citations per year. B) A histogram of the number of publications based on the average number of citations per year. The studies in the inset, highlighted in the histogram by the corresponding colour, are the most cited studies from our list (the Full literature review, see Data availability). C) The distribution of journals with more than one microfluidic characterization study publication, highlighting technology-focused (blue), crystallography (yellow) and multidisciplinary life science journals (green). D) The world map with highlighted countries developing or using microfluidic characterization techniques. The countries are colored based on the number of studies from that country, from light blue (1) to teal (~50). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

substrates serve to efficiently deliver the substrate to the reaction vessels (Buryska et al., 2019). In the first study, MicroPEX was validated on the temperature profiles of 10 HLDs and substrate profiles of 8 HLDs (Buryska et al., 2019). Afterwards, temperature profiles of altogether >50 HLDs were characterized in several studies, including both engineered variants (Markova et al., 2021; Schenkmayerova et al., 2021) and enzymes discovered by *in silico* analysis (Vasina et al., 2022a).

4.1.4. Substrate profiling

Substrate profiling helps to characterize the promiscuity of enzymes. Moreover, the collection of substrate specificity profiles is of great use for the machine learning (ML) community, e.g., to predict new substrates for enzymes for which activity has not been determined (Section 5). One of the notable examples of substrate profiling is the MicroPEX, which was applied for a systematic experimental analysis of substrate profiles of HLDs (Buryska et al., 2019; Vasina et al., 2022a) by performing measurements of a set of enzymes in a combinatorial manner with up to 27 halogenated substrates (Vasina et al., 2020). Another example of systematic microfluidic substrate profiling was shown by Xu et al. (Xu et al., 2017). Utilizing a previously developed proteomic microarray (Jeong et al., 2012), they searched for protein substrates for 3 isozymes of polypeptide *N*-acetylgalactosaminyltransferases. Out of more than 16,000 human proteins, they identified 570 potential substrates for their model enzyme. Using cluster analysis, they revealed functional redundancy and specialized roles of individual isoenzymes.

4.2. Enzyme kinetics

More than a century ago, enzyme kinetics was described by one of the most straightforward and well-known fundamental equations – the mathematical model of Victor Henri, Leonor Michaelis, and Maud Menten (Johnson and Goody, 2011). This way, they introduced a methodology for steady-state experiments that remains the gold standard to the present time (Table 1). Microfluidics can significantly increase the throughput and repeatability of kinetic data collection by offering low sample consumption and reaction parallelization under various conditions (e.g., substrate concentration, presence of inhibitors, pH, temperature). Here, we discuss steady-state, inhibition and transient-state kinetic microfluidic measurements.

4.2.1. Steady-state kinetics

Microfluidic systems for systematic analysis of enzyme kinetics typically employ fluorescence as the dominant detection method (Fig. 3). The highest throughput has been demonstrated by Markin et al., who reported a two-layer PDMS microfluidic platform with several pneumatic valves, called HT-MEK (High-Throughput Microfluidic

Enzyme Kinetics). This platform enables high-throughput expression, purification, and characterization of more than 1500 enzyme variants per experiment (Markin et al., 2021). More specifically, the device contains 1568 individual chambers that can each contain a mutated version of the enzyme and a microfluidic valve system that delivers reagents to all the mutants at the same time. The authors demonstrate the HT-MEK performance on the experimental analysis of 1036 single-site mutants of alkaline phosphatase PafA, yielding more than 5000 kinetic and thermodynamic constants from more than 670,000 reactions.

Another popular method is absorbance detection (Fig. 3), mostly demonstrated as a POC application. An exception is the droplet-on-demand microfluidic device developed by the Hollfelder group, allowing the generation of high-resolution concentration gradients for the determination of enzyme kinetics (Gielen et al., 2015). In a recent study, this system was upgraded by a multiplexed absorbance reader and validated by the characterization of a promiscuous metagenome-derived glycosidase active with a range of glycosidase substrates (Neun et al., 2022).

Alternatively, label-free detections are also increasingly employed systematically. The Cooks group recently reported an MS-based high-throughput screening platform, DESI-MS (desorption electrospray ionization mass spectrometry), for studies of enzymatic systems, such as acetylcholinesterase, nicotinamide *N*-methyltransferase, metabolites, lipids, or pharmaceutical molecules (Kulathunga et al., 2022; Morato et al., 2020, 2021). Combining DESI with liquid handling robotics, the system achieves throughputs of more than 1 sample/s, handling up to 6144 samples in a single run. A similar concept utilizing an acoustic droplet ejection (ADE) mechanism to deliver nL droplets into the ESI-MS was able to perform label-free in situ enzyme kinetics of an acyltransferase (Zhang et al., 2021b).

Similarly, Grant et al. developed a technique called "imaging self-assembled monolayers" for matrix-assisted laser desorption/ionization mass spectrometry for high-throughput quantitative mapping of 2592 unique experiments conducted in flow (Grant et al., 2018). The applicability of this technique toward providing kinetic constants for an enzyme was demonstrated by determining K_M values of human glutathione reductase. The same technique was applied for the cooperativity binding studies of calcium ions to peptidylarginine deiminase type 2 (Grant et al., 2022).

4.2.2. Inhibition kinetics

Several commercial microfluidic devices are available for kinetic measurements and were mostly applied for inhibition studies. Desktop Profiler (Caliper) and the second generation LabChip® EZ Reader (Perkin Elmer) use a microfluidic mobility shift assay (MMSA), which is based on the electrophoretic separation of charged molecules with a fluorescent tag. Despite showing considerable applicability, MMSA often requires in-house synthesis of fluorescently labeled substrates. Such substrates were, for example, needed to study the inhibition of β-secretase, an enzyme considered in the pathogenesis of Alzheimer's disease (Liu et al., 2017). The popularity of MMSA for biomedical studies has been shown especially in the characterization of various kinases (Fogarty et al., 2017; Gajiwala et al., 2017; Sawaguchi et al., 2021), which can be routinely characterized as a service by companies such as Carna Biosciences. A further increase in sensitivity and throughput can be achieved by single-molecule microarray techniques, such as Single Molecule Arrays (Simoa) (Rissin et al., 2010; Wang et al., 2020).

4.2.3. Transient-state kinetics

Most kinetic experiments in droplet-based microfluidics have traditionally relied on averaging over large droplet numbers, and the inability to resolve dynamics on the single-droplet level is a recognized limitation. Hess et al. (Hess et al., 2021) reported a novel imaging approach based on stroboscopic illumination to characterize enzyme–inhibitor reaction kinetics within a single experiment by tracking individual and rapidly moving droplets containing varying substrate concentrations. This approach allowed the evaluation of more than 1500 kinetic measurements in only 10 s. Furthermore, the Dyer group recently developed a rapid T-jump microfluidic mixer for the fast kinetic profiling of lactate dehydrogenase enzyme (Reddish et al., 2017). Mixing time of <100 μs allowed monitoring of enzyme kinetics and substrate-induced conformational changes at submillisecond resolution.

4.3. Enzyme stability

Poor stability, misfolding, or aggregation diminish enzyme production and prevent its further use in downstream applications. On the other hand, the stabilization of an enzyme and an increase in its robustness to sustain harsh conditions can promote its use in industrial processes (Liu et al., 2019). Such stabilization can be achieved, e.g., by immobilizing enzymes in so-called immobilized microfluidic enzyme reactors (IMERs). However, IMERs are not the focus of this article as their primary goal is not the characterization of enzymes, but rather intensification of a biocatalytic reaction with the focus on the product. Therefore, we refer the readers to excellent reviews about this topic (Kecskemeti and Gaspar, 2018; Meller et al., 2017; Zhang et al., 2021a).

Although both systematic studies and applications of commercial microfluidic instruments have been focused on elucidating enzyme thermostability and refolding (Fig. 3), the kinetic stability of enzymes, e.g., measuring the half-life of the biocatalyst at a given temperature, remains an underdeveloped area for microfluidic enzymologists. Similarly, to the best of our knowledge, the use of microfluidic devices to characterize protein solubility has not been explored.

4.3.1. Thermostability

Up to now, high-throughput technologies for enzyme thermostability characterization have been standardized to commercial instruments operating in microliter volume ranges, with the prominent example of nano-differential scanning fluorimetry (Magnusson et al., 2019). The only type of commercial microfluidic device for studying enzyme thermostability is the chip-based differential scanning calorimeter (DSC). The Schick group used the fast-scanning Flash DSC 1 (Mettler Toledo, Switzerland) instrument for studying lysozyme unfolding with a range of temperature scanning rates of 5 orders of magnitude (Mukhametzyanov et al., 2018). By applying the same technique, the Schick group also studied the reversible denaturation of lysozyme in a step-scan DSC regime, thus enhancing the resolution and signal-to-noise ratio of the calorimetric curves (Mukhametzyanov et al., 2022).

4.3.2. Refolding

While thermostability studies usually look at the thermodynamics of enzyme stability, folding and refolding studies explore the kinetics of protein folding. The Schick group used Flash DSC 2+ (Mettler-Toledo, Switzerland) to study lysozyme refolding in glycerol, revealing similarities in kinetic mechanisms for glycerol and water (Fatkhutdinova et al., 2022). Apart from commercial microfluidic calorimeters, the refolding kinetics was systematically studied by rapid-mixing continuous flow instruments, as discussed previously in Subsection 4.2.2. The Hagen group developed a rapid mixing instrument called Nanospec (Mitić et al., 2017; Srour et al., 2018), with which they achieved microsecond timescale analysis of cytochrome *c* refolding kinetics.

4.4. Enzyme structure

Since the structures of enzymes determine their functions, elucidating the relationships between enzyme structure and function will eventually accelerate their industrial applications. Therefore, determining the enzymes' structural properties aids in revealing the mechanism of enzyme reaction or exploring the possible binding modes of interesting ligands, ultimately leading to more effective protein engineering and drug discovery campaigns. Apart from the use of X-ray

crystallography, nuclear magnetic resonance (NMR), and cryogenic electron microscopy (Cryo-EM) for atomic structure determination, lower-resolution structural information can provide information about the secondary structure (e.g., circular dichroism) or quaternary structure/hydrodynamic parameters of proteins (small-angle x-ray scattering, SAXS). Within this diverse area of structural characterization, microfluidics has been predominantly utilized in X-ray crystallography, Cryo-EM, and SAXS studies.

4.4.1. X-ray crystallography

While several conventional crystallization techniques are actively pursued in both academic and industrial laboratories to produce high-quality protein crystals, microfluidic technology for structural biology was previously shown to improve protein crystallization over more traditional methods (Sui and Perry, 2017). There are excellent reviews targeting protein crystallization (Devos et al., 2021; Ghazal et al., 2016) and serial crystallography (Cheng, 2020; Hejazian et al., 2021), which provide an overview of microfluidic technology for crystallography studies. The following sections only describe selected recent protein crystallization and serial crystallography applications.

Starting with a screening of crystallization conditions, the Fang group employed their SODA system to screen model enzymes with a micro-batch (Zhu et al., 2014) and vapor diffusion technique (Liang et al., 2017). As these studies could only provide initial screening of crystallization conditions, they upgraded the SODA system to cover the whole process of crystallization analysis experiments (from the purified protein solutions to diffraction-quality crystals), studying the crystallization of 9 model proteins (Wang et al., 2019). Another system was developed by Ferreira et al. (Ferreira et al., 2018), who developed a droplet-based microfluidic platform for studying protein phase diagrams of the model protein lysozyme. This system also explored the effect of supersaturation ratios promoting crystallization (Ferreira et al., 2020a) and showed that uniform crystal sizes could be achieved by promoting protein nucleation in micron-sized droplets through low-frequency pulsed sonication (Ferreira et al., 2020b). Finally, several commercial microfluidic systems have been developed, for example, the free interface diffusion-based TOPAZ (Fluidigm) (Lee et al., 2009), the counter-diffusion-based CrystalSlide™ (Greiner Bio-One) (Ng et al., 2008), the droplet-based PlugMaker utilizing CrystalCard (Protein BioSolution) (Gerdts et al., 2008), or the valve-based Formulator or Mantis (Formulatrix). These were commercialized over 10 years ago and have been extensively used in many crystallographic studies (Martiel et al., 2018).

Serial crystallography that enables time-resolved protein dynamics at room temperature is witnessing an increasing utilization of microfluidic nozzles and mixers for sample delivery (Marinaro et al., 2022). By coupling acoustic droplet ejection (ADE) with a conveyor belt drive for serial femtosecond crystallography (SFX) experiments, Fuller et al. developed a drop-on-tape (DOT) technique to study photochemical (photosystem II) and chemical reactions (ribonucleotide reductase R2) over a wide range of time scales (Fuller et al., 2017). The DOT technique was then systematically applied, e.g., to capture the oxidation states of a methane monooxygenase (Srinivas et al., 2020) or to study the dynamics of Isopenicillin *N* synthase during synthesis of the precursor of all natural penicillins (Rabe et al., 2021). A different system was introduced by Knoška et al., who developed 3D-printed microfluidic nozzles and mixers coupled with megahertz X-Ray Free-Electron Laser (XFEL) to enhance the efficiency of mix-and-inject time-resolved SFX (Knoška et al., 2020). In a subsequent study, they further exploited the MHz pulse structure of the European XFEL to obtain two complete datasets from the same lysozyme crystal, paving the way for tracking sub-microsecond structural changes in individual crystals (Holmes et al., 2022).

Microfluidic sample delivery has found its use also for serial synchrotron crystallography (SSX). Monteiro et al. developed a 3D-printed X-ray-compatible microfluidic device (3D-MiXD) (Monteiro et al., 2020) which can be easily fabricated and used in a plug-and-play mode at X-ray crystallography beamlines. Apart from demonstrating the 3D-MiXD performance in the structural determination of aspartate α-decarboxylase and lysozyme (Monteiro et al., 2020), they further exploited the precipitation properties of ammonium sulfate for three different enzymes: L-aspartate α-decarboxylase, copper nitrite reductase, and copper amine oxidase (Stohrer et al., 2021).

4.4.2. Cryo-EM

Cryo-EM is increasingly used to solve biomolecular structures (Callaway, 2020). Microfluidics was successfully coupled with Cryo-EM for protein specimens preparation, yet due to the resolution limits of Cryo-EM, only rather large protein complexes have been characterized, such as apoferritin (Huber et al., 2022) or proteasomes (Huber et al., 2022; Schmidli et al., 2019). Recently, Mäeots et al. (Mäeots et al., 2020) utilized a modular microfluidic device for Cryo-EM sample preparation to capture early recombinase filament growth kinetics on sub-seconds timescales.

4.4.3. SAXS

Within the scope of this review, microfluidics coupled with SAXS has been mentioned for two relevant purposes: (i) to identify successful crystallization conditions for crystallization targets and (ii) to provide a structural characterization of target proteins. For further applications of microfluidic-based SAXS, we refer the readers to several detailed reviews (Ilhan-Ayisigi et al., 2021; Silva, 2017; Watkin et al., 2017). An example of crystallization conditions screening to improve crystal morphology was demonstrated by the Teychené group (Pham et al., 2017). The authors implemented a droplet-based microfluidics device to encapsulate and greatly enrich lysozyme crystallization conditions. In the following work, the authors integrated SAXS detection into a microfluidic platform to determine the radius of the gyration radius of lysozyme for calculating its structure envelope as a function of protein concentration (Rodríguez-Ruiz et al., 2017). In another study, Schwemmer et al. developed a centrifugal microfluidic platform entitled LabDisk, to characterize the radius of glucose isomerase gyration under varying protein and NaCl concentrations (Schwemmer et al., 2016). Finally, the Svergun group developed a 3D-printed microfluidic flow cell, transparent to terahertz frequencies (Schewa et al., 2020), that enabled the study of protein domain movements in solution (Schroer et al., 2021).

4.5. Overview of microfluidic analysis of biocatalysts

Our literature search provided insights into current trends for microfluidic characterization systems of biocatalysts. Based on the increasing number of citations, the bibliographic analysis revealed a growing interest in these systems and steady increments of new studies (Fig. 4A). The most cited articles offer microfluidic solutions for crystallography, highlighting the importance of microfluidics and its high level of development for structure determination of enzymes (Fig. 4B). In addition, the analysis revealed that most of the studies were published in technology-focused journals, such as *Analytical Chemistry* or *Lab on a Chip*, or field-specific journals, such as *IUCRJ*, for crystallography (Fig. 4C). However, there was a relatively high number of studies in multidisciplinary journals targeting the general scientific community, e.g., *Nature Communications*, *JACS,* or *PNAS*. The country-based analysis showed that most studies originate from the USA, China, and Europe. (Fig. 4D).

Concerning the systematic in-depth experimental analysis of biocatalysts, microfluidics has predominantly been employed for structural studies and enzyme kinetics (Fig. 3). As discussed above, commercial microfluidic solutions have been developed for all four characterization categories. Microfluidic approaches, including droplet-based, valve-based, and microwell arrays, have enabled massive parallelization of enzyme reactions (Shao et al., 2022). On the other hand, continuous flow and DMF devices have been mostly employed as POC experiments (Fig. 3). Concerning enzyme classes, experimental analyses using

microfluidic approaches have primarily focused on hydrolases (EC 3), oxidoreductases (EC 1), and transferases (EC 2), while other classes are less represented (Fig. 3). So far, most of the existing microfluidic systems have utilized their throughput/parallelization capabilities mostly on covering a wide range of experimental conditions, and less on the use of a wide range of different enzyme variants, except for the HT-MEK system (Markin et al., 2021). Large numbers of variants are typically processed in HTS campaigns rather than during the following characterization phase, which tends to be focused only on selected units or tens of hits. However, for efficient development of ML-based methods, in-depth experimental analysis of a larger number of enzyme variants will be necessary, e.g., to predict the function of the uncharacterized biocatalyst-encoding sequences (Mokhtari et al., 2021). Systematic implementation of microfluidic characterization methods for hundreds or thousands of enzyme variants would generate consistent and reliable datasets, critical for training of machine learning algorithms (Mazurenko et al., 2020).

5. Advanced data analysis by machine learning

In the previous sections, we have seen the exciting possibilities that microfluidics offers for high-throughput data collection. The generated data may help to identify suitable biocatalysts, determine the optimal enzymatic conditions, and understand the enzymatic mechanism, which can aid in finding better targets for protein engineering (Fig. 1). Microfluidic techniques accelerate data collection immensely and the challenge of exploring the vast space of possible candidates to be experimentally characterized prompts the use of advanced data analysis methods. A large group of methods, which have already shown significant potential in this respect, is based on machine learning (ML). ML aims to find patterns in the available data that can then be used, e.g., to predict labels for future input. For example, for a dataset consisting of an enzyme sequence and its label, such as an enzyme turnover number or temperature optimum, a generic algorithm can be trained to capture the relationship between the two. Once training is complete, the resulting model can predict labels for new sequences.

5.1. Machine learning in enzyme engineering

One notable example of recent successes of ML in enzyme engineering tasks is structure prediction, which has been attracting much attention recently due to the unprecedented power of AlphaFold 2 in predicting protein structures from sequences (Jumper et al., 2021). The EC number (Ryu et al., 2019), stability, protein solubility, and aggregation propensity have also often been targets for ML-based predictions (Marques et al., 2021). Furthermore, the success of deep learning in natural language processing and image classification has influenced the macromolecular X-ray crystallography domain, particularly for sequence-based protein crystallizability prediction and crystallization outcome image classification tasks (Klijn and Hubbuch, 2021; Vollmar and Evans, 2021).

The primary driver behind these successful ML applications for enzyme engineering tasks is increasing data availability: most advanced algorithms often use hundreds of thousands of data points for training. Large datasets usually come at the cost of pooling together multiple datasets, often collected using various techniques in many research groups. Heterogeneous data of this kind require significant effort to ensure data comparability and compatibility. Perhaps, this is why the most common targets for ML applications are more universal properties, such as protein folding or stability, rather than those determined by a particular enzymatic mechanism, which is more difficult to infer globally for multiple enzyme classes.

5.2. Machine learning for focused enzyme characterization

With the recent progress in high-throughput characterization methods, more enzymatic characteristics, such as specific activities, temperature optima, or kinetics, are attracting more and more attention from the ML community as the data sizes are starting to allow the training of ML models. The summary of the most recent studies using ML for experimental enzyme analysis is given in Table 3.

The bird's-eye view of the collected publications reveals the following observations. The sizes of datasets in Table 3 range from hundreds to thousands of measurements – the scale well within reach of modern microfluidic techniques. Surprisingly, most experimental datasets used in those studies were collected using conventional technologies such as 384-well microtiter plates. This implies that the tremendous potential of microfluidics in protein characterization covered in the previous sections is yet to be discovered by ML practitioners.

The lag in ML applications might be due to the current focus of the groups developing microfluidic devices on the engineering part. This is further supported by the fact that the hardware side of microfluidics has already long been benefiting from ML, e.g., for device design, flow or droplet control in microchannels, or image processing, e.g., we refer the readers to the latest reviews on intelligent microfluidics (McIntyre et al., 2022; Srikanth et al., 2021; Zheng et al., 2021).

Another possible explanation for the lag might be the apparent complexity of state-of-the-art ML methods. For instance, deep learning alone is a broad field of computer science with multiple subdomains. This complexity might seem intimidating for first-time users. However, only a small fraction of the studies in Table 3 uses complex methods primarily because the datasets are not large enough to train large complex neural networks. Most studies use conventional ML techniques, such as linear regressions, support vector machines, or decision trees, and ML libraries are becoming increasingly available for researchers with limited programming experience. Moreover, a recent effort to further improve the accessibility of the methods is directed toward developing general ML pipelines (Siedhoff et al., 2021). This availability, of course, does not obscure the fact that proper ML protocols and practices must be followed, e.g., according to the latest data, optimization, model, and evaluation (DOME) recommendations covering the fundamental aspects of ML application in life sciences (Walsh et al., 2021).

5.3. Prospects for ML-based microfluidic enzymology

Apart from exploring ML for the datasets generated by microfluidics, future perspectives for ML application for protein characterization include several directions. While the small size of a dataset usually precludes the use of complex deep learning architectures, recent approaches leveraging large unannotated protein databases such as UniProtKB/TrEMBL show promise in various protein engineering tasks (Bepler and Berger, 2021; Detlefsen et al., 2022; Kroll et al., 2022; Rao et al., 2019). The essence of these algorithms is to train large neural networks in a self-supervised manner, i.e., without any explicit labels provided, run these networks on the desired small set of protein sequences to generate protein features, and then use these features to train another predictor for the available labels. Many of these tools use protein sequences, although the recent deposition of structures predicted by AlphaFold2 for the whole UniProt database will also provide for self-supervised learning on structures (Jumper et al., 2021).

Another promising direction is knowledge transfer between datasets. Some of the studies in Table 3 already use aggregated data collected in enzymatic databases, such as BRENDA or SABIO-RK, and more such databases will likely be used as the community is still actively investigating reliability (Rembeza and Engqvist, 2021) or possible synergies, e.g., between the kinetic and structural data (Yan et al., 2022). However, a more ambitious transfer learning strategy is to train an ML predictor on one data set and then fine-tune it on the other, which starts to appear more often in drug discovery or other protein domains (Cai et al., 2020; Hanson et al., 2020). This strategy has the advantage of a more focused evaluation of a predictor for a particular target property or family, in

Table 3
The summary of studies applying machine learning algorithms to enzyme characterization using small datasets.

Characteristics	Specific characterization	Enzyme class	Dataset	Algorithm*	Reference
Activity	substrate profiles	phosphatases	218 enzymes vs. 167 substrates	Hierarchical clustering	(Huang et al., 2015)
	substrate profiles	ester hydrolases	147 enzymes vs. 96 substrates	Hierarchical clustering, linear regression	(Martínez-Martínez et al., 2018)
	substrate profiles	glycosyltransferase superfamily 1	54 enzymes vs. 13 sugar electrophiles and 91 nucleophiles (6318 data points)	Hierarchical clustering, Decision trees, KNN	(Yang et al., 2018)
	substrate profiles	glycosyltransferase GT-A fold	713 substrate profiles (6 classes) from CAZy GTand UniProt databases	Random forest, Gboost, SVM, Bayesian network, logistic regression, naïve Bayes, decision tree, shallow NN	(Taujale et al., 2020)
	substrate profiles	flavin-dependent halogenases	87 enzymes vs. 62 substrates	Hierarchical clustering	(Fisher et al., 2019)
	substrate profiles	nitrilases	12 enzymes vs. 20 substrates	Logistic regression, random forest, Gboost, SVM	(Mou et al., 2021)
	substrate profiles	OleA thiolases	73 enzymes vs 15 substrates	Random forest, naïve Bayes, shallow NN	(Robinson et al., 2020)
	substrate profiles	beta-keto acid cleavage enzymes	163 enzymes vs. 17 substrates	Hierarchical clustering	(Bastard et al., 2014)
	substrate profiles	seven enzyme families	seven datasets with 1000–23,000 enzyme-substrate pairs from the literature	Shallow NNs and Gaussian processes trained on enzyme-substrate embeddings	(Goldman et al., 2022)
	substrate profiles	four enzyme families	four datasets with 450–36,600 enzyme-substrate pairs from the literature	Convolutional NNs-trained on enzyme-substrate embeddings	(Xu et al., 2022)
	enantioselectivity (E value)	epoxide hydrolases	136 variants from the literature	SVM regression	(Zaugg et al., 2017)
	enantioselectivity (E value)	Nitric oxide dioxygenase	445 variants	Linear regression, elastic net, ridge regression, LASSO, SVM, shallow NN, KNN, decision trees, random forest, Gboost, Adaboost	(Wu et al., 2019)
	enantioselectivity ($\Delta\Delta G^{\ddagger}$)	an epoxide hydrolase	37 variants from the literature;	PLS regression	(Cadet et al., 2018)
	T_{opt}	various enzymes	2917 data points from BRENDA	Bayesian ridge, elastic net, decision tree, SVM regression, random forest, linear regression	(Gado et al., 2020; Li et al., 2019)
	T_{opt}	xylanases	145 data points from BRENDA and UniProt	Shallow NN, random forest, naïve Bayes, AdaBoost, SVM	(Shahraki et al., 2021)
	T_{opt} and pH_{opt}	cellulases	163 data points from BRENDA and UniProt	Shallow NN, decision tree, random forest, naïve Bayes, Gaussian process, Bagging, AdaBoost, KNN, SVM, XGBoost, Gboost	(Shahraki et al., 2020)
	T_{opt} and pH_{opt}	lipases	138 data points from BRENDA and UniProt	PCA, linear regression, ridge regression, random forest, LASSO, shallow NN, elastic net, SVM	(Shahraki et al., 2022)
	reaction rates based on experimental conditions	a BioH enzyme	125 reactions	Decision tree, SVM, Gaussian process, Bagging, Boosting, shallow NN	(Wan et al., 2021)
	redox cofactors and substrate specificities	dehydrogenases, reductases, and methyltransferases	8 datasets of 258–309 data points and 1 dataset of 953 data points taken from UniProt	Gboost	(Rappoport and Jinich, 2022)
Kinetics	k_{cat}, K_M, k_{cat}/K_M	β-glucosidase B variants	100 variants	Elastic net regression	(Carlin et al., 2016)
	k_{cat}, k_{cat}/K_M, and T_m	variants of eight enzymes	nine datasets of 8–33 variants per enzyme from the literature	pre-trained Maximum Entropy Model	(Xie et al., 2022)
	k_{cat}, K_M, k_{cat}/K_M	glucose oxidase variants	7 variants vs. 3 substrates at two pH levels	PLS regression	(Ostafe et al., 2020)
	in vitro and in vivo k_{cat}	*E.coli* K-12 enzymes	497 and 234 values of k_{cat} in vitro and $k_{app,\ max}$, respectively, from BRENDA, SABIO-RK, and MetaCyc databases	Linear regression, PLS regression, elastic net, random forest, deep NN	(Heckmann et al., 2018)
	in vivo k_{cat}	general	315 values of $k_{app,\ max}$ from BRENDA, SABIO-RK, and MetaCyc databases	Elastic net, random forest, deep NN	(Heckmann et al., 2020)
	reaction yields	phosphatases	218 enzymes vs. 157 substrates from the literature	graph convolutional NN	(Heid and Green, 2022)
	K_M for natural enzyme-substrate combinations	general	12,011 data points from BRENDA and SABIO-RK	Deep NN, Gboost, elastic net, graph NN	(Kroll et al., 2021)
	k_{cat}	general	16,838 data points from BRENDA and SABIO-RK	graph NN + convolutional NN	(Li et al., 2022)
Stability	T_{50}, T_m, T_{agg}	epoxide hydrolase	16 variants from the literature	PLS	(Li et al., 2021)
	T_{50}	P450	242 variants	Gaussian process	(Romero et al., 2013)
Structure	crystal morphology	lysozyme	6000 micrographs, out of which 272 manually labeled	convolutional NN	(Huang et al., 2022)

(*continued on next page*)

Table 3 (*continued*)

Characteristics	Specific characterization	Enzyme class	Dataset	Algorithm*	Reference
	Crystal yield, purity, and selectivity	Lysozyme, Ribonuclease A, Cytochrome C	3 datasets of 192 datapoints	PLS	(Wegner et al., 2022)
	Bragg spot detection	Thermolysin, Photosystem II, Hydrogenase, Cyclophilin A	5 datasets of 2000 images	convolutional NN	(Ke et al., 2018)

* Gboost = gradient boosting, KNN = K-nearest neighbors, SVM = support vector machines, NN = neural network, PLS = partial least squares, PCA = principal component analysis, LASSO = least absolute shrinkage and selection operator.

contrast to training and evaluating the predictor on an average large group of enzymes. Indeed, for enzyme engineering applications, one might be interested in the predictive power of an ML model for a particular enzyme, but the performance of global predictors may vary significantly for a particular case (Broom et al., 2020; Huang et al., 2020; Potapov et al., 2009). In general, the progress in ML technology holds great promise for complementing experimental determination of protein structures, functions, and many other properties with fast computational predictions. Nevertheless, it also requires a comparable acceleration in the capacity to validate those predictions and investigate new hypotheses experimentally, and microfluidics can be a pivotal technology to tackle this challenge.

Finally, many future targets are waiting to be tackled by ML once the appropriate datasets are made available: thermodynamic parameters (Vasina et al., 2022a), substrate or product inhibition constants (Kokkonen et al., 2021), protein solubility and aggregation in different conditions (Mazurenko, 2020), and optimal protein expression parameters. Apart from improving biotechnological workflows, they have the potential to elucidate the connections between various enzyme characteristics, e.g., melting temperatures and kinetic constants (Carlin et al., 2017). Moreover, the data availability fueled by microfluidics will allow investigation of such challenging topics as epistatic effects of mutations (Aghazadeh et al., 2021; Miton et al., 2021) or ensure data quality by reproducibility, which is critical for ML (Gygli, 2021). Last but not least, current algorithms often rely on the parameters derived from raw data, e.g., by fitting a particular model into it, which is typically the case of ΔG values (Mazurenko et al., 2018). This intermediate step introduces a source of subjectivity and human bias between the raw data and an ML predictor. Predicting raw signals has the potential to eliminate this bias, reveal new insights, and simplify data processing (Pucci et al., 2017; Zhang et al., 2021c).

6. Conclusions and perspectives

With the development of sustainable biotechnologies, the need for effective biocatalysts is constantly growing. While the artificial or natural sequence diversity can be explored efficiently using conventional experimental HTS protocols, the biochemical characterization of positive candidates requires introducing less laborious and more material-efficient techniques. Microfluidic devices have been successfully applied in NGS with a major effort to reduce the cost and reagent consumption. Similarly, microfluidic technology has successfully been applied in (ultra)HTS systems and is now commercially available in some instruments, e.g., droplet generation and manipulation by ONYX and droplet sorting by STYX from Atrandi Biosciences. However, only a few commercial systems have been introduced for microfluidic experimental analysis of enzymes. The "translation" of numerous reported microfluidic concepts and prototypes to commercial ready-to-use instruments widely available to the non-expert community is yet to be accomplished.

In the past, many technical aspects have been streamlined, leading to a rich toolbox of current microfluidic and optofluidic technologies, thus creating significant potential for its successful applications in enzyme analysis. We expect to see significant progress in this area soon as the first commercial technologies (e.g., LabChip® EZ Reader by Perkin Elmer or Formulator by Formulatrix) are already beginning to emerge, taking full advantage of the tremendous potential of microfluidics for systematic and rapid analysis of enzyme properties. To this end, an obvious next step will be implementing an automated operation. In this way, reaction conditions may be varied and controlled in a fully automated manner, allowing for a dramatic expansion of the parameter space within the reaction systems under study. Combining platform automation and real-time feedback algorithms with the described *in situ* monitoring methods will also allow for rapid reaction optimization. An important aspect of these microfluidic-based instruments will be their ease of operation without requiring extensive technical microfluidic knowledge.

Over the past two decades, microfluidics has evolved from emerging technology, with early studies focusing on developing platforms with robust functional components, to an important experimental tool that can process large amounts of experimental data produced at kilohertz frequencies. We envision that the next evolutionary phase will involve its association with data analysis tools, particularly those enabled by recent advances in ML. ML models have already been successfully applied for designing and operating microfluidic devices, but they are largely unexplored in the application of enzyme characterization datasets. The main problem is the absence of consistent data and gold standards for training the ML tools in this domain. Microfluidic platforms thus gain another value in their ability to produce enormous amounts of homogeneous datasets needed for a successful ML application. On the other hand, the broader availability of microfluidics for high-throughput experimental analysis of enzymes would also enable much faster validation of ML-designed proteins and would prevent tempting overestimation of the predictions without the actual testing. We assume that, in the nearest future, this strong synergy of microfluidics and ML will bring groundbreaking knowledge about enzyme function, evolution, and catalytic mechanism and enable the rapid search and design of highly efficient biocatalysts for successful industrial applications.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this review paper.

Data availability

The Full literature review is available in the figshare repository: https://doi.org/10.6084/m9.figshare.21946568.v1

Acknowledgments

The authors would like to acknowledge funding from the Czech Ministry of Education (grants INBIO CZ.02.1.01/0.0/0.0/16_026/0008451, TEAMING CZ.02.1.01/0.0/0.0/17_043/0009632, ESFRI RECETOX LM2018121, ESFRI ELIXIR LM2018131, EXCELES Oncology LX22NPO5102). The authors also acknowledge financial support from the Swiss National Science Foundation (grant no. 205321_176011) and ETH Zurich. This project has received funding from the European

Union's Horizon 2020 research and Innovation program (TEAMING 857560 and Sinfonia 814418). The article reflects the author's view, and the Agency is not responsible for any use that may be made of the information it contains. Michal Vasina acknowledges the financial support of his doctoral study by the scholarship Brno Ph.D. Talent.

Appendix A. Appendix

The following text describes the literature search. Since the domain of microfluidics is very extensive, we applied the following criteria focusing on the most recent studies relevant to biotechnology: (i) to ensure the recency of the research, we have covered the publications published from 2017 to 2022; (ii) studies describing microfluidic characterization of proteins other than enzymes were excluded; (iii) to dissect, which studies cover microfluidics and which do not, we imply the criterium of maximum reaction volume to be below 1 μL, (iv) we have used the keywords of "microfluidic" or "on-chip" with "enzyme" or "biocatalyst"; yet (v) we have excluded the microfluidic studies applying (ultra)high-throughput screening (UHTS), sorting, deep mutational scanning (DMS), biosensing, immunoassays, or immobilized microfluidic enzyme reactors (IMERs).

The reasons for excluding microfluidic applications of enzymes listed in criterion (v) are as follows. HTS and sorting of enzymes serve primarily for the identification of hits, as discussed in Section 2 and Fig. 1 of the main article. Moreover, this topic was covered in several excellent reviews (Longwell et al., 2017; Markel et al., 2020; Neun et al., 2020; Sesen et al., 2017). However, studies performing the so-called "quantitative high-throughput screening" can be regarded as a part of biochemical characterization, and they were, therefore, included. DMS aims to assess the effect of each amino acid at every position within the enzyme but is usually based on low-depth information from HTS. We refer the readers to several microfluidic DMS studies published so far (Nikoomanzar et al., 2019; Romero et al., 2015; Roychowdhury and Romero, 2022). Biosensing, immunoassays, and IMERs represent applications of previously characterized enzymes and, therefore, do not fit into the goal of biochemical characterization. We refer the readers to the reviews about microfluidic biosensing (Mross et al., 2015; Nadar et al., 2021), immunoassays (Giri et al., 2016; Shi et al., 2021), and IMERs (Kecskemeti and Gaspar, 2018; Meller et al., 2017; Zhang et al., 2021a).

The selected key studies are described in Table 2 of the main article (the full results of the extensive literature search can be found in an interactive table, the Full literature review, please, see Data availability). For each study, we have collected information about the detection technique applied, enzyme characteristics, and studied enzymes, including their enzyme commission (EC) numbers, type of microfluidic device as discussed in Section 3, and the number of enzyme variants characterized. Furthermore, we have determined the development level of microfluidic technologies based on the searches both in the articles themselves and in the citing literature. Based on this search, we have classified the articles into three categories: (i) proof-of-concept (POC) studies, which only developed and demonstrated the microfluidic system, (ii) systematic applications, i.e., studies that employed the developed technology in at least one other study for similar characterization, and (iii) studies employing commercially available microfluidic technologies. Finally, we have acquired the number of citations and the average number of citations per year from the Web of Science database (results as of 18th January 2023), while we do not include citations and publications from 2023.

References

Aghazadeh, A., Nisonoff, H., Ocal, O., Brookes, D.H., Huang, Y., Koyluoglu, O.O., Listgarten, J., Ramchandran, K., 2021. Epistatic net allows the sparse spectral regularization of deep neural networks for inferring fitness functions. Nat. Commun. 12, 5225. https://doi.org/10.1038/s41467-021-25371-3.

Alejaldre, L., Pelletier, J.N., Quaglia, D., 2021. Methods for enzyme library creation: which one will you choose? BioEssays 43, 2100052. https://doi.org/10.1002/bies.202100052.

Badenhorst, C.P.S., Bornscheuer, U.T., 2018. Getting momentum: from biocatalysis to advanced synthetic biology. Trends Biochem. Sci. 43, 180–198. https://doi.org/10.1016/j.tibs.2018.01.003.

Banerjee, A., Noh, J.H., Liu, Y., Rack, P.D., Papautsky, I., 2015. Programmable electrowetting with channels and droplets. Micromachines 6, 172–185. https://doi.org/10.3390/mi6020172.

Bastard, K., Smith, A.A.T., Vergne-Vaxelaire, C., Perret, A., Zaparucha, A., De Melo-Minardi, R., Mariage, A., Boutard, M., Debard, A., Lechaplais, C., Pelle, C., Pellouin, V., Perchat, N., Petit, J.-L., Kreimeyer, A., Medigue, C., Weissenbach, J., Artiguenave, F., De Berardinis, V., Vallenet, D., Salanoubat, M., 2014. Revealing the hidden functional diversity of an enzyme family. Nat. Chem. Biol. 10, 42–U77. https://doi.org/10.1038/NCHEMBIO.1387.

Bepler, T., Berger, B., 2021. Learning the protein language: evolution, structure, and function. Cell Syst. 12, 654–669.e3. https://doi.org/10.1016/j.cels.2021.05.017.

Berini, F., Casciello, C., Marcone, G.L., Marinelli, F., 2017. Metagenomics: novel enzymes from non-culturable microbes. FEMS Microbiol. Lett. 364 https://doi.org/10.1093/femsle/fnx211.

Beyerlein, K.R., Dierksmeyer, D., Mariani, V., Kuhn, M., Sarrou, I., Ottaviano, A., Awel, S., Knoska, J., Fuglerud, S., Jönsson, O., Stern, S., Wiedorn, M.O., Yefanov, O., Adriano, L., Bean, R., Burkhardt, A., Fischer, P., Heymann, M., Horke, D.A., Jungnickel, K.E.J., Kovaleva, E., Lorbeer, O., Metz, M., Meyer, J., Morgan, A., Pande, K., Panneerselvam, S., Seuring, C., Tolstikova, A., Lieske, J., Aplin, S., Roessle, M., White, T.A., Chapman, H.N., Meents, A., Oberthuer, D., 2017. Mix-and-diffuse serial synchrotron crystallography. IUCrJ 4, 769–777. https://doi.org/10.1107/S2052252517013124.

Bisswanger, H., 2014. Enzyme assays. Perspect. Sci. 1, 41–55. https://doi.org/10.1016/j.pisc.2014.02.005.

Bonner, W.A., Hulett, H.R., Sweet, R.G., Herzenberg, L.A., 1972. Fluorescence activated cell sorting. Rev. Sci. Instrum. 43, 404–409. https://doi.org/10.1063/1.1685647.

Bornscheuer, U.T., 2016. Protein engineering: beating the odds. Nat. Chem. Biol. 12, 54–55. https://doi.org/10.1038/nchembio.1989.

Broom, A., Trainor, K., Jacobi, Z., Meiering, E.M., 2020. Computational modeling of protein stability: quantitative analysis reveals solutions to pervasive problems. Structure 28, 717–726.e3. https://doi.org/10.1016/j.str.2020.04.003.

Buchegger, W., Haller, A., van den Driesche, S., Kraft, M., Lendl, B., Vellekoop, M., 2012. Studying enzymatic bioreactions in a millisecond microfluidic flow mixer. Biomicrofluidics 6, 012803. https://doi.org/10.1063/1.3665717.

Bunzel, H.A., Garrabou, X., Pott, M., Hilvert, D., 2018. Speeding up enzyme discovery and engineering with ultrahigh-throughput methods. Curr. Opin. Struct. Biol. 48, 149–156. https://doi.org/10.1016/j.sbi.2017.12.010.

Burke, K.S., Parul, D., Reddish, M.J., Dyer, R.B., 2013. A simple three-dimensional-focusing, continuous-flow mixer for the study of fast protein dynamics. Lab Chip 13, 2912–2921. https://doi.org/10.1039/C3LC50497B.

Buryska, T., Vasina, M., Gielen, F., Vanacek, P., van Vliet, L., Jezek, J., Pilat, Z., Zemanek, P., Damborsky, J., Hollfelder, F., Prokop, Z., 2019. Controlled oil/water partitioning of hydrophobic substrates extending the bioanalytical applications of droplet-based microfluidics. Anal. Chem. 91, 10008–10015. https://doi.org/10.1021/acs.analchem.9b01839.

Cadet, F., Fontaine, N., Li, G., Sanchis, J., Chong, M.N.F., Pandjaitan, R., Vetrivel, I., Offmann, B., Reetz, M.T., 2018. A machine learning approach for reliable prediction of amino acid interactions and its application in the directed evolution of enantioselective enzymes. Sci. Rep. 8, 16757. https://doi.org/10.1038/s41598-018-35033-y.

Cadwell, R.C., Joyce, G.F., 1992. Randomization of genes by PCR mutagenesis. Genome Res. 2, 28–33. https://doi.org/10.1101/gr.2.1.28.

Cai, C., Wang, S., Xu, Y., Zhang, W., Tang, K., Ouyang, Q., Lai, L., Pei, J., 2020. Transfer learning for drug discovery. J. Med. Chem. 63, 8683–8694. https://doi.org/10.1021/acs.jmedchem.9b02147.

Callaway, E., 2020. Revolutionary cryo-EM is taking over structural biology. Nature 578, 201. https://doi.org/10.1038/d41586-020-00341-9.

Carlin, D.A., Caster, R.W., Wang, X., Betzenderfer, S.A., Chen, C.X., Duong, V.M., Ryklansky, C.V., Alpekin, A., Beaumont, N., Kapoor, H., Kim, N., Mohabbot, H., Pang, B., Teel, R., Whithaus, L., Tagkopoulos, I., Siegel, J.B., 2016. Kinetic characterization of 100 glycoside hydrolase mutants enables the discovery of structural features correlated with kinetic constants. PLoS One 11, e0147596. https://doi.org/10.1371/journal.pone.0147596.

Carlin, D.A., Hapig-Ward, S., Chan, B.W., Damrau, N., Riley, M., Caster, R.W., Bethards, B., Siegel, J.B., 2017. Thermal stability and kinetic constants for 129 variants of a family 1 glycoside hydrolase reveal that enzyme activity and stability can be separately designed. PLoS One 12, e0176255. https://doi.org/10.1371/journal.pone.0176255.

Chatterjee, D., Hetayothin, B., Wheeler, A.R., King, D.J., Garrell, R.L., 2006. Droplet-based microfluidics with nonaqueous solvents and solutions. Lab Chip 6, 199–206. https://doi.org/10.1039/B515566E.

Chen, K., Arnold, F.H., 2020. Engineering new catalytic activities in enzymes. Nat. Catal. 3, 203–213. https://doi.org/10.1038/s41929-019-0385-5.

Chen, Y., Wijaya Gani, A., Tang, S.K.Y., 2012. Characterization of sensitivity and specificity in leaky droplet-based assays. Lab Chip 12, 5093–5103. https://doi.org/10.1039/C2LC40624A.

Chen, B., Lim, S., Kannan, A., Alford, S.C., Sunden, F., Herschlag, D., Dimov, I.K., Baer, T. M., Cochran, J.R., 2016. High-throughput analysis and protein engineering using microcapillary arrays. Nat. Chem. Biol. 12, 76–81. https://doi.org/10.1038/nchembio.1978.

Cheng, R.K., 2020. Towards an optimal sample delivery method for serial crystallography at XFEL. Crystals 10, 215. https://doi.org/10.3390/cryst10030215.
Clausell-Tormos, J., Griffiths, A.D., Merten, C.A., 2010. An automated two-phase microfluidic system for kinetic analyses and the screening of compound libraries. Lab Chip 10, 1302–1307. https://doi.org/10.1039/B921754A.
Colin, P.-Y., Kintses, B., Gielen, F., Miton, C.M., Fischer, G., Mohamed, M.F., Hyvönen, M., Morgavi, D.P., Janssen, D.B., Hollfelder, F., 2015. Ultrahigh-throughput discovery of promiscuous enzymes by picodroplet functional metagenomics. Nat. Commun. 6, 10008. https://doi.org/10.1038/ncomms10008.
Correa, A., Oppezzo, P., 2015. Overcoming the solubility problem in E. coli: Available approaches for recombinant protein production. In: García-Fruitós, E. (Ed.), Insoluble Proteins: Methods and Protocols, Methods in Molecular Biology. Springer, New York, NY, pp. 27–44. https://doi.org/10.1007/978-1-4939-2205-5_2.
Coughlan, L.M., Cotter, P.D., Hill, C., Alvarez-Ordóñez, A., 2015. Biotechnological applications of functional metagenomics in the food and pharmaceutical industries. Front. Microbiol. 6, 672. https://doi.org/10.3389/fmicb.2015.00672.
Culligan, E.P., Sleator, R.D., Marchesi, J.R., Hill, C., 2014. Metagenomics and novel gene discovery. Virulence 5, 399–412. https://doi.org/10.4161/viru.27208.
Detlefsen, N.S., Hauberg, S., Boomsma, W., 2022. Learning meaningful representations of protein sequences. Nat. Commun. 13, 1914. https://doi.org/10.1038/s41467-022-29443-w.
Devos, C., Van Gerven, T., Kuhn, S., 2021. A review of experimental methods for nucleation rate determination in large-volume batch and microfluidic crystallization. Cryst. Growth Des. 21, 2541–2565. https://doi.org/10.1021/acs.cgd.0c01606.
Ding, Y., Howes, P.D., deMello, A.J., 2020. Recent advances in droplet microfluidics. Anal. Chem. 92, 132–149. https://doi.org/10.1021/acs.analchem.9b05047.
Dong, Z., Fang, Q., 2020. Automated, flexible and versatile manipulation of nanoliter-to-picoliter droplets based on sequential operation droplet array technique. TrAC Trends Anal. Chem. 124, 115812 https://doi.org/10.1016/j.trac.2020.115812.
Doornbos, Richard M.P., de Grooth, Bart G., Greve, Jan, 1997. Experimental and model investigations of bleaching and saturation of fluorescence in flow cytometry. Cytometry 29, 204–214. https://doi.org/10.1002/(SICI)1097-0320(19971101)29:3<204::AID-CYTO3>3.0.CO;2-B.
Dörr, M., Fibinger, M.P.C., Last, D., Schmidt, S., Santos-Aberturas, J., Böttcher, D., Hummel, A., Vickers, C., Voss, M., Bornscheuer, U.T., 2016. Fully automatized high-throughput enzyme library screening using a robotic platform. Biotechnol. Bioeng. 113, 1421–1432. https://doi.org/10.1002/bit.25925.
Ebbinghaus, S., Dhar, A., McDonald, J.D., Gruebele, M., 2010. Protein folding stability and dynamics imaged in a living cell. Nat. Methods 7, 319. https://doi.org/10.1038/nmeth.1435.
Eydelnant, I.A., Betty Li, B., Wheeler, A.R., 2014. Microgels on-demand. Nat. Commun. 5, 3355. https://doi.org/10.1038/ncomms4355.
Fan, H.C., Wang, J., Potanina, A., Quake, S.R., 2011. Whole-genome molecular haplotyping of single cells. Nat. Biotechnol. 29, 51–57. https://doi.org/10.1038/nbt.1739.
Fatkhutdinova, A., Mukhametzyanov, T., Schick, C., 2022. Refolding of lysozyme in glycerol as studied by fast scanning calorimetry. Int. J. Mol. Sci. 23, 2773. https://doi.org/10.3390/ijms23052773.
Ferreira, J., Castro, F., Rocha, F., Kuhn, S., 2018. Protein crystallization in a droplet-based microfluidic device: hydrodynamic analysis and study of the phase behaviour. Chem. Eng. Sci. 191, 232–244. https://doi.org/10.1016/j.ces.2018.06.066.
Ferreira, J., Castro, F., Kuhn, S., Rocha, F., 2020a. Controlled protein crystal nucleation in microreactors: the effect of the droplet volume versus high supersaturation ratios. CrystEngComm 22, 4692–4701. https://doi.org/10.1039/D0CE00517G.
Ferreira, J., Opsteyn, J., Rocha, F., Castro, F., Kuhn, S., 2020b. Ultrasonic protein crystallization: promoting nucleation in microdroplets through pulsed sonication. Chem. Eng. Res. Des. 162, 249–257. https://doi.org/10.1016/j.cherd.2020.08.014.
Fidalgo, L.M., Maerkl, S.J., 2011. A software-programmable microfluidic device for automated biology. Lab Chip 11, 1612–1619. https://doi.org/10.1039/C0LC00537A.
Fisher, B.F., Snodgrass, H.M., Jones, K.A., Andorfer, M.C., Lewis, J.C., 2019. Site-selective C-H halogenation using flavin-dependent halogenases identified via family-wide activity profiling. Acs Cent. Sci. 5, 1844–1856. https://doi.org/10.1021/acscentsci.9b00835.
Fogarty, K., Kashem, M., Bauer, A., Bernardino, A., Brennan, D., Cook, B., Farrow, N., Molinaro, T., Nelson, R., 2017. Development of three orthogonal assays suitable for the identification and qualification of PIKfyve inhibitors. ASSAY Drug Dev. Technol. 15, 210–219. https://doi.org/10.1089/adt.2017.790.
Fowler, D.M., Fields, S., 2014. Deep mutational scanning: a new style of protein science. Nat. Methods 11, 801–807. https://doi.org/10.1038/nmeth.3027.
Fuller, F.D., Gul, S., Chatterjee, R., Burgie, E.S., Young, I.D., Lebrette, H., Srinivas, V., Brewster, A.S., Michels-Clark, T., Clinger, J.A., Andi, B., Ibrahim, M., Pastor, E., de Lichtenberg, C., Hussein, R., Pollock, C.J., Zhang, M., Stan, C.A., Kroll, T., Fransson, T., Weninger, C., Kubin, M., Aller, P., Lassalle, L., Bräuer, P., Miller, M.D., Amin, M., Koroidov, S., Roessler, C.G., Allaire, M., Sierra, R.G., Docker, P.T., Glownia, J.M., Nelson, S., Koglin, J.E., Zhu, D., Chollet, M., Song, S., Lemke, H., Liang, M., Sokaras, D., Alonso-Mori, R., Zouni, A., Messinger, J., Bergmann, U., Boal, A.K., Bollinger, J.M., Krebs, C., Högbom, M., Phillips, G.N., Vierstra, R.D., Sauter, N.K., Orville, A.M., Kern, J., Yachandra, V.K., Yano, J., 2017. Drop-on-demand sample delivery for studying biocatalysts in action at X-ray free-electron lasers. Nat. Methods 14, 443–449. https://doi.org/10.1038/nmeth.4195.
Gado, J.E., Beckham, G.T., Payne, C.M., 2020. Improving enzyme optimum temperature prediction with resampling strategies and ensemble learning. J. Chem. Inf. Model. 60, 4098–4107. https://doi.org/10.1021/acs.jcim.0c00489.
Gajiwala, K.S., Grodsky, N., Bolaños, B., Feng, J., Ferre, R., Timofeevski, S., Xu, M., Murray, B.W., Johnson, T.W., Stewart, A., 2017. The Axl kinase domain in complex with a macrocyclic inhibitor offers first structural insights into an active TAM receptor kinase. J. Biol. Chem. 292, 15705–15716. https://doi.org/10.1074/jbc.M116.771485.
Gerdts, C.J., Elliott, M., Lovell, S., Mixon, M.B., Napuli, A.J., Staker, B.L., Nollert, P., Stewart, L., 2008. The plug-based nanovolume microcapillary protein crystallization system (MPCS). Acta Crystallogr. D Biol. Crystallogr. 64, 1116–1122. https://doi.org/10.1107/S0907444908028060.
Ghazal, A., Lafleur, J.P., Mortensen, K., Kutter, J.P., Arleth, L., Jensen, G.V., 2016. Recent advances in X-ray compatible microfluidics for applications in soft materials and life sciences. Lab Chip 16, 4263–4295. https://doi.org/10.1039/C6LC00888G.
Gielen, F., Buryska, T., Vliet, L.V., Butz, M., Damborsky, J., Prokop, Z., Hollfelder, F., 2015. Interfacing microwells with nanoliter compartments: a sampler generating high-resolution concentration gradients for quantitative biochemical analyses in droplets. Anal. Chem. 87, 624–632. https://doi.org/10.1021/ac503336g.
Gielen, F., Hours, R., Emond, S., Fischlechner, M., Schell, U., Hollfelder, F., 2016. Ultrahigh-throughput–directed enzyme evolution by absorbance-activated droplet sorting (AADS). Proc. Natl. Acad. Sci. 113, E7383–E7389. https://doi.org/10.1073/pnas.1606927113.
Gilboa, T., Ogata, A.F., Reilly, C.B., Walt, D.R., 2022. Single-molecule studies reveal method for tuning the heterogeneous activity of alkaline phosphatase. Biophys. J. 121, 2027–2034. https://doi.org/10.1016/j.bpj.2022.05.005.
Girault, M., Beneyton, T., Pekin, D., Buisson, L., Bichon, S., Charbonnier, C., del Amo, Y., Baret, J.-C., 2018. High-content screening of plankton alkaline phosphatase activity in microfluidics. Anal. Chem. 90, 4174–4181. https://doi.org/10.1021/acs.analchem.8b00234.
Girault, M., Siano, R., Labry, C., Latimier, M., Jauzein, C., Beneyton, T., Buisson, L., Del Amo, Y., Baret, J.-C., 2021. Variable inter and intraspecies alkaline phosphatase activity within single cells of revived dinoflagellates. ISME J. 15, 2057–2069. https://doi.org/10.1038/s41396-021-00904-2.
Giri, B., Pandey, B., Neupane, B., Ligler, F.S., 2016. Signal amplification strategies for microfluidic immunoassays. TrAC Trends Anal. Chem. 79, 326–334. https://doi.org/10.1016/j.trac.2015.10.021.
Goldman, S., Das, R., Yang, K.K., Coley, C.W., 2022. Machine learning modeling of family wide enzyme-substrate specificity screens. PLoS Comput. Biol. 18, e1009853 https://doi.org/10.1371/journal.pcbi.1009853.
Grant, J., Goudarzi, S.H., Mrksich, M., 2018. High-throughput enzyme kinetics with 3D microfluidics and imaging SAMDI mass spectrometry. Anal. Chem. 90, 13096–13103. https://doi.org/10.1021/acs.analchem.8b04391.
Grant, J., Kimmel, B.R., Szymczak, L.C., Roll, J., Mrksich, M., 2022. Characterizing enzyme cooperativity with imaging SAMDI-MS. Chem. Eur. J. 28, e202103807 https://doi.org/10.1002/chem.202103807.
Guo, M., Xu, Y., Gruebele, M., 2012. Temperature dependence of protein folding kinetics in living cells. Proc. Natl. Acad. Sci. 109, 17863–17867. https://doi.org/10.1073/pnas.1201797109.
Guo, X.-L., Wei, Y., Lou, Q., Zhu, Y., Fang, Q., 2018. Manipulating femtoliter to picoliter droplets by pins for single cell analysis and quantitative biological assay. Anal. Chem. 90, 5810–5817. https://doi.org/10.1021/acs.analchem.8b00343.
Gygli, G., 2021. On the reproducibility of enzyme reactions and kinetic modelling. https://doi.org/10.48550/arXiv.2110.10419.
Ha, N.S., de Raad, M., Han, L.Z., Golini, A., Petzold, C.J., Northen, T.R., 2021. Faster, better, and cheaper: harnessing microfluidics and mass spectrometry for biotechnology. RSC Chem. Biol. 2, 1331–1351. https://doi.org/10.1039/D1CB00112D.
Hadd, A.G., Jacobson, S.C., Ramsey, J.M., 1999. Microfluidic assays of acetylcholinesterase inhibitors. Anal. Chem. 71, 5206–5212. https://doi.org/10.1021/ac990591f.
Hadwen, B., Broder, G.R., Morganti, D., Jacobs, A., Brown, C., Hector, J.R., Kubota, Y., Morgan, H., 2012. Programmable large area digital microfluidic array with integrated droplet sensing for bioassays. Lab Chip 12, 3305–3313. https://doi.org/10.1039/C2LC40273D.
Handelsman, J., 2004. Metagenomics: application of genomics to uncultured microorganisms. Microbiol. Mol. Biol. Rev. 68, 669–685. https://doi.org/10.1128/MMBR.68.4.669-685.2004.
Hanson, J., Litfin, T., Paliwal, K., Zhou, Y., 2020. Identifying molecular recognition features in intrinsically disordered regions of proteins by transfer learning. Bioinformatics 36, 1107–1113. https://doi.org/10.1093/bioinformatics/btz691.
Heckmann, D., Lloyd, C.J., Mih, N., Ha, Y., Zielinski, D.C., Haiman, Z.B., Desouki, A.A., Lercher, M.J., Palsson, B.O., 2018. Machine learning applied to enzyme turnover numbers reveals protein structural correlates and improves metabolic models. Nat. Commun. 9, 5252. https://doi.org/10.1038/s41467-018-07652-6.
Heckmann, D., Campeau, A., Lloyd, C.J., Phaneuf, P., Hefner, Y., Carrillo-Terrazas, M., Feist, A.M., Gonzalez, D.J., Palsson, B.O., 2020. Kinetic profiling of metabolic specialists demonstrates stability and consistency of in vivo enzyme turnover numbers. Proc. Natl. Acad. Sci. U. S. A. 117, 23182–23190. https://doi.org/10.1073/pnas.2001562117.
Heid, E., Green, W.H., 2022. Machine learning of reaction properties via learned representations of the condensed graph of reaction. J. Chem. Inf. Model. 62, 2101–2110. https://doi.org/10.1021/acs.jcim.1c00975.
Heinemann, J., Deng, K., Shih, S.C.C., Gao, J., Adams, P.D., Singh, A.K., Northen, T.R., 2017. On-chip integration of droplet microfluidics and nanostructure-initiator mass spectrometry for enzyme screening. Lab Chip 17, 323–331. https://doi.org/10.1039/C6LC01182A.
Hejazian, M., Balaur, E., Abbey, B., 2021. Recent advances and future perspectives on microfluidic mix-and-jet sample delivery devices. Micromachines 12, 531. https://doi.org/10.3390/mi12050531.

Hess, D., Rane, A., deMello, A.J., Stavrakis, S., 2015. High-throughput, quantitative enzyme kinetic analysis in microdroplets using stroboscopic epifluorescence imaging. Anal. Chem. 87, 4965–4972. https://doi.org/10.1021/acs.analchem.5b00766.

Hess, D., Yang, T., Stavrakis, S., 2020. Droplet-based optofluidic systems for measuring enzyme kinetics. Anal. Bioanal. Chem. 412, 3265–3283. https://doi.org/10.1007/s00216-019-02294-z.

Hess, D., Dockalova, V., Kokkonen, P., Bednar, D., Damborsky, J., deMello, A., Prokop, Z., Stavrakis, S., 2021. Exploring mechanism of enzyme catalysis by on-chip transient kinetics coupled with global data analysis and molecular modeling. Chem 7, 1066–1079. https://doi.org/10.1016/j.chempr.2021.02.011.

Ho, C., Lam, C., Chan, M., Cheung, R., Law, L., Lit, L., Ng, K., Suen, M., Tai, H., 2003. Electrospray ionisation mass spectrometry: principles and clinical applications. Clin. Biochem. Rev. 24, 3–12.

Höhne, M., Schätzle, S., Jochens, H., Robins, K., Bornscheuer, U.T., 2010. Rational assignment of key motifs for function guides *in silico* enzyme identification. Nat. Chem. Biol. 6, 807–813. https://doi.org/10.1038/nchembio.447.

Hollerbach, A., Ayrton, S., Jarmusch, A., Graham Cooks, R., 2017. Desorption electrospray ionization: Methodology and applications. In: Lindon, J.C., Tranter, G. E., Koppenaal, D.W. (Eds.), Encyclopedia of Spectroscopy and Spectrometry, Third edition. Academic Press, Oxford, pp. 401–408. https://doi.org/10.1016/B978-0-12-409547-2.12133-X.

Holmes, S., Kirkwood, H.J., Bean, R., Giewekemeyer, K., Martin, A.V., Hadian-Jazi, M., Wiedorn, M.O., Oberthür, D., Marman, H., Adriano, L., Al-Qudami, N., Bajt, S., Barák, I., Bari, S., Bielecki, J., Brockhauser, S., Coleman, M.A., Cruz-Mazo, F., Danilevski, C., Dörner, K., Gañán-Calvo, A.M., Graceffa, R., Fanghor, H., Heymann, M., Frank, M., Kaukher, A., Kim, Y., Kobe, B., Knoška, J., Laurus, T., Letrun, R., Maia, L., Messerschmidt, M., Metz, M., Michelat, T., Mills, G., Molodtsov, S., Monteiro, D.C.F., Morgan, A.J., Münnich, A., Peña Murillo, G.E., Previtali, G., Round, A., Sato, T., Schubert, R., Schulz, J., Shelby, M., Seuring, C., Sellberg, J.A., Sikorski, M., Silenzi, A., Stern, S., Sztuk-Dambietz, J., Szuba, J., Trebbin, M., Vagovic, P., Ve, T., Weinhausen, B., Wrona, K., Xavier, P.L., Xu, C., Yefanov, O., Nugent, K.A., Chapman, H.N., Mancuso, A.P., Barty, A., Abbey, B., Darmanin, C., 2022. Megahertz pulse trains enable multi-hit serial femtosecond crystallography experiments at X-ray free electron lasers. Nat. Commun. 13, 4708. https://doi.org/10.1038/s41467-022-32434-6.

Huang, H., Pandya, C., Liu, C., Al-Obaidi, N.F., Wang, M., Zheng, L., Toews Keating, S., Aono, M., Love, J.D., Evans, B., Seidel, R.D., Hillerich, B.S., Garforth, S.J., Almo, S. C., Mariano, P.S., Dunaway-Mariano, D., Allen, K.N., Farelli, J.D., 2015. Panoramic view of a superfamily of phosphatases through substrate profiling. Proc. Natl. Acad. Sci. 112, E1974–E1983. https://doi.org/10.1073/pnas.1423570112.

Huang, P., Chu, S.K.S., Frizzo, H.N., Connolly, M.P., Caster, R.W., Siegel, J.B., 2020. Evaluating protein engineering thermostability prediction tools using an independently generated dataset. ACS Omega 5, 6487–6493. https://doi.org/10.1021/acsomega.9b04105.

Huang, L., Yang, D., Yu, Z., He, J., Chen, Y., Zhou, J., 2022. Deep learning-aided high-throughput screening of time-resolved protein crystallization on programmable microliter-droplet systems. Chem. Eng. J. 450, 138267 https://doi.org/10.1016/j.cej.2022.138267.

Huber, S.T., Sarajlic, E., Huijink, R., Weis, F., Evers, W.H., Jakobi, A.J., 2022. Nanofluidic chips for cryo-EM structure determination from picoliter sample volumes. eLife 11, e72629. https://doi.org/10.7554/eLife.72629.

Hutchison, C.A., Phillips, S., Edgell, M.H., Gillam, S., Jahnke, P., Smith, M., 1978. Mutagenesis at a specific position in a DNA sequence. J. Biol. Chem. 253, 6551–6560. https://doi.org/10.1016/S0021-9258(19)46967-6.

Ilhan-Ayisigi, E., Yaldiz, B., Bor, G., Yaghmur, A., Yesil-Celiktas, O., 2021. Advances in microfluidic synthesis and coupling with synchrotron SAXS for continuous production and real-time structural characterization of nano-self-assemblies. Colloids Surf. B: Biointerfaces 201, 111633. https://doi.org/10.1016/j.colsurfb.2021.111633.

Jeffries, G.D.M., Lorenz, R.M., Chiu, D.T., 2010. Ultrasensitive and high-throughput fluorescence analysis of droplet contents with orthogonal line confocal excitation. Anal. Chem. 82, 9948–9954. https://doi.org/10.1021/ac102173m.

Jensen, E.C., Bhat, B.P., Mathies, R.A., 2010. A digital microfluidic platform for the automation of quantitative biomolecular assays. Lab Chip 10, 685–691. https://doi.org/10.1039/B920124F.

Jeong, J.S., Jiang, L., Albino, E., Marrero, J., Rho, H.S., Hu, J., Hu, S., Vera, C., Bayron-Poueymiroy, D., Rivera-Pacheco, Z.A., Ramos, L., Torres-Castro, C., Qian, J., Bonaventura, J., Boeke, J.D., Yap, W.Y., Pino, I., Eichinger, D.J., Zhu, H., Blackshaw, S., 2012. Rapid identification of monospecific monoclonal antibodies using a human proteome microarray*. Mol. Cell. Proteomics 11 (O111), 016253. https://doi.org/10.1074/mcp.O111.016253.

Jeong, H.-H., Lee, B., Jin, S.H., Jeong, S.-G., Lee, C.-S., 2016. A highly addressable static droplet array enabling digital control of a single droplet at pico-volume resolution. Lab Chip 16, 1698–1707. https://doi.org/10.1039/C6LC00212A.

Jiang, Y., Li, X., Morrow, B.R., Pothukuchy, A., Gollihar, J., Novak, R., Reilly, C.B., Ellington, A.D., Walt, D.R., 2019. Single-molecule mechanistic study of enzyme hysteresis. ACS Cent. Sci. 5, 1691–1698. https://doi.org/10.1021/acscentsci.9b00718.

Jiang, Y., Li, X., Walt, D.R., 2020. Single-molecule analysis determines isozymes of human alkaline phosphatase in serum. Angew. Chem. Int. Ed. 59, 18010–18015. https://doi.org/10.1002/anie.202007477.

Jochens, H., Bornscheuer, U.T., 2010. Natural diversity to guide focused directed evolution. ChemBioChem 11, 1861–1866. https://doi.org/10.1002/cbic.201000284.

Johnson, K.A., Goody, R.S., 2011. The original Michaelis constant: translation of the 1913 Michaelis-Menten paper. Biochemistry 50, 8264–8269. https://doi.org/10.1021/bi201284u.

Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A., Bridgland, A., Meyer, C., Kohl, S.A.A., Ballard, A.J., Cowie, A., Romera-Paredes, B., Nikolov, S., Jain, R., Adler, J., Back, T., Petersen, S., Reiman, D., Clancy, E., Zielinski, M., Steinegger, M., Pacholska, M., Berghammer, T., Bodenstein, S., Silver, D., Vinyals, O., Senior, A.W., Kavukcuoglu, K., Kohli, P., Hassabis, D., 2021. Highly accurate protein structure prediction with AlphaFold. Nature 596, 583–589. https://doi.org/10.1038/s41586-021-03819-2.

Katzke, N., Knapp, A., Loeschcke, A., Drepper, T., Jaeger, K.-E., 2017. Novel tools for the functional expression of metagenomic DNA. In: Streit, W.R., Daniel, R. (Eds.), Metagenomics: Methods and Protocols, Methods in Molecular Biology. Springer, New York, NY, pp. 159–196. https://doi.org/10.1007/978-1-4939-6691-2_10.

Ke, T.-W., Brewster, A.S., Yu, S.X., Ushizima, D., Yang, C., Sauter, N.K., 2018. A convolutional neural network-based screening tool for X-ray serial crystallography. J. Synchrotron Radiat. 25, 655–670. https://doi.org/10.1107/S1600577518004873.

Kecskemeti, A., Gaspar, A., 2018. Particle-based immobilized enzymatic reactors in microfluidic chips. Talanta 180, 211–228. https://doi.org/10.1016/j.talanta.2017.12.043.

Ki, M.-R., Pack, S.P., 2020. Fusion tags to enhance heterologous protein expression. Appl. Microbiol. Biotechnol. 104, 2411–2425. https://doi.org/10.1007/s00253-020-10402-8.

Klijn, M.E., Hubbuch, J., 2021. Application of ultraviolet, visible, and infrared light imaging in protein-based biopharmaceutical formulation characterization and development studies. Eur. J. Pharm. Biopharm. 165, 319–336. https://doi.org/10.1016/j.ejpb.2021.05.013.

Knoška, J., Adriano, L., Awel, S., Beyerlein, K.R., Yefanov, O., Oberthuer, D., Peña Murillo, G.E., Roth, N., Sarrou, I., Villanueva-Perez, P., Wiedorn, M.O., Wilde, F., Bajt, S., Chapman, H.N., Heymann, M., 2020. Ultracompact 3D microfluidics for time-resolved structural biology. Nat. Commun. 11, 657. https://doi.org/10.1038/s41467-020-14434-6.

Kokkonen, P., Beier, A., Mazurenko, S., Damborsky, J., Bednar, D., Prokop, Z., 2021. Substrate inhibition by the blockage of product release and its control by tunnel engineering. RSC Chem. Biol. 2, 645–655. https://doi.org/10.1039/D0CB00171F.

Kroll, A., Engqvist, M.K.M., Heckmann, D., Lercher, M.J., 2021. Deep learning allows genome-scale prediction of Michaelis constants from structural features. PLoS Biol. 19, e3001402 https://doi.org/10.1371/journal.pbio.3001402.

Kroll, A., Ranjan, S., Engqvist, M.K.M., Lercher, M.J., 2022. The substrate scopes of enzymes: A general prediction model based on machine and deep learning. https://doi.org/10.1101/2022.05.24.493213.

Kulathunga, S.C., Morato, N.M., Zhou, Q., Cooks, R.G., Mesecar, A.D., 2022. Desorption electrospray ionization mass spectrometry assay for label-free characterization of SULT2B1b enzyme kinetics. ChemMedChem 17, e202200043. https://doi.org/10.1002/cmdc.202200043.

Kuo, J.S., Chiu, D.T., 2011. Controlling mass transport in microfluidic devices. Annu. Rev. Anal. Chem. 4, 275–296. https://doi.org/10.1146/annurev-anchem-061010-113926.

Lafferty, M., Dycaico, M.J., 2004. GigaMatrix: A novel ultrahigh throughput protein optimization and discovery platform. In: Methods in Enzymology, Protein Engineering. Academic Press, pp. 119–134. https://doi.org/10.1016/S0076-6879(04)88011-X.

Lau, B.T.C., Baitz, C.A., Dong, X.P., Hansen, C.L., 2007. A complete microfluidic screening platform for rational protein crystallization. J. Am. Chem. Soc. 129, 454–455. https://doi.org/10.1021/ja065855b.

Leclerc, L.M.Y., Soffer, G., Kwan, D.H., Shih, S.C.C., 2019. A fucosyltransferase inhibition assay using image-analysis and digital microfluidics. Biomicrofluidics 13, 034106. https://doi.org/10.1063/1.5088517.

Lee, J.E., Fusco, M.L., Saphire, E.O., 2009. An efficient platform for screening expression and crystallization of glycoproteins produced in human cells. Nat. Protoc. 4, 592–604. https://doi.org/10.1038/nprot.2009.29.

Lee, B., Jin, S.H., Noh, Y.-M., Jeong, S.-G., Jeong, H.-H., Lee, C.-S., 2018. Scalable static droplet array for biochemical assays based on concentration gradients. Sensors Actuators B Chem. 273, 1572–1578. https://doi.org/10.1016/j.snb.2018.07.076.

Li, X., Jiang, Y., Chong, S., Walt, D.R., 2018. Bottom-up single-molecule strategy for understanding subunit function of tetrameric β-galactosidase. Proc. Natl. Acad. Sci. 115, 8346–8351. https://doi.org/10.1073/pnas.1805690115.

Li, G., Rabe, K.S., Nielsen, J., Engqvist, M.K.M., 2019. Machine learning applied to predicting microorganism growth temperatures and enzyme catalytic optima. ACS Synth. Biol. 8, 1411–1420. https://doi.org/10.1021/acssynbio.9b00099.

Li, G., Qin, Y., Fontaine, N.T., Ng Fuk Chong, M., Maria-Solano, M.A., Feixas, F., Cadet, X.F., Pandjaitan, R., Garcia-Borras, M., Cadet, F., Reetz, M.T., 2021. Machine learning enables selection of epistatic enzyme mutants for stability against unfolding and detrimental aggregation. Chembiochem 22, 904–914. https://doi.org/10.1002/cbic.202000612.

Li, F., Yuan, L., Lu, H., Li, G., Chen, Y., Engqvist, M.K.M., Kerkhoven, E.J., Nielsen, J., 2022. Deep learning-based kcat prediction enables improved enzyme-constrained model reconstruction. Nat. Catal. 1–11 https://doi.org/10.1038/s41929-022-00798-z.

Liang, Y.-R., Zhu, L.-N., Gao, J., Zhao, H.-X., Zhu, Y., Ye, S., Fang, Q., 2017. 3D-printed high-density droplet array chip for miniaturized protein crystallization screening under vapor diffusion mode. ACS Appl. Mater. Interfaces 9, 11837–11845. https://doi.org/10.1021/acsami.6b15933.

Liu, R., Liu, Y.-C., Meng, J., Zhu, H., Zhang, X., 2017. A microfluidics-based mobility shift assay to identify new inhibitors of β-secretase for Alzheimer's disease. Anal. Bioanal. Chem. 409, 6635–6642. https://doi.org/10.1007/s00216-017-0617-y.

Liu, Q., Xun, G., Feng, Y., 2019. The state-of-the-art strategies of protein engineering for enzyme stabilization. Biotechnol. Adv. Bioref. 37, 530–537. https://doi.org/10.1016/j.biotechadv.2018.10.011.

Livada, J., Vargas, A.M., Martinez, C.A., Lewis, R.D., 2023. Ancestral sequence reconstruction enhances gene mining efforts for industrial Ene reductases by expanding enzyme panels with thermostable catalysts. ACS Catal. 13, 2576–2585. https://doi.org/10.1021/acscatal.2c03859.

Longwell, C.K., Labanieh, L., Cochran, J.R., 2017. High-throughput screening technologies for enzyme engineering. Curr. Opin. Biotechnol. 48, 196–202. https://doi.org/10.1016/j.copbio.2017.05.012.

Lou, Q., Ma, Y., Zhao, S.-P., Du, G.-S., Fang, Q., 2021. A flexible and cost-effective manual droplet operation platform for miniaturized cell assays and single cell analysis. Talanta 224, 121874. https://doi.org/10.1016/j.talanta.2020.121874.

Lutz, S., 2010. Beyond directed evolution - semi-rational protein engineering and design. Curr. Opin. Biotechnol. 21, 734–743. https://doi.org/10.1016/j.copbio.2010.08.011.

Maceiczyk, R.M., Hess, D., Chiu, F.W.Y., Stavrakis, S., deMello, A.J., 2017. Differential detection photothermal spectroscopy: towards ultra-fast and sensitive label-free detection in picoliter & femtoliter droplets. Lab Chip 17, 3654–3663. https://doi.org/10.1039/C7LC00946A.

Mäeots, M.-E., Lee, B., Nans, A., Jeong, S.-G., Esfahani, M.M.N., Ding, S., Smith, D.J., Lee, C.-S., Lee, S.S., Peter, M., Enchev, R.I., 2020. Modular microfluidics enables kinetic insight from time-resolved cryo-EM. Nat. Commun. 11, 3465. https://doi.org/10.1038/s41467-020-17230-4.

Magnusson, A.O., Szekrenyi, A., Joosten, H., Finnigan, J., Charnock, S., Fessner, W., 2019. nanoDSF as screening tool for enzyme libraries and biotechnology development. FEBS J. 286, 184–204. https://doi.org/10.1111/febs.14696.

Mao, Z., Guo, F., Xie, Y., Zhao, Y., Lapsley, M.I., Wang, L., Mai, J.D., Costanzo, F., Huang, T.J., 2015. Label-free measurements of reaction kinetics using a droplet-based optofluidic device. SLAS Technol. 20, 17–24. https://doi.org/10.1177/2211068214549625.

Marinaro, G., Graceffa, R., Riekel, C., 2022. Wall-free droplet microfluidics for probing biological processes by high-brilliance X-ray scattering techniques. Front. Mol. Biosci. 9.

Măriuţa, D., Colin, S., Barrot-Lattes, C., Le Calvé, S., Korvink, J.G., Baldas, L., Brandner, J.J., 2020. Miniaturization of fluorescence sensing in optofluidic devices. Microfluid. Nanofluid. 24, 65. https://doi.org/10.1007/s10404-020-02371-1.

Markel, U., Essani, K.D., Besirlioglu, V., Schiffels, J., Streit, W.R., Schwaneberg, U., 2020. Advances in ultrahigh-throughput screening for directed enzyme evolution. Chem. Soc. Rev. 49, 233–262. https://doi.org/10.1039/C8CS00981C.

Markin, C.J., Mokhtari, D.A., Sunden, F., Appel, M.J., Akiva, E., Longwell, S.A., Sabatti, C., Herschlag, D., Fordyce, P.M., 2021. Revealing enzyme functional architecture via high-throughput microfluidic enzyme kinetics. Science 373, eabf8761. https://doi.org/10.1126/science.abf8761.

Markova, K., Kunka, A., Chmelova, K., Havlasek, M., Babkova, P., Marques, S.M., Vasina, M., Planas-Iglesias, J., Chaloupkova, R., Bednar, D., Prokop, Z., Damborsky, J., Marek, M., 2021. Computational enzyme stabilization can affect folding energy landscapes and lead to catalytically enhanced domain-swapped dimers. ACS Catal. 11, 12864–12885. https://doi.org/10.1021/acscatal.1c03343.

Marques, S.M., Planas-Iglesias, J., Damborsky, J., 2021. Web-based tools for computational enzyme design. Curr. Opin. Struct. Biol. 69, 19–34. https://doi.org/10.1016/j.sbi.2021.01.010.

Martiel, I., Olieric, V., Caffrey, M., Wang, M., 2018. Chapter 1:practical approaches for in situ X-ray crystallography: From high-throughput screening to serial data collection. In: Protein Crystallography, pp. 1–27. https://doi.org/10.1039/9781788010504-00001.

Martínez-Martínez, M., Coscolín, C., Santiago, G., Chow, J., Stogios, P.J., Bargiela, R., Gertler, C., Navarro-Fernández, J., Bollinger, A., Thies, S., Méndez-García, C., Popovic, A., Brown, G., Chernikova, T.N., García-Moyano, A., Bjerga, G.E.K., Pérez-García, P., Hai, T., Del Pozo, M.V., Stokke, R., Steen, I.H., Cui, H., Xu, X., Nocek, B.P., Alcaide, M., Distaso, M., Mesa, V., Peláez, A.I., Sánchez, J., Buchholz, P.C.F., Pleiss, J., Fernández-Guerra, A., Glöckner, F.O., Golyshina, O.V., Yakimov, M.M., Savchenko, A., Jaeger, K.-E., Yakunin, A.F., Streit, W.R., Golyshin, P.N., Guallar, V., Ferrer, M., The INMARE Consortium, 2018. Determinants and prediction of esterase substrate promiscuity patterns. ACS Chem. Biol. 13, 225–234. https://doi.org/10.1021/acschembio.7b00996.

Mazurenko, S., 2020. Predicting protein stability and solubility changes upon mutations: data perspective. ChemCatChem 12, 5590–5598. https://doi.org/10.1002/cctc.202000933.

Mazurenko, S., Stourac, J., Kunka, A., Nedeljković, S., Bednar, D., Prokop, Z., Damborsky, J., 2018. CalFitter: a web server for analysis of protein thermal denaturation data. Nucleic Acids Res. 46, W344–W349. https://doi.org/10.1093/nar/gky358.

Mazurenko, S., Prokop, Z., Damborsky, J., 2020. Machine learning in enzyme engineering. ACS Catal. 10, 1210–1223. https://doi.org/10.1021/acscatal.9b04321.

McIntyre, D., Lashkaripour, A., Fordyce, P., Densmore, D., 2022. Machine learning for microfluidic design and control. Lab Chip 22, 2925–2937. https://doi.org/10.1039/D2LC00254J.

Meller, K., Szumski, M., Buszewski, B., 2017. Microfluidic reactors with immobilized enzymes—characterization, dividing, perspectives. Sensors Actuators B Chem. 244, 84–106. https://doi.org/10.1016/j.snb.2016.12.021.

Miller, E.M., Wheeler, A.R., 2008. A digital microfluidic approach to homogeneous enzyme assays. Anal. Chem. 80, 1614–1619. https://doi.org/10.1021/ac702269d.

Miralles, V., Huerre, A., Malloggi, F., Jullien, M.-C., 2013. A review of heating and temperature control in microfluidic systems: techniques and applications. Diagnostics 3, 33–67. https://doi.org/10.3390/diagnostics3010033.

Mirete, S., Morgante, V., González-Pastor, J.E., 2016. Functional metagenomics of extreme environments. Curr. Opin. Biotechnol. 38, 143–149. https://doi.org/10.1016/j.copbio.2016.01.017.

Mitić, S., Strampraad, M.J.F., Hagen, W.R., de Vries, S., 2017. Microsecond time-scale kinetics of transient biochemical reactions. PLoS One 12, e0185888. https://doi.org/10.1371/journal.pone.0185888.

Miton, C.M., Buda, K., Tokuriki, N., 2021. Epistasis and intramolecular networks in protein evolution. Curr. Opin. Struct. Biol. 69, 160–168. https://doi.org/10.1016/j.sbi.2021.04.007.

Mokhtari, D.A., Appel, M.J., Fordyce, P.M., Herschlag, D., 2021. High throughput and quantitative enzymology in the genomic era. Curr. Opin. Struct. Biol. 71, 259–273. https://doi.org/10.1016/j.sbi.2021.07.010.

Monteiro, D.C.F., von Stetten, D., Stohrer, C., Sans, M., Pearson, A.R., Santoni, G., van der Linden, P., Trebbin, M., 2020. 3D-MiXD: 3D-printed X-ray-compatible microfluidic devices for rapid, low-consumption serial synchrotron crystallography data collection in flow. IUCrJ 7, 207–219. https://doi.org/10.1107/S2052252519016865.

Morato, N.M., Holden, D.T., Cooks, R.G., 2020. High-throughput label-free enzymatic assays using desorption electrospray-ionization mass spectrometry. Angew. Chem. Int. Ed. 59, 20459–20464. https://doi.org/10.1002/anie.202009598.

Morato, N.M., Le, M.T., Holden, D.T., Graham Cooks, R., 2021. Automated high-throughput system combining small-scale synthesis with bioassays and reaction screening. SLAS Technol. 26, 555–571. https://doi.org/10.1177/24726303211047839.

Mou, Z., Eakes, J., Cooper, C.J., Foster, C.M., Standaert, R.F., Podar, M., Doktycz, M.J., Parks, J.M., 2021. Machine learning-based prediction of enzyme substrate scope: application to bacterial nitrilases. Proteins-Struct. Funct. Bioinforma. 89, 336–347. https://doi.org/10.1002/prot.26019.

Mross, S., Pierrat, S., Zimmermann, T., Kraft, M., 2015. Microfluidic enzymatic biosensing systems: a review. Biosens. Bioelectron. 70, 376–391. https://doi.org/10.1016/j.bios.2015.03.049.

Mukhametzyanov, T.A., Sedov, I.A., Solomonov, B.N., Schick, C., 2018. Fast scanning calorimetry of lysozyme unfolding at scanning rates from 5 K/min to 500,000 K/min. Biochim. Biophys. Acta Gen. Subj. 1862, 2024–2030. https://doi.org/10.1016/j.bbagen.2018.06.019.

Mukhametzyanov, T.A., Fatkhutdinova, A.A., Schick, C., 2022. Step-scan differential calorimetry of protein denaturation: modeling and experiment. Thermochim. Acta 710, 179181. https://doi.org/10.1016/j.tca.2022.179181.

Nadar, S.S., Patil, P.D., Tiwari, M.S., Ahirrao, D.J., 2021. Enzyme embedded microfluidic paper-based analytic device (μPAD): a comprehensive review. Crit. Rev. Biotechnol. 41, 1046–1080. https://doi.org/10.1080/07388551.2021.1898327.

Neil, S.R.T., Rushworth, C.M., Vallance, C., Mackenzie, S.R., 2011. Broadband cavity-enhanced absorption spectroscopy for real time, in situ spectral analysis of microfluidic droplets. Lab Chip 11, 3953–3955. https://doi.org/10.1039/C1LC20854C.

Neun, S., Kaminski, T.S., Hollfelder, F., 2019. Chapter five - single-cell activity screening in microfluidic droplets. In: Allbritton, N.L., Kovarik, M.L. (Eds.), Methods in Enzymology, Enzyme Activity in Single Cells. Academic Press, pp. 95–112. https://doi.org/10.1016/bs.mie.2019.07.009.

Neun, S., Zurek, P.J., Kaminski, T.S., Hollfelder, F., 2020. Chapter thirteen - ultrahigh throughput screening for enzyme function in droplets. In: Tawfik, D.S. (Ed.), Methods in Enzymology, Enzyme Engineering and Evolution: General Methods. Academic Press, pp. 317–343. https://doi.org/10.1016/bs.mie.2020.06.002.

Neun, S., van Vliet, L., Hollfelder, F., Gielen, F., 2022. High-throughput steady-state enzyme kinetics measured in a parallel droplet generation and absorbance detection platform. Anal. Chem. 94, 16701–16710. https://doi.org/10.1021/acs.analchem.2c03164.

Ng, J.D., Clark, P.J., Stevens, R.C., Kuhn, P., 2008. In situ X-ray analysis of protein crystals in low-birefringent and X-ray transmissive plastic microchannels. Acta Crystallogr. D Biol. Crystallogr. 64, 189–197. https://doi.org/10.1107/S0907444907060064.

Ngara, T.R., Zhang, H., 2018. Recent advances in function-based metagenomic screening. Genomics Proteomics Bioinforma. https://doi.org/10.1016/j.gpb.2018.01.002.

Nightingale, A.M., Hassan, S., Makris, K., Bhuiyan, W.T., Harvey, T.J., Niu, X., 2020. Easily fabricated monolithic fluoropolymer chips for sensitive long-term absorbance measurement in droplet microfluidics. RSC Adv. 10, 30975–30981. https://doi.org/10.1039/D0RA05330A.

Nikoomanzar, A., Vallejo, D., Chaput, J.C., 2019. Elucidating the determinants of polymerase specificity by microfluidic-based deep mutational scanning. ACS Synth. Biol. 8, 1421–1429. https://doi.org/10.1021/acssynbio.9b00104.

Noji, H., Minagawa, Y., Ueno, H., 2022. Enzyme-based digital bioassay technology – key strategies and future perspectives. Lab Chip 22, 3092–3109. https://doi.org/10.1039/D2LC00223J.

Olmos, J.L., Pandey, S., Martin-Garcia, J.M., Calvey, G., Katz, A., Knoska, J., Kupitz, C., Hunter, M.S., Liang, M., Oberthuer, D., Yefanov, O., Wiedorn, M., Heyman, M., Holl, M., Pande, K., Barty, A., Miller, M.D., Stern, S., Roy-Chowdhury, S., Coe, J., Nagaratnam, N., Zook, J., Verburgt, J., Norwood, T., Poudyal, I., Xu, D., Koglin, J., Seaberg, M.H., Zhao, Y., Bajt, S., Grant, T., Mariani, V., Nelson, G., Subramanian, G., Bae, E., Fromme, R., Fung, R., Schwander, P., Frank, M., White, T.A., Weierstall, U., Zatsepin, N., Spence, J., Fromme, P., Chapman, H.N., Pollack, L., Tremblay, L., Ourmazd, A., Phillips, G.N., Schmidt, M., 2018. Enzyme intermediates captured "on the fly" by mix-and-inject serial crystallography. BMC Biol. 16, 59. https://doi.org/10.1186/s12915-018-0524-5.

Ostafe, R., Fontaine, N., Frank, D., Chong, M.N.F., Prodanovic, R., Pandjaitan, R., Offman, B., Cadet, F., Fischer, R., 2020. One-shot optimization of multiple enzyme parameters: tailoring glucose oxidase for pH and electron mediators. Biotechnol. Bioeng. 117, 17–29. https://doi.org/10.1002/bit.27169.

Pham, N., Radajewski, D., Round, A., Brennich, M., Pernot, P., Biscans, B., Bonneté, F., Teychené, S., 2017. Coupling high throughput microfluidics and small-angle X-ray scattering to study protein crystallization from solution. Anal. Chem. 89, 2282–2287. https://doi.org/10.1021/acs.analchem.6b03492.

Polinkovsky, M.E., Gambin, Y., Banerjee, P.R., Erickstad, M.J., Groisman, A., Deniz, A.A., 2014. Ultrafast cooling reveals microsecond-scale biomolecular dynamics. Nat. Commun. 5, 5737. https://doi.org/10.1038/ncomms6737.

Potapov, V., Cohen, M., Schreiber, G., 2009. Assessing computational methods for predicting protein stability upon mutation: good on average but not in the details. Protein Eng. Des. Sel. 22, 553–560. https://doi.org/10.1093/protein/gzp030.

Probst, J., Howes, P., Arosio, P., Stavrakis, S., deMello, A., 2021. Broad-band spectrum, high-sensitivity absorbance spectroscopy in picoliter volumes. Anal. Chem. 93, 7673–7681. https://doi.org/10.1021/acs.analchem.1c00587.

Pucci, F., Kwasigroch, J.M., Rooman, M., 2017. SCooP: an accurate and fast predictor of protein stability curves as a function of temperature. Bioinformatics 33, 3415–3422. https://doi.org/10.1093/bioinformatics/btx417.

Quince, C., Walker, A.W., Simpson, J.T., Loman, N.J., Segata, N., 2017. Shotgun metagenomics, from sampling to analysis. Nat. Biotechnol. 35, 833–844. https://doi.org/10.1038/nbt.3935.

Rabe, P., Kamps, J.J.A.G., Sutherlin, K.D., Linyard, J.D.S., Aller, P., Pham, C.C., Makita, H., Clifton, I., McDonough, M.A., Leissing, T.M., Shutin, D., Lang, P.A., Butryn, A., Brem, J., Gul, S., Fuller, F.D., Kim, I.-S., Cheah, M.H., Fransson, T., Bhowmick, A., Young, I.D., O'Riordan, L., Brewster, A.S., Pettinati, I., Doyle, M., Joti, Y., Owada, S., Tono, K., Batyuk, A., Hunter, M.S., Alonso-Mori, R., Bergmann, U., Owen, R.L., Sauter, N.K., Claridge, T.D.W., Robinson, C.V., Yachandra, V.K., Yano, J., Kern, J.F., Orville, A.M., Schofield, C.J., 2021. X-ray free-electron laser studies reveal correlated motion during isopenicillin N synthase catalysis. Sci. Adv. 7 https://doi.org/10.1126/sciadv.abh0250 eabh0250.

Rachinskiy, K., Schultze, H., Boy, M., Bornscheuer, U., Büchs, J., 2009. "Enzyme Test Bench," a high-throughput enzyme characterization technique including the long-term stability. Biotechnol. Bioeng. 103, 305–322. https://doi.org/10.1002/bit.22242.

Raj, A., Halder, R., Sajeesh, P., Sen, A.K., 2016. Droplet generation in a microchannel with a controllable deformable wall. Microfluid. Nanofluid. 20, 102. https://doi.org/10.1007/s10404-016-1768-4.

Rane, T.D., Puleo, C.M., Liu, K.J., Zhang, Y., Lee, A.P., Wang, T.H., 2010. Counting single molecules in sub-nanolitre droplets. Lab Chip 10, 161–164. https://doi.org/10.1039/b917503b.

Rao, R., Bhattacharya, N., Thomas, N., Duan, Y., Chen, X., Canny, J., Abbeel, P., Song, Y. S., 2019. Evaluating protein transfer learning with TAPE. Adv. Neural Inf. Proces. Syst. 32, 9689–9701.

Rappoport, D., Jinich, A., 2022. Protein Function Prediction from Three-Dimensional Feature Representations Using Space-Filling Curves. https://doi.org/10.1101/2022.06.14.496158.

Reddish, M.J., Callender, R., Dyer, R.B., 2017. Resolution of submillisecond kinetics of multiple reaction pathways for lactate dehydrogenase. Biophys. J. 112, 1852–1862. https://doi.org/10.1016/j.bpj.2017.03.031.

Reetz, M.T., 2013. Biocatalysis in organic chemistry and biotechnology: past, present, and future. J. Am. Chem. Soc. 135, 12480–12496. https://doi.org/10.1021/ja405051f.

Rembeza, E., Engqvist, M.K.M., 2021. Experimental and computational investigation of enzyme functional annotations uncovers misannotation in the EC 1.1.3.15 enzyme class. PLoS Comput. Biol. 17, e1009446 https://doi.org/10.1371/journal.pcbi.1009446.

Rissin, D.M., Kan, C.W., Campbell, T.G., Howes, S.C., Fournier, D.R., Song, L., Piech, T., Patel, P.P., Chang, L., Rivnak, A.J., Ferrell, E.P., Randall, J.D., Provuncher, G.K., Walt, D.R., Duffy, D.C., 2010. Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations. Nat. Biotechnol. 28, 595–599. https://doi.org/10.1038/nbt.1641.

Robinson, S.L., Smith, M.D., Richman, J.E., Aukema, K.G., Wackett, L.P., 2020. Machine learning-based prediction of activity and substrate specificity for OleA enzymes in the thiolase superfamily. Synth. Biol. 5, ysaa004. https://doi.org/10.1093/synbio/ysaa004.

Rodríguez-Ruiz, I., Radajewski, D., Charton, S., Phamvan, N., Brennich, M., Pernot, P., Bonneté, F., Teychené, S., 2017. Innovative high-throughput SAXS methodologies based on photonic lab-on-a-chip sensors: application to macromolecular studies. Sensors 17, 1266. https://doi.org/10.3390/s17061266.

Romero, P.A., Krause, A., Arnold, F.H., 2013. Navigating the protein fitness landscape with Gaussian processes. Proc. Natl. Acad. Sci. U. S. A. 110, E193–E201. https://doi.org/10.1073/pnas.1215251110.

Romero, P.A., Tran, T.M., Abate, A.R., 2015. Dissecting enzyme function with microfluidic-based deep mutational scanning. Proc. Natl. Acad. Sci. 112, 7159–7164. https://doi.org/10.1073/pnas.1422285112.

Chapter 7 Enzyme characterization. In: Roodyn, D.B. (Ed.), 1970. Laboratory Techniques in Biochemistry and Molecular Biology, Automated Enzyme Assays. Elsevier, pp. 102–113. https://doi.org/10.1016/S0075-7535(08)70515-1.

Roychowdhury, H., Romero, P.A., 2022. Microfluidic deep mutational scanning of the human executioner caspases reveals differences in structure and regulation. Cell Death Dis. 8, 1–8. https://doi.org/10.1038/s41420-021-00799-0.

Ryu, J.Y., Kim, H.U., Lee, S.Y., 2019. Deep learning enables high-quality and high-throughput prediction of enzyme commission numbers. Proc. Natl. Acad. Sci. 116, 13996–14001. https://doi.org/10.1073/pnas.1821905116.

Salmon, A.R., Esteban, R., Taylor, R.W., Hugall, J.T., Smith, C.A., Whyte, G., Scherman, O.A., Aizpurua, J., Abell, C., Baumberg, J.J., 2016. Monitoring early-stage nanoparticle assembly in microdroplets by optical spectroscopy and SERS. Small 12, 1788–1796. https://doi.org/10.1002/smll.201503513.

Sawaguchi, Y., Yamazaki, R., Nishiyama, Y., Mae, M., Abe, A., Nishiyama, H., Nishisaka, F., Ibuki, T., Sasai, T., Matsuzaki, T., 2021. Novel Pan-Pim kinase inhibitors with imidazopyridazine and thiazolidinedione structure exert potent antitumor activities. Front. Pharmacol. 12.

Schenkmayerova, A., Pinto, G.P., Toul, M., Marek, M., Hernychova, L., Planas-Iglesias, J., Daniel Liskova, V., Pluskal, D., Vasina, M., Emond, S., Dörr, M., Chaloupkova, R., Bednar, D., Prokop, Z., Hollfelder, F., Bornscheuer, U.T., Damborsky, J., 2021. Engineering the protein dynamics of an ancestral luciferase. Nat. Commun. 12, 3616. https://doi.org/10.1038/s41467-021-23450-z.

Schewa, S., Schroer, M.A., Zickmantel, T., Song, Y.-H., Blanchet, C.E., Gruzinov, A.Yu., Katona, G., Svergun, D.I., Roessle, M., 2020. A THz transparent 3D printed microfluidic cell for small angle x-ray scattering. Rev. Sci. Instrum. 91, 084101 https://doi.org/10.1063/5.0004706.

Schmidli, C., Albiez, S., Rima, L., Righetto, R., Mohammed, I., Oliva, P., Kovacik, L., Stahlberg, H., Braun, T., 2019. Microfluidic protein isolation and sample preparation for high-resolution cryo-EM. Proc. Natl. Acad. Sci. 116, 15007–15012. https://doi.org/10.1073/pnas.1907214116.

Schmitz, C.H.J., Rowat, A.C., Köster, S., Weitz, D.A., 2009. Dropspots: a picoliter array in a microfluidic device. Lab Chip 9, 44–49. https://doi.org/10.1039/B809670H.

Schroer, M.A., Schewa, S., Gruzinov, A.Y., Rönnau, C., Lahey-Rudolph, J.M., Blanchet, C. E., Zickmantel, T., Song, Y.-H., Svergun, D.I., Roessle, M., 2021. Probing the existence of non-thermal terahertz radiation induced changes of the protein solution structure. Sci. Rep. 11, 22311. https://doi.org/10.1038/s41598-021-01774-6.

Schwemmer, F., Blanchet, C.E., Spilotros, A., Kosse, D., Zehnle, S., Mertens, H.D.T., Graewert, M.A., Rössle, M., Paust, N., Svergun, D.I., von Stetten, F., Zengerle, R., Mark, D., 2016. LabDisk for SAXS: a centrifugal microfluidic sample preparation platform for small-angle X-ray scattering. Lab Chip 16, 1161–1170. https://doi.org/10.1039/C5LC01580D.

Sesen, M., Alan, T., Neild, A., 2017. Droplet control technologies for microfluidic high throughput screening (μHTS). Lab Chip 17, 2372–2394. https://doi.org/10.1039/c7lc00005g.

Shahraki, M.F., Ariaeenejad, S., Atanaki, F.F., Zolfaghari, B., Koshiba, T., Kavousi, K., Salekdeh, G.H., 2020. MCIC: automated identification of cellulases from metagenomic data and characterization based on temperature and pH dependence. Front. Microbiol. 11, 567863 https://doi.org/10.3389/fmicb.2020.567863.

Shahraki, M.F., Farhadyar, K., Kavousi, K., Azarabad, M.H., Boroomand, A., Ariaeenejad, S., Salekdeh, G.H., 2021. A generalized machine-learning aided method for targeted identification of industrial enzymes from metagenome: a xylanase temperature dependence case study. Biotechnol. Bioeng. 118, 759–769. https://doi.org/10.1002/bit.27608.

Shahraki, M.F., Atanaki, F.F., Ariaeenejad, S., Ghaffari, M.R., Norouzi-Beirami, M.H., Maleki, M., Salekdeh, G.H., Kavousi, K., 2022. A computational learning paradigm to targeted discovery of biocatalysts from metagenomic data: a case study of lipase identification. Biotechnol. Bioeng. 119, 1115–1128. https://doi.org/10.1002/bit.28037.

Shang, L., Cheng, Y., Zhao, Y., 2017. Emerging droplet microfluidics. Chem. Rev. 117, 7964–8040. https://doi.org/10.1021/acs.chemrev.6b00848.

Shao, F., Lee, P.-W., Li, H., Hsieh, K., Wang, T.-H., 2022. Emerging platforms for high-throughput enzymatic bioassays. Trends Biotechnol. https://doi.org/10.1016/j.tibtech.2022.06.006.

Shi, Y., Ye, P., Yang, K., Meng, J., Guo, Jiuchuan, Pan, Z., Zhao, W., Guo, Jinhong, 2021. Application of centrifugal microfluidics in immunoassay, biochemical analysis and molecular diagnosis. Analyst 146, 5800–5821. https://doi.org/10.1039/D1AN00629K.

Siedhoff, N.E., Illig, A.-M., Schwaneberg, U., Davari, M.D., 2021. PyPEF—an integrated framework for data-driven protein engineering. J. Chem. Inf. Model. 61, 3463–3476. https://doi.org/10.1021/acs.jcim.1c00099.

Silva, B.F.B., 2017. SAXS on a chip: from dynamics of phase transitions to alignment phenomena at interfaces studied with microfluidic devices. Phys. Chem. Chem. Phys. 19, 23690–23703. https://doi.org/10.1039/C7CP02736B.

Simon, C., Daniel, R., 2011. Metagenomic analyses: past and future trends. Appl. Environ. Microbiol. 77, 1153–1161. https://doi.org/10.1128/AEM.02345-10.

Sleator, R.D., Shortall, C., Hill, C., 2008. Metagenomics. Lett. Appl. Microbiol. 47, 361–366. https://doi.org/10.1111/j.1472-765X.2008.02444.x.

Song, H., Ismagilov, R.F., 2003. Millisecond kinetics on a microfluidic chip using nanoliters of reagents. J. Am. Chem. Soc. 125, 14613–14619. https://doi.org/10.1021/ja0354566.

Srikanth, S., Dubey, S.K., Javed, A., Goel, S., 2021. Droplet based microfluidics integrated with machine learning. Sensors Actuators A Phys. 332, 113096 https://doi.org/10.1016/j.sna.2021.113096.

Srinivas, V., Banerjee, R., Lebrette, H., Jones, J.C., Aurelius, O., Kim, I.-S., Pham, C.C., Gul, S., Sutherlin, K.D., Bhowmick, A., John, J., Bozkurt, E., Fransson, T., Aller, P., Butryn, A., Bogacz, I., Simon, P., Keable, S., Britz, A., Tono, K., Kim, K.S., Park, S.-Y., Lee, S.J., Park, J., Alonso-Mori, R., Fuller, F.D., Batyuk, A., Brewster, A.S., Bergmann, U., Sauter, N.K., Orville, A.M., Yachandra, V.K., Yano, J., Lipscomb, J.D., Kern, J., Högbom, M., 2020. High-resolution XFEL structure of the soluble methane monooxygenase hydroxylase complex with its regulatory component at ambient temperature in two oxidation states. J. Am. Chem. Soc. 142, 14249–14266. https://doi.org/10.1021/jacs.0c05613.

Srisa-Art, M., Dyson, E.C., deMello, A.J., Edel, J.B., 2008. Monitoring of real-time streptavidin−biotin binding kinetics using droplet microfluidics. Anal. Chem. 80, 7063–7067. https://doi.org/10.1021/ac801199k.

Srisa-Art, M., deMello, A.J., Edel, J.B., 2010. High-efficiency single-molecule detection within trapped aqueous microdroplets. J. Phys. Chem. B 114, 15766–15772. https://doi.org/10.1021/jp105749t.

Srour, B., Strampraad, M.J.F., Hagen, W.R., Hagedoorn, P.-L., 2018. Refolding kinetics of cytochrome c studied with microsecond timescale continuous-flow UV–vis spectroscopy and rapid freeze-quench EPR. J. Inorg. Biochem. 184, 42–49. https://doi.org/10.1016/j.jinorgbio.2018.04.011.

Stemmer, W.P.C., 1994. Rapid evolution of a protein in vitro by DNA shuffling. Nature 370, 389. https://doi.org/10.1038/370389a0.

Stohrer, C., Horrell, S., Meier, S., Sans, M., von Stetten, D., Hough, M., Goldman, A., Monteiro, D.C.F., Pearson, A.R., 2021. Homogeneous batch micro-crystallization of proteins from ammonium sulfate. Acta Crystallogr. Sect. Struct. Biol. 77, 194–204. https://doi.org/10.1107/S2059798320015454.

Suea-Ngam, A., Howes, P.D., Srisa-Art, M., deMello, A.J., 2019. Droplet microfluidics: from proof-of-concept to real-world utility? Chem. Commun. 55, 9895–9903. https://doi.org/10.1039/C9CC04750F.

Sui, S., Perry, S.L., 2017. Microfluidics: from crystallization to serial time-resolved crystallography. Struct. Dyn. 4, 032202 https://doi.org/10.1063/1.4979640.

Taujale, R., Venkat, A., Huang, L.-C., Zhou, Z., Yeung, W., Rasheed, K.M., Li, S., Edison, A.S., Moremen, K.W., Kannan, N., 2020. Deep evolutionary analysis reveals the design principles of fold a glycosyltransferases. Elife 9, e54532. https://doi.org/10.7554/eLife.54532.

Tawfik, D.S., Griffiths, A.D., 1998. Man-made cell-like compartments for molecular evolution. Nat. Biotechnol. 16, 652–656. https://doi.org/10.1038/nbt0798-652.

Tesauro, C., Frøhlich, R., Stougaard, M., Ho, Y.-P., Knudsen, B.R., 2015. Microfluidics-enabled enzyme activity measurement in single cells. In: Singh, A.K., Chandrasekaran, A. (Eds.), Single Cell Protein Analysis: Methods and Protocols, Methods in Molecular Biology. Springer, New York, NY, pp. 209–219. https://doi.org/10.1007/978-1-4939-2987-0_14.

Thorsen, T., Maerkl, S.J., Quake, S.R., 2002. Microfluidic large-scale integration. Science 298, 580–584. https://doi.org/10.1126/science.1076996.

Uchiyama, T., Miyazaki, K., 2009. Functional metagenomics for enzyme discovery: challenges to efficient screening. Curr. Opin. Biotechnol. 20, 616–622. https://doi.org/10.1016/j.copbio.2009.09.010.

Ufarté, L., Potocki-Veronese, G., Laville, É., 2015. Discovery of new protein families and functions: new challenges in functional metagenomics for biotechnologies and microbial ecology. Front. Microbiol. 6 https://doi.org/10.3389/fmicb.2015.00563.

Unger, M.A., Chou, H.P., Thorsen, T., Scherer, A., Quake, S.R., 2000. Monolithic microfabricated valves and pumps by multilayer soft lithography. Science 288, 113–116. https://doi.org/10.1126/science.288.5463.113.

Urbanski, J.P., Thies, W., Rhodes, C., Amarasinghe, S., Thorsen, T., 2006. Digital microfluidics using soft lithography. Lab Chip 6, 96–104. https://doi.org/10.1039/B510127A.

van Dijk, E.L., Auger, H., Jaszczyszyn, Y., Thermes, C., 2014. Ten years of next-generation sequencing technology. Trends Genet. 30, 418–426. https://doi.org/10.1016/j.tig.2014.07.001.

Vanacek, P., Sebestova, E., Babkova, P., Bidmanova, S., Daniel, L., Dvorak, P., Stepankova, V., Chaloupkova, R., Brezovsky, J., Prokop, Z., Damborsky, J., 2018. Exploration of enzyme diversity by integrating bioinformatics with expression analysis and biochemical characterization. ACS Catal. 8, 2402–2412. https://doi.org/10.1021/acscatal.7b03523.

Vasina, M., Vanacek, P., Damborsky, J., Prokop, Z., 2020. Chapter three - exploration of enzyme diversity: High-throughput techniques for protein production and microscale biochemical characterization. In: Tawfik, D.S. (Ed.), Methods in Enzymology, Enzyme Engineering and Evolution: General Methods. Academic Press, pp. 51–85. https://doi.org/10.1016/bs.mie.2020.05.004.

Vasina, M., Vanacek, P., Hon, J., Kovar, D., Faldynova, H., Kunka, A., Buryska, T., Badenhorst, C.P.S., Mazurenko, S., Bednar, D., Stavrakis, S., Bornscheuer, U.T., deMello, A., Damborsky, J., Prokop, Z., 2022a. Advanced database mining of efficient haloalkane dehalogenases by sequence and structure bioinformatics and microfluidics. Chem. Catal. 2, 2704–2725. https://doi.org/10.1016/j.checat.2022.09.011.

Vasina, M., Velecký, J., Planas-Iglesias, J., Marques, S.M., Skarupova, J., Damborsky, J., Bednar, D., Mazurenko, S., Prokop, Z., 2022b. Tools for computational design and high-throughput screening of therapeutic enzymes. Adv. Drug Deliv. Rev. 183, 114143 https://doi.org/10.1016/j.addr.2022.114143.

Vollmar, M., Evans, G., 2021. Machine learning applications in macromolecular X-ray crystallography. Crystallogr. Rev. 27, 54–101. https://doi.org/10.1080/0889311X.2021.1982914.

Wahler, D., Reymond, J.L., 2001. Novel methods for biocatalyst screening. Curr. Opin. Chem. Biol. 5, 152–158. https://doi.org/10.1016/S1367-5931(00)00184-8.

Walsh, I., Fishman, D., Garcia-Gasulla, D., Titma, T., Pollastri, G., Harrow, J., Psomopoulos, F.E., Tosatto, S.C.E., 2021. DOME: recommendations for supervised machine learning validation in biology. Nat. Methods 18, 1122–1127. https://doi.org/10.1038/s41592-021-01205-4.

Wan, Z., Wang, Q.-D., Liu, D., Liang, J., 2021. Accelerating the optimization of enzyme-catalyzed synthesis conditions via machine learning and reactivity descriptors. Org. Biomol. Chem. 19, 6267–6273. https://doi.org/10.1039/d1ob01066b.

Wang, J.-W., Gao, J., Wang, H.-F., Jin, Q.-H., Rao, B., Deng, W., Cao, Y., Lei, M., Ye, S., Fang, Q., 2019. Miniaturization of the whole process of protein crystallographic analysis by a microfluidic droplet robot: from nanoliter-scale purified proteins to diffraction-quality crystals. Anal. Chem. 91, 10132–10140. https://doi.org/10.1021/acs.analchem.9b02138.

Wang, X., Ogata, A.F., Walt, D.R., 2020. Ultrasensitive detection of enzymatic activity using single molecule arrays. J. Am. Chem. Soc. 142, 15098–15106. https://doi.org/10.1021/jacs.0c06599.

Watkin, S.A.J., Ryan, T.M., Miller, A.G., M. Nock, V., Pearce, F.G., Dobson, R.C.J., 2017. Microfluidics for Small-Angle X-Ray Scattering, X-Ray Scattering. IntechOpen. https://doi.org/10.5772/65678.

Wegner, C.H., Zimmermann, I., Hubbuch, J., 2022. Rapid analysis for multicomponent high-throughput crystallization screening: combination of UV–vis spectroscopy and chemometrics. Cryst. Growth Des. 22, 1054–1065. https://doi.org/10.1021/acs.cgd.1c00907.

Wu, H.-W., Huang, Y.-C., Wu, C.-L., Lee, G.-B., 2009. Exploitation of a microfluidic device capable of generating size-tunable droplets for gene delivery. Microfluid. Nanofluid. 7, 45–56. https://doi.org/10.1007/s10404-008-0359-4.

Wu, Z., Kan, S.B.J., Lewis, R.D., Wittmann, B.J., Arnold, F.H., 2019. Machine learning-assisted directed protein evolution with combinatorial libraries. Proc. Natl. Acad. Sci. 116, 8852–8858. https://doi.org/10.1073/pnas.1901979116.

Xia, Younan, Whitesides, George M., 1998. Soft lithography. Annu. Rev. Mater. Sci. 28, 153–184. https://doi.org/10.1146/annurev.matsci.28.1.153.

Xiang, L., Kaspar, F., Schallmey, A., Constantinou, I., 2021. Two-phase biocatalysis in microfluidic droplets. Biosensors 11, 407. https://doi.org/10.3390/bios11110407.

Xie, W.J., Asadi, M., Warshel, A., 2022. Enhancing computational enzyme design by a maximum entropy strategy. Proc. Natl. Acad. Sci. U. S. A. 119, e2122355119 https://doi.org/10.1073/pnas.2122355119.

Xu, Zhijue, Li, X., Zhou, S., Xie, W., Wang, J., Cheng, L., Wang, S., Guo, S., Xu, Zhaowei, Cao, X., Zhang, M., Yu, B., Narimatsu, H., Tao, S., Zhang, Y., 2017. Systematic identification of the protein substrates of UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase-T1/T2/T3 using a human proteome microarray. PROTEOMICS 17, 1600485. https://doi.org/10.1002/pmic.201600485.

Xu, Z., Wu, J., Song, Y.S., Mahadevan, R., 2022. Enzyme activity prediction of sequence variants on novel substrates using improved substrate encodings and convolutional pooling. In: Proceedings of the 16th Machine Learning in Computational Biology Meeting. Presented at the Machine Learning in Computational Biology, PMLR, pp. 78–87.

Yan, B., Ran, X., Gollu, A., Cheng, Z., Zhou, X., Chen, Y., Yang, Z., 2022. IntEnzyDB: An Integrated Structure-Kinetics Enzymology Database. https://doi.org/10.26434/chemrxiv-2022-k1n52.

Yang, G., Ding, Y., 2014. Recent advances in biocatalyst discovery, development and applications. Bioorg. Med. Chem. 22, 5604–5612. https://doi.org/10.1016/j.bmc.2014.06.033.

Yang, X., Liu, J., Xie, Y.L., Wang, Y., Ying, H., Wu, Q., Huang, W., Jenkins, G., 2014. A novel microfluidic system for the rapid analysis of protein thermal stability. Analyst 139, 2683–2686. https://doi.org/10.1039/C4AN00173G.

Yang, T., Stavrakis, S., deMello, A., 2017. A high-sensitivity, integrated absorbance and fluorescence detection scheme for probing Picoliter-volume droplets in segmented flows. Anal. Chem. 89, 12880–12887. https://doi.org/10.1021/acs.analchem.7b03526.

Yang, M., Fehl, C., Lees, K.V., Lim, E.-K., Offen, W.A., Davies, G.J., Bowles, D.J., Davidson, M.G., Roberts, S.J., Davis, B.G., 2018. Functional and informatics analysis enables glycosyltransferase activity prediction. Nat. Chem. Biol. 14 https://doi.org/10.1038/s41589-018-0154-9, 1109−+.

Yang, T., Villois, A., Kunka, A., Grigolato, F., Arosio, P., Prokop, Z., deMello, A., Stavrakis, S., 2022. Droplet-based microfluidic temperature-jump platform for the rapid assessment of biomolecular kinetics. Anal. Chem. 94, 16675–16684. https://doi.org/10.1021/acs.analchem.2c03009.

Yeh, A.H.-W., Norn, C., Kipnis, Y., Tischer, D., Pellock, S.J., Evans, D., Ma, P., Lee, G.R., Zhang, J.Z., Anishchenko, I., Coventry, B., Cao, L., Dauparas, J., Halabiya, S., DeWitt, M., Carter, L., Houk, K.N., Baker, D., 2023. De novo design of luciferases using deep learning. Nature 614, 774–780. https://doi.org/10.1038/s41586-023-05696-3.

You, L., Arnold, F.H., 1996. Directed evolution of subtilisin E in Bacillus subtilis to enhance total activity in aqueous dimethylformamide. Protein Eng. Des. Sel. 9, 77–83. https://doi.org/10.1093/protein/9.1.77.

Zaugg, J., Gumulya, Y., Malde, A.K., Boden, M., 2017. Learning epistatic interactions from sequence-activity data to predict enantioselectivity. J. Comput. Aided Mol. Des. 31, 1085–1096. https://doi.org/10.1007/s10822-017-0090-x.

Zeng, S., Li, B., Su, X., Qin, J., Lin, B., 2009. Microvalve-actuated precise control of individual droplets in microfluidic devices. Lab Chip 9, 1340–1343. https://doi.org/10.1039/B821803J.

Zeymer, C., Hilvert, D., 2018. Directed evolution of protein catalysts. Annu. Rev. Biochem. 87, 131–157. https://doi.org/10.1146/annurev-biochem-062917-012034.

Zhang, H., Bai, Y., Zhu, N., Xu, J., 2021a. Microfluidic reactor with immobilized enzyme-from construction to applications: a review. Chin. J. Chem. Eng. 30, 136–145. https://doi.org/10.1016/j.cjche.2020.12.011.

Zhang, Hui, Liu, C., Hua, W., Ghislain, L.P., Liu, J., Aschenbrenner, L., Noell, S., Dirico, K.J., Lanyon, L.F., Steppan, C.M., West, M., Arnold, D.W., Covey, T.R., Datwani, S.S., Troutman, M.D., 2021b. Acoustic ejection mass spectrometry for high-throughput analysis. Anal. Chem. 93, 10850–10861. https://doi.org/10.1021/acs.analchem.1c01137.

Zhang, H., Yang, Y., Zhang, C., Farid, S.S., Dalby, P.A., 2021c. Machine learning reveals hidden stability code in protein native fluorescence. Comput. Struct. Biotechnol. J. 19, 2750–2760. https://doi.org/10.1016/j.csbj.2021.04.047.

Zheng, J., Cole, T., Zhang, Y., Kim, J., Tang, S.-Y., 2021. Exploiting machine learning for bestowing intelligence to microfluidics. Biosens. Bioelectron. 194, 113666 https://doi.org/10.1016/j.bios.2021.113666.

Zhu, Y., Fang, Q., 2013. Analytical detection techniques for droplet microfluidics—a review. Anal. Chim. Acta 787, 24–35. https://doi.org/10.1016/j.aca.2013.04.064.

Zhu, P., Wang, L., 2016. Passive and active droplet generation with microfluidics: a review. Lab Chip 17, 34–75. https://doi.org/10.1039/C6LC01018K.
Zhu, Y., Zhang, Y.-X., Cai, L.-F., Fang, Q., 2013. Sequential operation droplet array: an automated microfluidic platform for picoliter-scale liquid handling, analysis, and screening. Anal. Chem. 85, 6723–6731. https://doi.org/10.1021/ac4006414.
Zhu, Y., Zhu, L.-N., Guo, R., Cui, H.-J., Ye, S., Fang, Q., 2014. Nanoliter-scale protein crystallization and screening with a microfluidic droplet robot. Sci. Rep. 4, 5046. https://doi.org/10.1038/srep05046.