# Introduction

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An enzyme assay is a test for enzyme function. The enzyme assay probes the chemistry of a single catalytic step in an enzyme and makes it return an answer, which may be a light signal or color change in the sample, or a biological selection event, or both. How to achieve this is left to the experimenter, who can, and usually must, combine various chemical insights and intuitions to arrive at a working assay system. It is a molecular game with plenty of degrees of freedom, but strict demands on efficacy. Ideally, the assay should be simple and free of mistakes – that is, no false positives or false negatives. Success is also rated in terms of which actual reaction is being assayed, some being more difficult than others, and in terms of ease of implementation, which often reduces to the price and availability of the reagents necessary to perform the assay.

Fortunately, the design and utilization of enzyme assays serve a useful purpose. Enzyme assays are indispensable tools for enzyme discovery and enzyme characterization. The present book aims to reflect the tremendous developments that have taken place in these areas over the last 10 years, particularly with regard to high-throughput screening assays and array experiments with multiple substrates. These developments have been discussed in several review articles [1].

The driving force for the invention of new enzyme assays comes in large part from the field of enzyme discovery and engineering [2]. In these areas of investigation enzyme assays are used to identify active enzymes from microorganism collections or randomly generated enzyme mutant libraries. This approach has been found to be very practical for discovering industrially useful catalysts. Enzyme engineering has led to an increased acceptance and utilization of enzymes for manufacturing, in particular in the area of fine chemicals synthesis [3].

## Enzyme Assays

What an enzyme assay does to visualize enzyme function is equivalent to what structural analysis tools do for visualizing enzyme structure. However, while

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one understands structure intuitively through its three-dimensional representation, there is no unified representation of function. Function can be described as a list of qualitative statements, or as a series of values for suitably defined parameters. For small molecules such as drugs, function correlates well with structure, and predictive quantitative structure–activity relationship (QSAR) models allow one to reduce function to structural elements. For macromolecules, however, the relationship between structure and function is blurred by complexity, and structural analysis delivers at best a crude insight into molecular function. This is particularly true for enzymes, where insignificant alterations in either protein or substrate structure can produce dramatic changes at the level of function, whether it is catalytic activity, selectivity or regulation of the enzyme. In this case the description of molecular function becomes largely independent of structure (Figure 1). Experience has shown that the functional information delivered by an enzyme assay on thousands of mutants is much more useful in showing how an enzyme could be improved than a detailed structure of a single enzyme.

While structural determination methods use physical principles, enzyme assays are mostly born out of chemical principles. Enzyme assay technology builds on classical bioorganic chemistry, and starts with a detailed analysis and understanding of an enzyme's reaction mechanism and the chemical properties of substrates and products. Engineering of substrate structure or the use of chemical sensors then allows the catalytic reaction to be translated into an observable signal. The assay design largely depends on intuition to formulate for each enzyme a working principle capable of turning enzymatic turnover into a signal.

In enzyme discovery and engineering the assay is used to select improved enzyme variants from pools of enzymes or enzyme mutants. The assay is critical in these experiments because "you get what you screen for". This adage summarizes the outcome of many experiments: the product of a selection procedure is only as good as the selection principle used. The detailed chemistry of the assay involved is therefore a key parameter for ensuring success in isolating the desired enzyme.

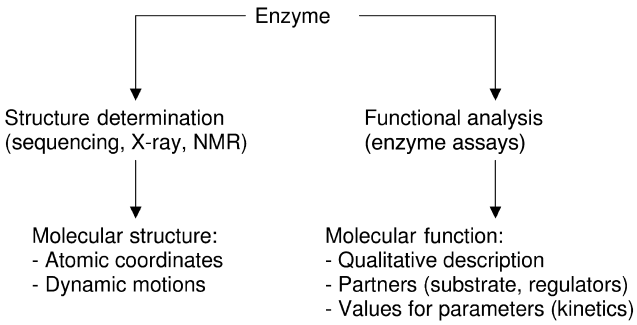


**Fig. 1** Enzyme assays as tools for functional analysis.

The principles and applications of enzyme assays are reviewed in this book. In Part I the chemistry of enzyme assays is discussed, in Part II the assays used in the context of genetic selection are covered, and in Part III multisubstrate assays for biochemical characterization of enzymes are discussed. Before even starting into these applications one should remember that positive hits from high-throughput screening assays must always be confirmed by an independent method before concluding that a new enzyme has been discovered (Figure 2).

There are many ways to connect the conversion of a substrate into a product with an observable signal (Figure 3). Enzyme activity can often be detected by the action of the enzyme on its natural substrate. An enzyme activity might lead to heat production if the reaction is exothermic, or induce a macroscopic change in the reaction medium, such as the clearing of an insoluble polymer substrate, or the precipitation of a reaction product. It is also possible to follow reaction turnover using standard analytical methods such as chromatography (gas chromatography or high-performance liquid chromatography) and mass spectrometry, or by nuclear magnetic resonance (NMR) spectroscopy. Several application examples of such methods, in particular with respect to assays for measuring enantioselectivity, are discussed by Manfred Reetz in Chapter 2 and by Theo Sonke and the DSM group in Chapter 4. Direct high-throughput screening assays for enantioselectivity are particularly important in the context of fine chemical synthesis because enantioselectivity is almost always the property being pursued in the course of developing a new catalyst. Electrochemical monitoring of enzyme activity is typically used for glucose-sensing mediated by glucose oxidase [4], and has recently been applied for the lipase cutinase using a hydroqui-

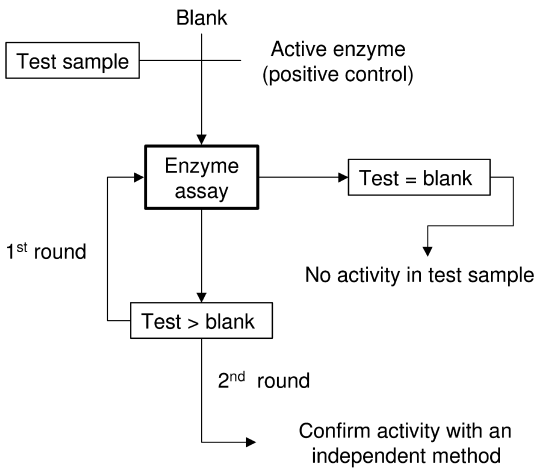


**Fig. 2** Principles of high-throughput screening enzyme assays. The signal produced by the assay on the test sample must be checked against a blank sample (medium only) and against an active enzyme as positive control. A positive identification must be repeated, and then confirmed by an independent method.

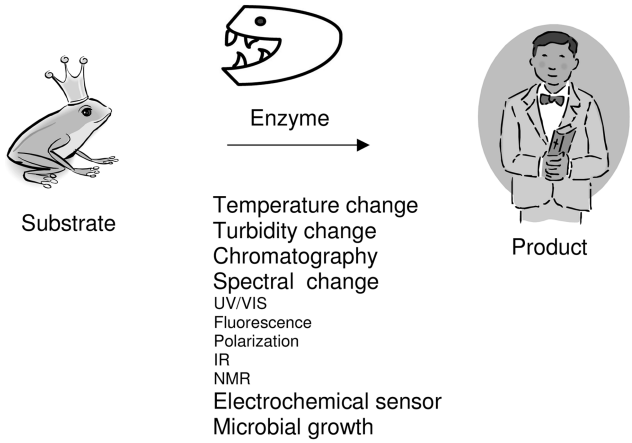


**Fig. 3** Signals for enzyme assays produced from enzymatic reactions. Note that observable spectral changes may occur either directly due to structural differences between substrate and product (e.g. Chapters 2, 4, 5, 9, 11 and 12), or indirectly, for example through an chemical indicator system (e.g. Chapter 1), by processing of the reaction product by secondary enzymes or reagents (e.g. Chapters 3, 6 and 10), or via the induction of gene expression by the reaction product (Chapter 7).

none monobutyrate ester substrate covalently linked to the surface of a gold electrode [5]. Microbial growth as a signal allows one to pick active colonies growing on substrates as carbon source, and also occurs upon genetic selection (Part II).

The largest group of enzyme assays are those that induce recordable changes in light absorbency or fluorescence in the assay medium. The simplest approach relies on colorimetric or fluorimetric chemosensors that respond to product formation or substrate consumption, such as pH indicators (Chapter 1). Such assays are particularly useful because they allow one to work with the substrate of synthetic interest. There are a number of strategies for inducing signals indirectly upon enzymatic turnover, as illustrated by the following examples.

In the copper-calcein assay in Figure 4 [6], an amidase releases a free amino acid as reaction product from the corresponding amide as substrate. Amino acids are strong chelators for metal ions, in particular $Cu^{2+}$ ions, while amino acid amides are not. The assay is based on a complex of $Cu^{2+}$ and the commercially available fluorescein derivative calcein (**3**), in which the calcein fluorophore is quenched by the metal ion. The deacetylation of *N*-acetyl-L-methionine (**1**) by acylase I induces a fluorescence increase because the free amino acid reaction product L-methionine (**2**) chelates $Cu^{2+}$, which releases free calcein, which regains its fluorescence. The copper-calcein assay can also be used to assay aminopeptidases and proteases using as substrate amino acid amides and bovine serum albumin, respectively. Following a similar principle, indirect product detection in a chemical transformation can also be realized by means of an

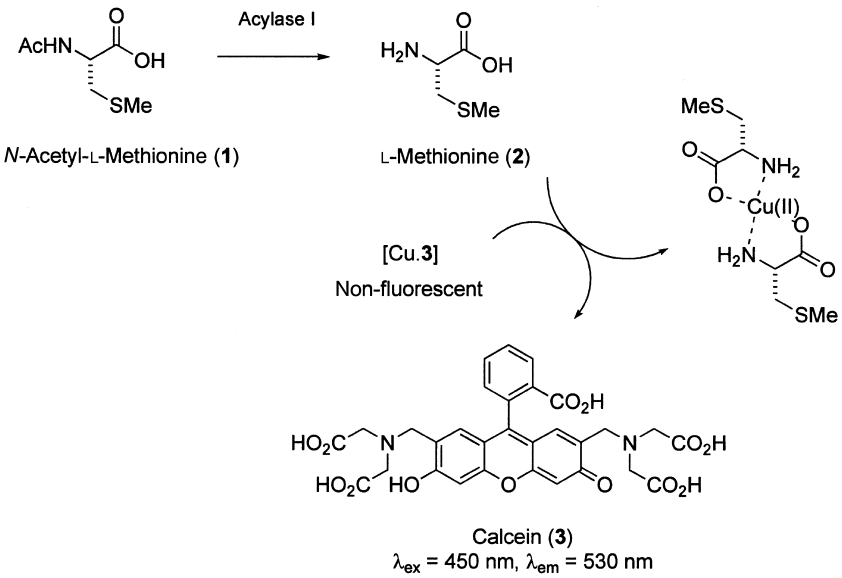


**Fig. 4** An indirect fluorogenic assay for acylase I. Calcein (**3**) is a commercially available inexpensive fluorescein derivative. The assay is also suitable for other amino acid-releasing enzymes, such as aminopeptidases and proteases, when using the appropriate substrate.

immunoassay using an antibody capable of differentiating product from substrate [7]. In these immunoassays the product-selective antibody plays the same role as the $Cu^{2+}$ ion in the amidase assay above.

Another elegant indirect assay by Matile and coworkers is based on vesicles containing a concentrated, autoquenched solution of fluorescein (Figure 5) [8]. The vesicles are equipped with synthetic pores for fluorescein. The pores are plugged by the enzyme substrate, but not by the reaction product. Reaction progress results in unplugging of the pore, which leads to diffusion of fluorescein outside the vesicles and an increase in fluorescence. The assay has been demonstrated for fructose bis-phosphate aldolase, alkaline phosphatase, galactosyltransferase, DNA exonuclease III, DNA polymerase I, RNase A, apyrase, heparinase I, hyaluronidase, papain, ficin, elastase, subtilisin, and pronase.

The most frequently used enzyme assays involve fluorogenic and chromogenic substrates. A synthetic substrate is designed such that the enzyme turns a nonfluorescent or colorless appendage of the substrate into a fluorescent or colored product. Thus, a color or fluorescent signal is created out of a dark or colorless solution by the direct action of the enzyme. This principle is realized by substrates with cleavable ethers or esters of electron-poor conjugated aromatic phenols. The conjugate bases of these phenols show very strong color and fluorescence properties not present in the protonated, alkylated or acylated derivatives. These include the well-known yellow nitrophenolate (**4**), the blue fluores-

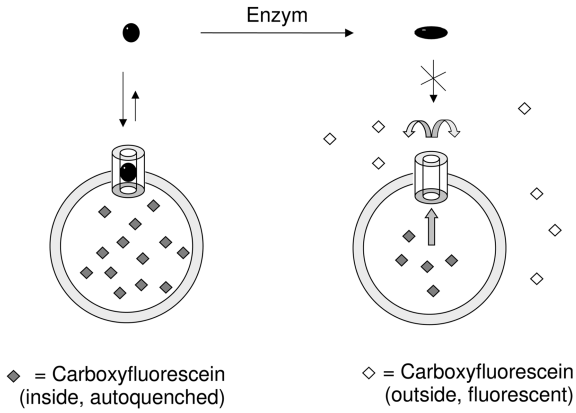


**Fig. 5** A general fluorescence enzyme assay using synthetic pores. Product turnover unplugs the pores, which allow fluorescein to diffuse to the outside of the vesicles and become fluorescent.

cent umbelliferone anion (**5**), the red fluorescent resorufin anion (**6**) and the green fluorescent fluorescein anion (**7**) (Figure 6). Many fluorogenic and chromogenic enzyme substrates are commercially available and serve as reference substrates for hydrolytic enzymes (see Chapter 1).

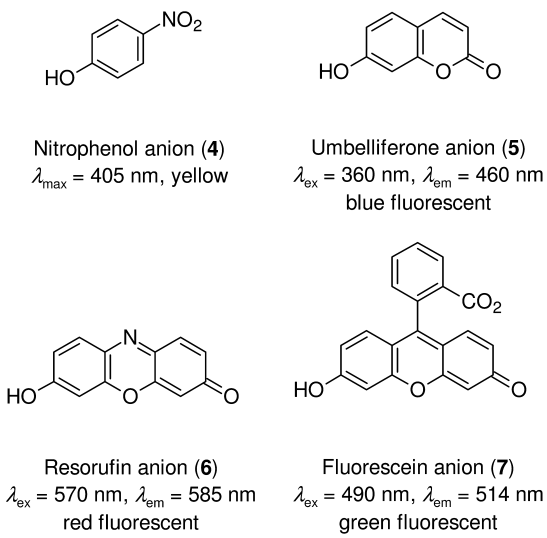


**Fig. 6** Acidic conjugated electron-poor phenols used in fluorogenic and chromogenic enzyme substrates. The corresponding neutral phenols are generally colorless and nonfluorescent.

### Part I: High-throughput Screening

The significance of an enzyme assay and its successful application depends on its chemical and analytical design. Part I discusses enzyme assays tailored to the problem of enzyme discovery, which requires high-throughput screening potential for relevant chemical transformations. In the context of fine chemical synthesis this means the ability to screen for enantio- and stereoselectivity of the targeted reactions.

In Chapter 1, Romas J. Kazlauskas describes the use of a colorimetric pH indicator together with reference fluorogenic substrates to carry out efficient high-throughput screening of esterolytic enzymes with chiral substrates. The method allows stereoselectivity information to be obtained directly from high-throughput screening with any substrate of synthetic interest.

In Chapter 2, Manfred T. Reetz reviews enzyme assays for screening enantioselective reactions. Analysis of isotopically labeled *pseudo*-enantiomeric mixtures by MS and NMR provides a practical approach for screening kinetic resolutions of racemic mixtures or the deracemization of prochiral substrates. For the case of asymmetric induction where a chiral product is formed from an achiral and nonprochiral substrate, the situation is more complex and requires indirect sensing of product chirality by enantioselective sensors.

In Chapter 3, Tyler W. Johannes, Ryan D. Woodyer, and Huimin Zhao review fluorogenic and chromogenic systems for redox enzymes. These assays are critical because redox enzymes have a particularly important and yet largely untapped potential for industrial applications. For example alkane monoxygenases can perform selective hydroxylation reactions on hydrocarbons that are simply not accessible at all to chemical catalysts [9]. In addition many chemical redox reagents are expensive, toxic, and difficult to handle, implying that economical enzyme replacements should be possible in almost all cases.

The best test bed for enzyme assays occurs in an industrial context, where practical catalysts need to be developed rapidly and applied in large-scale production. In Chapter 4, Theo Sonke, Lucien Duchateau, Dick Schipper, Gert-Jan Euverink, Sjoerd van der Wal, Huub Henderickx, Roland Bezemer, and Aad Vollebregt report their own experiences at the Dutch company DSM, where indirect colorimetric assays and high-throughput direct analyses such as HPLC and NMR have been used. This industrial contribution highlights the importance of screening for enantioselectivity, as also discussed in Chapters 1 and 2.

### Part II: Genetic Selection

Enzyme assays play a central role in the context of microbial screening and directed evolution experiments. In this field the catalysis signal is used as the selection criterion to accept or reject single genes or microbial colonies in the hope of isolating enzyme mutants with desirable catalytic properties. The genetic diversity undergoing selection through the enzyme assay consists either in

enzyme mutants generated artificially, or in biodiversity collections, such as gene libraries from the metagenome or microorganism collections (Figure 7).

Screening preferentially describes experiments in which the assay signal is used to direct an external device to pick individual active enzymes or enzyme-producing genes, such as manual picking from agar plates or microtiter plates or the use of fluorescence-activated cell sorting. The term "genetic selection" best describes systems where the expression of an active enzyme is linked to cell survival without external signal processing.

In Chapter 5, Nicholas J. Turner reviews the design and application of enzyme assays in the context of selecting active enzymes by colony picking on agar, which is the most common screen used for microbial cultures. The chapter discusses how much can be achieved quickly by implementing straightforward chemical reaction principles in a microbiological context. A critical overview of genetic selection methods used to isolate active enzymes is also presented.

Random mutagenesis protocols such as gene shuffling [10], error-prone polymerase chain reaction (PCR) [11], and the later improvements or variations of these methods [12] readily allow on the order of $10^{12}$ mutants of a given enzyme to be generated in a single experiment. However, high-throughput screening experiments in microtiter plates or even on agar plate can only test a few tens of thousands of mutants for catalytic activity. In recent years several groups have invented methods to allow efficient screening of such large numbers of mutants.

In Chapter 6, Amir Aharoni, Cintia Roodveldt, Andrew D. Griffiths, and Dan S. Tawfik provide a general overview of screening methods applicable in the context of both functional genomics and directed evolution. The authors discuss the critical problem of choosing the right expression system for a given enzyme, and the implementation of selection pressure that is able to distinguish between

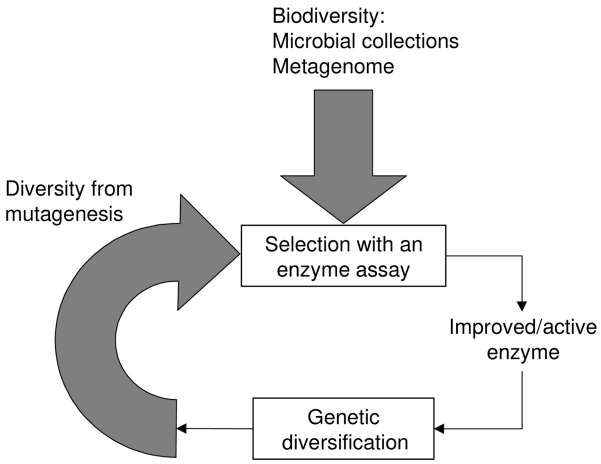


**Fig. 7** Selection of active enzymes from genetic libraries and biodiversity.

protein expression levels and protein function. A variety of high-throughput screening approaches for enzymes, such as phage-display and fluorescence-activated cell sorting, are reviewed, including the author's own elegant emulsion-based compartimentalization system for screening large genetic libraries.

In Chapter 7, Scott Lefurgy and Virginia Cornish review high-throughput selection methods by chemical complementation. The chapter includes an excellent review of genetic selection experiments that can be used to perform directed evolution, and emphasizes chemical complementation by the yeast three-hybrid system. In this system a synthetic chemical inducer of dimerization (CID) acts as a tether between a DNA-binding domain and a transcription activation domain. The CID either serves as a substrate cleavable by the enzyme, or is the product of an enzyme coupling. The experiment is set up such that activation or deactivation of gene expression in the presence of an enzyme cleaving or forming the CID is conditional for cell survival, allowing genetic selection to take place. Chemical complementation is demonstrated by various examples, including $\beta$-lactamases and glycosynthase enzymes.

In recent years microbiologists studying biodiversity have come to realize that the natural environment, in particular biotopes under extreme conditions, harbor a very large number of diverse microbes. In Chapter 8, Valéria Maia de Oliveira and Gilson Paulo Manfio present an overview of screening methods in the context of exploiting the genetic biodiversity available in microbial collections and in environmental DNA. Environmental DNA is recovered by direct PCR amplification and includes genetic material from noncultivable microbes, which is considered to be the vast majority (>99%), and is collectively called the metagenome [13]. In addition to screening for expressed enzyme activity in such libraries, it is also possible to analyze gene sequences for conserved sequence patterns indicative of certain enzyme activities.

## Part III: Enzyme Fingerprinting

An enzyme assay is a tool designed to visualize enzyme function. In its simplest expression, the resulting picture of enzyme activity is a single pixel in two colors (e.g. white = no activity, black = activity, with respect to the assay being performed). The picture can adopt higher levels of definition if the number of pixels is augmented, or if a color shading is allowed for each pixel. This can be realized by combining several different assays for the same enzyme into an array, and by obtaining quantitative rather than qualitative data from each assay. The resulting pictures of enzyme function are called activity profiles, or fingerprints (Figure 8).

The notion of fingerprint is associated with the possibility of using the activity profile as an identification mark for an enzyme or enzyme-containing sample, which is the prerequisite for all diagnostic applications of enzyme assays. Any device capable of recording an enzyme fingerprint is the equivalent of a camera for taking pictures of enzyme function. As for screening, enzyme assays for fin-

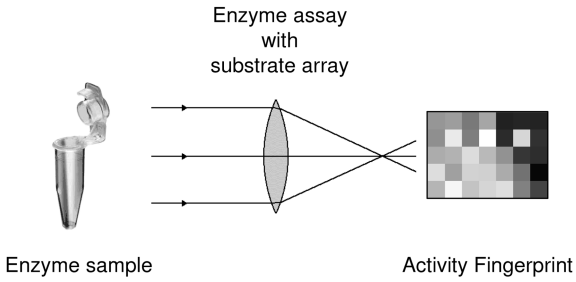


**Fig. 8** Enzyme activity fingerprinting.

gerprinting must be applicable in high-throughput. The goal here is to collect the enzyme activity data simultaneously on many different substrates or in many different reaction conditions.

In Chapter 9, Ruth Birner-Grünberger, Hannes Schmidinger, Alice Loidl, Hubert Scholze, and Albin Hermetter discuss assays used for the identification and biochemical study of lipases and esterases. These include fluorogenic substrates specifically designed for targeted hydrolases. The authors also review active-site labeling probes, which are used to covalently tag active enzyme for later identification by gel electrophoresis and mass spectrometry. Such active-site labeling probes have established themselves as useful reagents for the discovery of new disease-related enzymes.

In Chapter 10, Johann Grognux and Jean-Louis Reymond report a series of practical methods for recording activity fingerprints of enzymes, mostly in the case of hydrolytic enzymes such as lipases, esterases, and proteases. Enzyme fingerprinting involves recording a reproducible image of the reactivity profile of an enzyme, such that the images obtained from different enzymes can be used for functional classification. The principle derives from multi-enzyme profiling as used for phenotyping in microbiology and medical diagnostics. Methods of fingerprinting include arrays of indirect fluorogenic substrates acting by a common mechanism of fluorescence release, and substrate cocktail reagents, which allow recording of an activity fingerprint in a single experiment. Data acquisition and statistical analysis techniques leading to functional classification of enzymes are presented.

In Chapter 11, Jennifer L. Harris reviews the application of fluorogenic peptide substrate libraries for large-scale profiling of proteases, a method which is used to define the substrate specificities and the actual natural substrates of proteases. The chapter reviews a number of protease profiling methods and experiments. Protease profiling has proven to be a indispensable tool for the biochemical study of these enzymes. The concept of positional scanning peptide libraries is central to surveying the entire sequence space of peptide substrates within a reasonable experimental effort.

The promise of fingerprinting lies not only in multiparametric analysis for studying enzymes, but also in possible applications in the area of diagnostics

and quality control. To realize this promise it will be necessary to develop miniaturized technologies rivaling DNA-chip technology. Several groups have shown that enzyme fingerprinting experiments can be incorporated into microarrays for highly parallel assays. An overview of miniaturized parallel enzyme assay technologies is presented by Souvik Chattopadhaya and Shao Q. Yao in Chapter 12. Many of the future prospects for further development in enzyme fingerprinting reside in the exploration of microarray approaches, which will reveal how to produce reliable fingerprints with diagnostic value.

### Enzyme Assays in Other Areas

Enzyme assays are used in many other areas of investigation outside the scope of this book. For example, they are used routinely in the practice of medical diagnosis; clinical testing includes tests for enzymes that act as disease markers [14]. Enzyme assays are key components of bioanalytical systems, including signal amplification in the enzyme-linked immunosorbent assay (ELISA) [15], genetic analysis using PCR [16], and gene sequencing using DNA polymerases [17]. Enzyme assays play a critical role in drug discovery, where they are used to test enzymes as drug targets against potential inhibitors [18]. Enzyme assays furthermore form a core technology used for imaging, where they serve to localize active enzymes inside living cells or whole organisms [19]. In these imaging applications enzyme assays are used to complement immunofluorescence staining using fluorescence-labeled antibodies against cellular proteins [20]. Imaging in live organisms also includes the methods of magnetic resonance imaging (MRI) [21] and positron emission tomography (PET) [22].

Recent chemical developments in imaging include the introduction of reagents incorporating red or near-infrared (NIR) chromophores/quencher pairs. For example in Figure 9 peptide substrate **8** could serve for imaging proteases [23]. Further development of enzyme-specific fluorescent probes are also of importance in the context of imaging. A recent example is the aminocoumarin substrate **9**, which can be used to visualize monoamine oxidases (MAO) A and B by forming the fluorescent product **10** [24]. In another example, the fluorescent resonance energy transfer (FRET) substrates **11** and **12** have been used to image phospholipases in zebrafish larvae [25]. This area of imaging has been reviewed recently [26].

### How to Use this Book

The chapters that make up this book have been written by different authors and hence have different styles. Taken together, the book covers a large part of all enzyme assay advances in recent years, while also citing classical work. The index contains the names of enzymes for which an assay is described or at least cited, which will facilitate searches for a specific problem. The index also con-

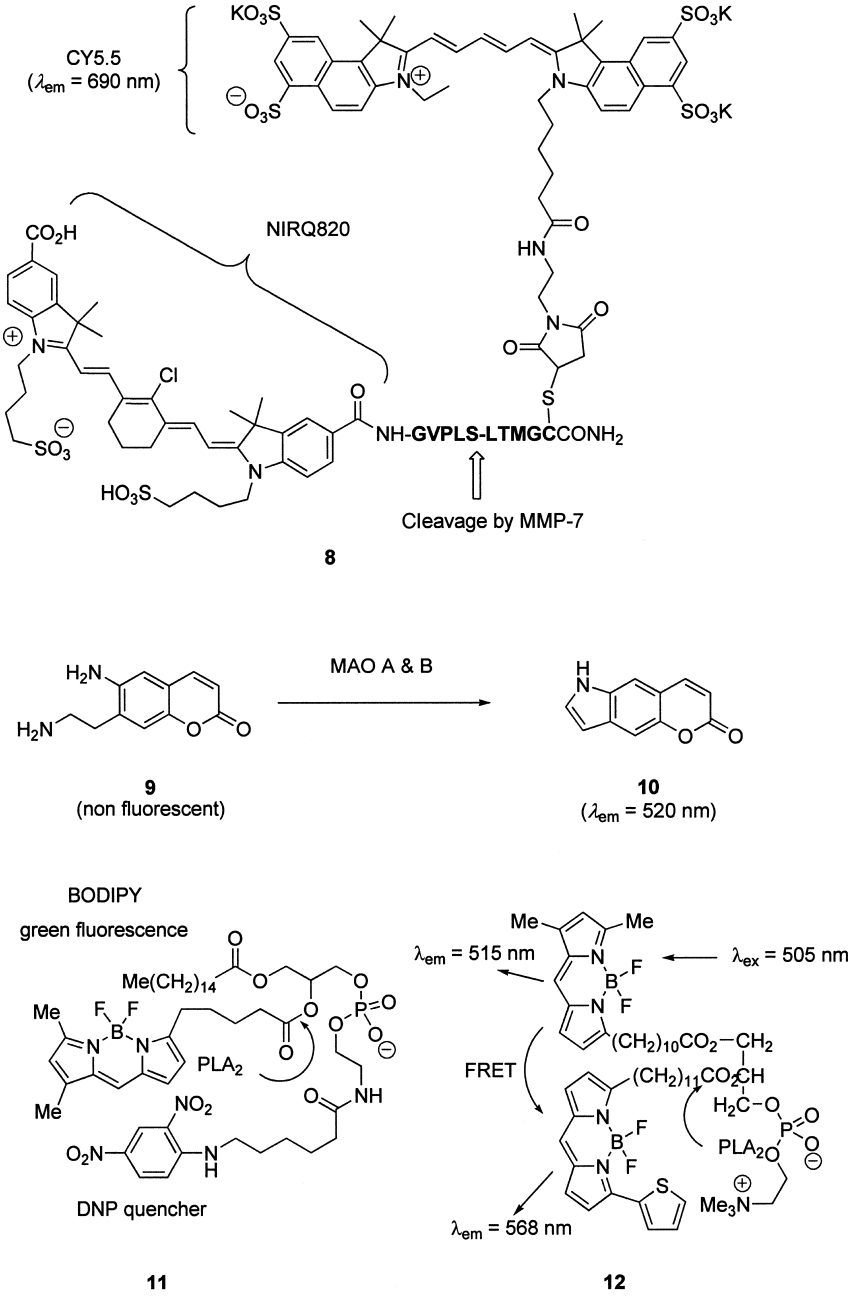


**Fig. 9** Example of fluorescent probes for enzyme imaging.

tains names of reagents and substrates. A number of experimental procedures are included. The book should serve as a useful reference to the original literature and as the basis for course material on the subject of enzyme assays.

## References


1 a) M.T. Reetz, *Angew. Chem.* **2001**, *113*, 292–320; b) M.T. Reetz, *Angew. Chem. Int. Ed. Engl.* **2001**, *40*, 284–310; c) D. Wahler, J.-L. Reymond, *Curr. Opin. Chem. Biol.* **2001**, *5*, 152–158; d) D. Wahler, J.-L. Reymond, *Curr. Opin. Biotechnol.* **2001**, *12*, 535–544; e) J.-P. Goddard, J.-L. Reymond, *Trends Biotechnol.* **2004**, *22*, 363–370; f) J.-P. Goddard, J.-L. Reymond, *Curr. Opin. Biotechnol.* **2004**, *15*, 314–322; g) M.T. Reetz, High-throughput screening of enantioselective industrial biocatalysts, in *Evolutionary Methods in Biotechnology*, eds S. Brakmann, A. Schwienhorst, Wiley-VCH, Weinheim, **2004**; h) H. Lin, V.W. Cornish, *Angew. Chem. Int. Ed.* **2002**, *41*, 4402–4425; i) M. Uttamchandani, D.P. Walsh, S.Q. Yao, Y. T. Chang, *Curr. Opin. Chem. Biol.* **2005**, *9*, 4–13; j) F.H. Arnold, *Nature* **2001**, *409*, 253–257; k) F.H. Arnold, P.L. Wintrode, K. Miyazaki, A. Gershenson, *Trends Biochem. Sci.* **2001**, *26*, 100–106.

2 a) K. Hult and P. Berglund, *Curr. Opin. Biotechnol.* **2003**, *14*, 395–400; b) G.J. Williams, A.S. Nelson, A. Berry, *Cell Mol. Life Sci.* **2004**, *61*, 3034–3046; c) D.E. Robertson, B.A. Steer, *Curr. Opin. Chem. Biol.* **2004**, *8*, 141–149.

3 a) A. Schmid, J.S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, *Nature* **2001**, *409*, 258–268; b) S. Panke, M. Held, M. Wubbolts, *Curr. Opin. Biotechnol.* **2004**, *15*, 272–279.

4 a) G. Leegsma-Vogt, M.M. Rhemrev-Boom, R.G. Tiessen, K. Venema, J. Korf, *Biomed. Mater. Eng.* **2004**, *14*, 455–464; b) S.A. Jaffari, A.P. Turner, *Physiol. Meas.* **1995**, *16*, 1–15.

5 W.-S. Yeo, M. Mrksich, *Angew. Chem. Int. Ed. Engl.* **2003**, *42*, 3299–3301.

6 a) G. Klein, J.-L. Reymond, *Angew. Chem. Int. Ed. Engl.* **2001**, *40*, 1771–1773; b) K.E.S. Dean, G. Klein, O. Renaudet, J.-L. Reymond, *Bioorg. Med. Chem. Lett.* **2003**, *10*, 1653–1656.

7 a) D.S. Tawfik, B.S. Green, R. Chap, M. Sela, Z. Eshhar, *Proc. Natl Acad. Sci. USA* **1993**, *90*, 373–377; b) G. MacBeath, D. Hilvert, *J. Am. Chem. Soc.* **1994**, *116*, 6101–6106; c) P. Geymayer, N. Bahr, J.-L. Reymond, *Chem. Eur. J.* **1999**, *5*, 1006–1012; d) C. Gauchet, B. Mohar, A. Valleix, P.Y. Renard, S. Meunier, F. Taran, A. Wagner, J. Grassi, C. Mioskowski, *Angew. Chem. Int. Ed. Engl.* **2002**, *41*, 124–127; e) F. Taran, P.Y. Renard, C. Creminon, A. Valleix, Y. Frobert, P. Pradelles, J. Grassi, C. Mioskowski, *Tetrahedron Lett.* **1999**, *40*, 1891–1894; f) S. Meunier, F. Taran, C. Mioskowski et al., *ChemBioChem* **2004**, *5*, 832–840; g) M. Matsushita, K. Yoshida, N. Yamamoto, P. Wirsching, R.A. Lerner, K.D. Janda, *Angew. Chem. Int. Ed. Engl.* **2003**, *42*, 5984–5987.

8 a) G. Das, P. Talukdar, S. Matile, *Science* **2002**, *298*, 1600–1602; b) N. Sorde, G. Das, S. Matile, *Proc. Natl Acad. Sci. USA* **2003**, *100*, 11964–11969.

9 a) Z. Li, J.B. van Beilen, W.A. Duetz, A. Schmid, A. de Raadt, H. Griengl, B. Witholt, *Curr. Opin. Chem. Biol.* **2002**, *6*, 136–144; b) W.A. Duetz, J.B. van Beilen, B. Witholt, *Curr. Opin. Biotechnol.* **2001**, *12*, 419–425; c) M. Alcalde, E.T. Farinas, F.H. Arnold, *J. Biomol. Screen.* **2004**, *9*, 141–146; d) M.W. Peters, P. Meinhold, A. Glieder, F.H. Arnold, *J. Am. Chem. Soc.* **2003**, *125*, 13442–13450; e) P.C. Cirino, F.H. Arnold, *Angew. Chem. Int. Ed. Engl.* **2003**, *42*, 3299–3301.

10 a) W. Stemmer, *Proc. Natl Acad. Sci. USA* **1994**, *91*, 10747–10751; b) A. Crameri, S.A. Raillard, E. Bermudez, W.P.C. Stemmer, *Nature* **1998**, *391*, 288–291; c) J.A. Kolkman, W.P.C. Stemmer, *Nat. Biotechnol.* **2001**, *19*, 423–428.

**11** a) H. Zhao, L. Giver, Z. Shao, J. A. Affholter, F. H. Arnold, *Nat. Biotechnol.* **1998**, *16*, 258–261; b) H. Tao, V. W. Cornish, *Curr. Opin. Chem. Biol.* **2002**, *6*, 858–864; c) V. Sieber, C. A. Martinez, F. H. Arnold, *Nat. Biotechnol.* **2001**, *19*, 456–460.

**12** C. Neylon, *Nucleic Acids Res.* **2004**, *32*, 1448–1459.

**13** R. I. Amann, W. Ludwig, K. H. Schleifer, *Microbiol. Rev.* **1995**, *59*, 143–169.

**14** a) Y. Li, C. Ronald Scott, N. A. Chamoles, A. Ghavami, B. M. Pinto, F. Turecek, M. H. Gelb, *Clin. Chem.* **2004**, *50*, 1785–1796; b) N. M. Verhoeven, B. Roos, E. A. Struys, G. S. Salomons, M. S. van der Knaap, C. Jakobs, *Clin. Chem.* **2004**, *50*, 441–443.

**15** G. J. Doellgast, M. X. Triscott, G. A. Beard, J. D. Bottoms, T. Cheng, B. H. Roh, M. G. Roman, P. A. Hall, J. E. Brown, *J. Clin. Microbiol.* **1993**, *31*, 2402–2409.

**16** S. M. Johnson, K. A. Simmons, D. Pappagianis, *J. Clin. Microbiol.* **2004**, *42*, 1982–1985.

**17** M. M. Albà, *Genome Biol.* **2001**, *2*, reviews3002.1-reviews3002.4.

**18** A. D. Rodrigues, J. H. Lin, *Curr. Opin. Chem. Biol.* **2001**, *5*, 396–401.

**19** a) N. J. Emptage, *Curr. Opin. Pharmacol.* **2001**, *1*, 521–525; b) R. J. Gillies, *J. Cell. Biochem. (Suppl.)* **2002**, *39*, 231–238.

**20** a) D. S. Smith, M. H. Al-Hakiem, J. Landon, *Ann. Clin. Biochem.* **1981**, *18*, 253–274; b) C.-H. Tung, N.-H. Ho, Q. Zeng, Y. Tang, F. A. Jaffer, G. L. Reed, R. Weissleder, *ChemBioChem* **2003**, *4*, 897–899.

**21** J. Hirsch, *J. Clin. Invest.* **2003**, *111*, 1440–1443.

**22** M. E. Phelps, *Proc. Natl Acad. Sci. USA* **2000**, *97*, 9226–9233.

**23** W. Pham, Y. Choi, R. Weissleder, C.-H. Tung, *Bioconjugate Chem.* **2004**, *15*, 1403–1407.

**24** G. Chen, D. J. Yee, N. G. Gubernator, D. Sames, *J. Am. Chem. Soc.* **2005**, *127*, 4544–4545.

**25** S. A. Farber, M. Pack, S. Y. Ho, I. D. Johnson, D. S. Wagner, R. Dosch, M. C. Mullins, H. S. Hendrickson, E. K. Hendrickson, M. E. Halpern, *Science* **2001**, *292*, 1385–1388.

**26** a) R. G. Blasberg, J. G. Tjuvajev, *J. Clin. Invest.* **2003**, *111*, 1620–1629; b) P. A. Binz, M. Muller, C. Hoogland, C. Zimmermann, C. Pasquarello, G. Corthals, J. C. Sanchez, D. F. Hochstrasser, R. D. Appel, *Curr. Opin. Biotechnol.* **2004**, *15*, 17–23.