Miniaturizing chemistry and biology in microdroplets

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Received (in Cambridge, UK) 7th November 2006, Accepted 19th January 2007
First published as an Advance Article on the web 23rd February 2007
DOI: 10.1039/b616252e

By compartmentalizing reactions in aqueous microdroplets of water-in-oil emulsions, reaction volumes can be reduced by factors of up to 10^9 compared to conventional microtitre-plate based systems. This allows massively parallel processing of as many as 10^{10} reactions in a total volume of only 1 ml of emulsion. This review describes the use of emulsions for directed evolution of proteins and RNAs, and for performing polymerase chain reactions (PCRs). To illustrate these applications we describe certain specific experiments, each of which exemplifies a different facet of the technique, in some detail. These examples include directed evolution of Diels–Alderase and RNA ligase ribozymes and several classes of protein enzymes, including DNA polymerases, phosphotriesterases, β-galactosidases and thiolactonases. We also describe the application of emulsion PCR to screen for rare mutations and for new ultra-high throughput sequencing technologies. Finally, we discuss the recent development of microfluidic tools for making and manipulating microdroplets and their likely impact on the future development of the field.

Introduction

Miniaturization in the electronics industry has driven remarkable advances in computing over the past few decades. Modern processors contain 40 million transistors or more in a device which can fit comfortably in a desktop computer, giving access to impressive computing power for even the average user. This rapid and sustained increase in computing power has facilitated advances across the whole spectrum of scientific and engineering fields. In contrast, miniaturization in chemistry and biology has been more modest: biochemical assays may now be performed in volumes of a few microlitres in a microtitre plate rather than a few millilitres in a test tube. Undoubtedly, the advent of high-throughput microtitre plate screening has yielded benefits for research and diagnostics, but practical considerations mean that it is rare for more than 10^5 assays to be performed in a single screen, and cost and availability of reagents sets a practical ceiling of 10^6–10^7 assays.

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Bernard T. Kelly was born near Liverpool, England, in 1974. He obtained his BA degree from the University of Cambridge in 1997 and moved on to the Medical Research Council's Laboratory of Molecular Biology to begin doctoral work on the newly-developed in vitro compartmentalization (IVC) technology with Dr Andrew Griffiths. He stayed on as a postdoctoral research associate, applying IVC technology to the study of fundamental evolutionary forces. Since 2005 he has been at the Cambridge Institute for Medical Research.

Jean-Christophe Baret was born in France in 1979. After graduation at ESPCI, Paris, he received his Master in fluid physics from the Université Paris 6 Jussieu. He obtained his PhD on electrowetting in 2005 from the University of Twente in the Netherlands in a collaboration with Philips Research Laboratories. He is currently a postdoctoral research fellow, holding an EMBO Long Term Fellowship, at the Institut de Science et d'Ingénierie Supramoléculaires (ISIS) of the Université Louis Pasteur, Strasbourg, working on droplet-based microfluidics for biological and chemical applications.

Valerie Taly (Abécassis) was born in France in 1975. After graduation as a Molecular Toxicologist, she did her PhD research at the Center of Molecular Genetics (CNRS, Gif-sur-Yvette, France) with Dr D. Pompon on applications of combinatorial methods (generally used for proteins directed evolution) to the study of the functional plasticity of human cytochromes P450. She obtained her PhD in Molecular Toxicology in 2003 and joined Andrew Griffiths group in the Medical Research Council in Cambridge (UK) to work on selection of enzymes using double-emulsions by FACS. She then worked as a seconded scientist of the MRC in the laboratory that Andrew Griffiths created in 2005 in ISIS (Strasbourg) where she works on directed evolution and HTS procedures using digital microfluidics. In 2006, she obtained a CNRS position as a permanent researcher in Andrew Griffiths new laboratory.

Andrew Griffiths received a BSc in Biochemistry from the University of Sheffield, UK, in 1985. He completed a PhD, studying messenger RNA splicing, under the supervision of Prof. Ian Eperon, at the University of Leicester, UK, in 1988. He then worked for 15 years at the MRC Laboratory of Molecular Biology, Cambridge, UK, first as a post-doc with Dr Greg Winter, where he worked on selection of human antibodies for therapy using phage display, and later as a group leader, where he became interested in developing systems based on compartmentalisation of reactions in emulsions to select for catalysis. He is currently a Professor in the Institut de Science et d'Ingénierie Supramoléculaires (ISIS), a new multidisciplinary research centre founded by Prof. Jean-Marie Lehn in Strasbourg.

The need to increase the throughput of laboratory assays can readily be seen by considering the vast combinatorial spaces inherent in the study of biological and macromolecular systems. The human genome, for example, contains ~30 000 genes and, since most cellular processes are mediated by intermolecular protein–protein and protein–nucleic acid interactions, it is desirable to dissect the interactions between the products of these genes; even a simple pairwise interaction search therefore requires the inspection of $\sim 10^9$ discrete gene combinations. The combinatorial spaces accessible to protein sequences dwarfs this figure, since a polypeptide chain may be composed of hundreds or even thousands of amino acids, each of which may be any one of twenty naturally occurring molecules, or numerous "artificial" alternatives. A short polypeptide of 100 amino acid residues, for example, may have any one of 20^{100} ($\sim 10^{130}$) different sequences. This fact poses problems for the engineering of novel proteins (or, indeed, other macromolecules such as nucleic acids), since it is still far from trivial to design biomolecules with specific properties from scratch, despite recent advances.[1–6]

Even for much smaller chemical entities, such as "drug-like" molecules of <500 Da, the potential chemical space is still overwhelming, containing perhaps in excess of 10^{60} molecules.[7] The desire to explore as much of this chemical space as possible in the search for new pharmaceuticals has led to the development of sophisticated robotic high-throughput screening programs.[8,9] Today, screening programs may process up to 100 000 compounds a day (slightly more than one per second), a thousand times as many as were processed in an entire week in 1990. Further reducing test volumes below the 1–2 μl capacity of 1536-well plates would enable both significant cost savings and higher throughput. However, using conventional technology, further miniaturisation is problematic: for example, evaporation becomes significant in microlitre volumes and capillary action causes "wicking" and bridging of liquid between wells.[8]

Alternative methods, based not on screening, but on selection, have been developed to allow the isolation of proteins and RNAs from large combinatorial libraries. These methods harness the power of selection to sift "blindly" through combinatorial libraries of macromolecules. For example, the sought-after property can be linked to the survival of a bacterial cell which harbours the molecule. Alternatively, a variety of technologies have been developed which physically link proteins to the genes that encode them, for example by display of proteins on filamentous phage[10] or ribosomes.[11] These "display-technologies", which allow proteins to be affinity purified together with the genes that encode them, have been highly successful for the selection of molecules for binding and have more recently been adapted to select for stability, catalysis and regulation.[12] Such techniques, whilst powerful, do not normally allow the direct observation and measurement of the properties of individual library members, which not only robs the experimenter of some degree of control over the search, but also exacerbates the problems of "false positives" and "false negatives". Thus, high-throughput screens conducted in microtitre plates continue to be used extensively to screen libraries.

In this review we describe a system which takes advantage of the natural tendency of small liquid structures to form droplets.[13] Compartmentalization of reactions in aqueous microdroplets in water-in-oil (w/o) emulsions can decrease volumes anything up to 10^9 times compared to the smallest assays in microtitre plates, making possible significant advances in diverse fields where large parameter spaces need to be explored. Emulsions are heterogeneous systems of two immiscible liquid phases, with one of the phases dispersed in the other as droplets of typically 1 μm to 100 μm diameter (~0.5 fl to 0.5 nl volume). Each droplet can be used as an independent microreactor. We will describe applications in the engineering of proteins and nucleic acids, and in ultra-high throughput DNA sequencing, describing certain specific experiments which exemplify different facets of the technique in some detail. Finally we will discuss the recent development of microfluidic tools for making and manipulating microdroplets and their likely impact on the future development of the field.

Directed evolution in emulsions

Natural selection works because genes and the molecules they encode are co-compartmentalized within cells, so that (at least in unicellular organisms) the activity of an RNA or protein determines the probability that the gene which encoded it will survive and replicate, but does not affect the survival and replication of other genes in other cells. In the late 1990s, in a collaboration with Dr Dan Tawfik, we started to compartmentalize genes not within cells, but in the tiny aqueous droplets of water-in-oil emulsions,[14] a technique we termed *in vitro* compartmentalization (IVC). The concentration of genes is set such that, statistically, few microdroplets will contain more than one gene. These aqueous compartments (Fig. 1(a)), which are about the same size as bacterial cells, have volumes as small as a femtolitre, and contain all the ingredients necessary for expression of the enclosed gene – cellular extracts, or purified components, allowing transcription of genes into RNA, and, if required, translation of RNA into protein. The expressed RNAs and proteins are unable to escape from the droplet in which they were created.

Selection for catalysts

We initially developed IVC to allow direct selection for catalysis. The selection was based on physically attaching substrates for reactions of interest to the genes themselves. Genes encoding RNA or protein molecules catalyzing the conversion of substrate to product thus become physically associated with product molecules (Fig. 1(b)), and may be retrieved by selecting for some property specific to the product. In this selection-based approach, the genes are sifted 'blindly' without any direct observation of the activities of individual variants. However, it has the advantage of allowing relatively large libraries of $>10^{10}$ genes to be selected using only a small volume of emulsion (a typical 1 ml emulsion contains $\sim 10^{10}$ droplets) as all the genes are processed in bulk.

This approach was initially developed to select a DNA methyltransferase (Fig. 2). In this case, the reaction is stopped, the emulsion is broken and the recovered genes are selected based

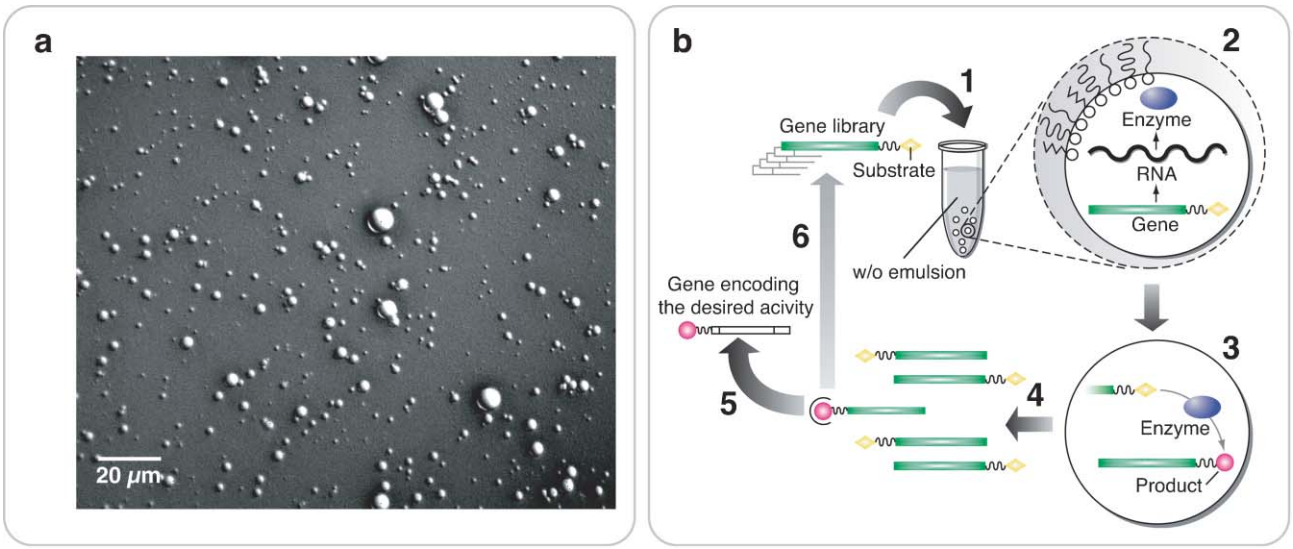

Fig. 1 *In Vitro* Compartmentalization (IVC). (a) A water-in-oil emulsion (generated by stirring) viewed under the microscope. Scale bar shown is 20 μm in length. (b) Selection for catalysis using IVC. (1) A library of genes, with substrate molecule(s) attached, is emulsified, isolating single genes in the aqueous compartments, which contain all the ingredients necessary for *in vitro* gene expression. The genes are expressed (2), producing enzymes or ribozymes which may be capable of converting substrate into product (3). Protein or RNA molecules expressed in this way are unable to leave the compartment, thus creating a linkage between genotype and phenotype (in this case, the catalytic activity of the gene); thus, only genes encoding active enzymes have their attached substrate molecules turned over into product molecules. The genes are recovered from the emulsion (4) and those genes encoding active enzymes or ribozymes are isolated (5) by, for example, specific binding of product molecules. Further rounds of mutagenesis and selection may also be performed (6). Adapted from ref. 14 (adapted with permission from D. S. Tawfik and A. D. Griffiths, *Nat. Biotechnol.*, 1998, **16**, 652–656. Copyright 1998, MacMillan Publishers Ltd).

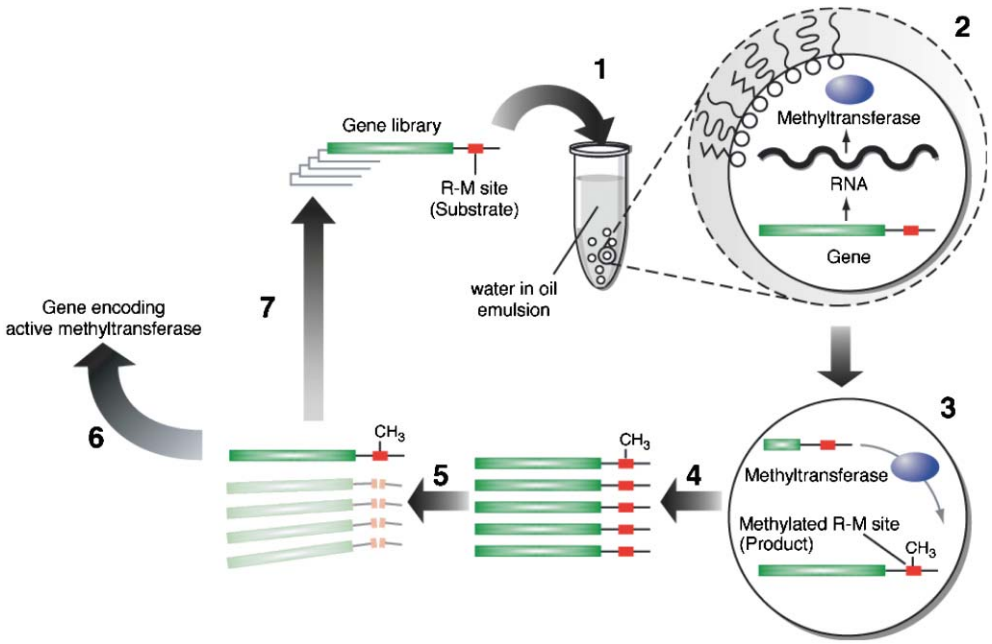

Fig. 2 Selection of DNA methyltransferases by IVC. (1) An *in vitro* transcription/translation mixture containing a library of genes encoding methyltransferase variants, appended to a restriction/methylation (R/M) site, is dispersed to form a water-in-oil emulsion under conditions that produce aqueous compartments which contain, for the most part, either a single gene, or no genes at all. (2) The genes are transcribed and translated within their compartments. (3) The target R/M site is methylated in the compartments containing a methyltransferase that recognizes it; since methyltransferase enzymes cannot leave the compartment in which they were expressed, they cannot methylate genes in other compartments. (4) The emulsion is broken, all reactions are stopped and the aqueous compartments combined. The recovered DNA is incubated with the cognate restriction endonuclease. (5) Unmethylated genes (*i.e.* those which do not encode a methyltransferase that can methylate the target R/M site) are digested whereas methylated genes (*i.e.* those which encode a methyltransferase that can methylate the target R/M site) remain intact and will thus survive. This surviving population is then amplified by the polymerase chain reaction and either characterized (6) or re-compartmentalized for further round of selection (7). (Adapted with permission from Y.-F. Lee *et al.*, *Nucleic Acids Res.*, 2002, **30**, 4937-4944. Copyright 2002, Oxford University Press.)

on the fact that the substrate, unmethylated DNA containing the target sequence, can be digested by a cognate restriction endonuclease with the same target sequence, whereas the product, the methylated target sequence, is resistant to digestion. Using this approach, initial experiments demonstrated model selection of wild-type HaeIII methyltransferase from a 10^7-fold excess of nonfunctional genes in only two rounds of selection.[14] Further experiments sought to alter the substrate specificity of the enzyme. The HaeIII methyltransferase efficiently methylates its canonical target sequence GGCC, but also promiscuously methylates certain non-canonical sequences, notably AGCC, at a much reduced rate.[15] The IVC technique outlined above was used to evolve HaeIII methyltransferases which efficiently methylate AGCC.[16] A two-step mutagenesis strategy, involving initial randomization of DNA-contacting residues followed by randomization of the loop that lies behind these residues, yielded a mutant with a 670-fold improvement in catalytic efficiency (k_{cat}/K_M^{DNA}) using AGCC. This is a rare example of a laboratory-evolved enzyme whose catalytic efficiency surpasses that of the wild-type enzyme with the principal substrate.

A similar selection strategy has been used to select DNA restriction endonucleases.[17] An active endonuclease, isolated within a compartment along with its encoding gene, cleaves the substrate sequence, which is appended to the coding sequence. Cleaved DNA is a substrate for a DNA polymerase which can incorporate a dUTP-16-biotin molecule at the site of the overhang produced by digestion. Thus, genes encoding an active endonuclease are cleaved, become labelled with biotin and are easily recovered by binding to streptavidin-coated beads, whereas genes encoding inactive endonuclease do not cleave the recognition sequence, do not become biotinylated, and consequently are not retained. This technique was used to select genes encoding the wild-type FokI restriction endonuclease from a $\sim 3 \times 10^5$-fold excess of mutated genes, encoding inactive FokI.

Below, we describe in more detail the recent use of this selection strategy to select Diels–Alderase ribozymes and the use of another variant of IVC, compartmentalized self-replication (CSR), to select DNA polymerases.

Directed evolution of novel Diels–Alderase ribozymes

We have used IVC to evolve ribozymes catalyzing the Diels–Alder [4 + 2] cycloaddition reaction between a 1,3-diene and an alkene dienophile. Despite its utility in synthetic chemistry, where it allows the formation of six-membered rings by making two simultaneous C–C bonds, at the same time generating up to four chiral centres,[18] the Diels–Alder cycloaddition mechanism seems to be rarely used in nature, which apparently prefers other routes to C–C bond formation such as the aldol reaction.

In the laboratory, however, both antibodies (reviewed in[19]) and ribozymes[20–22] have been generated which catalyze the Diels–Alder reaction. The Diels–Alderase antibodies were raised against a hapten that mimicked either the Diels–Alder adduct or the transition state of the desired reactant. Diels–Alderase ribozymes using both pyridyl-modified[21,22] and unmodified RNA[20] were generated using a variation on SELEX (systematic evolution of ligands by exponential enrichment).[23,24] One substrate is physically tethered to the members of an RNA library, and active sequences are enriched by selection for the formation of a product, which remains tethered to the RNA and allows either selective amplification or affinity purification of the RNA.

Advantageously, SELEX allows selection of large libraries of more than 10^{15} variants and, because selection is for an intramolecular single-turnover reaction (*in cis* reaction), it is possible to select for RNAs with very small rate accelerations (k_{cat}/k_{uncat}).[25] However, it is not a true selection for catalysis, and there is neither selection pressure for turnover of multiple substrate molecules, nor for the conversion of untethered substrates (*in trans* reaction). Indeed, the majority of ribozymes generated by SELEX do not catalyse the in *trans* reaction and those that do usually show poor turnover.

We therefore sought to evolve efficient ribozymes which demonstrate true intermolecular, multiple-turnover catalysis by exploiting the IVC technique (Fig. 3).

A substrate molecule, the UV-excitable dye anthracene (the diene) is attached to the *DNA gene* rather than the RNA catalyst. Genes are compartmentalized in emulsions (Fig. 3(a)) and the dienophile, biotin-maleimide, introduced through the oil phase. Thus, genes encoding ribozymes capable of catalyzing the Diels–Alder cycloaddition (Fig. 3(b)) *in trans* become biotinylated and may be selectively retrieved by binding to streptavidin (Fig. 3(a)). Pressure for multiple turnover could be achieved by adding free 9-anthracenylmethyl hexaethylene glycol to the droplets at a concentration of 100 μM, which is 200 times higher than the RNA concentration (~ 0.5 μM), thereby ensuring that only ribozymes capable of performing at least 10–100 turnovers were efficiently selected.

Previously, SELEX had been used to select Diels–Alderase ribozymes for intramolecular catalysis of an attached anthracene substrate to the cycloadduct product using the same biotin-maleimide dienophile.[20] We compared IVC and SELEX directly by using IVC to select Diels–Alderase ribozymes from the same library that was previously selected using SELEX.[20] The SELEX experiment started from a library of $\sim 2 \times 10^{14}$ RNA molecules, each 157 bases long with the central 120 nucleotides randomized. IVC was used to evolve novel Diels–Alderase ribozymes, starting from a pool of RNAs enriched by several rounds of SELEX. This strategy exploits the complementary characteristics of both SELEX and IVC: SELEX allows selection of very large libraries and for very small rate accelerations (k_{cat}/k_{uncat}) but only selects for intramolecular single-turnover reactions; selection of very large libraries by IVC is more difficult and the threshold for selection (k_{cat}/k_{uncat}) is higher, but IVC selects for true intermolecular catalysis and multiple turnover.[25] Despite the enrichment for active ribozymes being only five-fold per round of IVC, due to the relatively high rate of the uncatalysed Diels–Alder reaction ($k_{uncat} = 3\ M^{-1}\ min^{-1}$), it was possible to select active ribozymes with five rounds of SELEX then six to nine rounds of IVC.

All the RNAs selected using IVC showed true bimolecular, multiple turnover catalysts *in trans* and some contained a completely novel ribozyme fold that was not found using SELEX alone.[26]

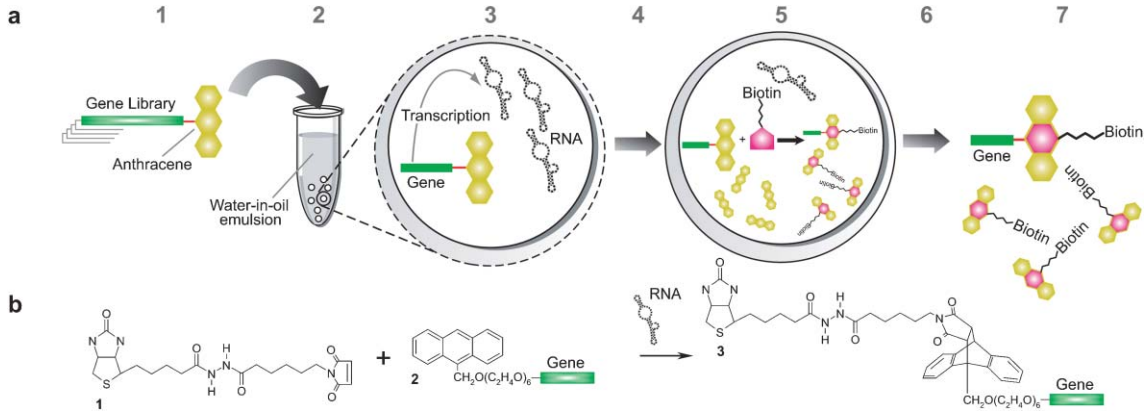

Fig. 3 Selection of Diels–Alderase ribozymes using IVC. (a) Schematic diagram of the selection procedure. A repertoire of genes (DNA) encoding ribozymes, each coupled to anthracene through a polyethylene glycol (PEG) linker, is created (1). Genes are compartmentalized within the aqueous droplets of a water-in-oil emulsion to give, on average, less than one gene per compartment (2). Genes are transcribed, giving ~60 RNA molecules per gene (3). Mg^{2+} and biotin-maleimide are allowed to diffuse into the compartments through the oil (4). In compartments containing active Diels–Alderase ribozymes, the formation of the cycloadduct by reaction of biotin-maleimide is catalyzed, thereby biotinylating genes encoding active ribozymes (5). The emulsion is broken (6), and active genes are enriched by binding to streptavidin-coated magnetic beads (7) and are amplified by PCR to allow further rounds of selection. For multiple-turnover selections, free anthracene is emulsified with the gene repertoire. (b) The Diels–Alder cycloaddition of biotin-maleimide (1) and 9-anthracenylmethyl hexaethylene glycol (AHEG) (2) covalently coupled to the gene to generate the adduct (3), thereby biotinylating the gene. Reprinted from ref. 26 (J. J. Agresti *et al.*, *Proc. Natl. Acad. Sci. USA*, 2005, **102**, 16170–16175. Copyright 2005, The National Academy of Sciences of the USA).

Interestingly, despite employing a technique capable of selecting for true multiple turnover catalysis, we found that the ribozymes evolved by IVC had values of $K_{M(diene)}$, $K_{M(dienophile)}$ and k_{cat} which were very similar to those of the ribozymes evolved by SELEX. Furthermore, none of the natural or artificial Diels–Alderases that have been characterised kinetically have k_{cat} of $\geqslant 1\ s^{-1}$, $k_{cat}/K_{m(diene)} \geqslant 10^4\ M^{-1}\ s^{-1}$, $k_{cat}/K_{m(dienophile)} \geqslant 10^3\ M^{-1}\ s^{-1}$, or $k_{cat}/K_{m(diene)}K_{m(dienophile)} \geqslant 10^6\ M^{-2}\ s^{-1}$. It may be difficult for Diels–Alderases to pass this upper limit due to the nature of the Diels–Alder reaction itself.

All of the Diels–Alderase ribozymes are strongly product inhibited; since the transition state of the reaction closely resembles the product, stabilizing the transition state – a hallmark of enzyme catalysis – is likely also to increase affinity for the product, decreasing the rate of product release and causing product inhibition.

Indeed, in the most efficient artificial Diels–Alderase described to date, the catalytic antibody 1E9, uncatalysed SO_2 elimination from the Diels–Alder adduct is programmed to avoid product inhibition[27] and the natural Diels–Alderase, macrophomate synthase, catalyses elimination of CO_2 from the cycloadduct to prevent product inhibition.[28] Thus, the requirement of circumventing product inhibition by evolving to catalyse, not only the Diels–Alder cycloaddition, but also a second reaction in which the cycloadduct is the substrate, may present an evolutionary barrier too high to enable widespread adoption of the Diels–Alder mechanism in natural enzymes.

Directed evolution of novel polymerases by compartmentalized self-replication

Holliger and colleagues employed emulsions in the directed evolution of DNA polymerases, a technique they termed compartmentalized self-replication, or CSR[29] (Fig. 4). In this approach, intact bacterial cells, each harbouring a DNA polymerase-encoding gene, are compartmentalized within the droplets of a thermostable emulsion, together with oligonucleotide primers to allow the amplification of the polymerase gene. The genes are transcribed and translated within the cells, which are then lysed by heating the emulsion, releasing the polymerase enzymes into the aqueous emulsion droplets. The emulsion is subjected to thermocycling to allow a polymerase chain reaction (PCR). Since the polymerase enzymes are isolated in the same droplets as the genes which encode them (which are released along with the enzymes when the bacterial host cell is lysed by thermocycling), the efficiency with which they copy DNA templates is reflected in the number of copies of their own gene which they are able to produce. This approach was used to evolve DNA polymerases with increased thermostability, resistance to PCR inhibitors, with modified base specificities, and which exhibit DNA polymerase, RNA polymerase and reverse transcriptase activity in a single polypeptide.[30,31]

Screening for catalysts

Using an approach based on screening has the advantage that the phenotype of every individual variant can be observed, allowing much greater control over the selection procedure. The disadvantage of this approach is the smaller number of variants that can be processed compared to a selection.

However, one way of greatly accelerating screening is to use fluorescence-activated cell sorting (FACS), which can routinely sort $>10^7$ clones per hour, and has a series of other advantageous features.[32] FACS has already proven a highly successful technique to select proteins (notably antibodies) with high binding affinities.[33–40] In addition, FACS has significant potential to select for catalysis;[41,42] however, so far, this approach has only been possible when the diffusion of product out of the cell can be restricted (*e.g.* refs. 43 and 44) or the product can be captured on the surface of the cell,[45,46] or onto microbeads.[47]

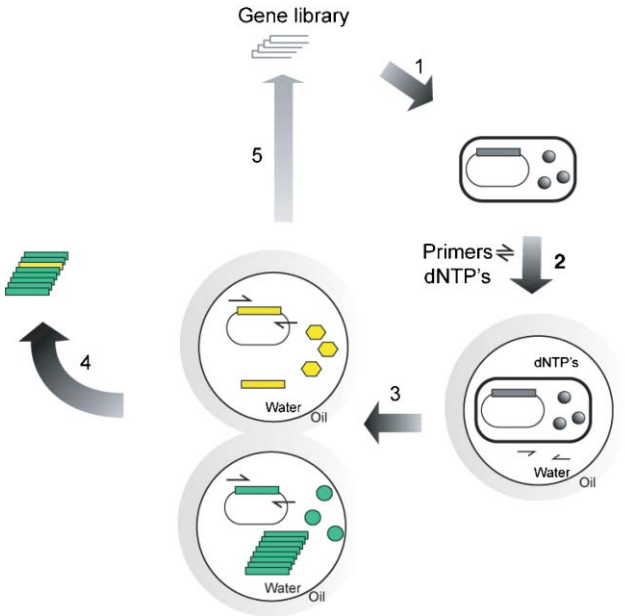

Fig. 4 Selection of polymerase variants by compartmentalized self-replication (CSR). (1) A library of genes encoding polymerase variants (grey spheres) is cloned and expressed in *E. coli*. (2) Bacterial cell containing the polymerases and the corresponding genes are dispersed in a buffer containing primers that flank the polymerase genes and dNTPs and compartmentalized within the aqueous compartments of a water-in-oil emulsion. (3) The emulsion is thermocycled resulting in the releasing of the polymerases and their encoding gene from the cell, allowing DNA-replication to proceed. Each polymerase replicates only its own encoding gene to the exclusion of those in other compartments. Genes encoding more active polymerases (green circles) are replicated more efficiently than genes encoding less active (yellow hexagons), or inactive, polymerases. (4) After breaking the emulsion the aqueous compartments are combined. The surviving population is then amplified by the polymerase chain reaction and either characterized (4) or submitted to further round of CSR (5). Adapted from ref. 29 (adapted with permission from F. J. Ghadessy *et al.*, *Proc. Natl. Acad. Sci. USA*, 2001, **98**, 4552–4557. Copyright 2001, The National Academy of Sciences of the USA).

IVC strategies were, therefore, developed based on ultra-high throughput screening using FACS. One such strategy involves attaching multiple substrate molecules to single biotinylated genes *via* streptavidin-coated microbeads with a diameter of $\sim$1 μm. These gene-bead-substrate complexes are then compartmentalised in a w/o emulsion at a concentration such that few microdroplets contain more than one gene. The genes are transcribed and, if necessary, translated. The substrate and product of the reaction catalysed by the RNA or protein encoded by the gene are physically linked to the gene *via* the microbead in the emulsion microdroplet. The emulsion is broken to recover the beads and the presence of product on the beads is detected using a fluorescent assay. The fluorescent, product-coated beads can then be sorted using FACS, at typically 20 000 beads per second, and the DNA amplified from the beads by PCR.

Evolution of RNA ligase

An example of this approach is the selection of a *trans*-acting RNA ligase ribozyme capable of ligating together two RNA oligonucleotide substrates.[48] A ribozyme capable of ligating RNA substrates had previously been selected using SELEX (the Bartel Class I ligase[49]), and this was used as the basis for a "doped" library of RNA mutants to validate the IVC/flow cytometry approach.

The genes encoding the ribozyme library were immobilized on microbeads along with one of the RNA oligonucleotide substrates. Compartmentalized transcription of the genes was carried out in an emulsion; the aqueous phase additionally contained the second RNA oligonucleotide substrate. Active ribozymes catalyzed the ligation of the two substrates. After recovery of the microbeads from the emulsion, ligation products were detected by hybridization of a fluorescently-labelled oligonucleotide complementary to the sequence of the second substrate RNA. Signal amplification was achieved by binding of fluorescently labelled antibodies to the fluorophore-labelled oligo, and the microbeads were sorted by flow cytometry. The technique successfully selected a *trans*-acting variant of the Bartel Class I ligase.

Evolution of phosphotriesterase

The naturally occuring phosphotriesterase (PTE) from the bacterium *Pseudomonas diminuta* is a highly enigmatic protein. It has no known natural substrate, but is capable of degrading organophosphate pesticides, such as paraoxon and parathion, highly efficiently. With its best substrate, the insecticide paraoxon (Fig. 5(a)), k_{cat} is high (2280 s^{-1}) and k_{cat}/K_M (6.2 $\times$

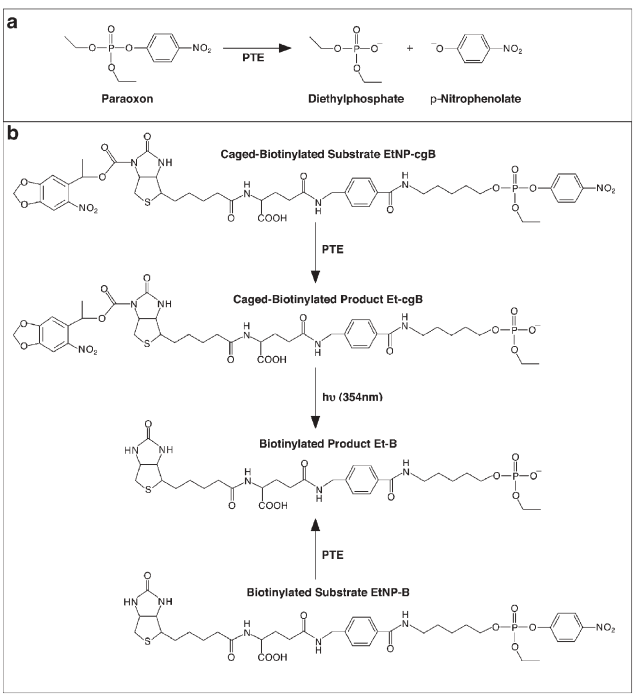

Fig. 5 PTE substrates. (a) PTE catalysed hydrolysis of paraoxon. (b) For selection, paraoxon was modified by substituting an ethyl group with a linker connected to caged-biotin.[55] PTE-catalysed hydrolysis of the resulting substrate (EtNP-cgB) gives *p*-nitrophenol and the corresponding phosphodiester Et-cgB. Irradiation at 354 nm releases the caging group and carbon dioxide to yield the (uncaged) biotinylated substrate (EtNP-B) or product (Et-B). Reprinted from ref. 47 (reprinted with permission from A. D. Griffiths and D. S. Tawfik, *EMBO J.*, 2003, **22**, 24–35. Copyright 2003, MacMillan Publishers Ltd).

10^7 M^{-1} s^{-1})[50] is close to the limit set by the diffusion-controlled encounter of the enzyme and the substrate.[51] Furthermore, Brønsted plots (V_{max} *vs.* pK_a of leaving group) created using a series of paraoxon analogues as substrates indicate that PTE may have evolved to use substrates (such as paraoxon) with *p*-nitrophenol leaving groups, since a change in the rate-limiting step from physical to chemical events occurs when the pK_a increases above 7.14 – the pK_a of *p*-nitrophenol – and the V_{max} decreases sharply with increasing pK_a ($\beta = -1.8$).[52] This would represent an evolutionary optimisation in only 60 years, since organophosphates with *p*-nitrophenol leaving groups were only released widely after the Second World War.

PTE can also catalyse the hydrolysis of the G-type organophosphorus nerve agents sarin and soman, and is the only enzyme characterised to date which can catalyse the hydrolysis of VX-type nerve agents. However, these nerve agents are much less efficient substrates than paraoxon.[53]

To select PTE we developed a version of IVC in which the translation step is completely separated from enzymatic catalysis, allowing selection under any chosen conditions[47] (Fig. 6). First, streptavidin-coated microbeads, each carrying a single gene and multiple copies of the protein they encode, were created by IVC (Fig. 6(a)). The beads recovered from this first emulsion were washed and resuspended in a buffer which contained zinc and carbonate ions to allow the captured inactive apo-enzyme to assemble into the catalytically active metallo-enzyme[54] before being dispersed amongst the aqueous compartments of a second emulsion (Fig. 6(b)). We wished to allow the reaction to be catalyzed in solution, so instead of attaching substrate molecules (paraoxon) to the microbeads from the outset, we synthesized substrate molecules containing a caged biotin moiety[55] (Fig. 5(b)). This caged-substrate was introduced into the droplets through the oil-phase to start the reaction. Active enzymes hydrolyse the paraoxon substrate, releasing *p*-nitrophenol and giving the phosphodiester product. Subsequently, the caging moiety is released by UV irradiation, allowing biotin to bind to the streptavidin-coated microbeads. After breaking the emulsion, fluorescent product-specific antibodies were added to the beads and FACS employed to sort out those microbeads which bear product molecules (and, therefore, genes encoding active proteins). The beauty of flow cytometry is that it is an ultra high throughput screening

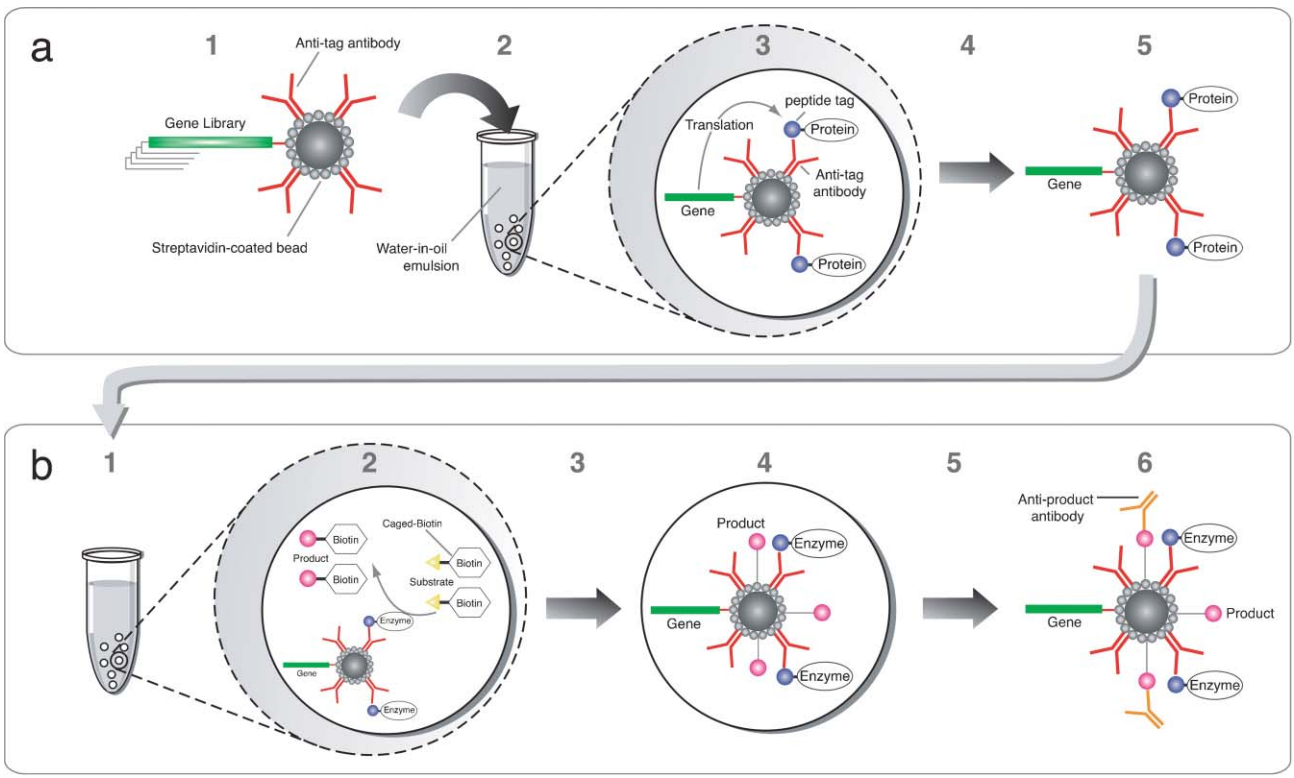

Fig. 6 Directed evolution of phosphotriesterase by IVC. (a) Creation of microbeads "displaying" the protein encoded by an attached gene. (1) A gene library, encoding variant proteins with a common peptide tag, is immobilized *via* the streptavidin–biotin interaction on microbeads, such that most microbeads carry at most a single gene. Additionally, the microbeads are coated with an anti-tag antibody. (2) The microbeads are dispersed amongst the compartments of an emulsion such that few compartments contain more than one microbead. (3) The genes are expressed and the encoded protein variants bind to the microbeads *via* a high-affinity interaction with the anti-tag antibody. (4) The emulsions are broken and (5) the microbeads recovered and washed. (b) Enzyme selection by compartmentalization. Microbead-display libraries (from (a)) are compartmentalized in an emulsion (1) and a soluble substrate attached to caged-biotin is added. The substrate is converted to product only in compartments containing beads displaying active enzymes (2). The emulsion is then irradiated to uncage the biotin (3). In a compartment containing a gene encoding an enzyme, the product becomes attached to the gene *via* the bead (4). In other compartments, in which the genes do not encode an active enzyme, the intact substrate becomes attached to the gene. The emulsion is broken (5) and the beads are incubated with fluorescently labelled anti-product antibodies (6). Product-coated beads are then enriched (together with the genes attached to them) using FACS. Reprinted from ref. 47 (reprinted with permission from A. D. Griffiths and D. S. Tawfik, *EMBO J.*, 2003, **22**, 24–35. Copyright 2003, MacMillan Publishers Ltd).

method, with throughputs of $>10^7$ h^{-1}; the criterion for retaining a gene may be adjusted manually by the experimenter, because the "phenotype" of every gene is measured directly by fluorescence.

Using this technique, the PTE enzyme was evolved into a variant which possesses a k_{cat} 63 times higher than that of the wildtype enzyme from which it was evolved. In fact, with a k_{cat} of $\sim 1.4 \times 10^5$ s^{-1} and a k_{cat}/K_M of 1.8×10^8, this mutant is one of the most efficient enzymes ever described.

Flow "cytometry" of double emulsions

Instead of selecting or screening (by FACS) genes and gene-bead complexes recovered from broken emulsions, what if the catalyzed reaction could be followed *in situ* within a droplet? One way to achieve this is by using FACS to sort intact emulsion droplets[56,57] (Fig. 7).

In this scheme, single genes, and all the components required for protein expression (either an *in vitro* coupled transcription and translation system (Fig. 7(a)), or, as described below, an intact *E. coli* cell (Fig. 7(b))), as well as a fluorogenic substrate, are compartmentalized within droplets of a w/o emulsion (Fig. 7(c)). If the translated enzyme is active, the non-fluorescent substrate is converted into a fluorescent product and, after conversion into a water-in-oil-in-water double emulsion, fluorescent droplets are sorted using FACS (Fig. 7(d)).

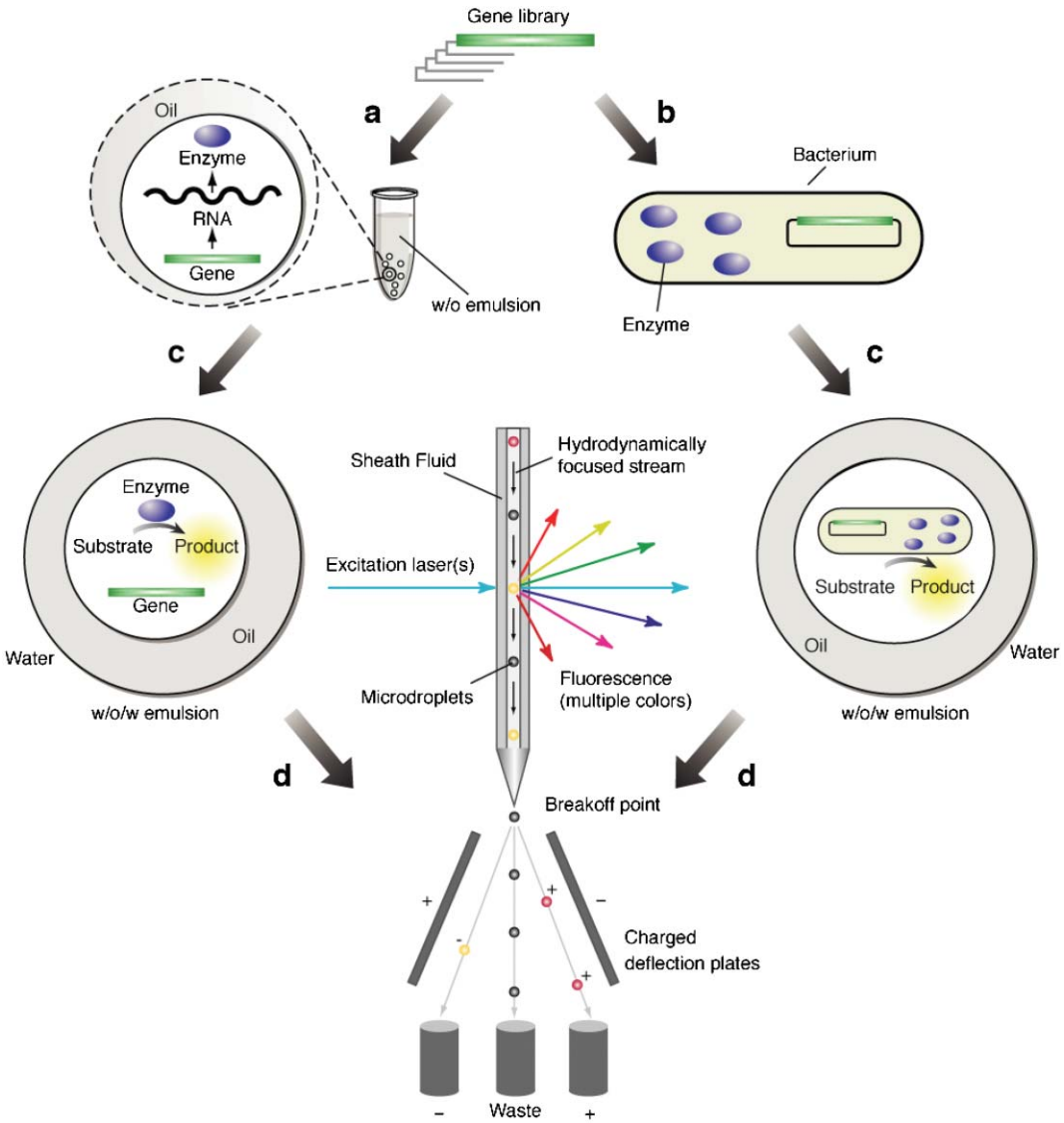

Fig. 7 FACS Selection of double emulsion droplets. (a) An *in vitro* transcription/translation reaction mixture containing a library of genes is dispersed to form a w/o emulsion with typically one gene per aqueous microdroplet and the genes are transcribed and translated within the microdroplets. (b) Alternatively, a library of gene variants is cloned and expressed in *E. coli* (in the cytoplasm, periplasm, or the surface of the cells). The bacteria are dispersed to form a water-in-oil (w/o) emulsion with typically one cell per aqueous microdroplet. (c) Proteins with enzymatic activity convert the non-fluorescent substrate into a fluorescent product and the w/o emulsion is converted into a water-in-oil-in-water (w/o/w) emulsion. (d) Fluorescent microdroplets are separated from non-fluorescent microdroplets using a fluorescence activated cell sorter (FACS). (Adapted from A. D. Griffiths and D. S. Tawfik, *Trends Biotechnol.*, 2006, **24**, 395–402. Copyright 2006, Elsevier.)

Directed evolution of β-galactosidases

We have used this double emulsion selection system to evolve the protein Ebg into an efficient β-galactosidase.[57] Ebg has been used extensively over the past three decades as a model to study the evolution of novel enzyme functions *in vivo*.[58–60] These studies demonstrated that Ebg, an *Escherichia coli* protein of unknown function and possessing negligible β-galactosidase activity, can evolve into an active β-galactosidase, allowing *E. coli* lacking the *lacZ* β-galactosidase gene to grow on lactose.

In vitro evolution of Ebg using flow "cytometry" of double emulsions allowed up to 4×10^7 variants to be screened in every generation and generated β-galactosidases with up to 1700-fold higher k_{cat}/K_M than wild-type Ebg. Only two specific mutations were ever seen to improve the β-galactosidase activity of Ebg *in vivo*. In contrast, nearly all the improved β-galactosidases evolved *in vitro* resulted from different mutations.

Directed evolution of thiolactonases

Alternatively, instead of generating proteins by *in vitro* translation in emulsion droplets, they can be generated by expression in the cytoplasm, periplasm, or on the surface of intact *E. coli*.

This strategy was used for the directed evolution of variants of the mammalian serum paraoxonase (PON1) with improved thiolactonase activity. PON1, which resides in HDL plasma particles (the "good" cholesterol), catalyses the hydrolysis of a broad range of substrates and has a profound impact on the onset and progression of atherosclerosis. It also hydrolyses thiobutyrolactones (TBLs), which are toxic metabolites, but they are rather poor substrates ($k_{cat}/K_M \leqslant 100\ M^{-1}\ s^{-1}$).

Single bacterial cells, each expressing a different PON1 variant were compartmentalized in the aqueous droplets of a w/o emulsion. The TBL substrate and a fluorogenic thiol-detecting reagent, *N*-(4-(7-diethylamino-4-methylcoumarin-3-yl)phenyl)-maleimide (CPM) were added through the oil phase. Hydrolysis of the TBL substrate generates a product with a free thiol which reacts with CPM to generate a fluorescent adduct. The emulsion was then converted into a w/o/w emulsion and sorted by FACS. Over 10^7 mutants were screened in this way resulting in variants with improvements in catalytic efficiency (k_{cat}/K_M) of 20- to 100-fold compared to the wild-type enzyme.

Compartmentalization of single cells in emulsion droplets provides unusually high enzyme concentrations ($>10^4$ enzyme molecules in <10 femtoliter) thus enabling detection and selection at extremely low signal-to-noise ratios. It also allows selection when the reaction product cannot be confined within the cell. Cell-free translation, on the other hand, enables the translation of essentially any protein, including proteins that are toxic to living cells. Both of these formats reduce the need to tailor the selection for each enzyme, substrate and reaction. This method greatly extends the range of applications of IVC, since fluorogenic subtrates are available for a wide range of enzymes, or can be readily synthesised.[61]

Selection and screening for binding and regulatory activities using IVC

IVC has also been used to select proteins and peptides for binding. Compartmentalization of genes in emulsions serves as a way of establishing a physical linkage between the gene and the protein it encodes. The expressed protein is coupled, either covalently[62] or non-covalently,[63–66] to the gene that encodes it within the emulsion droplet, either directly or through a microbead.[47,67] The emulsion is then broken and the gene–protein complexes recovered, and those with the desired activity enriched either by affinity purification using the target ligand, or by FACS of gene–bead–protein–ligand complexes.[67] In these applications, IVC allows the formation of large numbers of gene–protein complexes, akin to phage display, ribosome display, or similar "display" techniques.

However, in contrast to other "display" technologies, IVC is also well suited to selection for regulatory activities and its use to select highly potent inhibitors of DNA nucleases is described below.

Selection for DNA nuclease inhibitors

Regulatory functions can also be selected using IVC. For example, highly potent protein inhibitors of DNA nucleases have been selected by IVC directly for their inhibitory activity[68] and not simply for nuclease binding (as would be the case with a display-technology such as phage- or ribozyme-display).

The colicins form a class of plasmid-borne bacterial enzymes whose function appears to be to kill closely-related competing bacteria either by DNA degradation or by the formation of membrane pores. Colicins with DNA nuclease activity are paired with a cognate "immunity" protein, which inhibits the DNA-degrading activity of the nuclease.

Mutant libraries based on Im9, the cognate inhibitor of the colicin E9 nuclease, were selected for inhibition of a different nuclease, colE7. Genes encoding the mutant immunity proteins were compartmentalized in the presence of the colicin E7 protein, which is inactive in the absence of nickel or cobalt ions. After expression of the mutant immunity proteins, nickel or cobalt ions are added to the emulsion compartments *via* fusion with 100 nm-diameter solute-containing "nanodroplets", activating the nuclease molecules and allowing degradation of genes encoding Im9 mutants incapable of inhibiting colE7. In this way, novel immunity proteins with increases of well over 10^4 in affinity as well as selectivity were selected (Bernath *et al.* unpublished results).

Massively parallel PCR in emulsions

Compartmentalized gene expression has not been the only application of IVC technology. The CSR technique described above demonstrated the application of PCR to the directed evolution of DNA polymerase enzymes; other groups went on to demonstrate further applications of emulsion-PCR, or ePCR.[69–76] The advantage of ePCR is that it allows "clonal amplification" of DNA templates; in other words, individual DNA templates within a droplet are amplified in isolation from other templates. This has several advantages. First, it reduces the inherent bias of PCR towards shorter DNA fragments, which are amplified more rapidly than longer templates; secondly, it minimizes the generation of PCR artefacts caused by recombination between different DNA fragments in a complex mixture; and thirdly it allows multiple

copies of each fragment in a complex mixture to be amplified clonally, with all copies of the same fragment kept together in one physical location. PCR bias and inter-fragment recombination are particularly problematic in the preparation of genomic DNA libraries by PCR; ePCR can help to overcome these problems,[77] and clonal PCR amplification has enabled several novel applications, two of which we will describe in detail here. The first application, the so-called BEAMing strategy, allows the identification and quantification of rare mutant genes within large populations; the second enables ultra-high throughput DNA sequencing.

BEAMing

In the BEAMing strategy,[76,78] magnetic beads coated with oligonucleotide primers are compartmentalized along with the DNA fragments to be amplified; only 1 in 6 droplets contains a DNA fragment, thus reducing the occurence of multiple fragments in a single droplet. In addition to the beads and DNA fragments, the aqueous phase of the emulsion contains the ingredients of a PCR reaction, (including additional primers), so that, upon thermocycling of the emulsion, bead-bound primers anneal to a DNA fragment and are extended, resulting in beads coated with multiple copies of the co-compartmentalized DNA fragment. Recovery of the magnetic beads then allows hybridization of fluorescently-labelled oligonucleotide probes capable of specific detection of sequence variants, allowing the quantitation of allelic variants present in a population of DNA fragments (or transcripts, such as those obtained from tissue samples) by flow cytometry, as well as the identification of rare mutations.[78]

Emulsion PCR for ultra-high throughput pyro-sequencing

A similar technique forms the basis of two recent, novel sequencing technologies. In both cases, DNA fragments are initially amplified in a clonal manner by PCR on microbeads in emulsion compartments. In the ''multiplex polony sequencing'' approach of Church and colleagues these ''polonies'' or ''PCR colonies'' are short genomic fragments whose sequence is queried one base at a time by ligation of fluorescent oligonucleotides.[70] Using this technique, an evolved bacterial genome was sequenced at a fraction of the cost of traditional sequencing techniques. The second approach, termed ''picotiter plate pyrosequencing'', combines elements of microfabrication and ePCR;[69] we will discuss this approach in detail here.

Genomic DNA is first fragmented, ligated to adaptor sequences and separated into single strands (Fig. 8(a)). The DNA is then attached *via* the adaptor sequences to $\sim$28 μm diameter microbeads, at concentrations that ensure that the majority of microbeads carry at most a single DNA fragment; the microbeads are then compartmentalized in a thermostable w/o emulsion (Fig. 8(b) and (g)). The aqueous droplets contain the ingredients for a PCR reaction, and the majority of occupied droplets contain a single microbead (Fig. 8(c)), so that, after thermocycling, each DNA sequence fragment has been clonally amplified on a single microbead (Fig. 8(d)). The microbeads are subsequently loaded into picolitre-volume wells etched into the surface of a fibre-optic slide, which is imaged using a CCD camera (Fig. 8(e) and (h)) which can detect light emitted from the well during the course of a pyrosequencing reaction.[79] Enzymes necessary for pyrosequencing are loaded into the wells (Fig. 8(f)) and the slide mounted in a flow cell. Sequentially, each of the four nucleotides are washed through the flow cell. When a base is incorporated into a growing DNA strand, a molecule of pyrophosphate (PPi) is released. The enzyme ATP sulfurylase (APS) converts PPi into ATP in the presence of adenosine phosphosulfate. The ATP so created is used by the enzyme luciferase to mediate the conversion of luciferin to oxyluciferin, generating visible light in the process. Since the amount of light liberated is proportional to the quantity of ATP generated by APS, which in turn is equal to the amount of PPi released during DNA synthesis, the number of molecules of that particular base incorporated in each well of the slide can be inferred, and the clonal amplification of DNA fragments captured on microbeads enables a large signal amplification. In this way, with sequential washes to remove old reagents and deliver the next base, short sequencing reads (up to 100 bp) are built up for each well, and the genomic sequence built up by computational fragment reassembly. This technique allows the sequencing of a whole bacterial genome (25 million bases), at 99% or better accuracy, in one 4-h run. This represents an approximately 100-fold increase in throughput over current Sanger sequencing technology. High-throughput screening on this scale has rapidly produced advances in several fields, such as the study of ancient DNA,[80–82] whole-genome sequencing,[83–88] metagenomic analysis of microbial populations,[89–92] transcriptome analysis,[93–101] and the identification of sequence variation in evolved bacteria[88] and in heterogeneous cancer specimens.[102,81] The latest commercial version of this Technology, the Genome Sequencer 100™ System from 454 Life Sciences can read 100 million base pairs in a single run, with read lengths of 250 base pairs, and it is possible to have runs where the peak of the distribution is 460 base pairs, and perfect reads over 550 base pairs (Jonathan Rothberg, personal communication).

Digital microfluidics

The quest for ultra-high throughput screening is, of necessity, driving the development of ever smaller reaction vessels and the means to perform biochemical and genetic assays within them. Unfortunately, it is difficult to reduce the volume of assays in conventional microtitre plate based formats below 1–2 μl. As discussed above, flow ''cytometry'' of double emulsion droplets provides one route to enable the inspection and sorting of very large numbers (10^7–10^8) of femtolitre-volume reactions.

Parallel developments in the field of microfluidics promise to extend the level of precision with which such tiny assay compartments may be created, manipulated and inspected, offering the hope of unprecedented levels of control which could revolutionize the practice of molecular engineering, chemical biology and proteomics. Microfluidic systems consist of networks of channels of typically 10–100 μm diameter and are very versatile allowing a huge variety of different application (see *e.g.* the *Nature Insights* on the subject[103–110]). Small

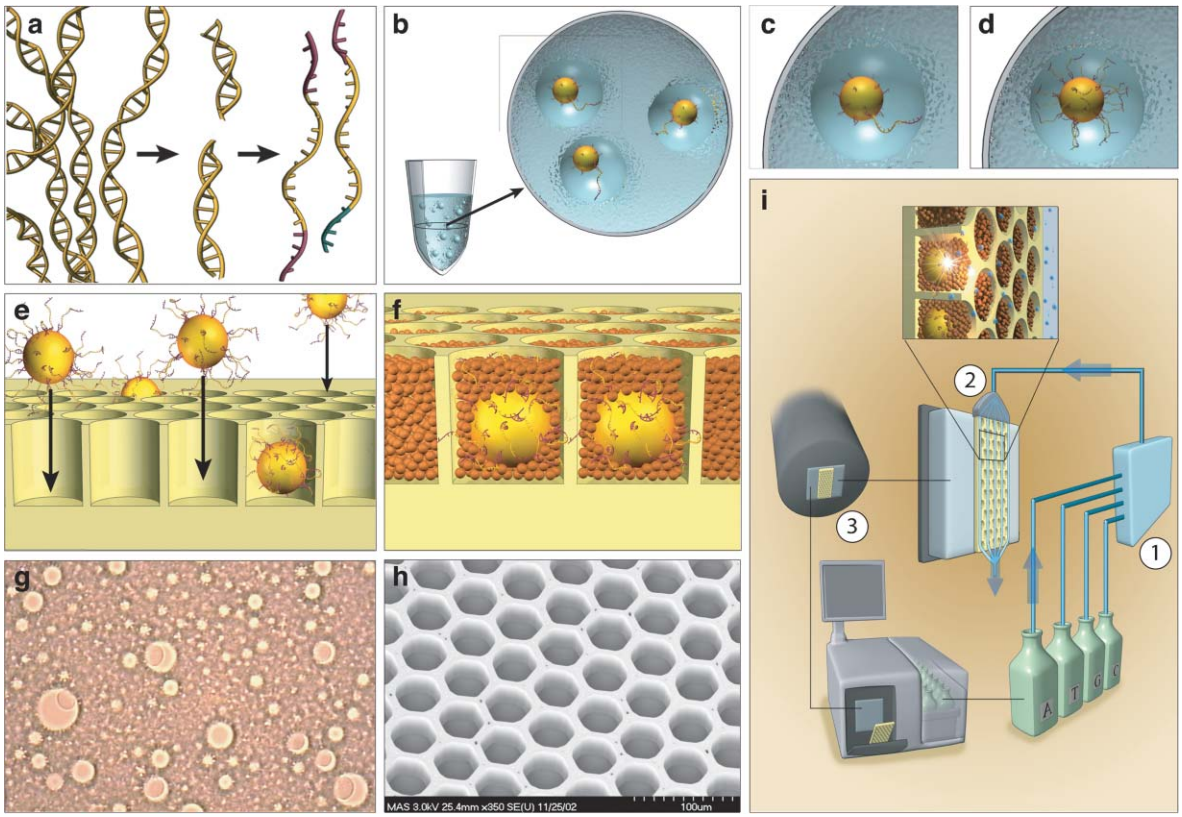

Fig. 8 Picotiter-plate pyrosequencing. (a) Genomic DNA is fragmented, ligated to adapter DNA sequences and separated into single strands. (b) DNA is immobilized on microbeads coated with a PCR primer such that the majority of beads carry at most a single DNA fragment. (c) The beads are then compartmentalized in a thermostable emulsion along with the ingredients of a PCR reaction and the mixture is thermocycled as in conventional PCR, leading to beads coated with ten million copies of the initial DNA fragment (d). The beads are recovered from the emulsion, the DNA strands denatured, and the beads (now carrying millions of single-stranded copies of the starting DNA fragment) are deposited in the wells of a fibre-optic slide (e). Smaller beads, carrying the enzymes required for pyrosequencing, are deposited into each well (f). (g) Microscope photograph of a thermostable emulsion. (h) Scanning electron micrograph of a fibre-optic slide, showing fibre-optic cladding and wells before bead deposition. (i) The pyrosequencing instrument, consisting of (1) a fluidic assembly, (2) a flow chamber which includes the fibre-optic slide containing deposited beads, and (3) a CCD camera-based imaging assembly and computer processing unit. Adapted from ref. 69 (adapted with permission from M. Margulies *et al.*, *Nature*, 2005, **437**, 376–380. Copyright 2005, MacMillan Publishers Ltd).

quantities of reagents can be brought together in a specific sequence, mixed and allowed to react for a specified time in a controlled region of the reactor channel network using electrokinetic and/or hydrodynamic pumping (for a review see ref. 111, and references therein).

Much recent progress in microfluidics has been driven by the development of soft-lithography, pioneered by George Whitesides.[112] Soft-lithography is based on micromolding using elastomeric polymers, the most important of which is poly(dimethylsiloxane) (PDMS). A positive-relief mould of the network of microchannels is created by using standard lithography: a photosensitive resist spin-coated on a silicon wafer is illuminated through a lithography mask. For a single layer of channels only one lithography step is required[113] but three dimensional arrays of microchannels can also be created with multiple photolithography steps.[114] After development, a layer of PDMS is cast on the three-dimensional structure of the mould and, after crosslinking, the PDMS slab is pulled off the wafer leading to the impression of the microchannels structure in the PDMS. Several PDMS devices can be made from a single mould, which makes PDMS-based microfluidics technology inexpensive and extremely versatile. The lateral dimensions of the channels are determined by the photolithography mask while the depth of the structure is controlled independently by the thickness of the spin-coated resist layers. Soft-lithography also allows liquids to be actuated using complex arrays of integrated pneumatic valves.[115] This technology allows the manipulation of small volumes of liquid as a continuous phase at a rate of about 1 nL s^{-1}. It has been used as a DNA sequencing tool,[116] and also for automated nucleic acid purification,[117] protein crystallography,[118] fluorescent immunoassays,[119] and even to build miniaturized flow sorters.[120,121]

This review, however, will focus on an alternative to this continuous flow approach, in which reactions are compartmentalized in droplets in the microfluidic system.

Several "digital microfluidic" systems exist that allow the manipulation of independent droplets. For example, thermocapillary pumping is used to move droplets in channels[122] and electrowetting-based actuation of droplets on arrays of electrodes has been used for glucose detection in different organic fluids[123,124] or analysis of peptides and proteins.[125] The most promising system in terms of throughput and universality of use is essentially a microfluidic version of IVC, based on the

manipulation of aqueous droplets in a hydrophobic carrier fluid flowing through arrays of microchannels. Droplets are insulated from each other and from the walls of the channels by carrier fluid. This both prevents cross-contamination and avoids the continual changes in concentration of reagents which result from parabolic flow-profile in continuous-flow systems. Such microfluidic devices allow the production of highly monodisperse emulsions at a rate greater than 10 kHz[126] using the instability of the interface between two streams of hydrophilic and hydrophobic phases.[13] Gel emulsions,[127,128] double emulsions,[129] polymerosomes[130] or bubbles[131] have also been produced using similar microfluidic devices.

After production, the trajectories of the droplets in the device are passively determined by the flow of the carrier fluid and the geometry of the channels, and droplets can be passively split and fused. It is, however, possible to superimpose additional active control of droplet behaviour in the carrier fluid in order to direct droplets in one direction rather than another by, for example, electrical forces acting on charged droplets[132] or dielectrophoresis on uncharged droplets.[133] These two systems presented in Fig. 9 are interesting for the realisation of sorting devices free of mechanical parts. Fusion between two droplets can also be actively controlled without mechanical parts by using electric fields *e.g.* by fusion of oppositely charged droplets[132] or electrocoalescence of uncharged droplets.[134]

PDMS devices, being transparent to visible light, are easily coupled to an optical set-up for detection of fluorescence in droplets and therefore many fluorescence-based biological assays can be adapted to digital microfluidic systems. Active actuation elements based on electric fields and coupled to optical detection are free of mechanical parts and therefore have much shorter response times than valves or pumps; it is possible to process droplets (production, manipulation, fusion and sorting) at rates larger than 1 kHz, leading to high-throughput manipulation of small liquid volumes (see http://www.raindancetechnologies.com).

Enzyme kinetic studies using digital microfluidic systems

Both continuous-flow and digital microfluidics devices allow the study of dynamic processes such as protein folding or the kinetics of enzyme-catalyzed reactions using very small volumes of reagent. A reaction mixture flowing at a given velocity along a microfluidic channel may be analysed over a timecourse dictated by the length of the channel and the speed of flow. For example, time-resolved NMR measurements of ubiquitin in a microfluidic device were employed to investigate changes in protein conformation after changes in solvent composition, using only microlitres of analyte to perform the experiment.[135] Kinetics of immobilized enzymes have also been studied in microfluidic devices.[136,137] For example, by flowing substrate over a packed bed of microbead-immobilized enzyme,[137] kinetics were determined for the horseradish peroxidase-catalyzed reaction between hydrogen peroxide and Amplex Red (*N*-acetyl-3,7-dihydroxyphenoxazine), yielding

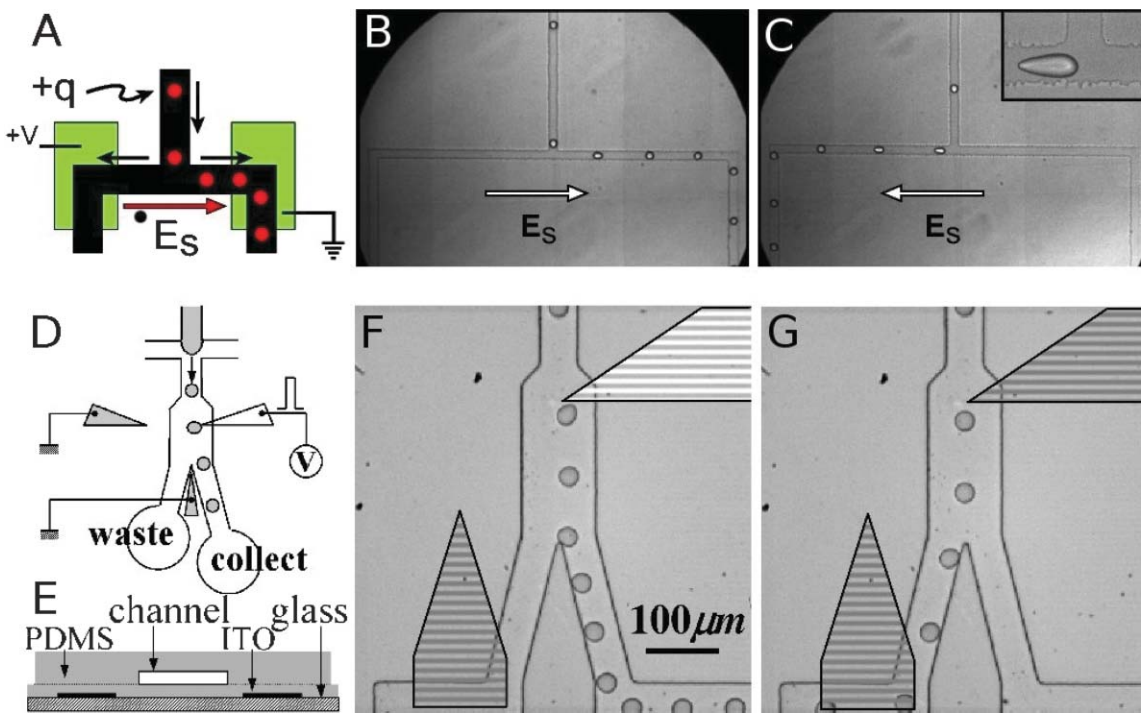

Fig. 9 Droplet sorting using electric fields. (A), (B) and (C): Charged droplet actuation using an electric field. A charged drop is oriented at a T junction by an applied electric field. Adapted from ref. 132 (reprinted with permission from D. R. Link *et al.*, *Angew. Chem., Int. Ed*, 2006, **45**, 2556–2560. Copyright 2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim). (D), (E), (F) and (G): Uncharged droplet actuation in an electric field gradient: the uncharged droplets are oriented at a Y junction using dielectrophoretic forces. Adapted from ref. 133 (reprinted with permission from K. Ahn *et al.*, *Appl. Phys. Lett.*, 2006, **88**, 024104. Copyright 2006, American Institute of Physics). (A), (D) and (E): Schematic representations of the sorting devices. (B) and (F), Sorting to the right. (C) and (G): Sorting to the left. These two systems are relevant for high-throughput screening due to the absence of mechanical parts which allows droplets to be sorted at kHz.

the fluorescent molecule resorufin, and for the β-galactosidase-catalyzed hydrolysis of (nonfluorescent) resorufin-β-D-galactopyranoside to D-galactose and resorufin; only 10 μl of substrate was required for each assay.

However, continuous flow approaches to measuring rapid reaction kinetics suffer from two problems which limit their use, both of which arise from the nature of laminar flow in microchannels. First, reagent mixing tends to be relatively slow, because separate reagent streams introduced into the same channel tend to remain as separate laminar flow streams, mixing only by diffusion. This makes it difficult to achieve rapid mixing of the sort employed by fast enzyme kinetic techniques such as stopped-flow methods. Secondly, the distribution of flow across a microfluidic channel is parabolic, so that solutes at the centre are transported more rapidly along the channel than solutes at the periphery, causing sample dispersion, which means that distance along the channel does not correlate exactly with reaction time.

Digital microfluidic systems allow the study of millisecond reaction kinetics.[138,139] Ismagilov and colleagues developed droplet-based microfluidic systems which employ winding channels to stimulate ''chaotic advection'', which rapidly mixes reagents brought together in the same droplet.[139,140] Reagent streams are brought together to form aqueous droplets separated by an immiscible phase; dispersion is therefore eliminated because the reagents are confined to an individual droplet or ''plug''. Rapid mixing, occurring over a few milliseconds, is achieved by passaging droplets through winding channels; fluid droplets moving around a curved section of channel are moving at different velocities relative to the two channel walls, which leads to a form of chaotic mixing which rapidly and efficiently mixes the constituents of the droplet.[140] This system was used to investigate rapid (millisecond), single-turnover kinetics of ribonuclease A by monitoring cleavage of a fluorogenic substrate.[139]

Droplet-based systems for studying kinetics have also been developed which exploit the ability to fuse surfactant-stabilised droplets in a controllable manner by electrocoalescence.[134] Two streams of uniformly sized droplets are made in a microfluidic device, one stream containing the enzyme, the other containing the substrate; the droplets are of two distinct sizes. When the streams are united in a single channel, size-dependent flow results in synchronization of the droplet streams, such that small drops containing enzyme become paired with larger drops containing substrate;[140] being stabilized by surfactants, the droplets do not coalesce spontaneously, but they may be forced to fuse in a controllable fashion by applying an electric field across the flow channel. After fusion, the droplets pass through a serpentine ''delay'' channel allowing images to be taken over a 2-s time period. In this way, the kinetics of the β-galactosidase-catalyzed hydrolysis of resorufin-β-D-galactopyranoside were measured by following fluorescence changes in droplets with a fluorescence microscope.

Directed evolution using digital microfluidics

Microfluidic devices such as these promise more than simply the recapitulation of model enzyme studies. Perhaps the most exciting opportunities lie in the application of high-throughput analysis of enzymatic processes to molecular engineering. *In vitro* expression of proteins and nucleic acids is already a well established procedure in millilitre-scale emulsions, and the use of emulsions generated in microfluidics is a logical extension; indeed, *in vitro* expression of proteins in a microfluidic droplet device has been reported.[141] Combined with the ability to monitor enzyme-catalyzed reactions with fluorogenic substrates, this approach could prove to be a powerful method for performing directed evolution of proteins and nucleic acids. In principle, genes encoding proteins or nucleic acids could be selected on the basis of precise kinetic specifications; expressed proteins or RNAs could be mixed with other molecules by droplet fusion to introduce substrates, inhibitors or effectors, in order to perform otherwise difficult or impossible selections. This approach was demonstrated recently in a standard emulsion-based selection for inhibitors of DNA nucleases (see above), where it was necessary to ensure expression of the potential nuclease inhibitors before the nucleases themselves were allowed to become active; in order to achieve this, nuclease-activating nickel or cobalt ions were introduced by fusion with ''nanodroplets''.[68] With controllable fusion of droplets in microfluidic devices already a reality,[134,140] this sort of procedure should be readily applicable to microfluidic-based selections.

Digital microfluidic machines for directed evolution hold out the possibility of having an unprecedented level of control over the procedure, enabling both detailed, quantitative studies of evolution at the molecular level and improved capacity to engineer proteins and nucleic acids for industrial and therapeutic applications.

Drug discovery using digital microfluidics

It is not only protein and nucleic acid engineering which should benefit from the rapid advance of microfluidic technology. Digital microfluidics allows extremely small volumes of compounds and other reagents to be screened, if necessary against multiple targets.[142,143] Screening techniques, perhaps using bead-based libraries encoded by fluorescent or other tags[144] combined with assays for enzyme or receptor inhibition, binding or regulation, could be employed to miniaturize lead optimization pipelines in the pharmaceutical industry. However, the synthesis of compound libraries could also benefit from the application of digital microfluidic technology. For example, Mitchell *et al*. demonstrated the synthesis of products of an Ugi multicomponent reaction (a reaction between an aldehyde, an amine, a carboxylic acid and an isocyanide) in a microchip device.[145] Digital microfluidic systems have since been used to perform large numbers of chemical reactions on small volumes in short times[146] which is of particular interest for combinatorial chemistry.[147] The possibility of combining compound synthesis and screening on a single microfluidic device is especially attractive. The compounds would be synthesised immediately before testing and would only exist transiently on chip. Only hits would need to be synthesised on a larger scale.

Conclusions

Since we introduced the use of emulsion microdroplets for *in vitro* protein selection and evolution, the technology has

diversified in several exciting new directions. Perhaps the most immediately appealing of these new applications lies in the field of DNA sequencing; here, two entirely novel ultra-high throughput sequencing technologies have been enabled by the use of clonal DNA amplification on microbeads, offering attractive new alternatives to traditional methods for whole-genome sequencing. The future prospects for microdroplet technology, however, are just as exciting. The precise control afforded by microfluidic platforms promises to open up new directions for molecular screening and engineering. It is even possible to envisage microfluidic systems capable of carrying out the entire process of molecular engineering: creation and screening of chemical or genetic libraries, selection and characterization of hits and reiteration of the whole process to further improve them; and the ultra-high throughput screening of protein or nucleic acid catalytic, binding or regulatory activities could have far-reaching implications for the field of functional genomics.

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